Secretory Structures of *Croton gratissimus* Burch. var. *gratissimus* (Euphorbiaceae): Micromorphology and Histo-phytochemistry

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A research dissertation submitted in fulfilment of the academic requirements for the degree of Master of Science in Biological Sciences.

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As the candidate's supervisor(s) I have approved this dissertation for submission.

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The National Research Foundation (NRF) is acknowledged for financial assistance towards this research.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION: PLAGIARISM

I, **Danesha Naidoo**, declare that:

(i)   the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii)   this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii)   this dissertation does not contain other persons' data, pictures, graphs or

other information, unless specifically acknowledged as being sourced from other persons;

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(vi)   this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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ABSTRACT

Croton gratissimus Burch. variety (var.) *gratissimus* (Euphorbiaceae) has a widespread distribution in tropical Africa and is frequently used in African traditional medicine to treat various ailments. In South Africa, dried leaves of *C. gratissimus* are smoked to treat influenza, colds and fever. Due to its extensive use in traditional medicine, research on the phytochemical composition of *C. gratissimus* has been documented. According to literature, these phytochemicals are possibly secreted or accumulated in secretory structures. However, little or no research is available on the structures involved in the production and/or accumulation of phytochemicals in *C. gratissimus*. Therefore, this study aimed to describe the micromorphology of trichomes and laticifers from the leaves and stems of *C. gratissimus* as well as to identify the possible site of synthesis of phytochemicals. Furthermore, the chemical composition and antibacterial properties of phytochemicals in the leaves and stems were also determined. In addition, the antibacterial activity of biosynthesised silver nanoparticles (AgNPs) from leaf and stem crude extracts was also investigated. Microscopic investigations revealed the presence of lepidote and glandular trichomes, and non-articulated unbranched laticifers on/in the leaves and stems of *C. gratissimus*. The lepidote trichomes formed a dense indumentum over the abaxial surface of leaves throughout all developmental stages, canopying the underlying glandular trichomes. Laticifers were present in the leaves and stems and were predominantly associated with the vascular tissue in both organs. All structures stained positive for alkaloids, phenolic compounds and lipids with histochemical tests. Phytochemical analyses of the leaves and stems revealed alkaloids, amino acids, phenolic compounds, flavonoids, carbohydrates, terpenoids, saponins and fixed oils and fats in both leaf and stem extracts. The methanolic leaf and stem extracts demonstrated weak to strong activities against various bacteria strains, which are attributed to the several bioactive compounds identified from Gas Chromatography-Mass Spectrometry (GC-MS) analyses. In addition, AgNPs were successfully biosynthesised from the methanolic leaf and stem extracts. Particles synthesised from both extracts were spherical in shape, but their size distribution differed between organs. Antibacterial assays demonstrated a stronger activity of particles from leaf extracts compared to those from stems. These findings corroborate the use of *C. gratissimus* in traditional medicine and indicate that various structures are involved in the production of bioactive compounds which contribute to the medicinal properties of this plant. Furthermore, the antibacterial activities exhibited by the extracts and AgNPs suggest that *C. gratissimus* is a potential source of antibacterial agents.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Adj	Adjacent cells
Ag/Ag^+	Silver/silver ions
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
ANS	Anisaldehyde-sulphuric acid
C	Chloroplast
C_L/C_S	*Croton* leaves/stems
CW	Cell wall
DPPH	2,2-diphenyl-1-picrylhydrazyl
Dr/Pr	Druse/Prismatic crystals
EDX	Energy dispersive X-ray
ER/RER	Endoplasmic reticulum/rough endoplasmic reticulum
Fig.	Figure
FTIR	Fourier-transform infrared spectroscopy
GB	Golgi body
GC-MS	Gas chromatography-mass spectrometry
GT	Glandular trichomes
HDL	High density lipoproteins
HPLC	High performance liquid chromatography
Id	Idioblast
LB	Lipid body
LDL	Low density lipoproteins
Lt	Laticifers

LT	Lepidote trichome
M	Mitochondria
MIC	Minimum inhibitory concentration
MRSA	Methicillin-Resistance *Staphylococcus aureus*
N	Nucleus
OD$_{620}$	Optical density at 620 nm
R	Radial cell/radii
S	Stalk
s.l	*sensu lato*
s.s	*sensu stricto*
SD	Standard deviation
SEM/FEGSEM	Scanning electron microscopy/Field emission gun SEM
Sm	Stoma
SPR	Surface plasmon resonance
Sr	Subradial cell
ST	Stellate trichome
TEM/HRTEM	Transmission electron microscopy/High resolution TEM
TLC	Thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate
U	Umbo
UV/UV-B	Ultraviolet/ultraviolet-B
UV-VIS	Ultraviolet-visible
V	Vacuole
VLDL	Very low density lipoproteins
Vs	Vesicles

CHAPTER 1: INTRODUCTION

1.1 Medicinal plants and traditional medicine

Plants play an important role in the survival of mankind as they provide food, medicine and other products and services, either directly or indirectly (Terashima, 2001; Hawkins, 2008; Ahvazi et al., 2012; Corlett, 2016). However, the expanding human population has led to an increase in exploitation of resources, resulting in the endangerment of many species and populations. One of the most frequent groups exploited is plants as they are collected for medicinal trade (Hawkins, 2008). Globally, the number of angiosperms being utilised for their medicinal value ranges between 50000 – 80000. Unfortunately, many of these species are faced with the risk of extinction due to overharvesting and habitat destruction (Chen et al., 2016). It is therefore imperative to enforce conservation practices and sustainable use in order to preserve medicinal plant biodiversity (Okigbo et al., 2008).

The use of plants for medicinal purposes dates back to ancient times, around 4000 – 5000 B.C. (Hosseinzadeh et al., 2015). Medicinal plants are those that contain essential active ingredients that are utilised for the treatment of diseases and pains (Okigbo et al., 2008). These plants are the source of medicines that are safe, beneficial and affordable (Heamalatha et al., 2011), and they constitute an abundance of compounds (Okigbo et al., 2008). Over 10000 compounds are produced by plants as a defence against predators, with many of these having the potential to be drugs (Okigbo et al., 2008). Consequently, due to the frequent use by people in underdeveloped countries, medicinal plants form the backbone of traditional medicine around the world (Devi et al., 2012; Singh, 2015).

Traditional medicine can be defined as "the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" (World Health Organisation (WHO), 2018).

Many people in developing countries lack access to modern drugs and therefore depend on traditional medicine as a primary source of healthcare due to their easy accessibility and affordability (Heamalatha et al., 2011; Hosseinzadeh et al., 2015; Masevhe et al., 2015). In Africa, traditional healers exploit the rich plant diversity for various treatments, hence indigenous plants are the key component in African traditional medicine (Okigbo et al., 2008; Masevhe et al., 2015). In South Africa, the trade in traditional medicines is a huge industry, with about 27 million

consumers. However, the majority of the species traded for traditional medicines are harvested from wild populations, leading to a decrease in biodiversity (Mander, 1998; Petersen et al., 2017).

The use of traditional medicine has retained its popularity due to cultural beliefs and historical use (Masevhe et al., 2015; Singh, 2015). This knowledge of medicinal plants is passed on to each generation through verbal exchange (Masevhe et al., 2015; Boadu and Asase, 2017). However, the loss of biodiversity and cultural inheritance threaten the survival of this information. Therefore, it is crucial that this knowledge be documented in order to preserve this cultural inheritance for current and future generations to utilise (Boadu and Asase 2017). In addition, traditional knowledge serves as a precursor for the discovery of new drugs or bioactive compounds that can be used for treating illnesses (Farnsworth et al., 1985; Boadu and Asase, 2017). Globally, about 25% of prescription drugs contain plant-derived ingredients (Sen and Chakraborty, 2017). According to a study by Fabricant and Farnsworth (2001), there are 122 plant-derived compounds originating from only 94 plant species that are used as modern drugs worldwide. Of these compounds, 80% are currently used to treat the similar or same ailment as used traditionally (Fabricant and Farnsworth, 2001; Yuan et al., 2016). As medicinal plants are important sources of novel plant compounds and new drugs (Boadu and Asase, 2017), and with a global approximation of 250 000 flowering plant species, there are possibly many drugs still undiscovered (Fabricant and Farnsworth, 2001). Finally, the availability of this information on harvested plants used for various treatments in specific regions can increase biodiversity conservation. Large-scale harvesting of medicinal plants for commercial trade results in adverse effects on population sizes and recovery following harvesting. Therefore, in order to achieve conservation, the quantities harvested need to be known and documented to ensure that this resource is maintained for future generations (Boadu and Asase, 2017).

1.2 *Croton gratissimus* Burch. variety (var.) *gratissimus*

Croton gratissimus Burch. (syn. *C. zambesicus* Müll. Arg.; *C. microbotryus* Pax., *C. amabilis* Müell. Arg.) commonly known as lavender *Croton* or lavender fever berry, belongs to the family Euphorbiaceae. This species comprises of two varieties, namely *C. gratissimus* Burch. var. *gratissimus* and *C. gratissimus* Burch. var. *subgratissimus* (Curtis and Mannheimer, 2005; Mulholland et al., 2010; Robert et al., 2010; PlantZAfrica, 2018). The former variety is the focus of this study. *Croton gratissimus* is a Guineo-Congolese species with widespread distribution in tropical, central and sub-Saharan Africa. This species grows in dry and warm areas on stony/rocky slopes of hills throughout the north east of the continent from South Africa to the horn of Africa (Ngadjui et al., 2002; Block et al., 2004; Mulholland et al., 2010). In South Africa, this plant is distributed over a wide range, being indigenous to six provinces (Pudumo et al., 2018).

The genus *Croton* is of Greek origin, being a derivation of the word Kroton meaning "tick", whilst *gratissimus*, the species name, means most pleasing/pleasant, in Latin (PlantZAfrica, 2018; Pudumo et al., 2018). The name is suitable as a lavender-like aroma is produced by the leaves when crushed (Mulholland et al., 2010; Pudumo et al., 2018). It is a semi-deciduous shrub or tree that can reach heights of up to 10-15 m (Block et al., 2004; Boon, 2010; Mulholland et al., 2010). The slender shaped trees have a V-shaped crown which extends upwards with drooping foliage and terminal branches. The leaves are simple with an alternate arrangement. The adaxial surfaces of the leaves are shiny, dark green in colour and lack hairs. The abaxial surface appears silver with orange-brown specks due to the presence of dense scales (Boon, 2010; Mthethwa et al., 2014; PlantZAfrica, 2018). The trees are monoecious with terminal racemes that give rise to small cream to golden yellow flowers. The fruit is a small three-lobed capsule (Boon, 2010; PlantZAfrica, 2018; Pudumo et al., 2018).

Croton gratissimus is used extensively in traditional medicine to treat various illnesses, with the whole plant having a reputation of being medicinally important (Ngadjui et al., 2002; Van Vuuren and Viljoen, 2008). The organs of *C. gratissimus* are used either independently, in combination with other parts or plants or co-administered with different species for a wide range of treatments (Van Vuuren and Viljoen, 2008; Mulholland et al., 2010; Pudumo et al., 2018). Table 1.1 provides a summary of the uses of *C. gratissimus*.

Table 1.1: Medicinal uses of *Croton gratissimus*.

Part/s used	Country	Uses	Preparation	References
Bark	South Africa	As purgative for stomach and intestinal disorders	Milk infusions of bark	Mulholland et al., 2010; Mthethwa et al., 2014
		Unspecified uterine disorder	Powdered bark blown into the womb	Mulholland et al., 2010
		Pleurisy	Powdered bark rubbed into chest incisions	Mulholland et al., 2010
	Nigeria	Malaria	Bark infusions	Langat et al., 2011
	Unspecified	Bleeding gums, abdominal disorders, skin inflammation, earache, chest complaints	Unspecified	Van Vuuren and Viljoen, 2008; Pudumo et al., 2018
		Swelling	Combination of bark with root of Amaryllidaceae species applied into incisions	Van Vuuren and Viljoen, 2008
Leaves	South Africa	Sores associated with STI's	Steam baths	Van Vuuren and Naidoo, 2010
		Influenza, colds and fever	Dried leaves smoked	Mulholland et al., 2010; Langat et al., 2011
	Zimbabwe/Botswana	Cough	Smoke from leaves, leaf decoction/tea	Mulholland et al., 2010; Langat et al., 2011
	Benin/Nigeria	Hypertension, urinary infection (as anti-microbial), malaria, dysentery, diarrhoea, convulsions, antidiabetic remedy	Leaf decoction	Robert et al., 2010; Okokon et al., 2011; Abdalaziz et al., 2016; Kumar et al., 2017
	Unspecified	Restlessness and Insomnia	Paste made with ground leaves, two other *Croton* species and goat fat heated on coals and fumes inhaled.	Mulholland et al., 2010; Langat et al., 2011
		Eye disorders, rheumatism	Unspecified	Mulholland et al., 2010
Roots	Zimbabwe	Abdominal pains and aphrodisiac	Root infusions	Mulholland et al., 2010; Mthethwa et al., 2014
	Sudan	Menstrual pain and constipation	Unspecified	Robert et al., 2010; Kumar et al., 2017
Root and bark	Unspecified	Respiratory disorders	Combination of root and bark	Van Vuuren and Viljoen, 2008

Figure 1.1: *Croton gratissimus* var. *gratissimus* occurring in the University of KwaZulu-Natal - Westville Campus (29°49'08.1"S 30°56'38.9"E).

1.3 Rationale for this study

Croton gratissimus has been used extensively in African traditional medicine for a wide range of treatments. For this reason, many phytochemical investigations have been carried out on various parts of the plant to validate its therapeutic value (Okokon et al., 2006; Van Vuuren and Viljoen, 2008; Okokon and Nwafor, 2009; Okokon and Nwafor, 2010; Robert et al., 2010; Okokon et al., 2011; Mthethwa et al., 2014; Kumar et al., 2017). According to Fahn (1979), these phytochemicals are possibly synthesised or accumulated by secretory structures (Vitarelli et al., 2015). However, limited or no research has been conducted on the micromorphology and ultrastructure of the structures responsible for the synthesis, secretion and/or accumulation of phytochemicals in the leaves and stems of *C. gratissimus*. Therefore, this study focussed on

identifying and describing the micromorphology and ultrastructure of the trichomes and internal secretory structures of the leaves (adaxial and abaxial surfaces) and stems, as well as determining the chemical composition and possible site of synthesis of the phytochemicals. In addition, another aim of this study was to synthesise silver nanoparticles (AgNPs) from crude extracts of leaves and stems of *C. gratissimus*.

1.4 Research aims and objectives

The aims and objectives for each chapter are outlined below:

Chapter 3

Aim: To determine the micromorphology and distribution of trichomes and laticifers of the leaves and stems; and to detect the possible location of phytochemicals of *C. gratissimus* using various microscopy techniques.

Objective:

- To identify, describe and compare the micromorphology and distribution of trichomes on leaves (adaxial and abaxial surfaces), at three developmental stages (emergent, young and mature), and stems using stereomicroscopy, light microscopy and scanning electron microscopy (SEM).
- To identify and describe the laticifers within the leaves and stems using SEM and light microscopy.
- To determine the ultrastructure of lepidote trichomes using Transmission Electron Microscopy (TEM).
- Elucidate the location of chemical compounds using various histochemical tests.

Chapter 4

Aim: Determine the chemical composition of phytochemicals in the leaves and stems of *C. gratissimus* and test for antibacterial activity of the methanolic extracts.

Objectives:

- Determine the chemical composition of the phytochemicals in the leaves and stems by preliminary qualitative phytochemical screening, Gas Chromatography-Mass Spectrometry (GC-MS) analysis and Thin Layer Chromatography (TLC).
- Determine the biological activity of the crude extracts from leaves and stems by conducting antibacterial tests.

Chapter 5

Aim: Synthesise, characterise and test for antibacterial activity of silver nanoparticles (AgNPs) using methanolic extracts of *C. gratissimus.*

Objectives:

- Synthesise AgNPs from the crude extracts of *C. gratissimus*.
- Characterise synthesised AgNPs using ultraviolet-visible (UV-VIS) spectroscopy, energy-dispersive X-ray (EDX) analysis, TEM, and Fourier-transform infrared spectroscopy (FTIR).
- Test for biological activity of the synthesised AgNPs by conducting antibacterial tests.

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CHAPTER 2: LITERATURE REVIEW

2.1 Euphorbiaceae

Euphorbiaceae Juss. (Stevens and Davis, 2001), also known as the "Spurge family", is one of the largest angiosperm families with about 300 genera comprising of approximately 8000 species (Mwine and Damme, 2011; Rahman and Akter, 2013). Members in this family are diverse in habit, being large woody trees, shrubs, climbing lianas and even simple herbs (Mwine and Damme, 2011; Rahman and Akter, 2013). They have a widespread distribution across the globe (Fig. 2.1) consisting of old and new world species (Mwine and Damme, 2011). They are predominant in the tropics, with the bulk inhabiting the Indo-Malayan realm and tropical America (Rahman and Akter, 2013).

Figure 2.1: Worldwide distribution of Euphorbiaceous species (Source: Angiosperm Phylogeny Website http://www.mobot.org/MOBOT/research/APweb/).

Member of this family are either monoecious or dioecious species. They possess simple leaves with alternate arrangements. However, palmate leaves do occur in certain species. (Rahman and Akter, 2013). Stipules are occasionally reduced to hairs, spines or glands, but may be absent in succulent species (Rahman and Akter, 2013). Inflorescences are either spicate or cyathium, in which flowers with reduced parts (example, the calyx and corolla) form a pseudanthium (Richardson et al., 1987). Flowers are unisexual with radial symmetry (Rahman and Akter, 2013). The number of stamens in staminate flowers can range from 1 to numerous, while pistillate flowers contain a superior ovary, which eventually gives rise to a schizocarp capsule or drupe (Richardson et al., 1987; Rahman and Akter, 2013). A characteristic feature of the family is the presence of latex, more specifically in the species belonging to subfamilies Euphorbioideae and

Crotonoideae (Richardson et al., 1987; Rahman and Akter, 2013) These plants possess a milky or colourless, acrid juice (Rizk, 1987).

2.1.1 Taxonomy

The systematics of Euphorbiaceae *sensu lato* (s.l.) has been very controversial due to the complexity and heterogeneity of its members (Wurdack et al., 2004; Mwine and Damme, 2011). Unlike other families, there is no one unique characteristic that distinguishes euphorbiaceous species (Mwine and Damme, 2011). Alternatively, many anatomical characters, such as laticifer type, wood anatomy, stomatal nature, trichomes, exine structure and inflorescence type are used to group species into the family, subfamilies, tribes and genera (Mwine and Damme, 2011).

The classification of the family can be dated back to 1824, with taxonomist Adrien de Jussieu classifying the genera of the family (Mwine and Damme, 2011). Throughout the years, several ground-breaking contributions led to the division of Euphorbiaceae into five subfamilies, based on the number of ovules per locule. Uniovulate subfamilies included Acalyphoideae, Crotonoideae, Euphorbioideae, whilst Phyllanthoideae and Oldfieldoiideae were bi-ovulate. (Wurdack et al., 2004; Mwine and Damme, 2011; Secco et al., 2012).

Eventually, lack of molecular evidence led to the division of the family into four families including Euphorbiaceae *sensu stricto* (s.s) (Acalyphoideae, Crotonoideae, Euphorbioideae), Phyllanthaceae (containing Phyllanthoideae), Picrodendraceae (containing Oldfieldoiideae) and Putranjivaceae (Secco et al., 2012). Further molecular investigations of Euphorbiaceae s.s has resulted in the current division of the family, which comprises the subfamilies Cheilosoideae, Acalyphoideae, Crotonoideae and Euphorbioideae (Stevens and Davis, 2001; Wurdack et al., 2005; Wurdack and Davis, 2009).

2.1.2 Medicinal importance

Many species in this family are poisonous (Mwine and Dame, 2011) whilst others are of economic importance, being used for food, medicine and poisons. Many products, such as various oils, waxes, rubbers, varnishes and paints are also derived from euphorbiaceous species (Rizk, 1987; Schultes, 1987). Notable species include *Ricinus communis* L. (castor oil), *Manihot esculenta* Crantz (cassava), *Hevea brasiliensis* Wild. ex. A. Juss. (Para rubber) and *Euphorbia antisyphylitica* Zucc. (Candelilla wax) (Schultes, 1987; Wurdack et al., 2005; Mwine and Damme, 2011; Rahman and Akter, 2013).

Euphorbiaceous species are used in the treatment of various ailments and diseases, being linked to traditional Indian, Chinese and Yucatan herbal systems (Mwine and Damme, 2011). Examples include, *Acalypha indica* L. for the treatment of ulcers, bronchitis, pneumonia and rheumatism,

Jatropha curcas L. for scabies, eczema, ringworm, toothache, diarrhoea and stomach aches (Sinhababu and Banerjee, 2018) and *Euphorbia tirucalli* for cancer, rheumatism, tumours, gonorrhoea and arthritis (Mwine and Damme, 2011). Ethnobotanically, species from *Croton* provide an amazingly broad range of uses, indicating that this genus is one of the most interesting in the Euphorbiaceae (Schultes, 1987).

2.2 The genus *Croton*

Croton L., belonging to subfamily Crotonoideae (Berry et al., 2005; Liu et al., 2013) and tribe Crotoneae (Vitarelli et al., 2015), comprises of approximately 1300 species (Stevens and Davis, 2001; Salatino et al., 2007). The plants exist as either trees, shrubs, herbs or sometimes lianas, with distributions in tropical and subtropical areas (Salatino et al., 2007; Liu et al., 2013).

Certain characters are used to distinguish species within this genus including petiolar glands, unisexual flowers in condensed inflorescences, inaperturate pollen and the presence of noticeable trichomes which are stellate or scale-like (Berry et al., 2005). The trichomes of species in *Croton* are highly variable. For this reason, the indumentum is an important character in this genus (Webster, 1993; Webster et al., 1996; de Sá-Haiad et al., 2009; Liu et al., 2013). Webster et al. (1996) identified and described seven trichomes types within *Croton* i.e. stellate, fasciculate, multiradiate/rosulate, dendritic, lepidote, papillate and glandular. Another characteristic of certain *Croton* species is the presence of latex, which is a clear or coloured sap. This feature has been linked to the medicinal properties of species as the latex contains many secondary compounds that may possess biological or pharmacological activity (Berry et al., 2005; Salatino et al., 2007; Lima et al., 2010).

2.2.1 Traditional uses

Croton species are known to possess a diverse range of compounds such as alkaloids, terpenoids, flavonoids and volatile oils (Webster, 1993; Berry et al., 2005; Salatino et al., 2007). Table 2.1 illustrates species within this genus that are used in traditional medicine (Salatino et al., 2007).

Table 2.1: Traditional uses of *Croton* species (Salatino et al., 2007).

Continent	Species	Plant part	Traditional uses
South America	*C. cajucara* Benth.	Leaves and stems	Diabetes, hypercholesterolemia, weight loss, gastrointestinal and hepatic problems.
	C. celtidifolius Baill.	Bark and leaves	Inflammatory diseases, leukaemia, ulcers and rheumatism.
	C. eluteria Bennett.	Bark	Bronchitis, diarrhoea, dysentery, fever and antimalarial remedy.
	C. lechleri L.	Bark (latex)	Wound healing, purgative and tonic, homeostatic regulation and to prevent infection from injury.
	C. nepetaefolius Baill.	Bark and leaves	Intestinal colic, as a carminative, stomachic and antispasmodic.
North America	*C. arboreous* Millsp.	Aerial parts	Anti-inflammatory for respiratory problems
	C. californicus Müll. Arg.	Leaves	Pain reliever for rheumatism.
	C. draco Cham. and Schltdl.	Latex	Cough, flu, diarrhoea, stomach ulcers, wound healer, herpes, anti-septic after tooth removal and oral sores.
Africa	*C. macrostachys* Hochst. ex Rich.	Roots and seeds	As an antidiabetic and purgative respectively.
Asia	*C. oblongifolius* Roxb.,	Leaves, flowers, fruit, seeds, bark and roots.	Tonic, treatment for flatworms, abdominal cramps, as a purgative, to treat indigestion and dysentery respectively. Bark also used to treat chronic hepatitis.
	C. roxburghii NP Balakr.	Various parts	Treatment against snake poisoning, for infertility, fever and wounds.
	C. tiglium L.	Unspecified	Laxative, tumours and cancer sores. Oil from seeds used as a purgative.
	C. tonkinensis Gagnep	Leaves	Abdominal pain, to treat burns, abscesses, impetigo, indigestion and gastric/duodenal ulcers.

2.2.2 Pharmacology

The traditional uses of *Croton* species are constantly being validated by pharmacological investigations (Lima et al., 2010; dos Santos Alves et al., 2017).

Jeeshna et al. (2011) investigated the antimicrobial properties of *C. bonplandianum* Baill. Phytochemical investigations of the leaf extracts revealed the presence of various metabolites

including alkaloids, flavonoids, glycosides, steroids, phenols, tannins, saponins and resins. In addition, leaf extracts exhibited antimicrobial activity.

Oil from the seeds of *C. tiglum* L. yielded the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Studies showed that TPA inhibited growth, promoted apoptosis or improved differentiation of human tumour cells for various cancer (leukaemia, lung, breast, colon, prostate and melanoma) (Nath et al., 2013). Salatino et al. (2007) also indicated that TPA, exhibited strong anti-HIV-1 activity.

A study by Teugwa et al. (2013) investigated the antioxidant properties of *C. macrostachyus* using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The methanolic extract exhibited antioxidant activity which was attributed to the flavonoids and phenols found in the fruits, leaves and roots (Maroyi, 2017).

A review by Salatino et al. (2007) on the chemistry and pharmacological activity of the crude extracts and pure compounds of *Croton* species revealed various metabolites, such as diterpenoids, volatile oils, alkaloids and phenolic substances. *Croton* species also display a multitude of pharmacological activities including anti-inflammatory, antihypertensive, anti-malarial, anti-cancer, anti-viral, antiulcer, cytotoxic, hypolipidemic, myorelaxant, antispasmodic, antimicrobial, anti-oestrogen and hypoglycaemic effects (Salatino et al., 2007).

2.3 Previous phytochemical studies of *C. gratissimus* var. *gratissimus*

2.3.1 Diterpenoids isolation

Diterpenoids from species in the genus have been reported to possess cytotoxic, anti-tumour and anti-HIV-1 activity (Ngadjui et al., 2002). Many studies have isolated several diterpenoid compounds from *C. gratissimus*. Block et al. (2002) isolated ent-trachyloban-3β-ol, a trachyloban diterpene from the dichloromethane leaf extract of *C. zambesicus* and demonstrated its cytotoxicity on carcinoma cells of the human cervix. The dichloromethane leaf extract also revealed two new trachylobane-type diterpenoids, one isopimarane-type diterpenoid, *trans*-phytol, β-sitosterol, α-amyrin and stigmasterol (Block et al., 2004). Ngadjui et al. (2002) identified three new clerodane diterpenoids from the stem bark extracts, whilst Mulholland et al. (2010) revealed four cembranolides. In addition, Langat et al. (2011) isolated ten new cembranolides from the leaf extracts.

