Isolation and characterization of bioactive compound from *Combretum erythrophyllum* leaf extracts

By

IMELDA PHUTI LEDWABA

Dissertation submitted in fulfilment of the requirements for the degree of Magister Technologiae Chemistry

In the

Faculty of Applied and Computer Sciences

Chemistry Department

At

Vaal University of Technology

August 2012

Supervisor: Dr. F.M. Mtunzi

Co-supervisor: Prof. A.M. Sipamla

Declaration

I, the undersigned hereby declare that the research data contained in this study is my own original work carried out at Phytomedicine Programme, University of Pretoria and Department of Chemistry, Vaal University of Technology. This dissertation has not been submitted at any university before. I declare that all information sources used or quoted have been indicated and acknowledged by means of complete references.

IMELDA PHUTI Ledwaba

Signature**.....**

Date: **.....**August 2012

Dedication

This work is dedicated to my late mother Mrs Rosina Kgabo Ledwaba and my kids Kwena and Siyathandwa.

Acknowledgements

First and formost I would like to thank God for giving me hope, strength and encouragement through the entire journey in this study.

The phytomedicine programme team at the University of Pretoria: Ms Belonnah Sakong, Ms Selaelo Raphatlelo, Mr Thanyani Ramadwa, Dr Lyndy McGaw and the great expert of all my research work, Dr Ahmed Aroke Shahid, and Professor Jacobus N. Eloff for the generous warm welcome to their team and assistance with all the work achieved.

I would like to extend my hand of appreciation to my supervisors at the Vaal University of Technology, Dr Fanyana Mtunzi and Professor Allworth Sipamla for giving me the attention when needed.

My sincere thanks to my family for looking after my kids and supporting me through this study, my partner Mr Bongani Buwa, my sisters (Mrs Daphney Phiri and family, Inocentia and MMaseipei) and all the Ledwaba's Family.

I would also like to thank my friends and colleagues for the support and motivation: Mrs Malesole Bucibo, Mr Thabo Brooms and Mr Mike Tshibangu.

A big thank you to the ladies in the post graduate office for assisting with the necessary information needed, guidance and support, Ms Beatrice Lekota, Ms Beatrice Toti and Ms Nomathemba Kenke.

My sincere thanks to Dr Johan Jordaan from the North West University (Potchefstroom campus) for helping with the NMR analyses and Mr Fletcher Hiten from the University of Stellenbosch for assisting with the MS analysis.

This study has been made successful by the financial support of Vaal University of Technology (VUT), University of Pretoria (UP) and the National Research Foundation (NRF).

Conferences and Proceedings

Poster Presentation

I.P. Ledwaba, A.S. Ahmed, F.M. Mtunzi, A.M. Sipamla and J.N. Eloff (2011). Biological activities of *Combretum erythrophyllum* extracts and fractions. Indigenous Plant Use Forum (IPUF), Durban (St Lucia) (4-7July 2011).

Progress Report Presentation

Biological activities of *Combretum erythrophyllum* leaf extracts. Vaal University of Technology (2nd March 2012).

Abstract

Combretum erythrophyllum has been used for medicinal purposes and several studies have been carried out to investigate the bioactive compounds present in the leaves of this plant. The World Health Organization reported that 80% of the people living in the developing countries almost exclusively use traditional medicine. *Combretum* species are used in many human medicines for the treatment of microbial infections and several anti-inflammatory conditions. Members of the Combretaceae family are widely traded in the traditional medicine market in Southern Africa. The family is also used for medicinal purposes in the rest of Africa and Asia for close to 90 medicinal indications. Many of these indications are related to infective agents. Traditional healers have long used plants to prevent or cure infectious conditions.

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties. This review highlights the current status of traditional medicines, its contribution to modern medicine, recent trends in the evaluation of anti-microbial activity with a special emphasis upon some tribal medicine, *in vitro* and *in vivo* experimental design for screening and therapeutic efficacy in safety and human clinical trials for commercial outlet. Many of these commercially available compounds are crude preparations administered without performing human clinical trials.

The leaves of *Combretum erythrophyllum* were extracted with acetone to obtain the crude extract. Liquid-liquid fractionation was performed on the crude using different solvents of different polarity. The crude and obtained fractions were investigated for antimicrobial activity. The crude and fractions were tested against certain bacterial and fungal microorganisms. The assay methods used included the microtitre dilution method for determining the minimum inhibitory concentration and bioautographic methods used to detect the inhibition of bacterial and fungal growth by active compounds separated from the crude and fractions. The antioxidant activity was performed using TLC-DPPH, DPPH, ABTS and hydroxyl radical scavenging.

The toxicity of crude extract and fractions was determined using MTT assay. Isolation of compounds was performed using column chromatography. Structural elucidation was done using NMR and MS spectrometry.

In microtitre dilution assay acetone fraction inhibited the growth of *S. aureus, E. faecalis, P. aeruginosa* with the lowest MIC value of 0.02, 0.32, 0.16 µg/ml and ethyl acetate fraction inhibited the growth of *E. coli* with the lowest MIC value of 0.16 µg/ml. All fractions were active against *C. neoformans* with the MIC value of 0.02 µg/ml. Dichloromethane was the least active against *C. albicans* with the MIC value of 0.16 µg/ml while the rest had the MIC value of 0.08 µg/ml. Dichloromethane was found to be active against *A. fumigatus* with the lowest MIC value of 0.16 µg/ml. Bioautography showed the presence of various inhibitory chemical compounds. Ethyl acetate and hexane fraction had a very good separation and showed various zones of inhibition on exposure to *E. faecalis, E. coli, S. aureus* and *P. aeruginosa* with the Rf values ranging from 0-0.98. Crude and fractions showed slight zones of inhibition against *C. neoformans, C. albicans* and *A. fumigatus* with the Rf values ranging from 0-0.76. TLC-DPPH assay displayed that ethyl acetate and water fraction had the highest antioxidant activity in CEF. Ethyl acetate fraction had a strong antioxidant activity in DPPH assay with the EC_{50} of 0.04272 µg/ml, water fraction showed a good antioxidant activity with the EC_{50} of 0.01825 µg/ml in ABTS assay and in the hydroxyl radical scavenging the crude extract scavenged 77.62 ± 1.41% at the highest concentration of 0.250 mg/ml and 47.21 ± 3.20% at the lowest concentration of 0.003 mg/ml. The toxicity level of the crude extract and fractions were found to be between 34 and 223 mg/ml which were all below doxorubicin (LC_{50} = 7.1855 µg/ml) which was used as the positive control.

Column chromatography was used in a bio-guided assay fractionation and led to isolation of one compound. The antimicrobial activity was determined against pathogenic bacteria. The isolated compound had a good activity against *Pa* and *Sa* with the lowest MIC value of 0.32 µg/ml. Nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy were used for the identification of isolated compounds. One compound was isolated and identified as Friedelin. The results obtained confirm that the leaves of *Combretum erythrophyllum* have a good antimicrobial activity and strong antioxidant activity.

List of abbreviations

ABTS acid	2, 2'-azinobis-3-ethylbenzotiazoline-6-sulphonic
Af	*Aspergillus fumigatus*
AIDS	Acquired immunodeficiency syndrome
BEA	Benzene/ethanol/ammonia solution
Ca	*Candida albicans*
CEF	Chloroform/ ethyl acetate/ formic acid
Cn	*Cryptococcus neoformans*
Cosy	Correlation Spectroscopy
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EA	Ethyl acetate
Ec	*Escherichia coli*
Ef	*Enterococcus faecalis*
EMW	Ethyl acetate/ Methanol/ Water
HEX	Hexane
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
INT	*p*-iodonitrotetrazolium violet

MIC	Minimum inhibitory concentration
MS	Mass Spectrometry
NMR (^{13}C and ^{1}H)	Nuclear magnetic resonance (Carbon 13 and proton)
Pa	*Pseudomonas aeruginosa*
Rf	Retention factor
Sa	*Staphylococcus aureus*
TLC	Thin layer chromatography
WHO	World Health Organization

Table of Contents

List of figures

List of tables

Chapter1

1.0. Introduction

Infectious diseases are a serious problem as significant cause of morbidity and mortality worldwide particularly in developing countries, accounting for approximately 50% of all deaths where access to health care is inadequate and as much as 20% of deaths in developed countries (Mahady, Fong & Farnsworth 2008). Despite the milestones reached in microbiology with the discovery and application of antibiotics, and the control of microorganisms, sporadic incidents of epidemics due to drug-resistant microorganisms and emergence of unknown disease-causing microbes pose an enormous threat to healthcare.

The development of resistant strains in infectious microorganisms to some existing antibiotics (National Institute of Health (NIH) 2001) has complicated the management of infectious diseases as available drugs are effective against only one-third of the diseases (Gerwick, Proteau, Nagle, Hamel, Blokhin &Slate 1994). Microbial drug resistance has developed due to overuse and abuse of antibiotics and many of them are no longer effective against diseases for which they had previously been used. The resistant pathogens may become more virulent and increase the risk of complications and mortality (Steckelberg 2009). All known antibiotics are natural products derived from fungi or other living systems and are produced on an industrial scale using a fermentation process (Crueger 1989).

The prevalence of other diseases such as cancer, human immunodeficiency virus (HIV) infection, haematological and autoimmune disorders and the consequential immune system dysfunction, may lead to symbiotic microorganisms becoming pathogens under certain conditions usually referred to as opportunistic infection. Opportunistic pathogens include viruses, bacteria, fungi and protozoa in immunocompromised patients and are posing new challenges in health care delivery worldwide. An opportunistic disease requires impairment of host defences, which may occur as result of genetic defects, exposure to antimicrobial drugs or immunosuppressive chemicals or as a result of an infectious disease with immunosuppressive activity (Ryan & Ray, 2004).

Some of the chemotherapeutic agents currently in use are toxic with associated adverse side effects (Covington, 1998). Therefore, there is a general need for new anti-infective and chemotherapeutic agents against various diseases pathoaetiologies that are highly effective, low toxicity with minor environmental impact. Plant-based medicines have many traditional claims including the treatment of ailments of infectious origin. The extracts of many plant species have been tested against hundreds of microbial strains using various *in vitro* models and some with good activity of pharmacological relevance. However, a limited number of these medicinal plant extracts have been tested in animal or human studies to determine safety and efficacy. Natural products and their derivatives represent about 50% of all drugs in clinical use (Farnsworth 1984; O'Neill & Lewis 1993).

Natural products derived from medicinal plants are classified in three major groups: terpenoids, alkaloids and phenolics. The mechanisms of these phytochemicals can be antimicrobial, antioxidant, anti-inflammatory and immunomodulatory. Alongside the production of beneficial phytochemicals, plants also produce potentially toxic substances; therefore incorporation of toxicity assays in bioactivity evaluation of medicinal plants is important. The largest users of medicinal plants in the world are in Chinese traditional medicine, with more than 5,000 plants and plant products listed in their pharmacopoeia (Bensky & Gamble 1993).

In South African traditional medicine, many plant species are being used to treat or serve as a prophylaxis against various forms of disease (infectious and non-infectious) (Steenkamp 2003). In South Africa, medicinal plant species are traded for use in traditional medicines, of which most are from ethnopharmacological guide (Williams 1996). The sustainable use and management of medicinal plants is of considerable challenge to all stakeholders. The stem, bark and roots of many medicinal plants are being harvested and traded in an unsustainable manner that may lead to accelerated death of the tree which is the source of medication. Evaluation and validation of bioactivity of the leaf extracts as a possible substitute for the use of stem, bark and roots provide a viable option for the conservation of medicinal plants.

Medicinal plant remedies also feature prominently in the treatment of ailments in domestic and production animals, and ethnoveterinary healing remains an important part of animal health care in developing countries (Cunningham & Zondi 1991). Among the medicinal plants used in South African traditional medicine, species of *Combretum* features prominently as agent for treating infectious diseases such as diarrhoea (*C. imberbe, C. vendae*), malaria (*C. ghasalense*), stomach disorders (*C. molle*) and coughs (*C. Molle, C. imberbe, C. erythrophyllum*) (Eloff, Katerere & McGaw 2008). *Combretum erythrophyllum* is a member of Combretaceae family, widely used for treatment of venereal diseases (Van Wyk & Gericke 2000). Roots are used as a purgative while dried and powdered gum can be applied to sores (Venter & Venter 1996).

The roots and bark decoctions of *Combretum erythrophyllum* are used for the treatment of cough and infertility as well as an aphrodisiac (Arnold & Gulimian 1984; Watt & Breyer-Brandwijk 1962). The leaves are used for the relief of cough and stomach pains while the seeds are used for the removal of intestinal worms in dogs and have been reported to be poisonous (Van Wyk & Van Wyk 1997). *C. erythrophyllum* is widely distributed in the Southern Africa region, mostly found in South Africa along the coast in the Eastern Province, through Kwazulu-Natal, Northern South Africa (Mpumalanga, Limpopo Province, Gauteng and the eastern parts of North West), Zimbabwe, Swaziland, Mozambique and slightly into the eastern parts of Botswana (Schmidt, Lötter & McCleland 2002).

Seven antibacterial phenolic compounds which include four flavonols (5,6,4'-trihydroxyflavononol (kaempferol), 5,4'-dihydroxy-7-methoxyflavonol (Rhamnocitrin), 5,4'-dihydroxy-7,5'-dimethoxyflavonol (Rhamnazin) and 7,4'-dihydroxy-5,3'-dimethoxyflavonol (quercetin-5,3'-dimethylether) and three flavones, 5,7,4'-trihydroxyfavone (apigenin), 5,4'-dihydroxy-7-methoxyflavone (genkwanin) and 5-hydroxy-7,4'-dimethoxyflavone) have been isolated from *C. erythrophyllum*. All compounds had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with the MIC value of <100 µg/ml. Rhamnocitrin and quercetin-5, 3'-dimethylether inhibited *Micrococcus luteus* and *Shigella sonei* with the MIC of 25 µg/ml (Martini, Katerere & Eloff 2004).

In this work a literature review of medicinal plants used ethnopharmacologically in treating infectious diseases is presented. The phytochemical profile, antibacterial, antifungal and antioxidant activities of extracts from *Combretum erythrophyllum* (Burch) were carried out. The bioactive compound was isolated, characterized and identified using various chromatographic and spectrophotometric methods. The results are hereby presented in the next chapters.

1.1. Aim of Research

To evaluate the biological activities of *Combretum erythrophyllum* leaf extracts against infectious diseases' pathogenesis and also to identify and isolate the active component responsible for the activity.

1.2. Specific objectives

1. Assess the biological activity of crude plant extract
2. Select and evaluate the best fractionation procedure for isolation
3. Isolate active compound
4. Determine the chemical structure of isolated compound
5. Determine the biological activity of isolated compound

Chapter2

Literature review

2.0. Importance of medicinal plants as therapeutic agents in disease treatment

Medicinal plants are well known natural sources of therapeutic agents used for the treatment of various diseases. About 20,000 plant species have been documented to be valuable for medicinal purposes by World Health Organisation (WHO) (Gullece, Aslan, Sokmen, Sahin, Adiguzel, Agar & Sokmen 2006). Natural products either as pure compounds or as standardized plant extracts provide unlimited opportunities for drug development or lead to new efficient drugs because of the unmatched availability of chemical diversity (Cos, Vlientinck, Vanden Berghe & Maes 2006). Increasing interest in medicinal plants can be attributed to a number of reasons such as cultural belief, affordability and unlimited access of traditional medicine. Long-time usage of herbal medicine have also increased people's positive attitude to the application of herbs (Maregesi, Ngassapa, Pieters & Vlientinck 2007). Many drugs currently used in orthodox medicine are medicinal plant isolates or derivatives. Examples include opium isolated from *Papaver somniferum* and aspirin, an acetyl derivative of 1-O-(2'-acetoxy) benzoyl-α-D-glucopyranose extracted from willow bark.

Aspirin is one of the most widely used compounds in the treatment of simple pain and inflammation while its toxicity is much lower than most compounds possessing similar pharmacologic activity (Hussain, Truelove & Kostenbauder 1980). World Health Organisation reported that over 80% of the world's population uses traditional medicine for some aspect of their primary healthcare need. Medicinal plants are the major components in all traditional medicine systems. Many research groups are engaged in multi-disciplinary evaluation of phytochemicals from medicinal plants for leads in developing drugs for treatment of various diseases (www.who.int/mediacentre/factsheet). The tradition of collecting, processing and applying plant-based medications have been handed down from generation to generation by adults using the oral method, especially in African countries (Herdberg & Staugard 1989). Medicinal plants have been used to cure a variety of human ailments since ancient times (Kamboj 2000).

A large number of anti-microbial agents derived from traditional medicinal plants are available for treating various diseases like meningitis, cholera and tuberculosis which are caused by micro-organisms (Jain 1994). Some of the life saving drugs from medicinal plants includes morphine, digoxin, emetine and ephedrine (Farnsworth & Morris 1976). Important factors for the anti-microbial potential of medicinal plant preparations include sensitivity of the infecting micro-organism, period of exposure, concentration, and structural features of the bioactive compound. Direct toxicity of compounds extracted from medicinal plants can be tested on animal cells because of the close association with human tissues or cells (Tyler 1997). Any substances with selective toxicity to pathogens and little or no toxicity to human and animal cells are considered good candidates for developing new antimicrobial drugs (Nimri, Meqdam & Alkofahi 1999; Saxena & Sharma 1999).

2.1. Plant for treatment of diseases

2.1.1. Diarrhoea

Diarrhoea is a condition of having three or more abnormal loose liquid bowel movements per day. Infection is one of the major causes of gastrointestinal disorders including diarrhoea, irritable bowel syndrome, constipation and intestinal pain and is estimated to be responsible for deaths of about 3-4 million individuals each year, (WHO 1996). The major microorganisms responsible for intestinal infection from food-borne are *Salmonella*, *Campylobacter jejuni* and *Escherichia coli* and water borne as a result of contamination of domestic water supplies include *Giardia intestinals* and *Cryptosporidium parvum* (Mathabe, Nikolova, Lall & Nyazema 2006). Traditional healers from different places use different medicinal plants for the treatment of diarrhoea (Dambisiya & Tindimwebwa 2003). Roots of *C. collinum* combined with *C. molle* and *Phyllanthus reticulates* are used for the treatment of acute diarrhoea. Leaves and roots of *C. imberbe* also have been used for the treatment of diarrhoea (Neuwinger 2000). Roots and stem bark of *C. zeyheri* are used for treatment of diarrhoea and root infusions are used for bloody diarrhoea. The following plants: Roots of *Punica granatum* L. (punicaceae) and stem bark of *Indigoferera daleoides* Benth. ex harv & Sond have been used for the treatment of diarrhoea with the MIC value of (0.039 mg/ml) (Mathabe *et al*. 2006).

2.1.2. Tuberculosis

Tuberculosis is another major infectious disease caused by *Mycobacterium tuberculosis* in humans and animals especially in immunocompromised situations (WHO 2007). Tuberculosis is a contagious disease in overpopulated areas among the malnutritioned and poor people (Pereira, Tripathy, Indamdar, Ramesh, Bhavsar, Date, Iyyer, Acchammachary, Mehendale & Risbund 2005). The treatment of Tuberculosis has become more complex because of the emergence of drug resistant *M*. *tuberculosis* strains (Balunus & Kinghorn 2005; Fabricant & Farnsworth 2001). An alternative method to find new drugs is in natural products isolated from medicinal plants. Natural products isolated from plants have played an important role in discovery of drugs against infectious diseases (Cragg, Newman & Snader 1997). Some plants from *Combretum* species and other species have been investigated for the treatment of tuberculosis. The bark of *C. molle* has been used for the treatment of tuberculosis with the MIC value of 1 mg/ml from the acetone extract against *M. tuberculosis*. Other plant species such as *Chenopodium ambrosioides* L*, Nidorella anomala* Steets*, Nidorella auriculata* DC*, Senecio serratuloides* DC var*. serratuloides, Cassine papilllosa* (Hochst) Kuntze*, Euclea natalensis A.DC* and *Polygala myrtifolia* L were found to be active against *M. tuberculosis* (MIC<100 mg/ml) (Lall & Meyer 1999).

