A STUDY OF PLANTS USED TO MANAGE *CANDIDA* INFECTIONS IN TANZANIA

By

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DECLARATION

AND

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I, **Deborah Kiumlo Balira Runyoro**, declare that this **thesis** is my own original work and that it has not been presented and will not be presented to any other University for similar or any other degree award.

Signature.....Date.....24/7/2021.....

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DEDICATION

In the memory of my loving parents

The late

Reverend Hezekiel Balira and Mrs. Penina Balira

also

To my precious and highly treasured gifts from God

Husband Gerald

and

Daughters

Aileen and **Jacqueline**

ABSTRACT

Candidiasis is one of the major opportunistic infections in individuals living with HIV infection especially those with severe immunosupression. The control of candidiasis is faced with several problems including, the limited number of effective drugs, the slow rate at which new drugs are being developed, the side effects and cost associated with these drugs. Furthermore relapse of candidiasis and resistance of *Candida* species to commonly used drugs are other important impeding factors in the management of candidiasis. These difficulties associated with the management of *Candida* infections necessitate the development of new anti-fungal agents in order to widen the spectrum of activities against *Candida* and combat strains expressing resistance to the available anti-fungal agents.

In view of the above facts, this study was carried out on 56 plantsspecies collected from four regions in the Eastern part of Tanzania namely Coast, Dar es Salaam, Morogoro and Tanga. The specific objectives of the study were; (i) to screen plants growing in Tanzania for anticandida activity, (ii) to isolate the compounds active against *Candida*, (iii) to elucidate their structures and (iv) to determine their minimum inhibitory concentrations (MICs).

A total of 63 aqueous methanolic extracts, prepared from 56 plant species collected based on interviews with traditional healers and literature search were screened against *Candida albicans* standard strain ATCC 90028 using bioautography agar overlay method. Twenty-eight out of the 63 plant extracts, belonging to 27 plant species and constituting 48% of all the plants collected were found to be active. Furthermore twenty (55.5%) out of 36 plants, obtained through interviewing traditional healers, were found active. *Albizia anthelmintica* root bark and *Combretum zeyheri* leaves were given priority for detailed chemical investigation and isolation of bioactive compounds. *Albizia anthelmintica* which was collected from two traditional healers, had not been previously evaluated for antifungal activity and became the most active of all plants screened. *Combretum zeyheri* was collected based on literature reports that it was active on several *Candida* species in three different countries in Africa and in this study it showed two

well resolved spots on the bioautogram. With these merits, the two plants were given priority for isolation of the active compounds.

Several separation techniques including partitioning, precipitation and chromatography were employed for the isolation of the compounds. Vacuum Liquid Chromatography (VLC), Centrifugal and normal Preparative Thin Layer Chromatography were found to be very much useful in the separation of compounds from *A. anthelmintica* aqueous extract, while the compounds from *Combretum zeyheri* ethylacetate extract were isolated by employing VLC.

The isolated compounds were characterized using 1D and 2D NMR experiments which included ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H -^{1}H COSY, HSQC and HMBC. Fourier transform infrared (FT-IR) spectroscopy and ES-MS were also used to confirm the structures of the characterized compounds. Six out of seven compounds isolated from *A. anthelmintica* root bark were saponins based on echinocystic acid. Two pure compounds and two isomeric mixtures of triterpenic acids belonging to a- and ß- amyrin groups were isolated from *Combretum zeyheri* leaves. With the exception of two saponins isolated from *A. anthelmintica* which are new the rest of the isolated compounds are known.

The MICs of the isolated compounds were determined using broth microdilution method for two standard strains of *C. albicans* (ATCC 90028 and MTCC1637) and clinical isolates of *C. albicans*. Of the isolated compounds, four were found to be active against at least one of the *C. albicans* strains. The active compounds were terminolic acid from *Combretum zeyheri* leaves, 3-O-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid, 3-O-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid and 3-O-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl from *A. anthelmintica* root bark. The MICs obtained were in the range of 25-125 µg/ml. However various combinations of the active saponins gave MICs as low as 12.5 µg/ml.

This study proved that plants growing in Tanzania are potential source of bioactive molecules active *in vitro* against *C. albicans*. Also the fact that 55.6% of plants obtained through interviewing traditional healers were found active, this study has shown the importance of ethnomedical information in drug discovery. For better results future studies should involve collection of more plants from different parts of Tanzania with climatic and seasonal variations. Also in view of the low activity showed by the isolated compounds when compared to the standard antifungal drugs it is suggested that the isolated compounds be used in the synthesis of analogues so as to study their structure activity relationships (SAR) aiming at obtaining more active analogues. This could be beneficial for a compound like terminolic acid, which though very abundant in the leaves of *C. zeyheri* it showed a weak activity with MIC of 62.5 µg/ml. Furthermore, experiments may be designed involving *in vivo* testing using animal models so as to predict the drug action in humans.

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ABREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
CD4	Cluster Designation 4
BAW	Butanol: Acetic acid: Water
BDH	British Drug House
BuOH	*n*-Butanol
CA- 4	Combretastatin A-4
CC	Column Chromatography
CFU	Colony-Forming Unit
CIMAP	Central Institute of Medicinal and Aromatic Plants
^{13}C NMR	^{13}C Nuclear Magnetic Resonance
CNS	Central Nervous Sysytem
COSY	Correlation Spectroscopy
CPTLC	Centrifugal Preparative Thin Layer Chromatography
DEPT	Distortionless Enhanced Polarization Transfer
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
DCCC	Droplet counter- current chromatography
EC	Esophageal candidiasis
ED_{50}	Median Effective Dose (produces desired effect in 50% percent of population
ESI	Electrospray ionisation
ES-MS	Electrospray Mass Spectroscopy
EtOAc	Ethyl acetate
EtOH	Ethanol
FCPC	Fast centrifugal partition chromatography
FTIR	Fourier transform infrared spectroscopy
5-FU	5-Fluorourasil
GIT	Gastro-Intestinal Tract

HAART	Highly Active Anti-Retoviral Therapies
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
^{1}H NMR	Proton Nuclear Magnetic Resonance
hR_f	Percentage relative flow
HSQC	Heteronuclear Single-Quantum Coherence
IC_{50}	Concentration resulting in 50% inhibition
IR	Infrared
ITM	Institute of Traditional Medicine
J	Coupling constant
LC-MS	Liquid Chromatography Mass-Spectroscopy
M^+	Molecular ion
MeOH	Methanol
MFC	Minimum Fungicidal Concentration
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MTCC	Microbial Type Culture collection
MTT	Methyl thiazolyl tetrazolium Chloride
m/z	Mass to Charge ratio
NCCLS	National Committee for Clinical Laboratory Standards
OPC	Oropharyngeal candidiasis
PNDC	Proton Noise Decoupled Carbon
RNA	Ribonucleic acid
PTLC	Preparative Thin Layer Chromatography
SAR	Structure Activity Relationship
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
TLC	Thin Layer Chromatography
UV	Ultra Violet
VLC	Vacuum Liquid Chromatography

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE STUDY

1.1 CANDIDIASIS

Despite the reduction of opportunisic infections (including fungal infections) due to highly active antiretroviral therapy (HAART) (Haddad and Powderly, 2001; Ruhnke, 2004; Sanchez- Vargas et al., 2005), candidiasis is still a major public health problem as an opportunistic infection in HIV and AIDS (Vazquez, 2000; Lattif et al., 2004; Vasques et al., 2006). However, this reduction of opportunistic infections has been observed in USA and Western Europe while the incidence of the infections (including candidiasis) in developing countries is still high (Ghannoum, 2001). Also, in a study on a Spanish cohort, it was observed that HAART caused enhancement of erythematous candidiasis while pseudomembranous candidiasis was suppressed (Ceballos- Salobrena et al., 2004). This observation has been shown to be associated with the immune reconstitution inflammatory syndrome (IRIS) (Cepeda et al., 2008).

The disease is a spectrum of opportunistic infections due to *Candida albicans* and other species of *Candida* which colonize the mucosal surface of all humans during birth or shortly after (Joklik et al., 1988). Thus the risk of endogenous infection is clearly ever present. When a person is infected with HIV, the virus disrupts the patient's immunity, predisposing him/her to secondary infections caused by organisms, which rarely cause infections in an immune competent individual (Chaisson and Volberding, 1995).

From the onset of the AIDS epidemic in the early 1980s, oropharyngeal candidiasis (OPC) and esophageal candidiasis (EC) were prominent features in the earliest clinical descriptions of the epidemic (Klein et al., 1984). Mucocutaneous candidiasis has continued to be a frequent factor in AIDS presentation and management (Vazquez, 1999; Lattif et al., 2004; Vasquez et al., 2006). Moreover, the first occurrence of OPC is recognized as an indicator of significant immunodeficiency, usually with CD4 counts dropping below the threshold level of 200/mm^3 (Badri et al., 2001; Antenyi et al., 2003).

1.1.1 The Genus *Candida*

The genus *Candida* includes around 200 species (Hazen and Howell, 2007). For a long time (up to early 1990s) *Candida albicans* was the main etiology of candidiasis, responsible for more than 70% of all *Candida* infections (Ghannoum, 2001). With the introduction of azoles (late 1980s and early 1990s) a shift in candidal species causing the infection started to occur with non-*albicans* becoming important in the disease process. Nine additional *Candida* species have been associated with infections and they include *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis*, *C. lusitaniae*, *C. rugosa* and *C. glabrata* and *C. stellatoidea* (Blinkhorn et al., 1989; Borg-von Zepelin et al., 1993; Edwards, 1995; Bonomo et al., 1996; Ghannoum, 2001). The diversity of *Candida* species that are encountered in infections is expanding and the emergence of other species that were rarely in play in the past is now likely.

Candida albicans was reported to be responsible for more than 90% of cases of OPC and vulvovaginitis. It has also been reported (Elmets, 1994) that infections due to *C. albicans* develop virtually in all HIV-positive patients and when *C. albicans* produces invasive candidiasis, other species begin to appear with increased frequency (Edwards, 1995).

Candida albicans can be found in soil, hospital environments, inanimate objects and food. Other species may live in non-animal environment, such as soil. It is very rare that *Candida* species are laboratory contaminants (Edwards, 1995). The organisms are commensals of man and can be recovered from many sources in normal and ill individuals. They are commonly found throughout the entire gastro-intestinal tract (GIT), mouth, the female genital tract and urine of patients with indwelling catheters (Edwards, 1995). The majority of *Candida* infections are endogenous in origin even though human-to- human transmission is possible as in the case of thrush of the newborn, which may be acquired from the maternal vagina (Edwards, 1995).

1.1.2 *Candida albicans*

These microorganisms are yeasts, that is, fungi that exist predominantly in unicellular, sexual and asexual forms (Joklik et al., 1988; Levinson and Jawetz, 2003). They are small (3-6 μm), thin walled, ovoid or ellipsoidal cells that reproduce by budding (Joklik et al., 1988; Edwards, 1995). They grow well on normal fungal media hence they do not require any special media for growth. Yeast form, pseudohyphae and hyphae may be found in cultures and tissue (Joklik et al., 1988; Edwards, 1995). *Candida albicans* can be differentiated from other species of *Candida* by placing the organism in serum and observing germ tube formation, small projections from the surface which appear within 90 minutes (Joklik et al., 1988; Levinson and Jawetz, 2003; Jawetz, 2004). Other distinguishing tests are based on physiological parameters rather than morphological characteristics. Metabolic tests include carbohydrate assimilation and fermentation reactions, nitrate utilization and urease production (Joklik et al., 1988; Levinson and Jawetz, 2003; Jawetz, 2004). Chlamydospore formation is also used to identify *C. albicans* (Levinson and Jawetz, 2003).

Strains of *C. albicans* vary in pathogenicity. Hyphal production and resistance to phagocytic killing are associated with virulence though *C. albicans* exhibits endotoxin-like activity, but this activity is not a prominent feature of its pathogenicity (Joklik et al., 1988). Cells of *C. albicans* and other species are capable of attaching to the epithelial cell membranes through an adhesion component thought to be a mannan or glycoprotein (Joklik et al., 1988). Yeast cells of *Candida* species also adhere to plastic surfaces, which facilitates adherence to catheters and prosthetic devices (Joklik et al., 1988).

Most strains of *C. albicans* secrete inducible proteases capable of digesting immunoglobulins and other substrates. The virulence of the *C. albicans* strains is correlated to both proteinase production and adherence to the epithelial cells. Another negative aspect of *C. albicans* is the immunomodulating activity through its glycoproteins located on the cell wall (Joklik et al., 1988).

1.1.3 Epidemiology of Candidiasis

There are many factors that predispose individuals to opportunistic *Candida* infections. Certain physiological changes in an otherwise healthy individual provide settings for opportunistic candidiasis, for instance a healthy pregnant woman is more likely to get *Candida* vulvovaginitis when compared to a non-pregnant woman of similar health status (Joklik et al., 1988; Jawetz, 2004). Infants are at risk if they are heavily exposed to *Candida* before the normal gastro-interstinal tract (GIT) microbial flora and the risk is increased if the mother has *Candida* vulvovaginitis. The infants usually develop oral thrush, perianal and genital infections, gastroenteritis and prolonged and painful diaper rash (Joklik et al., 1988). Other conditions like trauma, burn, abrasion or break of the epithelium of the skin or gut provides an opportunity for *Candida* to penetrate the skin mucosa or subcutaneous tissue. Excessive moisture and warmth increase the growth of *Candida* on the skin (Levinson and Jawetz, 2003). A decrease in a number of functional neutrophils lowers resistance to systemic infection. Defective T-cell immunity is evident in most patients with chronic mucocutaneous candidiasis. Endocrinological disturbances, such as diabetes mellitus, hypoparathyroidism, and Addison's disease result in an increased incidence of candidiasis (Joklik et al., 1988).

Many medical procedures designed to prolong life may lead to life threatening opportunistic infections. Immunosuppressants such as those given in organ transplantation or anti-cancer therapy, decreases resistance to *Candida*. Postoperative procedures such as indwelling catheters or use of prophylactic antibiotics increase the incidence of *Candida* infections (Joklik et al., 1988). Any medical procedure involving insertion of foreign bodies such as artificial heart valves and intravenous lines, which can be colonized by *Candida* increase the risk of the infection.

Patients with AIDS are highly susceptible to candidiasis, especially the type that involves mucosal surfaces of the esophagus and oropharynx (Joklik et al., 1988; Edwards, 1995).

1.1.4 Clinical Manifestations of Candidiasis

Many clinical forms of candidiasis are known, and can be divided into two broad groups:

1.1.4.1 Mucocutaneous candidiasis

This is an infection of the skin, mucous membranes and nails caused by endogenous *Candida*, which occur due to chronic maceration of these areas, physiological changes in the host and a compromised immune status. The risk factors include AIDS, pregnancy, diabetes, young or old age, use of birth control pills, trauma and treatment with corticosteroids or prolonged use of antibiotics. Mucocutaneous candidiasis is frequently one of the first signs in HIV infections (Vazquez, 2000) and it can variously be presented. Those frequently seen in HIV patients are oral candidiasis and esophageal candidiasis. *Candida* vulvovaginitis and onychomycosis incidences are also said to increase with HIV infection (Duerr et al., 1997; Gregory, 1996).

Oral candidiasis (thrush) can occur on the tongue, lips, gum, or palate. It is a patchy to confluent, whitish pseudomembraneous lesion composed of epithelial cells, yeast and pseudohyphae. Studies have shown that oral candidiasis is the most frequent AIDS associated opportunistic infection as up to 90% of HIV- infected individuals suffer at least one episode during the course of their disease (Vazquez, 1999). The high incidence of oral candidiasis in HIVand AIDS patients has made candidiasis a leading fungal infection in this immune-suppressed population (Vazquez, 2000; Jankowaska et al., 2001; Vazquez et al., 2006). It has been suggested that patients with thrush for no obvious reason should be evaluated for AIDS. Also, a number of studies indicate that oral candidiasis can be a useful marker for patients with high HIV loads (Campo et al., 2002). It has also been reported (Badri et al., 2001; Lal and Chussid, 2005) that oral candidiasis in HIV-infected patients provides an important prognostic information and can be used as a cost effective tool for screening patients in therapeutic interventions in resource-limited settings.

Esophageal candidiasis was believed to occur by direct spread from oral disease (thrush) but studies have shown that it can occur without thrush (Edwards, 1995). Chiou et al. (2000) noted that esophageal candidiasis is a debilitating infection, which develops in the setting of prior oropharyngeal candidiasis, low CD4 lymphocyte counts and previous antibiotics use. The most common symptoms of this *Candida* infection include painful

swallowing, a feeling of obstruction on swallowing, substernal chest pain and discrete ulceration of the esophagus (Connolly et al., 1989). The disease has also been associated with adult T-cell leukemia (Obata et al., 1988).

Candida vulvovaginitis is an infection seen in both HIV- positive and HIV- negative women. It is an infection common in pregnancy and other studies have shown that psychosocial factors, particularly stress, are the primary causes of *Candida albicans* vulvovaginitis (Meyer et al., 2006). However, it has also been reported that immunocompromised HIV-positive women with CD4 lymphocyte count below 200 are more susceptible to this *Candida* infection (Duerr et al., 1997). The majority of cases are caused by *Candida albicans*, but in recent years an increase has been observed in the frequency of non-*albicans Candida* infections, especially due to *C. glabrata* and *C. tropicalis* (Paulitsch et al., 2006).

Primary nail invasion by *Candida* is uncommon and almost exclusively seen in patients with an impaired immune function. The appearance of *Candida* onychomycosis in an adult who is not under immunosuppressive treatment always requires a laboratory evaluation of the immunologic function including HIV assays (Tosti et al., 1998). It has been reported that the number of affected individuals has increased concomitantly with an increased population of HIVand AIDS patients (Elmets, 1994; Gregory, 1996).

1.1.4.2 Invasive (systemic) candidiasis:

This is an infection that occurs when a person's own *Candida* organisms, normally found in the gastrointestinal tract, enter the bloodstream (candidemia). On rare occasions, it can also occur when medical equipment or devices become contaminated with *Candida*. In either case, the infection may spread throughout the body. The infections involve various organs such as the central nervous system, respiratory tract, cardiovascular system, gastrointestinal and urinogenital tract. The symptoms of invasive candidiasis are not specific. Fever and chills that do not improve after antibiotic therapy are the most common symptoms, additional specific symptoms may develop, which vary depending on the site of infection. If the infection does not respond to treatment, the patient's organs may fail and cause death.

Candida meningitis has been reported (Casado et al., 1997) to be associated with HIV and AIDS. In this infection, *Candida* infects both the parenchyma tissue and meninges. *Candida albicans* has been the responsible pathogen in 90% of *Candida* meningitis cases and other occasional cases are due to *C. tropicalis*. Overall, *C. tropicalis* is second to *C. albicans* in pathogenitic potential. *Candida guilliermondi*, *C. parapsilosis* and *C. tropicalis* are frequent causes of endocarditis (Joklik et al., 1988).

1.1.5 Mortality and Morbidity from Candidiasis

Most candidal infections are mucocutaneous and, as such, do not cause mortality. However, in patients with advanced immunodeficiency due to HIV infection, these mucosal infections can become refractory to antifungal therapy and may lead to severe oropharyngeal and esophageal candidiasis that initiates a vicious cycle of poor oral nutrition intake, malnutrition, wasting, and early death. Invansive candidiasis, regardless of its etiology, is usually associated with high mortality and morbidity rates (Liu et al., 2004; Cheng et al., 2006). For instance, invasive candidiasis is a leading cause of mycosis-associated mortality in the United States of America (Pfaller and Diekema, 2007).

1.1.6 Management of *Candida* infections

There are five classes of antifungal agents used in the treatment of candidiasis, which include; polyenes, azoles, pyrimidine analogs, allylamines and echinocandins.

Mucocutaneous candidiasis is usually treated by topical anti-fungal agents (Martindale, 1996). These agents include amphotericin B, nystatin, terbinafine and the azole derivatives. Esophageal infections are not normally accessible by topical therapy and are normally treated with oral azoles. Generally, topical therapy is not adequate in mucocutaneous candidiasis in immune compromised patients and is usually complimented with oral azoles. The most commonly used oral azoles are ketoconazole, fluconazole, itraconazole and voriconazole. Patients who, either do not respond or tolerate oral therapy may require intravenous amphotericin B treatment. Also, currently echinocandins are used in resistant cases of esophageal candidiasis.

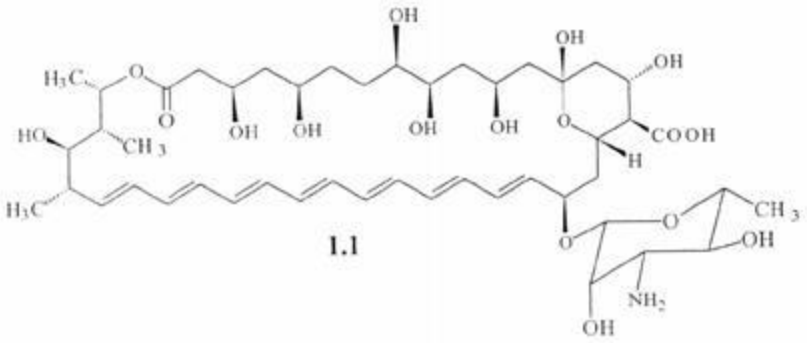
Deep and disseminated candidiasis requires systemic anti-fungal agents. Intravenous amphotericin B, with or without oral flucytosine, is the initial treatment of choice in most infections (Ghannoum, 2001). Other antifungals used include azoles and echinocandins a relatively new class of antifungal agents.

1.1.6.1 Polyenes:

From 1955 until the discovery of azoles, a polyene antibiotic, amphotericin B, which is known to cause significant nephrotoxicity, represented the standard therapy for systemic fungal infection (Sugar, 1986). The polyenes bind to fungal membrane sterol, resulting in the formation of aqueous pores through which essential cytoplasmic materials leak out and death of the organism ensures.

1.1.6.1.1. Amphotericin B

Amphotericin B **(1.1)** is a polyene antibiotic first isolated by Gold and colleagues from *Streptomyces nodosus* in 1955 (Gale, 1960). It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other. Amphotericin B is poorly soluble in water (Terrell and Hughes, 1992). The drug is widely distributed in tissues, but it however, penetrates poorly to the cerebral spinal fluid.



1.1

Since its discovery amphotericin B has been the most widely used antifungal agent to treat invasive or life threatening fungal infections (Ghannoum, 2001). Amphotericin B has a very broad range of activity, possessing fungicidal activity against most pathogenic

fungi containing a sterol, ergosterol (Ghannoum and Rice, 1999). Amphotericin B exerts its activity by binding to sterols, preferentially to the primary fungal cell membrane sterol, ergosterol. This binding disrupts osmotic integrity of the fungal membrane, resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites and then cellular death (Kerridge, 1985; Abuhammour and Habte-Gabe, 2004). At low levels, amphotericin B has immunostimulatory effect (Yamagaruchi et al., 1993)

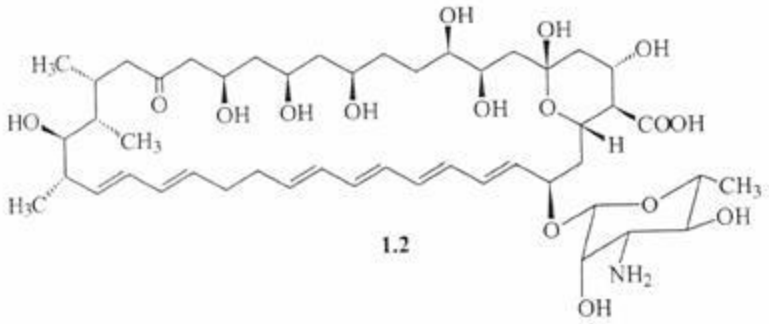
On occasion, however, isolates of any species may be found to be resistant. Among the *Candida* species, isolates of *C. albicans*, *C. guillermondii*, *C. lipolytica*, *C. lusitaniae*, *C. norvegensis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* have been reported to be relatively resistant to amphotericin B (Meyer, 1992; Terrell and Hughes, 1992; Karyotakis et al., 1993; Karyotakis, and Anaissie, 1994). Reduced susceptibility has been observed specifically at fungicidal levels for *C. parapsilosis* (Seidenfeld et al, 1983).

Despite the broad spectrum of fungicidal activity, amphotericin B has a narrow therapeutic index, limiting its clinical utility (Gallis et al., 1990). It weakly binds to the cholesterol in mammalian membranes, and this interaction may explain its toxicity. All patients have adverse reactions to amphotericin B, though this depends on the type of preparation; a lipid preparation is associated with fewer side effects. Nephrotoxicity is the major adverse effect with the following manifestations, azotemia, decreased glomerular filtration and loss of urinary concentrating ability. Others are renal loss of sodium, potassium and renal tubular acidosis (Meyer, 1992; Jawetz et al., 2004). The renal injury reduces erythropoietin production and leads to a normochromic normocytic anemia (Lin et al., 1990).

Other common side effects include fevers, nausea, vomiting and headache; also, thrombocytopenia, generalized seizure, shock and hyperbilirubinemia, are reported infrequently in patients using ampotericin B (Ghannoum, 2001; Olin and Spooner, 2006). In some patients, including children, amphotericin overdose has been found to cause arrhythmia, cardiac arrest or even death (Koren et al., 1990, Burke et al., 2006).

1.1.6.1.2. Nystatin

Nystatin **(1.2)** is the first polyene antibiotic produced by various strains of *Streptomyces noursei* and discovered in 1949 by Hazen and Brown (Rippon, 1982), it is structurally related to amphotericin B and having a similar mode of action. It can be used to treat local candidal infections (Ghannoum, 2001) of the mouth and vagina. Nystatin may also suppress subclinical esophageal candidiasis and gastrointestinal overgrowth of *Candida*. No systemic absorption occurs, and there are no side effects. However, nystatin is too toxic for parenteral administration.

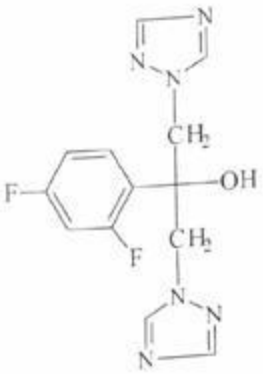


1.2

1.1.6.2 Azoles

The azoles have in common an imidazole or triazole ring with N-C substitution. The ring is responsible for the interaction of these drugs with certain target sites in the fungal cells. These are of two types, firstly, the oral drugs which are used to treat a wide range of systemic and localized fungal infections including; imidazole (ketoconazole), triazoles (fluconazole **(1.3)**, itraconazole **(1.4)**, voriconazole) and new azoles in development (posaconazole, and ravuconazole). Secondly, the other imidazoles, which include miconazole and clotrimazole, are used topically. The azoles interfere with sterol synthesis and compromise fungal cell membrane integrity. Integrity of the cell membrane requires that inserted sterols lack C-14 methyl groups (Ghannoum and Rice, 1999). They act by blocking the cytochrome P450-dependent 14- demethylation of lanosterol, which is a precursor of ergosterol in fungi and cholesterol in mammalian cells (Abuhammour and Habte-Gabe, 2004; Jawetz et al., 2004). However, the fungal cytochrome P450s, are

more sensitive to the azoles than the mammalian systems (Hitchcock et al., 1990; Jawetz et al., 2004). Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including α-14 methylated sterols (lanosterol, 4,14-dimethyl zymosterol and 24-methylenedihydrolanosterol), resulting in the formation of a cell membrane with altered structure and functions (Hitchcock et al., 1990).



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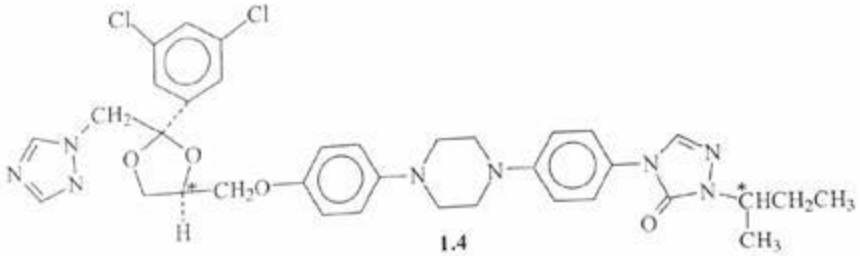

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Azoles have a broad spectrum of activity against several dermatophytes, *Candida*, *Cryptococcus* and other fungi that cause deep-seated infections. They are used against recurrent vaginal candidiasis, chronic infections of the skin, hair and nails caused by sensitive dermatophytes and yeasts, unresponsive to topical therapy; oropharyngeal candidiasis not responsive to local therapy; esophageal candidiasis; non-life-threatening,

non-meningeal coccidioidomycosis, paracoccidioidomycosis, blastomycosis and histoplasmosis (Terrel, 1999).

The adverse effects of azoles are related to their ability to inhibit mammalian cytochrome P450 enzymes. Ketoconazole is the most toxic and therapeutic doses may inhibit the synthesis of testeosterone and cortisol, which may cause a variety of reversible effects such as gynecomastia, decreased libido, impotence, menstrual irregularity and occasionally adrenal insufficiency (Ghannoum and Rice, 1999). Fluconazole and itraconazole at the recommended therapeutic doses do not cause significant impairment of mammalian steroidogenesis. All the antifungal azoles can cause both asymptomatic elevation of liver function tests and rare cases of hepatitis, itraconazole being the most hepatotoxic (Girois et al., 2006).

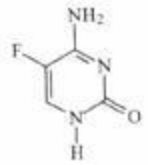
Induction and inhibition of cytochrome P450 enzymes at hepatic and extrahepatic sites are the mechanisms that underlie the most serious pharmacokinetic drug interactions of the azole antifungals. The azoles have been shown to notably decrease the catabolism of numerous drugs including histamine H-1 receptor antagonists, warfarin, cyclosporine, digoxin, felodipine, lovastatin, midazolam, triazolam, methylprednisolone, glibenclamide, phenytoin, rifabutin, ritonavir, saquinavir, nevirapine and *nor*triptylline (Albengres et al., 1998; Jawetz et al., 2004). Non-antifungal drugs like carbamazepine, phenobarbitone, phenytoin and rifampicin can induce the metabolism of azole antifungals (Albengres et al., 1998). The bioavailability of ketoconazole and itraconazole is also reduced by drugs that increase gastric pH, such as H_2 receptor antagonists, proton pump inhibitors, sucralfate and didanosine (Albengres et al., 1998). Serum monitoring of the co-administered drugs may be necessary to achieve a proper therapeutic dosage (Jawetz et al., 2004).

Frequent use of fluconazole has led to increasing reports of treatment failure due to resistance of *C. albicans* notably in oropharyngeal candidiasis in patients with AIDS (Rex et al., 1995; Hoang, 2001; Weig and Müller, 2001). Prolonged fluconazole prophylaxis in AIDS patients has also been found to result in reduced susceptibility to fluconazole and cross-resistance to itraconazole (Goldman et al., 2000). There has been a

noticeable shift toward non-*albicans* species with relative resistance to fluconazole and itraconazole. Some of the other *Candida* species resistant to fluconazole include; *C. glabrata, C. krusei* and *C. tropicalis* (Cuenca-Estrella et al., 2000). Magill et al. (2006) reported a case study of invasive candidiasis and candidemia due to a *C. glabrata* isolate that developed resistance to all currently available triazole antifungals after a course of fluconazole treatment. This is a cross-resistance among azoles, which was also reported by Müller et al., (2000).

1.1.6.3 Pyrimidine analogs

Pyrimidine analogs interfere with pyrimidine metabolism, DNA, RNA and protein synthesis in fungal cells (Ghannoum, 2001; Abuhammour and Habte-Gabe, 2004). Flucytosine (5- fluorocytosine) **(1.5)** is the only member of this class, which was approved for use in humans in the 1970s (Ghannoum, 2001). It is a synthetic fluorinated derivative of cytosine. It is an oral compound used in combination with amphotericin B with which it acts synergistically. Monotherapy with 5- fluorocytosine is limited because of the frequent development of resistance (Rapp, 2004). The combination with amphotericin B delays the emergence of flucytosine resistant mutants.



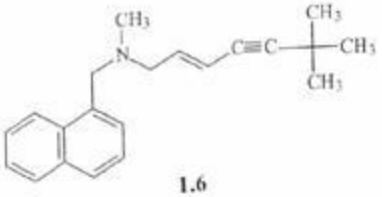
1.5

It is used to treat candidiasis and cryptococosis. It is effective against many dematiaceous fungi infections. It penetrates well in all tissues including cerebrospinal fluid. It is actively transported into fungal cells by a permease. It is converted by the fungal enzyme cytosine deaminase to 5-fluorouracil (5-FU) and incorporated into 5-fluorodeoxyuridine monophosphate a potent inhibitor of thymidylate synhetase, an enzyme involved in DNA synthesis and nuclear division (Diasio et al., 1978). Also, 5-FU is converted by uridine monophosphate pyrophosphorylase into 5-fluorouridylic acid, which is phosphorylated further and incorporated in RNA, resulting in disruption of protein synthesis.