2.3.2 Antidiabetic activity

Diabetes mellitus is a serious metabolic disease resulting in high blood glucose levels. This deficiency of insulin related to diabetes can also cause other complications such as hyperlipidemia as it promotes lipolysis. Although antidiabetic medication is available, diabetes and its associated

complications still remain a huge problem. Lately, certain medicinal plants have proved useful in the treatment of diabetes due to their antidiabetic and antihyperlipidemic properties (Kumar et al., 2017). Kumar et al. (2017) investigated the potential antidiabetic and hypolipidemic properties of the roots of *C. zambesicus*, whilst Okokon et al. (2006) evaluated the antidiabetic activity of the leaf extracts. The leaf and root extracts significantly reduced blood glucose levels in alloxan-induced hyperglycaemic experimental models. The effects of the extracts were comparable to that of the standard drugs tested in each study. These studies indicate that *C. zambesicus* possesses antidiabetic activity, supporting its use in traditional medicine. This activity is probably attributed to the alkaloids, terpenes and flavonoids in this species (Okokon et al., 2006). In addition, experimental models treated with the root extracts also demonstrated decreased levels in serum total cholesterol, triglycerides, low density lipoproteins (LDL) cholesterol and very low density lipoproteins (VLDL) cholesterol, whilst high density lipoproteins (HDL) cholesterol levels were increased thus confirming its traditional use as an antihyperlipidemic agent (Kumar et al., 2017).

2.3.3 Antimalarial activity

A study by Okokon and Nwafor (2009) determined the antiplasmodial activity of *C. zambesicus* root extracts to confirm its efficacy as an antimalarial agent. Experimental models (Swiss albino mice) were infected with *Plasmodium berghei* before being administered varying doses of the ethanolic root extracts and fraction gradients of the root extracts (n-hexane, chloroform, ethyl acetate and methanol). The root extracts demonstrated significant antiplasmodial activity which was comparable to the positive control (standard drug). This activity may be attributed to the alkaloids and terpenes, found in the root extracts. Thus, this study validates the ethnomedicinal treatment of malaria using *C. zambesicus.*

These investigations demonstrate the medicinal properties of *C. gratissimus*. However, Salatino et al. (2007) suggest that studies are needed on the structures involved in the production and accumulation of natural metabolites.

2.4 Secretory tissues of plants

Humans have exploited the natural chemicals from plant secretions for various applications (Fahn, 1988a; Fahn, 2000). In vascular plants, specialised secretory tissues, occurring as either single cells or secretory structures, are responsible for producing natural chemical compounds (Fahn, 1988a; Dickison, 2000; Fahn, 2000; Castro and Demarco, 2008). These are important as animal attractants, food rewards and defence against predators (Fahn, 1988a; Fahn, 2000).

Secretory tissues can occur either externally or internally (Fahn, 1988b; Tissier, 2018). They are differentiated by their structure, topography and substances they secrete. The classification of

these tissues is based on their secretory products (Fahn, 1988a; Demarco, 2017). Plant secretory products include essential oils, gums, resins, latex, mineral salts and chemical compounds (Dickison, 2000). The secretory structures may be directly involved in the synthesis and secretion of these metabolites, such as tissues that produce mucilage, gum, oil and latex, or they may serve as secretory vehicles for substances received from the vascular tissues (hydathodes, nectaries and salt glands) (Fahn, 1979; Fahn, 1988a). Secretions from external secretory structures are typically released onto the surface of the plant whilst internal secretory tissues secrete these substances into specialised intercellular air spaces or accumulate them within the secretory cell (Fahn, 1979; Fahn, 1988a; Fahn, 1988b). External secretory structures include trichomes/papillae and glands, nectaries, osmophors, hydathodes and colleters. Internal secretory structures include secretory cells/idioblasts, secretory spaces (cavities and ducts) and laticifers (Esau, 1965; Dickison, 2000).

2.5 Trichomes

Minute protuberances arising from specialised epidermal cells are known as trichomes (Marin et al., 2008; Schilmiller et al., 2008). The term trichome is a derivation of "trichos", the Greek word for hair (Glas et al., 2012). These structures, which range from a few microns to centimetres in size, occur on the surface of plant organs such as the leaves, stems and petals of most plants (Werker, 2000; Marin et al., 2008; Glas et al., 2012). Werker (2000) defines trichomes as "unicellular or multicellular appendages, which originate from epidermal cells only, and develop outwards on the surface of various plant organs".

These complex and diverse appendages vary in size, shape, cell number, origin, location, function, secretory ability, secretion mode and secreted materials (Werker, 2000; Weryszko-Chmielewska and Chernetskyy, 2005; Marin et al., 2008; Choi and Kim, 2013; Janošević et al., 2016). However, trichomes within a plant group display great consistency (Esau, 1965).

Their universal occurrence and great diversity makes them important diagnostic characters in plant taxonomy (Glas et al., 2012; Hu et al., 2012). Payne (1978) developed an illustrative and descriptive glossary on the different morphological variations of individual trichomes and the terminology used to characterise indumentum. However, one major criterion of trichome classification is determining whether they are glandular (secretory) or non-glandular/simple (non-secretory) (Glas et al., 2012; Tissier, 2012; Choi and Kim, 2013).

2.5.1 Non-glandular trichomes

Non-glandular trichomes are found on the majority of angiosperm species, with some occurrences in gymnosperms and bryophytes (Wagner et al., 2004; Glas et al., 2012). According to Werker (2000), these structures are unicellular or multicellular, branched or unbranched and symmetrical

or asymmetrical. They can also be uniseriate, biseriate or multiseriate, specifically in non-glandular trichomes which are multicellular and unbranched. Non-glandular trichomes display great variation in their morphology, anatomy and microstructure with these structures varying in size, length and cell shape. The width of non-glandular trichomes may be constant or change throughout the length of the hair, ending in a tapering or blunt tip. The diversity in their morphology is the basis for classification of these trichomes (Werker, 2000).

The various functions of non-glandular trichomes are dependent on their morphology, location and orientation (Werker, 2000). When non-glandular trichomes are present on leaf surfaces they function to reduce water loss, promote gaseous exchange for photosynthesis and prevent heat damage by reflecting solar radiation (Bhatt et al., 2010). In some instances, non-glandular trichomes form a dense "mat" that serve as a mechanical barrier, protecting the plant from external stresses such as herbivores and pathogens, extreme water loss and intense temperatures (Werker, 2000).

2.5.2 Glandular trichomes

Nearly 30% of vascular plants possess glandular trichomes, which are secretory structures that consist of a stalk and a glandular head (Wagner et al., 2004; Marin et al., 2008; Huchelmann et al., 2017). There are various types of glandular trichomes that differ by location, structure, production mode, function and chemical composition of secreted products. These many variations aid in the classification of these structures (Werker, 2000). Glandular trichomes are suggested to have developed from non-glandular trichomes through the evolution of apical cells into secretory cells (Fahn, 1988a, 1988b; Tissier, 2012). Similar to non-glandular trichomes, glandular trichomes are unicellular or multicellular, uniseriate or multiseriate and have great diversity in their shapes (Werker, 2000). They range from small appendages to large multicellular structures, consisting of a distinct base, stalk and secretory head (Werker, 2000; Schilmiller et al., 2008). However, a universal feature of glandular trichomes is their ability to synthesise, secrete or store vast quantities of specialised metabolites, such as terpenes and flavonoids (Glas et al., 2012; Tissier, 2012; Huchelmann et al., 2017).

Glandular trichomes function to aid in pollination and seed dispersal. These trichomes also provide chemical protection/defence against herbivores and pathogens by deterring or poisoning predators (Werker, 2000; Valkama et al., 2003; Choi and Kim, 2013). Phenolics and steroidal triterpenoids that are produced by glandular trichomes on the stems of birch species aid in the defence against mammalian herbivores (Valkama et al., 2004). Nepetalactone obtained from *Nepeta* species have been shown to function as animal attractants or repellents (Kaya et al., 2007).

Similar to non-glandular trichomes, glandular trichomes also function to protect against water loss, extreme temperatures and ultraviolet-B (UV-B) radiation (Werker, 2000; Valkama et al., 2004).

The essential oils and biological activity of the secreted compounds makes them commercially valuable with uses in the pesticide, food, pharmaceutical, fragrance and cosmetic industries (Serrato-Valenti et al., 1997; Schilmiller et al., 2008; Baran et al, 2010; Glas et al., 2012; Choi and Kim, 2013). For example, many sesquiterpenoid lactones are produced in the glandular trichomes of *Artemisia* species. *Artemisia annua* produces the sesquiterpenoid lactone from which the antimalarial drug, artemisinin, is derived (Duke and Paul, 1993). Gossypol and similar dimeric disesquiterpenes from the trichomes of *Gossypium hirsutum* (cotton) are strong antifungal agents. Lamiaceous species, *Mentha piperita* (mint), *Ocimum basilicum* (basil), *Lavandula spica* (lavender), *Origanum vulgare*, (oregano) and *Thymus vulgaris* (thyme) are cultivated for their essential oils which are produced by glandular trichomes (Glas et al., 2012).

Glandular trichomes are categorised into two major types, namely capitate and peltate, based on their head size and stalk length (Choi and Kim, 2013). Capitate trichomes usually possess a stalk that is more than double the size of its glandular head whilst peltate trichome comprise of a short stalk with a large secretory head (Ascensão and Pais, 1998; Glas et al., 2012; Choi and Kim, 2013; Huchelmann et al., 2017). In addition, peltate trichomes possess a subcuticular storage cavity which is located between the cell wall and the cuticle of the secretory head. The secretory products are stored in this space resulting in the "bulb-like" shape of peltate trichomes (Kaya et al., 2007; Huang et al., 2008; Glas et al., 2012).

2.5.3 Trichome variability and distribution

Sometimes whole families or even genera may only possess one type of trichome. Examples include the multicellular branched trichomes on *Verbascum thapsus* and *Platanus*, stinging hairs of species in Urticaceae, the T-shaped unicellular hairs of the Malpighiaceae and scale hairs in bromeliaceous species. Conversely, some families display a diverse range of trichome morphologies (Dickison, 2000). This diversity in trichome morphology may also exist in a single genus. Within *Croton*, five common trichomes types have been identified, namely lepidote, stellate, fasciculate or rosulate, fasciculate-stipitate and dendritic. However, between these trichome types, there are intermediate or transitional variations (Webster et al., 1996), as seen in Figure 2.2.

It is also not uncommon for glandular and non-glandular trichomes (with multiple types of each) to exist on the same individual or organ (Werker, 2000; Schilmiller et al., 2008). For example,

the study by dos Santos Alves et al. (2017) revealed stellate, simple and glandular trichomes on the leaves and petioles of *C. cordiifolius*.
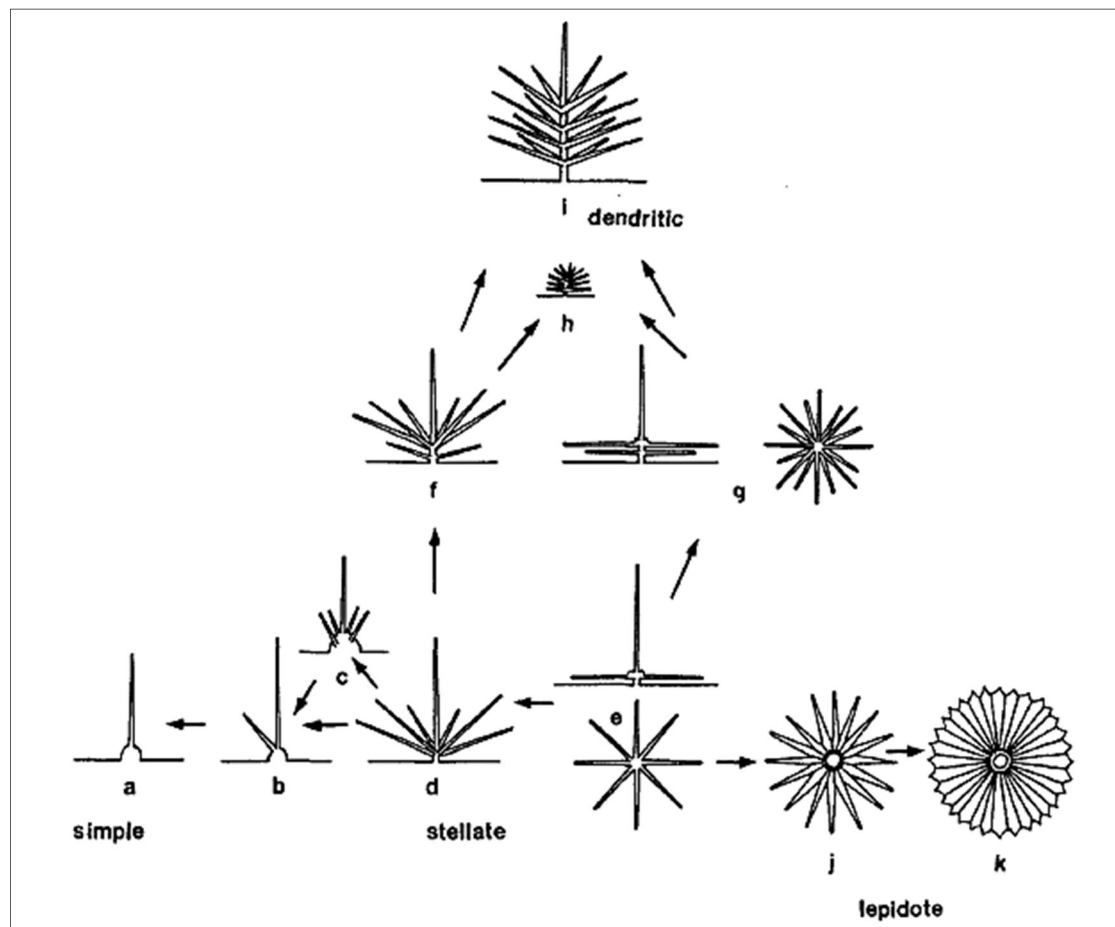
Figure 2.2: Possible transformational relationships between trichome types in *Croton*. a, simple; b, 2-5-radiate; c, rosulate (pin-cushion); d, fasciculate; e, stellate-rotate (lateral and frontal views); f, transition from multiradiate to dendritic; g, two-layered stellate (transitional to geminate); h, geminate; i, dendritic. Arrows indicate directions of apparent morphological change (Webster et al., 1996).

2.6 Laticifers

The term laticifer refers to an internal secretory system comprising a single specialised cell or rows thereof that produces and accumulates latex (Fahn, 1979; Furr and Mahlberg, 1981; Pickard, 2008). Latex, is derived from the Latin word for juice (Esau, 1965). This latex, which occurs in approximately 20,000 angiosperm species (Hagel et al., 2008; Lange, 2015), may be watery or sticky and viscous (Dickison, 2000; Lange, 2015). Latex also varies in colour, depending on the species, appearing white and milky (*Euphorbia, Lactuca, Taraxacum, Asclepias*), yellow-brown (*Cannabis*), orange (*Chelidonium*), red and even colourless (*Morus*, *Nerium*) (Fahn, 1979; Castro

and Demarco, 2008). It occurs in both monocotyledonous and dicotyledonous plants of varying habit, ranging from small herbs to shrubs, lianas and large trees (Esau, 1965; Lopes et al., 2009). Laticifers can exist in vegetative and reproductive organs and are typically associated with vascular tissues (phloem and xylem) but may also exist in the cortex, pith and foliar mesophyll (Dickison, 2000, Castro and Demarco, 2008; Lange, 2015). They may occur throughout the plant or be limited to specific tissues (Fahn, 1979; Castro and Demarco, 2008). When laticiferous tissue is damaged, the latex is released and functions as a wound sealant (Dickison, 2000, Castro and Demarco, 2008; Lange, 2015).

2.6.1 Laticifer classification

Laticifers can be simple, originating from a single cell, or compound, resulting from the fusion of cells (Esau, 1965). They are classified into two major types: non-articulated and articulated (Esau, 1965; Rudall, 1987; Fahn, 1979; Dickison, 2000; Lopes et al., 2009; Lange, 2015).

Non-articulated laticifers are multinucleated elongated cells. These laticifers arise from a single cell which elongates as the plant develops. However, cytokinesis does not occur during the successive nuclear divisions, resulting in a multinucleate cell (Mahlberg et al., 1987; Rudall, 1987; Dickison, 2000; Hagel et al., 2008; Lopes et al., 2009; Lange, 2015). This type of laticifers may be branched or unbranched (Dickison, 2000). The non-articulated branched laticifers develop through the intrusive growth of the individual cell into the surrounding tissues, giving rise to lateral branches (Dickison, 2000).

Articulated laticifers originate from interconnections with multiple cells to form a tube or vessel (Mahlberg et al., 1987; Dickison, 2000; Hagel, 2008). The walls at the end of each cell may continue to exist, become perforated or completely disintegrate. When complete lysis of the walls occur, the end result is a large multinucleated structure that is similar to the non-articulated type (Fahn, 1979). This type is categorised into non-anastomosing (unbranched) and anastomosing (branched), with the latter formed through lateral anastomoses with adjacent laticifers (Fahn, 1979; Hagel, 2008).

2.6.2 Latex

The chemical composition of latex is extremely variable (Castro and Demarco, 2008; Lange, 2015), being reported to contain various specialised metabolites such as terpenoids, alkaloids (*Papaver somniferum*), phenolics, proteins, polyisoprene hydrocarbons, starch grains (*Euphorbia*), triterpenols and sterols, fatty and aromatic acids, vitamin B1 (*Euphorbia*), tannins (*Musa*), cardiac glycosides, cannabinoids and phospholipids (Fahn, 1979; Castro and Demarco, 2008; Hagel, 2008; Lange, 2015). Latex is suggested to function in wound healing and as a

defence against herbivores and infectious diseases (Dickison, 2000; Hagel, 2008; Lange, 2015; Uday et al., 2015).

Latex has been used traditionally for a wide range of applications such as wound healing, burns, arthralgia and worm infections (Upadhyay, 2011; Uday et al., 2015). Examples include the bright yellow latex produced by *Argemone mexicana* (Mexican poppy) for the treatment of boils, dermatitis and wounds. The milky latex from the soft bark of *Jatropha curcas* for treating ulcers, bleeding gums, tumours and wounds (Uday et al., 2015).

The latex constituents possess anti-carcinogenic, proteolytic, anti-proliferative, anti-inflammatory, vasodilatory, antioxidant, antimicrobial, antiparasitic, anthelmintic, insecticidal and wound healing properties (Upadhyay, 2011; Uday et al., 2015). For example, the opiates morphine, codeine and thebaine are derived from the latex of *Papaver somniferum* (Opium poppy) (Hagel, 2008).

2.6.3 Laticifers in Euphorbiaceae

Laticifers occur in many members of the Euphorbiaceae (Mahlberg et al., 1987; Rudall, 1987). Their occurrence in the economically and medicinally important genera *Euphorbia, Jatropha, Hevea* and *Manihot* have been extensively studied. However, laticiferous studies on many other species of Euphorbiaceae are lacking (Rudall, 1987; Demarco et al., 2013). Within *Croton*, the latex from laticiferous species contain specialised metabolites with possible medicinal importance (Salatino et al., 2007; Lima et al., 2010).

2.7 Nanoparticles

Microscopic particles with sizes less than 100 nm are termed nanoparticles (Song and Kim, 2009; Thakkar et al., 2010). These particles have a broad range of applications in various fields including medicine, pharmaceutics, mechanics and energy science (Iravani, 2011). The use of nanoparticles for these various applications is attributed to their chemical, physical and biological properties (Ahmed et al., 2016a). However, these properties are dependent on the size, morphology and distribution of the particles (Song and Kim, 2009; Gabriella et al., 2017; Vetchinkina et al., 2018).

2.7.1 Nanoparticle synthesis

The production of nanoparticles is actively being researched and synthesised using either a "top-down" or "bottom-up" approach. In the "top-down" method, nanoparticles are synthesised through size reduction of suitable materials by means of physical or chemical treatments. The "bottom-up", or "self-assembly" approach, involves the formation of a structure from smaller units such as atoms, molecules and smaller particles/clusters, followed by the assembly of these

nanostructures into the final nanoparticle. This type of synthesis is achieved using chemical or biological processes (Thakkar et al., 2010; Mittal et al., 2013; De Matteis et al., 2018).

However, physical and chemical processes are expensive, labour-intensive and requires a high-energy input. In addition, toxic by-products produced through these processes are harmful to the environment and living organisms (Song and Kim, 2009; Thakkar et al., 2010; Mittal et al., 2013; Makarov et al., 2014; Ahmed et al., 2016b; Ibrahim, 2015; Parveen et al., 2016; Khan et al., 2018). Therefore, it is becoming increasingly important to develop environmentally friendly protocols for synthesising nanoparticles (Song and Kim, 2009; Thakkar et al., 2010; Makarov et al., 2014).

Biological or green synthesis (Khan et al., 2018; De Matteis et al., 2018) of nanoparticles is more advantageous over conventional procedures as it is environmentally friendly (Kuppusamy et al., 2016), simple (Mittal et al., 2013; Ahmed et al., 2016b), less expensive and does not require toxic chemicals (Iravani, 2011).

This method of synthesis utilises natural products such as a) microorganisms; (fungi, yeasts, bacteria, and actinomycetes); b) plants or plant extracts; c) membranes, viruses' DNA, and diatoms (De Matteis et al., 2018) and d) enzymes (Song and Kim, 2009) to produce various types of nanomaterials including, copper, titanium, magnesium, zinc, alginate, gold, silver (Logeswari et al., 2015) nickel and platinum (Kuppusamy et al., 2016).

2.7.2 Silver nanoparticles (AgNPs)

Silver nanoparticles have received a considerable amount of attention due to their extensive use in various fields including medical industries, optics, electronics, cosmetics, antimicrobials, drug delivery and bio-sensing (Song and Kim, 2009; Iravani, 2011; Ibrahim, 2015; Dhand et al., 2016; Ahmed et al., 2016a). In addition, these nanoparticles are known to possess antimicrobial, antitumor, anti-inflammatory and antioxidant properties (Vetchinkina et al., 2018).

The use of microorganisms for the synthesis of AgNPs has been reported (Vanaja and Annadurai, 2013; Ahmed et al., 2016a; Ahmed et al., 2016b). However, the use of plants or plant extracts is more superior over microorganisms as this method does not require the isolation, culturing and maintenance of cell cultures. (Song and Kim, 2009; Singhal et al., 2011; Ibrahim, 2015; Ahmed et al., 2016a). This method of biosynthesis is also much faster (Ahmed et al., 2016b) and safer for use in human health care (Ibrahim, 2015).

Biosynthesis of AgNPs using plants usually involves the addition of plant extracts to silver nitrate (AgNO$_3$) solution. Biomolecules from the plant extracts induce the reduction of silver ions (Ag$^+$) to AgNPs, which can then be measured using ultraviolet-visible (UV-VIS) spectroscopy (Ahmed et al., 2016a; De Matteis et al., 2018). The metabolites that are found in plants (proteins, amino

acids, enzymes, vitamins, terpenoids, flavonoids, tannins, alkaloids, phenolics, saponins and polyphenols) aid in the synthesis of AgNPs (Vinmathi and Jacob, 2015; Dhand et al., 2016; Jyoti et al., 2016; Kuppusamy et al., 2016; Gabriela et al., 2017; Raja et al., 2017; De Matteis et al., 2018). These are responsible for the reduction and stabilisation (capping agents) of fabricated particles (Iravani, 2011; Singhal et al., 2011; Mittal et al., 2013; Ibrahim, 2015; Vinmathi and Jacob, 2015; Ahmed et al., 2016a; Jyoti et al., 2016; Kuppusamy et al., 2016; Gabriela et al., 2017; Raja et al., 2017; De Matteis et al., 2018). The bioactive constituents that are attached to the AgNPs also enhance their therapeutic properties (Sangeetha, et al., 2016).

A considerable amount of literature is available on the synthesis of AgNPs using various plant extracts such as marigold flower, *Ziziphora tenuior, Solanum tricobatum, Artemisia nilagirica, Erythrina indica*, beet root, *Piper pedicellatum*, mangosteen and *Melia dubia* (Ahmed et al., 2016b; Raja et al., 2017).

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CHAPTER 3: MICROMORPHOLOGICAL AND HISTOCHEMICAL INVESTIGATION OF TRICHOMES AND LATICIFERS ON/IN THE LEAVES AND STEMS OF *CROTON GRATISSIMUS* BURCH. VAR. *GRATISSIMUS* (EUPHORBIACEAE)

3.1 Abstract

The leaves and stems of *C. gratissimus* possess three distinct structures, i.e. lepidote and glandular trichomes and non-articulated unbranched laticifers. The lepidote trichomes formed a dense indumentum on the abaxial surface of the leaves and canopied the glandular trichomes. Lepidote trichomes are assumed to be non-glandular. However, TEM of these structures indicated high metabolic activity within the stalk and radial cells. This suggested that lepidote trichomes play a role in the production and/or accumulation of secretory products. Glandular trichomes were embedded in the epidermal layer and consisted of a single cell which formed a prominent stalk and dilated head. Laticifers were observed in the mid-vein of leaves and were predominantly associated with the vascular tissue. In the stems, laticifers were associated with the phloem and pith. Both trichome types and laticifers stained positive for alkaloids, phenolic compounds and lipids with histochemical tests. Positive staining for these compounds in lepidote trichomes suggests their involvement in the production and/or accumulation of secondary metabolites. These results indicate that several structures are involved in the production and/or accumulation of secondary metabolites. These metabolites may provide mechanical and chemical defence for the plant, in addition to providing useful compounds for traditional medicine.

Keywords: Lepidote, glandular, non-articulated, secondary metabolites, indumentum.

3.2 Introduction

Plants are an integral component in traditional medicine (Sen and Chakraborty, 2017). Traditional medicine is used in the prevention and treatment of various ailments and is regarded as the world's most ancient form of healthcare (Yuan et al., 2016). These therapeutic properties are due to biologically active compounds, such as alkaloids, tannins, saponins, flavonoids, phenols, glycosides, terpenoids, anthocyanins, and coumarins, produced by plants (Chikezie et al., 2015). According to Lee and Ding (2016) and Demarco (2017), plant secretory structures are involved in the production of these natural bioactive compounds.

