Bio-guided isolation of biologically active compounds from seeds of selected South African medicinal plants

by

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DECLARATIONS

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DECLARATION 1 - PLAGIARISM

I, **Amanda Perumal** declare that

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

2. This dissertation has not been submitted for any degree or examination at any other university.

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

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

Not Applicable

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We, Dr Patrick Govender, Dr Sershen Naidoo and Dr Karen Pillay as supervisors of the MSc study hereby consent to the submission of this MSc Dissertation.

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SUMMARY

The use of synthetic drugs to treat infectious diseases is associated with many disadvantages. Some of these include high cost, severe side effects, antimicrobial resistance and addiction due to uncontrolled use. Drugs derived from natural sources have thus become the safer alternative. To this end, the World Health Organization (WHO) has recognized the potential utility of traditional remedies and strives to preserve the primary health care involving medicinal plants. There is ample archaeological evidence indicating that medicinal plants were regularly employed by people in prehistoric times. In several ancient cultures, botanical products were ingested for curative and psychotherapeutic purposes. South Africa boasts a variety of cultural groups who exploit its diverse flora for a multitude of purposes. One such purpose is rooted in the traditional health care system often involving diviners (sangomas) and herbalists. Parts of plants commonly utilised by traditional healers for medicinal purposes are leaves and roots. Other parts include bulbs, corms, fruits, tubers, and bark, neglecting the potentially medicinal seeds. After thorough evaluation of available literature on the Meliaceae and Anarcardiaceae families, two traditionally used tree species were selected. Due to scarcity of information, this study aimed to provide insight on the medicinal potential of the seeds of *Trichilia emetica* Vahl. (Meliaceae) and *Protorhus longifolia* (Bernh. ex C. Krauss) Engl. (Anacardiaceae), and the potential of their extracts to be subsequently developed into novel pharmaceuticals. Seeds were collected from each of the selected tree species and extracted via cold percolation using methanol, ethanol, ethyl acetate, chloroform, hexane and distilled water individually to ensure the extraction of phytocompounds across a broad range of polarities. This study aimed to determine the phytochemical profile and biological activity of crude seed extracts of *T. emetica* and *P. longifolia*. Phytochemical screening of *T. emetica* seed extracts via preliminary methods and gas chromatography-mass spectroscopy (GC-MS) showed the presence of alkaloids, cardiac glycosides, phenols, sterols and terpenoids. Good potential antioxidant activity (IC_{50} = 5.94 µg/mL) was observed for the methanol crude seed extract. Promising potential antifungal activity was also noted with methanol displaying the highest inhibition (MIC of 37.46 µg/mL). Phytochemical screening of *P. longifolia* seed extracts revealed the presence of phenols, flavonoids, cardiac glycosides, and sterols. The methanol and ethanol crude seed extracts displayed good antioxidant potential (IC_{50} = 5.00 and 32.61 µg/mL, respectively) as well as substantial potential anticancer activity (IC_{50} values below 30 µg/mL). Additionally, antibacterial activity was observed for majority of the extracts tested. No previous pharmacological testing has been conducted on the crude seed extracts of these tree species. The present study has produced novel results and has provided insight into the potential safety and efficacy of the seeds of *T. emetica* and *P. longifolia* as natural alternatives to synthetic drugs. These results warrant the integration of the traditional medicine system of these tree species into western medicine.

This dissertation is dedicated to my life’s greatest blessings, my parents.

BIOGRAPHICAL SKETCH

Amanda Perumal was born on the 8th of March 1986 and raised in KwaZulu-Natal, Durban. She matriculated in 2003 from Crossmoor Secondary in Chatsworth and achieved an exemption with merit. She then enrolled for a Bachelor of Science degree in 2006 at the University of KwaZulu-Natal, majoring in Biochemistry and Microbiology. In 2008, she had her son Aaron Cole and decided to put her studies on hold for a while. In 2011 she completed her undergraduate degree. Her keen interest in the field of Biochemistry stems from always being enthusiastic to learn new things. This led her to register for a Bachelor of Science Honours degree in 2012.

Amanda is passionate about research that could possibly aid in developing and potentially commercialising safe drugs of natural origin to treat cancers and other dreaded diseases with fewer side effects, where synthetic drugs have proved to be ineffective.

During her spare time, Amanda enjoys spending quality time with her babies (son and four dogs), scrapbooking and listening to classical and electronic dance music. She is also an avid reader and relishes anthology, satire and fantasy. Amanda is also obsessively environmentally conscience.

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- **Family and friends**, both on and off campus for providing a healthy distraction from the mayhem and foolishness.

PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1	**General Introduction and Project Aims**
Chapter 2	**Literature Review**
Chapter 3	**Research Results I** Pharmacological and chemical evaluations of crude extracts of *Trichilia emetic* seeds
Chapter 4	**Research Results II** Biological activity and chemical composition of crude extracts of *Protorhus longifolia* seeds
Chapter 5	**General Discussion and Conclusion**

CONTENTS

ABBREVIATIONS

°C	Degrees Celsius
ADS	Antioxidant Defence Systems
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
CANSA	The Cancer Association of South Africa
CFU	Colony forming unit
cm	Centimetre
DMAPP	Isopentenyl pyrophosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOXP/MEP	1-deoxyxylulose 5-phosphate/2-C-methylerithrytol 4-phosphate
DPPH	1,1′-Diphenyl-2-picrylhydrazyl
DSHEA	Dietary Supplement Health and Education Act
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's minimal essential medium
FBS	Fetal bovine serum
FPP	*(E), (E)*-farnesyl pyrophosphate
FTIR	Fourier-transform infrared spectroscopy
g	Gram
GC-MS	Gas Chromatography-Mass spectroscopy
GPP	*(E)*-geranyl pyrophosphate
GGPP	*(E), (E), (E)*-geranyl geranyl pyrophosphate
h	Hour
HBSS	Hanks balanced salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid
HIV	Human-immune deficiency virus
^{1}H-NMR	Proton nuclear magnetic resonance
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
ICU	Intensive care unit
INT	*p*-iodonitrotetrazolium chloride

IPP	Isopentenyl pyrophosphate
KZN	KwaZulu-Natal
L	Litre
LC-MS	Liquid Chromatography–Mass Spectrometry
M	Molar
m	Metre
mm	Millimetre
MCF-7	Human adenocarcinoma cells
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
min	Minute
mg	Milligram
MH	Mueller-Hinton
mL	Millilitre
mM	Millimolar
MOPS	Morpholinepropanesulfonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSD	Mass Selective Detector
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MVA	Mevalonate
m/z	Mass-to-charge ratio
mL/min	millilitre per minute
nm	Nanometre
OD	Optical density
PBS	Phosphate-buffered saline
ppm	Parts per million
rpm	revs per minute
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
Sec	Second
SOD	Superoxide dismutase
µg	Microgram
µL	Microlitre

µM	Micromolar
UKZN	University of KwaZulu-Natal
USA	United States of America
UV	Ultraviolet
Vero	Green monkey kidney epithelial cells
VREF	Vancomycin resistant *Enterococcus faecalis*
WHO	World Health Organisation

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Chapter 1

INTRODUCTION AND STUDY AIMS

1.1 INTRODUCTION

According to the World Health Organisation (WHO, 2005), 65-80% of the world's rural population relies on traditional medicine for their primary health care needs (Gurib-Fakim, 2006; Wendakoon *et al.*, 2012). In South Africa, many people from disadvantaged backgrounds turn to traditional healers for ethno-medicinal advice due to the high cost and inaccessibility of proper health care facilities (Keirungi and Fabricius, 2005). This indigenous medicinal knowledge that is passed down from traditional healers rarely appears in literature. Therefore, plants prescribed traditionally for the treatment of diseases needs to be scientifically validated for reputability and once this has been established, documented (Kaur and Arora, 2009). The scientific validation of traditional use of plants has led to the discovery of 74% of pharmacologically active plant-derived compounds (Ncube *et al.*, 2008). However, many plants used for traditional medicine in Africa have not been subjected to scientific validation (Prozesky *et al.*, 2001). This motivated the present study assessing the pharmacological activity of selected South African medicinal plants.

1.2 RESEARCH RATIONALE AND MOTIVATION

Infectious diseases caused by pathogenic organisms are the leading cause of premature deaths globally, with its effects being more rampant in developing countries (Wendakoon *et al.*, 2012)**.** Despite great advancements made since the advent of antibiotics, microbial resistance to some current antimicrobials is becoming a severe global challenge. Genetic modification and continuous, unsystematic use of present day antibiotics has also contributed to the materialisation and increase of antimicrobial resistance (Parekh and Chanda, 2007). A major challenge concerning many drug invention programmes is keeping up with the rate at which antimicrobial resistance develops. Hence, the critical need to uncover new antimicrobial agents with improved safety, better efficacy and novel modes of action against ever evolving diseases (Rojas *et al.*, 2006). Medicinal plants possess biologically active compounds comprising diverse chemical structures. These bioactives are often used to manufacture drugs capable of eliminating infectious diseases that are resistant to synthetic drugs (Dubey *et al.*, 2012). The continuous search for naturally-derived medicines is encouraged due to the high cost of synthetic drugs and the fact that long term use causes numerous side effects, while plant-derived medicines are better tolerated by the body, with drug resistance being less documented (Rankovic *et al.*, 2011).

There are 68 000 plant species in Africa, of which, approximately 35 000 are endemic to the continent (Freidberg, 2009). In Southern Africa alone, there are well over 30 000 species of higher plants (Xego *et al.*, 2016). The country is known to possess the most diverse temperate flora on earth with approximately 9 000 plant species (West *et al.*, 2012). It is estimated that between 3000 and 4000 species of plants are used medicinally throughout

South Africa, and of these, approximately 350 species are actively traded for this purpose (De Wet *et al.*, 2013; Xego *et al.*, 2016). Of the rich repository of known plant species in South Africa, only a few have been investigated for their pharmacological attributes, leaving many compounds of significant medicinal value undiscovered.

The scientific validation of South African plants used traditionally holds many potential advantages for the country:

- An increase in trade both nationally and internationally, thus increasing the global supply of herbal medicines;
- Providing economic prosperity to the country in the form of employment in many fields, viz. plant cultivation, drug manufacture, medical taxonomy, pharmacognosy (study of medicine from natural sources) etc.;
- South Africa becoming a primary source of new leads for drug discovery possessing greater efficacy than synthetic drugs and in doing so, placing South Africa on the map so to speak (Dauskardt, 1990; Hishe *et al.*, 2016).

In responding to the need to scientifically validate the use of South African plants in traditional medicine the present study investigated phytochemical composition as well as the antioxidant, antimicrobial and anticancer activity of *Trichilia emetica* Vahl. (Meliaceae) and *Protorhus longifolia* (Bernh. Ex C. Krauss) Engl. (Anacardiaceae). Both species were selected on the basis that they are endemic to South Africa and are well known for the medicinal properties of their barks, stems, roots and leaves (Verschaeve *et al.*, 2004; Germano *et al.*, 2005; Suleiman *et al.*, 2010; Mosa *et al.*, 2015). However, there is a paucity of information on the potential medicinal value of the seeds produced by these species. The focus on the seeds is based on the fact that other studies have shown seeds of a number of medicinally important species to produce novel therapeutic agents (Anwar *et al.*, 2007; Sirisena *et al.*, 2015; Timsina and Nadumane, 2015). Furthermore, both species investigated here produce recalcitrant, as opposed to orthodox seeds, which have been shown to possess a range of pharmacological properties (Othman *et al.*, 2007; Joshi *et al.*, 2013; Gannimani *et al.*, 2014).

1.3 AIMS OF STUDY AND SCOPE OF DISSERTATION

The aims of this study were to:

1. Evaluate the antibacterial, antifungal and antioxidant activity of seed extracts;
2. Investigate potential cytotoxicity and anticancer effects of seed extracts; and
3. Identify the phytochemical constituents present in each seed extract.

This dissertation is divided into five chapters, with this introduction being **Chapter 1** as it provides a brief background and rationale and motivation for this study.

In **Chapter 2**, a comprehensive literature review is presented, encompassing the use of plants in traditional medicine, its validation in modern scientific research, and a description of the plant species selected for this study.

Chapter 3 focuses on the *in vitro* antimicrobial, antioxidant and antitumour evaluation and the determination of the phytochemical composition of seed extracts of *T. emetica*. These were accomplished via various biological assays and Gas Chromatography-Mass Spectroscopy (GC-MS).

In **Chapter 4**, the *in vitro* biological activity and phytochemical constituents present in the seed extracts of *P. longifolia* was investigated using the same methods described in Chapter 3 of this dissertation.

Finally, **Chapter 5** presents a general discussion and conclusion as well as suggestions for future research studies.

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Chapter 2

LITERATURE REVIEW

MEDICINAL PLANTS

2 MEDICINAL PLANTS

2.1 INTRODUCTION

Phytomedicine, once territory of health food retailers and speciality stores only, has made its way onto the conventional shopping shelf as evidenced by the emergence of vast numbers of pharmaceutical products (Briskin, 2000). A major contributory factor to the growth in use of phytomedicine in the United States of America (USA) for example, has been the Dietary Supplement Health and Education Act (DSHEA) that endorsed the manufacture and marketing of these products (Brevoort, 1998). In recent years, a growing worldwide interest has been noted in "nutraceuticals" which refers to foods containing phytochemicals harbouring medicinal attributes (Manish *et al.*, 2015). The difference between medicinal plants and nutraceuticals is that the latter has a nutritional role in one's diet which may be beneficial to one's health after extended consumption (Korver, 1998). Medicinal plants, on the other hand, exhibit medicinal properties in response to an illness for a short/long duration without being consumed (Korver, 1998). The main foci for medicinal plant research are to understand their pharmacognosy and phytochemistry. The area of pharmacognosy involves bioactivity guided assays, documentation of mechanisms of action and target areas for phytochemicals. Phytochemistry research includes the characterisation of medicinal plants for possible biologically active compounds, their separation and structural elucidation (Briskin, 2000).

This chapter reviews some of the fundamental aspects of traditional medicine, phytochemical production and their antioxidant, antimicrobial and antitumour activity.

2.2 TRADITIONAL MEDICINE

The World Health Organisation (WHO, 2005) defines traditional medicine as "the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses." The use of plants as "herbal medicine" has been the basis of treatment for various diseases and physiological conditions long before recorded history (Solecki, 1975; Thillaivanan and Samraj, 2014). Fossil records date human use of plants to the Middle Palaeolithic age, approximately 60, 000 years ago (Fabricant and Farnsworth, 2001). The medicinal use of plants was described in ancient Chinese and Egyptian papyrus writings as early as 3,000 BC (Mamedov and Craker, 2011). Herbal medicine is the use of herbs (flowers, fruits, leaves, roots, seeds, etc.), substances resulting from herbs (essential oils, gums, resins, etc.) and plant preparations (fluid or dry extracts, tinctures, decoctions and infusions, oils etc.) (Efferth and Greten, 2014). Herbal remedies display therapeutic activity

as a consequence of their active constituents that act either alone or in combination. African and Native American cultures used herbs in healing rituals, while others developed traditional medical systems such as Ayurveda and Siddha in India, Kempo Medicine in Japan, Traditional Chinese Medicine in China and Unani Medicine in South Asia and the Middle East (Thillaivanan and Samraj, 2014).

Since prehistoric times men and women have been reliant on nature for food, shelter, clothing and medicines to treat a range of illnesses. Through trial and error, they sought to distinguish between those plants that were beneficial, poisonous or inactive. Methods to process these plants, in order to give optimal results were also quickly realised (Kunle *et al.*, 2012). Much of what is known today about modern medicine was acquired from herbal folklore of indigenous people (Fabricant and Farnsworth, 2001). In communities all over the world ethnomedicine is still the basis of their medicinal structure.

2.2.1 Traditional medicine in South Africa

A myriad of cultural groups exists in South Africa. Each of these relies on the flora of the land for a range of purposes (Arnold *et al.*, 2002). South Africa houses a wealthy assortment of plants with approximately 30 000 flowering species, of which, 80% are endemic (Fennell *et al.*, 2004). A large number of the country's inhabitants employ some form of traditional medicine in their daily lives. Traditional medicine makes use of bulbs, corms, herbs and trees (Eldeen, 2005). It has been estimated that approximately 20 000 tonnes of plant material is traded on an annual basis in the KwaZulu-Natal province alone in South Africa (Xego *et al.*, 2016). These plants are consumed in rural communities by an estimated 28 million people who turn to any one of 200 000 traditional healers for treatment (Verschaeve *et al.*, 2004). The exponential rise of the South African population in recent years has also led to a rise in the demand for medicinal plants (Fennell *et al.*, 2004), with an estimated 4-8% of annual income being spent on traditional healers (Freidberg, 2009).

2.3 SECONDARY METABOLITES

Plants produce primary and secondary metabolites. Primary metabolites are those responsible for primary metabolic processes viz. building and maintenance (Wink, 1999; Briskin, 2000; Wink, 2003). These include lipids, carbohydrates, proteins, chlorophyll, heme and nucleic acids (Wink, 1999; Briskin, 2000). Secondary metabolites (i.e. phytochemicals) are chemicals that play a variety of roles in plants such as defence against herbivory, inter-plant competition and pathogens, attracting pollinators and symbionts, protection against abiotic stress and as recently discovered, play a potential role at the cellular level (Wink and Schimmer, 1999). In recent years, the valuable medicinal effects of a combination of secondary plant metabolites have been investigated extensively. It has been determined that a specific therapeutic action is unique to a specific species/genus of plant/s. This is

consistent with the concept of combinations of secondary metabolites in a particular plant are often taxonomically distinct (Wink, 2003). A few of these secondary metabolites and their beneficial medicinal effects will be discussed below.

2.3.1 Antioxidants

An antioxidant can be described as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell and Gutteridge, 2015). Reactive oxygen species (ROS) are generally produced and perpetuated by normal oxidative metabolic processes that are invariably encountered by all aerobic organisms, specifically occurring at sites of respiratory and signalling events, e.g. mitochondria (Kermanizadeh *et al.*, 2015). This ROS includes the superoxide anion ($^{\cdot}O_2^-$), reactive nitrogen species (RNS), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) and are produced by the transfer of electrons and reduction of the ground state of oxygen (Gutteridge and Halliwell, 2010). Initially ROS were recognised chiefly as toxic by-products of aerobic metabolism and were only later discovered to be a diagnostic component of normal cellular metabolism due to their participation in redox reactions and transferral of single electrons (Chaitanya and Naithani, 1994). Despite these molecules having important physiological roles such as in phagocytosis and intercellular signalling (Olorunnisola *et al.*, 2012), ROS production needs to be stringently controlled.

Oxidative stress is referred to as the imbalance between ROS or free radicals and the antioxidants. If not quenched, ROS can oxidise (and in the process damage) proteins, lipids and nucleic acids (Battin and Brumaghim, 2009). Damage caused by ROS is responsible for the occurrence of many diseases including neurodegenerative and cardiovascular diseases and cancer (Battin and Brumaghim, 2009). Under stressful conditions, ROS production exceeds the capacity of ROS-scavenging systems (antioxidant mechanisms to maintain ROS levels) thereby disturbing homeostasis leading to oxidative stress and tissue damage.

Under physiological conditions, the disparaging effects of ROS are neutralised by the endogenous antioxidant defence system (Figure 2.1), thus, producing harmless molecules (Halliwell, 2006). This antioxidant defence system is made up of enzymatic (catalase, glutathione peroxidases, glutathione reductase and superoxide dismutase) and non-enzymatic (co-enzymes, minerals, vitamins A, C, E) components (Apel and Hirt, 2004). Many of these compounds are found naturally in plant tissue and are capable of quenching free radicals, thereby, shielding the body from damage by maintaining a redox state (Olorunnisola *et al.*, 2012).

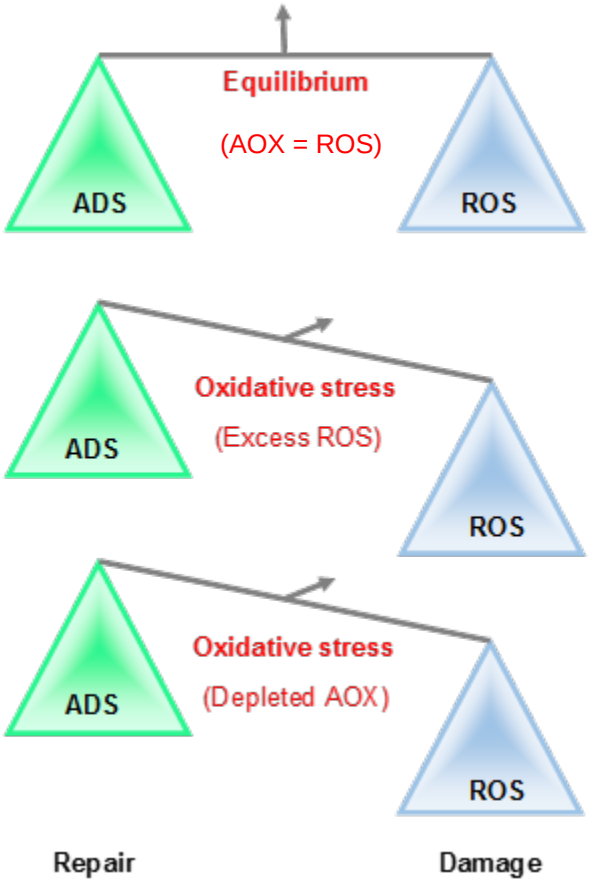

Figure 2.1 Diagrammatic representation of oxidative metabolism in a biological system. ADS = Antioxidant defence systems; AOX = Antioxidants; ROS = Reactive oxygen species (Emmons, 2016).

