Foliar Secretory Cavities of *Vepris lanceolata* (Lam.) G. Don (Rutaceae): Micromorphology and Chemical Composition of the Secretion.

by

NOZIPHO NTOMBIKAYISE NXUMALO

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PREFACE

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

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

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Signed: Professor Yougasphree Naidoo

Date: December 2017

Signed: Professor Gonasageran Naidoo

Date: 7 December 2017

DECLARATION: PLAGIARISM

I, Nozipho Ntombikayise Nxumalo, declare that:

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

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

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

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

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

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ABSTRACT

Secretory structures such as ducts, trichomes and cavities consist of cells that are primary sites synthesizing essential oils and other phytochemical compounds with medicinal properties. Little is known about the micromorphology of secretory structures and the composition of the chemical constituents. There was no information documented on the micromorphology of secretory structures of *Vepris lanceolata* (Lam.) G. Don (Rutaceae family). The aim of this research was to investigate the micromorphological characteristics of foliar secretory cavities, the chemical composition of the secretion, and the antibacterial activity of leaf extracts of *Vepris lanceolata*. Scanning electron microscope (SEM) images by chemical fixation and freeze drying revealed no external secretory structures on the surfaces of leaves. SEM images by freeze-fracture showed secretory cavities present in the leaf blade. The cavities were embedded amongst palisade and spongy parenchyma cells, next to the vascular bundle. Cavities were made up of the lumen surrounded by varying layers of epithelial cells, depending on the secretory phase of the cavity. Semi-thin and ultra-thin sections showed that foliar cavities develop schizo-lysigenously, i.e. cavities develop by both separation and degradation of epithelial cells. Transmission electron microscope (TEM) sections showed that during the secretory stage, secretory cells contained oil droplets, vacuoles and vesicles indicating active secretion. Histochemical assays of fresh leaves showed the localization of phytochemical compounds. Cavities turned orange red when stained with Sudan III indicating the presence of lipids and pink with NADI reagent to show essential oils. Cavities also stained positive for polysaccharides, sugars, phenolic compounds, proteins and alkaloids. Phytochemical screening showed the presence of alkaloids, glycosides, carbohydrates, proteins, tannins, phenolic compounds, flavonoids, fixed oils and fats. Preliminary thin layer chromatography (TLC) showed separation of bands indicating groups of active compounds in leaf extracts. Crude (ethanolic and methanolic) and water extracts of leaves showed antibacterial activity against gram positive bacteria *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (ATCC BAA-1683); and five strains of gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Escherichia coli* (carbapenem-resistant) (ATCC BAA 2340), *Klebsiella pneumonia* (ATCC 314588), *Pseudomonas aeruginosa* (ATCC 27853), as well as *Salmonella typhimurium* (ATCC

14026) according to the disc diffusion method. Leaf extracts have tannins, alkaloids, flavonoids, essential oil and flavonoids responsible for the antimicrobial activity of the plant.

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CHAPTER 1: INTRODUCTION

1.1 Rationale for the research

Plants have been used as medicinal herbs and products for many centuries to treat illnesses (Bourgaud et al. 2001; Balunas and Kinghorn, 2005; Atanassova et al. 2011; Maobe et al. 2013; Abdelhalim et al. 2017; Habtemarlam, 2017; Mathur and Hoskins, 2017; Mustafa et al. 2017; Xie and Zhou, 2017). Approximately, 80% of people globally who reside in developing countries cannot afford or have limited access to health infrastructure are dependent mainly on the use of traditional medicine as their primary health care to treat illness (Atanassova et al. 2011; Chang et al. 2017; Fenetahum et al. 2017; Iikasha et al. 2017; Mustafa et al. 2017).

Traditional practitioners use mineral material, plants, animals and other acceptable methods to provide sufficient health care by acting as health care facilitators to the community (Elujoba et al. 2005; Kyoshabire et al. 2017; Mustafa et al. 2017). These can be healers, bone setters, traditional psychiatrists, occult practitioners and herb sellers (Elujoba et al. 2005). Traditional practitioners serve the community by providing ointments, dried powders, concoctions, infusions, macerates, decoctions and tinctures which are used as oral, topical, rectal, subcutaneous and intra-uterine applications (Hamilton, 2004; Elujoba et al. 2005). The major part of herbal therapy, however, involves the use of plant extracts or their active compounds (Farnsworth et al. 1985; Mathur and Hoskins, 2017; Xie and Zhou, 2017). Although not legally authorised to practice, traditional healers are still considered as the primary health care givers in most rural areas in developing countries (Elujoba et al. 2005).

Medicinal plants have been found to produce many useful phytochemicals that have biological activities and can be used to treat diseases (Combrik et al. 2007; Nair and Chanda, 2007; Atanassova et al. 2011; Habtemarlam, 2017; Mathur and Hoskins, 2017). More than 30% of plant families are used in medicine, food, beverages, flavouring,

repellents, fragrance, cosmetics and for dieting (Edeoga et al. 2005; Djeridane et al. 2006; Atanassova et al. 2011; Kala et al. 2006; Mustafa et al. 2017; Rahman et al. 2017). A major part of aromatherapy involves the use of plant extracts or their active compounds (Farnsworth et al. 1985). Furthermore, medicinal compounds are used as taxonomic markers, and their natural compounds used as therapeutic agents and in studies to discover new synthetic compounds (Akerele, 1992; Calixto 2005; Habtemarlam, 2017). Various plant species are being investigated for potential medicinal cures. Therefore, there is still a lot of information that needs to be published.

1.2 Justification and research question

Phytochemical compounds possess biological or medicinal properties and are possible sources of new drugs. Secretory structures produce abundant useful phytochemical compounds. Secondary metabolites have different points of synthesis and localization and are found in vacuoles or specialized structures such as ducts, glandular trichomes, laticifers and secretory cavities (Palazzolo et al. 2013). These structures have specialized cells that are primary sites that synthesize essential oils and other phytochemical compounds and are located in leaves, bark, roots, stems, buds and fruits secretions (Palazzolo et al. 2013). Secretory cavities are used in taxonomy to identify particular families such as Rutaceae because cavities are found in all species of this family (Afoloyan and Meyer, 1995; Viera et al. 2001). The Rutaceae family produces essential oils and compounds that have high value in the commercial industry (Palazzolo et al. 2013). Many plants produce essential oils, steroids, saponins, phenolics, alkaloids, flavonoids, tannins and many other compounds. These chemical constituents often possess medicinal activities such as antimalarial, anticancer, antimicrobial and anti-oxidative properties. However, only about 6% in 250 000 plants have been screened and studied for biological activities and 15% have been evaluated phyto-chemically (Fabricant and Farnsworth, 2001).

Vepris lanceolata (Figure 1.1) is a 5 m shrub or 24 m tree that grows in tropical and subtropical regions (Lemmens, 2008; Kiplimo, 2012; Tekin and Eruygur, 2016). It has been used traditionally to treat fever, flu, colic, barrenness, menorrhagia and respiratory diseases (Steenkamp, 2003; Narod et al. 2004; Poullain et al. 2004; Fern, 2012).

Little is known about the morphology of secretory structures responsible for secreting phytochemical compounds and essential oils in most southern African plants. The chemical composition of the extracted compounds is also largely unknown. How does the micromorphology and chemical composition of the secretion assist in understanding the medicinal value of *Vepris lanceolata* was the research question that was addressed by this study? Hence, the focus was to evaluate the medicinal value of the plant. Therefore, microscopic examination of foliar secretory structures and histo-phytochemical analyses were conducted to identify and localize phytochemical compounds. In addition, extracts were evaluated for antibacterial activity. This study contributed towards a better understanding on the micromorphology and chemical composition of secretory structures of an important medicinal species.

1.3 Aims

The aim of this research was to investigate the morphological characteristics of foliar secretory cavities, the chemical composition of the secretion, and the antibacterial activity of leaf extracts of *Vepris lanceolata*.

1.4 Objectives

The objectives were to:

a) Describe and characterize the micro-morphology and structure of secretory cavities on leaves using stereomicroscopy and scanning electron microscopy (SEM).
b) Examine the ultrastructure of the foliar secretory structures to elucidate the development and intracellular organization. This was done by examining semi-thin and ultra-thin sections of resin-embedded leaf samples using transmission electron microscopy (TEM).
c) Perform histo-phytochemical assays and thin layer chromatography (TLC) to detect active compounds secreted in foliar secretory structures and localize the source of the secretions.
d) Investigate antibacterial activity of leaf extracts against gram positive and gram negative bacteria using agar disk diffusion.

Figure 1.1 *Vepris lanceolata* (Lam) G. Don growing in UKZN, Westville Campus

[A] Whole plant

[B] Trifolioate leaves

1.5 Outline of dissertation structure

Chapter 1 introduces the background information, justifies the need for the study and summative methods to investigate the morphology, secretion composition and medicinal importance of foliar secretory structures.

Chapter 2 reviews the literature published on the micro-morphology of secretory structures and compounds synthesised. In addition, biological activities of species under the Rutaceae family were stated.

Chapter 3 reveal the methodology used to investigate the morphology of secretory structures in *Vepris lanceolata* leaf and antibacterial activity of their secretion after secretions were detected and localized in the leaf.

Chapter 4 identifies and describes the type of secretory structures and the compounds secreted in these structures. Furthermore, this chapter reveals the extracts that have antibacterial activity against gram positive and gram negative bacteria.

Chapter 5 explains the findings and how the secretions play a role in understanding the medicinal value of the plant.

Chapter 6 incorporates all the finding and explanations as well as conclusions on the work done. Limitations, future studies and contributions are also stated.

CHAPTER 2: LITERATURE REVIEW

2.1 Drug discovery

Humans have relied on traditional medicine for thousands of years (Cai et al. 2004; Padhi and Panda, 2013; Mustafa et al. 2017). Plants have been used as a source of medicine in crude extracts or isolated compounds (Habtemarlam, 2017; Mathur and Hoskins, 2017; Mustafa et al. 2017). Approximately, 25-30% of prescribed drugs originate from natural sources (Bourgaud et al. 2001; Hostettamann and Marston, 2002; Hamilton, 2004; Calixto, 2005; Kala et al. 2006; Mahomoodally et al. 2010; Padhi and Panda, 2013; Bellah et al. 2015; Chang et al. 2017; Manju et al. 2017; Mustafa et al. 2017). This percentage can be doubled if over-the-counter medication is considered (Hostettamann and Marston, 2002). Cosmetic and food industries are progressively incorporating medicinal plants in their products (Kala et al. 2006). Over 21 000 plant species are used globally in herbal therapy (Bellah et al. 2015; Mathur and Hoskins, 2017).

Studies to obtain new active compounds which may lead to the discovery of new drugs encompasses the study of animals, fungi, animals, plants and bacteria (Balunas and Kinghorn, 2005; Calixto, 2005). Pharmacological and phytochemical studies are continuously conducted to discover new drugs from plant constituents to treat high mortality diseases, such as human immuno-deficiency virus (HIV), diabetes, tuberculosis and malaria (Padhi and Panda, 2013; Habtemarlam, 2017). This combination has become a standard protocol in identifying the safety of medicinal compounds (Habtemarlam, 2017). Drug discovery studies have now shifted on isolating pure compounds, as a specific compound would then treat a specific disease (Habtemarlam, 2017). Clinical trials are fuelled by the use of natural products to discovery new bioactive agents (Mathur and Hoskins, 2017; Mustafa et al. 2017). These agents can be natural, synthetic or partially synthetic depending on the source (Mathur and Hoskins, 2017; Mustafa et al. 2017). An anticancer drug, paclitaxel and penicillin are partial synthetic products, i.e. natural product was converted by chemical reactions into a final product (Mathur and Hoskins, 2017).

Medicinal plant research is prioritised in the discovery of new drugs and where the chemical entities are concerned, as most developed drugs are derived from plants (Balunas and Kinghorn, 2005). Aspirin which is derived from salicylate can be isolated from *Betula lenta* and *Spiraea ulmaria* (Bourgaud et al. 2001), vinblastine from *Catharanthus roseus*, reserpine from *Rauvolfia serpentine* (Cox and Balick, 1994) and taxol from *Taxus brevifolia* (Mathur and Hoskins, 2017). Tiotropium which is sold as Spiriva in the USA is derived from atropine (*Atropa belladona*) to treat chronic pulmonary diseases (Balunas and Kinghorn, 2005) and is anticholinergic (Akerele, 1992). Reminyl is derived from Galantamine (*Galanthus woronowii*) and used in the treatment of Alzheimer's disease (Balunas and Kinghorn, 2005).

Previously, pure herbal plants were used and in the 19th century synthetic drugs were manufactured (Djeridane et al. 2006). Active compounds isolated from medicinal plants led to the discovery of crucial medicinal drugs (Deans and Svoboda, 1990). These included vincristine for treating leukaemia; morphine isolated from *Papaver somniferus* L. is used as an analgesic; digitoxin or digitalin isolated from *Digitalis purpurea* L. in 1785 is used as a cardiotonic; ephedrine isolated from *Ephedra sinica* for asthma; strychnine isolated from *Strychnox nuxvomica* is used as a central nervous system stimulant; quinine isolated from *Cinchona ledgeriana* and *Cinchona officinalis* is used for treating tumor and malaria fever; codeine is used as an antitussic; *Claviceps purpurea* as an uterine stimulant; and cocaine as an anaesthesia (Farnsworth et al. 1985; Deans and Svoboda, 1990; Akerele, 1992; Fabricant and Farnsworth, 2001; Balunas and Kinghorn, 2005; Elujoba et al. 2005).

2.2 History and biodiversity of medicinal plants

China, Egypt, Greece and India are among the largest countries that are rich in medicinal plants used as source for modern drugs (Kala et al. 2006; Manju et al. 2017). The use of traditional medicine in India, Egypt, Syria, China and Greece date back to 5000 years (Kamboj, 2000; Rahman et al. 2017). First documented use of traditional plants was in 2600 B.C. from Sumerians Akkaidines (Manju et al. 2017). A Sumerian clay slab was discovered from Nagpur with a written proof of drugs prepared from 250 herbal plants

(Petrovska, 2012). In addition, Pen T'Sao written by Emperor Shen Nung in 2500 B.C. is a Chinese book referred to 365 cancer drugs derived from dried plants of *Cinnamomum camphora, Cinnamomum verum, Rheum officinale* Baill., *Gentiana lutea, Camellia sinensis, ephendra, Podophyllum peltatum* and *Datura stramonium* (Cai et al. 2004; Petrovska, 2012) The Ebers Papayrus (1500 B.C.) is the medical book that documented over 870 formulae and prescriptions, and 700 medicinal plants such as aloe, opium poppy and gentian used as powder, tea, herbs and other concoctions (Okigbo et al. 2009; Balunas and Kinghorn, 2005). Ayurveda was documented in 1000 B.C. in Charaka and Sushruta, India (Manju et al. 2017). Greece documented 600 medicinal plants in 100 AD (Manju et al. 2017).

India is reported to be among the few countries world-wide that is rich in traditional medicine and knowledge (Kamboj, 2000; Gupta et al. 2010). Two-thirds of the Indian population practice Ayurvedic medicine and the demand increases by 20% annually (Hamilton, 2004; Elujoba et al. 2005). India has about 17 000 plant species of which 7500 have been reported to have medicinal properties (Kala et al. 2006). This value is the highest known for countries with medicinal plants (Kala et al. 2006). There is no other region that matches this number because India has 16 various agro-climatic districts, 15 biotic provinces and 10 vegetative zones (Kamboj et al. 2000). The oldest Indian medical system, Ayurveda, has documented 2000 herbal plants followed by other medicinal systems such as Sidda and Unani (Kala et al. 2006).

In 2006, the Himalayas was reported to have an estimated 8000 angiosperms, 600 pteridophytes, 44 gymnosperms (Kala et al. 2006). Of these, 1748 are medicinal plant species indigenous to the Himalaya (Kala et al. 2006). These plants are consumed raw, boiled, cooked, as pickles, jam and spices such as *Terminalia berilica* and *Myrica esculenta* (Kala et al. 2006). Medicinal plants are also used by local communities as a source of timber and religion (Kala et al. 2006). Communities living in northern India have been reported to use plants when worshiping gods and goddesses (Kala et al. 2006). The Hindus use plants like *Cynodon dactylon, Ficus benghalensis, Musa paradisima, Saussurea obvallata, Aegle marmelos* and *Cedrus deodora* for religious reasons (Kala et al. 2006).

Mount Taibai in China has approximately 1850 plant species under 126 families and 25 species that are used in traditional medicine (Chang et al. 2017). Pakistan is rich in biodiversity of aromatic plants, with only 400-600 species from 5700 species investigated for medicinal properties (Mustafa et al. 2017). Ethopia has 7000 plant species of which 14% are used in traditional medicine (Kebebew et al. 2017).

2.3 Over-exploitation of medicinal plants

Biodiversity is decreasing each year, resulting in the loss of important indigenous plants (Muthar and Hoskins, 2017; Xie and Zhou, 2017). This reduction is due to the increase of alien species and overuse of plants as herbs, drugs, agriculture and fuel (Calixto, 2005; Kala et al. 2006; Kyoshabire et al. 2017; Xie and Zhou, 2017). The knowledge regarding the use and preservation of the biodiversity is also being lost (Calixto, 2005; Kala et al. 2006). The knowledge on the uses and significance of indigenous plants must always be documented and passed on from generation to generation (Kala et al. 2006).

Rare and endangered medicinal plant species are at the risk of becoming extinct (Muthar and Hoskins, 2017). Chemical toxins, global warming and harsh environmental conditions are amongst factors detrimental to plant biodiversity (Muthar and Hoskins, 2017). Another contributing factor is the increasing demand from many industries using plant-derived products (Calixto, 2005; Kala et al. 2006). In addition, with herbal therapy being cost-effective or affordable; and easily accessible, (Kala et al. 2006; Kebebew et al. 2017; Kyoshabire et al. 2017; Mustafa et al. 2017) over-harvesting of medicinal plants increases. Overharvesting may result in the extinction of highly valued herbal plants (Kala et al. 2006). Extinction may be accelerated when plants of low population density grow slowly and are restricted to specific regions (Kala et al. 2006). About 4000 to 10 000 medicinal plants are at high risk of becoming extinct locally, nationally and globally (Kala et al. 2006). Therefore, strategies to create awareness among people and companies using medicinal plants have to be put in place, effectively (Muthar and Hoskins, 2017).

2.4 Secondary metabolites

Medicinal plants produce many useful phytochemicals that have biological activities that are used in the treatment of illnesses (Atanassova et al. 2011, Bellah et al. 2015; Habtemarlam, 2017; Xhie and Zhou, 2017). Phytochemicals are secondary metabolites formed by plants during normal metabolic activities and include alkaloids, steroids, flavonoids, saponins, terpenoids, tannins (Okigbo et al. 2009; Bartwal et al. 2013; Maobe et al. 2013; Neilson et al. 2013; Bashir et al. 2016; Yadav et al. 2017). Primary metabolites are responsible for growth, development and reproduction (Bennett and Wallsgrove, 1994; Bourgaud et al. 2001; Bartwal et al. 2013; Neilson et al. 2013). Primary metabolites such as chlorophyll, carbohydrates, proteins and amino acids are responsible for plant metabolism (Maobe et al. 2013). Secondary metabolites are complex, natural products that are crucial in plant defence and pollination by emitting colour and odour that can either invite or deter insects (Bruneton, 2012; Bartwal et al. 2013; Saxena et al. 2013). Flavonoids assist the plant to cope with environmental and mechanical stresses by absorbing ultraviolet light to protect leaves from excessive sunrays (Bourgaurd et al. 2001; Bartwal et al. 2013). They are known to fight off illnesses in animals and humans as much as they also protect plants (Maobe et al. 2013).

Secondary metabolites were initially considered as insignificant by-products of primary metabolism and were not investigated (Bennett and Wallsgrove, 1994; Bourgaud et al. 2001). Spices and natural herbs with phytochemical compounds include leaves, seeds, buds, rhizomes, flowers and roots (Dean and Svoboda, 1990, Okigbo et al. 2009). There are approximately 200 000 phytochemical compounds of secondary metabolites (Neilson et al. 2013). These metabolites have different composition and concentrations dependent upon the developmental stage and site of synthesis (Neilson et al. 2013). Secondary metabolites are produced from intermediates of primary carbon metabolism by methyl erythritol phosphate, phenylpropanoic acid, mevalonic acid and shikimic acid pathways (Bennett and Wallsgrove, 1994; Bartwal et al. 2013). Herbal plants such as *Salvia officinalis*, *Thymus vulgaris*, *Rosmarinus officinalis*, *Laurus nobilis* and *Petroselinum crispum* are found in temperate regions while *C. verum*, *Vanilla planifolia*, *Syzygium*

aromaticum, *Myristica fragrans*, *Zingiber officinale* and *Piper nigrum* are found in tropical regions (Dean and Svoboda, 1990). There are large groups or classes of secondary metabolites, i.e. alkaloids, terpenoids, and phenolic compounds (Bourgaud et al. 2001). Phenolic compounds are found in all higher plants while alkaloids are scarce and found in specific genera (Bourgaud et al. 2001).

2.4.1 Phenolic compounds

Phenolics consist of secondary metabolites that are identified by one or more hydroxyl groups (Mustafa et al. 2017). They are synthesized from modified amino acids such as coumarins and those that are produced from condensed acetate units such as terpenoids (Bennett and Wallsgrove, 1994). Phenolic compounds are used in attracting pollinators, defence and antioxidants since they have the ability to slow down oxidation (Chan et al. 2016; Mustafa et al. 2017) These compounds are found in plants or food made from plant material such as herbs and spices (Chan et al. 2016). Free phenols are soluble while bound phenols are insoluble and have rigid, cell wall structural constituents comprised of covalent bonds (Chan et al. 2016). Conjugated phenols are esterified to constituents with low molecular mass or sugars (Chan et al. 2016). Phenolic compounds exhibit antioxidant effects and this is useful in natural therapy, since most chronic diseases are caused by oxidative stress that causes tissue damage (Atanassova et al. 2011).

2.4.1.1 Tannins

Tannins are polymeric phenolic elements localized throughout the plant (Cai et al. 2004; Compean and Ynalvez, 2014). Plants producing tannins have numerous biological activities (Harbone, 1998; Compean and Ynalvez, 2014). Tannins possess antibacterial activity (Mahomoodally et al. 2010; Kasimala et al. 2014). *Sanguisorba officinalis* and *Punica granatum* were found to contain high amounts of tannins (Cai et al. 2004; Bruneton, 2012). The two groups of tannins comprise condensed and hydrolysable tannins (Cai et al. 2004; Bruneton, 2012). Condensed tannins have complex constituents and are more available than hydrolysable tannins (Cai et al. 2004). Examples of

condensed tannins are proanthocyanidins and leucoanthocyanadins which are found in *Camellia sinensis* and *Areca catechu* (Cai et al. 2004).