Almost all plants possess tissues or organs which primarily produce and store secondary metabolites (Lee and Ding, 2016; Demarco; 2017; Tissier, 2018). These structures comprise of either single or multiple cells that vary in structure, topography and substance secreted (Demarco, 2017). Based on their location, they are classified into external and internal structures (Esau, 1965; Demarco, 2017; Tissier, 2018). Trichomes/papillae and glands, nectaries, osmophors, hydathodes and colleters are examples of external secretory structures. Secretory cells/idioblasts, cavities, ducts and laticifers are classed as internal secretory structures (Esau, 1965; Dickison, 2000).

Within the Euphorbiaceae, trichomes and laticifers are important characteristic features used for the taxonomic classification of the family (Mwine and Van Damme, 2011). Trichomes are minute appendages that arise from the epidermal cells of aerial plant parts (Marin et al., 2008; Schilmiller et al., 2008). These structures are categorised into non-glandular and glandular, based on their secretory abilities (Wagner et al., 2004; Choi and Kim, 2013; Huchelmann et al., 2017). Non-glandular trichomes are presumed to be non-secretory whilst glandular trichomes produce, accumulate and/or secrete copious amounts of secondary metabolites (Wagner et al., 2004; Glas et al., 2012; Tozin et al., 2016; Tissier, 2018).

Laticifers are internal secretory systems that comprise specialised cells (single or rows of multiple) (Fahn, 1979; Pickard, 2008; Castelblanque et al., 2016). They are categorised into two major types; non-articulated, which arise from a single cell, and articulated, that develop from multiple cells (Fahn, 1979; Hagel et al., 2008). These structures are responsible for synthesising and accumulating latex (Tan et al., 2011; Lange, 2015; Castelblanque et al., 2016) which is released upon damage of the laticiferous tissue (Castro and Demarco, 2008; Lange, 2015). The composition of latex is highly variable and contains various secondary metabolites (Castro and Demarco, 2008; Hagel et al., 2008; Lima et al., 2010; Lange, 2015). Latex-bearing *Croton* species have displayed medicinal potential and have been used traditionally for various treatments (Salatino et al., 2007; Lima et al., 2010; Uday et al., 2015).

Croton gratissimus Burch. var. *gratissimus* (syn. *C. zambesicus* Müll. Arg.; *C. microbotryus* Pax., *C. amabilis* Müell. Arg.) commonly known as lavender *Croton* or lavender fever berry, belongs to the family Euphorbiaceae (Mulholland et al., 2010; Robert et al., 2010; PlantZAfrica, 2018). It is a semi-deciduous shrub or tree with widespread distribution in tropical, central and sub-Saharan Africa (Ngadjui et al., 2002; Block et al., 2004; Mulholland et al., 2010).

This species has been used extensively in traditional medicine to treat various ailments such as fever, uterine disorder, dysentery, pleurisy, convulsions, chest complaints, bleeding gums and malaria (Ngadjui et al., 2002; Van Vuuren and Viljoen, 2008; Mulholland et al., 2010; Langat et al., 2011). Due to its extensive use in traditional medicine, many phytochemical investigations have been carried out on *C. gratissimus* to validate its therapeutic properties. However, research on the location of these metabolites and the structures involved in its production are scarce. Therefore, this study aimed to identify and describe the micromorphology of trichomes and laticifers from the leaves and stems of *C. gratissimus* and to determine whether these structures are responsible for the production and/or accumulation of metabolites.

3.3 Materials and methods

3.3.1 Plant collection and sampling

Leaves and stems of *Croton gratissimus* Burch. var. *gratissimus* were collected from the University of KwaZulu-Natal, Westville Campus (29°49'08.1"S 30°56'38.9"E). Leaves selected were classified into three developmental stages according to their lengths: emergent (<30 mm), young (30 – 60 mm) and mature (>60 mm). A voucher specimen (*Croton* 01 – Accession No. 18224) was prepared and deposited in the Ward Herbarium located in the School of Life Sciences at the University of KwaZulu-Natal, Westville Campus.

3.3.2 Stereomicroscopy

In order to obtain a general overview of the surfaces, fresh whole leaves (abaxial and adaxial) were viewed using a Nikon AZ100 (Japan), stereomicroscope equipped with a Nikon DS-F*i*3 camera. Images were captured at different magnifications using the NIS-Elements D 4.00 imaging software.

3.3.3 Scanning electron microscopy (SEM)

Scanning electron microscopy was used to examine the micromorphology of the trichomes and laticifers on/in the leaves and stems. Two methods were employed to prepare the material for viewing, i.e. chemical fixation and freeze-drying.

- **Chemical fixation**

Fresh whole leaves and stems were rinsed in a 1% JIK solution to remove excess dirt and the abaxial surface of some leaves were stripped with cellophane tape. Thereafter, leaf and stems segments were chemically fixed in 2.5% glutaraldehyde for 24 h at 4°C, before being subjected to three 5 min phosphate buffer (0.1 M with a 7.2 pH) washes. This was followed by post-fixation in 0.5% osmium tetroxide for 4 h at room temperature. Thereafter, the material underwent another three 5 min phosphate buffer rinses and was then dehydrated in a graded series of ethanol, 30%, 50%, 70% (each twice for 5 min) and 100% (twice for 10 min). Following dehydration, samples were critically point dried, using a Quorum K850 Critical Point Dryer. Segments were then mounted onto aluminium stubs, which were secured with carbon conductive tape, and sputter-coated with gold in a Quorum Q150 RES gold Sputter Coater. Samples were viewed using a Zeiss Leo 1450 SEM (Germany) at a working distance of 18 mm. Images were captured using the SmartSEM imaging software.

- **Freeze-drying**

Stem and leaf segments from whole leaves were rinsed in 1% JIK solution and quenched in liquid nitrogen. The segments were subsequently fractured on metal discs submerged in liquid nitrogen before being freeze-dried in an Edwards EPTD3 Freeze-Dryer at -60°C (vacuum pressure 10^{-2} Torr) for 96 h. Freeze-dried samples were then mounted onto aluminium stubs with carbon cement and sputter-coated with gold in a Quorum Q150 RES gold Sputter Coater. Viewing and imaging of the samples were achieved using a Zeiss Leo SEM at a 15 mm working distance and SmartSEM imaging software respectively.

3.3.4 Sample preparation for light and transmission electron microscopy (TEM)

Fresh leaf and stem segments were placed in buffered fixative (2.5% glutaraldehyde), for 24 h at 4°C. The material was then subjected to three 5 min washes with phosphate buffer (0.1 M with a pH of 7.2) before being post-fixed with 0.5% osmium tetroxide for 4 h at room temperature. Samples were subjected to another three 5 min wash with phosphate buffer following dehydration in a graded series of acetone solutions ranging from 30%, 50%, 75% and 100% (with two 10 min changes for each). An additional dehydration step was carried out by washing the material twice with propylene oxide for 10 min each. Following dehydration, the samples were gradually infiltrated with Spurr's resin (Spurr, 1969) (resin: propylene oxide; 1:3, 1:1, 3:1), before whole resin infiltration (100%) for 24 h. The segments were then orientated in silicon moulds with whole resin and allowed to polymerize for 8 h at 70°C. Glass knives, used for sectioning, were prepared using a LBK 7801A glass knife maker.

- **Light microscopy**

Monitor sections (1 µm) from the resin blocks were obtained using a Leica EM UC7 Ultra Microtome. The sections were fixed onto glass slides and stained with Toluidine-Blue. Prepared slides were viewed using a Nikon Eclipse 80*i* compound and fluorescent microscope equipped with a Nikon DS-Fi1camera. Images were captured using the NIS-Elements D 4.00 software.

- **Transmission electron microscopy (TEM)**

A Leica EM UC7 Ultra Microtome was used to cut ultra-thin sections (100 – 130 nm) which were picked up on copper grids and post-stained. The copper grids were placed onto drops of uranyl acetate and allowed to stain for 10 min before being rinsed with fresh distilled water. The grids were then placed onto drops of lead citrate enclosed in a petri dish with sodium hydroxide pellets and stained for a further 10 min. Thereafter, grids were rinsed with fresh distilled water and dried on filter paper. Stained sections were viewed using a JEOL 1010 TEM (Japan). Images were captured on the iTEM software.

3.3.5 Fluorescence microscopy

Transverse sections (80 – 100 µm thick) of fresh leaves and stems were cut using an Oxford® Vibratome Sectioning System. Sections were stained, mounted onto glass slides with distilled water and viewed using a Nikon Eclipse 80i compound and fluorescent microscope equipped with a Nikon Super High Pressure Mercury Lamp and a Nikon DS-Fi1camera. Images were captured on the NIS-Elements D 4.00 software.

- **Acridine orange**

Sections were stained with 0.01 % aqueous acridine orange for 20 min before being rinsed with distilled water for the detection of acidic compounds, such as nucleic acids and lignin. Stained sections were viewed under blue light. Lignified cell walls emitted a yellow-green fluorescence, whilst non-lignified cells fluoresced red (Demarco, 2017).

- **Auto-fluorescence**

Unstained sections were viewed with ultraviolet (UV) light to detect the presence of phenolic compounds and lignin. Two types of fluorescence are generated at UV excitation wavelengths between 340-360 nm. Phenolic compounds and lignin emit a blue or blue-green fluorescence. Chlorophyll emits a red fluorescence, which indicates the presence of chloroplasts (Talamond et al., 2015; Demarco, 2017).

3.3.6 Histochemistry

Fresh leaves and stems were sectioned transversely with a thickness of 80 – 100 μm using a Vibratome. Sections were stained, mounted onto glass slides and viewed using a Nikon Eclipse 80i compound and fluorescent microscope (Japan) equipped with a Nikon DS-Fi1camera. Images were captured on NIS-Elements D 4.00 software. The following compounds were identified and localised using appropriate histochemical stains:

- **Alkaloids**

Sections were stained with Wagner's reagent for 20 min before being rinsed with distilled water. A brown/orange colour indicated the presence of alkaloids (Furr and Mahlberg, 1981; Demarco, 2017).

- **Lipids**

Sudan III

Sections were placed in a saturated solution of Sudan III for 15 min and were then rinsed with 70% ethanol to remove excess stain. Lipids stained red/orange (Pearse, 1985; Guo et al., 2013).

Sudan black B

Sections were flooded with Sudan black B and stained for 20 min. They were subsequently rinsed with 70% ethanol before being washed with distilled water. Lipids stained dark blue to black (Demarco, 2017).

Nile blue

Sections were immersed in Nile blue and stained for 5 min at 60°C. Thereafter, the sections were washed twice in 1% acetic acid at 60°C followed by rinsing in distilled water. Acidic lipids stained blue whilst neutral lipids stained pink (Demarco, 2017).

- **Phenolic compounds**

Sections were placed in 10% ferric chloride and allowed to stain for 30 min. Thereafter, sections were washed with distilled water to remove excess stain. Brown/black precipitate was a positive indicator for the presence phenolic compounds (Demarco, 2017).

- **Lignin**

Sections were immersed in 10% phloroglucinol for 15 min. Stained sections were mounted in 25% hydrochloric acid and viewed microscopically. Lignin stained pink/red (Demarco, 2017).

- **Mucilage and pectin**

Sections were flooded with aqueous ruthenium red solution (1:5000) for 10 min. Mucilage and pectinaceous substances stained red/pink (Johansen, 1940).

- **Carboxylated polysaccharides, polyuronides, macromolecules with free phosphate groups and polyphenols**

Sections were placed in 0.05% Toluidine-Blue (metachromatic stain) for 1 min before being rinsed with distilled water. Carboxylated polysaccharides and polyuronides stained pinkish purple, macromolecules with free phosphate groups stained purple or green-blue and polyphenols (lignins) stained green/bright blue (O'brien et al., 1964; Zander, 2016).

3.4 Results and Discussion

The leaves and stems of *Croton gratissimus* possessed three types of structures, lepidote and glandular trichomes, and non-articulated unbranched laticifers. The presence of trichomes and laticifers in Euphorbiaceous species has been well documented (Rudall, 1994; Senakun and Chantaranothai, 2010; Vitarelli et al., 2015; dos Santos Alves et al., 2017). In the present study, the lepidote and glandular trichomes were present on the stems and abaxial surfaces of leaves (Fig. 3.1, 3.2, 3.3, 3.8, 3.12). Webster et al. (1996) revealed that these are two of the seven types of trichomes that have been identified and described in *Croton*. Lucena and Sales (2006) reported similar findings and indicated that trichome type is an important character in the taxonomic classification of *Croton* (dos Santos Alves et al., 2017). The lepidote trichomes formed a dense indumentum on the stems and abaxial surface of leaves at all developmental stages. The lepidote trichomes covered the underlying glandular trichomes (Fig. 3.1, 3.2, 3.3). The dense indumentum may provide protection for the leaf and for the smaller, energy-consuming glandular trichomes (Werker, 2000). The glandular trichomes were abundant on all leaf stages and on the stems (Fig. 3.8).

The adaxial surfaces also possessed non-glandular stellate trichomes along the sunken mid-vein of the leaves (Fig. 3.1, 3.2, 3.3). Although Webster et al. (1996) identified stellate trichomes as one of the commonly occurring trichome type in the genus, they were not studied further.

Non-articulated unbranched laticifers present *in C. gratissimus* were predominantly associated with the vascular tissue of the leaves and the phloem and pith in the stems (Fig. 3.9, 3.10, 3.13). Studies by Rudall (1989, 1994) indicated that non-articulated laticifers are common in *Croton*.

3.4.1 Surface overview

Stereomicrographs provided a general overview of the stems and leaf surfaces at the different developmental stages (Fig. 3.1, 3.2). At low magnifications, the adaxial surface of the leaves throughout all developmental stages appear glabrous and shiny, indicating the presence of a cuticle layer above the epidermis (Fig. 3.1a, 3.1c, 3.1e). In vascular plants, the cuticle layer plays an important role in preventing water loss from various organs (de Andrade et al., 2017). The ruptured cuticle layer on the adaxial surface of a leaf in Fig. 3.3a was probably due to rupture during the chemical preparation of the material.

The stems and abaxial surfaces of emergent, young and mature leaves were densely covered with lepidote trichomes resulting in a silvery appearance (Fig. 3.1b, 3.1d, 3.1f, 3.1g). This phenomenon was also recognised by Leandri (1972) who reported that many species in the genus possess a characteristic silver indumentum with copper specks, formed by scale-like trichomes on the abaxial surface of leaves (Berry et al., 2016). This dense indumentum protects developing leaves from desiccation as leaves are folded inwards, exposing the abaxial surface to the environment (Vitarelli et al., 2015). This abundance of lepidote trichomes did not decrease with maturity (Fig. 3.1b, 3.1d, 3.1f).

At higher magnifications, the lack of pubescence on the lamina of the adaxial surface was clearly visible (Fig. 3.2a, 3.3a). Stereomicrographs revealed translucent dots on this surface, which was not observed under SEM (Fig. 3.2a). Stellate trichomes were present on the adaxial surface of all developmental stages, occurring along the sunken mid-vein of the leaves (Fig. 3.2b, 3.3b).

Overlapping of lepidote trichomes on leaves and stems was observed at higher magnifications (Fig. 3.2c, 3.2d, 3.3c, 3,3d). Some of the lepidote trichomes contained an orange/brown secretion which can clearly be seen at higher magnifications (Fig. 3.2c, 3.2d, 3.2f, 3.4c). These secretory accumulations appeared as rust specks on the stems and abaxial surface of leaves (Fig. 3.1b, 3.1d, 3.1f, 3.1g). The trichomes also formed an indumentum over the petioles of the leaves.

Extrafloral nectaries were present on the mid-vein at the base of the leaf on the abaxial surface (Fig. 3.2e). These structures are common in the genus and are involved in mutualistic interactions. Their function is to provide rewards to insects that defend the plant against herbivores (Vitarelli et al., 2015). These structures were also covered with lepidote trichomes.
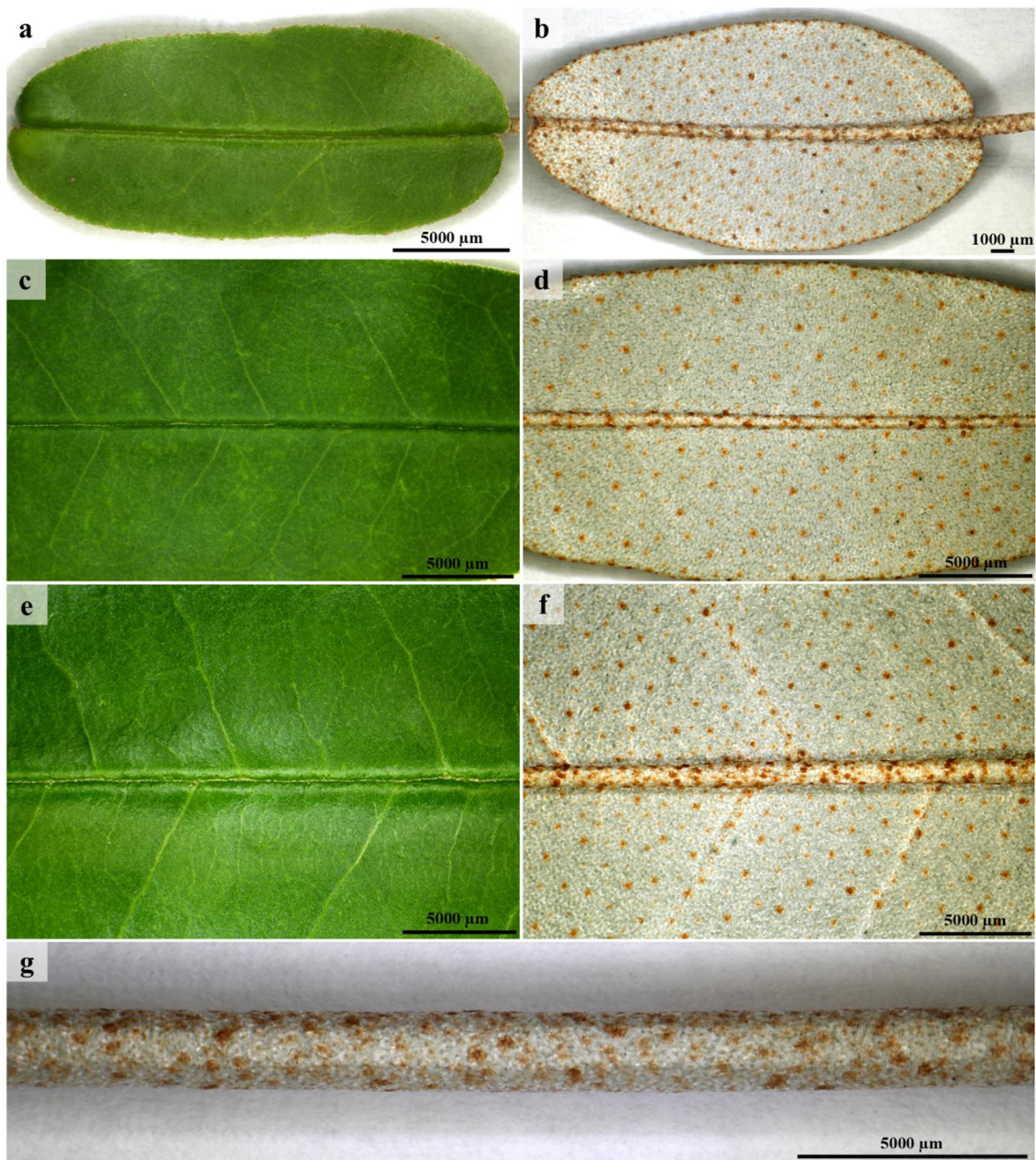
Figure 3.1: Stereomicrographs showing general overview of leaves and stems. a) Adaxial surface of emergent leaf. b) Abaxial surface of emergent leaf showing dense distribution of lepidote trichomes on lamina, mid-vein and petiole. c) Adaxial surface of young leaf. d) Abaxial surface of young leaf with dense indumentum of lepidote trichomes. e) Adaxial surface of mature leaf appearing shiny, indicating the presence of a cuticle layer. f) Abaxial surface of mature leaf showing lepidote trichomes densely distributed over the lamina and mid-vein. g) Stem covered with lepidote trichomes.
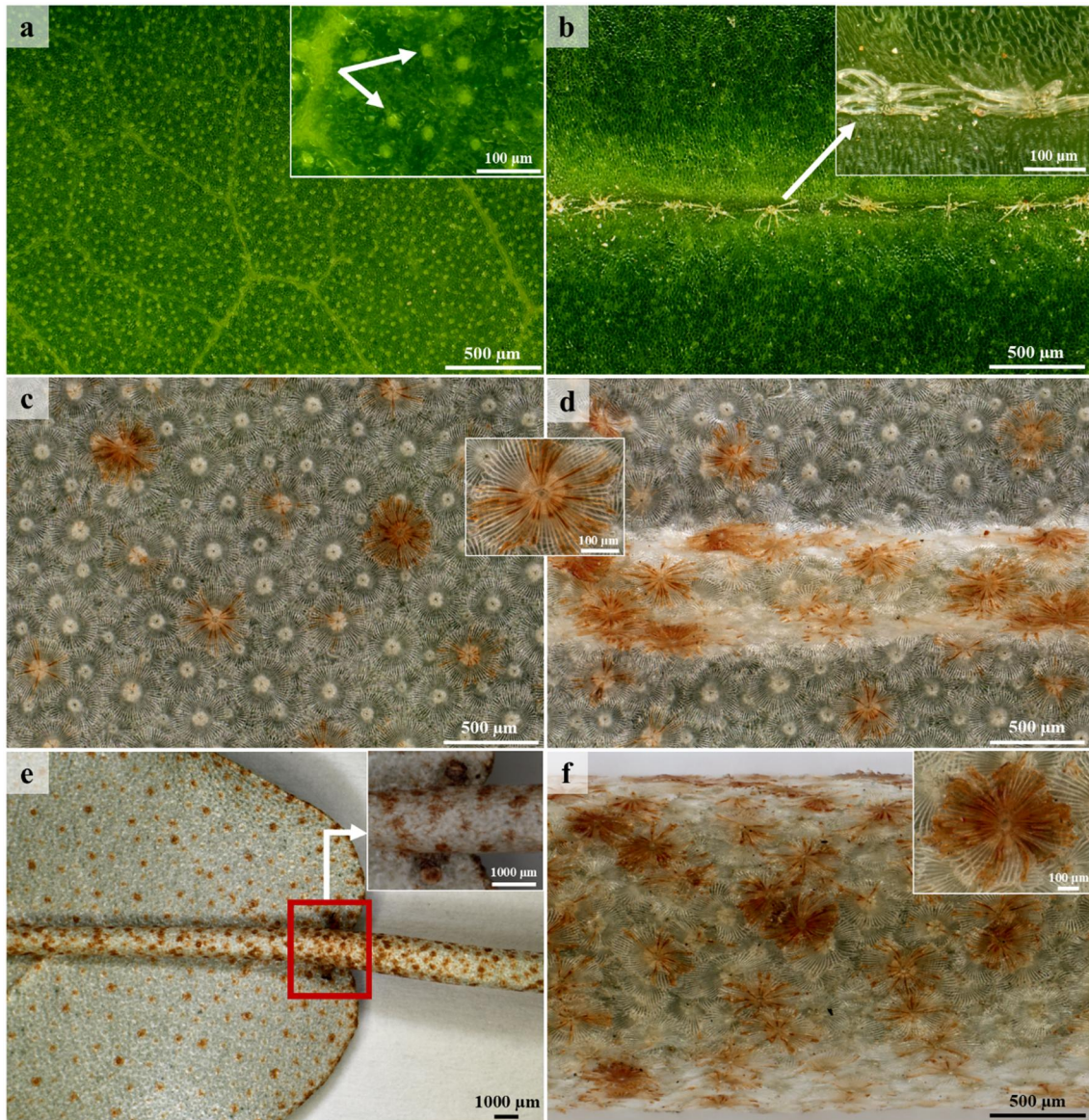
Figure 3.2: Stereomicrographs of leaf and stem surfaces. a) Glabrous lamina showing translucent dots on adaxial surface. b) Stellate trichome along sunken mid-vein on the adaxial surface. Note the glossy appearance of this surface which is indicative of a cuticle layer. c) Lamina of abaxial surface densely covered with lepidote trichomes. d) Mid-vein on abaxial surface covered with lepidote trichomes. e) Extrafloral nectaries present on the mid-vein at the base of the leaf. Note lepidote trichomes on petiole. f) Dense indumentum of lepidote trichomes on stem.
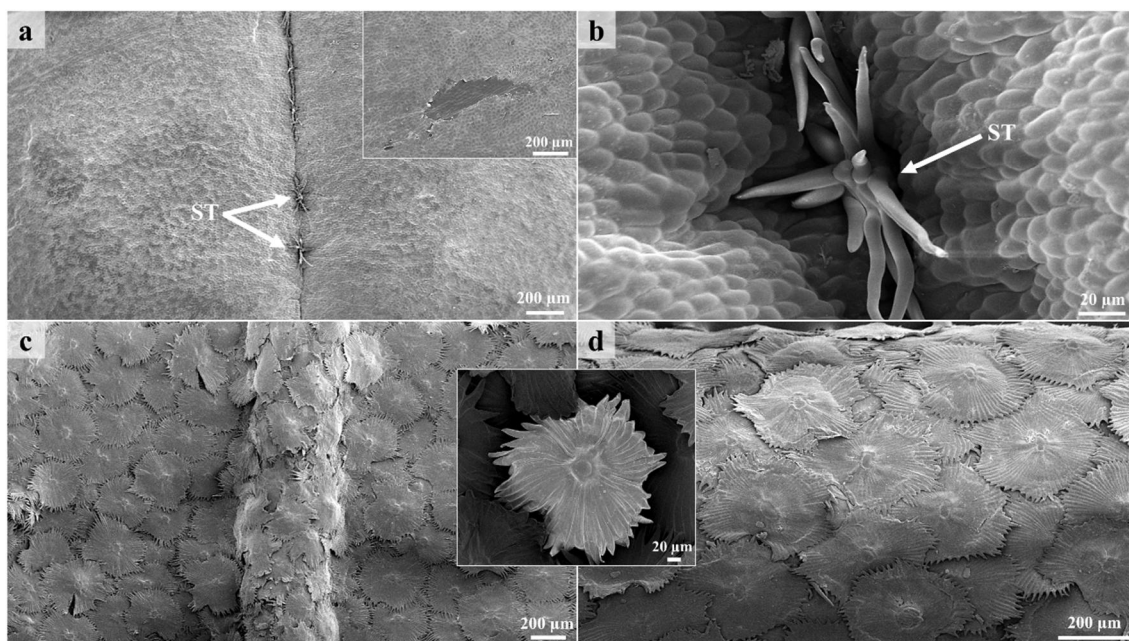
Figure 3.3: Scanning electron micrographs of leaves and stems. a) Adaxial surface showing stellate trichomes along the mid-vein of leaf. Note the peeled cuticle layer on this surface. b) Stellate trichome emerging from middle furrow (mid-vein) on adaxial surface. c) Dense indumentum formed by lepidote trichomes on the lamina and mid-vein on the abaxial surface. d) Lepidote trichomes fully covering stem. ST = Stellate trichome.

