THE MICROMORPHOLOGICAL AND ESSENTIAL OIL STATUS OF THE FOLIAR SECRETORY STRUCTURES OF *OCIMUM OBOVATUM* E. MEY. ex BENTH. SUBSP. *OBOVATUM* (LAMIACEAE)

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

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Durban 2011

DECLARATION

I, Nazeera Kasim, declare that this dissertation is my own work. It is being submitted in partial fulfilment 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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Nazeera Kasim

.....day of.....2011

DEDICATION

This dissertation is dedicated to the people in my life who have supported me in all my academic endeavours and to my loved ones who have always believed in my potential and supported me during my years of tertiary enlightenment.

ABSTRACT

Ocimum obovatum E. Mey ex Benth. var. *obovatum* is a traditionally used medicinal plant that grows along the KwaZulu-Natal coast and the western Cape of South Africa. The plant is noted for its hair restoration properties, remedy for infantile abdominal pain and cramps and its use as an enema to treat epigastric conditions in children. The aims of this study were to document the micromorphology and ultrastructure of the foliar secretory structures responsible for the production and secretion of the essential oils and chemical composition of the secretion, which gives the plant a distinct aroma. It is believed that these oils contain the active ingredients that contribute to the medicinal properties of the plant. A variety of microscopic methods and histochemical and phytochemical tests were used to achieve this. Leaves in all stages of development were pubescent and gland dots, characteristic of plants in the genus, were found on both adaxial and abaxial surfaces. Three types of trichomes were found on both leaf surfaces across all stages of development; non-glandular trichomes and two types of glandular trichomes. Non-glandular trichomes are single, multicellular and uniseriate with microornamentation and a supportive cellular pedestal. The glandular trichomes consisted of peltate and capitate trichomes. Peltate trichomes are made up of four head cells and a very small basal cell that gives the glands the appearance of being sessile. The capitate trichomes were further divided into two types based on the morphology of the glands. Type I capitate trichomes are smaller than the larger peltate trichomes and are composed of one basal cell and a head consisting of two broad head cells. Type II capitate trichomes consisted of one basal cell, one stalk cell and a single oval head cell. Histochemical tests showed that peltate and Type I capitate trichomes have cutinized or suberized walls in the stalk cell to prevent the apoplastic flow of secretory material into neighbouring mesophyll tissue. The histochemical stains also showed that the secretory material present in the glandular trichomes are lipid in nature and essential oils are present. Ultrastructural studies showed polymorphic leucoplasts, few Golgi bodies, numerous vesicles and mini vacuoles, mitochondria and short profiles of endoplasmic reticulum cisternae. Phytochemical tests revealed the presence of essential oils that are terpene-rich. Flavonoids, tannins, saponins, terpenoids, fixed oils and fat, phenolics and cardiac glycosides were also detected in a crude ethanolic extract of the leaves. These chemical compounds appear to be responsible for the medicinal properties for which the plant is traditionally exploited.

CONFERENCE CONTRIBUTIONS FROM THIS DISSERTATION

Kasim, N., Naidoo, Y., Nicholas, A., 2009. The micromorphological and essential oil status of the foliar secretory structures of *Ocimum obovatum* (Lamiaceae). 4th Global Summit on Medicinal and Aromatic Plants, Kuching, Sarawak, Malaysia.

Kasim, N., Naidoo, Y., Nicholas, A., 2009. Glandular trichomes in leaves of *Ocimum obovatum* L. (Lamiaceae). 47th Meeting of the Microscopy Society of Southern Africa, Westville, KwaZulu-Natal.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Yougasphree Naidoo, for her expertise, commitment, support and enthusiasm during this research. Her availability as a source of wisdom and guidance is highly appreciated.

I would like to extend a thank you to my co-supervisor, Prof. Ashley Nicholas, for his guidance and expertise throughout this research.

I would like to thank the following people:

Mr Isaac Dlamini for the propagation and care of the plants for the research.

The Electron Microscope Units at Westville and Pietermaritzburg (UKZN).

J. Wesley-Smith for assistance with Transmission Electron Microscopy.

C.T. Sadashiva for assistance with phytochemistry.

T. Karim for assistance in preparing phytochemical and histochemical reagents.

The National Research Foundation is acknowledged for financial assistance.

Finally, I would like to thank my Mum, Dad and Sister for their support and understanding as well as my grandparents for their moral support and financial assistance. A special thank you is extended to Z. Hussain and S.B. Kasim for moral support and assistance in the editing process of the research project.

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LIST OF ABBREVIATIONS

Adj	Adjacent epidermal cell
BC	Basal cell
Cap I	Type I capitate trichome
Cap II	Type II capitate trichome
CP	Cellular pedestal
Ct	Cuticle
CW	Cell wall
Cyt	Cytomatrix
ER	Endoplasmic reticulum
ESEM	Environmental scanning electron microscopy/micrograph
GC	Guard cell
GS	Golgi stack
L	Leucoplast
LB	Lipid body
LM	Light microscopy/micrograph
M	Mitochondrion
MO	Microornamentation
N	Nucleus
NG	Non-glandular trichome
P	Plasmodesmata
Pl	Plasmalemma
PT	Peltate trichome
SC	Stalk cell
SEM	Scanning electron microscopy/micrograph
SHC	Secretory head cell
sSC	Short stalk cell
SS	Subcuticular space
St	Stoma
TEM	Transmission electron microscopy/micrograph
V	Vesicles
Vac	Vacuoles
Vs	Vacuolated space
WHO	World Health Organization

PREFACE

Plants have been exploited as a source of medicine by nearly all cultures from ancient times (Hutchings et al.,1996; Trivedi 2006). Only a meagre percentage of the thousands of species of flowering plants have been studied in detail for chemical composition and medicinal value and, according to statistics, at least 80% of the world population still makes use of plant or plant extracts for their medicinal needs (Trivedi 2006). While developed countries have focused on the production of synthetic drugs, developing countries like South Africa have made significant discoveries in treating ailments using natural resources and indigenous knowledge.

As Ayurveda is practiced in India, *muthi* is traditionally prescribed medicinal remedies made up of plant or animal matter in South Africa. Specific plant species have been and are still prescribed to ailing patients; fuelling a market worth millions of rands in the country. Despite the advent and breakthroughs of western medicine, traditional medicine or *muthi* is still favoured due to its high cultural value (Dolds and Cocks 2002). It has been estimated that there are almost 27 million consumers of indigenous medicine in South Africa and research conducted thus far indicates that there are more than 700 species of plants that are actively traded for medicinal use (Mander 1998).

Plant material used for *muthi* is often boiled to extract the chemicals of medicinal value and it is generally only the extracts or the essential oils secreted by the glands on the aerial parts of the plants that are responsible for the medicinal properties of the plant (Werker 2000) that have been investigated thus far. With the extensive use of plants as medicinal remedies, a need for documentation and study of the plants, the ways in which they are administered and their pharmacological activities are needed. Recently, it has also been discovered that African marula extracts that have been prescribed to treat menstrual cramps for decades actually exacerbates the condition by causing increased uterine contractions in mice (unpublished Ojewole, 2009) indicating the need for pharmacological study of medicinal plant extracts.

The aims of this study were to document the micromorphology and ultrastructure of the foliar structures responsible for the secretion of essential oils in *Ocimum obovatum* E. Mey ex Benth var. *obovatum*, as well as to assess the chemical composition of the oils. The documentation of the foliar secretory structures on adaxial and abaxial surfaces of leaves was done using a variety of microscopic methods including stereomicroscopy, light microscopy, scanning-, and environmental scanning- and transmission electron microscopy; implementing already established preparatory techniques. The chemical nature of the secretions produced in the foliar secretory structures was also analysed using histochemical and phytochemical assays. The results from this research project provide insight into the biology of the foliar secretory structures of the plant as well as the preliminary chemical composition of the secretions produced by these structures. This information is vital to establish the safety and efficacy of the use of the plant in traditional medicine practices in the African region.

CHAPTER 1: INTRODUCTION

1.1 Traditional medicine

Traditional medicine encompasses the diagnosis and treatment of psychological and medical illnesses based on local knowledge, socio-cultural and religious beliefs (World Health Organization, 1978; Stangeland et al., 2008). These beliefs were developed over time by locals within the context of their belief systems and specific environmental conditions. In many parts of the world, including South Africa, traditional medicine is most often the initial choice for the provision of primary healthcare. This choice is almost always driven by the lack of access to conventional healthcare systems such as clinics or hospitals (Fabricant and Farnsworth, 2001; Elujoba et al., 2005; Gurib-Fakim, 2006; Stangeland et al., 2008).

In 1978 the World Health Organisation (WHO) defined traditional medicine as "*the sum total of knowledge or practices whether explicable or inexplicable, used in diagnosing, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience or observations handed down from generation to generation, verbally or in writing. It also comprises therapeutic practices that have been in existence often for hundreds of years before the development of modern scientific medicine and are still in use today without any documented evidence of adverse effects"*.

As Elujoba et al. (2005) explain, the "explicable" form of traditional medicine is described as the simple, scientific and direct application of the plant, animal or mineral material for the purpose of healing. In many cases this form of traditional medicine can be investigated, rationalized and sometimes explained scientifically. For example, the use of the willow plant, *Salix alba*, which contains salicylates for the treatment of fever and pains, led to the discovery of aspirin that possesses analgesic, antipyretic and anti-inflammatory properties (Farnsworth et al., 1985). Other modern medicinal drugs originally developed through traditional medicine, and that are currently being used in orthodox medical practices, include morphine, digoxin, quinine and atropine (Fabricant and Farnsworth, 2001; Elujoba et al., 2005, Gurib-Fakim, 2006).

According to the WHO (1978), the contribution of traditional medicine has been demonstrated by the reduction of high mortality, morbidity and/or disability caused by diseases such as HIV/ AIDS, tuberculosis, malaria, diabetes and mental disorders. It has been acknowledged that traditional medicine plays a significant role in the reduction of poverty by promoting the economic well-being of communities and developing health systems by extending health care and coverage to the masses (Elujoba et al., 2005).

A major challenge is the lack of development of scientifically-based evidence supporting the efficacy of traditional medicine, as well as the development of empirically based quality standards and regulations. A recommendation by WHO to member states of the African continent was to encourage the institutionalization of African Traditional Medicine in their respective health care systems (WHO, 1978). Efforts to meet this recommendation in South Africa have resulted in the implementation of a policy by the national government known as the Indigenous Knowledge Systems (South African Department of Science and Technology, 2005). This policy, which aims to preserve traditional medical knowledge and resources, has appealed to universities and industry to embark on collaborative projects aimed at documenting, bioprospecting and facilitating the discovery of potential compounds that can be utilised as drugs for commercial use, while preserving and conserving plant species traditionally exploited for medicinal purposes.

1.2 Description of *Ocimum obovatum*

Ocimum obovatum (E. Mey. ex. Benth) subsp. *obovatum* var. *obovatum* (Lamiaceae) is widely used in traditional African medicine, especially in southern African countries where it is believed to have great healing properties. The plant is commonly known as ‘Cat’s whiskers’ in English or ‘*Umathanjane*’ in isiZulu, and is used in South Africa and West Africa to treat ailments ranging from skin and chest infections, gastrointestinal illnesses and hair loss (Burkhill, 1985; Hutchings et al., 1996).

This plant, *O. obovatum,* chosen for the present investigation is widespread in tropical Africa, southern Africa and Madagascar; in grassland and open *Acacia* or *Brachystegia* woodland. The plants often grow in areas prone to burning or seasonal flooding and occur at altitudes ranging from 100- 2100 m (Paton et al., 1999). Plants are perennial and have a swollen woody rootstock that produces erect branched or unbranched flowering shoots between 0.15 m and 0.60 m high (Howard-Williams, 1970; Paton et al., 1999). Inflorescences consist of pink or lilac-white flowers with very long stamens (Paton et al., 1999). According to Burkhill (1985), *O. obovatum* is very variable in terms of leaf shape, pubescence and inflorescence structure but intermediates appear to connect all the West African forms of the plant (Figure 1).

While the traditional use of this plant has been described (Burkhill, 1985; Hutchings et al., 1996), and the chemistry of the leaf extract has, to some extent, been investigated (Burger et al., 1998), there are no studies describing the secretory structures responsible for the production of the volatile oils. These structures, known as trichomes, are an important diagnostic feature for taxonomists and they also secrete chemicals that contribute to the fragrance, flavour and pharmaceutical industries.

Figure 1: *Ocimum obovatum* (E. Mey. ex. Benth) subsp. *obovatum* var. *obovatum* growing in the grasslands of Pietermaritzburg, KwaZulu-Natal. Inflorescences include lilac-white flowers with long whisker-like stamens and polymorphic leaves.

1.3 The need for this study

Studies on trichome morphology and ultrastructure could prove to be very useful for taxonomy since current studies on the genus *Ocimum* have faced numerous problems due to the lack of specific sets of characteristics used in hypothesizing phylogenetic classifications (Paton et al., 1999). Therefore, a study of the trichomes could offer some clarity on the confused taxonomic status of *O. obovatum* and contribute to defining generic limitations in the Lamiaceae. Furthermore, preliminary phytochemical analysis of *O. obovatum* leaf extracts suggests that

volatile oils produced by the trichomes may be responsible for the hair restoration properties as well as antibacterial properties.

1.4 Aims and objectives

The aims and objectives of this study were to:

1) describe and characterize the morphology and distribution of the trichomes found on the leaves of *O. obovatum* at different stages of development using stereomicroscopy, scanning- and environmental scanning electron microscopy (SEM and ESEM).

2) investigate the anatomy of these foliar trichomes and chemical composition of the secretory material using histochemical stains.

3) describe the ultrastructure of the foliar trichomes of *O. obovatum* by investigating ultra-thin sections of resin-embedded leaf material using a transmission electron microscope (TEM).

4) determine the general phytochemical composition of the trichome secretions by phytochemical assays.

CHAPTER 2: LITERATURE REVIEW

2.1 The Lamiaceae

The Lamiaceae or 'mint family' is rich in aromatic species generally exploited as culinary herbs and medicinal plants (Paton et al., 1999; Werker, 2000). Typical characteristics of the family include a square stem, opposite and decussate, pellucid leaves (with many gland dots) (Paton et al., 1999). The leaf blades are simple, pinnately lobed or digitately compound, with entire or toothed margins. Flowers are arranged in compact axillary or verticillate cymes or, more rarely, solitary in the leaf axils. These inflorescences may be congested (*Pycnostachy*s). Flowers are sympetalous, pentamerous with didynamous stamens, bicarpellate, deeply divided gynoecium and a gynoblastic style. Fertilised carpels develop into small nutlets which are surrounded by a persistent calyx that has five lobes or teeth (Paton et al., 1999).

Lamiaceous plants are known by their characteristic zygomorphic flowers, although actinomorphic flowers that are four- or five- lobed do occur in some genera (Cantino et al., 1992; Paton et al., 1999). The corolla is typically tubular and bilabiate, with a two-lobed upper lip and a three-lobed lower lip that is coloured and often hairy. Flowers are bisexual, and rarely unisexual, with stamens arising at the corolla mouth or in the tube characteristically exserted from the tube of the corolla as in *Hemizygia* (Cantino et al., 1992). Another characteristic of plants in the family is a superior ovary located on an entire or lobed disc, with the style arising from the base of the pistil or terminal (Paton et al., 1999).

The Lamiaceae has approximately 240 genera and 6500 species and occur almost all over the world, except in the coldest Polar Regions (Cantino et al., 1992). The family is well represented in the tropical and temperate regions, with the majority of the species found in the Mediterranean and approximately 60 genera with about 980 species occurring in tropical upland savannas such as those found in the sub-Saharan African region (Cantino et al. 1992; Klopper et al., 2006). There are about 35 genera with approximately 255 species in South Africa occurring predominantly in the areas with summer rainfall. Habitats vary greatly from dry, rocky,

woodland or grassland to wet habitats, which include seasonally flooded regions and areas along riverbanks (Klopper et al., 2006).

Many plants of the family are of great economic importance in many countries because they are valued for the essential oils they produce (Werker, 1993; Paton et al., 1999; Fahn, 2000; Valant-Vetschera et al., 2003).

2.2 Essential oils

Essential oils, also known as ethereal or volatile oils are defined as aromatic oily liquids derived from a plant material (Guenther, 1948). Hedges and Lister (2007) argue that the term 'essential oil' is misleading since it is not vital or indispensable as the word 'essential' describes the importance of 'essential fatty acids'. Instead, the word describes the importance of the oil in representing the characteristic nature of the plant material, particularly in terms of its smell and taste. It is also pointed out that these oils are not food oils which are made up of a glycerol backbone and three fatty acids. These essential oils are comprised of terpenes, alcohols, phenols, ketones, acids, esters and oxides, amongst others and they are like other oils; immiscible in water and liquid at room temperature (Hedges and Lister, 2007).

In a review, Burt (2004) reported that approximately 3000 essential oils have been discovered thus far and from these, about 300 are of commercial importance. These oils have been exploited for use in the food and perfume industries where they provide flavours and fragrances. Other studies on the oils have determined that they possess antimicrobial and antifungal (Guenther, 1948; Holm, 1999; Fawole et al., 2009a), antiviral (Bishop, 1995), anticarcinogenic (Burger, 1998; Holm, 1999), antiparasitic (Pandey et al., 2000), and insecticidal (Konstantopoulou et al., 1992; Holm, 1999) properties. Antioxidant and anti-diabetic activities have also been observed (Edris, 2007). It has been suggested that the characteristics exhibited by these plant extracts are related to the function of particular compounds in the essential oils produced by the plants (Mahmoud and Croteau, 2002; Steenkamp, 2006).

Over 100 chemical compounds were identified at varying concentrations in the essential oils extracted from the plants studied in the genus *Ocimum* (Hiltunen, 1999). These compounds are responsible for the medicinal properties of most plants in the genus. The medicinal properties are attributed to the presence of compounds that are phenyl propane derivatives or terpenoids, such as linalool, eugenol, estragol, methyl chavicol, methyl eugenol, geraniol, geranial, 1,8-cineole, camphor, bornyl acetate, germacrene-D, E-myroxide, germacrene-B, caryophylene oxide and p-cymene (Grayer et al., 1996; Hiltunen, 1999; Carović-Stanko et al., 2010; Runyoro et al., 2010).

The essential oils of many plants, especially in the genus *Ocimum*, that were previously only used as traditional medicinal remedies have been investigated and now have a place in the commercial food, flavour or fragrance industries (Hiltunen and Holm, 1999). The commercial value of the essential oils produced and secreted by the glandular trichomes of *O. obovatum* that are only traditionally exploited as medicinal remedies need to be investigated. For example, Carović-Stanko et al. (2010) reported that essential oils that represent a true estragolchemotype are suggested to be carcinogenic and genotoxic in nature while compounds such as camphor which has been used as an antibacterial agent especially in ancient times where it was used in embalming practices and as an insect repellent. Numerous studies have been conducted on the anticarcinogenic properties of leaves of *O. sanctum*. Camphor is the major compound produced by *O. kilimandscharicum* (Carović-Stanko et al., 2010; Runyoro et al., 2010) and is the active ingredient in modern medicinal products including the Vicks® VapoRub® range (Paul et al., 2010).

The essential oil compound, eucalyptol (1,8-cineole) isolated in many *Ocimum* plants, is commonly used in the flavour, fragrance and cosmetic industries (Carović-Stanko et al., 2010). Limonene, one of the most common terpenes identified in nature and common in plants of the genus *Ocimum*, is the key constituent of citrus oils, eucalyptol and the major component of essential oils of tea tree (*Melaleuca alternifolia* L.) and is responsible for the suppression of inflammatory mediator production by activated human monocytes (Carović-Stanko et al., 2010). The importance of the antibacterial activity of the chemical constituents of the essential oils produced and secreted in the plants of the genus *Ocimum* are highlighted by their common use as natural preservatives in the food industry (Mäkinen and Pääkkönen, 1999; Carović-Stanko et al., 2010; Runyoro et al., 2010).

It is interesting to note that recent studies involve the use of compounds such as the terpenoids found in essential oils. Terpenoids have a significant ability to be absorbed through skin suggesting its possible implementation as a vector for transdermal drug delivery (Edris, 2007).

Basil oil is popular commercially with the highest production of 15 tons in India as recorded in 1999 (Hiltunen and Holm, 1999). The oils are also produced in smaller but significant quantities in countries like Bulgaria, Egypt, Pakistan and Israel (Lawrence, 1992). While the essential oils produced and secreted by *O. obovatum* have not been isolated and identified, Hiltunen (1999) and Hiltunen and Holm (1999) provided a general overview of the chemical composition of essential oils found in *Ocimum* species. Extensive studies on *Ocimum* oils, especially *O. basilicum*, have yielded sufficient data to characterize the oils produced and secreted by the plants. In general, the oils are made up of oxygenated monoterpenes that include linaool, camphor, cineole, geraniol and thymol and phenylpropane derivatives such as methyl chavicol and eugenol (Hiltunen and Holm, 1999).

Many species are also rich sources of terpenoids, iridoid glycosides and flavonoids that accumulate in considerable quantities in the various parts of the plants. These essential oils are produced in the specialised epidermal vestitures known as glandular hairs or trichomes that cover the aerial parts of the plants (Valant-Vetschera et al., 2003; Wagner et al., 2004; Hedges and Lister, 2007; Schilmiller et al., 2008).

2.3 Specialised epidermal cells and epidermal vestiture

An array of epidermal cells are found on the aerial and foliar organs of different angiosperms; the highly vacuolated bulliform cells present in the leaves of some monocotyledons (which are responsible for unrolling or rolling the leaves for the uptake or loss of water) and cytstoliths (which are complex, stalked, irregularly shaped mineral depositions of calcium carbonate over an internal cellulosic framework in the epidermis in hairs or enlarged cells) (Dickison, 2000). While some plants may have a perfectly smooth, non-sculptured or glabrous epidermal surface,

many other plant species are pubescent or hirsute, possessing a well-developed indumentum made up of hairs or trichomes (Wagner, 1991; Dickison, 2000).

According to Werker (2000), trichomes have been distinguished as hairs, scales and bristles according to their shape; exemplifying the liberal use of the term 'hair'. Based on their structure and development, trichomes constitute an intermediate group between papillae and emergences (Werker, 2000). Barthlott and Ehler (1977) attempted to distinguish trichomes from other epidermal appendages by using the ratio between the width and height of the appendage and the arbitrary decision on the boundary between the two. Others went on to distinguish a trichome on the basis of the length of the stalk; if the length of the stalk is more than half the height of the head then it is classified as a capitate trichome (Abu-Asab and Cantino, 1987).

2.4 Trichomes

Studies on homologous trichome morphology and distribution provide important information regarding specific, generic, tribal and subfamilial relationships of many plants. However, certain hair types are analogous and have evolved independently in different phyletic lines. Because they are a result of convergent evolution, they are deemed to be of lessened systematic value (Dickison, 2000). It is important to note that a distinction be made between the nature of the indument and the structure and colour of the individual hairs when describing trichomes since these play a significant role in the identification of plants in a genus, especially hybrids. Dickison (2000) has reported that despite the use of trichomes in classical systematics, there is still no satisfactory classification or categorisation for the vast structural diversity of the hairs on plant surfaces.

Trichomes are defined as small unicellular or multicellular protrusions of epidermal origin found only on the surfaces of leaves and various organs of many plant species (Werker, 2000). These trichomes are further distinguished by their growth on aerial or subterranean parts of the plant, with the latter consisting almost exclusively of root hairs (Werker, 2000). Trichomes vary greatly between tissue types and species in terms of their morphological characteristics (Wagner, 1991; Werker, 1993; Dickison, 2000; Fahn, 2000; Werker, 2000). Trichomes have been

described using a variety of terminology over the years. Some of the terms include dendritic, hooked, scaly, spiral and rounded (Metcalfe and Chalk, 1950, 1979; Uphof 1962; Fahn 1990). In 1978, Payne published the first English glossary describing and illustrating the different morphological characteristics of individual trichomes and terminology such as indumentums or pubescence (see Figure 3).

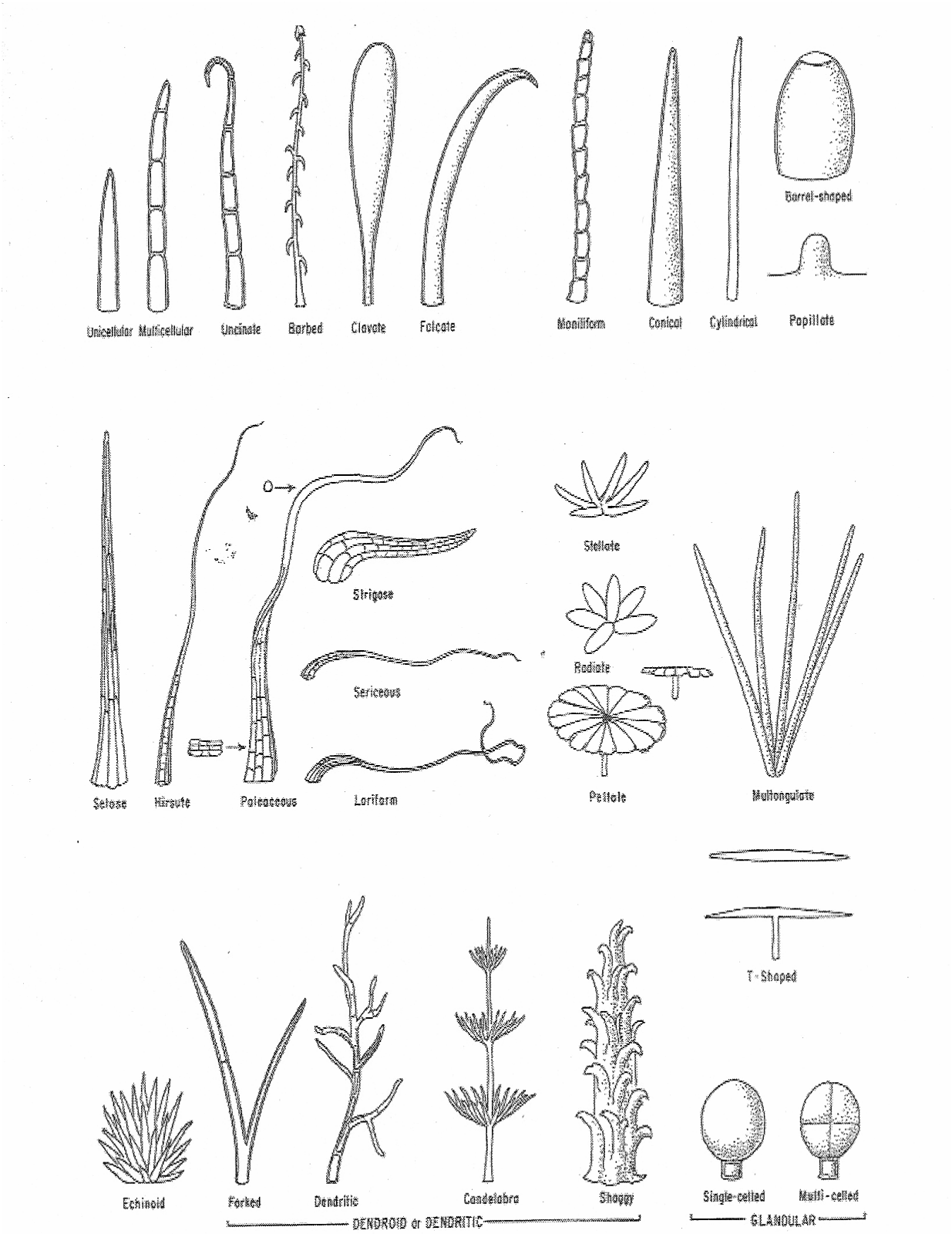


Figure 2: Varying morphologies of glandular and non-glandular trichomes characteristic of different plants. Trichomes can be unicellular, multicellular, uncinate, barbed, clavate, falcate, moniliform, conical, cylindrical, barrel-shaped, papillate, setose, hirsute, paleaceous, strigose, sericeous, loriform, stellate, radiate, peltate, multongulate, T-shaped, echinoid, dendroid (forked, dendritic, candelabra and shaggy) and single- or multi-celled glandular. (Adapted from Dickison, 2000 and Payne, 1978).

While entire families or genera may be delimited by a single trichome type, some families exhibit a great diversity in trichome morphology (Dickison, 2000; Werker, 2000). For example, the commonly cultivated tomato and potato plants possess uni- and multicellular and glandular trichomes on the same leaf, while the trichomes of *Verbascum thapsus* and *Platanus* possess multicellular, branched hairs (Dickison, 2000). Other examples include the stinging hairs of members of Urticaceae, the scale hairs that are typical of the Bromeliaceae, the stellate and tufted hairs of certain Malvaceae and the T-shaped unicellular hairs of the tropical Malpighiaceae (Dickison, 2000).

In the monocotyledonous family, Bromeliaceae (Pineapple family), all members possess trichomes on some part of the plant surface (Versieux et al., 2010). The epiphytic subfamily Tillandsioideae has leaves with its entire surfaces covered by a dense layer of elaborate absorbing trichomes that facilitate the rapid movement of water and minerals into the shoot (Dickison, 2000). This interesting example of a structural and functional relationship has enabled certain species to survive without any roots.

The morphology of the trichomes has been used to delineate species, especially in studies of hybrids. Therefore, the use of pubescent characters to support the generic relationships and differences together with other characters has been clearly established in families such as Asteraceae, Icacinaceae, Goodeniaceae and Poaceae (Dickison, 2000).

