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Ace	Acetone
AIDS	Acquired immune deficiency syndrome
Amp-B	Amphotericin-B
Ann.s	*Annona senegalensis*
AS	*Acokanthera schimperi*
ATCC	American type culture collection
BEA	Benzene/ethanol/ammonium hydroxide
BERB	Berberine chloride
BD	Bovine dermis cells
CC_{50}	Cytotoxicity (50% cell death)
CE	*Carissa edulis*
CEF	Chloroform/ethyl acetate/formic acid
CDV	Canine distemper virus
CHB	Chronic hepatitis B
CMV	Cytomegalovirus
CPE	Cytopathic effect
CPIV	Canine Parainfluenza virus
CRFK	Crandell feline kidney cells
DCM	Dichloromethane
DMEN	Dulbecos minimun essential medium

DMSO	Dimethyl sulfoxide
DNA	Dioxiribonucliec acid
DPPH	1-1-diphenyl-2-picryl-hydrazyl
EC	*Ekebergia capensis*
EC_{50}	Effective concentration 50
ELIZA	Enzyme linked immunosorbent assay
EMW	Ethyl acetate/methanol/water
EsbL	Extended spectrum b-lactamase
FCS	Fetal calf serum
FHV	Feline herpes virus
FMDV	Foot and Mouth Disease Virus
FMD	Foot and Mouth Disease
GI	Gastrointestinal
HAART	Highly active antiretroviral therapy
HCMV	Human cytomegalovirus
Hep G2	Human hepatoma cell line
Hex	Hexane
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive care unit
INT	*p*-iodonitrotetrazolium violet,

LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MDR	Multidrug resistance
MEM	Minimum essential medium
Met	Methanol
MH	Müller-Hinton
MIC	Minimum inhibitory concentration
MPs	Medicinal plants
MRSA	Methicillin- resistant *Staphylococcus aureus*
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NCCLS	National Committee for Clinical Laboratory Standards
NCE	New chemical entities
N/D	Not done
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
OIE	Office International des Epizooties
OPC	Oropharyngeal candidosis
PBS	Phosphate buffered saline
PH	*Podocarpus henkelii*
PPR	Peste des petits ruminants

Pz	*Plumbago zeylanica*
REACH	Registration, Evaluation and Authorisation of Chemicals
R_f	Retardation factor
RNA	Ribonucliec acid,
ROS	Reactive oxygen species
RP	Rinderpest
RS	Reactive species
RSV	Respiratory syncytial virus
RVF	Rift Valley fever
Sca	*Schrebera alata,*
SD	Sabrouraud dextrose broth,
SI	Selectivity index,
SV	Simian virus
$TCID_{50}$	Tissue culture infective dose 50
TLC	Thin layer chromatography
UK	United Kingdom
UV	Ultraviolet
VREF	Vancomycin resistant *Enterococcus faecalis*
VZV	Varicella-zoster virus
WHO	World Health Organisation
4-NQO	Nitroquinoline-1-oxide

Publications from this thesis

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Bagla, V.P., McGaw, L.J. and Eloff, J.N. Comparative cytotoxicity studies of extracts of selected medicinal plants on different cell types.

Abstract

Diseases caused by bacteria, fungi and viruses pose a significant threat especially to poor rural communities. Viral infections are frequently complicated by secondary bacterial and fungal infections which remain a major challenge globally and in particular, in sub Sahara Africa amongst humans and animals alike. The main aim of this study was to develop a low toxicity plant extract or isolated compound active against viral, bacteria and fungal pathogens from selected plant species.

Seven tree species that were investigated were *Acokanthera schimperi, Carissa edulis, Ekebergia capensis, Podocarpus henkellii, Plumbago zeylanica, Annona senegalensis* and *Schrebera alata* traditionally used in the treatments of various ailments were selected and extracted using solvents of varying polarity. Extracts of selected plants were tested for activity against two Gram positive and two Gram negative bacterial namely *Enterococcus faecalis* and *Staphylococcus aureus* and two Gram-negative species, *Pseudomonas aeruginosa* and *Escherichia coli* respectively, three fungal pathogens: *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigates* and four enveloped animal viruses: feline herpes virus–1 (FHV-1, dsDNA), canine distemper virus (CDV, ssRNA), canine parainfluenza virus-2 (CPIV-2, ssRNA) and lumpy skin disease virus strain V248/93 (LSDV, dsDNA). The presence of antioxidant constituents in the different extracts and cytotoxicity against three cell types CRFK, bovine dermis and Vero cells were determined. Bioautography and the serial microplate dilution methods were used to determine the number of antimicrobial compounds and antimicrobial activity of extracts against bacterial and fungal pathogens. Virucidal and attachments assays were used to determine the activity against viral pathogens. Qualitative antioxidant activities of extracts were tested using the DPPH reagent and cytotoxicity using the MTT assay.

Biological activity was observed in all the extracts against one or more organisms on bioautography. The intermediately polar system (CEF) separated more active constituents. Some extracts had compounds with similar R_f values active against one or more organisms. In both the antibacterial and antifungal assays, acetone extracts had the highest activity followed by DCM against one or more pathogens. Hexanes extracts were the least active. *P. henkellii* extracts had more active compounds against the bacteria and *Annona senegalensis* against the fungi. In the microdilution assay, *S. aureus* was the most susceptible bacterial organism to extracts of the different plant, followed by *P. aeruginosa* and *Escherichia coli,* and *E. faecalis* the least. *C. neoformans* on the other hand was the most susceptible fungal pathogen. In the antiviral assay, although activity was observed with hexane extracts of some plants in the virucidal assay, the most potent inhibition was observed with the acetone and methanol extracts of *Podocarpus henkelii* against CDV and LSDV in the virucidal assay and acetone extracts in the attachment assay.

In general the hexane was the least toxic while the intermediate polarity extracts were generally the most toxic indicating that highly polar compounds were possibly poorly or highly absorbed through membranes in the former and later respectively. Of the three cell types used CRFK was the most sensitive followed by bovine dermis and Vero cells the least. Cytotoxicity studies of extracts of the different plants revealed *A. senegalensis and A. schimperi* extracts were the most toxic plants in the cellular assay. These plants are toxic to animals and the cytoxicity is in line with the *in vivo* toxicity. The protective effects of antioxidant constituents in some extracts varied and appear to be influenced by the metabolism of the type of cell in culture. It also appears to suggest that metabolism in kidney-derived cells can be influenced by species variation in the origin of cells.

P. henkellii was selected for isolation of bioactive compound. Three compounds were isolated and their structure elucidated using ^{13}C and ^{1}H NMR and mass spectrometric data. The antibacterial, antifungal and antiviral activity of the isolated compounds 7', 4', 7'', 4''', tetramethoxy amentoflavone (C1), isoginkgetin (C2) and Podocarpusflavone–A (C3) were determined. Compound C2 was the most active against *E. coli* and *S. aureus* (MIC = 60 µg/mℓ) and a selectivity index (SI) value of 16.67. The compound was also active against *A. fumigatus* and *C. neoformans* (SI = 33.33) suggesting both antibacterial and antifungal activity with relative safety. Compound C3 had a broad spectrum of activity against *E. faecalis* and *P. aeruginosa* with SI values of 4. A less potent activity of the compounds was obtained in both the virucidal and attachment assays against test pathogens, indicating the lower activity of the compounds against tested viral pathogens. The studies further suggest structural activity relationship in the antimicrobial activity of biflavonoids. The compounds C1 and C2 had no toxic effect on the three cell types and mutagenicity studies indicated no activity of these compounds.

Podocarpusflavone-A occurs in every species of *Podocarpus* so far investigated, except *P. latifolius.* These studies represent the first isolation of bioactive compounds from *P. henkellii.* Although a different extractant was used than that used by traditional healers, the presence of antiviral compounds in *Podocarpus henkelii* against two unrelated viruses may justify on a chemotaxonomic basis the traditional use of related species *Podocarpus latifolius* and *Podocarpus falcatus* in the traditional treatment of canine distemper infection in dogs.

Chapter 1

Introduction

1.1. Introduction

Over the decades, there has been increasing evidence of infections caused by viruses and other pathogenic microorganisms. To date, viral infections still remain a major threat to humans and animals. As a metabolically inert particle, viruses reproduce only when they are within the host cell and as such require the metabolic pathway of living cells to replicate. This unique nature of viruses makes it difficult to design a drug that can either attack the virion or stages in the replication cycle, without affecting the host due to poor selective toxicity within host systems. Despite these drawbacks, substantial progress has been made in the development of antiviral agents for some viral infections in humans. On the contrary, despite the outbreaks in recent years of RNA viral infections in the livestock sector, little success has been achieved towards the development of antiviral agents against these diseases and very few, if any, are available for veterinary use.

Viral infections can be controlled either by prophylaxis or therapeutically. Although vaccines are available to protect against certain viral infections and advances are being made in DNA recombinant technology to produce new and safer vaccines, a comprehensive recent review indicates a possible vaccine-induced enhancement of infection in certain viral diseases (Huisman *et al*., 2009). These responses produced in vaccinates may possibly act as a deterrence in the development of vaccines against certain viral infections. Of equal concern is the use of old vaccine viral strains in the formulation of currently available vaccines in the face of emerging virulent strains in the field. Vaccines have also been associated with residual virulence and toxicity, contamination with other pathogens, allergic responses, disease in immunodeficient hosts (modified vaccines), neurological complications, and harmful effects on the foetus and vaccine failure. The situation has become even more complex in the past decade with the rise in viral latency and resistance development to existing antiviral drugs used in humans (Kott *et al*., 1999).

Bacteria and fungi on the other hand, apart from being causative agents in a variety of infections, play a significant role as opportunistic microbes in immunocompromised patients and nosocomial infections. In these infections, the majority of available chemotherapeutic agents rely on the immune competency of the infected host to fight infection. Coupled with this, of concern is the increase in the development of resistance by these pathogens to available antimicrobial agents (Novak *et al.,* 1999; Dessen *et al.,* 2001; White *et al.,* 1998; 2002; Jones *et al.,* 2004). Indeed with the increase in national and international trade as well as travel, resistant organisms can be transported easily across geographical boundaries leading to a global problem.

The structures of bacterial, fungal and viral pathogens differ in significant ways. As such, most chemotherapeutic agents aimed at inhibiting the continuous multiplication of these pathogens in infected hosts target structure or functions relevant for the continued survival of these pathogens within the host. For example, the majority of effective antibacterial agents inhibit steps important for the formation of peptidoglycan, the essential component of the bacterial cell wall. In contrast, most antifungal compounds target the formation or the function of ergosterol, an important component of the fungal cell membrane, while antiviral agents produce their effect either by inhibiting the formation of viral DNA or RNA or inhibiting the activity of viral reproduction (Jassim and Naji, 2003).

Despite these variations, some similarities do occur amongst agents used for treatment. Bauer *et al.* (1963) reported the use of a thiosemicarbazone derivative (morboran), which is effective in the treatment of tuberculosis as well as a prophylaxis in smallpox infection. On the other hand, antifungal nucleoside analogues absent amongst available antibacterial agents are present amongst antiviral agents (Ghannoum and Rice, 1999). Interestingly, the antibacterial RNA polymerase inhibitor rifampin, which demonstrates no intrinsic activity against fungi, appears quite active against several fungal species when used in combination with amphotericin B (Beggs *et al.,* 1976).

Plants provide an unmatched source of chemically diverse constituents (Cos *et al.,* 2006; Maregesi *et al.,* 2008), that may serve as new alternatives in the pursuit towards the development of potentially effective antimicrobial agents to counter the existing threat posed by these pathogenic microorganisms. As a result, some medicinal plants containing substances active against viruses, bacteria and fungi have been identified (Venkateswaran *et al.,* 1987; Hudson, 1990; Lee *et al.,* 1998; Thyagarajan *et al.,* 1990; Yam *et al*., 1998; Chattopadhyay and Naik, 2007; Masoko *et al*., 2008, Rybalchenko *et al.,* 2009). Historical evidence of the use of plants as a source of remedies to treat various disease conditions, coupled with scientific validation and the understanding that monotherapy results in drug resistance, and the growing interest in the use of medicinal plants creates no doubt that plants contain substances with therapeutic relevance. This chapter therefore presents an insight into the understanding of resistance development by microorganisms and the limitations associated with the use of presently available chemotherapeutic agents. It further highlights the economic impact especially of viral infections that pose a threat to food security in under-developed and developing countries.

1.2. Resistance development to antimicrobial agents

1.2.1. Viruses

Development of resistance to antimicrobial agents is a growing problem worldwide that causes difficulties in the treatment of important nosocomial and community-acquired infections. Currently there are available antiviral drugs

for the management of a range of viral infections caused by human immunodeficiency virus 1 (HIV-1), herpes simplex virus (HSV- 1 and HSV-2), cytomegalovirus (CMV), influenza A virus, respiratory syncytial virus (RSV), papilloma viruses and hepatitis B and C viruses in humans. Although considerable progress has been achieved in the past decades in this respect, the understanding of resistance development to antiviral agents is still in rudimentary stages, in large part because of the relatively recent advent of effective antivirals.

To combat the development of antiviral resistance requires knowledge of the mechanism by which these pathogens elude therapeutic agents. Prior to the discovery of antiretrovirals, an extensive and systematic analysis of herpes simplex virus and varicella zoster virus resistance to acyclovir was undertaken and these findings have provided a major insight into antiviral drug resistance (Coen, 1996; Gilbert *et al.,* 2002). With these viruses, the mutations that lead to resistance development to antiviral agents appear to be associated with reduced virulence and ability to cause infection. This phenomenon has served as a positive outcome for the majority of antivirals used in herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections. In contrast, the development of resistance by the human immunodeficiency virus to antiretroviral therapy results from mutations in the genome of the virus coding for structural changes in the target proteins that can affect the binding or activity of currently used antiretroviral drugs (Menéndez-Arias, 2010). Other resistance development mechanisms against effective treatment for influenza virus (Hill *et al.,* 2009), and chronic hepatitis B virus infection (Ghany and Liang, 2007) have been extensively reviewed. That notwithstanding, the processes by which viruses develop resistance to antiviral agents are increasingly being investigated and characterized for the growing number of antiviral agents.

1.2.2. Bacteria

Infectious diseases remain a leading cause of worldwide morbidity and mortality, whether in the general healthy population or in patients who are immunocompromised and are at risk of infection with invasive opportunistic pathogens. Even though antimicrobial drugs have played a major role in keeping these pathogens in check, the development of resistance to antibiotics currently remains one of the biggest challenges facing global health care systems. Available reports indicate that around 90–95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al.,* 2005) and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Chambers, 2001).

The development of resistance to antibiotics came to light from organisms that were exposed to the first commercially available antibiotics. The antimicrobial drug resistance of staphylococci to penicillin is one such example (Barber, 1947). Resistance develops either passively or actively as a result of attainment of new genetic material by the microbe or pre-existing innate mechanism (Summers, 2006; Wright, 2007). These resistance developments lead to

treatment failure which frequently has fatal consequences. It is worthy of note to recognize that resistance also affects the treatment of individuals with non-resistant organisms in areas with high rates of resistance thereby increasing overall treatment costs (Howard *et al*., 2003). The major mechanisms of antibiotic resistance include prevention of interaction of the drug with the target site, efflux of the antibiotic from the cell, and direct destruction or modification of the compound (Walsh, 2003; Levy and Marshall, 2004; Wright, 2005). Compounding the problem is the continued selective pressure by different drugs, resulting in bacteria acquiring additional kinds of resistance mechanisms that have given rise to multidrug resistance (MDR). Some of these resistance development mechanisms to antibacterial agents in Gram-positive and Gram-negative bacteria as well as molecular mechanisms of multidrug resistance have been extensively documented (Wright, 2005; Tenover, 2006; Rice, 2006; Alekshun and Levy, 2007; Matthew and Bliziotis, 2007).

Poverty, poor access and insufficient health care systems, civil conflicts and lack of commitment on the part of government in developing countries have partly contributed to the rise in treatment failure and have impacted negatively on efforts to control infectious diseases. Other factors within established clinical settings include inappropriate use of broad-spectrum antibiotics, lack of prudent judgement in instituting treatment (Hancock, 2005), colonisation pressure amongst infected patients as a source of the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) (Merrer *et al.,* 2000) and vancomycin resistant *Enterococcus faecalis* (VREF) (Bontem *et al.,* 1998). Prolonged intensive care unit (ICU) stay leads to exposure to hospital-acquired infections (Bontem *et al.,* 1998; Rahal *et al.,* 1998). The use of invasive devices such as endotracheal tubes has also been incriminated (Richards *et al.,* 1999; Kollef *et al.,* 1997).

Furthermore, antibiotics are not only used in human medicine but also for the treatment, mass prophylaxis and growth promotion in animals, thereby presenting a possible risk of resistant bacteria passed to humans via the food chain. Antibiotics used in both veterinary and human medicine are: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones (Prescott, 2000). It is considered that one way to prevent the transfer of antibiotic resistant strains from animals to the human population is to withdraw the use of antibiotics as production enhancers in veterinary practice (Hancock, 2005). This initiative has been recommended by the World Health Organisation (WHO) because of risk factors associated with their continued use.

The increased development of resistance is accompanied by medical and economic implications (Paladino, 2002; Cosgrove and Carmeli, 2003). The costs to bring a new drug onto the market are estimated at a minimum of US$300 million. Hence, the inability of developing economies to manage the spread of resistant strains through globalisation increases the demand for resources and services.

1.2.3. Fungi

Despite the increase in the prevalence of resistance to antibacterial and antifungal agents, not much attention has been devoted to the study of antibiotic resistance. Studies of resistance development specifically to antifungal agents have lagged even further behind. Resistance development to antifungal agents is a broad concept that describes failure of a fungal infection to respond to antifungal therapy (Sheehan *et al*., 1999). Traditionally, this resistance development is classified as either primary (intrinsic), where the organism is resistant prior to exposure to the antifungal, or secondary (acquired), due to a stable transient genotypic modification following exposure to an antifungal agent. A third type of antifungal resistance can be described as "clinical resistance", which arises from progression or relapse of an infection caused by a susceptible isolate in *in vitro* testing to an antifungal agent recommended for the treatment of the given infection. Such resistance development is common amongst immunocompromised patients or in patients where prosthetic materials have been used (Sheehan *et al*., 1999). In some cases, suboptimum drug concentrations in the blood might contribute to the development of clinical resistance.

The exposure of fungal pathogens to antifungal agents stimulates different responses in the metabolism of the organism. As a survival instinct, the fungal pathogen will strive to overcome the growth inhibitory effect of the antifungal agent by development of various mechanisms to counteract the inhibitory effect of the antifungal agent. These mechanisms will permit the growth of the pathogen at higher drug concentrations than is the case for normal susceptible pathogens, while in others where higher drug concentration results in growth inhibition, the fungal pathogen can alter the therapeutic potency of a given antifungal agent, which will determine if the agent will produce a static or a cidal effect (Sanglard, 2003). This property exhibited by fungal pathogens is termed antifungal drug tolerance. Prior to the validation by the United States National Committee for Clinical Laboratory Standards (NCCLS) now known as the Clinical and Laboratory Standard Institute (CLSI) in 1997, there was no widely accepted method for *in vitro* susceptibility testing of fungal pathogens. The method describes the determination of minimum inhibitory concentrations (MICs) of widely used antifungals against one species, which helps to evaluate with greater confidence whether *in vitro* susceptibilities are correlated with clinical response to therapy. Interpretive breakpoints of resistance with this standard method currently exist only for fluconazole, itraconazole, and flucytosine.

For many years, amphotericin B was the only drug available to control fungal infections until the advent in the 1980s and 1990s of the imidazoles and the triazoles. The introduction of these agents led to their widespread usage and to the development of drug resistant strains (Rex *et al.,* 1995). Resistance development mechanisms to the azoles have been most extensively investigated in recent years, as a large number of yeast isolates were available to research laboratories. Several reviews are available that describe in detail the different mechanisms resulting in resistance of

fungal pathogens to the azoles and other classes of antifungal agents including the molecular bases for such resistance (Sanglard *et al.,* 1998; Ghannoum and Rice, 1999; White *et al.,* 1998; Sanglard, 2002; Kontoyiannis and Lewis, 2002; Chamilos and Kontoyiannis, 2005; Prasad and Kapoor, 2005; Kanafani and Perfect, 2008).

With the increase in the incidence of systemic fungal infections, the choice of suitable antifungal agents remains relatively limited. The use of highly active antiretroviral therapy (HAART) has decreased the occurrence of mucosal candidiasis among acquired immune deficiency syndrome (AIDS) patients in the USA (Martins *et al.,* 1998). In under-developed and developing countries, the increase in the number and spectrum of fungal infections boosted by the AIDS pandemic coupled with poor access to HAART and problems associated with adherence to medication and toxicity, implies that resistance to antifungal agents still remains a major threat. Furthermore, advances in anticancer chemotherapy and organ transplants have attracted new interest in the development of new compounds with antifungal activity. Over the next decade, antifungal resistance may become an increasingly crucial determinant of the outcome of antifungal treatment.

1.3. Adverse effects associated with the use of antimicrobial agents

1.3.1. Antiviral agents

An adverse drug reaction is defined by the WHO as any response to a drug 'which is noxious and unintended and which occurs at doses used in man for prophylaxis, diagnosis or therapy' (WHO, 1984). As the incidence of drug resistance increases, the situation has become more alarming with the toxic effects associated with currently used antimicrobial agents. Presently, there is no cure for HIV infection and patients using therapies recommended either as immune boosters or for the reduction of viral load are expected to undergo treatment for life. The introduction of highly active antiretroviral therapy (HAART), which consists of a cocktail of drugs, has led to substantial reductions in morbidity and mortality associated with HIV-1 infection. Although considerable improvement has been made in the management of HIV infection (HIV/AIDS, 2008), drug related toxicity is increasingly being recognized because of the reduced incidence of HIV-1-associated opportunistic infections (Carr and Cooper, 2000). The present threat posed however does not seem to be life–threatening but can affect the quality of life and patient's compliance to treatment regimens and as a consequence will result in the development of resistant strains in the near future. Conversely, long-term toxic effects associated with prolonged therapy can also lead to changes in treatment regimens or discontinuation due to adverse effects that could not be foreseen in the short-term (Esté and Cihlar, 2010).

Numerous antiretrovirals that are currently used for the treatment of HIV infection have adverse effects, ranging from

nausea, headache, nail pigmentation, diarrhoea, mouth ulcers, central nervous stimulation, hypersensitivity, perioral paraesthesiae, renal calculi, hyperbilirubinaemia, reflux oesophagitis, retinoid effects and haemolytic anaemia, which are associated in some patients with the use of either nucleoside analogues, non-nucleoside analogues or HIV protease inhibitors. These effects and many more associated with the use of available therapies for HIV treatment have been documented (Carr and Cooper, 2000; Izzedine *et al.,* 2005; Hawkins, 2010).

In the management of hepatitis C viral infection, the side effect profile of combination therapy using standard interferon and ribavirin have been reported to be associated with fatigue, influenza-like symptoms, gastrointestinal disturbances, neuropsychiatric symptoms, and hematologic abnormalities which may call for the reduction in the dose of the treatment regimen or a total discontinuation of treatment depending on the severity of the effect (Poynard *et al.*, 1998; Maddrey, 1999; McHutchison and Poynard, 1999). In the case of chronic hepatitis B (CHB) virus infection, where patients are required to take treatment for a prolonged time period, it is important to strike a balance between long-term benefits and potential adverse effects. For instance, long-term lamivudine treatment in CHB virus infection in patients undergoing therapy is associated with an increased rate of drug resistant mutations (Guan *et al.,* 2001; Leung *et al.,* 2001) coupled with serious hepatitis flares and hepatic decompensation in isolated cases of individuals (Tipples *et al.,* 1996; Bartholomew *et al.,* 1997). Such risk may however be higher in older patients who are immunosuppressed and suffering from advanced liver diseases.

Furthermore, although foscamet is an effective treatment for acyclovir-resistant herpes simplex virus and acyclovir-resistant varicella-zoster virus, it significantly decreases the circulating levels of HIV antigens in AIDS patients with cytomegalovirus disease. The major adverse effect associated with the use of foscamet is renal dysfunction (Akesson *et al.,* 1986; Jacobson *et al.,* 1988) and acute renal failure when used alone or in combination with certain drugs (Cacoub *et al.,* 1988). The nephrotoxicity induced by this drug has limited its more widespread utilization, especially in transplant patients.

1.3.2. Antibacterial agents

Quinolones are widely used antibacterial agents with excellent activity against Gram-negative bacteria. Nausea, vomiting, diarrhoea and other reactions of the gastrointestinal tract are among the most common side effects associated with the use of quinolones. Compared to other groups of antibacterial agents with a broad spectrum (e.g. penicillins or cephalosporins), the incidence of diarrhoea is low and has been associated with the newly introduced quinolones such as levofloxacin, moxifloxacin and gatifloxacin. Depending on the type of quinolones, adverse effects on the cardiovascular system, phototoxicity (photocarcinogenicity) and connective tissue structures (chondrotoxicity

and tendinopathies) have been reported (Anderson *et al.,* 2001; Bailey *et al.,* 1983; Stahlmann, 2002).

With the β-lactams (comprising over 40 derivatives within the penicillin and cephalosporin families), paramount to their popularity has been their impressive safety profile. Although severe life threatening reactions occur with the use of these agents, most reported toxicities are mild and reversible in nature. The most common adverse effect associated with the use of these classes of drugs is hypersensitivity reactions, which can vary in severity from skin rashes to life-threatening anaphylaxis. The reported frequency of reactions ranges from 0.7% to 10% (Petz, 1978; Norrby, 1986). While anaphylactic reactions are exceedingly rare, death may result due to a combination of symptoms, which includes nausea, vomiting, abdominal pain, pallor, tachycardia, severe dyspnoea due to bronchospasm, rigors, loss of consciousness and peripheral circulatory failure due to vasodilatation and loss of plasma volume within minutes if not properly managed. In addition, mild to severe cases of haematological reactions, hepatotoxicity, nephrotoxicity, gastrointestinal reactions and neurotoxicity have been reported in some patients (Moake *et al.,* 1978; Yust *et al.,* 1982; Lurie *et al.,* 1970; Ruley and Lisi, 1974; Milman, 1978; Gardner *et al.,* 1978; Enat *et al.,* 1980; Onorato, 1978).

Due to a relatively low potential for Gram-positive organisms to develop resistance to aminoglycosides, their use in combination with the β-lactam antibacterials continues to be relevant for the treatment of serious Gram-negative infections despite the undesirable effects that are associated with their use. Both nephrotoxicity and ototoxicity are well described adverse effects associated with the use of aminoglycosides (Davey *et al.,* 1991; Kaloyanides and Pastoriza-Munoz, 1980). However, the damage caused to the tubes of the kidney as a result of aminoglycoside use is usually reversible, resolving soon after the drug is discontinued, while that due to damage to the inner ear may be permanent.

Toxic effects associated with the use of vancomycins have limited their use in the past decade, however, the increase in the incidence of infections involving methicillin-resistant staphylococci has once again led to an increase in the use of this drug in methicillin-resistance staphylococci infections. Reported adverse effects associated with vancomycin include: nephrotoxicity, ototoxicity, anaphylactoid reactions and phlebitis (Rybak *et al.,* 1990; Brummett and Fox, 1989; Farber and Moellering, 1983). Purification of the commercial product in the early 1980s was thought to have decreased the frequency of adverse effects; however, information evaluating the adverse-effect profile of the reformulated product is scanty. Additional risk factors include: increased age (i.e. geriatric population), liver disease, peritonitis, neutropenia, male gender and concurrent use of nephrotoxic agents (Pauly *et al.,* 1990).

The macrolides have been considered to be among the safest class of antimicrobials in clinical use due to the low incidence of severe toxic reactions. However, with the macrolides, gastrointestinal (GI) adverse effects were reported

to be responsible for the high rates of intolerance among treated patients (Itoh *et al.,* 1984; Zara *et al.,* 1985). The use of the newer macrolides has been associated with a lower incidence of the above adverse effects compared with erythromycin (Periti *et al.,* 1993). Apart from GI adverse effects, thrombophlebitis has been associated with intravenous administration of erythromycin. Other notable macrolide-induced adverse effects include transient sensorineural ototoxicity (Brummett and Fox, 1989). Clinical evidence indicates that ototoxicity is more likely to occur in patients who are elderly, renally or hepatically impaired or those receiving an erythromycin dosage exceeding 4 g/day. Furthermore, cardiovascular effects have been associated with intravenous infusion of erythromycin in critically ill patients (Tschida *et al.,* 1996).

1.3.3. Antifungal agents

Antifungal agents are mostly used in patients with severe underlying diseases, which make detection of adverse drug effects and that due to the underlying disease difficult. Amphotericin B deoxycholate has been the gold standard for the treatment of patients with invasive mycoses. Although impairment of liver function has not been considered to be a typical adverse effect associated with the use of amphotericin B, nephrotoxicity resulting from the cumulative effect of the drug leading to renal impairment, hypokalemia, hypomagnesemia, and acidosis have been reported (Gallis *et al*., 1990). As such, the use of the drug in combination with nephrotoxic drugs such as cyclosporine, vancomycin, aminoglycosides and cisplatin warrants regular monitoring of patients during and after treatment. Compared with conventional amphotericin B deoxycholate, the new formulations, namely lipid-based amphotericin B colloidal dispersion, amphotericin B lipid complexes, and liposomal amphotericin B, are less nephrotoxic.

Currently, newer classes of the third-generation triazoles have been introduced for clinical use, with less toxic effects, and in some cases are more effective than amphotericin B. One such drug, voriconazole, has been approved for first-line treatment of invasive aspergillosis. However, voriconazole is associated with mild elevation of the transaminase enzymes and visual disturbances (photophobia, blurred vision and altered colour discrimination), rash and gastrointestinal symptoms (Hoffman and Rathbun, 2002). Similarly, posaconazole has few side effects, the most common of which are nausea, vomiting, headache, abdominal pain and diarrhoea (Raad *et al.,* 2006) while in some cases, elevated liver enzymes may be observed. Another group of drugs, the echinocandins, have a mode of action that is different from all the other antifungals. These drugs produce their antifungal effect by inhibition of β-1, 3-D-glucan synthase, which forms an important component of the cell wall of many pathogenic fungi such as *Candida* and *Aspergillus* species. Drugs belonging to this group comprise caspofungin, micafungin, and anidulafungin, with fewer side effects ranging from headache, nausea, phlebitis, fever, rash, diarrhoea, leukopenia, anaphylaxis and haemolysis in isolated cases to hypokalaemia and elevated hepatic enzymes. In general this class of drugs is well tolerated.

1.4. Virus

Viruses are obligate intracellular parasites, which contain little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a lipid-containing envelope (Wagner and Hewlett, 1999). This gene strands, whether DNA or RNA, may be single or double stranded. Single stranded viral nucleic acid may be of positive or negative polarity (Duguid *et al.,* 1978). The molecular weight and type of nucleic acid are characteristic for each group of viruses. Depending on the type of virus, the nucleic acid can be linear or circular (Duguid *et al.,* 1978).

Although extremely simple in structure and composition, viruses are masters of camouflage and deception. Devoid of any means of independent locomotion, they disseminate by exploiting host cell organelles and metabolic pathways to propagate new viruses. They use the reproductive machinery of invaded cells, causing various ailments. Each strain of virus has its own unique configuration of surface molecules (Wagner and Hewlett, 1999). These surface molecules work like keys in a lock, enabling viruses to infect their hosts by precisely fitting the molecules on their surfaces to those on the membranes of target cells. Viral particles mediate the transfer of the viral genome and accessory proteins from an infected host cell to a non-infected host cell. This involves packaging the viral genome (RNA or DNA) and accessory proteins, releasing the package from the infected cell, protecting the essential components during extracellular transmission, and delivering them into a new host cell. Many viruses with a DNA genome must enter the nucleus, whereas RNA viruses, with a few exceptions, replicate in the cytosol.

The success of viruses over time has been established by four general attributes: genetic variation, variety in means of transmission, efficient replication within host cells, and the ability to persist in the host (Wagner and Hewlett, 1999). Consequently, viruses have adapted to all forms of life and have occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants alike. Unlike bacteria and fungi, viruses are the only pathogens in nature that use RNA as a store of genetic information (Duguid *et al.,* 1978). The body responsible for the classification of viruses is the International Committee on Taxonomy of Viruses (ICTV). Viruses have been classified into two broad groups based on the nature of the genome and the structure of the virion.

Viral diseases are still fatal and new viral diseases continue to emerge. Although some viral diseases can be kept under control by the use of vaccines or antiviral agents, the development of resistance in recent years to available antiviral drugs and the need to develop new therapies for the majority of viral infections, makes the search for new antivirals a virgin area for continued drug discovery. Furthermore, delays in the availability of vaccines and in the onset of protection (immunity gap) imply that during the critical initial stage of an outbreak, livestock will remain highly susceptible to infection. Many viruses have unique features in their structure or in their replication cycles, that can act as potential targets for antiviral drug testing. If antiviral agents were available to counter diseases in livestock this

could be a valuable adjunct to available disease control measures both in disease-free and disease-enzootic settings. In this study, greater attention will be given to literature on the antiviral activity of plants, as there are relatively few studies on the antiviral activity of plant compounds. Coupled with the fact that presently available antiviral drugs are still far beyond the means of rural populations in most developing countries, poverty, in terms of hunger eradication, child mortality reduction and improved human health, can be reduced by improved animal health (Perry and Sones, 2007).

1.4.1. Impact of viral disease outbreak on agriculture and livestock

"Livestock" is a collective term used for any breed or population of animals kept by humans for subsistence or commercial purpose. These sectors contribute to the livelihood of approximately 70% of the world's poor (DFID, 2000). Globally, the sector accounts for about 40% of the agricultural gross domestic product (Steinfeld *et al.,* 2006). Highly pathogenic viruses of livestock can be defined as those viruses that cause highly contagious or transmissible animal diseases and have the potential for very severe and rapid spread, irrespective of national borders, which are considered to be of serious socio-economic and/or public health significance, and which are of major importance in international trade of animals and their products (Domenech *et al.,* 2006). Such diseases have been listed by The Office International des Epizooties (OIE), which highlights the threat these agents pose to the livestock industry (OIE, 2004).

Foot and Mouth Disease Virus (FMDV) is an example of one such highly contagious virus that affects several species of animals and has an exceptionally high mutation rate (Domingo *et al.,* 2003), with high economic impact during an outbreak. The economic impact of the 2001 FMD epidemic in the United Kingdom (UK) rose to the tune of £8–9 billion, of which £3.1 billion represented direct losses to agriculture and the food chain while indirect losses to tourism were estimated to be as high as £5 billion (Thompson *et al.,* 2002; Campbell and Lee, 2003). Similarly, the direct economic consequences of the 1997 outbreak in Taiwan totalled US$ 3.31 billion (Yang *et al.,* 1999). As such, high revenue impact figures were predicted in case of FMD incursions in Australia, New Zealand and the United States of America (Belton, 2004; Garner *et al.,* 2002; Paarlberg *et al.,* 2002). The situation in Africa is of particular interest as five serotypes of the viruses are in circulation (Domenech *et al.,* 2006).

