PHYTOCHEMICAL SCREENING, ELEMENTAL ANALYSIS AND ANTIBACTERIAL INVESTIGATION OF *Rhoicissus tomentosa*: A MEDICINAL PLANT USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

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EXECUTIVE SUMMARY

Globally medicinal plants play a very significant role in health-promotion and the reduction of disease burden in our communities. Over 80,000 species of such plants and their secondary metabolites including tannins, terpenoids, alkaloids, terpenes, phenolic compounds, glycosides and flavonoids have demonstrated excellent antimicrobial properties in vitro. Phytochemicals constitute over 25 – 50% of all pharmaceutical drugs prescribed globally. In addition, most phytochemicals have antioxidant and anti-carcinogenic properties which are beneficial to humans. Furthermore, the vitamins and other substances in plants play a significant role in protecting the human body against diabetes and heart diseases. Many plants have been the source of many pharmacologically active principles that are now used in medicine, for example, vincristine and vinblastine found in *Catharanthus roseus* are prescribed for hypertension and can also act as chemotherapeutic drugs against leukemia, Hodgkin's disease, etc. Nature has provided us with a variety of very useful and important compounds but these have somehow been over looked in recent times and more research attention needs to be given to natural products to explore their many positive therapeutic attributes which is the purpose of this study on *Rhoicissus tomentosa*. *R. tomentosa* is a medicinal plant indigenous to Southern Africa. It is a vigorous, evergreen tendril climber with ornamental, vine-like leaves and bunches of purple grape-like fruits. The plant is mainly used by traditional healers to treat fertility related ailments.

The rhizomes *Rhoicissus tomentosa* were analyzed in this study. The dry powdered plant material was first screened for the presence of phytochemicals and the findings revealed the presence of alkaloids, flavonoids, saponins, steroids, reducing sugars and tannins. The ground plant material was subsequently extracted using 100% ethyl acetate and methanol/chloroform (1:1) and pressurized hot water extraction (PHWE) technique. The extracts were analyzed for antimicrobial activity against 14 common human bacterial pathogens using the disc diffusion method and the micro titer plate method and the findings revealed that the extracts showed moderate to high inhibitory activity against most of the test organisms. While different bacterial species were investigated, the most susceptible ones to the rhizome organic solvent extracts were *Staphylococcus aureus* (MIC 0.063 mg/mL) and *Bacillus subtilis* (MIC 0.125 mg/mL) and the ones most susceptible to the PHWE extract were *Staphylococcus aureus* (MIC 1.0 mg/mL) and

Bacillus cereus (MIC 4.0 mg/mL). A comprehensive Two-Dimensional Gas Chromatography coupled with Time-of-Flight (GC×GC-TOFMS) analysis of the different crude extracts revealed the presence of over 100 known phytochemical constituents and numerous unknown constituents.

Inductively coupled plasma atomic emission spectroscopy (ICP-OES) was also done to ascertain the trace metal composition of this plant and consequently ascertain whether the plant contains any heavy metals of concern. The findings revealed that calcium and iron among other metals contained in the plant have known health benefits. They were identified in amounts which are within the World Health Organization permissible limits for metals in medicinal plants.

The findings of this study support the use of this plant in traditional medicine as this study showed that the plant contains bioactive phytochemical compounds, has interesting antibacterial properties and contains metals in trace amounts.

Keywords: Antibacterial screening, GC×GC-TOFMS, ICP-OES, PHWE, Phytochemical screening, *R. tomentosa* rhizomes.

DECLARATION

I, Nkemdinma C. Uche-Okereafor hereby declare that the composition of this dissertation and the work herein described was carried out entirely by myself unless otherwise cited or acknowledged. It has not been submitted for degree purposes at any other University or institution. Every other source(s) used have been duly cited in text and acknowledged by complete references.

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Nkemdinma C. Uche-Okereafor

DEDICATION

I dedicate this work to God Almighty, for His grace. Also to my husband and family for their love and support.

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RESEARCH OUTPUTS

Nkemdinma Uche-Okereafor, Derek Ndinteh, Nicolette Niemann, Vuyo Mavumengwana (2016). **P**hytochemical screening, GC×GC TOF-MS analysis and antibacterial properties of crude *Rhoicissus tomentosa* rhizome extract. **International Conference on Advances in Science, Engineering, Technology and Natural Resources (Nov 24-25th, 2016). http://ia-e.org/images/proceedings_pdf/IAE1116436.pdf**

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LIST OF ABBREVIATIONS

ALT	Alanine Aminotransferase
ATCC	American Type Culture Collection
AST	Antimicrobial Susceptibility Test
ASP	Aspartate Aminotransferase
CCl_4	Carbon tetrachloride
CNS	Central Nervous System
$CHCl_3$	Chloroform
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DTD	Direct Thermal Desorption
EI	Electron impact ionization
GI	Gastro Intestine
GC	Gas chromatography
G-6-Pase	Glucose 6-Phosphatase
ICP	Inductively Coupled Plasma
ICP-OES	Inductively Coupled Plasma Atomic Emission Spectroscopy
LC-MS	Liquid chromatography Mass spectrometry
LPO	lipid peroxide
MC	Microbial culture
MS	Mass Spectrometry
MeOH	Methanol
MIC	Minimum Inhibitory Concentrations
MDR	Multi Drug Resistant
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
1D	One dimensional
1D GC	One Dimensional Gas chromatography
psi	Pounds per Square Inch
PHW	Pressurized Hot Water
PHWE	Pressurised Hot Water Extraction

RNA	Ribonucleic Acid
RT	Retention time
TOF	Time of Flight
TOF-MS	Time of Flight Mass Spectrometry
TM	Traditional Medicine
2D	Two-dimensional
2D GC	Two-dimensional Gas chromatography
GC×GC	Two-dimensional Gas chromatography
GC×GC-TOFMS	Two-dimensional gas chromatography coupled to time of flight mass spectrometry
UV	Ultra violet
UTIs	Urinary Tract Infections
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

As	Arsenic
Cd	Cadmium
Ca	Calcium
cm	centimeters
Cr	Chromium
Co	Cobalt
Cu	Copper
˚C	degrees Celsius
F	Fluorine
Ga	Gallium
g	gram
>	Greater than
I	Indium
Fe	Iron
Pb	Lead
Li	Lithium
Mg	Magnesium
Mn	Manganese
m/z	Mass to charge ratio
Hg	Mercury
m	meters
µL	microliter
µm	micrometer
mg	milligram
mg/kg	Milligram per kilogram
mg/mL	milligram per milliliter
mL	milliliter
mL/min	milliliter per minute
mm	millimeters

Mo	Molybdenum
nm	Nanometer
Ni	Nickel
%	percent
±	Plus or minus
K	Potassium
Rh	Rhodium
Se	Selenium
Na	Sodium
Sr	Strontium
V	Vanillin
V	Voltage
v/v	volume by volume
W	Watt
w/v	weight by volume
Zn	Zinc

OUTLINE OF DISSERTATION

The following chapters are presented in this dissertation:

Chapter One: General introduction

This chapter gives a general information on the background information of this study while highlighting the justification, aims and objectives of this study.

Chapter Two: Literature review

This chapter discusses the importance of plants in drug discovery and previous studies on the *Rhoicissus* genus together with the phytochemistry and biological activities of the genus. This chapter further reviews various techniques and aspects of this research on *R. tomentosa.*

Chapter 3: Phytochemical screening of the rhizomes of *Rhoicissus tomentosa* using chemical indicator tests

This chapter highlights the experimental and analytical techniques used for the phytochemical screening of the *Rhoicissus tomentosa* rhizomes used in this study.

Chapter Four: Antibacterial activity screening of the rhizomes of *Rhoicissus tomentosa* using disc diffusion and micro broth dilution assays.

This chapter highlights the antibacterial activity screening, experimental and analytical techniques used in this study.

Chapter 5: Phytochemical analysis of the rhizomes of *Rhoicissus tomentosa* using Two-Dimensional Gas Chromatography coupled to Time of Flight Mass Spectrometry (GC×GC TOF-MS).

This chapter highlights the phytochemical analysis of crude extracts of *Rhoicissus tomentosa* rhizomes highlighting their volatile content using GC×GC-TOFMS and the known biological activities of the identified volatiles.

Chapter 6: Elemental analysis of the rhizomes of *Rhoicissus tomentosa* using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

This chapter highlights the metals present in *R. tomentosa* rhizomes, the World Health Organization metal permissible limits for medicinal plants and some of the health benefits of the metals found.

Chapter 7: General discussions, conclusions and recommendations for future work

This chapter covers the general discussions and conclusion from this study and recommendations for future work.

CHAPTER ONE

1.0 BACKGROUND

Traditional medicine (TM), variously known as ethno-medicine, folk medicine, native healing, or complementary and alternative medicine, is the oldest form of health care system that has stood the test of time (Abdullahi, 2011). Prior to the introduction of modern medicine, TM used to be the dominant medical system available to millions of people in Africa in both rural and urban communities. It was the only source of medical care for a greater part of the population (Romero-Daza, 2002; Abdullahi, 2011).

Traditional medicine (TM) describes a group of health care practices and products with a long history of use. It frequently refers to medical knowledge developed by indigenous cultures that incorporate plant, animal and mineral-based medicines, spiritual therapies and manual techniques designed to treat illness or maintain wellbeing (WHO, 2002; Abbott, 2014). TM is mostly practiced outside of Western medicine and functions as a comprehensive system of health care which has been developed over hundreds or even thousands of years (Abbott, 2014). TM is not only a vital source of health care, but also an important source of income for many communities and also forms an integral part of a community's identity (Srivastava *et al*., 1996; Abbott, 2014).

The WHO defines traditional medicine as "the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2000). That is to say, traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine. The traditional preparations comprise medicinal plants, minerals, organic matter, etc. Herbal drugs constitute mainly those traditional medicines which predominantly use medicinal plant preparations for therapy (Kamboj, 2000).

The WHO estimates that up to 80% of the population in Africa makes use of traditional medicines (Shaw, 1998; WHO 2002; Azaizeh *et al*., 2003; Richter, 2003; Rivera *et al*., 2013; Oyebode *et al*., 2016) and ethnobotanical studies carried out throughout the continent of Africa has confirmed that native plants are the main constituent of African traditional medicines (Cunningham, 1993).

In recent years, research has been carried out to examine the chemistry of plants used by contemporary healers and elders to treat various illnesses. Data from literature have confirmed that traditional medicines possess chemical properties that can effectively and safely treat illness. More studies are also in progress to test the safety of traditional medicines when used in combination with biomedical-based treatments (National Aboriginal Health Organization, 2012).

In light of the global health care demand and the significant role of traditional medicine in meeting the public health needs of developing countries, traditional medical knowledge is experiencing increased attention worldwide (Abbott, 2014). Pre-industrial communities have been responsible for the discovery of most of the medicinal plants in use today, and many communities are still involved in the wild collection, domestication, cultivation and management of medicinal plant resources (Abbott, 2014). TM also contribute to the development of pharmaceutical treatments (Barrett *et al*., 1999; Abbott, 2014), for example the anti-cancer drug taxol was derived from the bark of the Pacific yew tree, and aspirin was isolated from willow bark (Avendaño & Menéndez, 2008; Abbott, 2014).

Some of the advantages of TM which has sustained its practice till this present time include: availability and proximity (WHO, 2002; Pal & Shukla, 2003; WHO, 2004; UNESCO, 2013), affordability (WHO 2000a; WHO, 2000b; UNESCO, 2013), familiarity and cultural acceptability (Pal & Shukla, 2003; UNESCO 2013), effective treatment of particular disorders (UNESCO, 2013), holistic and person-centered approach (Hedberg *et al*., 1983; Anyinam, 1987; Cunningham, 1993; UNESCO, 2013), protection of biodiversity (Kayne, 2009; WHO, 2008; UNESCO, 2013), etc.

Traditional medicine does more than provide raw materials for pharmaceuticals, most traditional medicine practitioners still hold valuable traditional knowledges which could prove important for new drug development. These traditional knowledges could provide valuable guidance in selecting and obtaining plant material of potential therapeutic interest. Bioactive compounds derived from currently used herbal medicines are more likely to have minimal toxicity, and a long history of clinical use suggests that herbal medicine may be clinically effective (Fabricant & Farnsworth, 2001; Koehn & Carter, 2005). Plant-derived compounds used as drugs are generally

used in ways that correlate directly with their traditional uses as plant medicines (Fabricant & Farnsworth, 2001; Koehn & Carter, 2005; Abbott, 2014).

1.2 JUSTIFICATION

Infectious diseases are among the leading causes of morbidity and mortality on our planet. The development of resistance of microbes (bacteria, viruses, fungi and other parasites) to available drugs is not surprising neither is it new (Choffnes *et al*., 2010). Infectious diseases caused by bacteria, fungi, viruses and parasites are major public health concerns, despite the remarkable progress in human medicine. Their impact is exceptionally great in developing countries due to the relative unavailability of medicines and the rise of widespread drug resistance (Okeke *et al*., 2005) (Cos *et al*., 2006). Research on new antimicrobial substances must, therefore, be continued and all possible strategies should be explored (Cos *et al*., 2006) and the use of ethno-pharmacological knowledge is one sure way to reduce experimentation and enhance the likelihood of success in new drug-finding efforts (Cordell & Colvard, 2005; Patwardhan, 2005; Cos *et al*., 2006).

Disease-causing microorganisms have above all been vulnerable to man's determination for survival and man has sought to deprive them of their habitat by using antimicrobial agents (Anibal de *et al.,* 2009). These microorganisms in turn have responded by developing resistance mechanisms to fight off this attack on them. Currently antimicrobial resistance among bacteria, viruses, parasites, and other disease-causing organisms is a serious threat to effective infectious disease management globally (Bloomfield, 2002; McEwen & Fedorka-Cray, 2002; Vidaver, 2002; Anibal de *et al.,* 2009; Monte *et al*., 2014). Microbial resistance to antibiotics is one of the biggest problems facing public health (Byarugaba, 2004; Okeke *et al*., 2005; Monte *et al*., 2014). Bacteria have developed resistance to all known antibiotics, hence, the high cost associated with the burden of microbial drug-resistance (Monte *et al*., 2014).

Bacterial resistance to antibiotics increases mortality, likelihood of hospitalization and length of stay in the hospital (Boucher *et al*., 2009; Giamarellou, 2010). Scientific studies of medicinal plants and plant based compounds for the purpose of treating various infectious disorders started in the late 19th century (Ogie-Odia *et al*., 2014). As such, plants are important sources of

antimicrobial agents, most of them with effectiveness against varied organisms including fungi, yeasts and bacteria, insects, nematodes and other plants (Abreu *et al*., 2013; Monte *et al*., 2014).

In the last three decades, pharmacological industries have produced a number of new antibiotics yet resistance to these drugs by microorganisms is still on the increase (Nascimento *et al*., 2000; Sani, 2014). According to the World Health Organization in 2014, antimicrobial resistance had reached alarming levels in many parts of the world. As such, very few, if any, of the available treatments remain effective for common infections (O'Neill, 2014; WHO, 2014; WHO, 2015). This has, thus, become a public health problem and as such, there have been calls for the development of new and efficacious antimicrobials. A huge requirement for these antimicrobials is that they must not be generally cytotoxic, they must be cost-effective and most importantly, should be active at minimal concentrations (WHO, 2014; WHO, 2015).

Since antibiotic resistance is a major problem in the treatment of bacterial infections, there is a need to find substitute way(s) to deal with infectious diseases. Thus, the use of plants and their extracts which are abundant in nature to overcome the problem of antibiotic resistance and serve as a source of novel drugs for the treatment of diseases have been proposed (Sani, 2014). The use of phytochemicals with known antimicrobial properties, can be of great significance in therapeutic treatments (Nascimento *et al*., 2000). Screening of *R. tomentosa* for antibacterial activity and identifying the various bioactive compounds in the plant that could be responsible for its antibacterial properties are part of the aims of this project.

1.3 HYPOTHESIS

It was hypothesized in this research work that:

1. *Rhoicissus tomentosa* is a plant that is rich in bioactive phytochemicals and can be a source for possible drug leads to address the issue of antibacterial resistance.
2. *R. tomentosa* contains compounds that may be beneficial to human health.

1.4 AIM

As antibacterial resistance remains a public health challenge in most countries of the world, the use of plant based natural products have received considerable attention. Specialized metabolites from plants serve as rich resources of chemicals for drug development (Wurtele *et al*., 2012).

This project is, thus, aimed at extracting and screening the rhizomes of *Rhoicissus tomentosa* for different phytochemicals, screening the crude extracts for antibacterial activity, screening the crude extracts for different specific bioactive constituents and metal content analysis of the plant to ascertain their toxicity/safety and health benefits.

1.5 OBJECTIVES OF THE STUDY

To achieve the aim stated in Section 1.4, the following objectives were set:

1. Extraction of plant material using the Pressurized Hot Water Extraction (PHWE) technique and organic solvents.
2. Phytochemical screening using chemical indicator tests.
3. Screening for bioactive compounds using GC×GC-TOFMS
4. Determination of antibacterial properties of extracts using disc diffusion assay and the micro broth dilution assay.
5. Metal analysis of the plant using ICP-OES to ascertain the safety of the plant for human consumption.

CHAPTER TWO

2.0 LITERATURE REVIEW

This chapter deals with the literature survey of medicinal plants and plant natural products; the botanical description of the Vitaceae family; botanical description, phytochemistry and medicinal uses of *Rhoicissus tomentosa* and other related species of the *Rhoicissus* genus. It was summarized with a brief conclusion and the need for the research in this study.

2.1 INTRODUCTION

Over the centuries humans have relied on plants for their basic needs such as food, clothing, and shelter which are all produced or manufactured from plant matrices, leaves, woods, fibers and storage parts, fruits and tubers. Plants have also been utilized for other purposes, namely as arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance, and hunger suppression, as well as inebriants and medicines (Salim *et al*., 2008). Historically, plants have served as drugs used as the result of accumulated knowledge and experience handed down from generation to generation (Mokgethi, 2006).

These plants are either "wild plant species"- those plants growing freely in self-conserved habitats in natural or semi-natural ecosystems and have the capacity to exist independently of direct human actions or the contrasting "domesticated plants species"- those that are grown through human actions such as selection or breeding and depend on management for their existence (Nwachukwu *et al*., 2010; Nwachukwu *et al*., 2011). Quite a number of definitions have been suggested for the term 'medicinal plant'. According to the World Health Organization, "a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis" (Saroya, 2011). The foundation of traditional healing system in many developing countries is formed by the use of medicinal plants (Steenkamp *et al*., 2013). The interaction between human beings and animals with plants is believed to have started off with the beginning of life on earth, when plants provided much of the shelter, oxygen, food and medicine needed by other living things (Mamedov, 2012).

Plant based antimicrobials are a vast unexploited source. The use of plant extracts for therapeutic purposes became popular when people realized that the effective life span of antibiotic is limited and over prescription and misuse of traditional antibiotics are causing microbial drug resistance (Alam *et al*., 2009; Jayalakshmi *et al*., 2011). Plants have formed the foundation of sophisticated traditional medicine practices that have been used for thousands of years by people in China, India, and many other countries (Cragg & Newman, 2005; Salim *et al*., 2008). These plants have given Western pharmacopoeia about 7000 different pharmacologically important compounds and a number of modern day top selling drugs (Jayalakshmi *et al*., 2011).

Understanding the relationship among medicinal plants used in traditional medicine systems can help distinguish plant materials with potential constituents which are applicable to modern-day medicine (Mamedov, 2012). Medicinal plants could be considered as plants (whole or in parts) which are used for therapeutic purposes owing to the active ingredients contained in them (Nemeth, 2012). These plants play a very significant role in health-promotion and the reduction of disease burden in our communities. Nature has been a reservoir of medicinal agents for thousands of years, and a remarkable number of modern drugs have been isolated from natural sources, particularly plants, with many based on their traditional medicinal uses (Cragg & Newman, 2005). With the isolation of quinine from Cinchona in 1820, an ancient herbal cure was transformed into a chemical drug. This was the spur for a new scientific discipline: ethnopharmacology - as Western scientists began to reinvent traditional herbal cures by extracting their active principles to create new and profitable drugs (Saroya, 2011). According to preliminary definition, ethnopharmacology may be defined as "a multidisciplinary study of biologically active agents used in traditional medicine" or, "Ethnopharmacology is the scientific study correlating ethnic groups, their health, and how it relates to their physical habits and methodology in creating and using medicines" (Saroya, 2011). Drug discovery from medicinal plants has evolved to include many fields of inquiry. Since medicinal plants typically contain an array of chemical compounds such as alkaloids, flavonoids, lignans, fatty acids, polyphenols, triterpenoids and quinones (Cowan, 1999; Mokgethi, 2006) that may act individually, additively, or in synergy to improve health, most studies aim to standardize the herbal remedies and to isolate and characterize the pharmacologically active compounds from these medicinal plants (Balunas & Kinghorn, 2005).

Medicinal plants can, therefore, play an important role as sources of new drugs, drug leads and new chemical compounds (Butler, 2004). The chemical substances derived from medicinal plants can play a role in the treatment of various diseases including cancer, AIDS, malaria and Tuberculosis (Cowan, 1999; Mokgethi, 2006).

2.1.1 South African Medicinal Plants

South Africa boasts of a unique and diverse botanical heritage with over 30,000 plant species of which about 3000 species are used therapeutically (van Vuuren, 2008). With up to 19,581 indigenous species, South Africa has the richest temperate flora in the world (Taylor *et al*., 2001; Nielsen *et al*., 2012; Mabona, 2013) A minimum of 11,700 species are endemic to South Africa and nearly 3,000 species are used as medicines with approximately 350 species forming the most commonly traded and used medicinal plants (Taylor *et al*., 2001; Nielsen *et al*., 2012). Not only is the South African flora rich in diversity but it is also mostly endemic (Mulholland, 2005; van Vuuren, 2008). In addition to this unique botanical heritage, South Africa has a cultural diversity with traditional healing being an integral part of each ethnic group (van Vuuren, 2008). Medicinal plant usage usually forms the backbone in numerous southern African rural communities for treating ailments with varying severity, this is due to limited access to conventional medicines in many remote areas (Naidoo & Coopoosamy, 2011; Mabona, 2013).

The reliance of a large portion of the population of southern Africa on plants can also be attributed to a number of factors such as good accessibility to the medicinal plants from the markets and mostly the wild, affordability and extensive knowledge and expertise amongst the local communities (Street *et al*., 2008; Mabona, 2013). Southern Africa is said to contain approximately 10% of the world's plant diversity (George *et al*., 2001; Mabona, 2013).

2.1.2 Natural Products from Plants

Previous literature has reported that natural products play an important role in the chemotherapy of various diseases. A large number of substances used in modern medicine for the treatment of serious diseases originated from scientific studies carried out on medicinal plants (Lall & Meyer, 1999; Mokgethi, 2006). There are undoubtedly many more secrets still hidden in the world of plants (Mendelsohn & Balick, 1995; Hamilton. 2004).

Recently, there has been renewed interest with regards to plants and their medicinal value (Briskin, 2000; Dahanukar *et al*., 2000; Abruzzo, 2005). According to the World Health Organization, about 70% of the world's population depends on medicinal plants for their primary health care and about 35,000 to 70,000 species have been used as curatives (Akerele, 1993; Mamedov, 2012). Those medicinal plants are either preparations of or natural product substances from plants that have potentially efficacious pharmaceutical agents (Balunas & Kinghorn 2005; McChesney *et al*., 2007). In today's global market, about 50 major drugs are believed to have originated from tropical plants (Mamedov, 2012), an important example is the antimalarial drug, quinine, which created the foundation for the synthesis of the commonly used antimalarial drugs, chloroquine and mefloquine which were originally isolated from the bark of *Cinchona officinalis* (Cragg & Newman, 2005).

Plant-derived natural products have for a long time been and will continue to be very significant as sources of medicinal agents and models for the design, synthesis, and semi-synthesis of novel substances for the treatment of diseases (Hamilton, 2004). Many of the medicinally important plant-derived pharmaceuticals have been instrumental and essential in introducing the era of modern medicine and therapeutics (Balandrin *et al*., 1993). However, despite these many important past contributions from the plant kingdom, there are still a number of plant species that have never been studied, thus, it is reasonable to expect that new plant sources of valuable and pharmaceutical interesting materials remain to be discovered and developed (Balandrin *et al*., 1993).

The plant chemicals used for these purposes are principally the secondary metabolites, which are derived biosynthetically from plant primary metabolites (i.e., carbohydrates, amino acids, and lipids) and are not directly involved in the growth, development, or reproduction of plants. These secondary metabolites can be classified into several groups based on their chemical classes, such as alkaloids, terpenoids, and phenolics (Salim *et al*., 2008). In 1805, morphine became the first pharmacologically active compound to be isolated in pure form from a plant, although its structure was not elucidated until 1923 (Salim *et al*., 2008).

In spite of numerous past successes in the development of plant-derived drug products, it has been estimated that only 5 to 15% of the 250,000 existing species of higher plants have been systematically investigated for the presence of biologically active compounds. Because many plant secondary metabolites are genus- or species-specific, the chances are therefore good to excellent that many other plant constituents with potentially useful biological properties remain undiscovered, uninvestigated, and undeveloped (Balandrin *et al*., 1993; Cragg & Newman, 2013).

2.2 PHYTOCHEMICALS: OVERVIEW

Phytochemicals are naturally occurring chemical compounds found in plants which might provide health benefits for humans in addition to the benefits from macronutrients and micronutrients (Hasler & Blumberg, 1999; Saxena *et al*., 2013). These phytochemicals also help protect plants from diseases and they contribute to plants' colour, aroma and flavour (Gibson *et al*., 1998; Saxena *et al*., 2013).

Phytochemicals can be found in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Saxena *et al*., 2013). Such phytochemicals are known as plant secondary metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property (King & Young, 1999; Saxena *et al*., 2013) . There are over a thousand known and numerous unknown phytochemicals. Plants usually produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect humans against diseases (Saxena *et al*., 2013).

Phytochemicals can come singly or as a mixture of important substances to form active principle responsible for its activity (synergy). When singly active, the processes of their separations are of great practical advantages, which in many cases the isolated phytochemical have better and higher activity (Olufunke, 2012). After their isolation and chemical characterization, phytochemicals have to be tested on animal models and human studies to evaluate their safety and efficacy and also their absorption, distribution, metabolism, excretion and mechanisms of action. As the knowledge of these compounds grow, researchers learn how best to design new products through

modifying their concentrations, combinations and/or their bioavailability (Hasler & Blumberg, 1999).

2.2.1 Classes of Phytochemicals

A plant cell produces two types of metabolites (Table 2.1): primary metabolites which are involved directly in growth and metabolism and secondary metabolites considered as end products of primary metabolism and not involved in metabolic activity. These are summarized in Table 3.1. They act as defense chemicals. Their absence does not cause bad effects in the plants (Irchhaiya *et al*., 2015)

Table 2.1: Classification of phytochemicals (Rao, 2003; Irchhaiya *et al*., 2015; Shenoy *et al*., 2016).

Primary metabolites	**Secondary metabolites**
Common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll, carbohydrates, lipids.	Alkaloids, terpenes, flavonoids, lignans, tannins, plant steroids, curcumines, saponins, phenolics, essential oils, glucosides, etc

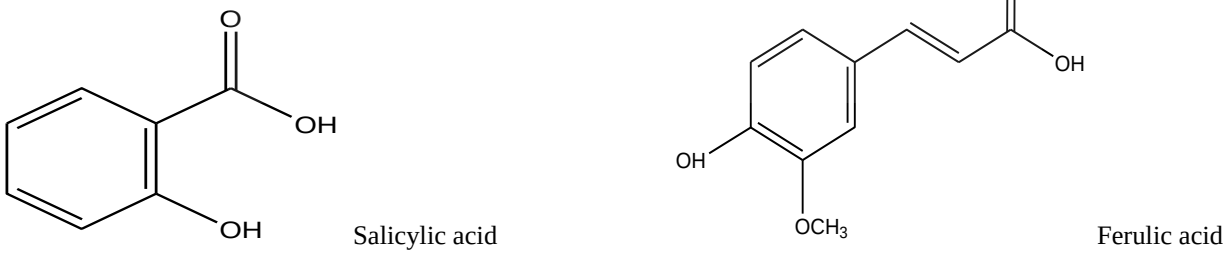
Primary Metabolites: Primary metabolites are widely dispersed in nature, occurring in one form or another in nearly all organisms. In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are essential for physiological development based on their role in basic cell metabolism. Primary metabolites obtained from higher plants for commercial use are high volume-low value bulk chemicals e.g. vegetable oils, fatty acids, carbohydrates etc. (Chandra & Khan, 2013).

