

THE SECRETORY APPARATUS OF
CERATOTHECA TRILOBA (PEDALIACEAE):
MORPHOLOGY AND CHEMICAL
COMPOSITION OF THE SECRETION

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A research report submitted to the Faculty of Science and Agriculture, University of KwaZulu-Natal, in fulfilment of the requirements for the degree of Master of Science in Biological Sciences (Plant Science).

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DECLARATION

I, Taariq Karim, declare that this dissertation is my own work. It is being submitted in fulfillment of the requirements for the degree of Master of Science in Biological Sciences (Plant Sciences) at the University of KwaZulu-Natal. It has not been submitted before for any degree or examination at this or any other university.

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Taariq Karim

.....day of.....2012

DEDICATION

This dissertation is dedicated to my parents who have supported me and always motivated me to do my very best.

ABSTRACT

Ceratotheca triloba (Bernh.) E. Mey. ex Hook. f., commonly known as the wild foxglove, is a fast growing annual that is indigenous to southern Africa. The surface of the plant is covered with fine, hair-like trichomes, which exude sticky, aromatic substances. The plant is traditionally used to treat various abdominal ailments, insect infestation of the skin and leaf extracts are administered to induce abortion. In order to provide a scientific basis for the medicinal properties of *C. triloba*, this study was initiated to characterise the morphology and ultrastructure of the foliar trichomes and to determine the chemical composition of the secretion. Phytochemical tests and a range of microscopic techniques including stereo microscopy, scanning electron microscopy, transmission electron microscopy, histochemistry and fluorescence microscopy were used. Leaf surface imaging with stereo microscopy and SEM indicated the presence of two morphologically distinct glandular trichomes, capitate and peltate. The capitate trichomes are tall, consisting of a single basal cell, 2-4 stalk cells, a neck cell and a head that is made up of four secretory cells. Capitate trichomes of up to 14 stalk cells were observed on the abaxial side of flower petals. The peltate trichomes consisted of a basal cell, a single stalk cell and a multicellular head. The peltate trichome head generally consisted of four cells, but occasionally were seven or eight-celled. Fully developed trichomes were found to be concentrated on emergent leaves and probably serve protective function. The density of trichomes decreased as the leaf expanded. TEM showed that the secretory cells of peltate trichomes contained centralised nuclei and numerous peripheral vacuoles. Numerous mitochondria and ER cisterna were found throughout the cytoplasm. Osmiophilic plastids which were found near nuclei and golgi apparatus were in close proximity to the peripheral vacuoles. Similar observations were made for the stalk cells of both trichomes. The head cell of capitate trichomes however, contained large amounts of osmiophilic substances, ER cisternae and mitochondria. The head cells of peltate trichomes store the secretory product in vacuoles and secrete them upon cell rupture or cell dissolution. The secretory product of capitate trichomes is secreted through the cell membrane via an eccrine mode of secretion. Peltate and capitate trichomes appear to be secreting similar compounds, but the composition of each compound in the secretory material may vary between the trichomes. Histochemical and phytochemical tests reveal that the secretions are comprised of mucilage, phenolic compounds, lipids, flavonoids, tannins, saponins

and fixed oils which may contribute to the medicinal properties of *C. triloba*. The observations made in this study provide useful information for additional research in the Pedaliaceae, and specifically in *C. triloba*. Future studies should isolate the active compounds for antimicrobial and antioxidant testing. Cytotoxicity testing should also be undertaken to test safety and efficacy of the active compounds.

CONFERENCE CONTRIBUTIONS FROM THIS DISSERTATION

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LIST OF ABBREVIATIONS

Sec	Secretory material
Bc	Basal cell
C	Capitate trichome
Ch	Chloroplast
Cp	Cellular pedestal
CPS	Capitate trichome in the post secretory phase
Cw	Cell wall
DC	Developing capitate trichome
DP	Developing peltate trichome
ER	Endoplasmic reticulum
ESEM	Environmental scanning electron microscopy/micrograph
Gb	Golgi body
L	Lipid drop
LM	Light microscopy/micrograph
M	Mitochondria
Mc	Mucilage
Me	Multimembranous structure
N	Nucleus
Nc	Neck cell
Nm	Nuclear membrane
Nu	Nucleolus
O	Oil drop
P	Peltate trichome
Pd	Plastids
Pn	Phenolic Compounds
PPS	Peltate trichome in the post secretory phase
SC	Subcuticular chamber
Sc	Secretory cell

SEM	Scanning electron microscopy/micrograph
Sh	Secretary head
St	Stalk cell
TEM	Transmission electron microscopy/micrograph
V	Vacuole
Vc	Vesicle
Vn	Vein
WHO	World Health organisation

CHAPTER 1: INTRODUCTION

1.1 ETHNOBOTANICAL MEDICINE

Traditional medicine can be defined as any non-western form of medication or medical practice. It is a very broad term as it incorporates the use of animal, plant and mineral based products for the diagnosis and treatment of ailments. Therefore, to describe medicinal practices involving the use of plants and plant products only, the term ethnomedicine or ethnobotanical medicine will be used instead.

The use of plants for medicine can be dated back in fossil records some 60 000 years ago to the Middle Paleolithic age and the development of ethnomedicinal practices can be traced back through documents described in ancient Ayurvedic and Chinese literature. The World Health Organisation (WHO) estimates that 85% of the world population still rely on some sort of plant product for medicine (Obi *et al.*, 2006). Even in developed countries, the use of drugs containing plant products is also common (Fabricant and Farnsworth, 2001). In Germany the consumption of plants for medicinal purposes has risen by 20%. In the USA, 25% of all drugs dispensed by pharmacies contained plant active compounds (Farnsworth *et al.*, 1985). Despite this, pharmaceutical companies in the USA have not shown much interest in ethnomedicinal research. Developing countries, on the other hand, have invested in ethnomedicinal research which is beneficial as they have a rich source of information on traditional medicinal plant species which are used by the local people (Fabricant and Farnsworth, 2001).

An approach to researching plants for medicinal practices is by utilizing the information acquired from traditional healers. The information obtained from ancient ethnomedicinal literature has been useful in modern medicinal research. A survey carried out by Fabricant and Farnsworth (2001) to determine if ethnomedicinal information had any use in modern day medicinal research showed that 122 plant-derived pure compounds were identified as established drugs used in modern medicine. Of these, 80% are still being used to treat the original ailments as described in ethnomedicinal literature. It was also found that these compounds were isolated from only 94 plants. The number of flowering plant species occurring globally is estimated to be 250 000,

leaving many plants to be studied and disease treating compounds still to be discovered (Ayensu and DeFilipps, 1978).

1.2 DESCRIPTION OF *Ceratotheca triloba*

Ceratotheca triloba (Bernh.) E. Mey. ex Hook. f., commonly known as the wild foxglove, is a fast growing annual that is indigenous to southern Africa (Fig. 1). The plant is also known as the South African foxglove because it is the only species of *Ceratotheca* in South Africa. The plant thrives in disturbed habitats and is commonly observed in dry grasslands along roadsides.

Plants of *C. triloba* may be bushy or may grow as a single stem that can reach a height of 2 m, depending on the soil moisture. The entire plant is covered in fine hair-like structures which exude sticky, aromatic substances. The plants display either pink or white flowers that grow in pairs along the uppermost part of stems. The petals have five lobes with the lowermost being longer than the rest and displaying streaks of thin lines leading into the flower. The soft leaves expand to about 50 mm in length and divide into three lobes at the base with bluntly serrated margins. Seeds of the plant are protected by a capsule that displays three horns at the tip.

In South Africa, leaf extracts of *C. triloba* are used by traditional healers to treat diarrhoea, nausea and painful abdominal and menstrual cramps, whereas in Zimbabwe, the same leaf extracts are administered to induce abortion. Furthermore, in Botswana, the entire plant of *C. triloba* is used to treat insect infestations of the skin and pediculosis. Despite its potential medicinal value, no significant scientific research has been carried out on this plant.

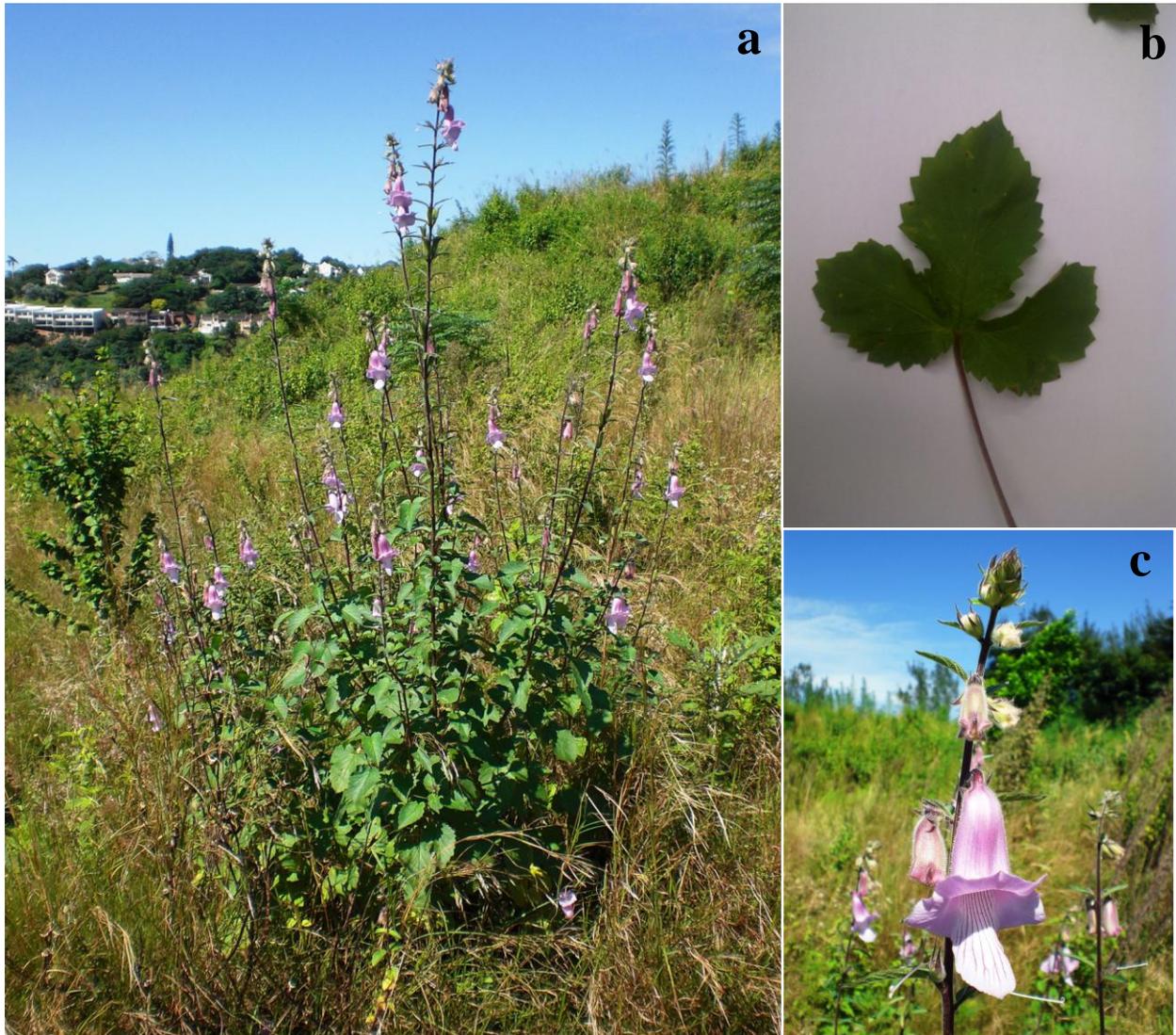


Figure. 1. *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook. f. growing along a roadside at the University of Kwa Zulu-Natal, Westville Campus. a) whole plant, b) mature three lobed leaf, and c) pink flower with five lobes; the lower most lobe has streaks of lines running into the corolla tube.

1.3 RATIONALE OF THIS STUDY

Trichomes have been studied previously because of their functional usefulness in nature and the economic value of the secretory product in industry (Valkama *et al.*, 2003). Trichomes are highly variable structures that provide a mechanical and chemical barrier against herbivores and the secretory product that they produce has been used in the food, fragrance and pharmaceutical industry (Fabricant and Farnsworth, 2001; Valkama *et al.*, 2003). Trichome morphological traits have also been key characteristics in plant taxonomic studies. Therefore the morphology of trichomes, chemical nature of the secretory product, and how the secretory product is produced are vital aspects that need to be addressed.

1.4 AIMS AND OBJECTIVES

The genus *Ceratotheca* has been neglected in terms of trichome research. Not much is known about the trichomes or the chemical properties of the secretions they produce. Since *C. triloba* has been used by traditional healers for a variety of ailments, this study was undertaken with the following aim and objectives:

Aim

To provide a scientific basis for the medicinal properties of *C. triloba*.

Objectives

- a. characterise the foliar trichomes of *C. triloba* according to morphology and distribution
- b. determine the chemical composition of the secretory product
- c. provide a description of the secretory process
- d. provide a phytochemical assessment of crude leaf extracts

CHAPTER 2: LITERATURE REVIEW

Secretory tissues in plants can be defined as multicellular structures or specialised single cells that eliminate specific substances to the external environment. These structures secrete specialised compounds which may aid in pollination, defence against herbivory and pathogens, and regulation of water and ions. In all cases, secretory structures serve as part of the metabolic pathway of plants, and in plant interrelations with the external environment. These structures include salt secreting glands, laticifers, nectaries, mucilage secreting cells, and trichomes (Fahn, 1979). There is great variation in structure and function of secretory tissues between species of plants. In addition, development and composition of the secretion can also vary. This review will focus on plant trichomes which are the most diverse group of secretory structures. The botanical literature has listed more than 300 morphologically distinct types of trichomes which have been used in plant classification (Kelsey *et al.*, 1984).

Trichomes, derived from the Greek word *trikhoma* which means ‘growth of hair’, are fine hair-like outgrowths found on the aerial regions of most angiosperms, some gymnosperms and bryophytes (Johnson, 1975). They are highly variable structures that are in contact with the external environment and therefore function in response to various biotic and abiotic stimuli (Tooker *et al.*, 2010). Trichomes serve as the first line of defence against plant natural enemies and they may attract and guide pollinators (Young *et al.*, 1984; Wagner, 1991). They also reflect UV radiation, and assist in drought tolerance by reducing leaf temperature and preventing excessive water loss (Levin, 1973; Wagner, 1991; Werker, 2000). In addition to variation in structure and function, differences may also occur in trichome development (Werker, 2000; Serna and Martin, 2006).

2.1. EVOLUTION OF PLANT TRICHOMES

Co-evolution between species is widely accepted as one of the fundamental mechanisms that produced the Earth’s diversity (Ehrlich and Raven, 1964). The process of co-evolution is a closely linked interaction between species. Interactions such as pollination, predation and mutualism between species may bring about genetic and morphological changes in one species

triggered by changes in the other (Ehrlich and Raven, 1964; Janzen, 1980). According to the co-evolutionary hypotheses, characteristics of plants that offer protection such as spines, thorns and trichomes evolved due to selection pressures imposed by natural enemies on plants (Juniper and Southwood, 1986). Therefore, plants evolved trichomes as a defence strategy against herbivores and plant pathogens.

Arabidopsis thaliana grown in an environment free of herbivores and pathogens displayed a decrease in production of two known characteristics that offer protection against natural enemies i.e. glucosinolate concentration and trichome density (Mauricio and Rausher, 1997). Glucosinolate is a secondary compound that is toxic to herbivores and pathogens (Mauricio and Rausher, 1997; Rosa and Rodrigues, 1999; Tierens *et al.*, 2001) and trichome density provides defence against plant's natural enemies (Ambrosio *et al.*, 2008). These results indicate that selection pressure enforced by plant natural enemies causes an increase in defence characteristics, which is supportive of the co-evolution hypothesis.

In contrast to co-evolution, the sequential evolution hypothesis considers the resistant features of trichomes as incidental and points to abiotic selection pressures, such as temperature and UV radiation, as the underlying cause of trichome evolution (Mauricio and Rausher, 1997). Unlike the co-evolution hypothesis, evidence to support the sequential evolution hypothesis is circumstantial.

2.2. TRICHOME CLASSIFICATION

Trichomes are highly diverse structures that vary in function, location, density, distribution, ability to secrete and timing of secretion. These differences can occur between species of plants, between plants within a single species and between organs of an individual plant. Classification of trichomes using one of the above characteristics is difficult because of overlap between species of plants and neither characteristic on its own is sufficient (Werker, 2000). The function of a trichome may vary according to its location on the plant. Trichomes located on vegetative organs may serve to repel herbivory, whereas trichomes located on reproductive organs may serve a dual role as attractants to pollinators as well as repellents to herbivores.

2.2.1 Morphological variation

a) Non-glandular trichomes

Non-glandular trichomes are generally classified according to their morphology. They range from unicellular to multicellular structures that can be uniseriate, biseriate or multiseriate, branched or unbranched (Werker *et al.*, 1994). The tips of non-glandular trichomes may be tapering or blunt (Werker, 2000). Thickness of cell walls may vary within and between trichomes and the hardening material within the walls may vary resulting in varying strength (Werker, 2000). Trichome length may differ between different plants, between organs of the same plants or within a single organ (Economou-Amilli *et al.*, 1982).

Occasionally more than one type of non-glandular trichome may be found on leaves as in the case of *Salvia blepharophylla* and *Coridothymus capitatus* (Economou-Amilli *et al.*, 1982; Bisio *et al.*, 1999). In *S. blepharophylla*, the three types were found: 1. multicellular, long trichomes with tapering tips, located along the leaf margins; 2. multicellular, uniseriate, short trichomes (usually 4-5 celled) with swollen bases located on the midrib and veins; and 3. unicellular papillae with enlarged bases and tapering tips, which were common on the abaxial surface of leaves. The reason for this variation in trichome morphology, however, is not known.

b) Glandular trichomes

Glandular trichomes include a variety of glands that synthesise, accumulate and secrete essential oils (Croteau, 1987; Gershenzon *et al.*, 1989). In order to classify these trichomes, the chemical composition of the secretion and the secretory process are considered in relation to trichome location and function.

The structure of glandular trichomes range from unicellular to multicellular and these can be uniseriate or multiseriate (Werker, 2000). The uniseriate, multicellular glandular trichome comprises of a base of one to a few cells, a stalk of one to many cells and a secretory head made up of one to many secretory cells (Fahn, 1988). Occasionally, a neck cell is located between the secretory head cells and stalk cells, and is morphologically different from the two.

In species of Lamiaceae, glandular trichomes are generally characterised in two major groups, the capitate and the peltate trichomes, named according to the shape of their secretory heads (Fahn, 1988). Capitate (Latin – *capitis* meaning “of a head”) trichomes usually have a globose secretory head made up of 1-4 cells, subtended by a stalk of one to many cells. They have been termed “short term trichomes” because they develop and release their secretion early in leaf development, thereby providing protection against herbivory even before any form of mechanical disturbance occurs (Werker, 2000). Peltate (Latin – *peltata* meaning “shield shaped”) trichome secretory heads are usually flattened and round made up of 4-18 cells subtended by a single stalk cell located at the centre of the secretory head. These have been defined as “long term trichomes” since they accumulate oil that is only release upon mechanical disruption (Werker, 2000). Similar forms of trichomes have been observed in other families such as the Cannabaceae (Dayanandan and Kaufman, 1976), Fabaceae (Zoric *et al.*, 2009), Curcubitaceae (Kolb and Müller, 2004) and Pedaliaceae (Naidoo *et al.*, 2006).

Variation in trichome structure may occur between species within a family or genus. *Mentha peperita* and *Leonotis leonurus* of the family Lamiaceae possess both capitate and peltate trichomes (Fahn, 1988; Ascensão *et al.*, 1995, Fig. 2). Both contain peltate trichomes consisting of a single basal cell, a stalk cell and a secretory head made up of eight secretory cells arranged in a circle (Fahn, 1988; Ascensão *et al.*, 1995). However, other species of the same family such as *Satureja thymbra*, have peltate trichomes of similar structure but the head contains more cells arranged in two concentric circles (Bosabalidis, 1990).

Differences in trichome structure may also be found within a single genus as in the case of *Salvia blepharophylla* (Bisio *et al.*, 1999) and *S. aurea* (Serrato-Valenti *et al.*, 1997), which contain peltate trichomes with secretory heads of four cells arranged in a quadrant, and eight cells arranged in a circle respectively. The capitate trichomes of *S. aurea* and *S. blepharophylla* are however, similar (Serrato-Valenti *et al.*, 1997; Bisio *et al.*, 1999) comprising of a single stalk cell and a bicellular secretory head. In contrast, those found on leaves and flowers of *S. scleria* and *S. dominica* are highly diverse, comprising of six different forms differing in structure, size, location, and manner of secretion (Werker *et al.*, 1985a). In addition, the short stalk capitate trichomes of *Plectranthus madagascariensis* (Ascensão *et al.*, 1998) and *P.annua* (Ascensão *et*

al., 1999) also differ with respect to the number of head cells, with the former possessing a bicellular head and the latter a unicellular one.

