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Medicinal properties and micromorphology of *Rauvolfia caffra* Sond.

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences in the School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by The National Research Foundation.

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.

_____ _____

Signed: Professor Y. Naidoo Signed: Professor G. Naidoo

Date: Date:

DECLARATION 1: PLAGIARISM

I, Valentina Govender, 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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a) their words have been re-written but the general information attributed to them has been referenced;

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(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

Medicinal plants are effective treatments for various ailments and conditions due to the fact that they are easily accessible, cost effective, trusted and have little to no side effects. Plants produce bioactive or secondary compounds that serve as a defence mechanism to deter pests, attract pollinators and continue to assist in the survival of the species. These compounds or phytochemicals are useful to humans in the form of natural medicine, *Rauvolfia caffra* Sond. (Apocynaceae) is no exception. The traditional use of the bark is to alleviate skin ailments. A decoction of the bark is consumed for the treatment of abdominal discomfort, abscesses, pneumonia and fever. The research problem is that *R. caffra* has not been fully documented and the traditional uses cannot be supported scientifically. Furthermore, micromorphological characteristics remain to be described. This is a key component since *R. caffra* synthesizes latex and transports it through laticifers. The micromorphological characteristics were described by stereomicroscopy, scanning electron microscopy, light microscopy and histochemical tests on the leaves and petioles. This detected the presence of laticifers. To investigate the medicinal value, preliminary phytochemistry and antibacterial screening was performed on crude extracts of the stems and leaves. Methanol, chloroform and hexane were used as solvents of extraction and the classes of compounds detected were alkaloids, glycosides, sterols, flavones and flavonones. Thin layer chromatography provided a visualization of the classes of compounds present. The methanolic stem extract was found to inhibit seven strains of bacteria including *E. coli* and methicillin-resistant *Staphylococcus aureus*. The findings do not discredit the traditional utilization of this plant. Additionally, silver nitrate was combined with the crude methanol and water extracts of the stem and leaves to determine if silver nanoparticles (AgNPs) can be formed using a protocol that is safe, not toxic to the environment and simple to carry out. Three different mixing ratios (1:1, 1:2 and 1:4) were used to discover the optimum conditions for synthesis and the extracts screened for their antibacterial activity. The AgNPs synthesized ranged from15.84nm to 34.99nm in diameter which falls within a range that is preferred in nanoscience. The water stem 1:4 AgNPs was found to inhibit two different strains of bacteria, viz. methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia*. *Rauvolfia caffra* does have the potential to be used in drug formulation and in nanotechnology to treat prevalent health problems in South Africa.

Keywords: *Rauvolfia caffra*; laticifers; alkaloids; glycosides; sterols; AgNPs

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CHAPTER 1: INTRODUCTION

1.1 Rationale for the research

In using herbal medicine against disease, it is possible to limit side effects that manufactured synthetic drugs from the pharmaceutical industry can induce (Briskin, 2000; Lee and Bae, 2017). Commercially available medicines and their generic forms are expensive, especially as poverty is an increasing concern globally and affects more than five billion people in underdeveloped countries (Afzal *et al*., 2011; Hoang *et al*., 2017). With the naturally available flora, inexpensive treatments that may benefit a large portion of the global community can be formulated (Afzal *et al*., 2011; Amuka *et al*., 2017).

Traditional medicine systems form a large part of the health expenditure in South Africa. More than eighty percent of the black ethnic group consult traditional healers regularly while other ethnic groups have specific cultural traditions (Jäger *et al*., 1996; Kelmanson *et al*., 2000; van Vuuren, 2008; Xego *et al*., 2016; Petersen *et al*., 2017). Of the thirty thousand plants that make up the distinctive floral diversity, only about three thousand species are employed in a therapeutic manner (van Vuuren, 2008; van Wyk *et al*., 1997; Xego *et al*., 2016). The scientific information on these medicinal plants is scarce amidst the considerably recorded written reports on the relationship between the people and the plants they choose to use (Hutchings *et al*., 1996; van Vuuren, 2008).

The indigenous medicinal plants of South Africa offer a wide array of natural plant compounds for the purposes of treating common illnesses that are prevalent in third world countries (Drewes, 2012; Moteetee and Kose, 2017). The need for confirmation and approval by the scientific method comes from the heavy dependence of the destitute to utilize inexpensive forms of medication (Hutchings *et al*., 1996; van Vuuren, 2008). In the standard functioning of plants, secondary metabolites or phytochemicals are formed which is not vital in the survival of the plant but may have other functions (Drewes, 2012; Okigbo *et al*., 2009). Plants may release toxins to deter insects or animals from consuming them, while others may develop characteristic colours or features to mimic the sexual partners of the pollinators they want to attract (Drewes, 2012). Importantly for humans, plants may also produce compounds that serve as therapy or healing for common and fatal human illnesses such as cancer, tuberculosis and malaria (Drewes, 2012; Ramamurthy and Sathiyadevi, 2017). Drewes (2012) estimates that about half of all medicine available commercially is from plants or derived from plant sources.

Many illnesses have come to light in recent years. Of these, the most prominent and extensive disease is HIV/AIDS which is responsible for more than forty percent of deaths in KwaZulu-Natal (Drewes, 2012; Motsei *et al*., 2003). Natural sources have been effective in providing cures for many other prevalent illnesses (Drewes, 2012; Boadu and Asase, 2017; Doffana, 2017; Kinda *et al*., 2017). According to Kelmanson *et al*. (2000) and Ginovyan *et al.* (2017), it is imperative to institute the regulation and reliability of customary medical care, which highlights the importance of testing the antibacterial and antimicrobial activities of the plant species used.

In the Kingdom Plantae, microscopically distinguishable plant structures carry out the activities of either secreting or excreting biochemical substances (Cutter, 1978; Thanh *et al*., 2017). Secretory components may occur in various locations in plant tissues and cells. By characterizing and identifying secretory structures, distinguishing features may arise and in only a particular family of plants (Cutter, 1978; Demarco, 2017). The cytoplasm may be the site of the production of the substance and its transportation towards the outside of the cell is defined as 'secretion' (Cutter, 1978). Excretion on the other hand refers to the substances that the plant has no benefit from (Cutter, 1978). The substances secreted by plants have potential to be used for medicinal and economic gain (Cutter, 1978).

The medicinal components of plant tissue can be found in oil glands and glandular structures of leaf hairs, also called trichomes. The latter are thread-like outgrowths that originate from the epidermal layer of plant tissues (Levin *et al*., 1973; Barthlott *et al*., 2017). They occur in many different conformations across the surfaces of leaves, stems and roots. They are also responsible for carrying out a variety of fundamental functions required by the plant to adapt and thrive in its environment (Weryszko-Chmielewska *et al.,* 2005; Barthlott *et al*., 2017). Generally, trichomes function to enhance wind protection to prevent the epidermal layer from dissipating water from the tissues, decreasing the absorbance of sunlight and increasing the absorption of moisture (Levin *et al*., 1973; Dalin *et al*., 2008).

The secretory structures present in plants serve as a vital component of indicating the presence of phytochemicals, secondary metabolites and other secretions that form part of the plants biochemical defense system (Umah *et al*., 2017). Effectively, the biological activity of the phytochemicals determines the degree of pharmacological assistance (Umah *et al*., 2017). These secretions are synthesized, stored and transported by a variety of specialized secretory structures within and throughout the plant (Fahn, 1988; Khan, 2017). Secretory structures are

usually described by their aerial presence and play a role in distinguishing taxa (Buchel *et al*., 2015). Interestingly, it is mostly plants that are known for their traditional medicinal uses that are the ones selected for morpho-anatomical microstructural analysis (Fahn, 1988; Buchel *et al*., 2015; Ascensao and Pais, 1998). Buchel *et al*. (2015), further elaborate that the physicality of microstructures can be a quick and facile method to determine the medicinal value of a plant species. Secretory structures which may be internal or external are involved in the processing of metabolic compounds such as resins, gums, latex, alkaloids, glycosides and related compounds (Umah *et al*., 2017). These internal and external secretory structures may be trichomes, resin ducts, idioblasts, laticifers, secretory cavities, myrosin cells, oil cells and nectaries (Fahn, 1988; Umah *et al*., 2017). The secretory apparatus provides indications of similarity with classifications, just as the morphology of pollen (Rodrigues *et al*., 2016).

Laticifers are elongated secretory cells that occur in a series to form ducts that are distributed throughout the plant body (Dghim *et al*., 2015). Laticifers conduct latex which are rich in metabolites and plays a part in plant defence by deterring herbivores, specifically insects (Cho *et al*., 2009; Dghim *et al*., 2015). The latex and laticifers are specific to each species. The latex shows colour variations for different families while the laticifers have structural differences (Cho *et al*., 2009). Interestingly, laticifers are proposed to serve as storage space for surplus atmospheric carbon (Hagel *et al*., 2008; Cho *et al*., 2009).

It is well known that plants from the Apocynaceae family contain latex and have been used medicinally (Lopes *et al*., 2014). Similar to other Apocynaceae species, *Rauvolfia caffra* has characteristic white latex which is used to treat stomach problems (Mnxati, 2011). This plant has been used traditionally for its medicinal benefit; however there is scarce information on the scientific background. As it is a popular treatment for sexually transmitted infections and general health problems such as skin infections, fever and pneumonia; scientific investigations should follow (Njau *et al*., 2014). On this note, accurate record keeping has not been a priority in South Africa. This leaves gaps in research areas especially with regard to indigenous plants such as *Rauvolfia caffra*.

1.2 Justification

There is a lack of knowledge on the micromorphology and medicinally valuable components of *Rauvolfia caffra.* The research questions to be addressed are:

- Do *R. caffra* leaves and stems possess secretory structures that produce chemical secretions that have medicinal value?

1.3 Aims

The aim of this research was to investigate secretory structures and phytochemical composition of *R.caffra*.

1.4 Objectives

The objectives of this study are to:

- Describe the surface morphology of the leaves and petioles at different developmental stages with stereo microscopy, scanning electron microscopy (SEM) and light microscopy (LM)
- Describe the phytochemical constituents and relate this to the medicinal uses
- To use histochemical staining techniques and light microscopy to reveal internal structures.
- To investigate the preliminary antibacterial activities of the extracts from leaves and stems
- To introduce a facile approach for silver nanoparticle biosynthesis from aqueous and methanolic crude extracts of the stems and leaves of *R. caffra.*

1.5 Outline of dissertation

Chapter 1 provides an overview to the research along with the aims, motivation and details of the research. The literature review in Chapter 2 provides an in depth understanding of the genus and species (*Rauvolfia caffra*) that is native to South Africa. Chapter 3 focuses on the micromorphology of the leaves and stem bark by microscopy while histochemical staining

techniques are used to detect the phytochemical compounds. Chapter 4 provides a phytochemical evaluation of the extracts, an analysis of the leaf latex, using thin layer chromatography and preliminary antibacterial screening. Chapter 5 discusses a rapid silver nanoparticle biosynthesis protocol for both the leaves and stem crude extracts, which is a first for the species. Chapter 6 will provide a general discussion and identify gaps for future research.

1.6 Outline of Methodology

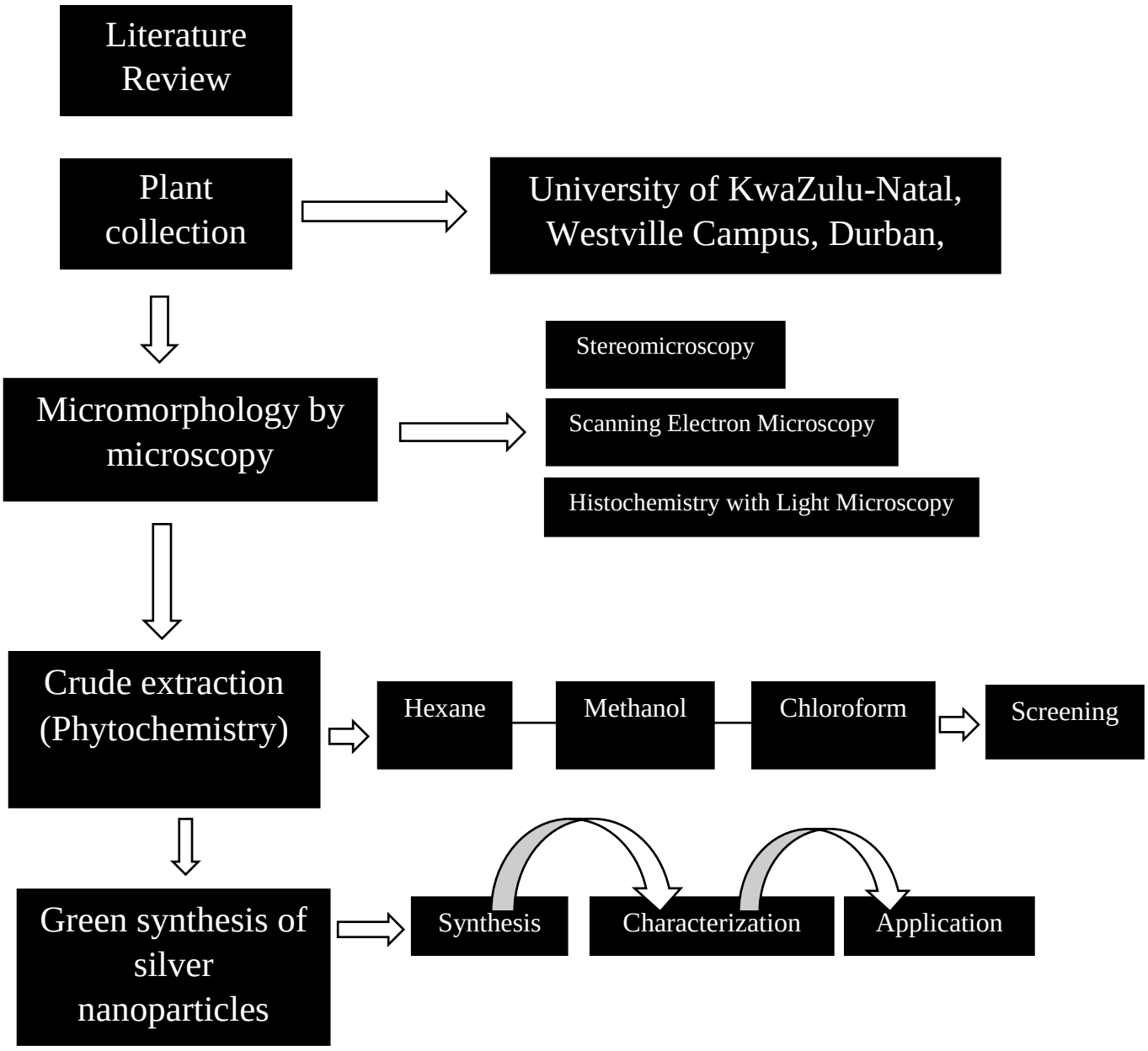

Figure 1: Flow diagram representing outline of the methodology

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

There is an increasing interest and demand for medicinal plants as their extracts are used to treat many diseases in pharmaceuticals, traditional medicine, pharmacognosy and ethnopharmacology (Maganha *et. al*. 2010; Nair and Chanda, 2007; Gairola *et. al*. 2009). There is interest in this sector due to potential uses in treating a range of human and animal health conditions effectively and naturally (Nair and Chanda, 2007). A large proportion of the world's population relies heavily on medicinal plants (Maganha *et. al*. 2010). African cultures still incorporate plant-derived cures as vital sources of medicine. More than 80% of the African population depend on traditional medicines (Fyhrquist, 2007). South Africa houses about 200,000 indigenous traditional healers (Gericke, 2002). Medicinal plants have been categorized according to the organs that contain compounds that may be used therapeutically (Gericke, 2002). Plant extracts are important in traditional medicine as they contain active compounds that can be isolated and synthesized for modern medicine by being screened biologically and pharmacologically (Balunas and Kinghorn, 2005).

Rauvolfia belongs to the Apocynaceae family and has sixty species in the genus (Mnxati, 2011). It is commonly called the 'quinine tree' as the bark is of a bitter taste. The tree is also known as 'umHlambamanzi' in isiZulu and 'kinaboom' in Afrikaans (Mnxati, 2011). *Rauvolfia caffra* can grow up to thirty meters in length with a strong bark is grey to brown (Mnxati, 2011). The leaves are simple and may be in whorls of three to six. Leaves have a leathery exterior that's tapering, smooth and dark-green in colour (Mnxati, 2011).

According to Njau *et al*. (2014), the bark decoctions are consumed for the alleviation of fever and for the treatment of pneumonia. The powdered bark is used for the treatment of skin ailments while chewing the bark can cure coughs (Njau *et al*., 2014). Njau *et al*. (2014) reported that extracts from this tree may have antimicrobial properties (in addition to antimalarial, antidiabetic and antitumor pharmacological remedies). The extracts apparently cause a decrease in the progression of reduction- oxidation processes in the cellular components of bacteria.

The South African variety of *R. caffra* needs to be investigated with regard to the medicinal, antimalarial, antidiabetic and antitumor properties described.

The commonly studied species of the genus is *Rauvolfia serpentina* (L.) Benth. ex Kurz, *Rauvolfia micrantha* Hook f., *Rauvolfia vomitaria* and *Rauvolfia tetraphylla* L. (Nanthini *et al*., 2011; Rahman and Ahfuza, 2015). The species, *Rauvolfia caffra* Sond. needs to be described, especially in the South African context. This review focuses on the phylogeny, botanical description and classification, micromorphology, chemical composition, ethnobotany and pharmacognosy of the genus *Rauvolfia*, focusing especially on *R. caffra*.

2.2 Phylogeny of the Apocynaceae family

The family Apocynaceae has gained popularity over the last two hundred years with new described species and distinguishing features being uncovered (Endress, 2004). The phylogenetic relationships, based on morphology, have resulted in the repositioning of certain tribes from new molecular data (Endress, 2004). The current systematics of the Apocynaceae places this family in the Gentianales (Rapini, 2012). According to Rapini (2012), it is one of the largest angiosperm families. The popularity of this family has increased as the species *Allamanda cathartica* L. and *Nerum oleander* among others, have been used traditionally as ornamentals (Rapini, 2012). Many of the Apocynaceae species possess phytochemicals such as the *Rauvolfia* while others are used for their wood and even fewer for fruit, *Aspidosperma* spp. and *Hancornia speciose* Gomes, respectively (Rapini, 2012).

The growth habit of the family is varied as some may be trees, vines, herbs and succulents, e.g. the tribe Stapeliinae (Rapini, 2012). Latex and a bicarpelar gynoecium are prominent features in this family (Rapini, 2012). Patterns of the lineage show a progressive move towards the synorganization of floristic features, the development of pollinaria being a specific example as part of the pollination mechanism of the Asclepiadoideae, Secamonoideae and Periplocoideae – subfamilies of the Apocynaceae (Endress, 2004; Rapini, 2012). Adanson, in 1763, originally identified this family as ‘Apocyna’ before Jussieu in 1789 officially confirmed it as ‘Apocineae’ (Rapini, 2012). Later, Robert Brown made descriptions of over forty genera which are still sound at the present time, in the family (Endress, 2004). He divided the asclepiads from Jussieu’s Apocineae and acknowledged it as a different family (Endress, 2004). According to Rapini (2012), the presence of pollinaria classifies taxa into the Asclepiadaceae while the absence of pollinaria classifies taxa into the Apocynaceae. Another of Brown’s significant contributions was further division within the asclepiads into three categories, rooted mostly on the quantity of pollinia present on each flower (Endress, 2004). Brown classified 170 species

across 53 genera both in the Apocynaceae and Asclepiadaceae together, now the Apocynaceae consists of 395 genera and about 5100 species (Endress, 2004).

The five subfamilies of the Apocynaceae are the Rauvolfioideae (10 tribes, 83 genera), Apocynoideae (8 tribes, 80 genera), Periplocoideae (33 genera), Secemonoideae (8 genera) and the Asclepiadoideae (4 tribes, 172 genera (Rapini, 2012). The Rauvolfioideae and Apocynoideae have a paraphyletic relationship while the Periplocoideae, Secamonoideae and Asclepiadoideae share a monophyletic relationship (Rapini, 2012). The genus *Rauvolfia* is part of the subfamily Rauvolfioideae and the tribe Vincaea with 85 species, most of which are found in tropical areas (Endress and Bruyns, 2000; Jyothi *et al*., 2012; Rodrigues *et al*., 2016).

2.3 The genus *Rauvolfia*

This genus comprises evergreen shrubs and trees with the exception of *R. purpurascens* of Panama which is a vine. The distribution of *Rauvolfia* spans across Africa, Asia, Oceania and the Americas (Rao, 1956; Milugo *et al*., 2013; Njau *et al*., 2014). Rao (1956) reported that all plants exude latex in the aerial components, when wounded. In the genus *Rauvolfia, R. serpentina* L. Benth ex Kurz. (commonly called 'snake root') has been extensively researched due to its wide availability in Bhutan, Cambodia, China, India, Laos and Indonesia (Rahman and Ahfuza, 2015). In this case, Mamgain *et al*. (1998), Singh *et al*. (2010), Sukumaran and Raj (2008), Mao (2009) and Dey and De (2011) report that this species is classified as threatened and critically endangered in India. Botanically, this plant exists as an erect shrub less than a meter tall (Rahman and Ahfuza, 2015).

The roots of *R. serpentina* have been used in the treatment of insect and snake bites, mental illness (more especially schizophrenia and insanity), other nervous system disorders such as anxiety, to alleviate high blood pressure and insomnia, gastrointestinal pains, pneumonia, malaria, skin diseases and breathing problems (Rai, 2004; Behera *et al*., 2006; Yusuf *et al*., 2006; Anisuzzaman *et al*., 2007; Singh, 2008; Meena *et al*., 2009; Pattanaik *et al*., 2009; Sen *et al*., 2008; Dey and De, 2011; Rahman and Ahfuza, 2015). Decoctions of the roots are administered to pregnant women to elevate uterine contractions and advance the release of the fetus (Rahman and Ahfuza, 2015). In Bangladesh and India, there have been reports of traditional healers utilizing *R. serpentina* to treat Acquired Immune Deficiency Syndrome (AIDS) (Rahamatullah *et al*., 2010; Dey and De, 2011). Rahamatullah *et al*. (2010) further

emphasized the potential of the plant as a cost-effective treatment while acknowledging the negative associations.

Rauvolfia tetraphylla L., commonly called 'devil pepper', can be found on the Indian continent in the form of a woody shrub between half a meter to a meter in height (Rahman and Ahfuza, 2015). Similar to the traditional treatment that *R. serpentina* provides, *R. tetraphylla* is used in the alleviation of elevated blood pressure, mental and nervous system disorders, treating skin ailments and aids in uterine contractions (Jyothi *et al.,* 2012; Rahman and Ahfuza, 2015). The root structures of this species may be used interchangeably with those of *R. serpentina* (Jyothi *et al*., 2012). Additionally, the plant produces anti-inflammatory effects, promotes diuresis and acts as a narcotic and tranquilizer with usage in treating malaria, syphilis and gingivitis, among other ailments (Jyothi *et al*., 2012; Sangram, 2012).

Rauvolfia vomitaria (Afzel), commonly called 'African serpent wood' or 'swizzle stick', has been used traditionally in Nigeria for treating elevated blood pressure and mental illness (Fapojuwami and Asinwa, 2013; Ojo *et al*., 2012). The root extracts and decoctions are used as sedatives, aphrodisiacs, abortive agents and as an antimycobacterial antioxidant (Ojo *et al*., 2012; Paul *et al*., 2011). Omole *et al*. (2009) reported that the leaf extract of *R. vomitaria* contained alkaloids, reducing sugars, tannins and saponins. Ojo *et al.* (2012) emphasizes the value of this plant in biological activities, specifically in acting against bacteria, viruses, diabetes and damage to liver cells. This species along with *R. tetraphylla* was once the source of the majority of marketable reserpine (Rao, 1956). Fapojuwami and Asinwa (2013) report that all parts of the plant are used traditionally: the leaves to treat malaria, hypertension and stomach-ache; the stem for treatment of malaria and barrenness; the root to treat jaundice; the seeds to treat symptoms of measles and the bark for the treatment of typhoid. A study carried out by Ekong *et al.* (2015) investigated the effect of *R. vomitaria* together with *Gongronema latifolium* in the neurological activities of mice. They found that *R. vomitaria,* by itself or together with *G. latifolium,* resulted in a rapid increase of cerebral cortical cells. Eteng *et al.* (2009) compared the leaf and root extracts of *R. vomitaria.* They found that the ethanolic root extract produced more harmful effects than the ethanolic extracts of the leaves in renal functioning.

Rauvolfia micrantha Hook. F is rare and endemic in southern India (Ramasubbu *et al*., 2008). The roots of *R. micrantha* are used as a substitute for *R. serpentina* (Sudha *et al.,* 2003; Ramasubbu *et al*., 2008). In the treatment of hypertension, the metabolites of *R. micrantha*

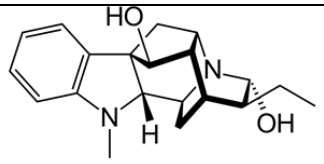
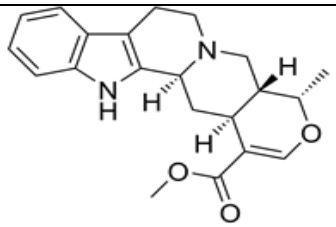
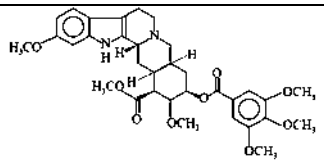
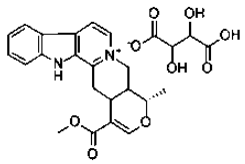
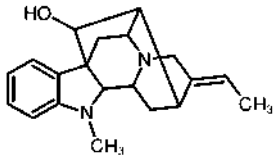
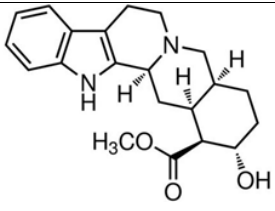
were identified as ajmalicine and ajmaline (Guillon *et al*., 2006). Sudha *et al.* (2003) proposed an innovative protocol to manufacture ajmaline and ajmalicine with the aid of auxins in *R. micrantha*. This work had previously been restricted to *Catharanthus roseus* (Sudha *et al*., 2003; Barrales-Cureno and Soto, 2012; Barrales-Cureno *et al*., 2017). Sudha *et al.* (2003) found that cultures of *R. micrantha* roots could produce indole alkaloids.

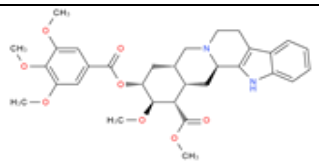
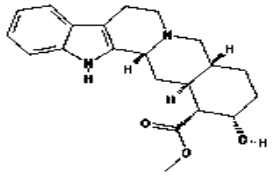
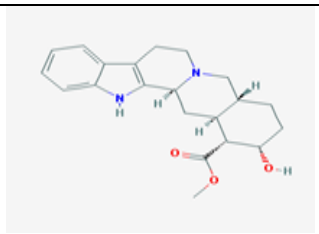
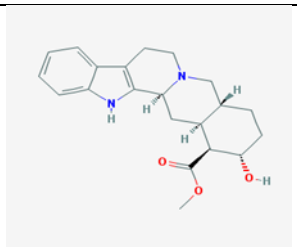
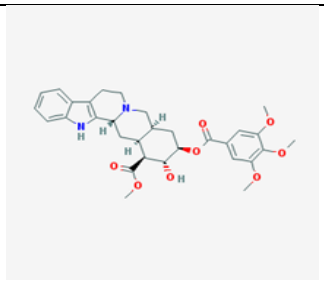
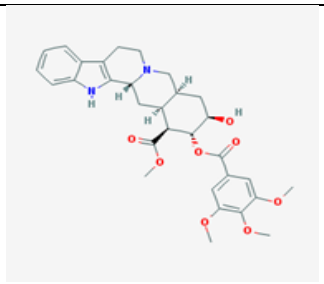
The findings of Nair *et al*. (2012) provide scientific support to the traditional uses of *R. serpentina, R. beddomei, R. micrantha, R. densiflora* and *R. tetraphylla* from the southern western Ghats of India. Their study revealed that the total antioxidant activity increased with higher concentrations of methanolic leaf extracts (Nair *et al*., 2012). Interestingly, the method of preparation of the leaves such as exposure to specific temperatures, exposure to sun or shade, oven drying, air drying or duration of drying may cause an increase or decrease in antioxidant activity (Nair *et al*., 2012). These factors should be subjected to testing to reveal the optimum conditions for extraction.