In contrast, hydrolysed tannins are comprised of a fundamental polyhydric alcohol like glucose, and hydroxyl sets that are esterified moderately or entirely by gallotannins or ellagitannins (Cai et al. 2004). Gallotannins are isolated from Chinese galls while ellagitannins are isolated from *Punica granatum* peels and *Termibalia chebula* fruits (Cai et al. 2004; Bruneton, 2012; Mustafa et al. 2017). In addition, complex mixtures with both types of tannins are synthesized in *Rosa chinesis*, *Aacia catechu* and *Sanguisorba officinalis* (Cai et al. 2004).

Phenolic acids such as gallic acid, vanillic acid, coumaric acid, cinnamic acid and ferulic acid are the largest group of phenolic compounds found in plants (Cai et al. 2004; Saxena et al. 2013). Coumarins and lignans are phenolics with a basic structure of C_6+C_3 isolated in species of Leguminosae, Rutaceae, Compositae, Oleaceae and Ranunculaceae (Cai et al. 2004). Components of coumarins include isocoumarins, furanocoumarins and pyranocoumarins (Cai et al. 2004; Bruneton, 2012). These are found in *Agrimonia pilosa*, *Psoralea corylifolia* and *Citrus aurantium*, respectively (Cai et al. 2004). Lignan components are found in *Magnolia officinalis* (neolignans), *Polygala tenuifolia* (cyclolignanolides), *Arctium lappa* (lignanolides) and *Forsythia suspensa* (bisepoxylignans) (Cai et al. 2004).

2.4.1.2 Flavonoids

Phenols are separated into two classes, non-flavonoids and flavonoids (Mustafa et al. 2017). Flavonoids are phenolic compounds localized in photosynthetic cells (Compean and Ynalvez, 2014). These compounds are produced by palatable organs like seeds, vegetables and fruit (Compean and Ynalvez, 2014). Flavonoid compounds have a heterocyclic pyrane ring connecting two benzene rings that makes up the 2-phenyl-benzopyrane or flavine nucleus (Harbone, 1998; Compean and Ynalvez, 2014; Mustafa et al. 2017). Flavonoids have anticancer, anti-inflammatory, antiviral, antibacterial, and antioxidant properties (Mahomoodally et al. 2010; Compean and Ynalvez, 2014;

Kasimala et al. 2014; Mustafa et al. 2017). There are 14 classes of flavonoids that are characterised by chemical structure and ring localization of substituents (Compean and Ynalvez, 2014).

Most common flavones such as baicalein and luteolin are found in *Scrutellaria baicalensis* roots and *Artemisia annua* aerial parts (Cai et al. 2004). Flavonols such as myritecin and quercetinare found in *Crataegus pinnatifioda* fruits, *Rosa chinensis* flower and *Alpina officinum* rhizomes (Cai et al. 2004). The families Rutaceae and Leguminosae possess hesperetin liquiritin, and naringenin flavanonols and flavanones (Cai et al. 2004). Flavonols (catechins) were found in *Acacia catechu* and *Astragalus mongholicus* and anthocyanins in *Prunella vulgans* inflorescences (Cai et al. 2004).

2.4.2 Alkaloids

Alkaloids are basic, organic compounds with heterocyclic nitrogen and are soluble in water (Compean and Ynalvez, 2014; Mustafa et al. 2017). Nitrogen is synthesized from amino acids (Compean and Ynalvez, 2014). There are numerous groups of alkaloids such as pyrroldines, purine, phenylalkylamines, atropane and pyrrolizidines which are assembled into three classes (Harbone, 1998; Compean and Ynalvez, 2014; Mustafa et al. 2017). These three classes of alkaloids are dependent on the initial and final structure of the molecule. There are true alkaloids which are basic and have nitrogen in a heterocyclic ring and are synthesized from amino acids. Pseudoalkaloids such as caffeine are not synthesized from amino acids. Proto-alkaloids such as mescaline are made from amino acids but does not have nitrogen in a heterocyclic ring (Bennett and Wallsgrove, 1994; Compean and Ynalvez, 2014).

Alkaloids are poisonous against fungi, bacteria and also deter herbivores (Bennett and Wallsgrove, 1994). Alkaloids such as morphine are used as narcotics, antimalarial, stimulant, analgesic and to relieve discomfort (Compean and Ynalvez, 2014; Mustafa et al. 2017). Plants extracts with antibacterial activity have a high concentration of alkaloids (Mahomoodally et al. 2010; Compean and Ynalvez, 2014; Kasimala et al. 2014).

Crinane alkaloids such as buphanidrine and distichamine isolated from *Boophone disticha* (Amaryllidacae) possess antibacterial activity (Babiaka et al. 2015). Indole alkaloids such as voacangine and dregamine isolated from *Tubernaemontana elegans* (Apocynaceae) have antimicrobial activity (Babiaka et al. 2015). Aeridone alkaloids such as tegerrardin isolated from *Teclea gerrardii* (Rutaceae) have antiplasmodial activity (Babiaka et al. 2015). *S. officinalis, Melissa officinalis* and *Mentha Piperita* possess high concentrations of total flavonoids and phenolic compounds (Atanassova et al. 2011). It is known that these compounds are good antioxidants and have scavenging ability and hence, minimize oxidation of free radicals (Atanassova et al. 2011). *Clausena excavate* belonging to the Rutaceae family also has antioxidant activity (Arbab et al. 2011).

2.4.3 Essential oils

There are two types of oils, volatile (85-99%) that do not persist in the environment and non-volatile (1-15%) oils that dissolve in organic solvents (Chaubey, 2012; Palazzolo et al. 2013). Volatile components are made up of mono-terpene (limonene, sesquiterpenes), oxygenated component like alcohol (linalool), citral, acids, esters and ketones (Palazzolo et al. 2013). The non-volatile components are fatty acids, waxes, polymethoxylated flavonoids, sterols, coumarins and carotenoids (Palazzolo et al. 2013). Essential oils (ethereal or volatile oils) are natural secondary metabolites with a strong scent and low density compared to water (Harbone, 1998; Bakkali et al. 2008; Bruneton, 2012; Chaubey, 2012; Palazzolo et al. 2013). These oils are produced by many plants ranging from perennials, shrubs, evergreen, trees to annuals (Svoboda and Hampson, 1999). Essential oils are synthesized in flowers, bark, seeds, twigs, herbs, roots, leaves, fruits, buds and wood (Sangwan et al. 2001; Palazzolo et al. 2013). Essential oil production is linked to secretory structures such as trichomes, ducts and cavities (Venkatachalam et al.1984).

Essential oils consist of various natural compounds which are important in the fragrance industry, in traditional medicine and in the production of insecticides (Russin et al. 1988). Oils are composed of lipophilic material ranging from 20-60 constituents at various concentrations, though only 2-3 of these components give essential oils their biological

properties (Bruneton, 2012; Chaubey, 2012; Palazzolo et al. 2013). About 3000 essential oils have been identified, of which 10% are significant in commercial industries like perfume, food, pharmaceutics, aromatherapy, sanitary, household products, cosmetics and agronomy (Rana et al. 1997; Barrata et al. 1998; Sangwan et al. 2001; Bakkali et al. 2008; Chaubey, 2012; Palazzolo et al. 2013). Essential oils have antimicrobial, anaesthetic, anti-inflammatory, sedative, antifungal and analgesic activities (Rana et al. 1997; Barrata et al. 1998; Liang, 2006; Bakkali et al. 2008; Palazzolo et al. 2013). Plants producing essential oils grow in temperate and tropical countries (Bakkali et al. 2008). Essential oils are used as food preservers, herbal remedies and additives (Bakkali et al. 2008).

The main components of essential oils are terpenes and their derivative terpenoids while other aliphatic and aromatic components may also be present in large quantities (Chaubey, 2012; Compean and Ynalvez, 2014). Terpenoids such as monoterpenoids, sesquiterpenoids and phenylpropanoids are the main components that are found in essential oils (Sangwan et al. 2001). Phenylpropanoids are not usually found in essential oils (Sangwan et al. 2001). However, those plants that do produce phenylpropanoids synthesize them in very large quantities (Sangwan et al. 2001). Methyl cinnamate, euginol, elemicin, dillapiole, chavicol, apiole, methyl euginol, anethole, methyl chavicol, estragole and myristicin are components of phenylpropanoids that have been recognised in essential oils (Sangwan et al. 2001). Geranyl acetate, d-limonene and d-carvone are used in flavourings, additives, creams, fragrance, perfumes and cleaning products (Bakkali et al. 2008).

Examples of terpenes are: α-pinene which is a four-ring monoterpene and β-caryophyllene which is a bicyclic sesquiterpene with a cyclobutane ring (Chaubey, 2012). They are recognized by referring to the number of isoprene units; e.g. hemiterpene has one isoprene unit, monoterpene has two isoprene units and sesquiterpenes has three isoprene units (Venkatachalam et al. 1984; Goodger et al. 2009; Bruneton, 2012; Compean and Ynalvez, 2014; Mustafa et al. 2017). These can be irregular, cyclic, regular or linear (Compean and Ynalvez, 2014). Alcohols, aldehydes, esters, ketons and phenols are monoterpene derivatives identified from plants (Compean and Ynalvez, 2014).

Terpenes can be synthesized either by the mevalonic-acid pathway producing sesquiterpenes, ubiquinones and sterols (Compean and Ynalvez, 2014). They can also be synthesized by the methyl-erythritol pathway synthesizing hemiterpenes, diterpenes and monoterpenes (Compean and Ynalvez, 2014). Terpenes and their derivatives have been shown to confer resistance to plants against pathogen and herbivorous attacks due to their insecticidal activity (Bennett and Wallsgrove, 1994). Furthermore, terpenoids are responsible for plant defence, wound healing, pollinating seed plants, preventing excess heat, and provide flavour and fragrance to fruits and flowers, respectively (Mustafa et al. 2017).

2.5 Secretory cavities

Plants consist of various structures that secrete primary and secondary metabolites (Fahn, 1988; Svoboda and Svoboda, 2000; Palazzolo et al. 2013). Primary metabolites are sugars, nucleic acids and amino acid, while secondary metabolites are made up of all other chemical compounds (Svoboda and Svoboda, 2000; Mustafa et al. 2017). Secondary metabolites attract pollinators and provide mechanical protection against herbivores as well as confer microbial resistance (Svoboda and Svoboda, 2000; Bakkali et al. 2008; Palazzolo et al. 2013; Mustafa et al. 2017). These secretory structures vary in structure, location and secreted substances (Fahn, 1988). Secretory tissues are usually classified according to the material they produce (Fahn, 1988). Secondary metabolites are synthesized and localized in vacuoles or specialized structures such as ducts, glandular trichomes and secretory cavities (Fahn, 1988; Naidoo et al. 2009; Palazzolo et al. 2013). These structures occur in leaves, roots, flowers, bark, buds and fruit (Palazzolo et al. 2013).

Various secretions can be produced by the same structure (Fahn, 1988). Secretion occurs when secreted materials are liberated into particular cavities (endotropic secretion) or to the outer surface (exotropic secretion) (Svoboda and Svoboda, 2000). Some plants have both internal and external secretory structures such as *Artemisia dracunculus* L. which has trichomes and cavities (Sangwan et al. 2001). The difference between these structures is that cavities secrete internally and secretions are released upon physical damage while

those in trichomes are released when cuticle ruptures (Sangwan et al. 2001). The former process is facilitated by specialized cells that exude mucilages, salts, resins, flavonoids, essential oils, waxes, gums, latex, fats and sugars (Svoboda and Hampson, 1999).

The secretory structure that secretes lipophilic substances can be comprised of an intercellular space (lumen) which is surrounded by epithelial (secretory) cells (Naidoo et al. 2009), which when the lumen is elongated is known as a secretory duct and when isodiametric-shaped is called a secretory cavity (Fahn, 1988; Turner et al. 1998; Svoboda and Hampson, 1999). Plant secretory cavities and ducts are glands comprised of specialized glandular cells lining huge spaces that are filled with secretions (Turner et al. 1998; Svoboda and Hampson, 1999). These structure secrete mucilage, resin or essential oils (Turner et al. 1998). Secretions are mostly mucilaginous or lipophilic (Teixeira et al. 2000). Epidermal cells or mesophyll structures are responsible for the production, storage and secretion of volatile oils (Sangwan et al. 2001).

Early families of the basal Ranalean complex are characterized by secretory cavities and idioblastic secretory cells to support the early development of these secretory structures (Curtis and Lersten, 1986). Gigantopteridales fossil group and Ginkgoaceae microsporophylls have been found to have these secretory structures (Curtis and Lersten, 1986). There are 40 families that have cavities with un-identified secretions while 16 and 14 families have mucilages and tannins, respectively (Curtis and Lersten, 1986). Secretory cavities are found in numerous families like Rutaceae, Myrtaceae, Hypericaceae, Cactaceae, Myrsinaceae, Olacaceae, Myoporaceae, Bombacaceae, Fabaceae, Anacardiaceae, Asteraceae, Ebenaceae and Leguminosae (Curtis and Lersten, 1986; Fahn, 1988; Kalachanis and Psaras, 2005). Cavities are also used in identification or characterization of two distinguished species (Turner et al. 1998). Furthermore, nature and localization of cavities can be used as a diagnostic character in different genera within the family (Turner et al. 1998). Reports have shown that foliar secretory cavity is a dominant trait that characterizes families like Theaceae, Myrtaceae, Flacourtiaceae and Rutaceae (Curtis and Lersten, 1986; Viera et al. 2011). Secretory cavities are described in Rutaceae as a common structure that produce and accumulate essential oil and other secretions (Liang et al. 2006).

Secretory cavities form either by schizogenous, lysigenous and schizo-lysigenous development (Turner et al. 1998; Teixeira et al. 2000). Schizogenous cavities are formed as a result of the secretory epithelium-lined space that forms following the division or separation of glandular cell initials (Turner et al. 1998; Svoboda and Hampson, 1999; Teixeira et al. 2000). Lysigenous cavities form after degradation of glandular cells, with secretory cells discharging their secretion into the expanding space (Turner et al. 1998; Svoboda and Hampson, 1999; Teixeira et al. 2000). Schizo-lysigenous cavities form when a storage space commences schizogenously, and then lysigenously, further widening the storage cavity (Turner et al. 1998; Teixeira et al. 2000). Secretory cavities store essential oil in the extracellular compartment to prevent toxic substance to penetrate adjacent cells (Russin et al. 1998; Goodger et al. 2009).

There is still a conflict among published literature that secretory cavities develop lysigenously, schizogenously or both (Fahn, 1988; Turner et al. 1998; Turner, 1999; Liang et al. 2006; Teixeira et al. 2000; Kalachanis and Psaras, 2005; Liang, 2006). Lysigenous development was first reported by Karsten in 1857 and it was known as the "cell wall resorption" theory (Turner, 1999). This theory described how all secretions are formed by combining products that resulted from the separation of cell wall components (Turner, 1999). This concept was cited in many publications, until Anton de Bary in 1877 used the term "lysigeny" instead of resorption (Turner, 1999).

In 1868, this theory was questioned by A.B. Frank in an article where he assessed that cavities or ducts can be formed by resorption or by separation of cells, i.e. schizogenously (Turner, 1999). Although Frank found schizogenous development, some species did show lysigenous development, hence it was shown that both forms exist separately (Turner, 1999). Thereafter, publications that followed supported schizogenous, until Martinet in 1870s published an article criticizing schizogenous and wrote that all glands develop lysigenously (Turner, 1999). Most of these arguments were on the Rutaceae family (Turner, 1999). Dissimilar procedures of sample preparations resulted in different findings (Turner et al. 1999).

2.6 Biological activities of Rutaceae

Numerous groups of secondary metabolites such as limonoids, pyranocoumarins, coumarins, alkaloids and carbazole alkaloids have been reported to occur in different species of the Rutaceae family (Arbab et al. 2011). Carbazole have antifungal activity; limonoids and coumarins have anti-HIV-1, antiplatelet, insecticidal, antioxidant and anti-malarial activities (Arbab et al. 2011). *Vepris ampody* (Rutaceae) growing in Madagascar and *Vepris glomerata* in Kenya showed anti-malarial activity (Babiaka et al. 2015). Furthermore, furoquinoline alkaloids (maculosidine) from *Vepris uguenensis* (Rutaceae) showed anti-parasitic activity against *Plasmodium falciparum* strains (Babiaka et al. 2015). Leaf extract of *Feronia limonia* (Rutaceae) growing in Bangladesh has antimicrobial activity against gram positive bacteria such as *Staphylococcus saprophyticus* and *Staphylococcus pyogenes* and gram negative bacteria such as *Escherichia coli, Shigella boydii, Shigella dysenteriae* and *Shigella flexneri* Bellah et al. 2015). Furthermore, it showed anti-diarrhoeal activity and was previously reported to have hepatoprotective, antioxidant, antidiabetic and antidiabetic activity (Bellah et al. 2015).

Esenbeckia leiocarpa (Rutaceae) produces compounds such as alkaloids, coumarins and lignans that inhibit acetylcholinesterase (AChE) and can be used in treating Alzheimer's disease (AD) during the early stages (Cardoso-Lopes et al. 2010). Alkaloids such as flindersiamine, skimmianine, leiokinine A, isolated from *Esenbeckia leiocarpa* stem using ethanol extracts showed anticholinesterasic activity. Saponins, tannins, flavonoids, steroids and anthraquinones were present in leaves of *Todalia asiatica* (Rutaceae) and used for treating diabetes and malaria (Maobe et al. 2013).

2.6.1 Antimicrobial activity

Approximately, 20000 plants have phytochemical compounds and have been used in folk medicine to treat microbial diseases (Compean and Ynalvez, 2014). The use of common commercial drugs to treat microbial infections has resulted in multiple drug resistance (Palombo and Sample, 2001; Parekh et al. 2005; Compean and Ynalvez, 2014; Kasimala

et al. 2014; Mustafa et al. 2017). Also, hostile side effects from prolonged use of antibiotics include allergy, hypersensitivity and repressed immune system (Parekh et al. 2005). Therefore, plant-derived antimicrobials are an alternative to the common antibiotics (Parekh et al. 2005). Active components of phytochemicals have been used for many years to synthesise antimicrobial agents (Compean and Ynalvez, 2014; Mathur and Hoskins, 2017). The cost of treatment of fungal infections is a major medical problem (Aoudou et al. 2010). Plant based antimicrobials are accessible, affordable and have fewer side effects since they are synthesized from natural products (Parekh et al. 2005).

It has been reported that one-third of the population in the world is infected by *Mycobacterium tuberculosis* (T.B.) (Gupta et al. 2010). Resistance to prescribed drugs that manage *Mycobacterium tuberculosis* has led to the occurrence of extensive drug resistance (XDR) and multi-drug resistance (MDR) strains (Gupta et al. 2010; Fankam et al. 2014). Medicinal plants offer an alternative treatment to manage TB strains and can eliminate or minimize resistance to drugs (Gupta et al. 2010; Fankam et al. 2014). *Acalypha indica, Allium cepa, Aloe vera, Adhatoda vasica* and *Allium sativum* are among those plants with anti-MDR (H37Rv) properties (Gupta et al. 2010).

Strong antibacterial activity was exhibited by *Beilschmiedia obscura* (Lauraceae), *Vepris soyauxii* (Rutaceae), *Pachypodanthium staudtii* (Annonaceae), and *Peperomia fernandopoiana* (Piperaceae) against MDR gram-negative microbes due to flavonoids, sterols, anthraquinones, tannins, triterpenes, saponins, anthocyanines and phenols (Fankam et al. 2014). Stems and leaves of *Rumex nervosus* (Polygonaceae) and *Ruta chalapensis* (Rutaceae) showed antibacterial activity using ethanol and methanol extracts against *Staphylococcus aureus* and *Escherichia coli* (Kasimala et al. 2014). *Pittosporum senacia* (Pittosporaceae), *Ocimum tenuiflorum* (Lamiaceae), *Momordica charantia* (Cucurbitaceae) and *Faujasiopsis flexuosa* (Asteraceae) showed antimicrobial activity against *Bacillus subtilis, Enteroccous faecalis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus,* methillin resistant *Staphylococcus aureus* (MRSA), *Salmonella typhimurium*; fungi *Aspergillus niger* and *Candida albicans* (Mahomoodally et al. 2010).

Vepris heterophylla (Ngamo et al. 2007), *Vepris unifoliata* (Garcia et al. 2017), *Vepris leandriana* (Rakotondraibe et al. 2001), *Vepris uguensis* (Cheplogoi et al. 2008) and *Vepris macrophylla* (Maggi et al. 2013) secrete essential oils active against gram positive and gram negative bacteria. Components of essential oils in Rutaceae that have antibacterial activity are mainly geranid, geranyl acetate, menthol, euginol, carvone, p-cymene, limonene, thymol and y-terpinene (Palazzolo et al. 2013; Silva et al. 2016; Trung et al. 2016; Brophy et al. 2016).

2.7 Rutaceae

The Rutaceae is a large family that is comprised of 162 genera and 1900-2096 species of shrubs (Maola et al. 2008; Pan, 2012; Kiplimo, 2012; Groppo et al. 2012; Roy et al. 2015; Tekin and Eruygur, 2016). The family is distributed in subtropical and tropical Australia, Asia, southern Africa and America (Pan, 2010; Groppo et al. 2012; Tekin and Eruygur, 2016). It grows in woodlands and deciduous forests (Pan, 2010). The family is noted for producing edible fruits, drugs, aromatic oil and bitter, medicinal concoctions (Groppo et al. 2012). Species under Rutaceae contributes significantly in the economy, as they are used for food, ornamental, gardening and medicine (Tekin and Eruygur, 2016; Ulukus et al. 2016). Some species are used for timber (Groppo et al. 2012). Leaves and stem axis are characterised by secretory cavities (Pan, 2010). Cavities produce rutaceous compounds, volatile oils, hesperidin and alkaloids that are known to possess antifungal, pesticides, herbicides and antimicrobial properties (Groppo et al. 2012; Tekin and Eruygur, 2016).

2.7.1 Overview of the genus *Vepris*

The genus comprises of 80-100 species of shrubs and trees (Kiplimo, 2012; Maggi et al. 2013). *Vepris* species are characterized by a smooth, pale to dark grey bark and plants are prickle-less (Kiplimo, 2012). The leaves are shiny green, alternate, large and have an aromatic smell when ground (Kiplimo, 2012).

Vepris species are known to have secondary metabolites such as alkaloids, limonoids, volatile oils, coumarins and flavonoids (Kiplimo, 2012). The plant has been used in traditional medicine to treat dermatological and respiratory infections (Kiplimo, 2012). In addition, the genus has antipyretic, antifungal, antiviral, anthelmintic, anti-malarial and anti-inflammatory properties (Kiplimo, 2012). Furthermore, it possesses antifungal and antiplasmodial properties (Kiplimo, 2012).

