# Isolation and characterization of compounds from *Calodendrum capense* (Rutaceae) and *Lydenburgia cassinoides* (Celastraceae) for treatment of fungal and bacterial infections in immunocompromised patients

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Declaration

I Bellonah Motshene Sakong declare that the thesis hereby submitted to the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria for the degree of Magister Scientiae has never been submitted by me for a degree at this or any other university. The results obtained from this study are my own investigation, except where the work of others is specifically acknowledged.

Signed: .....

Dated: .....

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# List of Abbrevations

| | |
|---|---|
| AIDS | Aquired immune deficiency syndrome |
| MRSA | Methicilllin-resistant *Staphylococcus aureus* |
| HIV | Human immunodeficiency virus |
| PRSP | Penicillin-resistant *Streptococcus pneumonia* |
| VRE | Vancomycin-resistant enterococci |
| CNS | Central nervous nystem |
| Cc | Calodendrum capense |
| Lc | Lydenburgia cassinoides |
| C. a | Candida albicans |
| C. n | Cryptococcus neoformans |
| A. f | Aspergillus fumigates |
| T-helper cells | Thymus-helper cells |
| ROS | Reactive oxygen species |
| RNS | Reactive nitrogen species |
| $CD4^+$ | cluster of Differentiation Four Plus |
| ARVs | Antiretroviral |
| PAMPs | Pathogen associated molecular patterns |
| PRRs | Pathogen |
| TLR | TOLL-like receptor |
| NK | Natural killer |
| DCs | Dendritic cells |
| LPS | Lipopolysaccharide |
| O-PS | O-linked polysaccharide |
| TB | Tuberculosis |
| TNF | Tumour necrosis factor |

| | |
|---|---|
| *S. a* | *Staphylococcus aureus* |
| *P. a* | *Pseudomonas aeruginosa* |
| *E. c* | *Escherichia coli* |
| *E. f* | *Escherichia faecalis* |
| *M. tuberculosis* | *Mycobecterium tuberculosis* |
| *M. africanum* | *Mycobacterium africanum* |
| *M. bovis* | *Mycobacterium bovis* |
| *M. microti* | *Mycobacterium microti* |
| *M. canetti* | *Mycobacterium canetti* |
| MDR | Multi-drug resistant |
| XDR | Extremely resistant |
| GIT | Gastrointestenal tract |
| DNA | Deoxyribose nucleic acid |
| RNA | Ribose nucleic acid |
| IFN-γ | Interferon gamma |
| *T. gondii* | *Toxoplasma gondii* |
| *S. stercoralis* | *Strongyloides stercoralis* |
| /mm$^3$ | Per millimetre cubed |
| Kg/ml | Kilogram per millilitre |
| Ml/g | Millilitre per gram |
| NS | Non-steroidal |
| PUFA | Polyunsaturated fatty acids |
| α | Alpha |
| WHO | World Health Organisation |
| $^1O_2$ | Singlet oxygen |
| $O_2^{\bullet}$ | Superoxide |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| •OH | Hydroxyl radical |
| $O_3$ | Ozone |
| HOCl | Hypochlorous acid |
| NO• | Nitric oxide |
| $ONOO^-$ | Peroxynitrite |
| COX | Cyclooxygenases |
| LOX | Lipoxygenases |
| MAPKs | Mitogen-activated protein kinases |
| PPARs | Peroxisome proliferator-activated receptors |
| TM | Traditional medicine |
| TLC | Thin layer chromatography |
| MTT | (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium |
| DPPH | 2, 2-diphenyl-1-picrylhydrazyl radical |

# Abstract

Infectious diseases are a serious concern worldwide especially in immune-compromised patients. Some of these diseases are considered to be contagious and are spread via airborne transmission, while others are not contagious, i.e. non-communicable diseases. The problem is compounded by the emergence of pathogens resistant to currently used antimicrobial drugs. A wide range of microbes including bacteria, fungi, parasites, viruses and protozoans are implicated as causative agents of various diseases. Many patients without ready access to Western medical facilities rely on medicinal plants for the cure of various ailments including infectious diseases.

Two plant species used in South African traditional medicine for treating infectious diseases, namely *Calodendrum capense* Thunb. (Rutaceae) and *Lydenburgia cassinoides* N. Robson (syn. *Catha transvaalensis,* Celastraceae) were screened for antimicrobial activities against a range of fungi, bacteria and mycobacteria. The test organisms included *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *Enterococcus faecalis*, *Mycobacterium smegmatis* and *M. bovis* BCG.

The *L. cassinoides* acetone extract generally had good activity, with MIC values ranging from 0.04 to 0.15 mg/ml while the *C. capense* extract MIC values ranged from 0.31 to 0.62 mg/ml against the fungi. The hexane extract of *L. cassinoides* had good activity (MIC = 0.04 mg/ml) against *M. smegmatis* and the methanol extract had MIC = 0.16 mg/ml against *M. bovis* BCG. The two plant species had reasonable antibacterial activity against *S. aureus* and *P. aeruginosa*, with MIC values ranging from 0.16 to 0.32 mg/ml. Antibacterial activity against *Escherichia coli* and *Enterococcus faecalis* resulted in MIC = 0.63 mg/ml for both plants. However in the bioautography assay, the hexane extracts of *C. capense* and *L. cassinoides* had good activity against *S. aureus*, showing active zones of bacterial growth inhibition. The aim of this study was to isolate and characterize the active compounds, with emphasis on antifungal activity, from *Calodendrum capense* and *Lydenburgia cassinoides* that may be useful in treating opportunistic infections in immunocompromised patients.

Bioassay-guided evaluation of the antimicrobial active components of both hexane fractions using *C. neoformans* and *C. albicans* as test organisms led to the isolation and characterization of lupeol from *C. capense* and ß-amyrin from *L. cassinoides*. The MIC values of lupeol and ß-amyrin ranged from 1.5 to 6.2 µg/ml against all the tested organisms. Both compounds were also tested against a resistant strain

of *Candida albicans* which resulted in MICs of 3.2 and 6.2 µg/ml respectively. Crude extracts and compounds were also tested for cytotoxicity against human liver (C3A) cells. The crude plant extracts had a low cytotoxicity with average $LC_{50}$ values of 205.8 ± 8.38 µg/ml for *L. cassinoides* and 83.07 ± 44.66 µg/ml for *C. capense.* $LC_{50}$ values for the isolated compounds were greater than 200 µg/ml, the highest concentration tested. Selectivity index (SI) values were calculated using the formula SI = $LC_{50}$/MIC. The SI values of the crude extracts of the two plant species ranged between 0.18 and 0.91, showing that these extracts were relatively toxic compared to the antimicrobial activity as the SI values were less than 1. However, the compounds ß-amyrin and lupeol had good activity and low toxicity with SI values greater than 10.

In conclusion both plant species showed broad-based antimicrobial activity against the standard ATCC strains of bacterial and laboratory isolates of fungal pathogens. Purified compounds with very good antifungal activity and negligible detectable cytotoxicity, namely lupeol from *C. capense* and ß-amyrin from *L. cassinoides* (both pentacyclic triterpenoids) were isolated. This is apparently the first report of these two compounds from these two plant species. In our research group, lupeol has been isolated from various other plant species and it is known to have antimicrobial and anti-inflammatory activities. The second compound, ß-amyrin, reportedly has anti-inflammatory, antitumor, antibacterial, gastroprotective and hepatoprotective effects.

The findings from this study show that the two isolated compounds were highly active against fungal and bacterial pathogens, with the lowest MIC value of 0.015 mg/ml. Both compounds showed much better selectivity index values with regard to antifungal activity compared to those of the crude extracts. The compounds also had good activity against the two *Mycobacterium* strains tested, indicating potential application in antimycobacterial therapy. The results may validate to an extent the use of these two plants as anti-infectious agents in traditional medicine. The compounds have potential for development into therapeutic agents, but various factors will need to be investigated further, including *in vivo* efficacy and safety, as well as other aspects such as mode of administration.

Keywords: *Lydenburgia cassinoides*, *Calodendrum capense*, microbial pathogens, β-amyrin and lupeol.

# Conferences and Proceedings

South African Association of Botany (SAAB), 15-18 January 2012 at Pretoria University Hatfield Campus

Indigenous Plant Use Forum (IPUF),2-5 July 2012 at University of Venda, Limpopo Province, South Africa .

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# Chapter 1

## Introduction

### 1.1 Infectious disease challenges in immunosuppressed patients

Infectious diseases are illnesses caused by pathogens or their toxins that are transmitted to the host through various mechanisms such as contaminated food, water and/or direct contact with infected hosts. Infectious diseases have a range of aetiology including bacteria, fungi, parasites, viruses and protozoans (Hotez et al., 2007). Some of these diseases are considered to be contagious (and are passed on via airborne transmission) while others are not contagious (non-communicable diseases). It is also possible for disease to pass indirectly through unhygienic conditions or from animals to people in which case they are known as zoonotic diseases. Within the large categories of infectious organisms are numerous mechanisms of transmission and symptoms. Infectious diseases are the major cause of morbidity, mortality and economic disruption for millions of people worldwide (Morens 2004; McIntosh 2004). Despite the advancement in orthodox medicine many people are still vulnerable to infection due to non-accessibility to adequate health care, especially in developing countries where co-infection is common (Basch, 1991). The adverse impact of infectious diseases is most severe among the poor populace who have few resources to attend hospital (Chatelain et al., 2009). Approximately 15 million people die annually due to infectious diseases, with children under the age of five (5) and the elderly (older than 60 years) being particularly vulnerable (Cullinan, 2006). The four leading infectious diseases in humans and animals are pneumonia, diarrhoea, tuberculosis and malaria, with high rates of disability or death (Louw, 2002).

Many infectious pathogens make the body more vulnerable to secondary infections by weakening of the body's immune system (where the host becomes immunosuppressed or immunocompromised). Other organisms then invade the host to take advantage of the weakened immune system to cause various health problems commonly referred to as opportunistic infections (Chatelain et al, 2009). An immunocompromised host has altered phagocytic, cellular, or humoral immunity that increases the risk of an infectious complication or an opportunistic process. Severe immunosuppression can be the result of human immunodeficiency virus (HIV) infection, congenital immunodeficiency, leukaemia, lymphoma, generalised malignancy or therapy with alkylating agents, anti-metabolites, radiation, or large doses of corticosteroids (2 mg/kg body weight) or 20 mg/day of prednisolone, hyposplenism and renal failure.

Patients may also be immunocompromised due to breached skin or defective mucosal defence barriers that permit microorganisms to cause either local or systemic infection (Tlaskalová-Hogenová 2011). Old age causes dysfunction of the immune system (immunosenescence) that contributes to an increased susceptibility of the elderly to infection and also contributes to autoimmune disease and cancer.

The major microorganisms associated with opportunistic infections include viral, bacterial, parasitic and fungal pathogens. The high morbidity and mortality rate due to acquired immunodeficiency syndrome (AIDS), an immunosuppressive disease, as a result of life-threatening opportunistic infections and sometimes undefined malignant tumour is still a great challenge in healthcare delivery systems (Garbino et al., 2001). However the mortality rate of immunocompromised patients is declining due to an increase in the understanding of disease mechanisms, awareness and a positive way of living.

Antibiotic-resistant infectious diseases have created a public health crisis worldwide. Infectious diseases are responsible for one-quarter of all the deaths in the world, second only to cardiovascular diseases. They are associated with the new strains of bacteria and viruses emerging within the past twenty-five years, which are also highly resistant to drugs and antibiotics such as methicilllin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumonia* (PRSP) and vancomycin-resistant enterococci (VRE) ((Fyhrquist et al., 2002).

Infections become more difficult to treat, the severity of illness increases, as does the duration of infectiousness, adverse reactions, the length of convalescence and costs. The screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective compounds. The antimicrobial compounds from plants may inhibit bacteria through different mechanisms than conventional antibiotics, and could therefore be of clinical value in the treatment of resistant microbes (Fyhrquist et al., 2002; ISIS Report, 2001) and medicinal plants have been part and parcel of human society to combat diseases from the dawn of civilization (Biswas et al., 2002).

*Candida albicans* and *Cryptococcus neoformans* are the predominant opportunistic invasive fungal pathogens from the group of yeasts associated with life-threatening infections in immunocompromised hosts (Fan-Harvard et al., 1991). *Candida albicans* organisms usually exist as commensals in the gastrointestinal tract and genital tract of healthy hosts and become pathogens when the host's resistance to infection is impaired locally or systemically. *Cryptococcus* species colonise human beings through the respiratory and gastrointestinal tracts. The most common sites of infection are the bloodstream and CNS, followed by pulmonary sites and the skin, eyes and gastrointestinal tract.

The treatment of infectious diseases is becoming a general health problem and is more challenging in immune-suppressed patients due to the emergence and spread of resistant strains of pathogenic organisms. Pathogens resistant to conventional antibiotic, anti-parasitic and antiprotozoal drugs have developed largely because of the indiscriminate use and abuse of such medications (Debruyne, 1997; Traeder et al., 2008). As a result many anti-infectious drugs used in controlling these organisms previously are no longer efficient. Therefore there is an urgent need for new, efficient, affordable and safer anti-infectious agents to combat the increasing cases of infectious diseases in human and animal.

Many known antibiotics are natural products. Medicinal plants and other natural product sources such as fungi have provided a wide range of therapeutic drugs useful in humans and animals, e.g. pneumocandins, penicillin and terrequinone (Vincente et al., 2003; Drosten 2003). The therapeutic properties of medicinal plants are associated with the presence of diverse phytochemicals useful in healing various diseases.

The uses of medicinal plants are recognised as an important part of the culture and tradition of mankind worldwide. Most of the population in urban South Africa as well as smaller rural communities rely on herbal medicines for their health care needs (Mander, 1998). Apart from the cultural significance, the popularity of medicinal plants among rural dwellers is generally due to their accessibility and affordability (Balick et al., 1994). As a consequence, there is an increasing move worldwide to integrate traditional medicine with primary health care (Akerele, 1987).

In South Africa, the use of orthodox therapies functions alongside traditional medicines to heal many ailments (Elujoba et al., 2005). Many of the medicinal plants used in South African traditional medicine have been investigated for their therapeutic effectiveness against opportunistic infectious pathogens (Mathabe et al., 2006; Buwa and Van Staden, 2006).

World-wide, 80% of the community uses medicinal plants for their primary health care. Many of the plants are used for treating fungal and bacterial infections. Plants are therefore an important resource for discovering antimicrobial compounds. In a wide random screening Mokoka et al. (2010) found that two plant species *Calodendrum capense* and *Lydenburgia cassinoides* had promising antifungal activity and high total activities against *Cryptococcus neoformans*. The MIC values for the crude extracts averaged 0.07 to 0.09 mg/ml. The biologically active components of these species were not yet isolated and characterised, and this formed the rationale for selecting these two plant species for further in-depth evaluation in this study.

Renewed interest in traditional medicines has meant that researchers are concerned not only with determining the scientific rationales for plant use, but studies are also aimed at discovering novel compounds of pharmaceutical value. Several methods are applied in the identification, isolation and characterization of bioactive compounds from medicinal plants and other natural sources through bioactivity-guided screening (Ley and Baxendale, 2002).

### 1.2 Problem statement

HIV patients are very susceptible to opportunistic fungal infections, which reduce the quality and potentially the duration of life. Many infectious fungi are resistant to currently used antifungal agents, making it difficult to treat fungal infections. The currently used antifungal agents also often have severe side effects. Plant extracts may help to resolve this problem by acting as a source of novel antifungal compounds with potentially fewer side effects, good activity and lower toxicity. *Calodendrum capense* and *Lydenburgia cassinoides* may be useful in inhibiting the growth of *Candida albicans* and *Cryptococcus neoformans*, and may be a source of interesting antifungal compounds with high activity and low cytotoxicity.

### 1.3 Hypothesis of the study

Plants extracts have chemical compounds that may inhibit the growth of opportunistic infectious microorganisms in immunocompromised patients.

### 1.4 Aim

To isolate and characterize antifungal compounds from *Calodendrum capense* and *Lydenburgia cassinoides*, plant species with known antifungal efficacy, that may be useful in treating fungal infections caused by *Candida albicans* and *Cryptococcus neoformans* as well as other infections in immunocompromised patients.

### 1.5 Objectives

1. To evaluate the antimicrobial activity, with emphasis on *Candida albicans* and *Cryptococcus neoformans,* of *Calodendrum capense* and *Lydenburgia cassinoides* leaf extracts

2. To determine the number of antimicrobial compounds present in the extracts using bioautography

3. To potentiate the activity of the extracts through fractionation using solvents of varying polarities

4. To determine the cytotoxicity of the extracts and the fractions

5. To isolate the active compounds from the potentiated fractions

6. To characterize the isolated compound(s)

7. To test the isolated compounds for antimicrobial activity and cytotoxicity

# Chapter 2: Literature review

## 2.1. Conditions and mechanism of immune suppression

Many human and animal illnesses are caused by infectious pathogens which are able to survive, multiply and exert their deleterious activities after overcoming the protective mechanisms of the host's immune system. Some of the disease aetiologies do not cause recognised illness but facilitate immune system dysfunctions to provide a good environment for opportunistic pathogens such as fungi, bacteria, viruses and parasites to infect and survive in hosts (Mehmood et al., 1999). The invading organisms may injure the host, resulting in various forms of health disorders.

The immune system is made of a network of cells, tissues, and organs that constitute the body's defence mechanism which fights against infectious organisms and negative stimuli. This includes white blood cells, or leukocytes, and lymphocytes called thymus (T)-helper cells that work together to protect the body (Salgame, 1991). Leukocytes made of neutrophils and macrophages circulate through the body between the organs and nodes via lymphatic vessels and blood vessels. In this way, the immune system works in a coordinated manner to monitor the body for germs or extraneous substances that cause infection or cell injury. In the presence of a pathogen, the leukocytes release the activated neutrophils and macrophages producing reactive oxygen species (ROS), reactive nitrogen species (RNS) and inflammatory mediators (eicosanoids and cytokines) as microbicidal agents in the phagocytosis process (Tipoe 2007). Efficient elaboration of the phagocytic agents during infection and proper dissipation of the same after the removal of the offending organisms is the optimal functionality of the immune system. However, any disturbance of the delicate balance between generation and dissipation of phagocytic agents can result in immune disorders.

## 2.2. Human Immunodeficiency Virus (HIV) infection

HIV infection disrupts tissue homeostasis as it directly affects the host immune system by killing $CD4^+$ T cells. Decreased $CD4^+$ T cell numbers affect the individual microbiota composition. Immune deficiency resulting from HIV infection and use of immune suppressant therapy is referred to as either secondary or acquired immunodeficiency, in which case the levels of lymphocyte T-helper cells are diminished. With low levels of T-helper cells, the immune system is unable to defend the body against normally harmless organisms, which can cause life-threatening infections

(opportunistic infection). HIV patients are also vulnerable to an increased risk of immune hypersensitivity disorders with associated enteropathy effects. HIV patients are susceptible to various diseases such as diarrhoea, tuberculosis, cancer, and oral candidiasis (Baker and Graham, 2002). The level of T-helper cells in the body are quantified as clusters of differentiation four (CD4) count, which is a measure of a certain type of protein in the blood critical to the body's ability to produce an efficient immune response to infection (Bettens et al., 1989). As HIV progresses, a person's $CD4^+$ count will be lower, indicating that the presence of the virus is stronger. The $CD4^+$ count is usually expressed in terms of the number of cells/$mm^3$ of blood. A person who is free of HIV normally has around 750 T-cells, while someone who is HIV positive may have a $CD4^+$ count of 500 cells/$mm^3$ T-cells. If the person's T-cells are below 200, it is clearly an indication of serious immune damage.

Life-long therapy with antiretroviral drugs (ARVs) is the only current treatment with established efficacy against HIV infection. Antiretroviral therapy successfully leads to re-establishment of the $CD4^+$ T-cell population. It is estimated that less than 50% of the patients requiring ARVs are actually receiving the drugs and treatment sites are located in towns and urban centres, whereas access to treatment in rural areas remains disproportionately low. These shortcomings in the distribution of ARVs and drugs to treat opportunistic ailments, as well as the problems of resistance development to currently used anti-infective agents, results in a need for alternative therapies which may be provided by medicinal plants. In conventional medicine, ARV's are only offered to decrease the virus load while traditional medicine aims to boost the immune system and to treat the opportunistic infections. HIV infection may basically be asymptomatic for extended periods and most patients seek treatment only at the later stages when AIDS-related symptoms and opportunistic infections occur.

### 2.3. Mechanisms of immune system invasion by opportunistic microbial pathogens

Vulnerability to infection and disease development, including the progression and severity of disease, result from the interplay of several factors such as age, nutritional status and genetics, all of which are dependent on the integrity of the immune system. The immune system continuously works to limit pathogen invasion and tissue damage through natural (innate) and acquired (adaptive) immunity, both of which intimately interact with each other by close cooperation between their cellular components (Borghetti et al., 2009). The first defensive line of protection against infectious organisms is provided by the innate response which is activated immediately after infection. An

important aspect of immunology is the early recognition of pathogens by innate immune cells through pattern recognition receptors (surface, humoral and cytoplasmic PRRs) binding pathogen associated molecular patterns (PAMPs) that function as "danger signals". The major pathway in this process is based on the TOLL-like receptor (TLR) expression on the surface of innate and inflammatory cells [neutrophils, macrophages, endothelial cells, natural killer (NK) cells, and dendritic cells (DCs)]. The concerted activity of epithelial, endothelial, inflammatory and innate cells expressing TLRs constitutes the first barrier against pathogens at the site of infection (Borghetti et al., 2009).

For successful resolution of infection, an efficient innate, inflammatory and adaptive immunity is required to block pathogen survival, replication and invasion, as well as to facilitate the tissue clearance of the pathogens and/or infected cells. However, most pathogenic microorganisms have various diverse mechanisms of invading the elaborate body defence strategy to proliferate and cause harm to the tissues, resulting in a disease state. On the other hand, immunosuppression leads to increased susceptibility to infection, and consequently disease development. The mechanisms of infection by pathogenic microorganisms include:

- Attachment: Attachment of microbes to host tissues involves complex interactions between molecules present on the cell surface of the host and the pathogen, through which the pathogen sticks to the host cell. Typical microbial factors involved in this step are the pili, flagellae, exopolysaccharides, lipopolysaccharide (LPS) and outer membrane proteins. This determines the effect of microbial pathogens on the tissue tropism and subsequent infection. The attachment to epithelial surfaces is a critical step for most microbial pathogens to cause an infection (Wilson et al., 2009).

- Microbial adherence: Adherence is a cell- and tissue-dependent process of microbial ability to somehow withstand the normal flux or flow within the particular tissue and to ''stick'' to the surfaces of the intended target. This phenomenon ensures a close contact between the host and microbial cells as one of the first steps in the infection process (Shoaf-Sweeney and Hutkins, 2009). In recognition of this, elucidation of the mechanisms involved and identification of agents which may interfere with, or potentially block the process, is essential. The approach may include the use of sub-inhibitory concentrations of antibiotics, isolated anti-adhesions, and non-antibiotic antimicrobial agents including plant extracts (Jones et al., 1997).

- Invasion of mucosa: Epithelial cell invasion by enteric bacterial pathogens can involve the activation or inhibition of different signal transduction pathways in host cells (Cossart et al., 2003). Manipulation of host signaling pathways affects the host cell response to a variety of external stimuli such as hormones, cytokines, neurotransmitters or pathogenic microbes. These processes assist the organism to invade the defence mechanisms mounted by the immune system of the host. Invasive microbial pathogens trigger an orchestrated signal transduction cascade in host cells that results in bacterial internalization and pathogenesis (Hu et al, 2006).

- Colonization: Tissue colonization by pathogenic microbes involves the proliferation of such organisms on the surface to form a colony. This process is consequently followed by haematogenous spread and systemic toxicity which is responsible for triggering the host immune response (Fernandez et al, 1999). After successful colonization, invading microbes may be eliminated spontaneously or due to the host immune defence mechanism and/or pharmacological intervention. However, disease may occur as a direct consequence of infection if there is sufficient damage to the host causing altered homoeostasis. Disease may also result when the degree of host damage resulting from the state of colonization is sufficient to alter homeostasis, leading to clinically detectable symptoms (Casadevall and Pirofski, 2002).

❖

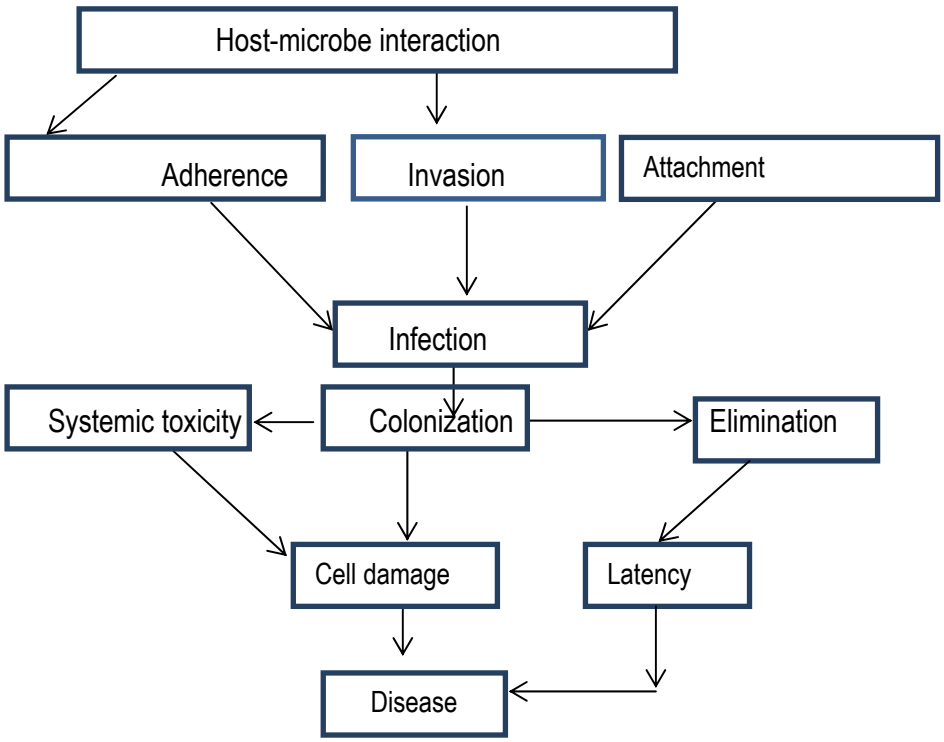


**Figure 2.1.** Schematic diagram of disease outcome from microbial–host interaction (modified from Pirofski and Casadevall, 2002)

### 2.4. Opportunistic infections in immunocompromised patients

Some organisms are opportunistic pathogens because they do not cause disease unless offered an unexpected chance by failure of the host defence system. The four broad categories of opportunistic infectious microorganisms involved in pathogenesis of various forms of diseases in immunocompromised individuals include bacteria, fungi, viruses and parasites.

