Structure, Biology and Chemistry of *Plumbago auriculata* (Plumbaginaceae)

By

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DEDICATION

To my daughter Ardraya Naidoo, she has given me the strength and encouragement to excel and be a positive role model for her.

"Laying Down the Footsteps She Can Be Proud To Follow"

ABSTRACT

Plumbago auriculata Lam. is endemic to South Africa and is often cultivated for its ornamental and medicinal uses throughout the world. Belonging to the family Plumbaginaceae this species contains specialized secretory structures on the leaves and calyces. This study focused on the micromorphological, chemical and biological aspects of the species. Micromorphological studies revealed the presence of salt glands on the adaxial and abaxial surface of leaves and two types of trichomes on the calyces. “Transefer cells” were reported for the first time in the genus. The secretory process of the salt glands was further enhanced by the presence of mitochondria, ribosomes, vacuoles, dictyosomes and rough endoplasmic reticulum cisternae. Histochemical and phytochemical studies revealed the presence of important secondary metabolites that possess many medicinal properties which were further analyzed by Gas chromatography-mass spectrometry (GC-MC) identifying the composition of compounds in the leaf and calyx extracts. A novel attempt at synthesizing silver nanoparticles proved leaf and calyx extracts to be efficient reducing and capping agents that further displayed good antibacterial activity against gram-positive and gram-negative bacteria. Biological studies revealed for the first time the presence of three variants of flower colour (white, pale blue and deep blue) and each colour had a characteristic sex-morph described as “Pin” or “Thrum.” Due to the “Pin” and “Thrum” scenario, plants are reported to be self-incompatible. However, the findings of this study suggested that plants were also self-compatibile. Graphical demonstration of calyx trichomes showed their involvement in insect entrapment often resulting in the death of the insect due to the struggle to free itself. This study concludes that *P. auriculata* is of good medicinal value and can contribute towards drug development and other medicinal uses in traditional markets as well as in the cosmetic and pharmacological industries. This species has striking morphological and biological features and possesses good value for future perspectives.

PREFACE

The experimental work described in this Thesis was carried out in the School of Life Sciences, University of Kwa-Zulu Natal, Westville Campus, Durban, from March 2015 to November 2017, under the supervision of Prof Yougasphree Naidoo and co-supervision of Prof Himansu Baijnath.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the thesis.

As the candidate's supervisors we have approved this thesis for submission.

Supervisor:

Signed: _____ Name: Prof Yougasphree Naidoo Date: _____

Co-Supervisor:

Signed: _____ Name: Prof Himansu Baijnath Date: _____

DECLARATION 1- PLAGIARISM

I, Mrs Karishma Singh declare that:

1. The research reported in this Thesis, except where otherwise indicated, is my original research.

2. This Thesis has not been submitted for any degree or examination at any other tertiary institution.

3. This Thesis does not contain other person's data, graphs, pictures or other information, unless specifically acknowledged as being sourced from other persons.

4. This Thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sourced have been quoted, then:

    a. Their words have been re-written but the general information attributed to them had been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and within quotation marks, and referenced.

5. This Thesis does not contain texts, tables, graphs, figures or graphics copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the thesis and in the References sections.

Signed……

DECLARATION 2- PUBLICATIONS

DETAILS TO CONTRIBUTION TO PUBLICATIONS that form part and/include research presented in this thesis (include publications in preparation, submitted, *in press*, and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

Karishma Singh, Yougasphree Naidoo, Himansu Baijnath. 2018.

A comprehensive review on the genus *Plumbago* with focus on *Plumbago auriculata* (Plumbaginaceae). Submitted 1/06/2017 to *African Journal of Traditional, Complimentary and Alternative Medicine.* Accepted for publication 02/10/2017. Published

Contributions: Karishma Singh collected data and wrote manuscript. Prof Naidoo and Prof Baijnath edited the manuscript.

Publication 2

Karishma Singh, Yougasphree Naidoo, Vishal Bharuth, Himansu Baijnath. 2017.

Micromorphology and histochemistry of the secretory apparatus of *Plumbago auriculata* Lam. Submitted to *South African Journal of Botany*. Awaiting comments.

Contributions: Karishma Singh carried out experimental work, recorded data and wrote manuscript. Vishal Bharuth recorded the Transmission Electron Microscopy aspect of the study. Prof Naidoo and Prof Baijnath provided comments and edited the manuscript.

Publication 3

Karishma Singh, Yougasphree Naidoo, Channangihalli Thimmegowda Sadashiva, Himansu Baijnath. 2017.

Gas chromatography-mass spectrometry analysis and phytochemical screening of *Plumbago auriculata* Lam. leaf and calyx extracts. Submitted to *Asian Pacific Journal of Tropical Medicine.* Awaiting comments.

Contributions: Karishma Singh carried out experimental work, recorded data and wrote manuscript. Dr Sadashiva assisted in writing the results section of the manuscript. Prof Naidoo and Prof Baijnath were supervisors and edited the manuscript.

Publication 4

Karishma Singh, Yougasphree Naidoo, Chunderika Moktar, Himansu Baijnath. 2017.

Biosynthesis of silver nanoparticles using *Plumbago auriculata* Lam. leaf and calyx extracts and evaluation of their antimicrobial activities. Submitted to *Advances in Natural Science: Nanoscience and Nanotechnology.* Article in Press

Contributions: Karishma Singh carried out experimental work, recorded data and wrote manuscript. Prof Naidoo and Prof Baijnath were supervisors and provided comments. Dr Moktar edited the manuscript and allowed the use of her facilities.

Publication 5

Karishma Singh, Yougasphree Naidoo, Himansu Baijnath.

Floral biology of *Plumbago auriculata* (Plumbaginaceae) and associated plant- insect interactions. Submitted to *Turkish Journal of Botany.* Awaiting comments.

Contributions: Karishma Singh carried out experimental work, recorded data and wrote manuscript. Prof Naidoo and Prof Baijnath were supervisors and provided comments and edited the manuscript.

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Chapter 1

Introduction

1.1 Rationale for the Research

The family Plumbaginaceae comprises 20 genera and over 400 species distributed and utilized throughout the world (The Plant List, 2017). The genus *Plumbago* is the largest genus in Plumbaginaceae with 18 species and is distributed in warm tropical regions (The Plant List, 2017). Plants in the genus are cultivated and utilized in many parts of the world for their ornamental and medicinal properties (Foden and Potter, 2015). In this genus, *Plumbago indica* L. and *Plumbago zeylanica* L. have been extensively researched (Saha and Paul, 2012; Chauhan, 2014; Saha and Paul, 2014; Tyagi and Menghani, 2014). They are mostly exploited for their medicinal uses. Research on *Plumbago auriculata* is limited in comparison with *Plumbago zeylanica* and *Plumbago indica.* A well-structured approach in documenting the genus *Plumbago* and its possible uses as phytomedicine has not been extensively studied. This together with the lack of knowledge focusing on *P. auriculata* in South Africa was the basis of the first rationale for this study.

Plumbago auriculata endemic to South Africa is a perennial shrub covered with trusses of pale blue flowers, greyish-green leaves and a hairy calyx (Figure 1) (Batten 1974; Ferrero et al., 2009, Foden and Potter, 2015). The plant contains specialized secretory structures on the leaves and calyces. The minute glands found on the surface of the leaves are reported to be salt glands (Sakai 1974, Faraday and Thomson, 1986). These salt secreting glands assist in the regulation of salt and ion concentrations (Amarasinghe, 1986; Kobayasi, 2008). Salt glands are often referred to as specialized trichomes (Fahn, 1988). However, some publications classify salt glands and trichomes as separate structures, depending on the position of the gland. For example salt glands of *Tamarix sp.* were reported to be sunken in epidermal crypts whilst those of the mangrove *Avicennia marina* project from the leaf and appear as trichomes (Campbell and Thomson, 1975; Fahn and Shimony, 1977). Salt glands found on leaf epidermis are common in the family Plumbaginaceae and are similar in ultrastructure and morphology amongst members of the

family (Faraday and Thomson 1986; Grigore and Toma, 2016). These glands were initially referred to as "*chalk glands*" due to the secretion of insoluble carbonate salts (Metcalfe and Chalk, 1972; Grigore and Toma, 2016), but it was later discovered that the glands of some species secrete sodium chloride, thus then referred to as "*salt glands*" (Faraday and Thomson, 1986; Grigore and Toma, 2016). However, no conclusive distinctions have been drawn between these two terms.

Panicker and Haridasan (2016) reported two types of trichomes (glandular and non-glandular) with a wide range of functions on the calyx of *P. auriculata*. Their trichomes bear a close resemblance to the trichomes of *Drosera* L. (Droseraceace) in their ability to trap and digest insects (Stoltzfus *et al*., 2002; Naidoo and Heneidak, 2013). Secretory structures occurring on the leaves and calyces of *P. auriculata* yielded interesting information that led the research to the second rationale for this study. The intent was to seek new insight into the way the secretory structures function, by interpreting images of their structure.

The investigation of the leaves and calyces to effectively synthesize nanoparticles and testing its antibacterial activity has not been attempted previously. In this regard, the study was novel. *P. auriculata* is of high medicinal importance possessing a wide range of secondary metabolites with plumbagin being the marker compound showing various pharmacological activities (Tyagi and Menghani, 2014). Previous studies highlighted the use of different plant parts for the treatment of human and animal ailments (Joy, 1998; Elgorashi *et al*., 2003; McGaw and Eloff, 2008; Deshpande *et al*., 2014). However, plants tend to exhibit toxic properties and need to be administered with caution (Jose *et al*., 2014). The Gas Chromatography-Mass Spectrometry analysis (GC-MS) of the extracts of these structures has also not been reported prior to this investigation. This technique sought to confirm the presence of medicinally important phytochemicals.

This species is reported to be heterostylous (style and stamen differing in length between morphs and positioned reciprocally in flowers of both morphs) (Ferrero *et al*., 2009). Previous studies reported that this species is primarily pollinated by insects especially butterflies (Ferrero *et al*.,

2009; Panicker and Haridasan, 2016). Knowledge in the floral biology of *P. auriculata* is lacking. In this regard, the intent was to expand on an understanding of the species which is characterized by different flower colours.

This study sought to provide a greater insight into the structure, biology and chemistry of *P. auriculata*. This research around these disciplines is novel and will contribute to the South African medicinal plant database. Conclusions made from this study will add to the body of data and stimulate further research especially in the oriental region of the world.

1.2 Justification

Plumbago auriculata is cultivated in tropical and subtropical regions around the world (Foden and Potter, 2015). This species yields many bioactive compounds for which it has been medicinally exploited especially in Asian countries where it is believed to have great healing properties (Deshpande *et al*., 2014; Lakshmanan *et al*., 2016). Infectious diseases are the world's leading cause of health problems often resulting in a large number of deaths per day (Ahmad and Beg, 2001). Over the years human resistance to pathogenic microorganisms has been reported on a large scale worldwide. Since the 19th century western medicine has been the primary source of treatment for medical conditions employed by many healthcare practitioners (Boozang, 1998). Western medicine is administered under the care of a healthcare professional, based on laboratory tests and diagnosis. However, treatment is often too expensive, exhibits harsh side effects and not readily available especially in rural communities (Boozang, 1998; Hassan, 2012). Therefore the use of alternative medicine affords a cheaper option due to the pressures from their competitors (western medicine). Plants (to lesser degree animals) have been used as a source of alternative medicine and form an important part of human healthcare in the modern era (Hassan, 2012).

Research over the years has provided reasonable data proving that plants possess active compounds that have medicinal properties (Ahmad and Beg, 2001; Rios and Recio, 2005; Kumar *et al*., 2006; Hassan, 2012). Estimates by Drewes (2012) report that about 50% of commercially available medicine is plant- derived. Plants have been used for many years as a source of alternate medicine (Ahmad and Beg, 2001; Kumar *et al*., 2006; van Vuuren, 2008; Hassan,

2012). According to the World Health Organization (WHO), 80% of the worlds' population relies on plants as a source of traditional herbal medicine (WHO, 2012). In developing countries the use of herbal medicine is now increasing as a primary source of health care because it is affordable, readily available, generally exhibits minimal side effects and is most often culturally accepted (Kumari *et al*., 2016). However, in order to be accepted as a viable alternative to western medicine, the same vigorous methodologies of scientific and clinical validation need to be applied to prove the safety and effectiveness of the therapeutic product (Cragg and Newman, 2012).

Over the last decade scientists have also explored the potential use of plants to synthesize metal nanoparticles as an alternate source of drug development (Salunke *et al*., 2014). Green synthesis is now established as an emerging trend in research (Dwivedi and Gopal, 2010; Ahmed *et al*., 2016). Biosynthesis using plants has gained much attention for their effectiveness as reducing and capping agents of metals and exhibits a wide range of pharmacological uses (Savithramma *et al*., 2011). The use of plants in nanoparticle synthesis is gaining popularity because plants are readily available and less-toxic, hence making this method cost effective, environmentally-friendly and low toxicity (Lee et *al*., 2011). Salunke *et al* (2014) and Ahmed *et al* (2016) have highlighted that the most commonly used metal for synthesizing nanoparticles is silver. This is due to its low toxicity to humans, activity at low temperatures, displays marked antimicrobial properties and use in many disciplines (Jain *et al*., 2009; Savithramma *et al*., 2011; Prabhu and Poulose, 2012; Venkata and Savithramma, 2013). It is for these reasons that silver nanoparticles have gained much attention in scientific research.

Plants and plant-based medicines are considered the oldest source of pharmacologically active compounds and have provided mankind with many useful compounds throughout the years (Omwenga *et al*., 2005). Plants possess a diverse category of phytochemicals which can be utilized in medicine fomulation and other applications (e.g. cosmetics, insecticides, food preparation) (Joseph *et al*., 2013). Techniques such as histochemistry and GC-MS have been used for decades to identify the composition of compounds that may be present in medicinal plants (Ascensao *et al*., 1997; Kalimuthu and Prabakaran, 2015).

Histochemical analysis is essential for the study of plant secretory structures and the composition of their secretions. As each secretory structure may produce one or more types of compounds,

various histochemical tests can be performed to detect metabolites of different chemical classes (Ascensao *et al*., 1997). Phytochemicals can be further screened using different qualitative chemical analyses for establishing the profiles of given extracts (Harborne, 1973; Joseph *et al*., 2013). GC-MS analysis provides an assessment of the various compounds with different chemical structures present in the plant.

Several classes of compounds such as alkaloids, tannins, carbohydrates, terpenoids, proteins, phenolics, flavonoids, naphthaquinones, saponins, glycosides, fixed fats and oils are phytochemically/ biologically active compounds that are naturally produced by plants (Kennedy and Wigthman, 2011; Pourmorad *et al.*, 2006). These compounds exhibit a broad range of medicinal properties such as antimicrobial, anticancer, antidiabetic, antiulcer, anti-inflammatory, antimalarial, antifertility and antioxidant (Seigler and Price, 1978; Joseph *et al*., 2013). Alkaloids and phenolic compounds are probably the two most important phytochemical compounds that are of medicinal value (Garba and Okeniyi, 2012). These compounds are produced, stored or excreted in specialized secretory plant structures (Ascensao *et al*., 1997).

Secretory structures occur in the form of trichomes, salt glands, idioblasts, resin ducts, laticifiers, colleters and nectaries located in various reproductive and vegetative organs of the plant (Thomas, 1991). Secretory structures are species specific and their function is characteristic to that species. These structures occur in various forms as a result of living conditions, genetic processes and phylogenetic characteristics. Visible changes in these structures are noted in varying growth conditions due to environmental factors. Conversely, it is not possible to observe such changes through the naked eye (Fahn, 1988; Umah *et al*., 2017)

Micromorphological studies on plants enable micro-level analyses of secretory structures through the use of light and electron microscopy (Yigit, 2016). Techniques employing microscopy, analyze characteristics such as the presence or absence of trichomes, canals, oil glands, salt-secreting glands, seed or pollen morphology and particular cell types (Subramanion and Sreenivasan, 2012). The types of electron microscopes used for these micro-structural studies are Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). These microscopes provide high resolution imaging making it possible to obtain morphological, structural, and elemental information from the plants at high magnification.

In nature plant-animal interactions are one of the most fundamental laws of nature that provide a basis on how these organisms influence the plants distribution, phenotypes, abundance and genotypic composition (Strauss and Irwin, 2004). Pollination, which is the transfer of pollen from the anther to the stigma, is a requirement for reproductive success in most flowering plants and is often the most common plant-animal interaction (McMullen and Close, 1993). Plant-animal interactions do not always result in pollination. Depending on the plant species animals are sometimes trapped and killed by plant structures in their quest to feed on nectar and other rewards. The floral biology of a plant provides the basis for a clear overview of the plant developmental stages, plant structures attributing to plant-animal interactions and their respective functions. The breeding system of plants has been well recognized since the 1800's by Hildebrand and Darwin in the mid nineteenth century highlighting heterostylous breeding systems (Ganders, 1979). Heterostyly is a genetically controlled floral polymorphism occurring in 28 families of flowering plants (Guvensen *et al*., 2013). The floral morphs differ reciprocally in style and stamen length known as a "Pin" (long-style, short stamen) morph with the stigmas above the anthers and the "Thrum" (short-style, long stamen) morph with the anthers above the stigma (Baker, 1966, Ganders 1979). Differences in other morphological features are also evident between morphs such as pollen grain size and production, stigmatic papillae, and corolla size or morphology. There are two morphs in distylous plants and three morphs in tristylous plants (Baker, 1966). Heterostylous plants display self-incompatibility i.e. the pollen from a flower of one morph cannot fertilize another flower of the same morph (Baker, 1966; Lord and Russell, 2002).

1.3 Aims and Objectives of this study

Aim 1: To investigate the micromorphology and the chemical composition of the secretory structures of *P. auriculata.*

Objectives:

1. To characterize the glandular structures of the leaves and calyces with the use of stereo and electron microscopy.

2. To determine the chemical make-up of the secretory product by conducting histochemical tests and light microscopical analyses to identify compounds.

3. To identify individual compounds present in the crude extracts.

4. To provide a description of the secretory process with the use of light and electron microscopy.

Aim 2: To investigate the potential use of *P.auriculata* leaf and calyx extracts as a bioreduction agent of metals.

Objectives:

1. To determine the optimum conditions for the synthesis of nanoparticles.

2. To characterize the structure of the nanoparticles.

3. To determine the potential antibacterial application of the newly synthesized nanoparticles.

Aim 3: To determine the linkage amongst the variation in flower colour with the associated sex-morphs.

Objectives:

1. To determine the sex-morph of each of the flower colours.

2. To determine the morphological differences between “Pin” and “Thrum” flowers.

3. To determine the trapping mechanism of the calyx trichomes.

1.4 Tables and Figures

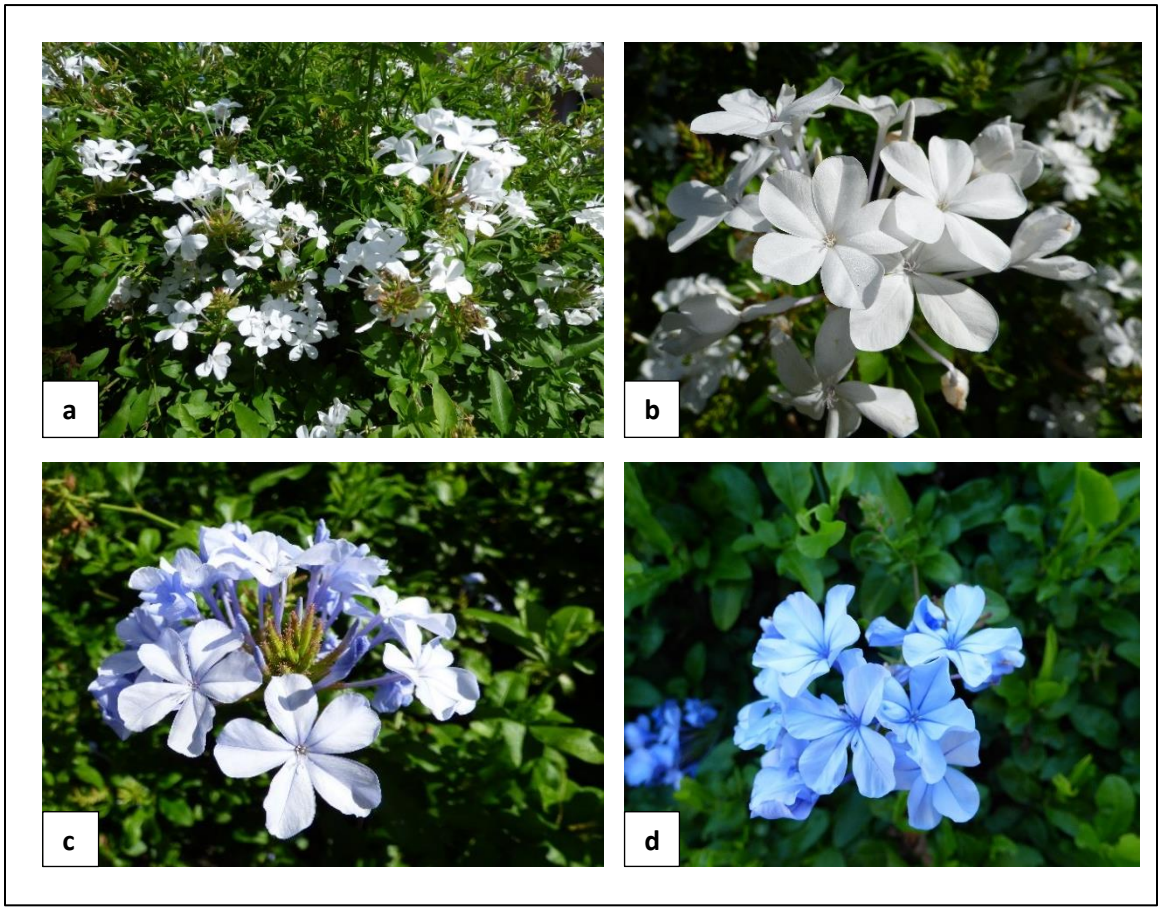

Figure 1: Shrub of *Plumbago auriculata* with different flower colours a & b) white flowers; c) pale blue flowers; d) deep blue flowers. Images captured at the University of Kwa-Zulu Natal, Westville, Durban, South Africa.

1.5 Outline of thesis structure

In order to achieve the aims and objectives of this study, different microscopical techniques, chemical and field methods were used. Following the findings of the analyses, this thesis is outlined as follows: Chapter 1 gives and brief introduction to this study. Chapter 2 gives a comprehensive review into the genus *Plumbago* with primary focus on *Plumbago auriculata.* Chapter 3 outlines the micromorphology of the secretory structures of *P. auriculata* and provides insights on the phytochemical compounds present in the structures. Chapter 4 identifies the phytochemical compounds present in the plant extracts and their composition. Chapter 5 evaluates the potential of the plant extracts as bio-reducing agents and their efficacy in antibacterial activity. Chapter 6 investigates the linkage amongst the variation in flower colour with the associated sex-morphs and provides an overview of plant-insect interactions. Chapter 7 concludes the study and provides recommendations for future research.

The design implemented for the thesis includes chapters in the form of research articles where each article has been presented in accordance to the style of the particular journal guidelines for which the manuscript has been written-up/ submitted. Chapter 1, Chapter 7, conclusion, and recommendation for future research have been formatted uniformly in accordance to UKZN guidelines.

1.6 Outline of thesis methodologies

The detailed methodologies for each chapter in this thesis are outlined accordingly in the chapters. A basic overview of how the research was conducted is as follows:

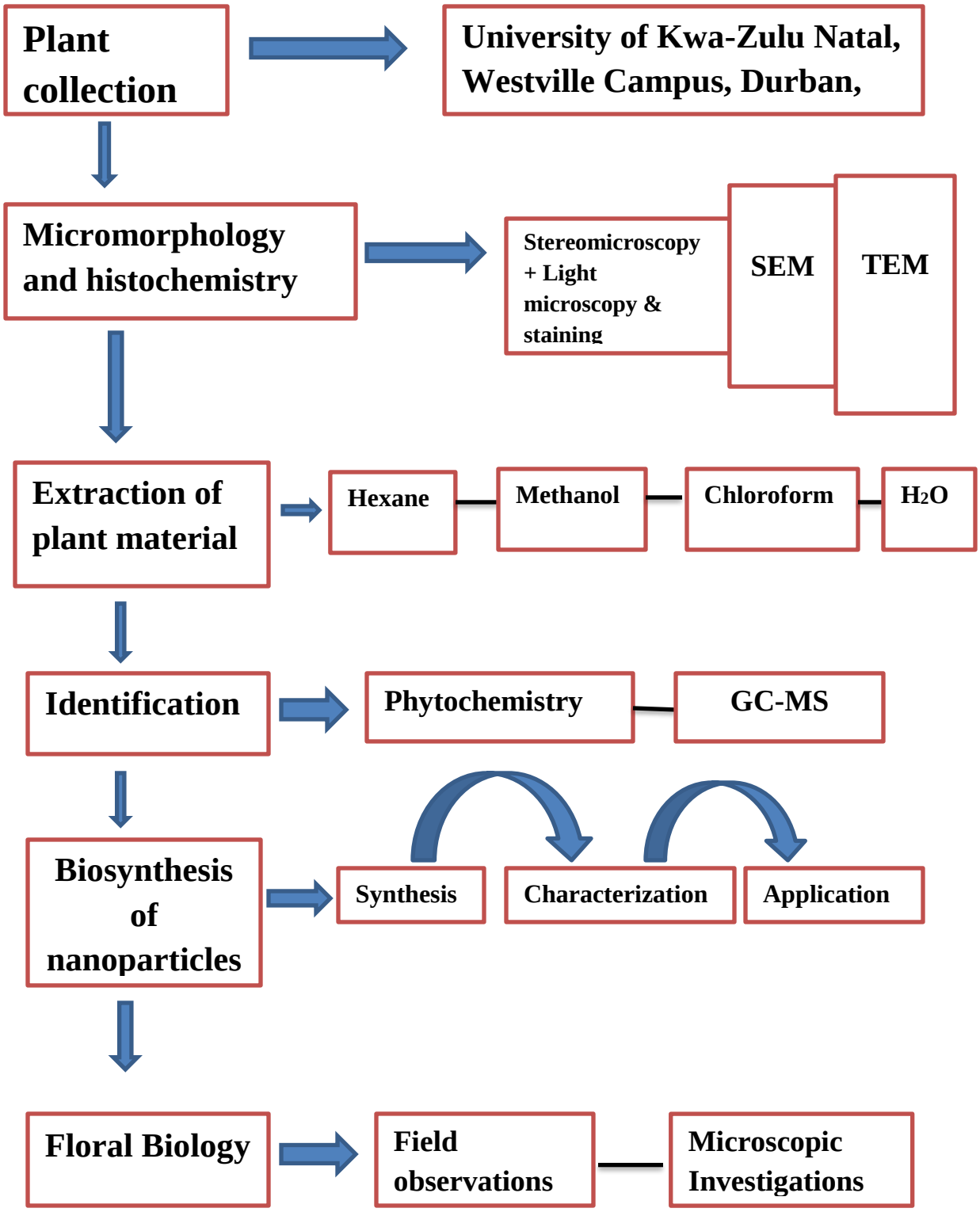

Figure 2: Flow diagram of the methodology employed in this study.

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Chapter 2

Review Article for Publication 1:

A Comprehensive Review on the genus *Plumbago* with focus on *Plumbago auriculata* (Plumbaginaceae).

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Abstract

Background: The genus *Plumbago* distributed in warm tropical regions throughout the world is the largest genus in Plumbaginaceae. Medicinal plants are characteristic to the genus *Plumbago* and are cultivated and utilized worldwide. *Plumbago auriculata* Lam. is endemic to South Africa and is often cultivated for its ornamental and medicinal uses throughout the world.

Materials and Methods: A comprehensive review of the genus *Plumbago* with focus on *P. auriculata* was carried out and information was gathered using scientific publications, conference proceedings, the internet and books. Articles based on the morphology, pharmacological and medicinal uses of *P. auriculata* was analysed thoroughly.

Results: *Plumbago auriculata* plant parts possess a wide range of phytochemicals with plumbagin being the marker compound showing various pharmacological activities. Different plant parts are claimed to be used for the treatment of human and animal ailments, however they do exhibit toxic properties and need to be administered with caution. Salt secreting glands and trichomes are characteristic of Plumbaginaceae.

Conclusion: This study reveals new insights on the genus *Plumbago* and the potential use of species in the genus as medicinal plants. *P. auriculata* possess the bioactive compound plumbagin and secondary metabolites, thus, it is of high medicinal importance. *P. auriculata* is a poorly nor a favourite studied species in the genus *Plumbago* and further research needs to be carried out to explore specific details of the species.

Keywords: *Plumbago,* salt glands, trichomes, pharmacological activities, plumbagin, micropropagation.

Introduction

Over the years, human health problems have been increasing at an alarming rate and are now becoming life threatening (WHO, 2012). Conventional medicine used to control health problems is often too expensive and has many side-effects. Therefore, many people have turned to the use of medicinal plants for the control and treatment of health problems. Plant extracts have been used for hundreds of years to cure ailments (Balunas and Kinghorn, 2005). The vast resource of thousands of medicinal plants and their contribution to human health are to be discovered. Exploiting traditional medicine systems has served as a promising approach in an era of increasing demand for drug production and rising costs of western medicine, globally.

It has been reported that many plants naturally produce secondary metabolites, commonly referred to as phytochemicals or biologically active compounds which are essential for plant metabolism but play a great role in the plants' protection mechanism (Ascensao et al., 1997). In addition to plant protection, these bioactive compounds also serve as precursors for the development of natural, environmentally friendly and low toxicity pharmaceuticals, flavourants, fragrances, cosmetics and pesticides due to their therapeutic and aromatic properties. Bioactive compounds are either produced, excreted or stored in small amounts in specialized cells, termed secretory structures in the form of salt glands, trichomes, resin ducts, idioblasts, laticifiers, colleters and nectaries located in various reproductive and vegetative organs of the plant (Thomas, 1991). In order to understand the biological activity and composition of the secreted exudate, it is vital to know the morphology, distribution and secretory processes of the main secretory structures involved. However, these structures vary in form, distribution, function and type of secretion across different plant families.

The family Plumbaginaceae Juss (a.k.a the leadwort family) has about 24 genera and about 400 species (The Plant List, 2017). This is debatable because some publications reported that there are 700-800 species in the family (APG II, 2003; Simpson, 2010; Renner and Specht, 2011). Plumbaginaceae was first described in 1789 by Antoine Laurent de Jussieu and it was the only family in the order Plumbaginales. However, in 2003 the APG system of plant classification placed Plumbaginaceae in the order Caryophyllales due to certain species having carnivorous characteristics and is sister to the family Polygonaceae (Kubitzki, 1993; Perveen and Qaiser, 2004; Simpson, 2010). Plumbaginaceae comprises mainly herbs, lianas and shrubs, often

occurring in saline habitats. The presence of secretory glands is characteristic of the family (Wilson, 1980; Faraday and Thomson, 1986a). Species of the Plumbaginaceae are of ornamental and medicinal importance. *Plumbago auriculata* Lam. an evergreen shrub is indigenous to South Africa (Figure 1) but is distibuted in other parts of the world in tropical and subtropical regions (Foden and Potter, 2015). *P. auriculata* occurs in thicket and shrub valley bushveld. It is often called Cape *Plumbago* or Cape Leadwort because it is widely found in the Cape regions of South Africa (Batten, 1986; Kubitzki, 1993; Foden and Potter, 2015). *P. auriculata* is an easy to manage plant as it is tolerant to high humidity, high temperature and diseases and can be treated as an ornamental as well as a medicinal plant as it contains many potent bioactive compounds (Chen and Gao, 2013). *P. auriculata* bears a close resemblance to *Drosera,* a carnivorous plant, which plays a role as an attractant as well as a repellant to many insects (Plachno et al., 2006). The genus *Plumbago* comprises of 18 species, three commonly studied species that is *Plumbago rosea* L. syn *indica* L., *Plumbago zeylanica* L., and *Plumbago auriculata*. In all these three species, *P. auriculata* is the least-studied especially in South Africa. This review focuses on information about the morphology, biology, chemical composition, pharmacology, medicinal uses and toxicity of the genus with a particular emphasis on *Plumbago auriculata*.

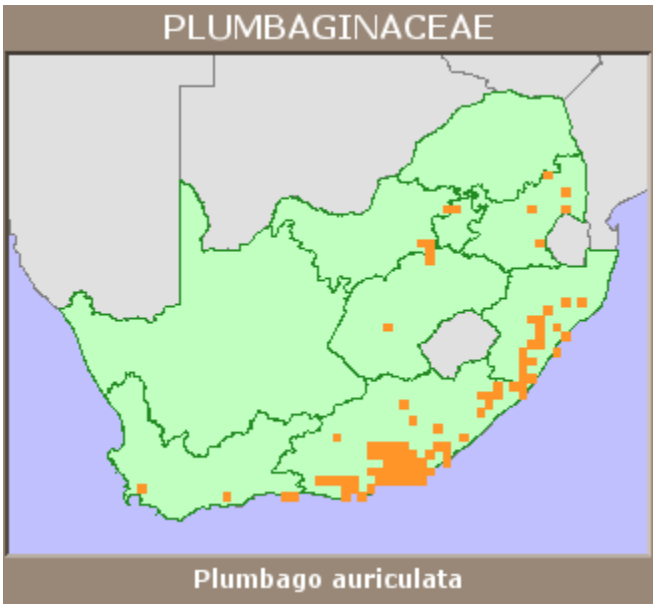


Figure 1: Distrubution of *Plumbago auriculata* in South Africa (After Foden and Potter, 2015).

An overview of the genus

The genus *Plumbag*o comprises 18 species distributed and utilised throughout the world in warm regions (APG, 2009; The Plant List, 2017). It is the largest genus in the family (APG, 2003, 2009). In Sourthen Africa 5 species are native and 2 are cultivated (Glen, 2002). Species are cultivated and utilized worldwide mostly for their medicinal and pharmacological properties due to the presence of plumbagin amongst other phytochemical compounds (Table 1)(Craven and Craven, 2000; Galal et al., 2012; Jose et al., 2014; Saha and Paul, 2012, 2014; Omwenga and Paul, 2012; Purger et al., 2012; That et al., 2012; Thamaraj and Antonysamy, 2013). The genus comprises shrubs or perennial herbs and the presence of a hairy calyx is characteristic of the genus (Batten, 1986). The hairy calyx is associated with insect entrapment amongst other functions. The most commonly cultivated and utilized species is *Plumbago zeylanica* with several publications (Abera et al., 2008; Chauhan, 2014; Demma et al., 2009; Jeyachandran et al., 2009; Tyagi and Menghani, 2014).