Rift valley fever (RVF) is a viral zoonosis that affects sheep, goat, buffalo and cattle (Van Tongeren, 1979). Human diseases due to infection with the virus also occur, especially during periods of intense epizootic activity, which occur after heavy rainfall, when there is an increase in the vector (mosquitoe) population (Arthur, 2000). The burden of human morbidity and mortality due to an outbreak often has a direct impact on economic loss of livestock, which can be as high as 70% of all affected animals. Many sub-Saharan tropical and sub-tropical countries in Africa have

reported outbreaks of RVF and the disease is encountered in an enzootic or epizootic form along the east and south coast of Africa and also in Madagascar. The 1970s saw the most severe outbreaks in South Africa in 1975 and in Egypt in 1977 (Gear, 1979; Hoogstraal *et al.,* 1979). Outbreaks of the disease in East Africa in the late 1970s and in 2000 caused livestock losses and human deaths and seriously affected international trade in livestock to the Middle East (Otte *et al.,* 2004). Other such outbreaks in past decades include rinderpest (RP) in Africa in the 1980s (Rweyemamu *et al.,* 2000), lumpy skin disease (Hunter and Wallace, 2001), African swine fever (Rweyemamu *et al.,* 2000), and Peste des petits ruminants (PPR) in India and Bangladesh (Roeder and Obi, 1999).

Of an equal severe consequence was the avian influenza epidemic in Asia caused by the highly pathogenic H5N1 strain in poultry in 2003. The virus has a high potential for rapid spread and is characterized by high mortality in chickens of between 75% and 100%. The outbreak of the disease resulted in the deaths and culling of about 40 million birds resulting in an economic loss to South East Asia to the tune of more than US $60 billion (Lokuge *et al.,* 2005). In Africa, routine vaccination of livestock has been prohibitively expensive to the common farmer, making the continent a refuge for endemism of some of these diseases.

The economic impact of animal diseases is complex and can go beyond the immediate impact on the directly affected animal producers. The most direct impact is the loss or reduced efficiency of production, which reduces farm income. These effects can create a negative impact on variation of prices determined by supply and demand, which in turn can pose a heavy burden on international trade. Of concern is the effect of these diseases and their negative impact on food security and nutrition in developing countries amidst associated health and environmental concerns where the majority of livestock production occurs in areas close to human populations (Otte *et al.,* 2004). A further limitation is the fact that most of these diseases occur in areas where resources are minimal, and where routine vaccination is seldom practiced.

Local remedies used in the treatment of various ailments have a long history in rural Africa and traditional medicines that have antimicrobial activity could have important benefits in communities where they are widely used. As traditional knowledge of disease treatment erodes in the face of socioeconomic change, the urgency to document indigenous remedies increases (Farnsworth, 1993). In the next chapter, a literature review of the antimicrobial activity of plants will be presented, followed by the aims and objectives of this study.

Chapter 2

Ethnomedicine in infections disease treatment and the relevance of cell culture in toxicity studies of medicinal plants

2.1. The value of ethnomedicine in drug discovery

In broad terms, ethnomedicine can be defined as the use of plants by humans as medicines (Farnsworth, 1990; 1994), while traditional medicine refers to any non-Western medical practice (Bannerman *et al.,* 1983). On the other hand, ethnopharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biological activities. It encompasses several disciplines that contribute to the discovery of natural products with biological activity (Rivier and Bruhn, 1979). The purpose of using plants as natural sources in drug development is multifaceted. These include a) the isolation of bioactive compounds for direct use as drugs, b) the production of bioactive compounds of novel or known structures as lead compounds to synthesize entities of higher activity and/or lower toxicity, c) to use agents as pharmacologic tools, and d) to use an extract of the whole plant or part of it as a herbal remedy.

Different approaches have been employed when selecting higher plants in the drug discovery process. These selections can be done either randomly, or by chemical screening, or ethnomedical claims involving the targeted disease. In either case, there are shortfalls associated with the selection process since plants, as biological systems, inherently vary in their chemistry and resulting biological activity.

There are advantages and disadvantages of using plants as the starting point in any drug development program. If the selection process is based on the ethnomedicinal approach, one can presume that any extract of the plant is likely to be safer than extracts or active compounds from plants with no history of human use. However, this presumption should be treated with caution as long-term toxic effects are often not linked to the use of the plant. If the active principle derived from such an investigation yields novel structures with useful biological activity, or novel biological activity of known compounds, patent protection may be possible.

Other problems encountered when plants are used as starting materials in the drug discovery process are mix-ups in labelling of plant samples or inaccurate taxonomic identification and the choice of inappropriate bioassay systems. Selection of plants based on ethnomedical use in conjunction with rational biological assays that correlate with the ethnomedical uses would be most appropriate in the drug discovery process (Fabricant and Farnsworth, 2001). Figure 2.1 illustrates a flow chart of sequence for the study of plants used in traditional medicine.

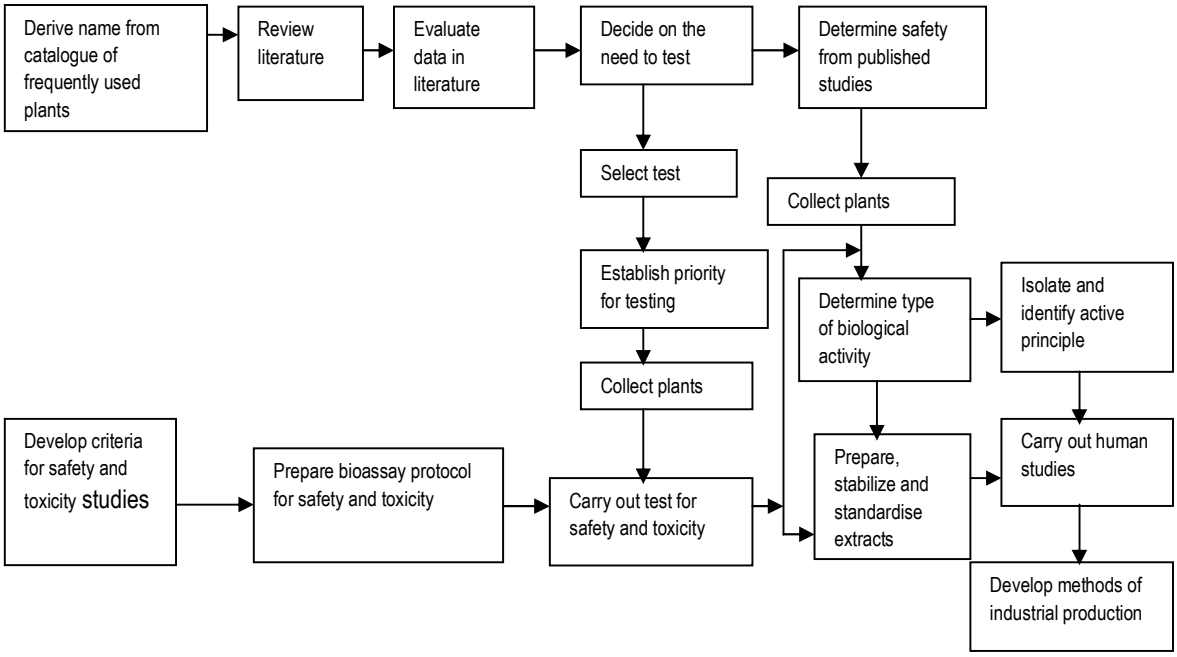

Figure 2.1: Flow chart of sequence for the study of plants used in traditional medicine (adapted from Farnsworth *et al.* (1985).

Several molecules of great interest because of their therapeutic usefulness, novel biological activity, or potential as pharmacologic probes have already been introduced in recent years following the approaches outlined above. One such is the antimalarial artemisinin from the Chinese medicinal herb Qing hao su (*Artemisia annua*), which is poorly soluble in water, making it unsuitable for administration. However, a derivative, sodium artesunate, has been developed, which retains the antimalarial activity but has much improved water solubility (Haynes and Vonwiller, 1994). More importantly, at least 119 compounds derived from plants have been considered as important drugs currently in use in one or more countries, with 77% of these derived from ethnomedical sources (Farnsworth *et al.,* 1985).

2.2. Use of plants as antimicrobial agents

Plants have been used as medicines for thousands of years (Samuelsson, 2004) and that they indeed contain pharmacologically active constituents is widely acknowledged. To ensure continued use, the specific plants to be used and the methods of application for particular ailments were passed down from generation to generation through oral history. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and

other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004).

It is estimated that today, plant materials are present in, or have provided the models for, 50% of Western drugs (Robbers, 1996). The role of plant-derived medicine is two fold in the development of new drugs. Firstly, they may serve as a natural blueprint for the development of new drugs, or secondly, as a phytomedicine to be used for the treatment of disease. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, leading to the early development of drugs from natural sources before the advent of their synthetic counterparts (Table 2.1).

Table 2.1. Some drugs of natural origin (adapted from Tulp and Bohlin, 2002)

Natural compound	Mode of action	(Semi) synthetic drug
Amphotericin B	Cell membrane permeability	Not yet described
Artemisinin	Interaction with haem	Arthemether
Atropine	Muscarinic acetylcholine receptor antagonist	Butylscopolamine
Avermectin	Cl– channel activator	Selamectin
Camptothecin	Topoisomerase I inhibitor	Irinotecan
Dicoumarol	Vitamin K antagonist	Acenocoumarol
Digoxin	Na+–K+-ATPase inhibitor	Acetyldigoxin
Ephedrine	β-Adrenoceptor agonist	Isoprenaline
Ergocornine	Dopamine receptor agonist	Bromocryptine
Gentamicin	Protein synthesis inhibitor	Metamycin
Griseofulvin	Mitosis inhibitor	Not yet described
Morphine	Opioid receptor agonist	Pethidine, fentanyl
Papaverin	Phosphodiesterase inhibitor	Sildenafil
Penicillin G	Transpeptidase inhibitor	Ampicillin, cyclacillin
Physostigmine	Cholinesterase inhibitor	Neostigmine
Podophyllotoxin	Topoisomerase II inhibitor	Etoposide
Vincristine	DNA polymerase inhibitor	Vindesine
Tetracyclines	Protein synthesis inhibitor	Doxycycline

Plant-based antimicrobials have huge therapeutic potential. They are effective in the treatment of infectious diseases and may mitigate side effects that are often associated with synthetic antimicrobials. An important issue with regard to drug discovery research is the role of unexpected observations. Herbal remedies usually have multiple effects on the body with activities that often act beyond the symptomatic treatment of disease. Such an effect can be observed with *Hydrastis canadensis*. Hydrastis not only has antimicrobial activity, but also increases blood supply to the spleen, promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

A great deal of the exploration and utilization of natural products as antimicrobials began from microbial sources. The discovery of penicillin created inroads for the later discoveries of streptomycin, aureomycin and chloromycetin (Trease and Evans, 1972). Although most of the antibiotics in clinical use were discovered from microorganisms in

the soil or fungi, plants have also been a source of antimicrobial agents (Table 2).

Table 2.2. Plants containing antimicrobial activity (adapted from Cowan, 1999)

Common name	Scientific name	Compound	Class	Activity
Oregon grape	*Mahonia aquifolia*	Berberine	Alkaloid	Bacteria, fungi
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, fungi
Olive oil	*Olea europaea*	Hexanal	Aldehyde	Bacteria, fungi
Lavender-cotton	*Santolina Chamaecyparissus*			Bacteria, fungi
Hemp	*Cannabis sativa*	b-Resercyclic acid	Organic acid	Bacteria and viruses
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide Sulfated terpenoids	Bacteria, fungi
Echinacea	*Echinacea Angustifolia*			Bacteria, fungi
Cascara sagrada	*Rhamnus purshiana*	Tannins	Polyphenols Anthraquinone	Viruses, bacteria, fungi
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivative	Bacteria, fungi
Woodruff	*Galium odoratum*		Coumarin	Viruses, bacteria, fungi
Purple prairie clover	*Petalostemum*	Petalostemumol	Flavonol	Bacteria, fungi
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids	Bacteria, fungi
Caraway	*Carum carvi*		Coumarins	Bacteria, fungi, viruses
Thyme	*Thymus vulgaris*	Caffeic acid Thymol Tannins	Terpenoid Phenolic alcohol Polyphenols Flavones	Viruses, bacteria, fungi
Burdock	*Arctium lappa*	Polyacetylene, tannins, terpenoids		Bacteria, fungi, viruses

The antimicrobial properties of plants have been associated with the presence of secondary metabolites produced by these plants that act as a deterrence against predation by microorganisms, insects, and herbivores. These secondary metabolites play a huge role as antimicrobial agents. Useful antimicrobial phytochemicals can be divided into several categories, summarized in Table 2.3.

Of these, the phenolics represent an extensive metabolic family given that this group of molecules is involved in lignin synthesis, making them common to all higher plants. However, other compounds such as alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genera and species. This narrower distribution of secondary compounds constitutes the basis for chemotaxonomy and chemical ecology

Table 2.3. Major classes of antimicrobial compounds from plants (adapted from Cowan, 1999)

Class	Subclass	Examples	Mechanism of action
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesins
	Flavones		Complex with cell wall
		Abyssinone	Inactivate enzymes
			Inhibit HIV reverse transcriptase
	Flavonols	Totarol	
	Tannins	Ellagitannin	Bind to proteins
			Bind to adhesins
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Membrane disruption
			Metal ion complexation
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or DNA
		Piperine	
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption
		Fabatin	Form disulfide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	-

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2.3. Common classes of antiviral compounds present in medicinal plants

Presently, it is doubtful that available mainstream antiviral drugs can solve the problems posed by viruses and emerging viral infections. Historically, scores of traditional medicinal plants have been used to treat viral infections the world over. Depending on the mode of application, different combinations of medicinal plants that have been used for this purpose may cause variations in therapeutic effect. Some of these variations have resulted in the therapeutic success of many medicinal plant extracts in several unrelated syndromes by virtue of their synergistic effects. Hence, several medicinal plants have been reported to have strong antiviral activity, some of which have been used to treat disease in humans and animals that suffer from viral infection (Hudson, 1990; Venkateswaran *et al.,* 1987; Thyagarajan *et al.,* 1988; 1990).

With an ever-growing potential, compounds isolated from natural sources are increasingly investigated for their ability to inhibit the replication cycle of various types of DNA or RNA viruses. A wide variety of active phytochemicals, including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulfides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyenes, thiophenes, proteins and peptides have therapeutic applications against different genetically and functionally diverse viruses (Chattopadhyay and Naik, 2007; Chattopadhyay and Bhattacharya, 2008; Naithani *et al.,* 2008). The antiviral mechanism of these agents may be related to their antioxidant activities, scavenging capacities, inhibition of DNA or RNA synthesis, inhibition of viral entry, or inhibiting viral reproduction (Christopher and Wong, 2006; Chattopadhyay and Naik, 2007; Naithani *et al.,* 2008). As a result, large numbers of candidate substances, such as phytochemicals and their synthetic derivatives, have been identified by a combination of *in vitro* and *in vivo* studies in different biological assays (Christopher and Wong, 2006; Naithani *et al*., 2008). Representatives of some active antiviral compounds from medicinal plants are shown in Table 2.4.

Table 2.4. Mechanism of action of the most active antiviral compounds from medicinal plants (adapted from Jassim and Naji, 2003)

Class of compound	Mechanism of antiviral activity / target
Furyl compounds: furocoumarins, furanochromones	DNA and RNA genomes: Interaction require long wave ultraviolet light
Alkaloids:B-carbolines, furanoquinolines, camptothecin, atropine, caffeine, indolizindine, swainsonine, castanospermine, colchicine, vinblastine	DNA and other polynucleotides and virion proteins. Some interactions are enhanced by ultraviolet light
Polyacetylenes (polyines)	membrane interaction. Phototoxic activity frequently required ultraviolet light
Polysaccharides	blocking viral binding
Thiophenes	membrane interaction. Phototoxic activity frequently required ultraviolet light
Flavonoids: amentoflavone, theaflavine, iridoids, Phenylpropanoid, agathisflavone, robustaflavone succedaneflavanone, chrysosplenol C, morin, coumarins galangin, baicalin	blocking RNA synthesis. Exhibits HIV inhibitory activity
Terpenoids: sesquiterpines, triterpeniods (moronic acid, ursolic acid, maslinic acid and saponin)	membrane mediated mechanisms. Inhibition of viral DNA synthesis
Lignans: podophyllotoxin and related lignans (schizarin-B, rhinacanthin E and F)	blocking virus replication: hepatitis B and influenza A virus
Miscellaneous phenolic compounds: caffeic acid, tannins, eugeniin, hypericin, quinine, salicylate etc	inhibition of virus DNA and RNA replication
Proteins and peptides:	
a) Single chain ribosome inactivation protein	interacts with ribosome function in infected cells thereby inhibiting viral protein synthesis
Pokeweed antiviral protein	inactivate infective HIV and HIV infected cells
Panaxagin	inhibits HIV-1 reverse transcriptase
Alpha and beta antifungal proteins	inhibits HIV-1 reverse transcriptase
b) Dimeric cytotoxins	interacts with ribosome function in infected cells thereby inhibiting viral protein synthesis
c) Lectins	viral membrane interaction
d) Antiviral factor	mechanism not clear
e) Meliacin	affects viral replication cycle

2.4. Cell cultures and toxicity studies

The development of a novel drug requires the evaluation of three major areas in drug design i.e. the efficacy, bioavailability and safety of the drug. About 30% of failures in the development of drugs have been associated with toxicity and safety issues (Kola and Landis, 2004). Among these, toxic effects imposed on the liver by these substances are one of the major issues encountered. Moreover, off-target or idiosyncratic toxicity, resulting in the post market withdrawal of drugs, is of increasing concern. This is to a certain extent due to the unavailability of adequate *in vitro* screening design that can effectively correlate with animal studies and its application to humans. However, Ekwall and co-workers have shown that a test battery of *in vitro* methods can predict human toxicity and that *in vitro* IC_{50} values correlate with *in vivo* LD_{50} data (Ekwall *et al.,* 1998; Clemedson *et al.,* 2000).

The rationale behind using cytotoxicity assays to predict *in vivo* toxicity stems from the concept of 'basal cell cytotoxicity'. It was suggested that for most chemicals, toxicity is an end result of non-specific change in cellular functions. In light of this, assessing the cytotoxic potential of compounds may possibly give an indication of their toxic potential *in vivo*. Cytotoxicity has been defined as the adverse effects resulting from interference of agents with structures and processes essential for cell survival, proliferation and function (Ekwall, 1983). One major factor that warrants consideration in a drug discovery programme is the toxic potential of new chemical entities (NCEs). At this stage, the rationale behind the screening for toxicity would not be directed towards predicting the extent and nature of all possible toxic effects *in vivo* but at the assessment of the risk of failure in *in vivo* studies.

The value of vertebrate cells in testing the toxic effect of substances came to light in the late 1960s/1970s when mammalian cell cultures were used to investigate potential effects posed to humans by chemicals in general (Rees, 1980). The term *in vitro* (literally in glass) refers to keeping entities, such as isolated cells of an organism, outside the living body in an artificial environment, in contrast to *in vivo*, i.e. in the organism. As such, cell cultures are used as *in vitro* models to mimic possible effects in the live animal. According to the use of *in vitro* terminology suggested by Schaeffer (1990), vertebrate cell lines are derived from primary cultures, taken directly from cells, tissues or organs of an organism. When a primary culture is successfully propagated into new culture vessels, it becomes a cell line and may be propagated a limited number of times (finite), or indefinitely, in which case it becomes an immortal or continuous (or permanent) cell line.

The use of vertebrate cells for predicting the toxicity of a substance in whole animals is based on the understanding that any interaction of a substance with an organism is initiated at the level of the cells. Toxic effects on cells by substances can translate to changes in tissue or organ function resulting in a detrimental effect on the whole organisms. Drug regulatory authorities in the USA and Europe emphasize the need for better non-animal alternative

test methods (Collins *et al.,* 2008; Xia *et al.,* 2008). Based on the central role of cells in the expression of toxicity, several mammalian *in vitro* models have received regulatory acceptance by the Organisation for Economic Cooperation and Development (OECD) as alternatives to whole animal tests. The European Commission also encourages the application and development of alternative models to the use of live animals in line with the new European legislation on the Registration, Evaluation and Authorisation of Chemicals (REACH) to be executed in an ethically and financially acceptable manner (Castaño *et al.,* 2003).

Besides their potential to replace or reduce animals in toxicity tests, cell cultures have several advantages compared to whole animal tests. For instance, small amounts of potentially toxic substances can be rapidly screened and analyzed in large numbers. With the quantity of test substance involved, less toxic waste is produced. The use of cells can also help identify the mechanisms underlying a toxic response. For instance, Noor *et al.* (2009), in a study using frequently used cytotoxicity assays, compared the toxic effect of different hepatotoxins on the human hepatoma cell line (HepG2) and rat hepatocytes. The study revealed that careful selection of assay parameters and inclusion of a kinetic time based assay improved prediction for non-metabolism mediated toxicity using HepG2 cells. With a better characterization of a wider range of cell culture models for use in *in vitro* tests, a more selective approach can be adapted in the choice of cells most suitable for the type of test to be conducted and the substance to be tested. Thus, to study the effect of medicinal plants (MPs) in cell culture, testing of extracts with a wide range of cell types should be undertaken, since cytotoxicity to a certain degree is cell-type-specific (Tang *et al.,* 2004). One should however, keep in mind that changes in the animal gut or pharmacokinetic issues such as absorption, distribution, metabolism and excretion may break the link between cellular and whole animal toxicity.

2.5. Antioxidants and cell culture

An antioxidant is defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge, 2007). Certain groups of compounds like flavonoids and other polyphenols have powerful antioxidant activities *in vitro*, by scavenging a wide range of reactive species, including hydroxyl radicals, peroxyl radicals, hypochlorous acid and superoxide radical (Rice-Evans, 2000). Flavonoids can also inhibit biomolecular damage by peroxynitrite *in vitro* (Pannala *et al.,* 1997; Heijnen *et al.,* 2001; Santos and Mira, 2004). However, this biomolecular damage is said to be less effective in the presence of physiological levels of HCO_3^-/CO_2 (Ketsawatsakul *et al.,* 2000; Santos and Mira, 2004). Figure 2.2. illustrates how cells respond to oxidative stress. Oxidative stress is an end result of a serious imbalance between reactive species (RS) production and antioxidant defence. Under such circumstances, cells elicit a wide range of responses as a result of this imbalance, ranging from increased proliferation, prevention of cell division, senescence, necrosis, apoptosis, or cell death mechanisms.

As such, antioxidants can be helpful to control the levels of free radicals and other RS to minimize oxidative damage, especially where imbalance between stress and protective elements *in vivo* is considered to play a role in disease development (Halliwell and Gutteridge, 2007). The beneficial effects of many medicinal plants may be via antioxidant activity due to high polyphenolic contents of these plants.

Cell culture is one of the most popular and commonly used methods to study the cellular effects of medicinal plants and chemical constituents isolated from them. In the human body, with the exception of the cells lining the corneal, skin and respiratory tract, most of the cells are exposed to O_2 concentrations in the range of 1–10 mm Hg while cells cultured under laboratory conditions of 95% air or 5% CO_2 experience about 150 mm Hg of O_2. Therefore, cells cultured under laboratory conditions are constantly under oxidative stress because the rate of reactive oxygen species (ROS) production from cellular enzymes increases when O_2 levels are increased (De Groot and Littauer, 1989; Halliwell, 2003). This phenomenon is most likely to increase their rates of ROS formation.

Due to the rich phenolic content of MPs, their oxidation in cell culture media can provoke biological effects in cultured cells. Such effects can make results of cell culture studies often confusing because of the oxidation process in the medium, especially when iron and other transition metals are present in the medium (Halliwell, 2003). Oxidation in culture media has also led to uncertain results in at least some studies of the effects of ascorbate, phenolic compounds and other antioxidants on cells. The observed effects due to oxidation following the addition of these compounds to culture media resulted in the production of H_2O_2 and other oxidation products (e.g. quinines and semiquinones from polyphenols) that were the true mediators of the effects observed (Halliwell, 2003; Long *et al.,* 2000; Lapidot *et al.,* 2002; Clement *et al.,* 2001; Chai *et al.,* 2003). Also H_2O_2 generation rates can be affected in complex ways when two or more antioxidants are present (Wee *et al.,* 2003). Some studies have shown that phenolic compounds are more stable in culture media such as F-10 and F-12 and can help minimize artefacts in cell culture associated with the use of DMEM (Long *et al.,* 2007).

To prevent oxidation in culture media, the addition of catalase or presence of pyruvate in some culture media can help scavenge H_2O_2 when generated. However, the absence of H_2O_2 in such media does not necessarily imply the lack of oxidation by medicinal plants in cell culture media (Long and Halliwell, 2009).

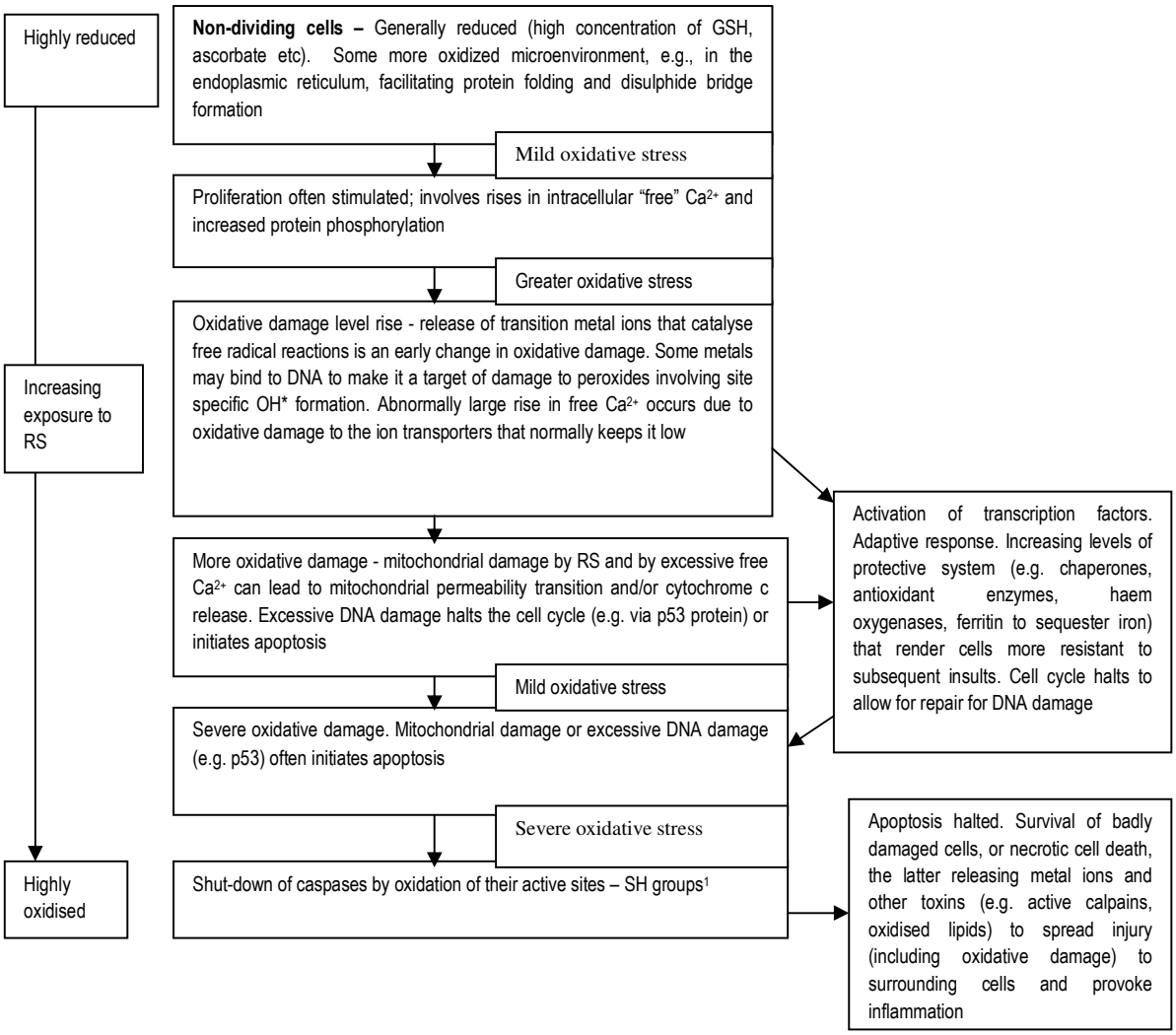

Figure 2.2: Response of cells to oxidative stress (Adapted from Halliwell and Gutteridge, 2007)

2.6. Hypothesis

1. Antibacterial and antifungal compounds can be isolated using bioassay-guided fractionation since activity against these pathogens is much easier to determine than antiviral activity.

2. The antibacterial and antifungal compounds isolated may have antiviral activity with relatively low toxicity. Some plant species have been shown to have antibacterial, antifungal and antiviral activity and an antibiotic has been reported as an effective prophylaxis in smallpox infection (Bauer *et al*., 1963).

2.7. Aim of the study

The aim is to develop a low toxicity plant extract or isolated compound that is effective against bacteria, fungi or viruses.

2.8. Objective

The aim of this study will be reached by addressing the following:

1. Determining antibacterial and antifungal activity of different leaf extracts of selected plant species
2. Determining the cytotoxic effect of different extracts on selected cell types compatible for the group of viruses chosen for later testing
3. Determining the antiviral activity of the different extracts on selected viruses
4. Selection of plant species to be investigated
5. Selection of the best extractant for high activity of the plant material
6. Isolation of antibacterial and antifungal compounds
7. Determining the chemical structure of isolated compounds
8. Determining the cytotoxic and genotoxic effects of isolated compounds
9. Determining the antibacterial, antifungal and antiviral activity of isolated compounds
10. Evaluating correlations between antiviral and antimicrobial activity

Chapter 3

Materials and Methods

3.1. Plant collection

The leaves of selected plants were collected when they were still green from the Pretoria National Botanical Garden (Gauteng province) and the Lowveld National Botanical Garden in the Mpumalanga province of South Africa in the month of April 2007. Voucher specimens are deposited in the herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

3.2. Plant preparation and storage

Due to fewer complications when working with dried plant material (Eloff, 1998a), leaves of plant materials were carefully examined and dried leaves, or fungus-infected leaves and twigs removed. Leaves were then evenly spread out in a drying room at room temperature for about 15 days. When completely dried, the leaves were milled into fine powder in a Jankel and Kunkel mill (Model A10) and properly sealed airtight in glass jars and stored in the dark.

3.3. Extraction of plant material

Milled plant materials were individually extracted (1 g in 10 mℓ) using acetone, hexane, dichloromethane (DCM) and methanol (technical grade, Merck) in glass centrifuge tubes. Tubes were vigorously shaken for 30 min on a Labotec model 20.2 shaking machine at moderate speed. Thereafter, the tubes were centrifuged at 5000 rpm (Rotofix 32 A-Germany) for 5 minutes and filtered through Whatman No. 1 filter paper into pre-weighed labelled containers. The process was repeated three times on the marc to exhaustively extract the plant material and the extracts were combined. The extracts were dried under a stream of cold air in a fume cupboard at room temperature and the quantity extracted per solvent was measured.

3.4. Thin layer chromatography (TLC) analysis of crude extracts

Extracts were reconstituted in acetone (10 mg/mℓ) and 10 µℓ of the reconstituted extract was spotted onto aluminium-backed TLC plates (Merck, silica gel 60 F254). The plates were developed for separation of constituents under saturated conditions in eluent solvent systems of varying polarities developed in the Phytomedicine

Programme, namely: ethyl acetate/methanol/water [EMW] [40:5.4:5] (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] [5:4:1] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide [BEA] [90:10:1] (non-polar/basic) (Kotze and Eloff, 2002).

Separated constituents were visualised under UV light at wavelengths of 254 nm and 365 nm (Camac Universal UV lamp TL-600). Vanillin, 0.1 g, was dissolved in 28 mℓ methanol and 1 mℓ of sulphuric acid was carefully added. Plates were then sprayed with the vanillin sulphuric acid spray reagent and heated at 100°C for five minutes to allow for development of colour.

3.5. Determination of qualitative antioxidant activity of extracts

The qualitative antioxidant activity of selected plants was carried out using the method of Deby and Margotteaux (1970). Thin layer chromatographic plates were prepared as described (section 3.4) to separate active constituents in the different extracts. For the determination of antioxidant activity, the plates were sprayed with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma®) in methanol as an indicator. A positive reaction is indicated by the appearance of a yellow spot against a purple background.

3.6. Solvent-solvent fractionation

The solvent-solvent group procedure employed by the United States National Cancer Institute as described by Suffness and Douros (1979) was used in this study. This procedure fractionates the components of an extract based on polarity and is represented in Fig 3.1.

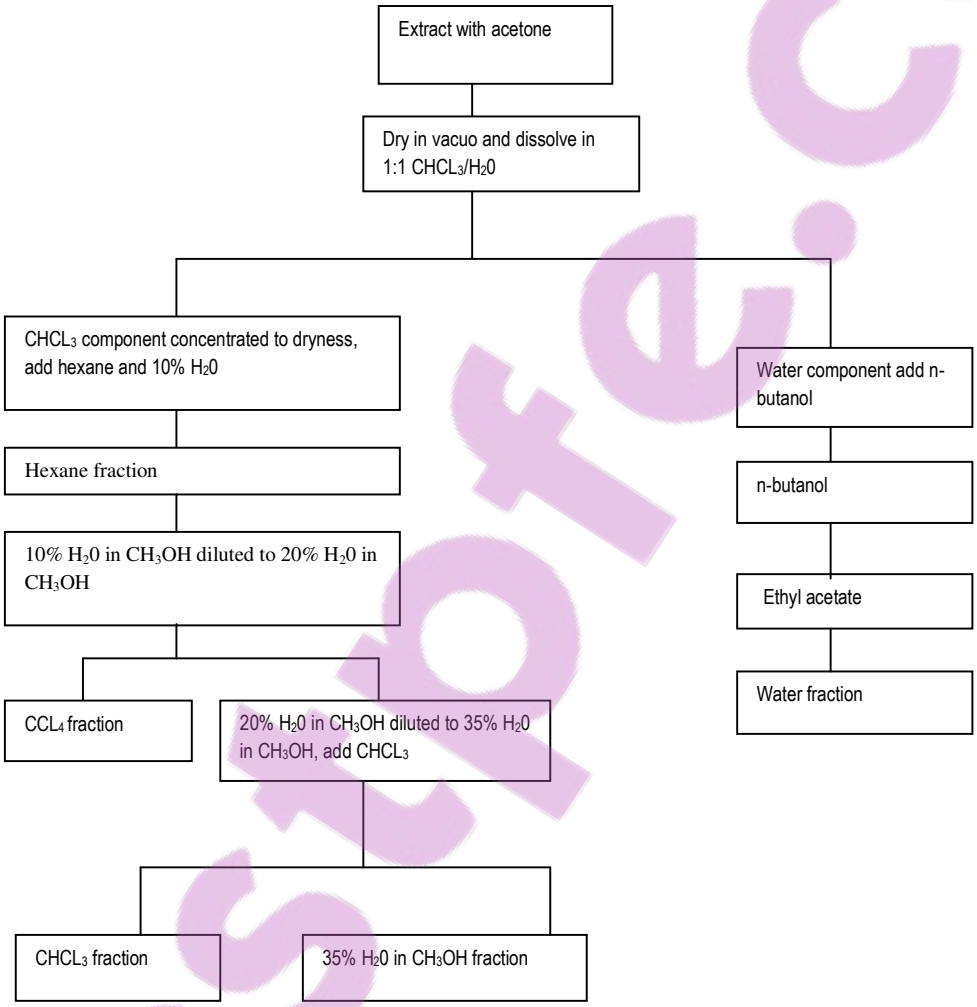

Figure 3.1: Flow chart of solvent-solvent fractionation of leaves

The grinded material (500 g) was extracted using acetone (1 g/10 ml) for 24 hours and dried in vacuo and dissolved in 1:1 chloroform/water. The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to

yield the n-butanol fractions and water. Ethyl acetate was added to the water fraction to yield the ethyl acetate and water fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water: methanol mixture. This yielded the hexane fraction and water methanol mixture was diluted to 20% water: methanol by addition of water. This was then mixed with carbon tetrachloride in a separatory funnel giving the carbon tetrachloride fraction. The 20% water methanol fraction was further diluted to 35% water: methanol and mixed with chloroform to yield the chloroform and water: methanol fractions. In all cases, equal volume of solvents was used and separation repeated with small quantities of solvents to facilitate separation.