Most trichomes can be classified as glandular or non-glandular. However, there are some trichomes that remain ambiguous. An example is the compound glandular hairs of the lamiaceous genera *Meriandra*, *Phlomis*, *Hyptis* and *Rosmarinus officinalis*. These hairs appear to be similar to branched non-glandular hairs but they all possess one glandular branch (Bokhari and Hedge, 1971; Rudall, 1980; Azizian and Cutler, 1982; Werker et al., 1985a). Werker et al. (2000) also distinguishes between non-glandular and glandular trichomes based on the degree of cutinisation of the cells; non-glandular trichomes have thick, highly cutinized cells while glandular trichomes have thin, non-cutinized cells.

2.4.1 Non-glandular trichomes

Non-glandular trichomes are classified according to their morphology, anatomy and microstructure since they display great diversity (Fahn, 2000; Werker, 2000). These trichomes may be uni- or multi-cellular, uniseriate, biseriate or multiseriate; they may differ in size, length and cell shape and can be symmetrical or asymmetrical (Payne, 1978). They may be uniform in width or tapering and they may be present articulating on the leaf surface or their cross-walls may not be as easily distinguishable on the surface. The wall thickness and materials embedded in the walls may vary contributing to the hairs being soft or stiff as in bristles. Hairs may also have micro-ornamentation such as small granules on them giving them a 'warty' appearance (Payne, 1978; Werker, 2000).

Non-glandular trichomes are often found at different regions on leaves and this has implications for their functions. These hairs are thought to play a major role in protecting the plant from external factors such as herbivores and pathogens (Werker, 1993; Fahn, 2000; Dickison, 2000; Werker, 2000). The hairs also play a role in deflecting light when the leaf is extensively exposed and they also trap air to prevent freezing during excessively low temperatures and prevent excessive water loss on a hot day (Werker, 2000). Levin (1973) has shown a correlation between non-glandular trichome density and insect pest resistance highlighting the function of the trichome as a mechanical barrier.

2.4.2 Glandular trichomes

Glandular trichomes are secretory structures classified according to chemical composition of the secretion, their mode of production, structure, location and, most importantly, function (Wagner, 1991; Werker, 2000). They can be uni- or multi-cellular, uniseriate or multiseriate and vary in shape and size. Unicellular glandular trichomes are morphologically distinguished by observing the apical and basal area of the trichome. However, this distinction may only be at the ultrastructural level (Werker, 2000). Multicellular, uniseriate glandular trichomes normally

display morphologically different cells along the hair while multiseriate glandular trichomes may display this characteristic across the trichome.

While multicellular glandular trichomes exhibit numerous structural variations, Werker (2000) describes a typical one to consist of a terminal secretory head with one or more secretory cells, a stalk of one or more cells and a base with one or more cells. Some trichomes also possess a neck cell that is morphologically different from the secretory head and the stalk that it separates (Werker, 2000). Differences between the groups of cells in trichomes are based on morphological and ultrastructural studies, but this is insufficient since there is a lack of information about the ultrastructure and function of the different groups of cells and the different cells of each group that make up the hair (Werker, 2000). Werker and Fahn (1981) accounted for some differences that exist between the different cells of a stalk and those within a secretory head. Their study on the foliar secretory trichomes of *Inula viscosa* revealed that the lowermost stalk cells can be highly vacuolated and the uppermost cells may have a dense cytoplasm. Different organelles (plastids, vesicles and Golgi bodies) were also observed for each pair of cells of the secretory head.

Many species possess both glandular and non-glandular trichomes and more than one type of each may also be present on the same part of the plant (Werker and Fahn, 1981). It is speculated that the non-glandular hairs, when present as a dense indumentum, also serve to protect glandular trichomes that are more energy-consuming and found below the hairs (Werker, 2000). In most species of the Lamiaceae, there are two main types of glandular trichomes present; peltate and capitate glands that have been so-named according to the shape of their secretory heads (Werker, 2000).

2.5 Taxonomic History

The taxonomy and nomenclature of *Ocimum* and its associated species is somewhat confusing despite being an economically and medicinally important herb (Paton et al., 1999). In order to communicate information about a plant, its uses and relationships, it is imperative that each species is clearly identified and has only one name. Rules are thus set in place to ensure this (*The International Code of Botanical Nomenclature*) (Paton et al., 1999). However, literature concerning *Ocimum* has failed to adhere to these rules resulting in the same species being referred to by more than one name (Paton et al., 1999; Williamson and Balkwill, 1995).

First described by Linnaeus in 1753 who listed five species, *Ocimum* was later recognised with about 40 species by Bentham (1832) who went on to divide the genus into three sections:

- *Ocimum* [*Ocymodon* Benth.] with appendiculate posterior stamens,
- *Hierocymum* Benth. with hairs at the base of posterior stamens, and
- *Gymnocymum* Benth. with glabrous posterior stamens.

Hierocymum and *Gymnocymum* consisted of a few species that have now been placed into the genus *Endostemon* N.E.Br. or *Hemizygia* (Benth.) Briq. In 1848, Bentham further subdivided *Ocymodon* into three subsections based on the morphology of the calyx: *Ocimum* (*Basilica sensu* Briquet, 1897) with an open and bearded throat of the fruiting calyx, *Gratissima* with the throat closed by the median lobes of the lower lip which is being pressed against the under surface of the upper lip, and *Hiantia* Benth. with truncate lateral calyx lobes that included species that were sometimes placed in *Becium* (Paton, 1995). A fourth section, *Hemizygia*, was also added (Bentham, 1948) but Briquet (1897) considered it to be a separate genus due to the fused anterior stamens.

A revision of African species (Paton, 1992) resulted in the recognition of approximately 30 species using Bentham's infrageneric classification of *Ocimum* with the section *Hemizygia* and subsection *Hiantia* removed. This classification was supported by Cantino et al. (1992) who studied the nutlet characters and then conducted a morphological analysis of the pollen; which Bentham did not consider. Despite these revisions, this classification is still plagued by confusion. For example, *Ocimum circinatum* A.J. Paton and *O. lamiifolium* are still anomalous with neither conforming to the classification categories appropriately (Paton, 1992). Other infrageneric classifications have been made (Pushpangandan, 1974; Pushpangandan and Bradu, 1995; Sobti and Pushpangandan, 1979). However, these have also been plagued by many problems (Paton et al, 1999). According to Paton et al. (1999), the study of New World species of *Ocimum* by Epling in 1936 is the most recent. Epling (1936) recognised 12 species, of which four are regarded as pantropical that are widely cultivated (Paton et al, 1999).

Ocimum L. belongs to the subfamily Nepetoideae, which are strongly aromatic due to the production of essential oils made up of monoterpenes, sesquiterpenoids and phenylpropanoids (Paton et al., 1999). *Ocimum* (along with *Lavandula*, *Orthosiphon* and *Plectranthus*) placed in the tribe Ocimae, which differs from other economically important herbs such as *Rosmarinus*, *Salvia* and *Thymus* that are placed in the tribe Mentheae. According to Paton et al. (1999), plants of the tribe Ocimae have declinate stamens; the stamens lie over the anterior lip of the corolla instead of ascending under the posterior lip.

The tribe Ocimae was divided into three subtribes in 1897 by Briquet where *Ocimum* was placed in the subtribe Ociminae. Characteristics of this subtribe include a small, flat anterior corolla lip with stamens and style ascending towards the posterior lip of the corolla (Paton et al., 1999). In 1992, Ryding further divided the subtribe Ociminae into three informal groups that saw *Ocimum* being grouped with *Becium* Lindl., *Erythrochlamys* Gürke, *Hemizygia* (Benth.) Briq., *Syncolostemon* E. Mey. ex Benth. and *Catoferia* (Benth.) Benth. with Paton (1992) insisting the addition of *Orthosiphon* Benth. subgen. *Nautochilus* (Bremek.) Codd due to the possession of many of the characters found in *Ocimum*.

The tribe is also essentially a tropical one, requiring warmth for growth and protection from frost, with *Ocimum* occurring naturally in tropical America, Asia and Africa (Cantino et al., 1992; Paton et al., 1999).

Species that are most heavily exploited for essential oil production and use as pot herbs include *O. basilicum* (Sweet Basil), *O. americanum* and their hybrid *O.* X *citriodorum* (Lemon Basil) (Paton et al., 1999). Plants exploited in the tropics include *O. kilimandsharicum*, which is extensively cultivated for camphor production, and *O. forskolei* (among others), which is used in traditional medicine and as a pot herb (Demissew and Asfaw 1994). *Ocimum viride* and *O. suave* are also indigenous to parts of Africa where they are traditionally prescribed as expectorants and also exhibit a range of antimicrobial properties (Hiltunen and Holm, 1999). Another species of economic importance is *O. gratissimum*. Essential oil produced from this plant is used in traditional medicine as well as an insecticide (Lawrence, 1992; Githinji and Kokwaro, 1993).

The essential oils of *Ocimum* also exhibit insect repellent activity that could be useful in countries with a warmer climate. Recent studies on the fixed oils found in *Ocimum* seeds have also identified anti-inflammatory activity (Singh and Majumdar 1997; Singh et al., 1996; Mediratta et al., 2002). However, despite the various pharmacological activities attributed to essential oils of *Ocimum*, the plants are mainly used as aromatic ornamentals and spices (Hiltunen and Holm, 1999).

2.6 *Ocimum obovatum*

Ocimum obovatum was transferred to *Becium* in 1901 by N.E. Brown who also reduced *O. galpinii* Gürke and *O. hians* Benth. to varieties of *Becium obovatum* (E. Mey ex Benth.) N.E. Brown. The genus *Becium* (described by Lindley in 1842) contained 35 species distributed in Africa, Madagascar, Arabia and a single species in India (Paton et al., 1999). *Becium obovatum* was later reduced to a variety of *Becium grandiflorum* (Lam.) *Pic. Serm.*by Sebald in 1989; since he failed to find any diagnostic characters to distinguish the two species. Despite this, Paton (1995) and Williamson and Balkwill (1995) continued to recognise the two species as distinct

based on the presence of a nectar gland at the base of each cyme and elongated, parallel thecae (Paton et al., 1999). Paton et al. (1999) examined the classificatory relationships of *Ocimum* and deduced that the distinctions between *Ocimum* and *Becium* cannot be maintained when New World species of *Ocimum* are considered. A number of New World species of *Ocimum* possess the nectar gland but also possess divergent thecae. These difficulties in classification were resolved by parsimony analysis that transferred species from *Becium* to *Ocimum* while recognising the level of subgenus and section of groups rather than the genus (McCallum and Balkwill, 2004; Paton, 1999).

2.6.1 Distribution

Ocimum obovatum is generally variable in habitat being widespread in tropical and eastern southern Africa. In tropical Africa, *O. obovatum* grows in wetland or dambo and riparian areas and is an indicator species for these areas (Pote, 2008). These areas are characterized by the presence of grasses, segdes and rushes when contrasted to the woodland and miombo areas (Pote, 2008). Dambos are substantially dry with grey or black clay soils and retain wet lines of drainage through the dry season. During the wet season, these areas are waterlogged but not above the height of the vegetation. Riparian areas are characterized by their close proximity to rivers (Pote. 2008). Howard-Williams (1970) reported the presence of *O. obovatum* (referred to as *B. obovatum* in the study) together with *B. homblei* (De Wild.) Duvign. & Plancke in areas of central Africa with high copper concentrations in the soil. The study concluded that while the plants can tolerate a wide range of edaphic conditions, *B. obovatum* appeared to be less tolerant to toxic conditions than the more competitive *B. homblei* (Howard-Williams, 1970).

Ocimum obovatum has been reported to occur in areas of South Africa referred to as serpentine, a term applied to describe rocks derived from serpentinite with high concentrations of heavy metals (nickel and chromium and high magnesium to calcium ratios) (Williamson and Balkwill, 2006). There are about 80 serpentine outcrops in south-eastern Mpumalanga and the vegetation present in this area is distinct (Williamson and Balkwill, 2006). In KwaZulu-Natal, the species is a common early flowerer in mesic grasslands where the grasses are relatively short. In South Africa, the plant is a common sight along the grasslands of the KwaZulu-Natal midlands and in the Western Cape (Mukoma, 2005).

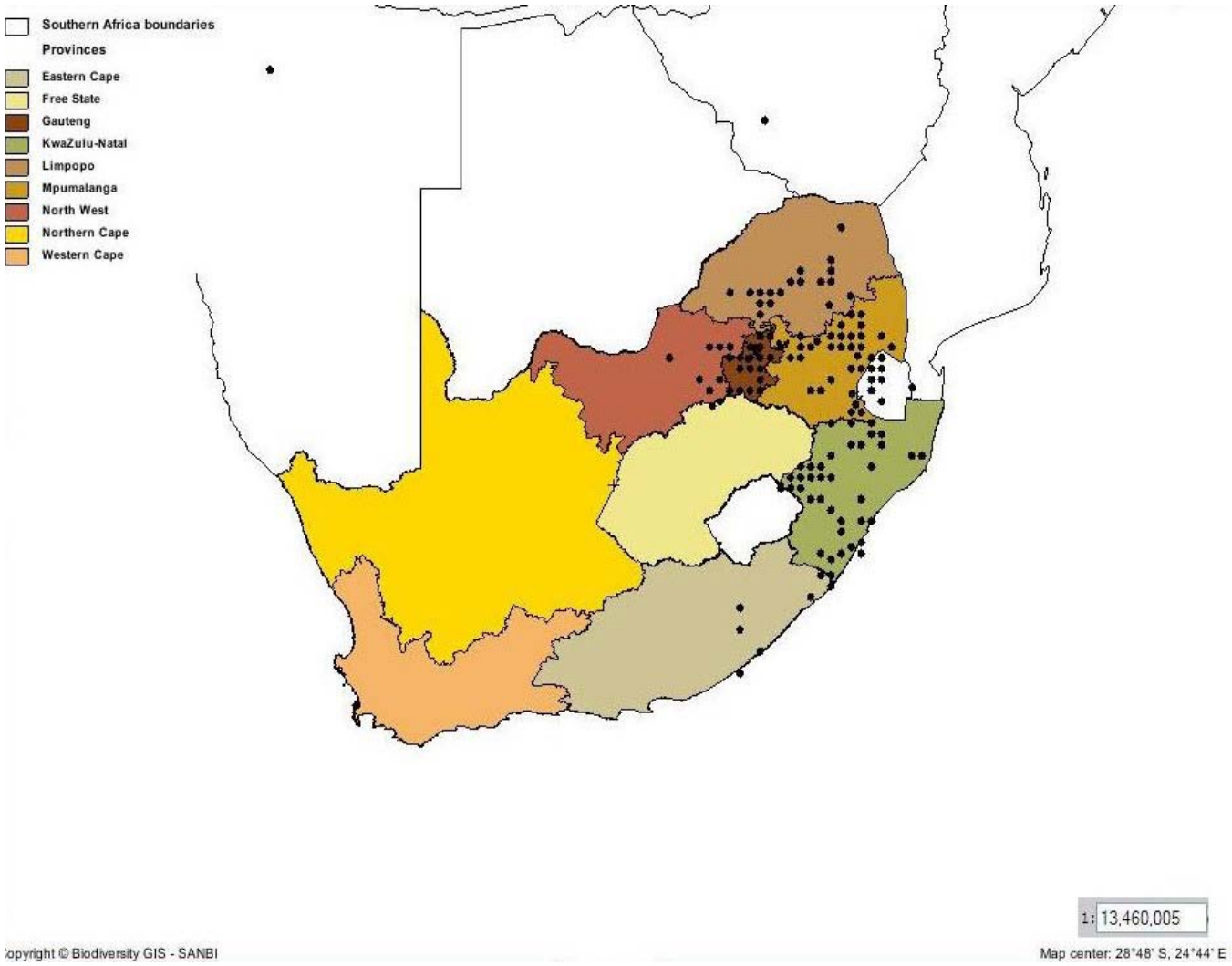


Figure 3: Map of South Africa showing distribution of *O. obovatum* (black circles). Constructed by the author using PRECIS distribution data. The map was generated using the Biodiversity-GIS distribution of species tool (www.sanbi.org).

2.6.2 Medicinal properties, uses and preparations

A preliminary study on the antimicrobial activity of *O. obovatum* by Fawole et al. (2009a) indicated that a 70% ethanolic extract of the leaves of the plant showed a pronounced effect against bacteria most often implicated in gastro-intestinal ailments, especially diarrhoea in children (Hutchings et al., 1996). This finding is consistent with the leaf extracts of other plants in the genus. Carović-Stanko et al. (2010) and Runyoro et al. (2010) studied the composition and antibacterial activities from several *Ocimum* taxa. The essential oils of *Ocimum* species *O. americanum* L., *O. basilicum* L., *O. chamechianum* Mill., *O. x citriodorum*, *O. kilimandscharicum* Baker ex. Görke, *O. lamiifolium* and *O. suave* and three botanical varieties and cultivars of *O. basilicum* ('Genovese', var. *purpurascens* and var. *difforme*) were analysed using gas chromatography coupled with mass spectrometry. These were then assessed for antimicrobial activity on cultures of gram-positive bacteria that included *Staphylococcus aureus, S. epidermidis, Listeria monocytogenes, L. ivanovii, Escherichia faecalis, E. faecium, Streptococcus mutans, S. viridans* and gram-negative cultures of *E. coli, Proteus mirabilis, Enterobacter cloacae, Klebsiella pneumoniae and Pseudomonas aeruginosa*. *Candida albicans, C. tropicalis* and *C. glabrata* were also assessed. The results indicate a great difference in the composition of the essential oils produced in the various plants, as well as varying degrees of antibacterial activity. It has been suggested that the antibacterial activity displayed by *O. obovatum* leaves could be the result of the presence of a particular compound or many different compounds (Steenkamp et al., 2006). Records from as far back as 1960 confirm the presence of antimicrobial substances in the essential oils of basil plants with eugenol responsible for the inhibitory effect on *Bacillus subtilis, Salmonella enteritidis, S. aureus, P. aeuruginosa, P. vulgaris* and *E. coli* (Mäkinen and Pääkkönen, 1999).

Other medicinal properties of the plant *O. obovatum* include its role in the treatment of cancer as well as its traditional use as a remedy for hair loss. While *O. obovatum,* then recognised as *B. grandiflorum,* has been documented to be used by the Zulu for its hair restorative properties (Hutchings et al., 1996), there has been no studies on the extract and its biochemical activities in the role of hair restoration. The chemicals isolated from *O. obovatum* (referred to as *B. grandiflorum* in the study)include isothymusin, an anti-fungal constituent that could also contribute to the hair restorative properties of the leaf extract (Grayer and Veitch, 1998).

Ocimum obovatum has been used in conjunction with four other traditionally used plant species indigenous to the KwaZulu-Natal province of South Africa to successfully treat two cases of cancer (invasive breast cancer and buccal cavity cancer) in the mid-1900s (Burger et al., 1998). Since then, studies on human lung carcinoma cells have reported a pronounced *in vitro* response to the extract of the plant with the induction of the formation of numerous intra-cytoplasmic vesicles in cancer cells when exposed to a hot water extract of the powdered root bark of *O. obovatum* (Burger et al., 1998). These intra-cytoplasmic vesicles were discovered to grow until they eventually inhibited cell division and cell growth of the tumours. Different combinations of the hot water extracts of the five plant species were also tested on melanoma cells and it was found that a combination of the extracts of *O. obovatum* and a *Crinum* sp. displayed a synergistic cytoxicity (Burger et al., 1998). Chemical screening of the root bark of the plant, *B. grandiflorum*, has revealed the isolation and characterisation of two triterpenoid saponins, beciumecine 1 and 2, that are most likely responsible for the typical response in cancer cells (Burger et al., 1998).

In west tropical Africa, products from the root are used to produce medicines that promote general healing as well as to treat intestinal infections by serving as laxatives or painkillers. Some people also prescribe the root as a genital stimulant or depressant, while infusions of the plant root have been used to treat tumours and cancers (Burkhill, 1985).

2.6.3 Chemistry

Earlier chemical studies on *O. obovatum* (referred to as *B. grandiflorum*) by Grayer and Veitch (1998) revealed the presence of a major vacuolar flavonoid, the 8-*O*-glucoide of isothymusin (5,8,4'-trihydroxy-6,7-dimethoxyflavone) and major external flavonoids, isothymusin and cirsimaritin (5,4'-dihydroxy-7,8-dimethoxyflavone) present in the diethyl ether surface wash of the plant. The presence of 8-methoxylated surface flavones, and their corresponding 8-hydroxylated derivatives as precursors, is common in the Lamiaceae but the accumulation of 8-hydroxylated external flavones is a rare occurrence (reported only in the genus *Scutellaria*). Therefore, the presence of isothymusin in *O. obovatum* is both biogenetically and chemotaxonomically significant (Grayer and Veitch, 1998).

Grayer and Veitch (1998) debated the possible explanations for the presence of a hydroxylated isothymusin, isothymusin and cirsimaritin and settled on the probability of hydroxylation at the C-8 of cirsimaritin, which is common in the genus *Ocimum*. It was also presumed that isothymusin is a precursor of 5,4'-dihydroxy-6,7,8-trimethoxyflavone, commonly known as xanthomicrol, that has been found in some *Ocimum* species (Grayer and Veitch, 1998).

2.6.4 Toxicology and pharmacology

According to the database of the Medical Research Council of South Africa (MRC, 2010), no proper study has been conducted on the toxicology or pharmacology of *O. obovatum* extracts. This gap in the information needs to be bridged in the light of recent discoveries of adverse reactions and potentially fatal side- or long-term effects of the use of traditionally prescribed medicinal plants.

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant Material

Established plants of *O. obovatum* were purchased from a local nursery (Tropical Nursery, Durban) and propagated in a shaded nursery. Leaves from these plants were used for the microscopic analyses. Leaves selected were classified as emergent (length: 10 to 29 mm from the first node), young (length: 30 to 50 mm from the second to fifth node), and mature (> 50mm from the fifth node). During September 2009, plants were harvested from the wild at the site of World's View in Pietermaritzburg, KwaZulu-Natal (29°37′0″S, 30°23′0″E) and a voucher specimen (Kasim 1) was deposited in the Ward Herbarium at the University of KwaZulu-Natal (Westville campus).

3.2 Microscopy

3.2.1 Stereomicroscopy

Leaves at the different stages of development were analysed using a Nikon AZ100 stereomicroscope equipped with a Nikon Fiber Illuminator (Nikon, Japan). Images of both adaxial and abaxial leaf surfaces were captured at a magnification of 4x with a light exposure of 400 ms on the NIS-Elements Software (NIS-Elements D 3.00, SP 4 (Build 502) Copyright © 1991-2008 Laboratory Imaging. Hasp ID: 55A 99202).

3.2.2 Scanning and Environmental Scanning Electron Microscopy (SEM and ESEM)

Leaf specimens from the different developmental stages were super-cooled in boiling liquid nitrogen at -260°C in an EMSCOPE SP 2000 and then placed in liquid nitrogen before they were

freeze dried in an Edwards Modulyo freeze dryer for five nights at -60 °C at a vacuum pressure of 10^{-2}Torr. The specimens were then secured onto brass stubs with double-sided carbon tape and sputter-coated with gold (at a vacuum of 0.1 Torr for 2.5 minutes) using a Polaron SC 500 Sputter Coater (Quorum Technologies Ltd., United Kingdom). The specimens were viewed using a LEO 1450 (Jeol, Germany) scanning electron microscope at an EHT of 5.00 kV and a probe size of 5 pA at varying working distances.

In addition, fresh leaves were also viewed with a Philips XL 30 environmental scanning electron microscope (ESEM). Leaves were excised (5 mm^2) and mounted on brass stubs and imaged in the microscope while still biologically functional under a low vacuum of 0.9 Torr at an EHT of 15.00 kV with a probe size of 3.9 pA at varying working distances. This had to be done quickly to acquire images before the leaves began deteriorating under the low vacuum.

3.2.3 Light Microscopy/ Transmission Electron Microscopy (TEM)

Two methods were employed to prepare the leaf material for sectioning and viewing with a TEM:

Fresh leaves sections (at approximately 3 mm^2) were excised from the apical, mid and basal regions of the leaves at the different developmental stages, i.e. emergent, young and mature, using a sterile blade. These sections were primarily fixed in a phosphate buffered solution (1 M) of 2.5% glutaraldehyde for 24 hours at 4°C. The sections were then subjected to three 5 minute washes with phosphate buffer before being post-fixed with 0.5% osmium tetroxide (made up in 0.1 M phosphate buffer) for 1 hour. The sections were then washed another three times with phosphate buffer for 15 minutes and then dehydrated by being placed into two 5 minute acetone baths each at the different concentrations (30%, 50%, and 75%). The sections were then subjected to two 10 minute dehydrations in 100% acetone before they were infiltrated with resin by placing the sections in equal parts of Spurr's resin (Spurr, 1969) and 100% acetone for 4 hours. The samples were then placed into 100% resin (Spurr's, 1969) and allowed to infiltrate

for 20 hours. Infiltrated samples were then orientated in a mould in whole resin and polymerized for 8 hours at 70°C.

Another batch of fresh leaves was also prepared using Todd's fixative (Todd, 1986) as a primary fixative. The excised sections (3 mm^2) were left in the fixative overnight before being washed with phosphate buffer and post-fixed in osmium tetroxide. The tissue was then infiltrated with resin as above; samples were orientated in the moulds with 100% Spurr's (1969) resin and polymerized for 8 hours at 70°C

The prepared resin blocks, were then sectioned with glass knives in a Reichert Jung Ultracut-E ultramicrotome (Reichert, Inc., USA). Monitor sections were stained with Toluidine Blue-O (Feder and O'Brien 1968) and viewed with a Nikon Eclipse ATI compound light microscope (Nikon, Japan) equipped with a Nikon DS-Fi1 camera and NIS-Elements imaging software package (NIS-Elements D 3.00, SP 4 (Build 502) Copyright © 1991-2008 Laboratory Imaging. Hasp ID: 55A 99202). Once trichomes were isolated, ultrathin sections of 80 nm were picked up on copper grids before being stained with a 2.5% saturated solution of uranyl acetate and then Reynold's (1963) lead citrate. The copper grids supporting the ultrathin sections were placed onto large drops of 2.5% uranyl acetate and allowed to stain for 8-10 minutes at room temperature before being rinsed with cooled, freshly boiled distilled water. The copper grids were then placed on drops of lead citrate solution (Reynold, 1963) in a closed Petri dish with dry NaOH pellets (to prevent any moisture or excessive oxygen from causing precipitation of the stain) and allowed to stain for 5-10 minutes at room temperature. The grids were then rinsed with distilled water and placed on filter paper, ready for viewing for with a Jeol 1010 (Japan) TEM equipped with an Olympus MegaView III CCD camera (Soft Imaging System GmBH, Münster, Germany).

3.3 Wax Sections

Fresh, hand-cut sections and wax sections were used for histochemical tests. Wax sections were prepared by fixing excised leaf samples (~ 3 mm^2) in formaldehyde (70%; aqueous) for 48

hours. These were then subjected to dehydration with three incubations in 70% ethanol for 30 minutes each, 2 hours in 80% ethanol, and 4 hours in 90% ethanol. The excised leaf samples were then subjected to incubations in 100% ethanol for 90 minutes, 1 hour, overnight and then 2 hours. The leaf 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 minutes each. Paraplast Plus® wax pellets (McCormick Scientific) were then added and left for 3 hours at a time for 12 hours in total. A final addition of wax was added for three hours readying blocks for sectioning.

Sections were de-waxed by subjecting them to three 2 minute baths of xylene (100%), three 2 minute baths of ethanol (100%), a 2 minute bath of ethanol (70%) and a final 2 minute bath of tap water. The de-waxed sections were then subjected to the histochemical stains.

3.4 Histochemistry

De-waxed and fresh, hand-cut sections were stained to detect the presence of alkaloids, essential oils, lipids, pectinaceous substances and phenolics.

Alkaloids (Furr and Mahlberg, 1981)

To test for the presence of alkaloids, leaf sections were stained for 10 minutes each with Wagner's reagent and Dittmar reagent and an orange/brown colour was indicative of alkaloids. Wagner's reagent is made up of 1.27 g iodine and 2 g potassium iodide dissolved in 100 ml dH_2O and Dittmar reagent constitutes 1 g of potassium iodide, 1 g of sodium nitrite, 30 ml HCl and 30 ml dH_2O.

Leaf sections pre-treated with tartaric acid were used as negative controls (Johansen, 1940).

Essential Oils (David and Carde, 1964)

Essential oils and terpenoids were determined by using NADI reagent and antimony trichloride, respectively. NADI reagent is made up of two solutions mixed in equal volumes just prior to use; Solution 1 requires 1% alpha-naphthol in 95% ethanol and Solution 2 requires 1% N, N-dimethyl-p-phenylenediamine HCl in water. Sections were incubated in the dark for 60 minutes at room temperature in NADI reagent and rinsed with sodium phosphate buffer (0.1 M, pH 7.2) for 2 minutes. This reagent oxidizes to form indophenol blue that changes colour with variation in pH, enabling the distinction between essential oils that stain blue and resin acids that appear red.

Leaf sections pre-treated with 2N HCl for 10 minutes were used as a negative control (Mace et al., 1974)

Lipids (Brundrett et al., 1991; Pearse, 1968)

Sudan III and IV (Sudan Red) and Sudan Black B were used to test for lipids. Leaf sections were immersed in Sudan III and IV for 15 minutes before being rinsed with 70% ethanol (to remove any excess stain) and then mounted in 70% glycerol. Cutinized or suberized walls or lipid inclusions stained orange to red.

Sections were flooded with a saturated and filtered solution of Sudan Black B for approximately 30 minutes and then rinsed with 70% ethanol and mounted in glycerine. All cell lipids (fats, oils, waxes, free fatty acids and phospholipids) stained dark blue to black.