2.1.3. Pneumonia

Pneumonia refers to lung inflammation, usually due to infections but sometimes non-infectious, that has the additional feature of pulmonary consolidation (Stedman's' medical dictionary 2006). Antibiotics improve outcomes in those with bacterial pneumonia (Kabra, Lodha & Pandey 2010). Initially antibiotics depend on the characteristics of the person affected, such as age, underlying health and the location where the infection was acquired (Lim, Baudouin, George, Hill, Jamieson, Le Jeune, Macfarlane, Read, Roberts, Levy, Wani &Woodhead 2009). The majority of deaths in children under the age of five due to pneumococcal diseases occur in developing countries (World Health Organisation).

Pneumonia can be caused by infectious agents like bacteria *(Streptococcus pneumonia, Haemophilus influenza)*, fungi (*Pneumocystis jiroveci*, *Cryptococcus neoformans*), viruses (influenza virus, Corona viruses) and Parasites (*Toxoplasma gondii*, *Strongyloides stercoralis*), (Pomerville 2010). Symptoms include cough, chest pain, fever and difficulty in breathing (Ashby & Turkington 2007).

2.1.4. Malaria

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus *Plasmodium*. The signs and symptoms of malaria include fever, shivering, joint pain, vomiting, anaemia and retinal damage (Beare, Taylor, Harding, Lewallen & Molyneux 2006). *P. falciparum, P. malariae, P. ovale, P. vivax* and *P. knowlesi* are the main causes of malaria in human beings (Mueller, Zimmerman & Reeder 2007; Singh, Kim Sung & Matusop 2004). Malaria has since been one of the main health hazards in the world (Newman 1976). The global extent and consequences of a disease such as malaria are disturbing especially with the higher number that the clinical cases of malaria is at present, being amongst the world's devastating infectious diseases infecting a lot of people and causing deaths (Sachs & Malaney 2002). Most of the drugs that have been used to cure malaria are derived from natural products (Christensen & Kharazmi 2001) and the other reason being that traditional medicine is a medicine of proximity and less constraining and less expensive (Gbéassor, Kossou, Amegbo, Koumaglo & Denke 1989; Pousset 1989). Artemisinin is one of the compounds which was isolated from the leaves of *Artemisia annua* and used for the treatment of malaria. The following *Combretum* species have been used for the treatment of malaria: Decoctions of roots and leaves of *C. collinum* are used as well as root decoctions of *C. micranthum*, stem bark of *C. molle*, Roots and leaves of *C. molle* with *Ochna pulchra* (Ochnaceae), *Burkea Africana* (Caesalpiniaceae) and *Diospyros chamaethamnus.*

2.1.5. AIDS

As the AIDS crisis leads an increasing number of countries to question their priorities in health expenditures, there is an emerging awareness that the traditional health practitioners can play an important role in delivering an Aids prevention message.

There are concerns about unsafe practices and a growth in claims of traditional cures for AIDS. Partnerships between modern and traditional health sectors are a foundation for building a comprehensive strategy to manage the AIDS crisis (Bodeker, Dvorak-Little, Carter & Burford 2006). Although most HIV/AIDS-infected people that need treatment can access antiretroviral therapy from local hospitals and health centres, several constraints of the antiretroviral program compel many HIV infected people to use medicinal plants to manage HIV/AIDS-related opportunistic infections (Chinsembu 2009).

Other people use medicinal plants to offset side effects from antiretroviral therapy. Documentation of anti-HIV plant species will help preserve this important indigenous knowledge resource and may also lead to the isolation of novel chemical compounds that can be developed into newer antiretroviral drugs. Due to high rates of utilization of traditional healers in sub-Saharan Africa, it is believed that traditional medicines are used for the treatment of HIV and related symptoms (Morris 2001; Bodeker 2003; Langlois-Klassen, Kipp & Rubaale 2008). In Africa, some research has raised concern that the African potato may inhibit antiretroviral therapy drug metabolism and transport (Mills, Foster, Van Heeswijk, Phillips, Wilson, Leonard, Kosuge & Kanfer 2005). Fungal infections have been found to be the major cause of mortality in patients with severely impaired immune mechanisms (Kelberg 1997).

Opportunistic fungal pathogens have become a common cause of morbidity and mortality with the rise in HIV (Garbino, Kolarova, Lew, Hirschet & Rohner 2001). *Candida* and *Aspergillus* infections are usually found mostly in immunocompromised persons such as cancer, transplant and AIDS patients (Kourkoumpetis, Manolakaki & Velmahos 2010). In immunocompromised patients, *candida* infections can affect the oesophagus with the potential of becoming systematic (Fidel 2002; Pappas 2006). Patients with late-stage HIV disease are at risk of acquiring aspergillosis (Meerseman, Vandecasteele, Wilmer, Verbeken, Peetersman & Van Wijngaerden 2004). Most people with weakened immune systems develop a systemic illness caused by *Candida* species (Choo, Chakravarthi, Wong, Nagaraja, Thanikachalam, Mak, Radhakrishnan & Tay 2010). In HIV patients, the presence of oral candidiasis is the initial opportunistic infection in most cases (Fan-Havard, Capano, Smith, Mangia & Eng 1991).

2.1.6. Skin

Infectious dermatological diseases are a common occurrence in the rural parts of South Africa. Traditional healing plays an important role in black African culture with the majority of people consulting traditional healers (Lindsey, Jager, Raidoo & Van Staden 1999). Plants having dermatological properties are highly required due to their ability to stop bleeding, speed up wound healing and soothe skin exposed to burns (Lewis & Elvin-Lewis, 1977). The fleshy leaves and roots of most species within the aloe family are used in many traditional treatments (Mabberley 1987). Traditional healers and indigenous people utilise mainly the leaf sap of this genus widely for the treatment of wounds, burns, rashes, cracked lips and cracked skin (Cera, Heggers, Robson & Duraccio 1980).

2.2. Plant as anti-inflammatory

The immune system response to a microorganism often causes symptoms such as high fever and inflammation, and has the potential to be more disturbing than direct damage caused by a microbe (Ryan & Ray, 2004). Inflammation is a branch of the complex biological resistance of vascular tissues to harmful stimuli such as pathogens, damaged cells or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation is one of the responses of the organism to the pathogen, without inflammation wounds and infections would never heal (Ferrero-Milani, Nielsen, Andersen & Girardin 2007). The leaf, bark and root of *C. kraussi* were used for the treatment of inflammation with the MIC range of 0.195-1.56 µg/ml against *M. aurum* and 1 µg/ml against *M. tuberculosis*.

2.3. Plant as immune modulator

2.3.1. Oxidation

The increase in the oxidation state of an atom through a chemical reaction is known as oxidation. Oxidation state is an indicator of the degree of oxidation of an atom in the chemical compound. The formal oxidation state is the hypothetical charge that an atom would have if all bonds to atoms of different elements were 100% ionic.

Oxidation state is typically represented by integers, which can be positive, negative or zero (Jensen 2007). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. A variety of endogenous free radical scavenging antioxidants exists in the human body while others are derived from dietary sources like fruits, vegetables and teas. It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppressant and neurodegenerative diseases. The consumption of antioxidants has several health benefits; this includes oxidative damage associated with free radical damage and their roles to diseases (Diaz, Frei & Keaney 1997).

Antioxidant nutrients, either exogenous or endogenous, whether synthetic or natural, have the capacity to search for free radicals in the system and neutralize them before they can cause any damage in the body cells (Maxwell 1995).Free radicals are natural by-products of human metabolism. These are reactive species with an unpaired electron which attack macromolecules such as protein, DNA and lipid. Oxidative stress is an imbalance between generation and elimination of reactive oxidative species (ROS) or reactive nitrogen species (RNS) in favour of ROS. Oxidative stress is capable of causing cells to lose their structure, function and eventually result in cell dysfunction. The endogenous source of ROS/RNS can be generated within the airway epithelial cells in response to pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α). Exogenous sources include environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc. (Li & Trush 1994).

ROS and RNS have a variety of functions including regulation of gene expression (Jabs 1999) and induction of apoptosis (Stewart 1994). The production of ROS/RNS may have very harmful effects and is neutralised by the antioxidant defences under normal circumstances in healthy individuals. Oxidative stress occurs when there is a change in balance in favour of ROS/RNS and may occur in several circumstances, like in disease or malnutrition where there are insufficient micronutrients to meet the needs of the antioxidant defences (Zidenberg-Cherr & Keen 1991).

Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Owen, Giacosa, Hull, Haubner, Spigelhalder & Bartsch 2000). Up to so far there is only one antioxidant compound isolated from *Combretum erythrophyllum* which is 5-hydroxy-7,4'-dimethoxyflavone+ but it had the weakest activity (Martini *et al*. 2004).

2.4. Plants for oxidative stress

2.4.1. Combretaceae family

Combretaceae is a large family with at least 600 species. *Combretum* is one of the most commonly occurring genera and widely used in African traditional medicine. The various parts of *Combretum* species are widely used for treating numerous ailments and diseases (Eloff *et al*. 2008)

2.4.2. Description of *Combretum erythrophyllum*

Combretum erythrophyllum is a small to large deciduous to semi-deciduous tree, usually densely or sometimes sparsely (Van Wyk & Gericke 2000) foliated with a roundish crown and drooping branches. Showy reddish and yellow leaves in autumn are characteristic and sometimes with whitish spring colours (Van Wyk & Van Wyk 1997). The tree is single- or multi trunked, wide spreading, usually about 12m high but can reach up to 20m height. *Combretum erythrophyllum* occurs in bushveld and grasslands usually along streams and river banks. Since *Combretum erythrophyllum* is a deciduous tree leaves can be found on the tree from September to May. The leaves often turn bright red and yellow in autumn.

Fig 2.1: *Combretum erythrophyllum* (Picture by I.P Ledwaba), 21/06/2010, University of Pretoria Onderstepoort campus

Table 2.1: Antimicrobial activity of compounds isolated from *Combretum* species and their uses in traditional medicine

Combretum species	Common name	Ethnobotanical use	Biological activity	Bioactive compounds isolated
Combretum adenogonium (Steud.ex A Rich)	Four leaved bushwillow	Leaves and stem are used for arterial hypertension, hepatitis, anti-inflammatory and urinary disease		
*Combretum apiculatum (*Sond)	Red bushwillow	Roots are chewed and the sap is swallowed for the treatment of snakebite. leaves are used for abdominal disorders and stem bark for conjunctivitis	Quercetrin and Kaempferol had strong antioxidant activity with EC_{50} values of 11.81 ± 85 and 47.36 ± 0.03 µM	Flavokawain, Alpenitin and Pinocembrin (Serage 2004), Cardamomin, Pinocembrin, Quercetrin and Kaempferol (Kgatle 2007)
Combretum bracteosum (Hochst) Brandis	Hiccup-nut	Leaves are used for the treatment of backache, earache, toothache, headache, menstrual pains, fever		

Combretum caffrum (Eckl and Zeyh) Kuntze	Cape bushwillow	Bark is used as a general tonic for production of general well being; decoctions of the roots are added to bath water before bedtime to treat pains in the body.	Methanol (MeOH) extract active against gram negative bacteria, Acetone, MeOH, water extracts and water decoction of the stem bark antimicrobial against gram positive bacteria and some fungal pathogens (Masika & Afolayan 2002).	Combretastatins A-1, A-2, A-3, A-4, B-1, B-2, B-3 and B-4 from the wood (Pettit , Smith & Singh 1987; Pettit, Singh & Schmidt 1988; Pettit, Singh, Hamel, Lin, Alberts & Garcia-Kendall 1989; Pettit, Singh & Boyd 1995 & Pettit, Toki, Herald, Boyd, Hamel, Pettit & Chapuis 1999)
*Combretum collinum (*Fresen)	Kalahari bushwillow	The powdered stem bark is mixed with porridge or put in tea and used against rectal collapse. Decoctions of roots and leaves are drunk for the treatment of malaria.		Triterpenoids in the leaves (Rogers & Coombes 1999)

*Combretum erythrophyllum (*Burch) Sond	River bush willow	The roots are used to treat veneral disease. Leaves are used as a cure for coughs and stomach pains. The seeds which are poisonous are used to purge dogs of intestinal worms.	Isolated compounds had the MIC values of < 200 µg/ml against *Staphylococcus aureus (Sa)*, *Escherichia coli (Ec)*, *Enterococcus faecalis (Ef)* and *Pseudomonas aeruginosa (Pa)*. Rhamnocitrin and Rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity.	Four flavonols: Kaempferol, Rhamnocitrin, Rhamnazin, quercetin 5,3'-dimethylether, Three flavones: Apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone (Martini *et al*. 2004) Combretastatin A-1 and (-)-Combretastatin (Schwikkard, Zhou, Glass, Sharp, Mattern, Johnson & Kingston 2000)
Combretum fragrans (F.Hoffm)	Four-leaved bushwillow	Root decoctions are drunk for the treatment of leprosy; cough and syphilis. Leaf decoctions are used for cleansing chronic wounds.	Roots antibacterial against *Sa*, *Klebsiella pneumonia* and *Shigella boydii* (Chhabra, Uiso & Mshiu 1984)	Roots contain anthracene glycosides, coumarins, flavonoids, starch, steroids/Triterpenoids and tannins (Chhabra *et al*. 1984)

Combretum imberbe (Wawra)	Lead wood	Leaves and roots are used for the treatment of cough and diarrhoea. Ashes of the wood are used for toothpaste.	Five pentacyclic Triterpenoids active against *Ec* and *Sa* (Angeh, Huang, Sattler, swan, Dahse, Härtl & Eloff 2006). Pentacyclic triterpenes (Rogers & Subramony 1988); glycosides based on imberbic acid (Rogers 1988), glycosidic derivatives of hydroxyimberbic acid, imberbic acid against *Mycobacterium fortuitum* and *Sa* (Katerere, Gray, Nash & Waigh 2003).	1,3-Dihydroxy-12-oleanen-29-oic1-Hydroxy-12-olean-30-oic acid 3,30-Dihydroxyl-12-oleanen-22-one 1,3,24-Trihydroxyl-12-oleanen-29-oic acid 1,23-Dihydroxy-12-oleanen-29-oic acid—3-0-2,4-di-acetyl-1-rhamnopyranoside (Angeh, Huang, Sattler, Swan, Dahse, Härtl & Eloff 2007a)
Combretum kraussii (Hochst)	Forest bushwillow	Bark, roots and leaves are used for fever and inflammation.		

Combretum micranthum (G.don)		Leaf infusions drunk for colds, fever, colic, vomiting and gastrointestinal problems. Roots decoctions as anthelminthics and for washing wounds	Aqueous acetone extracts of leaves bactericidal against *Shigella dysenteriae*, *S. Paratyphi B* and *Klebsiella ozenai* (Karou, Dicko, Sanon, Simpore & Traore 2005)	Polyphenols with antioxidative effects from leaves (Karou *et al*. 2005)
Combretum microphyllum (klotzsch)	Flame climbing bushwillow	The extract from the plant is used to treat mentally disturbed people		
Combretum mkhuzense	Maputaland bushwillow	Decoction of the roots is used as an aphrodisiac , to treat venereal disease and also to rid patients of intestinal worms	Leaf extracts active against *Sa* and *Bacillus subtilis* (Gaidamashvili & Van Staden 2002)	Lectins from leaf extracts(Gaidamashvili & Van Staden 2002)
Combretum molle (R. Br. Ex G. Don.)	Velvet bushwillow	The root is powdered and used as a wound dressing. Decoctions of the roots are used for hookworm, stomach pains, snake bite, leprosy, fever, general	Leaf extracts active against *Ef*, MIC < 0.2 mg/ml (Pegel and Rogers 1985, Eloff 1998b and 1999). Acetone fractions of stem bark inhibit the growth of *Mycobacterium*	Antifungal mollic acid-3-β-D-glucoside from leaf extract effective against *Penicillium expansum* (Pegel & Rogers 1985) Hydrolysable tannin, punicalagin,

		body swelling and abortion.	*tubercolosis* typus humanus (ATCC 27294) (Asres, Bucar, Edelsbrunner, Kartnig, Höger, & Thiel 2001a).	gives antimycobacterial effects (Asres *et al*. 2001a)
Combretum padoides (Eng.& Diels)	Thicket bushwillow	Leaves are used for snake bites and roots for eliminating hookworms	Acetone extracts of leaves antimicrobial, MIC 0.8 mg/ml against *Ec* and *Ef* (Eloff 1999)	1α, 23β-Dihydroxy-12-oleanen-29oicacid-23β-0-α-4 acetyl rhamnopyranoside, 1, 22-dihydroxy-12-oleanen-30-oic acid, Ethylcholesta-7, 22, 25-trien-0-β-*D*-glucopyranoside (Angeh, Huang, Swan, Möllman, Sattler & Eloff 2007b)
Combretum paniculatum (Vent.)	Forest Burning-bush Combretum	The flower and the root is used for the treatment of anti-inflammatory and antitumoral deficiencies		
Combretum psidioides (Welw)	Peeling bushwillow	Roots infusion can be used to treat coughs		
Combretum vendae	Venda		MIC values of 160 µg/ml (*Sa*), 320	Apigenin (Komape 2005)

(A.E. van Wyk)	bushwillow		µg/ml (*Ef*), > 320 µg/ml (*Pa*) and 320 µg/ml (*Ec*)	
*Combretum woodii (*Duemmer)	Large-leaved forest bushwillow	Leaves and bark are used for treatment of chest complaints	Combretastatin B5 had an MIC value of 16 µg/ml against *Sa*. 125 µg/ml against *Pa* and *Ef*. Ethyl acetate fraction had an average MIC value of 50 µg/ml against (*Sa, Ec*, *Ef* and *Pa*), acetone and methylene dichloride fractions had an MIC value of 140 µg/ml. The total activity of acetone and methylene dichloride fractions were found to be 1279 and 1309 ml/g.	Combretastatin B5 (Eloff, Famakin & Katerere 2005b) Combretastatin B5 (Zishiri 2005)

Combretum zeyheri (Sond)	Large-fruit bushwillow. Raas blaar	The smoke of burnt leaves is inhaled for treatment of coughs. Colic is cured with the bitter tasting water extracts of dried milled leaves.	Acetone leaf extracts have some antibacterial potential (Eloff 1999)	

EC_{50} = half maximal effective concentration, MeOH = methanol, MIC = minimal inhibitory concentration, *Sa* = *Staphylococcus aureus*, *Ec* = *Escherichia coli*, *Ef* = *Enterococcus faecalis*, *Pa* = *Pseudomonas aeruginosa*, mg/ml = milligram per millilitres, µg/ml = microgram per millilitres, ml/g = millilitres per gram

2.5. Classification of the phytochemical constituents of biological importance from *Combretum* species

2.5.1. Phenolics

Phenolic compounds are classified as secondary metabolites rather than primary metabolites. Primary metabolites include proteins, nucleic acids, carbohydrates and lipids, and are involved in the synthesis of material essential for the growth of all organisms (Haslam 1993). Some of the phenolic compounds include alkaloids, terpenoids and flavonoids.

Alkaloids are a group of naturally occurring chemical compounds which contain basic nitrogen atoms. This group also includes some related compounds with neutral (Mc Naught & Wilkinson 1997) and even weakly acidic properties (Manske 1965). Some synthetic compounds of similar structure are attributed to alkaloids (Lewis 1998). In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulphur and more rarely other elements such as chlorine, bromine and phosphorus (Chemical encyclopaedia).

Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants and animals and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid base extraction. Alkaloids often have pharmacological effects and are used as medications, as recreational drugs or in entheogenic rituals (Rhoades 1979). Most alkaloids are weak bases, but some are amphoteric, for example theobromine and theophylline. Mostly are poorly soluble in water but readily dissolve in organic solvents, such as diethyl ether, chloroform and 1, 2-dichloroethane (Spiller Caffeine 1997).

