Isolation and characterization of compounds active against *Cryptococcus neoformans* from *Maytenus undata* (Thunb.) Blakelock (Celastraceae) leaves

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DECLARATION

I declare that the experimental work described in this thesis was conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. These studies are the results of my own investigations, except where the work of others is acknowledged and has not been submitted to any other University or research institution.

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LIST OF ABBREVATIONS

5FC	5-Fluorocytosine
AmB	Amphotericin B
BEA	Benzene, ethanol, ammonia
CEF	Chloroform, ethyl acetate, formic acid
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
EMW	Ethyl acetate, methanol, water
EtOAc	Ethyl acetate
HA	Hemagglutination assay
INT	p-Iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
MeOH	Methanol
MEM	Minimal essential medium
MH	Müller Hinton
MIC	Minimum Inhibitory Concentration
MS	Mass spectrometer
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered solution
RBC	Red Blood Cells (Erythrocytes)
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
SD	Sabouraud Dextrose Broth
TLC	Thin Layer Chromatography

ABSTRACT

Microbial infections are a major threat to public health particularly in developing countries due to the relative unavailability of medicine and the emergence of widespread drug resistance. Serious invasive fungal infections caused by *Candida albicans, Cryptococcus neoformans* and *Aspergillus* spp. represents an increasing threat to human health. They have increased significantly during the past decade, especially in immunocompromised individuals, due to the increased occurrence of HIV infections and resistance development. The toxicity of available antifungal drugs/agents has contributed greatly to the need for new antifungal drugs.

Cryptococcus neoformans is a yeast organism that causes cryptococcosis in both humans and animals. This disease develops following inhalation and dissemination of the organism from the lungs to the central nervous system. Infection with *C. neoformans* often produces pneumonia and cryptococcal meningitis in HIV-infected patients. The problem with this fungus is that AIDS patients respond poorly to treatment and need lifelong therapy to suppress the infection and the drug treatment may be expensive in developing countries. This indicates an urgent need to develop new specific fungicidal antimicrobial agents for the treatment of cryptococcosis.

Plants synthesize a large number of secondary metabolites for protecting themselves against microbe infections caused by bacteria, fungi and viruses. These substances may be useful in the treatment of microbial infections in humans and animals. Plants can be considered as potential sources of therapeutic extracts or active pure chemical compounds for the development of medicines.

During this project, ten plant species *(Zanthoxylum capenses, Morus mesozygia, Calodendron capenses, Catha transvaalensis, Cussonia zuluensis, Ochna natalitia, Croton sylvaticus, Maytenus undata, Celtis africana and Cassine aethiopica)* were screened for activity against *C. neoformans* using both bioautography and the microdilution assay. The most active plant species was selected for the isolation of active metabolites. The selection of plant species was based on the lowest MIC value, presence of clear zones on bioautograms indicating antifungal activity, and high total activity against *C. neoformans*. *M. undata* indicated the presence of clear zones on bioautograms, a low average MIC value of 0.09 mg/ml and high total activity. *C. sylvaticus* and *C. transvaalensis* had lower or equal average MIC values to *M. undata* of 0.07 mg/ml and 0.09 mg/ml respectively. However a lack of clear bands to identify the position of active compounds on bioautography plates disqualified them for further analysis in this study.

The leaves of *M. undata* were exhaustively extracted with hexane, dichloromethane, acetone and methanol respectively. The hexane extract indicated the lowest MIC value of 0.02 mg/ml and was used for isolation of the active constituents. Column chromatography and bioassay-guided fractionation led to the isolation of six triterpene-like compounds.The structure of the isolated compounds was elucidated using the NMR and MS techniques and the compounds were identified as friedelin (**1**), epifriedelanol (**2**), taraxerol (**3**), 3-oxo-11α-methoxyolean-12-ene-30-oic acid (**4**), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) & 3,11-dihydroxyolean-12-ene-30-oic acid (**6**). Friedelin (**1**) and epifriedelanol (**2**) belong to the friedelane group of triterpenoids, taraxerol (**3**) belongs to the taraxerane group and 3-oxo-11α-methoxyolean-12-ene-30-oic acid (**4**), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) & 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) belong to the 12-oleanene group. These groups have been isolated previously from plants that belong to the Celastraceae family.

Four of the six isolated compounds **1,3,5** and **6** were isolated in sufficient quantity to be assayed against two fungal species (*Candida albicans* and *Cryptococcus neoformans*), two Gram-positive bacterial species (*Staphylococcus aureus*, ATCC 29213 and *Enterococcus faecalis*, ATCC 29212) and two Gram-negative bacterial species (*Escherichia coli*, ATCC 27853 and *Pseudomonas aeruginosa,* ATCC 25922).Two of the compounds, 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) & 3,11-dihydroxyolean-12-ene-30-oic acid (**6**), showed clear bands against all the tested organisms on bioautograms indicating microbial growth inhibition. MIC values ranged from 24 µg/ml to 63 µg/ml except for *S. aureus* which was resistant. All the tested microorganisms showed resistance against friedelin (**1**) and taraxerol (**3**) with MIC values of >250 µg/ml, except for *E. faecalis* with an MIC value of 130 µg/ml for taraxerol.

The cytotoxicity of the hexane extract and the isolated compounds were investigated using the tetrazolium-based colorimetric assay (MTT) using Vero monkey kidney cells and the hemagglutination assay using formaldehyde-fixed erythrocytes (RBCs). The hexane plant extract indicated toxicity towards the Vero monkey cells with an LC_{50} of 0.076 mg/ml. Compounds **1** and **3** indicated no toxicity against the cells with an LC_{50} greater than 200 µg/ml. However compounds **5** and **6** indicated toxicity with an LC_{50} of 6.16 µg/ml and 3.36 µg/ml, respectively. Also the hemagglutination assay indicates that hexane extract is toxic towards the RBCs with a HA titer value of 1.6. Both compounds **1** and **3** indicated no agglutination and compounds **5** and **6** indicated HA titer values of 1.33 and 0.67, respectively.

CONFERENCES AND PROCEEDINGS

2005

Indigenous Plant Use Forum (IPUF), Rhodes University, Grahamstown, South Africa

Paper: <u>TA Mokoka</u>, LJ McGaw and JN Eloff. Screening of anti-*Cryptococcus neoformans* compounds in selected South African plants.

2006

Indigenous Plant Use Forum (IPUF), University of Botswana, Gaborone, Botswana

Paper: <u>TA Mokoka</u>, LJ McGaw and JN Eloff. Anti-*Cryptococcus neoformans* activity of *Maytenus undata* extracts.

2007

Postgraduate Meeting, Faculty of Veterinary Science, University of Pretoria, Onderstepoort

Paper: <u>TA Mokoka</u>, LJ McGaw and JN Eloff. Antimicrobial activity of *Maytenus undata* extracts.

Indigenous Plants Used Forum (IPUF), University of Johannesburg, Gauteng, South Africa

Paper: <u>TA Mokoka</u>, LJ McGaw, V Bagla, EO Iwalewa and JN Eloff. *In vitro* antifungal, antibacterial and cytotoxicity activities of the isolated compounds from *Maytenus undata* leaves (Celastraceae)

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CHAPTER 1

INTRODUCTION

For many centuries indigenous people have used natural products, mainly from plants, to combat ailments caused by a variety of microorganisms. The use of medicinal plants in rural and developing countries to maintain good health has been largely passed from generation to generation verbally; hence the importance of medicinal plant usage is not well documented in many cases (Van Wyk *et al.*, 1997). Medicinal plants are the basis of traditional medicine for indigenous people living in rural and developing countries simply because many of them cannot afford conventional pharmacological treatment. Many medicinal plants have provided pharmaceutical companies worldwide with new pharmaceuticals, significantly contributing towards the economic worth of the company.

Presently, the world is faced with a tremendous problem of pathogens with increased antimicrobial resistance due to the abusive use of antibiotics. Antimicrobial agents are often unaffordable or unavailable in developing countries and the highly toxic effects of several presently used antifungal agents is another factor contributing to this problem. Medicinal plants may provide new therapeutical solutions in the shape of extracts or compounds which may be active against pathogens. They may also be less costly, with lower toxicity. There is therefore justification to study the efficacy and safety of medicinal plant extracts.

1.1 LITERATURE REVIEW

1.1.1 Microbial infection: a renewed threat

In recent years, many remarkable changes have occurred in our way of life, producing opportunities for colonization by microbes leading to emergence of unrecognized diseases, or the resurgence of diseases that, at least in developed countries, were thought to be under control (Cohen, 1998). Infectious diseases, particularly skin and mucosal infections, are common in many rural inhabitants due to lack of sanitation, potable water and awareness of hygienic food habits (Caceres *et al.*, 1993; Desta, 1993). Fungi have only emerged as significant pathogens during the past few decades when they have become more frequently diagnosed as opportunistic infections in immunocompromised hosts (Bodey and Anaissie, 1989).

In the last few years the number of immunocompromised and immunosuppressed patients, who frequently develop opportunistic systemic and superficial mycoses (Rahalison *et al.*, 1994; Li *et al.*, 1995) such as candidiasis, dermato-mycosis and other fungal infections, have dramatically increased (Odds, 1988; Ribbon, 1988; Diamond, 1991). This is mainly due to the non-availability of effective cheap antifungal drugs for systemic fungal infections and toxicity of available drugs like amphotericin B (Maddux and Brarriere, 1980;

Saral, 1991). Diamond (1991) indicated that up to 90% of all HIV patients contract fungal infections at some point during the course of the disease and that 10-20% die as a direct consequence of fungal infection (Drouhent and Dupont, 1989). This results in increased morbidity and mortality, and hence a reduction of the length and quality of life (Klein *et al.*, 1984). There is an increased need for the development of alternative antifungal substances.

Infections induced by pathogenic fungi are increasingly recognized as an emerging threat to public health worldwide (Wu, 1994; Walsh *et al.*, 1996). Although there are several synthetic and natural product based drugs available for the treatment of fungal infections, they are not consistently effective against potentially pathogenic fungal infections (Lazar and Wilner, 1990; Gearhart, 1994; Goa and Barradell, 1995). Excessive use of antibiotics has resulted in the emergence of antimicrobial resistance, which has had, and will continue to have, profound effects on human and animal health. These developments and the associated increase in fungal infections (Beck-Sagué and Jarvis, 1993) have intensified the search for new, safer, and more efficacious agents to combat serious fungal infections (Ghannoum and Rice, 1999).

1.1.2 *Cryptococcus neoformans*: an emerging problem agent

Cryptococcus neoformans was discovered 110 years ago when it was isolated from peach juice in Italy by Sanifelice and from the tibial lesion of a patient in Germany by Busse and Buschke (quoted by Mitchell and Perfect, 1995). The genus *Cryptococcus* consists of 19 species, characterized as encapsulated budding yeasts. *C. neoformans*, an encapsulated, usually spherical yeast, is a major pathogen. Two varieties are recognized, *C. neoformans* var. *neoformans and C. neoformans* var. *gattii.* The capsular serotypes of *C. neoformans* have been defined as serotypes A through D and serotypes AD (Evans, 1949; Evans, 1950; Evans and Kessel, 1951; Vogel, 1966). Isolates of *C. neoformans* var. *neoformans* may reveal capsular serotypes A, D, or AD, and isolates of *C. neoformans* var. *gattii* are serotypes B or C (Mitchell and Perfect, 1995).

C. neoformans var. *neoformans* has long been isolated from soil and avian habitats (Levitz, 1991). The ability of *C. neoformans* to use low molecular weight nitrogenous compounds such as creatine, partially explains its ecological niche in avian guano (Mitchell and Perfect, 1995). Birds do not become infected, probably because their relatively high body temperature is inimical to *C. neoformans*, but birds are likely to distribute the yeast in nature (Bulmer, 1990; Neilson, *et al.*, 1977; Ruiz and Bulmer, 1980). White to cream colonies of *C. neoformans* develop within 36 to 72 hrs on laboratory media. Strains of *C. neoformans* isolated from patients grow well at 37ºC and produce diphenol oxidases, which may function as antioxidants and enhance survival of the yeast in the host (Jacobson, *et al.,*, 1994; Jacobson and Tinnell, 1993). The presence of these enzymes, which oxidize a number of diphenolic substrates and lead to the production of melanin, has been used for identification and is considered a potential target for anticryptococcal chemotherapy (Ikeda, *et al.*,

1993; Jacobson, 1993; Williamson, 1994). At 41ºC, *C. neoformans* is inhibited or killed, and this temperature restriction may be an important determinant in its pathogenicity. Generally, *C. neoformans* does not grow as rapidly as yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* under similar conditions (Mitchell and Perfect, 1995).

1.1.3 Cryptococcosis in humans

Invasive mycoses have long been recognized as important opportunistic infections in immunocompromised hosts (Singh, 2003). By the late 1990s, fungal infections had become the seventh leading cause of infectious disease-related deaths in humans (Pinner *et al.*, 1996). Despite an expanded availability of antifungal drugs for the treatment of these infections, the mortality rate remains unacceptably high (Singh, 2003). *Cryptococcus neoformans* causes cryptococcosis in humans and animals. This is a life-threatening disease that develops following inhalation and dissemination of the organism from the lungs to the central nervous system (Uicker *et al.*, 2005). The emergence of the HIV pandemic has resulted in an increased number of immunocompromised patients which are susceptible to contracting cryptococcosis. Infection with the pathogenic fungus *C. neoformans* is a serious infection that often produces pneumonia and meningitis in HIV-infected patients (Brol *et al.*, 2002). *C. neoformans* is the second most important fungus causing disease in Aids patients. In many patients, cryptococcosis is the first indication of AIDS. It has a prevalence of about 6-10% in the United States, Western Europe, Australia and 10-30% in Sub-Saharan Africa (Powderly, 1993; Brol *et al.*, 2002). Van der Horst *et al.* (1997) indicated that a mortality rate in AIDS-associated cryptococcal meningitis ranging from 14% to 25% has been reported with most deaths in patients with AIDS-associated cryptococcosis occurring during the first two weeks of therapy. Patients with AIDS-complicated cryptococcosis often respond poorly to treatment (Zuger *et al.*, 1986). Cryptoccosis in AIDS patients is considered incurable and needs lifelong therapy to suppress the infection (Dupont *et al.*, 1992; Dupont *et al.*, 1994; Ellis, 1994; Kappe *et al.*, 1998).

Transplant recipients are also a major group of immunosuppressed hosts at risk of contracting cryptococcosis (Husain *et al.*, 2001). The cryptococcosis mortality rate is two to five times higher in solid organ transplant patients than in HIV-infected patients (Van der Horst *et al.,* 1997). Kretschmar *et al.* (2001) indicated that 55% of organ-transplant recipients with cryptococcosis have infections of the central nervous system (CNS) with a mortality rate of 50% and this has remained unchanged over the past two decades (Singh *et al.*, 2002). Cryptococcal meningitis still has a treatment failure rate of 10 to 25% (Velez *et al.*, 1993, Paugam *et al.*, 1994) and hence there is a clear and urgent need to develop new, specific fungicidal agents for the treatment of cryptococcosis.

1.1.4 Cryptococcosis in animals

Systemic fungal infections in animals often pose substantial management problems for veterinarians (Hector, 2005; Pinner *et al.*, 1996) because the majority of antifungal drugs are only approved for human use and the information that could help the veterinarian's choice of drug and treatment regimen is largely unavailable (Hector, 2005). Fungal infections affecting the central nervous system (CNS) of small animals such as dogs and cats are uncommon, with the possible exception of cryptococcosis (Lavely and Lipsitz, 2005). *Cryptococcus* has a predilection for the CNS of dogs (Berthelin *et al.*, 1994; Sutton, 1981) and cats (Jacobs and Medleau, 1998). Cryptococcosis is the most common systemic fungal disease in cats. CNS involvement has been reported to be as high as 55% in cats (Lester *et al.*, 2004). No significant age or sex predilection to the disease appears to be present in cats (Kerl, 2003), although males may be slightly more affected than females (Gerds-Grogan and Dayrell-Hart, 1997). Affected dogs are typically less than 4 years of age (Kerl, 2003). The incidence of cryptococcosis in dogs and cats with seizures in Florida was 5.9% (Barnes *et al.*, 2004), whereas 5.6% of dogs with meningoencephalomyelitis had CNS cryptococcosis (Berthelin *et al.*, 1994).
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The treatment of systemic fungal infections in veterinary medicine involves the administration of polyene antibiotics (Amphotericin B), the azoles, imidazoles and triazoles (itraconazole, fluconazole, voriconazole and ketoconazole) and the pyrimidines (flucytosine, 5-FC). Amphotericin B deoxycholate is the most widely used antifungal agent in veterinary medicine. The use of echinocandin antifungals (caspofungin and micafungin) is currently too expensive in most veterinary species (Wiebe and Karriker, 2005). In 1998 the total cost of the first year of treatment of systemic fungal infections in the United States was $2.6 billion. Total cost is highest for candidiasis ($1.7 billion) because of its high incidence. Aspergillosis accounts for $674 million and cryptococcosis and histoplasmosis cost are $151 million and $53 million, respectively (Wilson *et al.*, 2002).

1.1.5 Antifungal drugs in current use

The treatment of fungal infections in immunocompromised patients provides a challenge for infectious disease clinicians (Edmond *et al.*, 1999, Rees *et al.*, 1998). Invasive fungal infections are associated with an unacceptably high mortality rate in patients with suppressed immune systems. There are four major classes of systemic antifungal compounds currently in clinical use: polyenes, the azole derivatives, allylamines/thiocarbamates and the fluoropyrimidines (Georgopapadakou, 1998). The first three classes are targeted against ergosterol, the major fungal sterol membrane, they are ineffective against *Pneumocystis carinni* because this fungal species lacks ergosterol and instead has cholesterol (Kaneshiro *et al.*, 1989).

1.1.5.a Polyenes

Polyene antibiotics were discovered in 1956 from *Streptomyces* species and have been considered the gold standard for the treatment of most serious systemic fungal infections for a long time (Georgopapadakou and Walsh, 1996). This class of compounds has the broadest spectrum of activity of any clinically useful antifungal compounds (Brajtburg *et al.*, 1990; Gallis *et al.*, 1990). The clinically useful polyenes e.g. amphotericin B, nystatin (Figure 1.1) and natamycin have a greater binding affinity for ergosterol, the principal sterol in fungal membranes, over cholesterol (Warnock, 1991), and hence they are less toxic to mammalian cells (Georgopapadakou, 1998). Amphotericin B interacts with ergosterol in the fungal plasma membrane, resulting in aqueous pore formation which causes membrane disruption, leading to increased permeability, leakage of vital cytoplasmic components and death of the organism (Holz, 1974; Kerridge, 1980). Brajtburg *et al.* (1990) reported that oxidative damage was caused by polyenes, which may contribute to the fungicidal activity of these compounds.

Even if amphotericin B has been considered as the standard treatment for fungal infections, it has been associated with various serious toxic effects to mammalian cells. Berliner *et al.*, (1985), Martin *et al.*, (1994) and Pallister *et al.*, (1992) have reported the increased aggregation, adherence and fungicidal action of polymorphonuclear leukocytes associated with amphotericin B. Some of the side effects caused by amphotericin B include nephrotoxicity, reduction of renal blood flow, nausea, vomiting and anorexia. Nystatin, although too toxic for systemic use, is mainly applied topically in cases of mucosal membrane candidiasis (Gupte *et al.*, 2002).

1.1.5.b Azoles and their derivatives

The azole (Figure 1.1) antifungals discovered in the 1960s are totally synthetic and are the most rapidly expanding group of antifungal agents (Fromtling, 1988; Georgopapadakou and Walsh, 1996). They have two or three nitrogens in the 5-membered azole ring, and hence they are classified as imidazoles or triazoles respectively. Systemic azoles have fungistatic, broad-spectrum activity against most yeasts and filamentous fungi. Triazoles are systemic azoles in clinical use (Georgopapadakou, 1998). The clinical safety and efficacy of fluconazole in particular has resulted in its widespread use (Ghannoum and Rice, 1999).

Azoles act on ergosterol biosynthesis at the C-14 demethylation stage. This is a three step oxidative reaction, which is catalyzed by the cytochrome P-450 enzyme 14α-sterol demethylase (P450$_{DM}$) (Georgopapadakou, 1998). Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors including 14α-methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol) (Ghannoum and Rice, 1999). This inhibition causes disruption in the structure and function of the plasma membrane, making it more vulnerable to further damage, and alters the activity of several membrane-bound enzymes, such as those associated with nutrient transport and chitin synthesis (Georgopapadakou *et al.*, 1987, Georgopapadakou and Bertasso, 1992, Vanden Bossche *et al.*, 1994). Greater than 99% ergosterol

depletion affects cell growth and proliferation (Parks *et al.*, 1992). Also, inhibition of P-450$_{DM}$ by the azoles sensitizes fungal cells to oxidative metabolites produced by phagocytes (Shimokawa and Nakayama, 1992). In addition to inhibiting the 14α-demethylase in *C. neoformans*, fluconazole and itraconazole affect the reduction of obtusifolione to obtusifoliol, which results in the accumulation of methylated sterol precursors (Ghannoum *et al.*, 1994; Vanden Bossche *et al.*, 1993). Most of the azoles are toxic and cause hepatic necrosis and abdominal cramping. They are normally used topically in the treatment of candidiasis, coccidiodal meningitis, cutaneous dermatophytes and histoplasmosis (Iyer, 1998).
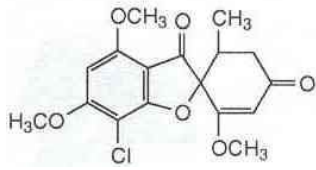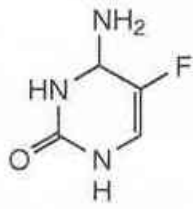
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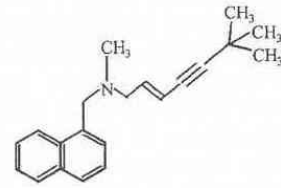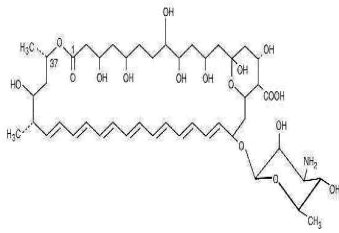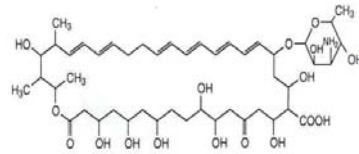
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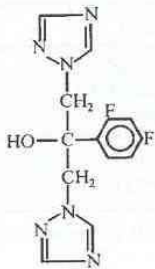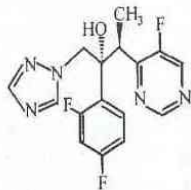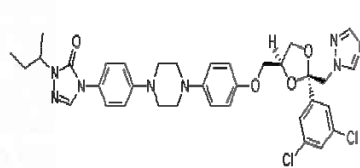
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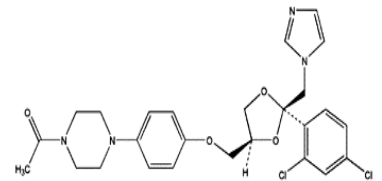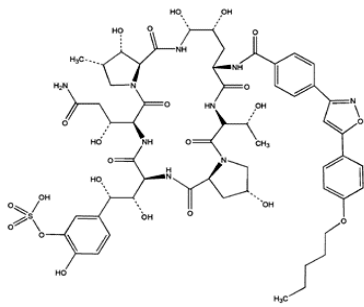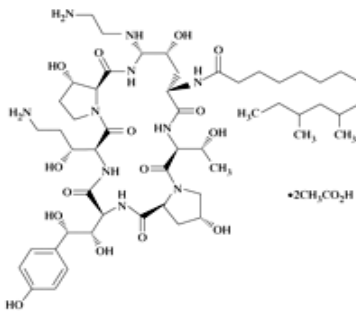
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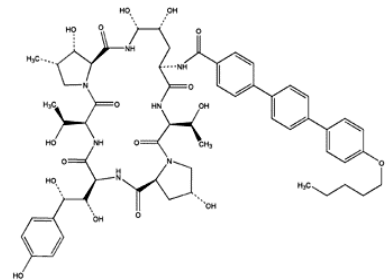
Fig 1.1 Structures of representative antifungal agents (Ghannoum and Rice, 1999; Odds *et al.*, 2003)

1.1.5.c Allylamines and thiocarbamates

This class of compounds was discovered in the 1970s and they are totally synthetic. Terbinafine (Figure 1.1) and naftifine are ergosterol biosynthetic inhibitors that are functionally and chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents. Allylamines act by inhibiting early steps of ergosterol biosynthesis. The allylamine inhibition of sterol synthesis occurs at the point of squalene epoxidation, a reaction catalyzed by squalene epoxidase (Ryder and Favre, 1997). This inhibition coincides with the accumulation of the sterol precursor squalene and the absence of any other sterol intermediate (Kerridge, 1980). Fungal cell death is associated primarily with the accumulation of squalene rather than with ergosterol deficiency (Ryder and Favre, 1997). High levels of squalene may increase membrane permeability (Lanyi *et al.*, 1974) and lead to disruption of cellular organization.

Ryder and Favre (1997) showed that terbinafine is highly effective against dermatophytes *in vivo* and *in vitro*. Terbinafine has high activity against *C. neoformans* and good activity against some azole resistant *C. albicans* strains (Ryder and Favre, 1997). Pharmacokinetic properties limit the clinical efficacy of terbinafine to skin and nail infections, despite broad-spectrum *in vitro* activity (Orth *et al.*, 1990).

