

Full Length Research Paper

# Structure and histochemistry of the glandular trichomes on the leaves of *Isodon rubescens* (Lamiaceae)

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The structure and histochemistry of the glandular trichomes on the surface of *Isodon rubescens* leaves were investigated. The main components in the secretion were identified. Morphological and histochemical study revealed one type of non-glandular and two types of glandular trichomes on the leaves of *I. rubescens*. These two types of glandular trichomes differed both anatomically and in the components of respective secondary metabolites. The glandular trichomes were classified into two subpopulations, namely the peltate and capitate glandular trichomes. The former was characterized by a short stalk and a large four-celled secretory head, while the latter was further subdivided into two groups; one has a short unicellular stalk and two-cellular head (type I), the other has a multicellular stalk and a globose unicellular head (type II). Peltate and capitate type I trichomes were constitutively present, whereas capitate type II was rarely found in all leaf samples. The secondary metabolites secreted by peltate trichomes were examined histochemically, which included terpenoids, flavonoids, carbohydrates, phenolics and alkaloids. The peltate trichomes are not only sites of oridonin accumulation, but also sites of oridonin biosynthesis in *I. rubescens*.

**Key words:** *Isodon rubescens*, Lamiaceae, glandular trichomes, histochemistry, secondary metabolites.

## INTRODUCTION

*Isodon rubescens* (Hemsley) H. Hara is a lamiaceous perennial herb native to the Yellow River valley of China. The leaves of *I. rubescens*, known as the local name "donglingcao" in China, has a long history of pharmaceutical use for the treatment of respiratory and gastrointestinal bacterial infections, inflammation and cancer (Sun et al., 2006). It is used in Chinese folk medicine to treat stomachache, pharyngitis, sore throat, cough and as an antitumor medicine for the treatment of esophageal and cardiac carcinoma, as the whole plant of *I. rubescens* has strong anticancer activity (Sartippour et al., 2005). Pharmacological study has shown that the major constituents of *I. rubescens* are diterpenoids, especially oridonin and ponidicin, which have significant antiangiogenic activity (Meade-Tollin et al., 2004). Other

types of compounds found in *I. rubescens* include mono-, sesqui-, triterpenes and flavonoids.

Oridonin, a bitter tetracycline diterpenoid (Figure 2) compound from *I. rubescens*, was found to have broad spectrum anti-tumor and antibacterial activities *in vitro* and *in vivo* and considered to be a potential new chemoprevention agent of cancer (Sun et al., 2006). As a medicinal plant, the plant is cultivated in Henan province. The concentrations of oridonin vary in leaves and stems at different growth stages (Chen, 2007). Previous investigation showed that the optimum harvest time of *I. rubescens* should be prior to florescence (Wu et al., 2005) in terms of its oridonin concentrations. The contents of oridonin in leaves and stems were close to 0.55 and 0.025%, respectively, thus, oridonin yields in leaves are up to 20 times as high as from stems (Lu et al., 2000). Consequently, the quality of *I. rubescens* is mainly determined by the contents of oridonin in leaves.

Glandular and non-glandular trichomes are widely

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distributed over the aerial reproductive and vegetative organs of plants of the Lamiaceae. Glandular trichomes are specialized in secretory structures. In many Lamiaceae species, these glandular trichomes produce, accumulate and secrete the constituents of terpenoids (monoterpenes, sesquiterpenes, diterpenes, etc.), in addition to accumulation of other secondary metabolites such as flavonoids, alkaloids, etc. A growing body of experimental evidence shows that terpene biosynthesis takes place within these glandular trichomes (Croteau and Johnson, 1984; Hay and Svoboda, 1993; Duke et al., 2000; Hallahan, 2000; Siebert, 2004). In the Lamiaceae family, there are two types of glandular trichomes, peltate and capitate trichomes, which are distinguished by respective structure and mode of secretion. The peltate trichomes compose of a basal epidermal cell, a stalk cell, and a head composed of four to 18 cells arranged in a single or two concentric disks (Werker et al., 1985; Bosabalidis, 1990; Ascensão et al., 1995, 1999; Ascensão and Pais, 1998). In mature peltate trichomes, secretion accumulates in a large subcuticular cavity that is developed above the secretory cells. The capitate trichomes are of various shapes (short, long, with a unicellular or pluricellular head). The most popular types present probably among all of the Lamiaceae species are short capitate trichomes, which is composed of a basal epidermal cell, a short unicellular stalk and a one- or two-celled head.

The secretions (monoterpenes, sesquiterpenes, phenolics, etc.) of glandular trichomes have been reported to be repellent to pests (Levin, 1973; Werker, 1993), toxic when eaten (Klingauf et al., 1983; Bestmann et al., 1987), inhibitory to egg hatching (Sharaby, 1988; Konstantopoulou et al., 1992) and to function as sticky traps (Kowalski et al., 1988). Another important role has been attributed to the secretions of glandular trichomes, such as phenolic compounds, and to their implication in the protection of mesophyll cells against UV-B radiation (Fahn and Shimony, 1996; Bosabalidis and Skoula, 1998; Manetas, 1999). Of significance in addition are the antimicrobial and allelopathic properties of the terpenoid components in the essential oils secreted by the glandular trichomes (Vokou and Margaris 1986; Sivropoulou et al., 1996). Diterpenoids have been further found to be deterrent, toxic and severe skin irritants to herbivorous mammals (Rosenthal and Berenbaum, 1991).