Mammalian cells lack cytosine deaminase and are therefore protected from the toxic effects of fluorouracil. The drug itself has very few side effects including nausea, vomiting and anorexia (Ghannoum, 2001), but conversion into fluorouracil results into a highly toxic compound probably responsible for the seen side effects, which include bone marrow suppression, hair loss and abnormal liver function. If conversion, occurs due to enteric bacteria then this could result into colitis. HIV and AIDS patients are more susceptible to bone marrow suppression by flucytosine (Jawetz et al., 2004).

1.1.6.4 Allylamines

The class is represented by terbinafine **(1.6)**, a drug having a fungicidal action against many fungi as a result of its specific mechanism of squalene epoxidase inhibition (Ryder, 1992; Jawetz et al., 2004). Treated fungi accumulate squalene while becoming deficient in ergosterol, an essential component of fungal cell membranes. The cidal action is closely associated with the development of high intracellular squalene concentrations, which are believed to interfere with fungal membrane function and cell wall synthesis (Darkes et al., 2003). In the case of *C. albicans*, growth inhibition with terbinafine appears to result from the ergosterol deficiency. The filamentous form of this fungus is more susceptible than the yeast form.



1.6

Terbinafine is given orally to treat dermatophyte infections. It is quite effective in treating nails and other dermatophyte infections (Darkes et al., 2003). It has been found to have a good activity against azole resistant *C. albicans* strains (Ghannoum and Rice, 1999) and has also demonstrated *in vivo* synergism with voriconazole on azole resistant *C. albicans* isolated from HIV patients (Weig and Müller, 2001). Side effects are not very common. The few observed include gastrointestinal distress, headaches, skin

reactions and loss of sense of taste (Gupta and Shear, 2000; Jawetz et al., 2004; Doty and Haxel, 2005).

1.1.6.5 Echinocandins

Echinocandins are lipopeptides and a relatively new class of antifungal agents targeting the fungal cell wall. The principal mechanism of action of the echinocandins is the noncompetitive inhibition of 1, 3-ß-D- glucan synthetase, an essential component of the cell wall of many fungi that is not present in mammalian cells (Morris and Villmann, 2006). The inhibition of glucan synthesis leads to increased cell wall permeability and lysis of the cell. Echinocandins exhibit fungicidal activity against *Candida* species, including triazole-resistant isolates, and fungistatic activity against *Aspergillus* species (Morris and Villmann, 2006). The class includes caspofungin, anidulafungin and micafungin, which have been approved by the U.S. Food and Drug Administration for the treatment of patients with candidemia, peritonitis, intra-abdominal abscesses, esophageal candidiasis and other forms of invasive candidiasis (Cohen-Wolkowiez et al., 2006; Messer et al., 2006; Dela la Torre and Reboli, 2007). Aminocandin is another echninocandin in development (Ghannoum et al., 2007).

1.1.6.5.1 Caspofungin

Caspofungin (**1.7**) is the first representative of echinocandins (Letscher-Bru and Herbrecht, 2003). It has activity against *Candida* (including azole resistant strains), *Aspergillus*, and certain dimorphic fungi, such as *Histoplasma*, *Blastomyces* and *Coccidiodes* (Petraitiene et al., 2002; Deresinski and Stevens, 2003; Letscher-Bru and Herbrecht, 2003; Magiorakos and Hadley, 2004). It also possesses activity against *Pneumocystis carinii* (Letscher-Bru and Herbrecht, 2003).

Caspofungin is recommended for the treatment of esophageal candidiasis, candidemia and other serious invasive *Candida* infections (Arathoon et al., 2002; Mora-Duarte et al., 2002; Magiorakos and Hadley, 2004). It shows additive or synergistic activity with amphotericin B and triazoles. In oropharyngeal and esophageal candidiasis it shows efficacy similar to amphotericin B and fluconazole and in invasive candidiasis with

homology among the genes and proteins (Jawetz et al., 2004). This causes a big problem in drug discovery in this area. The fungal cell wall may be considered to be a prime target for selective toxicity of antifungal agents because of its chitin structure, absent in human cells. It is only recently that echinocandins, a class of antifungal agents, acting on fungal cell wall have been approved for use (Letscher-Bru and Herbrecht, 2003). Secondly most of these compounds are not very stable, are poorly absorbed and very toxic, hence, have limited application. Nausea and vomiting associated with some anti-fungal agents especially nystatin, results into poor patient compliance.

The third problem is the relapse of *Candida* infections and resistance of *Candida* species to commonly used anti-fungal agents. When relapses occur, the infections tend to be increasingly recalcitrant to treatment (Sangeozrzan et al., 1994). To minimize the incidence of relapses in HIV and AIDS patients prophylaxis with oral azoles was reported to be effective (Reents et al., 1993; Goldman et al., 2000). Fluconazole was found to be a better choice for the treatment of relapsing oropharyngeal candidiasis in HIV and AIDS patients, resulting in either better cure rates or better prevention of relapse (Albougy and Naidoo, 2002).

Another serious problem facing the management of *Candida* infections is the fact that, the increased use of antifungal agents has resulted in the development of resistance to these drugs. There are reports of resistant strains to all classes of antifungal agents in use including echinocandins, the new antifungal agents. Much, worse, there is cross-resistance of fungal species to antifungal drugs and also cross-resistance among the antifungal drugs. The cross-resistance of fungal species to antifungal drugs must be considered as a potential problem to future antifungal treatment, and thus, the determination of susceptibility of fungal species to antifungal agents is an important component of information in development of new antifungal agents (Gupta and Tomas, 2003). Different mechanisms contribute to the fungal resistance to antifungal agents. These mechanisms include modification of a gene at the molecular level (gene mutation, conversion and overexpression), over expression of specific drug efflux pumps, alteration in sterol biosynthesis, and reduction in the intracellular concentration of target enzymes (Balkis et al., 2002).

The resistance of *Candida* species to the available anti-fungal agents necessitates the use of higher profile anti-fungal agents, which may be, both, more toxic and expensive than the available antifungals. Resistance, also, reduces the number of effective drugs available.

The fourth problem is associated with the patient's adherence to the treatment is the ever-increasing prices of drugs, especially in developing countries. Besides the price of the drug, the cost of antifungal therapy includes costs of mortality associated with failed treatment, prolonged hospitalization and treatment related to complications, and additional antifungal treatment to compensate for primary treatment failure. For a common Tanzanian this burden is unbearable.

This study aims at obtaining antifungal agents from plants so as to widen the spectrum of activities against *Candida* and combat strains expressing resistance to the available anti-fungal agents.

1.3 RATIONALE OF THE STUDY

In Tanzania there are several plant species, which are used traditionally in the treatment of various infections including candidiasis. For instance, in Bukoba, *Rauwolfia vomitoria* known as "omunyabusindi" in the Haya dialect is used in ringworm infection (Mutayabarwa, 2003). The root bark of the plant is grounded and suspended in cooking oil for five days after which the supernatant oil is applied to the affected area. Another plant *Vitex floribunda* from the same region is also employed in treating vaginal and oral candidiasis. The leaves of the plant, *Myrothamnus flabellifolia* from Songea are used in treating both oral and vaginal candidiasis. Also, earlier studies (Sawhney et al., 1978b; Khan et al., 2000) have shown the presence of anti-fungal activity in various Tanzanian plants, suggesting that the search for anti-fungals of plant origin should continue to explore their potential.

1.4 OBJECTIVES OF THE STUDY

1.4.1 Broad Objective

To obtain anticandida compounds from medicinal plants used to manage *Candida* infections in Tanzania.

1.4.2 Specific Objectives

1.4.2.1 To screen plants growing in Tanzania for anticandida activity

1.4.2.2 To isolate the compounds active against *Candida.*

1.4.2.3 To elucidate the structures of the isolated compounds.

1.4.2.4 To determine the minimum inhibitory concentrations of the active compounds

CHAPTER 2

SCREENING OF TANZANIAN PLANTS FOR ANTICANDIDA ACTIVITY

2.1 INTRODUCTION

In Africa and many other developing countries traditional medicine still forms the backbone of the primary health care (Khan and Nkunya, 1991). A large number of people in these countries depend on medicinal plants because they have no access to modern medicine (Elmi, 1991). African traditional medicine serves about 80% of the population (Elujoba et al., 2005). In Tanzania over 60% of the health care seeking population have a traditional healer as their first contact and majority of the people depend on traditional medicines for their primary health care. It is estimated that there are about 75,000 traditional healers in Tanzania with an average ratio of one traditional healer per 400 persons (Mhame, 2000). According to Tanzania service availability mapping 2005-2006 of the Ministry of Health and Social Welfare, in Tanzania the average number of persons served by one doctor was 25,000. . The corresponding number of persons per pharmacist was 125,000. The World Health Organization has since urged developing countries to utilize the resource of traditional medicine for achieving the goals of health care. This is due to various advantages of traditional medicine namely, affordability, ready availability, accessibility, acceptability and perhaps low toxicity (Elmi, 1991; Elujoba et al., 2005). Traditional medicine in Africa has demonstrated its contribution to reduction of excessive mortality, morbidity and disability due to diseases such as malaria, tuberculosis, sickle cell anaemia, diabetes and mental disorders (Elujoba et al., 2005).

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. The history of medicine dates back particularly to the existence of human civilization. The current modern medicine has its roots in traditional medicine and therapies. Nevertheless, the indigenous knowledge will remain as an important source of future medicine and therapies.

Plants, for thousands of years, were originally instrumental to early pharmaceutical drug discovery. The history of drug discovery or even drug chemistry is largely bound to the

plant kingdom. Numerous drugs have entered the international pharmacopoeia through the study of ethnopharmacology and traditional medicine. Plant based, life saving drugs, such as quinine, reserpine, digoxin, aspirin, narcotic analgesics, ergometrine, pilocarpine and tubocurarine were introduced in medicine several centuries ago. Therefore the process of deriving drugs from plant sources is certainly not new.

An analysis of the origin of drugs developed between 1981 and 2002 showed that natural product derived drugs comprised 28% of all new chemical entities launched into the market (Newman et al., 2003). Also, 24% of the new chemical entities were synthetic or semi-synthetic based on natural products. This suggests that natural products are an important source of potentially useful new compounds for development of chemotherapeutic agents. The first reason for this is the fact that each organism including plants is a unique chemical reservoir, capable of synthesising large numbers of highly complex and unusual chemical compounds. The second reason is that secondary metabolites from natural sources have been developed within living systems, they are often perceived as showing more drug-likeness and biological friendliness (Koehn and Carter, 2005), making them good candidates for further development of drugs (Balunas and Kinghorn, 2005).

Despite the expense involved in development of a drug, and a life span of 10-20 years (Farnsworth, 1984), nature remains the most reliable and the most important source of novel drug molecules. The first step towards this goal is the screening of plants used in traditional medicine. In this approach, local knowledge about potential use of plants is very useful as compared to the random approach where the indigenous knowledge is not taken into consideration.

In Chapter 1 it was emphasised that increased spread of HIV and AIDS has increased the occurrence of opportunistic infections including fungal infections and also that the number of effective antifungal agents is limited. In addition, a number of negative aspects associated with the available antifungal therapies including toxicity, drug interactions, resistance, relapse and high cost were discussed. In view of these factors it is clear that there is a need to look for effective anticandida agents from plants. Tanzania

is a tropical country with good environmental conditions to support growth of varied plant species. Being blessed with a rich biodiversity and natural forests containing 10,000 species (Mahunnah and Mshiu, 1991) the possibility of getting active principles from plants is immense.

2.2 PLANTS AS SOURCE OF ANTICANDIDA COMPOUNDS

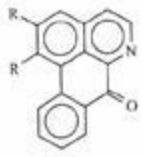
Many plant species from various plant families, obtained from different parts of the world have been screened and found to have activity against a number of human pathogenic fungi including *Candida* species. These plants are so numerous that they cannot be exhaustively discussed here and there are many more to be screened. The following are examples of plants, which were screened and found active against *Candida* species. Screening for anti-fungal activity of the medicinal plants used by the natives of British Columbia (Canada) revealed that 23 methanol extracts were active against *C. albicans* (McCutcheon et al., 1994). Non-polar and semi-polar extracts prepared from the leaves and stems of South African plants of the Asteraceae family that included *Eriocephalus africanus*, *Fellicia erigeroides* and *Helichrysum crispum* were reported to be active against *C. albicans* (Salie et al., 1996).

Several plants including *Alangium salvifolium* (Alangianaceae), *Acacia catechu* (Fabaceae), *Eclipta alba* (Asteraceae), *Plumbago indica* (Plumbaginaceae), *Tachyspermum ammi* (Apiaceae) and *Terminalia bellerica* (Combretaceae) from India were reported to have activity against *C. albicans* (Valsaraj et al., 1997). Among fifty Malaysian plants screened for antimicrobial activity 21 were found to be active against *C. albicans* (Wiart et al., 2004). The plants included *Eleusine indica* (Poaceae), *Neonauclea pallida* and *Hedyotis congesta* (Rubiaceae), *Solanum torvum* (Solanaceae), *Peristrophe tinctoria* (Acanthaceae), *Celosia argentea* (Amaranthaceae) and *Polyalthia lateriflora* (Annonaceaae). Others were *Ancistrocladus tectorius* (Ancistrocladaceae), *Trevesia burkii* (Araliaceae), *Apama corymbosa*, *A. tomentosa* (Aristolachiaceae), *Eclipta prostrata* (Asteraceae) and *Commelina communis* (Commelinaceae). Also included were *Euphorbia hirta* (Euphorbiaceae), *Cinnamomum iners* (Lauraceae), *Knema malayana* (Myristicaceae), *Peperomia pellucida*, *Piper porphyrophyllum*, and *P.*

xylosum (Piperaceae). Brazilian Plants used in traditional medicine including *Schinus terebinthifolius* (Anacardiaceae), *Ocimum gratissimum* (Lamiaceae), *Rosmarinus officinalis* (Lamiaceae), *Cajanus cajan* (Fabaceae), *Eugenia uniflora* (Myrtaceae), *Piper aduncum* (Piperaceae), *Plantango lanceolata* (Plantaginaceae) and *Solanum americanum* (Solanaceae) were reported active against *C. albicans* (Braga et al., 2007).

In many other studies the active compounds have been isolated and identified. Liriodenine **(2.1)**, an alkaloid isolated from *Liriodendron tulipifera* (Magnoliaceae), was found to have a four-fold lower, minimum inhibitory concentration, for several of the tested fungi, when compared to griseofulvin, a standard antifungal drug (Hufford et al., 1980). Isomeric compounds, lapachol **(2.2)** and β-lapachone **(2.3)**, isolated from the heartwood, of a tropical tree *Tabebuia avellanedae* (Bignoniaceae) were found to be active against a number of microbes, including *C. albicans* (Guiraud et al., 1994). Phenyl propanoid compounds **(2.4** and **2.5)** isolated from the root bark of *Cordia alliodora* (Boraginaceae) showed activity against *C. albicans* and a phytopathogenic fungus (Ioset et al., 2000). Hydroxyanthracenones **(2.6-2.9)** isolated from plants of the genus *Karwinskia* (Rhamnaceae) were reported to have antimicrobial activity against various bacteria and fungi including *C. albicans, C. boidinii* and *C. glabrata* (Salazar et al., 2006).

Besides being active, some of the compounds isolated from plants potentiated the activity of available antifungal agents. For instance, Antimicrobial screening of the plants *Warbugia ugandesis* and *W. stuhlmannii* (Canellaceae) used by East African traditional healers enabled isolation of polygodial **(2.10)**, which inhibited the growth of *C. utilis* at a concentration of 1.56 μg/ml and increased the potency of Actinomycin D, a drug proved to be devoid of activity against *C. utilis* when used alone (Kubo and Taniguchi, 1988). Also, nyasol **(2.11)**, an anti-fungal agent isolated from *Anemarrhena asphodeloides* (Liliaceae) showed synergistic effects with several azoles, against *C. albicans* (Lida et al., 2000).



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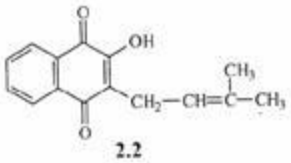

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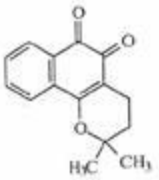

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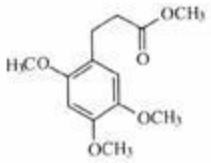

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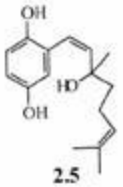

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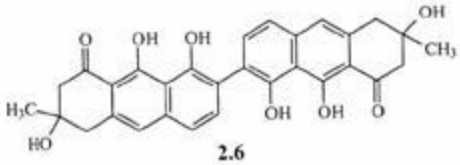

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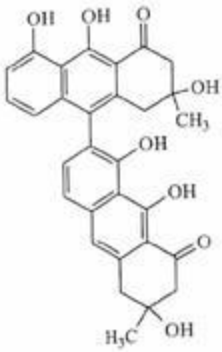

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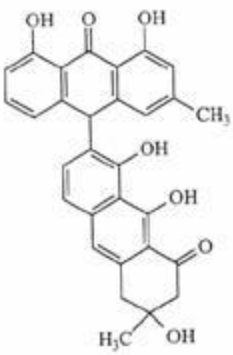

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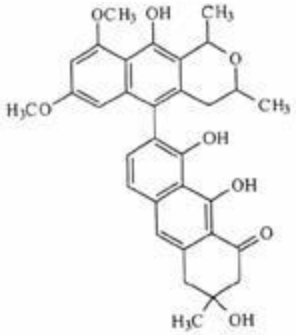

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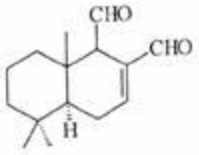
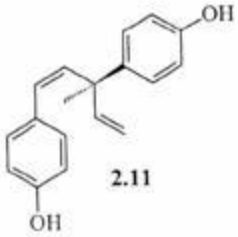
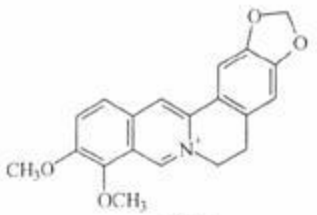


2.10

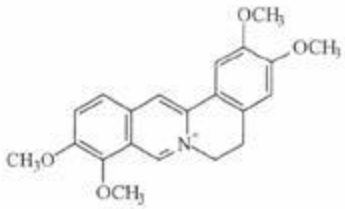


2.11

In other studies attempts have been made to determine the mechanism of action of plant based antifungal compounds. This is illustrated by the protoberberine alkaloids, berberine **(2.12)** and palmatine **(2.13)** isolated from Korean plants, *Coptis rhizoma* (Ranunculaceae) and *Phellodendron amurense* (Rutaceae), which exhibited potent growth inhibition on *C. albicans, C. glabrata, C. krusei* and *C. parapsilosis* (Park et al., 1999). Both compounds were shown to inhibit sterol synthesis in *C. albicans*, however palmatine also inhibited chitin synthetase (Park et al., 1999).



2.12

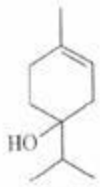


2.13

Essential oils are a class of plant products, which have been extensively investigated for their antimicrobial activity, in most cases revealing promising results. The essential oil of *Cinnamomum cassia* (Lauraceae) showed anticandida activity at a concentration of 0.169 µl/mL and it also potentiated the anticandida activity of amphotericin B by reducing the MIC of amphotericin B by 70% at a concentration of 0.1 microL/mL (Giordani et al., 2006). The oil from the rhizomes of *Zingiber nimmonii* (Zingiberaceae) from South India showed significant inhibitory activity against the fungi, *C. glabrata, C. albicans* and *Aspergillus niger* (Sabulal et al., 2006). Similarly, the essential oils obtained from Tanzanian *Satureja* species *S. biflora, S. masukensis* and *S. pseudosimensis* (Lamiaceae), also, *Plectranthus laxiflorus* (Lamiaceae) and *Vernonia smithiana* (Asteraceae) were found to be active against *C. albicans, C. tropicalis* and *C. glabrata* (Vagionas et al., 2007a; b).

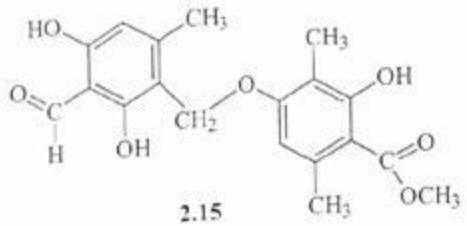
Some essential oil components have been isolated and evaluated for their anticandida activity. For example terpinen-4-ol **(2.14)** isolated from *Melaleuca alternifolia* (Myrtaceae) was found to have *in vivo* activity on vaginal candidiasis particularly the

azole-resistant forms (Mondello et al., 2006). Also, an attempt has been made, to determine the mechanisms of action for the low molecular weight, essential oils components. It was observed that the main specific targets of these components were the ergosterol pathway, respiratory chain, and chitin biosynthesis (Pauli, 2006).



2.14

In addition to human pathogens, studies have also involved plant pathogenic fungi; for instance, a phenolic compound **(2.15)** isolated from the stem bark of *Gordonia dassayakei* (Theaceae) was said to be responsible for the observed activity against plant pathogenic fungi *Curvularia* species, *Colletotricum glaeosporiodes*, *Rhizoctonia solani*, *Corynespora cassicola* and *Fusarium* species (Athukoralage et al., 2001).



2.15

Earlier studies on plants growing in Tanzania (Sawhney et al., 1978b; Rahalison et al., 1991, Khan et al., 2000, de Boer et al., 2005; Hamza et al., 2006, Vagionas et al., 2007a; b) had shown the presence of anticandida activity in various plants (Table 2:1), suggesting that the search for anticandida agents from plants growing in Tanzania should continue to explore their potential.

Table 2:1 Some Tanzanian plants reported to have anticaandida activity

Species	Family	Plant part *	References
Acacia nilotica	Fabaceae	S	Hamza et al., 2006
Acacia robusta subsp *usambarensis*	Fabaceae	L	Hamza et al., 2006
Agauria salicifolia	Ericaceae	L	Hamza et al., 2006
Asparagus falcatus	Liliaceae	L	Sawhney et al., 1978b
Bonamia mossambicensis	Convolvulaceae	R	Sawhney et al., 1978b
Boscia salicifolia	Capparidaceae	B	Sawhney et al., 1978b
Bridelia cathartica	Euphorbiaceae	S	Sawhney et al., 1978b
Cassia auriculata	Fabaceae	B	Sawhney et al., 1978b
Ceiba pentandra	Bombacaceae	L	Sawhney et al., 1978b
Cineraria grandiflora	Asteraceae	S, L	De Boer et al., 2005
Clausena anisata	Rutaceae	Sb	Hamza et al., 2006
Clutia abyssinica	Euphorbiaceae	R	De Boer et al., 2005
Combretum molle	Combretaceae	R	Khan et al., 2000
Combretum zeyheri	Combretaceae	Wp	Sawhney et al., 1978b
Coccinia adoensis	Cucurbitaceae	R	De Boer et al., 2005
Cyphostemma hildebrandtii	Vitaceae	L	Hamza et al., 2006
Deinbollia borbonica	Sapindaceae	R	Sawhney et al., 1978b
Dictyophleba lucida	Apocynaceae	L, T	Sawhney et al., 1978b
Drymaria cordata	Caryophyllaceae	Wp	Hamza et al., 2006
Elaeodendron buchannanii	Celastraceae	Sb	Hamza et al., 2006
Elaeodendron schlechteranum	Celastraceae	Sb	Hamza et al., 2006
Emilia sagittata	Asteraceae	Wp	Sawhney et al., 1978b
Eriosema psoraleoides	Fabaceae	B	Khan et al., 2000
Euphorbia heterophylla	Euphorbiaceae	Wp	Hamza et al., 2006
Harrisonia abyssinica	Simaroubaceae	Rb, Tw	Sawhney et al., 1978b
Hibiscus micranthus	Malvaceae	Wp	Sawhney et al., 1978b
Holarrhena febrifuga	Apocynaceae	L	Sawhney et al., 1978b
Hymenodictyon parvifolium	Rubiaceae	R	Hamza et al., 2006
Jatropha multifida	Euphorbiaceae	S	Hamza et al., 2006
Parvonia urens	Malvaceae	R	De Boer et al., 2005
Phyllanthus reticulatus	Euphorbiaceae	Wp	Sawhney et al., 1978b
Plectranthus laxiflorus	Lamiaceae	Ap	Vagionas et al., 2007b
Pseudolachnostylis maprouncaefolia	Euphorbiaceae	B	Sawhney et al., 1978b
Pteridium aquilinum	Dennsstraediaceae	L	Hamza et al., 2006
Rapanea melanophloeus	Myrsinaceae	Sb	Hamza et al., 2006
Rhoicissus tridentata	Vitaceae	Tu	Hamza et al., 2006
Satureja biflora	Lamiaceae	L, Fl	Vagionas et al., 2007a
Satureja mastukensis	Lamiaceae	L, Fl	Vagionas et al., 2007a
Satureja pseudosimensis	Lamiaceae	L, Fl	Vagionas et al., 2007a
Sclerocarya birrea	Anacardiaceae	R	Hamza et al., 2006
Securinega virosa	Euphorbiaceae	P	Sawhney et al., 1978b
Senecio deltoidea	Asteraceae	Wp	Hamza et al., 2006
Sida serratifolia	Malvaceae	L	Sawhney et al., 1978b
Solanum incanum	Solanaceae	L	Hamza et al., 2006
Spirostachys africana	Euphorbiaceae	S	Hamza et al., 2006

Sterculia africana	Sterculiaceae	L	Hamza et al., 2006
Swartzia madagascariensis	Fabaceae	R	Rahalison et al., 1991
Tagetes minuta	Asteraceae	L	Hamza et al., 2006
Turraea holstii	Meliaceae	L	Hamza et al., 2006
Vangueria infausta	Rubiaceae	R, F	De Boer et al., 2005
Vernonia smithiana	Asteraceae	Ap	Vagionas et al., 2007b
Vitex fischeri	Vitaceae	L	Sawhney et al., 1978b
Xeroderris stuhlmannii.	Fabaceae	Wp	Sawhney et al., 1978b
Zanthoxylum chalybeum	Rutaceae	Rb	Sawhney et al., 1978b
Ziziphus pubescens	Rhamnaceae	L	Sawhney et al., 1978b

*Ap: aerial parts; B: bark; F: fruits; Fl: flowers; L: leaves; P: pulp; R: roots; Rb:root bark; S: stem; Sb: stem bark; T: trunk; Tu: tuber; Tw: twig; Wp: whole plant

This study was intended to collect some plants used by traditional healers in treating *Candida* infections in four regions of Tanzania and also those plants reported in the literature to have anticandida activity, screen them for anticandida activity before proceeding with isolation of the compounds responsible for activity.

2.3 METHODOLOGY

2.3.1 Plant collection and identification

Plants were collected from Coast, Dar es Salaam, Morogoro and Tanga regions of Tanzania (Figure 2:1) and were identified in the field by a botanist. These regions were chosen due to convenience, particularly proximity to the laboratories where the extraction process and screening was to be carried out.

Twenty two traditional healers, including fourteen men and eight women, were identified through information given by leaders in visited villages in the four regions These were interviewed on plants they used to treat *Candida* infections. Symptoms of the various forms of *Candida* infections were described to the traditional healers so as to enable them give the appropriate plants they used in the management of these conditions.

Figure 2:1 Map of Tanzania showing Regions where plants were collected

The symptoms, which have been described in the literature, (Laskaris et al., 1992) included oral thrush, mouth ulcers and lesions of epithelial cells of the lips, erythematous lesion on the dorsum of the tongue and angular chellitis. Oral thrush, which is the commonest form of oral candidiasis in Tanzania (Matee et al., 2000) is known as "*Utando wa mdomoni*" in the Swahili language. Symptoms for esophageal candidiasis are painful swallowing, a feeling of obstruction on swallowing, substantial chest pain and discrete ulceration of the esophagus (Connolly et al., 1989) and that for vulvovaginal candidiasis is a cuddle milk discharge (Namkinga et al., 2005) known as "*Maziwa ya mgando*" in Swahili. Traditional healers were asked to provide or show the plants they used, give the vernacular names, the part of the plant used and how they prepared the medicine and the mode of administration. All the information gathered was recorded in an ethnobotany form (Appendix 1). During the interview the traditional healers were also required to sign and agree to the terms given in the agreement form (Appendix 2 and Appendix 3). This form, in short, explained the importance of the information they were providing and the research to be done on the plants provided; it, also, informed them that results and any profitable outcome will be communicated to them. They were, however, asked not to use the name of the researcher or the institution where the researcher came

from, in advertising their business. The agreement form was for the purpose of safeguarding the interests of both parties. A monetary honorarium of 10,000 Tanzanian shillings was given for each herbal drug and information obtained from the traditional healers. Only the plant parts used by the traditional healers were collected.

Additional plants collected were those reported in the literature (Sawhney et al., 1978b; Khan et al., 2000) as active against *Candida albicans*. Plants in this category were collected so that the compounds responsible for the activity could be isolated and have their MICs determined. In this category of plants several plant parts were collected in addition to those previously studied as indicated in the literature.

Also, two, additional plants, *Vitex fischeri* and *Elaeodendron buchananii*, were obtained as a courtesy of Dr. Z. Mbwambo of Institute of Traditional Medicine (ITM), MUHAS. These plants were collected from Kagera Region and they were included in the study because they were used to treat bacterial and fungi infections in this region.

For each plant collected a herbarium specimen was also prepared and deposited at the herbarium, Department of Botany, College of Natural and Applied of Sciences, University of Dar es Salaam (UDSM). Photographs of the plants were taken using a digital camera.

2.3.2 Drying and processing of the plant materials

The collected plant parts were sorted and unwanted materials removed before drying. The adhering soil was removed from the roots by using water and larger plant parts were chopped into smaller pieces to facilitate the drying process. The drying was done outdoors; however, delicate plant parts, such as leaves, were dried in the shade. The plant materials were turned occasionally during drying so as to enable uniform drying. Depending on the plant part the plant materials were dried for three to seven days.

2.3.3 Particle size reduction

A Fitz mill Type 6 (Manesty machines Ltd. Liverpool England) was used to grind the plant materials so as to facilitate the extraction process. The extent of fineness of the ground plant materials depended on their botanical structures. It ranged from a bruised state for leaves, to a coarse powder state for hard structures like stems and roots. The ground plant materials were stored in polythene containers until required for extraction.

2.3.4 Extraction of the plant materials

Ground plant materials ranging from 100 to 200 g were macerated with 80% methanol, shaking the flasks occasionally in order to facilitate the extraction process. The amount of plant materials used varied depending on both their availability and bulkiness and the amount of solvent used varied depending on the nature and amount of the plant materials. In each case the amount of solvent used was just enough to cover the plant materials. Maceration was done for two days at room temperature. The procedure was repeated three times so as to increase the yield of the extracts. Separation of the extracts from the marc was done by decantation followed by filtration using filter funnels fitted with Whatman No. 1 filter papers (Whatman International Limited, Maidstone, England). The weight of plant materials extracted and amount of solvent used for each extraction, and for the various plant materials were recorded. The extracts were pooled together and concentrated using a Buchi rotary evaporator set at 40-50°C to get rid of methanol. The resultant aqueous extracts were freeze-dried using Edwards freeze drier (Edwards High Vacuum International Crawley, Sussex, England). The dried extracts were kept in a deep freezer (-20°C) until required for screening for anticandida activity.

2.3.5 Screening of the extracts for anticandida activity

The extracts were screened for anticandida activity using bioautography agar overlay (Rahalison et al., 1991)..

2.3.5.1 Preparation of the samples to be screened

For each extract 1 g was weighed and dissolved in 5 ml of methanol to produce a solution with a concentration of 200 µg/µl. Amphotericin B (Calbiochem, USA and Canada), (0.002 µg/µl) was used as the standard anti-fungal agent

2.3.5.2 Preparation of *Candida albicans* inoculum

Sabouraud dextrose agar (SDA; Biotec Laboratories Ltd. UK) was used to prepare the culture medium according to the manufacturer's directions. *Candida albicans* ATCC 90028 (kindly provided by Prof. Paul Verweij, Department of Medical Microbiology, University Medical Center, St. Radboud Hospital, Nijmegen, The Nertherlands) was aseptically inoculated on petridishes containing autoclaved cooled and settled medium. The petridishes were incubated at 31 °C for 48 h to give white round colonies against a yellowish background (Figure 2:2). These were aseptically sub-cultured on SDA slants.