2.7.1.1 Description of *Vepris lanceolate*

Vepris lanceolata (Lam.) G. Don (figure 1.1a) is about two to five metres evergreen shrub or tree which can also reach a height of up to 24 metres in South African dry forests (Fern, 2012). It is commonly known as White Iron wood in English, *Witysterhout* in Afrikaans and *umoZane* in isiZulu (Lemmens, 2008). It is also known as *V. undulata* (Lemmens, 2008). The leaves are trifoliate, hairless, glossy green (figure 1.1b) and produce a lemony scent when grounded (Fern, 2012). The flowers are green with short auxiliary heads, and produce drupaceous fruits with smooth seeds (Kiplimo, 2012). Ripened fruits are black (Kiplimo, 2012). The tree is found in riverine forests and dunes (Fern, 2012).

2.7.1.2 Distribution of *V. lanceolata*

V. lanceolata is a diverse species that is distributed in Cameroon, Ghana, Zimbabwe, Mauritius, Zambia, Democratic Republic of Congo, Rwanda, Swaziland, Ethiopia, Zanzibar, Tanzania, Madagascar and South Africa (Kiplimo, 2012; Maggi et al. 2013).

2.7.1.3 Biological activities of *V. lanceolata*

The powdered roots of *V. lanceolate* are utilized in the treatment of colic and flu (Poullain et al. 2004). The plant has been used traditionally to treat asthma and respiratory illness (Narod et al. 2004). Roots are used to treat menorrhagia and infertility (Steenkamp, 2003). It is also used to treat skin ailments, wounds, fever, injuries, gout, sprain, rheumatism and influenza (Poullain et al. 2004). The crushed leaves are used externally as a dressing to

relieve headaches and clean sores or wounds (Fern, 2012). The plant has been reported to possess antioxidant and antibacterial activity against *Staphylococcus aureus* and *Shigella dysentrieae* (Narod et al. 2004; Poullain et al. 2004; Kiplimo, 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant collection

Fresh leaves of *Vepris lanceolata* plant were collected from the University of KwaZulu-Natal (Westville Campus), Durban, South Africa located on 29.831°S 30.925°E. The plant material was identified and verified by Mr. Edward Khathi and the specimen was deposited at the School of Life Sciences (UKZN) Herbarium with the voucher specimen X4076000**.** Leaves for phytochemical and antibacterial assays were air-dried for two months at room temperature, while fresh leaves were used for microscopy and histochemical staining.

3.2 Microscopy

3.2.1 Stereomicroscopy

Stereomicroscopy was used to study the basic overview of the distribution and morphology of secretory structures in leaves on both adaxial and abaxial surfaces. Five replicates per sample were examined with the Nikon AZ100 (Nikon, Japan) stereomicroscope and images were captured using the Nikon DXM1200C colour camera. The images were taken at 4x, 10, 20x and 40x magnification using the NIS-Element Software (NIS-Element D 3.00, 1991-2008 Laboratory Imaging).

3.2.2 Scanning electron microscopy (SEM)

3.2.2.1 Chemical fixation

For scanning electron microscopy, samples were prepared using chemical fixation. Leaves were trimmed into segments and fixed overnight in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) at 4°C. The samples were washed three times with the phosphate buffer. The samples were post-fixed in 0.5% osmium tetroxide for 1 hour. Dehydration was accomplished with a graded series of 25%, 50%, 75%, 90% and 3x100% ethanol. The ethanol was replaced from samples by critical point drying in liquid CO_2 using Quorum K850 (Zeiss, United Kingdom). The leaf segments were mounted onto the brass stubs and secured with double-sided carbon conductive tape.

3.2.2.2 Freeze fracture

Fresh leaves from different developmental stages (young and mature) were collected and used for freeze fracture. Leaves from each stage were crushed whilst immersed in liquid nitrogen at -260°C in an EMSCOPE SP 2000. Samples were then retained in liquid nitrogen and freeze dried in an Edwards Modulyo freeze dryer at -60°C in a vacuum of 10^{-2} Torr for 48 hours.

3.2.2.3 SEM viewing

Freeze fractured and chemically fixed samples were mounted onto the brass stubs secured with double-sided carbon conductive tape and sputter coated with gold for four minutes using Quorum Q150R ES (Zeiss, United Kingdom). Specimens were viewed with a Leo 1450 (Zeiss, England) SEM at an EHT of 12.00 Kv, working distance of 12 mm. Images were captured using the Smart SEM version 5.03.06.

3.2.3 Transmission electron microscopy (TEM)

For transmission electron microscopy, samples were prepared using chemical fixation. Samples were trimmed into 5 mm^2 segments and fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. The samples were washed three times with the phosphate buffer. The 2.0% aqueous osmium tetroxide was used during secondary fixation, in which the samples were fixed for 5 hours. The samples were rinsed again three times with phosphate buffer. Dehydration was accomplished with increasing concentrations of acetone of 25%, 30%, 50%, 75% for 10 minutes each and 2 x 100% acetone for 15 minutes each. Samples were pre-infiltrated twice for 15 minutes each time with 100% propylene oxide. Overnight infiltration was achieved with 75: 25% ratio of propylene oxide: Spurr's resin (Spurr, 1969). Samples were then infiltrated with 100% resin for 24 hours. Infiltrated samples were placed in a silicon mould with whole resin and polymerized at 70°C for 8 hours.

Resin blocks were sectioned in a Leica EM UC7 (England) using glass knives prepared in a LKB knife maker 7801A (Sweden). Semi-thin sections were stained using Toluidine

Blue-O and viewed with a Nikon Eclipse 80i compound light microscope (Nikon, Japan) and images captured with a Nikon DS-Fi1 camera and NIS-Elements imaging software package (NIS-Elements D 3.00, 1991-2008 Laboratory Imaging). Ultrathin sections of 100 nm were picked up on copper grids and then stained using 2.5% saturated solution of uranyl acetate and there after lead citrate. The copper grids were immersed in drops of 2.5% uranyl acetate for 8-10 minutes at room temperature. They were then washed with distilled water. The copper grids were then placed on drops of lead citrate solution in a closed Petri dish containing dry NaOH pellets for 5-10 minutes at room temperature. The grids were then rinsed with distilled water and placed on filter paper and samples were viewed with a Jeol 1010 (Japan) TEM with an Olympus MegaView III CCD camera (Soft Imaging System, Germany).

3.3 Phytochemical Analysis

3.3.1 Phyto-extraction

The leaves were collected and cut into pieces and air-dried for two months at room temperature (23 °C) to preserve volatile compounds. After two months had elapsed, the dried leaves were crushed and ground into fine powder with the Cyclotec 1093 sample mill. A total of 50 g leaf powder was obtained. The phyto-extraction was carried out on the finely ground powder using three different solvents, i.e. hexane, chloroform and methanol. The ratio used for this phyto-extraction was 1:10 ratio, i.e. 500 ml solvent for 50 g powder. Each solvent was extracted for four hours in a Soxhlet extractor at 60 °C. Each extract was filtered through a Whatman filter paper number one to remove considerable quantities of residuals. The rotary evaporator was used to dry out solvents and dried extract was stored until further use.

3.3.2 Phytochemical tests

Chemical tests were performed separately on the hexane, chloroform and methanol extracts using the standard procedures to test for the presence of different chemical compounds (Harbourne, 1973; Trease and Evans, 1978).

3.3.2.1 Alkaloids

Dragendroff's test: To 1 ml of filtrate, two drops of Dragendroff's reagent were added. Reddish orange precipitate indicated the presence of alkaloids.

Hager's test: To 1 ml of filtrate, two drops of Hager's reagent were added. Appearance of yellow precipitate indicated alkaloids.

Wagner's test: To 1 ml of extract, two drops of Wagner's reagent were added. Brown colouration indicated the presence of alkaloids.

3.3.2.2 Carbohydrates

Fehling's test: 1 ml of filtrate was boiled for 3 minutes on water bath with 1 mL each of Fehling's solution A and B. A red precipitate indicated the presence of carbohydrates.

Benedict's test: 1 ml of filtrate and 1 mL of Benedict's reagent was added. The mixture was heated on a boiling water bath for two minutes. A green, brick red to brown precipitate indicated the presence of carbohydrates.

Molisch's test: Two drops of alcoholic solution of α-naphthol were added to two ml of extract. After shaking well, one ml of concentrated sulphuric acid was added at the sides of the test tube and was left to stand to form two layers. A violet or purple ring between the layers indicated the presence of carbohydrates.

3.3.2.3 Proteins and amino acids

Two drops of Ninhydrin solution were added to 2 ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acids.

3.3.2.4 Detection of fixed oils and fats

Spot test: 1 ml extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil.

3.3.2.5 Saponins

Foam test: 2 ml of extract was added in 2 ml of distilled water. The suspension was shaken in a graduated cylinder for 10 minutes. Formation of foam layer indicated the presence of saponins.

3.3.2.6 Phenolic compounds

Lead acetate test: 1 ml of 10% lead acetate solution was added to 1 mL of plant extract. A bulky white precipitate indicated the presence of phenolic compounds.

3.3.2.7 Tannins

Ferric trichloride test: 0.5 ml of neutral 5% ferric chloride solution was added to 1mL extract. An intense black or blue colour indicated the presence of tannin compounds.

3.3.2.8 Flavones and flavonones

Concentrated sulphuric acid: 2 ml of extract was added to 1 ml of concentrated sulphuric acid. A yellowish orange colour indicated the presence of anthocyanins. A yellow to orange colour indicated flavones and orange to crimson colour indicated flavonones

3.3.3 Thin Layer Chromatography (TLC)

To observe the separation of different chemical compounds, TLC analysis was carried out. Hexane, chloroform and methanol solvents were used for phyto-extraction. For each solvent, the leaf extract was filtered into 250 ml beakers and cooled down. The TLC plates were made of aluminium and pre-coated with silica gel 60 F_{254}. A small spot of each extract was pipetted at about 1.5 cm from the bottom edge of a 10x10 cm TLC plate which was then dipped into a mobile phase solution of toluene: ethyl acetate: glacial acetic acid v/v (9:1:0.3) ml. Different compounds of secondary metabolites in the sample mixture migrated at various rates because of the variation in solubility in the solvent. The bands on the plate were viewed at 254 nm and 366 nm in a chamber of a UV spectroscope (Sethi, 1996).

3.4 Histochemical Analysis

Histochemistry is a technique used to observe the localization of chemical components within cells and tissues of plants.

3.4.1 Leaf Preparation

Fresh leaf sections were cut using the Oxford vibratome. The sections were 85-100 μm thick. Leaf sections were stained using different histochemical stains. The leaf sections were viewed with Nikon Eclipse 80I compound light microscope equipped with a Nikon DS-Fil camera and NIS-Elements imaging software package.

3.4.2 Histochemical tests

3.4.2.1 Lipids

The sections were immersed in 1% Nile Blue at 37°C for 1 minute and then in 1% acetic acid at 37°C for another minute before being rapidly rinsed with distilled water. Neutral lipids stain red while acidic lipids stain blue (Cain, 1947).

Other leaf sections were placed in the Sudan III and IV stain for 10-20 minutes. They were briefly rinsed with 70% ethanol to remove excess stain. The leaves were then placed in slides with 70% glycerol. Lipids stain orange or red (Pearse, 1985).

3.4.2.2 Polysaccharides

The leaf sections were mounted in 0.05% toludine blue for 1 minute and then in 70% glycerol. A pink colouration is indicative of the presence of carboxylated polysaccharides while nucleic acids stain purplish-greenish blue and polyphenols show blue colour. Hydroxylated polysaccharides are not stained (O'Brien et al. 1964).

3.4.2.3 Phenolic compounds

Fresh leaf sections were placed in 10% ferric trichloride with 0.3 ml of sodium carbonate for 15 minutes. Green or black deposits indicate the presence of phenolic compounds (Johansen, 1940).

3.4.2.4 Fehling's solution

The leaf sections were mounted in a mixture of equivalent volumes of solution A (1 L water and 79.28 g $CuSO_4$) and solution B (1 L water and 346 g sodium potassium tartrate and 100 g NaOH) and was then heated to the boiling point. Brick red precipitate indicates the presence of reducing sugars (Purvis et al. 1964).

3.4.2.5 Proteins

The leaf sections were immersed in mercuric bromophenol blue for 15 minutes. The sections were then rinsed in 0.5% acetic acid for 20 minutes and treated with 0.1 M sodium phosphate buffer (pH 7.0) for 3 minutes. Blue colour indicates the presence of proteins (Mazia et al. 1953).

3.4.2.6 Alkaloids

Fresh leaf sections were immersed in Dittmar's reagent and Wagner's reagent for 10 minutes each. Alkaloids stain orange or brown (Furr and Mahlberg, 1981).

3.4.2.7 Essential oil

Fresh leaf sections were placed in 1-naphthol and *N*,*N*-dimethyl-*p*-phenylene (NADI) reagent and incubated for 60 minutes in room temperature. Sections were rinsed in sodium phosphate buffer for 2 minutes. Essential oil stain blue and resin acids appear red (David and Carde, 1964).

3.4.2.8 Cutin and lignin

Fresh leaf sections were immersed in Safranin O for a few seconds and rinsed with distilled water. Lignified structures or cutinized cell walls stain bright red (Johansen, 1940).

3.5. Antibacterial activity

Ethanolic, methanolic and water extracts were tested against two strains of gram-positive bacteria:*Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1683); and five strains of gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Escherichia coli* (carbapenem-resistant) (ATCC BAA 2340), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 314588), as well as *Salmonella typhimurium* (ATCC 14026) according to the disc diffusion method (Rasoanaivo and Ratsimamananga-Urveg, 1993). Mueller Hilton agar (MHA) (Biolab, South Africa) was prepared (38 g in 1 L of water), poured into sterile petri dishes and allowed to set and dry at room temperature. Bacterial cultures were grown in Nutrient Broth (Biolab, South Africa) at 37°C for 18 hours in a shaking incubator and then standardized using a 0.5 McFarland standard turbidity. The bacterial cultures were further diluted with sterile distilled water in the ratio 1:150 v/v to yield a final concentration of 1 x 106 and then swabbed onto MHA plates. The samples to be tested were dissolved in 1000 µg/ml Dimethyl sulfoxide (DMSO). Antibiotic Assay discs (6 mm in diameter) were loaded with 20 µl of the prepared samples and placed onto the prepared agar plates which were inverted and incubated at 37 °C for 24 hours. The diameter of the zone of inhibition was measured in mm.

CHAPTER 4: RESULTS

4.1 Micro-morphology

Stereomicroscopy (fig. 4.1) and SEM of chemical fixed leaf samples (fig. 4.2) showed no external secretory structures. However, upon further examination SEM images of freeze-fractured leaf samples secretory cavities were observed (figure 4.3). A cavity is spherical with two layers of cell types, i.e. inner, larger epithelial cells surrounding a lumen and external parenchyma cells (fig. 4.3 and 4.4). Fig. 4.3a shows the cavity before secretory phase revealing a well-developed lumen while cavity containing secretions during a secretory phase is illustrated in figure 4.3b. Fig. 4.2 shows that the density of stomata on the abaxial surface is higher (a) than in adaxial surface (b).

Fig. 4.4 shows different stages of development of secretory cavities that are abundant on the lamina. There are upper elongated palisade parenchyma and rounded lower spongy parenchyma with intercellular spaces. Cavities appeared to be equally distributed in palisade and spongy parenchyma cells. Some cavities were found near the epidermis embedded in palisade cells. Those cavities found in palisade cells were enclosed by epithelial cells which were also surrounded by a layer of epidermal cells (fig. 4.4a, b). Cavities found amongst spongy mesophyll had two layers of cells. The inner layer consisted of flat, thin-walled epithelial cells that disintegrate at maturity and the outer layer is comprised of thick-walled spongy parenchyma cells (fig. 4.4). Fig. 4.4e shows that cavities in spongy cells are also close to the vascular bundle.

Meristematic cells are initial cells that later give rise to the cavity. These initial cells are cavity precursors and were small in size, characterized by a large nuclei (N), small vacuoles (V), thin cell walls and dense cytoplasm (fig. 4.5a). When inner cells were subjected to schizogenous formation, organelles and cytoplasm were disturbed creating a space amongst organelles (fig. 4.5a,b). As inner cells separated, other cells were seen to undergo lysis, as shown by fig. 4.5d and 4.4a. As the cavity became more pronounced, cell expansion took place in inner cells. The flatter part of the epithelial layer encapsulated

cells forming a lumen (fig. 4.4b; 4.5e). The lumen enlarged as the inner cells were subjected to schizo-lysigenous mechanism (fig. 4.5f). During maturation of secretory cavities, a large lumen, surrounded by epithelial cells were observed (fig. 4.4). Secretions of *Vepris lanceolata* foliar cavities were deposited straight to the cytoplasm (figure 4.5b).

Hence, the *Vepris lanceolata* leaf cavity develops from the separation and degeneration of epithelial cells. Epithelial cells produce secretions which are later stored in the lumen (fig. 4.5c-f). The mature cavity had a large central lumen accumulating essential oil and other secretions (fig. 4.5f). There were no special opening structures, which means that the oil is not dispelled at maturity. Furthermore, cavities were synthesized continuously as depicted in fig. 4.4, which shows cavities at different developmental stages for each leaf section.

Leaves were made up of outer cell layers, i.e. upper and lower epidermis that are enclosed by a protective layer called the cuticle (Fig. 4.7a). These epidermal layers consisted of stomata which are openings that allows for gaseous exchange (Fig. 4.2a). The parenchyma tissue was divided into palisade and spongy cells that were found between epidermal layers (Fig. 4.7a). Palisade parenchyma cells were cylindrical in shape and located below the upper epidermis while spongy parenchyma cells were round in shape and found below palisade cells. Spongy cells were also characterized by the presence of intercellular spaces as they were loosely packed to facilitate gaseous exchange (Fig. 4.7a).

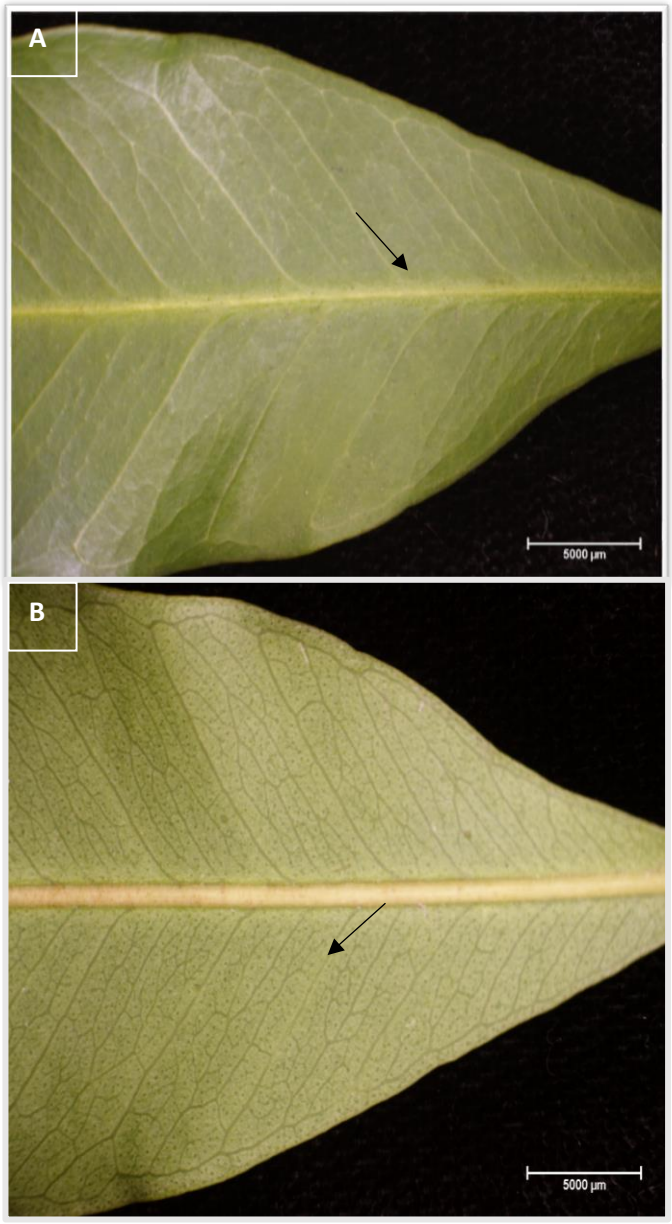


Figure 4.1 Stereomicroscopic images showing the overview of the leaf surface of the *Vepris lanceolate.* Both adaxial and surfaces appears to be smooth, no external secretory structures (arrows) are visible on both surfaces. [A] The adaxial surface. [B] The abaxial surface

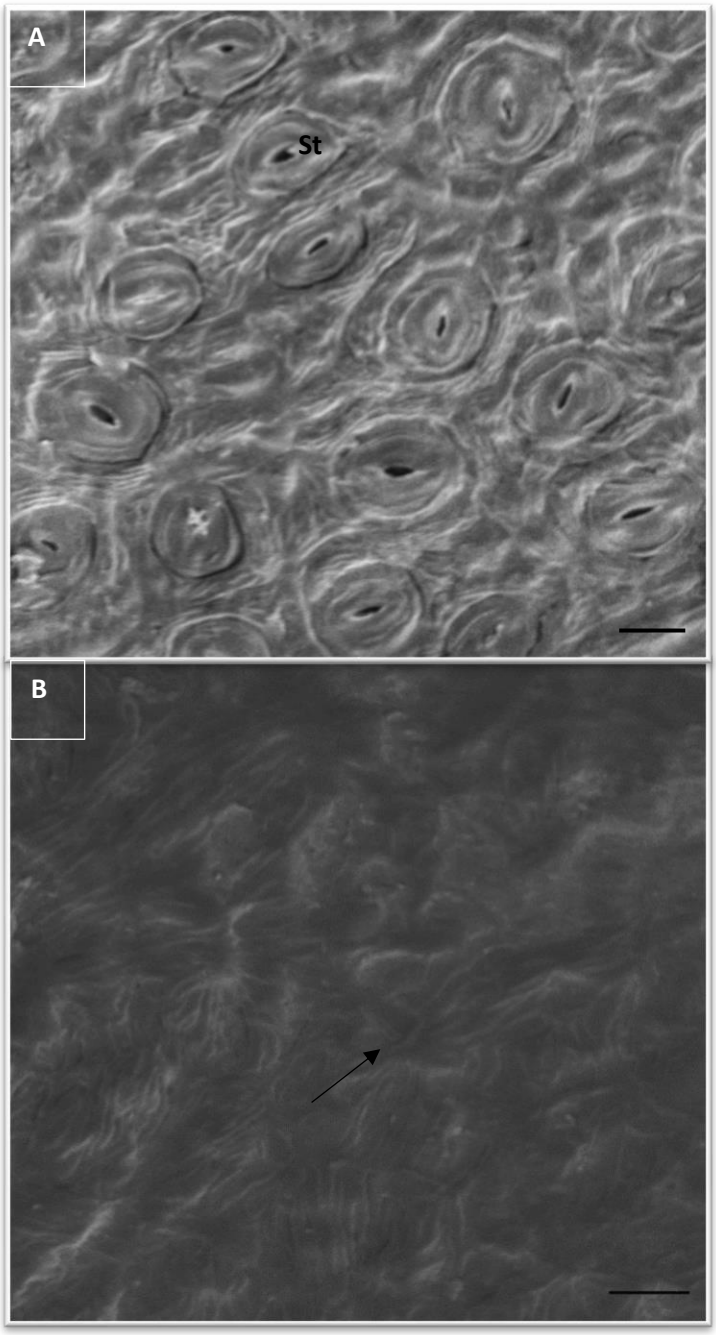


Figure 4.2 SEM image of leaves of *Vepris lanceolata* showing: [A] Stomata (**St**) on the abaxial surface, no specialized structures are observed. Scale bar= 20 µm. [B] No external specialized structures (arrow) on the adaxial surface. Scale bar= 200 µm.