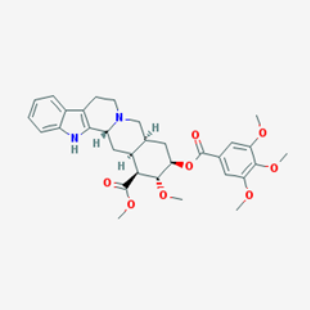
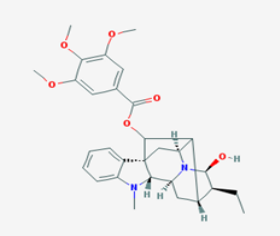
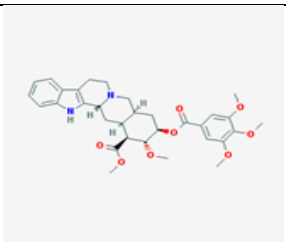
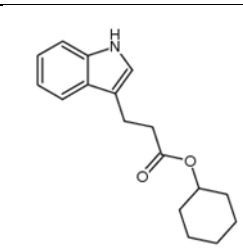
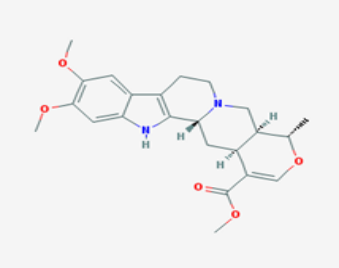
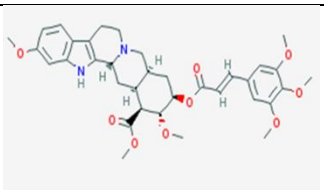
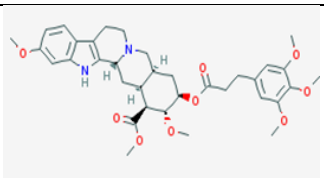
According to Balunas and Kinghorn (2005), medicinal plants provide new drugs in a natural form and can provide new ideas for the combinations of various chemicals to enhance the natural product and produce the desired effect. In the treatment of cancer, forty percent of the drugs produced to combat the disease from 1940 to 2002 were natural products (Balunas and Kinghorn, 2005). Traditional knowledge of natural medicine is still effective in combating current health problems. The drugs derived from *Rauvolfia* are ajmalicine, deserpidine, rescinnamine and reserpine (Fabricant and Farnsworth, 2001).

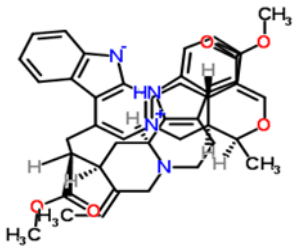
The table below provides a list of alkaloids that have been reportedly isolated from *Rauvolfia* species together with the chemical structures and applications.

Table 1: Alkaloids identified from *Rauvolfia* species with structures and applications.

Alkaloid	Structure	Application	Reference
Ajmaline $C_{20}H_{26}N_2O_4$		Cardiac dysrhythmia agent, identifies Brugada syndrome	(NCBI, 2017; Jyothi *et al*., 2012; Kumari *et al.*, 2013; Iqbal *et al*., 2013)
Ajmalicine $C_{21}H_{24}N_2O_3$		Remedies diseases of the circulatory system, discourages strokes, relieves hypertension	(NCBI, 2017; Jyothi *et al*., 2012; Kumari *et al.*, 2013)
Reserpine $C_{32}H_{40}N_2O_9$		Relieves high blood pressure, sedative, tranquilizer, remedies cardiovascular disease, treats neurological symptoms	(Iqbal *et al*., 2013; Jyothi *et al*., 2012; Kumari *et al.*, 2013; Singh *et al*., 2016)
Serpentine $C_{21}H_{20}N_2O_3$		Treats psychosis	(Iqbal *et al*., 2013; Jyothi *et al*., 2012; Kumari *et al.*, 2013)
Tetraphyllicine $C_{20}H_{24}N_2O$		Energizes muscular tissue	(Iqbal *et al*., 2013; Jyothi *et al*., 2012; Yu *et al*., 2013)
Rauvolscine $C_{21}H_{26}N_2O_3$		Analog of alpha-yohimbine	(Jyothi *et al*., 2013; NCBI, 2017)

Canescine $C_{32}H_{38}N_2O_8$		Analog of deserpidine	(Jyothi *et al.*, 2013; NCBI, 2017)
Pseudoyohimbine $C_{21}H_{26}N_2O_3$		Isomeric with yohimbine	(Jyothi *et al.*, 2013; NCBI, 2017)
Yohimbine $C_{21}H_{26}N_2O_3$		Treats improper erectile functioning	(Iqbal *et al.*, 2013; Jyothi *et al.*, 2012; Kumari *et al.*, 2013; NCBI, 2017)
Corynanthene $C_{21}H_{26}N_2O_3$		Diastereoisomer of yohimbine	(Iqbal *et al.*, 2013; Jyothi *et al.*, 2012; NCBI, 2017)
Raunescine $C_{31}H_{36}N_2O_8$		Lowers concentration of noradrenaline in rat brain studies	(Paasonen and Dews, 1958; Iqbal *et al.*, 2013; Jyothi *et al.*, 2012; NCBI, 2017)
Isoraunescine $C_{31}H_{36}N_2O_8$		Approximately ten times lower in effect than raunescine	(Paasonen and Dews, 1958; Iqbal *et al.*, 2013; Jyothi *et al.*, 2012; NCBI, 2017)

Recanescine $C_{32}H_{38}N_2O_8$		Analog of deserpedine	(Jyothi *et al.*, 2012; NCBI, 2017)
Ajmalimine $C_{30}H_{36}N_2O_6$		Unknown	(Kumari *et al.*, 2013; NCBI, 2017)
Deserpedine $C_{32}H_{38}N_2O_8$		Lowers blood pressure, treats psychosis	(Iqbal *et al.*, 2013; Kumari *et al.*, 2013; NCBI, 2017)
Indobinine $C_{17}H_{21}NO_2$		Aldose reductase inhibitor to prevent diabetes	(Kumari *et al.*, 2013; Pathania *et al.*, 2013; NCBI, 2017)
Reserpiline $C_{23}H_{28}N_2O_5$		Relieves high blood pressure, treats psychosis, remedies gastric symptoms	(Kumari *et al.*, 2013; Yu *et al.*, 2013; NCBI, 2017)
Rescinnamine $C_{35}H_{42}N_2O_9$		Lowers blood pressure	(Kumari *et al.*, 2013; NCBI, 2017)
Rescinnamidine $C_{35}H_{44}N_2O_9$		Unknown	(Kumari *et al.*, 2013; NCBI, 2017)

Serpentinine $C_{42}H_{44}N_4O_6$		Relieves hypertension	(Isharwal and Gupta, 2006; Iqbal *et al*., 2013; Kumari *et al*., 2013; NCBI, 2017)

2.4 *Rauvolfia caffra* Sond.

Botanical classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Gentianales

Family: Apocynaceae

Genus: *Rauvolfia*

Species: *Rauvolfia caffra*

Common Names: Kinaboon, Koorsboom, Munadzi, Muraldi, Nshongo, Quinine Tree, Umhlambamanzi, Umhlambamaze, Umjela, Umjele, Umjelo, Umkhadluvungu, Umthondisa, Umthundisa, Waterboekenhout. (Foden and Potter, 2015).

2.4.1 Description and distribution

Rauvolfia caffra is classified as 'Least Concern', as per the conservation status (Mnxati, 2011). *Rauvolfia caffra* is widespread in tropical Africa (Gbonjubola *et al*., 2010). It has aesthetic value, traditional and medicinal uses and is used as a source of wood for furniture (Mnxati, 2011).

Figure 1: *Rauvolfia caffra* (A) whole plant (B) leaves (Courtesy of: http://www.plantzafrica.com/plantqrs/rauvolfiacaffra.htm and http://www.plantsinstock.co.za/upload/4878.jpeg)

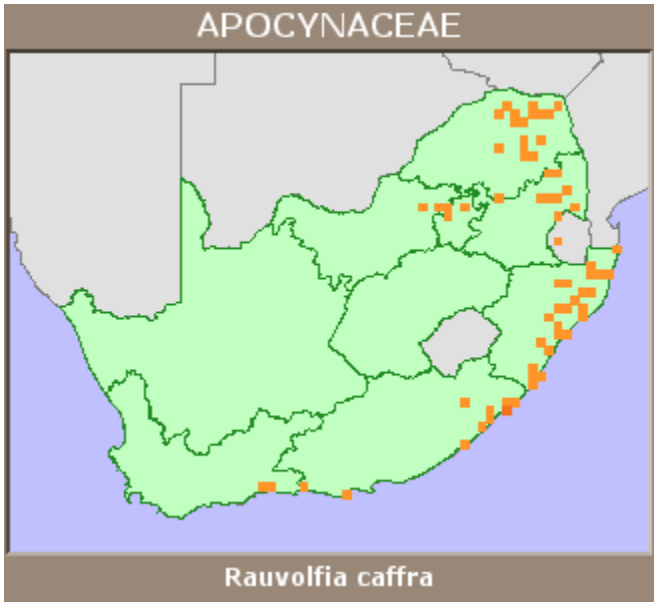


Figure 2: Distribution of *Rauvolfia caffra* in South Africa (Courtesy of Foden and Potter, 2015)

2.5 Secretory structures and micromorphology

The secretory structures in the leaves may contain essential oils, tannins, resins, latex, salts, enzymes and other secretions that are of pharmacological and taxonomic importance (Turner *et al*., 1998). The Apocynaceae is one of two that has laticiferous structures (Appezato-Da-Gloria and Estelita, 1997). Laticifers are latex conducting ducts that occur in almost six percent of all tracheophyte species (Freitas *et al*., 2010). The constituents of latex have been thoroughly investigated for the species *Hevea brasiliensis,* owing to its commercial value of providing natural rubber (Freitas *et al*., 2010). Latex is a liquid substance with a milky constituency and colour variations of clear, white, yellow, orange or red (Freitas *et al*., 2010; Mahlberg, 1993). Laticifers are the sites of latex synthesis, accretion and in the case of tissue injury- exudation (Freitas *et al*., 2010). Morphologically, laticifers can take an articulating or non-articulating form. Articulated laticifers are made up of a series of cells while non-articulating laticifers are made up of one long cell (Agrawal and Konno, 2009).

Further, differences among the types of laticifers that exist suggest that non-articulated laticifers are able to form an organized channel unassisted. Simple laticifers are those that have single cells (non-articulated). Compound laticifers arise as a result of cells organized in series (articulated). Non-articulated and articulated laticifers may occur as branched or unbranched (Freitas *et al*., 2010; Razak and Bahri, 2000). The anatomical characteristics of laticifers are thought to influence the rate and velocity of the flow of latex after tissue damage (Freitas *et al*., 2010). Latex is also referred to as the cytoplasm of laticifers (Kitajma *et al*., 2013).

Figure 3: Latex exudates from a leaf of milkweed (Courtesy: http://carex.tumblr.com/post/53884535831/malformalady-milkweed-latex-milkweed-is-named)

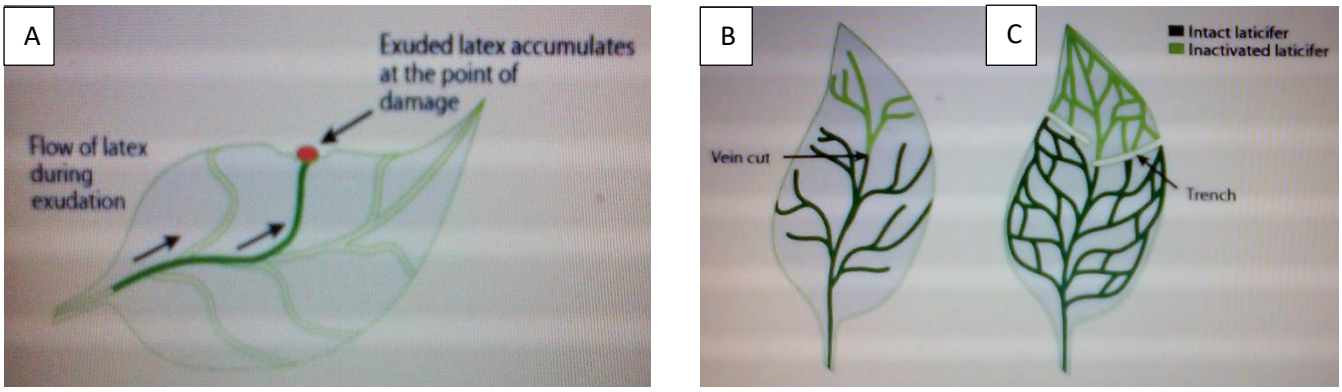


Figure 4: Description of laticifers and the flow of latex at a point of damage (A) The role of laticifers in the movement of latex at tissue damage site (B) non-articulated laticifers (C) articulated laticifers (Courtesy: Agrawal and Konno, 2009)

Laticifers play a significant role commercially, especially in the case of *Hevea brasiliensis*. The enzymes found in the latex contribute to the development of natural rubber (Aoki *et al*., 2014). Aoki *et al*. (2014) aimed developed a laticifer-specfic protocol that increased the outputs of laticifers and, in turn elevated the production of the highly sought after rubber. Sethi *et al*. (2008) described laticifers as occurring with vascular tissue and branching out into the inner tissue where they eventually reach the cutis. Barrato *et al.* (2010) reported that laticiferous ducts occur within the tissues of the cortex, phloem and pith of *R. sellowii*. The authors further described the anatomical features of laticifers as being of a larger cell size than neighbouring cells and have a noticeable nucleus and a compact cytoplasm (Barrato *et al*., 2010). Castelblanque *et al.* (2016), investigated the functioning of laticifers and their role in the enhancement of plant survival. They reported that much of this information is unknown.

2.6 Ethnobotany and Pharmacognosy

According to Njau *et al*. (2014), bark decoctions are consumed for the alleviation of fever and for the treatment of pneumonia. The powdered bark is used for the treatment of skin ailments while chewing of the bark can cure coughs (Njau *et al*., 2014). Tshikalange *et al.* (2015) reported that *R. caffra* is used to treat sexually transmitted diseases (STD's). Specifically, the stem bark is used for this purpose. The plant is also used to treat diarrhea and abdominal pains (Tshikilange *et al*., 2008). Mbunde *et al*. (2016) note that in Tanzania, a root extract is used in the treatment of mental illness and epilepsy, while the stem bark extract is used to relieve

diseases of the joints and muscles and alleviate pain in the chest. For the treatment of coughs, the bark and latex are prepared while the bark extracts are used for accelerated healing of wounds and skin ailments (Lall and Kishore, 2014). Milugo *et al.* (2013) reported that *R. caffra* is also used in the treatment of scabies, parasites, high blood pressure, dysentery and malaria.

2.7 Chemical composition and Toxicity

Latex is an important biochemical feature in *R. caffra* since it makes up part of the plant defence system to deter herbivorous insects (Agrawal and Konno, 2009). The bioactive compounds present in the latex are transported by laticifers. Latex may act as potential storage units for excess atmospheric carbon dioxide in the mitigation of climate change (Agrawal and Konno, 2009). According to Freitas *et al.* (2010), laticifers are specialized cells for particular functions. They display explicit DNA sequences to facilitate the synthesis of particular enzymes, metabolites and amino acids. Furthermore, the protein constituents in laticifers exhibit great variations and differences in *Cryptostegia grandiflora, Plumeria rubra* and *Euphorbia tirucalli* (Freitas *et al*., 2010).

Mousinho *et al.* (2011) extracted latex from *Himatanthus drasticus* (Apocynaceae). These researchers investigated the latex proteins effect on tumors and found that this protein was effective in diminishing tumors (Mousinho *et al.*, 2011). Gidrol *et al*. (1994) determined that a protein similar to lectin, called hevein was involved in contributing to the coagulation of latex at injury sites which is thought to act as a blockade for potential pathogens. Zou *et al.* (2015) reported that water comprised sixty percent of latex and adequate water acquisition allows for optimum latex movement and renewal. Furthermore, aquaporins are the intrinsic channel proteins that facilitate water movement to the laticifers (Zou *et al*., 2015).

Roots *R. caffra* extracts were found to restrain the growth of Gram-positive bacteria, although water and chloroform extracts showed a general inhibition effect against bacteria. Milugo *et al.* (2013) emphasizes the antioxidant activity of *R. caffra* and the detection of cardiac glycosides, a number of alkaloids, saponins and steroids. Fabricant and Farnsworth (2001) caution that toxic effects should be considered with use of these traditional remedies. In the case of latex, the defense mechanisms by the plant may be physical due to its viscosity (Sethi *et al*., 2008). The toxicity can be attributed to alkaloids, terpenoids and cardiac glycosides (Sethi *et al*., 2008). Research conducted by Mlala (2015) suggests that methanolic fractions of *R. caffra* stem bark may be toxic at elevated concentrations.

2.8 References

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CHAPTER 3: LEAF MICROMORPHOLOGY AND ANATOMY

3.1 Abstract

The members of the Apocynaceae family share a peculiar characteristic of synthesizing latex. Known as a plant protection agent, this substance also has medicinal and industrial applications. The objective of this study was to investigate the micromorphological feature of *Rauvolfia caffra,* a South African medicinal plant. Micromorphological observation by stereomicroscopy, light microscopy (LM) and scanning electron microscopy (SEM) was accomplished by free-hand and vibratome sections of the leaves and petioles. This provided information on the latex conducting ducts called laticifers, present in the pith and near the vascular tissue. Laticifers appeared to be articulated and unbranched from the paradermal sections of the midvein. The findings of this study are novel and contribute to the knowledge on laticifers in the Apocynaceae.

Keywords: Articulated laticifer; latex; laticifers; micromorphology; *Rauvolfia caffra*

3.2 Introduction

The Apocynaceae is one of the largest families with 360 genera and around 5000 species (Endress, 2004; Endress *et al*., 2014). The family comprises large trees, shrubs, wild herbs and even vines, however the common feature among them is the presence of latex (Capelli *et al*., 2017). Latex is a fluid exudate with colour variations for different species of plants (Dghim *et al*., 2015). The *Euphorbia* genus has characteristic white latex while the *Cannabis* genus has yellow – brown latex. The *Chelidonium* genus has yellow-orange while *Morus* genus has clear to colourless latex (Dghim *et al*., 2015). The colour of latex may differ among the organs of the same plant and may even change upon ablation, as is the case in *Hevea brasiliensis* (Dghim *et al*., 2015). Apart from colour, latex also contains an array of enzymes and proteins (Kajii *et al*., 2014). An example is the latex synthesized by the *Ficus* genus, which contains ficin. This protease plays a part in inhibiting pathogens and herbivorous fauna (Agrawal and Konno, 2009; Kajii *et al*., 2014). Another example is *Nerium oleander* L. (Apocynaceae) which was documented by Threophrastus in early 1916 to have characteristic poisonous 'juices' (Mahlberg, 1993).

Apart from its significance in plants, latex has industrial applications for the natural rubber that can be obtained (Aoki *et al*., 2014). Natural rubber as a raw material is resilient, elastic and has abrasive and impact resistant properties (Aoki *et al*., 2014). Of the 2500 higher plants that produce natural rubber, *Hevea brasiliensis* is preferred, based on the high yield of latex produced (Aoki *et al*., 2014). The yield is directly linked to the functioning of laticifers which are specialized cells that occur among vascular tissue (Aoki *et al*., 2014; Wiedenhoft *et al*., 2009). Laticifers may occur as a series of elongated cells that contain latex (Razak and Bahri, 2000; Dghim *et al*., 2015). Wiedenhoft *et al*. (2009) suggest that latex is produced in the laticifers and usually found near the xylem and phloem in the body of the plant. When the laticifers are damaged, they may release a large quantity of latex due to the positive turgor pressure that the cellular components are under (Bird *et al*., 2003). Laticifers may be simple and made up of single cells (non-articulated) or compound and composed of a series of cells (articulated) (Razak and Bahri, 2000).

Rauvolfia caffra Sond. is a South African medicinal plant that is used to treat pneumonia, fever and skin problems among others (Njau *et al*., 2014). It is a large tree that grows in the provinces of the Eastern Cape and KwaZulu-Natal. Records suggest that the bark has been used to make

furniture (Mnxati, 2011). There appears to be a lack of knowledge on the latex conducting ducts of *R. caffra* and no histochemical investigations have ensued. The purpose of this study is to detect the presence of laticifers in *R. caffra*, investigate the micromorphology by electron microscopy and detect the presence of chemicals by histochemistry (light microscopy).

3.3 Materials and Methods

3.3.1 Plant Collection

Fresh leaves of *R. caffra* were collected from the Westville campus (UKZN) located at 29.831°S 30.925°E. The plant material was verified by Mr. E. Khathi and a voucher specimen was deposited at the UKZN Westville Herbarium, voucher number X4076057.

3.3.2 Stereomicroscopy

Five replicates per developmental stage (young and mature) were used and fresh leaves, stems and petioles were examined on both the abaxial and adaxial leaf surface. Stereomicroscopy was accomplished using the Nikon AZ100, samples were observed and images captured with the Nikon DXM 1200C colour camera.

3.3.3 Scanning Electron Microscopy (SEM)

Ten replicates (five replicates per category) of fresh leaf and petiole sections was analysed. The samples were trimmed into 5mm X 5mm segments for freeze drying. The sections were prepared by rapid quenching in subcooled liquid nitrogen. Freeze drying was done by using the Edwards-Modulyo freeze dryer at -60°C in a vacuum of 10-2 Torr for 72 hours. Freeze fracture was accomplished by quenching 5cm X 5cm leaf segments in subcooled liquid nitrogen and fracturing the material to obtain smaller segments. The sections were mounted onto brass stubs and secured with carbon conductive tape. The samples were sputter coated with a Polaron SC500 Sputter coater and viewed using a Leo 1450 SEM at a working distance of 12mm. The images were captured using the smart SEM version 5.03.06.

3.3.4 Semi-thin sections with Light Microscopy (LM)

Leaf and petiole segments of sizes less than 5mm X 5mm were prepared by prefixing in a solution of 3 % glutaraldehyde (4°C) with 0.05 M cacodylate buffer for 24 hours followed by post-fixation at room temperature in 2% Osmium Tetroxide for 3 hours. The samples were washed thrice in 15 minute intervals before and after fixation in cacodylate buffer. This was followed by dehydration in a graded alcohol series of 10 minutes each in 10%, 30%, 50%, 70% and 100% ethanol. The samples were allowed to stand in 100% ethanol overnight at room temperature. The samples were then washed twice in propylene oxide for 15 minutes and then embedded in a mould with equal parts of Spurr's resin and acetone for 4 hours and then 100% resin for 24 hours at 70°C (Spurr, 1969). Semi-thin sections were prepared with Reichhert Ultracut E microtome. Glass knives were used for the sectioning process and was prepared on the LKB Knifemaker 7801A (Sweden). Sections were stained with toludine blue before fixing on glass slides. Images were obtained by viewing under the Nikon Eclipse ATI light microscope

3.3.5 Histochemistry with Light Microscopy (LM)

Thin vibratome sections (80-100microns) of both leaves and petioles were made and stained with the following reagents to detect the localization of specific cellular compounds and chemicals within the leaves. Images were examined and captured using the Nikon Eclipse ATI light microscope and appropriate controls were performed.

- Acridine orange – detects lysosomes

Sections were immersed in a dilute solution of the stain for ten minutes and then rinsed with distilled water. The colour change reaction is orange-red for cells that undergo apoptosis which gives an indication on cell viability (Gupta and De, 1983).

- Ferric trichloride – detects phenolics

Sections were immersed in 10% aqueous Ferric trichloride with a dash of sodium carbonate for five minutes and then rinsed with 70% ethanol. The colour change reaction is blue-black (Zarate and Yeoman, 1994).

- Toluidine Blue – detects carboxylated polysaccharides and phosphate groups

Sections were immersed in the stain for one minute and then rinsed with distilled water. This is a metachromatic stain where the carboxylated polysaccharides gives a pink colour reaction and the phosphate groups give a blue-purple colour change (Mitra and Loqué, 2014).

- Sudan III and IV– detects lipids

Sections were immersed in a saturated solution of the stain for fifteen minutes and then rinsed briefly with 70% ethanol to remove excess stain. Lipid inclusions show a colour change of orange- red (Buda *et al*., 2009).

- Mercuric bromophenol blue – detects proteins

Sections were immersed in bromophenol blue for ten minutes and then rinsed with 0.5% acetic acid to remove excess stain. The colour change reaction is blue (Hornatowska, 2005).

- Safranin – detects lignin, cutin and suberin (cell walls)

Sections were immersed in safranin for 30 seconds and then rinsed with distilled water. The colour reaction is bright red (Bond *et al*., 2008).

- Fast green – detects cellulose cell walls

Sections were immersed in fast green stain for one minute and then rinsed thoroughly with distilled water. The colour reaction is bright green (Tos *et al*., 1980).

- Safranin and fast green double stain

Sections were immersed in safranin for 30 seconds and then rinsed thoroughly with distilled water. The sections were then immersed in fast green for one minute and rinsed thoroughly with distilled water. The colour reaction is bright red for lignin, suberin and cutin and bright green for cellulose cell walls (Periasamy, 1967).

- NADI (Naphthol and diamine) – detects essential oils and resin acids

Sections were immersed in NADI for 20 minutes in the dark at room temperature and then rinsed with sodium phosphate buffer. The colour reaction is blue for essential oils and red for resin acids (Endo, 1972).

- Phloroglucinol – detects lignin and cuticle components

Sections were immersed in phloroglucinol for 10 minutes and then rinsed with distilled water. The colour reaction is red (Jensen, 1962).

- Ruthenium red – detects mucilage and pectin

Sections were immersed in ruthenium red for 10 minutes and then rinsed with distilled water. The colour reaction is pink (Colombo and Rascio, 1977).

3.4 Results and Discussion

The functions of laticifers lies in protecting the plant from herbivores, inhibiting pathogenic bacteria, viruses and fungi and acting in wound healing since the latex has quick coagulation time (Dghim *et al*., 2015). This acts as a block when the plant is wounded and seals off the damaged site, preventing entry of any disruptive microorganism (Dghim *et al*., 2015). Stereomicroscopy images of the emergent, young and mature leaves shows the adaxial surfaces as glabrous, leathery and shiny with a dark green colour while the abaxial leaf surface is pale green with a prominent and raised midrib (arrows) (Fig. 1.1 and Fig. 1.2). The secondary and tertiary veins are thick and prominent. The description by Mnxati (2011) is similar to these findings.