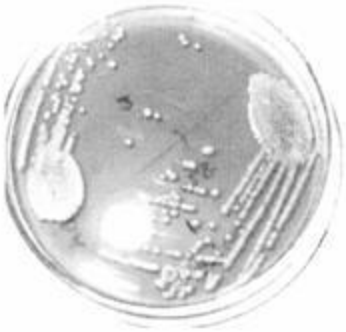


Figure 2:2 Culture of *Candida albicans* ATCC 90028

Candida albicans colonies from SDA slants were suspended in sterilised 0.9% sodium chloride solution (normal saline), which was compared with a 5 McFarland solution. The microbial suspension (1 ml) in normal saline was added to 74 ml of sterile medium, kept at 45°C, to give a concentration of 2×10^7 cells /ml.

3.1.2.5 . Bioautography agar overlay assay

Five microliters (5 µl) of the solution of each extract (200 µg/µl) was applied on a glass backed Silica gel G_{60} F_{254} TLC plate (20 x 20 cm, 250 µm thickness, Merck, Darmstadt,

Germany) and a reference plate was similarly prepared. Both plates were developed to a distance of about 10 cm in the same tank using pre-determined mobile phases. The mobile phase was removed from the plate by drying with a stream of cool air from a heating gun. The reference plate was observed in UV light to see if the separated spots were UV active after which it was sprayed with vanillin- sulphuric acid solution followed by heating using a heating gun.

Five microliters (5 µl) of the positive control, amphotericin B (0.002 µg/µl) equivalent to 0.01µg per spot was applied on the test plate in triplicate followed by removal of solvent using a stream of cold air. About 22 ml of the freshly prepared inoculum was uniformly spread using a sterile Pasteur pipette. After solidification the plates were placed in a polythene container lined at the bottom, with moist cotton wool. The plates were incubated at 31°C for 18 to 24 h, after which they were removed and sprayed with an aqueous solution (2.5 mg/ml) of thiazolyl blue (3-(4,5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (MTT) (BDH, Poole England). They were incubated for a further 4 h, after which the inhibition zones appeared colourless against a purple background. The colour change is due to the reduction of MTT to a formazan dye (Figure 2:3) by action of the dehydrogenase enzymes from *C. albicans* hence a colourless spot indicates absence of *C. albicans*.

Spots showing any inhibition were noted and their hR_f values and inhibition zones measured. The test was performed in duplicate.

+

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

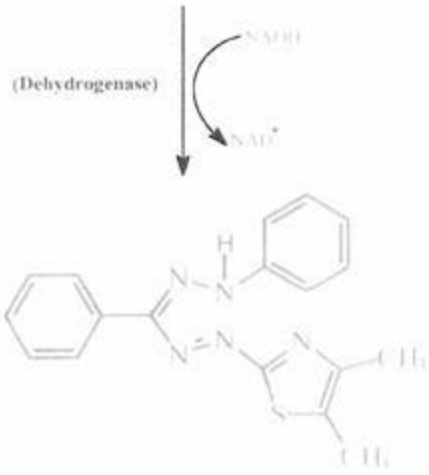


Figure 2:3 Reduction of (3-(4, 5 dimethylthiazol-2-yl), 5-diphenyl tetrazolium bromide) (MTT) to Formazan dye.

2.4 RESULTS AND DISCUSSION

2.4.1 Plants collected

Fifty-four (54) plants were collected and are as listed in Table 2:2. Plant parts collected and their numbers in brackets included a whole plant (1), aerial parts (6), Leaves (20), Stems (2), Roots (15), stem bark (14) and root bark (18).

2.4.2 Plants from traditional healers

A relatively higher number of plants used in the management of *Candida* infections, were collected from Tanga region when compared to the other three regions (Table 2:3). The region is in the northeastern zone of Tanzania, with approximately 670 traditional healers, one traditional healer per 343 people in Tanga city and one healer per 146 people in the rural area (Scheinman, 2002). Many traditional healers in this region were interviewed but only a few of them used plant remedies alone. Most of them were diviners rather than herbalists. The diviners treat patients after consulting their supernatural powers in the presence of patients. They did not relate the symptoms to the medicines given to patients. In this study the herbalists were the most resourceful group.

Underground organs including roots and root barks were the most frequently used plant parts followed by leaves, stems and entire herbs. The route of administration depended on the type of *Candida* infection; however, the most common route was oral. In most cases the medicines were boiled in water and drunk or a powdered drug was added to porridge and/or tea and drunk. Other routes of administration included topical application in form of gargles, douches, pastes and chewing sticks. Slightly more than 25% of the plants were used in combination with other plant materials.

Among the collected plants, twenty-two were reported elsewhere to be used for related infections or have proven antifungal activity (Table 2:4).

Seventeen plants representing 47.2% of the collected plants have been evaluated for antifungal activity before and found to be active against various fungi including *Candida* species and *Cryptococcus neoformans*, both causative agents of opportunistic fungal

Table 2:2 Plants collected

S/N	Plant Species	Family	Voucher specimen No.	Locality (Region)	Source[1]	Plant[1] part
1.	*Abrus precatorius* L.	Fabaceae	R/S 51	Dar es Salaam	T	L
2.	*Acacia nilotica* (L.) Del.	Fabaceae	R/S 47	Tanga	T	Rb
3.	*Acacia zanzibarica* S. Moore) Taub.	Fabaceae	R/S 38	Dar es Salaam	T	Rb
4.	*Agathisanthemum bojeri* Klotzsch	Rubiaceae	R/S 52	Dar es Salaam	T	R, Ap
5.	*Albizia anthelmintica* Brongn	Fabaceae	R/S 3	Morogoro and Coast	T	Rb, Sb
6.	*Allophyllus africanus* P. Beauv.	Sapindaceae	R/S 15	Coast	T	S
7.	*Asparagus africanus* Lam.	Liliaceae	R/S 39	Dar es Salaam	Lit*	Ap, R
8.	*Balanites aegyptiaca* (L.) Delile	Balanitaceae	R/S 2	Morogoro	T	Rb, Sb
9.	*Bonamia mossambicensis* Klotzsch	Convolvulaceae	R/S 41	Dar es Salaam	Lit*	R
10.	*Boscia salicifolia* Oliv.	Capparidaceae	R/S 21	Morogoro	Lit*	L, Sb
11.	*Bridelia cathartica* Bertol.	Euphorbiaceae	R/S 30	Morogoro	Lit*	L, Sb
12.	*Cajanus cajan* (L.) Millsp	Fabaceae	R/S 40	Dar es Salaam	T	L
13.	*Carica papaya* L.	Caricaceae	R/S 56	Tanga	T	R
14.	*Cassia abbreviata* Oliv	Fabaceae	R/S 23	Dar es Salaam	T	Rb
15.	*Cassia auriculata* L.	Fabaceae	R/S 29	Morogoro	Lit*	L, Sb
16.	*Catunaregum nilotica* (Stapf) Tier.	Rubiaceae	R/S 8	Tanga	T	Rb
17.	*Ceiba pentandra* Gaertn	Bombacaceae	R/S 32	Morogoro	Lit*	L
18.	*Chassalia umbraticola* Vatke	Rubiaceae	R/S 28	Coast	T	L, Rb

19.	*Clutia abyssinica* Jaub. & Spach	Euphorbiaceae	R/S 55	Tanga	T	L
20.	*Combretum molle* R. Br.	Combretaceae	R/S 20	Tanga	T	R
21.	*Combretum zeyheri* Sond	Combretaceae	R/S 13	Morogoro	Lit*	L,Sb
22.	*Crabbea velutina S.* Moore	Acanthaceae	R/S 37	Tanga	T	Wp
23.	*Deinbollia borbonica* Scheff	Sapindaceae	R/S 42	Morogoro	Lit*	R
24.	*Dichrostachys cinerea* (L.) Wight & Arn	Fabaceae	R/S 48	Dar es Salaam	T	L
25.	*Dictyophleba lucida* (K. Schum.) Pierre	Apocynaceae	R/S 26	Dar es Salaam	Lit*	L
26.	*Ehretia amoena* Klotzsch	Boraginaceae	R/S 14	Morogoro	T	Rb, Sb
27.	*Elaeodendron buchananii* (Loes) Loes	Celastraceae	Mhoro 12579ZH	Kagera	***	Rb
28.	*Eriosema psoraleoides* (Lam.) G. Don	Fabaceae	R/S 35	Morogoro	Lit**	L, Sb
29.	*Harrisonia abyssinica* Oliv.	Simaroubaceae	R/S 18	Tanga, Dar es alaam	Lit*& T	Rb, Ap
30	*Hibiscus micranthus* L. f.	Malvaceae	R/S 33	Morogoro	Lit*	Ap
31	*Holarrhena febrifuga* Klotzsch	Apocynaceae	R/S 31	Morogoro	Lit*	L, Sb
32	*Lannea stuhlmannii* Engl.	Anacardiaceae	R/S 43	Dar es Salaam	T	R
33.	*Margaritaria discoidea* (Baill) G.L. Webster	Euphorbiaceae	R/S 46	Tanga	T	R
34.	*Ocimum suave* Willd	Lamiaceae	R/S 54	Tanga	T	R
35.	*Ozoroa insignis* Delile	Anacardiaceae	R/S 25	Morogoro, Tanga	T	Rb
36.	*Phyllanthus reticulatus* Poir	Euphorbiaceae	R/S 17	Dar es Salaam	Lit*	Ap, R
37.	*Physalis peruviana* L.	Solanaceae	R/S 44	Tanga	T	L
38.	*Plectranthus barbatus* Andrews	Lamiaceae	R/S 4	Tanga	T	R, L

39.	*Pseudolachnostylis maprouneaefolia* Pax	Euphorbiaceae	R/S 19	Morogoro	Lit*	L, Rb, Sb
40.	*Pseudovigna argentea* (Willd) Verde	Fabaceae	R/S 49	Dar es Salaam	T	L
41.	*Salvadora persica* L.	Salvadoraceae	R/S 10	Tanga	T	Rb
42.	*Sclerocarya birrea* (A. Rich) Hochst	Anacardiaceae	R/S 16	Morogoro	T	Rb, Sb
43.	*Securidaca longepedunculata* Fres.	Polygalaceae	R/S 45	Tanga	T	Rb
44.	*Securinega virosa* (Roxb. ex Wild.) Voigt.	Euphorbiaceae	R/S 22	Dar es Salaam	Lit*	S
45.	*Sida serratifolia* L.	Malvaceae	R/S 34	Dar es Salaam	Lit*	Ap
46.	*Suregada zanzibariensis* Baill.	Euphorbiaceae	R/S 7	Dar es Salaam	T	L
47.	*Synaptolepis kirkii* Oliv	Thymelaeaceae	R/S 27	Dar es Salaam	T	R
48.	*Tetracera boivintiana* L.	Dilleniaceae	R/S 50	Dar es Salaam	T	L
49.	*Uvaria acuminata* Oliv.	Annonaceae	R/S 53	Dar es Salaam	T	R
50.	*Vitex fischeri* Gürke	Vitaceae	Mhoro 12578 ZH	Kagera	***	Rb
51.	*Xeroderris stuhlmannii* (Taub) Mendonca	Fabaceae	R/S 36	Morogoro	Lit*	L, Rb, Sb
52.	*Ximenia Americana* L.	Olacaceae	R/S 12	Morogoro, Tanga	T	Rb
53.	*Zanha africana* (Radlk.) Exell.	Sapindaceae	R/S 24	Tanga	T	R
54.	*Zanthoxylum chalybeum* Engl.	Rutaceae	R/S 9	DaresSalaam, Tanga	T& Lit*	Rb, Sb
55.	*Ziziphus abyssinica* Hochst. ex A. Rich.	Rhamnaceae	R/S 1	Morogoro	Lit*	L, Sb
56.	*Ziziphus mucronata* Willd	Rhamnaceae	R/S 11	Tanga	T	R

[1] Ap: aerial parts; L: leaves; Lit: literature; R: roots; Rb: root bark; R/S: D. Runyoro / H.O Selemani; S: stem; Sb: stem bark; T: traditional healer; Wp: whole plant, *Sawhney et al. (1978b), **Khan et al. (2000); ***Obtained through courtesy of Dr. Z. Mbwambo.

Table 2:3 Plants used in the management of *Candida* infections by traditional healers in four regions of Tanzania

Plant name	Locality	Vernacular name (Tribe)	Part used*	Type of Candida* infection	Method of preparation	Route and method of administration
Abrus precatorius	Bunju, Dar es Salaam	Msipo (Zaramo)	L	OPC	Fresh leaves are crushed Dried leaves soaked in water	Topical, applied in the mouth twice daily
Acacia nilotica Syn. *Acacia arabica*	Handeni, Tanga	Kiroriti (Maasai)	Rb	OPC	The root bark is boiled with water	Oral, one glass is taken two times daily
Acacia zanzibarica Syn. *Pithecolobium zanzibaricum*	Bunju Juu, Dar es Salaam	Malula (Nyamwezi), Mgunga mweupe (Swahili)	Rb	OPC, SI	The root bark is mixed with that of *Lannea stuhlmannii* and boiled with water	Oral, half a cup is taken three times adaily
Agathisanthemum bojeri Syn. *Oldenlandia bojeri*	Bunju A, Dar es Salaam; Kisarawe, Coast	Chamaligo (Swahili), Kingobulele (Zaramo)	W	VVC	The fresh or dried herb is boiled in water	Topical, applied as douche twice daily
			L	SI	The fresh leaves are crushed	Topical, rubbed on the skin twice daily

Albizia anthelmintica	Melela, Morogoro; Chalinze, Coast	Mfulete (Swahili) Makotana (Maasai), Mjambele (Gogo)	Sb, Rb	VVC	The dried stem and root barks are boiled with water or can be added to soup The fresh bark is mixed with milk	Oral, one glass is taken twice daily
Allophyllus africanus	Chalinze, Coast	Mchuki (Gogo)	S	OPC	Branches are made into chewing sticks	Topical, used twice daily
Balanites aegyptiaca	Melela, Morogoro	Olug'oswai (Maasai)	Rb, Sb	EC	Powdered barks are mixed with other plants and are boiled with water	Oral, half a glass is taken three times daily
Cajanus cajan Syn. *Cajanus flavus, Cajanus indicus*	Bunju, Dar es Salaam	Mbaazi (Swahili)	L	OPC	Fresh leaves are pounded	Topical, locally applied in the mouth
Carica papaya Syn. *Carica hermaphrodita, Carica mamaya, Papaya carica, Papaya orientalis, Papaya sativa, Papaya vulgaris*	Korogwe, Tanga	Papai dume (Swahili)	R	VVC	Fresh roots of the male plants are mixed with fresh roots of *Ocimum suave* and boiled with water	Oral, half a cup is taken three times daily

Cassia abbreviata Syn. *Cassia bearcana*	Bunju, Dar es Salaam	Mkundekunde (Swahili)	Rb	VVC	The root bark is boiled with a certain gum	Oral, one glass is taken two times daily
Catunaregam nilotica Syn. *Randia nilotica*	Handeni Tanga	Mdasha (Zigua)	Rb	OPC	The powdered root bark is boiled with water	Oral, one glass is taken three times daily
Chassalia umbraticola	Pugu hills, Dar es Salaam	-	Rb, L	OPC, EC	The root bark and leaves are boiled with water	Topical, used as a gargle four times daily
Clutia abyssinica	Lushoto, Tanga	Mhende (Sambaa)	L	SI	The fresh leaves are crushed	Topical, the crushed leaves are rubbed on the affected part of the skin two times daily
Combretum molle Syn. *Combretum sokodense, Combretum trichanthum, Combretum velutinum.*	Korogwe, Tanga	Mlama (Nyamwezi)	R	VVC	The dried roots are powdered and the powder is added to porridge	Oral, a glass is taken three times daily
Crabbea velutina	Handeni, Tanga	Mkunga (Zigua)	Wp	OPC	The herb is boiled with water	Oral, half a glass is taken three or four times daily

Dichrostachys cinerea Syn. *Dichrostachys nutans*, *Mimosa nutans* *Ehretia amoena*	Bunju, Dar es Salaam	Kikulagembe (Zaramo)	L	OPC	The dried leaves are powdered and added into edible oil	Topical, applied in the mouth four times daily
	Melela, Morogoro	Njabalelo (Maasai), Mkilika (Swahili).	R	SI	Dried and powdered roots are boiled with water.	Oral, one glass is taken four times daily
Harrisonia abyssinica Syn. *Harrisonia occidentalis*, *Zanthoxylum guineense*	Korogwe, Tanga	Mkusu (Swahili)	R	VVC	The roots are boiled with water	Oral, two spoonful taken three times daily
Lannea stuhlmannii	Bunju Juu, Dar es Salaam	Msayu (Nyamwezi), Mjenga ua (Swahili)	Rb	OPC	The powdered roots are boiled with water	Topical, used as a gargle two to three times daily
Margaritaria discoidea Var. *discoidea*.	Handeni, Tanga	Muhungulu (Zigua)	Rb	OPC	The fresh or dried root bark is boiled and drunk alone or mixed with porridge	Oral, half a glass is taken two times daily
Ocimum suave	Korogwe, Tanga	Mzumbasha (Sambaa)	R	VVC	Fresh roots are mixed with fresh roots of the male plant of *Carica papaya* and boiled with water	Oral half a cup is taken three times daily

Ozoroa insignis Syn. *Anaphrenium abyssinicum*	Handeni, Tanga; Mgeta, Morogoro	Muhombe (Zigua), Lokununu (Maasai), Mkomachuma (Zigua)	Rb, Sb	VVC	The barks are powdered, boiled with water alone or in combination with tea, honey, porridge and/or other plants including *Cordia* species	Oral, a quarter of a glass is taken three times daily
					The barks are powdered	Topical, applied locally on the affected areas twice daily
Physalis peruviana Syn. *Physalis edulis*	Lushoto, Tanga	Msupu (Sambaa)	L	SI	The leaves are squeezed to obtain the juice	Topical, applied on the affected area two times daily
Plectranthus barbatus Syn. *Coleus barbatus*	Handeni, Tanga	Vuga (Zigua)	L	OPC	The fresh leaves are squeezed to obtain the juice	Topical, used as a gargle two times daily
			R	VVC	The fresh roots are boiled with water and cooled	Oral, half a cup is taken three times daily
Pseudovigna argentea	Bunju A, Dar es Salaam	Nyingilila (Ngindo)	L	VVC	Fresh leaves are crushed	Topical, inserted in the vagina at night and removed in the morning.
				SI	The dried leaves are powdered and incorporated into petroleum jelly	Topical, applied on the skin three times daily

Salvadora persica	Handeni, Tanga	Mkunghuni (Gogo)	Rb	OPC	The powdered root bark is made into a paste using cooking oil The powdered root bark is added to porridge	Topical, applied locally in the mouth three times daily Oral, one glass is taken three times daily
Sclerocarya birrea Syn. *Poupartia birrea*	Melela, Morogoro	-	Rb, Sb	OPC, EC	The root and stem barks are boiled with water	Oral, a glass is taken three times daily Inhalation, the vapour is inhaled twice daily.
Securidaca longepedunculata	Handeni, Tanga	Masuke mengi (Zigua)	Rb	OPC	A little amount of the powdered root bark is added to either tea or porridge	Oral, taken at least four times daily
Suregada zanzibariensis Syn. *Gelonium zanzibariense*	Changanyikeni, Dar es Salam	Mdimu pori (Swahili)	L	VVC	The leaves are boiled with water	Topical, douching at least twice daily

Synaptolepis kirkii	Kisarawe, Coast	-	R	SI	The peeled roots are mixed with castor seeds, crushed and boiled with water	Oral, a quarter a cup is taken twice daily
Tetracera boiviniana Baill	Bunju A, Dar es Salaam	Mpingapinga (Matumbi)	L	SI	The fresh or dried leaves are boiled with water	Oral, a quarter of a cup is taken three times daily
Uvaria acuminata Oliver Syn. *Uvaria holstii, Uvaria leptocladon*	Kisarawe, Coast	Mzizimia (Swahili)	R	OPC	Powdered roots are boiled with tea	Oral, one cup is taken three times daily
Ximenia americana	Melela, Morogoro Handeni, Tanga	Ngomai (Masai), Mtundwe (Gogo)	Rb	OPC	The powdered bark is added to local brew The powdered root bark is boiled with water	Topical, applied as a gargle three times daily Inhalation, the vapour is inhaled three times daily
Zanha africana Syn. *Dialiopsis africana*	Handeni, Tanga	Mdahula (Zigua)	R	OPC, VVC	The dried roots are powdered and boiled with water	Oral, two glasses are taken twice daily
Zanthoxylum chalybeum. Syn. *Fagara chalybea*	Handeni, Tanga	Mkunungu (Swahili)	Rb	EC	Root bark is powdered and added to tea A little bit of salt is added to the powdered root bark	Oral, two cups are taken twice daily Oral, a little bit is licked thrice daily

Ziziphus mucronata Willd.	Handeni, Tanga	Mnyangwe (Gogo)	Rb	OPC	The root bark is powdered and mixed with milk	Oral, one cup is taken three times daily
					The root bark is powdered	Topical, applied locally in the mouth four times daily

*EC: esophageal candidiasis; L: leaves; OPC: oral pharyngeal candidiasis; R: roots; Rb: root bark; S: stem; Sb: stem bark; SI: skin fungal infections; VVC: vulvovaginal candidiasis; Wp: whole plant

Table 2:4 Literature review on plants collected through interviews with traditional healers

SN	Plant species	Literature reports of related ethnomedical uses and/or proven antifungal activity	References
1.	*Abrus precatorius*	Fresh bark is used in India for skin diseases and the entire plant is used in preparing medicated oils	John, 1984
		Fresh leaves are used in Thailand for inflammation	Panthong et al., 1986
		Ethanol and aqueous extracts of dried seeds of the Indian plant were reported to have antifungal activity against *Cryptococcus neoformans*	Sirsi, 1963
2.	*Acacia nilotica*	The dried fruits of Tanzanian plants are used for sore throat	Chhabra and Uiso, 1991
		Different extracts of the bark are reported to have antifungal activity against yeasts and other fungi	Gupta and Bilgrami, 1970; Sinha and Anjana, 1984
		The dried fruits of Sudanese plants are reported to be active against *C. albicans*	Almagboul et al., 1988; Nabi et al., 1992
		The root bark of a Tanzanian plant was reported active against *C. tropicalis, C. parapsilosis* and *C. krusei*	Hamza et al., 2006

3.	*Agathisanthemum bojeri*	The dried flowers are used for sore throat	Chhabra et al., 1991
4.	*Balanites aegyptiaca*	The fresh leaves, dried barks and roots of the Kenyan plant were reported active against *Bacillus subtilis, Penicillium crustosum, Saccharomyces cerevisiae, Epilachna varivestis, Biomphalaria glabrata* and *Lymnaea natalensis*	Taniguchi et al., 1978; Liu and Nakanishi, 1982
		The saponin fraction from the mesocarp of the Egyptian plant had a weak activity against *Aedes aegypti, Aspergillus niger* and *C. albicans*	Saeed et al., 1995
5	*Cajanus Cajan*	Decoction of the leaves is used for skin infection and rashes, sore throat and as a mouth wash to heal sore gums and halt toothache	Weniger et al., 1986; Coce and Anderson, 1996a, b
		The decoction prepared from the leaves is drunk or used as a gargle for infected gums	Singh, 1986
		The methanolic extract of the leaves of the plant from Rwanda was found to have an in vitro activity against *C. albicans*	Boily and Van Puyvelde, 1986
6.	*Carica papaya*	Latex, seeds, and leaves are used for ringworm infection	Holdsworth, 1991; 1992; Singh, 1986; Le Grand, 1989
		The fruit of the plant from Angola is used for eczema and psoriasis	Bossard, 1993
		The extracts of fruits, roots, latex and leaves of the plant from different countries were active against a number of microorganisms, including *C. albicans*, other species of *Candida* and other fungi	Emeruwa et al., 1982; Gundidza, 1986; Caceres et. al., 1995; Giordani et al., 1991; 1996; 1997
7.	*Clutia abyssinica*	Ethanol extract of the dried leaves exhibited antifungal activity against *Trichophyton mentagrophytes*	Vlietinck et al., 1995
		The roots of a Tanzanian plant were reported to have weak activity against *C. albicans*	De Boer et al., 2005
8.	*Combretum molle*	Twigs of the plant are used in Tanzania as chewing stick and the methanolic extract of the dried bark exhibited antifungal activity against *C. albicans*	Khan et al., 2000

		A 50% ethanol extract of the leaves exhibited antifungal activity against *Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum*, and *Epidermophyton floccosum*	Baba-Moussa, et al., 1999
9.	*Dichrostachys cinerea*	Pounded leaves of the East African plants are applied locally for skin ulcers	Hedberg et al., 1983b
		The leaves and roots of the Somalian plants are used for sore throat, venereal diseases, and skin ulcers and as a vaginal douche	Samuelsson et al., 1992a
		The dried stem was reported active against *Aspergillus niger* and *C. albicans*	Almagboul et al., 1988
10.	*Ehretia amoena*	The dried stem bark is used in Tanzania for treatment of skin diseases	Chhabra et al., 1984
11.	*Harrisonia abyssinica*	Hot water extract of fresh and dried root bark is used in Tanzania to treat skin diseases	Sawhney et al., 1978a; b
		Methanol extract of dried root bark exhibited activity against *Trichophyton mentagrophytes* and *C. albicans*	Sawhney et al., 1978b
		Chloroform extract of the stem bark exhibited antifungal activity against *Aspergillus niger, Microsporum canis, Trichophyton mentagrophytes* and *Aspergillus fumigatus*	Balde et al., 1995
12.	*Lannea stuhlmannii*	Fresh leaves are used in Tanzania as a dressing for sores, boils, carbuncles and abscesses	Chhabra et al., 1984
		The dried bark, leaves and roots of the plant are used in Kenya against a number of ailments including skin eruptions in children	Johns et al., 1990
13.	*Ocimum suave*	In Tanzania the scrapping of the roots are mixed with *Zingiber officinalis* and used for inflamed tonsils	Hedberg et al., 1983a
		The dried twigs are used as a chewing stick	Khan et al., 2000
		The essential oil isolated from the aerial structures of the plant was reported active against a number of microorganisms	Janssen et al., 1989
		The ethanol extract of the leaves of Rwandese plants were found to be active against *Bacillus subtillis* and *Microsporum canis*	Vlietinck et al., 1995

14.	*Ozoroa insignis*	Dried stem bark showed antifungal activity against *C. albicans* but not *Aspergillus niger*	Abreu et al., 1999
15.	*Salvadora persica*	The stem of the plant is used in Jordan for oral hygiene The roots are used as toothbrush The seeds of Tanzanian plants are used for oral hygiene The roots of the plant from Saudi Arabia were found to be fungistatic against *C. albicans*	Taha and Hani, 1995 Al-Said, 1993 Johns, et al., 1996 Al-Bagieh et al., 1994
16.	*Physalis peruviana*	The whole plant has been reported to be inactive against both *Cryptococcus neoformans* and *Candida*	Dhawan et al., 1977
17.	*Sclerocarya birrea*	The ethanolic extract of dried stem bark showed antifungal activity against *Candida albicans*	Adoum et al., 1997
		The roots and leaves of Tanzanian plants were reported active against *C. albicans, C. tropicalis, C. parapsilosis* and *C. krusei*	Hamza et al., 2006
18.	*Securidaca longepedunculata*	The aqueous, dichloromethane and ethanol extracts were reported to have activity against *C. albicans*	Desta, 1993; Taniguchi et al., 1978
		The Petroleum ether extract exhibited antifungal activity against *Candida albicans, Aspergillus fumigatus* and *Aspergillus niger*, while the dichloromethane extract was active against *Aspergillus fumigatus, Aspergillus niger* and the *Penicillium* spp .	Joseph et al., 2006
19.	*Suregada zanzibariensis*	The fresh leaves of the Tanzanian plants are mixed with that of *Acalypha fruticosa* and *Zanthoxylum chalybeum* pounded and rubbed on the skin for treatment of skin infections	Hedberg et al., 1983a
20.	*Ximenia americana*	The seeds are used in Angola for throat infections	Bossard, 1993

21	*Zanha africana*	Root barks of the Tanzanian plant are mixed with petroleum jelly and used externally to treat fungal and other skin infections and the root barks were reported active against *Trichophyton* species	Chhabra et al., 1982; Chhabra et al., 1991
		The stem bark was reported to be active against various species of *Candida*	Fabry et al., 1996
		A methanol extract of the root bark exhibited a weak anti-inflammatory activity	Cuellar et al., 1997
22.	*Zanthoxylum chalybeum*	Fresh leaves of the plant from Tanzania are pounded with leaves of *Acalypha fruticosa* and *Suregada zanzibariensis* and the resulting juice is used for skin infections	Hedberg et al., 1983b
		The fresh twigs of the plant from East Africa are used as toothbrush, air fresheners and for skin infections	Hedberg et al., 1983b; Johns et al., 1990
		The bark of the Kenyan plants was reported active against *Bacillus subtilis*, *Penicillium crustosum* and *Saccharomyces cerevisiae*	Taniguchi et al., 1978
		The extract of the root bark was repoted active against *C. albicans*	Sawhney et al., 1978b
23	*Ziziphus mucronata*	Aqueous and methanol extracts of stem bark showed antifungal activity against *C. albicans*	Gundidza, 1986

2.4.3 Bioautography agar overlay assay

Out of the 76 plant samples collected only 63 were extracted. The percentage yield of the extracts ranged from 2% for *Plectranthus barbatus* leaves to 26.5 % for *Securidaca longependunculata* root bark (Table 2:5). *Candida albicans* ATCC 90028 (Figure 2:2) was used in this study.

Twenty-eight out of the sixty-three extracts, belonging to 27 plant species and constituting 48% of all the plants screened, could be resolved into TLC spots exhibiting activity against *C. albicans* (Table 2:5). The number of active resolved spots for each extract ranged from 1 to 4. Table 2:5 also shows the TLC bioautography results for all the extracts developed using various solvent systems and Figure 2:4- Figure 2:6 show the chromatograms and bioautograms of some of the most active extracts.