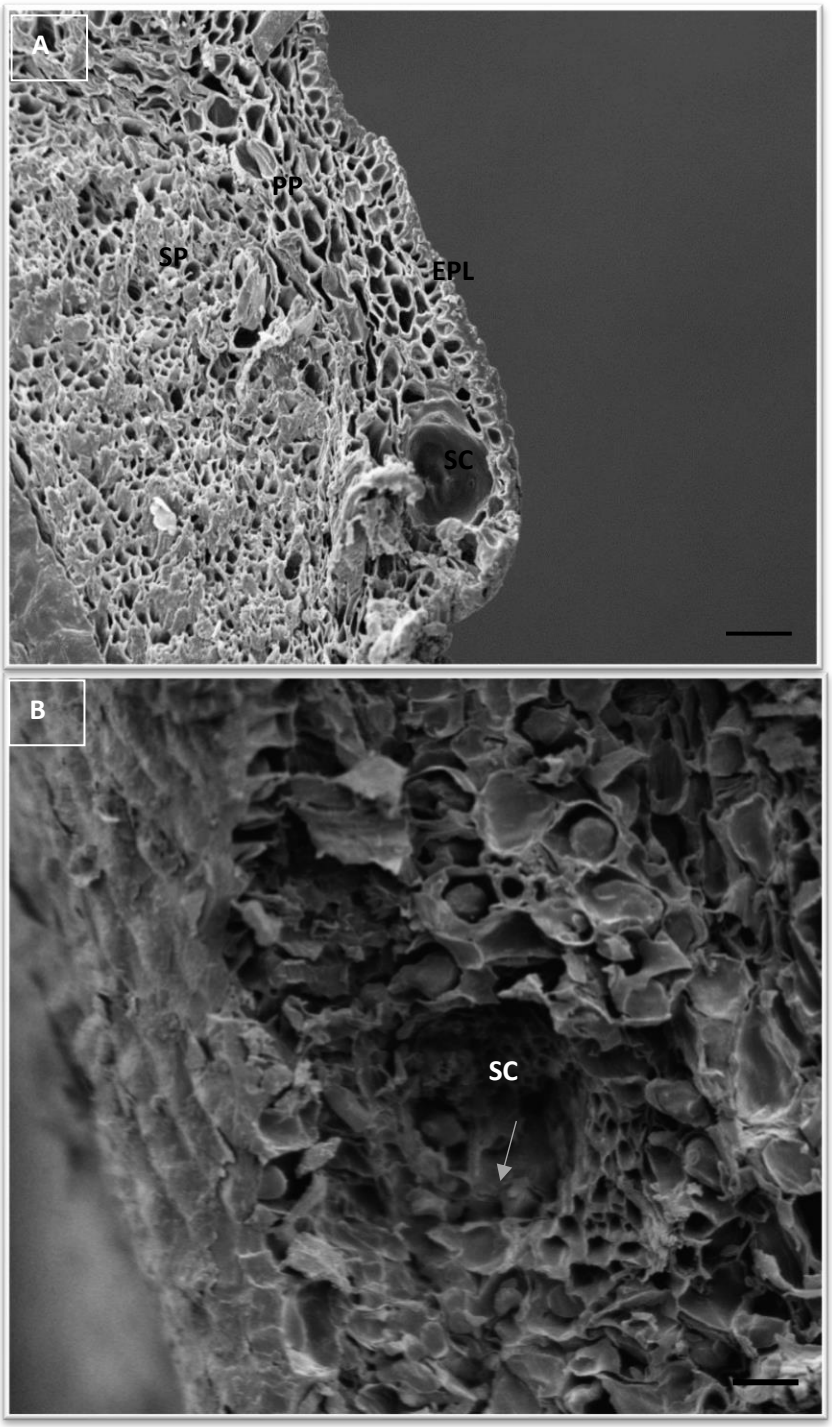
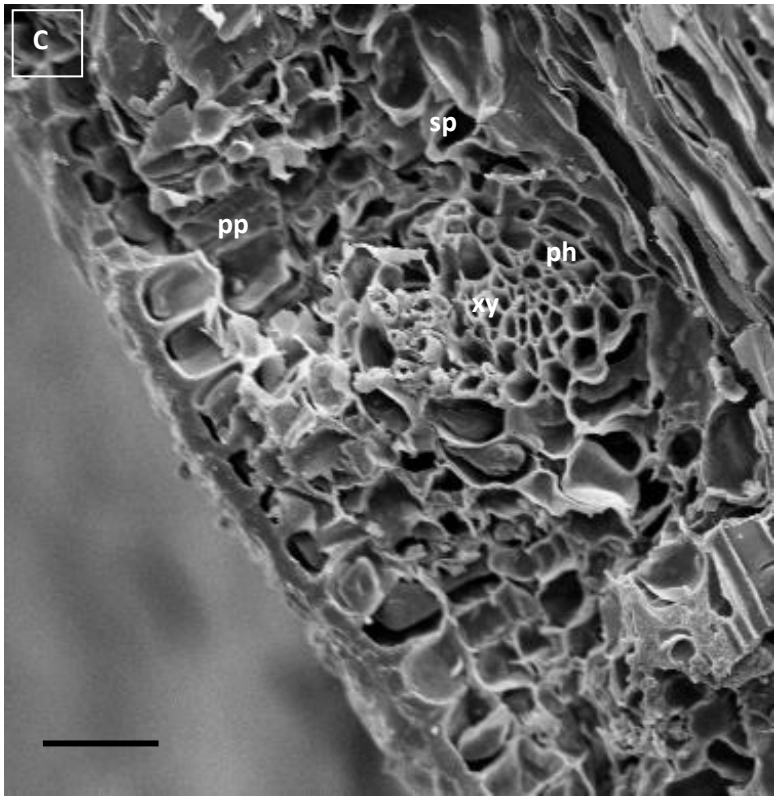


Figure 4.3 Cross sections of a freeze-fractured leaf of *Vepris lanceolata* showing: [A] Palisade parenchyma (**pp**), spongy parenchyma (**sp**), epidermal layer (**epl**) and secretory cavity (**sc**) located in palisade parenchyma. Scaler bar= 20 µm. [B] Fully developed cavity (**sc**) with pronounced lumen surrounded by epithelial cells during secretory phase plunged with secretion (arrow) formed within spongy cells. Scale bar= 20 µm.



[C] Vascular bundle illustrating phloem (**ph**) and xylem (**xy**) and located amongst spongy parenchyma (**sp**) below the palisade parenchyma (**pp**). Scaler bar= 20 µm.

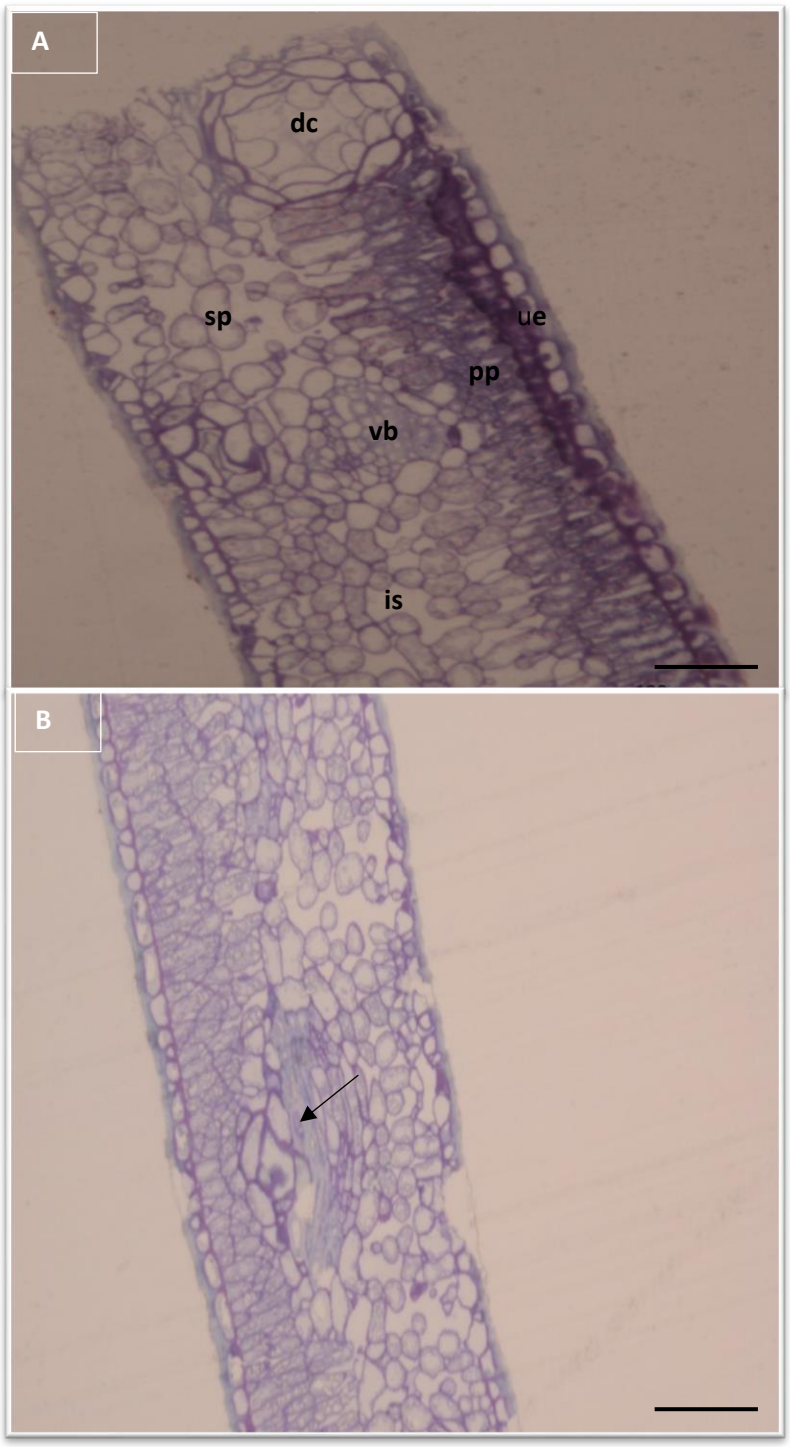
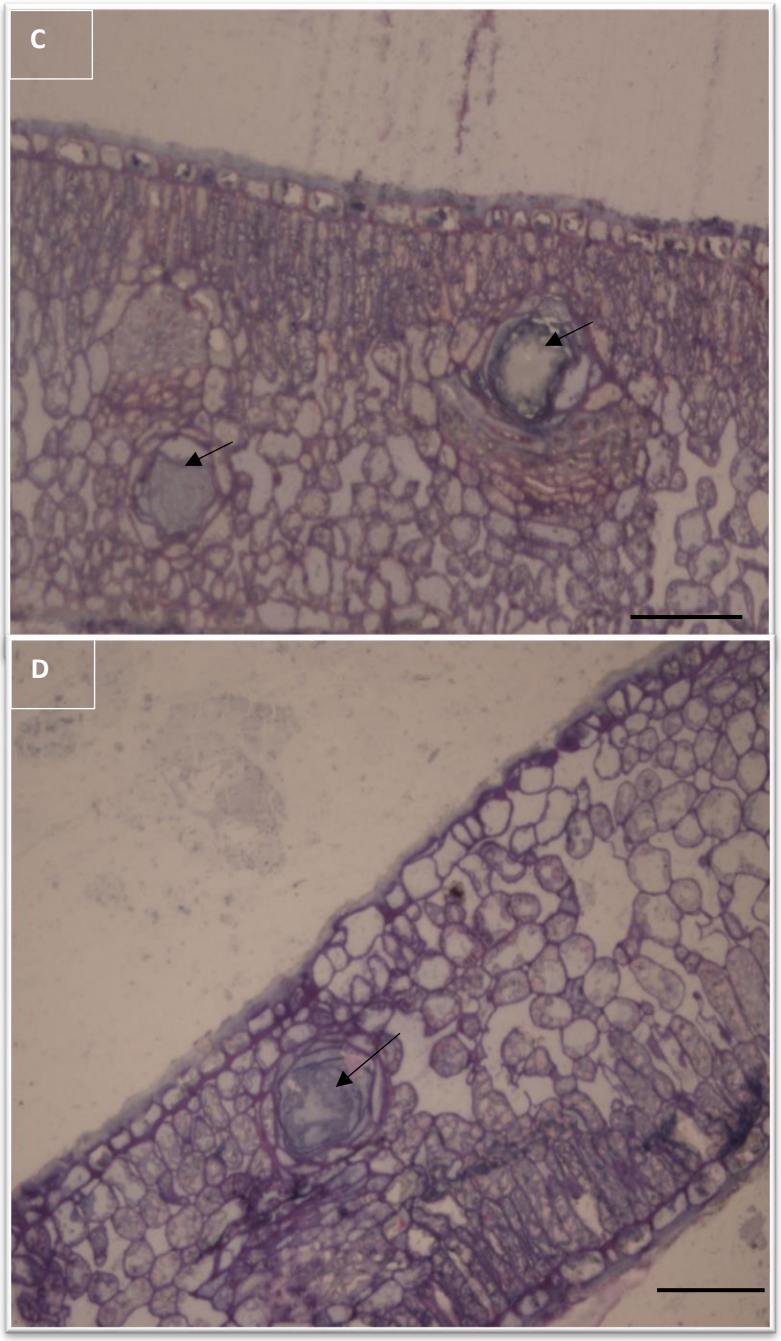
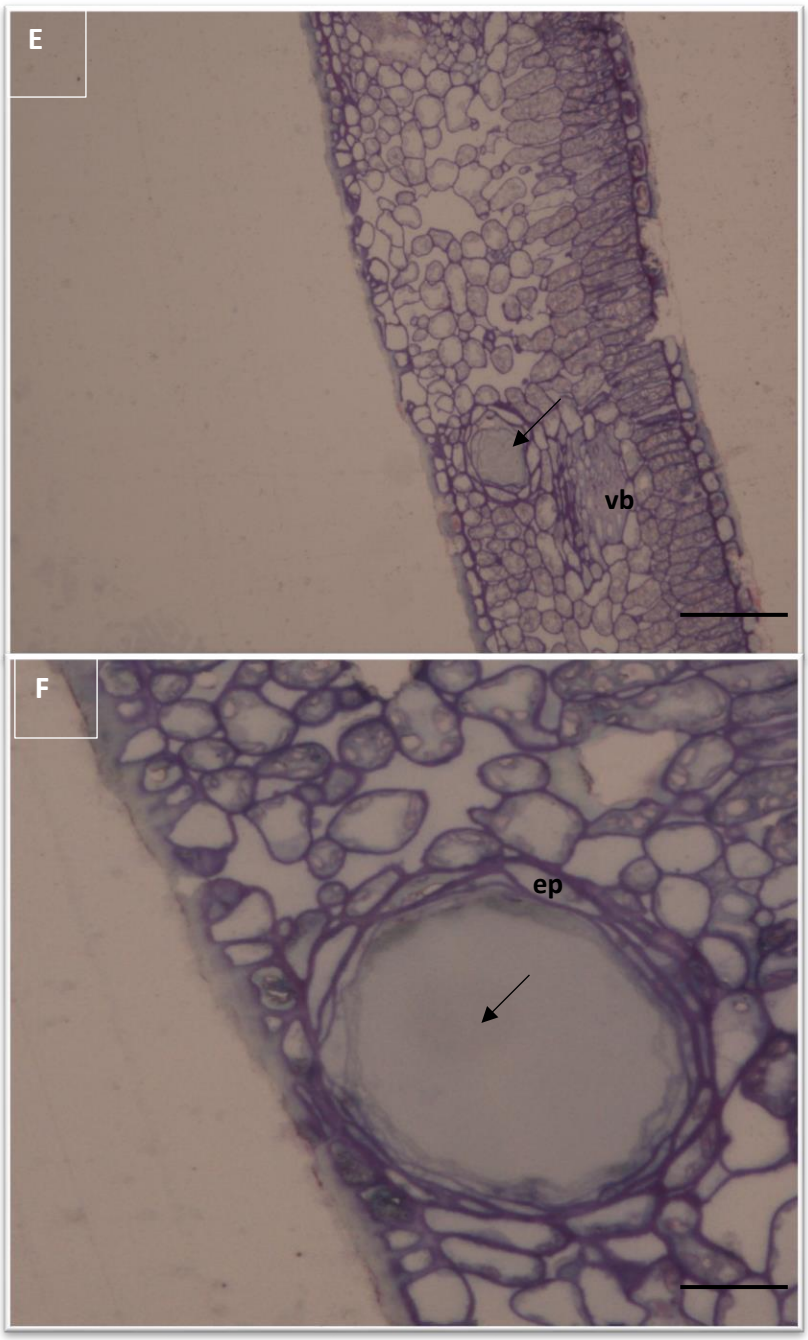


Figure 4.4. Light micrographs showing semi-thin sections of *Vepris lanceolata* leaf stained with toluidine blue. Scale bars = 100 µm [A] Developing cavity (**dc)** in palisade parenchyma, surrounded by upper epidermal layer (**ue**). Vascular bundle (**vb**) is located among spongy parenchyma (**sp**) and intercellular spaces (**is**). Meristematic cells during lysis can be observed in the developing cavity located amongst palisade mesophyll. [B] Stacks of surrounding epithelial cells (arrow) start to accumulate secretion which is observed by enlarging cells. The lumen at this this stage is still small and vacant, resembling intercellular spaces. This lumen can be distinguished from intercellular spaces by secreting cells surrounding it.



[C] As depicted by this image, cavities (arrows) can appear in both types of parenchyma tissue. Interestingly, the lower cavity located amongst spongy cells has been positioned next to the vascular bundle (**vb**). Lumen has now been filled with secretions, although some secretion was still present in several stacks of epithelial cells. [D] Another cavity (arrow) with two stacks of epithelial cell layers during secretory stage. Lumen is filled with secretions.



[E] Mature cavity (arrow) that is plunged with secretion located next to the vascular bundle (**vb**). [F] Enlarged image of cavity (arrow) surrounded by an epithelial cell layer (**ep**) after secretion has been accomplished. Secretion has been deposited into the lumen.

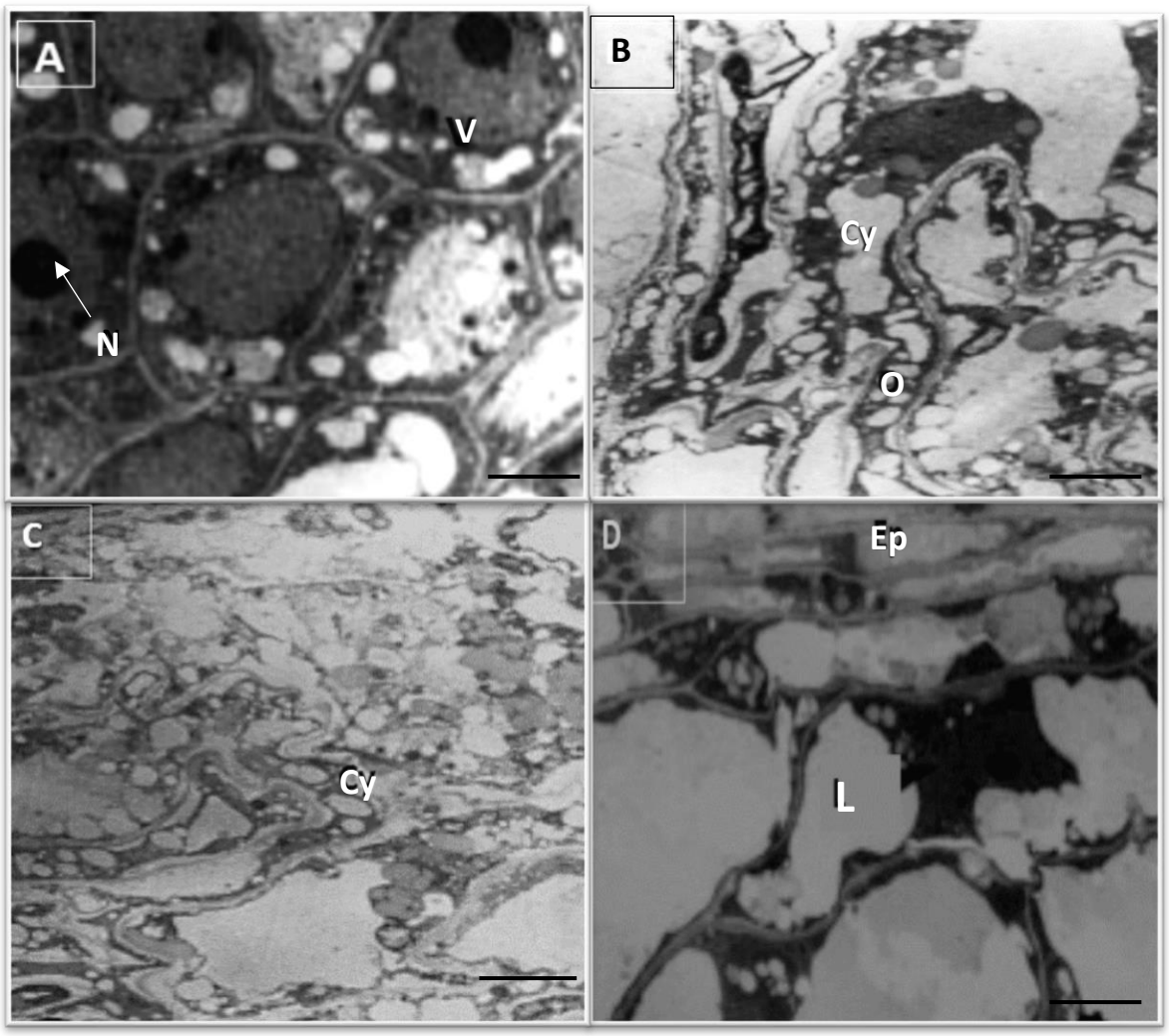
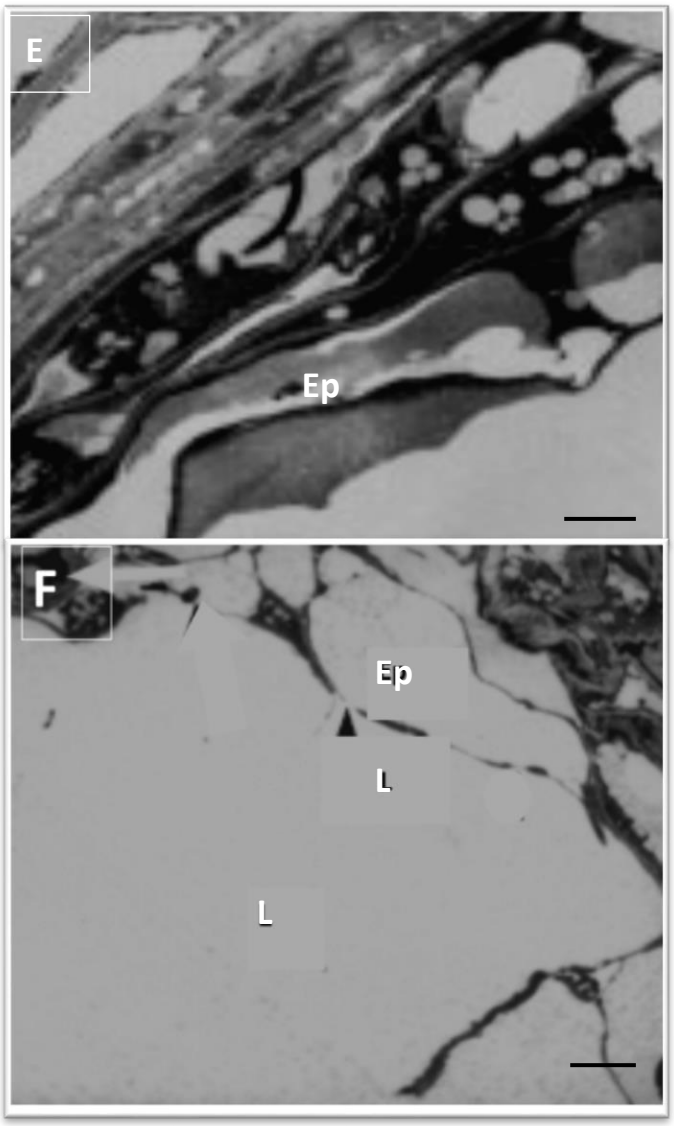


Figure 4.5 TEM images showing the ultra-structure and different stages of *Vepris lanceolata* cavity formation. [A] Micrograph showing ultrastructure of cell precursors located in the inner region of the cavity, at the beginning of the schizogeny. Small vacuoles (**V**) and nuclei (**N**) can be observed. Scale bar= 500nm. [B] Cytoplasm (**Cy**) and organelles appears to be distorted during schizogenous process. Oil droplets (**O**) are visible. Scale bar= 500nm. [**C**] Cell interior is highly disorganized and the cytoplasm (**Cy**) is completely degraded, when lysigenous process takes place. Scale bar= 500nm. [D] Stacks of epithelial cells (**Ep**) that surround the degrading inner region (L). Secretions that appears to be oil contents is observed as lysigenous process continuous to overlap with lysigeny. Scale bar= 500nm.