3.7. Antibacterial activity

3.7.1. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 µℓ (10 mg/mℓ) of the extracts or fractions and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4). The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. Thereafter, the eluted TLC plates were sprayed with a concentrated suspension of the test organism prepared in Müller-Hinton (MH) broth in a Biosafety Class II cabinet (Labotec, SA). In this study, two Gram-positive bacteria *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213), and two Gram-negative species, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used. The sprayed plates were placed in a humid chamber (100% relative humidity) and incubated overnight at 37°C. Plates were then sprayed with a 2 mg/mℓ solution of *p*-iodonitrotetrazolium violet (INT, Sigma) and further incubated at 37°C until a purple-red colour change was evident. Retardation factor (R_f) values of inhibitory zones, depicted as white areas (Begue and Kline, 1972) where reduction of INT to formazan did not occur, were recorded.

3.7.2. Microdilution assay for MIC determination

The serial microtitre dilution method described by Eloff (1998b) was used to screen the plant extracts for antibacterial activity. This method is used to determine the minimal inhibitory concentration (MIC) of plant extracts against bacteria by measuring reduction of INT to a red formazan in wells where biological activity against test organisms is evident. Growth inhibition is observed in wells where there is non-reduction of INT to a red colouration. The organisms (section 3.7.1) were used to test the MIC of the extracts, fractions or pure compounds. The bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C. Overnight cultures were diluted 1:100 in MH and plated onto agar plates. The densities of the bacterial cultures before antimicrobial testing were approximately:

Enterococcus faecalis, 1.5 × 10^{10} cfu/mℓ; *Staphylococcus aureus*, 2.6 × 10^{12} cfu/mℓ, *Pseudomonas aeruginosa*, 5.2 × 10^{13} cfu/mℓ, and *Escherichia coli*, 3.0 × 10^{11} cfu/mℓ.

The assay was conducted in 96-well flat bottomed microtitre plates. Sterile water (100 µℓ) was added to all the wells of the microtitre plate. In row A, 100 µℓ of extract, fraction or pure compound was added using a micropipette. The contents of row A were thoroughly mixed using a micropipette and 100 µℓ transferred to row B. The process of dilution was continued until all the rows down the column were completed and 100 µℓ of the last dilution was discarded. After the serial dilution process, 100 µℓ of test organisms were added to all the wells except for the negative control wells and incubated at 37°C overnight. Thereafter, 40 µℓ of 0.2 mg/mℓ INT were added and reincubated to ensure adequate colour change. Two wells were used as sterile control containing only water. The growth control contained both water and test organism. Gentamicin was used as a positive control. MIC readings were recorded after 12 and 24 hours of incubation. Tests were carried out in triplicate and each experiment was repeated three times.

3.8. Antifungal assay

3.8.1. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 µℓ (10 mg/mℓ) of the extracts or fraction and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4). The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. The fungal pathogens used in this study were *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. The pathogens were obtained from clinical cases of disease in animals and were kindly provided by Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *C. albicans* was isolated from a Gouldian finch, *C. neoformans* from a cheetah, and *A. fumigatus* from a chicken. Eluted TLC plates were sprayed with a concentrated suspension of the actively growing organisms prepared in Sabouraud Dextrose (SD) broth in a Biosafety Class II cabinet (Labotec, SA). The sprayed plates were placed in a humid chamber (100% relative humidity) and incubated overnight at 30°C. Plates were then sprayed with a 2 mg/mℓ solution of INT and further incubated at 30°C until a purple-red colour change was evident. R_f values of inhibitory zones, depicted as white areas where reduction of INT to formazan did not occur, were recorded.

3.8.2. Microdilution assay for MIC determination

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko and Eloff (2005) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of extracts, fractions or isolated compounds. Two-fold serial dilutions of test substances (10 mg/mℓ) dissolved in acetone were prepared in 96-well microtitre plates (section 3.9.1.1). Actively growing organisms (section 3.8.1) were transferred from SD agar plates using a sterile cotton swab into fresh SD broth. Densities of fungal cultures used in bioautography and for MIC determinations were as follows: *C. albicans*, 2.5×10^6 cfu/ml; *C. neoformans*, 2.6×10^6 cfu/ml; *A. fumigatus*, 8.1×10^6 cfu/ml. *Candida albicans* was diluted to a density of about 2.5×10^4 cfu/ml, *C. neoformans*, 2.6×10^4 cfu/ml, and *A. fumigatus* 8.1×10^4 cfu/ml. This suspension (100 µℓ) was added to each well. INT was then added and plates were incubated. Microtitre plates were incubated at 30°C for 24 to 48 hours. INT was used as an indicator of growth as previously described. Sterile controls were included as described (section 3.7.2). Amphotericin B (0.08 mg/mℓ), a standard antifungal agent, was included as a positive control. Each experiment was repeated three times.

3.8.3. Determination of total activity

The total activity of plant extracts and fractions was determined using the method of Eloff (2004). Total activities of an extract or fraction give an indication of the efficacy at which active constituents present in one gram can be diluted and still inhibit the growth of test organisms. This value is calculated in relation to the MIC value of the extract, fraction or compound and expressed mathematically as follows:

Total activity = amount extracted from 1 g (mg) or amount present in fraction (mg) / MIC (mg/mℓ)

3.9. Determination of cytotoxicity of extracts, fractions and pure compounds (MTT)

The cytotoxic effect of extracts, fractions or pure compounds was tested against the Vero monkey kidney cell line, Crandell feline kidney cells (CRFK) and bovine dermis cells. The cells used in this study were kindly provided by the Department of Veterinary Tropical Diseases, University of Pretoria. Cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cultures for the assay were prepared from confluent monolayer cells, seeded at a density of 4.8×10^4 cells per well in 96 well microtitre plates and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow attachment of the cells.

Dried crude plant extracts (100 mg) were reconstituted in 1ml DMSO and 10 fold serially diluted while pure compound (2 mg) was reconstituted in 0.1 ml of DMSO, and 2-fold serial dilutions of each test substance were prepared in growth medium. The growth medium on sub-confluent monolayer cells grown overnight in microtitre plates was removed and cells were exposed to 200 µℓ of the extracts, fractions or pure compound at different concentrations and incubated at 37°C in a 5% CO_2 atmosphere for 5 days. Thereafter, the extract-containing medium on the cells was replaced with fresh culture medium. Viability of cells was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983). The assay is based on mitochondrial dehydrogenase activity which is assessed by the reductive cleavage of the tetrazolium salt MTT (3-Ž4, 5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) by the succinic dehydrogenase present in living cells to yield a purple formazan dye. Cells were then exposed to 30 µℓ of 5 mg/mℓ MTT dissolved in phosphate buffered saline (PBS) and reincubated for four hours under the same conditions. The medium containing MTT was then removed and 50 uℓ DMSO was added to each well and the plates were gently rocked to dissolve the formazan crystals. The optical density (OD) was measured at a wavelength of 570 nm and a reference wavelength of 630 nm. The cytotoxicity was expressed as 50% cytotoxic concentration (CC_{50}) of substances to inhibit the growth of cells by 50%, when compared to untreated cells, calculated from the linear regression equation. Berberine chloride (Sigma) was used as a positive control; wells containing only cells without extract treatment were the negative control and a solvent control was also included.

3.10. Genotoxicity testing of isolated compounds

The isolated compounds were investigated for their potential mutagenic effect using the plate incorporation procedure described by Maron and Ames (1983). The assay was performed using *Salmonella typhimurium* strains TA98 and TA100. Aliquots of bacterial stock (100 µℓ) were incubated in 20 mℓ of Oxoid Nutrient broth for 16 h at 37°C on an orbital shaker. The overnight culture (0.1 mℓ) was added to 2 mℓ top agar (containing traces of biotin and histidine) together with 0.1 mℓ test solution (pure compounds, solvent control or positive control) and 0.5 mℓ phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 h at 37°C. Following incubation, the number of revertant colonies (mutants) was counted. All cultures were prepared in triplicate (except the solvent control where five replicates were prepared) for each assay. The assays were repeated twice. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/mℓ.

3.11. Antiviral assay

3.11.1. Cell cultures and viruses

Viruses and host cells were kindly provided by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Enveloped viruses, namely feline herpes virus–1 (FHV-1, dsDNA), canine distemper virus (CDV, ssRNA), canine parainfluenza virus-2 (CPIV-2, ssRNA) and lumpy skin disease virus strain V248/93 (LSDV, dsDNA) were used in the study. These groups of viruses were chosen for this study because entry of enveloped viruses into their host cells involves several successive steps, each one being open to therapeutic intervention. Inhibitors that prevent entry of the virus into host cells act by targeting viral and/or cellular components, through either the inhibition of protein-protein interactions within the viral envelope proteins or between viral proteins and host cell receptors, or through the inhibition of protein-lipid interactions.The susceptible cell types compatible for the growth of the viruses were Crandell feline kidney cells (CRFK), Vero cells and bovine dermis cells, respectively.

Cells were cultured in 75 cm^3 culture flasks and maintained in minimum essential medium (MEM, Highveld Biological) containing 5% fetal calf serum (FCS, Highveld Biological) supplemented with 0.1% gentamicin (Virbac) and incubated at 37°C in an atmosphere of 5% CO_2. Compatible cells for the growth of each virus were inoculated using 0.5 mℓ of infective virus, reincubated and observed daily for evidence of cytopathic effect (CPE). Following the development of 90% CPE, infected culture flasks were frozen at -70°C for 20 min and thawed. The process was repeated three times to ensure adequate release of viruses from cultured cells into the growth medium. The contents of the flask containing cells, virus and medium were centrifuged at 800 rpm for 10 min. The supernatant containing the virus was collected in cryotubes and preserved in liquid nitrogen until use. The effective titre ($TCID_{50}$/mℓ) of each virus was determined using the method of Reed and Muench (1938) prior to each assay.

3.11.2. Virucidal assay

The virucidal activity of plant extracts was evaluated using the method described by Barnard *et al.* (1992) with slight modifications. Non-cytotoxic concentrations of extracts, solvent–solvent fractions or pure compound were serially diluted 10-fold in MEM containing 5% FCS in 2 mℓ Eppendorf tubes and an equal volume of virus (20 µℓ) at an infective titre of 10^2 $TCID_{50}$ /mℓ was added to each concentration and incubated at 37°C for various time intervals ranging from 1 to 3 h. Growth medium on confluent cell monolayers grown in 96-well plates was removed and cells were exposed to 200 µℓ of the extract-virus mixture at each concentration in quadruplicate and incubated until CPE was observed, which was mostly between 1 and 5 days. The negative control comprised non-infected and untreated

cells while positive controls consisted of infected and untreated cells. The extent of cell damage was determined by the presence of CPE when compared to infected untreated and uninfected untreated controls by microscopic examination as well as the MTT colorimetric assay. The degree of cell destruction by microscopic examination was scored using reduction indexes described by Vanden Berghe *et al*., (1993). Plant extracts exhibiting reduction of viral infectivity at concentrations of 10^3 and 10^4 dilutions indicate strong activity while those with 10^2 to 10 as moderate to weak activity respectively. For the MTT assay, antiviral activity was expressed as a selectivity index (SI), which is the value of cytotoxic concentration (CC_{50}) divided by effective concentration (EC_{50}). Selectivity index values of more than three indicate potential antiviral activity of the test extract (Chattopadhyay *et al.*, 2009). The EC_{50} was calculated from the regression equation as follows:

EC_{50} = [(ODt)v – (ODc)v] / [(ODc)mock – (ODc)v] * 100

where (ODt) v is the optical density of the cells treated with virus and substance, (ODc) v is the optical density of the cells treated with virus (virus control), and (ODc) mock is the optical density of the mock infected cells (cell control).

3.11.3. Attachment assay

The ability of the viruses to attach to the host cell was tested using the method of Barnard *et al*., (1993) with slight modifications. Cells appropriate for the growth of each virus were seeded in 96-well flat-bottomed microtitre plates and incubated at 37°C in a 5% CO_2 incubator to attain an 80% confluent monolayer. Medium on the cells was removed and equal volumes of virus (50 µℓ) at an infective titre of 10^2 $TCID_{50}$/ml was added to cells and incubated at different time intervals from 1-3 h. Thereafter, cells were washed with phosphate buffered saline (PBS) to remove the unattached virus. Ten-fold serially diluted extracts, solvent-solvent fractions or pure compound at non-cytotoxic concentrations were added to cells and incubated again at 37°C in an atmosphere of 5% CO_2 and observed daily for evidence of CPE. The ability of the extract to prevent subsequent replication of the virus in host cells was scored microscopically and by the MTT assay. Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells. Antiviral activity by CPE reduction and MTT assay was determined as previously described (section 3.11.2).

Chapter 4

Comparative cytotoxicity studies of extracts of selected medicinal plants on different cell types

4.1. Introduction

Naturally derived plant products with medicinal value play an important role in health care systems, both in humans and animals. This is evident with the growing interest in their utilisation on a global perspective in the treatment of different ailments (Farnsworth and Morris, 1976; Farnsworth and Soejarto, 1985; Farnsworth, 1988; Balandrin, *et al.,* 1993). In the crude form, medicinal plants contain a diverse structural array of compounds with varying chemotherapeutic relevance that is harnessed traditionally through various modes of preparation. However, our current perspective of the cytotoxic effects of most medicinal plants utilised in traditional health systems is at a rudimentary stage including their long term effects on the majority of the population in resource poor settings who rely on these plants for solving their health problems.

The utilization of medicinal plants for the treatment of various ailments and the actualization of a non-toxic effect of these remedies depends on the contribution of various organs in the body. Even though the liver is the principal organ of metabolism-mediated clearance, the kidney possesses a distinctive physiology and metabolic pathways (Lohr *et al*., 1998) that help in the uptake, metabolism and elimination of various drugs and other chemicals from the body. A breakdown in the normal functioning of the kidney will result in renal selective toxicity, leading to the accumulation of these chemicals within the cells of the kidney.

The skin on the other hand is directly exposed to ultraviolet light, ozone and other environmental stress. These stress conditions can result in the generation of free radicals and reactive oxygen species (ROS) which are considered to be involved in inflammatory disorders and aging of the skin (Cross *et al.,* 1987). The skin is the first area of contact of any topically applied substance. Skin disorders such as burns, wounds, psoriasis, eczema, and fungal infections are some of the diseases for which traditional medicine has played a significant role and the relevance of the practice remains high (Alemayehu, 2001; Subbarayappa, 2001). Metabolism of substances in the

skin may play a role in the manifestation or amelioration of adverse effects through the topical route. It is of relevance under these circumstances to examine the effect of substances on the epidermis and dermis owing to the fact that different pathologies affect different layers, and the different layers of the skin have different functions.

A practical approach is needed for the safe evaluation of medicinal plants that can have potentially fatal adverse effects and have been presumed to contain acceptable toxicity profiles due to long term use. The proposition that drug toxicity should not only be defined solely by dose – response relationship, but also as a function of pharmacology, chemistry, metabolism, environmental and genetic risk factors (Li, 2004) warrants a thorough investigation of the toxic effects of medicinal plants. Since different constituents are present in crude plant extracts, drug–drug interactions based on pharmacological properties of inherent constituents can play a significant role in the safety or cytotoxic effects of extracts. As such, the understanding that drug toxicity invariably correlates with or the lack of metabolic conversion within the body (Koppal, 2004) may serve as a useful tool in the testing of the toxic effect of medicinal plants in vitro.

In work done in the Phytomedicine Programme we have focussed on selecting plants with high antimicrobial activities. Due to the difference in polarity of solvents and type of active constituents extracted from crude plant material (Eloff, 1998 Kotze and Eloff, 2002), the possibility is high that a similar phenomenon may influence the toxic effect of a plant extract depending on the type of solvent used for extraction. In previous projects, we have usually only determined the cellular toxicity at the end of the study. In many cases extracts with very promising activity were too toxic to use in further studies. The approach in this study was to determine toxicity at an early stage to select the best species for in depth further work entailing either isolating the bioactive compounds or by manipulating the extract to increase activity. If an extract contains a general metabolic toxin it would affect fungal as well as animal cells. It would be better to have an extract with a lower activity and higher safety because that indicates selective toxicity against the pathogen. The measurement of the viability of cells in culture has been evaluated by the metabolic reduction of soluble tetrazolium salt to insoluble formazan as a means of histochemical localization of enzyme activity in viable cells (Mosman, 1983; Alley *et al*., 1986). Hence, simultaneous cytotoxicity testing using different test systems is one way of testing the toxic effect of plant extracts which can provide information on the selective activity of the test substances on pathogens. This study was therefore aimed at evaluating the toxic effects of different extracts of seven South African medicinal plant species on Vero, Crandell feline kidney and bovine dermis cells in an *in vitro* toxicity study.

The antioxidant activity of extracts was also determined to investigate a possible correlation between good antioxidant activity and lower cytoxicity.

4.2. Materials and Methods

4.2.1. Plant collection and preparation

The plants used in this study were selected based on their traditional use in the treatment of various ailments and are represented in Table 4.1, together with their ethnomedicinal indication. The plants were collected and extracted using solvents of varying polarity as described in sections 3.1 and 3.2.

4.2.2. Determination of qualitative antioxidant activity of extracts

Thin layer chromatography plates (10 x 10 cm) were spotted with 100 µg (10 µℓ of 10 mg/mℓ) of the extracts as described in section 3.4 and sprayed with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma®) in methanol as an indicator of antioxidant activity as described in section 3.5. Masoko, *et al*., (2005).

4.2.3. Determination of cytotoxicity of extracts

The cytotoxic effect of the different extracts of plants selected for the study was evaluated on Vero, CRFK and bovine dermis cells using the method of Mosmann (1983) as described in section 3.9.

Table 4.1. Plants used in the study and their ethnomedicinal indication

Plant name	Family	Voucher specimen number	Plant part	Indication	Reference
Acokanthera schimperi (A.DC) Benth. Var.rotundata Codd	Apocynaceae	NBG 584177	Leaves	For the treatment of headache, epilepsy, amnesia, eye disease syphilis, rheumatism	Abebe and Ayehu, 1993
Carissa edulis (Forssk.) Vahl.	Apocynaceae	PBG841631		Schistosomiasis	Ndamba *et al.,* 1994
Ekebergia capensis Sparrm	Meliaceae	NBG1322	Roots	Gastritis, hyperacidity, coughing	Pujol, 1990
Podocarpus henkelii Stapf ex Dallim. & jacks	Podocarpaceae	PBG818945	Bark and Sap	Canine distemper	Watt and Breyer-Brandwijk, 1962; Dold and Cocks, 2001
Plumbago zeylanica L.	Plumbaginaceae	NBG307004	Roots	Chest pain, gall-sickness (animals) Pneumonia	Van der Merwe *et al*., 2001
Schrebera alata (Hochst.) Welw.	Oleaceae	PBG584579	Leaves	-	-

4.3. Results and Discussion

4.3.1. Effect of extracting solvents on yield of extracts

Water is mostly used in folk remedies for extraction; however, this solvent does not extract a wide range of active constituents contained in plants (Eloff, 1998c). To target polar and non-polar constituents for bioactivity testing in this study, leaves of each selected plant were extracted using hexane, DCM, acetone and methanol separately. The yield of extracts varied with the type of solvents used (Figure 4.1). In most cases, methanol extracted the highest quantity followed by acetone, and hexane the least. Only with DCM extracts of *Annona senegalensis* and *Plumbago zeylanica,* and the hexane extract of *Carissa edulis,* was the yield higher than extracts prepared with acetone (Figure 4.1). The high extraction yield obtained with methanol may be related to the possible presence of a large quantity of more polar compounds in the selected plants. It may also correlate with the season of the year with leaves containing larger quantities of carbohydrates.

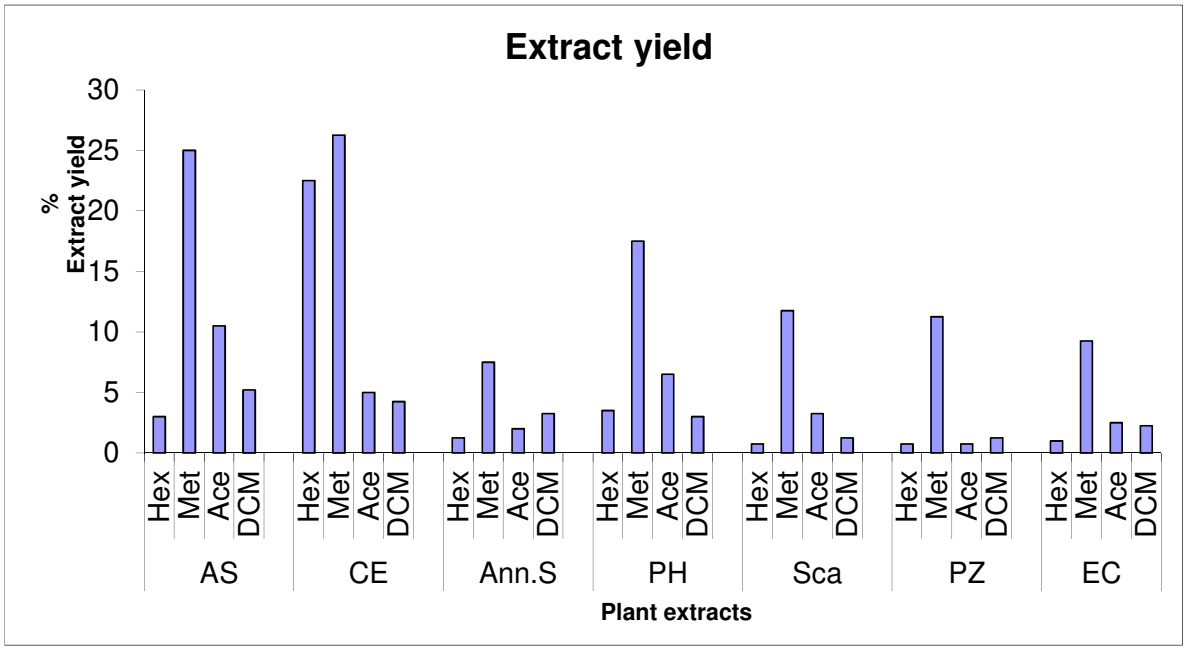

Figure 4.2. Percentage yield of plant material extracted using four different solvents for extraction. *As = Acocanthera schimperi, CE = Carissa edulis, Ann.s = Annona senegalensis, PH = Podocarpus henkelii, SCa = Schrebera alata, Pz = Plumbago zeylanica, EC = Ekebergia capensis, Hex = hexane, Met = methanol, Ace = acetone, DCM = Dichloromethane*

4.3.2. Cytotoxic effect of extracts on cells

4.3.2.1 Microscopic determination of cytotoxic effect of extracts of all the plants on different cell type

Apart from maintaining the stability of compounds in plant extracts during the extraction process (depending on whether they are thermo stable or labile), the choice of solvent used for extraction of plant material can also depend on what is intended with the extract (Eloff, 1998c). Different solvents, depending on their polarity, extract varying quantities of components in crude plant material that may be beneficial or harmful to biological systems. Hexane for instance extracts waxes, fats, and fixed oils while acetone extracts alkaloids, aglycones and glycosides. On the other hand, methanol extracts sugars, amino acids and glycosides while DCM will commonly extract alkaloids, aglycones and volatile oils (Houghton and Raman, 1998).

The cytotoxic effects of the hexane, DCM, acetone and methanol extracts of selected plants at concentrations ranging from 1 mg/ml to 0.001 mg/mℓ were tested on Vero, bovine dermis and CRFK cells by microscopic evaluation and using the MTT assay. Percent cell viability by microscopic evaluation was scored on a 5-point scale at different extract concentrations (5 = excellent and 1 = poor cell viability). According to the 5-point scale score (Table 4.2).

Table 4.2. Comparison of the cytotoxic effect of extractants at varying concentrations on different cell types based on a five point safety scale after microscopic evaluation (1–5)

Concentration (mg/ml)		1			0.1			0.01			0.001		
		Cells											
Plants		Vero	CRFK	B.D	Vero	CRFK	B.D	Vero	CRFK	B.D	Vero	CRFK	B.D
Plumbago zeylanica	Hexane	1	1	1	5	3	4	5	5	5	5	5	5
	DCM	1	1	1	1	2	1	5	5	5	5	5	5
	Acetone	1	1	1	1	1	2	5	3	5	5	4	5
	Methanol	3	1	2	3	1	4	5	5	5	5	5	5
Carissa edulis	Hexane	1	1	1	5	2	5	5	5	5	5	5	5
	DCM	1	1	1	1	2	1	1	5	3	3	5	5
	Acetone	1	1	1	5	1	5	5	3	5	5	3	5
	Methanol	1	1	1	1	5	5	4	5	5	4	5	5
Ekerbergia capensis	Hexane	1	1	1	3	5	1	5	5	5	5	5	5
	DCM	1	1	1	1	1	1	5	5	3	5	5	4
	Acetone	1	1	1	2	1	1	4	3	4	5	4	4
	Methanol	2	1	1	4	1	1	4	4	4	5	5	5
Annona senegalensis	Hexane	1	1	1	2	1	1	3	1	1	4	2	2
	DCM	1	1	1	1	1	1	4	2	1	5	2	1
	Acetone	1	1	1	2	1	1	3	1	1	4	1	1
	Methanol	1	1	1	1	1	1	1	1	1	1	2	2
Podocarpus henkelii	Hexane	1	1	1	5	1	3	5	4	4	5	4	5
	DCM	1	1	1	1	1	1	5	4	5	5	4	5
	Acetone	1	1	1	5	1	4	5	4	5	5	4	5
	Methanol	1	1	1	1	1	4	5	1	5	5	4	5
Schrebera alata	Hexane	1	1	1	5	3	3	5	5	5	5	5	5
	DCM	1	1	1	5	1	1	5	2	4	5	4	5
	Acetone	1	1	1	5	1	3	5	3	5	5	4	5
	Methanol	1	1	1	4	2	4	5	5	5	5	5	5
Acokanthera shimperi	Hexane	1	1	1	4	2	2	4	4	5	5	4	5
	DCM	1	1	1	1	1	1	1	1	1	3	1	1
	Acetone	1	1	1	1	3	1	1	4	1	1	4	1
	Methanol	1	1	1	1	3	2	1	5	5	1	5	5

To compare data for the different extractants, values for all the cell types and each plant specie at different concentrations from Table 4.2, were added (Table 4.3).

Table 4.3. Relative cytotoxicity of different extractants of plants on the different cell types and plant species at different concentrations

	Concentration (mg/mℓ)				
Extractant	1	0.1	0.01	0.001	Total
Hexane	21	65	91	96	273
DCM	21	27	72	83	203
Acetone	21	47	75	80	223
Methanol	25	50	81	89	245

In general, total values indicate the degree of toxic effect of solvent, plant or susceptible nature of cells to the different extracts. Low values represent high toxicity. In general the hexane extracts were the least toxic indicating that highly polar compounds were not toxic, possibly because they could not be absorbed through membranes. The intermediate polar extracts were generally the most toxic, possibly again because these compounds are better absorbed.

To determine which of plant had the lowest toxicity value for all the cell types at different concentrations, percent cell viability score by microscopic evaluation of the three cells of each plant specie from Table 4.2, were added (Table 4.4)

Table 4.4. Relative cytotoxicity of extracts of different extractants and cell types of different plant species at different concentrations

Plants	Concentration (mg/mℓ)				Total
	1	0.1	0.01	0.001	
Plumbago zeylanica	15	28	58	59	160
Carissa edulis	12	38	51	55	156
Ekebergia capensis	13	22	51	57	143
Annona senegalensis	12	14	20	27	73
Podocarpus henkelii	12	28	52	56	148
Schrebera alata	12	37	54	58	161
Acokanthera shimperi	12	22	33	36	103

Because low values are associated with toxicity, *Annona senegalensis* and *Acokanthera shimperi* extracts were the most toxic of plants of all the plants evaluated. (Table 4.4). These plants are toxic to animals and the cytoxicity is in line with the *in vivo* toxicity. P zeylanica and S. alata were the least toxic with C. edulis and P. henkelii having close to the same safety. C. edulis fruit are edible and the cytotoxicity data reflect this. Cells were more tolerant to the toxic effect of extracts at 0.01mg/mℓ and below in those plants that had moderate toxicity.

To determine which cells were the most sensitive all values for the different plant species and extractants from Table 4.2 were added (Table 4.5).

Table 4.5. Relative cytotoxicity of extracts of different extractants and different plant species on three cell types tested at different concentrations

Cell types	Concentration in (mg/mℓ)				Total
	1	0.1	0.01	0.001	
Vero	31	76	111	121	339
CRFK	28	49	100	111	288
B.D	29	64	108	116	317

Of the three cell types used CRFK was slightly sensitive followed by BD and Vero cells. This pattern was valid for all the concentration tested (Table 4.4). Vero and CRFK cells are both kidney derived cells, and may therefore be expected to show a similar response to the toxic effect of the extracts, but this was not the case in this study. At the highest concentration (1 mg/mℓ), all the extracts were very toxic to the cells with three exceptions where the methanol extracts were less toxic (Table 4.2). At the lowest concentration tested 0.001 mg/ml in most cases there was little cytotoxicity.

4.3.2.2. Determination of cytotoxicity by MTT assay

4.3.2.2.1. *Plumbago zeylanica*

Vero cell viability based on MTT assay following exposure to the different extracts of the same plant is presented in Figure 4.2. At the highest concentration (1 mg/mℓ), the hexane, DCM and acetone extracts of *Plumbago zeylanica* exhibited deleterious effects on the viability of Vero cells. However, with this species, the methanol extract at the

same concentration showed sustained cell viability of more than 40% (Fig. 4.2). At concentrations below 0.01 mg/mℓ, all the extracts had little effect on Vero cell viability. Unlike in the case of Vero cells, where the methanol extracts sustained cell viability, all the extracts showed deleterious effects on CRFK cells at 1 mg/mℓ (Fig. 4.3). The toxic effect at this concentration (1 mg/mℓ) was observed with the different extracts of all the tested plant extracts on CRFK cells. At concentrations where viability was sustained, a variation in the cytotoxic effect of extracts was observed between cells as could be seen with the methanol and hexane extracts on Vero, CRFK and bovine dermis cells at 0.1 mg/mℓ (Figs. 4.2, 4.3 and 4.4). At this concentration, the acetone and DCM extracts were toxic to all the cell types, with sustained cell viability only at lower concentrations (Table 4.2). Worthy of note is the cytotoxic effect of the methanol extract of *Plumbago zeylanica* on CRFK cells (Fig 4.3), which was not the case with Vero and bovine dermis cells at 0.1 mg/mℓ. A plausible reason for the observed effect may be related to the presence or lack of metabolizing enzymes that may influence the toxic effect of constituents present in extracts by CRFK cells, or a difference in bio-metabolism processes of substances in a variety of cell types. The hexane and acetone extracts of this plant at concentrations of 0.01 and 0.001 mg/mℓ showed increased cell viability of CRFK and Vero cells of more than 100% (Fig 4.2). This increase in cell viability at similar concentrations was also observed with extracts of the different plants and cell (Figs 4.2, 4.3 and 4.4). The proliferation in viable cells at these concentrations was very interesting to note. Some plant extracts may reduce MTT in the absence of cells (Shoemaker *et al*., 2004). Thus, the extracts were incubated in the absence of cells and the absorbance values subtracted from absorbance values after incubation in the presence of cells. Since medium-containing extracts were removed prior to the addition of MTT, it is unlikely that the extracts may have reduced MTT. This increase in viable cells at low concentrations may suggest a possible mitogenic effect or induction of expression of growth-stimulating substances evident by an increase in mitochondrial dehydrogenase activity as measured by MTT reduction.