The control for histochemical lipid tests was the pre-treatment of leaves with methanol and chloroform (1:1) (Lison, 1960).

Pectinaceous substances (Johansen, 1940)

Ruthenium red was used to identify pectinaceous substances in the leaf sections. Sections were immersed in an aqueous Ruthenium red solution (1:5000) for 10 minutes. A pink colouration is indicative of the presence of acidic polysaccharides such as mucilage and pectinaceous substances.

Phenolics (Johansen, 1940; Gabe, 1968)

Sections were placed in 10% ferric trichloride and a dash of sodium carbonate was added and left to stain for 15 minutes at room temperature. Ferric trichloride causes *orto*-dihydroxyphenols to react with the ferric ions resulting in the production of deep green or black deposits. Leaf sections were immersed in potassium dichromate for 10 minutes at room temperature before being rinsed with distilled water and mounted in 70% glycerol. Phenols appear brown after staining.

3.5 Crude ethanolic extract (European Phamacopoiea, 1975)

Fresh leaves were air-dried at room temperature for three days and milled to a fine powder. Approximately 5 g of powdered leaves were extracted with 50 ml of ethanol that was boiled and distilled by reflux for 45 minutes. The extract was filtered and the precipitate was reconstituted in ethanol and boiled and distilled by reflux again for 45 minutes and then added to the initial extract. This extract was stored in a light sensitive bottle at room temperature until needed.

3.6 Phytochemical analysis

Qualitative analyses on phytochemical constituents were conducted on the crude ethanolic extract of the leaves of *O. obovatum* using various standard chemical methods (Sofowara, 1982; Trease and Evans, 1987, Krishnaiah et al., 2009; Ramesh et al., 2010).

Alkaloids

The crude ethanolic extract was analysed for alkaloids using a modified Draggendorff's test, Hager's reagent and Wagner's reagent.

A drop of crude ethanolic leaf extract was spotted onto a small piece of pre-coated silica gel 60 F_{254} TLC plate (Merck) and the plate was sprayed with a modified Draggendorff's reagent that was made up by mixing two stock solutions: a) 0.6 g bismuth nitrate in 2 ml concentrated HCl and 10 ml distilled water, and b) 6 g potassium iodide in 10 ml distilled water in 7 ml concentrated HCl and 15 ml distilled water. This whole solution was diluted with 400 ml distilled water. An orange colouration of the TLC spot was indicative of the presence of alkaloids in the extract.

Hager's reagent was made up by mixing 1 g picric acid in 100 ml distilled water. The extract was treated with a few millilitres of Hager's reagent and a yellow precipitate was indicative of the presence of alkaloids.

The extract was also treated with a few drops of Wagner's reagent that was made up by dissolving 2 g of iodine and 6 g of potassium iodide in 100 ml distilled water. A reddish-brown precipitate indicated the presence of alkaloids.

Anthraquinones

The extract (50 ml) was heated with a 10% aqueous solution of ferric chloride and 1 ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. This was then further extracted with a strong ammonia solution. A deep red or pink colouration of the aqueous layer was regarded as a positive reaction for the presence of anthraquinones.

Amino acids

The extract was dissolved in a small quantity of water and subjected to ninhydrin. A positive reaction for amino acids was indicated by a blue colouration of the solution.

Carbohydrates

Carbohydrates were tested for using Molisch's test, Fehling's solution (reducing sugars) and Benedict's solution (monosaccharides).

About 300 ml of the ethanolic extract was dissolved in 4 ml distilled water and filtered. The filtrate was subjected to Molisch's test which consists of 15 g α-Naphthol in 100 ml chloroform. The formation of a reddish-brown ring is indicative of the presence of carbohydrates.

A few drops of freshly made up Fehling's solution was added to a small amount of the ethanolic extract. Fehling's solution is made up of two solutions mixed in equal volumes just prior to use: a) Copper sulphate solution prepared by dissolving 34.66 g copper sulphate in distilled water to 500 ml and; b) Alkaline tartaric solution, made up by dissolving 173 g of potassium sodium tartrate and 50 g NaOH in water, and was diluted when cold to make 500 ml.

The ethanolic extract was treated with a few drops of Benedict's solution that consists of two solutions: the first solution was made up by dissolving 173 g of sodium citrate and 100 g of sodium carbonate in 800 ml of water while heating. The solution was filtered and diluted to 850 ml. A second solution made up of 17.3 g copper sulphate dissolved in 100 ml of distilled water was added to the first with constant stirring and made up to 1 L. A reddish-brown precipitate is indicative of the presence of monosaccharides such as glucose and sucrose.

Phenolics

Approximately 50 mg of the crude ethanolic extract was diluted with 5 ml of distilled water and two drops of 5% ferric trichloride was added to the solution. A dark green colouration is taken as a positive indicator for the presence of phenolics.

Flavonoids

A few drops of 1% ammonia solution were added to the crude ethanolic extract of the leaf sample. The observation of a yellow colouration was regarded as a positive reaction for the presence of flavonoids.

The extract (5 ml) was also treated with 1 ml of 5% lead acetate solution. A flocculent, white precipitate indicates the presence of flavonoids.

Cardiac glycosides (Kellar Killiani test)

The ethanolic extract (5 ml) was mixed with 2 ml glacial acetic acid containing a drop of ferric chloride. This mixture is slowly added to 1 ml of concentrated sulphuric acid such that the acid

remains underneath the mixture. If cardiac glycoside constituents are present in the sample, a brown ring will appear.

Saponins

A foam test was conducted on the extract. The extract (1 ml) was diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. A constant layer of foam is indicative of the presence of saponins.

Steroids/Terpenoids

The crude ethanolic extract (5 ml) was mixed with 2 ml of chloroform and then 3 ml of concentrated sulphuric acid was carefully added to the mixture forming a layer. A reddish-brown colouration at the interface is indicative of the presence of terpenoid constituents in the extract.

Tannins

Braemer's test was used to test for the presence of tannins in the crude ethanolic extract. The extract (2-3 ml) was mixed with 10% alcoholic ferric chloride solution. A dark blue or greenish-grey colour indicates the presence of tannins.

Fixed oils and fats

A small quantity of the extract was pressed between filter papers (Whatman No. 1). Oil stains on the filter paper is a positive indication of the presence of fixed oils in the extract.

3.7 Gland counts, trichome frequency and peltate trichome diameter

Images acquired from stereomicroscopy and scanning electron microscopy were analysed with the iTEM software programme (Soft Imaging System GmBH, Münster, Germany). Glandular and non-glandular trichomes were counted along the lamina on both adaxial and abaxial surfaces. The diameters of the peltate trichomes were also measured on both surfaces.

3.8 Statistical analysis

The frequency of trichomes at each region of the lamina, and on each surface, was compared using a one-way ANOVA on the statistical software package SPSS for Windows (Rel. 15.0.0. 2006. Chicago: SPSS Inc). The same was done to compare the diameter of peltate and Type I capitate trichomes on both leaf surfaces across all stages of development. Post-hoc tests (Bonferroni and Tukey's) were done to make a pair-wise comparison of the trichome frequencies. A p-value of <0.05 was recognised as significant.

CHAPTER 4: RESULTS

Morphology and Distribution

The plants of *O. obovatum* subsp. *obovatum* var. *obovatum* range in habit from being little shrubs of about 20 cm found in open grasslands to larger shrubs found in dense grasslands that grow up to 30 cm high with polymorphic leaves (Figure 1). It was observed that the leaves of these herbaceous plants that grow closer to the coast are much larger and rounded when compared to the leaves of plants that grow inland at much higher altitudes (Figure 4 a and b). The leaves are simple, opposite and decussate with involuted leaf margins (Figure 5 a). The inflorescence is verticillate with flowers that are zygomorphic and labiate (flowers with corollas divided into two lip-like parts; a characteristic of the Labiatae) and appear white to lilac (Figure 5 b).

The pubescent, amphistomatous leaves of *O. obovatum* bear non-glandular and glandular trichomes on both adaxial and abaxial surfaces in all stages of development (Figure 6 a and b). The leaves appear iridescent, suggesting the presence of an oily substance on leaf surfaces (Figure 7 a). Closer examination of the leaf surface with a stereomicroscope revealed glistening and viscid globules at the tips of trichomes (Figure 7 b).

Non-glandular trichomes are single and uniseriate (Figure 8 a). These trichomes are acicular and multicellular, consisting of one to seven thick-walled cells with micro-ornamentation that gives the trichome a warty appearance (Figure 8 b). The cell closest to the basal region of the trichome is smooth and free of micro-ornamentation. This trichome base is surrounded by special subsidiary cells that protrude above the level of the epidermis and is collectively referred to as a cellular pedestal. This pedestal is formed by a group of five to eight epidermal cells arranged around the base of the non-glandular trichome (Figure 8 c).

Glandular trichomes are of two main types- peltate and capitate. When examined *in vivo* using a stereomicroscope, peltate trichomes appear as gland dots on both surfaces of leaves across all stages of development (Figure 6). Environmental scanning electron microscopy revealed that these trichomes are made up of four head cells and a very small basal cell that gives the glands the appearance of being sessile (Figure 9 a). Upon maturation of the leaf, peltate trichomes became sunken in shallow depressions (Figure 9 b).

The head cells of peltate trichomes display a smooth, bulbous, surface or a wrinkled surface (Figure 9 b and 10 a). This indicates the close attachment of the cuticle to the upper cell walls of the secretory head cells. This also emphasizes the outline of the secretory head cells or illustrates the presence of a large sub-cuticular space that is formed by the detachment of the cuticle together with the outer part of the cell wall. Cuticular rupture occurs along a predetermined line of apparent weakness in the equatorial plane of the head (Figure 10 b). However, neither cuticular pores nor pre-established regions for secretion release were observed.

The capitate trichomes of *O. obovatum* can be divided into two types based on the morphology of the glands. Type I capitate trichomes are smaller than the larger peltate trichomes and are composed of one basal cell and a head consisting of two broad head cells (33.45 ± 0.85 µm for emergent leaves and 30.2 ± 0.6 µm for young leaves) (Figure 11 a). These capitate trichomes were also observed in shallow depressions along the lamina of both surfaces. Type II capitate trichomes consisted of one basal cell, one stalk cell and a single oval head cell (Figure 11 b). Type II capitate trichomes were very rarely observed on the leaf surfaces. They were observed along the midrib or alongside veins on the abaxial surface. The heads of these trichomes were large (21.7 SD: ± 3.25µm) and appeared ovoid or globular.

The diameters of mature peltate trichomes are larger on the abaxial surface (about 50 ± 0.9µm) than those peltate trichomes found on the adaxial surface of the leaf (about 40 ± 6µm) (Table 2). There are significant differences in peltate trichome diameter between the various regions of the leaf surfaces across different developmental stages. However, post-hoc tests revealed that there is only a significant difference in the diameter of trichomes at the mature tip and mid blade region on the adaxial surface ($F_{1,8}$=2.229; $p<0.05$). The average diameter of the peltate trichomes

found in the basal region of mature leaves on the abaxial surface was also found to be significantly different from the rest of the samples analysed ($F_{1,8}$=21.209; $p<0.05$) (Table 2).

The diameter of the Type I capitate trichome was measured across samples of emergent and young leaves only. The presence of these trichomes on mature leaves was rare and those that were present were in the post secretory phase, making measurements difficult. An independent samples t-test was performed to determine if there were any significant differences in the size of these trichomes on leaves from the different developmental stages. It was found that Type I capitate trichomes were significantly larger on both surfaces of emergent leaves than on young leaves (t_{78}=8.826; $p<0.05$) (Table 2).

Type II capitate trichomes were rare on both surfaces of leaves across all developmental stages. The frequency and size of these trichomes could not be statistically analysed due to insufficient data. However, from the few trichomes observed, it can be established that the average diameter of the head cell of a Type II capitate trichome on an emergent leaf is 24.51 SD: ±3.70 µm and the average length of the stalk cell is 8.34 SD: ±1.47µm while on a young leaf the diameter of the head cell is 17.83 SD: ±3.85µm and the average length of the stalk cell is 13.82 SD: ±4.25 µm.

Non-glandular trichomes are found throughout the lamina on both surfaces but appear to be more concentrated above the veins and on the leaf margins (Figure 6). The trichomes also appear to be more densely populated along the basal region of the abaxial surfaces of leaves of all developmental stages (Figure 6 b). An analysis of variance (ANOVA) revealed that non-glandular trichomes had a significantly higher frequency of distribution along the basal region of the adaxial surface of young and mature leaves ($F_{1,8}$= 8.785; $p<0.05$) (Table 1). On the abaxial surface, it was found that there was a significantly higher frequency of non-glandular trichomes along the mid-blade and basal region of the leaves ($F_{1,8}$= 20.851; $p<0.05$)(Table 1).

Significant differences were also found in the distribution of glandular peltate and Type I capitate trichomes. Peltate trichomes on the adaxial leaf surface were found to be least frequent

along the mid blade region of young leaves while the frequency of peltate trichomes was greatest in the basal region of mature leaves when compared to that of the emergent and young leaves ($F_{1,8}$=82.346; $p<0.05$) (Table 1). The frequency of peltate trichomes on the abaxial surface of mature leaves was significantly greater than that of the emergent leaves ($F_{1,8}$=8.103; $p<0.05$) (Table 1).

Similar observations were recorded for Type I capitate trichomes. An ANOVA confirmed that there were significant differences in the frequency of these type of trichomes on the adaxial ($F_{1,8}$=4.745; $p<0.05$) and abaxial ($F_{1,8}$=267.004; $p<0.05$) surfaces of the leaves. These trichomes appeared to be more abundant in the mid region on both surfaces of mature leaves with the lowest frequency being recorded on the emergent leaves (Table 1).

Table 1: Frequencies of different types of trichomes on adaxial and abaxial surfaces of leaves of *O. obovatum* across different developmental stages at various regions of the leaf blade. Values are means (± SEM).

	Emergent	Young	Mature
Peltate trichome frequency (trichomes/µm^2) {n=5}			
Adaxial surface			
Tip	4.11 (±0.348)^{ns}	4.51 (±0.126)^{ns}	4.45 (±0.197) {n=2}^{ns}
Mid	4.25 (±0.263)^{a,b}	1.99 (±0.154)^{b,c}	4.88 (±0.107)^{a,c}
Base	3.94 (±0.219)^{d}	3.98 (±0.216)^{e}	4.81 (±0.043) {n=4}^{d,e}
Abaxial surface			
Tip	4.10 (±0.104)^{f}	4.21 (±0.078)^{ns}	4.60 (±0.268)^{f}
Mid	4.02 (±0.169)^{g}	4.30 (±0.122)^{ns}	4.69 (±0.211)^{g}
Base	3.96 (±0.296) {n=2}^{h}	4.10 (±0.214)^{ns}	4.30 (±0.216) {n=3}^{h}
Type I capitate trichome frequency (trichomes/µm^2) {n=5}			
Adaxial surface			
Tip	7.70 (±0.169)^{ns}	7.67 (±0.176) {n=2}^{ns}	7.91 (±0.052) {n=4}^{ns}
Mid	7.66 (±0.032)^{i}	7.71 (±0.055){n=2}^{ns}	8.45 (±0.189) {n=3)^{i}
Base	7.56 (±0.151)^{j}	7.60 (±0.035){n=2}^{ns}	8.15 (±0.092)^{j}
Abaxial surface			
Tip	4.65 (±0.119)^{k}	4.83 (±0.079)^{k}	4.71 (±0.155) {n=2)^{k}
Mid	7.48 (±0.104)^{l}	7.66 (±0.084)^{m}	8.38 (±0.129)$^{l, m}$
Base	4.37 (±0.077)^{n}	4.57 (±0.079)^{n}	5.29 (±0.072) {n=4)^{n}
Non-glandular trichome frequency (trichomes/µm^2) {n=50}			
Adaxial surface			
Tip	4.97 (±0.138)	4.98 (±0.197)	5.02 (±0.250)
Mid	4.79 (±0.218)	4.89 (±0.149)	5.21 (±0.357)
Base	4.24 (±0.204)$^{n, o}$	4.77 (±0.123)^{n}	4.72 (±0.027)^{o}
Abaxial surface			
Tip	4.69 (±0.198)	4.85 (±0.158)	4.87 (±0.138)
Mid	4.45 (±0.157)^{p}	4.65 (±0.160)	4.83 (±0.059)^{p}
Base	3.95 (±0.208)^{q}	4.19 (±0.142)^{r}	4.53 (±0.132)$^{q, r}$

Means with different letters (a-r) within rows are significantly different at $p \leq 0.05$ using the Tukey's-D and Bonferroni multiple comparisons tests.

ns- not significant.

Figure 4: *Ocimum obovatum* growing in the:

(a) Pietermaritzburg area of KwaZulu-Natal. The plants found in this inland region at a higher altitude appear to possess slender, obovate leaves (arrows).

(b) Krantzkloof region closer to the KwaZulu-Natal coast. The leaves of these plants appear to be larger, broader and ovate (arrows).


a
b

Figure 5: Plants of *O. obovatum*.

(a) Leaves are simple, opposite and decussate with involuted leaf margins.

(b) Verticillate inflorescences with zygomorphic, labiate white to lilac flowers; stamens lie over the anterior lip of the corolla and resemble “cat’s whiskers”.


a
b

Table 2: Comparison of glandular trichome diameters in different stages of leaf development and along different regions of the leaf blade on adaxial and abaxial surfaces of *O. obovatum*. Values are means (± SEM)

	Emergent	**Young**	**Mature**
Peltate trichome diameter (µm) {n=10}			
Adaxial surface			
Tip	42.2 (± 1.37)	39.9 (± 1.85)	36.8 (± 1.63)
Mid	43.3 (± 1.79)	39.3 (± 2.69) {n=9}	47.4 (± 2.25)
Base	40.1 (± 2.48)	39.6 (± 1.59) {n=9}	42.4 (± 2.39)
Abaxial surface			
Tip	54.9 (± 0.77)	50.6 (±0.86)	50.7 (± 0.93)
Mid	52.6 (± 0.75)	57.0 (±0.93)	51.7 (± 0.99)
Base	47.8 (± 1.11) *	49.2 (±0.99) *	39.5 (± 1.91) *
Type I Capitate trichome diameter			
Adaxial surface	33.5 (± 0.83) {n=39} *	30.1 (± 0.85) {n=41} *	-
Abaxial surface	33.4 (± 0.85) {n=23} **	30.2 (± 0.60) {n=49} **	-

Mean glandular trichome diameters with symbols (*/**) within rows are significantly different at $p \leq 0.05$ using the Tukey's-D and Bonferroni multiple comparisons test.

- Sufficient data could not be obtained for statistical analysis

Figure 6: Stereomicroscopic images of leaves of *O. obovatum* showing distribution of non-glandular trichomes and peltate trichomes. The non-glandular trichomes resemble hairs while the peltate trichomes appear as gland dots (arrow heads).

(a) Adaxial leaf surface.

(b) Abaxial leaf surface.

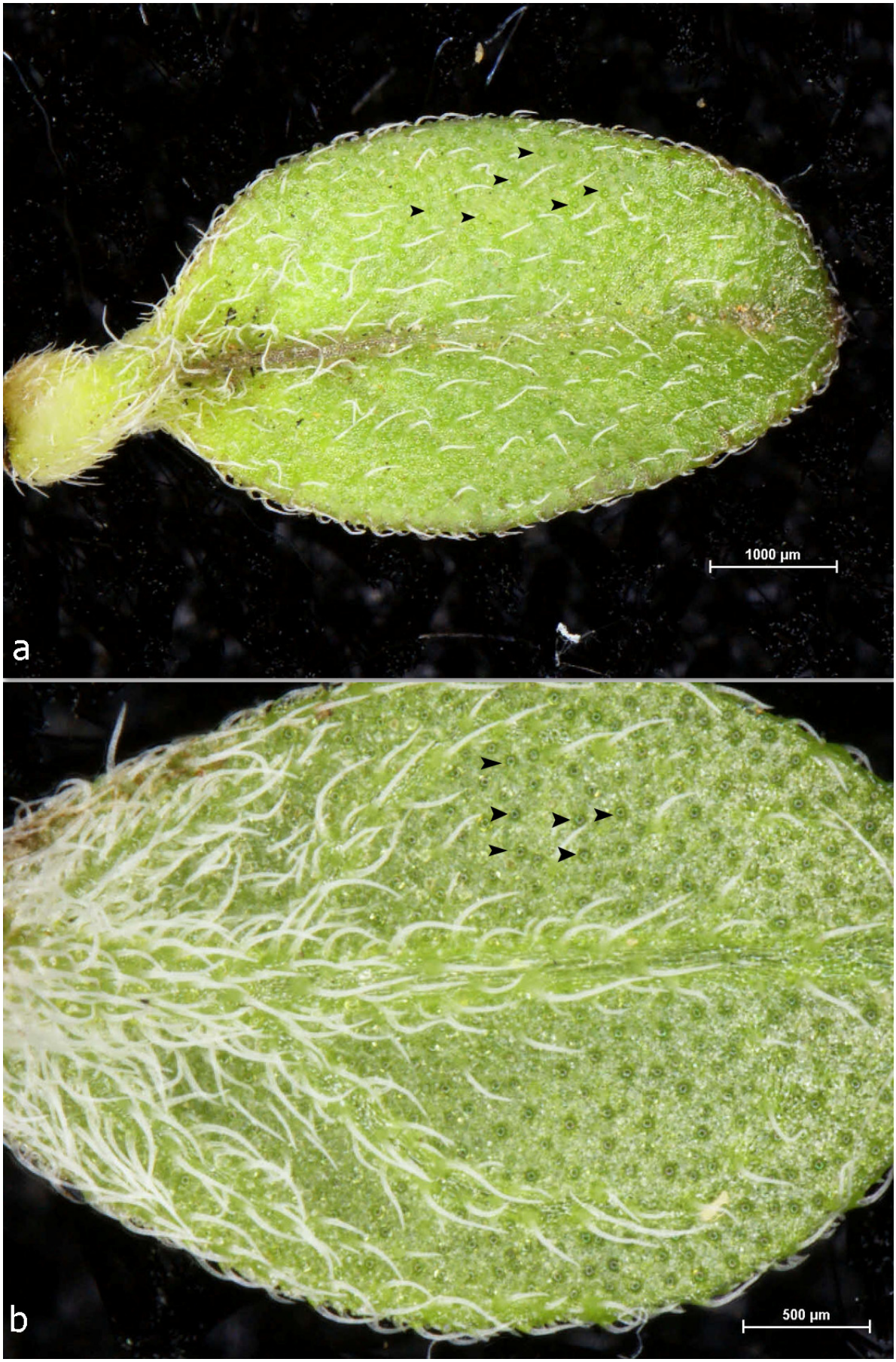

1000 µm
a
500 µm
b

Figure 7: Stereomicroscopic images of the leaves of *O. obovatum*.

(a) Mature leaf illustrating iridescence (arrowheads) emanating from adaxial surface.

(b) Globules that appeared to be viscid and glistening were found on the gland dots (arrows).


a
500 µm
b
500 µm

Figure 8: Environmental SEM of leaves of *O. obovatum* showing:

(a) non-glandular trichomes (NG) on abaxial leaf surface.

(b) microornamentation (MO) gives the trichome a warty appearance that decreases towards the basal region of the trichome.

(c) a cellular pedestal (CP) made up of a group of five to eight special subsidiary cells arranged around the base of the trichome raised above the leaf epidermis.

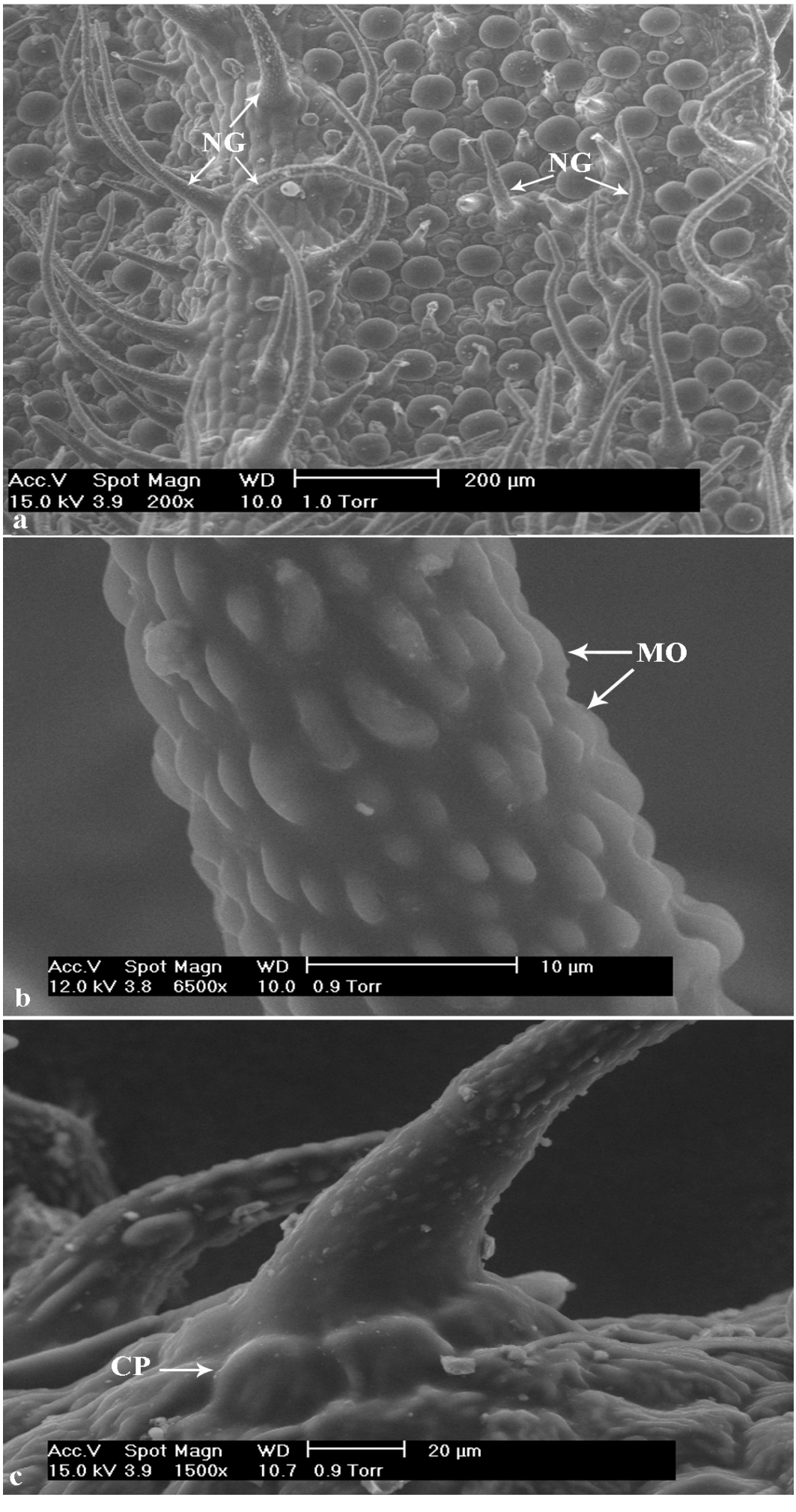

NG
NG
Acc.V Spot Magn WD 200 µm
15.0 kV 3.9 200x 10.0 1.0 Torr
a
MO
Acc.V Spot Magn WD 10 µm
12.0 kV 3.8 6500x 10.0 0.9 Torr
b
CP
Acc.V Spot Magn WD 20 µm
15.0 kV 3.9 1500x 10.7 0.9 Torr
c

Figure 9: Environmental SEM showing glandular trichomes on adaxial leaf surface of *O. obovatum*.

(a) Large, relatively smooth, four-celled peltate trichome (PT), Type I capitate trichome (Cap I) and non-glandular trichome (NG) with microornamentation were observed.

(b) Amphistomatous leaves of *O. obovatum* with stoma (St) in close proximity to glandular trichomes.