Variation may also extend to trichomes located on the same organ of a plant and between groups of cells within a single trichome. Meyberg *et al.*, (1991), reported that capitate trichomes found on leaves of *Nicotiana tobaccum* secreted different products even though they appeared to be morphologically analogous. Some trichomes secreted a resinous compound while the others secreted oil droplets. The exact functions of the different groups of cells that make up a trichome, and between cells of the same group are not clear. Ultrastructural studies indicated that trichomes with multicellular secretory heads may contain cells that secrete different substances than their neighbouring head cells (e.g. *Artemisia annua*, Duke and Paul, 1993) whereas other studies have highlighted the possibility that some head cells may not even be active in secretion (e.g. *Mallotus philipinensis*, Roth, 1977). Ultrastructural studies have also shown differences in cells of the stalk with the lowermost cells being highly vacuolated and the uppermost cells having dense cytoplasm (Turner *et al.*, 2000).

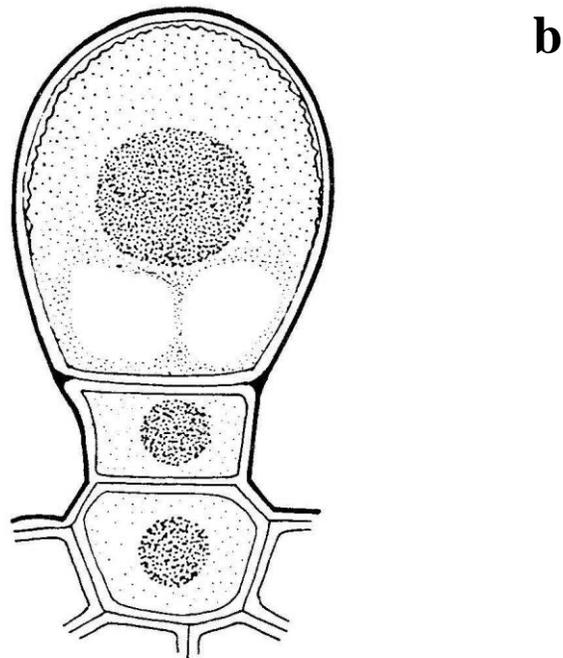
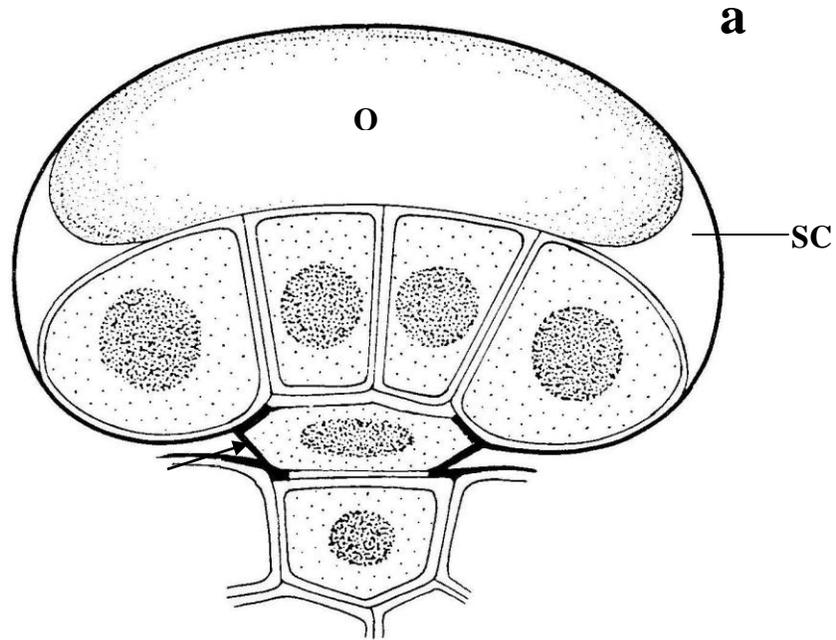


Figure 2. Glandular trichomes of Lamiaceae. a) Peltate trichome of *Mentha peperita* with oil drop (O) in subcuticular chamber (SC). The side walls of the stalk are highly cutinised (arrow) to prevent apoplastic backflow. b) Capitulate trichome with a single stalk cell and unicellular secretory head. (from Fahn, 1988)

c) Integrated forms of glandular and non-glandular trichomes

In most cases, trichomes can be clearly differentiated into glandular and non-glandular. However, there exist trichomes that cannot be characterised explicitly into either group. In the case of certain species of Lamiaceae, unbranched glandular trichomes are clearly differentiated from branched non-glandular trichomes. However, there are instances in which compound glandular trichomes are branched having many non-glandular branches with one glandular branch (e.g. *Phlomis*, Azizian and Cutler, 1982; *Rosmarinus officinalis*, Werker *et al.*, 1985c; *Hyptis*, Rudall, 1980). In addition, Ascensão *et al.*, (1995) reported that the stalk walls of capitate trichomes in *Leonotus leonurus* displayed cuticular warts which are a characteristic of the non-glandular trichomes of that species. It was concluded by Ascensão *et al.*, (1995) that these capitate trichomes represented an intermediate form between glandular and non-glandular trichomes. Fahn (1988) suggested that these findings may also provide some answers with regards to the evolution of these structures.

2.2.2 Variation in the process of secretion

In Lamiaceae, the peltate trichomes accumulate the secretory product in a subcuticular chamber, which forms when the cuticle detaches from the cell wall of the secretory cells (Werker *et al.*, 1985a, b). These peltate trichomes are assumed to release their contents when the elevated cuticle is damaged, usually by plant herbivores. *Ceratotheca sesamoides* of the family Pedaliaceae has peltate trichomes that lack a subcuticular chamber. They secrete mucilage when the secretory cell ruptures (Abels, 1975) or dissolves (Ihlenfeldt, 2001), resulting in cell death.

Capitate trichomes may also accumulate oils in a subcuticular chamber and release them either by cuticular rupture or by diffusion through micropores in the cuticle. The secretory product of the “short stalked capitate trichome” of *Plectranthus madagascariensis* accumulates in a small subcuticular space and is probably released by diffusion through micropores, whereas “the long stalked capitate trichome” releases its secretory product via cuticular rupture (Ascensão *et al.*, 1998). Similar variations were also reported for other species of Lamiaceae (e.g. *Ocimum basilicum*, Werker *et al.*, 1993; *Plectranthus annua*, Ascensão *et al.*, 1999) The “capitate Type I hairs” of *S. sclarea* however lack a subcuticular chamber and release their contents through

micropores in the cell wall and cuticle (Werker *et al.*, 1985a). Accumulation of secretory product in the cell lumen and direct diffusion through the cell wall were also observed for capitate trichomes in *S. aurea* (Serrato-Valenti *et al.*, 1997) and *O. basilicum* (Werker *et al.*, 1993). Kolb and Müller (2004) observed that the capitate trichomes found on leaves of *Cucurbita pepo* var. *styriaca* released their secretion when they interacted with the non-glandular trichomes and bristles which indicates that secretion in this species is dependant on trichome density.

2.2.3 Trichome function

a) Protection

Plants may require protection from a variety of abiotic (extreme temperature, intense light and water loss) and biotic (herbivores, pathogens and alleopathy from plant competitors) factors. Trichomes provide protection against all of the above threats, especially when they densely cover plant organs. The effect of trichome density on herbivore resistance has been noted in *Thithonia diversifolia* (Ambrosio *et al.*, 2008).

In the case of abiotic factors, a direct correlation has been observed between environmental stresses and the density of trichomes found on the plant organ (Clausen *et al.*, 1940). Johnson, 1975 reported that *Potentilla* (Rosaceae) planted in sunny sites contained more pubescent trichomes than those grown in shady sites. Warming (1909) has already emphasised the point that a greater number of trichomes will appear in plants that are subjected to a harsher moisture regime. He also noted the differences in trichome cover between plants of the same species which are found in xeric and mesic habitats. He observed that the plants which are found in xeric habitats contained more trichomes. In addition, the resin secreted by glandular trichomes, in *Betula pendula* found at high altitudes, serves as a moisture repellent, protecting the plant against the harsh winter and spring (Lapinjoki *et al.*, 1991). Non-glandular trichomes may serve as structural defences against plant herbivores. Gilbert (1971) observed a highly effective defence strategy of *Passiflora adenopoda* against the heliconiine butterfly larvae. The hook-like trichomes killed the larvae by entrapping them, followed by inducing many puncture wounds in the larval integument.

Glandular trichomes provide a variety of defence strategies by secreting specialised compounds that may either trap or poison herbivores, or provide an antimicrobial effect against pathogens. These compounds include terpenoids (Dawson *et al.*, 1966; Bakker *et al.*, 1972; Turner *et al.*, 2000), flavonoids (Kelsey *et al.*, 1984), tannins (Feeny, 1968) alkaloids (Rodriguez *et al.*, 1984) and phenolics (Yu *et al.*, 1992). Non-volatile compounds such as alkaloids, tannins and phenols may act as a deterrent to herbivory. In a study carried out by Harley and Thorsteinson (1967), several alkaloids (including nornicotine, lobeline, tomatine and hordinine) that were added to the diet of the two striped grasshopper, *Melanoplus bivittatus*, either repelled the insects or killed them. Similarly, phenolic compounds such as the coumarins, which are produced by *Melilotus* species, have an inhibitory effect on the vegetable weevil (Matsumoto, 1962). Some glandular trichomes secrete mucilage that interacts with phenols to trap herbivorous insects. Tingey and Gibson (1978) reported a defence strategy of *Solanum polyadenium* and *S. berthaultii* against the aphid *Empoasca fabae*. It was reported that the mouthparts and tarsi were encased by a sticky substance which hardened, resulting in starvation and death of the aphid. This is probably due to the release of phenolic compounds which are oxidised to quinines by polyphenol oxidase. The resulting quinines polymerise and trap insects (Yu *et al.*, 1992).

Tannins interact with proteins to form tannin-protein complexes, which reduce the total protein that can be ingested by herbivores. Since these complexes reduce the dietary content in plants and most angiosperms contain tannins, it is highly possible that insects use tannins as a negative feeding cue. In addition, chemoreceptors found on legs and mouthparts of insects detect possible fatal chemicals on the elevated head cells of trichomes (Dethier, 1963). This prevents feeding as the insect realises the host is unsuitable for consumption. In this way trichomes serve as a deterrent to herbivores by advertising the presence of highly toxic chemicals.

b) Attraction

Much of the research characterising trichomes has focused on the vegetative organs with the reproductive organs being neglected. Trichomes on flowers serve conflicting roles in deterring natural enemies and attracting pollinators (possibly the natural enemies of other plants). In some species of Lamiaceae, large glandular trichomes were observed on stamens, under the anther lobes (*Cannabis sativa*, Mahlberg *et al.*, 1984; *Leonotus leonurus*, Ascensão *et al.*, 1995).

Whether these trichomes secrete compounds that attract pollinators or whether they deter herbivores, or both, is not known.

Many authors have observed differences in trichome structure and distribution between vegetative and reproductive organs. This may be indicative of differences in function. In *Cannabis sativa*, stalked capitate trichomes were observed on floral organs, whereas the vegetative organs displayed various other trichomes (Mahlberg *et al.*, 1984). In many species, the calyx, which covers the flower during development, is covered in glandular and non-glandular trichomes similar to those found on the vegetative organs. These trichomes are probably for protection as described previously. The trichomes on the calyx of *Origanum vulgare* (Werker *et al.*, 1985b) are different from those on vegetative organs with respect to the ratio of the various types of glandular trichomes and the proportion of the chemical constituents they secrete, thus indicating that trichomes on the calyx may also serve as attractants. In *Salvia dominica* and *S. sclarea*, both the reproductive and vegetative organs are covered in capitate and peltate trichomes. The morphology of peltate trichomes is uniform between the two organs whereas the capitate trichomes differ in structure, size and mode of secretion. The main components of the essential oil, neryl acetate and linalyl acetate, also differed between vegetative and reproductive organs (Werker *et al.*, 1985a).

Variations in trichome occurrence and distribution were also observed within a flower. In some species of Lamiaceae such as *Origanum vulgare* (Werker *et al.*, 1985b) and *Leonotus leonurus* (Ascensão *et al.*, 1995), peltate trichomes were the only types observed on the outside of corolla tubes whereas the inside of the tubes displayed a variety of other glandular trichomes. Variation in trichome structure and distribution between and within organs may indicate differences in function. However, evidence to support this is inferred and more work is required to clarify these issues.

2.3 TRICHOME DEVELOPMENT

Most of the information regarding trichome development is drawn from studies on mutants of *Arabidopsis thaliana*. These studies show that the first cells (protodermal cells) to differentiate from the epidermis develop into trichomes. Trichomes are therefore generated in early leaf development and the rate of differentiation is rapid (Duke and Paul, 1993; Werker, 1993). This often provides protection to the leaf primordia which are more susceptible to herbivory. In *Inula viscosa*, fully developed trichomes were observed on leaves not more than 2 mm long (Werker and Fahn, 1981). Whether all the trichomes produced on leaf primordia are predetermined or develop as the leaf ages is still not clear.

The various processes in trichome development are discussed in the following section. Development of other protuberances, such as emergences and ‘false hairs’ will not be considered as they are not true trichomes.

2.3.1 Development of single cells

The epidermal cell, with a dense cytoplasm and a large nucleus, divides periclinally to form a protrusion above the epidermis. These cells can then undergo further periclinal divisions to form a stalk and anticlinal divisions to form secretory head cells (Werker, 2000). Uphof (1962) characterised the periclinal development of a multicellular, uniseriate trichome according to direction of division of the existing stalk cells. Acropetal division occurs when the uppermost stalk cell divides to form a new cell, whereas basipetal division occurs when the lowermost stalk cell undergoes division. In *Pelargonium scabrum*, capitate stalked trichomes result from a development of the protodermal cell which divides periclinally to form a single stalk cell (Oosthuizen and Coetzee, 1983). Further stalk cells develop from acropetal division. The peltate trichome in species of Lamiaceae undergoes two periclinal, acropetal divisions to form a stalk cell and a single secretory cell which undergoes further anticlinal division (e.g. *Origanum dictamnus*, Bosabaladis and Tsekos, 1982a; *Leonotus leonurus*, Ascensão *et al.*, 1995).

A common misconception with biseriate and multiseriate trichomes is that they all develop from more than one cell. In most biseriate trichomes, especially those of the Asteraceae, initial division occurs from a single protodermal cell which undergoes an anticlinal division to form two cells along the leaf epidermis (e.g. *Holocarpha virgata*, Carlquist, 1958; *Artemisia campestris*, Ascensão and Pais, 1987; *Stevia rabaudiana*, Montiero *et al.*, 2001). These cells then divide periclinally to form a fully developed biseriate trichome. There are variations to this form of development. In *Helichrysum aureonitens*, the first division is periclinal but the anticlinal division occurs soon after (Afolayan and Meyer, 1995).

In most trichomes, in addition to differentiation of the protodermal cell, other epidermal cells may differentiate around the protodermal cell. These generally form a ring of differentiated cells normally referred to as a cellular pedestal, which provides support and anchors the trichome in the epidermis (Werker, 2000). Some cellular pedestals may even grow above the epidermis thereby elevating the trichome.

2.3.2 Development from multiple cells

Initially only one protodermal cell divides anticlinally to form four cells, which undergo numerous periclinal divisions to form a developed trichome. In addition to the single protodermal cell, many epidermal cells also differentiate and divide together to form the developing trichome, and in most cases add to the structure as in *Helicteres hirsute* (Uphof, 1962).

2.4 THE FAMILY PEDALIACEAE

The Pedaliaceae is a small family, consisting of 17 genera and 80 species. It is a predominantly African family with the exception of a few species, such as *Pedaliium murex*, which is found in Yemen (Cronquist, 1981; Wood and Haig-Thomas, 1997). *Sesamum* is the largest genera consisting of 20 species, most of which are well documented for their oily seeds. Taxonomically,

the genera *Sesamum* and *Ceratotheca* are the closest, with the horns on seed capsules of *Ceratotheca* the only characteristic separating the two (Bruce, 1953).

Much of the research on this family focused on the oil producing seeds of *Sesamum*. In terms of research on trichomes, the Pedaliaceae has been somewhat neglected. Solereder (1908) described the mucilage hairs found on leaves of *Sesamum indicum* and *Ceratotheca melanosperma*. These trichomes were peltate, consisting of a head made up of four cells arranged in a cross (Figure 3). A capitate trichome was also described as having a multicellular stalk and a small spherical head of four cells which were shaped like small palisade cells. Similar observations were made for *C. sesamoides*. Naidoo *et al.*, (2006) described glandular trichomes found on leaves of *Harpagophytum procumbens* consisting of a multicellular stalk and head made up of four cells that were arranged in a quadrant, which secreted viscous material.

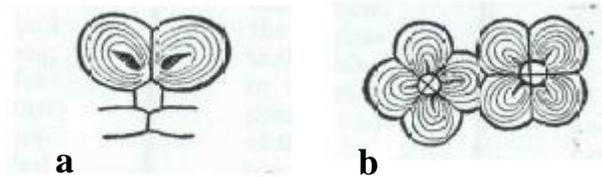


Figure 3. Mucilage hairs of *Sesamum indicum* and *Ceratotheca melanosperma*. (from Solereder, 1908)

CHAPTER 3: MATERIALS AND METHODS

3.1 COLLECTION AND PROPAGATION OF PLANT MATERIAL

Intact plants of *Ceratotheca triloba* were collected from the University of KwaZulu-Natal (UKZN), Westville campus and Umhlanga Rocks (29° 43' 33.6" S, 31° 4' 12" E) and transported to the greenhouse for propagation in pots. Voucher specimens were prepared and kept at the School of Biological and Conservation Sciences Ward Herbarium (Voucher specimen no. KARIM - 1).

3.2 SAMPLING

For light microscopy and scanning and transmission electron microscopy, samples were taken from mature plants in the field from the tip, mid and basal regions of leaves. Three stages of leaf development were sampled in this study i.e. the emergent (width < 2 mm, length < 9 mm) young (width 2 < 18 mm, length 9 < 26 mm) and mature (width > 18 mm, length > 26 mm) leaves.

3.3 PREPARATION FOR SCANNING ELECTRON MICROSCOPY (SEM)

Three techniques were used for SEM studies: freeze-drying, chemical fixation and environmental SEM.

3.3.1 Freeze-drying

Leaf segments were prepared by quenching in liquid nitrogen slush and freeze drying for 96 h in an Edwards Modulyo freeze dryer at -40 °C to -60 °C at a vacuum of 10⁻¹ Torr. After securing samples on brass stubs with carbon conductive tape, they were sputter coated with gold in an atmosphere of argon with a Polaron Sputter coater unit SC500. The leaf segments were viewed with a Leo 1450 SEM at 5 kV and a working distance of 15 mm.

3.3.2 Chemical fixation

Segments of leaf blades and petals were prepared for chemical fixation for SEM. These samples underwent a primary fixation in a solution of 2.5 % glutaraldehyde (4 °C) buffered with 0.1 M phosphate buffer (pH 7.2) for 2-24 h followed by a post fixation at room temperature in 0.5% osmium tetroxide (OsO₄) for 1 h. Samples were then dehydrated in a graded alcohol series of 30 %, 50 %, 75 % and 100 % ethanol. Critical-point-dried samples were then secured on brass stubs with carbon conductive tape and sputter coated with gold and viewed as described previously.

3.3.3 Environmental scanning electron microscopy (ESEM)

Segments of fresh leaves and petals were placed on brass stubs with carbon conductive tape and viewed under a Philips XL 30 ESEM at 15 kV and a working distance of approximately 13 mm.

3.4 PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

Two standard methods (differing in embedding time and the embedding medium) of sample preparation were used for TEM.

3.4.1 Method 1

Leaf segments (< 0.5 mm²) were prepared by prefixing in a solution of 2.5% glutaraldehyde (4 °C) buffered with 0.1 M phosphate buffer (pH 7.2) for 2-24 h followed by a post fixation at room temperature in 0.5% osmium tetroxide (OsO₄) for 1 h. The samples were washed three times for 5 min before and after post fixation in phosphate buffer followed by dehydration in a graded acetone series (i.e twice for 5 min each in 30 %, 50 %, 75 % and 100 % acetone). The leaf segments were allowed to stand in equal parts Spurr's (1969) resin and acetone for 4 h followed by Spurr's resin only for 24 h. Samples were then polymerized in whole resin at 70 °C for 8 h. Ultrathin sections were obtained with a Reichert Ultracut E microtome using glass knives and collected on uncoated 200 square mesh copper grids. The sections were stained with 2 % aqueous uranyl acetate (10 min) followed by Reynold's (1963) lead citrate (10 min). Sections were viewed with a Jeol 1010 TEM.