Recent morphological study (Liu et al., in press) revealed that *I. rubescens* leaves have one type of non-glandular and two types of glandular trichomes, that is, peltate and capitate. Both peltate and capitate trichomes consist of one basal cell, one stalk cell and one head. Most studies on glandular trichomes apply histochemical methods (Ascensão et al., 1999; Bisio et al., 1999; Corsi and Bottega, 1999; Bottega and Corsi, 2000; Nikolakaki and Christodoulakis, 2004, 2007) because they are considered useful for an initial investigation of the presence of some substances. Histochemical tests were useful to

localize *in situ* the main chemical classes of metabolites present in plant secretions. Preliminary histochemical results showed that terpenoid secretion was found to be restricted in peltate trichomes, which were probably the main organ of oridonin accumulation (Liu et al., in press). The use of *I. rubescens* in traditional Chinese 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.

In this article, the morphology and distribution of leaf trichomes of *I. rubescens* were studied using light microscopy (LM) and scanning electron microscopy (SEM). In addition, the chemical constitution of glandular secretions was investigated by means of histochemistry. The purpose of the present study was to probe into the structural, functional and ecological points of the glandular trichomes and their secretions. Direct proof that peltate trichomes are the only site of oridonin accumulation is provided.

## MATERIALS AND METHODS

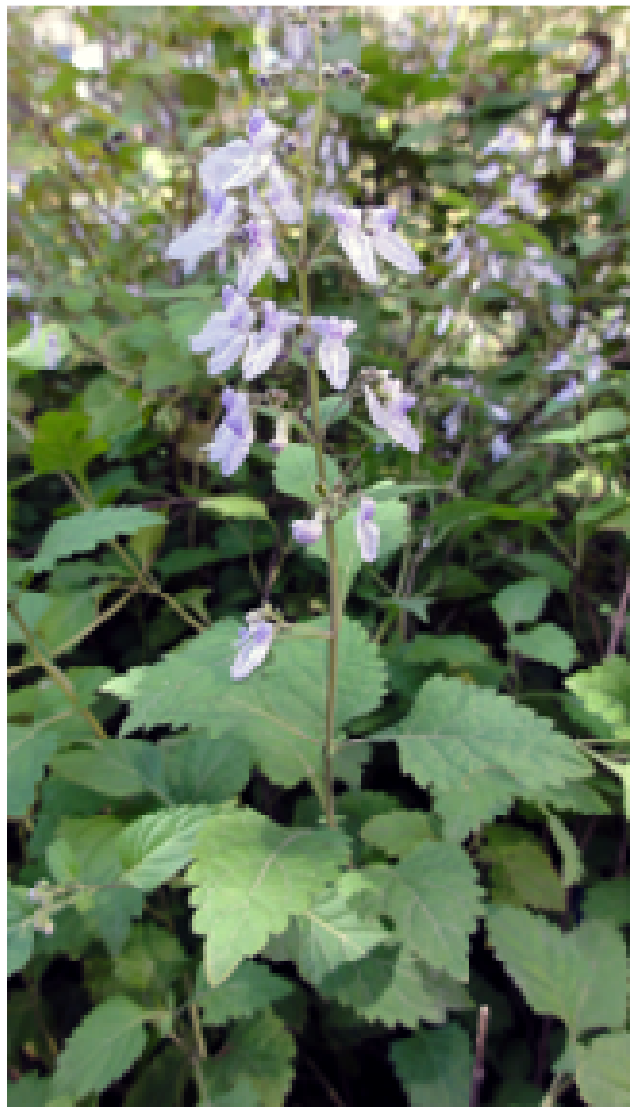
Fully developed leaves were collected from flowering *I. rubescens* plant (Figure 1) growing in open air during the summer months, in the Chinese Herb Garden of Henan University of Traditional Chinese Medicine, Zhengzhou, China. Voucher specimens were deposited in Herbarium of Henan University of Traditional Chinese Medicine.

### Microscopical investigations

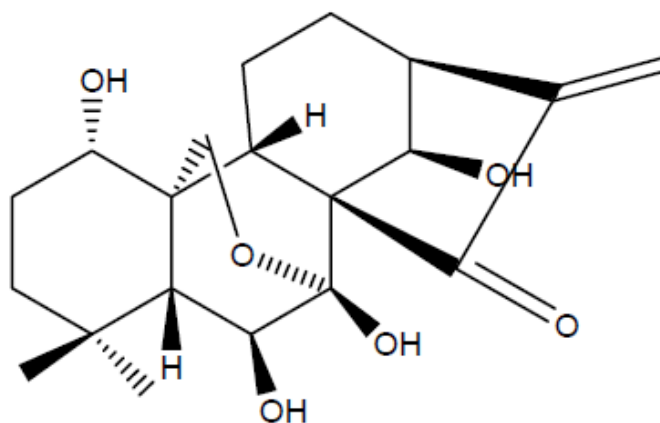
For light microscopy (LM), free-hand transverse sections of leaves were prepared and mounted in water on glass slides, and then examined with a Leica DM3000 light microscope. While for SEM, small pieces of leaves were fixed in formaldehyde + acetic acid + alcohol (FAA) for 24 to 36 h, dehydrated in an ethanol series, critical point dried, mounted on stubs with self-adhesive double-sided carbon discs and sputter coated with gold. Observations and digital photographs were taken with an S-4800 scanning electron microscope (Hitachi, Japan) at 10 kV.