1.1.5.d 5-Fluorocytosine (5FC)

The compound, 5-Fluorocytosine **(**5FC) is a fluorinated pyrimidine with inhibitory activity against many types of yeasts, including *Candida* species and *Cryptococcus neoformans***.** This agent is fungicidal (Ghannoum and Rice, 1999). 5FC (Figure 1.1) is mainly used in combination with other antifungals, such as amphotericin B and fluconazole in cryptococcal meningitis and in cases of disseminated candidiasis, but rarely as a single agent (Ghannoum and Rice, 1999). Aided by cytosine permease 5FC enters the fungal cells and, once inside the cell, is converted to 5-fluorouracil (5FU) by the enzyme cytosine deaminase. Subsequently, 5FU is converted by UMP pyrophosphorylase into 5-fluorouridylic acid (FUMP) which is phosphorylated further and incorporated into RNA, resulting in protein synthesis disruption (Polak and Scholer, 1975). 5FU is also converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division (Diasio *et al.*, 1978). Thus 5FC acts by interfering with pyrimidine metabolism, as well as RNA, DNA and protein synthesis in the fungal cells (Ghannoum and Rice, 1999).

Most filamentous fungi lack cytosine permease, cytosine deaminase and uracil phosphoribosyl transferase, and hence the useful spectrum of 5FC is restricted to pathogenic yeasts such as *Candida* species and *C. neoformans* (Odds *et al.*, 2003). Due to the absence or very low activity of cytosine deaminase, 5FC is relatively nontoxic to mammalian cells (Georgopapadakou, 1998). The chemical 5FC is also a potent and widely used anticancer agent (Georgopapadakou, 1998). However, 5FC has been reported to interfere with DNA synthesis and it has been implicated in bone-marrow toxicity, leucopenia and liver enzyme elevations (Iyer, 1998)

1.1.6 Development of resistance against antifungal agents

The term "resistance" is used to describe a microorganism that continues to produce clinical disease despite obtaining high concentrations of the drug at the site of infection (Perfect and Cox, 1999). Ghannoum and Rice (1999) indicated that resistance to antimicrobial agents has important implications for morbidity, mortality and health care costs in US hospitals, as well as in the community. An increasing number of reports of clinical resistance to antifungal agents highlights the need for understanding the molecular mechanisms responsible for the development of drug resistance (Yun-Liang and Hsiu-Jung, 2001). Perfect and Cox (1999) indicated that research on the mechanisms of antifungal drug resistance have focused on several areas (i) drug entry; (ii) drug efflux; (iii) target activity; (iv) inactivation of the drug within cells (i.e. degradation); and (v) effects on other enzymes in the target pathway that may circumvent the need for the target.

There are several antifungal agents currently available and new ones are in development, but the azoles remain the major class of antifungal compounds used to treat human infections (Pierson *et al.*, 2004). Resistance to azole antifungals such as fluconazole, clotrimazole, itraconazole and ketoconazole continues to be a significant problem among hospitalized patients. Hence new ways to treat or overcome fungal infections are strongly awaited (Roeder *et al.*, 2004).

Fungal resistance to polyenes is associated with altered membrane lipids, particularly sterols (Kelly *et al.*, 1994, Pierce *et al.*, 1978, Woods, 1971). Also, phospholipid alteration and increased catalase activity with decreased susceptibility to oxidative damage may contribute to fungal resistance against polyene antifungals (Sokol-Anderson *et al.*, 1988). The mechanisms of fungal resistance against azole antifungal agents involve (i) decreased accumulation of the drug from enhanced efflux; (ii) interference of their action on the target enzyme lanosterol 14α-demethylase, (iii) alterations in other enzymes of the biosynthetic pathway of ergosterol and (iv) decreased permeability of the fungal membrane to the drug. Generally, the mechanism of resistance against azole antifungals is the failure of drug accumulation inside the cell resulting from upregulation of multidrug efflux transporter genes (Sanglard and Odds, 2002, White *et al.*, 1998). The development of resistance against 5-flucytosine involves a reduction in 5FC uptake, prevention of 5FC from being converted to 5FU, and the prevention of 5FU from being converted to 5FdUMP and 5FdUTP. Primary resistance to 5FC is the result of a defect in cytosine deaminase which prevents the drug from entering the fungal cells (Vanden Bossche *et al.*, 1994). Secondary resistance is due to a defect in uracil phosphoribosyl transferase, thus preventing 5FU from converting to 5FdUMP or 5FdUTP (Whelan, 1987).

Plants may be potential sources of new classes of antifungal agents with novel mechanisms of action and represent an attractive strategy for the treatment of infections caused by resistant fungi in the near future. The safety and efficacy of medicinal plant extracts and isolated compounds needs to be validated. The need

to develop effective alternative medicine which will be useful in future to eliminate problems associated with fungal resistance development is on the rise. Medicinal plants provide a potential answer to this problem.

1.1.7 Overview of medicinal plants

Plants have been the subject of human curiosity and use (Ram *et al.*, 2004) for thousands of years. They have played an important role in African countries for centuries by providing food, shelter, clothing, means of transportation, fertilizers, flavours & fragrances, and not the least medicines (Gurib-Fakim, 2006). Indigenous people have relied on medicinal plants to combat ailments such as renal infection, abdominal pains, chest problems, headaches, toothaches, skin infections and many others, and this information has been handed down from generation to generation (von Maydell, 1996). A large proportion of these ailments occur owing to the presence of microorganisms such as fungi, bacteria and viruses that infect the body. In many African countries, medicinal plants are sold in market places or prescribed by traditional healers in their homes (Fyhrquist *et al.*, 2002) to help improve human and animal health. Plants have formed the basis of sophisticated traditional medicine systems and continue to provide mankind with new remedies (Gurib-Fakim, 2006).

Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute no less than 25% to the total (Gurib-Fakim, 2006). Natural products, particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (NCEs) (Newman *et al.*, 2000; Newman *et al.*, 2003; Butler, 2004). Butler (2004) indicated that in 2001 and 2002 approximately one-quarter of the best selling drugs worldwide were natural products, or were derived from natural products. Bioactive natural products have enormous economic importance as specialty chemicals. They can be used as drugs, lead compounds, biological or pharmaceutical tools, feed stock products, excipients and nutraceuticals (Pieters and Vlietinck, 2005).

More than 80% of the population in developing countries depends on plants for their medical needs (Farnsworth, 1988; Balick *et al.*, 1994). Samy *et al* (1999) mentioned that nearly 88% of the global population turns to plant-derived medicine as their first line of defense for maintaining health and combating disease. Because of this strong dependence on plants as medicines it is important to study their safety and efficacy (Farnsworth, 1994). With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of medicinal plants for bioactive compounds is important (Masoko *et al.*, 2005). Cowan (1999) estimated that one-quarter to one half of all pharmaceuticals dispensed in the United States have higher plant origins. The use of plant extracts, as well as other alternative forms of medical treatments, is currently enjoying great popularity.

The number of higher plant species on this planet is estimated at 250 000 (Ayensu and DeFilipps, 1978). Of these, only about 6% have been screened for biologic activity and a reported 15% have been evaluated phytochemically (Verpoorte, 2000). However due to the increased popularity of medicinal plant research this number may have risen.

1.1.8 Future antimicrobial sources

Medicinal plants constitute an important source of active bio-molecules (Sharma, 2002). Plants synthesize secondary metabolites as defense mechanisms to protect them against microbial infections. The presence of these compounds provides an invaluable resource that has been used to find new drug molecules (Gurib-Fakim, 2006).

Compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al.*, 1996). It is estimated that 60% of anti-tumor and anti-infectious drugs already on the market or under clinical trials are of natural origin (Yue-Zhong, 1998). Hardly any are from higher plant original. Many of these chemical compounds cannot yet be synthesized economically and are still obtained from wild or cultured plants (Rates, 2001). Recently there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank *et al.*, 1982; Vulto and Smet, 1988; Mentz and Schenel, 1989). This interest in drugs of plant origin is due to several reasons, for example conventional medicine can be ineffective or may result in side effects and other problems. A huge percentage of the world's population does not have access to conventional pharmaceutical products (Rates, 2001). There are, however, misconceptions about the harmlessness of medicinal plants, so safety as well as efficacy needs to be investigated.

1.1.9 Biologically active constituents from plants

Nature produces an amazing variety and number of natural products. Natural products have long been recognized as an important source of therapeutically effective medicines. Of the 520 new drugs approved between 1983 and 1994, 39% were natural products or derived from natural products (Cragg *et al.* 1997). Newman and Cragg (2007) mentioned that the number of new active substances (NASs), also known as New chemical Entities (NCEs) including biologics and vaccines hit a 24-year low of 25 in 2004, hence indicating that only 28% were derived from natural products and are usually a semisynthetic modification. However the number of NCEs rebound to 54 in 2005, with 24% being natural products or derived from natural products (Newman and Cragg, 2007). In the order of 100 000 secondary metabolites with a molecular weight less than 2500 mainly produced by microbes and plants have been characterized, (Roessner and Scott, 1996). Natural compounds are likely to continue to be sources of new commercially viable drug leads (Henkel *et al.* 1999).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives (Geissman, 1963). Most compounds are secondary metabolites, of which at least 12 000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1978). These substances serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. These compounds may also have other ecological significance, for example terpenoids are responsible for plant odors and quinines and tannins are responsible for plant pigments.

Among the array of secondary metabolites produced by plants are many compounds exhibiting antifungal activity. Well-known examples of these compounds include flavonoids, phenols and phenolic glycosides, unsaturated lactones, sulphur-containing compounds, saponins, cyanogenic glycosides and glucosinomates (Gomez *et al.*, 1990; Bennett and Wallsgrove, 1994; Grayer and Harborne, 1994; Osbourne, 1996).

1.1.10 Economic impact of natural products from medicinal plants

In 1997, the world market for over the counter (OTC) phytomedicinal products was US$10 billion, with an annual growth of 6.5% (Soldati, 1997). Brevoort (1997) estimated that the North American market for products of plant origin reached about US$ 2 billion in 1997. The needs of the pharmacological market and the recognition that research on medicinal plants used in folk medicine represents a suitable approach for the development of new drugs (Elisabetsky, 1987a; Calixto, 2000). In Germany, 50% of phytomedicine products are sold on medical prescription, the cost being refunded by health insurance (Gruenwald, 1997). Private and governmental institutions have recognized the importance of plants, hence they are financially supporting research programmes worldwide. Large pharmaceutical companies, such as Merck, CIBA, Glaxo, Boehringer and Suntex, were reported in 1993 to have specific departments dedicated to the study on new drugs from natural sources (Reid *et al.*, 1993). Pharmaceutical research into natural products has experienced a slow decline during the past two decades because of long product-development cycles encountered in the pharmaceutical industry (Koehn and Carter, 2005). Koehn and Carter (2005) indicated that the worldwide pharmaceutical research and development (R & D) spending tripled roughly from US$ 10 billion to US$ 30 billion from 1984-2003. Basic procedures for the validation of drugs from plant origin in developing countries are urgently needed because many people residing in these countries rely on plants for their therapeutic needs. The isolation of bioactive compounds or potentising of extracts from indigenous medicinal plants may enhance the production of new pharmaceuticals for the treatment of various infectious diseases caused by micro-organisms.

1.1.11 AIMS

The aim of this work was to isolate and characterize antifungal compounds with potential value to protect animals and humans against fungal infections. This aim will be addressed by attempting to attain a number of objectives.

1.1.12 OBJECTIVES

The objectives of this study were to:

1. Identify a plant extract with high antifungal activity against *Cryptococcus neoformans* from a selected group of species.
2. Determine the best extractant and investigate preliminary steps for isolation of the active compounds from the chosen plant species.
3. Isolate the compounds active against *C. neoformans.*
4. Determine the structure of the isolated compounds.
5. Determine the activity of the isolated compounds against other microbial pathogens such as *Candida albicans* and bacterial species.
6. Determine the cytotoxicity of the extracts and isolated compounds.
7. Evaluate the potential use of isolated compounds or extracts of *Maytenus undata*.

CHAPTER 2

PRELIMINARY SCREENING OF MEDICINAL PLANTS FOR ANTIFUNGAL ACTIVITY

2.1 INTRODUCTION

Plants have played an important role in the treatment of infectious diseases caused by a variety of pathogenic micro-organisms for centuries, mainly in rural populations. Gullo and Hughes (2005) held that 75% of new drugs originate from natural sources and that they are useful in combating infectious diseases. Eloff (2004) outlined three main reasons for performing screening studies on medicinal plants, i.e. to find new lead compounds for developing pharmaceuticals, to confirm the ethnomedicinal use of plants, and to develop the phytomedicines for use as herbal medicines.

The Phytomedicine Programme at the University of Pretoria [www.up.ac.za/phyto] has for several years undertaken the random screening of leaf extracts of trees against bacteria and fungi of medical importance. A database of close to 400 tree species and their activities against eight fungi and bacteria has been developed. The plant species selected for comparative preliminary screening in the present study were based on the Phytomedicine laboratory database, taking several aspects into consideration. Firstly, plants were selected based on their minimal inhibitory concentration (MIC) values against *Cryptococcus neoformans* i.e. plants with very low MIC values against a pathogen obtained during random screening were considered. Secondly, plants with very high total activity against *C. neoformans* compared to activity of the other plants were also considered. Lastly, the literature was evaluated to determine if any related work was carried out on any of these species.

2.2 MATERIALS AND METHODS

2.2.1 Collection and preparation of plant material

The plants used for screening were obtained from the Phytomedicine laboratory at the University of Pretoria in a powdered form. These plants were stored in glass storage containers in the dark in the Phytomedicine laboratory. The plant species chosen were: *Zanthoxylum capense* (Thunb.) Harv. (Rutaceae), *Morus mesozygia* stapf (Moraceae), *Calodendrum capense* (L.f.) Thunb. (Rutaceae), *Catha transvaalensis* Codd (Celastraceae), *Cussonia zuluensis* Strey (Araliaceae), *Ochna natalitia* (Meisn.) Walp. (Ochnaceae), *Croton sylvaticus* Hochst. ex C.Krauss (Euphorbiaceae), *Maytenus undata* (Thunb.) Blakelock (Celastraceae), *Celtis africana* Burm.f. (Ulmaceae) *and Cassine aethiopica* Thunb. (Celastraceae).

2.2.2 Extraction

Separate aliquots of 4 g of the leaves of the ten powdered plant species, listed in section 2.2.1 were extracted with 40 ml of four different solvents of increasing polarity (hexane, dichloromethane, acetone and methanol) in 50 ml centrifuge tubes. The tubes were shaken on a Labotec shaking machine for an hour. The extracts were centrifuged at 300 × g for 10 minutes and the supernatant was filtered through Whatman No. 1 filter paper into pre-weighed glass vials and placed under a stream of cold air to dryness.

2.2.3 TLC fingerprinting

Plant extracts were resuspended in acetone to give a concentration of 10 mg/ml. Aliquots of 10 µl (100 µg) were loaded on each of three aluminium-backed thin layer chromatography (TLC) plates (Silica gel 60 $F_{254,}$ Merck) and developed in three mobile systems of differing polarity developed in the Phytomedicine Laboratory, University of Pretoria (Kotzé and Eloff, 2002). The TLC systems used were as follows:
Benzene:Ethanol:Ammonia (18:2:0.2) (BEA, non polar)
Chloroform:Ethyl acetate:Formic acid (10:9:2) (CEF, intermediate polarity)
Ethyl acetate:Methanol:Water (EMW, 10:1.35:1) (polar).

The developed TLC plates were visualized under UV light at 254 µm and 365 µm to detect UV active absorbing spots / plant constituents. The plates were then sprayed with vanillin spraying reagent (0.1g vanillin dissolved in 28 ml methanol, add 1 ml sulphuric acid) and heated at 110ºC to optimal colour development.

2.2.4 Fungal cultures

Fungal cultures of *Cryptococcus neoformans* isolated from a cheetah were obtained from the Microbiology laboratory (Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria). The cultures were maintained in Sabouraud Dextrose (SD) agar at 4 ºC and were inoculated in SD broth at 37 ºC and incubated prior to conducting bioautography and microdilution assays. The fungal inoculum (adjusted with fresh medium to approximately 2×10^6 cells/ml) used during microdilution and bioautography assays was quantified using a haemocytometer.

2.2.5 Bioautographic assay of the extracts

TLC plates prepared as described in **section 2.2.3,** but not sprayed with vanillin spray reagent, were left for several days to allow the eluting solvent to evaporate from the plates before being sprayed with a day-old actively growing suspension of *Crytpococcus neoformans.* The TLC plates were then incubated for 24 hours at 37˚C under 100% relative humidity to allow the microorganism to grow on the plates. After overnight

incubation the bioautograms were sprayed with an aqueous solution of 2 mg/ml p-iodonotrotetrazolium violet (INT, Sigma) and incubated for 30 minutes for observation of clear zones indicating inhibition of fungal growth by bioactive compounds in the extract.

A set of TLC plates sprayed with vanillin was used as reference chromatograms for the bioautography plates displaying areas of inhibition. The R_f values of active zones were correlated with those of bands on the reference chromatograms.

2.2.6 Microdilution assay

The two fold serial dilution microplate method of Eloff (1998c) was used to determine the MIC values of plant extracts. This method has been used to evaluate antibacterial activities of plant extracts (Eloff, 1998c; McGaw *et al.*, 2001) and Masoko *et al.*, (2005) has modified the method for evaluating antifungal activity. Residues of plant extracts were resuspended into acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) in triplicate for each experiment were serially diluted two-fold with water in 96-well microtitre plates. A 100 µl of *C. neoformans* suspension (2×10^6 cells/ml) was added to each well. Acetone was used as a solvent control and distilled water was used as a negative control. Amphotericin B (160 µg/ml) was the positive control. As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in water was added to each well and the covered microtitre plates were incubated at 35°C overnight to ensure adequate colour development. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth. The colorless tetrazolium salt acts as an electron acceptor and is reduced to a formazan product by biologically active organisms (Eloff, 1998c). The MIC values were read after 24 hours and 48 hours. Where fungal growth is completely inhibited (lethal concentration), the solution in the well remains clear after incubation with INT, but inhibition of growth is measured as the first concentration of plant extract that causes a decrease in colour intensity indicated by the formazan salt (inhibitory concentration). The experiment was repeated three times to confirm the results, and three replicates were included in each experiment.

2.2.7 Total activity

Eloff (2000) mentioned that not only MIC but also the quantity extracted should be taken into account to compare the activity of different plants. Total activity indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit the growth of the tested fungal microorganism. This takes into account the quantity extracted from plant material and is calculated as follows:

Total activity = quantity extracted (mg/g)/ MIC value (mg/ml), the units are ml/g.

The higher the total activity of a plant extract, the more effective the original plant is (Eloff, 2000). If the total activity is calculated at each step of a bioassay-guided fractionation procedure it is easy to determine if there is a loss of biological activity during isolation, and also synergistic effects can be discovered very easily. This situation is equivalent to the terms efficacy and potency used in pharmacology (Eloff, 2004).

2.3 RESULTS AND DISCUSSION

2.3.1 Quantity extracted

Acetone (5.44 %) was the best extracting solvent amongst the four solvents because it generally extracted the highest quantity of plant material compared to the other solvents, followed by methanol (MeOH) with 3.39 %, hexane with 0.68% and with dichloromethane (DCM) (0.59 %) extracting the lowest quantity. The acetone extract of *Catha transvaalensis* (4) produced the highest amount of extract of all the plant species (14.6 %) *Z. capense* (1) extracts weighed the least of all the plant species with most of the solvents.
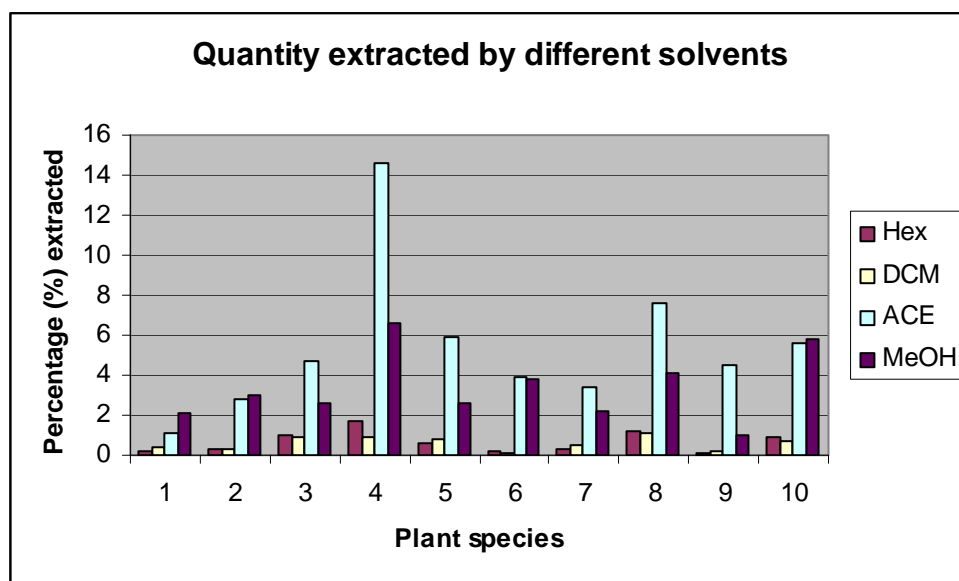
Fig 2.1 Quantity of extracts of ten plant species *(1 = Zanthoxylum capensis,* 2 = *Morus mesozygia,* 3 = *Calodendron capensis,* 4 = *Catha transvaalensis,* 5 = *Cussonia zuluensis,* 6 = *Ochna natalitia,* 7 = *Croton sylvaticus,* 8 = *Maytenus undata,* 9 = *Celtis africana and* 10 = *Cassine aethiopica)* extracted using different solvents

2.3.2 TLC fingerprinting

BEA was the best mobile phase system for separation of almost all the extracts, compared to CEF and EMW, because many compounds were distributed, or separated, efficiently using BEA (Fig 2.2). It is expected that the more polar extractants would extract few of the very non-polar compounds. In general, the separations of the extracted compounds in both the CEF and EMW systems were not as efficient because the majority of the extracted compounds were too non-polar for these solvent systems and moved to the top of the TLC plate making it difficult to count the number of extracted compounds visible after spraying with vanillin sulphuric acid spray reagent (Fig 2.2). Kotzé and Eloff (2002) obtained similar results after extracting *Combretum microphyllum* with different solvents of differing polarities. This can probably be explained by the presence of saponin-like components present in the plant material. Similar to a soap with a polar and non-polar ends, these compounds could make non-polar compounds soluble in polar extractants (Kotzé and Eloff, 2002). In BEA it appeared that the methanol extract contained the lowest number of compounds compared to hexane, DCM and acetone extracts, with DCM extracting the greatest number of compounds (50) followed by hexane (45) and acetone with 36 bands indicating the extracted compounds. Both CEF and EMW mobile system separation of the acetone and methanol extracts of *Catha transvaalensis* showed yellowish separated compounds with a low R_f value. This indicates that the separated compounds are polar. These compounds were also not extracted by the non polar hexane and DCM.
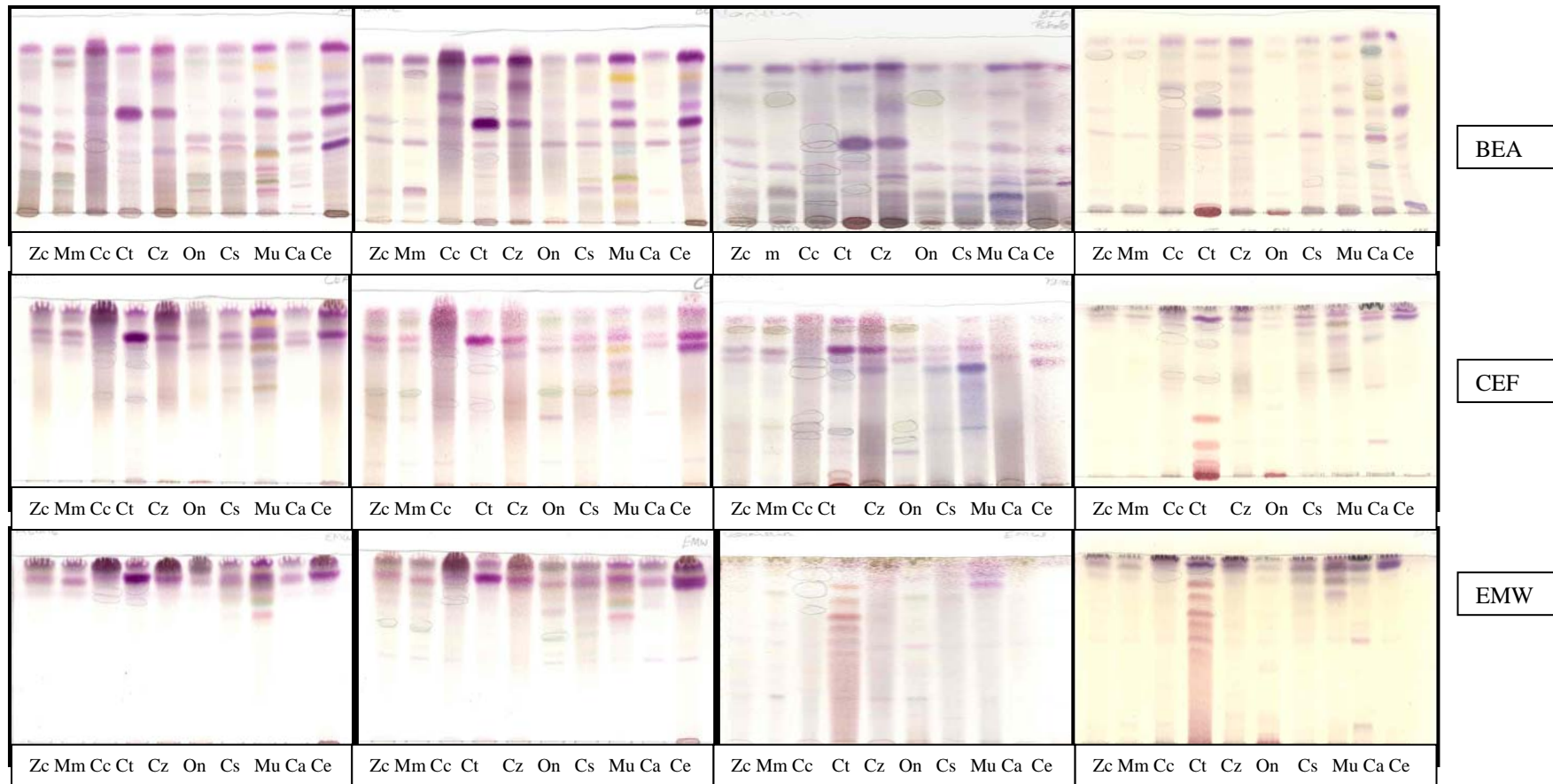
Fig 2.2 TLC chromatograms of ten plant species {**left to right**: *Zanthoxylum capensis* (Zc), *Morus mesozygia (*Mm), *Calodendron capensis* (Cc), *Catha transvaalensis* (Ct), *Cusssonia zuluensis* (Cz), *Ochna natalitia* (On), *Croton sylvaticus* (Cs), *Maytenus undata* (Mu), *Celtis africana* (Ca) *and Cassine aethiopica* (Ce)} extracted with hexane, DCM, acetone and methanol (**left to right**), developed in BEA, CEF and EMW (**top to bottom**), sprayed with vanillin sulphuric acid in methanol.

