POTENTIATION OF ANTIMICROBIAL ACTIVITY AND ISOLATION OF ACTIVE COMPOUNDS FROM *LOXOSTYLIS ALATA*

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Declaration

This research was carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof JN Eloff and Dr LJ McGaw.

This thesis represents work done by Selaelo Ethel Raphatlelo, except where the work of others is acknowledged and the results have not been submitted anywhere else before.

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List of Abbreviations

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
Ace	acetone
AIDS	Acquired immune deficiency syndrome
ATCC	American tissue culture collection
BEA	benzene:ethanol:ammonia
C	Crude extract
CDT	cytolethal distending toxin-producing *Escherichia coli*
$CD4^+$	Cluster of differentiation four plus
CEF	chloroform:ethyl acetate:formic acid
CFU	colony forming unit
Chlr	chloroform
CSIR	Council for Scientific and Industrial Research
DCM	dichloromethane
DHEC	diarrhoea-associated haemolytic *Escherichia coli*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EAEC	enteroaggregative *Escherichia coli*
Ec	*Escherichia coli*
Ef	*Enterococcus faecalis*
EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*

EMW	ethyl acetate:methanol:water
EPEC	enteropathogenic *Escherichia coli*
ET	ethyl acetate
ETEC	enterotoxigenic *Escherichia coli*
EtOAc	Ethyl acetate
Hex	hexane
HIV	Human immunodeficiency virus
INT	Iodonitrotetrazolium violet
LC_{50}	Lethal Concentration 50%
Met	methanol
MEM	Minimal Essential Medium
MIC	Minimal inhibitory concentration
MTT	3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear Magnetic Resonance
NO	nitrogen oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
O_{2^-}	Superoxide
Pa	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PD	Potato Dextrose

P.malariae	*Plasmodium malariae*
P. orale	*Plasmodium orale*
P. vivax	*Plasmodium vivax*
R_f	Retardation factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sa	*Staphylococcus aureus*
SARS	severe acute respiratory syndrome
STD	sexually transmitted diseases
TLC	Thin layer chromatography
T-lymphocyte	Thymus-helper cells
UV light	ultra violet light
WHO	world health organisation
5-FC	5-fluorocytosine

Abstract

The development of antimicrobial resistance has provided momentum to the search for potential alternatives for treatment of microbial diseases in both humans and animals. Medicinal plants are a useful alternative source of new medicines, pharmaceutical entities and bioactive compounds that may be used for not only treating human diseases but also for enhancing animal production and health (Cowman, 1999). *Loxostylis alata* from the family Anacardiaceae is a traditional medicinal plant which is used by indigenous people as a pain reliever during childbirth and as an immune modulator (Pooley, 1993; Pell, 2004). This species was found to have good antifungal activity against *Aspergillus fumigatus* in a previous study. The problem was that the same extract that had activity was also found to be toxic *In vivo* using broiler chicks. Hence the aim of this project was to increase the reported activity and reduce the toxicity of the extract by selective techniques, and also to isolate bioactive compounds from the plant. The objectives were to evaluate the antimicrobial activity of extracts prepared using different solvents, to use bioassay-guided fractionation to develop an extract with good antimicrobial activity, to determine *in vitro* toxicity of *Loxostylis alata* extracts, and also to isolate and identify biologically active compounds and determine their toxicity.

The leaf plant material was extracted in separate aliquots with four solvents (hexane, dichloromethane or DCM, acetone and methanol). Antifungal activity was tested using a microdilution method to determine Minimum Inhibitory Concentration (MIC) values. Bioautography was also performed against three animal fungal species, namely *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Candida albicans.* The antibacterial activity was determined against ATCC strains of the Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis* and the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa.* Solvent-solvent fractionation was conducted with the aim of increasing activity of the active extract and reducing its toxicity. Bioactive compounds were isolated using bioassay-guided column chromatography. Antimicrobial activity and cytotoxicity of the bioactive compounds was evaluated. Toxicity was evaluated using the colorimetric MTT (3-(4.5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide) assay on C3A human liver cells.

All four extracts prepared using different solvents showed different antimicrobial activity in the microdilution assay. The acetone extract had the highest antimicrobial activity with the lowest MIC value of 0.02 mg/ml, compared to the other extracts. The acetone extract was the only extract that showed one compound with antimicrobial activity in the bioautography assay. Based on these results, acetone was used for bulk extraction for isolation of active compounds.

From the bulk extract, solvent-solvent fractionation was performed and five fractions (hexane, DCM, ethyl acetate, acetone and methanol) were obtained. Antimicrobial activity of the fractions was evaluated to determine if there was a loss or a gain in activity after solvent-solvent fractionation. The hexane and DCM fractions had a high antimicrobial activity with MIC values of 0.02 mg/ml and 0.04 mg/ml respectively. However this activity was not observed with the hexane and DCM extracts. This demonstrated that solvent-solvent fractionation helped to increase the activity of the fractions. The ethyl acetate and acetone fractions had antifungal and antibacterial compounds with the same Rf values. Their lowest MIC value was <0.02 mg/ml. The ethyl acetate fraction had a high total activity followed by the hexane fraction compared to the other fractions.

Based on the MIC values, the presence of bioactive compounds in bioautography and a high total activity, the hexane and ethyl acetate fractions were subjected to further column chromatography for isolation of bioactive compounds using silica gel as a stationary phase. Two compounds with antimicrobial activity were isolated from the hexane fraction. One compound was isolated from the ethyl acetate fraction. The structural elucidation of the isolated compounds was done using NMR spectroscopy and Mass Spectrometry (MS). Compound 1 from the hexane fraction was identified to be lupeol while compound 2 was a mixture of lupeol, alpha-amyrin and beta amyrin. The two compounds had antifungal activities with MIC values ranging from 63 - 125 µg/ml against the tested fungal pathogens. The compound from the ethyl acetate fraction had both antifungal and antimicrobial activity with an MIC value of 4 µg/ml.

Cytotoxicity of the active fractions and compounds was tested to evaluate toxicity of fractions or compounds compared to the acetone crude extract. The compound isolated from the ethyl acetate fraction had low toxicity in *vitro* against C3A cells compared to the crude extract and fractions, with LC_{50} = 110.2834 µg/ml. This compound had the highest activity with the lowest MIC value of 4 µg/ml and selectivity index of 27.57. The increased activity of the compound compared to that of the ethyl acetate fraction from which it was isolated shows that there was a removal of inhibitory compounds from the ethyl acetate fraction during the isolation process, and a concentration of the active compound from a relatively low concentration in the fraction.

Suleiman (2010) reported that the acetone extract of *L. alata* at a dose of 200 mg/kg was as effective as ketoconazole which was used as a positive control. The acetone extract was able to treat broiler chicks which were infected with *Aspergillus fumigatus*. Thus the plant showed it has therapeutic values against *Aspergillus fumigatus.* However the active acetone extract was also found to be toxic to the chicks. The aim of the current study was to increase the activity of the acetone extract and reduce its

toxicity. This aim was fulfilled since we were able to develop ethyl acetate fraction through bioassay-guided fractionation which and isolate a compound from it with a high antimicrobial activity (4 µg/ml) and a low toxicity.

CHAPTER 1

POTENTIATION OF ANTIMICROBIAL ACTIVITY AND ISOLATION OF ACTIVE COMPOUNDS FROM *LOXOSTYLIS ALATA*

1.1 INTRODUCTION

Medicinal plants are the primary source of ingredients used in preparation of remedies to treat various forms of diseases and ailments (infectious and non-infectious) in traditional medicine. Some conventional drugs consist of purified plant compounds, or derivatives of such compounds (Amusan et al., 2007). The basic therapeutic mechanisms of phytochemicals include antimicrobial and anti-inflammatory, antioxidant, immunostimulant, antidiabetic and anti-parasitic activities (Shah et al., 2010). South Africa has one of the richest cultural and biological diversities in the world (De Wet and Van Wyk, 2008). The use of medicinal plants in the country is an integral part of the people's culture and about 80% of the population use medicinal plant preparations for their health care delivery (Shinwari et al., 2006).

Disease management, especially of infectious diseases, is a serious concern in South Africa, and the world at large, because of new cases of microbial pathogenicity and emergence of microbial drug resistant strains with less susceptibility to conventional antimicrobial drugs (Moore and Chaisson, 1996). Microbial infections are the most common cause of high morbidity and mortality in developed and underdeveloped countries (Garbino et al., 2001). The common bacterial pathogens associated with reported cases of drug resistant strains are *Escherichia coli* (urinary tract infections, diarrhoea, neonatal meningitis, pneumonia, sinusitis) (Donnenberg and Kaper, 1992); *Staphylococcus aureus* (osteomyelitis, pneumonia, endocarditis, septic athritis) (Chambers, 2005); *Pseudomonas aeruginosa* (meningitis, urinary tract infections, malignant otitis externa, endocarditis) (Shorr, 2009); *Enterococcus faecalis* (urinary tract infections, septic joint infections, endocarditis) (Hall et al., 1992; Plosker and Figgit, 2005); *Bacillus subtilis* and *Salmonella typhi* (typhoid fever, malaise, constipation) (Hensel, 2000). Common fungal pathogens include *Candida albicans* (candidiasis) (Yang et al., 2006), *Cryptococcus neoformans* (cryptococcal meningitis), *Aspergillus fumigatus* (aspergillosis, respiratory allergies) (Cohen et al., 1993) and *Sporothrix schenckii* (sporotrichosis) (Kauffman, 1999).

Microbial infections or infectious diseases also affect animals with serious detrimental effects. Infectious diseases are a major threat to livestock and poultry industries as they are known to cause low productivity in animals. For example, in poultry, infectious diseases cause decreased rates of egg

hatchability due to environmental infection and high mortality in chicks (Van Cutsem and Paj, 1988). *Cryptococcus*, *Candida* and *Pseudomonas* species have been reported to cause diarrhoea in calves, lambs and pigs. *Escherichia coli* is known to affect the urinary system of cattle, causing focal interstitial nephritis, and it also causes watery mouth and watery spotted kidney in lambs. *Staphylococcus aureus* has been reported to cause meningitis in calves, swollen joints and kidney abscesses in goats and sheep (Quinn et al., 1994).

Pathogenic mechanisms of microbial infection include adhesion, which involves the binding of the microorganism glycoproteins to the surface receptors of the host epithelial cells, as well as penetration and invasion of epithelial cells causing the release of prostaglandins and bradykinins that stimulate inflammatory reactions in the invaded cells (Ferrer, 2000).The body's immune response to infectious pathogens or any adverse stimuli typically involves the recruitment of white blood cells (phagocytes) to the site of infection. Phagocytes include immune effector cells such as monocytes, neutrophils and macrophages (Premack and Schall, 1996). The process of phagocytosis includes the release of reactive oxidative species (ROS) and reactive nitrogen species (RNS) as microbicidal agents. However, excessive generation of ROS/RNS without concomitant removal results in oxidative stress with its associated harmful effects such as damage to DNA, lipid peroxidation of polyunsaturated fatty acids of the cell membrane and damage to proteins (Splettstoesser and Schuff-werner, 2002).

Infectious diseases are treated using antibiotics, but some side effects such as vomiting, nausea, diarrhoea and allergies have been reported with the use of antibiotics (Norrby 1991). In addition to these side effects, microorganisms have developed resistance against many commonly used antibiotics. The development of antibiotic resistance has been exacerbated by the use of antibiotics as animal feed additives to promote growth of animals. Resistant strains can be transferred from animals to humans through food and hence the use of antibiotics as animal feed has been banned in many countries (Phillips et al., 2004). The cost and the emergence of resistant strains of microorganisms to antibiotics provide motivation for the continued search for new, safer and more efficient anti-infectious agents (Kunin, 1993).

Medicinal plants are a reservoir of diverse phytochemicals broadly classified as terpenoids, alkaloids and phenolics. These phytochemicals often have various bioactivities, which provide an attractive source of possible new therapeutic agents. Many medicinal plants have been screened for antimicrobial, antioxidant, antiparasitic and anti-inflammatory activity. *Loxostylis alata* is one such traditionally used plant species reported to possess good biological activities *in vitro* (including

antibacterial, antifungal, antioxidant and antiplatelet) and *in vivo* (dose-dependent protection against aspergillosis in chicks infected with *Aspergillus fumigatus*) (Suleiman et al., 2010). In the latter study, the extract of *L. alata* produced similar results to those obtained with ketoconazole in reducing severity of lesions in pathological scoring and reducing development of fungal infections in chemotherapeutic trials. However, the active extract was also found to be toxic (Suleiman et al., 2010). The aim of this project is to continue with the research on *Loxostylis alata* in relation to its activity against *A. fumigatus*. Efforts will be made to increase the reported activity of the plant extract using selective extraction and potentiation techniques, to reduce the toxicity and to isolate additional bioactive compounds that have not previously been isolated from the species. It is important to know what the bioactive compounds are in an extract, for example as potential biomarkers or for quality control purposes.

Potentiation of crude plant extracts is the removal of inactive components to increase the concentration of the active compounds. The use of plant extract mixtures rather than only purified compounds is important to investigate because if there is more than one bioactive compound in the extract, the possibility of a microorganism developing resistance against the extract is lower than the likelihood of resistance developing against a single active chemical (Harbilas et al., 2009). The development of a potentiated extract is much cheaper than the isolation of individual compounds. Several methods may be used to develop such an extract, such as selective extraction of plant material with solvents of different polarities in which the target compounds are more soluble. Solvent-solvent fractionation using two immiscible solvents with different polarity as a means of concentrating the target compounds in a particular fraction may also be used. Solid-liquid extraction via column chromatography using different stationary supports with the elution of target compounds using an isocratic or gradient mobile phase is a further method of potentiation (Coutinho et al., 2010). Toxicity is also another factor to consider when potentiating plant extracts or drugs, and a major aim should be to identify a means of eliminating deleterious effects while enhancing the efficacy of the preparations.

1.2 PROBLEM STATEMENT

Microbial infectious diseases caused by fungal and bacterial pathogens are threats to the well-being of animals as well as humans, affecting production and productivity in farm animals, in turn affecting the economy of the country. Microorganisms have developed resistance against many commercial antimicrobials and some currently used antimicrobials are expensive and have adverse side effects, so alternatives are needed.

1.3 AIM

- To potentiate the biological activity of the crude leaf extract of *Loxostylis alata*, to reduce the toxicity and to isolate the bioactive compounds from the extract.

1.4 OBJECTIVES

- To evaluate the antimicrobial activity of extracts of *Loxostylis alata* prepared using different solvents
- To use bioassay-guided fractionation and an enrichment procedure to develop an extract with good antimicrobial activity from *Loxostylis alata*
- To determine *in vitro* toxicity of *Loxostylis alata* extracts
- To isolate and identify active compounds from *L. alata* and to determine their toxicity

1.5 SIGNIFICANCE OF THE STUDY

The results obtained from this study will highlight the importance of medicinal plants in terms of their use as possible alternative sources of therapeutic antimicrobial agents, particularly in this case in animal health. The active antifungal and antibacterial compounds in *Loxostylis alata* will be identified and a potentiated extract with low toxicity will be developed. This study will possibly result in a product with potential for use as a commercial antifungal agent to protect against fungal infections in poultry.

CHAPTER 2

LITERATURE REVIEW

2.1 Infectious diseases

Due to poor sanitation, unhygienic conditions and spreading of microbial pathogens in the environment, many people and animals are exposed to infectious diseases caused by such microbial pathogens. Infectious diseases are illnesses caused by specific infectious pathogens (fungi, bacteria, viruses, protozoa or parasites) that result from transmission of the agent or its products from an infected person, animal or reservoir to a susceptible host (Murphy et al., 2008). Infectious diseases develop when pathogenic biological organisms invade, survive and multiply inside a host, causing harm to the host organs or tissues. Infectious diseases are the commonest causes of mortality and morbidity in Africa and worldwide (Algar and Novelli, 2007). The ability of the microorganism to infect the host and cause disease development and disease progression is influenced by several factors in the host, namely age, genetics, immune response and nutritional status as well as the ability of that pathogen to resist the immune mechanisms of the host (Ryan and Ray, 2004). For example immune-compromised patients are severely affected by infectious diseases because of their weak immune systems and poor ability to combat infections (Algar and Novelli, 2007).

The immune system of the host is responsible for fighting against pathogens by means of different mechanisms. The body's immune system fights infections via white blood cells. White blood cells are triggered or activated to the site of infection to modulate the immune system and to prevent development of disease (Piccirillo, 2008). Cellular immunity is most important in the defence against pathogenic microbes, and its impairment may induce extreme susceptibility to infection, or allow activation of dormant and otherwise asymptomatic microorganisms (opportunistic infections). Opportunistic infections, reactivation of dormant and asymptomatic microorganisms that cause infectious disease are associated with low CD4 T-lymphocyte counts. However, suppression of the immune system, whether acquired by natural infection with immunosuppressive pathogens such as HIV, or induced by therapy as in the case of cancer or organ transplants, may change the host response to infectious agents.

The development and administration of antibiotics and vaccines, along with improved hygiene, sanitation, and vector control enabled dramatic improvements in health and control of previously feared

contagious infectious diseases (Hamburg, 2008). Antibiotics are therapeutic drugs that are used for the treatment of infectious diseases. Antibiotics have different modes of action which include interference with cell wall synthesis, inhibition of prokaryotic protein and nucleic acid synthesis, and inhibition of prokaryote-specific metabolic pathways (Hawkey, 1998).

Successful treatment of infectious diseases by antibiotics has been diminishing because microorganisms have developed antibiotic resistant genes. Antibiotic resistance occurs usually when there is a change in the genetic composition of the bacteria that were previously inhibited by the antibiotic used. These changes are usually due to mutations or the expression of a latent chromosomal resistance gene or the introduction of new genetic information. The expression of these genetic changes results in changes in one or more biological mechanisms of the affected bacteria and ultimately determines the specific type of resistance that the bacteria develop, resulting in many possible biological forms of resistance. The three major mechanisms of resistance are production of an enzyme that will inactivate or completely destroy the antibiotic, preventing the accessibility of antibiotics to the target site or through alteration of the antibiotic target site to evade action of the antibiotic (Neu, 1992).

A variety of factors can be cited for the spreading of antibiotic resistance, for example antibiotic abuse, because often antibiotics are prescribed before the causative agent of an infection has been properly identified, and therefore, the most appropriate medication is not prescribed. Interrupting antibiotic use before completion of treatment can also contribute to antibiotic resistance (Okeke et al., 1999). Another factor that has contributed to antimicrobial resistance has been the use of antimicrobials as growth promoters in animals to increase their growth levels and prevent development of diseases (Cogliani et al., 2011). The use of antimicrobial growth promoters in production animals resulted in the emergence, spread, and transfer of resistance genes from microorganisms associated with those animals to bacterial pathogens that infect humans. The spreading and transfer of the resistant genes to the human population has limited the medical value of these antimicrobials (Aarestrup, 2000). These are some of the discoveries that caused the European Union to ban the use of antimicrobials as growth promoters. The aim was to reduce the pool of resistance genes in farm animals and in other non-human settings (Cogliani et al., 2011).

2.2 Modes of infectious disease transmission

Infectious pathogens can be transmitted from one host to another through body fluids (sexually transmitted diseases (STD), blood transfusion or orally through saliva), airborne inhalation (respiratory diseases), contaminated foods (gastrointestinal diseases), and vectors such as insect bites (mosquitoes for malaria).The mode of transmission of potential infectious pathogens varies widely, as they may be food-borne, water-borne, airborne, vector-borne or spread through contact.

- Water-borne diseases: The pathogenic agents of many diseases contaminate the aquatic environment through the faeces or wastes of symptomatic or asymptomatic infected persons or animals. Use of such water contaminated with human and animal excreta containing pathogenic organisms poses health risks to the population. Some water-borne and water-related diseases include cholera (*Vibrio cholerae*), diarrhoea *(Cryptosporidium parvum, Campylobacter* species), liver abscess and dysentery (*Entamoeba histolytica*), malabsorption (*Giardia duodenalis*) and dermatitis (*Pseudomonas aeroginosa*) (Karanis et al., 2007).

- Food-borne diseases: Food can be a means of transmitting infectious pathogens, providing a direct infection hazard following ingestion or food handling. Implicated foods are typically beef, pork, poultry, dairy products, but also eggs and fresh produce. Many pathogens can successfully invade and reach an appropriate colonisation site of a susceptible host. These pathogens include bacteria such as *Salmonella*, *Campylobacter*, *Shigella* and *Vibrio* spp., methicillin resistant *Staphylococcus aureus*, *E. coli*, Enterococci, and *Listeria monocytogenes*, parasites such as *Ascaris* spp., *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Trichuris trichiura*, *Trypanosoma cruzi* and *Trichinella* spp., and viruses such as norovirus, hepatitis A and rotaviruses (Newell et al*.,* 2010). Food-borne viruses are capable of infecting a host following oral ingestion, with the ability to pass unaffected through the stomach and other proximal parts of the small intestine which are rich in digestive enzymes and bile salts (Webber, 2005).

- Airborne infectious diseases: Airborne transmission is known to be the route of infection for a number of diseases including smallpox, measles, tuberculosis, influenza and severe acute respiratory syndrome (SARS). In order to transmit a microbial infection by air, the organism has to be excreted in the form of an aerosol. Infectious aerosols might be produced directly in the exhaled air of an infected animal or indirectly by sneezing and coughing from the infected host. Next, the organism has to maintain infectivity in aerosol form and be present in large enough dosage to initiate infection in another host. This mode of disease transmission may be

attributed to the movement of both people and fine aerosol droplets suspended in the air, and pathogen load (Mangili and Gendreau, 2005).

- Contact transmitted infectious diseases: Contact transmissions require relatively close interaction between the infected individual and susceptible host for efficient disease transmission. The spatial spread via this route of transmission is driven by human movement and behaviour. Body fluid exchange (sexually transmitted diseases (STD), blood transfusion and/or orally through saliva) between an infected individual and susceptible persons is the primary means of transmitting these infectious disease pathogens (Riddell and Morris, 2002). Particular pathogens transmitted by this means include blood-borne viruses such as hepatitis and the human immunodeficiency virus (HIV), gonorrhoea, *Candida* and *Cryptococcus*. Transmission of diseases by contaminated needles and blood transfusions, rather than by their natural vectors, may also affect the distribution patterns of bacterial and protozoal infectious agents. Transplantation of solid organs is frequently associated with infection due to the immuno-suppressive therapy administered to the recipients, contaminated blood transfusions or contaminated donated grafts (Riddell and Morris, 2002).

- Vector-borne infectious diseases: These are caused by the pathogens transmitted by insects such as *Anopheles* spp. which, as vectors of *Plasmodium falciparum*, *P. vivax*, *P. orale* and *P. malariae,* cause disease with clinical symptoms of malaria, and arthropods such as ticks which are vectors of, for example, *Borrelia burgdorferi* that causes lyme disease, and *Triatoma infestans, a vector of Trypanosoma cruzi* causing American trypanosomiasis (Gubler, 1998). The global spread of infectious agents by vectors is affected by natural factors such as movement of vectors by wind, carriage on transport hosts like migrating birds, and by means of transportation, e.g. flying or walking (Ferguson et al., 2001).

2.3 Microbial infectious pathogens

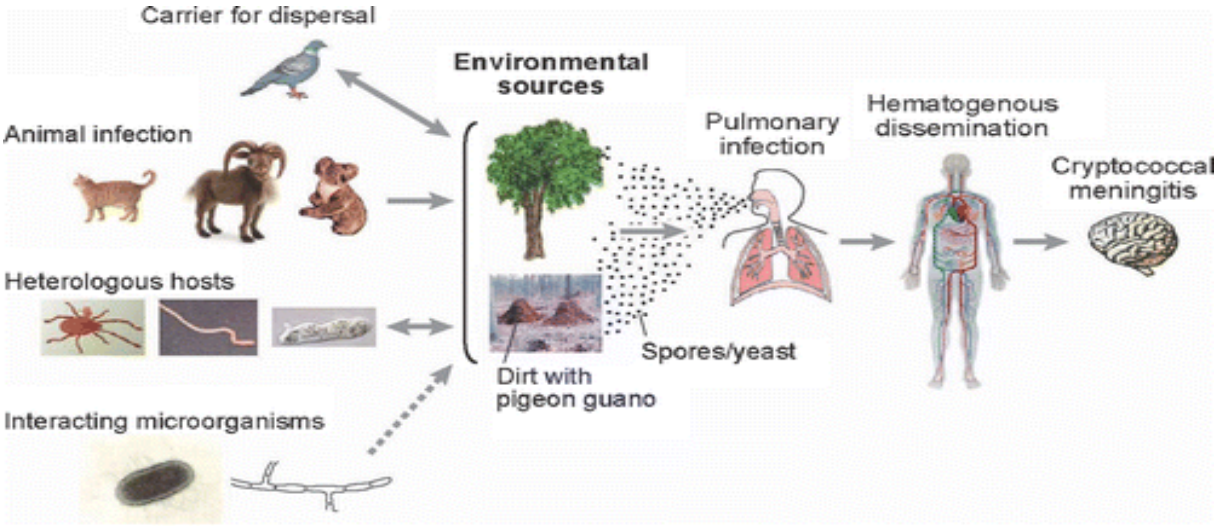
2.3.1 Fungi

Fungi are eukaryotic, single-celled, heterotrophic, multinucleated organisms characterized by a chitinous cell wall. In most cases fungi occur as filamentous growth and grow in multicellular colonies (grouped together as a mycelium) compared with yeasts, which are single cells. Fungal infections are major causes of disease with high rates of morbidity and mortality in immune-compromised patients such as those living with human immunodeficiency virus (HIV), cancer and organ transplants. In particular, systemic infections caused by *Candida albicans* and *Aspergillus fumigatus* are associated

with high mortality (Ascioglu et al., 2002). The innate responses provided by phagocytes and the adaptive response that includes humoral and cellular immunity are essential for effective anti-fungal defense. Fungal infections most frequently affect organs such as the lungs and urinary tract. *Candida* species, especially *C. albicans*, account for the majority of all fungal infections, followed by *Aspergillus* species, *Cryptococcus neoformans*, and *Histoplasma capsulatum*. Chemo- and radiotherapies for leukemia treatment or organ transplantation are associated with *Aspergillus* infections, whereas HIV infection is more often associated with *C. albicans*, *Pneumocystis carinii* or *Cryptococcus. neoformans* infections (Latge and Calderone, 2002).