Terpenoids are common constituents of the resins of higher plants and they are useful chemosystematic characteristics of existing plants especially conifers (Gershenzon & Dudareva 2007).The Combretaceae has yielded mainly pentacyclic triterpenoids varying from oleanoic and ursanoic acids to friedelins, cycloartanes and dammaranes. Arjunolic acid and glycosides have been isolated from *C. Molle* and *T. Arjuna* (Panzini, Pelizzoni, Verotta & Rogers 1993; Kumar & Orabhakar 1987).

Sericic acid and sericoside have been isolated from the roots of *T. sericea* (Eldeen, Elgorashi, Mulholland & Van Staden 2006). Friedelin, epifriedelin and betulinic acid from the bark of *C. imberbe* and oleanene-based pentacyclic triterpenes (imberbic acid) and its glycosides have been reported (Rogers & Subramony 1988; Rogers 1989; Angeh *et al*. 2007a). Other oleanene-type pentacyclic triterpenoids bearing 29-carboxy and 1α-hydroxy substituent have been isolated from *C. molle*, *C. edwardsii*, *C. eleagnoides*, *C. apiculatum*, *C. kraussi*, *C.padoides* and *Anogeissus leiocarpus* (Rogers & Verotta 1996; Katerere *et al*. 2003, Angeh *et al*. 2007b; Chaabi, Benayache, Benayache, N'Gom, Koné, Anton, Weniger & Lobstein *et al*. 2008). These compounds demonstrate the close chemotaxonomic relationships among the species and also between African and South American *Combretum* species (Facundo, Andrade, Silveira, Braz-Fihlo & Hufford 1993; Rogers 1995).

Cycloartane-type triterpenoids have been isolated from *C. erythrophyllum* (Rogers & Verotta 1996) and *C. quadrangulare* (Banskota, Tezuka, Kim, Tanaka, Saiki & Kadota 2000), while acidic dammarane arabinofuranosides have been reported from *C. rotundifolium* (Facundo *et al*. 1993). Co-occurance of tetracyclic and pentacyclic classes of these triterpenoids is unusual but *C. molle* contains both (Panzini *et al*. 1993) reported on the isolation of acetylated rhamnosides of 1, 3 hydroxylated pentacyclic triterpenoids from *C. imberbe* and *T. stulhmanii*. These compounds have good activity against *Mycobacterium fortuitum*. Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom (Narayana, Sripal, Chaluvadi & Krishna 2000).

Preliminary research indicates that flavonoids may modify allergens, viruses and carcinogens and so may be biological response modifiers. They exhibit several biological effects such as anti-allergic, anti-inflammatory, anti-microbial (Cushnie & Lamb 2005) and anti-cancer activities (De Sousa, Queiroz, Souza, Gurgueira, Augusto, Miranda, Peppelenbosch, Ferreira & Aoyama 2007).In preliminary studies UCLA cancer researchers have proposed that study participants who ate foods containing certain flavonoids, such as catechins found in strawberries and green and black teas; kaempferol from brussel sprouts and apples and quercetin from beans, onions and apples may have reduced risk of obtaining lung cancer (UCLA news 2008).

Flavonoids were found to be strong topoisomerase inhibitors and induce deoxyribonucleic acid (DNA) mutations in the MLL gene, which are common findings in neonatal acute leukaemia (Thirman, Gill, Burnett, Mbangkollo, McCabe & Koboyashi 1993; Strick, Strissel, Borgers, Smith & Rowley 2000). The DNA changes were increased by treatment with flavonoids in cultured blood stem cells (Barjesteh van Waalwijk van Doorn-Khosrovani, Janssen, Maas, Godschalk, Nijhuis & Van Schooten 2007). A high flavonoid content diet in mothers is suspected to increase risk particularly of acute leukaemia in neonates (Ross 1998; Ross 2000; Spector, Xie, Robinson, Heerema, Hilden & Lange 2005). Polyphenols were found to be strong topoisomerase inhibitors, similar to some chemotherapeutic anticancer drugs including etoposide and doxorubicin (Bandele, Clawson & Osheroff 2008). This property may be responsible for both an anticarcinogenic proapoptic effect and a carcinogenic, DNA damaging potential of the substances.

Chapter3

General Materials and Methods

3.0. Introduction

Acetone was used as an extracting solvent as it was found that it is not visibly toxic and it is also miscible with polar and non-polar solvents. The current chapter discusses the materials and methods used in preparing extracts of *Combretum erythrophyllum* leaves, as well as performance of antimicrobial activity and screening of the plant extracts using different methods. Determination of minimum inhibitory concentration (MIC) using microtitre plates, thin layer chromatography (TLC) fingerprints were used to detect the inhibition of bacterial and fungal growth by active compounds. Antioxidant activity was evaluated using several methods such as: the Trolox equivalent antioxidant capacity assay (TEAC assay), the total-trapping antioxidant parameter assay (TRAP assay), Ferric reducing antioxidant power (FRAP) and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. In this study 2, 2 azinobis-3-ethylbenzotiazoline-6-sulphonic acid (ABTS) assay, a DPPH free radical scavenging assay and DPPH assay were used. The inspiration for this study is to find the highest biological activity from *Combretum erythrophyllum* extracts. The extract with the highest activity will be considered for further analysis and purification to produce a pure biologically active compound.

3.1. Materials and Methods

3.1.1. Plant collection and treatment

Leaf materials of *Combretum erythrophyllum* were collected at the University of Pretoria botanical garden (Onderstepoort campus) in January 2010. The plant was identified by Prof. J. N. Eloff from University of Pretoria. Leaves were dried in the shade at room temperature for two weeks. Stems and thick veins were sorted and the dried leaves were ground to a fine powder form using a pulveriser and stored in a dry, air tight container away from moisture and direct sunlight.

3.1.2. Extraction procedure

Leaf material was extracted (1:10 w/v) using acetone (technical grade-Merck) as extractant with constant shaking on Labotec model 20.2 shaking machine for 6 hr. The supernatant was removed from the residue by filtration. This process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under vacuum using a rotary evaporator and the remaining extract was then transferred into a weight bottle. The mass of the extract obtained per 100 g of the plant material after the crude extract has dried was found to be about 10 g.

3.2. Qualitative assay

3.2.1 Phytochemical analysis

3.2.1.1. TLC fingerprint

Qualitative screening of the constituents in the leaf extracts of *Combretum erythrophyllum* for antioxidant activity was performed using thin layer chromatography (TLC) analysis. About 10µl of the crude extract and fractions was loaded on the TLC. The chromatograms were developed in the following solvent systems: Ethyl acetate/ Methanol/ Water (EMW) 10:1.35:1; Chloroform/ Ethyl acetate/ Formic acid (CEF) 10:8:2; Benzene/ ethyl acetate/ ammonia (BEA) 18:2:0.2. The developed plates were sprayed with DPPH in methanol and vanillin in methanol acidified with sulphuric acid (Kotze & Eloff 2002).

3.2.1.2. Bioautographic Methods

Bioautography was used to determine the number of active compounds in the crude and different fractions. The representative bioautograms are shown in figures below.TLC plates were loaded with about 10 µl of the extracts. The prepared plates were developed in the three different solvent systems which are: CEF, BEA and EMW. The chromatograms were dried at room temperature under a stream of air to remove the solvent, for 2 days. Cultures of bacteria were prepared overnight in Mueller Hinton (MH) broth and the plates were sprayed with a suspension of actively growing cells of gram positive and gram negative bacteria and fungi. The plates were

then incubated at a relative humidity at 37 ^{o}C overnight (18 hours) in a closed plastic container.

The plates were sprayed with 2 mg/ml of INT (*p*-Iodonitrotetrazolium violet, Sigma) solution. After spraying with INT the plates were then incubated at 100 % relative humidity at 37 ^{o}C for 1 hour. Inhibition of bacterial growth is indicated by clear zones on the chromatogram. Bacterial growth causes reduction of the colourless tetrazolium salt to a red formazan (Begue & Kline 1972). The following test organisms were used for the bioautography and MIC of the plant extracts: *Staphylococcus aureus* (gram positive) [ATTC 29213], *Enterococcus faecalis* (gram positive) [ATTC 29212], *Escherichia coli* (gram negative) [ATTC 27853], *Pseudomonas aeruginosa* (gram negative) [ATTC 25922], *Candida albicans, Cryptococcus neoformans* and *Aspergillus neoformans*.

3.3. Quantitative assays

3.3.1. Minimum Inhibitory Concentration

A concentration of 10 mg/ml of the plant extract was prepared and dissolved in acetone in a vial. It was then put in the shaker until all the extract was properly dissolved in acetone. 100 µl of distilled water was added to the 96-well microplates using a multichannel micropipette. The plant extract (100 µl) was added to the first well of the column and serially diluted by two-fold. The fresh bacterial culture was prepared from an overnight culture and diluted with fresh MH broth (1:100). The bacterial culture (100 µl) was added to the test sample in each well of the microtitre plate. The organism and the extract mixtures were incubated for 16 h at 37 ^{o}C and 40 µl of 0.2 mg/ml iodonitrotetrazolium (INT; Sigma) solution was added to each well (Eloff 1998a).

3.3.2. Evaluation of antioxidant activity

Oxidative stress can be serious especially if the individual is exposed to environmental challenges which increase the production of reactive species above their normal levels. Oxidative stress symbolise an inequity between systemic appearance of reactive oxygen species and a biological system's ability to readily

detoxify the reactive intermediates or to repair the resulting damage. In humans, oxidative stress is involved in the development of many diseases or sometimes it may intensify their symptoms. These include cancer (Halliwell 2007), atherosclerosis, heart failure (Singh, Dhalla, Seneviratne & Singal 1995) and bipolar disorder (Dean, van den Buuse, Berk, Copolov, Mavros & Bush 2011). The free radical can be evaluated using the following assays: ABTS assay, Antioxidant DPPH assay, Hydroxyl radical scavenging. Method of physiological relevance includes: DPPH, ABTS and Hydroxyl radical scavenging.

Plants (fruits, vegetables, medicinal herbs) may contain a wide variety of free radical scavenging molecules such as phenolic compounds; nitrogen compounds vitamins, terpenoids (Zheng & Wang 2001). The antioxidant effects of crude plant extract, solvent fractions and isolated compounds from *Combretum erythrophyllum* was analyzed on the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical.

3.3.2.1. Antioxidant DPPH assay

The 96 well microtitre plates were filled with 40 µl of methanol. The plant extract (40 µl) was added to all the wells followed by 160 µl of DPPH and the absorbance was measured immediately using a microplate reader at a wavelength of 516 nm. The plate reading was repeated every 5 min until 40 min (Brand Williams, Cuvelier & Berset 1995).

3.3.2.2. ABTS assay

The 96 well microtitre plates were filled with 40 µl of methanol. The plant extract (40 µl) was added to all the wells followed by 160 µl of ABTS and the absorbance was measured immediately using microplate reader at a wavelength of 734 nm. The plate reading was repeated after 6 min (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans 1999).

3.3.2.3. Hydroxyl radical scavenging assay

The 96 well microtitre plates were filled with 66µl of methanol. The plant extract (66 µl) was then added to the first well and serial dilution was then performed. $FeSO_4$, H_2O_2 and salicylic acid (120µl) were added to each well. The 96 well microtitre plates

were incubated in an oven at 37 ºC for 30 min. The absorbance was measured using microplate reader at a wavelength of 532nm (Kunchandy & Rao 1990).

3.4. Evaluation of cytotoxicity using (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay

Viable cell growth after incubation with the test compound was determined using the tetrazolium-based colorimetric assay (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT assay) described by Mosmann (1983). Cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5min and resuspended in growth medium to 5 x 10^4 cells/ml. The growth medium used was Minimal Essential Medium (MEM, Sigma) supplemented with 0.1 % gentamicin (Virbac) and 5% foetal calf serum (Sigma). A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well micro titre plate. Growth medium (200 µl) was added to wells of columns 1 and 12 to minimize the edge effect and maintain humidity. The plates were incubated for 24 h at 37 ºC in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells and replaced with 200 µl of test compound at different concentrations. The cells were disturbed as little as possible during the aspiration of the medium and addition of the test compound. Each dilution was tested in quadruplicate. The microtitre plates were incubated at 37 ºC in a 50% CO_2 incubator for 2 days with the test compound and extracts. Untreated cells and positive control (doxorubicin, Pfizer Laboratories) were included. After incubation, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37 ºC. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Versamax, Molecular Devices) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The LC_{50} values were calculated as the concentration of the test compound resulting in a 50 % reduction of absorbance compared to untreated cells (Mosmann 1983).

3.5. Bioguided isolation of antimicrobial compound

The crude extract of *Combretum erythrophyllum* was suspended in water in a 2 l separating funnel and partitioned using 300 ml of each of the following solvents: Hexane, dichloromethane, water and ethyl acetate in increasing order of polarity (Suffness & Douros 1979).

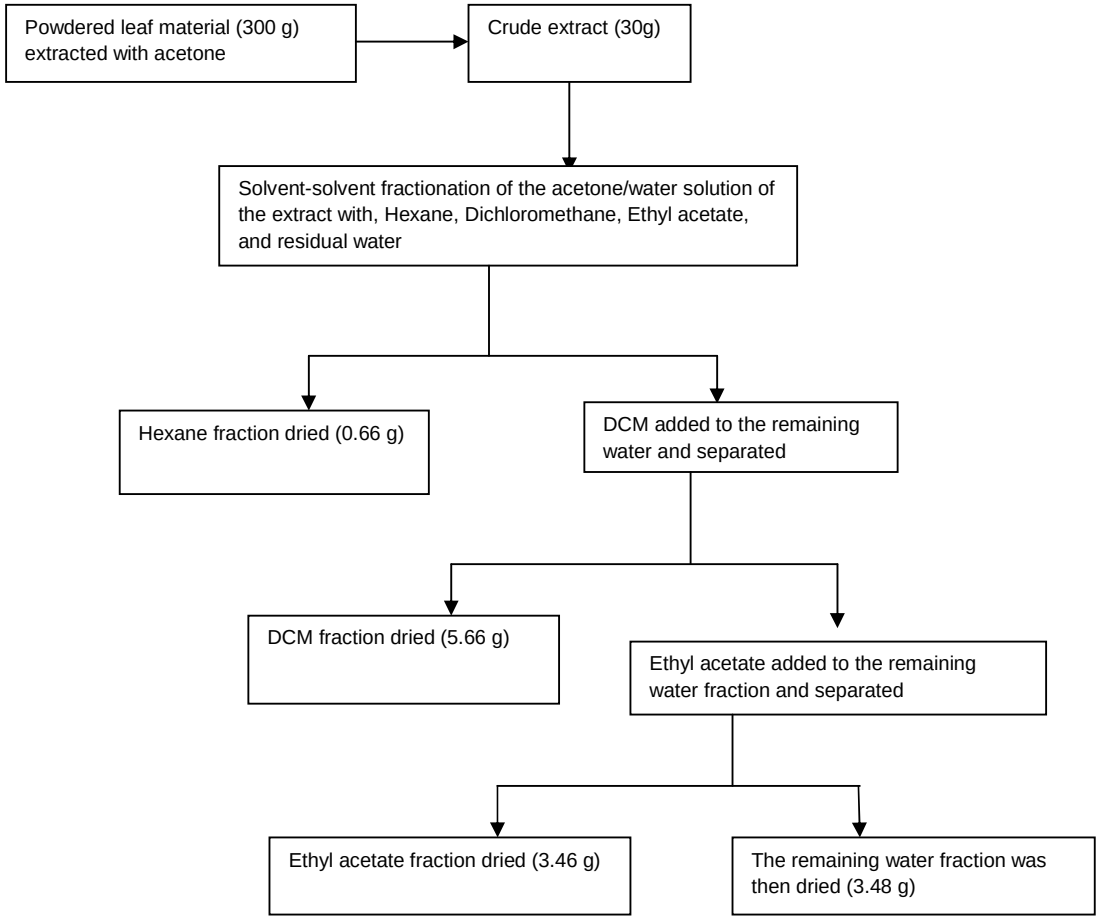


Figure 3.5.1: Protocol used for the solvent-solvent fractionation of the components in the acetone leaf extracts of *Combretum erythrophyllum.*

3.6. Column Chromatography

Crude extract (± 10 g) was used for the column with the following solvent ratios of hexane: ethyl acetate [100:0, 98:2, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 0:100]. The air dried fraction (1.7 g) of 95:5 fractions from the crude was re-dissolved in acetone. Silica gel (63 g) was used to pack the column and the dried mixture was poured on top then closed with cotton wool. Elution of the column was done using different ratios of hexane: ethyl acetate. 1000ml of the following ratios [100:1, 100:2, 100:3, 100:4, 100: 5 and 100:10] were used to elute the column. The eluants were collected using test tubes. TLC analysis was performed on all test tubes used for collection. Test tubes which showed single bands and the same R_f values were pooled together and the solvent was evaporated. This gave a clear glassy compound of about 30 mg. The pure compound was then sent for Nuclear magnetic resonance (NMR) analysis.

3.7. Spectroscopic analysis of isolated compound

Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) was performed at the North West University (Potchefstroom Campus) using Avance III Bruker 600MHZ, ultra shield plus with the software Topspin 2.1 PL 6. NMR system was executed with the suitable deuterated solvents to obtain the spectroscopic data and elucidate the structures of the isolated compounds.

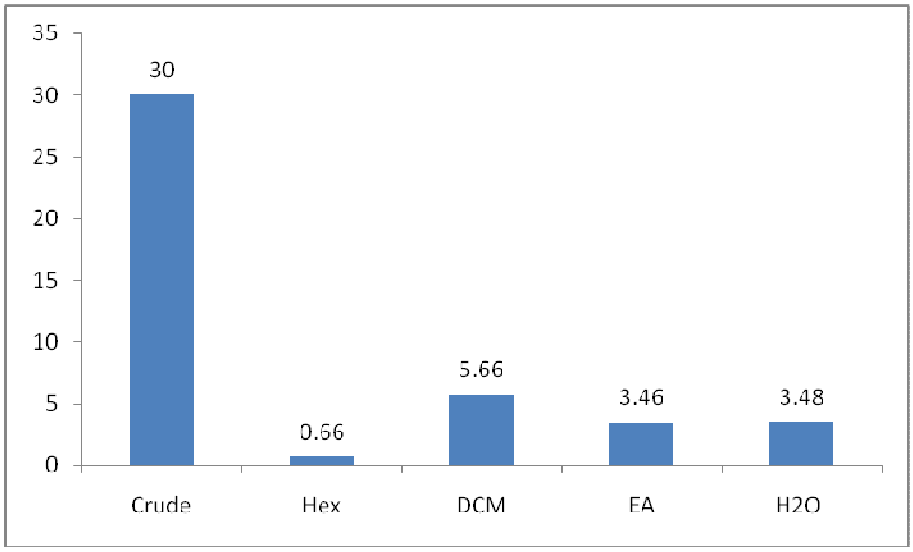
3.8. Mass spectrometry (MS)

Mass spectra of each of the isolated compounds were obtained using LC-MS at Stellenbosch University CAF. LC-MS data was obtained using Waters Synapt G2 instrument. The MS was running in an electrospray positive mode with capillary voltage 3kV, cone voltage 15 V.

Chapter 4

Results

4.1. Yield



g = grams, Hex = Hexane, DCM = Dichloromethane, EA = ethyl acetate, H_2O = Water.

