ISOLATION AND CHARACTERIZATION OF BIO-ACTIVE COMPOUNDS FROM *EUPHORBIA INAEQUILATERA* AND *DICEROCARYUM SENECIOIDES*.

By

NGOBENI ALISTER

RESEARCH DISSERTATION

Submitted in fulfilment of the requirements for the degree of

MASTER of SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE & AGRICULTURE

(School of Molecular & Life Sciences)

at the

UNIVERSITY OF LIMPOPO

Supervisor: Prof L.J. Mampuru

Co-supervisors: Prof P. Masoko

Prof M.P. Mokgotho

2012

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted in fulfillment of the Degree of Masters of Science at the University of Limpopo, Turfloop campus. It has not been submitted before for any degree or examination in any other university.

Signature ……..

Signed at …….. on …….. day of …..... 2012

TABLE OF CONTENTS

DEDICATION

To my family at large but, most importantly to my wife, Glenda Ngobeni; my son Ntwanano Ngobeni, and my mother, Linah Ngobeni.

ACKNOWLEDGEMENTS

I would like to sincerely acknowledge the following:

- Special thanks to the Almighty My Heavenly Father GOD for granting me the opportunity to study this degree, for His love, strength and courage throughout my life.

- My supervisor Prof. L.J. Mampuru; thanks for your patience, intelligence, guidance, advice, and encouragement.

- My co-supervisors Prof. P. Masoko and Dr. M.P. Mokgotho, guys you are the backbone of this work, thanks.

- Also special thanks to Dr. L. Mdee for assisting with the isolation process and chemical structural characterisation.

- My gratitude to Biochemistry and Microbiology staff members who contributed to my success and the knowledge.

- The postgraduate students in the Department of Biochemistry, Microbiology, and Biotechnology I would like to thank you for everything you have done to assist me throughout this study, it has been nice working with you.

- Finally, I would like to thank the University of Limpopo and the National Research Foundation (NRF) for their financial support.

ABSTRACT

This study was carried out to investigate antioxidant and antibacterial properties of 9 indigenous medicinal plants, viz., *Euclea undulata (mogweregwere), Momordica balsamia (mogapu badimo), sefapa badimo, Senecio asperulus (makgonatšohle), Stiburus alopecuroides (mošalašuping), serolana, Euphorbia inaequilatera (kgama-maswana), mokgagapitsi* and *Clerodendrum glabrum (mohlokohloko)* and to further isolate compounds that relate to these properties. Four extracting solvents with varying polarities viz. *n*-hexane, dichloromethane, acetone and methanol were used to extract the bioactive compounds from the ground powdered plant materials. The TLC plates, developed in three solvent systems *viz*., benzene, ethanol and ammonia (BEA, 18:10:0.2, v/v/v); ethyl acetate, methanol and water (EMW, 10:1.35:1, v/v/v) and chloroform, ethyl acetate and formic acid (CEF, 10:8:2, v/v/v), were visualised using DPPH, vanillin-sulphuric acid, visible light at 366 nm, UV light at 254 nm and bioautography for the presence of potential antioxidant and antibacterial compounds. The results of the screening process showed that only four plants possessed antioxidant compound(s) while six plants had antibacterial activity against *Staphylococcus aureus*. *Euclea undulata "*MKK" was observed to possess both antibacterial and antioxidant active compounds. Two antioxidant active compounds were isolated from two plants, viz., *Euphorbia inaequilatera* and *Dicerocaryum senecioides*. Solvent-solvent extraction, column chromatography and preparative TLC were used to further isolate and characterise target compounds. The antioxidant active compounds were found to separate well under EMW, an indication that the compounds are polar and intermediate-polar. The NMR spectra of the compound isolated from the *D. senecioides* revealed that the compound is a stilbenoid. For the first time, we report that the anti-inflammatory, antioxidant and antiproliferation properties of the *D. senecioides* reported by other studies performed in this laboratory could be due to this isolated stilbenoid compound. However, further studies are still necessary to confirm this assertion.

LIST OF ABBREVIATIONS

A	acetone
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
BAW	butanol, acetic acid and water
BEA	benzene, ethanol and ammonia
BHA	butylated hydroxyanisole
BHT	butylated hydroxyltoluene
CDC	centre for disease control
CEF	chloroform, ethyl acetate and formic acid
cm	centimetres
COX I & II	Cyclooxygenase-I & II
DCM	dichloromethane
DNA	deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
D1	hexane fraction
D2	dichloromethane fraction
D3	butanol fraction
EHEC	enterohaemorrhagic *Escherichia coli*
EMW	ethyl acetate, methanol and water
Fr	fraction
g	grams
GPx	glutathione peroxidase
GST	glutathione S-transferase

Hex	hexane
HPLC	high performance liquid chromatography
INT	*p*-iodonitrotetrazolium violet
IR	infrared
KM	*Euphorbia inequilatera (kgama-maswana)*
M	methanol
MBL	*Momordica balsamia (mogapu badimo)* leaves
MBR	*Momordica balsamia* (*mogapu badimo)* roots
mg	milligrams
MHH	*Clerodendrum glabrum (mohlokohloko)*
MKK	*Euclea undulata (mogweregwere)*
MKP	"*mokgagapitsi"*
ml	millilitres
ML	*Stiburus alopecuroides* (*mošalašuping)* leaves
mm	millimetres
MR	*Stiburus alopecuroides* (*mošalašuping)* roots
MS	mass spectrometry
MT	*Senecio asperulus (makgonatšohle)*
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
ROS	reactive oxygen species
RPM	revolutions per minute
SBL	"*sefepa badimo"* leaves
SBR	"*sefapa badimo"* roots

SOD	superoxide dismutase
SR	"*serolana"* roots (*Solanum spp*)
TLC	thin layer chromatography
UV	ultraviolet
V	volume
VRE	enterococci resistant to vancomycin

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Herbal medicines

Herbal medicines are plant-derived remedies that are used for their therapeutic properties and they have been an important tradition of many cultures and beliefs of African people [1]. Sanitation and hygiene levels for the majority of people in Africa are not comparable to those of the first world countries. This exposes African people to a wider array of microbial pathogens, which increases their susceptibility to bacterial, fungal and viral infections. Indigenous plants are often the only available means of treating such infections [2]. Since there is an increasing resistance to antibiotics by many pathogenic and opportunistic bacteria, plant extracts and plant-derived compounds have emerged as potential and promising antimicrobial agents [3].

Empiriological and experimental evidence suggest that free radicals and reactive oxygen species (ROS) are implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome (AIDS), heart disease, stroke, arteriosclerosis, diabetes, cancer and gastric ulcer [4, 5]. Antioxidants can protect the human body from free radicals, ROS effects and may retard progression of many chronic diseases as well as lipid oxidative rancidity in foods [4, 6, 7]. However, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), the most commonly used antioxidants at present, are suspected of being responsible for liver damage and carcinogenesis [8]. As a result scientists are trying to incorporate traditional medicine within primary health care [1]. This renewed interest in traditional medicines means that scientists are not only concerned in determining the scientific rationale of traditional practice of medicine plant usages, but also aspire to discover novel and safe plant compounds of pharmacological importance [9].

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives which are mainly secondary metabolites [10]. Currently identified secondary plant metabolites exceed 100 000 substances, belonging to a variety of chemical classes, including terpenoids, phenolics and alkaloids [11]. There is an interest in these secondary metabolites since they are known to demonstrate various biological activities that encourage positive health effects, such as antibacterial, anticancer, antifungal, antioxidant and antiviral activities that can be used in the food, agricultural and pharmaceutical industries.

The present study is intended to investigate nine indigenous medicinal plants for the presence of antioxidant and antibacterial compounds. The leaves and roots of *Euclea undulata (Mogweregwere), Momordica balsamia (mogapu badimo), sefapa badimo, Senecio asperulus (makgonatšohle), Stiburus alopecuroides (mošalašuping), Solanum spp (serolana), Euphorbia Inaequilatera (kgama-maswana), mokgagapitsi* and *Clerodendrum glabrum (mohlokohloko)*. These indigenous plants were mainly collected in the surrounds of the Limpopo province. Most of the selected plants are used to treat different infections and also for blood purifying purposes by traditional healers. For antibacterial activity, the plant extracts were tested against four pathogenic microorganisms, *i.e*., *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus* using bioautography approach. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, TLC-DPPH antioxidant screening and reducing power assays were used to evaluate the potential antioxidant activity of these plant extracts.

1.2. Problem statement

The increase in antibiotic resistance by microorganisms and the often lethal diseases caused by free radicals is posing serious ramifications to the lives and health of humans. Thus, there is a need to identify and process naturally occurring compounds which possess antibacterial and antioxidant activities.

1.3. Motivation of the study

Plants are chemical store houses of many chemical compounds which offer protection to the plants harbouring them from free radicals and pathogenic microorganisms. This makes plants a good source of natural antioxidants and antibacterial compounds. Most of these compounds are produced as secondary metabolites. Due to an increase in antibiotic resistance of many microorganisms and numerous incidences in diseases that are associated with the presence of free radicals, plants are therefore studied to discover novel compound(s) which can be used as antibiotics and/ or antioxidants. The selected medicinal plants in this study are often used by practitioners of traditional medicines to treat a variety of bacterial infections and other ailments and for blood purifications.

1.4. Aim

The aim of this study was to screen, isolate and identify antibacterial and antioxidative compounds from the nine indigenous medicinal plants from Limpopo province and to further isolate antioxidant compounds from plants with potent antioxidative.

1.4.1 Objectives

The specific objectives of this study were to:

(i) Screen the antibacterial and antioxidant activity of the nine indigenous medicinal plants, using four different solvents of varying polarities (*n*-hexane, dichloromethane, acetone and methanol) to obtain the crude extracts.

(ii) Evaluate the phytochemical profiles of the resultant crude extracts using vanillin-sulphuric acid.

(iii) Screen the crude extracts for antioxidant compounds using 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

(iv) Screen for antibacterial activity of the crude extracts using bioautography activity assay.

(v) Isolate and elucidate the chemical structure of the antioxidant compound(s) from *E. inaequilatera* and *D. senecioides*.

1.5. Hypothesis

The selected traditionally used indigenous medicinal plants possess inherent antioxidant and/or antibacterial compounds that account for their ethnobotanical use in traditional medicine.

1.6. Significance of the study

This study revealed the scientific information in terms of antibacterial and antioxidant activity of the selected indigenous medicinal plants; it also led to the discovery of a potential novel bio-active compound which can help in the fight against diseases caused by free radicals.

CHAPTER 2

LITERATURE REVIEW

2.1. Medicinal plants

Plants have long provided mankind with herbal remedies for many infectious diseases and they continue to play a major role in primary health care as therapeutic remedies in developing countries [12]. Plant-based drugs have been used worldwide in traditional medicines for the treatment of various diseases. Approximately 80% of world's population still relies on medicinal plants for their primary healthcare. According to a survey by the National Cancer Institute, USA, 61% of the 877 small-molecule new chemical entities introduced as drugs worldwide during 1981-2002 were inspired by natural products [13]. Plant species still serve as a rich source of many novel biologically active compounds. Very few plant species have been thoroughly investigated for their medicinal properties [8,14]. Plants are complex chemical storehouses of undiscovered biodynamic compounds with unrealized potential for use in modern medicine [15]. Plants produce a lot of antioxidants to control the oxidative stress, making them a good natural source of antioxidant and antibacterial activity, which can be observed in fruits, vegetables, roots, leaves and seeds [12].

2.2. Bacterial infections

Bacteria are microscopic, single-celled organisms found in air, water, soil, and food. They live on plants, insects, animals, pets, and even in the human digestive system and upper respiratory tract. There are thousands kinds of bacteria, but only a few actually cause disease in humans [16]. The fight against bacterial infection represents one of the high points of modern medicine. The development of antibiotics in the 1940s offered physicians a powerful tool against bacterial infections that has saved the lives of millions of people [17]. However, because of the widespread and sometimes inappropriate use of antibiotics, strains of

bacteria have begun to emerge that are antibiotic resistant. These new, stronger bacteria pose a significant threat to general welfare and health of people and a challenge to researchers. In the United States, bacterial infections are a leading cause of death in children and the elderly. Hospitalized patients and those with chronic diseases are at high risk of bacterial infection [17]. Common bacterial infections include pneumonia, ear infections, diarrhoea, respiratory tract infections, urinary tract infections and skin disorders.

2.2.1. *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium that inhabits the intestines of animals and humans. *E. coli* is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Some strains such as enterohaemorrhagic *E. coli* (EHEC) can cause severe food borne infections. The incidence of EHEC infections varies by age group, with the highest incidence of reported cases occurring in children aged under 15 years (0.7 cases per 100 000 in the United States) [18]. Sixty-three (63%) to 85% of cases are a result of exposure to the pathogen through food. EHEC produces toxins, known as verotoxins. EHEC can grow in temperatures ranging from 7°C to 50°C, with an optimum temperature of 37°C. Symptoms of the diseases caused by EHEC include fever, vomiting [18,19], abdominal cramps and diarrhoea that may progress to bloody diarrhoea (haemorrhagic colitis).

2.2.2. *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive, facultative anaerobic, cocci which occurs singly, in pairs or short chains. It is a normal inhabitant of the intestinal tract and female genital tracts. *Enterococcus faecalis* is an opportunistic bacterium that has become one of the most challenging hospital pathogens to treat. It has an intrinsic resistance mechanism against many antibiotics and a remarkable capacity for developing resistance against other antibiotics [20]. Enterococci are the leading cause of nosocomial infection. They are responsible

for approximately 110,000 cases of urinary tract infection, 25,000 cases of bactereamia, 40,000 wound infections, and 1,100 cases of endocarditis yearly in the United States [21]. The first strains of enterococci resistant to vancomycin (VRE) and teicoplanin, which were due to the presence of the *van*A resistance gene, were described in South Africa in 1996. The incidence of VRE in South Africa remains low but is expected to increase due to increasing prevalence of antibiotic resistance strains [22]. Enterococci infect its host by primarily colonizing the mucosal surfaces and also evade the host defenses, although little is known about the actual mechanism of evasion. The pathogenicity of the organism is believed to be closely associated with its ability to produce cytolysin, a toxin that causes rupture of a variety of target membranes, including bacterial cells, erythrocytes, and other mammalian cells [21].

2.2.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic rod belonging to the family Pseudomonadaceae. The family includes other genera, which together with certain other organisms, constitutes the bacteria informally known as pseudomonads. These bacteria are common inhabitants of soil and water. They occur regularly on the surfaces of plants and occasionally on the surfaces of animals. *Pseudomonas aeruginosa* is an opportunistic pathogen, that exploits the weak host defences to initiate an infection. *Pseudomonas aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteraemia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns, cancer and acquired immunodeficiency syndrome (AIDS) patients who are immunosuppressed. *Pseudomonas aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients is 50%. This bacterium is primarily a nosocomial pathogen. According to the Centre for Disease Control (CDC), the overall incidence of *P. aeruginosa* infections in US hospitals averages about 0.4% (4 per 1000 discharges), and the bacterium is the fourth most commonly-isolated nosocomial

pathogen accounting for 10.1% of all hospital-acquired infections. *Pseudomonas aeruginosa* produces a number of toxic proteins which not only cause extensive tissue damage, but also interfere with the human immune system's defence mechanisms. These proteins range from potent toxins that enter and kill host cells at or near the site of colonisation to degradative enzymes that permanently disrupt the cell membranes and connective tissues in various organs [23]

2.2.4. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive spherical (cocci) which, on microscopic, examination appears in pairs, short chains, or bunched grape-like clusters. *Staphylococcus aureus* is commonly found on the skin and in the nose of healthy people. The major habitats of the pathogen are the nasal membrane and skin of warm-blooded animals. It is one of the major causes of community-acquired and hospital-acquired infections and causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furunculosis; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis [24]. The incidence of methicillin-resistant *S. aureus* in South Africa is alarming, with up to 50% of nosocomial isolates being methicillin-resistant [22]. No vancomycin-intermediate *S. aureus* strains, reported in the United States and Japan, have been isolated in South Africa to date [22]. *Staphylococcus aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream [24].

2.3. Major groups of antimicrobial compounds from plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total [25]. In many cases, these aromatic substances serve as plant defence mechanisms against predation by microorganisms,

insects, and herbivores. Some, such as aromatic, give plants their odours; others (quinones and tannins) are responsible for plant pigments. The antimicrobial compounds produced by plants can be divided into several categories as follows:

2.3.1. Phenolics and polyphenols

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria, and fungi. Some other phenolic compounds are catechol and pyrogallol, both hydroxylated phenols, are toxic to microorganisms. Catechol has two hydroxyl groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms. Quinones on the other hand, are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being coloured, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin [26]. Kazmi *et al*. [27] described an anthraquinone from *Cassia italica*, a Pakistani tree, which showed bacteriostatic activity for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Some examples of phenolic and polyphenoloic compounds are flavones, flavonoids, flavonols, tannins and coumarins.