2.3.1.1*Cryptococcus neoformans*

C. neoformans is encapsulated yeast found in clinical settings and the environment, causing diseases like meningoencephalitus (cryptococcal meningitis) and cryptococcosis. The mode of infection is through inhalation of spores which can easily penetrate the airways infecting lower and upper respiratory systems causing asymptomatic and symptomatic infections (Dusmukes, 1988). Two varieties which are identified to be responsible for disease in humans include *C. neoformans* var.*gattii and C. neoformans* var.*neoformans* .*C. neoformans* var.*gattii* is the strain that commonly infects immunocompetent hosts and sometimes causes visual loss (Levitz, 1991). Cryptococcal meningitis is treated with a combination of liposomal amphotericin B or amphotericin B lipid complex and flucytosine (5-FC) for at least 2 weeks for the induction regimen, followed by fluconazole for 8 weeks for consolidation therapy, and fluconazole for 6–12 months for maintenance treatment (Perfect et al., 2010).



Lin X, Heitman J. 2006.
Annu. Rev. Microbiol. 60:69–105

Figure 2.1: The infection cycle of *Cryptococcus neoformans* spores (Lin and Heitman, 2006).

2.3.1.2 *Candida* species

Candida species are yeast-like fungi that form part of the normal human and animal flora but become pathogenic in certain defined conditions, resulting in superficial or invasive candidiasis. Superficial infections affecting the skin, nails, or mucous membranes such as oral and genital parts are termed chronic mucocutaneous candidiasis when the effect becomes persistent or recalcitrant. Invasive disease affects viscera or other sterile sites such as blood and cerebrospinal fluid. *Candida albicans* is the most common species and is responsible for approximately 50-60% of infections. Other species such as *Candida glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* are becoming important due to resistance issues with commonly used therapies such as fluconazole (Persons et al., 1991). Although *C. albicans* infections are high in immune-compromised individuals, statistics have also shown that 30-35% of healthy individuals are vulnerable to infection by the organism causing oesophageal candidiasis, and three of every four women experience vulvovaginal candidiasis at least once in their lifetime (Repentignyl et al., 2004). *C. albicans* invades the body by disrupting the host tissues through a process called morphogenesis where it transforms its hyphae secondary to adhesion to the host cell via cell surface adhesions (Calderone, 2001). Fluconazole is the most commonly used antifungal agent. Selective digestive decontamination with non-absorbable antibiotics, including nystatin, clotrimazole, and ketoconazole, can maintain anaerobic bacterial growth and reduce overgrowth of *Candida* (Patton et al., 2001).

2.3.1.3 *Aspergillus* spp.

Aspergillus spp. are airborne, filamentous and ubiquitous fungi found in the soil, plant debris and indoor air environment. Inhalation of *Aspergillus* spores, especially those of *A. fumigatus,* results in life threatening pulmonary infection in both normal and immunocompromised hosts, with extra-pulmonary dissemination to the central nervous system (CNS) and virtually any other organ. Some of the associated problems include respiratory allergies like allergic bronchopulmonary mycoses with a risk of asthma development (Knusten, 2003) and saprophytic diseases. Superficial and invasive infections such as pulmonary aspergillosis occur in 10 to 20% of leukaemia patients with high mortality rates of about 80 to 90%. Invasive aspergillosis is also responsible for about 30% of fungal infections in patients with terminal cancer (Verweij et al., 1997).

Animals that are mostly affected by this disease are chicks, quails, pheasants, pigeons and turkeys, as *A. fumigatus* spores are commonly found in breeder and broiler farms or hatcheries. Aspergillosis in poultry causes mortality and morbidity and this is a great concern in terms of the economy (Clemons et al., 2000). *A. fumigatus* is able to grow in hatcheries because of the level of moisture, nutrients and

temperatures found in the hatchery which supports its growth (Suls, 1995). Chicks younger than three days are most susceptible to *A. fumigatus* infection in the hatchery environment contaminated with spores because their respiratory systems are still immature and not strong enough to fight aspergillosis. Older chicks can also become infected in the breeder farms due to the presence of damp poultry feed and wood which is used to build the huts for poultry that supports growth of the microorganism. At the hatchery level the embryos may also get infected because of contaminated egg shell which allows transfer of spores into the embryos. Aspergillosis causes huge economic losses in poultry because of a decrease in productivity and cost of prevention or treatment of aspergillosis in broiler farms (Van Cutsem and Paj, 1988). Thus there is loss of interest in having breeder or broiler farms often because of the effects that are caused by aspergillosis, leading to fewer poultry farms.

The standard therapeutic agent for the treatment of invasive aspergillosis is conventional amphotericin B and itraconazole .However, some major deleterious effects associated with the use of these drugs include nausea, hypokalaemia, nephrotoxicity, rash, fever, chill and fluid retention (Myers, 2006).

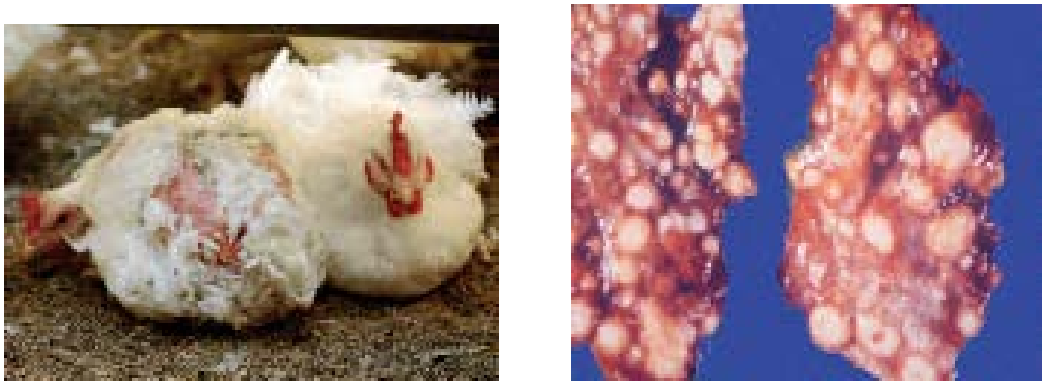


Figure 2.2. Infected broiler and aspergilloma lungs (Van Cutsem and Paj 1988).

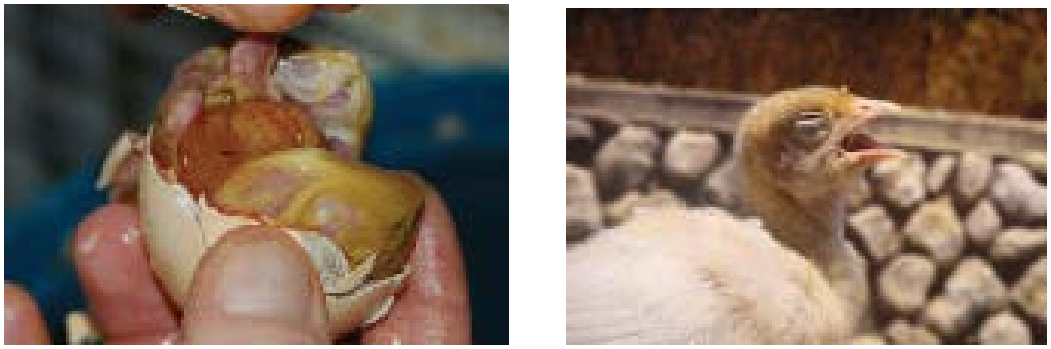


Figure 2.3. Chick mortality (Van Cutsem and Paj 1988).

2.3.1.4 Conventional therapeutic treatment

The treatment of fungal infections in orthodox medicine involves the application of antibiotics. Antibiotics are divided into different groups according to their mode of action and their structure (Ford,

2004). The polyene group of antibiotics includes amphotericin B, nystatin and natamycin, which possess a broad spectrum of activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Vanden Bossche et al., 2003). Polyenes prevent the growth of fungi by binding to the building blocks in the cell wall of the fungi called ergosterol, and cause cell death as a result of damage to the cell wall (Myers, 2006).

The azoles constitute another group of antifungal antibiotics. This group includes ketoconazole, eniconazole and econazole which are commonly used against *Cryptococcus, Aspergillus* and *Candida* species (Fromtling, 1988). Their mode of action is to inhibit fungal cell membrane synthesis (ergosterol) through interaction with the C-14 alpha demethylase. C14-alpha demethylase is dependent on P-450 that is associated with converting lanosterol into ergosterol. The interaction inhibits the production of ergosterol necessary to maintain membrane fluidity, thereby reducing the activity of membrane-associated enzymes resulting in increased permeability, inhibition of cell growth and replication (Ford, 2004).

2.3.2 Bacterial pathogens

Bacterial infections manifest into a disease state when invading bacteria escape the natural host defenses causing deleterious damage to tissue or organs disrupting normal homeostasis. Bacterial infections are a serious concern world-wide as they are emerging and spreading because the infectious bacterial pathogens respond poorly to the treatment (Morse, 1995). Bacteria are divided into Gram-negative and Gram-positive species. Examples of Gram-negative bacteria are *Escherichia coli* and *Pseudomonas aeruginosa* while Gram-positive species include *Staphylococcus aureus* and *Enterococcus faecalis*. Some of the commonly encountered bacterial pathogens include species described in the following paragraphs.

2.3.2.1 *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped facultative anaerobic bacterium. It is non-spore forming and motile, and lives in the intestines of host organisms. It is an important cause of food poisoning worldwide. Most strains of *E. coli* are harmless; however, virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections and neonatal meningitis (Todar, 2007). Seven groups of *E. coli* are currently known to cause diarrhea and they are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diarrhoea-associated haemolytic *E. coli* (DHEC) and cytolethal distending toxin (CDT)-producing *E. coli* (Clarke, 2001).

2.3.2.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod shaped bacterium with unipolar motility. It is a free-living bacterium, commonly found in soil and water, and it also inhabits the surfaces of plants and animals. *P. aeruginosa* is recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies indicate its occurrence as a nosocomial (hospital) pathogen. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed (Todar, 2008).

2.3.2.3 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive coccus, and is a non-motile, non-spore forming facultative anaerobe. It is frequently part of the normal skin flora. *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome and septicemia. It is still one of the four most common causes of nosocomial infections, often causing post-surgical wound infections (Ogston, 1984).

2.3.2.4 *Enterococcus faecalis*

Enterococci are facultatively anaerobic, ovoid or slightly elongated, oxidase- and catalase-negative, non-spore forming Gram-positive cocci. The two most common species of enterococci causing clinical infection are *Enterococcus faecalis* and *E. faecium.* Other species such as *E. avium*, *E. casseliflavus/E. flavescens*, *E. durans*, *E. raffinosus*, *E. gallinarum*, and *E. mundtii* are less involved in human and animal infection. *E. faecalis* can cause life threatening infections in humans, especially in the nosocomial environment where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity (Ryan and Ray 2004). In the gastrointestinal tract, enterococci cause cholangitis, peritonitis, and intra-abdominal abscess. Enterococci are also an infrequent cause of pneumonia, meningitis, and osteomyelitis, usually in the immunocompromised host. Enterococci can also cause endocarditis as well as bladder, prostate and epididymal infections (Pelletier 1996). Ampicillin or penicillin are antibiotics of choice for treating enterococcal infections.

2.3.2.5 *Mycobacterium* spp.

Mycobacterium species are a complex group of organisms, which can cause highly contagious and fatal disease in humans and animals such as tuberculosis. Members of the species include *Mycobacterium tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, and *M. canetti.* Mycobacteria are aerobic, acid-fast, non-motile, non-encapsulated, non-spore forming bacilli that grow most successfully in tissues with high oxygen content, such as the lungs. *M. tuberculosis* is an obligate intracellular pathogen that can infect several animal species, although human beings are the principal hosts (Schluger, 2005).

M. tuberculosis divides every 15–20 h, which is extremely slow compared with other bacteria (*Escherichia coli* divides every 20 minutes). The slow replication rate and ability of the *M. tuberculosis* to persist in a latent state result in long durations of both drug therapy of tuberculosis and for preventive therapy in infected people. Most immunocompetent individuals (over 90% of those infected) either eliminate *M tuberculosis* or contain it in a latent state (Gandhi et al., 2006).

2.3.2.5 Conventional therapeutic treatment of bacterial infections

Antibiotics that have been used for the treatment of bacteria are divided into two groups according to their effect on microorganisms. Those that kill bacteria are bactericidal agents, while those that only impair bacterial growth are known as bacteriostatic agents. Antibiotics which target the bacterial cell wall (penicillins, cephalosporins), or cell membrane, or interfere with essential bacterial enzymes usually are bactericidal in nature. Those which target protein synthesis, such as the aminoglycosides and tetracyclines are usually bacteriostatic (Finberg et al., 2004).

2.3.3 Parasitic infections

Parasitism is a mutual relationship between two organisms in which the parasite benefits for growth and reproduction to harm the host. Parasitism is caused by many organisms such as protozoa and helminths. Protozoa are unicellular eukaryotes, which replicate inside, but can also survive outside, their host. Helminths are eukaryotic parasitic worms, ranging from a few millimetres to several meters in length, and include cestodes (tapeworms), trematodes (flukes) and nematodes (roundworms). Enteric parasitic infections can be transmitted by the faecal–oral route by eating intrinsically contaminated food or via uptake of free-living parasitic stages from the environment (eggs, cysts and oocysts).

Contamination of food products (e.g. fruits, vegetables and meat) can be introduced via faeces, soil, irrigation water, sewage, human handling or improper processing of infected meat (Baron, 1996). Parasites differ from many bacterial food-borne pathogens in that they do not replicate outside the host, and they are generally not susceptible to the antibiotics that kill microorganisms. Furthermore, most parasites have an environmental resting stage (egg, cyst or oocyst), which is resistant to desiccation, disinfectants and other stresses. The probability of causing infections with very low doses of parasites is high. Many parasitic infections are asymptomatic, while others cause short-lived effects, and others may persist in the body for years, causing chronic disease (Quadir et al., 2010).

2.3.4 Viral infections

Virus infections are often associated with a transient or long lasting generalized suppression of the host immune response. Viruses are capable of causing significant illness and mortality in humans. Viruses need living cells to replicate. Noroviruses, astroviruses, and rotaviruses for instance multiply in the human intestines and cause acute gastro-enteritis with vomiting as well as diarrhoea (Le Guyader et al., 2008), whereas hepatitis A and E viruses may cause hepatitis that can be recognized by the development of abdominal pains and jaundice (Rabe et al., 2005).

2.4 Challenges in the treatment of infectious diseases

One of the most serious challenges in management of infection is the high incidence of the development and spread of microbial resistant strains to otherwise susceptible drugs without concomitant discovery of new anti-infectious agents. Antibiotic resistance can cause high hospital costs, high rates of mortality and secondary bacteremia. Antibiotic resistance also affects the economic outcomes of farms since antimicrobials are used for therapeutic purposes and to enhance animal growth and productivity. Other problems associated with the usage of antibiotics include side-effects, inaccessibility and expense, environmental contamination and ineffectiveness against some resistant strains (Kunin, 1993). The increase in resistance is associated with the abuse and over use of antibiotics and the mechanism involved is associated with changes in the genetic composition of the microbes. These changes are usually due to mutations or the expression of a latent chromosomal resistance gene or the introduction of new genetic information. The expression of these genetic changes in the cell ultimately determines the specific type of resistance developed by the organism. The three major mechanisms of resistance are production of an enzyme that will inactivate or completely destroy the antibiotic, prevention of the accessibility of antibiotics to the target site, or alteration of the antibiotic target site to evade action of the antibiotic (Neu, 1992).

As a result of the aforementioned problems with conventional antibiotics used for treatment of some infectious diseases, efforts are on-going to discover and develop safer and more efficient anti-infectious agents. Medicinal plants are a useful source of new medicines, pharmaceutical entities and bioactive compounds that may be used for treating human and animal diseases. Compounds found in plants may have a potential antimicrobial activity.

2. 5 Role of free radicals in infectious disease prevention and aetiology

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the mitochondria as a by-product of normal cellular metabolism. ROS/RNS are involved in cellular activity regulation which includes defence against infectious pathogens and cellular signalling systems at a physiological level (Gulcin et al., 2003). In the presence of infection, burns, muscle damage or inflammation the enzyme called phagocyte NADPH oxidase facilitates the production of ROS from leukocytes. The phagocyte NADPH oxidase is a multicomponent enzyme present in phagocytic leukocytes which consist of neutrophils, eosinophils, and monocytes/macrophages. This response triggers neutrophils, eosinophils, and macrophages/monocytes phagocytic cells to the site of infection. The phagocytic cells will then generate ROS which will play a role in cell signalling, killing of the infectious organism, stimulating of antioxidant repair process (Roos et al*.,* 2003) fulfilling the role of defence against infectious pathogens mainly by internalizing (phagocytosing) and exposing them to two destructive principles: reactive species (ROS/RNS) generated by the phagocyte NADPH oxidase (Roos et al., 2003).

ROS can be harmful if overproduced. Excessive generation of ROS/RNS without adequate annihilation mechanisms causes oxidative stress which involves the disturbance of endogenous antioxidant mechanisms in living organisms (Gulcin et al., 2003). Oxidative stress leads to diseases due to cellular damage and the resultant loss of structural integrity and functionality. Oxidative stress causes damage of DNA and mitochondria and this is associated with diseases such as rheumatoid arthritis, cancer, Alzheimer's disease, Parkinson's disease, non-degenerative diseases and ischemic reperfusion (Droge, 2002). When considering rheumatoid arthritis for example it is believed that ROS such as NO together with superoxide O_2^- and their interaction product are responsible for tissue injury because of the toxic oxidative reaction that they initiate. Reactive oxygen intermediates are thus mediators of inflammation and are responsible for the pathogenesis of tissue damage in rheumatoid arthritis (Valentao et al., 2002).

2.6 Role of inflammation in disease aetiology

Systemic inflammation characterised by the release of a wide range of pro- and anti-inflammatory polypeptides such as cytokines into the circulation is initiated by pathogens and non-infectious disease states. Inflammation is a local or systemic response to damaged tissue in the body characterized by pain, swelling, redness and loss of function. During inflammatory responses the body releases chemical mediators like prostaglandins, cytokines, leukotrienes, vasoactive amines and nitric oxide. *Pneumocystis carinii* infection elicits an inflammatory response in the lung (Kasper and Buzoni-Gatel, 1998). Chemical mediators are released in the form of plasma proteins or cells like mast cells, platelets, neutrophils and monocytes or macrophages. These substances bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and/or mediate oxidative damage (Coleman, 2002).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used to relieve inflammation and associated pain by inhibiting cyclooxygenase enzymes involved in the production of prostaglandins. Examples of NSAIDs are ibuprofen, indomethacin, meclofenamic acid, diclofenac, piroxicam, etadolac, meloxicam. These anti-inflammatory agents are associated with side effects including damage of the kidneys, worsening asthma, and damage to the upper gastrointestinal tract (Gale et al., 2007). Medicinal plants are used to treat diseases associated with the inflammatory response by interfering with inflammatory response pathways such as release of pro-inflammatory mediators and migration of leukocytes under inflammatory stimulus. Pharmacological evidence for anti-inflammatory activity of some medicinal plants like *Culcasia scandens* (Araceae) has been reported (Okoli et al., 2006).

2.7 Plant extracts as potential therapeutic agents in infectious diseases

One of the greatest accomplishments of modern medicine has been the development of antimicrobial pharmaceuticals for the treatment of infectious diseases. Medicinal plants are regarded as components of ancient healing processes that were not recognised by many people in many countries but now because of the demand of the health services and the development of resistance to antibiotics used to treat many diseases, among other considerations, they are highly recommended (WHO, 2000). About 35 000 to 70 000 plant species are known as medicinal plants. However it is estimated that only 5% of plant species have been evaluated for their potential as medicinal plants (Hassan et al., 2007).

Marginalized, rural and indigenous people, who cannot afford or access formal health care systems, are especially dependent on culturally familiar, technically simple, financially affordable and often effective traditional medicines. Traditionally used medicinal plants produce a variety of compounds which can either inhibit the growth of pathogens or kill them and have no or little toxicity to host cells (Cowan, 1999). Recently there has been widespread interest in promoting traditional health systems to meet primary health care needs. This is especially true in South Africa, as prices of modern medicines spiral and governments find it increasingly difficult to meet the cost of pharmaceutical-based health care. As a result there are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases (Azaizeh, 2003). Plants possess an inherent ability to synthesize phytochemical constituents of diverse structure, some of which exhibit good biological activity against one or more disease-causing agents (Farnsworth, 1985). These provide a large spectrum of natural and possibly new chemical constituents for scientific research engaged in drug discovery and development. The biologically active constituents of medicinal plants have served as templates for the synthesis of pharmaceuticals, agrochemicals, flavours, fragrances, biopesticides and food additives (Balandrin and Klocke, 1988). About 121 of the present clinically used drugs are primarily derived from medicinal plants or derivatives of purified compounds from medicinal plants (Payne et al., 1991). Hence, plants will continue to provide novel products as well as chemical models for new drugs in the coming centuries because the chemistry of the majority of plant species is yet to be characterized (Cox and Balick, 1994).

2.8 Bioactive phytochemical components in plant extracts

Plants have biologically active compounds called secondary metabolites which are responsible for their characteristics. Secondary metabolites help plants with adaptation to environmental changes and also play a role in protecting the leaves by absorbing UV light. The beneficial medicinal value of plant materials results from the combinations/ types of secondary products present in the plant. Due the discovery of these compounds, plants have been used for pharmaceutical purposes, cosmetics and for production of chemicals. In addition to the importance of secondary metabolites, some of these compounds may be toxic and this toxicity serves as a defense against herbivores. Examples of secondary metabolites are phenolics, alkaloids, quinones, tannins and terpenes (Ciocan and Bara, 2007).

2.8.1 Phenolics

Phenols are natural products that consist of a hydroxyl group attached to an aromatic hydrocarbon group. The greater the number of hydroxyl groups on the phenol, the more toxicity the compound will have towards the microorganism (Ciocan and Bara, 2007). Phenols are classified into four groups, namely simple phenols, benzoic acids, phenylpropanoids and flavonoids. The four groups are differentiated by the number of constitutive carbon atoms in conjunction with the basic structure of the phenolic skeleton (Rice-Evans, 1997). Every group of phenols has a certain function in the plant, for example flavonoids are sometimes synthesised when the plant is injured or infected and they also prevent absorption of UV into plant tissues. Flavonoids have antimicrobial activity against *Vibrio cholerae*, *Streptococcus mutans* and *Shigella* spp. (Ciocan and Bara, 2007). Phenylpropanoids are synthesised when the plant is under unfavourable conditions. Phenolics are known to have antimicrobial, anticarcinogenic and antioxidant activities (Ullah and Khan, 2008).

2.8.2 Alkaloids

Alkaloids are nitrogen-containing secondary metabolites. Many alkaloids are formed from amino acids while a few are formed from purines, pyrimidine or terpenes. They are classified into three groups depending on how the heterocyclic rings are distributed on the structure. The three groups are indole alkaloids, pyridine alkaloids and isoquinoline alkaloids. Alkaloids usually have a bitter taste and are known to be toxic (Stary, 1998). This group of secondary metabolites often has pharmacological activity which leads to alkaloids being used as therapeutic drugs because of their analgesic, antispasmodic, and bactericidal effects (Ciocan and Bara, 2007).

2.8.3 Quinone

Quinones are aromatic rings with ketones known to have antihaemorrhagic and antimicrobial activity. The antihaemorrhagic activity is due to their ability to prevent oxidation of tissues in the body. The target site in the organism is exposed to the surface and as a result quinones are able to form complexes with the extracellular, soluble proteins and cell wall of the microorganism, causing inactivation of proteins and hence antimicrobial activity (Kazmi et al., 1994).

2.8.4 Tannins

Tannins are divided into hydrolysable tannins (derivatives of gallic acid i.e. a hydroxyl group of a glucose molecule is esterified to a gallic acid) and condensed tannins (formed from monomers of flavonoids). Tannins have been reported to be toxic to microorganisms by binding cell walls of the organism, preventing growth and protease activity (Jones et al., 1994).