3.4.2 Lepidote trichomes

Individual lepidote trichomes were observed at higher magnification (Fig. 3.4). According to Webster et al. (1996), lepidote trichomes are scale-like hairs that are common in *Croton* species. They are similar to the appressed-stellate trichomes. However, the radial cells of lepidote hairs are fused, resulting in their shield-like appearance (Webster et al., 1996).

Vitarelli et al. (2016) suggest that lepidote trichomes function to increase water uptake from atmospheric moisture as the shield-like structure provides a larger surface area for absorption. In addition, the dense indumentum formed by lepidote trichomes may also function to protect the plant from herbivores, pathogens, excessive water loss and increased temperatures, as reported by Werker (2000).

Several authors (Inamdar and Gangadhara, 1977; Liu et al., 2013; Feio et al., 2018) indicated that lepidote trichomes are non-secretory. However, TEM (Fig. 3.6, 3.7) and histochemical analyses (Fig. 3.11) suggest that these structures may have a role in the synthesis and/or accumulation of secretory products. de Sá-Haiad et al. (2009) demonstrated that numerous non-glandular trichomes in several *Croton* species stained positive for various compounds with histochemical

tests. Therefore, the secretory products within the lepidote trichomes may also function as a chemical defence (Levin, 1973; Vitarelli et al., 2016).

The lepidote trichomes develop through a series of anticlinal and periclinal divisions. The resultant structure comprises a multiseriate, multicellular stalk, a multicellular subradial disc, numerous radial cells and a unicellular umbo/central cell (Fig. 3.4, 3.5). This is similar to the structure and development of the lepidote trichomes in *Croton erythroxyloides* described by Vitarelli et al. (2016).

The radial cells of the lepidotes are connected by webbing (Fig. 3.4a, 3.4b), ranging between 80 – 100%. Webster et al. (1996) developed an arbitrary scale to distinguish between the various types of lepidote trichomes. This scale included lepidote trichomes transitioning from stellate types with little webbing to hairs with radii that are completely fused.

Fully developed and developing lepidote trichomes were present on leaves (all developmental stages) and stems. According to Vitarelli et al. (2016) this occurs because of the asynchronisation and early development of these emergences. Younger/developing trichomes appeared to be canopied by the mature/developed lepidote trichomes (Fig. 3.5).
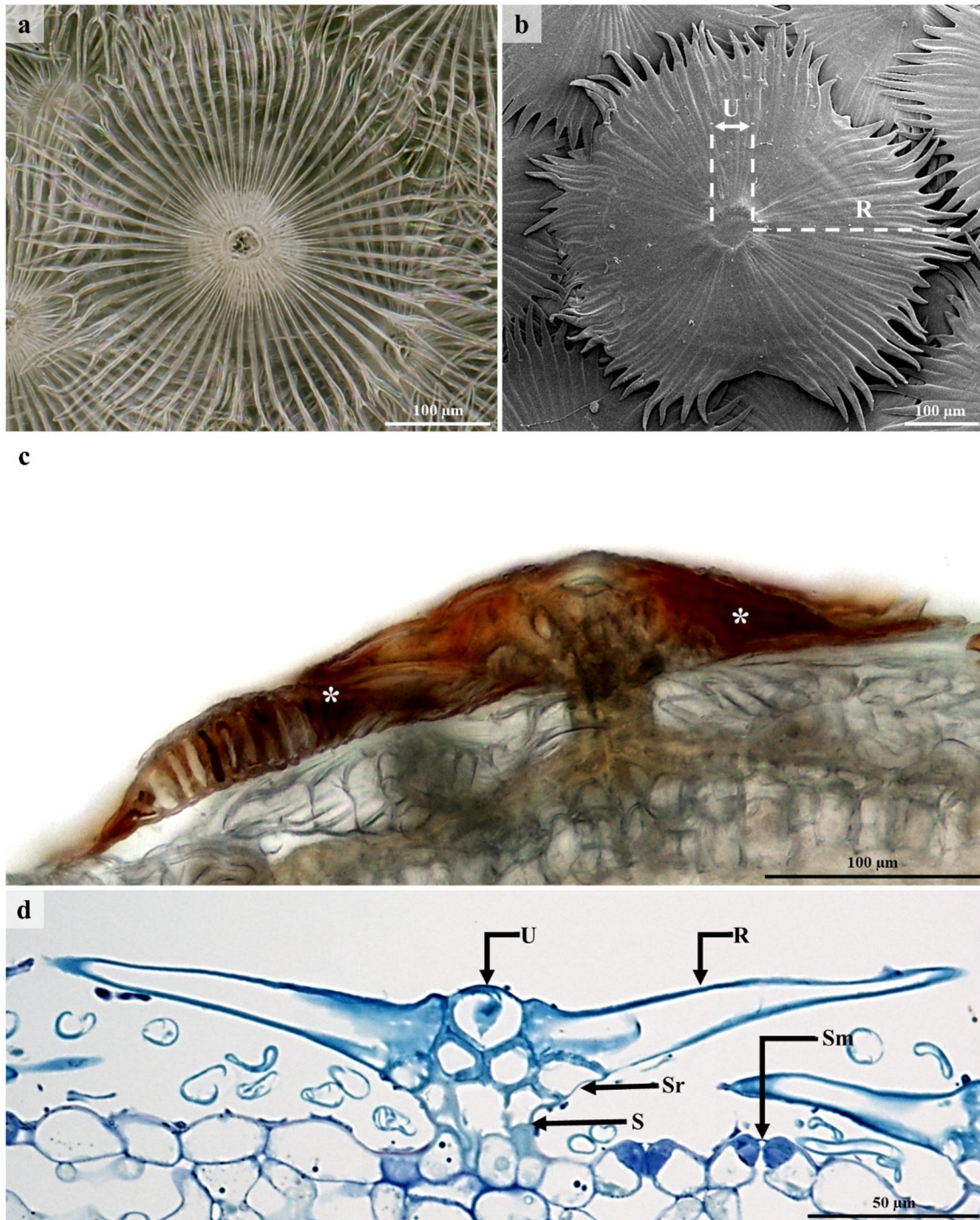
Figure 3.4: Morphology of lepidote trichomes. a) Stereomicrograph of lepidote trichome. b) SEM of lepidote trichome showing umbo/central cell and numerous webbed radial cells. c) Lepidote trichome with accumulated secretory substance. d) Light micrograph of lepidote trichome showing stalk cells, subradial cells, radial cells and umbo/central cell. U = Umbo, R = Radii/Radial cell, Sr = Subradial cell, S = Stalk, Sm = Stoma, * = Secretion.
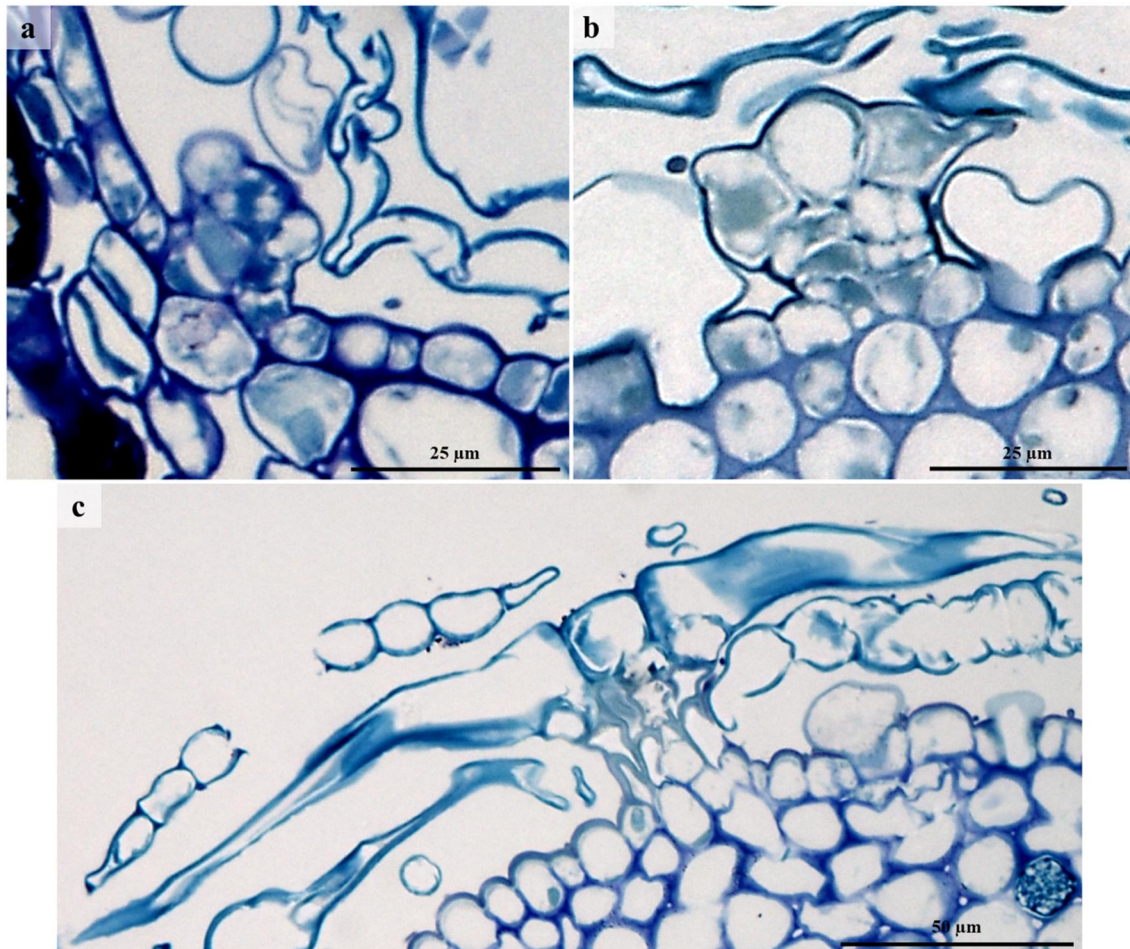
Figure 3.5: Development of lepidote trichomes. a) Emergence of protodermal cells giving rise to lepidote trichome through periclinal and anticlinal divisions. Note the periclinal divisions initiating the development of the stalk and the anticlinal divisions of the radial cells surrounding the central cell. b) Developing lepidote trichome. Note the increased number of stalk cells brought about by additional periclinal divisions and the stretching of the lateral radial cells. c) Fully developed lepidote trichome with stalk, subradial, radial and central cells. Note the elongation of stalk cells into a prominent stalk, the developed subradial cells, the distinct central cell and the extended radial cells.

3.4.3 Ultrastructure of lepidote trichomes

Transmission electron microscopy revealed the presence of various organelles within the stalk and radial cells of lepidote trichomes (Fig. 3.6, 3.7). Stalk cells contained numerous large and small vacuoles which appeared to occupy the bulk of the cell (Fig. 3.7a). Vacuoles were present in the radial cells (Fig. 3.7c). The vacuoles are reported to play a role in processing secretory material (Machado et al., 2005; Huang et al., 2008). Large nuclei with dense nucleoplasm were present in the stalk cells (Fig 3.6a, 3.6b). However, they were not very prominent because of the

surrounding dense cytoplasm (Fig 3.6a, 3.6b). The large vacuoles also constricted the space for the nuclei and appeared appressed to these organelles. Numerous chloroplasts were also observed in the stalk cells. Fahn (1979) states that plastids are the most common organelles involved in the production of lipophilic substances. Werker and Fahn (1981) suggested that large amounts of secretory substances may be produced by chloroplasts.

Stalk and radial cells contained lipid bodies, several vesicles, rough endoplasmic reticulum, Golgi bodies and numerous mitochondria (Fig. 3.6, 3.7). However, within the radii, the cytoplasm and the various organelles were restricted to the periphery of the cell (Fig. 3.7).

Many of the vesicles appeared translucent whilst others contained dense material (Fig. 3.6c, 3.6d, 3.6e, 3.7a, 3.7d). These vesicles in the stalk and radial cells indicate the secretion of hydrophilic substances. Their occurrence close to the plasmalemma suggests that they undergo granulocrine secretion (Tozin et al., 2016). The plasmalemma also appeared sinuous, indicating vesicle fusion (Ascensão et al., 1997). Fahn (1979) indicates that granulocrine elimination of secretions occurs in all secretory cells. Granulocrine secretion is described as the collection of secretory substances in membrane-bound vesicles that either fuse with the plasmalemma or are eliminated by invaginations of the plasmalemma (Fahn, 1979).

According to several authors (Ascensão and Pais 1998; Turner and Croteau 2004; Huang et al., 2008), Golgi bodies in secretory trichomes play a role in the production of acidic and neutral polysaccharides (Werker and Fahn, 1981). It has been suggested that endoplasmic reticulum is also involved in the production of polysaccharides (Werker and Fahn, 1981), by producing the protein component of the secretory product which is then transferred to the Golgi body (Ascensão and Pais 1998). The Golgi body produces the polysaccharide component which is then transported by the vesicles (Ascensão and Pais 1998). Huang et al. (2008) suggest that vesicles that are close to the plasmalemma and Golgi body (Fig. 3.6d, 3.7a, 3.7d), transport the polysaccharide material which is released through granulocrine secretion.

Stalk cells contained normal walls with visible plasmodesmata (Fig. 3.6e). However, the lateral cell walls of the stalk appeared highly cutinised (Fig. 3.6). Ascensão and Pais (1998) suggested that the presence of plasmodesmata enabled the symplastic transport of precursors. The cutinised walls act as an apoplastic barrier to prevent the back-flow of secreted substances as these may be toxic to mesophyll cells (Fahn, 1979; Ascensão et al. 1997; Werker, 2000).

Although lepidote trichomes are regarded as non-secretory, numerous organelles within the stalk and radial cells (Fig. 3.6 and 3.7) indicate high metabolic activity (Naidoo et al, 2014). However, much of the activity was in the stalk of the lepidote trichome. According to Fahn (1979), the

endoplasmic reticulum and Golgi body are involved in the secretion of hydrophilic substances. On the other hand, various organelles, including the nucleus, mitochondria, Golgi body, endoplasmic reticulum, plastids and ground cytoplasm may be responsible for the secretion of lipophilic substances. All these organelles were present in the lepidote trichomes of *C. gratissimus*. Therefore, these observations together with the positive staining for various compounds by histochemical tests, suggest that they are involved in the synthesis and/or accumulation of secondary metabolites. However, more studies are needed to confirm the secretory process in these trichomes.
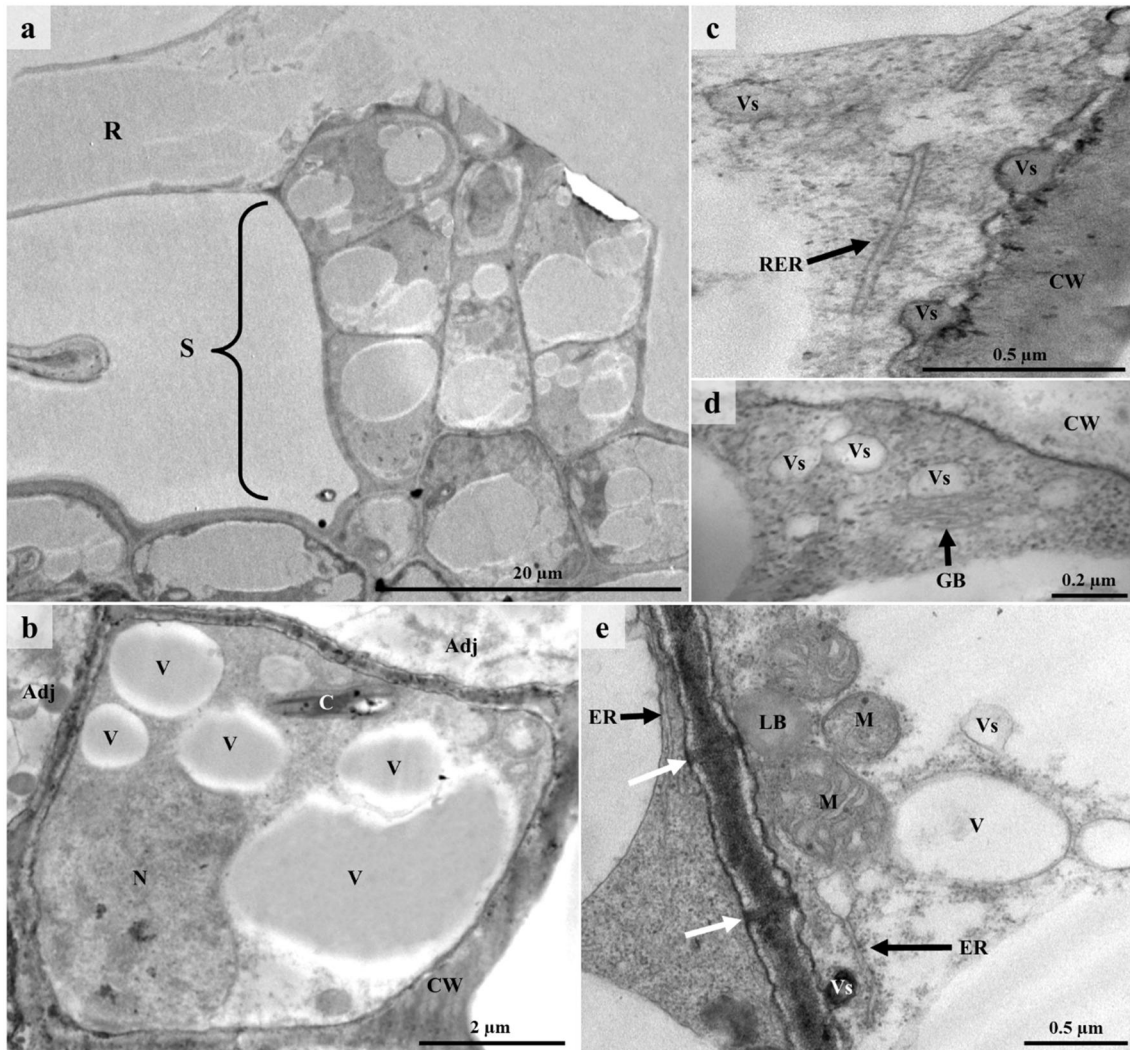
Figure 3.6: Transmission electron micrographs of lepidote trichome stalk cells. a) Section through the stalk cells and radial cell. Large and small vacuoles surrounded by dense cytoplasm and other organelles can be seen in the stalk cells. b) Single stalk cell containing dense cytoplasm with numerous vacuoles, a large nucleus and a chloroplast. c) Rough endoplasmic reticulum and vesicles at the periphery of a stalk cell wall. d) Vesicles and Golgi body present in stalk cell. e) Thick cell wall between two adjacent stalk cells with visible plasmodesmata (white arrows). Vacuoles, numerous mitochondria, endoplasmic reticulum and vesicles can be seen at the periphery of these cells. Note the presence of the electron dense vesicle next to the cell wall. R = Radial cell, S = Stalk, CW = Cell wall, Vs = Vesicle, V = Vacuole, N = Nucleus, M = Mitochondria, RER/ER = Rough Endoplasmic Reticulum/Endoplasmic Reticulum, C = Chloroplast, GB = Golgi body, LB = Lipid body, Adj = Adjacent cells.
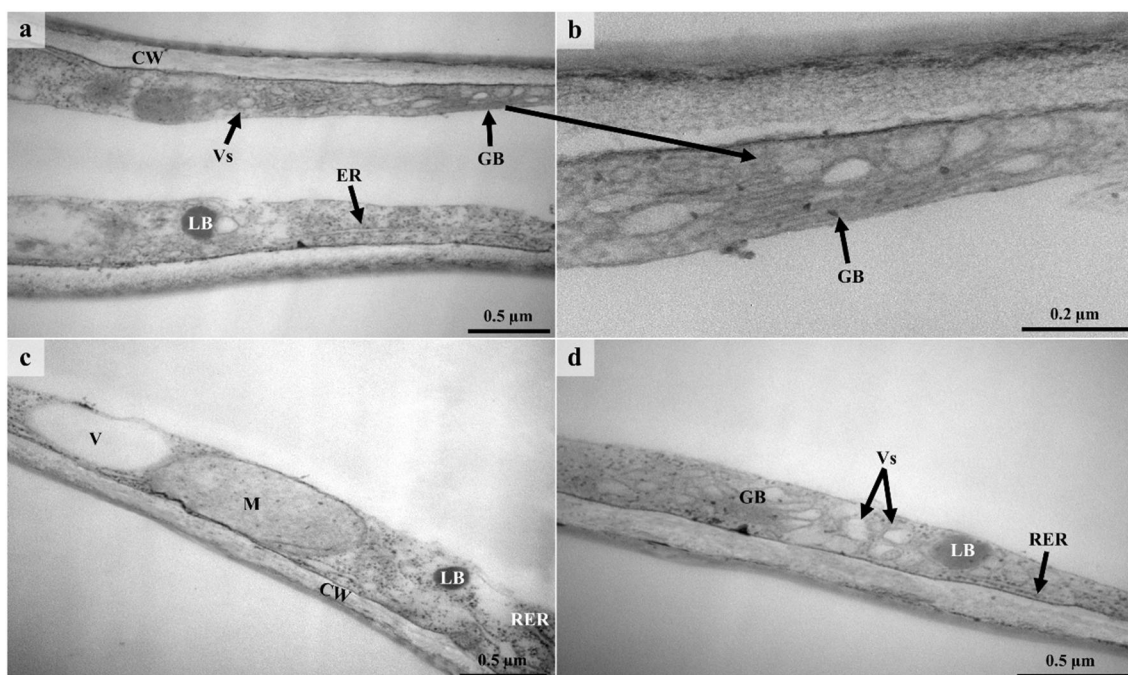
Figure 3.7: Transmission electron micrographs of lepidote trichome radial cells. a) Radial cell with thickened cell wall containing dense cytoplasm with vesicles, Golgi body, a lipid body and rough endoplasmic reticulum at the periphery. b) Higher magnification of Golgi body surrounded by dense cytoplasm. c) Vacuoles, mitochondria, lipid body and rough endoplasmic reticulum present along the radial cell wall. d) Golgi body, rough endoplasmic reticulum, a lipid body and numerous vesicles along the periphery of a radial cell wall. CW = Cell wall, Vs = Vesicle, V = Vacuole, M = Mitochondria, RER/ER = Rough Endoplasmic Reticulum/Endoplasmic Reticulum, GB = Golgi body, LB = Lipid body.

3.4.4 Glandular trichomes

Glandular trichomes were randomly distributed on the abaxial surfaces of emergent, young and mature leaves and stems (Fig. 3.8). According to several authors (Wagner et al., 2004; Choi and Kim, 2013; Glas et al., 2012; Huchelmann et al., 2017), glandular trichomes are involved in the production, secretion and accumulation of various secondary metabolites. A study by Vitarelli et al. (2015) also reported secretory trichomes on the abaxial surfaces of *Croton* species. In the present investigation, they also occurred on the extrafloral nectaries of the leaf. According to Webster et al. (1996), glandular trichomes exist in a limited number of *Croton* species and may occur on either one or both leaf surfaces. Webster et al. (1996) described glandular trichomes as "small embedded epidermal glands" and suggested that they contain terpenes which are responsible for the aroma when the leaves are crushed.

These glandular trichomes were canopied under layers of lepidote trichomes (Fig. 3.8d). Light micrographs indicate that they comprise a single cell and are embedded in the epidermal layer

(Fig. 3.8d). These unicellular glandular trichomes formed a prominent stalk and dilated head (Fig. 3.8d). This is consistent with the secretory trichomes of *Croton* species identified by Vitarelli et al. (2015). Unicellular glandular trichomes consisted of a narrow short stalk and an expanded distal end. The glandular trichomes existed in various forms as there is limited space to develop beneath the dense lepidote trichomes.

Light microscopy and SEM indicated paracytic stomata on the adaxial surface of leaves (Fig. 3.4d, 3.8d). These stomata are a common character in Euphorbiaceae, occurring in many of its species. Thakur and Patil (2014) state that paracytic stomata are primitive, whilst other types present in the family are a derivative of this. A study by de Sá-Haiad et al. (2009) revealed that paracytic stomata are predominant in *Croton* species.
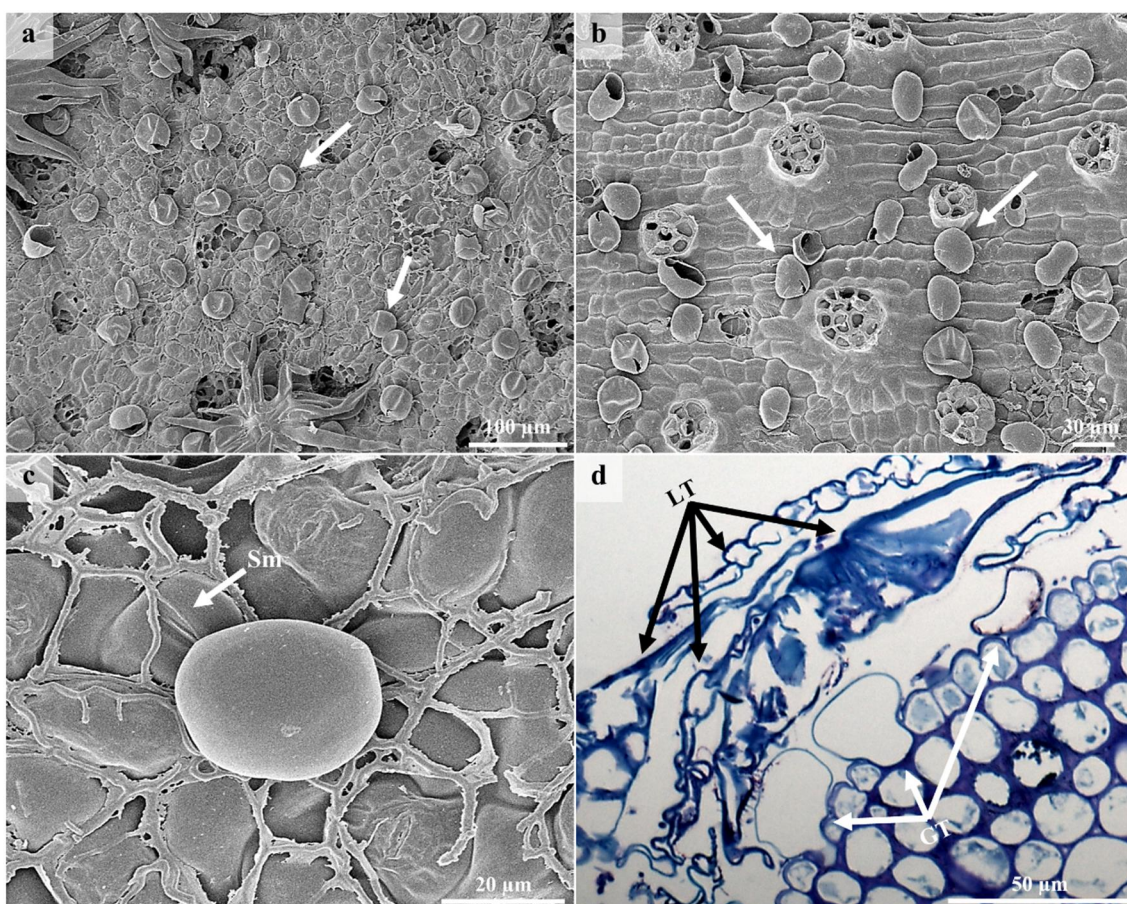
Figure 3.8: Micrographs showing glandular trichomes on the leaves and stems. a) Glandular trichomes on abaxial surface of leaves beneath lepidote trichomes. b) Stem showing glandular trichomes after removing lepidote trichomes. High magnification of single glandular trichome on abaxial surface. Note the presence of paracytic stomata. d) Light micrograph showing unicellular glandular trichomes of different forms canopied by several layers of lepidote trichomes. Sm = Stoma, LT = Lepidote Trichome, GT = Glandular Trichome.