3.4.2 Method 2

Leaf segments (< 0.5 mm²) were prepared by prefixing in a solution of 3 % glutaraldehyde (4 °C) buffered with 0.05 M cacodylate buffer for 8 h followed by post fixation at room temperature in 2 % OsO₄ for 2 – 4 h. The samples were washed twice for 30 min before and after post fixation in cacodylate buffer (0.05 M), followed by dehydration in a graded alcohol series of 10 min each in 10 %, 30 %, 50 % and 70 % ethanol. The samples were allowed to stand in 70 % ethanol overnight at 4 °C and further dehydrated for 10 min each in 80 % and 90 % ethanol followed by three 10 min changes in 100 % ethanol. The leaf segments were then washed twice in propylene oxide for 30 min and taken through increasing concentrations of Epon resin, diluted with propylene oxide (i.e. 25 % - 2 h, 50 % - 2 h, 75 % - 12 h and 100 % - 24 h). Leaf material was then embedded in 100 % resin at 70 °C for 48 h. Ultrathin sections were obtained with a Reichert Ultracut E microtome using glass knives and collected on uncoated 200- square mesh copper grids. The sections were stained with 2 % aqueous uranyl acetate (10 min) followed by Reynold's (1963) lead citrate (10 min). Sections were viewed with a Jeol 1010 TEM.

3.5 LIGHT MICROSCOPY (LM)

For light microscopy, thin, hand cut sections and sections embedded in wax or Spurr's (1969) resin were used.

3.5.1 Wax embedded material

Excised leaf samples (~ 3 mm²) were initially placed in formaldehyde (70 %) for 48 h. Thereafter, the leaf samples were dehydrated with eight incubations in a graded ethanol series (3 x 70 % - 30 min, 80 % - 2 h, 90 % - 4 h, 100 % - 90 min, 100 % - overnight, 100 % - 2 h). The samples were then placed in a graded series of xylene: ethanol mixtures (25:75, 50:50, 75:25) for an hour each before being placed in two xylene incubations for 15 min each. Paraplast Plus® wax pellets (McCormick Scientific) were added four times at 3 h intervals, followed by a final addition of wax before sectioning.

Wax sections were de-waxed by exposing them to three 2 min baths of xylene (100 %), three 2 min baths of ethanol (100 %), a single 2 min bath in 70 % ethanol and a final 2 min wash in tap water.

3.5.2 Spurr embedded material

Leaf material was prepared in Spurr's (1969) resin (according to the standard protocol 3.4.1) and semi-thin sections were placed on pre-cleaned glass slides. Monitor sections (1 μm) were stained with 1 % Toluidine Blue - O (Feder and O'Brien, 1968)

3.6 FLUORESCENCE MICROSCOPY

3.6.1 Cellulose cell walls

Excised leaf segments were placed in a non-toxic, 0.01 % solution of Cellufluor for 5 h, followed by washing in tap water for 30 min. Thereafter, the leaf segments were hand sectioned transversely and the sections mounted in 70 % glycerine on pre-cleaned slides before viewing with ultraviolet light. Untreated sections, viewed under UV light, were used as controls.

3.6.2 Phenols and lignin

Untreated freehand cross sections of leaves were viewed with UV light for the detection of phenols (Mabry *et al.*, 1970) and lignins. Sections for fluorescence microscopy were viewed and imaged with a Nikon Eclipse 80i Microscope fitted with an Ultra Violet (UV) filter assembly (excitation wavelength 330 – 380 nm).

3.7 HISTOCHEMISTRY

3.7.1 Pectin and mucilage (Johansen, 1940)

Freehand cross-sections of fresh leaves were stained with ruthenium red for 10 min for detection of mucilage in the secretion and pectins in the cell walls.

3.7.2 Phenolic compounds (Johansen, 1940)

For the detection of phenolic compounds, transverse sections of fresh leaves and wax embedded material were stained with 10 % ferric trichloride. A stock solution of ferric trichloride was prepared by diluting 10 g ferric trichloride pellets in 100 ml distilled water, followed by filtration through Whatman No.1 filter paper.

3.7.3 Lipids, cutin and suberin (Pearse, 1968)

For the detection of lipids, hand sections and wax embedded sections of leaf material were stained with aqueous solution of Sudan III and IV for 15 min. The stain was washed off with 70 % ethanol and the sections were mounted in 70 % glycerol.

Sudan Black B was also used to detect lipids. Sections were stained in an alcoholic solution of Sudan Black B for 30 min in a moist environment. The stained sections were washed with 70 % ethanol and mounted in 70 % glycerine. Controls leaves were pre-treated with methanol and chloroform (1:1) (Lison, 1960)

3.7.4 Alkaloids (Furr and Mahlberg, 1981)

Sections of wax embedded leaf material were stained with Dittmar reagent (1 g potassium iodide, 1 g sodium nitrite, 30 ml HCl and 30 ml H₂O) for the detection of alkaloids. Leaf sections that were pre-treated with tartaric acid were used as controls (Johansen, 1940)

3.7.5 Terpenoids (David and Carde, 1964)

Leaf sections, embedded in wax, were treated with Nadi reagent (David and Carde, 1964) for the detection of terpenoids. Stock solutions of solution A (1 % α -naphthol in 95 % ethanol) and solution B (1 % N, N-26 dimethyl-p-phenylenediamine HCl in water) were prepared and a mixture of equal volumes of each were used for staining. Sections stained with Nadi reagent were incubated in the dark for 60 min followed by a wash with sodium phosphate buffer for 2 min. Leaf sections that were pre-treated with 2 N HCl were used as controls (Mace *et al.*, 1974)

All sections for histochemistry were examined with a Nikon Eclipse 80i Microscope using bright field illumination. Images were viewed and saved with NIS Elements – D (version 3.0) software.

3.8 PRELIMINARY PHYTOCHEMICAL TESTS

3.8.1 Preparation of crude leaf extract (European Pharmacopiea, 1975)

Fresh leaves were dried at room temperature for one week and then ground down to a powder. Extracts were prepared by continuous extraction method a soxhlet apparatus and methanol as the organic solvent. The extracted solution was filtered, concentrated with a rotary evaporator, and stored at 4 °C for further use.

3.8.2 Phytochemical screening of leaf extracts

Preliminary phytochemical tests were carried out for the following phytochemicals:

a) Monosaccharides

Fehling's solution

For the detection of monosaccharides, extracts (3 ml) were mixed with an equal volume of Fehling's solution. Copper sulphate solution (34.66 g copper sulphate dissolved in 500 ml distilled water) and alkaline tartrate solution (173 g potassium sodium tartrate and 50 g NaOH dissolved in 500 ml water) were prepared. Both solutions were mixed with equal volumes before use. A brown colour reaction was indicative of monosaccharides.

Benedict's solution

Extracts were also mixed with Benedict's solution for the detection of monosaccharides. Benedict's solution is made up of two solutions: solution 1 (173 g sodium citrate and 100 g sodium carbonate in 800 ml distilled water while heating) and solution 2 (17.3 g copper sulphate in 100 ml distilled water). Solution 2 is added to solution 1 and made up to 1 L. The development of a reddish-brown precipitate is indicative of monosaccharides.

b) Oligosaccharides and polysaccharides

Plant methanolic extracts (3 ml) were added to water and filtered. The filtrate was then subjected to Molisch's reagent (15 g α -naphthol dissolved in 100 ml absolute ethanol). The formation of reddish-brown rings indicated the presence of di- and polysaccharides.

c) Flavonoids

Methanolic extracts (5 ml) were mixed with a solution of lead acetate (2 ml). Flocculent white precipitate indicated the presence of flavonoids.

d) Tannins

Breamer's test was performed for the detection of tannins. Plant methanolic extract (3 ml) was mixed with a 10 % alcoholic ferric trichloride solution. A dark blue or greenish grey colouration of the solution indicated the presence of tannins.

e) Alkaloids

Draggendorf's reagent

A drop of extract was spotted on a silica gel plate, followed by spraying with Draggendorf's reagent. An orange colouration of the spot indicated the presence of alkaloids.

Stock solutions were prepared as follows: Solution A – bismuth nitrate (0.17 g) dissolved in glacial acetic acid (2 ml) and distilled water (8 ml), and Solution B – potassium iodide (4 g) dissolved in glacial acetic acid (10 ml) and distilled water (20 ml). Both solutions were mixed in equal volumes and diluted to 100 ml.

Hager's reagent

Methanolic extracts were treated with Hager's reagent for the detection of alkaloids. Stock solution of Hager's reagent was prepared by dissolving 1 g of picric acid in 100 ml distilled water. The development of a yellow precipitate indicated the presence of alkaloids.

Wagner's reagent

Methanolic extracts were treated with Wagner's reagent (2 g iodine and 6 g potassium iodide dissolved in 100 ml distilled water). A reddish-brown precipitate was a positive result for alkaloids.

f) Saponins

The foam test was carried out for the detection of saponins. Methanolic extract (2 ml) was mixed with 20 ml distilled water and shaken. A 1 cm layer of foam, occurring at the top of the mixture, indicated the presence of saponins.

g) Amino acids

Methanolic extract (1 ml) was mixed with distilled water and ninhydrin solution (1 ml) was added. A blue colour change indicated the presence of amino acids.

h) Fats and fixed oils

A drop of extract was placed between two pieces of filter paper. Occurrence of oil stains indicated the presence of oils.

3.9 IMAGE ANALYSES – TRICHOME COUNTS AND MEASUREMENTS

Images obtained from SEM were analysed with iTEM software (Soft Imaging System GmbH, Münster, Germany). Trichomes were counted in quadrants for each treatment (differentiated according to region on the adaxial and abaxial surfaces of leaves and the stage of development) with a total number of 20 images per treatment. The length of the stalk of 100 capitate trichomes and the diameter of the secretory head were measured randomly from developing and mature peltate trichomes.

3.10 STATISTICAL ANALYSES

All statistical analyses were performed with the SPSS statistical software package for windows (Rel. 15.0.0. 2006. Chicago: SPSS Inc.). The frequency (count mm^{-2}) of trichomes was compared between treatments with a one-way analyses of variance (ANOVA) using the Tukeys Multiple Comparisons Test to compare differences among treatments. The diameter of peltate trichomes between developing and mature trichomes was compared with an independent sample t-test. A value of $p < 0.05$ was considered statistically significant.

4.1 TRICHOMES

4.1.1 Trichome distribution

The leaves of *C. triloba* possess two morphologically distinct trichomes, capitate (Fig. 4 a) and peltate (Fig. 5), both of which appear to be glandular. Trichomes occur on the adaxial and abaxial surfaces of leaves, with capitate dominant on the adaxial surface and peltate predominant on the abaxial surface. Distribution of capitate trichomes is uniform on both surfaces of the leaf. However, peltate trichomes were mostly observed along the grooves on the adaxial surfaces and on the veins on the abaxial surface (Fig. 7 a-f).

Emergent leaves were densely covered in trichomes and trichome density decreased as the leaf matured. The frequency (count mm⁻²) of trichomes on the emergent leaves was significantly higher on both side of the leaf surface as compared to the frequency on the young and mature leaves (Fig. 8 and 9). On the abaxial surface, the frequency of trichomes was significantly higher at the tip (82.17 ± 3.61 mm⁻², Mean \pm SE) but this decreased as the leaf matured (22.07 ± 2.79 mm⁻²). Trichome frequency was similar on the mid and base regions of the adaxial and abaxial surfaces. Capitate and peltate trichomes also occur on the abaxial surface of flower petals (Fig. 10), while the adaxial surface was bare.

4.1.2 Capitate trichomes

Capitate trichomes on leaves were approximately 248.10 ± 4.53 μ m in length, uniseriate and normally comprised of a single basal cell, 3-5 stalk cells, a neck cell and a secretory head containing vertical walls, arranging the head in a quadrant of four small secretory cells (Fig. 4 a, b and c). The stalk is made up of long cells at the base which gradually become shorter towards the secretory head. Trichomes with up to 8 stalk cells were observed along the leaf margin but these were not common. Wax sections stained with Toluidine Blue showed that the basal cells of capitate trichomes appeared to be highly vacuolated while the cells closer to the secretory head were denser with cytoplasm and probably organelles (Fig. 4 b). The trichome is anchored to the leaf epidermis by a pedestal made up of a ring of approximately 8-12 cells with very thick cell

walls (Fig. 4 d). Capitate trichome outer walls appear to lack cuticular warts and other forms of microornamentation.

There were two morphologically distinct types of capitate trichomes found on petals and therefore, for the purpose of petals alone these trichomes were classified as capitate Type I and Type II. The morphology of the capitate Type I trichome was similar to the capitate trichomes found on leaves, whereas the capitate Type II trichomes had stalks that were longer in length and comprised of approximately 14 cells (Fig. 10 a and b). The secretory heads of both types of capitate trichomes were arranged in quadrants, similar to those found on leaves.

Capitate trichomes appear to develop rapidly on leaf primordia since fully developed forms were only observed on emergent through to mature leaves whereas developing capitate trichomes were rare (Fig. 7 a-f). Development appears to occur from a single epidermal cell which probably undergoes many periclinal divisions to form the stalk and two anticlinal divisions to form the four-celled secretory head. This development was not observed directly. However, observations of developing trichomes on young leaves lead us to this assumption (Fig. 4 a).

4.1.3 Peltate trichomes

Fully developed peltate trichomes comprise a basal cell, a unicellular stalk and a multicellular secretory head (Fig. 5). The peltate trichome head consisted typically of four cells although some were seven or even eight-celled (Fig. 6 e and f). Developing trichomes had a secretory head made up of 2 to 4-cells (diameter = 31.21 μm), and these were significantly smaller than the fully developed 4-celled peltate trichome (diameter = 74.32, $t = 8.263$, $p < 0001$). Development appears to occur from a single epidermal cell which undergoes 3 periclinal divisions to form a basal cell, a stalk cell and a unicellular secretory head. The secretory head then divides periclinally to form a four celled head which expands in size resulting in a developed four celled peltate trichome. This four celled head may undergo further periclinal division to form a head of up to eight cells (Fig. 6). The peltate trichomes found on petals were morphologically similar to those found on leaves.

PLATE 1

Figure 4. Capitate trichomes observed on leaves of *C. triloba*.

- (a) SEM of a developed capitate trichome (C) and developing capitate (DC) trichome on a young leaf. Stalk cells (arrows) are differentiated early in leaf development and then expand in size.

- (b) Toluidine Blue O stained section of capitate trichome. A single basal cell (Bc), four stalk cells (St), a neck cell (Nc) and a multicellular secretory head (Sc) can be seen. Cells closer to the secretory head appear to contain more cytoplasm and probably more organelles than the cells closer to the epidermis. A cellular pedastal is also visible (Cp)

- (c) SEM showing the secretory head (S) of capitate trichome with four secretory cells.

- (d) SEM of the basal region of capitate trichomes showing a single basal cell (Bc) attached to an epidermal ring of cells called the cellular pedastal (Cp). A stalk cell (St) and stomata (arrows) can also be seen.

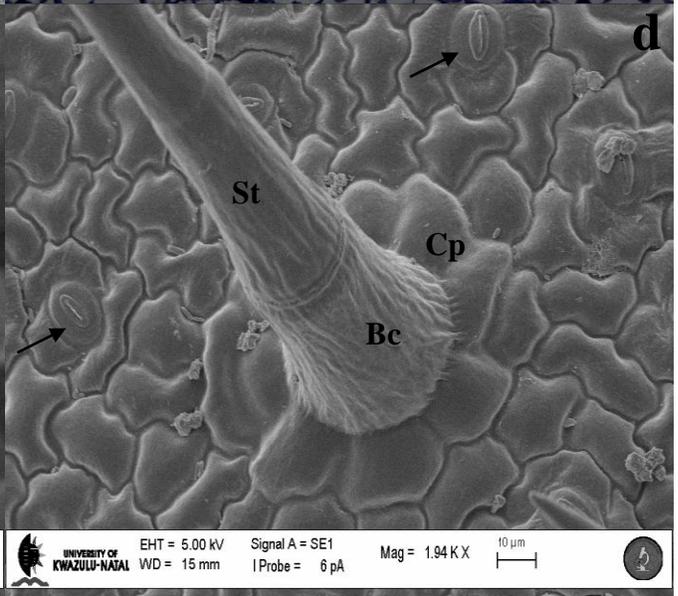
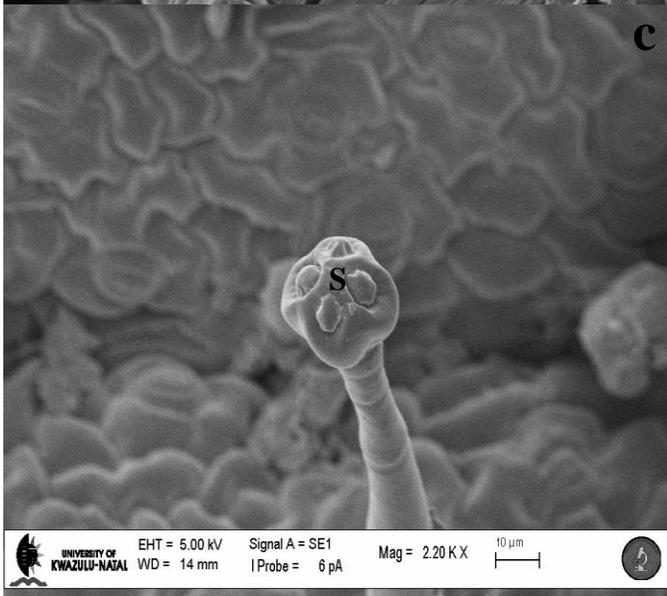
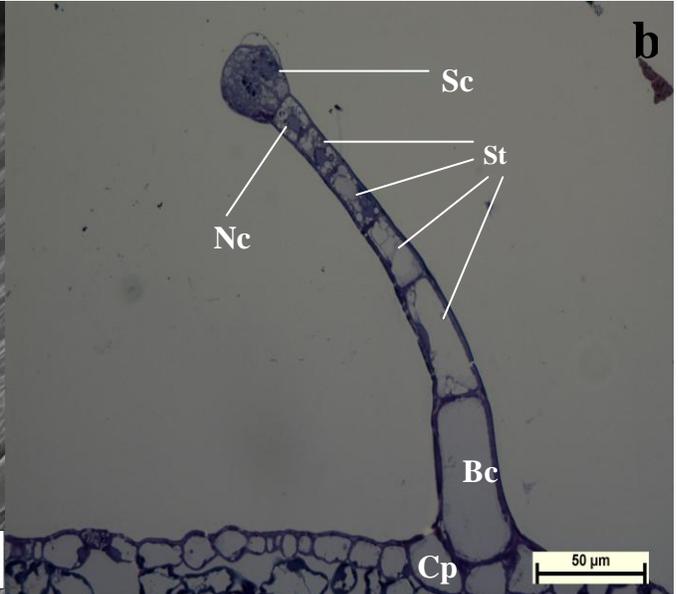
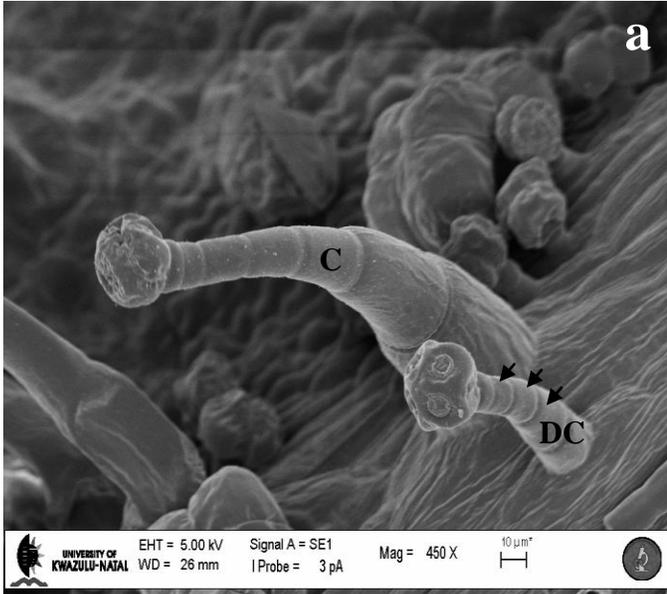


PLATE 2

Figure 5. Peltate trichome on leaves of *C. triloba*.

(a) ESEM of developed four-celled peltate trichome (P) and developing 2-celled peltate trichome (DP) with a single stalk cell (St).

(b) Toluidine Blue O stained section of two peltate trichomes showing a single basal cell (Bc), a unicellular stalk (St) and a multicellular secretory head (Sh).