Secondary Metabolites: Over the years, medicinal plants or their secondary metabolites have played important roles directly or indirectly in the human society to fight diseases (Wink *et al*., 2005). Secondary metabolites are often accumulated by plants in smaller quantities than the primary metabolites (Karuppusamy, 2009). In contrast to primary metabolites, they are synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products than the primary metabolites (e.g. steroids, quinines, alkaloids, terpenoids and flavonoids), which are used in drug

manufacture by the pharmaceutical industries. These are generally obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents (Chandra & Khan, 2013).

2.2.1.1 Phenolic compounds

Phenolic compounds from plants are one of largest group of secondary plants constituents synthesized by fruits, vegetables, teas, cocoa and other plants that have certain health benefits. They are characterized by the antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties, and have the capacity to protect from oxidative stress and some diseases (Pengelly, 2004; Kabera *et al*., 2014). Simple phenolics are bactericidal, antiseptic and anthelmintic. Phenol itself is a standard for other antimicrobial agents (Pengelly, 2004). They are distributed in almost all plants and subject to a great number of chemical, biological, agricultural, and medical studies (Dai & Mumper, 2010; Kabera *et al*., 2014). They are diverse in structure, and present in common hydroxylated aromatic rings (e.g., flavan-3-ols). Most of phenolic compounds are polymerized into larger molecules such as the proanthocyanidins, condensed tannins and lignans. In addition, phenolic acids may occur in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyl fatty acids, sterols, and glucosides (Dai & Mumper, 2010). Hydroxybenzoic and hydroxycinnamic acids present two main phenolic compounds found in plants. In tea, coffee, berries and fruits, the total phenolic compounds could reach up to 103 mg/100 g fresh weigh (Manach *et al*., 2004; Kabera *et al*., 2014).



Salicylic acid Ferulic acid

Figure 2.1: Structures of phenolic compounds

2.2.1.2 Flavonoids

They are structural derivatives of flavones, containing conjugated aromatic systems, often bound to sugar(s) as glycosides, and they are phenolic and water soluble in nature (Harborne, 1973).

They exert their roles as anti-oxidants, and hence protecting against degenerative diseases. Flavonoids such as quercetin, act as chain breaking anti-oxidants, and by preventing oxidation of low-density lipoprotein by macrophages and metal ions like copper. This reduces oxidative stress (Ngoci *et al*., 2011). They are referred to as 'nature's biological modifiers' because they act as anti-allergens, anti-inflammatory, and they induce phase two enzymes that eliminate mutagens and carcinogens (Ogunwenmo *et al*., 2007; Ngoci *et al*., 2011). They also act as anti-microbial by complexing extracellular and soluble proteins, and by complexing bacteria cell wall. More lipophilic flavonoids may also disrupt microbial membranes (Navarro *et al*., 2003; Al-Bayati & Al-Mola, 2008; Samy & Gopalakrishnakone, 2008; Kaur & Arora, 2009; Ngoci *et al*., 2011). Flavonoids are also known to increase coronary flow, to reduce the myocardial oxygen consumption and to lower the arterial pressure (Dong *et al*., 2005; Ngoci *et al*., 2011) and reduce capillary fragility (Harborne, 1973). They are also known to be anti-allergic and anti-spasmodic hence they are applied to relief asthma and nose bleeding (Ngoci *et al*., 2011; Njeru *et al*., 2013).

Flavonoids lacking hydroxyl groups (-OH) on their structure are more active against the micro-organism than those having the -OH group, and this supports the idea that their microbial target is the membrane (Cowan, 1999; Samy & Gopalakrishnakone, 2008; Ngoci *et al*., 2011; Njeru *et al*., 2013).

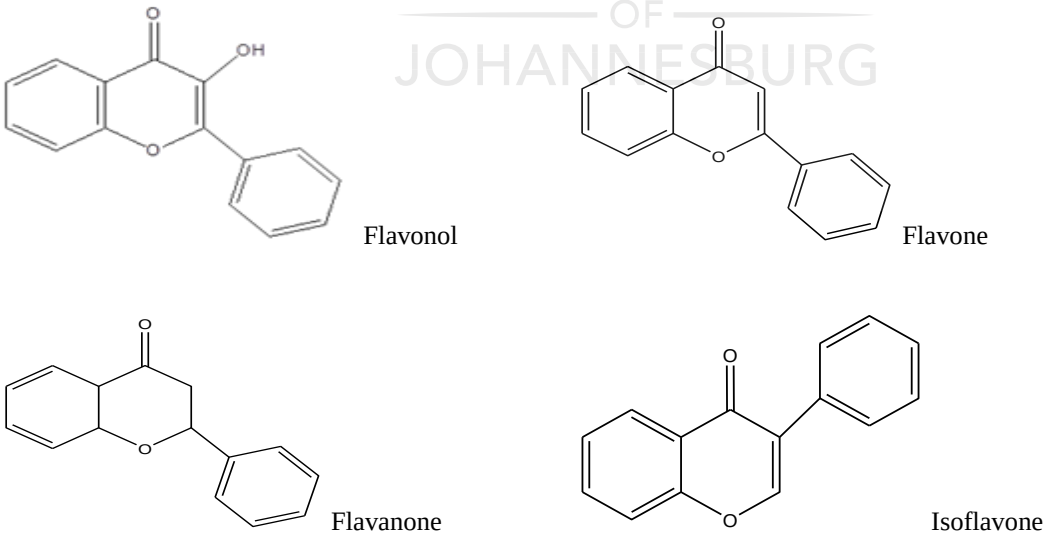
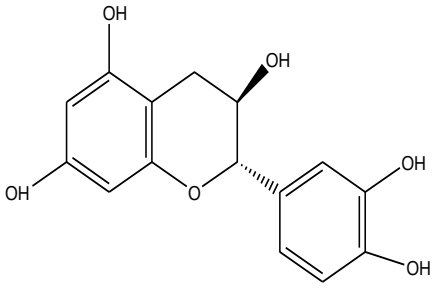


Figure 2.2: Structures of flavonoids.

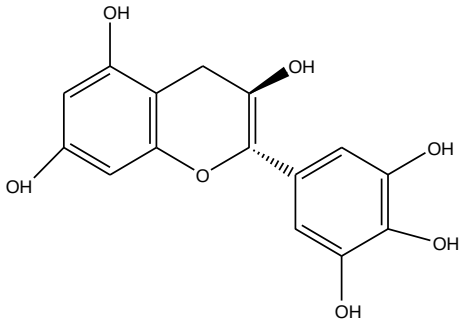
2.2.1.3 Tannins

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins. They have physiological role by acting as antioxidants through free radical scavenging activity, chelation of transition metals, inhibition of prooxidative enzymes and lipid peroxidation (Navarro *et al*., 2003; Koleckar *et al*., 2008; Ngoci *et al*., 2011), hence, modulating oxidative stress and preventing degenerative diseases. They also inhibit tumor growth by inducing apoptosis (Scalbert *et al*., 2005) and inhibiting mutagenicity of carcinogens (Okuda, 2005; Ngoci *et al*., 2011). They exhibit anti-microbial activity by complexing nucleophilic proteins by hydrogen bonding, covalent bonding, and nonspecific interactions. They also cause cell wall/membrane disruption (Cowan, 1999; Okuda, 2005; Biradar *et al*., 2008; Ngoci *et al*., 2011). They also inactivate microbial enzymes and cell envelope transport proteins by processes that may involve reaction with sulfhydryl groups of proteins (Samy & Gopalakrishnakone, 2008; Kaur & Arora, 2009; Ngoci *et al*., 2011). They also accumulate complexes metal ions (e.g. cobalt, manganese, iron, copper, etc.) necessary for microbial growth as co-factors and activators of enzymes. They also inhibit viral reverse transcriptase (Okuda, 2005; Ogunwenmo *et al*., 2007; Biradar *et al*., 2008; Ngoci *et al*., 2011).

Toxicity to microorganisms in phenolic compounds depends on the site and the number of hydroxyl groups, with evidence that increased hydroxylation results to increased toxicity (Przybylski *et al*., 1998; Cowan, 1999; Biradar *et al*., 2008; Samy & Gopalakrishnakone, 2008; Ngoci *et al*., 2011). They exhibit endocrine role activity by interacting with estrogen receptors. They are also anti-inflammatory, molluscicidal and hence important in the control of schistosomiasis. Tannins are known to have anti-diarrheal, anti-septic anti-fungal properties, anti-parasitic, anti-irritant properties and are also used in curbing hemorrhage, in wound healing, and improving vascular health by suppressing peptides that harden arteries (Awoyinka *et al*., 2007; Ogunwenmo *et al*., 2007; Ngoci *et al*., 2011). Also, they have economic role of tanning leathers in leather industry. Nevertheless they affect intake and digestibility of feeds among livestock, and excess of it can be carcinogenic on normal tissues (Scalbert *et al*., 2005; Ngoci *et al*., 2011; Njeru *et al*., 2013).



Catechin

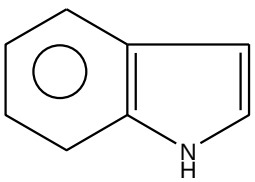


Gallocatechin

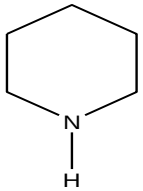
Figure 2.3: Structures of tannins

2.2.1.4 Alkaloids

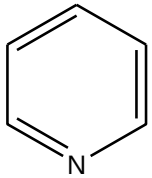
An alkaloid is a plant-derived compound that is toxic or physiologically active. Some alkaloids such as isopteropodine, pteropopine have anti-microbial activity whereby they act by promoting white blood cells to dispose harmful micro-organisms and cell debris (Ogunwenmo *et al*., 2007). Highly aromatic planar quaternary alkaloids like berberine, piperine and harmane work by intercalating the DNA and cell wall (Cowan, 1999). Others, by simulating neurotransmitters such as acetylcholine, dopamine and serotonin, they affect the central nervous system (CNS) at the synapses. They also act as narcotics, as anti-malaria, as topical anesthetic for ophthalmology; in treating hypertension, neuralgia, rheumatism, motion sickness, and also in extending the life of hormones (Ngoci *et al*., 2011). They have analgesic activity and hence used to alleviate pain in cases of boils, septic wounds, and complains such as headaches, abdominal pains and eye conditions. They also have anti-neoplatic activity, for example, indole alkaloids are used in leukemia and Hodgkin's disease chemotherapy. They act by terminating and depolymerization of protein microtubules that form the mitotic spindle in cell division. This process helps in terminating the tumor cells from separating or dividing and henceforth resulting to reduction of cancer. Nevertheless, some types of alkaloids are hallucinative, addictive, and toxic and hence used as arrow poison for hunting wild game (Ogunwenmo *et al*., 2007; Ngoci *et al*., 2011; Njeru *et al*., 2013).



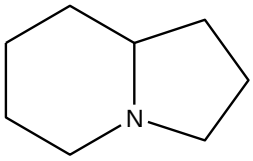
Indole nucleus



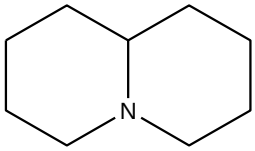
Piperidine nucleus



Pyridine nucleus



Indolizidine nucleus

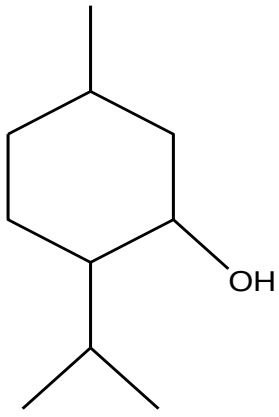


Quinolizidine nucleus

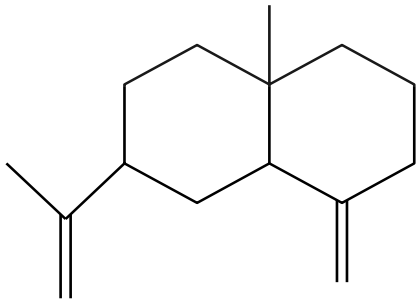
Figure 2.4: Structures of alkaloids

2.2.1.5 Terpenoids

Terpenoids are derivatives of isoprene molecule having a carbon skeleton built from one or more of C_{15} units (Harborne, 1973; Ngoci *et al*., 2011). They exert their roles as anti-bacterial, anti-fungal, anti-viral, anti-protozoan, anti-allergens, as immune boosters and as antineoplastic (Roberts, 2007) (Ngoci *et al*., 2011). The mechanism of action is speculated to involve membrane disruption by these lipophilic compounds (Cowan, 1999; Ogunwenmo *et al*., 2007; Samy & Gopalakrishnakone, 2008; Ngoci *et al*., 2011). This group of phytochemicals can cross the cell membranes, penetrating the interior of the cell and interacting with intracellular targets critical for antibacterial activity (Trombetta *et al*., 2005). They are also used to alleviate epilepsy, to relieve cold, influenza, cough and acute bronchial disease (Ngoci *et al*., 2011). Studies of terpenes has suggested that the possible target of these compounds involves hypothalamus-pituitary adrenal axis due to the observed effects on the levels of adrenocorticotropic hormone and corticosterone (Briskin, 2000; Ngoci *et al*., 2011; Njeru *et al*., 2013).



Menthol

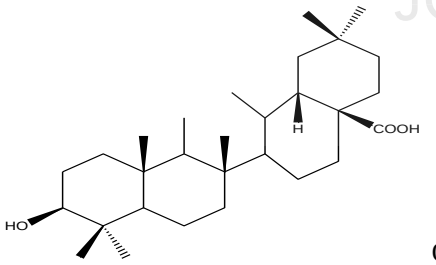


β-Selinene

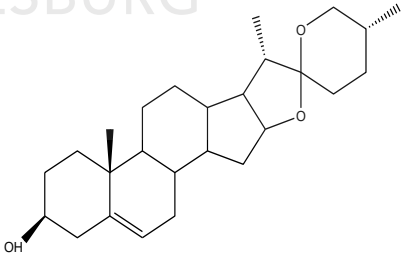
Figure 2.5: Structures of terpenoids

2.2.1.6 Saponins

Saponins are service active agents with soap-like properties and can be detected by their ability to cause foaming and to haemolyse blood cells (Harborne, 1973). They have a host of biological roles including boosting respiratory system as expectorant, and hence activity against cough. They also have anti-protozoa activity whereby they act by reacting with cholesterol in the protozoal cell membranes causing cell lysis. They serve as vaccine boosters by acting as adjuvant. They have anti- inflammatory, emetics, antiviral, antifungal, insecticidal, molluscicidal, piscidal and anti-bacterial activities (Ngoci *et al*., 2011). The mode of action for the anti-bacterial effects involve membranolytic properties of the saponins as well as lowering of the surface tension of the extracellular medium (Al-Bayati & Al-Mola, 2008). They have anti-neoplastic activity without killing normal cells. This is by reacting with cholesterol rich membranes of cancer cells, and inducing mitotic arrest that causes apoptosis of cell (Ngoci *et al*., 2011). This limits cell division and growth. They also bind to primary bile acids, which are metabolized by colon bacteria into secondary bile acids. Some of the secondary bile acids are promoters of colon cancer. They have economic values as source of cheap, environment friendly detergents, and cosmetics (Ngoci *et al*., 2011). Also, some saponins like Radix notoginseng have been reported to increase the blood flow of the coronary arteries, prevent platelet aggregation and to decrease the consumption of oxygen by heart muscles (Dong *et al*., 2005). They also have anti-oedema, antitussive, purgative and immunoregulatory properties (Ngoci *et al*., 2011; Njeru *et al*., 2013).



Oleanolic acid



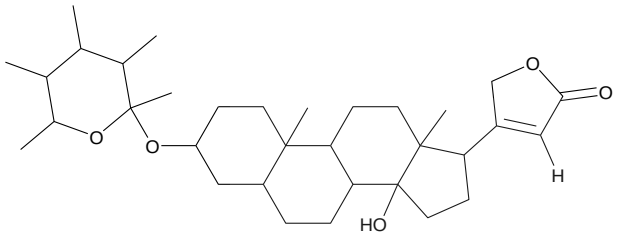
Diosgenin acid

Figure 2.6: Structures of saponins

2.2.1.7 Cardiac Glycosides

Cardiac glycosides occur as complex mixtures together in the same plant and most of them are toxic, however, many have pharmacological activity especially to the heart (Harborne, 1973).

They are used in treatment of congestive heart failure, whereby they inhibit Na+/K+-ATPase pump that causes positive ionotropic effects and electrophysiological changes. This strengthens heart muscle and the power of systolic concentration against congestive heart failure (Ogunwenmo *et al*., 2007) (Ngoci *et al*., 2011). They are also used in treatment of atrial fibrillation, flatter, and they acts as emetics and as diuretics (Harborne, 1973; Awoyinka *et al*., 2007; Ngoci *et al*., 2011; Njeru *et al*., 2013).

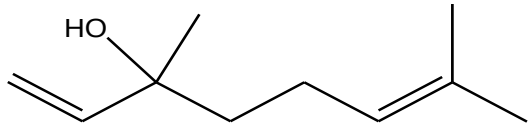


Cardenolides

Figure 2.7: Structure of cardiac glycoside

2.2.1.8 Essential Oils

Essential oils are the odorous and volatile products of various plant and animal species. Essential oils have a tendency evaporate on exposure to air even at ambient conditions and are, therefore, also referred to as volatile oils or ethereal oils**.** They mostly contribute to the odoriferous constituents or 'essences' of the aromatic plants that are used abundantly in enhancing the aroma of some spices (Martinez *et al*., 2008). Essential oils are secreted either directly by the plant protoplasm or by the hydrolysis of some glycosides (Doughari, 2012). Essential oils have been associated with different plant parts including leaves, stems, flowers, roots or rhizomes. Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour (Doughari, 2012).

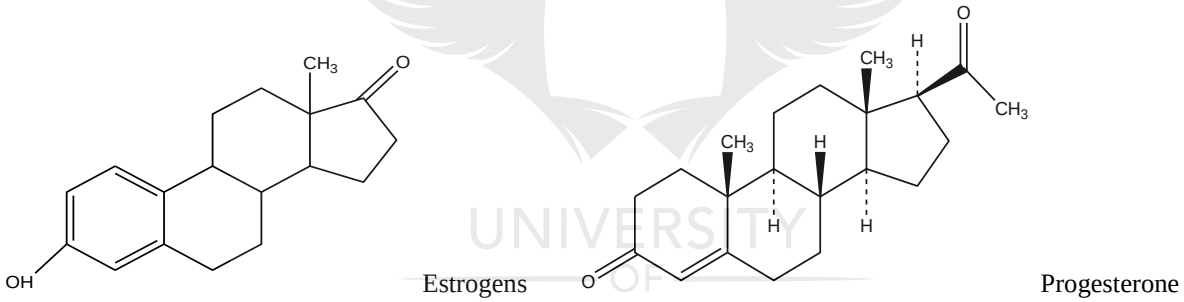


Lavender oil

Figure 2.8: Structure of an essential oil

2.2.1.9 Phytosteroids

Phytosteroids are plant steroids that may or may not act as weak hormones in the body. They share a common basic ring structure with animal steroids though they are not equivalent because of varying chemical groups attached to the main ring in different positions (Ngoci *et al*., 2011). They are mainly used to treat reproductive complications such as treatment of venereal diseases, used during pregnancy to ensure an easy delivery, as well as to promote fertility in women and libido in men. They also act as sex hormones derivatives, (for example, they can be metabolized to either androgen or estrogen-like substances) and hence they are potential source of contraceptives (Edeoga *et al*., 2005; Ngoci *et al*., 2011). They are also anti-microbial, analgesic, anti-inflammatory, and useful in treating stomach ailments and in decreasing serum cholesterol levels (Ngoci *et al*. 2011). They have also been indicated as potent inhibitors of macrophage activation, blocking the production of pro-inflammatory cytokines and LPS-induced lethality and therefore they have potential use as immunosuppressive agents (Soares *et al*., 2006; Ngoci *et al*., 2011; Njeru *et al*., 2013).



Estrogens Progesterone

Figure 2.9: Structures of steroids

2.3 THE FAMILY VITACEAE

Vitaceae (the grape family) represents the earliest diverging lineage within the rosids (Trias-Blasi *et al*., 2012; Lu *et al*., 2013). *Vitaceae* is a known group of flowering plants having a largely pantropical distribution in Asia, Africa, Australia, the neotropics, and the Pacific islands, with only a few genera in temperate regions (Nie *et al*., 2012). The family contains around 700-900 species, assigned to 13-15 genera (see table 2.3), making this a rather small family on global standards, and are primarily distributed in tropical and temperate areas throughout the world including woody and herbaceous vines and, in some cases, succulent trees (Soejima & Wen,

2006; Chen, 2009; de Sousa *et al*., 2011; Lu *et al*., 2013; Manchester *et al*., 2013; Najmaddin *et al*., 2013). A taxonomy of the *Vitaceae* family is summarized in Table 2.2.

Table 2.2: Taxonomy of the Vitaceae family (USDA, *n.d.*)

Taxonomic hierarchy	**Classification**
Kingdom	*Plantae* – Plants
Sub-kingdom	*Tracheobionta* –Vascular plants
Superdivision	*Spermatophyta* –Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Rhamnales*
Family	*Vitaceae* – Grape family

Vitaceae family comprises of woody climbers, vines, trees, shrubs and succulent's trees which are important food sources (Najmaddin *et al*., 2011). Leaf opposed tendrils, inflorescences and the unique seed morphology are the most useful characters to distinguish Vitaceae from other families. Morphologically the Vitaceae family is well delimited and easily recognized. The genera are delimited by floral morphology, inflorescence types and sometimes seed morphology (Najmaddin *et al*., 2013; Gerrath *et al*., 2015). In particular, the species of Vitaceae are characterized as woody, vines with leaf opposed tendrils which also includes shrubs and succulents inflorescence a cyme, corymb or panicle, usually with leaf opposed (Soejima & Wen, 2006; Najmaddin *et al*., 2013; Gerrath *et al*., 2015). Flowers of Vitaceae are relatively uniform in morphology at maturity and not particularly informative in systematic studies. Nectary morphology is highly variable in Vitaceae and has been emphasized in defining the genera (Soejima & Wen, 2006; Gerrath *et al*., 2015). Leaves in Vitaceae commonly bear ''pearl'' glands, and these glands are usually small spherical epidermal structures with a short stalk. Inflorescences of Vitaceae are typically paniculate systems (Soejima & Wen, 2006; Gerrath *et al*., 2015). Seeds of *Vitaceae* can be readily recognized by unique characters such as a dorsal chalaza and a pair of ventral in folds. Fossil seeds have been identified to the generic level and are potentially useful for inferring the past geographical distribution patterns of Vitaceae (Chen, 2009).

The family is economically important as the source for grapes, wine, and raisins (species of *Vitis* L.), as well as some ornamentals (Lu *et al*., 2013). Red wines of the Vitaceae family have biological properties favoring protection against major causes of adult mortality such as coronary heart disease (CHD) and cancer (Brookes, 2004). Proanthocyanidins are well-established components of the Vitaceae family which generally lacks ellagic acid derivatives. *Vitaceae* also lack anthraquinones, alkaloids, and iridoid compounds (Hutchings & van Staden, 1994; Brookes, 2004).

Species within the plant family Vitaceae (grape family) are commonly used in South African traditional medicine to prepare the tonic known as *isihlambezo* which is taken by pregnant women in their last trimester to promote maternal and fetal well-being and to facilitate a quick, uncomplicated labor (Lin *et al*., 1999; Mlambo *et al*., 2016).

2.3.1 The Genus *Rhoicissus*

The genus *Rhoicissus* consists of about 10 species endemic to tropical and Southern Africa (Naude, 2005; Nqolo, 2008; Nie *et al*., 2012). The species of the *Rhoicissus* genus are generally called "isinwazi" in Zulu and are used mostly in traditional medicine to treat fertility related ailments. The *Rhoicissus* species include *R. tomentosa, R. laetans, R. kougabergensis, R. microphylla*, *R. sessilifolia, R. digitata, R. Rhombedia, R. tridentate, R. revoilli* and *R. sekhukhuniensis* (Nqolo, 2008). *Rhoicissus* is a small genus of evergreen climbers, native to tropical and Southern Africa. In South Africa, the family Vitaceae is represented by 5 genera and 53 species, and the genus *Rhoicissus* is represented by 10 species (Agyare *et al*., 2013). This genus is made of slender plants and have cymose inflorescence that often bear tendrils and are most times mixed up with the genus *Amphelopsis.* The leaves are usually trifoliate or simply palmately lobed (Naude, 2005).

2.3.2 Medicinal Uses of Species of the *Rhoicissus* genus

The leaf, stem and root of *R. tomentosa* are mostly used by traditional healers in South Africa. In the KwaNibela Peninsula, St. Lucia, the boiled root mixed with other plants is used to enhance fertility (Corrigen *et al*., 2011). In some instances the Zulus traditionally boil the roots in milk and

administer as anthelmintic to calves, this ensures and facilitates uncomplicated delivery (Lin *et al*., 1999). Table 2.3 summarizes the traditional medicinal uses of the *Rhoicissus* genus.

Table 2.3: Therapeutic uses of some *Rhoicissus* species

Botanical name	English name	Zulu name	Medicinal use(s)
Rhoicissus digitata	Baboon grape or Dune grape	Isinwasi	The roots are used in traditional medicine to facilitate delivery during pregnancy and as anthelminthic for cattle in the same way as *R. tomentosa* (Nqolo, 2008). Tubers are used to treat cattle diseases (McGaw & Eloff, 2008) Different plant parts are used as enema for blood purification and intestinal cleansing, used to treat gastrointestinal complaints and used as an amulet that destroys gossip (Philander, 2011). It has also been reported that the plant is used to facilitate safe birth, increase fertility and treat painful menstruation and as a general pain reliever (Philander, 2011)
Rhoicissus kougarbegensis	NI	Isinwasi	Used during pregnancy to ensure safe delivery (Nqolo, 2008)
Rhoicissus microphylla	NI	Isinwasi	Used during pregnancy to ensure safe delivery (Nqolo, 2008)
Rhoicissus rhomboidea	Glossy forest grape	Isinwasi	The roots are used as traditional medicine during pregnancy to facilitate delivery and as anthelmintic for cattle in the same way as *R. tomentosa* (Nqolo, 2008)
Rhoicissus revoilli	Bitter bush grape	Isinwasi	Used during pregnancy to ensure a safe delivery (Nqolo, 2008). Root decoction is given to nursing mothers and cows to increase milk production. Pounded leaves are used to treat

Botanical name	English name	Zulu name	Medicinal use(s)
			wounds and ringworms. Pounded leaves decoction is used to treat intestinal worms (Omino & Kokwaro, 1993; Musyimi *et al*., 2008). In the Luo community of Kenya, pounded leaves decoction is applied to boils, this helps to ripen and heal the boils faster (Musyimi *et al*., 2008). Fresh leaf juice mixed with little water is applied topically to treat wounds. Fresh leaf and stem squeezed together with water are given orally and also nasally for livestock to treat leech infection (Enyew *et al*., 2014)
Rhoicissus sekhukhuniensis	NI	Isinwasi	Used during pregnancy to ensure safe delivery (Nqolo, 2008).
Rhoicissus sessilifolia	NI	Isinwasi	Used during pregnancy to ensure safe delivery (Nqolo, 2008).
Rhoicissus tomentosa	Common Forest Grape or Wild grape	Isinwasi	The boiled roots mixed in milk are given to calves to expel intestinal worms and also used during pregnancy to ensure safe delivery. Parts of the plant can also be used to treat abdominal pains, swellings, broken bones, lacerations, symptoms of epilepsy, convulsions and infertility. It may also be administered as an enema for delayed menstruation, dysmenorrhea, renal com-plaints, menorrhagia, bladder and kidney complaints, sprained ankles, stomach ailments and sores and used as anti-emetics in children (Veale *et al*., 1992;

Botanical name	English name	Zulu name	Medicinal use(s)
			Nqolo, 2008).
Rhoicissus tridentata	Bushman's Grape	Isinwasi	*R. tridentata* tubers are used for gynaecological purposes, stomach ailments, as well as kidney and bladder complaints (Steenkamp *et al*., 2013; Katsoulis, 1999; Brookes & Katsoulis, 2006), to prevent miscarriages, diarrhea (Samie *et al*., 2005). The herbal remedies prepared from this plant are taken orally by pregnant women to prevent premature delivery and growth of abnormal babies (Morris & Mdlalose, 1991; Dube, 2014). Root extracts of *R. tridentata* have a potential to stimulate and increase uterine contractility. Scientific reports have indicated that *R. tridentata*, promote childbirth by triggering hyperstimulation of the uterus (Dube, 2014; Brookes & Katsoulis, 2006). This plant is commonly used in the preparation of *isihlambezo*: a herbal remedy used by pregnancy women to prepare the uterus for childbirth (Varga & Veale, 1997; Dube, 2014). It used for the treatment of the tick-borne cattle disease, babesiosis (Naidoo *et al*., 2006). Roots and tubers are used to prepare concoctions, which are readily prescribed, for the treatment of ailments like epilepsy,

Botanical name	English name	Zulu name	Medicinal use(s)
			kidney and bladder complaints, etc. *(Opoku et al*., 2007); root, tubers and fruits are used to prevent miscarriages and treat diarrhea (Mabogo, 1990; Samie *et al*., 2005). The rootbark is used to treat erectile dysfunction (Mabogo, 1990; Rakuambo *et al*., 2006; Abdillahi & van Staden, 2012). In Kenya, it is used to treat malaria (Njoroge & Bussmann, 2006), boiled tubers are used to treat heartburn, diarrhoea, renal disorder and female infertility (Kigen *et al*., 2014), tubers are used to treat peptic ulcer (Kigen *et al*., 2014).