[E] Image showing epithelial cells in a close range in which contents of epithelial cells can be visualized. Scale bar= 200nm. [F] Lumen (L) is clearly visible surrounded by epithelial cells (arrow head) following schizo-lysigenous process. Scale bar= 200nm.

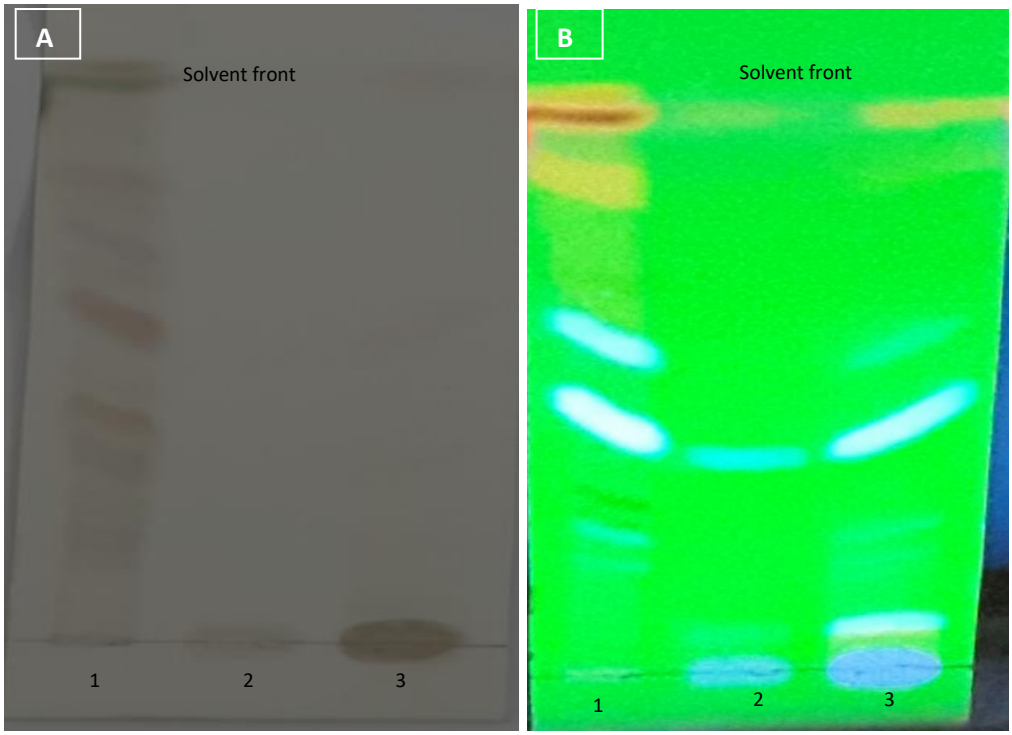
4.2 Histochemistry and Phytochemistry

Phytochemical analyses were carried out to determine the main groups of compounds present in hexane, methanol and ethanol leaf extracts of *Vepris lanceolata* which determine medicinal properties of the plant. Table 1 shows that leaf extracts reacted positive for alkaloids, glycosides, proteins, carbohydrates, phenolic compounds, volatile and non-volatile oils. Preliminary TLC studies showed separation of bands indicating the presence of main groups of active compounds in leaf extracts (fig. 4.6). Hexane showed more groups of compounds, while chloroform showed the least groups of compounds under UV light.

Histochemical analyses conducted on fresh leaf sections to show localization of the main groups of compounds (fig. 4.7). An unstained leaf section showed basic leaf anatomy and secretory cavities (fig. 4.7a). Foliar cavities stained with Wagner's and Dittmar reagent stained brownish orange showing the presence of alkaloids (fig. 4.7b). Nile blue stained the cavity red showing the presence of neutral lipids and the cavity lumen stained blue showing the compartmentalization of acidic lipids (fig. 4.7c). Epidermal layer stained orange to show the presence of cutin and cavity lumen also stained orange to indicate lipid secretion with Sudan III (fig. 4.7d). Fehling's solution stained leaf sections brick red showing the localization of reducing sugars (fig. 4.7e). Toluidine blue stained phosphate groups to purplish-greenish blue and lignin turned to blue while hydroxylated polysaccharides were not stained (fig. 4.7f). Blue-black colouration in the lumen of cavities indicated the presence of phenolic compounds during pre-secretory stage after staining with Ferric trichloride (fig. 4.7g). The lumen of developing cavities and epithelial cells stained black indicating the presence of phenolic compounds during post-secretory stage after staining leaf sections with Ferric trichloride (fig. 4.7h). Proteins turned greyish blue after staining leaf sections with Mercuric bromophenol blue (fig. 4.7i). The lumen of developing cavities stained blue showing the localization of essential oil while the epidermal and parenchyma cells turned reddish colour showing the secretion of resin acids after straining with the NADI reagent (fig. 4.7j). Safronin stained the cell wall and cavities bright red indicating the presence of cutin and lignin, respectively (fig. 4.7k).

Table 1 Results of phytochemical tests of hexane (**H**), chloroform (**C**) and methanol (**M**) extracts of *Vepris lanceolata* leaves

Test	**Compound**	**Present(+), Absent (-)**			**Reaction**
		H	**C**	**M**	
Dragendroff's test	Alkaloids	+	+	+	Reddish orange precipitate
Hager's test	Alkaloids	+	+	+	Yellow precipitate
Wagner's reagent	Alkaloids	+	-	-	Brown precipitate
Molisch's test	Glycosides	+	+	-	Violet ring
Fehling's test	Carbohydrates	+	+	+	Red precipitate
Benedict's test	Carbohydrates	-	+	-	Yellow-red precipitate
Ninhydrin	proteins	+	+	+	No reaction
Spot test	Fixed oils & fats	+	+	+	Oil stain on filter paper
Ferric chloride test	Tannins	-	-	-	Dark green
Lead acetate test	Phenolic compounds	-	+	-	Bulky white precipitate
Ruthenium red	Mucilage	-	-	-	No reaction
Conc. Suphuric acid	Flavonones	+	+	+	Yellow-orange



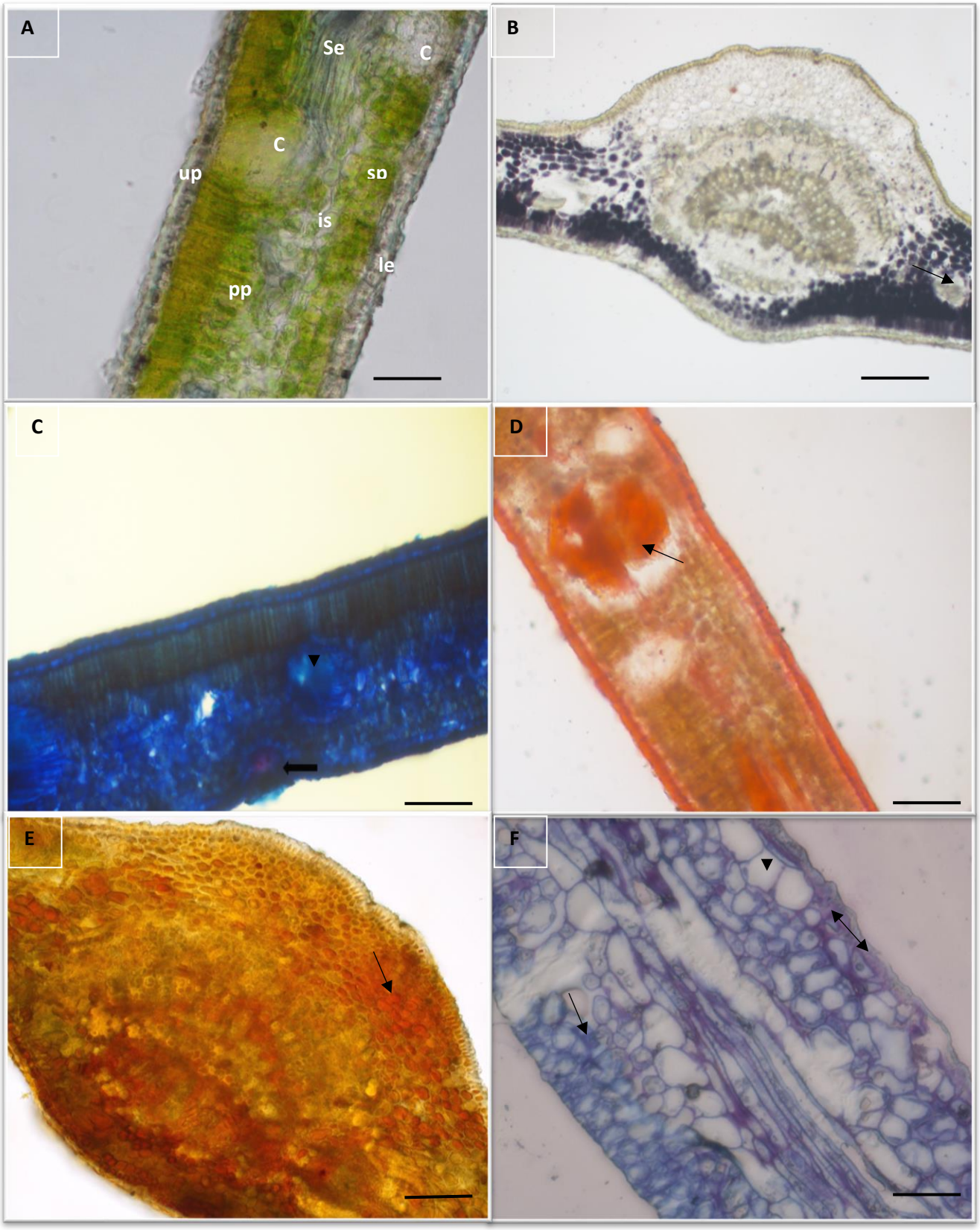
1-Hexane

2-Chloroform

3-Methanol

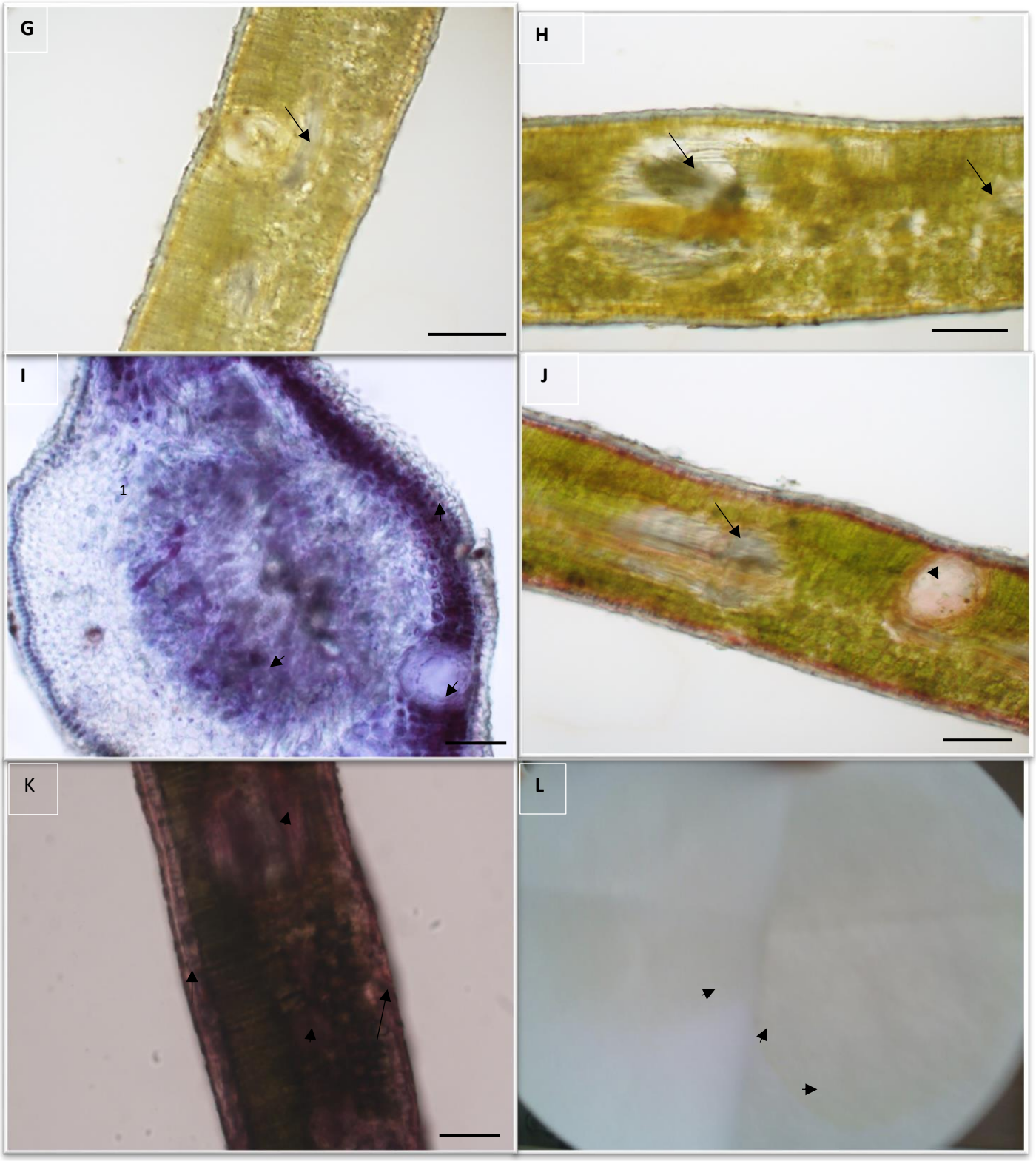
Mobile phase- Toluene: ethyl acetate: glacial acetic acid (9:1:0.3)

Figure 4.6. Thin Layer Chromatograph (TLC) of leaves of *Vepris lanceolata* hexane, chloroform and methanolic extracts [A] Visible light showing more bands for hexane than methanolic extracts. No bands were visible for the chloroform. [B] More bands are visible under 254nm UV for hexane than other solvents.



ue-upper epidermis, **le**-lower epidermis, **c**-cavity, **pp**-palisade parenchyma, **sp**-spongy parenchyma, **is**-intercellular space, **se**-secretion

Figure 4.7. Histochemical staining of fresh leaf sections of *Vepris lanceolate*. [A] Unstained fresh leaf section. Scale bar = 100 µm [B] Foliar cavities stained with Wagner's and Dittmar's reagent stained brownish orange showing the presence of alkaloids. Scale bar = 100 µm. [C] Leaf section stained with Nile blue. Oil gland stained red (arrow) showing the presence of neutral lipids and cavity lumen stained blue (arrow head) to show acidic lipids. Scale bar = 100 µm. [D] Fresh leaf section stained with Sudan III. Epidermal layer stain orange to show the presence of cutin and cavity lumen also stain orange to indicate lipid secretion. Scale bar = 100 µm. [E] Fresh leaf section stained brick red with Fehling's solution to show reducing sugars. Scale bar = 100 µm. [F] Toluidine blue stained phosphate groups to purplish-blue (double arrow), hydroxylated polysaccharides were not stained (arrow head) and lignin turned to blue (arrow). Scale bar = 100 µm.


G
H
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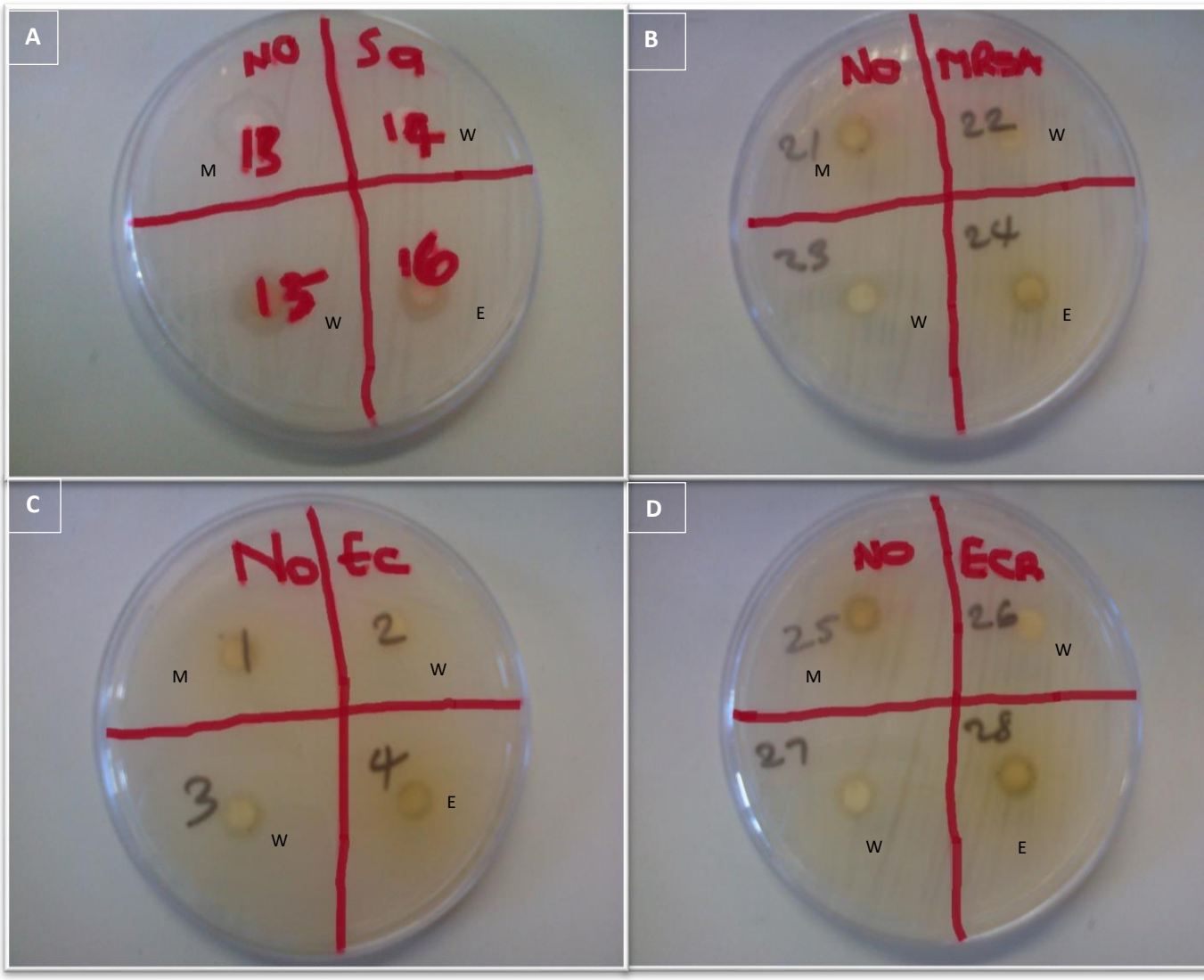
[G] Blue-black colouration in the epithelial cell layers (arrow) indicates the presence of phenolic compounds during pre-secretory stage after staining with Ferric trichloride. Scale bar = 100 µm. [H] The lumen of developing cavities and epithelial cells (arrows) stained black to indicate phenolic compounds during post-secretory stage following staining with Ferric trichloride. Scale bar = 100 µm. [I] Proteins turned greyish blue (arrow heads) following staining with Mercuric bromophenol blue. Scale bar = 100 µm. [J] Staining leaf section with NADI reagent; the lumen of developing cavities stained blue (arrow) to indicate essential oil; epidermal and parenchyma cells reddish colour (arrow head) to show resin acids. Scale bar = 100 µm. [K] Cutinized cell wall (arrows) and lignified structures (arrow heads) turned bright red after staining with safranin O. scale bar= 100 µm. [L] Spot test showing the presence of fixed oils and fats.

4.3 Antibacterial Activity

Methanolic, ethanolic and water extracts were used to screen antibacterial activity of *Vepris lanceolata* leaves against gram positive and gram negative bacteria (fig. 4.8; table 4.2; fig. 4.9). Gram positive bacteria used were *Staphylococcus aureus* (Sa) and methicillin-resistant *Staphylococcus aureus* (MRSa). Gram negative bacteria were *Escherichia coli* (Ec), *Escherichia coli* (carbapenem-resistant) (EcR), *Klebsiella pneumonia* (Kp), *Pseudomonas aeruginosa* (Pa) and *Salmonella typhimurium* (St). Inhibition zone of 1 cm and above was considered as showing definite activity while that of 0.6-0.9 cm zone was taken as displaying moderate activity. Slight activity was the inhibition zone of 0.1-0.5 cm in this study. Zero growth inhibition was taken as no activity against the bacterial strain.