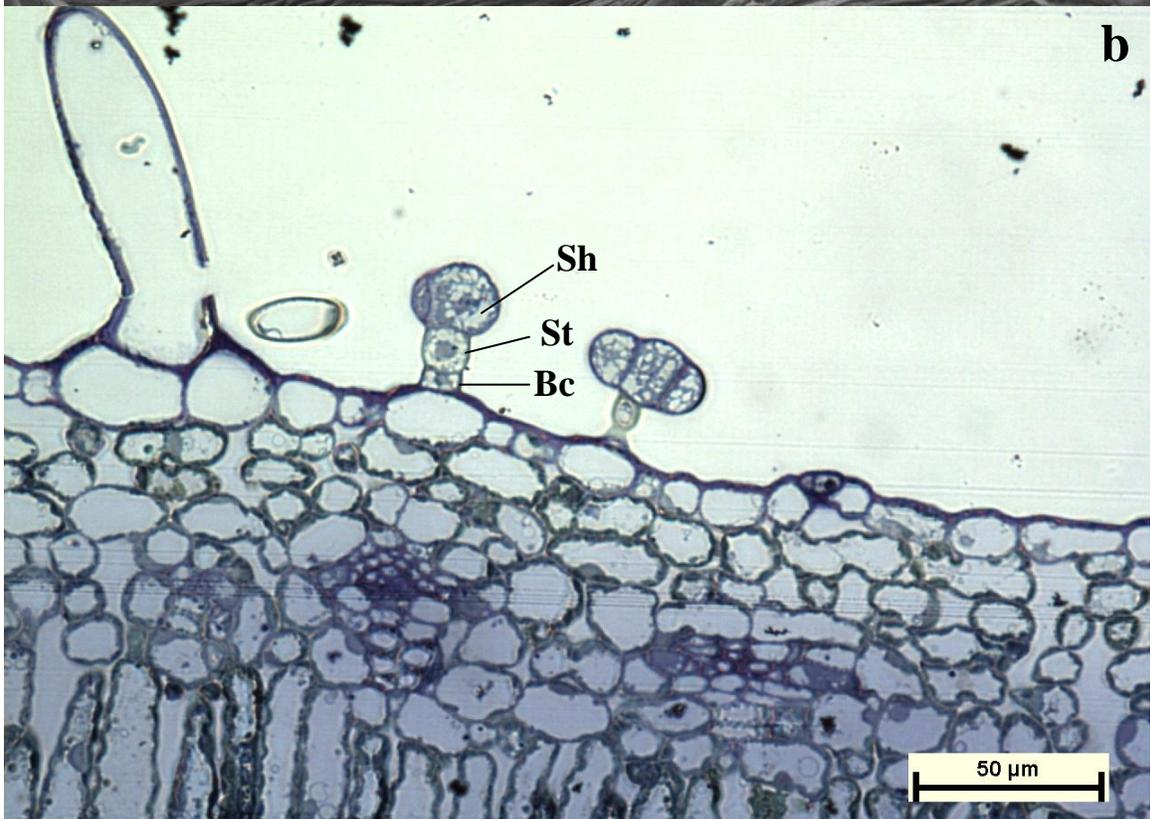
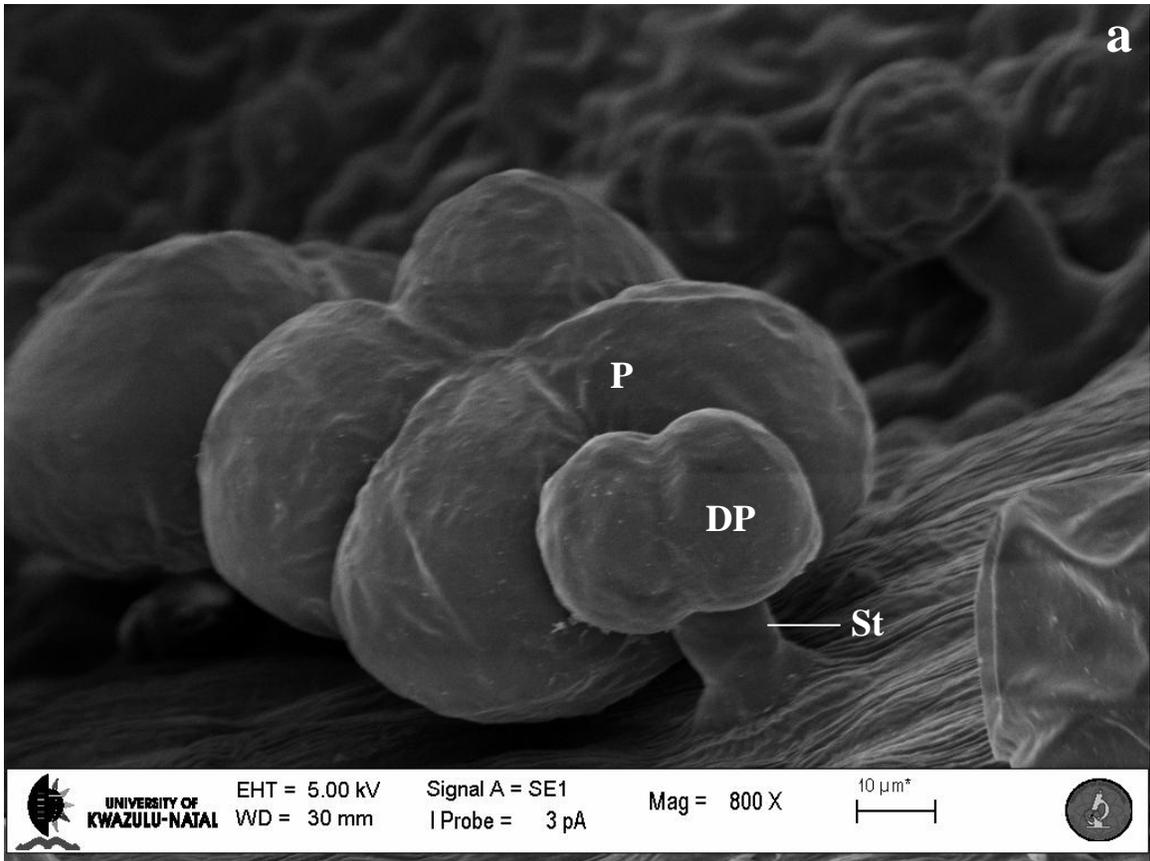


PLATE 3

Figure 6. Development of peltate trichomes on leaves of *C. triloba*.

(a) Developing peltate trichome (DP) with bicellular head. Trichome basal cell (Bc) and stomata (arrows) can also be seen.

(b) Developing peltate trichome with 3-celled secretory head.

(c) Developed 4-celled peltate trichome (P) and a peltate trichome in the post secretory phase (PPS) located on leaf vein (Vn).

(d) A peltate trichome with a secretory head made up of six cells.

(e) A 7-celled peltate trichome.

(f) Abaxial surface of leaf showing an 8-celled peltate trichome (arrow) on leaf vein (Vn).

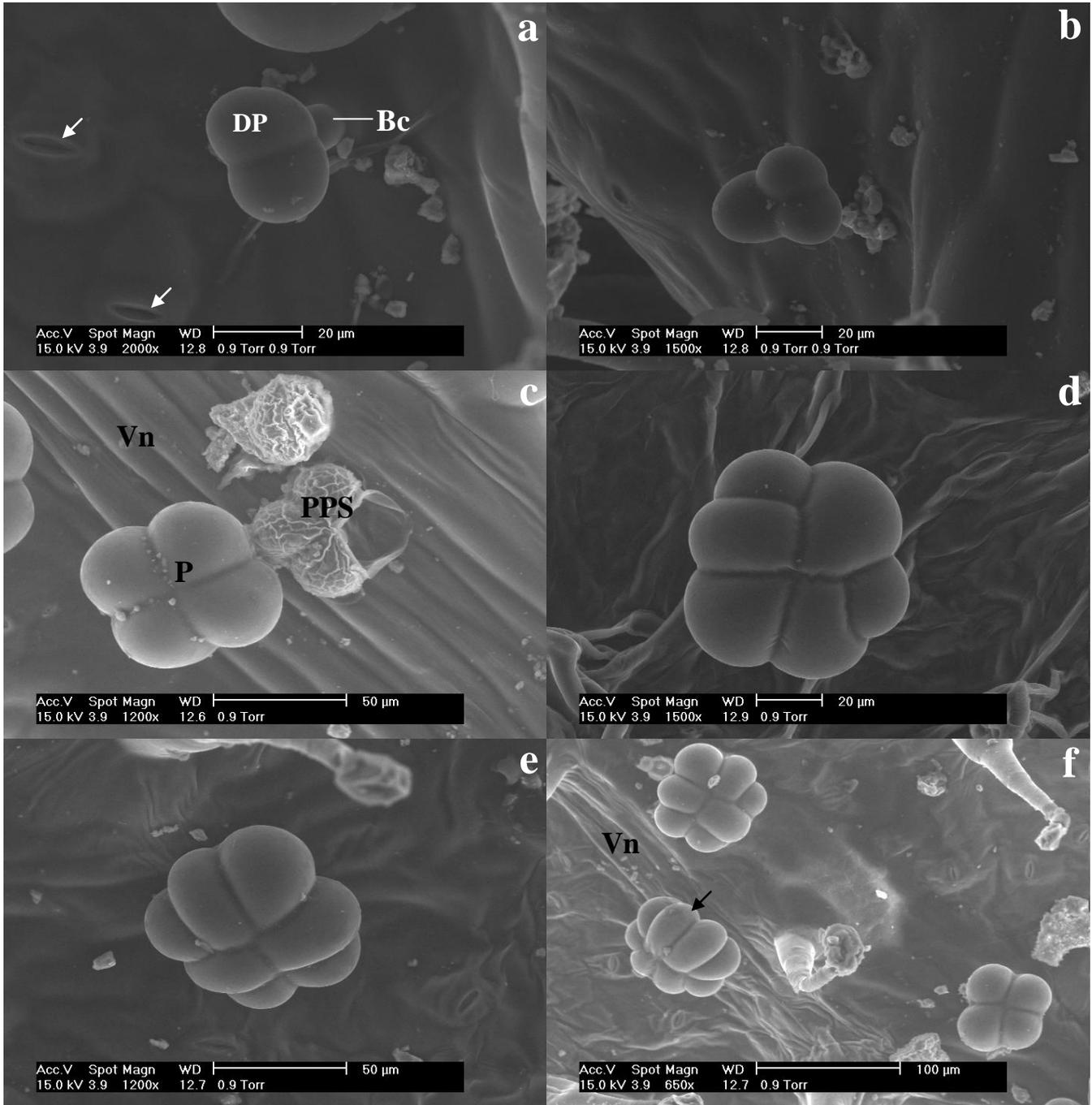


PLATE 4

Figure 7. SEM showing a decrease in density of trichomes as leaves mature.

(a) Emergent leaf – adaxial surface.

(b) Emergent leaf – abaxial surface.

(c) Young leaf – adaxial surface.

(d) Young leaf – abaxial surface.

(e) Mature leaf – adaxial surface.

(f) Mature leaf – abaxial surface.

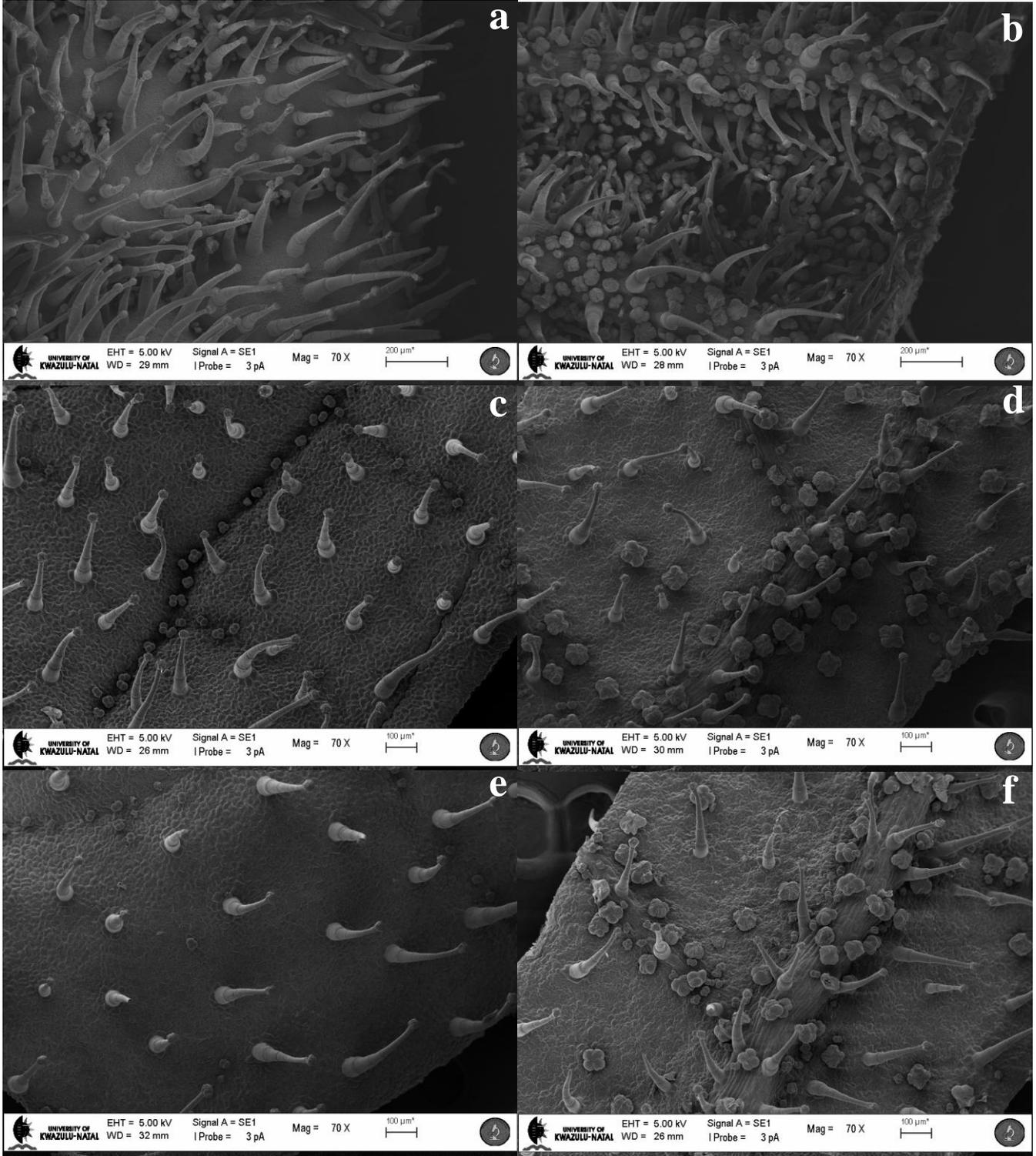


Figure 8. Trichome frequency on the adaxial surface of leaves at three stages of development, emergent, young and mature. The entire leaf area was sampled. Values are mean \pm SE. Bars with different letters are significantly different at $p < 0.05$ using Tukey's multiple range test.

Figure 9. Trichome frequency on the abaxial surface of leaves at three stages of leaf development, emergent, young and mature. The entire leaf area was sampled. Values are mean \pm SE. Bars with different letters are significantly different at $p < 0.05$ using Tukey's multiple range test.

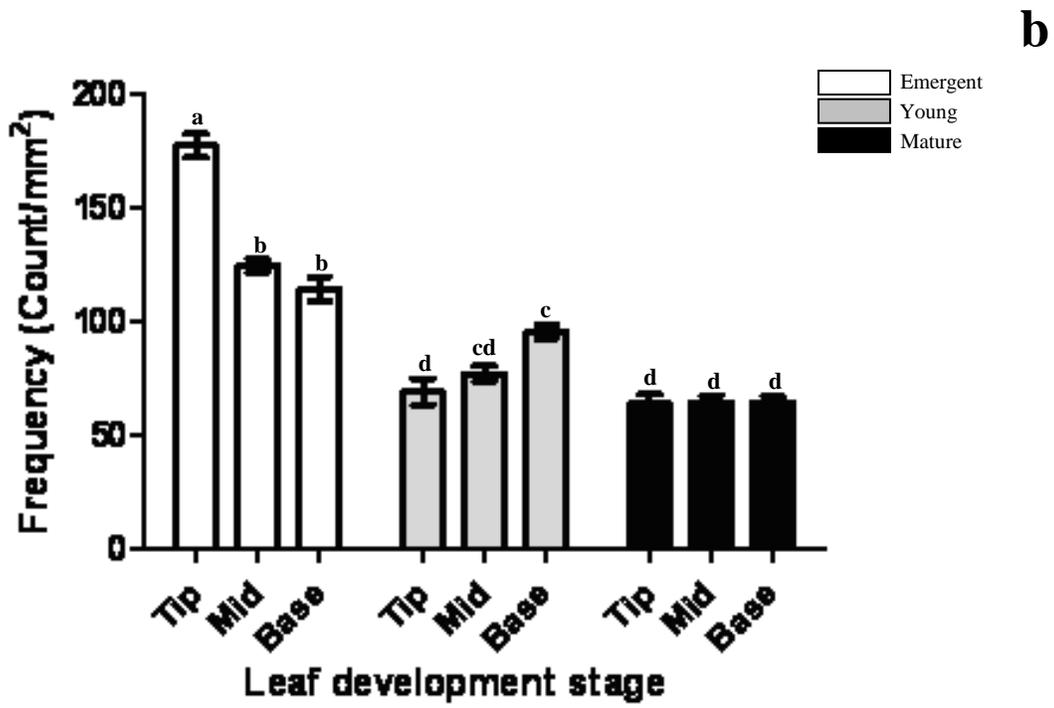
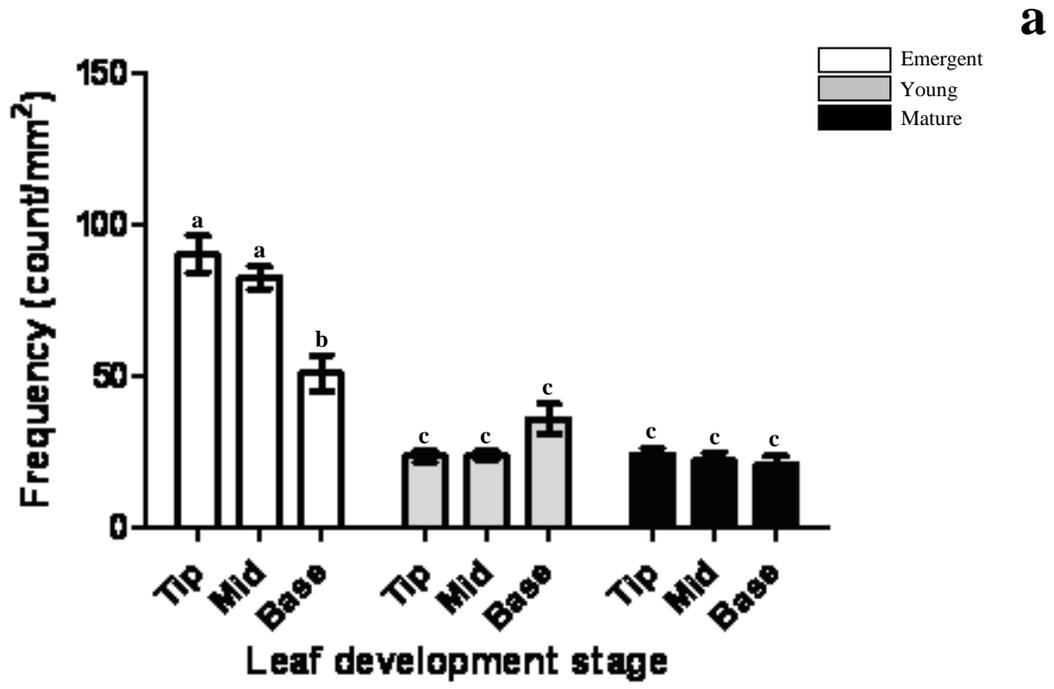
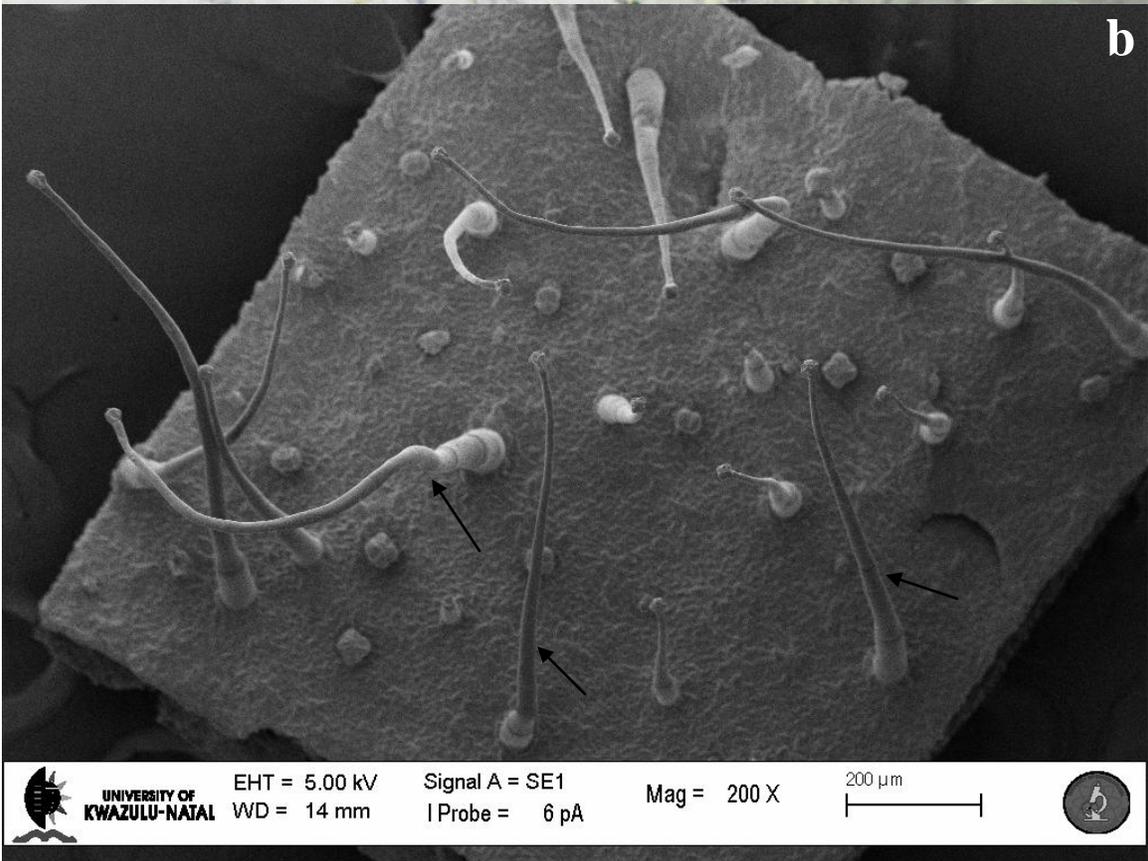


PLATE 5

Figure 10. Surface imaging of trichomes on flower petals of *C. triloba*.