### Histochemistry

Free-hand sections of fresh leaf tissue were stained with a series of histochemical reagents: osmium tetroxide (Lison, 1960) for unsaturated lipids; Sudan III for lipids; Sudan IV (Johansen, 1940) for lipids (except phospholipids); neutral red under UV (Clark, 1981) for total lipids; Nile blue for neutral and acidic lipids (Jensen, 1962); 2,4-dinitrophenylhydrazine for terpenoids (Ganter and Jollès, 1969); Nadi reagent (David and Carde, 1964) for terpenoids; 4-nitrosophenol in concentrated H<sub>2</sub>SO<sub>4</sub> (Gersbach et al., 2001) for monoterpene phenols; concentrated H<sub>2</sub>SO<sub>4</sub> (Cappelletti et al., 1986) for sesquiterpenes; vanillin-HCl, aluminium trichloride, lead neutral acetate (Guerin et al., 1971) and Neu's reagent (Neu, 1957) for flavonoids; antimony trichloride (Hardman and Sofowora, 1972) for terpene-containing steroids; potassium dichromate (Gabe, 1968) and ferric chloride (Johansen, 1940) for phenolic compounds; Ruthenium red (Johansen, 1940) for carbohydrates other than cellulose; and Wagner's reagent (Furr and Mahlberg, 1981) for



**Figure 1.** Flowering *Isodon rubescens* plant (photographed at the Chinese Herb Garden, Henan University of Traditional Chinese Medicine).



**Figure 2.** Chemical structure of oridonin.

alkaloids. All stains were matched by controls. The samples were observed under a Leica DM5500 fluorescence microscope.

#### Isolation of peltate trichomes from leaf surfaces

20 fully developed leaves collected from the fourth node below the terminal of the plant, were divided into two equal sets according to their opposite arrangement on the stem. Leaves from one of the sets were laid on a same flat surface. Adhesive tape was placed on both surfaces, gently rubbed to avoid removing any of the epidermis, and then removed (done twice). Although capitate trichomes and non-glandular trichomes are also present on both surfaces, the trichomes removed by the adhesive tape are predominantly the peltate trichomes because of their rugged leaf surface and large apex. By using this method, about 70% of the peltate trichomes were effectively removed by the adhesive. This set of treated and untreated leaves were dried separately and powdered. A 100 mg powder from each group was extracted in 0.5 ml of methanol for 30 min under occasional agitation. Equal samples (5  $\mu$ L) of both extracts were spotted on a thin layer chromatography (TLC) plate under the assistance of an automatic sample applicator (Germany, DESAGA AS30).

#### Extraction of leaves at seven different stages of development

20 *I. rubescens* plants were selected. Seven pairs of leaves were harvested from a single plant. They were harvested in pairs because these plants produce a pair of opposite leaves at each node. The leaves were harvested from the seven uppermost nodes. The seven pairs of leaves represented seven stages of leaf development. The leaves from each node were dried and powdered separately. A 100 mg powder from each leaf pair was extracted in 0.5 ml of methanol for 30 min. Equal samples (5  $\mu$ L) of seven extracts were spotted on a TLC plate.

#### Thin layer chromatography (TLC)

Thin layer chromatography was performed using a silica gel GF<sub>254</sub> TLC plate. The plates were developed in methylenechloride-ethanol-acetone (36:3:1). Spots were visualized by spraying the plate with ethanol containing 30% sulfuric acid and heated at 85°C for 5 min. The chromatograms were detected under UV and digitally scanned into a computer. To improve the visibility of spots, the images were processed using Adobe Photoshop 7.0. Oridonin on the plate was identified by chromatography with reference compound.

## RESULTS

### Trichomes

The leaves of *I. rubescens* bore both peltate and capitate trichomes, as well as uniseriate non-glandular trichomes, scattered over the abaxial and adaxial surfaces (Figures 3a to d). The density of both non-glandular and glandular trichomes on leaves varied depending on the developmental stages of leaves. Young leaves usually carried dense and obscure epidermal surfaces (Figures 3a and b), and the density of trichomes decreased progressively with aging (Figures 3c and e).

### Non-glandular trichomes

The non-glandular trichomes were multicellular and unbranched structures that consisted of two to five elongated cells. They were sharply pointed with nodules on the surface and 10 to 300  $\mu$ m in length (Figure 3e). Although non-glandular trichomes were present on both sides of the leaves, their quantity was higher along the veins of abaxial leaf surface.