#### 2.4.1. Bacterial pathogens

Bacteria are microscopic, single-celled organisms classified as prokaryotic cells due to the absence of a true nucleus. The prokaryotic nucleus lacks a nuclear membrane and the genetic constitution is contained in a single linear chromosome. These microorganisms are also classified according to their shape and grouping. Rod-like cells are referred to as bacilli, which occur in pairs as diplobacilli or chains as streptobacilli, spherical or ovoid cells are commonly referred to as cocci, which occur in

pairs as diplococci, in chains as streptococci or in clusters as staphylococci. The comma shape is referred to as vibrious.

Microbes in general and bacteria in particular are classified on the basis of oxygen use as aerobic (use oxygen) and anaerobic (do not use oxygen). A microbe that uses oxygen when available but can live anaerobically in the absence of oxygen is said to be a facultative aerobe. Those microbes that must have oxygen to survive are called obligate aerobes, while those that can grow only in the complete absence of molecular oxygen are called obligate anaerobes.Bacteria are also classified into two groups according to how they appear when stained in the laboratory using the multi-step Gram stain. One group of bacteria has cell walls with many layers of peptidoglycan interwoven with both lipotechoic and techoic acid. These relatively thick cell walls retain the dark purple colour imposed by the initial step of the staining procedure despite undergoing a later decolorizing step and counterstaining with the red dye safranin. Bacteria staining purple in this fashion are called “Gram-positive” bacteria. The other group, called “Gram-negative” bacteria, have a cell wall with a thin peptidoglycan layer that loses its integrity during the staining procedure, allowing decolourization to occur. The purple colour vanishes, allowing the cells to stain red with safranin (Feske 2006) As well as peptidoglycan, the Gram-negative cell wall has an outer plasma membrane containing lipopolysaccharide macromolecules composed of Lipid A (conserved among all Gram-negative species) and an O-linked polysaccharide (O-PS; variable among Gram-negative species)

Functionally, the O-linked polysaccharide confers virulence (the ability to infect host cells) on the bacterium. It is the Lipid A moiety that functions as an endotoxin capable of inducing endotoxic shock. Gram-negative and Gram-positive bacteria can also be distinguished by their differential susceptibility to physical assaults as well as various enzymes and drugs. For example, Gram-positive bacteria resist dry conditions better than Gram-negative bacteria and cannot be crushed as readily. Gram-positive bacteria are more resistant to lysozyme digestion and killing by streptomycin and tetracycline, but are highly susceptible to penicillin, anionic detergents, and basic dyes. In contrast, Gram-negative bacteria resist lysozyme digestion and killing by penicillin but dry out easily, are inhibited by basic dyes, and are susceptible to killing by tetracycline.

The three most common bacterial opportunistic diseases which influence morbidity and mortality in immunosuppressed patients are pneumonia, tuberculosis, and diarrhoea/enteritis.

- Pneumonia: Respiratory complications such as bacterial pneumonia are common in HIV patients with an overall rate of six times greater than the general population. A frequently

encountered pathogen is *Streptococcus pneumoniae*. Other species include *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Moraxella cattarhalis, Nocardia asteroides* and *Serratia. marcescens*. The clinical signs of these infections include productive cough, fever, dyspnoea, chest pain and weight loss.

- Tuberculosis: Tuberculosis is one of the commonest causes of morbidity and the primary cause of death in HIV-infected adults in sub-Saharan Africa, with southern Africa being the epicentre of the dual epidemic. Nine countries (South Africa, Swaziland, Lesotho, Namibia, Botswana, Mozambique, Zambia, Zimbabwe and Malawi) account for nearly 50% of the global TB/HIV burden. In comparison to HIV-free patients, people living with HIV are 20 times more likely to develop tuberculosis caused by *Mycobacterium tuberculosis* (Lawn and Zumla, 2011). Immunosuppressive drugs such as corticosteroids and tumour necrosis factor (TNF) antagonists for the treatment of rheumatological disorders have also been associated with a high risk of tuberculosis.

The genus *Mycobacterium* is a complex group of organisms which include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, and *M. canetti. M. tuberculosis* is an obligate intracellular pathogen that can infect several animal species, although human beings are the principal hosts. It is an aerobic, acid-fast, non-motile, non-encapsulated, non-spore forming bacillus. It grows most successfully in tissues with high oxygen content, such as the lungs.

Multi-drug resistant (MDR-TB) is caused by *Mycobacterium tuberculosis* that is resistant to at least isoniazid and rifampicin, and extremely resistant (XDR-TB) is caused by MDR tuberculosis strains that are also resistant to any fluoroquinolone and one of three injectable aminoglycosides (capreomycin, kanamycin, and amikacin).

- Gastro-intestinal disorders: Gastrointestinal infections caused by microbial pathogens in patients with low immunity may result in symptoms related to disorders such as diarrhoea, dysentery, intestinal inflammation and abdominal pains or cramps with or without vomiting. A broad range of potential aetiological agents include bacteria (*Campylobacter* species, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, *Clostridium difficile*, *Salmonella* species and *Staphylococcus aureus*), protozoa (*Cryptosporidium parvum*, *Giardia lamblia*) or viruses (rotavirus). In AIDS patients, *Cryptosporidia*, *Microsporidia* and *Isospora* species are the most common causes of diarrhoea and wasting syndrome (Dupont and Marshall, 1995). The

mechanisms of diarrhoeal disease in HIV/AIDS seropositive patients are enteric infection by pathogenic microbes (opportunistic enteric infection) and GIT architectural alteration secondary to local HIV infection (HIV enteropathy) (Dupont and Marshall, 1995).

### 2.4.2. Fungal pathogens

Fungi are eukaryotic organisms that can exist comfortably outside a host but that will invade and colonize a host if given the opportunity (Bastert, 2001). Fungi are either single-celled (yeasts) or multicellular (moulds). Fungal infections are well-known, causing life-threatening complications associated with morbidity and mortality in immunocompromised patients. For example, chemo- and radiotherapies for leukaemia treatment or organ transplantation are associated with *Aspergillus* infections, whereas HIV infection is more often associated with *Candida albicans*, *Pneumocystis carinii* and *Cryptococcus neoformans* infections. The diseases caused by this latter group of pathogens are referred to as AIDS-defining diseases. HIV positive patients have developed resistance to treatment with fluconazole, the currently used antifungal agent. In some cases, resistance to fluconazole triggers cross-resistance to other azoles or a pathogen shift from *Candida albicans* to less sensitive species such as *Candida glabrata* and *Candida krusei* (Bastert, 2001). Despite the existence of strong antifungal agents, the appearance of resistant or multi resistant strains imposes the need for a permanent search and development of new drugs (Silver and Bostian, 1993).

- Candidiasis is the most common opportunistic fungal infection in immunocompromised patients with *C. albicans* being the most frequently isolated species, followed by *Candida glabrata* and *Candida tropicalis. Candida* is a known colonizer of the human gastrointestinal tract. *Candida* infection may arise after liver transplantation because conditions that support super colonization or *Candida* overgrowth in the gut could promote the translocation of fungus to the extra luminal areas, resulting in subsequent intra-abdominal infections and further dissemination. *Candida* infections usually present as intra-abdominal abscesses, recurrent cholangitis due to biliary strictures, and peritonitis, all of which may be accompanied by fungemia.

- Invasive aspergillosis is the second most common fungal pathogen responsible for infections (Liu et al., 2011), the inhalation of airborne spores results in pulmonary infection, with extra pulmonary dissemination to the central nervous system (CNS) and virtually any other organ. The invasive aspergillosis-related mortality rate for these patients exceeds 90%.

- Cryptococcosis caused by *Cryptococcus neoformans* (unicellular yeast) is the third most commonly encountered pathogen as a result of the failure of the host's defences to contain the organism after inhaling aerosolized spores from an environmental source, and it manifests as symptomatic pneumonia or asymptomatic infection. The major *C. neoformans* infection sites in immunocompromised people include the CNS and lungs, but this microorganism can also infect other organs and disseminate to multiple sites. Clinical manifestations of infection included pneumonia only (46%), meningitis only (36%), dissemination to multiple distant organs (11%), or involvement of another single organ (e.g., lymph node) (7%). Cryptococcosis-associated mortality is estimated to be 25% and the infection usually presents with fever, cough, dyspnoea, haemoptysis and chest pain (Aguirre et al, 1995).

### 2.4.3. Viral pathogens

Viruses are acellular particles consisting of a protein coat (called a capsid) encasing a genome of either DNA or RNA. Due to their small size, viruses can only be observed with an electron microscope. To propagate itself, a virus enters the host cell with potent protein synthesis machinery which it can exploit to evade the immune system. Virus infections are characterized with a short time or long lasting generalized suppression of the host immune response. The high morbidity and mortality rate of virus infections is primarily as a consequence of opportunistic secondary infections (Mathers and Loncar, 2006) from defective production of type I interferons (IFN-γ). Some of the opportunistic viral infection pathogens include cytomegalovirus, herpes and hepatitis B and C virus.

### 2.4.4. Parasitic pathogens

Parasites are eukaryotic organisms that possess several chromosomes contained in a membrane-bounded nucleus. All parasites share the characteristic of being dependent on a host organism for both habitat and nutrition at some point in their life cycles, usually damaging the host but not killing it. The major classes of parasites are the protozoa, which are single-celled organisms, and the helminthic worms, which are multicellular. Some parasites are a few micrometres long while others are several meters in length. These parasites include *Toxoplasma gondii*, *Strongyloides stercoralis*, *Giardia lamblia*, *Cryptosporidium parvum*, and *Entamoeba histolytica*. Parasitic infections are more likely to cause prolonged diarrhoea in immunocompromised patients. The most common clinical presentation of *T. gondii* infection among people with compromised immune systems is focal encephalitis. *S. stercoralis* can produce overwhelming infection in immunocompromised persons

such as strongyloidiasis with clinical signs of non-inflammatory diarrhoea associated with eosinophilia.

### 2.5. Management of opportunistic infections and associated challenges

A $CD4^+$ count of lower than 200 cells/$mm^3$ is a threshold that puts immunocompromised individuals at greater risk of contracting opportunistic infections. There are various methods available for prevention and treatment of opportunistic infection such as living a healthy life, immune boosting or use of therapeutic regimes targeting the killing and removal of offensive pathogens.

#### 2.5.1. Immune system modulation

A healthy immune system is essential to ward off opportunistic infections pathogens as the host immune response plays a critical role in disease pathogenesis and illness-related outcomes. Immunomodulatory intervention in immune-compromised individuals may include immunosuppression (as in the case of organ transplants or inflammatory disorders) in which the immune suppression therapies are targeted at reducing proliferative responses of immune cells and also at repairing the impaired immunity. Alternatively immune stimulation may be employed (as in the case of HIV infections or full blown AIDS) where drugs are required for the proliferation of immune cells and the increased production of pro-inflammatory cytokines.

#### 2.5.2. Infection control

The application of antimicrobial drugs targeted at specific disease-causing pathogens can be useful in the management of opportunistic infections in HIV/AIDS patients. The use of antibiotics for treating most microbial infectious diseases is still the most successful intervention, despite the emergence of antibiotic-resistant strains (Rehalison et al., 1994). Opportunistic microbes have been characterised as the organisms which only cause disease in the host because of weakened immunity (Rehalison et al., 1994). However, these microbes lack unifying characteristics except that they cause disease in immunocompromised hosts. Oral fluoroquinolone therapy is generally administered to adult HIV patients with diarrhoeal symptoms to ensure that susceptible bacterial enteropathogens are excluded as the cause of the disease. In the absence of identifiable pathogenic aetiology, palliatives such as loperamide, diphenoxylate hydrochloride and tincture of opium can be useful.

### 2.5.3. Inflammatory optimization

The inflammatory process is a complex interaction of both the pro- and anti-inflammatory phases induced in the immune system to fight pathogens or adverse stimuli. Vigorous inflammatory responses provoked by invading microbes facilitate immediate recognition and eradication of pathogenic organisms. However, it can also be detrimental to the host if an optimal balance between the pro-inflammatory and anti-inflammatory reactions is disrupted. The pro-inflammatory phase induces pain, swelling, redness and heat, which are clinical signs of cellular destruction, while anti-inflammatory mechanisms of the inflammation process are involved in cellular repair and regeneration. The disproportionate treatment of inflammation is an important clinical medication for immunosuppression in order to alleviate pain, fever or tissue damage associated with inflammatory disorders. These two opposing mechanisms are continually balanced to maintain molecular wellness and overall wellbeing of humans and animals.The use of anti-inflammatory drugs like paracetamol and aspirin, regarded as safe and therefore regularly used even in early life, may also influence the immune system development (Colebatch et al., 2011).

A diverse arsenal of anti-inflammatory agents such as glucocorticoids (e.g. hydrocortisone/cortisone), non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit the biosynthesis of eicosanoids from polyunsaturated fatty acids (PUFA) of the membrane phospholipids, and tumour necrosis factor alpha (TNF-α) monoclonal antibodies (e.g. infliximab, adalimumab, golimumab) are used clinically to treat inflammation. Chronic application of these drugs leads to immune suppression, however. Immunosuppressive drugs used in organ transplantation, autoimmune diseases and chronic inflammatory conditions like arthritis include glucocorticoids such as cyclosporine and atacrolimus (Sharma 2010). For healthy living, the inflammatory response needs to be balanced and optimized in immunocompromised patients with adequate pharmacological intervention.

### 2.5.4. Nutritional status

Malnutrition is one of the major causes of decreased immune functionality generally and in immunocompromised patients in particular. It is also a major risk factor in infection while chronic infection is itself an important cause of malnutrition, because it increases metabolic demand over a long period. Inadequate nutrition intake deprives the immune system of the components needed to generate an effective immune response. The immune response, however, influences nutritional status, as some immune mediators such as TNF-α have a profound influence on nutrient absorption and metabolism.

Nutritional deficiency impairs cell-mediated immunity, and also decreases phagocyte function, cytokine production, secretory antibody affinity and response, and impairs the complement system. Immune disorders in response to nutritional deficiency range from increased opportunistic infections and degenerative diseases to suboptimal responses to vaccinations. Malnutrition is recognised as the risk factor for illness and death (Patel 2003). These immune changes predispose children to severe and chronic infections, most commonly infectious diarrhoea which further compromises nutrition causing anorexia, decreased nutrient absorption, increased metabolic needs and direct nutrient lossesTherefore, supplementation of food intake with essential micronutrients enhances the immune system and reduces susceptibility to opportunistic infections.

### 2.5.5. Prophylaxis

Identification of risk factors associated with opportunistic infections or diseases in immunosuppressed patients should facilitate timely intervention via the use of prophylactic agents. This helps in preventing the development of an overall incidence of proven microbial infections, and reduces the associated health care burden attributed to opportunistic infectious diseases such as morbidity and mortality.

Antimicrobial properties of pharmaceutical drugs and phytochemicals derived from food intake against pathogenic organisms can be beneficial in enhancing the immune reaction. Antibiotics are frequently used to counteract specific pathogens and serve as the most effective pharmacological intervention during infectious diseases. However, the emerging threat of drug resistant microbial strains is a serious concern using this method to manage opportunistic infections in immune-compromised people. Antibiotic treatments might also disturb the microbial community and homeostasis of the gut and may subsequently lead to an innate or adaptive immune reducing response towards infectious pathogens (Cattelan et al., 2004). The commensal microbial community is also recognised as being essential in shaping the immune response. This can be accomplished by oral administration of bacterial strains (probiotics) or by the use of special oligosaccharides that promote the growth of particular bacterial strains in the gut (prebiotics). Prebiotic oligosaccharides might act indirectly through microbiota-dependent mechanisms (i.e. rebalancing the microbiota composition in the gut) and/or have a direct effect via activation or inhibition of cellular receptors on immune competent cells (Jutel2009).

## 2.6. Use of medicinal plants in opportunistic infectious diseases

Aside from the application of orthodox drugs in immunocompetent and immunosuppressed individuals for treatment of various diseases, both infectious and non-infectious, medicinal plants also feature as alternative therapy. Medicinal plants form an important and integral part of the culture and tradition of many people worldwide. Today, most of the population in urban South Africa as well as smaller rural communities rely on herbal medicines for their health care needs. In addition to the cultural value, increased uses of medicinal plants occur due to their accessibility and affordability (Mander, 1998).

The increasingly widespread use of traditional medicine has prompted the WHO to encourage the development of national policy and regulations as essential indicators for the integration of such practice into national health care systems (Farnsworth, 1994). These issues pose challenges to scientists in validating the efficacy, and standardizing, of plant extracts used for a particular ailment with a view towards discovering any novel compounds of pharmaceutical value. The availability of new drugs with different mechanisms from currently used drugs that possibly also shorten the course of chemotherapy would improve patient adherence and affordability, thus enabling more favourable treatment outcomes of infectious diseases. In addition, alternative drugs are needed to counteract the spread of drug resistant microbes which threaten global control programmes against contagious diseases. In order to reduce time and material wastage on trial and error, as in random screening procedures, ethnopharmacological knowledge helps scientists to target plants that are medicinally useful (Balick et al., 1994).

Many drugs used in modern medicines have their origins in plants that were often used in the treatment of illness and diseases (Ram et al., 2004).The plant materials used include seeds, berries, roots, leaves, bark or flowers. The phytochemicals present in plant extracts of pharmacological relevance are broadly classified as phenolics, terpenoids, peptides, carbohydrates and alkaloids. In fact, plants and their derivatives contribute to more than 50% of drug needs worldwide, therefore traditional healers play an important role in developing natural medicines, as many drugs used in conventional medicine were originally derived from medicinal plants. (Balick et al, 1994). These include salicylic acid (precursor of aspirin) originally derived from white willow bark and the meadow sweet plant (*Filipendula ulmaria* (L.) Maxim).

Quinine and artemisinin (antimalarial drugs) are derived from *Cinchona pubescens* bark and *Artemisia annua* respectively. Vincristine (anticancer drug) is derived from periwinkle (*Catharanthus roseus*). Morphine, codeine and paregoric, derived from the opium poppy (*Papaver somniferum*),

are used in the treatment of diarrhoea and for pain relief. Some of the mechanisms through which medicinal plants exert their usefulness as therapeutic agents include antimicrobial, anti-inflammatory, antioxidant and immunomodulatory activity (Gurib-Fakim, 2006).

### 2.6.1. Antimicrobial activity

Plants and their extracts have been used by indigenous peoples for the treatment of infectious diseases for centuries. As a consequence of the complex, diverse composition of plant extracts, their use as antimicrobial agents provides a low risk of resistance development, making microbial adaptability very difficult. The potential antimicrobial effects of plant extracts on various infectious disease pathogeneses are outlined in Figure 2.2.

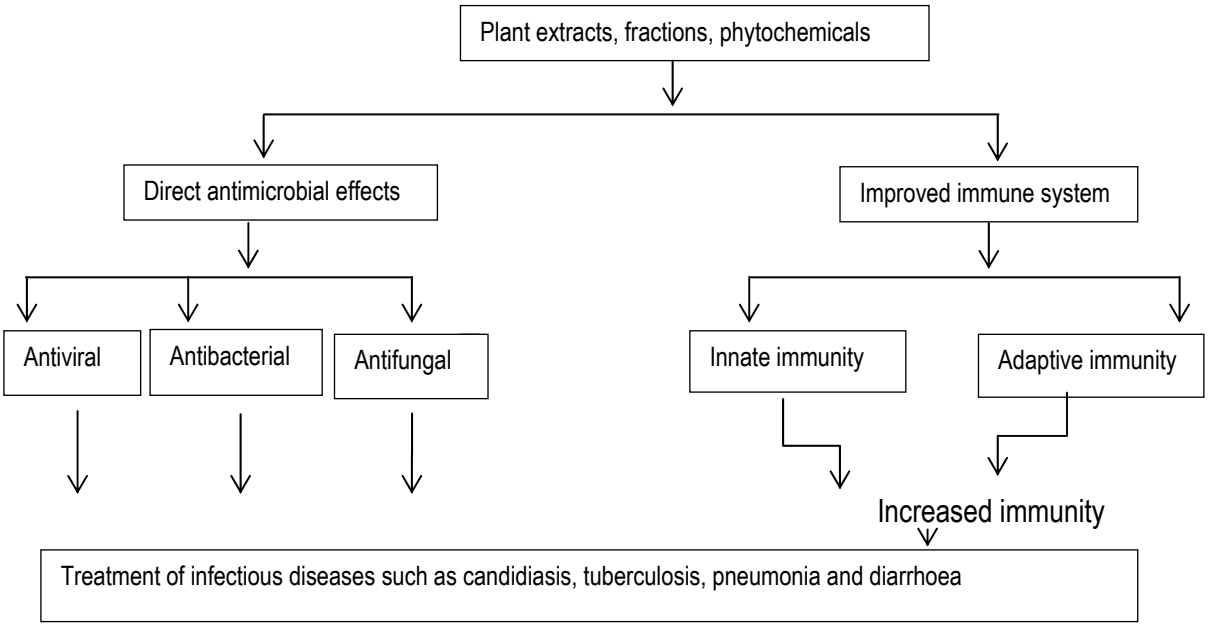


**Figure 2.2.** Schematic diagram of treatment of infectious diseases using medicinal plants

Gram-positive species of bacteria include *Enterococcus faecalis*, *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA). The mechanisms of action of plant extracts used for inhibiting the growth of microorganisms vary widely, and can be due in part to the hydrophobic nature of some components of the plant extracts (Gislene et al, 2000). These compounds can interact with the lipid bilayer of the bacterial cell membrane, or may disrupt the respiratory chain and the production of energy. Alternatively they can make the microbial cell membrane more permeable to antibiotics, leading to the interruption of vital cellular activity. The interference with bacterial enzyme systems can also be a potential mechanism of action. These

mechanisms of action can be attained by the synergistic effect of antibiotics with extracts at a sub-inhibitory concentration.

### 2.6.2. Antioxidant activity

Oxidative stress constitutes a unifying mechanism of increased oxidative damage in many types of disease processes resulting from imbalance between the generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the antioxidant defence systems in the body in favour of production. In pathophysiological conditions as in HIV infection, this can be caused by overproduction of reactive species or impairment of the endogenous antioxidant defence system (Gil et al., 2003).

Oxidative stress contributes to HIV infectious disease mechanisms through enhancing viral replication, elevating the inflammatory response, decreasing immune cell proliferation, loss of immune function, apoptosis of $CD4^+$-T cells, chronic weight loss, and increased sensitivity to drug toxicities (Valko et al., 2007). Physiologically important ROS/RNS include singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($^\bullet OH$), ozone ($O_3$), hypochlorous acid (HOCl), nitric oxide ($NO^\bullet$) and peroxynitrite ($ONOO^-$).

Exogenous antioxidants from dietary and medicinal plants have beneficial health effects in antioxidant-impaired individuals due to their ability to scavenge free radicals and neutralize reactive species. Strengthening of the antioxidant defence mechanisms with an exogenous antioxidant helps in reducing symptoms associated with increased levels of oxidative stress. Some of the exogenous antioxidant compounds include vitamins C and E, polyphenolics such as flavonoids, phenolic acids and tannins.

A natural xanthone glycoside (mangiferin) isolated from *Mangifera indica* potentiates the cellular antioxidant capacity with an immune-protective role mediated through inhibition of reactive species-induced oxidative stress in the lymphocytes, neutrophils and macrophages.

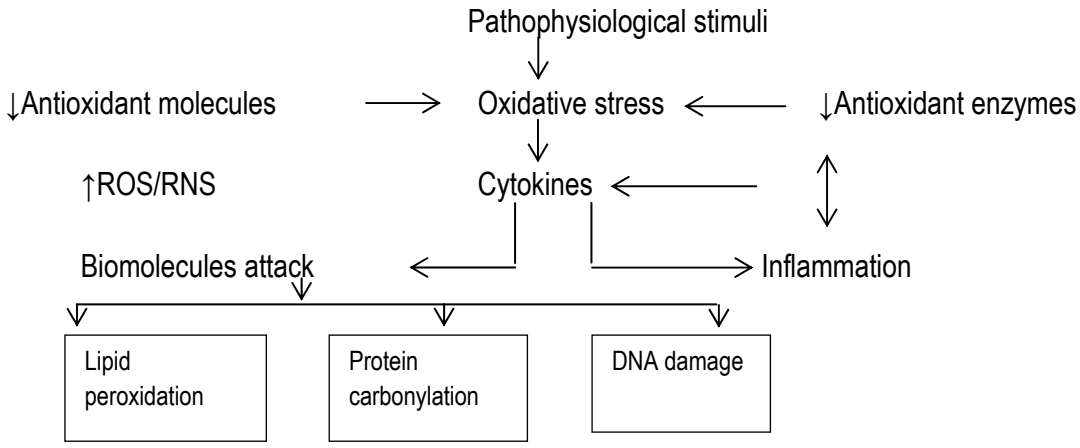


**Figure 2.3.** Oxidation mechanisms in disease initiation and progression (Rodrigo et al., 2011)

### 2.6.3. Inflammatory mediation

HIV infections cause chronic inflammation resulting in high plasma levels of inflammatory cytokines and production of ROS/RNS in seropositive patients (Granot and Kohen, 2004). Plant natural products (from medicinal and dietary plants) exert potent inflammatory mediatory effects *in vivo* through distinct molecular mechanisms. These include inhibition of cyclooxygenases (COX) and lipoxygenases (LOX), inhibition of mitogen-activated protein kinases (MAPKs), inhibition of certain transcription factors like NF-kB, activation of peroxisome proliferator-activated receptors (PPARs), TRPV channels, and activation of G-protein coupled receptors leading to attenuation of inflammation.