2.3.2. Terpenoids and essential oils

The fragrance of plants is carried in the so called *quinta essentia*, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually

oxygen, they are termed terpenoids. Terpenenes or terpenoids are active against bacteria, fungi, viruses, and protozoa [28]. In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria [29]. The ethanol-soluble fraction of purple prairie clover yields a terpenoid called petalostemumol, which showed excellent activity against *Bacillus subtilis* and *S. aureus* and lesser activity against Gram-negative bacteria as well as *Candida albicans* [30].

2.3.3. Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum*; the name morphine comes from the Greek Morpheus, god of dreams [29]. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family, are commonly found to have antimicrobial properties [31]. Berberine is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA [32].

2.3.4. Lectins and polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 [33]. They are often positively charged and contain disulfide bonds. Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors. Recent interest has been focused mostly on studying anti-HIV peptides and lectins, but the inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthus*, has long been known. Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues. They are toxic to yeasts and to both Gram-negative and Gram-positive bacteria. Thionins AX1 and AX2 from sugar beet were formal

to be active against fungi but not bacteria [30]. Fabatin, a newly identified 47-residue peptide from fava beans, appears to be structurally related to g-thionins from grains and inhibits *E. coli*, *P. aeruginosa*, and *Enterococcus hirae* but not *Candida* or *Saccharomyces* species [34].

2.4. Antioxidants and free radicals

2.4.1. Free radicals and their sources

Free radicals are organic molecules responsible for aging, tissue damage, and a wide variety of diseases. These molecules are very unstable, and thus bond with other molecules, destroying their strength and perpetuating the detrimental process. Oxidation reactions are an essential part of normal metabolism as oxygen is the ultimate electron acceptor in the electron flow system that produces ATP [35]. Problems may arise when electron flow and energy production become uncoupled so that oxygen free radicals, that is, reactive oxygen species (ROS), are produced [8, 36]. Actually, ROS are continuously produced within the cell as a result of mitochondrial electron transfer processes or as by-products of the enzymes xantine oxidase, lipoxygenases and cyclooxygenases [37]. Furthermore, ROS can be generated as a consequence of the intracellular metabolism of foreign compounds, toxins or drugs by cytochrome P450, monoxygenases, or because of exposure to environmental factors such as excessive iron salts or UV irradiation [38]. Other sources of ROS are macrophages and neutrophils that contain enzymes, such as NADPH oxidase complex, able to generate superoxide radicals and hydrogen peroxide. Reactive oxygen species thus play different positive roles *in vivo*, being involved in energy production, phagocytosis, cell growth and intercellular signaling regulation. Reactive oxygen species may also be highly damaging, as they can attack biological macromolecules, namely, lipids, proteins and DNA, induce oxidation and cause membrane damage, enzyme inactivation and DNA damage [39]. However, when the level of ROS level exceeds the antioxidant capacity of the

cell, the intracellular redox homeostasis is altered and oxidative stress ensues [40].

2.4.2. Free radicals associated diseases

There have been accumulating evidence that suggests that cellular damage arising from ROS can be involved in the aetiology and pathophysiology of human diseases such as neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson disease, Multiple sclerosis, Down's syndrome, etc.), inflammation, viral infections, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and ulcer [41]. In living systems, free-radicals are generated as part of the body's normal metabolic process and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes, through xanthine oxidase activity, atmospheric pollutants, and from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage, in particular, to the immune and nervous systems, while the stress hormones (adrenalin and noradrenalin) secreted by the adrenal glands under conditions of continuing and excessive emotional stress, are metabolised into simpler, although, free radical molecules [42].

Free radicals or oxidative injury appears to be the fundamental mechanism underlying a number of human neurologic and other disorders. For instance in diabetes, increased oxidative stress which co-exists with reduction in the antioxidant status has been postulated: Oxygen free-radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long-term complication of diabetes [43]. Similarly, in carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and

accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma [44].

2.4.3. The mechanism of antioxidant free radical scavenging

There is currently much interest in phytochemicals as bioactive components of food. The roles of fruit, vegetables and red wine in disease prevention have been attributed, in part, to the antioxidant properties of their constituent polyphenols (vitamins E and C, and the carotenoids). Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo.* It is now possible to establish the antioxidant activities of plant derived flavonoids in the aqueous and lipophilic phases, and to assess the extent to which the total antioxidant potentials of wine and tea can be accounted for by the activities of individual polyphenols [45].

Antioxidant compounds reduce or prevent the action of reactive oxygen species in tissue damage. The oxidation proceeds in lipids with polyunsaturated fatty acids, generating ROS such as hydroxyl radicals. Natural products with antioxidant activity are used to aid the endogenous protective system, increasing interest in the antioxidative role of nutraceutic products [46]. Antioxidants may act by decreasing oxygen concentration, intercepting singlet oxygen, or preventing first chain initiation by scavenging initial radicals [47]. Plants such as fruits, vegetables and medicinal herbs may contain a wide variety of free radical scavenging molecules, such as phenolic compounds, vitamins, terpenoids and some other endogenous metabolites, which are rich in antioxidant activity. Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antitumour, antibacterial or antiviral activities to a greater or lesser extent. The intake of natural antioxidants has been associated with reduced risks of cancer, diabetes and other diseases associated with ageing [48, 49]. These protective mechanisms either scavenge or detoxify ROS, block

their production, or sequester transition metals that are the source of free radicals, and include enzymatic and nonenzymatic antioxidant defenses produced in the body, namely, endogenous [50], and others supplied with the diet, namely, exogenous [51]. The two types of antioxidant defenses, the endogenous and exogenous antioxidants can be classified as follows:

2.4.3.1. Exogenous antioxidants

Many compounds in plants and vegetables have the ability of reacting with free radicals without generating further radicals, therefore, quenching chain reactions. Other compounds scavenge ROS and in so doing they become oxidized and need to be regenerated for further use. Antioxidant compounds react directly with radicals reducing oxidative stress and exerting their protective effects against cellular damage [52]. Polyphenols comprise a wide variety of compounds, divided into several classes (i.e., hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanindins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes and lignans), that occur in fruits and vegetables, wine and tea, chocolate and other cocoa products. Epidemiological studies showed that increased intake of polyphenols was associated with reduced risk of cardiovascular diseases, cancer and neurodegenerative disorders [53]. The beneficial effects of polyphenols are mainly ascribed to their capacity to counteract conditions of oxidative stress that accompany these pathologies. Several polyphenols have been demonstrated to have clear antioxidant properties in vitro as they can act as chain breakers or radicals scavengers depending on their chemical structures, which also influence their antioxidant power. A hierarchy has been established for the different polyphenolic compounds within each class on the basis of their capability to protect lipids, proteins or DNA against oxidative injury [54].

2.4.3.2. Endogenous antioxidants

Several antioxidant enzymes exist that convert ROS into less noxious compounds, for example, superoxide dismutase (SOD), catalase, thioredoxin

reductase, peroxiredoxin and glutathione peroxidase (GPx) [55]. Collectively, these enzymes provide a first line of defense against superoxide and hydrogen peroxides. They are of enormous importance in limiting ROS-mediated damages to biological macromolecules, but they are not able to be 100% effective because certain compounds generated by the interaction of ROS with macromolecules are highly reactive. It is then mandatory to detoxify these secondary products in order to prevent further intracellular damage, degradation of cell components and eventual cell death. This second line of defense against ROS is provided by enzymes such as GPx, glutathione S-transferase (GST), aldo-keto reductase and aldehyde dehydrogenase [56]. The detoxified metabolites produced by these enzymes are eliminated from the cell by efflux pumps such as the glutathione *S*-conjugate transporter [57].

2.5. Extraction and isolation of natural products

Extraction of natural products from crude material is one of the simple steps towards isolation whilst on the other hand isolation process of natural products remains a tough, extensive and a monotonous task [58]. Spectroscopic methods coupled with good separation techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. Sound strategies have helped in the isolation and characterization of many bioactive molecules [59]. Nowadays, bioassay-guided fractionation of medicinal plants is a routine feature in the attempt to isolate bioactive components from natural sources [60].

2.5.1. Extraction

In practice, as soon as the material is collected, in the case of plants, it needs to be identified by a taxonomist so as to ascertain the correct identity of the material. Various parts of the plant or the whole plant are collected (leaves, flowers, stem, wood, bark, root, root bark, etc.) and dried quickly in drying cabinets because fresh material has much water and this can lead to degradation

of the components of the plants by microbes. Good ventilation conditions or high speed fans can be used [60].

Once the material has been dried to constant weight, it is ground up to smaller particles and extracted usually using solvents of different polarities. The extraction process could either use one solvent or be stepwise extraction in which the same material is extracted using different solvents normally from non-polar solvents to polar solvents. There are different techniques which can be applied to extract natural products from crude samples; the following are some of the example:

2.5.1.1. Maceration

Here the plant material is extracted in solvents of differing polarity at room temperature, by leaving the plant material soaked in the selected solvents and/or shaking at room temperature and this allows for maximum extraction of most components.

2.5.1.2. Decoction

The plant material is boiled with the solvent usually under reflux. This method allows for extraction of a large number of metabolites, from the most insoluble material like the waxes to the lipophilic natural products.

2.5.1.3. Continuous extraction

Perhaps the most widely and commonly used technique for the extraction of natural products. The polarity gradient of the solvent is applied. Although some components may be destroyed in the process, it is still the best method of extraction used in natural product chemistry.

2.5.1.4. Infusion

In this technique hot liquid ‘solvent’ is poured on the plant material and this is different from decoction where the plant material is boiled with the solvent.

2.5.2. Partial purification and fractionation

Once the extraction is complete, the extract is usually concentrated under vacuum. The activity within the obtained extract can then be demonstrated by bioassay methods using both the crude and the fractionated or semi-purified extracts. Fractionation has the added advantage of getting to the biologically active material faster. There are techniques which can be applied to partially purify the desired components by removing some of the undesired components. The techniques which can be used are solvent/solvent partition and precipitation. Solvent/solvent partition needs two immiscible liquids and separates the components according to solubility which can be polarity or charge. This method relies on the ability of the components to be either soluble in water or the organic phase [60]. In precipitation/precipitation an excessive amount of the solvent e.g. ethanol, ammonium sulphate, lead etc. is added to the crude material to and some components in the crude material will precipitate.

2.5.3. Chromatographic techniques

For the separation of compounds within the extract, chromatographic techniques are employed. Chromatographic techniques have been instrumental in the separation of natural products. Chromatography is a process whereby a mixture of solutes may be resolved into components by exploiting differences in affinity of the solutes for particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase [60]. There are different types of chromatographic techniques which can be utilised to separate compounds here three chromatographic techniques will be discussed.

2.5.3.1 Thin layer chromatography

Thin layer chromatography (TLC) is one of the fastest and most widely used chromatographic techniques in the separation of natural products. TLC mostly used for phytochemical analysis of plant extracts and to check purity of isolated

compounds. TLC method employs glass or aluminium plates pre-coated with the sorbent (e.g., silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded on plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials. TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed simultaneously.

2.5.3.1.1 Preparative thin layer chromatography

Preparative thin layer chromatography is a technique which is usually employed to isolate bioactive natural compounds after column chromatography. Preparative thin layer chromatography uses the same principles to those of thin layer chromatography the difference is only that preparative thin layer chromatography has a thick stationary phase compared to TLC. This gives preparative TLC the advantage in that large quantity of sample is loaded on plates as a band and the developed in the chosen solvent system. After developing the plates they can be analysed using non-destructive detection e.g., UV and/or destructive chromogenic spray by exposing only a small portion of the plate. The band(s) with the compound(s) of interest now can be removed using a spatula or cut out with scissors. The compound can be cleaned by filtration, size exclusion in column chromatography, centrifugation, crystallisation etc. The purity of the compound(s) is checked using TLC or High Performance Liquid Chromatography (HPLC).

2.5.3.2. Column chromatography

Column chromatography (CC) is a popular technique which is used for fractionation and isolation of bioactive natural compounds. This technique is usually employed after solvent/solvent partition. To fractionate or isolate bioactive compounds the stationary phase normally used is silica gel with the mobile being

the solvent(s) of choice. There are eluting techniques which can be used which are isocratic elution and gradient elution. Isocratic elution employs only one mobile phase while gradient elution employs a sequence of mobile phases usually in order of polarity, increasing for normal phase chromatography and decreasing polarity for reverse phase chromatography. Gradient elution is normally employed when isolating and/or fractionating natural bioactive compounds from crude samples. After elution fractions collected are analysed using e.g., chemical tests, TLC, bioassays etc. to identify fractions of interest, similar fractions are grouped together for future work [61].

2.6. Structural characterisation of purified natural products

Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will attempt the structural characterization of the compounds [62]. Structural elucidation is crucial in assessing the biological activity of the molecule as it is a well-known fact that biological activity depends to a large extent on the 3-Dimensional (3-D) arrangement of functional groups on the molecule. Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR), Infra Red (IR) and UV-Visible, Mass Spectrometry (MS) and X-Ray analyses [63, 64].

Since the development of the high resolution NMR spectrometer in the 1950s, NMR spectra have been a major tool for the study of both newly synthesised and natural products isolated from plants, bacteria, etc. In the 1980's a second revolution occurred. The introduction of reliable superconducting magnets combined with newly developed, highly sophisticated pulse techniques and the associated Fourier transformation provided the chemist with a method suitable to determine the 3-D structure of very large molecules. Since drugs in clinical use are mostly synthetic or natural products, NMR spectroscopy has been mainly used for the elucidation and confirmation of structures [65]. In the present study,

NMR technique was used to characterise the chemical structure of the isolated biologically active compounds.

CHAPTER 3

Screening for antibacterial and antioxidant activities

3.1 Materials and methods

3.1.1. Extraction

The leaves and roots of *Euclea undulata (Mogweregwere), Momordica balsamia (mogapu badimo), sefapa badimo, Senecio asperulus (makgonatšohle), Stiburus alopecuroides (mošalašuping), Solanum spp (serolana), Euphorbia Inaequilatera (kgama-maswana), mokgagapitsi* and *Clerodendrum glabrum (mohlokohloko)* were collected from Zebediela (Lepelle-Nkumpi Municipality, Limpopo province) and the University of Limpopo . The identities of the medicinal plants were verified by a botanist at the University of Limpopo. The plant materials were allowed to dry completely at room temperature and later ground to a fine powder. The powdered materials were extracted in separate aliquots with solvents of varying polarities viz., *n*-hexane, dichloromethane, acetone and methanol by shaking for 24 hours at room temperature. The supernatants were filtered into pre-weighed beakers using 15.0 cm Whatman filter papers (Whatman). Supernatants were concentrated by drying under cold air using a fan; the residues were weighed daily until a constant mass was obtained.

3.1.2. Phytochemical analysis of extracted compounds

Thin layer chromatography was used to determine the chemical components of the plant extracts by UV light visualisation and staining with detecting reagents. The stock solutions of crude extracts were prepared by dissolving the extracts in 10 mg/ml acetone. The dissolved extracts (100 µg) were spotted on 10 x 10 cm ALUGRAM® SIL G/UV_{254} TLC plates by pipetting 10 µl of the 10 mg/ml stock solution at the baseline of the TLC plate. The plates were then developed in three different solvent systems, *viz*., benzene; ethanol; ammonia (BEA,

18:10:0.2, v/v/v); ethyl acetate; methanol; water (EMW, 10:1.35:1, v/v/v) and chloroform; ethyl acetate; formic acid (CEF, 10:8:2, v/v/v) (Kotze and Eloff 2002) [66]. The developed plates were inspected under UV light 254nm and later sprayed with H_2SO_4/vanillin and developed at 110°C for 5 min. The sprayed plates were scanned for analysis.

3.1.3. TLC-DPPH antioxidant screening

This method is generally used for the screening of potential antioxidant compounds in plants extracts. The chromatograms were prepared as mentioned above (Section 3.1.2) air-dried and then sprayed with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) to detect compounds that possess antioxidant activity (Es-safi *et al* 2007) [67]. An antioxidant compound reduces DPPH to colourless/ yellow spots on chromatograms.

3.1.4. Bioautography

This is an easy and quick bioassay used to screen bioactive compounds against microorganisms. This was achieved by preparing the chromatograms as mentioned in Section 3.1.2. The developed chromatograms were dried at room temperature for 2 weeks to evaporate organic solvents which may be toxic. The plates were then sprayed with the test microorganism (suspended in broth) and incubated at 37°C in a chamber at 100% humidity for 24 hours. After incubation, the plates were sprayed with 2 mg/ml *p*-iodonitrotetrazolium violet (INT) and incubated for 30 min at 37°C for colour development. The plates were then scanned for analysis this is the reference of the method followed by Begue and Kline 1972 [68]. An antibacterial activity will be shown by clear spots on the chromatogram and no activity will be coloured pink.