Table 1: Species List of the genus *Plumbag*o (The Plant List, 2017).

Species Name	Common Name	Locality	Description	Traditional uses	Pharmacological uses
Plumbago amplexicaulis Oliv.		Tanzania	Erect herb or subshrub, with glaborous stems and bright deep blue flowers.		
Plumbago aphylla Bojer ex Boiss.		Madagascar, Aldabra Islands and Tanzania	Slender shrub with erect stems, leaves simple, entire, and alternate. Flowers white with hairy calyx.	Used to treat diarrhoea, mouth infections and is applied as an eyewash for cataract.	Anti-bacterial and antifungal.
Plumbago auriculata Lam	Cape leadwort	South Africa,	Bushy perennial evergreen shrub,	Has many therapeutic	Antimicrobial, antiulcer,

		Africa, America, Asia, India	trusses of pale blue and white flowers, leaves have minute dots and persistent hairy calyx.	properties and can be used for warts, fractures, oedema, headaches, skin lesions, piles, rheumatism, diarrhoea and malaria and as emetics.	antimalarial, antifungal, anticancer.
Plumbago ciliata Engl.		Tanzania			
Plumbago caerulea Kunth	chileno	Chile	Shrub, with erect stem and blue flowers.	Ornamental value.	Roots yield plumbagin.
Plumbago dawei Rolfe		Ethiopia, Kenya, Uganda	Perennial herb with erect stems and white flowers.	Used to treat diarrhoea, stomach ache..	Antimicrobial, antimalarial
Plumbago europaea L.	Common leadwort/ European leadwort	Europe, Turkey	Herbaceous half shrubby, multi=branched plant with trusses of pink or purple flowers.	Used for treatment of blisters, inflammation, itching, toothache, skin disorders.	Antimicrobial, anticancer, antifungal, antimutagenic and insecticidal.
Plumbago glandulicaulis Wilmot-Dear		Kenya, Tanzania	Herb, erect hairy and sticky stem with leaves glaborous and white flowers.	Ornamental value.	
Plumbago indica L. syn.	Scarlet leadwort	Asia Africa,	Perennial herbaceous	Used to treat gastric acidity,	Anticancer, anti-inflammatory,

rosea L.		India, Indonesia ,Phillippines	plants with erect half woody stems and red flowers.	skin disease, constipation, , haemorrhoids, rheumatoid arthritis, paralysis, also used in horses to expel worms, also abortifacient.	antiatherogenic.
Plumbago madagascariensis M. Peltier		Madagascar	Shrub, erect stem and variations of blue, white and purple flowers.		
Plumbago montis-elgonis Bullock		Ethiopia, Kenya, Tanzania	Stout short perennial herb with pink flowers.		
Plumbago pearsonii (L.) Bolus		Namibia	Erect shrub, slightly branched with pink and purple-violet flowers.		Roots yield plumbagin.
Plumbago pulchella Boiss.	Cola de iguana	Mexico	White flowers.	Vesicant and caustic effect, used for body aches and pains, skin disorders, used as veterinary medicine..	Anticancer, antiulcer.
Plumbago stenophylla		Kenya, Tanzania	Small erect shrub or woody	Skin disorders and orally for	Antimalarial

Wilmot-Dear			herb, white flowers.	hookworms.	
Plumbago scandens L.	Louco; caataia; caapomonga	Brazil	Subshrub with white flowers.	Leaves used as nape compresses in mentally ill patients also used as local anastetic. Soothes toothaches and earaches. Reduces joint in flammation. -.	Antimicrobial, anti-cancer and antimalarial treatments.
Plumbago tristis Aiton	South Africa	Dark-flowered leadwort	Perennial shrub, dark-pink fowers at the end of pink hairy stems.		Roots yield plumbagin.
Plumbago wissii Friedr.	Brandberg *Plumbago*	Namibia	Multi-stemmed shrub, with violet to maroon purple flowers.	Ornamental value, used as a cure for lead disease or lead poisoning.	
Plumbago zeylanica L.	Ceylon leadwort, White leadwort	Africa, Asia, Australia, Ethopia, India, China	Herbaceous shruby plants with climbing or erect stems, petiolate leaves and white flowers.	Used to treat anemia, bronchitis, rheumatism, skin disorders, internal and external trauma, toxic swelling, and ulcers.	Antibacterial, antimalarial, antiplasmodial, anti-inflammatory, antiatherosclerotic, antidiabetic, hypolipidaemic, antifungal.

Blank spaces= no available information.

Classification of *Plumbago auriculata* (Foden and Potter, 2015).

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Caryophyllales

Genus: *Plumbago*

Species: *Plumbago auriculata* Lam.

Common names: Cape *Plumbago,* Cape Leadwort, Blue *Plumbago.*

Morphology

Plumbago auriculata is a perennial, bushy evergreen shrub (Figure 2a), up to 3m high with erect, climbing or trailing stems that are glabrous below becoming pubescent above (Batten, 1986). The leaves are simple, elliptic to obovate, slightly discolorous, greyish green beneath and often with whitish scales seemingly for light reflection (Aubrey, 2001). Leaves are thin in texture (Figure 2b), having miniscule glandular dots, with a winged petiole at the base and auriculate. The glands found on the both surface of the leaves are reported to be salt glands (Wilson, 1890; Sakai, 1974; Faraday and Thomson, 1986a). *P. auriculata* is covered with trusses of pale, sky blue flowers, however, there are variations of deep blue or white flowers (Figure 2b) (Batten, 1986; Ferrero et al., 2009). Flowers are salver-shaped (Figure 2d), actinomorphic and grouped in terminal inflorescences 2.5-3 cm long and flowering all year round (Luteyn, 1990; De Laet et al., 1995; Aubrey, 2001; Ferrero et al., 2009). Corolla is pale blue with the tube twice or more than twice the length of the calyx. Glandular and non-glandular hairs, often known as trichomes, are found on the calyx (Figure 2c). Stamens are free from the corolla, included or exserted and style exserted with 5 linear stigmas and superior 1-celled ovary. Fruit is a capsule, long-beaked, the valves coherent at the apex. The seed one, dark brown or black, oblong 7mm long and slightly flattened (Luteyn, 1990; Aubrey, 2001).

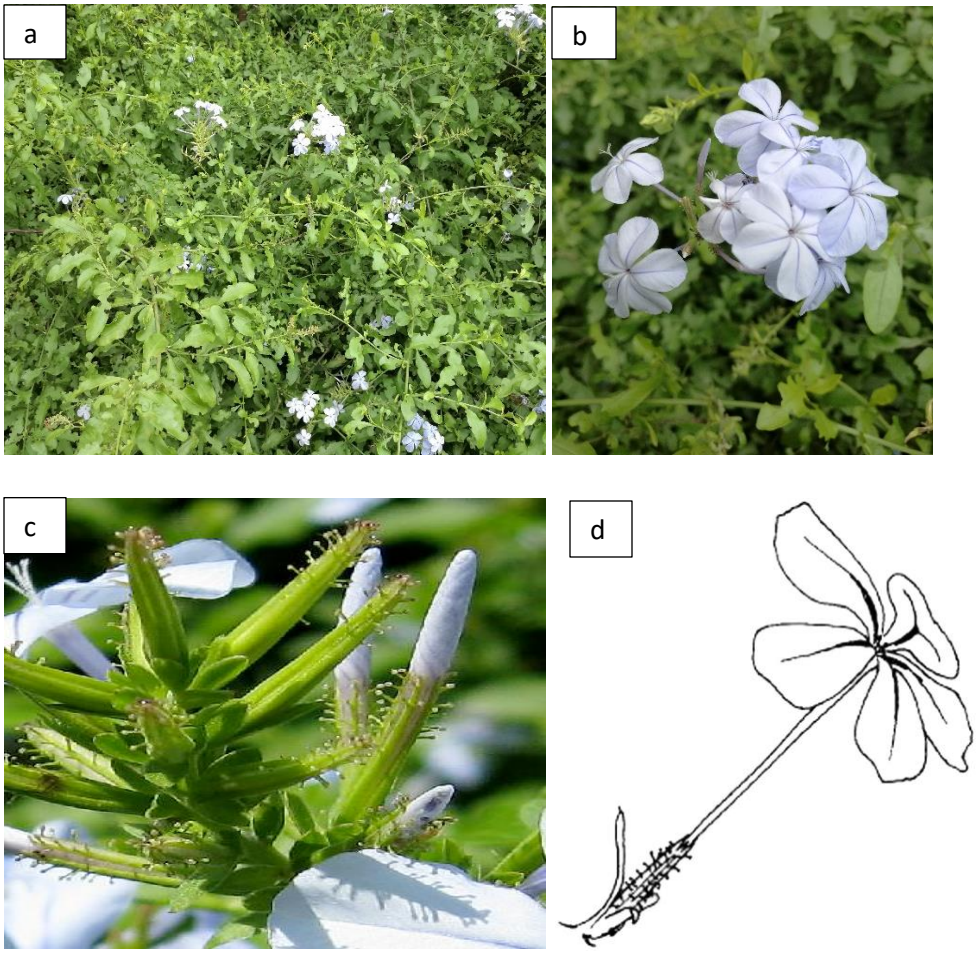


Figure 2: *Plumbago auriculata*: a) Bushy evergreen shrub image captured at the University of Kwa-Zulu Natal, Westville, Durban, South Africa; b) Variation of pale-blue flower and leaves (UKZN); c) Calyx showing trichomes; d) Illustration of flower showing calyx with trichomes (After Batten, 1986; ICPS, 1986).

Secretory Structures

Secretory structures are specialized plant structures associated with the release of substances produced in the cytoplasm and moved outside the cell (Cutter, 1978). Secretory structures appear in the form of trichomes, resin ducts, salt glands, idioblasts, laticifiers, colleters and nectaries located in various parts of the plant (Thomas, 1991). It has been reported that the primary function of secretory structures is related to defense responses against both herbivores and pathogens. (Fahn, 1988; Lange, 2015). These structures have the ability to produce or sequester secondary (or specialized) metabolites. Literature reports that salt glands occur sunken in the

epidermis of leaves and are associated with ion secretion whereas trichoms protrude from the surface of leaves or stems and are often responsible for the secretion of exudates (Cutter, 1978, Fahn, 1988; Naidoo and Naidoo, 1998). Although some researchers report salt glands to be specialized trichomes, based on available literature for the purpose of this review salt glands and trichomes are classified independently as secretory structures in Plumbaginaceae (Metcalfe and Chalk, 1972; Faraday and Thomson.1986b, Grigore and Toma, 2016).

Salt glands

A major constraint to plant growth is salinity, which causes plants to adapt to saline environments via specialized epidermal structures known as salt glands (Kobayashi, 2008). Salt glands are highly specialized organs consisting of several cells intended to excrete salt or shift ions from mesophyll tissue to the leaf surfaces where a layer of salt crystals is formed (Figure 3b). This serves an important role in regulating salt concentration in plant tissue (Fahn, 1988; Kobayashi, 2008; Hasanuzzaman et al., 2014). Salt glands are known to secrete a wide variety of ions, especially metal ions which contribute to metal tolerance in plants once these metal ions are eliminated (Fahn, 1988, Salama et al., 1999). The most commonly secreted ions via salt glands in flowering plants are sodium, potassium, calcium and magnesium (Kobayashi, 2008). These glands also secrete other salts in addition to sodium chloride that has a composition related to that of the root environment (Storey and Thomson, 1994). Salt glands occur in halophytic species, which include several families that are not taxonomically related, thus, providing a clear example of convergent evolution on a common adaptive device (Fahn, 1988; Naidoo and Naidoo, 1998). Salt glands occur on any aerial organ of the plant but are most abundant on leaves and are inserted in the epidermis (Fahn, 1988).

Salt glands function to eliminate salts to the outside of the plant or within the plant into vacuoles (Thomson, 1975). Structurally there are three types of salt glands described (Thomson, 1975); the bicellular glands of the grasses, the bladder cells of the Oxalidaceae, Chenopodiaceae and Mesembryanthemaceae and the multicellular gland found only in dicotyledons. Regardless of the fact that the structure of salt glands varies greatly among different species, it is, very similar in plants within the same family or genus (Salama et al., 1999). Salt glands of the family

Plumbaginaceae are multicellular and sunken in the leaf epidermal cells, consisting of basal and secretory cells with the cells varying in number from six up to forty (Thomson, 1975, Faraday and Thomson, 1986b). Glands were first described in the 1800's and were referred to as chalk glands due to the presence of insoluble carbonate salts found above the gland on the surfaces of stems and leaves (Grigore and Toma, 2016) . However, when it was later discovered that the glands of some species secrete sodium chloride (NACl) these glands were then referred to as salt glands (Faraday and Thomson, 1986a; Grigore and Toma, 2016). The salt glands (Mettenius glands or Licopoli glands), as described by Grigore and Toma (2016), based on historical facts by Metcalfe and Chalk (1972), occur inside the cavities on the inner side of stems and leaves, sometimes surrounded by simple hairs or groups of elongated cells. The glands are made up of 4 to 8 epidermal cells arranged in palisade surrounded by accessory cells made up of one or two layers (Figure 3a). Cutinized walls exist between the secreting cells and the accessory cells. The salt-secreting glands of the species within Plumbaginaceae are similar in ultrastructure and morphology and the primary pathways and basic mechanisms of salt movement through the glands is the same in all species of the family (Figure 3c) (Faraday and Thomson, 1986a). These glands are able to secrete a wide variety of ions and the secretions are similar in species of the family (Storey and Thomson, 1994). Salt glands are common in the family Plumbaginaceae and sometimes these glands secrete mucilage in addition to calcium carbonate and sodium chloride (Fahn, 1988; Grigore and Toma, 2016). Salt glands of *P. auriculata* are found on the epidermal axials of the leaves and are exo-recreto (excrete salt that form crystals on leaf surfaces) (Ceccoli et al., 2015).

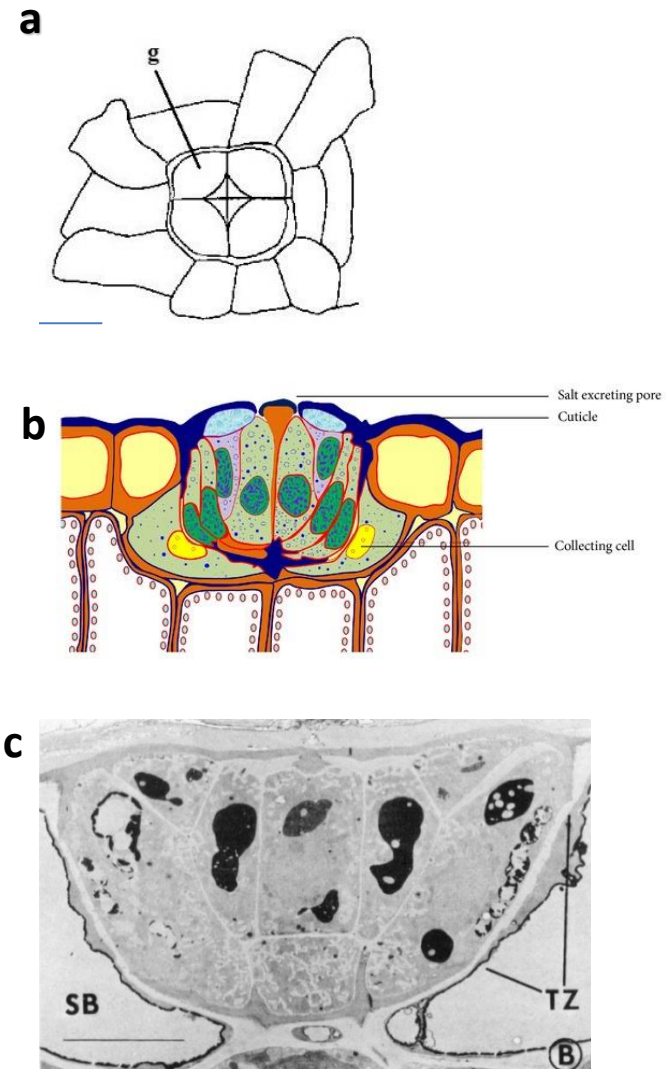


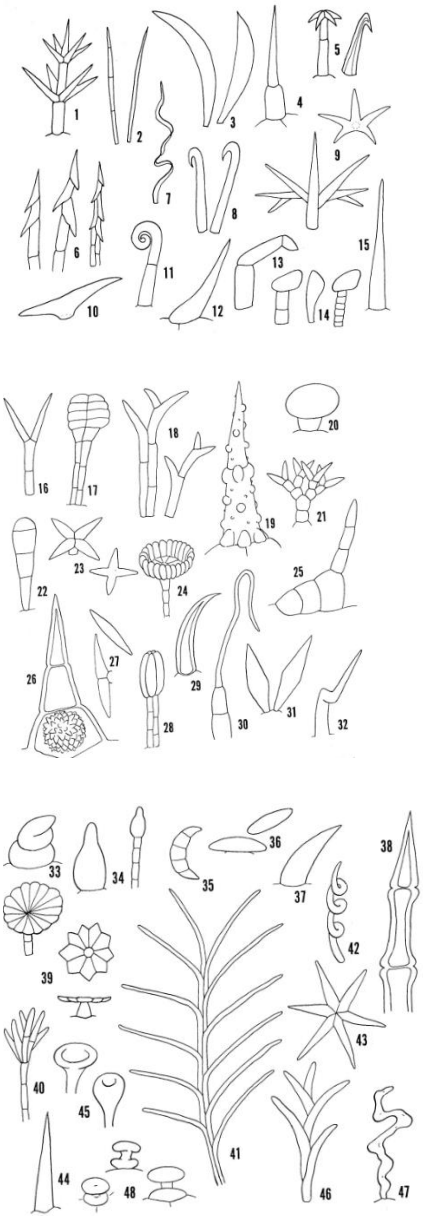
Figure 3: Structure of salt glands in various species: a) Salt glands (g) in the leaf of *Plumbago europaea*; Scale bar 100 µm (After Grigore and Toma, 2016); b)) Cross section of a generalized salt gland (After Hasanuzzaman et al., 2014); c) Electron micrograph showing the ultrastructure of an almost median longitudinal section from a secreting *Plumbago auriculata* salt gland (After Faraday and Thomson, 1986a); TZ-Transfusion zone; SB- subbasal cell; Scale bar 80 µm.

Trichomes

Trichomes are often referred to as epidermal appendages that can either be singular or multicellular, developing outwards on the surface of plant organs (Payne, 1978; Fahn, 1988). Their morphology varies greatly among tissue and species and according to botanical literature there are over 300 descriptions to characterize various morphological types, outlining a few in (Figure 4) (Payne, 1978; Wagner, 1991). Trichomes occur in all major groups of terrestrial plants and are needed to carry out the following functions: light reflectance; reduction of waterloss through transpiration; thermoregulation; herbivory; defence against radiation, pathogen attack. However, the role of trichomes can be considered species specific (Wagner, 1991; Bauer et al., 2015). As outlined by Payne (1978), trichomes can be either glandular or non-glandular and are often found occurring on different parts of the plant. Glandular trichomes are the primary secretory structures in most flowering plants and distributed over the vegetative aerial part of plants (Kaya et al., 2006). They vary in their structure, function and location as well as chemical compostion in the substances they secrete (Payne, 1978; Rusydi et al., 2013). Morphologically, glandular trichomes are classified as either peltate or capitate and generally consist of a foot, a stalk that is usually unicellular or bicellular and a secretory head (Payne, 1978; Ascensao et al., 1997; Salmaki et al., 2009). Capitate trichomes vary widely in stalk length, head shape and secretion type but a general rule is that the stalk length measures more than half the height of the head whereas peltate trichomes are usually made up of a short wide stalk, several head cells and one basal epidermal cell (Ascensao and Pais, 1998; Kaya et al., 2006). Capitate trichomes secrete a small amount of essential oils and some polysaccharides and these exudates are mostly excreted to the surrounding environment via pores in the cuticle of the head cell (Kamatou et al., 2007). The peltate trichomes are most important for essential oil production because most of the secretory cells are in the head and the exudates are stored in the subcuticular spaces between the head cell walls and the cuticle. They function as storage structures for the secreted exudates (Salmaki et al., 2009).

Non-glandular trichomes are also found occurring on the aerial vegetative parts of the plant as well as within plant tissue and can be described as follows (Fahn, 1988; Maclachlan and Carlquist, 1992): a) simple unicellular or multicellular, non-flattened hairs; b) squamiform hairs which are conspicuously flattened, multicellular hairs. These trichomes are termed ‘scales’ if

they are sessile or peltate hairs if they are stalked; c) branched, multicellular hairs which may be stellate; d) shaggy hairs which consist of a base and two or more contiguous rows of cells. The function and location of non-glandular trichomes are species-specific. Non- glandular trichomes of some *Kalanchoe* spp demonstrated how diverse these type of trichomes are in terms of length, number of cells in the stalk, shape of the upper part, density of occurrence , occurence of wax on their surface and cuticle ornamentation (Weryszko-Chmielewska and Chernetskyy, 2005). Trichome viability also acts as a determinant for the function of the trichome i.e. dead non-glandular trchomes found on the surface of *Kalanchoe* leaves provide a protective structure to the leaves enabling surface moisture. The function of trichomes is dependent on the species as well as the environment.



1. Abietiform; candelabra; orthocladous.
2. Acerate; acicular.
3. Acinaciform.
4. Acuminate terminal cell; belemonoid.
5. Anchor hairs, uncinate and with terminal fluke cells; barbed.
6. Ancistrous; anchor; barbed; glochids.
7. Aduncate; anfractuose; curly; ribbon-hair; serpentine.
8. Angler; hamate; hooked; uncinate.
9. Antler; pedate (sometimes termed stellate).
10. Anvil; malpighiaceous.
11. Apicircinatus; circinate.
12. Arrect.
13. Arthrodactylous.
14. Asciiform; hatchet-shaped.
15. Attenuate.
16. Bifid; dichotomous; forked; furcate.
17. Biseriate; colleter; gland.
18. Bootjack.
19. Bosselated; nodose; postulate.
20. Brevicollate.
21. Brevifurcate.
22. Clavate.
23. Cruciate; stellate.
24. Cup-shaped.
25. Cushion hair.
26. Cystolith hair (with cystolith in basal cell)
27. Dolabrate.
28. Doliform head of colleter.
29. Falcate.
30. Flagelliform; whip-like.
31. Fusiform.
32. Geniculate.
33. Heliciform; snail-shaped.
34. Lageniform; pulvinate.
35. Lunate; solenoid.
36. Limaciform.
37. Ornithorhynchous.
38. Osteolate cells of uniseriate hair.
39. Peltate.
40. Penicillate.
41. Plumose.
42. Spiral.
43. Stellate.
44. Subulate.
45. Surculate.
46. Sympodial.
47. Torulose

Figure 4: Trichome types (After Payne, 1978).

The calyx of *P. auriculata* bear large mucilage secreting trichomes (Figure 5a), which resemble those of the genus *Drosera* (Droseraceae) and *Drosophyllum* (Droserophyllaceae) that also occur in the order Caryophyllales (Stoltzfus et al., 2002; Madhavam et al., 2009; Panicker and Haridasan, 2016). The trichomes on the calyx can also be termed "colleters" meaning it secretes a sticky substance and usually consist of a multicellular stalk and head. However due to its close resemblance to *Drosera* the trichomes can also be considered as digestive glands due to their function in insect entrapment (Figure 5b) (Fahn, 1952). Naidoo and Heneidak (2013), studied the glandular hairs of *Drosera capensis* L. and reported that the leaves are characterized by eight types of glandular hairs with a red-coloured stalk and stalk head making it more attractive to insects These trichomes function to capture and entrap insects, absorb nutrients, produce mucilage and digestive enzymes, as well as secrete proteases in response to stimulation by certain salts (Stoltzfus et al., 2002; Naidoo and Heneidak, 2013; Bauer et al., 2015). Trichomes of Droseraceae were also found to exhibit phosphatase activity in the external glands (Plachno et al., 2006). Studies by Rashmilevitz and Joel (1976), mentioned in Stoltzfus et al. *(*2002), that trichomes of *Plumbago* have a resinous secretion and showed a positive response when stained with Sudan IV, therefore proving it has a substantial hydrophobic constituent. It was reported that trichomes of the genus *Plumbago* exclude crawling insects from the flowers, therefore favouring flying insects for cross pollination (Jose et al., 2014). Trichomes of *P. auriculata* produce a sticky transparent exudate which traps winged insects which resembles a cobweb. The exudate is known to turn brown upon maturity of the plant in *Plumbago* species; however nothing has been reported with regards to *P. auriculata* (Panicker and Haridasan, 2016). Non-glandular trichomes are also found on the calyx of *P. auriculata* (Figure 5a), but the function of the trichomes is unknown in this species. However, it has been reported in other species that these trichome types provide shade to the plant and serve as a mechanical barrier to prevent insects from piercing the leaf (Gonzales et al., 2008).

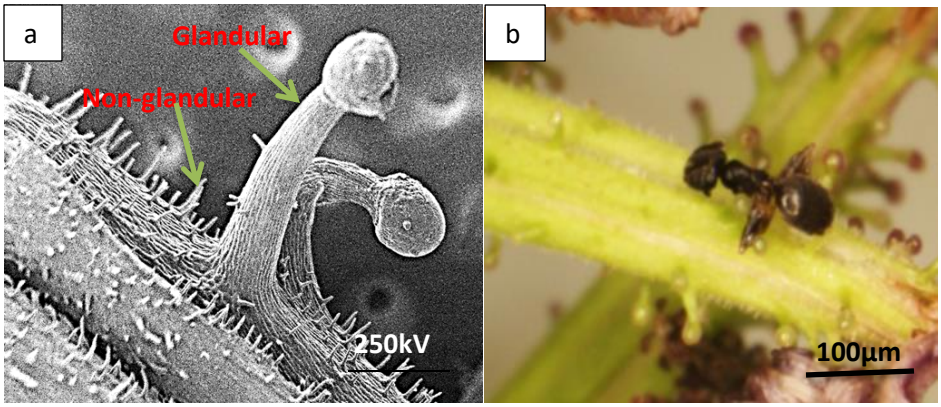


Figure 5: Types of trichomes on the calyx of *Plumbago auriculata:* a) Scanning electron (SEM) micrograph of glandular trichomes and non-glandular trichomes; b) Stereomicrograph of an ant captured on calyx.

Chemical Composition

Plants are of great importance to humans for use as ornaments, food preparations and medicines. The use of herbal medicines has always been in great demand (Balunas and Kinghorn, 2005). There is an intense interest in medicinal plants in which phytochemical constituents can have long-term health promoting properties (Kennedy and Wigthman, 2011). Plants naturally produce secondary metabolites, commonly referred to as phytochemicals or biologically active compounds that can contribute to a holistic healing approach (Kennedy and Wigthman, 2011). These include alkaloids, saponins, tannins, glycosides, flavonoids, terpenoids, naphthaquinones, carbohydrates, proteins, phenolic compounds, fixed oils and fats (Katsoulis et al., 2000; Pourmorad et al., 2006). Alkaloids and phenolic compounds are often the two major phytochemical compounds that are of medicinal importance. A wide range of medicinal properties of *P. auriculata* are attributed to plumbagin and other secondary metabolites.

Phytochemical Screening

Methanolic leaf extract of *P. auriculata* revealed the presence of the following phytochemicals-tannins, flavonoids, phenols, alkaloids, saponins, proteins, and carbohydrates with phenols being the most abundant compound (Lakshmanan et al., 2016). Aerial parts of *P. auriculata* extracted

in the following solvents: acetone, chloroform, petroleum ether, ethanol and ethyl acetate showed positive results when tested for steroids, carbohydrates, phenolics, tannins, saponins, flavonoids and terpenoids, however the aqueous extract only showed positive for the presence of tannins (Tharmaraj and Antonysamy, 2013). It can be concluded that aqueous extractions are not as effective as other solvents for phytochemical screening.

Bioactive Compounds

Systematic fractionation and phtochemical examination of the methanolic root extract of *P. auriculata* revealed the presence of the following compounds: α-amyrin, capensisone, α-amyrin acetate, isoshinanolene, β-sitosterol, diomuscinone, plumbagin and β-sitosterol-3β-glucopyranoside (Table 2) (Padhye et al., 2010; Ariyanathan et al., 2011; Saeidnia et al., 2014; Khan and Hossain, 2015). Capensisone is a novel quinone that was first to be characterized from the roots of *P. capensis* and, in their study, isoshinanolone and diomuscinone were reported for the first time for the genus *Plumbago* (Ariyanathan et al., 2011).

Table 2: Bioactive compounds reported in plant extracts of *Plumbago auriculata*

Compounds	**Medicinal/ pharmacological uses**
Plumbagin	Anticancer, antifungal, anti-inflammatory, antibacterial, antifertility, antimalarial, antidiabetic and antioxidant properties.
α-amyrin and α-amyrin acetate	
capensisone	
isoshinanolene	Antibacterial and antifungal properties.
β-sitosterol (Beta-sitosterol)	Heart disease and high cholesterol, gallstones, common cold and flu, rheumatoid arthritis, asthma, migraine headaches, menopause symptoms, enlarged prostrate, chronic fatigue syndrome, cervical cancer. It also boosts the immune system and prevents colon cancer as well as enhances sexual activity.
β-sitosterol-glucoside	Antioxidant, hypertension and antidiabetic properties (used to control menstrual bleeding.
diomuscinone	Wound healing, antimicrobial and anti-inflammatory properties.

Plumbagin

In the genus *Plumbago*, a highly potent and broad spectrum biological compound known as plumbagin is commonly found (Tyagi and Menghani, 2014). Plumbagin is a 5-hydroxy-2-methyl-1, 4-naphthoquinone-($C_{11}H_8O_3$) (Figure 6) present in the roots, stems and leaves of various *Plumbago* species; with the roots predominantly having the highest concentration of plumbagin (Padhye et al., 2010). Plumbagin is naturally a yellow pigment that was first isolated in 1829 via solvent extraction of powdered plant material and has since been of interest for research due to its high medicinal value (Tyagi and Menghani, 2014). Plumbagin is soluble in a variety of solvents such as acetone, benzene, alcohol, methanol, acetic acid and chloroform.

Plumbagin exhibits anticancer, antifungal, anti-inflammatory, antibacterial, antifertility, antimalarial, antidiabetic and antioxidant properties (Mallavadhani et al., 2002; Checker et al., 2009; Padhye et al., 2010; Jose et al., 2014). It is also reported to have other therapeutic properties such as stimulant action on the intestine, nervous system and heart as well as rheumatic pain relief (Galal et al., 2012). All parts of *P. auriculata* contain plumbagin with the highest amount of plumbagin accumulating in the leaves and stems of the plant in comparison to *P. zeylanica* and *P. rosea* (Jose et al., 2014). This shows that different species accumulate plumbagin in different parts of the plant.

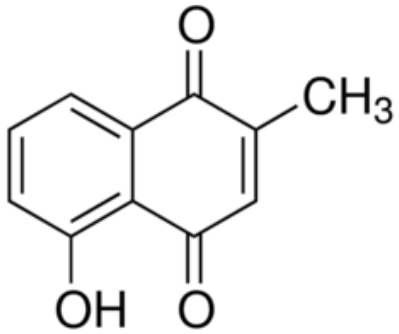


Figure 6: Structure of Plumbagin (After Padhye et al., 2010).

Mode of Action of Plumbagin

Anticancer activities

Plumbagin displays anticancer activities over a wide range of tumors such as breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, lung cancer, skin cancer, leukemia, liver cancer, renal cancer, cervical cancer. Studies have revealed that plumbagin is an effective inhihibitor of cell growth and when administered in combination with radiotherapy plumbagin has the ability to augment cell growth inhibition very effectively in comparison to a higher dose of radiation alone (Padhye et al., 2010). Extensive publications exist on the anticancer effect of plumbagin outlined in Padhye et al (2010).

Antifungal activities

The findings of the antifungal activity of plumbagin by Dzoyem et al (2007) suggested that the naphthoquinone delayed germination of the fungus and was capable of inhibiting growth when abministered at higher concentrations.

Anti-inflammatory

Plumbagin has been reported to be a topoisomerase-II inhibitor by displaying inhibitory activities of corresponding enzymes involved in rheumatoid arthritis (RA) (Jackson et al., 2008). Checker et al (2009) reported that plumbagin suppresses NF-kB (ubiquitous transcription factor) activation in tumor cells, and hence might have an effect on biological functions of leukocytes actively participating in various immune responses.

Antibacterial activity

Extensive publications reported the antibacterial activity of plumbagin as outlined by Padye et al (2010). However, to summarize those findings, plumbagin displays potent antibacterial activity against a range of bacterial strains such as: *Staphylococcus aureus*, *Myobacterium smegmatis* and various *Myobacterium tuberculosis* strains and *Escherichia coli*. However, plumbagin does display cytotoxic activity and needs to be administered at the appropriate concentration.

Antifertility activity

Plumbagin has been reported to be an irritant to the smooth muscle of the uterus when administered orally at high concentrations. Therefore it needs to be administered with caution. Madhavan et al (2009) reported that antifertility effects were found to be effective with doses of 1 mg/100 gm of body weight in female rats. Plumbagin also displays a significant effect on estrogen cycle and hormonal levels of female rats.

Antimalarial activity

Antimalarial activity of plumbagin was reported by Sumsakul et al (2014) by investigating in vitro antimalarial activity of plumbagin against K1 and 3D7 Plasmodium falciparum and in vivo

antimalarial activity in Plasmodium berghei-infected mouse model (a 4-day suppressive test). Their study concluded that plumbagin exhibited efficient antimalarial activity in both in vivo and in vitro experiments and displayed a fairly low toxicity at the dose levels up to 100 (single oral dose) and 25 (daily doses for 14 days) mg/kg body weight for acute and subacute toxicity.

Antidiabetic

It was reported that orally administered plumbagin significantly reduced the blood glucose levels and altered all other biochemical parameters to near normal. Further, it increased the activity of hexokinase and decreased the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase in diabetic rats. Plumbagin also enhanced GLUT4 mRNA and protein expression. It also contributed to glucose homeostasis (Sunil et al., 2012).

Antioxidant

Plumbagin contributes to EGFR activation in ROS-related mechanisms (Padhye ett al., 2010). It was also found to significantly reduce the catechol-induced DNA damage, and inhibit ascorbate and NADPH-dependent lipid peroxidation against mouse lymphoma cells (Demma et al., 2009).

