THE LEAF SECRETORY APPARATUS OF *HIBISCUS SURATTENSIS* AND *HIBISCUS SABDARIFFA* (MALVACEAE): MICROMORPHOLOGY, HISTO-PHYTOCHEMISTRY AND ULTRASTRUCTURE

Kashmira Raghu

Email address: 206500843@ukzn.ac.za

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Supervisor: Dr. Y. Naidoo

Email address: naidooy1@ukzn.ac.za

Co-supervisor: Prof. A. Nicholas

Email address: nicholasa@ukzn.ac.za

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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ABSTRACT

The research presented here forms the basis of the ethnobotanical and ethnopharmacological evaluation of *Hibiscus surattensis*, which is a widely distributed vegetable and medicinal shrub used by African and Indian traditional practitioners. Using light microscopy together with advanced electron microscopy techniques, the leaf topography and internal structure was examined. A closely related species, *Hibiscus sabdariffa* was also investigated for foliar and histophytochemical comparisons. *Hibiscus sabdariffa* is a widely consumed medicinal species with well-known biological activity and known chemical principles. Both species belong to the section Fucaria within the genus *Hibiscus*. Analyses of foliar secretory tissues showed that both *H. surattensis* and *H. sabdariffa* were characterised by capitate trichomes as well as mucilage-producing ducts and idioblasts. Head cells of capitate trichomes were between 5 and 7, while lignified stalk cells occurred in numbers of 2 or 3. A basal cell supporting the trichome was implanted in the epidermis comparative study between the two species showed some variation of chemical composition in trichome head cells. Polysaccharidic, alkaloids, phenolic and acidic lipid components were identified for both *H. surattensis* and *H. sabdariffa* whereas *H. sabdariffa* showed an intense staining of proteinaceous substances. Densities of capitate trichomes varied from emergent to mature developmental stages, with the highest number occurring on the emergent abaxial surface for both *H. surattensis* and *H. sabdariffa*. However, a distinct trend was observed for *H. surattensis*, where a decrease in capitate trichomes with progressive development was associated with the proliferation of mucilage idioblasts, particularly on the mature adaxial surface. Mucilage producing tissues are assumed to be associated with a protective role against dehydration in a number of plant families. Idioblasts observed in *H. surattensis* were visible leaf surface structures, embedded in the epidermal tissues. They contained considerable amounts of acidic polysaccharides and acidic lipids, and appeared to be implicated in reducing evaporative water loss in fully expanded leaves. Mucilage ducts were identified in vascular tissue within leaf veins. They occurred parallel with the conducting tissue and comprised of an epithelial layer of cells which seemed to be secreting a mucilage into an extracellular lumen. Crystal idioblasts were also identified in tissues of both *H. surattensis* and *H. sabdariffa.* The sequestration of calcium oxalate is assumed to be governed and regulated by specialised mesophyllous idioblasts that in turn appeared to be triggered by excess calcium within the plant body to differentiate into crystal forming cells. The supposed function of calcium oxalate crystals includes mechanical support and herbivory avoidance.

TEM revealed the cellular processes which involved crystal idioblast development in *H. surattensis*. This appeared to demonstrate that degeneration of organelles which are assumed to

occur when crystals reach maximal proportions. The non-glandular trichome component of each *Hibiscus* species was diverse in trichome type and might have accounted for differences in leaf texture and the leaf indumentum. The prickly texture of *Hibiscus surattensis* was attributed to rigid stellate and falcate trichomes as well as restrorse prickles, whereas the leaves of *H. sabdariffa* were glabrous with few falcate, bi- and trifurcate trichomes found mainly at the base or along the midvein. Preliminary phytochemical experiments which involved methanolic, chloroform and hexane extracts, yielded favourable results, which showed that leaves of *H. surattensis* and *H. sabdariffa* were chemically similar, in this regard, the author recommended further investigation into the phytochemical nature of *H. surattensis.*

CONFERENCES CONTRIBUTIONS FROM THIS THESIS:

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LIST OF ABBREVIATIONS

SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
MANOVA	Multiple Analyses of Variance
MMU	Microscopy and Microanalysis Unit
NGT	Non-Glandular Trichome
GST	Glandular Secretory Trichome
MC	Mucilage Cell
MD	Mucilage Duct
E	Emergent
Y	Young
M	Mature
V	Vacuole
V/Ve	Vesicle
LM	Lamellar material
M	Mitochondria
GB	Golgi Body
SCS	Subcuticular Space
N	Nucleus
C	Cuticle
CW	Cell Wall
ER	Endoplasmic Reticulum
RER	Rough Endoplasmic Reticulum
SM	Secreted Material
PM	Plasma Membrane
MVB	Multivesicular Body
EDD	Electron Dense Deposits
COC	Calcium Oxalate Crystal
SE	Standard Error

CHAPTER 1: INTRODUCTION

Ecosystems throughout the world are currently facing some of the most radical environmental changes seen in millennia, with evidence pointing to anthropogenic causes. The decline of botanical species in South Africa is a major cause for concern, especially since our biomes consist of intricately linked biotic and abiotic components. In addition, strong cultural ties with the natural environment also weighs heavily on the species comprising it, and as such harvesting of medicinal plants for traditional purposes has driven a large fraction of South African the flora to the brink of extinction. The identification, exploration and screening of traditionally consumed African medicinal plants are important for conservation and propagation schemes.

The worldwide concept of phyto-therapeutic medicine spans many generations and civilizations of humanity (Farnsworth *et al*., 1985; Gurib-Fakhim, 2006; Fabricant and Farnsworth, 2001). Current practices range from traditional herbal and alternative remedies to sophisticated compound isolation (Farnsworth *et al*., 1985). Despite the lengthy existence of this phyto-medicinal succession, the majority of earth's plant species are still to be screened for beneficial applications. Traditional knowledge systems are frequently the primary source of pharmacological research of novel compounds (Rybicki *et al*., 2012; Fabricant and Farnsworth, 2001) and there has been enormous success in the discoveries of commercially relevant compounds with proven biological activity from knowledge based on ancient cultural practices (digoxin, quinine and vinblastine; Gurib-Fakhim, 2006). At present, efforts in medicinal plant research are focused on the following objectives (Fabricant and Farnsworth, 2001):

a) extracting compounds of medicinal value that can be applied directly as a drug,

b) synthesizing organic compounds that might reduce the toxicity or increase the activity of present drug substances,

c) deriving substances that might improve the pharmacological efficiency of a drug,

d) utilizing plant components or entire plants in illness alleviation.

In a similar manner to many African countries, South African medicinal plant research is challenged by various factors: diminishing wild flora, as a result of habitat degradation, climate change, human encroachment and unsustainable utilization (Williams *et al*., 2013). A significant proportion of the South African population still rely on traditional healthcare providers (Williams *et al*., 2013), who's primary source of therapies is reported to emanate from the country's diverse flora. Uncontrolled harvesting by unskilled or unknowledgeable individuals

has resulted in deterioration of natural populations of many medicinally significant species (Taylor *et al*., 2001). The typical example is the natural stands of African Wild Ginger (*Siphonolochilus aethiopicus*) in KwaZulu-Natal which have been destroyed by overharvesting and destructive harvesting procedures (removal of rhizome and roots, Zschocke *et al.*, 2000). According to van Wyk (2008), propagation from rhizome segments is relatively simple, however, this species is a slow germinating annual and harvesting rhizomes in natural populations renders them severely susceptible to extinction (Zschocke *et al*., 2000) or reduced genetic diversity. This poses a challenge for the conservation of this species to the plant researchers and the government as the economic and cultural importance of this species has warranted its protection by South African conservation authorities (Zschocke *et al.*, 2000).

Restricted utilization of medicinal plant species has its drawbacks. Despite protection efforts, *S. aethiopicus* will not fare well in the wild given the present demands in traditional *muthi* markets. Therefore a different solution is required to maximize conservation attempts. Zschocke *et al*. (2000) examined plant part substitution as a remedy for invasive harvesting practices, and showed that aerial parts of plants often possess similar phytochemical properties as the exploited organ (roots, rhizomes, bark), and can therefore be substituted in place of the exploited plant part to either minimize damage to the plant or preserve the plant altogether. Though the exploited organ is usually rich in phytochemicals of interest, harvesting of them usually signals death of the entire plant. Plant part substitution might reduce the harvesting pressure on threatened species but enforcing a certain type of harvesting practice is problematic since no regular monitoring regime is in effect. Zschocke *et al*. (2000) also considers species substitution, but argues that consumers are less inclined to utilize new or different species, even if they might function in the same way as exploited species.

It is well known that medicinal plants exhibit certain chemical traits that are beneficial for the health and wellbeing of humankind, our livestock and pets. In recent decades, scientists have begun to enquire where these chemical and medicinal properties originate from within the plant, and what structures are responsible for conferring the pharmacological action of medicinal plant species (Svoboda and Svoboda, 2000). In most cases, it is found to be specialized structures that synthesize, secrete or store secondary metabolic products for the purposes of disease prevention, protection against water loss, and anti-herbivory tactics (Svoboda and Svoboda, 2000). In addition to functioning as biochemical factories, plant secretory structures also serve as taxonomic characters, aiding in the identification of plant families (Svoboda and Svoboda, 2000). Those which occur as external glands or hairs on the surfaces of plant organs are known

as trichomes and might be species or family specific (Svoboda and Svoboda, 2000) as are their chemical constituents.

In recent years, the elucidation of secretory pathways within trichomes has received considerable interest (Schilmiller *et al.* 2008). The secretory products derived from trichomes, are synthesized within the specialized cells comprising it, as there is no direct link between trichomes and the plant vascular system (Schilmiller *et al*., 2008; Fahn, 1988). Trichome secretions was reported to vary substantially, but generally consist of proteinaceous, polysaccharide or lipophilic material (Fahn, 1988). Further chemical classes comprising trichome exudation include terpenes, phenolics and alkaloids (Schilmiller *et al.,* 2008). Understanding of the activities and physiology of trichomes as well as functionally similar secretory structures, allows for further investigations into metabolic and genetic engineering of secretory processes, for the optimization of exudate output and quality (Schilmiller *et al*., 2008). Belonging to the family Malvaceae, the genus *Hibiscus* L. comprises approximately 200 species (Maganha *et al.*, 2010), a number of which have been medicinally exploited by many types of cultures and civilizations (Lim, 2012). In recent years, investigators have focused their pursuits on the phytochemical and pharmaceutical properties of some members of *Hibiscus* (Maganha *et al.*, 2010), but few have considered phytochemical secretion in relation to foliar micromorphology and physiology.

1.1. DESCRIPTION OF *Hibiscus surattensis* L.

Hibiscus surattensis L. is an indigenous scrambling annual commonly known as Wild Sour. Its distribution ranges from KwaZulu-Natal to the northern parts of South Africa bordering Zimbabwe. It was observed alongside rural homesteads around Nagel Dam in the Pietermaritzburg area as well as many roadside verges (personal observation). The reported ethnobotanical properties of *H. surattensis* span a range of illnesses and have been documented throughout various parts of Africa and India (Table 1).

The distribution of *H. surattensis* throughout Africa and Asia is depicted in Figure 2. The central and north western regions of Africa contain a greater number of *H. surattensis* populations than the extreme northern and southern regions of the continent. s*urattensis*, the species name, is derived from the Indian trade port, Surat (Ewan, 1970).

Table 1: Reported ethnomedicinal uses of *Hibiscus surattensis* in Africa and India.

Parts Used	Ailment	Country/Area	Reference
Aerial parts	Polyhydramnios	Nyong Valley, Cameroon	Jiofack *et al.*, 2009a
Leaves, flowers	Palpitation, gastralgia	South West Cameroon	Jiofack *et al.*, 2009b
Whole plant together with other herbs, prepared in calabash.	Anthrax	Northern Ghana	Oseni and Iddrisu, 2012
Flowers	Hypertension	Edo State, Nigeria	Gbolade, 2012
Unspecified, supplements the use of *Warburgia salutaris.*	Sores and skin irritations	Southern Africa	Mabona *et al.*, 2013
Leaves	Malaria	Benin, West Africa	Yetein *et al.*, 2013
Leaves	Venereal disease	South Africa	Pooley, 2013
Stems and Leaves	Venereal disease and Urethritis	Mizoram, India	Sharma *et al.*, 2001
Unspecified	Cough	Akwa Ibom State, Nigeria	Bassey and Effiong, 2011
Leaves	Wounds, Abscesses, Gonorrhoea	Morogoro, Tanzania	Amri and Kisangu, 2012

Thus far *H. surattensis* has received little scientific attention compared to its numerous relatives whose biological and pharmaceutical activities have been preliminarily reported (Maganha *et al.*, 2010; Salem *et al.*, 2014). Various ethnobotanical studies have highlighted its uses in traditional medicine in many countries, including Cameroon, Nigeria, Tanzania, South Africa and India (Table 1). In South Africa, it is used as part of a topical lotion together with the bark of *Warburgia salutaris* (G. Bertol.) Chiov. to treat inflamed dermal sores and irritations (Mabona *et al*., 2013) (Table 1). In Benin, West Africa, malaria is a prominent and often lethal killer and *H. surattensis* is one of the most commonly utilized species involved in malaria treatment, with the leaves ingested as a decoction (Yetein *et al*., 2013) (Table 1). The flowers of *H. surattensis* are consumed for treatment of hypertension in Edo state, Nigeria (Gbolade, 2012); and in Mizoram, India and the eastern regions of South Africa, the stems and leaves are used as a lotion to treat venereal disease and ureteritis (Sharma *et al*., 2001; Pooley, 2013) (Table 1).

Aerial parts of *H. surattensis* are covered in sharply pointed restrorse prickles (Akpan and Hossain, 1998) which might be injurious to large herbivores. Leaves, petioles, pedicels and stems are densely pubescent, with silvery hairs visible to the naked eye. The leaves occur alternately on the stem and petioles are often ensheathed with leafy stipules at the node. Some leaves are deeply lobed, appearing palmate, while others are broad and heart-shaped. Leaf margins are serrated and often appear reddish, suggesting the presence of anthocyanin compounds. Flowers are actinomorphic and bisexual, with most floral parts being pentamerous.

Stamens are monadelphous forming a tube through which the style emerges, as with other members of *Hibiscus* (Akpan, 2000). The ability of *H. surattensis* to self-pollinate (Akpan, 2000) demonstrates and evolutionarily advanced trait that promotes reproduction during unfavourable breeding conditions.

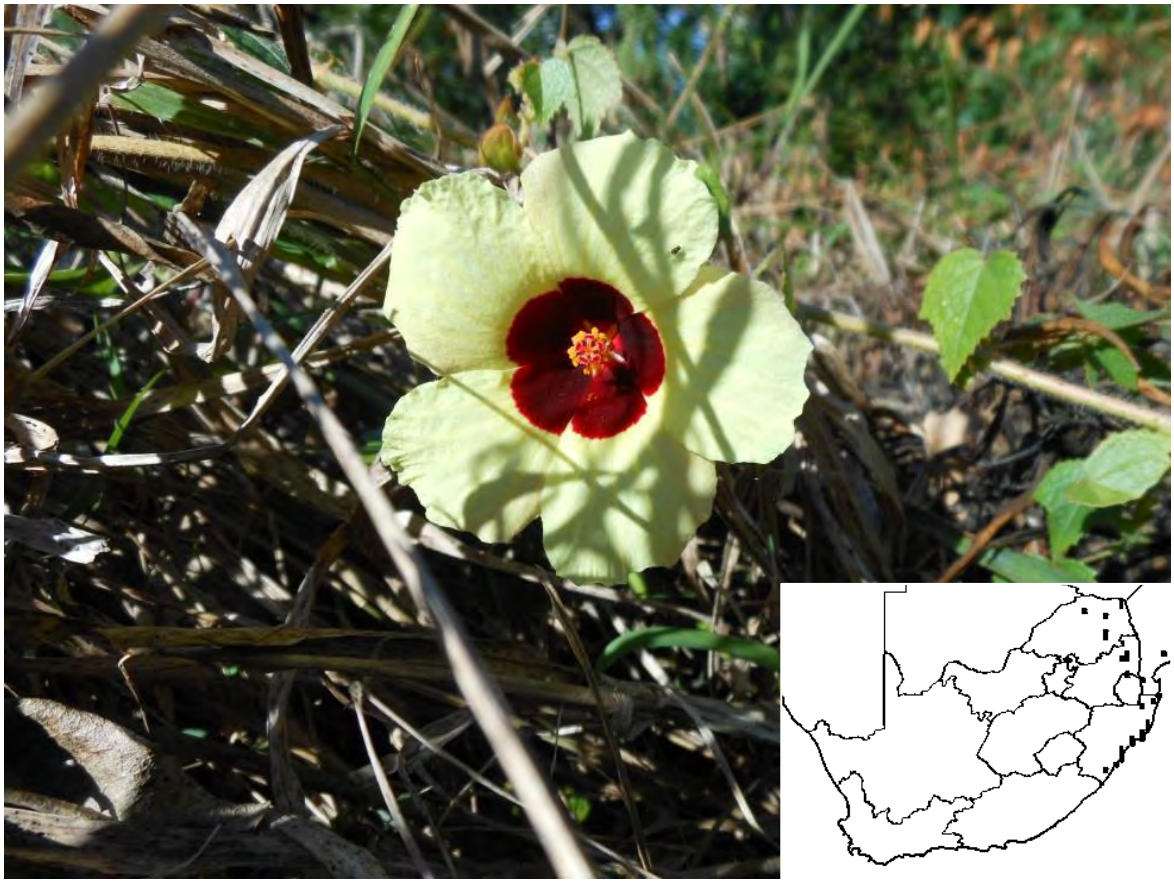

Figure 1: *Hibiscus surattensis*, growing in disturbed roadside shrubbery at the UKZN-Westville campus. Inset: showing distribution of *H. surattensis* in South Africa, image adapted from SANBI RED LIST webpage.

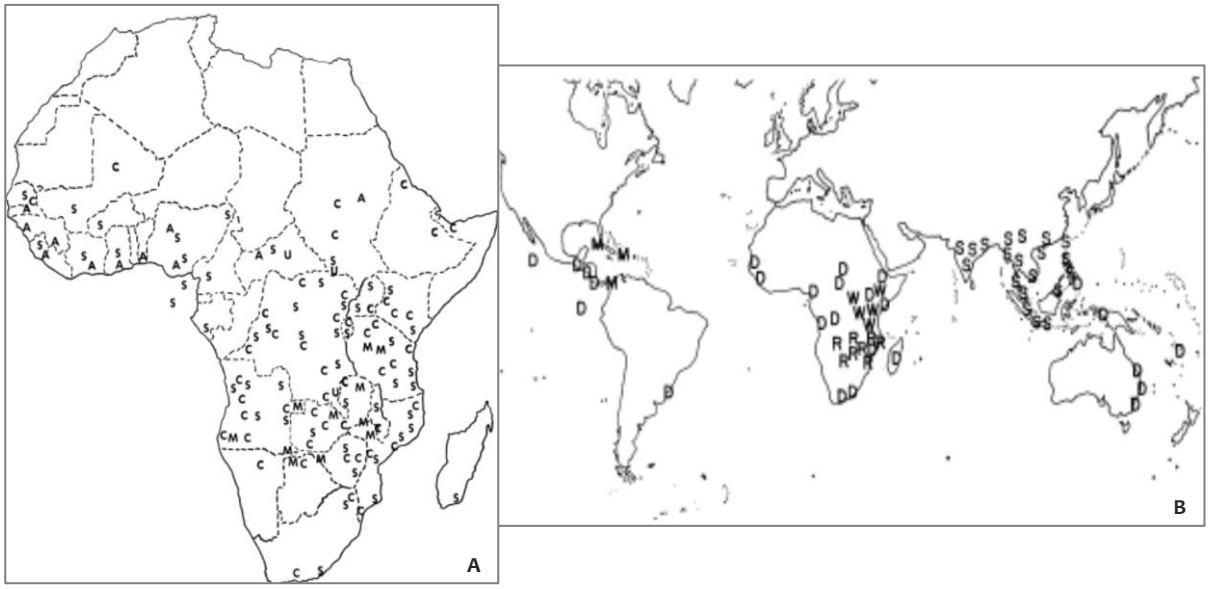

Figure 2: Distribution of *Hibiscus surattensis* in Africa (a), Asia and the Indo-Pacific Islands (b) (denoted by S), along with other species of *Hibiscus*. Image courtesy of Menzel and Wilson (1969).

1.2. DESCRIPTION OF *Hibiscus sabdariffa* L.

Hibiscus sabdariffa L., commonly known as Roselle, Java jute, Red sorrel, Jamaica sorrel and Florida cranberry, is famous for the sour tea produced from its fleshy calyces (Ali *et al*., 2005; Sindi *et al*., 2014). It is cultivated as part of the global agronomic industry (North Africa, Asia and Indonesia) where the calyces are traded for their culinary uses in hot and cold drinks (Fig. 3). China and Taiwan are the largest global suppliers of *H. sabdariffa* calyces (Lim, 2012).

The exact origins of *H. sabdariffa* remains unknown, since it has been propagated for over a thousand years, but many authors presume it to be India, Malaysia or tropical Africa (Mahadevan *et al.,* 2009; Da-Costa-Rocha *et al*., 2014). Lim (2012) considers the species to have originated in Africa and naturalized in other countries. The long list of common names as well as those in the vernacular demonstrates its worldwide popularity.

The medicinal properties of *Hibiscus sabdariffa* have been extensively studied and still continue to be the subject of various experimentation and applications in pharmacological, pharmacognostic and phytochemical research today. The red fleshy calyces of *H. sabdariffa* are harvested for the production of a sour tea, an infusion that is found to possess numerous medicinal properties (Ali *et al*., 2005; Sindi *et al*., 2014). *Hibiscus sabdariffa* calyces are used famously for the treatment of hypertension as well as for its cardio-protective effects (Ojeda *et al*., 2010). Two types of anthocyanins (delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside), by competing for active sites with the compound Angiotensin Converting Enzyme (ACE) were shown to be the main compounds involved in hypertension treatment (Ojeda *et al*., 2010). A study comparing the effectiveness of water extracts of *H. sabdariffa* calyces with Captopril (hypertension treatment), showed no significant differences between groups that were administered each of the treatments, demonstrating that the crude product was as effective in lowering moderate blood pressure as a leading pharmaceutical treatment (Herrera-Arellano *et al*., 2004). In east Africa this sour tea is also thought to help relieve cough (Lim, 2012) and its hydrating properties are said to bring relief to many Nigerian people who travel long distances to mosque. In Thailand a traditional sour tea is prepared which may be added to Ceylon tea and drunk to reduce cholesterol (Lim, 2012). Leaves of *H. sabdariffa* are also utilized as a potherb or vegetable, and serve as an accompaniment to various meat and fish dishes (described by Lim, 2012; personal observation, bunches of fresh leaves can be purchased in many Durban fresh produce markets in South Africa). Some studies indicate that *H. sabdariffa* plays an important role in the treatment of anaemia (Mugisha *et al*., 2014) particularly in immune-comprised HIV patients (Peter *et al*., 2014). Leaves of this species also

exhibits significant anticancer properties (Lin *et al*., 2011). Extracts derived from the leaves of *H. sabdariffa* were found to induce apoptosis in prostate cancer cell lines, reducing tumour proliferation and growth (Lin *et al*., 2011). Leaves of *H. sabdariffa* also show anti-hyperlipidemic effects. Gosain *et al*. (2010) demonstrated that in cholesterol-fed rat models, ethanolic leaf extracts of *H. sabdariffa* significantly reduced serum cholesterol, serum triglycerides and serum LDL. The extracts were tested over a 4 month period and showed no toxic effects (Gosain *et al*., 2010). The authors attributed the anti-hyperlipidemic effects of *H. sabdariffa* leaf extracts to hibiscus acid, sitosterol-β-D-galactoside, pectin, quercetin, kaempferol and flavonoids.

Hibiscus sabdariffa grows as an erect stem, with offshoots of leafy branches and large showy flowers which are reminiscent of the genus. Leaves and stems range from dark green to deep red in colour. They are serrated and arranged alternately on the stem, and might occur as simple or deeply palmately lobed. Leaves are mostly glabrous but the leaf base possesses an extra-floral nectary covered in fine hairs which is thought to attract phyto-protective fauna such as ants. *Hibiscus sabdariffa* flowers resemble those of *H. surattensis*, but the corolla colour might vary from light yellow to light pink.

Figure 3: Flower and calyx of *Hibiscus sabdariffa* (taken from visoflora.com). Inset: *H. sabdariffa* cultivation in India (Agarwal and Dedhia, 2014).
http://www.visoflora.com/images/original/hibiscus-sabdariffa-visoflora-8253.jpg

1.3. RATIONALE OF THE STUDY

Recently ethnomedicinal research has seen numeruous technological advances, and current phytomedicinal approaches to various illnesses is leading to the development of novel and often cheaper methods of treatment. Certain species within *Hibiscus* have been extensively examined for their pharmacognistic and phytochemical properties (Hanny *et al.*, 1973; Ogundajo *et al.*, 2014; Sindi *et al.*, 2014; Maganha *et al.*, 2010), but few are micromorphologically evaluated for the secretory mechanisms by which such phytochemicals are synthesized and exuded. Secretory structures documented within *Hibiscus* include glandular peltate and capitate trichomes, as well as specialized epidermal cells refered to as mucilage cells (Sawidis, 1991; Rocha and Machado, 2009; Shaheen *et al.*, 2007; Sayed *et al.*, 2012). This study seeks to evaluate two African medicinal species and review their present ethnobotanical usages in the context of a species substitution strategy. *Hibiscus sabdariffa* and *Hibiscus surattensis* are two closely related species, each extensively utilized throughout the African continent. The purpose of this investigation is to: (a) compare both histo-phytochemical and micromorphological properties of leaves of *Hibiscus sabdariffa* and *H. surattensis* and discuss these within the context of their ethnobotanical usages across the African continent (ecological considerations are also included), and (b) to determine if the secretory structures of *H. surattensis* demonstrates sufficient ethnopharmacological potential to warrant further phytochemical and biotechnological investigations.