2.8.5 Terpenes

Terpenes are a group of hydrocarbons that are derived from isoprene units. Terpenes consist of different types of structure, namely monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30). Terpenoids have been reported to prevent the activity of *Bacillus subtilis, Staphylococcus aureus, Vibrio cholerae*, *Pseudomonas aeruginosa* and *Candida* spp. (Ciocan and Bara, 2007).

2.9 Selected plant species for the study

Loxostylis alata A.Spreng family name Anacardiaceae(common names: wild pepper tree, teerhout, tar wood) was the plant species chosen for in depth study in this project.

Figure 2.4: Leaves of *Loxostylis alata* photographed by Dennis Stevenson, 2008, Botanical garden, Ruhr University, Bochum, Germany (www.plantsystematics.org/cgi-bin/dol/dol_term inal.pl?taxo)

Loxostylis alata from the family Anacardiaceae is a traditional medicinal plant which is used by indigenous people as a pain reliever during childbirth and as an immune modulator (Pooley, 1993; Pell, 2004). It is a small evergreen tree which grows to a height of about 5 m. It grows on rocky outcrops,

areas adjoining the Karoo, fringing forest and river banks. Suleiman et al. (2010) reported the antifungal activity of this plant and showed that the plant acetone extract has a high activity against *Aspergillus fumigatus* with an MIC value of 50 µg/ml. *Loxostylis alata* acetone extract produced a dose-dependent protection against aspergillosis in chicks infected with *Aspergillus fumigatus.* The extract produced similar results to those obtained with ketaconazole in reducing severity of lesions in pathological scoring and reducing development of fungal infections. Since then not much has been reported about this plant species. *Loxostylis alata* was chosen for the study because of its ability to inhibit the growth of *Aspergillus fumigatus* which has promising implications for the prevention and control of aspergillosis which usually affects domestic animals including poultry. The aim of this project was to increase the activity and reduce the toxicity of the crude plant extract, and to isolate other active compounds that have not previously been isolated from the species.

CHAPTER 3

PRELIMINARY SCREENING OF LEAF EXTRACTS FOR ANTIFUNGALAND ANTIBACTERIAL ACTIVITY

3.1. INTRODUCTION

Plants have been and continue to be the basis of many traditional medicines. They contain compounds that have fungistatic/ fungicidal and bacteriostatic/ bactericidal effects (Cowan, 1999). Screening of plant extracts is undertaken to discover novel compounds that have the potential to be used as pharmaceuticals, to develop herbal medicines which can be affordable and to verify the medicinal properties of plants (Eloff, 2004). Extraction of chemical components of plant material is done mainly to remove and separate any therapeutically, pharmaceutically useful constituents of plant material by using a selective solvent. There are different types of methods that are used for extraction of plant material. Examples of different extraction methods are maceration, percolation, decoction, counter-current extraction and Soxhlet extraction (Handa et al., 2008).

The nature of compounds extracted by solvents of different polarity can be determined by thin layer chromatography. A colour reaction in response to specific chromogenic reagents like vanillin-sulphuric acid reagent may indicate the presence of particular compounds. Such tests are useful when investigating the chemical constituents of the extract and also to monitor the effectiveness of the extraction process when a particular chemical class is being targeted. The behaviour of compounds in chromatographic systems is highly reproducible and can yield information on their identity or physico-chemical structures (Houghton and Raman, 1998).

The antimicrobial activity of individual compounds extracted can be evaluated using bioautography. The antimicrobial activity is indicated by the presence of a clear zone after spraying the TLC plates firstly with a bacterial or fungal culture followed by a solution of iodonitrotetrazolium chloride (INT). This method evaluates individual compounds active against the microorganism instead of the entire plant extract (Begue and Kline, 1972). Antibacterial activity can further be evaluated using broth dilution or microdilution methods which give the minimum inhibitory concentration (MIC) of an antimicrobial that will inhibit the visible growth of a microorganism (Eloff, 1998b).The minimum inhibitory concentration can be used to calculate the total activity of the plant extract. Total activity is the degree to which the active compound in one gram of plant material can be diluted and still inhibit the growth of the tested

pathogenic microorganism (Eloff, 2004). The plant species *Loxostylis alata* was selected for in depth screening and further investigation in this study based on its low MIC and the high total activity against *Aspergillus fumigatus* (Suleiman et al., 2010).

3.2. MATERIALS AND METHODS

3.2.1. Plant selection and collection

Loxostylis alata A.Spreng from Anacardiaceae family is the plant species selected for this study because it has proven antifungal activity (Suleiman et al., 2010). Plant material was obtained from the same collection site as Suleiman et al. (2010), at the Manie van der Schijff University of Pretoria Botanical Garden. The voucher specimen number PRU 96508 of the plant material is kept in the HGWJ Schweikerdt Herbarium of the Department of Plant Sciences, University of Pretoria.

3.2.2. Plant extraction

Three grams of the powdered leaf material was extracted sequentially with 30 ml of hexane, dichloromethane, acetone, and methanol for 30 min each at room temperature. The extraction process was facilitated by shaking on a Labotec model 20.2 shaker and then the extract was filtered through Whatman No.1 filter paper. The process was repeated three times on the marc to exhaustively remove all compounds. The plant extracts were concentrated under vacuum using a rotary evaporator, transferred into pre-weighed glass vials and air dried at room temperature. The masses of the recovered extracts were recorded.

3.2.3. TLC analysis and antioxidant activity screening

The dried extracts were redissolved in acetone to a final concentration of 10 mg/ml. Ten µl was loaded onto duplicate TLC plates and developed with solvent systems of different polarities, viz., BEA (benzene:ethanol:ammonia, 18:2:0.2; v/v/v), CEF (chloroform:ethyl acetate:formic acid, 10:8:2; v/v/v) and EMW (ethyl acetate:methanol:water 10:1.35:1; v/v/v) (Kotze and Eloff, 2002) The separated compounds were visualised under ultraviolet light and the TLC plates were subsequently sprayed with vanillin-sulphuric acid reagent (0.1 g vanillin, 28 ml of methanol and 1 ml sulphuric acid) and the plates heated at 100ºC to enhance colour development (Stahl, 1969). The other set of plates were sprayed with 0.2% DPPH (0.2 g DPPH in 100 ml of methanol) to test the antioxidant activity of different compounds isolated (Deby and Margotteaux, 1970)

3.2.4. Fungal species

Aspergillus fumigatus, Candida albicans and *Cryptococcus neoformans* cultures isolated from animal clinical cases were obtained from the collection of the Department of Veterinary Tropical Diseases, University of Pretoria. The organisms were maintained on Potato Dextrose (PD) agar and cultured for use in PD broth. Densities of fungal cultures used for screening procedures were as follows *Aspergillus fumigatus* (8.1×10^4 cfu/ml), *Candida albicans* (2.5×10^4 cfu/ml and *Cryptococcus neoformans* ((2.6×10^4). The colony forming unit for each fungal pathogen was calculated by using the following formular: $N \times 10^n \times 10$. N is the number of colonies on plate at the selected serial dilution.

3.2.5. Bacterial species

Bacterial species selected for the study included four nosocomial pathogens, namely two Gram-positive *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) and two Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) species. The selection of the specific bacterial strains is based on the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 1990). All the cultures were maintained on Mueller-Hilton agar at 4ºC. The cells were inoculated and incubated at 37ºC in Mueller-Hilton broth for 12 hours prior to the screening procedure. The densities of bacterial cultures for use in the screening procedures were as follows; *S. aureus* (2.6×10^{12} cfu/ml); *E. faecalis,* (1.5×10^{10} cfu/ml)*; P. aeruginosa* (5.2×10^{13} cfu/ml), *E. coli* (3.0×10^{11} cfu/ml).

3.2.6. Antimicrobial assays

3.2.6.1. Bioautography

TLC plates were prepared as in section 3.2.3 and were left to dry to allow all organic solvents to evaporate. *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans,* sub-cultured in PD broth and incubated for 4 hours before use, were sprayed onto separate TLC plates and incubated at 25ºC overnight in a closed chamber at 100% humidity. The plates were sprayed with a 2 mg/ml solution of iodonitrotetrazolium chloride (INT) and were incubated for an hour. The INT was converted to a coloured red-purple formazan by actively growing organisms and inhibition was indicated by clear zones on the TLC plates (Begue and Kline, 1972).

3.2.6.2. Microdilution assay

The microdilution assay was performed using the serial broth dilution method (Masoko et al., 2005; Eloff 1998) and the minimum inhibitory concentrations of the extracts were determined. Firstly 100 µl of distilled water was added to each well of a 96-well microtitre plate. Plant extract (100 µl of a 10 mg/ml solution) was added to the top well in the column, and after mixing, 100 µl was removed from this well and added into the next well. The process was repeated until the bottom of the column was reached, with 100 µl from the last well being discarded to ensure that all wells contained the same volume. Then 100 µl of *Candida albicans, Aspergillus fumigatus* or *Cryptococcus neoformans* culture (sub-cultured on PD broth and incubated for 4 hours before use) was added to all wells. Forty µl of a 0.2 mg/ml solution of iodonitrotetrazolium chloride (INT) was added to each well. The 96-well microtitre plates were sealed with lids and parafilm. Plates were incubated at 25ºC at 100% humidity overnight. The reduced INT is converted to a coloured red-purple formazan by actively metabolising microorganisms; inhibition of fungal growth is indicated by a less intense colour formation (Eloff, 1998b).

3.2.7. Total activity (TA)

Total activity indicates the degree to which the active compound in one gram of plant material can be diluted and still inhibit the growth of the tested bacterial or fungal microorganisms (Eloff, 2004). This was calculated as follows:

Total activity = quantity of material extracted from 1 g of plant material divided by MIC

TA = mg extracted from 1 g/ MIC (mg/ml). Unit ml/g.

3.3. RESULTS AND DISCUSSION

3.3.1. Mass extracted from plant extracts prepared using different solvents

The amount of plant material exhaustively extracted was measured in mg, and is shown in Figure 3.1, where 3 g of dried leaf material was sequentially extracted with 30 ml of the solvent. The highest quantity extracted was obtained using methanol (Figure 3.1). Hexane extracted the lowest quantities of the plant material.

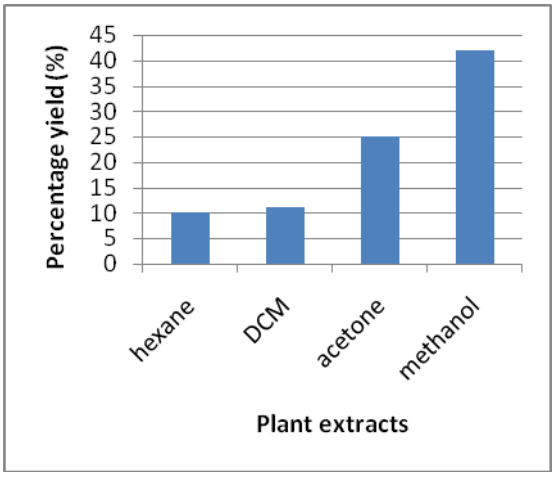


Figure 3.1: Percentage yield extracted from the leaves of *L. alata* by different solvents (hexane, dichloromethane (DCM), acetone and methanol).

3.3.2. TLC analysis and antioxidant activity

The greater the variety of the compounds that can be extracted by the various extracting solvents used, the better the chance that biologically active compounds will be extracted. In the TLC analysis, of the three mobile systems used, BEA showed the best separation of compounds compared to CEF and EMW in all the extracts (Figure 3.2). This shows that most compounds in the prepared plant extracts are non-polar because BEA is non-polar in nature.

Antioxidant activity of plant extracts was evaluated using the TLC-DPPH method as shown in Figure 3.2. DPPH is used as a free radical to evaluate antioxidant activity of some natural compounds. The degree of DPPH discolouration is attributed to the hydrogen-donating ability of test compounds which indicates their scavenging potential (Shimada *et al*., 1992). A clear zone on the TLC plate sprayed with DPPH indicates the presence of a compound with radical scavenging activity. Compounds with high antioxidant activity were observed in the acetone and methanol extracts but the hexane and DCM extracts did not show the presence of antioxidant compounds (Figure 3.2). This suggests that the antioxidant compounds found in *L. alata* tend to be more polar in nature as the more polar solvents extracted the antioxidant compounds.

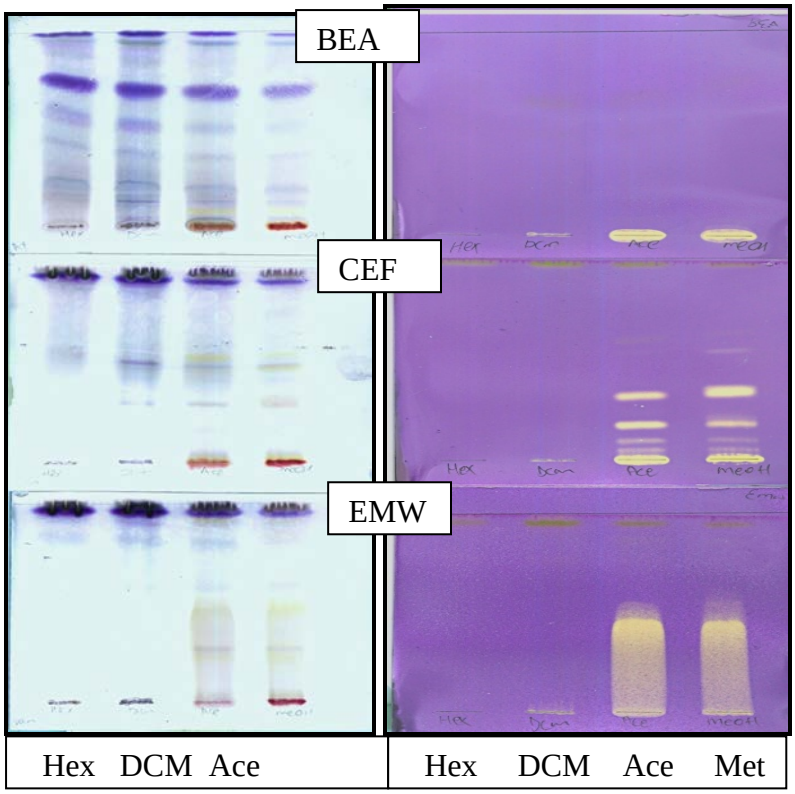


Figure 3.2: TLC chromatograms of *L. alata* leaves extracted from left to right with hexane (Hex), dichloromethane (DCM), acetone (Ace) and methanol (Met) developed in BEA (top); CEF (middle); EMW (bottom) and sprayed with vanillin-sulphuric acid reagent (left) to visualize different compounds in the extract, and DPPH (right) to evaluate antioxidant activity of different compounds.

3.3.3. BIOAUTOGRAPHY

Antifungal activity

Bioautography was used to evaluate antifungal activity of individual compounds localised on a TLC plate. Activity is indicated by clear zones on a reddish background after spraying with iodonitrotetrazolium chloride (INT). According to Figure 3.3, the acetone and methanol extracts showed compounds that inhibited *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. BEA and CEF eluents did not move the active compounds from the origin, because inhibition was only observed at the bottom of the plate. However EMW was able to move the active compound from the acetone and methanol extracts upwards on the TLC plate. This means that the active compound tends more to be polar in nature as EMW is a polar eluent. The hexane and DCM extracts did not show antifungal activity against the tested fungal pathogens.

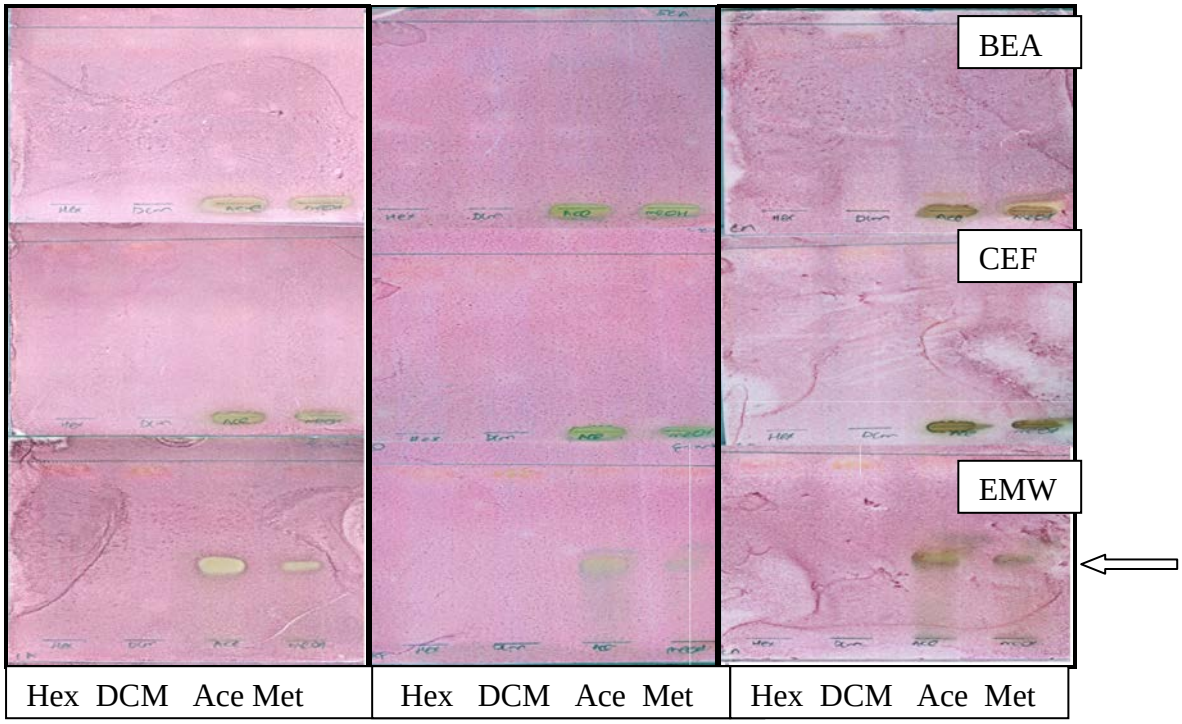


Figure 3.3: TLC bioautograms of hexane (hex), DCM (dichloromethane), acetone (Ace) and methanol (Met) extracts of *L. alata* sprayed with *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus.*

Antibacterial activity

Bioautography was used to evaluate antibacterial activity of individual compounds localised on the TLC plates. Activity is again indicated by clear zones on a reddish background after spraying with iodonitrotetrazolium chloride (INT), as for antifungal activity. According to Figure 3.4, the hexane, DCM, acetone and methanol extracts showed inhibition against *E. faecalis, E. coli, P. aeruginosa* and *S. aureus* in the BEA eluent system. Acetone and methanol extracts also had an antibacterial compound visible in the EMW eluent, however inhibition of this compound was only observed at the bottom of the plate in the BEA and CEF eluents. The active compound in the separation using EMW had the same Rf value of 0.37 as the active compound in the antifungal bioautography.

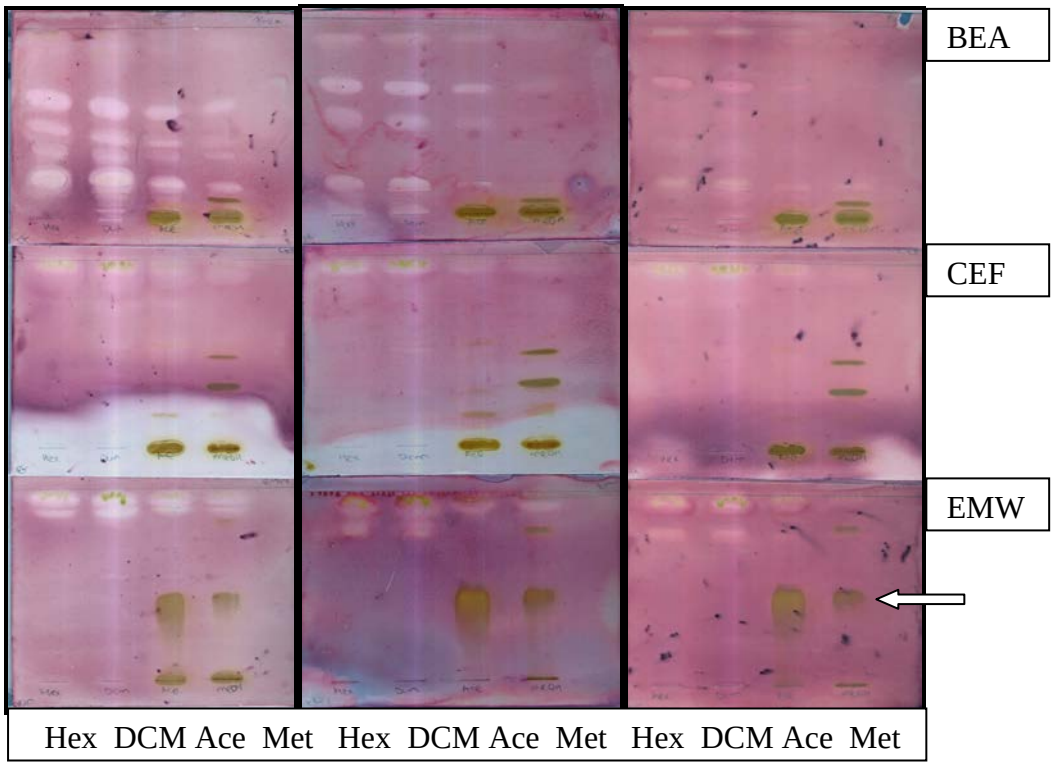


Figure 3.4: TLC bioautograms of hexane (hex), DCM (dichloromethane), acetone (Ace) and methanol (Met) extracts of *L. alata* sprayed with *E. faecalis, E. coli, P. aeruginosa* and *S. aureus.*

3.3.4. Minimum inhibitory concentration assay

Antifungal results

The antifungal activity of *L. alata* extracts was also evaluated by determining the lowest MIC value at which the growth of the test bacteria was inhibited and Table 3.1 shows the MIC values and total activity of *L. alata* extracts against all the tested fungal pathogens. The MIC values were read after 24 hours and 48 hours of incubation to determine if the extracts possess a fungicidal and fungistatic activity. The acetone extract showed a high antifungal activity with the lowest minimum inhibitory concentration of <0.02 mg/ml. The hexane extract had the lowest antifungal activity with the highest minimum inhibitory concentration of 2.5 mg/ml. Similar results of the acetone extract having a high activity and the hexane extract having low antifungal activity were reported by Suleiman et al. (2010).

The total activity indicates the volume to which the bioactive compounds present in the extract can be diluted and still inhibit growth of bacteria or fungi (Eloff, 2004). The methanol extract had the highest total activity of 10 575 ml/g, which shows that the acetone extract prepared from 1 g of plant material can be diluted in 10 575 ml of solvent and still inhibit the activity of *C. neoformans*.

Table 3.1: MIC values (mg/ml) and total activity (ml/g) of *L. alata* extracts against three fungi after incubation for 24 and 48 hours.

Microorganism	Time (hrs)	MIC (mg/ml)					Total activity (ml/g)			
		Hex	DCM	Ace	Met	Amp	Hex	DCM	Ace	Met
C. albicans	24	0.63	0.31	0.31	0.31	5	163	355	806	1365
	48	2.5	2.5	1.25	1.25	25	41	44	200	338
A. fumigatus	24	0.31	0.63	0.16	0.31	5	332	175	1563	1365
	48	>2.5	2.5	0.16	0.63	10		44	1563	671
C. neoformans	24	0.63	0.63	<0.02	0.04	0.63	163	175		10575
	48	0.63	1.25	<0.02	0.63	25	163	88		671

Hex (hexane), DCM (dichloromethane), Ace (acetone), Met (methanol), Amp (Amphotericin B-positive control)

Antibacterial activity

The acetone extract had the highest antibacterial activity against *S. aureus* and *E. coli* compared to the other extracts (Table 3.2). The hexane and DCM extracts had the lowest antibacterial activity against *E. faecalis* with MIC of 2.5 mg/ml.

The methanol extract had a high total activity of 2 644 g/ml against *P. aeruginosa,* followed by the acetone extract with a total activity of 1 562 g/ml against *S. aureus*, *E. coli* and *P. aeruginosa.*

Table 3.2: MIC values (mg/ml) and total activity (g/ml) of *L. alata* extracts against four bacterial species.

Organism	MIC values mg/ml					Total activity g/ml			
	hexane	DCM	acetone	MeOH	Gent	hexane	DCM	acetone	MeOH
E. faecalis	2.5	2.5	0.63	0.63	3.9	41	44	397	671
S. aureus	0.63	0.63	0.16	0.31	4.8	163	175	1562	1365
E. coli	0.63	1.25	0.16	0.31	3.9	163	175	1562	1365
P. aeruginosa	0.63	0.63	0.16	0.16	1.9	163	175	1562	2644

Gent (Gentamicin-positive control).

3.4 CONCLUSION

The acetone extract had compounds with antioxidant activity (Figure 3.1).The extract also had a compound with a good antifungal activity against all the fungal pathogens as seen in bioautography (Figure 3.2) and it also had the lowest MIC value of <0.02 mg/ml (Table 3.1). The acetone extract also had a high antibacterial activity against several bacterial species with an MIC value of 0.16 mg/ml (Table 3.2). It was due to this observation that acetone was selected for potentiation using solvent-solvent fractionation.