Medicinal Uses and Pharmacology of Plant Extracts

Plumbago auriculata has a strong medicinal value and for many years has been used in the traditional medicinal market as an alternate remedy for the prevention and control of certain human ailments. All parts of the plant are reported to have therapeutic properties for example; the leaves and roots have cardiotoxic, neuroprotective, anti-atherogenic and hepatoprotective properties (Deshpande et al., 2014). Roots and leaves are also reported to be used for warts, as emetics, fractures, oedema, headaches, skin lesions, piles, rheumatism, diarrhoea and malaria (Elgorashi et al., 2003; Chen and Gao, 2013). In Mozambique where malaria is endemic, aerial parts of *P. auriculata* are used for the treatment of symptoms associated with malaria such as vomiting and fever (Ramalhete et al., 2008). In Manchale, a rural village in India, it was reported that herbal healers use the root of the plant for the treatment of piles by forming a paste with water and applying directly (Poornima et al., 2012). Certain Indian tribes also make use of the

root extract by preparing fresh roots in rainwater and drinking for a week to combat acidity (Jain et al., 2010). Plants are not only used for the treatment and control of human ailments but it has been reported that plant extracts can also be used in ethnoveterinary as low-cost treatments for diseases of livestock and other animals (McGaw and Eloff, 2008). Root extracts of *P. auriculata* are used in as a treatment for diarrhaea in cows (Dold and Cocks, 2001). A fair amount of studies have been conducted to isolate and evaluate the pharmacological activities of the extracts of *P. auriculata.*

Antiulcer Activity

Different species of *Plumbago* plants were collected from different regions of Kerala, India. Roots of plants were dried and coarsely powdered, soaked in limewater and then extracted using ethanol as a solvent (Ittiyavirah and Paul, 2016). Goat intestines were used to test for antiulcer activity of the ethanolic root extracts by treating small pieces of the intestines (1.5-2 cm long) with 1ml *P.auriculata* extract. The results of that study reported that *P. auriculata* showed significant acid neutralizing capacities as well as excellent antioxidant properties observed from the DPPH assay. The use of goat intestine can be used as a standard test for in vitro antiulcer activities of plant extracts.

Antifungal Activity

Fresh plant material of *P. auriculata* such as roots, leaves, stems and flowers were extracted using distilled water as a solvent (Rajasekaran et al., 2015). Each extract was then mixed with a 1mM silver nitrate solution in a 1:10 ratio to form a silver nanoparticle. Only the root, flower and stem extracts were effective in the synthesis of silver nanoparticles and were further used in the application of the newly synthesized nanoparticles for antifungal activity. Antifungal activity was tested by spore germination assay on the following fungi: *Aspergillus fumigatus, Fucarium oxysporium, Aspergillus flavus, Curvularia lunata* and *Trichoderma* sp. Results of this study reported that the efficacy of the particle reflects on its size i.e. bigger paritcles were effective on larger spore such as *Curvularia lunata* and ineffective against smaller spore bearing fungi (Rajasekaran et al., 2015).

Antibacterial Activity

Roots of *P. auriculata* were dried and ground to a fine powder and, active components were extracted using the following solvents water, chloroform, methanol and ethanol (Muringani and Makwikwi, 2017). Antibacterial activity was tested using the kerbybaur disc diffusion method. Bacterial strains used were about 150 *E.coli* isolated from water and patient stool samples from the Mthata Region, Eastern Cape, South Africa. The results of that study reported that the ethanolic root extract showed the highest rate of activity against all the examined strains of the *E. coli* samples. Antibacterial activity of aerial parts of *Plumbag*o species has also been reported. Aerial parts of the plants were dried and extracted using various solvents and antibacterial assay was determined using the agar-well diffusion method (Tharmaraj and Antonysamy, 2015). The following bacterial strains used were *Streptococcus pyrogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Morganella morganii* and *Bacillus subtilis* and antibiotic amikacin (30 µg/ disc) was used as a standard to compare its effect on the bacterial strains with the plant extracts. The results of the study for *P.auriculata* reported that the ethanolic extract showed the highest activity (70%) and the highest zone of inhibition (23 ± 0.3 mm), followed by petroleum ether and chloroform extracts. The ethanolic extract of *P. auriculata* showed above 50% activity against Gram positive bacteria and 26% against Gram negative bacteria. The overall results of the study showed that *P. zeylanica* and *P. rosea* had the highest activity against Gram positive bacteria whilst *P. auriculata* had the least activity and the antibacterial potentials of *Plumbago* species are probably due to the presence of alkaloids and phenolic compounds (Tharmaraj and Antonysamy, 2015).

Plasmid-mediated multiple drug resistance is a serious and emerging problem in the treatment of infectious diseases because bacteria have become resistant to most of the drugs available. Scientists have developed a way to combat this problem by combining herbal extracts with antibiotics to inhibit the development and spread of R-plasmids (Patwardhan et al., 2015). *P. auriculata* root extracts were used to cure plastid-mediated antibiotic resistance, The plasmid curing activity of the root extract was determined by growing *Escherichia coli, Proteus vulgaris* and *Klebsiella pneumonia*, in the root extract. Ethanol root extract demonstrated the highest antibacterial activity as well as the maximum plastid curing activity (13-32%) compared to

chloroform, acetone or petroleum ether root extracts. *P. auriculata* root extracts can be used as plasmid curing agents in the treatment of nosocomial infections.

Cytotoxic activity

Gastric ulcer is one of the most common gastrointestinal disorders in recent times (Paul et al., 2013). Anti-*Helicobacter* and cytotoxic activity of detoxified ethanol root extracts of *P. auriculata* were assessed by preparing the roots in lime water and extracting them using ethanol as a solvent (Paul et al., 2013). Cell viability was assessed by Microculture tetrazolium (MTT) Assay in the presence and absence of different plant extracts. The study reported that the ethanol root extract has possible activity against *H.pylori*, cytotoxicity with MTT assay HGE-17 cell lines and the zone of inhibition test of *P. auriculata* ethanol root extracts showed significant activity. Paul et al. (2013), also revealed in their study that *Plumbago* species show cytotoxic activity, even in the absence of bioactive plumbagin.

Methanolic leaf extracts also showed significant cytotoxic activity when assayed against human lung cancer (A549) and ovarian cancer (PAI) cell lines using MTT assay (Lakshmanan et al., 2016). For A549, it showed a minimum cytotoxic activity at 45µg/ml and 10µg/ml cell line and for PAI 10µg/ml and 60µg/ml cell line at 24 hours and 48 hours, respectively.

Anticancer Activity

Cancer is a common, most dreadful life threatening disease reported worldwide and is often difficult to control and cure (Balunas and Kinghorn, 2005). Bioactive compounds extracted from plants are being extensively researched and utilized as a possible anticancer agent; in vitro *P. auriculata* leaves were dried and extracted using methanol as a solvent (Lakshmanan *et al.* 2016). The leaf extract was tested against ovarian (PAI), lung (A549) and malignancy cell lines for apoptotic and anti-proliferative activities. The GC-MC analysis of the methanolic extract revealed the presence of the compound sitosterol, hence showing good anticancer activity at minimal concentration 10-40µg. The study was successful in reporting that *P. auriculata* constitutes novel anticancer compounds.

Toxicity

Bioactive compounds found in plants often have many therapeutic uses and are of high medicinal value. Hoiwever, these compounds can exhibit toxic effects which can result in detrimental side-effects. Plumbagin, often referred to as the marker compound in the genus *Plumbago* is also toxic, as it generates superoxide anion reactive oxygen species that can damage various biomolecules (Jose et al., 2014). It is a powerful irritant in low doses that inhibits cell mitosis and, in higher than recommended doses, it can cause death from respiratory failure, paralysis as well as nucleotoxic and cytotoxic effects. Other commonly known side effects of plumbagin include skin rashes, diarrhoea, increase in neutrophil counts and white blood cells, hepatic toxicity and increase in acid phosphatase and serum phosphatase levels (Padhye et al., 2010). *P. auriculata* is assigned to toxicity class 4 meaning the juice sap or thorns of the plant can have adverse effects on the skin, resulting in dermatitis (Stone and King, 1997). It has been reported that traditional tribes in India that make use of roasted *P. auriculata* roots for wound healing use very small quantitites and for a limited time period because prolonged exposure and large quanties can lead to death by irritation (Jain et al., 2010).

Propagation Techniques

Plumbago auriculata makes a good ornamental plant that can be planted indoors or outdoors. Plant management is quite simple because of tolerance to high humidity, high temperatures and diseases and even though the plant secretes salt it is not limited to saline conditions (Joy et al., 1998; Aubrey, 2001). The common propagation method of this species is seed sowing. However, seed germination rate is very low and seed prices are often too expensive (Batten, 1986; Chen and Gao, 2013). Due to its high medicinal value, the conventional method of propagating *P. auriculata* is often difficult and cannot meet the growing demand for the plant in the traditional medicine and pharmaceutical markets. The plant displays poor germination and death of young seedlings under natural conditions. Therefore, researchers have developed plant tissue-culture as a method to produce seedlings which have certain advantages over conventional propagation methods. Chen and Gao (2013.), micropropagated 1 year old plants of *P.auriculata* using the nodals and leaves. They concluded that young nodals are best to use as explants because leaves

have a high differentiation grade and the ability of regeneration is weak. Deshpande et al. (2014), reported that *P.auriculata* increased callus in vitro mass production by using various growth hormones such as indoleacetic acid (IAA), 1-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA). IAA is a simple compound (auxin) that assists in plant growth and development (Davies, 2004). IBA is most commonly used commercially for plant propagation due to its efficacy to stimulate adventitious root growth and is often more stable than IAA against in vivo catabolism (Davies, 2004). NAA is a synthetic plant hormone widely used in agriculture, horticulture and plant tissue culture to increase cellulose fiber formation (Davies, 2004). However, it is toxic to the plant in high concentrations. Growth hormone NAA exhibited higher production of callus formation in all the explants; combination of hormones IBA and NAA showed best callusing from leaf explants. Combinations of BAP and NAA showed best callusing from stem and shoot apex explants. BAP are synthetic cytokinins used in tissue culture to promote cell division, bud formation and stem branching (Zhang et al., 2005). It has been reported that this species can also be propagated vegetatively by placing 15cm long stem cuttings in polybags and applying IAA and IBA treatments to improve rooting (Joy et al., 1998; Lakshmanan et al., 2016).

In related studies plumbagin production was enhanced using root cultures of *Plumbago indica* L. through precursor feeding using l-alanine followed by in situ adsorption of plumbagin on the nonpolar copolymer adsorbent, styrene–divinylbenzene resin (Diaion® HP-20) (Jaisi and Panichayupakaranant, 2017) Roots were fed with L-alanine (concentration 5 mM) for 14 days followed by the sequential addition of Diaion® HP-20 (10 g L−1) after 36 hours of L-alanine-feeding. Plumbagin production was significantly increased. Productivity levels obtainted were higher than that achieved using untreated root cultures or L-alanine feeding alone. Jaisi and Panichayupakaranant (2017) concluded that their study suggests the use of precursor feeding in combination with in situ adsorption as an easy and cost effective tool for the commercial production of medicinally important bioactive compounds like plumbagin. Overall tissue culture is a beneficial propagation technique for increasing plumbagin production in *Plumbago* spp as well as the production of other medicinal plant compounds.

Conclusion

The genus *Plumbago* yields many medicinally important species throughout the world. There is a lack in knowledge on some species within the genus thus providing an opportunity for future research. This review clearly shows the importance of *Plumbago auriculata* as a useful medicinal plant and also its level of toxicity. This species is used throughout the world; however limited research exists in South Africa where it is native. While the morphology of trichomes has been studied, the detailed trapping mechanisms of insects are still unknown. The structure and function of secretory structures occurring in this species are not well documented. Although many pharmacological studies have been conducted, studies on the secretory structures are limited to only a small number of publications dealing with secretory structures, especially the types of trichomes present and ultrastructure of the leaf glands. However, the nature of the secretions from these structures is unknown. Micropropagation results viewed in this study are aimed at encouraging and attracting researchers to promote rapid regeneration of *P. auriculata* to ensure easy availability of the plant for horticulture, medicinal and pharmacological uses. The use of plant hormones in tissue culture techniques proved to be effective in optimizing the production of medicinal plants, thus increasing the availability of bioactive compounds. Other techniques such as genetic engineering have not yet been explored for the genus and could prove useful in commercializing this medicinally important genus.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 3

Journal Article for Publication 2:

Micromorphology, and histochemistry of the secretory apparatus of *Plumbago auriculata* Lam.

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Abstract

This study interprets the structure and histochemical analysis of the leaves and calyces of *Plumbago auriculata* using light and electron microscopy and various histochemical tests. Glandular capitate and non-glandular uniserate trichomes were present on the surface of the calyx. Glandular trichomes bear a close resemblance to the digestive glands of the genus *Drosera*. Salt glands present on the leaf surface were abundant on the abaxial surface and revealed the presence of "transfer cells" reported for the first time in the genus. Abundant mitochondria, mini vacuoles, ribosomes, dictyosomes and rough endoplasmic reticulum cisternae were actively involved in the secretory process. Histochemical staining also revealed the presence of alkaloids and phenolic compounds that are of medicinal importance used to treat multiple ailments and also function as chemical deterrents in plants. Overall, this study contributes to the existing body of knowledge on the secretory structures of *P. auriculata* and provides new insights on the ultrastructural and histochemical aspects of the species.

Keywords: Salt glands, *Plumbago auriculata*, colleters, transfer cells, secretory structures.

Introduction

Worldwide, people are experiencing more health issues than ever before due to our changing lifestyle and environmental impacts. Plants have proved to be significant natural resources for effective therapeutic, agricultural and cosmetic agents, offering a broad spectrum of activity, minimal side effects and fewer cases of pathogen resistance (Joy et al., 1998; Balunas and Kinghorn, 2005). Plants contain specialized structures that produce, secrete and store secondary metabolites (Wagner, 1991). These metabolites are often referred to as, phytochemicals or bioactive compounds which function as precursors for the development of natural, environmentally friendly and low toxicity pharmaceuticals, flavourants, cosmetics and fragrances (Ascensao et al., 1997). Bioactive compounds are either produced, excreted or stored in small amounts in specialized cells, termed secretory structures in the form of trichomes, salt glands, idioblasts, resin ducts, laticifiers, stinging trichomes, colleters and nectaries located in various vegetative and reproductive organs of a plant (Thomas, 1991).

Plumbago auriculata is a perennial in the family Plumbaginaceae (Fig. 1) (Batten, 1986; Simpson, 2010). It is one of 18 species in the genus endemic to South Africa (The Plant List, 2017). However, it is distributed in other warm tropical regions of the world (Kubitzki, 1993; Foden and Potter, 2015). Considered as an ornamental and medicinal plant, morphologically, *P. auriculata* is described as a bushy evergreen shrub with trusses of pale, sky blue flowers. However, there are variations of white and deep blue flowers (Batten, 1986; Aubrey, 2001; Ferrero et al., 2010).The leaves are simple, thin textured and appear to be greyish green beneath and often have whitish scales apparently for light reflection (Aubrey, 2001). Leaves have miniscule glandular dots, with a winged petiole at the base and auriculate. The secretory structures found on both surfaces of the leaves are reported to be salt glands (Wilson, 1890; Sakai, 1974).

Figure 1: *Plumbago auriculata.* Image captured at the University of Kwa-Zulu Natal, Westville, Durban, South Africa.

Salt glands are highly specialized organs consisting of several cells intended to excrete salt or shift ions from mesophyll tissue to the leaf surfaces where a layer of salt crystals is formed. This serves an important role in regulating salt concentration in plant tissue (Fahn, 1988; Kobayashi, 2008; Hasanuzzaman et al., 2014). Salt glands are known to secrete a wide variety of ions, especially ions which contribute to metal tolerance in plants once these ions are eliminated (Fahn, 1988; Salama et al., 1999). The most commonly secreted ions via salt glands in flowering plants are sodium, potassium, calcium and magnesium (Kobayashi, 2008). Salt glands occur on any aerial organ of the plant, are most abundant on leaves and are embedded in the epidermis or raised from it (Fahn, 1988). The function of salt glands is to secrete salts to the outside of the plant or within the plant into vacuoles (Thomson, 1975; Amarasinghe and Watson, 1989). Salt glands of *P. auriculata* are found on the epidermal (abaxial/adaxial) surface of the leaves and are known to excrete salt that form crystals on leaf surfaces (Ceccoli et al., 2015). These glands are common to the family Plumbaginaceae (Grigore and Toma, 2016). In the 1800's these glands were initially referred to as chalk glands due to the secretion of insoluble carbonate salts (Metcalfe and Chalk, 1972; Grigore and Toma, 2016). However, when it was later discovered

that the glands of some species secrete sodium chloride (NaCl) these glands were then referred to as salt glands (Faraday and Thomson, 1986; Grigore and Toma, 2016). The term "salt glands and "chalk glands" tend to be interchangeable and to date no general distinctions have been drawn between the two terms (Dassanayake and Larkin, 2017). These glands are multicellular made up of 4 to 16 epidermal cells arranged in palisade with cells typically differentiated into collecting and secretory cells with a cuticular envelope covering the salt glands (Faraday and Thomson, 1986; Dassanayake and Larkin, 2017).

The calyx of *P. auriculata* bears large, mucilage secreting trichomes similar to those of the genus *Drosera* (Droseraceae) and *Drosophyllum* (Droserophyllaceae) (Stoltzfus et al., 2002; Panicker and Haridasan, 2016). The trichomes on the calyx can also be termed "colleters," due to the presence of a multicellular stalk and head. This type of trichome secretes a sticky exudate. These trichomes can also be termed digestive glands due to their function in insect entrapment which is similar to that of *Drosera* (Fahn, 1952; Stoltzfus et al., 2002; Naidoo and Heneidak, 2013; Bauer et al., 2015). Trichomes of *P. auriculata* produce a sticky, transparent exudate (which resembles a cobweb) and traps winged insects and are similar to that of *Plumbago zeylanica* L. (Panicker and Haridasan, 2016). Non-glandular trichomes are also found on the calyx of *P. auriculata* but the function of the trichomes is unknown in this species. However, previous studies have reported that these trichomes provide shade to the plant and serve as a mechanical barrier against insects (Gonzales et al., 2008).

The purpose of this study was to describe the micromorphology and fine-structure of the leaves and calyces. The nature of the secretions produced in the leaves and calyces was also investigated using histochemical assays to evaluate the composition of the compounds present and contribute to existing body of knowledge of the species.

Materials and Method

Plant Material

Fresh leaves and calyces of the *Plumbago auriculata* plant were collected at the University of Kwa-Zulu Natal in Durban, South Arica. A voucher specimen has been deposited in the Ward Herbarium, UKZN (Singh and Baijnath 1). Fresh samples were used for microscopy and

histochemical staining. Leaf stages were classified as emergent, young and mature.The calyx was classified as closed developmental stage (no flowers) and open developmental stage (flower present). Ten replicates for each developmental stage of the leaves and calyces were investigated microscopically.

Stereomicroscopy

Stereomicroscopy was used to study the distribution and morphology of calyx trichomes in open and closed developmental stages and the presence of salt crystals on the different leaf developmental stages of both the adaxial and abaxial surfaces. Samples were examined with the Nikon AZ100 stereomicroscope, Japan, equipped with Nikon Fibre Illuminator and images were captured using the Nikon DXM1200C colour camera. The images were taken using the NIS-Element Software.

Scanning Electron Microscopy (SEM)

For scanning electron microscopy, samples were prepared by chemical fixation according to Naidoo et al. (2013). Fresh leaf and calyx samples were trimmed into segments and fixed overnight in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) overnight at 4°C. The samples were washed three times (5 minutes per wash) with the 0.1M phosphate buffer. The samples were post-fixed in 0.5% osmium tetroxide for 1-2 hours. Thereafter, the samples were subjected to three five minute washes with phosphate buffer. Dehydration was accomplished with a graded series of 30%, 50%, and 75% alcohol (two changes, each of 5 minutes) followed by two changes for 10 minutes in 100% alcohol. The samples were then dried to their critical point in a Hitachi Critical Point Dryer. Critical point-dried samples were mounted onto brass stubs secured with carbon conductive tape and sputter coated with a Quorum Gold QISORES Sputter Coater, UK for 4 minutes. They were then viewed with a Leo 1450 SEM, Germany, at 10 kV, WD- 18nm. Images were captured using the Smart SEM version 5.03.06.

Transmission Electron Microscopy (TEM)

TEM preparation involved fixing small segments of fresh leaf and calyx samples in 2.5% glutaraldehyde for 24 hours. The segments were subjected to three 5 minute each phosphate buffer washes, followed by a 2 hour post-fixation with osmium tetroxide and caffeine. Thereafter the samples were subjected to three additional washes for 5 minutes each in phosphate buffer and

dehydrated in 30, 50 and 75% acetone (two changes, each of 5 minutes), followed by two 100% acetone changes for 10 minutes each. The dehydrated leaf and calyx segments were infiltrated with equal parts of Spurr's resin (Spurr, 1969) and acetone for 4 hours and thereafter in 100% resin for 24 hours. The sections were embedded in whole resin using silicone moulds and polymerized at 70 ºC in an oven for 8 hours.

Ultra-thin sections (100nm) sample sections were cut using a Reichert-Jung Ultracut-E ultramicrotome, UC 7, Germany (Munien et al., 2015). Sections were picked up onto copper grids and post-stained with 2.5% uranyl acetate followed with lead citrate solution. The sections were viewed and imaged using a Jeol 101 TEM, Korea, equipped with an Olympus MegaView III CCD camera at 100-200 kV.

Fluorescence Microscopy

Hand-cut sections of fresh young leaves were mounted with water on a slide, viewed and imaged at different wavelengths using a Zeiss LSM 710 confocal microscope, Germany. Cells containing phenolics emit a blue florescence at excitation wavelengths between 330 and 380 nm and plastids (e.g. chloroplast) emit a red fluorescence (Ascensao and Pais, 1987).

Acridine orange is a fluorochrome stain that binds to cell DNA, indicating cell viability by fluorescing yellow-orange (Winter et al., 2007). Hand-cut fresh calyx sections were also stained with 2% acridine orange for 2 minutes before rinsing with distilled water. The sections were then mounted in water, viewed and imaged at 488nm using a Zeiss LSM 710 confocal microscope.

Hand-cut sections of fresh leaf and calyx were stained with Calcofluor White for 2 minutes then rinsed with distilled water. Sections were then mounted in water and viewed with an epifluorescence microscope (Nikon Eclipse ATI) at excitation 365-410nm. Calcofluor White in plants is used for the microscopic observation of plant structures (Flores-Felix et al. 2015). Calcofluor white may not specifically bind to cellulose and can also stain callose, chitin and other cell wall polysaccharides (Krishnamurthy, 1999).

Histochemistry

Fresh leaf and calyx sections were sectioned using the Oxford vibratome sectioning system. The sections were 85-100 μm thick. They were stained with Nile blue for neutral and acidic lipids

(Cain, 1947; Ascensao and Pais, 1987); Ruthenium red for acidic polysaccharides (Johansen; 1940); Ferric trichloride for phenolic compounds (Johansen, 1940); Sudan III & IV and Sudan Black for lipids (Furr and Mahlberg, 1981); Dittmar's reagent and Wagner's reagent for alkaloids (Furr and Mahlberg, 1981); Mercuric Bromophenol Blue for total proteins (Ascensao and Pais, 1987); phloroglucinol for lignified cell walls (Rocha et al 2014) and Toluidine Blue for carboxylated polysaccharides (Furr and Mahlberg, 1981). Unstained leaf and calyx sections were used as controls by soaking the sections in methanol for 10 minutes. The sections were mounted on slides with distilled water and viewed with a Nikon Eclipse 80I compound light microscope, Japan, equipped with a Nikon DS-Fil camera and a NIS-Elements imaging software package. Appropriate controls were performed as described by Ascensao and Pais (1987).

Energy Dispersive X-ray Microanalysis (EDX)

Sample preparation for EDX was similar that of SEM. Spot analyses over the salt glands and area analyses of the trichomes were carried out to determine the chemical composition of the secretions. . EDX analysis was done using a Zeiss Ultra Plus FE-SEM, Germany, at 5 kV.

Trichome Density and Length

A selection of images obtained from the SEM was analyzed using Image analysis to count the number of the glandular and non-glandular trichomes on the calyx surface. Trichome surface area included in the image was also determined. The width and length of all trichomes were measured. This study focused only on the glandular trichomes because the dense arrangements of the non-glandular trichomes posed problems in counting. The differences in trichome density in different developmental stages as well as a difference between the length of the glandular and non-glandular trichomes on the calyx were analysed using a t-test.

The statistical software package IBM SPSS Statistics for Windows (Version 24.0) was used for data analysis.

Salt Gland Frequency

A selection of images obtained from the SEM were analyzed using Image analysis to count the number of salt glands present on both leaf surfaces of emergent, young and mature leaves. Leaf surface area included in the image was also determined. The differences in salt gland density in different developmental stages of the leaf as well as between the both surfaces were analysed such that there were six different locations: emergent adaxial, emergent abaxial, young adaxial, young abaxial, mature adaxial and mature abaxial. Salt gland density was compared using a Multivariate Analysis of Variance (One-way ANOVA) and Tukey's multiple comparisons test was used to compare differences between treatments. Statistics software IBM SPSS Statistics for Windows (Version 24.0) was used to analyse all data. The assumptions of normality and equality of variances were met for all ANOVA tests. A $P< 0.05$ was standard as being significant.

Results

Stereomicroscopy

Stereomicroscopy showed that in leaves the majority of the salt secretions occur on the abaxial surface (Fig. 2a) compared with the adaxial surface (Fig. 2b). Stereomicrographs of *P. auriculata* revealed two types of trichomes found on the calyx in both developmental stages (Fig. 2c and 2d).

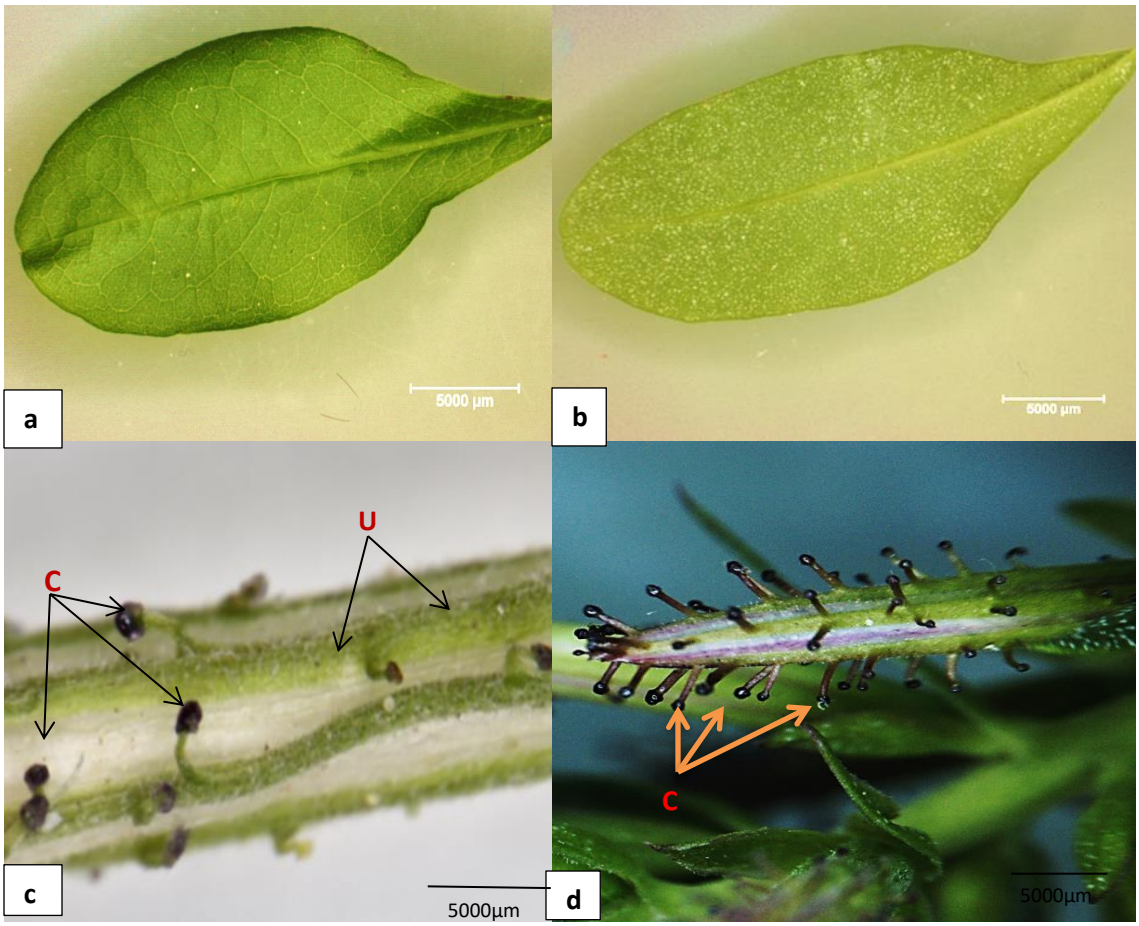


Figure 2: Stereomicrographs of *P. auriculata* secretory structures: a) adaxial leaf surface showing minimal salt secretion; b) abaxial leaf surface with salt secretions; c) Non-glandular uniserate (U) and glandular capitate (C) trichome types on open developmental stage of calyx; d) trichome structures on closed developmental stage of calyx. 40x magnification

SEM

Scanning electron microscopy of emergent, young and mature leaves showed that they contain salt glands on the epidermis (Fig. 3a). Salt glands appear to be more abundant on the abaxial surface of mature leaves in comparison to emergent and young leaves, based on SEM viewing. Scanning electron micrographs of the calyx revealed the two trichome types (Fig. 3b). The calyces bear large glandular capitate trichomes with visible mucilage secretions (Fig. 3c). Non-glandular trichomes were also observed (Fig. 3d). The capitate trichomes consisted of a basal cell, a single-celled stalk and a secretory head (Fig. 3c). The non-glandular trichome can be classified as uniserate (Payne 1978), consisting of a basal epidermal cell and is of a variable length.

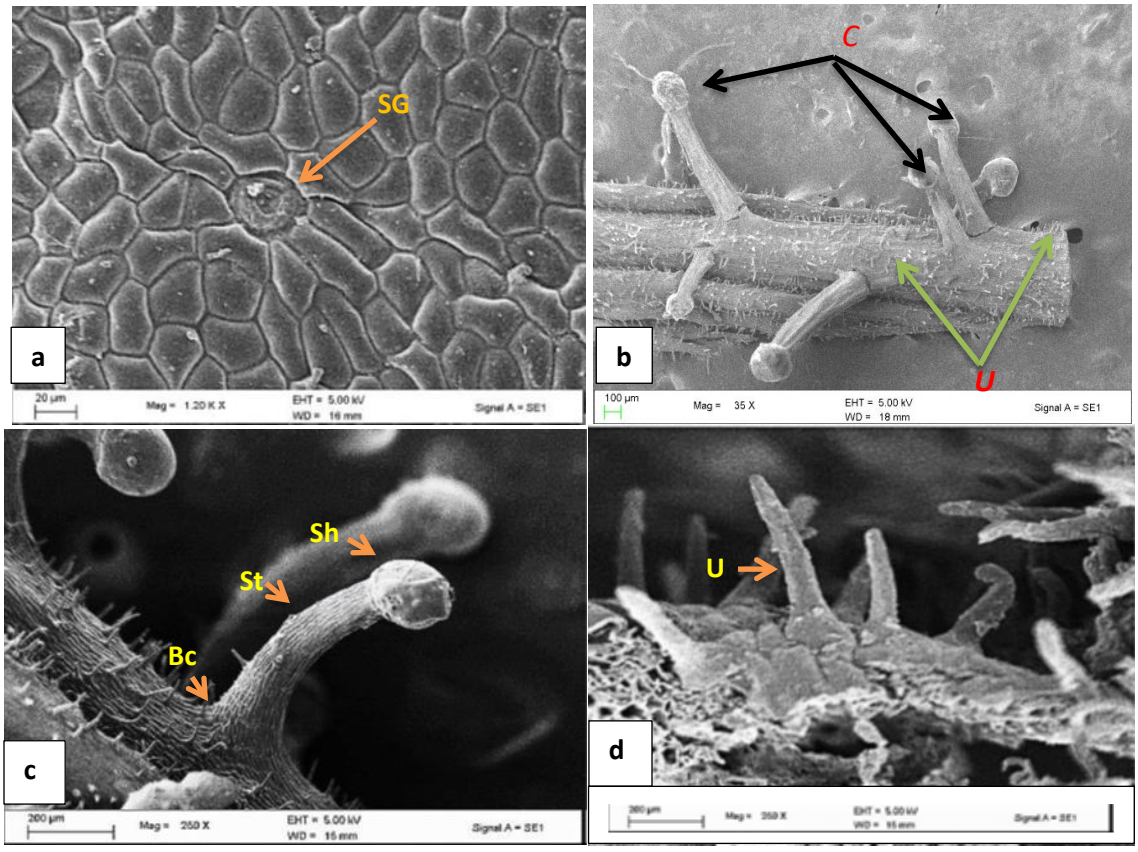


Figure 3: SEM of *P.auriculata* secretory structures showing: a) salt gland (SG) on mature leaf abaxial surface; b) glandular capitate (C) and non-glandular uniserate (U) trichome types on calyx, c) glandular capitate trichomes with a secretory head (Sh), a single-celled stalk (St), a

basal cell (Bc) on the epidermal layer of the calyx; d) non-glandular uniserate (U) trichomes on the surface of the calyx.

Light Microscopy

Light micrographs of trichomes on the calyx revealed that the non-glandular trichome type is uniserate and multicellular (Fig. 4b). The non-glandular trichomes protrude from the surface of the calyx (Fig. 4b). The glandular capitate trichomes possessing a multicellular stalk and a multicellular head protrude from the basal cell embedded in the epidermal cells (Fig. 4a).