(a) Stereo micrograph of abaxial surface of petal showing capitate Type II trichomes (arrows). These trichomes are longer in length as compared to the Type I capitate trichomes. A variation in morphology could indicate a difference in function.

(b) SEM of petal showing capitate Type II trichomes (arrows).



4.2 HISTOCHEMISTRY AND FLUORESCENCE MICROSCOPY

4.2.1 Cell walls

Histochemical analyses of capitate and peltate trichome cell walls revealed the presence of lignin (Fig. 14), pectin (Fig. 13) and cellulose (results not presented). Sections stained with Sudan III/IV and Sudan Black B tested positive for suberin or cutin in the single stalk cell of peltate trichomes and the neck cell of capitate trichomes. A continuous layer of cuticle along the leaf surface was observed with the Sudan dyes (Fig. 11 and 12).

4.2.2 Identification of secretory product

Histochemical analyses of the secretory cells of trichomes with the Sudan dyes, Ruthenium Red and ferric trichloride indicated the presence of lipids (Fig. 11 and 12), mucilage (Fig. 13) and phenolic compounds (Fig. 14 and 15) respectively (Table 1). Both capitate and peltate trichomes appeared to secrete copious amounts of mucilage which are released when the secretory cells burst or the cell wall dissolves (Fig. 13).

Sections stained with the Nadi reagent and Ditmarr reagent produced negative results, indicating the absence of terpenoids and alkaloids respectively (results not presented).

PLATE 6

Figure 11. Light micrographs of transverse sections of leaves of *C. triloba* stained with Sudan Black B.

(a) Capitulate trichome with lipid drop (L) in the secretory cell and a continuous layer of cuticle along the epidermis (long arrow).

(b) Peltate trichome stained with Sudan Black showing lipid drop (L) in secretory cell. Note continuous layer of cuticle along the epidermis (long arrows).

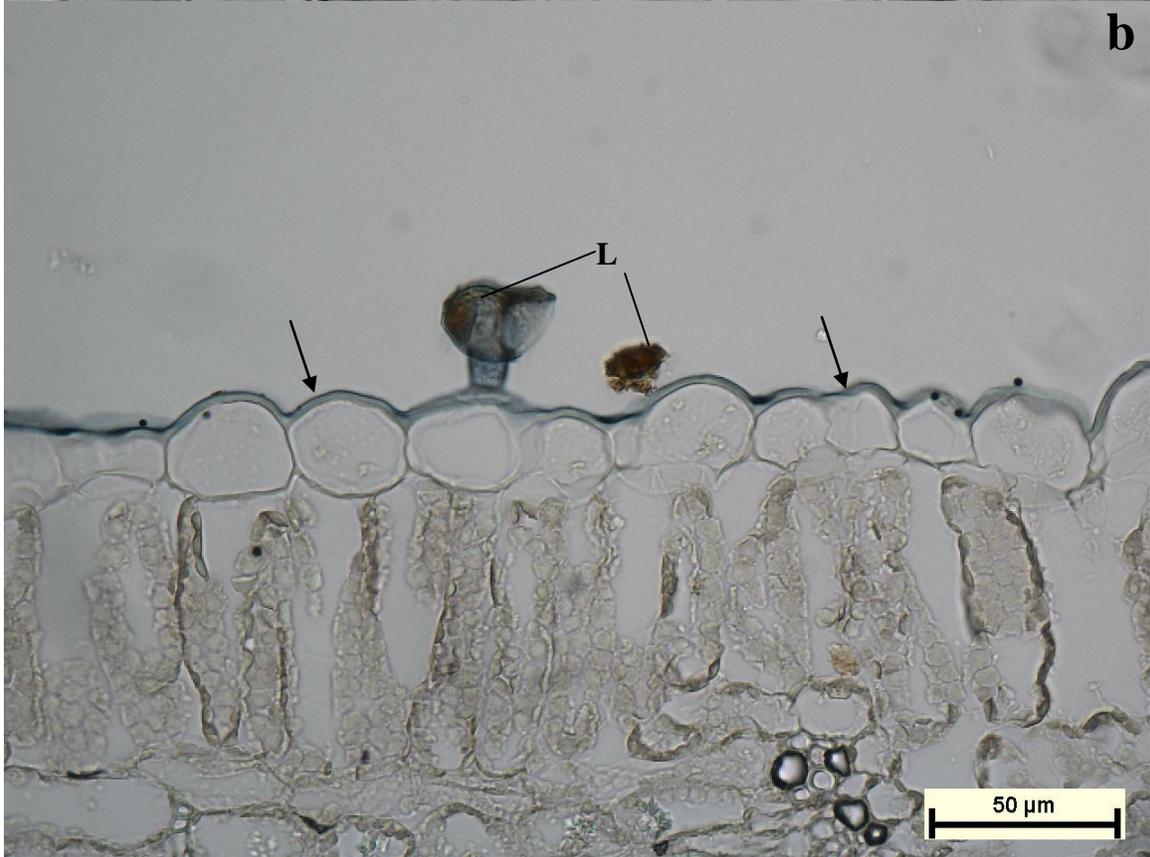


PLATE 7

Figure 12. Light micrographs of transverse sections of leaves of *C. triloba* stained with Sudan III and IV.

(a) Capitulate trichome with positively stained lipid (L) scattered in the secretory cell and a continuous layer of cuticle along the epidermis (arrow).

(b) Peltate trichome stained with Sudan III/IV showing lipid drop (L) in secretory cell. Note a continuous layer of cuticle along the epidermis (arrows).



PLATE 8

Figures 13. Light micrographs of transverse sections of leaves of *C. triloba* stained with Ruthenium Red.

(a) Free-hand cross section of capitate trichome showing secreted mucilage (Mc), stained pink, on secretory cells.

(b) Light micrograph of wax embedded transverse section stained with Ruthenium Red. Mucilage (Mc) stained pink can be seen around peltate trichomes that are in the post secretory phase (PPS). Pink stain in the cell walls was also observed which indicates the presence of pectins (arrows)



PLATE 9

Figures 14. Autofluorescence of transverse sections of leaves of *C. triloba*.

(a) Free-hand cross section of leaf showing capitate trichomes exhibiting a pale blue autofluorescence in the secretory cells, indicating the presence of phenolic compounds (Pn). Lignin can also be seen in cell walls indicated by a white fluorescence (arrows) and chloroplasts (Ch), which fluoresces red.

(b) Peltate trichome secretory cells. A pale autofluorescence is observed, which is indicative of phenolic compounds (Pn). Chloroplast (Ch) fluoresces red.

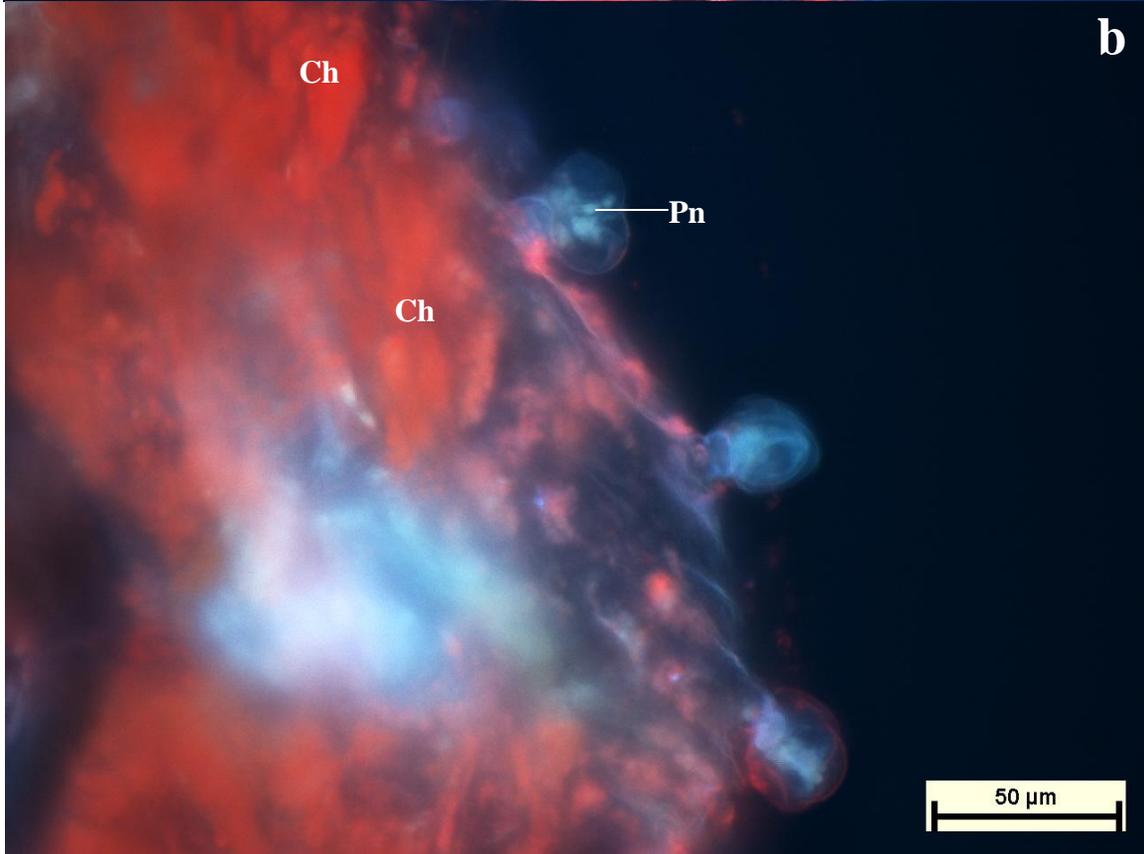
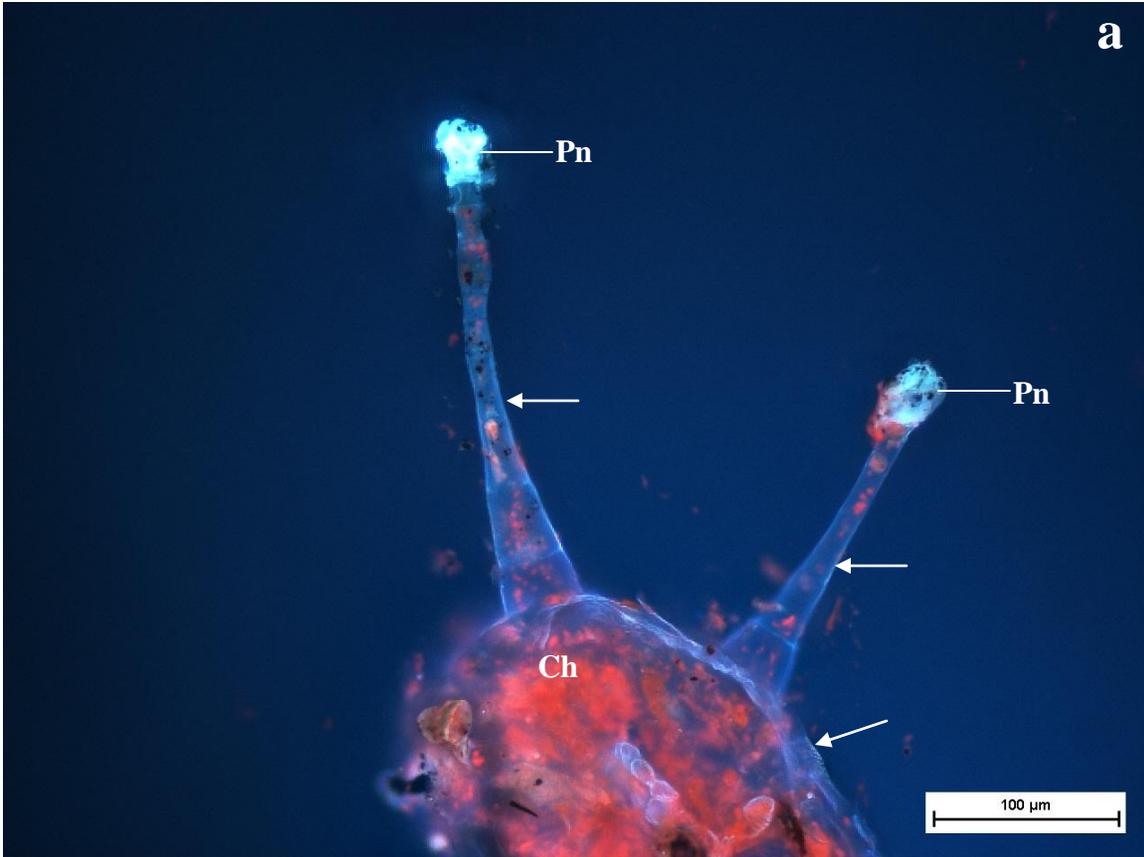


PLATE 10

Figures 15. Light micrographs of transverse sections of leaves of *C. triloba* stained with ferric trichloride.

(a) Free-hand section showing capitate trichome containing phenolic compounds (Pn) indicated by a green stain. A second capitate trichome can also be seen in the post secretory phase (CPS).

(b) Peltate trichomes containing phenolic compounds (arrows) indicated by a green stain.

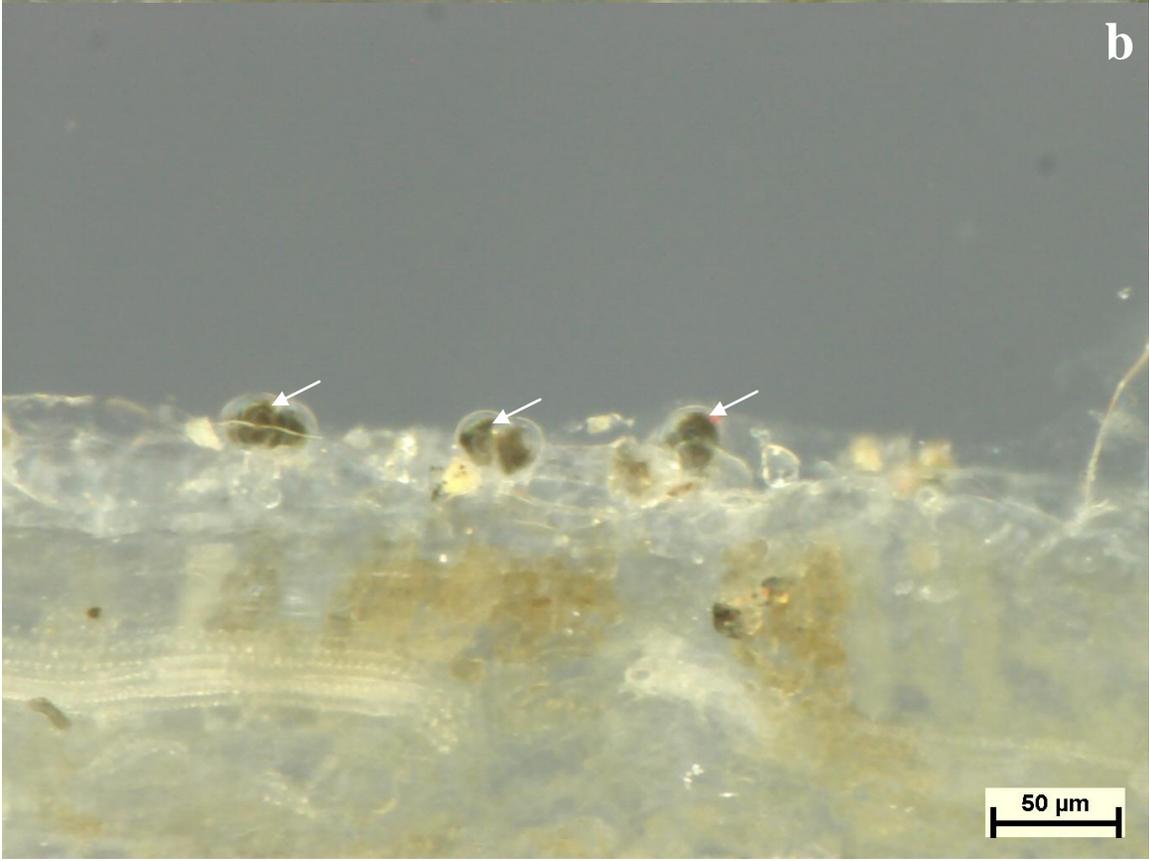
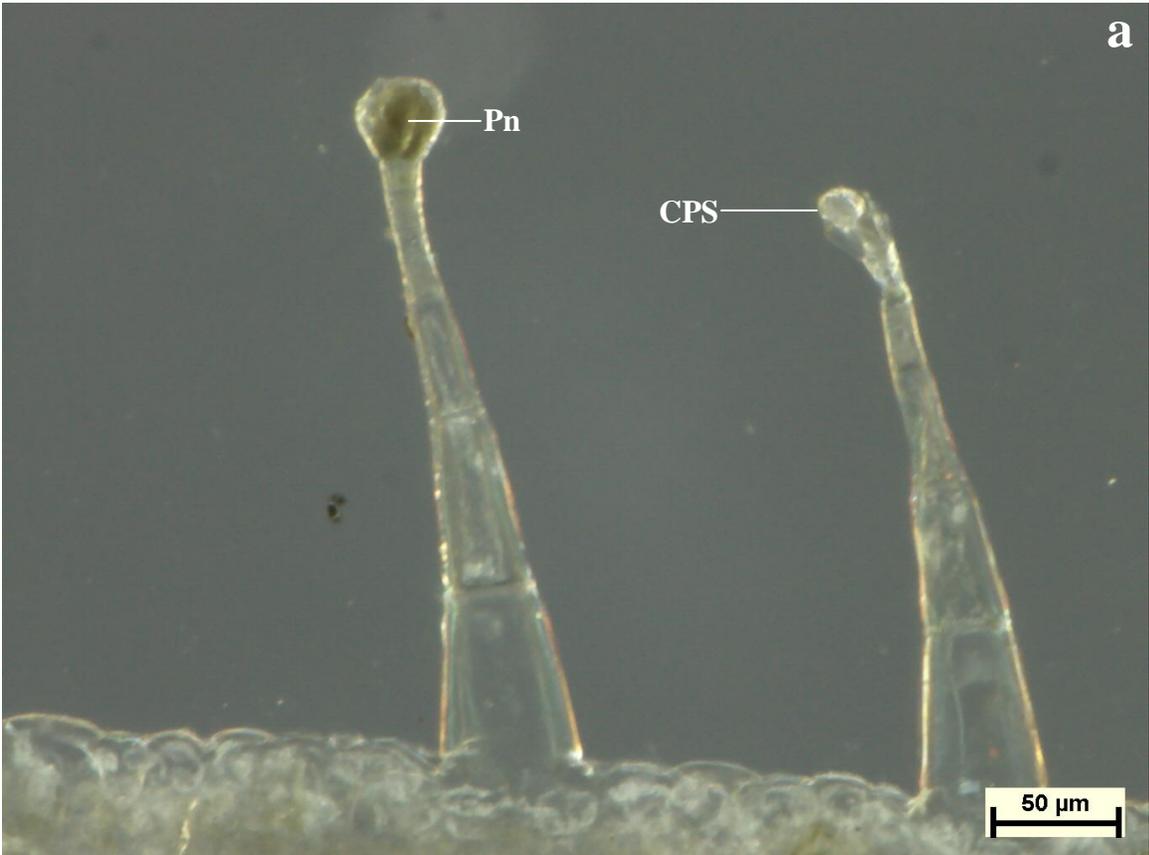


Table 1. Results of histochemical and fluorescence tests for the identification of compounds in the secretory product of *C. triloba*.