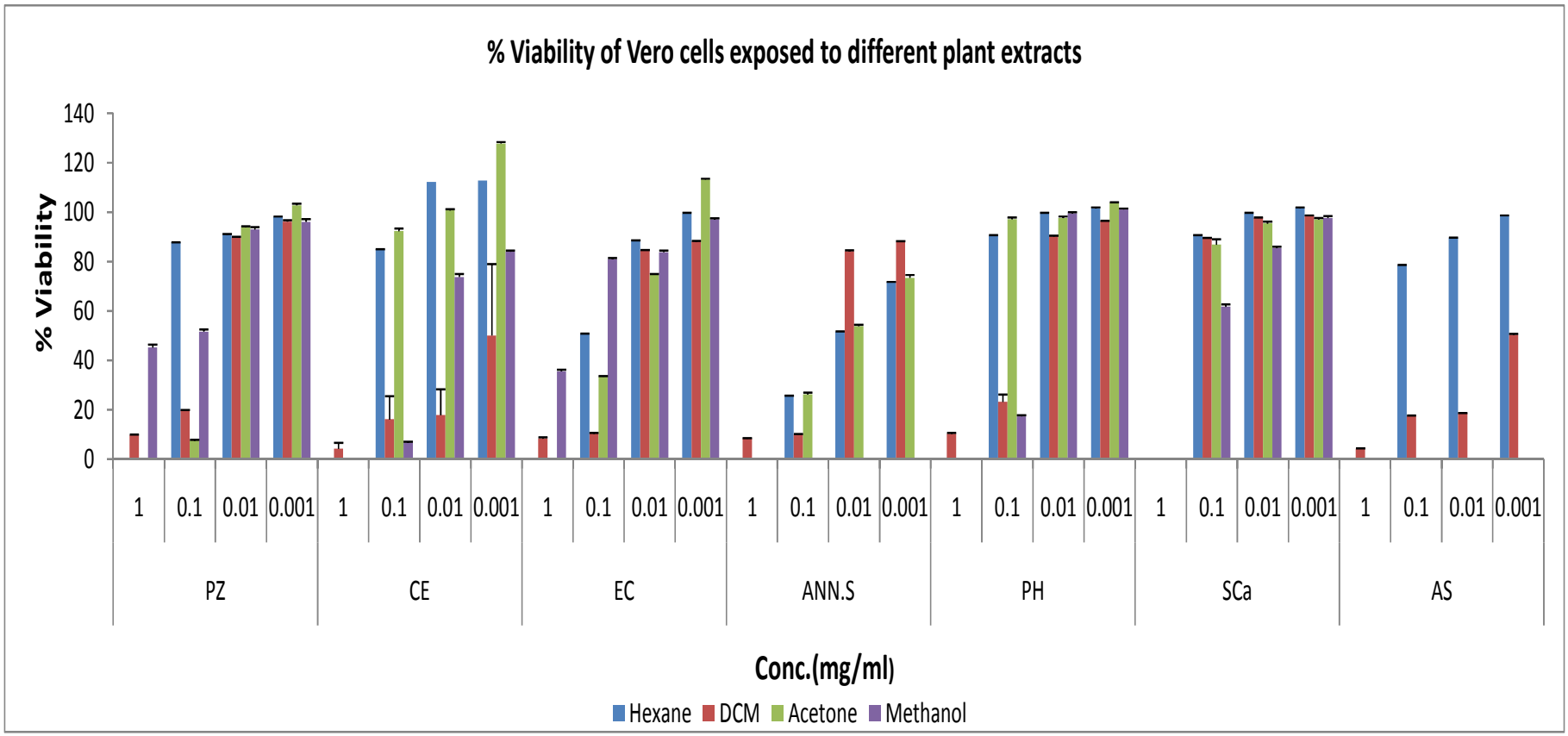

Figure.4.2: Viability of Vero cell exposed to extracts of different plant species extracted using solvents of varying polarity PZ = *Plumbago zeylanica*

CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* Sca = *Schrebera alata,*
AS = *Acokanthera schimperi*

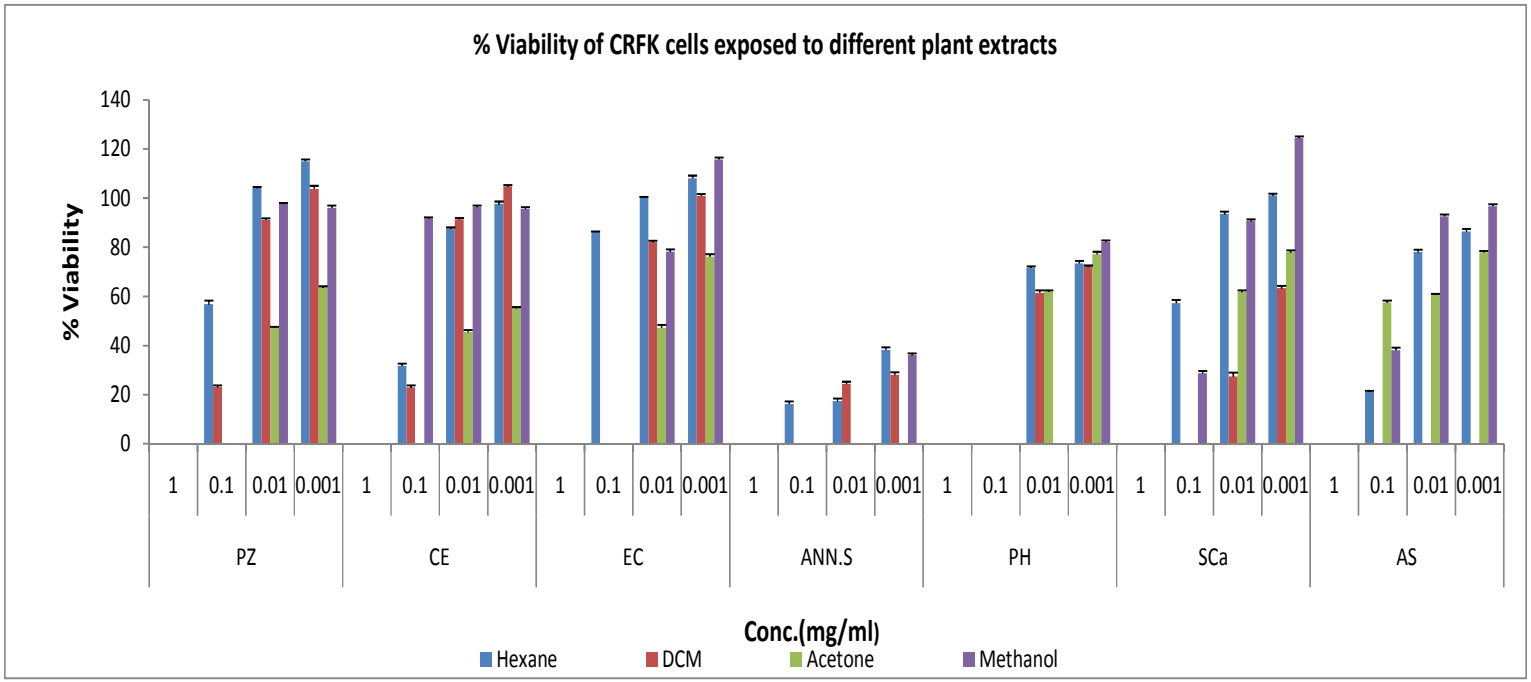

Figure 4.3: Viability of CRFK cell exposed to extracts of different plant species extracted using solvents of varying polarity, PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,*PH = *Podocarpus henkelii,* Sca = *Schrebera alata,* AS = *Acokanthera schimperi*

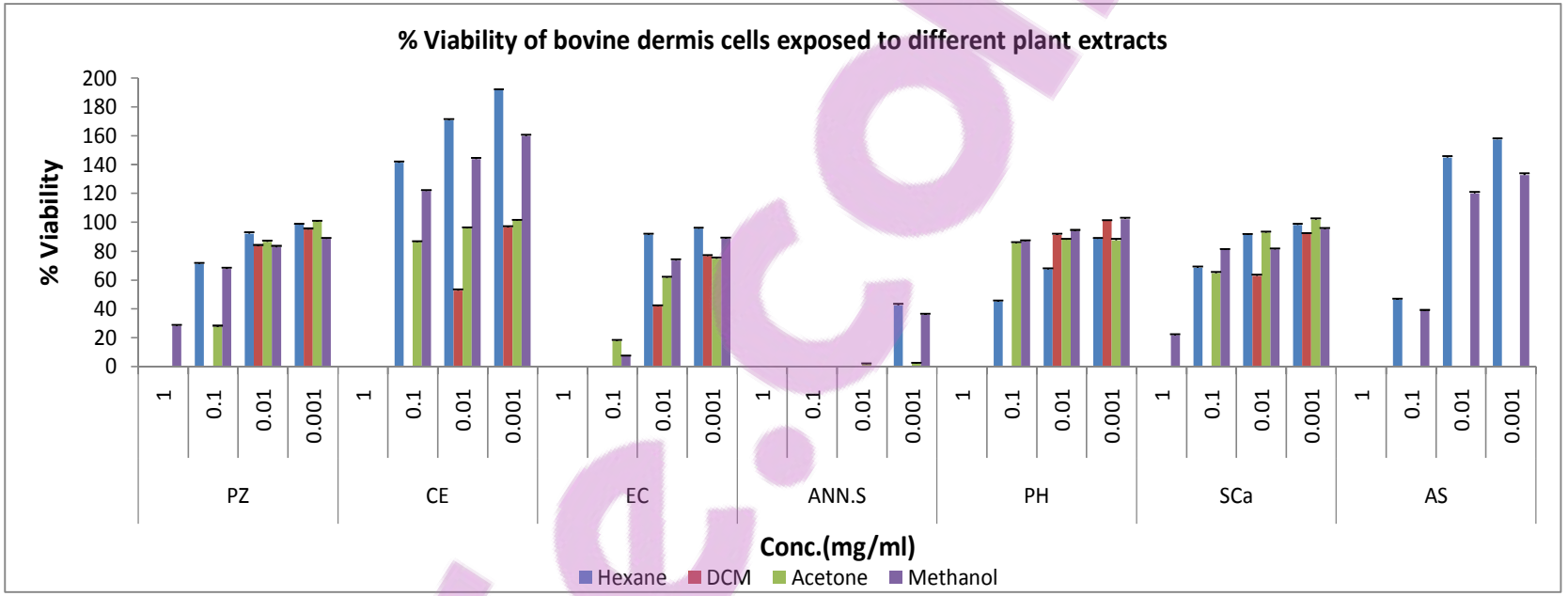

Figure.4.4: Viability of bovine dermis cell exposed to extracts of different plant species extracted using solvents of varying polarity PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* Sca = *Schrebera alata,* AS = *Acokanthera schimperi*

The influence of serum on the MTT assay using cultured smooth muscle cells at 5% and 10% serum concentration has been reported (Zhang and Cox, 1996). In that study, the increase of 20% cell viability at 10% serum concentration in the MTT assay when counted using a haemocytometer led to no difference in total mitochondrial activity per cell. It is however, not clear whether the increase in cell viability observed at this concentration is due to unknown factors in serum necessary for the maintenance of the homeostatic mechanisms of the cells or a protective or inductive phenomenon resulting from the presence of one or more constituents present in these extracts. Factors that may appear to have some mitogenic activity may make the cells responsive to growth factors present in the serum-extract medium. Nonetheless, earlier studies to investigate the growth stimulating effect on cells show that growth promoting substances exhibit a high degree of specificity with varying cell types (Temin *et al.*, 1972).

4.3.2.2.2. *Ekebergia capensis*

The pattern of cell viability observed with the hexane and methanolic extracts of *Ekebergia capensis* on Vero and CRFK cells was similar to that of *Plumbago zeylanica* although percent viability and CC_{50} values varied in some extracts (Table 4.3). With this species, only the hexane extract showed viability at 0.1 mg/ml on CRFK cells while on bovine dermis cells (Fig. 4.4), none of the extracts was shown to sustain cell viability at 0.1 mg/mℓ. At lower concentrations however, all the extracts exhibited sustained cell viability on all three cell types.

4.3.2.2.3. *Annona senegalensis*

Extracts of *Annona senegalensis,* at 1 mg/mℓ and 0.1 mg/mℓ were cytotoxic to the cell types used in this study. However, at concentrations below 0.1 mg/mℓ, where the hexane, DCM and acetone extracts of this plant were toxic to CRFK and bovine dermis cells (Figs. 4.3 and 4.4), these extracts sustained the viability of Vero cells within the ranges of 40 - 80% (Fig. 4.2). The methanol extract on the other hand was toxic to all the cells at the concentrations tested (Table 4.3). It is interesting to note that despite the inhibition of cell viability by the methanol extract of this specie, the hexane, acetone and dichloromethane extracts were able to sustain moderate to excellent Vero cell viability at similar concentrations, whereas all the extracts were toxic to bovine dermis cells even at the lowest concentrations tested (Fig. 4.4).

The metabolic variation leading to susceptibility of cells to constituents present in the extracts is not very clear. It is possible that constituents cytotoxic to Vero cells in the methanol extract of *Annona senegaliensis* may not be extracted by the other solvents. Alternatively, the presence of constituents in the hexane, DCM and acetone extracts with possible protective effects on Vero cells and/or a toxic effect on bovine dermis cells cannot be ruled out. Some authors have reported the activity of many antioxidants to be higher in the epidermis than dermis of hairless mouse and human skins with the difference being greater in human skin (Shindo *et al.,* 1993). Furthermore, substances with antioxidant effects have been suggested to be helpful in the removal of reactive oxygen species (ROS) and can equally be readily oxidized in culture media with deleterious effects on cells *in vitro* (Rice-Evans, 2000; Long *et al*., 2000; Halliwell, 2003).

4.3.2.2.4. *Carissa edulis*

All the extracts of *Carissa edulis* at the highest concentration were toxic to the different cell types (Figs. 4.2, 4.3 and 4.4), while the hexane and acetone extracts at a lower concentration of 0.1 mg/ml were less toxic to Vero cells with cell viability greater than 70% (Fig. 4.2). At this concentration (0.1 mg/mℓ) except for the methanolic extract of this plant, all other extracts had deleterious effects on CRFK cells whereas on bovine dermis cells (Fig. 4.4), only the DCM extract had a cytotoxic effect. The extent of reduced cell viability of the DCM extract was even more evident on Vero cells at a much lower concentration of 0.01 mg/ml, which was not the case with the other extracts at this concentration (Table 4.2). The susceptible nature of Vero cells to DCM extracts, even at a lower concentration where the other cells showed sustained cell viability, may be indicative of the susceptible nature of Vero cells to substances present in the DCM extract of this specie. In the case of *Carissa edulis,* the presence of antioxidant compounds was only evident in the acetone extract. The acetone extracts of this plant showed excellent cell viability of Vero and bovine dermis cells at an even higher concentration of 0.1 mg/mℓ but this was not the case for CRFK cells (Table 4.2).

Both acetone and DCM can extract alkaloids and aglycones. Pascaline *et al.* (2011) in a general screening programme of medicinal plants identified the presence of alkaloids, saponins, terpenoids, glycosides and phenolics from the chloroform and methanolic extracts of *Carissa edulis.* Similarly, other authors have reported the presence of biologically active cytotoxic alkaloids from the genus *Carissa* (Ganapaty *et al.,* 2010). It is therefore likely that the

acetone extract of this plant contains substances with deleterious effects on CFRK cells that could not be ameliorated by the presence of antioxidant constituents in the extract. There could also have been a possible oxidation of the antioxidant constituents in culture media leading to deleterious effects as could be seen with CRFK cells (Table 4.2.).

4.3.2.2.5. *Podocarpus henkelii*

The variation in susceptibility of cells to different extracts was also observed with the extract of *Podocarpus henkelii.* The viability of Vero cells exposed to extracts of *P. henkelii* was similar to that observed with *Carissa edulis.* While none of the extracts of this plant at 1 mg/mℓ and 0.1 mg/mℓ showed viability of CRFK cells, only the DCM extract at similar concentrations showed deleterious effects on bovine dermis cells (Fig. 4.4). A similar trend in the susceptibility of cells to extracts of *Schrebera alata* and *Acokanthera schimperi*, was observed with all the cell types (Table 4.2).

The cytotoxic concentrations of all the plants were also calculated on the different cell types (Table 4.6). Of all the hexane extract of the different plants against all the cell types, *Carissa edulis* had the best CC_{50} value, followed by *Schrebera alata, Ekebergia capensis, Acokanthera schimperi, Podocarpus henkelii, Plumbago zeylanica* and *Annona senegalensis* in that order. DCM extracts of *Plumbago zeylanica* had the best CC_{50} value followed by *Podocarpus henkelii* and *Acokanthera schimperi* the least, while the acetone extracts of *Carissa edulis, Schrebera alata* and *Podocarpus henkelii* had the best CC_{50} value in that order. With methanol extracts *Plumbago zeylanica* and *Ekebergia capensis* had the best CC_{50} value and *Annona senegalensis* the least.

Table 4.6. CC_{50} values of different extracts of the same plant on different cell types

	Hexane			DCM			Acetone			Methanol		
	CC_{50} µg/mℓ			CC_{50} µg/mℓ			CC_{50} µg/mℓ			CC_{50} µg/mℓ		
	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm
SCA	22	62	46	33	2	5	31	3	>1000	25	10	244
EC	43	43	14	27	14	2	30	5	12	678	4	13
PZ	36	5	21	43	44	7	32	3	14	>1000	11	243
PH	56	5	15	43	5	5	46	5	107	42	1	153
CE	76	89	>100	<0.001	6	4	>1000	>1000	71	10	28	112
ANN.S	16	1	1	27	<0.001	<0.001	8	<0.001	<0.001	<0.001	<0.001	<0.001
AS	30	30	37	<0.001	<0.001	<0.001	<0.001	50.1	<0.001	<0.001	16	52
BERB	10	9.8	3									

PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* SCA = *Schrebera alata,* AS = *Acokanthera schimperi*, BERB = berberine

4.3.2.3 Antioxidant activity

To investigate the presence of substances with a protective effect acting via an antioxidant mechanism, the different extracts were analyzed for the presence of antioxidant constituents by spraying chromatograms with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol. Figure 4.5. represents those plant that had antioxidant constituents. The antioxidant constituents in some plants were highly polar and could not move from the bottom of the TLC plates following elution in different solvent systems.

Qualitative antioxidant activity studies revealed the presence of antioxidant compounds in the acetone, and methanol extracts of *Podocarpus henkelii* with more than 90% viability of Vero cells at 0.1 mg/mℓ and at 0.1 mg/mℓ, less than 20% cell viability respectively, representing a huge difference in cell viability despite the presence of antioxidant constituents in both extracts (Fig. 4.2). Because antioxidant compounds are usually relatively polar compounds it is not surprising that the more polar solvents extracted the most antioxidant compounds. Heo and Jeon (2009) illustrated the protective effect of antioxidants derived from marine algae against H_2O_2-induced Vero cell damage. Other authors have shown that structure–activity relationships of some compounds may be related to effective radical scavenging (Harborne and Williams, 2000; Op de Beck *et al.,* 2003). On the other hand, Aderogba *et al.,* (2007) demonstrated the toxic effect of a flavonol glycoside, myricetin-3-*O*-galactopyranoside isolated from *Bauhinia galpinii*, on Vero and bovine dermis cells. Although crude extracts were investigated in the present study, it is likely that the process of cell damage may not be associated with generation of free radicals, or the concentration of antioxidant was too low and could not protect the cells from the toxic constituents contained in the extract. This may explain the toxic effect of the DCM and acetone extracts of *Acokanthera shimperi* on bovine dermis and vero cells as well as the acetone and methanol extracts of *Annona senegalensis* on the viability of CRFK and bovine dermis cells.

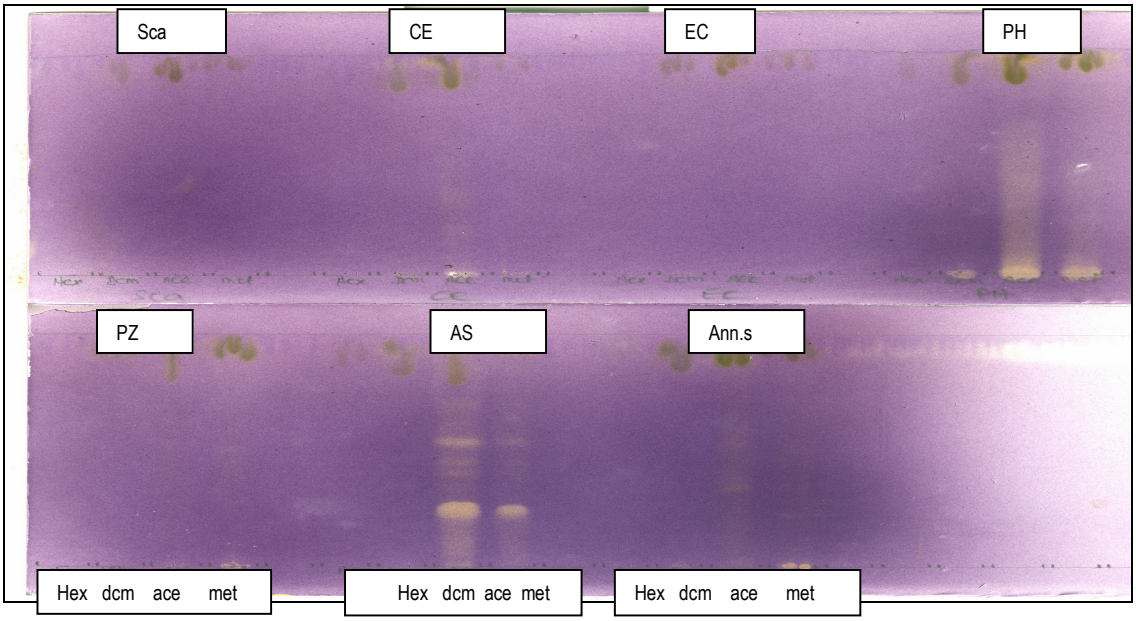

Figure 4.5. Thin layer chromatogram eluted in CEF indicating presence of antioxidant constituent in PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* SCA = *Schrebera alata,* AS = *Acokanthera schimperi*

The presence of compounds with antioxidant activity in the different extracts of plants used in the study did not seem to protect the cells from the toxic effect of substances present in the extract. This may suggest that the toxicity is not related to the generation of free radicals. It may also be likely that the protective effect of constituents with antioxidant activity may be related to the type of cell in culture under study. Synergism and mechanism of action between natural products in combating antimicrobial infections is well documented (Hemaiswarya *et al*., 2008). However, this is not the case in ascertaining the principal component responsible for cytotoxic effects, especially with crude plant extracts.

4.5. Conclusion

It is frequently stated that a plant is toxic without specifying the extractant used to prepare the extract tested for toxicity. In traditional medicine, mainly aqueous extracts are used because other extractants or solvents are not available. Extracts of many plants are administered in traditional medicine without prior knowledge of their chemical composition, toxicity and efficacy. Extracts in their crude form may contain toxic principles that may not be of therapeutic relevance. This study evaluated the cytotoxic effects of different extracts of the same plant and the possible protective effect of antioxidant constituents present in the different extracts.

Between cells and plant species, hexane extracts of the different plant species was by far the least toxic on the different cell types, followed by methanol, dichloromethane and acetone the most toxic. *Annona senegalensis* and *Acokanthera shimperi* extracts were the most toxic of plants of all the plants evaluated. Hexane is a solvent of low polarity and commonly extracts waxes, fats and fixed oils (volatile oils). Although the classes of volatile oils present and the lipophilic nature of the extracts were not determined in this study, available reports suggest the non-cytotoxic effect of a majority of these classes of compounds on different cell types (Allahverdiyev *et al*., 2004; Zai-Chang *et al*., 2005; Orhan *et al*., 2009; Al-Kalaldeh *et al*., 2010). This may possibly explain why the hexane extracts had a less deleterious effect on the viability of the cells. Between extracts of the plants species and cells, *Annona senegalensis* and *Acokanthera shimperi* were the most toxic on the different cell types while *Plumbago zeylanica, Carissa edulis*, *Ekerbergia capensis, Podocarpus henkelii* and *Schrebera alata* had moderate toxicity. Chrandell feline kidney cells were the most susceptible to the toxic effect of the different plants and extracts while Vero cells were the most tolerant.

The response of cells under culture conditions, especially where the addition of substances is required for evaluation of cytotoxic effects, and the mechanisms by which these cells withstand the potential toxic effects of these substances is complex. This is further complicated by the scantiness of information available on changes in metabolic activity of mammalian cells at different cell densities. Findings in this study suggest that the protective effect of substances with antioxidant activity in culture may be related to metabolism of the type of cell in culture. The studies also show a difference in susceptibility of kidney-derived cells used in this study, which may have been in part due to the metabolic efficiency of the cells being influenced by enzymatic conversion or degradation of cytotoxic components present in extracts or due to species variation in the origin of cells. It also suggests the presence of substances in some plant extracts depending on the solvent used for extraction that may induce viable cell proliferation. It further illustrates that the choice of solvent used in extraction can have an influence on the cytotoxic potential of a given plant. This should therefore be considered in the selection of solvent used for extraction of plant materials for biological activity testing. It is interesting that many publications in the Phytomedicine Programme have shown that acetone extracts are generally by far the best extractant to detect antimicrobial compounds (Kotze and Eloff, 2002), this extractant also yielded the most toxic extracts. This may be related to the bioavailability of compounds of intermediate polarity to cells of microorganisms and animals. Due to the cytotoxic effects of *Annona senegalensis* and *Acokanthera shimperi,* they will not be considered as potential candidates as possible microbial activity that maybe observed with these extracts may be due to their toxic effect on pathogens. The next chapter will focus on the evaluation of the different extracts of the plants specie for antibacterial activity.

Chapter 5

The antibacterial activity of different extracts of selected South African plant species

5.1. Introduction

The most widely and frequently used chemotherapeutic agents are antibiotics. Many such agents currently in use to treat bacterial and parasitic infections were first isolated from natural sources including ethnomedicinal plants (Coe and Anderson, 1996). The development of these drugs presented a huge breakthrough in the management of infections caused by bacteria with extraordinary clinical efficacy. However, the successes of these drugs over the decades have been compromised due to development of resistant strains of these pathogens with a commensurate negative impact on the treatment of disease. With the huge threat posed by these bacterial pathogens and the need to develop potent and less toxic alternatives to existing drugs, natural plant extracts and biologically active compounds isolated from plant species used in traditional medicine can be prolific resources for new antibacterial agents.

In most developing countries, the incidence of resistance development in humans is common amongst immunocompromised patients. Most important amongst these, on a global scale, are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus* species, and members of the Enterobacteriaceae, producing plasmid-mediated extended spectrum b-lactamase (ESbL), *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (Medeiros, 1997; Sajduda *et al.,* 1998). The United States Office for Technology Assessment for instance in 1992 estimated that the minimal hospital costs of 5 types of nosocomial infection acquired due to surgical wound infection and pneumonia that were resistant to antibiotics amounted to US$4.5 billion per year while the direct cost of managing antibiotic resistance in the United States was US$100 million to US$10 billion per year (Levy, 1992; US Office of Technology Assessment, 1995). This widespread emergence of resistant strains remains a clinical dilemma in hospitalized patients and raises a serious concern about the future of an antimicrobial approach. This has lead over the decades to the formulation of strategies by various countries to improve on the rational use of antibiotics in humans, aimed at reducing and eventually eliminating the use of antibiotics for purposes other than in human medicine and the treatment of infection in animals. The strategies further

seek to address the problem of spread of antibiotic-resistant organisms by improving hygienic practices and creating appropriate facilities in both hospital and public health settings (Swann Committee, 1969; Jungkind *et. al.,* 1995; US Office of Technology Assessment, 1995; Witte, 1998; Nikiforuk, 1996; Schwartz *et. al.,* 1997; Plotkin and Kimball, 1997; Government Official Report no. 132, Sweden, 1997; Williams and Heyman, 1998).

To solve the problems associated with the prevailing trend of resistance development, pharmaceutical companies have been involved in synthesizing derivatives of existing chemical classes to obtain drugs with 'expanded' spectrums of antibacterial activity. Besides this approach, current efforts in the development of new agents have made little headway with only two novel agents (a cyclic lipopeptide and an oxazolidinone) and a new streptogramin combination reaching clinical availability in the recent past (Shlaes, 2003). The biological cost associated with development of antimicrobial resistance compounds the problem (Gillespie and McHugh, 1997). Amidst these concerns, phytochemicals with inhibitory activity against b-lactamase-producing Gram-negative bacteria have been investigated (Yam *et al*., 1998), as well as those with inhibition of multidrug resistant (MDR) efflux pumps in *S. aureus* (Stermitz *et al*., 2001) and anti-antibiotic resistance properties (Lee *et al.,* 1998). Similarly the use of some plant phytochemicals in conjunction with conventional antibiotics is seen to potentiate the activity of some antibiotics (Zhao *et al*., 2001; Aqil *et al*., 2005). A recent report, by McGaw *et. al*. (2008) further illustrated the potential of some plants with activity against *Mycobacterium* infections.

Plants are considered to contain biologically active constituents with a wider safety margin than synthetic products (Davis, 1994) and developing countries have a wide diversity of flora with enormous potential that may provide solutions to many of the current resistance problems. This study was therefore aimed at the identification of antibacterial constituents in the selected species of plant chosen for the study (Table 4.1). Water is mostly used in folk remedies for extraction, but this solvent does not extract a wide range of active constituents contained in plants and antibacterial compounds (Eloff, 1998c, Kotze and Eloff). To target polar and non-polar constituents for bioactivity testing in this study, leaves of each selected plant species were extracted using hexane, DCM, acetone and methanol separately and tested for selective activity of the test on pathogens. The selectivity index (SI) is the ratio of the biological activity of the substance to the toxicity. The numbers of different antibacterial compounds present in different extract were determined by bioautography.

5.2. Materials and Methods

5.2.1. Thin layer chromatography (TLC) analysis of crude extracts

The plants were collected and extracted using solvents of varying polarity as described in sections 3.1 and 3.2. Extracted plant materials were spotted onto TLC plates and eluted in different solvent systems as described in section 3.4 for separation of constituents in the different extracts.

5.2.2. Bioautography on TLC plates

Thin layer chromatographic plates were prepared for the assay as described in section 3.4 without spraying with vanillin-sulphuric acid reagent, left to dry for 5 days and sprayed with test pathogens as described in section 3.7.1. Plates sprayed with pathogens were incubated in a humidified atmosphere at 37°C and thereafter sprayed with INT as described (section 3.7.1).

5.2.3. Microdilution assay for MIC determination

The serial microdilution method described by Eloff (1998b) was used to determine the minimum inhibitory concentration of the different plant extracts as described in section 3.7.2. Test pathogens used for the determination of MIC of extracts are described in section 3.7.1. The total activity of plant extracts was determined using the method of Eloff (2004) as described in section 3.8.2.

5.2.3. Determination of cytotoxic effect of the extracts on different cell types

The cytotoxic effect of the different extracts of plants selected for the study was evaluated on Vero, Crandell feline kidney (CRFK) and bovine dermis cells using the method of Mosmann (1983) as described in section 3.9. Selective activity of each extracts was calculated as follows: Selectivity index = CC_{50} / MIC

5.3. Results and Discussion

5.3.1. Chemical constituents of the crude extracts

Chromatograms eluted using CEF gave the best separation of constituents. The diversity of compounds extracted with different extracting solvents of the same plant is presented in Figure 5.1. A slight variation in the chemical composition of different extracts was observed in extracts of the same plant species eluted in CEF. This observation is illustrative of the presence of one or more constituents in different extracts of the same plant, which may serve as possible targets for isolation of biologically active compounds.

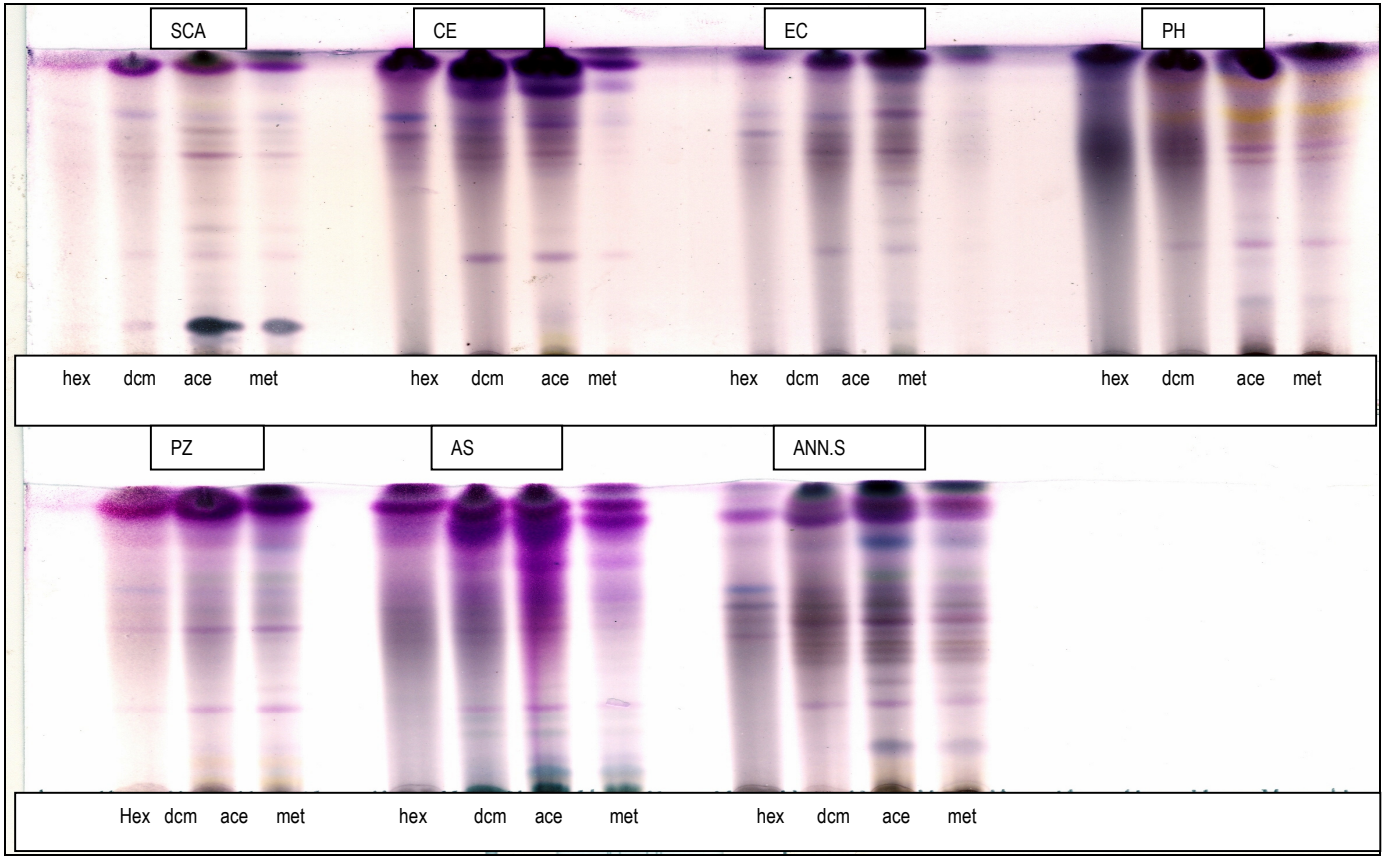

Figure 5.1: Thin layer chromatogram eluted in CEF of separated constituents of the same plant using different solvents for extraction. *SCa = Schrebera alata, CE = Carissa edulis, EC = Ekebergia capensis, PH = Podocarpus henkelii, Pz = Plumbago zeylanica As = Acocanthera shimperi, Ann.s = Annona senegalensis, HEX=Hexane, DCM = Dichloromethane, ACE = Acetone, MET = Methanol*

5.3.2 Inhibition of bacterial growth using bioautography

Bioautography was used to screen for antibacterial compounds to obtain more information on the diversity of compounds present in the different extracts. Inhibitory zones of antibacterial activity were observed as white spots on a purple background following spraying of bioautograms with INT. The R_f values of active constituents were recorded as those white areas on the bioautogram where reduction of INT to a coloured formazan did not occur due to the presence of compounds that inhibit the growth of the test pathogens (Table 5.1). In some cases the growth of the organisms was poor, making it difficult to detect zones of inhibition even when MIC values indicated good antibacterial activity. It is possible that the poor growth resulting in no activity of some extracts may be due to the evaporation or break-down of active compounds during removal of the TLC eluents to the disruption of synergism between active constituents caused by TLC separation or insufficient removal of the TLC eluents (Masoko and Eloff, 2005).

With the mobile systems used, biological activity was observed in all the extracts against one or more organisms. The intermediately polar system (CEF) separated more active constituents, a reflection of the difference in polarity of the systems used. In some cases, the four extracts showed compounds with similar R_f values active against one or more organisms. Bioautograms of *Podocarpus henkelii* extracts for instance displayed active compounds with similar R_f values of 0.21, 0.23, 0.28, 0.26, 0.33 and 0.96 in the different extracts. The extracts were active against *S. aureus* and *E. coli* respectively when eluted in BEA. Another compound, R_f 0.93, also had activity against *S. aureus* and *P. aeruginosa* when eluted in CEF. Likewise, the acetone and methanol extracts of *Acokanthera schimperi* and the dichloromethane extract of *Annona senegalensis* contained compounds active against *S. aureus* with R_f values of 0.72 and 0.88 respectively when eluted in CEF, which were not detected or separated with the other eluent systems.