### Peltate trichomes

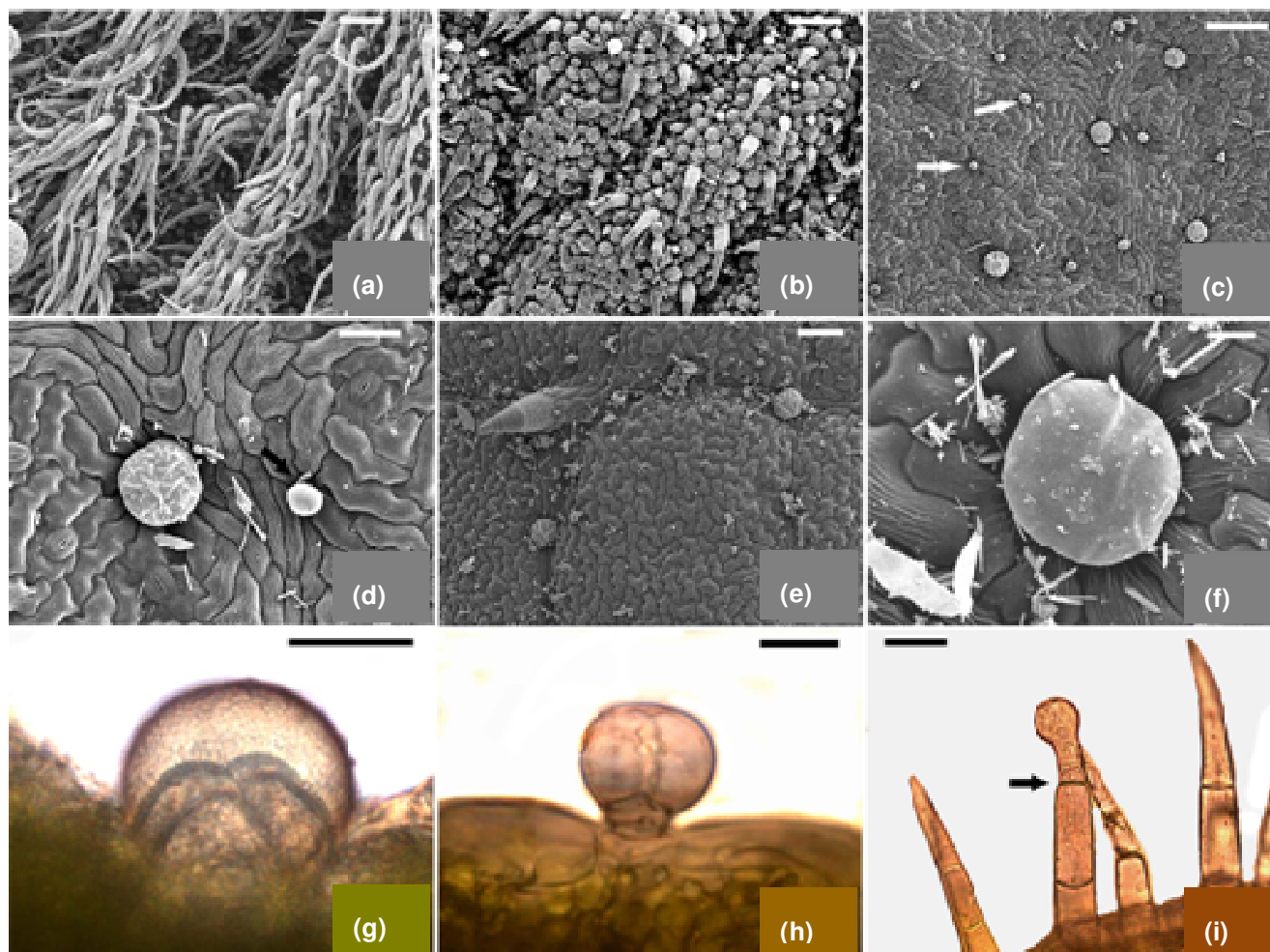
The peltate trichomes consisted of a basal cell, a short unicellular stalk and a broad head containing four secretory cells (occasionally the head consisted three, five or six cells). The secretory glandular trichomes have a characteristic spherical head, due to the development of large subcuticular spaces and accumulation of secretions (Figures 3f and g). The secretory head was about  $45 \pm 5$   $\mu$ m in diameter. The peltate trichomes were located in epidermal depressions in the mature leaf (Figure 3c). The surface of the mature peltate trichomes appeared sutureless because the accumulation of secretions in the subcuticular space distended the cuticle (Figure 3f). The secretion of peltate trichomes was colorless, abundant, and located in the subcuticular chamber. No chloroplasts were observed in the head cells of the peltate trichomes (Figure 3g).

### Capitate trichomes

There are two types of capitate trichomes which differ both in structure and size. Type I capitate consisted of one basal cell, a short stalk cell and a bicellular head (Figure 3h). It is typically  $18 \pm 2$   $\mu$ m tall, with a head that is  $18 \pm 2$   $\mu$ m in diameter. Type II capitate consisted of one basal cell, two to four stalk cells and a globose or pear-like unicellular head (Figure 3i). They are typically  $45 \pm 5$   $\mu$ m long, and the head is  $18 \pm 2$   $\mu$ m in diameter. Type I capitate trichomes was always present in all leaf samples, whereas type II capitate were only observed along the veins of the abaxial side of the leaf. Cuticle elevation was not remarkable either in the type I or II capitate trichomes (Figures 3h and i).

### Histochemistry of glandular trichomes

Table 1 shows the results of histochemical tests for peltate and type I capitate trichomes. No histochemical data is provided for capitate type II trichomes, as they are extremely rare. Both type I peltate and capitate trichomes were intensely black stained by osmium tetroxide (Figures 4a and b), indicating the presence of unsaturated lipids. Staining with Sudan III (Figures 4c and d) and Sudan IV (Figure 4e) showed positive lipid reaction both in the peltate and capitate type I trichomes.