2.3.2 Phenolic acids

Phenols are among the largest faction of secondary metabolites that occur in plants, with an estimated 8000 structures identified (Ezekiel *et al.*, 2013). Some are simple structures made up of one aromatic ring whilst others are more complex such as tannins and lignins (Weng and Yen, 2012). Polyphenolic compounds are found in both edible and non-edible plants and are known to possess antioxidant, antibacterial, antimutagenic, antiallergic and anti-inflammatory activity (Chanda *et al.*, 2014). They are also capable of modifying gene expression (Parekh and Chanda, 2007; Chanda *et al.*, 2014). Some classes of phenols that are of pharmaceutical value are discussed below.

2.3.2.1 Simple phenols

Simple phenols have one aromatic ring containing an alcohol, aldehyde or carboxyl group constituting a short hydrocarbon chain (Weng and Yen, 2012). Examples of simple phenols are Capsaicin and Eugenol (Figure 2.2). Capsaicin is isolated from *Capsicum* sp., and is used as a painkiller (Gurib-Fakim, 2006). Eugenol is known for its anti-inflammatory and antibacterial properties (Cseke *et al.*, 2006).

2.3.2.2 Phenylpropanoids

Phenylpropanoids (Figure 2.3) consist of a three-carbon side chains attached to an aromatic ring (Jain *et al.*, 2013). Common examples are hydroxycinnamic acids, hydroxycoumarins, lignans and phenylpropenes. These phenylpropenes are important constituents of essential oils (Jain *et al.*, 2013).

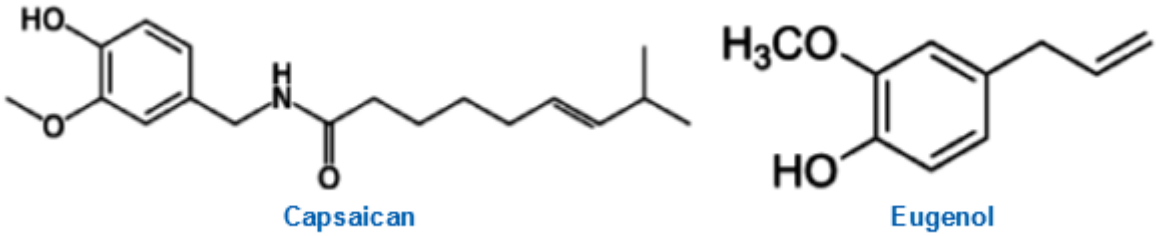

Figure 2.2 Common examples of simple phenols (Nakagawa and Hiura, 2014).

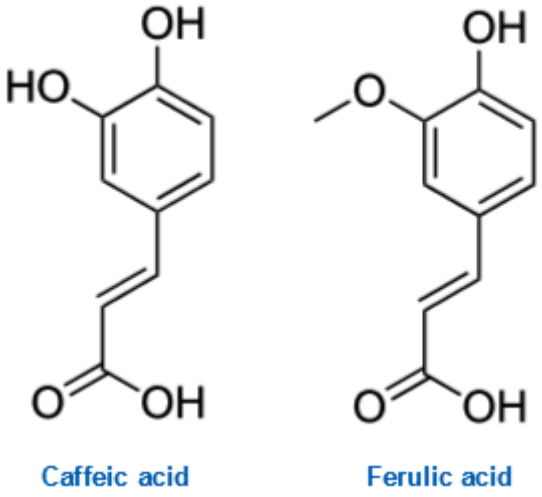

Figure 2.3 Chemical structures of some common phenylpropanoids (Kfoury *et al*., 2014).

2.3.2.3 Flavonoids

Flavonoids are commonly water soluble compounds made up of a pair of benzene rings separated by a propane component, a derivative of flavone (Cowan, 1999). Flavonoids are responsible for the bright colours of fruit, flowers and occasionally leaves (Gurib-Fakim, 2006). These colours attract pollinators. Flavonoids are also responsible for protecting the plant from UV damage (Gurib-Fakim, 2006). There are many subgroups of flavonoids that arise from slight changes in structure and these include anthocyanins, chalcones, isoflavones, flavonones, flavones and flavonols (Cseke *et al.*, 2006) (Figure 2.4). Flavonoids display potent antimicrobial, anticancer and antioxidant activity (Okigbo *et al.*, 2009). They are also known to diminish the threat of heart disease (Urquiaga and Leighton, 2000).

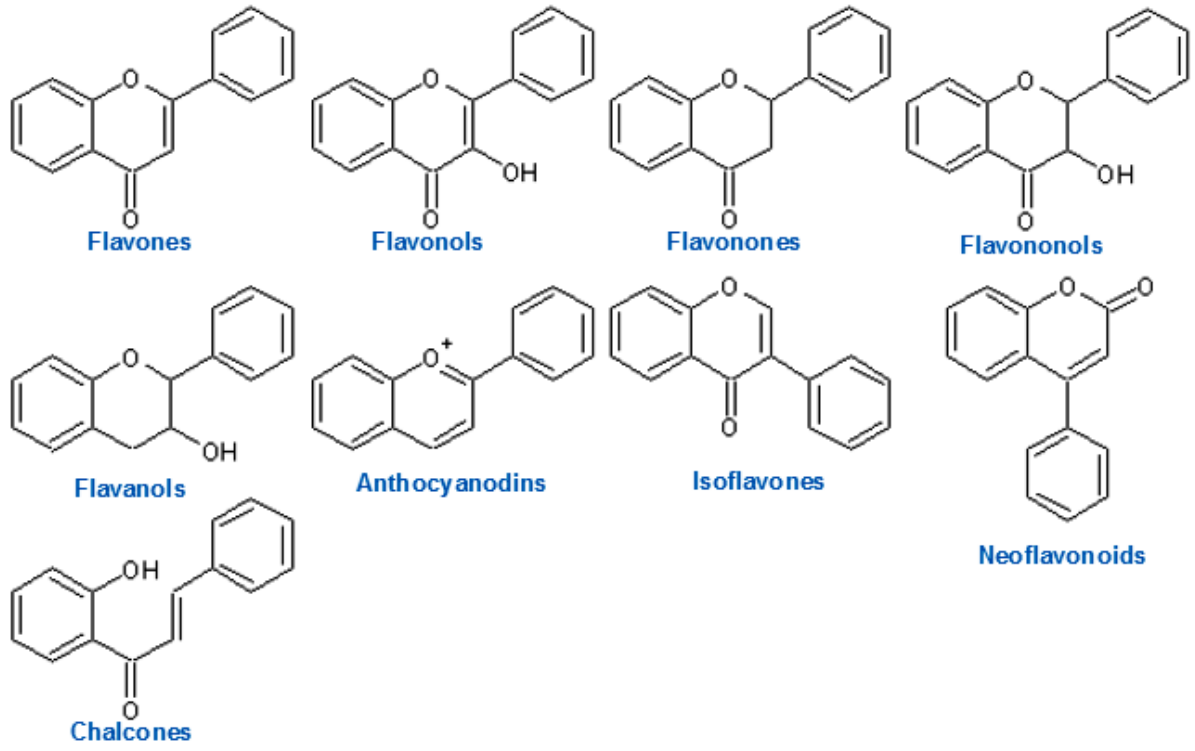

Figure 2.4 Basic structures of some flavonoids (Cseke *et al*., 2006).

2.3.2.4 Tannins

Tannins are water soluble oligomers that have the ability to bind to or precipitate water soluble proteins (Cseke *et al.*, 2006). Tannins are prevalent in vascular plants and are present chiefly in woody tissue but are also found in flowers, leaves and seeds (Aremu, 2009). Plant material with a high tannin content has a bitter taste thus deterring foragers. Tannins occur either in condensed or hydrolysable form, based on the ability of enzymes and acids to condense flavonols to polymers or hydrolyse them (Castillo *et al.*, 2012). Condensed tannins make up the largest group of polyphenols in plants and constitute approximately 50% of the leaf dry weight (Levin, 1976). Proanthocyanidins (Figure 2.5) are examples of condensed tannins. Corilagen (isolated from the leaves of eucalyptus and sumac) and geraniin (isolated from geranium) are examples of hydrolysable tannins (Figure 2.3). Both these have the ability to inhibit reverse transcriptase, thus potentially displaying anti-HIV activity (Cseke *et al.*, 2006).

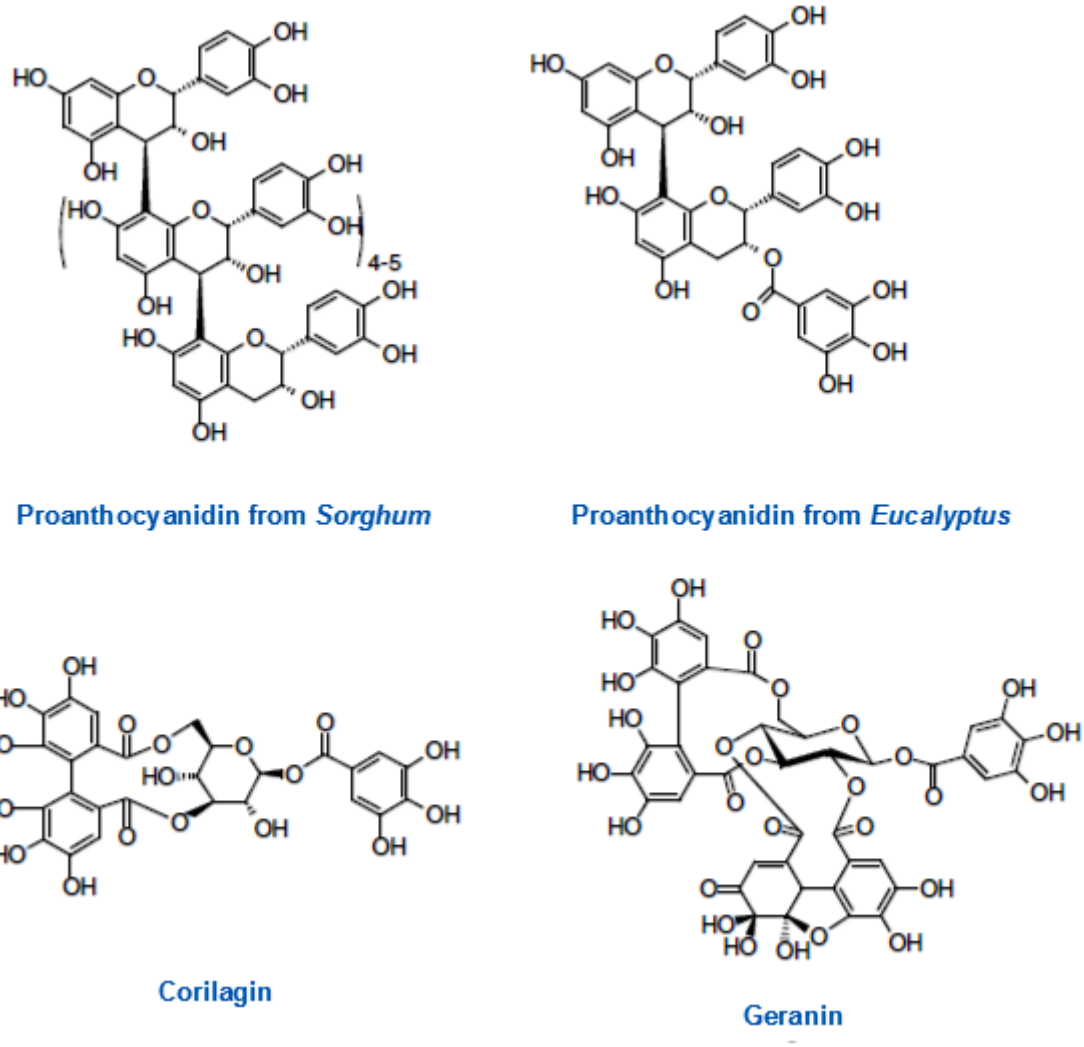

Figure 2.5 Common examples of condensed (proanthocyanidins) and hydrolysable tannins (Cseke *et al*., 2006).

2.3.3 Terpenes

Terpenes (Figure 2.6) have repeating five-carbon isoprene units which are made from acetate through the methylerythritolmevalonic acid pathway. Terpenes are well known for the use of their essential oils as fragrances (Freidberg, 2009). When other elements, usually oxygen, are added to their structure, they are termed terpenoids (Cowan, 1999).

2.3.3.1 Hemiterpenes – C_5

Hemiterpenes are the simplest terpenes made up of a single five carbon unit (Cseke *et al.*, 2006). Hemiterpenes aid in plant defence by acting as repellents to herbivores (Holopainen, 2004).

2.3.3.2 Monoterpenes – C_{10}

Monoterpenes are the major constituents making up essential oils and are of considerable economic importance due to their use as perfume (Cseke *et al.*, 2006). Common examples

include camphor, geraniol, iridoids, linalool, limonene, menthol, myrcene and pinene (Freidberg, 2009).

2.3.3.3 Sesquiterpenes – C_{15}

Similar to monoterpenes, most sesquiterpenes are also components of essential oils derived from plants (Gurib-Fakim, 2006). Common examples include artemisinin, bisabolol, caryophyllene, farnesol, humulene, santonin and the sesquiterpene lactones (bitter principles) (Cseke *et al.*, 2006; Gurib-Fakim, 2006). The medicinal properties of sesquiterpenes include antimicrobial, antihelmintic, molluscicidal and antimalarial (Gurib-Fakim, 2006).

2.3.3.4 Diterpenes – C_{20}

Diterpenes are found in both plants and animals and contain four isoprene units. Taxol is a well-known diterpene, famous for its antineoplastic activity. Other examples include cafestol, cambrene, forskolin, kahweol, stevoside and zoapatanol an abortifacient (Gurib-Fakim, 2006; Freidberg, 2009).

2.3.3.5 Sesterterpenes – C_{25}

Sesterterpenes consist of five isoprene units having 25 carbon atoms. Sesterterpenes are rare in comparison to the other classes of terpenes (Freidberg, 2009).

2.3.3.6 Triterpenes – C_{30}

Triterpenes are made of six isoprene units with 30 carbon atoms (Castillo *et al.*, 2012). Squalene, a linear triterpene, is formed from the reductive coupling of two farnesyl pyrophosphates from the cyclization of squalene. Squalene is subsequently processed to lanosterol (Gurib-Fakim, 2006). Lanosterol is a structural precursor to all sterols (Freidberg, 2009). When a sugar moiety is added to a triterpene or a sterol, a saponin is formed (Freidberg, 2009).

2.3.3.6.1 Sterols

Sterols are steroid compounds similar to cholesterol and differ in carbon side chain composition and the presence or absence of a double bond. The purpose of sterols is not well understood in plants but evidence suggests that some phytosterols are effective against cardiovascular disease due to its ability to lower cholesterol (Cseke *et al.*, 2006).

2.3.3.6.2 Saponins

Saponins are a part of a large group of glycosides occurring in plants. They are characterized by their bitter/astringent taste and their ability to behave as a surfactant and foam when added to water (Gurib-Fakim, 2006). Hence, the name saponin, derived from the Latin word ‘sapo’ meaning soap (Freidberg, 2009). Saponins are advantageous to human health as they confer haemolytic effects to erythrocytes, lowers cholesterol and possess

antimicrobial, anti-inflammatory and anticancer activity (Cseke *et al.*, 2006; Gurib-Fakim, 2006; Okigbo *et al.*, 2009).

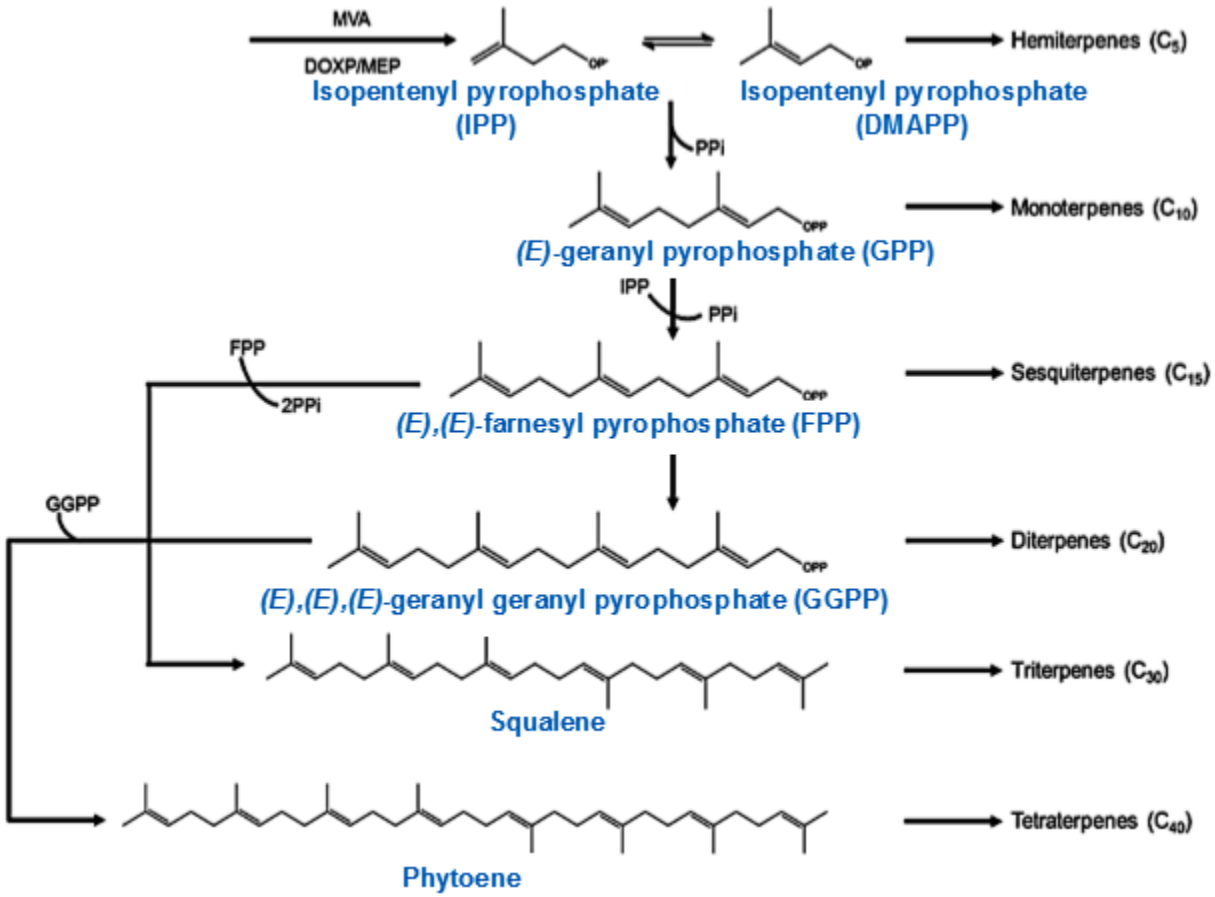

Figure 2.6 Schematic illustration of the biological synthesis of major classes of terpenes (Barbosa *et al*., 2014).

2.3.3.7 Tetraterpenes – C_{40}

Tetraterpenes are made up of eight condensed isopentenyl pyrophosphate (IPP) units and are derived from lycopene (Dornelas and Mazzafera, 2007). The most common and widely distributed tetraterpenes are carotenoids. Cyclisation at both ends results in β-carotene and cyclisation at one end only, results in γ-carotene. These pigments are found in leaves of higher plants and are responsible for imparting colour (yellow and red) to fruits and flowers which attract pollinators and herbivores, aiding in seed dispersal. Additionally, carotenoids also aid in photosynthesis by protecting plants from other pigments like chlorophyll which catalyse over-oxidation (Cseke *et al*., 2006).

2.3.4 Cardiac glycosides

Cardiac glycosides are steroid-like compounds (Newman *et al.*, 2008). They are composed of one/more sugars and a non-sugar moiety (aglycone) having a cyclopentanoperhydrophenanthrene nucleus (Hollman, 1985; Cseke *et al.*, 2006). Cardiac

glycosides are classified according to their steroid skeleton as C23 cardenolides or C24 bufadienolides (Figure 2.7) (De Padua *et al.*, 1999; Gurib-Fakim, 2006). Well known examples include digitoxin and digoxin from the genus *Digitalis* and ouabain from *Strophanthus gratus* (De Padua *et al*., 1999; Newman *et al*., 2008). They are known for their ability to treat congestive heart failure and are used as anti-arrhythmic agents (Gurib-Fakim, 2006).