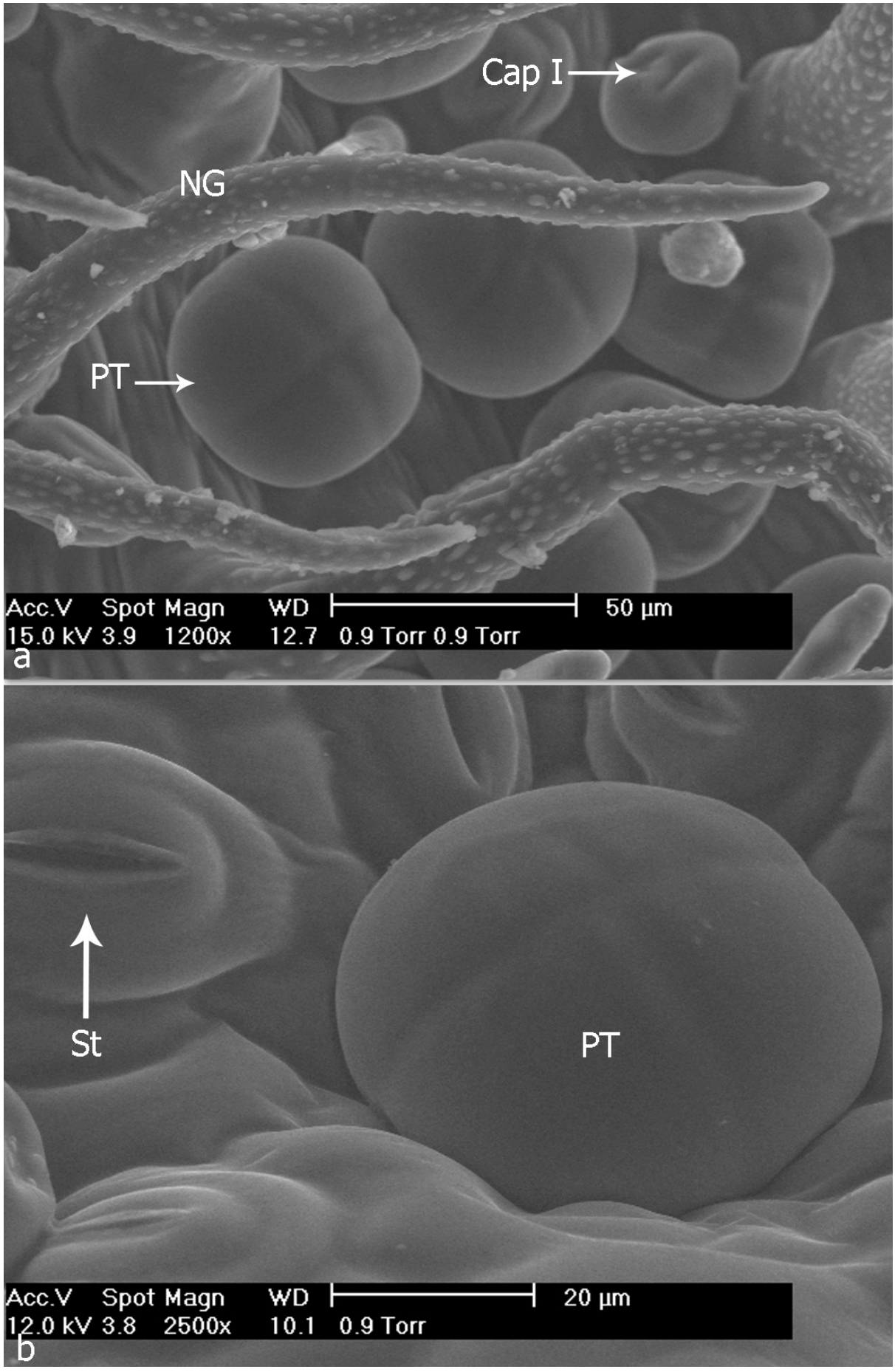

Cap I
NG
PT
Acc.V Spot Magn WD 50 µm
15.0 kV 3.9 1200x 12.7 0.9 Torr 0.9 Torr
a
St
PT
Acc.V Spot Magn WD 20 µm
12.0 kV 3.8 2500x 10.1 0.9 Torr
b

Figure 10: SEM of the adaxial leaf surface of *O. obovatum* showing

(a) the wrinkled surfaces of large peltate trichomes (PT) emphasizing the shape of the head cells (arrowheads).

(b) the secretion (Sec) that is exuded from the equatorial region of the gland where rupture occurs (arrows).

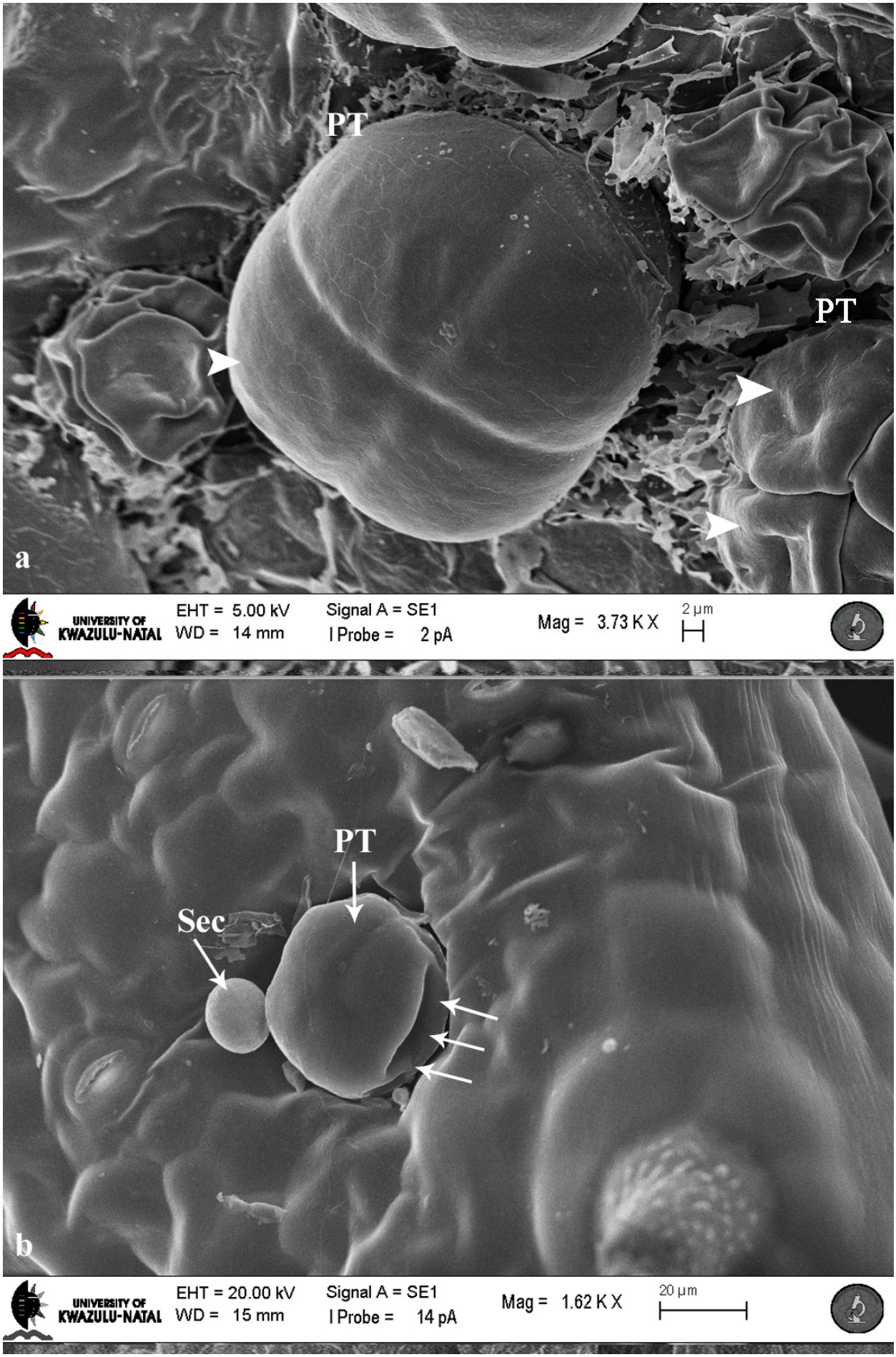
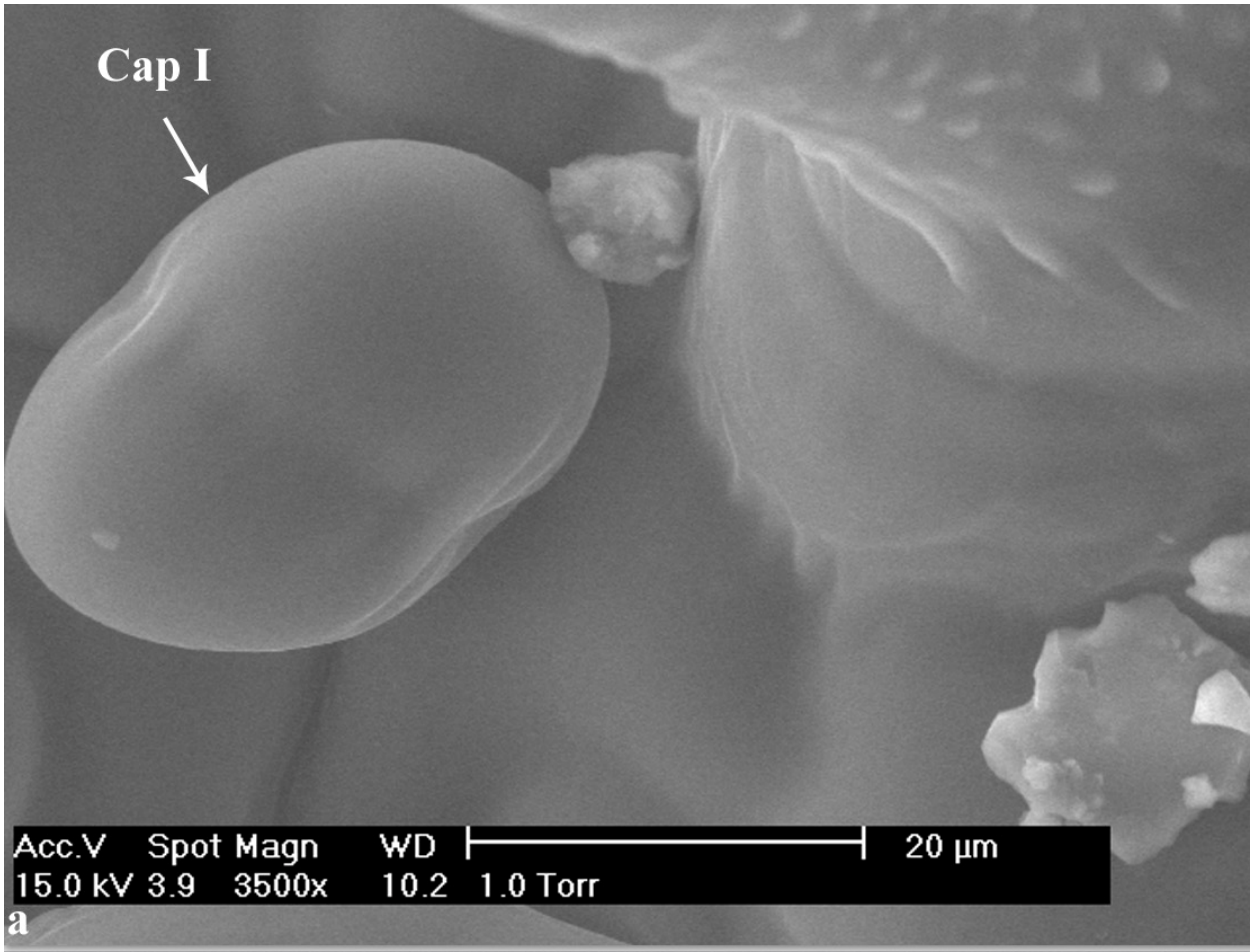
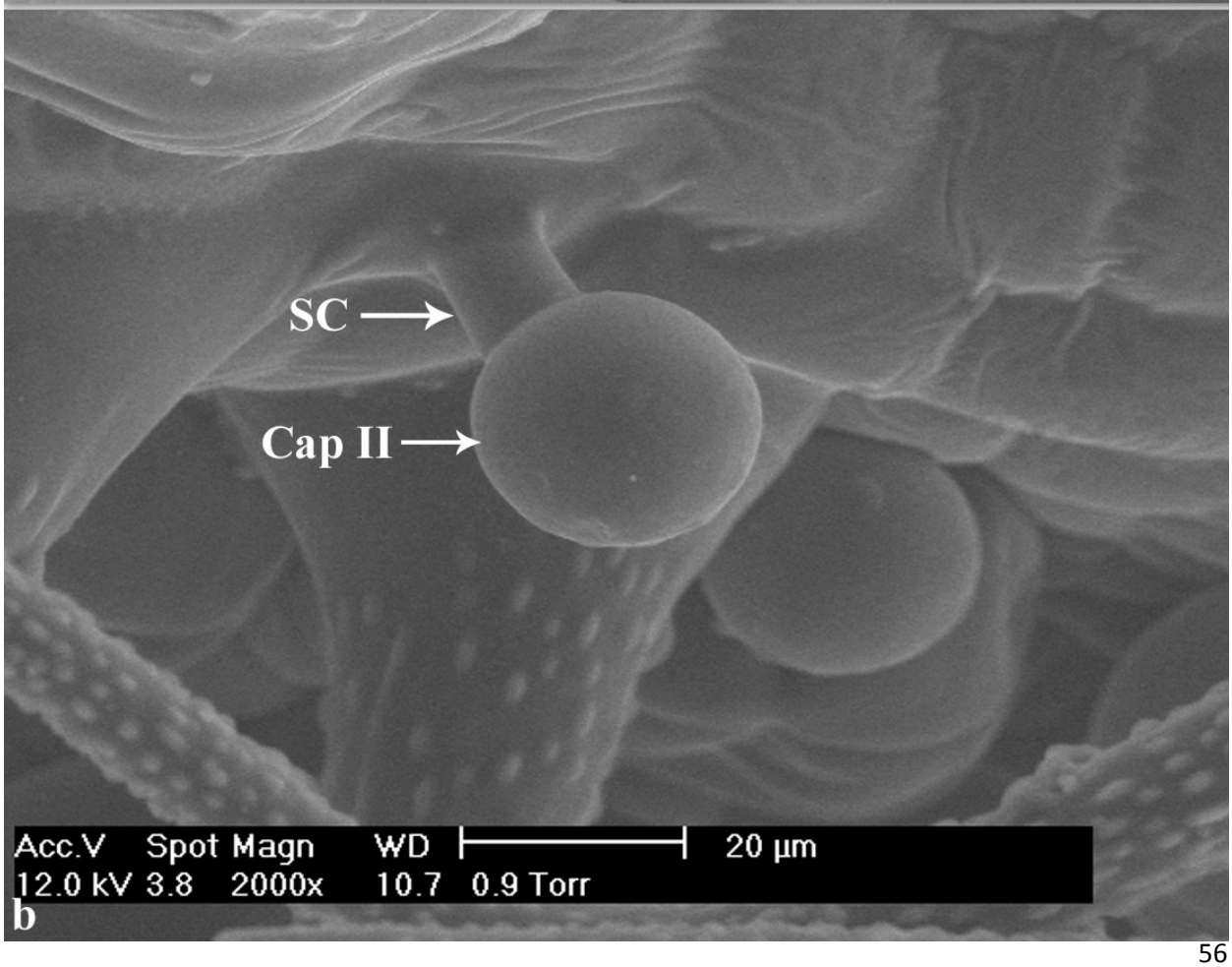

PT
PT
a
UNIVERSITY OF KWAZULU-NATAL
EHT = 5.00 kV
WD = 14 mm
Signal A = SE1
I Probe = 2 pA
Mag = 3.73 K X
2 µm
PT
Sec
b
UNIVERSITY OF KWAZULU-NATAL
EHT = 20.00 kV
WD = 15 mm
Signal A = SE1
I Probe = 14 pA
Mag = 1.62 K X
20 µm

Figure 11: Environmental SEM of leaf of *O. obovatum* showing:

(a) Type I capitate trichome (Cap I) with two secretory head cells

(b) Type II capitate trichome (Cap II) with a single oval head and a single stalk cell (SC) found on the abaxial surface of the leaves of *O. obovatum*.


Cap I
Acc.V Spot Magn WD 20 µm
15.0 kV 3.9 3500x 10.2 1.0 Torr
a


SC
Cap II
Acc.V Spot Magn WD 20 µm
12.0 kV 3.8 2000x 10.7 0.9 Torr
b

Histochemistry

Histochemical tests were carried out on fresh hand cut sections, and resin- and wax-embedded sections of leaf material to detect the main classes of compounds present in the secretions of the trichomes. Similar reactions were observed in both de-waxed and fresh, hand-cut sections. However, better images were obtained with de-waxed hand-cut sections. The trichomes appeared to have originated from a single protodermal cell that can be distinguished since it is larger than the neighbouring cells of the epidermis and their contents stain darker with Toluidine Blue-O (Figure 12) (Feder and O'Brien, 1968). The protodermal cell probably undergoes anticlinal division (Figure 13a) and subsequently divides periclinally in the case of larger peltate trichomes (Figure 13b). In *O. obovatum*, the peltate trichome develops a short stalk cell attached to four secretory head cells anchored to a single basal cell implanted in the epidermis (Figure 14).

Table 3 shows the results of the histochemical tests for the trichomes. Staining with Sudan Red (Figure 15a and b) and Sudan Black B (Figure 16 a and b) showed a positive lipid reaction in peltate trichomes and Type I capitate trichomes. The secretory product in the subcuticular space stained red and dark blue as is characteristic for Sudan Red and Sudan Black B, respectively. Ruthenium red was used to detect the presence of polysaccharides, such as pectinaceous substances and mucilage. It was observed that only the basal cells of peltate trichomes reacted positively to the stain while no reaction was recorded for the secretory product (Figure 17a). The secretory head cells of the Type I capitate trichomes showed a strong reaction to the stain, indicating the possible presence of mucilage. The stain was also taken up by the cuticle of the non-glandular trichome, especially by the microornamentation and the cell walls of multicellular trichomes (Figure 17 b).

Sections were stained with Wagner's and Dittmar reagent to test for alkaloids. Only the basal cells of the peltate trichomes reacted positively with both reagents (stained brown) while the head cells of the Type I capitate trichome stained positively for alkaloids with both reagents (stained light brown to orange) (Figures 18 and 19). NADI reagent was used to test for the

presence of essential oils. The oils were identified by the violet-blue colour following the reaction with NADI reagent (Figure 20).

Table 3: Results from histochemical tests on wax embedded sections and fresh, hand-cut sections of emergent leaves of *O. obovatum*.

Compound	Test	Peltate trichome	Type I Capitate trichome	Reaction observed
Lipids	Sudan Red	++	++	Secretory product in subcuticular space stained red.
	Sudan Black	++	++	Secretory product in subcuticular space stained dark blue.
Polysaccharides (Pectinaceous substances and mucilage)	Ruthenium Red	+	++	Only basal cell of peltate trichomes stained red while secretory head cells of Type I capitate trichome stained deep red.
Alkaloids	Wagner's	+	+	Only basal cell of peltate trichomes stained orange-brown while secretory head cells of Type I capitate trichomes reacted positively staining orange-brown.
	Dittmar's	+	+	Only basal cell of peltate trichomes stained orange-brown while secretory head cells of Type I capitate trichomes reacted positively staining orange-brown.
Essential oils	NADI	++	++	Both peltate and Type I capitate trichomes reacted positively staining brilliant or violet blue.
Phenols	Ferric trichloride	-	-	No reaction observed.
	Potassium dichromate	+	+	Only basal cells of peltate and Type I capitate trichomes reacted positively staining brown.

+/- indicates presence or absence of compound.

++ indicates intensity of reaction.

Figure 12: LM of transverse section through young leaf of *O. obovatum* stained with Toluidine Blue-O. Peltate trichome (PT) can be seen with a single basal cell (BC) which stains darker than the rest of the epidermal cells. The peltate trichome is sunken into a shallow depression on the leaf surface. A Type I capitate trichome (Cap I) with secretory head cell, single stalk cell, and basal cell embedded in the epidermis is also seen.

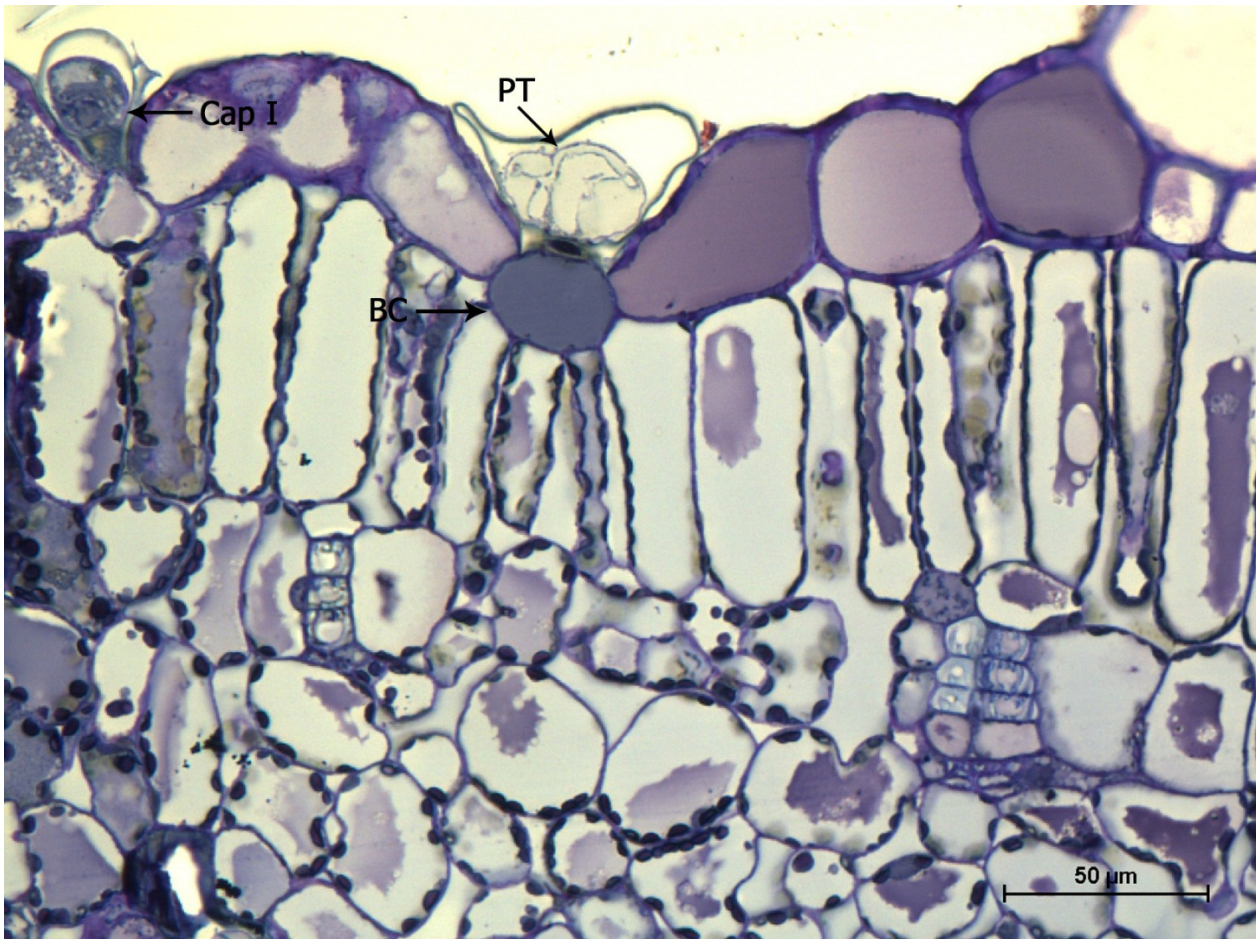

Cap I
PT
BC
50 µm

Figure 13: LM of transverse sections through young leaves of *O. obovatum* stained with Toluidine Blue-O showing:

(a) peltate trichome (PT) with two secretory head cells (SHC) and subcuticular space (SS).

(b) Type I capitate trichome (Cap I) with two distinct secretory head cells and a short stalk cell (sSC). The guard cells (GC) of a stoma (St) can be seen in close proximity to the trichome.

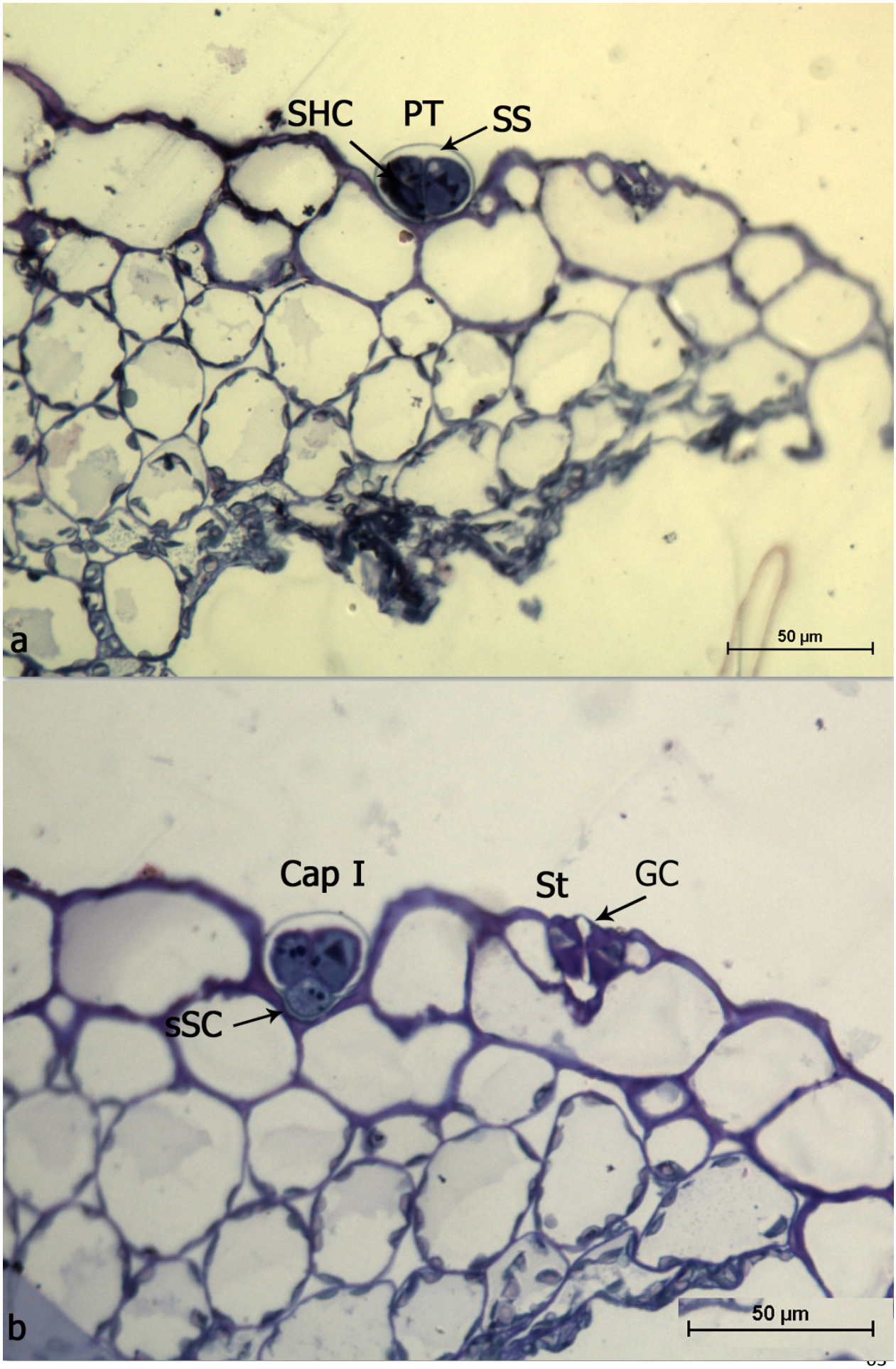

SHC
PT
SS
a
50 µm
Cap I
St
GC
sSC
b
50 µm

Figure 14: Oblique section stained with Toluidine Blue-O through peltate trichome on young leaf of *O. obovatum.* The four distinct secretory head cells (arrowheads) can be seen with an elevated subcuticular space (SS).

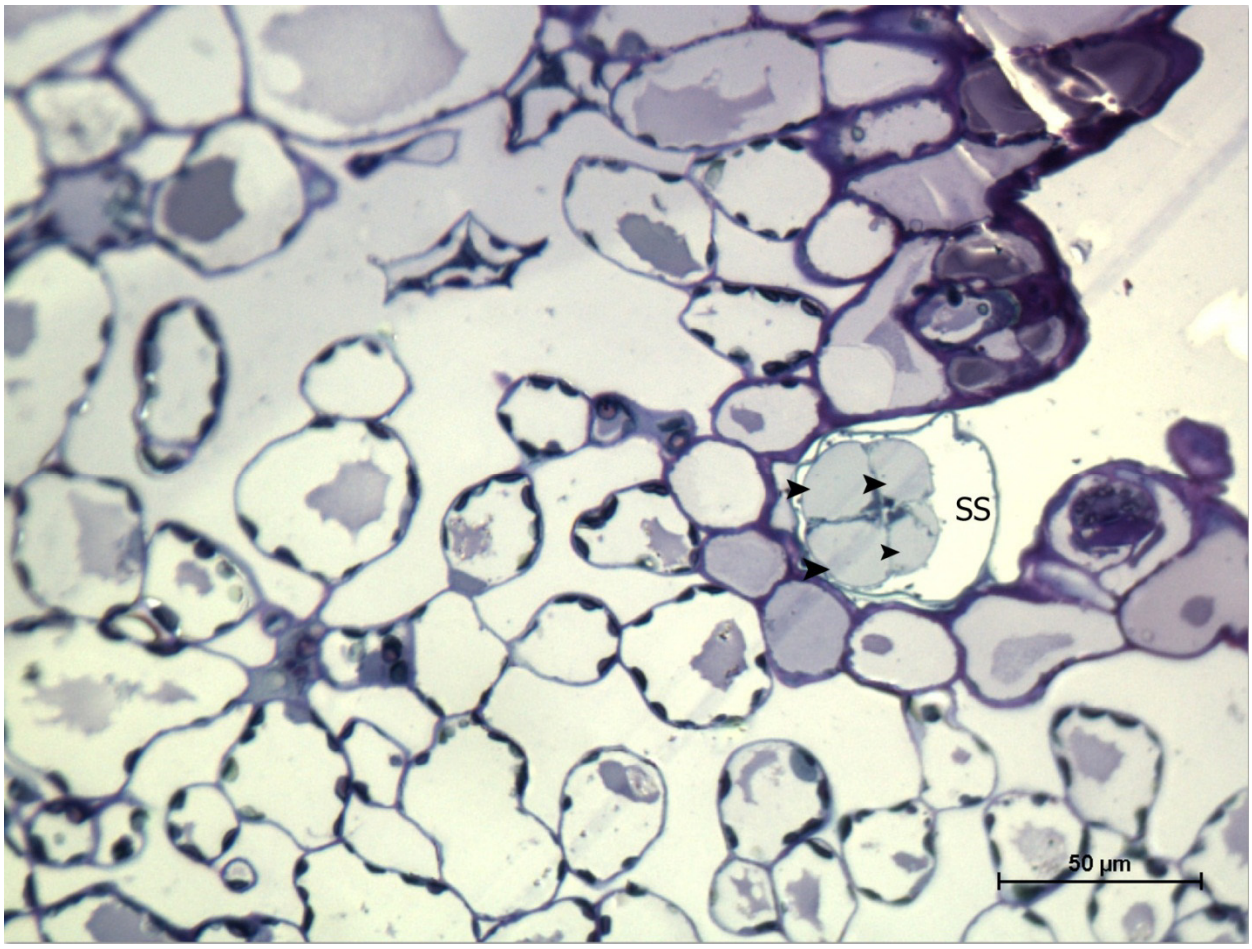

SS
50 µm

Figure 15: LM of sections through emergent leaf of *O. obovatum* stained with Sudan red to detect cutinised or suberized walls and other lipid inclusions.