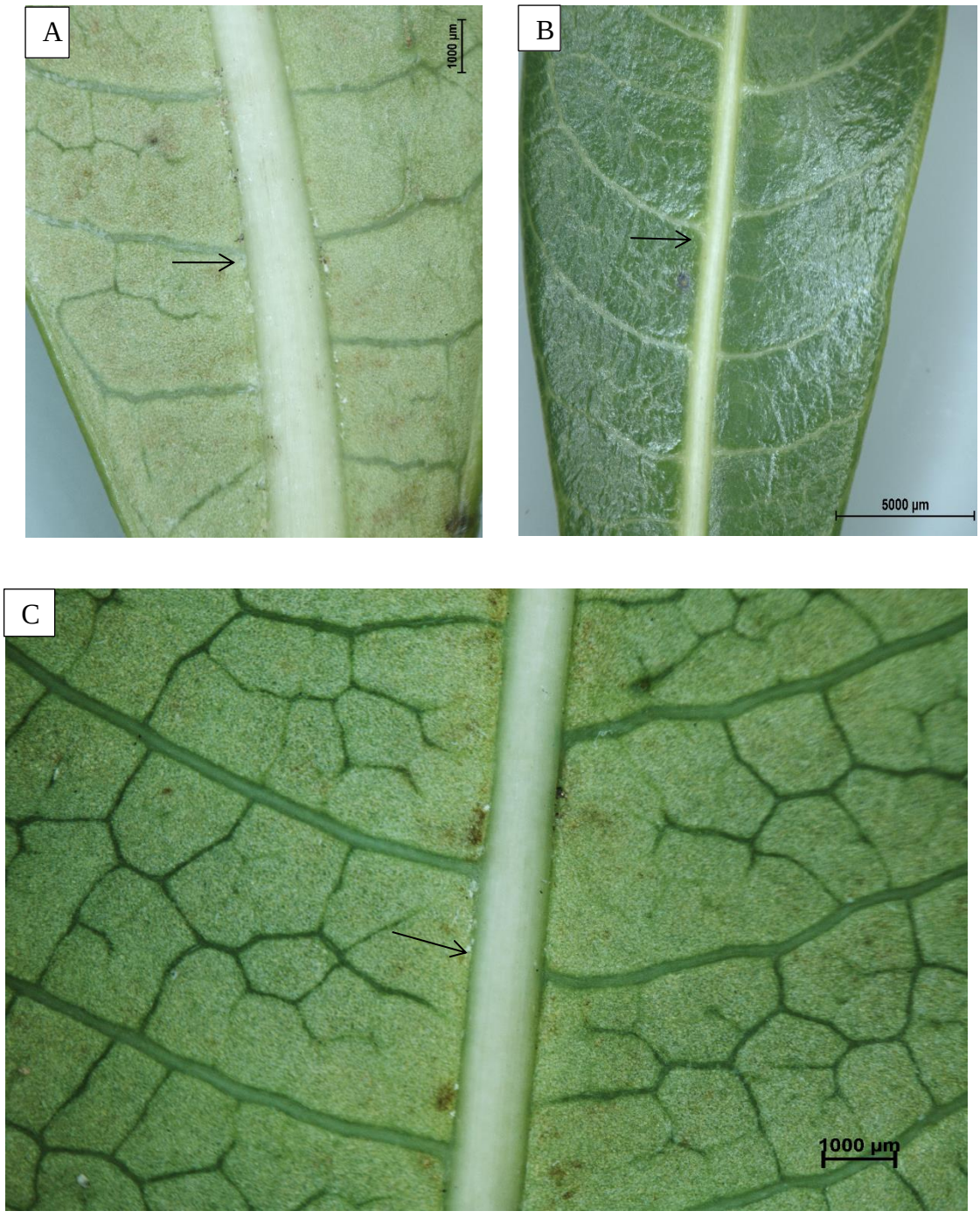


Figure 1.1: Stereomicroscope images of *Rauvolfia caffra* surface morphology (A) emergent abaxial leaf surface, (B) emergent adaxial leaf surface and (C) young abaxial leaf surface

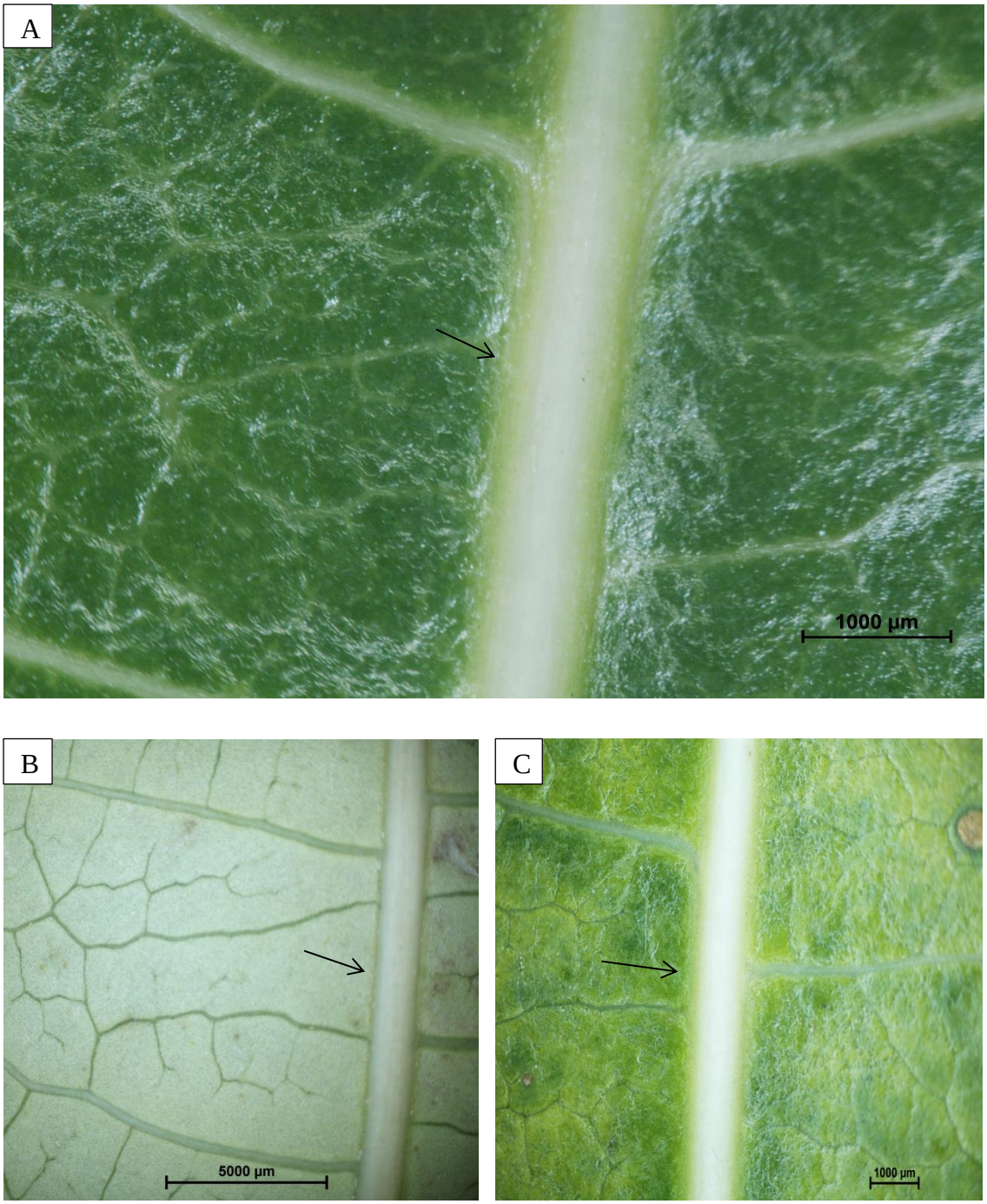


Figure 1.2: Stereomicroscope images of *Rauvolfia caffra* surface morphology (A) young adaxial leaf surface, (B) mature abaxial leaf surface and (C) mature adaxial leaf surface

The stereomicroscope images of the cross section of the stem and petioles are shown in Figure 2. Figure 2.1(A) shows the cross section stem. Figure 2.1(B) shows laticifers scattered across the pith. Figure 2.2(A) shows the exuding latex from the mature leaf upon being sectioned. The latex is the cytoplasmic content of the laticifer. There are no cytoplasmic connections between the laticifer and non-laticifer cells. It is suggested that the exudate should only be from that of the laticifer (Kush *et al*., 1990). The young and mature petiole was sectioned and shows the latex being dispelled (arrows) (Fig. 2.2(B) and Fig. 2.3).

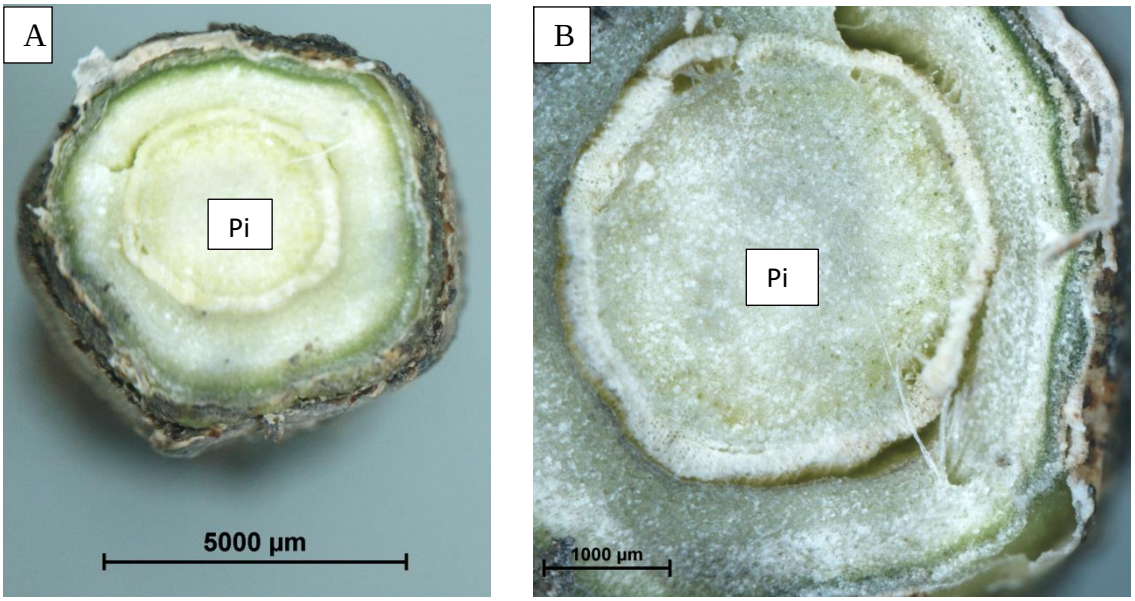


Figure 2.1: Stereomicroscope images of stem in cross section: (A) stem and (B) stem and laticifers among the pith (Pi)

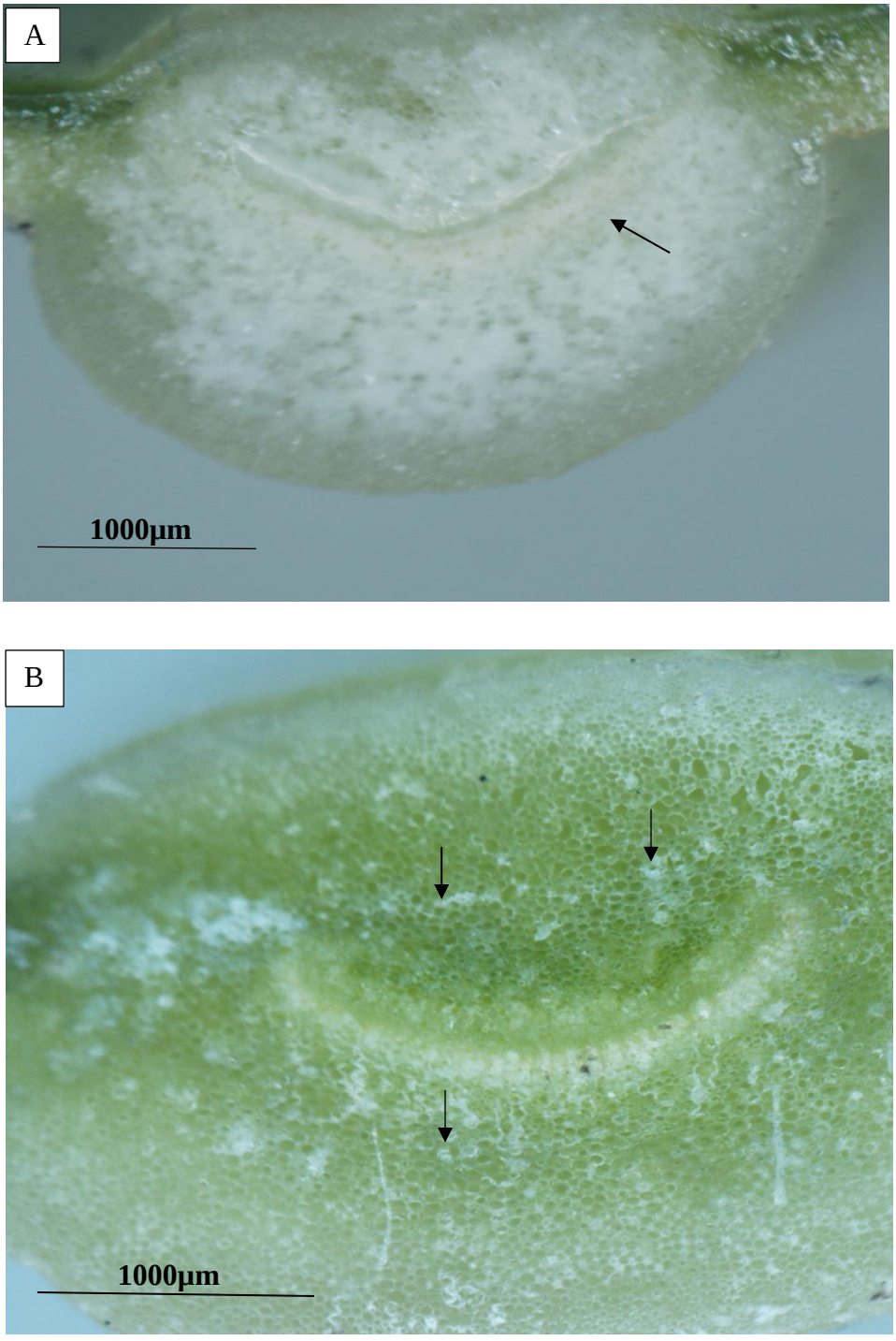


Figure 2.2: Stereomicroscope images of petioles in cross section (A) mature petiole showing latex appearing from site of sectioning and (B) mature petiole showing laticifers

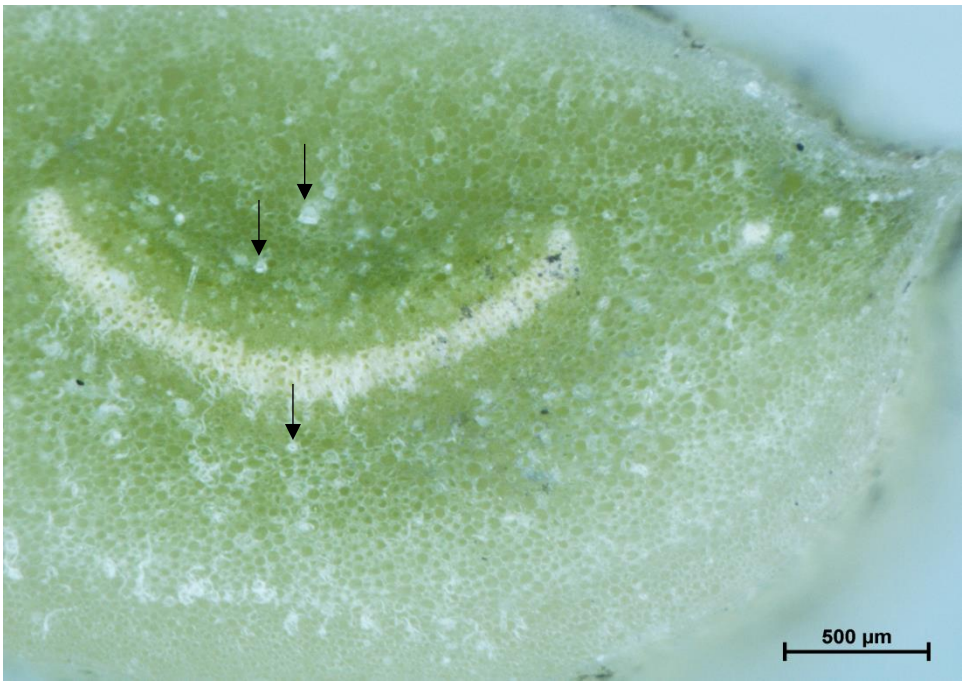


Figure 2.3: Stereomicroscope image of young petiole showing laticifers and latex

Scanning electron microscopy was performed on the emergent, young and mature leaves. The surface topography of leaves revealed the prominent midvein and the presence of stomata (Fig. 3(A) and Fig. 3(B)). However, the freeze fractured cross sections (Fig. 4) of the emergent (A) and mature (B) leaves showed what appeared to be laticifers. The technique of freezing the tissue was used by Fineran and Condon (1988) upon realization that the contents of laticifers are disturbed by chemical fixation methods, due to high tugor pressure. By incorporating this technique, latex particles seem to be preserved within the laticifer, specifically the central vacuole. The laticifer cells in Figure 4 appear to have exudates (arrows). The cells surrounding the laticifers appear almost bare in comparison. Similar results were found by Razak and Bahri (2000) upon investigation of mature *Musa* leaves and Demarco (2014).

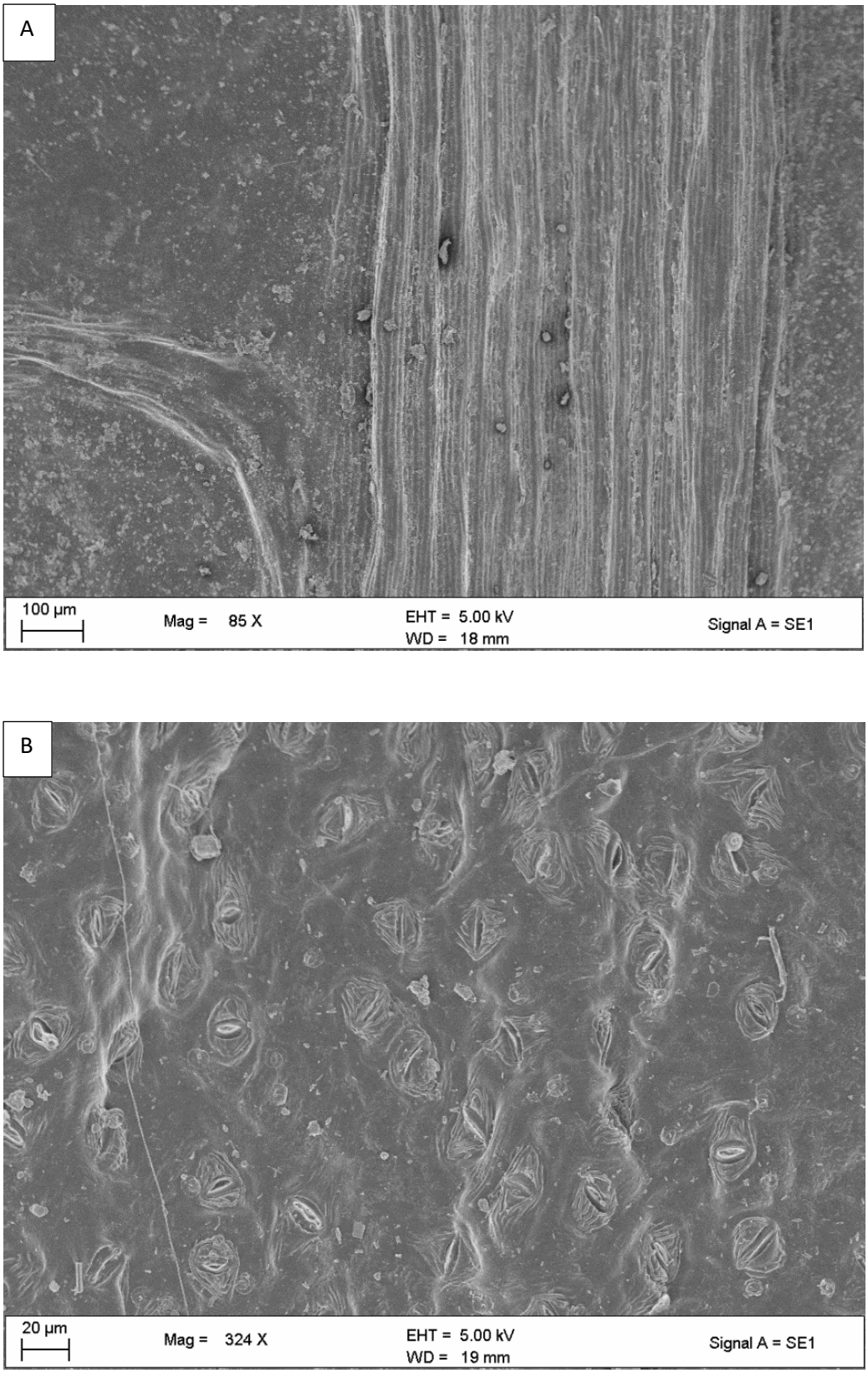


Figure 3: The scanning electron micrographs of the emergent leaves (A) showing the midvein and a lateral vein and (B) showing stomata on the leaf surface

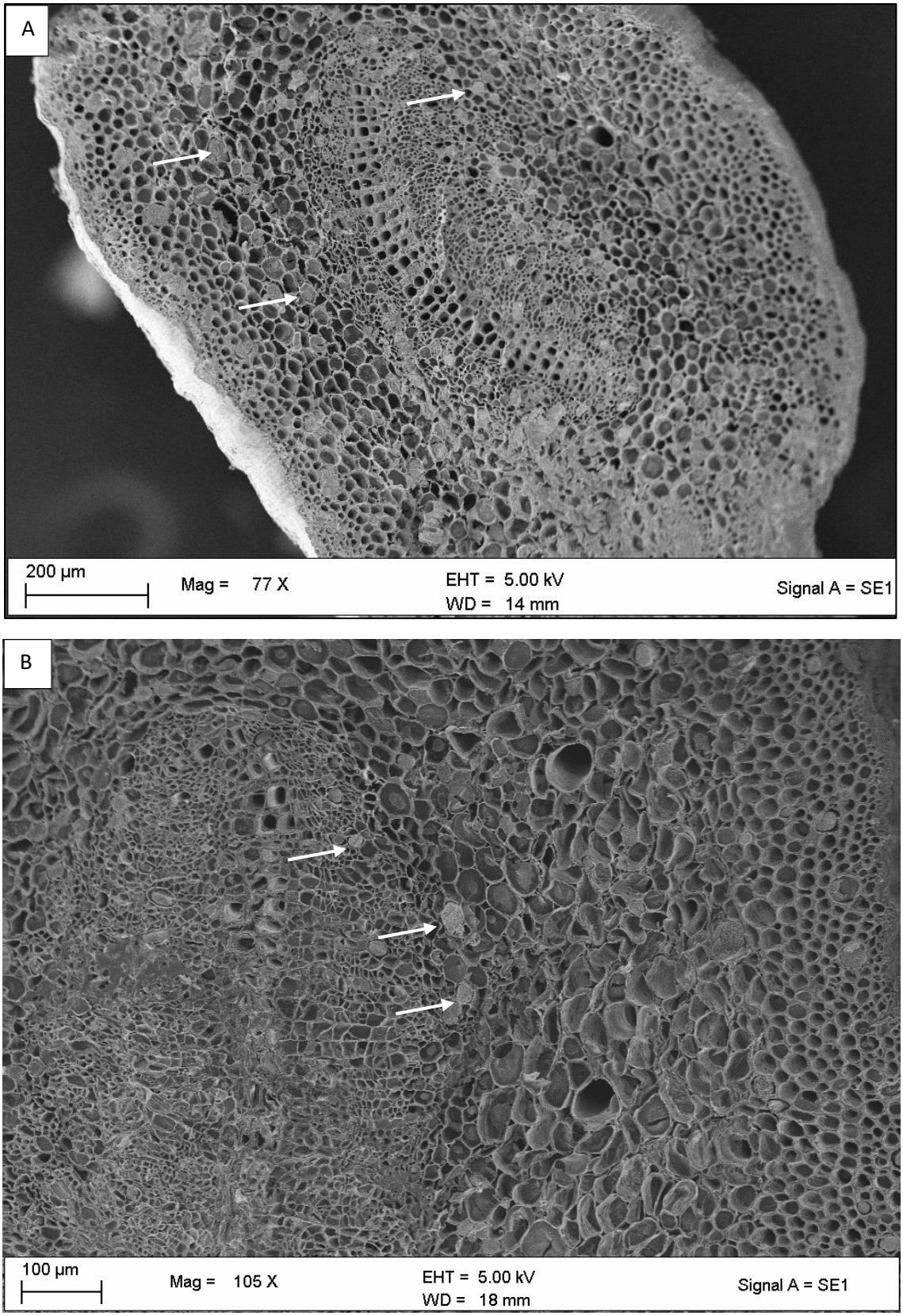


Figure 4: Scanning electron micrographs of the emergent (A) and mature (B) leaves showing freeze-fracture cross section with laticifers and exudates

The semi-thin survey sections from resin embedded emergent leaf and petiole (Fig. 5) shows that the latex was retained (arrow) in the laticifers which means that the latex does have the ability to coagulate (Razak and Bahri, 2000; Kajii *et al.*, 2014). The petiole shows more compact cells than the leaf section.