Figure 4.1.1: Quantity extracted (g) from crude extract using liquid-liquid fractionation

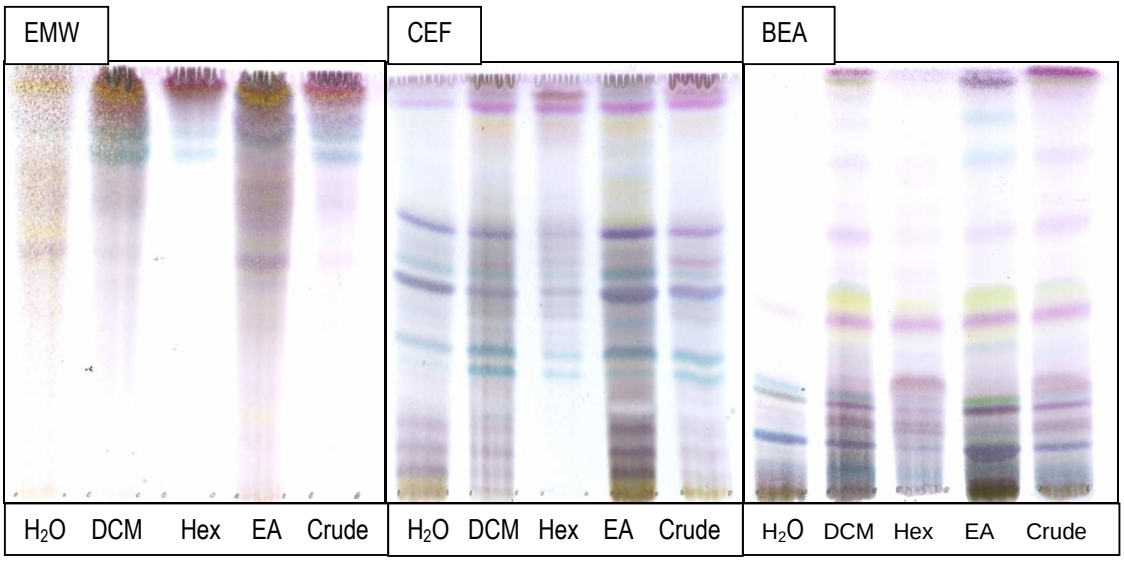
This graph indicates the amounts extracted from the leaves of *Combretum erythrophyllum* and according to this bar graph dichloromethane extracted the highest quantity of material from the crude extract and hexane extracted the lowest quantity.

4.2. Qualitative analysis

To determine the most active compounds from *Combretum erythrophyllum* leaves, TLC chromatograms were developed in different solvent systems and sprayed with Vanillin and DPPH. The figures of the chromatograms are displayed below (Figure 4.1.2 and 4.1.5). Retention factor (Rf) was calculated to compare if there is any similarities in compounds that were separated in the solvent system that was used, the retention factor was calculated as follows:

Rf = Distance moved by analyte / Distance moved by solvent front

4.2.1. Phytochemical Chromatogram



EMW = ethyl acetate, methanol, water, CEF = chloroform, ethyl acetate, formic acid, BEA = benzene, ethyl acetate, ammonia, EA = ethyl acetate, Hex = hexane, DCM = dichloromethane, H_2O = Water.

Figure 4.1.2: Phytochemical profile of crude extract and fractions separated with EMW, CEF and BEA (sprayed with vanillin)

Table 4.1.1: Rf values for plates sprayed with vanillin

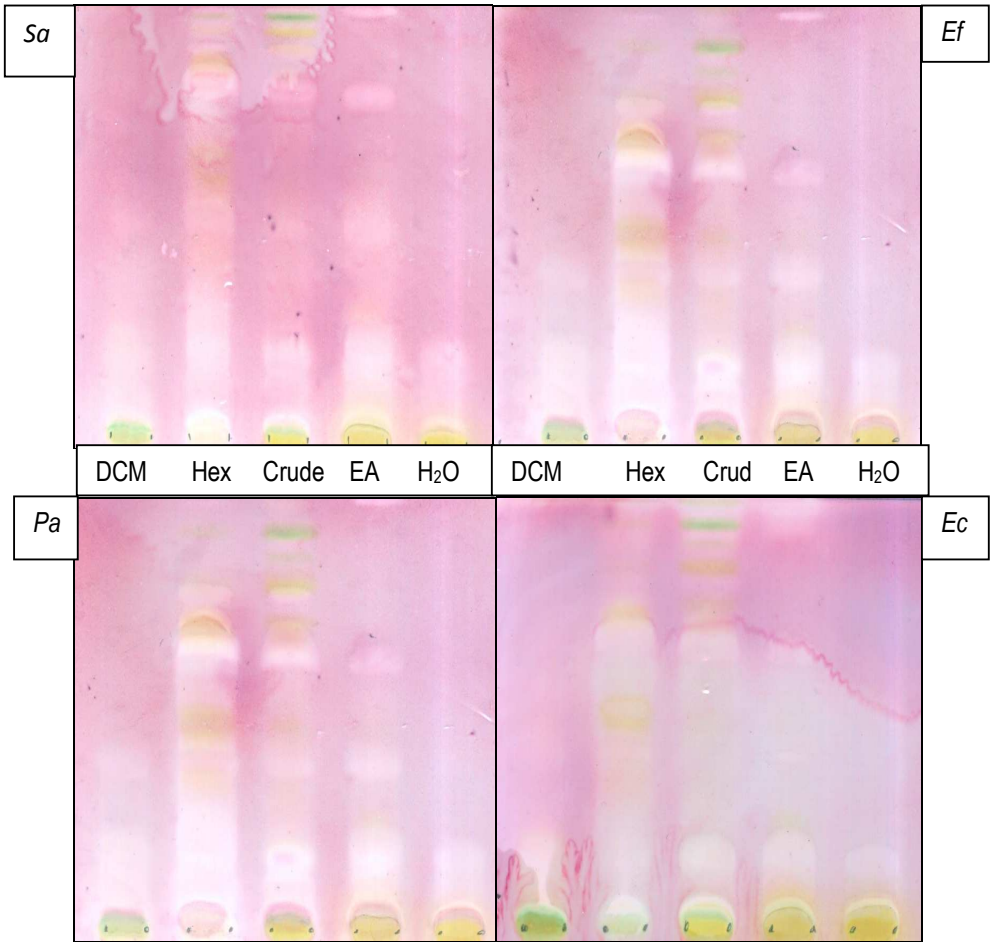
	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10	Band 11	Band 12
EMW												
H_2O	0.53	0.63	0	0	0	0	0	0	0	0	0	0
DCM	0.58	0.75	0.85	0.95	0	0	0	0	0	0	0	0
Hex	0.83	0.88	0.98	0	0	0	0	0	0	0	0	0
EA	0.27	0.53	0.58	0.75	0.87	0.93	0	0	0	0	0	0
Crude	0.58	0.72	0.82	0.87	0.97	0	0	0	0	0	0	0
CEF												
H_2O	0.08	0.13	0.20	0.32	0.38	0.45	0.52	0.55	0.65	0.96	0	0
DCM	0.08	0.13	0.20	0.32	0.37	0.50	0.53	0.62	0.90	0.95	0	0
Hex	0.30	0.35	0.50	0.63	0.92	0.97	0	0	0	0	0	0
EA	0.08	0.67	0.20	0.30	0.37	0.42	0.50	0.55	0.63	0.68	0.90	0.95
Crude	0.08	0.67	0.20	0.30	0.35	0.50	0.52	0.58	0.63	0.90	0.95	0
BEA												
H_2O	0.70	0.17	0.25	0.28	0.45	0	0	0	0	0	0	0
DCM	0.08	0.13	0.20	0.23	0.27	0.45	0.50	0.63	0.82	0.90	0.95	1
Hex	0.08	0.12	0.20	0.28	0.43	0.48	0.55	1	0	0	0	0
EA	0.05	0.13	0.22	0.25	0.47	0.50	0.67	0.83	0.97	1	0	0
Crude	0.08	0.15	0.20	0.23	0.27	0.32	0.47	0.50	0.68	0.85	0.95	1

Rf = retention factor, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate, H_2O = water, CEF = chloroform, ethyl acetate, formic acid, EMW = ethyl acetate, methanol, water, BEA = benzene, ethyl acetate, ammonia.

To screen the compounds present in the leaf material, the chromatogram was sprayed with vanillin to show compounds separated by CEF, BEA and EMW from Water (H_2O), dichloromethane (DCM), hexane (Hex), ethyl acetate (EA) fractions and crude extract. Observation of the TLC plates indicated that the different extract and fractions separated different compounds. Most compounds were sensitive against vanillin in all the solvents. There were also similarities elucidated in the chemical composition extracts this was observed by the same Rf values. Some Rf values were the same which means that the compound appearing on the TLC plate might be the same, but this can only be proven by isolation. Most compounds were visible in CEF and BEA. There are similarities in compounds appearing in CEF and BEA. EMW has a poor resolution against vanillin and only a few compounds were visible in the TLC plate developed in EMW.

4.3. Antimicrobial assays

4.3.1. Microbial bioautograms



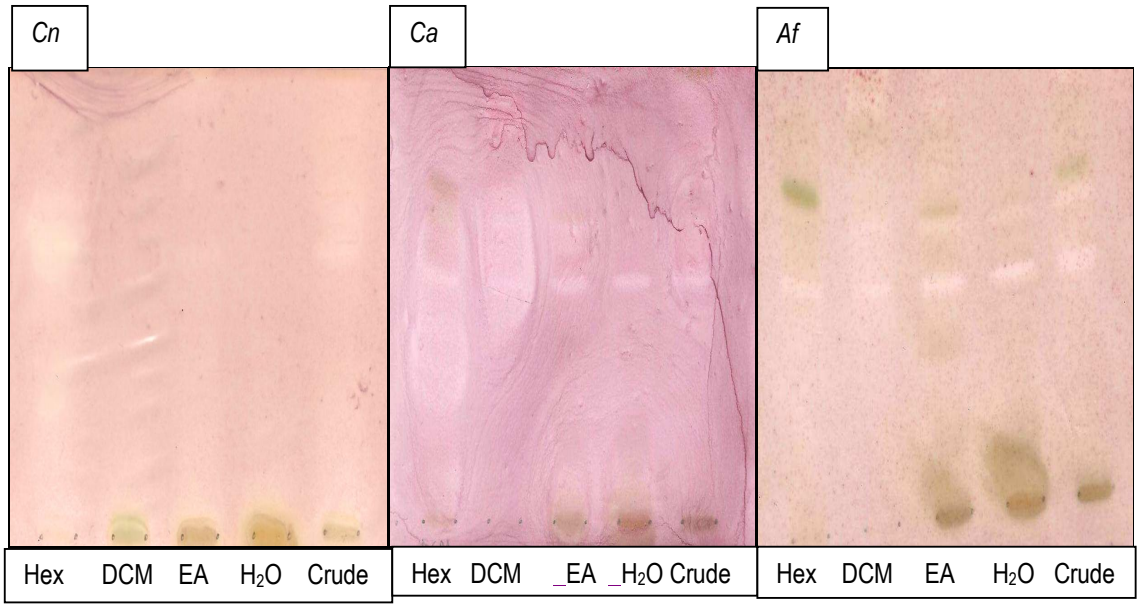
H = hexane, EA = ethyl acetate, *Sa* = *staphylococcus aureus*, *Ef* = *Enterococcus faecalis*, *Pa* = *pseudomonas aeruginosa*, *Ec* = *Escherichia coli,* hex = hexane, DCM = dichloromethane, H_2O = water.

Figure 4.1.3: Bioautography of crude extract and fractions developed in H: EA (60:40) and sprayed with *Sa, Ef, Pa* and *Ec*.

Table 4.1.2: Rf values for bacterial plates

	Band 1	Band 2	Band 3
Sa			
DCM	0.21	0	0
Hex	0.26	0	0
Crude	0.24	0	0
EA	0.26	0	0
H_2O	0.55	0	0
Ef			
DCM	0.13	0	0
Hex	0.19	0.31	0.66
Crude	0.21	0.66	0
EA	0.19	0.98	0
H_2O	0.21	0	0
Pa			
DCM	0.27	0	0
Hex	0.31	0.69	0
Crude	0.23	0.66	0
EA	0.21	0	0
H_2O	0.21	0	0
EC			
DCM	0.21	0	0
Hex	0.71	0	0
Crude	0.69	0.97	0
EA	0.98	0	0
H_2O	0	0	0

Rf = retention factor, DCM = dichloromethane, hex = hexane, EA = ethyl acetate, H_2O = water, *Ef* = *Enterococcus faecalis*, *Sa* = *Staphylococcus aureus*, *Pa* = *Pseudomonas aeruginosa*, *Ec* = *Escherichia coli.*



H = hexane, EA = ethyl acetate, *Cn* = *Cryptococcus neoformans*, *Ca* = *Candida albicans*, *Af* = *Aspergillus fumigatus*, Hex = hexane, DCM = dichloromethane, H_2O = water.

Figure 4.1.4: Bioautography of crude extract and fractions developed in H: EA (60:40) and sprayed with *Cn, Ca* and *Af.*

Table 4.1.3: Rf values for fungal plates

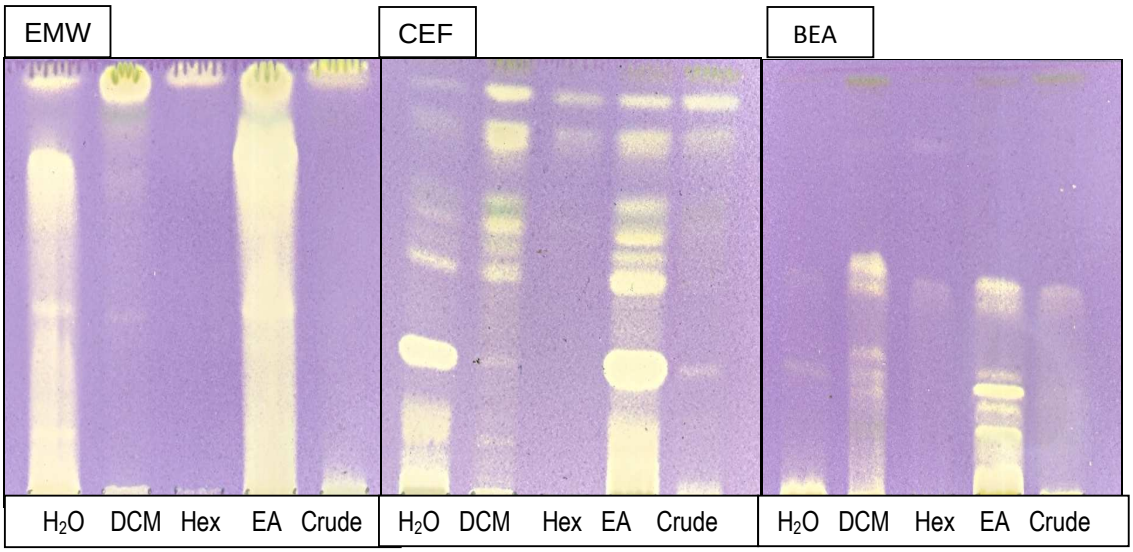
	Band 1	Band 2
Cn		
Hex	0.69	0
DCM	0	0
EA	0	0
H_2O	0	0
Crude	0.66	0
Ca		
Hex	0.59	0
DCM	0.59	0.74
EA	0.56	0
H_2O	0.56	0
Crude	0.59	0
Af		
Hex	0.56	0
DCM	0.53	0
EA	0.57	0.69
H_2O	0.56	0.71
Crude	0.76	0

Rf = retention factor, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate, H_2O= water, *Cn* = *Cryptococcus neoformans*, *Ca* = *Candida albicans*, *Af* = *Aspergillus fumigatus.*

The bioautography assay was used to determine the antibacterial and antifungal activity of compounds present in the plant extracts. The clear zones on the chromatogram indicate the inhibition of growth by the plant extract. Hexane fraction has the highest number of antibacterial compounds in all organisms followed by crude extract then ethyl acetate fraction. Water and DCM fractions have the poor activity in all organisms. Only a few compounds in the crude extract and other fractions inhibited the growth of antifungal microorganisms, in some cases it was found that the compound responsible for the activity is the same due to similar Rf values. The results obtained in this assay showed that the leaf extracts of *C. erythrophyllum* possess good antibacterial activity.

4.4 Antioxidant screening

4.4.1 TLC-DPPH chromatogram



EMW = ethyl acetate, methanol, water, CEF = chloroform, ethyl acetate, formic acid, BEA = benzene, ethyl acetate, ammonia, TLC = thin layer chromatography, DPPH = 2, 2-diphenyl-1-picrylhydrazyl, H_2O = water, DCM = dichloromethane, hex = hexane, EA = ethyl acetate.

Figure 4.1.5: TLC-DPPH assay of crude extract and fractions separated with EMW, CEF and BEA

Table 4.1.4: Rf values for plates sprayed with DPPH

	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8
EMW								
H_2O	0.37	0.44	0.77	0.95	0	0	0	
DCM	0.03	0.95	0	0	0	0	0	
Hex	1	0	0	0	0	0	0	
EA	0.84	0.98	0	0	0	0	0	
Crude	0.05	1	0	0	0	0	0	
CEF								
H_2O	0.08	0.21	0.35	0.55	0.63	0.87	0	0
DCM	0.11	0.29	0.52	0.63	0.85	0.94	0	0
Hex	0.84	0.92	0	0	0	0	0	0
EA	0.19	0.34	0.52	0.53	0.56	0.68	0.84	0.90
Crude	0.05	0.29	0.92	0	0	0	0	0
BEA								
H_2O	0.05	0.29	0	0	0	0	0	0
DCM	0.08	0.24	0.34	0.47	0.56	0	0	0
Hex	0.48	0	0	0	0	0	0	0
EA	0.15	0.19	0.24	0.27	0.44	0.48	0	0
Crude	0.48	0	0	0	0	0	0	0

Rf = retention factor, DPPH = 2, 2-diphenyl-1-picrylhydrazyl, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate, H_2O = water, CEF = chloroform, ethyl acetate, formic acid, EMW = ethyl acetate, methanol, water, BEA = benzene, ethyl acetate, ammonia.

Antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. The presence of antioxidant compounds was indicated by the yellow zones on the chromatograms which showed radical scavenger capacity against the purple background. The TLC chromatogram displayed that the water and ethyl acetate fractions in the CEF solvent system have sufficient antioxidant activity. The other fractions also have antioxidant activity but they have a lower activity, the same thing happens with the crude extract; the reason for low activity could be that the antioxidant present were not active in the solvent system used. Ethyl acetate fraction had the highest antioxidant activity in all the solvent systems. In general the ethyl acetate and water fraction had the highest antioxidant activity.

4.4.2. Quantitative analysis

4.4.2.1. MIC

Table 4.1.5: MIC values (mg/ml) of fractions and crude extract for bacterial assay

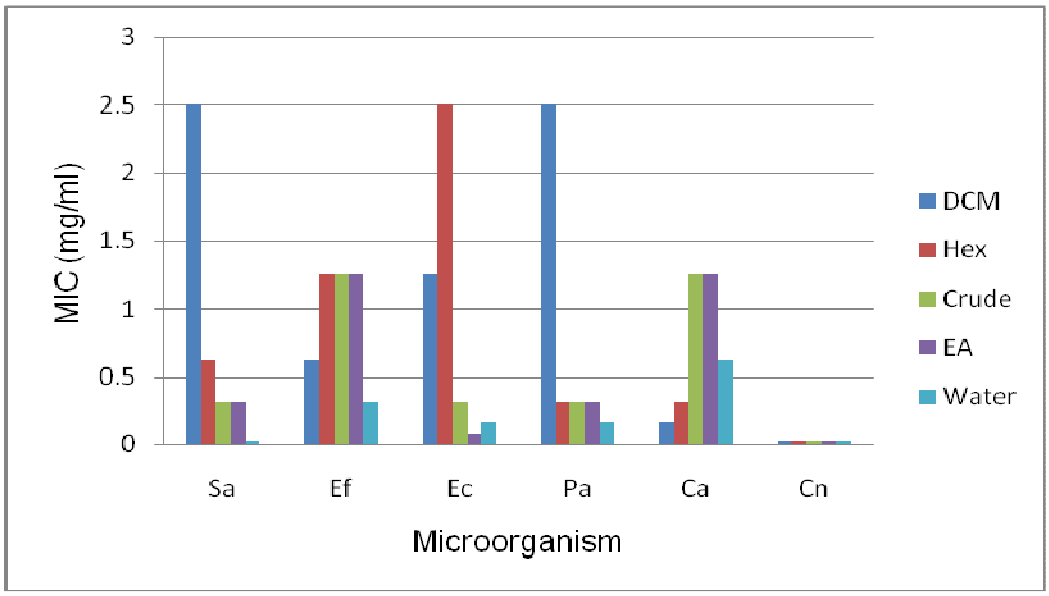
	DCM	Hex	Crude	EA	H_2O	H_2O control	Gent	Neg Con (Acet)
Sa	2.5	0.62	0.32	0.32	0.02	>2.5	0.04	>2.5
Ef	0.62	1.25	1.25	1.25	0.32	>2.5	0.04	>2.5
Ec	1.25	2.5	0.32	0.08	0.16	>2.5	0.32	>2.5
Pa	2.5	0.32	0.32	0.32	0.16	>2.5	0.04	>2.5

MIC = minimum inhibitory concentration, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate, H_2O = water, Gent = gentamicin, Neg = negative, Con = control, *Sa* = *Staphylococcus aureus*, *Ef* = *Enterococcus faecalis*, *Ec* = *Escherichia coli*, *Pa* = *Pseudomonas aeruginosa*.