(a) The stalk cell and cuticle of the peltate trichome (PT) and Type I capitate trichome (Cap I) showed a positive reaction.

(b) The cuticle of the non-glandular trichome (NG) also showed a positive reaction (arrow).

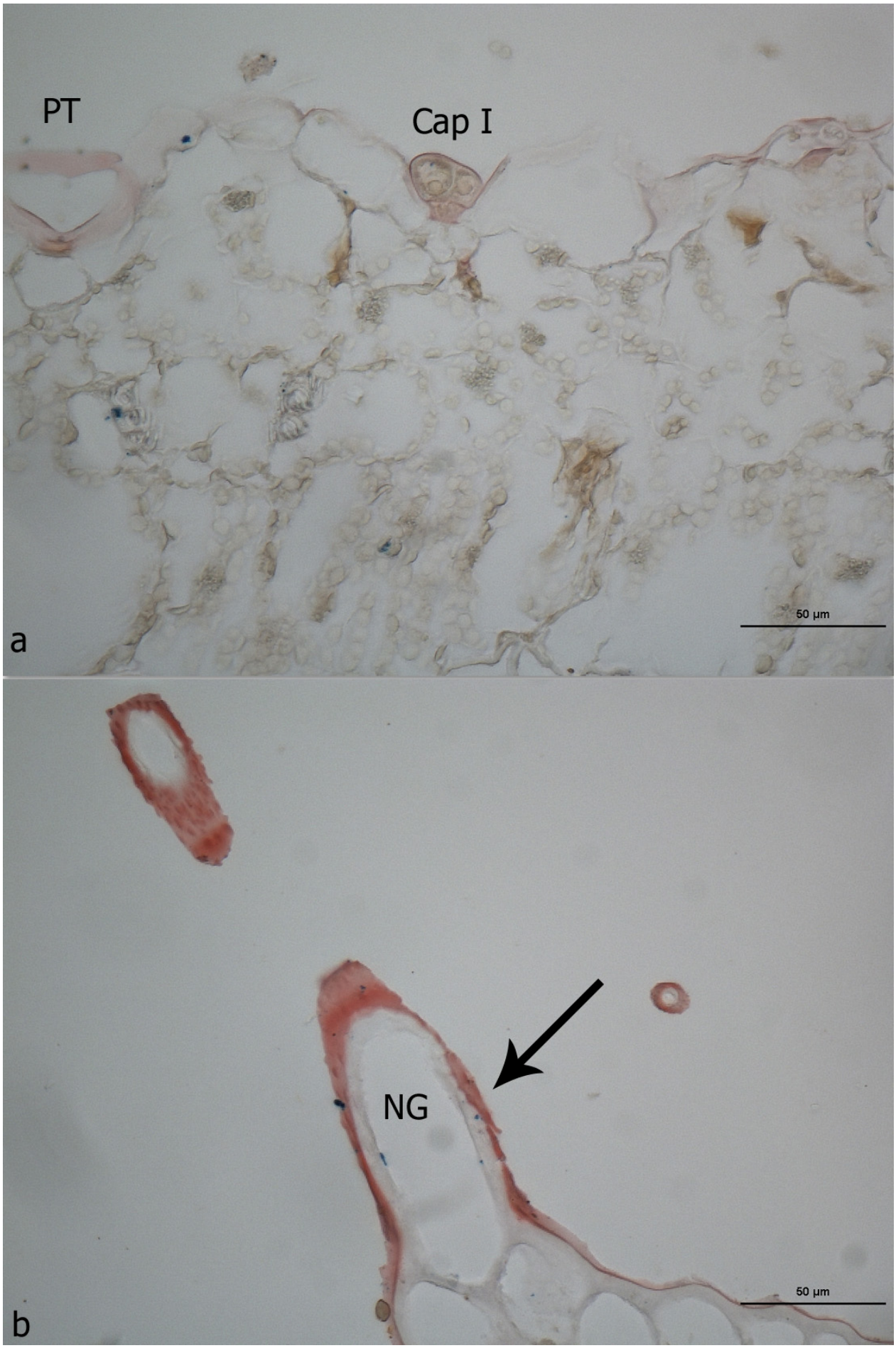

PT
Cap I
50 µm
a
NG
50 µm
b

Figure 16: LM of transverse section through emergent leaf of *O. obovatum* stained with Sudan Black B to identify lipids.

(a) The stalk cell and the cuticle of the Type I capitate trichome (Cap I) reacted positively with the stain.

(b) The peltate trichome (PT) reacted positively indicating the presence of lipids.

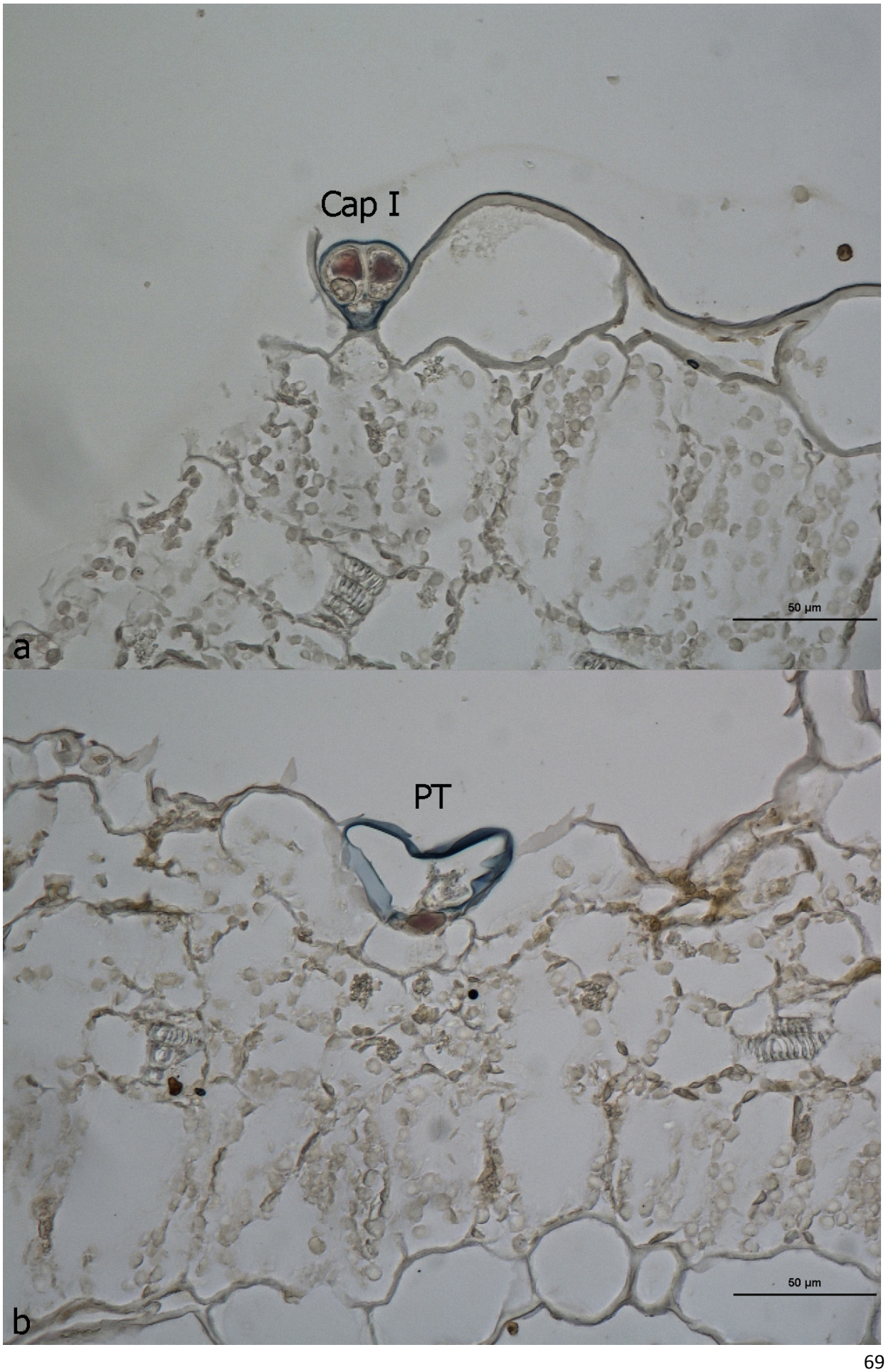

Cap I
50 µm
a
PT
50 µm
b

Figure 17: LM of sections through emergent leaves of *O. obovatum* stained with Ruthenium red to identify polysaccharides such as pectinaceous substances and mucilage.

(a) The basal and secretory head cells of peltate trichomes (PT) reacted positively with the stain, indicating the presence of pectinaceous substances or mucilage.

(b) In the Type I capitate trichome (Cap I) the stain exhibited a stronger reaction possibly indicating the presence of mucilage in the secretory head cells. The microornamentation on the surface of the non-glandular trichome (NG) as well as the cuticle reacted positively to the stain, indicating the presence of pectinaceous substances.

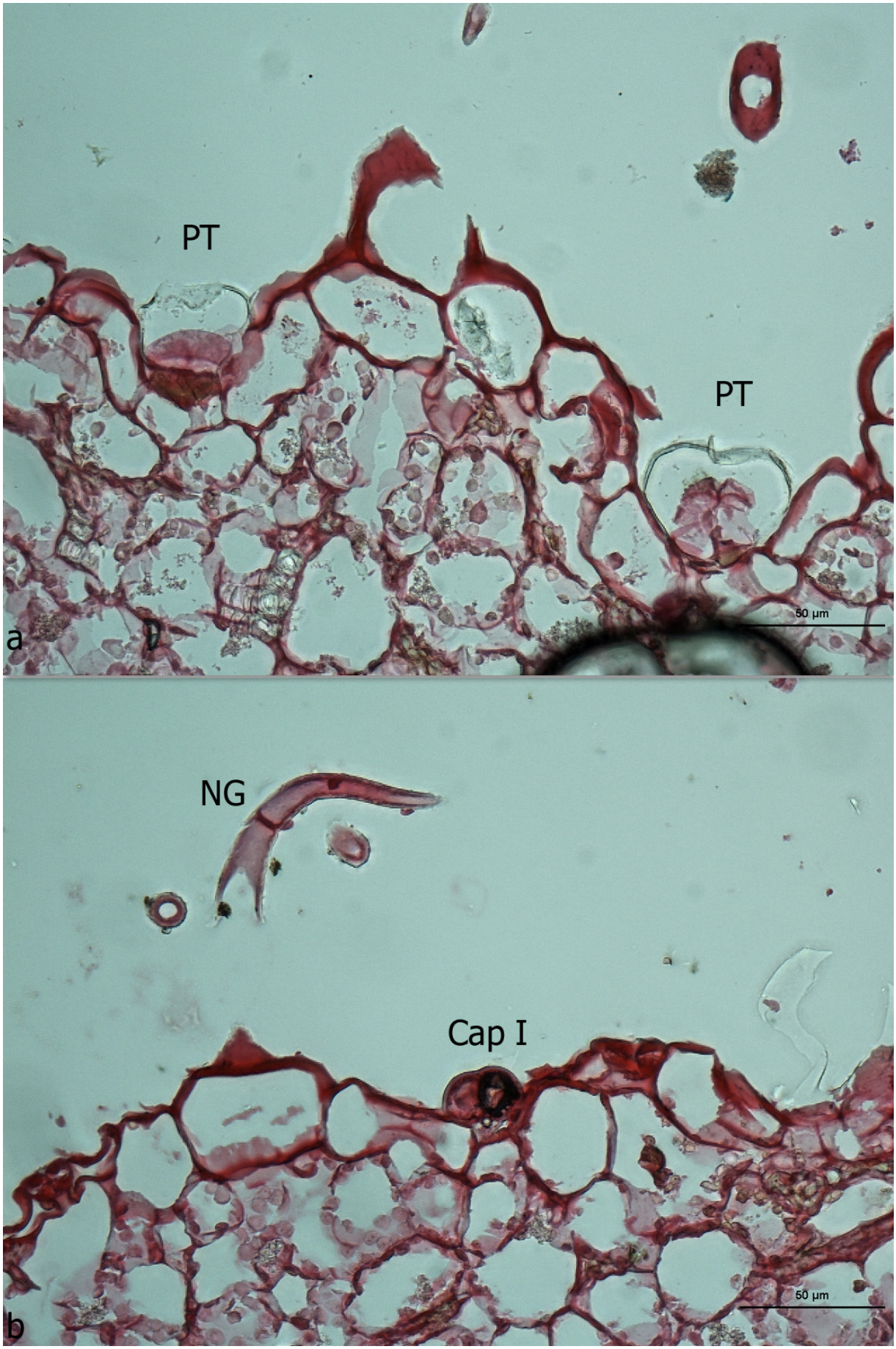
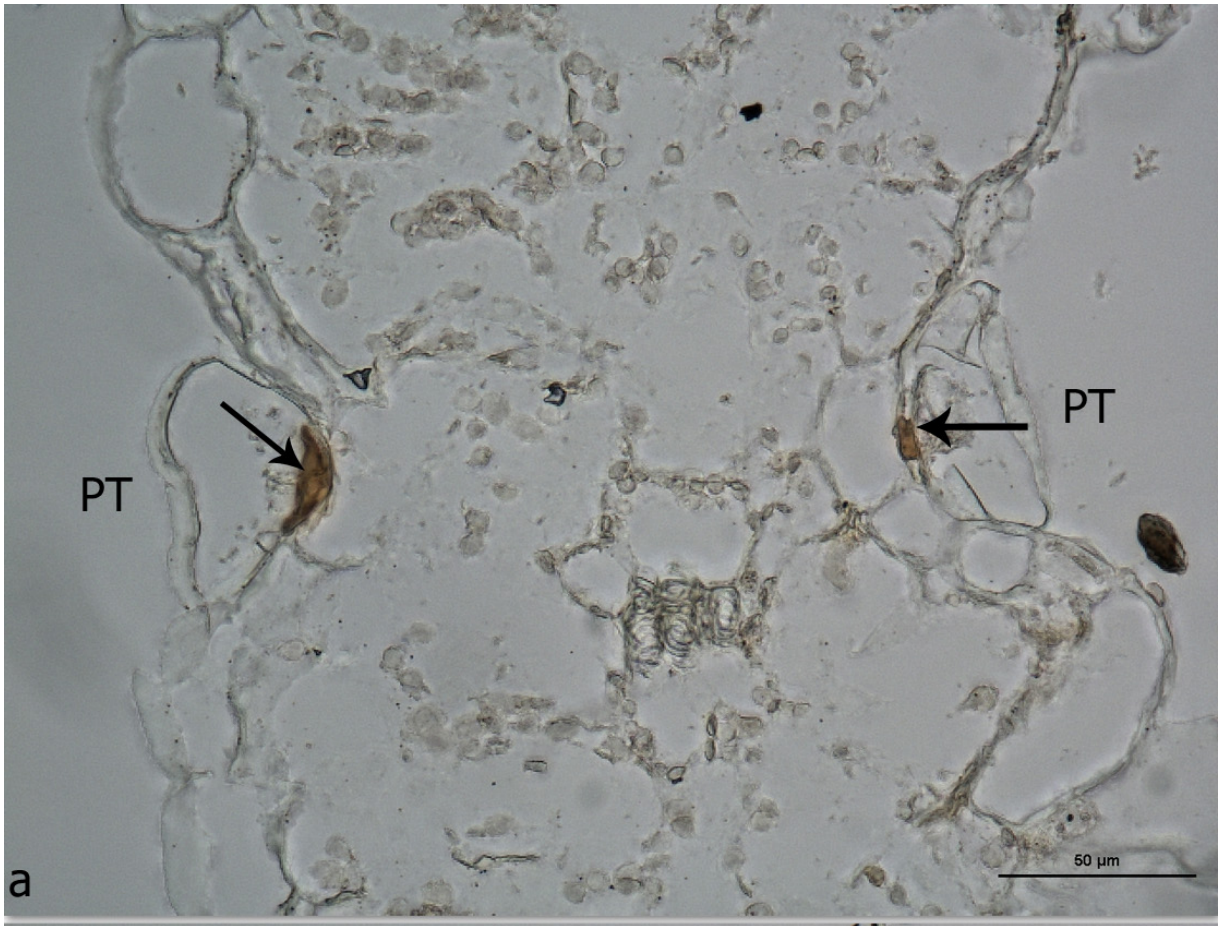

PT
PT
50 µm
a
NG
Cap I
50 µm
b

Figure 18: LM of sections through emergent leaves of *O. obovatum* stained with Dittmar reagent to detect the presence of alkaloids.

(a) The basal cells (arrows) of peltate trichomes (PT) reacted positively with the reagent.

(b) The secretory head cells of Type I capitate trichomes (Cap I) also showed a positive reaction with the reagent, indicating the presence of alkaloids (arrows).


PT
PT
50 µm
a

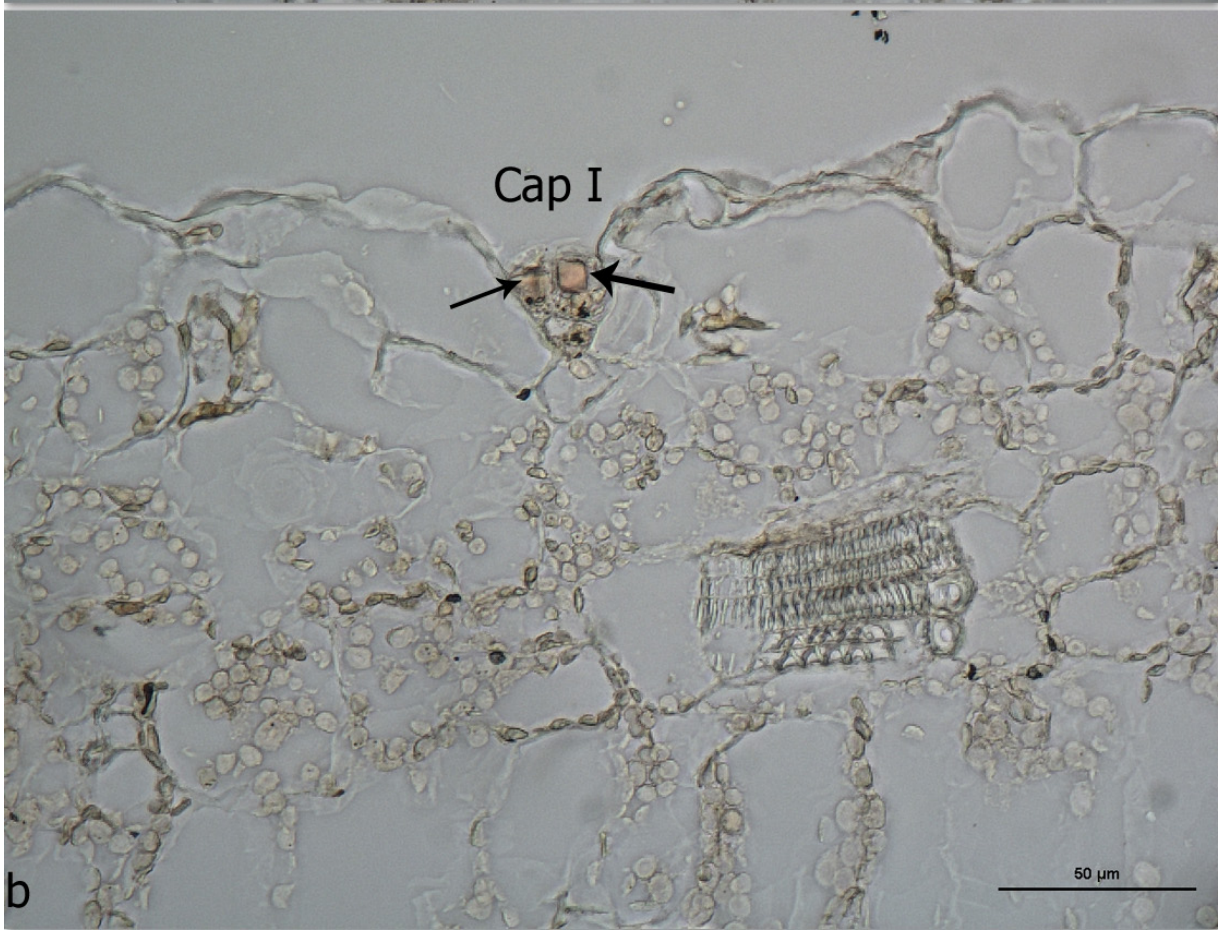

Cap I
50 µm
b

Figure 19: LM of emergent leaf of *O. obovatum* stained with Wagner's reagent to test for alkaloids.

(a) Only the basal cells (arrow) of peltate trichomes (PT) stained positively for the presence of alkaloids.

(b) The secretory head cells (arrow) of the Type I capitate trichome (Cap I) also showed a positive reaction to the reagent. No reaction was observed for non-glandular trichomes (NG).

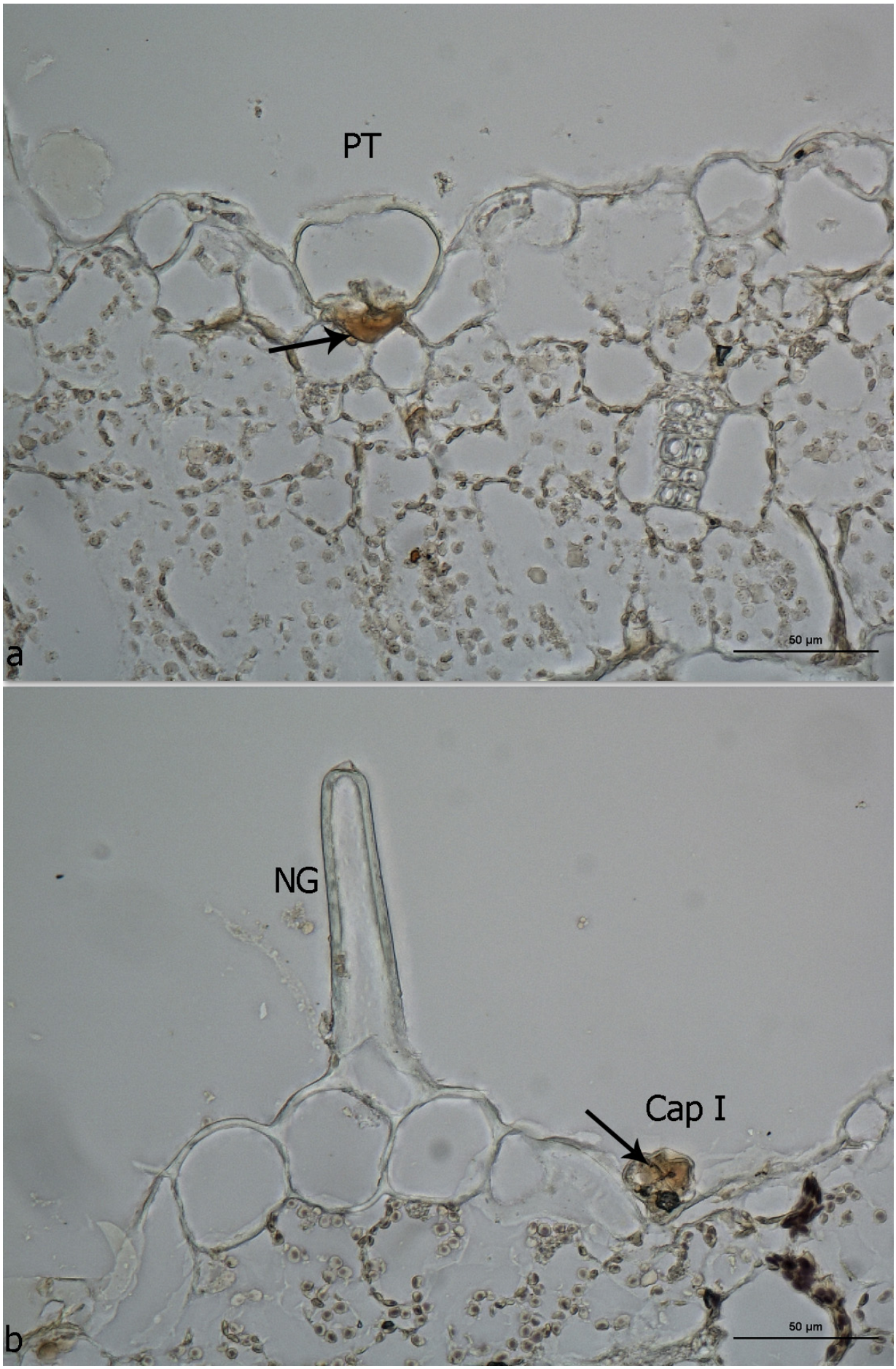
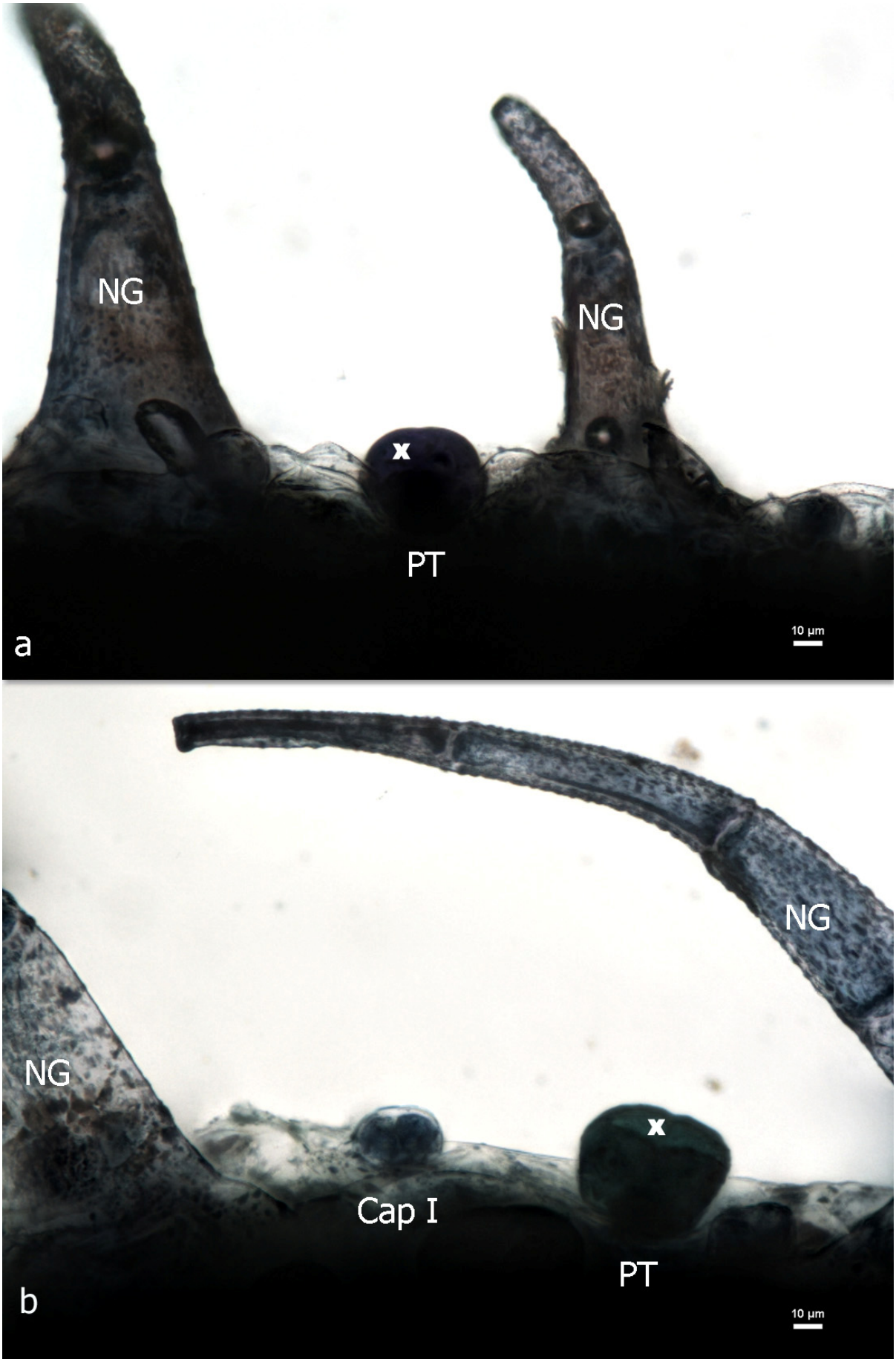

PT
50 µm
a
NG
Cap I
50 µm
b

Figure 20: LM of fresh sections of young leaf of *O. obovatum* stained with NADI reagent to detect the presence of essential oils.

(a and b) No reaction was observed for non-glandular trichomes (NG) while the subcuticular space (X) of the peltate trichome (PT) showed a positive reaction (violet blue) for terpenoids, a common constituent of essential oils. The subcuticular space in Type I capitate trichomes did not react with the reagent.