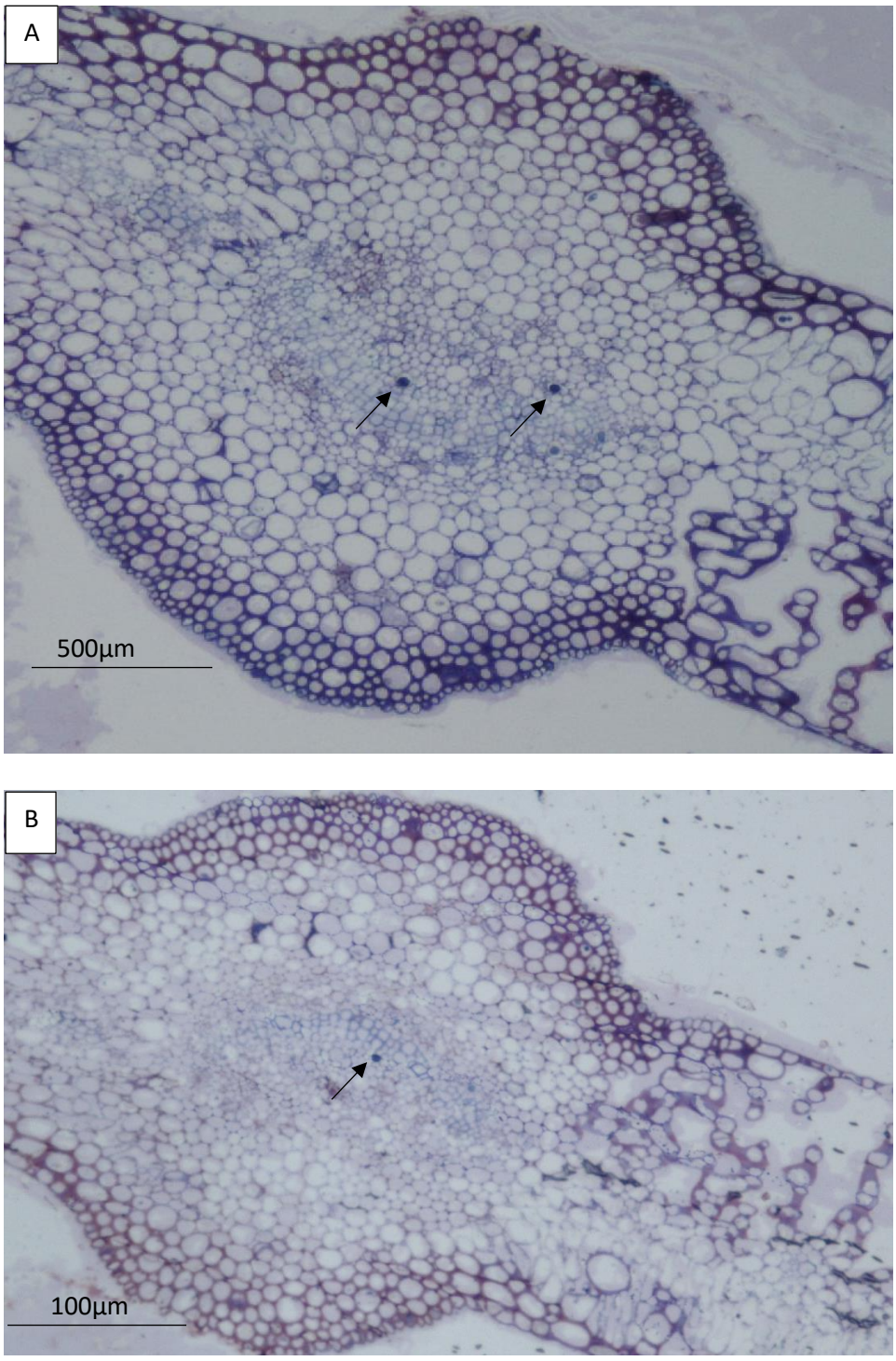


Figure 5: Semi-thin sections of resin embedded (A) emergent leaf and (B) emergent petiole viewed by light microscopy

The cross section of the mature leaf lamina (Fig. 6.1) shows the upper epidermis (UE) in a single layer with slightly thickened cell walls. The columnar palisade cells (P) occur on the upper surface of the leaf. Spongy tissue (S) occur above the lower epidermis (LE) as irregularly shaped cells with many intercellular air spaces (I). The palisade and spongy cells make up the mesophyll which carries out the functions of photosynthesis and gaseous exchange (Arshed and Agoo, 2017). There appears to be a stomatal opening (St) on the lower epidermis. The lower epidermis is composed of tightly compact, single layer of cells which is smaller in size than the upper epidermis.

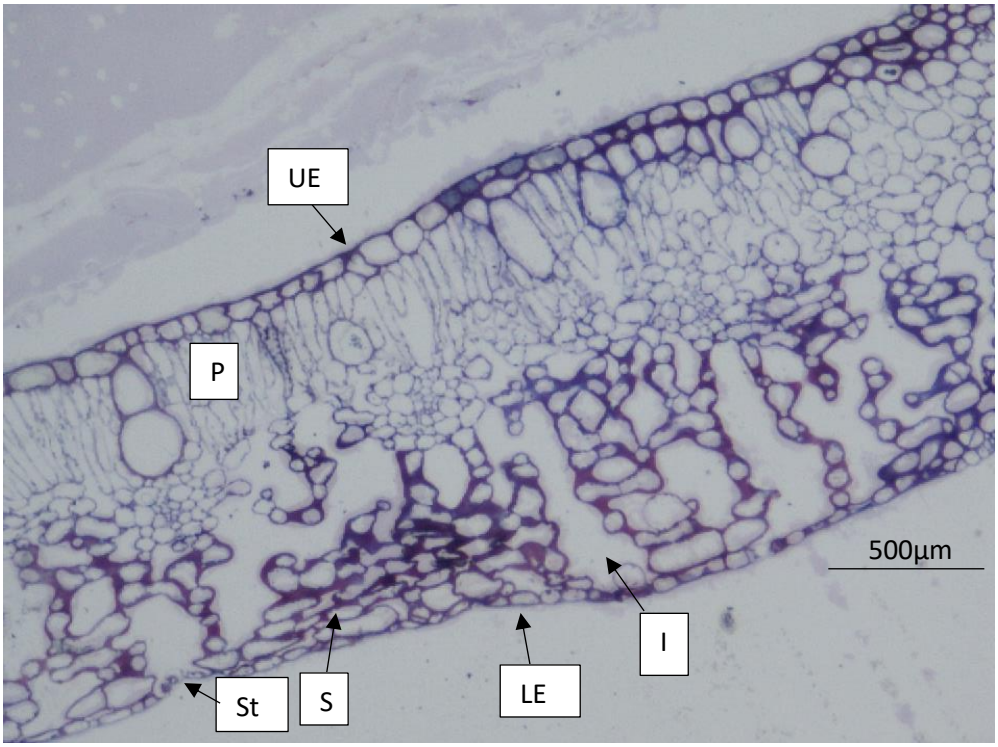


Figure 6.1: Semi-thin section of the mature leaf lamina showing: upper epidermis (UE), palisade (P), intercellular spaces (I), stomatal opening (St), spongy mesophyll (SP) and lower epidermis (LE)

The cross section of the midvein region of the mature leaf (Fig. 6.2) shows the upper epidermis (UE) in a single cell layer similar to the lamina (Fig. 6.1). The vascular tissue (V) is composed of xylem and phloem and the system is represented in an arc shape. The phloem has thin cell walls and are non-lignified. The xylem occurs as a band of lignified cells. Similar findings were reported by Allam *et al*., 2016). The parenchyma cells (P) around the vascular tissue has thin cell walls, are round in shape and vary in size (Martins *et al.,* 2012). The collenchyma cells (C) have a thickened cell wall which provides support and structure to the plant (Leroux, 2012). The spongy mesophyll (S) and intercellular spaces can be seen on the lamina. Additionally, the laticifer (L) appears to occur near the vascular tissue (Rajeswari *et al*., 2014; Murugan and Inamdar, 1987).

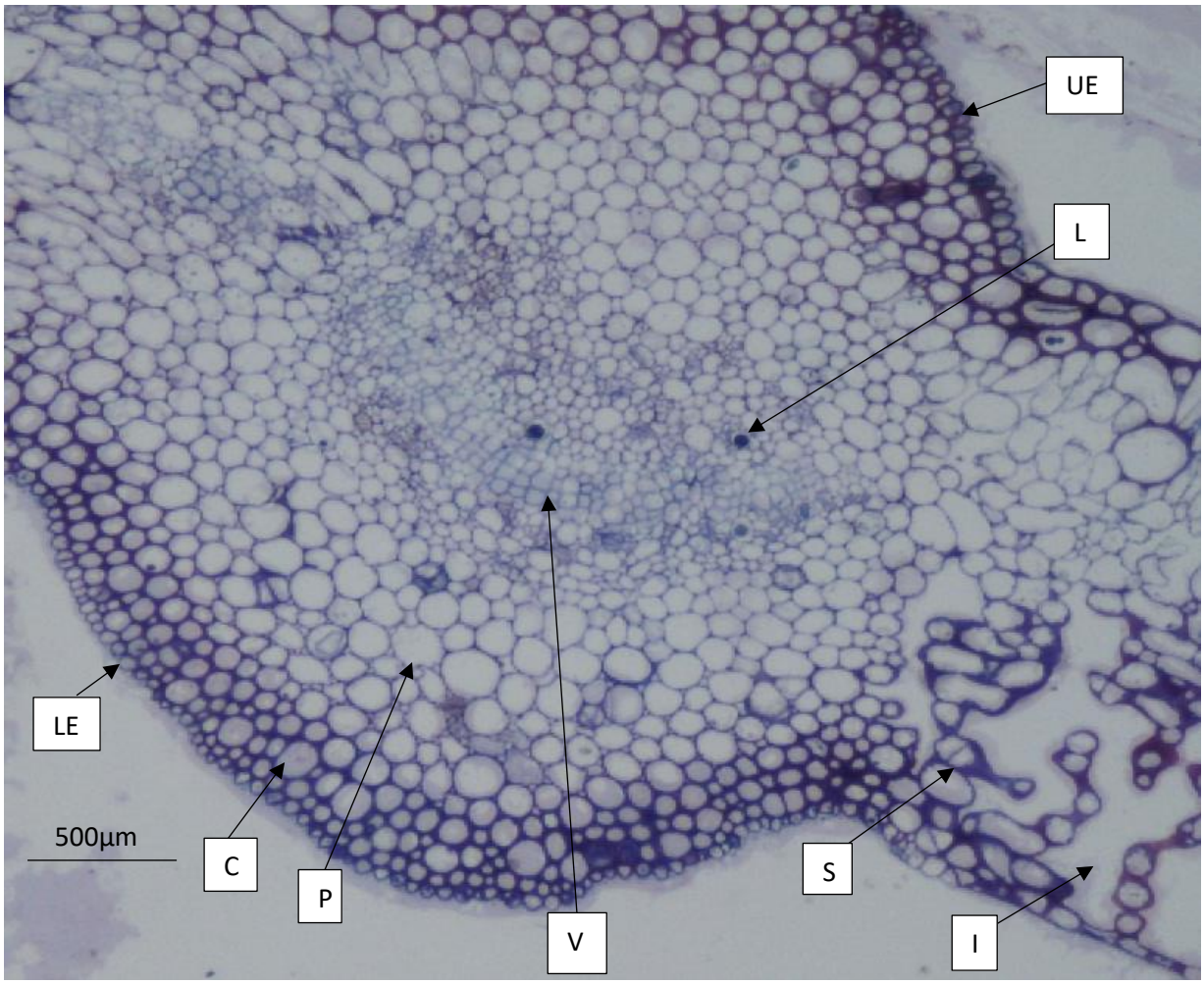


Figure 6.2: Semi-thin section of the midvein of the mature leaf showing: lower epidermis (LE), upper epidermis (UE), intercellular spaces (I), spongy mesophyll (S), collenchyma (C), parenchyma (P), vascular tissue (V) and laticifer (L)

Latex bearing plants have been found to have medicinal properties. Since *R. caffra* is used in traditional treatments, histochemical descriptions would be a basic and quick tool to determine the chemicals present in the plant (Wal *et al*., 2013; Budel *et al*., 2015). In terms of safe use, histochemical investigations are effective in standardizing medicinal plants which is crucial in herbal formulations as this is used to determine the quality of the plants used folk treatments (De Souza *et al*., 2017).

Laticifers are characterized as non-articulated when they develop from a single cell or articulated when they occur as branched or unbranched in a series of cells that are elongated (Lopes *et al*., 2009). The articulated laticifers may undergo lysis when their end walls become porous and thus may appear to be non-articulated (Lopes et al., 2009). The laticifers from the emergent leaf and petiole appear to be articulated (arrows) (Figure 7(A) and (B)). This is similar to the description of laticifers in *Ipomoea alba* L. by Pickard (2008). The articulated laticifers appear to be unbranched (Figure 7 (B)) with thick cell walls. Lopes *et al.* (2009) found similar results in *Mandevilla atroviolaceae* (Apocynaceae) and by Razak and Bahri (2000) in *Mandevilla splendens* (Apocynaceae). Interestingly, within the Apocynaceae, both types of laticifers have been found to occur in the same species (Pickard, 2008). Laticifers can be used for systematic classification as French (1988) found articulated laticifers in the leaves of 75 genera of the family Araceae.

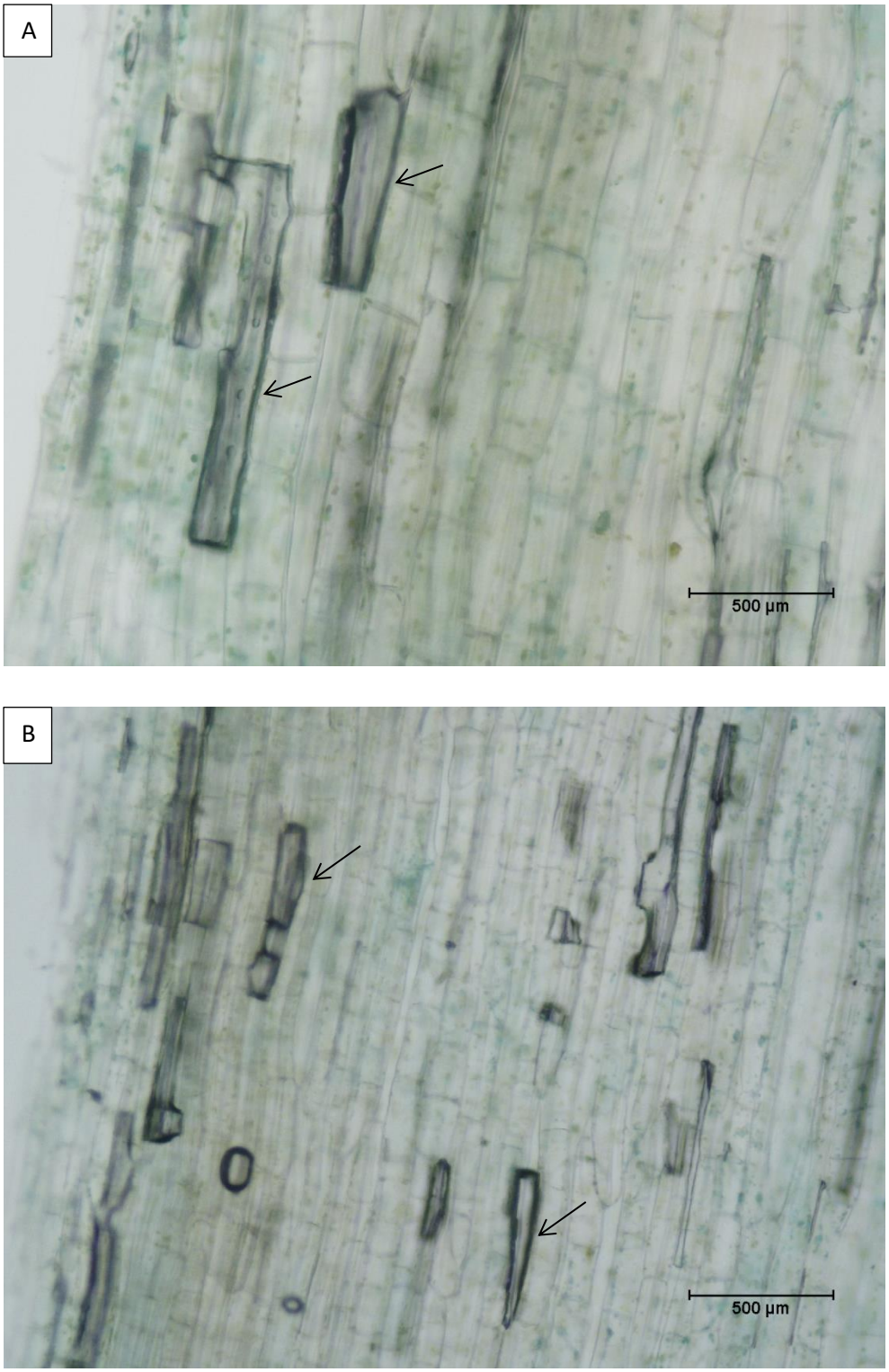


Figure 7: Longitudinal sections of (A) emergent leaf and (B) emergent petiole stained with fast green

Acridine orange stains lysosomes which contain enzymes that disintegrate macromolecules. This gives an indication on cell viability (Figure 8) (Pierzynska-Mach *et al*., 2014). The stain can be seen in the mature petiole (A), and in the mature leaf (B). The latex deposits stained a darker colour in the mature leaf (C) and emergent petiole (D). This revealed the presence of what appears to be the laticifers (arrows) (Lopes *et al*., 2009).

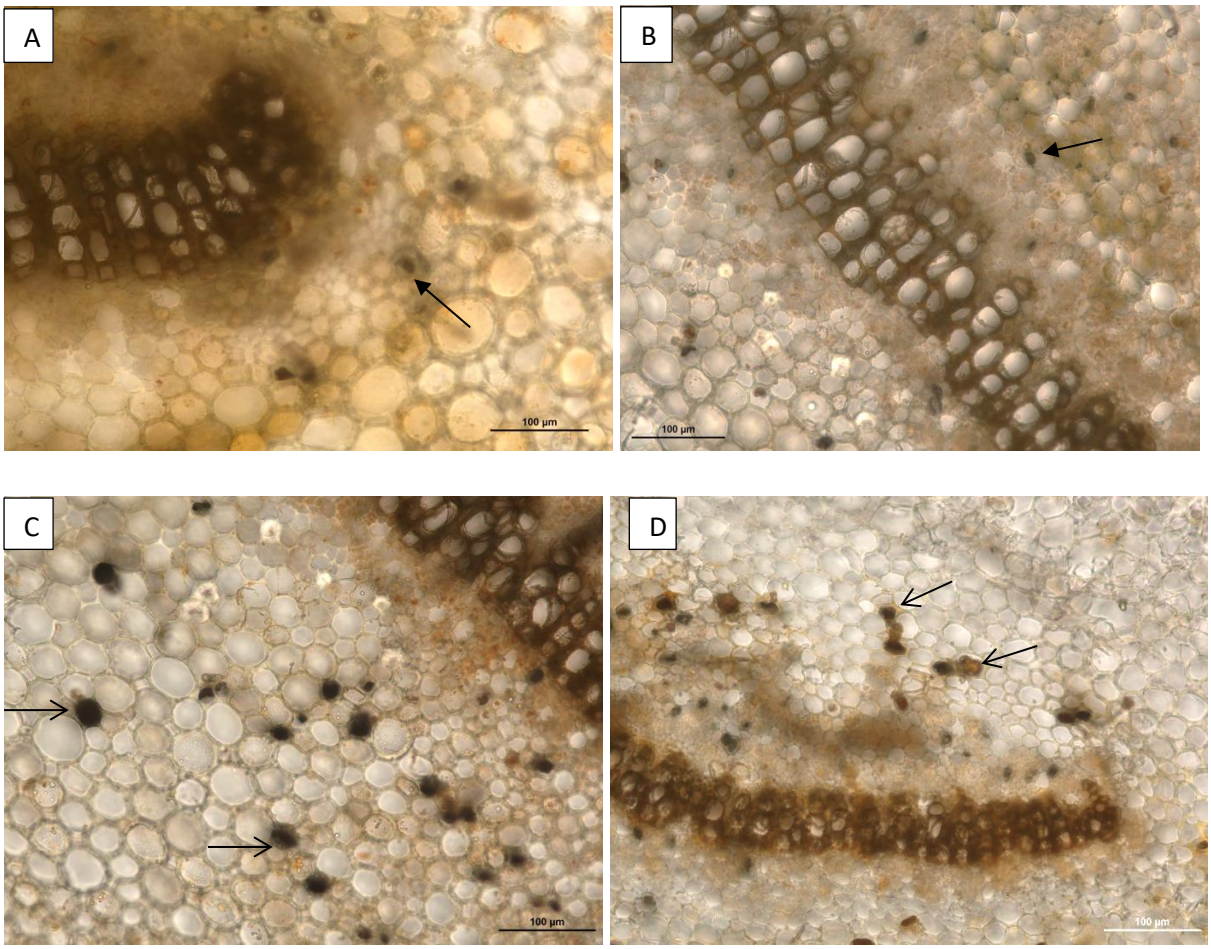


Figure 8: Transverse (cross) sections of the leaves and stems stained with acridine orange (A) mature petiole midrib, (B) mature leaf midrib, (C) mature leaf and (D) emergent petiole

Ferric trichloride stains phenolic compounds a dark colour (Figure 9(A) and (B)) (Kuster and Vale, 2016). The phenolics appear when glycosides that are non-toxic become hydrolysed. This releases phenolics that are noxious to microbial pathogens (Omwirhiren *at al*., 2017). This is consistent with plants that bear latex since it promotes self-healing and sealing of wounds (Agrawal and Konno, 2009).

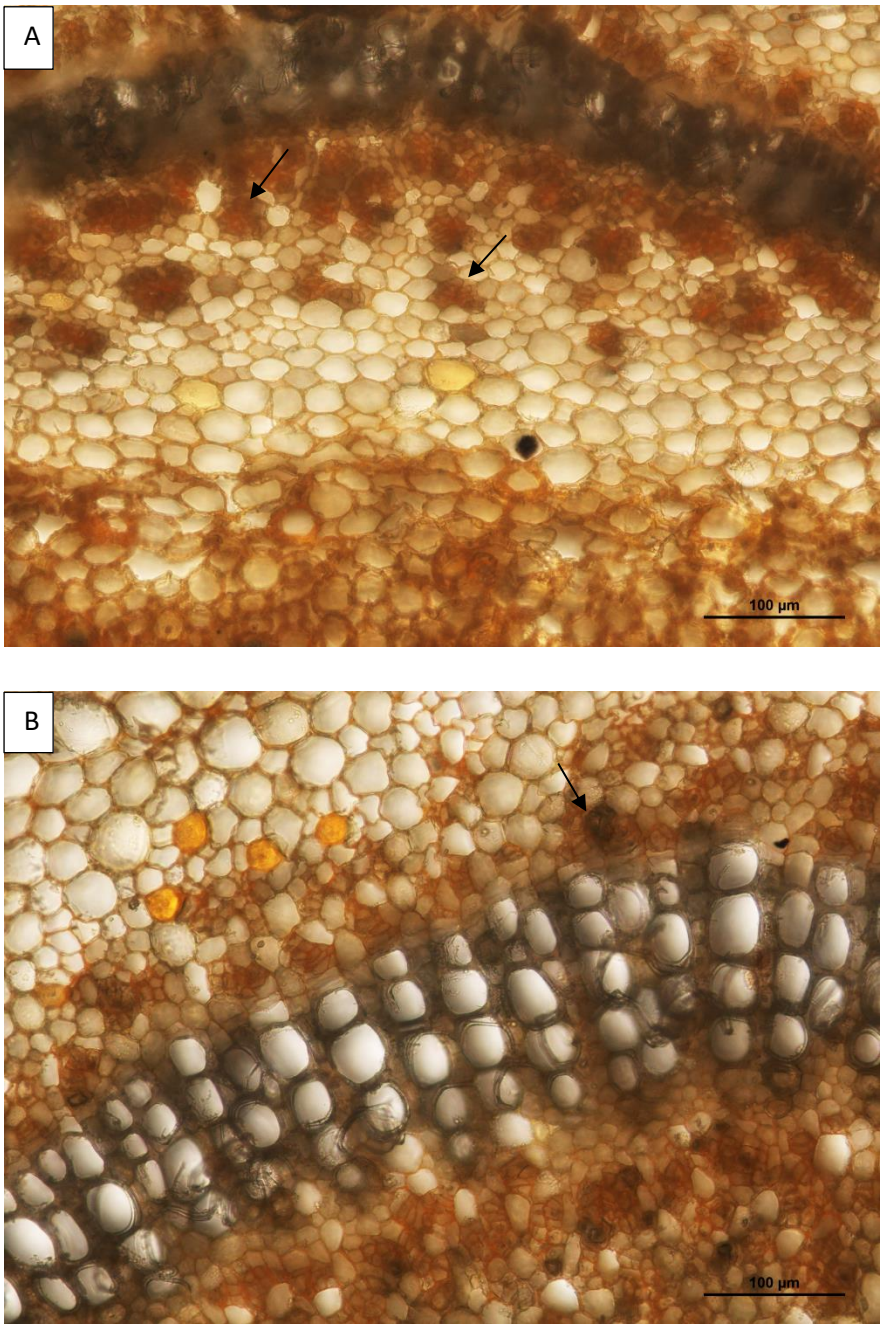


Figure 9: Transverse sections stained with ferric trichloride (A) emergent petiole, (B) mature leaf

Toluidine blue shows two different colour changes since it is metachromatic. The organelles stained pink-purple showing the presence of carboxylated polysaccharides while the blue shows phosphate groups on macromolecules (Figure 10(A) and (B)) (Sridharan and Shankar, 2012). The cell walls appear dark in colour due to the carboxylate groups (Wilson *et al*., 2000).

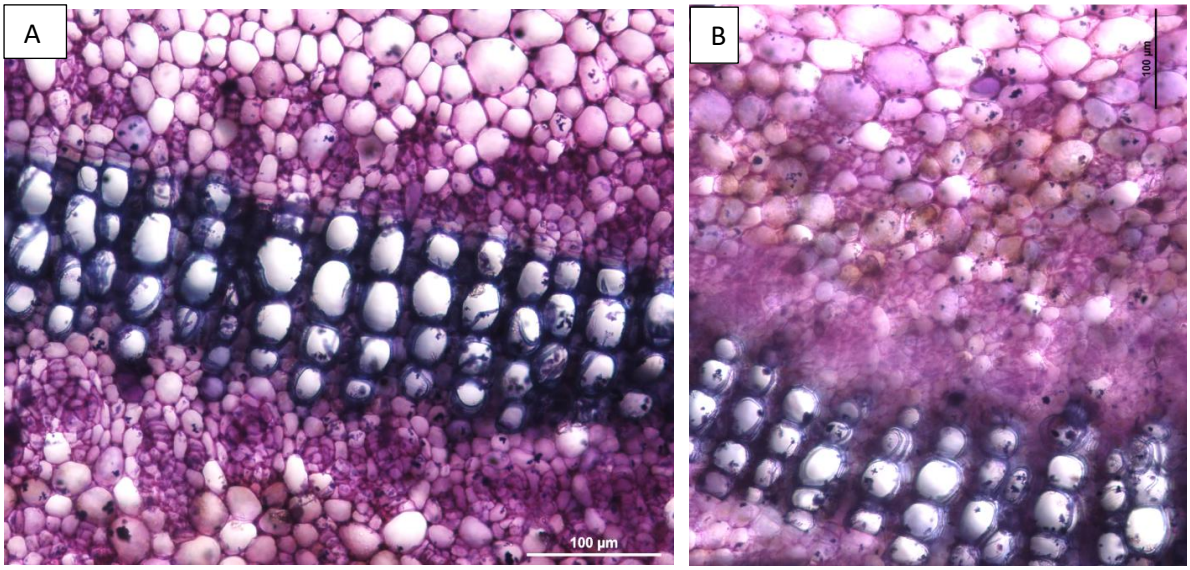


Figure 10: Transverse sections of the leaf and petiole stained with toluidine blue (A) mature leaf and (B) mature petiole

Sudan III and IV are stains for lipids and when applied to tissue, it stains the lipid inclusions red (Figure 11(A) and (B)) (Oliviera, 2015). Lipids are indicative of essential oils (Dhale, 2011).