3.2. Results

3.2.1. Yield of crude extracts

The results of the extraction of nine indigenous medicinal plants, using four solvent systems with different polarities, showed that the polar methanol resulted in high percentage yield of the extracts when compared to other solvents and *n*-hexane (a more non-polar solvent) had the lowest percentage yield of the extracts (Table 3.1).

Table 3.1: The yield in milligrams and percentage of crude extracts when 1 g of the twelve plant materials were extracted using four different solvents *n*-hexane, dichloromethane, acetone and methanol.

Plant	**Solvent**	**Yield Mass (mg)**	**Yield %**
MKK *Euclea undulata (Mogweregwere)* Whole plant	*n*-Hexane	15	1.47
	Dichloromethane	30	2.98
	Acetone	45	4.22
	Methanol	121	11.99
MBR *Momordica balsamia* (*Mogapu badimo)* roots	*n*-Hexane	6	0.59
	Dichloromethane	7	0.68
	Acetone	20	1.97
	Methanol	111	10.92
MBL *Momordica balsamia* (*Mogapu badimo)* leaves	*n*-Hexane	9	0.89
	Dichloromethane	18	1.78
	Acetone	19	1.88
	Methanol	95	9.26
SBR "*Sefapa badimo*" roots	*n*-Hexane	6	0.56
	Dichloromethane	8	0.79
	Acetone	34	3.39
	Methanol	136	13.28
SBL "*Sefapa badimo*" leaves	*n*-Hexane	29	2.83
	Dichloromethane	39	3.63
	Acetone	25	2.45
	Methanol	95	9.26
MR *Stiburus alopecuroides (Mošalašuping)* roots	*n*-Hexane	7	0.68
	Dichloromethane	10	0.97
	Acetone	8	0.78
	Methanol	216	20.93
ML *Stiburus*	*n*-Hexane	10	0.97

alopecuroides (Mošalašuping) leaves	Dichloromethane	23	2.27
	Acetone	27	2.65
	Methanol	155	15.33
SR *Solanum spp* "*Serolana*" roots	*n*-Hexane	8	0.79
	Dichloromethane	7	0.68
	Acetone	46	4.39
	Methanol	71	6.56
KM *Euphorbia inaequilatera (Kgama-maswana)*	*n*-Hexane	34	3.25
	Dichloromethane	60	5.67
	Acetone	37	3.61
	Methanol	116	11.1
MKP "*Mokgagapitsi*"	*n*-Hexane	29	2.78
	Dichloromethane	42	4.12
	Acetone	30	2.92
	Methanol	156	15.16
MT *Senecio asperulus* (*Makgona tšohle)*	*n*-Hexane	13	1.22
	Dichloromethane	24	2.24
	Acetone	92	8.93
	Methanol	203	19.2
MHH *Clerodendrum glabrum (Mohlokohloko)*	*n*-Hexane	33	3.25
	Dichloromethane	63	6.08
	Acetone	44	4.31
	Methanol	143	13.8

3.2.2. Phytochemical analysis of crude extracts using vanillin-sulphuric acid

TLC chemical fingerprinting analysis of the plant extracts developed using three different solvent systems BEA, CEF and EMW which were sprayed with vanillin-sulphuric acid. The chromatograms revealed the presence of many different compounds which demonstrated different polarities when visualised with vanillin-sulphuric acid (Figure 3.1). It should be noted that the all extracts were loaded onto the TLC plates in the order of polarity of solvents used to extract them, starting with *n*-hexane, dichloromethane, acetone and methanol.

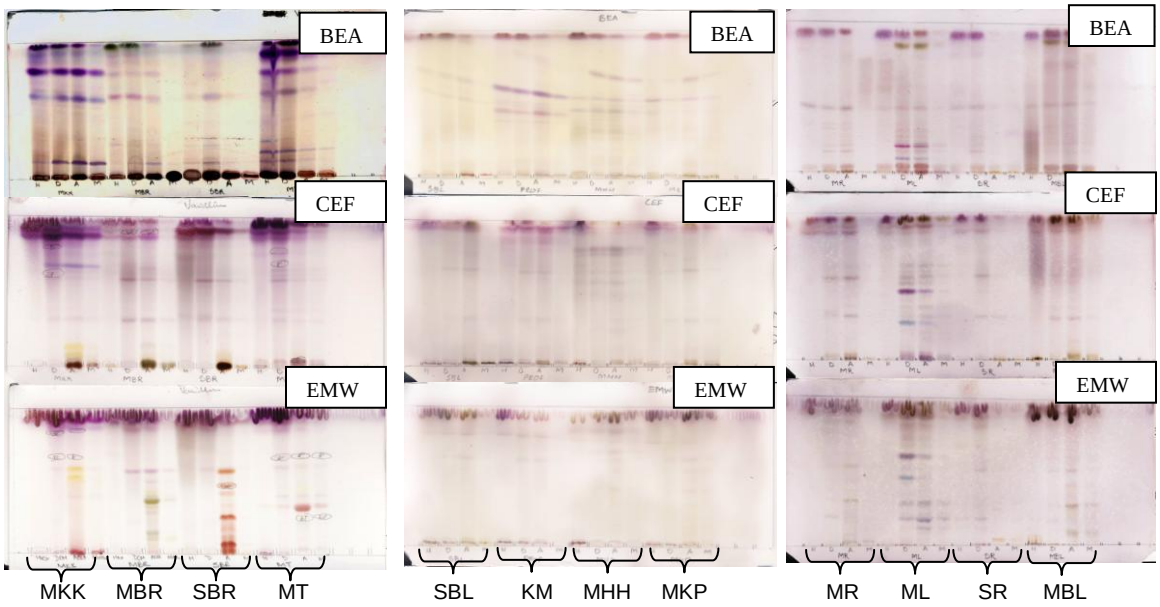


Figure 3.1 TLC chromatograms of different plant extracts developed using BEA, CEF and EMW solvent systems. Hundred micrograms of plant extracts redissolved in acetone was loaded on silica TLC plate and visualised with vanillin-sulphuric acid reagent and scanned.

3.2.3. TLC-DPPH of crude extracts

The antioxidant activity analysis of the plant extracts on TLC developed using three different solvent systems BEA, CEF and EMW and sprayed with DPPH. The chromatograms revealed the presence of numerous different antioxidant compounds which showed the characteristics of being intermediate to more polar when visualised using DPPH spray reagent (Figure 3.2).

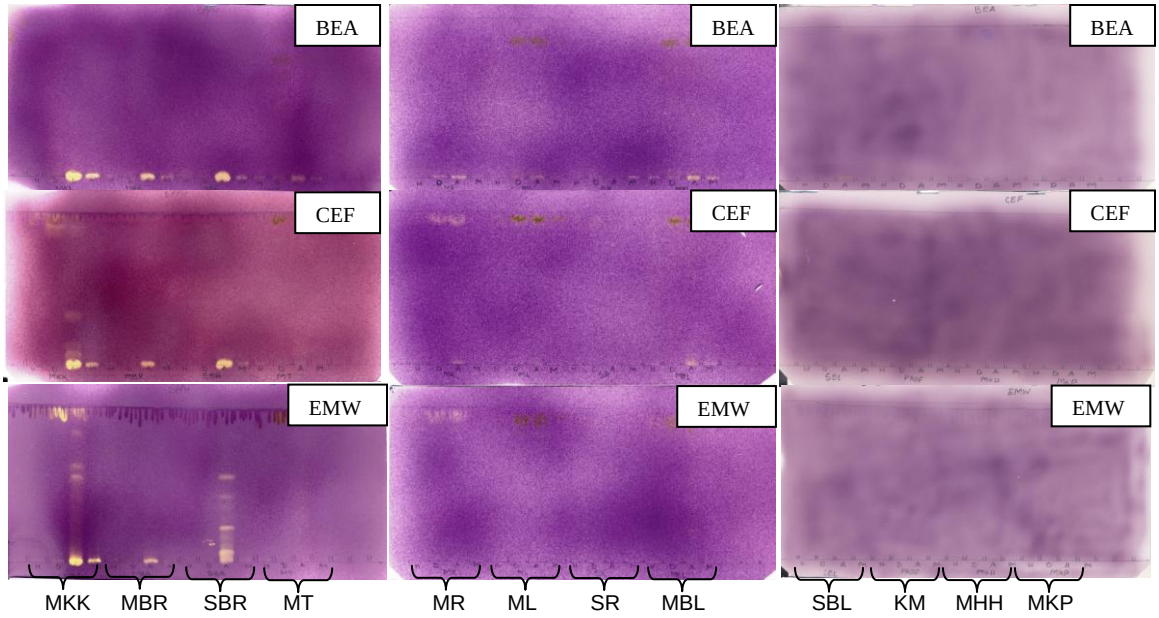


Figure 3.2 TLC chromatograms of different plant extracts developed using BEA, CEF and EMW solvent systems. Hundred micrograms of plant extracts redissolved in acetone was loaded on silica TLC plate and visualised with DPPH reagent and scanned.

3.2.4 Bioautography of crude extracts

The plant extracts were tested for antibacterial activity against four microorganisms viz., *S. aureus, E. faecalis, E. coli* and *P. aeruginosa* using the bioautography assay. Below is the bioautography analysis of plant extracts and three different solvent systems viz., BEA, CEF and EMW, where *S. aureus* was used as the test microbe. The chromatograms revealed the presence of different antibacterial compounds of different polarities (Figure 3.3).

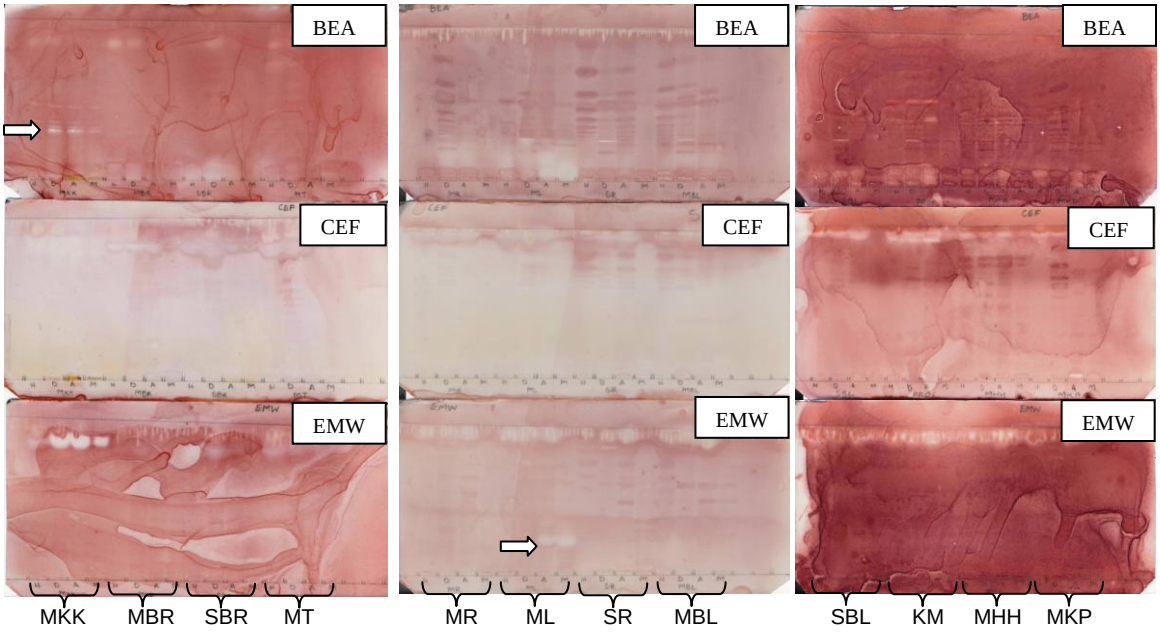


Figure 3.3 TLC chromatograms of different plant extracts developed using BEA, CEF and EMW solvent systems. Hundred micrograms of plant extracts redissolved in acetone was loaded on silica TLC plates. The plates were sprayed with *S. aureus* and incubated at 100% humidity for overnight. The plates were sprayed with INT observed after 30 min and scanned.

CHAPTER 4

Isolation of antioxidant compound from *Euphorbia inaequilatera*

4.1. Materials and methods

4.1.1. Extraction

Euphorbia inaequilatera plant materials (roots, stems and leaves) were collected at the University of Limpopo during the summer of 2007 and allowed to dry completely at room temperature. The dried plant materials were ground to a fine powder. Three hundred grams of the powdered plant material were exhaustively extracted by solvents of varying polarities; initially three times with 2 litre *n*-hexane followed by dichloromethane, acetone and methanol, by shaking for 1, 5, 24 hours at room temperature. The supernatants were filtered into labelled conical flasks and concentrated using a Büchii rotary evaporator at 40°C. The concentrated supernatants were then transferred into pre-weighed beakers which were dried under a fan and weighed daily until a constant mass was achieved.

4.1.2. Phytochemical analysis of *E. inaequilatera* crude extracts

TLC was used to check the chemical components of the plant extracts by visualising under the UV light and staining with detecting reagents. The stock solutions of the plant extracts were prepared by dissolving the extracts in 10 mg/ml acetone and spotted on 10 x 10 cm ALUGRAM® SIL G/UV_{254} TLC plates by pipetting 10 µl of the 10 mg/ml stock solution at the baseline of the TLC plate. The spotted plates were developed in three different solvent systems, viz. BEA (18:10:0.2 v/v/v); EMW (10:1.35:1 v/v/v) and CEF (10:8:2 v/v/v). The developed plates were inspected under UV light 254 nm and later sprayed with vanillin/H_2SO_4 and developed at 110°C for 5 min. The sprayed plates were scanned and analysed.

4.1.3. TLC-DPPH antioxidant screening of *E. inaequilatera* crude extracts

This method was used to potential antioxidant compounds in plants extracts. The chromatograms were prepared as mentioned before (Section 3.1.2) with butanol; acetic acid; water BAW (4:1:5 v/v/v) as an additional solvent system. After development, the chromatograms were air-dried and then sprayed with DPPH to detect compounds that possess antioxidant activity

4.1.4. Solvent-solvent extraction

The methanol crude extracts were re-dissolved in methanol: water (6:4 v/v) and absolute ethyl acetate was used to partition the desired material until a clear ethyl acetate layer was obtained. The ethyl acetate fraction was poured into the conical flasks and then concentrated using a Büchii rotary evaporator at 40°C to reduce the volume of ethyl acetate and then later transferred into a pre-weighed beaker. The extract was air dried under a fan and constant weight was achieved.

4.1.5. Fractionation and isolation of an antioxidant compound

Activated silica gel (60-120 mesh) was packed on to a glass column (450 mm × 40 mm) using *n*-hexane solvent and 21.44 g of ethyl acetate fraction was loaded on top of the packed silica gel. The column was eluted step-wise with 500 ml of *n*-hexane:dichloromethane (100:0, 95:5, 85:15, 80:20 75:25, 50:50, 25:75 and 0:100); dichloromethane:ethyl acetate (95:5, 90:10, 85:15 80:20, 75:25, 50:50, 25:75, 0:100); and ethyl acetate:methanol (95:5, 90:10, 85:15 80:20, 75:25, 50:50, 25:75, 0:100) to obtain 31 fractions (1-31) collected into 500 ml conical flasks. The fractions were concentrated using a Büchii rotary evaporator at 40°C to reduce the volume and then poured into a pre-weighed beaker and evaporated to dryness under a fan. The chromatograms of the fractions were prepared as mentioned in (Section 3.1.2) with one solvent system used EMW (10:1.35:1 v/v/v). The chromatograms were visualised using DPPH, vanillin/H_2SO_4 spray reagent and under UV light 254 nm. Fractions (23-25) were selected and combined for further separation. The combined fractions were fractionated on a silica gel, eluted with chloroform:ethyl acetate (100:0, 75:25, 50:50, 25:75) and

ethyl acetate:methanol (75:25, 50:50, 25:75, 0:100) 100 ml of each fit in well to obtain 66 fractions (1-66) collected into test tubes with 13 ml each. The fractions were left under a fan to concentrate.

The chromatograms of the fractions were prepared as mentioned in (Section 3.1.2) with one solvent system used EMW (10:1.35:1 v/v/v). The chromatograms were visualised by spraying with DPPH and then scanned. Fractions 44 and 45 were mixed and subjected to further isolation using preparative TLC. This was achieved by applying the mixed fractions on the baseline of the preparative TLC plate. The plates were developed in chloroform and ethyl acetate (75:25 v/v), this was performed three times every time using a fresh solvent system. The plates were visualised under UV light to locate the compound(s) of interest that were then scrapped off. The compound(s) were purified from silica gel by dissolving the mixture of the compound(s) and silica in acetone and centrifuged for 5 min at 2000 rpm then collecting the supernatant which had the compound(s) of interest. Phytochemical analyses of the isolated compound(s) were done to check their purity.