### 2.6.4. Immunomodulatory activity

The major cause of clinical disorders in HIV/AIDS patients is the progressive loss of CD4+ T-cell lymphocytes in blood. These cells mediate immune responses and their depletion is often associated with opportunistic infections (Kaslow et al., 1987). Many medicinal plants and their extracts have immunomodulatory activity acting on the complex network of mechanisms of the immune system. They either depress the host's vulnerability to tissue damage or potentiate defence mechanisms of the body against opportunistic infectious pathogens. Immunomodulators can stimulate or suppress any aspect of the immune system including the innate and adaptive components and are classified in Figure 2.4.

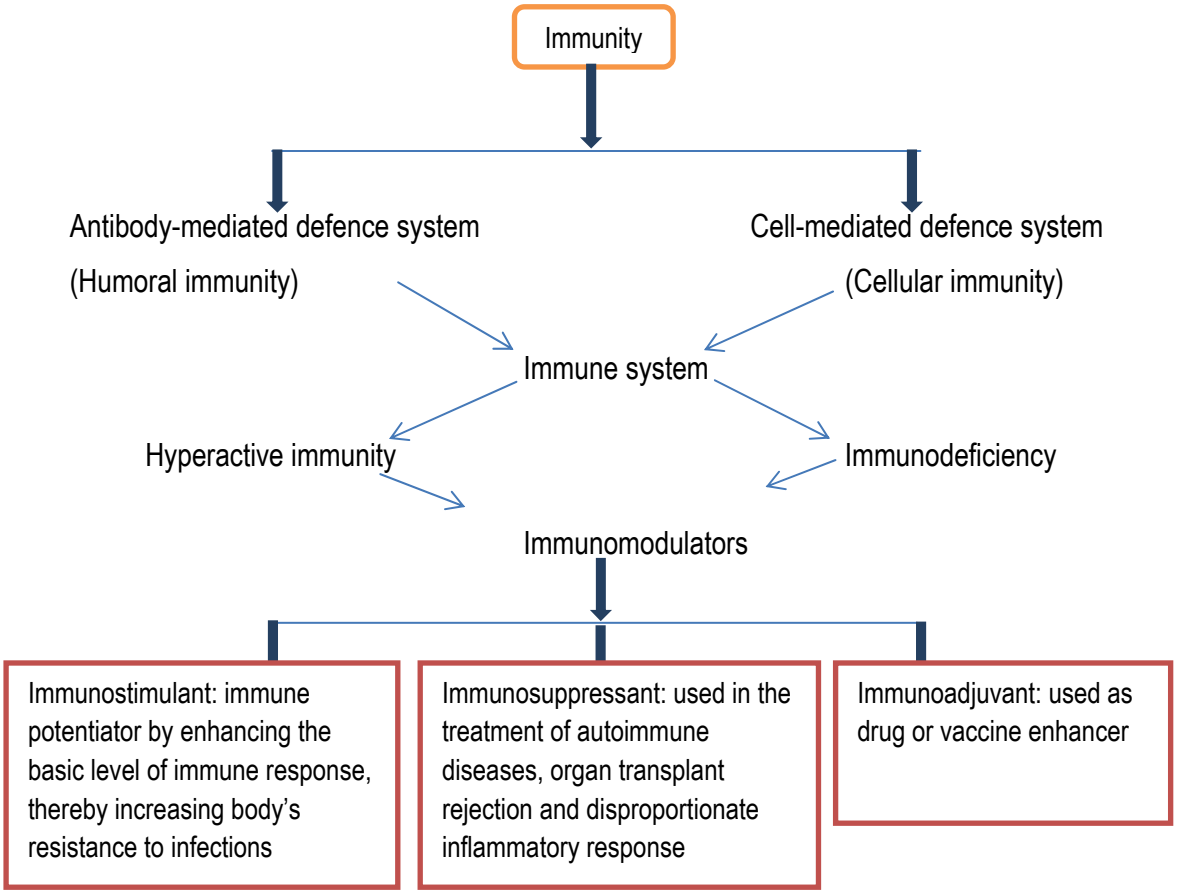


**Figure 2.4.** Graphical representation of immunity and immunomodulator classification

Phyto-extract activation of host defence mechanisms in the presence of an impaired immune response can provide supportive therapy to conventional chemotherapy. The mechanisms of immunomodulatory activity occur mainly via phagocytosis stimulation, macrophage activation, lymphoid cell stimulation, cellular immune function enhancement, antigen-specific immunoglobulin production increase, increased nonspecific immunity mediators and natural killer cell numbers, reducing chemotherapy-induced leukopenia, and increasing circulating total white cell counts and interleukin-2 levels (Gertsch et al., 2011).

Some medicinal plants with immune stimulatory effects include *Panax ginseng*, *Ocimum sanctum*, *Tinospora cordifolia* and *Terminalia arjuna,* while *Alternanthera heratenella* has immunosuppressive activity. The stem bark extract of *Mangifera indica* has the capacity to prolong survival of T-cell lymphocytes by opposing T-cell activation—a critical mechanism for T-cell depletion in HIV/AIDS patients (Hernandez et al., 2006). This suggests that *Mangifera indica* may have beneficial effects on the immune system of HIV/AIDS patients. A large number of phytochemicals including tannic acid, flavonoids, tocopherol, curcumin, ascorbate, carotenoids, polyphenols, and alkaloids acting individually or synergistically have potent immunomodulatory properties (DuPont and Marshall, 1995).

# Chapter 3: Extraction and phytochemical analysis

## 3.1. Plant selection and identification

The selection of medicinal plants for biological evaluations is commonly based on some established criteria. These include:

- The use of plants in indigenous medicine against the disease of interest (Carvalho et al., 1991). The general screening or taxonomic approaches can also yield very good leads.
- The availability of such medicinal plants in the area of study.
- The level of scientific information available in the literature validating the ethnopharmacological use of the medicinal plants.
- Phylogenetic relationships with plants of known medicinal value.
- Selection of medicinal plants from different families based on differences in chemical constituents which exhibit varying biological activities.

Scientific validation, and quality assurance, of medicinal plants requires a systematic approach, mainly including the ethnopharmacological assessment, botanical identification, phytochemical evaluation and fingerprinting of species. A proper ethnobotanical identification is the first step in validating the efficacy and subsequent discovery of new drug leads from the medicinal plants (; Zhao et al., 2010; Wangchuk et al., 2011). Authentication and identification of medicinal plants used in traditional medicine (TM) (Wangchuk et al., 2008, 2011) are usually done with the assistance of taxonomists and curators at established herbaria. Voucher specimens of the plants are also prepared and deposited in recognized herbaria with the assignment of an accession number for future reference.

Faulty identification of plant species is one of the challenges in traditional medicine. Other challenges include stability of phytochemical components, optimization of processing conditions through experimental design and identification of characteristic compounds potentially responsible for a measured activity. Quality control of medicinal plant preparations is extremely important as the effectiveness and safety of such remedies depend on the concentrations of their active ingredients. These are influenced by numerous factors, such as chemical, ecological and environmental effects (climate, cultivation conditions, harvest time, drying, storage, extraction procedure), as well as physiological and genetic factors (Kosuth et al., 2003). Deliberate or coincidental adulteration of medicinal plant preparations may cause a large variability in active ingredients and consequently put patient's health and safety at risk.

To overcome some of these critical problems, the World Health Organisation (WHO) recommends fingerprint analysis as a methodology for the quality assessment of natural products (Barnes et al., 2007). A fingerprint is a characteristic profile reflecting the complex chemical composition of the analysed medicinal plant extract and can be obtained by spectroscopic, chromatographic or electrophoretic techniques.

### 3.2. Plant parts used in traditional medicine, and conservation problems

Different parts of medicinal plants are harvested (whole plants, roots, stem bark, leaves and bulbs) and sold for drug preparation. The over-exploitation of roots, whole plants and stem bark without adequate programmes for replacement of such plants is destructive. This non-sustainable harvesting of medicinal plants threatens the survival of valuable species as well as the livelihoods of people that depend on them. The harvesting and use of leaves is considered less destructive, however, intense pruning can affect growth and reproductive performance (Gaoue and Ticktin, 2007). Therefore, a conservation strategy that involves consideration for sustainable harvesting which may simultaneously provide similar medicinal benefits can be the substitution of bulbs, roots and stem bark with leaves of the same plant (Lewu et al., 2006; Zschocke et al., 2000).

### 3.3. Qualitative evaluation of medicinal plant extracts

#### 3.3.1. Thin layer chromatography (TLC)

A plant is a complex and ever active chemical factory that produces a wide range of chemical entities that may comprise beneficial and protective agents against infectious pathogens, insect and animal attack, and adverse weather conditions (ultraviolet light and drought). These chemical moieties may have pharmacological properties required for treatment of various ailments in humans and animals. (Kotze and Eloff, 2002) The efficacy of medicinal plant preparations is based on the whole mixture of metabolites (synergism), rather than the presence of a single constituent.

Plant extracts may contain more than 100 000 different compounds with more than 4000 new compounds discovered every year(Verpoorte 1998) Many of these compounds are present in low concentrations and many have biological activities.. At least, 122 of, these compounds may be important for the quality, safety and efficacy of the herbal medicines due to synergetic interactions between numerous constituents (Kim, 2005).. Thin layer chromatography in combination with a suitable

detection technique offers a powerful tool for separating the individual compounds and developing a characteristic profile of compounds present in the sample at a high comcentration, called a fingerprint.

Thin layer chromatography (TLC) is a qualitative planar chromatographic technique for separating plant extract mixtures into their individual components. It is performed on a sheet of a glass or aluminium foil, which is coated with adsorbent material, usually silica gel or aluminium oxide. This layer of adsorbent is called the stationary phase. The sample is applied on the adsorbent, dried and eluted with solvent mixture (mobile phase) drawn up the plate via capillary action. Depending on the mobile phase, different components ascend the TLC plate at different rates as a function of the molecular weight and polarity. The chromatograms developed with various solvents under optimized reproducible conditions are stored as pictures or scanned images for future reference as fingerprints.

In addition, TLC can be used to monitor the stability of an extract and for the qualitative analysis of reaction products, to identify compounds present in a given mixture and also to determine the purity of a substance. TLC has the advantage of speed of analysis and comparison of many samples simultaneously, versatility of supports, solvent systems and detection reagents (Stahl, 1969). These attributes make TLC an ideal classic tool for the first stage of phytochemical analysis as well as for monitoring of column chromatography fractions during purification of natural plant products.

However, the widespread use of TLC in the analysis and quality control of herbal medicines has been hindered by its low reproducibility and resolution, the high compound concentration required for detection, and the semi-quantitative nature of the technique. Several factors in TLC are hard to control precisely, such as the sample spotting, the vapour environment inside the developing chamber and the unstable colorations when using chromogenic reagents for detection. Nonetheless, TLC is still being used as it is a readily available, easy to use and economical technique requiring low technology equipment and solvents.

### 3.3.2. High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is one of the most popular analytical techniques used for chemical profiling of medicinal plant products. HPLC has the advantage of easy operation and fully automatable technique with high resolution, selectivity and sensitivity, and also the possibility of coupling with analytical equipment or different detectors. For medicinal plant extract fingerprinting, some of the detectors or equipment which can be coupled with HPLC include ultra violet (UV) and

diode array detection (DAD) for UV absorbing compounds, evaporative light scattering detection (ELSD) and chemiluminescence detection (CL) for non-UV absorbing compounds, nuclear magnetic resonance (NMR) for metabolomic profiling and mass spectrometry (MS) for identification of the separated compounds. Furthermore, HPLC can be used for multi-compound quantification in complex biological samples. However, HPLC requires expensive machinery and often uses large volumes of environmentally unfriendly solvents. Other drawbacks include the undetected co-elution of compounds and the vulnerability of conventional (silica-based) columns to relatively basic (pH > 9) and acidic (pH < 2) mobile phases and high temperatures.

An important factor in fingerprint development is the choice of stationary phase and those commonly used for medicinal plant extract fingerprinting are particle-based C18 columns. The efficiency of these columns is highly dependent on their particle size (currently 3–5m). Smaller particle sizes drastically increase the back pressure: when the particle size is halved, the pressure quadruples. As the current HPLC instrumentation can withstand up to 400 bars, higher efficiencies and shorter separation times by decreasing particle size and increasing the flow rates are limited.

### 3.3.3. Gas chromatography (GC)

Gas chromatography (GC) is another analytical technique commonly used for the characterization and identification of compounds, especially the volatiles. GC has good separation efficiency and sensitive detection (flame ionization (FID) or mass spectrometry) making it a useful tool for the analysis of complex biological samples such as essential oils in medicinal plant preparations.

Despite its advantages, GC analysis of herbal products is usually limited to use with essential oils. However, problems such as the possible degradation of thermo-instable compounds and the pre-requisite to derivatize non-volatile to volatile compounds make GC unsuitable for many natural compounds for which derivatization cannot easily be accomplished. Current developments in GC–MS have led to reduced analysis times of essential oils, as short as 40–100 s, as well as decreased detection limits.

## 3.4. Extract preparation from medicinal plants (extraction)

The major step towards evaluating the therapeutic potential of a medicinal plant is preparation of a crude cellular lysate of the plant matrix followed by extraction of various components having

potential medicinal value. Extraction is the separation of biologically active compounds using selective solvents through standard procedures (Emea, 2006). Such extraction methods separate the soluble plant metabolites and leave behind the soluble cellular marc. The purpose of standardized extraction procedures for medicinal plants is to attain the therapeutically desired compounds and to eliminate unwanted material (WHO, 1998). The extraction methods used for plant material can influence the biological activities such as antimicrobial, antioxidant and anti-inflammatory activity as well as toxicity of the extract (Vargas et al., 2007).

An important factor to consider in the ethnomedical approach is the preparation of extract as described by the traditional healers in order to mimic as closely as possible the way in which the herbal remedy is indigenously used. The most common solvents of extraction used for preparation of indigenous medicine are water or ethanol. Unfortunately, some of these traditional methods have limitations of reproducibility and quality, consequently compromising the safety and efficacy of medicinal plant preparations. As such there is an urgent need to refine and further develop classical methodologies to obtain procedural consistency and highly pure plant components exhibiting medicinal value.

Traditional methodos are being replaced with modern sample preparation and extraction procedures. These involve the use of solvents such as hexane, methylene chloride, petroleum ether, acetone, methanol and liquefied carbon dioxide for extraction (Agbedahunsi et al., 1998; Bickiiet al., 2000; Goffin et al., 2002; Cimanga et al., 2006), which is not possible or acceptable in indigenous preparation of plant extracts and this, in a way, could affect the results obtained.

Traditional uses of *Lydenburgia cassinoides* and *Calodendrum capense*.

These two plant species has no much information, especially in traditionl medicine. The information provided below is from the genus of the plants.

Leaves and roots of the related Catha edulis (genus *Catha* changed to *Lydenburgia* for some species)are used as a remedy for influenza and treatment of coughs, asthma and other chest complaints. The root is used for stomach cramps and an infusion is taken orally to treat boils of infertility in men. In the northern countries of Africa, the leaves are brewed as a tea, the dried leaves smoked and green leaves are chewed to suppress the appetite.

One extraction technique aims to fractionate plant extracts by direct sequential extraction of phytochemicals from the same plant matrix with solvents of increasing polarities. The extraction process starts with the least polar solvent such as hexane and petroleum ether to the most polar

solvent such as water. Extracts of different polarities are obtained and consequently there is no need for liquid-liquid fractionation. In this case, each solvent extracts only the components that are soluble in it, therefore providing  of extraction and partial fractionation (Wojcikowski et al., 2007). A conventional extraction method is maceration, which involves the use of particular extractants that are capable of removing targeted phytochemicals maximally from the plant materials. The process can start with a highly polar solvent, followed by fractionation with solvents of different polarity to obtain subsequent fractions from the mother extract.

## 3.5. Materials and Methods

### 3.5.1. Plant collection

*Calodendrum capense* and *Lydenburgia cassinoides* leaves were collected from the University of Pretoria Botanical Garden (Hatfield Campus). Voucher specimens were prepared and assigned accession numbers and kept at the Schweickerdt Herbarium of the Department of Plant Sciences at the Faculty of Natural and Agricultural Sciences, University of Pretoria.

### 3.5.2. Preparation of the leaves

Leaves were separated from the stems and air dried at room temperature under shade for two weeks (Eloff, 1998b). The dried leaves were milled to a fine powder using a Macsalab mill (model 200 LAB), Eriez ®, Bramley and stored at room temperature in closed containers in the dark until needed.

### 3.5.3. General Extraction Procedure

Separate aliquots of plant material were extracted with hexane, acetone and methanol (technical grade - Merck) at a ratio of 1:10 (m/v) for 6 h. Extraction bottles were vigorously shaken on a Labotec model 20.2 shaking machine. The supernatants were filtered with Whatman No 1 paper, using a Buchner funnel. The process was repeated three times on the marc to exhaustively extract the plant material and all the volumes combined. The extracts were concentrated under vacuum

using a rotary evaporator (Labotec) and transferred into pre-weighed labelled containers and dried under a stream of air.

### 3. 5 4. Analysis (TLC fingerprinting)

Chemical constituents of the extracts were analysed by TLC using aluminium–backed TLC plates (Merck, silica gel 60 $F_{254}$). The TLC plates were developed under saturated conditions with the following eluents (Kotze and Eloff, 2002):

- Ethyl acetate, methanol, water [EMW] (40:6.5:5) (polar)
- Chloroform, ethyl acetate, formic acid [CEF] (50:40:10) (intermediately polar)
- Benzene, ethanol and ammonium hydroxide [BEA] (90:10:1) (non-polar)

To detect the separated compounds, the chromatograms were sprayed with vanillin-sulphuric acid solution in methanol (0.1 g vanillin (Sigma®): 28 ml methanol: 1 ml sulphuric acid) and heated to 110ºC to optimal colour development (Stahl, 1969).

## 3.6. Results

### 3.6.1. Extraction yield

The maceration method was used to obtain extracts of different polarities, namely hexane, acetone and methanol extracts. The highest yield was obtained using methanol as extractant (38% of dry material for *C. capense* and 69% of dry material for *L. cassinoides*). Extraction of the phytochemicals with hexane gave the lowest yield of 6% of dry material for *C. capense* and 17% of dry material for *L. cassinoides*.

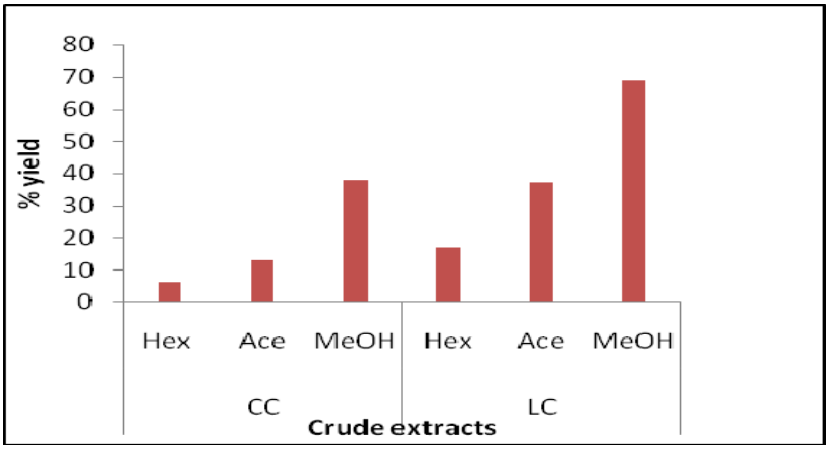


**Figure 3.1.** Percentage yield (%) of *Lydenburgia cassinoides* and *Calodendrum capense* leaf material extracted using hexane (Hex), acetone (Ace) and methanol (MeOH) as extractants

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### 3.6.2. Phytochemical analysis (TLC)

Thin layer chromatography fingerprints of the extracts covering a wide polarity range are presented in Fig 3.2. The extracts of both plant species showed the presence of many different phytochemicals with distinct $R_f$ values ranging from 0 to 9.5. The chromatogram of acetone and methanol extracts of *L. cassinoides* developed with ethyl acetate: methanol: water (EMW) indicates the highest diversity of compounds. The purple colourations of some spots indicate the possible presence of terpenoids while the pink colours of some spots may indicate the presence of flavonols (Shahid 2012). The chromatogram of *C. capense* developed with benzene: ethanol: ammonium hydroxide (BEA) revealed the diversity of non-polar compounds in the hexane and acetone extracts.

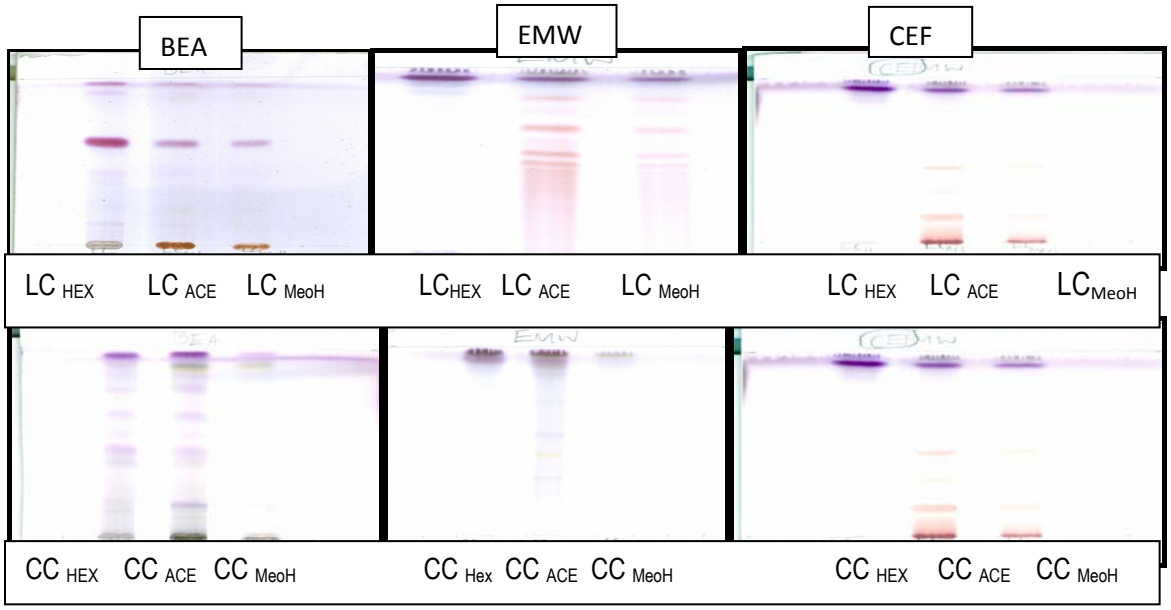



**Figure 3.2.** TLC profiles of *Lydenburgia cassinoides* and *Calodendrum capense* of Hex (hexane), Ace (acetone), MeOH (methanol) extracts developed in BEA (left), EMW (centre) and CEF (right) solvent systems and sprayed with vanillin–sulphuric acid

### 3.7. Discussion

Medicinal plants are rich resources for drug and food supplement development in modern medicines as well as traditional medicines. Many medicinal plants are traded in bulk from some developing countries for further value addition in developed countries (WHO, 1999). The important aspect of value addition is the identification, isolation and characterization of the active constituents either as single chemical compounds or as complementary mixtures of compounds using a variety of methods from simple traditional methods to advanced extraction techniques (WHO, 1999).

In South African traditional medicine, strategic programmes of managing opportunistic infections and complications in immunocompromised individuals, especially those living with HIV include a change of diet and behaviour, administration of herbal formulations, physiotherapy and spiritual healing. The most important component is the use of medicinal plant preparations and it has been postulated that the higher the diversity and the quantity of phytochemical classes in a plant the stronger and broader the spectrum of biological activities of such a plant extract (Cowan, 1999; Geyid et al., 2005).

To understand the complexity of compounds present in *Lydenburgia cassinoides* and *Calodendrum capense* extracts, TLC provides a powerful tool for separating the individual compounds and creating a characteristic fingerprint profile. Although the chemical profile of extracts by itself is insufficient in

determining the efficacy of a medicinal plant preparation, it can be linked to biological assays to provide assurance of efficacy and consistency.

The complexity of plant extracts and fractions depends on the extractants used to prepare the extract and also on the eluents used in developing the chromatograms. The TLC profiles of *Lydenburgia cassinoides* and *Calodendrum capense* extracts and fractions presented in Figure 3.2 revealed the presence of diverse compounds (non-polar and polar) with varied $R_f$ values.

### 3.8. Conclusion

In conclusion, thin layer chromatographic techniques can provide a relatively good picture of compounds present in medicinal plant preparations, producing chromatographic fingerprints of herbal medicines. Phytochemical fingerprints of herbal medicines can be used for the purpose of quality control and stability testing of bioactive components by comparing the integrated similarity difference of plant extract profiles in TLC chromatograms prepared at different times. In the following chapters, the ethnopharmacological uses or indications of each of the selected plant species is discussed to determine the appropriate bioactivity against organisms involved in opportunistic infections.