Table 4.1.6: MIC values (mg/ml) of fractions and crude extract for fungal assay

	DCM	Hex	Crude	EA	H_2O	H_2O con	Acet Con	Amphotericin B
Ca	0.16	0.32	1.25	1.25	0.62	>2.5	>2.5	>2.5
Cn	0.02	0.02	0.02	0.02	0.02	>2.5	>2.5	>2.5
Af	0.16	0.08	0.08	0.08	0.08	>2.5	>2.5	>2.5

MIC = minimum inhibitory concentration, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate, H_2O = water, Gent = gentamicin, Neg = negative, Con = control, *Ca* = *Candida albicans*, *Cn* = *Cryptococcus neoformans*, *Af* = *Aspergillus fumigatus*.



MIC = minimum inhibitory concentration, *Sa* = *staphylococcus aureus*, *Ef* = *Enterococcus faecalis*, *Ec* = *Escherichia coli*, *Pa* = *Pseudomonas aeruginosa*, *Ca* = *Candida albicans*, *Cn* = *Cryptococcus neoformans*, *Af* = *Aspergillus fumigatus*, DCM = dichloromethane, Hex = hexane, EA = ethyl acetate

Figure 4.1.6: MIC values against pathogenic microorganisms

MIC values are presented in table 4.1.5, 4.1.6 and are demonstrated in figure 4.1.6. Plant extracts with low MIC values could be a good source of bioactive compounds with antimicrobial strength. Acetone fraction has the highest activity against *Sa* (0.02 mg/ml), *Ef* (0.32 mg/ml) and *Pa* (0.16 mg/ml). Ethyl acetate fraction has the highest activity against *Ec* (0.08 mg/ml). Based on the MIC results water fraction and ethyl acetate fraction were the best extractants for MIC. The fractions with high MIC values do not mean that the plant is not active against a certain organism; it could be that the fraction contains a large number of compounds and they restrain each other's biological activity while the active compound is present in low concentration. Figure 4.1.6 gives clear indication that *Combretum erythrophyllum* has good antifungal activity.

4.4.2.2. Total activity

Total activity of an extract or fraction gives an indication of the efficacy at which active constituents present in one gram can be diluted and still inhibits the growth of the test organism. This value is calculated in relation to the MIC value of the extract. (Eloff 2004). In this study the total activity of extracts was calculated as follows:

Total activity (ml) = Amount extracted from 1 (mg) / MIC (mg/ml)

Table 4.1.7: Total activity of fractions and crude extract, amount extracted and their MIC values

Amounts extracted in mg							
Crude	0.3						
DCM	0.0566						
Hex	0.0066						
Ea	0.0346						
water	0.0348						
MIC values µg/ml							
	Sa	*Ef*	*Ec*	*Pa*	*Ca*	*Cn*	*Af*
Crude	0.32	0.32	1.25	0.62	1.25	0.32	0.08
DCM	0.32	0.32	0.63	0.32	0.16	0.08	0.16
Hex	0.62	0.32	0.32	0.32	0.32	0.16	0.08
Ea	0.32	0.32	0.16	0.32	0.62	0.08	0.08
H_2O	0.16	0.16	0.32	0.32	0.32	0.08	0.08
Average	0.348	0.288	0.536	0.38	0.534	0.144	0.096
Calculated total activity							
	Sa	*Ef*	*Ec*	*Pa*	*Ca*	*Cn*	*Af*
Crude	0.9375	0.9375	0.2400	0.4839	0.2400	0.9375	3.7500
DCM	0.1769	0.1769	0.0898	0.1769	0.3538	0.7075	0.3538
Hex	0.0106	0.0206	0.0206	0.0206	0.0206	0.0413	0.0825
Ea	0.1081	0.1081	0.2163	0.1081	0.0558	0.4325	0.4325
H_2O	0.2175	0.2175	0.1088	0.1088	0.1088	0.4350	0.4350
Average	0.2901	0.2921	0.1351	0.1797	0.1558	0.5108	1.0108

Mg = milligram, DCM = dichloromethane, hex = hexane, Ea = ethyl acetate, H_2O = water, *Sa* = *staphylococcus aureus*, *Ef* = *Enterococcus faecalis*, *Ec* = *Escherichia coli*, *Pa* = *Pseudomonas aeruginosa*, *Ca* = *Candida albicans*, *Cn* = *Cryptococcus neoformans*, *Af* = *Aspergillus fumigatus*.

To determine which extracts were the most efficient as sources of antibacterial compounds the total activity of the extracts were calculated. The value of the total activity indicates the volume to which the biologically active compounds present in one milligram of dried plant extract can be diluted and still kill the bacteria.

The total activity of all fractions and crude with the different bacterial and fungal strains are listed in table 4.1.7 above. For the bacterial strains the highest average total activity was found in *Ef* (0.2921) and the lowest was found in *Ec* (0.1351). For the fungal strains the highest average total activity was found in *Af* (1.0108) and the lowest was found in *Ca* (0.1558).

4.5. Antioxidant activity

4.5.1. DPPH assay

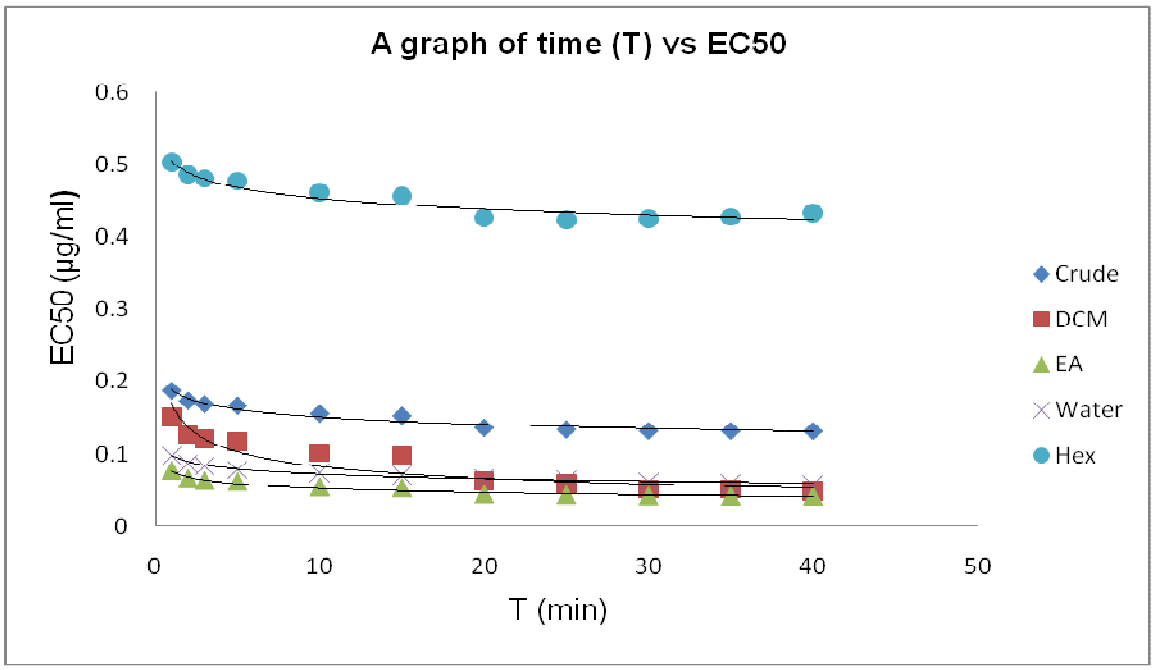
Table 4.1.8: Effective concentration (EC_{50}) of crude extracts and fractions (DPPH assay)

	Crude	Hex	DCM	EA	Water
T (min)	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}
1	0.1879	0.5028	0.1516	0.07755	0.09865
2	0.1737	0.4855	0.1269	0.06644	0.08754
3	0.169	0.4802	0.1211	0.06338	0.08413
5	0.1669	0.4768	0.1175	0.06199	0.07898
10	0.1558	0.4611	0.1017	0.05536	0.04262
15	0.1526	0.4566	0.09741	0.05389	0.07196
20	0.1361	0.4265	0.06289	0.04536	0.06725
25	0.1337	0.4228	0.05896	0.04406	0.06486
30	0.1318	0.4251	0.05205	0.04312	0.06246
35	0.1318	0.4273	0.05155	0.04271	0.05964
40	0.1314	0.4328	0.0489	0.04272	0.05814

T = time, min = minute, Hex = hexane, DCM = dichloromethane, EA = ethyl acetate.

DPPH assay is technically simple, rapid and needs only a UV-Vis spectrophotometer that might explain its widespread use in antioxidant screening. Analyses of a large number of samples could be made by using 96 well microtitre plates and results can be obtained within a short turnaround time. In this assay the radical scavenging activity was expressed as the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}). The EC_{50} is the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%.The reducing ability of antioxidants towards DPPH was evaluated by monitoring the absorbance decrease at 516 nm until the absorbance remained stable in the media.

A graphical representation of table4.1.8 is presented below in figure 4.1.7. During the course of a chemical reaction the concentrations of all samples change with time. With an increase in time the concentration of the DPPH decreases up to a point where by the reaction has reached equilibrium. According to this analysis the ethyl acetate fraction had a higher antioxidant activity with an EC_{50} 0.04272 µg/ml.



T = time, min = minutes, DCM = dichloromethane, EA = ethyl acetate, H_2O = water.

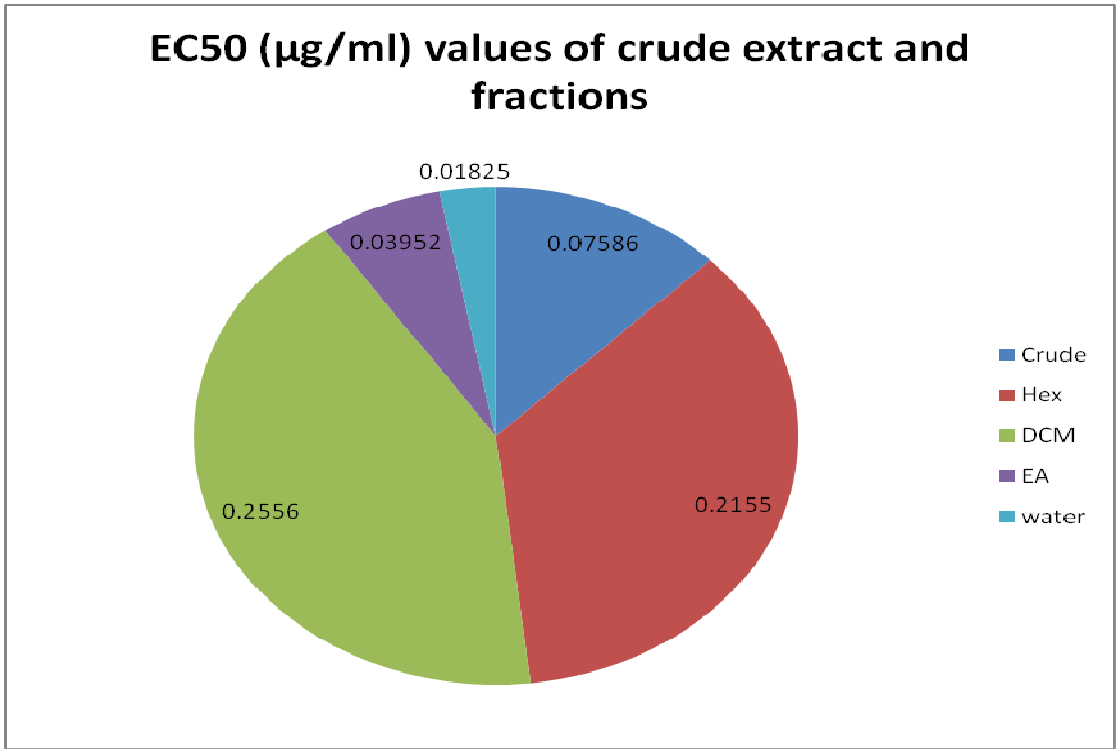
Figure 4.1.7: A graph of effective concentration (EC_{50}) of crude extract and fractions vs. Time

4.5.2. ABTS assay

Table 4.1.9: Effective concentration (EC_{50} µg/ml) of crude extract and fractions (ABTS assay)

	EC50 (µg/ml)
Crude	0.07586
Hex	0.2155
DCM	0.2556
EA	0.03952
water	0.01825

Hex = hexane, DCM = dichloromethane, EA = ethyl acetate



Hex = hexane, DCM = dichoromethane, EA = ethyl acetate

Figure 4.1.8: EC_{50} values of the crude extract and fractions (ABTS assay)

The graph pad prism was used to calculate the EC_{50} values of the crude and fractions. Antioxidant activity of plant extracts is of great importance, due to the ability of antioxidant to scavenge free radicals whereby cells in the human body could be protected against oxidative damage. The ABTS assay results showed that water fraction had the highest antioxidant activity. This proves that *C. erythrophyllum* leaf extracts have excellent antioxidant activity implying high polarity of active compounds. Some antioxidants have been isolated from *Combretum* species (Kgatle 2007).

4.5.3. Hydroxyl radical scavenging activities of the crude leaf extract and fractions of *C. erythrophyllum*

Table 4.1.10: Percentage hydroxyl radical scavenging of the crude extract and fractions

Concentration (mg/ml)	Crude	Hexane	DCM	Ethyl acetate	water
0.250	77.62±1.41	85.08±1.92	91.36±2.81	91.87±2.89	90.43±6.76
0.125	66.32±1.78	68.02±13.48	85.01±7.27	72.62±6.42	68.31±1.56
0.062	61.12±4.00	71.40±0.38	81.94±5.91	64.50±4.35	62.65±0.52
0.031	57.53±2.46	57.66±2.74	56.65±10.76	62.14±5.30	62.00±5.63
0.015	55.00±2.34	46.12±5.27	37.03±1.49	57.78±2.57	57.60±1.51
0.007	50.96±3.11	29.32±7.74	27.52±6.97	52.17±2.13	55.19±1.87
0.003	47.21±3.20	25.44±22.73	19.10±5.94	47.03±4.29	50.00±2.68

The results presented in Table 4.1.9 were the percentage hydroxyl radical scavenging with seven concentrations of the *C. erythrophyllum* leaf extract and fractions. These indicated that the extract and fractions may be a powerful hydroxyl radical scavenger. Indeed the crude extract was able to scavenge 47.21±3.20 %, water fraction 50.00±2.68% and ethyl acetate fraction 47.03±4.29% at a concentration as low as 0.003 mg/ml. At higher concentration, the scavenging of hydroxyl radical of the crude extract was more marked and reached 77.62±1.41 % at 0.25 mg/ml.

4.6. Cytotoxicity Assay

Table 4.1.11: Cytotoxicity average values and standard deviation values of Crude extract and fractions

Extracts	Average LC_{50} (mg/ml)	SD
Positive control (Doxorubicin)	7.1855 (µg/ml)	0.9659
Crude extract	34.8	0.0253
Hexane fraction	Nd	Nd
Dichloromethane fraction	36.6	0.0111
Ethyl acetate fraction	94.7	0.0010
water fraction	223.1	0.0334

Nd = not determined, SD = standard deviation, LC_{50} = lethal concentration 50, MTT= 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide.

The use of effective and safe plant extracts by traditional healers need to be encouraged. Toxicity testing is very important as some plants' extracts may be toxic, therefore safety testing is required (McGaw 2007). In this study toxicity testing of the crude extract and fractions showed no sign of toxic towards the Vero cells used. The toxicity of the isolated compound was not performed due to insufficient material.

Table 4.1.12: Calculated relative safety margin (RSM) using LC_{50} values from the MTT Cytotoxicity assay and MIC values

Microorganism	MIC (mg/ml)				
	Crude	Hexane fraction	Dichloromethane fraction	Ethyl acetate fraction	water fraction
Sa	0.32	0.32	0.62	0.32	0.16
Ef	0.32	0.32	0.32	0.32	0.16
Ec	1.25	0.63	0.32	0.16	0.32
Pa	0.62	0.32	0.32	0.32	0.32
Ca	1.25	0.16	0.32	0.62	0.32
Cn	0.32	0.08	0.16	0.08	0.08
Af	0.08	0.16	0.08	0.08	0.08
MTT assay LC_{50}(mg/ml)					
	34.8	Nd	36.6	94.7	223.1
LC_{50}/MIC					
Sa	108.75	Nd	59.03	295.94	1394.38
Ef	108.75	Nd	114.38	295.94	1394.38
Ec	27.84	Nd	114.38	591.88	697.19
Pa	56.13	Nd	114.38	295.94	697.19
Ca	27.84	Nd	114.38	152.74	697.19
Cn	108.75	Nd	228.75	1183.75	2788.75
Af	435.00	Nd	457.5	1183.75	2788.75

LC50 =, MTT = 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, MIC = minimum inhibitory concentration, Nd = not determined, *Sa* = *Staphylococcus aureus*, *Ef* = *Enterococcus faecalis*, *Ec* = *Escherichia coli*, *Pa* = *Pseudomonas aeruginosa*, *Ca* = *Candida albicans*, *Cn* = *Cryptococcus neoformans*, *Af* = *Aspergillus fumigatus*.

RSM = LC_{50}/MIC

The relative safety margin (RSM) indicates the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract. It is calculated using LC_{50} and the MIC values (LC_{50}/MIC). The extracts were found not to be toxic since their relative safety margin was large. This permits for the large quantities of the extract to be incorporated into treatment before it could cause any toxic reactions. Water fraction and Ethyl acetate fraction were found to be having the highest RSM values therefore a large quantity of material can be used in treatment of the test pathogen.

Chapter 5

Structural elucidation

5.1. Nuclear Magnetic Resonance (NMR)

NMR is a research technique that uses the magnetic properties of certain nuclei to determine physical and chemical properties of atoms or the molecules in which they are contained. NMR is used to provide detailed information about the structure, dynamics, reaction state and chemical environment of molecules. It can also be used to scrutinize the properties of organic molecules and it is also applicable to any kind of sample that contains nuclei possessing spin. Suitable samples for analysis range from small compounds analyzed with 1-dimensional protons (Hoffman 2003).

5.2. Mass Spectrometry (MS)

Mass spectrometer is an analytical technique that determines the mass to charge ratio of charged particles. It is used for determining masses of particles, elemental composition of a sample or molecule and for elucidating the chemical structures of molecules. The principle consists of ionizing chemical compounds to generate charged molecules and measuring their mass to charge ratios. The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule and determining the structure of a compound by observing its fragmentation (Sparkman 2000).