NG
NG
PT
a
10 µm
NG
NG
Cap I
PT
b
10 µm

Ferric trichloride and potassium dichromate were used to identify phenols in the sections. Ferric trichloride showed no reaction in any of the trichomes indicating the absence of phenols while potassium dichromate reacted positively with the components of the protoplasts by producing a brown colour (Figure 21). While the stains examined in this study illustrated slightly different colours in peltate and capitate trichomes, the exact differences between them cannot be deduced without detailed chemical analyses.

Ultrastructure

Samples for analysis with a TEM were prepared using two different fixation methods; conventional procedure and Todd's fixative. Sections of emergent leaves were favoured because the likelihood of isolating a developing or fully developed glandular trichome was higher than in sections from mature leaves which were more likely to possess glandular trichomes in a state of senescence. Non-glandular trichomes (fixed with Todd's fixative) appeared to be highly vacuolated with the cytomatrix and organelles restricted to the periphery of the non-glandular trichome wall (Figure 22 a). This narrow band of cytomatrix contained mitochondria and endoplasmic reticulum cisternae (Figure 22 d and e). A lipid body was also observed (Figure 22 f). Cuticular warts, or microornamentation observed were raised epidermal tissue surrounded by a thickened cuticle (Figure 22 b and c).

Peltate trichomes were extremely rare in resin embedded sections as determined at the level of light microscopy. However, a glancing section through a secretory head cell of a peltate trichome revealed the presence of a nucleus, numerous mini-vacuoles and vesicles as well as polymorphic plastids referred to as leucoplasts. A distinct separation was observed between the cell wall of the secretory head cell and the cuticle of the trichome that resulted in the creation of a subcuticular space. A closer examination of this secretory head cell revealed that the leucoplasts most probably contained secretory material (Figure 23 b).

Polymorphic leucoplasts had an almost dumbbell-shaped appearance and were most often observed along the periphery of the secretory head cells of Type I capitate trichomes. Vesicles,

vacuoles and short profiles of endoplasmic reticulum were observed in close proximity of the leucoplasts (Figure 24). Mitochondria were also were also observed (Figure 24 and 25 b). A Golgi body stack was recorded close to a vesicle and vacuole (Figure 25 a) and the density of the cytomatrix appeared to vary across the different samples examined. Vesicles were recorded to fuse with the cell wall of the secretory head cell (Figure 25 b).

The cell walls of the secretory head cells of the Type I capitate trichome appeared thicker than that of adjacent cells (Figure 26 a) and plasmodesmata were observed between adjacent secretory head cells (Figure 26 b). The organelles within the secretory head cells also appeared to be moving *en masse* toward the upper peripheral region of the cell (Figure 27). The subcuticular space also appears to be in a state of expansion (Figure 27 b). The fibrils of the cell wall of secretory head cells also appeared to be loosened when examined closely (Figure 28 a).

Figure 28 b shows a peltate trichome that appears to be in the post-secretory phase. The cell walls of the secretory head cells appear to have pulled away from the trichome cuticle. The organelles also seemed to have aggregated towards either the upper or lower region of the secretory head cell while the secretory material appears to have filled the space between the cell wall and cuticle maintaining the shape of the trichome.

Figure 21: LM showing sections of emergent leaves of *O. obovatum* stained to indicate the presence of phenols.

(a) Transverse section stained with ferric trichloride showed that phenols were localised only in the basal cell of the peltate trichome.

(b)Transverse section stained with potassium dichromate indicates a similar reaction in peltate trichomes.

No reaction was observed for capitate or non-glandular trichomes.

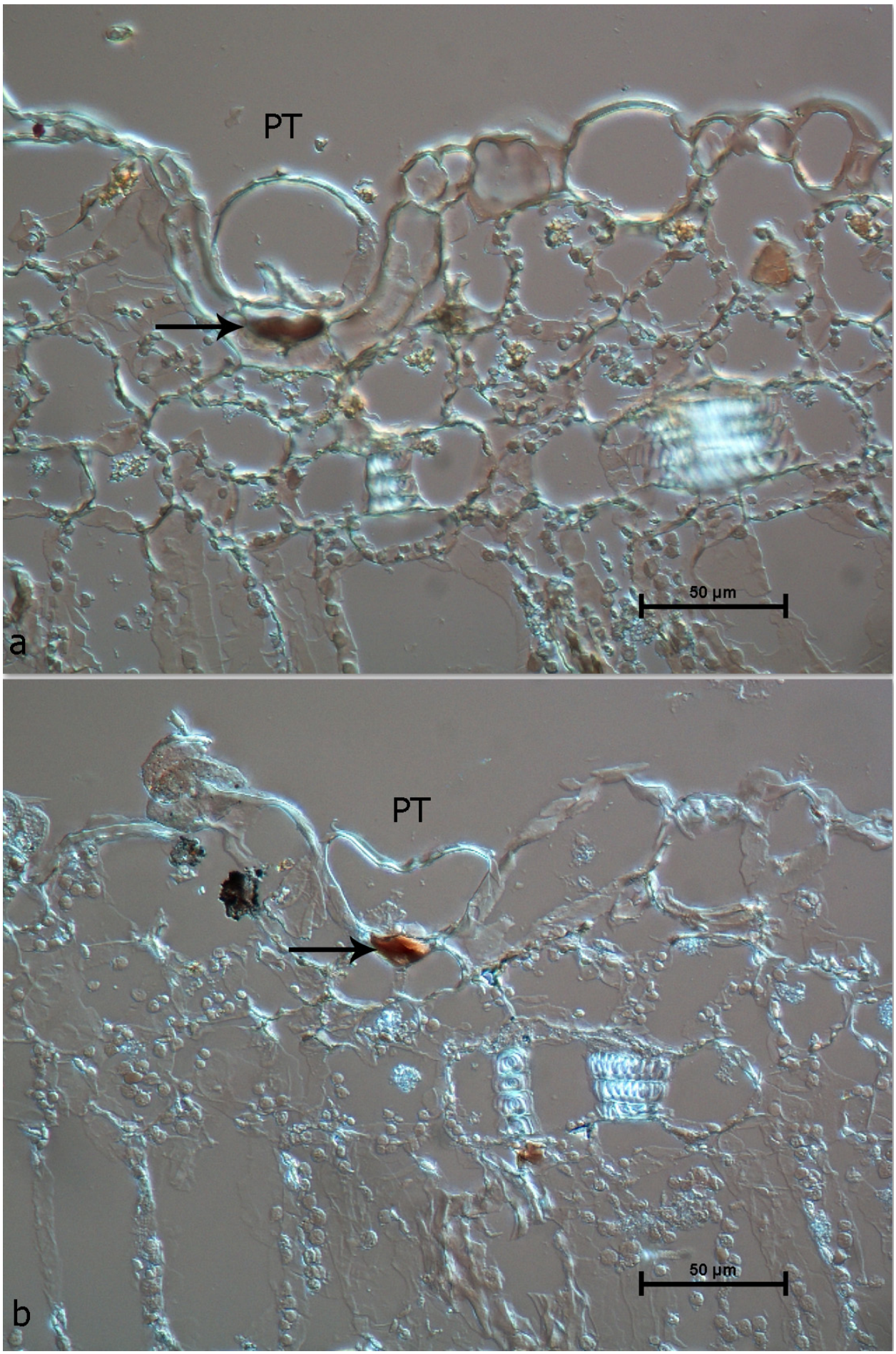

PT
50 µm
a
PT
50 µm
b

Figure 22: TEM of non-glandular trichome found on the leaf surface of *O. obovatum*.

(a) Part of a non-glandular trichome (NG) that is highly vacuolated (Vs).

(b and c) Microornamentation (MO) appears wavy or sinuous as extended growths from the epidermis with cuticular thickening over the raised areas (Ct).

(d-f) Numerous endoplasmic reticulum cisternae (ER), mitochondria (M) and a lipid body (LB) are present in the peripheral cytoplasm of the non-glandular trichome.

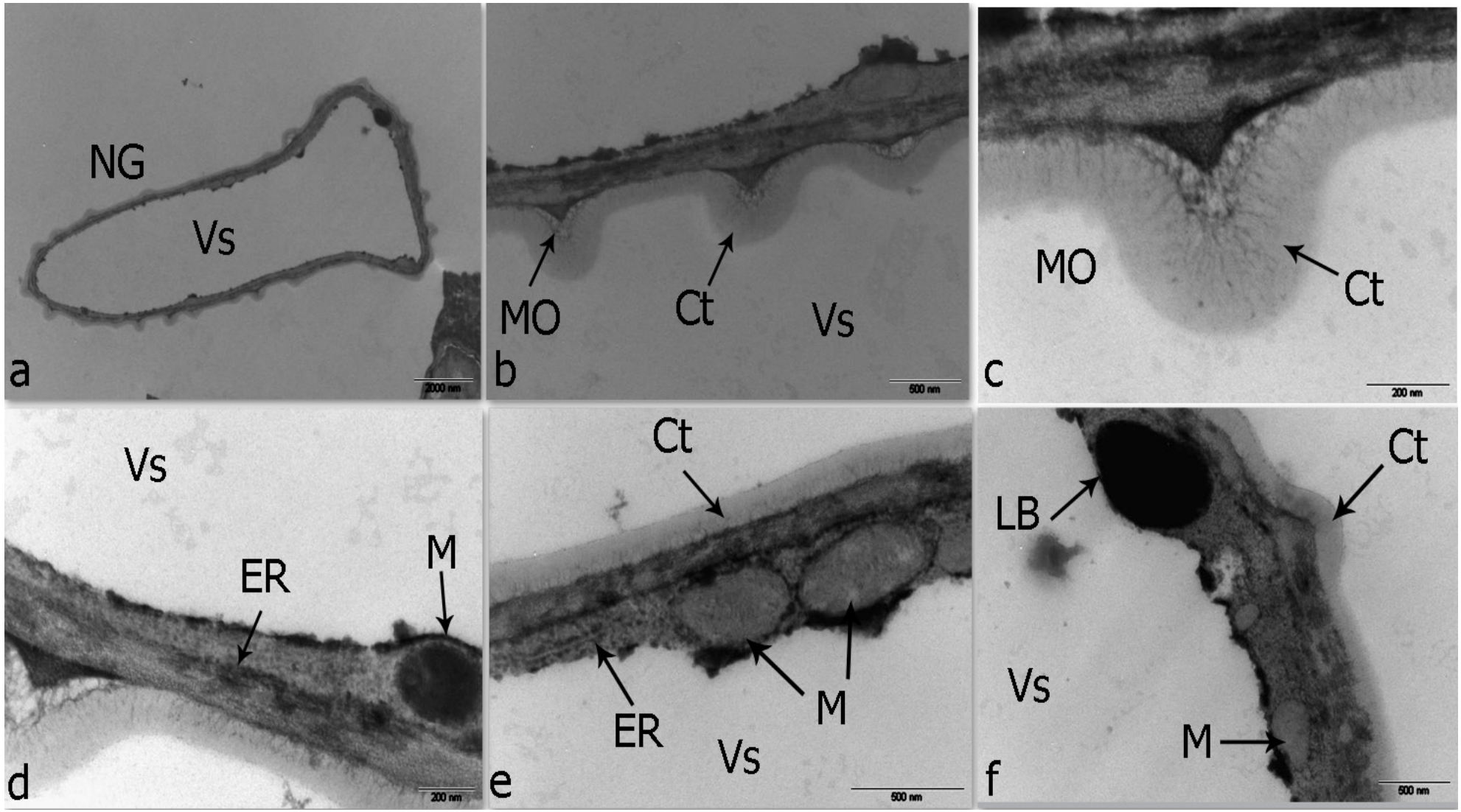

NG
Vs
a
2000 nm
MO
Ct
Vs
b
500 nm
MO
Ct
c
200 nm
Vs
ER
M
d
200 nm
Ct
ER
M
Vs
e
500 nm
LB
Ct
Vs
M
f
500 nm

Figure 23: TEM showing:

(a) a glancing section through a secretory head cell of a peltate trichome with subcuticular space (SS) that has developed as a result of the sequestration of secretory material via the cell wall (CW) causing the cuticle (arrow) to lift up and away from the cell wall of the secretory head cell. Organelles observed at this magnification included large vacuoles (Vac) with secretory material as well as numerous smaller vesicles (V) with secretory material. The nucleus (N) was present towards the centre of the secretory head cell and polymorphic plastids referred to as leucoplasts (L) were also observed. The ultrastructure of the trichome was also noted to be distinct from adjacent epidermal cells (Adj).

(b) at a higher magnification the distinct nucleus (N) was observed. Numerous vacuoles (Vac), vesicles (V) and leucoplasts (L) were also present and the cytomatrix (Cyt) appeared to be distinct from the darker staining and, hence, denser appearing secretory material (Sec) within the secretory head cell.

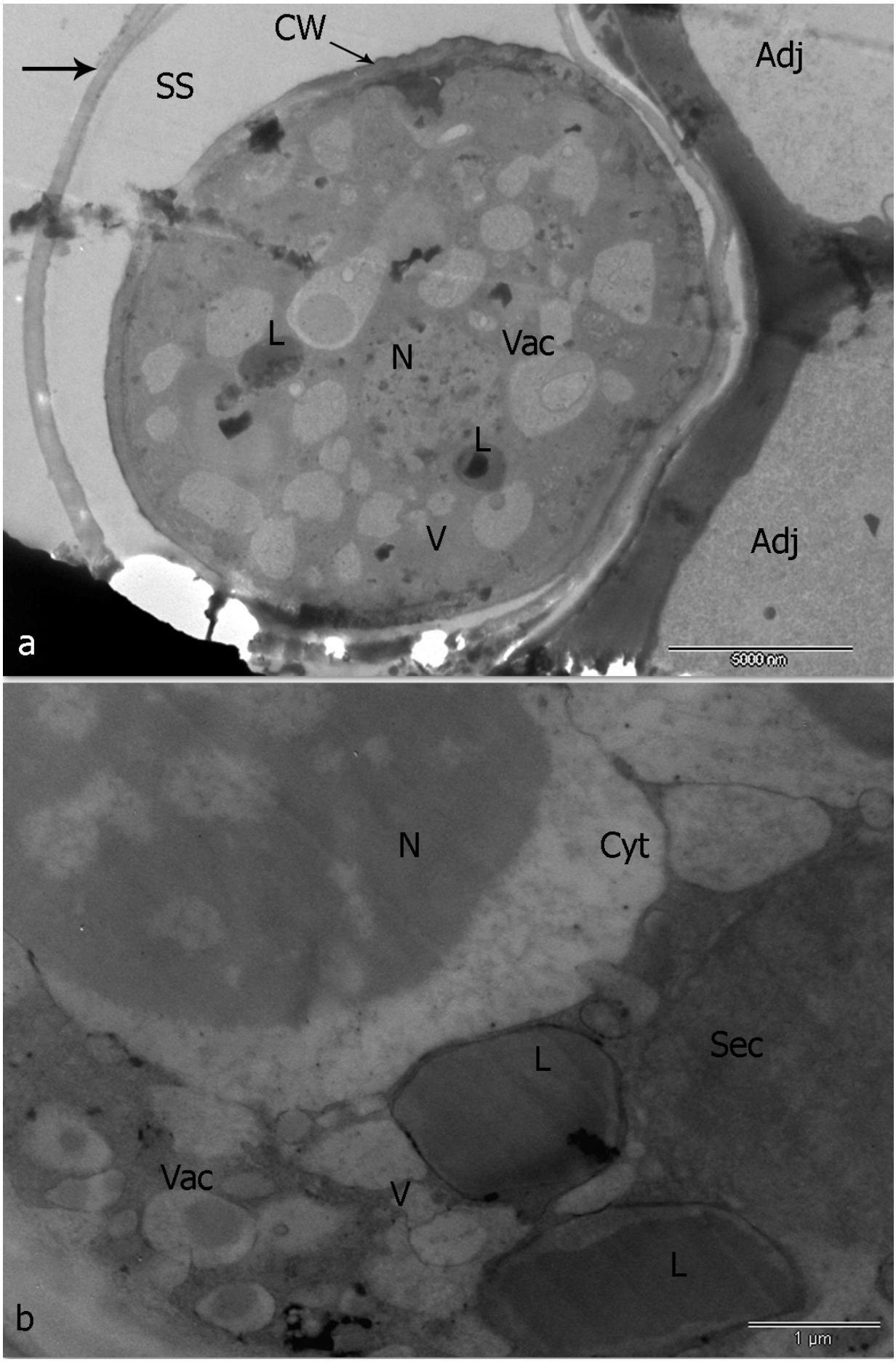
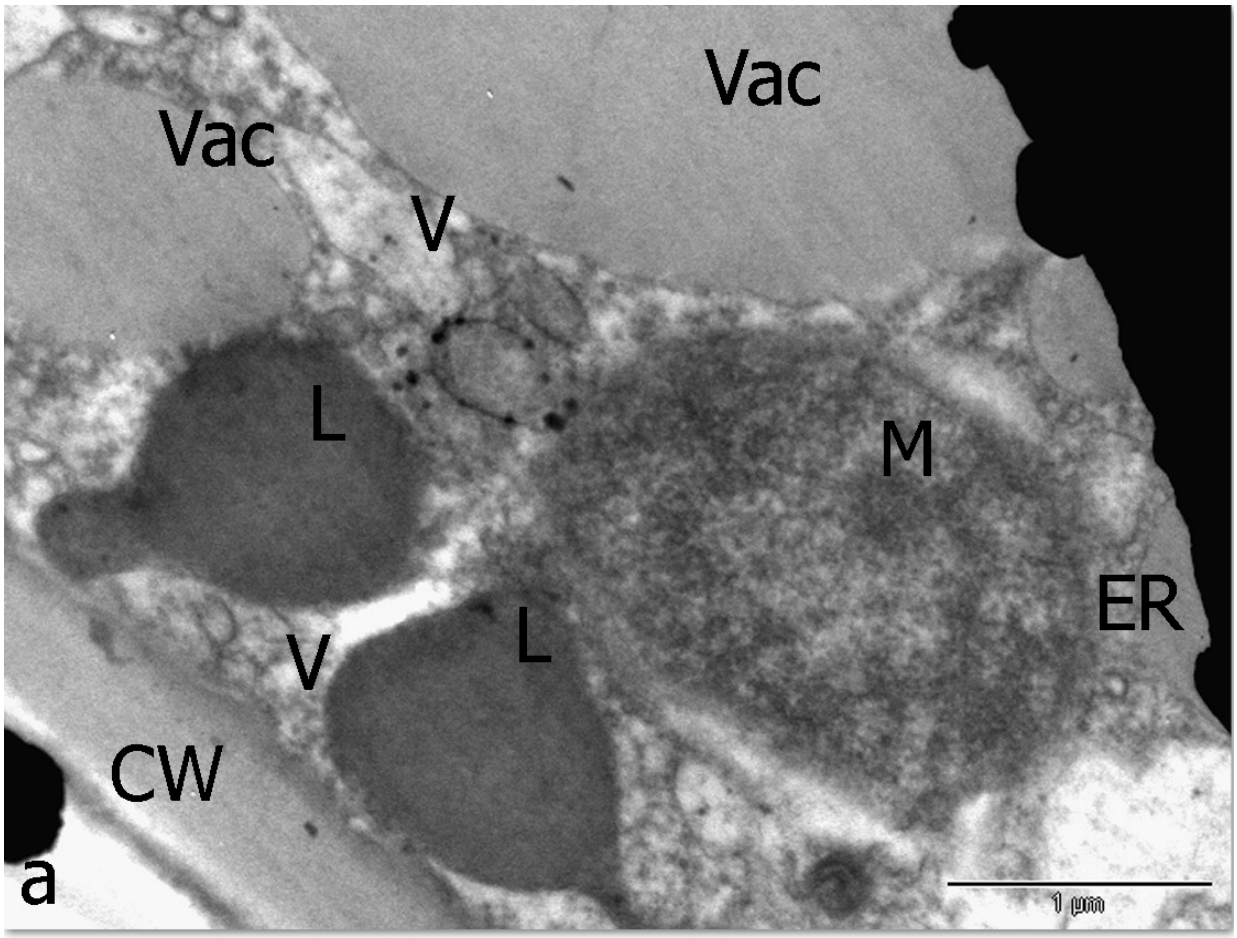

CW
SS
Adj
L
N
Vac
L
V
Adj
a
5000 nm
N
Cyt
Sec
L
Vac
V
L
b
1 µm

Figure 24: TEM of portion of Type I capitate trichome showing:

(a) polymorphic leucoplasts (L) with an almost dumbbell-shaped appearance. Vesicles (V) are closely associated with the leucoplasts and vacuoles (Vac). Secretory material and short profiles of endoplasmic reticulum cisternae (ER) were also visible. A mitochondrion (M) was also observed.

(b) a leucoplast (L) closely associated with vesicles (V) and profiles of endoplasmic reticulum cisternae (ER) implicated in the transport of secretory material to the cell wall (CW) for sequestration into the subcuticular space for storage.


Vac
Vac
V
L
M
ER
V
L
CW
a
1 µm

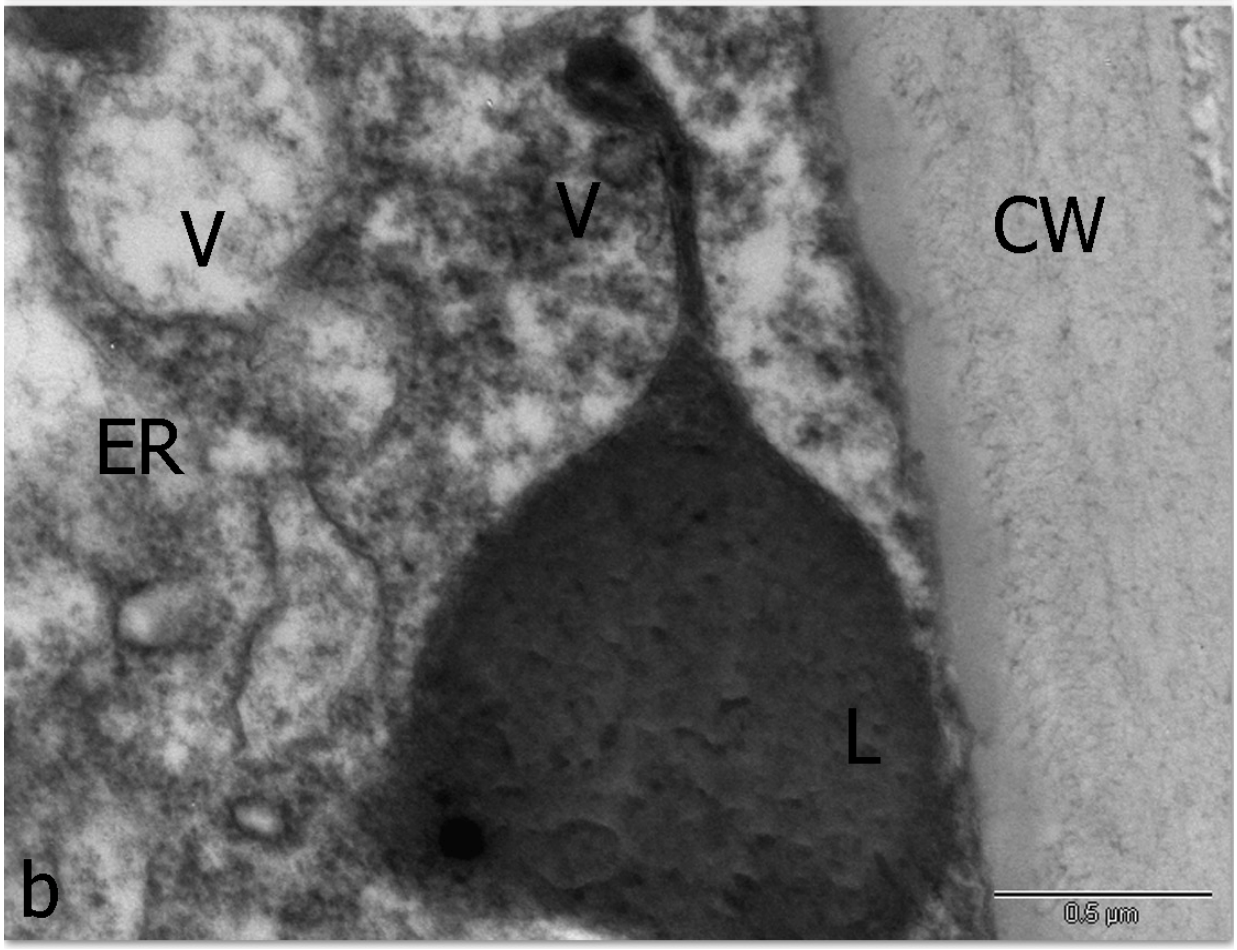
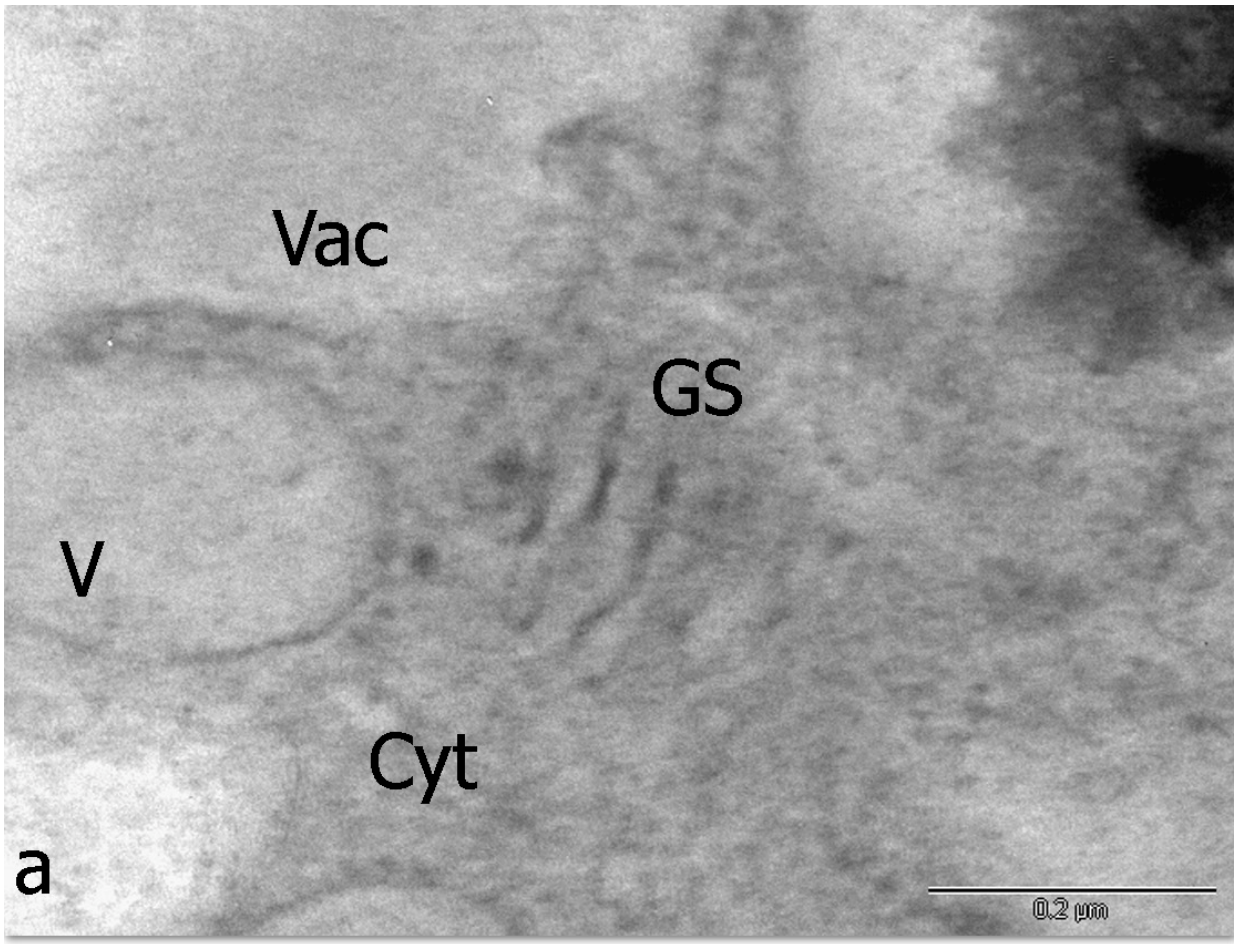

v
V
CW
ER
L
b
0.5 µm

Figure 25: TEM of ultrathin section through Type I capitate trichome showing:

(a) a vesicle (V) with secretory material in close proximity to vacuole (Vac) and a Golgi stack (GS). The cytomatrix (Cyt) was observed to be lightly stained.

(b) mitochondria (M) were observed along the periphery of the secretory head cell, close to the cell wall (CW). Numerous vesicles (V) were also observed and one was in the process of fusing (arrow) with the cell wall (CW). Part of a vacuole (Vac) with secretory material was also observed. The developing subcuticular space (SS) can be seen here.