# Chapter 4: Screening of *Calodendrum capense* and *Lydenburgia cassinoides* leaf extracts for antimicrobial activity

## 4.1. Introduction

### *4.1.1. Calodendrum capense*

Description:

*Calodendrum capense* (L.f.) Thunb is a native plant and a member of the Rutaceae, the buchu and citrus family, with approximately 290 species in southern Africa (Palgrave, 2002). Depending on the habitat, is not restricted to any specific habitat, though it is an evergreen to deciduous tree of up to 25m tall with a roundish dense canopy. *C. capense* is from the Greek word “meaning beautiful tree from the cape, while William Burchell (1782-1863) thought that the flower and fruit resembled the horse chestnut. (Figure 4.1). . Younger branches armed with robust spines up to 2 cm long. Leaves simple, opposite, more or less broadly elliptic, up to 22 cm long, hairless when mature, translucent gland-dots present, aromatic when crushed, margin entire and wavy. Flowers in dense axillary heads, large and showy, pale to deep pink with a darker purple marrom glandular spots; petals long and narrow, alternating with petal-like staminodes. Fruit a 5-lobed capsule up to 3.5 cm in diameter, covered in tubercles, yellow-green when young to brown and woody when ripe, dehiscing from the base, valves remaining connected to the stalk at the apex.

One of the diagnostic features of this family is the presence of oil glands on the leaves. The bark is used as an ingredient of skin ointments (Palmer, 1972) and is sold at traditional medicine markets while the roots are used medicinally for treating hypertension (Palmer, 1972).

The seed is yellowish and bitter and it contains about 60% oil. The fatty acid composition of the oil is approximately 18-24%, oleic acid 33-44% and linoleic acid 29-36%.

Distribution: This plant specie is from Kenya in the North to the Western Cape in the South.

**Figure 4.1.** Flowers (left) and tree (right) of *Calodendrum capense* from the Rutaceae family (htttp: //images.plantzafrica.co.za)

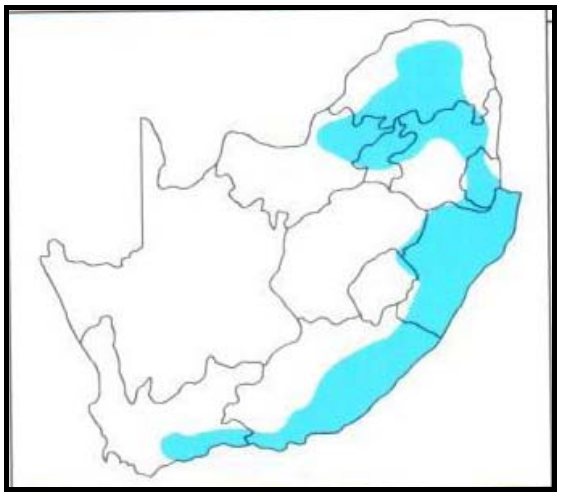


**Figure 4.1.1.** Distribution map of *Calodendrum capense* in Southern Africa (van Wyk & van Wyk, 1997)

### *4.1.2. Lydenburgia cassinoides*

*Is a shrub or small to medium-sized tree up to 9m in height, (Raimondo et al., 2009). Occurring in bushveld in ravines and on rocky hillsides often in groves. Lydenburgia cassinoides N. Robson belongs to the Celastraceae family and is known as Sekhukhune Bushman's tea, It was previously known as Catha transvaalensis Codd Leaves are spreading or erect widely elliptic 2.5-8 ×1.3-5 cm*

*shinny green above, dull and paler green below, hairless, more than 10 pairs of lateral veins and dense prominent net-veining on the undersurface. Flowers are brownish-green, small in dense axillary clusters protruding beyond the leaves.*

.

**Figure 4.2.** Leaves of *Lydenburgia cassinoides* from the Celastraceae family. Photograph by A Krige© SANBI.

Distribution:

*Lydenburgia cassinoides* is found in the woodlands and on rocky outcrops. It is scattered in the KwaZulu- Natal and Eastern Cape. Apart from this two provinces, it is also found in Western Cape, Limpopo, Mpumalanga, Swaziland , Mozambique and through to tropical Africa and the Arab countries.

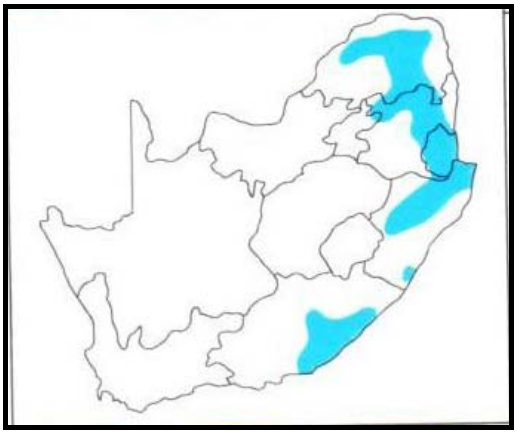


**Figure 4.2.1.** Distribution map of Lydenburgia cassinoises in Southern Africa (van Wyk & van Wyk, 1997)

Research on antimicrobial agents from higher plants faces many inherent problems due to various methodologies used in investigating the microbial growth inhibition. Lack of standardized and reliable methods such as inoculum size and medium type in many *in vitro* assays for testing potential antimicrobial activities of plant crude extracts, fractions and isolated bioactive compounds causes variations in results between research groups. This creates problems for direct comparisons and gives low reproducibility of results, often leading to false conclusions on the efficacy of extracts as antimicrobial agents (Sarker et al., 2007). Some of the methods widely employed in the quantitative evaluation of antimicrobial activities include disc diffusion, well diffusion and broth dilution. One of the most commonly used methods is the broth microdilution method (Eloff, 1998a). For the qualitative antimicrobial assay, three bioautographic techniques usually used include agar diffusion, direct bioautographic detection on a TLC plate and the agar overlay method (Choma and Grzelak, 2011).

Diffusion methods are based on the use of a reservoir containing the test substances which diffuse through an agar medium to be in contact with inoculated organisms. The size of the clear zone of microbial growth inhibition around the reservoir after incubation for a determined period is measured relative to the reference compounds. Different types of reservoirs are in use and the two most common include filter paper discs placed on the surface of the solid media previously inoculated with bacterial or fungal culture and holes punched in the medium. Diffusion methods are less labour intensive than some other methods, require small amounts of the test sample, and allow up to 5 or 6 substances to be tested against a single microorganism. However, diffusion methods are not the best choice for testing non-polar samples. The hydrophobic nature of these compounds may prevent the uniform diffusion of these substances through the agar medium and will therefore impact on the evaluation of their antibacterial capability (Rios et al., 1988; Rios and Recio, 2005).

A broth dilution technique consists of inoculating a suspension of the test organism into liquid growth media that incorporates a serial dilution of the test agent. After incubation, the minimal inhibitory concentration (MIC) is taken as the lowest concentration that will lead to a decrease in prevent perceptible growth of the organism. Both polar and non-polar compounds can be evaluated using the broth based microdilution assays. These methods offer advantages in economy, convenience and reproducibility of results.

### 4.2. Antimicrobial bioautography

This refers to qualitative bioassays such as antimicrobial activity of a substance on TLC chromatograms. Microbial bioautography involves growing a microorganism on the TLC plate containing previously eluted compounds, with the application of a chemical method such as p-iodonitrotetrazolium violet (INT) to detect a biological effect. TLC bioautography is a simple *in situ* method which permits the separation of a complex mixture and at the same time localizes the active constituents on the TLC plate (Martson, 2011). The major applications of TLC bioautography are to be found in the fast screening of a large number of samples for bioactivity and in the target-directed isolation of active compounds (bioactivity guided fractionation).

### 4.3. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is a quantitative endpoint measurement most commonly used for evaluating the antimicrobial activity of drugs or plant extracts. MIC values provide information on the lowest concentration of antimicrobial agents that reduces microbial growth under a visible threshold after 16 to 18 h. MIC is important to confirm the resistance of microorganisms to antimicrobial drugs and also to monitor the activity of the extracts. A lower MIC is an indication of a better antimicrobial activity. An MIC is generally regarded as the best measurement of the activity of extract against the microorganisms (Andrews, 2001).

### 4.4. Total activity

Total activities indicate the degree to which the active compounds extracted from one gram of plant material can be diluted and still inhibit the growth of the tested microorganisms. In proposing this measure, Eloff (2000) mentioned that not only MIC value but also the quantity extracted should be taken into account to compare the activity of different plants, and it is calculated as follows:

$$\text{Total activity} = \frac{\text{quantity extracted (mg/g)}}{\text{MIC value (mg/ml)}}$$

In this study a two-fold serial microdilution method was used to evaluate the antimicrobial activities (MIC and total activities) of the crude leaf extracts and fractions of *Calodendrum capense* and *Lydenburgia cassinoides*. Direct bioautography on TLC was also used for qualitative determination of the specific bioactive components of the extracts and fractions.

## 4.5. Material and Methods

### 4.5.1. Bacterial strains

The selection of organism strains was based on the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 1990). These bacterial species (Table 4.1) are the major cause of nosocomial infections in hospitals (Sacho et al., 1993). Two non-pathogenic mycobacterial species were also included to obtain an indication of antimycobacterial activity of the extracts. The bacteria were cultured in Müller-Hinton (MH) broth at 37°C and maintained on MH agar at 4°C while the mycobacteria were grown at 37°C in Middlebrook 7H9 broth supplemented with OADC supplement, and maintained on Middlebrook 7H10 agar supplemented with OADC supplement at 4°C.

**Table 4.1.** Bacterial microorganisms (ATCC number and Gram-positive or negative state)

| Organism | ATCC number | Gram classification |
|---|---|---|
| *Staphylococcus aureus* | 29213 | Gram-positive |
| *Enterococcus faecalis* | 29212 | Gram-positive |
| *Pseudomonas. aeruginosa* | 27853 | Gram-negative |
| *Escherichia coli* | 25922 | Gram-negative |
| *Mycobacterium smegmatis* | 1441 | Neither |
| *Mycobacterium bovis* BCG | P1172 | Neither |

### 4.5.2. Fungal strains

The fungi used in the testing procedure, namely the yeast species *Candida albicans* and *Cryptococcus neoformans* as well as *Aspergillus fumigatus*, were included in the screening process. They are among the most common and important disease-causing fungi of human beings and animals. The fungal strains used were cultured from clinical cases of fungal infectious diseases in animals (before treatment) in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *Candida albicans* was isolated from a Gouldian finch, *Cryptococcus neoformans* from a cheetah, while *Aspergillus fumigatus* was isolated from a chicken which suffered from a systemic mycosis. All fungal strains were maintained at 4°C on Potato Dextrose (PD) agar (Oxoid, Basingstoke, UK).

### 4.5.3. Microbial bioautography

The chromatograms of the extracts were developed using three mobile systems as described in Section 3.5.3 and dried for 72 h at room temperature under a stream of cold air to remove all traces of the solvent. Conidia of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* grown on PD agar were transferred into 80 ml of PD broth using sterile swabs. The chromatograms were inoculated with a fine spray (in a biosafety class II cabinet (Labotec, SA) of concentrated suspension of 2.6 x $10^6$ cfu/ml for *C. albicans,* 2.6 x $10^6$ cfu/ml for *C. neoformans* and 8.1 x $10^6$ cfu/ml for *A. fumigatus of* actively growing organisms. The fungal inoculated chromatograms were incubated at 37$^o$C for 16 h in a clean chamber at 100% relative humidity and sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in a sterilized water. The chromatograms were further incubated for another 6 h for the red colour, indicating fungal growth, and the white spots, indicating the zones of inhibition, to develop.

The procedure was repeated for the bacterial organisms, which were grown overnight in MH broth before being sprayed onto the TLC plates. The TLC plates were incubated overnight and then sprayed with 2 mg/ml of INT before further incubation for development of white zones of inhibition against a red background.

The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff, 1998b). Where fungal growth is inhibited, clear zones will appear on the TLC chromatograms after incubation with INT.

### 4.5.4. Minimum inhibitory concentration evaluation

The crude extracts of the two plant species were tested for antimicrobial activity using a broth microdilution method to determine minimum inhibitory concentration (MIC) values against eight microbial pathogens.

### 4.5.5. Antibacterial assay

To determine the MIC values, the microdilution assay described by Eloff (1998b) was followed. Plant extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted two-fold with distilled water in 96 well microtitre plates. Gentamicin (0.1 mg/ml, Virbac) was used as a positive control while acetone was the negative control.

One hundred microlitres of bacterial culture (grown overnight at 37°C and diluted to approximately $10^8$ cfu/ml with fresh broth) was then added to each well. The plates were covered and incubated overnight at 37ºC. To indicate the bacterial growth 40 µl of 0.2 mg/ml INT were added to each well and the plates were again incubated at 37ºC for 30 minutes. Bacterial growth in the wells was indicated by a red colour, whereas clear wells indicated inhibition of the bacterial growth by the plant extracts.

### 4.5.6. Antifungal assay

The two-fold serial microdilution assay (Eloff, 1998a) modified by Masoko and Eloff (2005) was used to determine the MIC values for plant extracts against the fungal strains. Dried extracts were reconstituted in acetone to a concentration of 10 mg/ml. All the wells of sterile 96-well microplates were filled with 100 µl of sterilized distilled water. Test plant extract samples were added to the first well and serially diluted two-fold down to the last well. Fungal cultures (containing approximately $10^9$ cfu/ml) in fresh PD broth were added to all the wells (100 µl in each well). As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in water were added to each microplate well (Eloff, 1998c) and incubated for 24 h at 37ºC at 100% relative humidity. The MIC was recorded as the lowest concentration of the extracts that inhibited fungal growth after 24-48 h. All determinations were carried out in triplicate. Amphotericin B was used as a positive control while acetone was used as negative control.

## 4.6. Results

### 4 6.1 Bioautography

The separation of the bioactive components of the extracts on TLC provides information on the numbers of compounds present in the mixture responsible for the observed antimicrobial activity as shown in Figures 4.3 and 4.4. The appearance of white spots on bioautograms of *L cassinoides* extracts represented zones of inhibition against *Candida albicans* with $R_f$ values of 0.58 developed in three mobile systems (Figure 4.4). Extracts of *C. capense* showed clear zones of inhibition in acetone extracts compared with hexane and methanol extracts against *Candida albicans*. Chromatograms of *L. cassinoides* also showed a good separation of compounds in acetone and methanol extracts against *Cryptococcus neoformans* with an $R_f$ value of 0.76 developed in BEA (left), EMW ( centre) and CEF (right).

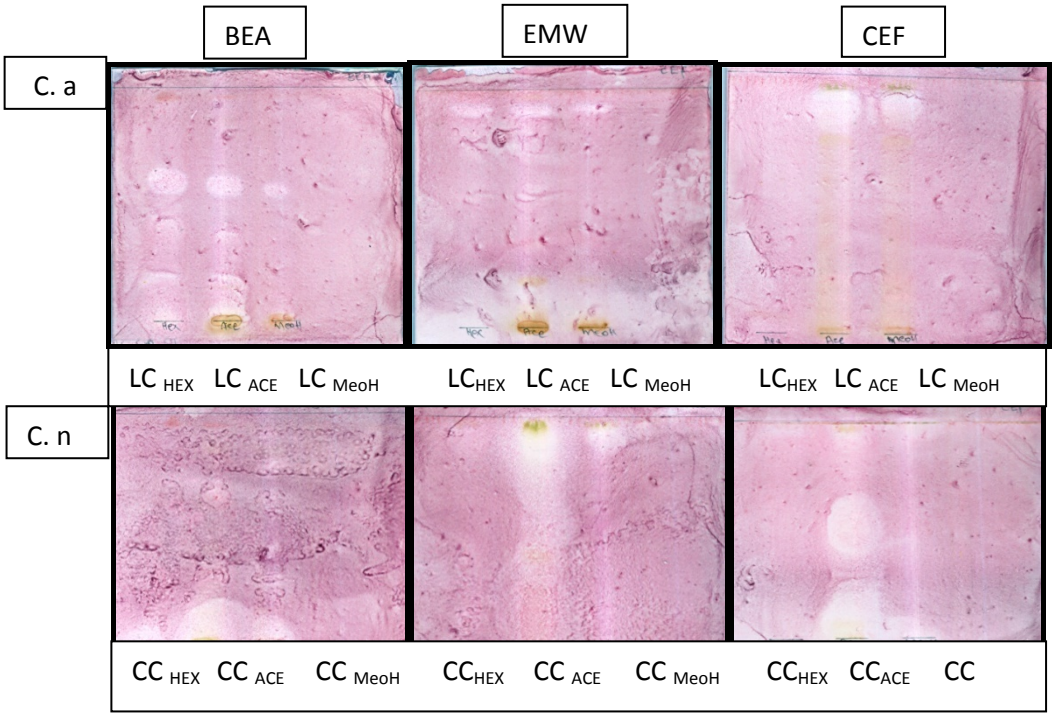



**Figure 4.3.** Bioautograms of hexane, acetone and methanol extracts of *Lydenburgia cassinoides* (LC) and *Calodendrum capense* (CC) developed in BEA (left), EMW (centre) and CEF sprayed with *Candida albicans* and *Cryptococcus neoformans*

*C. capense* hexane extract also showed clear zones of bacterial inhibition (Figure 4.4), but less so in both acetone and methanol extracts sprayed with *P. aeruginosa* (left), developed in BEA. *E. coli* was inhibited in plates developed in the EMW mobile system. Lastly *E. coli* was inhibited by compounds in the hexane, acetone and methanol extracts developed in CEF. Bioautograms of extracts were developed using three mobile phases. The two plant extracts were therefore active against bacterial organisms.

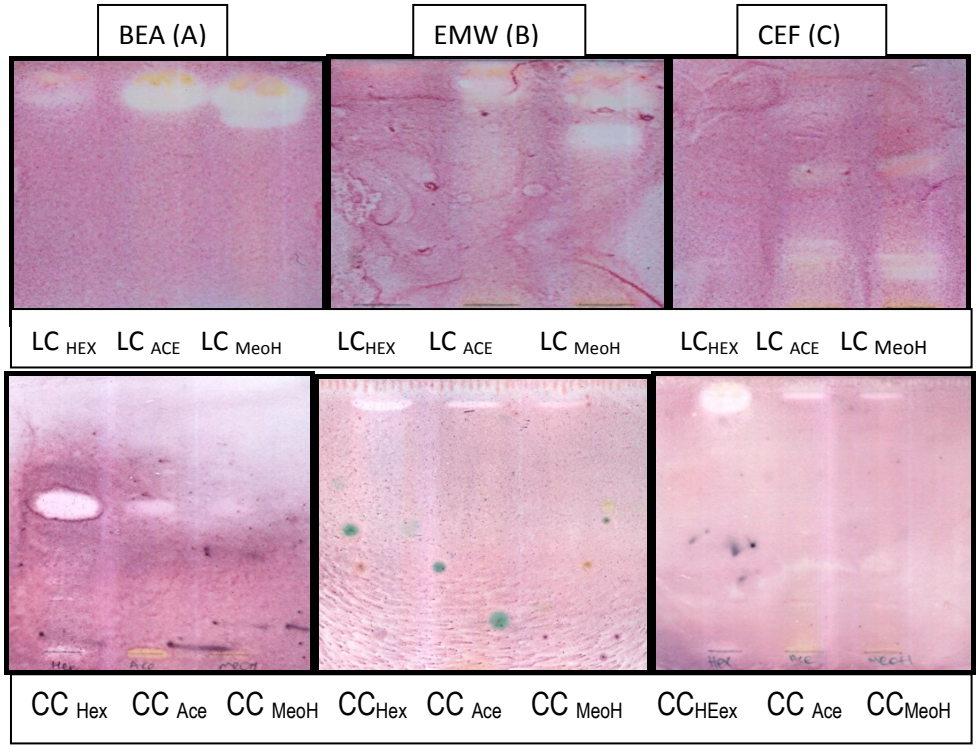



**Figure 4.4.**.Bioautograms of hexane, acetone and methanol extracts of *Calodendrum capense* (CC) and *Lydenburgia .cassinoides*(LC) developed in BEA (left), EMW (centre) and CEF (right), *Pseudomonas aeruginosa* ( A) and *Escherichia coli*.(B and C)

### 4.6.2. Minimum inhibitory concentration (MIC)

In the MIC microdilution assay, wells that appear turbid are indicative of microbial growth, while the wells that remain clear indicate no growth (Turnidge et al., 2003). A growth indicator such as INT can make this phenomenon more easily visible. Advantages of the broth microdilution method include that it is technically simple to set up, and it also allows one to quantify the antibiotic activity and to show the resistance of the organism.

The MIC values presented in Table 4.2 indicate that the best fungal inhibition was observed with MIC value of 0.04 mg/ml with the acetone extract of *L. cassinoides* against *C. neoformans,* followed by acetone extracts against *C. albicans* and *A. fumigatus*. The reference antibiotic (Amphotericin B) had MIC = 0.025 mg/ml against the tested microorganisms. The *C. capense* extract did not have much activity in all three extracts against the three tested organisms.

### 4.6.3. Total activity

Total activity is a measure of the quantity of antifungal compounds present and was calculated by dividing the quantity extracted in mg from 1 g leaves by the MIC value in mg/ml. The total activity (Table 4.2) values indicate the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the fungi (Eloff, 1999). Total activity values (Table 4.2) revealed that the crude methanol extract of *L. cassinoides* was the most active against *A. fumigatus, C. albicans* and *C. neoformans* as its antifungal component can be diluted in 1225, 612.9 and 612.9 ml of solvent and still inhibit the growth of these fungi (total activity of 1225.8, 612.9 and 612.9 ml/g respectively). This is followed by the acetone crude extract against *C. albicans* and *C. neoformans* (total activity of 419.3 and 209.6 ml/g, respectively).

The results from this study were not very comparable to those of the previous study (Mokoka et al., 2010), possibly owing to the fact that the plant material was collected from different places. Mokoka et al. (2010) reported much lower (almost ten-fold lower) MIC values and higher total activity values after 24 hours and 48 hours of incubation with *C. neoformans*.

**Table 4.2.** MIC values (mg/ml) and total activity in ml/g of two plant species (*L. cassinoides* and *C. capense*) extracts against three fungal microorganisms

| **Microorganisms** | **MIC values (mg/ml) 24 hours** | | | **Total activity (mg/ml)** | | | **MIC value (mg/ml) 48 hours** | | | **Total activity (mg/ml)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *L. cassinoides* | | | *L. cassinoides* | | | *C. capense* | | | *C. capense* | | |
| | Hex | Ace | MeOH | Hex | Ace | MeOH | Hex | Ace | MeOH | Hex | Ace | MeOH |
| *C. albicans* | 0.62 | 0.15 | 0.62 | 96.77 | 419.3 | 612.9 | 2.5 | 0.31 | 0.62 | 24 | 419.3 | 304 |
| *C. neoformans* | 0.31 | 0.04 | 0.31 | 96.77 | 209.6 | 612.9 | 0.31 | 0.31 | 0.62 | 193.5 | 419.3 | 612.9 |
| *A. aspergillus* | 0.31 | 0.15 | 1.25 | 96.77 | 419.3 | 1225.8 | 1.25 | 0.62 | 0.62 | 96.77 | 209.6 | 612.9 |
| Amphotericin B | 0.025 | 0.015 | 0.025 | | | | 0.025 | 0.025 | 0.025 | | | |

*M. smegmatis (*Table 4.3*)* was inhibited well by extracts of both plant species with MIC values of between 0.04 mg/ml and 0.16 mg/ml in both acetone and methanol extracts. *E. coli* and *P. aeruginosa* as Gram-negative bacteria also had low MIC values of 0.16 mg/ml for both acetone extracts of *L. cassinoides* and *C. capense.* The highest minimum inhibitory concentration values of 1.25 mg/ml were obtained against *M. bovis* BCG

**Table 4.3.** MIC (mg/ml) value of two plant species against six bacterial microorganisms

| | *L. cassinoides* **24 hours** | | | *C. capense* **24 hours** | | | **Gentamicin (mg/ml)** |
|---|---|---|---|---|---|---|---|
| **Microorganisms** | Hex[1] | Ace | MeOH | Hex | Ace | MeOH | |
| *E. coli* | 0.32 | 0.16 | 0.32 | 0.63 | 0.16 | 0.16 | 0.630 |
| *E. faecalis* | 0.32 | 0.16 | 0.32 | 0.63 | 0.32 | 0.32 | 0.10 |
| *P. aeruginosa* | 0.32 | 0.32 | 0.16 | 0.63 | 0.16 | 0.16 | 0.050 |
| *S .aureus* | 0.63 | 0.32 | 0.32 | 0.16 | 0.16 | 0.32 | 0.031 |
| *M. bovis* BCG | 1.25 | 0.32 | 0.16 | 1.25 | 0.32 | 0.16 | 0.019 |
| *M. smegmatis* | 0.04 | 0.16 | 0.32 | 0.04 | 0.16 | 0.32 | 0.078 |

## 4.7. Discussion

### 4.7.1 Bioautography

There are numerous microbial species that can cause opportunistic infections in immunocompromised patients, such as *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium* spp., *Candida albicans* and *Cryptococcus neoformans*. Antimicrobial substances from indigenous plants used as herbal medicine for curing various diseases ethnopharmacologically have been investigated to source new drugs or drug templates. The huge biodiversity in southern African plants and ethnobotanical knowledge could be a promising source for detecting new antibiotics. Bioautography techniques provide qualitative antimicrobial information of plant extracts, and hence supply a lead to targeting the active components in activity-directed isolation processes. Three solvent systems (BEA, EMW and CEF) were used to develop the TLC plates for microbial bioautograms. The BEA solvent system gave the best resolution of antimicrobial compounds with at least four visible active spots in the bioautograms and results for all the strains used were similar. Separation of the bioactive components of an extract on TLC provides information on the compounds present in the mixture and, as indicated by Figs. 4.3 and 4.4, both of the two species had chemical compounds responsible for antimicrobial activity.

Minimum Inhibitory Concentration (MIC) determinations are possible from quantitative assays used to test the antimicrobial properties of medicinal plant extracts and natural products used to treat or prevent infectious diseases. This involves measuring the Minimum Inhibitory Concentration (MIC) of each extract to assess the capacity of the test material to inhibit the growth of typical opportunistic pathogens, such as *Candida albicans* (a fungal species implicated in oral or vaginal candidiasis) and *Cryptococcus neoformans* (a fungal species implicated in central nervous system disorders).