Aims: To examine and compare the structures of phytochemical synthesis and exudation in the leaves of two medicinal plant species, *Hibiscus surattensis* and *Hibiscus sabdariffa*, as well as discuss the roles of secretory structures in ecological and biological contexts.

Objectives:

- To identify, characterize and compare foliar trichomes and secretory tissues on the abaxial and adaxial surfaces of different developmental stages of *Hibiscus surattensis* and *Hibiscus sabdariffa.*
- To compare histochemical features of secretory structures and crude phytochemical leaf extracts of *Hibiscus surattensis* and *Hibiscus sabdariffa* for differences and similarities in chemical composition between the related species.
- To investigate variances in trichome density between emergent, young and mature developmental stages of leaves of *Hibiscus surattensis* and *Hibiscus sabdariffa.*

Both study subjects display tremendous ethnobotanical value, indicating that phytochemical and ultrastructural analyses are required for further understanding of their medicinal value. Using scanning and transmission electron microscopy coupled with light microscopy techniques, this investigation sought to create a comprehensive understanding of the functioning of secretory structures of the foliar parts of *H. surattensis* and *H. sabdariffa*.

CHAPTER 2: LITERATURE REVIEW

Various structures functioning in the secretion of substances are reported to exist within vascular plants (Payne, 1978; Fahn, 1988). These are defined as specialized cells or complexes that synthesize, accumulate and exude secondary metabolites (Schilmiller *et al*., 2008; Fahn, 1988) for a range of different functions, from plant protection to pollinator attraction (Wagner *et al*., 2004). The term 'trichome' refers to any hair or appendage found on the epidermal surfaces of plants and might be of a secretory or non-secretory nature (Fahn, 1988; Schilmiller *et al*., 2008), although the usage of the term has broadened to encompass similar structures located internally (Levin, 1973) such as mucilage cells and oil idioblasts. More than one trichome type might be present in a particular plant organ (Bhatt *et al.*, 2010). The capacity for trichome formation is reported to be present in almost all major groups of terrestrial plants (Levin, 1973) and the location of trichomes on plant surfaces, the cellular mechanism of secretion and the ability to secrete often vary between species. The structure of plant trichomes might range in composition from a few cells to a complex arrangement of cells with advanced differentiation (Schilmiller *et al*., 2008). Secretory trichomes are often referred to as glandular, and contain specialized cells or cellular complexes that have an ability to produce phytochemicals with storage or protective functions. Exudate composition might include lipids, oils, mucilage, gums, nectar (Fahn, 1988), alkaloids, phenolics, proteinaceous substances (Duke, 1994; Ascensào and Pais, 1998) as well as flavonoids, cardiac glycosides, and other phytochemical compounds. In addition to trichomes, plants produce other forms of secretory structures which include mucilage ducts, resin ducts, hydathodes, nectaries and laticifers (Fahn, 1988).

2.1 PHYTOCHEMISTRY AND MICROMORPHOLOGY OF *HIBISCUS*

Species of *Hibiscus*, which are native to the tropical and subtropical parts of the world, are easily identified by marvellous blooms, especially those of the cultivated ornamental, *Hibiscus rosa-sinensis* (Pfeil *et al*, 2002; Maghanha *et al*., 2010). Various species which are utilised for nutritional, medicinal and beneficial applications have been investigated for their phytochemical and pharmacological properties. The constituents comprising the essential oil of *H. syriacus* flowers and buds were examined using GLC-MS (Hanny *et al.*, 1973). A comparison was made between the phytochemical components of cotton buds to that of H. *syriacus* to determine if the attraction of boll weevils to these species is due to their essential oil components, as it was suggested that essential oils might serve as insect attractants. However, it was found that the sesquiterpene hydrocarbon, 6-cadinene and the carbonyls α- and β-ionone, safranal, and pulegone were major consitituents of *H. syriacus* essential oil but that it did not resemble the essential oil composition of cotton buds (Hanny *et al.*, 1973) indicating a different role for

phytoconstituents of the essential oils of these species. An investigation of the phytochemical constituents of the medicinal species, *H. mutabilis* plant parts, showed that in leaves, the most abundant compounds detected were β-sitosterol , β-carotene and quercetin (Barve *et al*., 2010) whereas as floral parts contained quercetin, quercemeritrine, quercetin-3-D-xyloside, quercetin-3-sambubioside, isoquercetin, meratrin, hybridin, kaempferol, hyperin, guaijaverin, cyanidine-3-xlosyl glucose, cyanidin-3-monoglucoside, hibiscones and hibiscoquinones. The presence of these phytochemicals is suggested to account for the anti-inflammatory and antimicrobial activities observed within extracts of this species (Barve *et al*., 2010).

Together with phytochemistry, *Hibiscus* extracts have also been investigated for their ability to create nanoparticles through energy efficient green synthesis methods. This has been achieved with *Hibiscus rosa-sinensis, H. sabdariffa* and *H. cannabinus* (Philip, 2010; Kumar *et al*, 2014; Bindu and Umadevi, 2013). The recent interest in nanoparticles and nanomaterials is fuelled by their widespread applications from pharmacology and medicine to materials science (Philip, 2010). Philip (2010) indicated that gold nanoparticles synthesized from aqueous extracts of *H. rosa-sinensis* were bound to amine constituents of the plant extract whereas silver nanoparticles resulted from binding with carboxyl groups. For *H. sabdariffa*, the synthesis of silver nanoparticles was mediated by exposure to sunlight, and phenolic compounds as well as alkaloids and flavonoids were detected in the composition of nanoparticles using UV-Vis spectroscopy (Kumar *et al*., 2014). This highlights the importance of phytochemical profiling of species of *Hibiscus*, which together with elucidating mechanisms of phytochemical production, will aid in furthering biotechnological research of species within this genus.

Many species belonging to *Hibiscus* share similar foliar micro-architectural traits, which include similarities in stomatal features, aspects of the epidermis as well as trichome type and structure (Shaheen *et al*., 2007; Essiet and Iwok, 2014). Foliar secretory apparatus which were identified within the genus *Hibiscus* included peltate and capitate glandular trichomes, mucilage secreting idioblasts and extra-floral nectarfarious trichomes (Shaheen, *et al*., 2007; Sayed *et al*., 2012; Rocha and Machado, 2009; Sawidis, 1991). Limited investigations have been carried out on the foliar secretory and non-secretory structures of both *H. sabdariffa* and *H. surattensis.* Essiet and Iwok (2014) in a study comparing four species of the genus *Hibiscus*, noted the presence of glandular and non-glandular trichomes on the leaves of *H. surattensis*, with non-glandular types (curved and two-armed) frequently observed along leaf veins. No further information is provided on glandular trichome type, distribution or morphology. Foliar characteristics of *H. sabdariffa* were investigated by Shaheen *et al.* (2007) who conducted a comparative study of the micromorphological features of Pakistan's *Hibiscus* species. The authors observed the similar

prevalence of non-glandular trichomes located mostly along leaf veins as seen with *H. surattensis*, and described the trichome type as conical and spiralled. Cup-shaped peltate trichomes were also observed on surfaces of *H. sabdariffa*; however, the authors noted that eco-geographical differences might have rendered their observations of certain trichome types different to other studies on this species. It is important to note that both the studies mentioned above, examined fresh or dried leaf specimens using a compound light microscope, involving minimal preparation methods. Micrographs detailing certain micromorphological features were indistinct and unclear, but these studies served as a baseline for micromorphological research into *Hibiscus*, as they confirmed the presence and diversity of secretory and non-secretory appendages on the foliar surfaces.

Similar findings were also made for related genera of Malvaceae, however only external foliar appendages were considered together with non-secretory trichomes. A representative sampling of Pakistan's *Sida* species showed that stellate and peltate trichomes were the most prolific among leaves of this genus, with various forms of stellate trichomes providing taxonomically relevant diversity (Shaheen *et al*., 2009b). Similar findings were also made for the genera *Alcea* and *Althea* in which stellate trichomes were found across all taxa considered (Shaheen *et al*., 2010). Capitate trichome morphology, however, was uniform across these species and provided little taxonomic relevance, while peltate and non-glandular trichomes were identified within some species (Shaheen *et al*., 2010). The genus *Abutilon* demonstrated a greater diversity of secretory trichomes including peltate, which occurred on all studied species, capitate, flask-shaped and uniseriate, together with non-secretory stellate trichomes (Shaheen *et al*., 2009a). Given that many species of *Hibiscus* are medicinally and traditionally relevant (Da-Costa-Rocha *et al*., 2014; Gbolade, 2012; Jiofack *et al*., 2009a,b), research into their phytochemical and micromorphological properties, with emphasis on foliar trichomes, is necessitated.

2.2 TRICHOMES IN PLANT DEFENCE

Trichomes is reported to contribute significantly to the defence of plants from numerous biotic and abiotic threats (Levin, 1973). For over a century, insect-plant interactions among cultivated plants and their insect pests have been examined (Levin, 1973). It was found that plants negatively impacted insect fecundity and survival indicating a practical need for trichome research especially with economically important crop species (Levin, 1973). The success and proliferation of trichomes throughout various plant families lies in its multifunctional approach to plant defence (as demonstrated by Wagner *et al.*, 2004) as well as the ability to modify

trichome type and density through eco-geographically and environmentally driven selection (Johnson, 1975).

2.2.1 NON-GLANDULAR TRICHOMES

Non-glandular trichomes are often associated with the surface texture of plants and they play a vital role in defence against predators (Levin, 1973). Those involved in such defence strategies often appear sharply pointed or "hooked", either impaling insects which attempt to feed (Levin, 1973) or deterring larger browsers. Non-glandular trichomes are assumed to arise early in leaf development and senesce at maturity (Wagner *et al*., 2004) as in *Laurus nobilis* in the Lauraceae (Raghu and Naidoo, 2013). This suggests that non-glandular trichomes might play a role in the protection of emergent leaves until the accumulation of defence chemicals upon maturation of the leaf (Johnson, 1975). Many authors suggested that non-glandular trichomes are linked to protection against insect and animal herbivory, light reflectance, prevention of water loss, and seed dispersal (Wagner *et al*., 2004).

2.2.2 GLANDULAR TRICHOMES

In conjunction with the mechanical defence offered by non-glandular trichomes, plants have also adopted chemical approaches to defence tactics. Glandular trichomes are involved in the production, sequestration and accumulation of specialized phytochemicals that often possess antimicrobial as well as antioxidant properties (Duke, 1994; Schilmiller, *et al*., 2008). In many plants, the exudates of glandular trichomes were reported to be unpalatable to deter predators, or might increase plant lethality by inducing severe illness (Johnson, 1975; Levin, 1973). Glandular trichomes are structurally diverse (described by Payne, 1978), and phytochemically complex (Duke, 1994), with capitate and peltate types occurring more frequently in angiosperm families. Variation in trichome form and function was reported to be intra- or interspecific (Levin, 1973).

2.2.3 CAPITATE TRICHOMES

Capitate trichomes were found to occur within a large number of plant families including Lamiaceae, Malvaceae and Cucurbitaceae (Shaheen *et al*., 2009; Ascensào and Pais, 1998; Kolb and Müller, 2004). They usually consist of a single or multicellular bulbous head, atop a distinctive stalk composed of one or more cells (Ascensào and Pais, 1998; Kolb and Müller, 2004). The stalks cells are often cutinised to prevent the backflow of exudate into the leaf (Fahn, 1988, Naidoo *et al*., 2014), and in many species, they have been demonstrated to occur above a basal cell that is thought to provide structural support as well as an intercellular connection

between the epidermis and the trichome (Payne, 1978). In some species, they might be supported by a multicellular pedestal as in *Harpogophytum procumbens* and *Ceratotheca triloba* in the family Pedaliaceae (Naidoo *et al.,* 2014; Naidoo *et al*., 2012). Capitate trichomes might be classified by stalk length, which might allow for delineation of trichomes subtypes across a single surface (Gairola *et al*., 2009). In addition to foliar surfaces, they have also been observed on organs of flowers (Ascensào *et al*., 1995).

Secretion of capitate trichomes can vary with trichome subtype, family and eco-geographic location. Capitate trichomes of *Ceratotheca triloba*, family Pedaliaceae, are divided into two subtypes distinguished by stalk length, viz. long and short (Naidoo *et al*., 2012). Although similar in appearance, these trichomes were reported to differ significantly in secretory composition and mode. The head cells of long trichomes of *C. triloba* were demonstrated to synthesize and accumulate exudate with a high lipid and phenolic content, whereas those of short trichomes contained more polysaccharide constituents (Naidoo *et al*., 2012). In long trichomes, the active transportation of exudate from the head cells through micropores on the surface confirmed the mode of secretion to be eccrine (Naidoo *et al*., 2012). Secretion in short trichomes, however, was shown to be granulocrine since it involved vesicle-mediated transport to the secretory pore. Similar observations were made for the medicinal plant species, *Harpogophytum procumbens*, also of the family Pedaliaceae, which contained foliar secretory trichomes that secreted in a similar manner to short trichomes of *C. triloba* (Naidoo *et al,* 2014). Through microporous head cells, secretion composing of polysaccharides, lipids and phenolics were ejected. For the short trichomes of *C. triloba* and the trichomes of *H. procumbens*, numerous Golgi Bodies appeared to be involved in exudate synthesis.

In contrast to the mucilaginous secretions of the family Pedaliaceae, numerous members of Cannabinaceae and Lamiaceae were reported to secrete primarily oils and lipids (Hammond and Mahlberg, 1978; Ascensão and Pais, 1998). In *Cannabis sativa*, the capitate trichomes were shown to consist of an 8-13 celled discoid head with a multicellular stalk. Exudate composition was predominantly lipid in nature and exudate synthesis was attributed to numerous plastids that occured in the head cells during the secretory phase (Hammond and Mahlberg, 1978). Secretory products which were observed accumulating on the outer surfaces of plastids, coalesced and migrated to the region of the cell closest to the secretory cavity (Hammond and Mahlberg, 1978). The secretory cavity formed above the disc through separation of the cell walls of the head cells. The bulbous cavity accumulates secretion which is reported to be

compartmentalized into numerous membrane-bound spherical bodies (Hammond and Mahlberg, 1978).

Foliar trichomes of *Leonotis leonurus*, family Lamiaceae were reported to produce secretions of a predominantly lipid nature (Ascensão and Pais, 1998). In addition, however, the secretory contents of head cells tested positively for the presence of polysaccharides, proteins and phenolic compounds. Unlike with *C. sativa*, the cellular organelle implicated in exudate synthesis was the rough endoplasmic reticulum (RER), which proliferated during the secretory phase of trichome development (Ascensão and Pais, 1998). Following this Golgi Bodies began proliferating within trichome head cells. The authors suggested that proteinaceous elements of exudate might be synthesised in the RER cisternae, and transferred to the *cis*-Golgi where the polysaccharide component was manufactured (Ascensão and Pais, 1998). The secretion mode was determined to be granulocrine in nature as Golgi-derived vesicles merged with the plasma membrane releasing the secretory product into a periplasmic space between the plasma membrane and cell wall (Ascensão and Pais, 1998).

2.3 INTERNAL PLANT SECRETORY TISSUES

In addition to external secretory structures, a number of secretory tissues located within plant organs were reported to exist, viz. mucilage cells, mucilage ducts, oil idioblasts, crystal idioblasts and resin, latex and gum ducts (Fahn, 1988). Some of these structures, although internally located might be identified by surface features as demonstrated by Carpenter (2006) in Figure 4, with hydrapotes and ethereal oil cells.

2.3.1 MUCILAGE IDIOBLASTS AND DUCTS

According to Fahn (1989) mucilage in plants is comprised of complex polysaccharide polymers with a high molecular weight which are acidic or neutral in nature. The presence of mucilage cells and ducts has been observed in many plant families including Malvaceae, Polygalaceae, Lauraceae, Rhamnaceae and Araucariaceae (Bakker and Gerritsen, 1989; Clifford *et al*., 2002; Mastroberti and Mariath, 2008). Mucilaginous inclusions in plants were reported to include epistomatal mucilage plugs, mucilage idioblasts, mucilage ducts and subepidermal mucilage accumulations (De Aguiar-Dias and Cardoso-Gustavson -Dias and Cardoso-Gustavson, 2011; Zimmerman *et al.*, 2007; Bosabalidis, 2014; Christodoulakis *et al.,* 1990; Bredenkamp and Van Wyk, 1999) each thought to serve a unique function, including the prevention of water loss, water storage during dry periods, reserve food sources and trapping of insect by carnivorous plants (Fahn, 1989). Mucilage synthesized in seed coats of many plant species (Brassicaceae,

Solanaceae, Linaceae, and Plantaginaceae) acts to regulate germination or facilitate dispersal (Katayama *et al*., 2008; Fahn, 1989) whereas mucilage in developing root tips and germinating pollen tubes might facilitate growth and movement (Katayama *et al*., 2008).

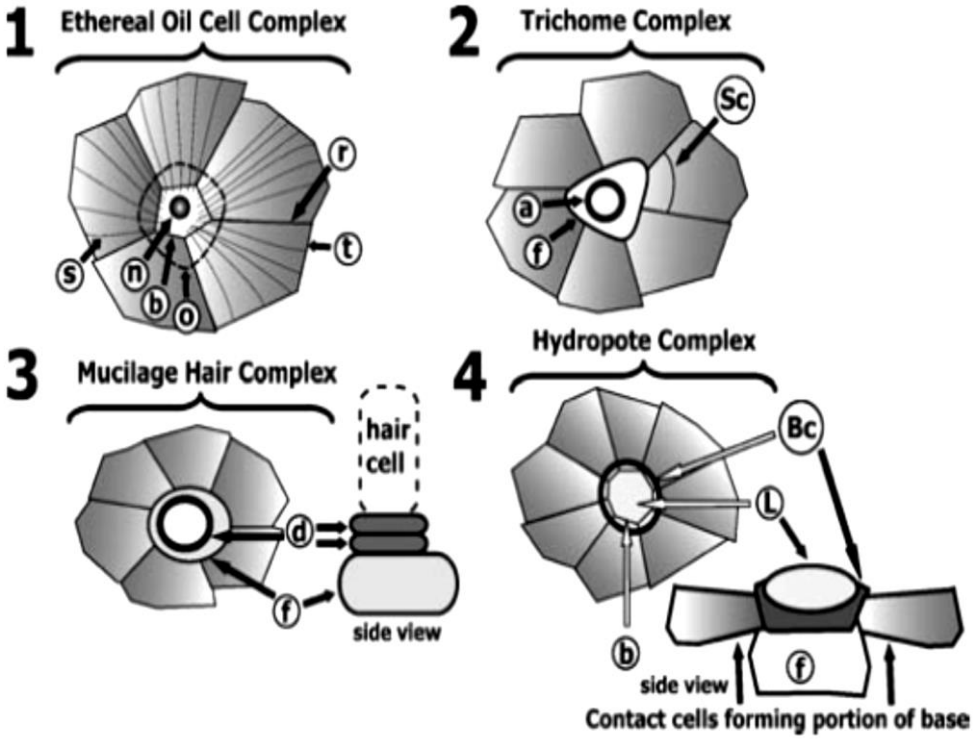

Figure 4: Various forms of epidermal secretory apparatus found in members of basal angiosperms, adapted from Carpenter (2006) 1) Ethereal oil cell complex typical of those in Austrobaileyales comprising oil cell (o), depicted with a dashed line to indicate that the majority of the cell situated below the epidermis, its nucleus (n), base (b) formed by anticlinal contact cell walls, and cuticular striations (s). A radial wall (r) and tangential wall (t) are indicated. 2) Trichome complex typical of Amborellaceae and Trimeniaceae showing abscission scar (a), foot cell to which the trichome was attached (f), and a strongly specialized contact cell (Sc). 3) Mucilage hair complex typical of Cabombaceae with two disk-shaped cells (d) to which the mucilage hair is attached, and a foot cell (f), level with the epidermis, upon which the disk-shaped cells rest. 4) Hydropote complex typical of Nymphaeaceae with base (b) formed by anticlinal contact cell walls, the lens-shaped cell (L), and the bowl-shaped cell (Bc). In surface view, the Bc often appeared as a dark ring surrounding the L. A subepidermal foot cell (f) situated beneath the Bc and L.

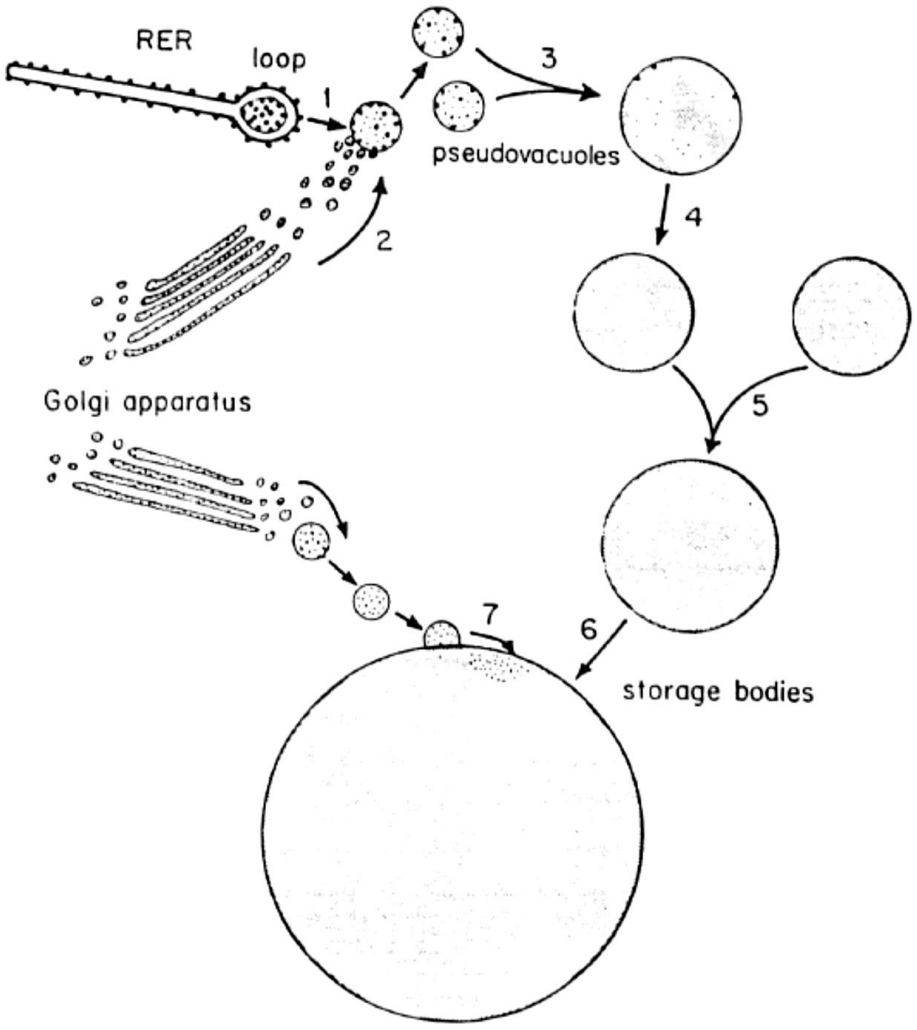

Figure 5: Diagram showing the likely origins of protein-carbohydrate mucilage in mango fruit ducts (From Joel & Fahn, 1980).

Mucilage idioblasts often initiate in the same manner as the surrounding paranchymatous or mesophyll cells, but during development the protoplast was shown to separate from the cell wall and the space between accumulates with mucilage (Fahn, 1989). The subcellular machinery responsible for mucilage secretion in Okra are Golgi Bodies, which appeared numerous in the protoplast. Upon maturity the mucilage idioblasts are observed to be larger than surrounding cells (Fahn, 1989). As with mucilage secreting idioblasts, mucilage ducts accumulated the secretion between the protoplast and cell wall, and similarly Golgi Bodies have been implicated in synthesis of the secretion (See Figure 4). Mucilage ducts, however, were shown to form a continuous network within the plant, often closely associated with the vascular tissue (Fahn 1989). In certain plant species, glycoproteinaceous secretions were reported to be synthesized

by the complimentary action of the RER and Golgi Body (Fig. 5). This is also seen in *Leonotis leonurus* (family Lamiaceae), where protein-polysaccharide secretion was shown to be synthesised in two types of capitate trichomes (Ascensão and Pais, 1998).

Mucilage duct systems in plants are a fairly understudied area of physiology (Pickard, 2007), and their role in plant systems is therefore unclear. However they are common features of the families Malvaceae, Polygalaceae and Rutaceae as well as members of Fabaceae (Fahn, 1988, De Aguiar-Dias and Cardoso-Gustavson -Dias and Cardoso-Gustavson -Dias, 2011). Mucilage duct formation has been speculated to be either schizonegous or lysigenous in nature, involving secretion of exudate into a lumen or conducting column that is externally located from the secreting cells (Pickard, 2007). Further investigation on mucilage duct formation is required to investigate their roles in plant defence systems and develop strategies to optimize exudate output and quality (Pickard, 2007), especially in species of medicinal value and potential pharmaceutical benefit.

2.3.2 CRYSTAL IDIOBLASTS

Crystal idioblasts have been reported accumulate calcium oxalate and form crystals of different shapes and sizes within their vacuole (Webb, 1999). They are a well-studied cytological component in plants and have been observed for over a hundred years. Though the exact functions of crystal idioblasts are unclear, many researchers have attempted to elucidate possible physiological and ecological roles for them. Schneider (1901) suggested that they might play a significant role in structural support of stems and leaves as it has been reported that that mucilaginous residue surrounding crystals might act as a bumper between the wall of the cell and the crystal (Schneider, 1901). Other authors have proposed that they might function in defence causing injury to herbivores, as well as a regulator for calcium and oxalate within the plant body (Webb, 1999). Different forms of calcium oxalate crystals have been identified in plants, and their sole occurrence in higher plants makes them an interesting taxonomic tool (Al-Rais *et al.*, 1971). Crystal form and development is highly specific, signifying genetic control of their presence within families (Webb, 1999).