Table 5.1: Retention factor (R_f) values of active constituents representing zones of inhibition of bacterial growth on bioautograms

Organism	Plant	BEA				CEF				EMW			
		Hex	DCM	Ace	Met	Hex	DCM	Ace	Met	Hex	DCM	Ace	Met
		0.26	0.26	0.26				0.72	0.72	-	-	-	-
	A.S	0.39	0.32	0.32			0.83	0.83	0.83				
		0.46	0.46	0.46									
S. aureus		0.98	0.98	0.98									
	C.E							0.72	0.72				
	Ann.S						0.83	0.83	0.83				
		-	-	-	-	-	0.88			-	-	-	-
	PH	0.21	0.21	0.21	0.21	0.47	0.47	0.47	0.47	0.97	0.97	0.97	0.97
		0.28	0.28	0.28	0.28	0.93	0.93	0.93	0.93				
		0.23	0.23	0.23	0.23		0.90	0.90	0.90	0.97	0.97	0.97	0.97
		0.46				0.93	0.93	0.93	0.93	0.97	0.97	0.97	0.97
	SCa	0.13	0.13	0.13									
	EC	0.26	0.52	0.52									
		0.32											
		-	0.19	0.19	-			-			-		
	PZ		0.26	0.26									
	A.S	-	-	-	-	-	0.88	0.88	0.88	-	0.91	0.91	0.91
	C.E						0.86	0.86	0.86		0.91	0.91	0.91
	Ann.S	-	-	-	-	-	-	-	-		-		
E. coli		0.26	0.26	0.26	0.26	0.91	0.91	0.91	0.91	0.97	0.97	0.97	0.97
	PH	0.33	0.33	0.33	0.33								
		0.96	0.96	0.96	0.96								
	SCa		0.26	0.26	0.26	0.88	0.88	0.88	0.88			0.38	0.38
										0.97	0.97	0.97	
	EC	0.47		0.47		0.91	0.91	0.91	0.91	0.97	0.97	0.97	0.97
	PZ	-	-		-		-				-		
	A.S						0.88	0.88	0.88	-	-	-	-
							0.93	0.93					
	C.E	-	-	-	-		0.84	0.84	0.84	0.97		0.97	
P. aeruginosa	Ann.S						-						
	PH								0.69	-	-	-	-
						0.93	0.93	0.93	0.93				
	SCa	-	-	-	-		0.93	0.93	0.93	-	-	-	-
	EC					0.9							
	PZ						-						

	A.S						0.8	0.8	0.8 0.9	0.92	0.92	0.92	0.92
						0.9		0.9					
	C.E	-	-	-	-		0.8	0.8	0.8	0.92	0.92	0.92	0.92
E. faecalis	Ann.S					0.91	0.91					0.91	0.91
	PH	-	-	-	-	0.95	0.95	0.95	0.95	-	-	0.34	0.34
	SCa					-	0.91	0.91	0.91			0.28	
												0.34	
										0.95	0.95	0.95	0.95
						0.92	0.92	0.92	0.92			0.34	0.34
	EC					0.96	0.96	0.96	0.96	0.92	0.92	0.92	0.92
	PZ	-	-	-	-	-	-	-	-	-	-	-	-

As = Acokanthera schimperi, CE = Carissa edulis, Ann.s = Annona senegalensis, PH = Podocarpus henkelii, SCa = Schrebera alata, Pz = Plumbago zeylanica, EC = Ekebergia capensis

N/A = no activity Hex = Hexane, DCM =Dichloromethane, Ace = Acetone, Met = Methanol

The presence of active zones with similar R_f values may not necessarily indicate the presence of identical compounds in these extracts. More active constituents were identified in extracts eluted in CEF and EMW against all the tested organisms. This suggests the presence of polar and non-polar active compounds in the extracts. The acetone and methanol extracts generally contained more active compounds than the DCM extracts, and hexane extracts showed the lowest number. Comparing the presence of active compounds, variation was observed between the eluent systems, plant species and susceptibility of microorganisms. In plants where BEA was used as eluent, fewer compounds active against *P. aeruginosa* and *E. faecalis* were separated although activity was observed against *S. aureus* and *E. coli* with the same plant extracts eluted in the same solvent system. A possible explanation for this may be that the non-polar and basic nature of BEA could not clearly separate the active non-polar constituents in these plants (Kotze and Eloff, 2002). In most cases, BEA could not move components of extracts from the origin of the TLC plate, indicating the polarity of these compounds. Favourable growth of cultures on bioautograms was observed with *S. aureus* and *E. coli* when compared with the other two microorganisms, which did not grow as well. It is possible that absence of growth may be associated with the sensitivity of these bacteria to residual eluent solvents on the bioautograms (Masoko and Eloff, 2005).

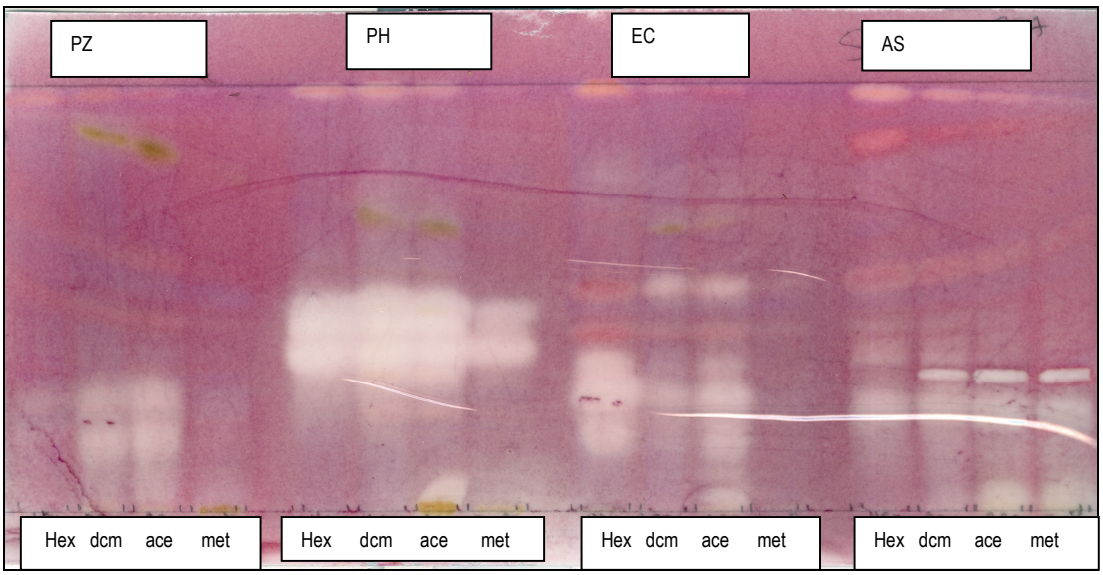

Figure 5.2. A representative bioautograph eluted in BEA indicating inhibition of growth of *S.aureus.*

White zones on a purple background represents inhibition of growth of the pathogen.

PH = Podocarpus henkelii, EC = Ekebergia capensis, As = Acokanthera schimperi,

Pz = Plumbago zeylanica, Hex = Hexane, DCM =Dichloromethane, Ace = Acetone,

Met = Methanol

5.3.3. Antibacterial activity of extracts in terms of MIC values

The MIC values of plant extracts incubated at different time intervals with four representative bacteria are represented in Table 5.2. Studies on South African medicinal plant extracts reporting MIC values below 0.1 mg/mℓ as deserving attention have been published (Eloff, 1999; McGaw *et al.,* 2000; Magee *et al.,* 2007). Thus, in this study, minimum inhibitory concentration values of up to 0.16 mg/mℓ were considered to reflect good antibacterial activity against tested pathogens. The high extraction yield of methanol did not correspond with strong antimicrobial activity except for *Carissa edulis* against *P. aeruginosa*, with MIC = 0.04 mg/mℓ after 24 hours of incubation. Overall, acetone extracts had good activity with MIC values within the range of 0.04 to 0.32 mg/mℓ, DCM 0.02 to 0.64 mg/mℓ and hexane the least after 24 hours of incubation in that order. This observation further supports the relevance of solvent type used in extraction, aimed at targeting bioactive constituents present in plants.

Differences in antibacterial activity were observed with varying times of incubation. The acetone and methanolic extracts of *Carissa edulis* for example, had an MIC of 0.08 mg/mℓ after 12 hours of incubation against *S. aureus*, but after 24 hours, the MIC values increased to 0.16 mg/ml, reflecting a decrease in antibacterial activity. A similar phenomenon was also found among other extracts, with the DCM extract of *Annona senegalensis* against *P. aeruginosa* showing MIC of 0.16 mg/mℓ at 12 hours and 0.02 mg/mℓ at 24 hours incubation. The decrease in MIC from 0.16 to 0.02 mg/mℓ was interesting to note. To investigate the variation in MIC values with time of incubation, contents of wells with MIC = 0.16 mg/mℓ were inoculated on MH agar and incubated overnight but no growth of the organism was apparent. It is possible that prolonged contact time may have resulted in a change in pH, which may be responsible for the observed activity at lower concentrations with this extract. These observations may suggest a possible concentration dependence of crude extracts from static to cidal effects produced *in vitro*.

A variation in susceptibility of Gram-positive and Gram-negative bacterial pathogens was observed between plants and type of extracting solvents (Table 5.3). The observed difference in the degree of susceptibility may be attributed to differences in active constituents extracted by each solvent, the presence of compounds that are acting in consonance with each other or morphological differences that exist between these organisms. Morphologically, the cell wall of Gram-negative bacteria is less permeable to antimicrobial substances than their Gram-positive counterparts (Nostro *et al.*, 2000; Hodges, 2002), which may be responsible for the observed differences in activity

Table 5.2: Antibacterial activity of selected plant species against Gram-positive and Gram-negative bacteria (MIC in mg/mℓ)

Plant species	Parts	Solvents															
		Hexane				Dichloromethane				Acetone				Methanol			
		S.a	*E.c*	*E.f*	*P.a*	*S.a*	*E.c*	*E.f*	*P.a*	*S.a*	*E.c*	*E.f*	*P.a*	*S.a*	*E.c*	*E.f*	*P.a*
Acokanthera schimperi	Leaves	2.2 2.5	1.25 1.25	0.64 0.64	0.64 0.64	0.32 0.32	2.5 2.5	0.16 0.16	0.16 0.16	0.08 0.08	0.08 0.08	0.32 0.32	0.32 0.32	0.16 0.16	0.32 0.32	0.32 0.32	0.64 0.64
Carissa edulis	Leaves	2.5 2.5	2.5 2.5	2.5 2.5	2.5 2.5	0.64 0.16	0.16 0.16	0.32 0.21	0.32 0.04	0.08 0.16	0.32 0.16	0.32 0.16	0.32 0.16	0.08 0.16	0.16 0.16	0.32 0.32	0.64 0.04
Annona senegalensis	Leaves	2.5 2.5	2.5 2.5	2.5 2.5	2.5 2.5	0.16 0.16	0.08 0.04	0.16 0.08	0.16 0.02	0.32 0.32	0.32 0.32	0.32 0.08	0.32 0.04	1.25 0.32	0.64 0.32	0.64 0.64	2.5 0.32
Podocarpus henkelii	Leaves	2.5 2.5	2.5 2.5	2.5 2.5	2.5 2.5	0.32 0.32	0.32 0.32	0.32 0.32	0.32 0.32	0.32 0.32	0.16 0.16	0.32 0.32	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.32 0.32
Schrebera alata	Leaves	2.5 2.5	2.5 2.5	2.5 2.5	2.5 2.5	0.16 0.16	0.32 0.32	0.13 0.13	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16
Ekebergia capensis	Leaves	2.5 2.5	2.5 2.5	2.5 2.5	2.5 2.5	0.13 0.13	0.16 0.16	0.32 0.32	0.32 0.32	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16	0.16 0.16
Plumbago zeylanica	Leaves	0.64 0.64	0.32 0.32	0.16 0.16	2.5 2.5	0.08 0.08	0.08 0.08	0.08 0.08	0.08 0.08	0.16 0.16	0.08 0.08	0.08 0.08	0.08 0.08	0.08 0.08	0.32 0.32	0.16 0.16	0.32 0.32
Gentamicin		0.003	0.003	0.006	0.006												

S.a = *Staphylococcus aureus*, E.c = *Escherichia coli,* E.f = *Enterococcus faecalis*, P.a = *Pseudomonas aeruginosa*

Table 5.3: Total activity values of different plant extracts against bacteria after 24 hours of incubation

Plant species	Solvents															Average
	Hexane				Dichloromethane				Acetone				Methanol			
	S.a	*E.c*	*E.f*	*P.a*	*S.a*	*E.c*	*E.f*	*P.a*	S.a	E.c	E.f	P.a	S.a	E.c	E.f	P.a	
Acocanthera schimperi	12	24	46.88	46.88	164.06	21	328.13	328.13	1312.5	1312.5	328.13	328.13	1562.5	781.25	781.2 5	390.63	485.5
Carissa edulis	9	9	9	9	265.63	265.63	202.38	1062.5	312.5	312.5	312.5	625	1640.6	1640.6	820.31	6562.5	878.7
Annona senegalensis	5	5	5	5	203.75	812.5	406.25	1625	62.5	62.5	250	500	234.38	234.38	117.19	234.38	297.7
Podocarpus henkelii	14	14	14	14	93.75	93.75	93.75	93.75	203.13	406.25	203.13	406.25	1093.75	1093.75	1093.75	546.88	342.3
Schrebera alata	3	3	3	3	156.25	78.13	192.31	156.25	203.13	203.13	203.13	203.13	734.38	734.38	734.38	734.38	271.6
Ekebergia capensis	4	4	4	4	173.08	140.63	70.31	70.31	156.25	156.25	156.25	156.25	578.13	578.13	578.13	578.13	212.9
Plumbago zeylanica	11.72	23.44	46.88	3	156.25	156.25	156.25	156.25	46.88	93.75	93.75	93.75	1406.3	351.56	703.13	351.56	240.6
Average	8.3	11.7	18.3	12.1	173.2	223.9	207.0	498.8	328.1	363.8	220.9	330.3	1035.7	773.4	689.7	1342.6	

Table 5.4: Selectivity index values relating observed activity of extracts with cytotoxicity

	HEXANE					DICHLOROMETHANE					ACETONE					METHANOL				
Plants	CC_{50} ug/ml	S.a	E.c	E.f	P.a	CC_{50}	S.a	E.c	E.f	P.a	CC_{50}	S.a	E.c	E.f	P.a	CC_{50}	S.a	E.c	E.f	P.a
SCA	22.0	0.01	0.01	0.01	0.01	32.0	0.20	0.10	0.25	0.20	30.0	0.19	0.19	0.19	0.19	25.0	0.16	0.16	0.16	0.16
EC	42.0	0.02	0.02	0.02	0.02	27.0	0.21	0.17	0.08	0.08	30.0	0.19	0.19	0.19	0.19	670.0	4.19	4.19	4.19	4.19
PZ	36.0	0.06	0.11	0.23	0.14	42.0	0.53	0.53	0.53	0.53	32.0	0.20	0.40	0.40	0.40	>1000	12.5	4.47	8.94	4.47
PH	56.0	0.02	0.02	0.02	0.02	42.0	0.13	0.13	0.13	0.13	45.0	0.14	0.28	0.14	0.28	42.0	0.26	0.26	0.26	0.13
CE	75.0	0.03	0.03	0.03	0.03	0.4	0.00	0.00	0.00	0.01	>1000	6.25	6.25	6.25	6.25	10.0	0.06	0.06	0.03	0.25
ANN.S	15.0	0.01	0.01	0.01	0.01	27.0	0.17	0.68	0.34	1.35	8.0	0.03	0.03	0.10	0.20	<0.001	0.003	0.003	0.001	0.003
AS	30.0	0.01	0.02	0.05	0.05	0.4	0.00	0.00	0.00	0.00	<0.001	0.01	0.01	0.003	0	<0.001	0.006	0.003	0.003	0.001
Berberine	10	10.00	0.17	0.33	0. 33															

As = *Acocanthera schimperi*, CE = *Carissa edulis*, Ann.s = *Annona senegalensis*, PH = *Podocarpus henkelii*, SCa = *Schrebera alata*, Pz = *Plumbago zeylanica*, EC = *Ekebergia capensis*, S.a = *Staphylococcus aureus*, E.c = *Escherichia coli*, E.f = *Enterococcus faecalis*, P.a = *Pseudomonas aeruginosa*

The quantity of antibacterial constituents present in each extract was determined by calculating total activity values (Table 5.3). This value indicates the volume to which the biologically active constituents originally present in 1 g of dried plant material can be diluted and still be potent enough to kill the microbial pathogen (Eloff, 1999). Here we chose to calculate total activity values from MIC values after 24 hours of incubation, the longest time the pathogens were in contact with plant extracts. From the 112 extracts tested, the bioactivity of the methanol extract of *Carissa edulis* against *P. aeruginosa* gave the highest total activity value 6562, followed by *Acokanthera schimperi* against *S. aureus.* Although MIC values of acetone extracts showed stronger antibacterial activity than methanol extracts, total activity values for methanol extracts were higher than those obtained for acetone extracts. Excellent selectivity index values (Table 5.4) were obtained for the methanol extract of *Plumbago zeylanica* against *S. aureus* and good to moderate activity against *Enterococcus faecalis, E.coli* and *P.aeruginosa*. The acetone extract of *Carissa edulis* on the other hand exhibited excellent SI values against all tested pathogens, while the methanol extract of *E. capensis* showed moderate activity against all the tested pathogens. This indicates that these plants have the highest inhibition of bacterial growth with relatively low toxicity to mammalian cells.

Overall, going by MIC values, acetone extracts of these plant species comparatively had the best activity followed by the DCM and hexane the least. The activity of the acetone extract is consistent with previous report (Eloff, 1999) were acetone is considered as the best extract for antimicrobial activity. However, methanol extracts had the best selectivity index value, followed by acetone and hexane the least. The susceptibility of pathogens was generally highest with methanol extracts of plants against *S.aureus* and *E. faecalis.* Hexane and methanol extracts of two plant species were less toxic , acetone extract, one plant species and DCM extracts none, indicating that constituents of DCM of these plants may generally be metabolic toxin (Table 5.4).

5.4. Conclusion

This study investigated the use of different extracts of the same plant to test for antibacterial activity. The separation of active constituents by TLC and subsequent exhibition of activity on bioautography by some compounds indicates the diversity of constituents present in extracts depending on the solvent used for extraction. Also, the presence of more than one active constituent on bioautography in extracts indicates synergistic effect of components of extracts

on test pathogens. All the plant species tested showed varying degrees of activity, with some having a broad spectrum of activity against test pathogens. Generally, acetone extracts had good antibacterial activity, followed by DCM extracts with MIC values as low as 0.08 mg/mℓ. The use of selectivity index values to determine the relationship of activity of a test substance to its cytotoxic concentration is important, especially where the use of a crude extract with synergistic activities is contemplated. This value can help decide whether the compounds active in an extract are general metabolic toxins. The antibacterial activity observed with most of the extracts according to selectivity index values, except for the methanol extract of *Plumbago zeylanica* against *S. aureus*, *E. faecalis, E. coli* and *P. aeruginosa* and the acetone extract of *Carissa edulis* against all tested pathogens, may be due to the toxic effect of the extracts on test pathogens. Results suggest that the choice of solvent used for extraction of a particular plant species can influence the biological activity of the extract.

In this study, plants with low to moderate toxicity and good total activity value, had antibacterial activity depending on the type of solvent used for extraction. Variation however exists in the susceptibility of bacteria, virus and fungal pathogens to the different extracts of these plant species. In the next chapter, the different extracts of these plants will be evaluated for antiviral activity against selected animal viruses.

Chapter 6

Evaluation of different extracts of selected south african plant species for antiviral activity

6.1. Introduction

Viral infections remain a major threat to humans and animals and there is a crucial need for new antiviral agents. Infections caused by viruses prevail and the unavailability of effective antiviral agents for the majority of viral infections is a serious problem. Although vaccines are available to control some of these infections, no effective antiviral therapy for the treatment of the majority of viral diseases in animals currently exists. With the difficulty in development of new vaccines, it can equally be presumed that easy viral candidates for vaccine development have been exhausted. In the midst of the threat posed to food security both in the developed and developing world, maintenance and sustenance of our zoological habitat as well as recreational privileges accorded to man by the well-being of animals, demand effort to find novel, specific and less toxic antiviral agents to counter the existing dilemma.

Feline herpesvirus-1 (FHV-1) is the most common viral pathogen of domestic cats worldwide. In cats, it causes infections of the eye characterised by conjunctivitis, and profuse ocular and nasal discharges. In severe cases, disease progression leads to keratitis and ulceration of the cornea as well as severe upper respiratory tract involvement (Gaskell and Willoughby, 1999; Andrew, 2001; Maggs, 2005). In contrast, canine distemper virus (CDV) infection affects predominantly canines, which serve as the natural host of the virus (Deem *et al.*, 2000). The virus causes highly contagious, systemic disease in dogs worldwide. The clinical signs and pathological lesions associated with the infection have been described by Jenner (Luader *et al.*, 1954). Despite the fact that infection of dogs may result in an array of clinical forms, immunosuppression and demyelinating leukoencephalitis characterize the main outcome in this species (Krakowka *et al.*, 1985). Dogs naturally infected with CDV have also been reported to serve as alternative animal models to study the pathogenesis of demyelinisation in various diseases, including multiple sclerosis (Baumgärtner and Alldinger, 2005; Vandevelde and Zurbriggen, 2005; Beineke *et al.*, 2009). Canine parainfluenza virus-2 (CPIV-2) is another pathogen that affects dogs. It is closely related to simian virus 5 (SV5),

human SV5 related isolates, porcine, ovine and feline parainfluenza viruses and to a lesser extent, the mumps virus (Randall *et al*., 1987, Ajiki *et al.,* 1982). The virus is one of several pathogens that causes kennel cough in dogs. Natural infection with CPIV-2 in dogs is self-limiting and restricted to the upper respiratory tract although some authors have reported the isolation of the virus from organs other than the respiratory tract (Evermann *et al*., 1980; Macartney *et al.,* 1985).

Lumpy skin disease virus (LSDV) affects cattle and is caused by a Capri pox virus. The disease is infectious, eruptive and occasionally fatal, affecting cattle of all ages and breeds. It is characterised by fever, skin nodules, necrotic plaques in mucosae and lymphadenopathy. During outbreaks, morbidity may be as high as 100% and mortality up to 40%. Severe economic losses during outbreaks are associated with emaciation, damage to hides, infertility in males and females, mastitis and reduced milk production (Barnard *et al*., 1994).

Although several hundreds of plants that have potential as novel antiviral agents have been studied, there still exist innumerable potentially useful medicinal plants waiting to be evaluated with biological activity that may be associated with a single phytochemical, or a number of different plant constituents. The objective of this study was to assess the antiviral effect of different extracts of selected medicinal plants with ethnobotanical indications in South African folk medicine, for *in vitro* activity against canine distemper virus (CDV), canine parainfluenza virus-2 (CPIV-2), lumpy skin disease virus (LSDV) and feline herpes virus-1 (FHV-1). Therapeutic inhibition of virus infection involves a number of strategies and targets a variety of steps in the life cycle of the virus such as cell entry, virus replication or the assembly and release of virions. Four enveloped viruses were selected for this study based on the postulation that targeting the entry of enveloped viruses may be a strategic approach for therapeutic interference, given that the site of action of the substances that will inhibit the virus is likely to be extracellular and practically open to the inhibitor, since potent antiviral activity is dependent on the identification and inhibition of viral specific events of replication.

6.2. Materials and Methods

6.2.1. Viral pathogens used in the study

The animal viruses used in the study were obtained from the Department of Veterinary Tropical Diseases, University of Pretoria, and selected for the study based on the reasons described in section 3.11.1. Two DNA and RNA viruses were used in the study. The viruses were as follows: feline herpes virus–1(FHV-1, dsDNA, enveloped), lumpy skin disease virus strain V248/93 (LSDV, dsDNA, enveloped), canine distemper virus (CDV, ssRNA, enveloped) and canine parainfluenza virus-2 (CPIV, ssRNA, enveloped). Viruses were propagated in monolayer cells and harvested as described in section 3.11.1. The effective titre ($TCID_{50}$/mℓ) of each of the viruses was determined using the method of Reed and Muench (1938), prior to each assay.

6.2.2. Cell cultures

The susceptible cell types compatible for the growth of the viruses were kindly provided by the Department of Veterinary Tropical Diseases, University of Pretoria. The cells used in this study were Crandell feline kidney cells (CRFK), Vero cells and bovine dermis (BD) cells, respectively. Cells were maintained in appropriate culture media in the study as described in section 3.11.1.

6.2.3. Determination of cytotoxic effect of extracts on cells

The selected plants (Table 4.1) were extracted with solvents of varying polarity as described in section 3.3. The cytotoxic effect of different extracts of each plant was tested against each cell type using the MTT colorimetric assay described by Mosmann, 1983 (section 3.9). The cytotoxicity was expressed as 50% cytotoxic concentration (CC_{50}) of substances to inhibit the growth of cells by 50%, when compared to untreated cells, calculated from the linear regression equation. Berberine chloride (Sigma) was used as a positive control; wells containing only cells without extract treatment were the negative control and a solvent control was also included.

6.2.4. Virucidal assay

The virucidal assay described by Barnard *et al.* (1992) with slight modifications was used to evaluate the antiviral potential of extracts of the selected plants as described in section 3.11.2. The extent of cell damage caused by infective virus was determined by the presence of CPE when compared to infected untreated and uninfected untreated controls by microscopic examination as well as the MTT colorimetric assay (section 3.11.2.) Plant extracts exhibiting reduction of viral infectivity at concentrations of 10^3 and 10^4 dilution were considered to possess strong activity while those with 10^2 to 10 as moderate to weak activity respectively by microscopic evaluation of infected cells. For the MTT assay, antiviral activity was expressed as a selectivity index (SI), where SI index values of more than three indicate potential antiviral activity (section 3.11.2.).

6.2.5. Attachment assay

The ability of the viruses to attach to the host cell was tested using the method of Barnard *et al*. (1993) with slight modifications as described in section 3.12.3. Antiviral activity by CPE reduction and MTT assay was determined as previously described (section 3.11.2).

6.3. Results and Discussion

In this study, the hexane, dichloromethane, acetone and methanol extracts of the different plants were tested for their antiviral activity against all four viral pathogens used in this study. Results are presented as those extracts of the different plants at the lowest dilution that exhibited reduced CPE by microscopic examination of infected cells in the virucidal and attachment assays (Tables 6.1 and 2). Prior to antiviral activity testing, the toxic effect of the different extracts was evaluated to ensure the extracts did not exert deleterious effects on cell viability.

In both the virucidal and attachment assays, the antiviral activity varied with the different extracts of the same plant as determined by virus-induced CPE by microscopic examination of infected cells (Tables 6.1 and 2). Some plant extracts exhibited moderate to good activity while others showed no evidence of reduced CPE. In the virucidal assay, the hexane extract of *Carissa edulis* inhibited by 75% FHV-1 and CDV induced CPE at 10^3 dilutions, while the DCM and methanol extracts at a dilution of 10^1 were able to inhibit viral-induced CPE by 25%. Despite the indication of reduced CPE of one or more of the tested viruses by these extracts, the acetone extract of this plant species was unable to inhibit viral-induced CPE of any of the viruses used in the study. A similar trend was also observed with the acetone extract of *Ekebergia capensis* and *Acokanthera schimperi* against all the tested pathogens (Table 1). While the DCM extract of *Ekebergia capensis* was able to inhibit viral-induced CPE of CPI-2 and CDV by 75% at 10^3 dilution of the extract, the hexane and methanol extracts inhibited viral-induced CPE of LSDV by 50% and 25% respectively at 10^1 dilution. With *Plumbago zeylanica*, all the extracts showed some degree of cell viability against either LSDV or CDV viruses, with the hexane extract exhibiting reduced CPE of CDV by 50% at 10^3 dilution. In contrast, only the acetone extract of *Schrebera alata* against LSDV and CDV and the methanol extract against LSDV induced CPE by 25% at 10^2 dilution while the hexane and DCM extracts could not. The acetone and methanol extracts of *Podocarpus henkelii* were able to inhibit viral-induced CPE of CDV and LSDV by 75% at 10^3 dilution while the DCM extract of this plant could not.

In the attachment assay were cells were exposed to viruses for various time intervals prior to addition of extracts. Variation was observed in the trend of inhibition of virus-induced CPE when compared to the virucidal assay. In this assay, only the DCM extract of *Carissa edulis* against CPI-2 exhibited reduced inhibition of viral infectivity by 25% as opposed to CDV in the virucidal assay, with the rest of the extracts being ineffective in reducing viral-induced CPE. The DCM and hexane extracts of *Ekebergia capensis* showed reduced LSDV and FHV-1 induced CPE by 50%, which was not the case in the virucidal assay (Table 6.2). The methanol extract of this plant exhibited no activity on all the tested pathogens in this assay. A similar variation was observed with extracts of *Acokanthera schimperi* in the attachment assay. While the hexane extract showed reduced FHV-1 induced CPE by 50% in the attachment assay, the effect was only observed against LSDV in the virucidal assay. Related trends were also observed with sensitivity of the viruses and activity of the different extracts of *Plumbago zeylanica, Schrebera alata* and *Podocarpus henkelii* (Table 6.2).

In the virucidal and attachment assays, SI values less than 1 represent weak, greater than 1 moderate, and greater than 3 good antiviral activities. The CC_{50}, EC_{50} and SI values of those extracts that exhibited reduced CPE are presented in Tables 6.4 and 6.5. In general, good selectivity index values were obtained in the virucidal when compared to the attachment assay (Tables 6.3 and 6.4). The hexane extract of *Carissa edulis* in the virucidal assay exhibited weak activity against FHV-1 with EC_{50} of 73.17 µg/mℓ and SI 1.22 while the same against CDV exhibited good activity with an EC_{50} 12.37 µg/mℓ and SI 6.14 (Table 6.3). However, this was not the case in the attachment assay. In the attachment assay, none of the extracts exhibited activity against the tested pathogens (Table 6.4). With *Ekebergia capensis,* weak activity was exhibited with the DCM extract against CPI-2 in both the virucidal and attachment assays while the hexane extract exhibited weak activity against FHV-1 in the attachment assay and not the virucidal assay (Tables 6.3 and 6.4). The hexane extract of *Plumbago zeylanica* on the other hand exhibited good activity against CDV with SI = 3.07 in the virucidal assay. Of all the plants used in this study the acetone extracts against CDV and the methanol extract of *Podocarpus henkelii* against LSDV exhibited good activity with SI values of 12.01 and 45.61 respectively in the virucidal assay (Table 6.3).

Vanden Berghe *et al*. (1993) suggested that the antiviral activity of a crude plant extract should be detectable in at least two subsequent dilutions of the maximum non-toxic concentration so as to be able to differentiate between virus-induced CPE and that due to the toxic effect of extracts. In addition, Cos *et al*. (2006) defined quality standards for primary evaluation for activity screening of natural products. The authors suggested a stringent endpoint of EC_{50} values < 100 µg/mℓ as a standard for antiviral efficacy of natural products, such as plant extracts. Apart from EC_{50} values, SI values of more than three are considered to be indicative of potential antiviral activity (Chattopadhyay *et al*., 2009).

Table 6.1. Virucidal activity of extracts of selected plants against test organisms following incubation of virus with extracts for 1-3 h prior to inoculation onto confluent host monolayer cells

Plants	Extracts	Virus	Time score (h) 1	2	3	Log concentration
Carissa edulis	Hexane	FHV-1, CDV	+++	+++	+++	10^3
	DCM	CDV	+	+	+	10^1
	Methanol	LSDV	+	+	+	10^1
Ekebergia capensis	Hexane	LSDV	++	++	++	10^2
	DCM	CPI-2, CDV	+++	+++	+++	10^3
	Methanol	LSDV	+	+	+	10^1
Acokanthera schimperi	Hexane	LSDV	+	+	+	10^1
	DCM	CDV	+	+	+	10^1
Plumbago zeylanica	Hexane	CDV	++	++	++	10^3
	DCM	LSDV	+	+	+	10^1
	Acetone	LSDV, CDV	+	+	+	10^1
	Methanol	LSDV	+	+	+	10^1
Schrebera alata	Acetone	LSDV, CDV	+	+	+	10^2
	Methanol	LSDV	+	+	+	10^2
Podocarpus henkelii	Hexane	LSDV	+	+	+	10^2
	Acetone	CDV	+++	+++	+++	10^3
	Methanol	LSDV	++	+++	+++	10^3

Plant extracts that show evidence of virucidal activity are represented. Log concentrations indicate the dilution at which the extract exhibited reduced viral- induced CPE. LSDV = Lumpy skin disease virus, CDV = Canine distemper virus, CPI-2 = Canine Para influenza virus-2, FHV-1 = Feline herpes virus, + + + = 75% inhibition, + + = 50% inhibition, + = 25% inhibition, h = duration in hours of incubation of virus with extracts prior to inoculation on to cells

Table 6.2. Antiviral activity of extracts of selected plants against test organisms following addition of virus onto monolayer cells and incubation for 1 – 3 h prior to addition of extracts

Plants	Extracts	Virus	Time score (h) 1	2	3	Log concentration
Carissa edulis	DCM	CPI-2	+	+	+	10^1
Ekebergia capensis	DCM	CPI-2, LSDV	+ +	+ +	+ +	10^3
	Hexane	FHV-1	+ +	+ +	+ +	10^3
Acokanthera schimperi	Methanol	CPI-2, LSDV	+ +	+ +	+ +	10^1 / 10^3
	Hexane	FHV-1	+ +	+ +	+ +	10^3
Plumbago zeylanica	Acetone	LSDV	+ +	+ +	+ +	10^3
	DCM	LSDV	+	+	+	10^2
Schrebera alata	Acetone	LSDV	+ +	+ +	+ +	10^1
	Methanol	CDV	+ +	+ +	+ +	10^1
Podocarpus henkelii	Acetone	LSDV	+ + +	+ + +	+ + +	10^3

Plant extracts that show evidence of antiviral activity are represented. Log concentrations indicate the dilution at which the extract exhibited reduced viral-induced CPE. LSDV = Lumpy skin disease virus, CDV = Canine distemper virus, CPI-2 = Canine parainfluenza virus-2, FHV-1 = Feline herpes virus, + + + = 75% inhibition, + + = 50% inhibition, + = 25% inhibition, h = duration of exposure of cells to virus prior to addition of extract

In the virucidal and attachment assays, no differences were observed in virus-induced CPE by microscopic examinations when the extracts were incubated for various time intervals. However, the trend and severity of virus-induced CPE by microscopic examination depending on the plant extracts varies between the two assays. This variation may possibly be related to the subjective nature of scoring associated with microscopic determination of CPE. In the virucidal assay, viruses were treated with extracts before infection of cells. Although some extracts were unable to completely inactivate the viruses, virus-induced CPE was markedly reduced following inoculation onto cell culture. Reduction in virus-induced CPE ranged from moderate to good antiviral activity against tested pathogens in one or more extracts of the plants tested when evaluated microscopically in both the virucidal and attachment assays. In the virucidal assay, the EC_{50} and SI values of extracts of the different plants exhibiting activity ranged between 3.36 and 73.17 µg/mℓ and 0.88 to 45.61 µg/mℓ respectively. The hexane extract of *Carissa edulis* had moderate to good activity against FHV-1 and CDV, with SI values of 1.22 and 6.14 respectively. Other authors have reported antiviral activity of an aqueous extract prepared from the roots of *Carissa edulis* with remarkable anti-herpes simplex virus (HSV) activity *in vitro* and *in vivo* for both wild type and resistant strains of HSV (Tolo *et al*., 2006). Although a different plant part and extract was used in that study, the activity observed with the hexane leaf extract against FHV-1 in this study supports earlier reports of the presence of substances with anti-herpes virus activity in *Carissa edulis. Carissa edulis* has been found to contain chemical constituents such as steroids, terpenes, tannins, flavonoids and cardiac glycosides (Ibrahim, 1997), benzenoids, lignans, phenylpropanoids, sesquiterpenes and coumarins (Achenbach *et al.,* 1983; Bentley *et al.,* 1984). Monoterpenes have been shown to exhibit low cytotoxicity and relatively strong anti-herpes simplex virus-1 action. The mechanism by which the monoterpene isoborneol inhibits antiviral activity involves the interactions of its hydroxyl groups with virus envelope lipids. Isoborneol may also inhibit virus replication and the glycosylation of viral proteins with resultant loss in HSV-1 infectivity (Armaka *et al*., 1999). Furthermore, potent anti-HSV-1 activities have also been reported for monoterpenes such as cineol and borneol, a stereoisomer of isoborneol. An analysis of diterpenes has shown that putranjivain A, isolated from *Euphorbia jolkini* (Euphorbiaceae), may interfere with HSV-2 ability to attach and penetrate cells and may also affect late stages in the replication of the virus (Khan *et al.,* 2005). Other groups of triterpenes have also been shown to inhibit infected cell polypeptide production in late stages of infection, reduce viral DNA synthesis or influence HSV-1 DNA synthesis and exhibit inhibitory effects on viral plaque formation (Khan *et al.,* 2005). Antiviral activity has also been reported for flavonoids (Li *et al.,* 2002), while sesquiterpenes have been demonstrated to exhibit moderate virucidal activity against a number of enveloped viruses including HSV-1, HCMV, measles virus and influenza A virus (Hayashi *et al,* 1996). Recent studies based on the evaluation of the antiviral activity of various synthetic coumarins have revealed that some of them serve as potent non-nucleoside RT-inhibitors, inhibitors of HIV-integrase or HIV-protease (Kostova *et al.,* 2006).