In this study, plant extracts were tested for antimicrobial activity at a highest test concentration of 2.5 mg/ml and only MIC values less than 0.5 mg/ml were considered as good activity while values less than 0.1 mg/ml were considered to be of pharmacological potential. The MIC values of 2.5 mg/ml and above were considered inactive or not having microbial growth inhibition (Ramadwa, 2010). The best antimicrobial activities were recorded with the hexane extracts of the two plant species against *E. coli* (0.08 mg/ml) and *P. aeruginosa* (0.04 - 0.16 mg/ml).

The low activity of most test samples against the Gram-negative bacteria indicates that these bacteria are more resistant to antimicrobial compounds than Gram-positive species, as has been the case in many other similar studies (Chariandy et al., 1999; Rabe and Van Staden, 1997). Gram-negative bacteria have an outer membrane which presents a barrier to various antimicrobial molecules (Sleigh

and Timbury, 1998). The MIC values of the standard antibiotics used as positive controls (gentamicin for bacteria and amphotericin B for fungi) in this study are given in Tables 4.2 to 4.3. The acetone used for dissolving the plant extracts was also tested to rule out the false positivity of the result.

### 4.8. Conclusion

From the bioautograms several antimicrobial compounds were observed in the plant extracts. Bioautography facilitates the *in situ* recognition of antimicrobial compounds on a TLC chromatogram. The overall results of the antimicrobial activity testing indicate that the crude extracts of *L. cassinoides* and *C. capense* contained some antibacterial and antifungal compounds. The presence of different classes of plant metabolites such as phenolics, terpenes, and alkaloids might explain the wide spectrum of activity of the tested extracts. However, the isolation of the active principles will confirm this hypothesis and provide some information on the identity of antimicrobial compounds in these extracts.

# Chapter 5

# Bioactivity guided isolation of compounds from *Lydenburgia cassinoides* and *Calodendrum capense*

## 5.1 Introduction

Recognition of medicinal plants as an alternative form of health care and the screening of medicinal plants for the presence of bioactive compounds is a vital exercise which is increasing in popularity (Masoko et al., 2005). It is crucial to study the safety and usefulness of natural products derived from plants (Farnsworth, 1994). Plants are important tools used in pharmacological and biochemical studies (Williamson et al., 1996). It is estimated that 60% of anti-infectious drugs which are already on the market are of natural origin (Yue-Zhong, 1998).

Recently there has been growing interest in alternative therapies and therapeutic use of natural products, especially those derived from plants (Mentz and Schenkel, 1989). Among the array of secondary metabolites produced by plants are compounds exhibiting antimicrobial activity. Examples of these compounds are flavonoids, phenols and phenolic glycosides comprising saponins and cyanogenic glycosides (Osbourne, 1996, Gomez et al, 1990, Grayer and Harborne, 1994). Many of these chemical compounds cannot be synthesized efficiently and are still obtained from natural or cultivated plants (Rates, 2001).

Column chromatography is a technique which is generally used in the isolation of natural products from plant extracts. Plant constituents are circulated between the solid phase and the mobile phase, which comprises an eluting solvent. The mobile phase is a liquid added to the top which flows down through the column while the stationary phase is a solid absorbent which is placed in a vertical glass column. Silica is a polar inorganic solid phase that is insoluble in most organic solvents and is one of the stationary phases that can be used in column chromatography. Separation of plant extract components on a column results from the difference in polarity or size of the compound to be separated and is an important tool for the isolation, purification and confirmation of natural products (Wen et al., 2004).

## 5.2 Liquid-liquid fractionation

Liquid-liquid fractionation is a technique to separate compounds in a complex mixture into fractions based on the polarity of the constituent compounds. Liquid-liquid fractionation is an effective

separation technique when all appropriate parameters and equipment are used. This method is effective especially when the compounds within the mixture are coloured as this gives the scientist the ability to see the separation of the compounds (Jones, 2000). If the compounds are not visible, certain components can be observed by other visualization methods. This makes it relatively easy to collect fractions (Skoog et al., 2007). It is also a less expensive procedure than other methods of separation like HPLC. This is because the most basic forms of liquid fractionation do not require expensive machines (Skoog et al, 2007). The addition of mobile phase, the detection of each separation and collection of the fractions are done manually.

## 5.3 Materials and Methods

### 5.3.1 Bulk extraction and bioassay-guided fractionation of *C. capense*

A schematic diagram of the isolation of an active compound from *C. capense* is shown in Figure 5.1. Powdered leaf material of *C. capense* (299 g) was extracted with 4L of acetone. The extract was concentrated using a Büchi rotavapor R114 (Labotec) and dried under a stream of air at room temperature. Dried plant material was subjected to liquid-liquid fractionation following the method of Suffness and Douros (1979) using hexane, dichloromethane, ethyl acetate, butanol and water. Bioautography of the various fractions against *Candida albicans* and *Cryptococcus neoformans* was performed.

The hexane fraction (with a mass of 16.5 g) was subjected to gravitational column chromatography on silica gel (column dimensions 2.5 cm×73 cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM). The eluting solvent mixture comprised hexane: ethyl acetate, starting with 100% hexane, and progressing to 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7 and finally 90:10 as mobile phase. Aliquots of the nine fractions were reconstituted in acetone (10 mg/ml) and 10 µl of the aliquot spotted on TLC. Three mixtures of hexane:ethyl acetate (90:10; 95:5, 97:3) as well as BEA (see section 8.3 was used to develop the plates. Fractions with similar chemical compositions were combined. The purification of the compounds was achieved by bioassay-guided fractionation and repeated column chromatography until a single spot was obtained for each active compound using three different mobile phases to develop the TLC.

Fraction with a mass of 240 mg had a compound with activity against *C. albicans* and *C. neoformans* in bioautography and was therefore subjected to column chromatography with silica gel as stationary phase using a gradient solvent of 100:0, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30 and 60:40 (hexane:ethyl acetate). Twenty eight fractions (28) were collected from combinations of similar profiles in chromatograms and bioautograms of collected fractions. Fraction 9 (467 mg) had an anticandidal

and anticryptococcal compound on bioautograms and was subjected to further column chromatography on silica gel using a gradient solvent system of 100:0, 98:2, 96:4, 94:6, 92:8 and 90:10 (hexane: ethyl acetate) and compound 1 (lupeol) was obtained.

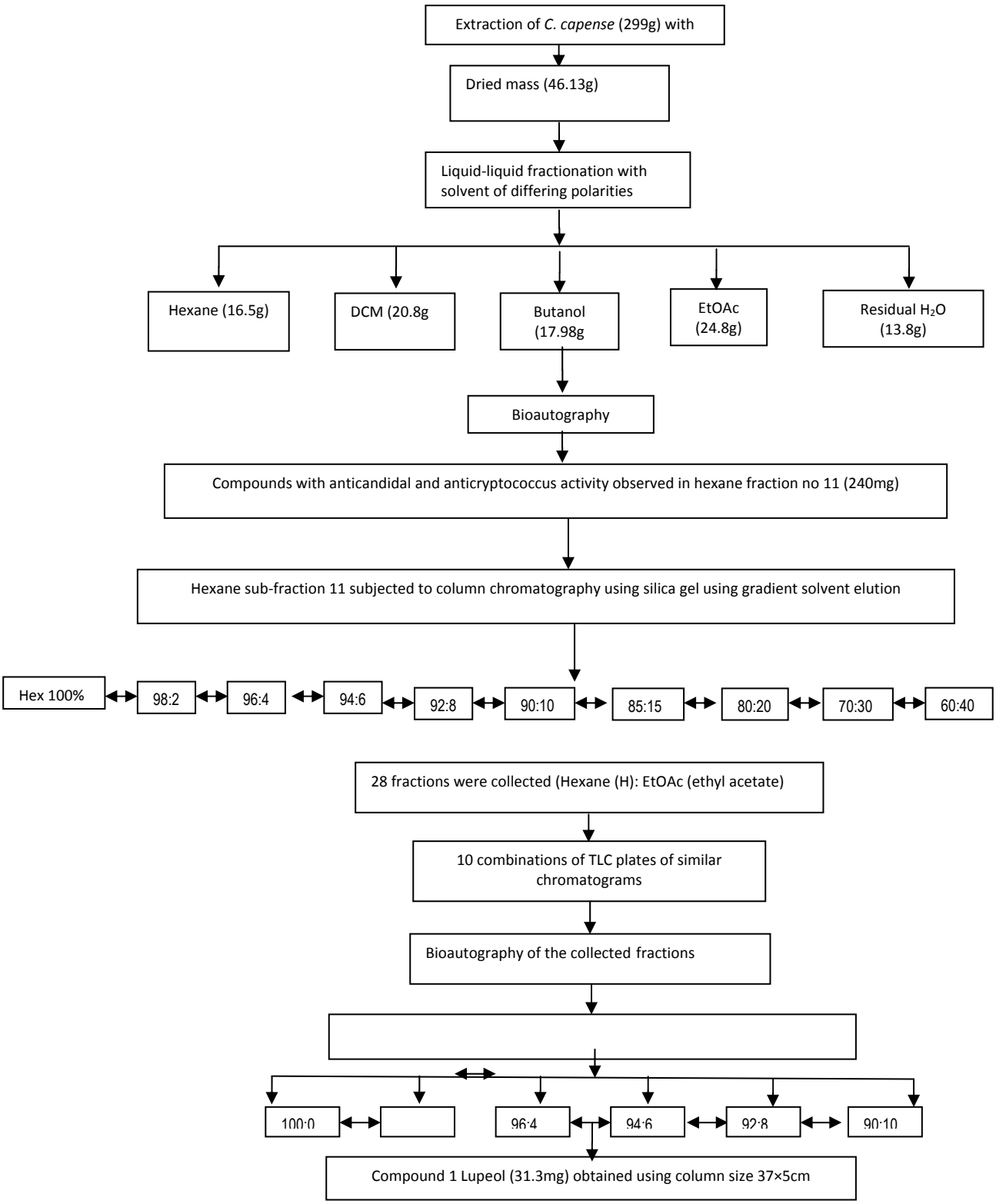



**Figure 5.1.** Schematic diagram of bioactivity-guided isolation of anticandidal and anticryptococcal compound from *C. capense* indicating the ratio hexane to ethyl acetate used as eluent to obtain different fractions

### 5.3.2 Bulk extraction and bioassay-guided fractionation of *L. cassinoides*

A schematic diagram of the isolation of an active compound from *L. cassinoides* is shown in Figure 5.2. Powdered leaf material of *L. cassinoides* (300 g) was extracted with 4L of acetone. The extract was concentrated using a Büchi rotavapor R114 (Labotec) and dried under a stream of air at room temperature. The mass of the dried extract was 44.10 g and the extract was subjected to liquid-liquid fractionation with hexane, dichloromethane, ethyl acetate, butanol and water (Suffness and Douros, 1979).

Bioautography of the various fractions against *Candida albicans* and *Cryptococcus neoformans* was conducted. The hexane fraction (with a mass of 12.1 g) had one anticandidal and anticryptococcal compound and was subjected to column chromatography on silica gel using a gradient solvent system of 100:0, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30and 60:40 (hexane:ethyl acetate).

Ten fractions were collected from the combination of similar fractions visualised by chromatograms, and bioautography of the collected fractions was performed. Fraction 8 (38.2 mg) showed the presence of an anticandidal and anticryptococcal compound on bioautography and was subjected to further column chromatography on silica gel using a gradient solvent system of 100:0, 98:2, 96:4, 94:6, 92:8 and 90:10 (hexane: ethyl acetate) and compound 1 (ß-amyrin)which yielded 29.9 mg was obtained.

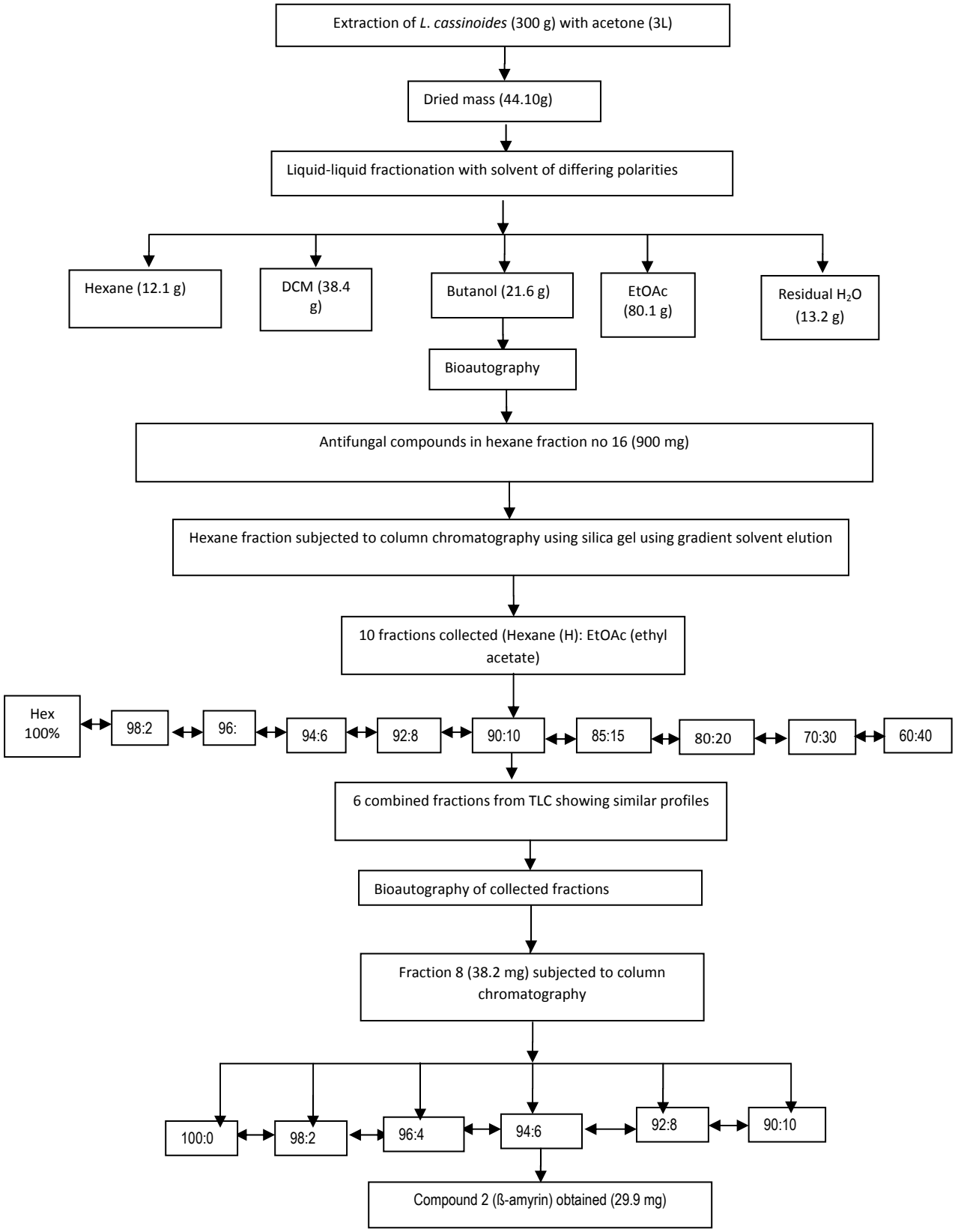



**Figure 5.2.** Schematic diagram of bioactivity-guided isolation of anticandidal and anticryptococcal compound from *L. cassinoides*

### 5.4 Results

Powdered leaf material of *C. capense* (299 g) and *L. cassinoides* (300 g) was respectively extracted with 4L of acetone. Liquid-liquid fractionation as described by Suffness and Douros (1979) was used to separate the components into fractions of various polarities. Column chromatography enabled the isolation of the active compounds.

#### 5.4.1 Percentage yield of fractions

The serial exhaustive extraction of *C. capense* and *L. cassinoides* leaves is described in section 3.5.3. Both plant materials were extracted with different solvents. The crude acetone extract of *L. cassinoides* resulted in the largest yield (85%) respectively of extracted chemical components, followed by *L. cassinoides* butanol fraction with 68% of the bulk extract, while *C. capense* butanol fraction resulted in a 40% yield (Figure 5.3). Generally, *C. capense* fractions yielded the least material.

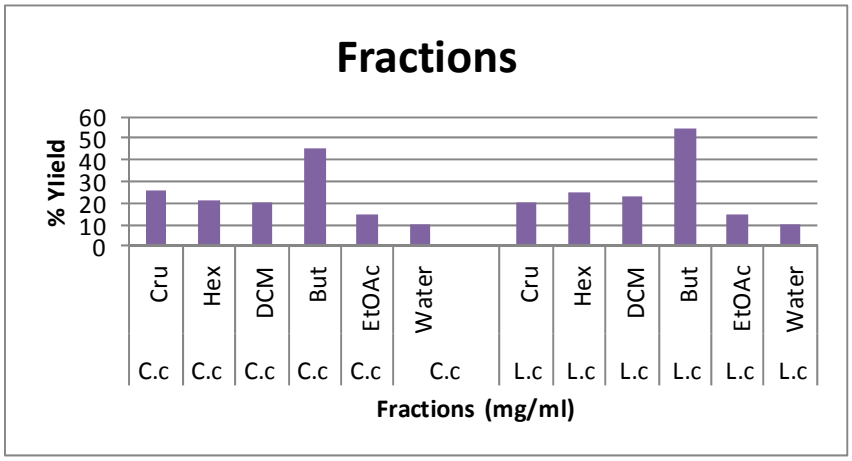



**Figure 5.3.** Percentage yield (%) of extracts and fractions of two plant species (*Lydenburgia cassinoides* (L.c.) and *Calodendrum capense( C.c.)*) fractionated with solvents of differing polarities(Cru = crude acetone extract, Hex =hexane, DCM = dichloromethane, But = n-butanol, EtOAc = ethyl acetate and water fractions)

TLC fingerprints of the extracts of *C. capense* and *L. cassinoides* fractions were prepared on one TLC plate by loading the extracts on 10 cm x 20 cm TLC plates and developing in hexane:ethyl acetate (70:30). The developed plates were then sprayed with vanillin-sulphuric acid solution to visualise compounds. The n-hexane fraction showed good activity in both plant species and was chosen for

further bioassay-guided fractionation by column chromatography. The Rf values of fractions ranged from 0.74 to 0.91. Each plant species was fractionated using a standard liquid-liquid fractionation procedure and five fractions were collected. Fractions and extracts showed similar compounds (Figure 5.4).

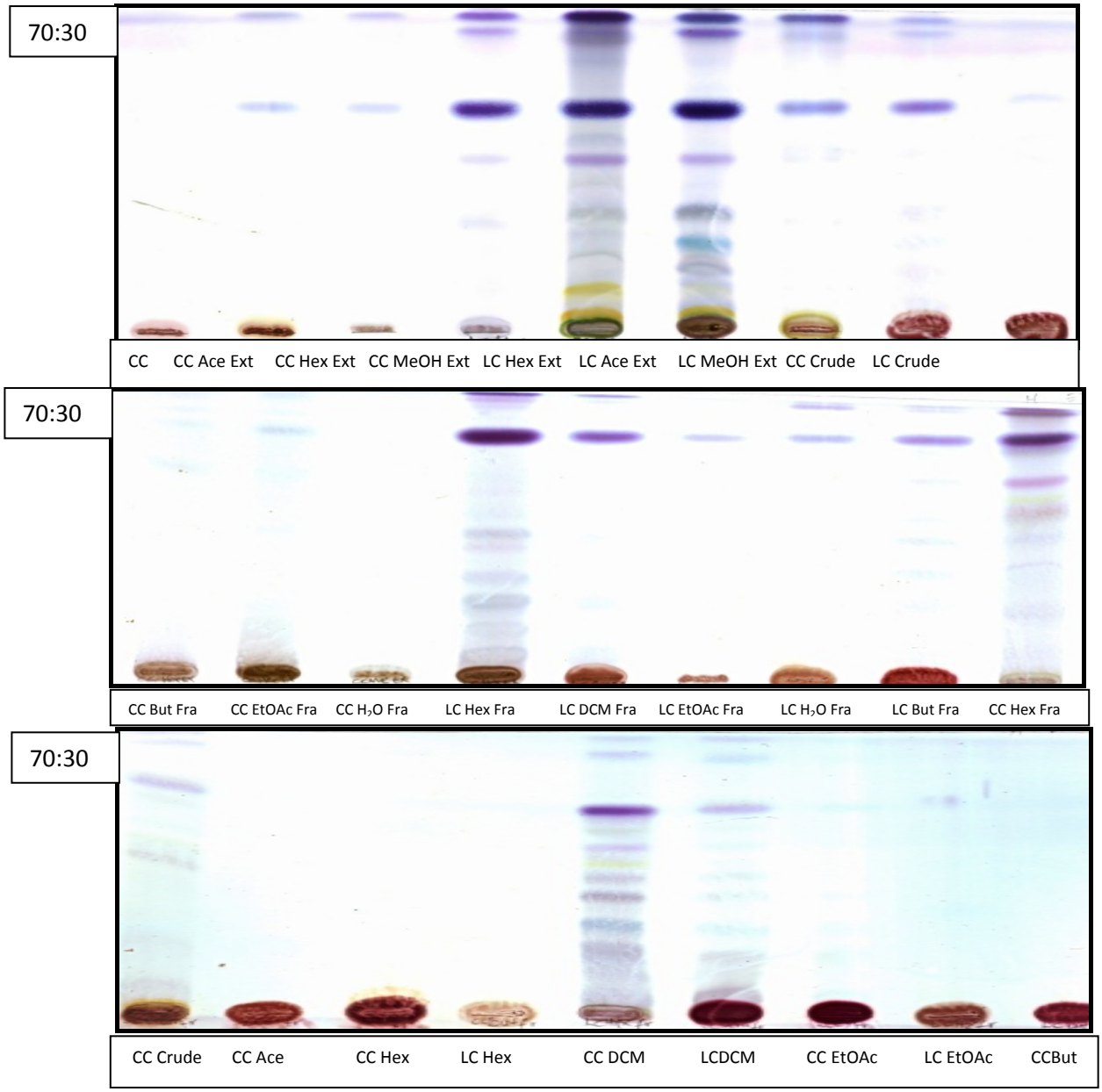
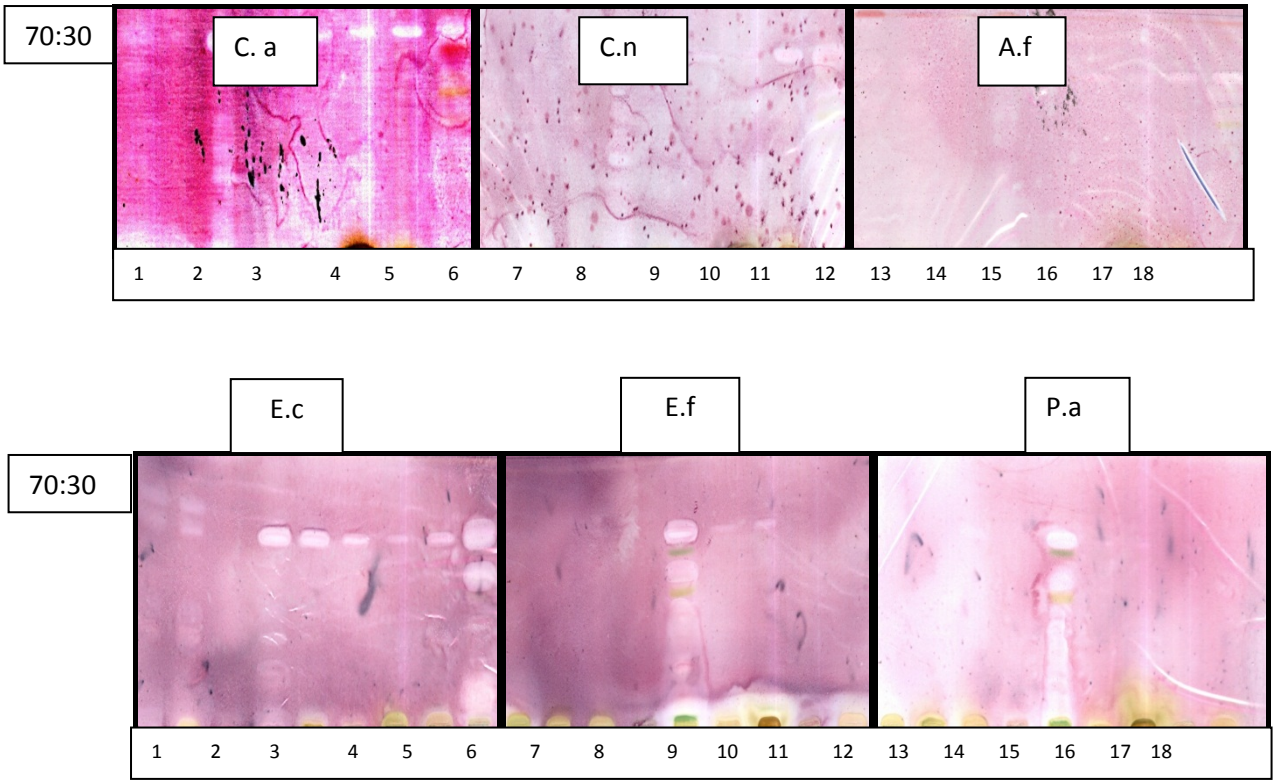



**Figure 5.4.** TLC chromatograms of the 70% acetone extracts and fractions developed in hexane: ethyl acetate (70:30%)

### 5.4.2 Bioautography

Bioautograms of fractions and extracts were prepared to visualise the compounds in the fractions and extracts (Figure 5.5) and were eluted in hexane: ethyl acetate (70:30). The hexane fractions of *C. capense* and *L. cassinoides* each had one zone of inhibition with Rf value of 0.91 against *Candida albicans* and *Cryptococcus neoformans*. The dichloromethane (DCM) fraction showed good activity against *E. coli*, E. *faecalis* and *P. aeruginos*a with a zone of inhibition at Rf = 0.74 followed by the hexane fraction and extract for *L. cassinoides* and *C. capense*. Butanol and ethyl acetate fractions were less active compared to the hexane and DCM fractions.