2.4 TAXONOMY

As well as functioning ecologically in plant defence, foliar and floral trichomes and secretory structures are considered important morphological characters in plant taxonomy (Johnson, 1975). Complimenting popular methodologies, the use of trichomes as taxonomic tools are assumed to be a reliable and efficient measure of morphological variation (Levin, 1973). They

are often reported to serve as a family level taxonomic tool and might prove helpful if reproductive organs are absent or damaged. Adebowale *et al.* (2014) demonstrated the use of foliar epidermal trichomes, stomatal features and epicuticular striations to investigate ecological and genetic relationships among 11 species belonging to the genus *Strychnos*. Despite being unable to significantly correlate stomatal length with genome size, they were able to make micromorphological distinctions among species that were otherwise difficult to distinguish between. It was asserted that coupled with molecular techniques, epidermal micromorphology was able to serve as an important accessory to plant taxonomic studies (Adebowale *et al*., 2014).

In addition to the classification of species using structural features, phytotaxonomy is another branch of plant taxonomy. Specialised secretions of plants vary with taxon, geographic region and environmental factors, but in many cases, closely related taxa might share similar secretion composition (Taylor *et al.,* 2001, Soejarto, 1996). Furthermore the authors considered the ethnobotanical information of a species to be of great importance in this regard, since it might lead to the discovery of compounds unique within related taxa that can be pharmacologically or biotechnologically exploited.

Reproductive features that taxonomically distinguish members of *Hibiscus* from the rest of the family Malvaceae included the persistence of the calyx and epicalyx after flowering with the epicalyx possessing 8 or more lobes, branching of the style into five parts, 5 apical teeth associated with the staminal column, stigmas that are capitate, cells of the ovary containing more than one ovule, petals that are fused with the staminal column at the base but do not form a tube around the basal region of the staminal column, and wingless fruit that contain 5-10 loculicidal cells (Pfeil *et al*, 2002). Most genetic studies have attempted to reconcile members of *Hibiscus* due to the long-standing debate of the polyphyletic parentage of the genus (Pfeil *et al*., 2002). Using 2 chloroplast DNA sequences as well reproductive micromorphological features, Pfeil *et al* (2002) elucidated relationships within the genus *Hibiscus* as well as the tribe Hibisciae. Their findings (Figure 6) demonstrated that that the genus *Hibiscus* is polyphyletic and encompasses certain members assigned to closely related genera signifying that the genus is misrepresented and might contain up to 300+ species. Figure 6 illustrates relatedness between members of *Hibiscus*, with *H. surattensis* appearing closely related to *H. sabdariffa*. The leafy epicalyx and small sizes of *H. surattensis* chromosomes, might signify primitive traits within the genus according to Akpan and Hossain (1998) who placed *H. surattensis* higher up on the evolutionary tree than *H. cannabinus* and *H. asper*.

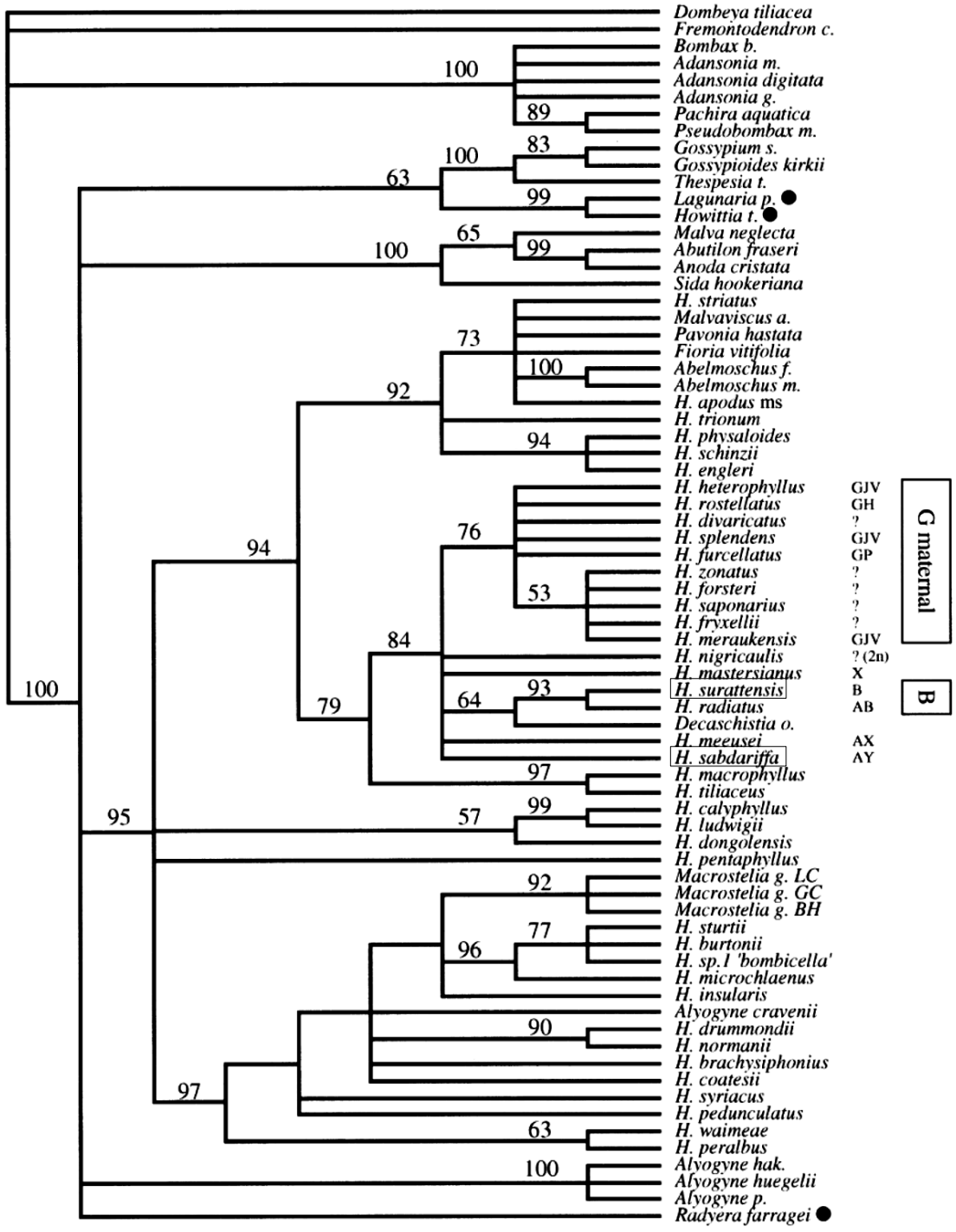

Figure 6: Strict consensus cladogram of 6,279 MP trees found using the *rpl16* intron. MP trees are each 371 steps, CI50.82 and RI50.91. Chromosome and chloroplast groups of *Hibiscus* section *Furcaria* are indicated (for chromosome groups one letter, e.g., 'B' indicates a diploid, two letters a tetraploid, three a hexaploid; for chloroplast groups see discussion. Bootstrap support for each branch above 50% is shown. (Taken from Pfeil *et al*, 2002).

CHAPTER 3: MATERIALS AND METHODS

3.1 PLANT MATERIALS

Hibiscus surattensis was collected from two locations, viz. Pigeon Valley Nature reserve (-29.864368, 30.987103) and the University of KwaZulu-Natal, Westville campus (-29.817897, 30.942771), Durban, South Africa. In most instances, leaves were collected and prepared directly for microscopy or phytochemical techniques, however a few plantlets were collected for propagation in the campus glasshouse. Species identification was confirmed with numerous herbarium specimens. Specimens of *H. sabdariffa* were either collected from a private residence in Chatsworth, Durban, or purchased from local fresh produce vendors in the Chatsworth area (-29.916089, 30.877114). For microscopy techniques, 3 stages of leaf development were compared: emergent, young and mature, which were distinguished by the level of leaf expansion as well as leaf colour and texture.

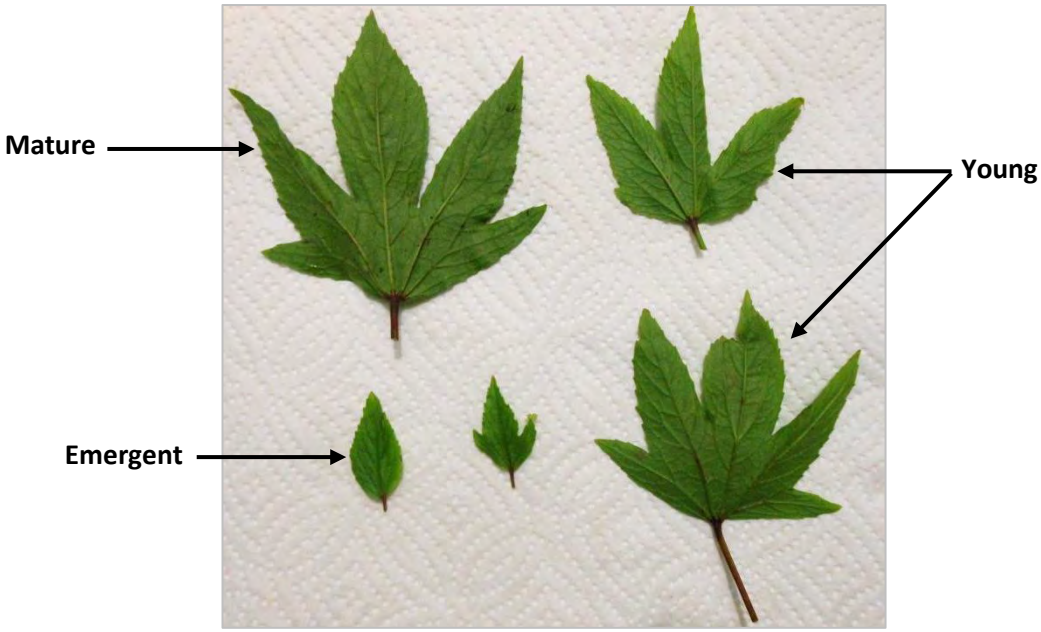

Figure 7: Leaves of *Hibiscus sabdariffa* illustrating the different stages of leaf development.

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3.2 STEREOMICROSCOPY

To gain a preliminary view of each leaf surface, fresh leaves were examined using the Nikon AZ100 Stereomicroscope fitted with Nikon Fiber Illuminator, and images were captured using NIS-Elements Software (NIS-Elements D 3.00). Both abaxial and adaxial surfaces for the 3

developmental stages were imaged, with particular emphasis on secretory and non-secretory structures.

3.3 SCANNING ELECTRON MICROSCOPY (SEM)

Using SEM, more detailed micromorphological observations were made of both leaf surfaces for each developmental stage of the species examined. Preparation involved firstly placing 2 mm^2 segments of fresh leaf tissue into 2.5% gluteraldehyde for 18 - 24 h before rinsing thrice for 5 min with 0.1 M sodium phosphate buffer (pH 7.0). Post-fixation involved placing samples in 0.5% osmium tetroxide for 1 - 2 h followed by a further three 5 min rinses with sodium phosphate buffer. Samples were then subjected to a graded ethanol dehydration series (30%, 50%, 75%, 100%; two 5 min sessions each, two 10 min sessions for 100%), after which they were critically point dried using Quorum K850 Critical Point Dryer with vertical chamber. Following this, samples were fixed to aluminium stubs using carbon conductive tape and gold sputter coated using either the Polaron SC 500 Sputter Coater or the Quorum Q150R ES gold sputter coater. Viewing and imaging of samples were carried out using the Jeol LEO 1450 SEM and a Zeiss Ultra-Plus FEG-SEM at 5 kV and a working distance (WD) of 15-17 mm.

3.4 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Ultrastructure of leaf tissue was viewed and imaged using TEM. Leaf segments (2 mm^2) were excised and fixed in 2.5% gluteraldehyde for 18 - 24 h. Samples were then rinsed thrice in sodium phosphate buffer before post-fixation for 1 - 2 h in 0.5% osmium tetroxide in darkness. Samples were then rinsed further three times for 5 min each in sodium phosphate buffer and dehydrated using a graded acetone series (30%, 50%, 75%, two 5 min sessions each, two 10 min sessions for 100%). Following this, samples were transferred to a clearing agent, propylene, and infiltrated with Spurr's resin (Spurr, 1969) using a graded series (25%, 50%, 100%, refers to percentage resin in propylene oxide solution). Samples were placed in silicon moulds and polymerized over 8 h at 70 °C.

Sectioning for TEM was carried out using the Reichert Jung Ultracut-E ultramicrotome or the Leica Ultramicrotome EM UC7 (Leica Microsystems, Germany). Survey sections were obtained to determine the regions of interest. Sections were stained with 1% Toluidine Blue and viewed using the Nikon ATi light microscope equipped with a Nikon DS-Fi1 camera and NIS-Elements imaging software package. Ultrathin sections were then cut at 90-130 nm and placed on copper grids. Sections were post-stained using 2.5% uranyl acetate and lead citrate and subsequently viewed using the Jeol JEM 1010 TEM. Images were obtained from emergent, young and mature leaf samples.

3.5 TRICHOME AND MUCILAGE CELL DENSITY COUNTS AND STATISTICAL ANALYSES

Random low magnification SEM micrographs were selected for trichome density counts and estimation of distribution. Using sophisticated image processing software Image J (Schneider *et al*., 2012), counts were made for glandular capitate trichomes, non-glandular falcate trichomes, non-glandular stellate trichomes, mucilage cells and prickles. Ten images were counted for both abaxial and adaxial surfaces at each stage of development, and this was achieved for both *Hibiscus* sp. A Multivariate Analysis of Variance (MANOVA) was performed for the *H. surattensis* dataset to determine differences between developmental stages for each of the trichome types counted. Assumptions of normality were not met but Pillais trace was used as a robust indicator of significance. Box's test of equality of variance was satisfied provided stellate and prickle trichome counts were omitted, since very few leaf stages contained these trichomes. LSD Post-hoc tests were used to make pairwise comparisons of trichome densities. For *H. sabdariffa,* only glandular trichomes were observed and counted, and comparisons between developmental stages were made using a One-Way ANOVA. A One-Way ANOVA was also used to determine differences in glandular capitate trichomes across all developmental stages between the two species. The statistical software package used was IBM SPSS Statistics for Windows (IBM Corp. Released 2012. Version 21.0. Armonk, NY). Significance was set at $p < 0.05$.

3.6 HISTOCHEMISTRY

Various histochemical tests were performed on fresh leaf sections of both study species. A segment of fresh leaf tissue was orientated between two segments of dental wax and secured in an Oxford® Vibratome. Sections of 60-100 µm thick were obtained and stained accordingly. Stained sections were viewed and imaged using the Nikon ATi compound light microscope.

a) *Mucilages and Polysaccharides:*

Sections were incubated for 10 min in aqueous (0.05%) Ruthenium Red solution, mounted and viewed. Polysaccharides and mucilage stained pink to red.

b) *Monochromatic staining*

Sections were stained in Toluidine Blue for approximately 1 min and rinsed in distilled water. Sections were then mounted and viewed. Carboxylated polysaccharides stained bright pink-purple, polyphenols stained blue to green and phosphate groups on macromolecules stained purple to blue.

c) *Phenolic compounds*

Sections were placed in 10% ferric trichloride for 15 min with a drop of aqueous sodium carbonate. Black or dark green deposits indicated the presence of phenolics.

d) *Alkaloids*

Sections were stained for 10 min each in Dittmars (1 g sodium nitrate and 1 g potassium iodide in 30 mL HCL and 30 mL dH_2O solution) and Wagners (1.27 g iodine and 2 g potassium iodide dissolved in 100 ml dH_2O) reagents before mounting and viewing. A brown-orange colour represented a positive test for alkaloids.

e) *Cutin, Suberin, Lipid and Lignin*

Sudan Black B: sections were immersed in the stain for approximately 10 min before being rinsed with 70% ethanol and mounted on a slide. Blue-black staining indicated lipids, cutin and suberin.

Sudan III/IV: sections were stained for 10 min and rinsed with 70% ethanol. Sections were mounted in glycerol and viewed. Tissues staining orange positively indicated the presence of lipids or cutin.

Phloroglucinol: Sections were immersed in Phloroglucinol for 1 min and viewed immediately. Orange to red colouration indicated the presence of lignin or cutin. Thirty minutes after staining, the red colouration changes to brown.

Nile Blue: Sections were stained for 1 min in 1% Nile Blue at 37 °C and then immersed in 1% acetic acid for a further 1 min. After rinsing with dH_2O, sections were mounted and viewed.

Red staining indicated the presence of fats, oils and waxes (neutral lipids) whereas blue staining indicated the presence of acidic lipids such as phospholipids.

f) *Total Proteins*

Sections were stained using Mercuric Bromophenol Blue (95% ethyl alcohol containing 10 g $HgCl_2$ and 100 mg bromophenol blue per 100 ml) for 15 min followed by immersion in 0.5% acetic acid for 20 min. sections were then rinsed in 0.1 M phosphate buffer for 3 min before mounting and viewing. Proteinaceous substances stained blue.

3.7 FLUORESCENCE MICROSCOPY

Fresh sections, cut using the vibratome, or using free hand sectioning methods, were viewed using fluorescence techniques for the detection of phenolic and lignin components. Sections were mounted in distilled water and viewed using the Zeiss 710 Laser Scanning Confocal Microscope, or the Nikon ATi fluorescence microscope. Sections were viewed using UV light with an excitation wavelength of 330 nm and DM wavelength of 400 nm.

Preliminary observations of cell viability were also carried out by staining with 2% acridine orange and 0.5% fluorescein diacetate.

3.8 PREPARATION OF LEAF EXTRACTS FOR PHYTOCHEMICAL TESTS

Leaves of each species were allowed to dry under ambient conditions for the preparation of crude extracts. Dried leaves were crushed using a mill, or by hand with a pestle and mortar. Powdered leaves (approximately 5 g) were placed in a round bottomed flask to which the relevant solvent (50 ml) was added. Methanol, chloroform and hexane were the preferred solvents of choice. For each solvent, 3 sessions consisting of 3 hrs each were carried out with the crude extract being filtered after each session. Phytochemical tests were carried out on the resultant extracts as follows.

Tests for Mucilage and Gums

Two drops of 0.5% Ruthenium Red solution were added to 1 ml of extract. A pink to red colour change indicated the presence of mucilage and/or polysaccharides.

Two ml of cold absolute ethanol was poured slowly down the sides of a test tube, to settle above 1 ml of extract. A white mucilaginous precipitate in the ethanol signified the presence of mucilage.

Tests for Carbohydrates and glycosides

A drop of α-naphthol solution was added to 1 ml of extract in a test tube. After mixing well, 0.5 ml of concentrated sulphuric acid was poured along the sides of the test tube to settle above the solution. The formation of a deep purple or violet ring indicated a positive test for carbohydrates.

One ml each of Fehlings solutions A and B were mixed with 1 ml extract and allowed to boil in a water bath. The formation of a red precipitate indicated the positive test for carbohydrates.

One ml of Benedicts reagent was mixed in a test tube with extract and boiled in a water bath for 2 min. A precipitate ranging in colour from yellow to red indicated a positive test for carbohydrates.

Test for Amino acids and proteins

A drop of Ninhydrin solution was added to 1 ml of extract. A colour change to purple indicated the positive test for amino acids or proteins.

Test for Sterols

Two ml of extract was mixed with 3 ml of chloroform. A few drops of sulphuric acid were poured down the side of the test tube. The formation of a red ring between the solvent layers and a green fluorescent ring below indicated a positive test for cholesterol.

Tests for Phenolics

Two drops of ferric trichloride were added to 1 ml of extract. The formation of a green or black precipitate or colour change indicated a positive test for phenolics.

Two drops of lead acetate were mixed with 1 ml of extract. The formation of a bulky white precipitate indicated a positive test for phenolics.

Test for Flavones and Flavonones

Half ml of concentrated sulphuric acid was added to 1 ml of extract. Yellow to orange colour change indicated a positive test for anthocyanins and/or flavones. A deeper orange to crimson colour change indicated a positive test for flavonones.

Test for Alkaloids

Two drops of Hagers reagent was added to 1 ml of extract. The formation of a yellow precipitate indicated a positive test for alkaloids.

Two drops of Wagners reagent was added to 1 ml of extract and an orange-brown precipitate indicated a positive test for alkaloids.

One ml of extract was mixed with two drops of Draggendorf reagent. A reddish precipitate signified a presence of alkaloids.

CHAPTER 4: RESULTS

4.1 STEREOMICROSCOPY

Leaves of *Hibiscus surattensis* and *H. sabdariffa* resembled each other in shape and orientation. However, they differed greatly in size, texture and colour. Stereo-microscopy images revealed major differences in the indumenta of both species (Fig. 9). *Hibiscus surattensis* appeared more densely hairy with high frequencies of stellate and falcate trichomes in emergent and young stages (Fig. 9 A and B), particularly on the abaxial surface. Prickle trichomes were observed at the young and mature stages of development and occurred only on main and lateral leaf veins (Fig. 9 F). Glandular trichomes were also observed (Fig. 9 B), occurring more frequently on the abaxial surfaces of young and emergent leaves. The surface view of mucilage cells was also distinguished by the radial arrangement of epidermal cells around a sunken core (Fig. 9 E, inset).

Leaves of *Hibiscus sabdariffa* possessed no distinct indumentum. They appeared glabrous, especially at the mature stage of development. Margins were reddish in appearance and were often punctuated by a falcate trichome (Fig. 10 F). At all developmental stages an extra-floral nectary was observed on the abaxial surface at the base of each leaf (Fig. 10 B). Non-glandular trichomes were also found to occur mostly in this region on both the abaxial and adaxial surfaces (Fig. 10 B and D). Just below the leaf base, the colour of the petiole was reddish, and in emergent leaves was densely pubescent (Fig. 10 D). Glandular trichomes appeared to be similar to those of *H. surattensis* (Fig. 10 G). However, the presence of mucilage cells was less obvious. In total, 8 foliar trichome types were identified between the two study subjects (Fig. 8).

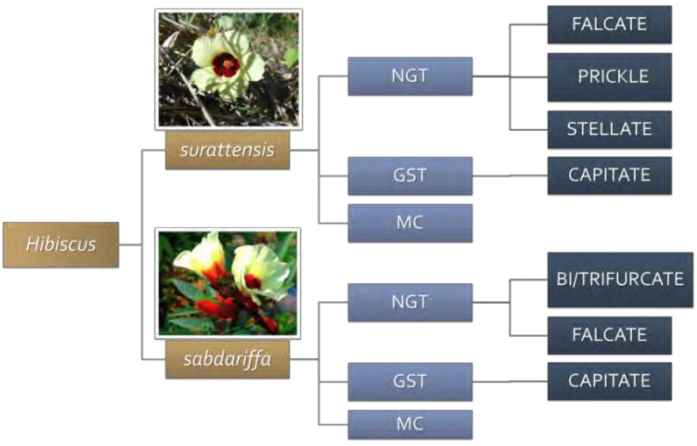

Figure 8: Diagram listing trichome types found to occur in the two *Hibiscus* specimens investigated. NGT – Non-glandular trichome; GST- Glandular secretory trichome; MC – Mucilage cell.

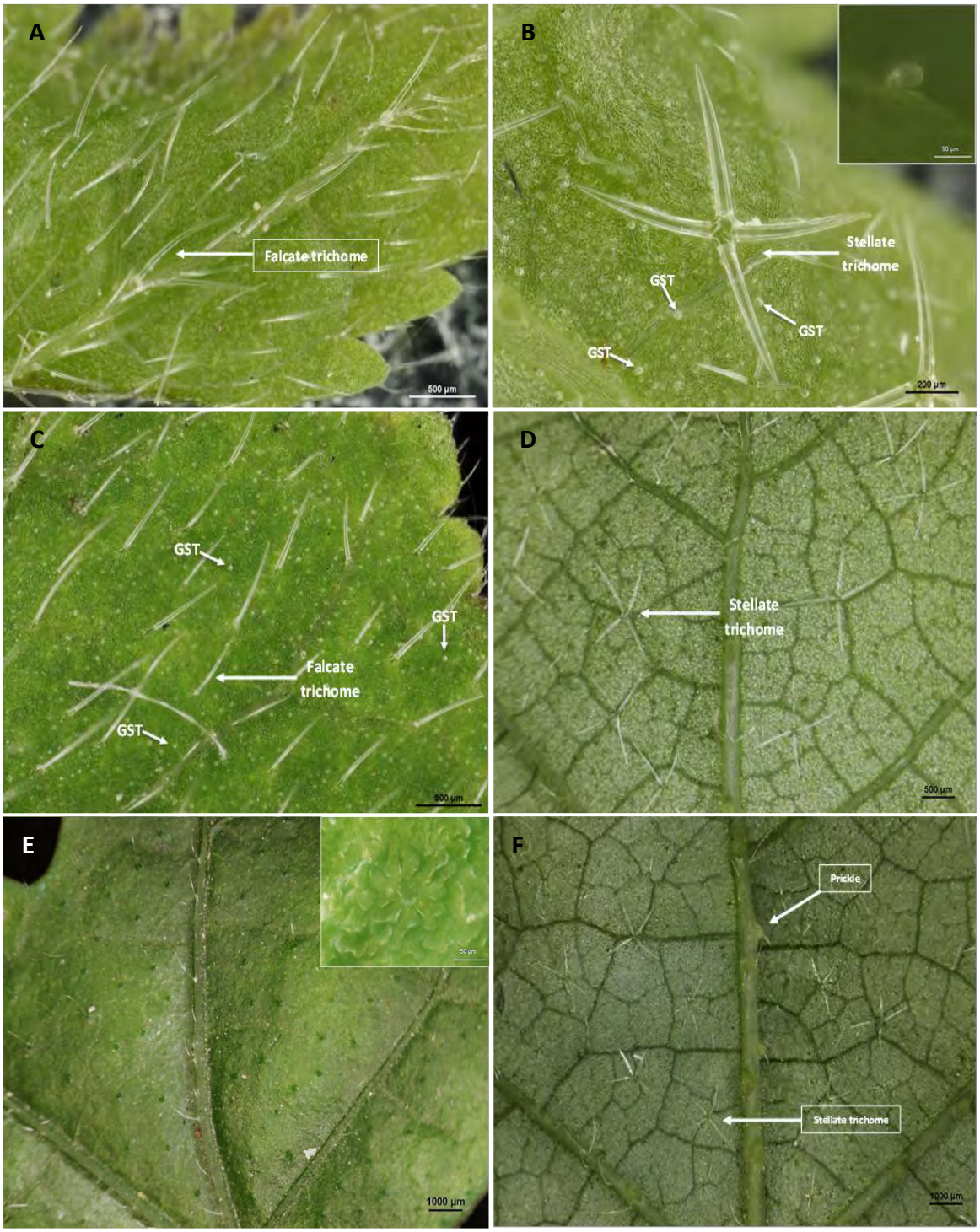

Figure 9: Stereomicrographs of leaves of *Hibiscus surattensis*: a) Adaxial surface of emergent leaf showing dense populations of falcate trichomes. b) Abaxial surface of emergent leaf showing stellate trichomes surrounded by glandular trichomes. c) Adaxial surface of young leaf showing numerous glandular and falcate trichomes. d) Stellate trichomes present on the abaxial leaf surface of a young leaf. e) Sparsely distributed trichomes on the adaxial surface of a mature leaf with falcate trichomes present mostly along leaf veins. f) Stellate and prickle trichomes present on the under-surface of a mature leaf.