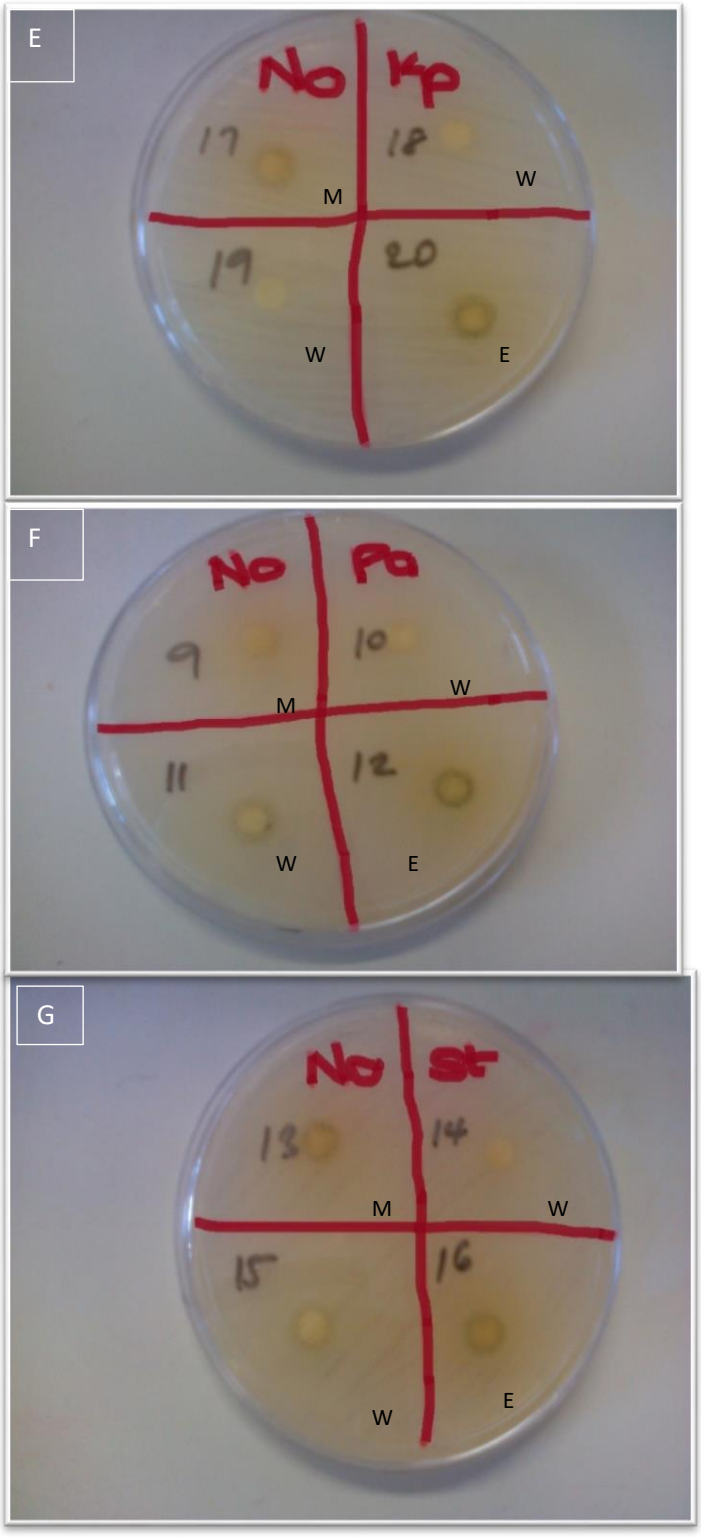
Crude extracts showed antibacterial activity against tested organisms, except for Kp. Crude extracts inhibited almost all strains when compared to water extract. Water extract only showed highest activity against Sa compared to all bacterial strains and extracts, with 2.1 cm inhibition zone. Furthermore, crude extracts showed highest activity against Sa, with inhibition zone of 1.9 cm and 1.6 cm for ethanolic and methanolic extracts, respectively. Gram positive MRSa exhibited moderate activity with 0.9 cm using ethanol and methanol and zero activity with water extract. Ec showed moderate activity with crude and water extracts as there was inhibition zone of 0.7 cm for both methanolic, water extracts; and 0.8 cm for the ethanolic extract.

Moderate activity was observed in EcR with slight activity of 0.8 cm and 0.9 cm for methanol and ethanol, respectively. Methanolic extract had 0.1 cm inhibition against Pa and was the only one with slight activity across all tests. Ethanolic (1 cm) and water (0.7 cm) extracts had definite and moderate activity against Pa strain, respectively. Kp only reacted to ethanolic extract observed by the zone of inhibition of 0.8 cm. No activity was observed for water and methanol against Kp. Moderate susceptibility to methanolic and ethanolic extracts was observed in St with 0.9 cm inhibition zone.



M- Methanol, W-water, E-ethanol

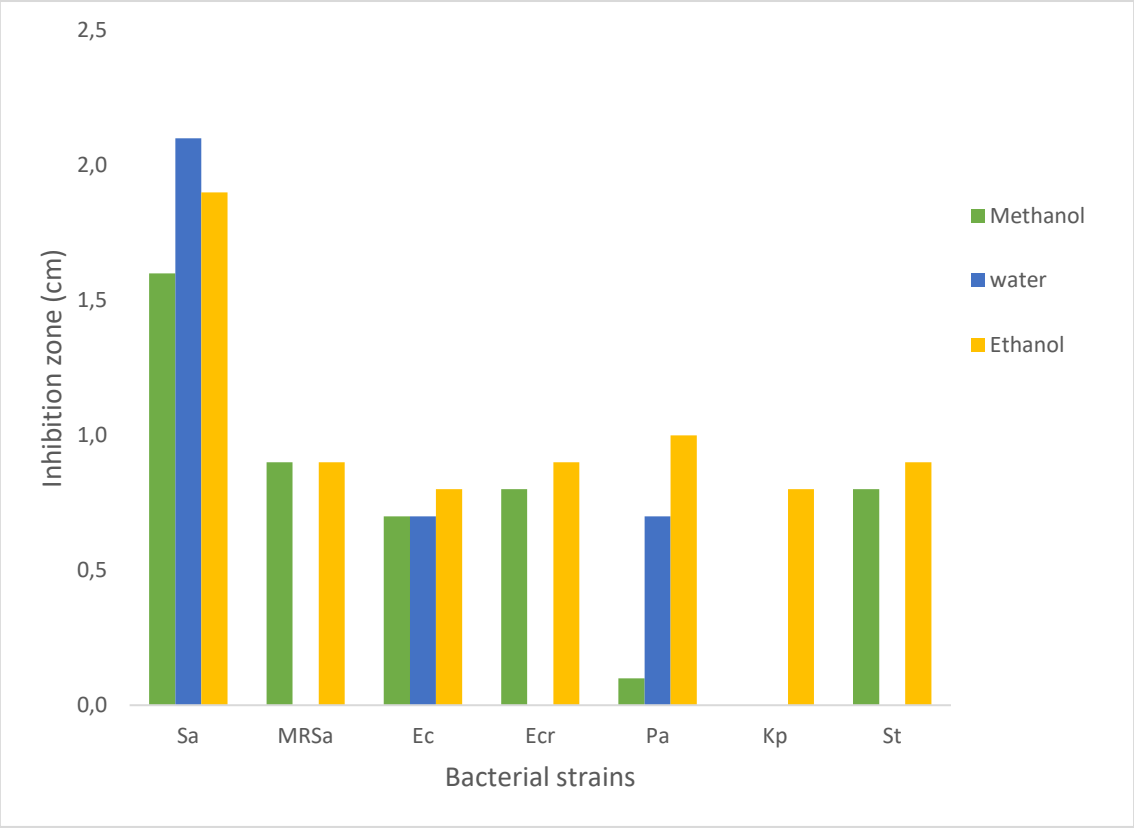
Figure 4.8. Antibacterial activity of *V. lanceolata* leaf using methanolic, water and ethanolic extracts . [A] Water extracts showed the highest activity against Gram positive *Staphylococcus aureus*. The strain was also susceptible to ethanolic and methanolic extracts. [B] Gram positive methicillin-resistant *Staphylococcus aureus* exhibited moderate activity using ethanolic and methanolic extracts only. [C] Moderate activity was observed in *Escherichia coli* (carbapenem-resistant) using methanolic and ethanolic extracts. Zero inhibition detected with water extract. [D] Definite activity with methanolic extract, moderate activity with ethanolic extract and slight activity was seen for *Escherichia coli*.



M- Methanol, W-water, E-ethanol

[E] *Klebsiella pneumonia* only reacted to ethanolic extract. No activity was observed with water and methanol. [F] Methanolic and ethanolic extracts were moderately and definitely,

respectively, active against *Pseudomonas aeruginosa* strain. Water extract showed slight activity. [G] *Salmonella typhimurium* was moderately susceptible to methanol and ethanol solvents.



Staphylococcus aureus (Sa), methicillin-resistant *Staphylococcus aureus* (MRSa), *Escherichia coli* (Ec), *Escherichia coli* (carbapenem-resistant) (EcR), *Pseudomonas aeruginosa* (Pa), *Klebsiella pneumonia* (Kp) and *Salmonella typhimurium* (St).

Figure 4.9. Graph showing antibacterial activity of *V. lanceolata* leaf using crude (methanolic, ethanolic) and water extracts

Staphylococcus aureus: Water had the highest inhibition (2.1 cm) while methanol and ethanol had 1.6 cm and 1.9 cm, respectively. Methicillin-resistant *Staphylococcus aureus*: Crude extracts showed 0.9 cm inhibition and no activity for the water extract. *Escherichia coli*: methanolic and water extracts had 0.7 cm inhibition, ethanolic extract showed 0.8 cm inhibition zone. *Escherichia coli* (carbapenem-resistant): no activity for water extract and moderate activity for methanolic (0.8 cm) and ethanolic (0.9 cm) extracts. *Pseudomonas aeruginosa*: slight activity (0.1 cm) for methanolic extract, moderate (0.7 cm) for water and definite activity (1 cm) for ethanolic extract. *Klebsiella pneumonia*: no activity for methanolic and water extracts, while ethanolic extract showed 0.9 cm inhibition. *Salmonella typhimurium*: no activity for water extract and moderate activity for methanolic (0.8 cm) and ethanolic (0.9 cm) extracts.

Table 2 Antibacterial activity of methanolic, ethanolic and water extracts of *V. lanceolata* leaf

BACTERIAL STRAIN	**LEAF EXTRACTS**		
	METHANOL	**WATER**	**ETHANOL**
Staphylococcus aureus	+++	+++	+++
methicillin-resistant *Staphylococcus aureus* (MRSA)	++	-	++
Escherichia coli	+++	++	++
Escherichia coli (carbapenem-resistant)	++	-	++
Pseudomonas aeruginosa	+	++	+++
Klebsiella pneumonia	-	-	++
Salmonella typhimurium	++	-	++

Key

+++ Definite activity: (1 and >2.5 cm)

+ + Moderate activity: (0.6-0.9 cm)

+ Slight activity : (0.1-0.5 cm)

No activity : (0cm)

CHAPTER 5: DISCUSSION

5.1 Micromorphology and development of the secretory cavity

In this study, epithelial cells surrounding the lumen of cavities contained small vacuoles, oil and dense cytoplasm showing that epithelial cells are active secretory cells (Fahn, 1988) for *V. lanceolata.* Essential oils and other phytocompounds are secreted as oil droplets in specialized cells (Caissard et al. 2004). Storage of secretions in cavities can occur in two ways, either within plastids or directly into the cytoplasm (Bosabalidis and Tseko, 1982). Secretions of *V. lanceolata* cavities were deposited straight to the cytoplasm (fig. 4.5b). Cavities of *Polygonum glabrum* were also reported to have developed in epidermis and sub-epidermal region (Curtis and Lersten, 1994) while *Myoporum bontiodes* cavities occurred in the upper parenchyma region (Das et al. 2013).

V. lanceolata foliar cavities produce large quantities of secretions (fig. 4.4b). The cavity lumen that secrete large amounts of secretions is generally surrounded by numerous layers of epithelial cells, thereby providing large surface area for the secretions to be stored (Svoboda and Hampson, 1999). Secretory cavities are used as a taxonomic feature in Rutaceae, as they are found in all the species of this family (Knight et al. 2001). Myrtaceae and Rutaceae secretory cavities have the same form but differ in origin of the secretory cavities (Kalachanis and Psaras, 2005).

Cavities can form in three ways, namely schizogenous, lysigenous and schizo-lysigenous development (Turner et al. 1998; Teixeira et al. 2000). Schizogenous cavities are formed as a result of the secretory epithelium-lined space that forms following the division or separation of initial cells (Turner et al. 1998; Svoboda and Hampson, 1999; Teixeira et al. 2000). Lysigenous cavities form after degradation of glandular cells, with secretory cells discharging their secretion into the expanding space (Turner et al. 1998; Svoboda and Hampson, 1999; Teixeira et al. 2000). Schizo-lysigenous cavities form when a

storage space commences schizogenously, and then lysigenously, further widening the storage cavity (Turner et al. 1998; Teixeira et al. 2000).

Although the *V. lanceolata* foliar cavity appears to have developed schizo-lysigenously, however, there is still a disagreement in the literature as to whether cavities develop schizo-lysigenously, lysigenously or schizogenously due to sample preparation methods (Fahn, 1988; Turner et al. 1998; Turner, 1999; Teixeira et al. 2000; Kalachanis and Psaras, 2005; Paiva and Machado 2007). Most of these arguments were on the Rutaceae family (Turner, 1999). Turner (1999) showed that different methods of sample preparations lead to different outcomes. Dry mounted samples showed schizogenous cavities with epithelial cells that were still intact while water mounted cavities had extensive bulging of epithelial cells similar to lysigenous cavities (Turner, 1999). All of these publications contradicted with Tschirch and Haberlandt (who suggested that all of the Rutaceae cavities develop schizo-lysigenously (Turner, 1999; Liang, 2006). Tschirch and Stock (1933) believed that cavities develop schizo-lysigenously and authors that suggest schizogenous and lysigenous development were due to incompletely assessing schizo-lysigenous development of cavities (Turner, 1999). Thompson et al. (1976) and Liang, (2006) reported that at an early stage, cavities are schizogenous and a later stage, damaged cells were observed indicating lysigenous cavities.

Rustia cavities originated schizogenously (Viera et al. 2001). *Citrus* have been reported to have lysigenous oil cavities, such as *Citrus deliciosa* fruit gland (Turner et al. 1998; Kalachanis and Psaras, 2005). However, there are other reports that suggest that cavities develop schizogenously for Rutaceae, such as in *Boronia* genus and *Citrus sinensis* (Turner et al. 1998; Kalachanis and Psaras, 2005). Ciccarelli et al. (2008) reported that cavities in *Myrtus communis* are formed schizo-lysigenously. However, in this publication, it was mentioned that other studies of *Myrtus communis* reported that cavities occur schizogenously.

Previous publications show that schizo-lysigenous and lysigenous developments are due to the artefacts of sampling preparation (Viera et al. 2001). Such an example was illustrated in the *Citrus limon* structure showing that the observation of lysigenous development was due to a fixation artefact, resulting from osmotic swelling of secretory cells submerged in salt solution (Viera et al. 2001). Cells from mature leaves bulge easily compared to those of younger leaves (Turner et al. 1998). This is misinterpreted as autolysis of cells following maturation (Turner et al. 1998; Viera et al. 2001). Secretory cells had thin walls compared to neighbouring cells causing them to break and rupture after fixation with hypertonic solution, causing an assumption that cavities form by lysis of schizogenous cavities (Turner et al. 1998). After modifying the protocol previously used, Turner et al. (1998) confirmed that *Citrus limon* cavities develop schizogenously and not lysigenously. Misconception of lysigenous development or incomplete assessment of schizo-lysigenous has been published in numerous articles and textbooks (Carr and Carr, 1970; Bosabalidis and Tseko, 1982; Fahn, 1982). However, some authors (Fahn, 1979) have demonstrated schizogenous development for *Citrus* and other Rutaceae species. Thompson et al. (1976) showed that *Citrus sinensis* cavities develop schizogenously.

5.2 Chemical composition of the secretions

Light micrographs, histochemical and phytochemical analysis confirmed that cavities of *V. lanceolata* are rich in essential oil and other phyto-compounds. Secretory cavities are involved in the synthesis of major phytochemical compounds (Fahn, 1988; Palazzolo et al. 2013). Phytochemical compounds are responsible for protection against pathogens and herbivores; communication signals between plants and beneficiary organisms; attract pollinators and shield against harmful ultraviolet (UV) rays and oxidative stress (Figueiredo et al. 2008; Bruneton, 2012). Treatment of disorders and ailments is dependent on medicinal properties of the plant (Banu and Cathrina, 2015; Devi et al. 2017; Waweru et al. 2017; Yadav et al. 2017).

Histochemical analysis showed the localization of volatile oil (terpenoids), alkaloids, neutral (fats, waxes, oils) and acidic (phospholipids, free fatty acids) lipids, proteins, phenolic compounds, reducing sugars, lignin, phosphate groups and polysaccharides. The Rutaceae, Asteraceae, Liliaceae, Malvaceae, Myrtaceae, Pinaceae, Lauracea and Rosaceae possess secretory cavities rich in secondary metabolites (Curtis and Lersten, 1986; Fahn, 1988; Kalacharis and Psaras, 2005; Figueiredo et al. 2008). *V. lanceolata* secretory cavities stained positive for lipids with Sudan III while Nile blue stained neutral lipids red and acidic lipids blue. Oil stain on filter paper showed the secretion of fixed oil and fats. Terpenoids which were detected in essential oil of *V. lanceolata* by a blue colouration are responsible for plant defence processes (Wittstork and Gershenzon, 2002). Terpenoids are localised within cytoplasm or plastids and secreted in specialized cells (Harbone, 1998). Essential oils have been reported in numerous studies to exhibit medicinal properties (Ascensao et al. 1997; Harbone, 1988; Brophy et al. 2016; Reddy and Al-Rajab, 2016; Silva et al. 2016; Vitali et al. 2017; da Silva et al. 2017; Deng et al. 2017; Garcia et al. 2017; Loizzo et al. 2018).

In this study, Wagner and Ditmar reagents reacted positively for alkaloids as *V. lanceolata* cavities and surrounding secretory cells stained brown while phytochemical tests of crude extracts turned brown. Studies have shown that alkaloids are used to treat depression, tumour, malaria, as stimulants, antibacterial and anaesthetic (Descallar et al. 2017). Alkaloids have anti-oxidative property and are crucial in combating cancer, ageing, inflammation, anxiety and cardiovascular diseases (Bruneton, 2012; Saxena et al. 2013; Descallar et al. 2017). According to Tiwali et al. (2011) and Waweru et al. (2017), alkaloids have anti-diuretic, anthelminthic and antidiarrheal activity. Acridone alkaloids were also isolated from leaf and root extracts of *Vepris verdoorniana* (Atangana et al. 2017) and *Vepris macrophylla* leaves (Maggi et al. 2013). Reddy et al. (2016) reported the presence of alkaloids in *Clausena dentata* (Rutaceae). Alkaloids were also detected in ethanolic and water extracts of *Lycopodium clavatum* (Descallar et al. 2017). Yadav et al. (2017) reported alkaloids in *Catharanthus roseus, Ocimum sanctum* and *Colotripis procera. Ruta chalpensis* (Rutaceae) was reported by Kasimala et al. (2014) to produce saponins, flavonoids, phenols, terpenes and non-volatile oils from crude extracts with

antioxidant effect and to treat colon cancer. Furoquinone alkaloids were reported in methanolic leaf extracts leaf extracts of *Oricia renieri* and *Oricia suaveolens* from the Rutaceae family (Nouga et al. 2017). Alkaloids can also be used as painkillers to relieve pain (Akinpelu and Onakoya, 2006).

Ferric trichloride was used in this study to detect the localization of phenolic compounds in *V. lanceolata* leaf sections. Ferric ions reacted with orto-dihydroxyphenols to produce a black or deep green deposits (Harborne, 1998). Epithelial cells surrounding the cavity were observed to have reacted positive in developing cavities while the lumen reacted positive in mature cavities. This phenomenon showed that epithelial cells do produce secretions which are later deposited into the lumen (Svoboda and Hampson, 2000).

Ferric chloride and lead acetate were used to screen the presence of tannins (dark green) and phenolic compounds (bulky, white precipitate), respectively in *V. lanceolata* leaves. Other authors have mentioned that herbs secreting tannins are used in the treatment of diarrhoea and dysentery (Akinpelu and Onakoya, 2006). Phenolic compounds are responsible for protection against microbes and UV radiation (Sharanabasappa et al. 2007). Phenols display anti-oxidant activity against tumour cells that have high concentration of reactive oxygen species (ROS) (Saxena et al. 2013). Phenolic acids are responsible for gastric hepatic and intestinal metabolism (Saxena et al. 2013). Phenolic compounds are polymerized into lignin and condensed tannins (Saxena et al. 2013). Tannins have astringent, anti-diarrhoeal, diuretic, anti-oxidant and antiseptic activities (Saxena et al. 2013). Poullain et al. (2004) reported that *V. lanceolata* displayed moderate antioxidant (β-carotene) and radical scavenging (DPPH) activity associated with the presence of phenolic compounds, tannins and flavonoids. These activities were observed in dichloromethane stem extracts and not in methanolic extracts (Poullain et al. 2004). *Anisomeles malabarica* has antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* due to alkaloids, tannins and flavonoids present in extracts (Kavitha et al. 2017).

Bromophenol blue stained *V. lanceolata* leaf section blue and ninhydrin turned crude extracts purple to show proteins while brick red was indicative of reducing sugars. A study by Ayoola et al. (2008) reported the presence of reducing sugars in ethanolic extracts of *Psidium guajava, Carica papaya.* In the present study, Molisch's test detected the presence of cardiac glycosides with a purple ring. Cardiac glycosides are steroidal glycosides that have been used in traditional medicine for centuries (Bruneton, 2012). Cutin detected in *V. lanceolata* leaves using Sudan III is responsible for plant growth and development (Curves et al. 2010). Lignin also detected in *V. lanceolata* is a polysaccharide that confers strength to cell components to handle mechanical and environmental pressure (Bischoff et al. 2010; Bruneton, 2012). Free phenols and phenolic acids are combined with lignin to form ester groups or they can be found in leaf components that are not soluble in alcohol (Harbone, 1998).