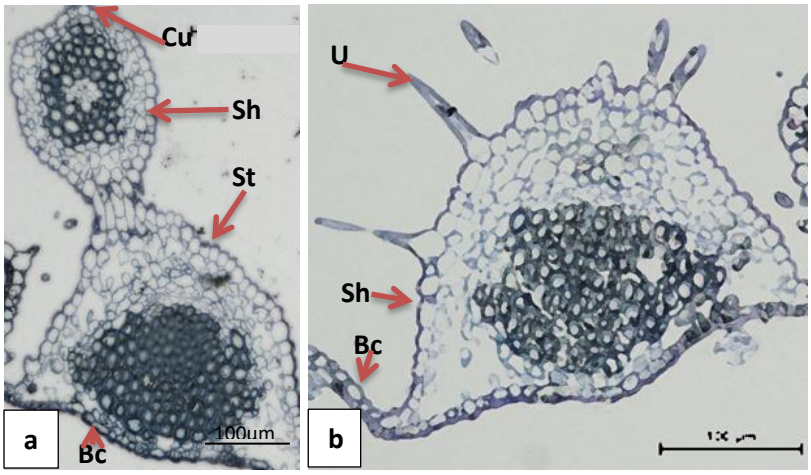
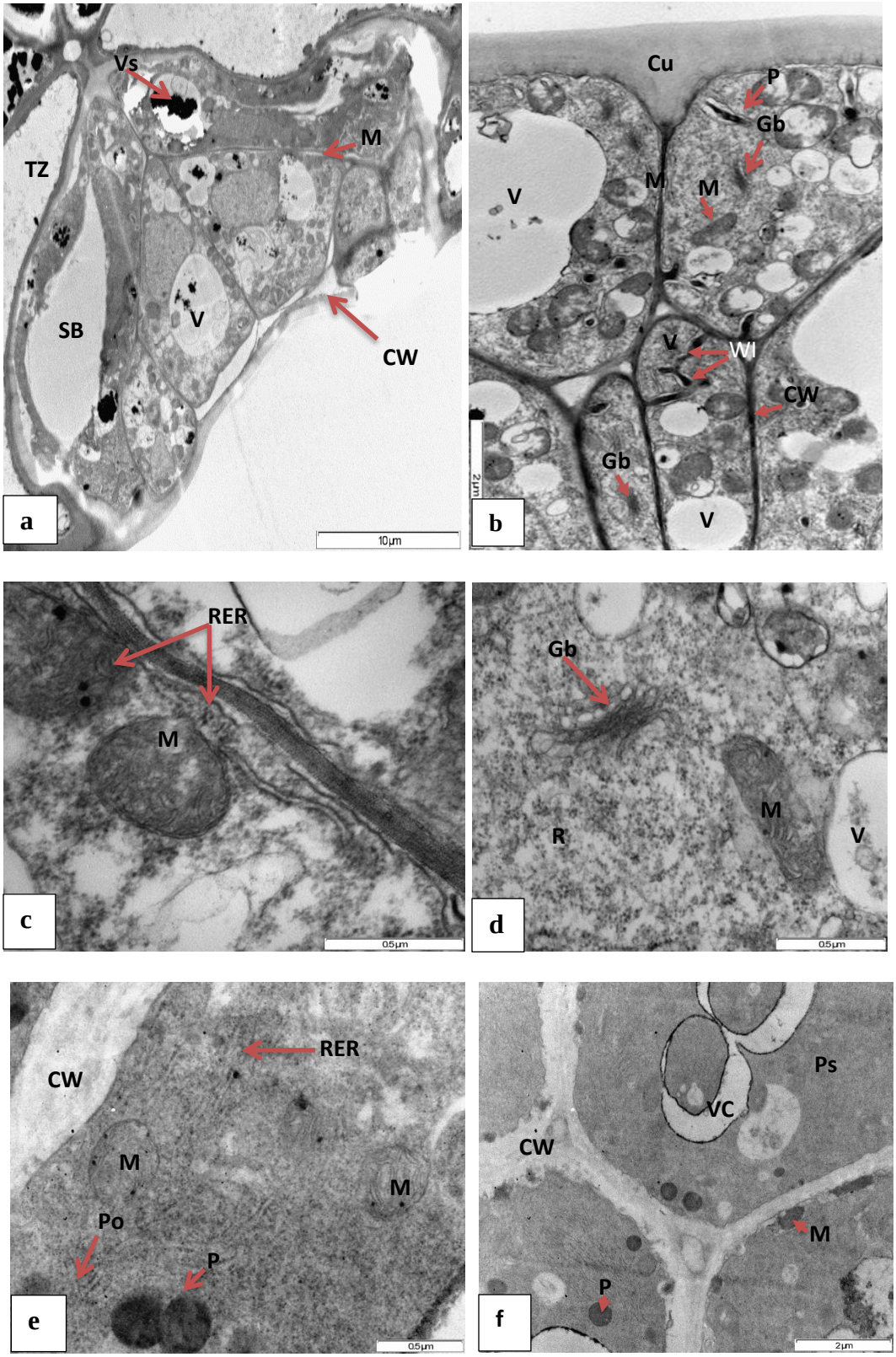


Figure 4: Light micrographs of trichome types found occurring on the calyx of *P. auriculata*. a) glandular capitate trichomes showing the cuticle (Cu) on a multicellular secretory head (Sh) and a multicellular stalk (St) protruding from the basal cell (Bc); b) Non-glandular uniserate (U) trichomes. 20x magnification

TEM

Ultrastructural studies reveal that the salt glands possess dense cytoplasm due to the presence of abundant mitochondria with a dense matrix showing a well developed cristae, abundant ribosomes grouping together forming polysomes, cisternae of the rough endoplasmic reticulum (RER), Golgi bodies with dictyosomes and a high frequency of plastids (Fig. 5 a-d). The presence of transfer cells have wall ingrows occurring throughout the salt gland (Fig. 5b). The cells are highly vacuolated with many mini vacuoles forming larger vacuoles (Fig. 5c). The

glandular capitate trichomes possess mitochondria, vacuoles, endoplasmic reticulum (RER) and many plastids (Fig. 5 e & f).


Vs
M
TZ
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c
Gb
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0.5µm
d
RER
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M
Po
P
0.5µm
e
Ps
VC
CW
M
P
2µm
f

Figure 5: TEM micrographs of ultrastructure of leaf salt glands of *P. auriculata* a) overview of a secreting salt gland showing notable features such as connecting cell walls (CW), sub-basal cell (SB), transfusion zone (TZ), vacuole with inclusions (VC), vesicles (Vs), mitochondria (M); b) cells are higly vacuolated (V) with many mitochondria (M) possessing a dense matrix, plastids (P), golgi bodies, thickened cell walls (CW) with wall ingrowths (WI) mainly in the upper region of the glands; c) cisternae of endoplasmic reticulum (RER) with ribosomes and mitochodia (M); d) dense cytoplasm with golgi bodies (Gb), mitochondria (M), ribosomes (R) and scattered dictyosomes; e) high magnification into the trichome showing plastids (P), endoplasmic reticulum (ER), polysomes (Ps) and mitochondria (M); f) trichome cells showing the presence of plastids (P), mitochondria (M), polysomes (Ps) and vacuoles with inclusion (VC). 20x magnification

Fluorescence Microscopy

Autofluorescence of fresh leaf sections emit a red fluorescence for chloroplast and blue fluorescence for phenolics under ultraviolet (UV) light (Fig. 6a). Sections stained with acridine orange depicted the viability of the glandular trichomes by fluorescing reddish orange. The non-glandular trichomes did not appear to be viable as they did not autofluoresce.

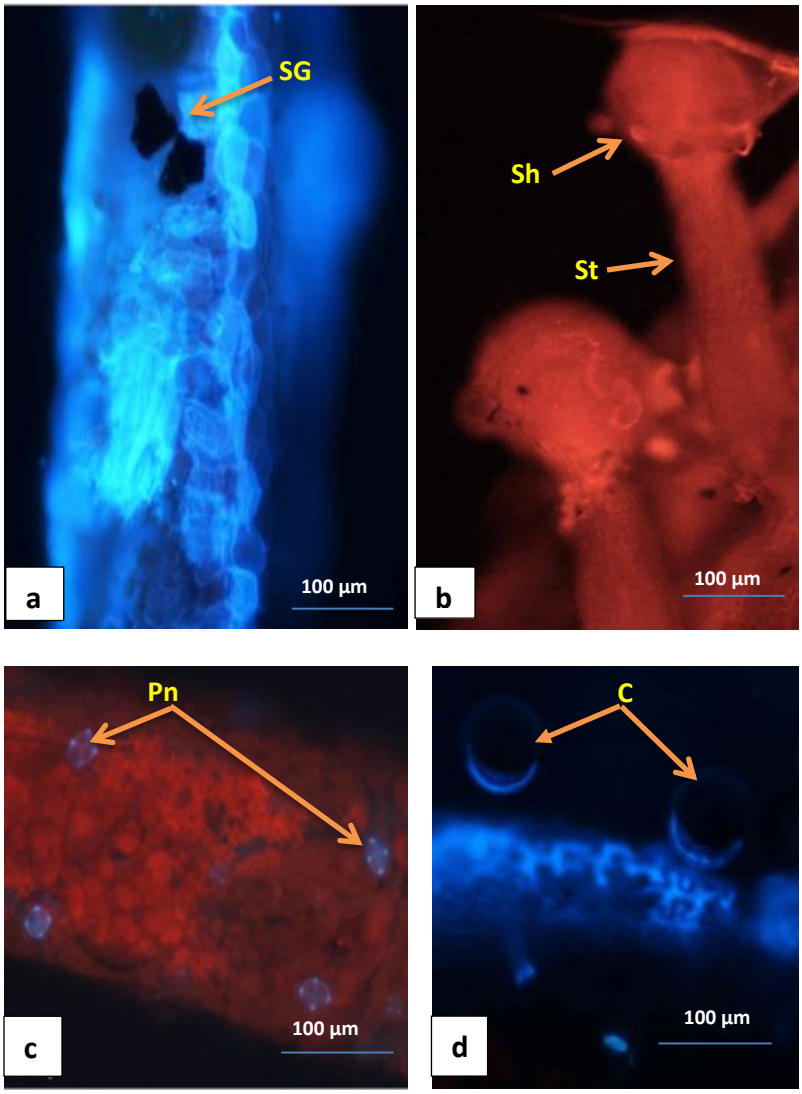


Figure 6: Fluorescence micrographs of the secretory apparatus of *P. auriculata* a) leaf section stained with calcofluor white; b) calyx stained with acridine orange showing trichome viability; c) autoflorescence of young leaf section showing chloroplasts and phenolics within the cells (Pn); d) calyx stained with calcofluor white. 20x magnification

Trichome density and length

On initial interpretation of the SEM images obtained, trichome density appeared similar in the developmental stages (Fig. 7). The closed calyx appeared to possess a slightly higher density of glandular capitate trichomes. The t-test confirmed that there was no statistical difference between the two developmental stages $p>0.05$. The glandular capitate trichomes on opened and closed calyces were approximately 506 ± 118 µm in length, with secretory heads having an average diameter of 176 ± 23 µm. Non-glandular trichomes were approximately 58 ± 23 µm in length.

The t-test confirmed that the glandular and non-glandular trichomes found on the calyx were significantly different in length $P<0.05$ (Fig. 8)

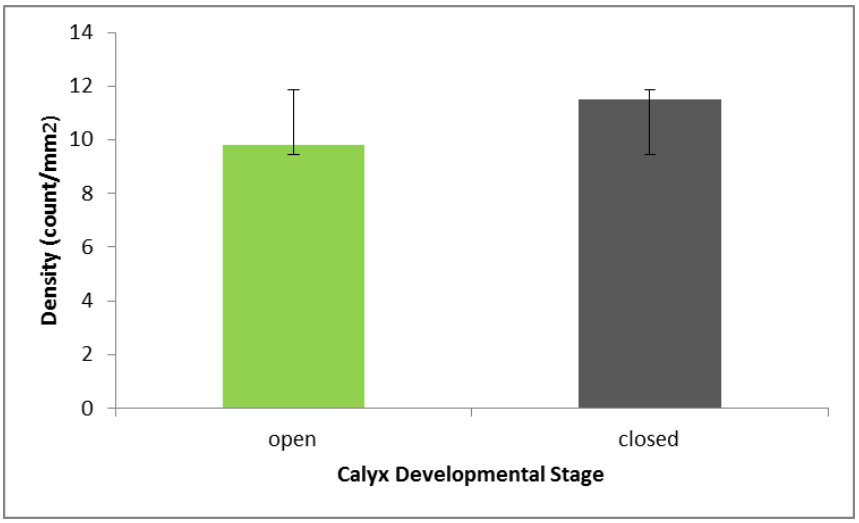


Figure 7: Frequency of glandular capitate trichomes at different developmental stages.

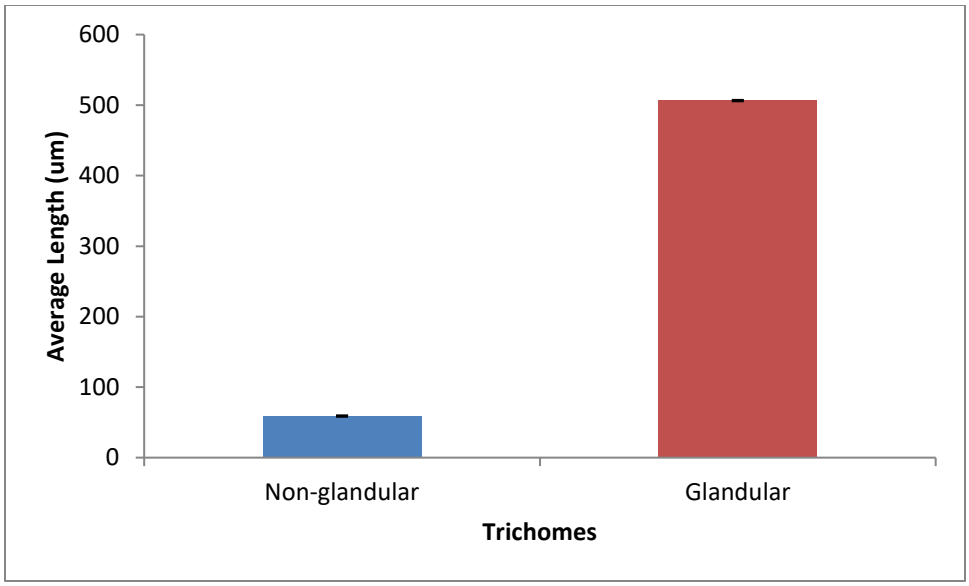


Figure 8: Average length of glandular capitate and non-glandular uniserate trichomes on the surface of the calyx.

Salt gland density

On initial interpretation of the SEM images obtained, salt glands appeared to increase with progressive leaf developmental stages (Fig. 9). Mature leaves showed a greater frequency of salt glands when compared to emergent leaves. Abaxial surfaces also appear to contain a higher salt gland frequency when compared to adaxial surfaces. Statistical analysis revealed a difference in salt gland frequency across the leaf developmental stages (df = 5, F= 38.9, $p < 0.05$). Further differences in frequency were found between leaf surfaces across the developmental stages: adaxial surfaces of mature leaves were significantly different from the adaxial surfaces of emergent and young leaves and the abaxial surfaces of mature and emergent leaves ($p = 0.002$); the abaxial surface of mature leaves was significantly different from the adaxial surface of young and emergent leaves and the abaxial surface of young and emergent leaves ($p = 0.02$); the abaxial surface of young leaves is significantly different from the adaxial and abaxial surfaces of emergent leaves ($p = 0.005$).

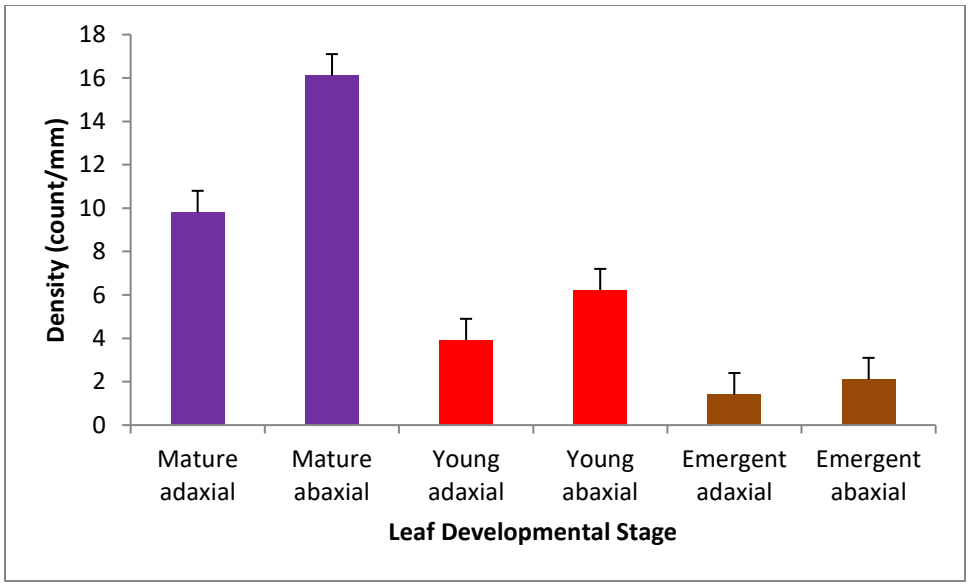


Figure 9: Frequency of salt glands at different leaf developmental stages on the adaxial and abaxial surfaces.

Histochemistry

Histochemical staining tests revealed the presence of lipids, phenolic compounds and alkaloids in the leaves and calyces (Fig. 10 &11) Black deposits indicated the presence of phenolic compounds (Fig 10b, 11a) and an orange or black colouration indicated the presence of lipids (Fig 10c, 11 c & f). The presence of alkaloids was positive with a brown colouration (Fig 10e) and a pink to red coloration indicated the presence of mucilage (Fig. 10f, 11e). These results correspond to histochemical tests in which various reactions gave different colourations indicating the presence of different compounds shown in Table 1.

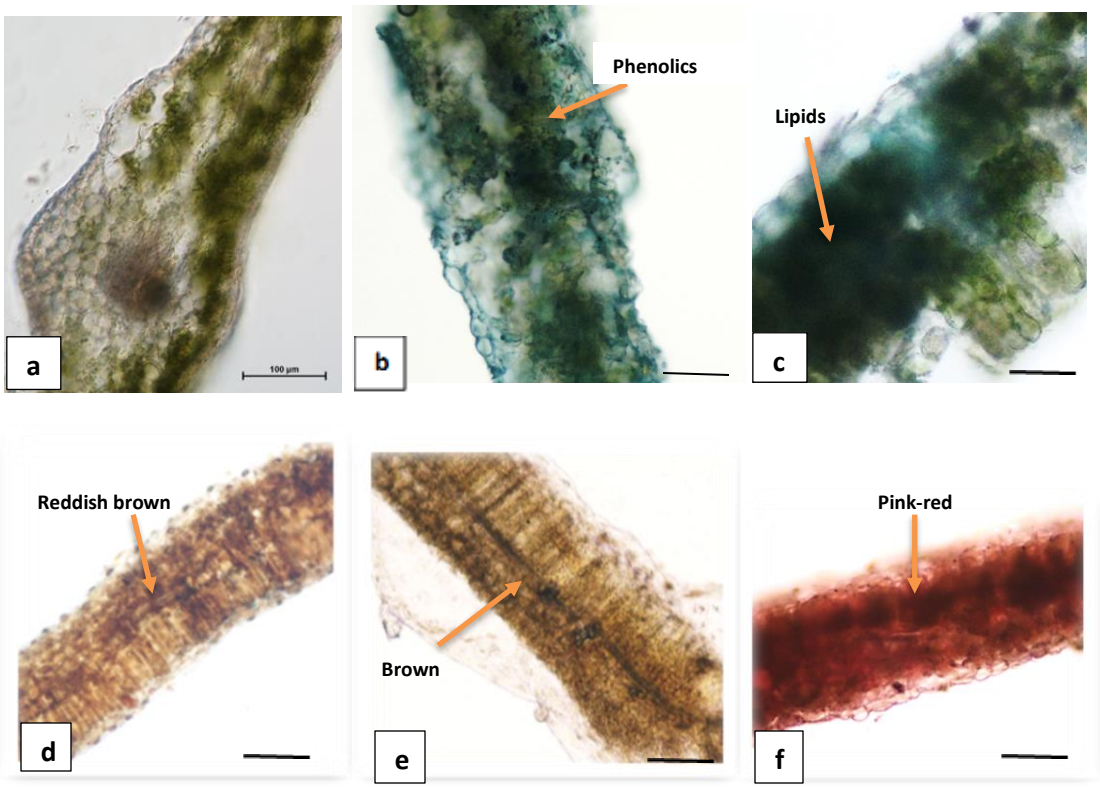


Figure 10: Light micrographs showing histochemical characterization of transverse leaf sections of *P. auriculata.* a) Unstained leaf section; b) Phenolic compounds stained black with Ferric trichloride; c) lipids stained black with Sudan black; d) Lignin aldehydes stained reddish brown with Phloroglucinol; e) alkaloids stained brown with Wagner's reagent; f) Mucilage stained pink-red with Ruthenium red. Scale bar =100 um.

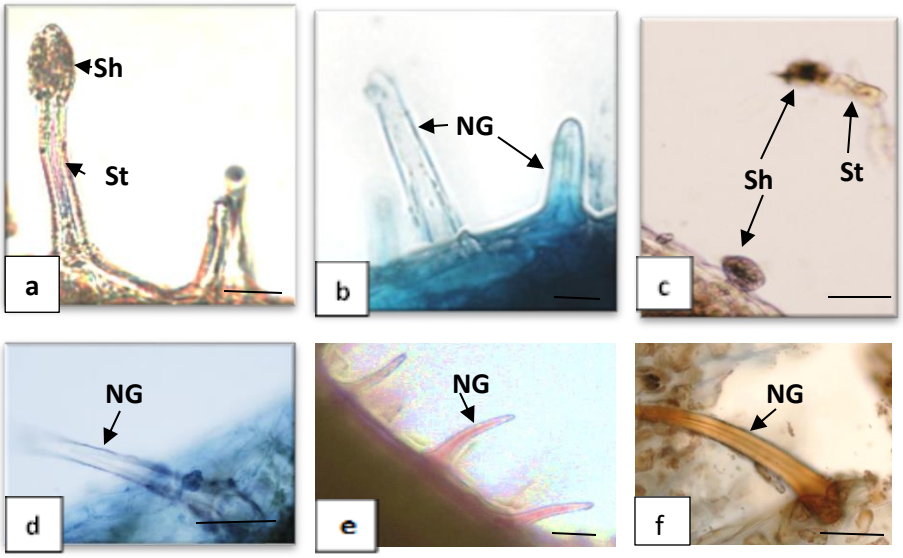


Figure 11: Light micrographs showing histochemical characterization of the trichomes of *P. auriculata*. a) phenolic compounds stained black in the capitate head (Sh) with Ferric trichloride ; b) neutral lipids stained in the non-glandular (NG) trichomes with Nile blue; c) Lipids stained black in the capitate head (Sh) with Sudan black; d) polysaccharides in non-glandular (NG) trichomes stained blue with toluidine blue; e) Mucilage stained red in the capitate head (Sh) and non-glandular (NG) trichomes with Ruthenium red; f) lipids stained orange in non-glandular trichomes (NG) with Sudan III and IV. Scale bar =100 µm.

Table 1: Observations of histochemical tests on fresh leaf and calyx sections of *P. auriculata*

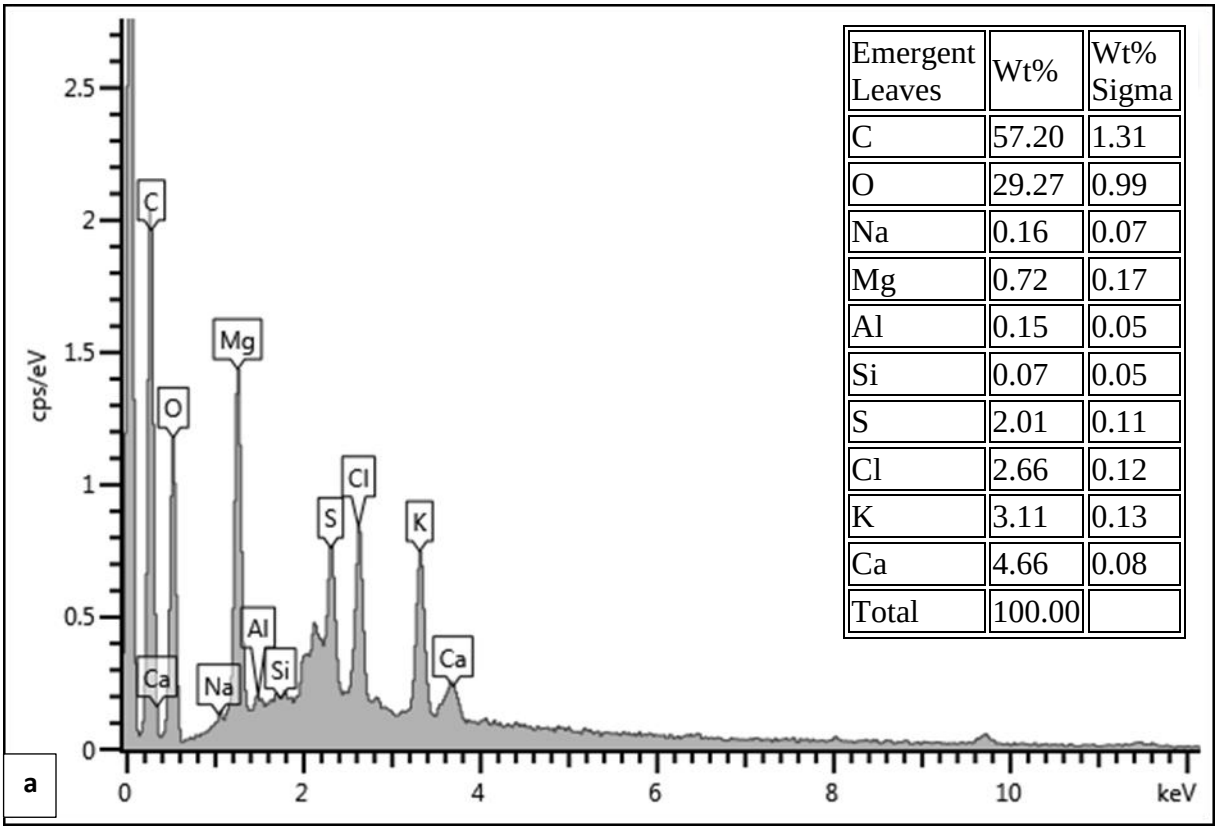
Compound Group	Stains	Leaves	Trichomes	Reaction observed
Alkaloids	Wagner's	+	+	Orange-brown colouration in leaf cells and trichomes
Cutin/subcutin/lipids	Sudan Black	+	+	Cells in leaf sections stained bla ck; head cells of capitate trichomes stained black
	Sudan III and IV	+	+	Orange colouration in trichomes.
Lipids	Nile Blue	+	+	Blue colouration in non-glandular trichomes.
Phenolic compounds	Ferric trichloride	+	+	Black deposits on the leaf section cells; head cells of glandular trichomes stained black.
Polysaccharides/ unesterified pectins	Ruthenium red	+	+	Cells in leaf sections stained dark pink-red, non-glandualr trichomes stained pink.
polysaccharides	Toludine blue	+	+	Non-glandualr trichomes stained blue.
Lignin aldehydes	Phloroglucinol	+	-	Cells in leaf sections stained reddish brown

+/- indicates presence or absence of compound groups

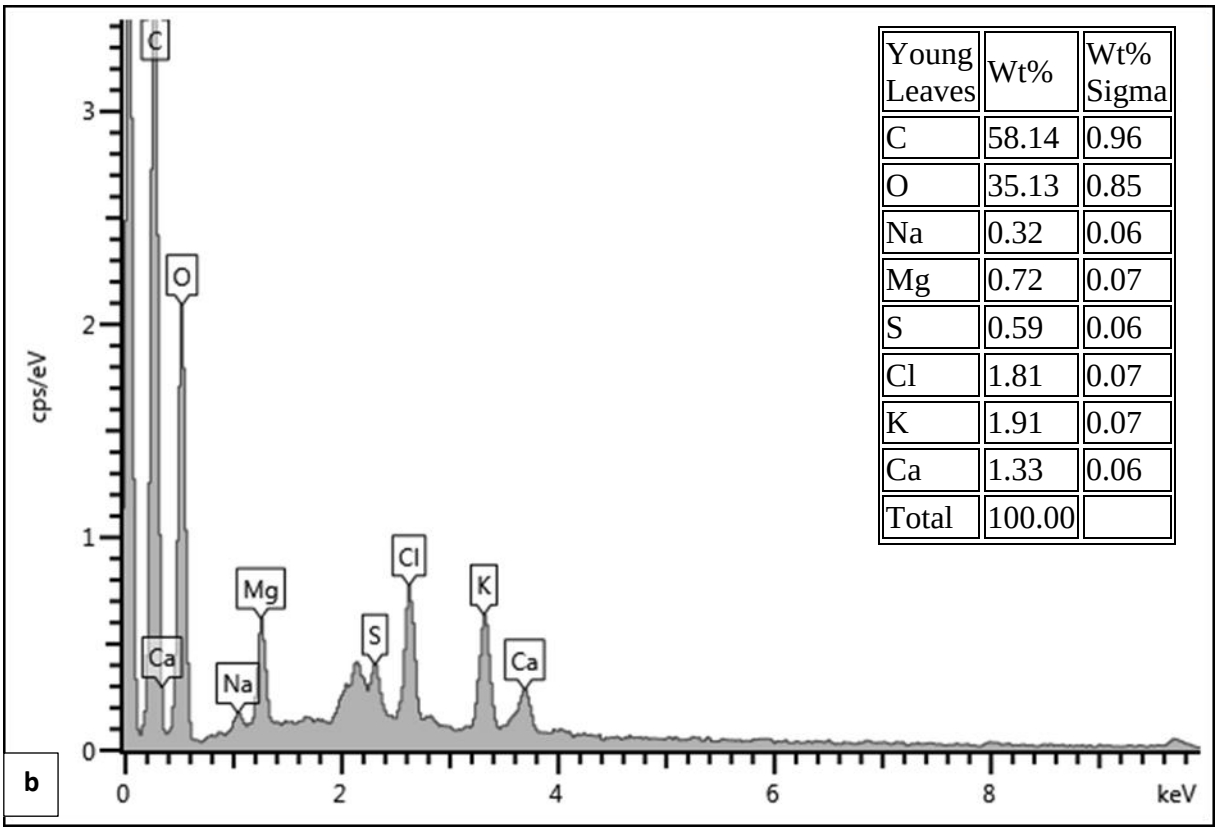
EDX

EDX analysis spectra revealed the presence of calcium and sodium salts within the leaf, but the presence of calcium was higher than sodium (Fig. 12) The mature leaves showed the highest amount of calcium salts (Fig. 12c) and emergent leaves the lowest (Fig. 12a). In the calyx

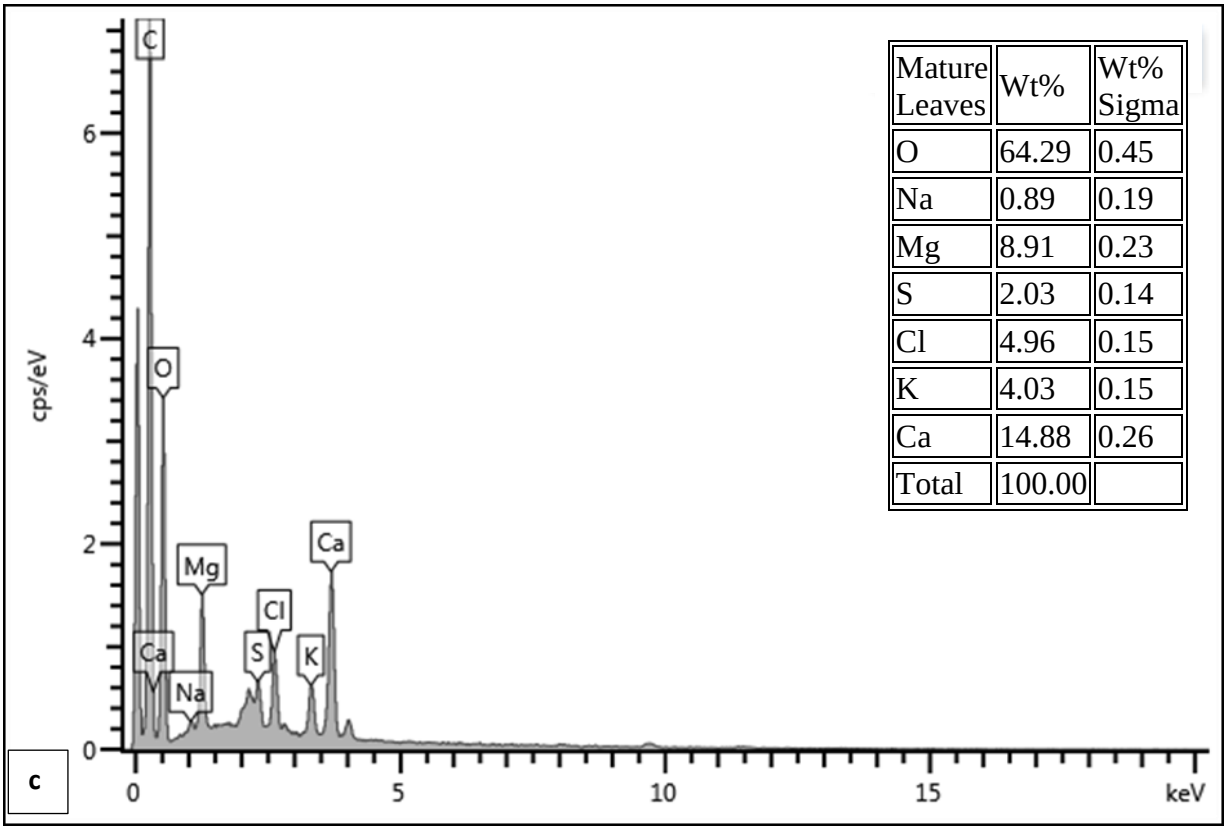
sodium salts were predominant (Fig. 12d). other elements found in the leaves of *P. auriculata* include: oxygen, magnesium, potassium, sodium and carbon (Fig. 12).