4.2. Results

4.2.1. Yield of crude extracts

The screening for bioactive compound(s) in the *E. inaequilatera* plant material had to be the initial step towards identification of potential compound(s) with antioxidant activity. During the exhaustive extraction process of *E. inaequilatera,* methanol yielded high quantity of extracts when compared to other extracting solvents (Table 4.1). And it was observed that the most non-polar *n*-hexane and most polar methanol extracting solvents extracted high quantity compounds when compared to intermediate extracting solvents dichloromethane and acetone.

Table 4.1: The mass in grams (g) of crude extracts and the percentage yield after exhaustive extraction of *E. inaequilatera* using solvents of different polarities.

Solvent	Extract Mass (g)	Yield %
n-Hexane 1	6.828	2.28
n-Hexane 2	3.321	1.11
n-Hexane 3	1.111	0.37
Dichloromethane 1	2.009	0.67
Dichloromethane 2	1.556	0.52
Dichloromethane 3	1.295	0.43
Acetone 1	1.804	0.60
Acetone 2	0.781	0.26
Acetone 3	0.528	0.18
Methanol 1	20.438	6.81
Methanol 2	7.167	2.39
Methanol 3	5.494	1.83

4.2.2. TLC-DPPH of crude extracts

TLC was used to analyse the antioxidant activity following exhaustive extraction of *E. inaequilatera*. Four different solvent systems, namely BEA, EMW, CEF and BAW were used to develop the TLC plates. The TLC-DPPH of the extracts revealed that only acetone and methanol solvents extracted the antioxidant compound(s), which showed better separation of the compounds when polar solvent systems EMW and BAW were used as shown in (Figure 4.1).

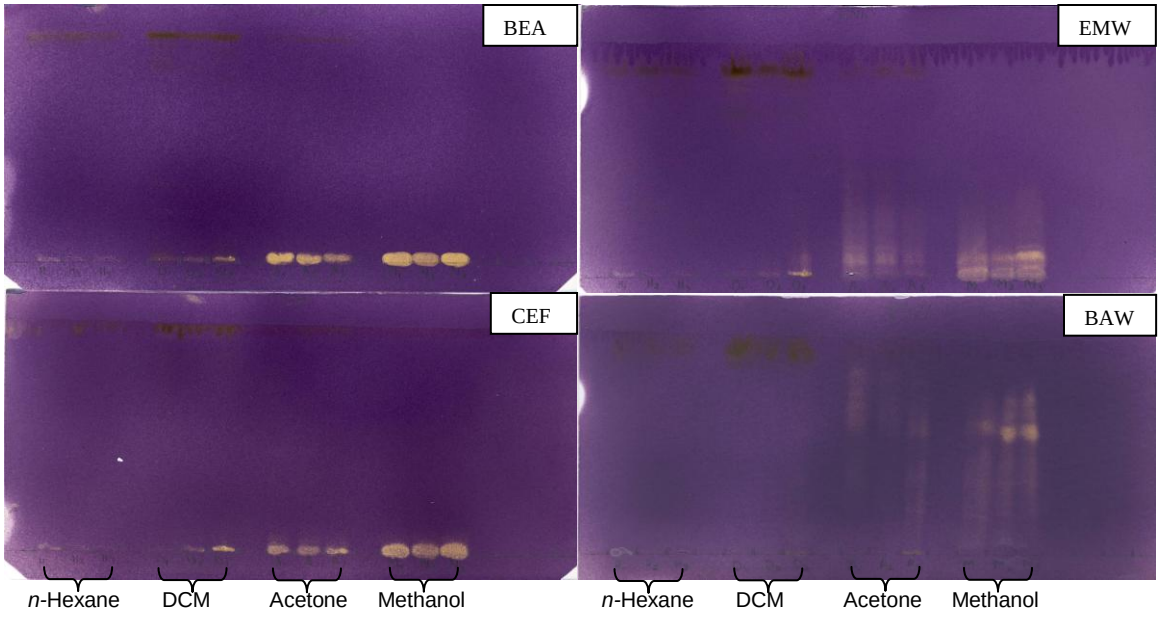


Figure 4.1 TLC chromatograms of exhaustive extraction extracts of *E. inaequilatera*. Extracts were loaded as a 10 mg/ml in 10 µl of acetone onto a silica TLC plates. The plates were developed in BEA, CEF, EMW and BAW and then visualised by DPPH reagent and scanned.

4.2.3. TLC-DPPH of fractions 1-31

In order to eliminate some of the unwanted compound(s), solvent/solvent extraction was applied and ethyl acetate was used to extract the desired portion from absolute methanol: water (6:4, v/v) solution. The ethyl acetate fraction was subjected to column chromatography and 31 fractions were obtained and TLC-DPPH revealed that only fractions 21-25 and 27-31 had antioxidant compound(s) (Figure 4.2). The antioxidant compound(s) were better separated with EMW and followed by CEF. This is because the compound(s) are intermediate-polar or polar compounds, and this is evident because they were eluted when methanol was gradually introduced in the column. The compound of interest was located in fractions 23 and 24; it reacted with vanillin-sulphuric acid to form a light brown colour as indicated in (Figure 4.3).

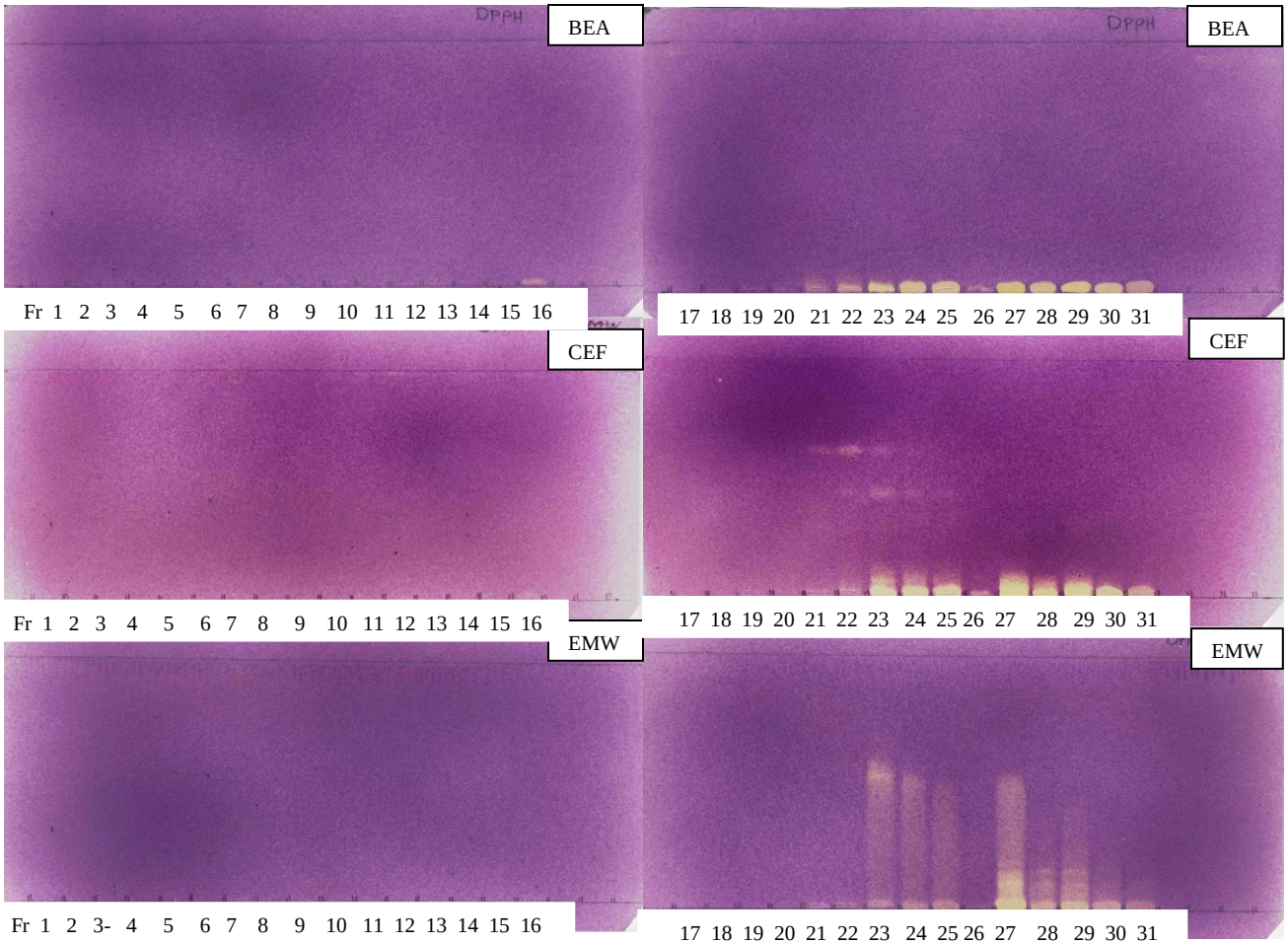


Figure 4.2 TLC chromatograms of fractions 1 to 31 from the column chromatography of *E. inaequilatera*. Ten microlitres of fractions were loaded onto the silica TLC plates and developed using BEA, CEF and EMW. The plates visualized by DPPH reagent and scanned.

4.2.4 Phytochemical analysis of fractions 1-31 using vanillin-sulphuric acid

TLC fingerprinting of 31 fractions following the column chromatography of the methanol extract of *E. inaequilatera* eluted using three different solvent systems. The chromatograms revealed the presence of numerous different compounds which showed the characteristics of being intermediate to more polar. The compounds showed a good separation when developed with EMW and visualised using vanillin-sulphuric acid spray reagent (Figure 4.3).

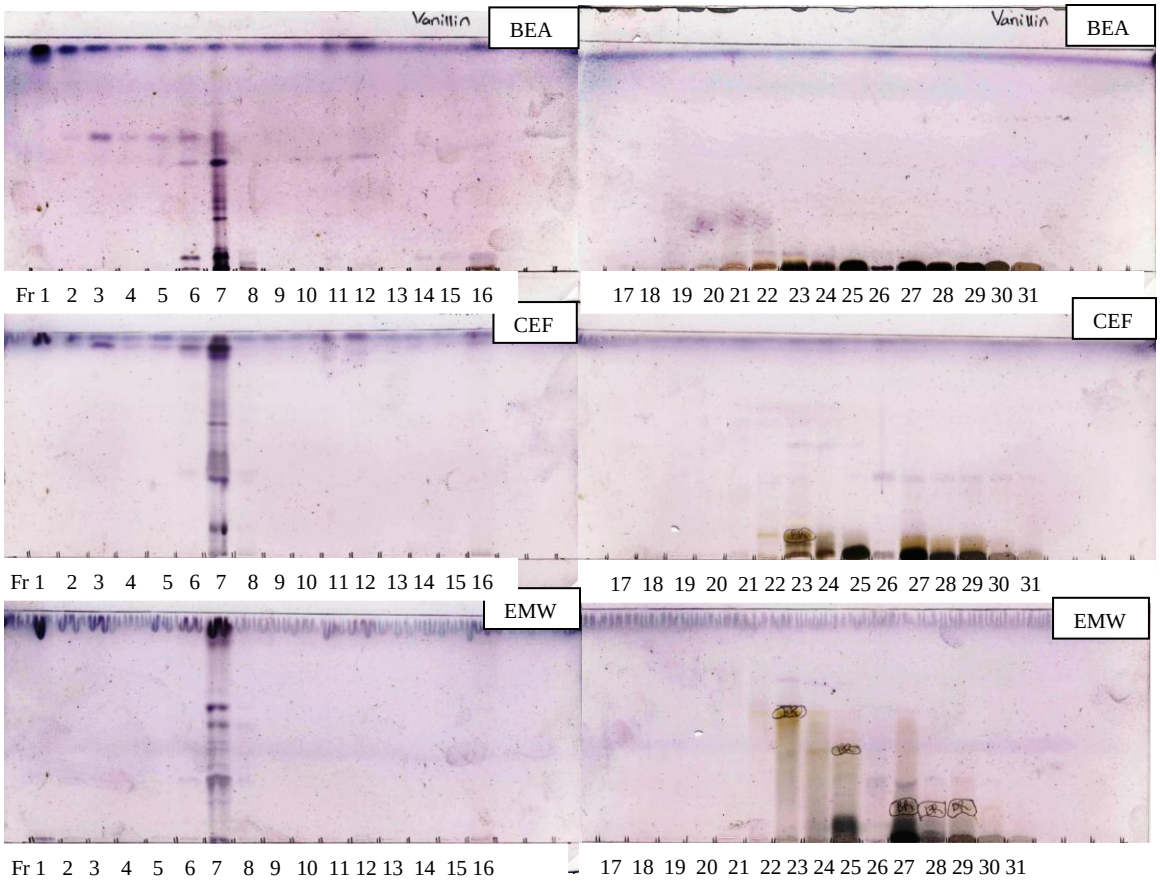


Figure 4.3 TLC chromatograms of fractions 1 to 31 from the column chromatography of *E. inaequilatera*. Ten microlitres of fractions were loaded onto the silica TLC plates and developed using BEA, CEF and EMW. The plates visualised by vanillin reagent.

4.2.5 Phytochemical analysis of fractions 1-31 under visible and UV light

The chromatograms revealed the presence of numerous different compounds with different polarities and chemical characteristics when visualised using UV and visible light (Figures 4.4a, b and c). UV light reveals the presence of fluorescent-quenching compounds with many double bonds and visible light reveals coloured compounds, usually with conjugated bonds in plant extracts. Compounds containing aromatic rings adsorb UV light at 254 nm and therefore quench the fluorescence of the pigment present in the silica gel [69]. When observed under visible light the compound of interest appeared to be brown coloured and under UV light it quenched the fluorescence of the silica gel (Figure 4.4.c).

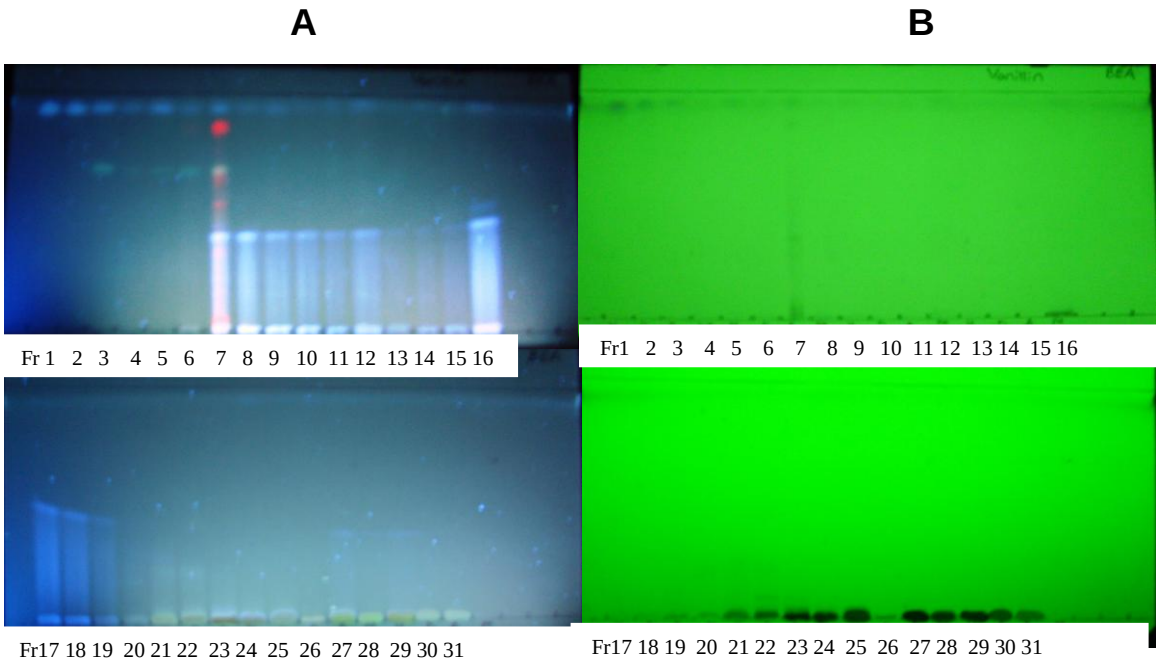


Figure 4.4a TLC chromatograms of fractions 1 to 31 from the column chromatography of *E. inaequilatera*. Ten microlitres of fractions were loaded onto the silica TLC plates and developed using BEA. The plates were visualised using visible light at 366 nm (**A**) and UV light at 254 nm (**B**).