Some degree of antiviral activity was also observed with the dichloromethane extract of *Ekebergia capensis* against CPIV-2 and CDV with SI values < 1, suggesting a less potent effect of the extracts against test pathogens. The triterpenoid class of compounds isolated from *Ekebergia capensis* has been found to possess anti-HSV-1 activity (Ryu *et.al.,* 1993). Nawawi *et al.* (1999) tested the aqueous and methanolic leaf extracts of *Plumbago zeylanica* for anti-HSV-1 activity in the plaque reduction assay. Although different methods were used, the methanol extracts of *Plumbago zeylanica* at 91 µg/ml in that study did not inhibit HSV-1 plaque formation in Vero cells. The report is consistent with our findings where the methanol extract of this plant species exhibited no inhibitory effect on FHV-1. Interestingly, the hexane extract exhibited promising activity against CDV with a good SI value of 3.07. Earlier reports from investigations of the chemical constituents of *Plumbago zeylanica* revealed the presence of two plumbagic acid glucosides, 3'-*O*-β-glucopyranosyl plumbagic acid and 3'-*O*-β-glucopyranosyl plumbagic acid methylester along with five naphthoquinones (plumbagin, chitranone, maritinone, elliptinone and isoshinanolone), and five coumarins (seselin, 5-methoxyseselin, suberosin, xanthyletin and xanthoxyletin) in root extracts of this species (Lin *et al.,* 2003). Some naphthoquinones like rhinacanthin-C and rhinacanthin-D isolated from *Rhinacanthus nasutus* have been shown to possess antiviral activity against cytomegalovirus (CMV) with EC_{50} values of 0.02 and 0.22 µg/mℓ respectively (Sendl *et al.,* 1996). Of all the extracts tested in the virucidal assay, the acetone and methanol extracts of *Podocarpus henkelii* against CDV and LSDV presented the best antiviral activity with SI value > 10 (Table 6.3). Biflavones of the amentoflavone and hinokiflavone groups, terpenoids and nor- and bisnorditerpenoid dilactones are major taxonomic markers in the family Podocarpaceae. Amentoflavone isolated from the ethanol extract of *Selaginella sinensis* showed potent antiviral activity against respiratory syncytial virus (RSV), with an IC_{50} of 5.5 µg/mℓ (Ma *et al*. 2002). Although the constituent responsible for the observed activity in the different extracts of these plants is not clear, the possibility that similar classes of compounds present in the extracts may be responsible for the observed activity cannot be ruled out.

In the attachment assay, where virus was incubated with cells for different time intervals prior to addition of extracts, the activity of the dichloromethane extract of *Ekebergia capensis* was similar to that obtained in the virucidal assay against CPIV-2, suggesting a less potent ability of the extract to inactivate the virus prior to inoculation on cell monolayers (Tables 6.3 and 6.4). Also, the hexane extract of this plant had some degree of activity against FHV-I with SI value < 1, which was not observed in the virucidal assay. The observed activity may be related to interference with the replication cycle of the virus in the attachment assay. Similar observations were also recorded for other plant extracts against the different pathogens (Tables 6.3 and 6.4). A degree of antiviral activity was also observed with the hexane extract of *Acokanthera schimperi* against FHV-1, the acetone extract of *Plumbago zeylanica* against LSDV

and the acetone extract of *Podocarpus henkelii* against LSDV with SI values ranging between 0.55 and 1.25 and EC_{50} values from 30.93 to 95.69.

Table 6.3. Selectivity index (SI) values (ug/mℓ) indicating virucidal activity of extracts of selected plants following incubation of virus with extracts prior to inoculation onto confluent host monolayer cells

Plants	Extracts	Virus	EC_{50}[a]	CC_{50}[b]	SI[c]
Carissa edulis	Hexane	FHV-1, CDV	73.17/12.37	89.41 / 76.00	1.22 / 6.14
	DCM	CDV	_	0.001	_
	Methanol	LSDV	_	111.66	_
Ekebergia capensis	Hexane	LSDV		14	
	DCM	CPI-2, CDV	30.93/ 30.93	27.27/27.27	0.88 / 0.88
	Methanol	LSDV	_	13.13	_
Acokanthera schimperi	Hexane	LSDV	_	36.58	_
	DCM	CDV	_	0.001	_
Plumbago zeylanica	Hexane	CDV	11.73	36.05	3.07
	DCM	LSDV	_	6.66	_
	Acetone	LSDV, CDV	_	14 /32.21	_
	Methanol	LSDV	_	243.26	_
Schrebera alata	Acetone	LSDV, CDV	_	>1000 / 30.93	_
	Methanol	LSDV	_	243.68	_
Podocarpus henkelii	Hexane	LSDV	_	14.52	_
	Acetone	CDV	3.76	45.17	12.01
	Methanol	LSDV	3.36	153.24	45.61

[a] EC_{50} = concentration of the sample required to inhibit virus-induced CPE by 50%, [b] CC_{50} = concentration producing 50% cytotoxic effect [c]SI = CC_{50} / EC_{50}, LSDV = Lumpy skin disease virus, CDV = Canine distemper virus, CPI-2 = Canine parainfluenza virus-2, FHV-1 = Feline herpes virus, - = extracts that exhibited no reduced CPE

Table 6.4. Selectivity index (SI) values (ug/mℓ) indicating antiviral activity of extracts of selected plants against test organisms following addition of equal volume of virus onto monolayer cells and incubation prior to addition of extracts

Plants	Extracts	Virus	EC_{50}[a]	CC_{50}[b]	SI[c]
Carissa edulis	DCM	CPI-2	_	0.001	_
Ekebergia capensis	DCM	CPI-2, LSDV	30.93 / _	27.27 / 2.48	< 1 / _
	Hexane	FHV-1	78.21	43.11	0.55
Acokanthera schimperi	Methanol	CPI-2, LSDV	_	0.001 / 51.66	_
	Hexane	FHV-1	51.89	30.29	0.58
Plumbago zeylanica	Acetone	LSDV	54.93	13.53	0.25
	DCM	LSDV	_	6.66	_
Schrebera alata	Acetone	LSDV	29.87	>1000	
	Methanol	CDV	_	25.49	_
Podocarpus henkelii	Acetone	LSDV	95.69	107.39	1.12

[a] EC_{50} = concentration of the sample required to inhibit virus-induced CPE by 50%, [b] CC_{50} = concentration producing 50% cytotoxic effect [c]SI = CC_{50} / EC_{50}, LSDV = Lumpy skin disease virus, CDV = Canine distemper virus, CPI-2 = Canine parainfluenza virus-2, FHV-1 = Feline herpes virus, - = extracts that exhibited no reduced CPE

Crude plant extracts contain a diversity of constituents that may exert their antiviral effect either singularly or in concert with each other. The fact that some degree of inhibition was observed with these extracts may suggest that the extracts contain an active component(s) in low concentrations that may be responsible for the observed activity. These substances may, if present at higher concentrations, be capable of inactivating the virus as well as preventing its replication in the host system.

6.4. Conclusion

Extracts of plants with antiviral activity were more potent in the virucidal than the attachment assay. Of the extracts tested in the virucidal assay, four extracts showed significant antiviral activity, two of which were different extracts of *Podocarpus henkelii* against two unrelated viruses. Although a different extractant was used than that used by traditional healers, the presence of antiviral compounds in *Podocarpus henkelii* against two unrelated viruses may justify on a chemotaxonomic basis the traditional use of related species *Podocarpus latifolius* and *Podocarpus falcatus* in the traditional treatment of canine distemper infection in dogs. In the next chapter, the extracts of the different plant species will be evaluated for their antifungal activity.

Chapter 7

Evaluation of different extracts of selected south african plant species for antifungal activity

7.1. Introduction

The remarkable rise in the incidence of life threatening systemic fungal infections has been a cause for concern in the last two decades. Although most of these pathogens have co-existed with man from time immemorial, the increase in opportunistic infections caused by these pathogens has become more alarming with the advent of HIV-AIDS (Groll *et al.,* 1996; Stevens, 1990; Denning, 1998). For example, oropharyngeal candidosis (OPC) was found to occur in more than 80% of all patients during the course of HIV infection (Torssander *et.al*., 1987; Feigal *et. al.,* 1991) while *Candida* infections following bone marrow transplants are said to occur in up to 11% of patients (Sable and Donowitz, 1994). On the other hand, invasive aspergillosis is said to affect between 10 and 20% of patients with leukaemia and between 5 and 25% of patients that have undergone heart or lung transplantation (Denning, 1994). A similar scenario has also been reported for *Cryptococcus neoformans* infection with an overall death rate in transplant recipients of 20 to 100% (Carlson *et al*., 1987; Jabbour, 1996; Singh *et al.,* 1997).

The common clinically used antifungals are associated with numerous drawbacks such as toxicity, efficacy and cost, and their frequent usage has resulted in the development and emergence of resistant strains of these pathogens. The challenge the world is faced with is the development of effective strategies for the treatment of infections caused by these pathogens. Furthermore, concerns in recent years have been raised about the environmental impact of the use of synthetic fungicides in agriculture and the potential health risks posed by their continuous use (Abad *et al*., 2007).

The existence of such problems generates the need to discover new classes of antifungal compounds to treat fungal infections. In recent years, investigation of active constituents present in plants and natural product-derived compounds has accelerated due to their importance in drug discovery. Currently, three out of the seven classes of clinically available antifungal agents are from natural sources (Arif *et al*., 2011). This places plants as a rich source of bioactive secondary metabolites of a wide variety, which can be exploited for their antifungal properties. The

molecules isolated can be used directly, or as a model for developing better molecules. In this study, different extracts of selected species of plants were evaluated for their antifungal activity against three fungal pathogens of clinical importance in an attempt to identify and isolate active compounds against these pathogens, especially when the prevalence of these pathogens in immunocompromised patients is high.

7.2. Materials and Methods

7.2.1. Thin layer chromatography (TLC) analysis of crude extracts

The plants were collected and extracted using hexane, acetone, dichloromethane and methanol as described in section 3.1 and 3.2. Extracted plant materials were dissolved in acetone and spotted onto TLC plates and eluted in different solvent systems as described in section 3.4 for separation of constituents in the different extracts.

7.2.2. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 µℓ (10 mg/mℓ) of the different extracts of each plant species and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4) without spraying with vanillin. The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. Chromatograms were sprayed with fungal pathogens as described (section 3.8.1).The fungal pathogens used in this study were *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Plates sprayed with pathogens were incubated in a humidified atmosphere at 30°C and thereafter sprayed with INT as described (section 3.8.1).

7.2.3. Microdilution assay for MIC determination

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko *et al*. (2005) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of extracts on selected fungal pathogens. Two-fold serial dilutions of test substances (initial concentration of 10 mg/mℓ) dissolved in acetone were prepared in 96-well microtitre plates as described (section 3.8.2). Actively growing organisms (section 3.8.1) were transferred from SD agar plates using a sterile cotton swab into fresh SD broth and the assay was conducted as described (section 3.8.2). Amphotericin B (starting concentration of 0.08 mg/mℓ), a standard antifungal agent, was

included as a positive control. The selectivity index (SI) was calculated by dividing the cytotoxic concentration of each extract of the different plant species by the MIC. i.e (CC_{50}) / (MIC).

7.2.4. Determination of total activity

The total activity of plant extracts was determined using the method of Eloff (2004). Total activities of an extract or fraction gives an indication of the efficacy at which active constituents present in one gram can be diluted and still inhibit the growth of test organism. This value is calculated in relation to the MIC value of the extract, expressed mathematically as follows: Total activity = amount extracted from 1g (mg) of plant material divided by the MIC (mg/mℓ)

7.2.5. Determination of cytotoxic effect of the extracts on different cell types

The cytotoxic effect of the different extracts of plants selected for the study was evaluated on Vero cells using the method of Mosmann (1983) as described in section 3.9.

7.3. Results and Discussion

7.3.1. Inhibition of bacterial growth using bioautography

Three solvent systems were used to separate the active components of the different extracts of each plant species. A lot of difficulties were experienced in the growth of pathogens on TLC plates. In some cases growth was poor depending on the solvent system used for elution, which was more evident in plates eluted in the non-polar and basic eluent system, BEA, as well as the polar and neutral eluent system, EMW. As such, zones of inhibition were not evident in a majority of extracts of the different plant species (Table 7.1). The non-activity recorded for most extracts eluted in BEA may be associated with the presence of more active polar constituents in the extracts that could not separate well in BEA. It is also possible that the poor growth of fungal pathogens observed in this study may be associated with residual traces of eluent solvent on chromatograms that inhibit fungal growth (Masoko and Eloff, 2005). In other cases, although growth was evident, the lack of zones of inhibitions on chromatograms may be associated with non-activity of the compounds, a possible evaporation or break-down of active compounds during the removal of TLC eluents or disruptions of synergism between active constituents caused by TLC (Masoko and Eloff,

2005). CEF and EMW were the solvent systems that best separated the active constituents and also supported growth of organisms, with the intermediately polar system CEF recording the highest number of active constituents (Table 7.1).

In general, where visible zones of inhibition were recorded, DCM extracts showed the highest number of active constituents against tested pathogens (although variation was observed between different species of plants) followed by the acetone, methanol and hexane extracts with the least number of active compounds. Between species of plants, some compounds were detected with similar R_f values in extracts of different plant species as could be seen with the acetone, methanol and DCM extracts of *A. schimperi* and *C. edulis* against *C. albicans* as well as *S. alata* against *A. fumigatus* (R_f 0.87) and *A. schimperi* and *A. senegalensis* against *C. neoformans* (R_f 0.91) on chromatograms eluted in CEF (Table 7.1). A similar phenomenon was also observed with extracts of some species of plants eluted in EMW.

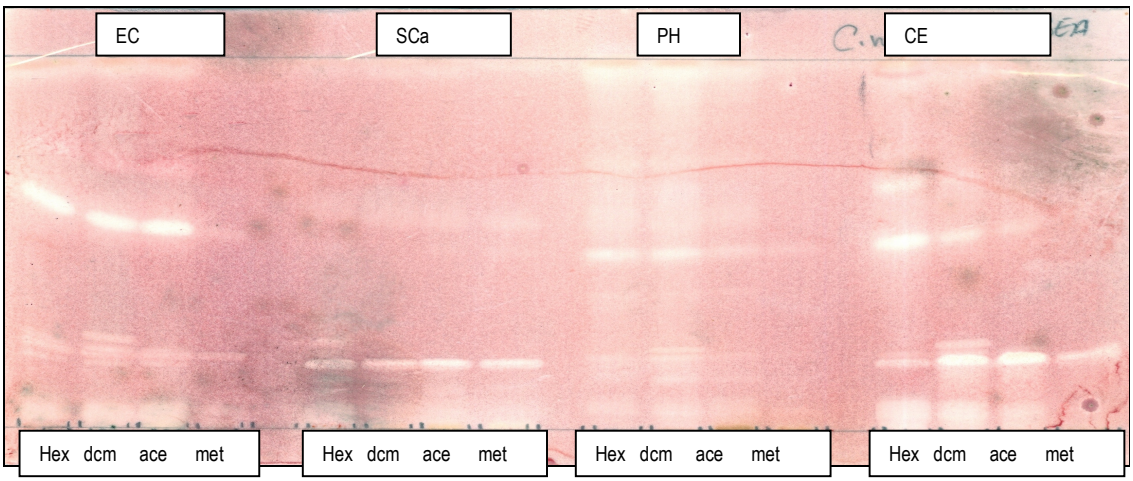

Figure 7.1. A representative bioautograph eluted in BEA indicating inhibition of growth of *C.neoformans.*

White zones on a purple background represent inhibition of growth of the pathogen.

PH = Podocarpus henkelii, EC = Ekebergia capensis, Schrebera alata, Carissa edulis

Hex = Hexane, DCM =Dichloromethane, Ace = Acetone, Met = Methanol

Table 7.1. R_f values of antifungal compounds detected[illegible] ts in bioautography

		BEA				CEF				EMW			
Organism	Plants	Acetone	Methanol	Hexane	DCM	Acetone	Methanol	Hexane	DCM	Acetone	Methanol	Hexane	DCM
	Acocanthera schimperi					0.87	0.87		0.87	0.93	0.93	-	0.93
	Carissa edulis			-					0.49				
						0.87	0.87		0.87	0.93	0.93	-	0.93
	Annona senegalensis			-		0.94			0.94				
	Podocarpus henkelii												
	Schrebera alata	0.33	0.33		0.33	0.51			0.51				
C. albicans						0.86	0.86		0.86				
	Ekebergia capensis									-	0.37	-	-
	Plumbago zeylanica			-				-					
	Acokanthera shimperi					0.85	0.85	0.85	0.85	0.93	0.93	-	0.93
						0.91	0.91	0.91	0.91				
	Carissa edulis	0.2	0.2	0.2	0.2	0.43	0.43	N/A	0.43				
		0.43		0.43	0.43		N/A		0.46				
				0.68								-	
						0.84	0.84	0.84	0.84				
				-		0.9	0.9	0.9	0.9				
	Annona senegalensis					0.5	0.5	N/A	0.5				
						0.91	0.91	0.91	0.91				
C.neoformans								-					
								-					
	Schrebera alata	0.2	0.2	0.2	0.2								
	Ekebergia capensis	0.2	0.2	0.2	0.2								
		0.54		0.24	0.24								
				0.54	0.54								
	Podocarpus henkelii	0.43		0.2	0.2								
				0.43	0.43								
					0.23								
	Plumbago zeylanica												
	Acokanthera schimperi					0.89	0.89	N/A	0.89	0.96	0.96	0.96	0.96
						0.93	0.93	N/A	0.93		N/A		
	Carissa edulis						N/A		0.52	0.96	0.96		0.96
						0.81	0.81	N/A	0.81				
						0.93	0.93	0.93	0.93				
	Annona senegalensis					0.26	0.26	N/A	0.26	0.97	0.97	0.97	0.97
				-		0.41	0.41	N/A	0.41				
A. fumigatus						0.5	0.5	0.5	0.5				
						0.93	0.93	N/A	0.93				
	Podocarpus henkelii					0.95	-	0.95	0.95				
	Schrebera alata					0.46				0.95	-	0.95	
						0.87	0.87	-	0.87				
	Ekebergia capensis								0.5	0.95			0.95
						0.96	0.96	-	0.96				
	Plumbago zeylanica												

Table 7.2. Antifungal activity of selected plant species against animal fungal pathogens (MIC mg/mℓ)

		Hexane			Dichloromethane			Acetone			Methanol		
Plant	Part	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f
Acokanthera schimperi	Leaves	0.16	2.5	0.32	0.08	0.32	0.64	0.08	0.32	1.25	0.08	0.43	0.64
Carissa edulis	Leaves	1.25	2.5	0.64	0.32	2.5	0.64	0.16	2.5	0.64	0.16	2.5	1.25
Annona senegalensis	Leaves	0.04	2.5	0.64	0.16	0.32	0.08	0.04	0.64	0.16	0.08	0.32	0.32
Podocarpus henkelii	Leaves	0.64	2.5	0.64	0.64	0.32	0.64	0.32	0.08	0.64	0.16	0.32	0.32
Schrebera alata	Leaves	0.64	2.5	1.25	0.32	0.32	0.64	0.64	0.16	0.64	1.25	0.16	1.25
Ekebergia capensis	Leaves	1.25	2.5	1.25	0.32	0.32	1.25	0.64	0.32	0.64	1.25	0.32	0.64
Plumbago zeylanica	Leaves	1.25	1.25	2.5	0.16	0.32	0.08	0.16	0.32	0.16	0.32	0.32	0.64

C.n = Cryptococcus neoformans, *C.a = Candida albicans*, *A.f = Aspergillus fumigatus*

(Table 7.1). Although these compounds were active against the same pathogens, the presence of compounds with similar R_f values in the different species of plants may not necessarily indicate the presence of the same active compounds in the different plants.

7.3.2. Antifungal activity of extracts in terms of MIC values

Various authors have used different assays to evaluate the antifungal activity of crude plant extracts. Tadeg *et al*. (2005) for instance used the agar well diffusion method while Abdillahi *et al.* (2008) used the M27-P broth dilution described by Espinel-Ingroff and Pfaller (1995) in evaluating the antifungal activity of plant extracts. While the agar well diffusion method is marked with limitations, especially when it comes to diffusion of extracts into the agar, MIC values obtained using the M27-P broth dilution method were found to be less senstive when compared with the serial microdilution method described by Eloff (1998b) in that study. On the other hand, Motsei *et al.* (2003) also used a serial microplate dilution assay in determining the antifungal activity of some South African medicinal plants against selected *C. albicans* isolates. In that study, an ELISA reader was used to measure the turbidity of organism exposed to plant extracts. Earlier reports by our group indicated several complications associated with measurement of growth using turbidity (Eloff, 1998b). In this study, we therefore used the serial microdilution method (Eloff, 1998b) as modified by Masoko *et al.* (2005) to evaluate the different extracts for antifungal activity since this method has been shown to be more reliable (Masoko *et al*., 2008).

In this study, MIC values were recorded following 24 and 48 hours of incubation. However, no differences in MIC values were observed with time of incubation and as such, only results for 24 hours are presented (Table 7.2). Since no validated criteria exist for MIC end points for *in vitro* testing of plant extracts, the proposed classification of Aligiannis *et al*., (2001) in this study was considered to be too high and as such, plant extracts with MIC values of < 0.1 mg/mℓ are considered as potential candidates for consideration. Minimum inhibitory concentration < 0.1 mg/mℓ in this study are considered to be of moderate antifungal activity, and < 0.08 mg/mℓ and below as excellent activity.

At 24 and 48 hours of incubation, MIC values of the different plant species ranged between 0.16 and 0.04 mg/mℓ. The acetone extract of the different plant species had the highest antifungal activity followed by the methanol and DCM, and hexane the lowest. Some extracts exhibited antifungal activity against one or more pathogens, which was

not observed in the other extracts. This could be seen with the acetone and methanol extracts of *S. alata* and *C. edulis* that was not the case with the DCM and hexane extracts (Table 7.2), indicating the presence of active constituents in some extracts depending on the type of solvent used for extraction.

The lowest MIC value indicating high activity for acetone extracts was obtained for *A. senegalensis* against *C. neoformans* at a concentration of 0.04 mg/mℓ (Table 7.2). Excellent activity with MIC = 0.08 mg/mℓ was also obtained with these extracts for *A. schimperi* and *P. henkelii* against *C. neoformans* and *C. albicans* respectively (Table 7.2). Although different assay methods were used, the non-activity observed with *A. schimperi* against *C. albicans* in this study, is consistent with previous reports (Tadeg *et al*., 2005) where the hydroalcoholic extract of this plant was inactive against the said pathogen. The finding further confirms the presence of consituents in *P.henkelii* active against *C. albicans* as reported by Abdillahi *et al*. (2008). Moderate antifungal activity (MIC = 0.16 mg/mℓ) was also observed with the acetone extracts of *C.edulis* and *P. zeylanica* against *C. neoformans, A. senegalensis* and *P. zeylanica* against *A. fumigatus* and *S. alata* against *C. albicans*.

Antifungal activities exhibited by the methanol extracts of the different plant species were most notable against *C. neoformans* in those plants that showed activity. *A. schimperi* and *A. senegalensis* exhibited excellent antifungal activity against *C. neoformans* with MIC = 0.08 mg/mℓ while moderate activity (MIC = 0.16 mg/mℓ) was obtained for *C. edulis* and *P. henkelii* against the same pathogen (Table 7.2). Of all the methanol extracts of the different plant species, only that of *S.alata* exhibited moderate activity against *C. albicans* while none showed activity against *A. fumigatus* (Table 7.2).

With DCM extracts, moderate to excellent activity was obtained for *A. senegalensis* and *P. zeylanica* against *C. neoformans* and *A. fumigatus* with MIC of 0.16 mg/mℓ and 0.08 mg/mℓ respectively, while *A. schimperi* exhibited excellent activity against *C. neoformans.* As observed with the methanol extracts of the different plant species against *A. fumigatus*, none of the DCM extracts of these plants exhibited activity against *C. albicans* (Table 7.2). Extracts prepared using hexane of the different plant species were less active against the different pathogens with moderate to excellent activity of MIC = 0.16 mg/mℓ and 0.04 mg/mℓ against *C.neoformans* obtained for *A. schimperi* and *A. senegalensis* respectively.

The total activity value, which was calculated by dividing extract quantity in mg from 1g crude material of the different extracts by the MIC are presented in Table 7.3. This value represents the volume at which active constituents present in 1g of crude plant extract can be diluted and still be potent enough to kill the pathogen. Despite the poor MIC values obtained with some extracts of the different plants, good total activity values were observed in some extracts as could be seen with the methanol extracts against *C. albicans* (Table 7.3). The highest total activity value was obtained for the methanol extract of *C. edulis*, acetone extract of *A. schimperi*, methanol extract of *P. henkelii* and DCM extract of *A. schimperi* against *C. neoformans* in that order. The total activity, on the other hand, of extracts against *C. albicans* were highest for the acetone extracts of *P. henkelii*, methanol extract of *S. alata* and the methanol extract of *P. henkelii*, while for *A. fumigatus* the DCM extract of *A. senegalensis* and the methanol extract of *A. schimperi* were most active in that sequence.

Table 7.3. Total activity values of different extracts of plants on pathogenic fungi after 24 hours of incubation

	Hexane			Dichloromethane			Acetone			Methanol		
Plant	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f
Acokanthera schimperi	187.5	12	93.75	656.25	164.06	82.03	1312.5	328.13	84	3125	581.4	390.63
Carissa edulis	180	90	351.6	132.8	17	66.41	281.3	20	78.13	1640.6	105	210
Annona senegalensis	312.5	5	19.53	203.13	101.56	406.3	500	31.25	125	937.5	234.38	234.38
Podocarpus henkelii	54.69	14	54.69	46.88	93.75	46.88	203.13	812.5	101.6	1093.8	546.87	273.4
Schrebera alata	11.72	3	6	78.13	78.13	39.6	50.78	203.13	50.78	94	734.38	94
Ekebergia capensis	8	4	8	70.31	70.31	18	39.06	78.13	39.06	60	234.38	117.19
Plumbago zeylanica	6	6	3	78.13	39.06	156.3	46.88	23.44	66.88	351.56	351.56	351.56
Average	108.6	19.1429	76.65286	180.8	80.55286	116.503	347.66	213.797	77.9214	1043.21	398.281	238.7371

C.n = *Cryptococcus neoformans,* C.a = *Candida albicans*, A.f = *Aspergillus fumigatus*

Table 7.4. Selectivity index values using Vero cell toxicity of extracts

	Solvents															
	Hexane				Dichloromethane				Acetone				Methanol			
Plants	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f
Acocanthera schimperi	30	0.02	0.02	0.01	0.4	0.00	0.00	0.01	<1	0.00	0.00	0.00	<1	0.00	0.00	0.00
Carissa edulis	75	0.12	0.03	0.06	0.4	0.00	0.00	0.00	>1000	24.23	96.9	24.2	10	0.01	0.06	0.01
Annona senegalensis	15	0.01	0.01	0.01	27	0.08	0.08	0.02	8	0.01	0.03	0.01	<1	0.00	0.00	0.00
Podocarpus henkelii	56	0.09	0.02	0.09	42	0.07	0.13	0.07	45	0.14	0.56	0.07	42	0.26	0.13	0.13
Schrebera alata	22	0.14	0.01	0.07	32	0.40	0.10	0.05	30	0.38	0.09	0.02	25	0.31	0.06	0.04
Ekebergia capensis	42	0.03	0.02	0.07	27	0.08	0.01	0.04	30	0.19	0.01	0.05	670	4.19	0.27	0.54
Plumbago zeylanica	36	0.90	0.01	0.06	42	0.26	0.13	0.53	32	0.80	0.05	0.20	>1000	17.88	4.47	4.47

C.n = *Cryptococcus neoformans*, C.a = *Candida albicans*, A.f = *Aspergillus fumigatus*

Despite less potent activity observed when evaluating MIC values, the SI value against test pathogens was highest for the acetone extract of *C. edulis* against *C. albicans* with SI value of 96.9, followed by those obtained for *C. neoformans* and *A. fumigatus* respectively (Table 7.4). With *P. zeylanica,* the methanol extract against *C.neoformans* had SI = 17.88 and 4.47 against *C. albicans* and *A. fumigatus.* On the other hand, only the methanol extract of *E. capensis* against *C. neoformans* showed a good SI value of 4.19. The high SI values obtained for some extracts of these plants indicate that these extracts are relatively less toxic with potent inhibitory activity on these pathogens.

Overall, going by MIC values, acetone extracts of these plant species comparatively had the best activity with more than one plant species having activity against all the pathogens used in the study followed by the methanol. With DCM extracts, activity was only observed against *C.albicans* while with the hexane extracts of the different plants, no activity was observed against any of the pathogens. The activity of the acetone extract is consistent with previous report (Eloff, 1999) were acetone is considered as the best extract for antimicrobial activity. Similarly, the acetone extracts had the best selectivity index value, followed by methanol. The susceptibility of pathogens was generally highest with methanol and acetone extracts of plants against *C.neoformans* respectively. *A.fumigatus* was the most non-susceptible pathogen against hexane, DCM and methonal extracts of the different plant species.

7.4. Conclusion

Plants are a relatively cheap source of biological material, consisting of a vast mixture of metabolites, primary or secondary, available for selecting molecules of desired biological activity. Hence in this study, solvents of varying polarity were used to extract selected plant species and each extract of the same plant was tested against three fungal pathogens. The antifungal activity of extracts of the different plant species varied with the type of organism. The acetone extracts of the different plant species exhibited the highest antifungal activity followed by the methanol and DCM extracts, and the hexane extracts were the least active. Except for *Ekebergia capensis*, extracts exhibited antifungal activity against one or more pathogens. *C. neoformans* was the most susceptible pathogen, followed by *A. fumigatus.* Despite the poor MIC values obtained with some extracts of the different plants, good total activity values were observed in some extracts suggesting the relevance of concentration of toxic principles present in an extract. It is also very important to determine selectivity index values so as to be able to differentiate between selective activity and activity due to toxic effects of the extracts. Thus, in the selection of plants for isolation of active compounds, the selectivity index value should also be considered.

Chapter 8

Plant selection and antimicrobial activity of solvent – solvent fractions of leaf material

8.1. Introduction

No *in vitro* method is available to localise antiviral compounds present in crude plant extracts, but bioautography remains a useful tool in revealing compounds with antibacterial and antifungal activity, localized on TLC plates. The compounds isolated using this technique in bioassay-guided fractionation can subsequently be tested against viral pathogens. *Podocarpus henkelii* was selected for isolation of bioactive compounds using acetone as the extractant. This plant, from the preliminary screening study was selected for further investigation based on the following reasons, 1) no compounds have been isolated from this plant and assayed for biological activity, 2) the acetone and methanol extracts exhibited good antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis,* 3) the acetone extract had excellent antifungal activity against *C. albicans* and good activity against *C. neoformans* and 4) the acetone extract had good to moderate activity against CDV and LSDV in both the virucidal and attachment assays, while the methanol extract was active against LSDV in the virucidal assay.

8.1.1. Description of the plant *Podocarpus henkelii* stapt ex Dallim. & Jacks.

Podocarpus henkelii stapt ex Dallim. & Jacks. (Podocarpaceae) is a large tree that grows up to 20m or more in height and often occurs in moist, evergreen mountain forests and less commonly in coastal forests. The leaves are dark green, shiny, long and slender, up to 17 x 1 cm, drooping, gradually tapering to a narrow apex and base. The leaf margin is entire and finely and tightly rolled under. The bark is yellowish grey, brown or dark grey. The male cones of the plant are large, about 3 x 0.4 cm, while in the female cone, the receptacle is not well developed and remains green. The plant has large, oval olive-green seeds measuring up to 2.5 x 2cm.

8.1.2.Taxonomy

Seven main genera make up the family Podocarpaceae; namely *Podocarpus* L' Her. ex Pers., *Dacrydium* Sol. Ex Forst., *Phyllocladus* Rich. Ex Mirb., *Acmopyle* Pilg., *Microcachrys* Hook.f., *Saxegothaea* Lindl. and *Pherosphaera* W. Archer bis (= Microstrobos J. Garden & L.A.S. Johnson, nom. inval.: Brummitt *et al.,* 2004) and accounts for most of the diversity in the Podocarpaceae. While some of these traditional genera form actively evolving complexes (i.e. *Podocarpus*, *Dacrydium*), others are of remote relevance (e.g., *Microcachrys*, *Saxegothaea*). The heterogeneity of

the genera *Podocarpus* and *Dacrydium* was documented a long time ago, but this diversity was commonly expressed taxonomically by means of subgenera, sections, and subgroups (Endlicher, 1847; Bertrand, 1874; Pilger, 1903; Florin, 1940; Buchholz and Gray, 1948).