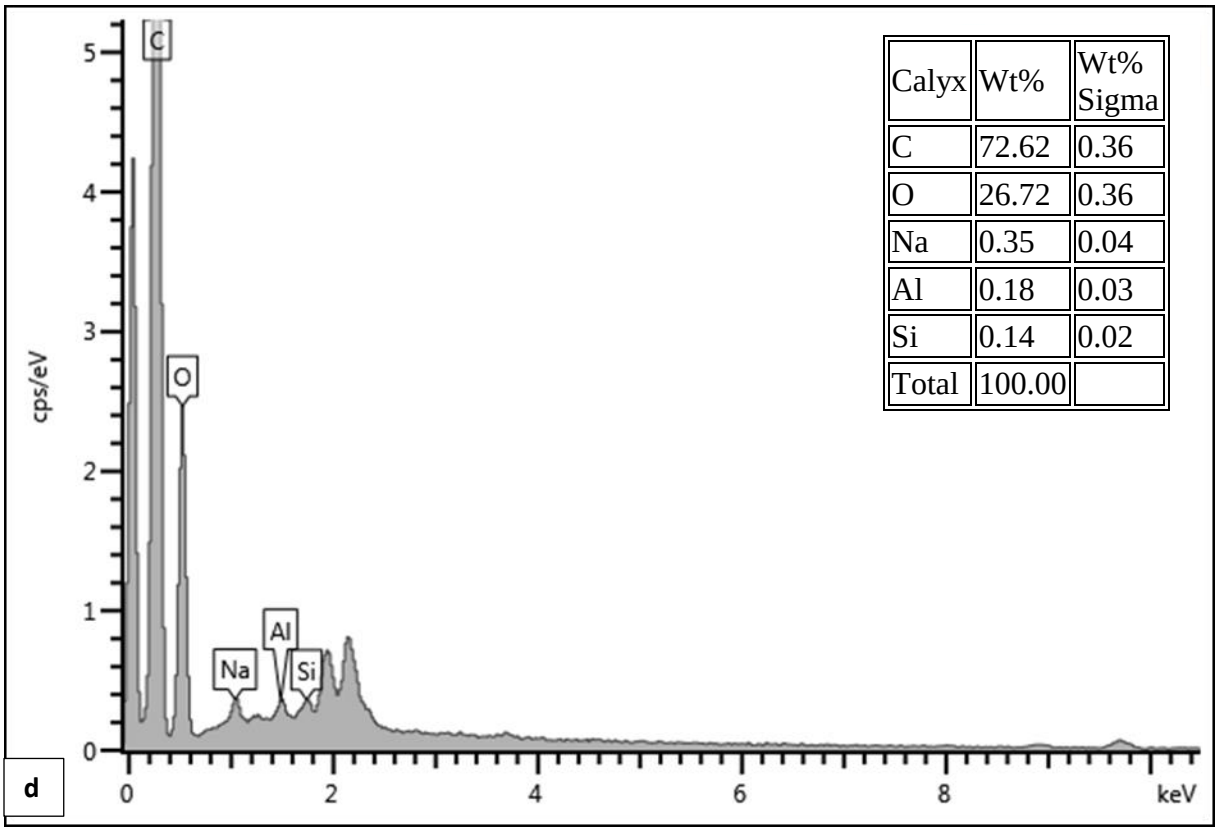
Emergent Leaves	Wt%	Wt% Sigma
C	57.20	1.31
O	29.27	0.99
Na	0.16	0.07
Mg	0.72	0.17
Al	0.15	0.05
Si	0.07	0.05
S	2.01	0.11
Cl	2.66	0.12
K	3.11	0.13
Ca	4.66	0.08
Total	100.00	



Young Leaves	Wt%	Wt% Sigma
C	58.14	0.96
O	35.13	0.85
Na	0.32	0.06
Mg	0.72	0.07
S	0.59	0.06
Cl	1.81	0.07
K	1.91	0.07
Ca	1.33	0.06
Total	100.00	



Mature Leaves	Wt%	Wt% Sigma
O	64.29	0.45
Na	0.89	0.19
Mg	8.91	0.23
S	2.03	0.14
Cl	4.96	0.15
K	4.03	0.15
Ca	14.88	0.26
Total	100.00	



Calyx	Wt%	Wt% Sigma
C	72.62	0.36
O	26.72	0.36
Na	0.35	0.04
Al	0.18	0.03
Si	0.14	0.02
Total	100.00	

Figure 12: EDX spectra showing the elemental composition of the secretions of *P. auriculata* leaf and calyx. a) emergent leaf; b) young leaf; c) mature leaf; d) calyx.

Discussion

Two morphologically different trichome types were observed on the calyx of *P. auriculata,* glandular capitate and non-glandular uniserate trichomes. The two trichome types have been previously reported for *P.auriculata* and *P. zeylanica* by Panicker and Haridasan (2016). Their studies reported that these trichome types may allow for the protection against herbivores and insect pathogens. Trichomes are ideal storage structures of secondary metabolites and ensure rapid release of these compounds at the time of damage (Wagner, 1991; Gonzales et al., 2008). This characteristic was extensively highlighted by members of the Lamiaceae family e.g the volatile compounds retained and stored in the secretory head of the peltate trichomes of *Salvia officinalis* L. were released upon rupture of trichomes by insect activity on the leaves and calyces of the species (Corsi and Bottega, 1999).

The glandular capitate trichomes bear a close resemblance to those of the genus *Drosera* Droseraceae) and *Drosophyllum* (Droserophyllaceae) and are thus considered as digestive glands due to their function in insect entrapment classified by the International Carnivarous Plant Society (ICPS) (2008) and further highlighted by Naidoo and Heneidak (2013) for *Drosera capensis* L. However, these trichomes can also be called colleters because they possess a multicellular stalk and head secreting a sticky exudate (Fahn, 1952). As the glandular trichomes of *P. auriculata* calyces are non-sessile whereas the trichomes of *Drosera* and *Drosophyllum* are sessile (ICPS, 2008; Naidoo and Heneidak, 2013) one can conclude that the trichomes of the genera are similar in function but differ in morphology.

The longer length of the non-glandular trichomes serves as a mechanical barrier and a defence mechanism for the plant as well as acts as a form of physical protection to the underlying glandular trichomes (Fahn, 1952). This was highlighted by Levin (1973) by correlating trichome density and insect pest resistance and further supported by Munien et al (2015) for Withania somnifera (L.) Dunal (Solanaceae). The results of this contrasted the findings by Levin (1973) and Munien et al (2015). In this study the length of the glandular capitate trichomes were longer than the non-glandular trichomes. Similar findings were reported by Panicker and Haridasan (2016) for *P. auriculata and P. zeylanica.* Therefore, it can be concluded that trichomes differ between families. However, the arrangement of the non-glandular trichomes bears no significance to the family.

The opened and closed calyx developmental stages showed no difference in density and size of the glandular trichomes indicating that the function of trichomes in the two developmental stages is similar. However, it has been reported by Panicker and Haridasan (2016) that in the genus *Plumbago* the exudate changes colour upon maturity from colourless to brown which was observed for *P. zeylanica* but this was not investigated in this study. Exudates produced by the glandular trichomes are sticky and may be toxic, allowing for insect entrapment and death of the insect whilst trying to free itself. Panicker and Harridasan (2016) reported that the trichomes on *P. auriculata* calyx are characterisric of this function.

Salt glands were observed on the abaxial and adaxial foliar surfaces of *P. auriculata* in all three developmental stages of the leaf; emergent, young and mature. The salt glands were predominantly more abundant on the abaxial leaf surfaces. This was based on visual observation

by the number of salt crystals present on the abaxial surface compared to the adaxial surface (Fig 2a-b) and was further confirmed by the EDX analysis of the leaf sections that attested the presence of calcium. Based on the EDX analyses these glands predominantly secrete calcium, sodium and traces of magnesium salts, thus can be viewed as salt glands based on the descriptions by Grigore and Toma (2016) and Dassanayake and Larkin, (2017). Gland density on the adaxial surface of *Pappophorum philippianum* (Poaceae) had a three times greater density of salt glands on the adaxial surface compared to the abaxial surface. It has been reported that salt glands are characteristic of the family Plumbaginaceae and are often made up of 16 to 24 cells (Thomson, 1975; Faraday and Thomson, 1986; Dassanayake and Larkin, 2017). Salt gland density may be determined in early leaf development, and as leaves mature, the density across the leaf area increases.

Fine structural observations (Fig. 5) showed the presence of plastids in the salt glands, and in head and stalk cells of glandular trichomes. Plastids produce microbodies which are formed by numerous unsaturated lipids after staining with osmium tetroxide and after conducting Sudan tests (Naidoo and Heneidak, 2013). Plastids in plants play a role in biosynthesis, accumulation and secretory processes of various compounds (Fahn, 1988). Yu et al (1992) reported that plastids found in the trichomes sometimes contain polyphenol oxidases (PPOs) which are copper metalloproteins involved in the reduction of phenols to quinines that function to entrap insect herbivores. The abundance of mitochondria in the cells, aligned along the cell walls is often associated with energy conversion, thus resulting in a higher respiration (Cutter, 1979). EDX analysis spectra (Fig. 11) depict the efficient respiration process as carbon levels were observed to be much higher than oxygen. Abundant mitochondria also provide an indication that the secretion by glands is an active process (Luttge, 1975). Cells are highly vacuolated with many mini-vacuoles that combine together to form larger vacuoles, that are implicated in the mechanism of secretion by serving as temporary collecting compartments where secreted salts accumulate prior to elimination as described for *Sporobulus virginicus* (Naidoo and Naidoo, 1998). These glands are surrounded with cutin-free “transfusion zones” symplastically connected by plasmodesmata to the surrounding sub-basal cells (Fig. 5a). Thomson (1975) reported that transfusion zone walls of salt glands of *Limonium* and other dicotyledonous plants have numerous plasmodesmata crossing the transfusion zone walls and this was further highlighted by Hill and Hill (1976) that occurrence of the plasmodesmata in these regions indicate symplastic

flow of solutes in the salt glands. The present study supports the findings of the previous statement and coincides with findings by Faraday and Thomson (1986).

This study was the first to report the presence of wall ingrowths in the cell walls of the salt secreting glands. Transfer cells are characterized by numerous plasma membrane invaginations (wall ingrowths) that increase the surface area, thereby facilitating intensive transport of ion solutes between interconnected protoplasts in or surrounding the plant. (Gunning and Pate, 1969; Iyer and Barnabas, 1993; McCurdy et al., 2008). These wall ingrowths ensure quick and efficient transport of salt to the outer surface of the plant as well as prevents backflow of salt because salt is toxic to the plant (Iyer and Barnabas, 1993; Kobayashi, 2008; Dassanayake and Larkin, 2017). The excess salt concentration in plants can be harmful by specific ion toxicities or osmotic influences (Eaton, 1942). Following Tozin and Rodrigues (2016) the presence of transfer cells in the secretory glands significantly improves the capacity of transport of the secretion. Ribosomes, dictyosomes and abundant cisternae of endoplasmic reticulum present in this study is reported to play a role in wall metabolism (Gunning and Pate, 1969; Cutter, 1978). The presence of these structures is in accordance with studies by Luttge (1975) and Hill and Hill (1976) highlighting the essential cell structures associated with secretion. Lignin has been suggested to be present in the wall ingrowths (Rocha et al., 2014).The findings in this study support this as lignified cell walls were positive when stained with phloroglucinol.

Fine structural observations of the glandular trichomes was presented for the first time in this study. Findings of this study showed that the mode of secretion of the sticky exudate from the trichomes is associated with many mitochondria in the head and stalk cells, dictyosomes, golgi bodies and an elaborate endoplasmic reticulum. Tozin and Rodrigues (2017) reported that the basal cell of the epidermal layer of a capitate trichome serves as a collecting cell for the secretory products which are then deposited into the secretory head via channels in the secretory stalk. The secretory products are gradually secreted from the head cells into a subcuticular space then released through pores in the cuticle. The cuticle often has one or more pores over each cell. This similar mechanism was reported by Naidoo et al (2012) for *Combretum molle* (Combretaceae). Qualitative EDX analyses indicated the presence of calcium carbonate in the capitate trichomes (Fig. 12).

Histochemical analysis showed that the leaves and trichomes of *P. auriculata* accumulate phytochemical compounds. Thus, these structures play a role in chemical defense against insect herbivores and pathogens. The major chemical compound groups found in these secretory structures were alkaloids and phenolic compounds. Which are often the two major phytochemical compounds that are of medicinal importance (Pourmorad et al., 2006). Alkaloids, derived from the word 'alkaline,' are naturally occurring chemical compounds containing nitrogen bases (Garba and Okeniyi, 2012). These compounds are reported to treat a range of ailments such as diarrhoea, fevers, inflammation, asthma and skin disorders (Edeoga et al., 2005). Alkaloids appear to be active metabolites but their usefulness to plants remains to be vague. However, some studies reported that alkaloids act as natural repellants against plant herbivores and competitors (Robinson, 1974, Edeoga et al., 2005). Phenolics are broadly distributed in plants and are the most abundant secondary metabolites (Dai and Mumper, 2010). Consisting of one or more aromatic rings with one or more hydroxly groups these compounds are stored within secretory structures (Fig. 11a) and are often released upon damage by insects (Dai and Mumper, 2010). They are released inside trichomes and are oxidized to quinines by polyphenol oxidase. In this regard phenolics function to glue insects to the surface of the calyx making it difficult for them to free themselves (Munien et al., 2015). The presence of phenolic compounds in the glandular trichomes shown in this study can be implicated in the trichome insect entrapment capabilities of *P. auriculata*. Phenolics assist the plant with defense against ultraviolet radiation, pathogens, predators and parasites, as well as contribute to the plant's colour (Dai and Mumper, 2010). Phenolic compounds are abundant in all plant organs and are, therefore, an essential part of human diet. This compound possesses strong antioxidant properties, among other medicinal and pharmacological uses, and is extensively exploited in the cosmetic industry (Negro et al., 2003).

Conclusion

This study provides additional information on the micromorphology and function of the secretory structures of *P. auriculata* and also helps in understanding the secretion modes of the salt glands and glandular capitate trichomes. The mitochondria, ribosomes, endoplasmic reticulum cisternae, dictyosomes and vacuoles are actively involved in the secretory process.

The presence of transfer cells in the salt glands was first to be reported in the family Plumbaginaceae. Histochemistry showed accumulation of phenolic compounds and alkaloids in the leaves and calyces of *P auriculata.*These compounds provide a chemical defense mechanism against insect herbivores and pathogens and are of medicinal importance. The secretory product of the calyx trichomes on the calyx should be further investigated and complete phytochemical analyses needs to be conducted to identify phytochemicals with potential medicinal properties.

Acknowledgements

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Chapter 4

Journal Article for Publication 3:

Gas chromatography-mass spectrometry analysis and phytochemical screening of *Plumbago auriculata* Lam. leaf and calyx extracts.

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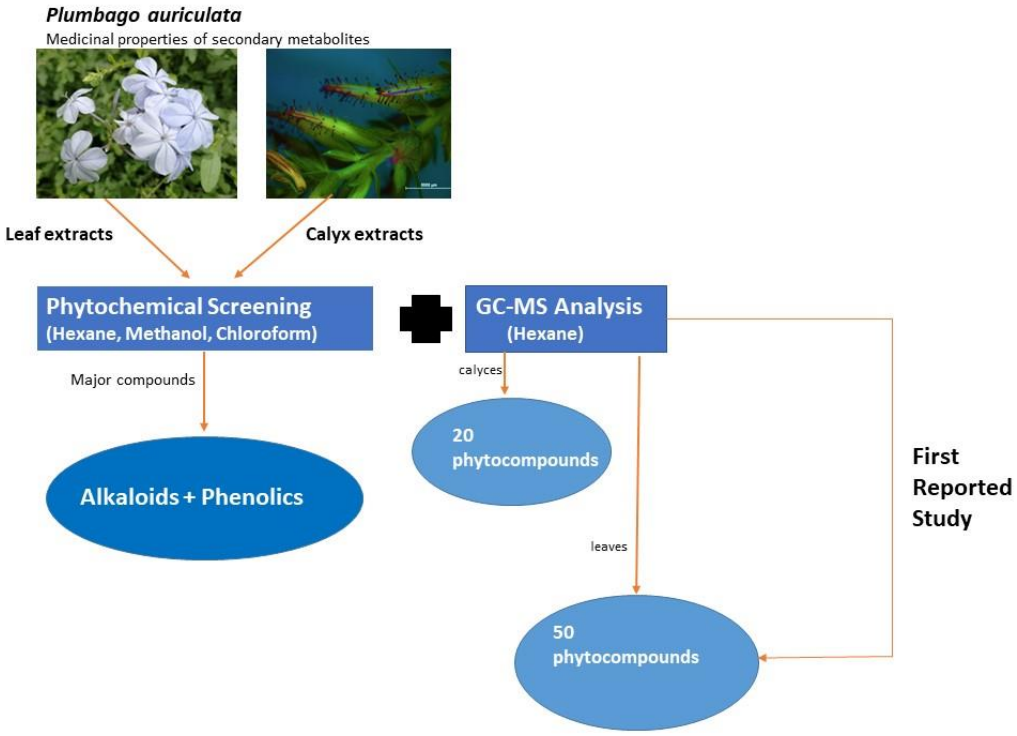
Objective: To identify the phytochemical compounds present in the leaf and calyx extracts of *Plumbago auriculata* and to evaluate its potential as a medicinal plant based on the possession of these compounds. **Methods:** The phytochemical components of the leaf and calyx in different solvent extracts (hexane, chloroform, and methanol) were screened by using various methods. Samples of the hexane extract of the leaf and calyx were subjected to GC-MS analysis. Compounds of interest were identified from the extract chromatograms by comparing the retention time of each peak and their respective fragmentation patterns with those of the reference samples. **Results:** The GC-MS analyses showed that the presence of 50 phytocompounds in the hexane extract of the leaf revealed the existence of 13-Docosenamide (9.81), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (9.44), n-Tetracosanol-1(9.3), 1, 10-Cycloicosanedione (8.41), 5, 7-Dihydroxy-4-methylcoumarin (6.94), Octadecanamide(5.20) and Nonadecanol (4.55) and 20 phytocompounds in the hexane extract of calyx included Bis(dodecanamido)methane (38.90), 9-Octadecenamide (33.71) and 1-Octadecanol (3.66) and 9,12-Octadecadienoic acid, methyl ester(3.31). The qualitative phytochemical analyses of the hexane, chloroform and the methanol extracts prepared from the leaves and calyces revealed the presence of secondary compounds with alkaloids and phenolics being the major compounds. **Conclusions:** The results obtained in this study were the first to be reported for this species and contributes to the body of knowledge for the potential use of *P. auriculata* as a medicinal plant. These identified phytochemicals presumed to be responsible for stimulating the pharmacological activity of this plant.

Keywords: *Plumbago auriculata*, GC-MS, phytochemicals, hexane extracts, medicinal plants

Graphical Abstract



Introduction

Gas Chromatography-Mass Spectrometry (GC-MS) is an analytical technique used to separate volatile components of complex mixtures, as well as to record mass spectrum of each component (Mtunzi et al., 2013). GC-MS is often used in disciplines such as food sciences, toxicology, forensics and environmental research. Medicinal plants are the richest bio-resources of traditional and alternate medicine (Kalimuthu and Prabakaran, 2015). Plants are also extensively used in the cosmetic and pharmaceutical industries, in food supplements, as chemical entities for drug development and nutraceuticals (Ajayi et al., 2011). According to the World Health Organization, approximately 80% of the world's population makes use of alternate medicine (Ajayi et al., 2011). The ethno-botanical knowledge and pharmacological studies on the aerial parts and roots of *Plumbago auriculata* suggested that the aerial parts and roots have a wide range of medicinal uses (Elgorashi et al., 2003; Ittiyavirah and Paul, 2016; Tharmaraj and Antonysamy, 2015). *P. auriculata* which belongs to the family Plumbaginaceae is a bushy, evergreen shrub common to South Africa and distributed and utilized in warm tropical regions in other parts of the world (Aubrey, 2001; Foden and Potter, 2015). The plant is covered with trusses of pale, sky blue flowers. However, there are variations of deep blue or white flowers (Batten, 1986; Ferrero et al., 2009). Species contain specialized secretory structures on the leaves and calyces such as salt glands found on the leaf epidermis and trichomes found on the calyx (Faraday and Thomson, 1986; Panicker and Haridasan, 2016). The plant parts possess a wide range of phytochemicals with plumbagin being the marker compound showing various pharmacological activities such as antifungal, antimalarial, antimicrobial, anticancer, antioxidant, antifertility, anti-inflammatory, hyperglycemic and cardiotonic (Jose et al., 2014; Mallavadhani et al., 2002; Padhye et al., 2010). Phytochemical screening which reported on the methanolic root extract of *P. auriculata* revealed the presence of the following phytochemicals: tannins, flavonoids, phenols, alkaloids, saponins, proteins and carbohydrates with phenols being the most abundant compound (Lakshmanan et al., 2016). In this study, further investigation was carried out to identify other active compounds present in the leaf and calyx extracts of the plant and explore the medicinal value of the extracts by phytochemical screening and GC-MS.

Materials and Method

Plant material

Leaf and calyx parts of *Plumbago auriculata* were collected at the University of Kwa-Zulu Natal in Durban, South Africa during May 2015. The plants were identified and authenticated by comparison with Herbarium specimens of the Ward Herbarium, School of Life Sciences, University of Kwa-Zulu Natal. A voucher specimen has been deposited in the Ward Herbarium (Singh and Baijnath 1).

Preparation of extracts

Leaves and calyces of *P. auriculata* were air dried for three months at room temperature and then powdered using a mill. The powdered material (50g) was "exhaustively extracted" by boiling leaves and calyces separately in hexane solvent in a round bottom flask using a soxhlet apparatus for 9 hours. The resultant extracts were filtered and concentrated under reduced pressure in a rotary evaporator. Ten milliliters of extract were measured out from the concentrate for phytochemical screening while the remaining extract was evaporated to complete dryness and stored at room temperature (24 ºC) for further use.

Phytochemical screening

One milliliter crude leaf and calyx extract was used for preliminary phytochemical screening. The phytochemical components in different solvent extracts were screened by using the methods described by Harborne (1973), as outlined in Table 1.

Gas chromatography–mass spectrometry (GC-MS)

GC-MS analysis was carried out on a Shimadzu GCMS-QP 2010SE instrument. The instrument was fitted with a Shimadzu HP-5MS capillary column (0.25 µm film thickness) having a dimension of 30 m (length) 0.25 µ (I.D.). Samples of the hexane extract of the leaf and calyx were subjected to GC-MS analysis. Helium was used as the carrier gas at 7.5 kPa pressure with oven temperature programmed at 60 ºC (for 2 min) to 270 ºC (for 30 min) at a ramping rate of 4

ºC per min. A 2 µL sample was manually injected at an injection temperature of 250 ºC with a split ratio of 1:5. For the GCMS detection, sample ionization energy of 70 eV was used. The system software was driven by Shimadzu GCMS solution workstation software (Japan). The compounds of interest were identified from the leaf and calyx chromatograms by comparing the retention time of each peak and their respective fragmentation patterns with those of the reference samples. The relative amount of each compound in the extracts as a percentage was then computed by comparing the area under a compound's peak to the total area.

Results and Discussion

The phytochemical components in different solvent extracts were screened by using the methods described by Harborne (1973). Qualitative phytochemical tests revealed the presence of alkaloids, anthocyanines, flavonoids, carbohydrates, phenols, amino acids and fixed oils in leaves and calyces. These results correspond to phytochemical tests in which various reactions gave different colourations indicating the presence of different chemical and phytochemical compounds presented in Table 1. It has been reported that plants are rich in secondary metabolites that exert a wide range of biological activities on physiological systems (Ajayi et al., .2011). It is therefore not unlikely that these phytochemicals found in *P. auriculata* may also play major roles in the biological activities and pharmacological properties of the plant.

Phytochemical compounds of the extracts analyzed by GC-MS are presented in Table 2 and 3. The major phytochemical components of the hexane leaf extract based on the peaks in Figure 1 were 13-Docosenamide (9.81), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (9.44), n-Tetracosanol-1(9.3), 1,10-Cycloicosanedione (8.41) and 5,7-Dihydroxy-4-methylcoumarin (6.94), Phytochemical components of the hexane calyx extract analyzed from the peaks in Figure 2 revealed the presence of 20 phytocompounds, including Octadecanamide(5.20), Nonadecanol (4.55), Bis(dodecanamido) methane (38.90), 9-Octadecenamide (33.71), 1-Octadecanol (3.66) and 9,12-Octadecadienoic acid, methyl ester (3.31). 13-Docosenamide, the major compound detected in the hexane extract of *P. auriculata* leaves, was first identified in the cerebrospinal fluid of sleep-deprived cats (Ben-Shabat et al., 1999). It has also been detected in the cerebrospinal fluid of rats and humans. 13-Docosenamide causes reduced mobility and slightly lessened awareness in rats whereas 9-octadecenamide

induces physiological sleep (Ben-Shabat et al., 1999). Another major compound, Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester, also known as 2-Palmitoylglycerol, is a fatty acid ester which increases the ability of 2-arachidonoylglycerol (sc-200794) to bind to CB1 and CB2 cannabinoid receptors (Gallily et al., 2000). Studies indicate that 2-Palmitoylglycerol also increases the inhibitory activity of 2-arachidonylglycerol on adenylyl cyclase (Gallily et al., 2000). The inhibitory effects of 2-arachidonylglycerol on TNF α (tumor necrosis factor-α) by mouse macrophages is enhanced in the presence of 2-Palmitoylglycerol. Furthermore, 2-Palmitoylglycerol may be a key component in modulating pain sensitivity as a result of its ability to interact with endocannabinoids. 2-monoacylglycerols are major end products of the intestinal digestion of dietary fats in animals through the enzyme pancreatic lipase (Walker et al., 2002). 9-Octadecenamide, the major compound detected in the hexane extract of *P. auriculata* calyx, is used for its anticancer properties (Abubaker and Majinda, 2016). 9,12- octadecadienoic acid methyl ester is a linolenic or fatty acid ester that has many pharmacological uses such as anti-inflammatory, anti-arthritic, antihistamine, antiandrogenic, cancer preventative, hepatoprotective, antieczemic, anticoronary, 5- Alpha-reductase- inhibitor, antiacne, hypocholesterolemic and can also be used as nematicide and insectifuge (Sharmila et al., 2012). Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, is a palmitic acid ester compound that can be used as an antioxidant, hypocholesterolemic, lubricant, antiandrogenic, pesticide, flavor, hemolytic and a 5-alpha-reductase- inhibitor (Sharmila et al., 2012). N-tetracosanol-1 is an alcoholic fragranced compound possessing antimicrobial, anticancer and anti-inflammatory activity (Kuppuswamy et al., 2013; Venkata et al., 2012). .Nonadecanol is an alcoholic compound that has potent antimicrobial and cytotoxic properties (Kuppuswamy et al., 2012). 1, 10 Cycloicosanedione is an organic compound that has strong antimicrobial activities and can be used in herbicides (Canli et al., 2014). Bis (dodecanamide) methane possesses antidiabetic, antioxidant and anti-inflammatory properties (Arya et al., 2012).

Conclusion

In summary, the findings of this study show that the leaves and calyces of *Plumbago auriculata* are a rich source of phytochemical compounds that can play an important role in preventing the progression of many diseases. These findings support the use of plants as an alternate source of medicine for the prevention and treatment of various human ailments. The use of leaf and calyx

extracts was the first reported study for *P. auriculata.* However, the phytocompounds found in this study were previously discovered in other plant species yielding many pharmacological properties. Further isolation of individual compounds and subjecting it to pharmacological activity will definitely produce beneficial results.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1: Phytochemical tests showing the presence and absence of major compounds within leaves and calyces of *Plumbago auriculata.*

		Leaves			Calyces		
Test	**Reagents/ Substrate**	**Hexa**ne	**Methanol**	**Chloroform**	**Hexane**	**Methanol**	**Chloroform**
Carbohydrates	**Mollsch**	+	+	+	+	+	+
	Fehling's	-	-	-	-	-	-
	Benedict's	-	+	-	-	+	-
Phenolics	**Ferric Trichloride**	+	+	+	-	+	-
	Potassium dichromate	-	+	+	-	+	-
Tannins	**Lead Acetate**	+	+	+	-	+	+
Alkaloids	**Mayer's**	-	+	-	-	-	+
	Hager's	-	-	+	-	-	+
Amino acids	**Ninhydrin**	-	+	+	-	+	-
Mucilage	**Ruthenium Red**	-	-	+	-	+	-
Cholesterol	**Salkowski's**	-	+	-	-	-	+
Anthocyanines Flavanones, Flavones, Flavanoids	**Concentrated sulphuric acid**	-	+	-	-	+	-
	Sodium hydroxide solution	-	+	+	-	+	-
Fixed oils and fats	**Filter paper**	+	-	+	+	-	+

*(+) presence and (-) absence

Table 2: Phytochemical composition of hexane leaf extract of *Plumbago auriculata*.

Sl.NO	Name of the compounds	% of compound	Retention Time	Retention Index	Molecular Weight
1	1-ethyl-3-methylbenzene,	3.75	4.0	1006	120
2	1,2,3-trimet hylbenzene	0. 63	4.4	1020	120
3	1-methyl-3-propyl- Benzene	0.63	4.7	1106	134
4	1,4-Diethylbenzene	0.83	4.8	1106	134
5	Tridecane	1.17	5.4	1115	184
6	2-Propyl-tetrahydropyran-3-ol	1.14	6.1	1156	144
7	Tetrahydropyran-2-carbinol	0.96	6.6	1012	116
8	4-Ethoxystyrene	1.09	7.1	1172	148
9	Dodecane	0.78	8.2	1214	170
10	1-Tridecene	0.90	9.4	1304	182
11	2-Decenoic acid	0.75	9.5	1380	170
12	2-Hydroxy-4-methylbenzaldehyde	2.58	10.0	1316	136
13	3-Hydroxy-4-methoxybenzoic acid	0.79	11.5	1560	168
14	1-Octadecene	1.31	11.9	1801	252
15	2-(Carboxymethyl)-6-methylbenzoic acid	0.79	12.0	1832	194
16	Z,E-2,13-Octadecadien-1-ol	1.39	13.0	2069	266
17	(3Z)-3-Octadecenyl acetate	0.47	13.1	2185	310
18	Octadecanal	0.54	13.3	1999	268
19	2-Ethyl-5-propylphenol	0.83	13.4	1426	164
20	Nonadecanol	4.55	14.1	2153	284
21	2-methyltetracosane	0.32	14.2	2442	352
22	Pentadecanoic acid	1.03	14.8	1869	242
23	3,7-Dimethyl-1-[2-(vinyloxy)ethyl]-3,7-dihydro-1H-purine-2,6-dione	0.68	15.0	2159	250
24	Heptadecyl dichloroacetate	1.57	15.1	2423	366
25	n-Octyl-.beta.-D-glucopyranoside	2.41	15.2	2409	292
26	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	0.94	15.3	2081	276
27	Z-2-Octadecen-1-ol	0.48	15.4	2061	268
28	Hexadecanoic acid, methyl ester	0.75	15.5	1878	270
29	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	0.85	15.6	2134	292
30	Eicosanoic acid	0.94	15.8	2366	312
31	Cyclopentadecanol	0.81	15.9	1987	226
32	5,7-Dihydroxy-4-methylcoumarin	6.94	16.1	1905	192
33	Hexadecyl pentafluoropropionate	1.33	16.2	1773	388
34	1-Heneicosanol	0.55	17.0	2351	256
35	Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester	0.89	17.7	2694	372
36	Hexadecanamide	2.52	17.9	2021	255
37	Phthalic acid, 3-methylbenzyl butyl ester	0.85	19.4	2525	312
38	13-Docosenamide	9.81	19.5	2625	337
39	Octadecanamide	5.20	19.7	2220	283
40	n-Tetracosanol-1	9.30	20.5	2650	354
41	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	9.44	20.7	2498	568
42	1,2-Benzenedicarboxylic acid, dicyclohexyl ester	1.24	20.8	2561	330
43	Bis (2-ethylhexyl) phthalate	1.27	21.0	2704	390
44	1,10-Cycloicosanedione	8.41	21.5	2741	308
45	9-Hexacosene	2.48	22.1	2614	364
46	cis-9,10-Epoxyoctadecanamide	0.58	22.5	2272	297
47	Stigmasta-4,7,22-trien-3.alpha.-ol	0.64	24.4	2750	410
48	19-Hydroxy-19-nortestosterone	1.15	24.6	2216	290
49	4,6-Cholestadien-3.beta.-ol	0.66	24.8	2579	384
50	Ergost-5-en-3-yl acetate	0.76	24.7	2771	442

Table 3: Phytochemical composition of hexane calyx extract of *Plumbago auriculata*.