NI= No information

2.3.3 Phytochemistry of some Species of the *Rhoicissus* Genus

Different species of the *Rhoicissus* genus have been shown to possess bioactive compounds which are proposed to be the reason for their effective use in traditional medicine. Crude extracts of *R. tomentosa* have previously been found to contain coumarins, flavonoids, phytosterols, essential oils, saponins, terpenoids and resveratrol (Nqolo, 2008). Compounds identified in the GC-MS analysis of the hexane extract of *R. tomentosa* include: carda-16, 20(22)-dienolide; 4, 4-dimethylcholestan-3-one; heptadecane; 1-heptadecene; 9-hexylheptadecane; 11-hydroxypregn-4-ene-3, 20-dione; lycoxanthin; Methyl- tetraacetylmannopyranoside; 3, 12-oleandione; 2-octadecyl-1, 3, 5-trimethylcyclohexane; 6, 10, 14-trimethyl-2-pentadecanone and 7, 8, 12-tri-*O*-acetylingol (Nqolo, 2008).

Some bioactive compounds identified in *R. tridentata* include, epigallocatechin, gallocatechin, hydrate, mollisacacidin, epicatechin, oleanolic acid, prostacyandin B3, prostacyandin B4, sitosterolin, garlic acid (Brookes & Katsoulis, 2006; McGaw & Eloff, 2008; Dube, 2014), vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid, ferulic acid, p-coumaric acid (bound phenolic acids); vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid, phthalic acid (free phenolic acids) (Steenkamp *et al*., 2013). Recently the presence of catechins were also confirmed in the acetone extracts of *R. tridentata* (Naidoo *et al*., 2006; Opoku *et al*., 2007).

Phenols, alkaloids, flavonoids, saponins and tannins have been shown to be present in *R. tridentata* (Mwangi *et al*., 2015). Proanthocyanidins were principal spasmogens in *Rhoicissus tridentata* extracts. Proanthocyanidins are amongst the most powerful antioxidants in nature and have well-documented health benefits for both the cardiovascular and immune systems (Brookes & Katsoulis, 2006). Irioids, stilbenes and triterpenoids were also identified in *R. tridentata* (Stark *et al*., 2013). The roots of *Rhoicissus revoilli* have also been found to contain flavonoids, anthraquinones, steroid glycosides, alkaloids, saponins and ketones while the leaves were found to contain anthraquinones, steroid glycosides, alkaloids, saponins, ketones and tannins (Musyimi *et al*., 2008).

These different groups of chemical compounds are responsible for a number of bioactive potentials of plants for example saponins have been found to be antibacterial agents and tannins

act as anti-inflammatory agents, control gastritis and irritating bowel disorder, they also contribute to antimicrobial power which heal wounds and stop bleeding (Ikezu *et al*., 2014).

2.3.4 Biological Activities of some Species of the *Rhoicissus* Genus

Although *R. tomentosa* is a known plant in South African traditional medicine, its scientific biological activities and those of other *Rhoicissus* species have not been fully studied.

2.3.4.1 Antimicrobial activity

The leaf and stem of *R. tomentosa* have displayed different levels of anti-microbial activities against some microbes of public health importance: *Bacillus subtilis, Candida albicans, Klebsiella pneumonia, Mycobacterium lutens, Mycobacterium phlei, Mycobacterium smegmatis, Streptococcus faecalis, Alcaligenes faecalis, Bacillus coagulans, Bacillus megaterium, Bacillus stearothermophilus, Proteus mirabilis, Proteus vulgaris, Bacillus cereus, Bacillus puntus, Proteus morganii, Pseudomonas syringae, Pseudomonas solanacearum, Salmonella sp, Salmonella boydii, Staphylococcus aureus, Streptococcus pyrogenes, Salmonella typhimurium, Salmonella marcescens* and *Staphylococcus epidermidis* (Lin *et al*., 1999).

The root extracts of *Rhoicissus revoilli* were found to be have antimicrobial activity against *Salmonella typhi, Streptococcus pyrogenes* and *Aspergillus niger* (Musyimi *et al*., 2008). It was reported that the methanolic extracts of *R. rhomboidea* root and *R. tomentosa*-leaf/stem showed different levels of anti-microbial activities against almost all microorganisms tested (Lin *et al*., 1999). The methanolic extract of *R. digitate* root mainly inhibited Gram positive micro-organisms and Gram negative micro-organisms such as *Pseudomonas* and *Alcaligenes faecalis*. Most of the extracts showed poor inhibitory activity towards *Salmonella* and *Shigella* and none inhibited *E. coli*. It was reported that the extracts of *Rhoicissus* species inhibited the Gram positive microorganisms better than Gram negative (Lin *et al*., 1999) an observation supporting previous studies by (Rabe & van Staden, 1997; Vlietinck *et al*., 1995) where it was reported that the plant extracts are more active against Gram positive than Gram negative bacteria. The anti-fungal activity was tested using *Candida albicans* and *Saccharomyces cerevisiae*. The methanolic leaf extract of *R. digitate* and *R. rhomboidea* exhibited the highest activity against *C. albicans*. It was reported that no significant inhibitory activity of the same extracts was observed against *S.*

cerevisiae. However, all the plant extracts that demonstrated good anti-inflammatory activities were reported in the literature to also show better inhibitory activity against *Candida albicans* (Nqolo, 2008).

2.3.4.2 Anti-inflammatory activity

Methanolic leaf and stem extracts of *R. tomentosa*, methanolic leaf extracts of *R. digitata* and methanolic root extracts of *R. rhomboidea* and *R. tridentata* have also been demonstrated to have inhibitory activity against COX-1, a prostaglandin-producing enzyme that promotes inflammation, pain and fever, activates platelets and protects the stomach and intestinal lining. Extracts of *R. digitata* leaf and of *R. rhomboidea* roots exhibited the highest inhibition of prostaglandin synthesis with values of 53 and 56%, respectively. It was reported that none of the aqueous extracts showed any significant anti-inflammatory activity (Lin *et al*., 1999). This supports the use of *R. tridentata* to treat pains, swellings, cuts and wounds by traditional practitioners. The results reported in the literature suggest that these *Rhoicissus* plants might have the potential to be used as anti-inflammatory agents (Nqolo, 2008).

2.3.4.3 Antioxidant activity

In vitro anti-oxidative properties of *R. tridentata* suggested that the plant contains compounds with strong radical scavenging and anti-radical generating activities (Opoku *et al*., 2002; Opoku *et al*., 2007). Studies by Steenkamp *et al*., (2013), revealed that the tubers of *R. tridentata* showed high antioxidant activity. Antioxidant activity of the tubers of *R. tridentate* has in part been ascribed to some of the bioactive chemical compounds like catechin, epicatechin, gallic acid and epigallo-catechin-gallate which are present in the plant (Naidoo *et al*., 2006). *Rhoicissus rhomboidea* and *Rhoicissus tridentate* inhibited the activities of NADPH free radicals, xanthione oxidase and also prevented production of thiobarbituric acid reactive substances and also free-radical mediated sugar damage. Related plants like *Rhoicissus digitata* and *Rhoicissus tomentosa* does not possess these inhibitory properties, except at very high concentrations (Atawodi, 2005).

2.3.4.4 Antispasmodic activity

Three *Rhoicissus* species: *R. tridentate, R. digitate* and *R. tomentosa* are used in the same manner by traditional healers (Corrigan *et al*., 2011) which also suggests that they may have similar

biological activities and possibly similar chemical components. *R. tridentata* have been shown to exhibit direct smooth muscle activity on the isolated uterus and ileum of rats (Kaido *et al*., 1997; Steenkamp, 2003). Although not established, *R. tomentosa* and *R. digitate* might have the same antispasmodic activity considering that they are both used to enhance female fertility and ensure safe delivery. *R. tridentata* extracts had been shown to have pharmacological actions on rat uterus and ileum (Katsoulis, 1999; Opoku *et al*., 2007) which supports the use of the roots and tubers of *R. tridentata* by traditional Zulu healers for the treatment of birth complications.

2.3.4.5 Anti-proliferative activity

Recent reports indicate that crude extracts of *R. tridentata* have anti-proliferative activities (Opoku *et al*., 2000; Opoku *et al*., 2007). The aqueous root extract of *R. tridentate* subsp. *Cuneifolia* showed high anti-proliferative with 96.27% inhibition of proliferation, whereas the methanol extract showed a lower anti-proliferative activity of 87.01 % (Opoku *et al*., 2000). The crude extract of *R. tomentosa* exhibited 80.35% and 70.40% inhibition of proliferation. The root extracts showed stronger inhibitory activities compared with the leaf and stem extracts with the exception of the stem extract of *R. rhomboidea*. The results from the literature suggest that *R. tridentate* subsp. *Cuneifolia* has a higher antineoplastic activity against the HepG2 cell line than the other crude plant extracts. *R. digitata, R. rhomboidea, R. tridentate* and *R. tridentate* subsp. *Cuneifolia,* showed potential antineoplastic activities. Since traditional healers mainly use aqueous decoctions it is possible that their preparations have been effective in some forms of cancer treatment (Nqolo, 2008).

2.3.4.6 Anti-peroxidative activity

Recent studies have shown that extracts of *R. tridentate* inhibited *in vitro* ascorbic acid/Fe^{2+} catalyzed lipid peroxidation in rat liver microsomes (Opoku *et al*., 2002). The recovery of the liver injury in the plant extract administered in rats could, therefore, be due to the anti-peroxidative properties of the plant extract.

2.3.4.7 Hepatoprotective activity

The administration of *R. tridentata* extracts after CCl_4 intoxication resulted in significantly reduced concentrations of alanine aminotransferase (ALT), and aspartate aminotransferase (ASP)

as well as the levels of lipid peroxide (LPO) while the concentrations of G-6-Pase were significantly increased. From the results obtained from this study, *R. tridentata* can be said to have bioactive components with hepatoprotective effects (Opoku *et al*., 2007).

2.3.4.8 Uterotonic activity

R. tridentata is reported to possess direct uterotonic activity. An interesting result was that seasonal effect on the potency of uterotonic activity of this plant was reported (Samie *et al*., 2005; Mwangi *et al*., 2015).

2.3.4.9 Anti-diabetic activity

The aqueous leaf extract of *R. tridentata* showed anti-diabetic activity via blood glucose lowering effect when administered intraperitoneally and orally in a dose-dependent manner (Mwangi *et al*., 2015).

2.3.5 The Specie: Rhoicissus tomentosa

Rhoicissus tomentosa, "isinwasi" in Zulu, "idiliya" in Xhosa or "wild grape" in English from the family *Vitaceae* is widely used by traditional healers in South Africa. *R. tomentosa* occurs in all the provinces of South Africa (Western Cape, Eastern Cape, KwaZulu-Natal, Mpumalanga and Limpopo Province) except for the Northern Cape (Nqolo, 2008; Agyare *et al*., 2013), as well as spreading to neighboring countries like Zimbabwe, Malawi and Mozambique (Watt & Breyer-Brandwijk, 1962).

It is a vigorous, evergreen tendril climber (Figure 2.1) with ornamental, vine-like leaves and bunches of purple grape-like fruits (Figure 2.2). *R. tomentosa* grows up to 20 meters in height and the top branches meander from one tree top to the next. It has large leaves (90-200 mm x 70-160 mm in size) which are circular to kidney-shaped, smooth and shiny above with rust-colored hairs below. The margins are usually toothed or slightly lobed. Its rounded fruits ripen from May to June, turning from green to red to purplish-black. They are edible by both birds and mammals with whitish flesh and a pleasant acidic flavor. The fruits of *R. tomentosa* are also used in the production of beverages such as jam, jelly and vinegar (Van Wyk, 2011). The flowers are small and grow in dense clusters on 2 cm flower stems. Flowers emerge in spring and mature into

summer. They are creamy-green in color. Propagation can be done via seeds, stem cuttings or by layering (Watt & Breyer-Brandwijk, 1962). The rhizomes of *R. tomentosa* (as shown in figure 2.3) which have not been previously studied were used in this research project.

Figure 2.10: Rhoicissus tomentosa plant tendril climber
(kumbula nursery, *n.d*)

Figure 2.11: Grape-like fruits and leaves of *R. tomentosa*
(kumbula nursery, *n.d*)

Figure 2.12: Rhizomes of *Rhoicissus tomentosa*.

2.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial activity of plant extracts and pure compounds can be detected by observing the growth response of various microorganisms to plant extract samples when they are in contact with them. There are several methods for detecting antimicrobial activity, but these methods are not equally sensitive and/or are not based upon the same principle, hence, results will be profoundly influenced by the method used (Cos *et al*., 2006). The most common techniques used in antimicrobial susceptibility testing are:

Disc Diffusion Assay: Disc diffusion has been the basis for antimicrobial susceptibility testing (AST) in most clinical microbiological laboratories since the technique was first described in the 1960s (Bauer *et al*., 1966; Hombach *et al*., 2013). Agar disc diffusion techniques have been widely used to assay plant extracts for antimicrobial activity (Salie *et al*., 1996; Ncube *et al*., 2008) although there are limitations with the technique. The disc diffusion technique is a suitable pointer of antimicrobial activity but not effective for quantification of bioactivity (Hammer *et al*., 1999; Nostro *et al*., 2000; Langfield *et al*., 2004; Ncube *et al*., 2008). Generally, the disc diffusion technique does not distinguish between bactericidal and bacteriostatic effects. The minimum inhibitory concentration cannot be determined and these are usually used for preliminary screening (Tepe *et al*., 2004; Parekh *et al*., 2006; Ncube *et al*., 2008) that is, as qualitative tests,

considering that the amount of extract that adheres to the disk is not quantitatively determined (Ncube *et al*., 2008).

Broth Microdilution: The broth microdilution method has provided a potentially useful technique for determining the minimum inhibitory concentrations (MICs) of large numbers of test samples. Its advantages over the disc diffusion techniques include increased sensitivity for small quantities of extract which is important for plant natural products; ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC (Langfield *et al*., 2004; Ncube *et al*., 2008). This method can also be used for a wide variety of microorganisms; it is not expensive and it presents reproducible results. In the micro-titer plate method, a stock solution of the extract is first obtained in solvent, usually the solvent used for extraction (Grierson & Afolayan, 1999) or in DMSO (Salie *et al*., 1996; Nostro *et al*., 2000; Baris *et al*., 2006; Ncube *et al*., 2008). Methanol and acetone are sometimes chosen as solvents because, in addition to dissolving the extracts completely they show no inhibition of the microorganisms even at 2% final concentration (Meyer & Afolayan, 1995; Afolayan & Meyer, 1997; Mathekga *et al*., 2000; Ncube *et al*., 2008).

2.4.1 Common Human Bacterial Pathogens

The most problematic bacterial pathogens include, but are not limited to, *Escherichia coli, Klebsiella pneumoniae, Enterobacteriaceae, Pseudomonas aeruginosa, Staphylococcus aureus* and *resistant Enterococcus* species (Tim-Cushnie & Lamb, 2005; Talbot *et al*., 2006; Giamarellou, 2010; Magiorakos *et al*., 2011; Marasini *et al*., 2015).

2.4.1.1 *Bacillus cereus*

B. cereus is a Gram-positive, motile (flagellated), spore-forming, rod shaped bacterium that belongs to the *Bacillus* genus (Rajkowski & Bennett, 2003). *B. cereus* is widespread in nature and readily found in soil, where it adopts a saprophytic life cycle; germinating, growing and sporulating in this environment (Vilain *et al*., 2006). Spores are more resistant to environmental stress than vegetative cells due to their metabolic dormancy and tough physical nature (Chandra & Khan, 2013).

2.4.1.2 *Bacillus subtilis*

B. subtilis is a Gram-positive, facultative, aerobic, sporulating bacteria normally found in soil and gastrointestinal tracts of humans and ruminants. *B. subtilis* is normally considered as being non-pathogenic; but it has been linked to food-borne illnesses, causing diarrhoea, nausea, vomiting, associated with rice dishes served in oriental restaurants and its infection is self-limiting (Prescott *et al*., 2008). *B. subtilis* produces "subtilism", which is an extracellular enzyme that catalyzes the breakdown of proteins into polypeptides, resembles trypsin in its action, and has been shown to be a potent occupational allergen (Prescott *et al*., 2008; Chandra & Khan, 2013).

2.4.1.3 *Enterobacter aerogenes*

E. aerogenes is a Gram-negative, facultative anaerobic bacterium that performs fermentative hydrogen production similar to *Escherichia coli* (Kurokawa & Tanisho, 2005; Mordi & Hugbo, 2011). *Enterobacter* species are emerging as important pathogens for a wide variety of nosocomial infections (Sanders & Sanders, 1997; Blot *et al*., 2003), including pneumonia, urinary tract infections, meningitis, wound infections, and infections related to intravascular and prosthetic devices. *E. aerogenes* is responsible for 15% to 25% of all *Enterobacter* infections. *E. aerogenes* can cause primary bacteremia in pediatric patients (Edwards *et al*., 1978; Loiwal *et al*., 1999), inflammation of the tissues around the mid-chest (mediastinitis) following cardiac surgery, and crepitant cellulitis (Chang *et al*., 2009).

2.4.1.4 *Enterobacter cloacae*

E. cloacae is a Gram-negative, rod shaped bacterium and an emerging opportunistic human pathogen that is associated with nosocomial infection (Humann *et al*., 2011). *E. cloacae* infections are seen commonly in burn victims, immunocompromised patients, and patients with malignancy. The urinary and pulmonary systems are the organ systems most commonly colonized in these patients. *E. cloacae* bacteremia can also occur depending on the extent to which an individual is immunocompromised (Musil *et al*., 2010).

2.4.1.5 *Enterococcus faecalis*

E. faecalis is a Gram-positive, cocci shaped, non-spore forming, fermentative, facultative anaerobic, bacterium. *E. faecalis* is responsible for chronic periradicular inflammation and failure

of endodontic treatment (Stuart *et al*., 2006; Suchitra & Kundabala, 2006). *E. faecalis* is a microorganism commonly detected in asymptomatic, persistent endodontic infections. They are also present in human female genital tracts and the oral cavity in lesser numbers. They catabolize a variety of energy sources including carbohydrates, glycerol, lactate, malate, citrate, arginine and many keto acids (Stuart *et al*., 2006). *E. faecalis* has an ability to survive harsh environments including extreme alkaline pH, salt concentrations (Stuart *et al*., 2006; Hedge, 2009). They resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation. They can grow in the range of 10 to 45°C and survive a temperature of 60°C for 30 minutes (Stuart *et al*., 2006).

2.4.1.6 *Escherichia coli*

E. coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium which is usually found in the gastro-intestinal tracts of warm blooded organisms. The most common cause of urinary tract infection in humans is *E. coli*, causing at least five types of gastro-intestinal diseases in humans (Chandra & Khan, 2013). Pathogenicity is generally due to the presence of one or more virulence factors, including invasiveness factors, heat-liable and heat-stable enterotoxins, verotoxins and colonization factors. Pathogenic strains are usually identified by detection of specific virulence factors or of a serotype associated with a virulence factor (Prescott *et al*., 2008; Chandra & Khan, 2013). *Escherichia coli* O157:H7 is a public health concern on a global scale and is found in a wide variety of foodstuffs including meat and meat products, milk, yogurt, water, salad vegetables, fruits, fruit juices and cider (Buchanan & Doyle, 1997; Burt & Reinders, 2003). *E. coli* is an emerging cause of food-borne infection which leads to bloody diarrhoea and occasionally to kidney failure. Most cases of the illness have been associated with eating under-cooked, contaminated, ground beef. Person-to-person contact in families and child care centers is also an important mode of transmission if hygiene is inadequate. *E. coli* infection can also occur after drinking raw milk and after swimming or drinking contaminated water (Chandra & Khan, 2013).

2.4.1.7 *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative, non-sporulating, facultative, aerobic, rod shaped, opportunistic bacterium that is normally found in the gastro-intestinal tract of humans (Zheng *et*

al., 2014) . An adhesion to a mucosal surface is often the first step in the development of an infection. A survey of the presence of *Klebsiella* in urban residents, hospital personnel, and newly admitted patients showed that 30-37% of individuals carried *Klebsiella*, including a 29-35% faecal carriage and a three-to-four-percent throat carriage. Strains of *K. pneumoniae* and *K. oxytoca* which have not acquired any resistance are determined as naturally resistant to ampicillin and carboxypenicillin but susceptible to other beta-lactam antibiotics. This is due to the production of a chromosomal penicillinase which is inhibited by clavulanic acid (Chandra & Khan, 2013).

2.4.1.8 *Klebsiella oxytoca*

K. oxytoca is a Gram-negative, rod-shaped opportunistic pathogen that causes primarily hospital-acquired infections, most often involving immunocompromised patients or those requiring intensive care (Lowe *et al*., 2012). *K. oxytoca* is closely related to *K. pneumoniae*.

2.4.1.9 *Mycobacterium smegmatis*

M. smegmatis is an acid-fast bacterial specie. It has the ability to enter human macrophages and survive inside them in a 'latent' or 'non-proliferating' form for a long period of time (Bentrup & Russell, 2001; Gomez & McKinney, 2004; Cordone *et al*., 2011). *M. smegmatis* is a fast growing, non-pathogenic mycobacterium frequently used as a model system to study its pathogenic counterpart *Mycobacterium tuberculosis*. *M. smegmatis* becomes dormant in low oxygen concentration conditions (Lim & Dick, 2001) and remains viable for over 650 days when it suffers carbon, nitrogen and phosphorous-starvation (Smeulders *et al*., 1999; Cordone *et al*., 2011). *M. smegmatis* is mostly found in soil, water, and plants (Brown-Elliott & Wallace, 2002; Singh & Reyrat, 2009). *M. smegmatis* has a cell wall, which is similar to that of *M. tuberculosis* (Singh & Reyrat, 2009). The bacterium is a resilient, non-hazardous, fast-growing and nonpathogenic soil and environmental microbe that can be cultured in any laboratory (Singh & Reyrat, 2009; Thuy *et al*., 2010). It shares many features and identical genomic sequences with pathogenic *M. tuberculosis* and has been suggested as a potential candidate for developing new vaccines for tuberculosis (Thuy *et al*., 2010).

2.4.1.10 *Proteus mirabilis*

P. mirabilis is a Gram-negative, facultatively anaerobic, rod-shaped bacterium. It is one of the most frequent etiological agents associated with urinary tract infections (UTIs), particularly in catheterized patients or individuals with structural abnormalities of the urinary tract (Pellegrino *et al*., 2003). It is a member of the normal microbiota of the mammalian intestinal tract and has been isolated from humans, dogs, monkeys, pigs, sheep, cattle, raccoons, cats, rats, and other mammals (Fernandez-Delgado *et al*., 2007). Additionally, the bacterium is widely distributed in the environment, occurring in polluted water, manure, and soil, where it plays an important role in decomposing organic matter of animal origin (Fernandez-Delgado *et al*., 2007).

2.4.1.11 *Proteus vulgaris*

P. vulgaris is a rod-shaped, Gram negative bacterium that inhabits the intestinal tracts of humans and animals. It can be found in soil, water and fecal matter. It is known to cause urinary tract infections and wound infections (Chandra & Khan, 2013). Patients with recurrent infections, those with structural abnormalities of the urinary tract, those who have had urethral instrumentation, and those whose infections were acquired in the hospital have an increased frequency of infection caused by *Proteus* and other organisms (eg, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Enterococci*, *Staphylococci*) (O'Hara *et al*., 2000; Chandra & Khan, 2013).

2.4.1.12 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped opportunistic pathogen that takes an advantage of the breakdown of host defense system to initiate an infection. It is a common environmental microorganism present in water and soil and is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen (Prescott *et al*., 2008; Chandra & Khan, 2013). The bacterium is naturally resistant to many antibiotics due to the impermeability characteristics of the outer membrane. Moreover, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics (Okemo *et al*., 2001; Chandra & Khan, 2013).

2.4.1.13 *Staphylococcus aureus*

S. aureus is a facultatively anaerobic, Gram-positive coccus and is the most common cause of *staphylococcal* infections. It is a spherical bacterium, frequently part of the skin flora found in the nose, and on skin (Cosgrove *et al*., 2009). It can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis (Kluytmans *et al*., 1997). Its incidence is from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections, often causing postsurgical wound infections. The treatment of choice for *S. aureus* infection is penicillin; but in most countries, penicillin resistance is extremely common and first-line therapy is most commonly penicillinase-resistant penicillin (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections like endocarditis (Bayer *et al*., 1998) but its use is controversial because of the high risk of damage to the kidneys (Cosgrove *et al*., 2009). The duration of treatment depends on the site of infection and on severity (Neely & Maley, 2000).

2.4.1.14 *Streptococcus epidermidis*

S. epidermidis is a Gram-positive coccus and is the most frequent coagulase negative *Staphylococcus* (CNS) isolated from bloodstream infections. Its prevalence is associated with its tendency to colonize central venous catheters and other implanted medical devices. It is a commensal inhabitant of human skin and mucosa that may cause bloodstream infections (Iorio *et al*., 2011).

2.5 CHROMATOGRAPHIC ANALYSIS OF PHYTOCHEMICALS: TWO-DIMENSIONAL GAS CHROMATOGRAPHY COUPLED TIME OF FLIGHT MASS SPECTROMETRY (GC×GC-TOFMS)

Comprehensive two-dimensional gas chromatography (GC×GC) is a multi-dimensional gas chromatography (GC) technique that has an increased separation power with reduced analysis time (Özel *et al*., 2010). GC×GC works by increasing peak capacity by applying two independent separations to a sample in one analysis. Typically, GC×GC involves press-fitted serial columns of

differing phases separated by a thermal modulator. One separation is performed on the first column, and the effluent from it is continually focused and "injected" onto the second column, where another separation occurs. By keeping the second column short a series of high-speed chromatograms are generated, and the first column separation is maintained (Cochran *et al.*, 2004). In GC×GC, all materials that enter the 1st dimension column (with stationery phase "A") pass through the 2nd dimension column (with stationery phase "B") to the same detector and then uses a "Modulator" to partition 1st column effluent as discrete plugs onto the 2nd dimension column. The effluent from the primary column is focused and segmented by the modulator into a discrete "plug". Each plug is then injected onto the secondary column by the modulator, where separation occurs. The GC×GC process is a succession of independent second column separations (Cochran *et al*., 2004; Gaquerel *et al*., 2009). GC×GC adds a second dimension of chromatographic resolution to the sample analysis. This is accomplished by using two orthogonal separation phases (such as non-polar and polar) within a single analysis. The use of these two separation mechanisms expands the chromatographic plane-thus creating additional peak capacity in which peaks can be resolved (Leco's Pegasus 4D, 2011). The use of a spectrometric detector, in particular a mass spectrometer (MS), is highly desirable for identification of the numerous separated compounds found during the GC×GC run (Shellie & Marriott, 2002). The focusing process of GC×GC leads to peaks on the order of 50 to 500ms wide, so a fast detector is required. When MS is used, only time-of-flight (TOF) has the necessary acquisition rates (hundreds of spectra/sec) (Cochran *et al*., 2004). At present time-of-flight mass spectrometry (TOF-MS) is the most compatible MS technique. TOF-MS with unit mass resolution can acquire the 100 or more mass spectra per second recommended for ideal GC×GC. The combination of Direct thermal desorption (DTD), GC×GC, and TOF-MS has allowed the detection of more than 10,000 individual organic compounds in aerosol samples (Hamilton *et al*., 2004; Özel *et al*., 2010). GC×GC is now recognized as the most suited analytical technique for the characterization of complex mixtures of volatile compounds; it is implemented worldwide in academic and industrial laboratories. However, in the frame of comprehensive analysis of non-target analytes, going beyond the visual examination of the color plots remains challenging for most users (Vial *et al*., 2011). GC×GC is a hyphenated technique and greatly improves the result of component separation and identification (Marriott & Kinghorn, 1998; Phillips & Beens, 1999) and it has been

successfully applied to the analysis of essential oils (Shellie & Marriott, 2002; Wu *et al*., 2004; Ma et al. 2007).