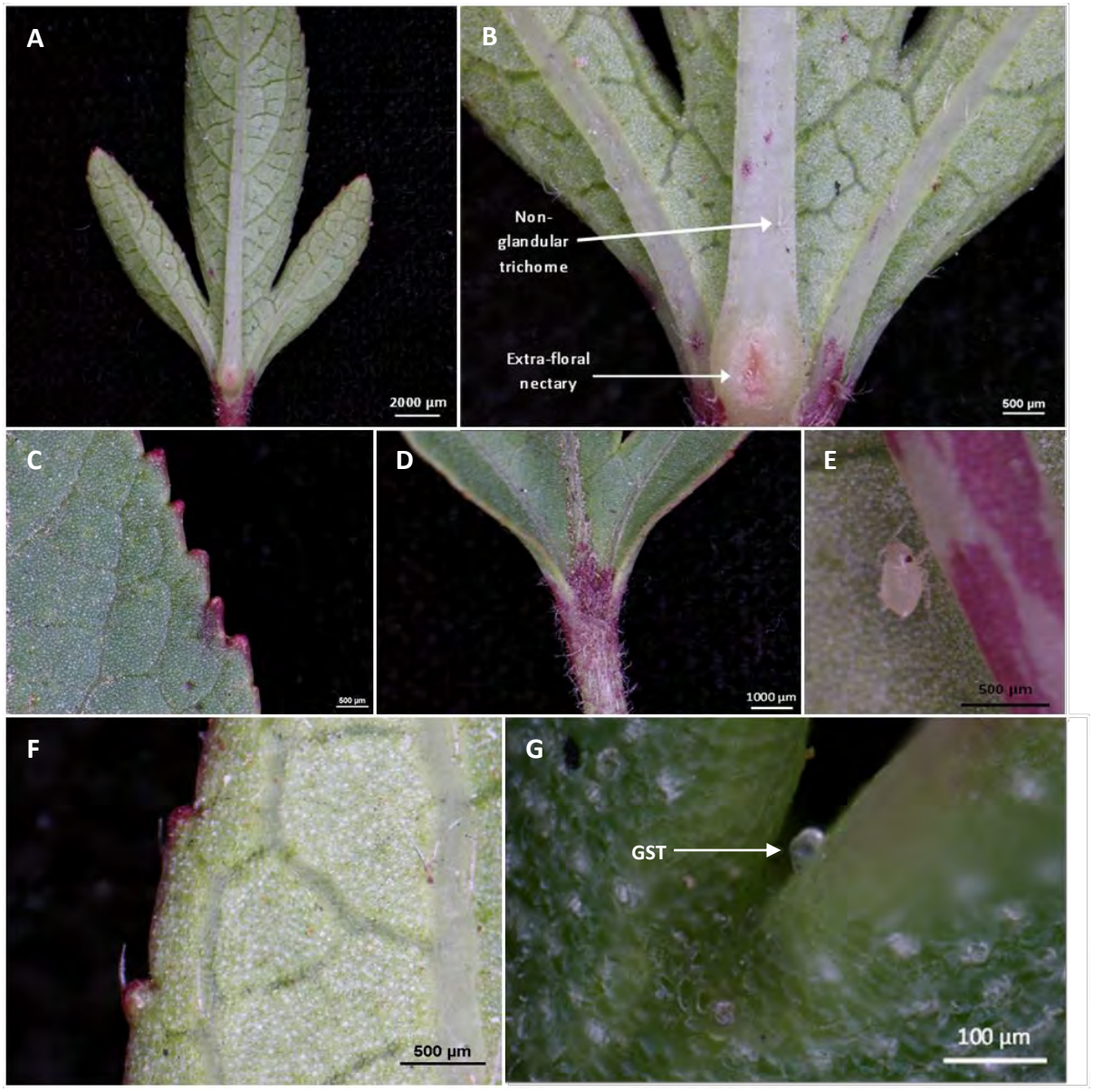

Figure 10: Steromicropgraphs of emergent leaves of *Hibiscus sabdariffa*: a) Overall abaxial view of emergent leaf. b) Extra-floral nectary present at the base of the leaf sparsely surrounded by non-glandular trichomes. c) Glabrous adaxial leaf surface showing reddish serrated margin. d) leaf base of the adaxial surface with pubescent petiole. e) Sap-sucking mite present on the abaxial leaf surface, along the lateral vein. f) Falcate trichome occurring on tapered end of leaf serrations. g) Glandular trichome on abaxial leaf surface. *Glandular secretory trichome* = GST.

4.2 GLANDULAR CAPITATE TRICHOMES

Glandular capitate trichomes of *Hibiscus surattensis* were found to occur on both leaf surfaces at all stages of development. However their frequency and distribution differed between upper and lower leaf surfaces and leaf developmental stage (Fig. 11). MANOVA statistics showed that there is a significant difference between trichome densities of emergent abaxial and emergent adaxial leaf surfaces as well as between young and mature abaxial surfaces and emergent and mature abaxial surfaces (df = 5; F= 3.737; p> 0.05). For *H. sabdariffa*, glandular trichome density of the abaxial surface of emergent leaves were shown to be significantly different to every other surface and stage investigated within that species (df = 5; F = 52.409 ; p > 0.05). Young and mature stages, however, did not show any significant differences in glandular trichome densities.

At maturity, capitate trichome heads consisted of 5 cells atop a 3-celled stalk and a basal cell embedded in the epidermis (Fig 12 B). From SEM the outlines of trichome head and stalk cells are clearly visible (Fig. 12 A), suggesting the absence of accumulated material in the aerial regions of the trichome. This was confirmed with light and TEM micrographs which showed a subtended cuticle on the ventral surface of the trichome (Fig. 12 B and E). Glandular capitate trichomes of *H. sabdariifa* were similar in structure to *H. surattensis* but differed in frequency across developmental stages (Fig. 14).

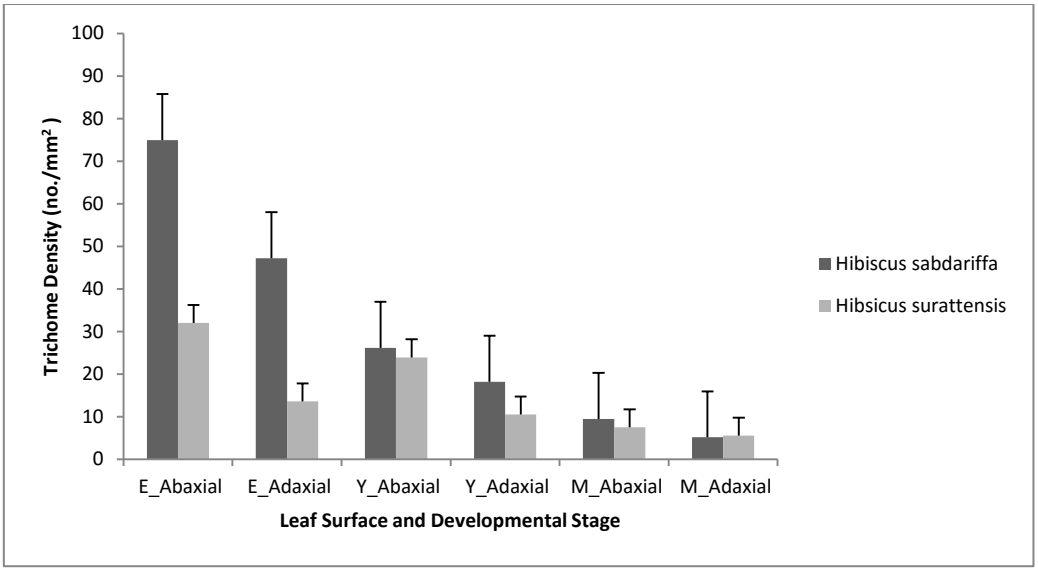

Figure 11: A comparison of glandular capitate trichomes across emergent, young and mature stages between *Hibiscus surattensis* and *Hibiscus sabdariffa.* Bars are means +SE. *Emergent* = E, *Young* = Y, *Mature* = M.

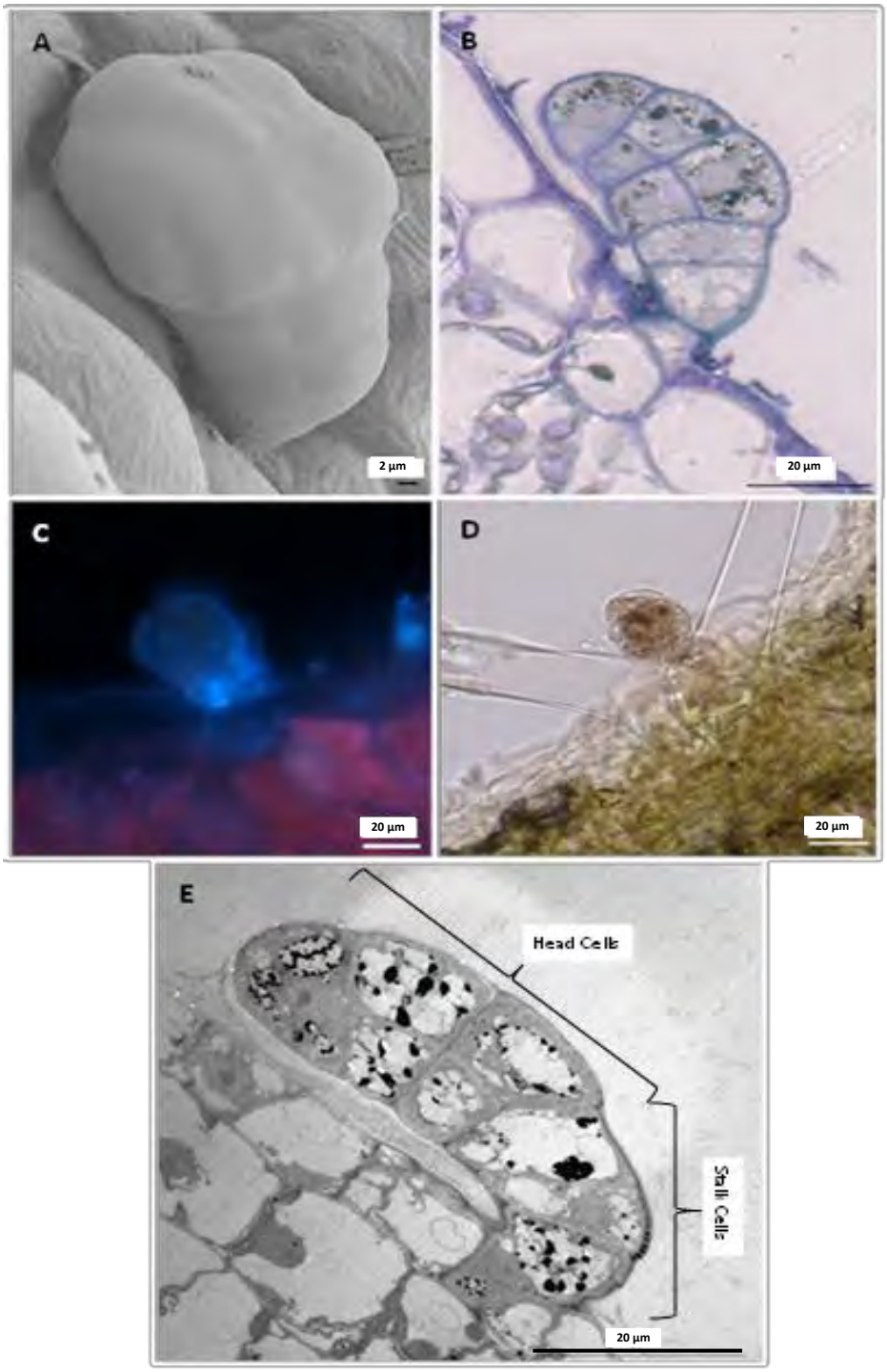

Figure 12: Glandular capitate trichome of *H. surattensis:* a) SEM of glandular capitate trichome showing distinct glandular head which consisted of approx. 5 cells. b) Light micrograph of resin embedded section stained with toluidine blue showing capitate trichome. Green deposits are present within trichome head cells as well as the basal cell. c) Autofluorescing capitate trichome showing faint fluorescence of organelles within the head cell and distinct fluorescence of the stalk cells. d) Fresh leaf section showing capitate trichome near non-glandular trichome, stained with ferric trichloride. e) TEM of capitate trichome showing electron dense material present within trichome head and stalk cells.

4.2.1 HISTOCHEMICAL OBSERVATIONS OF GLANDULAR CAPITATE TRICHOMES

Secretion composition and localisation was histochemically evaluated. Glandular trichomes of *H. surattensis* stained positively with Ruthenium Red, mercuric bromophenol blue, Wagner's and Dittmar's reagents, ferric trichloride and Nile Blue, for acidic polysaccharides and mucilage, proteins, alkaloids, phenolics and acidic lipids, respectively (Fig. 15). Similar observations were made for *H. sabdariffa* with glandular trichomes testing positive for the presence of acidic polysaccharides, proteins, alkaloids, phenolics and acidic lipids (Fig. 15).

Within capitate trichomes of *H. surattensis*, alkaloids were observed mainly in the head cells (Fig. 15 A). However, the entire leaf tissue stained positively. Similar observations were noted for Sudan Black B, Nile Blue, mercuric bromophenol blue and Ruthenium Red in which capitate trichome head cells stained along with varying degrees of positive staining of the entire leaf tissue (palisade and spongy mesophyll, epidermis; vascular tissue is excluded). The stalk cells of capitate trichomes stained intensely with Sudan III&IV and also auto-fluoresced intensely under UV light, indicating lignified anticlinal cell walls (Fig. 12 C). Toluidine Blue stained the basal cell intensely in the fresh leaf sections. However, resin embedded sections stained differently. Certain organelles within head cells of capitate trichomes as well as part of the basal cell stained green in sections prepared for TEM (Fig. 12). This might indicate the presence of phenolic substances within these cells.

For the capitate trichomes of *H. sabdariffa*, the test for alkaloids showed distinct staining of subcellular components (Fig. 16 A). This was also noted for the test for proteins and lipids, stained with mercuric bromphenol blue and Sudan III&IV, respectively. The stalk and basal cells stained more intensely for acidic polysaccharides than the head cells of capitate trichomes. Head cells of capitate trichomes stained more intensely with Toluidine Blue and ferric trichloride than trichomes of *H. surattensis*. As in *H. surattensis*, stalk cells of *H. sabdariffa* demonstrated intense autofluorescence, signifying the presence of lignified components in anticlinal cell walls. Phloroglucinol showed marginal staining of capitate trichomes of both species (Fig. 14 C).

Preliminary observations were made of trichome viability using Acradine Orange (Fig. 13) and Fluorescein diacetate (FDA not shown). Acradine orange demonstrates cell viability by visualising the intact nucleus with a fluorescent green colour whereas FDA stains the cytoplasm of viable cells. Both proved to be acceptable means of visualising viability of the studied glandular trichomes and will be investigated further in future studies.

Table 2: Histochemical investigations of the foliar structures of *Hibiscus surattensis* and *Hibiscus sabdariffa*.

Compound class	**Histochemical Test**	***Hibiscus surattensis***	***Hibiscus sabdariffa***
Alkaloids	Wagners and Dittmar	+ +	+ +
Proteins	Mercuric bromophenol blue	- +	+ +
Lipids	Sudan III&IV	- +	- +
	Sudan Black B	- +	- +
	Nile Blue	+ +	+ +
Viability	Acridine orange		
Polysaccharides and Mucilage	Ruthenium red	+ +	+ +
Ligin and cutin	Phloroglucinol	- +	- +
Metachomatic staining	Toluidine blue	+ +	- +
Phenolic compounds and Tannin	Ferric trichloride	+ +	+ +
	Autofluorescence	+ +	+ +

- + indicates weakly positive; + + indicates positive.

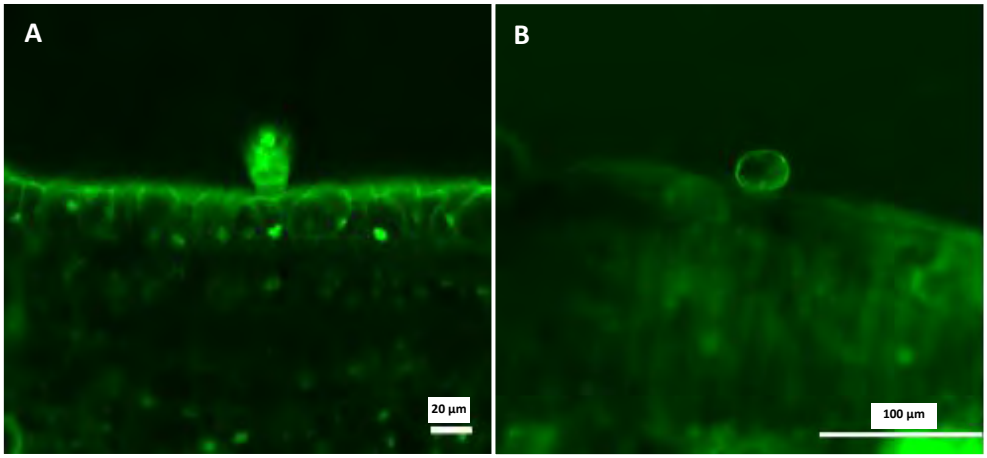

Figure 13: Fluorescence microscopy showing viability. a) Glandular capitate trichome of *H. surattensis* stained for viability with Acradine Orange. b) Glandular capitate trichome of *H. sabdariffa* stained for viability with Acradine Orange.

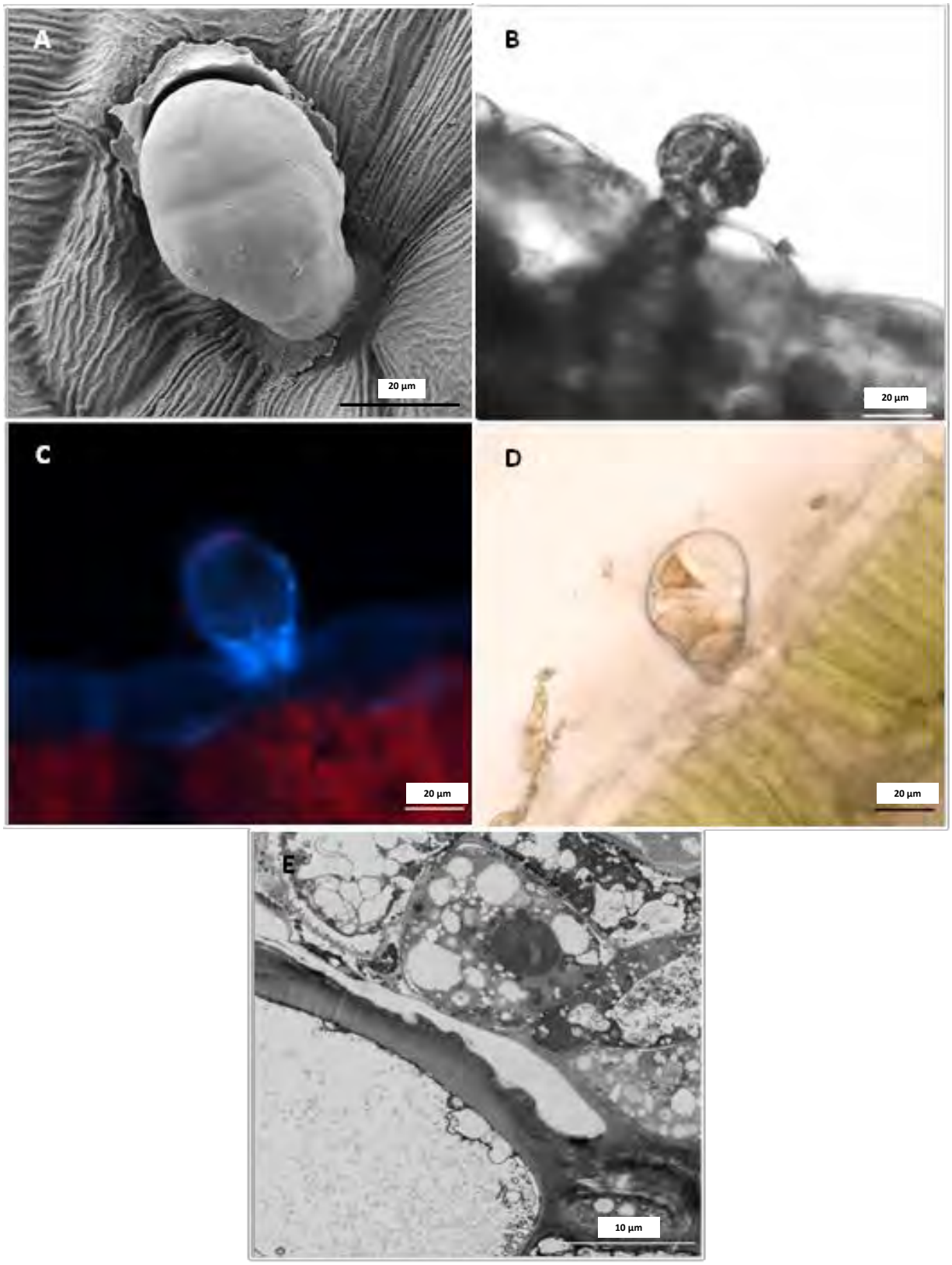
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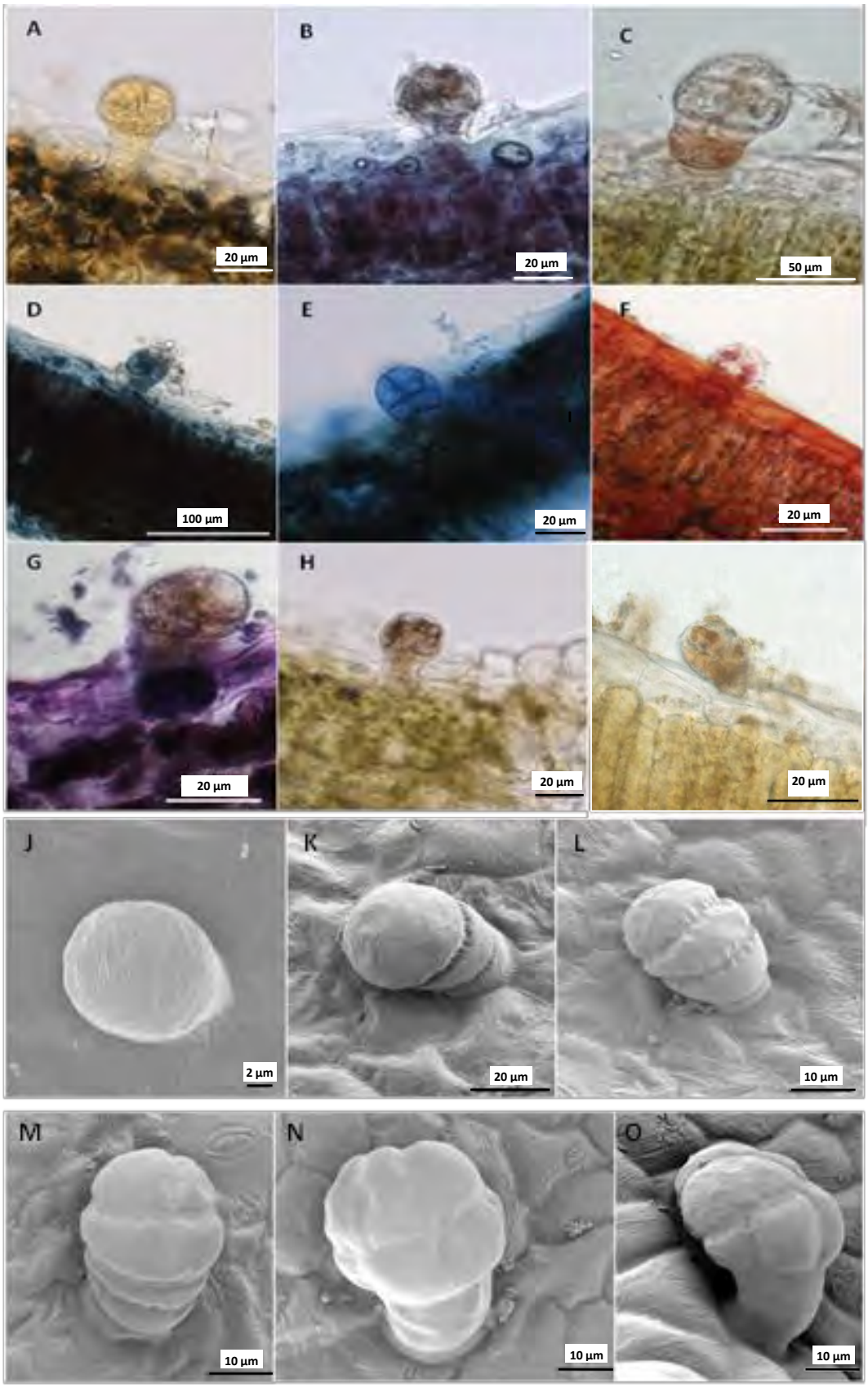
Figure 14: Glandular capitate trichomes of *Hibiscus sabdariffa*. a) SEM micrograph showing single capitate trichome together with secretion. b) Differential interference contract image of fresh leaf section showing dense cytoplasmic activity within capitate trichome head cells. c) Fluorescence micrograph of capitate trichome showing faint fluorescence of head cell organelles and stronger fluorescence of stalk cells. d) Light micrograph of unstained fresh leaf section showing capitate trichome with orange substance within head cells. e) TEM micrograph showing portion of capitate trichome and basal cell, with high levels of cellular activity and dense cytoplasm.