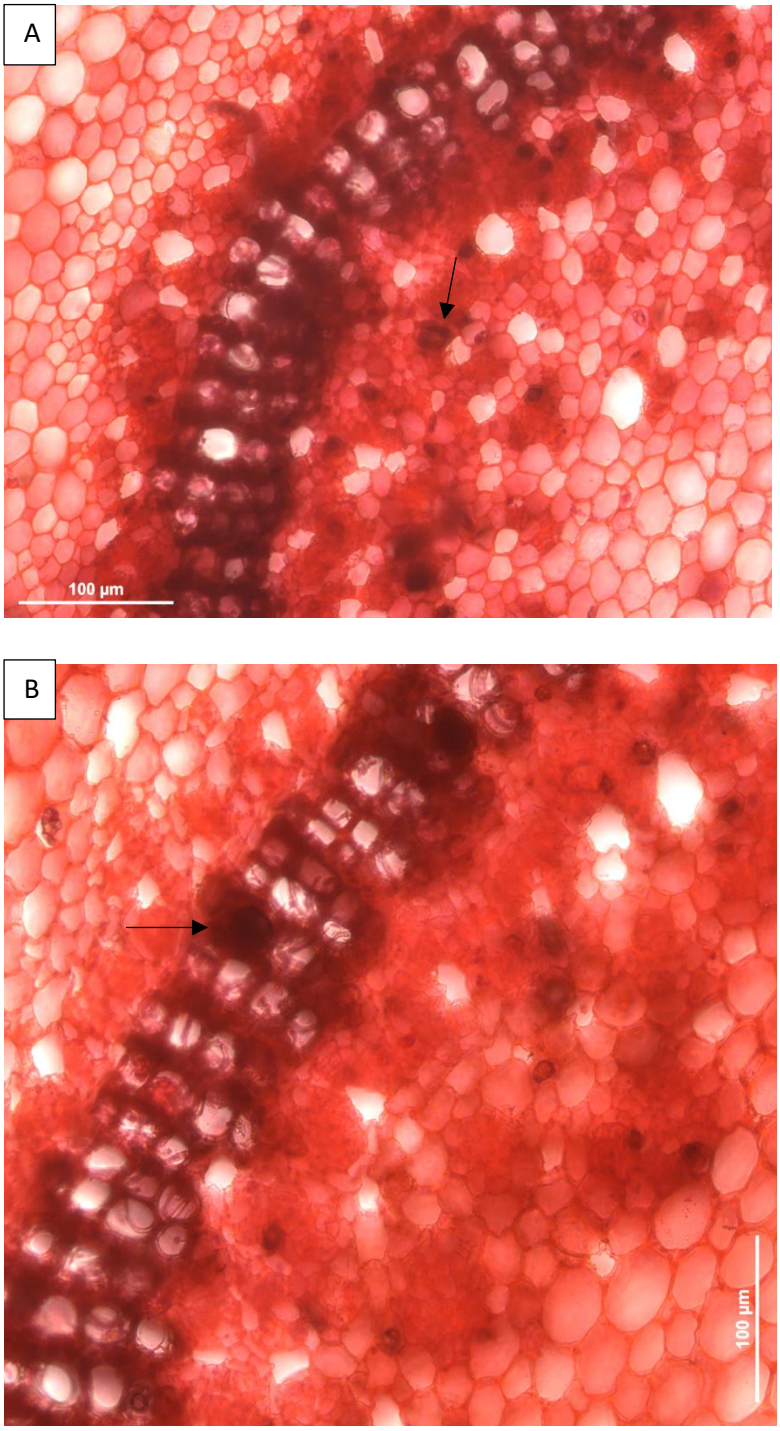
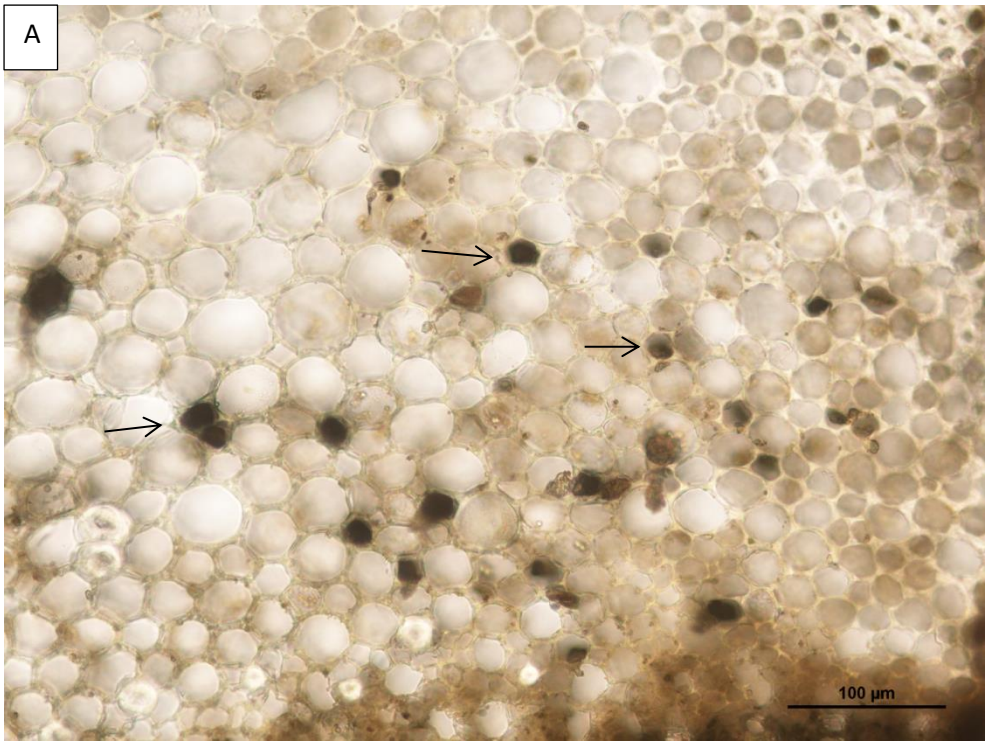
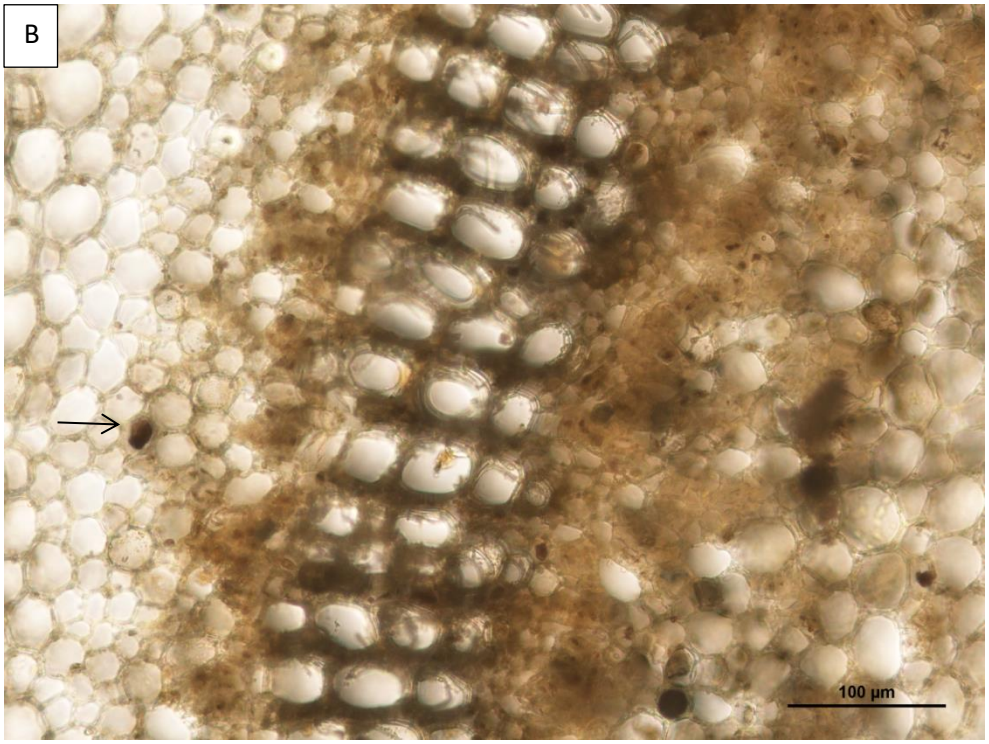


Figure 11: Transverse sections of the leaf and petiole stained with Sudan III and IV (A) young leaf and (B) young petiole

Mercuric bromophenol blue is a stain for the detection of proteins which is a predominant constituent of the protoplast (Mazia *et al*., 1953; Dhale, 2011). Figure 12(A) – (C) shows what appears to be latex which indicate the laticifers (arrows).





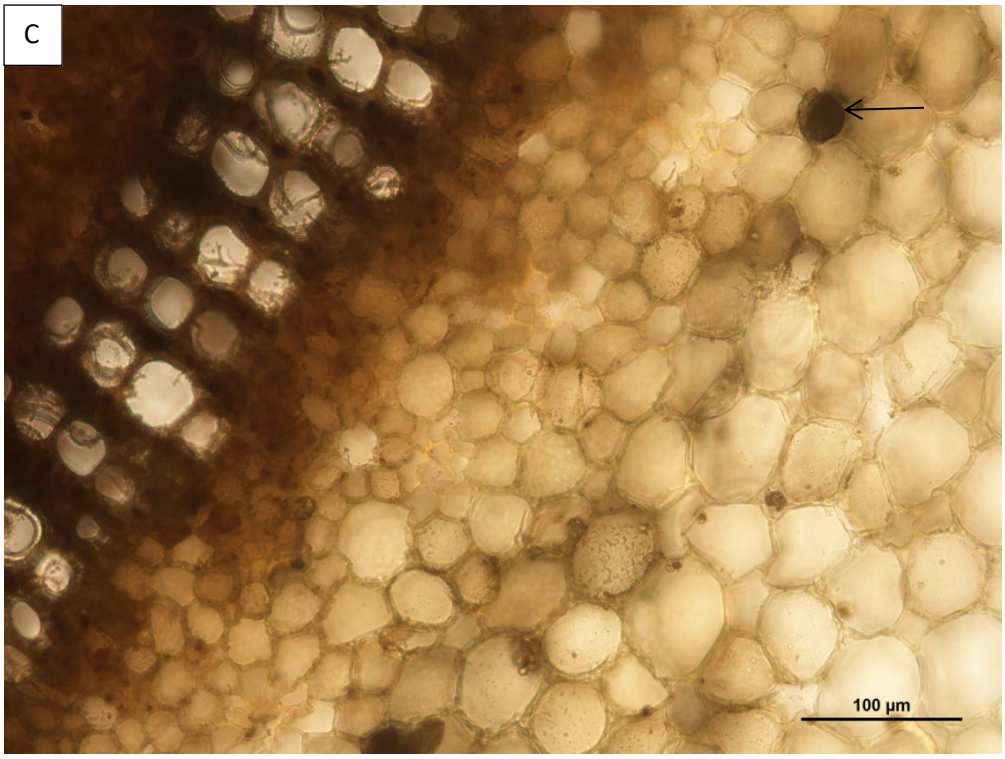


Figure 12: Transverse section of the leaf and petiole stained with mercuric bromophenol blue (A) mature leaf, (B) mature leaf midvein and (C) mature petiole

Safranin stains lignin, cutin and suberin in cell walls (Bond *et al*., 2008). Figure 13 shows a deep orange colouration on the cell walls of the vascular tissue and collenchyma tissue.

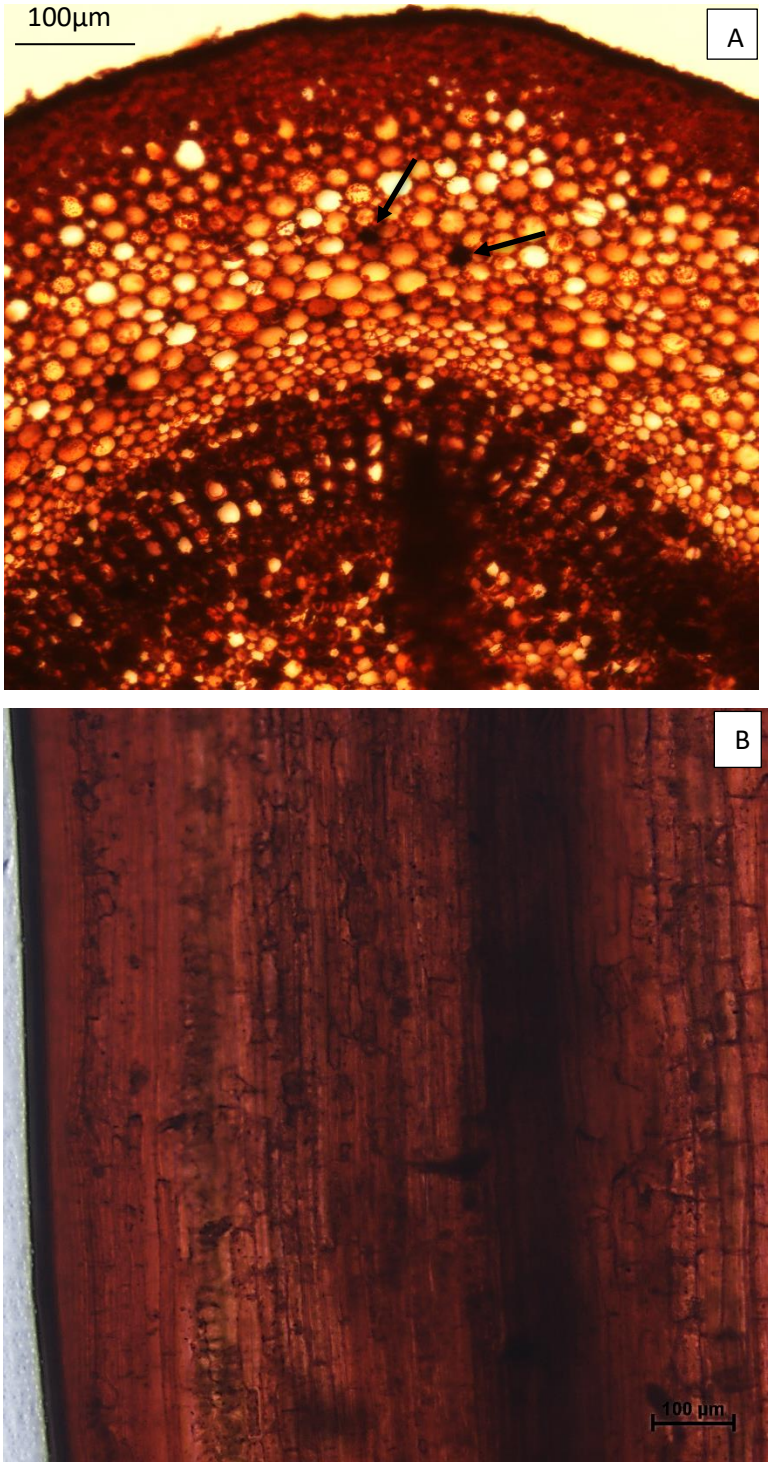


Figure 13: Leaf sections stained with safranin (A) transverse section and (B) paradermal section

The double staining technique using safranin and fast green shows the lignin, suberin and cutin components as red and the cell walls as green (Figure 14).

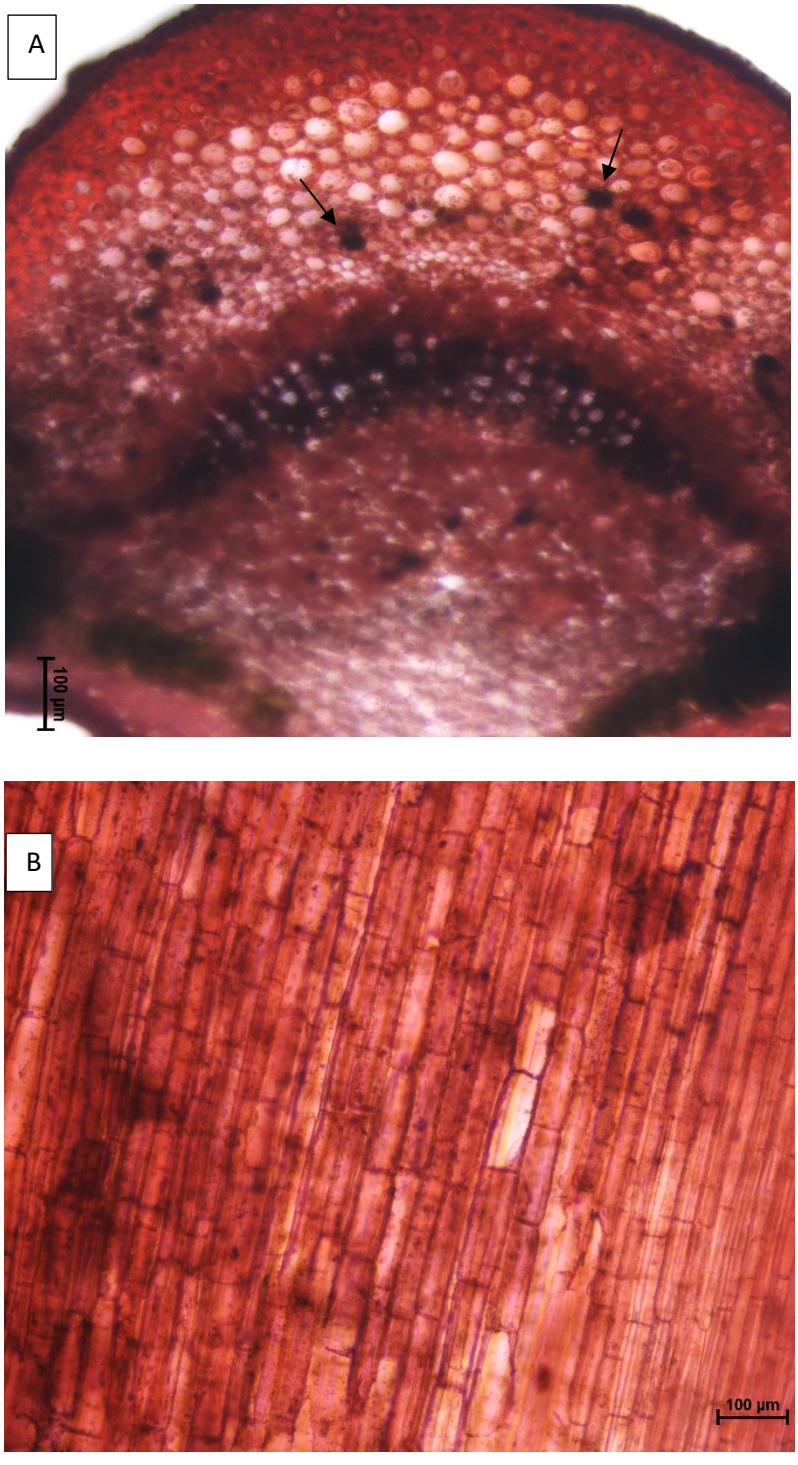


Figure 14: Leaf sections stained with safranin and fast green (A) transverse section and (B) paradermal section

NADI shows two colour reactions, blue indicates essential oil and red detects the presence of resin. Figure 15 shows the red staining on the cell walls of the parenchyma while the blue stain appears to be on the epidermis.

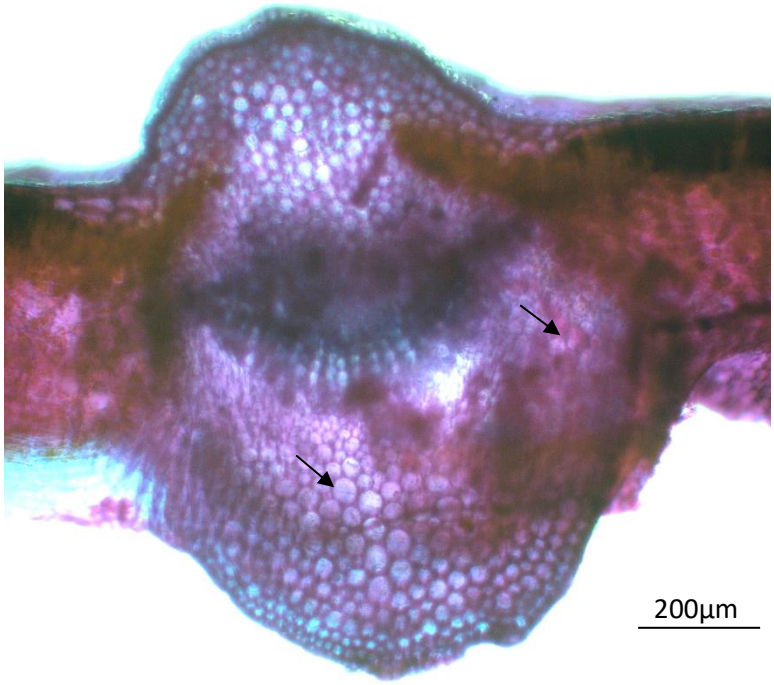


Figure 15: Transverse section of the emergent leaf stained with NADI

Ruthenium red stains mucilage and pectin a pink colour. The colour change appears in the transverse section (Fig. 16(A)) and in the lamina of the leaf blade (Fig. 16(B)).

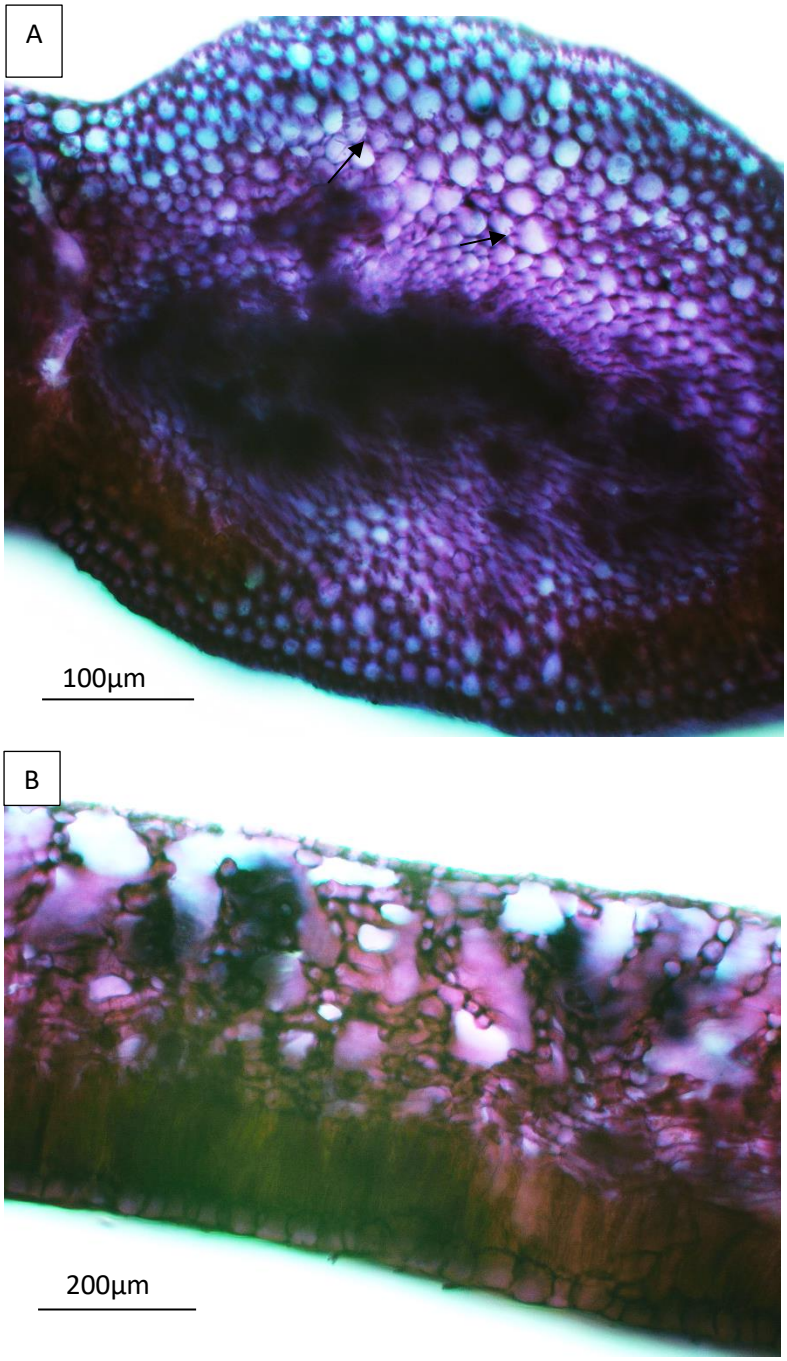


Figure 16: Young leaf stained with ruthenium red (A) transverse section and (B) lamina

Phloroglucinol stains lignin and cuticle components red. Figure 17 (A) shows the cross section of the young leaf with cells walls that have been stained. The paradermal section of the young leaf (Fig. 17(A)) shows the vascular tissue that have been stained. Figure 17 (C) shows the area above the vascular bundles where the cell walls have been stained.

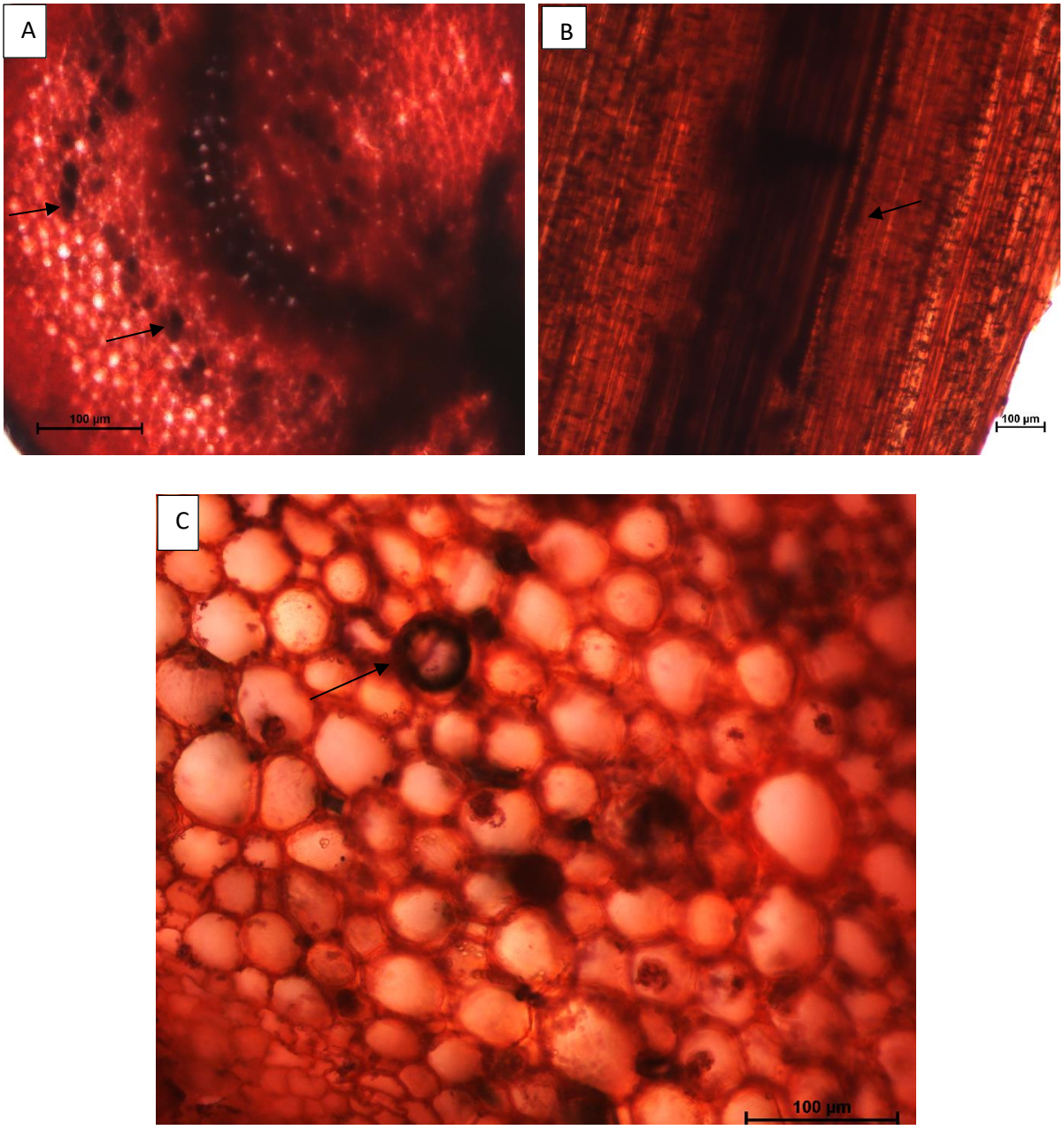


Figure 17: Young leaf stained with phloroglucinol (A) transverse section, (B) paradermal section and (C) higher magnification of the transverse section

Table 1: Histochemistry of *R. caffra* with the stain and reaction

Test / Stain	**Detection**	**Reaction (+/-)**
Acridine orange	Lysosomes	+
Ferric trichloride	Phenolics	+
Toluidine blue	Carboxylated polysaccharides and phosphate groups	+
Sudan III and IV	Lipids	+
Mercuric bromophenol blue	Proteins	-
Safranin	Lignin, cutin, suberin	+
Fast green	Cellulose cell walls	+
Safranin and fast green	Lignin, suberin, cutin and Cellulose cell walls	+
NADI	Essential oils and resin	+
Phloroglucinol	Cutin and lignin	+
Ruthenium Red	Mucilage and Pectin	+

3.5 Conclusion

The electron and light microscopy investigations revealed the presence of laticifers in the leaves and petioles of *R. caffra*. Scanning electron microscopy showed coagulated latex in the laticifers while light microscopy provided insight on the anatomy of the leaf. The longitudinal sections of the emergent leaf and petiole suggested that the laticifers are articulated and unbranched. Further work should include determining laticifers cellular contents and their length.