Time-of-flight mass spectrometry (TOF-MS) is probably the simplest method of mass spectrometric measurement by the physical principle. The key features of TOF-MS are extreme sensitivity (all ions are detected), practically unlimited mass range and as well as high-speed analysis (recent TOF-MS instruments can measure hundreds of full spectra per second). Thus all of this, make TOF-MS one of the most desirable methods of mass analysis (Schlag, 1994; Guilhaus, 1995; Hoskovec *et al*., 2012). The ions are introduced either directly from the ion source of the instrument as a very short pulse. This results in all the ions receiving the same initial kinetic energy. The pulsed nature of the TOF-MS source of ionization further enhances the system's accuracy by avoiding spectral skewing common in a continuous ionization mode.

The Comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC-TOFMS) is a technique that provides a more superior separation (more compounds can be separated and identified as compared to GC-MS. The data generated from GC×GC-TOFMS, however, amounts to thousands of compounds that cannot be interpreted in a fast and efficient way (Welthagen *et al*., 2004). GC×GC-TOFMS affords better selectivity and sensitivity compared to one-dimensional GC-MS (1D-GC-MS) (Schoenmakers *et al*., 2003). The increased selectivity is provided by the increase in chromatographic capacity of the two dimensional (2D) system and the increased sensitivity from the focusing effect of the modulator (Dimandja *et al*., 2003; Focant *et al*., 2004; Hoh *et al*., 2007; de Vos *et al*., 2011; de Vos *et al*., 2013). TOF-MS gives the acquisition rate necessary for accurate quantitation with 2D-GC and also provides the full range mass spectra necessary for sample screening for a broad range of analytes in one analytical run (de Vos *et al*., 2011; de Vos *et al*., 2013). GC×GC-TOFMS also provides full range mass spectra for all sample components, thus allowing for identification of non-target analytes with due consideration of the sample-preparation steps employed (de Vos *et al*., 2013).

The comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC-TOFMS) is the most advanced analytical technique of high sensitivity and

selectivity. It is also a technique which provides a substantial enhancement of peak capacity and signal intensity over conventional GC analysis (Hoskovec *et al*., 2012). Signal capacity and intensity enhancements are achieved by using a modulator which is capable to trap and concentrate effluent portions from the first column and deliver these "samples" into the second column (Hoskovec *et al*., 2012).

2.5.1 Advantages of Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry

Comprehensive 2D gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS) uses two GC columns connected via a thermal modulator. Compared with the first column, the second column is usually a short column with a different stationary phase and is operated at a higher temperature (Wei *et al*., 2013). The metabolites co-eluted from the first column are further separated in the second column and are directed to a time-of-flight mass spectrometer for detection. Consequently, the GC×GC TOF-MS system brings more accurate and rich information about compound retention times and mass spectrum than a 1D GC-MS system, representing a powerful technique for analysis of metabolites in complex biological systems (Wei *et al*., 2013). The GC×GC-TOFMS system generates a huge amount of high-dimensional data in metabolomics studies that require efficient and accurate data analysis algorithms to uncover the biological information. Many data analysis algorithms have been developed to process the GC×GC-TOFMS data for peak picking (Reichenbach *et al*., 2004; Sinha *et al*., 2004; Vivo-Truyols, 2012), chromatogram alignment (Pierce *et al*., 2005; van Mispelaar *et al*., 2003; Zhang *et al*., 2008) and peak list alignment (Almstetter *et al*., 2009; Kim *et al*., 2011; Wang *et al*., 2010; Wei *et al*., 2013). Compared with liquid chromatography mass spectrometry (LC-MS), the applications of GC×GC- TOFMS to metabolomics were not fully explored during the past decade. One significant problem limiting the usage of GC×GC-TOFMS in metabolomics is the lack of accurate and comprehensive data analysis tools (Wei *et al*., 2013). The comprehensive GC×GC-TOFMS provides a reliable basis for the automated analysis of complex samples (Ozel *et al*., 2006). Some of the advantages of comprehensive two-dimensional gas chromatography (GC×GC) include increased peak capacity, improved resolution, and unique selectivity compared to conventional one dimensional gas chromatography (1D GC) (Mostafa & Górecki, 2013).

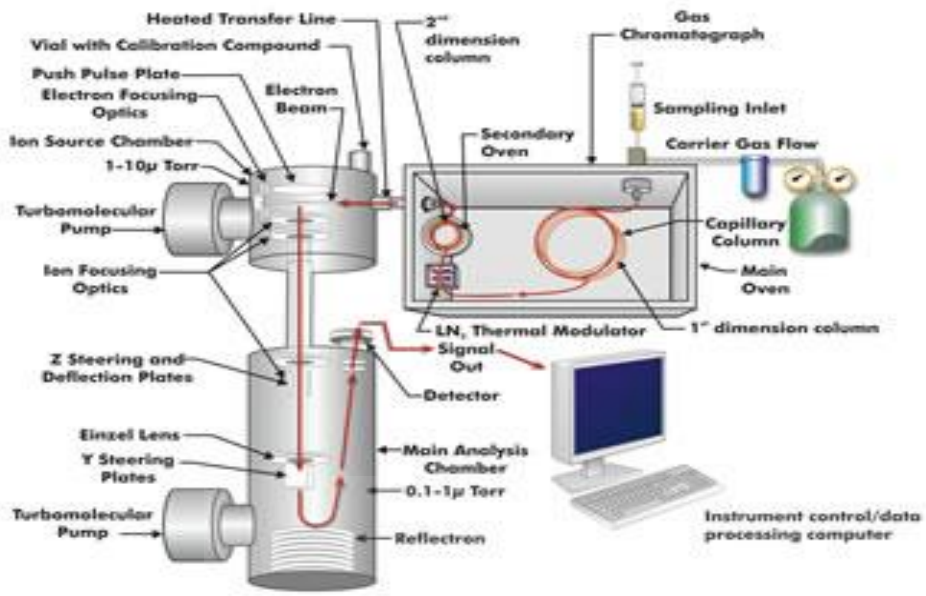


Figure 2.13: Schematic diagram of a typical GC×GC-TOFMS system, indicating the components (Heim *et al*., 2015)**.**

2.6 ELEMENTAL ANALYSIS: INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY (ICP-OES)

All elements in nature can be classified as metals or non-metals based on various sets of criteria. Several definitions reflect different properties of metals. A general definition based on physical properties is that metals are a large group of substances that are opaque, form alloys, conduct heat and electricity, and are usually malleable. More than 80% of the 125 known elements fit this definition. There are also several low-molecular-weight cations that do not have the physical properties of metals, such as calcium, sodium, potassium, and magnesium. Nevertheless, these cations are important in terms of human health because of their essential role in mammalian metabolism. A characteristic of this group of cations is that they are in themselves, rather than as members of metal-ligand complexes, responsible for several biological responses, including enzymatic reactions *in vivo* as well as nerve conduction and muscle contraction. They are also important (calcium in particular) in terms of risk assessment because of potential interactions with the principal metals. As with other essential metals, concentrations of cations in the body are controlled by homeostatic mechanisms (Goyer *et al*., 2004). A list of elements with their known beneficial effects is seen in Table 2.5.

Table 2.4: Classification of Metals Based on Characteristics of their Health Effects (Goyer *et al*., 2004)**.**

Nutritionally Essential Elements	Elements with Possible Beneficial Effects	Elements with No Known Beneficial Effects
Cobalt	Boron	Aluminum
Chromium III	Nickel	Antimony
Copper	Silicon	Arsenic
Iron	Vanadium	Barium
Manganese		Beryllium
Molybdenum		Cadmium
Selenium		Lead
Zinc		Mercury
		Silver
		Strontium
		Thallium

2.6.1 Trace Elements in Nature

Medicinal plants have been used for centuries to treat various ailments, ranging from common cold up to support of even cancer therapies in folk cultures throughout the world. Medicinal plants contain both the organic and inorganic constituents. Several studies on the organic constituents of medicinal plants have been reported but little attention has been made towards the investigation of the inorganic constituents and their role in the preparation of drugs (Mohanta *et al*., 2003; Garg *et al*., 2007; Bhanisana-Devi *et al*., 2015). It is very important to measure the trace elemental content of medicinal plants as they are essential for the metabolic functioning of the human body. Both the deficiency and excess of these elements may cause serious effects on human health (Rihawy *et al*., 2010; Bhanisana-Devi *et al*., 2015). Trace elements concentrations present in medicinal plants are of great importance to understand their pharmacological actions or detriments (Serfor-Armah *et al*., 2002; Selvaraju *et al*., 2011).

The human body requires both the metallic and the non-metallic elements within certain permissible limits for growth and good health. Therefore, the determination of elemental compositions in food, plants and related products is essential for understanding their nutritive

importance. Plants can easily be contaminated by metals during cultivation or later during the processing stage and therefore determining the content of the metals accumulated is of high importance. Accordingly, the presence of some metals in large quantities in the body may have a toxic effects (Khan *et al*., 2008; Sharma *et al*., 2009; Jabeen *et al*., 2010; Kostic *et al*., 2011).

Trace elements play an important role as catalysts or parts of prosthetic groups for enzymes, and, consequently, insufficient supply leads to element-specific deficiency symptoms. However, when present in excess, all of them can exert toxicity. In tissues and fluids, metals are mostly present as complexes with organic compounds like amino acids, proteins and peptides, organic acids or glutathione (Schümann, 2006; Prasad, 2008; Ebrahim *et al*., 2012). It is generally accepted that trace elements play an important role in the maintenance of human health. Besides, their concentrations and presence in medicinal plants are, aside from other organic plant compounds, of great importance in order to understand their pharmacological actions (Özcan, 2004; Yamashita, *et al*. 2005; Başgel & Erdemoğlu, 2006; Ebrahim *et al*., 2012). On the contrary, in some cases, plants may be contaminated with toxic concentrations of metals which may cause serious health hazard sequences, such as renal failure, symptoms of chronic toxicity and liver damage (Gomez *et al*., 2007; Ebrahim *et al*., 2012).

One very important feature when considering the health effects of trace elements is their slow accumulation in tissues, even at low doses. Hence, acute effects are reported very rarely, whereas chronic exposure can lead to the build-up of higher concentrations and onset of disease. Trace element toxicity can manifest with non-specific symptoms and, often, epidemiology is the only possible approach to ascertain their role (Prasad, 2008; Ebrahim *et al*., 2012).

2.6.2 Biological Roles of Trace Elements

Trace elements have at least five roles in living beings and they are as follows (Nielsen, 2014):

1. In close association with enzymes, some trace elements are integral parts of catalytic centers at which the reactions for life occur. Working in concert with a protein, and frequently with other organic coenzymes, the elements are involved in attracting substrate molecules and converting them into specific end-products.

2. Some trace elements donate or accept electrons in reactions of reduction and oxidation. In addition to the generation and utilization of metabolic energy, reactions of oxidation and reduction frequently involve chemical transformation of molecules.
3. One trace element, iron, is involved in binding, transporting, and releasing oxygen.
4. Some trace elements have structural roles; that is, imparting stability and three-dimensional structure to important biological molecules.
5. Some trace elements have regulatory roles. They control important biological processes through actions such as making hormones active, facilitating the binding of molecules to receptor sites on cell membranes, altering the structure or ionic nature of membranes to prevent or allow specific molecules to enter a cell, and inducing gene expression.

2.6.3 Nutritional Importance, Requirements, and Toxicity of some Trace Elements

Cadmium (Cd): Cadmium appears to mimic zinc, and to a lesser extent cadmium compounds are classified as human carcinogens (Waalkes, 2003; Krejpcio *et al*., 2007). The high levels of cadmium possess a serious toxicological effect on human health. The kidney is the critical target organ in the exposed population. Excretion of cadmium is very slow and it accumulates in human kidneys for a relatively long time, resulting in an irreversible impairment of the renal tract (Li *et al*., 2012; Griswold & Martin, 2009; Maobe *et al*., 2012). At high concentrations, cadmium produces serious effects on the liver and vascular and immune system (Maobe *et al*., 2012; Dghaim *et al*., 2015). Therefore, increasing this toxic element content in food/medicinal herb could be harmful (Krejpcio *et al*., 2007).

Calcium (Ca): Calcium ions are the most resourceful and common signaling agent in the human body (Berridge *et al*., 1998). It is important for strong bones, teeth, maintains proper blood pressure and for blood clotting. Its deficiency can lead to very serious problems like arthritis. It plays important function in nerve transmission, hormonal functions and metabolism of Vitamin D. The average human contains about 1 kg of calcium in our bodies. Children and pregnant women are encouraged to eat foods rich in calcium, such as cheese, milk and white bread (Pednekar & Raman, 2013). The recommended daily allowance for taking Ca is 800 mg for adults and for children 500-1000 mg (Karpiuk *et al*., 2016).

Chromium (Cr): Chromium is one of the abundant elements on the earth. It can exist either as trivalent and hexavalent. The trivalent is safe for human and the hexavalent is carcinogenic (Khuda *et al*., 2012). Over 50 years ago, trivalent chromium was reported to be the active component of the glucose tolerance factor' that alleviated impaired glucose tolerance in rats fed diets based on torula yeast and sucrose. There is much evidence that indicates chromium can have beneficial bioactivity in humans. Supranutritional amounts of chromium have been found to enhance insulin sensitivity or action in some people, especially insulin-resistant individuals with type II diabetes and highly elevated fasting plasma glucose and hemoglobin A1 concentrations. A low-molecular-weight chromium-binding substance or chromodulin has been reported to be an amplifier of insulin signaling. Chromodulin has been proposed to be formed and responsible for the beneficial effects of chromium supplementation on glucose metabolism. Chromium supplements have been touted as useful for building muscle and strength, inducing fat loss, and reducing blood cholesterol (Nielsen, 2014). Chromium is widely distributed throughout the food supply, but is highly variable among different lots of the same food. Chromium content of foods also is subject to increases and decreases during food processing. Whole grains, pulses, some vegetables (e.g., broccoli and mushrooms), liver, processed meats, ready-to-eat cereals, spices, and beer are generally good sources of chromium. The low order of toxicity for trivalent chromium apparently is because its complexes with oxygen-based ligands are usually electrochemically inactive and have a poor ability to cross cell membranes (Cefalu *et al*., 2010; Nielsen, 2014).

Copper (Cu): Copper is essential as a cofactor for enzymes involved in several fundamental processes including angiogenesis, neuropeptide signaling, iron metabolism, oxygen transport, energy production, antioxidant defense, and immune function (Nielsen, 2014); it also plays a significant role in a wide range of physiological processes including iron utilization, free radicals elimination, bone and connective tissues development, melanin production, and many others (Dghaim *et al*., 2015). Well established consequences of copper deprivation in humans have come mainly from studies of special populations whose sources of intake contained deficient amounts of copper (e.g., parenteral nutrition solutions, and milk or formulas with no supplemental copper for infants); consuming drugs (e.g., penicillamine) or undergoing dialysis resulting in excessive loss of copper; or having gastric bypass surgery, high intakes of zinc, or a disease (e.g., celiac

disease) or a genetic disorder (e.g., Menke's disease) that results in defective copper metabolism. Other consequences of inadequate copper intakes for humans such as impaired brain development for fetuses and children, and cardiovascular disease, osteoporosis, and increased infections for adults, have been suggested based on findings from epidemiological, animal, and short-term copper deprivation studies. Although copper is well established as an essential trace element, the prevalence of its dietary deficiency causing pathology in the general population has not been definitively determined (Nielsen, 2014). Nevertheless, excessive intake of copper can cause dermatitis, irritation of the upper respiratory tract, abdominal pain, nausea, diarrhea, vomiting, and liver damage (Griswold & Martin, 2009; Ullah *et al*., 2012; Dghaim *et al*., 2015).

Chronic copper toxicity is unusual. It occurs mainly in infants with immature bile flow and a high intake of copper; liver diseases in which bile flow is impaired; or with genetic disorders such as Wilson's disease that impairs the elimination of copper through the bile. The impairment of elimination causes copper to accumulate in the liver, renal tubules, cornea, and brain, resulting in damage to these organs (Nielsen, 2014). Good food sources of copper include legumes, whole grains, organ meats (e.g., liver), seafood (e.g., oyster, crabs), peanut butter, chocolate, mushrooms, and ready-to-eat cereal (Nielsen, 2014).

Indium (In): Indium has no known biological role but has been shown to cause birth defects in unborn children (Pednekar & Raman, 2013).

Iron (Fe): Iron has several key functions in the human body including oxygen supply, energy production, and immunity. Iron overdose is associated with symptoms of dizziness, nausea and vomiting, diarrhea, joints pain, shock, and liver damage. Iron toxicity has an adverse effect on various metabolic functions and cardiovascular system (Griswold & Martin, 2009; Dghaim *et al*., 2015). Iron is used to make tendons and ligaments and is important for maintaining healthy immune system. It is an essential part of haemoglobin. Its deficiency causes anaemia. But accumulation of iron in the body typically damages cells in the heart and liver which can cause cancer, coma, metabolic acidosis, liver failure, circulatory shock and long-term organ damages. The average human contains about 4 mg of iron. If the diet does not contain the 10 - 18 mg of iron needed each day, anaemia will eventually develop. Molasses, brewer's yeast, cocoa and

liquorice contain a lot of iron (Pednekar & Raman, 2013). The daily requirement of Fe for a child is 10 mg/day, whereas for an adult is 20 mg/day.(Karpiuk *et al*., 2016).

Lead (Pb): Lead is the most recognized toxic environmental pollutant (Dghaim *et al*., 2015). It can complex with various biomolecules and adversely affect their functions. Lead exposure may have an adverse effect on the blood, nervous, immune, renal, skeletal, muscular, reproductive, and cardiovascular systems causing poor muscle coordination, gastrointestinal symptoms, brain and kidneys damage, hearing and vision impairments, and reproductive defects (Johnson, 1998). Exposures to lead at early childhood and prenatally are associated with slowed cognitive development, learning deficits, and many other effects (Johnson, 1998; Krejpcio *et al*., 2007; Dghaim *et al*., 2015). Lead poisoning occur when the concentration reach between 100-140 µg/L (Uddin *et al*., 2013).

Manganese (Mn): Descriptions of signs of manganese deficiency in humans are very limited. The most convincing case of manganese deficiency is that of a child with postoperative short bowel on long-term parenteral nutrition with low manganese content (Nielsen, 2014). The child developed short stature and diffuse bone demineralization resulting in brittle bones. However, correlation studies have associated low manganese intake and/or plasma and tissue concentrations with several pathological conditions including osteoporosis, diabetes, and epilepsy, atherosclerosis, impaired wound healing, cataracts, childhood asthma, and Alzheimer's disease (Nielsen, 2014). Also, low maternal blood manganese concentration has been associated with increased risk of fetal intrauterine growth retardation and low birth weight. Based on human absorption and animal studies, high dietary intakes of calcium, phosphorus, iron, fiber, and phytate might increase the need or be deficiency predisposing factors for manganese. Because documented human manganese deficiency is so rare and manganese was considered one of the least toxic of the essential trace elements, manganese in the twentieth century was perceived mostly as an irrelevant nutrition concern for humans. However, manganese now receives significant attention as a toxicological concern for individuals whose normal homeostatic mechanisms are undeveloped, bypassed, or ill functioning. High retention of manganese in tissues, especially brain, has been reported for individuals receiving parenteral nutrition high in manganese and in individuals undergoing hemodialysis, especially when normal excretion via the

hepatobiliary system is impaired (Nielsen, 2014). In addition, biliary excretion of manganese is poor in neonates; thus, high intakes of manganese may increase tissue and brain concentrations in infants. Manganese toxicity has clinical features similar to those of Parkinson disease, including disequilibrium, tremor, muscle spasms, tinnitus, and hearing loss. Iron deficiency may exacerbate manganese toxicity because it enhances manganese absorption. Good food sources of manganese include unrefined grains, nuts, and leafy vegetables (Nielsen, 2014).

Mercury (Hg): Main effects of Mercury on human health and the environment effects on human health toxicity of mercury is dependent on whether it takes the form of elemental mercury, inorganic mercury or organic mercury compounds (particularly alkylmercury compounds such as methylmercury and ethylmethyl salts and dimethylmercury) (Pednekar & Raman 2013). Accordingly, the exposure situation varies considerably for these different forms of mercury and complicates toxicity assessment. In terms of methylmercury, dietary ingestion is the major source of human exposure, especially for seafood and fish. Around 80% of inhaled elementary mercury vapour is retained in the tissue of the lungs where it goes on to penetrate the blood-brain barrier where neurological effects take place (Mahurpawar, 2015). Ingestion of elementary mercury does not always lead to high levels of absorption but deaths have been reported. Inhalation of elementary mercury vapour has been observed to lead to symptoms including tremors, emotional lability, insomnia, memory loss, neuromuscular changes, and headaches as well effects on the kidney and thyroid. High exposures have led to death but the critical effects are neurotoxic and renal (Mahurpawar, 2015). The main route of exposure to inorganic mercury for humans is dietary although for some sub-sections of the population products such as skin-lightening creams, soaps and the use in traditional medicine and/ritualistic practices can result in significant exposures to both inorganic an elemental mercury. Methylmercury is a well-known potent neurotoxin which causes adverse impacts on the developing human brain. It passes readily through the placental barrier and the blood-brain barrier making any exposure during pregnancy of great concern. Methylmercury is considered possibly carcinogenic by the International Agency for Research on Cancer (Mahurpawar, 2015). Allopathic medical practitioners are skeptical about the use of mercury for therapy, a perception not supported by traditional medicine practitioners (Pednekar & Raman, 2013). Mercury has no known biological role. It is a virulent poison, readily

absorbed through the respiratory tract, the gastrointestinal tract or through the skin (Pednekar & Raman, 2013).

Nickel (Ni): As far as non-essential elements are concerned; nickel is known to cause cancer. Nickel and zinc, when present in low concentrations are important micronutrients, while in high concentrations, these two metals become toxic to plants (Lester, 1997). Its toxicity in humans is not very common occurrence because its absorption by the body is very low. Nickel is an essential element for plants such as the navy bean, which is used for baked beans (Pednekar & Raman, 2013).

Potassium (K): Potassium ions are the most abundant cations in the human body. It is extremely important for the cells in the body (Inam *et al*., 2011). It is essential for smooth flow of communication signals from cell to cell and its deficiency can contribute to diseases like stroke, heart problem, diabetes and hypertension. It acts in the intercellular fluid as the primary ion. Potassium together with sodium helps to regulate the water balance within the body. It regulates the transfer of nutrients to the cell, transmits electrochemical impulses and is necessary for normal growth and enzymatic reactions (Annalakshmi *et al*., 2012). The average human consumes potassium up to 7 mg a day and has a store of some 140 mg in the human body, mainly in the muscles. Normal diets contains enough potassium, but some foods such as instant coffee, sardines, nuts, raisins, potatoes and chocolate have more than average potassium (Pednekar & Raman, 2013).

Sodium (Na): Sodium is a vital element to human life. A healthy human body contains 90 to 130 gm of sodium. Potassium, Chlorine and Sodium together forms a part of blood plasma. Without sodium, cells will not get the nutrients to survive. Nervous system functioning depends on it. Loss of Sodium from body can leads to dehydration and weakness. The average person consumes about 10 mg of salt a day although all that is needed is about 3mg. Any excess may contribute to high blood pressure. Sodium performs the transmission of electrical impulses and the regulation of water content in tissue and blood (Pednekar & Raman, 2013).

Strontium (Sr): It is the fifteenth most abundant element on earth. Its occurrence in plants is a due to the type and chemical composition of soil, rainfall, agricultural practice and kind of plant.

Strontium has no known biological role and it is non-toxic. It replaces and mimics calcium (Pednekar & Raman, 2013).

Zinc (Zn): Zinc is a cofactor of over 200 enzymes involved in metabolic pathways but its high levels in human body can be toxic due to its interference with copper metabolism (Salgueiro *et al*., 2000; Krejpcio *et al*., 2007). Zinc has ability to occupy low symmetry site in enzymes, cause disturbances in enzymatic function and is capable of influencing immune system (Olivares & Uauy, 1996; Ashraf *et al*., 2010; Pednekar & Raman, 2013). Zinc is an essential trace element necessary for proper growth, blood clotting, thyroid function, and protein and DNA synthesis. Little information is available on Zn toxicity; however, high zinc intake beyond permissible limits produces toxic effects on the immune system, blood lipoprotein levels, and copper level (Serfor-Armah *et al*., 2001; Dghaim *et al*., 2015). It maintains various reactions of the body which help to construct and maintain DNA, required for the growth and repair of body tissues, important element of ligaments and tendons (Annalakshmi *et al*., 2012; Pednekar & Raman, 2013). It has an anti-diarrhoea activity and regulates fertility. The average human body contains about 2.5 mg and takes in about 15 mg per day. Herring, beef, lamb, sunflower seeds and cheese have above average levels of zinc. Zinc can be carcinogenic in excess (Pednekar & Raman, 2013). Therefore, dietary zinc intake should be appropriate (Krejpcio *et al*., 2007).

It is necessary to note that many curative effects of medicinal plants used in traditional medicine are due to the presence of very minute quantities of trace elements. Important constituents of the body such as enzymes are intimately associated with the chemical elements. Elements, particularly essential trace elements play both curative and preventive roles in fighting diseases such as Fe in anemia and iodine in goiter. At present about 14 such elements are considered to influence the state of health and diseases of animals, plants and human beings. These elements are Fe, Cu, Co, Ni, Zn, Mg, Mn, Mo, Cr, V, Li, Se, F and I (Shirin *et al*., 2010). The deficiency of trace elements in human subjects can occur under most practical dietary conditions. Many diseases which have been considered incurable may now possibly be treated by balancing the disequilibrium of these elements in the human body (Shirin *et al*., 2010).

In general, the geography, the geochemical soil characteristics, contaminants in the soil, water, and air, and other growth, transport, and storage conditions can significantly affect the properties

and the quality of the herbal plants and their formulations (Saad *et al*., 2006; Dghaim *et al*., 2015). The bioavailability of metals is influenced by several factors among which are the soil pH, the metal levels in the soil, the oxidation reduction potential of the soil, and other chemical and physical factors (Verma *et al*., 2007; Orish *et al*., 2012; Ullah *et al*., 2012; Dghaim *et al*., 2015).

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) is increasingly becoming a preferred source in analytical atomic adsorption (Greenfield *et al*., 1964; Wendt & Fassel, 1965). This is due to its efficiency and ability to analyze multi-elements in solution for a variety of samples which range from biological, ecological environmental and organic samples. It was first utilized by Greenfield *et al*., 1964, followed by Wendt & Fassel, 1965. Since then, it has been updated and recommended as the best analysis method compared to its older spectroscopic counterparts such as atomic adsorption and x-ray fluorescence because of its high precision, accuracy and adaptability (Greenfield *et al*., 1964; Wendt & Fassel, 1965; Hauser, 1980; Makita, 2014).

2.6.4 The ICP-OES Instrument

The basic components associated with ICP-OES are the sample introducing system (which consists of a peristaltic pump, nebulizer, spray chamber and drain assembly, the gas supply, ICP torch and the plasma), transfer optics and optical spectrometer; detectors and computer as shown in Figure 2.4 (Makita, 2014).

Instrument description: The instrument is used in atomic spectroscopy, and during analysis the sample is decomposed by intense heat into a cloud of hot gases containing free atoms and ions of the element(s) of interest. The high temperatures cause significant amounts of collisional excitation and ionization of the sample atoms. Once the atoms or ions are in their excited state, they can decay to lower states through thermal or radiative (emission) energy transitions. During ICP-OES analysis the intensity of the light emitted at specific wavelengths is measures and used to determine the concentration of the element(s) of interest. In ICP-OES analysis the thermal excitation sources can populate a large number of different energy levels for several different elements at the same time. All of the excited atoms and ions can then emit their characteristic radiation at the same time. This results in the flexibility to choose from several different

emissions concurrently and allows detection of multiple elements concurrently (Fitzsimmons, 1996).