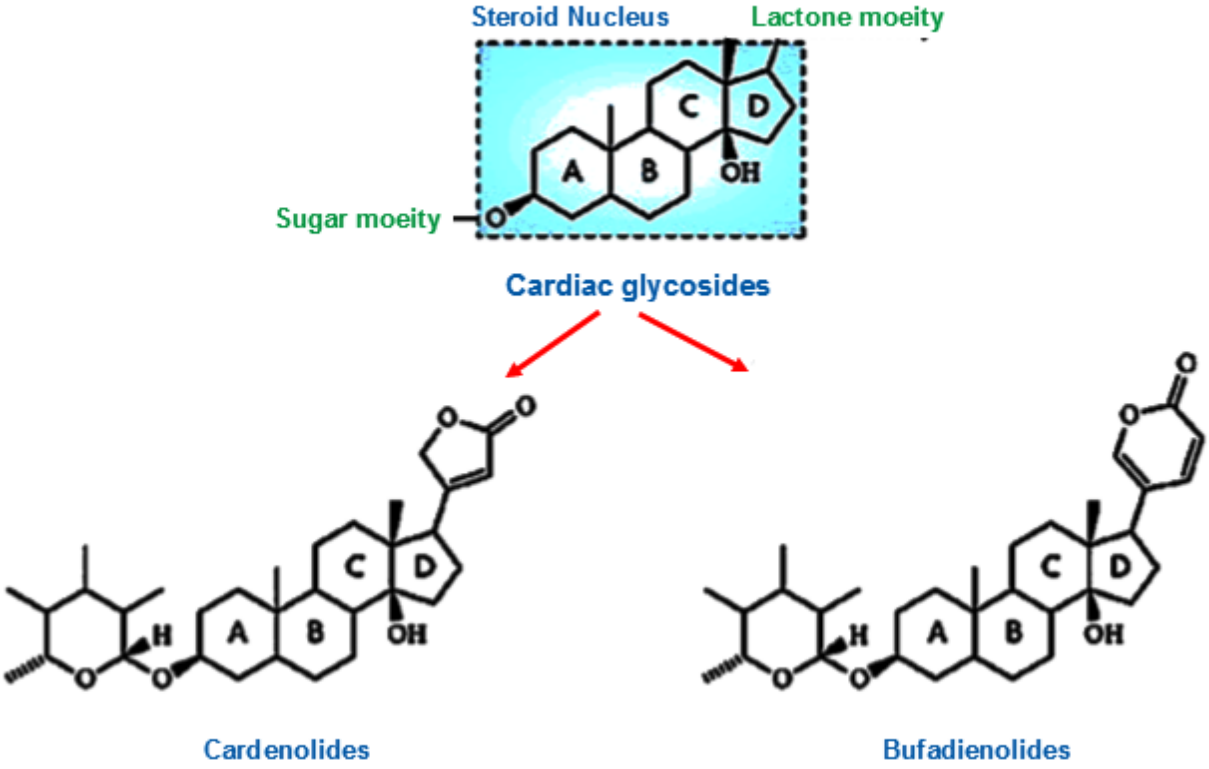

Figure 2.7 Structural types of cardiac glycosides (Parisi and Ventrella, 2014).

2.3.5 Alkaloids

Alkaloids are basic cyclic nitrogen-containing compounds that form salts when combined with acids and produce alkaline solutions when soluble (Harborne, 1973; Gurib-Fakim, 2006; Okigbo *et al.*, 2009). The nitrogen atom/s present in alkaloids are derived from an amino acid and is responsible for the chemical nature and behaviour of the molecule in a biological system (Cseke *et al.*, 2006). Alkaloids are divided into different subgroups based on the ring structure present, i.e. non-heterocyclic and heterocyclic (Cseke *et al.*, 2006).

Table 2.1 Classification of alkaloids

Types	Common Examples	Activity	Plant Source	Reference
Non-heterocyclic alkaloids	Colchicine	anti-inflammatory	*Colchicum* spp.	(Gurib-Fakim, 2006)
	Erythromycin	antibiotic	*Streptomyces erythreus*	(Weber *et al.*, 1985)
	Taxol	antitumour	*Taxus brevifolia*	(Cragg and Newman, 2005)
Heterocyclic alkaloids				
Pyrrolidine	Hygrine	hepatotoxic	*Coca* sp.	(Pictet, 1904)
Piperidine	Piperine	antibacterial	*Piper* spp.	(Scott *et al.*, 2008)
Pyridine	Nicotine	anti-herbivore	*Nicotiana tabacum*	(Ujváry, 1999)
Pyrrolizidine	Senecionine	hepatoxic	*Senecio homoiensis*	(Aniszewski, 2015)
Indolizidine	Castanospermine	anti-HIV	*Castanospermum australe*	(Taylor *et al.*, 1992)
Tropane	Atropine	anticholinergic	*Atropa belladonna*	(Kamada *et al.*, 1986)
Quinolizidine	Lupinine	nutrition	*Lipinus palmeri*	(Kinghorn and Balandrin, 1984)
Indole	Reserpine	antihypertensive	*Rauwolfia serpentina*	(Wilkins and Judson, 1953)
Quinolone	Quinine	flavouring-tonic water and bitter lemon	*Cinchona pubescens*	(Minor and Date, 2007)
Isoquinoline	Morphine	narcotic antagonist	*Papaver somniferum*	(Unterlinner *et al.*, 1999)
Acridine		antibiotic	*Balsamocitrus paniculata*	(Wainwright, 2001)
Purine	Caffeine	stimulant	*Coca* sp.	(Cseke *et al.*, 2006)

2.4 PROBLEMS ASSOCIATED WITH MICROBIAL INFECTIONS

2.4.1 Bacteria

Infectious disease caused by intrusive opportunistic pathogens are one of the leading causes of disease and mortality globally (Bagla, 2011). Despite the use of antimicrobial drugs to combat infectious diseases, some pathogens develop antibiotic resistance to drugs, creating a myriad of challenges for the global health sector. For example, between 70-80% of *Staphylococcus aureus* strains are resistant to methicillin and between 90-95% of these strains are penicillin resistant (Hemaiswarya *et al.*, 2008). Resistance comes about by active or passive means as a result of horizontal gene transfer from another microbe or an inherent mechanism, which often leads to disastrous consequences. Antibiotic resistance is characterized by contact inhibition of the drug with the active site, efflux of the antibiotic from the cell and complete destruction or modification of the compound (Wright, 2005) (Figure 2.8). An additional challenge is selective pressure posed by various antibacterial drugs which results in molecular mechanisms that produce multi-drug resistance (MDR) in bacteria (Wright, 2005; Abreu *et al.*, 2012).

Poverty, limited access to proper medical care, political conflicts and an absence of commitment from governments of third world countries are partly responsible for treatment struggles encountered. This negatively impacts efforts to control communicable diseases (Hancock, 2005). Additional influences within well-established medical environments include misuse of broad-spectrum antibiotics, absence of cautionary judgement when administering treatment (Hancock, 2005) and pressure from the numbers of diseased patients that are able to spread a number of resistant microbes, e.g. vancomycin resistant *Enterococcus faecalis* (VREF) (Bonten *et al.*, 1998) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Merrer *et al.*, 2000; Bagla, 2011). Lengthy intensive care unit (ICU) stays (Bonten *et al.*, 1998) and the use of intrusive devices such as endotracheal tubes and catheters (Richards *et al.*, 1999), increases exposure time to hospital-acquired infections. Since the same antibiotics are also used to treat infections, promote growth and mass prophylaxis in animals, there is also a risk of resistant bacteria being passed to humans via the food chain (Prescott and Dowling, 2013). This increased prevalence of resistance in recent years has innumerable economic and medical consequences increasing the demand for novel drugs of natural origin (Cosgrove and Carmeli, 2003).

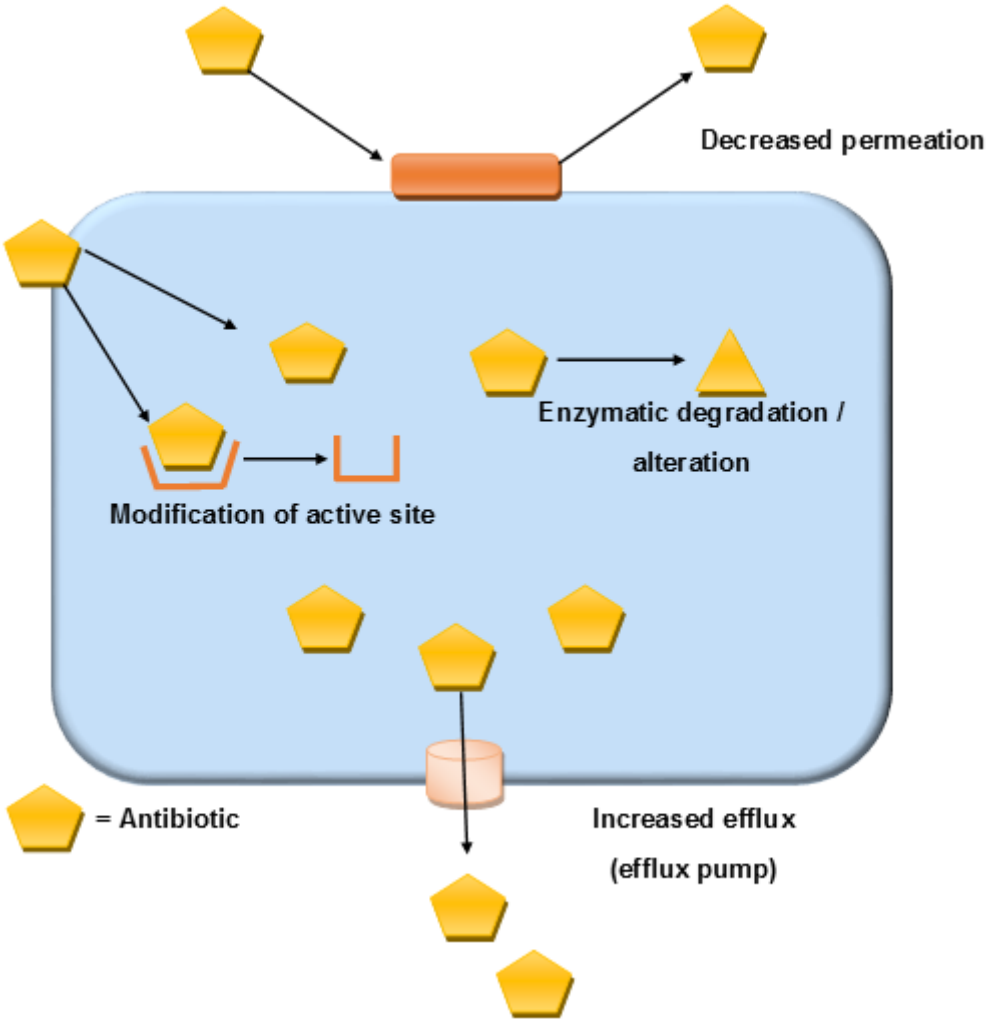

Figure 2.8 Mechanism of action of antimicrobial resistance by a cell (Abreu *et al*., 2012).

2.4.2 Fungi

In spite of the large increase of antibacterial and antifungal resistance, little attention has been given to antibiotic resistance research, particularly in terms of antifungal resistance. Antifungal resistance refers to a fungal infection that remains unaffected by antifungal treatment (Suleiman *et al.*, 2010). The development of resistance can be primary (intrinsic), where an organism is resistant prior to antifungal treatment or secondary (acquired), where the organism undergoes transient genotypic modification after exposure to an antimycotic (Figure 2.8) (Suleiman *et al.*, 2010). Another type of antifungal resistance is what is referred to as 'clinical resistance'. This type of resistance occurs during *in vitro* testing and stems from recurrence or progression of a fungal infection due to an isolate's susceptibility to an antifungal agent that was used to treat an infection (Bagla, 2011). This type of resistance is common amongst patients that have been dosed at substandard levels, fitted with prosthetic material or immuno-compromised (Sheehan *et al.*, 1999).

When exposed to a fungal pathogen, an antifungal agent stimulates various responses in an organism's metabolism (Bagla, 2011). As a survival strategy, the fungal pathogen develops mechanisms to circumvent the growth inhibitory action of the antifungal agent. This in turn, allows the growth of a typical susceptible fungal pathogen to occur at a higher drug concentration. In cases where growth is inhibited by higher drug concentrations, the pathogen is capable of altering the effectiveness of the antifungal agent which may result in either a fungicidal or fungistatic effect. This is known as antifungal drug tolerance (Sanglard, 2003).

Amphotericin B was the only drug available in the early 1960s for the treatment of systemic mycoses until the imidazoles and triazoles were introduced in the 1980s and 1990s (Pappas *et al.*, 2015). Amphotericin B is still known as the "gold standard" drug for the treatment of severe fungal infections though (Sanglard, 2003). The emergence of these antifungal agents led to their extensive use and subsequent evolution of resistant strains (Rex *et al.*, 1995). In recent years, mechanisms of azole resistance has been widely researched but resistance to echinocandin and polyene are poorly understood (Kanafani and Perfect, 2008).

The limited number of antifungal agents available is inadequate to counteract the rise of invasive fungal infections. Inaccessibility in some countries, coupled with toxicity and poor uptake of medication, antifungal resistance remains a pronounced threat and can become a fundamental factor in determining the future outcome of antifungal therapy.

2.4.3 Phytocompounds implicated in antimicrobial activity

Due to the chemical diversity and defence mechanisms of phytocompounds against microbes in their natural environment, plant extracts are investigated for the treatment infectious diseases caused by bacteria and fungi (Abreu *et al.*, 2012). The antimicrobial activity of these phytocompounds is probably due to their ability to complex with cell wall components of microbes (Mumbengegwi *et al.*, 2016). Increased side effects, antimicrobial resistance and the exorbitant prices associated with synthetic drugs has promoted human interest in phytochemicals and subsequently increased popularity of herbal remedies (Abreu *et al.*, 2012). Plants that possess antimicrobial activity have the potential to elucidate a novel molecule that could be further derived by chemical means for potential treatment of drug resistant strains of pathogenic microbes, thus, possessing greater efficacy than synthetic drugs.

2.5 CANCER AND PLANT-DERIVED ANTICANCER AGENTS

Cancer is the second leading cause of premature deaths worldwide (Stratton *et al.*, 2009) and is characterised by the uncontrolled abnormal proliferation of cells (Richard *et al.*, 2015). It results in malignant tumours that attack connecting regions of the body and has the ability to metastasise (WHO, 2015). In 2012, cancer was responsible for 8.2 million deaths and the

number of cases is expected to rise by 70% within the next 20 years (WHO, 2015). Over 200 types of cancer exist with colorectal, breast, lung, stomach, oesophageal and liver cancer being amongst the most common (Siegel *et al.*, 2015). Cancer is caused by a number of factors which include genetic predisposition, ionising and ultra violet radiation, smoking, alcohol, obesity, environmental toxins, oxidative stress and diseases caused by microorganisms (Richard *et al.*, 2015). Cancer treatment involves chemotherapy, radiation and/or surgery (Simoben *et al.*, 2015). These procedures are accompanied by resistance and side effects whilst the use of medicinal plants provides a safer and more comfortable alternative (Weber *et al.*, 1985).

The first plant-derived anticancer agents to advance to clinical use were the vinca alkaloids, vinblastine and vincristine (Figure 2.9), isolated from the Madagascar periwinkle, *Catharanthus roseus* (Johnson, 1968). Vinblastine is used in the treatment of Kaposi's sarcoma, lymphomas, leukemias, breast, lung and testicular cancers. Vincristine is used to treat lymphomas and leukemias, particularly, acute lymphocytic leukemia in children (Pui and Evans, 2013). Semi-synthetic analogues of the vinca alkaloids most recently discovered are vinorelbine and vindesine which when used in conjunction with other anticancer agents, are able to treat many cancers. Vinorelbine has exhibited activity against advanced breast carcinoma and small cell lung cancer (Newman and Cragg, 2012; 2015).

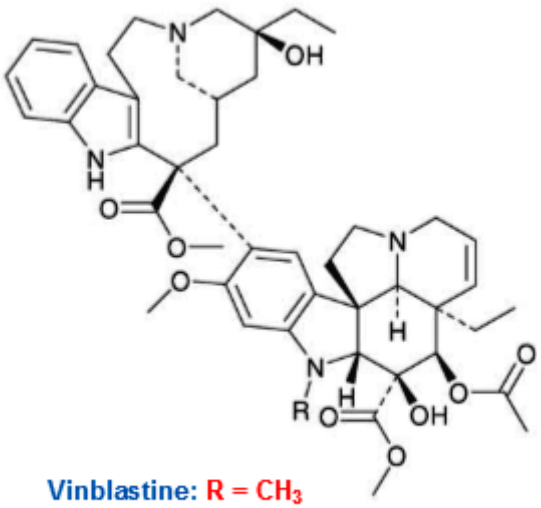

Figure 2.9 Vinca alkaloids, vinblastine and vincristine, isolated from *Catharanthus roseus* (Cragg and Newman, 2005).

Podophyllotoxin is a cyclolignan isolated from a plant resin (podophyllin) produced in a species belonging to the genera *Podophyllum* (Gordaliza, 2007; Stratton *et al.*, 2009). It is employed in the treatment of various types of genital tumours, Wilms' tumours, lung cancer, and non-Hodgkin's lymphomas (Gordaliza, 2007). Podophyllotoxin is also used in combined

therapy for enhanced efficacy. Examples of these include the treatment of neuroblastomas, where podophyllotoxin is used in conjunction with cisplatin. When used in combination with methotrexate and general polychemotherapy, podopyllotoxin is effective against multiple myeloma (Gordaliza, 2007).

On investigation of podophyllotoxin, three semi-synthetic derivatives with antineoplastic activity were synthesised from its isomer, epipodophyllotoxin, viz. etoposide, etopophos (prodrug of etoposide) and teniposide (Figure 2.10) (Cragg and Newman, 2005). They are used to treat many types of cancers, including leukemia, Kaposi's sarcoma, small cell lung cancer, testicular cancer and colon cancer (Gordaliza, 2007; Cragg and Newman, 2013).

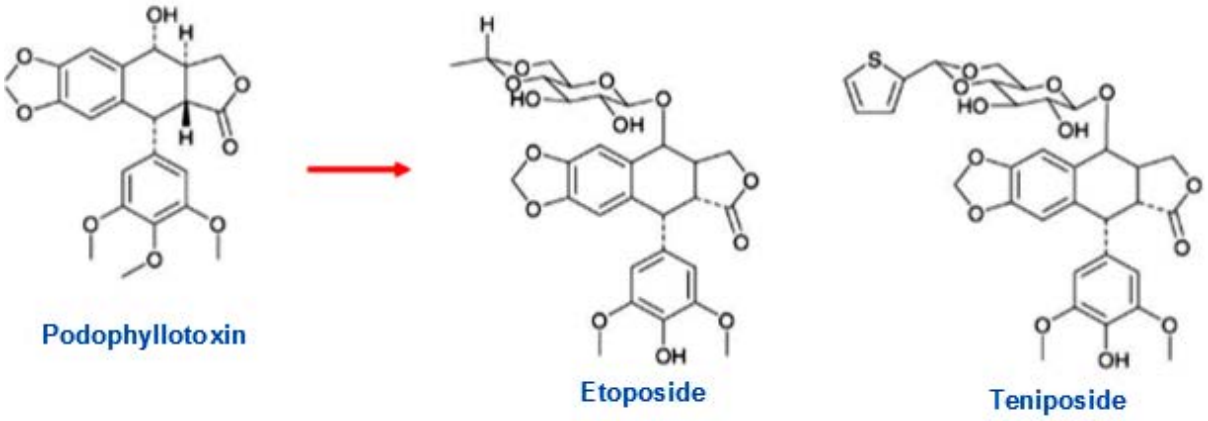

Figure 2.10 Podophyllotoxin isolated from *Podophyllum peltatum* with two semi-synthetic derivatives employed in cancer treatment (Lakshmi *et al*., 2015).

Camptothecin is a quinoline alkaloid whose anticancer activity was ascertained in 1958 (Wall *et al.*, 1966). It is isolated from the *Camptoteca acuminata* tree found in China and Tibet (Gordaliza, 2007). Camptothecin along with its derivatives inhibit DNA topoisomerases, thereby inhibiting DNA replication, and thus cellular proliferation (Wall *et al.*, 1966). Camptothecin was subsequently chemically modified to decrease its toxicity (Kingsbury *et al.*, 1991) and more efficient derivatives, topotecan and irinotecan (Figure 2.11) were developed (Cragg and Newman, 2013; Lakshmi *et al.*, 2015). Topotecan is used in the treatment of ovarian cancer and small cell lung cancer (Cragg and Newman, 2013) whilst irinotecan is used in the treatment colorectal cancer (Cersosimo, 1998).

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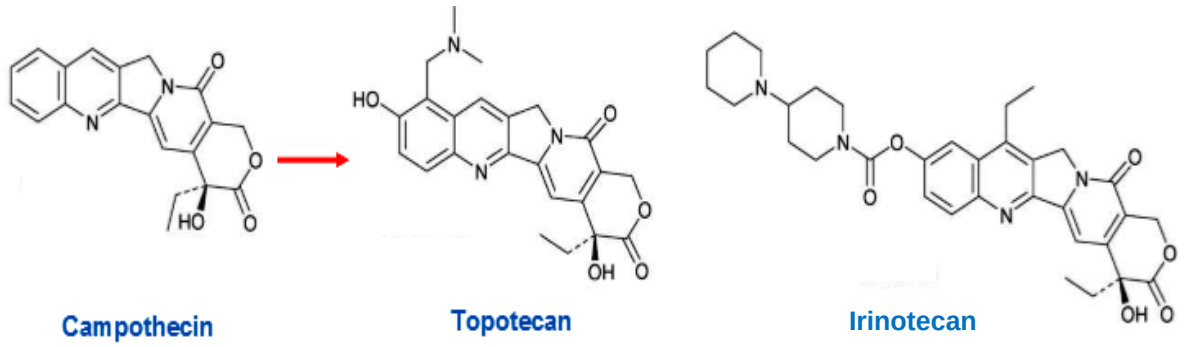

Figure 2.11 Campothecin isolated from *Camptoteca acuminata* and two semi-synthetic derivatives used to treat many types of cancers (Lakshmi *et al*., 2015).

Paclitaxel (taxol) (Figure 2.12), from the class taxanes, is a diterpene isolated from the tree bark of *Taxus brevifolia* found in North America (Cragg and Newman, 2015). It was later found that paclitaxel together with several other precursors (baccatins) were present in other species of the genus *Taxus* (Cragg and Newman, 2013). Taxol is currently used in the treatment of ovarian cancer and shows promising potential in the treatment of breast, head, lung and neck cancer.

Docetaxel (Figure 2.12) is a semi-synthetic derivative of paclitaxel that displays potent neoplastic activity against breast cancer and small cell lung cancer (Gordaliza, 2007). This potency is attributed to its improved water solubility (Gordaliza, 2007).