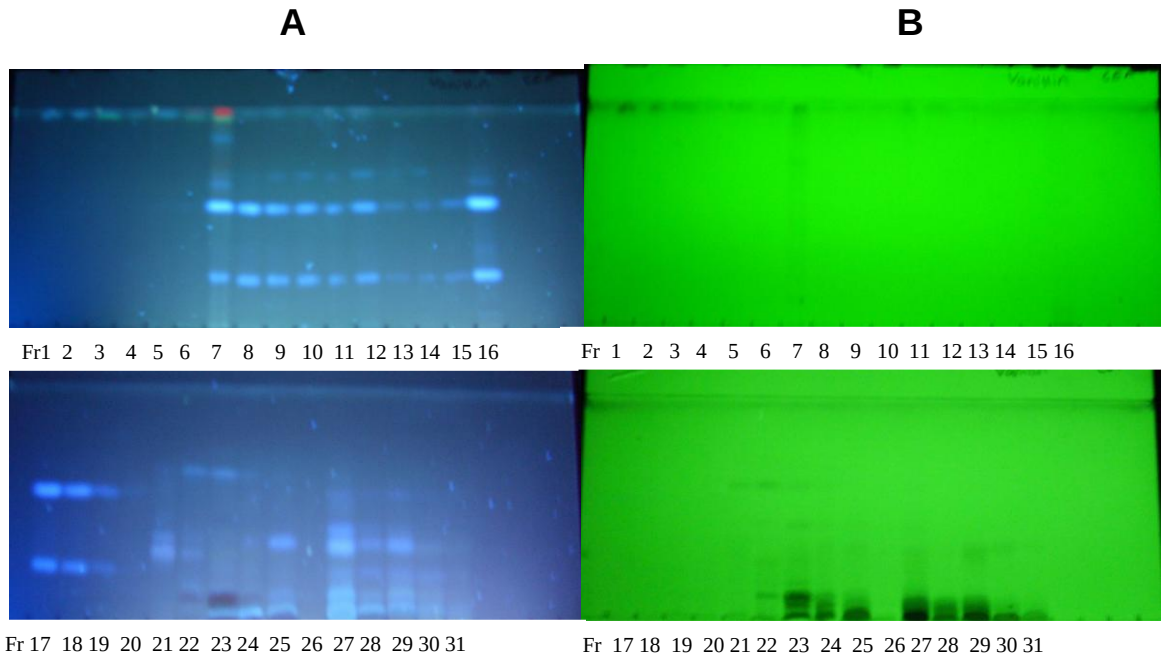


Figure 4.4b TLC chromatograms of fractions 1 to 31 from the column chromatography of *E. inaequilatera*. Ten microlitres of fractions were loaded onto the silica TLC plates and developed using CEF. The plates were visualised using visible light at 366 nm (**A**) and UV light at 254 nm (**B**).

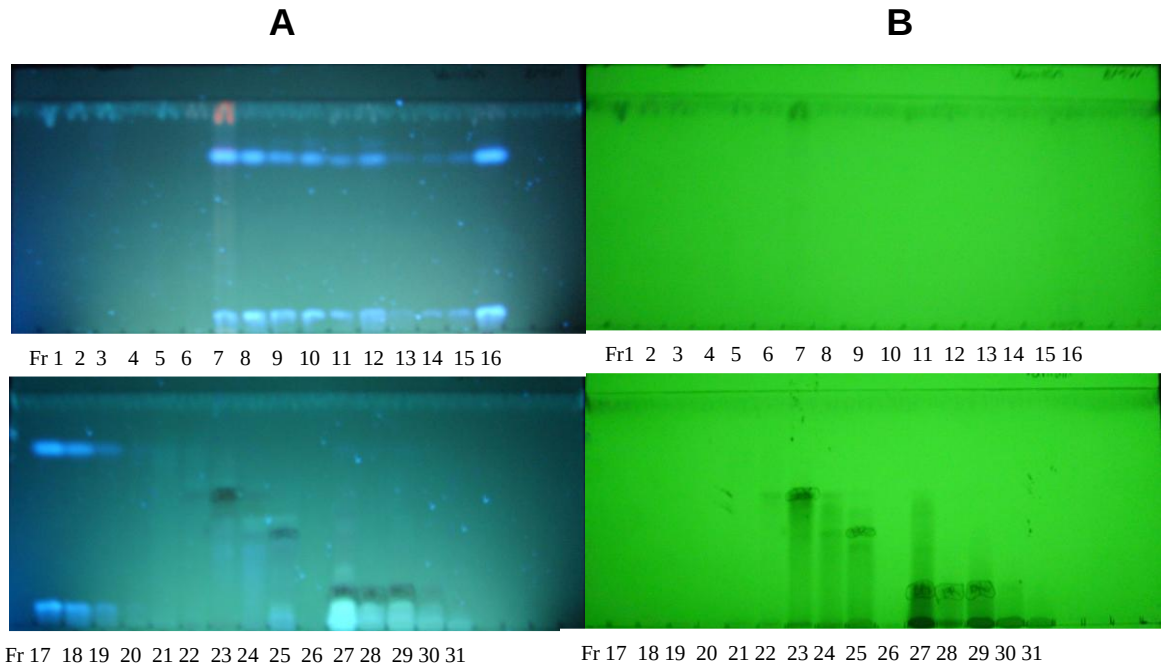


Figure 4.4c TLC chromatograms of fractions 1 to 31 from the column chromatography of *E. inaequilatera*. Ten microlitres of fractions were loaded onto the silica TLC plates and developed using EMW. The plates were visualised using visible light at 366 nm (**A**) and UV light at 254 nm (**B**) and pictures taken.

4.2.6 TLC-DPPH of fractions 1-66

After the first column chromatography, the fractions which contained the compound of interest appeared to be contaminated with other compounds. To get a pure compound, the mixture of fractions 23 and 24 were subjected to another column chromatography. In the second column chromatography 66 fractions were collected and subsequently subjected to TLC-DPPH analysis. The compound of interest was spotted to be present in fractions 44 and 45 as indicated in (Figure 4.5). To further purify the compound, fractions 44 and 45 were mixed and subjected to preparative TLC. The phytochemical analysis of the isolated compound revealed that the compound was about 90% pure, but could not be further characterised because the isolated compound was lost when it was sent for spectral analysis.

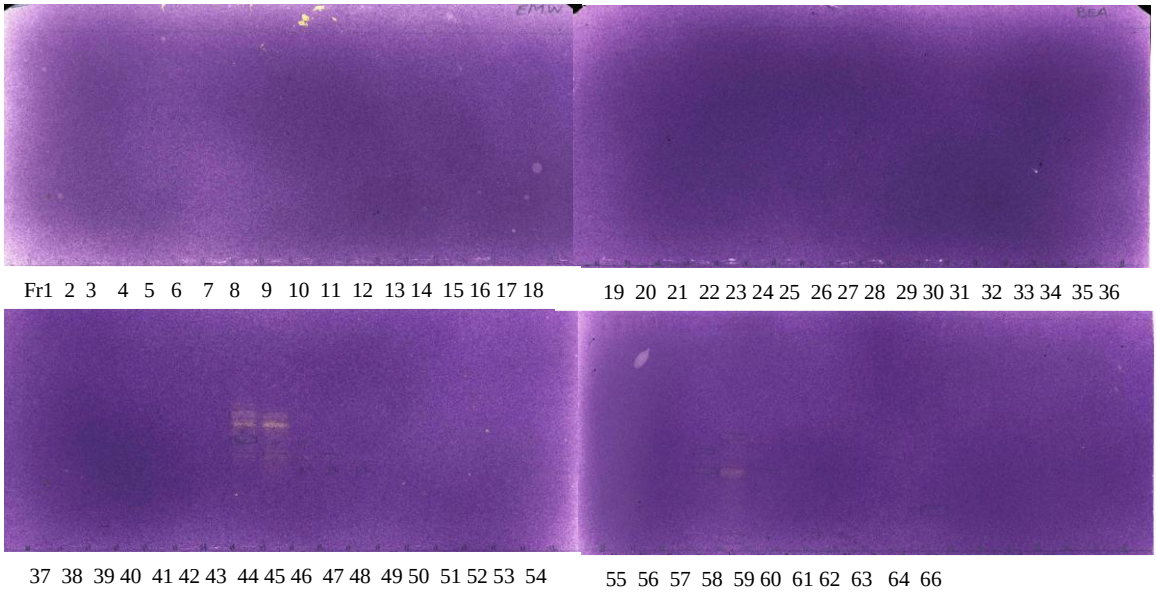


Figure 4.5 TLC chromatograms of fractions 1 to 66 of column chromatography using silica gel 60 of mixed fractions 23 and 24 plates developed using EMW solvent system. Ten microlitres of fractions were loaded onto a silica TLC plate. The plates were visualized by DPPH reagent and scanned.

CHAPTER 5

Isolation of antioxidant compound(s) from *Dicerocaryum senecioides*

5.1. Materials and methods

5.1.1. Extraction

Dicerocaryum senecioides plant materials (leaves) were collected during the summer of 2008 from the University of Limpopo and allowed to dry completely at room temperature. The dried leaves were ground to a fine powder. One hundred grams of the powdered leaves was exhaustively extracted with 1 litre of *n*-hexane by shaking for 1, 5, and 24 hours at room temperature. This process was repeated three times. The supernatants were filtered into conical flasks, concentrated using a Büchi rotar vapour at 40°C, poured into pre-weighed beakers and concentrated by drying under a fan and weighed daily until a steady mass was achieved.

5.1.2 Solvent-solvent extraction of *Dicerocaryum senecioides*

The *n*-hexane extracts were mixed and dissolved in a solution of 75% ethanol in water (3:1, v/v). The dissolved extracts were partitioned, respectively, with *n*-hexane (D1), dichloromethane (D2) and butanol (D3) until clear phases were achieved with all the partitioning solvents. The fractions were filtered into conical flasks, concentrated using a Büchi rotary evaporator at 40°C and subsequently poured into pre-weighed beakers. The fractions were concentrated by drying under a fan and weighed daily until a steady mass was achieved. The butanol D3 fraction was chosen for further analysis and isolation of an antioxidant compound as guided by the work which was reported previously by Madiga [70].

5.1.3 Phytochemical analysis of the D3 fraction

TLC was used to check the chemical components of the crude extracts by visualizing the developed plates under the UV light and staining with detecting reagents. The stock solution was prepared by re-dissolving the extract in acetone at concentration of 10 mg/ml. The dissolved extract was spotted on 10 x 10 cm ALUGRAM® SIL G/UV_{254} TLC plates by pipetting 10 µl of the 10 mg/ml stock solution at the baseline of the TLC plate. The plates were developed in EMW (10:1.35:1 v/v/v) and inspected under UV light, visible light, later sprayed with vanillin-sulphuric acid and further developed at 110°C for 5 min. The developed plates were scanned and analysed.

5.1.4 TLC-DPPH antioxidant screening of the D3 fraction

TLC-DPPH was used for the screening of the D3 fraction for compounds with potential antioxidant activities. The chromatograms were prepared as mentioned in Section 5.1.3. After development, the chromatograms were air-dried and sprayed with DPPH solution to detect compounds that possess inherent antioxidant activity

5.1.5 Fractionation and isolation of an antioxidant compound from D3 fraction

Activated silica gel (60–120 mesh) was packed onto glass column (450 mm × 40 mm) using chloroform as a packing solvent. A 4.69 g of the D3 fraction was loaded on top of the silica gel. The D3 fraction was fractionated by elution with 100 ml each of chloroform/ethyl acetate (100:0, 75:25, 50:50, 25:75 and 0:100) and ethyl acetate/methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, 0:100) to obtain 90 fractions (1-90) that were collected into properly measured test tubes. The fractions were left under the fan to concentrate. The chromatograms of the fractions were prepared as mentioned in section 3.1.2 using EMW (10:1.35:1, v/v/v) as solvent system. The developed plates were inspected under UV light, visible light and later sprayed with vanillin-sulphuric acid and further developed at 110°C for 5 min. The sprayed plates were scanned

and analysed. Fractions 50-70 were mixed for further processing using preparative TLC; this was achieved by applying the mixed fractions on the baseline of the preparative TLC plates. The plates were developed using the EMW solvent system and air-dried under a fan. This process was repeated three times, using a fresh solvent system every time. The plates were inspected under UV light to locate the compound of interest and then collected by scrapping it off the plate. The collected compound was then purified from the silica gel by dissolving the mixture with acetone and centrifuging at 2000 rpm for 5 min. The yellow coloured supernatant was collected with the compound of interest and the process was repeated until a clear supernatant was obtained. Phytochemical analysis of the isolated compound was performed to confirm its purity.

5.2 Results

5.2.1 Phytochemical analysis of the D3 fraction

The D3 fraction showed a strong antioxidant compound which reacted with vanillin-sulphuric acid to produce a dark brown colour. When the compound was visualised under visible light, a light blue colour was observed and it quenched the fluorescence under UV light as shown in Figure 5.1., this alludes to the presence of an aromatic ring(s) in its structure.

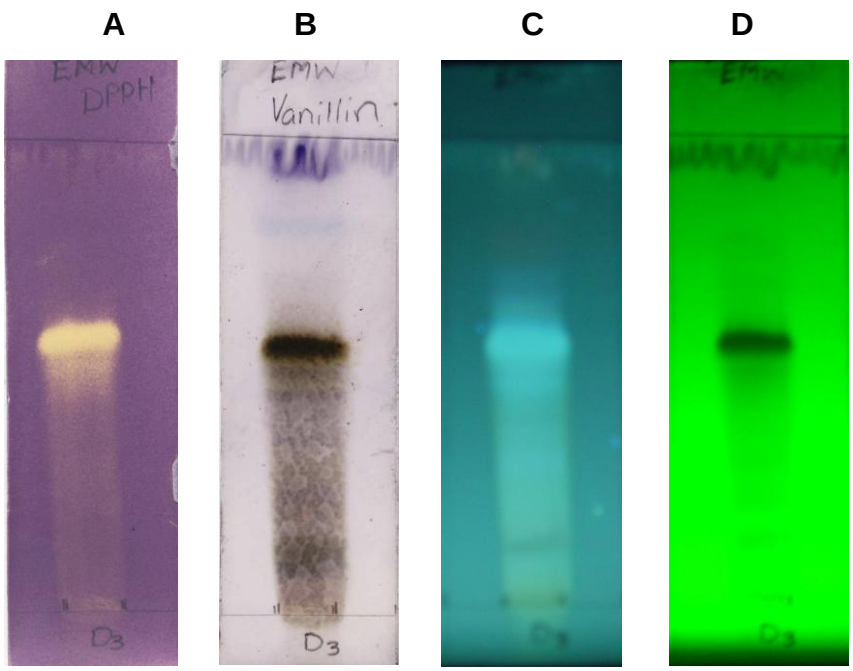


Figure 5.1 TLC chromatograms of the D3 fraction from *D. senecioides*. The plates were visualised using DPPH (**A**), vanillin-sulphuric acid (**B**), visible light at 366 nm (**C**) and UV light at 254 nm (**D**).

5.2.2 Phytochemical analysis of fractions 1-90 using vanillin-sulphuric acid

TLC chemical fingerprinting of the 90 fractions subsequent to column chromatography of the D3 fraction vanillin-sulphuric acid. The chromatograms revealed the presence of numerous different compounds which showed characteristics of being intermediate to more polar in nature. The compound of interest appeared to be distributed across fraction 50-70 (Figure 5.2).