Compound group	Test	Peltate	Capitate	Reaction Observed
Lipids	Sudan III/IV	+	+	Red colouration in head cells
	Sudan Black B	+	+	Blue colouration in head cells of trichomes
Mucilage	Ruthenium Red	++	+	Pink stained mucilage in and around peltate trichomes and on capitate trichome heads.
Phenols	Ferric trichloride	+	++	Deep green colouration
	Autofluorescence	+	++	Pale blue fluorescence
Alkaloids	Dittmar's reagent	-	-	Pink colouration
Terpenoids	Nadi reagent	-	-	Black colouration

+/- indicates presence/absence of compound

++ indicates intense reaction

4.3 ULTRASTRUCTURE

Samples for TEM were prepared using two methods that differed with the type of fixative used and the duration that the samples were fixed for and dehydrated. Observations of capitate trichomes using TEM indicated that the secretory cells of these trichomes contained large amounts of electron dense lipophilic material that hindered the ability to observe cellular detail (Fig. 16 a). Despite the fact that cellular detail was difficult to observe, mitochondria and endoplasmic reticulum cisternae were observed in secretory head cells.

The cytoplasm of stalk cells of capitate trichomes differed from head cells, being characterised by less lipophilic material and more vacuoles (Fig. 16 a). Oval shaped plastids were observed bordering the nucleus and containing smaller, light and dark stained lipophilic material (Fig. 16 a and b). Numerous mitochondria and endoplasmic reticulum were also observed in stalk cells in close proximity to the nucleus (Fig. 16 b). The outer cell walls of the secretory head cells were thicker as compared with the cell wall of the stalk cells (Fig. 16 a). However, the cell wall of the head cells appeared to be arranged with loose fibrils in contrast to the more sturdy cell walls of the stalk. There appeared to be cell wall intrusion between the head and neck cells (Fig. 16 a).

The secretory head cells of the peltate trichomes contained centralized nuclei and numerous large vacuoles in the cytoplasm (Fig. 17 a). Oval shaped, light and dark stained electron dense plastids, endoplasmic reticulum cisternae and mitochondria were observed in close proximity to the nucleus (Fig. 17 b). Endoplasmic reticulum cisternae, golgi bodies and mitochondria were also observed scattered throughout the cytoplasm, especially around vacuoles that were closer to the cell wall (Fig. 18 b). These vacuoles were closely associated with multimembranous structures found in the cytoplasm, which appeared to push against the vacuoles (Fig. 18 a and b). Small vesicles developed in the cytoplasm with the simultaneous degradation of the multimembranous structures (Fig. 19 a). The vesicles appeared to fuse with the vacuoles (Fig. 19 b). Vesicle formation and translocation into vacuoles could indicate the production and storage of secretory product respectively. Loose fibrils in the cell walls in peltate trichomes were not observed, as in the case of the outer cell walls of capitate trichomes.

The cytoplasm of stalk cells of peltate trichomes appeared to be similar to the secretory cells (Fig. 17 a). These cells contained a centralized nucleus surrounded by electron dense plastids and mitochondria, numerous cytoplasmic vacuoles and golgi bodies along the periphery of the cells. The walls of the stalk cells appeared to be thicker than the cell walls of secretory cells.

4.4 PHYTOCHEMICAL TESTS

4.4.1 Phytochemical screening of plant extract

Plant extracts tested positive for oligosaccharides, polysaccharides, flavonoids, saponins, tannins and fixed oils. Monosaccharides, amino acids and alkaloids were absent in extracts (Table 2).

PLATE 11

Figure 16. Transmission electron micrographs of a capitate trichome.

(a) Section through the head (Sh) and stalk cells (St). Copious amounts of electron dense secretory material (Sec) and dense cytoplasm can be seen in the secretory cells of the head. A thin cell wall (Cw) separates the two head cells, and stalk and head cells. The outer cell wall of the secretory cells appears to be arranged with loose fibrils. Between the head and stalk cells, an intrusion of the cell wall was observed (arrows). Stalk cells contain a central nucleus (N), bordered by electron dense plastids (Pd). Numerous vacuoles (V) were observed throughout the cytoplasm.

(b) Section through the stalk cell showing nucleus (N) with nuclear membrane (Nm). Electron dense plastids (Pd), numerous mitochondria (M), small vacuoles (V) and endoplasmic reticulum (ER) were also observed surrounding the nucleus .

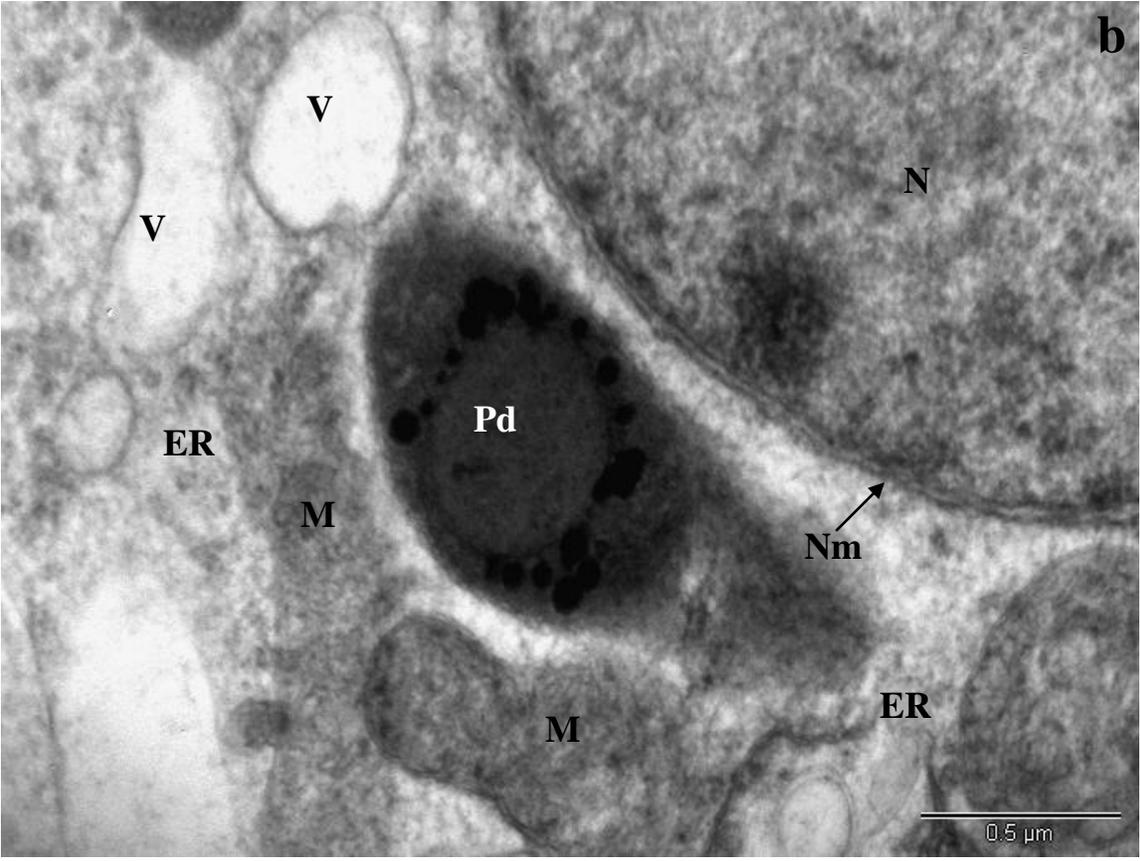
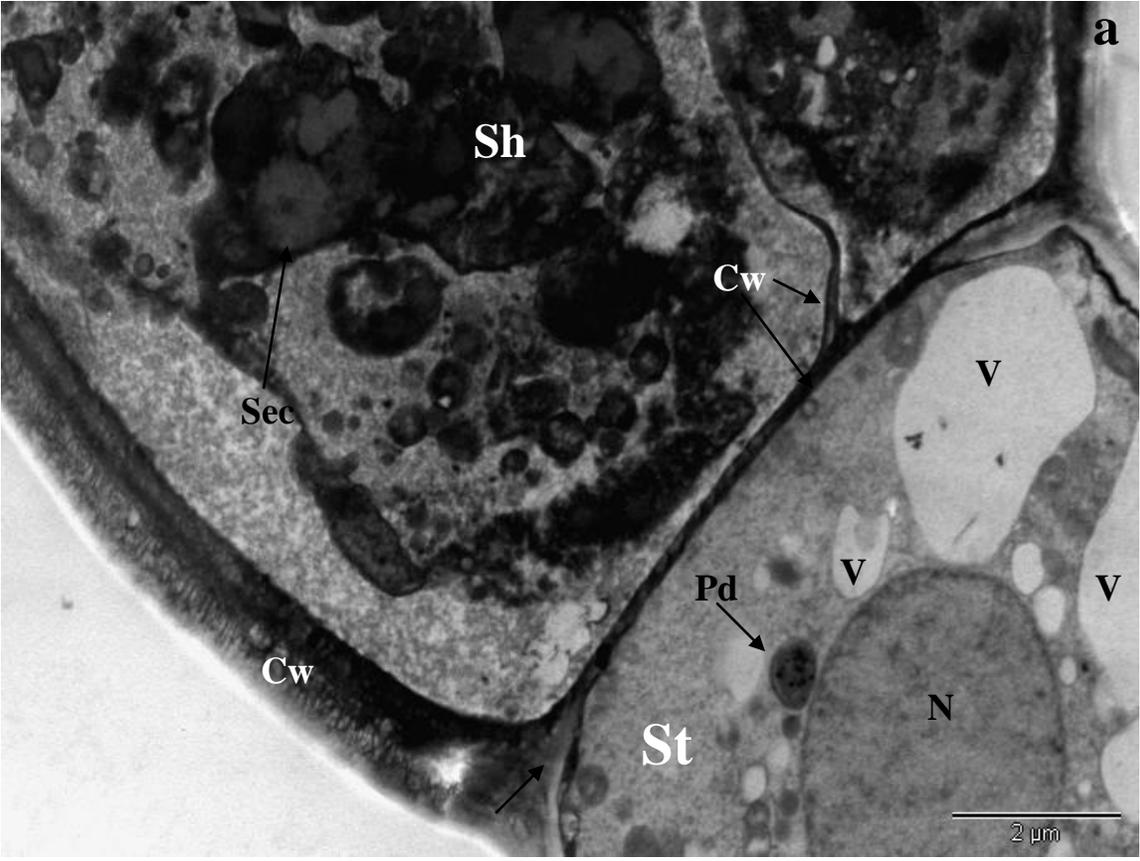


PLATE 12

Figure 17. Transmission electron micrographs of head cells of peltate trichomes.

- (a) Section showing two head cells (Sh) and the stalk cell (St). Central nucleus (N) with nucleolus (Nu) can be seen in the head cell, with numerous peripheral vacuoles (V) in the cytoplasm. Light and dark stained electron dense plastids (Pd) surround the nucleus.

- (b) Section through the secretory head cell showing nucleus (N) with nucleolus (Nu) and nuclear membrane (Nm). Light and dark stained plastids (Pd), mitochondria (M), endoplasmic reticulum (ER) cisternae and vacuoles (V) can also be seen surrounding the nucleus.

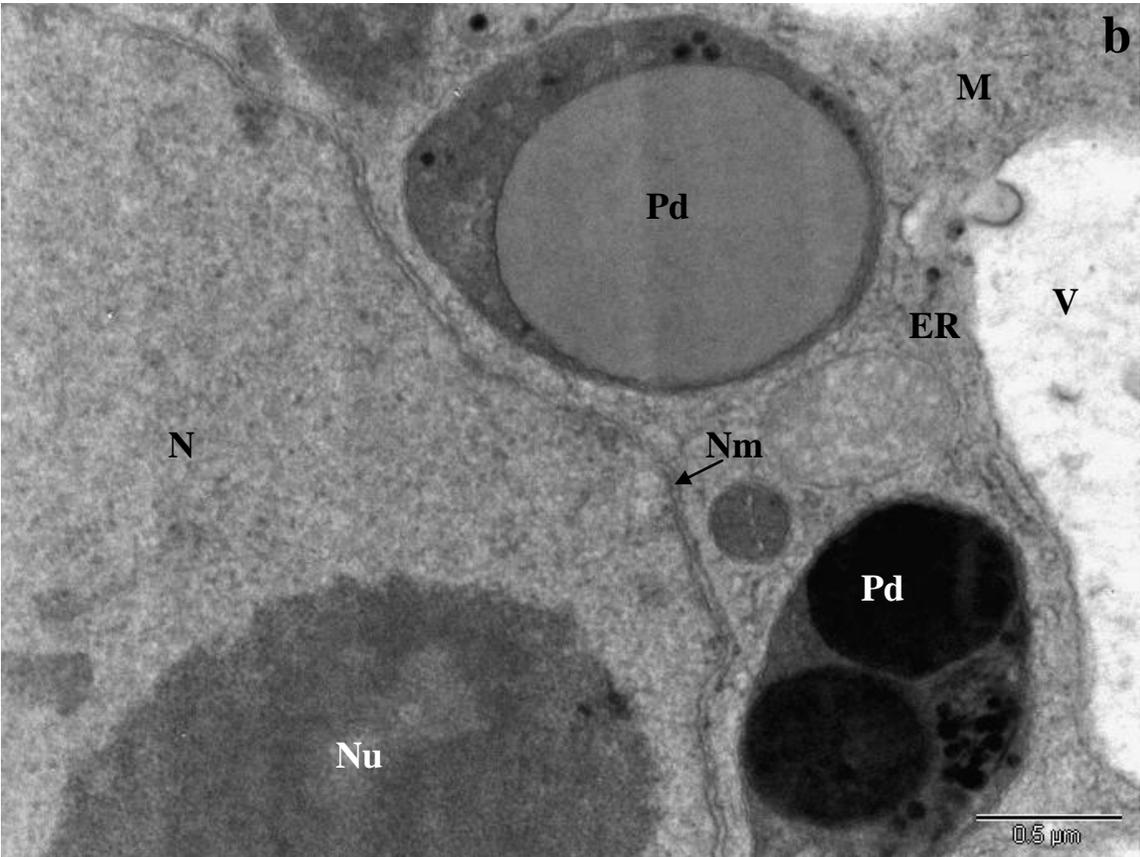
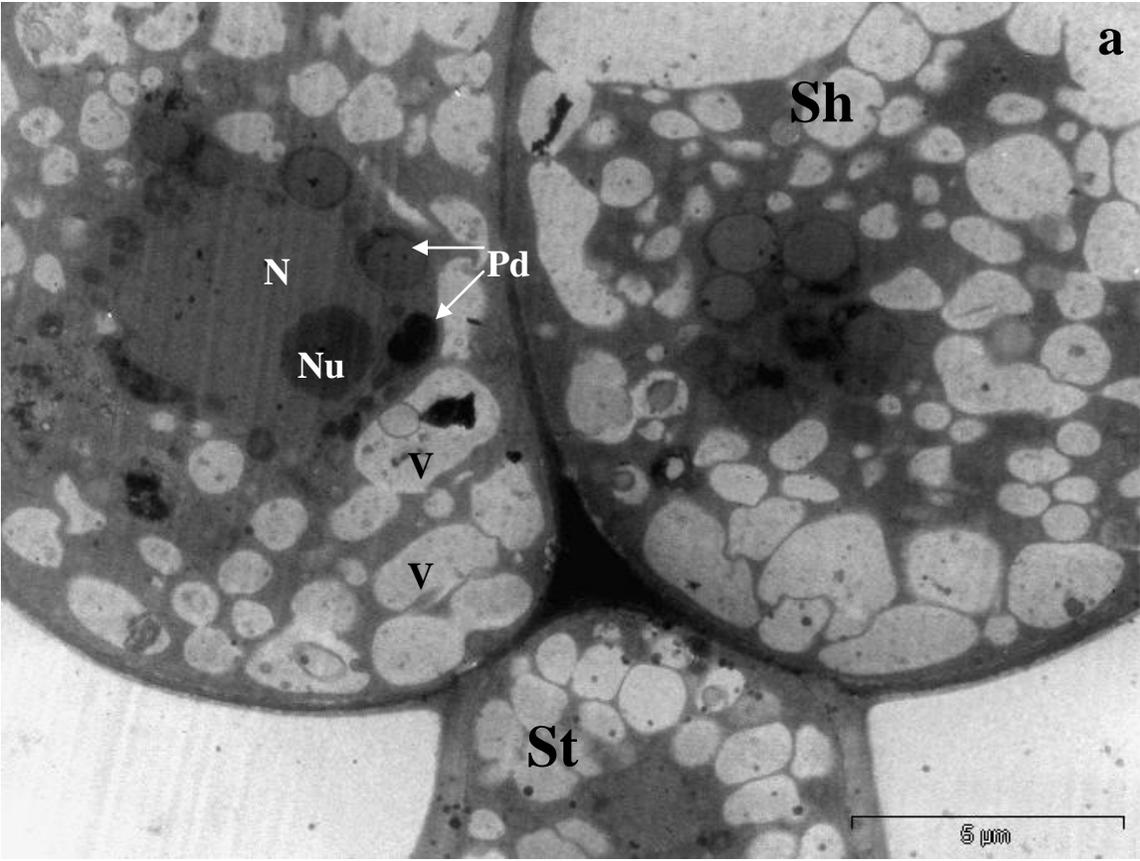


PLATE 13

Figure 18. TEM of cell wall region of a peltate trichome.

(a) Section showing outer cell wall (Cw) of peltate trichome with invagination of the cytoplasm (arrows) into vacuoles (V).

(b) High magnification section showing loosening of the cell wall material (arrows) in the cell wall (Cw). The cytoplasm, which appears to be pushing into the vacuole (V), contains vesicles (Vc) and a multimembranous structure (Me). Mitochondria (M) and endoplasmic reticulum cisternae (ER) cisternae can also be seen.