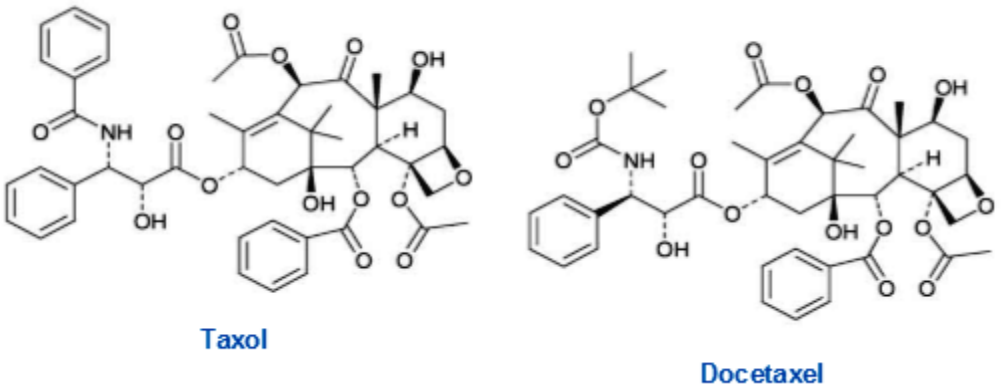

Figure 2.12 Taxol isolated from *Taxus brevifolia* and the semi-synthetic derivative Docetaxel used to treat breast and small cell lung cancer (Cragg and Newman, 2005).

2.6 EXTRACTION, ISOLATION AND CHARACTERISATION OF BIOACTIVES FROM PLANT MATERIAL

2.6.1 Extraction of biologically active compounds

Once the desired plant material has been harvested, extraction is the first crucial step to obtaining bioactive compounds possessing pharmacological potential. This process includes the washing, drying (air- or freeze-drying) and grinding of plant material (Sasidharan *et al.*, 2011). Selection of a solvent system also occurs at this stage and is based on the desired end product one wishes to attain. Hydrophilic compounds are extracted using polar solvents like ethanol and methanol while lipophilic compounds are extracted using dichloromethane (Brusotti *et al.*, 2013). Methods of extraction commonly employed are soxhalation, sonication or cold percolation, amongst many others. Various new age extraction techniques are also employed and some of these include solid-phase extraction, microwave-assisted extraction and supercritical-fluid extraction (Sasidharan *et al.*, 2011).

2.6.2 Screening of biologically active compounds

Assessing the biologically activity of a particular plant extract is implemented to scientifically validate the use to that plant in traditional medicine (Brusotti *et al.*, 2013). These *in vitro* bioassays are commonly employed to assess the antimicrobial, antioxidant, antitumour and enzyme activity of a particular plant extract or pure compound. The activity of a crude extract or pure compound is generally considered noteworthy if the inhibitory concentration required to achieve half maximal inhibition (IC_{50}) is below 100 µg/mL or 25 µM respectively (Cos *et al.*, 2006). Once biological activity has been established, the extract is then subjected to purification and isolation of the biologically active compound/s (Azmir *et al.*, 2013).

2.6.3 Identification and characterisation of biologically active compounds

Plant extracts occur as a combination of biologically active compounds or phytocompounds with differing polarities. These compounds are sometimes difficult to separate rendering their isolation and characterisation challenging (Sasidharan *et al.*, 2011). It is common practice to use a variety of separation techniques in order to obtain pure bioactive compounds and these include column chromatography, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) to name a few. These pure compounds subsequently undergo structural elucidation via Proton Nuclear Magnetic Resonance (^{1}H-NMR) Gas Chromatography-Mass Spectroscopy (GC-MS), Liquid Chromatography-Mass Spectroscopy (LC-MS) or Fourier-transform infrared spectroscopy (FTIR) to associate activity with structure, thus creating a basis for drug development (Sticher, 2008) (Figure 2.13).

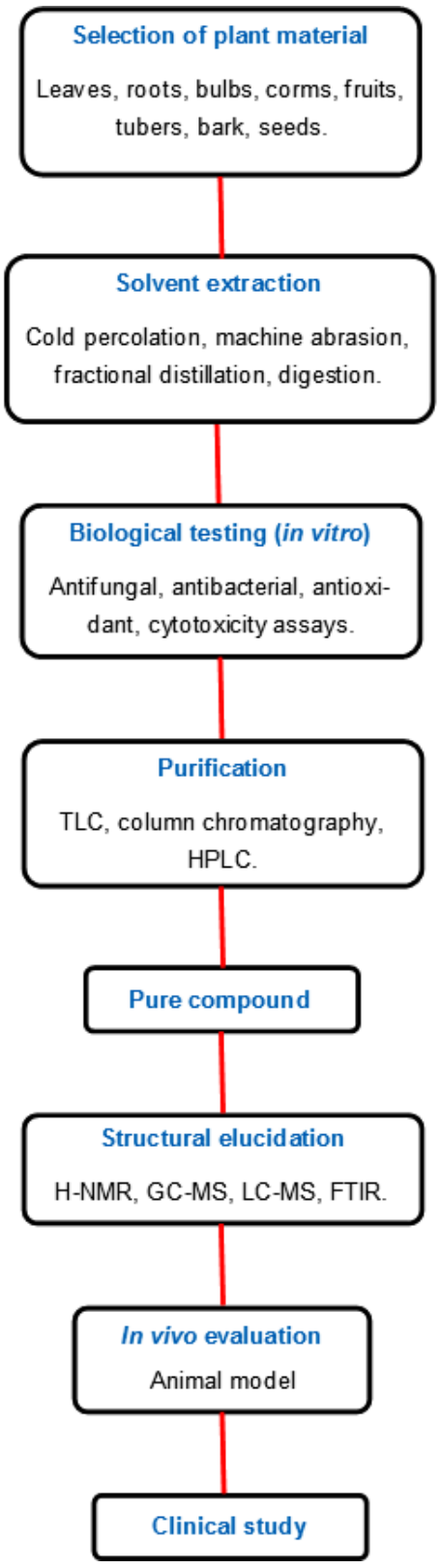

Figure 2.13 Summary of the general approaches in extraction, isolation and characterisation of biologically active compounds (Brusotti *et al*., 2013).

2.7 *Trichilia emetica* Vahl.

Trichilia emetica commonly known as the Natal mahogany is a member of the Meliaceae family and is derived from the Greek word “tricho” making reference to the three-lobed fruits while “*emetica*” refers to the trees emetic properties (Allaby, 2012). It is an evergreen tree reaching up to 20-35 m in height and has red-brown or grey-brown bark and the leaves are dark glossy green on the upper surface and covered with brownish hairs on the lower surface (Allaby, 1998). The flowers are small, creamy to pale yellow-green, and fragrant. The furry, rounded, red-brown fruit capsules (±3 cm across), contain 3-6 shiny black seeds (1.4-1.8 cm) with a large fleshy scarlet or orange-red aril (Figure 2.14) (Orwa *et al.*, 2009). *T. emetica* is widely distributed and grows naturally throughout sub-Saharan Africa extending from KwaZulu-Natal in the south, through Swaziland, Mpumalanga and Limpopo Provinces (in South Africa), into Zimbabwe and northwards into Cameroon, Sudan and Uganda (Germishuizen and Meyer, 2003). It grows in warm and frost free environments and prefers areas with high rainfall with moist, heavy soil and is therefore abundant along rivers in low altitude areas (Cronquist, 1981; Orwa *et al.*, 2009).

T. emetica is a multipurpose tree that has been used throughout Africa for many centuries. The seeds are rich in oil which is used for the manufacture of natural soaps, candle making, lip balm therapy and various other cosmetic purposes (Von Breitenbach, 1987; Orwa *et al.*, 2009). *T. emetica* has a suite of uses in African folk medicine. *T. emetica* combined with *Cyathula natalensis* Sond. is known to treat leprosy and in Senegal, is used to treat a range of ailments affecting the skin due to its oil being rich in essential fatty acids (Oliver-Bever, 1986). In South Africa, the Zulu people use the leaves and stem bark to provide relief from severe backache whilst the Xhosa people use the stem bark to treat kidney-related issues and as an enema (Watt and Breyer-Brandwijk, 1962). In some instances, *T. emetica* oil is combined with coconut oil and is used as a moisturiser by people in rural areas. The oils produced by *T. emetica* was originally used in the production of cocoa butter derivatives and served as the starting material in its lipase catalysis (Grace *et al.*, 2008). The types of oil found in the pressed seeds include solid butter and mafura oil extracted from the fleshy seed and the kernel respectively (Grace *et al.*, 2008).

Although there are many reports on the bioactivity of various parts of *T. emetica* (Tahir *et al.*, 1999; Komane *et al.*, 2011; Vieira *et al.*, 2014), to the best of our knowledge, this is the first report on the biological screening of the crude seed extracts.

Figure 2.14 Seeds of *T. emetica* (Phytotrade Africa, 2012).

2.8 *Protorhus longifolia* (Bernh. Ex C. krauss) Engl.

P. longifolia is a tall (can grow up to 15 m), evergreen tree belonging to the Anarcardiaceae family and is commonly known as Red-beech (Mosa *et al.*, 2014a). It is the only species of the genus *Protorhus* indigenous to Southern Africa whilst the other species are found mainly in Madagascar (Archer, 2000). *P. longifolia* is found in forests, open woodlands and on riverbanks of the Northern Province, Mpumalanga, Eastern Cape and KwaZulu-Natal and is extremely resistant to desiccation (Mosa *et al.*, 2014a). It has glossy, dark green leaves, greenish-white flowers and purple fruit, each containing a single seed (Figure 2.15) (Mosa *et al.*, 2014a).

No previous literature has reported on the biological screening of the seeds of *P. longifolia* except for that of the aqueous seed extract. It has successfully been manipulated in the synthesis silver and gold nanoparticles, having displayed potential antibacterial activity (Gannimani *et al.*, 2014).

Figure 2.15 Seeds of *P. longifolia* (Mosa, 2014b).

2.9 CONCLUSION

The identification of pharmacologically active compounds exhibiting antimicrobial, antioxidant and anticancer activity is indicative of the tremendous nutraceutical potential of plant sources. The main objectives of most current day research on plant phytochemicals are:

- To isolate biologically active compounds for subsequent use as pharmaceutical drugs.
- To produce bioactives, of known or novel molecular structures, as innovative compounds employed in semi-synthesis to enhance activity and/or diminish toxicity.
- To successfully use the entire plant or segments of it as herbal medicine (Fabricant and Farnsworth, 2001)

Bioactivity guided isolation from crude extracts have the potential to provide fractions or constituents with pharmacological activity which can substitute synthetics drugs with naturally-derived drugs of equal efficacy.

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Chapter 3

RESEARCH RESULTS 1

Pharmacological and chemical evaluations of crude seed extracts of *Trichilia emetica*

Pharmacological and chemical evaluation of crude seed extracts of *Trichilia emetica*

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3.1 ABSTRACT

Trichilia emetica Vahl. is a tree species belonging to the Meliaceae family. It is commonly used throughout South Africa in ethnomedicine to treat a variety of ailments. The phytochemical profile, antimicrobial, anticancer and antioxidant properties of the leaves, roots and stem bark extracts have been reported. However, the same cannot be said for the seed extracts of *T. emetica*. This study assesses the biological activity and the phytochemical profile of the seed extracts of *T. emetica*. The seed samples were extracted using solvents of different polarity to obtain crude seed extracts. Phytochemical screening was performed qualitatively via a series of reactions and quantitatively using gas chromatography-mass spectroscopy (GC-MS). The crude seed extracts were subjected to antimicrobial testing using disc diffusion and the broth microdilution assay. The free radical scavenging potential of crude seed extracts was determined using the DPPH test. Cytotoxicity testing was performed using the human breast adenocarcinoma (MCF-7) and the green monkey kidney (Vero) cells. The phytochemical investigation on the different crude seed extracts indicated the presence of phenols, flavonoids, terpenes, sterols, alkaloids and glycosides. No bactericidal activity was noted. Antifungal activity was noted for methanol, hexane and chloroform crude seed extracts with the methanol extract displaying the greatest inhibition (37.46 µg/mL). The methanol seed extract was the only extract to display strong antiradical activity with an inhibitory concentration required to achieve a half maximal inhibition (IC_{50}) of 5.94 µg/mL. No cytotoxic activity was noted towards MCF-7 and Vero cells. The observed antioxidant and antifungal activities of a few crude seed extracts are worthy of further investigation. These findings support the use of *T. emetica* in traditional medicine.

3.2 INTRODUCTION

By the late 1980s, approximately 65% of the global population relied on/used plants as a means of primary health care (Farnsworth, 1988; Shai *et al.*, 2008). In South Africa, 60-80% of the population depend on the traditional use of plants as a source of medical relief from both human and animal ailments (Dauskardt, 1990). An estimated 65% of plants used medicinally throughout the world are tree species, many of which are slowly becoming endangered due to unsustainable harvesting (Gates, 2000). Approximately 10% of the documented South African tree species are threatened (Golding, 2002; Siebert and Smith, 2005). This is of great concern as some indigenous tree species may become extinct before their potential curative value has been investigated.

T. emetica is an evergreen tree, native to Africa (Oliver-Bever, 1986). The surfeit of applications established for *T. emetica* in traditional medicine has engaged the curiosity of scientists, thus encouraging screening for a broad range of biological and pharmacological activities, before incorporation into the country's official health care system. Different plant material of *T. emetica* are used traditionally for the treatment of different diseases (Diallo *et al.*, 2003). Powder obtained from roots are used to treat abdominal pains, dysmenorrhoea and hepatic disorders (Mashungwa and Mmolotsi, 2007). The stem bark is employed in the treatment of bronchial inflammation and fever (Mashungwa and Mmolotsi, 2007). The leaves are used to treat malaria and the fruit act as a diuretic (Sanogo, 2011).

A study conducted on leaf extracts of *T. emetica* displayed good antioxidant activity (Frum and Viljoen, 2006). Leaves (Shai *et al.*, 2008) and root extracts of *T. emetica* exhibited promising antibacterial activity (Germano *et al.*, 2005; Komane *et al.*, 2011) whilst fruit extracts showed inhibition of fungal growth (Geyid *et al.*, 2005). Root extracts of *T. emetica* also exhibited proliferation inhibition of MCF-7 and murine sarcoma (S180) cells (Traore *et al.*, 2007). Additionally, *T. emetica* also exhibited anti-inflammatory (McGaw *et al.*, 1997), antischistosomal (Sparg *et al.*, 2000), antiplasmoidal (Prozesky *et al.*, 2001), anticonvulsant (Bah *et al.*, 2007), antitrypanosomal (Hoet *et al.*, 2004), antitussive (Sutovska *et al.*, 2009), antimutagenic (Verschaeve and Van Staden, 2008) and hepatoprotective properties (Germano *et al.*, 2005).

The only known traditional use of *T. emetica* seeds are for cosmetic purposes (Van Wyk, 2015). This has resulted in the potential medicinal use of these seeds being an unexplored area of interest. The aim of the present research study was to determine the phytochemical constituents, *in vitro* antimicrobial, antioxidant, and antitumour activity of crude seed extracts of *T. emetica.*

3.3 MATERIALS AND METHODS

3.3.1 Reagents

Chemicals and growth media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa unless otherwise stated. Solvents used for extraction were of high performance liquid chromatography (HPLC) grade and were purchased from Sigma-Aldrich (USA). The CellTiter 96® nonradioactive cell proliferation assay kit was purchased from Promega Corporation (USA).

3.3.2 Seed material

Mature seeds were collected from *T. emetica* trees growing in St. Lucia, KwaZulu-Natal, South Africa (13 53 N, 60 58 W). The aril was removed and the seeds were air-dried at room temperature (25°C) for several days; after which, they were crushed to a fine powder and stored in air tight containers for subsequent use in solvent extractions.

3.3.3 Extract preparation for *in vitro* assays

Six seed extracts were prepared by the cold percolation method (Parekh and Chanda, 2007) using several organic solvents, viz. methanol, ethanol, ethyl acetate, hexane, chloroform and distilled water. For each extraction, 1 mL of distilled water and 1 mL of the respective solvent was added to 100 mg of dried powdered seed material in several microcentrifuge tubes, vortexed for 1 min, parafilmed and left at room temperature. After 24 h, the microcentrifuge tubes containing the extract was centrifuged (centrifuge 5417R, Eppendorf, Germany) at 5000 rpm for 10 min, the supernatant was collected and the solvent left to evaporate for a further 24 h. After 24 h, any solvent that did not evaporate was put into a concentrator (Eppendorf, Germany) until there was no trace of solvent.

3.3.4 Preliminary phytochemical analysis

To determine the presence of secondary metabolites, extracts were also prepared by cold percolation (Parekh and Chanda, 2007) as described in section 3.3.3 but the supernatants were not allowed to evaporate, but rather used directly. For these studies 5 mL of each solvent and 5 mL of distilled water were added to 500 mg of dried powdered seed material in several microcentrifuge tubes, vortexed for 1 min, parafilmed and left at room temperature. After 24 h, the microcentrifuge tubes containing the extract was centrifuged at 5000 rpm for 10 min, the supernatant was collected used in a range of phytochemical assays described below. All these qualitative analyses were carried out in triplicate for all solvent extracts, with

each experiment being repeated twice according to the methods of Harborne (1973) and Trease and Evans (1978).

3.3.4.1 Test for alkaloids

Dragendorff's reagent test

Two mL of Dragendorff's reagent (potassium bismuth iodide solution) and 2 mL of diluted hydrochloric acid were added to 1 mL of seed extract. A reddish brown precipitate was considered to be indicative of the presence of alkaloids.

3.3.4.2 Test for flavonoids

NaOH test

One mL of 1N NaOH solution was added to 1 mL of seed extract. Formation of a yellow colour demonstrated a positive presence of flavonoids.

3.3.4.3 Test for cardiac glycosides

Keller-Killani test

One mL of glacial acetic acid was carefully added to 2 mL of seed extract and mixed well. Thereafter, 2 drops of 5% ferric chloride ($FeCl_3$) solution was added after cooling to room temperature. This solution was transferred carefully to a test tube containing 2 mL of concentrated sulphuric acid ($H_2SO_{4)}$. The formation of a reddish brown ring at the junction of two liquid layers was considered to be indicative of the presence of glycosides.

3.3.4.4 Test for terpenoids

Salkowski test

Five mL of seed extract was added to 2 mL of chloroform. Thereafter, 3 mL of concentrated H_2SO_4 was slowly added to form a layer at the interface. A reddish brown colour at the interface was considered to be indicative of the presence of terpenoids.

3.3.4.5 Test for steroids

Lieberman-Buchard test

Two mL of acetic anhydride was added to 5 mL of seed extract. Thereafter, 1 mL of H_2SO_4 was carefully added. The formation of a blue-green colour indicated the presence of steroids.

3.3.4.6 Test for saponins

Foam test

Five mL of seed extract was shaken vigorously in 20 mL distilled water. Formation of a stable honeycomb-like foam was an indication of the presence of saponins.

3.3.4.7 Test for phenols

Phenol test

On the addition of 0.5 mL $FeCl_3.6H_2O$ (w/v) solution to 2 mL of extract, the formation of an intense dark green colour was considered to be indicative of the presence of phenols.

3.3.4.8 Test for tannins

Gelatin test

The test solution was evaporated to dryness and the resultant residue was dissolved in 1% (w/v) liquefied gelatin; to this was added 10% (w/v) sodium chloride (NaCl) solution. A white precipitate was considered to be indicative of the presence of tannins.

3.3.5 *In vitro* antimicrobial susceptibility testing

3.3.5.1 Test organisms

Gram-negative bacterial strains (*Escherichia coli* American Type Culture Collection® (ATCC®) 35218™, *Klebsiella pneumoniae* (ATCC® 700603™), *Pseudomonas aeruginosa* (ATCC® 27853™)) and Gram-positive bacterial strains (*Staphylococcus aureus* (ATCC® 43300™), *Enterococcus faecalis* (ATCC® 5129™), were procured from the Biochemistry Department, School of Life Sciences, University of KwaZulu-Natal, Westville, South Africa.

Yeast strains (*Candida albicans* (ATCC® 10231™)*, Candida krusei* (ATCC® 6258™) and *Candida parapsilosis* (ATCC® 22019™) were procured from the National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Hospital, Durban, South Africa.

3.3.5.2 Storage and maintenance of microbial cultures

Test organisms were preserved in 15% (v/v) glycerol solution within sterile cryovials (Greiner, Germany) and stored at -80°C until required. Mueller-Hinton (MH) agar and Sabouraud Dextrose (SD) agar were prepared for bacterial and fungal cultures, respectively. Media was sterilised and poured into plastic Petri dishes and allowed to solidify. Plates were then sealed using parafilm and stored at room temperature overnight to ensure sterility before stock cultures were sub-cultured by adding 10 µL of culture to a petri dish and a four-way streak performed. Petri dishes were then incubated (at 37°C and 30°C for bacteria and fungi, respectively) for 24 h, and subsequently stored at 4°C until required for bioassays. Mueller-Hinton and SD broth were prepared for the bioassays. Single colonies of each bacterial and fungal culture were used to inoculate 20 mL of MH broth and SD broth,

respectively. These cultures were then incubated (37°C and 30°C for bacteria and fungi, respectively) in an Infors HT Multitron environmental shaker (United Scientific, South Africa) at 160 rpm for 24 h.

3.3.5.3 Screening for antimicrobial activity

All the antimicrobial assays described below were carried out in a Class II, Type A2 microbiological safety cabinet (Airvolution, South Africa).