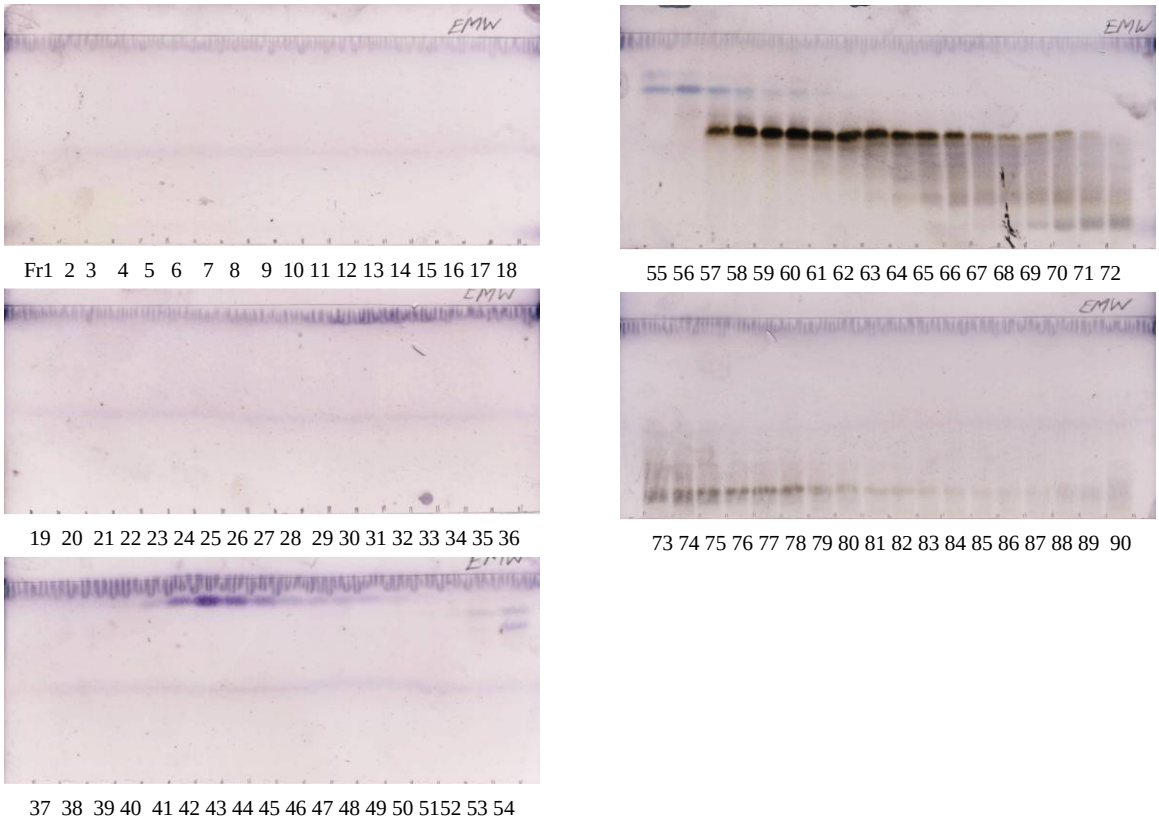


Figure 5.2 TLC chromatograms of fractions 1 to 90 from the column chromatography of the D3 fraction. Ten microlitres of each fraction were loaded onto a silica TLC plate and developed using EMW solvent system. The plates were visualised by vanillin-sulphuric acid spray reagent.

5.2.3 Phytochemical analysis of fractions 1-90 under visible and UV light

TLC chemical fingerprinting of the 90 fractions revealed the presence of numerous different compounds under UV and visible light which suggested the characteristics of an intermediate to more polar chemical property. The compound of interest was distributed across fractions 50-70 (Figures 5.3a-b).

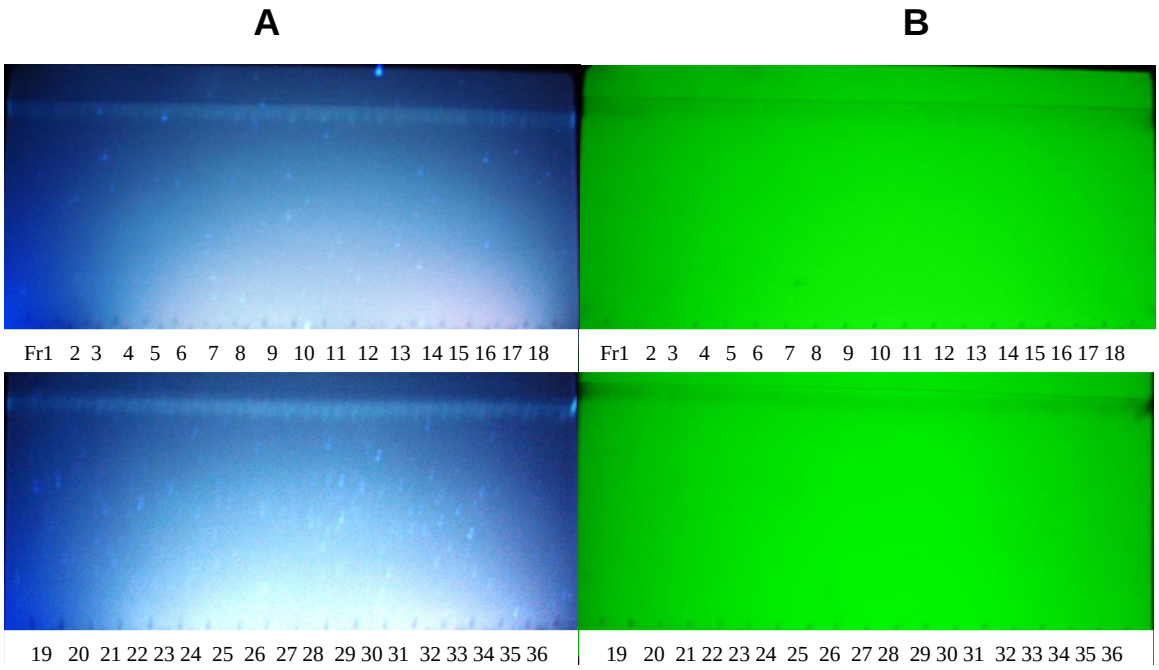


Figure 5.3a TLC chromatograms of fractions 1 to 36 from the column chromatography of the D3 fraction. Ten microlitres of each fraction were loaded onto a silica TLC plate and developed using EMW. The plates were visualised using visible light at 366 nm (**A**) and UV light at 254 nm (**B**).

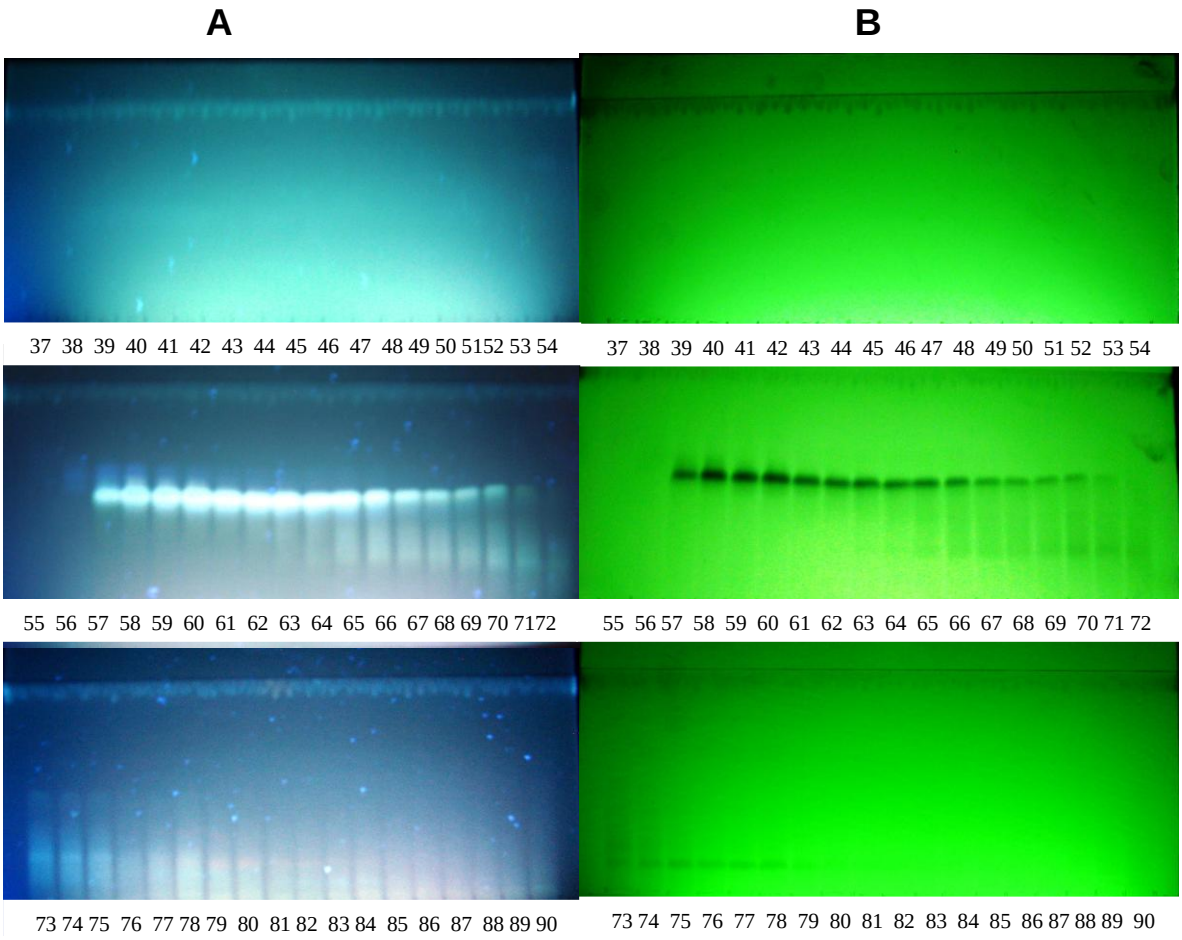


Figure 5.3b TLC chromatograms of fractions 37 to 90 from the column chromatography of D3 fraction. Ten microlitres of fractions were loaded onto a silica TLC plate and developed using EMW. The plates were visualised using visible light at 366 nm (**A**) and UV light at 254 nm (**B**).

5.2.4 Phytochemical analysis of the isolated compound

To further isolate and purify the compound of interest, fractions 50-70 were mixed and subjected to a preparative TLC. Subsequent to the preparative TLC, an antioxidant compound was isolated which was coloured yellow when dissolved in acetone. The isolated compound was not 100% pure, but it was nevertheless characterised because it constituted about 85% of the total mixture when compared to the other compounds in the mixture (Figure 5.4).

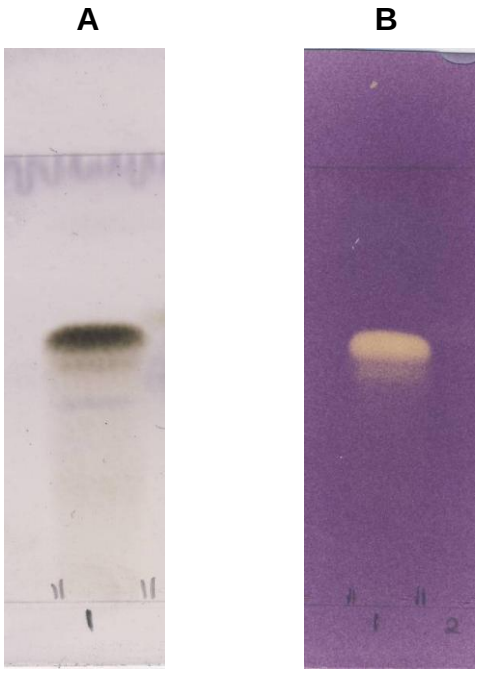
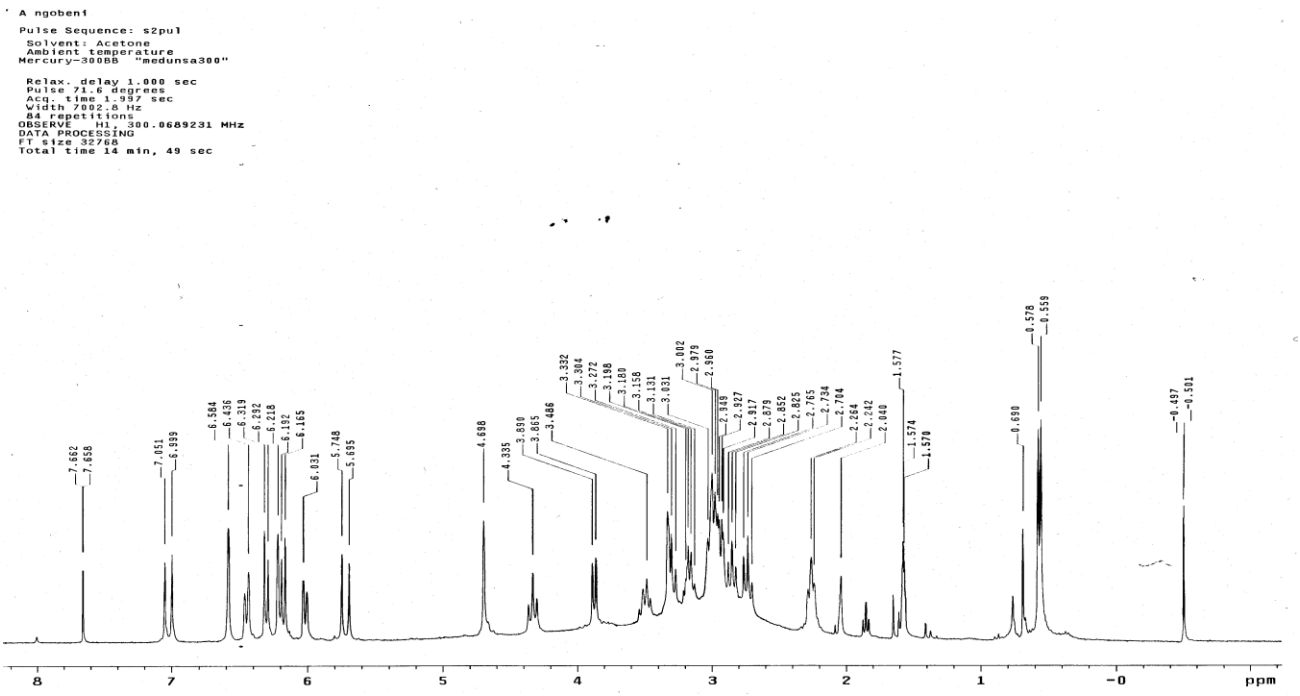


Figure 5.4 TLC chromatograms of the isolated compound of interest. Ten microlitres of the isolate was loaded onto a silica TLC plate and developed using EMW. The plates were visualised using vanillin-sulphuric acid (**A**) and DPPH (**B**).

5.2.5 NMR spectra of the isolated compound

The results of ^{1}H-NMR and ^{13}C-NMR of the isolated compound alluded to a compound with aromatic groups and sugar moieties attached to it. The observations suggested that the compound could be in the category stilbenoid compounds (Figures 5.5a-c).

A



B

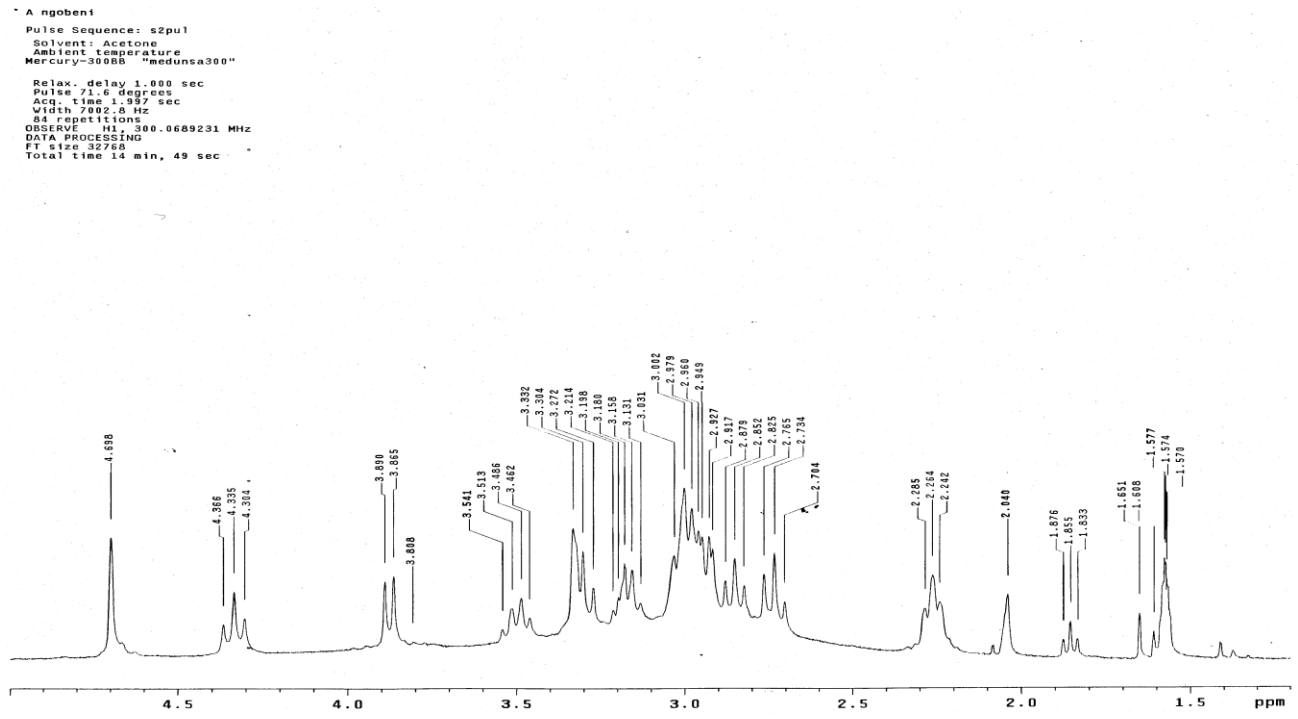
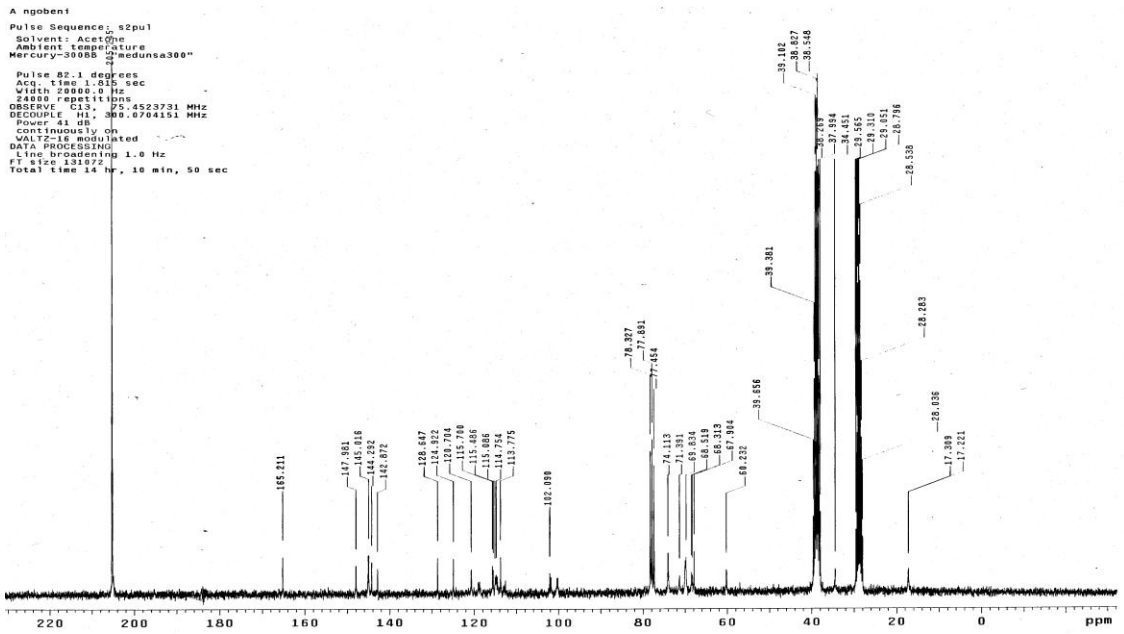