2.3.4 Bioautography of plant extracts

The bioautograms of ten plant extracts against *C. neoformans* are shown in Fig 2.3. *M. undata* showed a wide clear band on the bioautograms of DCM and hexane extracts developed in BEA and EMW respectively. Other plant extracts did not show any well-defined activity against *C. neoformans* on bioautography. Acetone extracted a high quantity of plant constituents from *M. undata* compared to both hexane and DCM, however this extract did not show any zones of fungal growth inhibition. These compounds were extracted by more non-polar solvents like hexane and DCM and not by acetone or methanol, implying that nature of the active constituents tends more towards non-polarity.
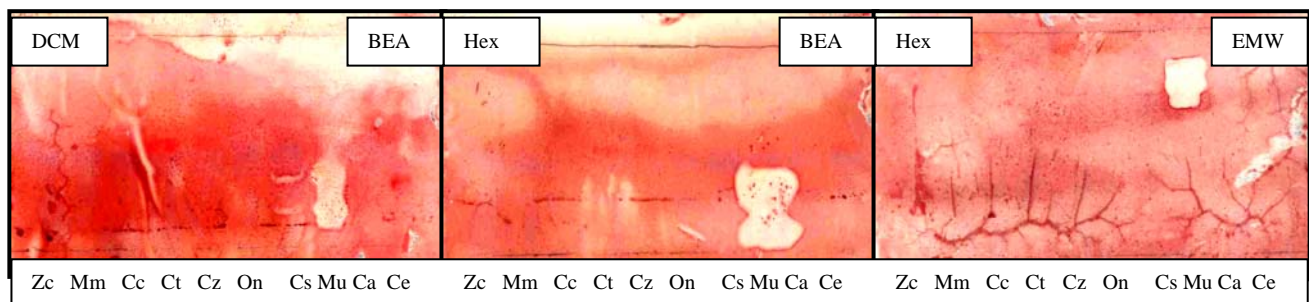
Fig 2.3 TLC bioautogram of ten plant species (from **left to right**: *Zanthoxylum capensis* (Zc), *Morus mesozygia* (Mm), *Calodendron capenses* (Cc), *Catha transvaalensis* (Ct), *Cusssonia zuluensis* (Cz), *Ochna natalitia* (On), *Croton sylvaticus* (Cs), *Maytenus undata* (Mu), *Celtis africana* (Ca) and *Cassine aethiopica* (Ce)) extracted with DCM, Hexane developed in BEA and EMW and sprayed with *Cryptococcus neoformans*. Clear zones on the bioautogram indicate fungal growth inhibition.

2.3.5 Microdilution assay

The MIC values and the total activity of the ten plant species extracted with four different solvents against *Cryptococcus neoformans* are presented in Table 2.1. This table shows that the *M. undata* extracts had very low MIC values compared to those of the other plant species. Also what is evident from the table is the high total activity of *M. undata* extracts, especially the methanol extract with 2050 ml/g and DCM extract with 550 ml/g respectively after 24 hours incubation with the test organism. This shows that 1 g of *M. undata* methanol and DCM extracts can be diluted with 2050 ml and 550 ml respectively and still inhibit *C. neoformans* growth. After 48 hours *M. undata* DCM and MeOH extracts showed a moderate total activity of 636 ml/g and 128 ml/g, respectively. This indicates that the inhibition was fungistatic and that *C. neoformans* was able to overcome the activity of the extracts after 24 hours.

Maytenus undata showed an average MIC value of 0.09 mg/ml after 24 hours and 0.18 mg/ml after 48 hours of incubation (Table 2.2). Also some of the plant species such as *C. sylvaticus* and *C. transvaalensis*

indicated lower/equal average MIC values (0.07 mg/ml and 0.09 mg/ml, respectively) than those of *M.undata* but their activity was not detected using bioautography (Fig 2.3).

A possible reason may be that some compounds are volatile and may have evaporated when the TLC plates are left for a few days to remove the TLC eluting solvents. The active compounds could also have decomposed to produce compounds of low or no antifungal efficacy. *M. undata* indicated a higher average total activity of 769 ml/g than any other plant species tested after 24 hours. After 48 hours both *C. transvaalensis* and *C. capenses* showed higher average total activities of 659 ml/g and 323 ml/g, respectively than *M. undata* with an average total activity of 234 ml/g (Table 2.2). In general this result indicates that, after 48 hours, *C. transvaalensis* extracts were almost three times more active than *M. undata* extracts against *C. neoformans*.

TABLE 2.1 Quantity extracted from 4 g of plant material, average MIC values and total activity of ten plant species against *C. neoformans*

Plant species	Incubation period				24 hours								48 hours							
	Quantity Extracted (mg)				MIC values (mg/ml)				Total activity (ml/g)				MIC values (mg/ml)				Total activity (ml/g)			
	HEX	DCM	ACE	MeOH	HEX	DCM	ACE	MeOH	HEX	DCM	ACE	MeOH	HEX	DCM	ACE	MeOH	HEX	DCM	ACE	MeOH
Z. capensis	9	15	46	83	0.04	0.02	0.08	0.32	56	188	144	65	0.64	0.18	0.08	0.945	4	21	144	22
M. mesozygia	13	11	111	122	0.08	0.04	0.12	0.24	41	69	232	127	0.64	0.12	0.16	0.64	5	123	174	48
C. capensis	39	38	190	104	0.08	0.02	0.03	0.32	122	475	1583	81	0.64	0.20	0.04	0.64	15	48	1188	41
C. transvaalensis	69	36	584	264	0.02	0.04	0.12	0.16	865	225	1217	413	0.64	0.24	0.06	0.48	27	38	2433	138
C. zuluensis	24	31	238	103	0.16	0.02	0.16	0.32	38	388	372	81	0.64	0.20	0.12	0.64	9	39	496	40
O. natalitia	9	5	155	152	0.16	0.02	0.08	0.32	14	63	485	119	1.25	0.24	0.08	0.32	2	5	485	119
C. sylvaticus	13	20	137	89	0.02	0.02	0.12	0.12	163	250	286	186	0.24	0.24	0.64	0.32	14	21	54	70
M. undata	50	44	305	164	0.08	0.02	0.24	0.02	156	550	318	2050	0.16	0.12	0.12	0.32	78	92	636	128
C. africana	6	7	182	41	0.32	0.02	0.12	0.40	5	90	379	26	0.32	0.36	0.06	0.08	5	5	758	128
C. aethiopica	38	28	225	233	0.04	0.02	0.12	0.40	238	350	469	146	0.64	0.48	0.12	1.25	15	15	469	47

Table 2.2 Average MIC values and total activity of *C. neoformans* against the different plant extracts

Plant species	24 hours		48 hours	
	MIC value (mg/ml	Total activity (ml/g)	MIC value (mg/ml)	Total activity (ml/g)
Z. capense	0.15	113	0.46	48
M. mesozygia	0.12	117	0.39	88
C. capense	0.11	565	0.38	323
C. transvaalensis	0.09	680	0.36	659
C. zuluensis	0.17	220	0.40	146
O.natalitia	0.15	170	0.47	153
C. sylvaticus	0.07	221	0.36	41
M. undata	0.09	769	0.18	234
C. africana	0.22	125	0.21	224
C. aethiopica	0.15	300	0.62	137

2.4 CONCLUSIONS

Different solvents extracted different types of plant constituents owing to their different polarities. This feature enables the extraction of plant constituents to be more selective. Bioautography (Fig 2.3) indicated fungal growth inhibition by *M. undata* extracts, especially the hexane and DCM extracts compared to the remaining ten plant species screened. Table 2.2 revealed that the lowest average MIC value was observed with the *M. undata* extracts. *M. undata* showed a well-defined fungal growth inhibition against *C. neoformans* on TLC plates in bioautography, and good antifungal activity in the microdilution assay. Because active constituents from other plant species were not revealed during bioautography, the plant constituents responsible for low MIC values observed during the microdilution assay against *C. neoformans* could not be localized on the TLC plates. A means of avoiding this problem would be to use more volatile solvents as TLC eluents that would evaporate quickly from the TLC plate. Decreased exposure to oxygen in air and light may reduce chemical changes or volatilization of potentially active compounds that are exposed on TLC plates.

The *M. undata* extracts indicated the presence of antifungal constituents using bioautography, and low MIC values in the microdilution assay against *C. neoformans*. This facilitates the identification and isolation of active constituents, and hence *M. undata* was selected for isolation and characterization of plant compounds active against *C. neoformans*.

CHAPTER 3

ACTIVITY OF *MAYTENUS UNDATA* LEAF EXTRACTS AGAINST *CRYPTOCOCCUS NEOFORMANS*

3.1 Introduction

The family Celastraceae includes 98 genera with approximately 1264 species (Simmons *et al*., 2001), and has a long history of use in traditional medicine (Gonazález e*t a*l., 2000). These plants are widespread in tropical and subtropical regions including North Africa, South America and East Asia, particularly in China (Spivey *et al*., 2002). Pentacyclic triterpenes from the friedelane, oleanene, lupane and ursane (De Oliveira *et al*., 2006) as well as the taraxerane, glutinane, dammarane and baccharane series are commonly isolated from species of the Celastraceae family (Núñez et al., 2004). These compounds are likely to be responsible for the various biological activities observed in the plants.

Plants of the genus *Maytenus* belong to the family Celastraceae (Sneden *et al*., 1980) and are either trees or shrubs growing to a height of 1-9 m (Robson *et al*., 1994). This genus has been widely known since the isolation of the antitumor compound maytansine in 1972 (Sneden *et al*., 1980). It has been reported that *Maytenus* species are widely used in folk medicine as anti-tumor, anti-asthmatic and anti-ulcer agents, and as treatment for stomach problems, as analgesics, anti-inflammatories and antimicrobials (Orabi *et al*., 2001; Ghazanfar, 1994; Muhammad *et al*., 2000 and Al Haidari, 2002). Species of *Maytenus* contain compounds with potential pharmacological activity associated with various groups of secondary metabolites such as triterpenoids, sesquiterpenes and alkaloids (Brüning and Wagner, 1978; Hutchings *et al*., 1996). El Tahir *et al.* (2001) found pristemerin, a pentacyclic triterpene of the friedelane type in *M. senegalensis* extracts and activity *in vivo* was demonstrated by Gessler *et al* (1995). Maytenoic acid has been isolated from *M. aquifolium* extracts (Abraham *et al*. 1971, Nossack *et al*., 2004) and has antiplasmodial activity. The compound 3-oxo-friedelan-20α-oic acid occurs in *M. senegalensis,* and has antibacterial and antimitotic activity (Monache *et al*., 1972).

Maytenus undata (Thunb.) commonly known as kokoboom, koko-tree or South African holly, is a shrub or tree between 1.5 to 10 m high (figure 3.2), widespread in tropical southern Africa and in south and southwestern Arabia (Collenette, 1985). This plant is called idohame, egqwabali, ikhukhuze, indabulovalo, inqayi-elibomvu in Zulu (Hutchings *et al*., 1996) and occurs in forest, at forest margins, in ravine forest among boulders and also in open woodland and bushveld, often on termite mounds (figure 3.1). It is often multi-stemmed but can become well-branched, single-stemmed. It has smooth, grey-brown bark with angular purplish young branches. The leaves are ovate, oblong, elliptic to almost circular, 2-13 × 1-9 cm, pale green through to dark green and frequently glossy, thinly leathery, net-veining dark and distinct below. Flowers are small, pale yellow to greenish yellow, inconspicuous, in very shortly stalked axillary clusters barely 10 × 10

cm (September - May). It has a three-lobed capsule fruits about 5-7 mm in diameter, white to yellow becoming red-brown around March – September. The seeds are enveloped by an orange-brown or yellow aril (Figure 3.2). The wood of *M. undata* is red, heavy, close-grained and was once used in wagon construction and for farm tools (Palagrave, 2002). The bark of this plant is used in Zulu as traditional medicine (Hutchings *et al.*, 1996).
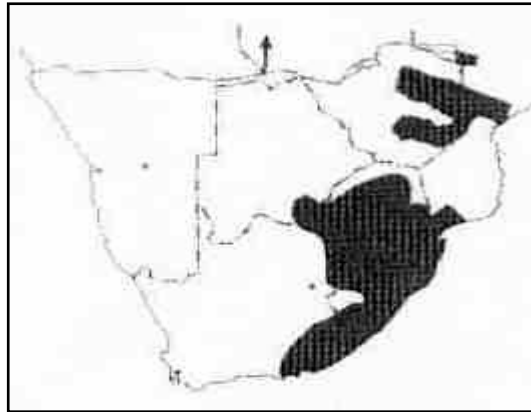
Fig 3.1 Geographical distribution of *maytenus undata* (Palagrave, 2002)

Muhammad *et al.* (2000) investigated the chemical constituents of this plant species and were able to isolate 12-oleanene and 3, 4-seco-12-oleanene triterpene acids which indicated antibacterial activity with MIC values ranging from 50 µg/ml to 3.25 µg/ml. Assessment of the current literature revealed no information on the antifungal activity of this plant, especially activity against *C. neoformans.* As a result, the activity of this plant species against *Cryptococcus neoformans* was investigated in this study.
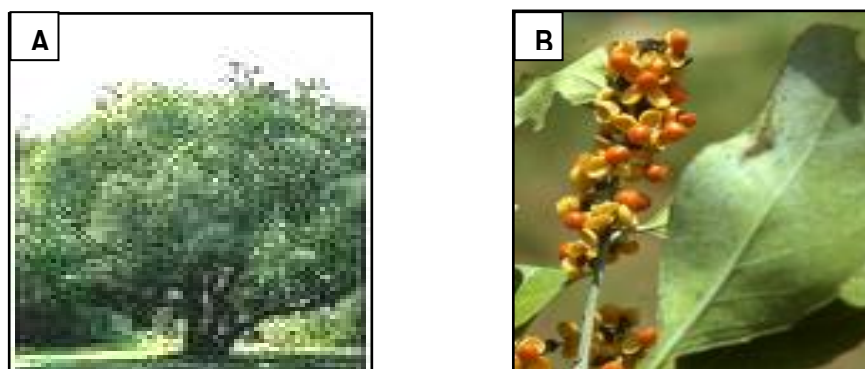
Fig 3.2 *Maytenus undata* shrub (A), leaves, fruits and seeds (B) (httt://images.google.co.za)

3.2 MATERIALS AND METHODS

3.2.1 Plant collection

Fresh *Maytenus undata* leaves were collected from the Lowveld National Botanic Gardens in Nelspruit during summer months (November 2005). The plant leaves were stored in a well-ventilated, dark room until dryness

to avoid photo-oxidation of metabolites from direct sunlight. A voucher specimen has been kept in the Faculty of Veterinary Science University of Pretoria, Medicinal Plant herbarium of the Department of Paraclinical Sciences. Dried plant leaves were ground to a very fine powder using a Jankel and Kunkel Model A10 Mill and stored in a dark place at room temperature until used.

3.2.2 Exhaustive sequential extractions of plant material

Approximately 600g of powdered plant material was exhaustively extracted successively with 6 liters of different solvents of increasing polarity (hexane, dichloromethane, acetone and methanol respectively) three times with fresh 6 liters of solvent each time, and shaken vigorously for six hours on a Labotec shaking machine to facilitate the extraction process. The supernatant was filtered through Whatman No. 1 filter paper using a Buchner funnel and most of the solvent evaporated at 45ºC using a Büchi rotavapor R-114 (Labotec). The reduced extracts were poured into pre-weighed glass vials and left under a stream of cold air to dryness. The quantity extracted was weighed.

3.2.3 Preparation of extracts for TLC fingerprinting and bioautography

The dried extracts were resuspended to a concentration of 10 mg/ml in acetone and TLC was performed as described in **Section 2.2.3**. Three mobile systems were again used, namely BEA (non-polar), CEF (intermediate polarity) and EMW (polar). A duplicate set of TLC plates was sprayed with vanillin and placed in an oven at 110ºC for a few minutes until optimal colour development. These TLC plates served as reference chromatograms. The TLC plates not sprayed with vanillin were left for five days under a stream of air to allow the mobile phase to evaporate completely from the plates to avoid lethal effects of solvents during bioautography. TLC plates were sprayed with *Cryptococcus neoformans* suspension (2×10^6 cells/ml), and incubated overnight in a humid environment. After incubation, the TLC plates were sprayed with 2 mg/ml of INT solution and incubated for 30 minutes until optimum color change, where the colourless INT solution turns red indicating growth of the microorganisms on the TLC plate surface. Clear zones of inhibition can be observed against a red background, indicating fungal growth inhibition of separated plant constituents on the TLC plates.

3.2.4 Microdilution assay

The microdilution method of Eloff (1998c) modified by Masoko *et al.* (2005) was used to determine the MIC values of the *M. undata* extracts against *C. neoformans.* This method is fully described in chapter 2, Section 2.2.6. A well known antifungal agent, amphotericin B, was used as a positive control.

3.3 RESULTS AND DISCUSSION

3.3.1 Quantity of *Maytenus undata* extracted using sequential exhaustive extraction

Maytenus undata leaves were sequentially extracted with hexane, DCM, acetone and methanol respectively. Methanol was the best extractant in terms of quantity extracted amongst the four solvent systems used because it extracted the highest quantity of 13.69% (82.16 g) followed by hexane with 4.09% (24.55 g), DCM with 2.54% (15.23 g) and acetone with 0.75% (4.47 g) respectively. In total about a quarter of the original 600 g was extracted.

3.3.2 TLC fingerprints and bioautograms

TLC plate fingerprints of *M. undata* extracts after sequential exhaustive extraction are shown in Figure 3.1a. The separation in BEA indicates a number of separated non-polar compounds. Some of the extracted compounds did not move on the TLC plate and they have remained at the base of the TLC plates, indicating that they are polar and unable to develop in a more polar system. As expected, the separation of the hexane extract in the polar CEF and EMW systems was poor because the extracted compounds moved to the top of the TLC plates, and hence it is not easy to identify the number of the extracted compounds. The DCM extract indicated the same problem in CEF but the separation was improved in EMW. The separation of the acetone extract was most efficient in almost all the systems used except that in BEA many of the extracted compounds did not develop. The methanol extract did not indicate many compounds extracted from *M.undata* because it did not dissolve well in acetone. From Figure 3.1a it was evident that the same compounds were extracted by both hexane and DCM, and even in the acetone extract the same compounds were observed. This implies that sequential exhaustive extraction is perhaps not the best technique to use when initially separating the components of a crude plant extract. An alternative method such as solvent-solvent extraction would possibly have resulted in a better separation of compounds based on polarity although it is more time demanding. Methanol extracted the highest quantity of all the extractants, but these compounds did not react with the vanilln-sulphuric acid spray reagent used on the chromatograms.
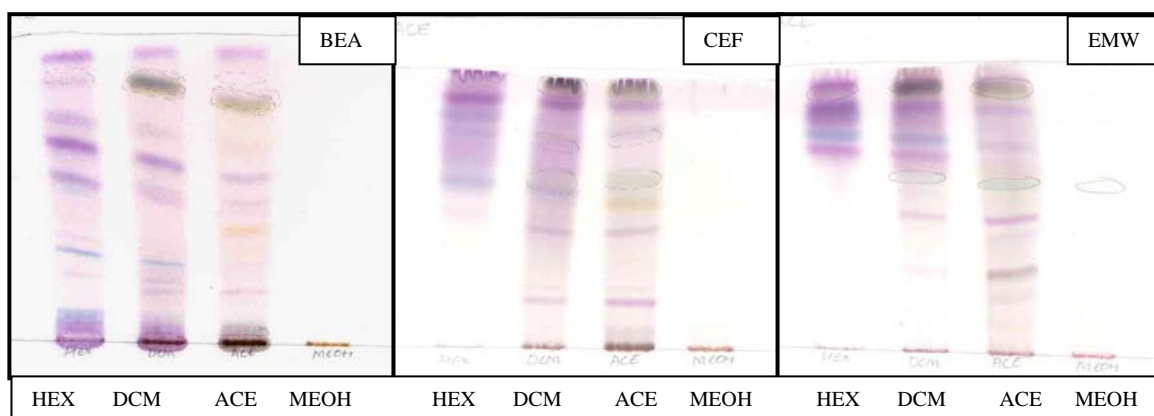
Fig 3.3a TLC fingerprints of *Maytenus undata* extracts, serially extracted with hexane (HEX), dichloromethane (DCM), acetone (ACE) and methanol (MeOH), developed in BEA, CEF and EMW respectively and sprayed with vanillin (0.1% vanillin in sulphuric acid).

TLC bioautograms of *M. undata* extracts against *C. neoformans* are presented in Figure 3.1b. The TLC plates were developed in three mobile systems (BEA, CEF and EMW) as mentioned above. From Figure 3.1b it shows that hexane, DCM and acetone extracts were active against *C. neoformans*. As was the case in Figure 3.1a, some of the compounds did not move in BEA. Figure 3.1b indicates clear zones in the hexane, DCM and acetone extracts at the base of the TLC plate which were not efficiently developed in a non-polar system. This supports the fact that these compounds are relatively polar and that they interact strongly with the matrix on the TLC plate. The clear zones indicate inhibition of *C. neoformans* growth because in these areas the INT is not reduced to the red product by actively growing organisms. With both CEF and EMW solvent systems, a broad clear band indicating *C. neoformans* growth inhibition is present in the hexane and DCM extracts. The identification of the number of compounds active against *C. neoformans* was made difficult by the fact that the separation of these compounds was not efficient. It is also possible that the compounds separated well and that this would have been detected with a shorter incubation period. The possibility of both hexane and DCM extracting similar active constituents was mentioned in the case of Figure 3.1a, and this is supported by the presence of clear bands in both hexane and DCM with the same R_f values. The fact that active compounds are relatively polar is evident because they were not able to develop in BEA. However they developed from the origin in CEF and EMW even though they were not well separated in both systems.
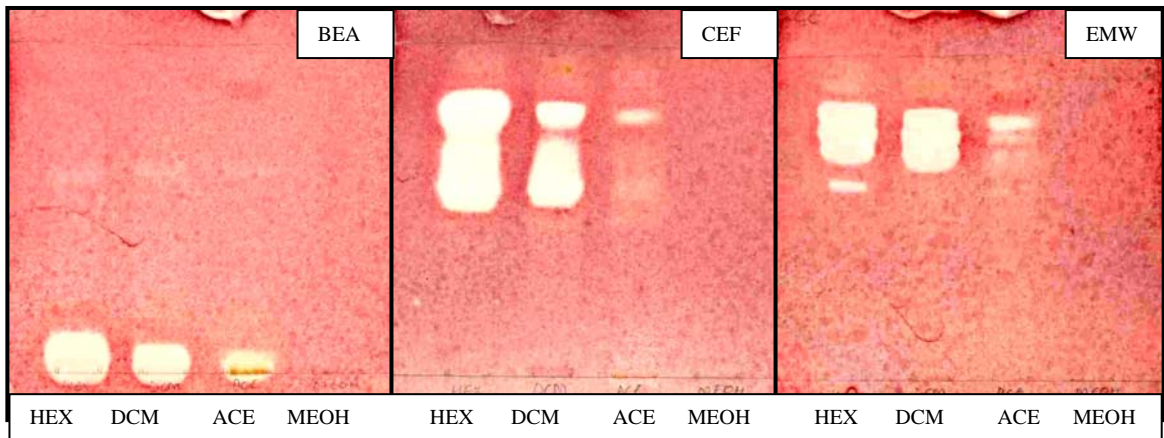
Fig 3.3b Bioautograms of *Maytenus undata* extracts, extracted with hexane (HEX), dichloromethane (DCM), acetone (ACE) and methanol (MeOH), developed in BEA, CEF and EMW respectively and sprayed with an actively growing *C. neoformans* suspension and INT solution. Clear zones on chromatograms indicate fungal growth inhibition.