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CHAPTER 4: PRELIMINARY PHYTOCHEMICAL AND ANTIBACTERIAL EVALUATION

4.1 Abstract

The leaves and stems of *Rauvolfia caffra* were extracted with methanol, chloroform and hexane to obtain crude extracts. Phytochemical screening was carried out on these extracts for the detection of bioactive compound classes by colour change indications. Carbohydrates, phenolics and tannins, flavones and flavonones and saponins were detected from the extracts of either leaves or stems. Thin layer chromatography (TLC) was performed to identify compounds from the extracts. The hexane and chloroform extract of both stems and leaves had compounds with the largest retention factor (Rf) values. The hexane and methanolic extracts of both leaves and stem were subjected to antibacterial testing by disc-diffusion against seven strains of bacteria which were *K. pneumonia, P. aeruginosa, S. aureus, E. coli* (carbapenem-resistant), methicillin-resistant *S. aureus, S. typhimurium* and *E. coli.* The methanolic extract of the stems was found to inhibit all seven strains. The overall results show that *R. caffra* does possess bioactive compounds that may be used medicinally.

Keywords: Antibacterial activity; Phytochemical analysis; *Rauvolfia caffra*; Retention factor; TLC analysis

4.2 Introduction

Plants form the primary basis of food and medicines for thousands of years (Joseph *et al*., 2013). Medicinal plants have been reliable, efficient, cost-effective and readily available for the treatment of a wide range of ailments and diseases (Benli *et al*., 2008; Ramaswamy *et al*., 2014). Bioactive compounds produced by plants are the sources for drug discovery (Ramaswamy *et al*., 2014). The first and most primal source of healthcare comes from herbal origins for approximately 80% of the entire population of earth, as recorded by the World Health Organization (WHO) (Ramaswamy *et al*., 2014; Sharma *et al*., 2016). Since people with limited access to western medicine rely heavily on traditional plants, Srinivasen *et al*. (2001) calls for the urgent documentation of medicinal plants in third world countries - presumably, for their identification, efficient and optimum use and conservation. Panday and Tripathi (2014) emphasize the importance of evaluating drug safety before consumption to reduce side-effects and incidence of mortality.

Secondary metabolites are described as one of plants greatest energy investments, surpassing the synthesis of essential metabolites (Kennedy and Wightman, 2011). Defence and protection are the major functions of secondary metabolites (Kennedy and Wightman, 2011). The investigations on medicinal plants should be in constant revision considering the high rate of occurrence of new infections and more resistant infection strains, which lead to high mortality (Islam *et al*., 2015). Industries of research, academia and pharmaceuticals all have a role to play in devising new methods of combating prevalent diseases (Islam *et al*., 2015).

Rauvolfia caffra is an indigenous South African species, of the Apocynaceae family, occurring in KwaZulu-Natal and the Eastern Cape. Known as the quinine tree, the bark has been reported to have antihypertensive effects (Milugo *et al*., 2013). The chewing of the bark is thought to alleviate coughs and gastric problems (Milugo *et al*., 2013; Njau *et al*., 2014). *Rauvolfia caffra* has an abundance of alkaloids, including reserpine and reserpiline as documented on the stem barks by Nasser and Court (1984).

The Apocynaceae family is well-known for containing latex. According to Mahajan and Badgujar (2008), plants that synthesize latex have been used in the treatment of high blood sugar, asthma, dysentery, malaria and skin ailments. The latex of *R. caffra* occurs as a white, low viscosity liquid that discolours within a short period of time and evaporates. Latex may be made up of alkaloids, proteins, tannins, terpenes, resins and enzymes (Mahajan and Badgujar, 2008).

The phytochemical screening of indigenous medicinal plants provides a foundation for the discovery of new antibiotics, although a large number of these plants remain undiscovered (Joseph *et al*., 2013). *Rauvolfia caffra* has been used traditionally and this study aims to screen the leaves and stem extracts of *R. caffra* and determine if the extracts possess antibacterial activity that could be used in healthcare.

4.3 Material and Methods

4.3.1 Plant Collection

Fresh leaves and stems of *R. caffra* were harvested from UKZN Westville campus (29.831°S 30.925°E) and left to dry for two months at room temperature. The material was authenticated by Mr. E. Khathi and a voucher specimen (X4076057) was deposited at UKZN Westville campus herbarium.

4.3.2 Phytochemistry

The air-dried material was then ground to a fine powder and 33g dissolved in approximately 50ml of hexane, in a round bottom flask and connected to a hot continuous Soxhlet apparatus for distillation by reflux (European Pharmacopoiea, 1975). The flask was heated at 40°C and solvent extraction proceeded for three hours. The solution was filtered and the extract retained. This process was repeated thrice. Successive extractions using chloroform followed by methanol were carried out thrice after thirty minute intervals for both the stem and leaf material. The resultant solvent extracts were used for phytochemical tests according to Kodangala *et al.* (2010) and Joshi *et al*. (2011) which were:

Detection of carbohydrates

- Benedict's test – to one ml of extract, one ml of Benedict's reagent was added and then heated for two minutes on a boiling water bath. A yellow to red precipitate is indicative of the presence of carbohydrates.
- Molisch test – two drops of alpha-naphthol was added to two ml of filtrate and shaken well. One ml of concentrated sulphuric acid was gently poured along the sides of the tube and allowed to stand. A violet or purple ring is indicative of the presence of carbohydrates.

- Fehling's test – one ml of each Fehling's solution A and B was boiled in a water bath together with one ml of filtrate. A red precipitate is indicative of the presence of carbohydrates.

Detection of alkaloids

- Dragendroff's test – two drops of Dragendroff's reagent was added to one ml of extract. A reddish-orange precipitate indicated the presence of alkaloids.
- Hager's test – two drops of Hager's reagent was added to one ml of filtrate. A yellow precipitate indicated the presence of alkaloids.
- Wagner's test – two drops of Wagner's reagent was added to one ml of filtrate. A brown precipitate indicated the presence of alkaloids.
- Mayer's test – two drops of potassium mercuric iodide solution was added to 2ml of filtrate. Pale yellow to orange precipitate indicated the presence of alkaloids.

Detection of amino acids

- Ninhydrin test – two drops of Ninhydrin reagent was added to two ml of dilute extract. A deep purple colour change indicated the presence of amino acids.

Detection of sterols

- Salkowski's test – three ml of chloroform and two drops of concentrated sulphuric acid was added to two ml of filtrate. A red ring and green precipitate indicated the presence of cholesterol.

Detection of fixed oils and fats

- Spot test – two drops of extract was pressed between two filter papers, oil stains were indicative of fixed oil.

Detection of phenolic compounds and tannins

- Ferric chloride test – five ml of filtrate was dissolved in 0.5ml of 5% ferric chloride solution. A dark green colour change is indicative of the presence of phenolic compounds.

Detection of saponins

- Foam test – five ml of extract was diluted with distilled water to 20ml. The solution was shaken in a graduated cylinder for fifteen minutes. The presence of saponins in the extract was identified by the formation of a persistent two centimetre foam layer.

Detection of gums and mucilage

- Ruthenium red test – five ml of extract was added to water along with two drops of ruthenium red. A pink colour change was indicative of the presence of mucilage.

Detection of flavones and flavonones

- Aqueous sodium hydroxide – five ml of extract was added to two ml of aqueous sodium hydroxide solution. A blue to violet colour change is indicative of the presence of anthocyanins. A yellow to orange colour change indicated the presence of flavones and orange was indicative of the presence of flavonones.

4.3.3 Thin Layer Chromatography (TLC)

The crude hexane, chloroform and methanol extracts of the stems and leaves from the phytochemical extraction were filtered for the TLC analyses. The solvent system used was 9.5 ethyl acetate: 0.5 Toluene. Glass capillaries were used to spot each of the sample solutions on pre-coated silica gel 60 F254 TLC plate (E.Merck) of uniform thickness of 0.2mm. The solvent was allowed to develop to a distance of 8 cm on the plate before spraying with anisaldehyde-sulphuric acid reagent and heated at 105°C for five minutes and then examined. The plate was photographed and Retention factor (Rf) values were calculated by measuring the distance travelled by the sample divided by the distance travelled by the solvent.

4.3.4 EDX Analyses

Energy dispersive X-ray analysis (EDX) was carried out at the Electron Microscope Unit at UKZN – Westville campus. Fresh leaves were harvested and cut to allow latex to flow onto cover slips which were left to dry for 24 hours. After this time, the cover slips were mounted onto brass stubs using carbon conductive tape, sputter coated with gold by Polaron SC500 Sputter Coater and elements detected by EDX detector Zeiss Ultra Plus FEG-SEM at 5kV.

4.3.5 Antibacterial assay

The antimicrobial activity of the prepared samples were tested against two strains of gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1683); and five strains of gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Escherichia coli* (carbapenem-resistant) (ATCC BAA 2340), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 314588), as well as *Salmonella typhimurium* (ATCC 14026) according to the disc diffusion method.

Mueller Hilton agar (MHA) (Biolab, South Africa) was prepared (38g in 1 L of water), poured into sterile petri dishes and allowed to set and dry at room temperature. Bacterial cultures were grown in Nutrient Broth (Biolab, South Africa) at 37°C for 18 hours in a shaking incubator and then standardized using a 0.5 McFarland standard turbidity. The bacterial cultures were further diluted with sterile distilled water in the ratio 1:150 to yield a final concentration of 1 x 106 and then swabbed onto MHA plates. Antibiotic assay discs (6mm in diameter) were loaded with 20µl of the prepared samples and placed onto the prepared agar plates which were inverted and incubated at 37°C for 24 hours. After this time, the plates were viewed and photographed with their zones of inhibition to determine if the extract displayed any activity against the bacteria (Nascimento *et al*., 2000).

4.4 Results and Discussion

Phytochemical screening of the leaves revealed the presence of alkaloids, carbohydrates and glycosides, sterols, fixed oils and fats, phenolics and tannins, saponins, flavones and flavonones (Table 1). Phytochemical screening of the stems revealed the presence of alkaloids, carbohydrates and glycosides, proteins and amino acids, sterols, phenolics and tannins, saponins, mucilage, flavones and flavonones (Table 2). The dried leaf extracts showed less compounds per extract compared to the stems (Table 1 and Table 2). The stem extracts did not indicate the presence of fixed oils and fats, however it did reveal the presence of proteins and amino acids, and mucilage which was not detected in the leaf extracts.

According to Tiwari *et al.* (2011), alkaloids, tannins and phenols have antimicrobial, antidiarrheal and anthelmintic effects while saponins have anticancer activity. Phenolic compounds have properties that prevent cell death, lessen the effects of aging and reduce inflammation and cancerous tissue (Gopalakrishnan and Udayakumar, 2017). Flavonoids display antioxidant properties while tannins have astringent effects (Gopalakrishnan and Udayakumar, 2017; Najib *et al.*, 2017; Omwirhiren *et al.*, 2017). This supports the traditional uses of the bark for treating digestive problems and the applying the leaves on wounded skin (Milugo *et al*., 2013). The detection of mucilage in the stem extracts functions in wound healing and reducing inflammation (Morton, 1990).

Table 1: Preliminary phytochemical profile from three extractions of the leaves with alcoholic solvents

Test	Compound	Reaction	Methanolic Extract *	Chloroform Extract *	Hexane Extract *
Dragendroff	Alkaloids	Reddish-Orange Precipitate	--	--	+
Wagner's	Alkaloids	Brown Precipitate	--	--	--
Hager's	Alkaloids	Yellow Precipitate	+	--	++
Molisch	Carbohydrates	Violet Colour Change	++	++	++
Fehling's	Carbohydrates	Red Precipitate	+	+	+
Benedict's	Carbohydrates & Glycosides	Yellow – Red Precipitate	++	++	++
Ninhydrin	Proteins & Amino acids	Purple Colour Change	--	--	--
Salkowskis	Sterols	Red Ring & Green	+	--	--
Spot Test	Fixed oils & fats	Oil stain	+	+	--
Ferric Chloride	Phenolics & Tannins	Dark Green Colour Change	++	++	+
Foam Test	Saponins	2cm layer of foam	++	--	--
Ruthenium Red	Mucilage	Pink Colour Change	--	--	+
Sodium Hydroxide	Flavones & Flavonones	Yellow-Orange Colour Change	++	++	++

*Intensity of reaction: (--) No observed changes, (+) low-medium intensity, (++) high intensity.

Table 2: Preliminary phytochemical profile from three extractions of the stems with alcoholic solvents

Test	**Compound**	**Reaction**	**Methanolic Extract ***	**Chloroform Extract ***	**Hexane Extract ***
Mayer's	Alkaloids	Pale yellow Precipitate	**+**	**++**	**++**
Molisch	Carbohydrates	Violet Colour Change	**+**	**++**	**++**
Fehling's	Carbohydrates	Red Precipitate	**+**	**++**	**++**
Benedict's	Carbohydrates & Glycosides	Yellow – Red Precipitate	**++**	**++**	**++**
Ninhydrin	Proteins & Amino acids	Purple Colour Change	**++**	**++**	**++**
Salkowskis	Sterols	Red Ring & Green	**--**	**++**	**++**
Spot Test	Fixed oils & fats	Oil stain	**--**	**--**	**--**
Ferric Chloride	Phenolics & Tannins	Dark Green Colour Change	**+**	**++**	**++**
Foam Test	Saponins	2cm layer of foam	**++**	**++**	**++**
Ruthenium Red	Mucilage	Pink Colour Change	**+**	**+**	**+**
Sodium Hydroxide	Flavones & Flavonones	Yellow-Orange Colour Change	**++**	**++**	**++**

*Intensity of reaction: (--) No observed changes, (+) low-medium intensity, (++) high intensity.

Figure 1 and Table 3 depict the results obtained from TLC analysis. The bands take on different colours as the sample progresses up the plate. This provides a visualization of the separation of compounds (Fig. 1). The Rf values quantifies the movement of the samples up the plate. The hexane and chloroform extracts from the stem had six bands, the hexane extract of the leaves had four bands, the chloroform extract of the leaves had three bands while the methanol extract of the stem had two bands and methanol extract of the leaves had one. The hexane and chloroform stem extracts showed a higher number of bands according to the TLC profile (Fig. 1 and Table 3). The hexane leaf extract also had a higher number of bands. This is indicative of a high number of phytochemicals and better separation of the secondary metabolites (Francis and Sudha, 2017; Yahyaoui *et al.*, 2017).

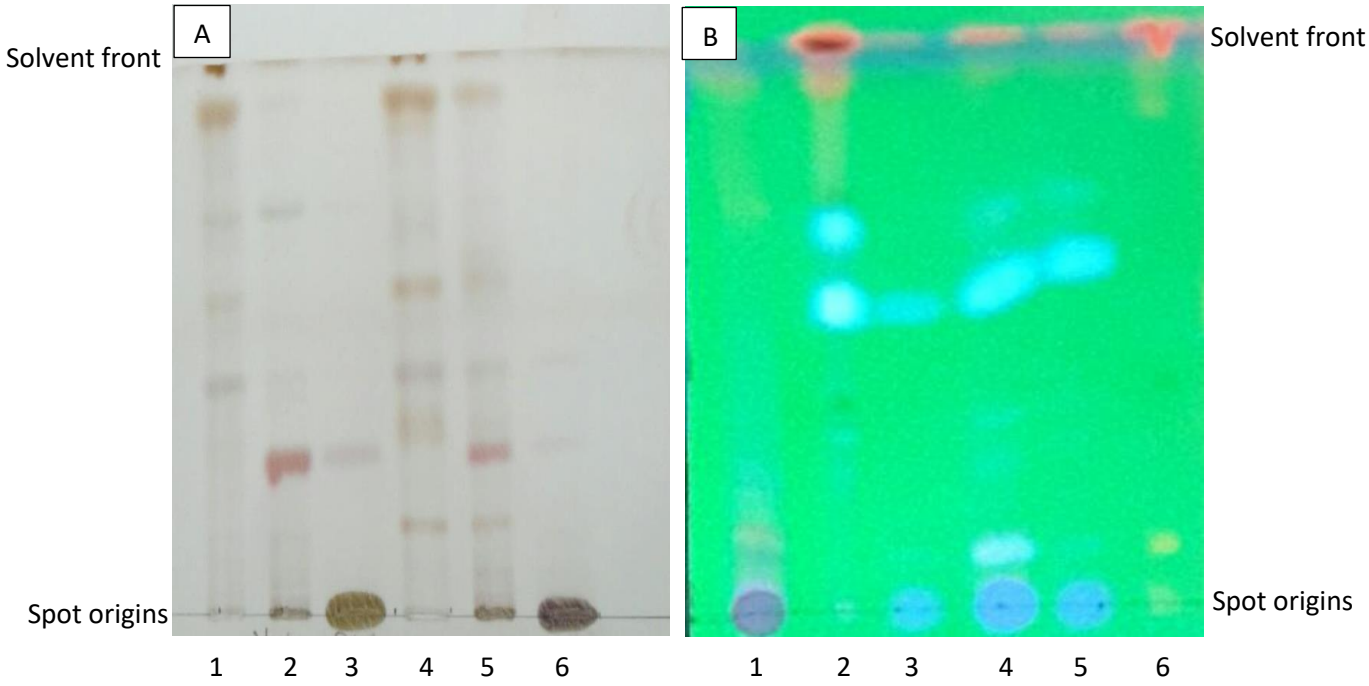
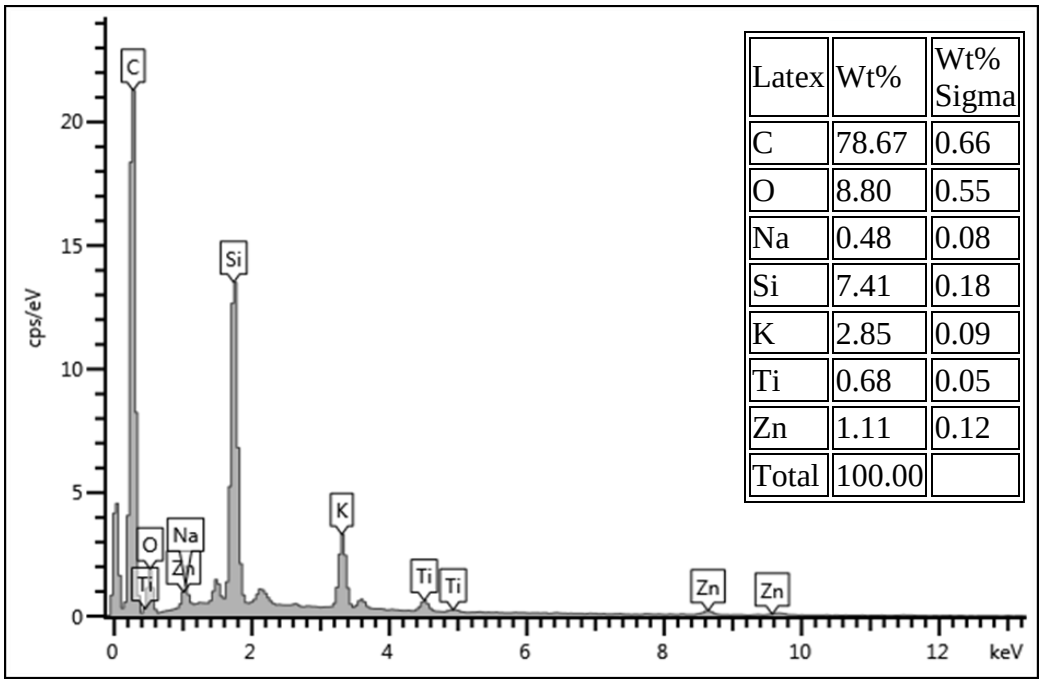


Figure 1: TLC profile of *R. caffra* crude extracts under visible light (A) and UV at 254nm (B), 1- hexane extract of leaves, 2- chloroform extract of leaves, 3- methanolic extract of leaves, 4- hexane extract of stems, 5- chloroform extract of stems, 6- methanolic extract of stems

Table 3: TLC profile of *R. caffra* crude extracts with retention factor (Rf) values

Extract	**Stem methanol**	**Stem hexane**	**Stem chloroform**	**Leaves methanol**	**Leaves hexane**	**Leaves chloroform**	**Bands**
Rf values	0,27	0.16	0.16	0.28	0.41	0.28	1
-	0.41	0.36	0.29	-	0.57	0.42	2
-	-	0.43	0.42	-	0.71	0.72	3
-	-	0.58	0.58	-	0.91	-	4
-	-	0.71	0.74	-	-	-	5
-	-	0.93	0.93	-	-	-	6

Titanium (Ti), Zinc (Zn), Sodium (Na) and Potassium (K) was detected from the EDX analysis of the fresh latex. The leaf latex FEG-SEM EDX data indicated the presence of zinc which has positive implications for humans in the case of normal brain and bone development (Sharma and Mukundan, 2014). Okubo and Utsunomiya (1996) describes potassium content as related to sodium chloride which allows for osmotic adjustments within the laticifer.



Latex	Wt%	Wt% Sigma
C	78.67	0.66
O	8.80	0.55
Na	0.48	0.08
Si	7.41	0.18
K	2.85	0.09
Ti	0.68	0.05
Zn	1.11	0.12
Total	100.00	

Figure 2: EDX Spectrum of the fresh leaf latex of *R. caffra*

Methanol and hexane extracts were used for preliminary antibacterial testing based on their extreme polarity; hexane is non-polar and methanol is a polar solvent (Zazouli *et al*., 2016). The methanolic extracts of the stem showed clear zones of inhibition against all seven bacteria tested. The hexane extracts of the leaves and stems showed limited activity while the methanolic extracts of the leaves had no activity against any bacteria. Gopalakrishnan and Udayakumar (2017) states that plant extracts have been used traditionally as natural medication for years. Antimicrobial products from plants have been determined to be efficient treatments with lower risk of side effects than synthetic chemical based medicine (Islam *et al*., 2015; Kadirvelmurugan *et al*., 2017).

From Table 4 and Figure 4, it appears that the methanolic stem extract showed activity against all seven strains of bacteria (gram-positive and gram-negative strains) probably due to the many classes of compounds present in the stems. The hexane stem extract showed slight activity in inhibiting *Escherichia coli* (gram-negative), *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (gram-positive) while the hexane leaf extract showed slight activity against methicillin-resistant *Staphylococcus aureus*. The inhibiting effect of the methanolic extract, particularly of the stem, shows potential as a drug lead (Sharma *et al.,* 2016; Sonam *et al.,* 2017). According to Dharajiya *et al*. (2017), the many phytochemicals present in the crude extract which were extracted in three different types of solvents may play the role of the antimicrobial agent. Seetharaman *et al*. (2017) implicates the alkaloid group as having antibacterial activities. From Table 2, all three stem extracts have alkaloids while the leaves (Table 1) had alkaloids present for two of the extracts only.

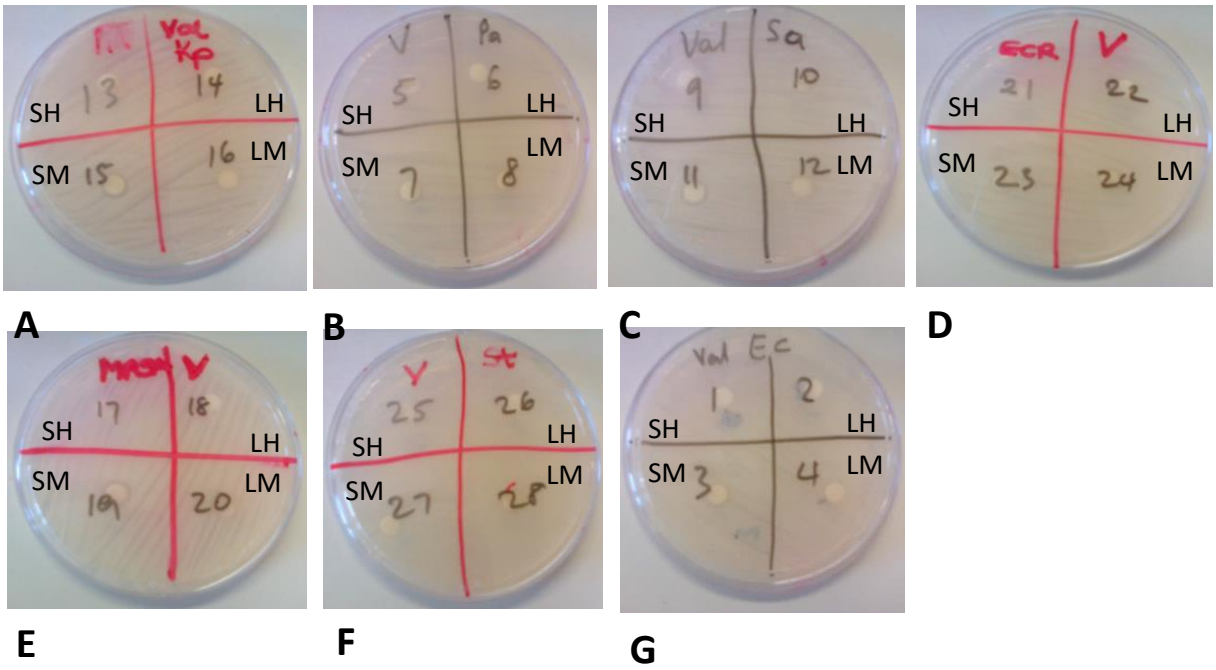


Figure 4: Bactericidal effects of the methanolic (M) and hexane (H) extracts of the stems (S) and leaves (L) against human pathogens A – *Klebsiella pneumonia*, B - *Pseudomonas aeruginosa*, C - *Staphylococcus aureus*, D - *Escherichia coli* (carbapenem-resistant), E - methicillin-resistant *Staphylococcus aureus*, F - *Salmonella typhimurium*, G - *Escherichia coli*

Table 4: Preliminary antibacterial activity of the hexane and methanol extracts of *R. caffra* leaves and stems

Extract / **Bacteria**	**Stem hexane**	**Leaves hexane***	**Stem methanol***	**Leaves methanol***
Escherichia coli	+	--	++	--
Staphylococcus aureus	+	--	++	--
Pseudomonas aeruginosa	--	--	++	--
Salmonella typhimurium	--	--	++	--
Klebsiella pneumonia	--	--	++	--
Methicillin-resistant *Staphylococcus aureus*	+	+	++	--
Escherichia coli (carbapenem-resistant	--	--	++	--

*Intensity of inhibition: (--) No activity, (+) affected the bacteria, (++) clear zone of inhibition.