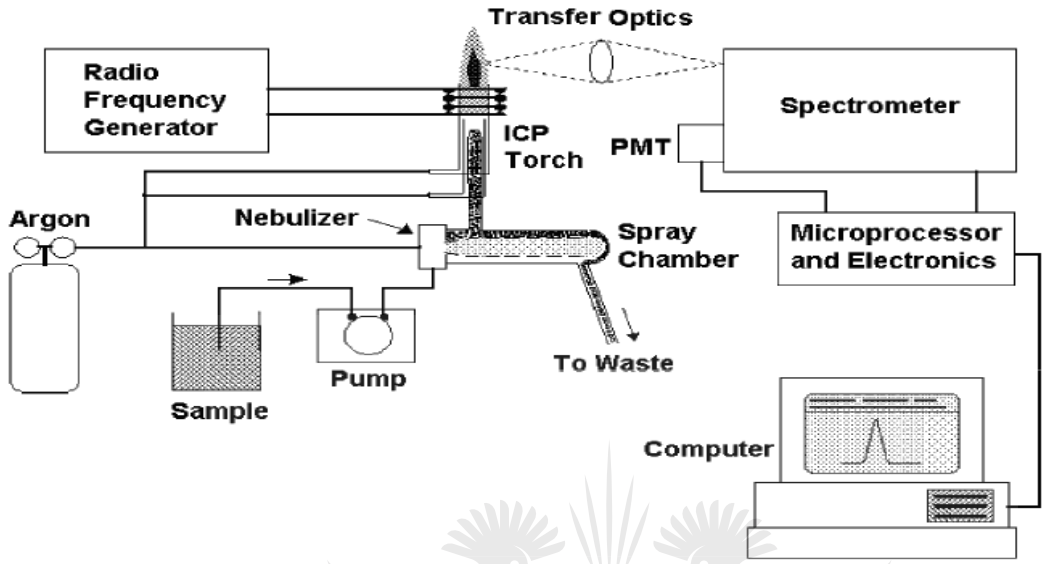


Figure 2.14: Schematic diagram of a typical ICP-OES system, indicating the key components. (Boss & Fredeen, 2004; Makita, 2014).

2.7 CONCLUSION

From this literature review, we see the medicinal importance of *R. tomentosa* and other species of the *Rhoicissus* genus. The presence of different biologically active phytochemicals in the *Rhoicissus* genus and their known biological activity shows that species of this genus although mostly used traditionally to for fertility related ailments might be sources for drug discovery. Some of the bioactive compounds previously identified in the different species of the *Rhoicissus* genus show that the different species might have similar bioactivity and hence can be used to treat similar ailments. These biological activities give some credence to the traditional medicinal uses of the *Rhoicissus* genus. The presence of elements in medicinal plants is an important issue that requires investigation because some of these elements as much as they have beneficial health effects might also have detrimental effects on humans when they are accumulated.

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CHAPTER THREE

3.0 PHYTOCHEMICAL SCREENING OF THE RHIZOMES OF *Rhoicissus tomentosa* USING CHEMICAL INDICATOR TESTS.

ABSTRACT

Many medicinal plants have been the source of various pharmacologically active compounds that are now used in medicine. The advancement of science into the search for antibiotics largely depends on plants as raw source material. These plants are known to contain chemical compounds which are responsible for the various bioactivities of these plants. Phytochemical screening of the rhizomes of *R. tomentosa* was conducted using different chemical indicator tests to ascertain the presence of bioactive phytochemicals. The results showed that the rhizomes of the plant contain alkaloids, flavonoids, saponins, steroids, reducing sugars and tannins. The presence of flavonoids which are known phytoestrogens confirm the traditional medicinal use of this plant to treat female infertility. The other phytochemicals identified in the plant have been reported to have several biological activities which could mean that *R. tomentosa* can be used to treat symptoms of some other ailments.

Keywords: Biological activities, Medicinal plants, phytochemical screening, *R. tomentosa*

3.1 INTRODUCTION

Phytochemical studies offer revelation and understanding of phytoconstituents (Hamburger & Hostettmann, 1991). Phytochemicals are secondary plant metabolites and have biological properties such as antioxidant activity, antibacterial effect and anticancer property. Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common bacterial infections (Saxena *et al*., 2013).

Preliminary tests and screenings on plant extracts are quicker and easily carried out following standard procedures and methods in manuals and literature. They detect the presence and in some occasions amount of basic phytoconstituents like terpenoids, alkaloids, flavonoids, saponins,

glycosides, steroids, tannins, anthraquinones, etc. (Olufunke, 2012). These are the groups of secondary metabolites known to be responsible for the medicinal property of most plants (Ogie-Odia *et al*., 2014).

Tannins are polyphenolic compounds which possess anti-inflammatory, antiseptic and antioxidant properties (Dolara *et al*., 2005; Saxena *et al*., 2013). In Chinese and Japanese natural healing, most tannin-containing plants are used as astringents against diarrhoea, as diuretics and against stomach duodenal tumors (De Bruyne *et al*., 1999; Tona *et al*., 1999; Saxena *et al*., 2013).

Flavonoids are also polyphenolic compounds and have been reported to have multiple biological activities which include antimicrobial (Ogie-Odia *et al*., 2014), cytotoxicity, anti-inflammatory, anti-tumor, anti-allergy, vascular- and oestrogenic activities (Saxena *et al*., 2013). Flavonoids' most important bioactivity is that they are powerful antioxidants thus allowing them to scavenge free radicals and protect the body from reactive oxygen species (Tapas *et al*., 2008; Saxena *et al*., 2013).

Steroids have been reported to possess antibacterial properties and they are very important compounds especially due to their relationship with sex hormones (Epand *et al*., 2007; Saxena *et al*., 2013).

Alkaloids have a variety of biological activities including cytotoxicity which is the most common. Their other biological activities include antimicrobial (Ogie-Odia *et al*., 2014), analgesic (Harborne, 1973; Saxena *et al*., 2013), antispasmodic, antibacterial, antihypertensive, antimalarial and anti-cancer (Wink, 1988; Saxena *et al*., 2013).

Saponins have bioactivities which include haemolytic activity and cholesterol binding property, antimicrobial, anti-protozoan, immunostimulant, anti-carcinogenic and antioxidant activities (Morrissey & Osbourn, 1999; Takechi *et al*., 1999; Saxena *et al*., 2013)

Reducing sugars act as anti-diabetics by reducing blood glucose levels (Nelson & Cox, 2008).

The nature and distribution of these compounds can vary subject to the plant tissue in which they are located (Cassidy & Kay, 2013).

The phytochemical screening of the rhizomes of *R. tomentosa* was done as described below:

3.2 MATERIALS AND METHODS

3.2.1 Sample Collection

Plant material (rhizomes of *R. tomentosa*) was purchased from the Faraday Muthi market in Johannesburg in February 2015. The rhizomes of *R. tomentosa* was submitted to the herbarium at the University of Johannesburg with reference number BTNNU01. The collected plant material was cut into smaller pieces and dried at 40˚C in an oven for five days.

3.2.2 Experimental Processes

3.2.2.1 Phytochemical screening

Phytochemical tests of the powdered rhizomes were carried out following the methods outlined by Harborne, (1984) and modified by Tamilselvi *et al*. (2012).

3.2.2.2 Water extraction

Powdered plant material (10 g) was weighed and added to distilled water (200 mL) and was placed in a water bath to boil. The mixture was filtered using Whatman No. 1 filter paper and the filtrate was used to carry out the phytochemical tests that required water extract.

3.2.2.3 Test for Tannins

The water extract filtrate (2 mL) was put in a vial, 2-3 drops of Iron (III) Chloride was added to the filtrate in the vial and colour change was observed.

3.2.2.4 Test for Alkaloids

Powdered plant material (0.5 g) was weighed into a beaker, 5 mL of a 1 % hydrochloric acid solution was added and the beaker was placed in a boiling water bath for 2-3 minutes. 1 mL of the solution was collected in a vial and few drops of Dragendorff's reagent was added to it.

3.2.2.5 Test for Flavonoids

Powdered plant material (0.5 g) was weighed into a beaker, 10 mL of ethyl acetate was added and the beaker was placed over a steam bath for 3 minutes. The mixture was filtered and 4 mL of the filtrate was collected in a vial and 1 mL dilute ammonia was added to it.

3.2.2.6 Test for Steroids

Powdered plant material (0.5 g) was weighed into a beaker, dissolved in chloroform (5 mL) and filtered. The filtrate was collected in a beaker, concentrated sulfuric acid was added to it. The beaker was swirled gently to mix properly then it was allowed to stand for few minutes for layers to separate.

3.2.2.7 Test for Cardiac glycosides

Powdered plant material (0.5 g) was weighed into a beaker, glacial acetic acid (2 mL) containing 1 drop of 1 % $FeCl_3$ was added. 1 mL of concentrated sulfuric acid was slowly added and the beaker was left to stand for a while to allow the phases to separate.

3.2.2.8 Test for reducing sugars

The water extract filtrate (10 mL) was put in a beaker, Benedict's reagent (2 mL) was added to it and the solution was heated over a boiling water bath.

3.2.2.9 Test for Saponins

The water extract (10 mL) was put in a closed vial and shaken vigorously to allow the formation of froth. The froth was collected carefully into another vial and 3 drops of olive oil were added and the mixture was shaken vigorously again to observe for the formation of a stable froth and emulsion.

3.3 RESULTS AND DISCUSSIONS

The results obtained from the phytochemical screening of *R. tomentosa* are presented in Table 3.1. The rhizomes tested negative for cardiac glycosides but positive for tannins, flavonoids, steroids, reducing sugars, alkaloids and saponins.

Table 3.1: Result of the phytochemical screening of *R. tomentosa*

Phytoconstituents	Observation	Results
Tannins	Blackish-blue/Blackish-green coloration.	Positive
Flavonoids	Yellow coloration	Positive
Steroids	Reddish brown ring	Positive
Reducing sugars	Bright blue	Positive
Alkaloids	Turbidity/ Precipitation	Positive
Saponins	Persistent froth	Positive
Cardiac glycosides	No brown ring at interface	Negative

Medicinal plants are rich sources of bioactive chemicals and these compounds have been known to possess beneficial properties to human health in addition to the nutritional benefits they may have (Hasler & Blumberg, 1999). The results of the phytochemical screening carried out on the rhizomes of *R. tomentosa* revealed the presence of many known groups of bioactive compounds: tannins, flavonoids, steroids, reducing sugars, alkaloids and saponins. The phytochemical results for the presence of flavonoids and saponins support previous findings by (Nqolo, 2008), but the other phytoconstituents to the best of our knowledge are being reported present in the *R. tomentosa* rhizomes for the first time. Phytochemical studies carried out on related plants of the same genus revealed the presence of phenols, alkaloids, flavonoids, saponins and tannins (Mwangi *et al*., 2015), irioids, stilbenes and triterpenoids (Stark *et al*., 2013) in *R. tridentata*; the roots of *R. revoilli* were found to contain flavonoids, anthraquinones, steroid glycosides, alkaloids, saponins and ketones, while the leaves were found to contain anthraquinones, steroid glycosides, alkaloids, saponins, ketones and tannins (Musyimi *et al*., 2008); all these phytoconstituents were also identified in the rhizomes of *R. tomentosa* but the presence of reducing sugars are being reported for the first time in *Rhoicissus* genus as much scientific research studies have not been carried out on *R. tomentosa* and other species of the *Rhoicissus* genus.

3.4 CONCLUSION

The identification of flavonoids in this plant and the previous literature stating their oestrogenic activities (Havsteen, 2002) gives some credence to the traditional use of this plant to enhance

fertility. The presence of other phytochemicals tannins and alkaloids in *R. tomentosa* suggests that besides the known traditional medicinal uses of the plant, it could also have other medicinal uses considering that each identified phytochemical has its own biological activity.

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CHAPTER FOUR

4.0 ANTIBACTERIAL ACTIVITY SCREENING OF THE RHIZOMES OF *Rhoicissus tomentosa* USING DISC DIFFUSION AND MICROBROTH DILUTION ASSAYS.

ABSTRACT

Medicinal plants have always been considered as a source for healthy life for people. Therapeutic properties of medical plants are very useful in healing various. In many parts of the world, medicinal plants have been used for their antibacterial, antifungal and antiviral activities for hundreds of years. Methanol/chloroform (50:50, v/v), ethyl acetate (100%) and Pressurized Hot Water (PHW) extracts of the rhizomes were tested against 14 bacterial strains using the agar disc diffusion and microdilution minimum inhibitory concentration (MIC) assay methods. The study revealed that both organic solvent extracts showed moderate to high inhibitory activity against most of the test organisms. The minimum inhibitory concentrations of the organic solvent extracts ranged from 0.0063 mg/mL to 16 mg/mL, while the PHW extracts had moderate inhibitory activity. The MIC values for the PHW extract ranged from 1 mg/mL to 16 mg/mL. *R. tomentosa* showed potential as a possible source of drug lead for the treatment of diseases caused by bacterial pathogens.

Keywords: Disc diffusion, Medicinal plants, MIC, *R. tomentosa*

4.1 INTRODUCTION

In ethnopharmacology research, antimicrobial susceptibility test (AST) is used to determine the effectiveness of potential antimicrobials from biological extracts against a number of different microbial species. AST methods are used to screen plant extracts for antimicrobial activity but are largely used to determine the usefulness of an antimicrobial in combating infections (EUCAST Definitive Document, 2000; Ncube *et al*., 2008). Antimicrobial susceptibility standard tests can be conveniently divided into diffusion and dilution methods. Common diffusion tests include agar well diffusion, agar disk diffusion and bioautography, while dilution methods include agar dilution and broth micro/macrodilution. The broth and agar based methods are the conventional reference methods for AST (Tenover *et al*., 1995; Ncube *et al*., 2008).

The search for novel antimicrobial agents from plants has of recent been great interest. Medicinal plants have been said to contain a rich variety of bioactive compounds and in some cases these compounds are responsible for the antimicrobial properties if the plants. of antimicrobial agents. The rhizomes of *R. tomentosa* which to the best of our knowledge has been not been previous tested for antimicrobial activity was studied in this project. The leaves and stem had been studied previously and they showed impressive inhibitory activities against a good number of human pathogenic bacteria (Lin *et al*., 1999).

The rhizomes of *R. tomentosa* were tested against 14 strains of bacteria to ascertain the effects of the plant on these different bacteria species. *R. tomentosa* and the other species of the *Rhoicissus* genus are mainly used for fertility treatment (among a few other uses) by traditional healers. Considering that medicinal plants contain several active ingredients with different bioactivities, it is therefore necessary to screen *R. tomentosa* for antibacterial activity despite it not being used by traditional healers to treat bacterial infections. This can help give insight on other medicinal uses of the plant and provide ideas on further research to isolate the different compounds contained in the plant, identify compounds with interesting bioactivity at very low concentrations, find out if these compounds work better individually or in combination hence making the compounds in the plant a possible drug lead.

4.2 MATERIALS AND METHODS

4.2.1 Sample Collection

Rhizomes of *R. tomentosa* were collected and prepared as described in Chapter 3.

4.2.2 Extract Preparation

4.2.2.1 Organic Solvent extraction

The method described by (Fomogne-Fodjo *et al*., 2014) with slight adjustments was used for the preparation of the crude extracts. The dried rhizomes were blended into a fine powder with a shop-bought coffee mill and 200 g of plant powder was weighed into two separate Schott bottles, 1000 mL of a methanol/chloroform (50:50, v/v) solution was added to one and 1000 mL of 100% ethyl acetate was added to the other and both were left on a platform shaker for three days. The

extract suspensions were filtered through Whatman No. 1 filter paper and evaporated to dry out the solvent using a rotatory evaporator with consideration to the boiling points of the extracting solvents. The process was repeated another two times to ensure maximal extraction of compounds. The crude extracts were collected in beakers and placed in a desiccator to dry completely for up to 2 weeks.

4.2.2.1 Pressurized Hot Water Extraction (PHWE)

Pressurized Hot Water Extraction was done using a makeshift laboratory scale PHWE unit described by Khoza *et al*. (2016).

In this procedure, a ratio of 3.0 g of *Rhoicissus tomentosa* powder and 3.0 g of diatomaceous earth (1:1) were weighed and placed in an extraction cell. The extraction cell was connected inside a Gas Chromatography oven which is programmed for heating the sample contained inside the stainless-steel cell. Deionized water was pumped through the cell at a flow rate of 1.0 $mL.min^{-1}$. The pressure was between 1000-3000 psi and was regulated by a valve. Prior to the start of the extraction procedure, the oven is allowed to preheat for approximately 10 minutes until equilibration is reached. Deionized water was also heated prior to extraction to 80 ˚C to minimize the temperature gradient and is regulated by a thermometer. After pumping of the system was completed, the extract was collected in a 50 mL microtubes and was taken for further analysis. With regards to optimization studies, the extraction cell was heated at temperature of 100 ˚C (Gbashi, 2016).

4.2.3 Experimental Processes

4.2.3.1 Determination of anti-bacterial activity

Two methods were used to determine the anti-bacterial activity of *R. tomentosa*: The Disc diffusion method and the Minimum Inhibitory Concentrations method.

4.2.3.2 Bacterial strains used

Crude extracts of *R. tomentosa* were tested against 14 specific bacteria strains: *Bacillus cereus (ATCC10876), Bacillus subtilis (ATCC19659), Enterobacter aerogenes* (*ATTC13048*), *Enterobacter cloacae (ATCC13047) , Enterococcus faecalis (ATCC13047),*

Escherichia coli (ATCC25922), Klebsiella oxytoca (ATCC8724), Klebsiella pneumonia (ATCC13882), Mycobacterium smegmatis (MC^2 155), Proteus mirabilis (ATCC7002), Proteus vulgaris (ATCC6380), Pseudomonas aeruginosa (ATCC27853), Staphylococcus aureus (ATCC25923), Streptococcus epidermidis (ATCC14990).

4.2.3.3 Disc Diffusion

The method described by Othman *et al*. (2011) was followed. The experiment was done in triplicate and the bacterial strains were inoculated into nutrient broth and allowed to grow overnight in an incubator at 37˚C for 24 to 36 h depending on the growth rate of each bacteria and compared with a 0.5 McFarland's standard which was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4). Nutrient agar was prepared according manufacturer's instructions; 20 mL was poured into disposable petri-dishes and allowed to solidify. Filter papers discs of 6 mm were made and autoclaved to ensure sterility.

The antibiotic, streptomycin purchased from Sigma-Aldrich was used as the positive control and was prepared by weighing 0.032g in 1 mL of distilled water. Sterile distilled water was used as negative control.

Organic Solvent extract: The plant's crude solvent extracts (0.2 g) was weighed into 2 mL micro tubes and re-dissolved in 2 mL of dimethyl sulfoxide (DMSO) using a vortex. The discs were impregnated with 20 µL of the dissolved plant extract under sterile conditions and the plates were left open for the solvents to evaporate. Separate discs were impregnated with 10 µL of each of the positive and negative controls. The bacteria were diluted to match a 0.5 McFarland's standard where necessary and streaked on plates using sterile cotton swabs. Impregnated discs were placed on appropriate plates and incubated overnight at 37˚C.

Pressurized Hot Water Extract: Pressurized hot water (PHW) extract of *R. tomentosa* (25mL) was aseptically measured into a 50mL micro tube. The extract was put in a -80˚C refrigerator for 24 hours. The frozen extract was then freeze-dried for 48 h. The freeze-dried PHW extract (0.2g) was weighed into 2mL Eppendorf tubes and re-dissolved in 2 mL of sterile distilled water using a

vortex. The discs were impregnated with 20µL of the dissolved plant extract under sterile conditions and the plates were left open for the solvents to evaporate. Separate discs were impregnated with 10 µL of each of the positive and negative controls. The bacteria were diluted to match a 0.5 McFarland's standard where necessary and streaked on plates using sterile cotton swabs. Impregnated discs were placed on appropriate plates and incubated overnight at 37˚C.

4.2.3.4 Minimum Inhibitory Concentrations (MICs)

MICs were carried out according to the method outlined by Andrews, (2001). The experiment was done in five repeats using 96-well micro titer plate and the bacterial strains were inoculated into Mueller Hinton broth and allowed to grow overnight in an incubator at 37˚C for 24 to 36 hours depending on the growth rate of each bacteria and compared with a 0.5 McFarland's standard. Mueller Hinton (MH) broth was prepared according to the manufacturer's instructions and 5.5 mL was dispensed into McCartney bottles and autoclaved at temperature of 121˚C, pressure of 15 psi for 15 minutes. The antibiotic, Streptomycin was used as the positive control and was prepared by weighing 0.032g in 1 mL of distilled water while DMSO was used as a negative control.

Each of the three different crude extracts: methanol:chloroform 50:50 (v/v) extract, 100 % ethyl acetate extract and PHW extract (0.176 g) were weighed into empty autoclaved MacCourtney bottles to ensure sterility. The crude extracts were dissolved in a minimal amount of DMSO and the remaining volume was made up to 5.5 mL using MH broth. Serial dilutions were carried out from 16 mg/mL down to 0.03125 mg/mL. The outer wells of the plate were filled with sterile distilled water. Standardized overnight bacterial cultures (100 µL) were added into each well horizontally and vertically in 5 repeats for each bacterium. In vertical order, 100 µL of the diluted samples were added in the wells from 16 mg/mL down to 0.03125 mg/mL. The plates were covered and incubated overnight. Resazurin sodium salt solution, 10µL of 0.02 % (w/v) was added to the wells and incubated for another two hours. The wells were visually inspected for color changes.

4.3 RESULTS AND DISCUSSIONS

4.3.1. Disc diffusion

4.3.1.1 Solvent extracts

Different solvents were used to extract compounds from *R. tomentosa* rhizomes and both extracts were tested for antibacterial properties. The results from the disc diffusion experiments are shown in Table 2. The methanol/chloroform (50:50 v/v) crude extract showed significant zones of inhibition on the agar plates against *B. cereus, E. faecalis, K. pneumoniae, M. smegmatis, P. mirabilis* and *P. vulgaris*. The ethyl acetate extract (100%) showed zones of inhibition against the same bacterial strains as the methanol/chloroform extract and against *S. epidermidis.* When compared to streptomycin, the extracts had noteworthy zones of inhibition since they did not contain a single pure compound but a mixture of chemicals. On average the 100 % ethyl acetate extract showed better zones of inhibition.

Table 4.1: Disc diffusion test result showing measurements of zones of inhibition of the crude solvent extracts.

Test organism	**Gram-stain reaction**	**MeOH/$CHCl_3$ (50:50) extract zones of inhibition (mm)**	**100% Ethyl acetate extract zones of inhibition (mm)**	**Streptomycin zones of inhibition (mm)**
B. cereus	**Positive**	**10**	**11**	**28**
B. subtilis	Positive	NI	NI	20
E. faecalis	**Positive**	**7**	**11**	**20**
M. smegmatis	**Positive**	**10**	**11**	**23**
S. aureus	Positive	NI	NI	25
S. epidermidis	**Positive**	NI	**9**	**25**
E. aerogenes	Negative	NI	NI	21
E. cloacae	Negative	NI	NI	20
E. coli	Negative	NI	NI	25
K. oxytoca	Negative	NI	NI	24
K. pneumoniae	**Negative**	**8**	**8**	**29**
P. mirabilis	**Negative**	**11**	**10**	**24**
P. vulgaris	**Negative**	**13**	**14**	**27**
P. aeruginosa	Negative	NI	NI	24

NI = No Inhibition

4.3.1.2 PHW extract

The results from the disc diffusion experiments are shown in Table 4.2. The PHW extract showed zones of inhibition on the agar plates against *B. cereus, B. subtilis, K. pneumoniae, M. smegmatis, P. mirabilis* and *P. vulgaris*. When compared to streptomycin, the extracts had less significant zones of inhibition unlike the solvent extracts.

Table 4.2: Disc diffusion test result showing measurements of zones of inhibition of the PHW extract**.**

Test organism	PHW extract zones of inhibition (in mm)	Streptomycin zones of inhibition (in mm)
B. cereus	**8**	**28**
B. subtilis	**9**	**20**
E. cloacae	NI	20
E. aerogenes	NI	21
E. faecalis	NI	20
E. coli	NI	25
K. oxytoca	NI	24
K. pneumoniae	**8**	**25**
M. smegmatis	**8**	**23**
P. mirabilis	**9**	**24**
P. vulgaris	**10**	**27**
P. aeruginosa	NI	24
S. aureus	NI	25
S. epidermidis	NI	25

NI= No Inhibition

4.3.2. Minimum Inhibitory Concentration (Microdilution method)

The results of the MIC assay of the rhizomes of *R. tomentosa* both for the organic solvent extracts and PHW extracts are displayed on Table 4.3.

4.3.2.1 Solvent extracts

The Minimum Inhibitory Concentration (MIC) results showed that the methanol/chloroform extracts of *R. tomentosa* rhizomes were very antibacterial against *B. cereus* (MIC at 0.500 mg/mL), *B. subtilis* (MIC at 0.125 mg/mL) and *S. aureus* (MIC of 0.063 mg/mL). The ethyl

acetate extract showed better antibacterial properties against a number of bacteria, most notably *Mycobacterium smegmatis* (MIC 0.063 mg/mL), *B. cereus* (MIC 0.5 mg/mL), *B. subtilis* (MIC 0.125 mg/mL) *S. aureus* (MIC 0.063 mg/mL).

4.3.2.2 Pressurized Hot Water Extract

The Minimum Inhibitory Concentration (MIC) results showed that the PHW extract of *R. tomentosa* rhizomes showed following results *B. cereus* (MIC 4 mg/mL), *B. subtilis* (MIC at 8 mg/mL), *E. faecalis* (MIC 8 mg/mL), *K. pneumoniae* (MIC 8 mg/mL), *M. smegmatis* (MIC 16 mg/mL), *P. vulgaris* (MIC 8 mg/mL), *S. aureus* (MIC of 1 mg/mL) and *S. epidermidis* (MIC 16 mg/mL).

Table 4.3: MIC values of solvent and PHW extracts of *R. tomentosa.*

Test organism	MeOH/$CHCl_3$ extract (MIC mg/mL)	Ethyl acetate extract (MIC mg/mL)	PHW extract (MIC mg/mL)	Streptomycin Positive Control (MIC mg/mL)
B. cereus	**0.5**	**0.5**	**4**	**0.125**
B. subtilis	**0.125**	**0.125**	**8**	**0.125**
E. cloacae	>16	>16	>16	0.5
E. aerogenes	>16	>16	>16	8
E. faecalis	>16	**2**	**8**	**0.125**
E. coli	>16	>16	>16	8
K. oxytoca	>16	>16	>16	4
K. pneumoniae	>16	>16	**8**	**0.5**
M. smegmatis	>16	**0.063**	**16**	**0.125**
P. mirabilis	**16**	>16	>16	**0.5**
P. vulgaris	**16**	**8**	**8**	**0.125**
P. aeruginosa	>16	>16	>16	8
S. aureus	**0.063**	**0.063**	**1**	**0.032**
S. epidermidis	>16	**2**	**16**	**0.5**

MICs are considered the "gold standard" for testing the susceptibility of microorganisms to antimicrobials hence, it is used to judge all other methods of susceptibility testing. It was used in this study to ensure accurate results since the disc diffusion method is not exactly 100 % accurate

and reliable due to factors like the sample on the disc not diffusing properly into the agar. Crude extracts are interpreted as having antimicrobial activity when the MIC values are between 8 mg/mL and 1 mg/mL, with an MIC value below 1 mg/mL interpreted as a good indicator of the antibacterial properties of crude extracts (Valgas *et al*., 2007).

The two different extracts also showed varying results when compared to each other. For the methanol/chloroform extract, disc diffusion test results showed that growth in *B. subtilis* and *S. aureus* were not inhibited but in the MIC test they were inhibited. The MIC tests on the other hand have showed no inhibitory activity against *E. faecalis, M. smegmatis* and *K. pneumonia* but their growth was inhibited during the disc diffusion test. The MIC results and disc diffusion results for the ethyl acetate extract were similar except that in the disc diffusion test it did not show activity against *B. subtilis* and *S. aureus* but in the MIC test it could inhibit these two species. The MIC test showed no inhibitory activity against *K. pneumonia* and *P. mirabilis* but the disc diffusion test showed inhibition from this extract. The discrepancy observed between the two tests could be due to either the extracts precipitating out when diluted particularly to the point of achieving very low concentrations considering that the dilution for the MIC started from 16mg/mL while the disc diffusion dilution was 20µL and was not diluted further.

Of the 14 bacterial strains screened for antibacterial activity, 8 were susceptible to the crude extracts of *R. tomentosa* at the starting concentration (16 mg/mL) and below while the rest did not display sensitivity at the concentrations tested. It is still possible for those bacterial strains to be susceptible to the plant material at higher concentrations.

The disc diffusion test is a method used to screen for bacterial susceptibility to a particular antibacterial agent or an agent suspected of having antibacterial activity. Due to some factors like: the sample impregnated on the disc not being able to diffuse properly into the agar, hence zones of inhibition may be absent even though the extract is active. Also, since it can only indicate if samples are active against particular bacterial strains but will not give the concentrations at which the bacteria are susceptible therefore MIC is done to know the concentrations at which the bacteria are susceptible.