Table 2:5 TLC Bioautography results

S/N	Plant species	P[1]	%Y[1]	M[1]	hR_f	Iz[1] in mm
1	*Abrus precatorius.*	L	18.3	3	-	-
2	*Acacia nilotica*	Rb	16.5	3	63	<4
					75	<4
					85	<4
3	*Acacia zanzibarica*	Rb	12.5	2	-	-
4	*Agathisanthemum bojeri*	Ap	6.5	3	-	-
5	*Agathisanthemum bojeri*	R	16.0	4	80	<4
					100	10
6	*Albizia anthelmintica*	Rb	9.0	5	25	15
					40	4
					50	5
					60	6
7	*Allophyllus africanus*	Ap	3.0	3	-	-
8	*Asparagus africanus*	Ap	16.4	2	0	<4
					50	<4
					75	<4
9	*Balanites aegyptiaca*	Rb	22	5	30	13
10	*Bonamia mossambicensis*	R	6.0	3	-	-
11	*Boscia salicifolia*	L	14.5	4	-	-
12	*Bridelia cathartica*	Sb	10.0	2	-	-
13	*Cajanus cajan*	L	8.0	3	37	4
					53	10
					78	10
14	*Carica papaya*	R	6.0	3	-	-
15	*Cassia abbreviata*	Rb	11.5	1	-	-

16	*Cassia auriculata*	Sb	10.0	4	60	10
					85	8
17	*Catunaregum nilotica*	Rb	6.0	3	0	<4
					40	<4
					55	<4
					80	<4
18	*Ceiba pentandra*	L	5.5	3	-	-
19	*Chassalia umbraticola*	Rb	6.0	3	0	5
					80	6
20	*Clutia abyssinica*	L	9.5	1	-	-
21	*Combretum molle*	R	13.5	4	33	7
					48	4
22	*Combretum zeyheri*	L	3.5	3	46	9
					61	6.5
23	*Crabbea velutina*	Wp	8.0	3	-	-
24.	*Deinbollia borbonica*	R	5.0	3	-	-
25.	*Dichrostachys cinerea*	L	12.0	1	-	-
26	*Dictyophleba lucida*	L	13.0	2	10	5
					40	5
					85	<4
27	*Ehretia amoena*	Sb	6.5	3	-	-
28	*Elaeodendron buchananii*	Rb	13.5	3	-	-
29	*Eriosema psoraleoides*	Sb	4.5	3	-	-
30	*Harrisonia abyssinica*	Rb	12.5	1	90	10
31	*Harrisonia abyssinica.*	Ap	10.5	1	-	-
32	*Hibiscus micranthus*	Ap	3.5	3	-	-
33	*Holarrhena febrifuga*	L	3.0	5	45	10
34	*Lannea stuhlmannii*	R	9.5	3	-	-
35	*Margaritaria discoidea*	R	15.0	3	50	5
					60	4
36	*Ocimum suave*	R	5.5	3	-	-
37	*Ozoroa insignis* (Morogoro)	Rb	11.5	1	-	-
38	*Ozoroa insignis* (Tanga)	Rb	9.0	3	-	-
39	*Phyllanthus reticulatus*	Ap	11.5	1	-	-
40	*Physalis peruviana*	L	13.6	1	0	7
					45	6
					80	<4
41	*Plectranthus barbatus*	R	5.5	3	72	12.5
42	*Plectranthus barbatus*	L	2.0	1	85	7
43	*Pseudolachnostylis maprounecefolia*	S	12.0	4	-	-
44	*Pseudolachnostylis maprouneaefolia*	R	12.0	1	-	-
45	*Pseudovigna argentea*	L	4.0	3	sf	<4
46	*Salvadora persica*	Rb	8.5	3	80	10
47	*Sclerocarya birrea*	Rb	9.0	3	-	-
48	*Securidaca longepedunculata*	Rb	26.5	3	72	<4
					85	<4
49	*Securinega virosa*	S	2.5	3	-	-
50	*Sida serratifolia*	Ap	4.0	1	85	10
51	*Suregada zanzibariensis*	L	8.0	1	0	10
					80	5
					100	<4

52	*Synaptolepis kirkii*	R	5.7	3	-	-
53	*Tetracera boiviniana*	L	4.0	3	-	-
54	*Uvaria acuminata*	R	7.0	3	70	7
55	*Vitex fischeri*	Rb	6.0	3	52	<4
					65	<4
56	*Xeroderris stuhlmannii*	Sb	6.5	3	-	-
57	*Ximenia americana (Morogoro)*	Rb	18.5	4	-	-
58	*Ximenia americana (Tanga)*	Rb	8.5	4	-	-
59	*Zanha africana*	R	6.5	3	11	4
					41	5
60	*Zanthoxylum chalybeum*	Rb	9.0	4	30	<4
					85	<4
61	*Ziziphus abyssinica*	L	11.5	6	37	9
62	*Ziziphus abyssinica.*	Sb	9.0	3	-	-
63	*Ziziphus mucronata*	R	8.0	3	75	<4

[1] Ap: aerial parts; Iz: inhibition zone ; L: leaves; M: mobile phase; P; plant part; R: roots; Rb: root bark; S: stem; Sb: stem bark; Sf: several faint spots;Wp: whole plant; %Y: % yield; -: no inhibition

Mobile phases: **1**: Chloroform: Methanol 9:1; **2**: Chloroform: Methanol 5:1; **3**: Chloroform: Methanol 4:1; **4**: Chloroform: Methanol: Ethylacetate 4:1:1; **5**: Chloroform: Methanol: Ethylacetate: water 28:30:35:5; **6**: Dichloromethane: Methanol: Water 30:10:1

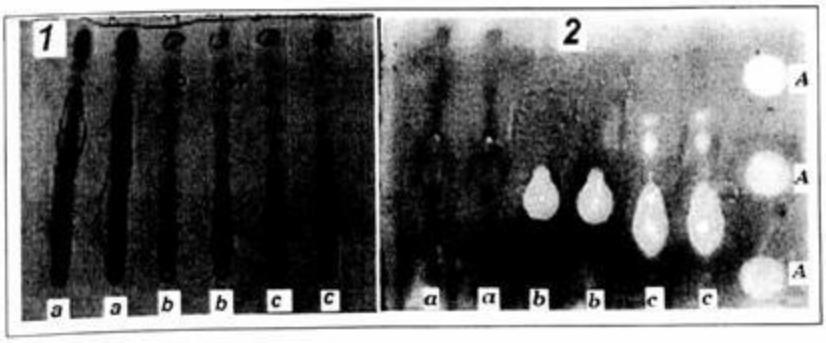


Figure 2:4 Chromatograms (1) and bioautograms (2) for *Holarrhena febrifuga* leaves (a), *Balanites aegyptiaca* root bark (b) and *Albizia anthelmintica* root bark (c). Mobile phase: Chloroform: Methanol: Ethyl acetate: water 28:30:35:5; Amphotericin B 0.01 µg (A).

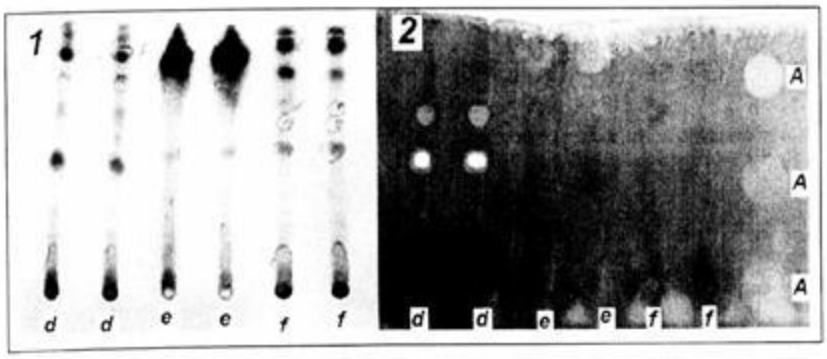


Figure 2:5 Chromatograms (1) and bioautograms (2) for *Combretum zeyheri* leaves (d), *Plectranthus barbatus* roots (e) and *Vitex fischeri* root bark (f) mobile phase: Chloroform: Methanol 4:1 3; Amphotericin B 0.01µ g (A)

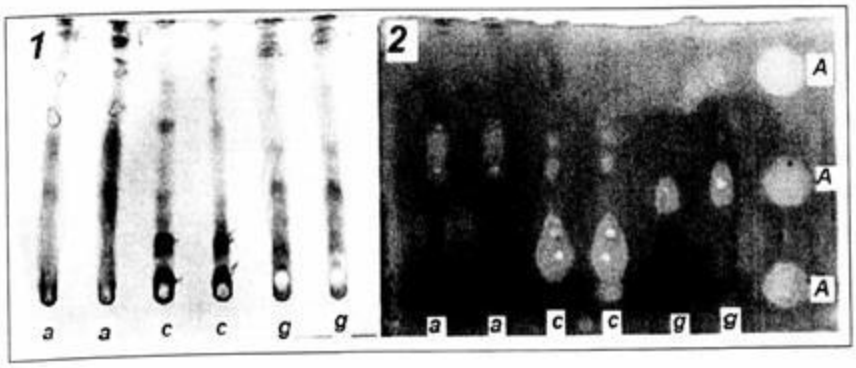


Figure 2:6 Chromatograms (1) and bioautograms (2) for *Holarrhena febrifuga* leaves (a) *Albizia anihelmintica* root bark (c) and *Ziziphus abyssinica* leaves (g) mobile phase: Dichloromethane: Methanol: water 30:10:1; Amphotericin B 0.01µ g (A)

Bioautography agar overlay method is considered as one of the most efficient methods for the detection of antimicrobial compounds (Rahalison et al., 1991). It involves the transfer of the active compounds from the stationary phase into the agar layer through a diffusion process. From Table 2:5 it can be seen that a total of 27 plants, screened by using this method, exhibited anticandida activity. However, only 8 out of the 18 plants previously reported to be active (Sawhney et al., 1978b), were found to be active in this

bioassay. *Ziziphus abyssinica* and *Asparagus africanus*, which were collected as substitutes for *Ziziphus pubescens* and *Asparagus falcatus*, respectively, previously reported active (Sawhney et al., 1978b), were also found to be active. This proves the fact that allied species may contain similar or related active compounds (Evans, 1989). *Eriosema psoraleoides*, a plant, reported (Khan et al., 2000) to be active was found to be inactive in this bioassay.

For most plants screened the plant parts used were more or less similar, with very few peculiar exceptions, such as *Xerroderris stuhlmannii*, which Sawhney et al. (1978b) reported that the whole plant was active. However, this plant is a tree, and, in this study only the stem barks was evaluated. The observed discripances in the activity could be due to the bioassay methods employed. In the present study the bioautography method was used on partially resolved extracts, whereas Sawhney et al. (1978b) used the agar dilution method for unresolved crude extracts. Also it was not possible to compare concentrations because in the bioautography method absolute amounts of extracts were chromatographed while in the agar dilution method concentrations of the extracts were used. Such differences could have contributed to the observed differences in the bioassay results.

Twenty out of 36 plants (55.6%), obtained through interviewing traditional healers, were found active in this bioassay. Ten of these have also been reported to be active in previous studies; these include; *Ziziphus mucronata* (Gundidza, 1986), *Cajanus cajan* (Boily and Van Puyvelde, 1986), *Acacia nilotica* (Almagboul et al., 1988), *Harrisonia abyssinica* and *Zanthoxylum chalybeum* (Sawhney et al., 1978). Others were *Securidaca longepedunculata* (Taniguchi et al., 1978; Desta, 1993; Joseph et al., 2006), *Salvadora persica* (Al-Bagieh et al., 1994), *Balanites aegyptiaca* (Saeed et al., 1995), *Zanha africana* (Fabry et al., 1996) and *Combretum molle* (Khan et al., 2000). *Harrisonia abyssinica* and *Z. chalybeum* were selected through the literature and were also mentioned by the interviewed traditional healers. *Physalis peruviana* and *Uvaria acuminata* were reported previously as inactive (Dhawan et al., 1977, Sawhney et al., 1978b), but were found to be active in this study. The remaining eight active plants, including *Margaritaria discoidea*, *Suregada zanzibariensis*, *Plectranthus barbatus*,

Albizia anthelmintica, *Catunaregum nilotica*, *Pseudovigna argentea*, *Agathisanthemum bojeri* and *Chassalia umbraticola*, were screened for anticandida activity for the first time. Other plants obtained from the traditional healers, which were reported active previously were found to be inactive in this study and they include *Ozoroa insignis* (Abreu et al., 1999), *Carica papaya* (Gundidza, 1986) *Dichrostachys cinerea* (Almagboul et al., 1988) and *Clutia abyssinica* (De Boer et al., 2005. The discrepancy in the results could be due to a number of factors, including differences in the plant parts used, extracting solvents, time and season of collection of plant materials, geographical location, age of the plants and bioassay methods.

Hamza et al. (2006) screened a number of Tanzanian plants obtained through ethnomedical survey from Coast, Morogoro, Singida and Tanga regions. Their study was performed just after this study and among the 56 plants they collected and identified 10 plants species were similar to those we collected. They screened the plants against a number of *Candida* species and *Cryptococcus neoformans* using broth microdilution method of NCCLS. When their results were compared to the results obtained by bioautography method in this study (Table 2:6) a lot of discrepancies were observed. For instance, *Albizia anthelmintica* and *Balanites aegyptiaca* the most active plants in this study showed no activity at all in the study by Hamza et al. (2006). A reverse was observed for *Sclerocarya birrea*, a plant, which showed activity against four *Candida* species in the study by Hamza et al. (2006), was found to be inactive in this study. It should be noted that things like locality, season, age and stage of development of the plant at the time of collection play a great role in determining the amount and type of secondary metabolites present in the plant and hence, the biological activity. Therefore, among other things the observed differences may be due to one or more of these factors.

Table 2:6: Comparison of the bioautography results of this study with broth microdilution results of a study by Hamza et al., 2006

Plant Species	Bioautography Results using *C albicans* ATCC 90028			Broth microdilution method on a number of *Candida* species		
	Plant[1] part	hR_f and Iz[1] in mm		Plant part [1]	Sensitive *Candida* species	MICo/MFC[1]
Acacia nilotica	Rb	63	< 4	L	*C. parapsilosis*	31/-
		75	< 4		*C. tropicalis*	63/-
		85	< 4		*C. krusei*	1000/1000
Albizia anthelmintica	Rb	25	15	R	None	
		40	4			
		50	5			
		60	6			
Balanites aegyptiaca	Rb	30	13	Rb	None	
Cassia abbreviata	Rb		-	R, Sb	None	
Elaeodendron buchananii	Rb		-	Sb	*C. tropicalis*	250/-
					C. krusei	63/-
Lannea stuhlmannii	R		-	L	None	
Ocimum suave	R		-	L	None	
Salvadora persica	Rb	80	10	R	None	
Sclerocarya birrea	Rb		-	L, R	*C. albicans*	250/-
					C. parapsilosis	125/-
					C. tropicalis	63/-
					C. krusei	63/-
Zanthoxylum chalybeum	Rb	30	< 4	Rb	*C. albicans*	500/-
					C. parapsilosis	1000/-
		85	<4		*C. tropicalis*	2000/4000
					C. krusei	2000/2000

[1]Iz: Inhibition zone; L: Leaves; MFC: Minimum fungicidal concentration in µg/ml; MIC_O: minimum inhibitory concentration in µg/ml; R: root; Rb: root bark, S: stem; Sb: stem bark;

In this study extracts from eleven plants were resolved into several spots showing large inhibition zones (= 10 mm). The plants included *Agathisanthemum bojeri*, *Albizia anthelmintica*, *Balanites aegyptiaca*, *Cajanus cajan*, *Cassia auriculata*, *Harrisonia abyssinica*, *Holarrhena febrifuga*, *Plectranthus barbatus*, *Salvadora persica*, *Sida*

serratifolia and *Suregada zanzibariensis*. Eight among these were obtained through interviewing traditional healers including *A. anthelmintica*, *B. aegyptiaca* and *P. barbatus* the most active of the screened plants. This observation shows that ethnomedical information is important in drug discovery.

2.5 PRIORITIZATION OF PLANTS FOR ISOLATION OF ACTIVE COMPOUNDS

Plants to be used for isolation of the active compounds were selected based on the size of inhibition zones; those with inhibition zones greater than or equal to 9 mm were given priority for further investigation. A cut –off point of 9 mm was selected in order to have a wider choice of plants to study further especially those which have not been previously subjected to extensive phytochemical studies. This in turn, was expected to yield interesting new biologically active compounds. For instance *Combretum zeyheri* has not been studied extensively to yield biologically active compounds. Extracts obtained from plants growing in Tanzania, Zimbabwe and South Africa were reported active on several fungi, including *C. albicans*. Priority was also given to those plants, which have never been evaluated for anticandida activity before, obtained through interviews with traditional healers and for which no anticandida compounds had been isolated before. Five plants, which met the criteria were short listed for further investigation; these included *Albizia anthelmintica*, *Combretum zeyheri*, *Plectranthus barbatus*, *Ziziphus abyssinica*, and *Balanites aegyptiaca* in order of their merits. Due to limited time and difficulties associated with isolation of compounds from *A. anthelmintica* root bark only two plants were used for isolation of the active compounds.

CHAPTER 3

ISOLATION, CHARACTERIZATION AND ANTICANDIDA ACTIVITY OF COMPOUNDS OF *ALBIZIA ANTHELMINTICA* ROOT BARK

3.1 INTRODUCTION

3.1.1 The Genus *Albizia*

Albizia is a genus of about 150 species of mostly fast growing subtropical and tropical trees and shrubs in the subfamily Mimosoideae of the Leguminosae (Fabaceae) family, (Polhill and Raven, 1981). The genus is distributed in Asia, Africa, Madagascar, tropical and subtropical America and Australia. Some of the species found in Tanzania are as shown in Appendix 4 which include *A. europhylla*, *A. glaberrima* and *A. schimperiana* that are endemic (Selemani, 2007). Some of these species including, *A. euryphylla*, *A. forbesii*, *A. isenbergiana* and *A. zimmermannii* have not been investigated scientifically. Eleven *Albizia* species are said to be indigenous to South Africa (Candy et al., 1978). The plants are easily identified by bipinnately compound leaves, although the trees of the genus have frequently been confused with *Acacia* species (Anderson and Morrison, 1990).

Several species of *Albizia* are used as ornamental plants (Mazanti et al., 1983). It has been reported that some *Albizia* species are toxic to livestock (Gummow, 1992), though some are used as animal feed (Yoshikawa et al., 1998). Most species of the genus produce gum, some of which have been chemically investigated aimed at finding a non-*Acacia* source of natural emulsifiers for food and pharmaceuticals. For instance, Mhinzi (2002) studied gums from *A. amara*, *A. petersiana* and *A. harveyi* from Tanzania and found high tannin content as compared to Acacia gum used in the food industry. Anderson and Morrison (1990) noted that some of these gums find their way in official gums as adulterants.

According to the literature, only few of the *Albizia* species have undergone scientific investigation and the most studied species being *A. julibrissin*, also known as the silk tree (Hocking, 1997).

3.1.1.1 Ethnomedical uses of *Albizia* species

Several species of *Albizia* find use in African and Asian traditional medicine. Barks are the most used part of the plants whereas the other parts are used infrequently. The plants find use in varied types of ailments (Table 3:1) such as worm infestations, parasitic and microbial infections, cancer, sterility, contraception and some have effects on the nervous system.

Table 3:1 Ethnomedical uses of some *Albizia* species

Species	Plant part*	Country	Uses	References
A. adianthifolia	WP	S. Africa	Stomach ailments	Mc Gaw et al., 2000
	L, R		Dysentery	
	L	Rwanda	Anthrax	Boily and Van Puyvelde, 1986
	Rb		Yaws	
			Scabies, skin diseases, eye inflammations, intestinal parasites and snakebite	Mazzanti et al., 1983
A. amara	So	India	Leprosy	Chandra et al., 1956
A. anthelmintica	B	Africa	Tapeworm infestation	Tschesche and Forstmann, 1957
		E. Africa	Anthelmintic	Kokwaro, 1976 Weiss, 1979
		S. Africa	Contraceptive	Watt and Breyer-Brandwijk, 1962
		Sudan	Anthelmintic	Tschesche and Forstmann, 1957
		E. Africa	Contraceptive, anthelmintic, purgative, sexual stimulant, swelling of the body, childbirth, syphilis, gonorrhea, nervous complaints, rheumatism and fever	Hedberg et al., 1983a
	B, L		Anthelmintic	
	R		Sexual stimulant antimalarial, tapeworm infestations	Kokwaro, 1976
		Somalia	Purgative, anthelmintic, gonorrhea, sterility and rheumatism	Samuelsson et al., 1992a
	R, Rb	Tanzania	Antimalarial, antiemetic antipyretic and	Johns et al., 1994
			Constipation	Chhabra et al., 1987

	R	S. Africa	Venereal diseases	Arnold and Gulumian, 1984
		E. Africa	Purgative, anthelmintic, sexual stimulant and gonorrhea	Hedberg et al., 1983a
	Rb		Purgative, febrifuge and anthelmintic	
		Somalia	Taenifuge, antimalarial, venereal diseases, rheumatism, childbirth and gastrointestinal disorders	Caprani et al., 1989
			Anthelmintic, childbirth, antimalarial, gastrointestinal disorders, rheumatism and venereal diseases	Arnold and Gulumian, 1984
	Sb	Sudan	Tapeworm infestation, amoebic dysentery and antimalarial	Khalid et al., 1996
		Tanzania	Anthelmintic, antimalarial, venereal diseases and fever	Chhabra and Uiso, 1991
A. antunesiana	B	Zimbabwe	Aphrodisiac	Watt and Breyer-Brandwijk, 1962
	-		Urinary schistosomiasis	Ndamba et al., 1994
	R	Tanzania	Epilepsy	Mathias, 1982
		S. Africa	Amenorrhea	Arnold and Gulumian, 1984
A. chinensis	Wp	Uganda	Childbirth	Watt and Breyer-Brandwijk, 1962
	S, L	Thailand	Swellings, boils and removal of bad blood.	Anderson, 1986
A. coriarica	B	Angola	Astringent	Bossard, 1993
		E. Africa	Menorrhalgia and post-partum hemorrhage	Kokwaro, 1976
	B, R	Kenya	Gastrointestinal problems.	Johns et al., 1995
			Coughs, stomach and skin problems	Johns et al., 1990
	S		Chewing stick	
	R, B	Uganda	Diarrhoea, snake bite, amoebiasis, syphilis, uterine fibroid	Tabuti et al., 2003b
	B		Diarrhoea, stomachache	Geiseler et al., 2002
A. falcataria		Papua-New Guinea	Chest congestion	Holdsworth, 1993
			Topical ulcers	Holdsworth et al., 1983
	Wp		Insomnia and venereal diseases	Holdsworth and Wamoi, 1982

A. fulva	B		New cuts and sores	Holdsworth and Rali, 1989
A. glaberrima	Rb	Tanzania	Bilharziasis, blocked urinary passage	Hedberg et al., 1983a
A. grandibracteata	L	Uganda	Diarrhoea	Hamill et al., 2003
A. gummifera	B	E. Africa	Antimalarial	Kokwaro, 1976
	Sb		Fever, gonorrhea, coughs and malaria	Rukunga and Waterman, 1996, 2001
		Tanzania	Malaria	Gessler et al., 1994
	L, B	Uganda	Induction of labour	Hamill et al., 2000
A. harveyi.		Tanzania	Intestinal problems, prevention of abortion and infertility	Hedberg et al., 1983a
A. julibrissin	Sb	China	Tonic	Kinjo et al., 1991
			Sedative	Zou et al., 1998
		Korea	Lung cancer	Woo, 1985
		Japan	Tonic	Ikeda et al., 1997
A. lebbek	B	India	Astringent, tonic restorative piles and diarrhoea,	Lee et al., 1995
			Tuberculosis	Pal et al., 1995
			Gonorrhea	Diddiqui and Husain, 1993
			Eczema, insect bites, asthma	Tripathi et al., 1979b
	F		Spermatorrhoea	Watt and Breyer-Brandwijk, 1962
	L		Dropsy	Reddy et al., 1989
	-		Antivenin	Selvanayahgam et al., 1994
	R	Nigeria	Brain tonic, astringent	Adesina, 1982
	Se	India	Epileptic fits	Sharma et al., 1992
		Mozambique	Aphrodisiac	Amico, 1977
	S	India	Diabetes, anthelmintic	Rajurkar and Pardeshi, 1997
	Sb		Leprosy	Zafarullah et al., 1980
A. lophantha	Wp	S. Africa	Gynecological disorders	Watt and Breyer-Brandwijk, 1962
A. lucidior	B	Nepal	Anthelminthic, diarrhoea and dysentery	Manandhar, 1995b
A. malacophylla	R	Uganda	Migraine	Tabuti et al., 2003b
A. myriophylla	B	Vietnam	Sweetener	Ito et al., 1994
A. odoratissima		India	Stomachache, dysentery	Kshirsagar and Singh, 2001

A. procera		Bangladesh	Scabies, expulsion of threadworms	Alam, 1992
		Thailand	Rejuvenating and neurotonic remedies	Ingkaninan et al., et al., 2003
A. schimperiana	Sb	Tanzania	Warts	Chhabra et al., 1984
A. versicolor	R	E. Africa	Anthelminthic	Kokwaro, 1976
			Purgative, headache, anthelmintic, Scrofula and gonorrhea	Hedberg et al., 1983a
		Tanzania	Sinus infections	
	Rb	E. Africa	Headache, purgative	
	Sb		Scrofula, cough, removal of particles from eyes, anthelmintic, purgative, skin rashes	
		Tanzania	Anthelminthic, purgative headaches, coughs	Chhabra and Uiso, 1991
		Kenya	Anthelmintic, analgesic	Rukunga and Waterman, 1996, Kokwaro, 1976
A. zygia	B	E. Africa	Antimalarial	
		W. Africa	Yaws	Watt and Breyer-Brandwijk, 1962
	R, B	Uganda	Diarrhoea, Cataract	Tabuti et al., 2003b

*B: bark; F: flower; L: leaf; R: roots; Rb: root bark; S: stem; Sb: stem bark; Se: seeds; So: seed oil and Wp: whole plant

3.1.1.2 Biological activities of the genus *Albizia*

Out of several plants, only a small percentage of the *Albizia* species have been screened for biological activities (Appendix 5).

These species are reported to display a number of biological activities such as anthelmintic activity (Kaleysa, 1975; Galal et al., 1991a; Ibrahim, 1992; Koko et al., 2000; McGaw et al., 2000; Molgaard et al., 2001; Gathuma et al., 2004), molluscicidal activity (Okunji and Iwu, 1988), cytotoxicity, which was studied using brine shrimp lethality test (Lee and Kim, 1990; Orsini et al., 1991) and several cancer cell lines (Mar et al., 1991; Pezzuto et al., 1991; Park and Kim, 1992; Kim et al., 1996; Takatsuki et al, 1996; Freiburghaus et al., 1996; Haddad et al., 2002; Liang et al., 2005) and DNA binding effects (Pezzuto et al, 1991). Some species were also screened for their

antimalarial activity (Gessler et al., 1994; Leaman et al., 1995; Wanyoike et al., 2004, Lenta et al., 2007).

Other activities studied include antitrypanosomal (Freiburghaus et al., 1996; Kamanzi Atindehou et al., 2004; Lenta et al., 2007) and antimicrobial activity against several bacteria and fungi (Sawhney et al., 1978a, Chhabra and Uiso, 1991; Yadava and Reddy, 2001; Srinivasan et al., 2001; Geyid et al., 2005; Buwa and van Staden, 2006). Others are antioxidant (Lee et al., 1998; Lau et al., 2007), antidiarrheal (Besra et al., 2002), analgesic (Gupta et al., 1981), antianaphylactic and antiasthmatic activities (Barua et al., 1997), immunomodulation (Barua et al., 2000) and antihyperglycemic activities (Singh et al., 1976). Also the studies included insect repellency (Gupta, 1987) and antischistosomal (Elsheikh et al., 1990) activities. Some have been reported to have effects on the CNS, reproductive system and smooth muscles (Vohora and Khan, 1974; Setty et al., 1976; 1977; Kamboj et al., 1977; Banerji et al., 1979a, b; De Spires et al., 2000; Gummow et al.,1992; Khalid et al., 1996; Tanira et al., 1996; Chintawar et al., 2002; Gupta et al., 2005).

3.1.1.2.1 Anticandida activity of *Albizia* species

Most of the *Albizia* species studied for antifungal activity showed no activity against *Candida* and other fungi (Dhar et al., 1968; Dhawan et al., 1977; Naovi et al., 1991; Tanira et al., 1994; Ali et al., 2001). Anticandida activity was only reported for the water extract prepared from the entire plant of *Albizia lebbek* from India (Srinivasan et al., 2001) and the butanol extract of the bark of *A. gummifera* from South Africa (Buwa and van Staden, 2006).

3.1.1.3 Bioactive compounds of *Albizia* species

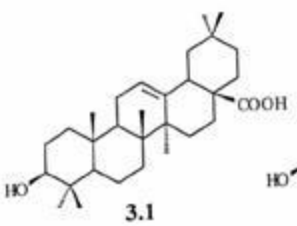
Alkaloids and triterpenoid saponins are the major groups of compounds occurring in the genus *Albizia*. The other compounds of limited occurrence, include, steroids (Orsini et al., 1991, Rawa et al., 1989, Debella et al., 2000), flavonoids (Yadava and Reddy, 2001), lignans (Kinjo et al., 1991), lipids (Agrawal and Singh, 1991), phenylpropanoids (Schaller and Schidknecht, 1992), sesquiterpenes (Ueda and Yamamura, 2000) and

tannins (Ma et al., 1997). Some of the compounds have been reported to exhibit a number of biological activities.

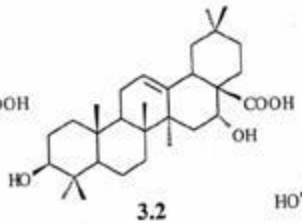
3.1.1.3.1 Saponins

Most of the *Albizia* species contain various triterpenes and their glycosides. Most of the species, including *A. lucida*, *A. versicolor*, *A. lebbeck*, *A. chinensis*, *A. gummifera* and *A. Schimperiana*, possess saponins with aglycone, based on olean-12-ene (Shrivastava and Saxena, 1988; Rawa et al., 1989; Orsini et al., 1991; Debella et al., 2000; Rukunga and Waterman, 2001). The aglycones may include oleanolic acid **(3.1)**, echinocystic acid **(3.2)** and acacic acid **(3.3)**. However, lupane based aglycones have also been reported in species like *A. lebbeck*, *A. gummifera* and *A. schimperiana* (Agrawal and Singh, 1991; Rukunga and Waterman, 1996; 2001). Some of the saponins contain an acetamido moiety in their sugar chain (Caprani et al., 1989; Orsini et al., 1991; Abdel- Kader et al., 2001; Haddad et al., 2002; Melek et al; 2007). Others like those isolated from the bark of *A. julibrissin* (Liang et al., 2005, Zou et al., 2005; 2006; Zheng et al., 2006) and the seeds of *A. procera* (Yoshikawa et al., 1998) include a monoterpene in their sugar chain.

Most of these saponins are cytotoxic, for instance isomeric saponins, albiatrioside (3-O-β-D-xylopyranosyl (1→2)- α-L-arabinopyranosyl (1→6)-2-acetamido -2- deoxy-β- D-glucopyranosyl oleanolic acid **(3.4)** and 3-O-[α-L-arabinopyranosyl (1→2)- α- L-arabinopyranosyl (1→6)-2-acetamido –2-deoxy-β- D-glucopyranosyl oleanolic acid **(3.5)** isolated from the stem and infructescence of *A. subdmidiata*. They were reported to have a significant cytotoxic activity against human ovarian cancer cell lines and a weak antiyeast activity against *Saccharomyces cerevisiae* (Abdel-Kader, 2001). Another saponin, based on echinocystic acid, 3-O-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid **(3.6)** a compound previously isolated from *A. anthelmintica* root bark (Caprani et al., 1989) was isolated from *A. procera* bark. The compound was reported to be cytotoxic against liver carcinoma cell line with IC_{50} of 10 μg/ml (Melek et al., 2007).



3.1



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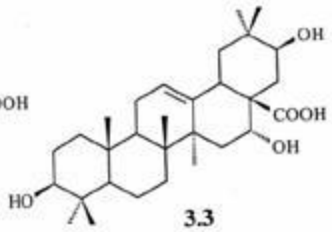
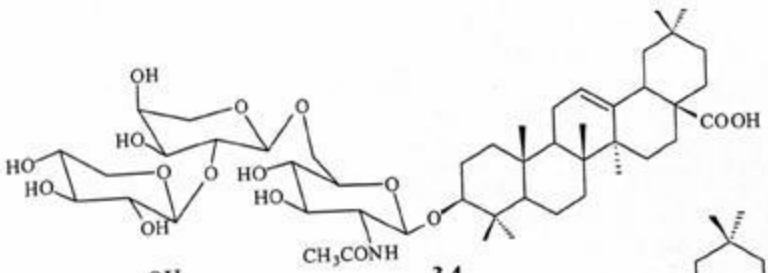


3.3

Several julibrosides having antitumor activity against several human cancer cell lines had been isolated from the stem and stem bark of *A. julibrissin* (Liang et al., 2005, Zou et al., 2005; 2006; Zheng et al., 2006). Adianthifoliosides A (**3.7**), B, and D isolated from the roots of *A. adianthifolia* were reported to have cytotoxic effect against human leukemia T cells (Jurkat cells) and its 2 prosapogenins had lymphoproliferative effects (Haddad et al., 2004). Grandibracteosides A, B and C (**3.8-3.10**) isolated from *A. grandibracteata* leaves from Uganda were reported to have antitumor activity against epidermal carcinoma and human breast cancer cell lines (Krief et al., 2005).