**Figure 3.** *I. rubescens* leaf trichomes under light micrograph and scanning electron micrograph. (a) Abaxial surface of a young leaf. Bar = 100  $\mu\text{M}$ . (b) Adaxial surface of a young leaf showing distribution of glandular and non-glandular trichomes. Bar = 100  $\mu\text{M}$ . (c) Abaxial surface of a mature leaf showing distribution of peltate and capitate type I (arrows) glandular trichomes. Bar = 100  $\mu\text{M}$ . (d) Peltate and capitate type I (arrow) on the abaxial surface of a mature leaf. Bar = 30  $\mu\text{M}$ . (e) Adaxial surface of a mature leaf showing distribution of glandular and non-glandular trichomes. Bar = 50  $\mu\text{M}$ . (f) Peltate trichomes on the abaxial surface of a mature leaf. Bar = 10  $\mu\text{M}$ . (g) A fully-developed peltate trichome with four-celled head. The cuticle becomes greatly detached from the head cell walls forming a large subcuticular space. Bar = 20  $\mu\text{M}$ . (h) Capitate type I trichome. Bar = 10  $\mu\text{M}$ . (i) Arrow showing a capitate type I trichome. Bar = 20  $\mu\text{M}$ .

The presence of lipids in type I peltate and capitate trichomes was confirmed by the intense yellow fluorescence with neutral red (Figures 4f and g). Acid lipids in type I peltate and capitate trichomes were identified by a blue color with Nile blue (Figures 4h and i). Terpenoids were present in the secretion of peltate trichomes, identified by the black color when stained with 2, 4-dinitrophenylhydrazine (Figure 4j) or by the yellow color with Nadi reagent (Figure 4k), whereas terpenoids were absent in type I capitate trichomes. The presence of monoterpene phenols and sesquiterpenes in peltate trichomes was confirmed by the black staining with 4-nitrosophenol in conc.  $\text{H}_2\text{SO}_4$  (Figure 4l) or conc.  $\text{H}_2\text{SO}_4$  (Figure 4m), respectively, whereas these were absent in capitate type I trichomes.

Flavonoids were present in the secretion of peltate trichomes as indicated by the yellow color, when staining with vanillin-HCl (Figure 4n). With aluminium trichloride, peltate trichomes showed intense green fluorescence (Figure 4o), indicating different component of flavonoids in comparison to capitate type I trichomes, with fluorescence blue under UV light (Figure 4p). Flavonoids were identified when stained with lead neutral acetate, fluorescing intense blue under UV light (Figure 4q). With Neu's reagent, peltate trichomes showed intense yellow fluorescence (Figure 4r), indicating different flavonoids in comparison to capitate type I trichomes, with fluorescence blue under UV light (Figure 4s). With antimony trichloride, peltate trichomes showed intense green fluorescence under UV light (Figure 4t), indicating the presence of

**Table 1.** The histochemical tests performed and the strength of the reaction observed in the glandular trichomes. (+), Faintly

Target compound	Reagent	Colour observed	Trichome type	
			Peltate	Capitate (type I)
Unsaturated lipids	Osmium tetroxide	Black	+ (Figure 4a)	+ (Figure 4b)
Lipids	Sudan III	Red	+ (Figure 4c)	(+) (Figure 4d)
Lipids	Sudan IV	Brown	+ (Figure 4e)	(+)(not shown)
Lipids (total)	Neutral red	Yellow	+ (Figure 4f)	+ (Figure 4g)
Acid lipids; Neutral lipids	Nile blue	Blue	+ (Figure 4h)	+ (Figure 4i)
Terpenoids	2,4-Dinitrophenylhydrazine	Black	+ (Figure 4j)	-
Terpenoids	Nadi	Black	+ (Figure 4k)	-
Monoterpene phenols	4-nitrosophenol in conc. H <sub>2</sub> SO <sub>4</sub>	Yellow	+ (Figure 4l)	-
Sesquiterpenes	Conc. H <sub>2</sub> SO <sub>4</sub>	Yellow	+ (Figure 4m)	-
Flavonoids	Vanillin-HCl	Yellow	+ (Figure 4n)	-
Flavonoids	Aluminium trichloride	Green/black; blue	+ (Figure 4o)	+ (Figure 4p)
Flavonoids	Lead neutral acetate	Blue	+ (Figure 4q)	n.d.
Flavonoids	Neu's reagent	Yellow; blue	+ (Figure 4r)	+ (Figure 4s)
Terpene-containing steroids	Antimony trichloride	Yellow	+ (Figure 4t)	n.d.
Phenolic compounds	Potassium dichromate	Yellow	+ (Figure 4u)	n.d.
Phenolic compounds	Ferric trichloride	Black	+ (Figure 4v)	-
Carbohydrates	Ruthenium red	Pink; red	+ (Figure 4w)	+ (Figure 4x)
Alkaloids	Wagner's reagent	Black	+ (Figure 4y)	-

positive; -, negative; +, positive, n.d., not determined.

terpene-containing steroids. Phenolic compounds were present in the secretion of peltate trichomes, confirmed by the yellow color when stained with potassium dichromate (Figure 4u) or by black color with ferric trichloride (Figure 4v). Both peltate and type I capitate trichomes showed polysaccharide compounds by the pink or red staining of the secreted material with Ruthenium red (Figures 4w and x). Only peltate trichomes showed the presence of alkaloids by the black staining of Wagner's reagent (Figure 4y).