3.3.3 Minimum inhibitory concentrations

The MIC values and total activity of *M. undata* extracts against *C. neoformans* after 24 hours and 48 hours are shown in Table 3.1. From Table 3.1 it can be seen that *C. neoformans* was most susceptible to hexane, then DCM and acetone extracts after 24 hours. After 24 hours, the hexane extract had a very low MIC value of 0.02 mg/ml followed by the DCM extract with an MIC value of 0.04 mg/ml. The acetone extract had an MIC value of 0.08 mg/ml. This shows that the hexane extract was more active than the other extracts because the lower the MIC value of an extract the more active the extract is. These results are comparable with the bioautograms because all three extracts that showed low MIC values indicated clear spots on the TLC bioautograms. *C. neoformans* was least susceptible to the methanol extract which indicated a very high MIC value and lower total activity of 147 l compared to the hexane and DCM extracts. If the quantity present in each extract is taken into account [Eloff, 2004], the hexane extract showed a high total activity of 1228 l followed by DCM with 381 l and the acetone extract showed a total activity of 56 l after 24 hours. The total activity of the methanol extract was higher than that of the acetone extract simply because methanol extracted a high quantity of about 13.69% compared to acetone which extracted only 0.75% from the same quantity of powdered leaf material.

After 48 hours incubation with *C. neoformans*, all the extracts showed an increase in MIC values and were for all practical purposes inactive. This indicates that the microorganism may have partly overcome the inhibition potential of the *M.undata* extracts. *C. neoformans* was more susceptible to the acetone extract than the other three solvent extracts. The acetone extract indicated an MIC value of 0.32 mg/ml with a total activity of 14 l. Because *C. neoformans* has possibly shown the potential to overcome the inhibition ability of *M. undata* extracts it illustrates that the active constituents may be fungistatic not fungicidal. Alternatively, a less likely

explanation may be that the active components in the plant extract could have decomposed during the longer incubation time to produce less active products, allowing surviving *C. neoformans* organisms to proliferate.

Table 3.1 MIC values and total activity in liter per fraction of *M. undata* serial extracts against *C. neoformans* after 24 hours and 48 hours incubation

Extracting solvents	24 hrs						48hrs					
	MIC values (mg/ml)				Average	Total activity	MIC value (mg/ml)				Average	Total activity
Hexane	0.02	0.02	0.02	0.02	0.02	1228	2.50	2.50	2.50	2.50	2.50	9.8
DCM	0.04	0.04	0.04	0.04	0.04	381	1.25	1.25	1.25	1.25	1.25	12
Acetone	0.08	0.08	0.08	0.08	0.08	56	0.32	0.32	0.32	0.32	0.32	14
MeoH	0.64	0.64	0.64	0.32	0.56	147	2.50	2.50	2.50	2.50	2.50	33
AmpB (µg/ml)	0.31	0.31	0.31	0.31	0.31	-	0.62	0.62	0.62	0.62	0.62	-

3.4 CONCLUSION

The antifungal activity results obtained in this chapter were comparable to the results achieved during the preliminary screening of the selected plant species. During the preliminary screening procedure, both the hexane and DCM extracts showed antifungal activity (Figure 2.3) indicating that plant constituents active against *C. neoformans* were extracted with those solvents. These results were comparable with those obtained after the serial exhaustive extraction of *M. undata* leaves. The hexane extract indicated a high total activity after 24 hours and it was selected for further isolation work because it was easy to localize the active constituents on the bioautograms. The isolation of the active constituents will be described in the following chapter.

CHAPTER 4

ISOLATION OF COMPOUNDS FROM *MAYTENUS UNDATA* LEAF EXTRACTS

4.1 Introduction

Plant extracts are usually complex mixtures containing hundreds or thousands of different constituents (Hamburger and Hostettmann, 1991). The separation and determination of the active components in medicinal plant extracts provide solutions in studying their pharmacological, pharmacokinetic and toxicological mechanisms (Sun and Sheng, 1998). The presence of other plant constituents (e.g. proteins, fats, sugars and tannins) in a crude extract makes the isolation and measurement of active constituents extremely difficult and powerful separation techniques with high efficiency and sensitivity are required (Wen *et al.*, 2004).

Chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) are frequently used for the analysis of plant medicines (Wen *et al.*, 2004). TLC is an important method for the isolation, purification and confirmation of natural products. This method is considered reproducible and accurate (Wen *at al.*, 2004) and it is also suitable for bioautography techniques (Iscan *et al.*, 2002).

Column chromatography is one of the most frequently used techniques in the isolation of natural plant constituents. In principle, plant constituents are distributed between the solid phase (for example silica gel or Sephadex) and the mobile phase, which comprises an eluting solvent. In silica gels the separation of compounds from each other in an extract is based on a number of factors including the polarity of compounds, hence compounds are eluted from the column with solvent systems of differing polarity. Silica gels constitute polar ends which interact strongly with polar compounds and they are eluted later from the column. In Sephadex gel filtration the separation of constituents in an extract depends on the size of the molecules. Constituents with a small molecular weight interact strongly with the matrix of the gel and tend to move slowly through the gel and they are eluted later while the large molecular weight constituents are eluted early because they move fast through the column.

The successful isolation of bioactive compounds from indigenous medicinal plants will validate indigenous knowledge adding value to plants and support plant conservation and knowledge preservation. It may also contribute to research and development in the production of new pharmaceutical drugs for the treatment of various infectious diseases caused by micro-organisms such as fungi, bacteria and viruses.

4.2 Materials and methods

A schematic representation of the procedure followed in the isolation of compounds from *Maytenus undata* is shown in figure 4.1
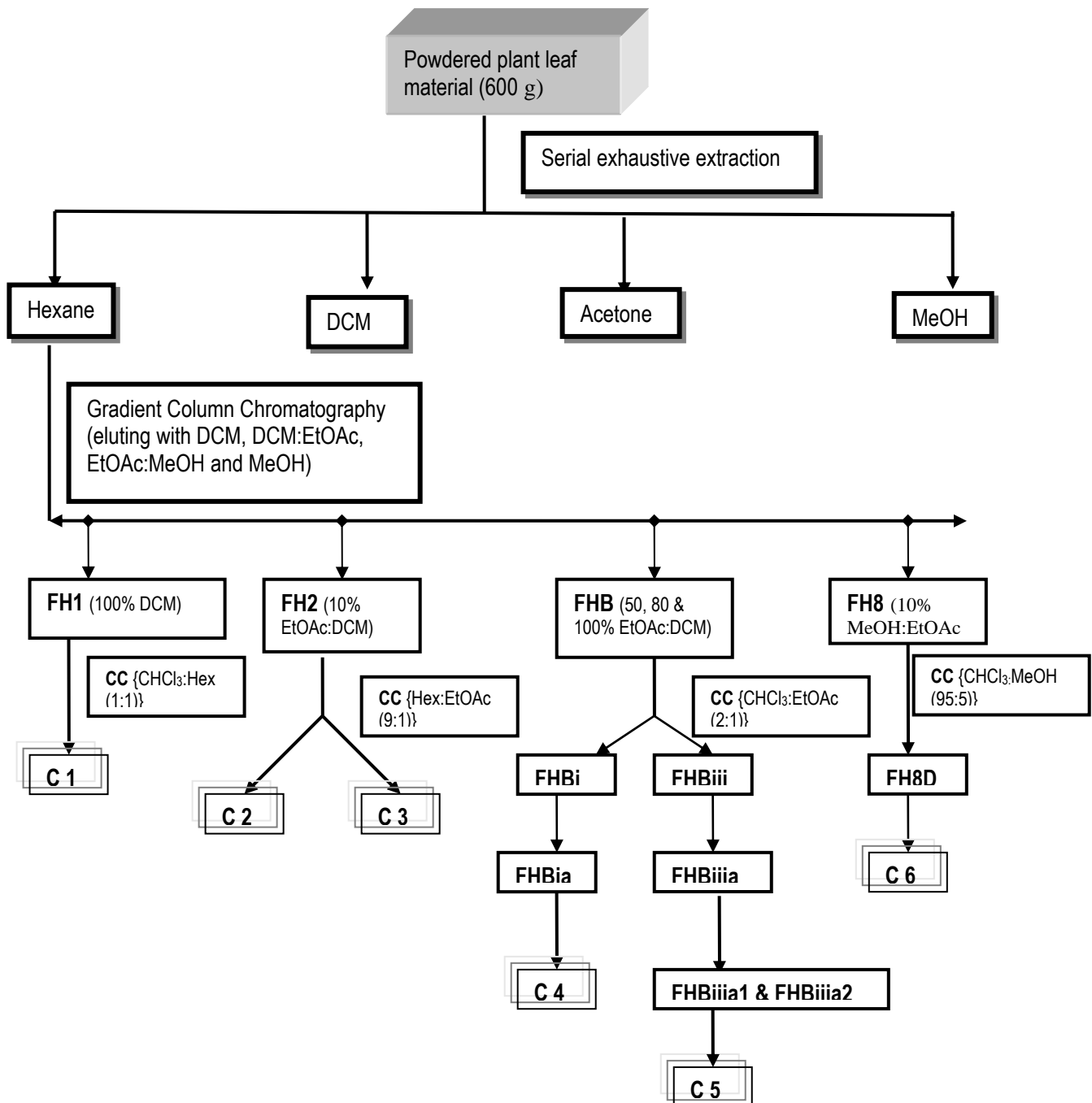
Fig 4.1 Schematic representation, indicating the isolation pathway of the compounds from *M. undata.*

4.2.1 Isolation procedure

4.2.1.1 Isolation of active compounds from hexane extract

Silica gel 60 (400 g) (Merck) was mixed with DCM to form a slurry and packed to a height of 37.0 cm in a 5.0 cm diameter glass column. Hexane extract (22 g) was dissolved in a small volume of DCM, mixed with 25 g of silica gel 60 (Merck), allowed to dry under a stream of cold air and loaded on top of the packed column. Initially the column was eluted with 100% DCM and subsequently, the polarity of the eluting solvent was sequentially increased with ethyl acetate (EtOAc) and methanol (MeOH) respectively. Essentially, a volume of 200 ml of 100% DCM was initially used, followed by the same volume of each of the following solvent mixtures: 10% EtOAc, 20% EtOAc, 30%EtOAc, 50% EtOAc, 80% EtOAc all in DCM, 100% EtOAC and 10% MeOH, 20% MeOH, 30% MeOH, 50% MeOH, 80% MeOH all in EtOAc, and finally the column was eluted with 100% MeOH. The fractions were labeled FH1 to FH13. Fractions of 200 ml each were collected, concentrated using a rotavapor at 45ºC and transferred to preweighed glass vials to dry completely under a stream of cold air. The collected fractions were resuspended to 10 mg/ml in $CHCl_3$, and 100 µg was loaded onto each of six 10×20 cm TLC plates. Two plates each were developed in BEA, CEF and EMW. One set of TLC plates eluted with each of the three solvent systems was sprayed with vanillin and served as reference chromatograms, and the remaining three plates were sprayed with *C. neoformans* suspension for bioautographic assay.

4.2.1.1a Isolation from FH1 (1)

Silica gel 60 (65 g) was mixed with $CHCl_3$:Hex (1:1) and packed in a 27.0 cm × 3.0 cm column. The 100% DCM fraction (**FH1)** from the first column (2.112 g) was dissolved in a small volume of $CHCl_3$ and mixed with 4 g of silica gel 60, dried and loaded on top of the packed column. The column was eluted with $CHCl_3$:Hex (1:1). Fractions of 10 ml were collected.

4.2.1.1b Isolation from FH2 (2)

Silica gel 60 (160 g) was mixed with Hex: EtOAc (9:1) solution and packed in a 27.0 cm × 4.5 cm column. The 10% EtOAc fraction in DCM (**FH2**) from the first column (4.452 g) was dissolved in $CHCl_3$ and mixed with 15 g of silica gel 60 (Merck), dried and loaded on top of the packed column and the column was eluted with Hex: EtOAc (9:1) solution. Fractions of 10 ml volume were collected.

4.2.1.1c Isolation from FHB (5, 6 & 7)

Fractions FH5, FH6 and FH7 were combined and the solvent removed. Silica gel 60 was mixed with CHCl$_3$: EtOAc (2:1) and packed in a 24.0 × 3.0 cm column. Plant extract   (FHB, 5.477 g) was dissolved in CHCl$_3$ and mixed with 3 g of silica gel 60, dried and loaded on top of the packed column. The column was eluted with a mixture of CHCl$_3$: EtOAc (2:1). Ten µl of the collected fractions were loaded onto 10 cm × 20 cm TLC plates and developed in CEF, sprayed with vanillin sulphuric acid in methanol and heated in an oven at 110ºC until optimal colour development.

4.2.1.1ci Isolation from FHBi

Silica gel 60 (15 g) was mixed with CHCl$_3$:MeOH (9:0.8) and packed in a 36.0×1.0 cm. Fraction **FHBi** (1.10 g) was dissolved in CHCl$_3$ and mixed with 1 g of silica gel 60, dried and loaded on top of the packed column before being eluted with CHCl$_3$:MeOH (9:0.8) and 1 ml fractions were collected.

4.2.1.1cia Isolation from FHBia

Silica gel 60 (20 g) was mixed with DCM: EtOAc (4:1) and packed in a 40.0 × 1.0 cm column. **FHBia** (400 mg) was dissolved in CHCl$_3$ and mixed with 4 g of silica gel 60, dried and loaded on the column. The column was eluted with DCM: EtOAc (4:1) and 1 ml fractions were collected.

4.2.1.1cia1 Isolation from FHBia5

Silica gel (10 g) was mixed with Hex: EtOAc (1:1) and packed in a 20.0 × 1.0 cm column. **FHBia5** (80 mg) was dissolved with CHCl$_3$, dried and loaded on top of the column. The column was eluted with Hex: EtOAc (1:1) and 1 ml fractions were collected.

4.2.1.1cii Isolation from FHBiii

Silica gel 60 (40 g) was mixed with CHCl$_3$ and packed in a 19 × 3.0 cm column. **FHBiii** (817 mg) was dissolved in CHCl$_3$ and mixed with 50 mg silica gel 60, dried and loaded on top of the packed column. The column was eluted with 200 ml of 100% CHCl$_3$ and the polarity of the eluting system was gradually increased with MeOH starting with 2.5%, 5%, and 7.5% until 10% MeOH and fractions (1 ml) were collected.

4.2.1.1ciia Isolation from FHBiiia

Silica gel 60 (Merck) was mixed with 2.5% MeOH in $CHCl_3$ and packed in 13.0 × 1.0 cm column. **FHBiiia** (500 mg) was dissolved in $CHCl_3$, dried and mixed with 5 g of silica gel 60 (Merck), left under a stream of air to evaporate the solvent and loaded on top of the packed column. The column was eluted with $CHCl_3$:MeOH (97.5:2.5) and fractions (1 ml) were collected.

4.2.1.1ciia1 Isolation from FHBiiia1 and FHBiiia2

Silica gel 60 (10 g) was mixed with $CHCl_3$:MeOH (90:5) and packed in separate 20.0 × 1.0 cm columns. Both **FHBiiia1** (118 mg) and **FHBiiia2** (94 mg) were dissolved in $CHCl_3$, mixed with silica gel 60, dried and each fraction loaded on top of one of the packed columns. The columns were eluted with $CHCl_3$:MeOH (90:5) and 1 ml fractions were collected.

4.2.1.1d Isolation from FH8 (8)

Silica gel 60 (50 g) was mixed with $CHCl_3$: MeOH (95:5) and packed in 100 cm × 1 cm column. The 10% MeOH in EtOAc fraction (1.697 g) was dissolved in $CHCl_3$ and mixed with 2 g of silica gel 60 (Merck), dried and loaded on top of the packed column. The column was eluted with $CHCl_3$: MeOH (95:5) solution.

4.2.1.1di Isolation from FH8D

Silica gel (10 g) was mixed with 10% MeOH in $CHCl_3$ and packed in a 20 cm× 1 cm column. **FH8D** (200 mg) fraction was dissolved in $CHCl_3$, mixed with 500 mg of silica gel 60 (Merck), dried and loaded on top of the packed column. The column was eluted with 10% MeOH in $CHCl_3$.

4.2.1.1ii Isolation from FH8D3

Silica gel 60 (5 g) was mixed with 5% MeOH in $CHCl_3$ and packed in a 17.0 cm × 1.0 cm column. **FH8D3** (120 mg) was dissolved in $CHCl_3$, mixed with 10 mg of silica gel 60, dried and loaded on the packed column. The column was eluted with 5% MeOH in $CHCl_3$ and fractions (1 ml) were collected.

4.2.2 Thin layer chromatography (TLC) profiles

After every subsequent isolation of the plant constituents the collected fractions were loaded onto 10 cm × 20 cm TLC plates and developed in an appropriate mobile phase which gave a good separation and sprayed

with vanillin spray reagent heated in an oven at 100 ℃ until colour development. Similar fractions were combined together and kept for further isolation until clean pure compounds were obtained.

4.3 Results and Discussion

The preliminary isolation of active constituents from the hexane extract using silica gel column chromatography resulted in 13 fractions. These fractions were reconstituted in an appropriate organic solvent (CHCl$_3$), developed by TLC in BEA and sprayed with vanillin spray reagent or *C. neoformans*. This TLC analysis enables the pooling of fractions which contain similar constituents and also makes it easy to target the active constituents.

Fractions containing plant extract constituents after the initial open column chromatography were developed in BEA and sprayed with vanillin spray reagent as depicted in Figure 4.2 (A). As expected, plant constituents were separated based on their polarity because some of the constituents were eluted using 100% DCM and other constituents are not eluted; however when the polarity of the eluting solvent was increased other constituents were eluted. Also it is clear that both 20% EtOAc and 30% EtOAc in DCM eluted similar constituents with slight differences which might be because of the overlapping of constituents of closely related polarities.

To ensure the isolation of antifungal compounds pooled fractions were analyzed by bioautography against *C. neoformans* (Figure 4.1B). Two clear bands on a bioautogram in fractions 3 & 4 indicate the presence of plant constituents which are able to inhibit fungal growth. These clear bands have the same R$_f$ values in BEA and other solvent systems that were used (CEF and EMW) which implies that they are the same constituents in both fractions. Therefore these two fractions were combined to yield the fraction called **FHA**. Again in Figure 4.2, fractions 4, 5, 6 and 7 indicated clear bands of the same R$_f$ value in BEA showing *C. neoformans* growth inhibition, except fraction 7 which showed an extra thin clear band under a broader clear band indicating the presence of two active constituents against *C. neoformans* in this fraction. Therefore, fractions 5, 6 and 7 were combined to yield the fraction called **FHB**.

By comparing a TLC chromatogram in Figure 4.2 (Left), fraction 8 contains a thin reddish band which is also present in fraction 7. This band in fraction 7 indicated a slight antifungal activity (Figure. 4.2, right) showing a thin clear band. Fraction 7 was therefore employed to serve as a guide in assuming the presence of an active constituent in fraction 8; hence it was further isolated and it will be discussed in the following sections. The poor separations of plant fractions on the TLC plate when using EMW, CEF and BEA made the identification of the exact active constituent more difficult. Subsequent separation of plant constituents from semi pure fractions was performed and the results are indicated below for each separation step.
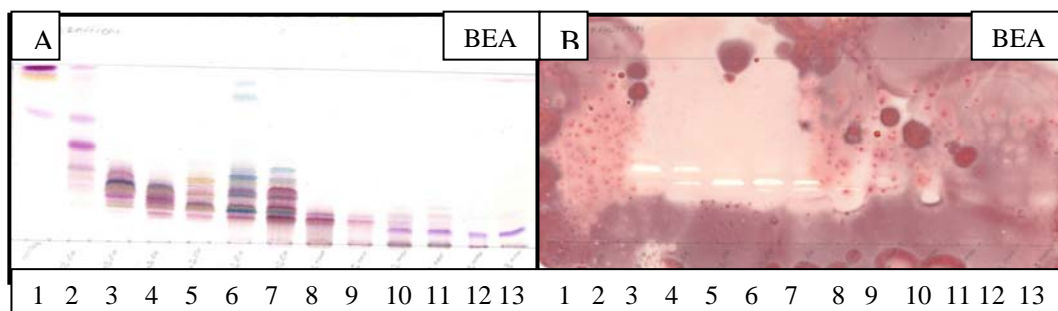
Fig 4.2 TLC of fractions of the hexane extract after open column chromatography.

[Fractions from **left to right**: 100% DCM (**1**), 10% EtOAc (**2**), 20% EtOAc (**3**), 30% EtOAc (**4**), 50% EtOAc (**5**), 80% EtOAc (**6**), 100% EtOAc (**7**), 10% MeOH (**8**), 20% MeOH (**9**), 30% MeOH (**10**), 50% MeOH (**11**), 80% MeOH (**12**) and 100% MeOH (**13**)]. TLC plates were developed in BEA and (A) sprayed with vanillin sulphuric acid spray reagent and (B) sprayed with *C. neoformans*.

Fractions of **FH1** were loaded onto 10 cm × 20 cm TLC plates and developed in CHCl$_3$:Hex (1:1) as shown in Figure 4.2a. The developed fractions were sprayed with vanillin spray reagent and heated in an oven at 110°C until optimal color development. Fractions 65-80 (**C1**) were combined and yielded 0.029% (175 mg) of a pure white precipitate which was subjected to NMR spectroscopy and MS for analysis.
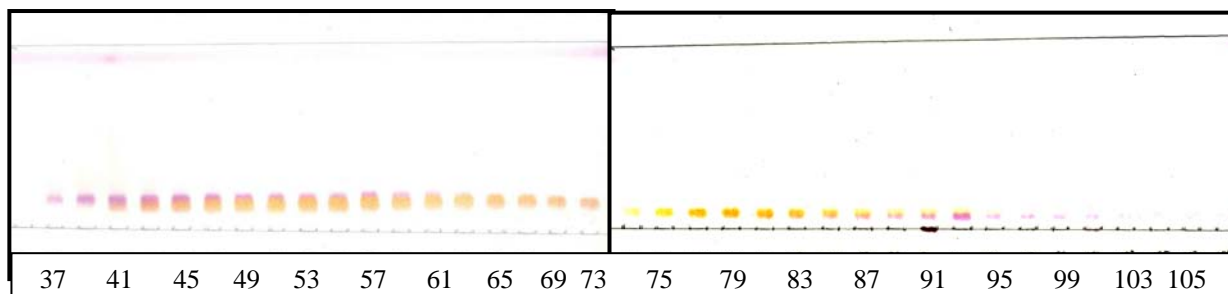
Fig 4.2a Fractions of **FH1** developed in CHCl$_3$:Hex (1:1), sprayed with vanillin spray reagent .