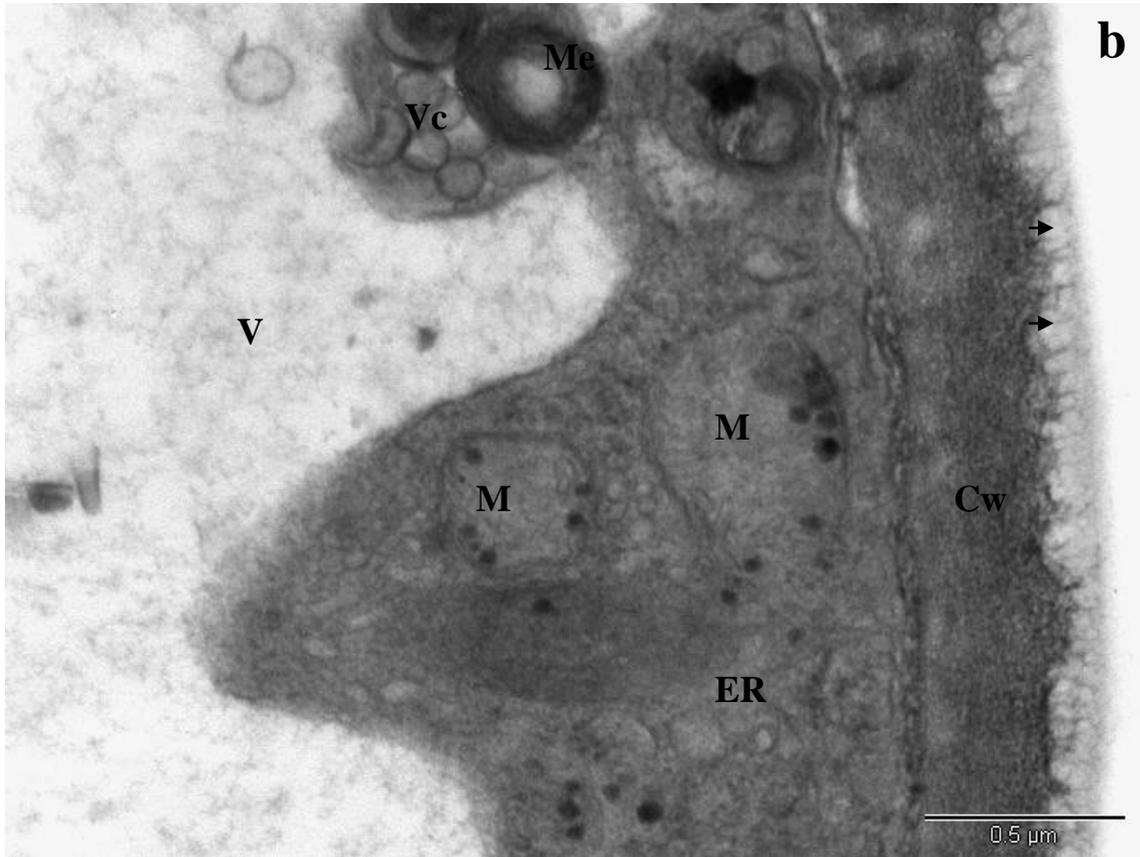
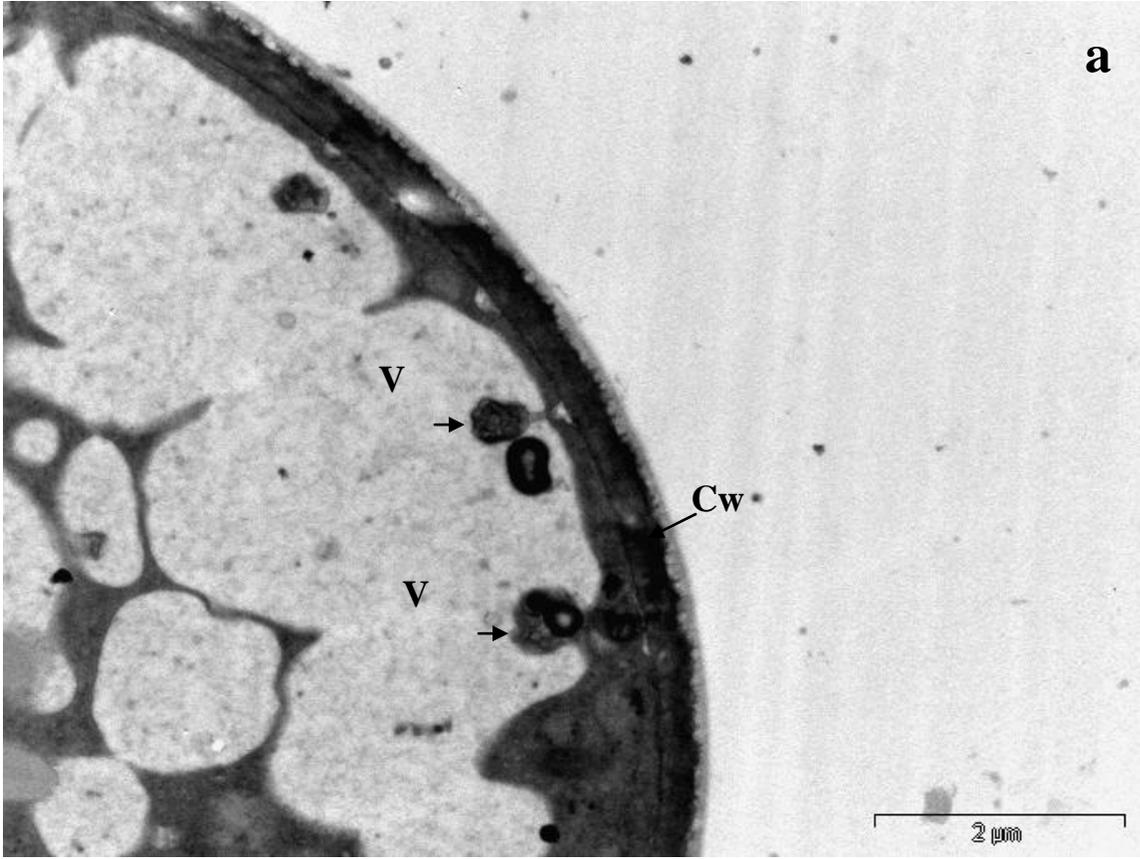


PLATE 14

Figure 19. TEM showing vesicle formation in peltate trichomes.

(a) Multimembranous structure (Me) appears to be invaginated into a vacuole (V) near the cell wall (Cw). Vesicles (Vc) are beginning to develop in the cytoplasm. These structures are surrounded by golgi body (Gb), mitochondria (M) and endoplasmic reticulum (ER).

(b) Vacuole (V) contains numerous vesicles (Vc) after multimembranous structure degrades. The degradation of the membranous structures could form the viscous secretory material which is then stored in vesicles and vacuoles before secretion.

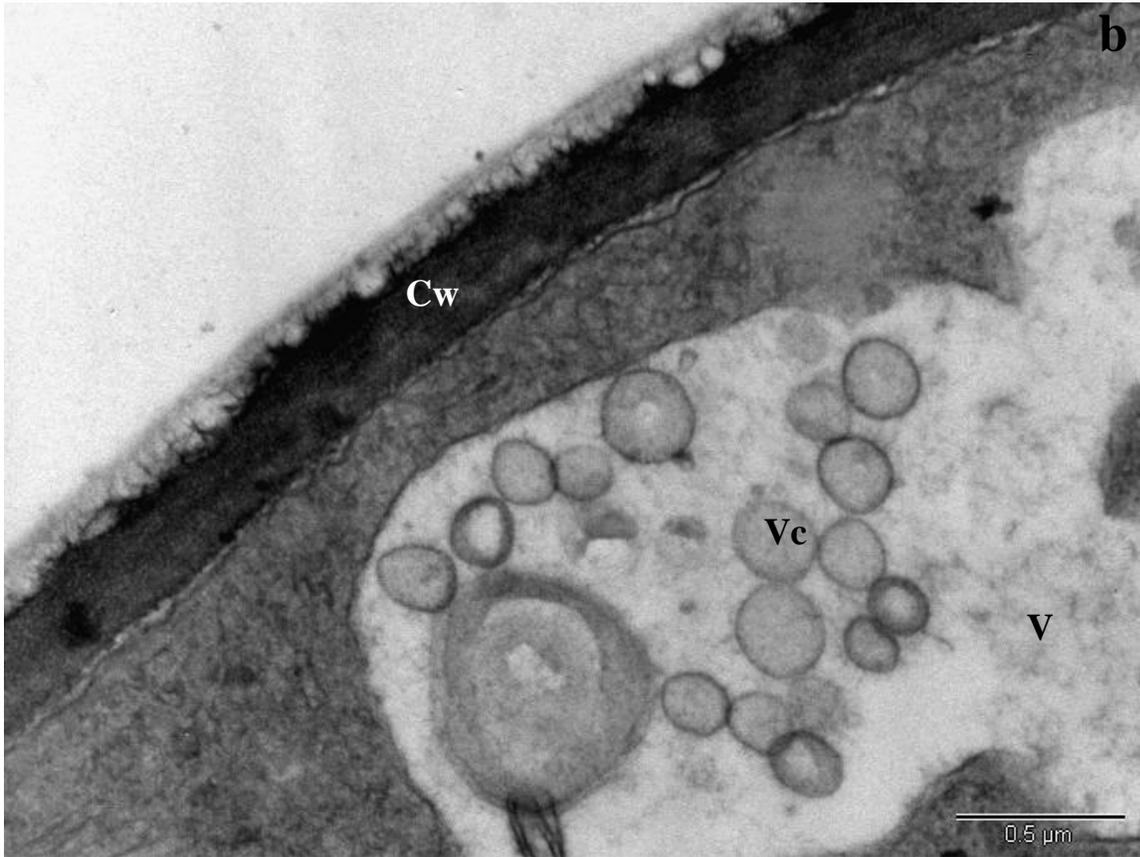
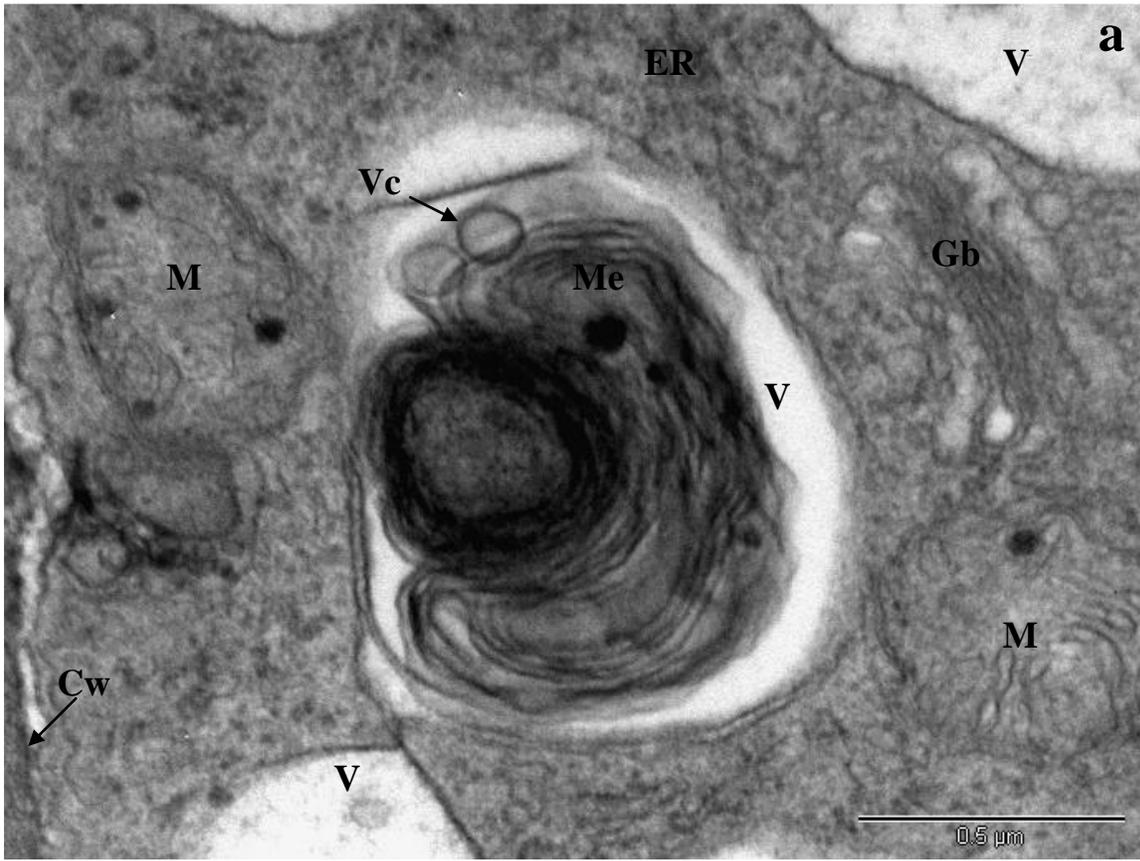


Table 2. Results of phytochemical tests of crude methanolic extracts of powdered leaves of *C. triloba*.

Compound group	Test	Reaction Observed	Present(+)/Absent(-)
Oligo- and Polysaccharides	Molisch's	Formation of a reddish ring	++
Monosaccharides	Fehling's	No reaction	-
	Benedict's	No reaction	-
Flavonoids	Lead acetate	Flocculent white precipitate	+
Tannins	Braemer's	Greenish to grey colouration	+
Alkaloids	Draggendorff's	Yellow colouration	-
	Hager's	No reaction	-
	Wagner's	No reaction	-
Saponins	Foam	Layer of foam after shaking an aqueous solution for 15 minutes	+
Amino acids	Ninhydrin	No reaction	-
Fixed oils and fats	Filter paper	Oily residue on filter paper	+

++ indicates intense reaction

CHAPTER 5: DISCUSSION

Reports of trichome morphology have been limited to *Harpagophytum procumbens* (Naidoo *et al.*, 2006), *Sesamum indicum* and *Ceratotheca melanosperma* (Solereeder, 1908) while phytochemical studies on the Pedaliaceae are limited to *C. sesamoides*, *Pedaliium murex* (Sahayaraj *et al.*, 2008), *H. procumbens* (Georgiev *et al.*, 2010) and a few *Sesamum* spp. including *S. indicum* (Bankole *et al.*, 2007), *S. radiatum* (Shittu *et al.*, 2006) and *S. angolense* (Potterat *et al.*, 1988).

This study was undertaken to investigate the morphology of foliar trichomes of *C. triloba* and to determine the chemical constituents of the secretory product. Two morphologically distinct trichomes were observed on leaves of *C. triloba*, capitate and peltate. A characteristic feature of Lamiaceae is the occurrence of peltate and capitate trichomes (Ascensão *et al.*, 1999), and since *C. triloba* displays both types, the characteristics of trichomes found on *C. triloba* will be discussed in conjunction with other species of Pedaliaceae and other families, including Lamiaceae.

The peltate trichomes found on leaves of *C. triloba* were uniseriate and comprised of a basal cell, a unicellular stalk and a “shield-like” secretory head made up of approximately four secretory cells arranged in a quadrant. Peltate trichomes with up to eight secretory cells were also observed but these were rare. Solereeder (1908), made a similar observation for foliar peltate trichomes found on *C. melanosperma* as consisting of a “single short stalk cell, although it may be exceptionally long and multicellular; the head is peltate, and consists of four cells (in isolated cases also of 5-8) arranged like a cross, their wall projecting convexly at the margin of the head” (see figure 3). Single stalk cells were a common feature for peltate trichomes, whereas multicellular long stalks were not. The stalk of peltate trichomes on leaves of *C. triloba* was always observed to be unicellular, similar to those observed in species of Lamiaceae (e.g. *Leonotis leonurus*, Ascensão *et al.*, 1995; *Salvia aurea*, Serrato-Valenti *et al.*, 1997). Multicellular long stalks in peltate trichomes have been observed in other species of the Pedaliaceae such as *Rogeria adenophylla* (Solereeder, 1908); whereas multicellular short stalks were the common feature in the bi- and multiseriate trichomes of the Asteraceae (e.g. *Artemisia*

campestris, Ascensão and Pais, 1987; *Stevia rabaudiana*, Montiero *et al.*, 2001). Four-celled peltate trichomes were also common among many species of Lamiaceae such as the *Ocimum* species (Werker *et al.*, 1993).

Capitate trichomes on leaves of *C. triloba* were uniseriate and comprised a single basal cell, multicellular stalk consisting of longer cells at the base and gradually shorter cells towards the head. The small spherical secretory head consisted of vertical walls that divided the head into a quadrant. Head cells similar to these were described for *C. melanosperma* as palisade-like in appearance (Solereeder, 1908). Capitate trichomes, comprising of a four-celled secretory head, were not common in Lamiaceae with capitate trichomes in species of this family having either a unicellular or bicellular head (Werker *et al.*, 1985a; Serrato-Valenti *et al.*, 1997; Bisio *et al.*, 1999). In *Harpagophytum procumbens* (Naidoo *et al.*, 2006), a close relative of *Ceratotheca*, the leaves lacked typical capitate trichomes since these trichomes comprised of a large, flat secretory head as compared to the small, spherical head observed in *Ceratotheca*. These trichomes appeared to be an intergraded form since they have a secretory head of a peltate trichome subtended by a long stalk. Intergraded forms of trichomes have been reported previously for capitate trichomes in species of Lamiaceae (e.g. *Salvia sclarea* and *S. dominica*, Werker *et al.*, 1985a). Non-glandular trichomes were not observed on leaves of *C. triloba*. This trend has been observed in other species of the Pedaliaceae (e.g. *C. melanosperma*, Solereeder, 1908; *Harpagophytum procumbens*, Naidoo *et al.*, 2006).

Both the capitate and peltate trichomes were observed on the adaxial and abaxial surface of leaves throughout leaf development. The higher frequency of trichomes on emergent leaves as compared to the frequency of trichomes on the young and mature leaves might indicate that trichomes served a more important role in preventing herbivory on the vulnerable emergent leaves (Fig. 8 and 9). In addition, the relative higher frequency of trichomes at the tip region of emergent leaves indicated that trichomes might be of higher value at the tips of leaves, which were relatively more exposed to herbivores and environmental factors. However, the basal region on the abaxial surface of young leaves was significantly higher than the tip region. Ascensão and Pais (1987) suggested that trichomes develop in early leaf development and the final number of trichomes remained constant throughout leaf expansion. Hence, trichome density on the

expanding leaf decreased as the leaf matured. However, this did not explain the variation in trichome frequency between the different regions (tip, mid and base regions) as observed on leaves of *C. triloba*. One could assume that different parts of a leaf expand at different rates, thereby presenting varying trichome frequencies in different regions. Werker *et al.*, (1993) suggested that in *Ocimum basilicum*, glandular hairs were produced in early organ development and the rate of differentiation appeared to be very rapid across the surface. Differentiation remains as long as an organ contains a meristematic region. Therefore, as in the case of leaves which contain both meristematic and mature regions, a difference in trichome frequency will be noted.

This difference might also relate to the function of the ‘short term’ capitate and ‘long term’ peltate trichomes as described by Werker (2000). The ‘short term’ capitate trichomes, which secreted material during early leaf development, could densely cover meristematic regions whereas the long term peltate trichomes might be found on the more established regions. This was noted on leaves of *C. triloba* in which peltate trichomes were concentrated on the abaxial surface along the veins and along the grooves on the adaxial surface. These regions were well established, being protected by epidermal cells that had thick pectinised walls. Another possible reason for the trichomes to develop on the veins is assumed that they might provide a quicker and energetically cheaper mode to transport raw materials to the secretory head cells.

The rapid differentiation of capitate trichomes in early leaf development might provide a protective barrier on emergent leaves, which were more susceptible to biotic and abiotic pressures (Woodman and Fernandes, 1991). There is a direct correlation between environmental stresses on a plant and trichome density on that same plant (Johnson, 1975). Plants of *Potentilla* (Rosaceae) grown in shady sites contained fewer trichomes than those grown in sunny sites indicating the importance of trichome density in protecting plants against high temperatures (Clausen *et al.*, 1940). Trichomes are associated with reflecting light and appeared to play a role in preventing excessive water loss. In addition to abiotic pressure, trichomes density appeared to be also important against biotic threats such as herbivory. Fernandes (1994) noted that density of capitate trichomes on leaves was one of the major factors influencing the number of herbivores

feeding on plants. Emergent leaves therefore required a higher density of trichomes to prevent or reduce herbivory. These mapped scenarios are also assumed to occur in *C. triloba*.

Segments of flower petals were also prepared for SEM to compare trichomes on petals with those observed on leaves. The occurrence of different trichomes on different plant organs might be due to difference in function between these trichomes (Werker *et al.*, 1985a). Since trichomes on vegetative organs helped to prevent herbivory, one must consider the potential dual role which is played by trichomes which are found on flowers in repelling herbivores while at the same time attracting pollinators. The capitate Type II trichome found only on flower petals of *C. triloba* could possibly be secreting substances that might attract pollinators, while the peltate and capitate Type I trichomes is assumed to function in a similar way to those found on leaves, in order to repel herbivores. If this is the case, then the main components of the secretory product should differ accordingly between the two types of capitate trichomes in order to provide the differences in function.

In *Salvia dominica* and *S. sclarea*, in addition to differences in shape and occurrence of capitate trichomes between vegetative and reproductive organs, there was also a difference in the main components of the essential oil, being neryl acetate and linalyl acetate (Werker *et al.*, 1985a). Both the reproductive and vegetative organs of these species were covered in capitate and peltate trichomes. The morphology of peltate trichomes was uniform between the two organs whereas the capitate trichomes differed in structure, size and mode of secretion. In addition, Mahlberg *et al.*, (1984) reported that capitate stalked trichomes were only observed on flower petals of *Cannabis sativa* while other glandular trichomes were found on leaves. Similar observations in differences in trichome occurrence and distribution between reproductive and vegetative organs were reported for *Salvia officinalis* (Venkatachalam *et al.*, 1984) while, in *Origanum vulgare*, no difference in trichomes occurrence was observed between the reproductive and vegetative organs (Werker *et al.*, 1985b).

The development of both capitate and peltate trichomes in *C. triloba* appeared to originate from a single protodermal cell which divided periclinally to form a protrusion above the epidermis. These cells then have gone through further periclinal divisions to form a stalk and anticlinal

divisions to form secretory head cells. Uphof (1962) characterised the periclinal development of a multicellular, uniseriate trichome according to direction of division of the existing stalk cells. Acropetal division appeared to occur when the uppermost stalk cell divided to form a new cell, whereas basipetal division is assumed to occur when the lowermost stalk cell has gone through division. In *Pelargonium scabrum*, capitate stalked trichomes resulted from a development of the protodermal cell which divided periclinally to form a single stalk cell (Oosthuizen and Coetzee, 1983). Further stalk cells occurred from acropetal division. The peltate trichome in species of Lamiaceae underwent two periclinal, acropetal divisions to form a stalk cell and a single secretory cell which underwent further anticlinal division (e.g. *Origanum dictamnus*, Bosabalidis and Tsekos, 1982a; *Leonotus leonurus*, Ascensão *et al.*, 1995). It is not clear whether the stalk in either the peltate or the capitate trichomes on leaves of *C. triloba* developed via acropetal or basipetal division. In addition to differentiation of the protodermal cell, other epidermal cells appeared to develop around the protodermal cell in capitate trichomes. These generally form a ring of differentiated cells normally referred to as a cellular pedestal, which provides support and anchors the trichome in the epidermis.