4.2.2 MICROMORPHOLOGICAL OBSERVATIONS OF CAPITATE TRICHOME DEVELOPMENT

Glandular capitate trichomes of both *Hibiscus* species appeared to follow similar steps in development (Fig. 15 and 16). At the emergent stage of leaf development, various stages of trichome development were observed whereas at the young and mature leaf stages, mostly mature trichomes occurred. This indicated that in young leaves no new trichomes were emerging, and trichomes present at this stage was assumed to persist to the mature stage of development.

Capitate trichomes of both *H. surattensis* and *H. sabdariffa* start of as single-celled protuberances emerging from the epidermis (Fig. 15 and 16 J). They appeared featureless until the first periclinal division produced the first visible site of cleavage resulting in the two-celled stage of development (Fig. 15 and 16 K). A further periclinal division resulted in the 3-celled stage, after which further periclinal and anticlinal divisions led to the formation of distinctive components of the capitate trichome, viz. the head cells and stalk cells (Fig. 15 and 16 L-M). Cells of the trichome head continued to divide until a total of 5-7 cells were present and the number of cells of the stalk in a mature trichome were three (Fig. 15 and 16 O). The developmental stages of capitate trichomes were observed for both the abaxial and adaxial surfaces of emergent leaves of both species.

Figure 15: Histochemical and developmental observations on the capitate trichomes of *H. surattensis*. a) Positive staining for alkaloids using Wagners and Dittmars reagents. b) Capitate trichome stained with mercuric bromophenol blue, positively staining for proteins. c) Sudan III & IV stained capitate trichome testing positively for lipids. d) Capitate trichome stained with Sudan Black B testing positively for lipids. e) Capitate trichome head staining positively with Nile Blue for acidic lipids and free fatty acids. f) Positive staining for mucilage and acidic polysaccharides using Ruthenium Red. g) Metachromatic stain, Toluidine Blue stained the basal cell of the glandular trichome deep purple indicating phosphate groups on macromolecules. h) Ferric trichloride staining a capitate glandular trichome dark brown to black, positively indicating the presence of phenolic compounds. i) Phloroglucinol staining for cutin and suberin. j) – o) Various stages of trichome development from single celled epidermal protrusion to mature trichome.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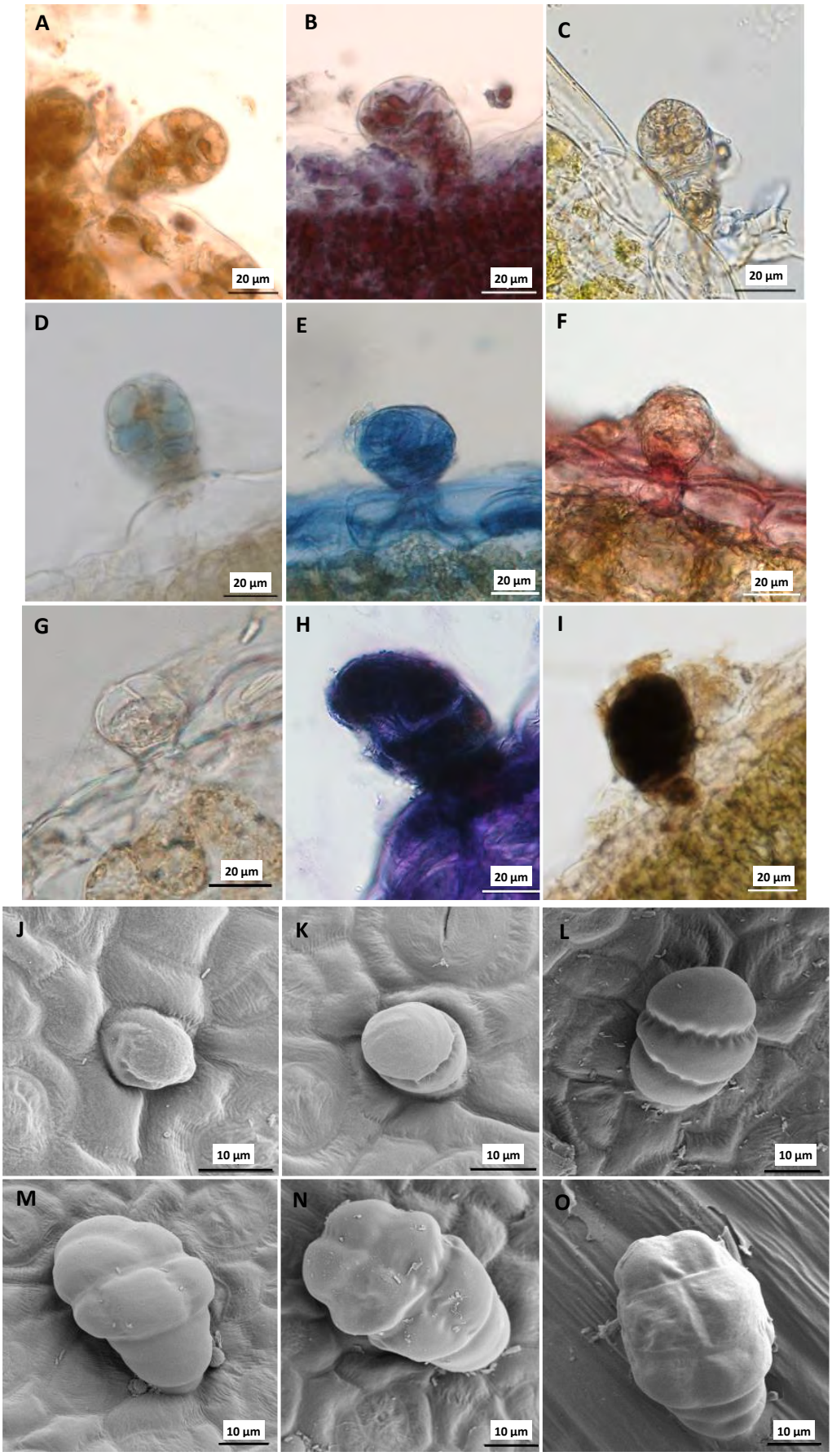
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Figure 16: Histochemical and developmental observations on the capitate trichomes of *H. sabdariffa*. a) Glandular trichome stained positively with Wagner's and Dittmar's reagents. b) Mercuric blue staining of glandular trichome, contents of head cells stained positively. c) Positive test for lipids in capitate trichome head, stained with Sudan III&IV. d) Sudan Black B stained positively for lipids in trichome head cells. e) Acidic lipids detected in capitate trichomes using Nile Blue. f) Ruthenium Red staining capitate trichome positively for the presence of mucilage. g) Cutinised and suberized trichome components stained with Phloroglucinol. h) Metachromatic staining using Toludine Blue showed the presence of phosphate groups on macromolecules within trichome head cells. i) Capitate trichome staining positively for phenolics with ferric trichloride. j) – o) Various stages of trichome development from single celled epidermal protrusion to mature trichome.

4.2.3 ULTRASTRUCTURE OF GLANDULAR CAPITATE TRICHOMES

Within capitate trichomes of *Hibiscus surattensis*, the presence of large quantities of electron-dense material was observed inside the vacuole (V) and in vesicles (V) of head and stalk cells (Fig. 17 A and H). This porous material was often observed as aggregations within or lining the interior surface of vesicles. The electron-dense material observed might be synthesized on the inner edges of vacuoles by constituents supplied by surrounding organelles and might migrate as it accumulates towards the centre of the vacuole (Fig. 17 H). It is proposed that this material might be phenolic or flavonoid compounds since histo-phytochemical tests have strongly confirmed the presence of both chemical classes (Tables 3 and 4). The head cells of *H. surattensis* appeared to be highly vacuolated with some vacuoles containing what appeared to be lamellar material (LM, concentric membranous inclusions within the vacuole or in the cytoplasm), indicating high membrane turnover and active metabolism (Fig, 17 F and H). Head cells of trichomes also displayed numerous mitochondria (M) and Golgi bodies (GB) located near vacuoles and on the cellular periphery (Figure 17 B and C).

One of the striking ultrastructural features that were difficult to detect in light and scanning electron microscopy was the presence of a subcuticular space (SCS) in the head of the capitate trichome (Fig 17 A). The cuticle of capitate trichomes of *H. surattensis* separated from the cell wall at the apex of the trichome and formed a subcuticular space on the ventral surface of the trichome in which an amorphous material was secreted (Fig 17 G). However, it appeared that the secreted material is not the same as the electron-dense material located within vacuoles, and might perhaps be synthesised by organelles outside the vacuole. Loosening of cell wall microfibrils was also observed, through which secreted material traversed.

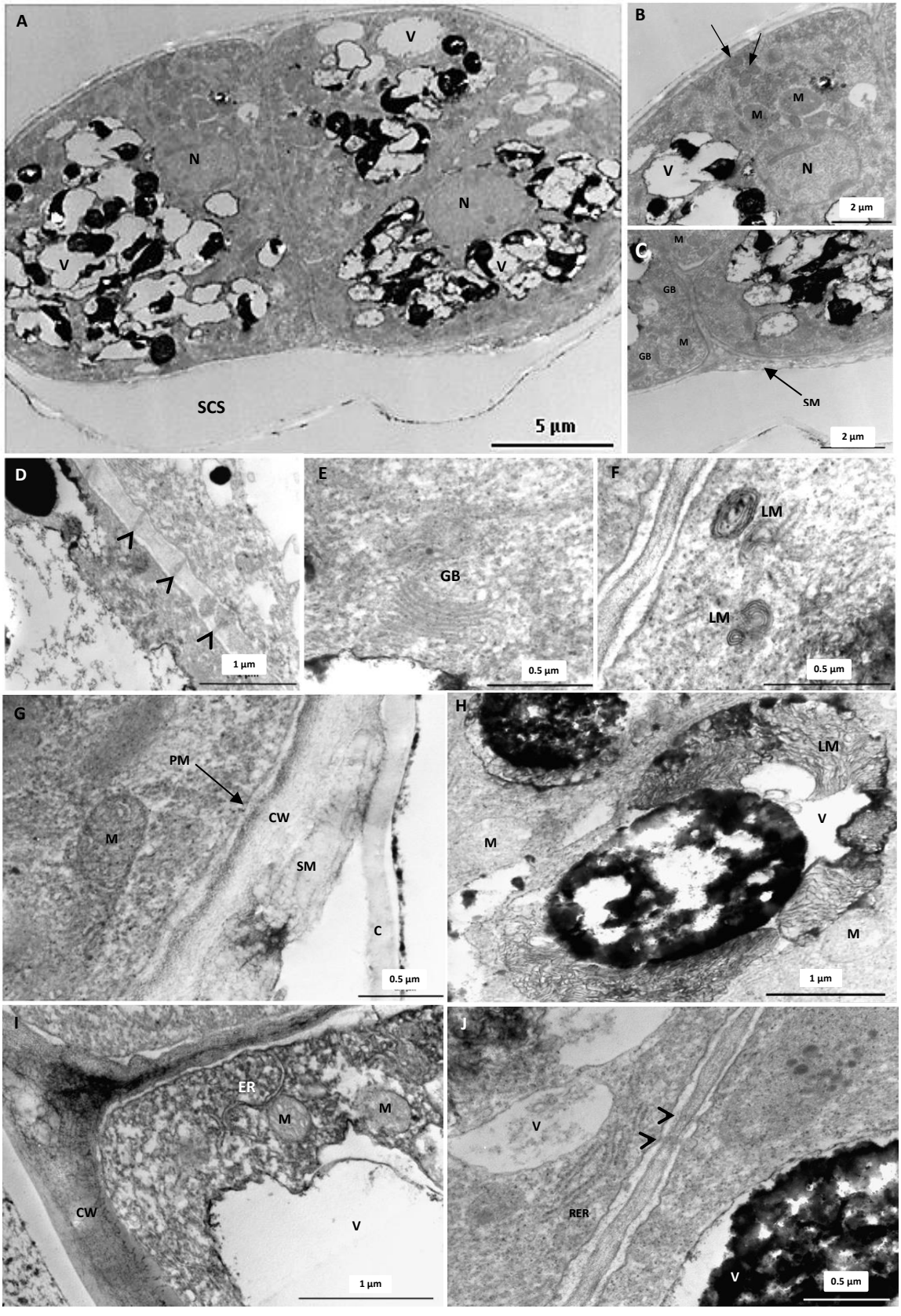
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Figure 17: TEM of capitate trichomes of *Hibiscus surattensis:* a) Glandular trichome head showing electron dense material within vacuoles and vesicles and the presence of the subcuticular space (SCS). b) Numerous mitochondria and dense cytoplasm of head cells. c) Golgi bodies and mitochondria located on the periphery of head cells as well as trichome secretion present in the subcuticular space along the cell wall. d) Numerous plasmodesmata were observed in the cell walls between the stalk and head cells. e) Golgi body in close proximity to vacuole. f) Lamellar bodies (LM) observed in the cytoplasm at various locations within the head cells. g) Region at which the cuticle separates from the cell wall to form the subcuticular space with possible amorphous secreted material located within the SCS. h) Porous electron dense material surrounding by lamellar material within the vacuole. i) ER and mitochondria located near the cell periphery and vacuole. j) Vesicle containing electron-opaque material and ER in association with plasmodesmata. Vesicle formation observed on one end of plasmodesmata. *Nucleus* = N, *Mitochondria* = M, *Vacuole, vesicles* = V, *Golgi body* = GB, *Endoplasmic reticulum* = ER, *Secreted material* = SM, *Subcuticular space* = SCS, *Lamellar body* = LM, *Plasma membrane* = PM, *Cuticle* = C, *Cell wall* = CW. *Plasmodesmata* = small arrows, *Small mitochondria* = large arrows.

Capitate trichomes of *H. sabdariffa* and *H. surattensis* appeared to be similar in structure, though, ultrastructurally many differences were noted. Glandular capitate trichomes of *H. sabdariffa* also bore a subcuticular space as in *H. surattensis*, but the mode and nature of secretion appeared quite different. At a glance, many electron dense deposits were visible throughout the head cells of the trichome (Fig. 18 A). These deposits did not appear porous as with those of *H. surattensis* but appeared more fluid in nature. They were observed within vacuoles but also on the cell periphery and in the subcuticular space (Fig. 18 B and C). At the apical end of the trichome, just below the dorsal surface, a large accumulation of electron dense material was observed that appeared to be compartmentalized from other cellular components (Fig. 18 A). Within the head cells, organelles were not as numerous or observable as in *H. surattensis.* Golgi bodies and mitochondria were noted Fig. 18 D and F), but fewer ER cisternae and no lamellar bodies were identified. Head cells appeared highly vesiculated with the vacuole and vesicles occupying a large proportion of the cell volume, especially in the apical head cell from which secretion ensued (Fig. 18 E and F). High levels of vesiculation were also observed in stalk cells and head cells other than the apical cell, where the cytoplasm appeared more electron dense with numerous organelles.

4.3 MUCILAGE IDIOBLASTS AND DUCTS

They are distinguished from one another primarily by their mode of secretion and formation.

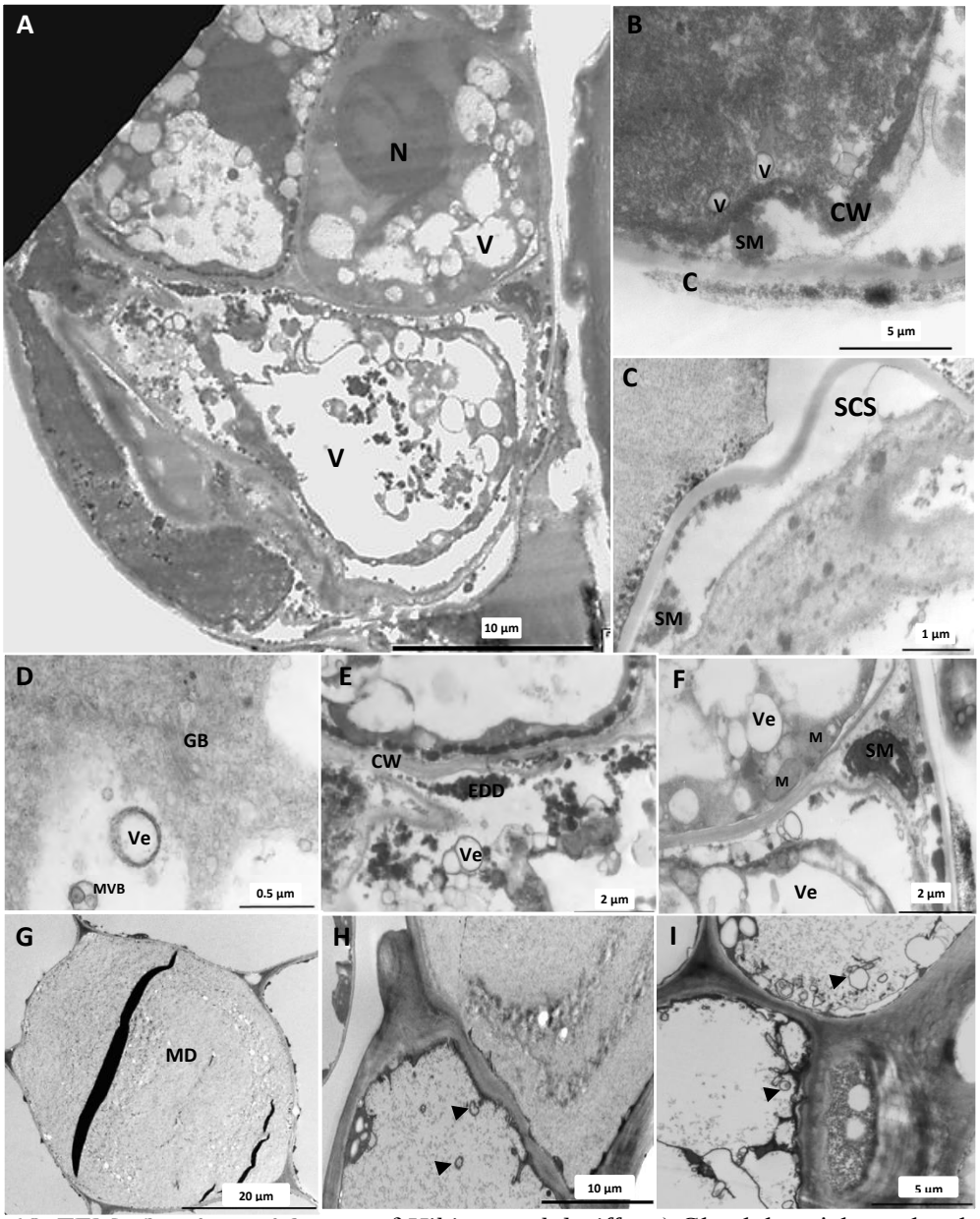

Figure 18: TEM of capitate trichomes of *Hibiscus sabdariffa:* a) Glandular trichome head at low magnification showing dense cytoplasmic deposits within vacuoles and vesicles as well as intracellular compartmentalization of electron dense deposits at the apical dorsal region of the head. b) Region at which the cuticle separates from the cell wall to form the subcuticular space. c) Presence of subcuticular space with electron dense material being deposited within. d) Golgi body and secretory vesicle in head cell of trichome. e) Electron dense deposits (EDD) lining the periphery of head cells as well as occurring in the vacuole. f) High levels of vesiculation at region where two head cells interacted as well as electron dense material deposited into the space between cell walls and the cuticle. g) Mucilage duct in *Hibiscus sabdariffa.* h) Epidermal mucilage cells in *H. sabdariffa* showing subcellular activity. i) Vesicle mediated mucilage deposition in mucilage cells of *H. sabdariffa.*

Mucilage idioblasts appeared to differentiate from mesophyll or epidermal cells whereas mucilage ducts formed by the schizogenous demise of certain cells near the vascular bundle to form a conducting lumen for secretion to enter.

Using scanning electron microscopy, the dorsal surface of mucilage idioblasts in the epidermis was visualised for both *H. surattensis* and *H. sabdariffa* (Fig 20 A). In *H. sabdariffa*, they were less obvious and often difficult to distinguish between surrounding epidermal tissue. Therefore, the density of mucilage cells across various developmental stages of the leaf was only considered for *H. surattensis.* Epidermal mucilage idioblasts were characterised by the radial arrangement of epidermal cells surrounding a cell with a wrinkled surface appearance (Fig 20 A). The cuticular striations present on the surrounding epidermal cells were directed towards the wrinkled surface of the mucilage cell. Mucilage ducts were only visualised with scanning (Fig 20 C) and transmission electron microscopy (Fig 18 G, Fig 22) and resin-embedded samples viewed with light microscopy. Therefore histochemical investigations on fresh leaf sections did not consider mucilage duct chemistry.

For *Hibiscus surattensis,* significant differences between mucilage cell density were found to occur between the mature adaxial surface and every other surface investigated (df = 5; F= 1.261; $p< 0.05$) apart from emergent abaxial which was shown not to be significantly different ($p = 0.54$). Due to the fact that the assumption of normality was not met for this sample subset in the MANOVA, a Kruskal-Wallis test was used to confirm significance (df = 5, Chi square = 32.690, $p < 0.05$). Fig. 19 depicts the trend of increasing mucilage cell density on the adaxial leaf surface with progressive development.

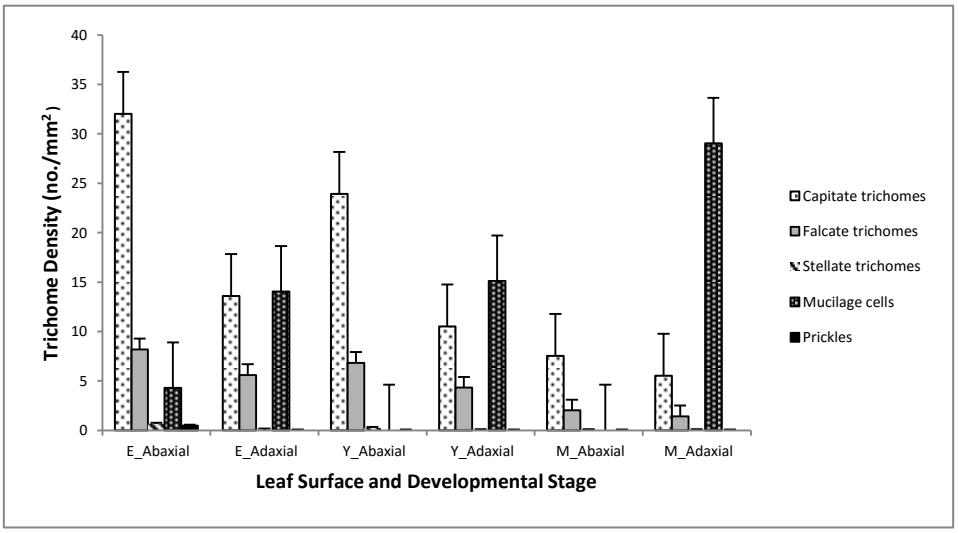

Figure 19: Mean trichome density across *Hibiscus surattensis* leaf surfaces for each of the developmental stages investigated (with +SE). *Emergent* = E, *Young* = Y, *Mature* = M.

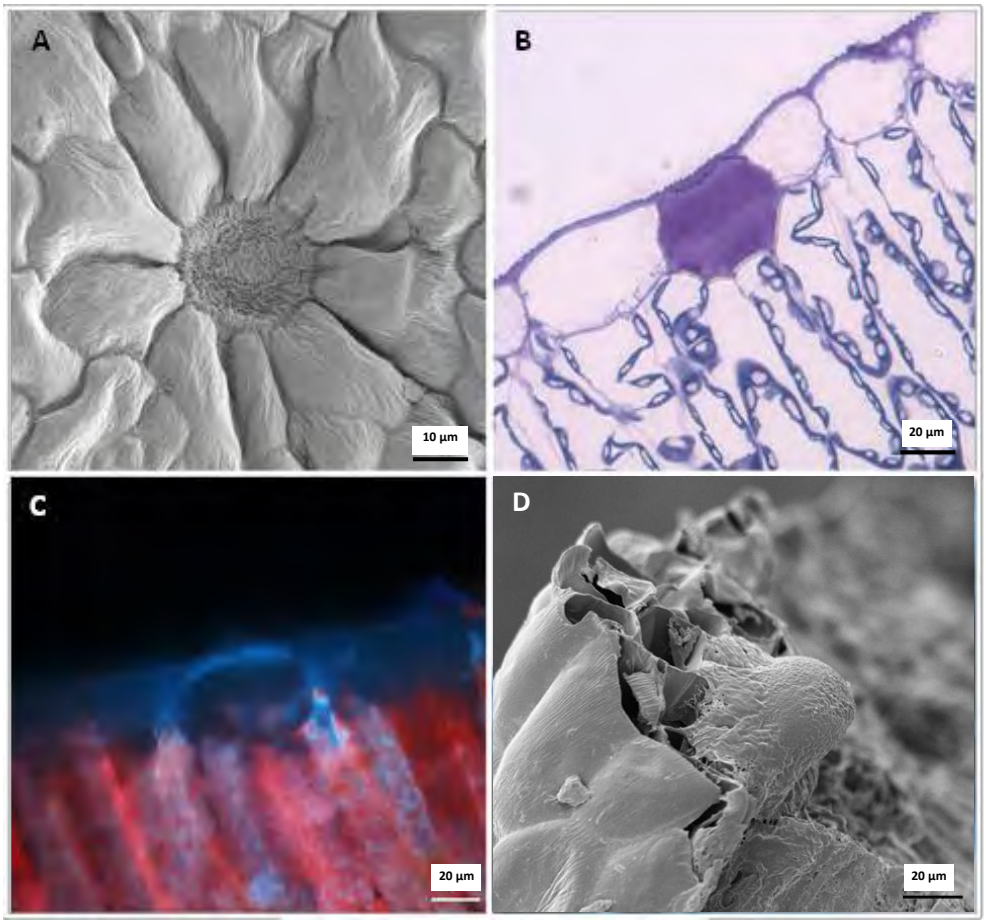

Figure 20: a) SEM of the adaxial leaf surface showing the surface view of a mucilage cell of *Hibiscus surattensis*. b) Resin embedded section stained with Toluidine Blue, showing *H. surattensis* mucilage cell staining bright purple. c) Strong autofluorescence of the upper regions of the *H. surattensis* mucilage cell. d) SEM of freeze fractured mucilage duct in *H. sabdariffa*.