3.4.5 Laticifers

A single laticifer type was observed throughout all leaf developmental stages and in stems (Fig. 3.9, 3.10). Within the Euphorbiaceae, latex and laticifer distribution are characters used to broadly classify the family (Wurdack et al., 2005), due to their presence in several genera (dos Santos Alves et al., 2017). The laticifers were present in the mid-vein of the leaf and were predominantly associated with the vascular tissue, with occasional occurrences in parenchyma (Fig. 3.9a, 3.9c). In the stems, the laticifers were predominant in the phloem and pith (Fig. 3.9b, 3.9d). This is consistent with other work as laticifers are typically associated with the vascular tissues, more specifically the phloem, but may also occur in the pith, cortex and foliar mesophyll (Dickison, 2000; Castro and Demarco, 2008; Lange, 2015). However, in this study, laticifers were not observed in the foliar mesophyll.

Both non-articulated (branched and unbranched) and articulated laticifers have been reported to occur in Euphorbiaceae (Hagel et al., 2008; Demarco et al., 2013). However, non-articulated laticifers are more common and widespread in the family compared to the articulated type (Demarco et al., 2013). In the present study, laticifers appeared non-articulated and unbranched as they were composed of a single row of cells without branches (Fig. 3.10a). According to Lange (2015), non-articulated laticifers are cells that develop from a single cell. It is suggested that they form through apical intrusive growth (Da Cunha et al., 1998). The cell divides ceonocytically, resulting in an elongated, multinucleated structure (Rudall, 1989; Lange, 2015). Studies by Rudall (1994), de Sá-Haiad et al. (2009) and Feio et al. (2016) revealed the presence of non-articulated laticifers in several *Croton* species.

Longitudinal and transverse monitor sections stained with Toluidine-Blue revealed latex within laticifer cells (dark stained contents) (Fig. 3.10a, 3.10b). Fresh latex from the leaves and stems of *Croton* was difficult to identify as the exudate was a clear watery sap. The latex of laticifers is actually the protoplast of these cells. This protoplast which contains the metabolites, is housed within a larger central vacuole (Castro and Demarco, 2008; Prado and Demarco, 2018). These compounds may function to protect the plant against herbivores and pathogens (Prado and Demarco, 2018).

Scanning electron micrscopy of freeze-fractured material also indicated latex within laticifer cells (Fig. 3.10c). Coagulation of the latex within the cells was probably due to decrease of turgor pressure within these cells during the preparation of the material. Generally, the pressure of latex within laticifer cells is high. When there is a sudden drop in pressure, the surrounding turgid cells compress the laticiferous cell, releasing the latex (Southorn, 1969) which polymerises when

exposed to air (Prado and Demarco, 2018). This coagulation of latex seals plant wounds, thus indicating its ability to act as a physical barrier (Demarco, 2015).
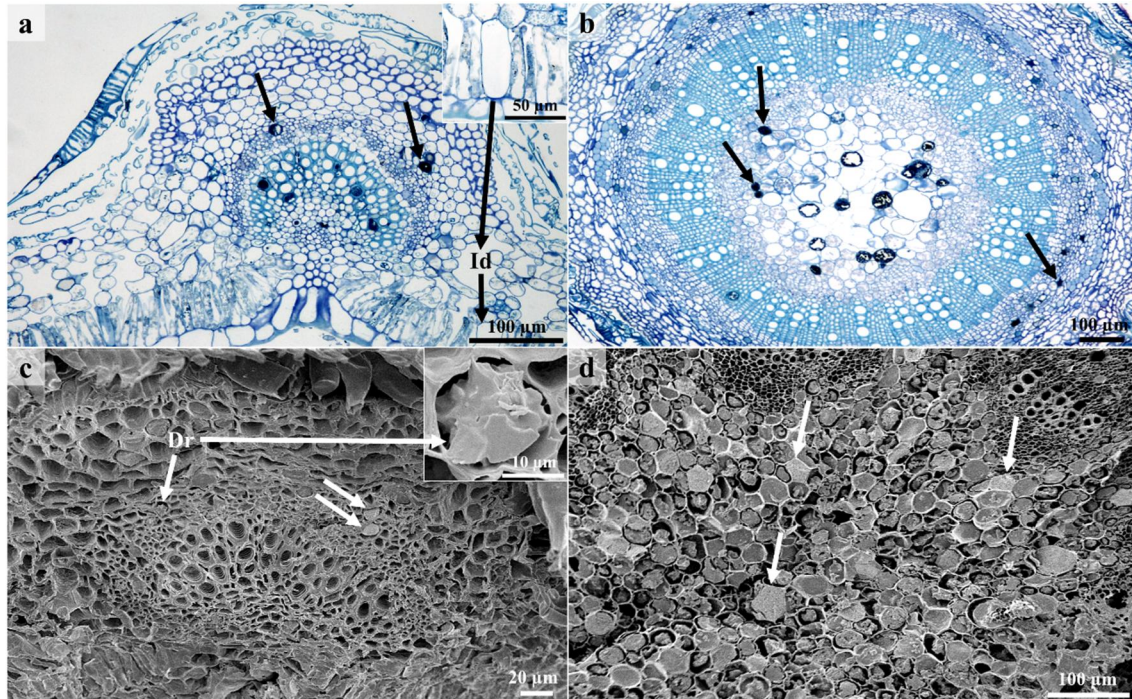
Figure 3.9: Laticifer distribution in leaves and stems. a) Transverse section of leaf stained with Toluidine-Blue showing distribution of laticifers predominantly in the vascular tissue. Note the idioblasts at the adaxial side of the leaf. b) Transverse section of the stem stained with Toluidine-Blue showing laticifers in the phloem and pith. c) Scanning electron micrograph of coagulated latex within laticifer cells (associated with phloem). Druse crystals are also present in the leaf section. d) Transverse section through stem showing latex containing laticifers in pith. Id = Idioblast, Dr = Druse crystal.
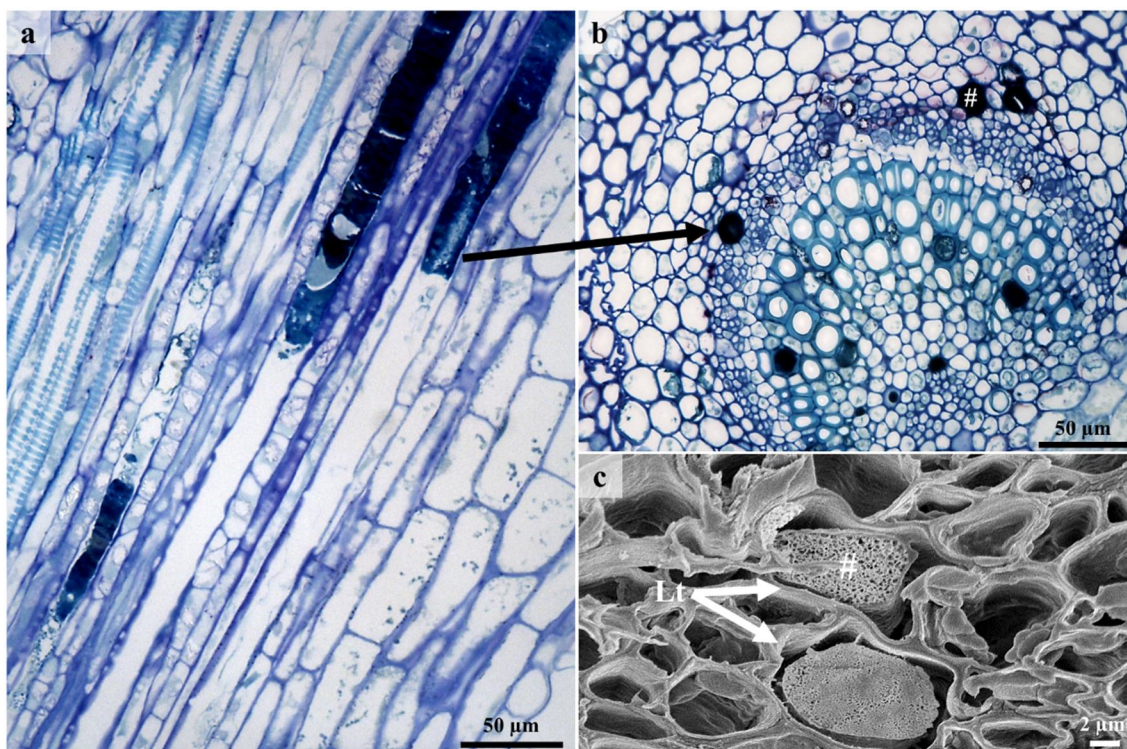
Figure 3.10: Laticifer cells showing secretory contents. a) Longitudinal section of leaf showing latex within non-articulated laticifers. b) Light micrograph of transverse section showing laticifer cells with latex contents. c) Freeze- fracture through laticifer cells containing coagulated latex. Lt = Laticifer, # = Latex.

3.4.6 Histochemistry and fluorescence microscopy

Histochemical and fluorescence analyses of the lepidote and glandular trichomes and laticifer cells revealed the presence of hydrophilic and lipophilic substances. Appropriate controls were also conducted (results not presented). The presence of these secondary metabolites indicates that these three structures may be responsible for the production of biologically active compounds that are used in traditional medicine (Salatino et al., 2007).

Lepidote trichomes are generally classed as non-glandular (Inamdar and Gangadhara, 1977; Liu et al., 2013; Feio et al., 2018), and non-secretory (Wagner et al., 2004). However, these trichomes tested positive for various compounds (Fig. 3.11).

In lepidote trichomes, subradial and central cells appeared lignified after staining with Toluidine-Blue and phloroglucinol (Fig. 3.11g, 3.11h). A yellow fluorescence emitted by these cells after staining with acridine orange also indicated the presence of lignified cells (Fig. 3.11j). Lignified cells were also observed in glandular trichomes (blue autofluorescence) indicated in Fig. 3.12e. The lateral walls of the lower stalk cells of lepidote trichomes stained orange with Sudan III, indicating cutinised walls (Fig 3.11e). Cells that are cutinised or lignified typically act like

endodermal cells/Casparian strips and prevent the apoplastic flow of water, or the back-flow of secreted substances (Fahn, 1979; Werker, 2000). The cell walls of the subradial, central and radial cells of lepidote trichomes contained pectinaceous substances as they stained pink with ruthenium red (Fig. 3.11d). The pectin provides support and strengthens the structure of these trichomes. Pectin may also aid in plant defence as it induces phytoalexin accumulation which possesses antimicrobial properties (Voragen et al., 2009).

Both trichome types and laticifers were found to possess various compounds, with alkaloids, phenolic compounds and lipids common among all three.

Lepidote trichomes (stalk, subradial cells, radii and central cell/umbo), glandular trichomes and laticifers stained orange/brown with Wagner's reagent, indicating the presence of alkaloids (Fig. 3.11a, 3.12a, and 3.13a). *Croton* species have been reported to contain an abundance of active alkaloids (Salatino et al., 2007). Alkaloids are common among angiosperms and are considered to be the most active, diverse and therapeutic secretory compounds (Wink, 2015; Roy, 2017). Their main function is to provide chemical defence against herbivores and pathogenic microorganisms due to their toxic nature (Wink, 2015; Roy, 2017; Debnath et al., 2018). In addition, plants containing alkaloids have been used for centuries in medicine to treat various ailments, due to their medicinal and pharmacological properties, with many of these now isolated and marketed as drugs (Roy, 2017; Bribi, 2018; Debnath et al., 2018).

Positive reactions for phenolic compounds were observed in the lepidote trichomes (stalk, subradial cells, radii and central cell/umbo) and glandular trichomes, and laticifers, which all produced a dark brown to black precipitate after staining with ferric chloride (Fig. 3.11b, 3.12b, 3.13c). Phenolics were also detected using auto-fluorescence as the stalk of lepidote trichomes fluoresced blue under UV light. Salatino et al. (2007) state that phenolic compounds are common among *Croton* species. These compounds defend the plant against pathogens, parasites and predators (Huang et al., 2009), and may also play a role in pollination (Lin et al., 2016). Furthermore, phenolic compounds from medicinal plants used in traditional medicine are known to possess biological and pharmacological activities (Huang et al., 2009; Maslennikov et al., 2014).

Lipidic compounds were detected using Sudan III, Sudan black and Nile blue. Laticifers (Fig. 3.13f) and cells comprising lepidote trichomes (Fig. 3.11e) stained orange with Sudan III, indicating the presence of lipid components. Demarco et al. (2013) suggested that the lipid component of latex has the ability to coagulate, demonstrating its function to seal wounds. A positive reaction was also observed in glandular trichomes with Sudan III which stained lipid inclusions orange-red (Fig. 3.12d). Sudan black stained lipids dark blue in the stalk, subradial,

radial and central cell of lepidote trichomes (Fig. 3.11f). Nile blue was used to detect acidic and neutral lipids. Subradial, radial and central cells of lepidote trichomes (Fig. 3.11g) and laticifer cells (Fig. 3.13b) stained blue, indicating a positive reaction for acidic lipids. The stalk cells of lepidote trichomes (Fig. 3.11c) and glandular trichomes (Fig. 3.12c) stained pink which was a positive indication for neutral lipids. The presence of lipids in these external structures is in accordance with literature as lipophilic substances are usually secreted by glandular trichomes (Valkama et al., 2003).

Alkaloids, lipids and phenolic compounds were also detected in the laticifers of *C. echinocarpus* and *C. urucurana* (Feio et al. 2016). Feio et al. (2016) suggested that the phenolic compounds and alkaloids present in these species are responsible for their biological activities.

Sections stained with ruthenium red revealed the presence of mucilaginous substances in laticifers which was indicated by a pink colouration in the cells (Fig. 3.13d). According to Demarco et al. (2013), mucilage is common in the latex of euphorbiaceous species and may play a role in wound sealing (Fisher et al., 2009; Kuster et al., 2016). Staining with Toluidine-Blue resulted in an intensely dark blue/purple colouration of laticifers (Fig. 3.13e), indicating that these cells contain macromolecules with free phosphate groups.

In the present study, light and SEM micrographs (Fig. 3.9c, 3.13d) indicated druse (Dr) and prismatic crystals (Pr) in the leaves of *C. gratissimus*. The organs and tissues of numerous plant species possess calcium oxalate crystals (Franceschi and Horner, 1980). These deposits are housed within vacuoles of specialised cells known as crystal idioblasts (Pennisi et al., 2001). Within a crystal idioblast, there is great variation in the number, shape and size of the crystals (Franceschi and Horner, 1980; Konyar et al., 2014). However, common shapes include the druse, styloid, raphide, prism and crystal sand (Franceschi and Horner, 1980; Konyar et al., 2014). These crystals have been used as taxonomic tools due to the specificity of the shape and location within a taxon (Franceschi and Horner, 1980; Konyar et al., 2014). Solereder (1908) stated that oxalate of lime (primitive term for calcium oxalate) is present in many forms in various genera of Euphorbiaceae. Calcium oxalate crystals appear to have various functions. These include removing excess calcium and oxalate to maintain ionic balance and prevent toxicity (metal detoxification) of the plant, tissue support, and protection against foraging herbivores (Franceschi and Horner, 1980; Nakata, 2002; Konyar et al., 2014).

In addition, secretory idioblasts were also identified at the adaxial side of leaves, as shown in Fig. 3.13a. Idioblasts are secretory cells that are noticeably different from surrounding cells, differing by size, form, metabolism, development and content (Esau, 1965; Castro and Demarco, 2008; Bosabalidis, 2014). These cells contain various substances such as oils, tannins, phenolic

compounds, mucilage, gums, resins and crystals (Esau, 1965; Dickison, 2000; Castro and Demarco, 2008; Bosabalidis, 2014). According to Solereder (1908) internal oil or resin secreting cells may result in the presence of transparent dots on the leaf surface. Idioblasts in *Croton* species have been reported to contain lipophilic substances (de Sá-Haiad et al., 2009; Vitarelli et al., 2015). This might suggest that the secretory idioblasts present in leaves, were probably responsible for the translucent dots observed on this surface (Fig. 3.2a). However, more detailed investigations need to be carried out on these cells to confirm this.
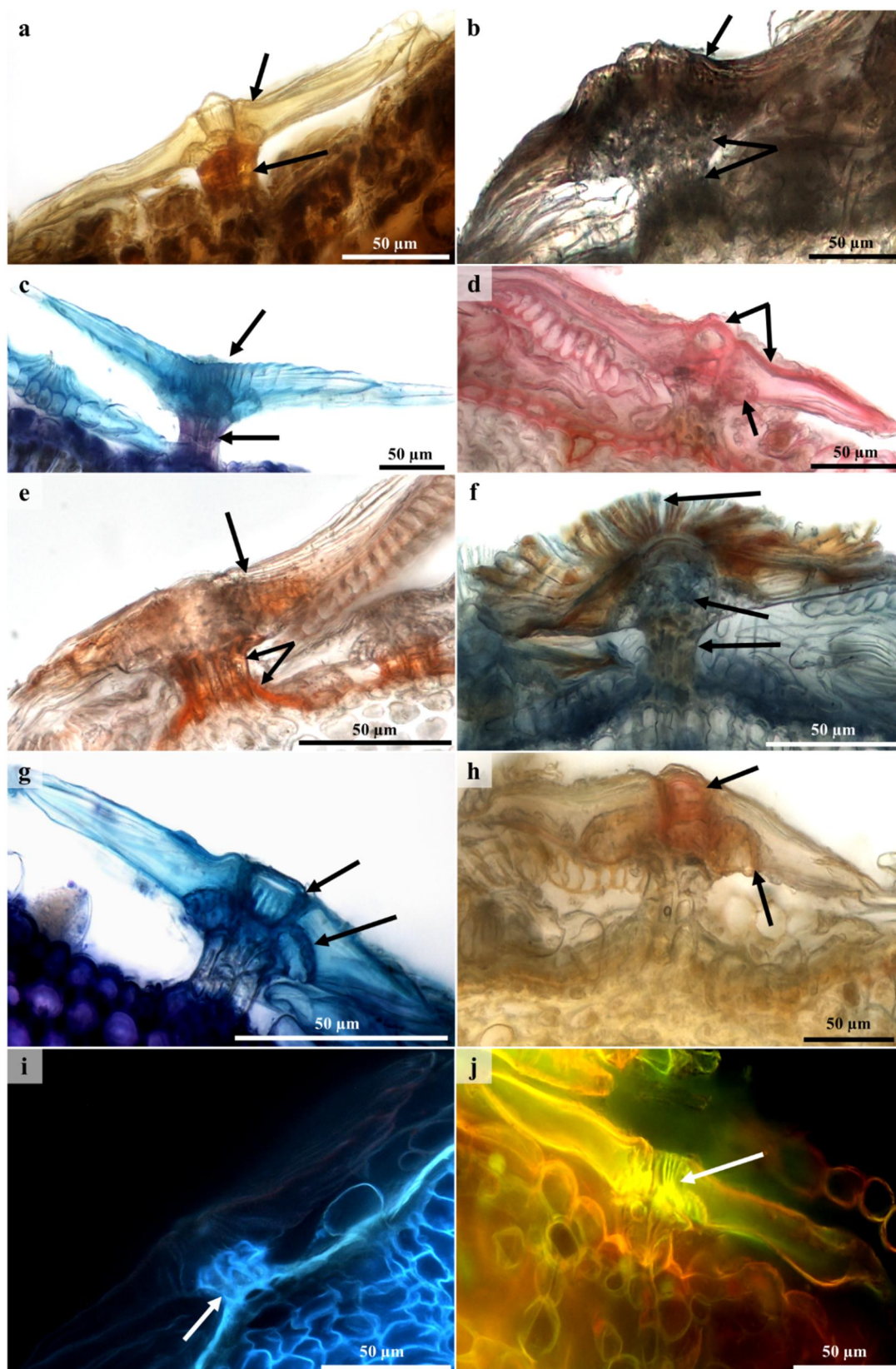
Figure 3.11: Histochemical and fluorescence micrographs showing chemical compounds of lepidote trichomes. a) Orange/brown colouration of stalk (intense), subradial, radial and central

cells (weak) suggest a positive indication for the presence of alkaloids with Wagner's reagent. b) Phenolics detected in stalk, subradial cells, radii and central cell with ferric chloride (brown to black precipitate). c) Pink colouration indicated neutral lipids in stalk cells and blue colouration of subradial, radial and central cells indicated acidic lipids with Nile blue. d) Pectin in the subradial, radial and central cell walls was indicated by a pink colour. e) Orange staining of the stalk and radii with Sudan III indicated the presence of cutinised walls and lipids. f) Positive staining for lipids in the stalk, subradial and radial cells with Sudan black. g) Toluidine-Blue revealed lignification of the subradial and central cells (blue colouration). h) Positive indication of lignin in the subradial and central cells with phloroglucinol. i) Blue autofluorescence indicated phenolic compounds in stalk cells. j) Yellow fluorescence with acridine orange revealed lignified subradial and central cells.
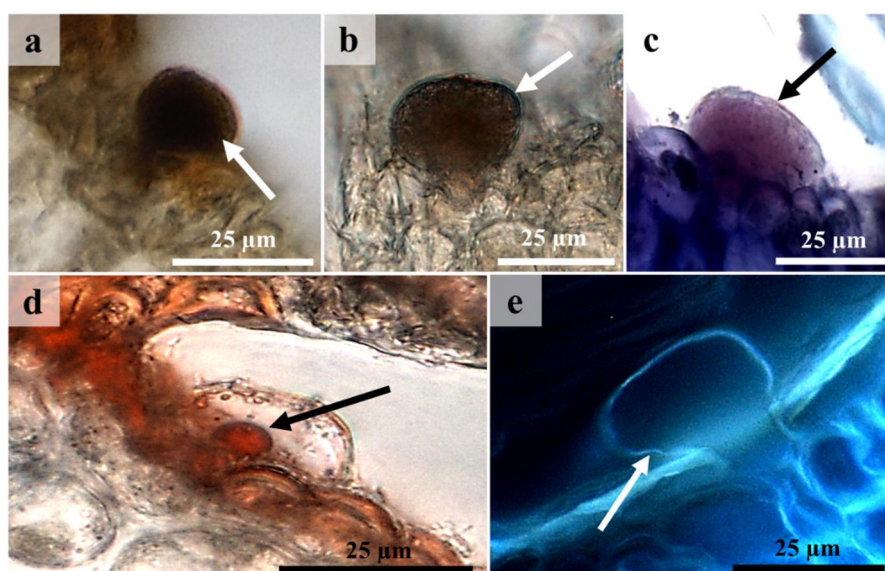
Figure 3.12: Histochemical and fluorescence micrographs showing chemical compounds of glandular trichomes. a) Positive staining for alkaloids (brown colour) with Wagner's reagent. b) Glandular trichomes tested positive for phenolic compounds with ferric chloride (indicated by brown/black precipitate). c) Pink colouration indicated neutral lipids with Nile blue. d) Lipid droplet stained red/orange with Sudan III. e) Lignified cell walls of glandular trichome detected with autofluorescence.
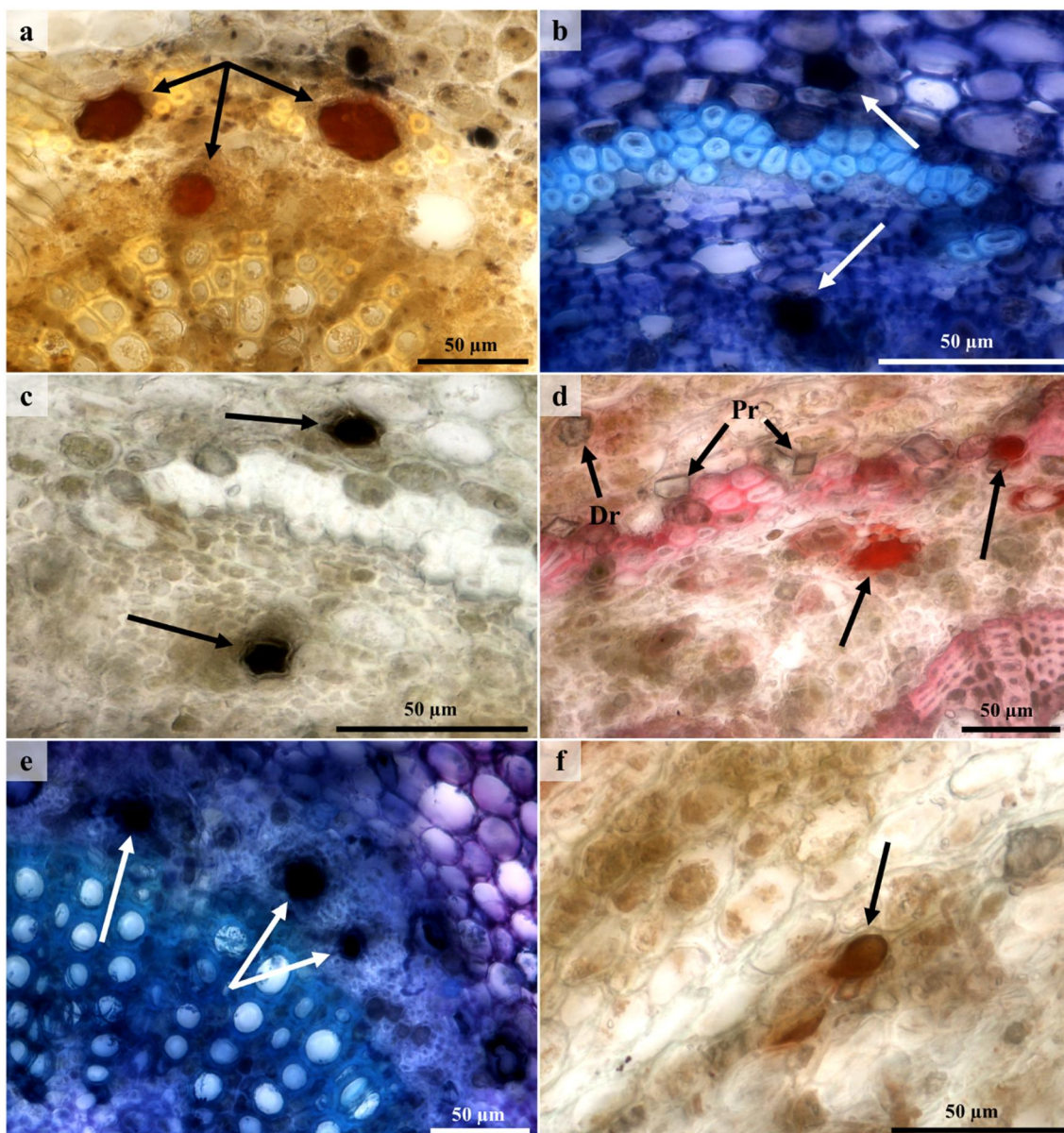
Figure 3.13: Histochemical and fluorescence micrographs showing chemical compounds of laticifer cells. a) Orange colouration a positive indication for alkaloids with Wagner's reagent. b) Blue colouration within laticifer cells indicated acidic lipids with Nile blue. c) Positive indication (dark brown to black) for phenolic compounds with ferric chloride. d) Pink colouration indicated mucilage with ruthenium red. Note the presence of druse and prismatic crystals. e) Blue staining of laticifer cells with Toluidine-Blue indicated macromolecules with free phosphate groups. f) Positive stain (orange colour) for lipids with Sudan III. Pr = Prismatic crystal, Dr = Druse crystal.