### Thin layer chromatography

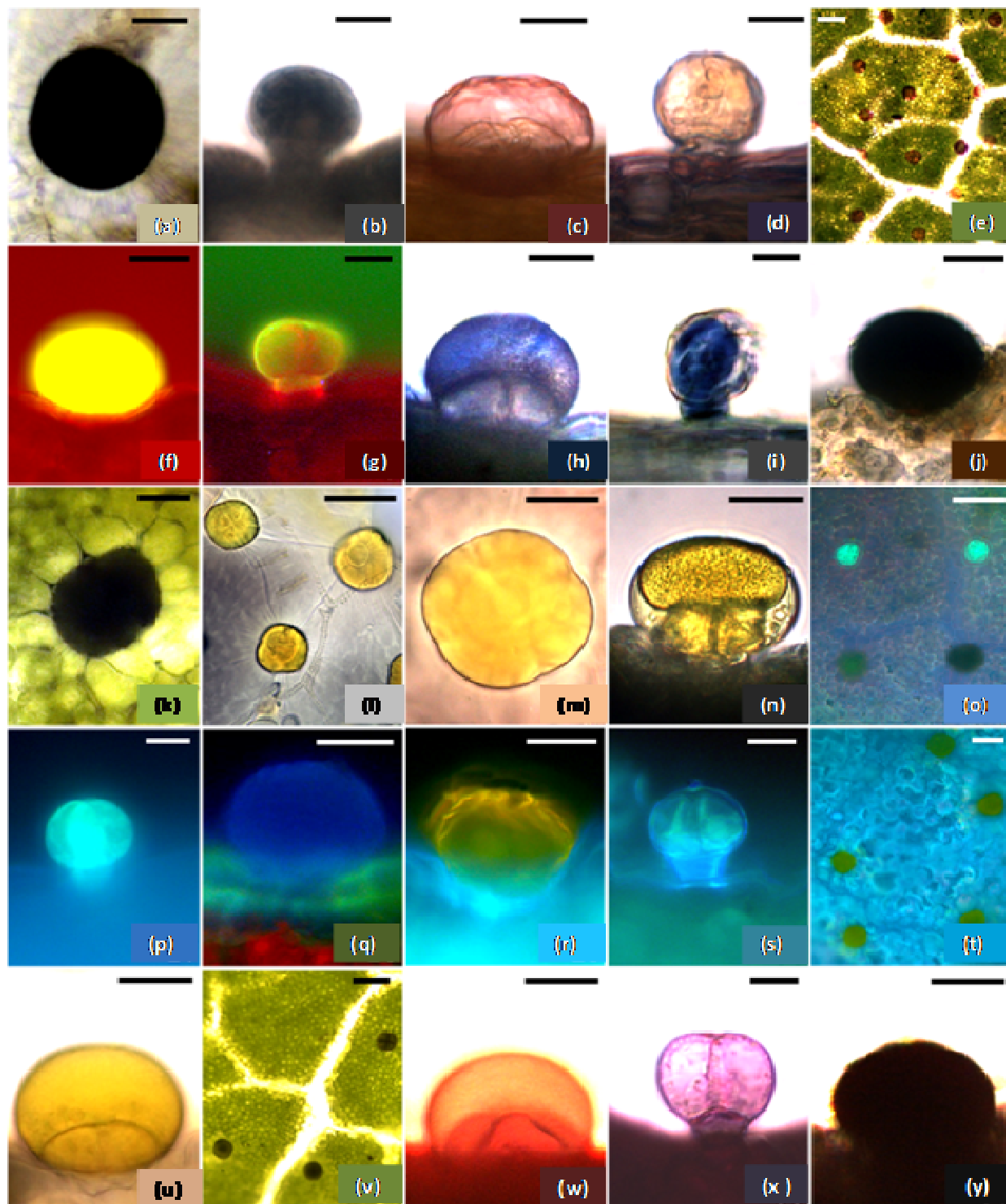
Comparative TLC of the extracts from the leaves that had its peltate trichomes 70% removed and leaves that had trichomes not removed showed that the oridonin content reduced in the former leaves in accordance with the percentage of peltate trichomes removed (Figure 5A). The concentration of oridonin appeared remarkably similar in samples of leaves from the uppermost to the fifth node (Figure 5B). Oridonin is apparently thin in older leaves and progressively diminish in concentration as the leaves become senescent.