5.3. Distortionless enhancement by polarization transfer (DEPT)

DEPT spectroscopy determines the number of hydrogen atoms attached to a carbon, whether the ^{13}C resonance will appear as a positive or a negative peak. DEPT spectroscopy differentiates CH_3 groups from CH_2 groups from CH groups from carbons that have no hydrogens attached (Doddrell, Pegg & Bendall 1982).

5.4. Heteronuclear single-quantum correlation spectroscopy (HSQC)

HSQC is a 2D proton-detected heteronuclear shift correlation experiment which provides the same information as the closely related Heteronuclear multiple quantum correlation (HMQC) that is one-bond H-X correlations. The main advantage of HSQC is that it has slightly better resolution that can be obtained in the X-dimension where the resonances are broadened by homonuclear proton couplings in the HMQC but not in the HSQC (Cavanagh, Frame and Lennox 2006).

5.5. Heteronuclear multiple quantum correlation (HMQC)

HMQC is a 2D experiment used to correlate directly bonded carbon-proton nuclei. Utilizes proton detection and has very high sensitivity. The correlation can be used to map known protons' assignments onto their directly attached carbons. The 2D spectrum can also prove useful in the assignment of the proton spectrum itself by dispersing the proton resonances along the ^{13}C dimension and so reducing proton multiplet overlap. It also provides a convenient way of identifying diastereotopic germinal protons since these will produce two correlations to the same carbon (Gan 2006).

5.6. Heteronuclear multiple bond correlation (HMBC)

HMBC identifies proton nuclei with carbon nuclei that are separated by more than one bond. The pulse sequence utilizes zero and double quantum coherence between J-coupled protons and carbons to label each proton with the frequency of a remote carbon in the F1 dimension of a two dimensional experiment. HMBC detects heteronuclear correlations over longer ranges of about 2 - 4 bonds. HMBC gives correlation between carbons and protons that are separated by two, three and sometimes in conjugated systems, four bonds (Loss, Kühn & Bruker Biospin 2005).

5.7. Correlation Spectrometry (COSY)

Correlation spectroscopy relies on J-coupling or bond coupling to correlate protons that are directly coupled. COSY is one of several types of two-dimensional nuclear magnetic resonance or 2 dimensional (2D)-NMR.

2D-NMR spectra provide more information about the molecule than a one dimensional NMR spectrum and are especially useful in determining the structure of the molecule, particularly for molecules that are too complicated to work using one-dimensional NMR (Martin & Zekter 1988).

5.8. NMR Sample preparation

The samples were collected from column chromatography, dried and weighed. The deuterated solvents NMR (Merck) were used to dissolve the samples and they were passed through Pasteur pipettes plugged with cotton wool to assist with the removal of impurities. The samples were pipetted into NMR tubes with the aid of Pasteur pipettes and the analysis was performed using Avance III Bruker 600MHZ, ultra shield plus with the software Topspin 2.1 PL 6. The samples were then dried and sent for LC-MS at Stellenbosch University CAF. LC-MS data was obtained using a Waters Synapt G2 instrument. The analyte was eluted on a Waters BEH C18, 2.1x50mm column using 0.1% formic acid to acetonitrile gradient using a Waters UPLC at flow rate of 0.4ml/min for 20 min. The MS was running in electrospray positive mode with capillary voltage 3 kV and cone voltage 15 V. Leucineenkaphelin was used for lock mass calibration. Structures were elucidated by Dr Ahmed Aroke and Dr David Katerere.

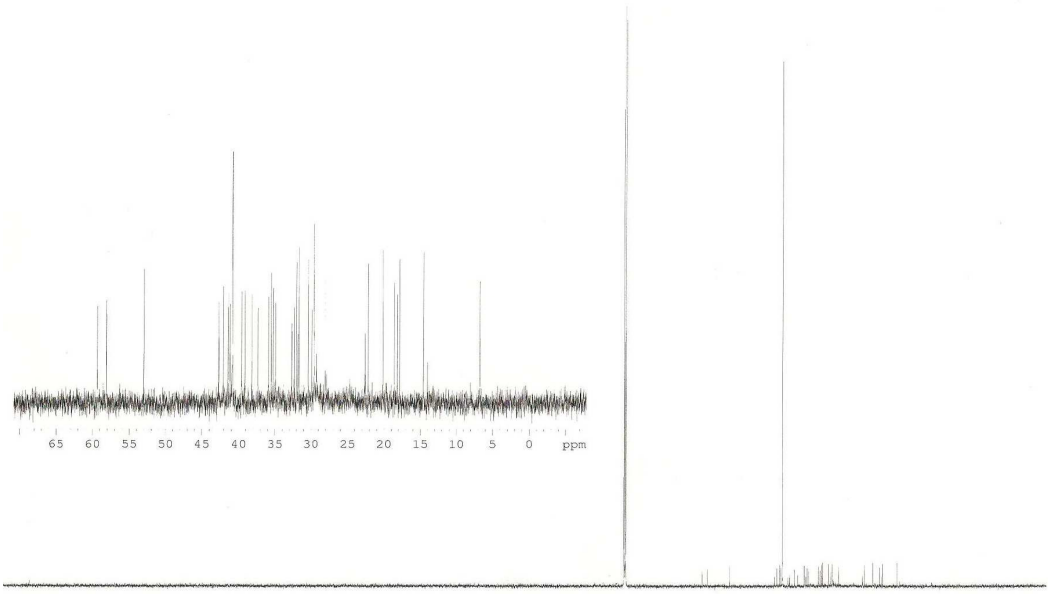


Figure 5.1: ^{13}C NMR spectrum of compound I, solvent used $CDCl_3$, magnet field strength 600 MHz

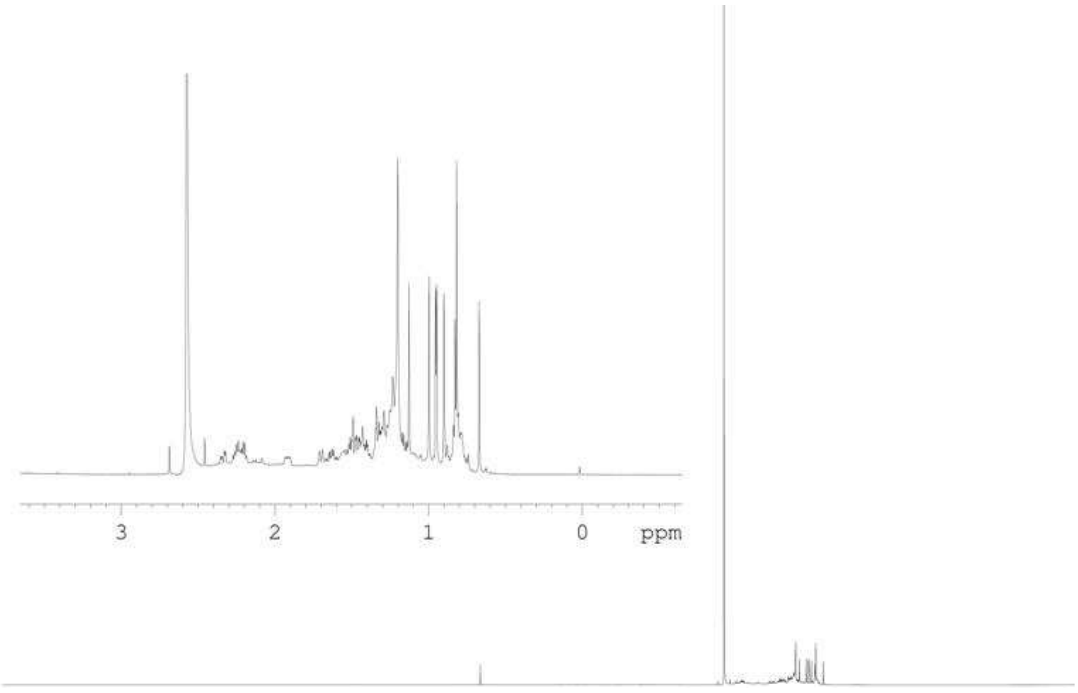


Figure 5.2; ^{1}H NMR spectrum of compound I, solvent used $CDCl_3$, magnet field strength 600 MHz

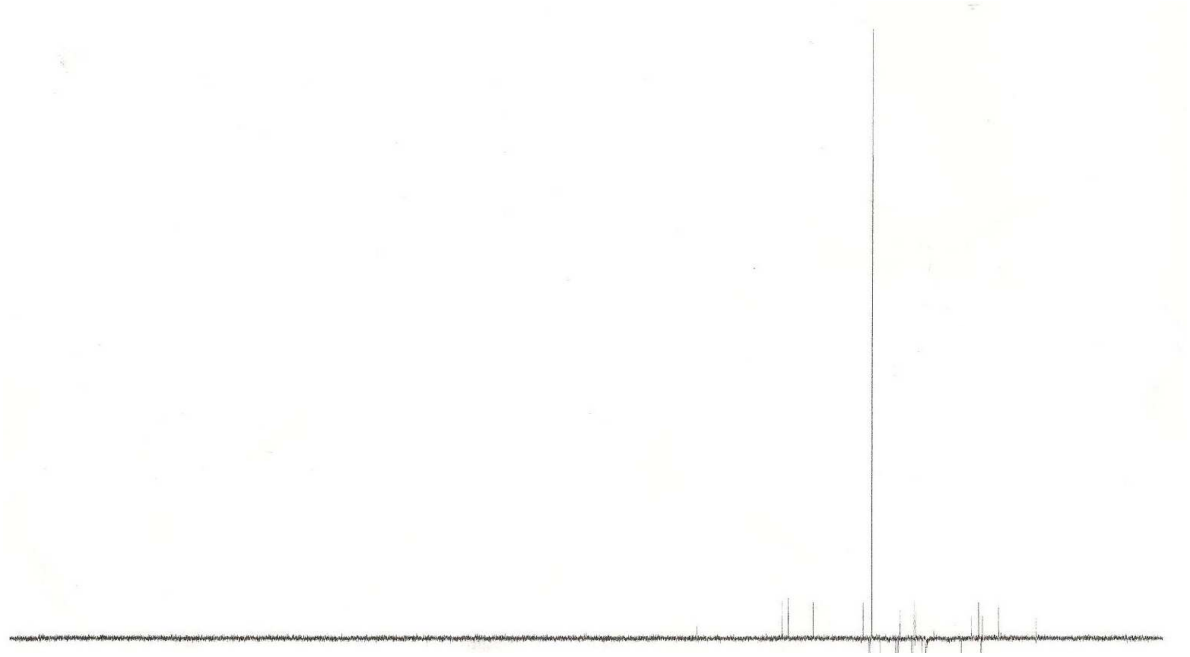


Figure 5.3: DEPT NMR 135 spectrum of compound I, solvent used $CDCl_3$, magnet field strength 600 MHz

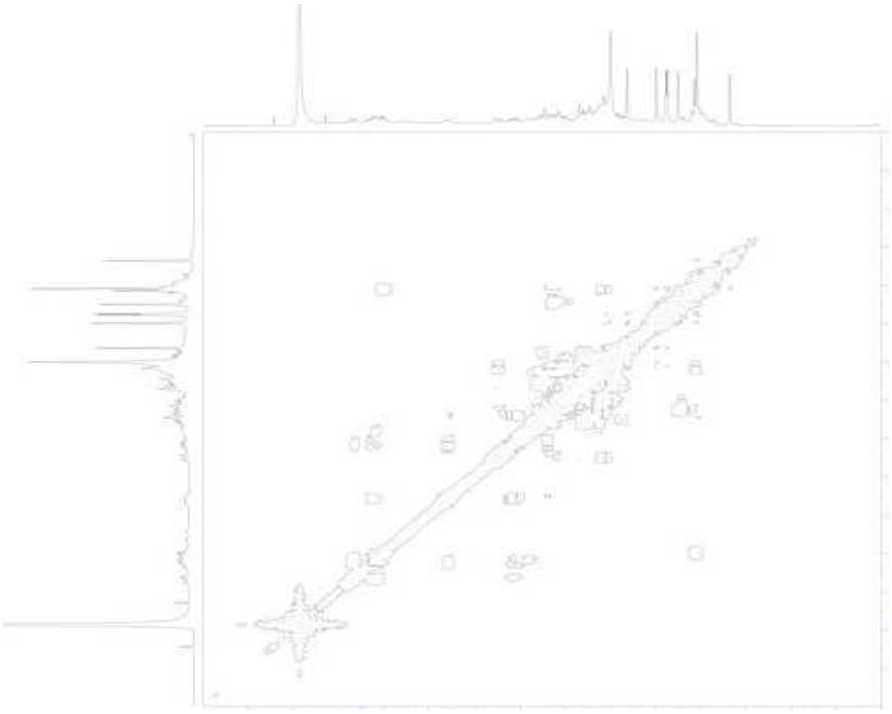


Figure 5.4: COSY NMR spectrum of compound I, solvent used $CDCl_3$, magnet field strength 600 MHz

Figure 5.5: HSQC NMR spectrum of compound I, solvent used $CDCl_3$, magnet field strength 600 MHz

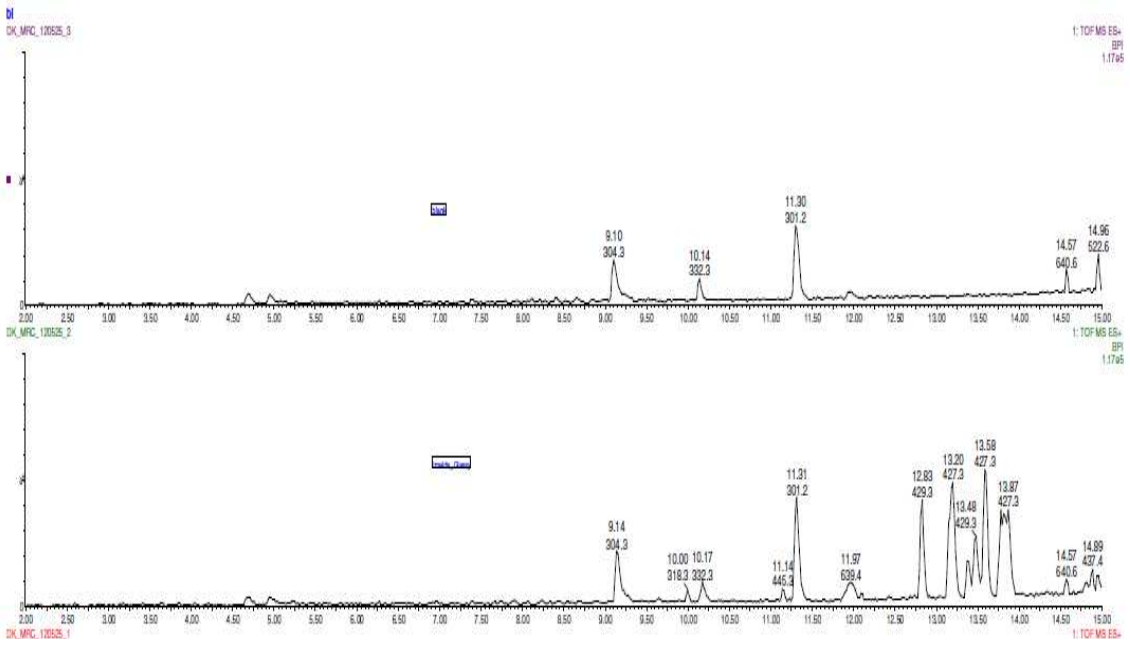


Figure 5.6: LC/MS spectrum of compound I

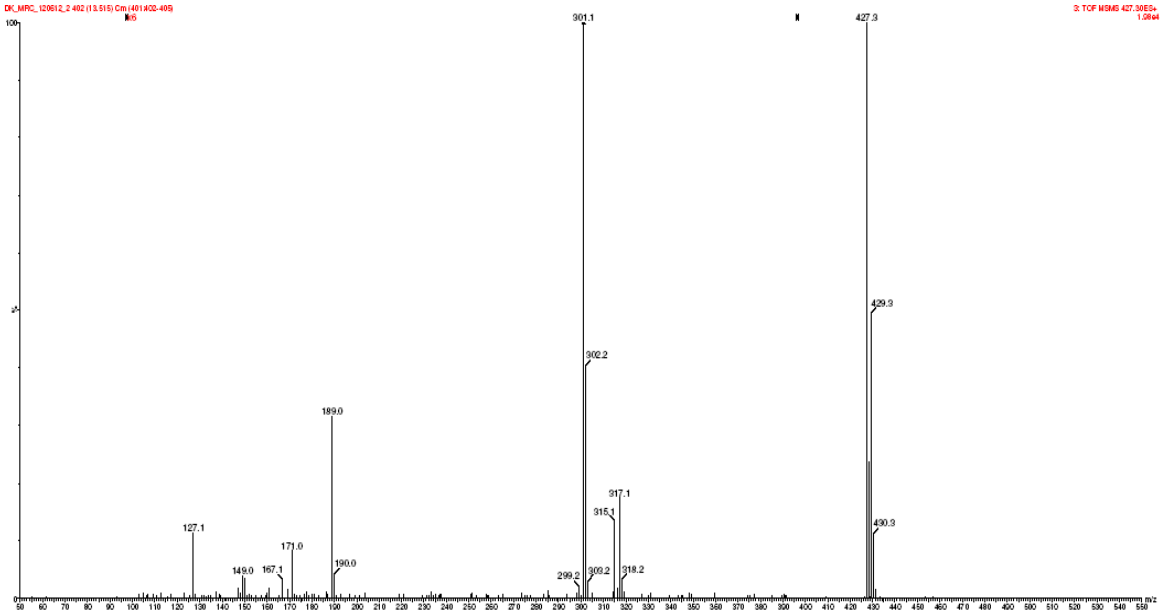


Figure 5.7: MS/MS spectra of compound I

5.9. Identification and Characterization of the isolated compound

Compound I was isolated as glassy compound (30mg). Acetone leaf extract of *C. erythrophyllum* was successively partitioned into hexane, dichloromethane and ethyl acetate-soluble fractions. Antimicrobial evaluation of the extract and fractions indicated that most of the activity was in the hexane-soluble fraction. Conventional open column chromatographic separation of the active fraction led to the isolation of friedelin. The IR spectrum of the isolated compound indicated the presence of carbonyl group (1714 cm^{-1}) while no carbon-carbon double bond was evidenced. Its molecular formula was determined as $C_{30}H_{48}O$ based on the molecular peak (M = 424) in the mass spectrum and corroborated by the NMR data. The ^{13}C NMR spectrum revealed 30 carbon signals, including characteristic signals for saturated ketone group (_δc = 217.21). The complete chemical shift assignments are listed in table 4.1. The compound was characterized using nuclear magnetic resonance (NMR) spectroscopy and comparison with literature values.