3.5 Conclusion

Three types of structures, lepidote and glandular trichomes, and non-articulated, unbranched laticifers are present on/in the leaves and stems of *C. gratissimus*. These structures were found to

contain various secondary compounds which are reported to protect the plant from herbivores and pathogens. Furthermore, the secretory products present within trichomes and laticifers may contribute to the medicinal properties of the plant as some of them are known to possess biological and pharmacological activities. Although these structures were found to play a role in the production and/or accumulation of secondary metabolites, further work is required to identify their mode of synthesis. In addition, crystal and secretory idioblasts, extrafloral nectaries and stellate trichomes were also identified on the leaves of *C. gratissimus*. However, further investigations need to be conducted to determine whether these structures are also involved in the production/accumulation of compounds that contribute to the biological activity of the plant.

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CHAPTER 4: PHYTOCHEMICAL AND ANTIBACTERIAL ANALYSES OF *CROTON GRATISSIMUS* BURCH. VAR. *GRATISSIMUS* (EUPHORBIACEAE) LEAF AND STEM EXTRACTS

4.1 Abstract

Croton gratissimus is commonly used in traditional medicine to treat various ailments. This study was conducted to determine the chemical composition of the leaf and stem extracts, evaluate its antimicrobial potential and identify compounds that are responsible for the biological activity of this species. Preliminary phytochemical screening revealed alkaloids, amino acids, phenolic compounds, flavonoids, carbohydrates, terpenoids, saponins and fixed oils and fats in the leaf and stem extracts. Antibacterial assays indicated weak to strong activities of the methanolic extracts, with stem extracts displaying stronger activity than the leaves. Several bioactive compounds were present in the leaf and stems extracts. Many of these possess antimicrobial and antibacterial activities. The presence of these compounds in the methanolic extracts was probably responsible for the activity demonstrated in this study. These findings validate the use of *C. gratissimus* in traditional medicine and indicate its potential as a source of antimicrobial agents.

Keywords: Terpenoids, bacterial strains, bioactive compounds, antimicrobial agents, biological activities.

4.2 Introduction

Plants are known to produce a diverse range of bioactive compounds such as alkaloids, terpenoids, saponins and phenolic compounds (Chikezie et al., 2015; Altemimi et al., 2017). These bioactive compounds are reported to possess therapeutic properties, some of which include antidiabetic, anticarcinogenic, anti-inflammatory, antioxidant, antimicrobial and antimalarial activity (Chikezie et al., 2015). The use of these plants in traditional medicine has led to the discovery of many important drugs such as morphine (*Papaver somniferum* L.), atropine (*Atropa belladonna* L.), colchicine (*Colchicum autumnale* L.), quinine *(Cinchona ledgeriana* Moens ex. Trimen) and reserpine (*Rauvolfia serpentina* (L.) Benth ex. Kurz) (Fabricant and Farnsworth, 2001). For this reason, studies on traditional medicinal plants are important precursors in the development of novel drugs (Heamalatha et al., 2011; Mustafa et al., 2017).

Plants used in traditional medicine to treat microbial related diseases are great sources of antimicrobial agents (Samy and Gopalakrishnakone, 2010). Several studies have demonstrated the antimicrobial properties of species in *Croton* (Fontenelle et al., 2008; Bayor et al., 2009; Selowa et al., 2010; Fernandes et al., 2013; Obey et al., 2016; Leite et al., 2017).

The species *C. gratissimus* Burch. var. *gratissimus* belonging to the Euphorbiaceae (Robert et al., 2010; PlantZAfrica, 2018) has a reputation of being medicinally important (Van Vuuren and Viljoen, 2008). Several parts of this plant have been used in traditional medicine for the treatment of various ailments (Van Vuuren and Viljoen, 2008; Mulholland et al., 2010; Robert et al., 2010; Pudumo et al., 2018). Previous studies on the plant extracts, isolated compounds and essential oils have revealed cytotoxic (Block et al., 2002; Block et al., 2004; Mulholland et al., 2010; Lawal et al., 2017), antiulcerogenic (Okokon et al., 2011), antidiabetic (Okokon et al., 2006; Kumar et al., 2017), analgesic (Okokon and Nwafor, 2010b) antipyretic (Okokon and Nwafor, 2010b), antimicrobial (Abo et al., 1999; Van Vuuren and Viljoen, 2008; Okokon and Nwafor, 2010a; Van Vuuren and Naidoo, 2010; Mthethwa et al., 2014; Lawal et al., 2017), antioxidant (Abdalaziz et al., 2016), antiplasmodial (Okokon and Nwafor, 2009) and ant-HIV-1 (Mthethwa et al., 2014) activities.

There is a growing need to develop new antimicrobials from natural sources such as medicinal plants due to the rise in antibiotic resistance and shortage of novel antimicrobial agents (Cowan, 1999; Abdallah, 2011; Elisha et al., 2017). Antibacterial screening is a good starting point in determining whether medicinal plants used in traditional medicine are sources of antimicrobial agents (Samy and Gopalakrishnakone, 2010). Therefore, this study determined the chemical composition of the leaf and stem extracts of *C. gratissimus* and evaluated the antibacterial activity

of methanolic extracts. In addition, the possible compounds responsible for antibacterial, anti-inflammatory, antioxidant and anticancer activities were also identified.

4.3 Materials and Methods

4.3.1 Plant collection and sampling

Leaves and stems of *C. gratissimus* Burch. var. *gratissimus* were collected from the University of KwaZulu-Natal, Westville Campus (29°49'08.1"S 30°56'38.9"E). The material was air dried for two months before being ground to a powder for phytochemical extraction. A voucher specimen (*Croton* 01 – Accession No. 18224) was prepared and deposited in the Ward Herbarium located in the School of Life Sciences at the University of KwaZulu-Natal, Westville Campus.

4.3.2. Crude extracts

Powdered leaf and stems were placed in separate round bottom flasks and boiled through continuous reflux with a graded series of solvents (hexane, chloroform and methanol) ranging from non-polar to polar. Three 3 h sessions were carried out for each solvent. The extracts were filtered and used for preliminary phytochemical screening and thin layer chromatography (TLC).

Methanolic leaf and stem extracts using high performance liquid chromatography (HPLC) grade solvent was obtained in the same way as above for Gas Chromatography-Mass Spectrometry (GC-MS) analysis and antibacterial assays.

4.3.3 Preliminary phytochemical screening

The hexane, chloroform and methanol crude extracts were used to carry out preliminary phytochemical screening using standard protocols (Raaman, 2006; Benmehdi et al., 2012; Hossain et al., 2013; Morsy, 2014). Identification of the following phytochemical compounds was as follows:

- **Alkaloids**

Wagner's

Extracts were treated with Wagner's reagent. A reddish/brown precipitate indicated the presence of alkaloids.

Dragendorff's

Dragendorff's reagent was added to a few mL of each extract. A red precipitate indicated the presence of alkaloids.

- **Carbohydrates**

Molisch's

For each extract, two drops of alcoholic α-naphthol solution were added to a test tube with one mL of filtrate. Thereafter, 2 mL of concentrated sulphuric acid were added to the side of the test tube. The formation of a violet ring at the junction indicated the presence of carbohydrates.

Benedict's

One mL of each extract was treated with Benedict's solution and heated in a water bath. A reddish precipitate indicated the presence of reducing sugars.

Fehling's

One mL each of Fehling's A and B solutions was added to one mL of each extract and boiled in a water bath. A red precipitate indicated the presence of reducing sugars.

- **Flavonoids**

Each extract was treated with a few drops of lead acetate solution. A yellow coloured precipitate was a positive indication for the presence of flavonoids.

- **Phenolic compounds**

Five mL of distilled water were added to each extract. Thereafter, a few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence if phenolic compounds.

- **Saponins**

Twenty mL of distilled water were added to each extract and shaken for 15 min. The formation of a foam layer was a positive indicator for saponins.

- **Fixed fats and oils**

A few drops of each extract were pressed between two filter papers. Oil stains on the filter paper was a positive indicator for fixed oils.

- **Terpenoids**

Chloroform (2 mL) was added to 5 mL of each extract. Subsequently, 3 mL of concentrated sulphuric acid were carefully added to form a distinct layer. A reddish brown colour at the interface was a positive indicator for the presence of terpenoids.

- **Amino Acids**

Ninhydrin reagent was added to each extract and boiled in a water bath for a few mins. A characteristic blue-violet colour indicated the presence of amino acids or proteins.

4.3.4 Thin Layer Chromatography (TLC)

Hexane, chloroform and methanol extracts of leaves and stems were used for TLC analysis. Each sample was pipetted (10 µL) onto a precoated silica gel 60 F_{254} TLC plate (E. Merck). The plate was then placed in a mobile phase of Toluene: Ethyl acetate: Formic acid (9.5: 0.7: 0.3) and allowed to develop. The plate was then visualised and imaged under 254 nm and 365 nm UV light. Thereafter the plate was dipped in anisaldehyde-sulphuric acid (ANS) reagent and heated before being imaged. The reagent was made up of 0.5 mL ANS, 10 mL Glacial acetic acid, 85 mL methanol and 5 mL concentrated sulphuric acid.

4.3.5 Gas Chromatography-Mass Spectrometry (GC-MS)

Analysis of methanolic leaf and stem extracts was carried out using GC-MS (QP-2010 SE Shimadzu, Japan), with an Rx_5Sil MS fused silica column (Restek) in scan mode. The flow rate of the carrier gas (helium) was 0.96 mL/min, with a total flow of 4.9 mL/min and linear velocity of 36.7 cm/s at 3.0 mL/min purge flow. An injection volume of 1 µL was used in a splitless injection mode. The ion source and interface temperatures were 240°C and 280°C respectively. The initial oven temperature was set at 50°C and held for 1 min. Thereafter it was increased to 310°C at a rate of 10°C/min and held for 10 min. Total running time of GC-MS analysis was 37 min.

4.3.6 Preliminary antibacterial assays

- **Bacterial strains**

The antibacterial properties of the methanolic leaf and stems extracts were evaluated against eight bacterial strains. Three gram negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia coli* – ATCC 25218) and five gram positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus aureus* – ATCC 29213, Methicillin-Resistance *Staphylococcus aureus* (MRSA) – clinical type, Methicillin-Resistance *Staphylococcus aureus* (MRSA) – environmental type) bacterial strains were used along with positive (Streptomycin – gram positive, gentamycin – gram negative) and negative (methanol) controls.

- **Inoculum preparation**

Bacterial strains were sub-cultured into test tubes with autoclaved (vertical type steam sterilizer HL-340, Taiwan) nutrient broth (16 g in 1 L) (Biolab, Merck). The test tubes were then placed on

a suspension mixer (SM-3600, Taiwan) and incubated overnight at optimum temperatures (gram positive = 30°C, gram negative = 37°C). Following incubation, bacterial strains were diluted with sterile nutrient broth and measured using a spectrophotometer to attain an optical density (OD_{620}) between 0.08 – 0.1, equivalent to the 0.5 McFarland turbidity standard.

- **Preliminary antibacterial activity of leaf and stem extracts**

The agar well diffusion method was used to determine antibacterial activity of the leaf and stem extracts. Each extract was dried down and re-dissolved in methanol to attain a 1mg/mL concentration. Autoclaved Mueller-Hilton agar (38 g in 1 L) (Biolab, Merck) was poured into sterile petri dishes and allowed to set before being inoculated with cultured broth. Thereafter, a sterile cork borer was used to punch 5 mm holes into the agar plates, which were then filled with the leaf and stem extracts (~ 90 µL). The plates were then incubated at relevant temperatures for 24 h before being viewed and imaged for inhibition zones to determine potential antibacterial activity.

4.4 Results and Discussion

4.4.1 Preliminary phytochemical screening

Within the Euphorbiaceae, several terpenoids, alkaloids, fatty acids and phenolic compounds have been isolated from various species (Rizk, 1987). The genus *Croton* is represented by a diverse range of secondary metabolites. The main constituent of the phytochemicals in *Croton* are terpenoids, consisting mainly of diterpenoids. Other metabolites reported in this genus include triterpenoids, volatile oils, alkaloids and phenolic compounds (Nath et al., 2013). In the present study, preliminary phytochemical screening revealed alkaloids, amino acids, phenolic compounds, flavonoids, carbohydrates, terpenoids, saponins and fixed oils and fats in the leaf and stem crude extracts, as indicated in Table 4.1. A study by Abdalaziz et al. (2016) reported similar compounds in the fruit and whole plant extracts of *C. zambesicus*.

Secondary metabolites are known to possess a wide range of biological and pharmacological activity (Wink, 2015). Several euphorbiaceous species displayed various activities such as antibacterial, antiviral, antifungal, anticancer and anti-inflammatory (Mwine and Van Damme, 2011).

Terpenoids are the largest class of organic compounds that exhibit various biological activities including anticancer, antimalarial, anti-inflammatory and antimicrobial (Wang et al., 2005). Several diterpenes have been isolated from *C. gratissimus*, with some of them displaying cytotoxic activity against cancer cell lines (Block et al., 2002, 2004; Mulholland et al., 2010).

The presence of flavonoids, saponins and phenolic compounds in the leaf and stem extracts indicated antimicrobial potential (Murugan et al., 2013; Azalework et al., 2017). Alkaloids are also known to possess antimicrobial activity (Kumar et al., 2009). In addition, alkaloids isolated from species in this genus have displayed anticancer, antioxidant and acetylcholinesterase inhibitory activities (Salatino et al., 2007; Xu et al., 2018).

A study by Salatino et al. (2007) indicated that the medicinal properties of *Croton* species are attributed to the presence of a diverse range of phytochemicals such as, diterpenoids, triterpenoids, steroids, volatile oils (mono- and sesquiterpenoids), alkaloids, flavonoids and phenolic compounds. Thus, the presence of these bioactive compounds support the traditional use of *C. gratissimus* for various treatments.

Table 4.1: Phytochemical compounds identified in the hexane, chloroform and methanolic crude extracts from leaves and stems of *C. gratissimus* var. *gratissimus*.

Compound Group	Test	Reaction Observed	Leaves			Stems		
			Hexane	Chloroform	Methanol	Hexane	Chloroform	Methanol
Alkaloids	Wagner's	Reddish/brown precipitate	++	++	++	++	++	++
	Dragendorff's	Red precipitate	++	++	+	++	++	+
Phenolics	Ferric Chloride	Dark green colouration	+	++	++	-	+	+
Fixed Fats and Oils	Filter Paper	Oil stains on filter paper	++	-	-	+	-	-
Terpenoids	Salkowski's	Reddish brown colour at interface	++	++	+	++	++	+
Saponins	Foam	Foam layer	++	++	+	+	++	+
Flavonoids	Lead Acetate	Yellow precipitate	-	+	++	-	+	++
Amino Acids	Ninhydrin	Blue violet colour	-	-	++	-	-	++
Carbohydrates	Molisch's	Violet ring at junction	++	++	+	++	++	+
	Benedict's	Reddish precipitate	++	++	++	+	-	-
	Fehling's	Red precipitate	-	++	-	-	++	-

+/- indicates presence/absence
++ indicates intense reaction

4.4.2 Thin layer chromatography (TLC)

Thin layer chromatography provided a visualisation of the various classes of compounds present in the leaf and stem extracts of *C. gratissimus*, as indicated in Fig. 4.1. Certain bands that were not detected under visible light (Fig. 4.1c) were identified under UV light as seen in Fig. 4.1a and 4.1b. According to Kagan and Flythe (2014), the separation of compound groups on a TLC plate is based on polarity. Polar compounds tend to remain closer to the spot origin while less polar compounds travel further up the plate (Kagan and Flythe, 2014). The mobile phase employed for the separation of compounds in this study worked best for phytochemicals extracted using non-polar solvents (hexane and chloroform). This indicated that several classes of lipophilic compounds were present in the leaf and stem extracts, represented by the numerous bands in Fig. 4.1. This mobile phase did not work well for hydrophilic compounds as only a few bands were visible in the methanolic extracts of leaves and stems. It has been suggested that the number and type of bands separated on a TLC plate is dependent on the solvent combinations of the mobile phase (Seanego and Ndip, 2012; Kagan and Flythe, 2014).
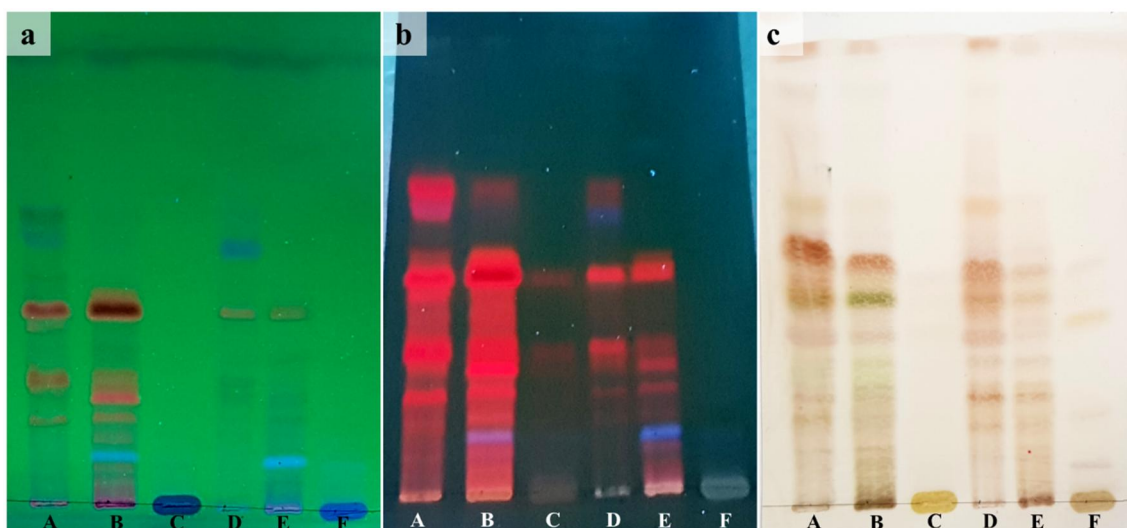
Figure 4.1: Separation of compounds on TLC plate spotted with hexane, chloroform and methanol extracts from leaves and stems. a) Viewed at 254 nm. b) Viewed at 365 nm. c) Viewed after heating with ANS reagent. A = hexane leaves, B = chloroform leaves, C = methanol leaves, D = hexane stems, E = chloroform stems, F = methanol stems.

4.4.3 Gas Chromatography-Mass Spectrometry (GC-MS)

Species from the genus *Croton* have been reported to contain numerous compounds with biological activities. Some of these include antidiabetic, anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, antimalarial, acetylcholinesterase inhibitory and cytotoxic activities (Salatino et al., 2007; Xu et al., 2018).

Analysis of the methanolic extracts in the current study revealed the presence of numerous compounds in the leaves and stems. The leaf extract was shown to contain 109 compounds with the major constituents being Isovellerdiol (8.69%), Cyclopropane, 1,1-dimethyl-2-(2-phenyl-1-pentenylidene)- (6.38%), 9-Octadecen-1-ol, (Z)- (5.68%), Bicyclo[3.1.1]hept-2-ene,2,2'-(1,2-ethanediyl)bis[6,6-dimethyl- (5.10%), β–Sitosterol (4.89%) and 1-Heptacosanol (4.57%). These major compounds accounted for 35.01% of the total composition, whilst the minor constituents and trace elements consisted of 34.33% and 30.66% respectively.

Ninety-seven compounds were detected in the methanolic stem extract. The major constituents included l-[-]-4-Hydroxy-1-methylproline (28.02%), 9-Octadecen-1-ol, (Z)- (5.35%), β–Sitosterol (3.92%), Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl- (3.46%) and Bicyclo[4.3.0]nonane, 7-methylene-2,4,4-trimethyl-2-vinyl- (3.33%), which accounted for 44.08% of the total composition. In addition to the major phytochemicals, an unidentified compound was detected which constituted for 5.70%. Minor compounds accounted for 26.19% and trace elements comprised 24.03%.

The analysis also revealed that five compounds were common in the leaf and stem methanolic extracts. These included n-Heptadecanol-1, 9-Octadecen-1-ol, (Z)-, n-Nonadecanol-1, Isovellerdiol and β–Sitosterol. Tables 4.2 and 4.3 summarise the major and minor compounds in leaves and stems respectively.

Several constituents in the leaf and stem extracts were reported to possess biological activities. Compounds that have been shown to possess antimicrobial activities included Phytol acetate (Sivakumar and Dhivya, 2015; Karthikeyan et al., 2016a), n-Heptadecanol-1 (Balamurugan et al., 2012), 9-Octadecen-1-ol, (Z)- (Gayathri and Sri, 2018), n-Nonadecanol-1 (Kuppuswamy et al., 2013; Ogukwe et al., 2016; Arora et al., 2017), Phytol (Wagay and Rothe, 2016; Ameachi and Chijioke, 2018; Mohiuddin et al., 2018), 1-Heptacosanol (Begum et al., 2016; Ogukwe et al., 2016; Roy et al., 2018), Cycloisolongifolene, 8,9-dehydro-9-formyl- (Zhang et al., 2017), β–Sitosterol (Sen et al., 2012; Bin Sayeed et al., 2016; Karthikeyan et al., 2016a), Caryophyllene (Raman et al., 2012; Padmashree et al., 2018; Rizwana, 2018), Tributyl acetylcitrate (Hussein et al., 2016a; Ibraheam et al., 2017) and 9-Octadecenamide, (Z)- (Selvin et al., 2009; Basa'ar and Farooqui, 2017).

Table 4.2: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanolic leaf extract showing major and minor compounds.

No.	Retention time	Compound name	Peak area %	CAS no.
1	14.766	Octanoic acid, octyl ester	1.29	2306-88-9
2	17.056	Phytol, acetate	1.24	10236-16-5
3	17.524	n-Heptadecanol-1	2.90	1454-85-9
4	19.288	9-Octadecen-1-ol, (Z)-	5.68	143-28-2
5	19.510	n-Nonadecanol-1	2.50	1454-84-8
6	19.743	Phytol	1.24	150-86-7
7	21.691	4,14-Retro-retinol	3.99	16729-22-9
8	22.059	Bicyclo[3.1.1]hept-2-ene, 2,2'-(1,2-ethanediyl)bis[6,6-dimethyl-	5.10	57988-82-6
9	23.046	Cyclopropane, 1,1-dimethyl-2-(2-phenyl-1-pentenylidene)-	6.38	0-00-0
10	23.363	2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-1,1,4a-trimethyl-	1.17	61141-19-3
11	23.696	delta.9(11)-Methyltestosterone	1.23	1039-17-4
12	23.744	Bufa-20,22-dienolide, 14,15-epoxy-3,11-dihydroxy-, (3.beta.,5.beta.,11.alpha.,15.beta.)-	3.09	39005-15-7
13	23.817	1,4-Piperazinediethanol, .alpha.,.alpha.'-bis(phenoxymethyl)-	1.19	0-00-0
14	24.068	(7,7-Dimethyl-1-oxo-2,3,4,5,6,7-hexahydro-1H-inden-2-yl)acetic acid, ethyl ester	1.42	139571-20-3
15	24.550	Tetratetracontane	1.22	7098-22-8
16	24.596	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1-methyl-, [1S-(1.alpha.,4a.alpha.,10a.beta.)]-	1.09	57378-57-1
17	25.986	1-Heptacosanol	4.57	2004-39-9
18	26.125	5(S),9(S),10(S)-15,16-Epoxycleroda-3,8,13(16),14-tetraene-19,18:20,12(S)-diolactone (swassin)	2.87	69749-03-7
19	26.501	5,8,11-Heptadecatriynoic acid, methyl ester	2.03	56554-57-5
20	27.059	Cycloisolongifolene, 8,9-dehydro-9-formyl-	1.89	59820-24-5
21	27.674	Cycloprop[e]indene-1a,2(1H)-dimethanol, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1a.alpha.,3a.beta.,6a.beta.,6b.alpha.)-(-)- (Isovellerdiol)	8.69	37841-93-3
22	27.903	Benzenesulfonamide, 4-cyclohexyl-N-(3-pyridyl)-	2.43	0-00-0
23	29.250	.beta.-Sitosterol	4.89	83-46-5
24	30.376	B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)- (beta-Simiarenol)	1.24	1615-94-7
		Total	**69.34**	

Table 4.3: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanolic stem extract showing major and minor compounds.