CHAPTER 4

POTENTIATION AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF ACETONE EXTRACTS FROM *LOXOSTYLIS ALATA*

4.1. INTRODUCTION

Plants produce chemical compounds that are important to the plants for various functions, but these compounds may also be useful for pharmaceutical purposes, cosmetics and for the production of chemicals (Harborne 1978). Solvent-solvent fraction is usually performed to separate compounds that make up an extract according to their polarities. Development of an active, useful extract rather than isolating purified active compounds is less time consuming and does not require a lot of resources (Zishiri, 2005). Thus the main aim of the fractionation method is to remove inactive compounds and increase the activity of the extract. Once different fractions have been obtained, effective separation methods for constituents in plant extracts can be used to achieve this objective.

In the Phytomedicine Programme, University of Pretoria, Zishiri (2004) potentiated the antimicrobial and antioxidant activity of *Combretum woodii* leaf extract using selective extraction. Selective extraction was also used by Chikoto (2003) to potentiate the antioxidant activity of grape seed extract. Angeh (2007) also potentiated the activity of *Melianthus comosus* extract against plant fungal pathogens to result in a potentially commercially useful antifungal preparation.

In this study, the acetone extract of *Loxostylus alata* in a preliminary screening procedure had good antimicrobial activity against several microorganisms. A bulk acetone extract was then used for solvent-solvent fraction to further potentiate its activity and remove inactive components to enhance the activity of the extract.

4.2. MATERIALS AND METHODS

4.2.1. Bulk extraction procedure

Powdered plant leaf material (200 g) was extracted with 2L of acetone three times. The acetone solvent of the combined extracts was evaporated using a Büchi rotavapor R-114 (Labotec). The concentrated extract was then poured into a pre-weighed beaker and left to dry under a stream of cold air. The quantity extracted was 41.67 g, a 20.84% yield. The acetone extract was subjected to solvent-solvent fraction following the procedure shown in Figure 4.1.

The acetone extract was washed three times with 200 ml of hexane, dichloromethane, ethyl acetate, acetone and methanol successively to yield six fractions. Each fraction was evaporated using a Büchi rotavapor R-114 (Labotec). The mass of each fraction extracted was recorded.

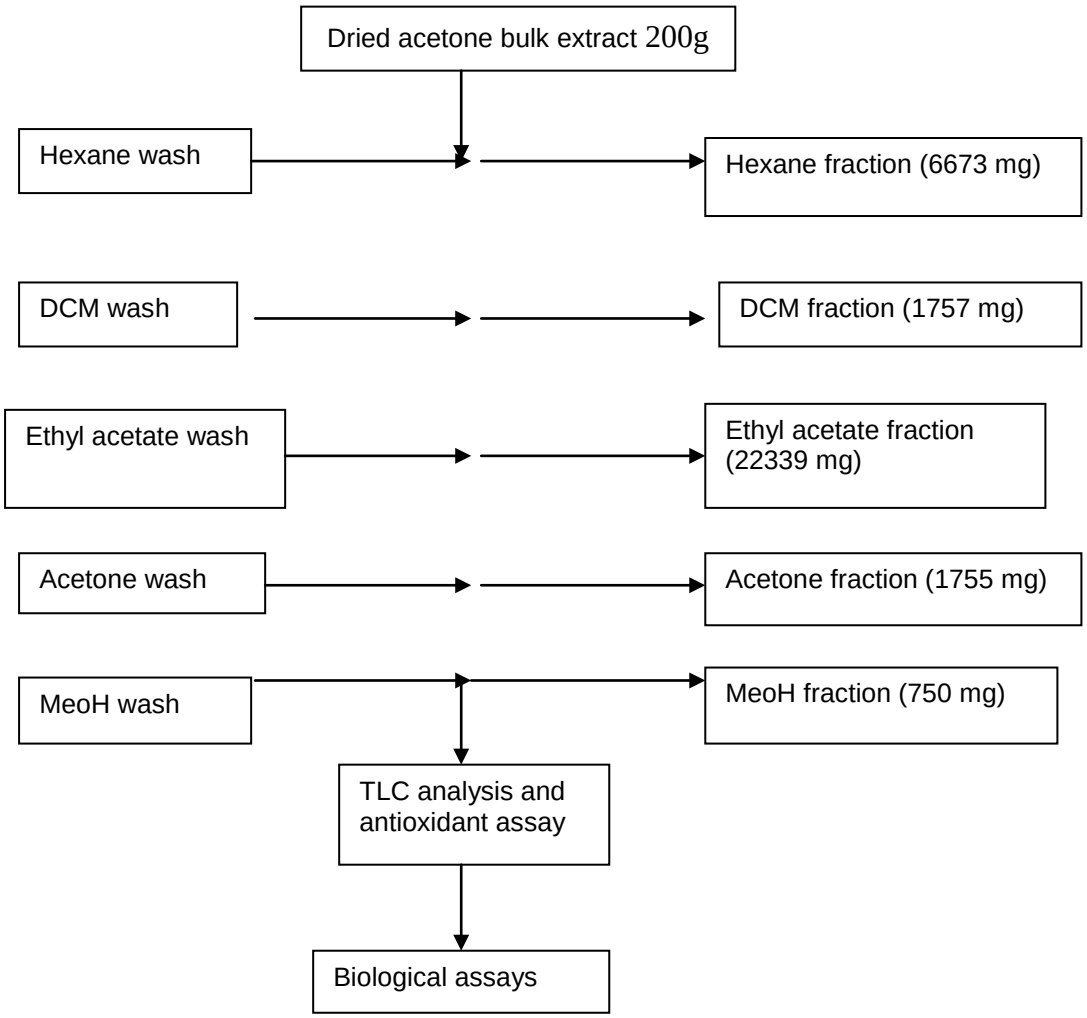


Figure 4.1: Schematic representation of solvent-solvent fractionation of bioactive compounds from *L. alata.*

4.2.2. TLC analysis

TLC analysis (Kotze and Eloff, 2002) and the antioxidant screening assay (Deby and Margotteaux, 1970) were done as in Chapter 3 section 3.2.3

4.2.3 Bioautography

Bioautography (Begue and Kline, 1972) and the microdilution assay (Masoko et al., 2005; Eloff 1998) were done as in Chapter 3 section 3.2.6.1 and 3.2.6.2

4.2.4 Antioxidant assays

4.2.4.1 DPPH radical scavenging assay

The antioxidant activity of the plant extracts was measured by their ability to scavenge the free radical DPPH (Brand-Williams et al., 1994). Dried samples were redissolved in methanol. About 40 µl of each fraction was added into wells of a 96 well microtitre plate. DPPH was dissolved in methanol and160 µl of the DPPH solution was added into the wells with fractions. The change in absorbance was measured at a wavelength of 516 nm at time intervals of 1, 5, and 10 minutes using a microtitre plate reader (Versamax).

4.2.4.2 ABTS radical scavenging assay

The ABTS cation decolourization method was used determine the free radical-scavenging activity as a measure of hydrogen donating capacity (Re et al., 1999). ABTS radical solution was prepared by dissolving 1.32 $\times 10^4$ µg of ABTS in 10 ml of 50% methanol. Potassium persulphate solution was made by dissolving 7.68$\times$ 10^4 µg of potassium persulphate in 10 ml of distilled water. The ABTS and potassium persulphate solutions were mixed together and made up to 200 ml with 50% methanolic solution and kept for 12 hours at room temperature in the dark. ABTS radical solution was diluted with 50% methanol to an absorbance between 0.7 to 0.8 at 734 nm before performing the experiment. The fractions were serially diluted from 19 to 2000 µg/ml in 96 well microtiter plates and 160 µl of ABTS radical solution was added to each well. A blank was prepared using extracts without ABTS radical. Absorbance of the test samples and the blanks were taken after in 6 minutes using a microtitre plate reader (Versamax).

4.3. RESULTS AND DISCUSSION

4.3.1 Solvent-solvent fractionation

4.3.1.1. Mass extracted

The acetone extract was fractionated by solvent-solvent fractionation and the percentage yield of each fraction is shown in Figure 4.2. Ethyl acetate extracted a high mass which was almost half that of the crude extract. The methanol extract had the lowest percentage yield compared to all the fractions. The overall mass of the fractions was 33.27 g whereas the total mass from the crude acetone extract was 41.67 g.

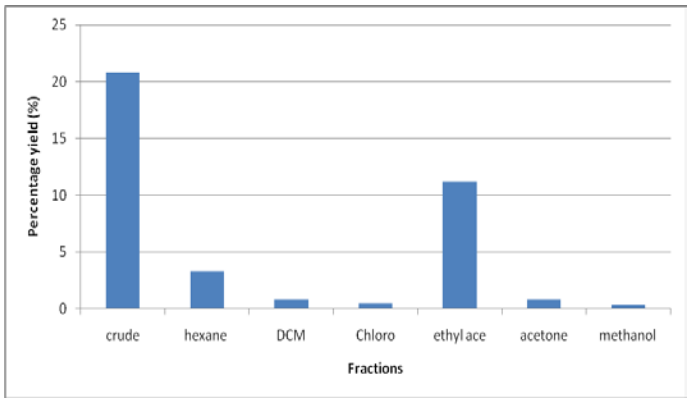


Figure 4.2: Percentage yield extracted from five fractions (crude extract, hexane, dichloromethane, chloroform, ethyl acetate, acetone and methanol) of the acetone extract of *L. alata* leaves.

4.3.1.2. TLC analysis and antioxidant activity

The greater the variety of the compounds that can be extracted by the various extracting solvents used, the better the chance that biologically active compounds will also be extracted. In the TLC analysis, of the three mobile systems used, BEA separated more compounds compared to CEF and EMW in all the extracts (Figure 4.3). This shows that most compounds in the prepared plant extracts are non-polar because BEA is non-polar in nature and separates non-polar compounds well.

Antioxidant activity of plant extracts was evaluated using the TLC-DPPH method as shown in Figure 2. DPPH is used as a free radical to evaluate antioxidant activity of some natural compounds. The degree of DPPH discolouration is attributed to the hydrogen-donating ability of test compounds which indicate the scavenging potential (Shimada et al., 1992). A clear zone on the TLC plate sprayed with DPPH indicates the presence of a compound with radical scavenging activity. Compounds with high antioxidant activity were observed in the crude extract, ethyl acetate, acetone and methanol fractions

but the hexane and DCM fractions did not show the presence of antioxidant compounds (Figure 4.3). This shows that most of the antioxidant compounds are intermediate to polar as the more polar solvents selectively extracted the antioxidant compounds.

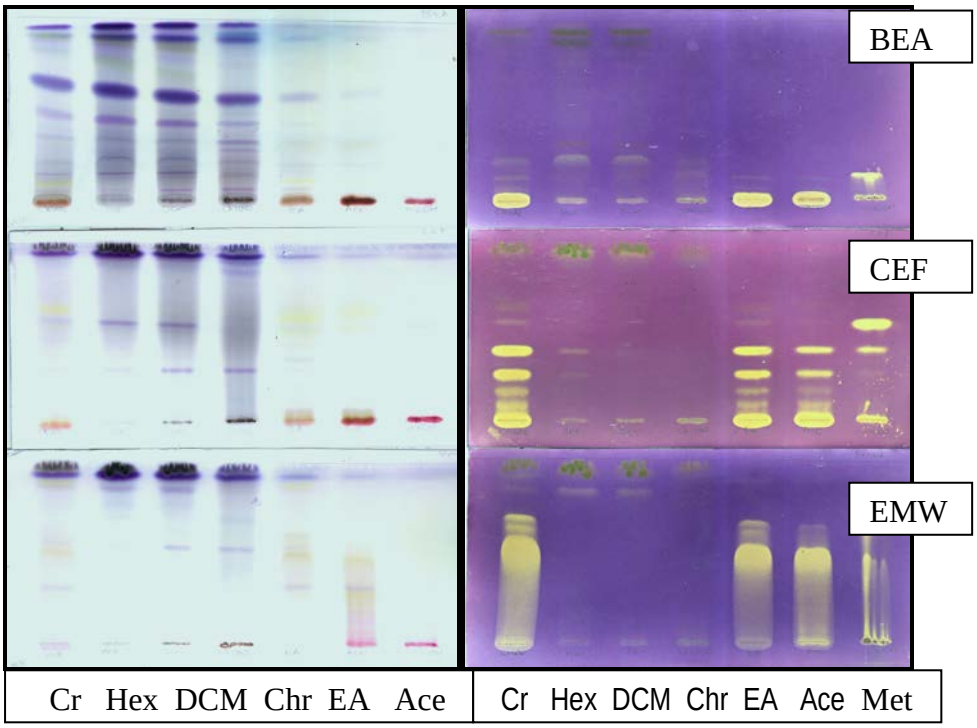


Figure 4.3: TLC chromatograms of *L. alata* leaves extracted from left to right with hexane (Hex), dichloromethane (DCM), Chloroform (Chr), Ethyl acetate (EA), acetone (Ace) and Methanol (Met) developed in BEA (top); CEF (middle); EMW (bottom). Sprayed with vanillin-sulphuric acid (left) and DPPH (right).

4.3.1.3.Bioautography

4.3.1.3.a Antifungal activity

TLC bioautograms of the six fractions of *L. alata* developed in BEA, CEF and EMW mobile systems and sprayed with *Aspergillus fumigatus* are shown in Figure 4.4. The crude extract, hexane, DCM and chloroform fractions showed clear zones of inhibition on the BEA mobile system against *Aspergillus fumigatus*. The same group of compounds also showed zones of inhibition against *Candida albicans* and *Cryptococcus neoformans.* The active compounds observed in the hexane, DCM and chloroform fractions are non-polar, because they moved to the top of the TLC plate using the BEA mobile system which is non-polar. This group of active compounds was not observed before in the hexane and DCM fractions. This indicates that their activity was increased during the potentiation process. The EMW

eluent showed another compound with activity in the crude extract, ethyl acetate and acetone fractions against the three fungal organisms tested but BEA and CEF did not move the active compound because inhibition was only observed at the bottom of the plate. This means that the active compound is more polar in nature because it was moved by EMW only, which is a polar solvent system.

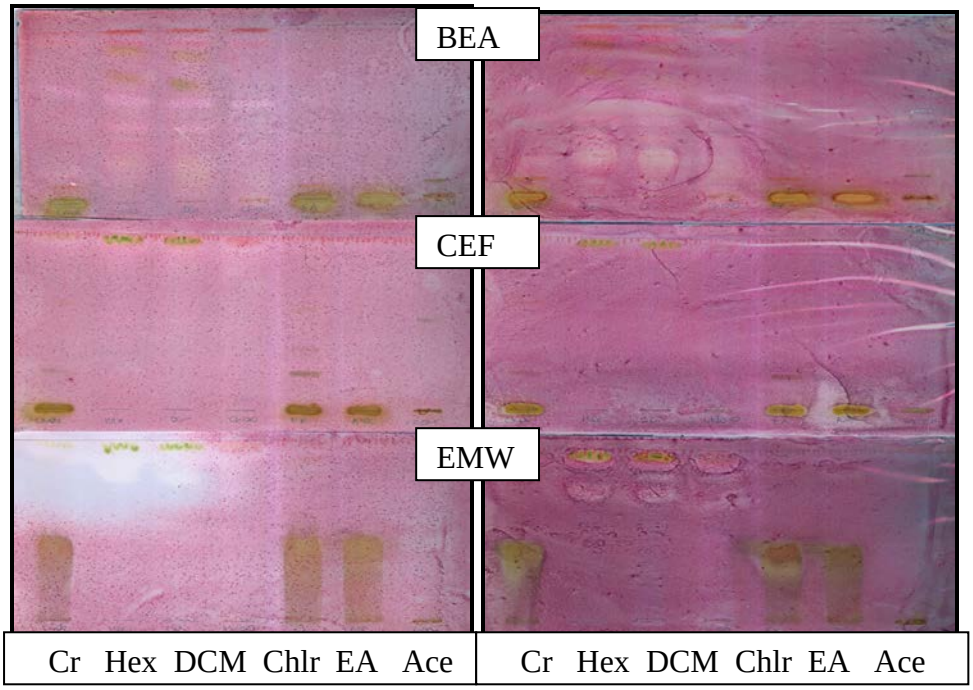


Figure 4.4: TLC bioautograms of six fractions of *L. alata* developed in BEA, CEF and EMW solvent systems sprayed with *Aspergillus fumigatus* (left) and *Candida albicans* (right). Lanes from left to right: C (Crude extract) Hex (hexane), DCM (dichloromethane), Chlr (chloroform), ET (ethyl acetate), Ace (acetone) and Met (methanol).

4.3.1.3.b Antibacterial activity

TLC bioautograms of the six fractions of *L. alata* developed in BEA, CEF and EMW mobile systems and sprayed with *Enterococcus faecalis* and *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* are shown in Figure 4.5. The crude extract, hexane, DCM and chloroform fractions showed clear zones of inhibition using the BEA mobile system against *E. faecalis*, *S. aureus, E. coli* and *P. aeroginosa.* The active compounds observed in the hexane, DCM and chloroform fractions are non-polar, because they moved to the top of the TLC plate using the BEA mobile system which is non-polar. This group of active compounds was not observed before in the hexane and DCM extracts. This indicates that their activity may have been increased during the potentiation process to allow their detection. The EMW eluent showed another compound with activity in the crude extract, ethyl acetate and acetone fractions against the three fungal organisms tested but BEA and CEF did not move the active compound because inhibition was only observed at the bottom of the plate. This means that the

active compound is more polar in nature because it was moved by EMW only, which is a polar solvent system. The antibacterial compounds in the hexane, DCM, ethyl acetate and acetone fractions have the same Rf values as the active compounds against the fungal pathogens.

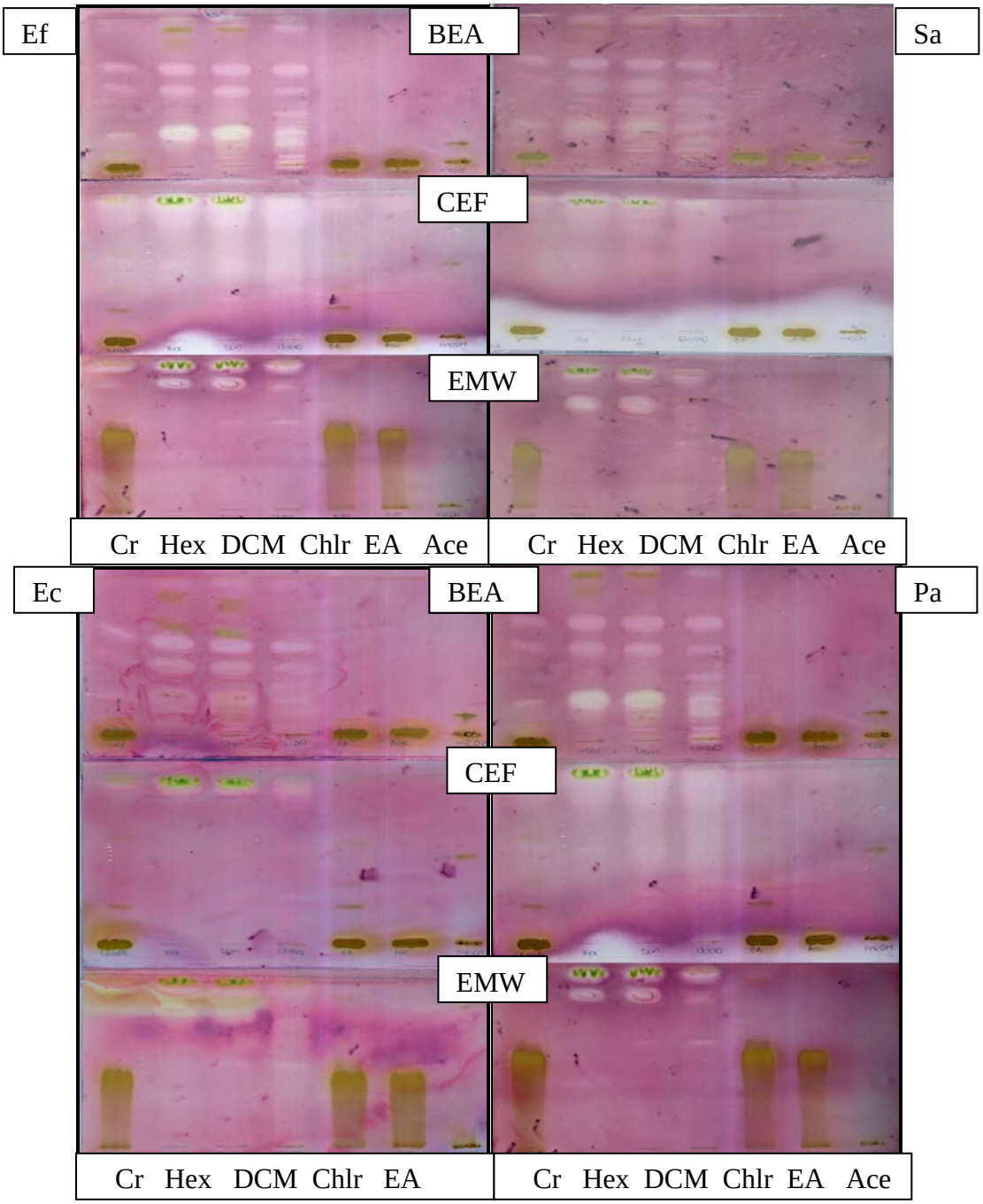


Figure 4.5: TLC bioautograms of six fractions of *L. alata* developed in BEA, CEF and EMW solvent systems sprayed with *Enterococcus faecalis* (Ef) and *Staphylococcus aureus* (Sa), *Escherichia coli* (Ec) and *Pseudomonas aeruginosa* (Pa). Lanes from left to right: C (Crude extract) Hex (hexane), DCM (dichloromethane), Chlr (chloroform), ET (ethyl acetate), Ace (acetone) and Met (methanol).

4.3.1.4. Minimum Inhibitory Concentration values of the crude extract and fractions

The antifungal activity of *L. alata* extracts was determined by evaluating the lowest MIC value at which the growth of the test fungi was inhibited and Table 4.1 shows MIC values (mg/ml) of the six fractions of *L. alata* tested against *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*. The increase or decrease in activity of the crude extract and fractions after solvent-solvent fractionation may be due to synergy or inhibitory compounds. If the inhibitory effects of the fractions are lower than that of the crude extract, the decreased MIC values in the crude extract may be due to synergism of active compounds in the crude extract since it is expected that the activity of individual compounds should be increased after fractionation. If the inhibitory activity of the fractions is higher than the activity of the crude extract, the increased MIC may be due to the removal of inhibitory compounds during the fractionation process. The potential inhibitory compounds in the crude extract may interfere with the absorption, assimilation and subsequent metabolism of the active compounds by the organism, or they may interfere with the active compounds by coupling with them to form inactive complexes. The lowest MIC value of 0.02 mg/ml was observed in the hexane fraction and an MIC of 0.04 mg/ml was noted by the DCM fraction against *A. fumigatus.* This activity was not observed in the hexane and DCM extracts and this indicates that the activity of the extracts has been potentiated. The acetone and ethyl acetate fractions also showed antifungal activity with the lowest MIC value of <0.02 mg/ml against *Cryptococcus neoformans*.

Table 4.1: MIC values (mg/ml) of the crude extract and five fractions against three fungal pathogens.

Plant extracts	24 hours				48 hours			
	MIC values mg/ml				MIC values mg/ml			
	C. Alb	*A. fum*	*C. Neo*	Average	*C. Alb*	*A. Fum*	*C. Neo*	Average
Crude	0.63	0.16	<0.02		2.5	1.25	0.16	1.3
hexane	0.16	0.02	0.16	0.11	2.5	1.25	0.63	1.46
DCM	0.16	0.04	0.31	0.17	1.25	1.25	0.63	1.04
Chloro	0.63	1.25	1.25	1.04				
EtAC	0.63	0.16	<0.02		1.25	0.63	0.16	0.68
Acetone	0.63	0.31	<0.02		1.25	0.63	0.16	0.68
MeOH	0.63	1.25	0.31	0.73	1.25	0.63	0.16	0.68

Table 4.2: Average MIC values (mg/ml) of crude extract and five fractions against four bacterial pathogens.

Organisms			MIC values mg/ml			
	Crude	hexane	DCM	Ethyl ace	Acetone	MeoH
E. faecalis	0.31	0.63	0.63	0.31	0.31	1.25
S. aureus	0.31	0.31	0.31	0.31	0.63	0.31
E. coli	0.31	0.63	1.25	0.63	0.63	0.31
P. aeruginosa	0.16	0.63	0.63	0.16	0.31	0.63
Average	0.27	0.55	0.705	0.35	0.47	0.63

4.3.1.5. Total activity of the crude extraction and five fractions

The total activity indicates the volume to which the active constituents present in the fraction can be diluted and still inhibit the growth of the test organism. Total activity is calculated by dividing the quantity present in the extract in mg with the MIC value in mg/ml. The comparison of the total activity of the crude extracts and fractions from solvent-solvent fractionation helps in evaluating if there is a change in the biological activity during the process (Eloff, 2004). Fractions with low MIC are expected to have a high total activity and this was the case with the ethyl acetate and hexane fractions. On average, the ethyl acetate fraction had the highest total activity compared to other fractions against the tested fungal pathogens. The average total activity of the crude extract (80 3360 ml) was higher than the sum of the average total activities of the fractions (62 3346 ml).