Table 5.1: The spectral data of friedelin isolated from *C. erythrophyllum*

Carbon number	DEPT	^{13}C	Literature
1	CH_2	22.20	22.27
2	CH_2	41.44	41.51
3	C	217.21	213.17
4	CH	58.13	58.19
5	C	42.71	42.13
6	CH_2	41.20	41.27
7	CH_2	18.57	18.23
8	CH_2	53.01	53.08
9	C	35.92	37.42
10	CH	59.38	59.45
11	CH_2	35.53	35.61
12	CH_2	30.41	30.50
13	C	40.92	39.68
14	C	39.23	38.28
15	CH_2	29.68	29.98
16	CH_2	35.32	36.00
17	C	32.07	32.76
18	CH	42.07	42.77
19	CH_2	35.01	35.32
20	C	27.95	28.16
21	CH_2	32.75	32.40
22	CH_2	39.16	39.24
23	CH_3	6.74	6.83
24	CH_3	14.64	14.68
25	CH_3	17.93	17.94
26	CH_3	20.17	20.26
27	CH_3	18.65	18.67
28	CH_3	31.97	32.09
29	CH_3	31.77	31.79
30	CH_3	34.94	35.03

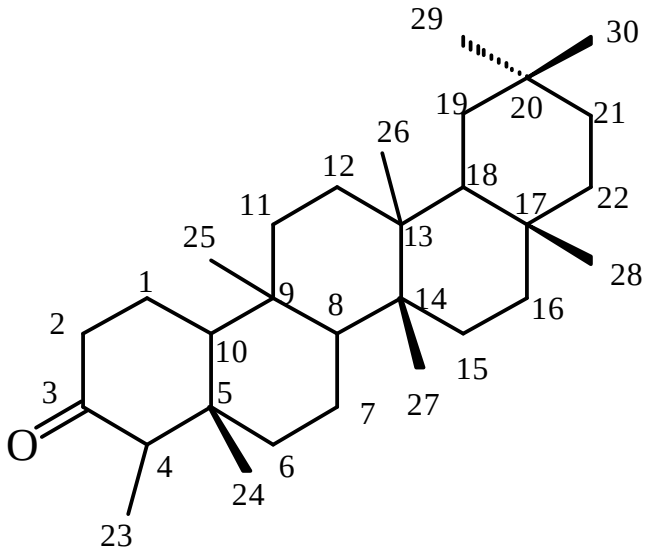


Figure 5.8: Chemical structure of Friedelin with antimicrobial properties isolated from the hexane fraction of acetone leaf extract of *Combretum erythrophyllum*

Table 5.2: MIC values for Friedelin

Microorganism	*Sa*	*Ef*	*Ec*	*Pa*	H_2O	Gent
MIC (µg/ml)	0.32	0.63	0.63	0.32	2.5	2.5

The antibacterial activity of the compound was performed and the compound was active against *Sa* (0.32 µg/ml) and *Pa* (0.32 µg/ml). This indicates that friedelin has mild antibacterial activity.

Chapter 6

Discussions

6.1. Extraction yield

The total mass extracted from the crude extract (30 g) of the leaves of *Combretum erythrophyllum* using different solvents are presented in figure 4.1.1. Dichloromethane fraction was found to be the best extractant, extracting the highest quantity of the plant material, more than any other solvents. Dichloromethane fraction extracted the highest mass (5.66 g) and the lowest mass was found in hexane fraction (0.66 g). The total masses extracted ranged between 0.66 g and 5.66 g. All fractions and crude extract were used for antifungal, antibacterial and antioxidant assay.

6.2. Phytochemical fingerprinting

Leaves of *Combretum erythrophyllum* were extracted with acetone to obtain a crude extract followed by liquid-liquid fractionation; four fractions were obtained from the crude extract. The chromatograms that were sprayed with vanillin revealed that most of the compounds from the crude extract and fractions were active in CEF followed by BEA and only a few were active in EMW. Compounds from the crude extract, water, ethyl acetate and dichloromethane fraction were most active in CEF and BEA; hexane fraction was slightly active in this solvent systems. Polar and neutral compounds easily separate in EMW, non-polar compounds separate in BEA and intermediate polarity and acidic compounds separate in CEF. R_f values were calculated to check the similarities in compounds present in the plant extract and fractions. In CEF and BEA, the blue, purple and green band is common in both solvent systems; it is only the water fraction that does not have the green band in BEA. In EMW, the blue band slightly appears in the crude extract, hexane and dichloromethane fraction and the purple band appears only in the ethyl acetate fraction.

6.3. Antimicrobial activity

Bioautography was performed using fungal and bacterial microorganisms in a 60:40, hexane: ethyl acetate solvent system. From the bioautography results the activity of the crude extract and fractions was observed by the clear zones on the chromatogram. The higher the inhibition zones the more active the extract/fraction. In this study, from the bacterial bioautography assay the hexane fraction had the highest number of zones of inhibition in all organisms. The crude extract and ethyl acetate had moderate zones of inhibition. DCM and water fraction had poor inhibition in all organisms. The growth of inhibition was found to be very poor in all in all fungal organisms, hexane fraction and crude extract were the only ones which had one zone of inhibition on the *CN* and other fractions and crude extract did not inhibit anything from the organism. Crude extract and all fractions were also not active against *Ca* and *Af*, since there were only few zones of inhibition on these organisms. MIC was determined on the crude extract and all fractions. The MIC values of the bacterial assay were between 0.02 – 2.5 mg/ml. The high MIC values were found in DCM (2.5 mg/ml) against *Sa*, *Ec* and *Pa*, also in hexane fraction with the MIC value of 2.5 mg/ml against *Ec* and 1.25 mg/ml against *Ef*. Crude extract and EA fraction had MIC 1.25 mg/ml against *Ef*. The most active fractions were water fraction with the lowest MIC 0.02 mg/ml against *Sa* and EA fraction with the lowest MIC 0.08 mg/ml against *Ec*. The MIC values of the fungal assay were between 0.02 – 1.25 mg/ml. The highest MIC values were found in the crude extract and EA fraction against *Ca* (1.25 mg/ml) and DCM fraction *Ca* and *Af* (0.16 mg/ml). Crude extract and all fractions had the lowest MIC of 0.02 mg/ml against *Cn*. Hexane, EA and water fractions and crude extract also had the lowest MIC of 0.08 mg/ml against *Af*.

The relative safety margin of water was found to be the highest compared to the other fractions; therefore the highest amount of its fraction must be used in treatment. The results of the antibacterial and antifungal analyses showed that the crude extract and all fractions produced zones of inhibition against most of the test organisms used with the lowest MIC value of 0.02 mg/ml and 0.08 mg/ml against *Af* and *Ec* respectively. This indicates that the leaves of *C. erythrophyllum* have strong antibacterial and antifungal activity which confirms their use as anti-infective agents.

This generally proves that the leaves of *C. erythrophyllum* have good antimicrobial activity.

6.4. Antioxidant

In qualitative analysis of antioxidant activity the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screening test for the radical scavenging ability of the compounds present in the crude and fractions. In TLC DPPH, water fraction and ethyl acetate indicated a high antioxidant activity. The DPPH method measures electron donating effect of the compounds present in the crude and fractions and provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. DPPH is reduced from a purple to a light yellow compound by electrons from oxidant compounds. In this study three methods were used to measure the antioxidant activities of crude extract and fractions (DPPH, ABTS and Hydroxyl radical scavenging). Crude extract and fractions exhibited the antioxidant activity with ethyl acetate having the highest antioxidant activity; this could be due to high polarity of active compounds.

In DPPH assay ethyl acetate fraction had the highest antioxidant activity with EC_{50} of 0.04272 µg/ml and hexane fraction had the lowest antioxidant activity with the EC_{50} 0.4328 µg/ml. In the ABTS assay the water fraction had the highest antioxidant activity with the EC_{50} 0.01825 µg/ml and the DCM fraction had the lowest activity with the EC_{50} 0.2556 µg/ml. In the hydroxyl radical scavenging the crude extract scavenged 77.62 ± 1.41% at the highest concentration of 0.250 mg/ml and 47.21 ± 3.20% at the lowest concentration of 0.003 mg/ml, the water fraction and ethyl acetate fraction as well scavenged 50.00±2.68% and 47.03±4.29% at 0.003 mg/ml.

6.5. Cytotoxicity

The toxicity of the crude extract and fractions against the Vero cells using the MTT assay was investigated and revealed that the evaluated extracts were not toxic. According to American National Cancer Institute (ANCI) the toxicity level should be below 200 µg/ml. The toxicity levels of the crude extract and fractions were found to be between 34 and 223 mg/ml which were all below doxorubicin (LC_{50} = 7.1855

µg/ml) which was used as positive control. The toxicity of the isolated compound was not performed due to an insufficient amount of the compound. The results of the crude extract and fractions provide a clear indication for the traditional use of *Combretum erythrophyllum* leaves in treating infections.

6.6. Compound isolation and characterization

In this study the antibacterial activity of the isolated compound was performed and found to be active against *Sa* and *Pa* with the MIC (0.32 µg/ml). The structure of the isolated compound was determined using (^{1}H-NMR, ^{13}C-NMR and MS). The compound was identified as friedelin with the molecular formula ($C_{30}H_{48}O$). Due to small quantity of the isolated compound the cytotoxicity was not performed. Friedelin was isolated from other Combretum species but it was isolated for the first time from *Combretum erythrophyllum*.

6.7. Conclusion

TLC chromatograms sprayed with vanillin, indicated that the DCM and EA fractions extracted the most compounds. All fractions contained a number of antioxidant compounds based on the TLC chromatogram that was sprayed with DPPH, even though the amount of antioxidant compounds were different in each fraction.

From the data and observations obtained it can be concluded that the leaves of *Combretum erythrophyllum* are good sources of antioxidant and can be used for treating ailments associated with oxidative stress. The highest number of antioxidant compounds was found in EA, DCM and water fraction.

The MIC values proved that the leaves of *Combretum erythrophyllum* have a potential to treat infections from bacterial and fungal pathogens.

From the toxicity assay it can be concluded that the leaves of *Combretum erythrophyllum* are safe for medicinal use in folk medicine in treating infections, therefore the results obtained in this study support several of the traditional uses of *Combretum erythrophyllum*.

Chapter 7

References

ANGEH, J.E., HUANG, X., SATTLER, I., SWAN, G.E., DAHSE, H., HÄRTL, A. & ELOFF, J.N. 2007a. Antimicrobial and anti-inflammatory triterpenoids from *Combretum imberbe* (Combretaceae) *Journal of Ethnopharmacology* 110: pp. 56-60.

ANGEH, J.E., HUANG, X., SATTLER, I., SWAN, G.E., DAHSE, H., HÄRTL, A. & ELOFF, J.N. 2006. Antimicrobial and anti-inflammatory activity of four known and one new triterpenoids from *Combretum imberbe* (Combretaceae). *Journal of Ethnopharmacology* 110(1): pp.56-60.

ANGEH, J.E., HUANG, X., SWAN, G.E., MÖLLMAN, U., SATTLER, I. & ELOFF, J.N. 2007b. Novel antibacterial triterpenoids from *Combretum padoides* (Combretaceae). Arkivoc (ix), pp. 113-120.

ARNOLD, H.J. & GULIMIAN, M. 1984. Pharmacopeia of traditional medicine in Venda. *Journal of Ethnopharmacology* 121: pp. 35-74

ASHBY, B. & TURKINGTON, C. 2007. The Encyclopaedia of Infectious Diseases. New York: InfoBase publishing, pp. 185. ISBN: 987-0-8160-6397-0.

ASRES, K., BUCAR, F., EDELSBRUNNER, S., KARTNIG, T., HÖGER, G. & THIEL, W. 2001a. Investigations on Antimycobacterial Activity of some Ethiopian Medicinal Plants. *Phytotherapy Research* 15: pp. 613-617.

BALUNUS, M.J. & KINGHORN, A. D. 2005. Drug discovery from medicinal plants. Life sciences 78: pp. 431-441.

BANDELE, O.J., CLAWSON, S.J. & OSHEROFF, N. 2008. “Dietary polyphenols as topoisomerase II poisons: B-ring substituents determined the mechanism of enzyme mediated DNA cleavage enhancement.” Chemical Research in Toxicology 6: pp. 1253-1260.

BANSKOTA, A.H., TEZUKA, Y., KIM, Q.T., TANAKA, K., SAIKI, I. & KADOTA, S. 2000. Thirteen novel cycloartanes-type triterpenes from *Combretum quadrangulare*. *Journal of Natural Products* 63: pp. 57-64.

BARJESTEH VAN WAALWIJK VAN DOORN-KHOSROVANI, S., JANSSEN, J., MAAS, L.M., GODSCHALK, R.W., NIJHUIS, J.G. & VAN SCHOOTEN, F.J. 2007. “Dietary flavonoids induce MLL translocations in primary human CD34+ cells”. Carcinogenesis 28 (8): pp. 1703-9.

BEARE, N.A., TAYLOR, T.E., HARDING, S.P., LEWALLEN, S. & MOLYNEUX, M.E. 2006. "Malarial retinopathy: a newly established diagnostic sign in severe malaria". *Am. J. Trop. Med. Hyg.* **75** (5): pp. 790–7.

BEGUE, M.J. & KLINE, R.M. 1972. The use of tetrazolium salts in bioautographic procedures. *J. Chrom*. 64: pp. 182-184.

BENSKY, D. & GAMBLE, A. 1993. Chinese herbal medicine. Materia medica, new ed. Eastland Press Inc., Seattle, Wash.

BODEKER, G. 2003. Traditional medicine, G. Cook, A. Zumla, Editors, Manson’s Tropical Disease, (21st edition.), WB Saunders, London pp. 33-48.

BODEKER, G., DVORAK-LITTLE, M., CARTER, G. & BURFORD, G. 2006. HIV/Aids: Traditional systems of health care in management of global epidemic, *Journal of alternative and Complementary medicine* 12: pp. 563-576.

BRAND-WILLIAMS, W., CUVELIER, M.E. & BERSET, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologiae* 28: pp. 25-30.

CAVANAGH, J.H., FRAME, B. & LENNOX, J. 2006. The sustainability assessment model (SAM): measuring sustainable development performance. *Australian Journal of Environmental Management* 13: pp. 31-34.

CERA, L.H., HEGGERS, J.P., ROBSON, M.C. & DURACCIO, M.R. 1980. The therapeutic efficacy of the Aloe vera cream (Dermaide Aloe) in dermal injuries: two cases reports. J. Am. Anim. Hos. Ass., 16: pp. 768-772.

CHAABI, M., BENAYACHE, S., BENAYACHE, F., N’GOM, S., KONÉ, M., ANTON, R., WENIGER, B. & LOBSTEIN, A. 2008. Triterpenes and polyphenols from Anogeissus leiocarpus (Combretaceae). Biochemical Systematics and Ecology 36: pp. 59-62.

CHEMICAL ENCYCLOPAEDIA: alkaloids

CHHABRA, S.C., UISO, F.C. & MSHIU, E.N. 1984. Phytochemical Screening of Tanzanian Medicinal Plants. I. *Journal of Ethnopharmacology* 11: pp. 157-159.

CHINSEMBU, K.C. 2009. Model and experiences of initiating collaboration with traditional healers in validation of ethnomedicines for HIV/AIDS treatment in Namibia. *J Ethnobio Ethnomed* 5: pp. 30.

CHOO, Z.W., CHAKRAVARTHI, S., WONG, S.F., NAGARAJA, H.S., THANIKACHALAM, P.M., MAK, J.W., RADHAKRISHNAN, A. & TAY, A. 2010. A comparative histopathological study of systemic candidiasis in association with experimentally induced breast cancer. *Oncology Letters* 1 (1): pp. 215-222.

COS, P., VLIENTINCK, A.J., VANDEN BERGHE, D. & MAES, L. 2006. Anti-inefective potential of natural products: How to develop a stronger in vitro 'proof of concept', *Journal of Ethnopharmacology* 106: pp. 290-302.

COVINGTON, E.C. 1998. Anticonvulsants for neuropathic pain and detoxification. *Cleve Clin J med* 65: pp. 121-129.

CRAGG, G.M., NEWMAN, D.J. & SNADER, K.M. 1997. Natural products in drug discovery and development. *Journal of Natural Products* 60: pp. 52-60.

CRUEGER, W. 1989. Biotechnology. A text book of industrial microbiology. Sunderland: Sinauer Associates, Inc.

CUNNINGHAM, A.B. & ZONDI, A.S. 1991. Cattle Owners and Traditional Medicines Used for Livestock. Investigational report No. 69: Institute of Natural Resources, Pietermaritzburg.

CUSHNIE, T.P.T. & LAMB, A.J. 2005. "Antimicrobial activity of flavonoids". International *Journal of Antimicrobial Agents* 26 (5): pp. 343-356.

DAMBISYA, Y.M. & TINDIMWEBWA, G. 2003. Traditional remedies in children around Eastern Cape, South Africa. *East African Medical Journal* 80: pp. 401-405.

DEAN, O.M., VAN DEN BUUSE, M., BERK, M., COPOLOV D.L., MAVROS, C. & BUSH, A.I. 2011. "N-acetyl cysteine restores brain glutathione loss in combined 2-cyclohexene-1-one and D-amphetamine-treated rats: relevance to schizophrenia and bipolar disorder". *Neurosci Lett*. 499 (3): pp. 149-153.

De SOUSA, R.R., QUEIROZ, K.C., SOUZA, A.C., GURGUEIRA, S.A., AUGUSTO, A.C., MIRANDA, M.A., PEPPELENBOSCH, M.P., FERREIRA, C.V. & AOYAMA, H. 2007. “Phosphoprotein levels, MAPK activities and NFKappaB expression are affected by fisetin”. *J. Enzyme Inhib Med Chem*. 22 (4): pp. 439-444.

Diaz, M.N., Frei, B. & Keaney, J.F. 1997. Antioxidants and atherosclerotic heart disease. *N.Engl.J.Med* 337: pp. 408-416.

DODDRELL, D.M., PEGG, D.T. & BENDALL, M.R. 1982. Distortionless enhancement of NMR signals by polarization transfer. *Journal of Magnetic Resonance* 48: pp. 323-327.

ELDEEN, I.M.S., ELGORASHi, E.E., MULHOLLAND, D.A. & Van STADEN, J. 2006. Anolignan B: A bioactive compound from the roots of *Terminalia sericea*. *Journal of Ethnopharmacology* 103: pp. 135-138.

ELOFF, J.N. 1998b. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: pp. 711-713.

ELOFF, J.N. 1999. The antibacterial activity of 27 Southern African members of the Combretaceae. *South African Journal of Science* 95: pp. 148-152.

ELOFF, J.N. 2004. Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. Phytomedicine 11: pp. 370.

ELOFF, J.N. 1998a. A sensitive and quick method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: pp 711-713.

ELOFF, J.N. FAMAKIN, J.O. & KATERERE, D.R. 2005b. Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves, *African Journal of Biotechnology* 4: pp. 1166-1171.

ELOFF, J.N., KATERERE, D.R. & McGAW, L.J. 2008. The biological activity and chemistry of the Southern African Combretaceae. *Journal of Ethnopharmacology* 119: pp. 686-699.

FABRICANT, D.S. & FARNSWORTH, N.R. 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives* 109: pp. 69-136.

FACUNDO, V.A., ANDRADE, C.H.S., SILVEIRA, E.R., BRAZ-FIHLO, R. & HUFFORD, C.D. 1993. Triterpenes and flavonoids from *Combretum leprosum*. Phytochemistry 32: pp. 411-415.

FAN-HAVARD, P., CAPANO, D., SMITH, S.M., MANGIA, A. & ENG, R.H.K. 1991. Development of resistance in *candida* isolates from patients receiving prolonged antifungal therapy. *Antimicrobial Agents and Chemotherapy* 35: pp. 2302-2305.

FARNSWORTH, N.F. 1984. The role of medicinal plants in drug development. In natural products and drug development, Krogsgaard-Larsen P., Brogger Christensen L., Kofold H.(eds). Munksgaard, Copenhagen, pp. 17-28.

FARNSWORTH, N.R. & MORRIS, R.W. 1976. Higher plants: the sleeping giants of drug development. Am. *J Pharm* 148: pp. 46-52.

FERRERO-MILLANI, L., NIELSEN, O.H., ANDERSEN, P.S. & GIRARDIN, S.E. 2007. Chronic inflammation importance of NOD2 and NALP3 in interleukin-1 betageneration. *Clin exp. Immunol*, 147(2): pp. 227-235.

FIDEL, P.L. 2002. Immunity to *candida*. Oral Dis 8: pp.69-75.

GAIDAMASHVILI, M. & VAN STADEN, J. 2002. Interaction of lectin-like proteins of South African medicinal plants with *staphylococcus aureus* and *Bacillus subtilis*. *Journal of Ethnopharmacology* 80: pp. 131-135.

GAN, Z. 2006. 13C/14N heteronuclear multiple quantum correlation with rotary resonance and REDOR dipolar recoupling, *journal of Magnetic Resonance* 184: pp. 39-43.

GARBINO, J., KOLAROVA, L., LEW, D., HIRSCHET, B. & ROHNER, P. 2001. Fungemia in HIV-infected patients, a 12-year study in a tertiary care hospital. *AIDS Patient-Care STDs* 15: pp. 407-410.