3.3.5.3.1 Disc diffusion (antibacterial and antifungal)

The sensitivity of various strains of bacteria and fungi were examined via a modified disc diffusion method (Bauer *et al*., 1966). Bacterial cultures prepared in sterile MH broth were measured spectrophotometrically (Analytik Jena Specord 210, Germany) at 630 nm to obtain an absorbance of 0.08-0.1, equivalent to the No. 0.5 McFarland constant. Cultures that did not meet this standard were diluted further with broth to obtain the desired absorbance. Petri dishes containing MH agar and SD agar were inoculated with either bacteria or fungi using a sterile swab. Discs (6 mm diameter) were prepared by punching holes into Whatman filter paper No.1. These discs were autoclaved and subsequently placed onto the agar using sterile forceps. Ten µL of each extract (400 µg/mL) was placed onto a single disc. The Petri dishes were incubated statically overnight at 37°C for bacteria and 30°C for fungi, after which, the zones of bacterial and fungal inhibition was recorded using callipers. Neomycin and Amphotericin B were used as positive control agents for bacteria and fungi, respectively whilst the various solvents used to prepare the seed extracts and 5% (v/v) dimethyl sulfoxide (DMSO) were used as negative control agents. Antimicrobial activity of each extract was assayed in triplicate and the experiment performed twice.

3.3.5.3.2 Minimum Inhibitory Concentration (MIC) for antibacterial determination

A modified broth microdilution assay (Eloff, 1998) was used to determine the MIC of extracts against the aforementioned bacteria. Dilutions of extracts were prepared using 5% (v/v) DMSO to give different concentrations (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL). Forty µL of each concentration of extract was added to a 96-well microtitre plate. Thereafter, bacterial cultures prepared in sterile MH broth were measured spectrophotometrically (Analytik Jena Specord 210, Germany) at 630 nm to obtain an absorbance of 0.08-0.1, equivalent to the No. 0.5 McFarland constant. Cultures that did not meet this standard were diluted further with MH broth to obtain the desired absorbance. Once diluted, 160 µL of bacterial culture in broth was added to wells containing extracts. Neomycin made up using sterile distilled water, at 200 µg/mL, 100 µg/mL, 50 µg/mL, 25

µg/mL, 12.5 µg/mL and 6.25 µg/mL was used as a positive control. Distilled water, different solvents used to prepare the seed extracts and 5% DMSO were used as negative control agents. After a 24 h incubation at 37°C, 40 µl of freshly prepared iodonitrotetrazoliumchloride2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H tetrazolium chloride (INT) solution was added to each microtitre well as an indicator of bacterial growth. The plates were thereafter incubated at 37°C for 4 h, after which, the MIC was assessed spectrophotometrically using a microtitre plate reader (BioTek, Synergy HT, Germany) at 630 nm. Cell growth was determined as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100 \qquad \text{(Equation 1)}$$

The control sample reading was obtained from the untreated wells. The effective inhibitory concentration (IC_{50}) was determined via linear regression analyses. All treated wells were assayed in triplicate and expressed as mean percentage viable cells. The experiment was repeated twice.

3.3.5.3.3 MIC for antifungal determination

The MIC of extracts against the aforementioned *Candida* species was determined using the broth microdilution assay (Eloff, 1998) with slight modifications. Seed extracts were diluted with 5% (v/v) DMSO to yield a range of concentrations: 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. Forty µL of each concentration of extract was added to a 96-well microtitre plate. *Candida* strains that were grown aerobically in sterile SD broth were centrifuged (centrifuge 5417R, Eppendorf, Germany) at 4000 rpm for 5 min, the supernatant removed and cells re-suspended in 1% sterile saline. Turbidity of the cells in saline was measured spectrophotometrically (Analytik Jena Specord 210, Germany) at 625 nm to ensure an absorbance of 0.08-0.1, equivalent to the No. 0.5 McFarland standard following the National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 guidelines (2002). Once the desired absorbance was achieved using saline, the working suspension was diluted 1:20 in a mixture containing RPMI (Roswell Park Memorial Institute) 1640 medium (BioWhittaker™, Lonza) with 0.165 M morpholinepropanesulfonic acid (MOPS) (BioWhittaker™, Lonza) buffered to pH 7.0. The working suspension was further diluted with RPMI (1:50) to obtain a final test inoculum of $1\text{-}5 \times 10^3$ CFU/mL. One hundred and sixty µL of the working inoculum suspension was dispensed into each well containing the extract. Amphotericin B made up using sterile distilled water at 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL, were used as a positive control whilst the different solvents used to prepare the seed extracts and 5% DMSO were used as negative control agents. Plates were then incubated in an aerobic environment at 35°C for 24 h. After

incubation, 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-18sulfophenyl)-2H-terazolium salt) was added directly to each well, incubated at 37°C for 4 h and the absorbance recorded at 490 nm on a microtitre plate reader (BioTek Synergy HT, Germany). Cell growth was determined using Equation 1. IC_{50} values were determined via linear regression analyses. Each extract at their various concentrations were assayed in triplicate and expressed as the mean percentage of viable cells. The experiment was repeated twice.

3.3.6 *In vitro* antioxidant activity

DPPH (2, 2'-diphenly-1-picrylhydrazyl) *free radical scavenging activity*

The DPPH assay was employed to determine the free radical scavenging activity of the all seed extracts, according to a modified method by Burits and Bucar (2000) and Melendez *et al.* (2014). DPPH solution was prepared by adding 3.7 mg of DPPH to 15 mL of methanol. To each well of a microtitre plate, 150 µL of DPPH solution and 50 µL of methanol extract at varying concentrations (200 µg/ml, 100 µg/ml, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL) were added to give a final volume of 200 µL. The plate was then left to incubate in the dark for 30 min, after which the absorbance was read using a microtitre plate reader (BioTek Synergy HT, Germany) at 517 nm. Ascorbic acid served as a positive control whilst methanol was used as a negative control. The different concentrations of each extract were assayed in triplicate and the experiment was repeated twice. Free radical activity was calculated using the following equation:

$$\text{Free radical scavenging activity (\%)} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100 \qquad \text{(Equation 2)}$$

Where A_{sample} = methanol extract of seeds

A_{blank} = methanol

$A_{control}$ = DPPH-methanol

IC_{50} of seed extract and ascorbic acid required to scavenge the DPPH free radical by 50% was calculated using linear regression analysis.

3.3.7 Tissue culture

3.3.7.1 Cell lines

Adherent Green monkey kidney epithelial cells (Vero) and human breast adenocarcinoma cells (MCF-7) were used in the studies described below. Cell lines were obtained from ATCC.

3.3.7.2 Tissue culture techniques

All cell culture techniques were carried out in a Class II, Type A2 biological safety cabinet (Airstream®, USA) under sterile conditions. Vero and MCF-7 cells were routinely cultured and maintained in Dulbecco's Modified Eagle's medium (DMEM) (BioWhittaker™, Lonza) and Eagle's Minimum Essential Medium (EMEM) (BioWhittaker™, Lonza), respectively. Both media contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and L-glutamine (BioWhittaker™, Lonza), supplemented with 10% (v/v) heat-activated fetal bovine serum (FBS) (HyClone™, Thermo Scientific™), 100 unit/mL penicillin, 0.1 mg/mL streptomycin (BioWhittaker™, Lonza) and 0.1% (v/v) sodium pyruvate using standard cell culture protocols.

3.3.7.2.1 Re-suspension of cells and subculturing procedure

Cryogenic vials containing Vero and MCF-7 cells were removed from liquid nitrogen and thawed within 4 min via rapid agitation in water bath set at 37°C. Immediately upon thawing, vials were disinfected with 70% ethanol, opened under aseptic conditions and the contents transferred to a sterile 15 mL Greiner® tissue culture tube. Cells were centrifuged (Hettich Universal Type 1200, Germany) at 2000 rpm for 3 min, the supernatant removed, and re-suspended in 3 mL of fully constituted medium. The cell suspension was transferred to a 75 cm^3 culture flask (Greiner®) containing 12 mL of the appropriate fully constituted medium. Cells were incubated at 37°C in a humidified incubator (Autoflow NU-4850, NuAire, USA) containing 5% CO_2. Cell growth was assessed using an inverted microscope (Olympus CKX41, Germany) at 40 X and 100 X magnification. Once the adherent cells were confluent, they were passaged by removing spent media, rinsing with 10 mL PBS (phosphate buffered saline) (1X) solution BioWhittaker™, Lonza) and the addition of 1.5 mL of Trypsin-EDTA (1X) solution (BioWhittaker™, Lonza). The appropriate culture medium was then added to neutralise action of the Trypsin-EDTA solution in a 1:1 ratio and the cells were split accordingly into the number of required flasks.

3.3.7.3 Cytotoxicity

Cell viability was determined using the Vero cell line by the CellTiter 96® AQueous One Solution Assay as outlined in the Promega Technical Bulletin (2012). It is composed of a tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt) and an electron coupling reagent PES, (phenazine ethosulphate). For cytotoxicity, cells were cultured in RPMI 1640 medium (BioWhittaker™, Lonza) supplemented with 10% FBS. Cells were seeded and incubated for 24 h. Thereafter, different extracts of varying concentrations (200 µg/mL, 150 µg/mL, 100 µg/mL, and 50

µg/mL) were added to wells in microtitre plates. These plates were further incubated for 24 h after which 20 µL of MTS dye was added to each well. After a 3 h incubation period, the absorbance at 490 nm was read using a standard microtitre plate reader (BioTek, Synergy HT, Germany). Cell viability was based on the conversion of the tetrazolium salt MTS by the enzyme hydrogenase to a coloured formazan. The fraction of surviving cells was calculated using Equation 1. The control sample reading was obtained from the untreated wells. All treated wells were assayed in triplicate and expressed as mean percentage viable cells. The experiment was repeated twice.

3.3.7.4 In vitro anticancer activity

Anticancer activity was determined using the MCF-7 breast carcinoma cell line by the CellTiter 96® AQueous One Solution Assay as outlined in the Promega Technical Bulletin (2012). Twenty-four hours after cells were seeded, seed extracts of varying concentrations (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) were added to 96-well microtitre plates. Plates were incubated for 24 h after which 20 µl of MTS dye was added to each well. After a 3 h incubation period, the absorbance at 490 nm was read using a standard microtitre plate reader (BioTek Synergy HT, Germany). Cell viability was calculated using Equation 1. The control sample reading was obtained from the untreated wells. All treated wells were assayed in triplicate and expressed as mean percentage viable cells. The experiment was repeated twice.

3.3.8 Gas Chromatography-Mass Spectroscopy (GC-MS)

Analysis of all the crude seed extracts by GC-MS was carried out using PerkinEmler® Gas Chromatography (Clarus® 580) functioning with Mass Selective Detector (MSD) mass spectrometer (Clarus® SQ8S) instrument that has a built-in auto-sampler. Analysis of all crude seed extract samples were carried out on an Elite-5ms (30 m x 0.25 mm internal diameter x 0.25 µm) column. Oven temperature was programmed to progress from 37-320°C at a rate of 18-25°C/min and detained for 0.5 and 1.85 min at 18 and 320°C, respectively. The temperature of the injector was 250°C with the MS Ion Source temperature being 280°C, with a full scan and solvent delay of 0-2.30 min. MS Scan Range was m/z 35-500 in 0.10 sec. One µL of each crude seed extract sample was injected at a split flow rate of 20 mL/min in helium carrier gas.

3.3.9 Statistical analyses

Statistical analyses were performed on SPSS software, Version 22. Percentage data obtained were arcsine transformed, analysed for normality and thereafter subjected to a One

Way Analysis of Variance (ANOVA). Results were considered significantly different if p values were less than 0.05 (IBM Corporation, 2013).

3.4 RESULTS

3.4.1 Preliminary phytochemical analysis

Preliminary phytochemical analyses, shown in Table 3.1, were performed to determine the presence of secondary metabolites that could potentially confer medicinal properties to the various crude seed extracts of *T. emetica*. The extracts variably displayed alkaloids, cardiac glycosides, phenols, sterols, terpenoids and flavonoids but there was no indication of saponins and tannins in all extracts. The chloroform extract resulted in the highest diversity of secondary metabolites while distilled water resulted in the least. The most commonly occurring secondary metabolites (across the various extracts) were sterols and glycosides, whilst terpenoids were only found in the methanol extract.

Table 3.1 Phytochemical analyses of crude extracts of *T. emetic* seeds

Secondary metabolites	Methanol extract	Ethanol extract	Ethyl acetate extract	Hexane extract	Chloroform extract	Distilled water extract
Alkaloids	-	-	+	+	+	-
Cardiac glycosides	+	+	+	-	+	-
Phenols	+	-	+	+	+	-
Sterols	-	+	+	+	+	-
Flavonoids	+	+	-	-	+	+
Saponins	-	-	-	-	-	-
Terpenoids	+	-	-	-	-	-
Tannins	-	-	-	-	-	-

Key: -: not detected; +: detected

3.4.2 *In vitro* antimicrobial activity

3.4.2.1 Disc diffusion

Disc diffusion is one of many methods employed to determine the ability of antibiotics in hindering microbial growth. It relies on the supposition that antibiotics have the ability to freely diffuse in a semi-solid nutrient enriched agarose medium (Bonev *et al.*, 2008). For this experiment, paper discs were impregnated with the six crude seed extracts at a concentration of 400 μg/mL. The formation of clear zones around the disc were indicative of antimicrobial growth inhibition.

The results for antibacterial and antifungal activity (Table 3.2) indicate that none of the crude seed extracts of *T. emetica* exhibited antibacterial activity. These results are validated by the fact that neomycin, which was employed as the positive control, and, inhibited the growth of all bacterial species used in this study at 400 μg/mL. The seed extracts did, however, exhibit antifungal activity: the ethyl acetate extract inhibited the growth of *Candida krusei* only (8 mm), while all *Candida* species were inhibited by the chloroform extract and the positive control, Amphotericin B.

3.4.2.2 Minimum Inhibitory Concentration (MIC)

The MIC is defined as the lowest concentration of extract that is responsible for an almost complete inhibition of microbial growth in a broth culture (Gulluce *et al.*, 2007; Lawal *et al.*, 2015). The reference drug Neomycin was used as a positive control in this study. None of the extracts in the tested concentration range inhibited bacterial growth (Table 3.3). The hexane and chloroform fractions exhibited good activity (40.95-100 and 76.27-100.11 μg/mL, respectively) against all three fungal pathogens, whilst the methanol extract inhibited *C. krusei* and *C. parapsilosis* and the ethyl acetate inhibited *C. parapsilosis* only. In contrast, the ethanol and aqueous extracts displayed no antifungal activity. These data suggest that the extracts were least effective against *C. albicans* and most effective against *C. parapsilosis* (particularly, in terms of the methanol, ethyl acetate and hexane extracts).

Table 3.2 Antimicrobial activity of crude extracts *T. emetica* seeds (active concentration of 400 µg/mL)

	Zone of inhibition (mm)						
Bacterial species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Neomycin
E. coli	0	0	0	0	0	0	15 ± 0.58[a]
K. pneumoniae	0	0	0	0	0	0	15 ± 0.58[a]
P. aeruginosa	0	0	0	0	0	0	14 ± 0.58[a]
S. aureus	0	0	0	0	0	0	7 ± 0.58[b]
E. faecalis	0	0	0	0	0	0	8 ± 0.58[b]
Fungal species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Amphotericin B
C. albicans	0	0	0[b]	0	16 ± 0.94[c]	0	11 ± 0.47[a]
C. krusei	0	0	8 ± 0.47[a]	0	8 ± 0.47[b]	0	12 ± 0.47[a]
C. parapsilosis	0	0	0[b]	0	13 ± 0.47[a]	0	11 ± 0.47[a]

Values labeled with different letters are significantly different when compared within extract type, across species (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each

Table 3.3 Minimum inhibitory concentrations (µg/mL) of crude extracts *T. emetica* seeds against pathogenic bacteria and fungi

Bacterial species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Neomycin
E. coli	-	-	-	-	-	-	3.13 ± 1.26^{d}
K. pneumoniae	-	-	-	-	-	-	6.26 ± 0.97^{b}
P. aeruginosa	-	-	-	-	-	-	5.74 ± 1.20^{c}
S. aureus	-	-	-	-	-	-	25 ± 1.26^{a}
E. faecalis	-	-	-	-	-	-	1.56 ± 0.97^{e}
Candida species	**Methanol**	**Ethanol**	**Ethyl acetate**	**Hexane**	**Chloroform**	**Distilled water**	**Amphotericin B**
C. albicans	-	-	-	100 ± 2.92^{a}	80.12 ± 1.03^{b}	-	0.62 ± 0.09^{b}
C. krusei	90.41 ± 1.22^{a}	-	-	77.40 ± 1.07^{b}	100.11 ± 0.95^{a}	-	1.25 ± 0.03^{a}
C. parapsilosis	37.46 ±2.35^{b}	-	40.62 ± 2.30^{a}	40.95 ± 0.82^{c}	76.27 ± 2.49^{c}	-	1.25 ± 0.06^{a}

Values labeled with different letters are significantly different when compared within extract type, across species (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.

3.4.3 *In vitro* free radical (DPPH) scavenging activity

The DPPH free radical assay has been used extensively as a model scheme to evaluate the scavenging activity of antioxidants *in vitro* (Oyaizu, 1986). From the (Table 3.4 and Figure 3.1), it was noted that except for the methanol extract, all the seed extracts of *T. emetica* demonstrated poor radical scavenging ability in the concentration range 6.25-200 µg/mL. The methanol seed extract exhibited good radical scavenging activity with an IC_{50} value of 5.94 µg/mL. However, despite this low IC_{50} value, the radical scavenging ability of the methanol extract was not dose-dependent: Free radical scavenging of 52.37% and 57.13% was obtained at extract concentrations of 6.25 µg/mL and 200 µg/mL (Figure 3.1). The free radical scavenging ability of the standard, ascorbic acid, increased in a dose-dependent fashion with an IC_{50} value of 4.67 µg/mL.

Table 3.4 IC_{50} (µg/mL) of crude extracts of *T. emetica* seeds and ascorbic acid

Sample	IC_{50} (µg/mL)
Methanol	5.94 ± 0.75
Ethanol	-
Ethyl acetate	-
Hexane	-
Chloroform	-
Distilled water	-
Ascorbic acid	4.67 ± 0.16

Values represent mean ± SD of 3 trials of 3 replicates each.

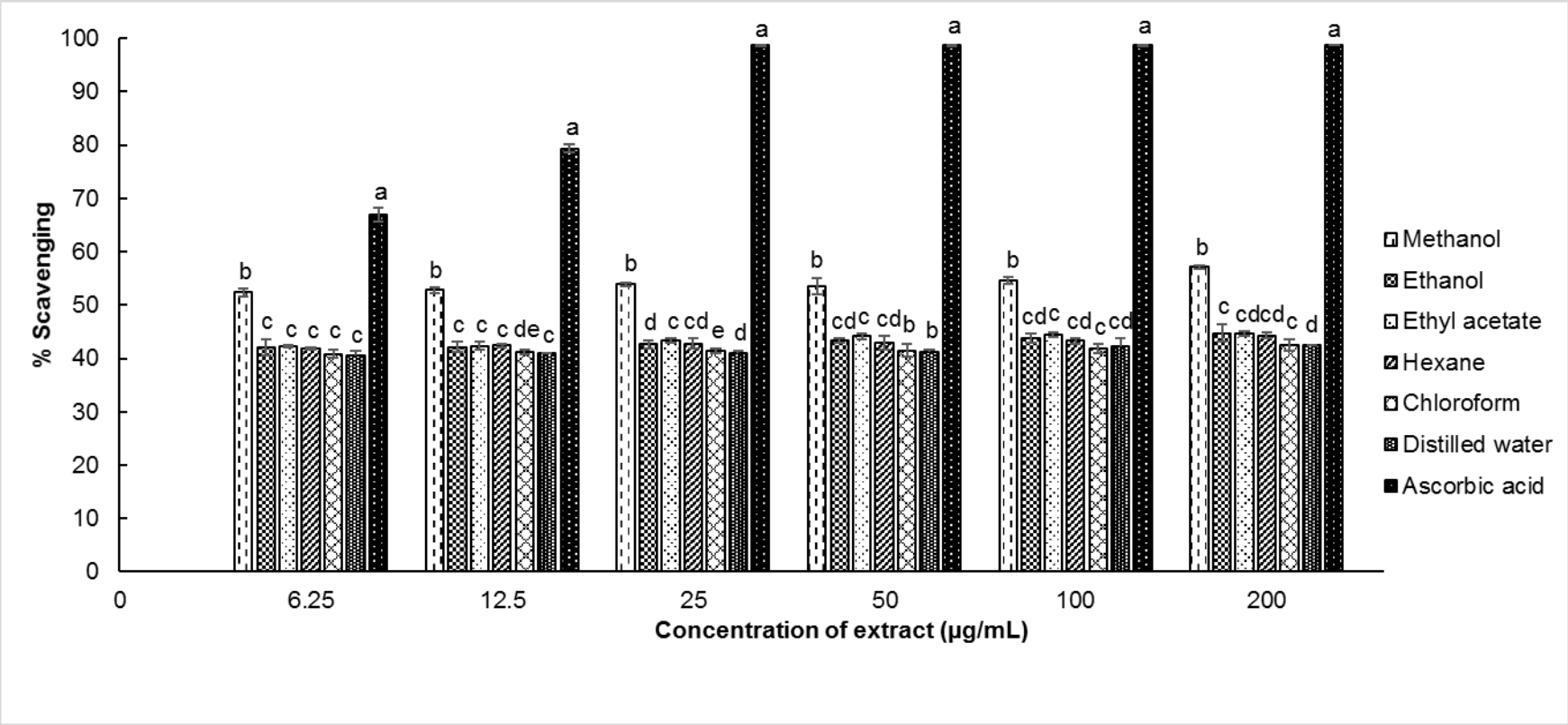

Figure 3.1 Radical scavenging activity of crude extracts of *T. emetica* seeds and ascorbic acid on DPPH. Bars labelled with different letters are significantly different when compared within extract type, across concentrations (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.