Vac
GS
V
Cyt
a
0.2 µm

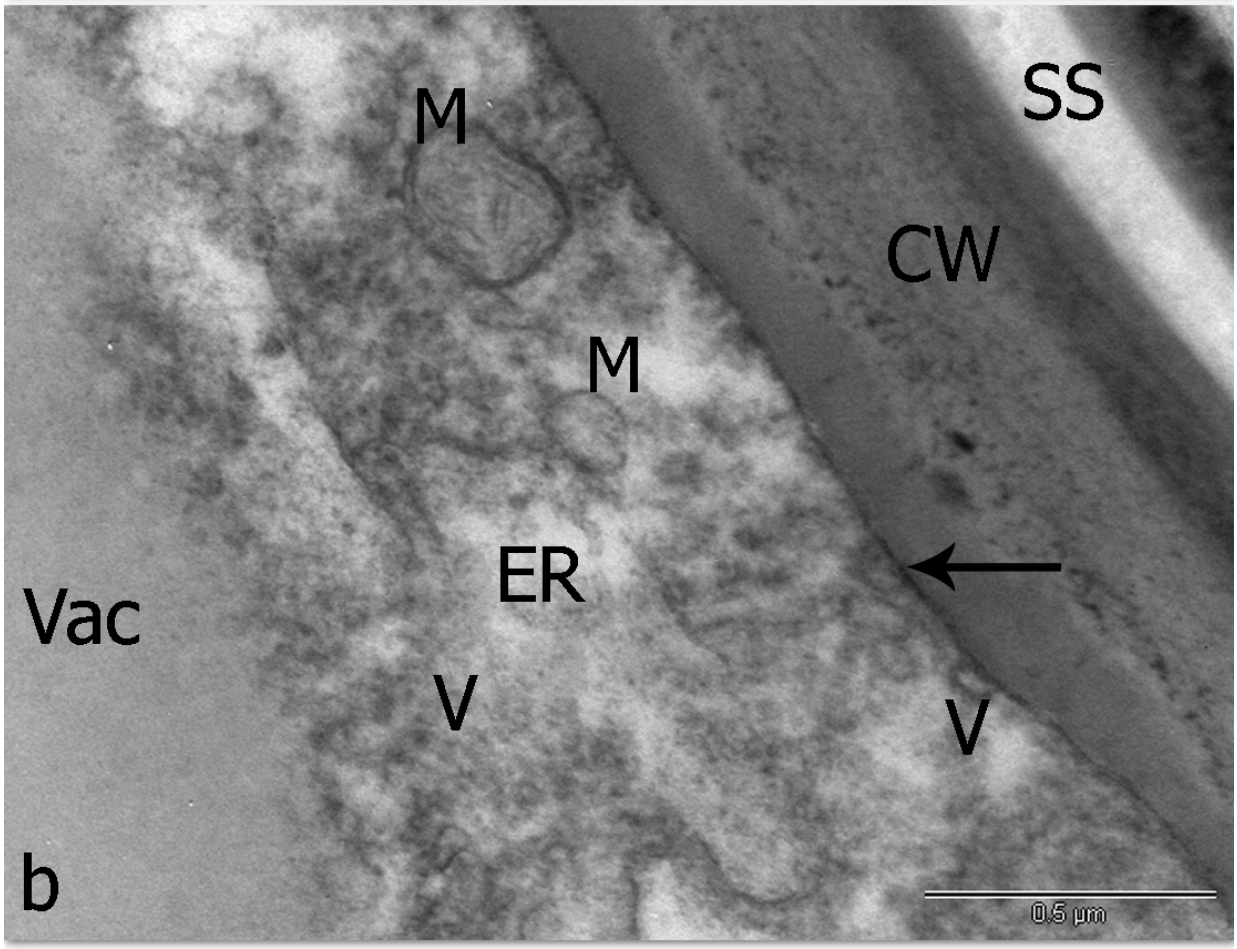

M
SS
CW
M
ER
Vac
V
V
b
0.5 µm

Figure 26: TEM of ultrathin section through portion of a Type I capitate trichome of *O. obovatum* showing:

(a) the basal region of two adjacent secretory head cells and an adjacent epidermal cell (Adj). Numerous organelles can be seen in the lower secretory head cell while it appears that the organelles in the upper secretory cell are aggregated toward the periphery of the cell. The cell wall (CW) of the trichome appears to be much thicker than that of the adjacent.

(b) plasmodesmata (P) were seen between the two secretory head cells with a mitochondrion (M) and short profiles of endoplasmic reticulum cisternae (ER). Vacuoles (Vac) and vesicles (V) were also observed and the cytomatrix (Cyt) appeared denser possibly indicating the secretory phase.

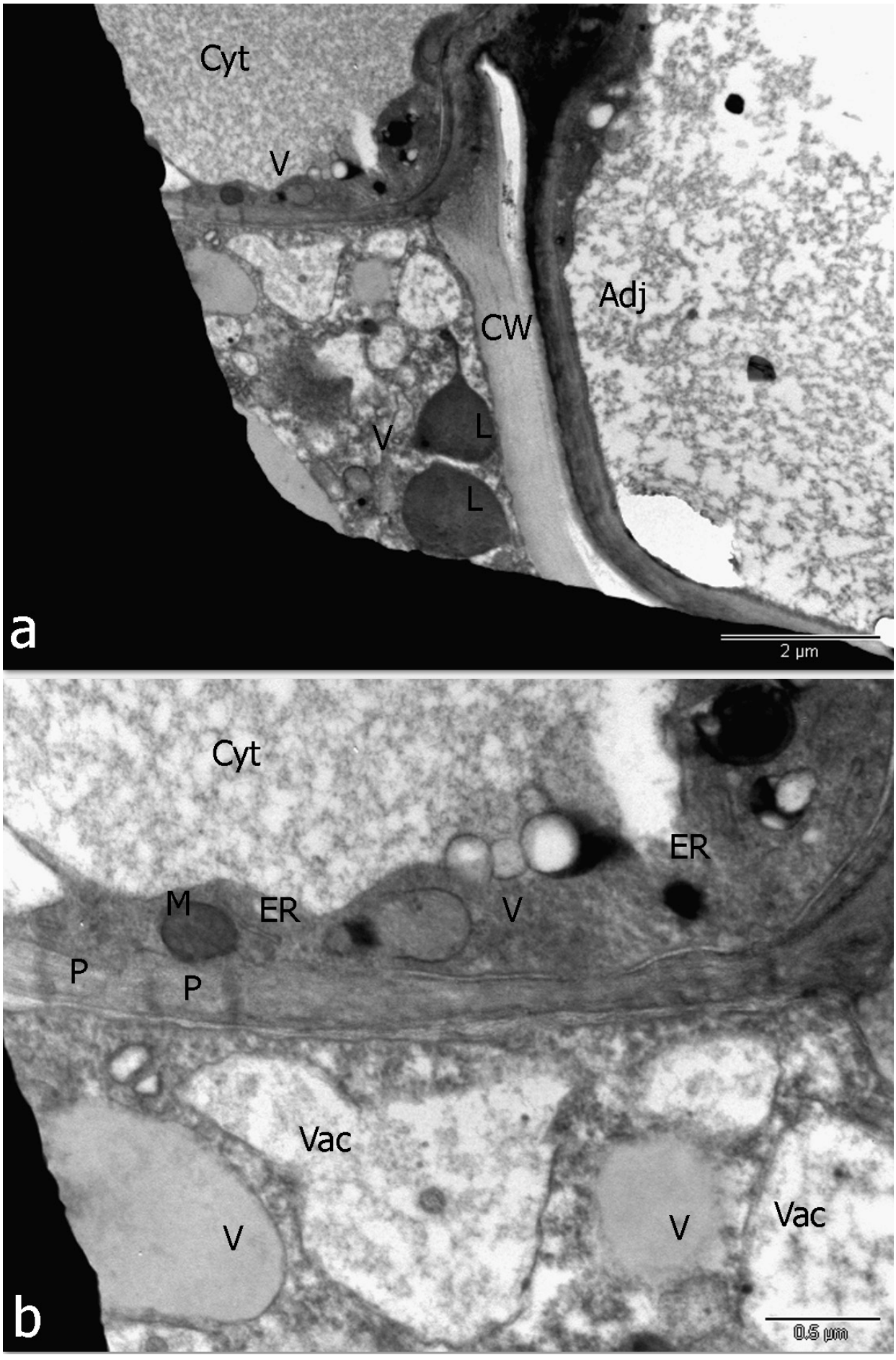
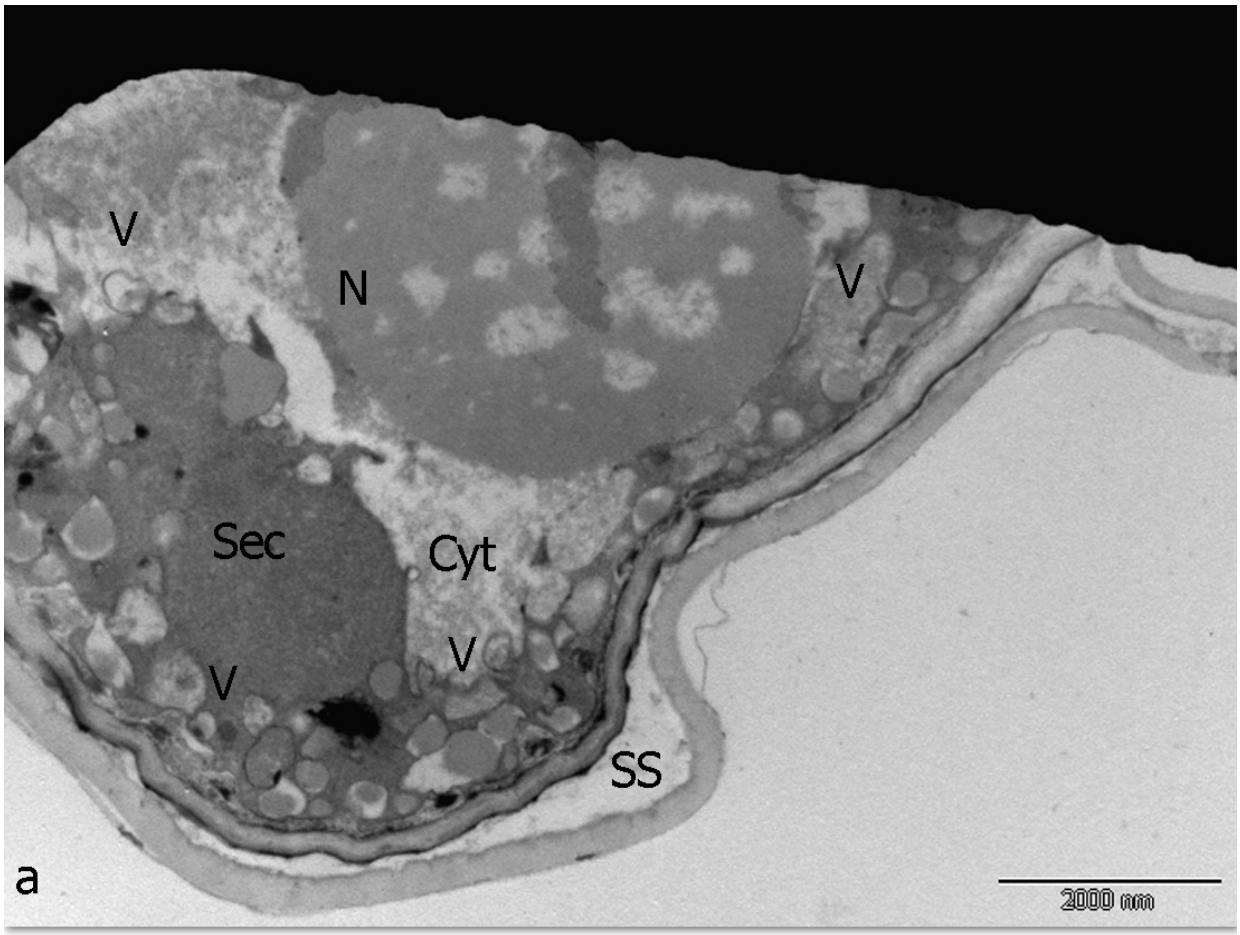

Cyt
V
Adj
CW
V
L
L
a
2 µm
Cyt
ER
M
ER
V
P
P
Vac
V
V
Vac
b
0.5 µm

Figure 27: TEM of Type I capitate trichome showing:

(a) nucleus (N) and dense cytomatrix (Cyt) indicating secretory phase. It appears that there is a mass movement of vesicles (V) and very dense secretory material (Sec) toward the top periphery of the secretory head cell wall to be sequestered into the developing subcuticular space (SS).

(b) at a higher magnification, endoplasmic reticulum cisternae (ER) were visible and vesicles appeared to contain secretory material being transported to the subcuticular space (SS) that has formed as a result of the separation of the cell wall (CW) and cuticle (Ct).


V
N
V
Sec
Cyt
V
V
SS
a
2000 nm

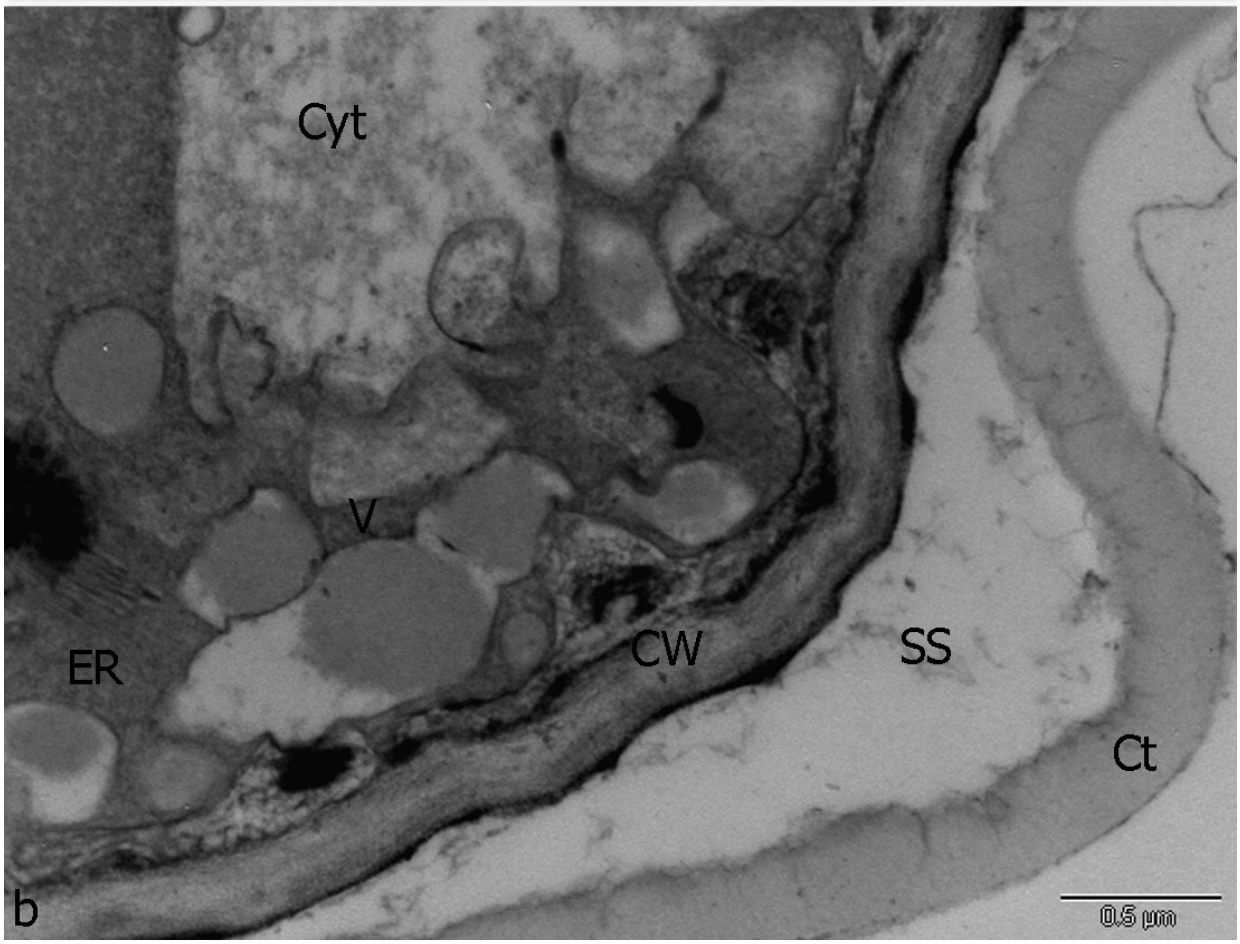
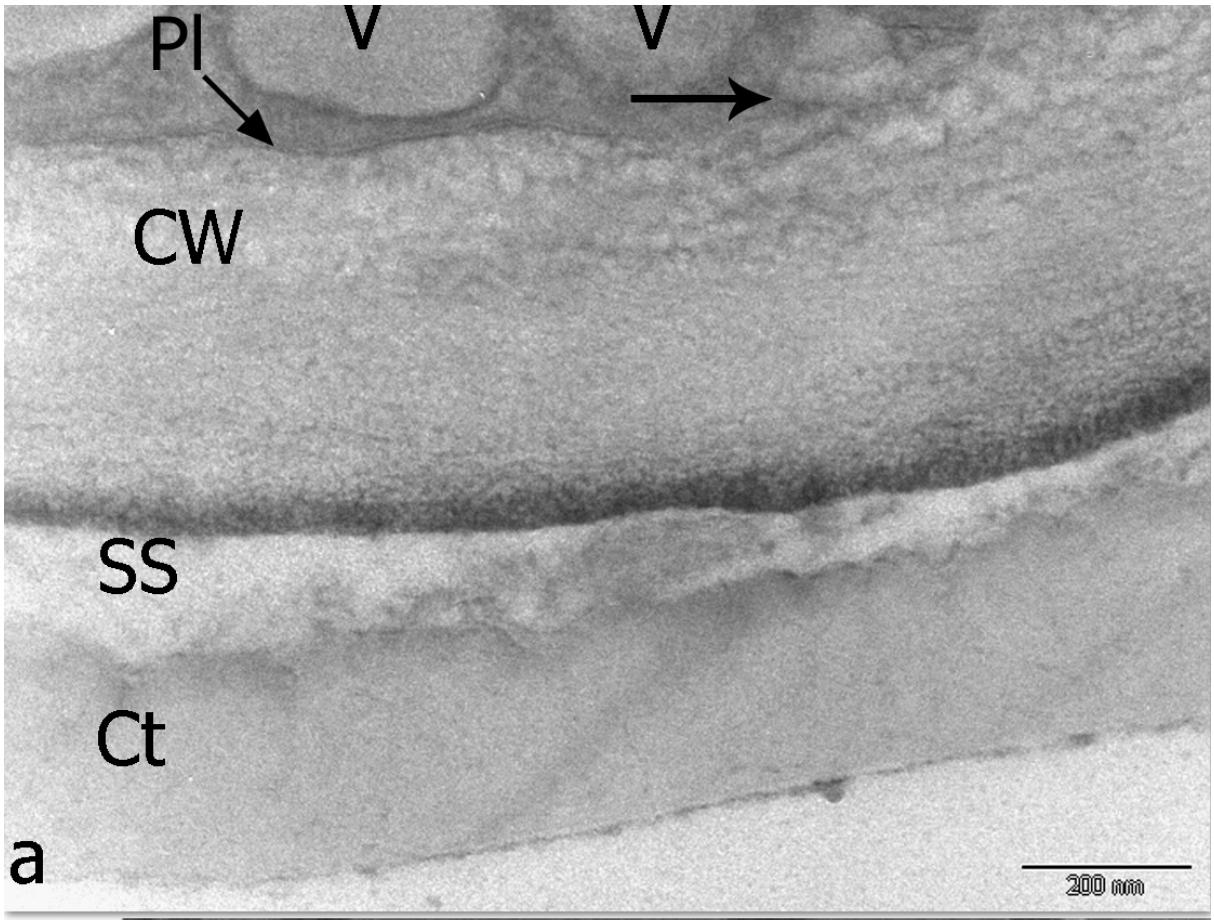
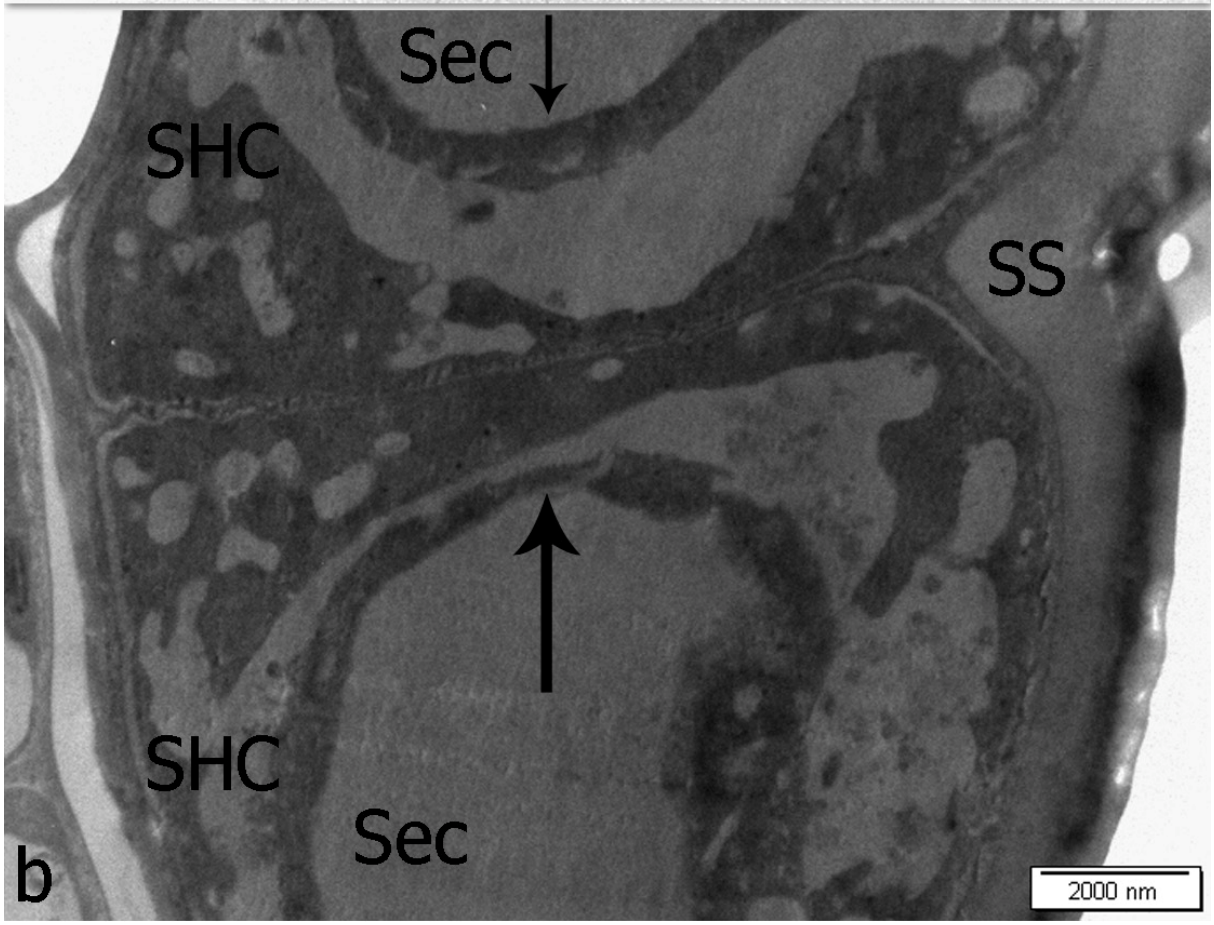

Cyt
V
ER
CW
SS
Ct
b
0.5 µm

Figure 28: TEM of ultrathin section of Type I capitate trichome showing:

(a) vesicles (V) close to the cell wall and vesicle (arrow) fusing with the plasmalemma (Pl) and cell wall (CW) that appears to be loosened in preparation for the storage of secretory material in the cell. The secretory product in the vesicle is sequestered into the subcuticular space (SS) and, upon mechanical stimulus, is released to the exterior of the trichome via cuticular (Ct) rupture.

(b) Type I capitate trichome that appears to be in post-secretory phase. The cell walls (arrows) of the secretory head cells (SHC) appear to have pulled away from the cuticle of the trichome giving the cells a plasmolysed appearance. The secretory material (Sec) can be seen in the subcuticular space (SS) between the cell wall and cuticle of the trichome.


Pl
V
V
CW
SS
Ct
a
200 nm


Sec
SHC
SS
SHC
Sec
b
2000 nm

Preliminary phytochemistry

The qualitative analysis of preliminary phytochemical screening of the extract is shown in Table 4. These assays revealed the identity of several major classes of compounds including carbohydrates, phenols, flavonoids, tannins, saponins, fixed oils and fats, cardiac glycosides and terpenoids. Secondary metabolites, including tannins, flavonoids, terpenoids and cardiac glycosides, were present in trace amounts in the extract. The extract did not contain sugars, alkaloids, anthraquinones and amino acids.

Table 4: Results of phytochemical screening of crude ethanolic extract of dried, powdered leaves of *O. obovatum*.

Compound	Test	Reaction observed	Present (+)/Absent (-)
Carbohydrates	Molisch's	Formation of a reddish-brown ring	+
Fructose	Fehling's	Precipitate formed but blue	-
Monosaccharides	Benedict's	No reaction	-
Phenolics	Ferric trichloride*	Dark green coloration	++
Flavonoids	Concentrated NH_3 was added to the ethanolic extract*	Yellow coloration	+
	Lead acetate	Flocculent white precipitate	+
Tannins	Braemer's	Greenish-grey coloration	++
Alkaloids	Draggendorff's	Yellow coloration	-
	Hager's	No reaction	-
	Wagner's	No reaction	-
Cardiac glycosides	The ethanolic extract was mixed with glacial acetic acid and concentrated sulphuric acid was slowly added*	Formation of a brick-red ring at the interface between sulphuric acid and mixed extract solution	+
Saponins	Foam test	Constant layer of foam after shaking aqueous solution of extract for 15 minutes	++
Anthraquinones	Borntrager's	Dark brown coloration of solution	-
Amino acids	Ninhydrin	Solution turned colourless	-
Fixed oils and fats	Filter paper	Oil patch	++
Steroids and terpenoids	Mixed ethanolic extract with chloroform and slowly added concentrated sulphuric acid**	Reddish-brown coloration at interface between sulphuric acid and mixed extract solution	++

+/- indicates presence/absence.

++ indicates intense reaction.

Tests were done as per methods outlined in Trease and Evans (1987); *Test from Krishnaiah et al., 2009; **Tests from Ramesh et al., 2010.

CHAPTER 5: DISCUSSION

This study was undertaken to provide useful information about the anatomy and morphology of the trichomes found on the leaves of *O. obovatum* subsp. *obovatum* var. *obovatum* as well as to assess the phytochemical content of the leaf extracts. This is the first report on the foliar secretory structure characteristics and preliminary phytochemistry of the species. Like other members of the Lamiaceae, *O. obovatum* was found to possess non-glandular trichomes and both peltate and capitate trichomes of two subtypes on both adaxial and abaxial surfaces of leaves in all stages of development.

Hallahan (2000) reported that peltate trichomes of the Lamiaceae comprise a broad head made up of several secretory cells, up to 16, a wide, short stalk and a basal epidermal cell. Peltate trichomes of *O. obovatum* were found to be made up of four head cells with a short stalk cell and single basal cell (see Figure 9). Type I capitate trichomes were observed to be comprised of two head cells, a short, unicellular stalk cell and a single basal cell. Rare Type II capitate trichomes were recorded to possess a single ovoid head cell, a single stalk cell and a single basal cell (see Figure 11). These findings are similar to those recorded for *O. basilicum* and several other species (Werker et al., 1993). However, for the morphology of trichomes for other members of the Lamiaceae such as *Origanum* species, *Plectranthus*, *Leonotis* and *Satureja thymbra*, Bosabalidis and Tseko (1984), Werker et al. (1985 a, b, c), Bosabalidis (1990), Ascensão et al. (1997) and Ascensão et al. (1999) report a variable number of head cells arranged differently.

It has been generally assumed that peltate trichomes are responsible for the majority of the essential oils produced in plants of the Lamiaceae (Maffei et al., 1989; Kokkini et al., 1994; Clark et al., 1997). However, dissidents refute this assumption because some studies have shown that various capitate trichomes also produce a significant amount of essential oils (Ascensão et al., 1999). The large size of the peltate trichomes and higher frequency, when compared to that of capitate trichomes, defines its role in determining the yield and quality of essential oils in *O. obovatum*. The presence of mature peltate trichomes across all stages of development suggests that oil gland neogenesis occurred throughout the ontogeny of the

leaves. In the pre-secretory phase, the neogenic peltate trichome appeared rigid and flat-topped and on the surface of the leaf in emergent leaves, whereas they were slightly sunken in shallow depressions on the surfaces of young and mature leaves. This rigid appearance is probably due to the production and accumulation of oil in the secretory head cells (see Figure 10 a). A developmental modification of these trichomes is the formation of a large subcuticular space that is used for the storage of secretory material. This space forms by the progressive elevation of the trichome cuticle together with a thin residual cell wall layer. The same observations were also recorded for the Type I capitate trichomes in the present study. Previous studies have also reported a similar pattern of cuticular elevation and subcuticular space formation in the secretory trichomes of *Cannabis sativa* (Kim and Mahlberg, 1995) and in some species in the Lamiaceae (Werker et al., 1993; Ascensão et al., 1997; Ascensão et al., 1999; Turner et al., 2000; Machado et al., 2006).

The morphology of peltate trichomes in the pre-secretory phase indicated that the cuticle appeared close to the wall of the secretory head cells, giving them an appearance of being devoid of oil with a depressed top, but still intact. These trichomes were present along the entire lamina on both surfaces yet they appeared to be most frequent in the basal region of emergent and young leaves. The wrinkled surface observed in some leaf samples could be an artefact or it could be related to the progressive subcuticular space formation (Hammond and Mahlberg, 1977; Venkatachalam et al., 1984). The distribution of peltate trichomes along the leaf surfaces of mature leaves of *O. obovatum* was sparse with the trichomes sunken into shallow depressions. Similar findings were reported for *O. basilicum* (Werker et al., 1993).