No.	Retention time	Compound name	Peak area %	CAS no.
1	12.356	Caryophyllene	1.14	87-44-5
2	15.337	l-[-]-4-Hydroxy-1-methylproline	28.02	67463-44-9
3	17.524	n-Heptadecanol-1	2.51	1454-85-9
4	19.286	9-Octadecen-1-ol, (Z)-	5.35	143-28-2
5	19.510	n-Nonadecanol-1	2.87	1454-84-8
6	20.932	Tributyl acetylcitrate	1.70	77-90-7
7	21.138	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	1.28	64566-18-3
8	21.242	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	1.75	7220-78-2
9	21.287	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	2.29	7220-78-2
10	21.370	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	1.35	2566-91-8
11	21.697	Bicyclo[4.3.0]nonane, 7-methylene-2,4,4-trimethyl-2-vinyl-	3.33	0-00-0
12	21.983	9-Octadecenamide, (Z)-	1.28	301-02-0
13	22.178	1-Naphthalenecarboxylic acid, decahydro-1,4a-dimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, methyl ester, [1S-[1.alpha.,4a.alpha.	1.01	10178-35-5
14	22.567	Ethyl stearate, 9,12-diepoxy	1.56	0-00-0
15	23.370	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	3.46	826337-63-7
16	23.449	1-Methyl-1-phenyl-1-silacyclobutane	1.79	3944-08-9
17	24.749	26-Dehydroxy-dihydropseudoprogenin-25-ene $$ Furost-25-en-3-ol #	1.52	0-00-0
18	27.657	Cycloprop[e]indene-1a,2(1H)-dimethanol, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1a.alpha.,3a.beta.,6a.beta.,6b.alpha.)-(-)- (Isovellerdiol)	2.45	37841-93-3
19	27.894	Cycloprop[e]indene-1a,2(1H)-dimethanol, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, (1a.alpha.,3a.beta.,6a.beta.,6b.alpha.)-(-)- (Isovellerdiol)	1.69	37841-93-3
20	29.247	.beta.-Sitosterol $$ Stigmast-5-en-3-ol, (3.beta.)-	3.92	83-46-5
21	30.214	Unknown	5.70	-
		Total	**75.97**	

Other notable activities reported for compounds that were present in *C. gratissimus* included anti-inflammatory, anticancer/cancer preventive and antioxidant. Compounds that were found to be responsible for anti-inflammatory activity are Phytol acetate (Sivakumar and Dhivya, 2015; Karthikeyan et al., 2016a), n-Nonadecanol-1 (Anburaj et al., 2016), Phytol (Silva et al., 2014; Wagay and Rothe, 2016; Mohiuddin et al., 2018), β–Sitosterol (Saeidnia et al., 2014; Karthikeyan et al., 2016a; Bin Sayeed et al., 2016), B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)-(Kuroshima et al., 2005), Caryophyllene (Legault and Pichette, 2007; Mohammed et al., 2016; Padmashree et al., 2018) and 9-Octadecenamide, (Z)- (Hussein et al., 2016a, 2016b; Hadi et al., 2016; Basa'ar and Farooqui, 2017).

Anticancer/ cancer preventive compounds included Phytol acetate (Sivakumar and Dhivya, 2015; Karthikeyan et al., 2016a, 2016b), n-Nonadecanol-1 (Anburaj et al., 2016), Phytol (Wagay and Rothe, 2016; Ameachi and Chijioke, 2018; Mohiuddin et al., 2018), 1-Heptacosanol (Raman et al., 2012), Cycloisolongifolene, 8,9-dehydro-9-formyl- (Mohiuddin et al., 2018), β–Sitosterol (Yinusa et al., 2015; Nyigo et al., 2016; Bin Sayeed et al., 2016), Caryophyllene (Legault and Pichette, 2007; Padmashree et al., 2018), Tributyl acetylcitrate (Hussein et al., 2016a) and (R)-(-)-14-Methyl-8-hexadecyn-1-ol (Mohansrinivasan et al., 2015).

Compounds such as Phytol acetate (Karthikeyan et al., 2016b), Phytol (Raman et al., 2012; Mohammed et al, 2016) Tetratetracontane (Sivakumar and Gayathri, 2015; Agarwal et al., 2017), 1-Heptacosanol (Raman et al., 2012; Begum et al., 2016; Roy et al., 2018), Cycloisolongifolene, 8,9-dehydro-9-formyl- (Zhang et al., 2017), β–Sitosterol (Yinusa et al., 2015; Saeidnia et al., 2014; Bin Sayeed et al., 2016) and Caryophyllene (Legault and Pichette, 2007; Mohammed et al, 2016; Padmashree et al., 2018) were reported to possess antioxidant activity.

Many of the ailments treated with *C. gratissimus* are possibly attributed to the biological activities of these compounds. Thus, the presence of these various bioactive compounds in the leaf and stem extracts, further demonstrates the usefulness of *C. gratissimus* in traditional medicine.

4.4.4 Preliminary antibacterial assays

Biological screening of plant extracts is an important precursor in the discovery of novel antimicrobial drugs (Sendeku et al., 2015). Antibacterial assays of *C. gratissimus* indicated that the methanolic leaf extracts inhibited the growth of almost all bacterial strains except for *P. aeruginosa,* which did not display any sensitivity to the extract. The leaf extract exhibited a weak to moderate activity against both *E. coli* strains, MRSA (environmental type), *S. aureus* and *B. subtilis*. Conversely, the extracts demonstrated strong activities against MRSA (clinical type) and *S. aureus* (ATCC 29213). The resistance from *P. aeruginosa* and the weak activities of the *E. coli*

strains indicated that gram negative bacteria were least sensitive to the leaf extracts, whilst gram positive ones were more susceptible. Similar results were reported for the leaf essential oils of *C. gratissimus* (Lawal et al., 2017). Antibacterial assays indicated that the oil exhibited a stronger activity on gram positive compared to the gram negative strains (Lawal et al., 2017). Gram negative bacteria are known to be more resistant to antibiotics due to the lactamase enzyme which is secreted into the periplasmic space (Elisha et al., 2017).

Stem extracts inhibited the growth of all investigated bacterial strains. However, the extracts exhibited weak to moderate antibacterial activity against *E. coli* (ATCC 25218), *P. aeruginosa* and MRSA (clinical type). In contrast, these extracts displayed a greater inhibition of bacterial growth against *E. coli*, MRSA (environmental type), *S. aureus* (ATCC 29213), *S. aureus* and *B. subtilis*. These results indicated that stem extracts had similar effects against gram negative and positive bacteria. This broad spectrum activity of stem extracts was also demonstrated in the ethyl acetate fraction of the roots from *C. gratissimus*. This root extract inhibited both gram negative and positive bacteria with some displaying a higher susceptibility than others (Okokon and Nwafor, 2010a).

The stem extracts were more effective in retarding bacterial growth than the leaves. This was demonstrated with *E. coli, P. aeruginosa*, MRSA (environmental type), *S. aureus* and *B. subtilis*. Table 4.4 is a summary of the activities exhibited by leaf and stem extracts against each bacterial strain.

Table 4.4: Antibacterial activities of leaf and stem extracts of *C. gratissimus* against eight bacterial strains.

Bacterial Strain	Leaves	Stems
E. coli (ATCC 25218)	+-	+-
E. coli	+-	++
P. aeruginosa	-	+
MRSA (environmental type)	+-	++
MRSA (clinical type)	++	+
S. aureus (ATCC 29213)	++	++
S. aureus	+	++
B. subtilis	+	++

(-) No activity, **(+-)** weak activity, **(+)** moderate activity, **(++)** strong activity.

Previous studies of *C. gratissimus* demonstrated antibacterial activity of various plant extracts (Abo et al., 1999; Van Vuuren and Viljoen, 2008; Okokon and Nwafor, 2010a; Van Vuuren and

Naidoo, 2010; Mthethwa et al., 2014; Lawal et al., 2017). A study by Van Vuuren and Naidoo (2010) on the antibacterial potential of leaf oil and solvent extract of *C. gratissimus* indicated a moderate to strong activity against STI-causing bacteria (*Ureaplasma urealyticum* and *Gardnerella vaginalis*). Mthethwa et al. (2014) demonstrated that the methanolic leaf extracts displayed good activity against *S. aureus* and *S. epidermidis*.

Abo et al. (1999) investigated the antibacterial activity of leaf and stem bark extracts. The stem bark extracts exhibited strong activities against *Proteus mirabilis, S. aureus, Bacillus megaterium* and *B. subtilis*. However, leaf extracts displayed little to no activity against all bacterial strains. A study by Van Vuuren and Viljoen (2008) demonstrated antibacterial activity of the essential oils and solvent (methanol: chloroform) extracts of leaves, roots, bark and combinations thereof. However, the activity against bacterial strains varied between plant part and extract combinations (Van Vuuren and Viljoen, 2008). These variations in the antibacterial activity of *C. gratissimus* may be attributed to several factors including extraction methods, bacterial strains and source of plant extracts (Mostafa et al., 2018).

The antimicrobial activities of plant extracts are likely attributed to phytochemical constituents such as terpenoids, alkaloids and phenolic compounds. These compounds are suggested to interfere with the enzymes and proteins of the microbial cell membrane, causing them to disrupt. This eventually leads to the death of the microbial cell or the inhibition of enzymes involved in the synthesis of amino acids (Mostafa et al., 2018).

The GC-MS analysis of leaf and stem extracts revealed various compounds with possible antibacterial and antimicrobial activities. These included Phytol acetate, n-Heptadecanol-1, 9-Octadecen-1-ol, (Z)-, n-Nonadecanol-1, Phytol, 1-Heptacosanol, Cycloisolongifolene, 8,9-dehydro-9-formyl- (Xiang et al., 2018), β–Sitosterol (Saeidnia et al., 2014; Yinusa et al., 2015), Caryophyllene, Tributyl acetylcitrate, 9-Octadecenamide, (Z)- (Hadi et al., 2016; Hussein et al., 2016a, 2016b; Ibraheam et al., 2017), Tetratetracontane (Deshmukh, 2015) and Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis- (Hussein et al., 2016b). The presence of these compounds probably contributed to the activity of the extracts against the bacterial strains.

4.5 Conclusion

The leaf and stem extracts of *C. gratissimus* were found to possess several phytochemical compounds including terpenoids, carbohydrates, flavonoids, alkaloids, amino acids, phenolic compounds, saponins and fixed fats. Antimicrobial assays of the extracts indicated weak to strong activities against various bacterial strains. The stem extracts however exhibited stronger activity than the leaf extracts. GC-MS analyses indicated several bioactive compounds in the leaves and

stems. Many of these were reported to possess antimicrobial and antibacterial properties which probably contributed to the activity demonstrated in this study. This study confirmed the usefulness of *C. gratissimus* in traditional medicine and indicated its potential as a source of antimicrobials. However, further studies are needed to investigate the antibacterial properties of individual compounds to determine their potency and potential as antibacterial agents. Minimum inhibitory concentration (MIC) assays could assist in determining the antibacterial potency of the extracts and compounds.

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CHAPTER 5: BIOLOGICAL SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES FROM LEAVES AND STEMS OF *CROTON GRATISSIMUS* BURCH. VAR. *GRATISSIMUS* (EUPHORBIACEAE)

5.1 Abstract

Silver nanoparticles (AgNPs) have received considerable attention due to their strong antimicrobial properties. This study set out to biosynthesise AgNPs from the methanolic leaf and stem extracts of the medicinally important species *Croton gratissimus*. Ultraviolet-visible spectroscopy, energy-dispersive X-ray (EDX) analysis and transmission electron microscopy (TEM) confirmed the production of AgNPs from both extracts. Transmission electron micrographs revealed spherical AgNPs from both leaf and stem extracts. However, the size distribution of these particles differed between organs. Silver nanoparticles from leaf extracts were monodispersed whilst those from stems displayed polydispersion. Antibacterial assays indicated that AgNPs from leaf extracts were more effective in inhibiting bacterial growth than particles from stems. These results suggest that AgNPs from leaf extracts could be potential sources of antibacterial agents. However, optimisation in the synthesis process may improve the potency of these silver nanoparticles.

Keywords: Peaks, wavelengths, capping agents, reducing agents, spherical.

5.2 Introduction

Microscopic particles with sizes ranging from 1 – 100 nm are termed nanoparticles (Song and Kim, 2009; Thakkar et al., 2010; Bagyalakshmi and Haritha, 2017). Studies on noble metal nanoparticles are becoming increasingly popular as they exhibit enhanced biological and physiochemical properties compared to the bulk material of equivalent composition. (He et al., 2001; Song and Kim, 2009; Thakkar et al., 2010; Ahmed et al., 2016c; Gharibshahi et al., 2017). In particular, silver nanoparticles (AgNPs) are of great interest because they are known to possess stronger antimicrobial properties than other metals (Ibrahim, 2015; Premasudha et al., 2015). For this reason, they are regarded as potential antimicrobial agents (Ibrahim, 2015; Singh et al., 2018).

Physical and chemical processes have been employed for synthesising AgNPs. However, these methods have proved costly, laborious, energy-consuming and harmful to environmental and human health due to the toxic by-products produced through these processes (Thakkar et al., 2010; Makarov et al., 2014; Ibrahim, 2015; Ahmed et al., 2016b; Dhand et al., 2016; Kuppusamy et al., 2016; Gabriela et al., 2017; Gharibshahi et al., 2017).

Biological methods using enzymes, plants or plant extracts and microorganisms are more advantageous over conventional processes as they are environmentally friendly, inexpensive and do not require toxic chemicals (Song and Kim, 2009; Iravani, 2011; Parveen et al., 2016). Conversely, the use of plants and plant extracts is more feasible than other biological methods as it provides natural reducing and capping agents for the formation of AgNPs (Ponarulselvam et al., 2012; Mittal et al., 2013; Gabriela et al., 2017; Khan et al., 2018). These natural capping agents from plants are also reported to improve the therapeutic properties of the nanoparticles (Sangeetha et al., 2016). A considerable number of plants have been used for the synthesis of AgNPs, some of which include *Azadirachta indica*, *Pelargonium graveolens*, *Aloe vera*, *Capsicum annuum* L, *Mentha piperita*, *Albizia adianthifolia*, *Ziziphora tenuior* and *Jatropha curcas* (Ponarulselvam et al., 2012; Gabriela et al., 2017).

Croton gratissimus Burch. var. *gratissimus* (syn. *C. zambesicus* Müll. Arg.; *C. microbotryus* Pax., *C. amabilis* Müell. Arg.) belonging to the family Euphorbiaceae (Mulholland et al., 2010; Robert et al., 2010; PlantZAfrica, 2018) is a medicinally important plant that has been used in traditional medicine for the treatment of various ailments (Ngadjui et al., 2002; Van Vuuren and Viljoen, 2008). Previous studies on *C. gratissimus* revealed antimicrobial activities of extracts from different plant organs (Abo et al., 1999; Van Vuuren and Viljoen, 2008; Okokon and Nwafor, 2010; Van Vuuren and Naidoo, 2010; Mthethwa et al., 2014; Lawal et al., 2017). Due to the extensive use in traditional medicine and the antimicrobial properties exhibited by various plant

organs, this study set out to investigate the antibacterial activity of AgNPs synthesised from leaf and stem extracts of *C. gratissimus*.

5.3 Materials and Methods

5.3.1 Plant collection and sampling

Leaves and stems of *Croton gratissimus* Burch. var. *gratissimus* were collected from the University of KwaZulu-Natal, Westville Campus (29°49'08.1"S 30°56'38.9"E). The material was air dried for two months before being ground to a powder for synthesis of AgNPs. A voucher specimen (*Croton* 01 – Accession No. 18224) was prepared and deposited in the Ward Herbarium located in the School of Life Sciences at the University of KwaZulu-Natal, Westville Campus.

5.3.2 Crude methanolic extraction

Powdered leaf and stems were placed in separate round bottom flasks with HPLC grade methanol and boiled through continuous reflux. Two 2 h sessions were carried out for leaves and stems. The obtained extracts were filtered and used for biological synthesis of AgNPs.

5.3.3 Biosynthesis of silver nanoparticles (AgNPs)

The synthesis of AgNPs was adapted from Khanra et al., 2016. Deionised water was used to prepare a 1 mM silver nitrate ($AgNO_3$) solution (Merck). Thereafter, 2 mL of the methanolic plant extracts were added to 18 mL of $AgNO_3$ solution (9: 1) and heated at 80°C for 90 min. This was carried out for both leaf and stem extracts. A colour change from clear/light green to light or dark brown indicated silver nanoparticle synthesis. These solutions were then centrifuged at 16000 rpm at 20°C for 60 min. Subsequently, the supernatant was discarded and the remaining pellet was washed several times with distilled water to remove any impurities. Pellets were resuspended in distilled water for silver nanoparticle characterisation using UV-VIS spectroscopy, energy-dispersive X-ray (EDX) analysis, Transmission Electron Microscopy (TEM), and Fourier-transform infrared spectroscopy (FTIR).

5.3.4 Ultraviolet-visible (UV-VIS) spectroscopy

The synthesis of AgNPs from each extract was measured using a SHIMADZU UV-1800 UV-VIS Spectrophotometer (Merck, Japan) at a range of 200 – 800 nm. The 1 mM $AgNO_3$ solution served as the blank in the analysis.

5.3.5. Energy-dispersive X-ray (EDX) analysis

Small amounts of the aqueous AgNPs were dropped onto aluminium stubs and left to dry. Thereafter, samples were sputter coated with gold in a Quorum Q150 RES gold Sputter Coater. The elemental composition of the nanoparticles was determined using the Aztec Software (Oxford

Instruments, United Kingdom) on the Zeiss Ultra Plus field emission gun scanning electron microscope (FEGSEM).

5.3.6 Transmission electron microscopy (TEM) and Image analysis

The size and shape of synthesised AgNPs were determined using a transmission electron microscope (Sorescu et al., 2016). Carbon coated formvar grids were dipped into the aqueous silver nanoparticle solution and set aside to dry. The dried grids were then viewed using a Jeol JEM 2100 High Resolution Transmission Electron Microscope (HRTEM) at a voltage of 200 kV. The iTEM Soft Imaging System Software (Olympus, Germany) was used to determine the size of AgNPs from obtained images.

5.3.7 Fourier-transform infrared spectroscopy (FTIR)

The aqueous solutions of AgNPs were placed in a Perkin Elmer Spectrum 100 spectrophotometer (USA) which allowed for the identification of functional groups that were attached to the AgNPs (capping agents) (Sorescu et al., 2016).

5.3.8 Preliminary antibacterial assay

- **Bacterial Strains**

The antibacterial properties of AgNPs from leaves and stems were evaluated against eight bacterial strains. Three gram negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia coli* – ATCC 25218) and five gram positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus aureus* – ATCC 29213, Methicillin-Resistant *Staphylococcus aureus* (MRSA) – clinical type, Methicillin-Resistant *Staphylococcus aureus* (MRSA) – environmental type) bacterial strains were used along with positive (streptomycin – gram positive, gentamycin – gram negative) and negative (methanol) controls.

- **Inoculum preparation**

Bacterial strains were sub-cultured into test tubes with autoclaved (vertical type steam sterilizer HL-340, Taiwan) nutrient broth (16 g in 1 L) (Biolab, Merck). The test tubes were then placed on a suspension mixer (SM-3600, Taiwan) and incubated overnight at optimum temperatures (gram positive = 30°C, gram negative = 37°C). Following incubation, bacterial strains were diluted with sterile nutrient broth and measured using a spectrophotometer to attain an optical density (OD_{620}) between 0.08 – 0.1, equivalent to the 0.5 McFarland turbidity standard.

- **Preliminary antibacterial activity of leaf and stem extracts**

The agar well diffusion method was used to determine antibacterial activity of the AgNPs synthesised from leaves and stems. The pellets obtained after centrifugation were re-dissolved in distilled water to attain a 1 mg/mL concentration. Autoclaved Mueller-Hilton agar (38 g in 1 L) (Biolab, Merck) was poured into sterile petri dishes and allowed to set before being inoculated with cultured broth. Thereafter, a sterile cork borer was used to punch 5 mm holes into the agar plates, which were then filled with aqueous silver nanoparticle solutions (~ 90 µL). The plates were then incubated at relevant temperatures for 24 h before being viewed and imaged for inhibition zones to determine potential antibacterial activity.

5.4 Results and Discussion

5.4.1 Biosynthesis of silver nanoparticles (AgNPs)

The use of plant extracts to synthesise nanoparticles is becoming increasingly popular (Mittal et al., 2013), with AgNPs having a wide range of applications in the medical industry (Song and Kim, 2009). In the current study, AgNPs were synthesised from the leaves and stems of *Croton gratissimus*. This was indicated by the colour changes in Fig. 5.1. Leaf and stem extracts displayed a light to dark brown colour following the 90 min reaction time. Silver nanoparticles produced from plant extracts are known to produce a brown colour (Sorescu et al., 2016). This colour change is suggested to be brought about by the excitation of surface plasmon vibrations of AgNPs (Singhal et al., 2011; Khanra et al., 2016; Sorescu et al., 2016).

Metabolites present in plant extracts such as amino acids, proteins, terpenoids, flavonoids, alkaloids, phenolic compounds and saponins, are responsible for the synthesis of AgNPs as they provide natural reducing and capping agents for the fabricated particles (Mittal et al., 2013; Ahmed et al., 2016a; Kuppusamy et al., 2016; Raja et al., 2017; De Matteis et al., 2018). The visible variation in the AgNPs produced from leaf and stem extracts is probably attributed to the different plant organs used for synthesis. Previous work suggested that extracts from different organs of the same plant can comprise varying combinations and concentrations of reducing agents (Mittal et al., 2013; Sigamoney et al., 2016).
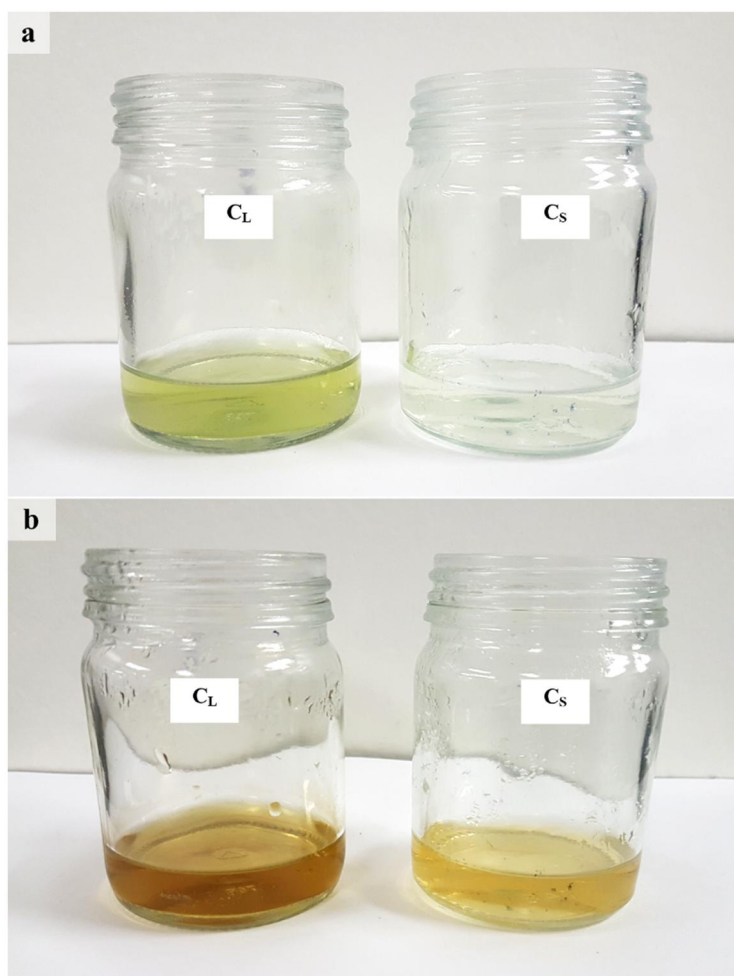
Figure 5.1: Visual representation of the leaf and stem extracts before (a) and after (b) the 90 min reaction time. C_L = *C. gratissimus* var. *gratissimus* leaves, C_S = *C. gratissimus* var. *gratissimus* stems.

5.4.2 Ultraviolet-visible (UV-VIS) spectroscopy

The synthesis of AgNPs was analysed by UV-VIS spectroscopy. UV-VIS spectroscopy is used to monitor the formation and stability of AgNPs (Logeswari et al., 2015; Khanra et al., 2016). Figure 5.2 shows the UV-VIS spectra of the AgNPs from the leaf and stem extracts at a range of 350 – 518 nm. Surface plasmon resonance (SPR) bands were observed at 414 nm and 421 nm in the leaves and stems respectively. According to Mittal et al. (2013), AgNPs are usually characterised by absorption wavelengths in the range of 400 – 450 nm. The absorption peaks observed in this range confirmed the presence of AgNPs. The analysis also indicated that the particles are spherical in shape as absorption bands between 400 – 450 nm indicate spherical metallic nanoparticles (Martinez-Castanon et al., 2008; Raja et al., 2017). The AgNPs from leaf and stem extracts displayed narrow and wide peaks respectively. It is suggested that the width of peaks is related to the size dispersion of the particles (He et al., 2001; Martinez-Castanon et al., 2008; Ponarulselvam

et al., 2012; Vanaja and Annadurai, 2013). The narrow peak at a lower wavelength from the leaves indicated monodispersal of small-sized particles (He et al., 2001; Martinez-Castanon et al., 2008; Vanaja and Annadurai, 2013; Bonnia et al., 2018). Conversely, the broadened peak at a slightly higher wavelength from stem extracts indicated polydispersion of AgNPs (He et al., 2001; Martinez-Castanon et al., 2008; Ponarulselvam et al., 2012). This wide peak is suggested to be formed by the combination of several bands (He et al., 2001; Martinez-Castanon et al., 2008). According to the literature, SPR bands are highly influenced by several factors including extract and AgNO$_3$ concentrations, type of biomolecules constituting the extract and the size and shape of nanoparticles (Gabriela et al., 2017; Raja et al., 2017).
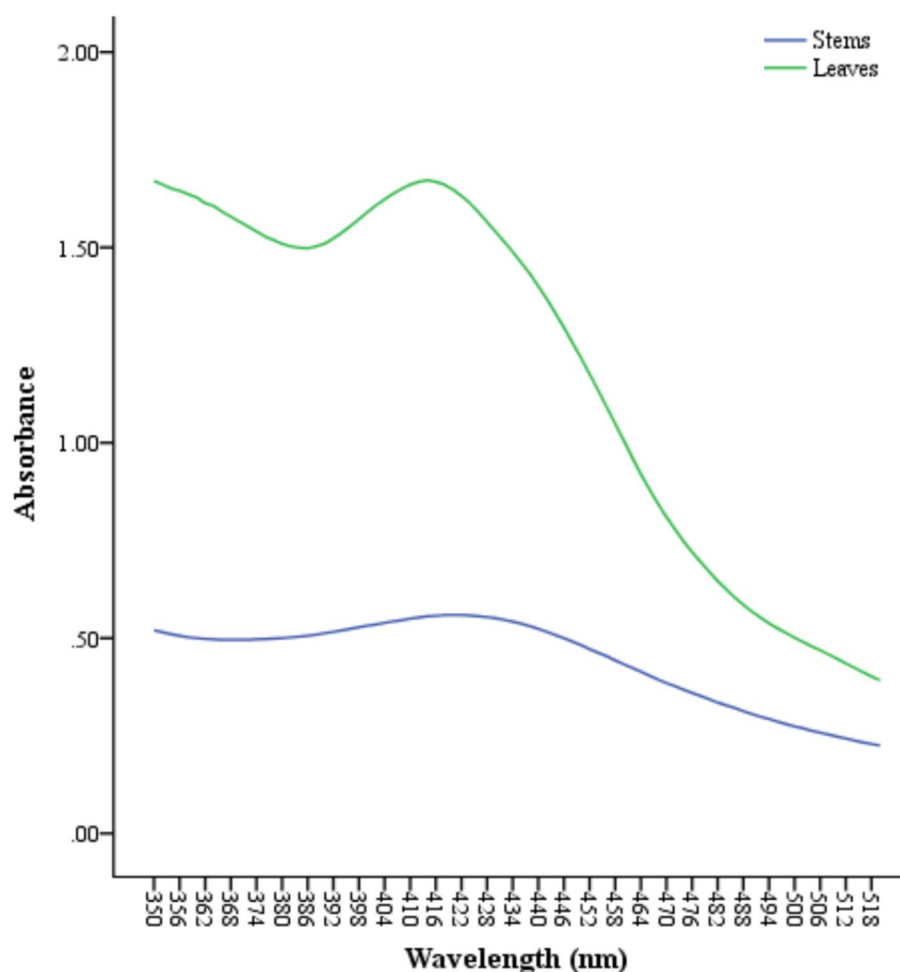
**Figure 5.2:** Ultraviolet-visible spectra of silver nanoparticles synthesised from leaves and stems of *C. gratissimus* var. *gratissimus* after the 90 min reaction time.