Figure 5.5a The ^{1}H-NMR spectrum of the compound isolated from the D3 fraction where, **A** is the full spectrum and **B** represents the magnification from 1-5 ppm section of the full spectrum.

A



B

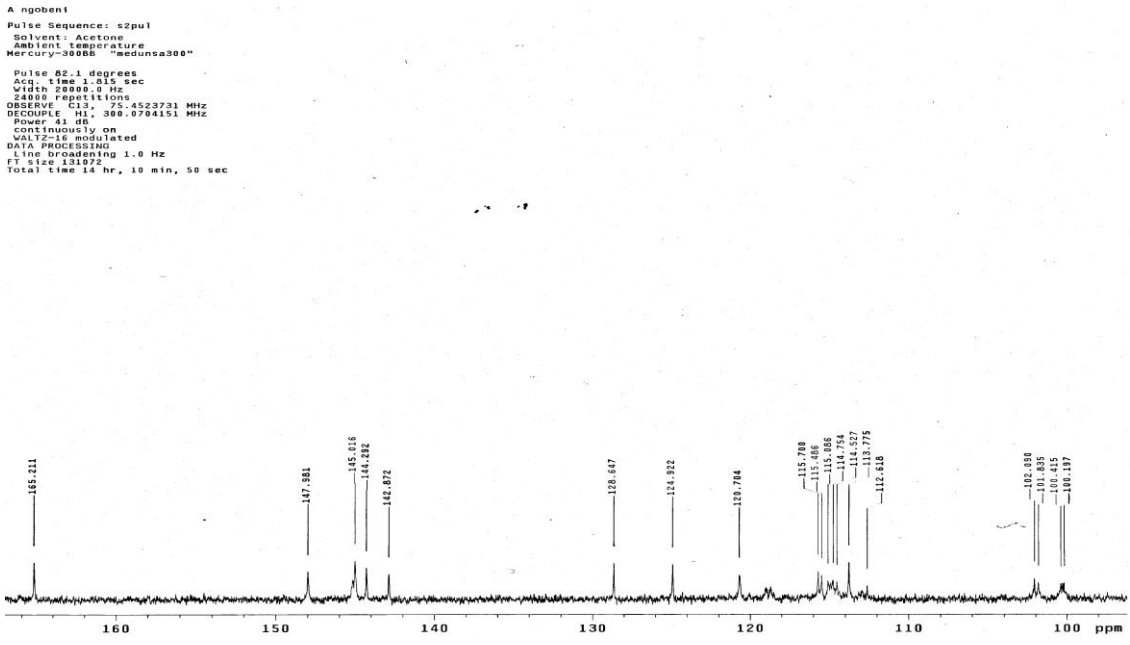


Figure 5.5b The ^{13}C-NMR spectrum of the compound isolated from the D3 fraction of where, **A** is the full spectrum, **B** represents the magnification from 100-170 ppm section of the full spectrum**.**

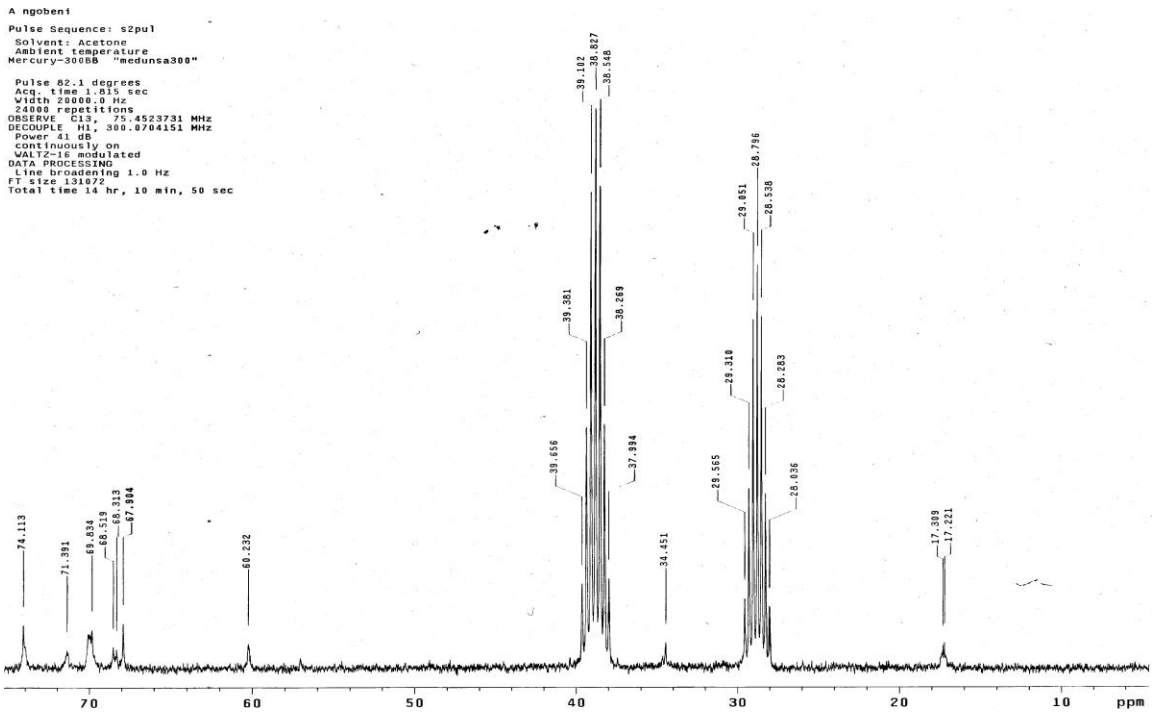


Figure 5.5c The ^{13}C-NMR spectrum of the compound isolated from the D3 fraction from 10-80 ppm section of the full spectrum.

5.2.6 Stilbenoid glycoside compound

Represented below is an astringin compound which illustrates the chemical features of a stilbenoid glycoside compound. Other stilbenoid glycosides have more than one sugar moieties attached to and this could be the case regarding the isolated compound. The NMR spectra supported the suggestion that the isolated compound could be a stilbenoid with one or more sugar moieties attached to it.

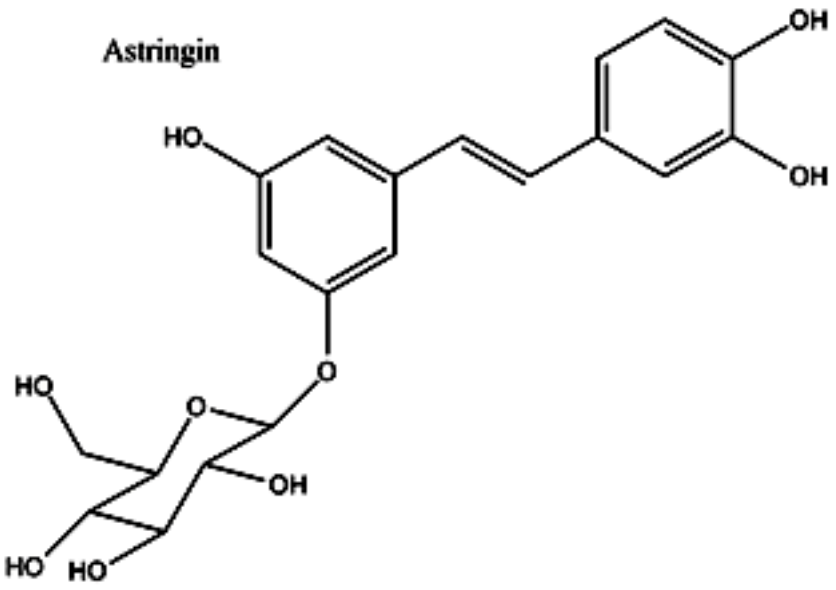


Figure 5.6 A representative of a typical stilbenoid glycoside compound.

CHAPTER 6

DISCUSSION

Since the discovery and introduction of antibiotics into clinical use, there has been an increase in reported cases of bacterial antibiotic resistance. Frequent multiple antibiotic resistance in bacteria makes it desirable to identify novel antimicrobial agents from plants. This study was aimed at screening nine medicinal plant materials for the presence of antibacterial and antioxidant compound(s). Screening medicinal plants is one of the first steps towards the isolation and characterisation of potentially bioactive compound(s), although this process does not suggest that all plants screened will have the antibacterial and bioactive compound(s). This was evident from the work done by Chomnawang *et al.* [71] where 19 Thai medicinal plants were screened for antibacterial effects against acne-inducing bacteria and found that only 13 medicinal plants could inhibit the growth of *Propionibacterium acnes* and a compound Mangostin was later isolated. Steenkamp *et al*. [72] screened aqueous and methanolic extracts of 32 Venda medicinal plants for antifungal activity against *Candida albicans* and found that only 17 plants had antifungal activity.

The use of different extracting solvents in this study resulted a substantial difference in the quantity of material extracted from each extracting solvent. The most polar extracting solvent, methanol, was found to have extracted the highest quantity of compounds throughout the extraction of the nine indigenous medicinal plant tested. Eloff *et al.* [73] and Kotze & Eloff [66] observed that solvents with intermediate polarity extracted much higher quantities of compounds when compared to the polar and non-polar extractants. The difference in the results observed in this study and those reported by other researchers could be due to the difference in the family and sub-family of the plants and the materials that were used.

When Eloff *et al.* [73] did the phytochemical analysis of *Combretum woodii,* which was extracted using 10 different solvents and developed the TLC plates in three solvent systems using the non-polar BEA, the intermediate polarity CEF and the more polar EMW, they observed that approximately 17 bands were separated with the non-polar BEA compared to 11 bands found with the intermediate polarity CEF and 9 bands with the more polar EMW solvent system. The observations in our current study have some similarities to the observations made by Eloff *et al.* [73]. When looking at Figures 3.1 A, B and C, where BEA showed a good separation of most bands with all the four extracting solvents, the most polar methanol extractant showed the least separated bands and no bands in some plant extracts. This observation was expected because BEA is a non-polar developing solvent system while methanol is a polar extracting solvent. Thus due to the difference in polarities one can expect few to no bands in these kind of combinations. Although CEF showed very few bands and no bands on *n*-hexane extractants throughout Figures 3.1 A, B and C, this is also similar to the observation made by Eloff *et al.* [73] and this is due to the difference in polarities between the extractants and the developing solvent.

The free radical scavenging activity of the indigenous medicinal plant extracts was then evaluated using DPPH. The principle of the DPPH method is based on the reaction of the antioxidant compound with the stable free radical which results in the discolouration from purple colour to yellow. The degree of discolouration indicates the scavenging potential of the sample's antioxidative activity and this depends on the hydrogen donating ability of the antioxidant compound(s) [67]. In this study only four indigenous plants showed the presence of compound(s) with antioxidative activity against the free radical, DPPH. This is evidenced by the yellow bands that appeared on the TLC plates against the purple DPPH background (Figures 3.2 A and B). As observed in Figure 3.2 A, the antioxidant compound(s) were extracted mostly with the intermediate polar extracting solvent acetone and the bands were separated well with EMW. The

intensity and reaction speed of the compounds were vey different, suggesting that the antioxidant compounds at these locations may possess different properties; some of which have a fast radical scavenging capacity, reducing DPPH very rapidly while others took a longer time to react. These compound(s) can therefore be presumed to be polar in nature. The plants which showed abundant antioxidant compound(s) that separated well with the EMW solvent system were MKK (*Euclea undulata)*, SBR and MBR (*Momordica balsamia)*. The other plants displayed little or no antioxidant activity as shown in Figures 3.2 B and C.

The antibacterial activities of the nine indigenous medicinal plants were determined against four pathogenic microorganisms' viz. *S. aureus, E. faecalis, E. coli* and *P. aeruginosa*. The clear zones on the plates, with pink background, indicate antibacterial property of the compound(s) when tested against the four pathogenic microorganisms. Out of the four microorganisms used, a clear zone was only observed where *S. aureus* was the test microbe. This observation was similar with the previous observations made by Eloff *et al*. [73], where the plant extracts used showed antibacterial activity against Gram-positive *S. aureus* and *E. faecalis*. This observation provides a comparable and plausible explanation that the isolated compounds may contain antibacterial activity that could be effective against Gram-positive cocci bacteria such as *S. aureus*. What was quite surprising in this study was that the compounds exhibited no antibacterial activity against *E. faecalis*, which is also a Gram-positive coccus. This observation could be due to the fact that the plants used do not produce compounds which can inhibit the growth *E. faecalis*. Other plant materials which showed antibacterial activity against *S. aureus* were ML (*Stiburus alopecuroides)*, MR (*Stiburus alopecuroides)*, MHH (*Clerodendrum glabrum)*, MKP and MBR (*Momordica balsamia)*.

The screening of antibacterial and antioxidant activities in all the medicinal plants tested revealed MKK (*Euclea undulate)* as the only plant that possesses both the

antibacterial and antioxidant compound(s). This plant was chosen for isolation and characterisation of its antibacterial and antioxidant compound(s), but due to the unavailability of enough plant material, isolation and characterisation of the antibacterial and antioxidant compound(s) was not done. MKK "*Euclea undulata"* if studied very well could be one of the solution to some of the problems faced by the primary health sector because it has the ability to produce both antibacterial and antioxidant compound(s). The unavailability of enough plant material from MKK (*Euclea undulata)* has led to two plant materials from *E. inequilatera* and *D. senecioides* being further used for the isolation and characterisation of bioactive compound(s).

Euphorbia inaequilatera is a medicinal plant that is used by African people to relieve indigestions. This application could provide a possible clue that the plant may contain beneficial therapeutic properties. Due to the lack of extensive research on this plant, the current study was aimed at understanding how the plant works, and the properties it contains in terms of antibacterial and antioxidant activity. The plant was therefore investigated to identify if it possess any potential antioxidant properties. A single antioxidant compound was successfully isolated, but could not be further characterised because the isolated compound was lost when it was sent for spectral analysis.