For antibacterial testing the ethyl acetate fraction had the highest average total activity compared to other fractions. The average total activity of the crude extract (165 924 ml) was higher than the sum of the total activities of the fractions (102 146 ml).

Table 4.3: Total activity (ml) of *L. alata* crude extract and five fractions against three fungal pathogens.

Plant extracts	24 hours incubation				48 hours incubation			
	Total activity ml				Total activity ml			
	C. Alb	*A. fum*	*C. Neo*	Average	*C. Alb*	*A. fum*	*C. Neo*	Average
Crude	66143	260438	2083500	803360	16668	33336	260438	103480
Hexane	41706	333650	41706	139021	2669	5338	10592	6199
DCM	10975	43900	5665	20180	1405	1405	2787	1866
EtAC	35458	139619	1116950	430676	17871	35458	139619	64316
Acetone	2786	5661	87750	32066	1404	2786	10969	5053
MeOH	1190	600	2419	1403	600	1190	4688	2159

Table 4.4: Average total activity (ml) of *L. alata crude* extract and five fractions against four bacterial pathogens.

Organisms	Total activity ml					
	Crude	hexane	DCM	Ethyl ace	Acetone	MeoH
E. faecalis	134419	10592	2787	72061	5661	600
S. aureus	134419	21525	5665	72061	2786	2419
E. coli	134419	10592	1405	35458	2786	2419
P. aeruginosa	260438	10592	2787	139619	5661	1190
Average	165924	13325	3161	79779	4224	1657

4.3.1.6 Evaluation of percentage activity recovered after fractionation

The percentage activity recovered during fractionation was calculated to evaluate the synergistic effects of the bioactive compounds in the crude extracts. The ethyl acetate fraction had an average activity recovery of 54% against the fungal pathogens and 48% against the bacterial pathogens followed by the hexane fraction with 17% against fungal pathogens and 8% average activity recovery against bacterial pathogens. This shows that the fungal pathogens were less sensitive compared to the bacterial species. The ethyl acetate and hexane fractions were further used for isolation of the bioactive compounds.

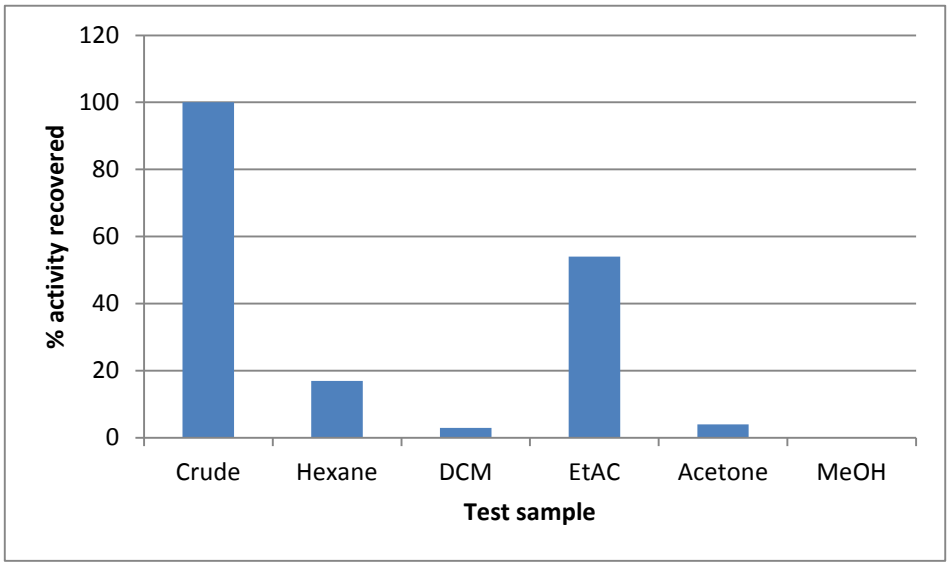


Figure 4.6: Average percentage of the activity of the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions against three tested fungal pathogens regained upon solvent-solvent fractionation.

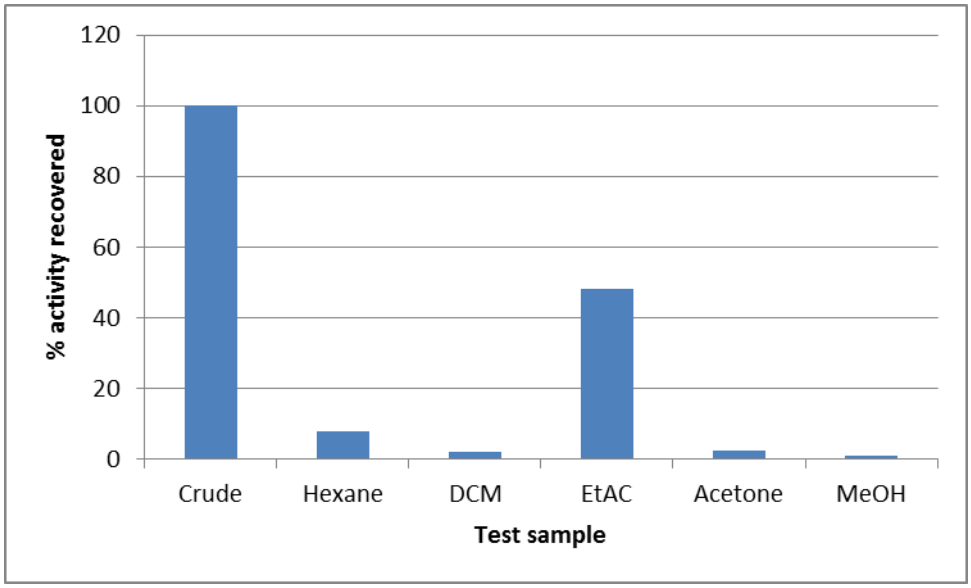


Figure 4.7: Average percentage of the activity of the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions against four tested bacterial pathogens regained upon solvent-solvent fractionation.

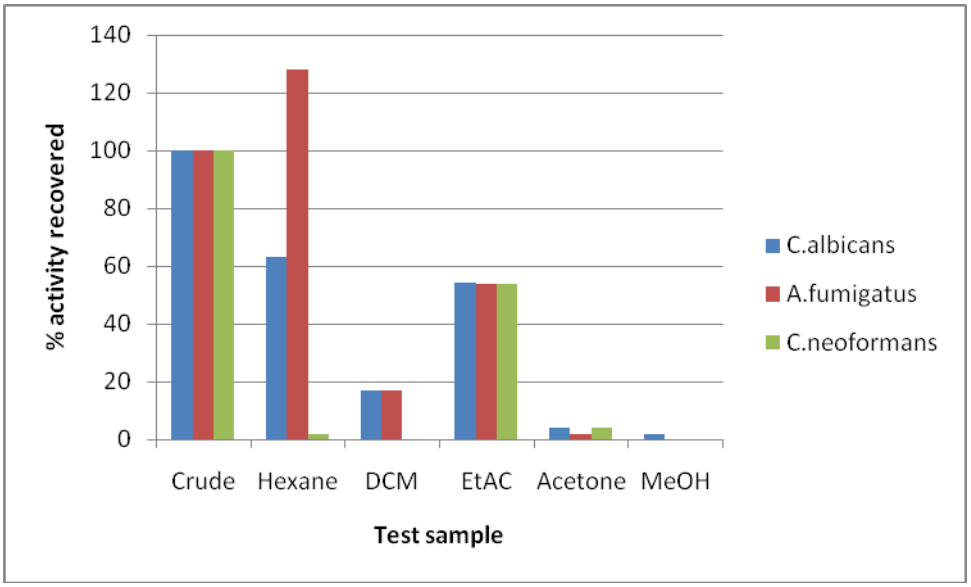


Figure 4.8: Percentage of the activity of the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions against three tested fungal pathogens regained upon solvent-solvent fractionation.

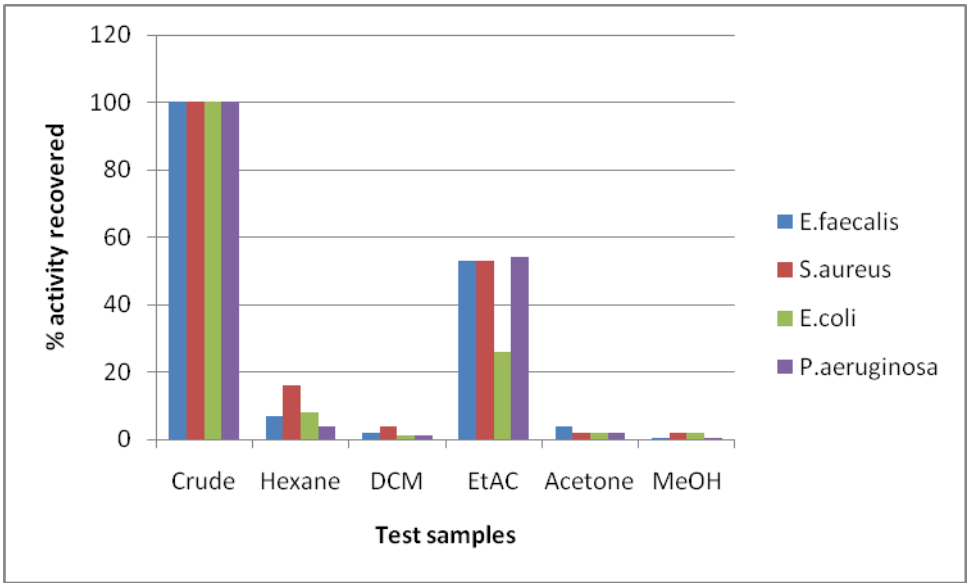


Figure 4.9: Percentage of the activity of the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions against four tested bacterial pathogens regained upon solvent-solvent fractionation.

4.3.2 Antioxidant evaluation

4.3.2.1 DPPH assay

Plant extracts were evaluated for antioxidant activity as shown in Figure 4.10. The DPPH assay was used to evaluate antioxidant activity of the crude extract and the fractions. DPPH forms a stable free radical, DPPH• (1, 1-diphenyl- 2- picrylhydrazyl). The reaction involves a colour change from violet to yellow that can be easily monitored using a spectrophotometer at 516 nm. Extracts that change the violet colour to yellow have hydrogen donating ability thus have antioxidant activity. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Mandal et al., 2009). The crude extract had a high antioxidant activity compared to the fractions. Polar fractions had higher antioxidant activity compared to non-polar fractions. This is expected because antioxidant compounds are often polar in nature. Antioxidant compounds are important in preventing/scavenging the effects of free radicals in our biological system. Antioxidants are also known to prevent oxidation of lipids and protect the body from the development of diseases.

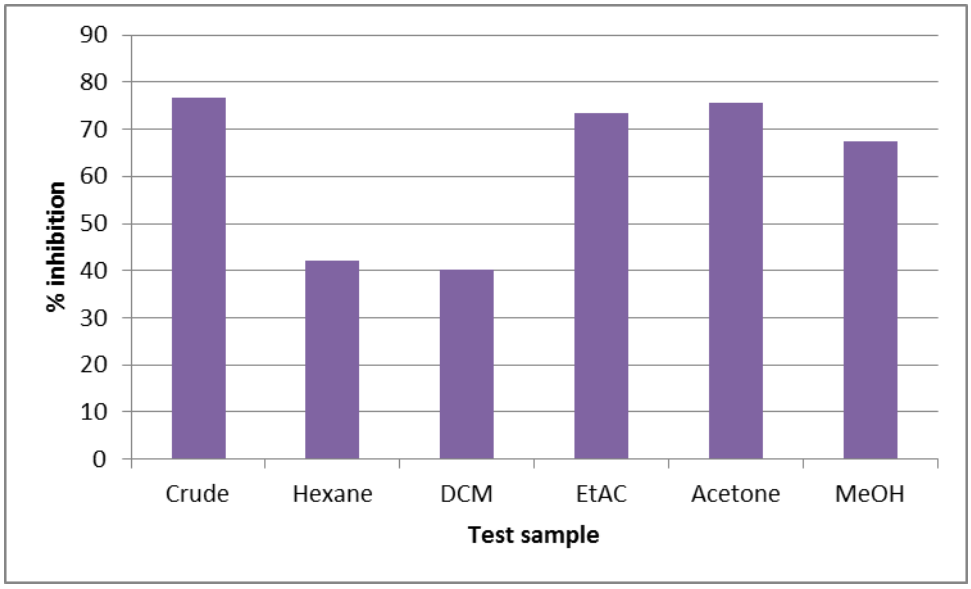


Figure 4.10: Percentage inhibition at which the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions were able to scavenge the DPPH free radical.

4.3.2.2 ABTS assay

The ABTS (2, 2'-azinobis [3-ethylbenzothiazoline-6- sulphonate]) assay was also used to evaluate the antioxidant activity of the plant extracts. The ABTS assay involves the oxidation of ABTS to an intensely-coloured nitrogen-centred radical cation, $ABTS^{\bullet+}$ (Re et al., 1999). The crude extract, acetone, ethyl acetate fractions and methanol had high percentage inhibition ranging from 85.79% and 91.62% while ascorbic acid and trolox had percentage inhibition values of 72.42 and 68.87% respectively. This shows that the crude extract, acetone, ethyl acetate and methanol fractions have a good antioxidant activity compared to ascorbic acid and trolox which were the standards used.

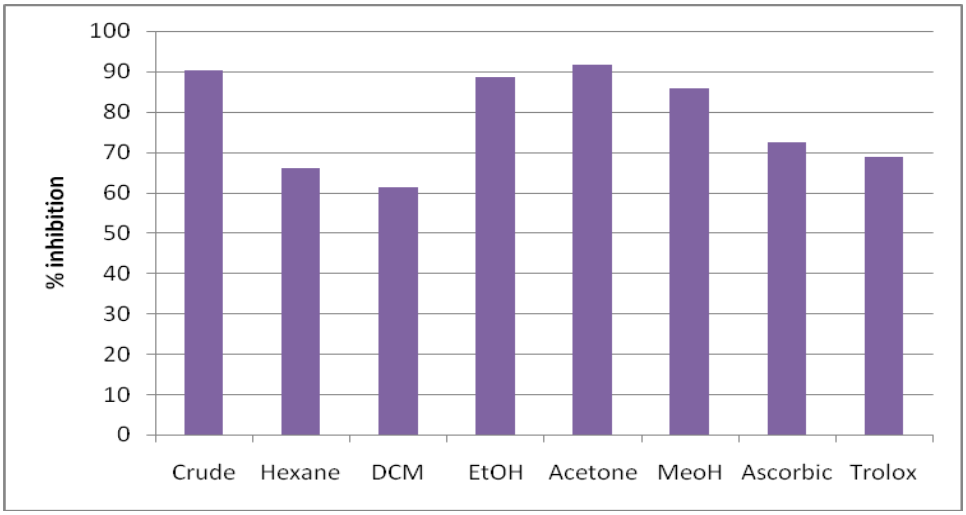


Figure 4.11: Percentage inhibition at which the crude extract, hexane, DCM, ethyl acetate, acetone and methanol fractions were able to oxidise ABTS.

4.4. CONCLUSION

Solvent- solvent fractionation was performed on the acetone extract to group compounds from the acetone extract according to different polarity, to remove inactive compounds and to increase the activity of bioactive compounds by increasing their relative concentrations. From the results obtained, the antimicrobial activity of the hexane and DCM fractions had increased with the lowest MIC value of 0.02 mg/ml compared to that of the hexane and DCM fractions. In bioautography there were zones of inhibition in the separated hexane and DCM fractions using the BEA mobile system, but there were no zones of inhibition in the hexane and DCM fractions in Chapter 3. The antimicrobial compound in the ethyl acetate fraction had the same Rf value as that in the acetone extract in Chapter 3. The active compounds in the hexane, DCM, ethyl acetate and acetone fractions in the antifungal bioautograms had the same Rf values as the active compounds against the fungal pathogens. The ethyl acetate and hexane fractions had the lowest MIC values on average against the tested organisms. Different compounds with antimicrobial activity against the tested pathogens were observed in the two fractions. The ethyl acetate and hexane fractions had the highest average activity recovered upon fractionation and as a result, they were further used for isolation of the bioactive compounds.

CHAPTER 5

ISOLATION AND STRUCTURAL ELUCIDATION OF THE ACTIVE COMPOUNDS

5.1. INTRODUCTION

Isolation of natural compounds from medicinal plants is a useful technique in the identification of natural compounds with potential pharmaceutical properties (Tsipouras et al., 1995). Well known techniques that have been used for the separation of natural products include chromatographic techniques (Gurib-Fakim, 2006). Examples of chromatographic techniques include column chromatography, gas chromatography, supercritical fluid chromatography, high performance liquid chromatography and thin layer chromatography (Wen et al., 2004).

Thin layer chromatography (TLC) is a rapid and widely used technique for isolation, purification and confirmation of chemical compounds (Wen et al., 2004). TLC is usually used together with column chromatography and may be used to determine the choice of adsorbent, solvent and working conditions in column chromatography. Basically TLC pre-optimizes the separation conditions while column chromatography is used for complete separation of chemical compounds (Schlitt and Geiss, 1972). The chromatographic systems consist of a stationary and a mobile phase. A sample is applied on the stationary phase, for example silica gel, and mobile phase is allowed to pass through the system. Different components in the mixture will interact differently with the stationary and mobile phases. Components will be carried along with the mobile phase according to different polarities and separation will be achieved (Peterson and Cummings, 2006).

After isolating compounds the structure of the isolated compounds has to be elucidated to determine and confirm the molecular structure and the purity of the isolated compound. The structure of isolated compounds may be determined using nuclear magnetic resonance (NMR) spectroscopy (^{1}H) and ^{13}C NMR-spectroscopy, and mass spectrometry (Celiz et al 2009).

5.2. MATERIALS AND METHODS

5.2.1. Isolation of active compound from the hexane fraction

5.2.1.1 Column 1

The procedure used to isolate the bioactive compounds from the leaves of *Loxostylis alata* is schematically shown in Figure 5.1.Column chromatography was chosen for separation of compounds using silica gel as a stationary phase. About 175 g of silica gel 60 (Merck) was mixed with hexane pure solvent to form a slurry and stirred using a stirring rod before being poured into a glass column. Twenty ml of hexane was added into the column of 40 cm length and 2.5 cm diameter whose vent was plugged with cotton to avoid bubbles when the slurry was packed into the column. The solvent was allowed to flow out the column to allow the gel to settle. The hexane fraction (8 g) was dissolved in 50 ml acetone mixed with 20 g silica gel and the mixture was allowed to dry under a stream of cold air for about 5 hours, before it was layered onto the column bed. Initially, hexane (1000 ml) was gradually added into the column to remove fats, waxes and some chlorophyll. The polarity was increased by addition of ethyl acetate (EtOAc) at intervals of 5% until 100% EtOAc.

Twenty-eight fractions (with quantity of evaporated mass ranging from 80 mg to 890 mg) were collected in glass jars and allowed to concentrate under a stream of cold air. TLC analysis was performed on all of the fractions and bioautography was done using *A. fumigatus, Candida albicans* and *Cryptococcus neoformans* as test organisms to locate antifungal active compounds.

5.2.1.2 Column 2

A second column (40 cm length and 1.5 cm diameter) was run to isolate the active compound in fraction 3 from column 1. Fraction 3 (520 mg) was dissolved in acetone and mixed with 6 g of silica. The mixture was allowed to dry. About 80 g silica gel was mixed with hexane and used to pack the glass column. The sample was then loaded on top of the column. The column was initially eluted with 200 ml of 100% hexane followed by 95:5, 90:10, 85:15 and 80:20 hexane:ethyl acetate. Fractions were collected in test tubes and allowed to dry. TLC chromatograms of all the fractions were prepared for visualisation of compound separation.

5.2.2. Isolation of active compound from the ethyl acetate fraction

5.2.2.1 Column 3

Silica gel (80 g) was mixed with chloroform and packed in a column (40 cm in length and 1.5 cm in diameter) to isolate bioactive compounds from the ethyl acetate fraction. The dried ethyl acetate fraction was dissolved in acetone and mixed with 6 g silica. The mixture was allowed to dry and was loaded on the column. The column was eluted with 100% chloroform followed by 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60 chloroform:ethyl acetate up to 100% ethyl acetate. Fractions were collected and allowed to dry. TLC plates of all the fractions were sprayed with ferric chloride (2 g of ferric chloride dissolved in 10ml of methanol) for visualisation because the compound of interest could not be visualised using vanillin and it was suspected to be a tannin.

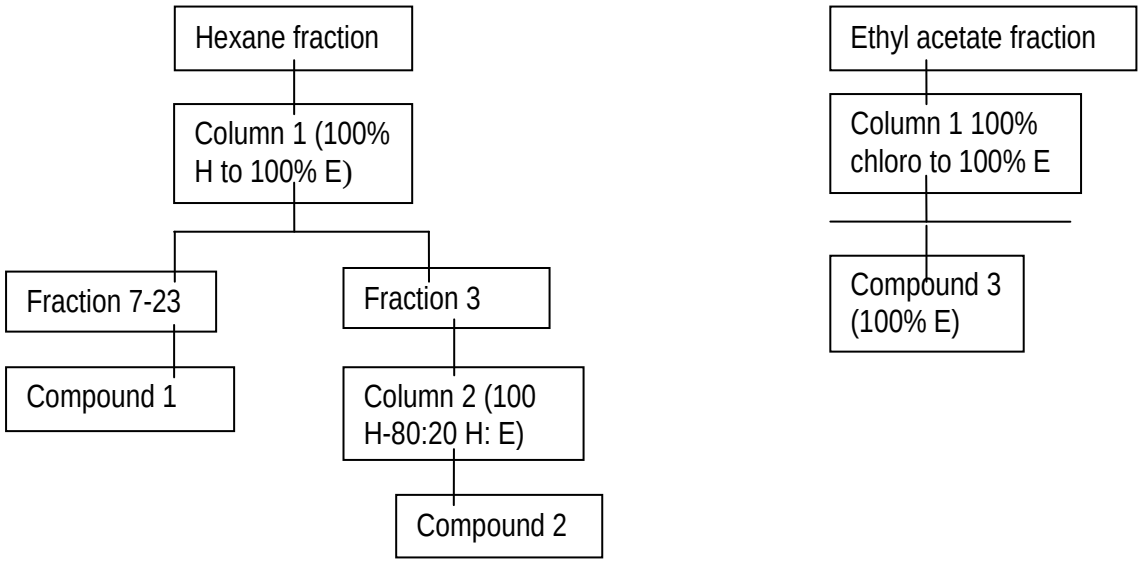


Figure 5.1: Schematic representation of isolation of bioactive compounds from *L. alata* using column chromatography with silica gel 60 as the stationary phase. (H = hexane, E = ethyl acetate, chloro = chloroform)

5.2.3 Structure elucidation

The structure of the isolated compounds from the hexane fraction of the acetone extract of *Loxosylis alata* leaves were elucidated using ^{1}H NMR and ^{13}C NMR spectral analysis using a Varian-NMR-vnmrs 600 located at the CSIR, Pretoria.

5.3. RESULTS AND DISCUSSION

5.3.1 TLC profiling of isolated compounds

5.3.1.1 Compound 1 from hexane fraction

The TLC chromatogram of the first column of the hexane fraction developed in 95:5 hexane: ethyl acetate mobile system is shown in Figure 5.2. Fractions 7- 23 showed the pure compound with the same Rf value of 0.18 in the first column when developed with 95:5 hexane:ethyl acetate mobile system. The compound from fractions 7-23 showed activity against the fungal organisms tested. This compound had an Rf value of 0.46 when developed using 70:30 hexane and ethyl acetate solvents. Suleiman et al. (2010) isolated a compound with an Rf value of 0.47 in this solvent system from *L. alata*. Fraction 3 contained an active compound but it was not pure. A second column was run to isolate the compound in fraction 3.

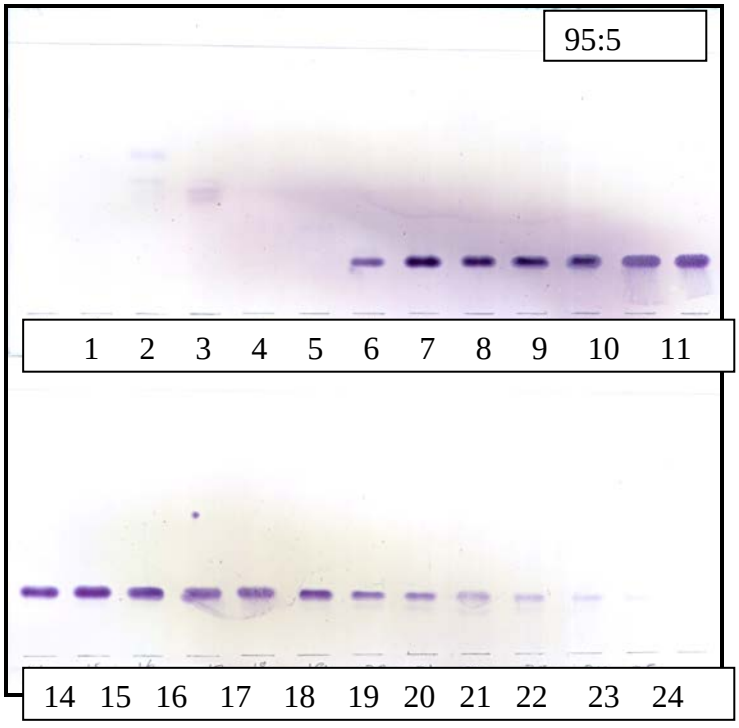


Figure 5.2: TLC plate of fractions from the first column of the hexane fraction developed in 95:5 hexane:ethyl acetate mobile system. The plates were sprayed with vanillin.