Fractions of **FH 2** loaded onto 10 cm × 20 cm TLC plate and developed in Hex: EtOAc (9:1), shown in Figure 4.2b. The developed fractions were sprayed with vanillin spray reagent and heated at 110°C in an oven until optimal color development. Fractions 37-40 (**C2**) and Fractions 55-61 (**C3**) were respectively combined and yielded 0.0025% (15 mg) and 0.026% (155 mg) respectively of pure white precipitates on which NMR spectroscopy and MS analysis was performed.

| 37 | 39 | 41 | 43 | 45 | 47 | 49 | 51 | 53 | 55 | 57 | 59 | 61 | 63 | 65 | 67 | 69 | 71 | 73 |
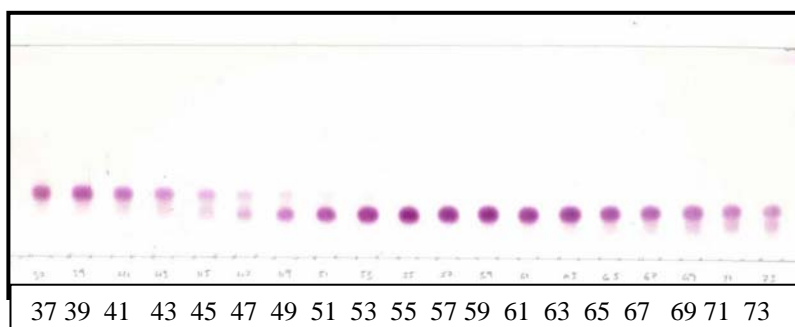
Fig 4.2b Fractions of **FH2** developed in Hex:EtOAc (9:1)

Fractions of **FHB** loaded onto 10 cm × 20 cm TLC plate and developed in CEF, depicted in **Figure** 4.2c. The developed fractions were sprayed with vanillin spray reagent and heated at 110°C in an oven until optimal color development. Fractions 8-12 (**FHBi**), 13-31 (**FHBii**), and 32-118 (**FHBiii**) were respectively combined and left under a stream of cold air to evaporate the solvent.
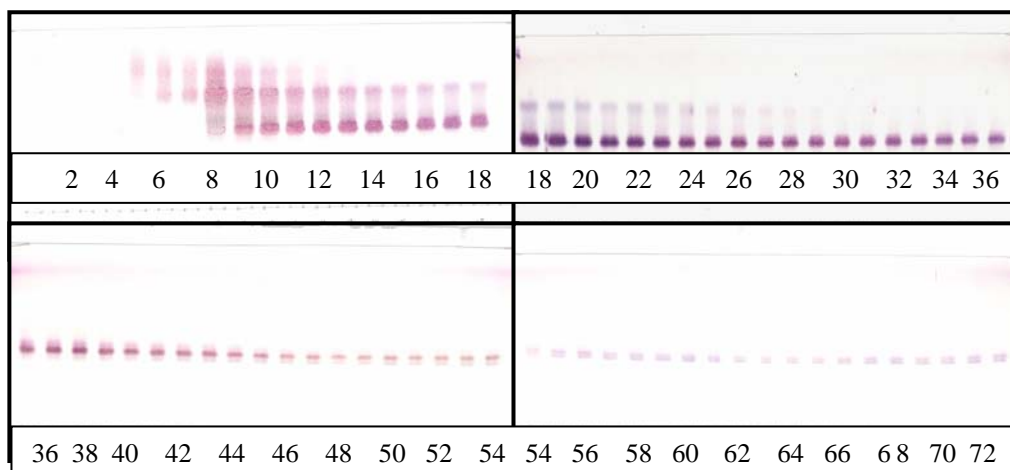
Fig 4.2c Fractions of **FHB** developed in CEF

Fractions of **FHBi** loaded onto 10 cm × 20 cm TLC plate and developed in $CHCl_3$:MeOH (9:0.8) as shown in Figure 4.2d. The developed fractions were sprayed with vanillin spray reagent and heated at 110°C in an oven until optimal color development. Fractions (1 ml) were collected and loaded onto TLC plates and developed in $CHCl_3$:MeOH (9:0.8). Fractions 11-23 (**FHBia**), 25-37 (**FHBib**) were combined.
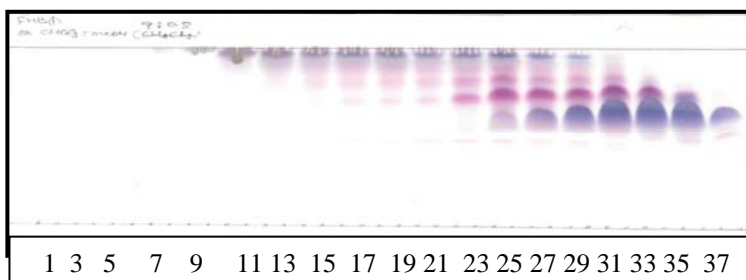
Fig 4.2d Fractions of **FHBi** developed in CHCl$_3$:MeOH (9:0.8)

Fractions of **FHBia** loaded onto 10 cm × 20 cm TLC plate and developed in DCM:EtOAc (4:1) as depicted in Figure 4.2e. The developed fraction were sprayed with vanillin spray reagent and heated at 110°C in an oven until optimal color development. Fractions (1 ml) were collected and aliquots loaded on TLC plates and developed in DCM: EtOAc (4:1). Fractions 65-83 (**FHBia5**) and fractions 84-105 (**FHBia6**) were combined. The streaking of some bands may be due to overloading the plate or more likely to ionization of compounds because ionization was not inhibited by this neutral pH solvent system.
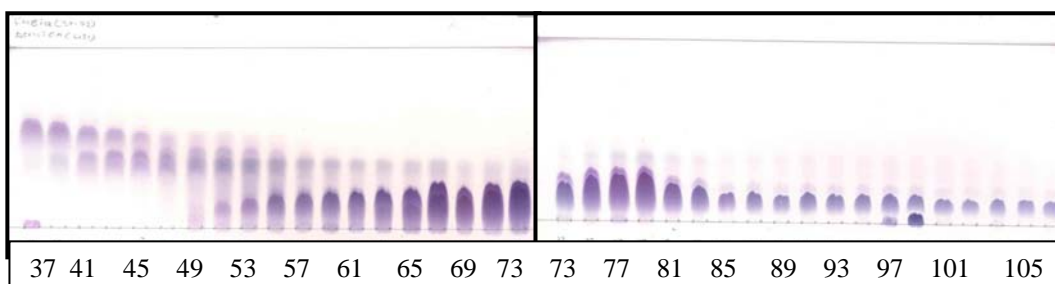
Fig 4.2e Fractions of **FHBia** developed in DCM:EtOAc (4:1)

Fractions of **FHBia5** loaded onto 10 cm × 20 cm TLC plate and developed in Hex: EtOAc (1:1) as shown in Figure 4.2f. Fractions (1 ml) were collected and developed on TLC plates in Hex: EtOAc (1:1) and sprayed with vanillin spray reagent and heated at 110°C in an oven until optimal color development. Fractions 31-73 (**C4**) were combined and yielded 0.0038% (23 mg) of a white powder which was subjected for NMR and MS analysis. Similar streaking as above was encountered here.
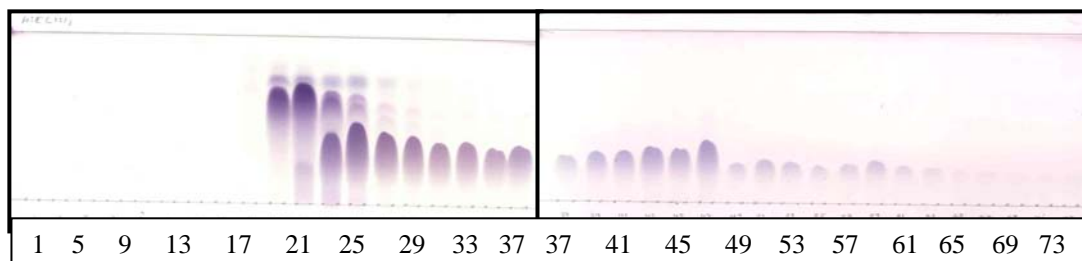
Fig 4.2f Fractions of **FHBia5** developed in Hex: EtOAc (1:1)

Fractions of **FHBiii** loaded onto 10 cm × 20 cm TLC plate and developed in 10% MeOH in CHCl$_3$ as depicted by Figure 4.2g. Collected fractions (10 ml) were loaded on TLC plates and developed in 10% MeOH in CHCl$_3$ solution, sprayed with vanillin spray reagent and heated in an oven at 110°C until optimal colour development. Fractions 33-36 (**FHBiiia**), 37&38 (**FHBiiib**) were combined.

Crude	30	32	34	36	38	40	42	44	48
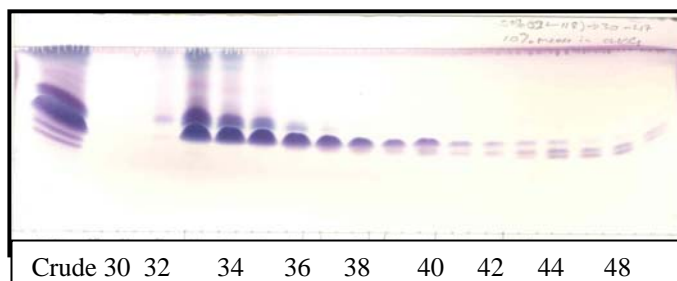
Fig 4.2g Fractions of **FHBiii** developed in 10% MeOH in CHCl$_3$

Fractions of **FHBiiia** loaded onto 10 cm × 20 cm TLC plate and developed in 5% MeOH in CHCl$_3$ as depicted by Figure 4.2h. Fractions (5 ml) were collected and loaded on 10 cm × 20 cm TLC plates and developed in 5% MeOH in CHCl$_3$ and sprayed with vanillin spray reagent. Fractions 15-24 (**FHBiiia1**), 25-72 (**FHBiiia2**) and 73-99 (**FHBiiia3**) were combined.

1	5	9	13	17	21	25	29	33	37	37	39	41	43	45	47	49	51	53	55
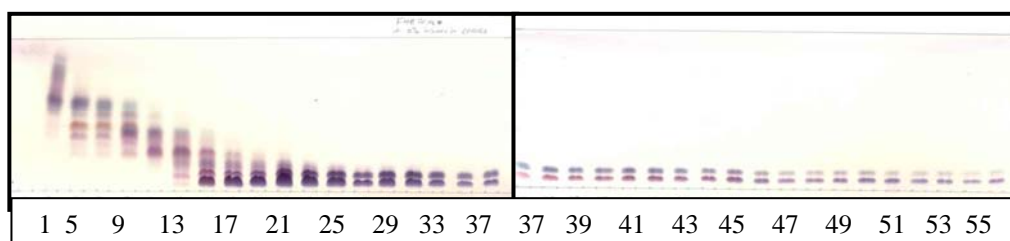
Fig 4.2h Fractions of **FHBiiia** developed in 5% MeOH in CHCl$_3$

Fractions of **FHBiiia1 and FHBiiia2** loaded onto 10 cm × 20 cm TLC plate and developed in CHCl$_3$:MeOH (90:5) as shown by Figure 4.2i and 1 ml fractions were collected. The collected fractions were then loaded on TLC plates and developed in CHCl$_3$:MeOH (90:5). Fractions 37-73 (**C5**) yielded 0.014% (82 mg) of pure white powder and which was subjected to NMR and MS analysis.
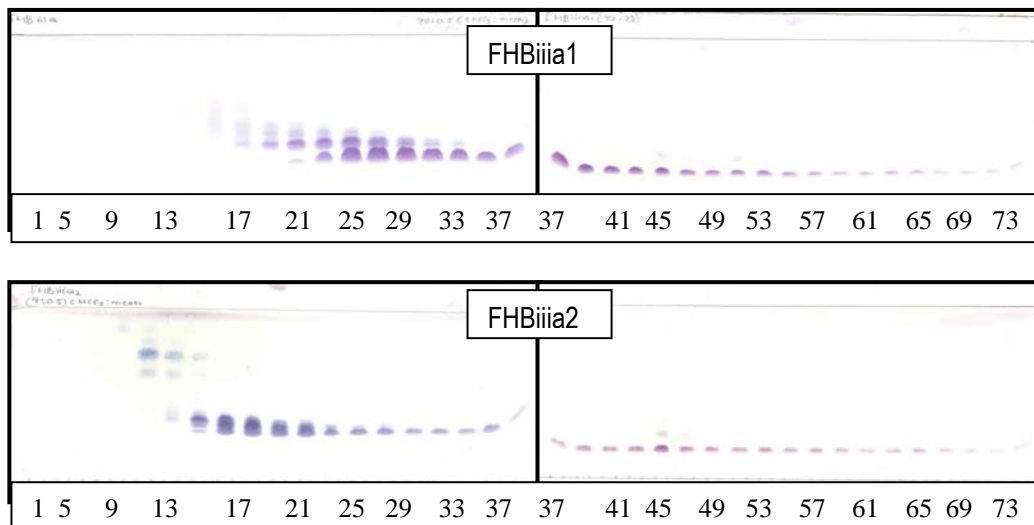
Fig 4.2i Fractions of **FHBiiia1** (**top**) and **FHBiiia2** (**below**) developed in CHCl$_3$:MeOH (90:5)

Fractions of **FH8** loaded onto 10 cm × 20 cm TLC plate and developed in 5% MeOH in CHCl$_3$ as shown by Figure 4.2j and Fractions (1 ml) volume were collected, loaded on TLC plates and developed in 5% MeOH in CHCl$_3$ before being sprayed with vanillin spray reagent. Fractions 113 to 156 (**FH8D**) were combined and used for further isolation.
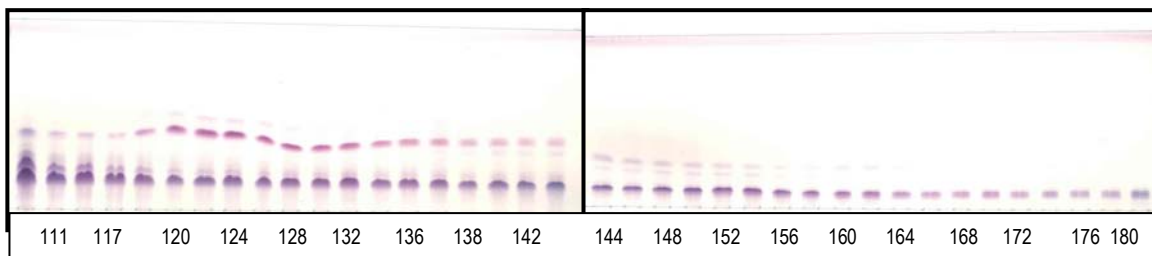
Fig 4.2j Fractions of **FH8** developed in 5% MeOH on CHCl$_3$

Fractions of **FH8D** loaded onto 10 cm × 20 cm TLC plate and developed in 10% MeOH in CHCl$_3$ as shown in Figure 4.2k and Fractions of 1 ml were collected, loaded on a TLC plate and developed in 10% MeOH in CHCl$_3$ before being sprayed with vanillin spray reagent. Fractions 27-37 (**FH8D3**) were combined and further isolated.
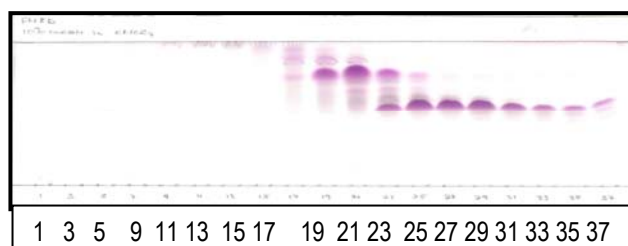
Fig 4.2k Fractions of **FH8D** developed in 10% MeOH in $CHCl_3$

Fractions of **FH8D3** loaded onto 10 cm × 20 cm TLC plate and developed in 5% MeOH in $CHCl_3$ as depicted in Figure 4.2l, Fractions (1 ml) were collected, loaded on TLC plates and sprayed with vanillin spray reagent. Fractions 39-41, 43-46 and 51-55 all together combined to make (**C6**) yielded 0.011% (68 mg) of pure white powder which was subjected to NMR and MS analysis.
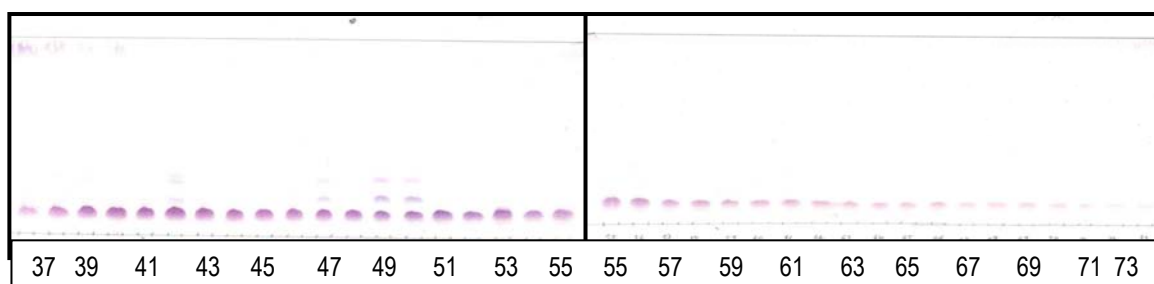
Fig 4.2l Fractions of **FH8D3** developed in 5% MeOH in $CHCl_3$

All the isolated compounds were white powders and were non detectable under UV light at 360 nm and 254 nm. Plant constituents are produced and stored in varying quantities in the plant, indicated by different quantities of active constituents isolated from the aerial parts of *M. undata*. Only 0.029% (**C1**), 0.0025% (**C2**), 0.026% (**C3**), 0.0038% (**C4**), 0.014% (**C5**) and 0.011% (**C6**) yields were isolated from *M. undata* aerial parts. **C1** was present in a relatively large quantity in the hexane extract, followed by **C3**, **C5**, **C6** and **C4**. The yield of **C2** was the lowest of all the compounds isolated from this extract. These values do not represent the exact quantity of a plant constituent present in the plant extract or even in the whole plant because a substantial percentage was lost during the isolation process. They approximate the relative concentration of these compounds in the plant extract; the higher the amount of the compound present in a plant or extract, the more likely that it will be isolated in a higher amount.

Conclusion

Six white powders were isolated from *M. undata* aerial parts. These compounds suspected to be triterpenes before further identification studies such as NMR analysis because they were white powdery substances that were not UV active and triterpenes have been found in this species. Further identification studies such as NMR analysis are necessary to confirm this supposition. During isolation of compounds from crude extracts, a large proportion of plant constituents are lost especially when using silica gel chromatography. The quantity of some plant constituents is higher than others hence they can be easily isolated. Plant constituents may be present in low quantities or could be difficult to isolate for various reasons, resulting in potentially valuable active compounds being lost during the isolation process. In the following chapter the structures of the isolated compounds are determined using NMR and MS spectroscopy techniques.

CHAPTER 5

STRUCTURAL ELUCUDATION AND CHARACTERIZATION OF ISOLATED COMPOUNDS

5.1 Introduction

The plant kingdom has an estimated 200,000 primary and secondary metabolites (Fiehn, 2002) with a diverse set of atomic arrangements. This property allows for wide variations in chemical properties such as molecular weight, polarity, solubility and physical properties like volatility (Dunn and Ellis, 2005). The analysis of drug metabolites and natural products requires the utilization of rapid assays and techniques to screen a maximum number of active components in small quantities of biological mixtures within the shortest possible time (Yang, 2006). Lack of rapid assays or techniques and inefficient methods of identification of unknown components results in valuable information on potential new drug candidates being neglected or inaccurately interpreted (Yang, 2006). The identification of compounds involves a diverse range of analytical techniques and methods such as nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared (IR) spectroscopy, and mass spectroscopy (MS). In this study NMR and MS techniques were used as tools for the analysis and identification of the compounds isolated from *M. undata*.

5.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is the most powerful spectroscopic tool for obtaining structural details of complex organic compounds. The structural assignment of all isomers is possible by considering chemical shifts, coupling constants, and integration ratios of the NMR data (Yang, 2006). This technique is extensively used in biomedical, pharmaceutical, environmental, food and natural products analysis, as well as for the identification of drug metabolites (Albert, 1999; Albert, 2004; Jaroszewski, 2005). It is also needed for unambiguous structure determination, especially for the stereo-specific identification of unknown active compounds that may be of interest for the development of pharmaceuticals and functional foods. All compounds having NMR-measurable nuclei can be detected including ^{1}H and ^{13}C, which are major structural elements of organic compounds (Yang, 2006). Yang (2006) indicated that NMR spectroscopic analysis requires time-consuming isolation and purification steps in order to acquire NMR spectra on individual components.

One of the setbacks of NMR technique is its low sensitivity and its inability to detect some NMR "silent" functional groups having poor or non-existent magnetic properties, such as SO_4 and NO_2 (Giinther, 1995), and hence it is not adequate for structure determination of such metabolites (Yang, 2006). Relatively high concentrations of analytes are required to achieve sufficient detection sensitivity (Yang, 2006).

5.1.2 Mass Spectroscopy (MS)

Mass spectroscopy (MS) is the most widely applied technology in metabolomics, as it provides a blend of rapid, sensitive, and selective qualitative and quantitative analyses with the ability to identify metabolites. Mass spectrometers operate by ion formation, separation of ions according to their mass-to-charge (m/z) ratio and detection of separated ions (Dunn and Ellis, 2005). A complete characterization of the chemical composition of complex natural products is impossible to obtain with MS alone due to the occurrence of complex mixtures of conjugates (glycosides) and aglycones present in natural extracts (Yang, 2006). This technique is useful in the determination of the molecular weight of natural products in the development of new pharmaceuticals.

5.2 Materials and methods

5.2.1 Sample preparation for NMR analysis

The Varian Unit Innova 300 MHz NMR system (Oxford instruments) and Brüker DRX – 400 instrument were used for ^{13}C and ^{1}H NMR. The compounds isolated from *Maytenus undata* (**Chapter 4**) were dried, weighed (10-20 mg) and dissolved in a maximum of 2 ml of deuterated chloroform (Merck) because the isolated compounds were soluble in $CHCl_3$. The solutions were then pipetted into NMR tubes (commonly of 5 mm diameter to a depth of 2-3 cm) using clean Pasteur pipettes and taken to the Department of Chemistry and Biochemistry, University of Limpopo (Medunsa Campus), and subsequently to the Department of Chemistry, University of Pretoria and the University of Botswana for proton (^{1}H), carbon 13 (^{13}C) and distortionless enhancement through polarization transfer (DEPT).

5.2.2 Sample preparation for MS analysis

The analytical VG 7070E mass spectrometer (Quattro (VG Biotech, England) using electron impact at 70 eV as ionization technique was used for mass spectroscopy. Approximately 2 mg of each isolated compound was dried, placed into a 2 ml glass vial and sent to the University of Pretoria and the University of Botswana for MS analysis.

5.3. Results and discussion

5.3.1 Identification of compounds

5.3.1.1 Compound 1

Compound **1** was isolated as a white precipitate, indicating a triterpene-like compound. The ^{13}C NMR spectrum (Figure 5.1) showed the presence of 30 carbons including seven quaternary carbons, four CH carbons, eleven CH_2 carbons and eight CH_3 carbons. The presence of a keto group was evident in the ^{13}C NMR spectrum from the appearance of a keto carbon signal at δ 213.5 (Figure 5.2 and Table 5.1). The ^1H and ^{13}C NMR spectral data for this compound indicates that it belongs to the friedelane group and was identified as friedelan-3-one (friedelin) (Figure 5.9) based on its identical ^1H and ^{13}C NMR data and physical constants reported in the literature (Hisham *et al.*, 1995). The molecular formula of this compound was identified as $C_{30}H_{50}O$ with a molecular weight of 426.72.

This compound has been widely isolated from hexane extracts of Celastraceae plant species, such as *Salacia beddomei* stem bark (Hisham *et al.*, 1995) and *M. aquifolium* leaves (Nossack *et al.*, 1998). Also compound **1** has been isolated from the hexane extract of *Garcinia smeathmannii* Oliver (Clusiaceae) stem bark (Kuete *et al.*, 2007) and the hexane extract of *Terminalia glabrescens* (Combretaceae) trunk bark (Garcez *et al.*, 2003) and also the hexane extract of *Heliotropium ellipticum* (Boraginaceaae) whole plant (Jain *et al.*, 2001).

5.3.1.2 Compound 2

Compound **2** was isolated as a white precipitate indicating a triterpene-like compound. The ^{13}C NMR spectrum (Figure 5.3) showed the presence of 30 carbons including six quaternary carbons, five CH carbons, eleven CH_2 carbons and eight CH_3 carbons (Table 5.2). The presence of a hydroxyl group was evident in the ^{13}C NMR spectrum from the appearance of a hydroxyl carbon signal at δ 72.8 (Table 5.2). The ^1H and ^{13}C NMR spectral data for this compound indicates that it belongs to the friedelane group and was identified as friedelan-3β-ol (epifriedelanol) (Figure 5.9) based on its identical ^1H and ^{13}C NMR data and physical constants reported in the literature (Kundu *et al.*, 2000). The mass spectrum showed a [M]+ ion peak at m/z 429 which was consistent with a molecular formula $C_{30}H_{53}O$.

This compound is widespread in plants and has been isolated from the hexane extract of *Maytenus truncata* Reiss leaves (Celastraceae) (Salazar *et al.*, 2000) and *Vitis trifolia* L. (Vitaceae) (Kundu *et al.*, 2000); the hexane extract of *M. aquifolium* leaves (Nossack et al., 1998) and also the hexane extract of *Heliotropium ellipticum* (Boraginaceaae) whole plant (Jain *et al.*, 2001).

5.3.1.3 Compound 3

Compound **3** was isolated as a white precipitate indicating a triterpene-like compound. The NMR spectrum showed the presence of 30 carbons. The ^{13}C NMR spectrum (Figure 5.4) showed the presence of a double bond (δ 158.1, 116.9), a hydroxyl group (δ 79.1), seven quaternary carbons, five CH carbons, ten CH_2

carbons and eight CH_3 carbons (Table 5.3). The ^{1}H and ^{13}C spectral data for this compound indicates that it belongs to the taraxerane group and was identified as taraxerol (Figure 5.9) based on its identical ^{1}H and ^{13}C NMR data and physical constants reported in the literature (Sakurai, 1987). The mass spectrum showed [M][+] ion peaks at m/z 302 and 204 which was consistent with the molecular formula $C_{30}H_{50}O$ and its molecular weight was identified as 426. This compound was previously isolated from the benzene extract of the stem bark of *Myrica rubra* (Sakurai *et al.*, 1987) and the hexane extract of *Terminalia glabrescens* (Combretaceae) trunk bark (Garcez *et al.*, 2003).

5.3.1.4 Compound 4

Compound **4** was isolated as a colourless powder, indicating a triterpene-like compound. The ^{13}C NMR spectrum (Figure 5.6) showed the presence of a double bond (δ 122.5, δ 148.5), a ketone group (δ 218.3), a carboxylic acid group (δ 182.7), a methoxy group (δ 53.8), nine quaternary carbons, five CH carbons, nine CH_2 carbons and seven methyl groups (CH_3) (Table 5.4). The ^{1}H and ^{13}C spectral data for this compound indicates that it belongs to the 12 oleanene group and was identified as 3-oxo-11α-methoxyolean-12-ene-30-oic acid (Figure 5.9) based on its identical ^{1}H and ^{13}C NMR data and physical constants reported in the literature (Muhammad *et al.*, 2000). The mass spectrum showed a [M][+] ion peak at m/z 485.3639 which was consistent with the molecular formula $C_{31}H_{49}O_4$. This compound was first isolated from the ethanol (95%) extract of *Maytenus undata* (Celastraceae) aerial parts (Muhammad *et al.*, 2000).

5.3.1.5 Compound 5

Compound **5** was isolated as a colourless powder indicating a triterpene-like compound and exhibited ^{1}H and ^{13}C NMR data that were closely related to compound 4, but it lacked the methoxy group at C- 11 which was hydroxylated instead. The ^{13}C NMR spectrum (Figure 5.7) showed the presence of a double bond (δ 126.2, δ 148.1), a ketone group (δ 218.3), a carboxylic acid group (δ 182.2), a hydroxyl group (δ 67.9), nine quaternary carbons, five CH carbons, nine CH_2 carbons and seven methyl groups (CH_3) (Table 5.5). The ^{1}H and ^{13}C spectral data for this compound indicates that it belongs to the12 oleanene group and was identified as 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (Figure 5.9) based on its identical ^{1}H and ^{13}C NMR data and physical constants reported in the literature (Muhammad *et al.*, 2000). The mass spectrum showed a [M][+] ion peak at m/z 471.34 which was consistent with a molecular formula $C_{30}H_{47}O_4$.

Compound **5** was dissolved in acetone for NMR analysis in the literature (Muhammad *et al.*, 2000) and it was dissolved in chloroform ($CHCl_3$) for NMR analysis during this project, hence there was a slight difference in chemical shifts than what is observed in the literature. This compound was first isolated from the ethanol (95%) extract of *Maytenus undata* (Celastraceae) aerial parts (Muhammad *et al.*, 2000).