Sl.NO	Name of the compounds	% of compounds	RT	RI	M.W
1	Tetradecyl trifluoroacetate	1.27	15.0	1613	310
2	Tridecanoic acid	0.69	15.8	1670	214
3	Tetradecanamide	0.77	15.9	1822	227
4	1-Octadecanol	3.66	17.0	2053	270
5	2-Tetradecyloxyethanol	0.73	17.4	1930	258
6	8-Methyl-6-nonenamide	1.78	17.7	1369	169
7	Hexadecanamide	2.73	17.9	2021	255
8	Oxiraneoctanoic acid,	0.89	18.9	2129	312
9	2-Hexadecoxyethanol	1.60	19.2	2129	286
10	9,12-Octadecadienoic acid, methyl ester	3.31	19.4	2093	294
11	9-Octadecenamide	33.71	19.51	2228	281
12	13-Docosenamide	3.01	19.55	2625	337
13	Octadecanamide	1.07	19.7	2220	283
14	1,3,3,3-Tetramethyldisiloxanyl tris(trimethylsilyl) orthosilicate	0.35	20.3	1297	444
15	2,2,19,19-Tetramethyl-3,6,10,14,18-pentaoxa-2,19-disilaicosane	0.81	20.5	1911	380
16	Oleanitrile	1.21	20.57	2064	263
17	1,2-Benzenedicarboxylic acid, Bis (6-methylheptyl) ester	1.70	20.8	2704	390
18	Nonadecanamide	1.56	21.17	2319	297
19	Bis (dodecanamido) methane	38.90	22.7	5295	690
20	4-Methoxy-6-methyl-5-nitro-2-benzofuran-1,3-dione	0.26	26.0	21.41	237

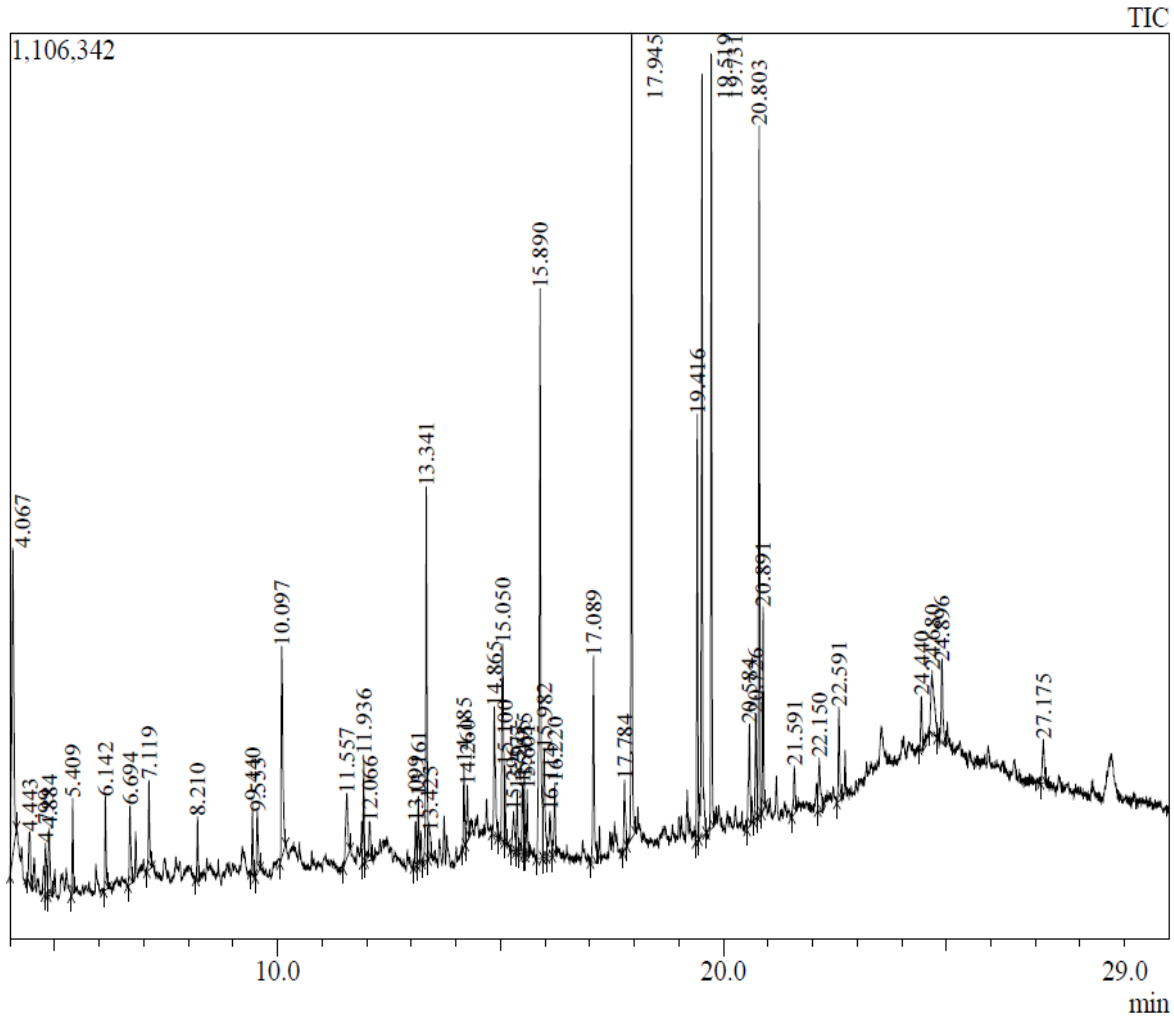

Figure 1: GC-MS analysis of hexane leaf extract of *P. auriculata*..

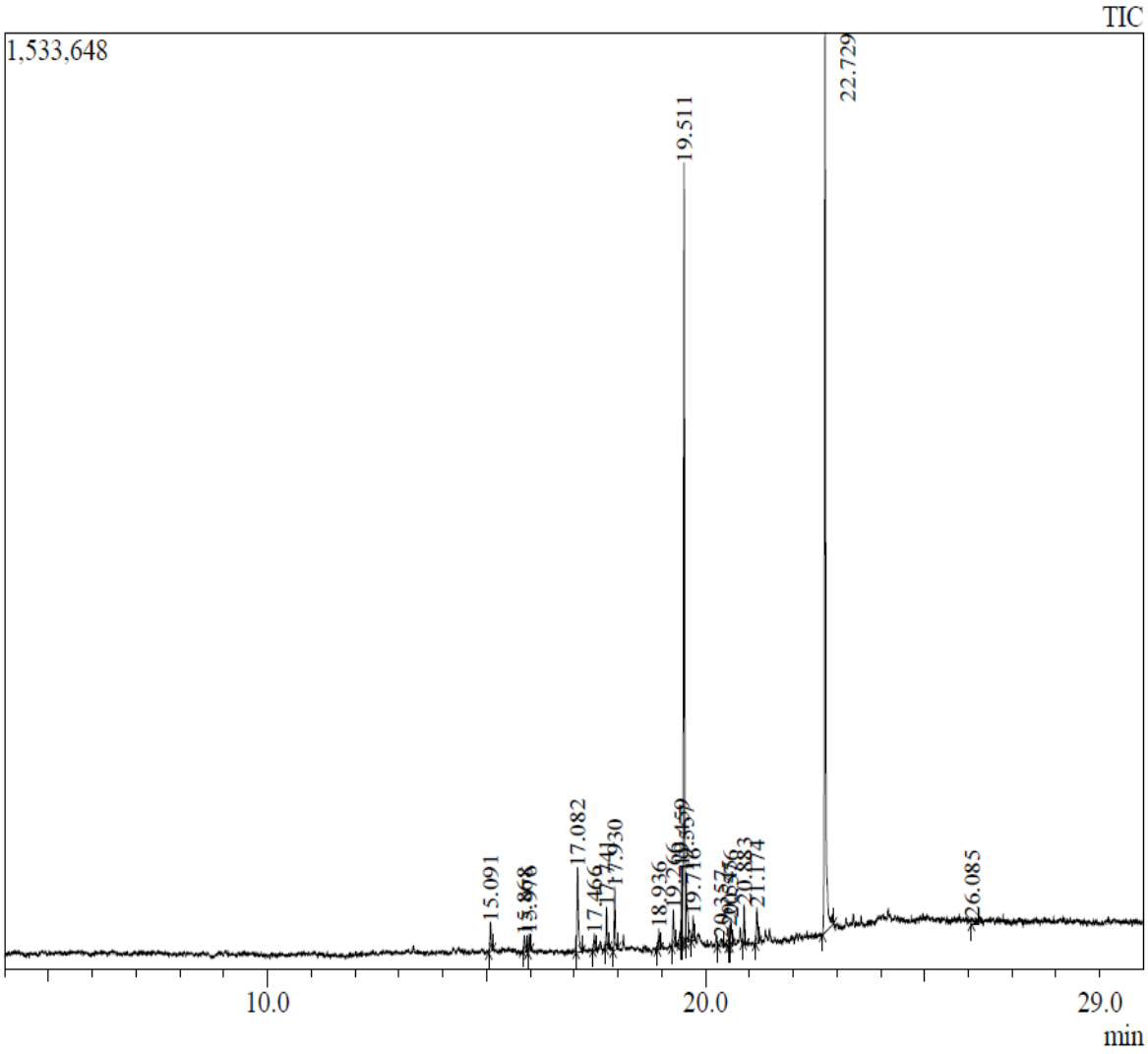

Figure 2: GC-MS analysis of hexane calyces extract of *P. auriculata***.**

Chapter 5

Journal Article for Publication 3:

Biosynthesis of silver nanoparticles using *Plumbago auriculata* Lam. leaf and calyx extracts and evaluation of their antimicrobial activities.

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Abstract

The use of plants in the biosynthesis of nanoparticles is a fast-growing technique and has gained much interest from researchers over the years. This study reports the utilization of leaf and calyx extracts of *Plumbago auriculata* for the biosynthesis of silver nanoparticles (AgNPs). The formation of AgNPs which was confirmed by the colour change in the plant extracts was characterized by UV-Vis spectrophotometric, TEM, SEM, EDX and FT-IR analysis. The water-soluble components of the extracts were responsible for the reduction of Ag^+ ions. FT-IR analysis revealed the efficient capping and stabilization properties of these particles and the nature of the capping agent. The antibacterial properties of the biosynthesized AgNPs were evaluated against both gram-negative and gram-positive bacteria and the results obtained showed good antibacterial activity against *Klebsiella pneumoniae*. This was the first reported study for *Plumbago auriculata* and contributes to the environmentally-friendly and cost effective technique of the biosynthesis of nanoparticles for drug development.

Keywords: biosynthesis, *Plumbago auriculata*, silver nanoparticles, antimicrobial activity, FT-IR.

Classification numbers: 4.02, 5.08

1. Introduction

Nanotechnology is a fast growing approach in the modern era. It has become one of the most active areas of research across the globe to explore the potential of synthesizing nanoparticles using different plants [11, 23]. Nanoparticles display characteristic properties in their distribution, size and morphology. Metals used to synthesize nanoparticles include copper, silver, gold, iron oxide, platinum, silica and nickel [12, 22]. Due to their unique properties and applications, metal nanoparticles have become quite popular over the years [15, 18]. Silver is the most commonly used metal for synthesizing nanoparticles. It is non-toxic to humans, active at low concentrations, and exhibits various applications in the following fields: antimicrobials and therapeutics, high sensitivity bimolecular diagnostics and detection and catalysis and micro-electronics [9, 18, 24, 27]. Previous studies have reported that silver ions and silver based compounds are highly toxic to many microorganisms [19]. Due to the high antimicrobial properties of AgNPs they are used in numerous household products, the medical industry and in cosmetic and pharmaceutical products.

Synthesis of nanoparticles can be achieved through various methods. Chemical methods are the most popular approaches but they are often too expensive and can be toxic [12]. Biosynthesis has become a more widely used approach as it is cost effective, ecofriendly and has low toxicity. Biological methods include the use of enzymes, plant extracts and microorganisms [13; 18]. Biosynthesis using plants is the best method because it a safe option and plants are more easily available and widely distributed [2]. Factors affecting biosynthesis include temperature and pH

with temperature variations in the synthesis process playing an important role in controlling the size and shape of the particles [22]. Aqueous (polar) mediums are reported to be more effective in biosynthesis than in non-polar media [22].

Plants have been used for centuries because of their medicinal properties. Currently, the biosynthesis of AgNPs with plant extracts is being exploited. This approach is more economical as it minimizes processing time and can be done on a small to large scale basis [12, 27]. Nanostructured systems are believed to potentiate the action of plant extracts, thereby improving their activity and reducing the required dose and possibly reducing adverse side effects [3, 22]. Plant-mediated biosynthesis is considered a widely acceptable technology for the rapid production of AgNPs but caution must be given to the type of extraction solvents used because of the hydrophobic nature of the capping agents that are used [2, 15].

Plants and plant metabolites exhibit strong antimicrobial properties and this has been confirmed by the number of scientific publications. The ethno-botanical knowledge and pharmacological studies on the aerial parts and roots of *Plumbago auriculata* suggest that the aerial parts and roots have a wide range of medicinal uses [6, 8, 25]. *P. auriculata* is a bushy evergreen shrub, common to South Africa and is also distributed in warm tropical regions in other parts of the world [7]. The various parts of the plant possess a wide range of phytochemicals. Plumbagin, the marker compound, shows various pharmacological activities including antimicrobial, anticancer, antifertility, antifungal, anti-inflammatory, antimalarial, antioxidant, hyperglycemic and cardiotonic [14, 17, 10]. It was reported that the aqueous root extract of *P. auriculata* (= *P. capensis* Thunb.) effectively synthesizes AgNPs and can be effectively applied as an antifungal agent [21]. Other species in the genus *Plumbago* which are also reported to effectively

synthesize nanoparticles include *Plumbago indica* L. and *Plumbago zeylanica* L. [11, 26]. The present study attempted to synthesize AgNPs with *P. auriculata* extracts and to characterize and evaluate the antimicrobial activity of the extracts.

2. Materials and Method

2.1 Plant material and preparation of the extract

Plumbago auriculata was collected on the Westville Campus of the University of Kwa-Zulu Natal in Durban, South Africa. A voucher specimen has been deposited in the Ward Herbarium (Singh and Baijnath 1). Aqueous extracts of leaves and calyces were prepared using fresh samples (25g) which were crushed in distilled water (100ml) using a mortar and pestle. Samples were then filtered through Whatman No.1 filter paper and stored at 4ºC for 14 days.

2.1.1 Preparation of 1mM Silver nitrate solution

One molar silver nitrate stock solution was prepared by dissolving $AgNO_3$ (0.17g) (BDH Chemicals Ltd. England) in distilled water (100ml). A 1mM solution was prepared by diluting 1M solution (10ml) in distilled water (90ml). This solution was stored in a dark bottle for further use at room temperature. The concentration of the extracts and $AgNO_3$ were 17000µg/ml and 10000µg/ml respectively.

2.2 Synthesis of AgNPs

Aqueous and methanolic extracts (leaf and calyx) (5ml each) were separately added to 1mM $AgNO_3$ solution (45ml) for reduction of Ag^+ ions. Synthesis of AgNPs occurred at room temperature (24 ºC) and at 60ºC by heating extracts in a water bath. The change in colour of the solution (this indicated the formation of the AgNPs) was observed and the time recorded.

2.3 Characterization of P. auriculata *AgNPs*

2.3.1 UV-Vis Spectrophotometric analysis

The reduction of pure Ag^+ ions was monitored by using a UV-Vis spectrophotometer (Spectrostar Nano BMG, Germany). Distilled water was used as blank. The reaction medium was analyzed for its maximum absorption at a wavelength range of 220-600 nm and the corresponding peaks were recorded. The absorbance of the reaction medium was measured within 24 hours.

2.3.2 pH

pH levels of the different extracts were measured before and after bioreduction using a pH meter (WS instruments, pH 50+, Italy) and calibrated at a two point calibration between 4 and 7. The pH readings of water and silver were also recorded.

2.3.3 Scanning Electron Microscopy (SEM) analysis and Energy Dispersive X-ray Microanalysis (EDX)

Scanning Electron Microscopy analysis was done using a Zeiss Ultra Plus field emission scanning electron microscope (FE-SEM, Germany) at 5 kV. Thin films of the sample were prepared on a carbon coated brass stub by adding a drop of the sample onto the coverslip mounted on the stub. The stub was allowed to air-dry for 10 minutes. Samples on the stubs were then sputter coated with gold for approximately 20 minutes. Shape and morphology of the nanoparticle clusters was identified. EDX was used to identify the composition of the synthesized AgNP and to confirm the presence of silver. EDX analysis was done using a Zeiss Ultra Plus FE-SEM with software Aztec (Oxford instruments, UK) at 5kV.

2.3.4 Transmission Electron Microscopy (TEM) analysis

Transmission electron microscopy analysis was performed to characterize the size and shape of the synthesized AgNPs. A drop of the nanoparticle solution was placed on a formvar coated copper grid and air dried for 10 minutes. Images were viewed using the Joel TEM 1010 (Japan) at 200 kV.

2.3.5 Fourier Transform Infrared (FT-IR) analysis

FT-IR measurements were carried out to identify the possible biomolecules responsible for reduction, capping and efficient stabilization of AgNPs. and the local molecular environment of the capping agents on the nanoparticles [4]. FT-IR of dried biomass after bioreduction was carried out by removing any free residue from the capping ligand. The residual solution of 50 ml after reaction was centrifuged at 5000 rpm for 15 minutes and the supernatant was decanted and the pellet formed was dried in an oven at 50 ºC. The dried nanoparticle was analyzed in order to evaluate the bioreducing and capping functional groups of the silver nanoparticles. Infra-red spectra of the crude extracts and their corresponding biosynthesized AgNPs were obtained on a Perkin Elmer Spectrum 100 FT-IR (USA) with universal attenuated total reflectance (ATR) sampling accessory.

2.4 Antibacterial assay

Preliminary antibacterial screening of the biosynthesized AgNPs was carried out against 2 gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* Rosenbach ATCC BAA-1683 (methicillin resistant *S. aureus*, MRSA*)* and 4 gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 31488, *Escherichia coli* ATCC 25922 and

Salmonella typhimurium). The bacteria were grown overnight in Nutrient Broth (Biolab, South Africa) at 37 ºC in a shaking incubator (100rpm). The bacterial concentration was adjusted to 0.5 McFarland's Standard with sterile distilled water using a DEN-1B McFarland densitometer (Latvia). Mueller-Hinton Agar plates (MHA) (Biolab, South Africa) were lawn inoculated with the prepared bacterial suspensions using a sterile throat swab and 5ul (17000 µg/ml) of the extracts were spotted onto the MHA plates. The plates were incubated at 37 ºC for 18 hours and after incubations the plates were read to determine antibacterial activity which was denoted by clear zones in the area where the extracts were spotted. Based on the preliminary screening results, the Minimum Inhibition Concentrations (MICs) were determined. The aqueous dispersions of AgNPs were serially diluted 2-fold with water ranging from 0.1-500 µg/ml) and 5 µl of each concentration was spotted on the lawn inoculated MHA plates and incubated at 37 ºC for 24h. The crude extracts and $AgNO_3$ solution served as controls.

3. Results and Discussions

In the biosynthesis of nanoparticles, a colour change in the reaction mixture is the first step that indicates that nanoparticles have been synthesized [22]. In this study, the reaction mixture displayed a wide range of colour changes resulting from the exposure to varying temperatures and extract mediums. When the reactions were placed in a water bath at 60º C, the colour changed gradually within the first 30 minutes. After 2 hours in the water bath at 60 ºC, the colour of the leaf extracts varied between crimson and dark orange (Figure 1a-d) and the colour of the calyx extracts ranged from crimson to dark orange (Figure 1 e and h). At room temperature, the reactions took longer. After 1 hour, a gradual change in colour was observed and after 2 hours the extracts reached the end colour. The methanol extracts for both leaves and calyces resulted in a darker colour when exposed to 60 ºC (Figure 1 b and 1 g). It was reported that with the formation of AgNPs with *P. capensis* root extract, the colour changed from clear to reddish orange [21]. The change in colour after bioreduction is due to the excitation of the surface Plasmon vibrations in AgNPs [9]. Similar changes have also been reported in previous studies and, hence, the bioreduction of Ag^+ ions in the reaction between the plant extract and $AgNO_3$ was confirmed [2].

Each metallic nanoparticle has its own characteristic absorption pattern [3, 4]. UV-Vis spectroscopy is a valuable technique to establish the formation and stability of metal nanoparticles. It is well documented that the optical absorption spectra of metal nanoparticles are dominated by surface Plasmon resonance that shift to longer wavelengths with increasing particle size [3]. In AgNPs, the appearance of surface Plasmon resonance peaks at 446 nm and provides a convenient spectroscopic signature for the formation of AgNPs. Absorption spectra of AgNPs formed have an absorption maximum in the range 440-460 nm. The UV-Vis

spectra recorded implied that most rapid bioreduction was achieved at 60 ºC for the leaf and calyx water extracts. This was denoted by broadening of the peaks that was indicative of the formation of large polydispersed nanoparticles due to slow reduction rates [15]. It was reported that maximum absorption rate for *P. capensis* aqueous root extract was at 420 nm. Reduction of Ag^+ ions in this study was evident by the UV-Vis spectroscopy [21]. The UV-Vis spectrum of AgNPs (Figure 2) was recorded from the reaction mediums, 24 hours after synthesis. The samples showed similar behavior with maximum absorption peaks ranging between 420-460 nm. Reduction of silver ions by the extracts was evident by the UV-Vis spectroscopy.

The pH levels for water (pH 7) and silver nitrate solution (pH 6) was fairly neutral (Figure 3A and B). The calyx crude extracts were slightly more alkaline prior to bioreduction (pH 7.9 and pH 7.6) (Figure 3 C and D) but after bioreduction of Ag^+ ions pH levels remained fairly neutral (Figure 3 E-H). The aqueous leaf extracts showed high levels of alkalinity before bioreduction (pH 8.4 and pH 7.8) (Figure 3 I and J) and after bioreduction (pH 7.6 and pH 7.61) (Figure 4 K and L) whilst the methanol solutions were more acidic after bioreduction (pH 4.6 and pH 4.8) (Figure 3 M and N). As previously reported, aqueous media are more effective in biosynthesis because pH affects the production and stability of the nanoparticles. [22].

Electron micrographs showed particles of varied shapes and sizes. Size and shape determine the efficacy of the particles. The smaller the particle sizes the greater are their properties. The TEM images showed relatively spherical to oblong shape nanoparticles formed with diameters in the range of 15-30 nm. Analysis of the TEM images revealed that at room temperature spherical to oblong AgNPs ranging in size from 15.22 nm to 26.5 nm of both leaves and calyces were formed (Figure 4

a and b) whilst at 60 ºC particles appear to be spherical but become aggregated with rising temperature. The size range was 18.33 nm to 29.48 nm for the leaves and calyces, respectively (Figure 4 c and d). The AgNPs synthesized at 60 ºC appeared slightly larger than the AgNPs synthesized at room temperature and were predominantly spherical to oblong in shape. Similar phenomena were also reported [11, 18, 21]. It is also reported that the shape of metal nanoparticles can considerably change their optical and electronic properties [20]. The nanoparticles observed in this study tend to aggregate with increased temperature. This is a result of a decreased binding force between AgNPs and the capping molecules that tend to decrease with increasing temperatures [5]. The TEM images showed that the high density AgNPs synthesized by the *P. auriculata* leaf and calyx extracts and this further confirmed the development of silver nanostructures.

From the EDX spectra (Figure 5), it can be seen that AgNPs reduced by *P. auriculata* have a weight percentage of silver as follows: 1.7% (Figure 5a), 8.5% (Figure 5b), 0.65% (Figure 5c), 2.56% (Figure 5d), 0.58% (Figure 5e), 0.82% (Figure 5f), 0.83 (Figure 5g) and 0.85 % (Figure 5h). From the EDX spectra of the AgNPs that was recorded, it can be deduced that AgNPs reduced by *P. auriculata* have varying weight percentages of silver. Previous studies have reported a much higher weight percentage of silver (16.41%) from synthesized AgNPs in comparison to the results of this study [20]. However, this could be a result of a larger quantity of $AgNO_3$ prepared and used in the biosynthesis process.

FTIR analysis was used to characterize the extracts and the resulting AgNPs. FTIR absorption spectra of methanol and water soluble extracts before and after reduction of Ag^+ ions are shown in Figure 6. Absorbance bands in Figure 6 a-d (before reduction) observed in the region 500- 4000 cm-1 and in Figure 6 e-l (after reduction) are approximately 1037, 1041,1083, 1100, 1158, 1205, 1258, 1261,

1343, 1396, 1447, 1514, 1609, 1619, 1637, 2108, 2852, 2920, 2934, 3224, 3277 and 3306. These absorbance bands are known to be associated with stretching vibrations for –C-O-C-, ether linkages, -C-O-, terminal methyls, -C-C- groups or from aromatic rings and alkyne bonds, respectively, and N-H, primary and secondary amines and O-H groups. These bands denote stretching vibrational bands responsible for compounds like terpenoids and flavonoids and revealed the capping of AgNPs. The absorption peak at 1640-1550 is close to that reported for proteins, suggesting that proteins are interacting with biosynthesized AgNPs. Absorption peaks occurring between 3650-3200 cm-1 denote alcohols (O-H bands) possibly arising from proteins and carbohydrates present in the sample which is in agreement with the value reported in the literature [15]. The main peaks (-C=O and O-H bands) present in the extracts are also present in the AgNPs, inferring the attachment of biomolecules which are present in the extract to nanoparticles. It has already been reported that the hydroxyl groups act as reducing agents and the carboxyl group promotes size and shape of the nanoparticles [1] The data presented in this study indicate the involvement of –C=O and –OH functional groups in the reduction and stabilization of AgNPs.

The MIC for gram-negative and gram-positive bacteria is presented in Table 1. The biosynthesized AgNPs showed antibacterial activity. The crude extracts displayed antibacterial activity at 17000µg/ml against all the bacteria tested. The synthesized nanoparticles and the $AgNO_3$ were active against all the bacteria tested. For *E.coli,* $AgNO_3$ had higher activity than the AgNPs for all extracts with the exception of nanoparticles synthesized from the calyx extract at 60 ºC (MIC of 1.25µg/ml). For *S. aureus*, the aqueous leaf extract AgNPs at 60 ºC displayed the highest activities whilst for *K. pneumoniae* the AgNPs showed higher or similar activity as compared with $AgNO_3$. AgNPs which synthesized with aqueous calyx extract at 60

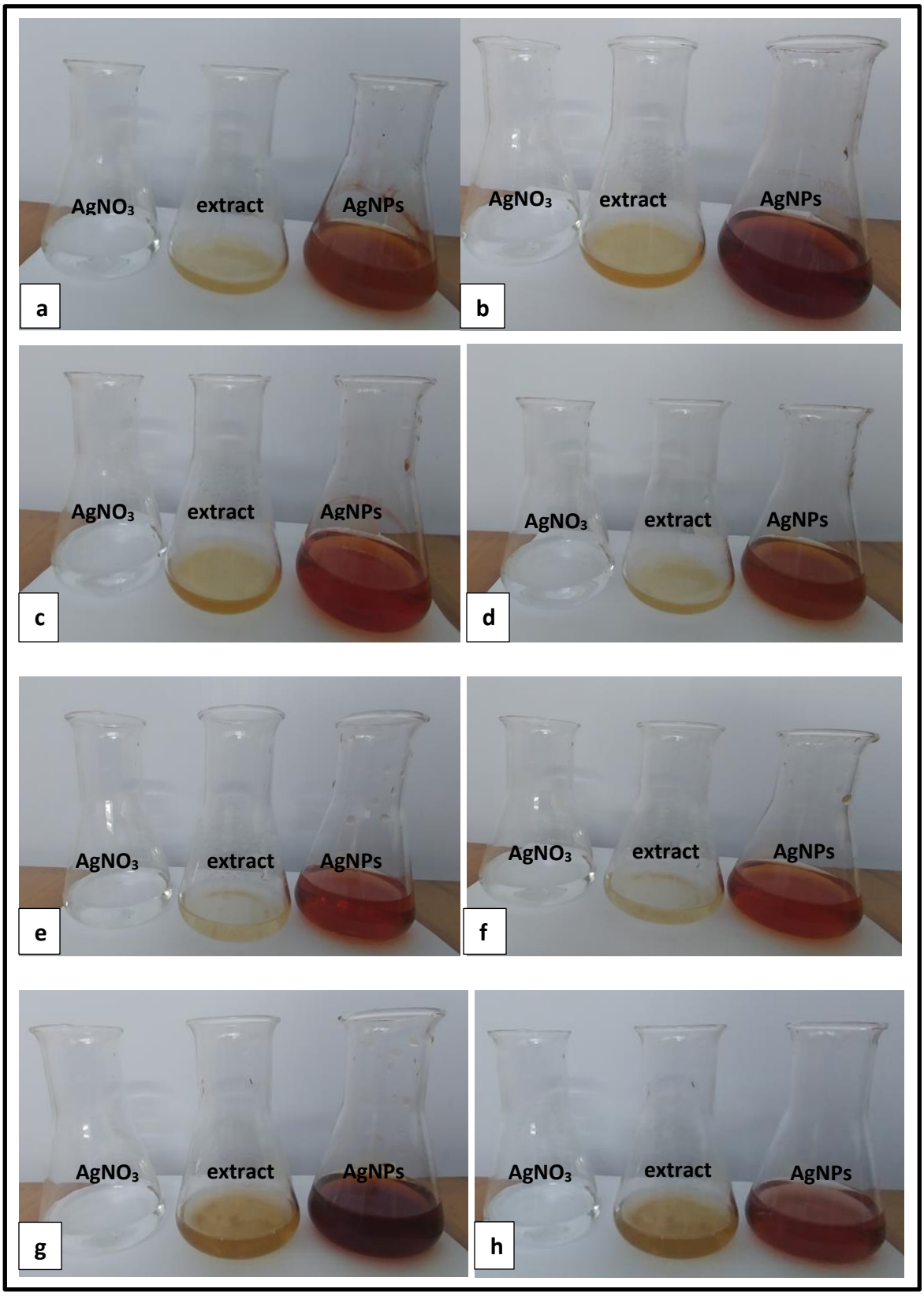
ºC showed the most activity against *K. pneumoniae* with an MIC of 62,5µg/ml. For *S. typhimurium*, the AgNPs displayed a higher activity than the $AgNO_3$ with both samples with a MIC of 250µg/ml. Results obtained in previous studies support the antibacterial potential of AgNPs obtained in this study [16, 23, 25, 26].

AgNPs prepared from leaf and calyx extracts showed maximum activity against *K. pneumoniae*. In this study, the biosynthesized AgNPs were found to have higher antibacterial activities as compared to the crude extracts. This is in accordance with previous studies that have reported better antibacterial activity of AgNPs than the crude extracts [22, 26]. As in previous studies, this study reveals that plant extracts and silver in their nano form are biologically active and can be used as antibacterial agents in drug development [1]. This is the first reported study for *Plumbago auriculata.*

4. Conclusion

In conclusion, AgNPs were successfully synthesized using *P. auriculata* leaf and calyx extracts. The synthesis of AgNPs was confirmed by the change in colour upon addition of the $AgNO_3$ solution to the extracts. Characterization by UV-Vis, TEM, SEM and EDX analysis further confirmed the reduction of Ag^+ ions. FT-IR spectra revealed that the –OH and >-C=O groups present in the biomolecules were responsible for the stabilization and reduction of the AgNPs. AgNPs had desirable antibacterial properties against both gram-negative and gram-positive bacteria in comparison to the crude plant extracts and the $AgNO_3$ solution and, hence, has a great potential in the preparation of drugs. Biosynthesis of AgNPs using *P. auriculata* extracts provides an environmentally friendly and cost-effective option in comparison to chemical and physical synthesizing techniques. The results

obtained in this study were the first to be reported for this species, thus adding to the current knowledge of biosynthesized metal nanoparticles.


AgNO3
extract
AgNPs
a
AgNO3
extract
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b
AgNO3
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Figure 1: Colour changes observed in the biosynthesis of AgNPs at two different temperatures of the different extracts: a) crimson of leaf methanolic extract (24 ºC); b) dark orange of leaf methanolic extract (60 ºC); c) crimson of leaf water extract (60 ºC); d) crimson of leaf water extract (24 ºC); e) crimson of calyx methanolic extract (24 ºC); f) crimson calyx water extract (60 ºC); g) dark orange of calyx methanolic extract (60 ºC); h) crimson of calyx water extract (24 ºC).

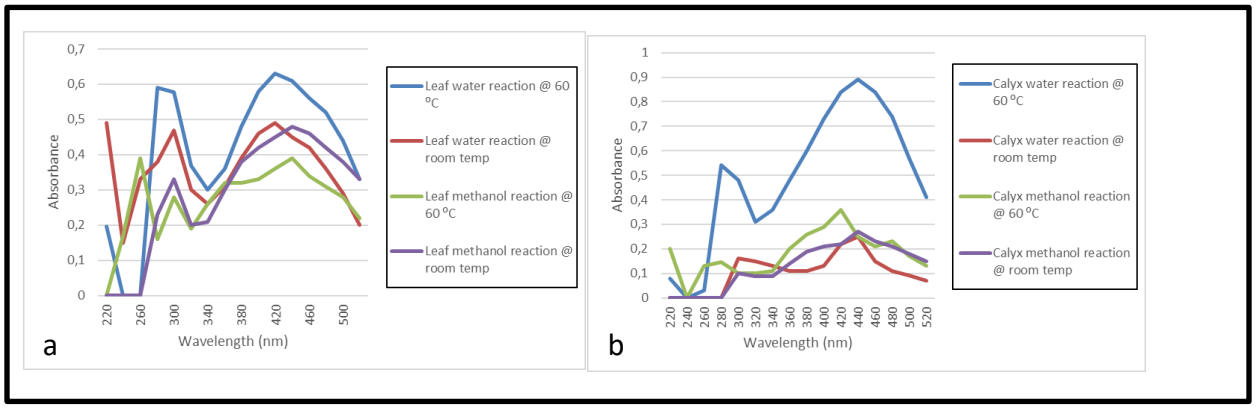


Figure 2: UV-Vis absorption spectra of aqueous silver nitrate with 1ml *P. auriculata* a) leaf and, b) calyx extracts 24 hours after synthesis.

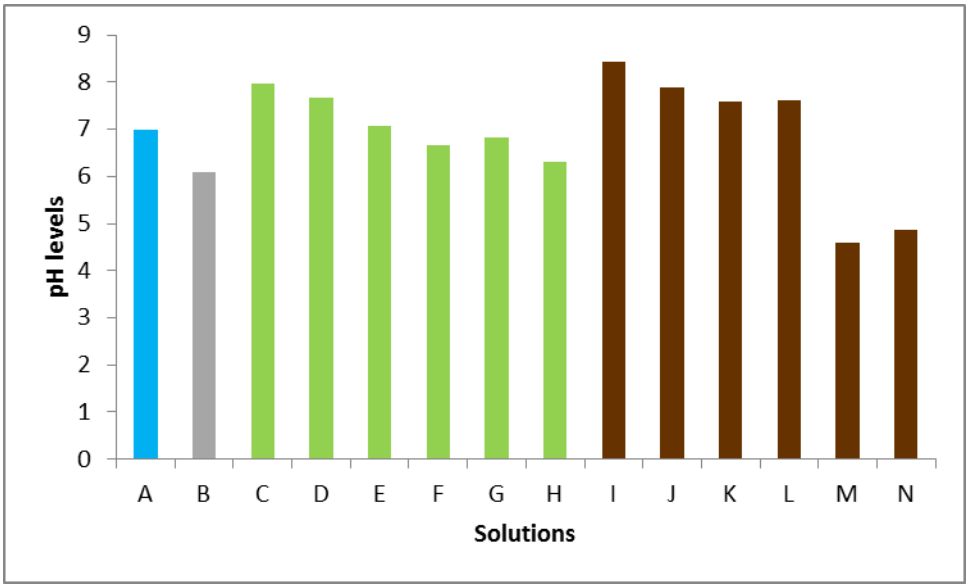


Figure 3: pH levels of the different solutions before and after synthesis of AgNPs, A) water; B) $AgNO_3$; C) calyx crude water extract; D) calyx crude methanol extract; E) AgNPs synthesized using calyx water extract (24 ºC); F) AgNPs synthesized using calyx water extract (60 ºC) ; G) AgNPs synthesized using calyx methanol extract (24 ºC); H) AgNPs synthesized using calyx methanol extract (60 ºC); I) leaf crude water extract; J) leaf crude methanol extract; K) AgNPs synthesized using leaf water extract (24 ºC); L) AgNPs synthesized using leaf water extract (60 ºC); M) AgNPs synthesized using leaf methanol extract (24 ºC); N) AgNPs synthesized using leaf methanol extract (60 ºC).