Flavonoids (flavonones) produced a yellowish orange colour with concentrated sulphuric acid test in *V. lanceolata* leaf extracts. Flavonoids are radical scavengers which have antioxidant activity and heal wounds (Bruneton, 1999; Ghafar et al. 2010). *Citrus wilsonii* and *Citrus medica* extracts have definite antioxidant activity due to flavonoids, limonoids and coumarins (Zhao et al. 2015). Flavonoids are phenolic compounds that are present in all vascular plants (Harbone, 1998). Flavones, flavonones and flavonols are known as flavonoid glycosides (Bruneton, 2012). These compounds have antioxidative, anti-inflammatory, cardiovascular, antiviral and anti-platelet properties (Bruneton, 2012; Saxena et al. 2013; Barreca et al. 2017). Flavonones have a bitter taste and are colourless in fruits and leaves (Harbone, 1998). They have been reported in *Citrus* sp. (Khan et al. 2010). *Graptophyllum pictum* leaves showed antioxidant activity using DPPH and ABTS assays due to flavonoids, reducing sugars, saponins, phenolics and tannins (Jiangseubchatveera et al. 2017). Flavonoids showed antioxidative, anti-inflammatory, antifungal, antifungal, anti-carcinogenic and cytotoxic activities of ethanolic and water extracts of *Lycopodium clavatum* (Descallar et al. 2017).

5.3 Antibacterial activity

In the present study, gram positive bacteria *Staphylococcus aureus* had the highest susceptibility than methicillin-resistant *Staphylococcus aureus* (MRSa) and all gram negative bacterial strains using methanolic, ethanolic and water extracts of *V. lanceolata* leaves. *S. aureus* had the highest sensitivity with 2.1 cm inhibition zone using water extract. Gram positive bacteria were more sensitive to antibacterial agents than gram negative bacteria due to the outer membrane of gram negative being resistant and impenetrable (Srinivasan et al. 2001; Shake et al. 1999; Fisher and Phillips, 2008; Orlanda and Nascimento, 2015). This is due to the outer membrane outlining the cell wall inhibiting the penetration of hydrophilic compounds (Khan et al. 2010). However, other authors suggest that gram negative bacteria grow very slowly, hence over time it would be affected the same as gram positive bacteria (Fisher and Phillips, 2008).

A study by Reddy and Al-Rajab, (2016) revealed that *Ruta graveolens* (Rutaceae) had antimicrobial activity against *Bacillus cereus S. aureus* and fungi *Candida albicans* and no activity against *Enterobacter aerogenes* due to high resistance of Gram negative bacteria. Mothana et al. (2008) reported activity of *Lippia ctriodora, Plectranthus hadiensis, Acalypha fruticose,* and *Dodonaea viscora* extracts against *B. subtilis, Micrococcus flavus,* MR *S. epidermidis*, MRSa and no activity against gram negative bacteria (*E. coli* and *P. aeruginosa*). Methanolic extracts of 21 plant species were reported by Rabe and van Staden, (1997) to have antibacterial activity than aqueous extracts, in which all gram positive were sensitive to the extracts and only *Warbugia salutaris* showed activity against *E. coli*. Matu and van Staden (2003) also mentioned studies in which *S. aureus* was the most sensitive bacterium to crude extracts.

V. lanceolata leaf extracts were active against *S. aureus*, MRSa, *E. coli*, EcR, *P. aeruginosa, P. klebsiella* and *S. tymphimurium*. Methanol and ethanol solvents were more appropriate to be used in antimicrobial assays than water since they are commonly used in antimicrobial studies since they show more activity against tested microorganisms (Ahmed and Beg, 2001). Ethanol was also the better solvent in this study compared to

methanol by inhibiting the growth of all tested micro-organisms using *Vepris lanceolata* leaf extracts. However, in some studies methanol was a better solvent than ethanol (Aumeeniddy-Elalfi et al. 2015). A study by Narod et al. (2004) showed that *Vepris lanceolata* and *Todalia asiatica* leaf and stem extracts had antibacterial and antifungal activity using agar dilution method. *Vepris lanceolata* chloroform: methanolic, methanolic and hexane extracts were effective against *P. aeruginosa, E. coli, C. albicans* and *A. niger;* while *T. asiatica* effective against *P. aeruginosa, S. aureus, A. niger* and *C. albicans* (Narod et al. 2004).

Most bacterial strains were resistant to water extract, except for Gram positive *S. aureus* and Gram negative *E. coli* and *P. aeruginosa*. Crude extracts of *Ruta chalpensis* (Rutaceae) leaves showed antimicrobial activity against *S. aureus* and *E. coli* using disk diffusion method and no activity observed for water extract (Kasimala et al. 2014). Water is a general solvent but crude extracts are more consistent to give reliable biological activities (Das et al. 2010). Other factors for negative results are due to the presence of other small concentration of components exerting an antagonistic reactions with tested compounds, thereby changing the positive results to non-reactive outcome (Saraf, 2010). Venkatesan et al. (2016) suggested that some bacterial strains are not sensitive to extracts and only reactive to purified compounds, due to inhibitory factors in extracts. Recommended extracting solvents are methanol, water and ethanol (Das et al. 2010). According to Das et al. (2010), tannins and phenolic compounds are soluble and hence, extracted better in water while chloroform is the best solvent for non-polar compounds.

Methanolic extracts of *Vepris verdoorniana* (Rutaceae) showed antimicrobial activity against *S. aureus*, *Candida albicans* and *Mucor meihei* (Atangana et al. 2017). Fankam et al. (2014) reported that *Vepris soyauxii* (Rutaceae) and other six plants species showed activity against *E. coli*, *Klebsiella pneumonia*, *Enterobacter aerogenes* and *Providencia stuartii* due to alkaloids, flavonoids, tannins, terpenes and saponins. A study by Rakotondraibe et al. (2001) showed that *Vepris leandriana* demonstrated high activity against *S. aureus, Bacillus subtilis, B. cereus, K. pneumonia*, *E. coli* and *C. albicans*; low activity against *P. aeruginosa*; and no activity against *Streptococcus faecalis*.

Aqueous, methanolic and hexane extracts of twelve plant species including *Zanthoxylum chalybeum* (Rutaceae) were investigated for antibacterial activity using the agar diffusion assay (Matu and van Staden, 2003). Gurib-Fakim et al. (2005) reported that stem and leaf extracts of *Vepris lanceolata* and *Todalia asiatica* revealed antibacterial activity against *S. aureus, Escherichia coli, P. aeruginosa, S. typhimurium* and antifungal activity against *C. albicans*. Shi et al. (2015) reported that methanolic extracts of *Todalia asiatica* roots had antimicrobial activity against *S. aureus* and MRSa. *Todalia asiatica* (Rutaceae) showed antibacterial activity (disk diffusion assay) against *E. coli, P. aeruginosa, S. aureus, Shigella boydii, Bacillus subtilis* and *Bacillus pumilis,* due to alkaloids, saponins, flavonoids, triterpenes and phenolic compounds (Balaji et al. 2016).

Crude extracts were active against *S. aureus, E. coli, Salmonella paratyphi, Shigella dysenteriae* and *Candida albicans* (Ahmed and Beg, 2001). A study by Vats et al. (2011) on *Murraya koenigii* (Rutaceae) roots showed antimicrobial activity against *S. aureus, B. subtilis, M. luteus, E. coli, P. aeruginosa* and no antifungal activity against A. *niger* and *C. albicans* using ethanolic, chloroform, ethyl acetate and petroleum ether. Methanolic extract of *Z. chalybeum* was active against *Bacillus subtilis, Micrococcus luteus* and exhibited highest antibacterial activity against *S. aureus* (Matu and van Staden, 2003).

The present study displayed results similar to those observed in a study by Saraf (2010) whereby methanolic extracts of *Costus speciosus* rhizome showed antimicrobial activity against *E. coli, S. aureus, K. pneumonia,* and *P. aeruginosa* using agar disk diffusion assay (Saraf, 2010). Water extract was only active against *S. aureus*. Kelmanson et al. (2000) reported methanolic extracts of *Dioscorea dregeana, Vernonia colorata* and *Cheilanthes viridis* had higher antibacterial activity against Pa and *Dioscerea sylavata* against *E. coli* compared to water and ethyl acetate extracts. A study by Racowski et al. (2017) on *Citrus latifolia* peel showed highest antimicrobial activity against *E. coli* using hot water extract and less activity against *S. aureus* and *Saccharomyces cerevisiae* using ethanolic extract; and no activity was observed with cold water extract. Hence, using

only cold water extract is unfavourable as most compounds are insoluble in water and can only be isolated with lipophilic or non-polar solvents (Kelmanson et al. 2000). However, Matu and van Staden (2003) and Shale et al. (1999) argue that since water extracts are commonly used in high concentrations by traditional practitioners, it is still appropriate and less harmful than crude extracts.

Studies show that antibacterial agents such as thymol and carvacrol (phenolic compounds) disrupt the components and functioning of bacterial strains by degrading outer membranes, hardening cell wall and depriving the cell of its cytoplasm (Fisher and Phillips, 2008; Khan et al. 2010; Orlanda and Nascimento, 2015). Terpenes enter the bacterial cell wall and disrupt the lipid layer causing the protein and membrane degradation, resulting in cytoplasmic leakage, leading to cell disintegration and finally, cell death (Fisher and Phillips, 2008; Orlanda and Nascimento, 2015). Furthermore, ions may leak out causing bacterial membrane to collapse (Fisher and Phillips, 2008; Orlanda and Nascimento, 2015). Saponins inhibit fungal growth (Khan et al. 2010). Tannins affect gram positive more than gram negative bacteria (Khan et al. 2010). Changes in antimicrobial activity is due to the presence and absence of certain components or compounds (Orlanda and Nascimento, 2015). All of these mechanisms are crucial in effectiveness of the extracts in the bacterial strains.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

6.1 Introduction

Plant synthesise secondary metabolites used to treat disorders and diseases. Plants have been used for many years by traditional people who cannot access or afford Western medicine. Fever, meno-pause, respiratory diseases, wounds, headaches are amongst many illness treated by medicinal plants in ethno-medicine. This is all due to these secondary metabolites produces by plants in specialized cells. Specialized cells are found in leaves, stem barks, roots and reproductive parts. Such structures can be cavities, ducts, canals, laticifers, salt cavitiess, trichomes. *Vepris lanceolata* leaves produce phytochemical compounds that are stored in cavities. These compounds have antibacterial activity against gram positive and gram negative bacterial strains.

6.2 Aims and objectives

The study was done to investigate the micromorphogical characteristics of secretory cavities in leaves, the chemical composition of the secretion and antibacterial activity of the leaf extracts of *Vepris lanceolata.* The micromophological and phytochemical studies were done for the first time in this reserach. The secretory cavities were found to develop schizo-lysigenously, distributed in the spongy and palisade parenchyma cells. In addition, some of the cavities were distributed closer to the epidermis. Histo-phytochemical analyses revealed that these cavities are secretory structures. Alkaloids, proteins, carbohydrates, glycosides, phenolic compounds, non-volatile and volatile oils were present in leaf extracts. The leaf extracts showed good antibacterial activity against both gram negative and positive bacteria using disk diffusion assay. Hence, the aims and objectives outlined in chapter 1 answered the research question which was as follows: how does the micromorphology and chemical composition of the secretion assist in understanding the medicinal value of *Vepris lanceolata*? Therefore, the findings of the

study has shown that the morphological characteristcs and chemical composition of the secretions can be used to ascertain the value and properties of medicinal plants.

6.3 Challenges

a) The main challenge was the sample preparation using the standard procedures for TEM. The protocol for chemically fixing the leaf samples had to be slightly adapted as the resin blocks were brittle and could not be sectioned.

b) Also, two methods were used while preparing samples to view with the SEM, i.e. chemical fixation and freeze drying. Samples tend to collapse when submerged under liquid nitrogen, hence, chemical fixation has to be used. Alternatively, chemical fixation damages fragile leaf material and freeze drying had to be used, instead.

c) Another challenge was freeze fracture viewing. This process is really time and resource consuming. This is because one cannot know definitely where secretory cavities are located as they are internal structures, more time was spent repeating freeze fracture, until cavities could be located within the cell.

d) Limited availability of resources and equipment was another shortcoming in this research.

e) Microtome sectioning was a challenge as there were times when resin blocks would break midway due to poor infiltration of the resin and new blocks had to be prepared from scratch.

6.4 Future studies

This research was conducted to investigate preliminary TLC and phytochemical tests and only reported groups of compounds. Future research should include the isolation of compounds by HPLC and identifying the bioactive components. Essential oil was detected and a need to isolate the oil using GC-MS is crucial in identifying composition of the essential oil. Antimicrobial activity was done on extracts. Hence, future study should investigate activity of each compound as some of the bacteria are only sensitive to purified compounds.

6.5 Final comments and summary conclusions

This research investigated the morphological characteristics of foliar secretory cavities, the chemical composition of the secretion, and the antimicrobial activity of leaf extracts of *Vepris lanceolata*. *Vepris lanceolata* is used for its medicinal properties in ethno-medicine to treat asthma, respiratory diseases, fever, pulmonary infections, influenza, infertility, colic, cardiac pain, wounds, stomach ache and headache. Phenolics compounds, flavonoids, tannins, alkaloids and essential oils have been detected in leaf extracts. Plants synthesize compounds to cope with ecological stressors and hence, adapt to the environment, e.g. flavonoids are produced as a defence mechanism during attack. Alkaloids are used to relieve pain and heal wounds. Antagonistic and synergistic effects of phyto-compounds result in biological activity of medicinal plants. Research question that asked how the micromorphology and chemical composition of the secretion of foliar secretory cavities assist in understanding the medicinal value of *Vepris lanceolata* has been fulfilled. This plant has a potential to be a source of active antimicrobial agents.

REFERENCES

Abdallah, E.M., 2016. Preliminary phytochemical and antibacterial screening of methanolic leaf extract of *Citrus aurantifolia*. Pharmaceutical Biotechnology: Current Research 1 (1), 1-5.

Afoloyan, A.J., Meyer, J.J.M., 1995. Morphology and ultrastructure of secreting and nonsecreting foliar trichomes of *Helichrysum aureonitens* (Asteraceae). International Journal of Plant Sciences 156(4), 481-487.

Ahmed, I., Beg, A.Z., 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 74, 113-123.

Akerele, O., 1993. Nature's medicinal bounty: don't throw it away. Traditional Medicine 14, 1-6.

Akinpelu, D.A., Onakoya, T.M., 2006. Antimicrobial activities of medicinal plants used in folklore remedies in south-western. African Journal of Biotechnology 5 (11), 1078-1081.

Aoudou, Y., Léopold, T. N., Michael, J.D.P., Xavier, F., Moses, C.M., 2010. Antifungal properties of essential oil and some constituents to reduce food borne pathogens. Journal of Yeast Fungal Research 1, 1-8.

Arbab, I.A., Abdul1, A.B., Aspollah, M., Abdullah, R., Abdelwahab, S.I., Mohan, S., Abdelmageed, A.H.A., 2011. *Clausena excavata* Burm. f. (Rutaceae): A review of its traditional uses, pharmacological and phytochemical properties. Journal of Medicinal Plants Research 5(33), 7177-7184.

Ascensão, L., Marques, N., Pais, M.S., 1997. Peltate glandular trichomes of *Leonotis leonurus* leaves: ultrastructure and histochemical characterisation of secretion. International Journal of Plant Science 158, 249-258.

Atangana, A.F., Toze, F.A.A., Langat, K.M., Happi, E.N., Wansi, J.D., 2017. Acridone alkaloids from *Vepris verdoorniana* (Excell & Mendonça) Mziray (Rutaceae). Phytochemistry Letters 19, 191–195.

Atanassova, M., Georgieva, S., Ivancheva, K. 2011. Total phenolic and total flavovoid contents, antioxidant capacity and biological contaminants in medicinal herbs. Journal of the University of Chemical Technology and Metallurgy 46, 181-88.

Aumeeruddy-Elalfi, Z., Gurib-Fakim, A., Mahomoodally, F., 2015. Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of Mauritius. Industrial Crops and Products 71, 197-204.

Ayoola, G.A., Coker, H.A.B., Adesegun, S.A., Adepoju-Bello, A.A., Obaweya, K., Ezennia, E.C., Atangbayila, T.O., 2008. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research 7 (3), 1019-1024.

Babiaka, S.B., Ntie-Kang, F., Lifongo, L.L., Ndingkokhar, B., Mbah, J.A., Yong, J.N., 2015. The chemistry and bioactivity of Southern African flora I: a bioactivity versus ethnobotanical survey of alkaloid and terpenoid classes. The Royal Society of Chemistry 5, 43242–43267.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils - A review. Food and Chemical Toxicology 46, 446–475.

Balaji, P., Senthilraj, R., Janakiraman, K., Venkatachalam, V., Sivakkumar, T., Kannan, K., 2016. Preliminary phytochemical screening and antimicrobial activity of aqueous extracts of *Enicostema axillare* leaves and *Toddalia asiatica* root bark. Pharma Science Monitor 7(2), 249-258.

Balunas, M.J., Kinghorn, A.D., 2005. Drug discovery from medicinal plants. Life Sciences 78, 431-441.

Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G., Ruberto, G., 1998. Antimicrobial and antioxidant properties of some commercial essential oils. Flavour and Fragrance Journal 13, 235-244.

Barreca, D., Gattuso, G., Bellocco, E., Calderaro, A., Trombetta, D., Smeriglio, A., Lagana, G., Daglia, M., Meneghin, S., Nabavi, S.M., 2017. Flavanones: Citrus phytochemical with health-

promoting properties. International Union of Biochemistry and Molecular Biology 43(4), 495-506.

Bartwal A., Mall, R., Lohani, P., Guru, S.K., Arora, S., 2013. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. Journal of Plant Growth Regulation 32, 216–232.

Bashir, U., Ali, A., Hafeez, R., 2016. Phytochemical screening and estimation of antimicrobial activity of *Citrus* peel. Pakistan Journal of Phytopathology 28(2), 185-191.

Bellah, S.F., Raju, I.H., Billah, S.M.S., Rahman, S.E., Murshid, G.M.M., Rahman, M., 2015. Evaluation of antibacterial and antidiarrhoeal activity of ethanolic extract of *Feronia limonia* leaves. The Pharma Innovation Journal 3(11), 50-54.

Bennett, R.N., Wallsgrove, R.M., 1994. Secondary metabolites in plant defence mechanisms. New Phytologist 127, 617-633.

Bhavana, A., Shilpa, P., Naveen, G., Laxmi, G., Varalaxmi, N., Satyanarayana, T., 2016. Phytochemical screening and possible pharmacological actions of *Citrus limonum* methanolic leaf extract. Indo American Journal of Pharmaceutical Research 6(9), 1-11.

Bischoff, V., Nita, S., Neumetzler, L., Schindelasch, D., Urbain, A. and Eshed, R., 2010. Trichome birefringence and its homolog AT5G01360 encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis*. Plant Physiology 153, 590–602.

Bosabalidis, A., Tsekos, I., 1982. Ultrastructural studies on the secretory cavities of *Citrus deliciosa* ten. I. Early stages of the gland cells differentiation. Protoplasma 112, 55-62.

Bourgard, F., Gravot, A., Milesi, S., Gontier, E., 2001. Production of plant secondary metabolites: a historical perspective. Plant Science 161, 839-851.

Brophy, J.J., Forster, P.I., Goldsack, R.J., 2016. Leaf oils of the Australian species of *Clausena* and *Micromelum* (Rutaceae). Journal of Essential Oil Research 28(5), 406–412.

Bruneton, J., 2012. Principles of herbal pharmacology. in: Bone, K., Mills, S. Principles and practice of phytotherapy: Modern herbal medicine, Churchill Livingstone, Elsevier, 45-82.

Calixto, J.B., 2005. Twenty-five years of research on medicinal plants in Latin America: A personal view. Journal of Ethnopharmacology 100, 131-134.

Cain, A.J., 1947. The use of Nile blue in the examination of lipids. Quarterly Journal of Microscopy Science 88, 383–392.

Caissard, J., Joly, C., Bergougnoux, V., Hugueney, P., Mauriat, M., Baudino, S., 2004. Secretion mechanisms of volatile organic compounds in specialized cells of aromatic plants. Recent Research Developments in Cell Biology 2, 1-15.

Cardoso-Lopes, E.M., Maier, J.A., da Silva, M.R., Regasini, L.O., Simote, S.Y., Lopes, N.P., Pirani, J.R., Bolzani, V.S., Young, M.C.M., 2010. Alkaloids from Stems of *Esenbeckia leiocarpa* Engl. (Rutaceae) as Potential Treatment for Alzheimer Disease. Molecules 15, 9205-9213.

Chan, C-L., Gan, R-Y., Corke, H., 2016. The phenolic composition and antioxidant capacity of soluble and bound extracts in selected dietary spices and medicinal herbs. International Journal of Food Science and Technology 51, 565-573.

Chang, N., Luo, Z., Li, D., Song, H., 2017. Indigenous uses and pharmacological activity of traditional medicinal plants in Mount Taibai, China. Evidence-Based Complementary and Alternative Medicine, Article ID 8329817, 1-11.

Chaubey, M.K., 2012. Fumigant toxicity of essential oils and pure compounds against *Sitophilus oryzae* L. (Coleoptera: Curculionidae). Biological Agriculture & Horticulture 28(2), 111-119.

Ciccarelli, D., Andreucci, A.C., Pagni, M., 2001. Translucent glands and secretory canals in *Hypericum perforatum* l. (Hypericaceae): morphological, anatomical and histochemical studies during the course of ontogenesis. Annals of Botany 88(4), 637-644.

Combrinck, S., Du Plooy, G.W., McCrindle, R.I., Botha, B.M., 2007. Morphology and histochemistry of the glandular trichomes of *Lippia scaberrima* (Verbenaceae). Annals of Botany 99, 1111–1119.

Compean, K.L., Ynalvez, R.A., 2014. Antimicrobial activity of plant secondary metabolites: A review. Research Journal of Medicinal Plant 8(5), 204-213.

Cunningham, B., 1993. African medicinal plants: setting priorities at the interface between conservation and primary healthcare. People and plants working paper 1. United Nations Educational, Scientific and Cultural Organization. 1-50.

Curtis, J.D., Lersten, N. R. 1994. Developmental anatomy of internal cavities of epidermal origin in leaves of *Polygonum* (Polygonaceae). New Phytologist 127, 761-770.

Curvers, K., Seifi, H., Mouille, G., de Rycke, R., Asselbergh, B., Van Hecke, Vanderschaeghe, D., Höfte, H., Callewaert, N., Van Breusegem, F., Höfte, M., 2010. Abscisic acid deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to *Botrytis cinerea*. Plant Physiology 154, 847–860.

Das, S., Sheue, C., Yang, Y., 2013. Leaf micromorphology and leaf glandular hair ontogeny of *Myoporum bontioides* A. Gray. Feddes Repertorium 124, 50–60.

David, R., Carde, J.P., 1964. Coloration diffe´rentielle des pseudophylles de Pin maritime au moyen du re´actif de Nadi. Comptes Rendus de l' Academie des Sciences, Paris, Serie D 258, 1338–1340.

Deans, S.G., Svoboda, K.P., 1990. Biotechnology and bioactivity of culinary and medicinal plants. Agbiotech News and Information 2(2), 211-216.