5.3.1.6 Compound 6

Compound **6** was isolated as a colourless powder, indicating a triterpene-like compound. The ^{13}C NMR spectrum (Figure 5.8) showed the presence of a double bond (δ 126.1, δ 147.7), two hydroxyl groups (δ 76.6, δ 67.1), a carboxylic acid group (δ 179.2), eight quaternary carbons, seven methyl groups (CH_3), nine CH_2 carbons and six CH carbons (Table 5.6). The ^{1}H and ^{13}C spectral data of this compound indicates that it belongs to the 12 oleanene group and was identified as 3,11-dihydroxyolean-12-ene-30-oic acid (Figure 5.9). The chemical formula for this compound was identified as $C_{30}H_{48}O_4$ with a molecular weight of 472. This is the first report of this compound isolated from any plant species according to the available literature.

This compound is closely related to glycyrrhetinic acid ($C_{30}H_{43}O_4$, MW = 470.67) which is produced by the hydrolysis of glycyrrhizinic acid ($C_{42}H_{62}H_{16}$, MW = 822.92), a major component of *Glycyrrhiza glabra* L. (licorice) (Salari and Kadkhoda, 2003). The difference between glycyrrhetinic acid and 3,11-dihydroxyolean-12-ene-30-oic acid is at C11 where glycyrrhetinic acid contains a keto group and 3,11-dihydroxyolean-12-ene-30-oic acid contains a hydroxyl group. It is unlikely that this is an artifact of the isolation procedure as articats as readily formed by oxidation not by reduction under the conditions used.
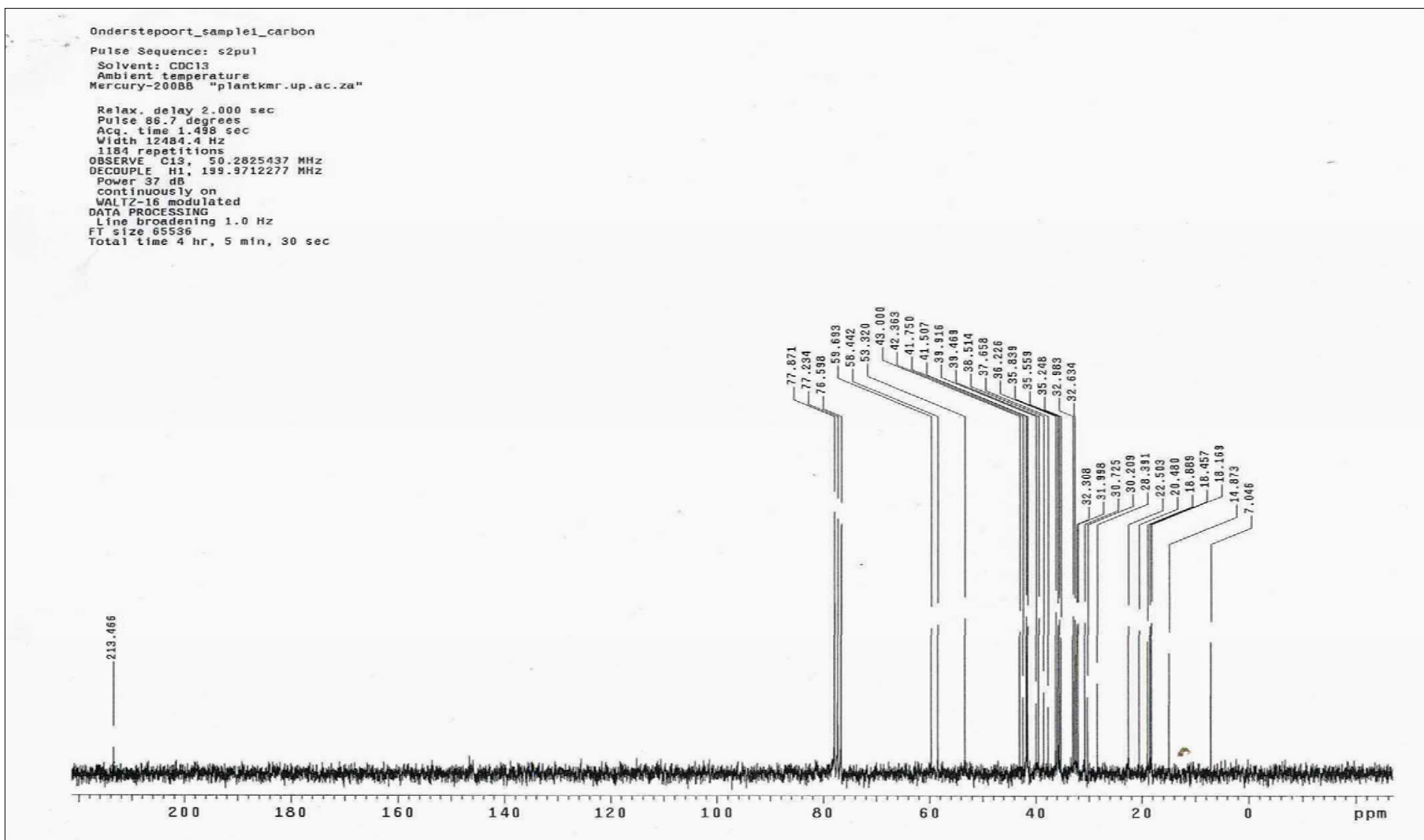
Fig 5.1 ^{13}C NMR spectrum of **Compound 1**
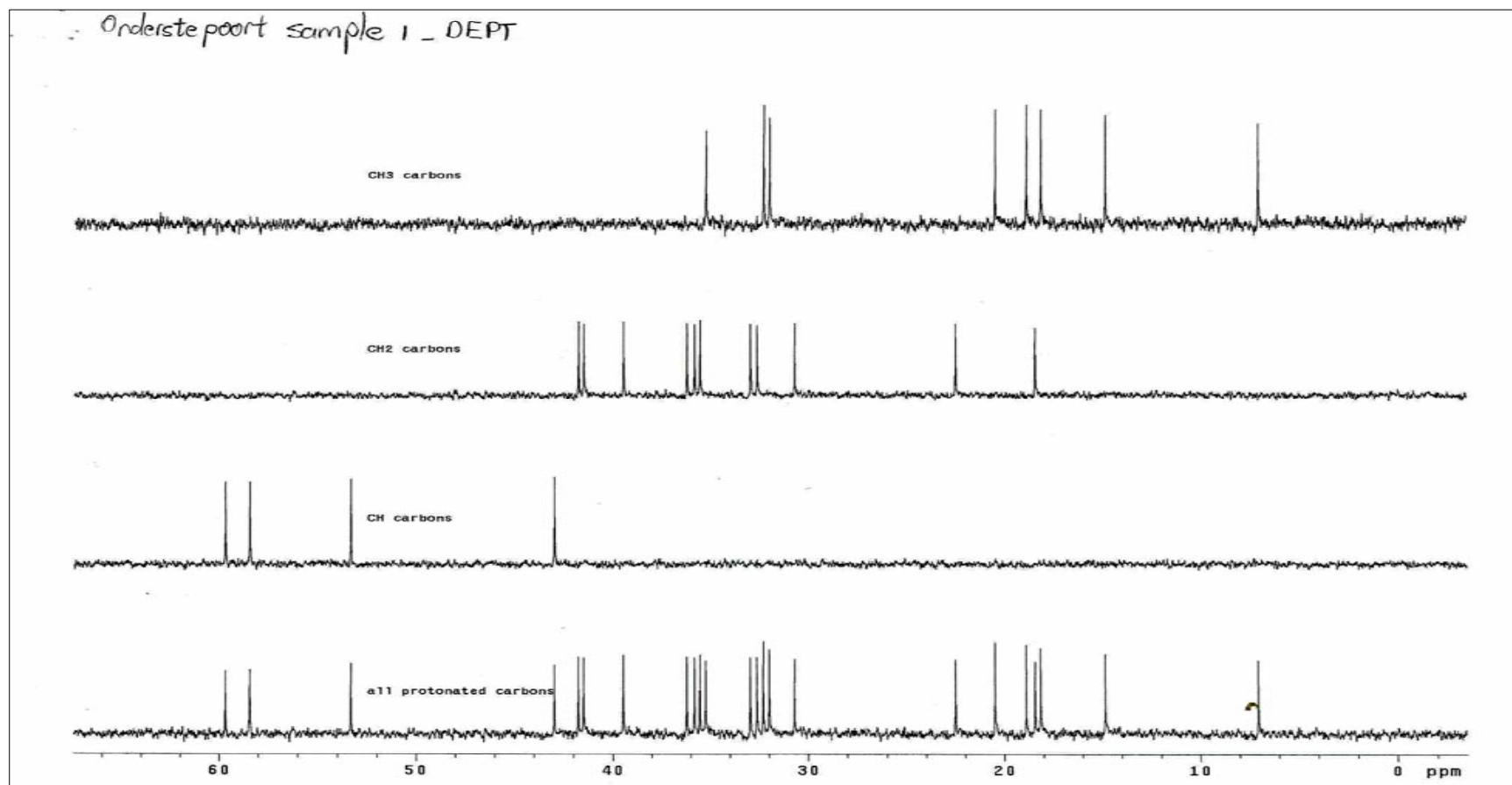
Fig 5.2 DEPT NMR for **Compound 1** differentiating CH$_3$-, CH$_2$- and CH- carbons

Table 5.1 ^{13}C NMR data for **compound 1 (TS1)** indicating chemical shifts, and comparison with chemical shifts published by *Hisham *et al.* (1995) for friedelin

Atom	δ_c (TS1)	δ_c (Friedelin)*
C		
3	213.5	213.2
5	42.4	42.1
13	40.0	39.7
14	38.5	38.3
9	37.6	37.4
17	30.2	30.0
20	28.4	28.2
CH		
10	59.7	59.5
4	58.4	58.2
8	53.3	53.1
18	43.0	42.8
CH$_2$		
2	41.7	41.5
6	41.5	14.3
22	39.5	39.2
16	36.2	36.0
11	35.8	35.6
19	35.6	35.4
15	33.0	32.8
21	32.6	32.4
12	30.7	30.5
1	22.5	22.3
7	18.5	18.2
CH$_3$		
30	35.2	35.0
28	32.3	32.1
29	32.0	31.8
26	20.5	20.3
27	18.9	18.7
25	18.2	17.9
24	14.9	14.6
23	7.0	6.8

ts2 in CDCl3 (Mdee, Maytenus undata)
C-13
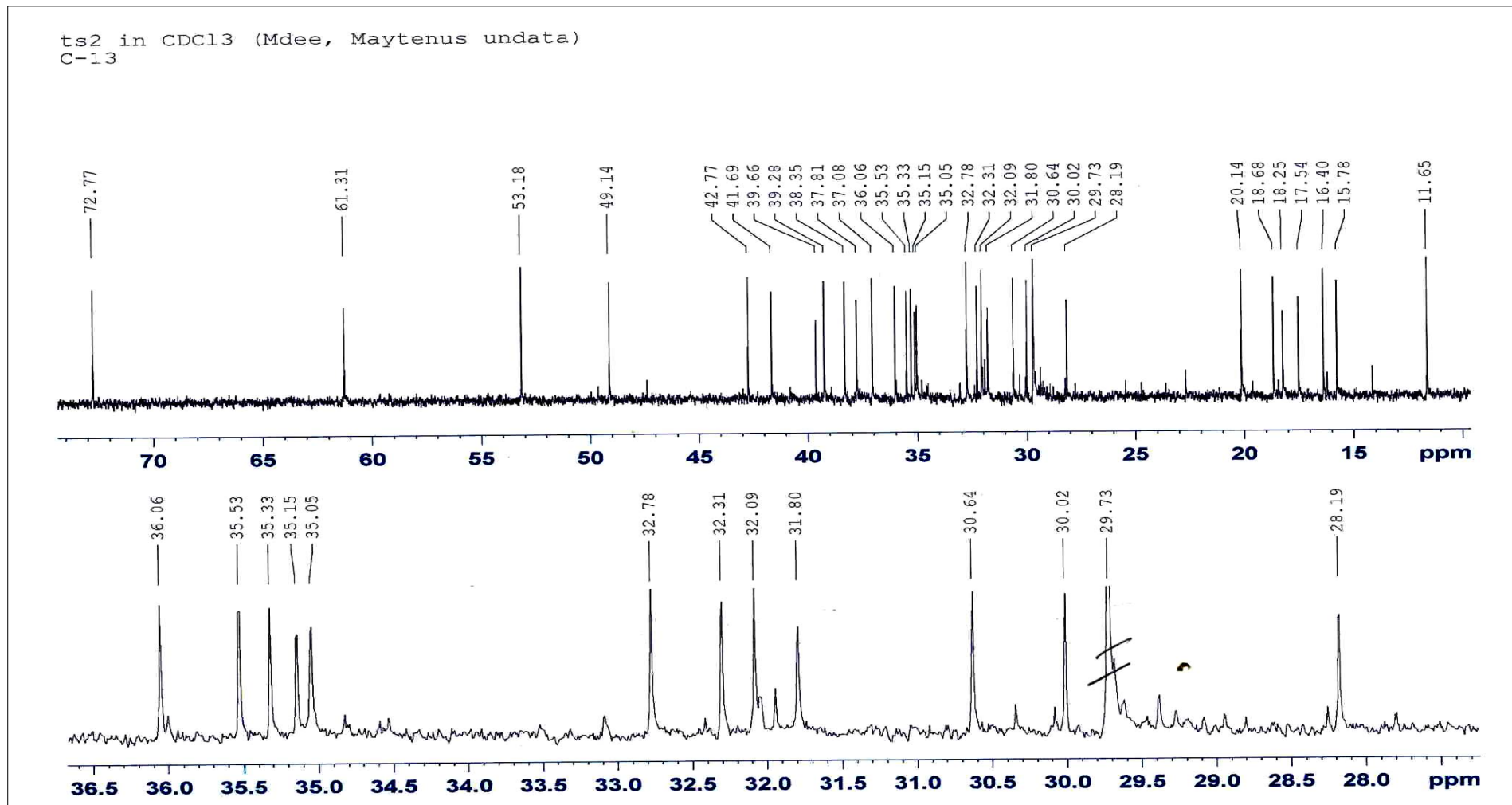
Fig 5.3 ^{13}C NMR spectra for **Compound 2**

Table 5.2 ^{13}C NMR data for **compound 2 (TS2)** indicating chemical shifts and comparison with chemical shifts published by *Kundu et al.* (2000) for epifriedelanol

Atom	δ$_c$ (TS2)	δ$_c$ (Epifriedelanol)*
C		
14	39.7	39.7
9	38.4	38.4
13	37.8	37.8
5	37.1	37.1
17	30.0	30.0
20	28.2	28.2
CH		
3	72.8	72.8
10	61.3	61.4
8	53.2	53.2
4	49.1	49.2
18	42.8	42.8
CH$_2$		
6	41.7	41.7
22	39.3	39.3
16	36.1	36.1
19	35.5	35.6
11	35.3	35.3
2	35.2	35.2
21	32.8	32.8
15	32.3	32.3
12	30.6	30.6
7	17.5	17.6
1	15.8	15.8
CH$_3$		
29	35.0	35.0
30	32.1	32.1
28	31.8	31.8
27	20.1	20.1
26	18.7	18.6
25	18.2	18.2
24	16.4	16.4
23	11.6	11.6
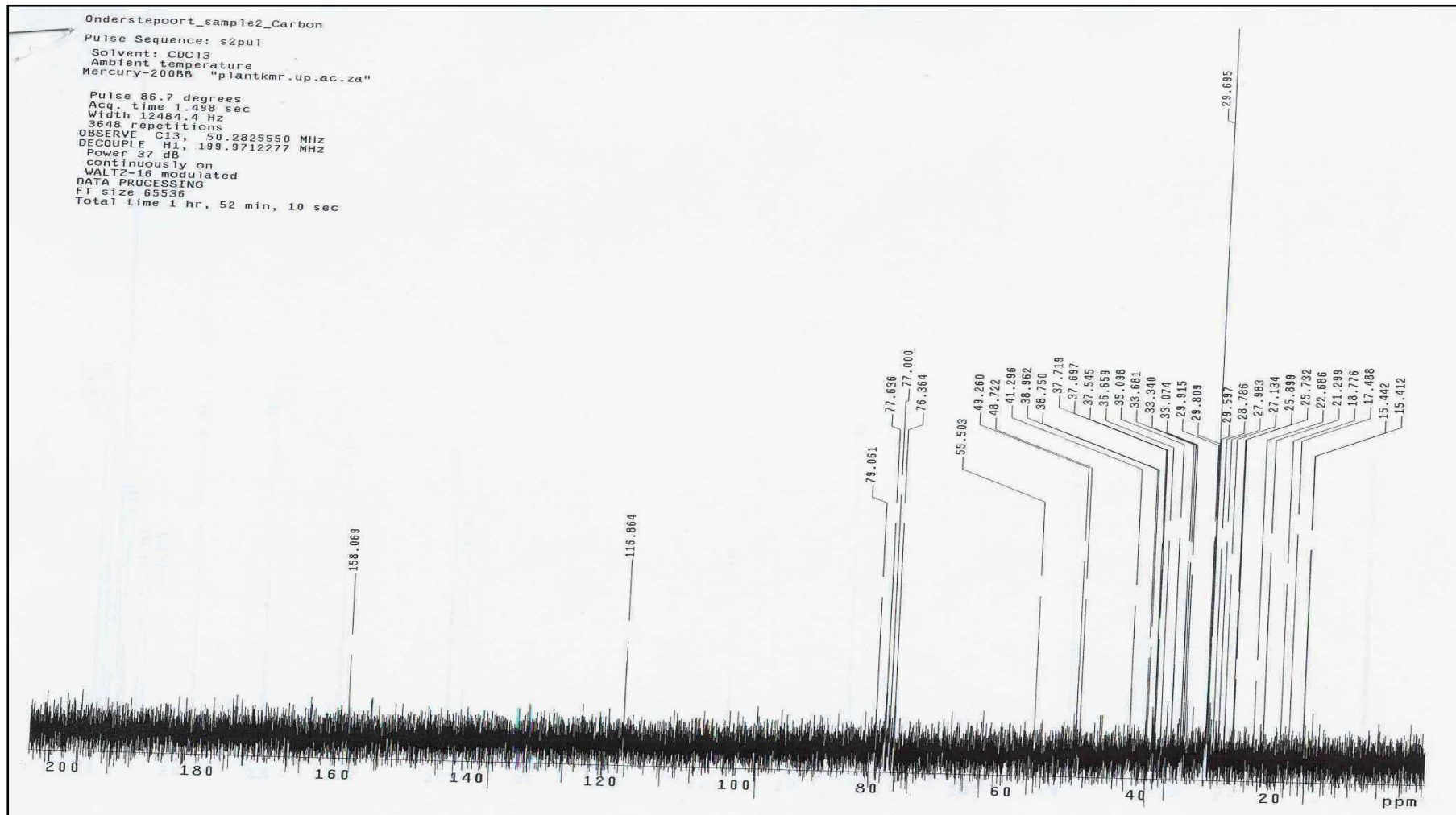
Fig 5.4 ^{13}C NMR spectrum for **Compound 3**
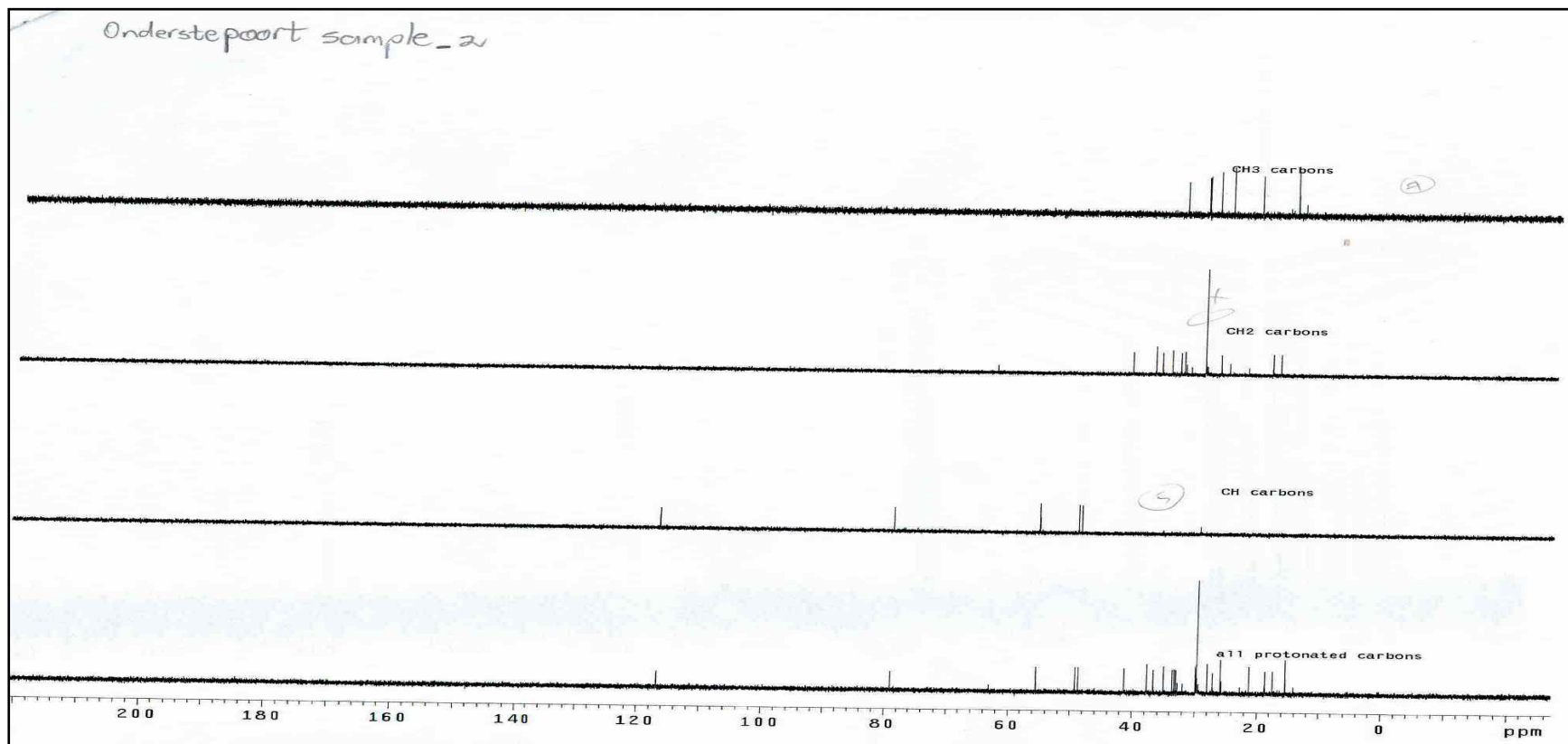
Fig 5.5 DEPT for **Compound 3** differentiating CH₃-, CH₂- and CH- carbons

Table 5.3 ^{13}C NMR data for **compound 3 (TS3)** indicating chemical shifts and comparison with chemical shifts published by *Sakurai *et al.* (1987) for taraxerol

Atom	δ$_c$ (TS3)	δ$_c$ (Taraxerol)*
C		
14	158.1	158.1
4	38.9	39.1
8	38.7	38.9
17	38.7	38.1
10	37.7	37.9
13	37.7	37.9
20	29.7	29.0
CH		
15	116.9	117.0
3	79.1	79.2
5	55.5	55.7
18	49.3	49.4
9	48.7	48.9
CH$_2$		
19	41.3	41.4
1	37.6	38.1
16	37.5	36.9
12	36.7	35.9
7	35.1	35.3
21	33.7	33.9
22	33.1	33.2
2	27.1	27.3
6	18.8	19.0
11	17.5	17.7
CH$_3$		
29	33.3	33.5
28	29.9	30.1
26	29.9	30.1
23	27.9	28.1
27	25.9	26.0
30	21.3	21.5
24	15.4	15.6
25	15.4	15.6
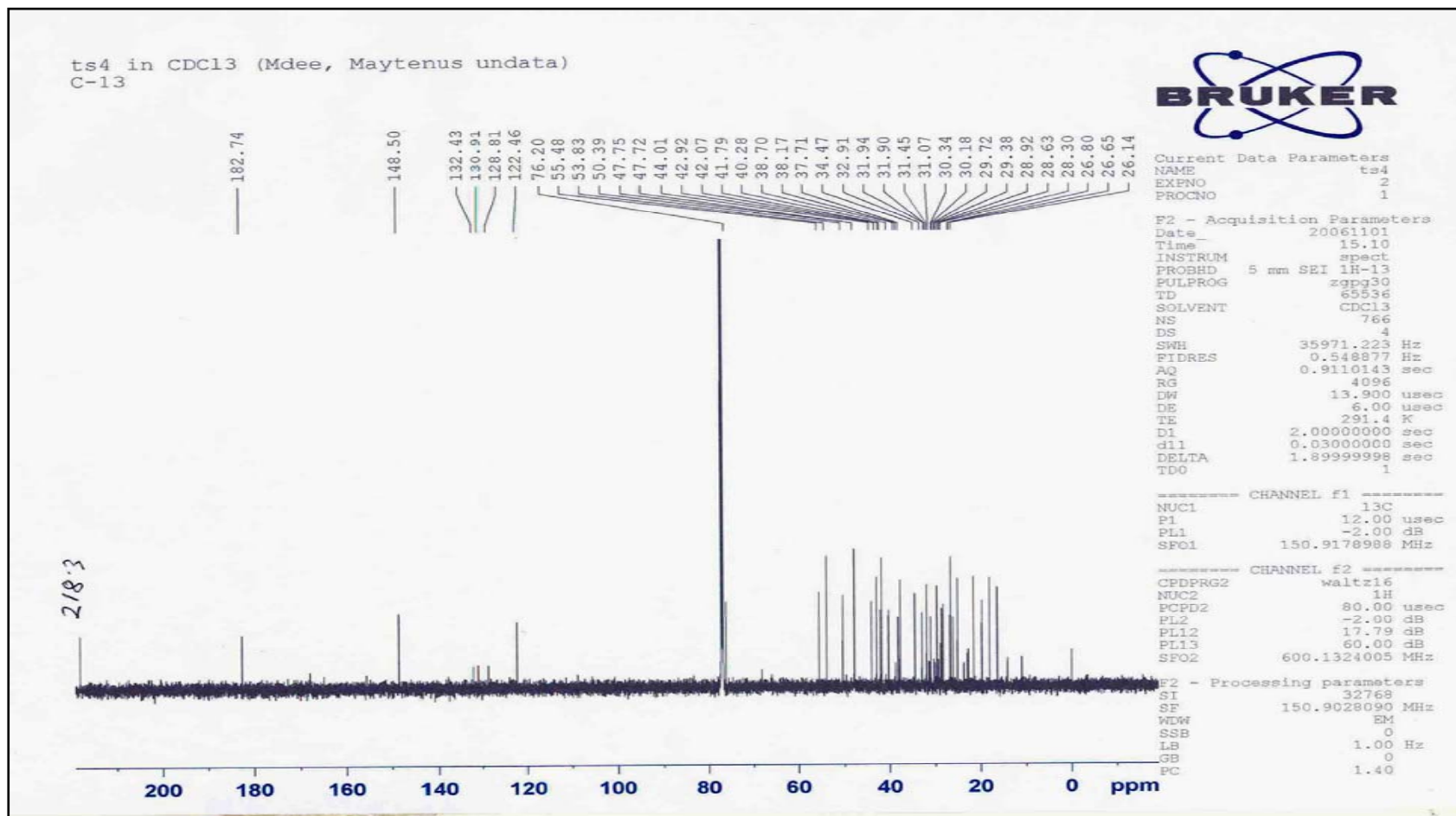
Fig 5.6 ^{13}C NMR spectra for **Compound 4**