3.4.4 *In vitro* cytotoxicity/anticancer activity

The anti-proliferative activity of *T. emetica* crude seed extracts were tested against the Vero and MCF-7 cell lines. Cadmium, a well known carcinogen, was employed as a positive control and exhibited a potent cytotoxic effect (0 % viability) on Vero and MCF-7 cells at a concentration of 4 µg/mL (Tables 3.5 and 3.6, respectively). Untreated cells served as the negative control. None of the seed extracts exhibited any cytotoxicity against both the Vero and MCF-7 cell lines at the tested concentrations (6.25-200 µg/mL).

Table 3.5 Vero cell viability after 24 h exposure to crude extracts of *Trichilia emetic* seeds

	% Viability					
Concentration (µg/mL)	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water
50	100 ± 1.56	100 ± 2.10	100 ± 0.69	100 ± 1.32	100 ± 2.96	100 ± 1.31
100	100 ± 2.39	100 ± 0.52	100 ± 0.98	100 ± 2.36	100 ± 3.56	100 ± 1.97
150	100 ± 2.09	100 ± 2.92	100 ± 0.88	100 ± 0.65	100 ± 3.11	100 ± 2.12
200	100 ± 1.36	100 ± 1.63	100 ± 1.24	100 ± 1.26	100 ± 1.25	100 ± 3.26

Values represent mean ± SD of 3 trials of 3 replicates each.

Table 3.6 Cytotoxicity of crude extracts of *T. emetica* seeds against MCF-7 breast cancer cells

	% Viability					
Concentration (µg/mL)	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water
6.25	100 ± 3.04	100 ± 3.45	100 ± 2.06	100 ± 2.87	100 ± 3.88	100 ± 1.26
12.5	100 ± 2.29	100 ± 0.94	100 ± 0.55	100 ± 0.59	100 ± 2.36	100 ± 0.96
25	100 ± 3.04	100 ± 3.45	100 ± 2.06	100 ± 2.87	100 ± 3.88	100 ± 1.26
50	100 ± 2.29	100 ± 0.94	100 ± 0.55	100 ± 0.59	100 ± 2.36	100 ± 0.96
100	100 ± 4.80	100 ± 1.24	100 ± 2.13	100 ± 2.14	100 ± 1.39	100 ± 0.97
200	100 ± 1.44	100 ± 1.95	100 ± 0.76	100 ± 3.15	100 ± 2.26	100 ± 4.84

Values represent mean ± SD of 3 trials of 3 replicates each.

3.4.5 Gas Chromatography-Mass Spectroscopy analysis

Gas Chromatography-Mass Spectroscopy analysis is a technique employed for the separation and identification of chemical components in an organic mixture (Gopalakrishnan and Finose, 2014). The GC-MS analysis of crude extracts of seeds of *T. emetica* revealed the present of 39 different phytocompounds. The results obtained for the six extracts are presented in Tables 3.7-3.12.

A total of 16 different chemical components were isolated from the methanol extract (Table 3.7) with pentanoic acid, 5-hydroxy-,2,4-di-T-butylphenyl esters (10.85%) and p-xylene (9.63%) occurring in the greatest abundance.

Table 3.7 Phytocompounds of crude methanol extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
3.67	1,1-dimethyl-1-silacyclobutane	$C_5H_{12}Si$	100	4.89
3.94	p-xylene	C_8H_{10}	106	9.63
5.33	Hentriacontane	$C_{31}H_{64}$	436	8.87
6.10	Nonadecane, 2,6,10,14-tetramethyl	$C_{23}H_{48}$	324	3.58
6.41	Dodecane, 1-fluoro	$C_{12}H_{25}F_2$	188	8.62
7.43	Sydnone, 3-(3,3-dimethylbutyl)-	$C_8H_{14}O_2N_2$	170	6.97
7.72	Benzaldehyde, 2,5-dimethyl-	$C_9H_{10}O$	135	5.72
9.79	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	518	4.16
10.26	Pentanoic acid, 5-hydroxy-,2,4,-di-T-butylphenyl esters	$C_{19}H_{30}O_3$	306	10.85
11.11	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	592	4.29
11.72	Disulphide, di-tert-dodecyl	$C_{24}H_5OS_2$	402	3.48
14.35	Trimethyl[-4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	$C_{17}H_{30}PSi$	435	5.78
15.74	Trimethyl[-4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	$C_{15}H_{24}O_2Si$	264	5.91
19.18	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	6.10
19.45	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	5.61
19.72	Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}O_3$ Si_3As	468	18.55

Fourteen phytocompounds were identified in the ethanol extract (Table 3.8) with pentanoic acid, 5-hydroxy-,2,4, -di-T-butylphenyl esters (12.54%) being markedly higher.

Table 3.8 Phytocompounds of crude ethanol extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
3.96	p-xylene	C_8H_{10}	106	9.61
5.24	N-heptyl acrylate	$C_{10}H_{18}O_2$	170	3.59
5.34	Dodecane, 1-fluoro	$C_{12}H_{25}F_2$	188	8.98
5.47	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	402	4.25
6.42	Hentriacontane	$C_{31}H_{64}$	436	9.27
7.35	4-Undecane, 5-methyl-	$C_{12}H_{24}$	168	3.21
7.72	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	4.79
7.96	Benzenepropanal, 4-(1,1-dimethyl)-	$C_{13}H_{18}O$	190	7.69
10.26	Pentanoic acid, 5-hydroxy-,2,4,-di-T-butylphenyl esters	$C_{19}H_{30}O_3$	306	12.54
14.01	Trimethyl[-4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	$C_{17}H_{30}OSi$	278	7.06
15.55	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	6.60
17.22	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	6.92
19.23	Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}O_3\ Si_3As$	468	8.33
19.54	4-methyl-2,4-bis(4'-trimethylsiloxyphenyl)pentene-1	$C_{24}H_{36}O_2Si_2$	412	7.16

A total of 12 phytocompounds were identified from the ethyl acetate (Table 3.9) with phenol, 2,4-bis(1,1-dimethylethyl)- (14.47%) occurring in the greatest abundance.

Table 3.9 Phytocompounds of crude ethyl acetate extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
3.02	Strychane, 1-acetyl-20.alpha.-hydroxy-16-methylene	$C_{21}H_{26}O_2N_2$	338	4.26
3.59	Cyclobutanone, 2,3,3,4-tetramethyl-	$C_8H_{14}O$	126	9.32
3.94	p-xylene	C_8H_{10}	106	10.92
5.33	Dodecane, 1-fluoro-	$C_{12}H_{25}F$	188	10.51
6.21	1R,2C,3T,4T-tetramethyl-cyclohexane	$C_{10}H_{20}$	140	5.08
7.43	Hentriacontane	$C_{31}H_{64}$	436	9.44
7.72	Benzaldehyde, 2,4-dimethyl-	$C_9H_{10}O$	134	10.18
7.96	Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	190	12.96
8.80	Benzaldehyde, 2,5-dimethyl-	$C_9H_{10}O$	134	5.73
10.27	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	14.47
12.18	Hexacosyl acetate	$C_{28}H_{56}O_2$	424	2.92
13.35	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	4.20

Four phytocompounds were identified in the chloroform extract (Table 3.10) with phenol, 2,5-bis(1,1-dimethylethyl) being most abundant (39.70%).

Table 3.10 Phytocompounds of crude chloroform extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
6.42	Dodecane, 1-fluoro-	$C_{12}H_{25}F$	188	27.71
7.72	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	23.73
10.27	Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	39.70
17.71	3-ethyl-3-methylnonadecane	$C_{22}H_{46}$	310	8.88

Nine compounds were identified in the hexane extract (Table 3.11). Ethylbenzene (14.77%) was found to be in greatest abundance.

Table 3.11 Phytocompounds of crude hexane extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
3.35	3-hexanol, 2,2-dimethyl-	$C_8H_{18}O$	130	13.70
3.86	Ethylbenzene	C_8H_{10}	106	14.77
4.21	Benzene, 1,3-dimethyl-	C_8H_{10}	106	12.76
4.96	Benzene, 1-ethyl-2-methyl	C_9H_{12}	120	13.29
5.34	Mesitylene	C_9H_{12}	120	9.89
6.42	Dodecane, 1-flouoro-	$C_{12}H_{25}F$	188	9.56
7.72	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	8.38
10.27	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	14.07
16.40	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	3.57

The distilled water extract contained 14 phytocompounds (Table 3.12) with 1-methoxy-1,4-cyclohexadiene (12.97%) being in excess.

Table 3.12 Phytocompounds of cude distilled water extract of *T. emetica* seeds acquired via GC-MS

Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area %
3.95	p-xylene	C_8H_{10}	106	9.71
4.33	1-methoxy-1,4-cyclohexadiene	$C_7H_{10}O$	110	12.97
5.34	Dodecane, 1-fluoro	$C_{12}H_{25}F_2$	188	10.54
6.11	11-methylnonacosane	$C_{30}H_{62}$	422	2.89
8.78	7-ethyl-5-phenyl-2,3,6,7-tetrahydro-5H-thiazolo[3,2-A]pirimidin-7-O	$C_{14}H_{18}N_2S$	262	3.70
9.23	2-isopropyl-5,6-dimethyl-1,3,2-oxathiaborinane	$C_8H_{17}O_3SB$	172	4.77
9.30	Hentriacontane	$C_{31}H_{64}$	436	7.55
9.80	Methyl 2,2-dimethyl-3-hydroxypropionate	$C_6H_{12}O_3$	132	3.92
13.94	Trimethyl[-4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	$C_{17}H_{30}OSi$	278	7.03
16.38	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	7.52
16.95	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	7.30
17.27	Trimethyl[-4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	$C_{15}H_{24}O_2Si$	264	6.89
18.60	4-methyl-2,4-bis(4'-trimethylsiloxyphenyl)pentene-1	$C_{24}H_{36}O_2Si_2$	412	7.89
19.71	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	298	7.34

The phytocompounds that were present in most extracts were dodecane, 1-fluoro, hentriacontane, p-xylene and octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (Figure 3.2).

The greatest diversity of phytocompounds was obtained when methanol (Table 3.7) was used as the extracting solvent. Chloroform (Table 3.10) did not appear to facilitate efficient extraction of secondary metabolites since smaller amounts of phytocompounds were obtained relative to all extracts.

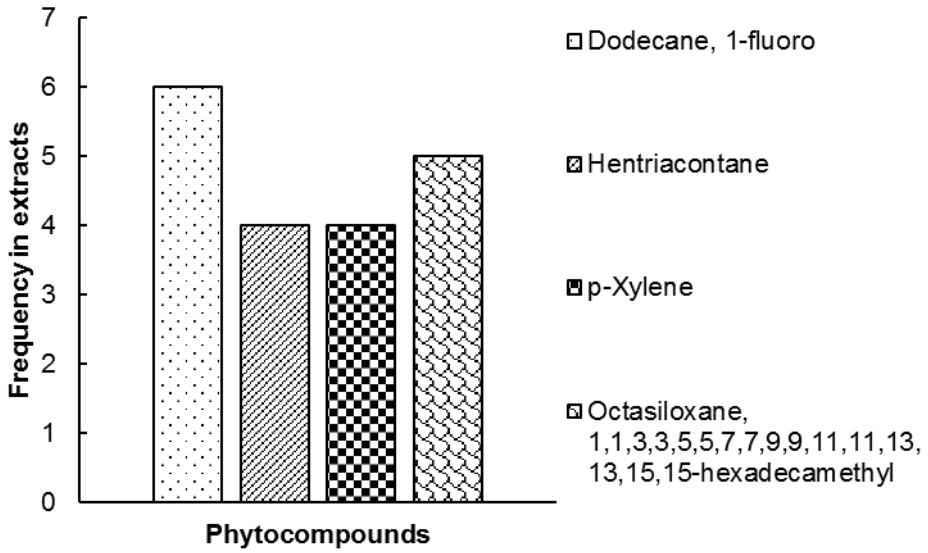

Figure 3.2 Common phytocompounds found in most crude extracts of *T. emetica* seeds.

3.5 DISCUSSION

This section details the antioxidant performance, antimicrobial effectiveness and cytotoxicity/anticancer activity of solvent and aqueous extracts of the seeds of *T. emetica*.

Preliminary phytochemical screening of the solvent and distilled water extracts of *T. emetica* revealed the presence of alkaloids, glycosides, phenols, sterols and flavonoids as its main phytocompounds (table 3.1). Chloroform proved to be the solvent of choice to employ during phytochemical extraction as it yielded a larger number of phytocompounds. The presence of several biologically active compounds was validated via GC-MS (tables 3.7-3.12) and suggests that the crude seed extracts of *T. emetica* possess pharmacological value, thus corroborating its use in herbal medicine. A study carried out by Nana *et al*., 2013 using the methanol, hexane and ethyl acetate fractions of the stem bark of *T. emetica* yielded similar phytocompounds to this study. Flavonoids, phenols and terpenoids were found to be the main phytoconstituents present, with no indication of the presence of saponins and sugars (Nana *et al.*, 2013). With regard to secondary metabolites of *T. emetica* obtained from other parts of the tree, numerous limonoids, commonly referred to as Trichilin (Nakatani *et al*., 1984), have been isolated Nakatani *et al.*, 1981; Nakatani *et al.*, 1985; Diallo *et al.*, 2003). Limonoids are metabolically transformed, oxygenated triterpene compounds containing four six-membered chain and a furan ring. Limonoids are known for their sometimes bitter taste

and scent of peels of citrus fruit. Limonoid occurrence is restricted to the Meliaceae and Rutaceae families of the plant kingdom (Roy and Saraf, 2006; Tundis *et al.*, 2014). Three seco-limonoids were isolated from the stem bark of *T. emetica* exhibiting insect antifeedant activity (Nakatani *et al.*, 1984; Nana *et al.*, 2013). Other compounds related to limonoids have also been isolated from the stem bark of *T. emetica* i.e. dregeana 4, nymania 1, rohituka 3, seco-A-protolimonoid and trichilin A (Gunatilaka *et al.*, 1998; Diallo *et al.*, 2003). Studies have shown the health benefits of limonoid administration to be numerous, including antineoplastic activity (Poulose *et al.*, 2005; Dzoyem *et al.*, 2015). Limonoid glucosides restrict HIV replication in infected human mononuclear cells (Battinelli *et al.*, 2003; Sunthitikawinsakul *et al.*, 2003; Poulose *et al.*, 2005), exhibits anticonvulsant, anti-inflammatory, antischistosomal, antitrypanosomal, antimutagenic and antimalarial properties (Bray *et al.*, 1990; Germano *et al.*, 2005; Poulose *et al.*, 2005; Nana *et al.*, 2013) and confers a cytotoxic effect on breast cancer cells (So *et al.*, 1996). Limonoid ingestion was also reported to lower serum cholesterol levels (Kurowska and Manthey, 2004; Germano *et al.*, 2005). Further investigation involving fractionation and characterisation of bioactives present in the extracts of *T. emetica* is needed to ascertain the presence of limonoids. This could potentially be a new source of limonoids to explore. It was also reported in earliest studies that the aqueous fraction of the stem bark is composed of 6.82 % tannins (Burkill, 1995) and the methanol-acetic acid fraction of the roots contain bound phenolic acids (Germano *et al.*, 2006).

The observed inactivity of the extracts against bacterial species (table 3.2 and 3.3) was not anticipated since antibacterial activity was noted in root, fruit and leaf solvent extracts in previous studies (Germano *et al.*, 2005; Shai *et al.*, 2008; Vieira *et al.*, 2014). Fungal inhibition may be due to the major or minor phytocompounds present in the seed extracts or the synergistic consequence of both (Rankovic *et al.*, 2011). The ability of the methanol, ethyl acetate, hexane and chloroform fractions of *T. emetica* (table 3.2 and 3.3) to exhibit strong activity on some/all *Candida* species indicates the potential of their bioactive compounds to be developed into effective antifungal agents.

It has been established that aromatic amines (*p*-aminophenol, *p*-phenylene diamine, etc.), ascorbic acid, flavonoids, glutathione, tannins and tocopherol have the ability to reduce and decolourise DPPH by their hydrogen donating ability (Blois, 1958; Kumaran and Karunakaran, 2007) resulting in potent antioxidant activity. All crude seed extracts, with the exception of methanol, exhibited poor antioxidant activity (figure 3.1) showing no IC_{50} in the concentration range tested (table 3.4). The methanol seed extract of *T. emetica* displayed good radical scavenging activity with an IC_{50} value of 5.94 µg/mL. It was reported in an earlier study, that seeds of *Trichilia* contains 40-60% fats that are made up of linoleic, oleic

and palmitic acids (Oliver-Bever, 1986). This antiradical activity of the methanol crude seed extract could be attributed to the presence phenols is in the fatty acids of the seeds. Good radical scavenging activity was also reported on the methanol leaf extract of *T. emetica* by Frum and Viljoen (2006) where an IC_{50} value of 17.9 µg/mL was noted. The phytocompounds identified in this study may be responsible for the antioxidant potential of the methanol seed extract, however, their precise mode of action is inadequately understood.

It is noteworthy, despite reports on the cytotoxicity of other extracts of *T. emetica* on mammalian cells in culture (Komane *et al.,* 2011; Traore *et al.,* 2007), the seed extracts did not exhibit cytotoxic effects on both Vero and MCF-7 cells (Tables 3.5 and 3.6). The non-toxicity of seed extracts observed for Vero cells in this study, reiterates the potential use of bioactives present in the seed extracts as a new, safe pharmacologically active antifungal drugs. Conversely, kurubasch aldehyde, a sesquiterpenoid isolated from the root extract of *T. emetica*, displayed high inhibitory effects towards murine sarcoma S180 and MCF-7 cells with IC_{50} values of 7.4 and 78 µM respectively) (Komane *et al.,* 2011; Traore *et al.,* 2007). These results reiterate the need for further investigation into the isolation and characterisation of bioactives in the crude seed extracts of *T. emetica*.

3.6 CONCLUSION

All extracts investigated did not exhibit cytotoxic effects on Vero and MCF-7 cells. However, the results in this study suggest that the methanol extract of the seeds of *T. emetica* is a potential source of antioxidants as it was the only solvent to exhibit radical scavenging ability. The solvent seed extracts (excluding the distilled water extract) of *T. emetica* are potential sources of antifungal agents with the methanol extract as the most promising fungicidal agent. Hence, more efforts are required to ascertain the fungicidal effects/principle and further, get it purified and characterised.

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Chapter 4

RESEARCH RESULTS 2

Biological activity and chemical composition of crude extracts of *Protorhus longifolia* seeds

Biological activity and chemical composition of crude extracts of *Protorhus longifolia* seeds

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4.1 ABSTRACT

This paper presents the results of phytochemical screening, antioxidant, antimicrobial and anti-proliferative activity studies of the crude seed extracts of *Protorhus longifolia* (Bernh. Ex C. krauss) Engl. (Anacardiaceae). Seeds were extracted via cold percolation using methanol, ethanol, ethyl acetate, chloroform, hexane and distilled water. Phytochemical screening was carried out using standard qualitative procedures and Gas Chromatography-Mass Spectroscopy (GC-MS). Antioxidant activity was ascertained using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay. Antimicrobial evaluation was performed using the disc diffusion method and the microbroth dilution assay. The anti-proliferative activity was evaluated on the human breast adenocarcinoma (MCF-7) and the green monkey kidney (Vero) cells. Phytochemical screening showed the presence of cardiac glycosides, phenols, sterols and flavonoids in the plant extracts with phytocompounds of pharmacological importance identified via GC-MS analysis. The methanol and ethanol extracts exhibited good antioxidant potential with IC_{50} of 5.00 ± 0.33 µg/mL and 32.61 ± 0.42 µg/mL, respectively. All seed extracts exhibited an antibacterial effect for the disc diffusion method against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The best antibacterial potential was noted for the ethanol extract against *P. aeruginosa*, with a minimum inhibitory concentration (MIC) of 98.61 ± 4.07 µg/mL using Neomycin as a standard. The anti-proliferative activity of the methanol and ethanol extracts against MCF-7 cells was within the limits (IC_{50} less than 30 µg/mL) stipulated by the American National Cancer Institute (NCI) for cytotoxicity. These results endorse the exploitation of seeds of *P. longifolia* in the search for new antimicrobial and anticancer drugs and a source of antioxidants.

4.2 INTRODUCTION

In humans, fungal and bacterial infections co-exist in various ailments (Morales and Hogan, 2010). Failure of drug treatment for microbial infections has drawn attention to the problem of antimicrobial resistance and its underlying mechanisms (Jose *et al.*, 2016). Reactive oxygen species (ROS) are usually associated with inflammation, aging, and cancer. Antioxidants play a pivotal role in scavenging ROS (Upadhyay *et al.*, 2010). Cancer is a major cause of concern for the public health sector in both developing and industrialised countries. Cancer chemotherapeutic agents can often provide temporary relief of symptoms with occasional cures. However, the effective doses of most of the agents also fall in the range of toxic dose (Bhavana *et al.*, 2016).

Medicinal plants possess many bioactive properties, usually antimicrobial, antioxidant, anticancer and anti-inflammatory. Medicinal plant extracts and their derived products offer considerable potential for the development of new agents effective against infectious diseases currently difficult to treat (Valgas *et al.*, 2007).

The family Anacardiaceae is made up of several plant species of economic value. Well known members of this family include cashew, mango, pink pepper and pistachio, together with many other plants of local significance. The Anacardiaceae family appears to be a rich source of plant species comprising a promising source of natural antioxidants that possess hypoglycaemic, antibacterial, analgesic and anti-inflammatory potential (Ojewole, 2004; Maiga *et al.*, 2005; Schulze-Kaysers *et al.*, 2015).

P. longifolia is an important medicinal plant frequently traded in the Eastern Province of South Africa and sold as *muthi*. Traditionally, the stem bark is used by the Zulus to treat diarrhoea and heart water in cows (Dold and Cocks, 2001), stomach bleeds, hemiplegic paralysis and heart burn, whilst other parts are believed to strengthen the heart (Mosa, 2014). In recent years, a combination of secondary plant metabolites has been investigated extensively for their medicinal significance. Lanosteryl triterpenes were isolated from the stem bark of *P. longifolia*, exhibiting anti-platelet aggregation (Mosa *et al.*, 2011b), anti-inflammatory, antihyperlipidemic (Mosa et al., 2015) and antibacterial activity (Mosa et al., 2014).