### DISCUSSION

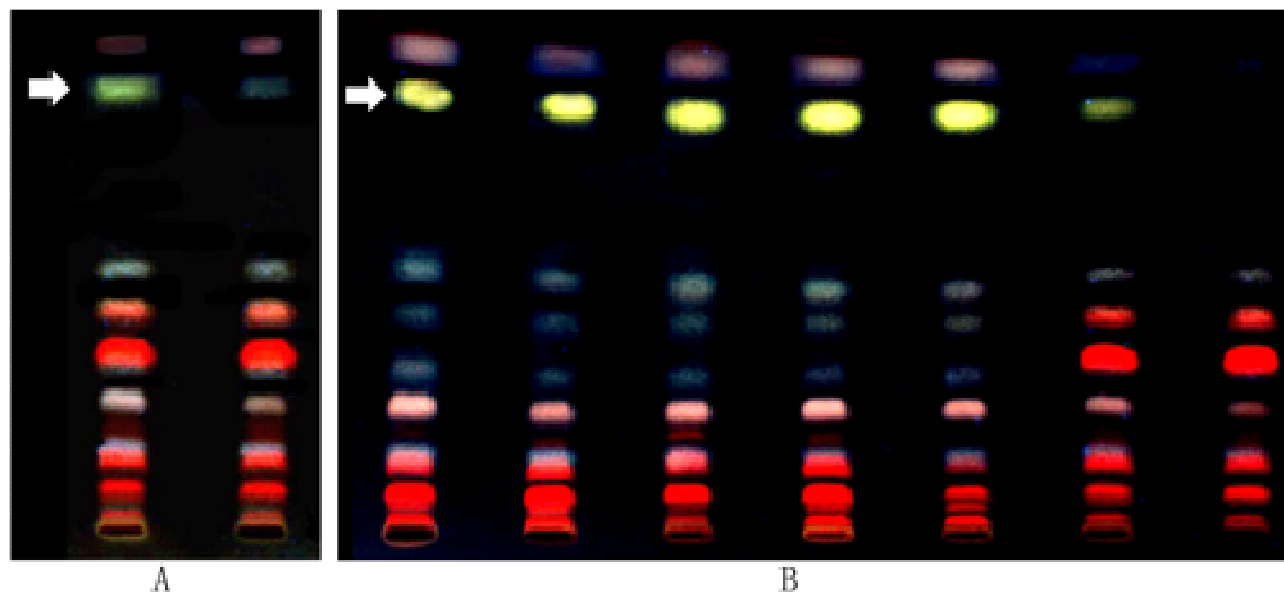
Similar to plants of most Lamiaceae species, the leaf surfaces of *I. rubescens* also have one type of non-

glandular trichomes and two types of glandular trichomes, including peltate and capitate (short and long stalked) ones, all of which possess an independently characteristic morphology, ontogeny, histochemistry and secreting process. These differences become evident only when fresh leaves are investigated histochemically or microscopically. The non-glandular trichomes are established early in leaf differentiation and their density decreases with leaf development and age. As to the functional and ecological roles of trichomes, non-glandular trichomes are thought to reduce the heat load of plants, increase tolerance to freezing, assist in seed dispersal, maintain water balance in leaves, deflect intense solar radiation and offer protection from insect herbivores (Johnson, 1975; Mauricio and Rauscher, 1997; Werker, 2000). The fact that non-glandular trichomes of *I. rubescens* did not give any positive reaction for substances involved in chemical defence indicates that their role is protection from water loss, temperature regulation and mechanical protection from herbivores (Ascensão et al., 1995, 1999; Yashodhara et al., 2001).

Capitate trichomes generally consist of rounded to pear shaped heads with one to two cells supported by stalks of variable length. The short-stalked capitate trichomes described by Liu et al. (in press) correspond to the capitate type I in the present work. The present study also revealed the presence of capitate type II. Capitate type I trichomes are the popular capitate trichomes of Lamiaceae. They consist of one basal cell, a short stalk cell and a two-cellular head. Usually, capitate type I



**Figure 4.** Light micrographs of glandular trichomes stained with different histochemicals and observed with either bright field or fluorescence microscope. (a) and (b) Osmium tetroxide for unsaturated lipids; (c) and (d) Sudan III for lipids; (e) Sudan IV for lipids (except phospholipids); (f) and (g) neutral red for total lipids under GF; (h) and (i) Nile blue for acid and neutral lipids; (j) 2,4-dinitrophenylhydrazine for terpenoids; (k) Nadi for terpenoids; (l) 4-nitrosophenol in conc.  $H_2SO_4$  monoterpene phenols; (m) concentrated  $H_2SO_4$  for sesquiterpenes; (n) Vanillin-HCl for flavonoids; (o) and (p) aluminium trichloride for flavonoids under UV; (q) lead neutral acetate for flavonoids under UV; (r) and (s) Neu's reagent for flavonoids under UV; (t) antimony trichloride for terpene-containing steroids under UV; (u) potassium dichromate for phenolic compounds; (v) ferric chloride for phenolic compounds; (w) and (x) ruthenium red for polysaccharides; (y) Wagner's reagent for alkaloids. 2, 4, 7, 9, 16, 22 and 24 are capitate trichomes (type I), while others are peltate trichomes. b, d, g, i, p, v and x: bars = 10  $\mu$ M; e, l, m, r: bars = 50  $\mu$ M; o and t: bars = 100  $\mu$ M. Others: bars = 20  $\mu$ M.



**Figure 5.** Methanol extracts of fresh leaves subjected to thin layer chromatograms. A. Comparative analysis of leaves with or without adhesive tape procedure. Arrows: oridonin. Lane 1, without adhesive tape processing; lane 2, with adhesive tape processing. B. Comparative analysis of leaves at seven progressive stages of development. Lane 1, leaves from the uppermost node of the stalk (the youngest leaves); lane 2, leaves from the second node down; lane 3, leaves from the third node down; lane 4, leaves from the fourth node down; lane 5, leaves from the fifth node down; lane 6, leaves from the sixth node down; lane 7, leaves from the seventh node down.

trichomes developed only small subcuticular spaces and the secretory material mainly accumulated in the cell lumen. The contribution of capitate trichomes to the total accumulation in this plant would be minor because their storage capacity, relative to that of peltate trichomes, is insignificant. The secretion is probably discharged via micropores; however, in *I. rubescens*, as well as in other species, pore formation has never been observed by SEM.