The genus *Podocarpus* was primarily subdivided into eight sections, with the division placing more emphasis on the structure of the leaf, namely: *Afrocarpus* J. Buchholz & N. E. Gray, *Dacrycarpus* Endl., *Eupodocarpus* Endl., *Microcarpus* Pilg., Nageia (Gaertn.) Endl., *Polypodiopsis* C. E. Bertrand, *Stachycarpus* Endl., and *Sundacarpus* J. Buchholz&N. E.Gray (Buchholz and Gray, 1948). The African taxa were placed in the *Podocarpus* section (Leistner, 1966). Later on, suggestions of raising the section *Podocarpus* to generic ranks was proposed by Quinn (1970) and accepted by De Laubenfels (1972), using data on embryology, gametophyte development, female cone structure and cytology. Page (1989) further raised some of the other sections to generic ranks. These changes have however brought about nomenclature complications as exemplified by the raising of section *Afrocarpus* to the rank of genus with resultant rejection by some botanists (Leistner *et al.*, 1995; Glen, 2000).

Subsequent to these studies, several phylogenetic classifications of the Podocarpaceae have been undertaken, based on both morphological and molecular (DNA sequence) data (Kelch, 1997; Conran *et al.*, 2000; Sinclair *et al.*, 2002; Barker *et al.*, 2004) in an effort to resolve these discrepancies in nomenclature. It thus appears from available reports that there is significant molecular and morphological proof in favor of the generic level recognition of *Afrocarpus* and the other genera as recommended by Page (1989). These studies have resulted in the change of nomenclature of species of *Podocarpus* (*P. falcatus*) to *A. falcatus* (Thunb) C.N.Page (Barker *et al.*, 2004). Similarly, the taxonomic statuses of *P. milanjianus* and *P. latifolius* have been in contention. However, recent studies (Barker *et al.*, 2004) invalidated these suppositions. However, there still remain other taxonomic complications within the African Podocarpaceae such as the delimitation of species pair and complex, which needs to be, resolved (Barker *et al.*, 2004). With these existing problems in nomenclature, care should be taken when selecting a plant or collecting literature on *Podocarpus* species for a particular study (Lourens *et al.*, 2008). A summary of traditional and proposed taxa in the Podoacarpaceae family is presented in Table 8.1.

Table 8.1. Traditional and proposed taxa in the Podocarpaceae (Adapted from Kelch, 1997)

Adapted from Dallimore *et. al.*, 1966; Buchholz and Gray, 1948c; Florin, 1931	Adapted from Page, 1990	Present geographical distribution
Acmopyle Pilger	*Acmopyle* Pilger	New Caledonia, western Pacific (Fiji)
Dacrydium Sol. ex Lam.		
group A	*Falcatifolium* De Laubenf	New Caledonia, Malesia
group B	*Dacrydium* Sol. ex Lam	New Zealand, New Caledonia, western Pacific, Malesia, Philippines, Southeast Asia
group C	*Halocarpus* Quinn	New Zealand
group C	*Lagarostrobos* Quinn	New Zealand, Tasmania
group C	*Lepidothamnus* Phil..	New Zealand, southern South America
Microcachrys Hook. f.	*Microcachrys* Hook. f.	Tasmania
Microstrobos Gard. et Johns	*Microstrobos* Gard. et Johns.	Tasmania, New South Wales
Phyllocladus Rich. ex Mirbel	*Phyllocladus* Rich. ex Mirbe	New Zealand, Tasmania, Malesia
Podocarpus L, Her. ex Persoon		
P. sect. *Podocarpus* Endl.	*Podocarpus* L, Her. ex Persoon	
subsect. A, C, D, & E	P. subg. *Podocarpus*	New Caledonia, Australia, Tas- mania, New Zealand, Africa, Madagascar, Central and South America, Mexico, Caribbean
subsect. B & F	P. subg. Foliolatus De Laubenf.	Southeast Asia, Japan, Malesia, Philippines, western Pacific, New Caledonia, Australia
P. sect. Nageia Endl.	Nageia Gaertn. N. sect. Nageia (Endl.) De Laubenf.	Southeast Asia, India, Malesia, Philippines, Japan
P. sect. Afrocarpus Buchh. et Gray	N. sect. Afrocarpus (Buchh. et Gray) De Laubenf.	Africa
P. sect. Polypodiopsis Bertr.	N. sect. Polypodiopsis (Bertr.) De Laubenf.	South America, New Caledonia, western Pacific, Malesia
P. sect. Dacrycarpus Endl.	Dacrycarpus (Endl.) De Laubenf.	Malesia, New Zealand, New Caledonia, western Pacific, Philippines
P. sect. Microcarpus Pilger	Parasitaxis DeLaub.	New Caledonia
P. sect. Stachycarpus Endl. p.p.	Prumnopitys Phil.	South America, New Zealand, Queensland, New Caledonia
P. sect. Sundacarpus Buchh. et Gray	Sundacarpus (Buchh. et Gray) Page	Malesia, Queensland
Saxegothaea Lindl.	Saxegothaea Lindl.	southern South America

8.1.3. Chemotaxonomy

Plants contain an array of chemical constituents, some of which occur in abundance or are unique to a particular species. Hence, the presence or absence of certain classes of compounds can be used as chemotaxonomic markers in plants. One such class of compounds is the biflavonoids. Biflavonoids are dimers of flavonoids, linked by a C–O–C or C–C bond. In nature, very few plants contain biflavonoids as major constituents, and they can be found in *Selaginella* species, *Ginkgo biloba* and *Garcinia kola* (Kim *et al*., 2008). These classes of compounds are the chemotaxonomic markers in a majority of families from the Gymnospermae, including the families Taxaceae and Ginkgoaceae (Geiger and Quinn, 1988). Consequently, the *Podocarpus* species contain a simple pattern of derivatives based on amentoflavone and hinokiffavone. Presence of the biflavonoids amentoflavone and hinokiflavone including nor- and bisnorditerpenes in *Podocarpus* has been shown to be a good taxonomic marker in these species (Cambie and James, 1967; Ito and Kodama, 1976; Roy *et al*., 1987). Podocarpusflavone A is present in every species of *Podocarpus* so far investigated, except *P. latifolius*. Of equal importance is the recognition of the *Podocarpus* segregated genera by the presence or absence of different monomer flavonoid glycosides. For instance, *Dacrycarpus* is differentiated by the presence of 3-methoxyflavones, while *Prumnopitys* and *Podocarpus* are characterized by the predominance of flavonol 3-O-glycosides and flavone C-glycosides respectively (Markham *et al.,* 1985). The phytochemistry of *Podocarpus* s.l. has been extensively reviewed by Abdillahi *et al*., (2010) and a summary of chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* is presented in Fig. 8.1.

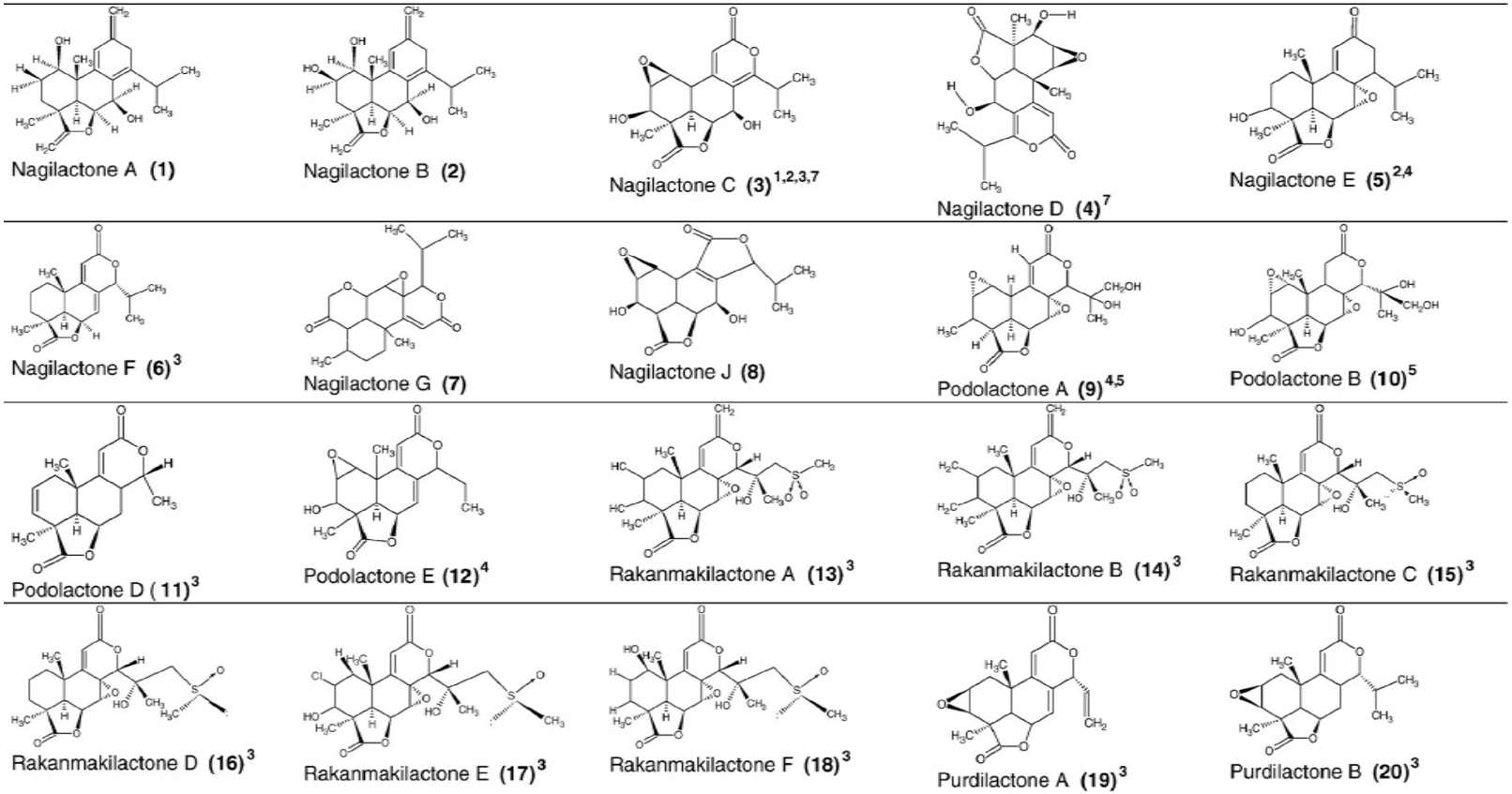

Figure. 8.1. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic; [13]anti-tyrosinase/melanin inhibition. (Adapted from Abdillahi *et al*., 2010)

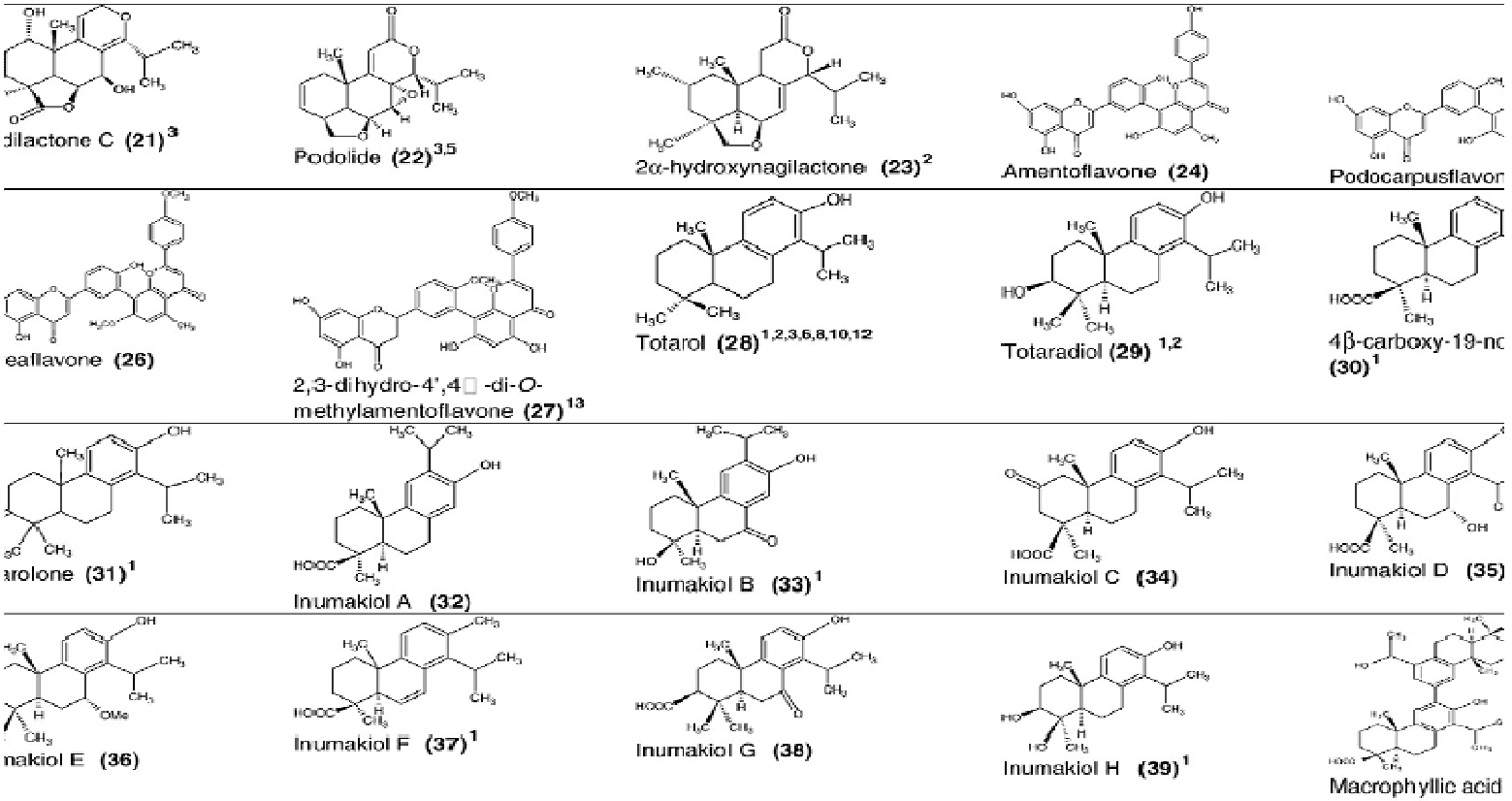

Fig. 8.1. cont. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic;[13]anti-tyrosinase/melanininhibition. (Adapted from Abdillahi *et al.*, 2010)

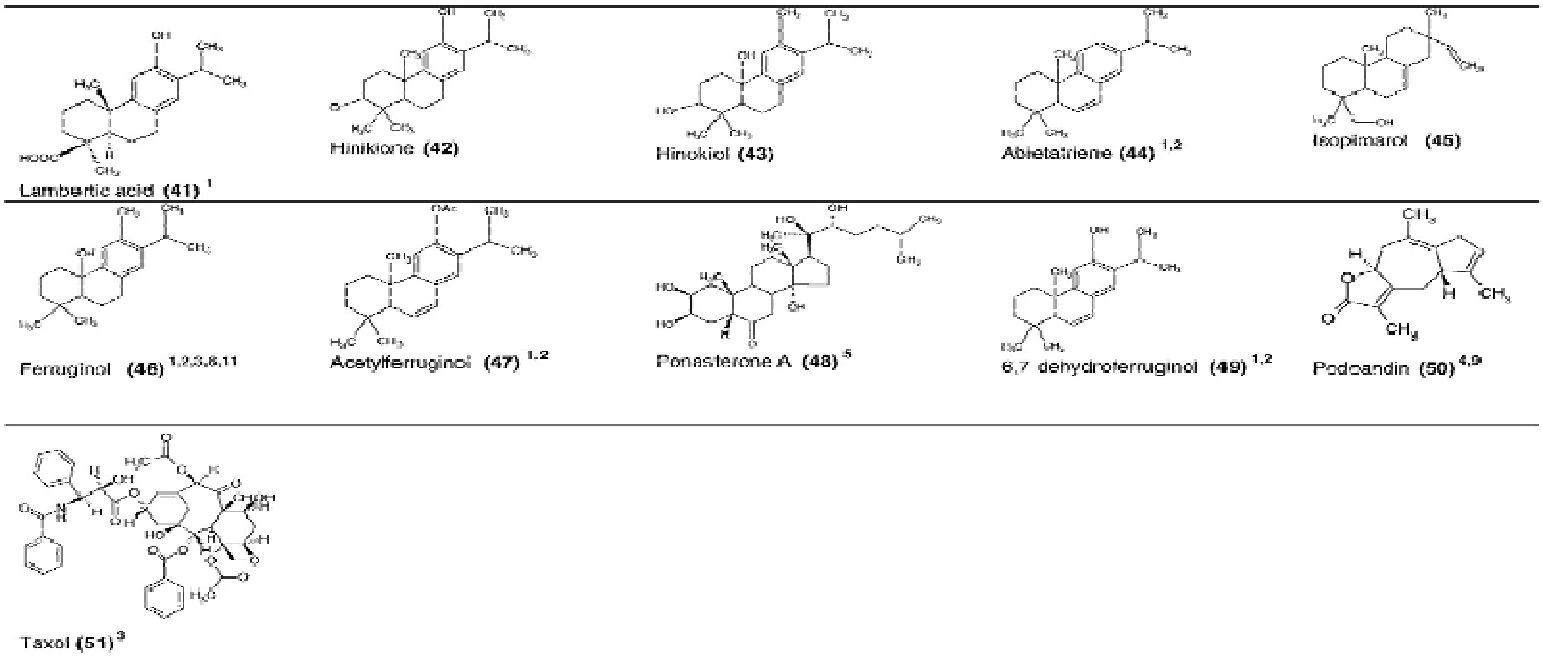

Fig. 8.1. cont. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic; [13]anti-tyrosinase/melanin inhibition (Adapted from Abdillahi *et al*., 2010)

8.1.4. Medicinal uses

Several species of *Podocarpus* s.l. have been used in different cultures around the world as a remedy for various ailments in human and animals. Depending on the type of ailments, different plant parts such as leaves, bark and fruit have commonly been used. A summary of the medicinal use of species of *Podocarpus* s.l is presented in Table 8.2. This species of plant has proved to be useful in the treatment of fevers, asthma and coughs (Chopra *et al*., 1986; Riley, 1994), cholera, heart ailments, kidneys, lungs, stomach diseases and for sweaty feet, worms and blood disorders (Duke and Ayensu, 1985). Other authors have reported their use in the treatment of rheumatism and painful joints (Chopra *et al*., 1986), as antitumor agent and in pest control (Nakanishi, 2006), treatment of distemper in dogs and gall sickness in cattle (Dold and Cocks, 2001; Masika and Afolayan, 2003), chest complaints and stomach ache (Watt and Breyer-Brandwijk, 1962; Beentje, 1994) and gonorrhoea (Pankhurst, 2000).

Table 8.2. Medicinal uses of *Podocarpus* species (Adapted from Abdillahi *et al.,* 2010)

Species	Geographical distribution	Plant part	Medicinal uses	References
Podocarpus henkelii Stapf ex Dallim. & Jacks.	South Africa	Sap	chest complaints	(Watt and Breyer-Brandwijk,1962; Hutchings *et al.,* 1996)
Podocarpus falcatus (Thunb.) R. Br. Ex Mirb.	South Africa, East Africa	bark sap Oil	gallsickness in cattle,distemper in dogs, head ache chest complaints gonorrhoea	Watt and Breyer-Brandwijk,1962; Sindiga, 1995;Hutchings *et al.*, 1996; Venter and Venter, 1996;Pankhurst, 2000; Dold and Cocks, 2001)
Podocarpus ferrugineus Don. (Miro)	New Zealand	Gum		(Uphof, 1968; Johnson, 1999)
Podocarpus latifolius (Thunb.) R. Br. Ex Mirb.	South Africa, East Africa	bark Sap	gallsickness in cattle,distemper in dogs stomachache, screeaned for anticancer and AIDS. chest complaints	(Watt and Breyer-Brandwijk,1962;Cunningham ,1993; Beentje, 1994;Sindiga,1995; Hutchings *et al*.,1996; Dold and Cocks, 2001)
Podocarpus macrophyllus (Thunb.) Sweet	China, Japan, E. Asia	stem bark fruit	ringworms and blood disorders tonic for heart, kidneys, lungs and stomach .	Duke and Ayensu (1985)
Podocarpus nagi (Thunb.) Zoll. & Moritz.	East Asia, Japan, Mexico, New Zealand	bark stem bark fruit seed	antiseptic,astringent, carminative and treatment of fevers, asthma, coughs, cholera arsenic poisoning, skin diseases and ulcers carminative, pectoral and stomachic. cholera, heartailments, stomach diseases and sweaty feet.	(Chopra *et al*., 1986; Duke and Ayensu, 1985)
Podocarpus nakaii Hayata	Taiwan		antitumor agent and pest control	Nakanishi (2006)
Podocarpus neriifolius D. Don.	Papua New Guinea, Himalayas and China	leaves	rheumatism and painful joints.	Chopra *et al.*, (1986)
Podocarpus totara G. Bennett ex D. Don	New Zealand	bark leaves berries	gonorrhoea and syphilis, splints on limbs, fever piles, sores and lesions laxative,constipation in women .	Riley (1994)
Podocarpus sp.	Java, Malaya		arthritis and rheumatism.	Johnson (1999)

8.2. Materials and Methods

8.2.1. Solvent-solvent fractionation of leaf material

Ground material (500 g) was extracted using acetone (1 g/10 ml) for 24 hours. The supernatant was filtered through Whatman No 1 filter paper using a Büchner funnel. The dried acetone extract (43 g) was subjected to solvent-solvent fractionation as described by Suffness and Douros (1979) and adapted by Eloff (1998a) to fractionate the components based on polarity (section 3.6). The components of the crude extract were separated into the n-butanol, hexane, ethyl acetate, carbon tetrachloride, chloroform and methanol: water fractions.

8.2.2. Analysis and concentration of fractions

All fractions were collected in glass jars following solvent-solvent fractionation and concentrated under a stream of air. Fractions were reconstituted to 10 mg/mℓ for TLC analysis and bioassay. For TLC analysis, fractions were spotted onto TLC plates, eluted in suitable solvent systems (section 3.4), viewed under UV light and sprayed with vanillin sulphuric acid reagent prepared as described (section 3.4) and heated at 100°C for five minutes to allow for colour development. Those fractions with similar TLC profiles were combined. The minimum inhibitory concentrations of the fractions were determined as well as bioautography using the different organisms. Plates for bioautography were not sprayed with the chromogenic spray reagent prior to the assay.

8.2.3. Bioassay–guided fractionation

The different fractions were spotted onto TLC plates and sprayed with concentrated suspensions of bacterial and fungal pathogens as described (Sections 3.7.1 and 3.8.1) while MIC was determined as described (section 3.7.2. and 3.8.2.). The retardation factor (R_f) values of compounds showing zones of inhibition were recorded.

8.3. Results and Discussion

The acetone leaf extract of *Podocarpus henkelii* was partitioned into six fractions and bioautography was carried out to identify active antibacterial constituents using *S. aureus* and *E. coli*, and antifungal constituents using *C. albicans, C. neoformans* and *A. fumigatus*. The carbon tetrachloride fraction had more active compounds, followed by chloroform, ethyl acetate and hexane fractions in that order against *S. aureus*. The n-butanol and methanol: water fraction had less active compounds (Fig. 8.2). The R_f values of compounds active against *S. aureus* in the carbon tetrachloride fraction were 0.5, 0.62, 0.8 and 0.9, for chloroform they were 0.6, 0.62 and 0.9, for ethyl acetate 0.6 and 0.62, and for the hexane fraction 0.8. On the other hand, R_f values for compounds active against *E.coli* for the carbon tetrachloride fraction were 0.43, 0.53, 0.62 and 0.9, for chloroform 0.43, 0.53, 0.62 and 0.9, for ethyl acetate 0.6 and 0.62, and for the hexane fraction 0.9 (Table 8.3a).

For the antifungal activity, the chloroform fraction had more active compounds against the three fungal pathogens, followed by the carbon tetrachloride and ethyl acetate fractions, while the n-butanol and methanol: water fractions had fewer active compounds (Fig 8.3). The R_f values for compounds in the carbon tetrachloride fraction active against *A. fumigatus* were 0.63 and 0.9, and against *C. albicans* 0.93. On the other hand, the chloroform fraction had compounds active against *A. fumigatus* with R_f values of 0.54, 0.63 and 0.9, against *C. albicans*, R_f values were 0.93, 0.6 and 0.7 and against *C.neoformans*, R_f values were 0.6 and 0.63. The ethyl acetate fraction had one compound active against *A.fumigatus* (R_f = 0.9) and *C. albicans* (R_f = 0.93) (Table 8.3b).

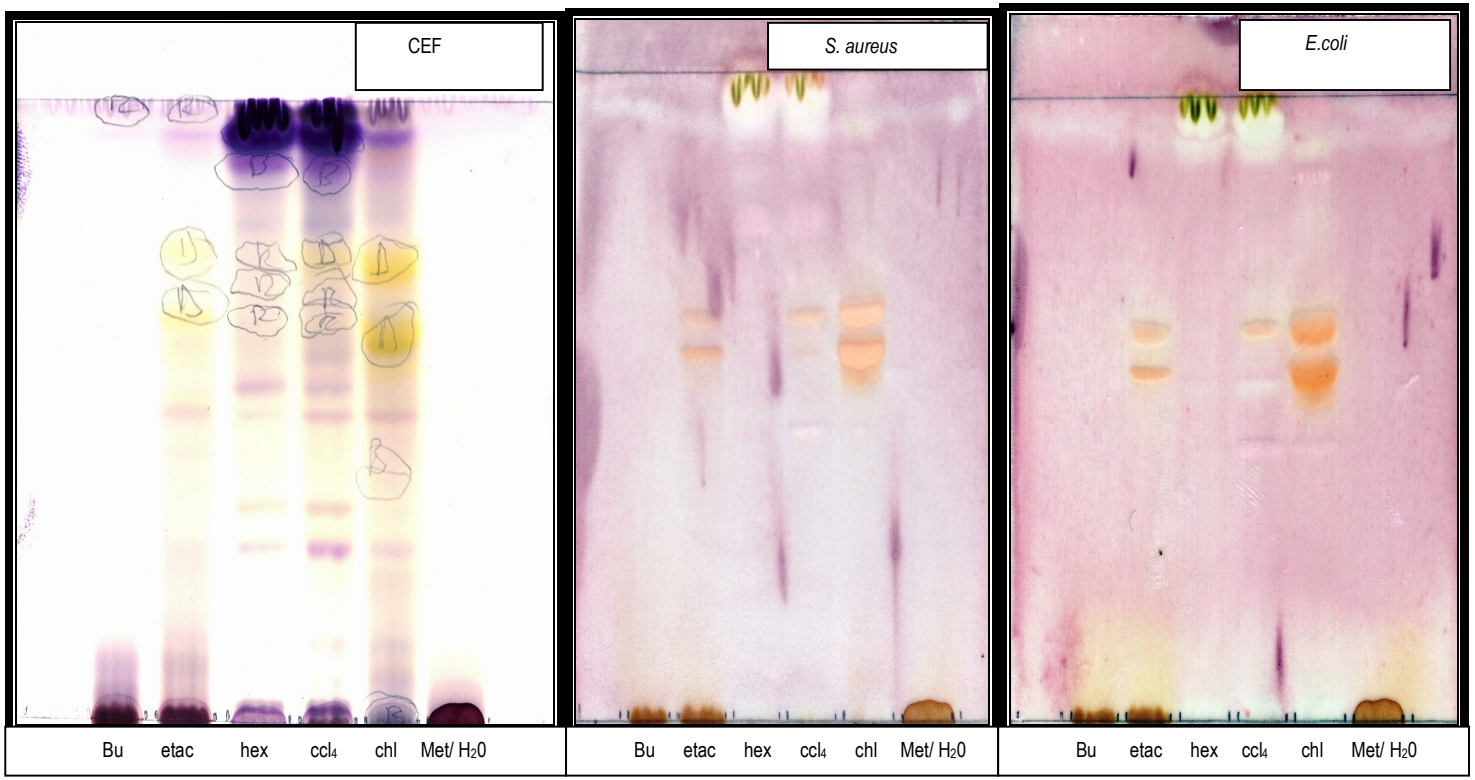

Figure 8. 2. Bioautography of solvent-solvent fractions indicating zones of inhibition on TLC plates against bacterial pathogens. Zones of inhibition against a purple background indicate activity of separated compounds on TLC plates eluted in CEF against *S. aureus* and *E. coli,* Bu = butanol, etac = ethyl acetate, hex = hexane, ccl4 = carbon tetrachloride, chl = chloroform, met = methanol

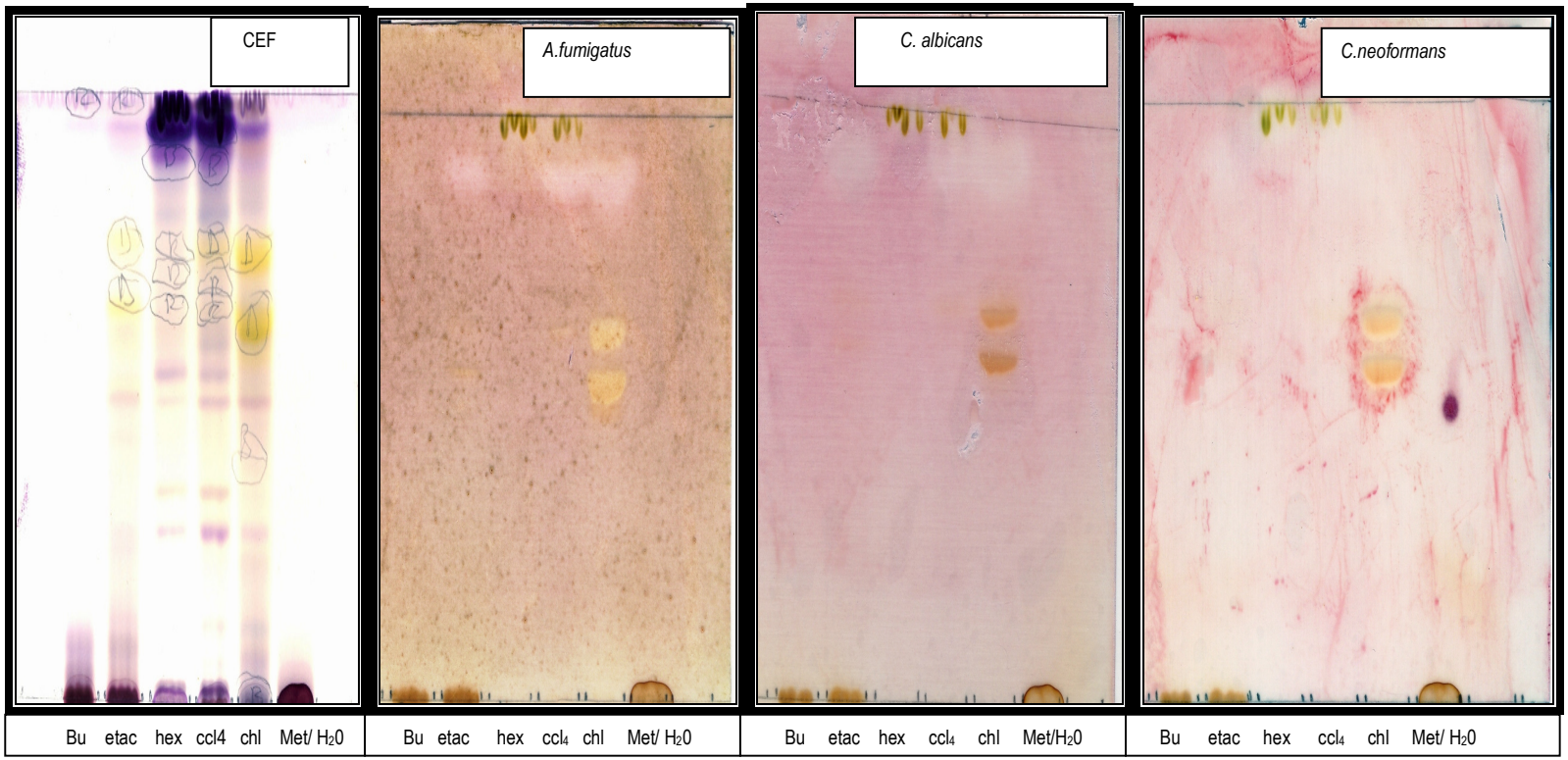

Figure 8. 3. Bioautography of solvent-solvent fractions indicating zones of inhibition on TLC plates against fungal pathogens.

Zones of inhibition against a purple background indicate activity of separated compounds on TLC plates eluted in CEF against *A. fumigatus, C. albicans* and *C. neoformans,* Bu = butanol, etac = ethyl acetate, hex = hexane, ccl4 = carbon tetrachloride, chl = chloroform, met = methanol

Table 8.3a. R_f values of compounds in solvent-solvent fractions active against bacterial pathogens

Organism	CCL_4	Ethyl acetate	$CHCL_3$	Hexane
S. aureus	0.5	0.6	0.6	0.8
	0.62	0.62	0.62	
	0.8		0.9	
	0.9			
E. coli	0.43	0.6	0.43	0.9
	0.53	0.62	0.53	
	0.62		0.62	
	0.9		0.9	

Table 8.3b. R_f values of compounds in solvent-solvent fractions active against fungal pathogens

Organism	CCL_4	Ethyl acetate	$CHCL_3$
A. fumigatus	0.63	0.9	0.54
	0.9		0.63
			0.9
C. albicans	0.93	0.93	0.93
			0.6
			0.7
C. neoformans			0.6
			0.63

The MIC values for the different fractions are presented in Tables 8.3a and 8.3b) against bacterial and fungal pathogens. In some fractions, variation was observed in susceptibility of pathogens with time of incubation. The hexane fraction against *S. aureus* for instance had a low MIC of 0.08 mg/mℓ at 12 hour but following prolonged incubation, a higher MIC of 0.16 mg/mℓ was obtained. This variation in susceptibility may suggest the ability of the organism to revive itself at lower concentrations and susceptibility to the extract at higher concentrations. The lowest MIC of 0.08 mg/mℓ was obtained for the carbon tetrachloride and ethyl acetate fractions against *S. aureus* followed by the chloroform and hexane fractions having an MIC of 0.16 mg/mℓ. With this pathogen, the presence of a high number of active compounds on bioautography correlated with a good MIC value. Unlike *S. aureus*, *E. coli* was more sensitive to the hexane extract with an MIC of 0.04 mg/ml, followed by the carbon tetrachloride and ethyl acetate fractions respectively. Although the chloroform fraction had active compounds comparable to those in the ethyl acetate fraction, the MIC value were higher (0.16 mg/mℓ) when compared to those obtained for the ethyl acetate fraction. The low activity obtained for the chloroform fraction suggests the presence of compounds in this fraction that may antagonize the activity of one another in the fraction. *E. faecalis* on the other hand was more sensitive to the carbon tetrachloride fraction, while the same MIC value of 0.16 mg/mℓ was obtained for both the ethyl acetate and hexane fractions against this pathogen. With *P. aeruginosa* the organism was most susceptible to the carbon tetrachloride fraction with an MIC of 0.08 mg/mℓ. Apart from the carbon tetrachloride fraction, the susceptibility of *P. aeruginosa* to the other fraction seems to require a high concentration with prolonged exposure time. Overall, the carbon tetrachloride and ethyl acetate fractions were the most active against the test bacterial pathogens.