The work done by Madiga [70] led to the quest to isolate an antioxidant active compound from *D. senecioides*. The D3 fraction had a strong antioxidant compound which reacted strongly with vanillin-sulphuric acid to produce dark brown colour. When the compound was visualised under visible light, it was light blue in colour and quenched the fluorescence under UV light (Figure 5.1). These observations may indicate that the compound contain aromatic ring(s) in its structure as reported by Masoko [69]. As an initial step to isolating the antioxidant compound, the D3 fraction was subjected to column chromatography and 90 fractions were collected. The fractions were then analysed using vanillin-sulphuric acid, visible light and UV light to locate the fractions that may contain

the compound of interest. The compound of interest was observed to be distributed from fractions 50 to 70 (Figure 5.2 and Figure 5.3b).

To further isolate and purify the compound of interest, fractions 50-70 were mixed and subjected to preparative TLC. Subsequent to preparative TLC, an antioxidant compound was isolated which was yellow when dissolved in acetone. The isolated compound was not 100% pure, but it was nonetheless characterised because it constituted 85% of the total mixture when compared to the other compounds in the mixture (Figure 5.4). However, the NMR spectra of the isolated compound could not conclusively ascertain the structure of the parent compound, but useful information about the compound was obtained that could account for the compound's antioxidant activity. The ^{1}H NMR spectra (Figure 5.5a) of the isolated compound showed that the isolated compound has a primary parent structure of a stilbene with several sugar moieties. Subsequent ^{13}C NMR spectra (Figure 5.5b) further established that the isolated compound could be a stilbenoid. Indeed stilbenes have been found in many families of higher plants, such as Vitaceae, Gnetaceae, Polygonaceae, Liliaceae, Moraceae and Cyperaceae. These compounds have attracted much attention for their beneficial biological effects, which include antioxidant, COX-I &-II-inhibitory, anti-platelet-aggregation, anti-fungal, tyrosinase-inhibitory, anti-HIV-1 and cytotoxic effects [74]. Stilbenoids are secondary products of heartwood formation in trees that can act as phytoalexins. In chemical terms, they are hydroxylated derivatives of stilbene. In biochemical terms, they belong to the family of phenylpropanoids and share most of their biosynthesis pathway with chalcones. An alternative bacterial stilbenoid, ketosynthase-directed pathway, exists in bacterial symbionts of nematodes [75]. Moreover, stilbenoids have triggered considerable interest for their bioactivities and are therefore regarded as one of the important candidates for drug development.

The findings by Madiga [70], where the anti-inflammatory properties of D3 fraction of *D. senecioides* were observed, could be attributed to the presence of

this isolated stilbenoid compound. Previous studies also revealed that D3 fraction of *D. senecioides* had antiproliferation activity against cancerous cells [76]. For the first time here we report that the antioxidant, anti-inflammatory and antiproliiferation properties observed in the D3 fraction of *D. senecioides* could be due to the presence of this isolated stilbenoid compound.

CONCLUSION

The study has demonstrated that MKK (*Euclea undulata)* possesses both antibacterial and antioxidant activities. Two antioxidant compounds were isolated, one from each plant, *E. inaequilatera* and *D. senecioides*. The isolated compound in the D3 fraction of *D. senecioides* was identified as a stilbenoid.

FUTURE WORK

The antioxidant and antibacterial active compounds of MKK (*Euclea undulate)* warrant further investigations by way of isolation and structural elucidation. Further studies still need to be carried out on this isolated stilbenoid compound from the D3 fraction to understand its biochemical and molecular function and to get its full chemical structure.

CHAPTER 7

REFERENCES

1. Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M and van Staden, J. (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology* **94:**205-217.

2. Taylor, J.L.S., Rabe, T., McGaw, L.J., Jäger, A.K. and van Staden, J. (2001). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* **34:**23-37.

3. Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K and Schempp, C.M. (2007). Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. *Phytomedicine* **14:**508-516.

4. Claudia, P., Daniele, M., Raffaella, R and Pasquale, P. (2009). Cardioprotection: A radical view Free radicals in pre and postconditioning. *Biochimica et Biophysica Acta* **1787:**781–793.

5. Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B and Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study.*The Lancet* **342:**1007-1011.

6. Kinsella, J.E., Frankel, E., German, B and Kanner, J.I. (1993). Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *The Lancet* **341:**454-457.

7. Beara, I. N., Lesjak, M. M., Joven, E. D., Balog, K. J., Anackov, G. T and Orcic, D. Z. (2009). Plantain (*Plantago L.)* species as novel sources of flavonoids antioxidants. *Journal of Agricultural and Food Chemistry* **57(19):**9268–9273.

8. Esmaeili, M. A and Sonboli A. (2010). Antioxidant, free radical scavenging activities of Salvia brachyantha and its protective effect against oxidative cardiac cell injury. *Food and Chemical Toxicology* **48:**846–853.

9. Cox, P.A and Balick, M.J. (1994). The ethnobotanical approach to drug discovery. *Scientific American* **270:**60-65.

10. Marjorie, M.C. (1999). Plant products as antimicrobial agents. *American Society for Microbiology* **12(4):**564-582.

11. Rates, S.M.K. (2001). Plants as source of drugs. *Toxicon* **39:**603-613.

12. Sokmen, A., Jones, B.M and Erturk, M. (1999). The in *vitro* antibacterial activity of Turkish medicinal plants. *Journal of Ethnopharmacology* **67:**79-86.

13. Newman, D.J., Cragg, G.M and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981–2002. *Journal of Natural Product* **66:**1022-1037.

14. Heinrich, M and Gibbons, S. (2001). Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. *Journal of Pharmacy and Pharmacology* **53:**425-432.

15. Wang M. Y., West B. J., Jensen, C. J., Nowicki, D., Chen, S., Palu, A. K and Anderson, G. (2002). *Morinda citrifolia* (Noni): A literature review and recent advances in Noni research. *Acta Pharmacologica Sinica* **23(12):**11271141.

16. Gold, H.S and Eisenstein, B.I. (2000).Introduction to bacterial diseases (5th ed.). Philadelphia, Pa: Churchill Livingstone 320-361.

17. Howard, B.J., Keisser, J.F., Weissfeld, A. S., Smith, T.F and. Tilton R.C. (1994*).* Clinical and Pathogenic Microbiology (2nd ed.). St. Louis: Mosby 280-295.

18. Helge, K. (2001). The Role of virulence factors in enterohemorrhagic *Escherichia coli* (EHEC) - Associated hemolytic-Uremic syndrome. *Seminars in Thrombosis and Hemostasis* **27:**207-214.

19. Reilly, A. (1988). Prevention and control of enterohaemorrhagic *Escherichia coli* (EHEC) infections: memorandum from a WHO meeting. WHO Consultation on prevention and control of enterohaemorrhagic *Escherichia coli* (EHEC) Infections. *Bulletin of the World Health Organization* **76(3):**245-55.

20. Sakka, V., Tsiodras, S and Galani, L. (2008). Risk-factors and predictors of mortality in patients colonised with vancomycin-resistant enterococci. *Clinical Microbiolial Infections* **14(1):**14-21.

21. Laupland, K., Zygun, D., Davies, H., Church, D., Louie, T and Doig, C. (2002). Incidence and risk factors for acquiring nosocomial urinary tract infection in the critically ill. *Journal of Critical Care* **17:**50-57.

22. Klugman, K.P. Emerging infectious diseases-South Africa, volume 4 number4. www.cdc.gov/ncidod/eid/vol4no4/klugman.htm. Date: 15-09-2007.

23. Govan, J. R and Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiology and Molecular Biology Reviews* **60(3):**539-579.

24. Mulligan, M.E., Murray-Leisure, K.A., Ribner, B.S., Standiford, H.C., John, J.F., Korvick, J.A., Kauffman, C.A and Yu, V.L. (1995). Methicillin-resistant *Staphylococcus aureus*: a consensus review of the microbiology, pathogenesis, and epidemiology with implications for prevention and management. *The American Journal of Medicine* **98(6):**599-600.

25. Geissman, T. A. (1963). Flavonoid compounds, tannins, lignins and related compounds. *Elsevier, New York* **9:**265-270.

26. Schmidt, H. (1988). Phenol oxidase (EC.1.14.18.1), a marker enzyme for defense cells. Progress in histochemistry and cytochemistry. *Gustav Fischer* **17:**123-128.

27. Kazmi, M. H., Malik, A., Hameed, S., Akhtar, N and Noor A. S. (1994). An anthraquinone derivative from *Cassia italica*. *Phytochemistry* **36:**761-763.

28. Barre, J.T., Bowden, B.F., Coll, J.C., Jesus, J., Fuente, V.E., Janairo, G.C and Ragasa, C.Y. (1997). A bioactive triterpene from *Lantana camara*. *Phytochemistry* **45:**321-324.

29. Chaurasia, S.C and Vyas, K.K. (1977). In vitro effect of some volatile oil against *Phytophthora parasitica* var. *piperina*. *Indian Journal of Medical Research* **1977:**24-26.

30. Hufford, C.D., Jia, Y., Croom, E.M., Muhammed, I., Okunade, A.L., Clark, A.M and Rogers, R.D. (1993). Antimicrobial compounds from *Petalostemum purpureum*. *Journal of Natural Products* **56:**1878-1889.

31. Omulokoli, E., Khan, B and Chhabra. S.C. (1997). Antiplasmodial activity of four Kenyan medicinal plants. *Journal of Ethnopharmacology* **56:**133-137.

32. Phillipson, J.D and O'Neill, M.J. (1987). New leads to the treatment of protozoal infections based on natural product molecules. *Acta Pharmaceutical* **1:**131-144.

33. Balls, A.K., Hale, W.S and Harris, T.H. (1942). A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chemistry* **19:**279-288.

34. Zhang, Y and Lewis, K. (1997). Fabatins: new antimicrobial plant peptides. *FEMS Microbiology Letters* **149:**59-64.

35. Davies, K.J. (1993). Oxidative stress: the paradox of aerobic life. *Biochemical Society Symposia* **6:**1-31.

36. Nohl, H., Gille, L and Staniek, K. (2005) Intracellular generation of reactive oxygen species by mitochondria. *Biochemical Pharmacology* **69:**719-23.

37. Szocs, K. (2004). Endothelial dysfunction and reactive oxygen species production on ischemia/reperfusion and nitrate tolerance. *General Physiology and Biophysics* **23:**265-95.

38. Ichihashi, M., Ueda, M., Budiyanto, A., Bito, T., Oka, M., Fukunaga, M., Tsuru, K and Horikawa, T. (2003). UV-induced skin damage. *Toxicology* **189:**21-39.

39. Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J and Telser J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Biochemistry and Molecular Cell Biology* **266:**37-56.

40. Halliwell, B. (1999). Antioxidant defense mechanisms: from the beginning to the end. *Free Radical Research* **31:**261-72.

41. Repetto, M.G and Llesuy, S.F. (2002). Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Brazilian Journal of Medical and Biological Research* **35(35):**523-534.

42. Atawodi, S.E. (2005). Antioxidant potential of African medicinal plants. *African Journal of Biotechnology* **4(2):**128-133.

43. Sabu. M.C and Kuttan, R. (2002). Antidiabetic activity of medicinal plants and its relationship with their antioxidant property. *Journal of Ethnopharmacology* **81:**155-160.

44. Tsao, A.S., Kim, E.S and Hong W.K. (2004). Chemoprevention of cancer. *CA: A Cancer Journal for Clinicians* **54:**150-180.

45. Rice-Evans, C.A., Miller, N.J and Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, **2(4):**152-159.

46. Halliwell, B and Gutteridge, J. M. C. (1989). Free radicals in biology and medicine: Antioxidant defences (2nd ed.). Oxford: Oxford University Press, 105-245.

47. Saha, K., Lajis, N.H., Israf, D.A., Hamzah, A.S., Khozirah, S., Khamis, S and Syahida, A. (2004). Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *Journal of Ethnopharmacology* **92:**263-267.

48. Cai, Y., Luo, Q and Corke, M. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* **74:**2157-2184.

49. Katsube, T., Tabata, H., Ohta, Y., Yamasaki, Y., Anuurad, E., Shiwaku, K and Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *Journal of Agricultural and Food Chemistry* **52:**2391-2396.

50. Hayes, J.D and McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research* **31:**273-300.

51. Porrini, M., Riso, P., Brusamolino, A., Berti, C., Guarnieri, S and Visioli, F. (2005). Daily intake of a formulated tomato drink affects carotenoid plasma and lymphocyte concentrations and improves cellular antioxidant protection. *British Journal of Nutrition* **93:**93-99.

52. Gaetke, L.M and Chow, C.K. (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **189:**147-163.

53. Huxley, R.R and Neil, H.A.W. (2003). The relation between dietary flavonoid intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. *European Journal of Clinical Nutrition* **57:**904-908.

54. Heijnen, C.G.M., Haenen, G.R.M.M., Oostveen, R.M., Stalpers, E.M and Bas, A. (2002). Protection of flavonoids against lipid peroxidation: structure activity relationship revisited. *Free Radical Research* **36:**575-581.

55. Hayes, J.D and McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defense against oxidative stress. *Free Radical Biology and Medicine* **31:**273-300.

56. Kuhn, H and Borchert, A. (2002). Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. *Free Radical Biology and Medicine* **33:**154-172.

57. Akerboom, T.P.M and Sies, H. (1989). Transport of glutathione, glutathione disulfide, and glutathione conjugates across the hepatocyte plasma membrane. *Methods in Enzymology* **134:**523-34.

58. Mahler, M and Thomason, V. (2005). Purification of natural products, Chromatography Application Note AN25. *A Teledyne Technologies Company*.

59. Rios, J.L., Recio, M.C and Villar, A. (1991). Isolation and Identification of the antibacterial compouds from from *Helichrysum stoechas*. *Journal of Ethnopharmacology* **34:**51-55.

60. Wei. L., Mouming, Z., Bao, Y., Guanglin, S and Guohua R. (2008) Identification of bioactive compounds in Phyllenthus emblica L. fruit and their free radical scavenging activities. *Food Chemistry* **114(2009):**499–504.

61. Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Journal of Molecular Aspects of Medicine* **27:**1-93.

62. Pieters, L and Vlietinck, A.J. (2005). Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds? *Journal of Ethnopharmacology* **100:**57-60.

63. Balunas, M.J and Kinghorn, A.D. (2005). Drug discovery from medicinal plants. *Journal of Life Sciences* **78:**431-441.

64. Lindsey, K.L., Budesinsky, M., Kohout, L and van Staden, J. (2006). Antibacterial activity of maytenonic acid isolated from root-bark of *Maytenus senegalensis*. *South African Journal of Botany* **72:**473-477.

65. Holzgrabe, U., Waver, I and Diehl, B. (1999). NMR Spectroscopy in Drug Development and Analysis (1st ed.). Wiley-VCH. 16-61.

66. Kotze, M and Eloff, J.N. (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae) *South African Journal of Botany* **68:**62-67.

67. Es-Safi, N.E., Kollman, A., Khlifi S and Ducrot, P.H. (2007). Antioxidative effect of compounds isolated from *Globularia alypum* L. Structure-activity relationship. *Lebensmittel-Wissenchaft und Technologie* **40:**1246-1252.

68. Begue, W.J and Kline, R.M. (1972). The use of tetrazolium salts in bioautographic procedure. *Journal of Chromatography* **88:**182-184.

69. Masoko, P. (2006). Characterization of antifungal compounds isolated from *Combretum* and *Terminalia* species (Combretaceae). PhD thesis- University of Pretoria, pp 48-60.

70. Madiga, M.C. (2007). Antioxidative, anti-inflammatory and antineoplastic potential of *Dicerocaryum species*. MSc dissertation -University of Limpopo, pp 45-46.

71. Chomnawang, M.T., Surassmo, S., Nukoolkarn, V.S and Gritsanapan, W. (2005). Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. *Journal of Ethnopharmacology* **101:** 3030-333.

72. Steenkamp, V., Fernandes, A.C and Van Rensburg, C.E.J. (2007). Screening of Venda medicinal plants for antifungal activity against *Candida albicans*. *South African Journal of Botany* **73**: 256–258.

73. Eloff, J.N., Famakin, J.O and Katerere, D.R.P. (2005). *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. *African Journal of Biotechnology* **4(10):**1161-1166.

74. Jong, P.L., Byung, S.M., Ren, B.A., Min, K.N., Sang, M.L., Hyeong, K.L., Jae, G.K., Ki, H.B and Sam, S.K. (2003). Stilbenes from the roots of Pleuropterus ciliinervis and their antioxidant activities. *Phytochemistry* **64:**759–763.

75. Sobolev, V. S., Horn, B. W., Potter, T. L., Deyrup, S. T and Gloer, J. B. (2006). Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. *Journal of Agriciulture and Food Chemistry* **54(10):** 3505–3511.

76. Mphahlele, R. P (2008). Evaluation of molecucar mechanism(s) associated with the antiproliferative and apoptosis-inducing properties of the crude methanolic and semi-purified extracts of *Dicerocaryum species* in Jukart T-cells. MSc dissertation-University of Limpopo, pp 35-46.