5.4.3 Energy-dispersive X-ray (EDX) analysis

Energy-dispersive X-ray (EDX) analyses (Fig. 5.3) revealed peaks for silver (Ag) in both samples, confirming the production of AgNPs from leaf and stem extracts. The SPR of AgNPs results in a

characteristic optical absorption at 3 keV (Ibrahim, 2015). Therefore, the peaks observed at 3 keV for leaves and stems indicated silver (Shahverdi et al., 2007; Raja et al., 2017; Bonnia et al., 2018). Table 5.1 represents the mean ± SD percentage of elemental silver from leaf and stem nanoparticles. A higher percentage of elemental silver was recorded for nanoparticles from leaf extracts compared to those from stems. However, statistical analyses revealed no significant difference between elemental silver from leaf and stem nanoparticles.
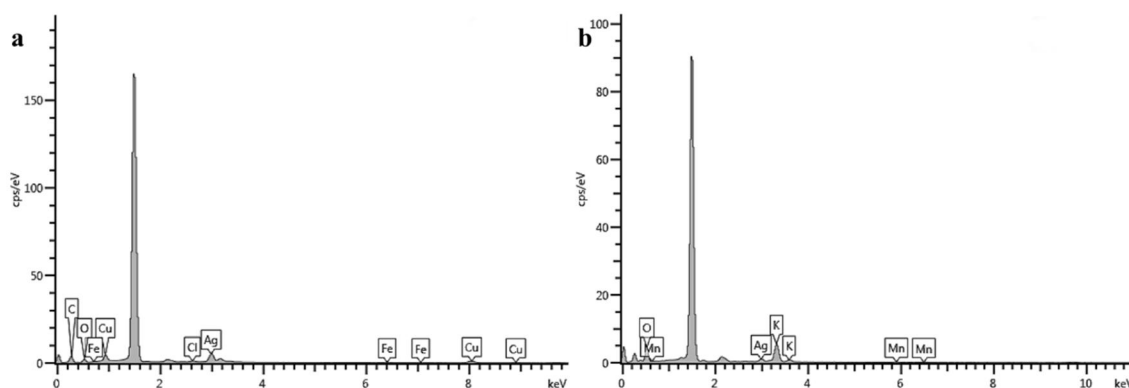
Figure 5.3: Energy-dispersive X-ray (EDX) spectra of silver nanoparticles synthesised from leaf (a) and stem (b) extracts of *C. gratissimus* var. *gratissimus*.

Table 5.1: Mean percentage of elemental silver from nanoparticles synthesised from leaf and stem extracts of *C. gratissimus* var. *gratissimus*.

Plant organ	Percentage of silver
Leaves	36.04 ± 17.64
Stems	23.24 ± 11.72

Mean ± standard deviation (SD), n = 3. *p* = 0. 354.

5.4.4 Transmission electron microscopy (TEM) and Image analysis

Transmission electron microscopy confirmed the formation and shape (spherical) of AgNPs from the leaf and stem extracts (Fig.5. 4). However, the size distribution of the AgNPs differed between leaves and stems. Silver nanoparticles from stem extracts varied in size. This corroborated the results from UV-VIS spectroscopy which indicated that the particles were monodispersed in leaves and polydispersed in stems. Figure 5.5 is an illustration of the particle size distribution from leaves and stems. The size of AgNPs synthesised from leaves ranged from 7.19 – 24.67 nm with a mean of 13.09 nm. The stem extracts produced AgNPs with sizes ranging from 6.24 – 40.95 nm with a mean of 13.64 nm. Table 5.2 shows the average particle size and standard deviations from leaf and stem extracts. There was a significant difference in particle size distribution between leaves and stems. Sigamoney et al. (2016) indicate that dissimilar plant parts comprise varying biochemical constituents which influences the synthesis, size, shape and yield

of AgNPs. Phytochemical studies revealed alkaloids, amino acids, phenolic compounds, flavonoids, carbohydrates, terpenoids, saponins and fixed oils and fats in leaf and stem extracts (Chapter 4). However, their concentrations probably differed contributing to the significant size differences of AgNPs between organs.

Figure 5.4: Transmission electron micrograph showing silver nanoparticles synthesised from the leaves (a) and stems (b) of *C. gratissimus* var. *gratissimus*.
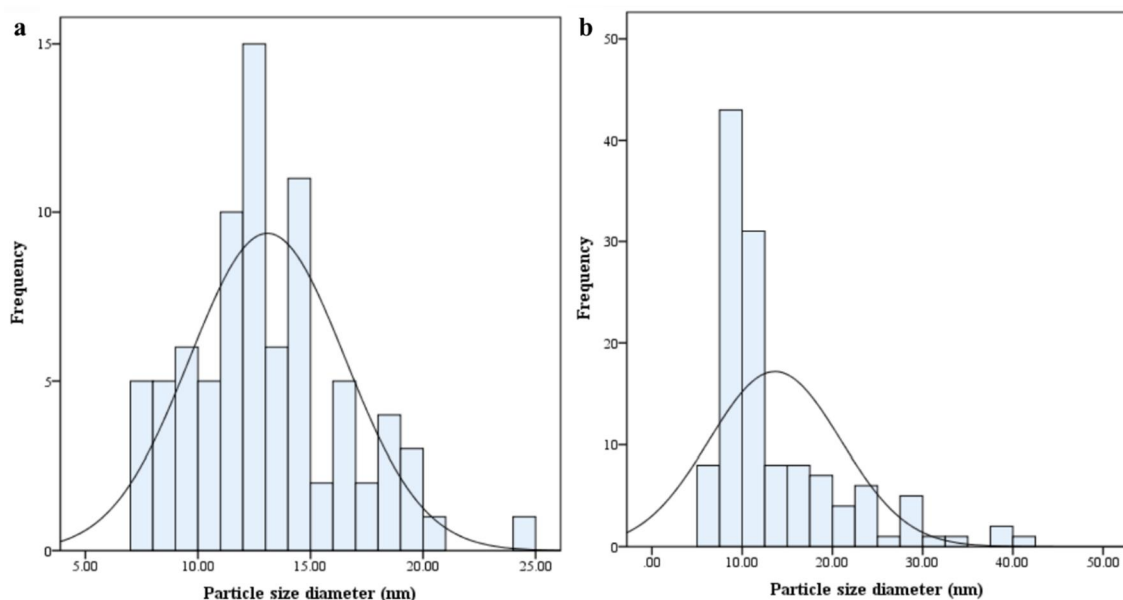
Figure 5.5: Particle size distribution from leaves (a) and stems (b) of *C. gratissimus* var. *gratissimus*.

Table 5.2: Mean particle size of silver nanoparticles synthesised from leaves and stems of *C. gratissimus* var. *gratissimus*.
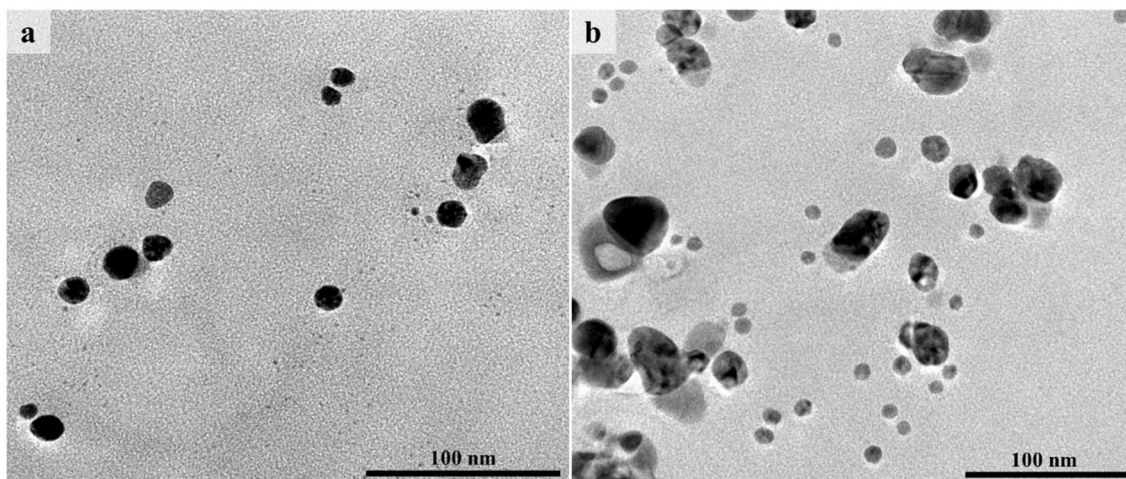
Plant organ	Particle size (nm)
Leaves	13.09 ± 3.45
Stems	13.64 ± 7.31

Mean $\pm$ standard deviation (SD), n = 6. $p = 0.035$.

5.4.5 Fourier-transform infrared spectroscopy (FTIR)

Figure 5.6 illustrates the results from FTIR spectroscopy. The FTIR spectra of AgNPs synthesised from leaf and stem extracts displayed peaks at 3269 – 3273, 2112 – 2120, 1637 – 1639, 1375, 1217, 1016, 569 – 577 cm^{-1}. The peaks between wave numbers of 3269 – 3273 cm^{-1} may correspond to the O-H stretching of alcoholic and phenolic compounds (Koyyati, et al., 2014; Bagyalakshmi and Haritha, 2017). Peaks at this region also indicated the C-H aldehyde stretch of alkanes (Koyyati, et al., 2014). Peaks between 2112 – 2120 cm^{-1} correspond to the C≡C stretch of alkynes (Coates, 2000). The peaks between wave numbers of 1637 – 1639 cm^{-1} are assigned to the C=O stretch or bend of amide I bond of proteins (Singhal et al., 2011; Ibrahim, 2015; Ahmed and Ikram, 2015; Ahmed et al., 2016b). The C-N stretch vibrations of aromatic amines correspond to the peak at 1375 cm^{-1} (Koyyati, et al., 2014; Balashanmugam and Kalaichelvan 2015; Gabriela et al., 2017). This peak displayed at 1375 cm^{-1} suggests that amino groups are involved in the encapsulation and stabilisation of AgNPs (Balashanmugam and Kalaichelvan 2015). The peak at 1217 cm^{-1} displays the C-O stretch of esters or C-N stretch of amines (Raja et al., 2017). The peak at 1016 cm^{-1} may be assigned to the C-O stretch of alcohol and ethers (Praba et al., 2014; Singh et al., 2018). The peaks between 569 – 577 cm^{-1} may correspond to the C-Br stretch of alkyl halides (Vinay et al., 2017).

According to literature, phytochemicals in plant extracts such as terpenoids, phenolics, glycosides, proteins and alkaloids are involved in the formation of AgNPs (Dhand et al., 2016; Raja et al., 2017). The results from FTIR spectroscopy indicated that AgNPs contained proteins, phenolic and alcoholic compounds in addition to functional groups such as alkanes, alkynes, amides, amines, ethers and alkyl halides. Similar results were reported for the AgNPs from aqueous leaf extracts of *C. zambesicus*. Ahmed et al. (2016c) identified amide, carboxylic acid, phenols, carbonyl and hydroxyl groups to be responsible for the capping and stabilisation of the synthesised AgNPs. This suggests that the functional groups identified in this study and the phytochemicals (Chapter 4) may act as natural reducing and capping agents in the synthesis and stabilisation of AgNPs (Ahmed et al., 2016c; Sorescu et al., 2016; Raja et al., 2017).
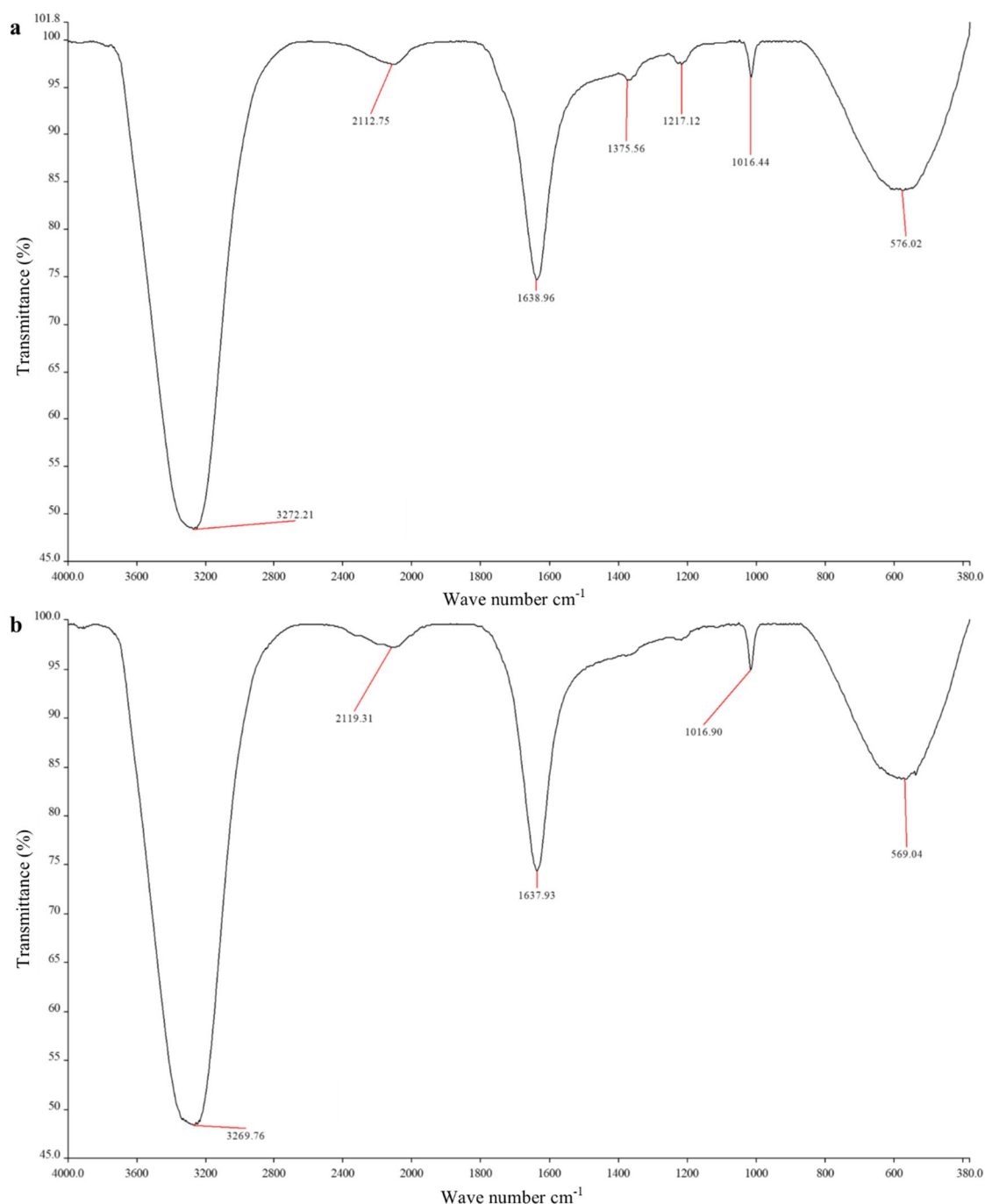
Figure 5.6: Fourier-transform infrared spectra of silver nanoparticles synthesised from a) leaf and b) stem extracts of *C. gratissimus* var. *gratissimus*.

5.4.6 Preliminary antibacterial assay

Silver is known to exhibit antibacterial activities against several bacterial strains (Singhal et al., 2011). In the medical industry, silver and AgNPs are utilised for a wide range of applications such as silver-containing topical creams and ointments used for the prevention of infection from burns/wounds (Singhal et al., 2011). Although microorganisms have been used for the synthesis

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of AgNPs (Vanaja and Annadurai, 2013; Ahmed et al., 2016a; Ahmed et al., 2016b), plant extracts are reportedly safer for use in human health care (Ibrahim, 2015). Antibacterial assays of *C. gratissimus* indicated that AgNPs synthesised from the leaf extracts had a stronger activity compared to those from stems. Table 5.3 is a summary of the activities exhibited by the AgNPs from leaf and stem extracts against the various bacterial strains.

Table 5.3: Antibacterial activities exhibited by silver nanoparticles from leaf and stem extracts of *C. gratissimus* var. *gratissimus* against eight bacterial strains.

Bacterial Strain	Leaves	Stems
E. coli (ATCC 25218)	+	-
E. coli	-	-
P. aeruginosa	+	-
MRSA (environmental type)	+	+-
MRSA (clinical type)	+	+-
S. aureus (ATCC 29213)	++	+-
S. aureus	+	+-
B. subtilis	+	+-

(-) No activity, (+-) weak activity, (+) moderate activity, (++) strong activity.

The AgNPs from leaf extracts were effective in inhibiting growth of most bacterial strains, except for *E. coli*, which did not display any sensitivity to the synthesised particles. These particles from the leaf extracts exhibited moderate activity against *E. coli* (ATCC 25218), *P. aeruginosa*, MRSA (environmental type), MRSA (clinical type), *S. aureus* and *B. subtilis*. The only strain to display high susceptibility against AgNPs from leaf extracts was *S. aureus* (ATCC 29213). This indicated that the AgNPs from leaf extracts had similar effects against gram negative and gram positive bacteria. A previous study on AgNPs from the aqueous leaf extracts of *C. zambesicus* indicated a minimum inhibitory concentration (MIC) at 30 mg/mL against both gram negative and positive bacterial strains (Ahmed et al., 2016c). According to Ahmed et al. (2016c), the AgNPs caused cell disruption which allowed penetration into the bacterial cells. The release of silver ions from the particles ultimately results in the death of the bacteria (Dhand et al., 2016). It is suggested that the phytochemical constituents from the leaves and the chemical groups identified in FTIR spectroscopy contributed to the antibacterial activity of the AgNPs (Ahmed et al., 2016c).

The differences between the activities of AgNPs from leaf extracts in the study by Ahmed et al. (2016c) and the current investigation, were probably attributed to the higher concentrations used in antibacterial assays (Ahmed et al., 2016c). Different extraction solvents and processes may have also played a role in the varying activities (Ahmed et al., 2016c). In addition, higher

concentrations of AgNO$_3$ solution and plant extracts used for synthesis may have also contributed to the differences in antibacterial activities (Ibrahim, 2015; Ahmed et al., 2016c).

Silver nanoparticles synthesised from stem extracts displayed little to no activity against the bacterial strains. *Escherichia coli* (ATCC 25218), *E. coli* and *P. aeruginosa* were resistant to the AgNPs from stems. These nanoparticles were also not very effective against MRSA (environmental type), MRSA (clinical type), *S. aureus* (ATCC 29213), *S. aureus* and *B. subtilis*, as indicated by the weak activities in Table 5.3. This indicated that the gram negative bacteria used in this study were resistant to the AgNPs synthesised from stem extracts, whilst gram positive bacteria displayed slight susceptibility. It is known that gram negative bacteria secrete a lactamase enzyme into their periplasmic space which increases their resistance to antibiotics (Elisha et al., 2017). In contrast, gram positive bacteria are more susceptible to attack from antimicrobial agents as they lack an outer layer (Ahmed et al., 2016c).

According to literature, the antibacterial activities of smaller AgNPs are stronger than those that are larger. This is due to the increased surface area of these particles which allows for greater surface contact resulting in the death of the bacterial cell (Gabriela et al., 2017). Therefore, the stronger antibacterial activity exhibited by AgNPs from leaf extracts was probably attributed to the monodispersion of small particles compared to the polydispersion of those produced from stems.

5.5 Conclusion

Silver nanoparticles were successfully synthesised from the methanolic leaf and stem extracts of *C. gratissimus*. The AgNPs from both extracts were spherical in shape but their sizes differed between the plant organs. Several groups were identified to play a role in the formation and stabilisation of AgNPs from leaves and stems. These included proteins, phenols, alcohols, alkanes, alkynes, amides, amines, ethers and alkyl halides. Antibacterial assays indicated that AgNPs from leaf extracts were more effective in inhibiting bacterial growth than those from stems. This was probably due to the monodisperal of AgNPs from leaf extracts. These results indicate that AgNPs from leaf extracts are potential sources of antibacterial agents as they display inhibitory properties. However, in order to consider these AgNPs as antibacterial agents, further studies are needed to improve their potency. This can be achieved by employing higher concentrations and combinations in the biosynthesis of AgNPs and determining their antibacterial activities. In addition, MIC assays should be conducted to determine their potential as antibacterial agents.

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CHAPTER 6: CONCLUSIONS AND FUTURE RECOMMENDATIONS

Traditional medicinal plants have been the subject of numerous investigation as they are responsible for the production of several natural bioactive compounds such as alkaloids, terpenoids and phenolic compounds. *Croton gratissimus* is a medicinally important species that has been used traditionally for the treatment of various ailments. This study aimed to identify and describe the structures from leaves and stems of *C. gratissimus* in order to determine their possible role in the production of secondary metabolites (Chapter 3). Furthermore, the study aimed to determine the chemical composition and antibacterial activity of leaf and stem extracts (Chapter 4). In addition, this study sought out to biosynthesise silver nanoparticles (AgNPs) from the methanolic leaf and stem extracts and determine its antibacterial properties (Chapter 5).

6.1 Major findings

Chapter 3: Microscopic investigations revealed three structures, i.e. lepidote and glandular trichomes, and non-articulated unbranched laticifers from the leaves and stems of *Croton gratissimus*. The lepidote and glandular trichomes were present on stems and the abaxial surfaces of leaves at all developmental stages. Lepidote trichomes comprised a multiseriate, multicellular stalk, a multicellular subradial disc, numerous radial cells and a single central cell. These trichomes formed a dense indumentum over the leaf surface, canopying the underlying glandular trichomes. Although this type of trichome is classed as non-glandular and non-secretory, transmission electron microscopy (TEM) revealed several organelles, such as vacuoles, vesicles, endoplasmic reticulum, golgi bodies and mitochondria, within stalk and radial cells suggesting that these structures may be involved in the synthesis of secondary metabolites. Glandular trichomes were embedded in the epidermal layer and comprised a single cell that formed a prominent stalk and distal head. Laticifers were predominantly associated with the vascular tissues in the leaves and stems. Histochemical analysis revealed alkaloids, phenolic compounds and lipids to be common among all three structures. The presence of alkaloids and phenolic compounds indicated that these structures are involved in the production of bioactive compounds which possibly contributes to the medicinal properties of the plant.

Chapter 4: Preliminary phytochemical screening of leaf and stem extracts revealed several compounds including terpenoids, carbohydrates, flavonoids, alkaloids, amino acids, phenolic compounds, saponins and fixed fats. Antibacterial assays revealed weak to strong activities against all bacterial strains investigated. However, stem extracts exhibited a stronger inhibition

of bacterial growth compared to leaf extracts. Several bioactive compounds identified from Gas Chromatography-Mass Spectrometry (GC-MS) analyses are suggested to contribute to the antibacterial activity of the extracts demonstrated in this study. This indicates that *C. gratissimus* is a potential source of antibacterial agents.

Chapter 5: Silver nanoparticles were successfully synthesised from the methanolic leaf and stem extracts of *C. gratissimus*. This synthesis was confirmed with ultraviolet-visible (UV-VIS) spectroscopy as both leaf and stem extracts displayed peaks between 400 – 500 nm. Ultraviolet-visible spectroscopy and TEM revealed spherical-shaped nanoparticles from both extracts. However, the size distribution of these particles differed between both extracts as leaves displayed monodispersion of particles whilst those from stems exhibited polydispersion. Phytochemical groups that were identified to play a role in the reduction and stabilisation of AgNPs included proteins, phenols, alcohols, alkanes, alkynes, amides, amines, ethers and alkyl halides. Antibacterial assays revealed that AgNPs from leaf extracts displayed stronger activities than those from stems. The activities exhibited by AgNPs from leaf extracts indicate that these particles are potential sources of antibacterial agents.

6.2 Challenges

Poor resin infiltration of leaf and stem tissue for TEM was the only major challenge experienced throughout this study. However, several protocols were employed to overcome these infiltration issues.

6.3 Future recommendations

The work conducted in this study serves as a precursor for future research on *C. gratissimus*. Although many structures were revealed to play a role in the production of phytochemical compounds, further research is required to identify their mode of synthesis. In addition, further investigations can be conducted on the crystal and secretory idioblasts, stellate trichomes and extrafloral nectaries identified from the leaves of *C. gratissimus*, as these structures may contribute to the medicinal properties of this plant. The antibacterial activity from crude extracts provides the basis for future studies as individual compounds can be isolated to investigate their potential as antibacterial agents. Furthermore, in order to consider AgNPs as potential antibacterial agents, additional research is required to improve the potency of these particles.

6.4 Final conclusion

Overall, this study confirmed the traditional use of *C. gratissimus* and revealed three structures that are involved in the production of some of the bioactive compounds. Furthermore, the plant extracts contain phytochemicals that aid in the synthesis and stabilisation of AgNPs. The

antibacterial activity of the extracts and AgNPs indicate that *C. gratissimus* is a potential source of antibacterial agents that could be utilised in the healthcare industry.