The antibacterial screening results of the rhizomes of *R. tomentosa* correspond with previous findings by Lin *et al*., (1999) on the antibacterial activity of the leaves and stem of *R. tomentosa*. This is the first report on the antibacterial activity of the rhizomes of *R. tomentosa*. From the results, we observed that the crude extracts of *R. tomentosa* showed better antibacterial activity against the Gram-positive organisms: *B. cereus, B. subtilis, E. faecalis, M. smegmatis, S. aureus* and *S. epidermidis* than Gram-negative ones: only *P. vulgaris* and *P. mirabilis* displayed sensitivity to the extracts. Both extracts did not have any effect on *E. coli* as was also seen in the studies of Lin *et al*, (1999).

The PHW extract of the rhizomes of *R. tomentosa* showed antibacterial activity against 8 bacterial species, of which some were the same bacteria inhibited by the solvent extracts but at different concentrations. The solvent extracts had lower, more significant MIC values than the PHW extract. All three extracts (ethyl acetate, methanol/chloroform 50:50 v/v and PHW extracts) showed inhibitory activity against *B. cereus, B. subtilis, P. vulgaris* and *S. aureus*. The ethyl acetate and PHW extracts showed inhibitory activity against *E. faecalis, M. smegmatis* and *S. epidermidis*. All three extracts showed inhibitory activity against *K. pneumoniae* in the disc diffusion assay was inhibited by both solvent extracts.

The higher MIC values seen with the PHW extract can be attributed to the polarity of water as compared to that of organic solvents. This difference in MIC values of the solvent and PHW extracts support the study of Mensah *et al*., (2013) that water is not a good solvent for extraction of solutes with inhibitory activities and that natural products are more extractable using chemical solvents (Mensah *et al*., 2013; Ogie-Odia *et al*., 2014). We also noted the PHW extract had the most significant effect against *S. aureus* (MIC 1 mg/mL), ethyl acetate and methanol/chloroform extracts (MIC 0.063mg/mL) also had a significant effect against the same bacterium, so it can be said that *S. aureus* is the most susceptible bacterium to *R. tomentosa* rhizomes. From all the antibacterial screening results, it can be seen that *R. tomentosa* is most active against the Gram-positive organisms than the Gram-negative ones and this could be attributed to the differences in the peptidoglycan layer of their cell wall.

4.5 CONCLUSION

R. tomentosa rhizomes showed that at low concentrations (0.5 mg/mL and 0.125 mg/mL respectively) there was inhibitory activity against *B. cereus* and *B. subtilis*; at 0.063 mg/mL it showed impressive inhibition of *S. aureus* and *M. smegmatis*. With MIC of 2 mg/mL it showed activity against *E. faecalis* and *S. epidermidis* too which suggests that the plant might be a potentially good source of raw material for possible antimicrobial medications. The ethyl acetate extracts showed bioactivities against more bacterial strains than the methanol and chloroform extracts which also suggests that ethyl acetate as a solvent most probably extracted some potent bioactive compounds which were not extracted by the methanol and chloroform. This study also helped to show that this plant might have other medicinal uses apart from the already known medicinal uses.

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CHAPTER FIVE

5.0 PHYTOCHEMICAL ANALYSIS OF THE RHIZOMES OF *Rhoicissus tomentosa* USING TWO-DIMENSIONAL GAS CHROMATOGRAPHY COUPLED TO TIME OF FLIGHT MASS SPECTROMETRY (GC×GC TOF-MS)

ABSTRACT

GCxGC-TOFMS is a multidimensional Gas Chromatography technique that combines two independent separations to accurately analyze highly complex samples Phytochemical analysis of the crude extracts of the rhizomes of *R. tomentosa* was carried out using GCXGC-TOFMS to ascertain the groups of bioactive chemical compounds present in the plant. Fatty acids (oleic acid, tetracosanoic acid, etc.), fatty acid esters (Docosanoic acid, methyl ester, Octadecanoic acid, methyl ester, etc.) and amino acid (D-Asparagine) which possess antimicrobial activities and other chemical compounds with varying biological activities were identified in the organic solvent extracts while the squalene with antioxidant activity and 9-Octadecenamide, (Z)- with anti-inflammatory activity and a host of other compounds were identified in the pressurized hot water extract. The presence of these bioactive compounds in *R. tomentosa* rhizomes and their known bioactive properties help support the antibacterial activity of this plant and suggests that this plant has potential as a possible drug lead.

Keywords: amino acids, fatty acids, GC×GC-TOFMS, *R. tomentosa*

5.1 INTRODUCTION

GC×GC is a technique used for complete analysis and characterization of complex samples. Comprehensive GC×GC appears nowadays to be the prime analytical tool for the study of complex mixtures of volatile compounds (Adahchour *et al*., 2006; Cordero *et al*., 2006; Vial *et al*., 2011). The major advantages of GC×GC are its non-specificity, i.e. ability to assign a range of compounds without previous knowledge (non-target compounds), as well as peak identification using mass spectra. Furthermore, GC×GC provides a broad fingerprint of oils or their fractions, which greatly improves the possibility of recognizing new compounds and opens up prospects for new geochemical indicators (Aguiar *et al*., 2011; Ventura *et al*., 2012; Oliveira *et al*., 2012).

Since the GC×GC system produces very narrow peaks (mostly narrower than 50ms, depending on the frequency of modulation) a Time of Flight Mass Spectrometry (TOF-MS) detector with a high acquisition rate (up to 500 full spectra per second) is required (Hoskovec *et al*., 2012). GCxGC with TOF-MS detection thus operates with high precision independent of concentration range (Hoskovec *et al*., 2012). The Comprehensive two dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC-TOFMS) is a recently developed analytical technique which offers a solution to the problem of co-elution and provides high sensitivity and selectivity (Dalluge *et al*., 2003; Dimandja *et al*., 2003; Mondello *et al*., 2008; Hoskovec *et al*., 2012). GC×GC-TOFMS technology has been shown to provide high-quality mass spectra with great sensitivity largely as a result of the enhanced resolution and zone compression obtained from the orthogonal separation (Gaquerel *et al*., 2009).

The GC×GC-TOFMS analysis of the rhizomes of *R. tomentosa* was done to ascertain the groups of secondary metabolites contained in the plant. Based on the results of this analysis, together with existing literature on the bioactivity of the secondary metabolites identified in this, the potential of compounds from this plant as a source for drug lead to help address the issue of antimicrobial resistance could be deduced. This is the first time GC×GC-TOFMS analysis is being done on any part of the *R. tomentosa* plant, in this case, the rhizomes.

5.2 MATERIALS AND METHODS

5.2.1 Sample Collection

Rhizomes of *R. tomentosa* were collected and prepared as described in Chapter 3.

5.2.2. Sample Preparation

Organic Solvent extracts: All reagents used in this analysis were of analytical grade and were purchased from Sigma Aldrich. The crude 100% ethyl acetate extract and 50:50 (v/v) methanol/chloroform extracts of *R. tomentosa* were analyzed. The extracts were first homogenized by weighing 0.5 g into two vials then 200 µL of cold methanol was added. The mixtures were thoroughly vortexed. The samples were then transferred to auto sampler vials and immediately analyzed.

Pressurized Hot Water Extract: All reagents used in this analysis were of analytical grade and were purchased from Sigma Aldrich. The PHW extract of *R. tomentosa* was prepared for analysis using the methods outlined by Politi *et al*., (2007) The extract was first homogenized by weighing 0.5 g of freeze-dried sample into a vial then adding 200 µL of cold methanol. The mixture was thoroughly vortexed. The sample was transferred to an auto sampler vial and immediately analyzed.

5.2.3 Instrumental Conditions

The sample was analyzed using a cryo jet Pegasus 4D GC×GC-TOFMS (Leco Corporation St. Joseph, MI, USA). Samples were run in a two-dimensional mode. Sample injection volume was 1 µL with helium as the carrier gas at a flow rate of 1 mL/min. The inlet temperature was set at 280 ˚C and a split mode injection type was used with a ratio of 10. The first dimension column was a Restek Rtx-5siLMS (30 m × 250 µm × 0.25 µm d.f.) and the temperature profile was 10 ˚C/min from 50 to 300 ˚C with a hold for 5 min at 300 ˚C. The second dimension column was Restek Rxi17siLMS (1 m × 250 µm × 0.25 µm d.f.) and the temperature profile was the same as the first dimension except for the temperature offset of +10 ˚C. The second dimension separation time was set to 4 seconds and controlled with an internal cryo-jet-modulator creating a hot/cold pulse. This instrument utilizes electron impact ionization (EI). The temperature of ion source was set at 250 ˚C. The detector voltage was set at 1564V and the filament bias at −70 V. The mass range collected was 40 to 550 m/z with an acquisition rate of 200 spectra/s (Ralston-Hooper *et al*., 2008). The data generated was analyzed using ChromaTOF® software.

5.3 RESULTS AND DISCUSSIONS

GC×GC TOF-MS analysis was done to determine the chemical make-up of the plant material and analyze the volatile components. The result showed prevalent presence of many bioactive chemicals of both known and unknown components as shown in the different chromatograms (figures 5.1 to 5.3). In Table 5.1 and 5.2 are some of the known volatile chemicals that were identified in the organic solvent and the pressurized hot water (PHW) extracts respectively, of *R. tomentosa* using the ChromaTOF® software and a NIST database. Table 5.3 and 5.4 shows some of the known chemical components identified in the organic solvent extracts and PHW extract respectively, of *R. tomentosa* and their known biological uses as reported in literature.

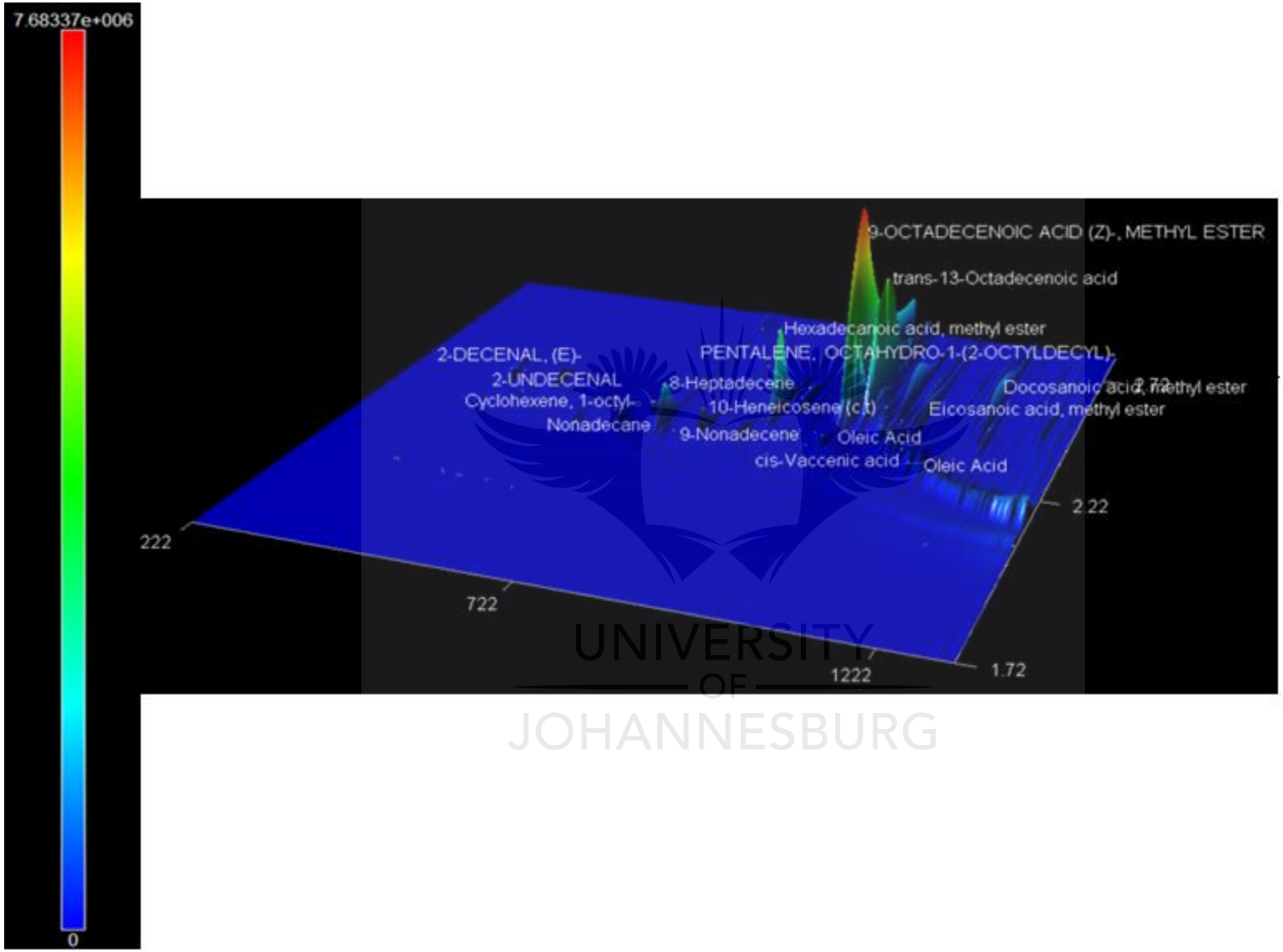

Figure 5.1: GC×GC-TOFMS Chromatogram showing most fatty acids identified in the rhizomes *of R. tomentosa*

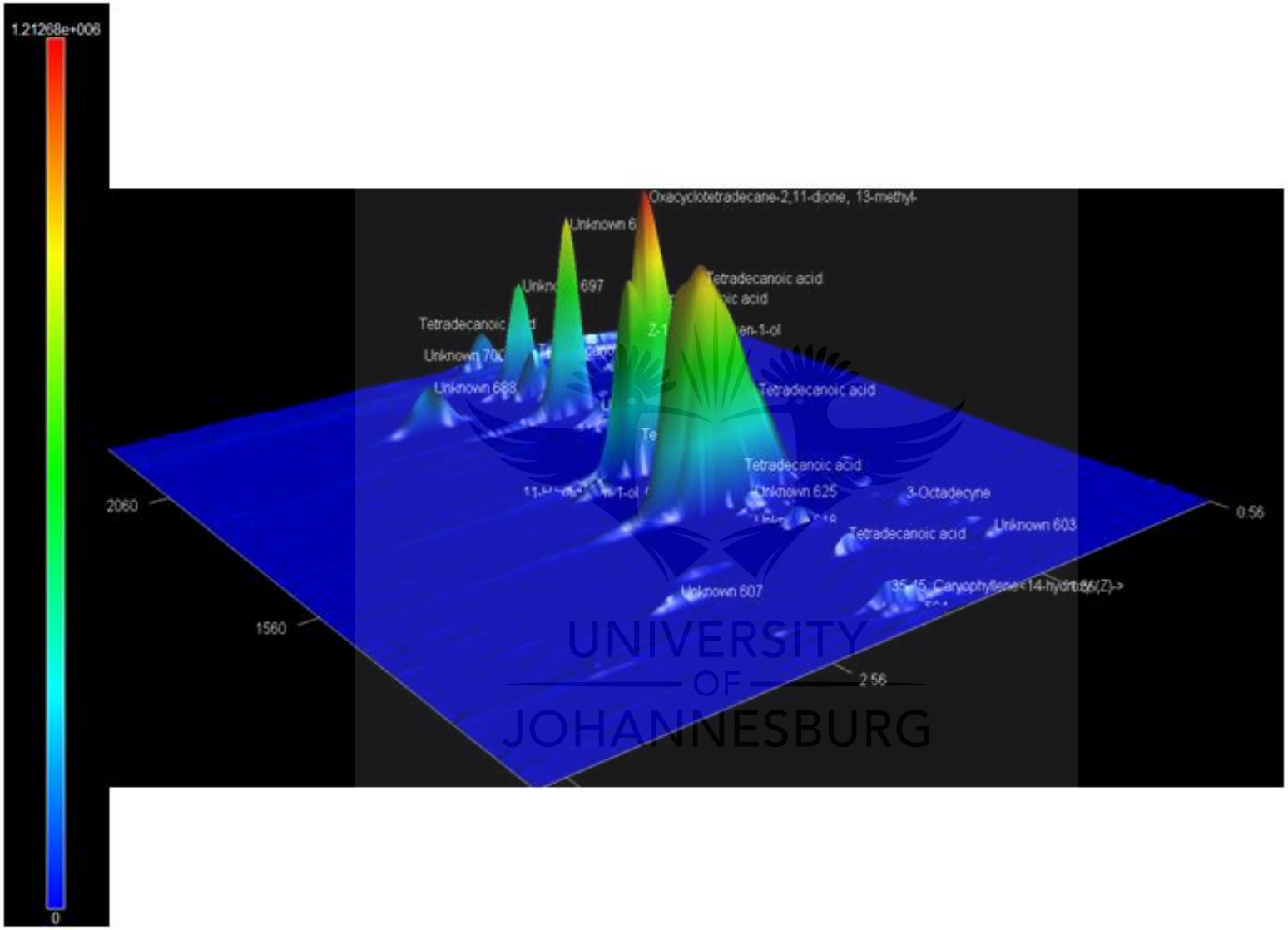

Figure 5.2: GC×GC-TOFMS chromatogram showing some fatty acid esters and unknown compounds identified in the rhizomes *of R. tomentosa*

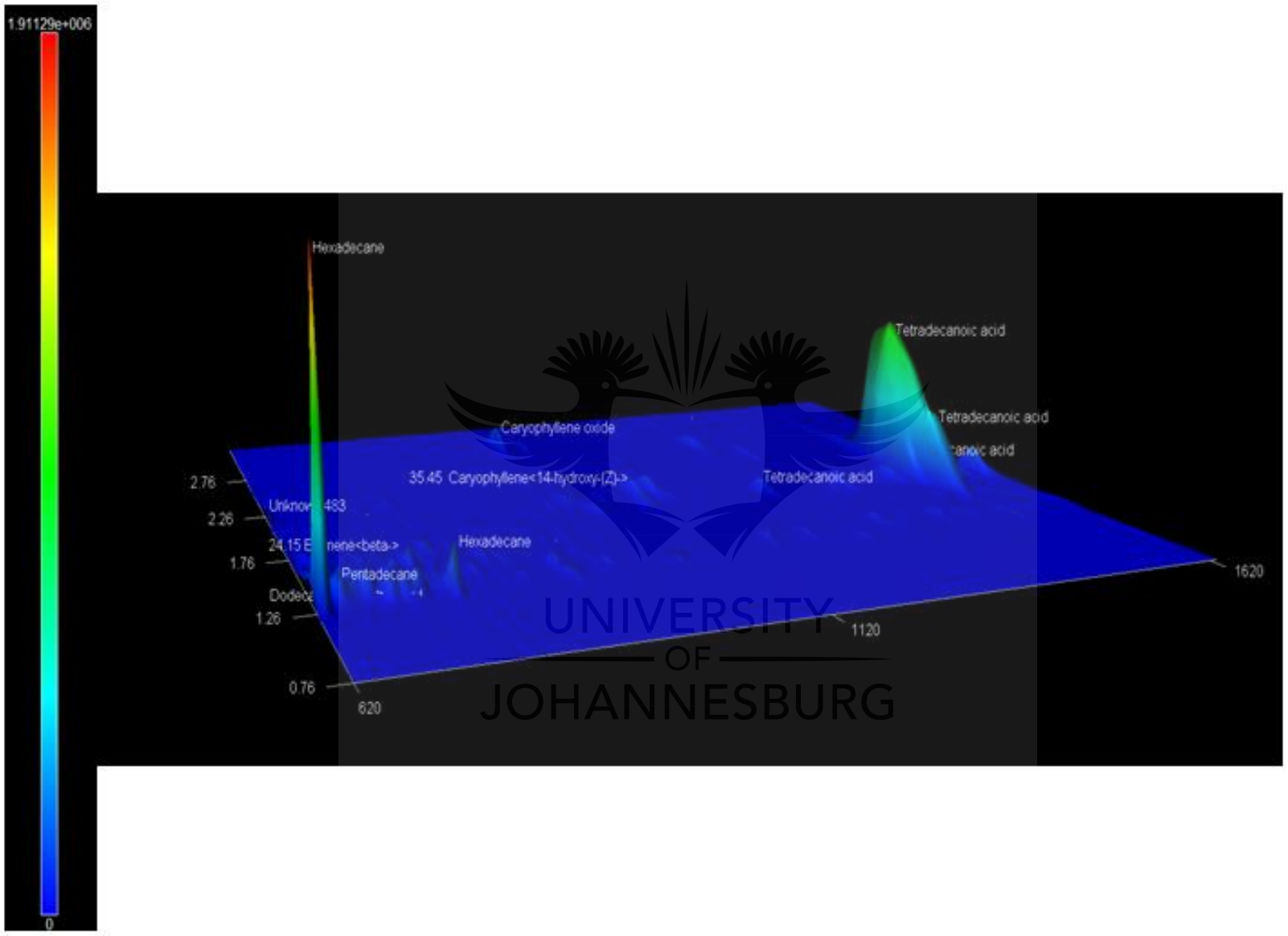

Figure 5.3: GC×GC-TOFMS chromatogram showing some of the alkanes and unknown compounds identified in the rhizomes *of R. tomentosa*

Table 5.1: Table showing the known compounds identified in the organic solvent extracts of *R. tomentosa* by GCxGC-TOFMS

Name	Exact Mass	Area %	Retention time (s)
Fatty acids			
Oleic Acid	282.256	27.296	1806, 2.110
n-Hexadecanoic acid	256.240	3.679	1845, 2.060
trans-13-Octadecenoic acid	282.256	1.213	999, 2.530
9-Octadecenoic acid (Z)-	282.256	0.380	1749, 2.060
Octadecanoic acid	284.272	0.264	2037, 2.090
cis-Vaccenic acid	282.256	0.465	2199, 2.110
Hexadecenoic acid, Z-11-	254.225	0.023	906, 2.770
trans-13-Octadecenoic acid	282.256	0.013	951, 2.580
9-Octadecenoic acid (Z)-	282.256	0.013	876, 2.830
(Z)-11-hexadecenoic acid	254.225	0.036	921, 2.630
Hexadecenoic acid, Z-11-	254.225	0.010	885, 2.460
9,12-Octadecadienoyl chloride, (Z,Z)-	298.206	0.010	1173, 2.590
cis-9-Octadecenoic acid	282.468	27.296	1050, 2.550
Eicosanoic acid	312.530	0.121	1140, 2.450
Docosanoic acid	340.334	0.238	1341, 0.440
Tetracosanoic acid	368.646	0.041	1809, 2.010
Tetradecanoic acid	228.376	0.448	1152, 2.500
Organic acids			
10-Undecenyl Chloride	202.112	8.754	1545, 2.050
1,3,5-Benzenetriol	126.032	0.030	756, 2.640
1,2,3-Benzenetriol	126.032	0.008	690, 2.320

Name	Exact Mass	Area %	Retention time (s)
Fatty acid esters			
Docosanoic acid, methyl ester	354.350	0.238	1188, 2.530
Octadecanoic acid, methyl ester	298.287	0.236	984, 2.420
Hexadecanoic acid, methyl ester	270.256	0.167	867, 2.390
Eicosanoic acid, methyl ester	326.319	0.003	1092, 2.440
9-Octadecenoic acid (Z)-, Methyl ester	296.272	1.790	969, 2.480
Octadecanoic acid, 2-propenyl ester	324.303	0.043	1257, 2.620
Tetracosanoic acid, Methyl ester	382.381	0.041	1281, 2.450
7-Hexadecenoic acid, methyl ester, (Z)-	268.240	0.045	855, 2.410
Nonanoic acid, Methyl ester	172.146	0.003	369, 2.330
Tridecanoic acid, methyl ester	228.209	0.002	741, 2.370
Dodecanoic acid, Methyl ester	214.193	0.001	600, 2.370
Undecanoic acid, Methyl ester	200.178	0.019	447, 2.330
Heptadecanoic acid, Methyl ester	284.272	0.004	927, 2.400
Heptadecanoic acid, 16-Methyl-, Methyl ester	298.287	0.004	981, 2.980
Hexadecanoic acid, Methyl ester	270.256	0.006	1236, 2.460
Alcohols			
Oleyl Alcohol	268.277	0.074	1200, 2.420
Z-10-Pentadecen-1-ol	226.230	0.066	2157, 1.990
9-Hexadecen-1-ol, (Z)-	240.245	0.006	1104, 2.390
6,8-Dodecadien-1-ol	182.167	0.013	636, 2.300
Phenolic acid			
2,4,6-Trihydroxybenzoic acid	170.022	0.029	810, 2.480

Name	Exact Mass	Area %	Retention time (s)
Phenol, 5-Methyl-2-(1-Methylethyl)-	150.105	0.005	435, 2.480
Amino acids			
D-Asparagine	132.054	0.025	1902, 2.200
L-Arginine	174.112	0.003	1218, 2.040
Uridine	244.203	0.033	1602, 2.310
Glycyl-L-valine	174.198	0.037	660, 2.220
Alkenes			
9-Nonadecene	266.297	0.025	840, 2.300
(ñ)-2-Hydroxyoctanoic acid	160.110	0.012	2052, 2.200
7-Hexadecene, (Z)-	224.250	0.045	642, 2.280
1-Nonadecene	266.297	0.034	1542, 2.010
10-Heneicosene	294.329	0.029	1605, 2.010
8-Heptadecene	238.266	0.073	1434, 2.010
1-Heptadecene	238.266	0.062	1551, 2.020
Alkanes			
Nonadecane	268.313	0.009	852, 2.290
Tetradecane	198.235	0.008	582, 2.240
Triacontane	422.485	1.789	969, 2.300
Eicosane	282.329	0.014	1077, 2.360

Table 5.2: Table showing the known compounds identified in *R. tomentosa* PHW extract by GCxGC TOF-MS

Name	Unique Mass	Area %	R.T. (s)
Reducing sugars			
Glyceraldehyde	60	8.1425	239.5
Heterocyclic aldehyde			
Furfural	95	3.1459	244.5
Alcohols			
2-Furanmethanol	98	3.8938	256.9
2,3-Butanediol	43	5.7436	215.4
Carbohydrate			
Dihydroxyacetone	72	6.0838	288.3
Organic acids			
Dimethyl phthalate	163	1.3604	768.7
Diisooctyl phthalate	149	2.4827	1411.6
Heptanoic acid, 4-methoxyphenyl ester	124	0.31584	1010.8
Phenolic acid			
Phenol, 2,4-bis(1,1-dimethylethyl)-	191	1.6065	810.4
Dodecyl acrylate	55	1.7699	1293.3
3,4,5-Trihydroxybenzoic acid	170	0.98982	1109.4
Alkane			
Heptacosane	41	0.65463	1393
Octadecane, 6-methyl-	57	1.3718	1483.9

Name	Unique Mass	Area %	R.T. (s)
Heptadecane, 2,3-dimethyl-	55	0.60336	1619.3
2-Bromotetradecane	57	0.21402	1344.8
Heptadecane, 2,6-dimethyl-	57	0.49151	1392.9
Fatty acid amide			
9-Octadecenamide, (Z)-	59	0.53626	1524
Triterpene			
Squalene	69	0.98221	1535.4
Alkyl aldehyde			
Hexanal	56	0.14984	221.5

Tables 5.3 and 5.4 contains a list of the volatile chemicals present at highest concentrations in the analyzed crude solvent extracts of *R. tomentosa*. Fatty acids and amino acids are present on this list, and they are usually the building blocks for cell membranes and proteins (enzymes). Yet many of these compounds have also been found to have antimicrobial effects, so they may contribute significantly to the antibacterial properties of the plant as seen in chapter four.

Table 5.3: Table showing the compounds identified in *R. tomentosa* crude solvent extracts by GCxGC-TOFMS at highest concentrations and any known bioactivity of the compounds.