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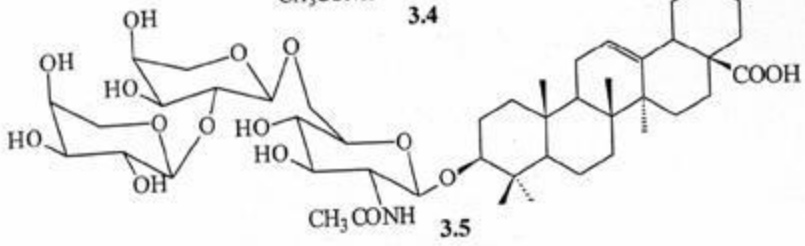

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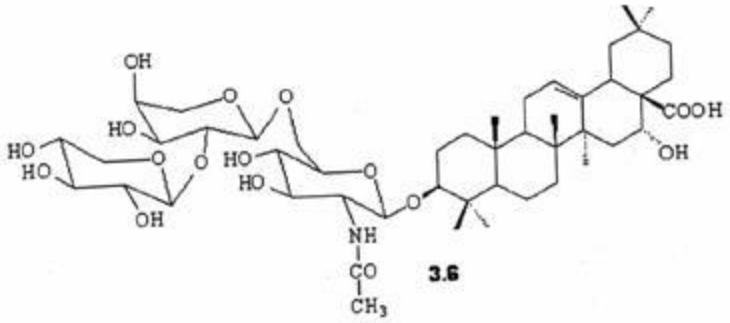

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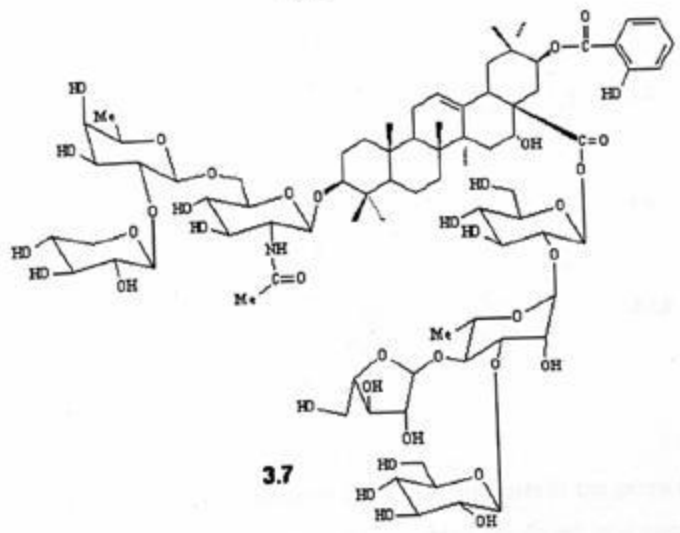

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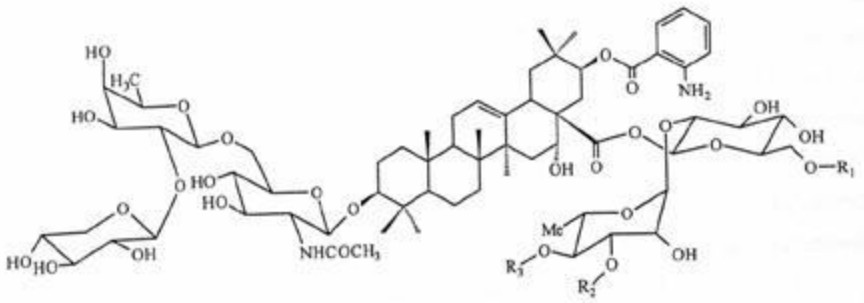

R_1	R_2	R_3	
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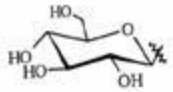
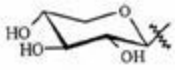
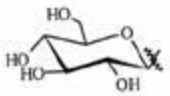
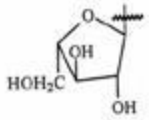
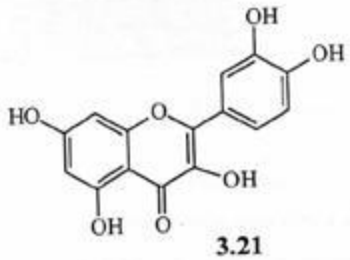
	H		**3.9**
H			**3.10**

3.1.1.3.2. Alkaloids

The other pharmacologically interesting class of compounds in the genus is the spermine alkaloids, in particular the budmunchiamines which are found in a number of species including; *A. adianthifolia*, *A. gummifera*, *A. Schimperiana.* , *A. amara* and *A. julibrissin* (Smith, 1977; Mazzanti et al., 1983; Pezzuto et al., 1991; Rukunga and Waterman, 1996). Several budmunchiamines have been reported to display a number of biological activities. The budmunchiamines L4 **(3.11)** and L5 **(3.12)** isolated from the stem bark and leaves of *A. adinocephala* from Panama were able to inhibit the *Plasmodium falciparum* plasmepsin II enzyme (Ovenden et al., 2002). Also, budmunchiamine K **(3.13)**, 6-hydroxyl budmunchiamine **(3.14)**, 5-normethyl budmunchiamine **(3.15)**, 6-hydroxyl-5-normethyl budmunchiamine **(3.16)**, and 9- normethyl budmunchiamine **(3.17)** isolated from the methanolic extract of the roots of *A. gummifera* displayed *in vitro* antiplasmodial activity using both chloroquine sensitive and resistant strains. These compounds were also active *in vivo* using the rodent parasite *Plasmodium berghei*

3.1.1.3.3. Flavonoids

Different types of flavonoids and their gycosides have been isolated from several *Albizia* species such as *A. adianthifolia, A. grandibracteata, A. lebbeck* and *A. julibrissin* (Candy et al., 1978; Gartlan et al., 1980; El Mousallamy, 1998; Yadava and Reddy, 2001). These compounds are important in that they may play a preventive role in development of cancer and heart diseases due to their antioxidant activity. Lau et al. (2007) had quantified three antioxidant constituents of *A. julibrissin*, which were all based on quercetin **(3.21).**



3.21

3.1.2 *Albizia anthelmintica* Brongn.

3.1.2.1 Morphology

Albizia anthelmintica (Figure 3:1) previously named as *Rottlera schimperi* Hochst and commonly known as a source of mussena bark (Hocking, 1997) is a deciduous tree or bush 3-9 m and sometimes up to 12 m. high. The bark is smooth and grey to brown in colour. Young branches are glabrous or sometimes shortly pubescent. The leaves and pinnae are glabrous to shortly pubescent they are obliquely obovate to suborbicular and mucronate at the apex. Flowers are usually borne on leafless twigs on pedicel. Calyx is pale greenish, glabrous to sparsely finely pubescent outside. Corolla is pale greenish, glabrous or puberulous on and near lobe margins. Staminal tube scarcely exserted beyond corolla, filaments are white in colour. Pods are oblong, glabrous, or occasionally puberulous all over and straw coloured. Seeds are flattened and round.

plant as a purgative and febrifuge (Hedberg et al., 1983a, Samuelsson et al., 1992a). Furthermore the plant is used for gynecological conditions including cure of sterility (Samuelsson et al., 1992a), control of haemorrhage during childbirth (Bally, 1937; Mazzanti et al., 1983; Caprani et al., 1989) and prevention of pregnancy (Watt and Breyer-Brandwijk, 1962). In many African countries the plant is also used as sexual stimulant (Bally, 1937, Watt and Breyer-Brandwijk, 1962). An overdose is fatal (Kokwaro, 1976, Samuelsson et al., 1992a).

3.1.2.3 Previously reported biological activities of *Albizia anthelmintica*

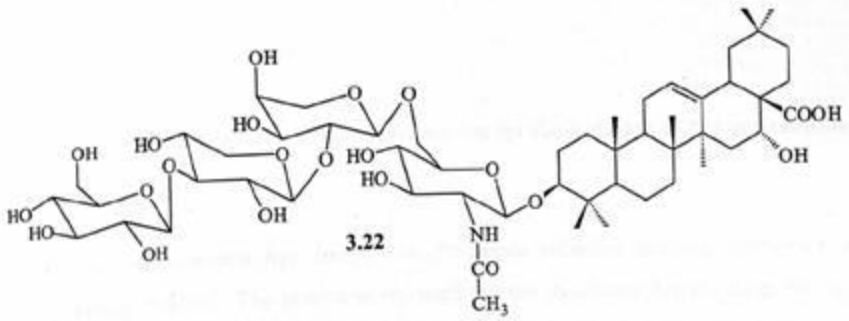
The plant is well known for its anthelmintic property, which has been shown by the water and methanolic extracts of the stem bark, roots and root bark on various worms (Galal et al., 1991a; b; Koko et al., 2000; Gathuma et al., 2004). In some cases the anthelminthic activity was comparable to that of albendazole (Koko et al., 2000). Other biological activities include antischistosomal (Elsheikh et al., 1990), spasmogenic (Khalid et al., 1996) and antimitogenic (Desta, 1995) activities. In Tanzanian the plant has been reported to have a weak antibacterial activity against *Neisseria gonorrhea* (Chhabra and Uiso, 1991). Also the plant exhibited antimalarial activity against *Plasmodium falciparum* (Weenen et al., 1990) but no activity in the brine shrimp lethality test (Massele and Nshimo, 1995). However, the plant has never been evaluated for antifungal activity.

3.1.2.4 Compounds previously isolated from *Albizia anthelmintica*

The earliest phytochemical work on the root bark of the plant was done before the advent of modern techniques of structure elucidation and hence it only gave partial structures of the compounds present in the plant. Tchesche and Forstmann (1957) and Tchesche et al. (1966) reported the isolation of musennin triterpene saponin. The triterpene aglycone was identified as echinocystic acid and the sugars were identified as three molecules of L-arabinose and one molecule of D-glucose. Not much was discussed regardingthe connectivity of these sugar moeties. It was not until 1969, when using NMR and other techniques that the actual structure of musennin was elucidated and another saponin, desglucomusennin, was isolated by Tchesche and Kammerer (1969). However, they only

gave the connectivity of the sugars but they did not give the type of anomeric configurations for the sugars.

Caprani et al. (1989) isolated three saponins from the root bark of *A. anthelmintica* a plant growing in Tanzania and provided their actual structures. These were; 3-O-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)] [α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid **(3.22),** 3-O-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl(1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid **(3.6)** and 3-O-)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid **(3.23)**. They also found that the saponin 3-O-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid **(3.6)** had molluscicidal activity against *Biomphalaria glabrata*. Mazzanti et al. (1983) reported the presence of histamine in the stem and root bark of Somalian plants. The root bark was also reported to be very rich in sucrose (Tchesche Forstmann, 1957) and the seeds from plants growing in Tanzania have been reported to be rich in certain amino acids including albizzine (L-α-amino-β-ureidopropionic acid) **[3.24]**, S- (β-carboxyl-isopropyl)-L-cystein, S- (β-carboxyl –ethyl)- L-cystein and 4-hydroxyl-pipecolic acid (Krauss and Reinbothe, 1970).



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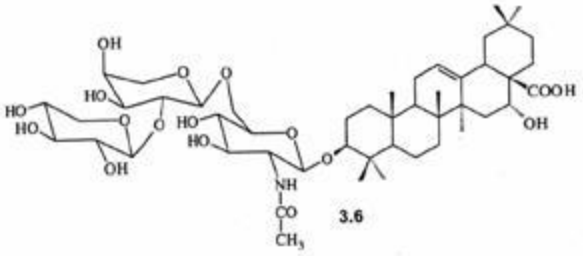

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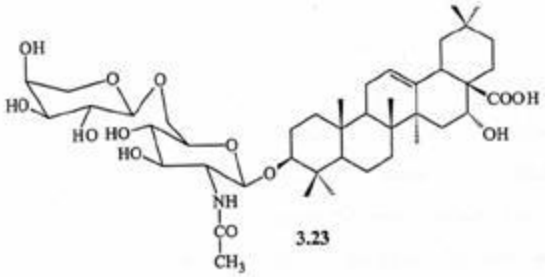

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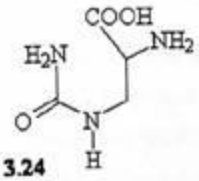


3.24

3.1.2.5 Prioritization of *Albizia anthelmintica* for the isolation of the anticandida compound

Albizia anthelmintica was among the 36 plants collected through interviews with traditional healers. The plant was reported by two traditional healers from Morogoro and Coast regions to be used for vaginal candidiasis. From the literature there is no indication of the plant being screened for antifungal activity before eventhough three saponins based on echinocystic acid have previously been isolated from the plant. This

plant showed the largest inhibition spot of 15mm (Chapte r 2; Table 2:5), furthermore it showed other 3 small inhibition spots (4-6mm). These two factors made this plant to be given the highest priority when compared with plants screened for anticandida activity in this study.

3.2 METHODOLOGY

3.2.1 Isolation of compounds from *Albizia anthelmintica*

3.2.1.1 Bulk plant collection

The root barks of *A. anthelmintica* were collected in August 2004, from plants growing wild in Melela, Morogoro region, Tanzania. The locality and time of collection were the same as those for the initial collection (Chapter 2). The plant was identified in the field by a botanist and a herbarium specimen was prepared and compared to the Herbarium specimen prepared in the initial collection. Prior to extraction the root barks were freed of the soil by shaking and dried in the open for a week.

3.2.1.2 Extraction and fractionation of *A. anthelmintica* root bark

Five kilograms of *A. anthelmintica* root bark were extracted by maceration using aqueous methanol (80%). In this method, the ground root bark was soaked in aqueous methanol (80%) for one week with occasional shaking to enhance the extraction process. The extract was then separated from the marc by decantation followed by filtration using filter funnels fitted with Whatman No. 1 filter papers (Whatman International Limited, Maidstone, England). The process was repeated twice so as to obtain enough extract. The resultant extract was concentrated *in vacuo* using a Buchi rotary evaporator set at 40-50° C to get rid of methanol. The aqueous extract was partitioned with *n*- hexane followed by ethylacetate (Figure 3:2). The *n*-hexane and ethylacetate extracts were dried *in vacuo* and the final aqueous extract was freeze-dried using Edwards freeze drier (Edwards High Vacuum International Crawley, Sussex, England). The three extracts were screened for anticandida activity using bioautography agar overlay (Rahalison et al., 1991). The final

aqueous extract was the most active and it was therefore used for the isolation of the active compounds.

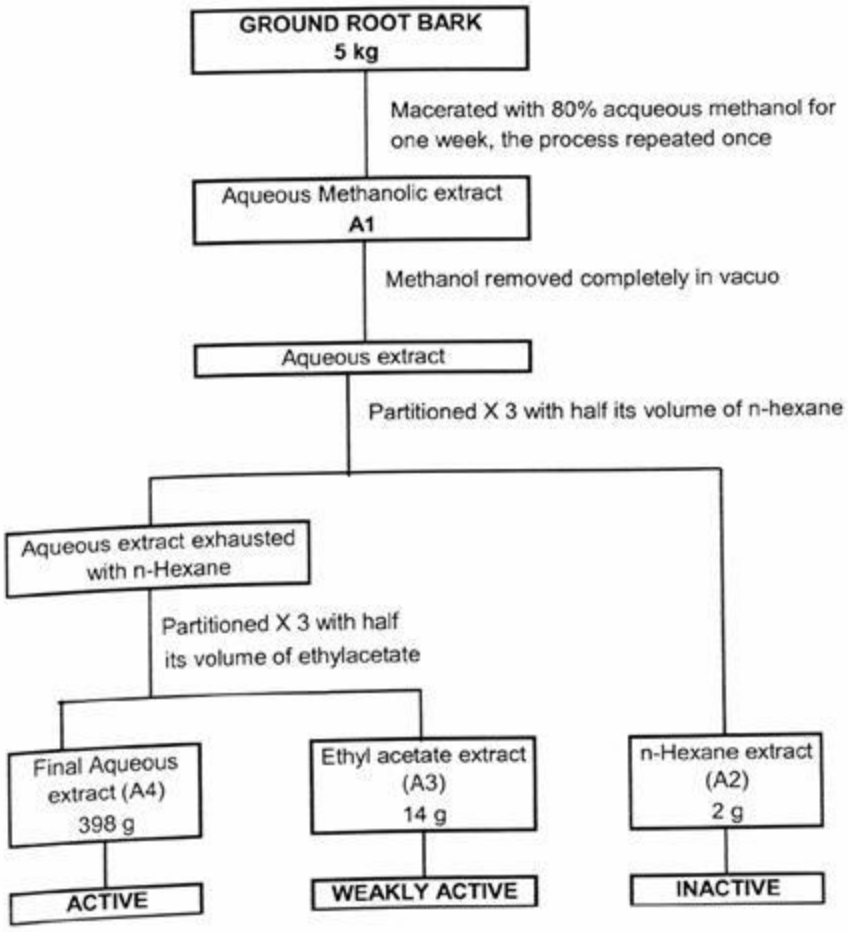


Figure 3:2 Scheme of extraction and partitioning of the extract of *Albizia anthelmintica* root bark

3.2.1.3 Isolation of compounds from the aqueous fraction of the aqueous methanolic extract of *A. anthelmintica* root bark

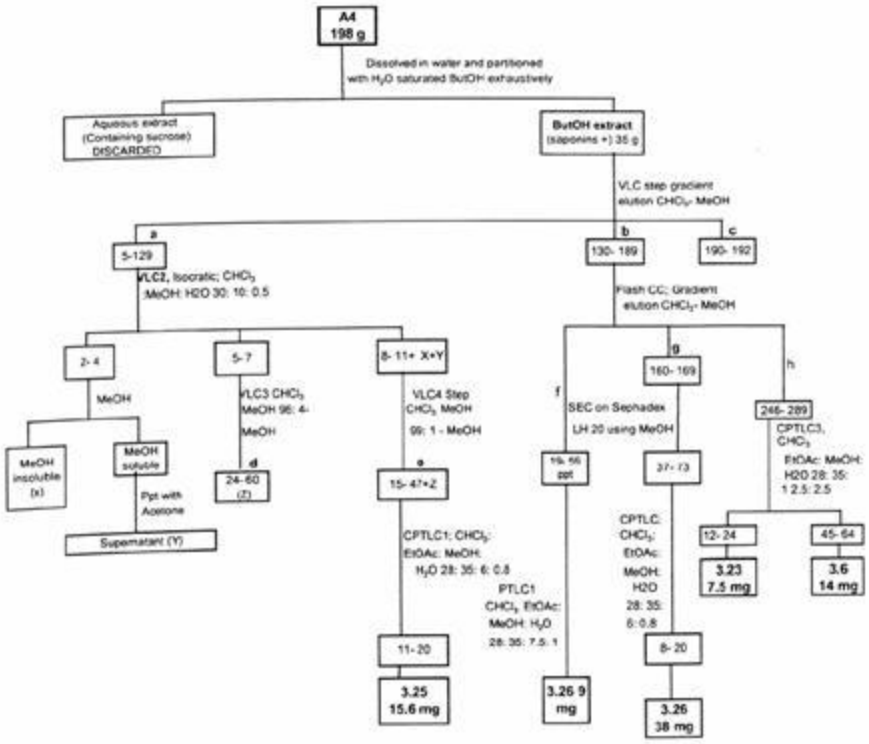
3.2.1.3.1. Initial isolation of compounds

The initial isolation of compounds was done at the School of Pharmacy, MUHAS, in Tanzania using normal column chromatography, preparative thin layer chromatography and size exclusion chromatography. Most of the compounds isolated were impure and insufficient, however, they were sent to Dr. C. Wright of the University of Bradford, UK for ^{1}HNMR, ^{13}CNMR, DEPT and ES-MS analysis. The ^{1}HNMR indicated that most of the compounds were saponins and also that the root bark contained a lot of sucrose.

3.2.1.3.2. Further fractionation and isolation of compounds

Further isolation of compounds was carried out at the Central Institute of Medicinal and Aromatic Plants (CIMAP), India. A number of separation techniques were employed for the isolation of the compounds from the root bark extract. These included partitioning of compounds between two immiscible solvent systems, precipitation and chromatography. Some of the techniques did not give the desired results, the techniques, which were able to give either separation or clean up of the mixture of compounds are the ones shown in the schemes of isolation of compounds (Figure 3:3 and 3:4).

Different types of chromatographic techniques were employed and these included open column chromatography (CC), vacuum liquid chromatography (VLC) run by Buchi type B-189 water aspirator, flash column chromatography using both descending and ascending modes operated by a Buchi pump manager C-615, size exclusion (gel filtration) chromatography using Sephadex LH 20, centrifugal accelerated radial thin layer chromatography (Chromatotron) [CPTLC] (Chromatotron, Harrison research, USA) and preparative thin layer chromatography (PTLC) on silica gel G_{60} F_{254} plates (0.5 mm thick, Merck, Darmstadt, Germany).



Eluting solvents: a: $CHCl_3$- $CHCl_3$: MeOH 92.5: 7.5; **b:** $CHCl_3$: MeOH 9:1- 1:1; **c:** $CHCl_3$:MeOH 1:1; **d:** $CHCl_3$:MeOH 94:6; **e:** $CHCl_3$:MeOH 98:2-95:5; **f:** $CHCl_3$:MeOH 99.5:0.5; **g:** $CHCl_3$:MeOH 98:2- $CHCl_3$:MeOH 9:1 **h:** $CHCl_3$:MeOH 3:1

Figure 3:3 Scheme oisolation of Compounds **3.6, 3.23, 3.25** and **3.26** from the final aqueous extract (A4) of the root bark of *A. anthelmintica*

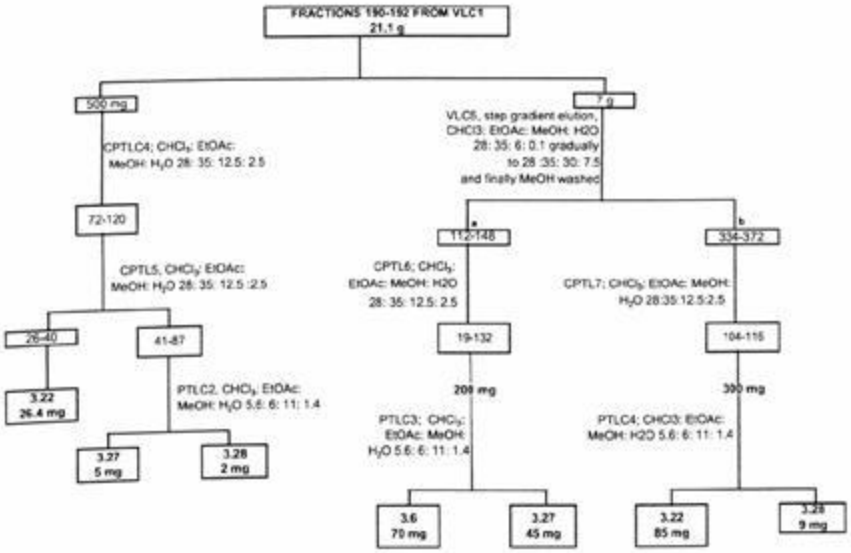


Figure 3:4 Scheme for isolation of Compounds **3.6**, **3.22**, **3.27** and **3.28** using fractions 190- 192 of VLC 1 (Figure 3:3)

For open column chromatography silica gel (60-230 mesh) for column chromatography (Sisco Research Laboratories PVT limited Bombay, India) was used while for flash chromatography silica gel, 230-400 mesh (Qualigens fine chemicals Division, Glaxo SmithKline Pharmaceutical limited Mumbai, India), and silica gel H for TLC (Thomas Baker; chemicals, Mumbai, India) were used. The latter was also used for VLC.

The rotar plates for CPTLC were prepared by using silica gel H, calcium sulphate and distilled water in recommended proportions. For instance, a 1mm thick plate was made by mixing well 36 g of silica gel H and 12 g of anhydrous calcium sulphate, which was chilled in a deep freezer at 0- 5 °C. The chilled mixture was mixed with 95 ml of chilled distilled water, shaken vigorously for about a minute and poured onto the clean rotar. The bottom of the rotar plate was tapped in order to reduce air bubbles and the rotar was placed on a flat surface, covered for one hour and allowed to settle and dry. It was then heated in an oven at 70 °C overnight after which the rotar was scrapped to a thickness of

1 mm. In order to obtain a good rotar, the mixture of silica gel, calcium sulphate and water had to be very cold so as to reduce air bubbles.

During chromatography separations, gradient, step gradient and isocratic elutions were carried out using either analytical or distilled solvents. The eluate or column separations were continuously monitored by thin layer chromatography using aluminium backed silica gel G_{60} F_{254} plates (200 μm thickness, Merck, Darmstadt, Germany), developed with appropriate solvent systems. During the course of column separations, especially in gradient elution, the TLC mobile phases had to be adjusted so as to suit the compounds eluting from the column. TLC plates were visualized using a UV lamp set at 254 and 365 nm as a non-destructive method followed by dipping in either 10% aqueous sulphuric acid, or vanillin sulphuric acid reagent and heated at 100 °C which resulted into visible coloured spots. The fractions were pooled according to their TLC profiles.

During PTLC, plates used were normally 0.5 mm thick and a maximum of 50 mg was applied on each plate. Multiple and continuous isocratic development in which the TLC plates were left in isocratic system for several hours after the solvent front had reached the top of the TLC plate were used to effect separation. This type of TLC development allowed the closely eluting bands to be separated. The compounds being separated could not give clear strong bands in UV hence during application of the mixture of compounds to be separated onto the plates, indicator spots were applied on far left and right sides of the plates. After development of the plates a clear demarcation was made between indicator spots and the rest of the plate by carefully scratching off the adsorbent using a clean spatula. The visualizing reagent was then carefully applied to the separated strips having the developed indicator spot on either side of the plate followed by heating. The resulting coloured spots were used to locate the appropriate sections containing the desired compounds being separated. After the appropriate sections of the plate were marked, the adsorbent containing the visualizing reagent was carefully scratched off and the plate wiped with a piece of cotton wool moistened with methanol, followed by scratching off the adsorbent containing the desired compounds which were then extracted from adsorbent with methanol.

All isolated compounds were usually developed on TLC plate by using at least three solvent systems. This was necessary to ensure that the compounds obtained were not mixtures. The compounds isolated from *Albizia anthelmintica* root bark were as shown on the TLC profile (Figure 3:5).

Figure 3:5 TLC profile of Compounds isolated from *Albizia anthelmintica* root bark*

3.2.2 Structure elucidation of the isolated compounds

Identification of compounds usually involves use of a combination of different techniques including Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), Infrared (IR) and Utraviolet (UV) Spectroscopy. In this study the first three techniques were used so as to identify the isolated compounds. In addition, the melting points for some of the isolated compounds were also determined.

The isolated compounds were routinely subjected to one dimensional (1D) NMR experiments including Proton NMR (^{1}HNMR), Carbon NMR (^{13}CNMR) and

* Mobile Phase: Chloroform: Ethyl acetate; Methanol: Water 28:35:55:7

distortionless enhancement through polarisation transfer (DEPT). In case where the structure of a compound could not be confirmed using the above techniques then two dimensional experiments including both homonuclear correlation and heteronuclear correlation experiments were performed. The homonuclear correlations involved ^{1}H -^{1}H correlation spectroscopy (COSY) and the heteronuclear correlations involved heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple bond connectivity (HMBC).

3.2.2.1 Nuclear magnetic resonance spectroscopy (NMR)

The NMR spectra were recorded at the NMR laboratory at the Central Institute of Medicinal and Aromatic Plants (CIMAP). A Bruker 300 spectrometer using 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR was used. The isolated compounds were dried and weighed (14- 100 mg) dissolved in a maximum of 0.2- 0.5 ml of deuterated solvents (Aldrich, USA). Deuterated Pyridine and Dimethylsulfoxide were used as solvents of choice since the isolated compounds were found to be very soluble in them. The resultant solutions were filtered and added to the Clean and dry NMR tubes (5 mm id). ^{1}H NMR, ^{13}C NMR and DEPT were performed for each compound but 2D experiments including COSY, HSQC and HMBC were recorded when necessary. For those compounds obtained in small amounts NMR spectra were recorded at Central Drug Research Institute (CDRI), Lucknow, India on a Bruker instrument operating at 400 MHz and 100 MHz for Proton and carbon NMR, respectively.

3.2.2.2 Determination of molecular weights of the compounds

Electrospray ionization (ESI) was employed using Shimadzu LC-PDA-MS instrument equipped with Mass detector LCMS 2010, Photo Diode Array (PDA) Detector SPD-20A, pump 20 AD, column Oven CTO 20A. One milligram of each compound was separately dissolved in 1ml of methanol (LC-MS grade, Riedel-de-Haën) and 10 µl of the resultant solution was injected and passed through SS tubing directly to PDA and to the Mass detector, after splitting in a ratio of 1:4, the column was not used for separation. The mobile phase used was water: methanol (3:7) at a flow rate of 1ml/min. Mass acquisition conditions were Acquit ion mode: SCAN, Ionization Interface: ESI, Ionization mode:

Both +ve and –ve simultaneously, CDL temperature: 250 °C, N_2 flow rate: 1.5ml/min and heat block temperature: 250 °C.

3.2.2.3 Infrared spectroscopy (IR)

The IR spectrum of some of the compounds was determined using FT-IR Spectrum BX, Perkin Elmer. Three to four milligrams of the compounds was used to prepare KBr pellets used for recording the IR spectra..

3.2.2.4 Determination of melting points for the isolated compounds

Melting points of the isolated compounds were determined by using Jain Scientific Glass work melting point determination apparatus, India and/or Ez melt Automatic melting point apparatus, Stanford Research System, UK.

3.2.3 Determination of Minimum Inhibitory Concentrations (MICs) for the isolated compounds

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints (Andrews, 2001).

3.2.3.1 Broth micro-dilution assay for MICs determination

The MICs of the isolated compounds were determined for two standard strains of *Candida albicans* (ATCC 90028 and MTCC1637), and clinical isolates of *C. albicans* using broth microdilution assay. A 96 well microtitre plate consisting of 12 columns (1-12) and eight rows (A-H) (Figure 3:6) was used. Rows A-F contained the different compounds to be tested, rows G and H were the positive and negative controls respectively. Amphotericin B and clotrimazole were used as reference antifungal drugs. One hundred and fifty microlitres (150 µl) of Sabouraud Dextrose Broth (SDB) was added to all wells of the microtitre plates. To the first six wells of the first column (A-F), an appropriate volume of the stock solutions of the test compounds dissolved in dimethyl

sulfoxide (DMSO) were separately added and the volume was adjusted to 300 µl by addition of SDB so as to obtain the required initial concentration. For example, 7.5 µl of the stock solutions of the test compounds (10 mg/ml) was used in order to obtain the initial concentration of 250 µg/ml. A two fold serial dilution of the test compounds was used across the plate so as to obtain lower concentrations of the test compound.

Ten (10) µl of an overnight inoculum of the test fungi equivalent to 0.5 McFarland, dilution and also quantified by colony plate count of 1.0 X 10^6 to 5 X 10^6 CFU/ml was added to the wells, except wells on the last row, to give a final concentration of inoculum of approximately 10^4 CFU/ml. The last row contained DMSO as a solublizing solvent. The trays were incubated at 28 °C for 24 hrs. The concentration of the test compound exhibiting no visible growth after 24 hrs when compared to the negative control tube was taken as the minimum inhibitory concentration.

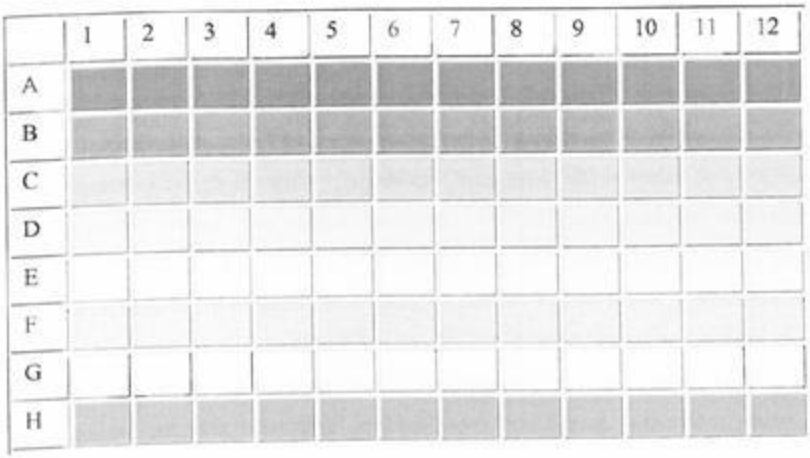
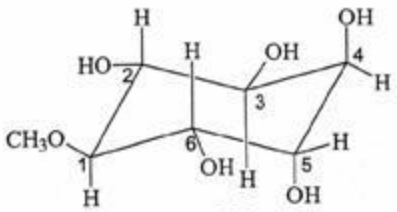


Figure 3:6 Microtitre plate used for determination of MICs

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and characterization of *O*-methyl cyclitol (3.25)



3.25

The active aqueous extract obtained after extraction and partitioning (Figure 3:2) was partitioned between water and water-saturated butanol. The saponin rich butanol extract (35 g) was subjected to vacuum liquid chromatography (VLC1) [Figure 3:3], step gradient eluted with chloroform and methanol in different proportions. Fractions 5- 129 (976.5 mg) eluted with chloroform 100% to chloroform: methanol 92.5: 7.5 were rich in compound **3.25** hence pooled together and subjected to further purification on to VLC2 (Figure 3:3). Isocratic elution of VLC2 was carried out with chloroform: methanol: water 30: 10: 0.5. Fractions 2- 4, 5- 7 and 8- 11 contained compound **3.25** in varied proportions and were separately pooled. The pooled fractions 2- 4 (189.8 mg) produced methanol insoluble (X) and soluble when methanol was added. The methanol soluble fraction was precipitated with acetone, to obtain a supernatant solution (Y). Both X and Y were rich in compound **3.25** and were added to the pooled fractions 8- 11, their combined weight was 358. 9 mg. These were further subjected to VLC 4 (Figure 3:3). Step gradient elution of VLC4 was carried out with chloroform and methanol in different proportions starting with 1% methanol in chloroform. Fractions 5- 47 (50 mg) eluted with 2 to 5 % methanol in chloroform were rich in compound **3.25,** hence were set apart. The pooled fractions 5- 7 of VLC2 were subjected to VLC3 (Figure 3:3) eluted with chloroform and methanol in different proportions. Fractions 24- 60 (Z) [11.6 mg] which were rich in **3.25** were combined with fractions 5- 47 from VLC4 and subjected to (CPTLC1) [Figure 3:3] eluted with chloroform: ethylacetate: methanol: water 28: 35: 6: 0.8. Fractions 11- 20

which showed a single spot in various solvent systems afforded 15.6 mg of a shiny amorphous compound **3.25**.