Ingle *et al*. (2017) and Sharma *et al.* (2016) emphasize that plant phytochemistry requires a combination of techniques and purification procedures to effectively isolate bioactive compounds. The identification and associated benefits of these compounds by extraction are the advanced steps that should be undertaken in treating prevalent health concerns (Ingle *et al.*, 2017; Yadav, 2017). Najib *et al.* (2017) reports that the secondary compounds produced by plants have a necessary part to play in medicine. Mulholland *et al.* (2015) and Bisi-Johnson *et al*. (2017) does not discredit the value of crude extracts, especially in the South African context as this gives rise to drug leads also called 'ethnobotanical leads'. Bisi-Johnson *et al*. (2017) states that South African people from both rural and urban backgrounds still rely heavily on traditional medicine.

4.5 Conclusion

This study attributes some merit to *R. caffra* as a South African traditional medicinal plant. The phytochemical screening detected more classes of compounds present in the stems than leaves. The antibacterial screening showed the methanolic stem extract to have more activity against the strains of bacteria than the other crude extracts. *Rauvolfia caffra* does contain bioactive compounds and further work would require higher concentrations of extracts to be evaluated for the determination of antibacterial effects; this will include the identification and purification from crude extracts to develop antimicrobial drugs.

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CHAPTER 5: SILVER NANOPARTICLE BIOSYNTHESIS

5.1 Abstract

Plants possess many phytochemicals that have medicinal properties making them beneficial to man. As a result the revolutionary idea of silver nanoparticle (AgNP) biosynthesis has enhanced the industrial applications of plants in healthcare. The active compounds in plants are able to reduce metal ions on a nano-scale. Silver in particular has antibacterial, anticancer and disease fighting properties. Coupling these effects with the plant extracts may have an inexpensive positive benefit in healthcare. This study introduces a protocol for the green synthesis of *Rauvolfia caffra* by varying the mixing ratios using two plant parts, leaves and stems, and two crude extracts, water and methanol. A simple and safe method of combining silver nitrate with the extract and heating the solutions in a water bath produced a positive colour change reaction for each of the solutions, indicating the initial success of biosynthesis. The samples were characterized by UV- spectroscopy, EDX and TEM. The size of the AgNPs were in the range of 15.84 to 35.99nm. Preliminary antibacterial screening revealed activity against *Klebsiella pneumonia.* The results presented in this study are novel.

Keywords: AgNP; biosynthesis; green synthesis; nanoparticle; *Rauvolfia caffra*

5.2 Introduction

Biotechnology is an evolving field which includes nanotechnology or nanoparticle synthesis as it is a new area of research with multiple applications (Mittal *et al*., 2014; Erjaee *et al*, 2017). The 'nano' nature of the particles implies a substantially small size (1-100nm) and an increased ratio of surface area to volume (Mittal *et al*., 2014; Devi and Selvan, 2017; Khatoon *et al.*, 2017). According to Vadivu *et al*. (2017), nanotechnology is defined as "the synthesis, characterization, exploration and application of nanosized materials for the development of science". Additionally, nanoparticles have distinguishable characteristics when compared to the original form of the same material (Vadivu *et al*., 2017). Nanosized materials are sought after based on their mechanistic, optic and biology-related attributes (Rajkuberan *et al*., 2016).

Mittal *et al*. (2013) report that metal nanoparticles (gold and silver in particular) are deemed to be antimicrobial. Their study further elaborates other noble metals such as platinum and palladium that are already incorporated into personal hygiene products for their effectiveness in fighting bacteria. Gold is known to be compatible with biological material. The nanoparticles synthesized from biological material have had success in eradicating bacteria and cancerous tissue (Teimuri-Mofrad *et al.,* 2017). Silver nanoparticles (AgNPs) are described as being most required in the field of medical technology due to the bacterial and inflammation fighting characteristics (Mittal *et al*., 2014). Tiwary and Jha (2017) report that silver also has antiviral and antifungal properties as well as anti-parasitic and antidiabetic activity. Furthermore, silver compounds are effective antiseptics and are used in surgery as protective clothing, in the dressing of wounds and as bandages (Gunasekaran *et al.,* 2017; Tiwary and Jha, 2017). Currently AgNPs can be synthesized by the use of reducing agents such as hydrazine and other toxic chemicals (Khan and Khan, 2017). However, the synthetic methods are expensive, noxious and environmentally hazardous (Iravani *et al*., 2014; Khan and Khan, 2017; Chinnasamy *et al*., 2017).

Additionally, Gunasekaran *et al.* (2017) point out that AgNPs reaction with mammalian cells shows a reduced level of noxiousness. With regard to the effectiveness of AgNPs, Tiwary and Jha (2017) report that these nanoparticles are lethal to about 650 different types of microorganisms coupled with a fast reaction time of thirty minutes. Khan and Khan (2017) report that fungi, bacteria and plants can be used as biological tools for the inexpensive and simple production of AgNPs, referring to this process as "nano-factories". However, plant material is the most suitable substance for the green synthesis of nanoparticles (Teimuri-

Mofrad *et al*., 2017). Plants naturally have medicinal properties and these have been attributed to the phytochemicals which are able to reduce, cap and stabilize Ag^{+} ions (Chinnasamy *et al*., 2017; Khatoon *et al*., 2017; Maddila and Hemalatha, 2017). This is because plants and plant extracts are simple to maintain, eco-friendly, rapid, affordable, easily available and their stability can be controlled (Kumar and Yadav, 2008; Chung *et al*., 2016; Hafez *et al*., 2017; Tiwary and Jha, 2017; Verma *et al*., 2017).

Rauvolfia caffra is used traditionally with plant extracts administered orally to relieve fever, pneumonia and high blood pressure (Njau *et al*., 2014). This species occurs in tropical Africa and is distributed along the borders of the provinces of KwaZulu-Natal and Eastern Cape in South Africa (Foden and Potter, 2015). The stem extracts are used indigenously to treat sexually transmitted diseases and it is proposed to have antibacterial properties (Tshikalange *et al*., 2015; Njau *et al*., 2014). Nanotechnology studies have not been undertaken on this species. The aim of this study was to introduce a protocol for biosynthesis of silver nanoparticles using *R. caffra* leaf and stem extracts.

5.3 Materials and Methods

5.3.1 Plant Collection

Fresh leaves and stems of *R. caffra* were collected from UKZN Westville campus (29.831°S 30.925°E) and allowed to dry for eight weeks at room temperature. The plant material was identified by Mr. E. Khathi and a voucher specimen (X4076057) was deposited at the herbarium at UKZN (Westville).

5.3.2 Methanol extraction

Each of the samples was ground to a fine powder. Thirty-three grams of ground material were dissolved in 50ml of methanol in a round bottom flask under hot continuous extraction by soxhlet apparatus. The flask was heated at 40°C and solvent extraction proceeded for 3 hours. The solution was filtered, the extract was retained and the procedure was repeated twice. The extracts were stored at room temperature (25°C) for seven days.

5.3.3 Fresh water extraction

Twenty grams of fresh leaf and stem material were collected and washed three times with distilled water. The material was cut into small pieces and separately mixed with 100ml

distilled water and allowed to stand for 24 hours in concealed jars. It was then filtered and prepared for synthesis (Sarkar and Paul, 2017).

5.3.4 Synthesis of silver nanoparticles using plant extracts

The silver nanoparticle solution was prepared as 1mM $AgNO_3$ (BDH Chemicals Ltd. England) with distilled water. Different mixing ratios of each extract (leaves methanol extract, leaves water extract, stem methanol extract and stem water extract) were prepared by combining each extract with the silver nanoparticle solution in the following ratios 1:1, 1:2 and 1:4. The solutions were incubated in a hot water bath at 60°C for 60 minutes and a colour change was observed (Gunasekaran *et al*., 2017).

5.3.5 Characterization

- UV – Visible spectroscopy

Each sample was aliquoted into cuvettes and placed into the spectrophotometer (Spectrostar Nano BMG, Germany). The preliminary characterization of silver nanoparticles was monitored in the range of 220-800nm.

- pH evaluation

The pH was measured for each sample by extending the probe into the sample and retrieving a final reading. The pH meter (WS instruments, pH 50+, Italy) was calibrated to a two point calibration between 4 and 7.

- Energy dispersive X-Ray analyses (EDX) via Scanning Electron Microscopy (SEM)

A drop of each sample was placed on a cover slip and left to dry for 24 hours. The cover slip with the sample was then mounted onto brass stubs with carbon conductive tape. The samples were sputter coated with gold in a Polaron SC500 Sputtercoater and viewed with Zeiss Ultra Plus field emission SEM (FE-SEM, Germany) at 5 kV. Silver nanoparticle information by EDX microanalysis of elements present was captured by using the software Aztec (Oxford instruments, UK).

- Transmission Electron Microscopy (TEM)

Fine structural studies was performed to gain information on the size, shape and overall morphology of the synthesized silver nanoparticles. A drop of each sample was placed on copper grids and left to dry for 15 minutes before viewing with Jeol TEM 1010 (Japan) at 200 kV.

- Antibacterial screening

The preliminary antibacterial effect of synthesized silver nanoparticles was evaluated against *Escherichia coli* (ATC 25922), *Staphylococcus aureus* (ATCC 25923), methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1683), *Klebsiella pneumoniae* ATCC 31488, *Salmonella typhimurium* (ATCC 14026). Mueller Hilton agar was prepared and poured into sterile petri dishes to settle at room temperature. The bacterial cultures were swabbed onto the plates and wells of 5mm was were made by gel puncture. The samples to be tested were pipetted at 5µl onto the agar which was incubated at 37ºC for 24 hours and the effect of the samples on the bacteria viewed.

5.4 Results and Discussion

Nanotechnology and particularly nanoparticle synthesis from phyto-materials have been selected as the safest and most preferred option when compared to other materials (Mittal *et al*., 2014). This is due to the massive implications it has for future applications in medicine and healthcare (Mittal *et al*., 2014). This study has shown that silver ions can be reduced with *R. caffra* extracts and the results show that it was a successful protocol. Figure 1 shows the combination of silver nitrate and the plant extracts. Initially there was no colour change. However after exposure at 60°C in a water bath for 60 minutes, the solutions changed from pale -yellow to pale red -brown and then intense red -brown. The colour change indicated that these were optimal conditions for the generation of silver nanoparticles of *R. caffra*, since this represents the change in morphology of the silver nanoparticles over time (Sithara *et al.*, 2017). This change is due to the excitation of the surface plasmon where vibrations occur due to the reduction that takes place (Jain *et al*., 2009). The methanol leaf and stem AgNPs produced a less intense colour in comparison to the water extracts. Previous studies on *R. tetraphylla* L. have reported similar findings where the crude leaf extract was able to reduce silver ions and generate AgNPs (Kalaiarasi *et al*., 2013).

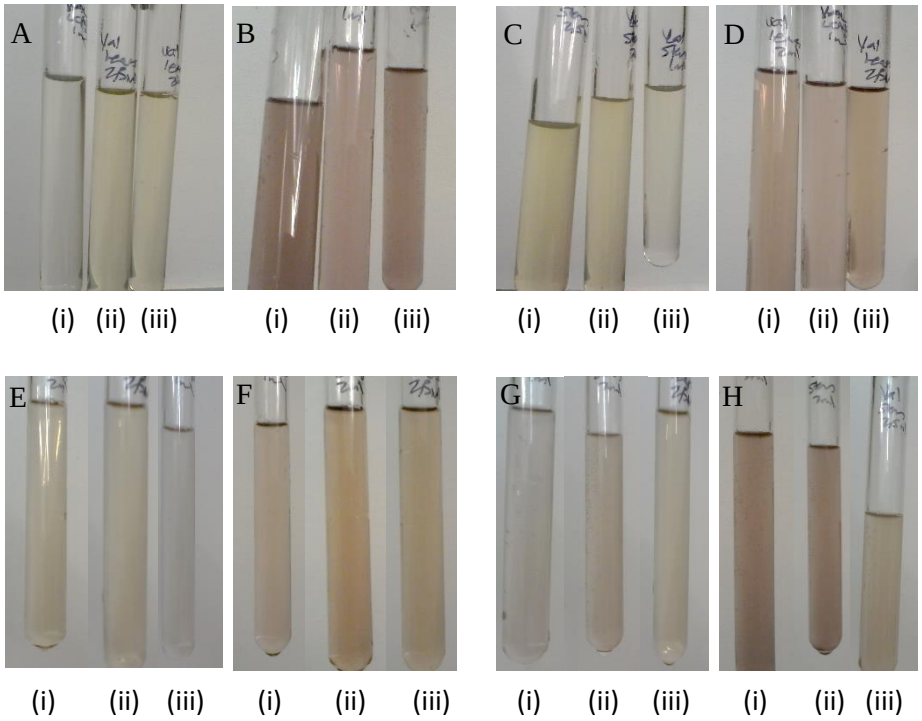

Figure 1: Photographic representation of silver nanoparticle biosynthesis for the three mixing ratios. (A) Silver nitrate with water extract of leaves (i) 1:1, (ii) 1:2, (iii) 1:4; (B) silver nitrate with water extract of leaves after treatment (i) 1:1, (ii) 1:2, (iii) 1:4; (C) Silver nitrate with

methanol extract of leaves (i) 1:1, (ii) 1:2, (iii) 1:4; (D) Silver nitrate with methanol extract of leaves after treatment (i) 1:1, (ii) 1:2, (iii) 1:4; (E) Silver nitrate with water extract of stems (i) 1:1, (ii) 1:2, (iii) 1:4; (F) silver nitrate with water extract of stems after treatment (i) 1:1, (ii) 1:2, (iii) 1:4; (G) Silver nitrate with methanol extract of stems (i) 1:1, (ii) 1:2, (iii) 1:4; (H) Silver nitrate with methanol extract of stems after treatment (i) 1:1, (ii) 1:2, (iii) 1:4

Ultraviolet-visible (UV-Vis) spectroscopy provides information on the formation and stability of the AgNPs (Pasupuleti *et al*., 2013). Absorption patterns are unique to each metal-reduced nanoparticle solution (Anandalakshmi *et al.,* 2015). The peaks of the UV -Vis spectra are indicative of the AgNPs surface plasma resonance (Anandalakshmi *et al.,* 2015). Figure 2 is indicative of the UV – Vis spectroscopy showing characteristic spectra per solution for all three mixing ratios. The methanolic extract from leaves (A) produced intense spectra at 460nm. The water extract from leaves (B), the methanolic extract from stem (C) and the water extract from stem (D) showed declining absorbance with an increase in wavelength. Figure 2(A) shows the peak at 460nm for all three mixing ratios which indicates intense synthesis of AgNPs (Paulkumar *et al*., 2017). Figure 2(B-D) shows a decline in absorbance with an increase in wavelength for all three mixing ratios. This indicates a small number of larger sized AgNPs since there is a decrease in reduction reactions between the silver nitrate solution and the plant extracts (Paulkumar *et al*., 2017). The UV analysis was conducted 24 hours after synthesis at room temperature which could have had an influence on the samples. According to Mittal *et al*. (2014), the rate of synthesis increases with an increase in temperature.

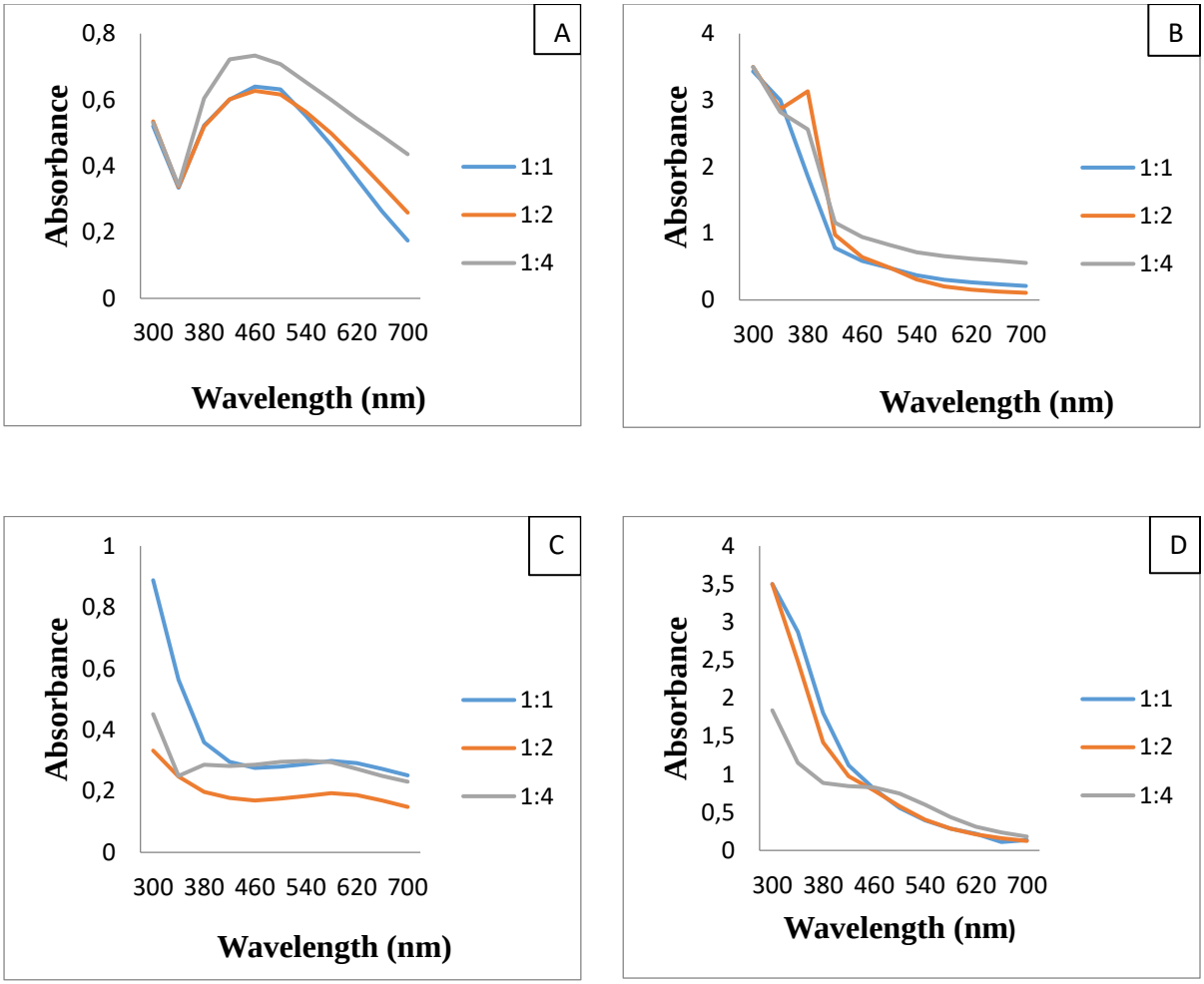

Figure 2: UV- Vis Spectroscopy recorded after 24 hours with silver nitrate solution (A) the methanolic leaves extract, (B) the water leaves extract, (C) the methanolic stem extract, and (D) the water stem extract

The pH level for water was 7 which is neutral and for the solver nitrate solution it was 6 which indicates a reasonably neutral solution. The methanolic extract from leaves and stems AgNPs showed pH to be around 3, the water AgNPs of the stems showed pH around 5.4 while the water AgNPs of the leaves showed pH in the range of 4-6.6. The methanolic leaves and stems AgNPs are acidic after bioreduction while the water extracts AgNPs are neutral (Fig. 3). The pH values may give an indication of the size of the nanoparticles that form (Mittal *et al*., 2014). In this case, the methanol extract from leaves AgNPs generated smaller particles than the water extract from leaves AgNPs which show that aqueous extract from leaves extract are more stable (Mtewa, 2017). Contrastingly, the water extract from stem AgNPs generated smaller sized nanoparticles than the methanol extract from stem AgNPs. The pH does not provide an indication of particle size however it does provide an insight on the stability of the NPs.

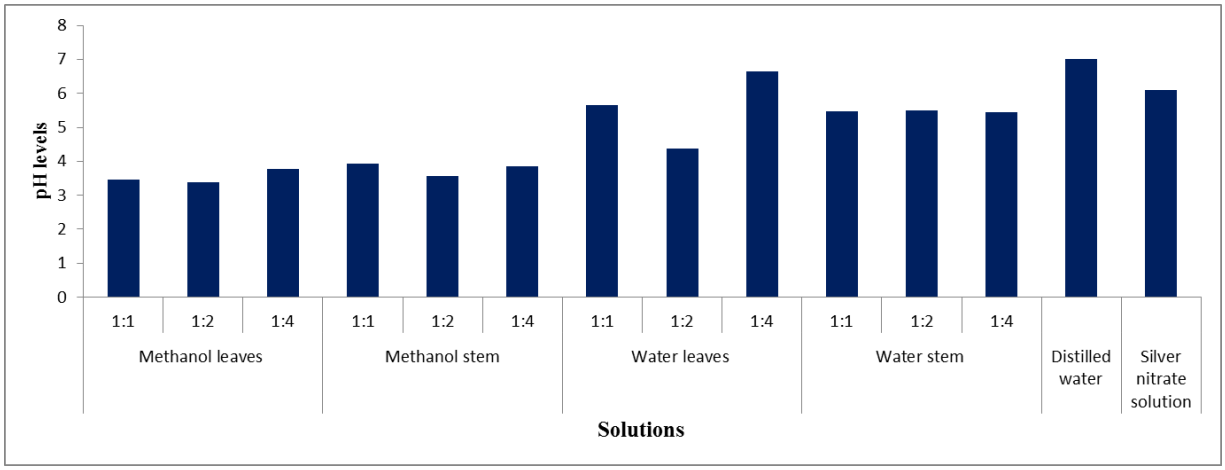

Figure 3: The pH levels of all three mixing ratios per *R. caffra* extract after synthesis of silver nanoparticles with the controls

The EDX analysis shows the reduction of AgNPs by *R. caffra* extracts (Fig. 4.1 and 4.2). The peaks for silver from Figures 4.1 and 4.2 confirm the biosynthesis of silver nanoparticles. Figure 4.1 (A) has a silver weight of 16.42% which is the highest out of all the samples. The weight percentages were: Figure 4.1 (B) 1.42%, (C) 0.98%, (D) 3.21%, (E) 0.65%, (F) 0.95% and Figure 4.2 (A) 1.73%, (B) 4.48% (C) 0.64%, (D) 2.83%, (E) 5.31% and (F) 0.36%. The mixing ratio of 1:4 for both the water extract from leaves AgNPs and water extract from stem AgNPs showed a highe weight percentage of silver. The mixing ratio of 1:2 for both methanol extract from leaves AgNPs and methanol extract from stem AgNPs showed the highest percentage of silver when compared to the other samples in the same category. The mixing ratio of 1:1 showed a low weight percentage. All samples showed a peak for the element silver. According to Smitha *et al*. (2009), the amount of extract used for the synthesis of AgNPs is a determining factor on the size and shape of particles that are formed. In this case, the samples with a 1:4 mixing ratio produced smaller sized particles with the exception of methanol extract from stem AgNPs. Since these samples had a higher amount of silver nitrate solution, it was expected to produce a higher weight percentage. However only this applies to water extract from leaves AgNPs and water extract from stem AgNPs. A number of factors such as extraction time and method could have influenced the AgNP synthesis in the methanolic samples (Do *et al*., 2014).

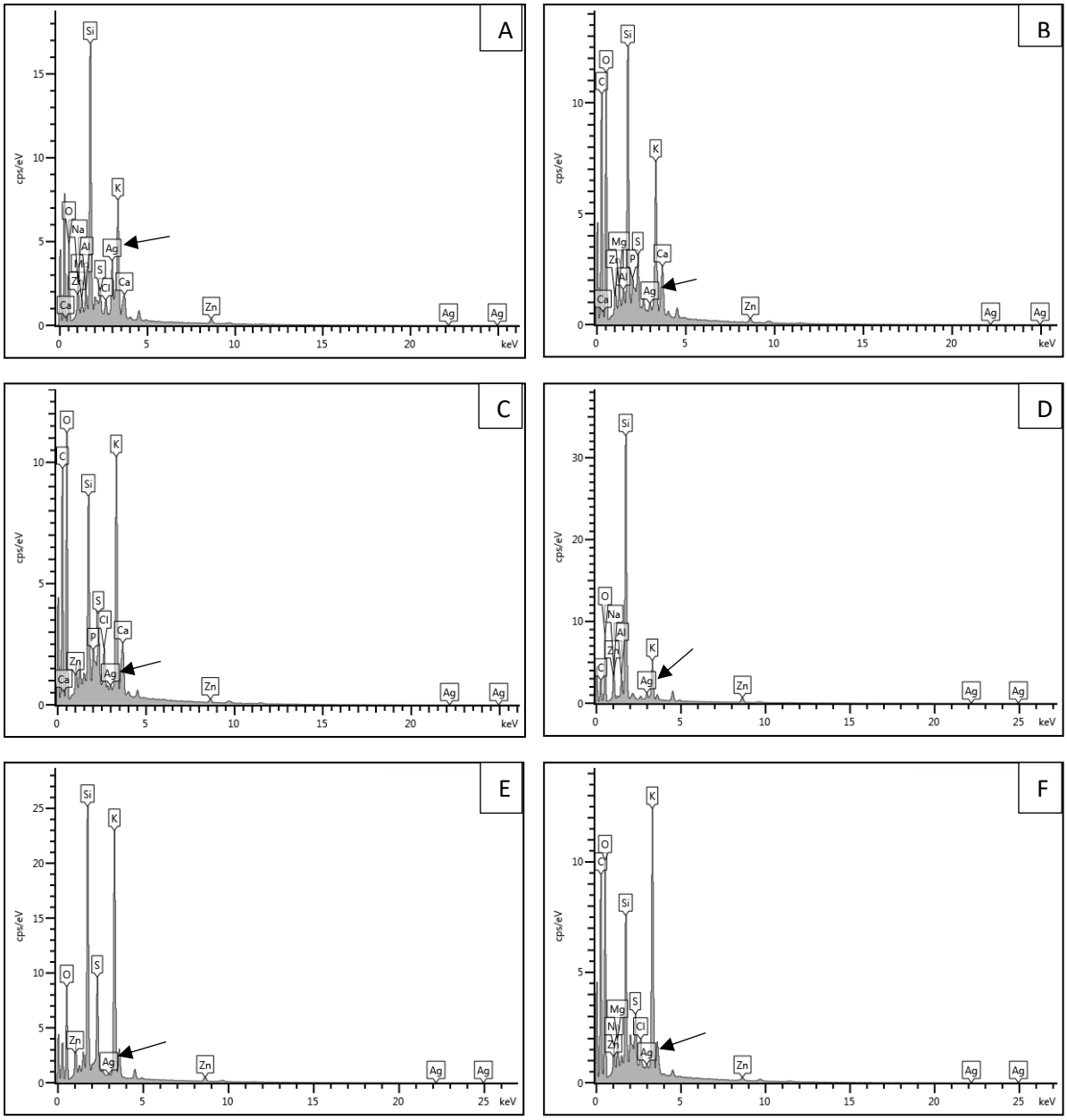

Figure 4.1: The Energy Dispersive X-Ray spectra of *R. caffra* water extracts from stem and leaves per mixing ratio, after silver nanoparticle biosynthesis showing silver peaks (arrows) (A) leaves extract AgNPs 1:4; (B) leaves extract AgNPs 1:2; (C) leaves extract AgNPs 1:1; (D) stem extract AgNPs 1:4; (E) stem extract AgNPs 1:2; (F) stem extract AgNPs 1:1