Compound	**Area %**	**Biological activity**	**Molecular formula**	**Nature of compound**
cis-9-Octadecenoic acid	27.296	Antioxidant , anti-inflammatory, anti-tumor, anti-plasmodial activity, anti-spasmodial and antimicrobial activities (Visioli *et al*., 2002; Llor *et al*., 2003; Waterman & Lockwood, 2007).	$C_{18}H_{34}O_2$	Fatty acid
9-Octadecynoic acid	0.004	Antimicrobial, cytotoxicity, anti-asthmatics, antidepressants and antimigraine activities (Li *et al*., 2008).	$C_{18}H_{32}O_2$	Fatty acid
Docosanoic acid	0.238	Antipruritic, antioxidant and anesthetic activities (SIDS Initial Assessment Report, 2001).	$C_{22}H_{44}O_2$	Fatty acid
Tetradecanoic acid	0.448	Antipruritic, antifungal, anti-infective and antioxidant activities (Sutha *et al*., 2011; Jadhav *et al*., 2014).	$C_{14}H_{28}O_2$	Fatty acid
Tetracosanoic acid	0.041	Antibacterial activity (Hussain *et al*., 2012).	$C_{24}H_{48}O_2$	Fatty acid
Eicosanoic acid	0.121	Anti-abortifacient, antioxidant, antibacterial, analgesic and antipyretic activities (Brash, 2001; Hsouna *et al*., 2011).	$C_{20}H_{40}O_2$	Fatty acid

Compound	Area %	Biological activity	Molecular formula	Nature of compound
Tetracosanoic acid, Methyl ester	0.041	Antioxidant activity (Hosseinihashemi *et al*. ,2015).	$C_{25}H_{50}O_2$	Fatty acid ester
Docosanoic acid, Methyl ester	0.047	Anti-infective, anti-ulcer and antibacterial (SIDS Initial Assessment Report, 2001).	$C_{23}H_{46}O_2$	Fatty acid ester
Decanoic acid, 2-propenyl ester	0.013	Analgesic, antipyretic, antibacterial, antifungal and anti-inflammatory activities (Asghari *et al*., 2012).	$C_{13}H_{24}O_2$	Fatty acid ester
Heptadecanoic acid, 16-methyl-, Methyl ester	0.004	Anti-acne agent and anti-infective agent; antibacterial, anti-cancer (skin cancer), antipyretic and anti-inflammatory activities (Suseem & Saral, 2013; Elaiyaraja & Chandramohan, 2015).	$C_{18}H_{36}O_2$	Fatty acid ester
Hexadecanoic acid, methyl ester	0.016	Anti-spasmodial, antioxidants, smooth muscle relaxant and anti-abortifacient activities (Cai *et al*., 2005; Lin *et al*., 2009; Wang *et al*., 2010; Ajoku *et al*., 2015).	$C_{17}H_{34}O_2$	Fatty acid ester
Octadecanoic acid, methyl ester	0.017	Antipruritic, anti-inflammatory, antifungal antipsychotic, anti-abortifacient and antibacterial activities (Abou-Elela *et al*., 2009).	$C_{19}H_{34}O_2$	Fatty acid ester
D-Asparagine	0.025	Immunostimulant, antibacterial, anti-infective, analgesic and antiviral activities (Karmali *et al*., 1986)	$C_4H_8N_2O_3$	Amino acid
L-Arginine	0.002	Anti-inflammatory, Immunostimulant, and antihypertensive activities (Angeli *et al*., 2007; Gad, 2010).	$C_6H_{14}N_4O_2$	Amino acid

Compound	Area %	Biological activity	Molecular formula	Nature of compound
Glycyl-L-valine	0.037	Analgesic, antipyretic, anti-inflammatory and antioxidant activities (Buschmann *et al*., 2003).	$C_7H_{14}N_2O_3$	Amino acid
Uridine	0.033	Neuroprotective activity, pyrimidine metabolism, antidepressants and anti-epileptic actions (Ulus *et al*., 2006; Wurtman *et al*., 2010; Dobolyi *et al*., 2011; Kondo *et al*., 2011).	$C_9H_{12}N_2O_6$	Amino acid

Table 5.4: Table showing few of the compounds identified in *R. tomentosa* PHW extract by GCxGC-TOFMS at highest concentrations and any known bioactivity of the compounds.

Compound	Area %	Biological activity	Molecular formula	Nature of compound
Furfural	3.1459	Anti-fungal activity (Gyawali & Kim, 2012)	$C_5H_4O_2$	Heterocyclic aldehyde
Squalene	0.98221	Antioxidant, anti-tumor and anti-cancer activities (Huang *et al*., 2009)	$C_{30}H_{50}$	Triterpene
9-Octadecenamide, (Z)-	0.53626	Anti-inflammatory and antibacterial activities (Hadi *et al*., 2016)	$C_{15}H_{35}NO$	Fatty acid amide
Hexanal	0.14984	Anti-fungal activity (Gyawali & Kim, 2012)	$C_6H_{12}O$	Alkyl aldehyde
Dihydroxyacetone	6.0838	Antioxidant (Windsor *et al*., 2012)	$C_3H_6O_3$	Carbohydrate

Most fatty acids (*cis*-9-Octadecenoic acid, oleic acid, tetracosanoic acid, etc.) and fatty acid esters (Docosanoic acid, methyl ester, Octadecanoic acid, methyl ester, etc.) and the amino acid: D-Asparagine have been reported to have antibacterial activity (Table 5.3) and from chapter four of this work, we could see that this plant had very interesting antibacterial activity even at low concentrations. This activity could have resulted from the presence of these fatty acids and fatty acid esters in the rhizomes of *R. tomentosa. Mycobacterium smegmatis* have been reported to be susceptible to oleic acid (Salem *et al*., 2014), hence, the inhibitory activity of the rhizomes of *R. tomentosa* on *M. smegmatis* (Table 4.3) could be attributed to the presence of the oleic acid.

The leaf and stem of *R. tomentosa* have been reported to possess anti-inflammatory activity (Lin *et al*., 1999). Although a biological test to ascertain the anti-inflammatory property of the rhizomes of *R. tomentosa* was not carried out, most of the compounds identified form the GC×GC-TOFMS analysis of the plant (fatty acids: *cis*-9-Octadecenoic acid, 9-Octadecenamide, (Z)- ; fatty acid ester: decanoic acid, 2-propenyl ester, heptadecanoic acid, 16-methyl-, methyl ester; amino acids: L-arginine, glycyl-L-valine) are known to have anti-inflammatory activity (Table 5.3) which suggests that the rhizomes of *R. tomentosa* might equally have anti-inflammatory activity like the stem and leaf.

The GCxGC TOF-MS results revealed several biologically active chemicals which might contribute to the plant's antibacterial activity and its uses in traditional medicine, however, the relationship between these phytoconstituents, the biological activities and ethnobotanical uses of *R. tomentosa* have not been established. Garlic acid for example (trihydroxybenzoic acid) contained in *R. tridentate* (Steenkamp *et al*., 2013) was also identified in *R. tomentosa* using GC×GC-TOFMS analysis. There is a possibility that other chemicals contained in *R. tomentosa* may still be found in *R. tridentate* and possibly other *Rhoicissus* species hence they could possibly be used to treat similar diseases and symptoms. This validates the studies of Corrigen *et al*., (2011) where they reported same medicinal uses for *R. tomentosa* and *R. digitate* and the studies of (Nqolo, 2008) where similar medicinal uses were reported for all species of the *Rhoicissus* genus.

5.4 CONCLUSION

GCxGC TOF-MS analysis gave an insight on the general chemical components of the plant. The results of this study give credence to the traditional use of this plant to treat diseases from the stand point of traditional medicine and gives insights into possible therapies or benefits the plant can have on other ailments not yet considered by traditional medicine practitioners or traditional/indigenous knowledge systems. 2-Dimensional gas chromatography coupled to time of flight mass spectra (GCxGC-TOFMS) analysis of the crude extracts (organic solvent extracts and PHW extracts) of *R. tomentosa* displayed the presence of over 100 known bioactive compounds. These bioactive constituents belong to different classes of compounds and have been previously reported to have anti-abortifacient, analgesic, anti-inflammatory, antifungal and antibacterial properties. In general, this study gave some level of validity to the ethnobotanical uses of *R. tomentosa* with consideration to the known biological activities of the compounds identified therein. In the light of the increasing problem of antibiotic resistance to existing antibiotics, the bioactive compounds identified in this plant can be isolated using column chromatography and tested for antimicrobial properties and other biological activities in anticipation for a possible drug lead for microbial infections and fertility treatment.

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CHAPTER SIX

6.0 ELEMENTAL ANALYSIS OF THE RHIZOMES OF *Rhoicissus tomentosa* USING INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY (ICP-OES)

ABSTRACT

The presence of elements in medicinal plants is an important aspect of ethnopharmacological research which in most cases is overlooked. Elements in medicinal plants can either have detrimental or beneficial effects on the health of the individuals consuming the plant. The elemental analysis of *R. tomentosa* rhizomes was done using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) and the concentration of each element was compared to the World Health Organization standards for elemental limits in medicinal plant. The result revealed the presence of important elements such as Calcium, Potassium, Sodium, and Iron which are known to have beneficial health effects, amongst others which have no beneficial health effects. Most the elements identified in the plant were below the WHO permissible limits for elements in medicinal plants but excessive accumulation of some of these elements can be detrimental to health. This plant is mostly only consumed for medicinal purposes, hence, the accumulation of these might be rare as the plant is not consumed frequently.

Keywords: ICP-OES, medicinal plants, metal analysis, *R. tomentosa*

6.1 INTRODUCTION

According to the World Health Organization (WHO), the use of traditional herbal medicine has become popular not only in the developing countries, but also in the developed regions, either as a complementary or alternative way to treat and to avert ailments. The pharmacological properties of medicinal plants have been ascribed to the presence of some active constituents which are accountable for important physiological activities in living organisms (Özcan 2004; Yamashita *et al*., 2005; Ebrahim *et al*., 2012). In recent years, research on the role of trace elements and minerals in various metabolic processes and their impact on human health has become an area of concern and high priority in environmental research and protection. The functional role of trace

elements is described in terms of their nutritionally essential role or their potential toxicity (Rahmatollah & Mahbobeh, 2010; Ebrahim *et al*., 2012).

One very important factor to consider with regards the health effects of trace elements is their slow accumulation in tissues, even at low quantities. Hence, acute effects are rarely reported, however chronic exposure can lead to the build-up of higher concentrations and subsequently a disease. Trace element toxicity can manifest with non-specific symptoms and, often, epidemiology is the only possible method to determine their role (Prasad, 2008; Ebrahim *et al*., 2012).

The most extensively used techniques for elemental analysis are Electrothermal Atomic Absorption Spectrometry (ETAAS) (Chuang *et al*., 1999), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Farias *et al*., 2002; Wuilloud & Gonzalez, 2001) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Matsuura *et al*., 2001). These methods are rapid and sensitive for the determination of trace amounts of metals in different matrices (Gomez *et al*., 2007).

The present study was conducted to determine the elemental composition of the rhizomes of *R. tomentosa*, which may assist in future researches to focus on the health benefits of these elements and how the added to the pharmacological properties of the plant and/or the toxicology of these elements and the negative effects they may have on humans and understand the need to avoid elemental contamination of soil via mining. The results are compared to the WHO permissible limits for elemental concentrations in medicinal plants according to literature and discussed with respect to the known actions of these trace elements. This study also helps us understand that although some of these elements have beneficial effects, others do not and even though found in low concentration may with time become toxic when accumulated in the body. The Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was used for the elemental analysis carried out on *R. tomentosa* rhizomes.

6.2 MATERIALS AND METHOD

The total metal content was analyzed using inductive coupled plasma optical emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro, Germany). All measurements of samples were done in triplicate and only the mean of the measured values are reported in this study.

6.3.1 Sample Preparation

Sample preparation was done according to the method of Marin *et al*, (2011). The rhizomes of *R. tomentosa* were cut into smaller pieces and dried at 40˚C in an oven for five days. The dried plant material was ground and sieved through a 100 microns mesh. The plant sample was stored in a closed plastics bags until analysis. The certified reference material was analyzed in the same experimental conditions used for sample analyses in order to evaluate the accuracy of the method.

6.3.2 Instrumental Conditions

A multi-elemental standard solution of 1000 mgL^{-1} containing all analyzed elements (Calcium, Cadmium, Chromium, Copper, Iron, Gallium, Mercury, Potassium, Manganese, Sodium, Nickel, Lead, Rhodium, Strontium, Zinc, Indium) supplied by Merck (Darmstadt, Germany) was used for calibration. HNO_3 65% and H_2O_2 30% from Merck (Darmstadt, Germany) analytical grade were used for sample digestion. Ultrapure water obtained by a Milli Q system (Millipore, France) was used for dilutions. A vegetable certified reference material IAEA-359 Cabbage (Vienna, Austria) was used for the quality control of metals determination. Determinations were carried out using a Perkin Elmer Model Optima 5300 DV spectrometer (Perkin Elmer, USA) ICP-OES equipped with an Ultrasonic Nebulizer CETAC U-6000AT+ (CETAC, USA) and an auto sampler AS 93-plus. Argon (purity higher than 99.995%) was used to sustain plasma and, as carrier gas. A closed-vessel microwave system Berghof MWS-3+ with temperature control mode, (Berghof, Germany) was used for wet digestion. All Teflon digestion vessels were previously cleaned in a bath of 10% (v/v) nitric solution for 48 h to avoid cross- contamination.

The plant sample (0.5 g) was digested in 10 mL HNO_3 and analyzed under the following conditions: Temperature, 200˚C; Power, 1800W; Ramp Time, 20 minutes; Hold Time, 10 minutes and Pressure, 800psi.

6.3 RESULTS AND DISCUSSIONS

Metals such as calcium and sodium respectively occurring at concentrations of 27.187 mg/kg and 65.264 mg/kg were detected in high amounts in the rhizomes of *R. tomentosa* and these are essential nutrients to the body. Gallium, rhodium and indium were not detected in the plant. The other metals like lead, iron, cadmium, etc. were detected in low amounts. Table 6.1 shows metal concentrations in *Rhoicissus tomentosa*.

The pharmacological modes of action of medicinal plants are believed to be multifactorial (Ebrahim *et al*., 2012). As mentioned before, trace elements play an important role as catalysts or parts of prosthetic groups for enzymes, and, consequently, insufficient supply may lead to element-specific deficiency symptoms (Ebrahim *et al*., 2012). Although trace elements confer some bioactive properties on medicinal plants, prolonged exposure to these elements can become toxic and pose health risks due to the capability of heavy metals to bioaccumulate and disrupt the functions of vital organs in the human body such as brain, kidneys and liver (Ray & Ray, 2009; Uddin *et al*., 2013).

Table 6.1: Metal concentrations in *Rhoicissus tomentosa*

Elements	**Wavelength (nm)**	**Mean concentration ±Standard deviation (mg/kg)**	**WHO Permissible Limits for medicinal plants (mg/kg)**
Calcium (Ca)	317.933	27.187 ±0.207	NI
Cadmium (Cd)	226.502	0.126 ±0.004	0.2-0.3 (Khan *et al*., 2013; Shah *et al*., 2013; Nazir *et al*., 2015)
Chromium (Cr)	205.618	6.662 ±0.015	1.50 (Shah *et al*., 2013; Nazir *et al*., 2015).
Copper (Cu)	224.700	0.022 ±0.003	10 (Shah *et al*., 2013; Nazir *et al*., 2015)
Iron (Fe)	239.562	5.756 ±0.03	20 (Shah *et al*., 2013; Nazir *et al*., 2015)
Gallium (Ga)	417.206	ND	NI
Mercury (Hg)	194.227	0.282 ±0.015	1.0 (Sarma *et al*., 2011)
Potassium (K)	766.491	10.365 ±1.493	NI
Manganese (Mn)	257.611	0.161 ±0.00	200 (Shah *et al*., 2013)
Sodium (Na)	330.298	65.264 ±0.229	NI
Nickel (Ni)	221.648	5.634 ±0.002	1.5 (Shah *et al*., 2013).
Lead (Pb)	168.215	0.757 ±0.026	10 (Shah *et al*., 2013).
Rhodium (Rh)	343.489	ND	NI
Strontium (Sr)	421.552	3.774 ±0.001	NI
Zinc (Zn)	206.200	0.472 ±0.01	50 (Shah *et al*., 2013; Nazir *et al*., 2015)
Indium (I)	230.606	ND	NI

ND= Not detected, NI= No information

Calcium (Ca) was present at a concentration of 27.187 mg/kg, although no information was found on the WHO permissible limit of Ca in medicinal plants, Ca is known to be an essential element responsible for building bones, teeth and muscles system and helps in heart functions. The high content of Ca in *R. tomentosa* might not be a risk to humans. Cadmium (Cd) which is known to be toxic was detected at a concentration of 0.126 mg/kg which is below the WHO permissible limit of 0.2-0.3mg/kg. Chromium (Cr) is known to have a significant effect in controlling sugar level in the blood of human beings but is also known to sometimes cause skin irritation when in high amounts, was detected at 6.662 mg/kg which is exceedingly high above the WHO limit of 1.50mg/kg hence, calls for caution when using this plant as obtained from the Faraday Muthi market for medicinal purposes. The reason for this is that the same medicinal plant growing in different geographical locations may contain different metals in different concentrations. As such, it is probable that this plant was harvested from a location with high Cr.

Copper (Cu) is a trace element and was detected in *R. tomentosa* at a concentration of 0.022 mg/kg which is below its WHO limit of 10mg/kg. Cu is needed in the body to facilitate iron uptake and Cu deficiency can lead to impaired growth. Iron (Fe) is necessary for growth, development, normal cellular functioning, and synthesis of some hormones and connective tissue in the body and it was detected at 5.756 ±0.03mg/kg and the WHO limit is 20mg/kg. Fe in *R. tomentosa* is not necessarily a health risk but can be an added advantage to the biological activities of the plant. Mercury (Hg) was detected at 0.282 mg/kg and is below the WHO limit of 1.0 mg/kg. Hg has no known health benefit but can be toxic if exposed to for a long period.

Potassium (K) was detected at 10.365 mg/kg. No information was found on the WHO permissible limit for K, but K is an essential element known to help manage blood pressure, keep heart functioning properly, and enhance muscle control, growth and health of the body cells. Manganese (Mn) was detected at 0.161 mg/kg and is very well below the WHO limit of 200mg/kg. Mn is an essential element that functions as a cofactor of enzymes involved in energy metabolism and protein synthesis. Sodium (Na) is an essential element necessary for blood regulation, regulation of cellular activity and nervous system function. Na was detected at a high concentration of 65.264 mg/kg and although no information was found on the WHO permissible

limit, its high content may not be a health risk considering that the plant is only consumed when necessary and for medicinal purposes.

Nickel (Ni) was detected at 5.634 mg/kg which is above the WHO limit of 1.5 mg/kg. Biochemical functions of Ni have not been demonstrated in humans and higher animals. Humans exposed to highly nickel-polluted environments are at higher risk to develop lung cancer. Lead (Pb) was detected at 0.757 mg/kg and is well below the WHO permissible limit of 10mg/kg. Exposure to high levels of Pb can cause severe damage to the brain and kidneys in adults or children and ultimately cause death. It is therefore encouraging that the Pb content of *R. tomentosa* was found to be very low. Strontium (Sr) was detected at 3.774 mg/kg and no information was found on the WHO permissible limit. Sr is sometimes absorbed by the human body as if it were calcium and it has been shown to inhibit sensory irritation when applied topically to the skin. Zinc (Zn) was detected at 0.472 mg/kg which is well below the WHO permissible limit of 50 mg/kg. Zn is an essential trace element and plays an important role in various cell processes, normal growth, brain development, behavioral response, bone formation and wound healing.

Although some of these elements do not have any known health benefits e.g. mercury, some of them have important health implications e.g. iron, calcium which could be of positive impact of humans consuming the plant.

In dealing with the elemental contents of medicinal plants, it is necessary to know that the geographical locations where the plants were originally harvested might have had impacts on the levels of metals detected therein. Plants can accumulate heavy metals from contaminated soil and humans are exposed to them through consumption. That is to say, that elemental contents of plants differ from location to location. Plants harvested from areas close to mines will tend to have high metal contents. Thus, the metal content of *R. tomentosa* specie would differ depending on the area where it was harvested from. It is, therefore, important to consider the geographical area of the plant before harvesting for medicinal use to reduce the risk of metal toxicity.

6.4 CONCLUSION

Most metals identified in *R. tomentosa* are known to have beneficial bioactivities. The metals (except for sodium and calcium which happen to be essential) were identified in low concentrations which were not beyond their permissible limits except for chromium, while no conclusion could be drawn for strontium as no permissible limits can be found in literature and many other authoritative sources. As such this may be an indication that consumption of this plant as obtained from its geographical location is not toxic to human health; although prolonged exposure to these metals might become harmful. Also, two known toxic metals rhodium and indium were not detected in the plant. This certifies that the consumption of *R. tomentosa* rhizomes for health benefits will have little or no side effects as there are no toxic metals in the plant and all the other metals identified therein were way below the permissible limits. If there are any side effects associated from the use of this plant for medicinal purposes, it might come from prolonged use of the plant considering that prolonged exposure to metals can over time become toxic, even if the metals are in small amounts. Also, considering that this plant was originally harvested from the wild in Kwa-Zulu Natal, South Africa, it could be concluded that the soil in the area has little metal contamination based on the results from this study.

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CHAPTER SEVEN

7.0 GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK.

The interest in phytochemicals from medicinal plants based on a wide range of scientific reports that link them to various protective health benefits have grown tremendously in recent years. *R. tomentosa*, a plant indigenous to Southern Africa is mostly used to treat female fertility ailments. It has been reported that most modern-day drugs have their active ingredients from plant sources, yet quite many medicinal plants are yet to be fully explored for their pharmacological benefits.

In this study, the results of phytochemical screening showed the presence of very important classes of phytochemicals with biological properties that support the use of the plant in traditional medicine (e.g. flavonoids which are phytoestrogens help enhance fertility in female mammals) and other phytochemicals which have biological properties not associated with the major traditional medicinal uses of *R. tomentosa* (e.g. tannins, alkaloids). The results of the GC×GC-TOFMS revealed the presence of numerous unknown compounds (which could not be identified from the NIST database used in the study) and several other known bioactive compounds which constituted mainly of fatty acids and their esters, amino acids and phenolic acids. Most of these compounds are also known to have biological properties which are not associated with the traditional medicinal uses of the plant. From these phytochemical studies, we identified compounds with known antimicrobial properties against some pathogenic bacteria. The results of the screening for antibacterial activity showed that the plant had inhibitory activity against some bacteria at very low concentrations of 0.063 mg/mL for *S. aureus* and *M. smegmatis*, 0.125 mg/mL for *B. subtilis* and 0.500 mg/mL for *B. cereus*. These recorded inhibitory activities at low concentrations indicates that the plant could be a potential source of drug leads. These studies confirm the first two hypotheses of this research.

Considering that the traditional healers administer this plant to their patients in its raw form (undifferentiated crude water constituents), the elements identified in the plant were within the limits mapped out by WHO for concentration of elements in medicinal plants except for chromium and strontium (no information is presently available on the limit). In considering the

traditional use of this plant: it is given to pregnant women to assist in safe delivery, the presence of iron (Fe) which is very important for pregnant women as it's deficiency can lead to anemia was considered noteworthy. Calcium (Ca) which helps to build bones and strong teeth was also identified, among other beneficial elements e.g. sodium (Na). Elements like mecury (Hg) and lead (Pb) which are harmful to human health were also identified but in very low concentrations which were below their permissible limits. These findings confirm the third hypothesis of this study.

In conclusion, based on the results of this study (on the rhizomes of *R. tomentosa*), it is apparent that the plant has interesting antimicrobial activities. This shows that this plant can be used to treat other bacterial infections besides being used for fertility treatment. The phytochemical screening and analysis revealed the presence of several bioactive compounds. Based on these, this plant shows to be a promising source of many potential pharmaceutically based phytochemicals in the area of drug lead research. The metal analysis showed the presence of several elements.

Most of the metals identified are known to have beneficial effects to humans although they can be toxic if exposed to for a long period. Except for strontium and chromium, the metals that were identified in *R. tomentosa* were all within the permissible limits. As discussed in chapter 6, the geographical area where a plant is harvested plays a major role in the metal content of the plant therefore, *R. tomentosa* plants will contain different elements at different concentrations depending on where they are grown/harvested. It is, thus, necessary to consider the industrial (e.g. mining) or agricultural (use of fertilizers) activities of a particular geographical region before harvesting plants for medicinal uses.

Based on the findings from this study, it is, therefore, recommended that further work be done on the rhizomes of *R. tomentosa* to isolate and identify the bioactive components of the plant in pure form. The GC×GC-TOFMS analysis of the plant revealed the presence of many unknown compounds. This is an indication that there might be some novel chemical compounds which still need to be identified, isolated and their structure elucidated and thus add to the wealth of scientific knowledge. Cytotoxicity tests should be carried out on compounds identified from the plant to determine their mode of action and concentrations at which they are most effective. It is

also recommended that caution is exercised with respect to heavy metals present in the soil when either planting or harvesting medicinal plants.

APPENDICES

CHAPTER 4

Disc diffusion plates used for antibacterial susceptibility test (Tables 4.1 and 4.2)