GBÉASSOR, M., KOSSOU, Y., AMEGBO, K., KOUMAGLO, K. & DENKE, A. 1989. Antimalarial effects of eight African medicinal plants. *J Ethnopharm* 25: pp. 115-118.

GERSHENZON, J. & DUDAREVA, M. 2007. The function of terpene natural product. Nature chem. Boil 3: pp. 408-414.

GERWICK, W. H., PROTEAU, P.J., NAGLE, D.G., HAMEL, E., BLOKHIN, A. & SLATE, L.D. 1994. Structure of curacin a, a Novel antimitotic, antiproliferative and brine shrimp toxic natural product from the marine cyanobacterium. Lyngbya Majuscula. *J. Org. Chem*. 59: pp. 1243–1245.

GULLECE, M., ASLAN, A., SOKMEN, M., SAHIN, F., ADIGUZEL, A., AGAR, G. & SOKMEN, A. 2006. Screening the antioxidant and antimicrobial properties of the lichens Parmelia saxatilis, Platismatia glauca, Ramalina pollinaria, Ramalima polymorpha and Umbilicaria nylanderian, *Phytomedicine* 13: pp. 515-521.

HALLIWELL, B. 2007. "Oxidative stress and cancer: have we moved forward?". *Biochem. J*. 401 (1): pp.1-11.

HASLAM, E. 1993. Shikimic Acid: Metabolism and Metabolites. (Chichester, UK: John Wiley and Sons).

HERDBERG, M. & STAUGARD, P. 1989. Traditional Medicine in Botswana. Traditional Medicinal Plants. Gaborone Ipelegeng Publishers.

HOFFMAN, R.E. 2003. Variations on the chemical shift of TMS. *Journal of Magnetic Resonance*, 163: pp.325-331.

HUSSAIN, A.A., TRUELOVE, J.E. & KOSTENBAUDER, H.B. 1980. Derivatives of Asprin. The University of Kentucky Research Foundation, Lexington, ky.

JABS, T. 1999. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochemical Pharmacology* 57: pp. 231-245.

JAIN, S.K. 1994. Ethnobotany and research on medicinal plants in India. Ciba found Symp, 185: pp. 153-164.

JENSEN, W.B. 2007. *The Origin of the Oxidation-State Concept* J. Chem. Educ., 84: pp. 1418.

KABRA, S.K., LODHA, R. & PANDEY, R.M. 2010. Kabra, Sushil K. ed. "Antibiotics for community-acquired pneumonia in children". *Cochrane Database Syst Rev* 3 (3): CD004874.

KAMBOJ, V.P. 2000. Herbal medicine. Curr Sci 78: pp. 35-39.

KAROU, D., DICKO, M.H., SANON, S., SIMPORE, J. & TRAORE, A.S. 2005. Antioxidants and Antibacterial activities of polyphenols from ethnomedicinal plants in Burkina Faso. *African Journal of Biotechnology* 4(8): pp. 823-828.

KATERERE, D.R., GRAY, A.I., NASH, R.J. & WAIGH, R.D. 2003. Antimicrobial activity of Pentacyclic triterpenes isolated from African Combretaceae. *Phytochemistry* 63: pp. 81-88.

KELBERG, B.J. 1997. Trends in immunotherapy of fungal infections. *European Journal of Clinical Microbiology Infectious Diseases* 16: pp. 51-55.

KGATLE, D.T. 2007. Isolation and characterization of antioxidant compounds from *Combretum apiculatum* (Sond.) subsp a*piculatum* leaf extracts. MSc Thesis. University of Pretoria, South Africa.

KOMAPE, P. 2005. Biological activity of leaf extracts of *Combretum vendae* and isolation of an antibacterial compound. MSc Thesis. Medunsa, South Africa.

KOTZE, M. & ELOFF, J.N. 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). *South African Journal of Botany* 68: pp. 62-67.

KOURKOMPETIS, T., MANOLAKAKI, D. & VELMAHOS, G. 2010. *Candida* infection and colonization among non-trauma emergency surgery patients. Virulence 1(5): pp. 359-366

KUMAR, D.S. & ORABHAKAR, Y. S. 1987. On the ethnomedical significance of the arjuna tree, *Terminalia arjuna* (ROXB) wight and Arnot. *Journal of Ethnopharmacology* 20: pp. 173-190.

KUNCHANDY, E. & RAO, M. N. A. (1990). Oxygen radical scavenging activity of curcumin. *Journal of Pharmacology* 58: pp. 237-240.

LALL, N. & MEYER, J.J.M. 1999. In vitro inhibition of drug-resistant and drug sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selects South African plants. *Journal of Ethnopharmacology* 66: pp. 347-354.

LANGLOIS-KLASSEN, D., KIPP, W. & RUBAALE, T. 2008. Who's talking? Communication between health providers and HIV-infected adults related to herbal medicine for AIDS treatment in western Uganda. *Social Science and Medicine* 67: pp. 165-176.

LEWIS, R.A. 1998. Lewis dictionary of toxicology. CRC Press pp.51, ISBN: 1-56670-223-2.

LEWIS, W.H. & ELVIN-LEWIS, M.P.H. 1977. Medicinal Botany plants affecting man's health. John Wiley & Sons, New York. pp. 515.

LI, Y. & TRUSH, M.A. 1994. Reactive oxygen dependent DNA damage resulting from the oxidation of Phenolic compounds by a copper redox cycle, Cancer Res 54: pp. 1895-1898

LIM, W.S., BAUDOUIN, S.V., GEORGE, R.C., HILL, A.T., JAMIESON, C., Le JEUNE, I., MACFARLANE, J.T., READ, R.C., ROBERTS, H.J., LEVY, M.L., WANI, M. & WOODHEAD, M.A. 2009. Pneumonia Guidelines Committee of the BTS Standards of Care, Committee. BTS Guidelines for the management of community acquired pneumonia in adults. *Thorax* 64 Suppl 3: pp. 1-55.

LINDSEY, K., JAGER, A.K., RAIDOO, D.M. & VAN STADEN, J. 1999. Screening of plants used by southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin synthesis inhibitors and uterine relaxing activity. *J. Ethnopharmacol* 64: pp. 9-14.

LOSS, S., KÜHN, T., BRUKER BIOSPIN, A.G. 2005, FALLANDEN. Introduction to 1- and 2- dimensional NMR 1.

MABBERLEY, D.J. 1987. The plant Book: a portal dictionary of the higher plants. Cambridge University Press, Cambridge, pp. 21.

MAHADY, G.B., FONG, H.H.S. & FARNSWORTH, N.R. 2008. WHO monographs on selected medicinal plants volume 4. WHO Publications Geneva, Switzerland: Fructus Macrocarponii. pp. 132-147.

MANSKE, R.H.F. 1965. The alkaloids, Chemistry and physiology. Volume VIII. New York: academic Press, pp. 673.

MAREGESI, S.M., NGASSAPA, O.D., PIETERS, L. & VLIETINCK, A.J. 2007. Ethnopharmacological survey of the bunda district, Tanzania: Plants used to treat infectious diseases, *Journal of Ethnopharmacology* 113: pp. 457-470.

MARTIN, G.E. & ZEKTER, A.S. 1988. Two dimensional NMR methods for establishing molecular connectivity VCH publishers INC: New York, pp. 59.

MARTINI, N., KATERERE, D.R.P. & ELOFF, J.N. 2004. Biological activity of five antibacterial Flavonoids isolated from *Combretum erythrophyllum* (Combretaceae). *J. Ethnopharmacol* 93: pp. 207-212.

MASIKA, P.J. & AFOLAYAN, A.J. 2002. Antimicrobial Activity of some plants used for the treatment of livestock disease in the Eastern Cape, South Africa. *Journal of Ethnopharmacology* 83: pp. 129-134.

MATHABE, M.C., NIKOLOVA, R.V., LALL, N. & NYAZEMA, N.Z. 2006. Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province, South Africa. *Journal of Ethnopharmacology* 105: pp. 286-293.

MAXWELL, S.R.J. 1995. Prospects for the use of antioxidant therapies. Drugs 49: pp. 345-361.

McGAW, L.J., STEENKAMP, V. & ELOFF, J.N. 2007. Evaluation of athrixia bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *Journal of Ethnopharmacology* 110: pp. 16-22.

McNaught, A.D. & Wilkinson, A. 1997. IUPAC. Compendium of chemical terminology, 2nd edition, (the Gold Book). Blackwell scientific publications, Oxford ISBN: 0-9678550-9-8 doi: 10.1351/ goldbook.

MEERSSEMAN, W., VANDECASTEELE, S.J., WILMER, A., VERBEKEN, E., PEETERSMAN, W.E. & VAN WIJNGAERDEN, E. 2004. Invasive *aspergillosis* in critically ill patients without malignancy. *Am J Respir Crit Care Med* 170(6): pp. 621-625.

MILLS, E., FOSTER, B.C., VAN HEESWIJK, R., PHILIPS, E., WILSON, B., LEONARD, B., KOSUGE, K. & KANFER, I. 2005. Impact of African herbal medicines on antiretroviral metabolism. AIDS 17: pp. 95-97.

MORRIS, K. 2001. Treating HIV in South Africa- a tale of two systems. *Lancet* 357: pp. 1190.

MOSMANN, T. 1983. Rapid colometric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: pp 55-63.

NARAYANA, K.R., SRIPAL, R.M., CHALUVADI, M.R. & KRISHNA, D.R. 2000. Bioflavonoids Classification, Pharmacological, Biochemical effects and therapeutic potential. *Indian Journal of Pharmacology* 2001, 33: pp. 2-16.

NATIONAL INSTITUTE OF HEALTH, 2001. NIAID global health research plan for HIV/AIDS, malaria and tuberculosis. U.S. Department of Health and Human Services, Bethesda, M.D.

NEUWINGER, H.D. 2000. African traditional medicine. A Dictionary of Plant Use and Applications. Medipharm Scientific Publishers, Stuttgart, Germany. pp. 589.

NIMRI, L.F., MEQDAM, M.M. & ALKOFAHI, A. 1999. Antibacterial activity of Jordanian medicinal plants. *Pharmaceutical Biology*, 37(3): pp. 196-201.

O'NEILL, M.J. & LEWIS, J.A. 1993. The renaissance of plant research in the pharmaceutical industry. In human Medicinal Agents from Plants, Kinghorn A.D., Balandrin M.F. (eds). ACS Symposium Series 534. American Chemical Society, Washington, DC, pp. 48-55.

OWEN, R.W., GIACOSA, A., HULL, W.E., HAUBNER, R., SPIGELHALDER, B. & BARTSCH, H. 2000. The antioxidant/anticancer potential of Phenolic compounds isolated from olive oil. *Eur. J. Cancer*, 36 (10): pp. 1235-1247

PANZINI, I., PELIZZONI, F., VEROTTA, L. & ROGERS, C.B. 1993. Constituents of the fruit of South African *Combretum* species. *South African Journal of Science* 89: pp. 324-327.

PAPPAS, P.G. 2006. Invasive candidiasis. *Infect. Dis. Clin. North Am*. 20: pp. 485-506.

PEGEL, K.A. & ROGERS, C.B. 1985. The characterization of mollic acid-3β-D-xyloside and its genuine aglycon mollic acid, two novel 1α-hydroxycycloartenoids from *Combretum molle. Journal of the Chemical Society of Perkin Transactions* 1: pp. 1711-1715.

PELLECURE, S., ALLEGRINI, J. & BUOCHBERG, S. 1976. Huiles essenticlles bactericides et fongicides. Revue del lnstitute Pasteur de lyon, 9: pp. 135-159

PERREIRA, M., TRIPATHY, S., INDAMDAR, V., RAMESH, K., BHAVSAR, M., DATE, A., IYYER, R., ACCHAMMACHARY, A., MEHENDALE, S. & RISBUND, A. 2005. Drug resistance pattern of *Mycobacterium tuberculosis* in seropositive and

seronegative HIV-TB patients in Pune, India. *Indian Journal for Medical Research* 121: pp. 235-239.

PETTIT, G.R., SINGH, S.B., HAMEL, E., LIN, C.M., ALBERTS, D.S. & GARCIA-KENDALL, D. 1989. Isolation and structure of the strong cell growth and tubulin inhibitor Combretastatin A-4, *Experientia* (Basel), 45: pp. 205-211.

PETTIT, G., SINGH, S.B. & BOYD, M.R. *et al*. 1995. Antineoplastic agents 291. Isolation and synthesis of Combretastatin A-4, A-5 and A-6. *Journal of Medicinal Chemistry* 38: pp. 1666.

PETTIT, G.R., SINGH, S.B. & SCHMIDT, J.M. 1988. Isolation, Structure, Synthesis and antimitotic properties of Combretastatin B-3 and B-4 from *Combretum caffrum. Journal of Natural Products* 51(3): pp. 517-527.

PETTIT, G.R., SMITH, C.R. & SINGH, S.B. 1987. Recent advances in the chemistry of plant antineoplastic constituents. In: Hosttemann K., and Lea P.J., Editors, *Biologically Active Natural Products. Proceedings of the Phytochemical Society of Europe-27,* Oxford Science Publications.

POMERVILLE, J.C. 2010. Alcamo's Fundamentals of microbiology (9th edition). Sudbury, mass: Jones and Barlett Publishers. pp. 323, ISBN: 0-7637-6258-x.

POUSSET, J.L. 1989. Medicinal Plants. Practical use. Ed. Ellipse, pp. 150.

RE, R., PELLEGRINI, N., PROTEGGENTE, A., PANNALA, A., YANG, M. & RICE-EVANS, C. 1999. Antioxidant activity applying ban improved ABTS radical cation decolorization assay. *Free radical Biology and Medicine* 26: pp. 1231-1237.

RHOADES, D.F. 1979. "Evolution of Plant Chemical Defense against Herbivores". In Rosenthal, Gerald A., and Jansen, Daniel H. Herbivores: Their interaction with secondary plant metabolites. New York: Academic Press. pp. 41.ISBN 0-12-597180-X

ROGERS, C.B. & COOMBES, P.H. 1999. Acidic triterpenes glycosides in trichome secretions differentiate subspecies of *Combretum collinum* in South Africa, *Biochemical Systematics and Ecology* 27: pp. 321-323.

ROGERS, C.B. & VEROTTA, L. 1996. Chemistry and biological properties of the African Combretaceae. In Hostettman K, Chinyanganga F., Maillard. M., Wolfender J.-L. (Eds), Chemistry, Biological and Pharmacological Properties of African Medicinal plants. University of Zimbabwe Publications, Harare, Zimbabwe.

ROGERS, C.B. 1988. Pentacyclic triterpenoids from *Combretum imberbe* leaves. *Phytochemistry* 27: pp. 3217-3220.

ROGERS, C.B. 1995. Acidic Dammarane arabinofuranosides from Combretum rotundifolium. *Phytochemistry* 40: pp. 833-836.

ROSS, J.A. 2000."Dietary flavonoids and the MLL gene: A pathway to infant leukaemia? Proc Natl Acad Sci USA 97 (9): pp. 4411-3.

ROSS, J.A. 1998. "Maternal diet and infant leukaemia: a role for DNA topoisomerase II inhibitors?" *Int J Cancer Suppl* 11: pp. 26-8

RYAN, K.J. & RAY, C.G. 2004. Sherris medical microbiology (4th edition). McGraw Hill.

SAXENA, V.K. & SHARMA, R.N. 1999. Antimicrobial activity of essential oil of Lantana aculeate. Fitoterapia, 70: pp. 59-60

SCHMIDT, E., LÖTTER, M. & McCLELAND, W. 2002. Trees and shrubs of Mpumalanga and Kruger National Park,

SCHWIKKARD, S., ZHOU, B.-N., GLASS, T.E., SHARP, J.L., MATTERN, M.R., JOHNSON, R.K. & KINGSTON, D.G.I. 2000. *Bioactive compounds from Combretum erythrophyllum*, *Journal of Natural Products* 63: pp.457-460.

SERAGE, A. 2004. Isolation and Characterization of antibacterial compounds in *Combretum apiculatum* Sond subsp. *apiculatum* Excell. MSc Thesis. University of Pretoria, South Africa.

SINGH, B., KIM SUNG, L., MATUSOP, A. *et al*. 2004. "A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings". *Lancet* 363 (9414): pp.1017–24.

SINGH, B., DHALLA, A.K., SENEVIRATNE, C. & SINGAL, P.K. 1995. "Oxidative stress and heart failure". *Molecular and cellular biochemistry* 147 (1): pp.77-81.

SPARKMAN O.D. 2000. Mass spectrometry desk reference. *Journal of Chemical Education* 78, pp.106.

SPECTOR, L.G., XIE, Y., ROBISON, L.L., HEEREMA, N.A., HILDEN, J.M. & LANGE, B. 2005. "Maternal diet and infant leukaemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group." *Cancer Epidemiol biomarkers Prev* 14 (3): pp.651-5.

SPILLER CAFFEINE, G.A. 1997. CRC Press ISBN 0-8493-2647-8

STECKELBERG, J.M. 2009. Mayo Clinic, Rochester, Minn.

STEDMAN'S MEDICAL DICTIONARY, 2006. 28th edition. Philadelphia: Lippincott Williams and Wilkins.

STEENKAMP, V. 2003. Traditional herbal remedies used by South African women for gynaecological complaints. *Journal of Ethnopharmacology* 86: pp. 97-108.

STEWART, B.W. 1994. Mechanisms of apoptosis: Integration of genetic, biochemical and cellular indicators. *Journal of National Cancer Institute* 86: pp. 1286-1296.

STRICK, R., STRISSEL, P.L., BORGERS, S., SMITH. S.L. & ROWLEY, J.D. 2000. "Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukaemia." *Proc Natl Acad Sci USA* 97 (9): pp. 4790-5.

SUFFNESS, M. & DOUROS, J. 1979. Drugs of plant origin. Methods in Cancer Research (26): pp. 73-126.

THIRMAN, M.J., GILL, H.J., BURNETT, R.C., MBANGKOLLO, D., McCABE, N.R. & KOBOYASHI, H. 1993. Rearrangement of the MLL gene in acute lymphoblastic and acutemyeloid leukaemia's with 11q23 chromosomal translocations". *N Engl J Med* 329 (13): pp. 909-914.

TRADITIONAL MEDICINE. http://www.who.int/mediacentre/factsheets/fs134/en/.

TYLER, V.E. 1997. Rational Phytotherapy, 3rd edition. Springer-Verlag, Berlin, Heidelberg, New York.

UCLA news May 2008-fruits, vegetables, teas may protect smokers from lung cancer.

VAN WYK, B. & VAN WYK, P. 1997. Field guide to the trees of southern Africa.

VAN WYK, B.E & GERICKE, N. 2000. Peoples plants. A guide to useful plants of Southern Africa.

VENTER, F. & VENTER, J.A. 1996. Making the most of indigenous trees.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd edition, E& S Livingstone LTD, London, pp.449-445.

WILLIAMS, V.L. 1996. The Witwatersrand muti trade, *Veld and flora* 82: pp. 12-14.

WORLD HEALTH ORGANISATION, 1996. Water and Sanitation: Fact sheet, vol. 112. Geneva, World Health Organisation.

WORLD HEALTH ORGANISATION, 2007. Tuberculosis, WHO, Fact sheet No. 104.

WORLD HEALTH ORGANISATION. Acute Respiratory Infections: *Streptococcus pneumonia*.

ZHENG, W. & WANG, S.Y. 2001. Antioxidant activity and Phenolic compounds in selected herbs. *J. Agricul.Food Chem*. 49: pp. 5165-5170.

ZIDENBERG-CHERR, S. & KEEN, C.L. 1991. Trace elements, micronutrients and free radicals. In Essential Trace Elements in Antioxidant Processes, pp. 107.

ZISHIRI, V.K. 2005. Potentising and application of a *Combretum woodii* leaf extract with high antibacterial and antioxidant activity. MSc Thesis. University of Pretoria, South Africa.