The head area of the capitate trichomes type I store lipids, predominantly unsaturated lipids and acid lipids, which could be confirmed by the staining reaction with osmium tetroxide (Figure 4b) and Nile blue (Figure 4g). Although the ferric trichloride test for phenolic compounds was negative, the aluminium trichloride and Neu's reagent (Figure 4s) test for flavonoid detection induced blue fluorescence. Thus, the only class of phenolic compounds histochemically identified in these trichomes, by fluorochromes under UV light, was flavonoids. Histochemical analyses showed the absence of terpenoids and alkaloids, whereas occurrence of polysaccharides in these trichomes (Table 1). Moreover, no chloroplasts were observed in the peltate trichomes, and the absence of chlorophyll fluorescence also reveals that chloroplasts were not present in the head cells; thus, they were unable to produce the photosynthates needed. According to the literatures available, the basal cell works as a collector of the mesophyll photosynthates. The photosynthetic products then move to the stalk and finally to head cells of the trichome, where the production of the

constituents of the essential oil and other substances takes place. This theory is enhanced by the presence of plasmodesmata and the absence of chloroplasts in the cells of the gland, as suggested by other authors (Ascensão et al., 1997; Ascensão and Pais, 1998). The secretory product of peltate trichomes was colorless, transparent, abundant, and stored in the subcuticular space. Cuticle detachment occurs only in the upper region of the glandular cell, the cuticle remaining firmly attached to the cell wall in the basal portion. The secretion seems to remain trapped in the intact subcuticular spaces unless external factors, such as the correct environmental conditions or mechanical damage (including grazing), which cause their rupture.

The present histochemical results indicate that the secretion of the peltate trichomes contained phenolics, flavonoids, terpenoids, carbohydrates and alkaloids. Autofluorescence is diagnostic of flavonoids, which, depending on the structural type, show dark yellow, green or blue fluorescence under UV light (Wagner and Bladt, 1996). The difference in the autofluorescence pattern under UV light for the two types of trichomes (peltate and capitate type I) also indicated differences in the secretory activity, with type I capitate trichomes having blue autofluorescence and peltate trichomes showing no autofluorescence (not shown). The appearance of the contents of peltate trichomes seems also to reflect their maturity. The observation that peltate trichomes belonging to the same leaf section react differently with Neu's reagent (Figure 4o), indicates that these



trichomes are at different stages of maturity or that peltate trichomes store products with different chemical composition. Histochemical tests were useful to localize *in situ* the main chemical classes of metabolites, which were present in plant secretions. The positive reaction with 2,4-dinitrophenylhydrazine (Figure 4j) and Nadi reagent (Figure 4k) indicate the presence of terpenoids in peltate trichomes. Histochemical analyses with free-hand transverse sections of leaves showed that oridonin is absent from other tissues other than peltate trichomes.

Furthermore, TLC analysis (Figure 5A) of the extracts from the leaves that had their peltate trichomes mostly removed showed that the oridonin content is reduced in accordance with the percentage of peltate trichomes removed. This result suggests that production of oridonin is specific to these trichomes. The above analyses indicate that oridonin is only present in parts of the plant that contain peltate trichomes. In conclusion, the experimental evidence presented here shows that oridonin is secreted as components of a complex secretion that accumulates in the subcuticular space of peltate trichomes. It seems highly probable that peltate trichomes are not the only sites of oridonin accumulation, but also sites of oridonin biosynthesis in *I. rubescens*. The dramatically lower yields of oridonin previously reported for whole stems compared with whole leaves (Lu et al., 2000) is primarily due to the fact that the stems are largely composed of woody vascular tissue and pith, which do not contain oridonin. Stems typically weigh about 10 times as much as leaves when dry sections of both organs with equal abaxial surface areas are compared. According to our observation, the density of peltate trichomes on the stem surface is similar to that on both leaf surfaces. As expected, the concentration of oridonin in whole stems and whole leaves differs in a manner that roughly corresponds to their different ratios of weight to abaxial surface area. Moreover, the peltate trichomes are densely distributed on the very young *I. rubescens* leaves. Although additional peltate trichomes continue to initiate and develop during leaf development, their overall density decreases as the leaves grow. The fact (Figure 5B) that the concentration of oridonin in the leaves remains relatively even throughout much of their development suggests that it progressively accumulates within the peltate trichomes as the leaves grow. Although peltate trichomes appear fully developed very early during leaf development, it is apparent that their chemical composition continues to change as the leaves mature. The fact that various compounds (phenolics, flavonoids, terpenoids, etc) are localized in the glandular trichomes that are distributed over much of the plant's exterior suggests that they serve a protective function. The compartmentalization of these compounds in extracellular spaces implies that they are not involved in the normal physiological processes of the plant. The density of glandular trichomes decreases with leaf maturity, indicating that the young *I. rubescens* leaves have denser

trichome coverings. The fact that there is high concentration of oridonin in the premature leaves suggests that this could be an adaptive feature of the plant, wherein the young, tender and attractive to herbivores, are given the highest level of protection. According to field observation, *I. rubescens* leaves are rarely consumed by herbivores. Considering the bitter taste of oridonin not pleasant to herbivores and the biological activity, most likely, these secretions function as deterrents to herbivorous animals. If so, it would likely discourage further consumption of the plant. This observation supports the argument that the trichomes in the epidermal layer of young developing organs are associated with defensive strategy to protect these organs against the insects, herbivores and also to changing environment (Tan et al., 2002; Auge et al., 2003; Nguefack et al., 2004).

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