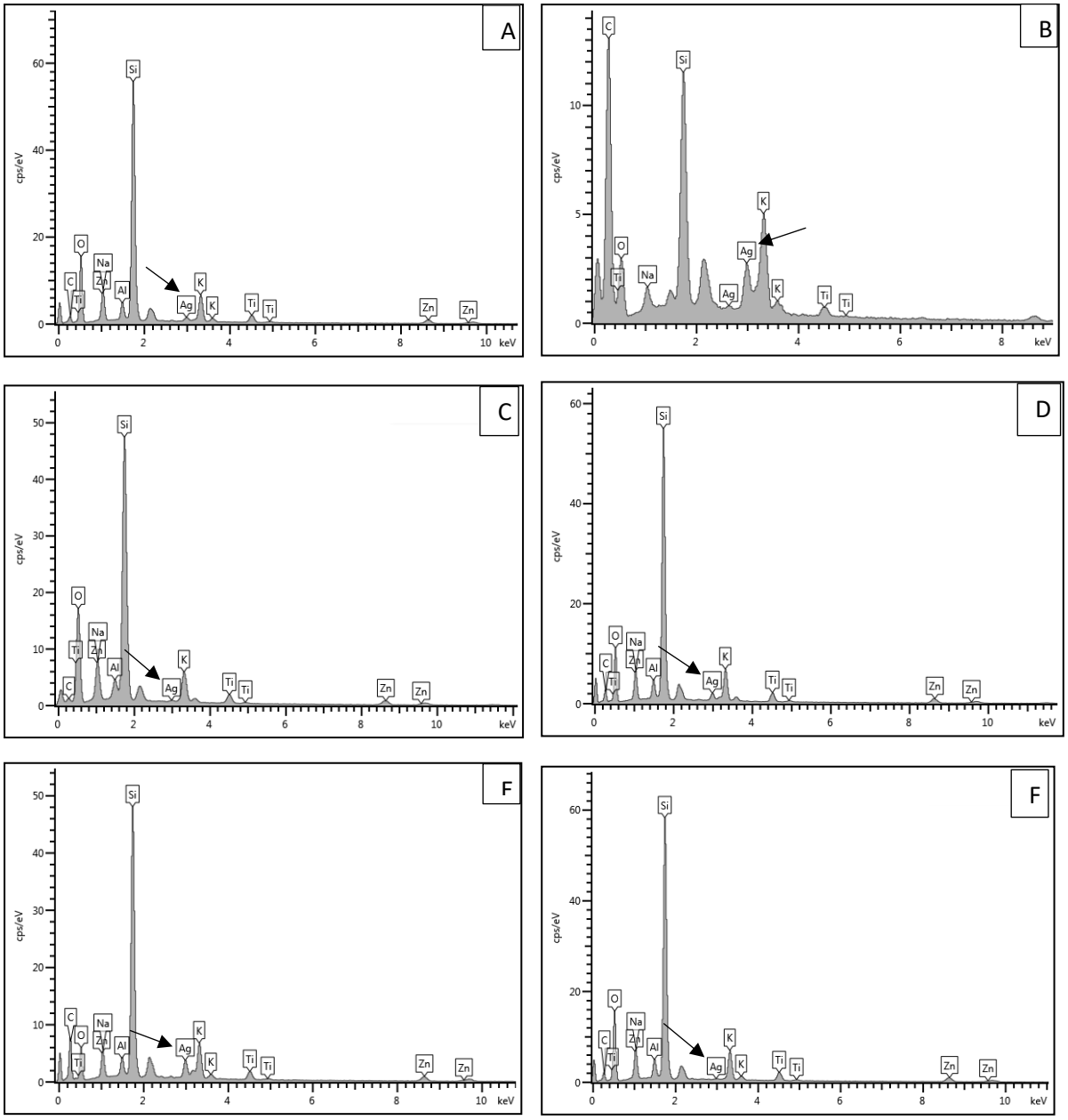

Figure 4.2: The EDX spectra of *R. caffra* methanol extracts from stem and leaves per mixing ratio, after silver nanoparticle biosynthesis showing silver peaks (arrows). (A) leaves extract AgNPs 1:4; (B) leaves extract AgNPs 1:2; (C) leaves extract AgNPs 1:1; (D) stem extract AgNPs 1:4; (E) stem extract AgNPs 1:2; (F) stem extract AgNPs 1:1

The size and shape of the newly synthesized nanoparticles using *R. caffra* extracts was shown in Figures 5.1 and 5.2. The electron micrographs show round to oblong shaped particles. Figure 5.1 A, B, D, E and F and Figure 5.2 D-F show a high density of particles while Figure 5.1 B and 5.2 A-C show a low density. The mean particle size (Fig. 5.3) shows that the water extract from stem 1:1 AgNPs are the smallest in diameter (15.84nm) while the water extract from leaves 1:1 AgNPs are the largest in diameter (35.99nm). The methanol extract from leaves AgNPs have a larger mean particle size than the methanol extract from stem AgNPs. Overall, the water extract from leaves AgNPs have the highest mean particle size in the range of 31.41nm to 35.80nm while water extract from stem AgNPs have the lowest in the range of 15.84nm to 19.66nm. Overall, the particles formed are in the size range of 15.84nm to 35.99nm which is similar to leaf broth AgNP synthesis of *R. tetraphylla* L. (Kalaiarasi *et al*., 2013). Optimally shaped nanoparticles has an influence on the properties of NPs (Khan *et al*., 2017). One of these properties is the antimicrobial effect the particles have based on their shape and size; and depending on the different surface areas of the NP that interact with microbes (Raza *et al*., 2016). The water extract from stem AgNPs are the smallest in size and range which is preferable in nanotechnology (Tran *et al*., 2013; Erjaee *et al.,* 2017).

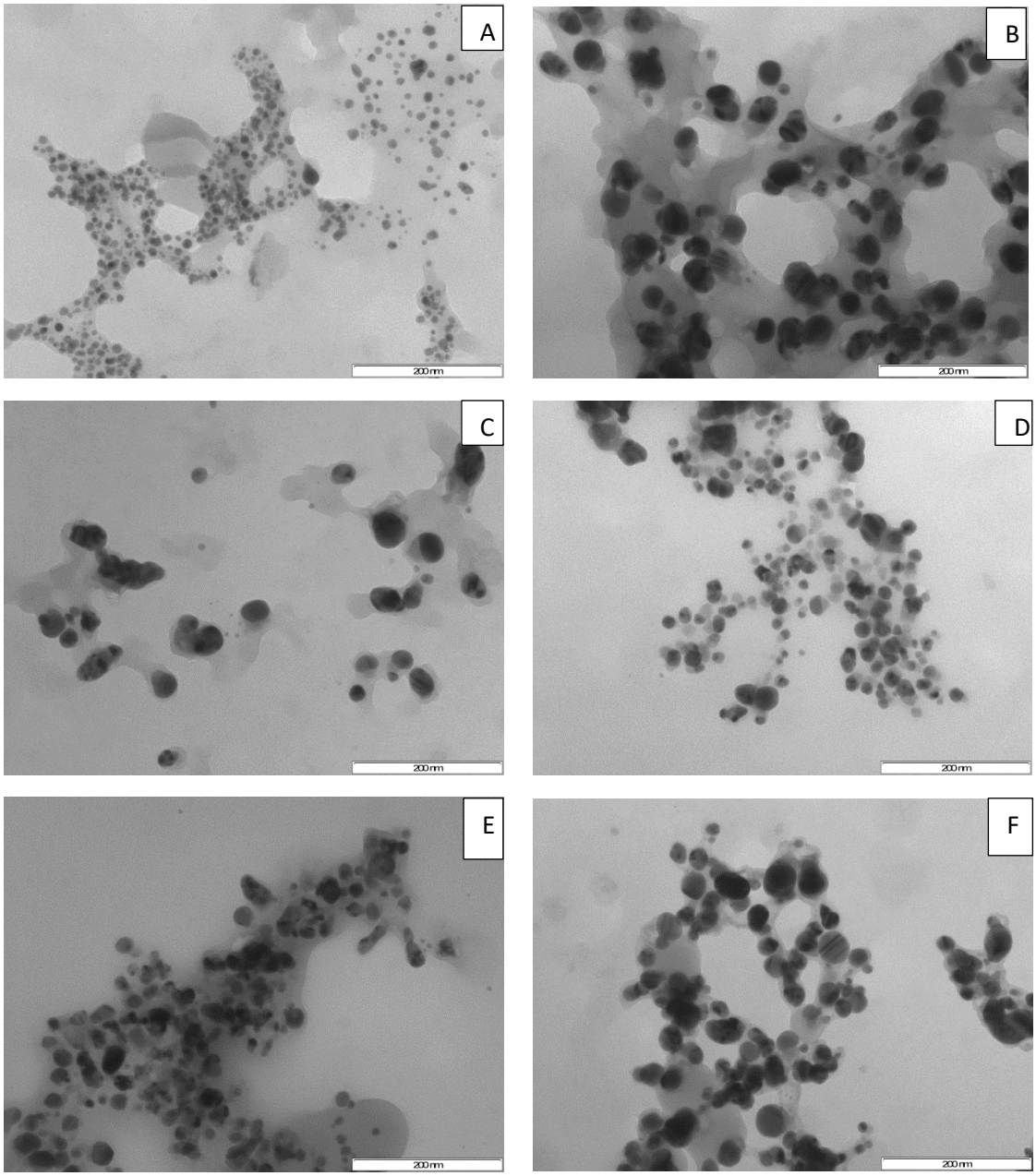

Figure 5.1: TEM of stem and leaves methanol extracts per mixing ratio, after silver nanoparticle biosynthesis. (A) leaves AgNPs 1:1; (B) leaves AgNPs 1:2; (C) leaves AgNPs 1:4; (D) stem AgNPs 1:1; (E) stem AgNPs 1:2; (F) stem AgNPs 1:4

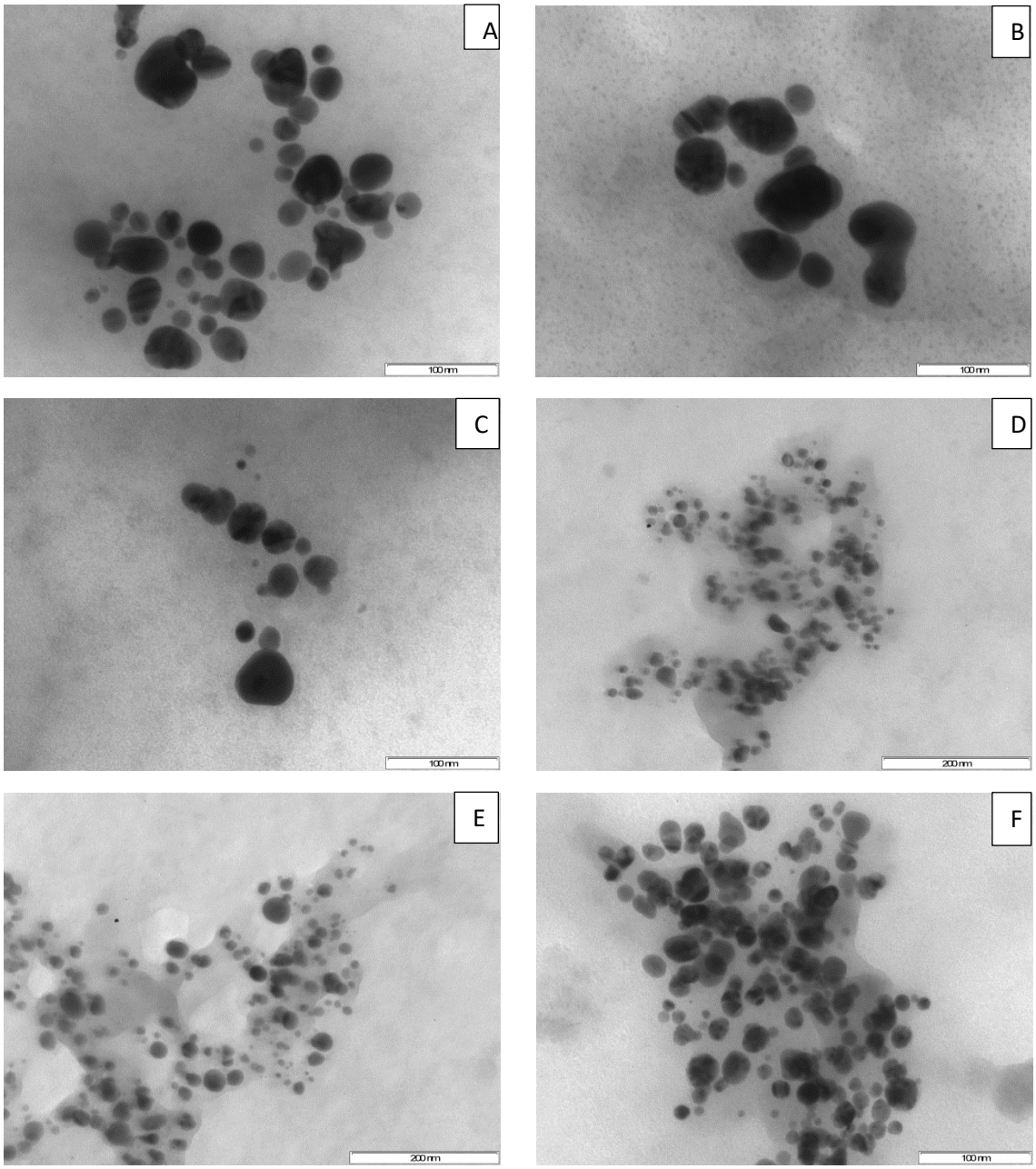

Figure 5.2: TEM of stem and leaves water extracts per mixing ratio, after silver nanoparticle biosynthesis. (A) leaves AgNPs 1:1; (B) leaves AgNPs 1:2; (C) leaves AgNPs 1:4; (D) stem AgNPs 1:1; (E) stem AgNPs 1:2; (F) stem AgNPs 1:4

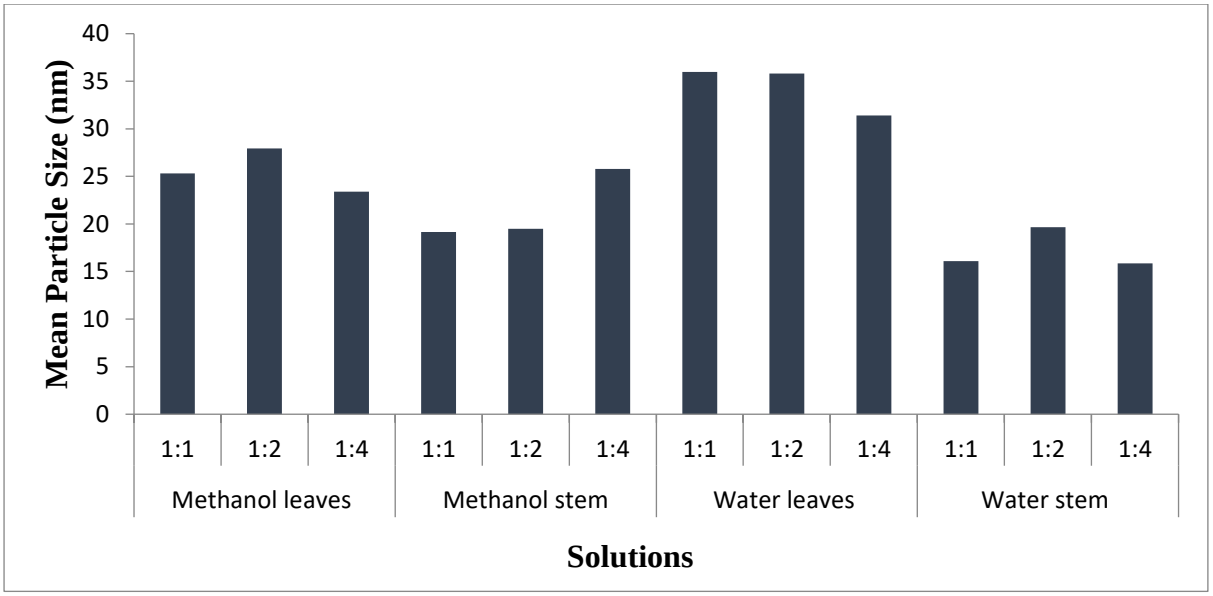

Figure 5.3: The mean particle size of *R. caffra* AgNPs from different extraction

The AgNPs with the lowest average diameter from each category were tested for activity against five strains of bacteria as the smallest size of nanoparticles is preferred. From Figure 6 and Table 1, the water extract from stem 1:4 AgNPs showed activity against methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumonia* (Fig. 6 and Table 1). The methanol extract from leaves 1:4 AgNPs and water extract from leaves 1:4 AgNPs also show activity against *Klebsiella pneumonia.* In a study by Raza *et al.* (2016), the smallest sized nanoparticles had the greatest bacterial efficacy against *Escherichia coli* and *Pseudomonas aeruginosa.* These findings support Raza *et al*. (2016) where the water extract from stem 1:4 AgNPs shows effectiveness against two bacterial strains. The methanol extract from stem 1:1 AgNPs did not show any activity despite having the smallest mean particle size in that category. *Klebsiella pneumonia* is gram-negative and has a single or double peptidoglycan layer which the AgNPs may have been able to penetrate (Roy *et al*., 2015). The cell membrane of the bacterial cells break open when silver ions are released from silver NPs. This damages cytoplasm membranes, degrades cellular transport, reduces intracellular ATP, prevents DNA replication and leads to bacterial cell death (Salam *et al*., 2012; Roy *et al.*, 2015; Yadav *et al*., 2016).

The small surface area to volume ratio that is characteristic of small NPs release a larger amount of silver cations, promoting their dissolution and increasing the ability to be detrimental to bacteria. This could also explain why there was no effect of the *R. caffra* AgNPs on the remaining bacteria (Fig. 6 and Table 1). The ability of AgNPs to release silver ions gives the

synthesized solution their antibacterial properties. This could have occurred at a slow rate and therefore did not affect the bacteria (Salam *et al*., 2012; Raza *et al.*, 2016; Yadav *et al*., 2016). This places emphasis on the morphological characteristics of AgNP formation from plant extracts. In this study, the water stem 1:4 AgNPs of *R. caffra* may be the preferred solution based on the aqueous extract, the phytochemical components of the stem, the efficacy of AgNP synthesis and the size and shape of AgNPs generated.

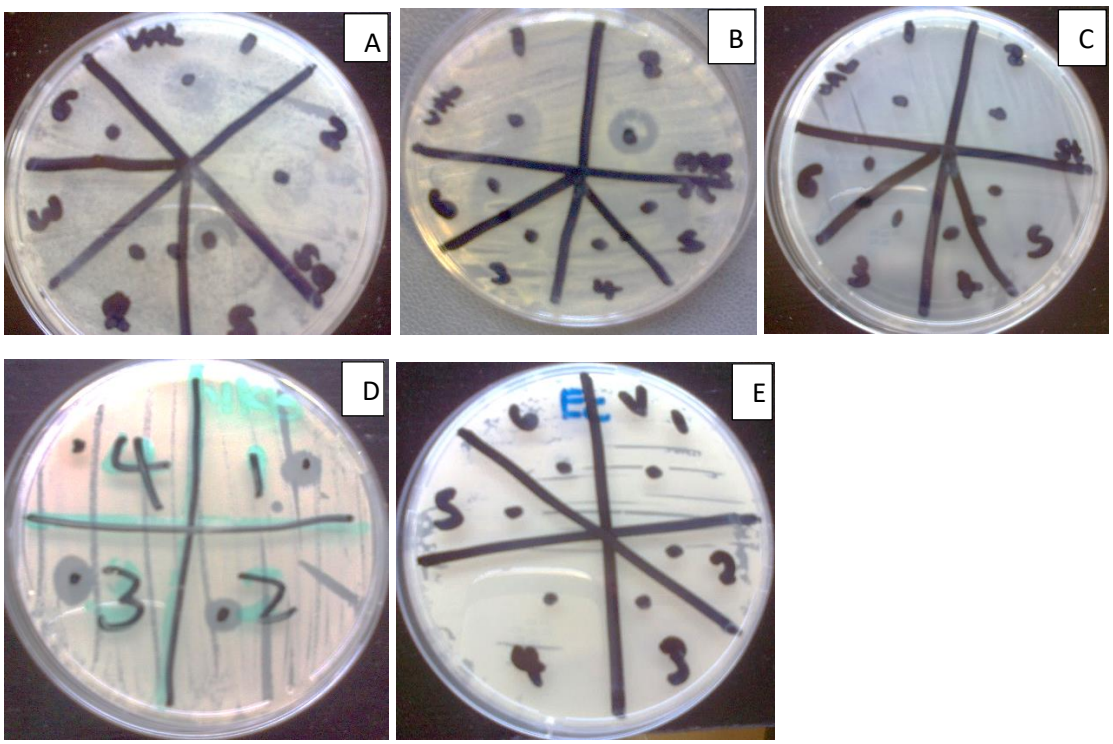

Figure 6: Bactericidal effects of *R. caffra* AgNPs (1 – Water leaves 1:4, 2 – Water stems 1:4, 3- Methanol leaves 1:4, 4- Methanol stems 1:1) against human pathogens (A) *Staphylococcus aureus,* (B) methicillin-resistant *Staphylococcus aureus,* (C) *Salmonella typhimurium,* (D) *Klebsiella pneumonia,* (E) *Escherichia coli*

Table 1: Preliminary antibacterial activity of *R. caffra* AgNPs

Bacteria	**Water leaves AgNPs 1:4**	**Water stems AgNPs 1:4**	**Methanol leaves AgNPs 1:4**	**Methanol stems AgNPs 1:1**
Staphylococcus aureus	--	--	--	--
methicillin-resistant *Staphylococcus aureus*	--	++	--	--
Salmonella typhimurium	--	--	--	--
Klebsiella pneumonia	++	++	++	--
Escherichia coli	--	--	--	--

*Intensity of inhibition: (--) No activity, (+) affected the bacteria, (++) clear zone of inhibition

5.5 Conclusion

This study demonstrated effective biosynthesis of AgNPs using *R. caffra* leaf and stem extracts. The AgNPs were spherical and oblong in shape but varied in size depending on the extracts. The AgNPs displayed good antibacterial activity with the smaller particles having greater activity against *Klebsiella pneumonia*. *Rauvolfia caffra* silver nanoparticles have potential use in the nanotechnology industry and can be used as an eco-friendly, affordable alternative to conventional medicine.

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CHAPTER 6: GENERAL DISCUSSION

Rauvolfia caffra Sond. is a large tree that is used in traditional medicine in South Africa. Despite use in treating sexually transmitted diseases, fever, pneumonia and skin infection, there is a lack of scientific information on the medicinal value of this species (Njau *et al*., 2014; Tshikalange *et al*., 2005). In addition to the medicinal benefit, the micromorphology has not been investigated. The aim of this research was to investigate the phytochemical components and describe the micromorphological structures present on the leaves and petioles.

Rauvolfia caffra possess laticifers that produce latex. The micromorphological data (Chapter 3) indicated that no external microstructures were present on the leaf surfaces. Transverse (cross) sections of the stems and leaves showed the exuding latex which is milky white in colour. The SEM micrographs showed exudates in the laticifers cells. This is related to the ability of latex to coagulate. The coagulation mechanism has not received much attention. There are two proposed mechanisms of coagulation that exist – chemical coagulation, which has been reported in *Ficus benjamina* and *Hevea brasiliensis* and physical coagulation that has been proposed for *Euphorbia* spp. (Bauer *et al*., 2014). The vast majority of literature supports the occurrence of non-articular laticifers in the Apocynaceae. However this study reveals the presence of articulated and unbranched laticifers which is similar to the study by Lopes *et al*. (2009) where articulated laticifers were reported in the Apocynoideae.

The preliminary medicinal investigations of the crude extracts of *R. caffra* were described (Chapter 4). The methanol extracts of the stem were effective against all strains of bacteria tested. The classes of compounds detected from the leaf and stem extracts add to the scientific knowledge on *R. caffra* (Tiwari *et al*., 2011; Gopalakrishnan and Udayakumar, 2017). The crude extracts have health benefits so that the effect of silver nanoparticles of the extracts was investigated (Chapter 5).

The protocol proposed for AgNP biosynthesis was novel and produced the ideal sized silver nanoparticles (Paulkumar *et al.,* 2017). The colour change, UV spectra and pH showed varied results for each sample. However, all of the samples showed the presence of silver in the EDX analysis which indicated the formation of AgNPs. This was confirmed by TEM. Three of the samples were observed to inhibit *K. pneumonia.* The study confirmed that *R. caffra* extracts can be used to synthesize AgNPs in an eco-friendly, safe and quick manner.

Both the crude and water stem extracts AgNPs showed inhibition against bacteria. This research has shown validity in terms of utilizing *R. caffra* for treating prevalent human ailments. Further studies should be carried out to investigate fully the potential of this species.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction

Rauvolfia caffra has not been subjected to scientific study in South Africa. The published literature has limited contextual information on scientific findings. The traditional uses suggested that this plant has pharmacological value. A limited number of species from the genus *Rauvolfia* has been documented. The Apocynaceae species have been found to synthesize latex which is stored and transported by laticifers. The micromorphological features were described in terms of the laticifers which occur in the pith and surrounding the vascular tissue. The laticifers were described as articulated and unbranched. Morphological characteristics of *R. caffra* were not reported in the literature.

Preliminary phytochemical screening was achieved by extraction of the leaves and stems with various solvents. The colour change reactions indicated the presence of several classes of compounds including phenolics, tannins, flavones and flavonones, alkaloids, glycosides, mucilage and proteins. The methanolic extract of the stem inhibited different strains of bacteria. This indicates that *R. caffra* possesses a number of bioactive compounds with potential to be used medicinally.

Silver nanoparticle biosynthesis combines plant extracts that have natural phytochemicals with silver nitrate to produce silver nanoparticles. This is intended to have a compounded effect to inhibit pathogens. *Rauvolfia caffra* AgNPs were synthesized successfully and screened against bacteria. The product had an inhibitory effect on *K. pneumonia*.

The results suggest that *R. caffra* shows promise as a potential drug lead and AgNP synthesizer to combat increasing health concerns.

7.2 Aims and Objectives

The aim of this research was to investigate the phytochemical composition of *R. caffra* leaves and stems in relation to traditional use and to describe the micromorphological structures present.

7.3 Challenges

In the microscopy investigations a major challenge was to apply different treatments to preserve the material without collapse of cellular structures.

7.4 Future possibilities

There are many possibilities for future research. In terms of microscopy, the different stages of laticifer development can be investigated. This information can be systematically applied to determine the taxonomic implications for subfamilies in the Apocynaceae. The physiological aspects of elucidating the coagulation mechanism for the latex would be an interesting area of research. Along with this, the percentage composition of rubber in the latex can also be determined. Gas-chromatography/mass spectroscopy can be carried out to determine the quantitative composition of the crude extracts and essential oils. The phytochemistry and antibacterial activity can be expanded by isolating the bioactive compounds and identifying any drug precursors that may be present. The minimum inhibitory concentrations can also be investigated on both the crude extracts and silver nanoparticles. Fourier-transform infrared spectroscopy can be applied to the AgNPs to identify organic and polymeric components.

7.5 Final comments and summary conclusions

This research provided insight on the micromorphology, ultrastructure, preliminary antibacterial effect and rapid silver nanoparticle biosynthesis potential of *R. caffra*. The medicinal potential of this species can be exploited in various industries including healthcare and nanotechnology. The findings of this study are mostly novel and contribute to the knowledge and understanding of South African ethnobotany.

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