1:CC But Fra, 2: CC EtOAc Fra, 3: CC $H_2O$ Fra, 4: LC Hex Fra, 5: LC DCM Fra, 6: LC EtOAc Fra, 7: LC $H_2O$ Fra, 8: LC But Fra, 9: CC Hex Fra , 10:CC Bu tFra, 11: CC EtOAc Fra, 12:CC $H_2O$ Fra,13: LC Hex Fra , 14: LC DCM Fra, 15LC EtOAc Fra, 16 :LC $H_2O$ Fra, 17: LC But Fra, 18:CC Hex Fra

**Figure 5.5.** Bioautograms of acetone extract and fractions of various polarities against *E. coli* and *E. faecalis* organisms

### 5.4.3 Minimum inhibitory concentration (MIC)

Five fractions with differing polarity were prepared from each plant species. Microdilution antifungal assays to determine MIC values were then performed using the fungal pathogens. The most activity was observed in the ethyl acetate (EtOAc) and butanol fractions with MIC values of 0.04 mg/ml for fractions of *L. cassinoides* (Table 6.1). Both hexane fractions (*L. cassinoides* and *C. capense*) showed activity when tested against *C. neoformans*, *A. fumigatus* and *C. albicans*. However, the

crude extract of *C. capense* had an MIC value of 0.16 mg/ml when tested against *C. neoformans* and *A. fumigatus*. The crude extract of *C. capense* was more active than that of *L. cassinoides*. The water fraction of *L. cassinoides* with an MIC value of 0.04 mg/ml was also active against *A. fumigatus* while *C. capense* water fraction was active against both *C. neoformans* and *A. fumigatus*. The butanol fraction was also active against *C. albicans* and *A. fumigatus.* Fractions of *L. cassinoides* did not show much activity against *Candida* and *Cryptococcus neoformans* while the DCM fraction of *C. capense* was more active against *Aspergillus fumigatus*.

**Table 5.1.** MIC values of fractions (mg/ml) of two plant species (*L. cassinoides* and *C. capense*) against three fungal microorganisms

| **MIC (mg/ml)** | | | | **MIC (mg/ml)** | | | |
|---|---|---|---|---|---|---|---|
| *Calodendrum capense* | | | | *Lydenburgia cassinoides* | | | |
| | *C. a* | *C. n* | *A. f* | | **C. a** | **C. n** | **A. f** |
| C. c Cru | 0.31 | 0.16 | 0.16 | L.. c Cru | 0.31 | 0.31 | 0.16 |
| C. c Hex | 0.16 | 0.08 | 0.08 | L.. c Hex | 0.16 | 0.16 | 0.16 |
| C. c DCM | 0.31 | 0.16 | 0.04 | L.. c DCM | 0.63 | 0.63 | 0.31 |
| C.c ETOAc | 0.31 | 0.08 | 0.08 | L.c ETOAc | 0.16 | 0.16 | 0.04 |
| C. c But | 0.16 | 0.31 | 0.16 | L.. c But | 0.31 | 0.16 | 0.04 |
| C. c Water | 0.63 | 0.16 | 0.08 | L .c Water | 0.31 | 0.31 | 0.04 |
| Amphotericin B (µg/ml) | 1.25 | 0.62 | 0.62 | | 1.25 | 0.63 | 0.62 |

[1]Microorganisms = *C.n = Candida albicans*, *C.n =Cryptococcus neoformans*, *A. f= Aspergillus fumigatus.* [2]Plant species: *L. cassinoides = Lydenburgia cassinoides, C. capense = Calodendrum capense.* [3]Extractants: Cru = crude extract, Hex =Hexane, DCM = Dichloromethane, EtOAc = Ethyl acetate, $H_2O$ = Water, But =Butanol

Fractions were then further tested for antibacterial activity. The crude extract of *L. cassinoides* against *E. coli* was more active compared to activity against other organisms while the MIC value against *S. aureus* was greater than 2.5 mg/ml for *C. capense* (Table 6.2). Both hexane fractions from the two plant species showed good activity when tested against the four bacterial organisms.

**Table 5.2.** MIC (mg/ml) values of fractions from two plant species against four bacterial microorganisms

| **Plant species** | **Antibacterial activity of fractions (mg/ml)** | | | |
|---|---|---|---|---|
| | *S. a* | *E. c* | *P. a* | *E. f* |
| L. c crude | 0.63 | 0.08 | 0.16 | 0.16 |
| C. c crude | 1.25 | 0.63 | 0.16 | 0.16 |
| L. c Hex | 0.63 | 0.08 | 0.16 | 0.16 |
| C. c Hex | 0.31 | 0.08 | 0.04 | 0.16 |
| L. c DCM | 1.25 | 0.16 | 0.63 | 0.31 |
| C. c DCM | 1.25 | 0.63 | 1.25 | 0.16 |
| L. c ETOAc | 0.63 | 0.16 | 0.31 | 0.16 |
| C. c ETOAc | 0.63 | 0.16 | 0.04 | 0.16 |
| L. c But | 1.25 | 0.63 | 0.63 | 0.31 |
| C. c But | ›2.5 | 1.25 | 0.16 | 0.63 |
| L. c $H_2O$ | 1.25 | 0.16 | 0.31 | 1.25 |
| C. c $H_2O$ | ›2.5 | 0.63 | 0.31 | 0.31 |
| Gentamicin (µg/ml) | 1.25 | 0.16 | 0.31 | 0.31 |

[1]Microorganisms = *E. coli*= *Escherichia coli*, *E. faecalis* =*Escherichia faecalis*, *P. aeruginosa* = *Pseudomonas aeruginosa*, *S. aureus* = *Staphylococcus aureus,* [2]Plant species: *L. cassinoides* = *Lydenburgia cassinoides, C. capense* = *Calodendrum capense.* [3]Extractants: Hex =Hexane, DCM= Dichloromethane, EtOAc= Ethyl acetate, But= Butanol and $H_2O$ = water

Figure 5.6 shows the fractions of *L. cassinoides* (A) and *C. capense* (B) separated using TLC. Similar fractions were combined for each plant species and loaded on the TLC plates (10 cm x 20 cm). Fractions were eluted in two solvent systems, namely hexane:ethyl acetate (100:7) and hexane:ethyl acetate (100:15). The TLC plates show polar and non-polar compounds with different $R_f$ values after spraying with vanillin-sulphuric acid spray reagent.

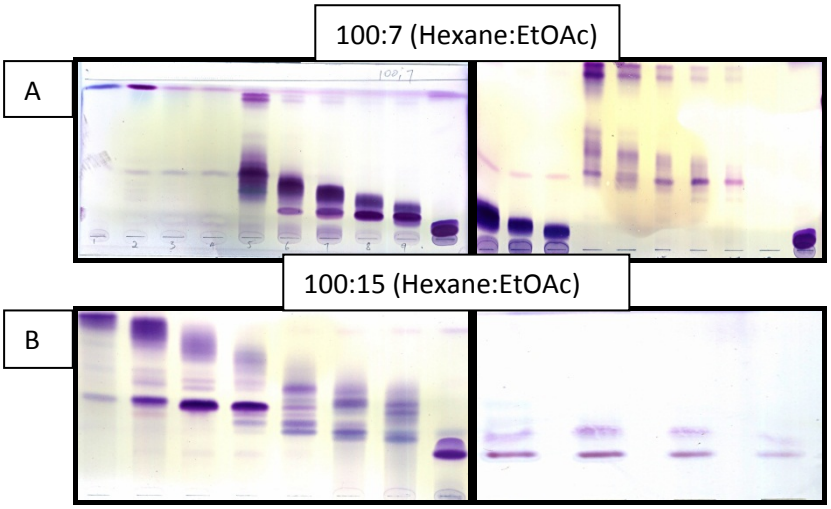



**Figure 5.6.** Fractions of *L. cassinoides* and *C. capense* developed in two solvent systems and sprayed with vanillin sulphuric acid

The TLC plate in Figure 5.7 shows the combined fractions from the two plant species developed in hexane:ethyl acetate (70:30) and sprayed with vanillin-sulphuric acid. Compounds were isolated after the second column of extracts from each plant species.

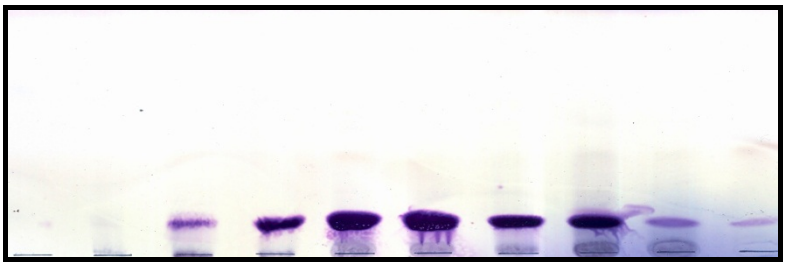


**Figure 5.7.** Compounds from *L. cassinoides* (β-amyrin) and *C. capense* (lupeol) developed in hexane:ethyl acetate (70:30) and sprayed with vanillin-sulphuric acid

The crude extracts of the two plant species and two isolated compounds were tested for antimicrobial activity against eight microbial pathogens. The method is described in sections 4. 2. 5 and 4. 2. 6.

The MIC values of the isolated compounds and crude extracts against fungal pathogens are shown in Table 6.3. The crude extracts and two isolated compounds were tested against four resistant clinical isolates of *Candida albicans* (designated numbers 1051608, 1051604, M0825 and 824), where the activity was average, one ATCC strain (10231) and three other laboratory isolates (*Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus).* It was observed that both compounds *(ß*-amyrin and lupeol*)* had good activity against *Cryptococcus neoformans* and *Aspergillus fumigatus,* followed by lupeol where activity was also seen against the resistant strain M0825.

**Table 5.3.** Minimum inhibitory concentrations of two isolated compounds (mg/ml) against three laboratory standard fungal strains and four resistant *Candida* strains.

| **Crude extract /compound** | **Different strains of *Candida albicans* isolates** | | | | | | *C. n* | *A. f.* | *Amph B* |
|---|---|---|---|---|---|---|---|---|---|
| | **laboratory isolate** | *ATCC10231* | *1051608* | *1051604* | *M0825* | *824* | | | |
| **L. C. extract** | 0.31 | 0.31 | ›2.5 | 125 | 0.31 | ›2.5 | 0.31 | 0.31 | 1.25 |
| **C.c. extract** | 0.31 | 0.62 | 1.25 | 1.25 | 0.62 | ›2.5 | 1.25 | 0.62 | ›2.5 |
| **ß-amyrin** | 0.062 | 0.031 | 0.062 | 0.062 | 0.032 | ›0.025 | 0.015 | 0.015 | 0.031 |
| **Lupeol** | 0.062 | 0.031 | 0.062 | 0.062 | 0.015 | ›0.025 | 0.015 | 0.015 | 0.031 |

[1]L. C. = *L. cassinoides*, C. c. = *C. capense;* [2]*C. n. = Cryptococcus neoformans, A.f. = Aspergillus fumigatus*, Amph B = amphotericin B

Both compounds were also tested against the four bacterial ATCC strains. ß-Amyrin and lupeol showed good activity against all the organisms. Both compounds had the same Rf values on TLC, and both belong to the pentacyclic sub-class of triterpenoids, so it is unsurprising that they show a similar profile of biological activity.

The microdilution assay of the hexane crude extracts from *L. cassinoides* and *C. capense* and the isolated compounds revealed that both crude extracts of plant species and the isolated compounds were active against all the tested bacterial microorganisms. The positive control gentamicin was used against the bacteria while ciproflaxin was the positive control for the two *Mycobacterium* species.

**Table 5.4.** Minimum inhibitory concentration (MIC) of two isolated compounds (µg/ml) against six bacterial organisms from *L. cassinoides* and *C. capense*

| **Crude extract / compound** | **Microorganisms** | | | | | |
|---|---|---|---|---|---|---|
| | *S. a.*[1] | *E. c.* | *E. f.* | *P. a.* | *M. smegmatis* | *M. bovis* **BCG** |
| **L. c. extract** | 63 | 80 | 16 | 16 | 16 | 31 |
| **C. c. extract** | 125 | 63 | 16 | 16 | 16 | 31 |
| **ß-amyrin** | 15 | 31 | 19 | 75 | 16 | 15 |
| **Lupeol** | 15 | 31 | 9. | 75 | 31 | 15 |
| **Gentamicin** | 20 | 80 | 80 | 20 | | |
| **Ciprofloxacin** | | | | | 19 | 75 |

[1]*S.a.* = *Staphylococcus aureus*, E. c. = *Escherichia coli*, E. f. = *Enterococcus faecalis*, P. a. = *Pseudomonas aeruginosa, M . smeg= Mycobacterium smegmatis, M. Bovis BCG= Mycobacterium bovis*

## 5.5 Conclusion

One active compound was isolated from each species of interest, namely lupeol from *C. capense* and β-amyrin from *L. cassinoides.* These two compounds eluted at the same Rf value using different TLC solvent systems, and are both pentacyclic triterpenoids.

The two compounds and both crude extracts had antimicrobial activity against all the tested organisms, and the two compounds indicated similar degrees of microbial growth inhibition.

# Chapter 6

# Structure elucidation of isolated compounds from *L. cassinoides* and *C. capense*

## 6.1 Nuclear Magnetic Resonance (NMR)

NMR spectroscopy (1D and 2D) is a very useful technique in structure elucidation of compounds from natural resources, including those isolated from plants. Data obtained from 1D ($^{1}$H- and $^{13}$C-NMR), and 2D spectra (HMQC, HMBC, COSY, etc) are enough to assign fully protons and carbons of a structure of the compound of interest.

The $^{1}$H- and $^{13}$C-NMR spectra usually exhibit signals in part per million (ppm), characteristic for protons and carbons present in the molecules. The experiment is called one-dimensional or 1D-NMR. Further measurements are required after 1D-NMR to establish correlations between protons and carbons (HMQC, HMBC) and between protons and protons (COSY, NOESY, TOCS). For example, COSY, which is a homonuclear correlation, is helpful to establish proton-proton coupling ($^{2}$J, $^{3}$J) while NOESY allows the assignment of the spatial orientation (stereochemistry) of protons within the same molecule. HMQC and HMBC are used to assigned direct and long corrections between protons and carbons and are very helpful to build up the chemical structure during elucidation.

When structure elucidation becomes more complicated, such as in the case of unusual or new compounds, other techniques such as mass spectrometry (MS), ultra-violet (UV), infra-red (IR) data and literature information are required to facilitate the structure determination (Bastert, 2001).

## 6.2 Structure elucidation of isolated compounds from *L. cassinoides* and *C. capense*

The structures of the compounds isolated in this study were determined by extensive NMR techniques and chemical methods, mainly by 1D NMR ($^{1}$H, $^{13}$C and DEPT) and 2D NMR (HSQC, HMBC and COSY) and by comparison with published literature data.

### 6.2.1 Structure elucidation of compound 1

Compound **1** was obtained as white powder and gave a positive result with the Liebermann Burchard test characteristic for triterpenes. Its $^{13}$C NMR spectrum (Figure 6.2a) exhibited a total number of 30 carbons with 3 downfields displayed at $\delta$ 145.4, 121.9 and 79.3 ppm assignable to a triterpene skeleton with one ethylenic double bond (C=CH) and one oxymethine (HCOH) groups characteristic for $\beta$-amyrin skeleton. The $^{1}$H-NMR data (Table 6.2.1, Figure 6.2b) of compound **1** had seven singlets assignable to

eight methyl groups (at $\delta$ 0.79, 0.83, 0.87, 0.93, 0.96, 0.99 and 1.13), one ethylenic proton (at $\delta$ 5.18, *m*, H-12), one oxygenated methine proton (at $\delta$ 3.19, *m*, H-3). The $^{13}$C-NMR data in combination with HSQC (Table 6.1, Figure 6.2.1a to Figure 6.2.1d) revealed the presence of a total number of 30 carbons including six quaternary carbons, seven methines, nine methylenes and eight methyl groups. Signals due to two ethylenic carbons were observed at $\delta$ 121.9 (C-12) and 145.4 (C-13) while signals owed to oxygenated carbon and methyl groups were found at $\delta$ 79.3 (C-3), 33.6 (C-29), 28.6 (C-28), 28.3 (C-23), 26.2 (C-27), 23.9 (C-30), 17.0 (C-26), 15.8 (C-25) and 15.7 (C-24). All these NMR data were similar to those published for $\beta$-amyrin (**1**, Figure 6.1), a compound commonly found in the plant kingdom (Mahato and Kundu, 1994).

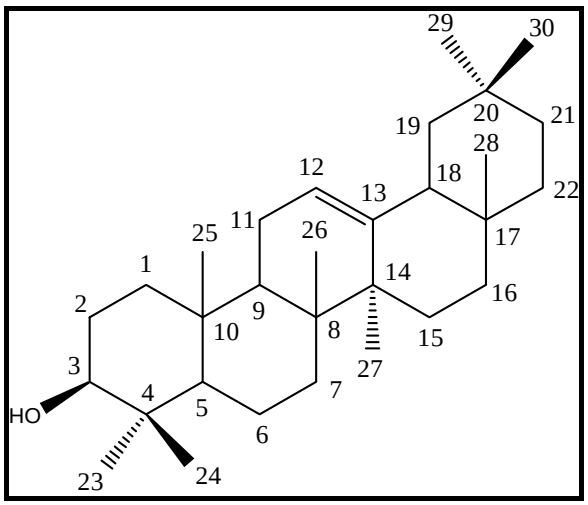



**Figure 6.1.** Structure of $\beta$-amyrin (**1**) isolated from *Lydenburgia cassinoides* leaves

**Table 6.1.** NMR (600 MHz) data for compound **1** (β-amyrin) in $CDCl_3$

| **Position** | **$^1$H** | **$^{13}$C** | **Published data (Mahato and Kundu, 1994)** |
|---|---|---|---|
| 1 | | 38.8 ($CH_2$) | 38.7 |
| 2 | | 27.5 ($CH_2$) | 27.3 |
| 3 | 3.19 (*m*) | 79.3 (CH) | 79.0 |
| 4 | | 39.0 (C) | 38.8 |
| 5 | | 55.4 CH | 55.3 |
| 6 | | 18.6 $CH_2$ | 18.5 |
| 7 | | 32.9 $CH_2$ | 32.8 |
| 8 | | 39.0 (C) | 38.8 |
| 9 | | 47.8 (CH) | 47.7 |
| 10 | | 37.4 (C) | 37.6 |
| 11 | | 23.8 $CH_2$ | 23.6 |
| 12 | 5.18 (*m*) | 121.9 (CH) | 121.8 |
| 13 | | 145.4 (C) | 145.1 |
| 14 | | 41.9 (C) | 41.8 |
| 15 | | 26.4 ($CH_2$) | 26.2 |
| 16 | | 27.2 ($CH_2$) | 27.0 |
| 17 | | 32.7 (C) | 32.5 |
| 18 | | 47.5 (CH) | 47.4 |
| 19 | | 47.1 (CH) | 46.9 |
| 20 | | 31.3 (CH) | 31.1 |
| 21 | | 34.9 ($CH_2$) | 34.8 |
| 22 | | 37.2 ($CH_2$) | 37.2 |
| 23 | 0.83 (*s*) | 28.3 ($CH_3$) | 28.2 |
| 24 | 0.87 (*s*) | 15.7 ($CH_3$) | 15.5 |
| 25 | 0.93 (*s*) | 15.8 ($CH_3$) | 15.6 |
| 26 | 0.79 (*s*) | 17.0 ($CH_3$) | 16.9 |
| 27 | 1.13 (*s*) | 26.2 ($CH_3$) | 26.0 |
| 28 | 0.96 (*s*) | 28.6 ($CH_3$) | 28.4 |
| 29 | 0.87 (*s*) | 33.6 ($CH_3$) | 33.3 |
| 30 | 0.99 (*s*) | 23.9 ($CH_3$) | 23.7 |