The compound **3.25** was amorphous, colourless and shiny, its electro spray mass spectra (ES-MS) showed *m/z* at 193 and 217 in –ve and +ve mode respectively. The *m/z* at 193 is a molecular ion less H (M^+ -H) and *m/z* at 217 was due to M^+ +Na.

The compound was characterized on the basis of its ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, COSY, HMBC and ES-MS data. The ^{1}H NMR spectrum of compound **3.25** (Figure 3:7, Table 3:2) showed a singlet at δ 3.92 (3H) for methoxyl protons, two triplets centred at δ 4.14 (*J* 9.6 Hz, 1H) and δ 4.62 (*J* 9.6 Hz, 1H) and two multiplets centered at δ 4.73 (2H) and δ 4.79 (2H) for 6 carbinolic methine protons in the molecule.

The ^{13}C NMR spectrum of compound **3.25** (Figure 3:8, Table 3:2) showed a total of 7 intense peaks indicating that compound **3.25** contained at least 7 carbon atoms. The DEPT-135, showed 1 methyl and 6 methine carbons while DEPT-90 (Figure 3:9) showed 6 methine carbons.

The ^{1}H NMR (Figure 3:7) of **3.25** showed a characteristic signal at δ 3.92 and the furthest upfield carbon (δ 60.9) in ^{13}C NMR (Figure 3:8) corresponding to the methoxyl group. The methoxyl was attached at C-1 (δ 85.9) as was indicated in HMBC (Figure 3:12) by the cross peak due to long- range correlation between methoxyl carbon (δ 60.9) and the H-1 (δ 4.14). The presence in the ^{1}H NMR of **3.25** (Figure 3:7) of the downfield signals at δ 4.14, 4.62, 4.73 and 4.79 suggested that compound **3.25** was polyhydroxylated which was further supported by the presence in the ^{13}C NMR spectrum of compound **3.25** of downfield signals at δ 72.4, 73.2, 73.8, 74.3, 74.8, and 85.9. From ^{13}C NMR and DEPT spectrum of compound **3.25**, the molecular formula of **3.25** was deduced as $C_7H_{14}O_6$, which was also supported by the ES-MS (Figure 3:13), which showed *m/z* at 193 corresponding to M^+ -H, which meant that the molecular weight of compound **3.25** was 194.

Table 3:2 NMR spectral data of compound 3.25 compared to that of D-bornestol

C	^{13}C NMR*	HSQC (δ values for attached Hs	HMBC C-H correlations	1H-1H COSY coupling Hs	D-bornestol**	
					^{1}H NMR	^{13}C NMR
1	85.9d	4.14t (1H)	All protons	4.14 and 4.62, 4.73	3.06	80.8
2	74.8d	4.62t (1H)	4.14 , 4.73 ^{2}J; 4.79 ^{3}J		3.49	72.0
3	72.4d	4.73m (1H)	4.14 ^{3}J		4.16	68.00
4	74.3d	4.79m (1H)			3.13	74.80
5	73.8d	4.79m (1H)			3.47	72.70
6	73.2d	4.73m (1H)	4.79 ^{2}J		3.35	71.50
CH_3O	60.9q	3.92s (3H)	4.14 ^{2}J		3.28	57.10

*Done in Pyridine D_5 at 400 MHz and 100 MHz for ^{1}H NMR and ^{13}C NMR respectively, DEPT was used to determine the multiplicities; **Obendorf et al., 2005

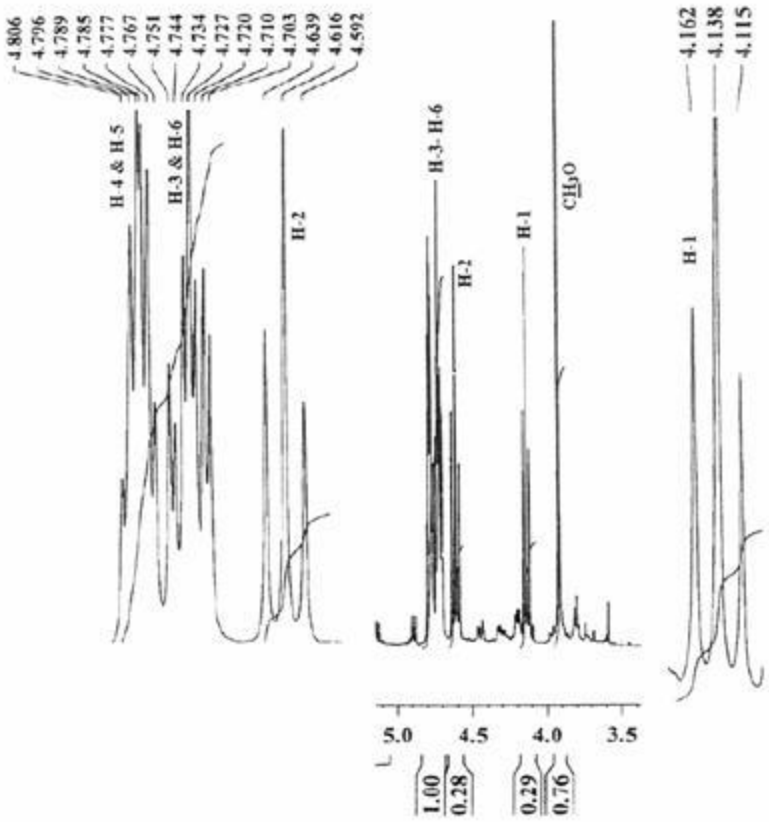


Figure 3:7 ^{1}H NMR Spectrum of Compound **3.25**

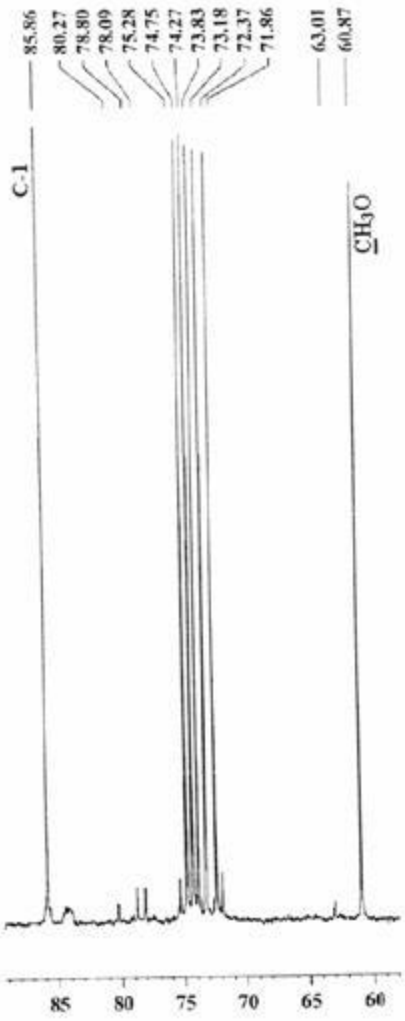


Figure 3:8 ^{13}C NMR Spectrum of Compound **3.25**

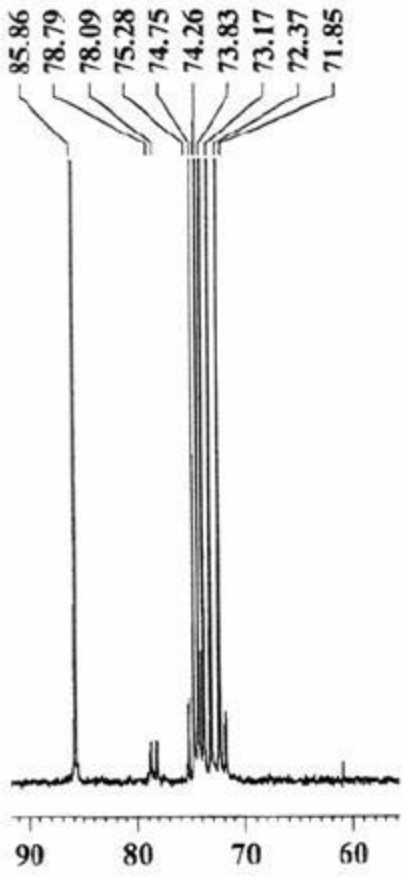


Figure 3:9 DEPT-90 Spectrum of Compound **3.25**

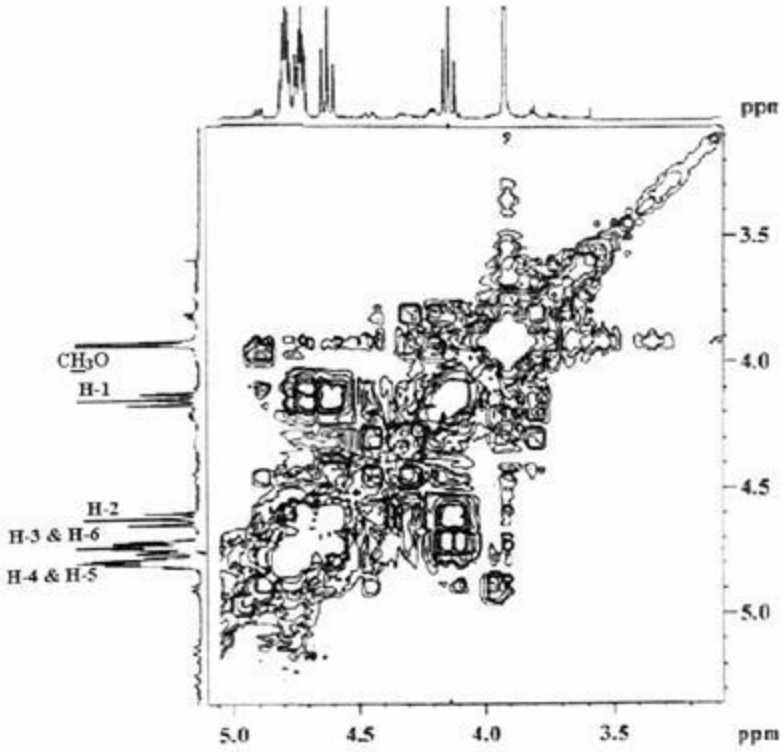


Figure 3:10 COSY Spectrum of Compound **3.25**

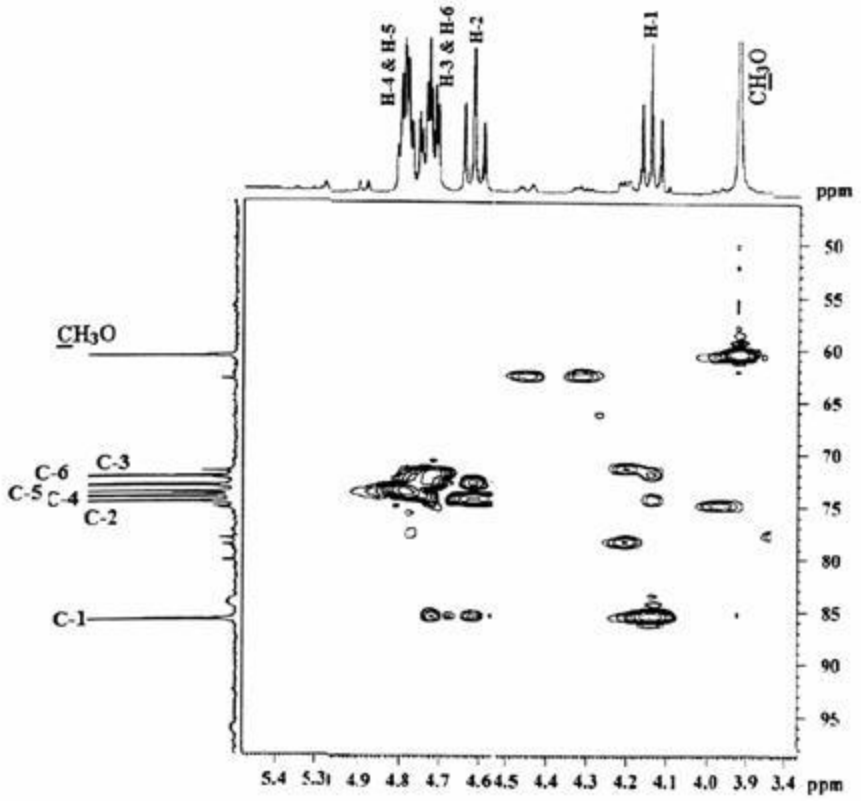


Figure 3:11 HSQC Spectrum of Compound **3.25**

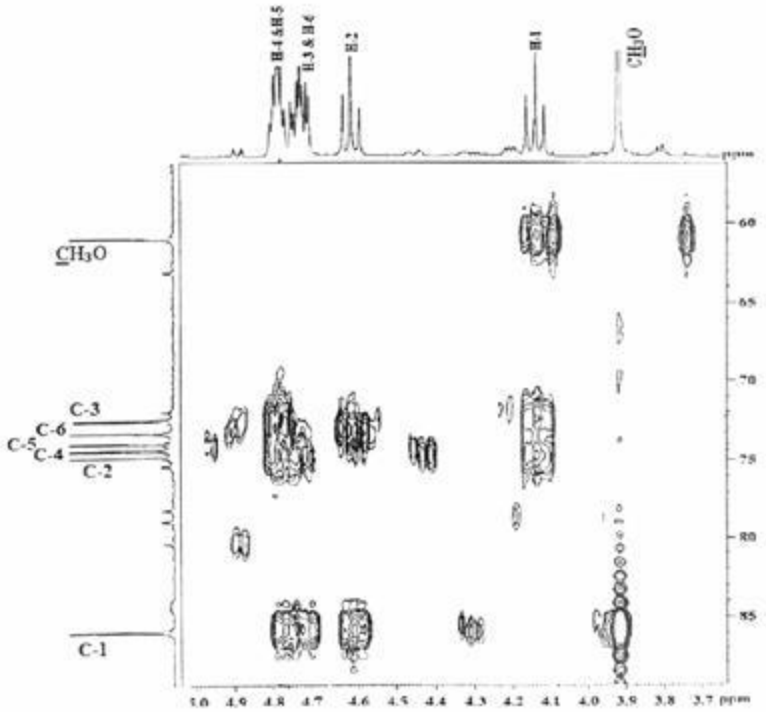


Figure 3:12 HMBC Spectrum of Compound **3.25**

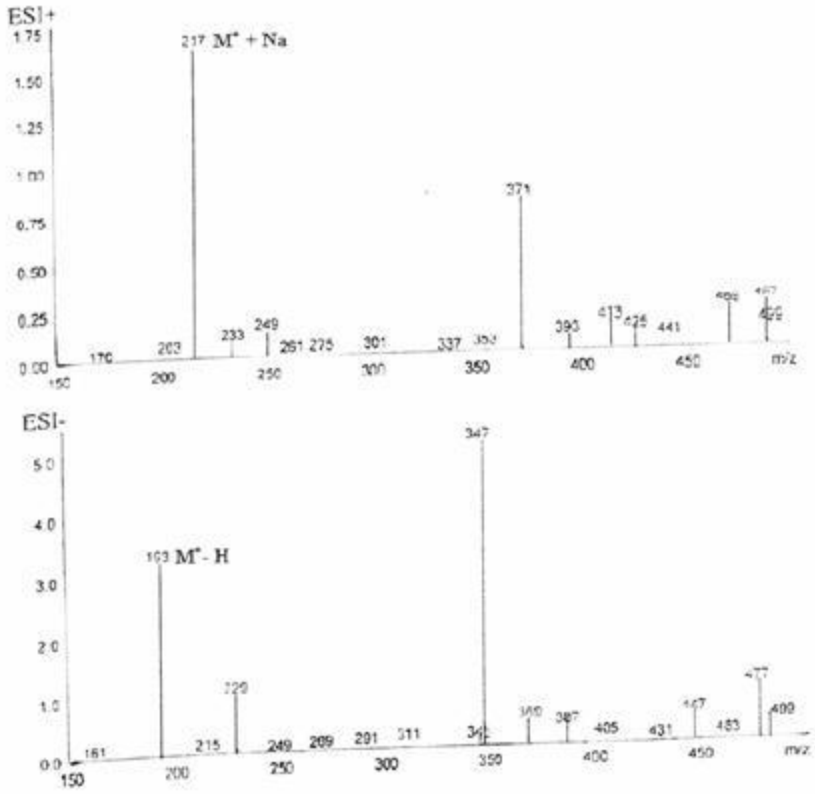


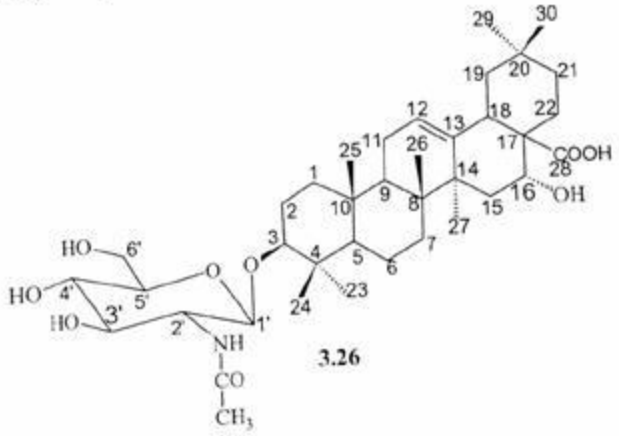
Figure 3:13 ES- MS of Compound **3.25**

The ^{1}H NMR (Figure 3:7) of compound **3.25** showed two clear triplets at δ 4.14 (*J* 9.6 Hz, 1H) [H-1] and δ 4.62 (*J* 9.6 Hz, 1H) [H-2] and also showed one other complex triplet and a multiplet. From the observed splitting pattern as well as HSQC spectrum (Figure 3:11) and COSY (Figure 3:10) of **3.25** it was deduced that H-1 (δ 4.14) was coupled to two protons at δ 4.62 [H-2] and 4.73 [H-6] on either side of it in the puckered ring. The presence of a triplet for H-2 meant that it was also coupled to another proton. Furthermore, the coupling constant of 9.6 Hz meant that the other proton was also axial. Furthermore, the integration value for the peak at δ 4.73 corresponds to two protons, H-6 and H-3 which couple with H-1 and H-2, respectively. The analysis of the complex

triplet at δ 4.73 indicated that the main triplet split by coupling with the protons at δ 4.79 (H-4, H-5) had a coupling constant of 9.6 Hz. The larger coupling constant indicates that these protons were also axially oriented. The complex triplet and the multiplet at δ 4.79 had coupling constants in the range of 1.6 Hz to 4.0 Hz, which meant that the remaining two protons at δ 4.79 were equatorially oriented and the observed coupling constants were due to a/e coupling with its neighbouring protons (H-3 and H-6) and e/e among themselves. From the spectroscopic data and the observed coupling constant the structure of **3.25** is as suggested above.

It should be noted, however, that compound **3.25** has 6 stereo centers, which means that there are 2^6 stereoisomers of which one is compound **3.25**. The ^{13}C NMR chemical shifts were closely related to those of methyl –1D-myo-inositol (D-bornestol) [Table 3:2] (Obendorf et al., 2005) meaning that compound **3.25** is one of the methyl -myo-inositol isomers. This type of compounds is being reported in this plant for the first time.

3.3.2 Isolation and Characterization of compound 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid (3.26)



3.26

Compound **3.26** was obtained by subjecting the fractions 130-189 obtained from VLC 1 to flash chromatography (Figure 3:3). Step gradient elution was carried out using

chloroform and methanol in different proportions. Fractions 19- 59 and 60- 169 which were eluted with 0.5% and 2- 10% methanol in chloroform respectively were rich in compound **3.26** hence were separately pooled. The pooled fractions 19-59 (23 mg) were subjected to preparative thin layer chromatography (PTLC1) developed in Chloroform: ethyl acetate: methanol: water 28: 35: 7.5: 1 which afforded **3.26** (9 mg) as a white powder. Also, fractions 60-169 (123 mg) were subjected to gel filtration (Sephadex LH 20) [Figure 3:3] eluted with methanol. Fractions 37- 73 (97 mg) were rich in compound **3.26** hence were pooled and subjected to centrifugal PTLC on a chromatotron (CPTLC1) [Figure 3:3]. Chromatotron fractions 8- 20 resulted in the isolation of 38 mg of a white powder **3.26**, which gave a single spot in several solvent systems.

Compound **3.26** had a melting point of 211 °C and in its electro spray mass spectra (ES-MS) showed base peaks at *m/z* 674 and 698 in –ve and +ve mode respectively, the *m/z* 674 is the molecular ion less H (M^+ -H) while m/z 698 is M^+ + Na. The IR spectrum of **3.26** showed a broad band centred at 3424 (overlapping O-H, N-H signals), 2945 (C-H), 1702 (COOH), 1655 (HNC=O), 1560, 1544, 1459, 1377, 1108, 1077 (C-O-C) 1027 and 981 cm^{-1}.

The structure of **3.26** was determined on the basis of its ^1H NMR, ^{13}C NMR, DEPT, IR and ES-MS spectroscopic data. Proton (^1H) NMR (Table 3:3) of **3.26** showed 6 upfield singlets at δ 0.82, 0.97, 1.00, 1.04, 1.17 and 1.80 for 7 tertiary methyl groups. One of the signals appearing at δ 1.17 represented two methyl groups as it was indicated by integration value for this peak. Other characteristic signals in the spectrum were a singlet at δ 2.15 ascribed to the methyl protons in the acetyl group, an olefinic proton at δ 5.61, carbinolic methine proton at δ 5.18, a doublet for anomeric proton at δ 5.03 (*J* 8.4 Hz), a doublet centred at δ 8.90 (*J* 8.8 Hz), several other sugar protons between δ 3.94- 4.53 and two doublets of doublets at δ 3.27 (*J* 3.2, 10.4 Hz) and δ 3.60. The ^{13}C NMR of **3.26** (Table 3:4) showed 34 intense signals suggesting the presence of at least 34 carbon atoms in the molecule. These peaks were for 8 methyl, 9 methylene, 11 methine and 8 quaternary carbons according to DEPT. A peak at δ 33.5 represent methyl and a methylene whereas a peak at δ 47.4 represented a methine and methylene carbon atoms

as indicated by DEPT. Six of the methine carbons appearing downfield at δ 72.2, 74.4, 76.2, 78.3, 89.5 and 105.0 were oxygenated. Similarly one of the methylene carbons appearing at δ 63.1 was oxygenated.

Table 3:3 **^{1}H NMR chemical shifts of selected peaks (in δ values) of 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid (3.26)**

H*	Observed δ Values	Reported δ Values**
3	3.27dd (J 3.2, 10.4Hz)	
12	5.61s	5.23?
16	5.18s	
18	3.60dd	
NHCOMe	2.15s	2.15
NH	8.90d (J 8.8 Hz)	8.95 (J 9Hz)
23, 24, 25, 26, 27, 29, 30	0.82s, 0.97s, 1.00s, 1.04s, 1.17s, 1.80s (21H)	0.82s, 0.99s, 1.02s,1.07s, 1.19s, 1.83s (21H)

*Experiments were done in C_5D_5N at 400 MHz; **Maillard et al., 1989

Table 3:4 **^{13}C NMR Chemical shifts (in δ values) of 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid (3.26)**

C	Observed*	Reported**	C	Observed*	Reported**
1	38.8t	38.5t	16	74.8d	74.8d
2	26.5t	26.3t	17	49.2s	49.0s
3	89.4d	89.0d	18	41.7d	41.5d
4	39.4s	39.2s	19	47.4t	47.3t
5	56.0d	55.7d	20	31.1s	31.0s
6	18.7t	18.5t	21	36.3t	36.1t
7	33.5t	33.4t	22	32.3t	32.5t
8	40.1s	39.8s	23	28.3q	28.0q
9	47.4d	47.1d	24	17.2q	16.9q
10	37.2s	36.9s	25	15.7q	15.5q
11	24.0t	23.7t	26	17.7q	17.5q
12	122.4d	122.1d	27	27.5q	27.2q
13	145.3s	145.2s	28	-	180.0s
14	42.3s	42.0s	29	33.5q	33.4q
15	36.3t	36.1t	30	25.2q	24.9q
C-3-O-sugar GlcNHAC					
1'	105.0d	104.8d			
2'	58.1d	57.8d			
3'	76.2d	76.2d			
4'	72.2d	72.5d			

5'	78.3d	78.2d
6'	63.1t	62.8t
C=O	170.7s	170.2s
Me (CONH)	23.8q	23.7q

*Experiments were done in C_5D_5N at 100 MHz; DEPT was used to determine the multiplicities;
**Maillard et al., 1989

The presence of 7 tertiary methyl groups upfield in the ^{1}H NMR of **3.26** suggested that compound **3.26** was a triterpene. The presence of a trisubstituted double bond in **3.26** was indicated by the presence of a downfield proton at δ 5.61 in the ^{1}H NMR. This was further confirmed by the presence of downfield signals at δ 122.4 and 145.3 in ^{13}C NMR of **3.26,** which are a characteristic set of signals for C-12 and C-13 in olean-12-ene (Mahato and Kundu, 1994). The fact that compound **3.26** contained an oleanene skeleton in its molecule was further confirmed by presence of a doublet of doublets at δ 3.60 in ^{1}H NMR corresponding to H-18, which couples with diastereotropic protons at C-19.

The presence of several signals between δ 3.94 and δ 5.03 in ^{1}H NMR suggested that compound **3.26** had an attached sugar moiety which was further confirmed by the presence of downfield signals at δ 5.03 in ^{1}H NMR and δ 105 in ^{13}C NMR of **3.26** corresponding to an anomeric proton and carbon, respectively.

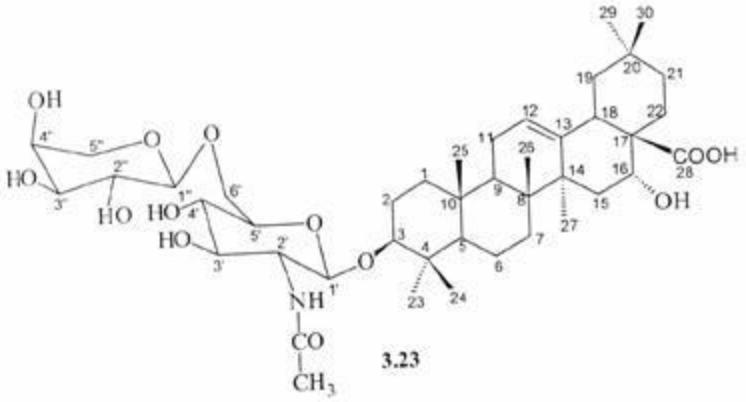
The presence of a *N*-containing sugar moiety was indicated by ES-MS spectra of **3.26**, which showed *m/z* at 674 attributable to (M^+ -H). Hence **3.26** had an odd number molecular weight of 675. Furthermore, a typical NH signal at δ 8.90d (*J* 8.8 Hz, 1H) in the^{1}H NMR was observed confirming the presence of nitrogen in compound **3.26**. The presence of a CH_3 signal at δ 23.8, a C=O signal at δ 170.7, and a characteristic signal at δ 58.1 due to C-2' in the ^{13}C NMR of **3.26** and, also, the presence of a singlet at δ 2.15 in the ^{1}H NMR clearly indicated that 2-acetamido-2-deoxy- β- D-glucopyranose is the sugar moiety in saponin **3.26** (Caprani et al., 1989; Maillard et al., 1989; Melek et al., 2007). The β- configuration was indicated by the large $^3J_{H\text{-}1'\text{-}H\text{-}2'}$ value (8.4 Hz) (Maillard et al., 1989; Melek et al., 2007).

The aglycone of **3.26** was identified as echinocystic acid by comparison of the ^{13}C NMR data with those of pentacyclic triterpenes and taking into consideration the glycosylation shifts (Mahato and Kundu, 1994). The C-3 (δ 89.3) to which the sugar moiety was attached was deshielded as expected by about 10 ppm due to α- effect, also C-2 (δ 26.5) was shielded by 1.5 ppm due to β- effects. These effects do not, however, affect the quaternary carbon atom C-4 (δ 39.4). This is consistent with the expected behaviour of glycosylated pentacyclic triterpenes. Also, the downfield chemical shift of δ 1.80 for the methyl group (Me-27) attached to C-14 in the ^{1}H NMR spectrum supports the presence of an axial hydroxyl group at C-16 (Melek et al., 2007). On the basis of above spectroscopic data, **3.26** was characterized as 3-*O* - [(2-acetamido-2-deoxy-β -D-glucopyranosyl) oxy]-16α-hydroxyolean-12-ene- 28-oic acid or 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid.

The ^{13}C NMR and ^{1}H NMR spectroscopic data of **3.26** correlated well with those reported in the literature (Maillard et al., 1989, Table 3:3 and Table 3:4). Compound **3.26** is related to the saponins previously isolated from the plant (Caprani et al., 1989) and it is reported for the first time in this plant.

3.3.3 Isolation and characterization of 3-*O*-[α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.23)

The fractions 246-289 (3.5 g) which were eluted from the flash column with 25% methanol in chloroform (Figure 3:3) were rich in compounds **3.23** and **3.6.** These were pooled and 100 mg of pooled fractions was subjected to chromatotron (CPTLC3, Figure 3:3). Isocratic elution with chloroform: ethyl acetate: methanol: water 28: 35: 12. 5: 2.5 at a flow rate of 4-10 mls/ min was carried out. Fractions 12-24 afforded 7.5 mg of a white powder **3.23**, which showed a single spot on TLC developed in several solvent systems. The CPTLC was repeated twice so as to obtain enough amounts of **3.23**.



3.23

Compound **3.23** had a melting point of 214°C and in its electro spray mass spectra (ES-MS) showed base peaks at *m/z* 806 and 830 in –ve and +ve mode, respectively. The *m/z* at 806 was for a molecular ion minus H (M^+ -H) while *m/z* at 830 was due to M^+ + Na. The IR spectrum of **3.23** showed bands at 3449 (overlapping signals for O-H, N-H), 2942 (C-H), 1702 (COOH), 1686, 1654 (HN-C=O), 1560, 1544, 1083 (C-O-C) and 671 cm^{-1}

The structure of **3.23** was determined on the basis of its ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC, IR and ES-MS spectroscopic data. Proton (^1H) NMR spectrum (Table 3:5) of **3.23** showed 6 upfield singlets at δ 0.82, 0.97, 1.05, 1.11, 1.17 and 1.80 for 7 tertiary methyl groups. The peak, at δ 0.97 represented two methyl groups, which was made clear from its integration ratio. Other characteristic signals in the spectrum were a singlet at δ 2.14, an olefinic proton at δ 5.58, carbinolic methine proton at δ 5.10, doublets centred at δ 8.94 (*J* 8.4 Hz), 4.89 (*J* 6 Hz), 4.95 (*J* 7.6 Hz), a doublet of doublets at δ 3.17 and several sugar protons between δ 3.71- 4.95. The ^{13}C NMR of **3.23** (Table 3:5) showed 37 intense signals suggesting the presence of at least 37 carbon atoms in the molecule. These peaks were for 8 methyl, 10 methylene, 14 methine and 7 quaternary carbons according to DEPT. The peak at δ 33.6 represented a methyl and a methylene whereas that at δ 47.4 represented a methine and a methylene carbon atom as indicated by DEPT. Eight of the methine carbons appearing downfield at δ 69.2, 72.4, 72.7, 74.4, 76.0, 76.7, 105.1 and 105.3 were oxygenated. Similarly two of the methylene carbons appearing at δ 66.5 and 69.9 were also oxygenated.

The ^{1}H NMR spectrum of **3.23** showed the presence of several signals between δ 3.71-4.95 indicating the presence of sugar molecules in the compound. Furthermore, the presence of sugar molecules was confirmed by downfield signals at δ 105.1 and δ 105.3 in ^{13}C NMR of **3.23** suggesting the presence of two anomeric carbons for at least two sugar units in **3.23**.