Table 5.4 ^{13}C NMR data for **compound 4 (TS4)** indicating chemical shifts, compared with chemical shifts published by *Muhammad *et al.* (2000) for 3-oxo-11α-methoxyolean-12-ene-30-oic acid

Atom	δ$_c$ (TS4)	δ$_c$ (3-oxo-11α-methoxyolean-12-ene-30-oic acid)*
C		
3	218.3	218.6
30	182.7	183.3
13	148.5	148.9
4	47.8	48.4
8	44.0	44.4
20	42.9	43.3
14	41.8	42.2
10	37.7	38.1
17	31.9	32.3
CH		
12	122.5	122.8
11	76.2	76.6
5	55.5	55.9
9	50.4	50.9
18	47.7	48.1
CH$_2$		
19	42.1	42.6
1	40.3	40.6
22	38.2	38.6
2	34.5	34.8
21	32.9	33.3
7	31.1	31.5
16	26.8	27.2
15	26.1	26.5
6	19.7	20.1
CH$_3$		
29	28.6	29.3
28	28.3	29.0
23	26.7	27.0
27	25.2	25.5
24	21.5	21.9
26	18.1	18.6
25	16.4	16.7
11-OMe	53.8	54.1
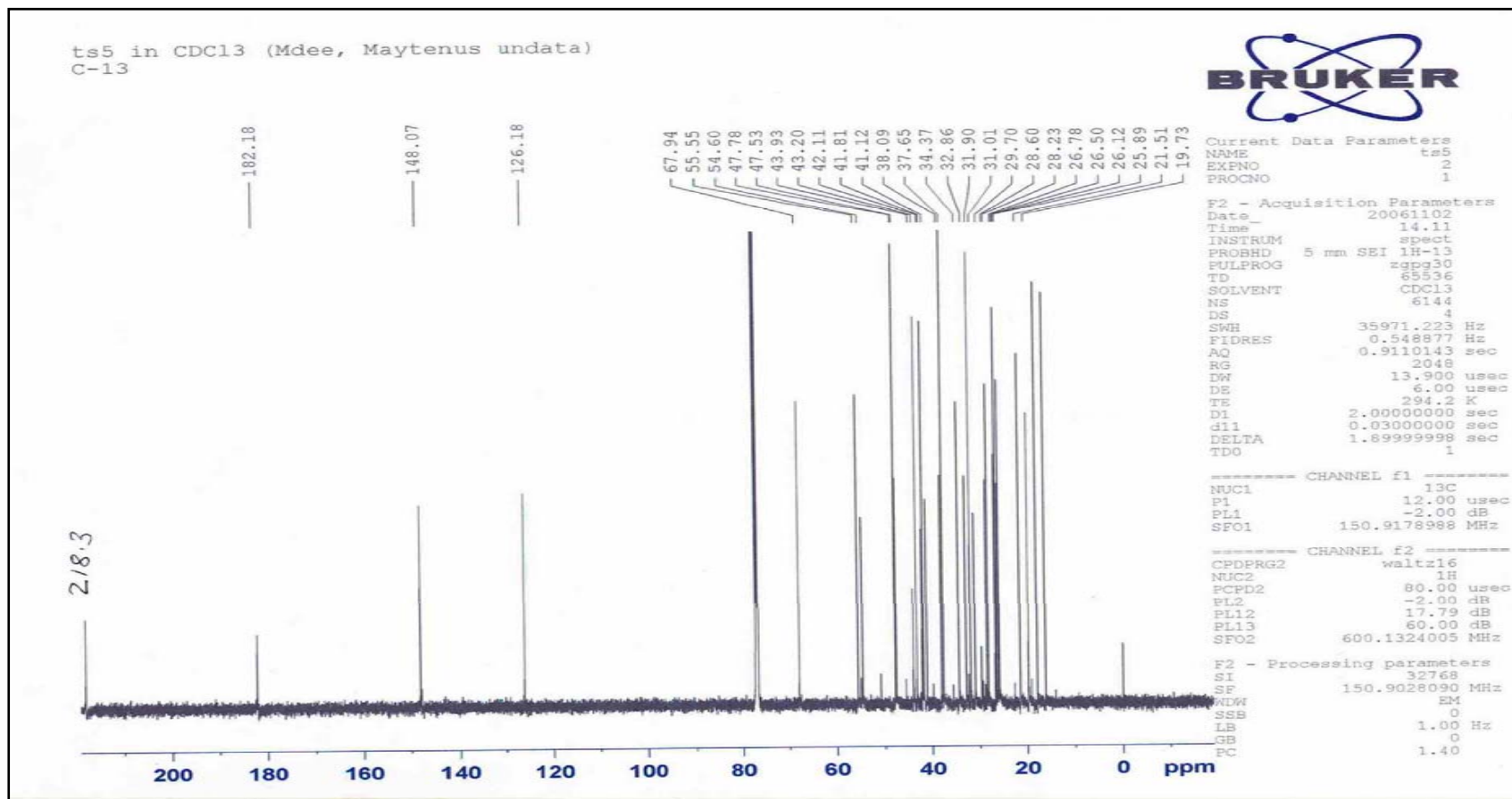
Fig 5.7 ^{13}C NMR spectrum of **Compound 5**

Table 5.5 ^{13}C NMR data for **compound 5 (TS5)** indicating chemical shifts, compared with chemical shifts published by *Muhammad *et al.* (2000) for 3-oxo-11α-hydroxyolean-12-ene-30-oic acid

Atom	δ_c (TS5)	δ_c (3-oxo-11α-hydroxyolean-12-ene-30-oic acid)*
C		
3	218.3	217.0
30	182.2	178.9
13	148.1	147.2
4	47.8	48.6
20	43.9	44.8
8	43.2	44.3
14	41.8	43.0
10	37.7	38.9
17	31.9	33.1
CH		
12	126.2	128.7
11	67.9	68.3
5	55.6	56.8
9	54.6	55.4
18	47.5	49.0
CH$_2$		
19	42.1	43.3
1	41.1	42.5
22	38.1	39.5
2	34.4	35.3
21	32.9	34.2
7	31.0	32.2
16	26.8	28.0
15	26.1	27.3
6	19.7	20.9
CH$_3$		
29	28.6	29.3
28	28.2	29.3
23	26.5	27.4
27	25.9	26.3
24	21.5	22.2
26	17.9	18.9
25	16.2	17.0

TS6

Pulse Sequence: s2pul

Solvent: CDC13
Ambient temperature
Mercury-300BB "medunsa300"

Pulse 82.1 degrees
Acq. time 1.815 sec
Width 20000.0 Hz
7616 repetitions
OBSERVE C13, 75.4520130 MHz
DECOUPLE H1, 300.0688577 MHz
Power 41 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 6 hr, 41 min, 23 sec
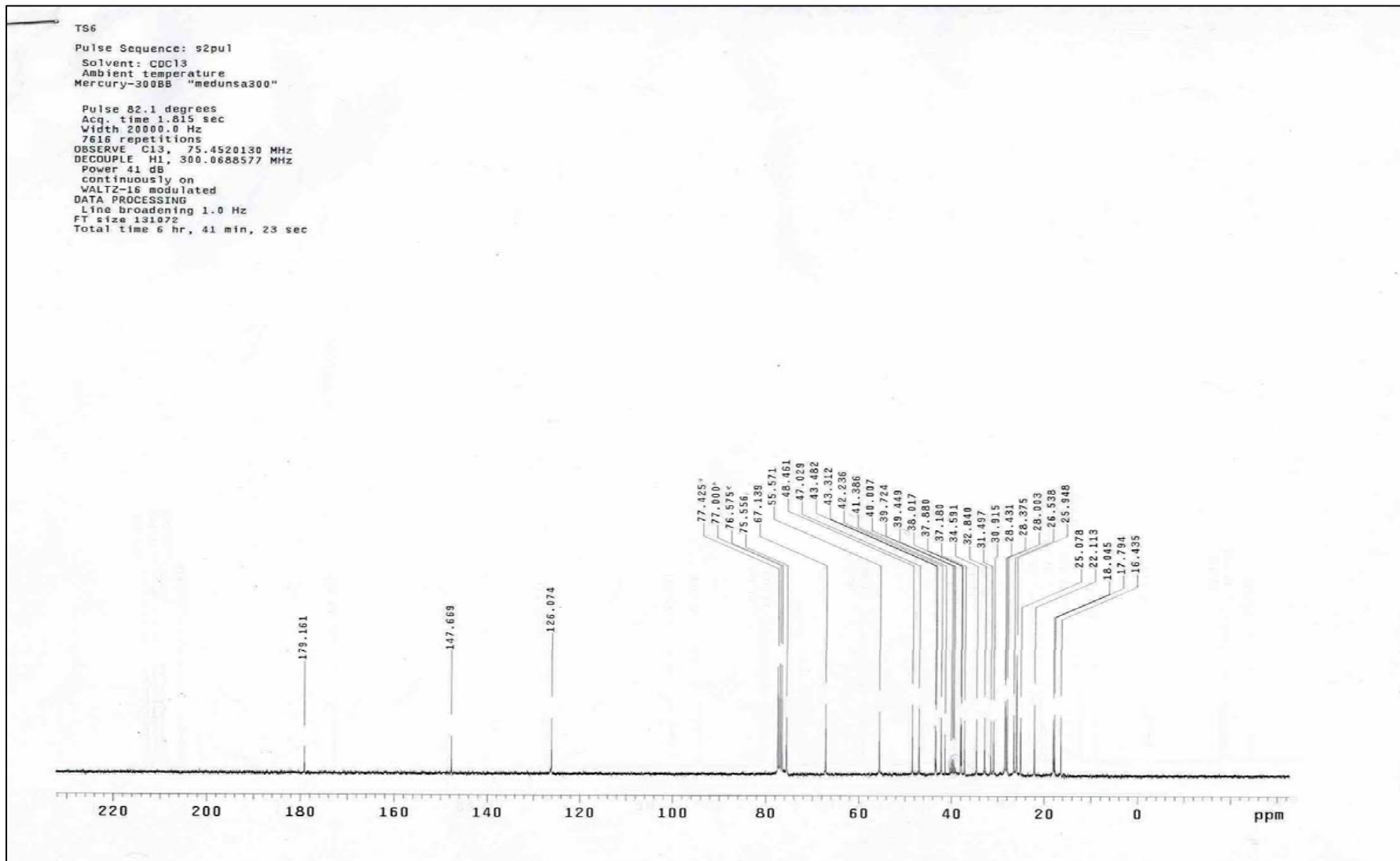
Fig 5.8 ¹³C NMR spectra for **Compound 6**

Table 5.6 ¹³C NMR data for **compound 6 (TS6)**

Atom	δc (TS6)
C	
30	179.2
13	147.7
20	43.5
9	43.2
14	41.4
6	37.9
4	37.2
17	31.5
CH₃	
28	28.4
23	28.4
29	28.0
27	26.5
24	22.1
26	17.8
25	16.4
CH₂	
2	42.2
22	38.0
1	34.6
8	32.8
21	30.9
19	30.9
16	26.5
15	25.1
7	18.1
CH	
12	126.1
3	76.6
11	67.1
10	55.6
5	48.5
18	47.0

Friedelin (1)

Epifriedelanol (2)

Taraxerol (3)

3-oxo-11α-methoxyolean-12-ene-30-oic acid (4)

3-oxo-11α-hydroxyolean-12-ene-30-oic acid (5)   **3,11-dihydroxyolean-12-ene-30-oic acid (6)**
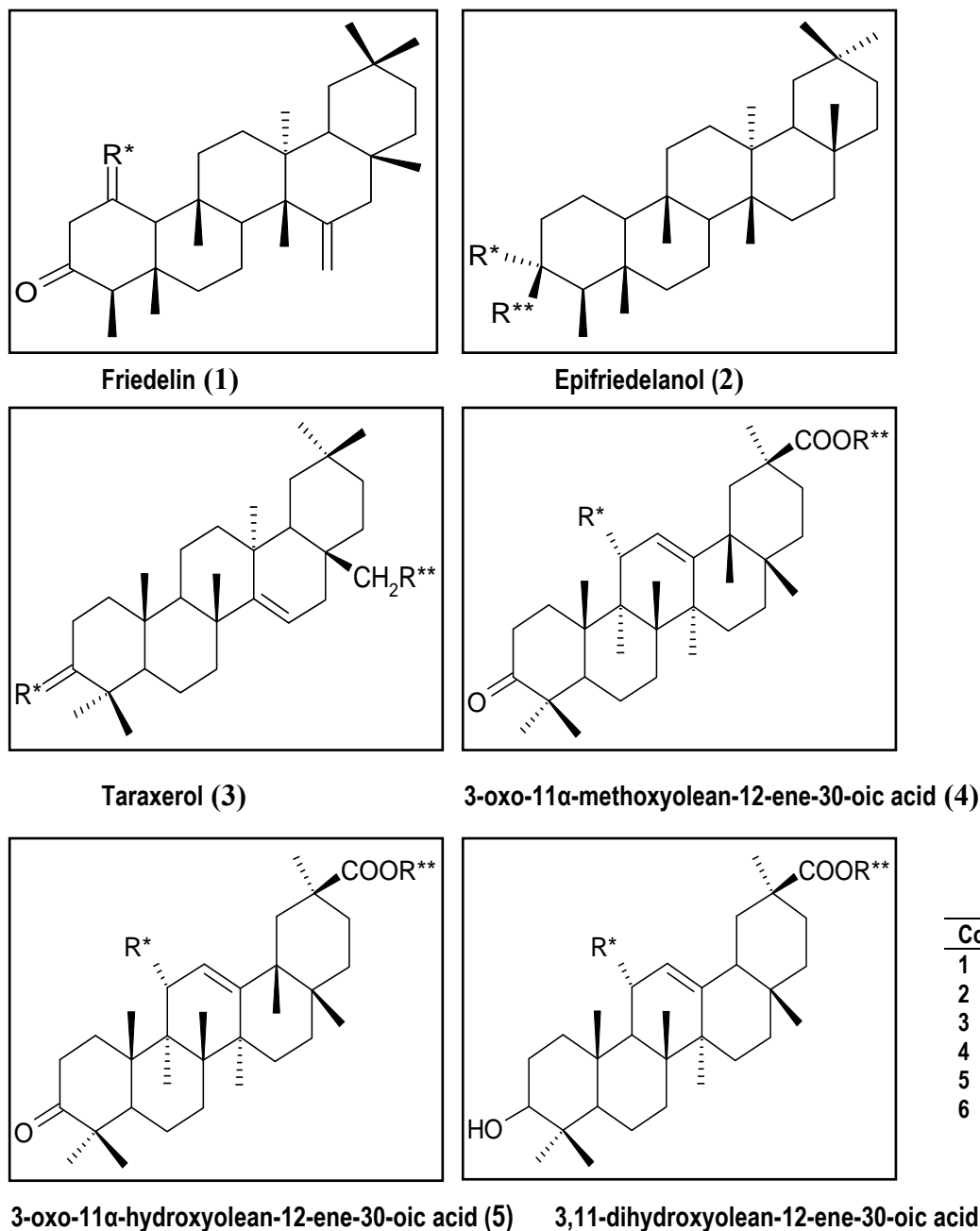
Compound	R*	R**
1	H₂	-
2	H	OH
3	H₂	H
4	OCH₃	H
5	OH	H
6	OH	H

Fig 5.9 Structures of the isolated compounds from *M. undata* aerial parts

5.4 Conclusion

The structure of the six isolated pentacyclic triterpenes was elucidated using NMR and MS techniques. Friedelin **(1)** and epifriedelanol **(2)** belong to the friedelane group, taraxerol **(3)** belongs to the taraxerane group and 3-oxo-11α-methoxyolean-12-ene-30-oic acid **(4)**, 3-oxo-11α-hydroxyolean-12-ene-30-oic acid **(5)** & 3,11-dihydroxyolean-12-ene-30-oic acid **(6)** belong to the 12-oleanene group. The compound 3-oxo-11α-methoxyolean-12-ene-30-oic acid **(4)** contains a methoxy group, and 3-oxo-11α-hydroxyolean-12-ene-30-oic acid **(5)** contains a hydroxyl group at C11. From the literature it is evident that the Celastraceae family

contains the friedelane, taraxerane and oleanene constituents, and hence it was not surprising to isolate these constituents from the aerial parts of *M. undata*, a member of the Celastraceae family. It is also evident from the literature that friedelin **(1)** and epifriedelanol **(2)** are abundant in many plant species, and have commonly been isolated from the hexane extract of those species. The first isolation and characterization of 3-oxo-11α-methoxyolean-12-ene-30-oic acid **(4)** and 3-oxo-11α-hydroxyolean-12-ene-30-oic acid **(5)** was reported by Muhammad and colleagues (2000) from *M. undata*. This is the first report of the isolation and characterization of 3,11-dihydroxyolean-12-ene-30-oic acid **(6)** from *M. undata*. However, a closely related constituent has been isolated from *Glycyrrhiza glabra* by the hydrolysis of glycyrrhizinic acid into glycyrrhetinic acid (Salari and Kadkhoda, 2003).

It is evident that plants contain numerous constituents which need to be isolated by bioassay guided fractionation and properly characterized to facilitate the determination of the specific pharmacological activity of the extract. In the next chapter the antifungal activity of the isolated compounds will be evaluated to determine if the antifungal compounds were indeed isolated. The safety of these isolated compounds will also be evaluated.

CHAPTER 6

IN VITRO **ANTIFUNGAL AND ANTIBACTERIAL AND CYTOTOXICITY ACTIVITY OF THE ISOLATED COMPOUNDS**

6.1 Introduction

Antimicrobials are compounds which, at low concentrations, exert an action against microorganisms and exhibit selective toxicity towards them (Goodyear and Threlfall, 2004). During isolation some compounds might undergo chemical modifications because of heat, photooxidation or the effect of organic solvents used during isolation. These modifications might increase or decrease the activity of the isolated compounds, hence it is important to conduct quantitative biological activity studies of the isolated compounds to investigate whether activity has been retained or lost during isolation. Also, activity of the isolated compounds against different microbes determines the specificity of the isolated compounds against various microorganisms. In many cases the biological activities of compounds isolated by phytochemists have not been determined.

The idea that herbal drugs are necessarily safe and free from side effects is false. Plants contain hundreds of constituents and some of them are toxic, such as the highly cytotoxic anti-cancer plant-derived drugs, as well as digitalis and the pyrrolizidine alkaloids (Calixto, 2000). In plant bioactivity studies, it is recommended to screen the isolated compounds for cytotoxicity against various cell lines to gain an indication of potential toxicity. Further investigations of the compounds prior to potential drug development, including acute toxicity and other *in vivo* toxicity trials, need to be undertaken.

6.2 Materials and methods

6.2.1 TLC fingerprinting of the isolated compounds

Aliquots (10 µl) of 1 mg/ml solution in CHCl$_3$ (equivalent to 10 µg) of each isolated compound were loaded on 3 × 10 cm TLC plates. TLC plates were prepared as described in **Section 2.2.3**.

6.2.2 Bioautographic assays of the isolated compounds

Aliquots 10 µg of each isolated compound were loaded on 5 × 10 cm TLC plates. TLC plates were prepared as described in **Section 2.2.3** and left for several days in a dark to allow evaporation of the eluting solvents. Biautographic assays were conducted as described in **Section 2.2.5.** Two fungal species (*Candida albicans* and *Cryptococcus neoformans*), two Gram-positive bacterial species (*Staphylococcus aureus*, ATCC 29213 and *Enterococcus faecalis*, ATCC 29212) and two Gram-negative bacterial species (*Escherichia coli,* ATCC

27853 and *Pseudomonas aeruginosa,* ATCC 25922) were used. Bacteria were cultured in Müller-Hinton (MH) broth at 37 ºC and maintained on MH agar at 4 ºC. Developed TLC plates were sprayed with actively growing suspension of the above mentioned organisms and incubated overnight at 37ºC.

6.2.3 Microdilution assay

6.2.3.1 Antifungal assay

Aliquots (100 µl) of 1 mg/ml solutions dissolved in acetone of the isolated compounds were serially diluted with distilled water in 96- well microtitre plates and the serial dilution assay was conducted as described in **Section 2.2.6**. A three to five day-old Sabouraud Dextrose (SD) broth culture of *C. albicans* was diluted 1:10 with fresh SD broth and *C. neoformans* was used as a concentrated suspension.

6.2.3.2 Antibacterial assay

The assay was conducted as described by Eloff (1998c). The method is basically the same as the one outlined in **Section 2.2.6** the only difference is that INT (0.2 mg/ml) was added following overnight incubation of compounds with bacteria. Overnight cultures of the bacteria were diluted 1:100 with fresh Mueller Hinton (MH) broth prior to use in the assay.

6.2.4 Cytotoxicity

6.2.4.1 Tetrazolium-based colorimetric assay (MTT)

The procedure described by McGaw *et al* (2007) was used to investigate cytotoxicity of the hexane plant extract and four of the isolated compounds. The plant extracts were tested for cytotoxicity against Vero monkey kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. Plates were incubated overnight at 37 ºC in a 5% CO_2 incubator and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts (200 mg/ml) and isolated compounds (20 mg/ml) were prepared by dissolving them in DMSO. Serial 10-fold dilutions of each extract and isolated compounds were prepared in growth medium and added to the cells.

The viable cell growth after 120 hours incubation with plant extracts and isolated compounds was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide

(MTT) assay, Sigma) described by Mosmann (1983). Briefly, after incubation, 30 µl of MTT (5 mg/ml in phosphate buffered solution, PBS) was added to each well and the plates were incubated for a further 4 hours. The medium was aspirated from the wells and 50 µl DMSO added to each well to solubilize the formazan produced by mitochondrial activity. The absorbance was measured on a Titertek Multiscan MCC/340 microplate reader at 540 nm test wavelength and reference wavelength of 690 nm. Berberine chloride (Sigma) was used as a positive control. The intensity of colour is directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

6.2.4.2 Preparation of formaldehyde fixed Equine erythrocytes (RBC)

Equine erythrocytes fixed with formalin were prepared according to Sadique *et al* (1989). Fresh blood was collected from a horse (Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria) into clean, dried glass tubes containing 3.8% sodium citrate (9 part of blood: 1 part of 3.8% sodium citrate). The blood was centrifuged at 4000 rpm for 10 min. The packed RBCs were washed with 10 mM phosphate-buffered saline (PBS), pH 7.2, until a clear supernatant was obtained. The washed and packed RBCs were suspended in 5% (v:v) formaldehyde in PBS (1:12.3 v:v). The mixture was left at room temperature for 24 hours. The final fixed RBC were washed and centrifuged with PBS three times (Iwalewa, 2005).

6.2.4.3 Hemagglutination assay (HA)

The hemagglutination assay was used to determine toxicity of hexane extract and the isolated compounds on erythrocytes membrane. The degree of toxicity is indicated by agglutination of the RBCs due to the change in the physiology of the blood cell membrane (Iwalewa *et al.,* 2005). The hemagglutination assay was conducted using the procedure described by Iwalewa *et al* (2005) and Sadique *et al* (1989). PBS (100 µl) was placed into U shaped 96-well microtiter plates. The first row was used as a control without extracts. *Maytenus undata* hexane extract (100 µl of 10 mg/ml solution in acetone) and isolated compounds (100 µl of 3 mg/ml solution in acetone) were added to the second row and were twofold serially diluted down the column. Then 50 µl of horse RBCs was added to all the wells and incubated at room temperature for 1 hour. The presence of buttons in the center of the well indicated no agglutination i.e. RBC membranes are not disrupted, hence no toxicity of the extract or isolated compounds. The HA titer value of the extracts was calculated as the reciprocal of the last dilution concentration showing agglutination, and was calculated using the following formula. A high hemagglutination value indicates the high level of toxicity which could cause serious toxic effects particularly on the RBC blood group (Iwalewa *et al.,* 2005). A test was carried out in triplicate and experiments were repeated three times.