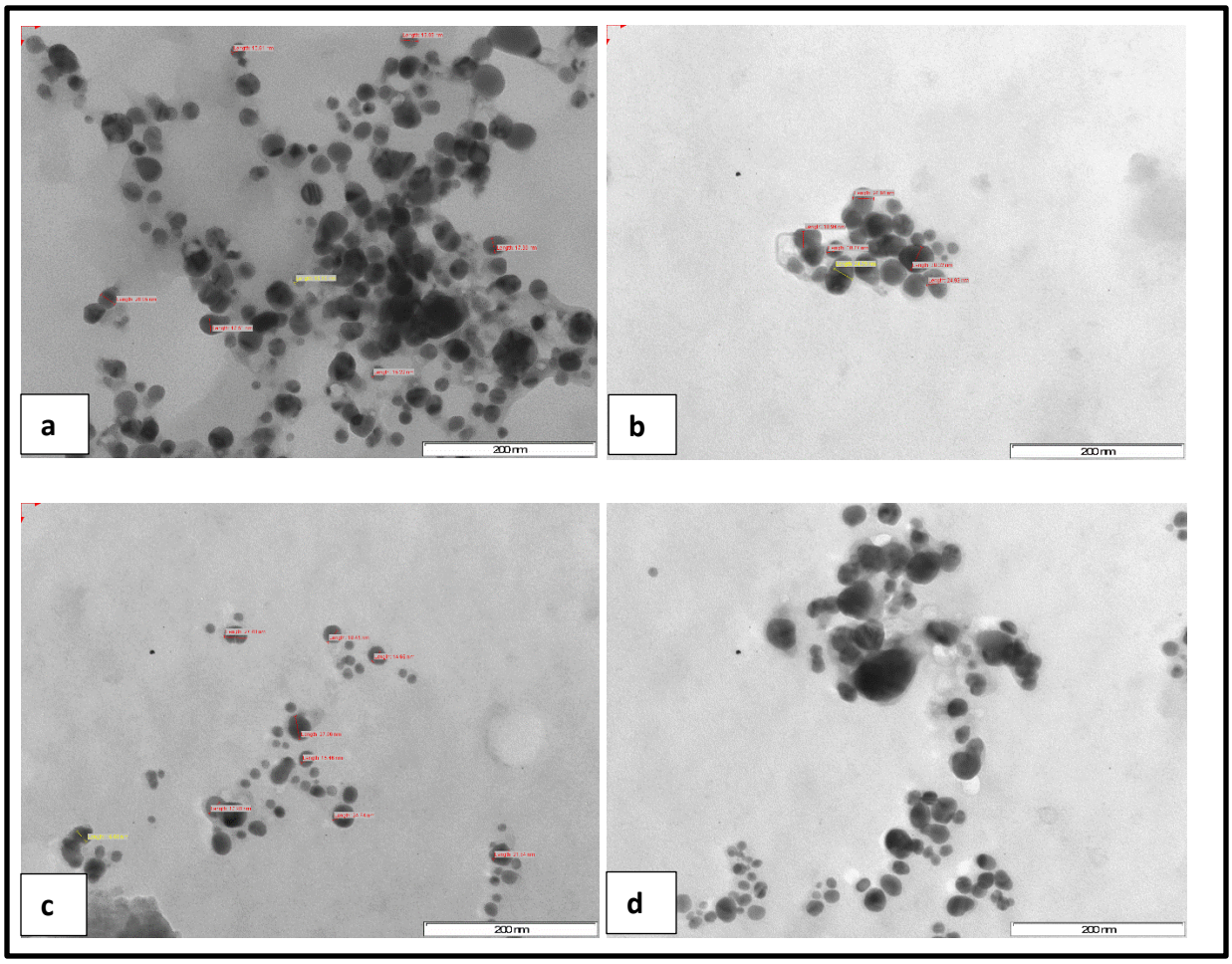


Figure 4: TEM micrographs of AgNPs synthesized at different temperatures using leaf and calyx extracts of *P. auriculata*: a) synthesis with leaf extract (24 ºC); b) synthesis with calyx extract (24 ºC); c) synthesis with leaf extract (60 ºC); d) synthesis with calyx extract (60 ºC). 200 nm scale at 200kV.

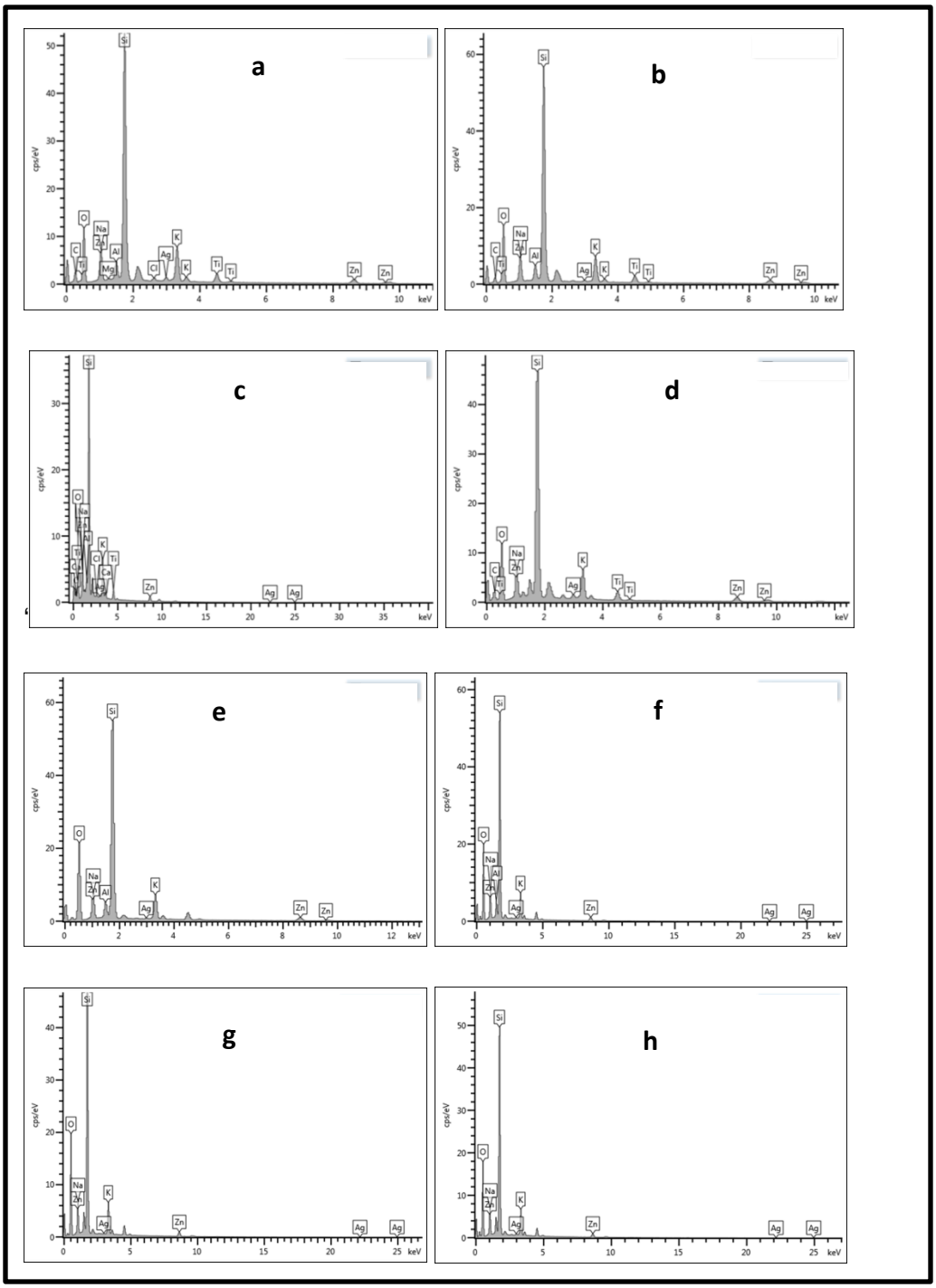
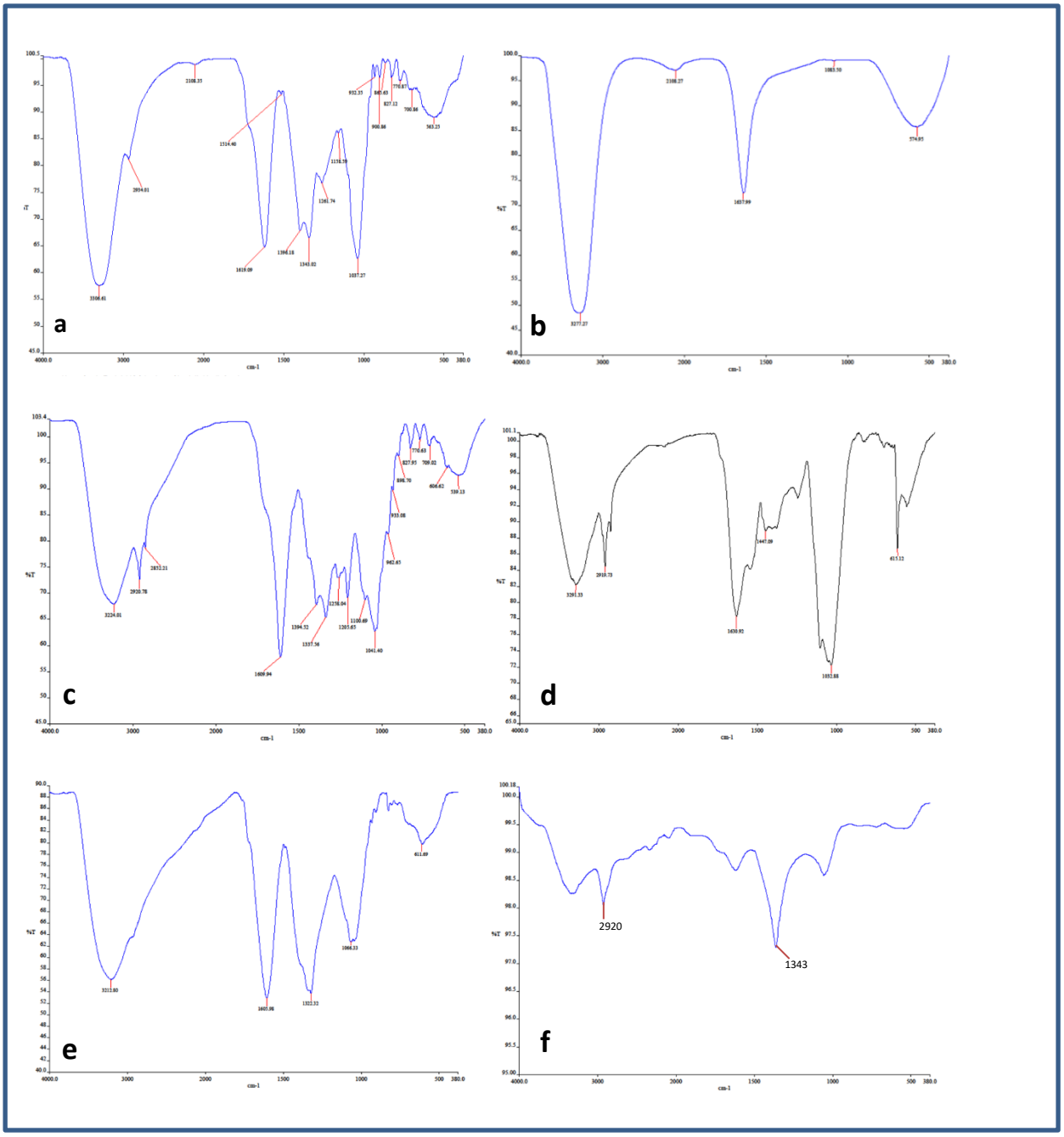


Figure 5: EDX spectra recorded after formation of AgNPs with different X-ray emission peaks labeled for the different extracts of *P. auriculata*: a) Leaf methanol (24 ºC); b) leaf methanol (60 ºC); c) leaf water (24 ºC); d) leaf water (60 ºC); e) calyx methanol (24 ºC); f) calyx methanol (60 ºC); g) calyx water (24 ºC); h) calyx water (60 ºC).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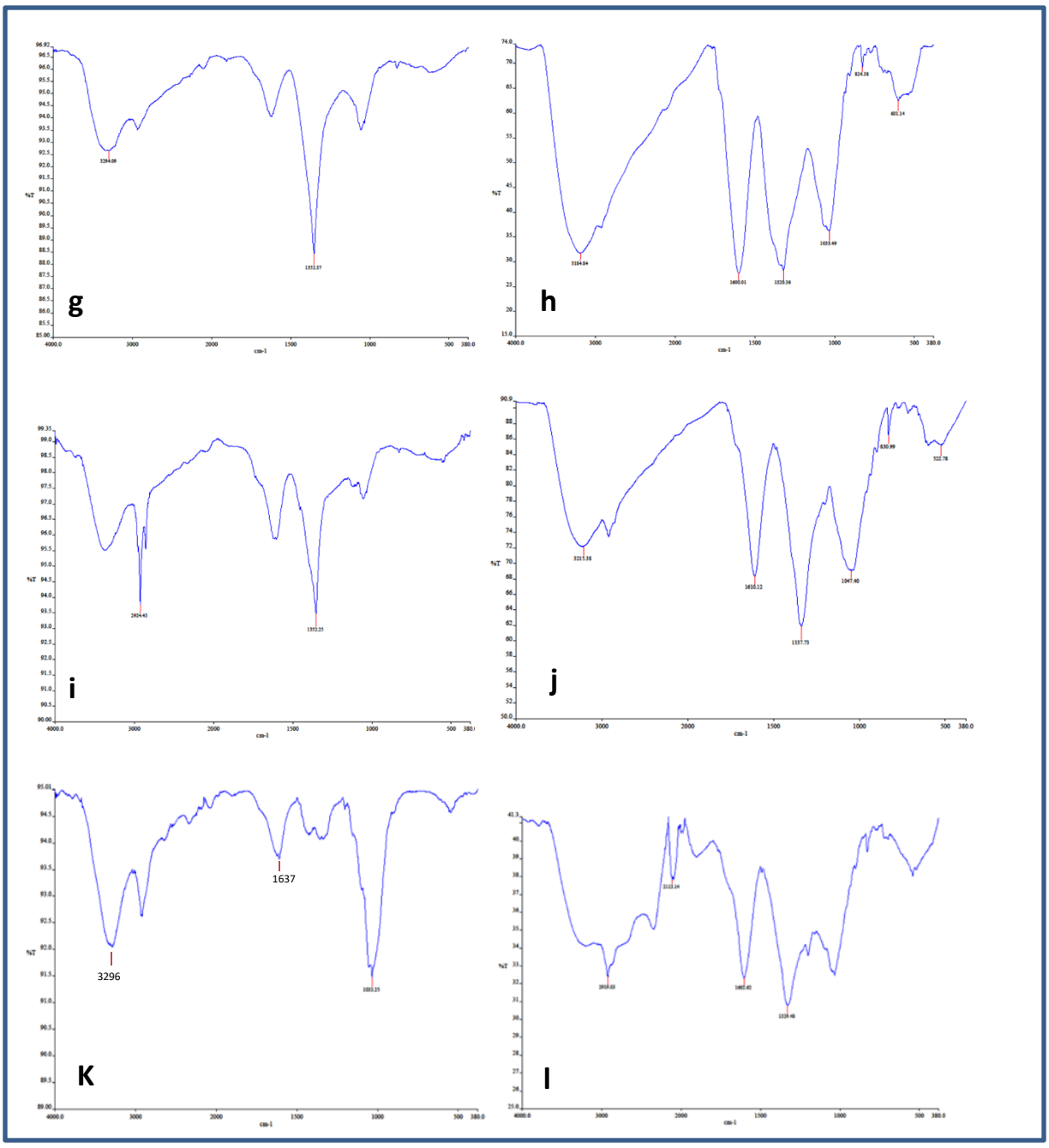

Figure 6: FT-IR spectra of crude plant extracts and biosynthesized AgNPs of *P. auriculata* a) crude leaf methanol extract; b) crude leaf water extract; c) crude calyx methanol extract; d) crude calyx water extract; e) synthesis with leaf methanol extract (60 ºC); f) synthesis with leaf water extract (60 ºC); g) synthesis with leaf methanol extract (24 ºC); h) synthesis with leaf water extract (24 ºC); i)

synthesis with calyx methanol extract (60 ºC); j) synthesis with calyx water extract (60 ºC); k) synthesis with calyx water extract (24 ºC); l) synthesis with calyx methanol extract (24 ºC).

Table 1: Minimum Inhibitory Concentration of biosynthesized silver nanoparticles.

Microorganism	**Sample**	**Minimum Inhibitory Concentration (µg/ml)**		
		Extract	**$AgNO_3$**	**AgNPs**
Escherichia coli	4	17000	15.63	15.63
	5	17000	15.63	125
	6	17000	15.63	250
	7	17000	15.63	31.25
	8	17000	15.63	1.95
Staphylococcus aureus	3	17000	10000	250
	4	17000	10000	125
Klebsiella pneumoniae	1	17000	500	250
	2	17000	500	500
	3	17000	500	125
	4	17000	500	125
	5	17000	500	125
	6	17000	500	62.5
	7	17000	500	500
	8	17000	500	125
Salmonella typhimurium	7	17000	10000	250
	8	17000	10000	250

Sample ID: 1- leaves methanol extract (60 ºC); 2- leaves methanol extract (24 ºC); 3- leaves water extract (24 ºC); 4- leaves water extract (60 ºC); 5- calyx water extract (24 ºC); 6- calyx water extract (60 ºC); 7- calyx methanol extract (24 ºC); 8- calyx methanol extract (60 ºC).

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Chapter 6

Journal Article for publication 5:

Floral Biology of *Plumbago auriculata* (Plumbaginaceae) and associated plant-insect interactions

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Abstract

Plumbago auriculata Lam. is a heterostylous species with two floral morphs that differ in the positioning of the style and stamen known as "pin" (L-morph) and "thrum" (S-morph). This species is common to South Africa but is cultivated throughout the world. It is an evergreen perennial shrub with trusses of different flower colours. This study investigated for the first time the structure of deep blue flowers, pale blue flowers and white flowers with each colour associated with different sex-morphs. Deep blue flowers were exclusively thrum morphs, white flowers exclusively pin morphs and pale blue flowers had both pin and thrum morphs. Pin flowers had a significantly larger corolla and produced bigger and a slightly larger number of pollen grains compared to the thrum flowers. Whilst the breeding system of *P. auriculata* is known to be self-incompatible, the sex-morphs of the exclusively deep blue flowers and white flowers suggest that the species might also be self-compatible. Floral visitors were mainly small insects especially butterflies that showed no colour preference towards the three colour forms. Whilst insect visitation is mainly to reap the benefits of the rewards from the flower, insects are often entrapped by the sticky exudate secreted by the glandular trichomes on the calyx. Trapped insects were mainly ants and often die in the struggle to free themselves.

Key words: Pin and Thrum, insect-entrapment, heterostylous, calyx, floral morphs, colour forms

1. Introduction

Since the 1800's, a considerable amount of research has been published on the breeding systems and pollination biology of plants. Pollination is a requirement for reproductive success in most flowering plants which involves the transfer of pollen from the anther to the stigma (McMullen and Close, 1993). The success rate of pollination is dependent on pollen viability i.e. the ability of pollen to effectively seed set (Dafni and Firmage, 2000). However, whilst seed set certainly indicates pollen viability, the lack of seed set does not necessarily indicate the lack of viability.

Heterostyly is a genetically controlled floral polymorphism occurring in at least 28 families of flowering plants, including Plumbaginaceae (Baker, 1966; Stone, 1995; Guvensen et al., 2013). Heterostyly was first investigated thoroughly by Charles Darwin in the mid nineteenth century (Ganders, 1979a). Heterostylous plants having two flower morphs are termed "distylous" (Stone 1995; Kulkarni and Baskaran, 2008). The sexual differences in sex organ levels are primarily characterized between a "Pin" (long-style, short stamen) morph with the stigmas above the anthers and the "Thrum" (short-style, long stamen) morph with the anthers above the stigma (Figure 1). Heterostyly functions to effectively increase pollen transfer between morphs (Lord and Russell, 2002; Kulkarni and Baskaran, 2008). However, the pollen from a flower of one morph cannot fertilize another flower of the same morph; this is termed heteromorphic self-incompatibility (Lord and Russell, 2002). Thus, pollen originating in a "Thrum" morph will reach, primarily, flowers with a "Pin" morph but when pollen is transferred between two flowers of the same morph no fertilization will take place due to the self-incompatibility mechanism.

Plumbago auriculata Lam. is a perennial, bushy evergreen shrub common in South Africa (Foden and Potter, 2015). It grows up to 3m high with erect, climbing or trailing stems that are glabrous below, becoming pubescent above (Batten, 1986). Thin textured, simple, elliptic to obovate leaves are slightly discolorous, greyish green beneath and often with whitish scales (Aubrey, 2001). Leaves have minute glandular dots with a winged petiole at the base and auriculate. *P. auriculata* is covered with trusses of flowers. However, there are variations of deep-blue, pale blue or white flowers (Figure 2) (Batten, 1986; Ferrero et al., 2009). Flowers are regular, salver-shaped, actinomorphic, 5-merous (pentamerous), bracteates, hypogynous, heterostylous and grouped in terminal inflorescences 2.5-3 cm long and flowering occurs throughout the year (Luteyn, 1990; Aubrey, 2001; Ferrero et al., 2009). The calyx is synsepalous,

plicate, 5-ribbed, sepals scarious, and the corolla marcescent, sympetalous or of nearly distinct petals, exserted beyond the calyx. Lobes are convolute-imbricate; 5 distinct, sometimes epipetalous stamens borne on the corolla tube opposite the lobes; anthers are in trorse and stigmas are 5 and linear, with the styles being either 1 or 5 and the ovary is 5-carpellate, 1-loculed, and usually 5-ribbed. Ovule is solitary, and the fruit a capsule or utricle, partly or totally enclosed by the persistent calyx (Luteyn, 1990). Secretory structures are found on the foliar surfaces of the plant. Salt glands occur on both leaf surfaces and trichomes are found protruding from the calyces (Faraday and Thomson, 1986; Panicker and Haridasan, 2016).

Flowers of *P. auriculata* display reciprocal herkogamy i.e style and stamen differing in length between morphs and positioned reciprocally in flowers of both morphs (De Laet et al., 1995; Ferrero et al., 2009). Heterostylous flowers in Plumbaginaceae were first decribed by Baker (1966) and Ferrero et al (2009) further demonstrated reciprocal herkogamy in the pale blue flowers (reciprocal positioning of the style and stamens in the different sex morphs) of *P. auriculata*. Studies by Ferrero et al (2009) focused on the pale blue flowers in which both sex-morphs were present. Heterostylous flowers are adapted for pollination by different pollinators or different body parts of the same pollinator (Lord and Russell, 2002).

Previous studies have found that members of this family are pollinated primarily by insects and wind. Kubitzki et al (1993) reported that pollination was carried out by bees and small beetles on the Mediterranean *Plumbago*. In 2009, Ferrero et al reported that the main pollinators of *P. auriculata* appeared to be long-proboscid flies and butterflies and the flowers were less frequently visited by birds and bees. Further studies by Panicker and Haridasan (2016) found that most insects that visited the species were winged, stingless bees, flea beetles, thrips and aphids. The stingless bees were the most abundant pollinators whilst the flea beetles and aphids were accidental pollinators because the flea beetle is a scavenger and the aphid a pest. Their study also reported that the trichomes found on the calyces secrete a sticky exudate that acts as an insect trap. Trapped insects struggle to escape and are often killed in the struggle. Due to their close resemblance to the genus *Drosera* L. the trichomes can also be considered as digestive glands due to their function in insect entrapment (Fahn, 1952; Naidoo and Heneidak, 2013). This study aimed to investigate distyly in *Plumbago auriculata* associated with the three different flower colour variants (deep blue, pale blue and white) and the trapping mechanism of the calyces.

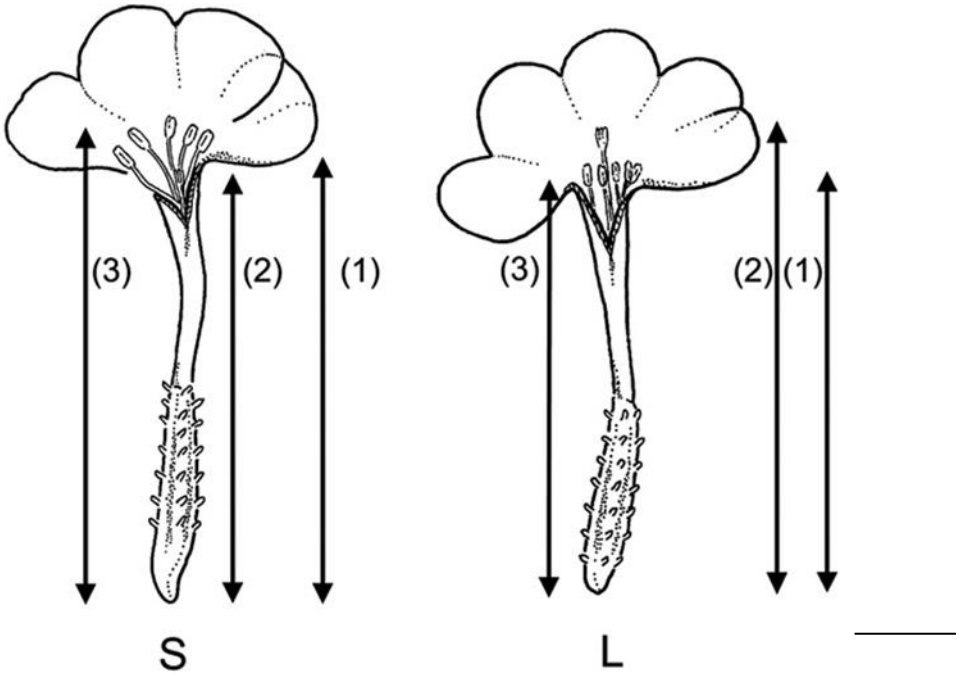

Figure 1: Diagrammatic representation of flowers of *Plumbago auriculata* showing different sex morphs a) Short-morph (S) and b) Long-morph (L) flowers. Numbers correspond to the morphometric measurements recorded for each flower: sideview: (1) corolla length; (2) style length; (3) stamen height; Scale bar =10 mm (after Ferrero et al., 2009).

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Caryophyllales

Genus: *Plumbago*

Species: *Plumbago auriculata* Lam.

Common names: Cape Plumbago, Cape Leadwort.

Figure 2: *Plumbago auriculata* showing pale blue flowers, image captured at the University of Kwa-Zulu Natal, Westville, Durban, South Africa.

2. Materials and Method

Study Species

The study species with the 3 colour forms were monitored in and around the Reservoir Hills (29.7937° S, 30.9360° E) and the Westville areas (29.8178° S, 30.9434° E) of Durban, Kwa-Zulu Natal, South Africa. Voucher specimens (Singh and Baijnath 1) were deposited in the Ward Herbarium, University of Kwa-Zulu Natal, Durban.

2.1 Floral morphometry

Flowers were observed throughout 2017 and collected during September 2017 from randomly selected shrubs (1 of each colour variant) (Figure 1) for morphometric measurements. Fifty fully developed flowers from each of the 3 colour variants were selected. Corolla tube length (distance from the base of the corolla to the corners of the corolla mouth), corolla width and stamen and style lengths were measured. Flower dissections also revealed the sex–morphs associated with each colour variant. Flowers descriptions were similar to descriptions by Luteyn (1990) and the colour of the anthers were uniform throughout the 3 colour forms.

Pollen characteristics of each colour variant were analyzed using 20 flowers each. Pollen grains were dusted onto a copper coated forvar grid and sputter coated with gold sputter coated with a Quorum Gold QISORES Sputter Coater, UK for 4 min. Pollen size, structure and exine ornamentation for each morph were then viewed with a Leo 1450 SEM, Germany, at 10 kV, WD- 18nm. Images were captured using the Smart SEM version 5.03.06.

2.2 Plant-insect interactions

Plant-insect interactions were observed in the field and colour observations with the use of stereomicroscopy. Plants were observed from 2016 till October 2017, thus allowing observations to take place at each seasonal period (winter, spring, summer, autumn). The flowering period was continuous throughout the year. The different colour variants were closely monitored for insect visitor colour preference to determine the identity and foraging patterns of the floral visitor species. Observations were carried out (30-minute per floral bush) at different periods of the day from 6:30- 7:30am (morning), 12:00- 13:00pm (mid- day),16:00- 17:00pm (afternoon) and 19:00 to 20:00pm (evening). A floral visitation event was defined as the arrival of any visitor at one of the flowers of the target bush. Stereomicroscopy was used to study insects entrapped on the sticky calyces of flowers and floral visitors. Insects were examined with the Nikon AZ100 stereomicroscope, Japan, equipped with a Nikon Fibre Illuminator and images were captured using the Nikon DXM1200C colour camera. The images were taken using the NIS-Element Software. Collected insects were provisionally identified using available manuals and published literature.

2.3 Statistical analysis

The differences between morphs and the 3 colour forms were tested by one-way ANOVA. All data were analysed using the statistical software package IBM SPSS Statistics for Windows (Version 24.0). The assumptions of normality and equality of variances were met for all ANOVA tests. A $P< 0.05$ was recognized as being significant.

3. Results

3.1 Floral morphometry

Flowers are distylous, being either pin morphs or thrum morphs, and each flower colour was associated with a specific sex-morph (Figure 3). Mean ± SD values of 4 floral characters are shown in Table 1. Measurements of corolla width and length of pin and thrum morphs of flowers revealed that there was a significant difference between pin and thrum flowers ($p < 0.05$). The corolla was larger in the pin-flowers ($p=0.003$) and, as expected, the style in the pin-flowers was significantly larger than that present in the thrum-flowers ($p= 0.012$_Table 1). There was also a significant difference for the stamen height between pin and thrum flowers with the anthers in the thrum-flowers placed higher than the pin-flowers ($p= 0.016$). (Table 1).

The characteristics of the pollen grain can be described as follows (Figure 4): shape- oblate-spheroidal; exine- sexine thicker than nexine; aperture- small to long; outline- more or less circular and ornamentation- tectum bacculate. However, pollen in the thrum-flowers was significantly larger and elongated (ovoid-shape) than pollen of the pin flowers (Figure 4b) (Table 1). Pollen grains in the pin-flowers appeared smaller, rounder (dumbbell-shaped) and slightly more grains porduced compared to thrum-flowers (Table 1; Figure 4a) Pollen grain exine- sexine was thicker than nexine, aperture- small to long; with a more or less circular outline and tectum bacculate ornamentation (Figure 4c).

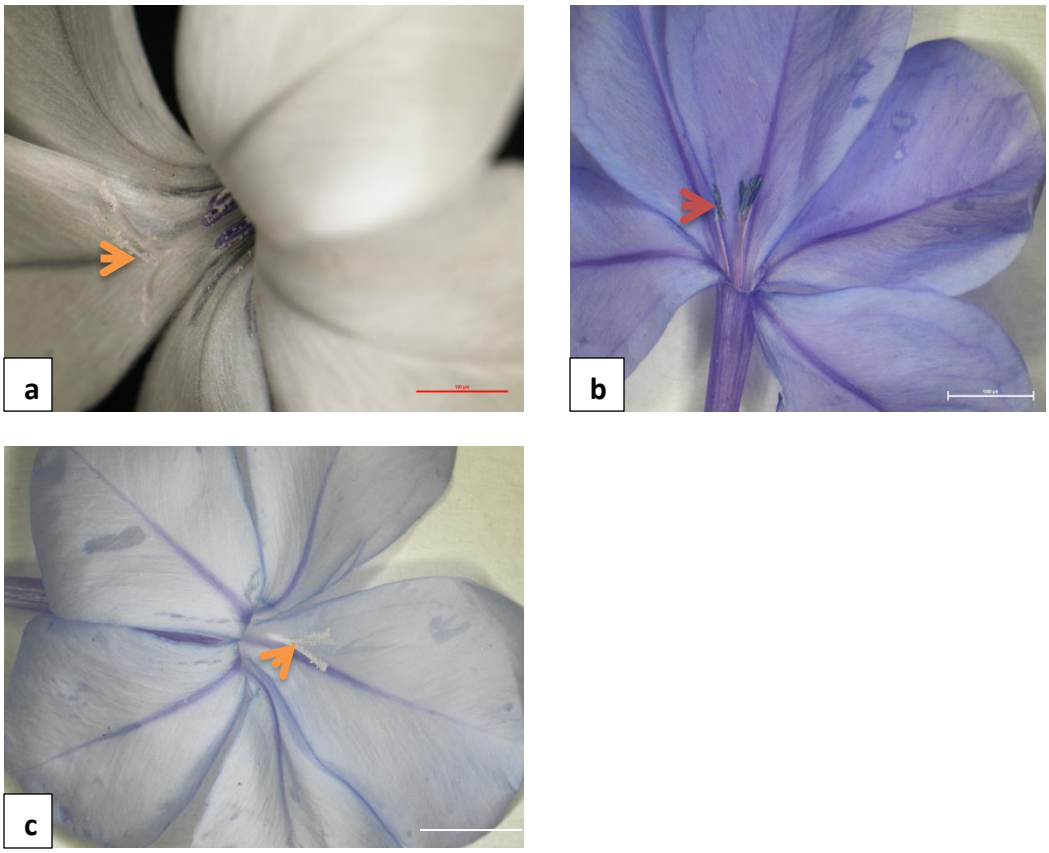

Figure 3: Sex-morphs associated with different flower colour in *Plumbago auriculata* a) Pin (L-morph) white flowers; b) Thrum (S-morph) deep blue flowers; c) Pin (L-morph) pale blue flowers. Orange arrow heads (style above stamen); red arrow heads (stamen above style). 20x magnification.

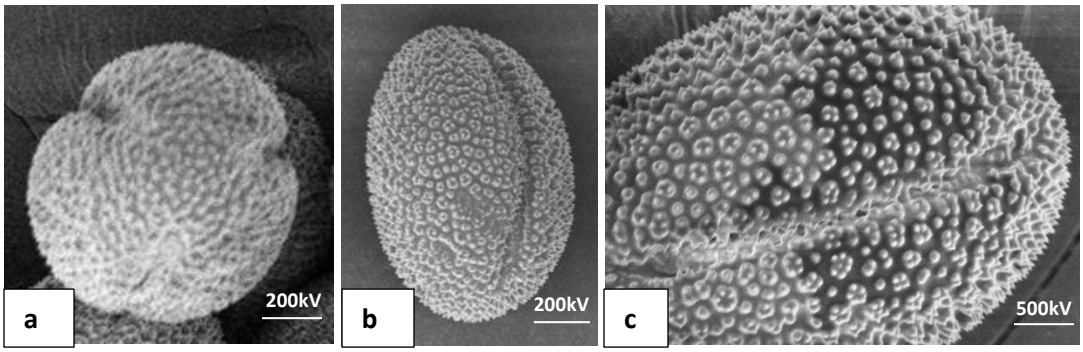

Figure 4: Pollen grains of *Plumbago auriculata* a) Pin flowers; b) Thrum flowers; c) Pollen grain exine sculpturing.