Similar to compound **3.26**, the ^{1}H NMR and ^{13}C NMR spectral data of **3.23** (Table 3:5) clearly indicated that echinocystic acid was the aglycone in this saponin. Also, the spectral data supported the presence of acetyl glucosamine as one of the sugars. The β-configuration of this sugar was indicated by its $^{3}J_{H\text{-}1'\text{-}H\text{-}2'}$ value of 7.6 Hz. Furthermore, apart from the diagnostic features for echinocystic acid discussed for compound **3.26**, the presence echinocystic acid was confirmed by 2D NMR experiments ^{1}H- ^{1}H (COSY) and C- H (HMBC) correlation spectral data (Table 3:5). Carbon 3 (δ 89.5 d) to which the sugar chain was attached showed C-H ^{3}J, correlations with H-23 (δ 1.11) and H-24 (δ 0.97). Another important correlation was seen in COSY between H-3 (δ 3.17) and the diastereotropic protons attached to C-2 (δ 2.3, 1.8). These correlations support the assignment of δ 89.5 to C-3.

Table 3:5 NMR spectral data of 3-*O*-[α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.23)

C	^{13}C NMR*, **	HSQC (δ Values for attached Hs**	HMBC C-H correlations	^{1}H-^{1}H COSY Coupling Hs
1	38.8t **(39.1)**	0.97,1.50		H-1 and H-1'
2	26.6t **(26.7)**	2.30, 1.80		H- 2 and H- 2'; H-2' and H-3
3	89.5d **(89.9)**	3.18	H- 23, H- 24 ^{3}J	
4	39.4s **(39.5)**	-	H- 23, H- 24 ^{2}J	
5	55.9d **(56.7)**	0.68	H- 23, H - 24 ^{3}J	
6	18.7t **(18.9)**			
7	33.6 **(33.7)**	0.94, 1.28 1.55		
8	40.1s **(40.4)**	-		
9	47.4d **(47.6)**	1.70		
10	37.1s **(37.4)**	-		

11	23.9t **(24.1)**	1.90		
12	122.3d **(122.6)**	5.58 **(5.65)**		
13	145.0s **(145.2)**	-		
14	42.3s **(42.5)**	-		
15	36.2t **(36.4)**	1.35, 1.74, 2.34		
16	74.4d **(74.9)**	5.10		
17	-	-		
18	41.8d **(42.0)**	3.62		
19	47.4t **(47.6)**	2.74, 1.31		
20	31.1s **(31.0)**	-		
21	36.2t **(36.4)**	1.35, 1.74, 2.34		
22	32.3t	1.22		
23	28.2q **(28.4)**	1.11 **(1.20)**	H- 24 ^3J	
24	17.2q **(17.1)**	0.97 **(1.0)**	H- 23 ^3J	
25	15.7q **(15.8)**	0.82 **(0.83)**		
26	17.8q **(17.1)**	0.97 **(1.0)**		
27	27.5q	1.80 **(1.83)**		
28	-	-		
29	33.6q **(33.4)**	1.05 **(1.22)**		
30	25.5	1.17**(1.10)**		
C-3-O-sugar GlcNHAC				
1'	105.1d **(104.6)**	4.95 (*J* 7.6Hz)		
2'	57.8d **(58.5)**	4.54		
3'	76.7d **(76.5)**	4.06		
4'	72.7d **(73.2)**	4.55		
5'	76.0d **(76.0**	4.34		
6'	69.9t **(69.9)**	4.24, 4.82		H- 5' and H- 6, & H- 6', H-6 ' and (H-6') '
Me (CONH)	171.0s **(171.0)**	-	H- Me (CONH) ^2J	
Me (CONH)	23.8q **(24.2)**	2.14 **(2.22)**		
Me (CONH)		8.94 **(8.85)**		N- H and H- 2' (GlcNAC)
Ara				
1"	105.3d **(104.9)**	4.89 (*J* 6 Hz)		
2"	72.4d **(72.3)**	4.45		H- 2" and H- 3"
3"	74.4d **(74.2)**	4.18		
4"	69.2d **(68.6)**	4.33		
5"	66.5t **(65.8)**	4.29, 3.71		H- 5" and (H- 5") '

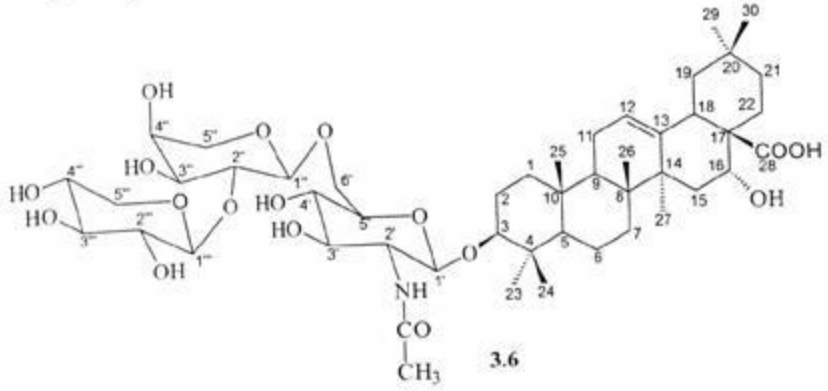
*Experiment was done in C_5D_5N at 300 MHz and 75 MHz for ^1H NMR and ^{13}C NMR, respectively. DEPT was used to determine the multiplicities **Reported data in parentheses and bolded (Caprani et al., 1989)

The ES-MS spectra of **3.23**, showed a base peak at *m/z* 806 due to (M^+-H), which meant that **3.23** had a molecular weight of 807. The molecular weight of 807, and the fact that compound **3.23** contained 3-O- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid in its moiety meant that the other sugar had to be a pentose. Furthermore, the ^3J H-

1"- H-2" value of 6.0 Hz involving the anomeric H at δ 4.89 clearly indicated that the second sugar had, a α-configuration.

The ^{13}C NMR spectral data of **3.23** correlated well with those of 3-O-[α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid, a compound previously isolated from the root bark of this plant (Caprani et al., 1989) [Table 3:5]. Hence compound **3.23** was characterized as 3-O-[α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid a known compound from this plant.

3.3.4 Isolation and characterization of 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.6)



3.6

Compound **3.6** was obtained from fractions 130-189 and 190-192 from VLC1 (Figure 3:3). The fractions 130-189 (7 g) were pooled and subjected to CPTLC3. The fractions 45-64 from the CPTLC3, which were eluted as above showed a single spot in several solvent systems. These were pooled and afforded 14 mg of a white powder **3.6**.

The compound **3.6** was also obtained by subjecting 7 g of fractions 190-192 obtained from VLC1 (Figure 3:3) to VLC5 (Figure 3:4) which was eluted with chloroform: ethyl

acetate: methanol: water 28: 35: 6: 0.1 and gradually increasing the polarity to 28: 35: 30: 7.5 by increasing the amount of methanol and water and finally the column was washed with methanol.

The fractions 112- 148 (976.3 mg) eluted with chloroform: ethyl acetate: methanol: water 28: 35: 11: 0.75 were rich in compound **3.6** and **3.27**. These fractions were pooled and subjected to CPTLC6. Isocratic elution of the chromatotron with chloroform: ethyl acetate: methanol: water 28: 35: 12.5: 2.5 at a flow rate of 5- 20 mls /min was carried out. Fractions 19- 86 which contained a mixture of compounds **3.6** and **3.27** were pooled and 200 mg were separated on PTLC3 (50 mg/ plate) by multiple development in chloroform: ethyl acetate: methanol: water 28: 35: 55: 7 as explained earlier. A white powder **3.6** (70 mg) was obtained which showed a single spot on TLC developed in several solvent systems.

Compound **3.6** had a melting point of 244 °C and its electro spray mass spectra (ES-MS) showed base peaks at *m/z* 938 and 962 in –ve and +ve mode, respectively. The *m/z* 938 is the molecular ion less H (M^+ -H) while the *m/z* 962 is M^+ + Na. The IR spectrum showed bands at 3424 (overlapping signals for O-H, N-H), 2945 (C-H), 1702 (COOH), 1655 (HNC=O), 1560, 1544, 1459, 1376, 1146, 1079 (C-O-C) 1061, 1008, 769 cm^{-1}.

The structure of **3.6** was characterized by analysis of its ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC, IR and ES-MS spectroscopic data (Figure 3:14- Figure 3:20). Thus, ^1H NMR spectrum (Figure 3:14, Table 3:6) showed 7 upfield singlets at δ 0.76, 0.90, 0.93, 0.98, 1.11, 1.12 and 1.77 (3H each) for 7 tertiary methyl groups. Other characteristic signals in the spectrum were a singlet at δ 2.11, an olefinic proton at δ 5.53, carbinolic methine proton at δ 5.17, doublets centred at δ 8.95 (*J* 9.0 Hz), 4.95 (*J* 6.0 Hz), 4.99 (*J* 5.1 Hz), several sugar protons between δ 3.6- 4.99 and a doublet of doublets at δ 3.28 ascribed to H-3. The ^{13}C NMR of **3.6** (Figure 3:15 and Table 3:6) showed 46 intense signals suggesting the presence of at least 46 carbon atoms in the molecule. These peaks were for 8 methyl, 11 methylene, 19 methine and 9 quaternary carbons according to DEPT-135 (Figure 3:16). The peak at δ 47.6 represented both a methine and a methylene carbon atom as indicated by DEPT. Fourteen of the methine carbons appearing

downfield at δ 68.1, 69.1, 72.8, 73.0, 73.1, 74.4, 75.0, 75.8, 76.2, 79.81, 89.6, 102.8, 104.8 and 105.6 were oxygenated. Similarly three of the methylene carbons appearing at δ 65.0, 66.8 and 69.8 were oxygenated.

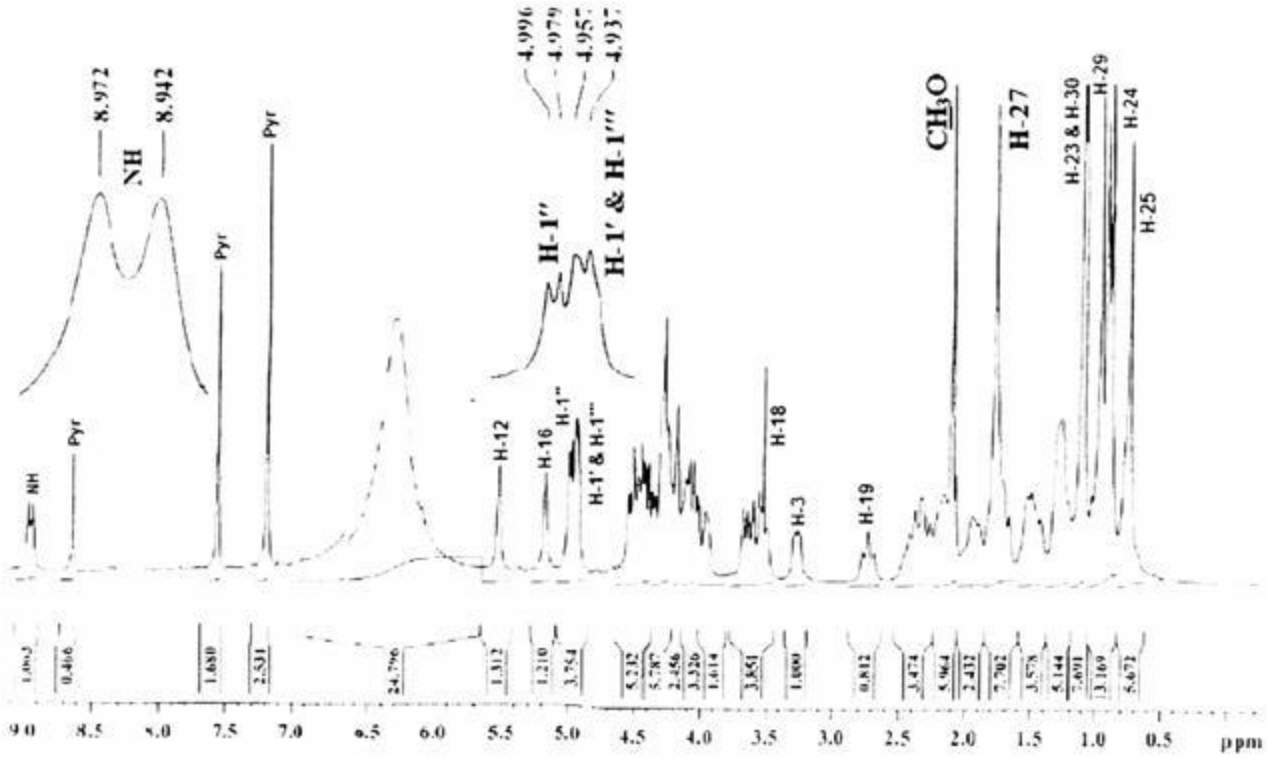


Figure 3:14 ^{1}H NMR Spectrum of Compound **3.6**

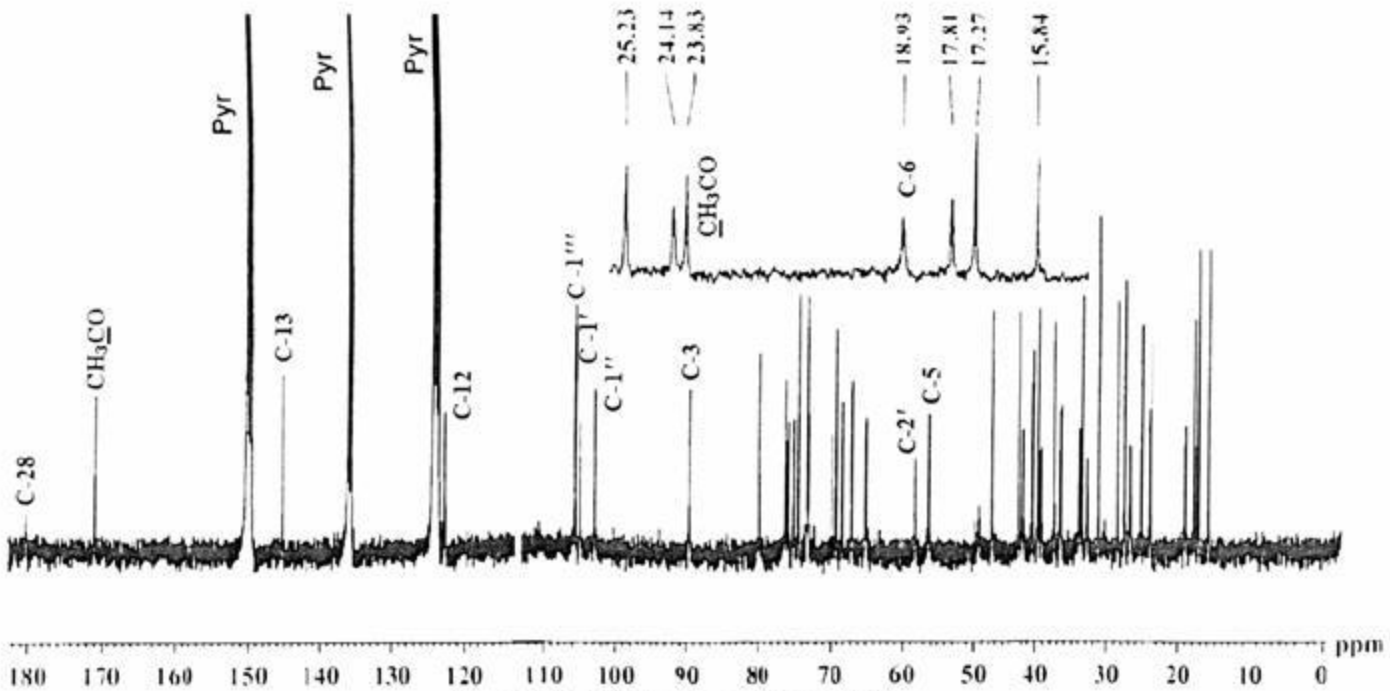


Figure 3:15 ^{13}C NMR Spectrum of Compound **3.6**

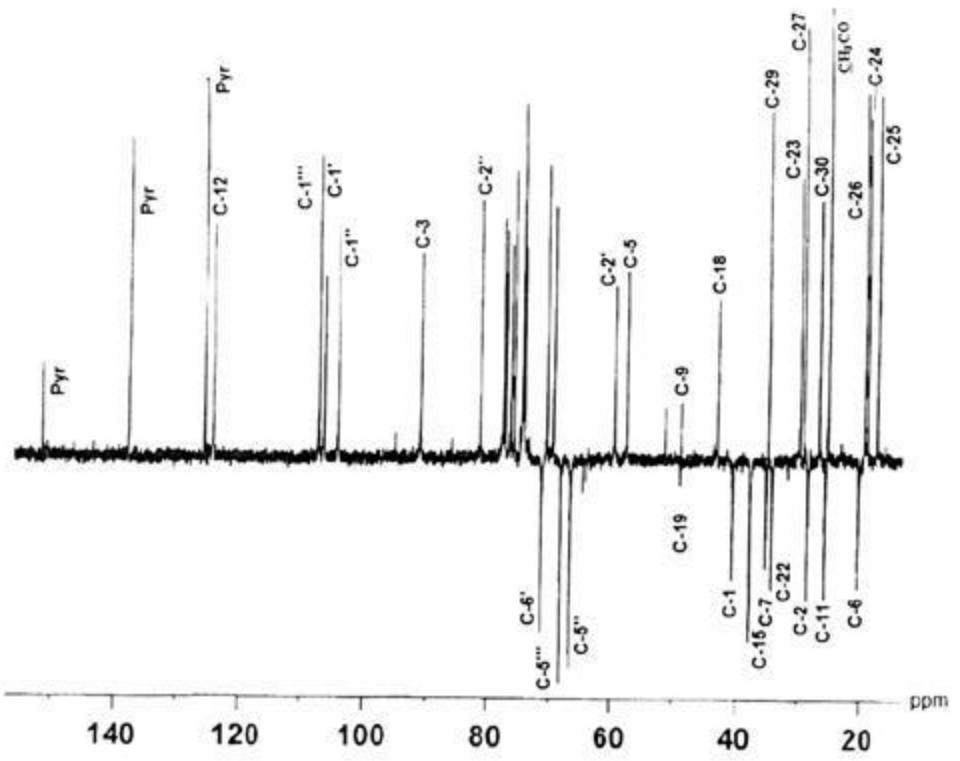


Figure 3:16 DEPT-135 Spectrum of Compound **3.6**

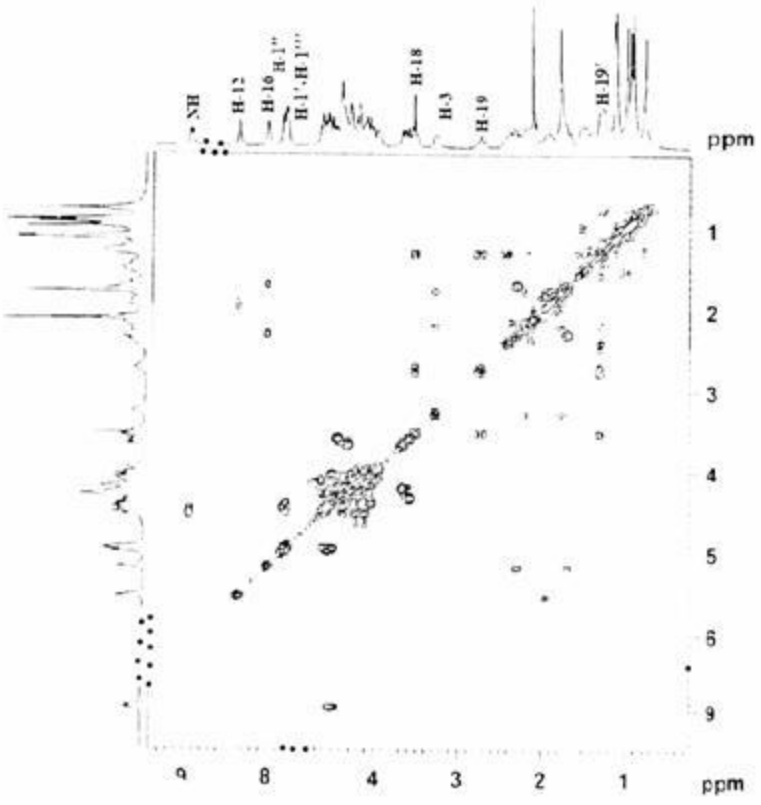


Figure 3:17 COSY Spectrum of Compound **3.6**

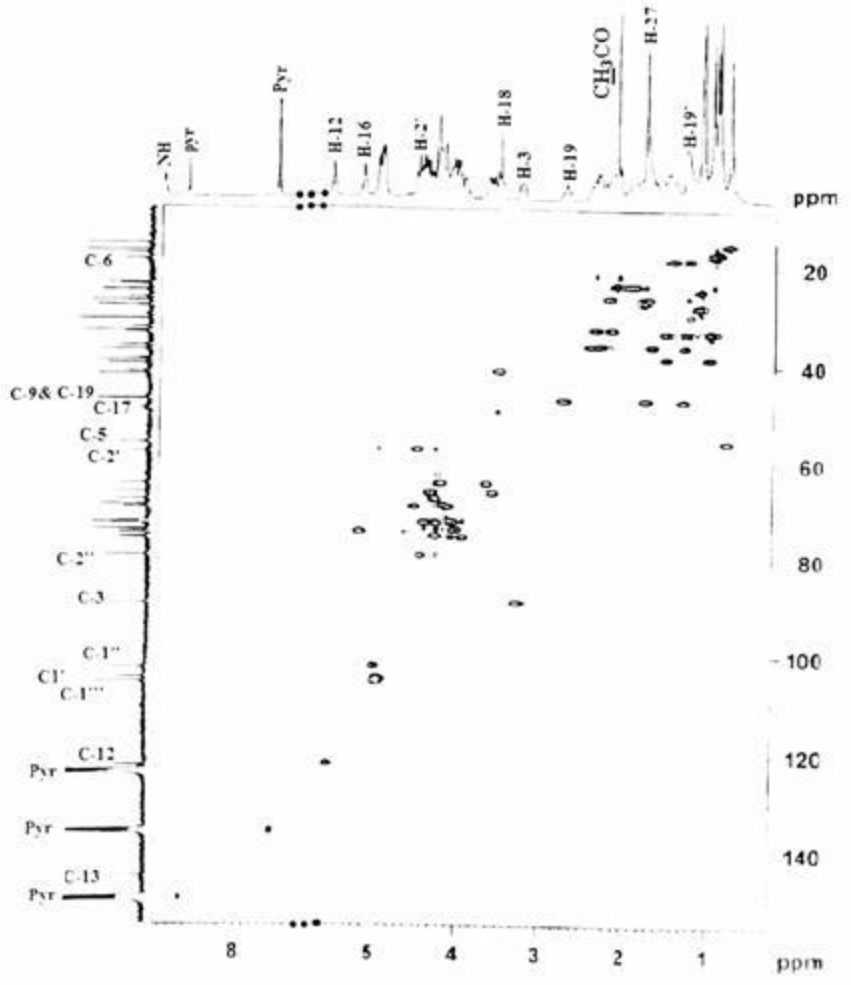


Figure 3:18 HSQC Spectrum of Compound **3.6**

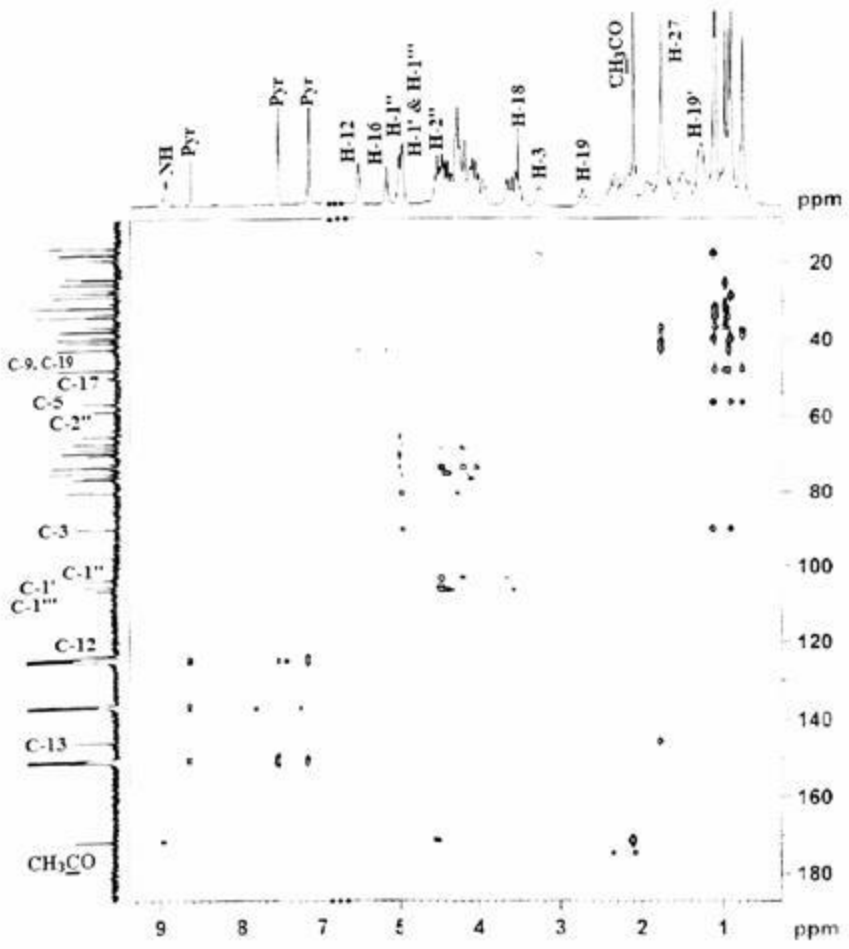


Figure 3:19 HMBC Spectrum of Compound **3.6**

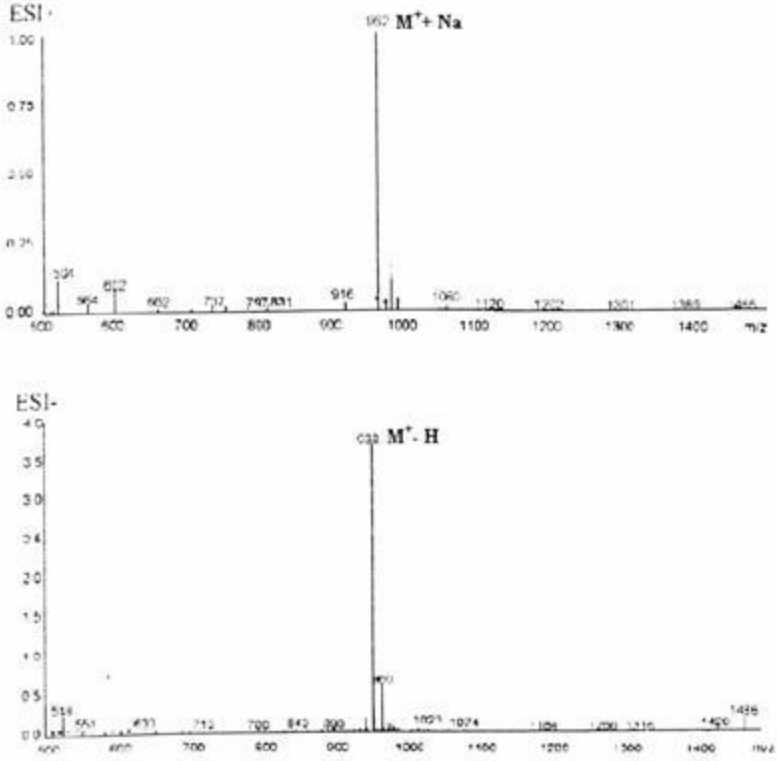


Figure 3:20 ES- MS of Compound **3.6**

Table 3:6 NMR spectral data for 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.6)

C	^{13}CNMR*,***	HSQC (δ Values for attached Hs) **	HMBC C-H correlations	^{1}H-^{1}H COSY coupling Hs
1	39.2t **(38.8)**	1. 50, 1.00		
2	26.8t **(26.6)**	2.2, 1.80		
3	89.6d **(89.2)**	3.28 **(3.37)**	H- 23, H- 24, GlcNHAC ^{3}J	H- 3 and H -2, H- 2'
4	39.6s **(39.4)**	-	H- 23, H- 24 ^{2}J	
5	56.3d **(55.9)**	0.83 **(0.89)**	H- 23, H- 24, H- 25 ^{3}J	

6	18.9.t **(18.6)**	1.43, 1.27		
7	33.9t **(33.6)**	1.27, 1.50	H- 26 ^{3}J	
8	40.3s **(40.0)**	-	H- 26 ^{2}J; H- 27 ^{3}J	
9	47.6d **(47.3)**	1.75	H- 25, H- 26 ^{3}J	
10	37.4 s **(37.1)**	-	H- 25 ^{2}J	
11	24.1t **(23.8)**	1.93		H-12 and H-11
12	122.7d **(122.5)**	5.53 **(5.61)**		
13	145.3s **(145.1)**	-	H- 27	
14	42.4s **(42.1)**	-	H- 26 ^{3}J; H- 27 ^{2}J	
15	36.4t **(36.3)**	2.33, 1.68	H- 27 ^{3}J	
16	75.0d **(74.8)**	5.20 **(5.25)**		H-16 and H-15, H-15'
17	49.3s **(48.9)**	-		
18	41.8d **(41.5)**	3.50 **(3.60)**	H-12, H-16 ^{3}J	H-18 and H-19, H-19'
19	47.6t **(47.4)**	1.26, 2.73 **(1.37, 2.81)**	H- 29, H- 30 ^{3}J	H-19 and H-19'
20	31.3s **(31.1)**	-	H- 29, H- 30 ^{2}J	
21	36.4t **(36.2)**	2.40, 1.3 **(2.50, 1.37)**	H- 29, H- 30 ^{3}J	
22	32.8t **(32.8)**	2.33, 2.15		
23	28.5q **(28.2)**	1.12 **(1.19)**	H- 24 ^{3}J	
24	17.3q **(17.1)**	0.90 **(0.98)**	H- 23 ^{3}J	
25	15.8q **(15.6)**	0.76 **(0.86)**		
26	17.8q **(17.5)**	0.93 **(1.03)**		
27	27.5q **(27.3)**	1.77 **(1.85)**		
28	180.3s **(180.0)**	-		
29	33.6q **(33.4)**	0.98 **(1.07)**	H- 30 ^{3}J	
30	25.2q **(24.8)**	1.11 **(1.18)**	H- 29 ^{3}J	
C-3-O-sugar GlcNHAC				
1'	104.8d **(104.9)**	4.95 **(5.03)**		H-1' and H-2'
2'	58.3d **(58.0)**	4.47 **(4.53)**		H-2' and N-H, H-3'
3'	75.8d **(75.7)**	4.25 **(4.33)**		
4'	72.8d **(72.8)**	4.07 **(4.13)**		
5'	76.2d **(76.1)**	3.95 **(4.02)**		
6'	69.80t **(69.6)**	4.54, 4.13 **(4.61, 4.22)**	H-1" ^{3}J	H- 6' and H- (6') '
Me (CONH)	171.0s **(170.2)**		H- Me (CONH), N-H ^{2}J; H-2'; (GlcNHAC) ^{3}J	
Me (CONH)	23.8q **(23.7)**	2.08 **(2.13)**		N- H and H- 2'
Me (CONH)		8.96 **(8.79)**		
Ara				
1"	102.8d **(102.5)**	4.99 **(5.08)**	H- 2" ^{2}J	H-1" and H- 2"
2"	79.8d **(79.5)**	4.45 **(4.52)**	H-1"' ^{3}J	H-2" and H-3"
3"	73.1d **(72.9)**	4.27 **(4.33)**	H- 2" ^{2}J	
4"	68.1d **(67.7)**	4.28 **(4.33)**		
5"	65.0t **(64.6)**	3.65, 4.23 **(3.73, 4.30)**	H-1" ^{3}J	H-5" and (H-5") '

Ara				
1'''	105.6d **(105.6)**	4.95 **(5.05)**	H- 2 ^{3}J; H- 5, H- 5'^{3}J	H-1''' and H-2'''
2'''	73.0d **(72.8)**	4.40 **(4.47)**		H-2''' and H-3'''
3'''	74.4d **(74.3)**	4.03 **(4.10)**	H- 2 ^{3}J	
4'''	69.1d **(68.9)**	4.16 **(4.25)**		
5'''	66.8t **(66.6)**	3.57, 4.35 **(3.67,4.40)**		H- 5''' and (H- 5''')'

* Done in C_5D_5N at 300 MHz and 75 MHz for ^{1}H NMR, and ^{13}C NMR respectively, DEPT was used to determine the multiplicities; **Reported data in paretheses and bolded (Melek et al., 2007)

The spectral data of compound **3.6** (Table 3:6) had all the diagnostic features supporting the presence of 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid in its moiety as discussed for compound **3.23** and **3.26**. Similar to compound **3.23** the heteronuclear correlation HMBC spectrum (Figure 3:19) and homonuclear correlation ^{1}H-^{1}H COSY, spectrum (Figure 3:17) of **3.6** supported the presence of echinocystic acid. An additional evidence to support the presence of the hydroxyl group at position C-16, which was not evident in the spectra of compound **3.23** (due to the small sample), was indicated by the homonuclear ^{1}H-^{1}H COSY correlations of **3.6** (Figure 3:17). The carbinolic proton H-16 (δ 5.20) was seen to be coupling with the diastereotropic protons H-15 and H-15' [δ 2.33, 1.68] (Figure 3:17 and Figure 3:21). Also the connectivity of the different parts of the acetylglucosamine sugar, which was not evident from the spectral data of compound **3.23** due to the same reason were clearly shown by the HMBC spectrum of **3.6**. Cross peaks due to long range correlations between the carbonyl signal at δ 171.0 and H- Me (CONH) [δ 2.14], H-N (δ 8.94) and H-2' (δ 4.47) were seen in the HMBC spectrum of **3.6** (Figure 3:19), which confirmed further the presence of acetyl glucosamine as one of the sugar in the molecule of **3.6**.