Deng, G., Craft, J.D., Steinberg, K.M., Li, P.L., Pokharel, S.K., Setzer, W.N., 2017. Influence of different isolation methods on chemical composition and bioactivities of the fruit peel oil of Citrus medica L. var sarcodactylis (Noot) Swingle. Medicines 4(1), 1-11.

Devi, K., Santhi, M., Umadevi, U., 2017. Phytochemical analysis of selected wound healing medicinal plants. International Journal of Pharmaceutical Sciences and Research 8(2), 852-855.

Descallar, A.L., Nuñez, M.P.S., Cabrera1, M.L.N., Martin, T.T.B., Obemio1, C.D.G., Lañojan, R.S., 2017. Phytochemical analysis and antioxidant capacity of *Lycopodium clavatum* Linn. from

Lake Sebu, South Cotabato, Philippines. AIP Conference Proceedings 1803, 020021 (2017); doi: 10.1063/1.4973148.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P., Vidal, N., 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry 97, 654-660.

Edeoga, H.O., Okwu, D.E., Mbaebie, B.O., 2005. Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology 4 (7), 685-688.

Elujoba, A.A., Odeleye, O.M., Ogunyemi, C.M., 2005. Traditional medicine development for medical and dental primary health care delivery system in Africa. African Journal of Traditional, Complementary and Alternative medicines 2(1), 46- 61.

Fabricant, D.S., Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug discovery. Environmental Health Perspectives 109(1), 69-75.

Fahn, A., 1979. Secretory tissues in plants. Academic Press, London and New York, 158-221.

Fahn, A. 1988. Tansley Review No. 14 Secretory tissues in vascular plants. New Phytologist 108, 229-257.

Fahn, A. 2002. Functions and location of secretory tissues in plants and their possible evolutionary trends. Israel Journal of Plant Sciences 50, 59-64.

Fankam, A.G., Kuiate, J.R., Kuete, V., 2004. Antibacterial activities of Beilschmiedia obscura and six other Cameroonian medicinal plants against multi-drug resistant Gram-negative phenotypes. Complementary and Alternative Medicine 14 (241), 1-9.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z., 1985. Medicinal plants in therapy. Bulletin of the World Health Organization 63(6), 965-981.

Fisher, K., Phillips, C. Potential antimicrobial uses of essential oils in food: is citrus the answer? Trends in Food Science & Technology 19, 156-164.

Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Scheffer, J.C., 2008. Factors affecting secondary metabolite production in plants: volatile components and essential oils. Journal of Flavour Fragrance 23, 213–226.

Furr, M., Mahlberg, P.G., 1981. Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. Journal of Natural Products 44, 153-159.

Garcia, G., Charmillon, J.M., Roux, E., Sutour, S., Rakotozafy, J.B., Désiré, O., Paoli, M., Tomi, F., Rabehaja, D. J.R., 2016. Chemical composition of leaf and bark essential oils of *Vepris unifoliolata* from Madagascar. Journal of Essential oil Research http://dx.doi.org/10.1080/10412905.2016.1251982.

Ghafar, M.F.A., Prasad, K.N., Weng, K.K., Ismail, A. 2010. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. African Journal of Biotechnology 9(3), 326-330.

Gómez-Cansino, R., Guzmán-Gutiérrez, S.L., Campos-Lara, M.G., Espitia-Pinzón, C.I., Reyes-Chilpa, R., 2017. Natural compounds from Mexican medicinal plants as potential drug leads for anti-tuberculosis drugs. Anais da Academia Brasileira de Ciências 89(1), 31-43.

Goodger, J.Q.D., Cao, B., Jayadi, I., Williams, S.J., Woodrow, I.E., 2009. Non-volatile components of the essential oil secretory cavities of *Eucalyptus* leaves: Discovery of two glucose monoterpene esters, cuniloside B and froggattiside A. Phytochemistry 70, 1187–1194.

Groppo, M., Kallunki, J.A., Pirani, J.R., Antonelli, A., 2012. Chilean *Pitavia* more closely related to Oceania and Old World Rutaceae than to Neotropical groups: evidence from two cpDNA non-coding regions, with a new subfamilial classification of the family. PhytoKeys 19, 9–29.

Gupta, R., Thakur, B., Singh, P., Singh, H.B., Sharma, V.D., Katoch, V.M., Chauham, S.V.S., 2010. Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant *Mycobacterium tuberculosis* isolates. Indian Journal of Medical Research 131, 809-813.

Gurib-Faim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine 27, 1-93.

Habtemariam, S., 2017. Going back to the good old days: the merit of crude plant drug mixtures in the 21st century. International Journal of Complementary & Alternative Medicine 6(2).

Hamdan, D., El-Readi, M.Z., Tahrania, A., Kaufmann, F.H.D., Farrag, N., El-Shazly, A., Winka, M., 2011. Secondary metabolites of ponderosa lemon (*citrus pyriformis*) and their antioxidant, anti-infl ammatory, and cytotoxic activities. Z. Naturforsch 66, 385 – 393.

Hamilton, A.C., 2004. Medicinal plants, conservation and livelihoods. Biodiversity and Conservation 13, 1477–1517.

Harborne, J. B., 1973. Phytochemical methods, Chapman and Hall Ltd., London.

Harborne, J.B., 1998. Phytochemical methods: A guide to modern techniques of plant analysis. Chapman and Hall, 3rd edition, London, UK.

Hostettmann, K., Marston, A., 2002. Twenty years of research into medicinal plants: Results and perspectives. Phytochemistry Reviews 1, 275–285.

Iikasha, A.M.N., Bock, R., Mumbengegwi, D.R., 2017. Phytochemical screening and antibacterial activity of selected medicinal plants against laboratory diarrheal bacteria strains. Journal of Pharmacognosy and Phytochemistry 6(5), 2337-2342.

Jabin, F., Nasreen, S., 2016. Phytochemical analysis of some medicinal plants. International Journal of Applied Research 2(8), 293-295.

Jiangseubchatveera, N., Liawruangrath, S., Teerawutgulrag, A., Santiarworn, D., Pyne, S.G., 2017. Phytochemical screening, phenolic and flavonoid contents, antioxidant and cytotoxic activities of Graptophyllum pictum (L.) Griff. Chiang Mai Journal of Science 44 (1), 193-202.

Johansen, D.A., 1940. Plant microtechnique, McGraw-Hill, New York.

Kala, C.P., Dhyani, P.P., Sajwan, B.S., 2006. Developing the medicinal plants sector in northern India: challenges and opportunities. Journal of Ethnobiology and Ethnomedicine 2(32), 1-15.

Kalachanis, D., Psaras, G.K., 2005. Structure and development of the secretory cavities of *Myrtus communis* leaves. Biologia Plantarum 49 (1), 105-110.

Kamboj, V.P., 2000. Herbal medicine. Current Science 78(1), 35-51.

Kasimala, M.B.K., Tukue, M., Ermias, R., 2014. Phytochemical screening and antibacterial activity of two common terresterial medicinal plants *Ruta chalepensis and Rumex nervosus.* Bali Medical Journal (Bali Med. J.) 3(3), 116-121.

Kavitha, T., Nelson, R., Thenmozhi, R., Priya, E., 2017. Antimicrobial activity and phytochemical analysis of *Anisomeles malabarica* (L) R.BR. Journal of Microbiology and Biotechnology Research 2 (1), 1-5.

Kebebew, M., Dadi, K., Mohammed, E., 2016. Diversity, knowledge and use of traditional medicinal plants in Guduru District, Horo Guduru Wollega zone, Oromia region of Ethiopia. Journal of Medicinal plants studies 5(1), 364-371.

Kelmanson, J.E., Jager, A.K., van Staden, J., 2000. Zulu medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69, 241-246.

Khan, R.A., Khan, M.R., Sahreen, S., Bohari, J., 2010. Antimicrobial and phytotoxic screening of various fractions of *Sonchus asper*. African Journal of Biotechnology 9(25), 3883-3887.

Kiplimo, J.J., Islam, S., Koorbanally, N.A., 2012. Ring A-seco limonoids and flavonoids from the Kenyan *Vepris uguenensis* Engl. and their antioxidant activity. Phytochemistry 83, 136-143.

Knight, T.G., Klieber, A., Sedgley, M., 2001. The relationship between oil gland and fruit development in Washington navel orange (*Citrus sinensis* L. Osbeck). Annals of Botany 88, 1039-1047.

Kyoshabire, M., Katuura, E., Cunningham, A.B., Hoeft, R., 2017. Medicinal plants and herbalist preferences around Bwindi Impenetrable National Park. Journal of Medicinal Plants Research 11(8), 161-170.

Lemmens, R.H.M.J., 2008. *Vepris nobilis* (Delile) Mziray. In: Louppe, D., Oteng-Amoako, A.A. & Brink, M. (Editors). PROTA (Plant Resources of Tropical Africa / Resources végétales de l'Afrique tropicale), Wageningen, Netherlands. Accessed 27 November 2017.

Liang, S., Wu, H., Lun, X., Lu, D., 2006. Secretory cavity development and its relationship with the accumulation of essential oil in fruits of *Citrus medica* L. var. *sarcodactylis* (Noot.) Swingle. Journal of Integrative Plant Biology 48(5), 573−583.

Loizzo, M. R., Falco, T., Bonesi, M., Sicari, V., Tundis, R., Bruno, M., 2018. Ruta chalepensis L. (Rutaceae) leaf extract: chemical composition, antioxidant and hypoglicaemic activities. Natural Product Research 32(5), 521-528.

Maggi, F., Randriana, R.F., Rasoanaivo, P., Nicoletti, M., Quassinti, L., Bramucci, M., Lupidi, G., 2013. Chemical composition and in vitro biological activities of the essential oil of *Vepris macrophylla* (Baker) I.Verd. Endemic to Madagascar. Chemistry and Biodiversity 10, 356-366.

Mahomoodally, M.F., Gurib-Fakim, A., Subratty, A.H., 2010. Screening for Alternative Antibiotics: An investigation into the antimicrobial activities of medicinal food plants of Mauritius. Journal of Food Science 75(3), 173-177.

Maobe, M.A.G., Gatebe, E., Gitu, L., Rotich, H., 2013. Preliminary phytochemical screening of eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in Kisii region, southwest Kenya. European Journal of Applied Sciences 5 (1), 01-06.

Manju, K., Jat, R.K., Anju, G., 2012. A review on medicinal plants used as a source of anticancer agents. International Journal of Drug Research and Technology 2(2), 177-183.

Mathur, S., Hoskins, C., 2017. Drug development: Lessons from nature (Review). Biomedical Reports 6, 612-614.

Mazia, D., Brewer, P.A., Alfert, M., 1953. The cytochemistry staining and measurement of protein with mercuric bromophenol blue. Biology Bulletin 104, 57–67.

Mothana, R.A.A., Abdo, S.A.A., Hasson, S., Althawab, F.M.N., Alaghbari, S.A.Z., Lindequist, U., 2008. Antimicrobial, antioxidant and cytotoxic activities and phytochemical screening of some Yemeni medicinal plants. Advance Access Publication 7(3), 323-330.

Mustafa, G., Arif1, R., Atta, A., Sharif, S., Jamil, A., 2017. Bioactive Compounds from Medicinal Plants and Their Importance in Drug Discovery in Pakistan. Matrix Science Pharma 1(1), 17-26.

Narod, F.B., Gurib-Fakim, A., Subbratty, A.H., 2004. Biological investigations into *Antidesma madagascariense* Lam. (Euphorbiaceae), *Faujasiopsis flexuosa* (Lam.) C. Jeffrey (Asteraceae), *Toddalia asiatica* (L.) Lam. and *Vepris lanceolata* (Lam.) G. Don (Rutaceae). Journal of Cell and Molecular Biology 3, 15-21.

Neilson, E.H., Goodger, J.Q.D., Woodrow1, I.E., Møller, B.L., 2013. Plant chemical defense: at what cost? Trends in Plant Science 18(5), 250-258.

Nouga, A.C., Ndom, J.C., Mpondo, E.M., Nyobe, J.C.N., Meva'a, L.M., Cranwell, P.B., Howell, J.A.S., Harwood, L.M., Wansi, J.D., 2017. New furoquinoline alkaloid and flavanone glycoside derivatives from the leaves of *Oricia suaveolens* and *Oricia renieri* (Rutaceae). Natural Product Research 30(3), 305-310.

O'Brien, T.P., Feder, N, McCully, M.E., 1965. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 49, 367–373.

Okigbo, R.N., Anuagasi, C.L., Amadi, J.E., 2009. Advances in selected medicinal and aromatic plants indigenous to Africa. Journal of Medicinal Plants Research 3(2), 086-095.

Orlanda, J.F.F., Nascimento, A.R., 2015. Chemical composition and antibacterial activity of *Ruta graveolens* L. (Rutaceae) volatile oils, from São Luís, Maranhão, Brazil South African Journal of Botany 99, 103-106.

Padhi, L. and Panda, S.K., 2013. Medicinal plants: potential to fight superiority diseases. Annals of Pharma Research, 1(1), 8-17.

Paiva, E.A.S., Machado, S.R., 2007. Structural and ultrastructural aspects of ontogenesis and differentiation of resin secretory cavities in *Hymenaea stigonocarpa* (Fabaceae-Caesalpinioideae) leaves. Nordic Journal of Botany 24(4), 423-431.

Palazzolo, E., Laudicina V.A., Germanà, M.A., 2013. Current and potential use of citrus essential oils. Current Organic Chemistry 17, 3042-3049.

Palombo, E.A., Semple, S.J., 2001. Antibacterial activity of traditional Australian medicinal plants. Journal of Ethnopharmacology 77, 151–157.

Petrovska, B.B., 2012. Historical review of medicinal plants' usage. Pharmacognosy Reviews 6(11), 1-6.

Poullain, C., Girard-Valenciennes, E., Smadja, J., 2004. Plants from Reunion Island: evaluation of their free radical scavenging and antioxidant activities. Journal of Ethnopharmacology 95, 19-26.

Purvis, M.J., Collier, D.C., Walls, D., 1964. Laboratory techniques in botany, Butterworths, London.

Pearse, A.G.E., 1985. Histochemistry: theoretical and applied. Vol. II, 4th ed. Longman.

Prathapa, R.M., Rama, R.V., Shantha, T. R., Kishore K.R., Shiddamallayya N., Sulochana B., 2016. Phytochemical analysis and thin layer chromatography of *Clausena dentata* (willd.) roem. – Leaf. World Journal of Pharmacy and Pharmaceutical Sciences 5(9), 1706-1716.

Rabe, T., van Staden, J., 1997. Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56, 81-87.

Rahman, T., Roy, P.R., Chhanda, N.N., Seraj, S., Rahmatullah, M., 2017. Combination of Aryuveda and folk medicine: plants and formulations of a traditional medicinal practitioner in Noakhali district, Bangladesh. Journal of Medicinal Plants Studies 5(1), 292-297.

Rawoski, I., Piotto, J., Procopio, V., Freire, V.T., 2017. Evaluation of antimicrobial activity and phytochemical analysis of Thaiti lemon peels (*Citrus latifolia* Tanaka). Journal of Microbiology Research 2017, 7(2), 39-44.

Rakotondraibe, L. H.R., Rakotovao, M., Ramanandraibe, V., Ravaonindrina, N., Frappier, F., Brouard, J., 2001. Composition and antimicrobial activity of leaf oils of *Vepris leandriana* H. Perr. Journal of Essential Oil Research 13(6), 467-469.

Rasoanaivo, P., Ratsimamanga-Urverg, S., 1993. Biological evaluation of plants with reference to the Malagasy flora. Monograph for the IFS-NAPRECA Workshop on Bioassays, Antananarivo, Madagascar, 72–79.

Reddy, D.N., Al-Rajab, A.J., 2016. Chemical composition, antibacterial and antifungal activities of *Ruta graveolens* L. volatile oils. Cogent Chemistry 2, 1-11.

Russin, W.A., Uchytil, T.F., Feistner, G., Durbin, R.D., 1988. Developmental changes in content of foliar secretory cavities of *Tagetes erecta* (Asteraceae). American Journal of Botany 75(12), 1787-1793.

Salomi, M.V., Manimekalai, R., 2016. Phytochemical Analysis and Antimicrobial Activity of Four Different Extracts from the Leaves of *Murraya koenigii*. International Journal of Current Microbiology and Applied Sciences 5(7), 875-882.

Sangwan, N.S., Farooqi, A.H.A., Shabih, F. and Sangwan, R.S., 2001. Regulation of essential oil production in plants. Plant Growth Regulation 34, 3–21.

Saraf. A., 2010. Phytochemical and antimicrobial studies of medicinal Plant *Costus Speciosus* (Koen.). E-Journal of Chemistry 7 (1), 405-413.

Saxena, M., Saxena, J., Nema, R., Singh, D. and Gupta, A., 2013. Phytochemistry of medicinal plants Journal of Pharmacy and Phytochemistry, 1(6), 168 - 82.

Shale, T.L., Stirk, W.A., van Staden, J., 1999. Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. Journal of Ethnopharmacology 67, 347-354.

Sethi, P.D., 1996. High performance thin layer chromatography (HPTLC), 1st edition. CBS Publishers & distributors, New Delhi, India, 1-74.

Sharanabasappa, G.K., Santosh, M.K., Shaila, D., Seetharam, Y.N., Sanjeevrao, I., 2007. Phytochemical studies on *Bauhinia racemosa* Lam. *Bauhinia purpurea* Linn. and *Hardwikia binate* Roxb. E-Journal of Chemistry 4(1), 21-31.

Shi, L., Ji, Z., Li., Y., Li, Y., Guo, C., 2015. Antioxidant and antibacterialactivity of *Toddalia asiatica* (linn) lam root extracts. African Journal of Traditional Complementary and Alternative Medicine 12(6), 169-179.

Silva, F.S., Menezes, P.M.N., de Sa, P.G.S., Souza, E.A.A, Almeida, J.R.G,S. de Lima, J.T., Uetanabaro, A.P.T., Silva, T.R.S., Peralta, E.D., Lucchese, A.M., 2016. Chemical composition and pharmacological properties of the essential oils obtained seasonally from Lippia thymoides. Pharmaceutical Biology 54(1), 25–34.

da Silva, F.B., dos Santos, N.O., Pascon, R.C., Vallim, M.A., Figueiredo, C.R., Martins, R.C.C., Sartorelli, P., 2017. Chemical composition and in vitro cytotoxic and antimicrobial activities of the essential oil from leaves of *Zanthoxylum monogynum* St. Hill (Rutaceae). Medicines 4(31), 1-8.

Srinivasan, D., Nathan, S., Suresh, T., Perumalsamy, L., 2001. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharmacology 74, 217–220.

Steenkamp, V., 2003. Traditional herbal remedies used by South African women for gynaecological complaints. Journal of Ethnopharmacology 86, 97–108.

Svoboda, K.P., Hampson, J.B., 1999. Bioactivity of essential oils of selected temperate aromatic plants: antibacterial, antioxidant, antiinflammatory and other related pharmacological activities. Plant biology department SAC Auchincruive, Ayr, Scotland, UK., KA6 5HW. 1-17.

Parekh, J., Jadeja, D., Chanda, S., 2005. Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turkish Journal of Botany 29, 203-210.

Thomson, W.W., Platt-Aloia, K.A., Endress, A.G., 1976. Ultrastructure of Oil Gland Development in the Leaf of Citrus sinensis L. Botanical Gazette 137(4), 330-340.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. and Kaur, H., 2011. Phytochemical screening and Extraction: A Review. Internationale Pharmaceutica Sciencia, 1(1).

Teixeira, S. de P., Castro, M. de M., Tozzi, A.M.G. de A., 2000. Secretory cavities and pellucid dots in leaflets of *Lonchocarpus* (Leguminosae, Papilionoideae, Millettieae). Plant Systematics and Evolution 221, 61-68.

Trease, G.E., Evans, W.C., 1978. A text book of Pharmacognosy, 14th edition. Bailliere Tindall Ltd. London, UK.

Tschirch, A., Stock, E., 1933. Die Harze. Gebruder Borntraeger, Berlin, 418(1).

Turner, G.W., Alison A.M., Gifford, E.M., 1998. Schizogenous secretory cavities of citrus Limon (l.) burm. F. and a reevaluation of the lysigenous gland concept. International Journal of Plant Sciences 159 (1), 75-88.

Turner, G.W., 1999. A brief history of the lysigenous gland hypothesis. The Botanical Review 65(1), 76-88.

Vaghasiya, Y., Chanda, S.V., 2007. Screening of methanolic and acetone extracts of fourteen Indian medicinal plants for antimicrobial activity. Turkish Journal of Botany 31, 243-248.

Vats, M., Singh, H., Sardana, S., 2011. Phytochemical screening and antimicrobial activity of roots of *Murraya koenigii* (linn.) Spreng. (Rutaceae). Brazilian Journal of Microbiology 42, 1569-1573.

Venkatesan, S., Balamurugan, A., Susindran, P., Vasanthi, K., Subashini, E., Anithakumari, M., 2016. Phytochemical screening, FT-IR analysis and antimicrobial activity of *Wattakaka volubilis*. International Journal of Biological Research 4(2), 165-169.

Venkatachalama, K.V. Kjonaas, R., Croteau, R., 1984. Development and Essential Oil Content of Secretory Glands of Sage (*Salvia officinalis*). Plant Physiology 76(1), 148-50.

Vieira, R.C., Delprete, P.G., Leitao, G.G., Leitao, S.G., 2001. Anatomical and chemical analyses of leaf secretory cavities of *Rustia formosa* (Rubiaceae). American Journal of Botany 88(12), 2151–2156.

Vitali, L.A., Dall'Acqua, S., Maggi, F., Martonfi, P., Papa, F., Petrelli, D., Sut, S., Lupidi, G., 2017. Antimicrobial and antioxidant activity of the essential oil from the Carpathian *Thymus alternans* Klokov. Natural Product Research Research, 31 (10), 1121-1130.

Waweru, W.R., Osuwat, L.O., Wambugu, F.K. 2017. Phytochemical analysis of selected indigenous medicinal plants used in Rwanda. Journal of Pharmacognosy and Phytochemistry 6(1), 322-324.

Wubetu, M., Abula, T., Dejena, G., 2017. Ethnopharmacologic survey of medicinal plants used to treat human diseases by traditional medical practictioners in Dega Damot district, Amhara, North western Ethiopia. BMC Research Notes 10(157), 1-13.

Yadav, R., Khare, R.K., Singhal, A., 2017. Qualitative phytochemical screening of some selected medicinal plants of Shivpuri District (M.P.). International Journal of Life Sciences Scientific Research 3(1), 844-847.

Xie, S., Zhou, J., 2017. Harnessing plant biodiversity for the discovery of novel anticancer drugs targeting microtubules. Frontiers in Plant Science 8(720).

Zhao, P., Duan, L., Guo, L., Dou, L., Dong, X., ZHOU, P., Li, P., Liu, E. Chemical and biological comparison of the fruit extracts of *Citrus wilsonii* Tanaka and *Citrus medica* L. Food Chemistry 173, 54–60.