Figure 1

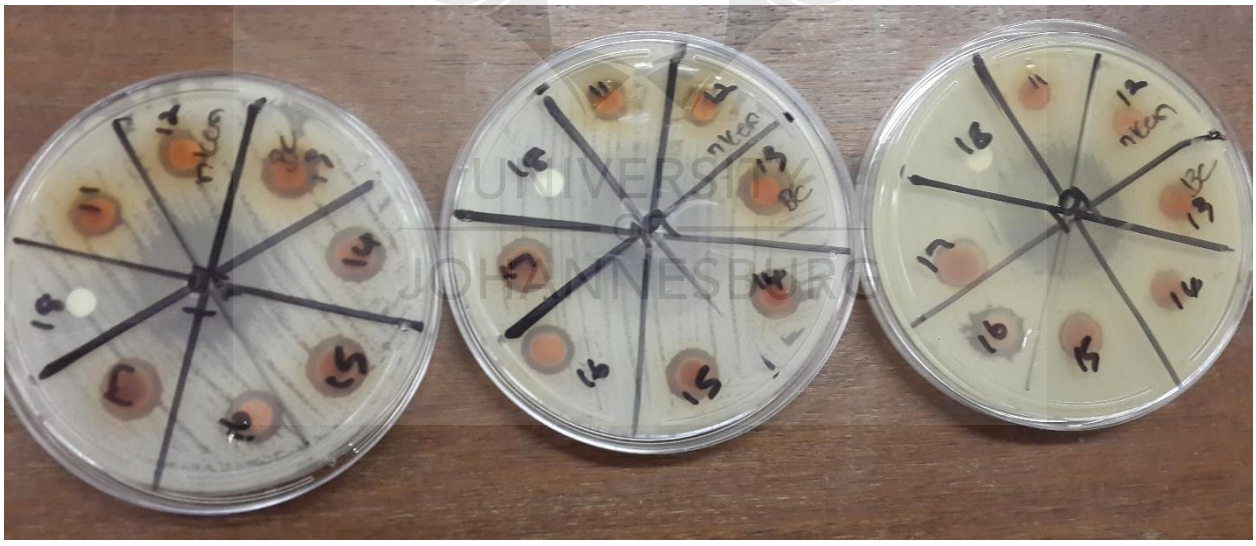


Figure 2

Figures 1-2: Disc diffusion plants showing zones of inhibition for *B. cereus*

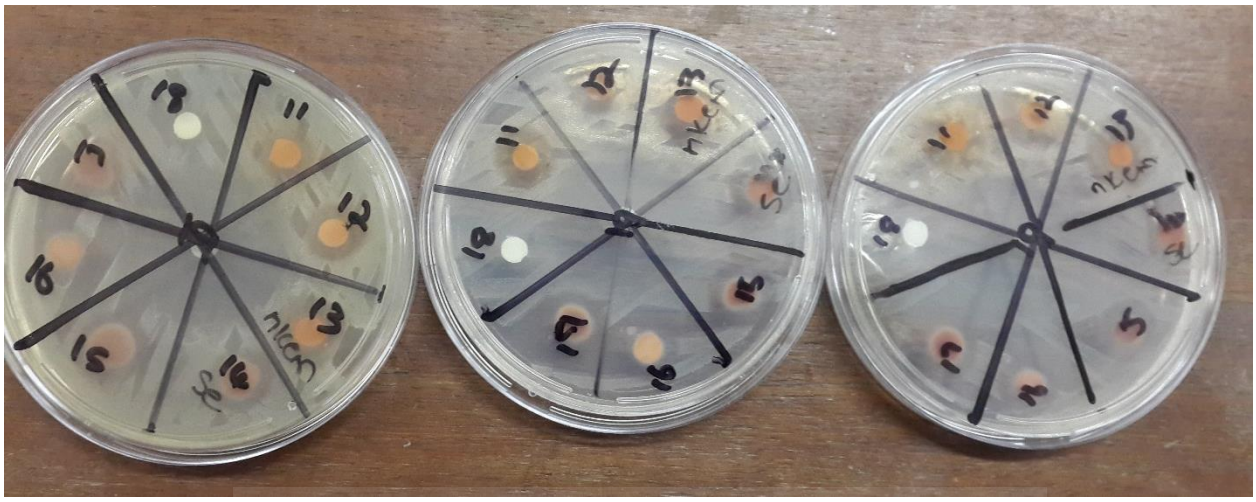


Figure 3: Disc diffusion plants showing zones of inhibition for *S. epidermidis*

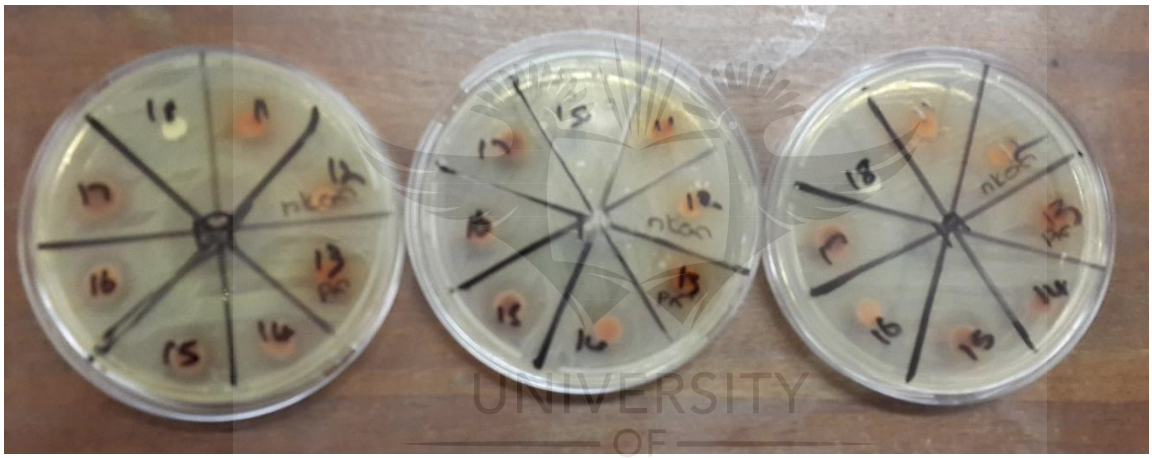


Figure 4: Disc diffusion plants showing zones of inhibition for *P. mirabilis*

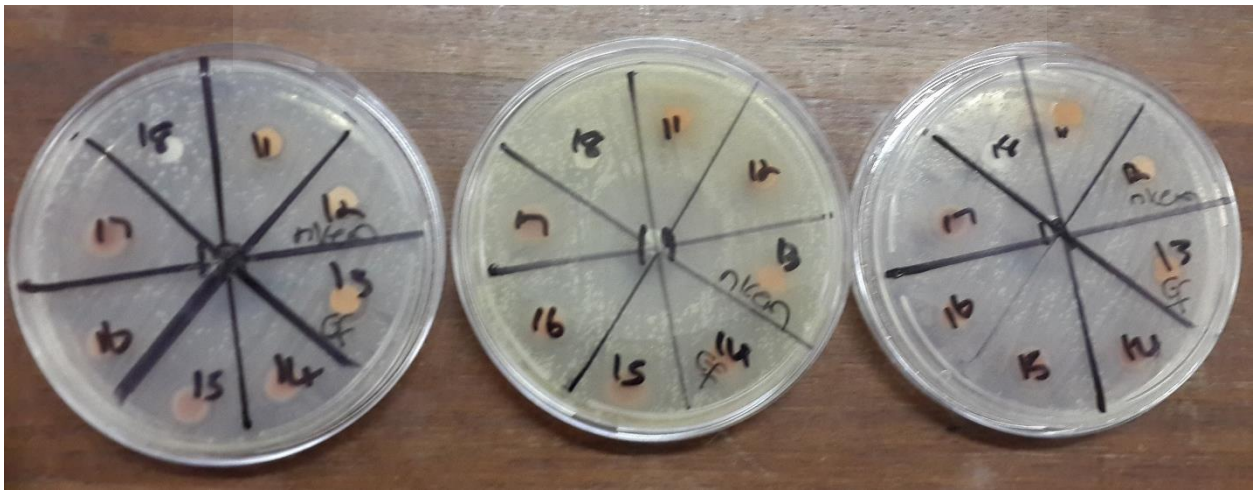


Figure 5: Disc diffusion plants showing zones of inhibition for *E. faecalis*

Figure 6: Disc diffusion plants showing zones of inhibition for *K. pneumonia*

Figure 7

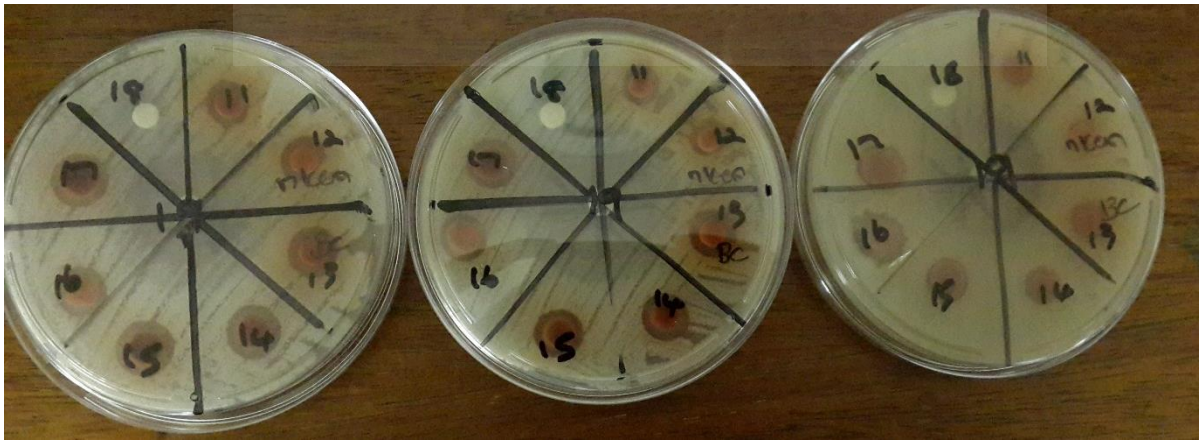


Figure 8

Figures 7-8: Disc diffusion plants showing zones of inhibition for *B. subtilis*

96 well microdilution plates used for antibacterial susceptibility test (Table 4.3)

Figure 9: Micro titer plate showing inhibition for *S. aureus, B. subtilis and P. mirabilis*

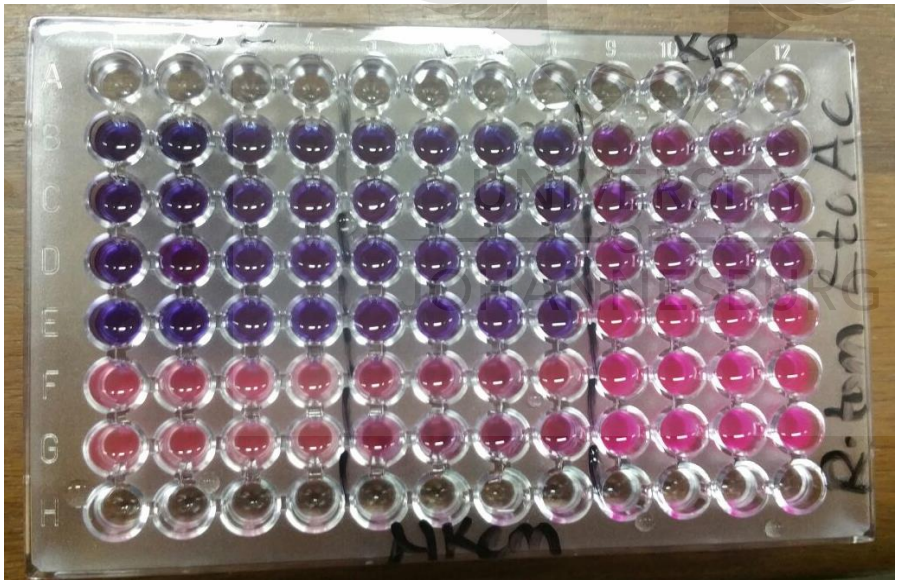


Figure 10: Micro titer plate showing inhibition for *E. faecalis and K. pneumonia*

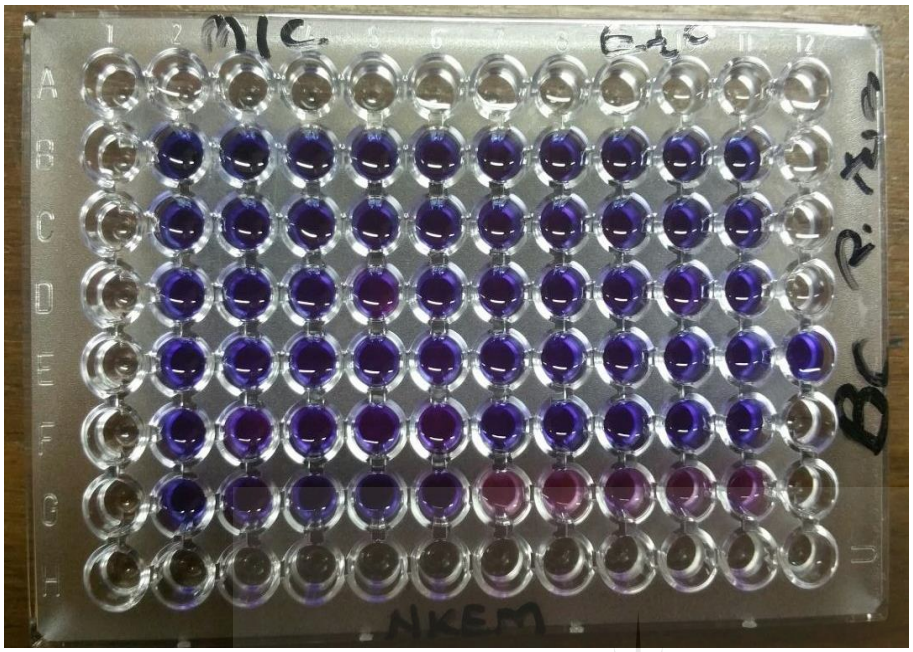


Figure 11: Micro titer plate showing inhibition for *B. cereus*

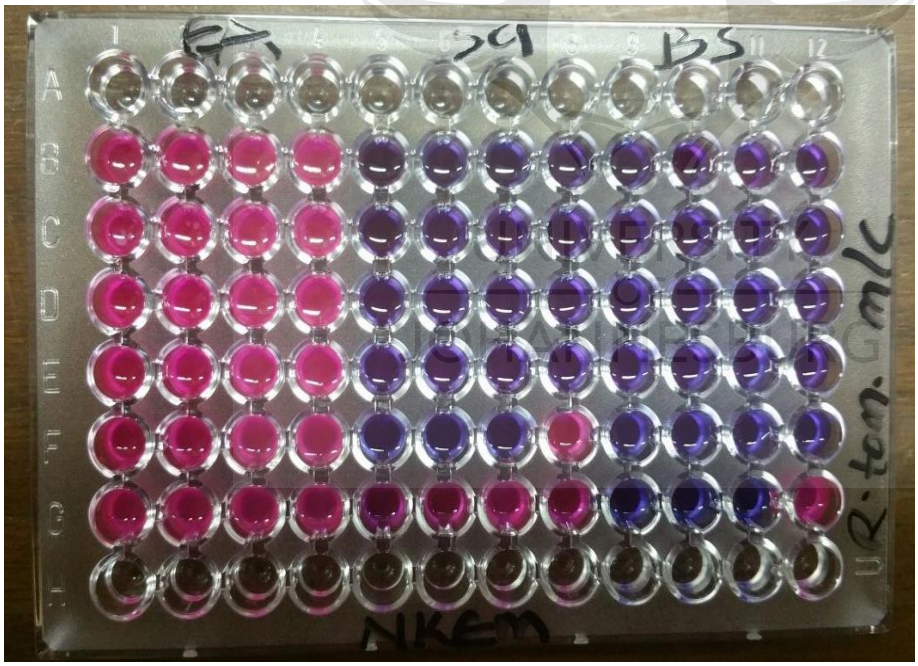


Figure 12: Micro titer plate showing inhibition for *E. aerogenes, S. aureus and B. subtilis*

Figure 13: Micro titer plate showing inhibition for *E. coli, S. aureus and B. cereus*

Figure 14: Micro titer plate showing inhibition for *E. coli, M. smegmatis and B. cereus*

Figure 15

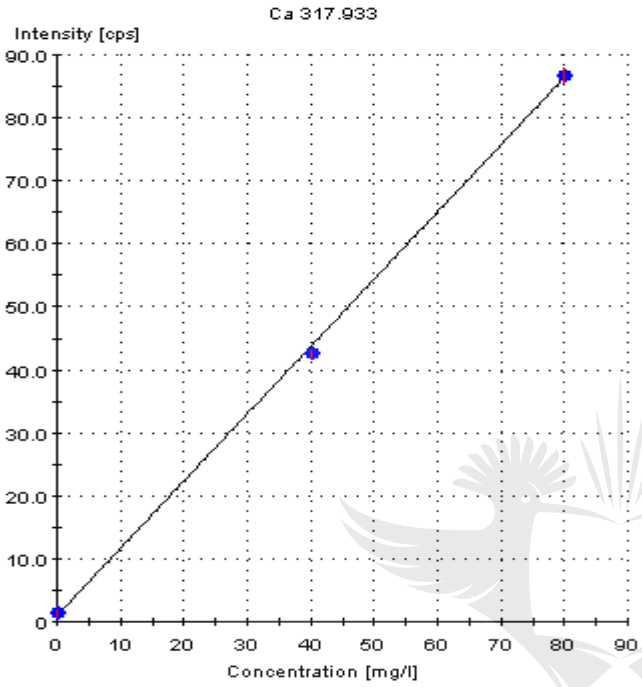
Figure 16

Figures 15 - 16: Micro titer plates showing inhibition for all 14 strains of bacteria used in this study.

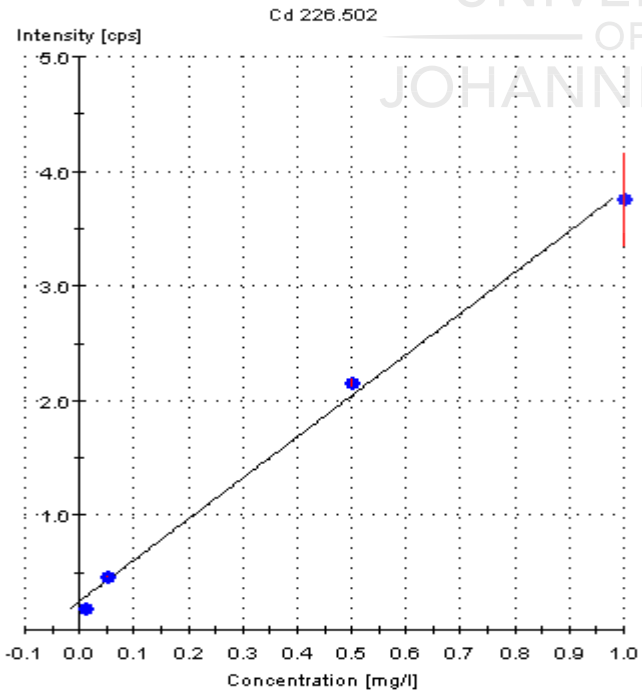
CHAPTER 6

Regression line graphs showing the different wavelengths for the elemental analysis results (Table 6.1).

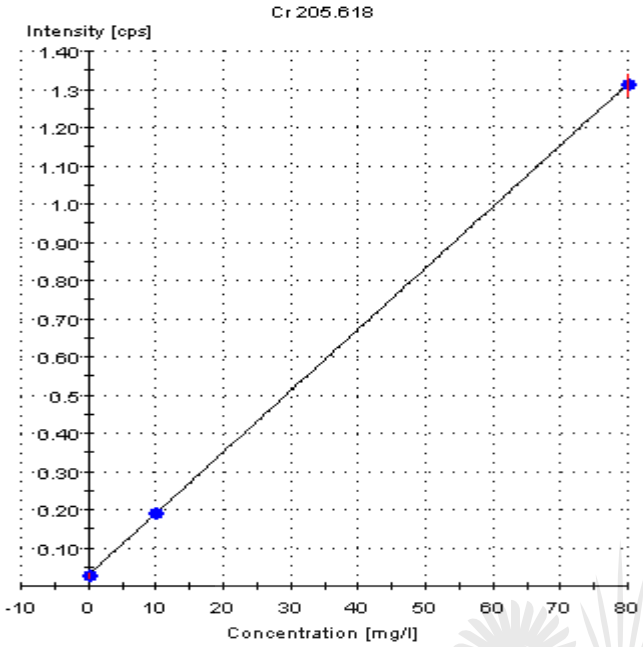
Calcium



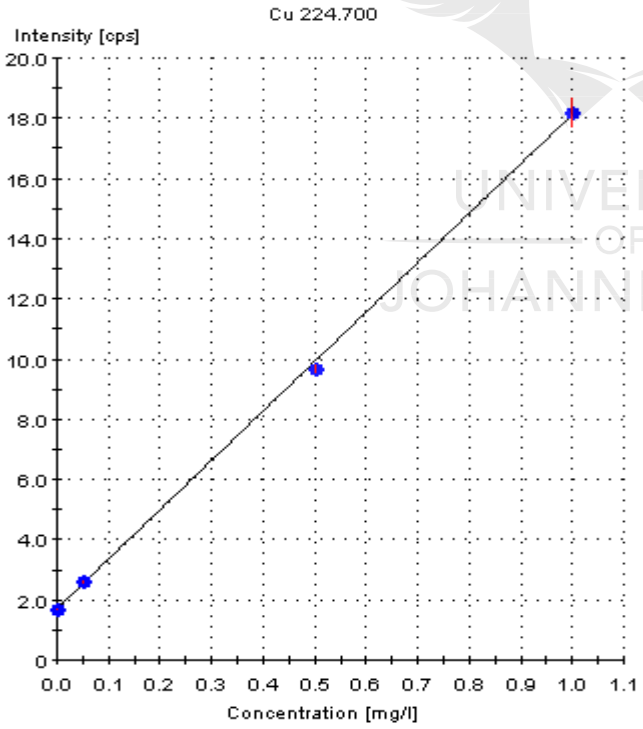
Cadmium



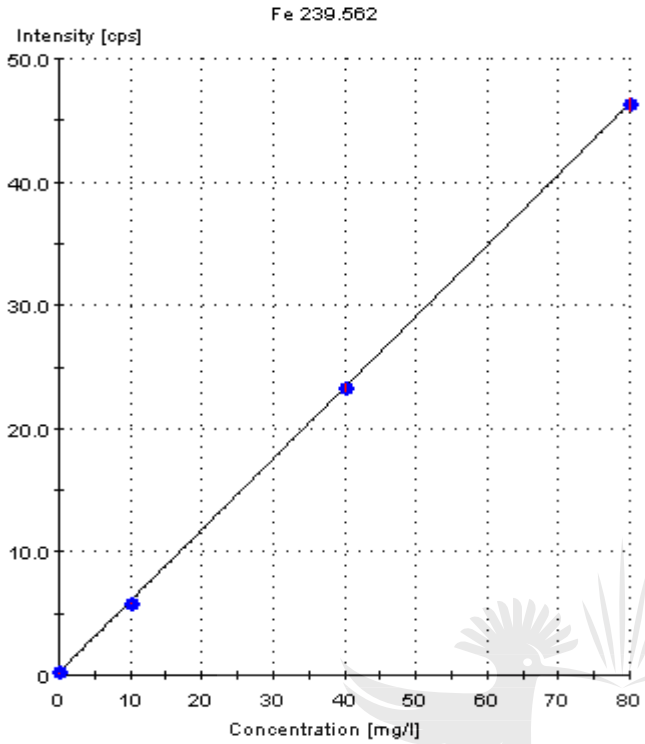
Chromium



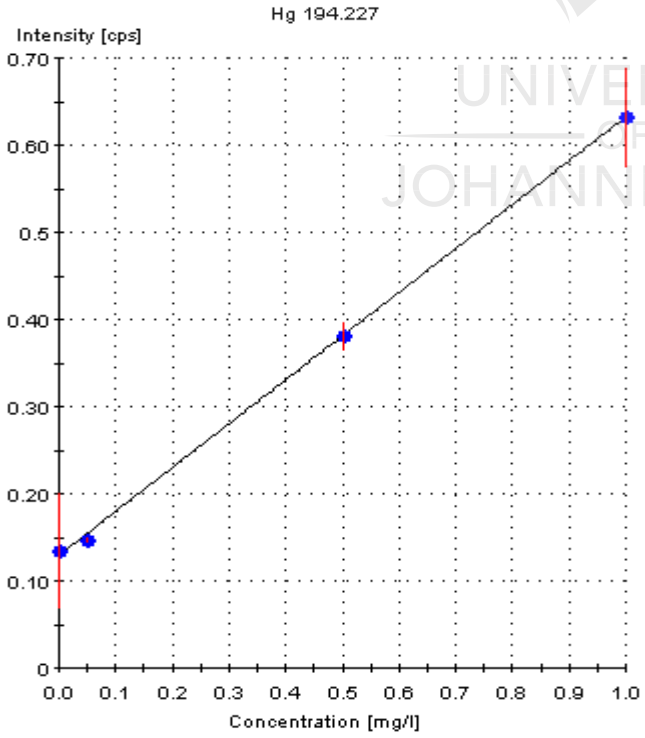
Copper



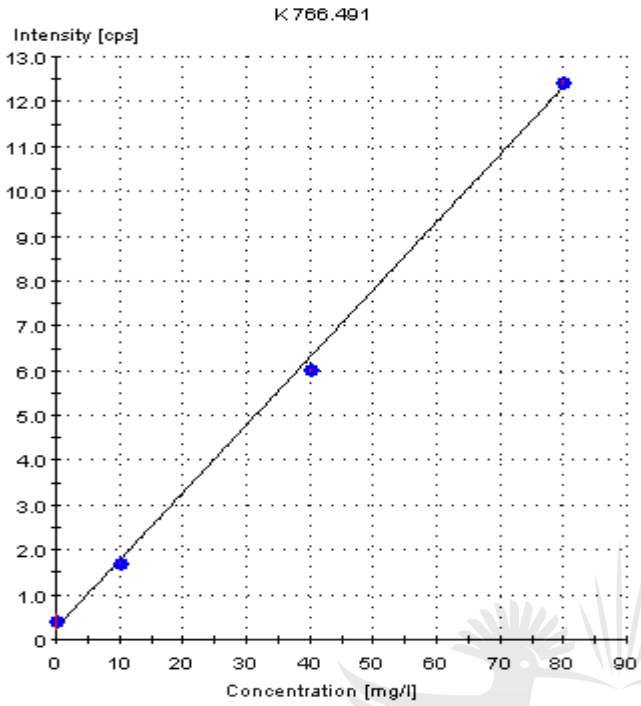
Iron



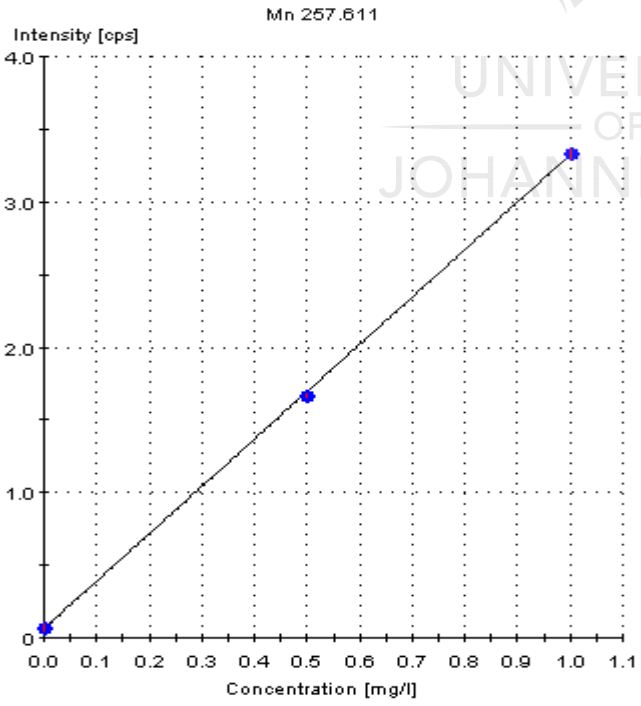
Mercury



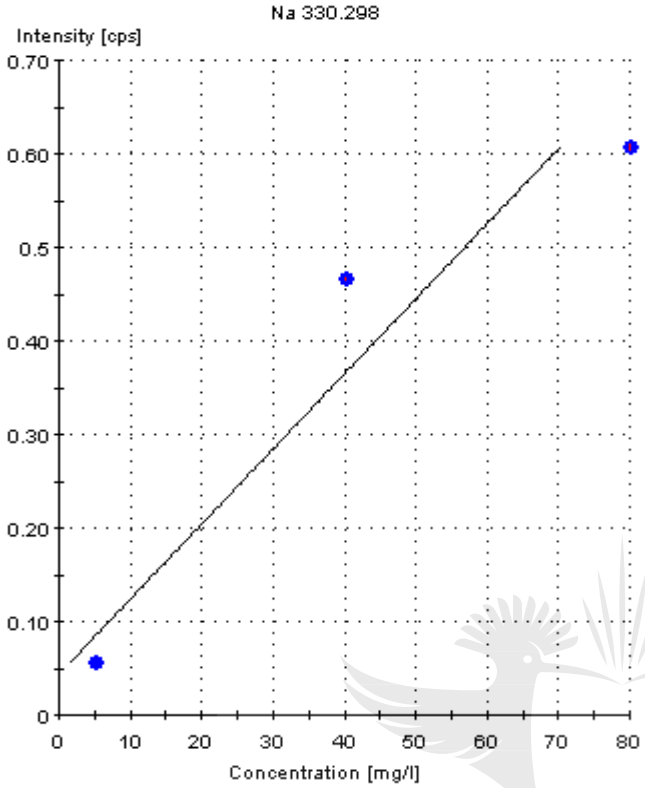
Potassium



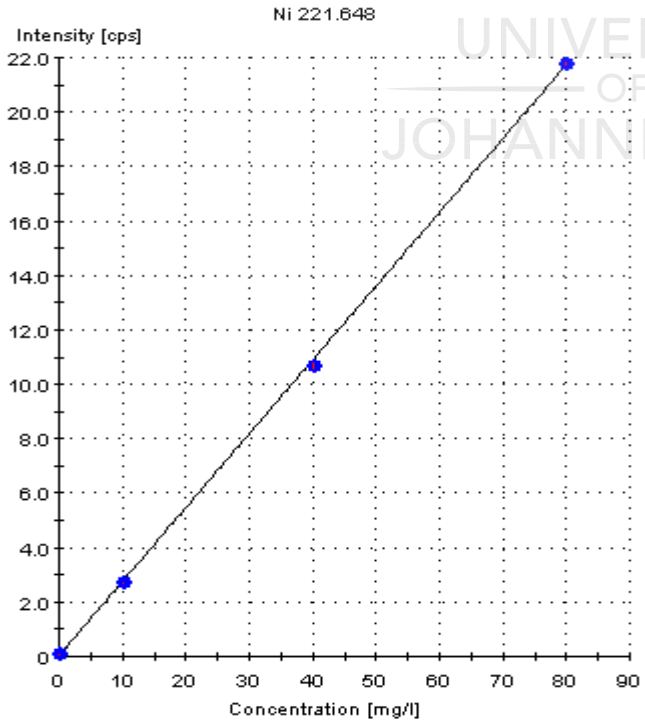
Manganese



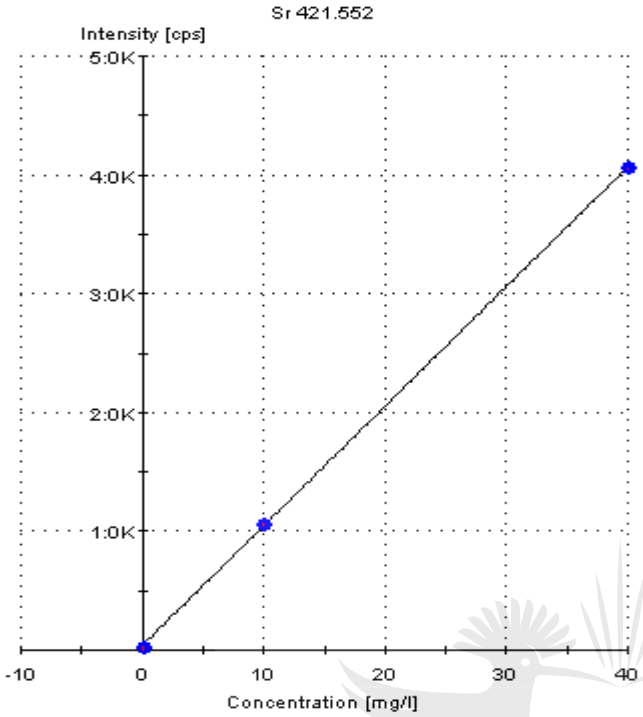
Sodium



Nickel



Strontium



Zinc