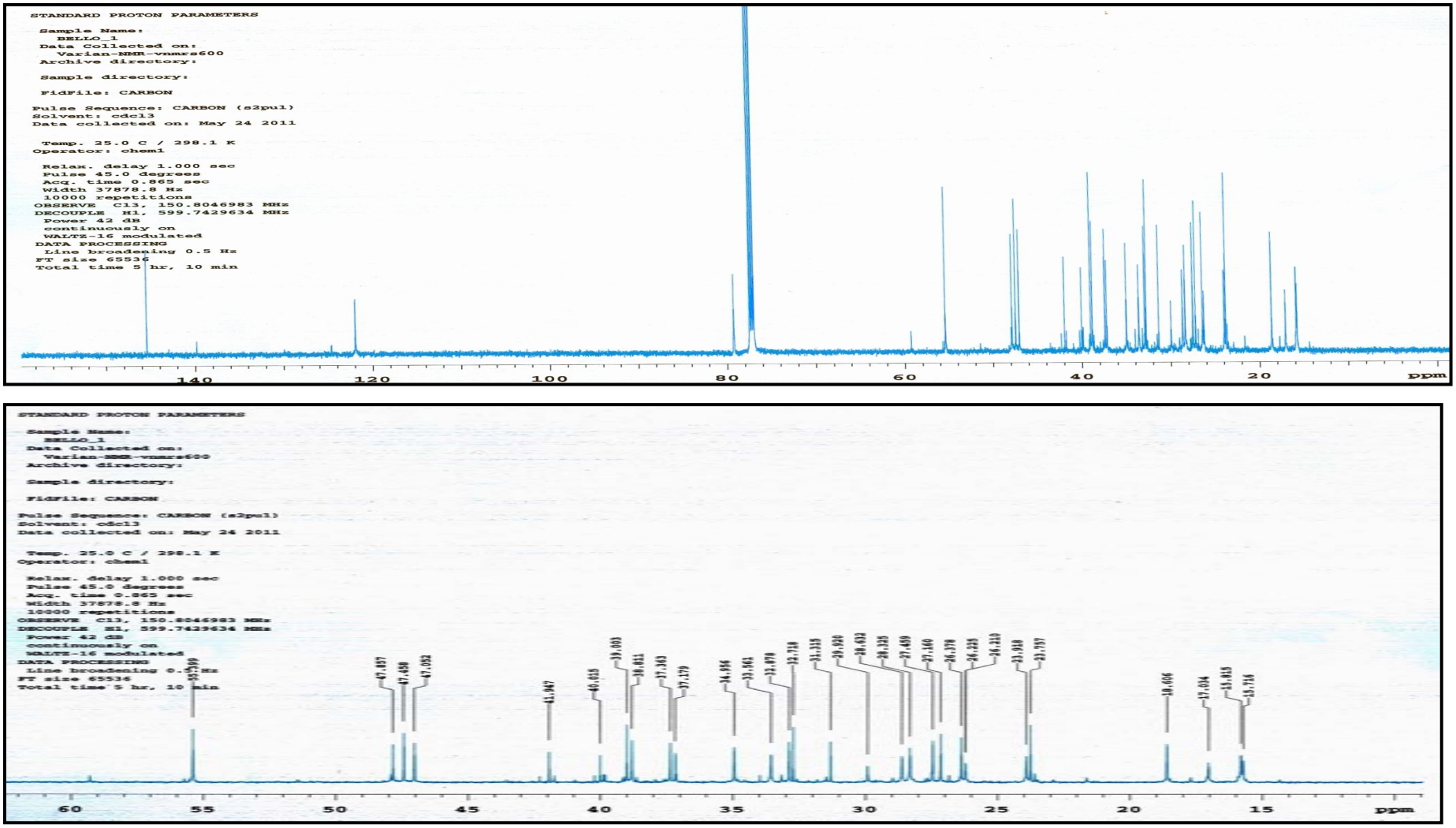



**Figure 6.2a.** $^{13}$C NMR spectra of Compound **1** (β amyrin)

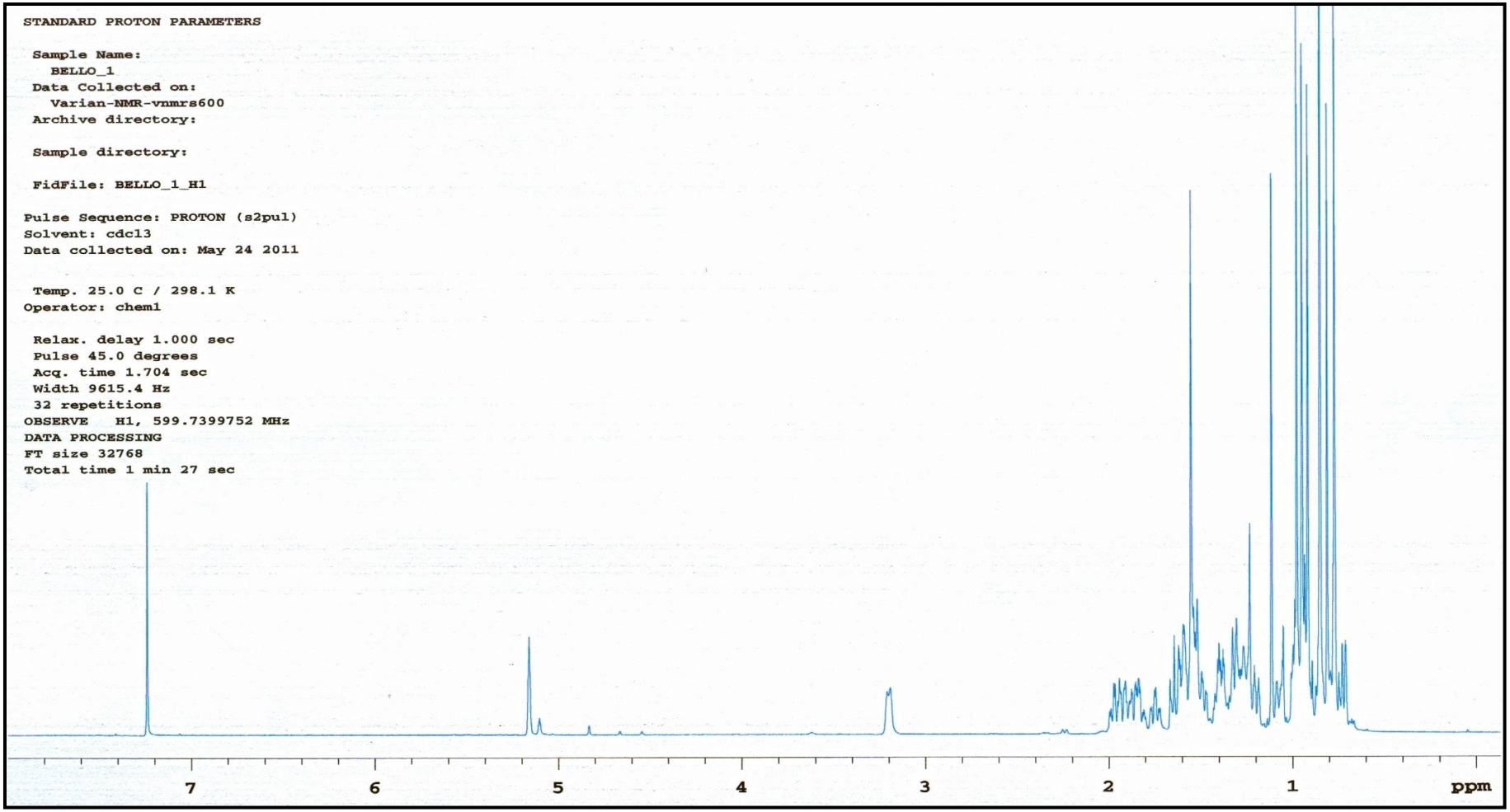



**Figure 6.2b.** $^{1}$H NMR spectrum of compound **1** (β amyrin)

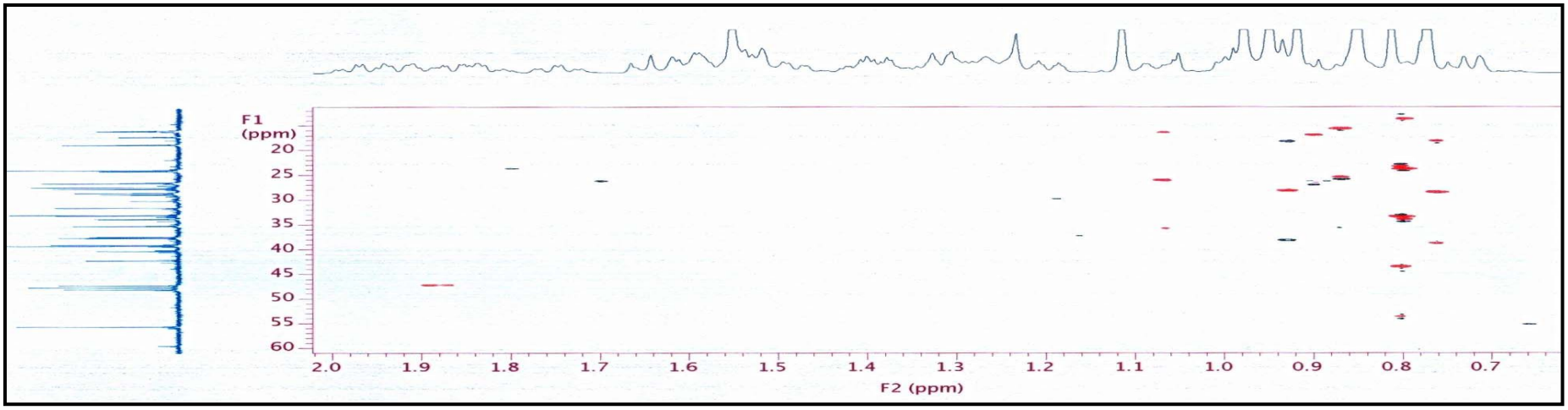



**Figure 6.2c.** HSQC spectrum of compound **1** (β amyrin)

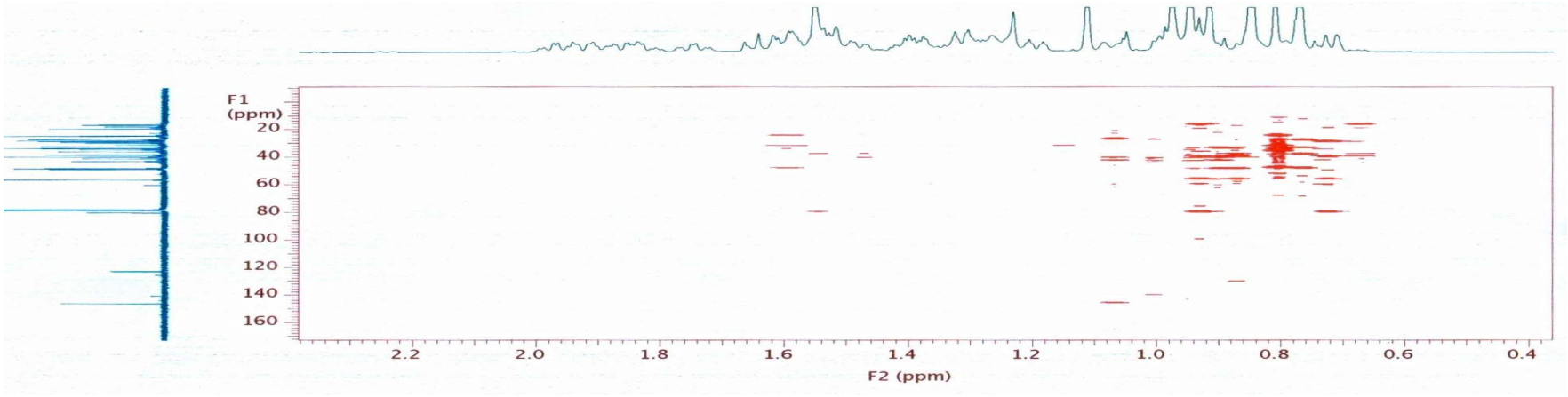



**Figure 6.2d.** HMBC spectrum of compound **1** (β amyrin)

### 6.2.2 Structure elucidation of compound 2

Compound **2** was obtained as a white powder and gave a positive result with the Liebermann Burchard test characteristic for triterpenes. Its $^{1}H$ and $^{13}C$ NMR spectra (Table 6.2, Figures 6.3 and 6.4b) exhibited signals at $\delta$ 4.67 (*brs*, H-29$_a$), 4.54 (*brs*, H-29$_b$), 3.18 (*m*, H-3), 109.3 (C-29), and 79.0 ppm (C-3) assignable respectively to protons and carbons of a methylene and oxymethine groups at positions 29 and 3 of a lup-20(29)-en-3-ol class of triterpenes (Mahato and Kundu, 1994). Furthermore, one characteristic signal of carbon C-20 of lupeol structure was observed on the $^{13}C$ NMR spectrum (Figure 6.4b) at $\delta$ 151.0 ppm while seven singlet signals relevant for angular methyl groups appeared on the $^{1}H$ NMR spectrum between 0.65-1.60 ppm. All the NMR data (Table 6.2, Figures 6.4a to 6.4e) were in agreement with those published for lupeol (**1**) (Mahato and Kundu, 1994).

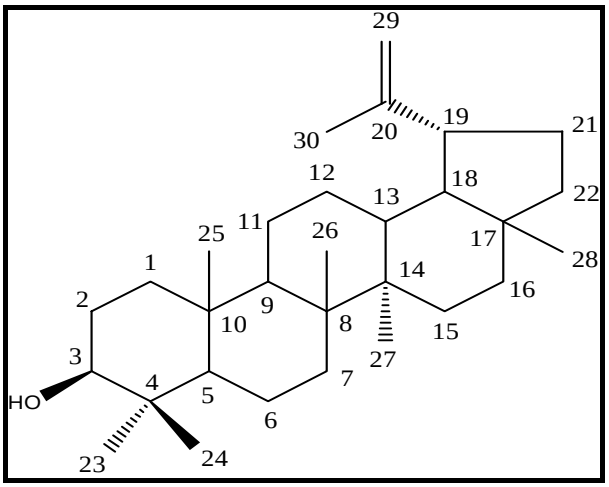



**Figure 6.3.** Structure of lupeol (**2**) isolated from *Calodendrum capense* leaves

**Table 6.2.** NMR (500 MHz) data for compound 2 (lupeol) in $CDCl_3$

| Position | $^{1}H$ | $^{13}C$ | Published data (Mahato and Kundu, 1994) |
|---|---|---|---|
| 1 | | 38.7 ($CH_2$) | 38.7 |
| 2 | | 27.4 ($CH_2$) | 27.4 |
| 3 | 3.18 (*m*) | 78.9 (CH) | 78.9 |
| 4 | | 38.9 (C) | 38.8 |
| 5 | | 55.3 (CH) | 55.3 |
| 6 | | 18.3 ($CH_2$) | 18.3 |
| 7 | | 34.3 ($CH_2$) | 34.2 |
| 8 | | 40.8 (C) | 40.8 |
| 9 | | 50.4 (CH) | 50.4 |
| 10 | | 37.2 (C) | 37.1 |
| 11 | | 20.9 ($CH_2$) | 20.9 |
| 12 | | 25.1 ($CH_2$) | 25.1 |
| 13 | | 38.0 (CH) | 38.0 |
| 14 | | 42.8 (C) | 42.8 |
| 15 | | 27.4 ($CH_2$) | 27.4 |
| 16 | | 35.6 ($CH_2$) | 35.5 |
| 17 | | 43.0 (C) | 43.0 |
| 18 | | 48.3 (CH) | 48.2 |
| 19 | | 47.9 (CH) | 47.9 |
| 20 | | 151.0 (C) | 150.9 |
| 21 | | 29.8 ($CH_2$) | 29.8 |
| 22 | | 40.0 ($CH_2$) | 40.0 |
| 23 | 0.94 (*s*) | 28.1 ($CH_3$) | 28.0 |
| 24 | 0.73 (*s*) | 15.4 ($CH_3$) | 15.4 |
| 25 | 0.80 (*s*) | 16.1 ($CH_3$) | 16.1 |
| 26 | 0.91 (*s*) | 16.0 ($CH_3$) | 15.9 |
| 27 | 0.95 (*s*) | 14.5 ($CH_3$) | 14.5 |
| 28 | 1.19 (*s*) | 18.3 ($CH_3$) | 18.0 |
| 29 | 4.67 (*brs*), 4.54 (*brs*) | 109.3 ($CH_2$) | 109.3 |
| 30 | 1.60 (*s*) | 19.3 ($CH_3$) | 19.3 |

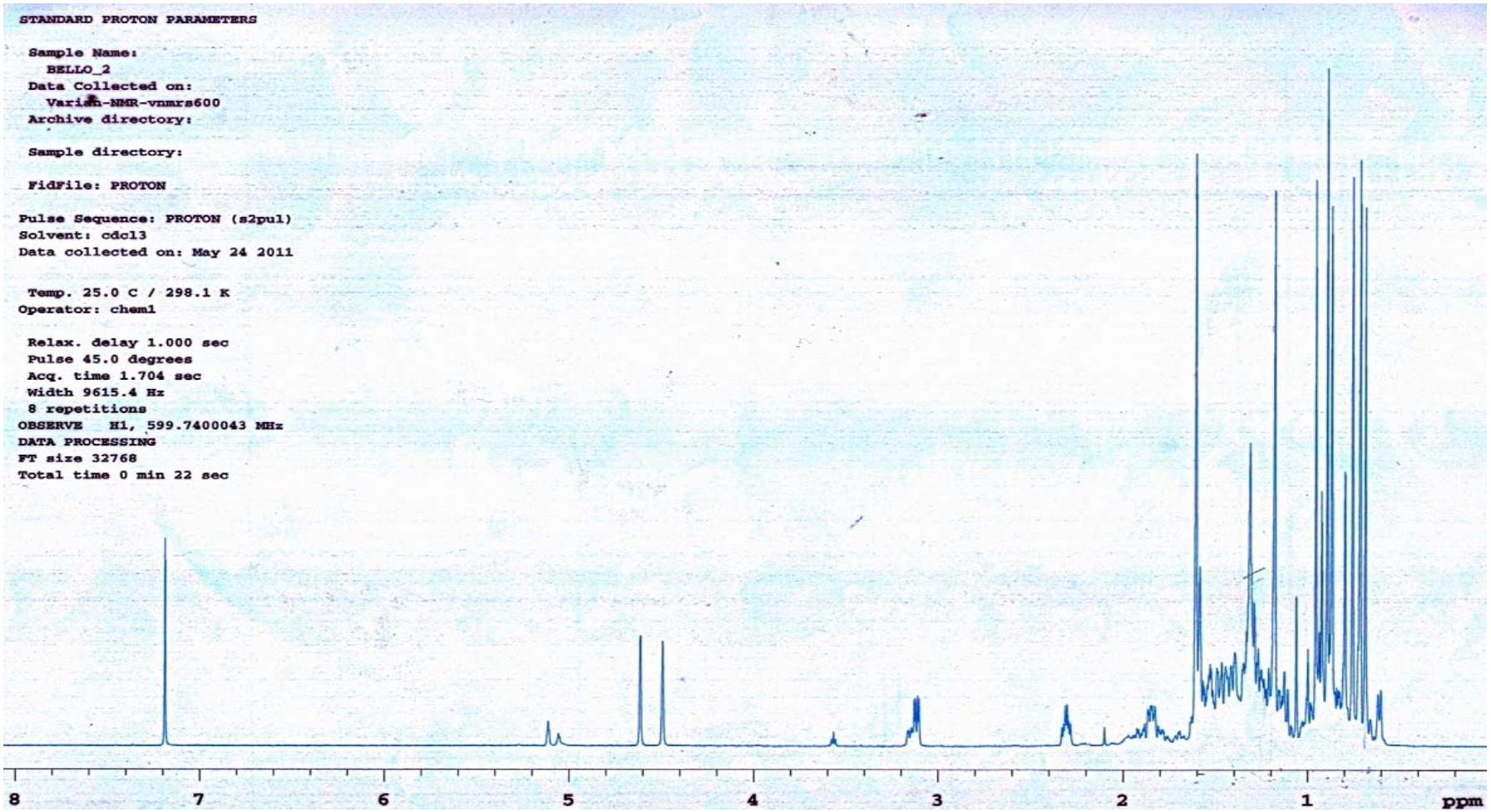



**Figure 6.4a.** $^{1}$H NMR spectrum of compound **2** (lupeol)

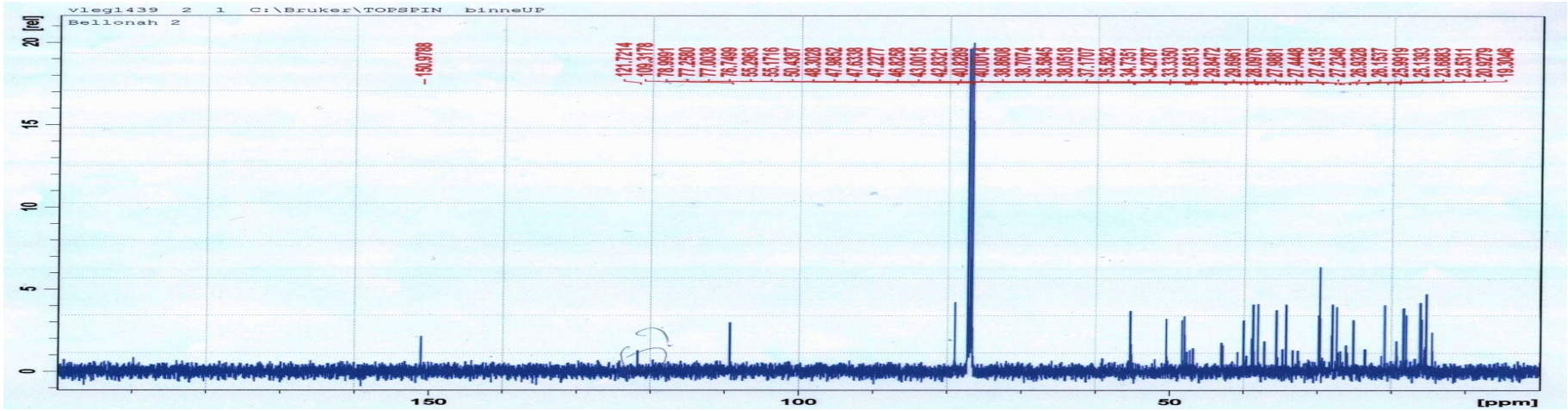



**Figure 6.4b.** $^{13}$C NMR spectrum of compound **2** (lupeol)

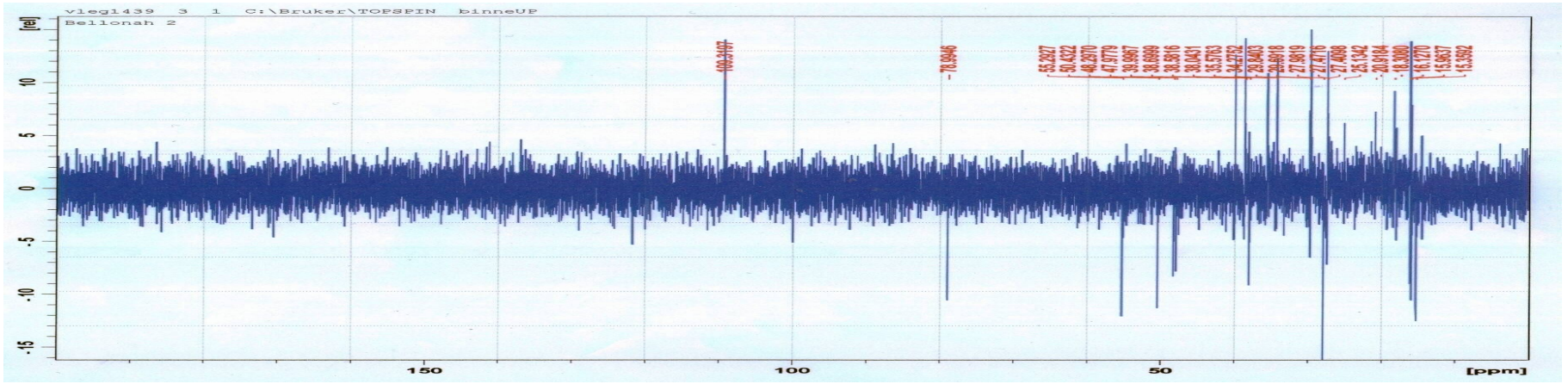



**Figure 6.4c.** DEPT NMR spectrum of compound **2** (lupeol)

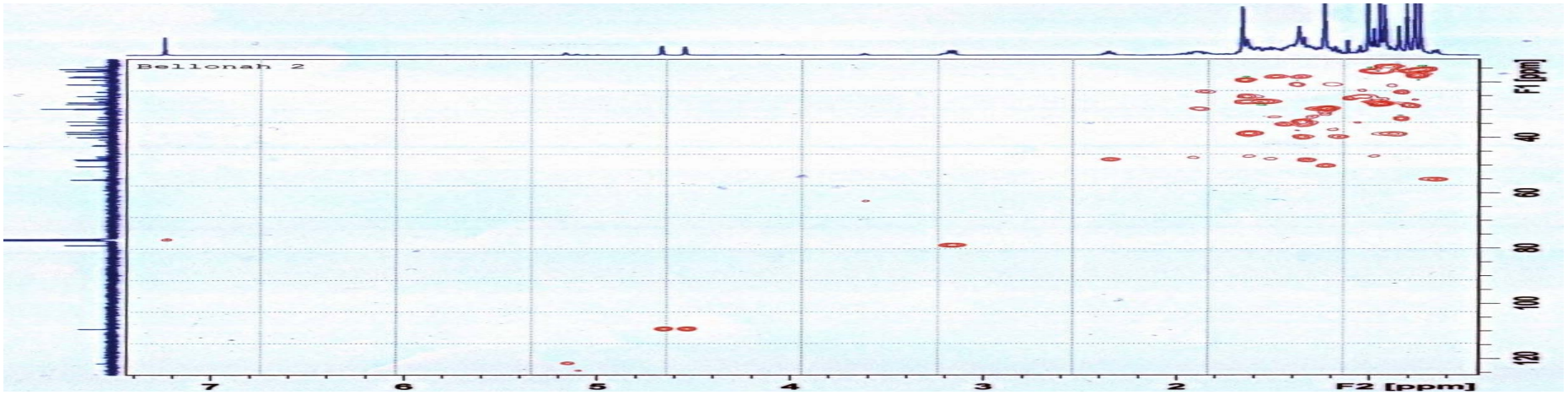



**Figure 6.4d.** HSQC spectrum of compound **2** (lupeol)

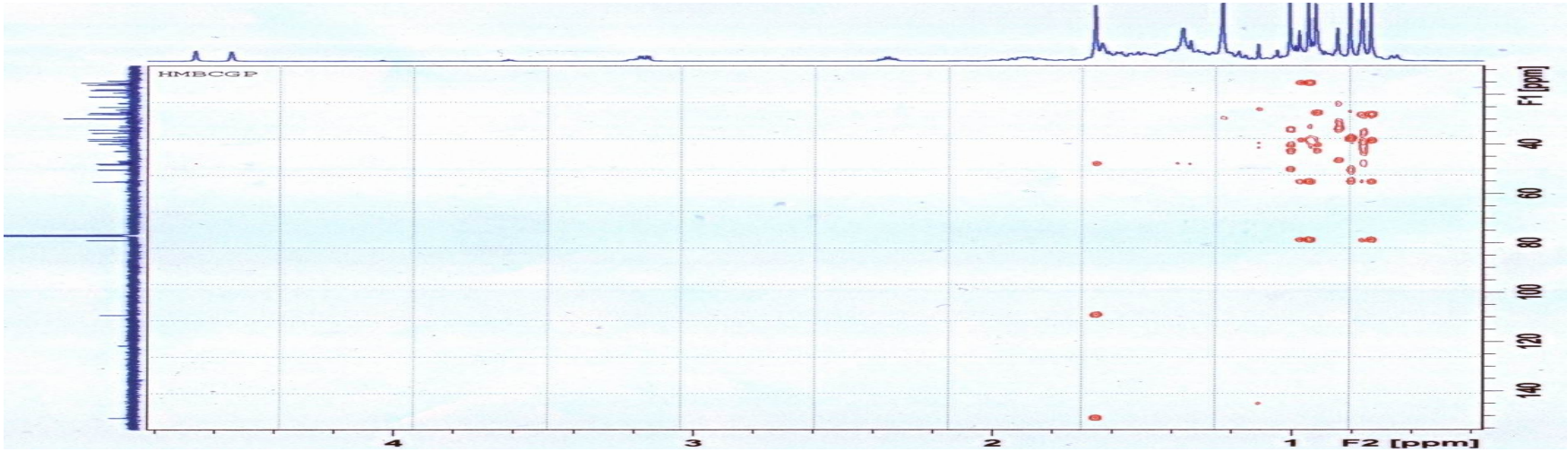



**Figure 6.4e.** HMBC spectrum of compound **2** (lupeol)

# Chapter 7

# Biological activity of isolated compounds from *Lydenburgia cassinoides* and *Calodendrum capense*

## 7.1 Introduction

Pentacyclic triterpenoids are triterpenes which belong to the group of saponin compounds Triterpenes belong to a large group of compounds arranged in a four or five ring configuration of 30 carbons with several oxygens attached. They are assembled from C5 isoprene units through the cytosol mevalonate pathway to make a C30 compound and are steroidal in nature. This group of compounds is commonly found in plants (Mahato and Kundu, 1994). Around 4000 triterpenoids are known and usually occur freely, but others may occur as glycosides (saponins). Triterpenoids have a wide spectrum of biological activities and are common active compounds in plants used in traditional medicine (Ponou et. al, 2008, Mahato et al., 1992; Singh et al., 2002; Garcez et al., 2006; Cao et al., 2010). Triterpenoids are secondary metabolites of mixed biosynthesis, as are alkaloids, flavonoids and oligosaccharides. Examples of common terpenoids are geranial and camphor (monoterpenes), farnesol and artemisinin (sesquiterpenoids). Artemisinin and its derivative α-arteether, also known by the name qinghaosu, find current use as antimalarials especially in the treatment of cerebral malaria (Cotton, 2011; Cowan, 1999).

Triterpenoid compounds were isolated from the two species under study, namely *Lydenburgia cassinoides* and *Calodendrum capense*. These compounds were tested for antimicrobial activity against a range of fungal and bacterial species.

## 7.2 Materials and Methods

TLC plates were prepared as described in section 3.5.4. Aliquots of 5 µl of a 1 mg/ml solution of each isolated compound were loaded on TLC plates. The plates were developed in BEA and hexane:ethyl acetate (95:5 and 90:10 or 97:3). One set of plates was sprayed with vanillin-sulphuric acid spray reagent as a reference set of chromatograms and further sets of plates were used for bioautography, using fungal and bacterial test organisms.

The crude hexane leaf extracts of *Lydenburgia cassinoides* and *Calodendrum capense*, and the two compounds isolated from these plant extracts were tested for antimicrobial activity in the

microdilution assay against nine microbial pathogens. The method is described in sections 4. 2. 5 and 4. 2. 6.

### 7.3 Results and Discussion

The two isolated compounds showed the same $R_f$ values (Figure 7.1) but were confirmed to be not the same compounds by their differing NMR structures.