Observations indicated that the mode in which the trichomes on leaves of *C. triloba* secreted their product differed substantially from that in the Lamiaceae. In most species of Lamiaceae, the peltate trichomes accumulated secretory product in a subcuticular chamber which formed when the cuticle detached from the cell wall of the secretory cells (Werker *et al.*, 1985a, b). These peltate trichomes appeared to release their contents either through the elevated cuticle or when the elevated cuticle was damaged, which usually occurred via mechanical disturbances which might be caused by herbivores (Schnepf, 1969). Peltate trichomes of *C. triloba*, however, lacked a subcuticular chamber and secretory material which appeared to be stored in numerous vacuoles in the cell lumen is assumed to be only secreted upon breakage of the cell wall. Similar observations were found in *C. sesamoides* where the peltate trichomes secrete mucilage when the secretory cell ruptured (Abels, 1975) or dissolved (Ihlenfeldt, 2001), resulting in cell death. In other species of the Pedaliaceae such as *Harpagophytum procumbens* (Naidoo *et al.*, 2006), the glandular trichomes, which also appeared to lack a subcuticular chamber, secreted viscous mucilage through micropores to the exterior. Peltate trichomes of *C. triloba* therefore appeared to secrete substances through a deviation of the granulocrine pathway of secretion, by employing

vesicles and vacuoles for storage only but not fusing with the plasmalemma as the final step in secretion. The secretory process of peltate trichomes will be discussed in more detail using TEM data.

Similarly, the capitate trichomes found on leaves of *C. triloba* lacked a subcuticular chamber which is also a common feature for capitate trichomes of the Lamiaceae. In Lamiaceae, capitate trichomes are also reported to accumulate oils in the subcuticular chamber and released them either by cuticular rupture or diffusion through micropores in the cuticle (*Ocimum basilicum*, Werker *et al.*, 1993; *P. annua*, Ascensão *et al.*, 1999). The secretory product of the “short stalked capitate trichome” of *P. madagascariensis* appeared to accumulate in a small subcuticular space and is probably released by diffusion through micropores whereas “the long stalked capitate trichome” is assumed to release its secretory product via cuticular rupture (Ascensão *et al.*, 1998). The capitate trichomes of *C. triloba* appeared to produce copious amounts of secretory material which are not stored in vacuoles as in the case of peltate trichomes. It therefore appeared that the capitate trichomes employed an eccrine pathway of secretion through the plasmalemma and cell wall. The process of secretion for capitate trichomes will be discussed further with TEM.

Although histochemical tests are not absolutely specific, they can be useful in indicating the major components of cells walls and the secretory material of trichomes *in situ*. Histochemical analyses with Sudan III/IV and Sudan Black indicated the presence of suberin or cutin in the lateral walls of the single stalk cells of peltate trichomes and neck cell of capitate trichomes. The cell walls of the stalk cells of both trichomes also tested positive for pectins (see figure 13) and cellulose with the Ruthenium Red and Calcofluor White tests respectively. The process occurring could be similar to that of the walls of developing endodermis cells in root hairs, which might have highly complex wall arrangements to prevent the backflow of water. Similarly, the cell walls in stalk cells could prevent the backflow of secretory product (Bruni and Modenesi, 1983; Fahn, 1988). This might be important since secretory material which is produced by trichomes could be potentially harmful to the rest of the plant (Fahn, 1988).

Sections tested with Ruthenium Red indicated that the secretory cells of peltate trichomes secreted copious amounts of mucilaginous polysaccharides while the capitate trichomes appeared to secrete it to a lesser degree (see figure 13). Similar observations were made by Solereder (1908) who referred to the peltate trichomes on *C. melanosperma* as ‘mucilage hairs’. The function of the mucilage is not clear but the presence of viscous secretions could act as a lubricant in facilitating leaf growth and expansion (Modensi *et al.*, 1984). The slimy layer on leaves might even reflect light and thus prevent excessive water loss (Gaff, 1997). Viscous secretions have also been shown to trap phytophagous insects. Gibson (1971) demonstrated the resistance of *Solanum polyadenium*, *S. berthaultii* and *S. tarijense* to the aphid *Empoasca fabae*. The glandular trichomes on these plants secreted viscous substances that trapped the aphids which were eventually immobilized and starved to death. Tingey and Gibson (1978) showed that the viscous substances polymerized to trap the aphids, encasing the legs and tarsi. Polymerization was shown to occur from the interaction of polysaccharides with phenolic compounds.

There have been many reports on the medicinal uses of relatives of *C. triloba*, such as *C. sesamoides* and *Sesamum angustifolium* because of their mucilaginous nature. In Sudan, food is served with a gelatinous sauce made from these plants to make food more palatable (Bedigian, 2004). The same has been found in Nigeria and Kenya. These plants have also been used for their gelatinous property as lubricants to ease difficult childbirth (Adjanohoun, 1986).

Positively stained wax sections with Sudan III/IV and Sudan Black in the secretory cells of both capitate and peltate trichomes indicated the presence of lipids. The ferric trichloride test for phenolic compounds is based on the reaction of orto-dihydroxyphenols with ferric ions to form deep green or black deposits. A positive reaction for phenols was observed in the head cells of capitate and peltate trichomes (see figure 15). In addition to this, blue autofluorescence was observed in head cells which might also indicate the presence of phenolic compounds (Mabry *et al.*, 1970). Ecologically, phenolic compounds act as deterrents to herbivory. Matsumoto, (1962) showed that a coumarin which is produced by *Melilotus* species, had an inhibitory effect on the vegetable weevil. Gilbert *et al.*, (1967) demonstrated the inhibitory feeding effect of juglone which is produced by *Carya ovata*, to the bark beetle, *Scolytus multistriatus*. Medicinally, phenolic

compounds are known for their antioxidant activities as free radical scavengers (López-vélez *et al.*, 2003). Plant phenolics are also well known for preventing cancer and coronary heart disease (Rice-Evans *et al.*, 1997; Roginsky, 2003). This is suspected to justify a use of *C. triloba* as a traditional medicine; however, this suggestion might be subject of future extensive investigation which is not in the scope of this study.

TEM of peltate trichomes showed that secretory head cells contained centralized nuclei and numerous large vacuoles that appeared to contain mucilage taking up most of the space in the cell lumen (see Figure 17 a). Solereder (1908) similarly described the lumina of peltate trichomes in *C. melanosperma* with mucilage occupying the larger part of the cell lumen and the remains of the protoplast making up a very small space. The presence of endoplasmic reticulum cisternae, golgi apparatus and mitochondria scattered throughout the cytoplasm, especially around vacuoles could be an indication of mucilage production (see Figure 18 b). It is well known from many investigations that the golgi apparatus is involved in mucilage production and transport and that many dictyosomes are present in mucilage producing cells. Many of these studies described a granulocrine method of secretion in which mucilage was transported via vesicles to the apoplast (Trachtenberg and Fahn, 1981; Schnepf *et al.*, 1983). Figure 20 describes the different modes of secretion, including granulocrine secretion which employed vesicles that fused with cell membranes to release substances to the apoplast. Granulocrine secretion however, did not appear to occur in peltate trichomes of *C. triloba*. In these trichomes, mucilage appeared to be stored in vacuoles and was only released once the cell wall broke. An elevated cuticle and subcuticular chamber which were characteristics of the secretory process for trichomes of Lamiaceae (Ascensão 2010) were not observed in this study. The above observations emphasised the fact that secretory products in the peltate trichomes were not secreted through the cell membrane, but rather stored until the cell wall ruptured (Abels, 1975) or dissolved (Ihlenfeldt, 2001) as in the case of *C. sesamoides*.

Cell wall rupture can be caused by direct mechanical disturbance whereas cell wall dissolution, resulting in cell death, is most probably caused by the plant itself. A recent study by Tooker *et al.*, (2010), on the tomato plant *Solanum lycopersicum*, showed that trichomes on the leaf surface acted as ‘early warning’ senses. Any disturbance, such as movement on the surface of plants,

induced rapid molecular signalling to prepare the plant for herbivore attack. Similarly, peltate trichomes on *C. triloba* could have been informed of a potential attack and thus took the necessary steps for cell wall dissolution and apoptosis. Both the capitate and peltate trichomes could act as sensors. This process appeared to be efficient in preventing or reducing feeding before it actually happened.

Mucilage appeared to be produced from an interaction between vacuoles and multimembranous structures, which appeared to push against the vacuoles (Figure 18 a and b). Small vesicles developed in the cytoplasm with the simultaneous degradation of the multimembranous structures. The vesicles appeared to then fuse with each other to form pseudovacuaoles or established vacuoles (Figure 19 b). This was observed for the glandular trichomes of *Pharbitis nil* (Unzelman and Healey, 1974) in which many protein-carbohydrate filled vesicles were enclosed by a network of ER. The rough endoplasmic reticulum (RER), which extended to the plasmalemma, sequestered vesicles to the apoplast while smooth endoplasmic reticulum (SER) promoted vesicle fusion into larger vesicles, probably for storage. Kristen (1976) noted that in placental papillae of *Aptenia cordifolia* ER cisternea, which have lost their membrane-bound ribosomes, appeared to promote pseudovesicle formation in the organelle-free parts of the cytoplasm. There was no evidence of vesicle transport into the apoplast. Figure 21 shows how pseudovacuaoles are produced for the synthesis and storage of mucilage in mango fruits (Joel and Fahn, 1980).

In the secretory head cells of peltate trichomes oval shaped, light and dark stained electron dense plastids, endoplasmic reticulum and mitochondria in close proximity to the nucleus is probably responsible for the production of lipids and phenolic compounds. The most common ultrastructural feature of a cell secreting lipophilic substances was the occurrence of osmiophilic plastids which were surrounded by ER and mitochondria (Fahn and Evert, 1974; Joel and Fahn, 1980; Bosabalidis and Tsekos 1982b; Fahn, 1988). Two kinds of plastids, differing in shape and osmiophilic density, were observed in head cells of peltate glands. Whether each plastid produced a different substance is not known. However, Fahn (1988) suggested that in a cell different substances were produced by different organelles.

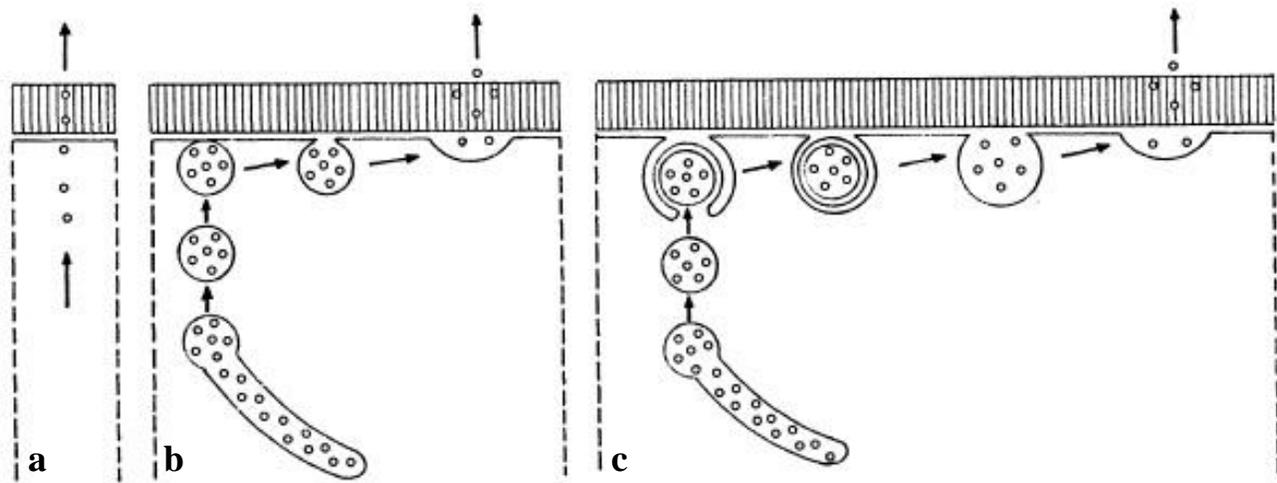


Figure 20. Illustration showing different pathways of secretion in secretory cells. (a) Eccrine secretion. (b and c) Granulocrine secretion, substances are secreted via vesicles that fuse with the plasmalemma. (From Fahn 1979).

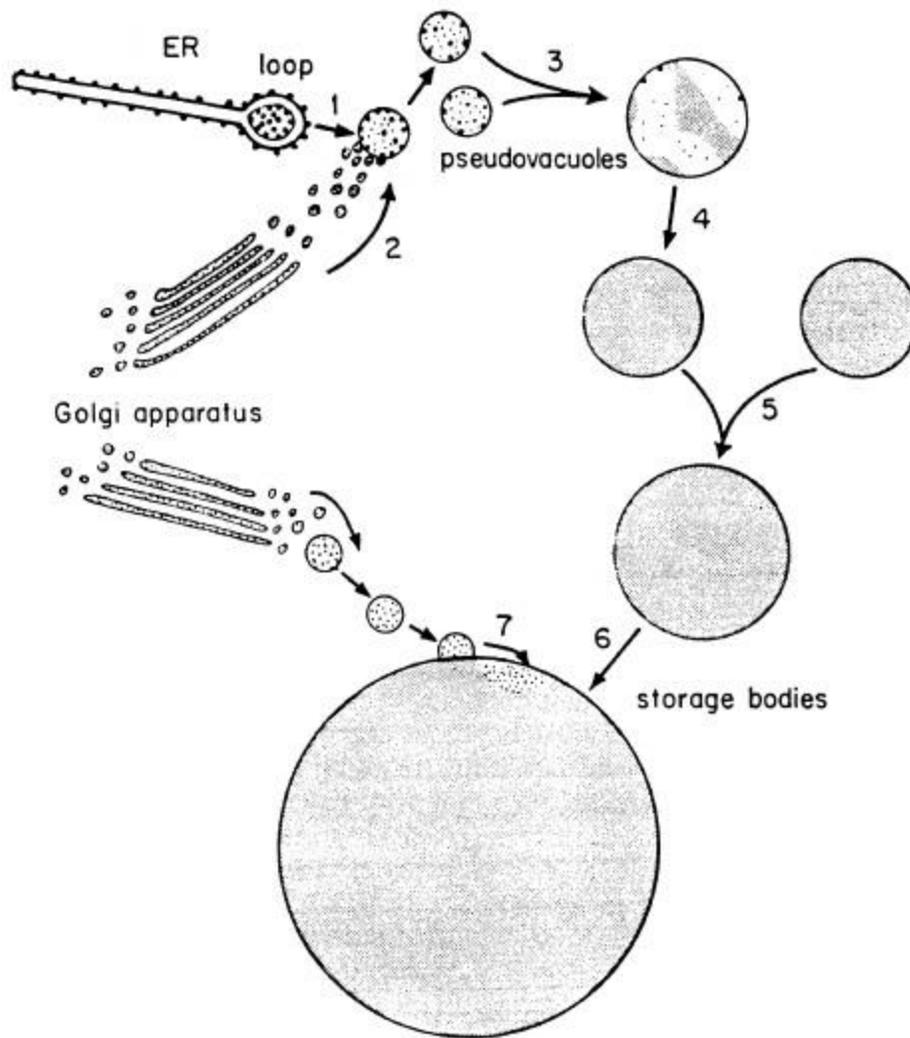


Figure 21. Illustration showing likely origin of protein-carbohydrate mucilage in mango fruit ducts. (From Joel and Fahn, 1980)

The stalk cells of peltate trichomes were ultrastructurally similar to the secretory head cells. Even though plasmodesmata were not observed between the head cells and stalk cells, there is still a possibility that they are present. This could mean cell to cell transport via a symplastic pathway.

The observations noted for the secretory cells of capitate trichomes, having abundant amounts of osmiophilic material in the cytoplasm (see Figure 16 a), indicated that the secretory product such as lipids and phenolics are produced in organelles and are released into the ground cytomatrix. This has also been noted in the oil gland of *Origanum dictamnus* (Bosabalidis and Tseko, 1982a) and the calyx glands of *Plumbago capensis* (Rachmilevitz and Joel, 1976). The observed mitochondria and ER probably produced lipophilic substances (Vassilyev, 1970; Fahn and Benayoun, 1976).

The ultrastructure of stalk cells of capitate trichomes differed from capitate head cells and was ultrastructurally similar to the cells of peltate trichomes. The stalk cells of capitate trichomes contained peripheral vacuoles and a centralized nucleus which was surrounded by osmiophilic plastids, numerous mitochondria and ER (see Figure 16 a and b). The occurrence of loose fibres in the outer cell wall of secretory cells seemed to indicate that these cells secreted substances in a similar way to capitate trichomes of Lamiaceae (Ascensão, 2010) even though capitate trichomes in *C. triloba* also lacked an elevated cuticle. A basic eccrine pathway could be an explanation for secretion in the capitate trichomes of *C. triloba*. There appeared to be cell wall intrusion between the head cells and neck cells, probably to prevent backflow of the secretory product (Fahn, 2000).

Preliminary phytochemical tests on crude methanolic extracts indicated the presence of oligo- and polysaccharides, flavonoids, saponins, tannins and fixed oils. The oligosaccharides and polysaccharides are probably mucilage or mucilaginous products. The flavonoids, which consist of a large group of compounds that normally occur as glycosides, contain numerous phenolic hydroxyl functional groups attached to a ring structure (Rice-Evans *et al.*, 1997). They are known antioxidants since vitamins C and E belong to this group (Rice-Evans *et al.*, 1997). Flavonoids were also reported to play a role in inhibiting diarrhoea and easing painful abdominal

cramps (Schuier *et al.*, 2005). These ailments have been treated by traditions healers with leaf extracts of *C. triloba* (Steenkamp, 2003).

Ecologically, tannins prevented herbivory by interacting with proteins to form tannin-protein complexes which reduced the total protein that could be ingested by herbivores. Since these complexes reduced the dietary content in plants and most angiosperms contain tannins, it was highly possible that insects used tannins as a negative feeding cue. Medicinally, tannins have been reported to have anti-cancer and antioxidant properties (Motar *et al.*, 1985; Erdèlyi *et al.*, 2005). They were also used in the treatment of skin lesions and ulcers by protecting the wound and preventing excessive blood loss (Bruneton, 1999; Aiyegoro and Okoh, 2010). Tannins are another group of compounds that are known for treating diarrhoea and other stomach ailments (Bruneton, 1999). Saponins prevent inflammation and display antimicrobial properties (Bruneton, 1999; Fawole *et al.*, 2009). This evidence is indirectly supported the possible contention that *C. triloba* is used in traditional medicine due to its tannin and saponin content.

Characterisation of trichomes on leaves of *C. triloba* has highlighted the potential uses of this plant for future research. The morphological characteristics of trichomes observed on leaves of *C. triloba* could be used in plant systematic studies. Both the capitate and peltate trichomes secreted compounds that were medicinally important and could be used commercially. The mode of secretion of peltate trichomes, in which mucilage appeared to be produced and stored, was unique and the same mode could be manipulated using biotechnological tools for the production of other commercially important compounds.

CHAPTER 6: CONCLUSION

This study was initiated to characterise the morphology and ultrastructure of the foliar trichomes and to determine the chemical composition of the secretion in order to provide a scientific basis for the medicinal properties of *C. triloba*.

Leaf surface imaging indicated the presence of two morphologically distinct glandular trichomes: capitate and peltate. These trichomes were of similar morphology to those observed on *C. melanosperma* and *Sesamum indicum*. The capitate trichomes were tall, consisting of a single basal cell, 2-4 stalk cells, a neck cell and a head that is made up of four secretory cells. The peltate trichomes consisted of a basal cell, a single stalk cell and a multicellular head. In most cases the peltate trichome head consisted typically of four cells, but some were seven or even eight-celled. Fully developed trichomes were found to be concentrated on emergent leaves probably to provide protection against herbivory and to prevent excessive water loss. The density of these trichomes decreased as the leaf expanded because capitate trichomes, also known as short term trichomes, matured early in leaf development and appeared to provide protection in emergent leaves against various biotic and abiotic factors. Peltate trichomes however, developed as the leaf matured and probably provided long term protection to leaves against herbivory.

The ultrastructure of the stalk cells of both trichomes and the secretory head cells of peltate trichomes were similar. These cells consisted of centralised nuclei surrounded by osmiophilic plastids which are known producers of lipophilic secondary product. The cytomatrix contained numerous vacuoles surrounded by dictyosomes. Mitochondria and ER cisternea were found throughout the cytoplasm, in close proximity to the nucleus and vacuoles. The head cell of capitate trichomes however, contained large amounts of osmiophilic substances, ER and mitochondria. Peltate and capitate trichomes appeared to be secreting similar compounds, but the composition of each compound in the secretory material might vary between the trichomes.

Histochemical and phytochemical tests indicated that the major components of the secretory product were mucilage and phenolic compounds. Other components included tannins, saponins

and flavonoids. It is assumed that these compounds contribute to the medicinal value of *C. triloba*.

Future studies should focus on isolating and quantifying the compounds in the secretory product. Antioxidant screening coupled with cytotoxicity tests should be undertaken to evaluate the safety of the secretory product for commercial and medicinal use. Similar studies on other species of the Pedaliaceae could identify interesting plant compounds that may be of medicinal value.

CHAPTER 7: REFERENCES

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