The aim of this research study was to evaluate the potential antimicrobial, antioxidant and anticancer activity of the crude seed extracts of *P. longifolia* and to determine the phytochemical composition of these extracts.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

As described in Chapter 3 (section 3.3.1).

4.3.2 Seed material

Mature seeds were collected from *P. longifolia* trees growing at the University of Kwa-Zulu Natal (Westville campus), KwaZulu-Natal, South Africa (29 52 S, 30 58 E). The seeds were air-dried at room temperature for several days; after which, they were crushed to a fine powder and stored in air tight containers for subsequent use in solvent extractions.

4.3.3 Extract preparation for *in vitro* assays

Extracts were prepared via cold percolation method by Parekh and Chanda (2007) as described in Chapter 3 (section 3.3.).

4.3.4 Preliminary phytochemical analysis

All qualitative analyses were carried out according to the methods Harborne (1973) and Trease and Evans (1978) as described in Chapter 3 (section 3.3.4).

4.3.4.1 Test for alkaloids

Dragendorff's reagent test

As described in Chapter 3 (section 3.3.4.1).

4.3.4.2 Test for flavonoids

NaOH test

As described in Chapter 3 (section 3.3.4.2).

4.3.4.3 Test for cardiac glycosides

Keller-Killani test

As described in Chapter 3 (section 3.3.4.3).

4.3.4.4 Test for terpenoids

Salkowski test

As described in Chapter 3 (section 3.3.4.4).

4.3.4.5 Test for steroids

Lieberman-Buchard test

As described in Chapter 3 (section 3.3.4.5).

4.3.4.6 Test for saponins

Foam test

As described in Chapter 3 (section 3.3.4.6).

4.3.4.7 Test for phenols

Phenol test

As described in Chapter 3 (section 3.3.4.7).

4.3.4.8 Test for tannins

Gelatin test

As described in Chapter 3 (section 3.3.4.8).

4.3.5 *In vitro* antimicrobial susceptibility testing

As described in Chapter 3 (section 3.3.5).

4.3.5.1 Test organisms

As described in Chapter 3 (section 3.3.5.1).

4.3.5.2 Storage and maintenance of microbial cultures

As described in Chapter 3 (section 3.3.5.2).

4.3.5.3 Screening for antimicrobial activity

As described in Chapter 3 (section 3.3.5.3).

4.3.5.3.1 Disc diffusion (antibacterial and antifungal)

Antimicrobial activity was assessed via a modified method of Bauer *et al*. (1966) as described in Chapter 3 (section 3.3.5.3.1).

4.3.5.3.2 Minimum Inhibitory Concentration (MIC) for antibacterial determination

The MIC for bacteria was determined using a modified broth microdilution assay by Eloff (1998) as described in Chapter 3 (section 3.3.5.3.2).

4.3.5.3.3 MIC for antifungal determination

The MIC for fungi was determined using a modified broth microdilution assay by Eloff (1998) as described in Chapter 3 (section 3.3.5.3.3).

4.3.6 *In vitro* antioxidant activity

Antioxidant activity was assessed using a modified method by Burits and Bucar (2000) and Melendez *et al*. (2014) as described in Chapter 3 (section 3.3.6).

4.3.7 Tissue culture

4.3.7.1 Cell lines

As described in Chapter 3 (section 3.3.7.1).

4.3.7.2 Tissue culture techniques

As described in Chapter 3 (section 3.3.7.2).

4.3.7.2.1 Re-suspension of cells and subculturing procedure

As described in Chapter 3 (section 3.3.7.2.1).

4.3.7.3 Cytotoxicity

Cell viability was determined using the Vero cell line by the CellTiter 96® AQueous One Solution Assay as described in Chapter 3 (section 3.3.7.3).

4.3.7.4 In vitro anticancer activity

Cell viability was determined using the MCF-7 cell line by the CellTiter 96® AQueous One Solution Assay as described in Chapter 3 (section 3.3.7.4).

4.3.8 Gas Chromatography-Mass Spectroscopy (GC-MS)

As described in Chapter 3 (section 3.3.8).

4.3.9 Statistical analyses

As described in Chapter 3 (section 3.3.9).

4.4 RESULTS

4.4.1 Preliminary phytochemical analyses

Preliminary phytochemical investigation of six crude seed extracts of *P. longifolia* for its secondary metabolites revealed the presence of cardiac glycosides, phenols, sterols and flavonoids with no indication of alkaloids, terpenoids, tannins and saponins (Table 4.1). The most commonly occurring secondary metabolites were sterols and glycosides and the rarest were phenols. Ethanol proved to be the best solvent for extraction as it yielded the greatest diversity of secondary metabolites. The distilled water extract did not show the presence of any secondary metabolites and is therefore a poor choice for phytochemical extraction.

Table 4.1 Phytochemical analyses crude extracts of *P. longifolia* seeds

Secondary metabolites	Methanol extract	Ethanol extract	Ethyl acetate extract	Chloroform extract	Hexane extract	Distilled water extract
Alkaloids	-	-	-	-	-	-
Cardiac glycosides	+	+	+	+	-	-
Phenols	-	-	-	-	+	-
Sterols	-	+	+	+	+	-
Flavonoids	+	+	-	-	-	-
Saponins	-	-	-	-	-	-
Terpenoids	-	-	-	-	-	-
Tannins	-	-	-	-	-	-

Key: -: not detected; +: detected

4.4.2 *In vitro* antimicrobial activity

4.4.2.1 Disc diffusion

Antimicrobial activity at 400 µg/mL of the six extracts of *P. longifolia* seeds were assayed *in vitro* by the disc diffusion method against five bacterial strains and three fungal strains. All extracts exhibited fungicidal activity against *C. albicans*, *C. krusei* and *C. parapsilosis* (Table 4.2). However, all seed extracts had a bactericidal effect on the three Gram negative bacteria considered but only inhibited *S. aureus* from the two Gram positive bacteria tested. Among the extracts screened, methanol displayed the best antibacterial activity. Highest antibacterial activity was demonstrated by the methanol seed extract against *S. aureus* with an inhibition zone of 16 mm, as compared with an inhibition zone of 9 mm displayed by the standard (neomycin). The most susceptible bacterium to the extracts was *P. aeruginosa*, whilst the most resistant bacterium was *E. faecalis*.

4.4.2.2 Minimum Inhibitory Concentration (MIC)

The extracts of *P. longifolia* seeds were additionally evaluated for their antimicrobial potential using the microbroth dilution assay. Table 4.3 summarises the results obtained. The reference drug, neomycin, used against bacterial strains in this study exhibited MICs in the range of 1.56-25 µg/mL. Amphotericin B, the positive control against fungal strains, displayed MICs in the range of 0.62-1.25 µg/mL. All crude extracts of *P. longifolia* seeds were found to be inactive against all tested fungal strains. The highest antibacterial potential was displayed by the ethanol extract against *P. aeruginosa* with a MIC of 98.61 µg/mL. *S. aureus* was the most susceptible bacterium to *P. longifolia* seed extracts. This contradicts the results obtained for the disc diffusion assay which is purely qualitative. The microbroth dilution assay is quantitative and thus more reliable (Eloff, 1998). *E. faecalis* remained the most resistant bacterium.

Table 4.2 Antimicrobial activity of crude extracts of *P. longifolia* seeds (active concentration of 400 µg/mL)

	Zone of inhibition (mm)						
Bacterial species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Neomycin
E. coli	13 ± 0.82^{b}	11 ± 0.94^{c}	7 ± 0.47^{b}	7 ± 0.47^{c}	7 ± 0.47^{c}	8 ± 0.47^{b}	15 ± 0.58^{a}
K. pneumoniae	13 ± 0.47^{b}	10 ± 0.94^{c}	7 ± 0.82^{b}	8 ± 0.94^{c}	7 ± 0.47^{c}	9 ± 0.82^{ab}	15 ± 0.58^{a}
P. aeruginosa	15 ± 0.47^{a}	15 ± 0.47^{a}	11 ± 0.82^{a}	14 ± 0.47^{a}	12 ± 0.47^{a}	9 ± 0.47^{a}	14 ± 0.58^{a}
S. aureus	16 ± 0.47^{a}	13 ± 0.47^{b}	10 ± 0.47^{a}	10 ± 0.47^{b}	9 ± 0.47^{b}	8 ± 0.94^{ab}	9 ± 0.58^{b}
E. faecalis	0^{c}	0^{d}	0^{c}	0^{d}	0^{d}	0^{b}	8 ± 0.58^{b}
Fungal species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Amphotericin B
C. albicans	0	0	0	0	0	0	11 ± 0.47^{a}
C. krusei	0	0	0	0	0	0	12 ± 0.47^{a}
C. parapsilosis	0	0	0	0	0	0	11 ± 0.47^{a}

Values labeled with different letters are significantly different when compared within extract, across species (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.

Table 4.3 Minimum inhibitory concentrations (µg/mL) of crude extracts of *P. longifolia* seeds against pathogenic bacteria and fungi

Bacterial species	Methanol	Ethanol	Ethyl acetate	Chloroform	Hexane	Distilled water	Neomycin
E. coli	140.44 ± 0.92^{b}	142.50 ± 2.95^{a}	≤400	≤400	≤400	≤400	3.13 ± 1.26^{d}
K. pneumoniae	≤400	≤400	≤400	≤400	≤400	≤400	6.26 ± 0.97^{b}
P. aeruginosa	≤400	98.61 ± 4.07^{b}	≤400	≤400	≤400	≤400	5.74 ± 1.20^{c}
S. aureus	188.94 ± 0.66^{a}	142.05 ± 3.10^{a}	≤400	159.88 ± 5.06^{a}	115.35^{a}	≤400	25 ± 1.26^{a}
E. faecalis	-	-	-	-	-	-	1.56 ± 0.97^{e}
Candida species	Methanol	Ethanol	Ethyl acetate	Hexane	Chloroform	Distilled water	Amphotericin B
C. albicans	-	-	-	-	-	-	0.62 ± 0.09^{b}
C. krusei	-	-	-	-	-	-	1.25 ± 0.03^{a}
C. parapsilosis	-	-	-	-	-	-	1.25 ± 0.06^{a}

Values labeled with different letters are significantly different when compared within extract type, across species (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each. –: no minimum inhibition in the tested range (6.25-200 µg/mL).

4.4.3 *In vitro* free radical (DPPH) scavenging activity

The lower the IC_{50} value of a substance, the more effective its free radical scavenging effect (Suleiman *et al.*, 2010a). In the present study free radical scavenging activity was determined via the DPPH assay (summarised in table 4.4). The methanol extract of seeds displayed the highest radical scavenging activity with an IC_{50} value of less than 10 µg/mL. This value was similar to that of the positive control (ascorbic acid) used, exhibiting a concentration dependent reduction potential (Figure 4.1). The ethanol, ethyl acetate and distilled water extracts displayed moderate radical scavenging activity with IC_{50} values 32.61, 64.43 and 138.16 µg/mL, respectively. The chloroform and hexane extracts did not show any antioxidant activity.

Table 4.4 IC_{50} (µg/mL) of crude extracts of *P. longifolia* seeds and ascorbic acid

Sample	IC_{50} (µg/mL)
Methanol extract	5.00 ± 0.33
Ethanol extract	32.61 ± 0.42
Ethyl acetate extract	64.43 ± 0.42
Chloroform extract	-
Hexane extract	-
Distilled water extract	138.16 ± 0.76
Ascorbic acid	4.67 ± 0.16

Values represent mean ± SD of 3 trials of 3 replicates each. – : no minimum inhibition in the tested range (6.25-200 µg/mL).

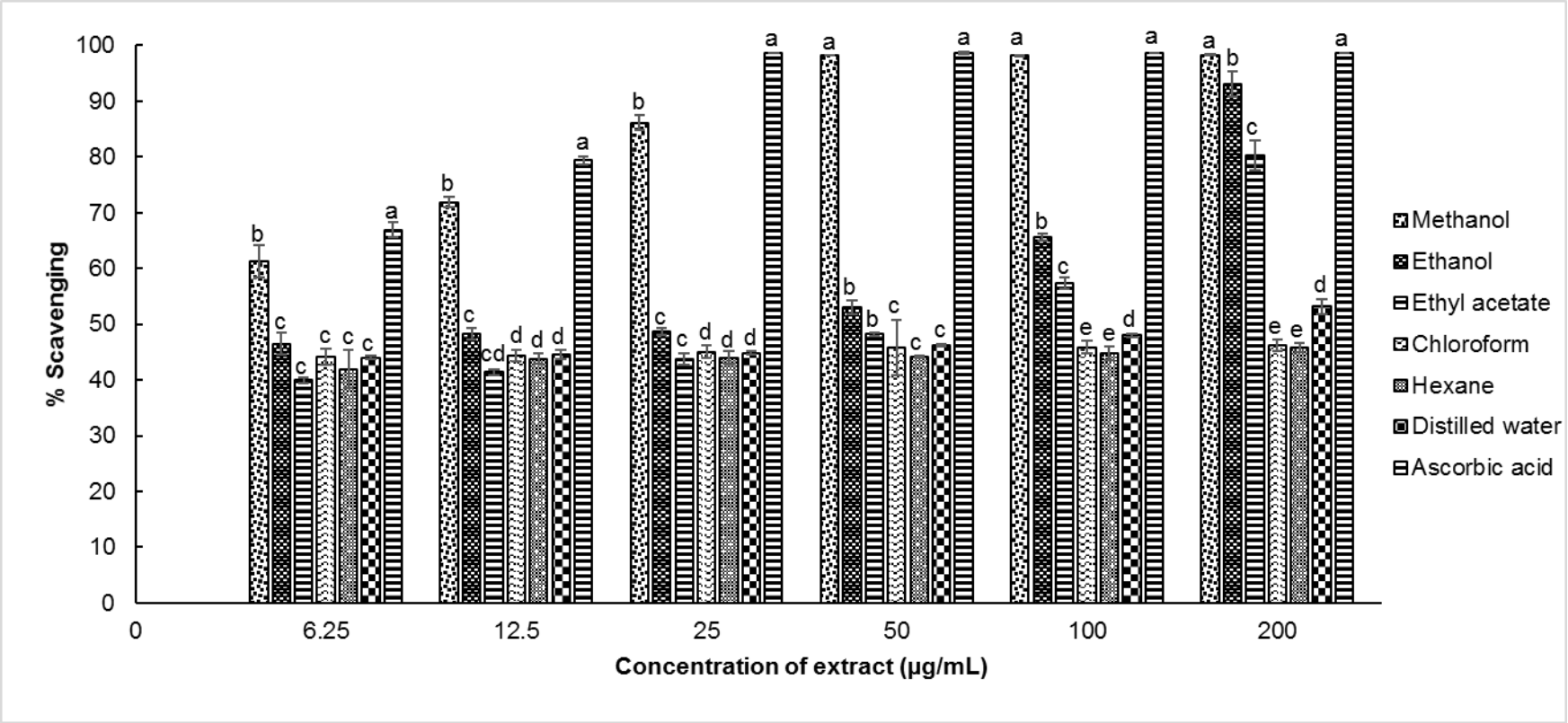

Figure 4.1 Radical scavenging activity of the crude extracts of *P. longifolia* seeds and ascorbic acid on DPPH. Bars labelled with different letters are significantly different when compared within extract type, across concentrations (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.

4.4.4 *In vitro* cytotoxicity and anticancer activity

All crude seed extracts were subjected to *in vitro* cytotoxic screening against Vero and MCF-7 cells. Both cell lines were exposed to the aforementioned extracts at varying concentrations to determine percent viability (Table 4.5 and Fig. 4.2) and IC_{50} (Table 4.6) using the MTS assay. None of the seed extracts exhibited any cytotoxicity against the Vero cell line at the tested concentrations (50-200 µg/mL). However, the methanol and ethanol extracts exhibited high anti-proliferative activity towards the MCF-7 cell line, displaying IC_{50} values below 30 µg/mL (Table 4.6). Poor cytotoxic activity (IC_{50} value above 30 µg/mL) was noted for the hexane extract whilst the ethyl acetate, chloroform and distilled water extracts showed no anticancer activity. In excess of 90% of Vero and MCF-7 cells exposed to the reference cytotoxic agent, cadmium, at 4 µg/mL were not viable. Untreated cells served as the negative control and exhibited a viability of 100%.

Table 4.5 Vero cell viability after 24 h exposure to crude extracts of *P. longifolia* seeds

	% Viability					
Concentration (µg/mL)	Methanol	Ethanol	Ethyl acetate	Chloroform	Hexane	Distilled water
50	100 ± 3.08	100 ± 4.36	100 ± 2.06	100 ± 2.80	100 ± 0.70	100 ± 2.04
100	100 ± 4.27	100 ± 3.21	100 ± 3.00	100 ± 3.00	100 ± 3.33	100 ± 2.56
150	100 ± 3.10	100 ± 2.98	100 ± 1.63	100 ± 3.64	100 ± 2.61	100 ± 4.12
200	100 ± 1.56	100 ± 1.50	100 ± 1.80	100 ± 1.56	100 ± 3.98	100 ± 2.48

Values represent mean ± SD of 3 trials of 3 replicates each.

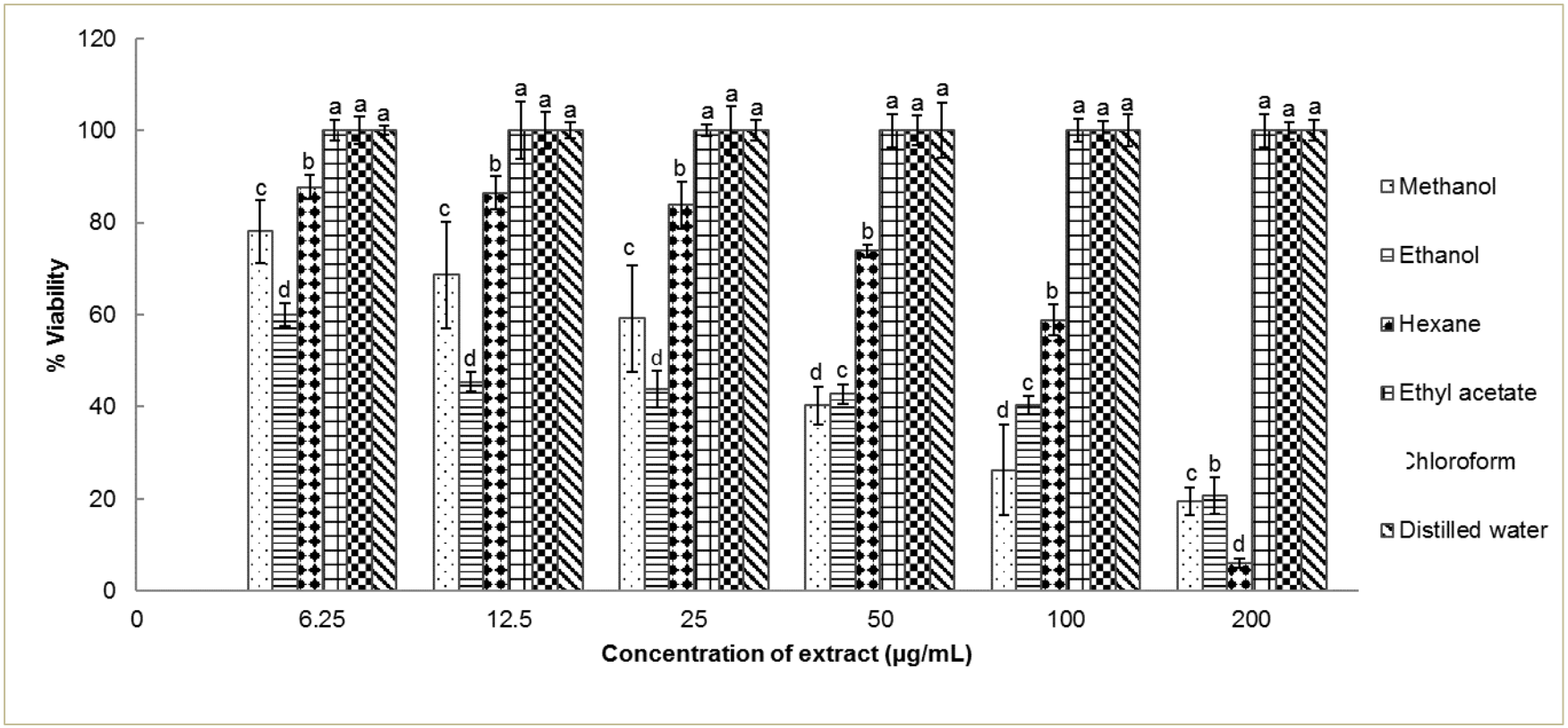

Figure 4.2 Cytotoxicity of crude extracts of *P. longifolia* seeds against MCF-7 breast cancer cells. Bars labelled with different letters are significantly different when compared within extract type, across concentrations (ANOVA; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.

Table 4.6 Anticancer activity of MCF-7 after 24 h exposure to crude extracts of *P. longifolia* seeds

Extract	IC_{50} (µg/mL)
Methanol	24.35 ± 4.80
Ethanol	8.53 ± 1.34
Ethyl acetate	-
Chloroform	-
Hexane	33.43 ± 3.15
Distilled water	-

Values represent mean ± SD of 3 trials of 3 replicates each. – : no minimum inhibition in the tested range (6.25-200 µg/mL).