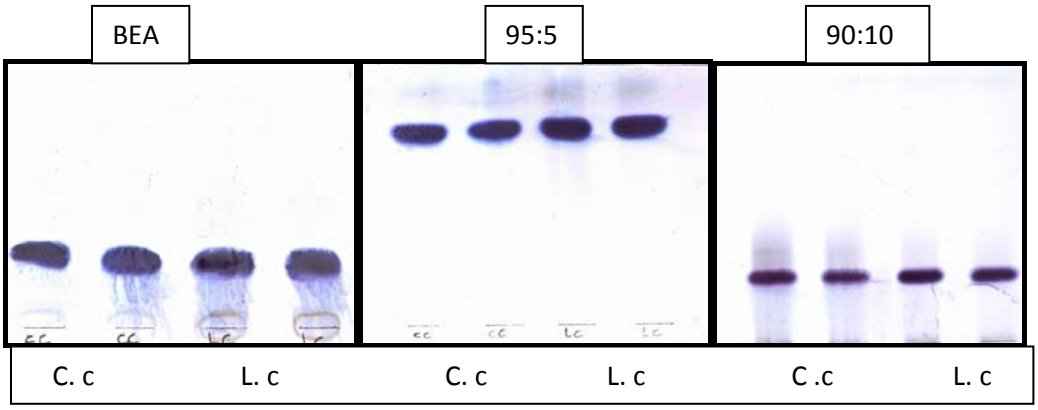



**Figure 7.1.** TLC chromatograms of isolated compounds developed in hexane:ethyl acetate (90:10), BEA, and hexane:ethyl acetate (95:5) of β-amyrin from *Lydenburgia cassinoides* (L. c) and lupeol from *Calodendrum capense* (C. c). The chromatograms were sprayed with vanillin-sulphuric acid spray reagent.

The bioautograms of the compounds showed that they both had $R_f$ values of 0.14 in BEA, 0.66 in hexane:ethyl acetate (95:5) and 0.59 in hexane:ethyl acetate (97:3) mobile systems (Figure 7.2). The results also validate that the two compounds were active against the three tested fungal isolates as clear zones of inhibition were visualised against all the tested organisms.

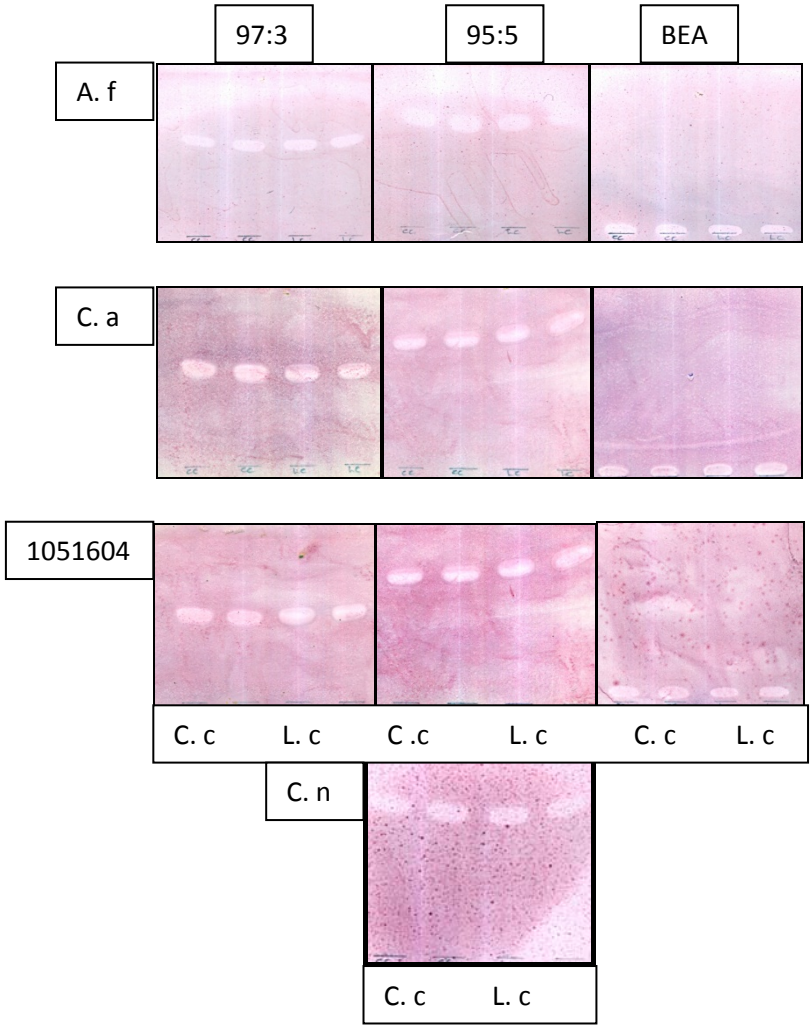



**Figure 7.2.** Bioautograms of ß-amyrin from *Lydenburgia cassinoides* and lupeol from *Calodendrum capense* developed in BEA, hexane:ethyl acetate (97:3) and hexane:ethyl acetate (95:5). The bioautograms were also sprayed with three fungal organisms and one resistant laboratory fungal isolate (A. f = *Aspergillus fumigatus*, C. a = *Candida albicans*, C. n = *Cryptococcus neoformans* and 10516044 = *C. albicans* resistant strain M0825).

The compounds β-amyrin and lupeol showed good activity against the four bacterial strains included in the testing (Figure 7.3). Clear zones of inhibition were observed, showing as white spots against a reddish background. The $R_f$ values ranged between 0.39 and 0.95.

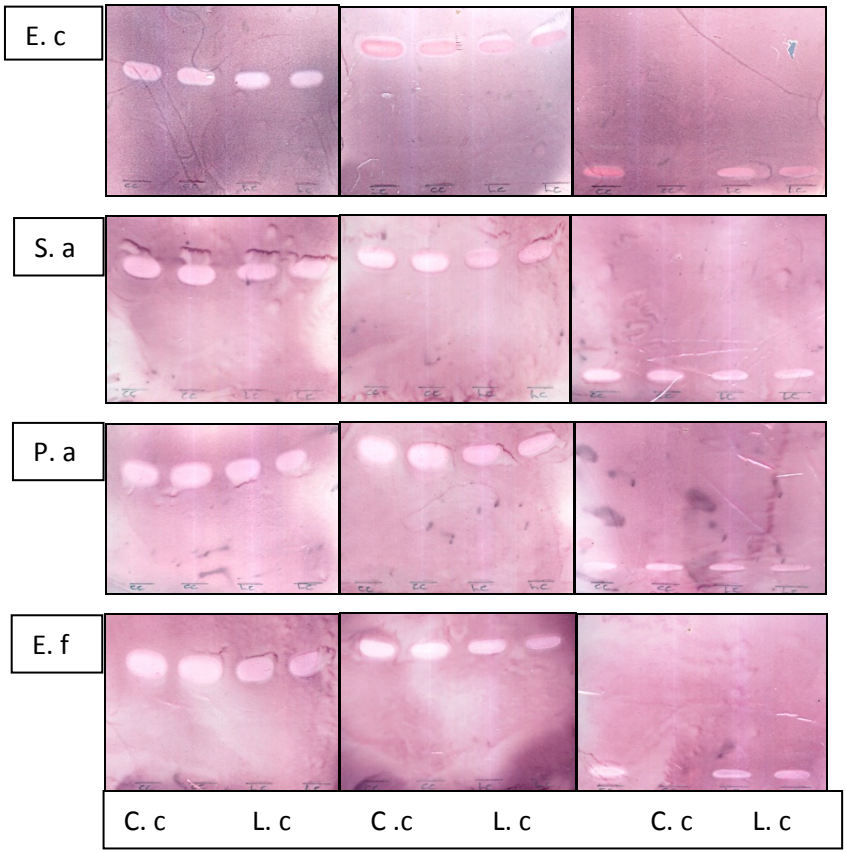



**Figure 7.3.** Bioautograms of ß-amyrin from *Lydenburgia cassinoides* (L. c) and lupeol from *Calodendrum capense* (C. c) developed in BEA, hexane:ethyl acetate (97:3) and hexane:ethyl acetate (95:5). The bioautograms were sprayed with four bacterial organisms (E. c = *Escherichia coli,* P. a = *Pseudomonas aeruginosa,* S. a = *Staphylococcus aureus* and E. f = *Enterococcus faecalis*).

The MIC values of the isolated compounds against fungal pathogens are shown in Table 7.1. It was observed that both compounds *(ß*-amyrin and lupeol) had good activity against *Cryptococcus neoformans* and *Aspergillus fumigatus,* and lupeol was also active against the resistant strain M0825 of *C. albicans.*

**Table 7.1.** Biological activity of isolated compounds tested against the three fungal pathogens (MIC values in µg/ml)

| Isolated Compounds | Microorganisms (3 Laboratory isolates, 1 ATCC and 4 resistance strains of C. albicans) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. albicans* | ATCC 10231 | 1051608 | 1051604 | M0825 | 824 | C. n | A. f |
| ß-amyrin | 62 | 31 | 62 | 62 | 31 | ›2.5 | 16 | 16 |
| Lupeol | 62 | 31 | 62 | 62 | 16 | ›2.5 | 16 | 16 |
| Amph B | 31 | 31 | 1.25 | 1.25 | 16 | 1.25 | 8 | 8 |

Both compounds were also tested against the four bacterial ATCC strains. *ß*-Amyrin and lupeol showed good activity against all the organisms with similar MIC values (Table 7.2). These isolated compounds are from one class of compounds and had the same Rf values in various TLC solvent systems but they were isolated from two different plant species.

**Table 7.2.** Biological activity of isolated compounds tested against four bacterial microorganisms

| **Microorganisms** | **β-amyrin** | **lupeol** | **Gentamicin (µg/ml)** |
|---|---|---|---|
| *E. coli* | 31 | 31 | 16 |
| *S. aureus* | 16 | 16 | 19 |
| *P. aeruginosa* | 78 | 78 | 78 |
| *E. faecalis* | 19 | 19 | 80 |

The two isolated compounds were further tested for antimycobacterial activity. The results shown in Table 7.3 revealed that β-amyrin and lupeol were active against *Mycobacterium smegmatis* and *M. bovis* BCG. The positive control, ciprofloxacin, also showed activity against both pathogens.

**Table 7.3.** MIC values (µg/ml) of isolated compounds tested against two *Mycobacterium* species

| **Microorganisms** | **β-amyrin** | **lupeol** | **Ciprofloxacin** |
|---|---|---|---|
| *Mycobacterium smegmatis* | 16 | 31 | 19 |
| *Mycobacterium bovis* BCG | 16 | 16 | 78 |

## 7.4 Conclusion

The findings from this study show that the two isolated compounds were highly active against fungal and bacterial pathogens, with the lowest MIC value of 16 µg/ml, comparable to MIC values achieved with the positive controls. The compounds also had good activity against the two *Mycobacterium* strains included in the study, indicating potential application in antimycobacterial therapy. Immunocompromised patients are often affected by secondary infections with mycobacterial species that are not normally infectious to healthy individuals so it was of value to investigate antimycobacterial activity of the crude extracts and isolated compounds.

# Chapter 8

# Cytotoxicity studies and selectivity index values of crude extracts and isolated compounds

## 8.1 Introduction

Medicinal plant preparations are used for treating various disease conditions with increasing justifications that a long application in traditional practice and being a natural product is healthy and safe. Plants are sessile with the ability to synthesize a vast array of secondary metabolites as defence mechanisms for protecting themselves against pathogen infections and some of these metabolites can potentially be toxic to humans and animals. Scientific evaluations of many plant extracts have validated potential cytotoxic, genotoxic, and carcinogenic effects of many compounds within plants (Ernst, 2004). Typical examples of the potential danger of plant compounds are the pyrrolizidine alkaloids and their N-oxide derivatives, which are found in thousands of vegetable species and can be genotoxic and carcinogenic, and thus pose significant health risks to humans and animals (Mei et al., 2007).

Therefore, plants utilized ethnobotanically need to be checked for their toxic potential from the perspective of understanding how they may alter cell functions and other severe consequences from side effects (Gurib-Fakim, 2006). Some of the side effects may occur through several different mechanisms, including direct toxic effects of the herbs, effects of contaminants, and interactions with drugs or other herbs. In addition, factors such as microorganisms, microbial toxins, and genetic factors may also affect the content of active constituents in the herbal product. Rash, fever, abdominal pain, dyspnoea, vision problems and palpitations are common adverse effects that may be induced by plant extracts.

Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. For this reason, their joint administration with “conventional medicines” can produce variations in the magnitude of the effect. This type of interaction, just like that produced between two or more medicines, can produce pharmacokinetic interactions if they affect the processes of absorption, distribution, metabolism and excretion, or exert pharmacodynamic interactions. Poisonous plants can affect the entire spectrum of organ systems, with some plants having several toxic principles that affect different systems. The dominant effect may depend on the condition, growth stage or part of the plant, the amount consumed, and the species and susceptibility of the victim. Poisoning can result from contamination of non-toxic food plants with

mycotoxins-synthesizing fungi and other interactions with organisms including insects, helminths and bacteria that result in the elaboration of toxins.

## 8.2. Materials and Methods

### 8.2.1. Preparation of extracts

Extracts were prepared to final concentrations of 10 mg/ml and the purified compounds were prepared to 2 mg/ml in acetone.

### 8.2.2 Cytotoxicity assay

Viability of cells in the presence of the test sample was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, or MTT, assay) described by Mosmann (1983) with slight modifications by McGaw (2007). To determine the cytotoxic concentration of the extracts, human liver C3A (obtained from the American Type Culture Collection, number CRL-10741) cells were seeded in 96-well plates (Corning Incorporated) at a cell density of 5 x $10^4$ cells/ml in 96-well flat bottom culture plates and then incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 24 h to allow attachment of cells. Thereafter, the cells were exposed to various concentrations of the extracts (1.0 to 0.0075 mg/ml), in quadruplicate for each concentration and incubated at 37°C in a humidified $CO_2$ atmosphere (5% CO2) for 48 h. At the end of the incubation period, the medium on the cells was removed; the wells were rinsed with PBS and fresh medium pipetted onto the cells.

To each well, 30 µl of 5 mg/ml MTT (Sigma), dissolved in PBS (pH 7.4) was added, and the plates were re-incubated for another 4 h. The medium containing MTT was removed and 50 µl DMSO was added to each well and the 96-well plates were gently swirled for 5 min at room temperature to dissolve the formazan crystals. The cytotoxic effect of extracts on cells was indicated by a clear appearance or lack of purple formazan colour in the wells.

The optical density (OD) was measured at a wavelength of 570 nm and a reference wavelength of 620 nm using a Versamax microtitre plate reader (Molecular Devices). The cytotoxicity was expressed as 50% cytotoxic concentration ($CC_{50}$) of substances inhibiting the growth of cells by 50% as calculated from the linear regression equation. C3A cells, without extract treatment served as negative controls and the positive control was doxorubicin hydrochloride (Pfizer).

## 8.3. Results

**Table 8.1.** Cytotoxicity, $LC_{50}$ and Selectivity Index (SI) values of crude extracts and isolated compounds (β-amyrin and lupeol)

| **Plant extracts and compounds** | **$LC_{50}$ (µg/ml)** | **MIC (µg/ml)** | | **Selectivity Index (SI)** | |
|---|---|---|---|---|---|
| | | *M. smegmatis* | *M. bovis* **BCG** | *M. smegmatis* | *M. bovis* **BCG** |
| *L. cassinoides* crude extract | 205.814 | 16 | 31 | 12 | 6.63 |
| *C. capense* crude extract | 83.078 | 16 | 31 | 0.71 | 5.19 |
| β-amyrin | ›200 | 15 | 15 | 13.33 | 13.33 |
| lupeol | ›200 | 15 | 13.33 | 13.33 | 13.33 |
| doxorubicin | 3.20 µM | | | | |

The cytotoxic effects of the crude leaf extracts of the selected plants on C3A cell lines were examined in this study by the MTT assay. The cytotoxic activities of the crude extracts expressed as $LC_{50}$ values from the MTT assay were 83.08±44.67 µg/ml and 205.81±8.38µg/ml for *Calodendrum capense* and *Lydenburgia cassinoides* crude acetone extracts respectively. The percentage viability of cells (0–100%) was plotted against concentrations of 7.5–1000 µg/ml of the extracts and presented in Figures 8.1 and 8.2. The extracts had lower cytotoxicity to the C3A cells (>50 µg/ml) than the positive control (doxorubicin) which had $LC_{50}$ of 3.2±0.46 µM. The negative control (acetone) was not toxic to the cells at the highest concentration tested.

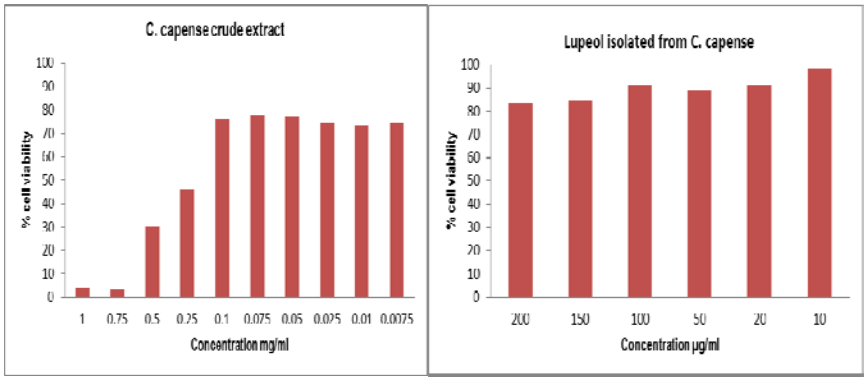



**Figure 8.2.** Percentage cell viability of the crude acetone extracts of *Calodendrum capense* and isolated compound (lupeol) against C3A cells

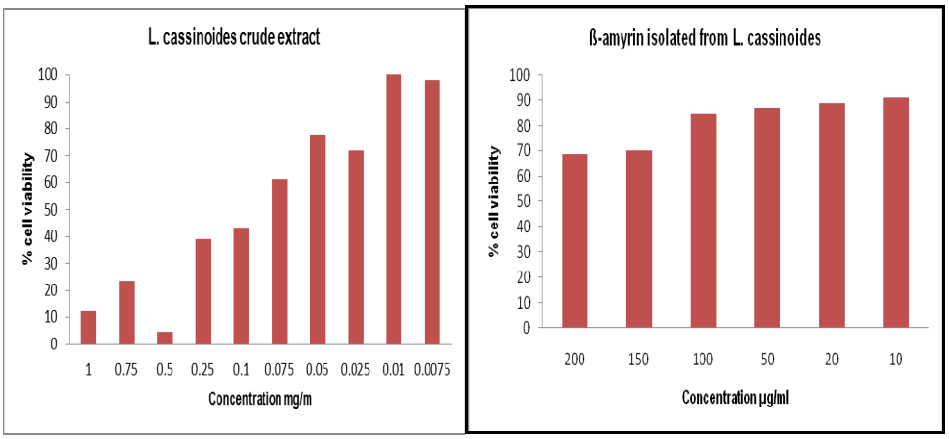



**Figure 8.3.** Percentage cell viability of the crude acetone extracts of *Lydenburgia cassinoides* and isolated compound (β-amyrin) against C3A cells

Table 8.1 shows the cytotoxicity and selective activity of ß-amyrin and lupeol. The two compounds were not toxic even when tested at the highest concentration (200 µg/ml), so the $LC_{50}$ values of ß-amyrin and lupeol were greater than 200 µg/ml. For calculation of SI values though, the $LC_{50}$ values were taken as being equal to 200 µg/ml. The two crude extracts from the two plant species showed a relatively low degree of toxicity. The selectivity indexes (SI) of the crude extracts when taking the mycobacterial species in to account, were between 0.71 and 6.63 while selectivity index values for ß-amyrin and lupeol were 13.33 for both compounds.

**Table 8.1.** Cytotoxicity, $LC_{50}$ and Selectivity Index (SI) values of crude extracts and isolated compounds (β-amyrin and lupeol)

| **Plant extracts and compounds** | | **MIC (µg/ml)** | | **Selectivity Index (SI)** | |
|---|---|---|---|---|---|
| | | *M. smegmatis* | *M. bovis* **BCG** | *M. smegmatis* | *M. bovis* **BCG** |
| *L. cassinoides* crude extract | 205.814 | 16 | 31 | 12 | 6.63 |
| *C. capense* crude extract | 83.078 | 16 | 31 | 0.71 | 5.19 |
| β-amyrin | ›200 | 15 | 15 | 13.33 | 13.33 |
| lupeol | ›200 | 15 | 13.33 | 13.33 | 13.33 |
| doxorubicin | 3.20 µM | | | | |

Both compounds showed much better selectivity index values with regard to antifungal activity compared to those of the crude extracts.

## 8.4 Discussion and Conclusion

In this chapter, the cytotoxicity of the crude acetone leaf extracts and isolated compounds on C3A cell lines *in vitro* was examined using the MTT assay. Cytotoxicity assay results revealed that the acetone extracts of *Calodendrum capense* and *Lydenburgia cassinoides* have $LC_{50}$values of over 50 µg/ml against C3A cell lines after 48 h incubation. Thus, the crude extracts of these two plants were relatively low toxicity in inducing cell death towards the C3A human liver cell line, following guidelines of the American National Cancer Institute. The pure compounds both had $LC_{50}$ values greater than 200 µg/ml which was the highest concentration tested. This implies that their cytotoxicity against this liver cell line was negligible, but further testing on a number of cell lines as well as using different parameters of endpoint cytotoxicity testing would need to be carried out to confirm the lack of cytotoxicity of these compounds.

*In vitro* cytotoxicity assays can be used to some extent to predict human toxicity and for the general screening of chemicals for potential toxic effects (Scheers et al., 2001). It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (George and John, 2006). In order to avoid overestimation or underestimation of the toxicity of a

substance, incubations with various concentrations using different assays are required to distinguish between effects on specific organelles or general cytotoxicity.

# Chapter 9

# General Discussion and Conclusions

Opportunistic infections are a serious threat to public health. These illnesses are caused by pathogens or their toxins that are transmitted to the host through various mechanisms. Some of these diseases are considered to be contagious (airborne transmission) while others are not contagious (non-communicable diseases). The four leading infectious diseases in humans and animals are pneumonia, diarrhoea, tuberculosis and malaria, with high rates of disability or death (Louw, 2002).

Many infectious pathogens make the body more vulnerable to secondary infections by weakening the body's immune system. Other organisms then invade the host to take advantage of the weakened immune system to cause various health problems commonly referred to as opportunistic infections (Chatelain et al, 2009). There are numerous microbial species that can cause opportunistic infections in immunocompromised patients, such as *E. coli*, *S. aureus*, *Mycobacterium* spp., and various fungal organisms.

The aim of this study was to isolate and characterize antifungal compounds from *Calodendrum capense* and *Lydenburgia cassinoides*, species with known antifungal efficacy that may be useful in treating fungal infections caused by *Candida albicans* and *Cryptococcus neoformans* in immunocompromised patients. Various solvents were used to extract dried, ground leaf material collected from trees of the two species. The highest yield was obtained using methanol (MeOH) in both plant species, while extraction with hexane gave the lowest yield.

The extracts of both plants showed the presence of many different compounds with distinct Rf values when visualised using thin layer chromatography (TLC). The chromatograms of the acetone and methanol extracts of *L. cassinoides* indicated the highest diversity of compounds, while those of *C. capense* revealed the best diversity of compounds in the hexane and acetone extracts. The purple colour of some spots possibly indicated the presence of terpenes while the pink spots indicated the potential presence of flavonol compounds.

Acetone extracts of *L. cassinoides* and *C. capense* each had average MIC values of 0.16 mg/ml against the two Gram-negative bacteria included in the antimicrobial screening, namely *E. coli* and *P. aeruginosa*. Good activity against *M. smegmatis* was shown by both plant species with low MIC values

of 0.04 mg/ml in acetone and methanol extracts. The highest MIC value of 1.25 mg/ml was obtained against *M. bovis* BCG.

Three solvent systems were used to develop TLC chromatograms for microbial bioautography and to establish phytochemical fingerprints for the extracts. The BEA system gave the best separation of antimicrobial compounds for *L. cassinoides* against both fungal and bacterial pathogens.

To initiate the isolation of bioactive compounds, solvent–solvent fractionation using solvents of differing polarities was performed on the bulk acetone extract of both plant species. Each plant species yielded five fractions where the hexane extract was the most active. For both plant species therefore, the hexane fraction was used to perform repeated column chromatography for isolation of the active compound(s). One active antimicrobial compound from each plant species was isolated, and these both showed the same Rf values, visualised in different TLC eluent systems, ranging from 0.39 to 0.95. Lupeol was isolated from *Calodendrum capense* and β-amyrin was isolated from *Lydenburgia cassinoides*. This appears to be the first report of the isolation of these compounds from these plant species.

The isolated compounds were further tested in various biological activity assays. In antimicrobial assays, the compounds were active against the four bacterial and several fungal organisms tested. The compounds were very active against *Cryptococcus neoformans* and *Aspergillus fumigatus*, but anticandidal activity was average. Isolated compounds were also tested against resistant strains of *Candida albicans* and ATCC strains but the activity was again minimal.

The MTT assay to test the crude extracts and compounds for cytotoxicity was conducted using the human liver C3A cell line. In this assay, the crude samples were tested in ten concentrations against the cells, while the isolated compounds were tested in six concentrations. The results revealed that when the concentration is high, the cell viability is low; meanwhile, when the concentration is low, the cell viability is high. The $LC_{50}$ values of the isolated compounds were greater than 200 µg/ml, even at the highest concentration tested. The selectivity index (SI) values of both crude extracts ranged from 0.18 to 0.91 for all the organisms tested. This means that the two crude extracts were relatively toxic compared to the antimicrobial activity because the SI index values were less than one. However ß-amyrin and lupeol showed good activity and low toxicity with SI values greater than 10.

With their good antimicrobial activity and relatively low toxicity, the compounds isolated in this study have potential for development as antibiotic agents to combat common illnesses encountered in immunocompromised patients. Such patients often fall victim to diseases caused by opportunistic

pathogens such as *Cryptococcus neoformans* and *Aspergillus fumigatus* as well as many bacterial pathogens. Lupeol and β-amyrin showed excellent activity against several of these fungal and bacterial organisms implicated in causing opportunistic infections, and as such are worthy of further investigation for efficacy and safety in animal models as therapeutic agents.

# Chapter 10

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