Bioautography

Bioautograms of isolated compounds against *E. coli* and *P. aeruginosa* are shown in Figure 5.3. Antimicrobial activity is indicated by clear zones on a reddish background after spraying with iodonitrotetrazolium chloride (INT). Both compounds showed clear bands against *E. coli* and *P. aeroginosa* which indicates that the compounds were able to inhibit the growth of the tested microorganisms.

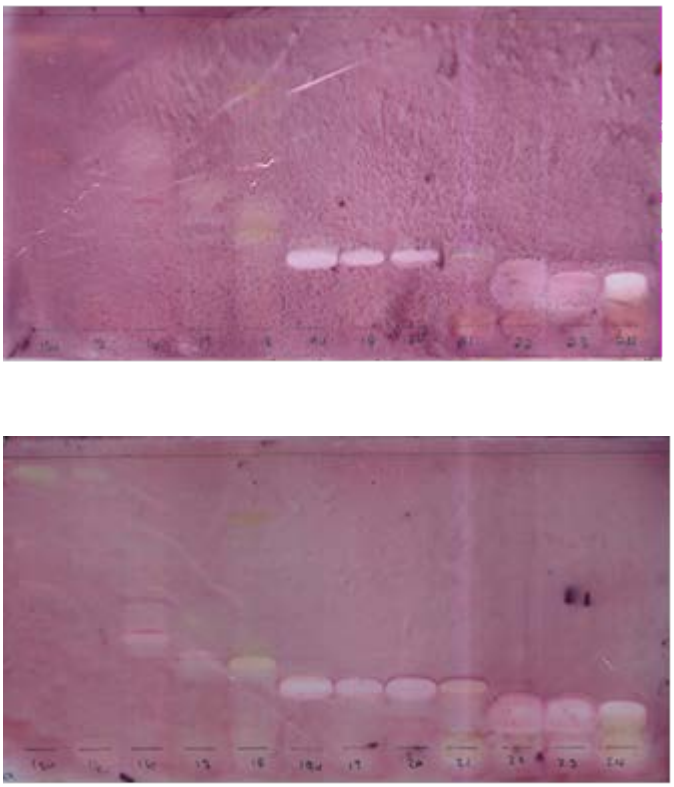


Figure 5.3: TLC plate of fractions from the first column of the hexane fraction developed in 90:10 hexane: ethyl acetate mobile system. The plates were sprayed with *Escherichia coli* and *Pseudomonas aeruginosa.*

5.3.1.Compound 2 from hexane fraction

The TLC plate of fractions of the second column showing constituents of fraction 3 from the first column was developed in 90:10 hexane:ethyl acetate mobile system. Fractions 1-3 are shown in Figure 5.4.

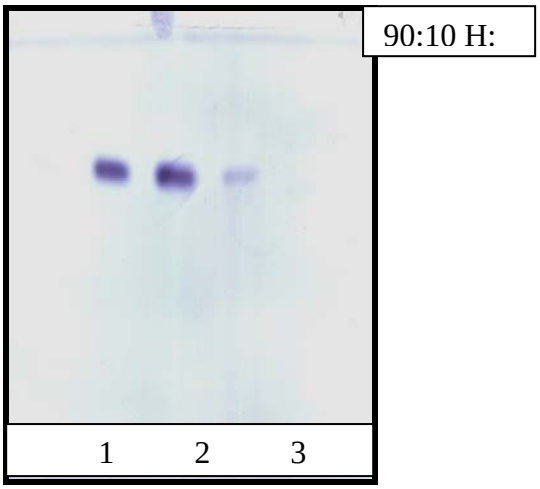


Figure 5.4: TLC plate of the second column from fraction number three from the first column of the hexane fraction developed in 90:10 hexane:ethyl acetate mobile system and sprayed with vanillin-sulphuric acid spray reagent.

5.3.1.3 Compound 3 from ethyl acetate fraction

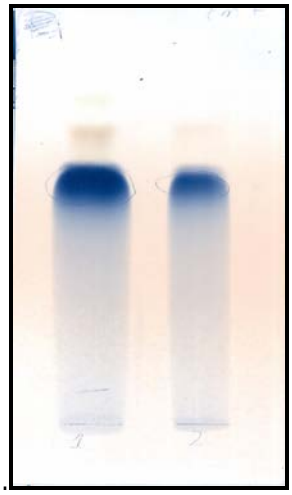


Figure 5.5: TLC plate of the compound from the ethyl acetate fraction developed in EMW mobile system and sprayed with ferric chloride.

5.3.2 Structural elucidation

Compound 1 (hexane fraction compound)

Compound 1 was isolated from the hexane fraction. The ^{13}C NMR showed the presence of 7 methyl, 11 methylene and 6 methane carbons whereas ^{1}H NMR showed the presence of seven tertiary methyl groups at 0.98, 0.78, 0.84, 1.06, 0.96, 0.78 and 1.67. The ^{1}H NMR and ^{13}C NMR spectral data of the compound showed features of a pentacyclic triterpene. The compound was identified as lupeol when comparing spectral signals with previous published reports (Burns et al., 2000). Lupeol was isolated from *L. alata* (Suleiman et al., 2010).

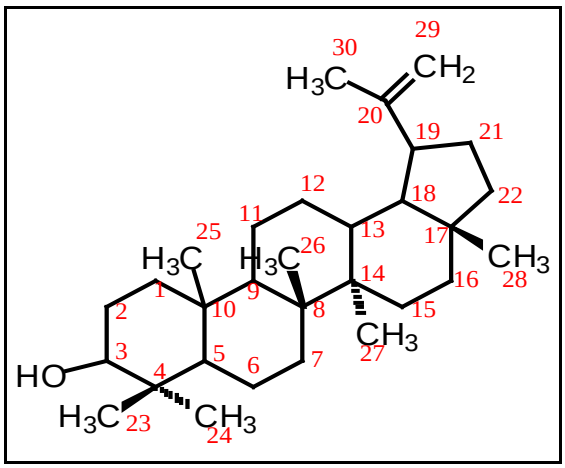


Figure 5.6: Structure of lupeol isolated from the hexane fraction of *Loxostylis alata* leaves.

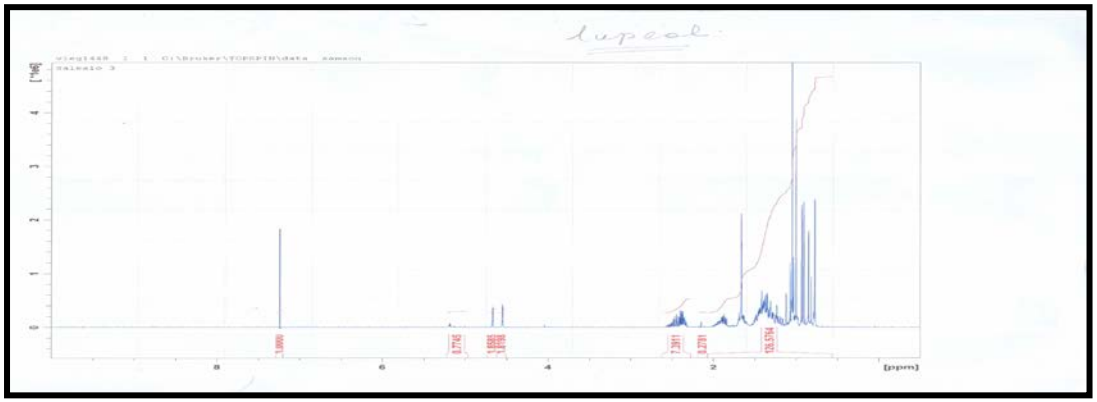


Figure 5.7: ^{1}H NMR spectrum of lupeol isolated from the hexane fraction of *Loxostylis alata* leaves.

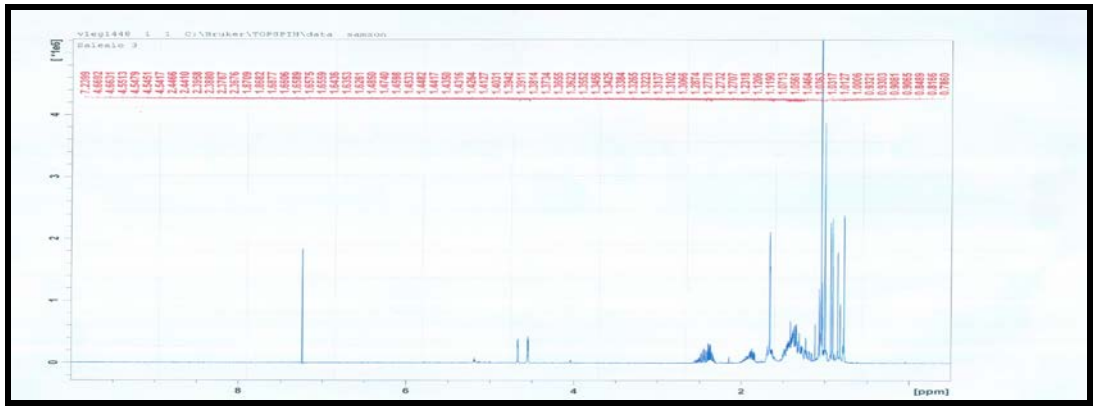


Figure 5.8: ^{13}C NMR spectrum of lupeol isolated from the hexane fraction of *Loxostylis alata* leaves

Compound 2

NMR spectra herewith showing a mixture of three compounds (lupeol, beta amyrin and alpha amyrin

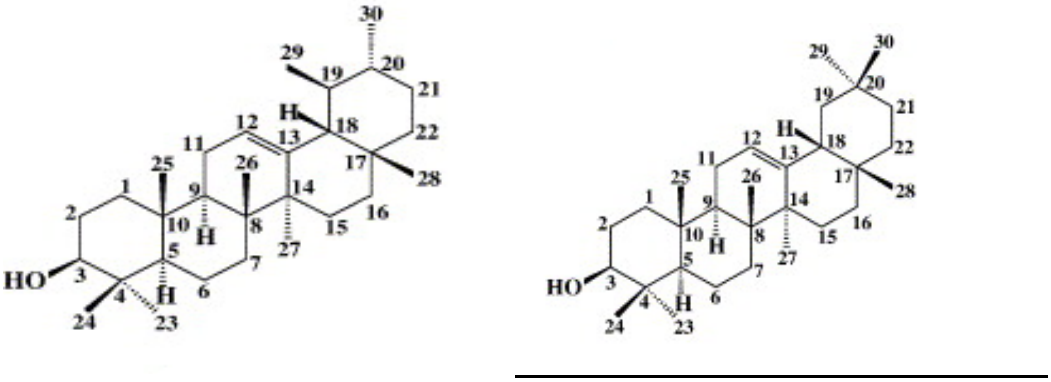


Figure 5.9. Structure of alpha-amyrin and beta-amyrin isolated from *L.alata*

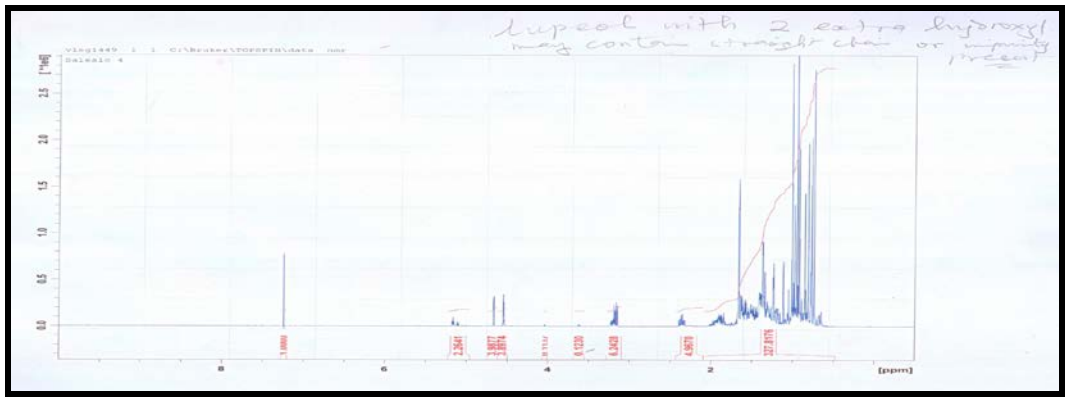


Figure 5.10: ^{1}H NMR spectrum of lupeol, alpha amyrin and beta amyrin isolated from hexane fraction of *Loxostylis alata* leaves.

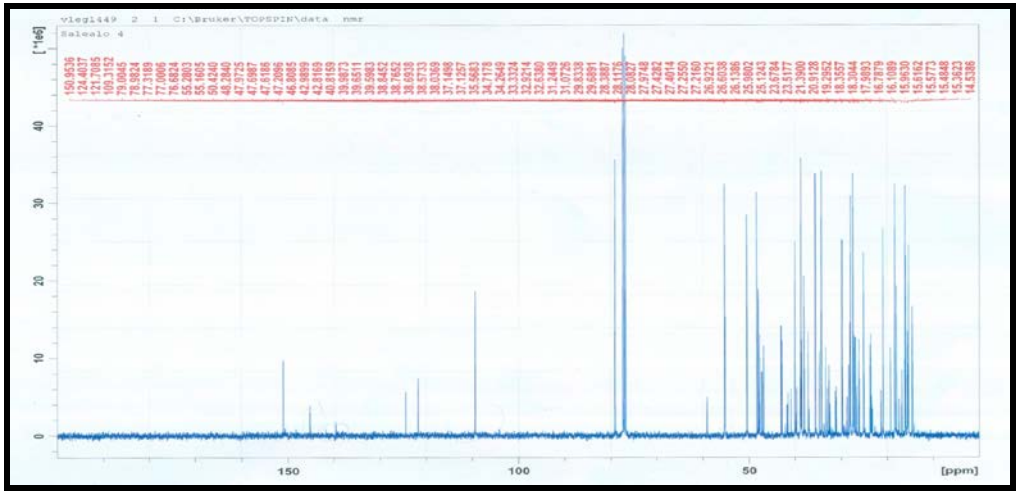


Figure 5.11: ^{13}C NMR spectrum of lupeol, alpha amyrin and beta amyrin isolated from the hexane fraction of *Loxostylis alata* leaves.

Table 5.1: NMR data of isolated lupeol

Peak number	Compound 1	Published (Burns et al., 2000)
1	37.72	38.72
2	27.42	27.43
3	79.05	79.02
4	38.86	38.87
5	55.28	55.31
6	18.32	18.33
7	34.28	34.29
8	40.84	40.84
9	50.43	50.35
10	37.17	37.18
11	20.93	20.94
12	25.15	25.16
13	38.05	38.07
14	42.83	42.85
15	27.94	27.46
16	35.59	35.60
17	43.00	43.01
18	48.29	48.32
19	47.98	48.00
20	150.97	150.98
21	29.69	29.86
22	40.00	40.02
23	28.03	28.00
24	15.40	15.38
25	16.08	16.13
26	16.08	15.99
27	15.32	14.56
28	17.96	18.02
29	109.31	109.32
30	20.92	19.32

Ethyl acetate compound

Figure 5.11 to 5.14 shows the NMR spectra of the compound isolated from ethyl acetate fraction. This compound was a mixture of phenolic compounds and some impurities because there were a lot of oxygenated aromatic carbons around 166.4 to 164.3 ppm. There were also other aromatic carbons observed between 147.0 to 115.0 ppm. Moreover there were carbon signals around 110 and 93 ppm as well as 77.5 to 62.86 ppm and that could be associated with further aromatic carbons and a sugar moiety.

The proton NMR had signals around 8.47 to 6.05. Protons from a sugar moiety could also be observed from 5.71 to 3.06 ppm. However signals due to impurities were observed on the spectra from 0.83 to 2.68 ppm. A conclusive structure could not be assigned to this compound as a result.

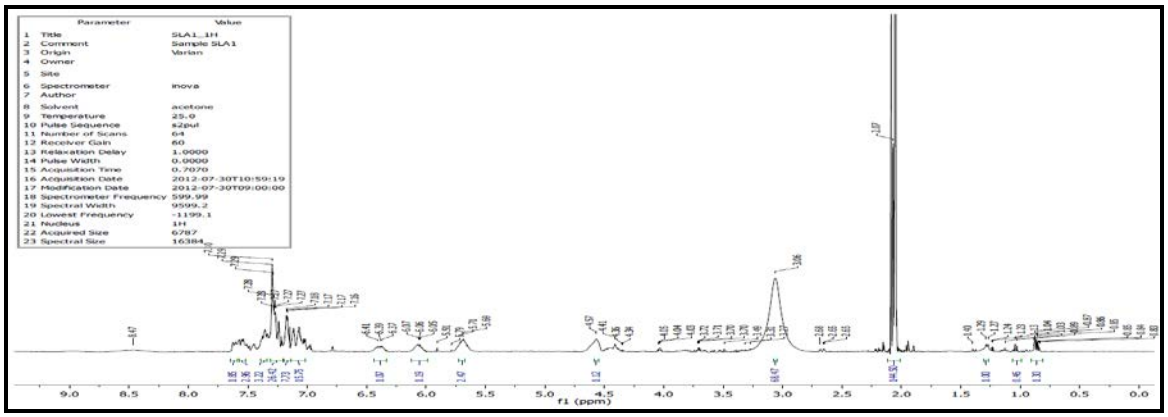


Figure 5.12: ^{1}H NMR spectrum of ethyl acetate compound isolated from the ethyl acetate fraction of *Loxostylis alata* leaves.

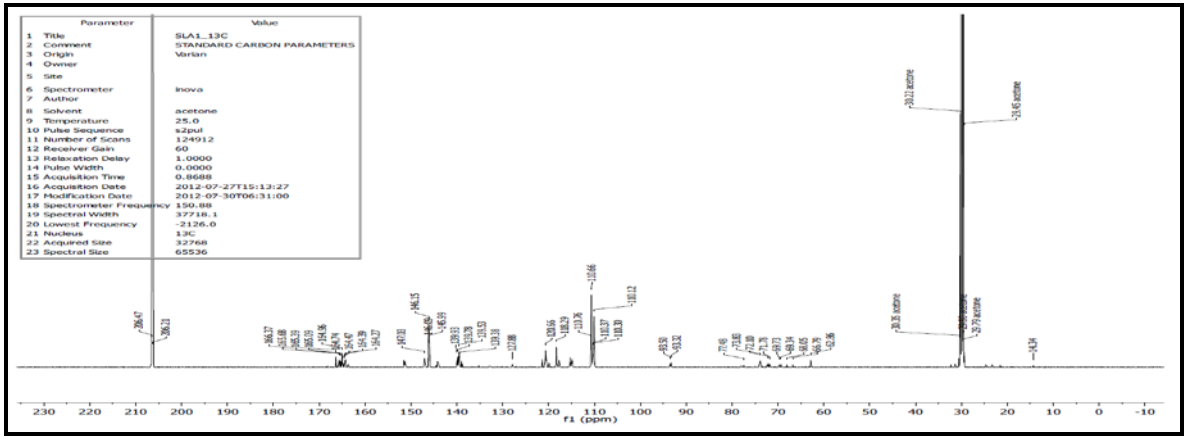


Figure 5.13: ^{13}C NMR spectrum of ethyl acetate compound isolated from the ethyl acetate fraction of *Loxostylis alata* leaves.

5.4 CONCLUSION

Two compounds were isolated from the hexane fraction of *L. alata* using column chromatography with silica gel as the stationary phase. The structures of the two compounds were elucidated as lupeol and lupeol with two extra hydroxyl groups. One compound was isolated from the ethyl acetate fraction but owing to the presence of impurities which could not be removed in the present study, it was not possible to elucidate the structure of this compound which is likely to be phenolic in nature. It is of importance to evaluate the biological activities of the isolated compounds to check if activity was lost during column chromatography or not. Cytotoxicity of the isolated compounds also needs to be evaluated.

CHAPTER 6

EVALUATION OF THE BIOLOGICAL ACTIVITIES OF THE ISOLATED COMPOUNDS AND CYTOTOXICITY OF THE CRUDE EXTRACT, FRACTIONS AND BIOACTIVE COMPOUNDS

6.1 INTRODUCTION

Evaluation of the biological activity of the isolated compounds is important to determine if the individual compound has greater or less activity and whether it is toxic or not when compared to the crude extract. This gives an idea of whether the compound has the potential to be used as a therapeutic agent or antimicrobial when compared to the crude extract because during the isolation process there might be chemical changes to the compounds due to heat or effect of organic solvents used in the isolation process. The active compounds may also be used as markers when assessing quality control of potentiated extracts for possible commercial use.

Cytotoxicity of the extracts and isolated compounds is also an important aspect to consider. The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is a commonly used measure of cell viability. *In vitro* assays of a cell population's response to external factors is based on measuring cell viability and multiplication of cells. Active cells reduce the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH and produce a purple formazan (Berridge and Tan, 1993). The purple formazan formed after the reaction is quantified using a spectrophotometer (Niks and Otto, 1990).

In the absence of cells or when there is cell death, the MTT reagent yields low absorbance values when measured on a spectrophotometer. From the absorbance values measured, the number of cells and the signal produced should have a linear relationship (Mosmann, 1983; Figure 6.4), and this allows accurate quantification of changes in the rate of cell proliferation. Advantages of the MTT assay are sensitivity and reproducibility, elimination of the requirements of radioactive compounds; it is easily performed and precisely quantifiable (Ferrari et al., 1990).

6.2 MATERIALS AND METHODS

6.2.1 MIC determination of active compounds

The microdilution assay for antifungal activity (Masoko et al., 2005) and antibacterial activity (Eloff, 1998) was done as described in Chapter 3 section 3.2.6.2

6.2.2 Cytotoxicity assay

Viable cell growth after incubation with test sample was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983). C3A human liver cells were used for cytotoxicity determination. The cells of a subconfluent culture were harvested and resuspended in growth medium to 5 $\times 10^4$ cells/ml. The growth medium used was Minimal Essential Medium (MEM) supplemented with 0.1% gentamicin and 5% foetal calf serum. A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Exactly 200 µl of MEM was added to wells of columns 1 and 12 to minimize the 'edge effect' and maintain humidity. The plates were incubated for 24 hours at 37°C in a 5% CO_2 incubator, until the cells were attached to the microtitre plates and in an exponential phase of growth. The MEM was aspirated from the cells and replaced with 200 µl of test compound at differing concentrations (serial dilution prepared in growth medium). The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in quadruplicate. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours with test compound. Untreated cells and positive control (berberine chloride, Sigma) were included.

After incubation, 30 µl MTT (stock solution of 5 mg/ml in Phosphate Buffered Saline, PBS) were added to each well and incubated for a further 4 hours at 37°C. The medium in each well was then carefully removed, without disturbing the MTT crystals in the wells, before washing with PBS and replacing with fresh MEM. Following the washing step to remove traces of plant extract which may non-specifically reduce the MTT, 50 µl of DMSO were added to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader at a wavelength of 570 nm (VersaMax microplate reader, Molecular Devices). The wells in column 1, containing no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

6.2.3. Selectivity index

Selectivity index gives the relative safety of each extract and it is the ratio of LC_{50} and MIC (minimum inhibitory concentration).

6.3 RESULTS AND DISCUSSION

6.3.1. Antifungal activity

The antimicrobial activity of isolated compounds was determined by evaluating their MIC values against three fungal pathogens, which are shown in Table 6.1. Isolated compounds with MIC values ranging from 64-100 µg/ml are regarded as potential therapeutic agents (Gibbons, 2004). Compounds with MIC values of 10 µg/ml and less are regarded as remarkable and require more attention (Rios and Recio, 2005). All microorganisms tested were sensitive to the isolated compounds. The ethyl acetate compound had the highest antifungal activity against *Candida albicans* with an MIC of 4 µg/ml and good activity against *Cryptococcus neoformans* with an MIC of 16 µg/ml. Increased inhibitory effects of the compound towards *C. albicans* and *C. neoformans* in comparison with the crude extract shows that there was a removal of inhibitory compounds from the crude extract during the fractionation process. The compound also had a good activity compared to the positive control amphotericin B against *C. albicans* and *C. neoformans. A. fumigatus* was the least sensitive to the isolated compounds and the positive control.

Lupeol and lupeol, alpha-amyrin and beta-amyrin mixture had good activity against *C. albicans* and *C. neoformans* with MIC of 63 µg/ml. However, the lupeol compounds had low antifungal activity compared to the crude extract, and this might be due to the synergistic effects of different compounds present in the crude extract. Lupeol has been reported to have antifungal activity against *Sporothrix schenckii, Microsporum canis, Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans* and *Candida guilliermondi* with MIC values ranging from 12- 250 µg/ml (Ajaiyeoba et al., 2003). Ajaiyeoba et al. (2003) further reported that the lupeol-rich extract of *Buchholzia coriacea* exhibits a dose-dependent antifungal activity where the antimicrobial effect of lupeol was shown to be more than well-known antibiotics such as ampicillin and tioconazole. In addition to antifungal activity, lupeol has been reported to have anti-inflammatory and anti-cancerous activities because of its ability to interact with multiple molecular targets affecting and modulating inflammatory and carcinogenic responses (Gallo and Sarachine, 2009). Lupeol, alpha-amyrin and beta-amyrin mixture had the same activity as lupeol against *C.albicans* and *C.neoformans*.This shows that there was no synergistic effect of the three compounds combined.