4.4.5 GC-MS analysis

GC-MS profiling of the methanol, ethanol, ethyl acetate, chloroform, hexane and distilled water crude seed extracts of *P. longifolia* revealed the presence of various compounds with potential therapeutic properties. The major compound names, together with their retention time, molecular formula, molecular weight and their relative abundance in terms of peak area percent are presented in Tables 4.7-4.12. A total of 40 different phytocompounds were identified across the six extracts.

The methanol extract revealed the presence of 14 phytocompounds (Tables 4.7) with hentriacontane (10.71%) and cyclotrisiloxane, hexamethyl (10.66%) being the most abundant.

Table 4.7 Phytocompounds of methanol crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
3.96	p-xylene	C_8H_{10}	106	10.40
4.68	3-carene	$C_{10}H_{16}$	106	7.65
5.18	Cyclohexene, 4-methylene-1-(1-methylethyl)-	$C_{10}H_{16}$	136	4.89
5.34	Hentriacontane	$C_{31}H_{64}$	436	10.71
5.76	Pyridine, 2-(phenylmethyl)-	$C_{12}H_{11}N$	169	3.84
9.39	Benzene, 1,1'-(1-methylethylidene)bis(4-methoxy)	$C_{17}H_{20}O_2$	256	6.72
10.01	Alloaromadendrene	$C_{15}H_{24}$	204	2.82
10.17	Cis-muurola-4(14),5-diene	$C_{15}H_{24}$	204	4.61
14.07	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	7.39
15.32	Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}O_3Si_3As$	342	5.82
18.46	Trimethyl[-4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	$C_{15}H_{24}O_2Si$	264	6.98
18.76	Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}O_3\ Si_3As$	468	7.94
19.08	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	10.66
19.16	4-methyl-2,4-bis(4'-tetramethylsiloxyphenyl)pentene-1	$C_{24}H_{36}O_2Si_2$	412	9.58

A total of 22 phytocompounds were identified from the ethanol extract (Table 4.8). Dodecane-1-fluoro (7.82%), cyclotrisiloxane, hexamethyl (7.77%) and hentriacontane (7.67%) were found in greatest abundance.

Table 4.8 Phytocompounds of ethanol crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
3.02	Pyridine, 2-(phenylmethyl)-	$C_{12}H_{11}N$	169	4.96
3.61	4-undecane, 5-methyl	$C_{12}H_{24}$	168	3.91
3.96	p-xylene	C_8H_{10}	106	7.37
4.68	3-carene	$C_{10}H_{16}$	136	4.92
4.92	2-ethyl-1-hexanol, heptafluorobutyrate	$C_{12}H_{17}O_2F_7$	326	2.43
5.18	Pyridine, 2-[(2-methoxyphenyl)methyl]-	$C_{13}H_{13}ON$	199	3.24
5.34	Dodecane, 1-fluoro-	$C_{10}H_{25}F$	188	7.82
5.73	(Z)-8-hydroxy-4,7-dimethyl-oct-6-enoic acid lactone	$C_{10}H_{16}O_2$	168	1.99
6.42	Hentriacontane	$C_{31}H_{64}$	436	7.67
7.36	2,3-dimethyl-3-pentanol, trifluoroacetate	$C_9H_{15}O_2F_3$	212	2.32
7.72	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	4.75
8.50	1R,2C,3T,4T-tetramethyl-cyclohexane	$C_{10}H_{20}$	140	2.52
9.23	Methyl 10,12-octadecadiynoate	$C_{19}H_{30}O_2$	290	2.20
9.39	Benzene, 1,1'-(1-methylethylidene)bis(4-methoxy)	$C_{17}H_{20}O_2$	256	3.62
10.01	Alloaromadendrene	$C_{15}H_{24}$	204	1.61
10.17	Cis-muurola-4(14),5-diene	$C_{15}H_{24}$	204	3.37
13.48	Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}O_3Si_3As$	342	3.58
14.43	4-methyl-2,4-bis(4'-tetramethylsilyloxyphenyl)pentene-1	$C_{24}H_{36}O_2\ Si_2$	412	6.14
14.76	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7\ Si_8$	578	5.31
17.02	Trimethyl[4-(2-methyl-4-oxo-2-phenyl)phenoxy]silane	$C_{15}H_{24}O_2\ Si$	264	5.79
19.03	Cyclotrisiloxane, hexamethyl]-	$C_6H_{18}O_3Si_3$	222	7.77
19.31	Trimethyl[-4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	$C_{17}H_{30}OSi$	278	6.67

The ethyl acetate extract contained 14 different compounds (Table 4.9). Phenol, 2,4-bis (1,1-dimethylethyl) was present in greatest abundance (13.17%).

Table 4.9 Phytocompounds of ethyl acetate crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
3.02	3,4-altrosan	$C_6H_{10}O_5$	162	2.51
3.61	Cyclobutanone, 2,3,3,4-tetramethyl-	$C_8H_{14}O$	126	7.50
3.96	Benzene, 1,3-dimethyl-	C_8H_{10}	106	9.90
4.68	3-carene	$C_{10}H_{16}$	136	7.48
7.44	Dodecane, 1-fluoro-	$C_{10}H_{25}F$	188	9.46
7.72	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	8.17
7.97	Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	190	10.69
8.42	1R,2C,3T,4T-tetramethyl-cyclohexane	$C_{10}H_{20}$	140	5.14
9.31	Hentriacontane	$C_{31}H_{64}$	436	8.75
10.17	Cis-muurola-4(14),5-diene	$C_{15}H_{24}$	204	4.14
10.27	Phenol, 2,4-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$	206	13.17
11.81	(5-methyl-2-phenyl-1,3-dioxan-4-yl)methanol	$C_{12}H_{16}O_3$	208	2.13
12.47	Heptadecane, 7-methyl-	$C_{14}H_{38}$	254	4.62
15.39	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	6.33

The chloroform extract contained three phytocompounds (Tables 4.10) with phenol, 2,4-bis (1,1- dimethylethyl) (39.81%) being the most abundant phytocompound.

Table 4.10 Phytocompounds of chloroform crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
7.72	Benzaldehyde, 2,5-dimethyl-	$C_9H_{10}O$	134	31.32
10.27	Phenol, 2,4-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$	206	39.81
11.88	Hentriacontane	$C_{31}H_{64}$	436	28.87

The hexane extract contained 11 phytocompounds (Tables 4.11) with phenol, 3-hexanol, 2,2-dimethyl- (12.64%) and benzene, 1-ethyl-4-methyl- (12.50%) being the most abundant phytocompounds.

Table 4.11 Phytocompounds of hexane crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
3.35	3-hexanol, 2,2-dimethyl-	$C_8H_{18}O$	130	12.64
3.97	Benzene, 1,3-dimethyl	C_8H_{10}	106	11.16
4.22	p-xylene	C_8H_{10}	106	10.88
4.29	Carbonic acid, neopentyl cyclohexyl ester	$C_{12}H_{22}O_3$	214	7.89
4.97	Benzene, 1-ethyl-4-methyl-	C_9H_{12}	120	12.50
5.34	Mesitylene	C_9H_{12}	120	8.05
6.29	1,2-ethanediol, dipropanoate	$C_8H_{14}O_4$	174	10.25
7.73	Benzaldehyde, 2,4-dimethyl	$C_9H_{10}O$	134	7.32
10.17	Cis-muurola-4(14),5-diene	$C_{15}H_{24}$	204	4.26
10.28	Phenol, 2,4-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$	206	9.89
16.41	Hentriacontane	$C_{31}H_{64}$	436	5.15

The distilled water extract contained four phytocompounds (Table 4.12). Benzene, 2-methoxy-1-(2-nitroethenyl)-3-(phenylmethoxy)-, phenol, 2,4-bis (1,1- dimethylethyl)- and Chromium, bis(anisole)- exhibited a 25.45% peak area.

Table 4.12 Phytocompounds of distilled water crude extract of *P. longifolia* seeds acquired via GC-MS

Retention time	Compound name	Molecular formula	Molecular weight	Peak area %
3.96	Benzene, 2-methoxy-1-(2-nitroethenyl)-3-(phenylmethoxy)-	$C_{16}H_{15}O_4N$	285	25.45
5.34	Hexane, 3,4-bis(1,1-dimethylethyl) 2,2,5,5-tetramethyl-	$C_{18}H_{38}$	254	23.65
10.27	Phenol, 2,4-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$	206	25.45
11.50	Chromium, bis(anisole)-	$C_{14}H_{16}O_2Cr$	268	25.45

The phytocompounds present in most extracts were hentriacontane, cis-muurola-4(14),5-diene and phenol, 2,4-bis(1,1-dimethylethyl)- (Figure 4.3). Comparatively, the maximum extraction yield was achieved when ethanol was used as the extracting solvent. Chloroform was the least efficient solvent at extraction as a low yield of phytocompounds were obtained.

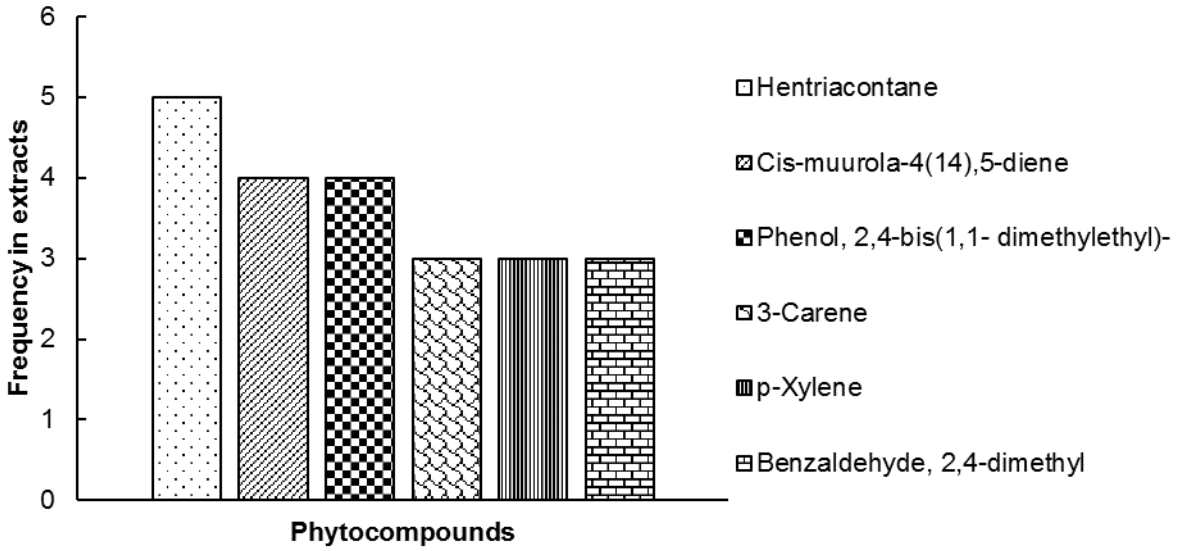

Figure 4.3 Common phytocompounds found in most crude extracts of *P. longifolia* seeds.

3.5 DISCUSSION

The current study was undertaken to provide relative data on the phytochemistry, *in vitro* antioxidant, antitumour and antimicrobial activity of six different crude seed extracts of *P. longifolia*.

The phytochemical screening, by qualitative estimation (Table 4.1) and GC-MS (Tables 4.7-4.12) has revealed the presence of a diverse number of phytochemical constituents in the crude seed extracts tested. This demonstrated seeds of *P. longifolia* to be a rich source of phenols, flavonoids, cardiac glycosides, and sterols. These are known to exhibit various biological activities (Vijayarathna and Sasidharan, 2012) and consequently suggests that the seeds of *P. longifolia* has the potential to be used as a herbal remedy. Although the qualitative assessment of these seed extracts did not indicate the presence of terpenoids, in a previous study, two triterpenoids, 3-oxo-5α-lanosta-8,24-dien-21-oic acid and 3β-hydroxylanosta-9,24-dien-24-oic acid exhibiting antibacterial and anti-platelet aggregation activity were isolated from chloroform stem bark extract of *P. longifolia* (Mosa *et al.*, 2011a). Tannins (7%) and tanning material (10.2-18%) of the stem bark of *P. longifolia* has also been reported previously (Hutchings *et al*., 1996).

Despite the absence of potential antifungal activity, all crude extracts of *P. longifolia* seeds exhibited considerable potential antibacterial activity against all bacterial test organisms except *E. faecalis*, as indicated by zones of inhibition for the disc diffusion assay (Table 4.2). In an attempt to rank the MIC of plant extracts, Holetz *et al* (2002) posed that good antimicrobial activity was for MICs below 0.1 mg/mL, intermediate activity for MICs between 0.1-0.5 mg/mL, poor activity for MIC between 0.5-1 mg/mL and MIC above 1 mg/mL was inactive. In view of this, MIC values for this study suggests ethanol crude extract of *P. longifolia* seeds to be good antimicrobials against Gram negative *P. aeruginosa* (98.61 µg/mL), while intermediate activity was displayed by methanol and ethanol extracts against *E. coli* and by the methanol, ethanol, chloroform and hexane fractions against *S. aureus* (Table 4.3). In a previous study carried out by Suleiman *et al* (2010b) to ascertain the antimicrobial potential of the acetone, dichloromethane, hexane and methanol crude leaves extracts of *P. longifolia*, *P. aeruginosa* was also the most susceptible bacterium, displaying a MIC of 0.08 mg/mL for the acetone leaf extract with no fungicidal effects observed for all solvent extracts in the range of 6.25-200 µg/mL. However, these results were not clear since the external membrane of Gram negative bacteria behaves as a barrier to antibiotics and the periplasmic space possesses enzymes that break down foreign bodies from the external environment (Duffy and Power, 2001). These results suggest that the extracts of *P. longifolia* have the potential to behave as broad spectrum antibiotics.

Although many studies report on the DPPH scavenging ability of plants from all over the world, only a few studies report low IC_{50} values, as was illustrated by the methanol, ethanol, ethyl acetate and distilled water crude seed extracts of *P. longifolia* (Table 4.4). The highest DPPH radical scavenging ability was observed for the methanol crude seed extract (IC_{50} = 5.00 µg/mL). This was comparable to the standard antioxidant, ascorbic acid, used (IC_{50} = 4.67 µg/mL (Figure 4.1). These results suggest that the extract possesses phytocompounds that serve as free radical scavengers and consequently potentially ameliorate oxidative stress related to metabolic ailments (Ibrahim *et al.*, 2013). In a study conducted by Mosa *et al*. (2011b), the methanol bark extract of *P. longifolia* displayed the highest radical scavenging ability (IC_{50} = 0.07mg/mL). Conversely, triterpenes isolated from the methanol stem bark extract of *P. longifolia* exhibited poor antioxidant activity due to inadequate scavenging of DPPH radicals (Mosa *et al.*, 2011a).

The IC_{50} limit displaying good anticancer activity for plant crude extracts set by The American National Cancer Institute (NCI) is 30 µg/ mL after a 72 hour exposure period to extracts. Additionally, plant crude extracts displaying an IC_{50} of 20 µg/mL is thought to be potently cytotoxic. In the present study, the results clearly demonstrate that all crude extracts of *P. longifolia* seeds did not confer cytotoxicity to the Vero cells (Table 4.5). Additionally, whilst ethyl acetate, chloroform and distilled water extracts were not cytotoxic, methanol, ethanol and hexane seed crude extracts induced a significant ($p<0.05$) reduction in MCF-7 cell proliferation numbers (Figure 4.2). The methanol extract displayed good antitumour activity with an IC_{50} of 24.35 µg/mL whilst the ethanol extract displayed toxicity against MCF-7 well below that indicated by the NCI (IC_{50} = 8.53 µg/mL), thus classifying the ethanol seed crude extract of *P. longifolia* as a potential pure anticancer compound. This differential cytotoxic effect amongst seed extracts may be due to each extracts chemical diversity and in some instances, good biological potency. Conversely, lack of cytotoxicity of *P. longifolia* stem bark extracts has previously been demonstrated during preliminary toxicity screening using the brine shrimp lethality bioassay (Mosa *et al.*, 2011b).

Overall, 40 different phytocompounds were identified from the crude extracts of *P. longifolia* seeds. The phytochemical profile of these seeds were predominantly made up of sesquiterpenes and monoterpenes. A few compounds of known biological importance were identified. The cyclopropane-containing monoterpene, 3-carene was identified in the methanol (Table 4.7), ethanol (Table 4.8) and ethyl acetate (Table 4.9) fractions and is known for its anesthetic activity (Librowski *et al*., 2004). Cis-muurola-4(14),5-diene, a component of essential oils was identified in the methanol, ethanol, ethyl acetate and hexane (Table 4.10) fractions. The compound 3,4-altrosan was identified in the ethyl acetate fraction and is known for its antibacterial and antifungal activity (Nirubama *et al*., 2014). The essential oil alloaromadendrene was identified in methanol and ethanol fractions. Alloaromadendrene

from the leaves of *Cinnamomum osmophloeum* has demonstrated *in vivo* antioxidant activity on the nematode *Caenorhabditis elegans.* Not only did it suppress juglone-induced oxidative stress in *C. elegans*, alloaromadendrene also extended the life expectancy of the nematode (Yu *et al*., 2014). This suggests that alloaromadendrene could be responsible for the noted radical scavenging ability of the methanol and ethanol crude seed extracts (Figure 4.1 and Table 4.4) and could potentially be used as a source of antioxidants to delay aging.

3.6 CONCLUSION

In conclusion, it can be stated that tested crude seed extracts of *P. longifolia* have strong antioxidant, antimicrobial and anticancer activities *in vitro*. On the basis of these results, *P. longifolia* appears to be a safe pharmacological agent and could be of significance in human therapy, animal and plant diseases.

3.7 ACKNOWLEDGEMENTS

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Chapter 5

General discussion and conclusion

5.1 GENERAL DISCUSSION AND CONCLUSIONS

The objectives of this study was to investigate the phytochemical composition as well as the antimicrobial, antioxidant and anticancer activity of the crude seed extracts of *T. emetica* and *P. longifolia*. The seed material of these tree species were extracted using six different solvents (methanol, ethanol, ethyl acetate, chloroform, hexane and distilled water) via cold percolation.

In the first study, phytochemical analyses of extracts of *T. emetica* seeds showed the presence of alkaloids, cardiac glycosides, phenols, sterols flavonoids and terpenoids. Some extracts of *T. emetica* exhibited an inhibitory effect on a few fungal pathogens displaying the potential to prevent microbial infections in susceptible individuals. Free radical scavenging ability was only observed for the methanol extract of *T. emetic* seeds. No cytotoxic effect was noted for all extracts of *T. emetica* seeds against both tested cell lines. The lack of toxicity against the Vero cell line endorses safe application of these extracts as potential antifungal agents. The GC-MS analysis validates the results obtained for the preliminary phytochemical analysis, antioxidant and antifungal activity by providing molecular information of the crude seed extracts of *T. emetica*. These results are in agreement with previous outcomes that suggest the genus *Trichilia* possesses antioxidant and antimicrobial activity (Ayo *et al.*, 2013; Frum and Viljoen, 2006; Geyid *et al.*, 2005)

In the second study, preliminary phytochemical analysis of *P. longifolia* seeds revealed the presence of cardiac glycosides, phenols, sterols and flavonoids. Even though these seed extracts failed to display fungal inhibition, broad-spectrum antibacterial effect was noted against clinical isolates. This antibacterial activity may be ascribed to the presence of phytocompounds that confer medicinal properties to treat bacterial infections. Bacteria are more susceptible to antimicrobial activity than fungi because of variation in arrangement, permeability and composition of their cell walls (Tepe *et al.*, 2004; Rankovic *et al.*, 2011). Fungal cells walls contain microfibrillar polysacharrides (chitin), adding to its rigidity, thus making the wall poorly penetrable to antimicrobial agents (Ruiz-Herrera, 1991). Cell walls of Gram positive bacteria are composed of peptidoglucanes and teichoic acids and the cell walls of Gram negative bacteria are composed of peptidoglucanes, lipopolysacharides and lipoproteins and thus more penetrable than fungal cell walls (Van Heijenoort, 2001). The greatest free radical scavenging activity was noted for the methanol seed extract of *P. longifolia*. Additionally, these seed extracts displayed considerable toxicity on MCF-7 cells thus displaying potential anticancer activity on breast cancer. All extracts had no effect on the Vero cell line, encouraging their use in concoctions that manage blood-clotting associated ailments (Mosa *et al.*, 2015)**.**

The present study demonstrated varied phytochemical content between the tested extracts of *T. emetica* and *P. longifolia* seeds. Extracts of *P. longifolia* seeds eluted more phytochemicals of therapeutic value than those of *T. emetica*. In addition, the DPPH scavenging activity of both species were found to be significantly correlated with the tree species overall biological activity. These findings also suggested that extracts of *P. longifolia* seeds produced better toxicity effects on the tested cell lines than those of *T. emetica*. Moreover, a good correlation was determined between the two toxicity models. Seeds of *T. emetica* and *P. longifolia* are unexplored parts of highly investigated trees and are storehouses of valuable bioactive phytochemicals. Preliminary investigations into the antibacterial, antifungal, antioxidant and anticancer investigations of various solvent extracts are highly promising.

Considering the range of traditional uses of *T. emetica* and *P. longifolia*, it is pertinent to examine the seeds of these species for other equally important biological potential such as antiviral, anti-inflammatory, anthelmintic, laxative and analgesic activity. A range of extraction approaches should also be tested in an effort to ascertain the method conferring unsurpassed activity. Another important aspect that requires further investigation is the isolation, purification and characterisation of the bioactive molecules responsible for eliciting the activities demonstrated in this study. These preliminary findings will contribute to the advancement in the production of novel and more potent phytochemical based antimicrobial, antioxidant and anticancer agents. Further studies are required to evaluate the clinical significance of these findings.

This study served to highlight the acclaimed therapeutic potential of *T. emetica* and *P. longifolia* and lends credence to their application in traditional medicine.

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