4.3.1 HISTOCHEMICAL OBSERVATIONS OF MUCILAGE IDIOBLASTS

Histochemical reactions of epidermal mucilage cells yielded strongly positive results for acidic polysaccharides, acidic lipids and lipids with Ruthenium Red, Nile Blue, Toluidine Blue and Sudan III and IV respectively (Fig. 21 C, E, F, H). Weaker reactions were observed for ferric trichloride, mercuric bromophenol blue, Sudan Black B and Wagners and Dittmar reagents. There was no distinct reaction observed for the phloroglucinol test. For certain tests, the upper region of the mucilage cell stained, particularly in the tests for alkaloids and proteins. Autofluorescence microscopy also exhibited a similar pattern with the surface region of the mucilage cell fluorescing as a light blue layer (Fig. 20 C) which might indicate polyphenolic or lignified components.

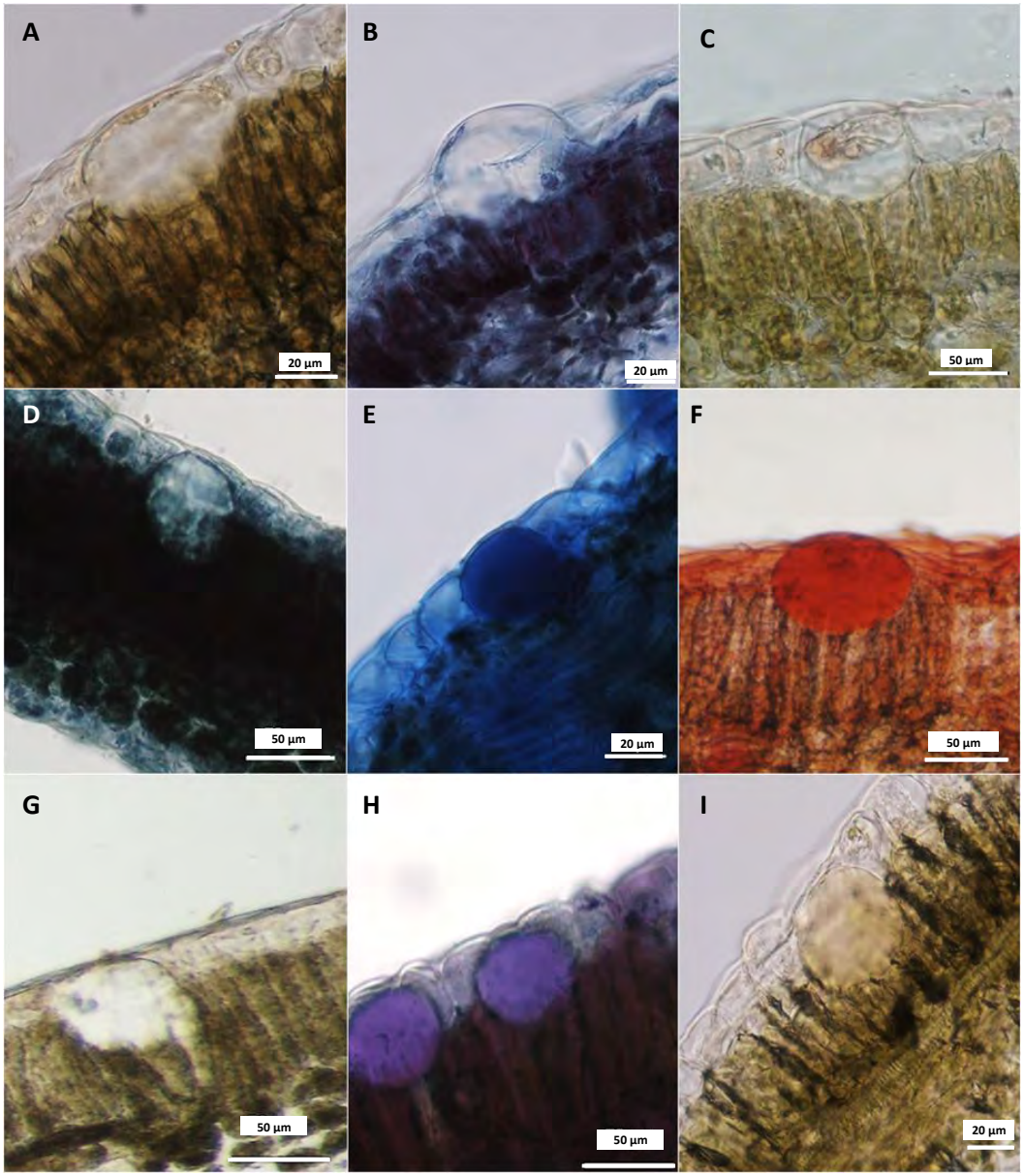

Figure 21: LM of histochemical observations of mucilage idioblasts of *Hibiscus surattensis*. a) Absence of alkaloids within mucilage cell stained using Wagner's and Dittmar's reagent. b) No protein detected within mucilage cell stained with mercuric bromophenol blue. c) Positive staining of lipids with mucilage cells using Sudan III&IV. d) Slight staining of lipids within mucilage cells using Sudan Black B. e) Intense staining of acidic lipids within mucilage cells using Nile Blue. f) Intense staining of acidic polysaccharides and mucilage within mucilage cells using Ruthenium Red. g) Phloglucinol detected cutinised and suberized components of mucilage cells. h) Metachromatic staining using Toluidine Blue stained mucilage bright purple. i) Ferric trichloride faintly stained mucilage cells a brownish-yellow.

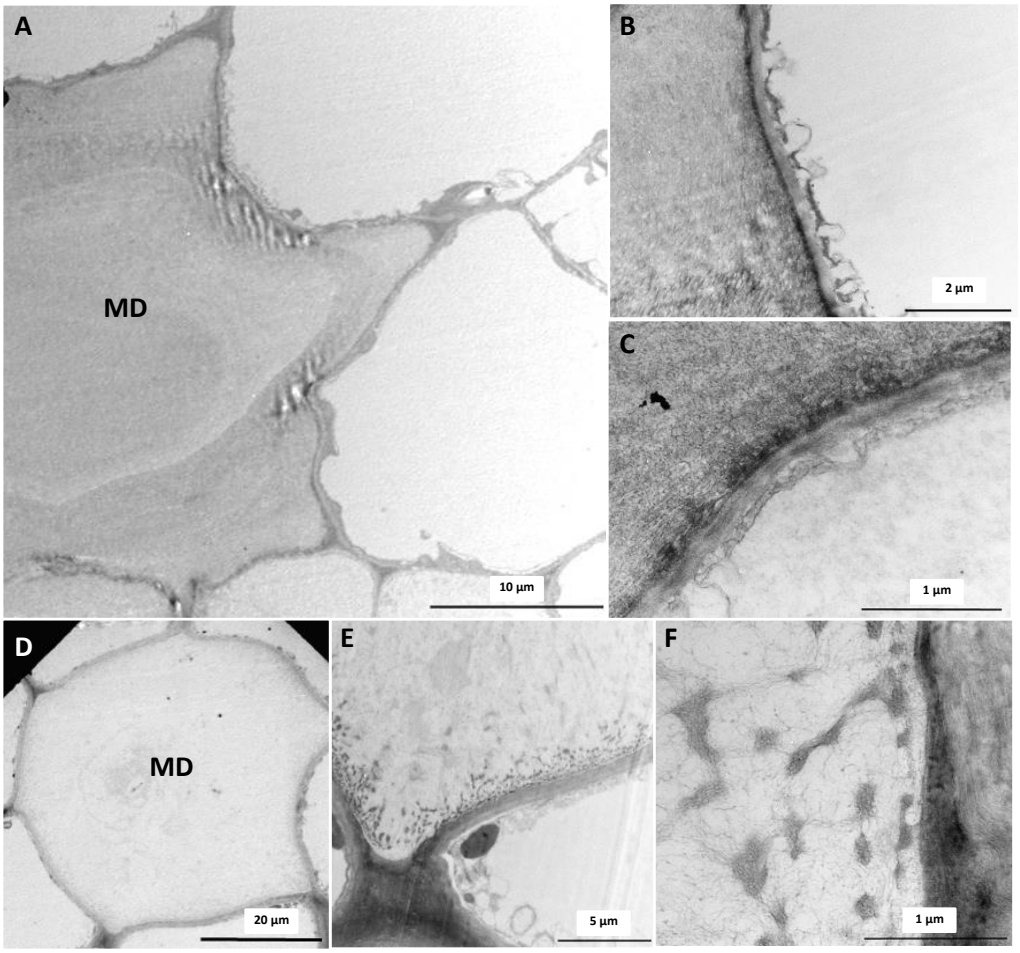

Figure 22: TEM of mucilage ducts in *Hibiscus surattensis*: a) Mucilage duct and supporting cells. b) Vesicular activity observed on the inner edge of the cell adjacent to the duct. c) Further vesicular activity within peripheral cytoplasm and dense electron opaque material at duct edge. d) Mucilage duct demonstrating lack of subcellular organelles. e) Possible mucilage deposition emanating from edge of mucilage duct. f) String-like nature of mucilage viewed at high magnification. *Mucilage duct* = MD.

4.3.2 ULTRASTRUCTURE OF MUCILAGE IDIOBLASTS AND MUCILAGE DUCTS

In mucilage ducts, the secreted material appeared to be synthesised in the surrounding tissue and is assumed to be transferred into the lumen of the duct. Those of *H. surattensis* (Fig. 17) displayed an irregular structure which was not enclosed by a plasma membrane. Also, within the lumen of the duct no subcellular structures were observed (Fig. 17 A and D). The substance within the duct was electron opaque and slightly granular with certain regions showing increased density of the mucilaginous substance than others, particularly in the duct periphery.

In cells surrounding the duct, few to no observable organelles were identified as with most xylem and phloem parachymatous tissues of the vascular bundle. However, on the duct-facing edge of the surrounding cells vesicular activity was observed on the cell periphery, indicating deposition and transfer of mucilage precursors to the mucilage duct (Fig. 22 B and C). Stratification of mucilage on the periphery of the duct is also observed with the duct's edges appearing denser and string-like mucilaginous components which seemed to traverse from the edge of the duct to the centre (Fig. 22 E and F). Ducts of *Hibiscus sabdariffa* demonstrated similar ultrastructural features whereas mucilage idioblasts displayed high levels of intracellular compartmentalization and vesicle formation (Fig.18 H and I).

4.4 CRYSTAL IDIOBLASTS

Crystal idioblasts were identified as specialised cells of the leaf mesophyll that differentiated to form a druse crystal within a vacuole. Initially detected with a light microscope, they were later observed with greater detail using SEM on freezed fractured specimens (Fig 23 A). During ultrastructural observations the crystal has separated from the resin, allowing for the remaining encircled cell remnants to be investigated. The crystals were identified as star-shaped druse crystals with many protruding pointed edges and were enclosed within a cell of the mesophyll (Fig. 23 A). They were found to be numerous in both species of *Hibiscus* and were often observed throughout the leaf mesophyll.

Immature crystal idioblasts differed from surrounding mesophyll cells by fewer chloroplasts and active cellular processes which occured in the central region of the cell which in most mesophyll cells is usually occupied by a water-storage vacuole (Fig 23 B). High levels of vacuolation and vesicle formation were observed in developing crystal idioblasts whereas those in which crystals were formed cellular activity was restricted to the cell periphery (Fig. 23 C). Numerous Golgi bodies, mitochondria and ER were observed in the cytoplasm as well as the active formation of vesicles that fused with or entered the central vacuole which contained the crystal (Fig 23 D and E). In mature crystal idioblasts in which the crystal encompassed most of the cellular volume, the cytoplasm appeared almost non-existent (Fig. 23 F). Few organelles such as plastids and ER were observed to occur in an electron-transparent medium that was similar to that within the vacuole (Fig. 23 G-I). Perhaps vacuole enlargement and subsequent degeneration appeared to result in cytoplasm dilution and submersion of organelles in the vacuolar contents. Numerous multivesicular bodies were also observed, indicating high membrane turnover (Fig. 23 I).

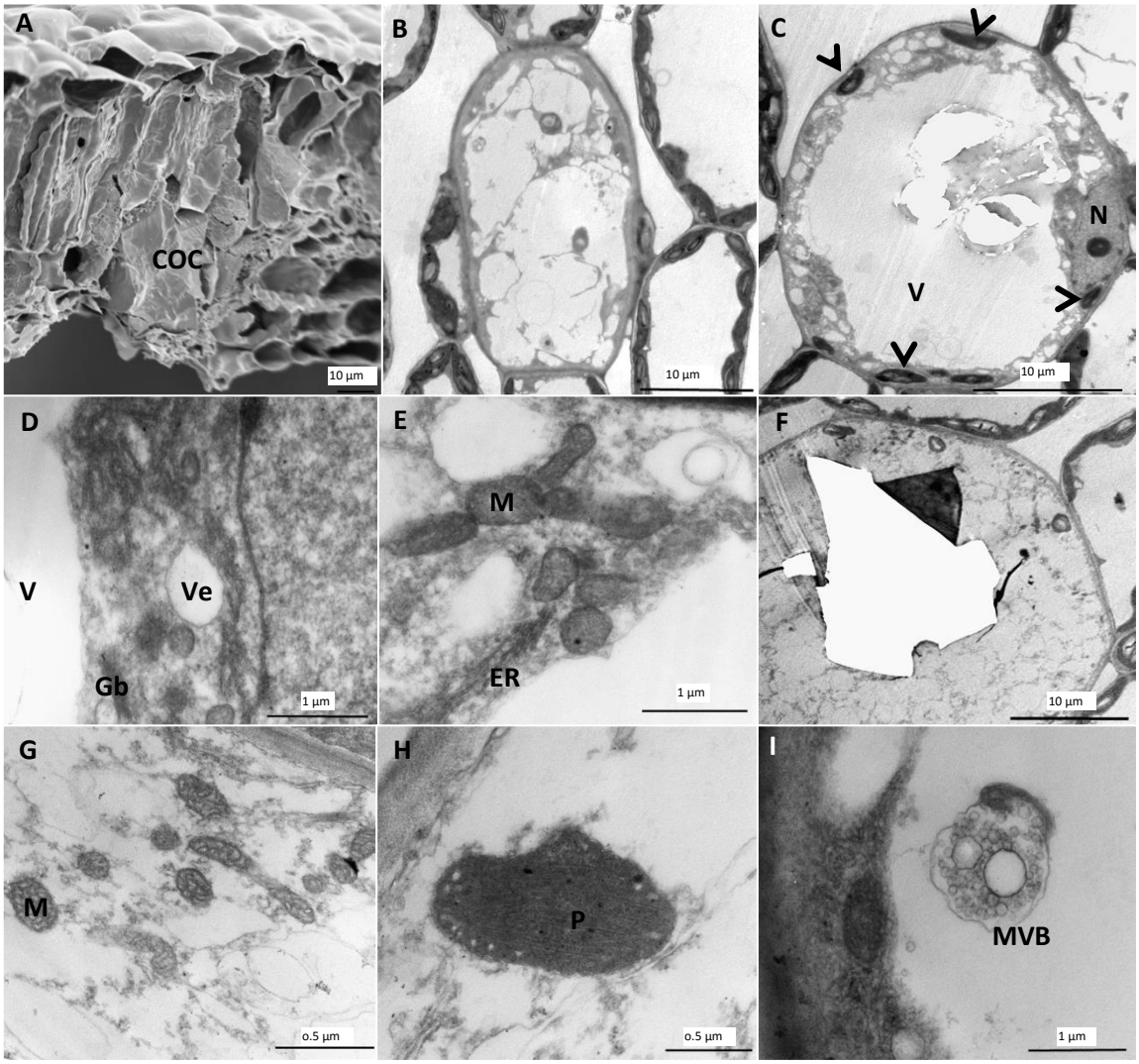

Figure 23: Crystal idioblast of *Hibiscus surattensis*: a) freeze fracture SEM of crystal within leaf mesophyll. b) Immature idioblast within mesophyll. c) Intermediate stage of crystal development; large vacuole with scar left by crystal; organelles restricted to cell periphery. d) Numerous mitochondria observed at intermediate stage with Golgi bodies and vesicles within dense cytoplasm. e) Elongated large mitochondria with rough ER and vesicular activity. f) Mature crystal idioblast marked by large scar in the central region of the cell. g) Vacuolar membrane appears to have broken down, cytoplasm is electron light; mitochondrial features more prominent. h) Plastid showing electron dense stroma. i) Multivesicular body observed at cell periphery. *Crystal* = COC, *Nucleus* = N, *Mitochondria* = M, *Vacuole* = V, *vesicles* = Ve, *Golgi body* = GB, *Endoplasmic reticulum* = ER, *Chloroplasts* = arrows , *Plastid* = P, *Multivesicular body* = MVB.

4.5 NON-GLANDULAR TRICHOMES

The types of non-glandular trichomes found in *Hibiscus surattensis* included stellate, falcate and prickle types whereas *H. sabdariffa* displayed falcate, bi- and trifurcate trichomes.

Stellate trichomes of *H. surattensis* were large and observed frequently on the emergent abaxial surface. They consisted of 4-5 acutely pointed arms converging to a sessile base (Fig. 24 A). The direction of the arms appeared to be situated parallel to the leaf surface. Prickle trichomes were also most identifiable and frequent on the emergent abaxial surface. They consisted of a rigid structure which was wider at the lower region of the trichome and tapered to a sharply pointed tip (Fig. 24 B). The base of prickle trichomes consisted of two rows of numerous epidermal cells arranged in a concentric ring. This, as well as the wider region of the lower part of the trichome, is thought to provide support for the trichome as it attaches onto surfaces. Falcate trichomes were observed to taper more acutely than the stellate and prickle trichome types but remained rigid (Fig. 24 C). They were also supported by concentric epidermal cells forming a basal pedestal (3-4 rows of approximately 8 cells each). Highest densities of falcate trichomes were observed on the emergent and young abaxial surfaces which decreased with the developmental stage (Fig. 19). Significant differences in falcate trichome density were only observed between the emergent abaxial leaf surface and both surfaces of the mature leaf ($df = 5$, $f = 1.584$, $p < 0.05$).

Non-glandular trichomes of *H. sabdariffa* appeared similar in shape and structure to those of *H. surattensis.* Stellate trichomes were very few and there were no prickle trichomes observed on any of the leaf surfaces investigated. Falcate trichomes were observed mostly towards the base on the abaxial surface of leaves of various developmental stages and were therefore not statistically considered in this investigation. Falcate trichomes possessed a basal pedestal similar to that in *H. surattensis*, however, fewer concentric rings of epidermal cells were observed. Bi- and trifurcate trichomes were also present, especially at the basal region of the leaf. They consisted of two or more arms and appeared sessile (Fig. 24 D-F).

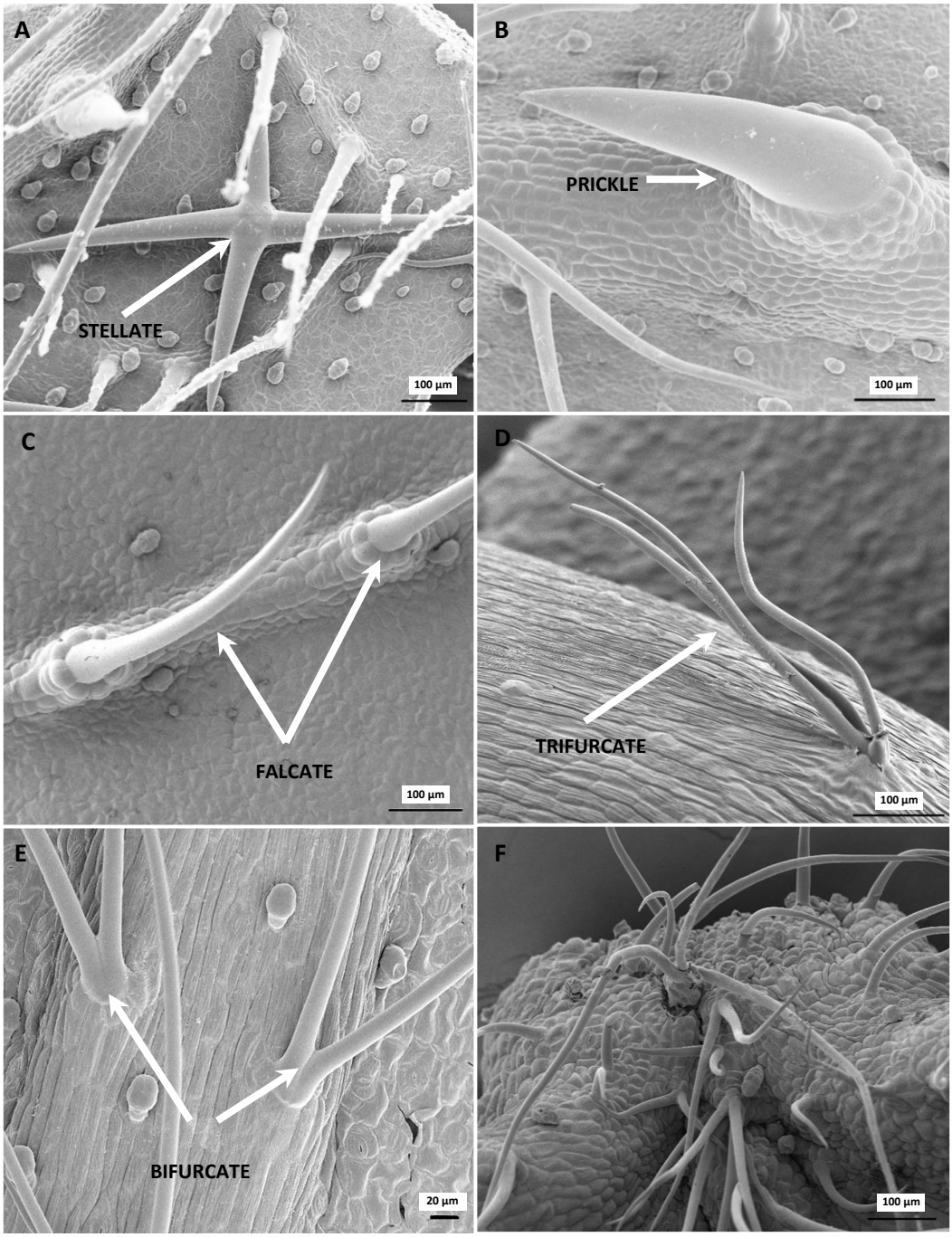

Figure 24: SEM of non-glandular trichomes of *Hibiscus surattensis* and *H. sabdariffa*: a) Stellate trichome observed on the abaxial surface of *H. surattensis*. b) Prickle trichome observed on the abaxial surface of *H. surattensis.* c) Falcate trichomes on the lateral vein of *H. surattensis*. d) Multi-furcate trichome with 3 arms on a lateral vein of *H. sabdariffa.* e) Two bifurcate trichomes on a lateral vein at the base of a young leaf of *H. sabdariffa*. f) High density of non-glandular trichomes at the adaxial leaf base.

4.6 PRELIMINARY PHYTOCHEMISTRY

Phytochemical tests were carried out on methanolic, chloroform and hexane leaf extracts of each of the species investigated. The most prominent classes of compounds which were identified in the methanolic extract of *H. surattensis*, were carbohydrates, sterols, phenolic compounds and flavones and flavanones, as identified with Fehlings and Benedicts tests, Salkowski test, Ferric trichloride and lead acetate tests, and the aqueous sodium hydroxide test respectively (Table 3). Carbohydrates were also detected in chloroform and hexane extracts. Tests for alkaloids, fixed oils and fats, gums and mucilage were not as strongly positive whereas proteins and saponins were not detected in any of the extracts (Table 3).

Hibiscus sabdariffa exhibited similar results with carbohydrates, phenolic compounds and flavones and flavanones displaying strongly positive reactions with the methanolic extract (Table 4). Alkaloids tested strongly positive in chloroform and hexane extracts. Chloroform and hexane extracts of *H. sabdariffa* also tested strongly positive for phenolics, flavones and flavanones. Weak but detectable amounts of mucilage and fixed oils and fats were observed in methanolic extracts of *H. sabdariffa* (Table 4).

Table 3: Preliminary phytochemical tests for various classes of compounds in methanolic, chloroform and hexane leaf extracts of *Hibiscus surattensis.*

Phytochemical	Test	*Methanol*	*Chloroform*	*Hexane*
Alkaloids	Draggendorf	- +	- +	- +
	Hagers	- +	- +	-
	Wagners	- +	- +	- +
Carbohydrates and glycosides	Molisch's	- +	+ +	+ +
	Fehling's	+ +	+ +	+ +
	Benedict's	+ +	-	-
Proteins	Ninhydrin	-	-	-
Sterols	Salkowski's	+ +	-	-
Fixed oils and fats	Spot	- +	-	-
Phenolic compounds and Tannin	Ferric chloride	+ +	-	-
	Lead acetate	+ +	-	-
Saponins	Foam	-	-	-
Gums and Mucilage	Precipitation	-	-	-
	Ruthenium Red	- +	-	-
Flavones and Flavanones	Aqueous sodium hydroxide	+ +	-	-
	Conc. Sulphuric acid	- +	- +	- +

- Indicates negative; - + indicates weakly positive; + + indicates positive.