HA titer value = 1/concentration value

6.3 Results and Discussion

6.3.1 TLC fingerprints of the isolated compounds

Some of the isolated compounds (compounds **1**, **3**, **5** and **6**) from *Maytenus undata* aerial parts (Figure 6.1). Compounds **2** and **4** were not isolated in sufficient quantities for TLC analysis, but were used for structure elucidation only. The location of these compounds on the TLC chromatogram is shown by an arrow (compound **2**) and a red circle (compound **4**) on the crude extract separation in the BEA solvent system.
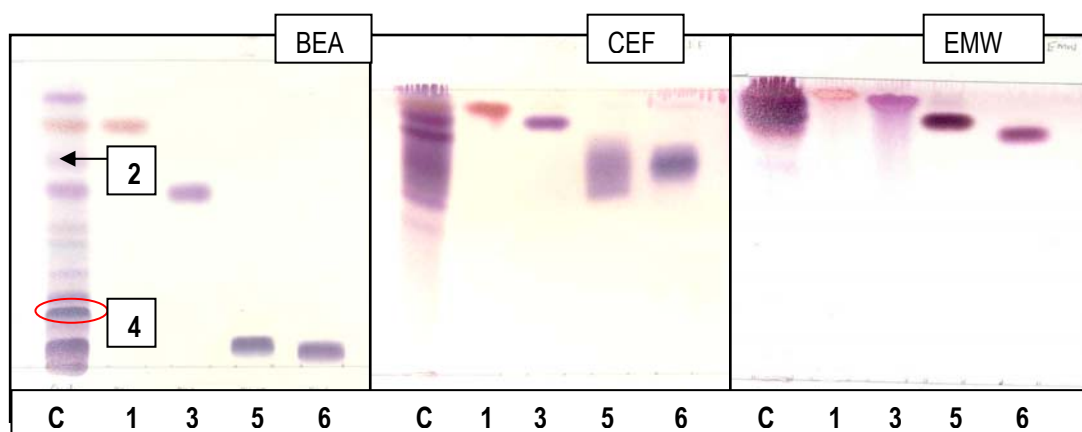
Fig 6.1 TLC fingerprints of 10 µg of the isolated compounds developed in BEA, CEF and EMW respectively and sprayed with vanillin sulphuric acid. From left to right **C** = Crude extract, **1** = compound 1, **2** = compound 2, **3** = compound 3, **4** = compound 4, **5** = compound 5 and **6** = compound 6

As an aid to dereplicating isolation of antifungal compounds from other plant species the R_f in our commonly used TLC solvent systems were determined. The R_f values of the isolated compounds ranged from 0.95 to 0.03 in BEA, 0.95 to 0.78 in CEF and 0.93 to 0.83 in EMW (Table 6.1). The R_f values of compounds **2** and **4** in CEF and EMW were not calculated since the separation of constituents in the crude extract was poor. Both compounds **5** and **6** contain carboxylic groups which might be responsible for them streaking in an acidic mobile phase such as CEF, hence the separation and resolution of bands was poor. However compounds separated well in both BEA and EMW, producing distinct bands.

Table 6.1 R_f values of the isolated compounds in different mobile systems. Friedelin (**C1**), epifriedelanol (2), taraxerol (**C3**), 3-oxo-11α-methoxyolean-12-ene-30-oic acid (4), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**C5**), 3,11-dihydroxyolean-12-ene-30-oic acid (**C6**)

Mobile phase	R_f values of the isolated compounds					
	1	**2**	**3**	**4**	**5**	**6**
BEA	0.95	0.85	0.73	0.14	0.04	0.03
CEF	0.95	-	0.91	-	0.77	0.78
EMW	0.93	-	0.92	-	0.87	0.83

6.3.2 Bioautographic assays of the isolated compounds

Bioautograms of the isolated compounds against *C. neoformans*, *E. coli*, *P. aeruginosa* and *S. aureus* as depicted in Figure 6.2. A clear band on the bioautogram indicates activity of the crude extract or isolated compounds against a certain microorganism. Compound **1** did not indicate any clear bands against any of the tested microorganisms, meaning that all the tested microorganisms were resistant to compound **1** at the tested concentration. Compound **3** showed clear bands against both *C. neoformans* and *E. coli*; however *P. aeruginosa* and *S. aureus* showed resistance against compound **3** at the tested concentration. All the microorganisms were sensitive to compounds **5** and **6**. Compounds **5** and **6** also indicated activity against *Candida albicans* and *Enterococcus faecalis*.
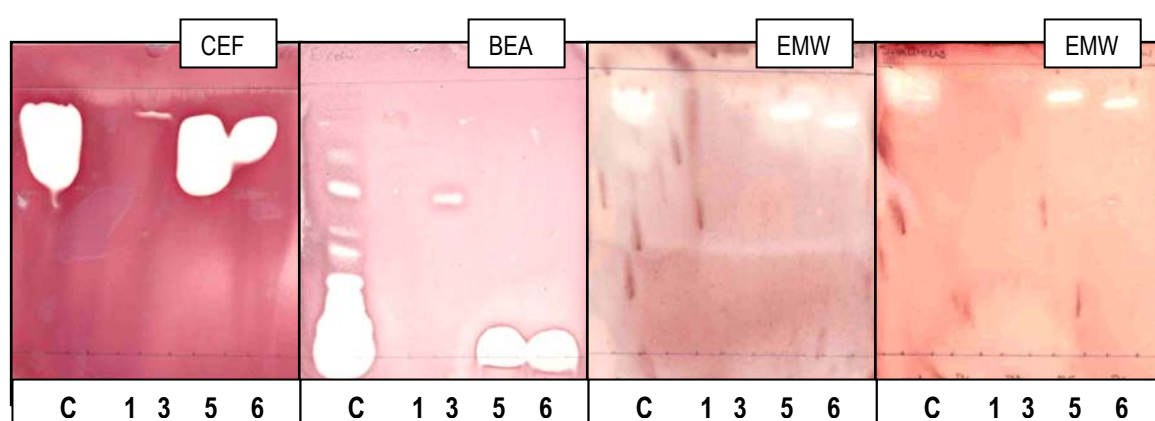
Fig 6.2 TLC bioautograms of the isolated compounds against *Cryptococcus neoformans*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (from left to right), TLC plates developed in CEF, BEA and EMW.**C** = Crude extract, **1** = compound 1, **3** = compound 3, **5** = compound 5 and **6** = compound 6

6.3.3 Minimum inhibitory concentrations

The MIC values of the isolated compounds against two fungal species and four bacterial species as shown in Table 6.2. All the microorganisms were resistant against friedelin (**1**) and taraxerol (**3**) at the highest concentration tested except *E. faecalis* which indicated some sensitivity to taraxerol (**3**) with an MIC of 130 µg/ml. Bioassay guided fractionation did therefore not work in the case of these two compounds. It is possible that compounds with similar R_f values do have antifungal activity and were not isolated.
All the fungal species were sensitive to 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) and 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) with MIC values ranging between 63 µg/ml and 47 µg/ml. However both 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) and 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) were more active against *C. neoformans* than *C. albicans*. A review of the current available literature indicates that not one of the isolated compounds have been tested for antifungal activity against *C. neoformans*, but some of the compounds have been tested against *C. albicans* and other bacterial species. Kuete *et al.* (2007) showed that friedelin possesses inhibitory activity against *C. albicans* with an MIC value of 2.44 µg/ml. Our results could not confirm the antifungal activity of this compound against *C. albicans*.

Staphylococcus aureus was less sensitive to the tested compounds with an MIC value of > 250 µg/ml, the highest concentration tested. All the tested Gram–negative bacterial species were sensitive to 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) with MIC values of 36 µg/ml and 32 µg/ml for *P. aeuroginosa* and *E. coli,* respectively. The compound isolated for the first time 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) was more active against *E. coli* (MIC = 24 µg/ml) than any of the other microorganisms tested. Kuete *et al.* (2007) showed that friedelin possesses inhibitory activity against *E. faecalis* with an MIC value of 0.61 µg/ml, but *E. coli*, *P. aeuroginosa* and *S. aureus* were less susceptible to friedelin at the tested concentrations (Kuete *et al.*, 2007). These results were consistent with the results observed during this study except for *E. faecalis*. Muhammad *et al.* (2000) showed that 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) was active against *S. aureus* and *P. aeruginosa* with MIC values >10 µg/ml and 6.25 µg/ml respectively and it could not inhibit BMΦ thromboxane B_2 superoxide anion generation.

It was mentioned in **chapter 5** that 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) is closely related to glycyrrhetinic acid. Kim *et al.* (2002) reported that glycyrrhetinic acid did not inhibit the growth of *E. coli* (KCTC 1682) and *C. albicans* (KCTC 7270), but 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) showed substantial activity against all the tested microorganisms in this study with MIC values ranging from 63 µg/ml to 24 µg/ml, except for *S. aureus* which was resistant. The reason for this difference in activity might be due to the presence of two hydroxyl groups in 3,11-dihydroxyolean-12-ene-30-oic acid (**6**) compared to glycyrrhetinic acid which contains a keto group and lacks a hydroxyl group. Inhibition of the inception and growth of skin tumors (Wang *et al.*, 1991) and the growth and differentiation of mouse melanoma cells *in vitro* (Abe *et al.*, 1987) associated with glycyrrhetinic acid have been reported.

There is no noteworthy difference between the MIC of the hexane crude extract containing a large percentage of inactive compounds and the isolated compounds. The compounds are not highly active against the tested microorganisms, especially against *C. neoformans,* in pure form. This suggests the possibility of synergistic effects between active or even non-active plant constituents in *M. undata* extract which might influence the uptake of active constituents by microorganisms. This phenomenon is possible in plant extracts containing numerous constituents responsible for different properties within the plant. Some of the compounds which showed activity on bioautograms were not isolated in this study because they were present in quantities too low to isolate using these methods. These compounds may contribute to possible synergistic effect of the crude extract.

Table 6.2 MIC values of the isolated compounds and positive controls against fungal and bacterial species Friedelin (**C1**), taraxerol (**C3**), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**C5**), 3,11-dihydroxyolean-12-ene-30-oic acid (**C6**)

Microorganisms	MIC values of the isolated compounds (µg/ml)						
	Crude (mg/ml)	1	3	5	6	AmB (µg/ml)	Gentamicin (mg/ml)
C. albicans	0.30	>250	>250	63	63	0.16	-
C. neoformans	0.08	>250	>250	47	47	0.16	-
E. coli	0.12	>250	>250	32	24	-	0.0063
E. faecalis	0.08	>250	130	32	63	-	0.025
S.aureus	0.63	>250	>250	>250	>250	-	0.015
P. aeruginosa	0.30	>250	>250	36	32	-	0.0032

6.3.4 Tetrazolium-based colorimetric assay (MTT)

The percentage (%) cell viability increased with a decrease in concentrations of both hexane extract and berberine (Figure. 6.3). The highest concentrations of both hexane extract and berberine indicated very low percentage (%) cell viability this implies that the hexane extract at concentrations from 200 µg/ml are toxic to the Vero cells. The same held for berberine at concentrations from 100 ppm (100 µg/ml). The hexane extract indicated an LC_{50} of 76 µg/ml and berberine indicated an LC_{50} of 12.35 ppm (1.2 µg/ml), calculated from the regression curve. The crude extract therefore had a low level of toxicity.
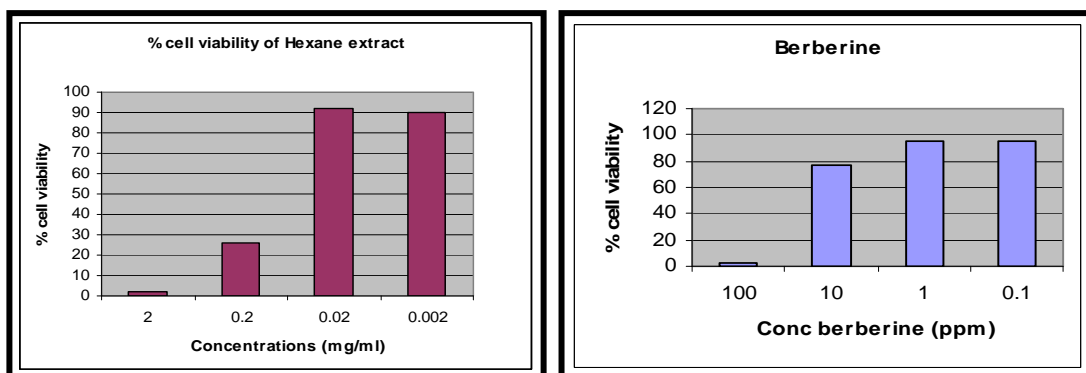
Fig: 6.3 Percentage (%) cell viability of hexane extract and berberine

Two of the isolated compounds friedelin (**C1**) and taraxerol (**C3**) indicated slightly lower cell viability at high concentrations and cell viability increased with a decrease in compound concentrations (Figure 6.4). Friedelin (**C1**) showed higher cell viability as compared to taraxerol (**C3**). Both friedelin (**C1**) and taraxerol (**C3**) indicated $LC_{50} > 200$ µg/ml indicating that there was little to no cytotoxicity towards the Vero monkey kidney cells. Compounds 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (C**5)** and 3,11-dihydroxyolean-12-ene-30-oic acid (C**6)** showed toxicity towards the Vero cells at concentrations from 10 µg/ml with LC_{50} values of 6.03 and 2.98 µg/ml, respectively. The degree of toxicity of the hexane extract may be attributed to presence of the relatively toxic compounds **5** and **6.** Also some compounds which were not isolated could have toxic effects towards the Vero monkey kidney cells. Compounds **5** and **6** were more toxic to Vero cells more than compounds **1** and **3**. The toxicity of compounds **2** and **4** could not be determined as they were not isolated in sufficient quantity, but it is possible that they may also contribute to the cytotoxic effect of the hexane extract.
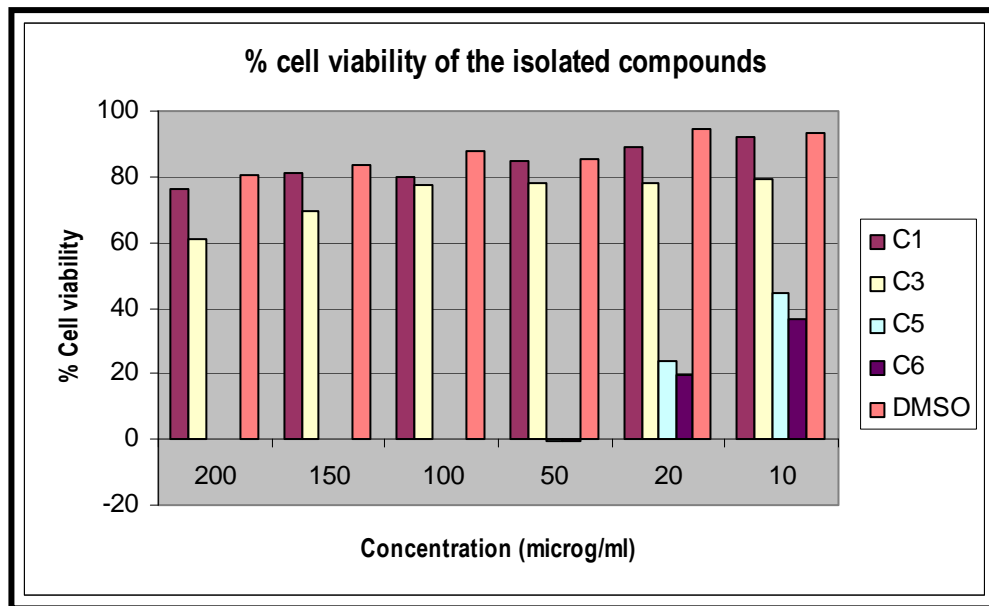
Fig: 6.4 Percentage (%) cell viability of friedelin (**C1**), taraxerol (**C3**), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**C5**), 3,11-dihydroxyolean-12-ene-30-oic acid (**C6**) and DMSO

6.3.5 Hemagglutination assay (HA)

The *Maytenus undata* hexane extract caused agglutination and the concentration that caused agglutination of the formaldehyde-fixed equine erythrocytes was 0.625 mg/ml. This resulted in a high HA titer value which indicates toxicity of the extract towards the RBCs. Compounds **1** and **3** indicated the presence of buttons at the bottom of the well even at the highest concentration tested meaning that they did not show any toxicity towards the RBCs. However compounds **5** and **6** caused agglutination of the RBCs. This indicates that these compounds are toxic at 0.75 mg/ml and 1.5 mg/ml towards the formaldehyde-fixed RBCs. Compound **5** is more toxic to the RBCs than compound **6** (Table 6.3).

The *M. undata* hexane extract contains constituents that are able to alter/change the physiology of the RBC membrane to cause agglutination. Since both compounds **5** and **6** indicated agglutination at relatively high concentrations, this shows their involvement in causing agglutination in the crude extract. It is evident that this plant extract at high concentrations might cause toxic effects on RBCs.

Table 6.3 Cytotoxicity activities of hexane extract and the isolated compounds on formaldehyde-fixed equine erythrocytes (RBCs). Friedelin (**C1**), taraxerol (**C3**), 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**C5**), 3,11-dihydroxyolean-12-ene-30-oic acid (**C6**)

Crude extract & isolated compounds	Concentration value where agglutination occurred (mg/ml)	HA titer value
Hexane extract	0.625	1.6
C1	*	*
C3	*	*
C5	0.75	1.33
C6	1.5	0.67
Control (Ace)	*	*

* No activity

Both of the toxicity studies that were performed (MTT assay and hemagglutination assay) indicated that the hexane crude extract and compounds **5** and **6** were toxic. However compounds **1** and **3** did not show any signs of toxicity in either of the toxicity assays. The hexane crude extract and compounds **5** and **6** displayed antimicrobial activity against the tested microbes and compounds **1** and **3** were not convincingly active. Interestingly the hexane crude extract and compounds **5** and **6** showed activity at concentrations below the concentrations that indicated toxicity. The average MIC value of the *M. undata* hexane extract against the tested microorganisms was 302 µg/ml, except for *S. aureus* against which an MIC value of 630 µg/ml was obtained.

The hexane extract concentration that caused agglutination was 625 µg/ml; this indicates that the concentration needed to inhibit microbial growth is lower than the RBC toxic concentration, but higher than the cytotoxic concentration (against Vero cells). The same applies to compounds **5** and **6** which indicated toxic concentrations to RBC of 750 µg/ml and 1500 µg/ml, respectively. These concentrations are much higher than the MIC concentrations (ranging between 63 µg/ml – 24 µg/ml) except for *S.aureus* which was resistant. The isolated compounds **5** and **6** prevent microbial growth at concentrations lower than 750 µg/ml and 1500 µg/ml. Compounds **5** and **6** indicated LC_{50} of 6.03 and 2.98 µg/ml, respectively. These concentrations indicated high cytotoxicity and little potential of therapeutic use to protect animals against fungi.

6.4 Conclusion

The species *Maytenus undata* contains antimicrobial compounds which possess activity against different microorganisms. All the tested microorganisms showed resistance against friedelin **(1)** and taraxerol **(3)** with MIC values of >250 µg/ml except for *E. faecalis* with an MIC value of 130 µg/ml. The compounds 3-Oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) and 3,11-dihydroxyolean-12-ene-30-oic acid **(6)** showed clear bands against all the tested organisms on bioautograms, indicating microbial growth inhibition. MIC values of compounds **5** and **6** ranged from 63 µg/ml to 24 µg/ml except for *S. aureus* which was resistant .The compounds that were isolated generally showed lower activity against the microbes employed in the screening tests than the crude extract. This implies that it may be more productive to use the entire plant extract to combat microbial infection than to use compounds in pure form.

The potential synergism between active plant constituents present in an extract, as well as other biochemical interactions between the constituents, may explain this occurrence. This supports the use of crude plant extracts by people in developing countries who rely heavily on medicinal plants. The use in antimicrobial therapy of crude extracts, which contain a vast number of compounds, may act to reduce the occurrence or development of resistant microbes. It is far easier for a pathogenic microorganism to develop resistance against a single purified compound rather than against a suite of compounds acting simultaneously possibly with different targets to result in antimicrobial activity. This supports the development of efficacious herbal products derived from biologically active plant extracts, rather than pharmaceuticals comprising a single active constituent isolated from the plant extract.

Toxicity of hexane crude extract and compounds **5** and **6** indicated that their antimicrobial activity is due to their toxic effects. This reduces value in the use of these compounds as antimicrobials. However compounds **1** and **3** were not effective in combating microbial growth and also were not toxic.

Chapter 7

GENERAL CONCLUSIONS

Medicinal plants have been used for centuries as the primary mode of treatment of ailments caused by various organisms such as bacteria, fungi and viruses. This dependency on medicinal plants for such a long time has raised questions of efficacy and safety of traditional medicine. On the other hand, it has provided the opportunity for researchers and scientists to evaluate the biological activity and safety of medicinal plants to promote their usage, and for the development of new pharmaceuticals which may be less toxic and more potent than currently used antimicrobials.

Plants produce a vast number of secondary constituents some of which may act to protect the plants against invading pathogens. These constituents are important for human and animal lives because they can be used in the production of new pharmaceuticals or feed additives. The extraction of medicinal plants with appropriate solvents can result in extracts rich in potential antimicrobially active constituents. The phytochemical and biological activity analysis of the plant constituents follows, and therefore the solvent used to extract the plant material plays a crucial role in obtaining active constituents in soluble form.

The aim of this study was to isolate antifungal compounds with potential value to protect animals and humans against fungal infections. During this study, extracts of ten plant species were screened against *Cryptococcus neoformans. Maytenus undata* was selected as the most promising species for further work based on the presence of clear zones on bioautograms, a low average MIC value of 0.09 mg/ml and high average total activity of the extracts. Some plant species had lower MICs, but lack of clear bands and lower average total activity disqualified it from further analysis.

The bulk serial extraction of *Maytenus undata* leaves with various organic solvents including hexane, dichloromethane, acetone and methanol was performed. The hexane extract was the most active against *C. neoformans* with an MIC value of 0.02 mg/ml. The DCM extract indicated an MIC value of 0.04 mg/ml however active constituents were not isolated. Column chromatography led to the isolation and identification of six constituents present in the *M. undata* leaves namely friedelin **(1)** epifriedelanol **(2)**, taraxerol **(3),** 3-oxo-11α-methoxyolean-12-ene-30-oic acid **(4)**, 3-oxo-11α-hydroxyolean-12-ene-30-oic acid (**5**) and 3,11-dihydroxyolean-12-ene-30-oic acid **(6)**. This has contributed to the base of phytochemical knowledge by providing knowledge of some of the compounds present in *M. undata* aerial parts. Depending on the quantity of active metabolites in an extract, other active constituents are often not isolated resulting in the loss of valuable information needed for the development of new, effective and safer pharmaceuticals.

The activity of the isolated compounds in comparison with the original hexane extract was evaluated against various fungal and bacterial species to obtain an idea of the specificity of the isolated compounds. By conducting these studies the efficacy of the isolated compounds and the *M. undata* crude extract was

evaluated. Compounds **1** and **3** were isolated in relatively high amounts (0.029% and 0.026% respectively) however they were inactive with an MIC value of >250 µg/ml against almost all the tested microorganisms, except for compound **3** against *E. faecalis* with MIC = 130 µg/ml. Compound **5** was isolated in larger amounts (0.014%) than compound **6** (0.011%). Advanced standardized techniques and methodologies are needed to further isolate and develop new plant products required for ailments caused by infectious pathogens.

The cytotoxicity of the hexane extract and the isolated compounds were investigated using the tetrazolium-based colorimetric assay (MTT) on Vero monkey kidney cells and the hemagglutination assay on formaldehyde-fixed erythrocytes (RBCs). The hexane plant extract had a low toxicity towards the Vero monkey cells at concentrations greater than 0.2 mg/ml with an LC_{50} of 76 µg/ml and HA titer value of 1.6 when using RBCs. Compounds **1** and **3** had low toxicity against the cells with an LC_{50} greater than 200 µg/ml and they did not indicated any agglutination of the RBCs hence no toxic effects were observed at the tested concentrations. . However compounds **5** and **6** were toxic with an LC_{50} of 6.16 µg/ml and 3.36 µg/ml, respectively towards the Vero cells and the HA titer values of 1.33 and 0.67, respectively. The most active compound were toxic, supporting the need for toxicity testing to be included as part of a programme investigating antimicrobial activity. Non-specific effects are common; selective antifungal or antibacterial activity in the absence of mammalian cell toxicity is the ultimate aim of a screening project.

The objectives formulated for this study listed on page 13 were largely obtained, but the aim to isolate compounds with potential use against infections in animals and humans was only partially obtained. The isolated compounds had lower antifungal activity and higher toxicity than the drugs currently in the market. Even though active compounds were toxic towards the cell lines some chemical modifications might provide more effective constituents with lower toxicity. This highlights the need to chemically synthesize new derivatives of the active constituents. Another approach would be to use extracts containing several antifungal products. This may limit the development of resistance. *In vitro* antifungal activity or toxicity does not necessarily mean that the extracts or compounds will be active or toxic when provided to animals because parameters such as uptake, inactivation, excretion or even synthesis of an active compound from a pro-drug may play a role. Future studies may therefore involve animal experiments to verify the results obtained here.

This study proved that even though plants are neglected they contain compounds which can be useful in the challenges facing modern medicine when it comes to combating infectious diseases. Authorities should start to recognize medicinal plants and the influence that they have especially in developing countries. Research on these natural resources should be encouraged and funding should be made available for the development and production of new pharmaceuticals or herbal extracts of therapeutic value. Today the world is faced with devastating ailments such as malaria, cancer, diabetes and tuberculosis, and biological resources such as plants might provide solution to this misery.

Chapter 8

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