The compound isolated from the ethyl acetate fraction has noteworthy high activity and has potential to be used in the treatment of diseases if it has low toxicity.

Table 6.1: MIC values (µg/ml) of the isolated compounds and positive control amphotericin B against three tested fungal pathogens.

Organism		MIC values µg/ml			
	Crude (µg/ml)	Lupeol	L, α and β amyrin	EC comp	Amphotericin B
C. albicans	630	63	63	4	16
A. fumigatus	160	125	63	125	91
C. neoformans	20	63	63	16	16

L, α and β amyrin- lupeol, alpha-amyrin and beta-amyrin EC comp= ethyl acetate compound

6.3.2 Antibacterial activity

MIC values of the isolated compounds against four tested bacterial pathogens are shown in Table 6.2. Isolated compounds had lower inhibitory effects than the crude extract with MIC values ranging from 31 - 250 µg/ml. Low activities of isolated compounds might be due to the synergistic effects of different compounds present in the crude extract. *P. aeruginosa* was more sensitive to the isolated compounds compared to the other tested pathogens with MIC ranging from 31 - 63 µg/ml. The activity of lupeol against *P. aeruginosa* at a concentration of 30 µg / 100 µl was also reported by Ahamed et al. (2007). Armed et al. (2010) reported the antibacterial activity of lupeol against *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Sarcina lutea, Salmonella paratyphi, Salmonella typhi, Shigella boydi, Shigella dysenteriae, Vibrio mimicus, Vibrio parahemolyticus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Myotis flavus.*

Table 6.2: MIC values in µg/ml of the isolated compounds and positive control gentamicin against four tested bacterial pathogens.

Organism		MIC values µg/ml			
	Crude (µg/ml)	Lupeol	L, α and β amyrin	EC comp	Gentamicin
E. faecalis	310	250	250	125	25
S. aureus	310	125	125	125	15
E. coli	310	125	125	125	6.3
P. aeruginosa	160	63	31	63	0.3

L, α and β amyrin- lupeol, alpha-amyrin and beta-amyrin , EC comp= ethyl acetate compound

6.3.3 Cytotoxicity assay

Cytotoxicity of the crude extract, ethyl acetate fraction and hexane fraction was tested on C3A human liver cells. Figure 6.1 shows cell viability when exposed to different concentrations of the test samples. The percentage cell viability increased with a decrease in the concentration of the test sample. A high concentration (1 mg/ml) of the hexane fraction indicated a low percentage cell viability which implies that the hexane fraction at 1 mg/ml is toxic to the C3A cells. Toxicity is indicated by the concentration that kills 50% of cells (LC_{50}). The lower the LC_{50} value the higher the toxicity of the tested extract or compound. The hexane fraction had an LC_{50} of 31 µg/ml. The ethyl acetate fraction had an LC_{50} of 68.9 µg/ml whereas the crude extract had an LC_{50} of 78.1 µg/ml. Thus the crude extract was less toxic than the hexane and ethyl acetate fractions.

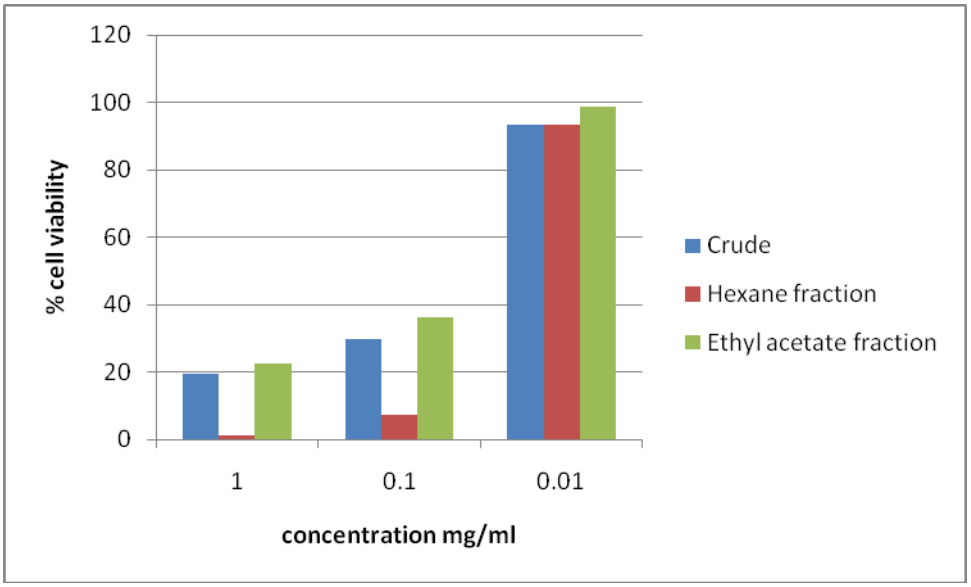


Figure 6.1: Percentage cell viability after being exposed to various concentrations of crude extract, hexane fraction and ethyl acetate fraction of *Loxostylis alata*.

Figure 6.2 shows cell viability when the cells were exposed to different concentrations of the isolated compounds. Lupeol (compound 2) showed a low percentage cell viability at the highest concentration tested of 200 µg/ml and it had an LC_{50} of 49.06 µg/ml. Lupeol was less toxic than the hexane fraction and this may be due to the toxic compounds that were present in the hexane fraction which were removed during the isolation process. Geetha and Varalaxmi (1998) reported that the toxicity of lupeol is very low because when administered orally in a dose of 2 g/kg it produced no adverse effects in rats and mice, and after 96 hours of observation no mortality was recorded. Toxicity of lupeol, alpha-amyrin and beta-amyrin mixture was not tested because of low quantity of the compound isolated. The ethyl acetate compound (compound 1) was less toxic to the cells with an LC_{50} of 111.69 µg/ml. The ethyl acetate compound was also less toxic than the ethyl acetate fraction from which it was isolated. The isolated compounds were less toxic than the doxorubicin positive control which had an LC_{50} of 3.20 µM.

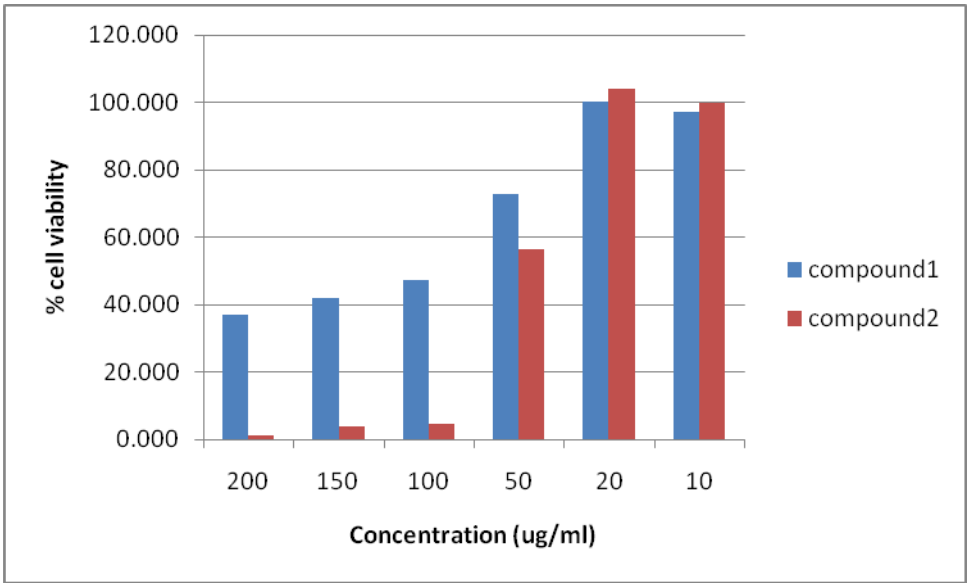


Figure 6.2: Percentage cell viability after being exposed to various concentrations of compound 1 from the ethyl acetate fraction and compound 2 (lupeol) from the hexane fraction of the acetone extract of *Loxostylis alata*.

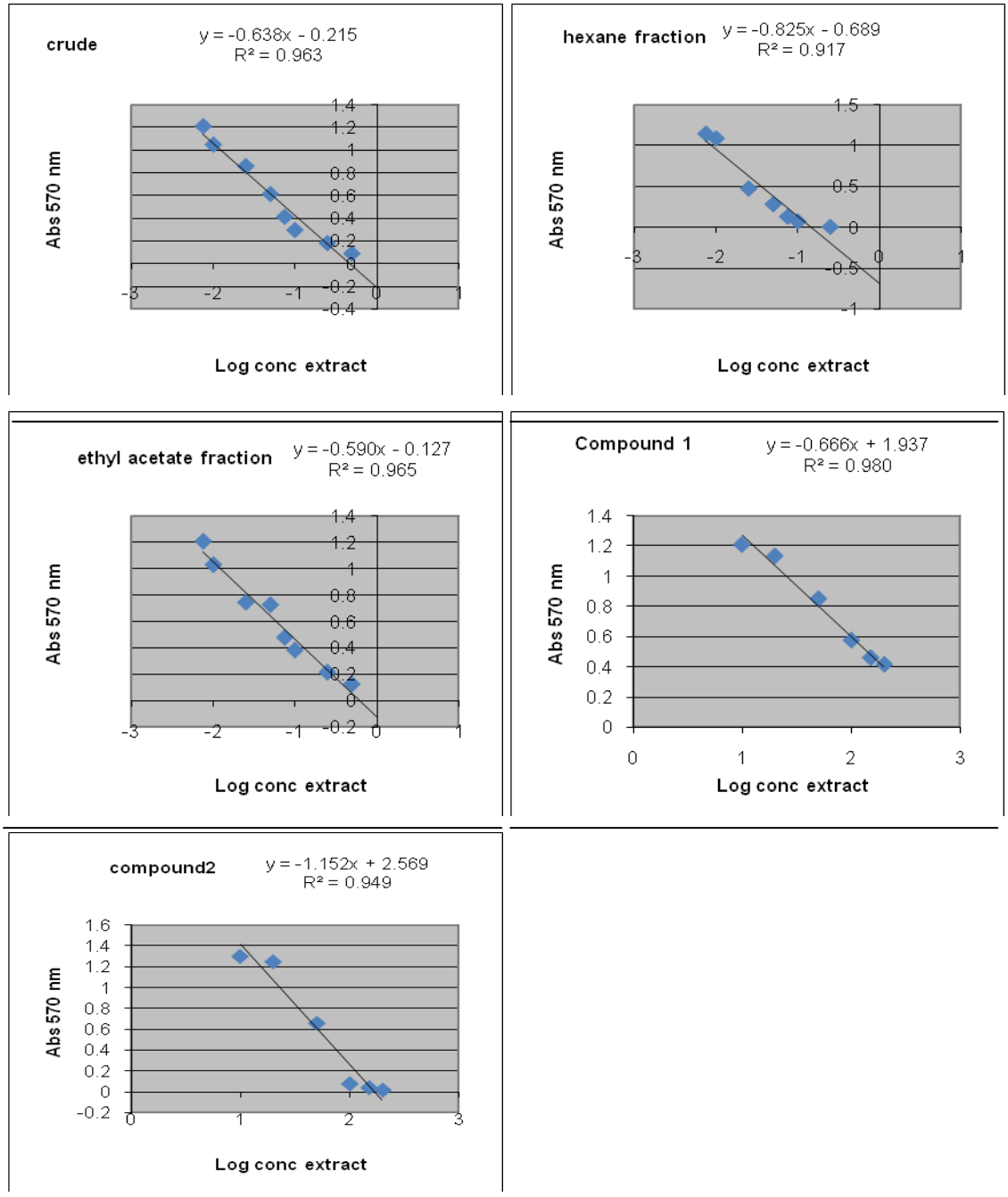


Figure 6.3: Cytotoxicity of the crude extract (LC_{50} =78.7 µg/ml), hexane fraction (LC_{50} 31.1 µg/ml), ethyl acetate fraction (LC_{50} = 68.9 µg/ml), compound 1 from ethyl acetate fraction (LC_{50}= 111.69 µg/ml), compound 2 (lupeol) (LC_{50}= 49.1µg/ml) against C3A cells.

6.4. Selectivity index

Selectivity index (SI) values give an idea of whether the tested extract may be safe or not. The high the selectivity index the less cytotoxic the plant extract in this case as cytotoxicity values were used to calculate the SI values.

Table 6.3: MIC (µg/ml), LC_{50} µg/ml) and Selectivity Index values of the crude extract, fractions and isolated compounds against tested fungal pathogens

	C. albicans			*A.fumigatus*			*C. neoformans*		
	MIC(µg/ml)	LC50(µg/ml)	SI	MIC(µg/ml)	LC50(µg/ml)	SI	MIC(µg/ml)	LC50(µg/ml)	SI
Crude	630	63.52	0.1	160	63.52	0.39	20	63.52	3.18
Hex	160	32.87	0.21	20	32.87	1.64	160	32.87	0.21
EtAC	630	57.06	0.09	160	57.06	0.36	<0.02	57.06	2.85
Lupeol	63	48.85	0.78	125	48.85	0.39	63	48.85	0.78
EC	4	110.28	27.57	125	110.28	0.88	16	110.28	6.89

Table 6.4: MIC (µg/ml), LC_{50} µg/ml) and Selectivity Index values of the fractions against tested bacterial pathogens

	E.faecalis			*S.aureus*			*E.coli*			*P.aeruginosa*		
	MIC(µg/ml)	LC50(µg/ml)	S.index	MIC(µg/ml)	LC50(µg/ml)	S.index	MIC(µg/ml)	LC50(µg/ml)	S.index	MIC(µg/ml)	LC50(µg/ml)	S.index
Crude	310	63.52	0.2	310	63.52	0.2	310	63.52	0.2	160	63.52	0.397
Hex	630	32.87	0.05	310	32.87	0.11	630	32.87	0.05	630	32.87	0.05
EtAC	310	57.06	0.18	310	57.06	0.18	630	57.06	0.09	160	57.06	35.66
Lupeol	250	48.85	0.195	125	48.85	0.39	125	48.85	0.39	63	48.85	0.78
EC	125	110.28	0.88	125	110.28	0.88	125	110.28	0.88	63	110.28	1.75

Hex-hexane, EtAC- ethyl acetate, EC-ethyl acetate compound, S.index- selectivity index

6.4 CONCLUSION

The isolated compounds had good antifungal activity and low toxicity in *vitro* against C3A cells compared to their crude fractions. Compound 1 (compound isolated from the ethyl acetate fraction) had the lowest toxicity with LC_{50} of 111.69 µg/ml. The same compound had the highest antifungal activity and the lowest MIC value of 0.004 mg/ml. This active compound was not isolated before from the species and it is has the potential to be used as an antimicrobial because of its good inhibitory activity and relatively low toxicity. Further work needs to be done to purify this compound and conclusively identify its structure.

CHAPTER 7

GENERAL DISCUSSION

Alleviating microbial infections is a serious concern in South Africa and the world at large because of new cases of microbial pathogenicity and the emergence of microbial drug resistant strains with decreased susceptibility to conventional antimicrobial drugs (Moore and Chaisson, 1996). This provides motivation for the continued search for new, safer and more efficient anti-infectious agents (Kunin, 1993). The presence of antibacterial, antifungal and other biological activities has been demonstrated in extracts of different plant species used in traditional medicine practices (Martini and Eloff, 1998; McGaw et al., 2000, 2001; Masoko et al., 2005; Masoko and Eloff, 2005; Masoko et al., 2007; Shai et al., 2008). Traditionally used medicinal plants produce a variety of compounds which can either inhibit the growth of pathogens or kill them and may have little toxicity to host cells but research is needed to confirm this for each species under investigation (Cowman, 1999).

Loxostylis alata from the family Anacardiaceae is a traditional medicinal plant which was used by indigenous people in Kwa Zulu-Natal as a pain reliever during childbirth and as an immune modulator (Pooley, 1993; Pell, 2004). Suleiman (2010) reported that at a dose of 200 mg/kg the crude acetone extract of *L. alata* had the same inhibitory ability against *Aspergillus fumigatus* as the ketoconazole positive control *in vivo* in broiler chicks. This shows that *L. alata* crude acetone extract has the potential to be used as an antifungal agent. However at a higher dose of 300mg/kg the extract was found to be toxic to the chicks. Hence there is rationale to develop a safe and effective antimicrobial against infectious diseases from this plant species. Thus the aim of this research was to potentiate the biological activity of the crude acetone leaf extract of *Loxostylis alata*, to reduce the toxicity potential and to isolate the bioactive compounds from the extract.

Extracts of the leaves of *Loxostylis alata* were tested for antibacterial and antifungal activity to evaluate if there was an increase or gain in the antimicrobial activity of the plant material. The fungal pathogens used were *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*. The bacterial species included *Enterococcus faecalis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. Solvents of different polarities were used to extract the leaves for screening purposes, namely hexane, dichloromethane, acetone and methanol. The methanol extract had the highest mass compared to the extracts resulting from the other solvents. TLC analyses were conducted using three mobile systems, BEA, CEF and EMW, to separate different compounds in the extracts. BEA separated more compounds compared to CEF and EMW in all the

extracts. This shows that most compounds in the prepared plant extracts are non-polar because BEA is non-polar in nature and it is expected that a polar eluent will elute better more polar compounds.

The three mobile systems were further used for bioautography, where activity of compounds separated on a TLC plate is indicated by clear zones on a reddish background after spraying with the bacterial or fungal culture and the indicator iodonitrotetrazolium chloride (INT). The acetone and methanol extracts showed zones of inhibition against the three fungal species. However, the hexane and DCM extracts did not have any visible active compounds.

The antimicrobial activity of *L. alata* extracts was further determined by evaluating the lowest MIC value at which the growth of the test bacteria or fungi was inhibited. The acetone extract showed a high antifungal activity with the lowest minimum inhibitory concentration of 0.02 mg/ml. The hexane extract had the lowest antifungal activity with minimum inhibitory concentrations of 2.5 mg/ml. The acetone extract was then selected for potentiation (solvent-solvent fractionation) because of its good antifungal activity compared to that of the other extracts.

Bulk extraction of *L. alata* leaf material (200 g) was done using acetone (2 L). The dried acetone extract was then used for solvent-solvent fractionation. The dried material was washed three times with hexane, DCM, chloroform, ethyl acetate, acetone and methanol. The five resulting fractions (hexane, DCM, ethyl acetate, acetone and methanol fraction) were tested for antifungal and antibacterial activity to evaluate if the activity increased or decreased during the solvent-solvent fractionation process. The extracts were also tested for antioxidant activity to determine whether antioxidant activity could be correlated with antibacterial or antifungal activity. The crude extract, ethyl acetate, acetone and methanol fractions had compounds with antioxidant activity but the hexane and DCM fractions did not have compounds with antioxidant activity. In bioautography, the hexane and DCM fractions had compounds with antifungal activity in the BEA eluent system but these active compounds were not observed before in the extracts. This indicates that their activity was increased during the potentiation process, or the concentration may have increased by potentiation to allow visualisation using the bioautography method. This is supported by Houghton et al. (2007) who reported that fractionation leading to isolation of individual compounds should result in active fractions having a higher activity than the original crude extract. The EMW eluent separated another compound with activity in the crude extract, ethyl acetate and acetone fractions against the three fungal organisms tested but BEA and CEF did not move the active compound because inhibition was only observed at the bottom of the plate. This means that the active compound is more polar in nature because it was moved by EMW only, which is a polar solvent system. The

same group of antifungal compounds had the same Rf value as the compounds that showed antibacterial activity against the tested bacterial species.

The potentiated activity of the fractions was further determined by the MIC assay. The lowest MIC value of 0.02 mg/ml was observed in the hexane fraction, and 0.04 mg/ml in the DCM fraction against *A. fumigatus.* The acetone and ethyl acetate fractions also showed antifungal activity with the lowest MIC value of <0.02 mg/ml against *Cryptococcus neoformans.* This activity was not observed in the hexane and DCM extracts, indicating that the activity of the extracts was potentiated. Thus the therapeutic activity of plant preparations can be manipulated in several ways to increase the efficacy (via potentiation) and also potentially to reduce the toxicity.

Based on the MIC values, presence of bioactive compounds in bioautography and a high total activity, the hexane and ethyl acetate fractions were subjected to isolation of bioactive compounds using column chromatography and silica gel as a stationary phase. Two compounds with antimicrobial activity were isolated from the hexane fraction. One compound was isolated from the ethyl acetate fraction.

Structures of the isolated compounds were elucidated using NMR and MS techniques. Compound 1 from the hexane fraction was identified as lupeol while compound 2 was lupeol, alpha-amyrin and beta-amyrin mixture . Lupeol falls into the group of triterpene secondary metabolites. Lupeol has been investigated previously for biological activities and reported to have therapeutic effects against inflammation, arthritis, diabetes, cardiovascular ailments, renal disorders, hepatic toxicity, microbial infections and cancer (Chaturvedi et al., 2008).

In this study lupeol had good antifungal activity against *C. albicans, C. neoformans* and *Aspergillus fumigatus* with MIC values ranging from 63 - 125 µg/ml. Ajaiyeoba et al. (2008) reported that lupeol has antifungal activity against *Sporothrix schenckii, Microsporum canis, Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans* and *Candida guilliermondi* with MIC values ranging from 12 - 250 µg/ml. Lupeol had good antibacterial activity against *P. aeruginosa* with MIC values of 31 and 63 µg/ml respectively. Thus lupeol has a broad spectrum of antimicrobial activity. The compound isolated from the ethyl acetate fraction had good antifungal activity with the lowest MIC value of 4 µg/ml. The compound was more active than the crude extract against *C. albicans.* The increased activity of the compound implies that the crude extract may contain inhibitory compounds which were removed during fractionation, or merely that the concentration of the isolated compound is low in the crude extract. The inhibitory compounds in the ethyl acetate fraction may interfere with the absorption, assimilation and subsequent metabolism of the active compounds by the

organism. The compound was more active than amphotericin B against *C. albicans*. Furthermore it had the same inhibitory activity as amphotericin B against *C. neoformans.* Amphotericin B is the positive control that was used in the study. It falls into the group of polyene antibiotics which are used for the treatment of fungal diseases (Frattarelli et al., 2004). The high inhibitory action of the compound against *C. albicans* and *C. neoformans* shows that the compound has the potential to be used as an antifungal if it is found to be non-toxic *in vivo*.

Cytotoxicity of the active fractions and compounds was evaluated because the plant extract was found to be toxic in chickens in the previous study (Suleiman, 2010). Lupeol was less toxic than the hexane fraction from which it was isolated. This shows that some of the toxic compounds were removed during column chromatography. In addition lupeol has been reported as being non-toxic as it does not cause systemic toxicity in animals at doses ranging from 30 to 2000 mg/kg (Murtaza et al., 2009). The compound isolated from the ethyl acetate fraction had low toxicity in *vitro* against C3A cells compared to the crude extract and ethyl acetate fraction with an LC_{50} of 111.69 µg/ml. The same compound had the highest activity with the lowest MIC value of 4 µg/ml and a very good selectivity index of 27.57. Not only were the inhibitory and inactive compounds in the crude acetone extract of *L. alata* removed to enhance the activity of the compound isolated from the ethyl acetate fraction, but toxic compounds were also removed during the solvent- solvent fractionation and isolation process. Hence the aim of the study was fulfilled because we were able to potentiate the activity of the acetone extract by developing the ethyl acetate fraction and isolating the active compound from the fraction. The compound isolated from the ethyl acetate fraction had a good antimicrobial activity with the lowest MIC of 4 µg/ml. The same compound was also found to be relatively non-toxic *in vitro*. Hence this compound has the potential to be used as a pharmaceutical agent due to its activity and low toxicity. This investigation proves that medicinal plants such as *Loxostylis alata* are important sources in the investigation of potential antimicrobials since they contain compounds which have therapeutic value in combating infectious diseases. Further studies are recommended to test the purified compound from the ethyl acetate fraction, as well as the ethyl acetate fraction in animal studies to verify the results *in vivo*.

REFERENCES

Aarestrup F.M. 2000. Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark: Chapter 1: Introduction. Acta Pathologica, Microbiologica et Immunologica Scandinavica. 108 :5-6.

Ahamed B.K.M., Krishna, V., Gowdru, H.B., Rajanaika, H., Kumaraswamy, H.M., Rajshekarappa, S., Dandin, C.J., Mahadevan, K.M. 2007. Isolation of bactericidal constituents from the stem bark extract of Grewia tiliafolia Vahl. Research Journal of Medicinal Plants. 1:72-82.

Ahmed Y., Sohrab, M.H., Al-Reza, S.M., Tareq, F.S., Hasan, C.M., Sattar, M.A. 2010. Antimicrobial and cytotoxic constituents from leaves of Sapium baccatum. Food and Chemical Toxicology. 48:549–52.

Ajaiyeoba E.O., OnochaP.A., Nwozo, S.O., Sama, W. 2003. Antimicrobial and cytotoxicity evaluation of Buchholzia coriacea stem bark. Fitoterapia. 74:706–709.