Table 4: Preliminary phytochemical tests for various classes of compounds in methanolic, chloroform and hexane leaf extracts of *Hibiscus sabdariffa.*

Phytochemical	Test	Methanol	Chloroform	Hexane
Alkaloids	Draggendorf	- +	- +	- +
	Hagers	- +	- +	- +
	Wagners	-	+ +	+ +
Carbohydrates and glycosides	Molisch's	- +	+ +	+ +
	Fehling's	- +	+ +	+ +
	Benedict's	+ +	-	-
Proteins	Ninhydrin	-	-	-
Sterols	Salkowski's	-	-	-
Fixed oils and fats	Spot	- +	-	-
Phenolic compounds and Tannin	Ferric chloride	+ +	-	-
	Lead acetate	+ +	++	-
Saponins	Foam	-	-	-
Gums and Mucilage	Precipitation	-	-	-
	Ruthenium Red	- +	-	-
Flavones and Flavanones	Aqueous sodium hydroxide	+ +	- +	-
	Conc. Sulphuric acid	-	++	-

- Indicates negative; - + indicates weakly positive; + + indicates positive.

CHAPTER 5: DISCUSSION

A comprehensive understanding of the types of foliar plant defence structures utilized by *H. surattensis* and *H. sabdariffa* are discussed; as well as the ecological, ethnobotanical and pharmacological implications of the phytochemical findings.

Leaves of *H. surattensis* had 4 types of foliar trichomes:

a) Large stellate trichomes,
b) Large prickle trichomes,
c) Acutely pointed falcate trichomes,
d) Glandular capitate trichomes.

In addition to external micromorphological appendages, internal specialisation of mesophyll and epidermal structures were encountered, viz. mucilage cells, mucilage ducts and calcium oxalate crystal idioblasts. Leaves of *H. sabdariffa* possess similar structures but differed in the types of non-glandular trichomes identified. Bi- and trifurcate trichomes, consisting of two and three arms respectively, together with falcate trichomes comprised the non-secretory trichome component of leaves of *H. sabdariffa*. For the purposes of this report, mature capitate trichomes are characterised micromorphologically by achievement of maximum trichome size (40-50 μm). Ultrastructurally, their identification is further substantiated by active secretory processes and by the accretion of exudate into the subcuticular space.

5.1 TAXONOMIC SIGNIFICANCE

Few studies consider the systematic significance found in the diversity of foliar trichomes present within the polyphyletic genus *Hibiscus*; which comprises over 300 species. Given that South Africa possesses many species, studies on indigenous *Hibiscus* should focus on corroborating genetic studies with (micro-)morphological traits (as per Pfeil *et al*., 2002). In systematically evaluating leaf micromorphology within *Hibiscus*, Shaheen *et al* (2007) identified capitate glandular trichomes present on most leaf surfaces but observed a profound diversity of non-glandular foliar appendages. This suggests that capitate glandular trichomes might be a significantly conserved feature of the genus (Shaheen *et al*., 2007) whereas non-glandular trichomes might serve as a species level tool for classification. Results of this study support this notion as observations made between *H. surattensis* and *H. sabdariffa* display a similar trend. Glandular trichomes and mucilage secreting tissues appeared to be similar in structure and chemical composition whereas the distribution and types of non-glandular

trichomes accounted for vast differences in the leaf landscape. In order to make valid taxonomical comparisons, both the leaf indumentum and examination of leaf microstructures need to be considered (Dickison, 2000).

5.2 LEAF INDUMENTUM

The indumenta of leaves are often overlooked or their ecological role underestimated, but they have demonstrated greater elasticity to changing environments than that shown by actual trichome structure (Johnson, 1975). Leaves of *Hibiscus surattensis* appeared highly pubescent at all developmental stages especially at the emergent stage. This is attributed to the high density of non-glandular structures present on the leaf surface. When leaves were handled or touched, the sharply pointed hairs hook and break off into the skin causing pain and irritation. In this regard, they might act as an important first line of defence against large herbivores. Among the many functions of non-glandular trichomes highlighted by Wagner *et al*. (2004), there are few that might apply to *H. surattensis*. These include decreased mobility of phytophagous insects and temperature, water loss and light regulation, and also to assist in climbing. This is associated with the taxonomic description of *H. surattensis* as it is a scrambling creeper and is often entwined around surrounding vegetation. The thorny, hook-like trichomes that comprise the indumentum of the leaves is assumed be important in hooking onto surfaces to align the plant in favourable positions for optimal growth conditions. Unlike leaves of *H. surattensis*, those of *H. sabdariffa* possessed no distinct indumentum. Leaves appeared to be completely glabrous except for a tuft of hairy trichomes which were located at the base of the leaf on the midvein area, particularly on the adaxial surface. It was noted that certain leaf margins appeared ciliate-dentate (Payne, 1978) with a single hair present on each tooth. The reddish petiole also appeared sheathed in non-glandular trichomes, perhaps to confine insect movement between plant parts (Ramalho *et al*., 1984).

5.3 GLANDULAR TRICHOMES

Prominent capitate glandular trichomes of varying densities were observed on both species across all leaf surfaces. Capitate trichomes were characterised by a bulbous head consisting of 5-7 cells, a supporting stalk of 3 cells with cutinised sidewalls and a basal cell embedded in the epidermis. Curvature of the stalk cells resulted in the antrorse orientation of capitate trichomes on the leaf surface (term antrorse derived from Payne, 1978).

5.3.1 CAPITATE TRICHOME MICROMORPHOLOGY

Studies detailing micromorphology within *Hibiscus* described the secretory trichomes comprising extrafloral and floral nectaries (Sawidis *et al*., 1987; Rocha and Machado, 2009) but none have so far attempted to detail the fine structure of foliar glandular appendages or the phytochemicals they contain. Trichomes identified within nectarfarious tissues of other *Hibiscus* share certain similar morphological characteristics to those observed on the foliar surfaces of *H. surattensis* and *H. sabdariffa* but the functions of the secretory apparatus differed significantly. Trichomes responsible for nectar synthesis and secretion in *H. pernambucenis* were multicellular and clavate and appeared to be similar to the capitate trichomes of *H. surattensis* and *H. sabdariffa*. They occurred within the nectary furrow located at the base of the abaxial leaf surface (Rocha and Machado, 2009). Production of nectar spanned the lifetime of the trichome and ceased at the start of leaf senescence indicating that active secretion in nectary trichomes are not regulated by environmental circumstances (Rocha and Machado, 2009). As such, head cells of these trichomes displayed large nuclei, dense cytoplasm and active cellular processes (Rocha and Machado, 2009). Developmental observations of *H. surattensis* and *H. sabdariffa* capitate trichomes also demonstrated trichome longevity since it was during early leaf ontogenetic stages that trichomes of different developmental stages (ranging from simple epidermal protrusions to fully formed trichomes) were observed. The lack of trichome scars on mature leaves supported the notion that the density of glandular trichomes is controlled by the expansion of leaves (Werker *et al.*, 1993) and that capitate trichomes most likely persist on the leaf surface until leaf senescence. Secretion, however, was not observed continuously as in nectary trichomes. Comprehensive viability assessments of capitate trichomes would provide a better understanding of trichome longevity and could assist in leaf developmental studies within *Hibiscus*.

5.3.2 DIFFERENCES IN DENSITY OF FOLIAR CAPITATE TRICHOMES

Comparisons between developmental stages of each species as well as those between *H. surattensis* and *H. sabdariffa* demonstrated important trends. Overall abaxial surfaces of leaves exhibited higher frequencies of capitate trichomes but a negatively sloping decline was encountered for *H. sabdariffa* when comparing abaxial to adaxial leaf surfaces from emergent to mature developmental stages. The relatively rapid development of glandular trichomes on emergent leaf surfaces and persistent longevity appeared to indicate an increased resource investment in early leaf development which is assumed to reduce the biological cost of defence in mature leaves (Levin, 1973). A similar trend was observed for leaves of *H. surattensis*, but the frequencies of capitate trichomes per unit area are fewer than for leaves of *H. sabdariffa*.

This is assumed to be attributed to the multiple avenues of micromorphological defence which appeared to be adopted by *H. surattensis* in the form of the diverse non-glandular components of anti-herbivore strategies. Leaves of *H. sabdariffa* lacked prominent non-glandular structures, perhaps focusing resources on phytochemical lines of defence. González *et al*. (2008) examined the effects of herbivory and water restriction on the trichome density of leaves of *Madia sativa*. They showed that trichome density, as a whole, was affected under conditions of leaf damage and water shortage. Glandular trichome density increased only under conditions of leaf damage when water was controlled for whereas an increase in non-glandular trichome density was observed with leaf damage only under experimentally induced drought conditions (González *et al*., 2008). This is an indication that the induction of non-glandular trichomes required higher levels of stressful factors than that required for the promotion of glandular trichomes and might suggest that ecological factors drive the development of non-glandular and glandular trichome differentiation and development on leaves of *H, surattensis* and *H. sabdariffa*.

5.3.3 CAPITATE TRICHOME HISTOCHEMISTRY

Chemical features of glandular trichomes of *H. surattensis* and *H. sabdariffa* displayed distinct histochemical reactions observed with light and fluorescence microscopy. The main secretory constituents identified were polysaccharidic, phenolic and alkaloidic for capitate trichomes of both *Hibiscus* species. This appeared to be consistent with other findings within the family Malvaceae for trichomes of nectaries (Rocha and Machado, 2009) and leaves (Garcia *et al*., 2014). Trace amounts of lipids and proteins were also detected in head cells of *H. surattensis* capitate trichomes whilst those of *H. sabdariffa* demonstrated a stronger presence of proteinaceous substances. In nectary trichomes of *H. rosa-sinensis* L., the weak, positive detection of lipid components were attributed to ER elements and associated membrane activities that occurred during secretory processes (Sawidis, 1991). The most prominent reactions detected in nectary trichomes of *H. pernambucenis* Arruda and *H. rosa-sinensis* confirmed high levels of polysaccharide compounds (Rocha and Machado, 2009; Sawidis, 1991). This was not the case for *H.surattensis* and *H. sabdariffa*. Polysaccharidic components did contribute largely to trichome head cells but more significant reactions were observed for acidic lipids, alkaloids and phenolics. This might be due to the functionality of the secretion in plant defence against herbivory for *H. surattensis* and *H. sabdariffa* rather than as an insect attractant as in the nectaries of *H. rosa-sinensis* and *H. pernambucenis* (Rocha and Machado, 2009; Sawidis, 1991).

The strong presence of phenolic compounds within head cells of capitate trichomes was further substantiated with autofluorescence microscopy. Similar observations were made for long stalked capitate trichome head cells of *Plectranthus ornatus* Codd (Ascensão *et al*., 1999) that fluoresced blue under UV light, indicating the presence of phenols. Fluorescence microscopy also revealed thickening or lignification of stalk cell sidewalls of capitate trichomes, perhaps providing structural support. Sawidis (1991) suggested that the sidewalls of clavate trichomes of *H. rosa-sinensis* which were impregnated with cutin-like material might be structurally suited for the promotion of symplastic transport of solutes, preventing cell-to-cell apoplastic backflow (Fahn, 1988). Cardoso-Gustavson & Davis (2014) attributed the autofluorescence of trichome sidewalls in nectaries to cutinised stalk cells which also contained lipophilic impregnations in lateral walls. They showed that thickened anticlinal sidewalls of the stalk cells appeared to prevent significant reabsorption of nectar, restricting reabsorption to the trichomes. This appeared to be important since foliar trichomes also exhibited similar anticlinal thickening of trichome sidewalls, perhaps ensuring the unidirectional flow of secretions or secretory precursors from the stalk to the cells of the head as well as preventing the backflow of exudate from the head to stalks cells.

5.3.4 GLANDULAR TRICHOME ULTRASTRUCTURE

Ultrastructural comparisons of capitate trichomes between *H. surattensis* and *H. sabdariffa* yielded many similarities and differences in terms of cellular composition and organisation at the secretory phase.

The presence of dense porous vacuolar material deposited along the inner peripheral membrane and within the vacuole appeared to be a distinct feature of capitate trichomes of *H. surattensis*. A similar observation was previously reported for peltate trichomes of Finnish birch species (Valkama *et al*., 2003) where the electron dense material appeared to form whorl-like arrangements within vacuoles and thought to likely be phenolic in nature (Valkama *et al*., 2003). It was also observed to occur exclusively in vacuoles and was not located within secretory vesicles or the periplasmic or subcuticular spaces which suggested that this substance might have only accumulated within trichome head cells to serve as a deterrent if the trichome is ruptured by mechanical or physical means (Valkama *et al*., 2003); as it might be the case for capitate trichomes of *H. surattensis*.

Valkama *et al.* (2003) also reported on the presence of electron dense droplets contained within vacuoles in close association with myelin-like membranous structures. A similar observation is made for head cells of *H. surattensis* where lamellated material was often seen within vacuoles

surrounding the porous electron-dense vacuolar material. This appeared to suggest a secretory role for this organellar arrangement; as previously described for trichomes of Finnish birch (Valkama *et al*., 2003) and *H. rosa-sinensis* (Sawidis, 1991). Within the head cells of trichomes of Finnish birch, lamellated material appeared as large aggregations of a membranous nature. They also occurred as smaller structures located between the plasmamembrane and cell wall (Valkama *et al*., 2003). Myelin-like lamellar material was also identified in nectary trichomes of *H. rosa-sinensis* but they were located in stalk cells rather than the head cells as in trichomes of *H. surattensis* (Sawidis, 1991). Due to the high electron density of these structures aggregated single lamellae were difficult to distinguish unless they occurred singly (Valkama *et al*., 2003). Lamellar bodies in animal cells are well studied cytological subjects, and their functions include the maintenance and provision of lipidic or lipid-associated material for specialized secretory purposes within cells (Schmitz and Müller, 1991). They function closely in conjunction with Golgi bodies, ER and mitochondria (Schmitz and Müller, 1991). Their role in the capitate trichomes of *H. surattensis* is assumed to be involved in lipid recycling, and vacuolar and vesicular development.

Trichomes of *H. sabdariffa*, however, demonstrated high vacuolation in head cells with an amorphous electron dense substance which occured within scattered vacuoles. Trichomes of *H. sabdariffa* exhibited electron dense material within vacuoles, vesicles and the subcuticular and periplasmic spaces, suggesting that this accumulated substance is synthesized for secretion. For *H. surattensis,* cell wall microfibril loosening was observed at the point at which the cuticle separated from the cell wall. An amorphous secreted material was observed within the space just below (Fig 11 g). Therefore, it appeared that secreted material traversed through parts of the loosened cell wall and into the subcuticular space by osmotic pressure within head cells. The exact mechanisms of secretion synthesis appear unclear at present but it is thought that Golgi bodies and endoplasmic reticulum cisternae might be involved as they appeared to occur around the inner edges of all head cell plasmamembranes. Secretory vesicles containing anything other than electron-dense material were sparse. Active secretion into a subcuticular or periplasmic space is a well-known trait of many secretory trichomes and has been previously observed in *Ceratotheca triloba*, *Cannabis sativa*, *Ocimum obovatum* as well as in various members of Lamiaceae (Naidoo *et al*., 2012; Hammond and Mahlberg, 1978; Naidoo *et al*., 2013; Ascensão and Pais, 1998; Huang *et al*., 2008).

5.4 MUCILAGE CELLS AND DUCTS

Epidermal mucilage cells and mucilage conducting ducts were identified in both species of *Hibiscus* investigated. Their presence was established by structural and histochemical dissimilarities from surrounding leaf tissue.

Typically, both mucilage cells and ducts were stained with Toluidine blue during semi-thin sectioning for TEM. This confirmed the presence of acidic polysaccharides within secretions of these specialised cells. Since the significance of acidic polysaccharides is not known within these tissues, further investigation is recommended. SEM imaging revealed the topographical morphology of epidermal mucilage cells which has not been reported on. The surface view of mucilage epidermal cells displayed significant invagination of the cuticle, with surrounding epidermal cells which were radially arranged. These structures exhibited similar features to those observed by Carpenter (2006) on leaves of fossilized basal angiosperms. He described hydrapote, glandular trichome, mucilage hair and oil cell topography on the leaf landscape which comprised of radially arranged epidermal cell apices converging to a specialised cell at the centre. The presence of mucilage cells within *Hibiscus* has been well established in fruit (okra - *Abelmoschus esculentus* (L.) Moench [syn. *Hibiscus esculentus* L*,* Fahn, 1989) and leaves (*H. pernambucenis*: Rocha and Machado, 2009 and *H. rosa-sinensis*: Sawidis, 1991) but this study, to the knowledge of the author, is the first to report on histochemical reactions and fine structure as well as the density of mucilage idioblasts across different foliar developmental stages.

5.4.1 MUCILAGE IDIOBLAST HISTOCHEMISTRY

Histochemical observations of mucilage idioblasts of *H. surattensis* revealed that mucilage composition was mostly polysaccharidic in nature with traces of lipid, phenolic, alkaloid and protinaceous components. Reports on foliar mucilage idioblasts within Malvaceae mainly considered metachromatic staining of mucilage, and highlighted the presence and localization of polysaccharidic compounds within mucilage idioblasts (Martini *et al.,* 2008, Garcia *et al*., 2014) but few authors explored a spectrum of tests to consider the presence of further chemical classes. The importance of wider histochemical testing of mucilage idioblasts was demonstrated by Bosabalidis (2014) with the Lamiaceaous species *Teucrium polium* L. Mesophyllous mucilage idioblasts of *Teucrium polium* produced exudate of a glycoproteinaceous nature, staining positively with both PAS and Coomasie Brilliant Blue. Staining with Sudan Black B yielded negative results, eliminating the possible presence of lipidic compounds (Bosabalidis, 2014) as similarly observed for *H. surattensis* and *H. sabdariffa*.

5.4.2 DENSITY OF MUCILAGE CELLS ON LEAVES OF *H. Surattensis*

In contrast to the decreasing trend of capitate trichome density, foliar developmental progression was marked by increasing numbers of mucilage cells, particularly on the adaxial surfaces of all ontogenetic stages. This trade-off between capitate trichome and mucilage cell development towards the latter developmental stages in leaf ontogeny is assumed to signify a similar relationship between defence strategies offered by capitate and non-glandular trichomes (discussed above, González *et al.*, 2008).

5.4.3 MUCILAGE DUCT ULTRASTRUCTURE

Ducts of *H. surattensis* and *H. sabdariffa* displayed very similar ultrastructural findings. They consisted of cells which are arranged in a concentric manner surrounding an extracellular lumen (De Aguiar-Dias and Cardoso-Gustavson -Dias, 2011; Pickard, 2007). It is suggested, from the size and shape of the lumen, as well as the lack of flattened epithelial cells, that lysigenous breakdown of a central column of cells is assumed to be responsible for the formation of the duct (Pickard, 2007). Other findings of ducts examined by De Aguiar-Dias and Cardoso-Gustavson -Dias (2011) of *Polygala angulata* DC. showed that schizolysigenous processes governed duct development in stem initials. Foliar duct epithelial cells of *H. surattensis* appeared to contain mostly vacuole, with no plastids or few distinct organelles; however, they showed activity at the peripheral cytoplasmic regions. Vesicle formation and shuttling were observed at the duct facing edge of epithelial cells, indicating that mucilage or mucilage precursors were transported to the duct via exocytosis (Battey *et al*., 1999). Further investigation and exploration of stem conducting tissue are needed to elaborate on the current findings on *H. surattensis* and *H. sabdariffa* mucilage ducts.

5.4.4 FUNCTION OF MUCILAGE IN HIGHER PLANTS

Mucilage in plants has been widely purported to provide insurance against severe water loss due to the high retention of water by polysaccharidic complexes (Fahn, 1989; Zimmerman *et al*., 2007; De Aguiar-Dias and Cardoso-Gustavson -Dias, 2011), thereby facilitating adaptive responses to environmental stress (Bosabalidis, 2014). As in xerophytic plants such as succulents (Landrum, 2002), mucilage-derived structures which are also play a role in trees in highly saline environments, functioning in xylem vessels and stomatal plugs to draw up and retain soil-borne and air-borne water within these plants for longer periods (Zimmerman *et al*., 2007). Futhermore, mucopolysaccharides are reported to be thermodynamically compelled to reduce the activity of water (Zimmerman *et al.,* 2007), thus controlling and regulating water mechanics in respective plant species. It is assumed that within xylem, they played an important

role in water lifting, and have been shown to be as effective in creating osmotic gradients as those created by low-molecular weight solutes in classic osmotic pressure models (Zimmerman *et al*., 2007). The present research detected duct channels running alongside foliar conducting tissue in both study species, but the duct channel association with xylem and/or phloem is still to be determined. *Hibiscus surattensis* and *H. sabdariffa* both grow in tropical habitats with variable water availability and environmental conditions, but the prominence of mucilage ducts and idioblasts might signify adaptation to intermittent xeric conditions. Bosalidis (2014) also suggested that mucilage in plants might serve an important role in coping with heat or drought stress as well as cold stress, by causing water to be less susceptible to evaporation in hot dry environments and lowering the freezing point of water during winter.

5.5 CRYSTAL IDIOBLASTS

In addition to mucilage ducts and cells, other significant internally located secretory structures observed within the species investigated were calcium oxalate crystals. Their prevalence in the tissues of leaves of both study species, are attributed to specialised idioblasts, which differentiated from regular mesophyll cells, as described by Webb (1999). In addition, it has been shown that intravacuolar formation of crystal aggregates within these idioblasts (Webb, 1999), is the primary mechanism of crystal formation in modern angiosperms. Using TEM the present study was able to examine development of crystal idioblasts within leaves of *H. surattensis*, and found that idioblasts were ultrastructurally distinct from surrounding mesophyll tissue upon differentiation. Including the decline in chloroplast numbers, numerous organellar processes were observed, perhaps developing the necessary cellular mechanics preceding crystal formation. Webb (1999) reported on the formation of membraneous crystal chambers within the vacuole that separated and controlled crystal formation, independently from regular vacuolar function. The formation of such crystal chambers were indistinguishable as the scars in the resin of ultrathin sections created by the crystals might have prevented their imaging in cells where crystal formation had commenced. This could be alleviated with fixation of plant material in potassium permanganate to preserve membraneous crystal chambers (Webb, 1999). In idioblasts of *H. surattensis* where crystal nucleation had not yet been initiated, compartmentalisation of the vacuole was observed (Figure 17b). It is not known whether that led to the development of specific chambers responsible for crystal nucleation and formation.

Despite there being a vast wealth of knowledge of crystal development in higher plants, there is no clear evidence of the mechanisms of calcium and oxalate deposition within crystal cells (Webb, 1999), however Monje and Baran (2002) argued that the presence of proton pumps and

ion channels regulated crystal formation through a tightly genetically controlled process and that crystal type, shape and size was not randomly achieved. It is also thought that increasing levels of calcium within plants might promote the differentiation of mesophyll into idioblasts as a means to eliminate excess calcium from the plant body (Webb, 1999; Monje and Baran, 2002), but their role in plants is speculated to be involved in the regulation of pH, plant defence, gravi-perception, mechanical support and water retention strategies (Monje and Baran, 2002). Further research into calcium oxalate crystals might point to evidence of multileveled strategies of excretion, secretion, defence and regulation of these structures within plants.

5.6 PHYTOCHEMICAL FINDINGS AND ETHNOPHARMACOLOGICAL IMPLICATIONS

Hibiscus sabdariffa is a well-known medicinal and pharmacological species used in Indian and Chinese medicinal preparations. Various studies discussed the phytochemical and related pharmacological properties of extracts of *H. sabdariffa* calyces (Garg *et al*., 2012; Ochani and D'Mello, 2009; Sarkar *et al*., 2014; Yang *et al*., 2012; Jovan *et al*., 2014; Linn *et al*., 2011). Phytochemical testing carried out on leaf extracts aimed to determine if *H. surattensis* might possess similar chemical constituents to *H. sabdariffa* and therefore potentially possess some of the pharmacological principles demonstrated by *H. sabdariffa*. Our findings showed that carbohydrates, phenolics, alkaloids, flavones and flavanones, fixed oils and fats were detectable in leaf fractions of *H. sabdariffa* and *H. surattensis*, whereas proteins and saponins were undetectable. Sterols were found to positively react in extracts of *H. surattensis* but did not occur in *H. sabdariffa*. The similarity of the foliar phytochemical signatures of these two related species permits further research into extracts of *H. surattensis*, preferably along the current methods utilized for *H. sabdariffa*. It is expected that leaves of *H. surattensis* will demonstrate similar biological and chemical activities to that of *H. sabdariffa* if not greater reactivity.

Comparisons of essential oil composition between leaves of *H. surattensis* and calyces of *H.* sabdariffa were considered by Inikpi *et al.* (2014). *Hibiscus sabdariffa* calyces yielded high quantities of fatty acids, especially hexadecanoic acid (64.3%) and linoleic acid (22.7%). Leaves of *H. surattensis* consisted mainly of β-caryophyllene (12. 9%), menthol (10.6%), methyl salicylate (9.7%), camphor (9.2%) and lower quantities of hexadecanoic acid (4.3%) (Ogundajo *et al*., 2014). Advanced phytochemical comparisons of ethanolic and aqueous fractions of the leaf extracts are required as well as comprehensive investigations into compound isolation and bioactivity. The exploration of medicinal activity among related plant taxa has previously yielded positive outcomes (reserpine, Gurib-Fakhim, 2006; hibiscus-specific

compounds, Maghanha *et al*., 2010) and should be investigated more vigorously for potential drug candidates.

Biotechnological advances have allowed for greater methods of discovery and exploration within the field of medicinal plant biology. From utilizing and manipulating glandular trichomes as biofactories for optimized production of economically important phytocompounds (Schilmiller *et al*., 2008) to improving drug discovery strategies through wide scale screening and processing practices (Brusotti *et al*., 2014). The future of phytomedicinal research will now rely heavily on advancing technologies and practices culminating from various disciplines of science. Data presented here might form the basis of a comprehensive ethnopharmacological survey into *H. surattensis*, with the potential of isolating compounds or compound classes that are responsible for the traditional reputation of this species. Species decline in South Africa is a factor that will lead to long term detrimental ecological impacts, and current utilization patterns of ethnobotanical species will exacerbate predicted trends. The concept of species substitution for medicinal usage is not novel; however, such changes in practices might influence the successful conservation of severely threatened species.