Table 1: Comparison of floral characters between flowers from 3 colour variants of *Plumbago auriculata.*

	White Flowers ('Pin")			**Pale Blue Flowers ("Pin"/ "Thrum")**			**Deep Blue Flowers ("Thrum")**			***df***	**p**
Floral Size	Mean ± SD	Range	n=	Mean ± SD	Range	n=	Mean ± SD	Range	n=		
Corolla Length(mm)	25 ± 2.2	24-26	50	22 ± 3.5	22-28	50	29 ± 1.2	27-30	50	24	0.026
Corolla Width(mm)	27.5± 2.1	25-29	50	23.3 ± 1.6	21-26	50	20.1 ± 4.2	18-22	50	23	0.003
Style Length(mm)	29.4 ± 6.8	27-31	50	26.8 ± 2.7	20-28	50	21.6 ± 0.6	20-22	50	25	0.021
Stamen Length(mm)	19.4 ± 1.8	17-21	50	22.1 ± 0.8	18-26	50	24.5 ± 2.1	21-25	50	21	0.016
Pollen width(µm)	55.3 ± 2.6	53-55	20	52.7 ± 2.4	47-55	20	48.4 ± 3.6	45-50	20	29	0.032
Pollen Length(µm)	56.7± 1.2	54-57	20	53.2 ± 2.8	52-75	20	74.6 ± 4.2	73-76	20	18	0.02
Pollen Production	1325 ± 422		20	1280± 388		20	1309 ± 315		20	33	0.04

P< 0.05 differ significantly (between morphs), SD- Standard deviation

3.2 Plant-insect interactions

Preliminary field observations revealed that the primary insect visitors are butterflies, bees, ants and beetles. Butterflies, in particular, were seen mainly foraging around the flowers but no visuals are available. Trichomes on the calyces of *P. auriculata* secrete a sticky exudate involved in insect entrapment (Figure 5). Insects entangled in a cob web of sticky exudate struggle to free themselves and often die in the process (Video 1).

Figure 5: Ant entrapment on the calyx of *P. auriculata.* 40x magnification.

Video 1: Ant entrapment on the calyx of *P. auriculata.* (Supplementary attachment Video 1).

4. Discussion

Flowers of *P. auriculata* are distylous and display reciprocal herkogamy, with style and anthers differing in length in the different sex morphs. This study was in agreement with data presented by Ferrero et al (2009). Our observations revealed that the different sex morphs are associated with the different flower colour variants. The self-incompatibility breeding system of Plumbaginaceae highlighted by Baker (1966) suggested that the reciprocal positioning of the anthers and stigmas favours outbreeding which was first recognized by Darwin in 1877. Based on the Mendelian inheritance, self-incompatible and distylous plants are controlled by a single supergene with two alleles. Ihus, thrum-flowers are heterozygous and pin-flowers are homozygous (Ganders, 1979). Thrum-flowers are known to be largely self-incompatible compared with pin-flowers because pollen from thrum anthers can germinate on a thrum stigma, rarely penetrating its surface. Hence fertilization is not achieved. This was observed in *Erythroxylum coca* (Erythroxylaceae) (Ganders, 1979). However, pollen grains from pin-flowers readily germinate on and penetrate the surface of a pin stigma but only in the absence of thrum

pollen. Ferrero et al (2009) reported pin and thrum morphs in pale blue flowers of *P. auriculata* with significant seed set in inter-morph pollination and no seed set in intra-morph pollination. The pale blue flowers in our study were in accordance with Ferrero et al (2009) but the unusual pattern observed for the white pin-flowers and deep blue thrum-flowers was the first to be reported for *P. auriculata.* Observations of the pale blue flowers in our study revealed varying gradients of the colour between the pin and thrum flowers as opposed to a uniform colour throughout the both sex-morphs. Although *P. auriculata* displays self-incompatibility, it seems likely that it is also self-compatible. Similar results were observed in *Fagopyrum esculentum* in which the species displayed both types of breeding systems by means of a supergene, the components of which control both physiological and structural characteristics of distyly (Matsui et al 2007). A higher frequency of pin/thrum flowers of *Primula veris* L. also revealed self-compatibility of wild populations, especially in the absence of thrum-flowers (Lees, 1971). Ganders et al (1979b) suggested that in species containing both breeding systems, self-incompatibility is primitive and the self-compatibility represents a derived breeding system.

Based on observations by Ganders (1979) and Ferrero et al (2009), the width of corolla of the pin-flowers was significantly greater than that of the thrum-flowers. This morphological feature promotes reciprocal positioning of the anthers to the stigmas in the L-morph because of a slight bulge low in the tube where the anthers are positioned. The morphometric description of the corolla in the present study coincides with that of Ferrero et al (2009). The stigmas of the sex morphs may differ in overall shape, size or, more commonly, in the size of the stigmatic papillae (Baker, 1966; Ganders, 1979) It has been reported that in heterostylous species pin stigmas are hemispherical and larger while thrum stigmas are somewhat flattened and smaller (Dulberger, 1975; Ganders, 1979). Differences in the length of stigmatic papillae are known in members of Plumbaginaceae with the stigmatic papillae of the pin morph being longer than those of the thrum morph. (Dulberger 1975). This was comparable with the present study. Pollen size and production difference between pin and thrum flowers highlighted for Plumbaginaceae by Baker (1966) and further reported by Ferrero et al (2009) were similar to the present study. Similar results were reported for distylous *Hedyotis salzmannii* (Rubaiaceae) but this species is self-compatible (Riveros et al., 1993). The morphological description of the pollen grains coincides with studies by Perveen and Qaiser (2004) and is uniform throughout the genus *Plumbago.*

Although the data is somewhat limited, observations made in this study provided supplementary information to findings reported by Ferrero et al (2009) and Panicker and Haridasan (2016). The main floral visitors of *P. auriculata* from their research were small insects and butterfly species *Pieris sp.* and *Papilio demodocus* Esper. However, the precise role of the butterflies with regards to pollination is not clearly understood. Lunau and Maier (1995) described that flower colours signaled floral visitors. Flower visitors showed no preferential behavior towards the three colour variants of *P. auriculata* found in our study. This aspect certainly warrants further investigation.

Capture of insects occurs on the calyx of *P. auriculata* by large glandular secreting trichomes. Studies reported by Stoltzfus et al. (2002) indicated that trichomes of *Plumbago* have a resinous secretion. It was later highlighted by Stoltzfus et al (2002) that carnivorous plants tend to have a more mucilaginous adhesive secretion which was further confirmed by Naidoo and Heneidak (2013). Graphical representation of our findings showed captured ant species trying to break free from the sticky exudate which acts as a final retention of the insect. Similar findings were reported by Naidoo and Heneidak (2013) for *Drosera capensis* L. and Panicker and Haridasan (2016) for *P. auriculata.* Although it is not certain how many insects are captured, it is clear that the most common insects entrapped on the calyx, irrespective of their species, were ants. The large amount of exudate secreted can be attributed to the large size of the glandular trichomes (Voigt et al., 2009; Naidoo and Heneidak, 2013). Stoltzfus et al. (2002) reported that *P. auriculata* is capable of secreting protease in response to stimulation by several factors, including captured insects, but no conclusions were made. However, Naidoo and Heneidak (2013) reported the protease activity for *Drosera* species.

5. Conclusion

Plumbago auriculata is a heterostylous species with “Pin” and “Thrum” flowers. Plants have always been characterized as having pale blue flowers with either pin or thrum plants. This study was the first to report additional flower colour forms with each having a separate morph (white-pin or deep blue-thrum). In this regard, the study is novel. The breeding system of this species is reported to be self-incompatible. However, based on our findings with regards to flower colour, it can be inferred that this species is also self-compatible. Pin-flowers had a larger corolla, style, pollen width and produced slightly more pollen grains compared to the thrum-lowers. Small insects and butterflies were the main floral visitors and showed no preference to flower colour.

Ants were the main insects entrapped on the calyx and this was mainly as a result of the sticky exudate secreted by the large glandular trichomes. We propose a comprehensive monitoring measure to survey the number of individuals of pin and thrum plants of the different flower colours within smaller populations to find out the exact mechanism of their breeding system.

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Chapter 7

Conclusions and Recommendations for Further Research

7.1 Introduction

The lack of knowledge focusing on *P. auriculata* in South Africa formed the foundation for this study that sought to provide a greater insight into the structure, biology and chemistry of this species by documenting in a structured manner the genus *Plumbago* and its possible uses as phytomedicine. A greater understanding of the species, which is characterized by secretory structures and different flower colours was also provided. The investigation of the leaves and calyces to effectively synthesize nanoparticles and, testing its antibacterial activity makes this study unique.

The family Plumbaginaceae has approximately 24 genera and about 400 species (The Plant List, 2017). *Plumbago* is the largest genus in the family comprising 18 species distributed and utilized throughout the world in warmer regions (APG II, 2009; The Plant List, 2017). The review on the genus presented in this study provided resourceful information on the distribution of species and highlighted the medicinal value of the genus as a whole. Like other members of the genus, *Plumbago auriculata* also has high medicinal importance (Deshpande *et al*., 2014; Singh et al., 2018). Plants are rich in secondary compounds that offer therapeutic benefits against a wide range of human ailments (Liew and Keong Yong, 2016). The usage of plants as an alternative to western medicine has been accepted on a global scale (Hassan, 2012). With the naturally available flora, affordable treatments that may benefit a large portion of the global community can be formulated (Afzal *et al*., 2011). The medicinal properties of *P. auriculata* were attributed to the combination of phytochemical compounds in the plant which was previously reported by Tharmaraj and Antonysamy, (2013).

In the present study preliminary phytochemical screening of *P. auriculata* and various histochemical tests confirmed the presence of phytochemical compounds found in the leaves and

calyces responsible for the medicinal properties for which the plant is traditionally and pharmacologically exploited (Deshpande *et al*., 2014; Lakshmanan *et al.*, 2016; Singh *et al*., 2018). Gas chromatography-mass spectrometry results further revealed the presence of 50 phytocompounds in the hexane extract of the leaves and 20 phytocompounds in the hexane extract of the calyces. The presence of various phytocompounds confirmed the application of *P. auriculata* for various diseases by traditional practitioners. Although the GC-MS analyses has been reported for the first time in this study, the phytocompounds found in the extracts have previously been isolated from other medicinal plants and were believed to play an important role in plant defense as well as possess many pharmacological properties (Sharma *et al*., 2015; Abubaker and Majinda, 2016). Thus, this type of study provides information on the nature of active compounds present in the medicinal plants.

The structural features of this study showed that *P. auriculata* possesses secretory structures on the surface of the leaves and calyces. Salt glands were found on both surfaces of the leaves, but were more abundant on the abaxial leaf surface based on visual observations due to the presence of secreted salt crystals. The function of salt glands is to secrete ions to the outside of the plant or within the plant sequestered into vacuoles (Thomson, 1975; Amarasinghe and Watson, 1989). The presence of abundant mitochondria in this study supported Luttage (1975) who stated that "ion secretion is an active process." In an attempt to understand the secretory process, this study reported for the first time the presence of "transfer cells" characterized as wall ingrowths in cell walls that facilitate ion transport between interconnected protoplast by increasing the surface area (Gunning and Pate, 1969). Abundant mitochondria, ribosomes, mini vacuoles, dictyosomes and rough endoplasmic reticulum cisternae appeared to be actively involved in the secretory process. Salt glands in the *Plumbaginaceae* have been described by Faraday and Thomson (1986) and revised by Grigore and Toma (2016). The results presented in this study provides supplementary information to the existing body of knowledge.

This study revealed two trichome types on the calyx of *P. auriculata,* i.e. non-glandular and glandular capitate trichomes. These trichome types were reported by Panicker and Haridasan (2016) but their fine structure and mode of secretion was lacking in knowledge. The non-glandular trichomes were uniserate and it is generally assumed that these types of trichomes serve as a mechanical barrier and a defence mechanism for the plant (Payne, 1978; Gonzales *et*

al., 2008). The length of the non-glandular trichomes in this study was similar to that reported for *Plumbago zeylanica* (Panicker and Haridasan, 2016) but contrasted in length for members of the Lamiaceae (e.g *Pogostemon cablin* Benth.) (Rusydi *et al*., 2013) and Solanaceae (e.g *Withania somnifera* (L.) Dunal) (Munien et al., 2015).

The second type of trichome was of the glandular capitate type. Ultrastrutural observations of these trichomes confirmed the secretory function in the head cells by the presence of many mitochondria, dictyosomes, golgi bodies and elaborate endoplasmic reticulum cisternae. On the basis of morphology, these trichomes can be termed "*colleters*" because they are made up of a multicellular stalk and a multicellular head that secrete a sticky exudate (Fahn, 1952). However, based on functionality these trichomes can be termed "*digestive glands*" due to their function in insect entrapment (ICPS, 2008). Findings in this study demonstrated the insect entrapping mechanism of the glandular trichomes by secretion of the sticky exudate causing death of the insect in a struggle to free itself. Similar findings for *P. auriculata* were reported by Stoltzfus et al (2002) and Panicker and Haridasan (2016). Naidoo and Heneidak (2013) also reported the resemblance of these trichomes to *Drosera capensis* L. Histochemical tests revealed the presence of phenolic compounds which apart from their medicinal properties, have been suggested to play a role in insect entrapment by gluing the insect to the surface of the calyx (Dai and Mumper, 2010). In this study ferric trichloride confirmed the presence of phenolic compounds in the secretory head of the glandular capitate trichomes. This further substantiates the insect entrapment mechanism of the glandular trichomes highlighted in this study. However, a comprehensive study is needed to verify the precise insect entrapping mechanism.

The next sector of the study was designed for green synthesis of silver nanoparticles and their antibacterial evaluation. In the present investigation, novel approach for biosynthesis of AgNPs from leaf and calyx extracts of *P. auriculata* was given. The silver nanoparticles synthesized by a bio-reduction method exhibit all the characteristic features of nanoparticles (Banarjee *et al*., 2014; Mohammed, 2015). FT-IR analysis revealed the possible involvement of phytoconstituents as effective capping and stabilizing agents of AgNPs. Most importantly, the AgNPs demonstrated good antibacterial activity against both gram-negative and gram-positive bacteria which make them a reliable source of antibacterial agents (Appendix 1). This enhanced

antibacterial effect can be attributed to the presence of the several phytochemical compound classes highlighted in this study. Nanostructured systems are suggested to have the ability to potentiate the action of plant extracts thereby improving its activity whilst reducing its required dosage and side effects (Bonifacio et al., 2014). This study confirmed bacterial activity at a lower AgNP concentration than the crude extracts. Results obtained from this study confirmed this protocol to be simple, rapid, convenient, environmentally safe and non-toxic. Although synthesis in this study was shown on a small scale, due to the effectiveness of this method it can easily be used for larger scale particle synthesis due to the simplicity of this method as opposed to chemical and physical particle synthesizing methods (Jain et al., 2009; Subbenaik, 2016). Therefore, these studies would certainly be useful in the development of AgNPs for potential drug development opening doors for a new range of human multi-pathogenic drugs.

Despite their interest as alternative drug plants, very little is known of the biology of this *P. auriculata*. The final trial of this study linked flower colour and floral morphs as revealed for the first time, three flower colour variants (white, pale blue and deep blue). Visual interpretation of data in this study confirmed that white flowers were exclusively "Pin" flowers; deep blue flowers were exclusively "thrum" flowers and pale blue flowers had a mixture of "Pin" and 'Thrum" flowers on separate plants. Whilst the results for the pale blue flowers presented in this study were in accordance with previously reported studies (Ferrero et al., 2009); the results for the white flowers and deep blue flowers were reported for the first time - in this regard the study was novel. In the family Plumbaginaceae heterostyly and the self-incompatibility system were extensively studied by Baker (1966) and afterwards several studies have been carried out in a number of genera of this family (e.g. Ferrero & al., 2009; Morretti et al., 2015). According to Baker, the elaborate expression of evolution of these systems is represented by *Limonium vulgare* Mill. which presents reciprocal herkogamy and other characteristic features of heterostylous plants. In this species it is explainable that, based on the reactions of heteromorphic incompatibility systems typical of Plumbaginaceae, pin flowers are compatible to thrum flowers and vice versa. However, the sex-morphs of the white flowers and the deep blue flowers suggest that flowers are likely to also be self-compatible (pin + pin; thrum + thrum) by means of a supergene. The stereomicroscope interpretation of the three flower colour variants confirmed that the pin-flowers had a broader corolla regardless of the flower colour in order to accommodate for the low positioning of the anthers in the corolla tube according to Ganders (1979). Consequently,

the pin stigmas were larger than the thrum stigmas as commonly described for the Plumbaginaceae (Baker, 1966). SEM survey of pollen grains highlighted a comparable difference in pollen size and production between pin and thrum-flowers but showed no obvious differences in exine sculpturing. This study further explored preferential behavior of floral visitors towards the three flower colours. Although the main floral visitors were small insects, including butterflies, this study indicated that no definite colour preference was noted and the role of these insects in pollination was undefined due to the inability to quantify the precise role of each pollinator type in promoting legitimate pollen transfer. In addition to floral visitation for the benefits of the rewards insects are often trapped on the calyx by the large capitate trichomes as previously highlighted. In Chapter 6 it is emphasized that ants were the common insects entrapped in the sticky exudate which was confirmed by means of graphical illustrations in Figure 5 and Video 1.

7.2 Aims and objectives

This thesis set out to investigate the structure, biology and chemistry of *P. auriculata* using a variety of microscopic, chemical and field methods

In order to develop a better understanding of the structure and functioning of the secretory apparatus, micromorphological studies were accomplished using a variety of microscopic methods such as stereomicroscopy, light microscopy, SEM, TEM, EDX and implementing already established preparatory techniques. The chemical composition of these secretory structures was also analyzed using histochemical and phytochemical assays and GC-MS analysis in order to substantiate the biological/medicinal properties of the species.

This study further attempted to synthesis silver nanoparticles under optimum conditions with the use of leaf and calyx extracts endowed with potential antibacterial properties. Biosynthesized nanoparticles were characterized with an array of techniques (UV-Vis Spectrophotometry, SEM, TEM, EDX, FT-IR).

Floral investigations aimed at interlinking flower colours with floral morphs. This was achieved through stereomicroscopy and SEM. In addition to microscopic methods, field observations were undertaken to develop a better understanding of the plant-insect interactions associated with pollination and insect entrapment.

7.3 Challenges

The greatest challenge in this study was the floral biology. Besides being knowledgeable about the different sex-morphs associated with the three colour variants, the time spent becoming familiar with the floral phenology (timing of biological events) was limited and subjected to an array of environmental conditions that often left flowers in a poor condition for monitoring. Studies on the phenology of medicinal plants is vital in developing a basic knowledge with regards to the right season of the plant collection for medicinal purposes. Furthermore, environmental conditions also posed problems for plant pollinators as cold/rainy weather in particular has been reported to reduce insect activity (Frost, 2014). Some pollinators were mobile, in flight and sometimes difficult to capture or identify therefore a large portion of possible pollinators remain undocumented. In order to overcome some of these challenges plants were monitored under laboratory conditions and microscopic techniques were used for detailed visual observations and imaging.

7.4 Future possibilities

The genus *Plumbago* is of high medicinal importance throughout the world. This genus comprises 18 species but only 40% of the species has been researched for their medicinal uses. This lack of knowledge of some species provides an opportunity for further research.

Micromorphology of the salt glands contributes to the existing body of knowledge. However, to the best of my knowledge their physiological mechanisms are not documented. Therefore it would be interesting to determine their physiological responses to stress factors, the salt tolerance levels of the species, the effects of increased rates of salinity on growth, as well as the productivity and photosynthetic characteristics of *P. auriculata.*

Although there are currently few studies based on the morphology of the trichomes of *P. auriculata* to better understand their trapping mechanisms, further studies are needed to link morphological features of trichomes with physiological or chemical profiles.

Phytochemical screening of the plant extracts revealed the presence of several compound classes of medicinal importance highlighting *P. auriculata* as a good candidate for further isolation and purification of the therapeutically active compounds towards sustainable drug development.

The extracts of *P. auriculata* effectively synthesized silver nanoparticles. The use of this plant extracts can further be explored for the biosynthesis of other metals such as copper or gold. Applications of the biosynthesized nanoparticles can be tested against a wider range of pathogens. Further studies can explore the potential use of biosynthsized nanoparticles on plant pathogens as a means of an eco-friendly, less toxic biological control method. Further studies are required to highlight the biosynthesis process of AgNPs and determine its toxicity levels. Also there is a need to verify whether microorganisms develop resistance towards the biosynthesized nanoparticles and vigourously examine cytotoxicity levels of these nanoparticles towards human cells before proposing or administering their therapeutic use.

Described as a heterostylous, self-incompatible species, the biology of *P. auriculata* displayed interesting features of the plant that needs to be further explored in detail to gain a better understanding on the precise breeding mechanism of the species. Genetic studies will hopefully provide an opportunity to better understand various factors that influence flower colour evolution.

7.5 Final comments and summary conclusions

Plumbago auriculata is an important medicinal plant globally that is used in the treatment of various diseases. This study definitively proved that *P. auriculata* leaf and calyx extracts contain many biologically active compounds. It also provides a detailed insight about the phytochemical profile which could be exploited for plant-based drug development.

Microscopical interpretation of the leaves and calyces revealed the presence of salt glands and trichomes on these structures with interesting features characteristic to each structure. “Transfer cells” were reported for the first time in the salt glands of *P. auriculata.* The glandular capitate trichomes on the calyces were implicated in insect entrapment.

For the first time leaf and calyx extracts proved to be effective in synthesizing AgNPs and showed good antibacterial activity against gram negative and gram positive bacteria. Therefore,

this method can be applied for rapid, cost effective, and an eco-friendly way of synthesizing AgNPs which can further be used in various biological and medical applications. The work adds to the confirmation of previous reports on biosynthesis of metal nanoparticles using plant extracts.

The discovery of three flower colour variants and the associated sex-morphs has set in motion an array of questions regarding the breeding system of *P. auriculata.* This study provides new insights on the breeding system of heterostylous flowers.

Overall *P. auriculata* has proved to have a complex diversity of phytochemical compounds that could potentially play an important role in preventing the progression of many human ailments.

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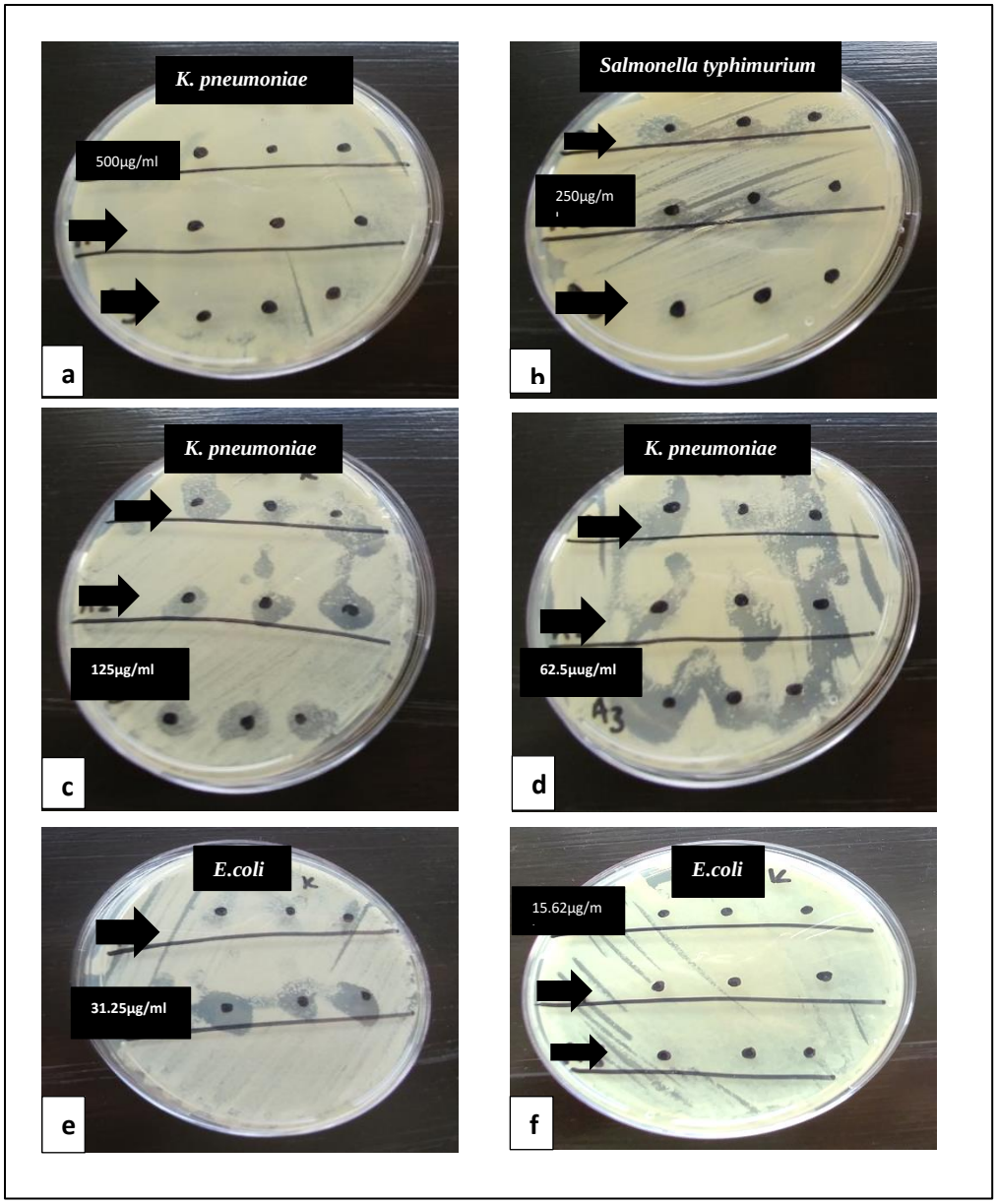
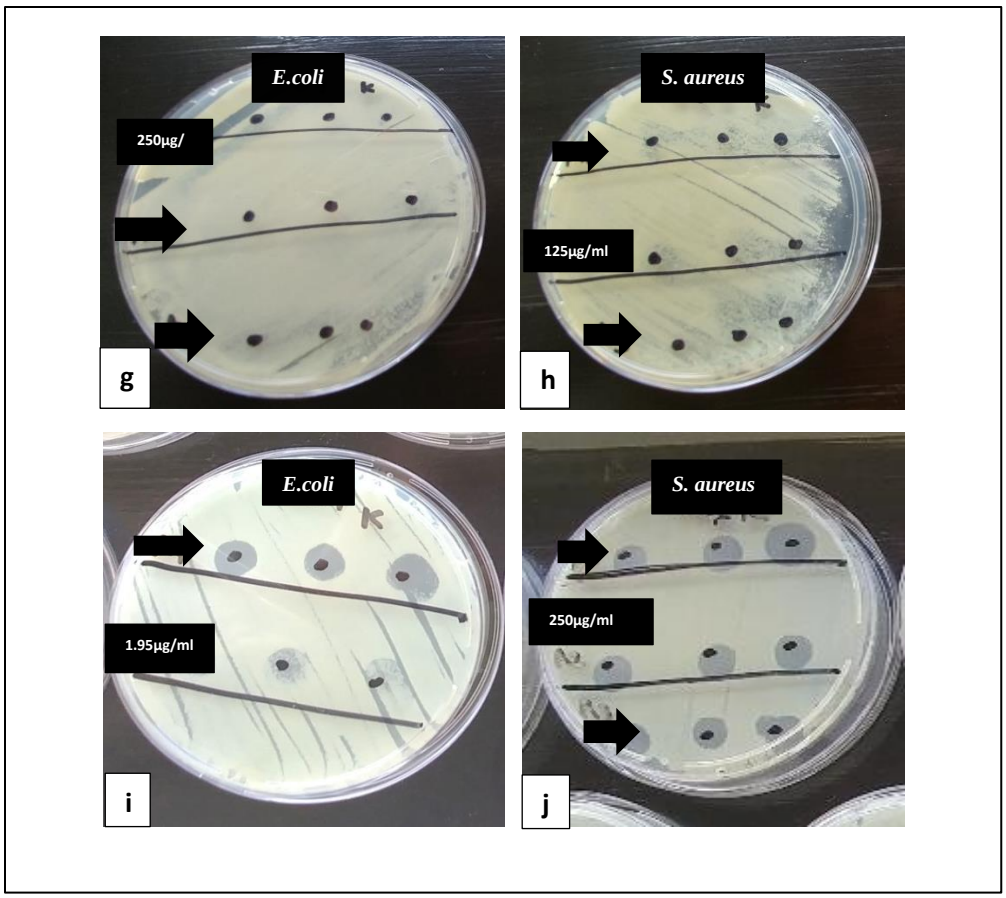
Appendix 1

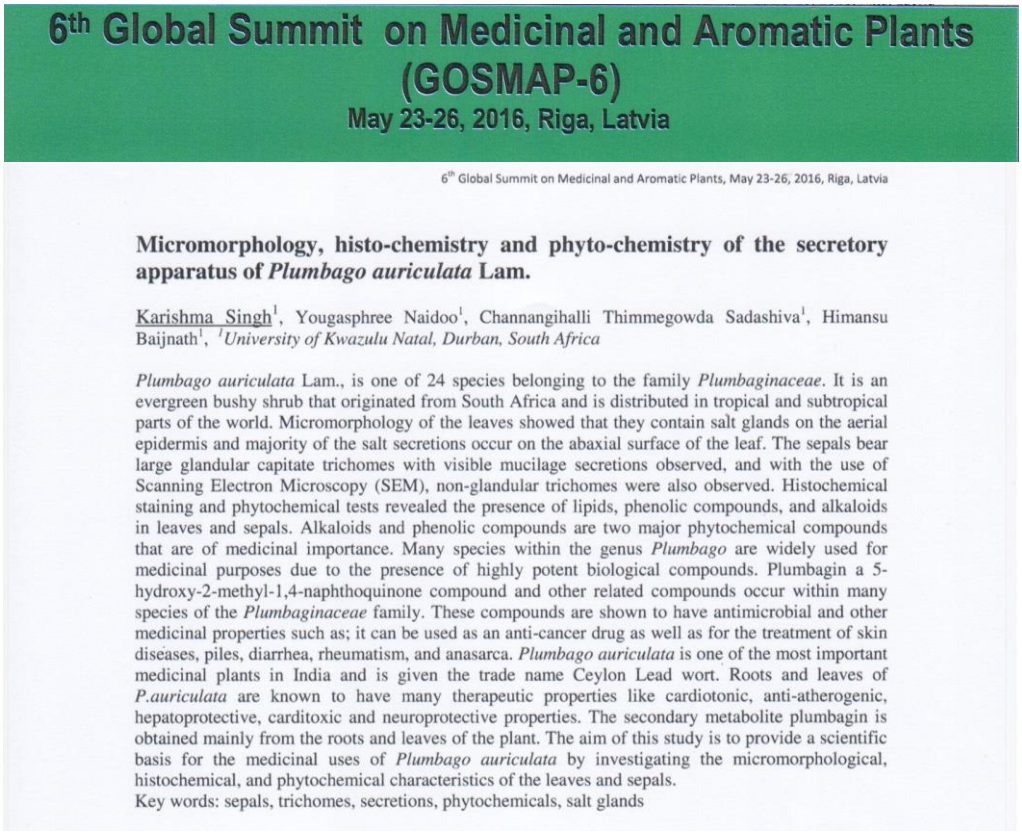
Representative Minimum Inhibition Concentration of biosynthesized AgNPs using extracts of *P. auriculata* against bacterial strains.




E.coli
250µg/
g
S. aureus
125µg/ml
h
E.coli
1.95µg/ml
i
S. aureus
250µg/ml
j

Appendix B

International Conference Attendance & Certificate



6th Global Summit on Medicinal and Aromatic Plants (GOSMAP-6)
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Micromorphology, histo-chemistry and phyto-chemistry of the secretory apparatus of *Plumbago auriculata* Lam.

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Plumbago auriculata Lam., is one of 24 species belonging to the family *Plumbaginaceae*. It is an evergreen bushy shrub that originated from South Africa and is distributed in tropical and subtropical parts of the world. Micromorphology of the leaves showed that they contain salt glands on the aerial epidermis and majority of the salt secretions occur on the abaxial surface of the leaf. The sepals bear large glandular capitate trichomes with visible mucilage secretions observed, and with the use of Scanning Electron Microscopy (SEM), non-glandular trichomes were also observed. Histochemical staining and phytochemical tests revealed the presence of lipids, phenolic compounds, and alkaloids in leaves and sepals. Alkaloids and phenolic compounds are two major phytochemical compounds that are of medicinal importance. Many species within the genus *Plumbago* are widely used for medicinal purposes due to the presence of highly potent biological compounds. Plumbagin a 5-hydroxy-2-methyl-1,4-naphthoquinone compound and other related compounds occur within many species of the *Plumbaginaceae* family. These compounds are shown to have antimicrobial and other medicinal properties such as; it can be used as an anti-cancer drug as well as for the treatment of skin diseases, piles, diarrhea, rheumatism, and anasarca. *Plumbago auriculata* is one of the most important medicinal plants in India and is given the trade name Ceylon Lead wort. Roots and leaves of *P.auriculata* are known to have many therapeutic properties like cardiotonic, anti-atherogenic, hepatoprotective, carditoxic and neuroprotective properties. The secondary metabolite plumbagin is obtained mainly from the roots and leaves of the plant. The aim of this study is to provide a scientific basis for the medicinal uses of *Plumbago auriculata* by investigating the micromorphological, histochemical, and phytochemical characteristics of the leaves and sepals.

Key words: sepals, trichomes, secretions, phytochemicals, salt glands

6th Global Summit on Medicinal and Aromatic Plants

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CERTIFICATE

This to certify that Ms. Karishma Singh (South Africa) has presented paper(s) during the 6th Global Summit on Medicinal and Aromatic Plants, May 23-26,2016 Radisson Blu Hotel, Riga, Latvia.

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