Algar V., Novelli, V. 2007. Infections in Immune compromised host. Paediatrics and Child health. 17:132-136.

Amusan O.O.G., Sukati N.A., Dlamini P.S., Sibandze, F.G. 2007. Some Swazi phytomedicines and their constituents. African Journal of Biotechnology. 6:267-272.

Angeh J.E., Huang X., Sattler I., Swan G.E., Dahse H., Härtl A., Eloff J.N. 2007.Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from Combretum imberbe (Combretaceae). Journal of Ethnopharmacology. 110:56-60.

Ascioglu S., Rex J.H., De Pauw B., Bennett J.E., Bille J., Crokaert F., Denning D.W., Donnelly J.P., Edwards J.E., Erjavec Z., Fiere D., Lortholary O., Maertens J., Meis J.F., Patterson T.F., Ritter J., Selleslag D., Shah P.M., Stevens D.A., Walsh T.,J. 2002. Defining Opportunistic Invasive Fungal Infections in Immunocompromised Patients with Cancer and Hematopoietic Stem Cell Transplants: An International Consensus. Clinical Infectious Diseases. 34:7–14.

Azaizeh H., Fulder S., Khalil K., Said O. 2003. Ethnomedicinal knowledge of local Arab practitioners in the Middle East Region. Fitoterapia. 74:98-108.

Balandrin M.J., Klocke J.A. 1988. Medicinal, aromatic and industrial materials from plants. Biotechnology in agriculture and forestry. Medicinal and aromatic plant. 4:1–36.

Baron S. 1996. Medical Microbiology, 4th edition. University of Texas medical branch at Galveston.

Begue M.J., Kline R.M. 1972. The use of tetrazolium salts in bioautographic procedures. Journal of Chromatography. 64:182-184.

Berridge M.V., Tan, A.S. 1993. Characterisation of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT): subcellular localization, substrate dependence and involvement of mitochondrial electron transport in MTT reduction. Archives of Biochemistry and Biophysics. 303:47.

Brand-Williams W., Cuvelier M.E., Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensm.-Wiss. u. -Technol. 28:25–30.

Burns D., Reynolds W.F., Buchanan G., Reese P., Enriquez R. 2000. Assignment of 1H and 13C NMR spectra and investigation of hindered Side-chain rotation in lupeol derivatives. Magnetic Resonance in Chemistry.38:488–493.

Calderone R.A., Fonzi W.A. 2001. Virulent factors of *Candida albicans*. Trends in Microbiology. 9:327-335.

Celiz M.D., Tso J., Aga D.S. 2009. Pharmaceutical Metabolites in the Environment: Analytical Challenges and Ecological Risks. Environmental Toxicology and Chemistry. 2 :2473–2484.

Chambers H.F. 2005. Community-Associated MRSA — Resistance and Virulence Converge. Journal of medicine. 352:1485-1487.

Chaturvedi P.K., Bhui K., Shukla Y. 2008. Lupeol. Connotations for chemoprevention. Cancer Letters. 263:1-13.

Chikoto H., Eloff J.N. 2003. Extraction of grape seed to produce proanthocyanidin rich extract. International Society of Ethnopharmacology. South African Journal of Botany. 69:227.

Ciocan I.D., Bara I. 2007. Plant Products as Antimicrobial Agents. Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza", Secţiunea Genetică şi Biologie Moleculară, TOM VIII.

Clarke S.C. 2001. Diarroeagenic Escherichia coli- an emerging problem. Diagnostic Microbiology and Infectious Diseases. 41:93-8.

Clemons K.V., Calich V.L.G., Burger E., Filler S.G., Graziutti M., Murphy J., Roilides E., Campa A., Dias M.R., Edwards J.E., Fu Y., Fernandes-Bordignon G., Ibrahim A., Katsifa H., Lamaignere C.G., Meloni-Bruneri L.H., Rex J., Savary C.A., Xidieh C. 2000. Pathogenesis I: interactions of host cells and fungi. Medical Mycology. 38:99–111.

Cogliani C., Goossens H., Greko C. 2011. Restricting Antimicrobial use in food Animals: Lessons from Europe. Microbe. 6:274–279.

Cohen J. D. W., Denning M. A. 1993. Epidemiology of invasive aspergillosis in european cancer centers. European Journal Clinical Microbiology Infectious Diseases. 12:392–393.

Coleman J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation. Clinical and Experimental Immonology. 129:4-10.

Coutinho I.D., Kataoka V.M.G., Honda N.K., Coelho R.G., Vieira M.C., CAL C. 2010. Influência da variação sazonal nos teores de flavonóides e atividade antioxidante das folhas de Campomanesia adamantium (Cambess.) O. Berg, Myrtaceae. Reviews in Brazilian Farmacognosy 20:322-327.

Cowan M.M. 1999. Plant products as antimicrobial agents. ClinicalMicrobiology Reviews. 12:561-582.

Cox P.A., Balick, M.J. 1994. The ethanobotanical approach to drug discovery. Scientific American. 270:82–7.

Deby C., Margotteaux G. 1970. Relationship between essential fatty acids and tissue antioxidant levels in mice. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales. 165:2675-2681.

De Wet H., Van Wyk B.E. 2008. An ethnobotanical survey of southern African Menispermaceae . South African Journal of Botany. 74:2–9.

Dismukes W.E. 1988. Cryptococcal meningitis in patients with AIDS. Journal of Infectious Diseases. 157: 624-8.

Donnenberg M.S., Kaper J.B. 1992. Enteropathogenic Escherichia coli. Infect. Immunology. 60:3953.

Droge W. 2002. Free radicals in the physiological control of cell function. Physiological Reviews. 82:47–95.

Eloff J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica. 64:711–713.

Eloff J.N. 1998b. A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. Plant Medica. 60:1-8.

Eloff J.N. 2004. Quantifying the bioactivity of plants extracts during screening and bioassay-guided fractionation. Phytomedicine 11:370.

Farnsworth N.R. 1984. Traditional medicine. In: World health forum. International Journal of Health Development. 5:373.

Famsworth N. R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z.G. 1985. Medicinal plants in therapy. Bull. WHO. 63:965-981.

Ferguson N.M., Donnelly C.A., Anderson R.M. 2001 the foot and mouth epidemic in Great remove Britain: pattern of spread and impact of intervensions. science. 292:1155-1160.

Ferrari M., Fornasiero M.C., Isetta A. M .1990. Mtt colorimeric assay for testing macrophage cytotoxic activity invitro. Journal of immunological Methods. 131:165-172.

Ferrer J. 2000. Vaginal candidosis: Epidemiological and etiological factors. International Journal of Gynecological pathology. 71:7-21.

Finberg R.W., Moellering, R.C., Tally, F.P., Graig, W.A., Pankey, G.A.,Dellinger, E.P., West, M.A., Joshi, M., Linden, P.K., Rolston, K.V., Rotschafer, J.C., Rybak, M.J. 2004. The Importance of Bactericidal Drugs:Future Directions in Infectious Disease. Clinical Infectious Diseases. 39: 1314–1320.

Ford M.M. 2004. Antifungals and their use in veterinary Ophthalmology.2004. Veterinary Clinics of Small Animal. 34:669–691.

Frattarelli D.A.C., Reed M.D., Giacoia G.P. 2004. Antifungals in systemic neonatal candidiasis. Drugs. 64:949–68.

Fromtling R. 1988. Overview of medically important antifungal azole derivatives. Clinical Microbiology Reviews. 1:187–217.

Gale G. A., Kirtikara K., Pittayakhajonwut P., Sivichai S., Thebtaranonth Y., Thongpanchang C. Vichai V. 2007. In search of cyclooxygenase inhibitors, anti-Mycobacterium tuberculosis and anti-malarial drugs from Thai flora and microbes, Pharmacology & Therapeutics. 115:307-351.

Gallo M.B.C., Sarachine M.J. 2009. Biological activities of lupeol. International Journal of Biomedical and Pharmaceutical Sciences. 3:46-66.

Gandhi N.R., Moll A., Sturm A.W., Pawinski R., Govender T., Lalloo U., Zeller K., Andrews J., Friedland G. 2006. Extensively drug resistant tuberculosis as a cause of death in patients co-infectted with tuberculosis and Hiv in a rural area South Africa. The lancet. 368:9547.

Gantois I., Ducatelle R., Pasmans F., Haesebrouck F., Van Immerseel F., 2008. Salmonella enterica serovar Enteritidis genes induced during oviduct colonization and egg contamination in laying hens. Applied and Environmental Microbiology. 74:6616–6622.

Gantois I., Ducatelle R., Pasmans F., Haesebrouck F., Gast R., Humphrey T.J., Van Immerseel f. 2009. Mechanisms of egg contamination by Salmonella Enteritidis. FEMS Microbiology Reviews. 33:718–738.

Garbino J., Kolarova L., Lew D., Hirschel B., Rohner, P. 2001. Fungemia in HIV-Infected patients: A 12-Year study in a tertiary are hospital. AIDS patient care and STDs. 15:407-410.

Garbino J., Lew D.P., Romand J.A., Hugonnet S., Auckenthaler R., Pittet D. 2001.Prevention of severe Candida infections in non neutropenic, high-risk, critically ill patients: a randomized, double-blind, placebo-controlled trial in patients treated by selective digestive decontamination. 28:1708-1717.

Geetha T., Varalaxmi, P. 1998. Anti-inflammatory activity of lupeol and lupeol linoleate in adjuvant-induced arthritis. Fitoterapia. 69:13–19.

Georg M., Lauer M.D., Bruce D., Walker M.D. 2001. Hepatitis C virus infection. New England Journal of Medicine. 345:41-52.

Gibbons S. 2004. Anti-staphylococcal plant natural products. Natural Products Report . 21:263-277.

Gubler D.J. 1998. Resurgent Vector-Borne Diseases as a Global Health Problem. Emerging Infectious Diseases .3:442-450.

Gulcin I., Oktay M., Kıreçci E., Küfrevio L. 2003. Screening of antioxidant and antimicrobial activities of anise (Pimpella anisum L.) seed extracts. Food Chemistry. 83:371-382.

Gurib-Fakim A. 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular aspects of Medicine. 27:1-93.

Hall L.M., Duke B., Urwin G., Guiney M. 1992. Epidemiology of Enterococcus faecalis urinary tract infection in a teaching hospital in London, United Kingdom. Journal of Clinical Microbiology. 30:1953–1957.

Hamburg M.A. 2008. Considerations for infectious disease research and practice. Technology in Society. 30:383– 387.

Handa S.S., Singh S.P., Longo, K.G., Rakesh D.D. 2008. Extraction Technologies for Medicinal and Aromatic Plants. International Centre for Science and High Technology. Trieste. Italy. 22-25

Harbilas D., Martineau L. C., Harris C. S., Adeyiwola-Spoor D. C. A., Saleem A., Lambert J., Caves D., Johns T., Prentki M., Cuerrier A., Arnason J. T., Bennett S. A. L., Haddad P. S., 2009. Evaluation of the antidiabetic potential of selected medicinal plant extracts from the Canadian boreal forest used to treat symptoms of diabetes: Part II. Canadian Journal of Physiology and Pharmacology. 87:479-492.

Harborne J.B. 1978. Phytochemical methods, 3rd edition. Chapman and Hall, London. 60:135- 203.

Hassan S.W., Umar R.A., Yakubu A.S., Dabai Y.U., Abbas A.Y. 2007. Wound -healing, Antibacterial and chemical properties of stem bark extracts of *Kingelia- africana* (Bignoniaceae). Nigeria Journal of Biochemistry and Molecular Biolology. 22:5-12.

Hawkey P.M. 1998. The origins and molecular basis of antibiotic resistance. British Medical Journal. 317:657-660.

Hensel M. 2000.Salmonella Pathogenicity Island 2. Molecular Microbiology. 36:1015-1023.

Houghton P., Fang R., Techatanawat I., Steventon G., Hylands P.J., Lee C.C. 2007. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. Methods. 42:377-387.

Houghton P.J., Raman A. 1998. Analysis of Crude Extracts, fractions and isolated compounds: Laboratory Handbook for the fractionation of Natural extracts. 1st edition., Thomson Publishing, USA.

Jones G. A., McAllister T.A., Muir A.D., Cheng K.J. 1994. Effects of sainfoin (Onobrychis vicifolia Scop) Condesed Tannins on Growth and Proteolysis by four Strains of ruminal bacteria. Applied and Environmental Microbiology. 60:1374–1375.

Karanis P., Kourenti C., Smith H. 2007. Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt. Journal of Water and Health. 5:1-38.

Kasper L.H., Buzoni-Gatel D. 2001. Ups and downs of Mucosal Cellular Immunity against protozoan parasites. Infection and Immunity. 69:1–8.

Kauffman C.A. 1999. Sporotrichosis. Clinical Infectious Diseases. 29:231-6.

Kazmi M. H., Malik A., Hameed S., Akhtar N., Noor A. S. 1994. An anthraquinone derivative from Cassia italic. Phytochemistry .36:761–763.

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

Knusten A.P. 2003. Immunopathogenesis of allergic bronchopulmonary aspergillosis. Frontiers in Bioscience. 8:589-602.

Kunin C.M. 1993. Resistance to antimicrobial drugs a world-wide calamity, Annals of internal medicine. 118:557-561.

Latge J.P., Calderone, R. 2002. Host–microbe interactions: fungi Invasive human fungal opportunistic infections. Current Opinion in Microbiology. 5:355–358.

Latge J.P., Calderone, R. 2002. Host–microbe interactions: fungi Invasive human fungal opportunistic infections. Current Opinion in Microbiology. 5:355–358.

Le Guyader F.S., Le Saux J.C., Ambert-Balay K., Krol J., Serais O., Parnaudeau S., Giraudon H., Delmas G., Pommepuy M., Pothier P., Atmar R.L. 2008. Achi Virus, Norovirus, Astrovirus, Enterovirus, and Rotavirus Involved in Clinical cases from a French Oyster-related gastroenteritis outbreak. Journal of Clinical Microbiology. 46:4011.

Levitz S.M. 1991. The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. Reviews of Infectious Diseases. 13:1163-1169.

Lin X., Heitman, J. 2006. The biology of *Cryptococcus neoformans* species. Annual review of Microbiology. 60:69-105.

Mandal P., Misra T.K., Ghosal M. 2009. Free- radical scavenging activity and phytochemical analysis in the leaf and stem of *Drymaria diandra* Blume. International Journal of Intergrity Biology, 7:80-84.

Mangili A., Gendreau M.A. 2005. Transmission of infectious diseases during commercial air travel. Lancet . 365:989–996.

Martini N.D., Eloff J.N. 1998. The preliminary isolation of several antibacterial compounds from *Combretum erythrophyllum* [Combretaceae]. Journal of Ethnopharmacology . 62:255–263.

Masoko P., Eloff J.N. 2005.The diversity of antifungal compounds of six South African Terminalia species (Combretaceae) determined by bioautography. African Journal of Biotechnology. 4:1425-1431.

Masoko P., Picard J., Eloff J.N., 2005. Antifungal activities of six South African Terminalia species (Combretaceae). Journal of Ethnopharmacology. 99:301–308.

Masoko P., Picard J., Eloff J.N. 2007. The antifungal activity of twenty-four Southern African Combretum species (Combretaceae). South African Journal of Botany. 73:173-183.

McGaw L.J., Jager A.K., Van Staden J.V. 2000. Antibacterial, anthelminticand anti-amoebic activity of South African medicinal plants. Journal of Ethnopharmacology. 72:247-263.

McGaw L.J., Rabe T., Sparg S.J., Jager A.K., Eloff J.N., van Staden J. 2001. An investigation on the biological activity of Combretum species. Journal of Ethanopharmacology. 75:45-50.

Morse S.S. 1995. Factors in the Emergence of Infectious Diseases. Emerging Infectious Diseases. 1:1.

Moore R.D., Chaisson, R.E. 1996. Natural history of opportunistic diseases in an HIV-infected urban clinical cohort, Annals of Internal Medicine. 124:633–642.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferarion and cytotoxicity assays. Journal of Immunology ethods. 65:55-63.

Murphy K.M., Travers P., Walport M., 2008. Janeway's Immunobiology, Seventh edition.

Murtaza I., Saleem M., Adhami V.M., Hafeez B.B., Mukhtar H. 2009. Suppression of cFLIP by lupeol, a dietary triterpene, is sufficient to overcome resistance to TRAIL-mediated apoptosis in chemoresistant human pancreatic cancer cells. Cancer Research.69:1156–1165.

Myers R.S. 2006. VI.antifungal agents. In immunizing and Antimicrobial agents page. 4-5.

Neu H.C. 1992. The crisis in antibiotic resistant. Science. 257:1064-1073.

Newell D.G., Koopmans M., Verhoef L., Duizer E., Aidara-Kane A., Sprong H., Opsteegh M., Langelaar M., Threfall J., Scheutz F., Van der Giessen J., Kruse H. 2010. Food-borne diseases — The challenges of 20 years ago still persist while new ones continue to emerge. International Journal of Food Microbiology. 139:S3–S15.

Niks M., Otto M. 1990. Towards an optimized MTT-assay. Journal of Immunological Methods.130:149-151.

Norrby S.R. 1991. Side-effects of quinolones: Comparisons between quinolones and other antibiotics. European Journal of Clinical Microbiology and Infectious Diseases. 10:378-383.

Ogston A. 1984. “On Abscesses”. Classics in Infectious Diseases". Reviews of Infectious Diseases. 6:122–28.

Okeke I.N., Lamikanra A., Edelman R. 1999. Socio economic and behavioral factors leading to Acquired Bacterial Resistance to Antibiotics in developing countries. Emerging Infectious Diseases. 5:18-27.

Okoli C.O., Akah P.A., Egbuniwe O.N. 2006. Analgesic activity of leaf extracts of *Culcasia scandens* P. Beauv. Indian Journal of Experimental Biology. 44:422-424.

Patton L L., Bonito A. J., Shugars D. A. 2001. A systematic review of the effectiveness of antifungal drugs for the prevention and treatment of oropharyngeal candidiasis in HIV-positive patients. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics. 92:170-179.

Payne G., Bringi V., Prince C., Shuller M. 1991. The quest for commercial production of chemicals from plant cell culture, Plant Cell and Tissue Culture in Liquid Systems, Oxford University Press, Oxford.

Pell S.K. 2004. Molecular systematiucs of the cashew family. Anacardiaceae. Phd thesis, Graduate Faculty OF Louisiana State University of Agriculture and Mechanical College, United States of America

Pelletier L.L. 1996. Microbiology of the Circulatory System. In Baron’s Medical Microbiology (Baron et al., eds). 4th edition. University of Texas Medical Brach.

Perfect J.R., Dismukes W.E., Dromer F., Goldman D.L., Graybill J.R., Hamill R.J., Harrison T.S., Larsen R.A., Lortholary O., Nguyen M., Pappas P.G., Powderly W.G., Singh N., Sobel J.D., Sorrell T.C. 2010. Clinical Practice Guidelines for the Management of Cryptococcal Disease. Clinical Infectious Diseases. 50:291–322.

Persons D.A., Laughlin M., Tanner D., Perfect J., Jon P., Gockerma M.D., Hathorn J.W. 1991. Fluconazole and *Candida krusei* Fungemia. New England Journal of Medicine. 325:1315.

Peterson B.L., Brian S. 2006. A review of chromatographic methods for the assessment of phospholipids in biological samples. Cummings Biomedical Chromatography. 20:227–243.

Phillips I., Casewell M., Cox T., De Groot B., Friis C., Jones R., Nightingale C., Preston R., Waddell J. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy.53:28–52.

Piccirillo C.A. 2008. Regulatory T cells in health and disease. Cytokine 43:395-401.

Plosker G.L., Figgitt D.P. 2005. Linezolid: a pharmaco-economic review of its use in serious Gram-positive infections. Pharmaco-economics. 23:945-964.

Pooley E. 1993. Trees of Natal, Zululand and Transkei, Natal Flora Publications Trust, Durban. 244–246.

Premack B.A., Schall T.J. 1996. Chemokine receptors: gateways to inflammatory and infection. Nature Medicine. 2:1174-1178.

Qadir S., Dixit A.K., Dixit P. 2010. Use of medicinal plants to control Haemonchus contortus infection in small ruminants. Veterinary World. 3:515-518.

Quinn P.J., Carter ME., Markey B., and Carter GR. 1994. Clinical Veterinary Microbiology . USA Virginia. 498.

Rabe C., Musch A., Schirmacher P., Kruis W., Hoffmann, R. 2005. Acute hepatitis induced by an Aloe vera preparation: A case report. World Journal of Gastroenterology. 11:303–304.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine. 26:1231–1237.

Repentignyl D.E., Lewandowski D., Jolicoeur P. 2004. Immunopathogenesis of orophangeal candidiasis in human immunodeficiency virus infection. Clinical microbiology reviews. 17:729-759.

Rice-Evans C.A., Miller N.J., Paganga G.1997. Antioxidant properties of phenolic compounds. Trends in Plant Science. 2:152.

Riddell L.A., Morris K. 2002. Surgery in Carriers of Blood-borne Infections. Haemolymphoid System.208-211.

Rios J.L., Recio M.C. 2005. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology. 100:80-84.

Roos D., Van Bruggen R., Meischl C. 2003. Oxidative killing of microbes by neutrophils. Microbes and Infection Journal. 5:1307–1315.

Ryan K.J., Ray C.G. 2004. Sherris medical microbiology. McGraw Hill 261-271.

Schlitt H., Geiss, F. 1972. Journal of Chrornatography. 67:261.

Schluger N.W. 2005. The pathogenesis of tuberculosis: the first 100 (and 23) years. American Journal of Respiratory cell and Molecular biology. 32:251-256.

Shah K.A., Patel M.B., Patel R.J., Parmar P.K. 2010. *Mangifera Indica* (Mango). Pharmacognosy review (The New England Journal of Medicine). 4:42-48.

Shai L.J., McGaw L.J., Aderogba M.A., Mdee L.K., Eloff J.N. 2008. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentate* (Burm.f) C.A. Sm. leaves. Journal of ethnopharmacology. 119:238–244.

Shimada K., Fujikawa K., Yahara K., Nakamura T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. Journal of Agricultural and Food Chemistry. 40:945-948

Shinwari Z.K., Watanabe T., Rehman M., Yoshikawa T.A. 2006. Pictorial Guide to Medicinal Plants of Pakistan. KUST. Kohat, Pakistan.

Shorr A.F. 2009. Review of studies of the impact on Gram-negative bacterial resistance on outcomes in the intensive care unit. Critical Care Medicine. 37:1463-1469.

Splettstoesser W. D., Schuff-Werner P. 2002. Oxidative stress in phagocytes – 'the enemy within'. Microscopy Research and Technique. 57:441-455.

Stahl E. 1969. Thin Layer Chromatography, Springer-Verlag, New York. 40.

Stary F. 1998. The National Guide to Medicinal Herbs and Plants. Tiger Books International London. 12–16.

Suleiman M. M., McGaw L. J., Naidoo V., Eloff J.N. 2010. Evaluation of several tree species for activity against the animal fungal pathogen *Aspergillus fumigatus*. South Africa Journal of Botany. 76:64-71.

Suls L. 1995. Aspergillosis: A common but dangerous diseases. World's Poultry Science Journal. 111:56-59.

Todar K. 2007. Pathogenic E. coli. University of Wisconsin-Madison Publishers Madison, WI. 120

Todar K. 2008. *Pseudomonas aeroginosa*. Online text book for Bacteriology

Tsipouras A., Ondeyka J., Dufresne C., Lee S., Salituro G., Tsou N., Goetz M., Singh S.B., Kearsley S.K. 1995. Using similarity searches over databases of estimated 13C NMR spectra for structure identification of natural product compounds. Analytica Chimica Acta. 316:161-171.

Ullah M.F., Khan M.W. 2008. Food as Medicine: Potential Therapeutic Tendencies of Plant Derived Polyphenolic compounds. Asian Pacific Journal of Cancer Prevention. 9.

Valentao P., Fernndes E., Canvalho E., Andrade P.B., Seabra R.M., Bastos M.L., 2002. Antioxidant activity of *Hypericum androsaenium* infusion scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. Biological and Pharmaceutical Bulletin. 25:1324–1327.

Van Cutsem J., Paj J. 1988."Experimental Aspergillosis." 6th Mediterranean Congress of Chemotherapy, Taormina, Giardini Naxos, Italy. 22-27.

Vanden Bossche H., Engelen M., Rochette F. 2003. Antifungal agents of use in animal health- chemical, biochemical and pharmacological aspects. Journal of Veterinary Pharmacology Therapy. 26:5-29.

Verweij P.E., Denning D.W. 1997. Diagnostic and therapeutic strategies for invasive aspergillosis. Respiratory and critical care medicine. 18:203-215.

Webber R. 2005. Communicable Disease Epidemiology and Control, 2nd edition.

Wen D., Liu Y., LiW., Liu H. 2004. Separation methods for antibacterial and antirheumatism agents in plant medicines. Journal Chromatography. 812:101-117.108-123.

World Health Organization. (2000). Western Pacific Region.

Yang C.R., Zhang Y., Jacob M.R., Khan S.I., Zhang Y.J., Li X.C. 2006. Antifungal activity of C-27 Steroidal Saponins Antimicrobial agents and chemotherapy. 50:1710–1714.

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