5.7 FUTURE WORK

Further investigations of secretory tissues within *H. surattensis* and *H. sabdariffa* might include ultracytochemical techniques to detect specific organelles and pathways of secretion synthesis. Identification of such pathways might open new avenues to engineering secretory processes and structures for biotechnological or pharmaceutical applications. The use of ultracytochemistry in the elucidation of secretory mechanisms, especially for plant trichomes, is not novel. In 1988, Meyberg identified mucilaginous secretions emanating from glandular trichomes of *Nymphoides peltata* emergent leaves. Using ultrastructural and ultracytochemical techniques Meyberg (1988) was not only able to implicate the RER cisternae and Golgi Body in synthesis of protein-polysaccharide components, but was also able to detect that Golgi vesicles furthest from the *cis*-cisternae stained more intensely for mucilaginous contents, thereby identifying secretory vesicles at varying stages of progression. In 2006, Liao *et al.* elucidated the cytological pathway of aloin in mature leaves of *Aloe arborescens,* which is an important anthraquinone that contributes to the medicinal activity of this species. Labelling with lead acetate, cytological and ultracytostructural observations showed aloin was produced by plastids in assimilating tissues in the leaf but transported to vascular bundle sheath cells. Our research group has previously employed methods of ultracytochemistry and was able to describe secretory processes of *Bulbine inflata* staminal hairs with greater detail and understanding than

ultrastructural methods alone (Naidoo *et al*., 2011). It is therefore intended that the elucidation of secretory processes in glandular trichomes, and possibly mucilage idioblasts, of *H. surattensis* be investigated using these techniques together with further cytochemical labelling methods.

Current high resolution microscopy techniques such as super resolution fluorescence microscopy and cryo high resolution scanning electron microscopy, have led to researchers now being able to visualise previously undetectable interactions of tissues, as well as chemical principles at the atomic level which has been instrumental in advancing the present understanding of drug delivery mechanisms. Using high resolution cryo-SEM, a recent study described the structure of the protein, beta-galactosidase, which is a bacterial enzyme that binds to the inhibitory drug, phenylethyl-beta-D-thiogalactopyranoside (PETG) (Bartesaghi *et al*, 2015). According to the National Health Institute (NHI, 2015), researchers were able to derive angstrom level resolution and accurately describe the mechanisms of action of the enzyme, thereby aiding in drug design and execution. The use of super resolution fluorescence microscopy has greatly improved research in the health sciences, and its application in plant sciences is generating considerable interest. Nuclear microprobe analysis also allows for the elemental mapping of biological structures at the microscopic level allowing for the elucidation of chemical localisation within complex secretory tissues.

The author believes that biological and chemical evaluations of extracts of *H. surattensis* will contribute to understanding the reported ethnomedicinal applications of this species in the future. Tests of antimicrobial, antioxidant, antianalgesic and anti-inflammatory properties need to be conducted to validate the wound healing, anti-malarial and anti-hypertensive effects of *H. surattensis*. Advanced phytochemical comparisons between *H. surattensis* and *H. sabdariffa* could lead to investigations of biological and chemical activity that might emulate findings of *H. sabdariffa* as this species has a number of widely reported pharmaceutical and biological applications that are currently being explored. Furthermore, the author believes that nanotechnology involving the green synthesis of nanoparticles from botanically derived extracts is a trending area of interest. The synthesis of silver nanoparticles from aqueous extracts of *H. sabdariffa* has already been successfully achieved (Kumar *et al*., 2014). Nanoparticles demonstrated both anti-microbial and environmental heavy metal detection properties. Both *H. surattensis* and *H. sabdariffa* are annual shrubs that die off during winter months. The exploration of tissue culture techniques to propagate these species will ensure the availability of

plant material for scientific investigation as well as explore potential optimization techniques for commercial uses throughout the year.

In conclusion, the data obtained from this study should be used to further investigations on *H. surattensis* as a potentially viable candidate for drug discovery methods. By isolating the drivers of medicinal characteristics of *H. surattensis*, these can be identified in plant species that are severely overharvested for traditional uses, thereby confirming that *H. surattensis* might function as a substitute for threatened species in *muthi* practices.

It is therefore recommended that *H. surattensis*, due to its great ethnobotanical reputation and wide distribution, be evaluated as a species substitute for threatened species exhibiting similar activity, to curb harvesting pressure on severely threatened medicinal flora.

CHAPTER 6: CONCLUSION

In recent decades, medicinal plant biology has made bold leaps in terms of bettering screening and isolation methods of potential drug principles, as well as developing biotechnological techniques to optimize compound output and quality. Studies focusing on evaluating species with pharmaceutical potential, however, should also focus efforts on the structures that synthesize phytochemicals of interest, as well as ecological roles for which they developed, as this might lead to furthering biotechnological and phytomedicinal research efforts. In many plants, trichomes are seen as the biofactories that produce important secondary compounds with a range of beneficial applications, and currently a number of researchers are involved in elucidating the metabolic pathways of exudate production. Also, trichome isolation and micropropagation of secretory tissues are receiving considerable attention as these techniques minimize production costs of active principles while ensuring sample purity and integrity.

The findings presented here demonstrated the diversity of both secretory and non-secretory foliar structures present on and within *Hibiscus surattensis* and *Hibiscus sabdariffa*. Capitate trichomes together with mucilage producing idioblasts and ducts formed the secretory component, demonstrating active cellular processes responsible for the production of exudates. Together with ER cisternae and Golgi Bodies, lamellar bodies were also implicated in secretion synthesis and accumulation in capitate trichomes of *H. surattensis*, and histochemical tests detected the presence of polysaccharidic, alkaloid and phenolic compounds in trichome head cells. Glandular capitate trichomes of *H. sabdariffa*, exhibited a higher intensity of protein and alkaloid staining, and ultrastructural findings indicated that high levels of vesiculation together with compartmentalisation of trichome head cells characterised the secretory phase of development. Mucilage idioblasts of *H. surattensis* stained intensely for acidic polysaccharides, and marginally for various other compounds. They occurred primarily on the adaxial surfaces of leaves at the mature stage of development. Ducts synthesising and translocating mucilage in the leaf, comprised of a lining of epithelial cells with vesicle formation which occurred at the duct facing edge. Mucilage synthesis and accumulation has been noted in various other plants with water regulation strategies (Zimmerman *et al*., 2007), implying that mucilage might increase the chances for survival under reduced water conditions. Non-glandular trichomes were identified for *H. surattensis*. They included falcate, stellate and prickles which were rigid structures found on both abaxial and adaxial leaf surfaces. In contrast, non-glandular trichomes of *H. sabdariffa* were not rigid, and consisted on falcate, bi- and trifurcate types. They were less apparent on the leaf lamina but more pronounced at the leaf base. It appears that for *Hibiscus surattensis*,

numerous measures of plant defence have been put in place, both chemical and mechanical. *Hibiscus sabdariffa*, in contrast, lacked rigid thorny structures that conferred mechanical defence, but did possess a number of chemical constituents present within secretory structures, that might assist in defence mechanisms. The medicinal reputations of the species studied here are built on their wide cultural and traditional uses across many countries. Advancing research into the production and potential of the phytochemicals that confer the medicinal properties of these species might assist in furthering drug discovery efforts while expanding on conservation strategies to ameliorate the harvesting pressure on medicinal species that are in decline.

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APPENDIX

Table 1: Phytochemical tests on Methanolic extracts of *Hibiscus surattensis* and *Hibiscus sabdariffa*:

Phytochemical	Test	*H. sabdariffa*	*H. surattensis*	Description
Alkaloids	Draggendorf	- +	- +	*H. sabdariffa:* white precipitate. *H. surattensis:* Murky green with precipitate.
	Hagers	- +	- +	*H. sabdariffa:* green yellow precipitate. *H. surattensis:* Murky green.
	Wagners	-	- +	*H. sabdariffa:* green precipitate. *H. surattensis:* formation of yellow layer at top.
Carbohydrates and glycosides	Molisch's	- +	- +	*H. sabdariffa:* Red solution below, brown precipitate above. *H. surattensis:* Light green ring, overall dark solution.
	Fehling's	- +	+ +	*H. sabdariffa:* green bulky precipitate. *H. surattensis:* Green precipitate in green solution.
	Benedict's	+ +	+ +	*H. sabdariffa:* yellow precipitate in green solution. *H. surattensis:* Green precipitate.
Proteins	Ninhydrin	-	-	*H. sabdariffa:* no change. *H. surattensis:* no change.
Sterols	Salkowski's	-	+ +	*H. sabdariffa:* no change. *H. surattensis:* Ring at the top of solution and green ring in the middle.
Fixed oils and fats	Spot	- +	- +	*H. sabdariffa:* green spot. *H. surattensis:* green spot.
Phenolic compounds and Tannin	Ferric chloride	+ +	+ +	*H. sabdariffa:* dark green to black solution. *H. surattensis:* solution turned black.
	Lead acetate	+ +	+ +	*H. sabdariffa:* Bulky white precipitate. *H. surattensis:* Bulky white precipitate.
Saponins	Foam	-	-	*H. sabdariffa:* no formation of foam. *H. surattensis:* no formation of foam.
Gums and Mucilage	Precipitation	-	-	*H. sabdariffa:* no precipitate. *H. surattensis:* no precipitate.
	Ruthenium Red	- +	- +	*H. sabdariffa:* solution changed lighter green. *H. surattensis:* Solution turned darker.
Flavones and Flavonones	Aqueous sodium hydroxide	+ +	+ +	*H. sabdariffa:* orange colour change. *H. surattensis:* Green-orange precipitate.
	Conc. Sulphuric acid	-	- +	*H. sabdariffa:* no colour change. *H. surattensis:* Deep green.

- Indicates negative; - + indicates weakly positive; + + indicates positive.

Table 2: Phytochemical tests on Chloroform extracts of *Hibiscus surattensis* and *Hibiscus sabdariffa.*

Phytochemical	Test	*H. sabdariffa*	*H. surattensis*	Description
Alkaloids	Draggendorf	- +	- +	*H. sabdariffa:* orange solution, orange layer above. *H. surattensis:* orange solution below reddish ring.
	Hagers	- +	- +	*H. sabdariffa:* yellow layer above. *H. surattensis:* yellow layer above.
	Wagners	+ +	- +	*H. sabdariffa:* brown precipitate. *H. surattensis:* Peach coloured solution with orange droplets suspended.
Carbohydrates and glycosides	Molisch's	+ +	+ +	*H. sabdariffa:* Red solution above, separated from clear bottom layer by brownish precipitate. *H. surattensis:* Deep burgundy solution above a brownish ring.
	Fehling's	+ +	+ +	*H. sabdariffa:* red precipitate in upper layer. *H. surattensis:* yellow and green precipitate above murky layer.
	Benedict's	-	-	*H. sabdariffa:* blue solution. *H. surattensis:* greenish-blue solution.
Proteins	Ninhydrin	-	-	*H. sabdariffa:* no change. *H. surattensis:* no change.
Sterols	Salkowski's	-	-	*H. sabdariffa:* no change. *H. surattensis:* no change.
Fixed oils and fats	Spot	-	-	*H. sabdariffa:* no spot. *H. surattensis:* no spot.
Phenolic compounds and Tannin	Ferric chloride	-	-	*H. sabdariffa:* no reaction. *H. surattensis:* no reaction.
	Lead acetate	++	-	*H. sabdariffa:* Bulky white precipitate. *H. surattensis:* no reaction.
Saponins	Foam	-	-	*H. sabdariffa:* no formation of foam. *H. surattensis:* no formation of foam.
Gums and Mucilage	Precipitation	-	-	*H. sabdariffa:* no precipitate. *H. surattensis:* no precipitate.
	Ruthenium Red	-	-	*H. sabdariffa:* stain did not dissolve. *H. surattensis:* stain did not dissolve.
Flavones and Flavonones	Aqueous sodium hydroxide	- +	-	*H. sabdariffa:* clear layer above slight murky layer. *H. surattensis:* no reaction.
	Conc. Sulphuric acid	++	- +	*H. sabdariffa:* yellow precipitate in yellow solution. *H. surattensis:* formation of orange layer below light orange solution.

- Indicates negative; - + indicates weakly positive; + + indicates positive.

Table 3: Phytochemical tests on Hexane extracts of *Hibiscus surattensis* and *Hibiscus sabdariffa.*

Phytochemical	Test	*H. sabdariffa*	*H. surattensis*	Description
Alkaloids	Draggendorf	- +	- +	*H. sabdariffa:* pink solution, orange droplets at the bottom. *H. surattensis:* pink solution with red bottom layer.
	Hagers	- +	-	*H. sabdariffa:* yellow layer below. *H. surattensis:* no reaction.
	Wagners	+ +	- +	*H. sabdariffa:* brown precipitate at bottom. *H. surattensis:* pink coloured solution with orange droplets suspended.
Carbohydrates and glycosides	Molisch's	+ +	+ +	*H. sabdariffa:* formation of black precipitate below orange solution. *H. surattensis:* formation of black precipitate below orange solution.
	Fehling's	+ +	+ +	*H. sabdariffa:* formation of blue precipitate below blue solution. *H. surattensis:* formation of blue precipitate below blue solution.
	Benedict's	-	-	*H. sabdariffa:* green blue solution. *H. surattensis:* bright blue solution.
Proteins	Ninhydrin	-	-	*H. sabdariffa:* no change. *H. surattensis:* no change.
Sterols	Salkowski's	-	-	*H. sabdariffa:* no change. *H. surattensis:* no change.
Fixed oils and fats	Spot	-	-	*H. sabdariffa:* no spot. *H. surattensis:* no spot.
Phenolic compounds and Tannin	Ferric chloride	-	-	*H. sabdariffa:* no reaction. *H. surattensis:* no reaction.
	Lead acetate	-	-	*H. sabdariffa:* no reaction. *H. surattensis:* no reaction.
Saponins	Foam	-	-	*H. sabdariffa:* no formation of foam. *H. surattensis:* no formation of foam.
Gums and Mucilage	Precipitation	-	-	*H. sabdariffa:* no precipitate. *H. surattensis:* no precipitate.
	Ruthenium Red	-	-	*H. sabdariffa:* stain did not dissolve. *H. surattensis:* stain did not dissolve.
Flavones and Flavonones	Aqueous sodium hydroxide	-	-	*H. sabdariffa:* no reaction. *H. surattensis:* no reaction.
	Conc. Sulphuric acid	-	- +	*H. sabdariffa:* formation of light yellow layer below light clear solution. *H. surattensis:* formation of yellow layer below light clear solution.

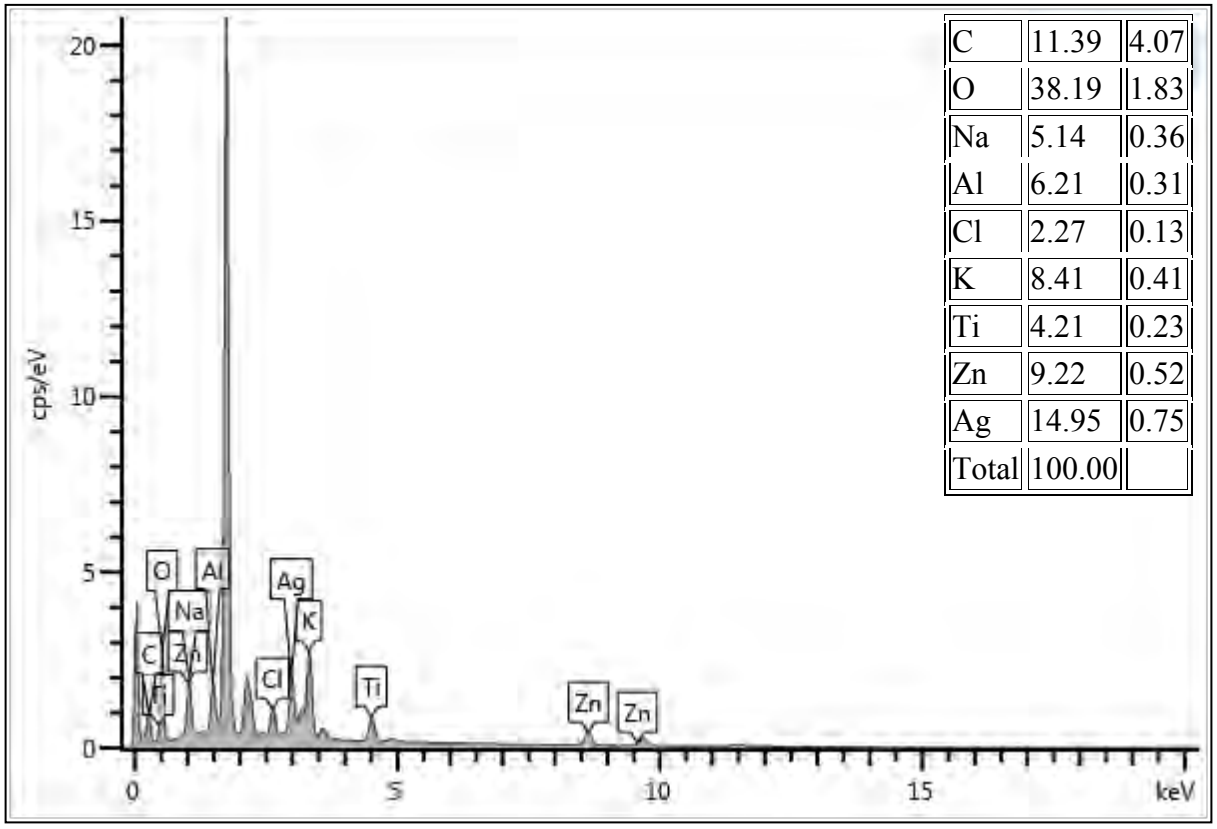
- Indicates negative; - + indicates weakly positive; + + indicates positive.

Table 4: Histochemical tests for chemical localisation in the foliar trichomes of *Hibiscus surattensis.*

Phytochemical	Histochemical Test	*Hibiscus sabdariffa*	Description
Alkaloids	Wagners and Dittmar		➢ Glandular trichomes: intense orange staining of whole trichome. ➢ Mucilage cells: mostly unstained, the apex of some stained faint orange. ➢ Non-glandular trichomes: faint staining. ➢ Entire tissue stained light brown-orange.
Proteins	Mercuric bromophenol blue	- +	➢ Glandular trichomes: some trichome heads retained original colouration whereas others stained slightly. ➢ Mucilage cells: remained largely unstained, but few showed staining of cellular material within. ➢ Non-glandular trichomes: showed no significant staining. ➢ Tissue stained intense blue.
Lipids	Sudan III&IV	- +	➢ Glandular trichomes: stalk and cuticle of trichome stained light orange. ➢ Mucilage cells: apex showed marginal orange staining. ➢ Non-glandular trichomes: remained mostly unstained. ➢ Tissue showed partial orange staining mostly in the epidermal cells and cuticle.
	Sudan Black B		➢ Glandular trichomes: some heads stained blue. ➢ Mucilage cells: remained mostly unstained but showed some staining of cellular components. ➢ Non-glandular trichomes: Basal cells stained intensely. ➢ Tissue showed intense staining.
	Nile Blue	+ +	➢ Glandular trichomes: Glandular heads show blue staining. ➢ Mucilage cells: stained intense dark blue. ➢ Falcate trichomes appear mostly unstained.
Viability	Acridine orange		➢ Nuclei intensely stained.
Polysaccharides and Mucilage	Ruthenium red	+ +	➢ Glandular trichomes: Glandular heads show distinct staining of certain intracellular components. Basal cell stains deeply. ➢ Mucilage cells: Mucilage cells stain positively and strongly for the presence of pectins and polysaccharides (acidic). ➢ Ruthenium red stains stellate trichomes very marginally, perhaps excess mucilage might have coated stellate prong which stained slightly red.
Ligin and cutin	Phloroglucinol	- +	➢ Glandular trichomes: Glandular trichomes do not stain. ➢ Mucilage cells: did not stain. ➢ Non-glandular trichomes: Falcate trichomes stain pink, indicating the presence of cutin.
Metachomatic staining	Toluidine blue	+ +	➢ Glandular trichomes: showed some purple blue staining but remained unstained mostly. Trichome base sometimes stains intense purple. ➢ Mucilage cells: Intense pink staining indicating the presence of acidic polysaccharides. Staining varied from intense to vaguely stained. ➢ Non-glandular trichomes: Some falcate trichome apexes stain bright blue
Phenolic compounds and Tannin	Ferric trichloride	+ +	➢ Glandular trichomes: Distinct dark staining within head cells. Stalk stained faint orange. ➢ Mucilage cells: slight orange staining. ➢ Non-Glandular trichomes: cuticle component of falcate trichomes stained red or faint orange. Content sometimes stained orange.
	Autofluorescence	+ +	➢ Glandular trichomes: stalk showed strong blue autofluorescence, whereas cuticle of head cells fluoresced marginally. Certain components of the head cells fluoresced faint green and red. ➢ Mucilage cells: overall faint blue fluorescence, with higher fluorescence at the apex of cell. ➢ Non-glandular trichomes: Cutin and lignin components fluoresced blue-green. ➢ Chlorophyllous tissue fluoresced red.

Table 5: Histochemical tests for chemical localisation in the foliar organs of *Hibiscus sabdariffa*.

Phytochemical	Histochemical Test	*Hibiscus sabdariffa*	Description
Alkaloids	Wagners and Dittmar	+ +	➢ Glandular trichomes: stained positively for the presence of alkaloids. ➢ Mucilage cells: Did not stain for the presence of alkaloids. ➢ Tissue also stained a lighter brown.
Proteins	Mercuric bromophenol blue	+ +	➢ Glandular trichomes: Contents stained slight blue. Some retained red/orange colouration. ➢ Mucilage cells: parts of cells stained deep blue. ➢ Tissue showed intense staining.
Lipids	Sudan III&IV	- +	➢ Glandular trichomes: showed strong orange colouring of parts of the trichome head indicating the presence of lipid inclusions. ➢ Mucilage cells: did not stain. ➢ Non-glandular trichomes: showed slight to no orange colouration. Slight orange might have signified the presence of cutin or suberin.
	Sudan Black B	- +	➢ Glandular trichomes: stalks stained deep blue but trichome heads remained their original brownish colour. Certain trichomes showed traces of blue within head cells. ➢ Mucilage cells: show traces of blue. ➢ Tissue stained dark blue.
	Nile Blue	+ +	➢ Glandular Trichomes stained blue indicating the presence of fatty acids or phospholipids. Some glandular trichomes remained orange/brown. ➢ Mucilage cells: stain deep blue indicating the strong presence of acidic lipids. ➢ Mesophyll retained characteristic green shade, however tinged with blue stain.
Viability	Acridine orange		➢ Nuclei intensely stained.
Polysaccharides and Mucilage	Ruthenium red	+ +	➢ Glandular trichomes: showed red staining throughout the trichome in some, whereas others showed certain cellular components staining red. ➢ Epidermal tissue stained red.
Ligin and cutin	Phloroglucinol	- +	➢ Glandular trichomes: stain brown. ➢ Mucilage cells: do not stain. ➢ Non-glandular trichomes: stained intense brown suggesting presence of cutin.
Metachomatic staining	Toluidine blue	+ +	➢ Glandular trichome: some stained intensely. Some glandular trichomes remained orange. ➢ Mucilage cells: stained a deep blue and purple indicating the presence of polyphenols or the phosphate groups on macromolecules such as nucleic acids. ➢ The cuticle and epidermal tissues also stain blue/purple while the inner palisade tissues remain green. In some the entire tissue stains.
Phenolic compounds and Tannin	Ferric trichloride		➢ Intense staining of trichome head cells.
	Autofluorescence	+ +	➢ Glandular trichomes: blue-ish green autofluroescence with the stalk autofluorescing intensely. Appears that's cuticle of trichome autofluoresces and that at post secretory phase, no autofluorescence is seen in the head. ➢ Mucilage cells: Mild to no autofluorescence, however, it appears certain mucilage cells do contain substances that fluoresce. The upper parts of mucilage cells show some fluorescence.



C	11.39	4.07
O	38.19	1.83
Na	5.14	0.36
Al	6.21	0.31
Cl	2.27	0.13
K	8.41	0.41
Ti	4.21	0.23
Zn	9.22	0.52
Ag	14.95	0.75
Total	100.00	

Figure 1: EDX spectrum of silver nanoparticles synthesized from aqueous extracts of *Hibiscus sabdariffa* as per Kumar *et al*. (2014).

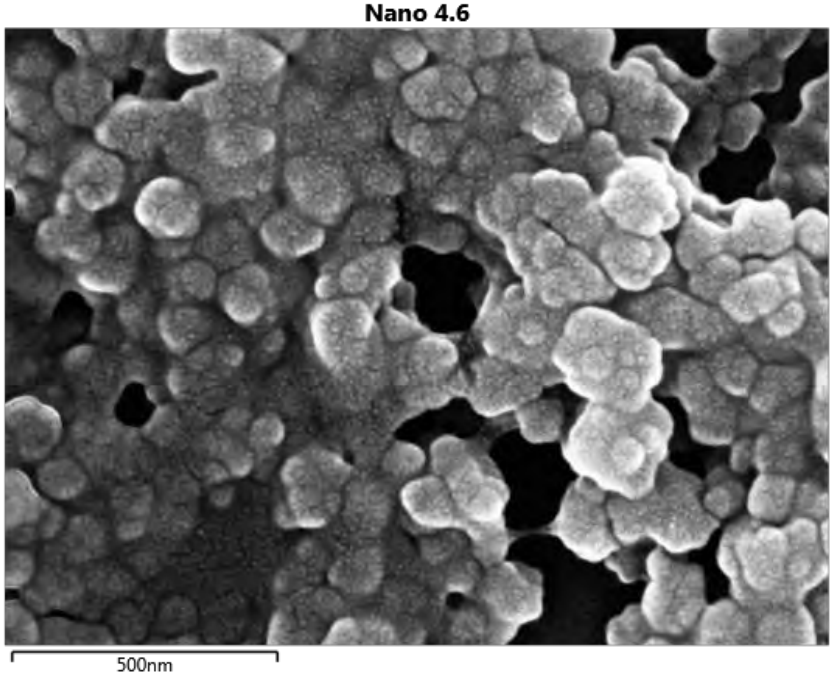


Figure 2: SEM of silver nanoparticles synthesized from aqueous extracts of *Hibiscus sabdariffa* as per Kumar *et al*. (2014).