The capitate trichomes also vary in structure and size within the family, as well as within species (Werker, 1985a; Werker, 2000). The trichomes in *Salvia* spp. differ in terms of the number of head cells and stalk cells while those of *Coridothymus capitatus* only possess one type of capitate trichome (Werker et al., 1985a; Werker, 2000).The leaves of *O. obovatum* have capitate trichomes that are divided into two subtypes: Type I with two head cells, one to two stalk cells, and a single basal cell and Type II with a single ovoid head cell and one to two stalk cells and a single basal cell. These capitate trichomes were not as prominent as the peltate trichomes on both leaf surfaces across the different stages of development. However, Type II capitate trichomes were very rarely encountered and, if they were, they were recorded

in the vicinity of the midrib on the abaxial surface of the leaf. Werker et al. (1993) recorded similar observations in *O. basilicum*.

The non-glandular trichomes were also observed to have a significantly different distribution along the leaf laminas across all stages of development in *O. obovatum* when compared to *O. basilicum*. Non-glandular trichomes in *O. basilicum* occur only along the veins and margin of the leaves (Werker et al., 1993) while those of *O. obovatum* occur across the entire lamina on both surfaces of the leaf, across all stages of development. These trichomes also appear to be concentrated along the veins, margin and basal region of the leaves. The high frequency of non-glandular hairs that appear mostly along the basal regions of leaves of all stages of development, and predominantly on the abaxial surface, suggest that they serve a protective function. They shield the numerous peltate trichomes that appear to be concentrated in this region on the leaves of emergent and young leaves. This is one of the many suggested functions of this type of trichome (Werker, 2000).

The observed cellular pedestal provides mechanical support to the trichome as well as to serve as a point of attachment. The microornamentation present on the surface of these trichomes appear to be similar to those described for *Leonotis leonurus*, *P. ornatus* (Ascensão et al., 1999; Werker, 2000), *Xysmalobium undulatum* and *T. flavum* (Lakusic et al., 2005; Naidoo et al., 2009). They are apparently indicative of the age of the leaves. However, in *O. obovatum* microornamentation was observed in emergent leaves, suggesting a function other than an indicator of maturity. Evolutionary changes in the cell wall and cuticle of plant trichomes which usually serve to protect the plant surfaces from environmental stresses, such as excessive sunlight or increased rates of transpiration, include the development of mechanisms unique to different plants. One such example is the lotus plant, *Nelumbo nucifera*. The leaves of this plant are superhydrophobic, which is defined as one that has a static contact angle that is equal to or above 150° and a low hysteresis or low tilting angle of less than 10°. This physical phenomenon, dubbed ‘The Lotus Effect’, allows a water droplet to roll off, collecting dirt or dust particles in its wake thus providing a self-cleaning property (Koch et al., 2009). It is hypothesized that the microornamentation present on the cuticular surface of non-glandular trichomes of *O. obovatum* could serve a similar function to that of

N. nucifera. However, more observation and tests need to be done to confirm this phenomenon in *O. obovatum*.

The uneven distribution of all trichome types and their variable frequencies on the leaf surfaces at the different developmental stages begs the question: does the number of trichomes increase with maturity or if it remains fixed? Ascensão and Pais (1987) have suggested that the number of trichomes is established during the early stage of leaf differentiation and that this number remains fixed throughout the life span of the leaf. Others have concluded that the frequency of trichomes increases throughout all stages of foliar development (Croteau et al., 1981; Maffei et al., 1986; 1989). Werker et al. (1993) reported an increase in the production of trichomes for the duration of which a meristematic zone remains in a growing leaf in *O. basilicum.* Furthermore, it is suggested that the leaf ceases the production of new trichomes at the onset of leaf expansion and differentiation (Werker et al., 1993). There has been no other report on the issue since Werker et al. (1993), but some studies have suggested that the frequency of trichomes could vary due to contamination or pollution in a region (Sharma et al., 2003) indicating that trichome production could cease and initiate at various stages in response to environmental conditions.

Histochemical tests revealed that the secretory product within the subcuticular spaces of the peltate and Type I capitate trichomes of *O. obovatum* are lipid in nature, as indicated by the positive reaction to Sudan Red and Sudan Black B. The positive reaction to Sudan Red in the stalk cell of Type I capitate trichomes and cuticle of head cells of both peltate and Type I capitate trichomes and negative reaction of Ruthenium red also indicated the presence of structural lipids such as cutin or suberin. According to Fahn (1979 and 1988) and Werker (2000), this is an endodermal feature common in oil-secreting trichomes, preventing the backflow of secretory material into mesophyll tissue. Ruthenium red confirmed the presence of acid polysaccharidic substances, such as pectinaceous substances (Johansen, 1940), in the basal cells of peltate trichomes and secretory head cells of Type I capitate trichomes (see Figure 17). Despite the positive reaction in the trichomes, Ventrella and Marinho (2008) suggest that acid polysaccharides may be present in the cell protoplasts as a precursor of the secretory product. This was suggested because the positive reaction was not observed in the secretory material in the subcuticular space of peltate trichomes and was not specific to the

head cells of the Type I capitate trichome as was similarly observed in the Brazilian medicinal plant, *Cordia verbenaceae* (Ventrella and Marinho, 2008).

The presence of terpenes in the essential oils of the glandular trichomes was indicated by the positive reaction with NADI reagent and confirmed by the characteristic scent of the leaves. These essential oils rich in terpenes are most probably responsible for the medicinal properties displayed by the plant as recorded in studies on other plants in the Lamiaceae and Boraginaceae (Werker et al., 1985a; Ascensão and Pais, 1987; Ascensão et al., 1997; Harbone, 1997; Ventrella and Marinho, 2008). Despite the low specificity of the histochemical tests employed, NADI reagent has been used in many previous studies to locate terpenes in plant secretions (Ascensão et al., 1997; Corsi and Botega, 1999; Sacchetti et al., 1999; Machado et al., 2006). Ferric trichloride and potassium dichromate were used to identify the presence of phenolic compounds. The ferric trichloride test is based on the reaction of orto-dihydroxyphenols with ferric ions to form deep green or black deposits. No reaction was observed for this test in both peltate and Type I capitate trichomes. The potassium dichromate test is based on the formation of a product coloured by the condensation of free phenolic hydroxyl groups with the chromium of the reagent (Gabe, 1968). A positive reaction was observed when stained with potassium dichromate but only in the basal cells of peltate and Type I capitate trichomes. Wagner's and Dittmar reagents form red-brown precipitates with alkaloids (Furr and Mahlberg, 1981) and a positive reaction was only observed in the basal cells of peltate and Type I capitate trichomes. Like the reaction for Ruthenium red, this result could possibly indicate that this class of compound could be a precursor in the protoplasts for the secretory product since no alkaloids were detected by the phytochemical tests performed on a crude ethanolic extract of the leaf material of *O. obovatum*. Schilmiller et al. (2008) report that alkaloids are rare among the specialized metabolites produced and found in glandular trichomes and that no studies have reported their *de novo* synthesis in glandular trichomes in plants.

Ultrastructural observations of the glandular trichomes of *O. obovatum* confirmed the essential oil production and secretory function in the secretory head cells by the presence of polymorphic plastids with short profiles of ER cisternae and few Golgi bodies in close proximity, as well as numerous vesicles. These ER cisternae, Golgi bodies and vesicles have

been implicated in the transport of synthesized substances, especially triterpenes in plants of aromatic importance (Ascensão et al., 1999; Ascensão; 2010). Fewer Golgi bodies are present in the cells during triterpene synthesis (Ascensão, 2010). These triterpenes are most likely biosynthesized in the polymorphic, almost bell-shaped plastids, referred to as leucoplasts (Fahn, 1979; Croteau et al., 1981; Ascensão, 2010).

Despite the morphological differences between the different types of glandular trichomes, the ultrastructure is remarkably similar (Fahn, 1979; Ascensão, 2010). According to Fahn (1979), organelles involved in the process of secretion of hydrophilic substances include the endoplasmic reticulum and the Golgi apparatus. In cells responsible for the secretion of lipophilic substances almost all organelles are involved, i.e. nucleus, mitochondria, plastids, Golgi bodies, ER and the ground cytomatrix (Fahn, 1979). The plastid has been the most commonly mentioned organelle in literature associated with the synthesis of lipophilic substances (Fahn, 1979). Early ultrastructural studies undertaken on peltate trichomes of some plants of the Lamiaceae revealed that typical characteristics included well developed endoplasmic reticulum with dilated cisternae and vacuoles filled with strongly osmiophilic material (Amelunxen, 1965). During the initiation of the formation of the subcuticular space, the cytomatrix was observed to be relatively dense with an increased number of ribosomes (Amelunxen, 1965).

Plasmodesmata present between the two secretory head cells are indicative of intercellular communication and symplastic transport between the two cells (Fahn, 1979). There also appears to be a marked difference in the cell wall of trichomes and those of adjacent epidermal cells. The thicker cell walls of the trichomes could be a possible adaptation to prevent apoplastic flow of secretory material into neighbouring cells, as recorded for secretory trichomes (Fahn, 1979). Ascensão (2010) has also reported that, in most secretory trichomes, the fibrils of the secretory head cell walls appear to be loosened to accommodate the storage of secretory products. A similar phenomenon was observed in the cell wall of the secretory head cells of *O. obovatum* (Figure 28 a).

Cytoplasm densification (most probably due to the increase in the number of ribosomes) was observed in the glandular trichomes of *O. obovatum* and according to Ascensão (2010), this is indicative of the secretory phase. Other ultrastructural features of this phase, observed in the family, include the extensive development of plastids and ER cisternae proliferation (Ascensão, 2010). The numerous mini vacuoles and vesicles observed throughout the secretory head cells, especially along the wall bordering the subcuticular space in the glandular trichomes of *O. obovatum*, are probably responsible for the transport of secretory material out of the secretory head cells. In the Lamiaceae this mode of secretion has been speculated to be granulocrine in nature; i.e. the vesicles containing the cellular products destined for secretion travel to the cell surface where the vesicular membrane fuses and becomes integrated with the plasma membrane. The plasma membrane then pulls apart and discharges the vesicle contents to the exterior of the cell (Fahn, 1979; Ascensão et al., 1999; Sharma et al., 2003; Becker et al., 2006; Ascensão, 2010

It has been reported that secretion in glandular trichomes can occur in one of three ways; the rupture of the cuticle in weaker regions (Ascensão and Pais, 1987; Figueiredo and Pais, 1994; Ascensão et al., 1995; Serrato-Valenti et al., 1997; Bisio et al., 1999), through pores in the cuticle (Bisio et al., 1999; Ascensão et al., 1999) or via diffusion through the cuticle (Serrato-Valenti et al., 1997). While the elimination of secretory products to the subcuticular space is not fully understood, the results suggest that ER vesicles transport the secretory material to the wall of the secretory head cell for sequestration into the subcuticular space or this occurs via transient ER-plasma membrane fusion (Ascensão, 2010). In the glandular trichomes of *O. obovatum* vesicles were observed fusing with the plasma membrane (Figure 25 b) and a mass movement of vesicles filled with secretory material was also observed (Figure 27 a). This mass aggregation is closely associated with a developing subcuticular space created by the elevation of the trichome cuticle away from the wall of the secretory head cell (Figure 27). Evidence from previous studies suggests that the movement of the terpene oils across the plasma membrane to the subcuticular space is a result of directional lipid transfer or polarization (Sharma et al., 2003). This occurs in either membrane vesicles or exocytosed vacuoles or is mediated through a large family of lipid-transfer proteins (on a molecular level) as recorded to occur in many plants (Sharma et al., 2003). Essential oil discharge to the exterior of the trichome is most likely to be released by rupture of the cuticle as no cuticular micropores were observed (Ascensão, 2010).

During the secretory phase, active biosynthesis of the terpene oil probably occurred in the glandular trichomes of *O. obovatum*, resulting in the head cells accumulating the oil. When the cuticles of the trichomes were distended, they appeared almost smooth and balloon-like (see Figure 9). This distended surface is indicative that the trichome cuticular cavity is filled with essential oils. Sharma et al. (2003), state that this particular phase in the glandular trichome is indicative of the developmental progression towards the termination of oil production and, thus, secretion. Maturation of peltate trichomes was suggested by the creation of a subcuticular space over the four secretory head cells and the progressive sequestration of the terpene oil into this subcuticular space (see Figure 14).

Subsequent to the filling of essential oil, the post-secretory phase was marked by the rupture of the peltate trichome cuticle along what appears to be a predetermined line of weakness along the equatorial region of the trichome head (see Figure 10 b). This rupture probably resulted from an increased internal hydrostatic pressure within the subcuticular space and from within the secretory head cells. Transmission electron micrographs revealed that there appeared to be a re-distribution of secretory material during senescence of the peltate trichome (see Figure 23 b). This could possibly account for the way in which the trichome ruptures since no pores or areas indicating the release of secretions were observed. Previous studies have reported that under natural conditions, mechanical stimulus by herbivores caused the cuticle of glandular trichomes to rupture, releasing the contents of the secretory trichomes on to the various surfaces of the plants (Werker, 1993; Werker et al., 1993; Ascensão et al., 1995; 1997; Serrato-Valenti et al., 1997; Ascensão et al., 1999; Sacchetti et al., 1999; Pichersky and Gershenzon, 2002).

Further studies should be conducted to measure the thickness of the peltate trichome cuticle. It is suspected that a particular area of the cuticle is thinner than the rest and this could offer an explanation for the rupture along a seemingly predetermined line of weakness along the equator of the trichome. Similar observations were made in *P. ornatus* (Ascensão et al., 1999).The consequence of this rupture was the extrusion of the secretory material to the external periphery of the trichome and the subsequent collapse of the trichome head (see Figure 10 b). Mechanical stimulus or injury could also be responsible for the collapse of these

trichomes. For example, insects crawling on the leaf surface could rupture the peltate trichomes and release the essential oils which probably act as a repellent.

Consistent with studies on other members of the Lamiaceae such as *Mentha piperita*, *Salvia officianalis*, *L. leonurus*, *Sideritis syriaca*, *P. ornatus* and *P. laxifloris*, and several *Teucrium* species (Croteau et al., 1981; Werker et al., 1985a; Ascensão et al., 1995; Ascensão et al., 1999), the glandular trichomes of *O. obovatum* have a large subcuticular space. Secretory products are synthesized in the head cells and secreted into the subcuticular space where they accumulate and undergo some changes in the constituents of the essential oils (Werker, 1993; Ascensão et al., 1999). Rapid and permanent turnover of monoterpenes have been recorded in *M. piperita*. This has resulted in the loss of quality and quantity of essential oils present in the glands due to catabolism (Croteau and Martinkus, 1979; Croteau, 1987; Werker, 1993; Ascensão, 1999).

Triterpenoids are a prominent group of terpenoids in plants and are reported to be the main constituent of the essential oils in *O. obovatum* (Fawole et al., 2009a). This group of terpenoids are reported to exhibit numerous properties ranging from mammal toxicity, molluscidal activity, anti-feedant activity to hormonal interference in insects (Harbone, 1997). These triterpenoids have been recorded in *O. obovatum*. Burger et al. (1998) have reported two triterpenes, beciumecine 1 and 2. These triterpenoid saponins have shown anti-cancer activity when tested on H522 human lung carcinoma cells (Burger et al., 1998). Other chemical compounds that have already been isolated and identified in *O. obovatum* and other *Ocimum* species include 8-oxygenated leaf-surface flavones, cirsimaritin, isothymusin and xanthomicrol (Grayer and Veitch, 1998; Grayer et al., 2001).

The medicinal properties of cirsimaritin, isothymusin and xanthomicrol isolated and identified in other traditionally used medicinal plants in the different parts of the world (Abdalla and Zarga, 1987; Suksamrarn et al., 2003; Jahaniani et al., 2005; Agarwal et al., 2009), support the use of *O. obovatum* in the treatment of ailments as outlined in Burkhill (1985) and Hutchings et al. (1996) in the areas where the plant is found. However, there is still a gap in the knowledge of the exact chemistry and pharmacological effects of the

essential oils. Future studies need to include cytotoxicity tests as well as screening for antimicrobial activity.

Results obtained from preliminary phytochemical screening conducted on a crude ethanolic extract of the leaves of *O. obovatum* indicated the presence of phenolics, tannins, flavonoids, steroids and saponins. Studies conducted on these secondary metabolites reveal the wide-ranging medicinal properties exhibited (Hausteen et al., 1983; Motar et al., 1985; Ruch et al., 1989; Just et al., 1998; Grierson and Afolayan, 1999; Ferguson, 2001; Li et al., 2003). Phenolic compounds have been suggested to play an important role in the leaves of the plants by protecting them from the damaging effects of UV rays and also against certain phytopathogenic microorganisms (Sharanabasappa et al., 2007). Studies have also reported that the antioxidant activity in higher plants is closely correlated with the plant phenolic content (Thabrew et al., 1998; Kähkönnen et al., 1999; Akinmoladun et al., 2010). Akinmoladun et al. (2010), report that the risk of chronic, degenerative diseases may be reduced with the inclusion of phenolics, such as flavonoids, in our diet.

Flavonoids have been identified as health promoters due to its anion radicals, accounting for the antioxidative properties displayed in wound healing (Bruneton, 1999; Aiyegoro et al., 2010). This secondary metabolite has been suggested to play a key preventative role in the development of cancer and heart disease (Kähkönnen et al., 1999). (Schuier et al. (2005) reported that flavonoid derivatives play a significant role in the inhibition of the development of fluids that lead to diarrhoea and abdominal cramps. This, therefore, further substantiates the use of *O. obovatum* in traditional medicinal remedies, especially in the treatment of gastric ailments in children as noted by Hutchings et al. (1996).

Aiyegoro et al. (2010) reported that tannins play a role in the treatment of inflamed and ulcerated tissues. This chemical compound promotes waterproofing of the external layers of skin and mucosa, offering protection to the underlying layers (Bruneton, 1999). Tannins have also been reported to cause vasoconstriction in small superficial blood vessels promoting regeneration in cases of superficial wounds and limiting the loss of fluid (Bruneton, 1999). Studies on tannins isolated from plant material have also shown significant activity in the

prevention of cancer and as an anti-cancer treatment (Motar et al., 1985; Ruch et al., 1989; Erdèlyi et al., 2005). This secondary metabolite has also been cited to possess anti-diarrhoeal properties (Bruneton, 1999).The presence of tannins in the leaves of plants such as *H. longifolium*, highlights the plant as a potential source of bioactive compounds in the treatment of cancer (Erdèlyi et al., 2005; Aiyegoro et al., 2010). The use of *O. obovatum* in the treatment of cancer cases in KwaZulu-Natal could be attributed to the presence of tannins in the leaf extract.

Saponins, which are large forms of glycosides, were detected in the ethanolic leaf extract of *O. obovatum* and have been reported to be present in the plant (Burger et al., 1998). This important class of compound is reportedly responsible for most of the observed biological effects. Saponins are divided into two distinct groups on the basis of their aglycones; steroidal saponins and triterpenoid saponins (Bruneton, 1999; Aiyegoro et al., 2010). Steroidal saponins are almost exclusively present in monocotyledonous angiosperms while triterpenoid saponins are common in eudicotyledonous angiosperms (Bruneton, 1999). Saponins prevent inflammation and display antibacterial, anti-oedema, antifungal and anti-amoebic properties and could thus play a significant role in prevention of hair loss and the treatment of skin infections for which *O. obovatum* is traditionally valued (Bruneton, 1999; Fawole et al., 2009b). Cardiac glycosides were also detected in the plant extract of *O. obovatum*. These secondary metabolites have been implicated in the treatment of various heart disorders (Bhowmik et al., 2010) and their bitter taste renders the leaves unpalatable to insects thus deterring herbivory (Gökçe et al., 2009). Alkaloids were not detected in the plant leaf extract of *O. obovatum*.

The preliminary phytochemical findings of this study are similar to those identified by Fawole (2009b) in *B. obovatum*. Phenolics, tannins, flavonoids, steroids and saponins present in the leaf material of *O. obovatum* are responsible for the medicinal properties for which the plant is exploited. Preliminary phytochemical tests are thus helpful in the determination of the nature of the compounds present in the chemical constituents found in plant material. For example, quantification of phenols and flavonoids in the plant could provide sufficient data to understand the basic pattern of growth and metabolism as well as serve as chemical markers

in taxonomic studies. These tests may also lead to the location of the source of pharmacologically active chemical compounds.

The presence of peltate and Type I capitate trichomes filled with terpenoids in leaves of *O. obovatum* from emergence through to maturity is suggestive of a protective function. Nishida (2002) reports that terpenoid substances function as toxicants, antibiotics and serve as anti-feedants. The high frequency of non-glandular trichomes on both surfaces of leaves across all developmental stages also appear to be a protective adaptation against oviposition and herbivory, which is further assisted by the production of terpenoids (Nishida, 2002; Machado et al., 2006).

Micromorphological characters such as trichomes are important diagnostic characters in the differentiation of taxa (Cantino, 1990; Paton et al., 1999). These can assist in characterization of various taxa in other genera of the Lamiaceae, especially in the case of hybrids (Giuliani et al., 2008). Histochemical and ultrastructural assessments are also useful in the determination of the nature of the secretions produced by the glandular trichomes. The importance of phytochemical studies has been emphasized due to their significant role in the study of systematics, as well as in the search for additional resources of raw materials for the pharmaceutical industry.

While primary metabolites contribute to the functional integrity of plant cells and tissue, secondary metabolites do not appear to be vital to the immediate survival of the plant (Sharanabasappa et al., 2007). Further physico-chemical and spectroscopic studies need to be conducted to isolate and identify components of the various classes of phytochemical compounds. The *in vivo* potential of the plant in the treatment of gastrointestinal diseases, resulting from bacterial infections in humans as well as anti-cancer activity, also needs to be clarified. The results generated from this study may be useful in clarifying the confusion surrounding the generic position of *O. obovatum* subsp. *obovatum* var. *obovatum* in the family Lamiaceae. There is still debate by taxonomists as to whether this species should be classified as belonging to the genus *Ocimum* or the genus *Becium*. Apart from genetic studies, studies on trichome morphology, function and development could provide valuable

information that may contribute to a more phylogenetically correct classification for *O. obovatum*. Micromorphological studies, particularly of trichome structure and phytochemistry, are important if we are to have a holistic understanding of other South African medicinal plants.

CHAPTER 6: CONCLUSION

This study set out to investigate the foliar secretory structures of a traditionally used indigenous medicinal plant, *Ocimum obovatum.* The investigation was conducted using a variety of microscopic methods, including light and electron microscopy, and histochemical and phytochemical assays.

Results of the study indicates that there are three types of trichomes present on the adaxial and abaxial surfaces of the leaves across all developmental stages; non-glandular and two types of glandular trichomes. Non-glandular trichomes that are uniseriate, multicellular and covered in micro-ornamentation probably function as a physical barrier against adverse environmental conditions and herbivory while the cuticular warts possibly promote self-cleaning via the physical phenomenon known as 'The Lotus Effect'. Glandular trichomes consisted of peltate trichomes and capitate trichomes further divided into two subtypes; Type I capitate trichomes and Type II capitate trichomes. Peltate trichomes comprised of four head cells, a very short, almost non-existent stalk cell and a large basal cell and appear to be sunken into the epidermis as the leaves mature. Type I capitate trichomes were made up of two head cells, a single stalk cell and a basal cell. Type II capitate trichomes were rare, found mostly along the midrib of the abaxial surfaces of leaves. These trichomes were comprised of a single large, ovoid head cell, one to two stalk cells and a single basal cell.

Histochemical tests showed that peltate and Type I capitate trichomes have cutinized or suberized walls in the stalk cell to prevent the apoplastic flow of secretory material into the neighbouring mesophyll tissue. The histochemical stains also showed that the secretory material present in the glandular trichomes are lipid in nature and essential oils are present. These essential oils are most likely responsible for the medicinal properties of the plant. Acid polysaccharides are also suggested to be the precursor of the secretory product. The ultrastructural study revealed polymorphic leucoplasts, few Golgi bodies, numerous vesicles and vacuoles, mitochondria and short profiles of ER cisternae. The cytomatrix appeared to become denser during the active process of secretion and a mass movement of vesicles filled with secretory material and other organelles seem to be associated with the developing

subcuticular space formed by the elevation of the cuticle away from the wall of the secretory head cell. Phytochemical tests revealed the presence of essential oils that are terpene-rich. Flavonoids, tannins, saponins, terpenoids, fixed oils and fat, phenolics and cardiac glycosides were also detected in a crude ethanolic extract of the leaves. These chemical compounds are probably responsible for the medicinal properties for which the plant is traditionally exploited.

Future research should embark on studies to isolate the active compounds in the essential oils of the leaves. The isolated essential oils, crude ethanolic and hot water extract of the leaves should be screened for antimicrobial and antioxidant activity, as well as cytotoxicity to establish the safety of the plant for use as a traditional remedy and potential commercial use.

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Appendix A

Essential oil extraction (European Pharmacopoiea, 1975)

Approximately 80 g of fresh leaves were subjected to hydro-distillation using a Clevenger-type apparatus. The leaves were boiled for 4 hours and the essential oils were collected in a hexane fraction. The collected essential oils were dried with anhydrous sodium hydroxide and stored in a light sensitive vial at 4°C until required for use.

Appendix B

Thin Layer Chromatography (TLC) and Preliminary High Performance Thin Layer Chromatography (HPTLC)

A sample of the crude ethanolic extract (10µl) was spotted onto pre-coated silica gel 60 F254 TLC plates (Merck) and developed in an optimized mobile phase of toluene: ethyl acetate: glacial acetic acid (9:0.8:0.2) to a distance of 8 cm. The plates were then imaged in light and then stained with anisaldehyde reagent made up of 0.5 ml anisaldehyde, 10 ml glacial acetic acid, 85 ml ethanol and 5 ml concentrated sulphuric acid. After staining, the plate was dried and imaged with a digital camera.

Results from the HPTLC of a crude ethanolic extract of dried, powdered *O. obovatum* leaves visualized at 254 nm, 366 nm and after being sprayed with anisaldehyde reagent revealed the presence of numerous classes of compounds (Figure 1).

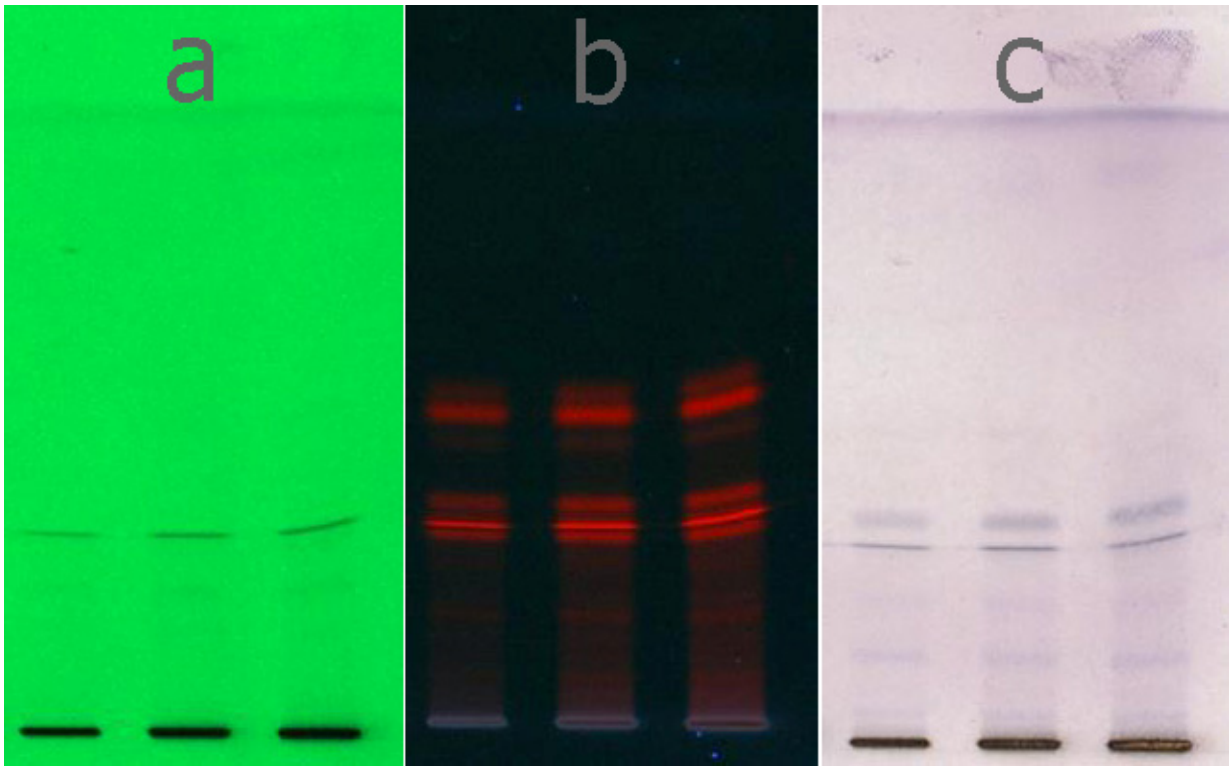

Figure 1: Results from high performance thin layer chromatography (HPTLC) of crude ethanolic extract of dried, powdered leaves of *O. obovatum* viewed under UV-light at (a) 254 nm, and (b) 366 nm and (c) after being sprayed with anisaldehyde reagent. These results indicate that the extract is comprised of a number of different compounds with varying class sizes when allowed to separate in a mobile phase of toluene: ethyl acetate: glacial acetic acid (9.5:0.4:0.1).