In the antifungal assay, *C. neoformans* was the most susceptible of the three pathogens tested with MIC of 0.08 mg/mℓ obtained for the carbon tetrachloride and chloroform fractions followed by *A. fumigatus* with an MIC of 0.16 mg/mℓ (Table 8.3b). Comparing the presence of active constituents on bioautography in the different extracts, the susceptibility of *C. neoformans* to the carbon tetrachloride fraction does not appear to be related to the presence of single active components in the fraction. A similar effect was observed with this fraction against *C. albicans*. Although no active compound was identified in the carbon tetrachloride fraction against this pathogen, the low MIC obtained suggests the presence of compounds with synergistic effects in the fraction responsible for the activity observed.

8.4. Conclusion

Of the different fractions evaluated, the ethyl acetate, carbon tetrachloride and chloroform fractions contained the highest numbers of antibacterial and antifungal compounds that were active against one or more organisms tested in the study. The study also suggests that synergism and antagonism between different compounds contained in a fraction may potentiate or reduce the activity of the fraction. The fractions identified containing active compounds will be combined. The bioactive compounds will be isolated and evaluated for biological activity.

Chapter 9

Isolation and determination of chemical structure of compounds from *Podocarpus henkelii* Stapt ex Dallim. & Jacks

9.1. Introduction

After solvent-solvent fractionation of the *P. henkelii* acetone leaf extract, and identification of fractions with activity against bacterial and fungal pathogens, the next step was to isolate and identify the bioactive compounds. The chemical structure of a compound can be elucidated using a combination of different techniques such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. The information acquired from the various techniques can then be put together to obtain key structural facts.

The approach in acquiring these data is of paramount importance. A good start will be to obtain information on the molecular formula from the high resolution mass spectrum, or from the molecular ion mass and the number of signals present in the carbon and proton NMR spectra. One can then proceed to determine the number of double bonds and/or rings present by calculating the degree of unsaturation and thereafter that of the functional groups and other molecular fragments present from the ^{1}H and ^{13}C NMR spectra. This information is then assembled, wrong structures eliminated and the correct structure is verified by reanalyzing the NMR spectra against the proposed structure.

9.2. Materials and Methods

9.2.1. Column chromatography

Following bioassay-guided fractionation, the ethyl acetate, carbon tetrachloride and chloroform fractions containing the highest numbers of antibacterial and antifungal compounds were combined and dried under a stream of air. The fraction (mass/g) was mixed with silica gel as stationary phase. The silica gel column (60cm x 5cm) as eluted in a gradient system of chloroform: methanol (9:1) to separate the bioactive compounds. Fractions (109, of 30 ml volume)

were collected and combined to produce four fractions based on similar compounds noted in TLC fingerprints. Compound 2 crystallized out of the eluting solvent while compounds 1 and 3 were obtained on final purifications with equal volume of hexane: ethyl acetate (1:1).

9.2.2. Structural elucidation

The compounds isolated were subjected to instrumental analysis. The compounds were dissolved in methanol, chloroform and dimethyl sulfoxide depending on solubility of the compound for analysis. The structures of the three compounds were elucidated using ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectrometry.

9.3. Results and Discussion

Bioassay-guided fractionation of the acetone leaf extract of *Podocarpus henkelii* using column chromatography led to the isolation of three biflavonoids. The three compounds are structurally related C-C linked biflavonoids. The NMR spectra indicated two flavone units linked through the C-3 of a flavone ring to the C-8 of the second flavone. This class of compound consists of three ring systems: A, B, and C. Ring B, which has a hydroxyl substitution at C-4, often gives a typical 4 peak pattern of two doublets (AA'BB' system) with a characteristic coupling constant; this pattern was clearly shown in C-3, C-5, C-2 and C-6 with a coupling constant of 9 Hz at ring IIB. The protons at H-6 and H-8 in ring A, which are metacoupled also showed a characteristic coupling constant of 2.1 Hz. The isolated compounds (Figure 9.1) were identified as 7,4'7",4"'- tetramethoxy amentoflavone (**1)**, isoginkgetin (**2)** and Podocarpus flavones-A (**3)** based on comparison of NMR and MS data with literature values (Krauze-Baranowska *et al*., 2004; Amaro-Luis *et al*., 2008). The NMR and MS date is included in the appendix.

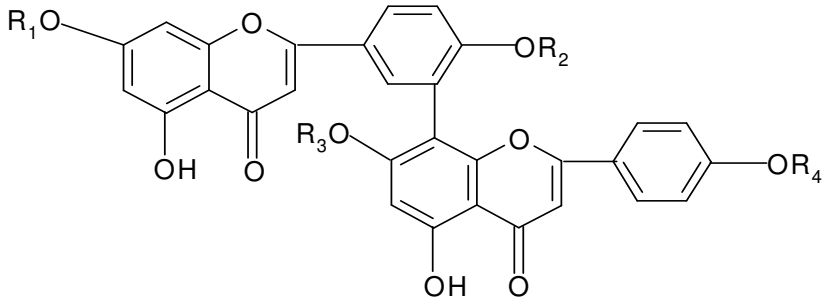

Figure 9.1: Structure of compounds isolated from *Podocarpus henkelii*

Compound 1: $R_1 = R_2 = R_3 = R_4 = CH_3$ 7', 4', 7'', 4''', tetramethoxy amentoflavone

Compound 2: $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$ isoginkgetin

Compound 3: $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$ podocarpusflavone –A

9.4.Conclusion

Three biflavonoids were isolated from *Podocarpus henkelii* and the structures elucidated using data obtained from ^{1}H NMR, ^{13}C NMR and MS analysis. The isolated compounds were identified as 7,4'7'',4'''- tetramethoxy amentoflavone (**1**), isoginkgetin (**2**) and Podocarpusflavones-A (**3**). Available reports indicate the presence of Podocarpusflavone-A in every species of *Podocarpus* so far investigated, except *P. latifolius*. This study represents the first report of isolation of compounds from *Podocarpus henkelii,* as well as the presence of *Podocarpus* flavones-A in this species.

Chapter 10

Biological activity and toxicity studies of isolated compounds from *Podocarpus henkelii* Stapf ex Dallim. & Jacks.

10.1. Introduction

In nature, different types of plants produce certain chemicals (phytoalexins) that are naturally toxic to microorganisms. These chemicals produced by plants play an essential role in the natural defence and well-being of plants, and belong to a wide range of classes, which include the flavonoids and isoflavanoids (Smith, 1996). Flavonoids can be classified into flavanones, flavones, flavonols, and biflavones (Beecher, 2003). Biflavonoids are linkages of flavone–flavone, flavanone–flavones or flavanone–flavanone subunits. Naturally occurring flavonoids are polyphenolic compounds, which can be found in different parts of plants such as flowers, fruits, nuts, seeds, stems and vegetables. They can also be found in wine, honey and commonly consumed beverages such as tea (Grange and Davey, 1990; Middleton and Chithan 1993).

Apart from the phytonutritional role of flavonoids in providing beneficial health effects by the alteration of various metabolic processes, these classes of compounds have been acclaimed for their neuroprotective effect (Kang *et al*., 2005), antiparasitic activity (Mbwanbo *et al.,* 2006), protective effect against DNA damage and lipoperoxidation (Yamaguchi *et al.,* 2005), antiviral activity (Lin *et al.,* 1997; Miki *et al.,* 2007), antimicrobial activity (Lin *et al*., 2001; Xu and Lee, 2001; Yenjai *et al*., 2004*;* Martini *et al*., 2004), anti-inflammatory activity (Selvam and Jachak, 2004), antioxidant activity (Cardoso *et al.,* 2005) and many more.

The biflavonoids Podocarpusflavone-A and isoginkgetin have previously been isolated from *Podocarpus neriifolius* D.Don (Podocarpaceae) (Rizvi *et al.,* 1974). The compound 7', 4', 7'', 4''', tetramethoxy amentoflavone on the other hand has been isolated from *Dacrydium cupressinum* and *Araucaria cooki* (Hodges, 1965). Isoginkgetin has been reported to be less toxic to rat skeletal muscle myoblasts *in vitro* (Weniger *et al*., 2006) in addition to its inhibition of tumour cell invasion by regulating phosphatidylinositol 3-kinase/Akt -dependent matrix metalloproteinase-9 expression (Yoon *et al*., 2006). It has also been found to possess an inhibitory effect on pre-mRNA splicing (O'Brien *et al*., 2008) and neuroprotective effects *in vitro* (Kang *et al.,* 2005).

The presence of diverse molecules represented in the class of biflavonoids and their symmetrical or asymmetrical nature, offers a huge leeway for manipulation by synthetic chemists to further potentiate the biological activity of these useful classes of compounds. Despite the promise and potential therapeutic relevance of this class of compounds, very few biflavonoids have been investigated either for their biological activity, toxicity or as leads for the development of new drugs.

To date no information on antimicrobial activity of these biflavonoids is available. Hence, this study was aimed at evaluating the antibacterial and antifungal activity of the compounds, namely isoginkgetin, Podocarpusflavone-A and 7', 4', 7'', 4''', tetramethoxy amentoflavone isolated from *Podocarpus henkelii*. Their cytotoxic effects on the viability of Vero, bovine dermis and CRFK cells were also assessed in order to determine their selective inhibitory activity, as well as their ability to cause genetic damage with resultant gene mutations as measured by the Ames test.

10.2. Materials and Methods

10.2.1. Determination of minimum inhibitory concentration (MIC) of isolated compounds against bacterial pathogens

The serial microtitre dilution method described by Eloff (1998b) was used to determine the minimum inhibitory concentration (MIC) values of the isolated compounds. The activity of the isolated compounds (1 mg/mℓ) dissolved in DMSO was evaluated against test pathogens (section 3.9.1) using the dilution procedure described in section 3.9.1.1. After incubation for 10 h, 40 µℓ of 0.2 mg/ml INT was added and the plates were further incubated for 2 h. MIC readings were recorded after 12 and 24 hours incubation. Solvent controls and 0.1 mg/mℓ of the standard antibiotic gentamicin (50 mg/mℓ, Virbac) were included in each experiment.

10.2.2. Determination of minimum inhibitory concentration (MIC) of isolated compounds against fungal pathogens

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko *et al*. (2008) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of isolated compounds. The activity

of the isolated compounds were tested at 1 mg/ml dissolved in DMSO against test pathogens (section 3.9.2.1) using the method described in section 3.9.2.2. INT was used as an indicator of growth. A solvent control and amphotericin B (0.08 mg/ml), a standard antifungal agent, was included as a positive control.

10.2.3. Virucidal assay

The virucidal activity of isolated compounds was evaluated using the method described by Barnard *et al.* (1992) with slight modifications as described in section 3.12.2. The activities of the compounds were tested at 2 mg/mℓ since this concentration did not show toxic effects on cells in the cytotoxicity assay. Antiviral activity was evaluated by the ability of the compound to reduce viral-induced CPE by microscopic examination as well as the MTT colorimetric assay. Antiviral activity was determined as described in section 3.12.2. Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells.

10.2.4 Attachment assay

The ability of the viruses to attach to the host cell was tested using the method of Barnard *et al*. (1993) with slight modifications as described in section 3.12.3. Antiviral activity by CPE reduction and MTT assay was determined as previously described (section 3.12.2). Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells.

10.2.5. Cytotoxicity assay using MTT

The cytotoxic effects of compounds at 2 mg/ml dissolved in DMSO were tested against the Vero monkey kidney cell line, CRFK cells and bovine dermis cells as described in section 3.10. Compounds 1 and 2 (2 mg) were dissolved in 0.1 ml DMSO to produce a stock concentration of 20 mg/ml solution. Compound 3 was not isolated in sufficient quantity to allow testing for cytotoxicity. The cytotoxicity was expressed as 50% cytotoxic concentration (CC_{50}) of substances to inhibit the growth of cells by 50%, when compared to untreated cells, calculated from the linear regression equation. Berberine chloride (Sigma) was used as a positive control; wells containing only cells without compound treatment were the negative control and a solvent control was also included. For the purpose of calculating selectivity index (SI), cytotoxicity values greater than 1000 µg/mℓ were taken as being 1000. Selective activities of the compounds were calculated as follows:

Selectivity index (SI) = CC_{50} / MIC

10.2.6. Genotoxicity testing of isolated compounds

The compounds 1 and 2 were investigated for their potential mutagenic effect using the plate incorporation procedure as described in section 3.11. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/mℓ.

10.3. Results and Discussion

10.3.1. Antibacterial activity of compounds

The identified biflavonoids were assayed for antibacterial activity against two Gram-positive and two Gram-negative bacterial strains. Results of antibacterial activity of compounds against test organisms are represented in Tables 10.1a and 10.1b. There were no changes in MIC values with an extended time of incubation, suggesting that the activity was bactericidal rather than bacteriostatic. The Gram-positive organisms were more sensitive to test compounds than their Gram-negative counterparts. This finding is consistent with reports ascribing the effectiveness of antimicrobial agents against Gram-positive bacteria to the porous nature of the outer peptido-glycan layer (Nostro *et al*., 2000; Hodges, 2002). However, with flavonoids, two factors have been reported to be important in their antibacterial activity, namely the lipophilicity of the compounds and the presence of a hydroxyl substitution on the phenolic ring, especially at the 4th and the 5th positions. The lipophilic nature, which is enhanced by increasing the number of methoxy substitutions, is responsible for the trapping of flavonoids in the lipophilic cell wall of the bacteria (mainly Gram-negative). This possibly explains why compound **1** is the least active of the isolated biflavonoids against all the tested pathogens with MIC range of 130-250 ug/mℓ. The MIC values of compounds **2** and **3** ranged between 60 and 250 ug/mℓ. Compound **2** was the most active against all the test pathogens with good activity against *S. aureus* and *E. faecalis* (MIC = 60 ug/mℓ) and a higher selectivity index value (Table 10.1b). The three compounds have a 5th hydroxy substituent, which possibly explains some measure of activity. Compound **3**, which had the highest number of hydroxy substituents, had a broader spectrum of activity than the other compounds against *E. faecalis* and *P. aeruginosa* (MIC = 60 ug/mℓ). Previous reports (Lin *et al*., 2001) showed that some biflavones with hydroxyl substituents were completely inactive against *M. tuberculosis*. In this study, the high number of hydroxyl substitution of compound **3** may be responsible for the uptake of this compound by the organisms. This

finding is in agreement with previous reports ascribing structural activity relationships of flavonoids with antibacterial activity (Cushnie and Lamb, 2005).

Table 10.1a. Minimum inhibitory concentration values (ug/mℓ) of isolated compounds against two Gram-positive and two Gram-negative bacteria after 12 and 24 h incubation

Organism	Time(h)	C1	C2	C3	Gentamicin
S. aureus	12	130	60	130	3
E. faecalis		250	60	60	
E. coli		250	130	250	6
P. aeruginosa		250	130	60	
S. aureus	24	130	60	130	3
E. faecalis		250	60	60	
E. coli		250	130	250	6
P. aeruginosa		250	130	60	

Table 10.1b. Selectivity index values of compounds against bacterial pathogens after 12 and 24h incubation

		SI		
	Time (h)	C1	C2	C3
S. aureus	12	7.69	16.67	7.69
E. faecalis		4.00	16.67	16.67
E. coli		4.00	7.69	4.00
P. aeruginosa		4.00	7.69	16.67
S. aureus	24	7.69	16.67	7.69
E. faecalis		4.00	16.67	16.67
E. coli		4.00	7.69	4.00
P. aeruginosa		4.00	7.69	16.67

However, these factors do not completely explain the activity of biflavonoids because Lin *et al.* (2001) found that the methylation or acetylation of these compounds caused no significant change in their activity in that study. It may therefore be possible that the broad spectrum of activity observed with compound **3** may be associated with multiple effects rather than with a specific cellular target.

10.3.2. Antifungal activity of compounds

The activities of the test compounds against fungal pathogens are represented in Tables 10.2a and 10.2b. The trend of activity did not follow the pattern observed in the antibacterial studies. The best antifungal activity was obtained with compound **2** against *A. fumigatus* and *C. neoformans* with MIC of 30 ug/mℓ and excellent selectivity index values of greater than 30. A similar result was obtained for compound **1** against *A. fumigatus*. Compound **3** was less active against the test fungal pathogens with MIC ranging between 130 and 250 ug/mℓ.

Antimicrobial activity exhibited by naturally occurring flavonoids is attributed to the presence of a phenolic group, and the addition of more such groups might potentiate the activity (Harborne and Williams, 2000). However, a study by Picman *et al*. (1995) indicated that increasing the number of hydroxyl, methoxyl or glycosyl substituents resulted in a steady loss of antifungal activity. This observation may possibly explain the low activity exhibited by compound **3** in this study. Other reports (Gafner *et al.,* 1996) suggest that the organism *Verticillium albo-atrum* used by Picman and co-workers (1995) may be exceptional in its response to hydroxyl/methoxyl substitution. Although the fungal pathogens used in that study were plant pathogens, it is not clear whether the response of the pathogens used in this study were influenced by such substitutions. It may be likely that the structure-activity relationship of antifungal compounds could possibly be associated with multiple factors, unlike in bacteria where cell wall interactions are most critical. Variation in time interval and susceptibility of *C. albicans* to compound **1** was observed. This difference in time of incubation suggests that *C. albicans* might have overcome the antifungal effect of compound **1** and was only susceptible at higher concentrations. This observation may suggest a possible fungistatic effect of compound **1** on *C. albicans* after 24 h of incubation. It is also noteworthy that the very slight change in MIC with prolonged time of incubation suggests that the antifungal effect of the compound is long lived.

Table 10.2a. Minimum inhibitory concentration values (ug/mℓ) of compounds against selected fungal pathogens after 24 and 48 h incubation

Organism	Time (h)	C1	C2	C3	Amp-B
C. albicans	24	130	250	250	40
A. fumigatus		30	30	250	80
C. neoformans		130	30	130	20
C. albicans	48	250	250	250	
A. fumigatus		30	30	250	
C. neoformans		130	30	130	

Amp-B = Amphotericin B

Table 10.2b. Selectivity index values of compounds against fungal pathogens after 24 and 48h incubation

		SI		
	Time (h)	C1	C2	C3
C. albicans	24	7.69	4.00	4.00
A. fumigatus		33.33	33.33	4.00
C. neoformans		7.69	33.33	7.69
C. albicans	48	4.00	4.00	4.00
A. fumigatus		33.33	33.33	4.00
C. neoformans		7.69	33.33	7.69

10.3.3. Antiviral activity of compounds

In the virucidal assay, compounds 1, 2 and 3 were incubated with FHV-1, LSDV, CDV and CPI-2 prior to inoculation onto cells, while in the attachment assay, cells were infected with virus prior to addition of test compounds for 1, 2 and 3 hours. In the virucidal assay, viruses were able to induce cytopathic effect when observed by microscopic examination in cells following exposure to test compounds at 10^{-1} dilution prior to inoculation onto cells, suggesting a non-cidal effect on test pathogens. A similar lack of activity of test compounds was observed in the attachment assay. Amentoflavone isolated from the ethanol extract of *Selaginella sinensis* showed potent antiviral activity against respiratory syncytial virus (RSV), with an IC_{50} of 5.5 µg/ml (Ma *et al*. 2001).

Various factors such as lipophilicity and the presence of a hydroxyl substitution on the phenolic ring for antibacterial activity of naturally occurring flavonoids have been suggested. In this study however, the presence or lack of substitution did not seems to potentiate the activity of the isolated compounds. The lack of activity may, in part, relate to the type of viruses used in the study where structural activity relationships are multifactorial rather than targeting a single component.

10.3.4. Toxicity studies of compounds

The mutagenic properties of organic substances, whether synthetic or natural, can be tested using the Ames test (Ames *et al*., 1975). The Ames test is based on a short-term bacterial reverse mutation assay aimed at detecting ranges of chemical substances capable of producing genetic damage with resultant gene mutations. The results from the Ames test performed on the isolated compounds are presented in Table (10.3) as the mean number of revertants per plate in *S. typhimurium* strains TA98 and TA100 ± S.E.M. Compounds **1** and **2** were tested for their potential genotoxic effects in independent repeated assays. Compound **3** was not tested in the Ames test due to the limited quantity isolated. Substances are considered active if the number of induced revertant colonies is twice the number of revertant colonies of the negative control (blank) (Maron and Ames, 1983). None of the compounds investigated was mutagenic in the *Salmonella*/microsome tester strains TA98 and TA100. Flavonoid-induced mutation in the Ames test is reported to more or less match that of structurally related compounds, e.g. naphthalene derivatives, and the pathological consequences of mutation occurring from the eating of flavonoid-containing foods is said to be low

(Habs *et al*., 1984; Bent, 2002). The observations are consistent with findings in this study where compounds tested exhibited no mutagenic effect (Table 10.3).

Table 10.3. Number of his+ revertants in *Salmonella typhimurium* strains TA98 and TA100 produced by isolated compounds

	TA98			TA98		
	No. of colonies			No. of colonies		
	Concentration (µg/ml)			Concentration (µg/ml)		
Compounds	1000	100	10	1000	100	10
C1	23±5.3	28.3±3.2	26.7±3.8	176±31.8	139±2.5	138±67
C2	25.3±4.6	25.3±4.6	25.5±2.1	170.3±225	169±14.6	154±4.4
C3	-	-	-	-	-	-
Spontaneous	19.3±4			152±10		
4NQO	170.3±20			960±35.1		

A similar non-toxic effect was also observed in the cytotoxicity assay when CRFK, Vero and bovine dermis cells were exposed to the test compounds indicating no differences between the three cell lines with regard to their sensitivity to the compounds. Although the influence of structural-activity relationships on cytotoxicity is not well understood, Kuo *et al*. (2008) suggested that OMe and hydroxyl groups in biflavonoids and monoflavonoids play a crucial role in mediating cytotoxic activity. This may possibly explain the observed non-toxic effect of the test compounds.

10.4. Conclusion

Compound C2 was the most active against *E. coli*, *S. aureus, A. fumigatus* and *C. neoformans*, exhibiting both antibacterial and antifungal activity with good selectivity index values. Compound C3 presented a broad spectrum of activity against *E. faecalis* and *P. aeruginosa*. It could therefore be ascertained that the relationship between structures of the compounds and observed biological activity and toxic effect could support the relevance of functional group substitution in the biological activity of biflavonoids. Compounds C1 and C2 showed no deleterious effect in the cytotoxicity assay on various cell lines, and mutagenicity studies indicated the putative non-genotoxic effect of these compounds. Further studies, including those incorporating a metabolic activation step, are necessary to confirm this conclusion. Naturally occurring pure compounds exhibiting good antimicrobial activity which can

selectively kill microorganisms without being significantly toxic to host cells can be a useful tool in evaluating the potential toxic effect of compounds *in vivo*.

Chapter 11

General discussion and conclusion

The aim of this study was to develop a low toxicity plant extract or isolated compound that is effective against selected bacteria, fungi or animal viruses from leaves of the most promising plant and validate its ethnomedicinal use.

11.1. Antibacterial and antifungal activity of different extracts of selected plant species

The antibacterial and antifungal activity of the hexane, DCM, acetone and methanol extracts of seven selected plant species were determined**.** On bioautography, the presence of visible zones of microbial inhibition varied with some extracts. The presence of visible zones of inhibition was influenced by the polarity of solvents used for extraction of plant active constituents. In general, the acetone and methanol extracts of the different plants species had more active compounds (25 each) followed by DCM extracts. This suggests that the active compounds have intermediate polarity or polar characteristics.

The acetone extracts had the best antifungal activity followed by the methanol extracts. Extracts from more than one plant species had activity against all the pathogens. With DCM extracts, activity was only observed against *C.albicans* while with the hexane extracts no substantial activity was observed against any of the pathogens. The activity of the acetone extracts for those plants that had antifungal constituents ranged between 0.16 to 0.08 mg/mℓ against *C. albicans* and *C. neoformans*. The acetone extract of *Podocarpus henkelii* had an MIC of 0.08 mg/mℓ *against C. neoformans* as did the acetone and DCM extracts of *Acokanthera schimperi* against *C. neoformans*. The DCM and methanol extract of *Annona senegalensis* against *A. fumigatus* and *C. neoformans* respectively had MICs of 0.08 mg/mℓ while the DCM extract of *Plumbago zeylanica* had an MIC 0.08 mg/mℓ against *A.fumigatus*. The

activity of the acetone extract is consistent with previous report (Eloff, 1999; Kotze and Eloff, 2002) where acetone was the best extract for antimicrobial activity. The fact that extracts from several plant species had a wide activity against several bacteria and fungi may indicate the presence of a general metabolic toxin. Consequently the cellular toxicity was investigated.

With bacterial pathogens, acetone extracts of the different plants had the best activity with MIC ranging between 0.16 to 0.08 mg/mℓ followed by the DCM and hexane the least. The DCM extract of *Carissa edulis* had the best MIC (0.04 mg/mℓ) against *P. aeruginosa*.

11.2. Determining the cytotoxic effect of the different extracts on different cell types

In general the hexane was the least toxic indicating that highly polar compounds were not toxic, possibly because they could not be absorbed through membranes. The intermediate polarity extracts were generally the most toxic, possibly again because these compounds are better absorbed.

Annona senegalensis and *Acokanthera schimperi* extracts were the most toxic of all the plants evaluated. These plants are toxic to animals and the cytotoxicity is in line with the *in vivo* toxicity. *P zeylanica* and *S. alata* were the least toxic with *C. edulis* and *P. henkelii* having close to the same safety. *C. edulis* fruit are edible and the cytotoxicity data reflect this. Cells were more tolerant to the toxic effect of extracts at 0.01 mg/mℓ and below in those plants that had moderate toxicity.

To determine which cells were the most sensitive, all values for the different plant species and extractants were combined. Of the three cell types used CRFK was slightly sensitive followed by BD and Vero cells. This pattern was valid for all the concentration tested. Vero and CRFK cells are both kidney derived cells, and may therefore be expected to show a similar response to the toxic effect of the extracts, but this was not the case in this study. At the highest concentration (1 mg/mℓ), all the extracts were very toxic to the cells with three exceptions where the methanol extracts were less toxic. At the lowest concentration tested 0.001 mg/ml in most cases there was little cytotoxicity.

Despite the good antibacterial activity recorded, for extracts of many plant species, only *Podocarpus henkelii* and *Plumbago zeylanica* extracts had moderate toxicity on the different cell types used in the toxicity assay. Similarly, the acetone extracts had the best selectivity index value, followed by methanol. The susceptibility of fungal pathogens was generally highest with methanol and acetone extracts of plants against *C.neoformans* respectively. *A.fumigatus* was the most non-susceptible pathogen against hexane, DCM and methonal extracts of the different plant species.

The protective effect of antioxidant constituents in some extracts varied and appears to be influenced by the metabolism of the type of cell in culture. It also appears to suggest that metabolism in kidney-derived cells can be influenced by species variation in the origin of cells. More studies are required to understand the factors responsible for the difference in susceptibility, to provide more insight on possible contra-indication of some herbal remedies in different animal species. The presence of substances in some extracts at low concentrations that induce viable cell proliferation *in vitro* is also worthy of further investigation, which can help understand the positive or negative outcomes of these substances *in vivo*. As a positive outcome, the presence of such substances in certain plants can serve as useful tonics for organ revitalization which can in turn help balance biochemical and physiological events within the body or act as adaptogens that will help enhance a non-specific resistance of the organism to stress factors and thereby promote its adaptation to stressful external conditions (Mowrey, 1998; Antoshechkin, 2001).

11.3. Determining the antiviral activity of different extracts of selected plant species

The antiviral activities against four viral pathogens in the virucidal and attachment assay were determined for the extracts of the different plant species. In general, good selectivity index values were obtained in the virucidal assay: indicating a more potent antiviral activity when compared to the attachment assay. Of the extracts tested in the virucidal assay, four extracts had significant antiviral activity, two of which were different extracts of *Podocarpus henkelii* against two unrelated viruses. The acetone extracts of *Podocarpus henkelii* against CDV and the methanol extract against LSDV had good activity with SI values of 12.01 and 45.61 respectively in the virucidal assay. The hexane extract of *Plumbago zeylanica* on the other hand had good activity against CDV with SI = 3.07 in the virucidal assay. The hexane extract of *Carissa edulis* in the virucidal assay had weak activity against FHV-1 with EC_{50} of 73.17 µg/mℓ and SI 1.22 while the same against CDV exhibited good activity with an EC_{50} 12.37 µg/mℓ and SI 6.14.

Although a different extractant was used than that used by traditional healers, the presence of antiviral compounds in *Podocarpus henkelii* against two unrelated viruses may justify on a the traditional use of related species *Podocarpus latifolius* and *Podocarpus falcatus* in the traditional treatment of canine distemper infection in dogs.

11.4. Selection of plant species for further investigation

From the pool of initially selected plant species, *P. henkelii* was chosen for further investigation based on the following reasons, 1) no compounds have been isolated from this plant and assayed for biological activity, 2) the

acetone and methanol extracts had good antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis,* 3) the acetone extract had excellent antifungal activity against *C. albicans* and good activity against *C. neoformans* and 4) the acetone extract had good to moderate activity against CDV and LSDV in both the virucidal and attachment assays, while the methanol extract was active against LSDV in the virucidal assay.

11.5. Isolation and biological activity of isolated compounds

Using bioassay-guided fractionation, three biflavonoids were isolated from the leaves of *P. henkelii.* ^{13}C and ^{1}H NMR and mass spectrometric data led to the identification of the compounds as 7', 4', 7'', 4''', tetramethoxy amentoflavone (C1), isoginkgetin (C2) and Podocarpusflavone–A (C3). Podocarpusflavone-A and isoginkgetin have previously been isolated from *Podocarpus neriifolius* D.Don (Podocarpaceae) (Rizvi *et al.,* 1974). The compound 7', 4', 7'', 4''', tetramethoxy amentoflavone on the other hand has been isolated from *Dacrydium cupressinum* and *Araucaria cooki* (Hodges, 1965). Podocarpusflavone-A has been found to occur in every species of *Podocarpus* so far investigated, except *P. latifolius.* Reports on biological activity of these compounds are scanty. Isoginkgetin has low toxicity on rat skeletal muscle myoblasts *in vitro* (Weniger *et al*., 2006) in addition to its inhibition of tumour cell invasion by regulating phosphatidylinositol 3-kinase/Akt -dependent matrix metalloproteinase-9 expression (Yoon *et al*., 2006). It has also an inhibitory effect on pre-mRNA splicing (O'Brien *et al*., 2008) and neuroprotective effects *in vitro* (Kang *et al.,* 2005). Apart from the aforementioned, no information is available on the antimicrobial activity of any of these compounds.

Compound C2 was the most active against *E. coli* and *S. aureus* (MIC = 60 ug/mℓ) and SI value of 16.67. The compound was also active against *A. fumigatus* and *C. neoformans* (SI = 33.33) suggesting both antibacterial and antifungal activity with very good selectivity index values. Compound C3 presented a broad spectrum of activity against *E. faecalis* and *P. aeruginosa* with SI values of 4. A less potent activity of the compounds was obtained in both the virucidal and attachment assays against test pathogens, indicating the non-activity of the compounds against tested viral pathogens. The antibacterial and antifungal activity of flavonoids has been reported to be influenced by structural activity relationships (Harborne and Williams, 2000; Cushnie and Lamb, 2005). Structural activity relationships and antiviral activity have also been reported for biflavonoids where methylation of the hydroxyl groups of biflavonoids resulted in diminished activity (Lin *et al*., 1997). It could therefore be necessary to investigate the relationship between structures of the compounds with the aim of synthesizing a nontoxic and still potent derivative.

11.6. The cytotoxicity and genotoxic activity of isolated compounds

The cytotoxicity of the isolated compounds was tested on vero, CRFK and bovine dermis cells using the MTT assay and mutagenic effect in the Ame's test. Compound 3 was not tested due to the small quantity of the compound isolated. In the cytotoxicity assay. Compound C1 and C2 had no deleterious effect on Vero, bovine dermis and CRFK cells and had no mutagenic activity based on the Ames test.

11.7. Evaluating the correlation between antiviral and antimicrobial activity

Correlating toxicity and biological activity of crude extracts, the antimicrobial activity of extracts against test bacterial, fungal and viral pathogens was influenced by the polarity of solvents used for extraction of plant active constituents. The observed activity was consistent against bacterial and fungal pathogens in some plants. In plants such as *C. edulis* and *P. zeylanica* the acetone and methanol extracts were active against all the bacterial and fungal pathogens. On the other hand, only the hexane extracts of these plants had activity in the virucidal assay against CDV with SI values of 6.14 and 3.07 respectively. This suggests that with these plants, antiviral activity is associated with the non-polar constituents rather than with the intermediate and polar constituents. In contrast, good SI values of 12.01 and 45.61 were obtained for the acetone and methanolic extracts of *P. henkelii* against CDV and LSDV respectively. This finding suggests that polarity of solvents used for extraction may not necessarily dictate the presence of antiviral constituents in a given extract. However, the fact that more active constituents are present in the acetone and methanol extracts with significant activity against bacterial, fungal and viral pathogen, suggests that intermediate and polar solvents do extract a range of constituents active against these pathogen. These solvents can therefore be used to extract biological active constituents with a wide range of activity across a broader spectrum.

Many microbes are causative agents for a majority of life threatening diseases worldwide, with significant economic impact to national economies. With the dwindling per capita income of most third world economies, it has become challenging to strike a balance between establishing good health care systems and providing food by sustained growth in the agricultural sector. Coupled with this is the cost associated with treatment of diseases in resource poor settings. The identification and validation, of traditionally use medicinal plants can play a significant role in alleviating health problems within communities; improve livestock production and food security. Plants are a relatively cheap source of biological material consisting of a vast number of metabolites, primary or secondary. These plants can be prepared in different forms for a desired therapeutic effect or for selecting the molecule of desired biological activity. While a desired therapeutic effect is key behind the rationale for its continuous use, a plant may contain substances with effects that may be unfavourable, depending on the solvents used to extract the desired biologically active component.

It would therefore be worthwhile to invest financial resources in investigating the medicinal potential of our rich flora to combat prevailing microbial infections with the hope of finding potential lead targets for use or that can act as templates for the synthesis of effective and cheap antimicrobials or develop low technology extracts based on good science that can address the primary health care needs of poor communities. The use of crude or potentised

extracts, which more often than not retain their therapeutic efficacy as opposed to single lead compounds due to availability and cost in resource poor settings, provides an exciting challenge using plants such as *P. henkelii*.

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