From the ES-MS spectral data (Figure 3:20), the molecular weight of **3.6** was deduced as 939. Based on this molecular weight, the ^{13}C NMR and DEPT spectral data the molecular formula of **3.6** was deduced as $C_{48}H_{77}NO_{17}$. According to the molecular formula $C_{48}H_{77}NO_{17}$ for **3.6** the remaining two sugars had to be pentoses and from the ^{13}C NMR in conjunction with the literature (Caprani et al., 1989) it was evident that both sugars were L-arabinose. The configuration of anomeric center (δ 4.99) of one of the arabinose

units was determined to be α- as it was revealed by $^{3}J_{H\text{-}1''\text{-}H\text{-}2''}$ value of 5.1 Hz. For the other arabinose and acetyl glucosamine the configuration was difficult to determine due to poor resolution of ^{1}H NMR signals, which gave the same chemical shift of δ 4.95 for both of the remaining anomeric protons. From the literature (Melek et al., 2007; Caprani et al., 1989) the acetyl glucosamine was assigned a β-configuration whereas L-arabinose was allocated an α- configuration.

The connectivity between echinocystic acid and the sugar chain was confirmed by the HMBC spectra of **3.6**. The cross peak was seen between C-3 of echinocystic acid and H-1' indicating the presence a glycosidic bond at this carbon atom. Furthermore, the resonances of C-3 and C-2 were shifted as expected.

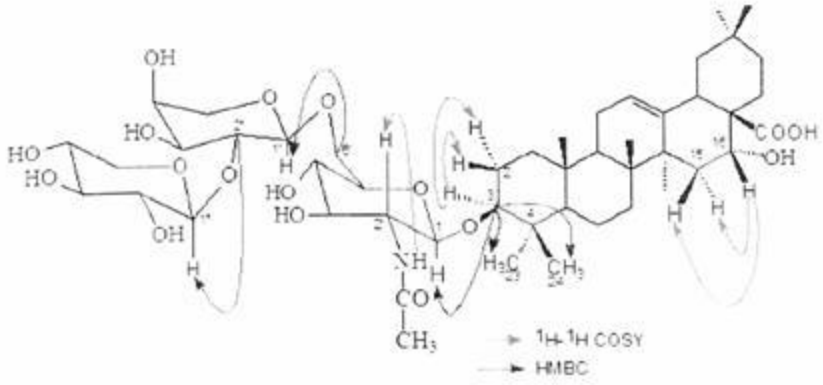
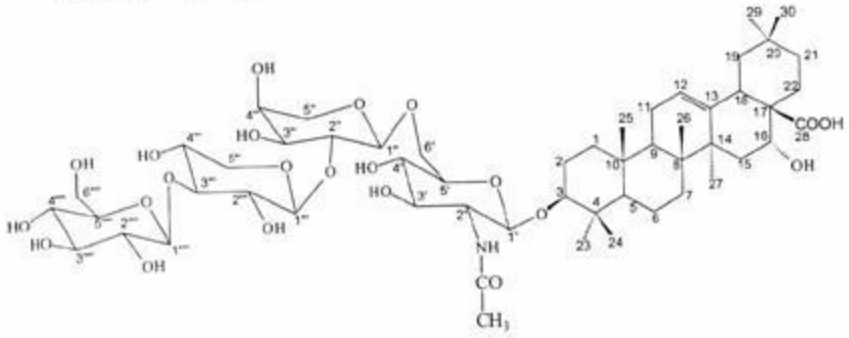


Figure 3.21 Important homonuclear and heteronuclear correlations of compound **3.6**

The linkages between the sugars were determined through HMBC experiment; the linkage between acetyl glucosamine and arabinose was indicated by a cross peak observed between C-6' (δ 69.8) and H-1" (δ 4.99). This was further confirmed by the downfield shift of about 6.7 ppm for C-6' and upfield shift of about 2.1 ppm for C-5' due to glycosylation. The cross peak between C-2" (δ 79.8) and H-1"' indicated the linkages between these two pentoses. Again the resonance of C-2" was shifted downfied by 6.9

ppm due to glycosylation. From these spectroscopic data compound **3.6** was characterized as 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid, a known compound of *A. anthelmintica* (Caprani et al., 1989). ^{1}H NMR and ^{13}C NMR data correlated very well to those previously reported for this compound [Table 3:6] (Caprani et al., 1989, Melek et al., 2007).

3.3.5 Isolation and characterization of 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)] [α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.22)



3.22

Compound **3.22** was isolated from fractions 190- 192 (21.1 g), which were eluted from VLC1 (Figure 3:3) as discussed earlier. Five hundred milligrams of the pooled fraction was subjected to CPTLC. Isocratic elution of the rotar was carried out with chloroform: ethyl acetate: Methanol: water 28: 35: 12.5: 2.5 at a flow rate of 5- 15 mls /min (Figure 3:4). Fractions 72- 120 (219 mg), which were rich in compound **3.22** were pooled and re-subjected to CPTLC under the same conditions (Figure 3:4). Fractions 26- 40 afforded 26.4 mg of a white powder, **3.22**, which showed a single spot on TLC plate run in different solvent systems. Similarly, compound **3.22** was obtained from VLC5 (Figure 3:4), fractions 344- 372 (715 mg), eluted from the column with chloroform: ethyl acetate: methanol: water 28: 35: 15: 1.75. These fractions were rich in compound **3.22** and **3.28**

and they were further subjected to CPTLC7 under isocratic elution with chloroform: ethyl acetate: Methanol: water 28: 35: 12. 5: 2.5 at a flow rate of 5- 20 mls /min. Fractions 104- 116 (347.2 mg) which contained compounds **3.22** and **3.28** were pooled and separated on PTLC developed with chloroform: ethyl acetate: methanol: water 28: 35: 55: 7 (Figure 3:4) to afford 85 mg of a white powder **3.22**.

Compound **3.22** had a melting point of 249 °C and in electro spray mass spectra (ES-MS) showed base peaks at *m/z* 1100 and 1124 in –ve and +ve mode, respectively. The *m/z* 1100 is the molecular ion less H (M^+ -H) while the *m/z* 1124 is M^+ + Na. The IR spectrum showed bands at 3424 (overlapping signals for O-H, N-H), 2944 (C-H), 1702 (COOH), 1654 (HNC=O), 1577, 1560, 1522, 1376, 1146, 1078 (C-O-C) 1044 and 767 cm^{-1}.

The structure of **3.22** was characterized by analysis of its ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC, IR and ES-MS spectroscopic data. Proton (^1H) NMR of **3.22** showed 6 upfield singlets at δ 0.81, 0.94, 0.98, 1.03, 1.16 and 1.82 [21H] corresponding to 7 tertiary methyl groups. Other characteristic signals in the spectrum were a singlet at δ 2.15, an olefinic proton at δ 5.60, a doublet for carbinolic methine proton centered at δ 5.27 (*J* 7.5), broad singlets for carbinolic methines at δ 5.10 and δ 5.23, doublet centered at δ 9.04 (*J* 8.4 Hz), several sugar protons between δ 3.6- 5.27 and a doublet of doublets at δ 3.27. The ^{13}C NMR of **3.22** (Table 3:7) showed 49 intense signals suggesting the presence of at least 49 carbon atoms in the molecule. These peaks were for 8 methyl, 12 methylene, 21 methine and 9 quaternary carbons according to DEPT. The peak at δ 47.5 represented two carbons, a methine and a methylene carbon atom as indicated by DEPT. Sixteen of the methine carbons, appearing downfield at δ 67.7, 68.7, 71.8, 72.8, 74.9, 75.7, 75.9, 78.3, 78.6, 79.9, 83.3, 89.6,102.5, 104.7, 105.5, and 105.7 were oxygenated. Similarly, four of the methylene carbons appearing at δ 62.8, 64.5, 66.8 and 69.7 were oxygenated.

The spectral data (Table 3:7) of compound **3.22** like those for compounds **3.6**, **3.23** and **3.26** had characteristic features indicating the presence of 3-*O*- [(2-acetamido-2-deoxy-β-

D-glucopyranosyl echinocystic acid in its moiety. Similar to compound **3.6** as discussed earlier, HMBC and COSY spectral data (Table 3:7) of **3.22** gave the connectivity within the entire 3-O- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid in its moiety.

Table 3:7 NMR spectral data of 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)] [α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.22)

C	^{13}C NMR *, **	HSQC (δ Values for attached Hs	HMBC C-H Correlations	^{1}H-^{1}H COSYcoupling Hs
1	39.1t **(39.1)**	0.98, 1.54		
2	26.7t **(26.6)**	2.21, 1.8		H-2 and H-2'
3	89.6d **(89.6)**	3.27	H- 23, H- 24, H-1' GlcNHAC ^{3}J	H-3 and H-2, H-2'
4	39.5s **(39.4)**	-	H- 23, H- 24 ^{2}J	
5	56.2d **(56.2)**	0.83	H- 23, H- 24, H- 25 ^{3}J	
6	18.8t **(18.8)**	1.30, 1.48		
7	33.8t **(33.7)**	1.33, 1.55	H- 26 ^{3}J	
8	40.3s **(40.2)**	-	H- 26 ^{2}J; H- 27 ^{3}J	
9	47.5 **(47.5)**	1.77	H- 25, H- 26 ^{3}J	
10	37.3s **(37.3)**	-	H- 25 ^{2}J	
11	24.1t **(24.0)**	1.95		H-11 and H-12
12	122.6d **122.5)**	5.60 **(5.65)**		
13	145.0s **(145.2)**	-	H- 27 ^{3}J	
14	42.4s **(42.3)**	-	H- 26, H- 27 ^{2}J	
15	36.3t **(36.3)**	2.34, 1.74		
16	74.9d **(74.8)**	5.23		H-16 and H-15, H-15'
17	49.2s **(49.4)**	-		
18	41.9s **(41.8)**	3.68		H-18 and H-19, H-19'
19	47.5t **(47.5)**	2.8, 1.42	H- 29, H- 30 ^{3}J	H-19 and H-19'
20	31.1s **(31.0)**	-	H- 29, H- 30 ^{2}J	
21	36.3t **(36.3)**	1.34	H- 29, H- 30 ^{3}J	
22	32.2t **(32.3)**	2.4, 2.25,		
23	28.5q **(28.4)**	1.16 **(1.20)**	H- 24 ^{3}J	
24	17.2q **(17.1)**	0.94 **(1.00)**	H- 23 ^{3}J	
25	15.8q **(15.7)**	0.81 **(0.95)**		
26	17.8q **(17.7)**	0.98 **(1.05)**		
27	27.5 **(27.4)**	1.82 **(1.80)**		
28	181.3 **(180.6)**	-		
29	33.4q **(33.3)**	1.03 **(1.08)**	H-30 ^{3}J	
30	25.3q **(25.2)**	1.16 (1.22)	H-29 ^{3}J	
C-3-O-sugar GlcNHAC				
1'	104.7d **(104.5)**	4.97		H-1' and H-2'

2'	58.3d **(58.1)**			H- 2' and H-NCOMe
3'	75.9d **(75.8)**	3.98		
4'	72.8d **(72.7)**			
5'	75.7d **(75.5)**	3.35		
6'	69.7t **(69.6)**	4.14, 4.55		
Me (CONH)	171.2s **(171.2)**	-	H- Me (CONH) ^2J	
Me (CONH)	23.7q **(23.5)**	2.15 **(2.20)**		
Me (CONH)		9.04 **(8.55)**		
Ara				
1"	102.5d **(102.4)**	5.10		H-1" and H-2"
2"	79.9.d **(79.9)**	4.53		H-2" and H-3"
3"	72.8d **(72.4)**	4.20		
4"	67.7d **(67.7)**	4.40		
5"	64.5 t **(64.6)**	3.68, 4.28		H- 5" and H-5"
Ara				
1"'	105.5 **(105.4)**	4.97		H-1"' and H-2"'
2"'	71.8 **(71.7)**	4.55		H-2"' and H-3"'
3"'	83.3 **(83.3)**	4.16		
4"'	68.7d **(68.6)**	4.36		
5"'	66.8t **(66.7)**	3.6, 4.30		H- 5"' and (H-5"')'
Glc				
1""	105.7d **(105.4)**	5.27		H-1"" and H- 2""
2""	75.9d **(75.8)**	4.05		
3""	78.6d **(78.3)**	3.95		
4""	71.8 **(71.7)**	4.23		
5""	78.3d **(78.1)**	4.23		
6""	62.8t **(62.8)**	4.35, 4.49		

*Done in C_5D_5N at 300 MHz and 75 MHz for ^1H NMR and ^{13}C NMR respectively, DEPT was used to determine the multiplicities; **Reported data in parentheses and bolded (Caprani et al., 1989)

The sugar chain present in compound **3.22** was determined on the basis of NMR, IR and ES-MS spectral data. From the ES-MS spectral data the molecular weight of **3.22** was deduced as 1101. Based on this molecular weight, the ^{13}C NMR, DEPT and HSQC spectral data the molecular formula of **3.22** was deduced as $C_{54}H_{87}NO_{22}$. According to the molecular formula $C_{54}H_{87}NO_{22}$ for **3.22** and the fact that this compound contained 3-*O*- [(2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid in its moiety the other remaining sugars had to be 2 pentoses and one hexose. From the ^{13}C NMR of the sugar part the literature (Caprani et al., 1989) and taking into consideration the shifts due to glycosylation, the other sugars were suggestive of two L-arabinose units and one D-glucose unit. The proton NMR spectrum of **3.22** was not well resolved so as to provide

anomeric configurations for all the sugars. However, for the terminal sugar, glucose, with an anomeric carbon at δ 105.7 and an anomeric proton at δ 5.27 had a β- configuration as indicated by a large coupling constant ($J_{H\text{-}1'''\text{-}H\text{-}2'''}$) of 7.5.

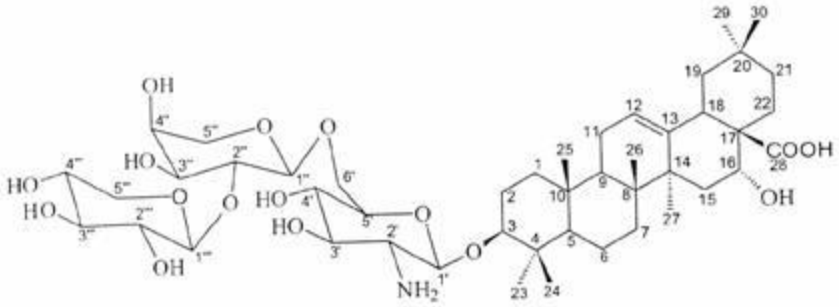
Similar to compound **3.6** discussed above the connectivity between echinocystic acid and the sugar was confirmed by the HMBC spectra of **3.22** and the shifts due to glycosylation (Table 3:7). The presence of a glycosidic bond in the compound was further confirmed by the presence of a peak at 1078 cm^{-1} in the IR spectrum of **3.22** due to the ether linkage (C-O-C).

The linkages between the sugars were determined from the ^{13}C NMR chemical shifts of **3.22** for the sugar part (Table 3:7), which were similar to those of compound **3.6**. The extra sugar molecule, glucose, in **3.22** when compared to compound **3.6**, was connected to the second arabinose unit through a linkage between its C-1'''' and C-3''' (δ 83.3) of the arabinose as indicated by the downfield shift of the resonance for C-3'''.

These spectroscopic data for compound **3.22** correlated well with the known *A. anthelmintica* compound, 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)] [α-L-arabinopyranosyl (1→6)]-2-acetamido-2-deoxy -β- D-glucopyranosyl echinocystic acid (Caprani et al., 1989, Table 3:7).

3.3.6 Isolation and characterization of 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.27)

The compound **3.27** was obtained along with compound **3.6** from PTLC 3 (Figure 3:4) as discussed earlier. The CPTLC3 afforded 45 mg of shiny colourless amorphous compound **3.27**, which showed one spot on TLC developed in several solvent systems. Other smaller amounts (5 mg) of compound **3.27** were obtained during isolation of compound **3.22** on CPTLC5. Fractions 41- 87 (92.1 mg), which were obtained from CPTLC5 (Figure 3:4), were rich in compounds **3.27** and **3.28**. These were pooled and separated on PTLC2 double developed in chloroform: ethylacetate: methanol: water 28: 35: 55: 7; compound **3.27** was obtained as a shiny colourless amourphous compound.



3.27

Compound **3.27** in its electro spray mass spectra (ES-MS) showed base peaks at *m/z* 896 and *m/z* 920 in –ve and +ve mode, respectively, the *m/z* 896 is the molecular ion less H (M^+ -H) while the 920 is M^+ + Na.

The structure of **3.27** was characterized by analysis of its ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and ES-MS spectroscopic data (Figure 3:22- Figure 3:28). It should be noted that the NMR spectra of compound **3.27** were all determined in deuterated DMSO and signals were calibrated based on the chemical shifts of this solvent, most of the signals as indicated in Table 3:7 were upfield due to solvent effects. The shifts due to solvent effects were confirmed by observing the chemical shifts of compound **3.22** in both pyridine and DMSO.

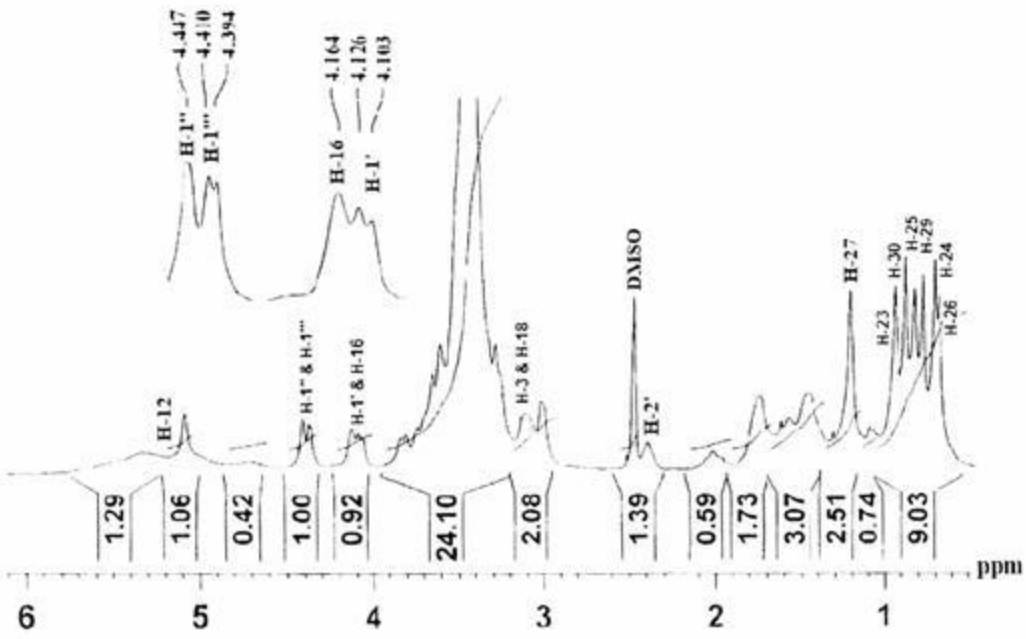


Figure 3:22 ^{1}H NMR Spectrum of Compound **3.27**

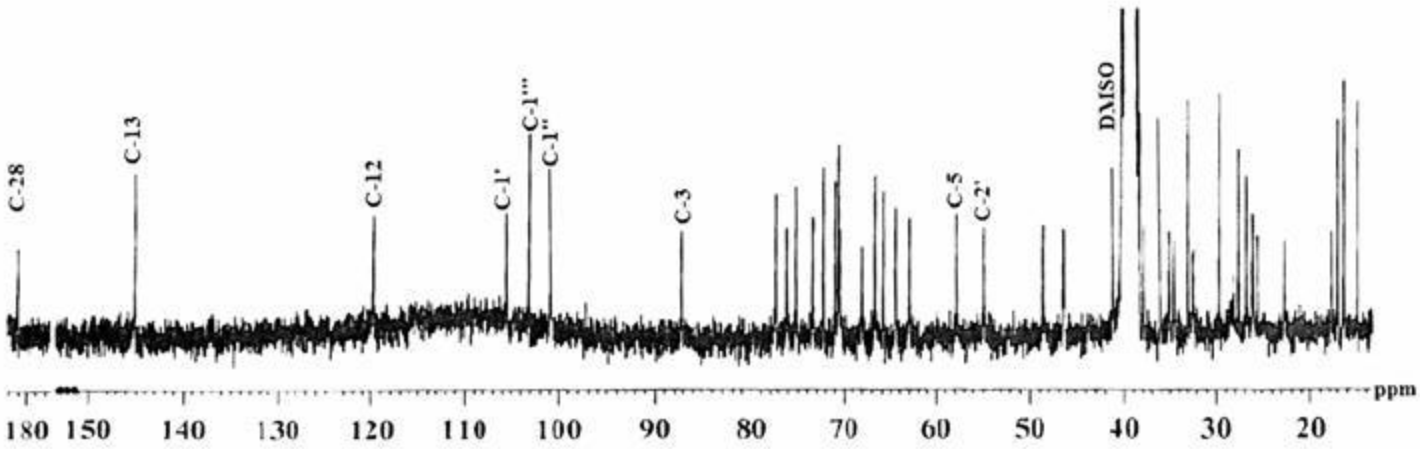


Figure 3:23 ^{13}C NMR Spectrum of Compound **3.27**

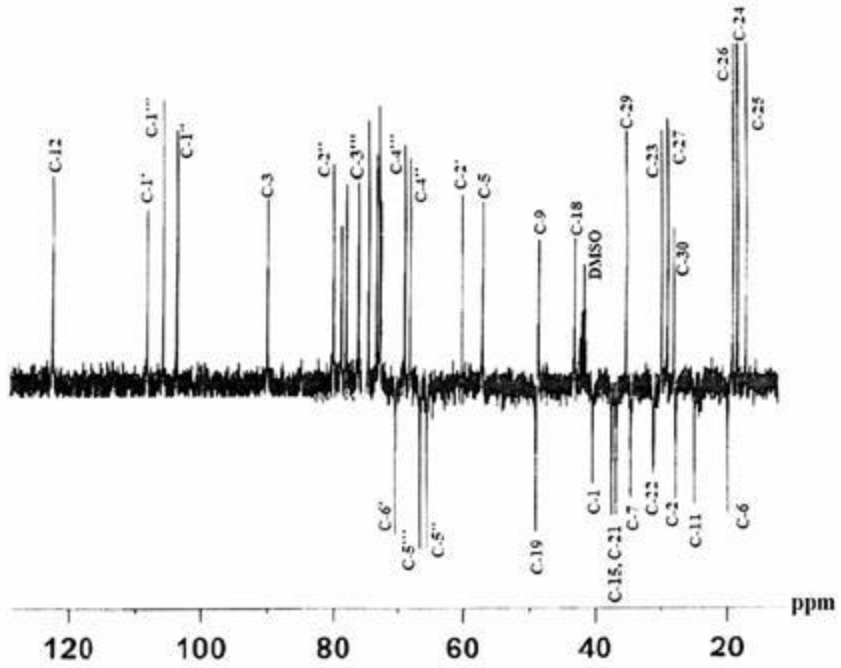


Figure 3:24 DEPT-135 Spectrum of Compound **3.27**

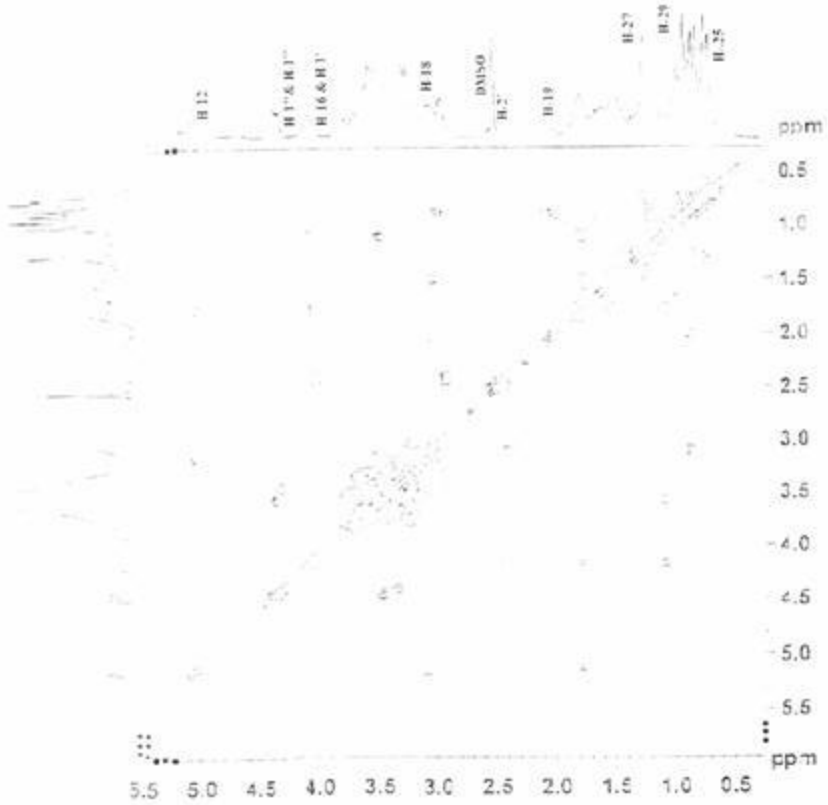


Figure 3:25 COSY Spectrum of Compound **3.27**

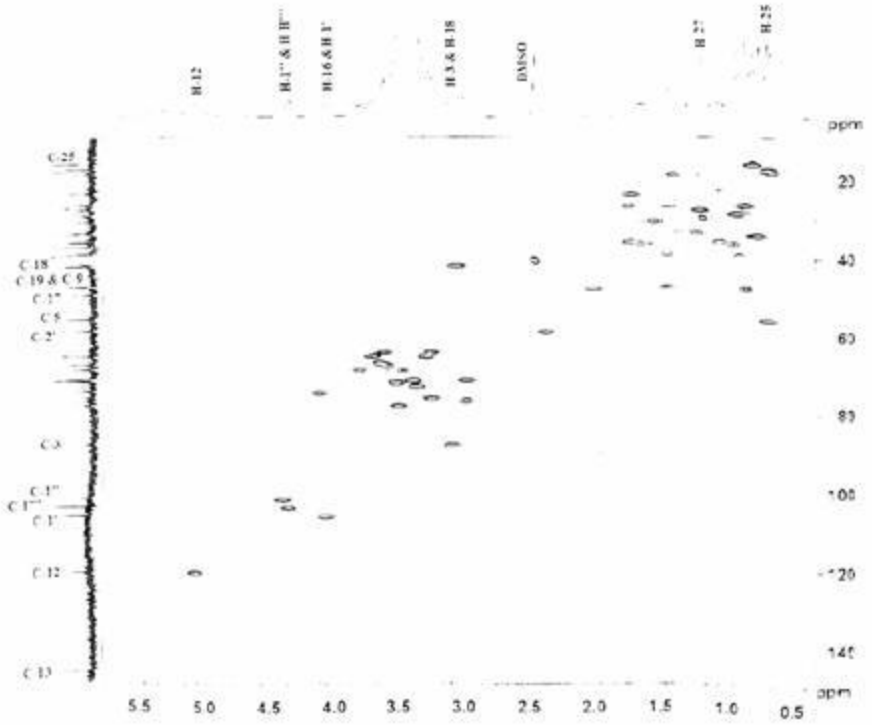


Figure 3:26 HSQC Spectrum of Compound **3.27**

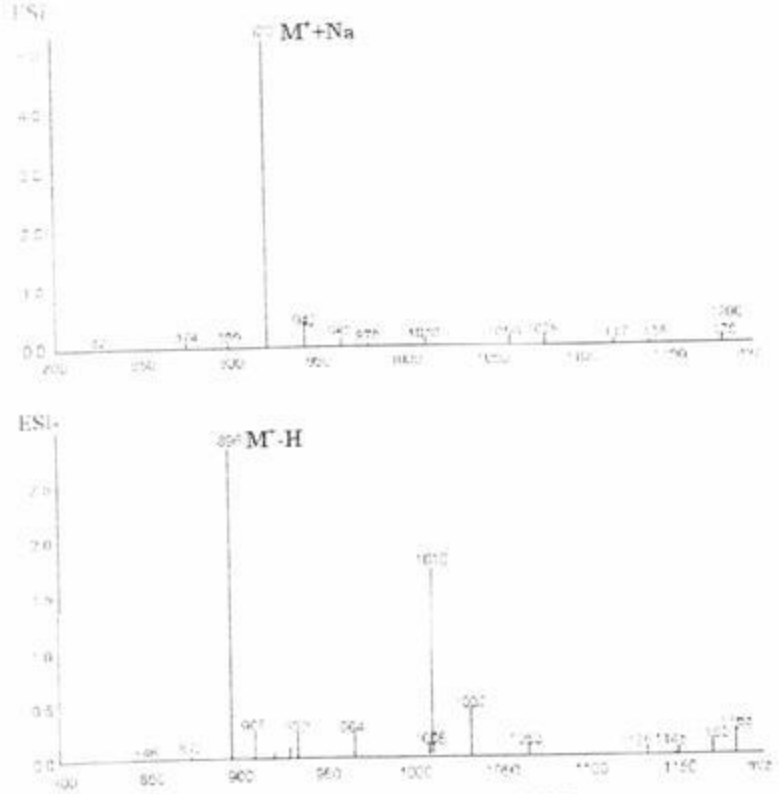


Figure 3:28 ES-MS of Compound **3.27**

The ^{1}H NMR of **3.27** (Figure 3:22) showed 7 upfield singlets at δ 0.70, 0.74, 0.80, 0.85, 0.91, 0.97 and 1.27 (3H each) for 7 tertiary methyl groups. Other characteristic signals in the spectrum were an olefinic proton at δ 5.12, a broad singlet at δ 2.39 for H-2', carbinolic methine proton at δ 4.16 (H-16), doublets centered at at δ 4.11 (*J* 6.9 Hz) and 4.40 (*J* 4.8 Hz), several sugar protons between δ 3.01- 4.45 and a multiplet at δ 3.14. The ^{13}C NMR of **3.27** (Figure 3:23) showed 46 intense signals suggesting the presence of at least 46 carbon atoms in the molecule. DEPT-135 spectrum (Figure 3:24) of **3.27** showed 7 methyl, 12 methylene, 19 methine and 7 quaternary carbons. Fourteen of the methine carbons appearing downfield at δ 65.9, 66.7, 70.5, 70.6, 70.9, 72.2, 73.4, 75.2, 76.2, 77.2,

87.1, 100.9, 103.1 and 105.4 were oxygenated. Similarly three of the methylene carbons appearing at δ 63.0, 64.4 and 68.0 were oxygenated.

Table 3:8 NMR spectral data for 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.27)

C	^{13}C NMR*	HSQC (δ Values for attached Hs	HMBC C-H Correlations	^{1}H-^{1}H COSY coupling Hs
1	38.0t	1.49, 0.95	H- 25 ^{3}J	
2	25.5t	1.78, 1.5		H- 2 and H-1 H-1'
3	87.1d	3.14	H- 23, H- 24, GlcNH$_2$ H'-1 ^{3}J	
4	38.5s	-	H- 23, H- 24 ^{2}J	
5	55.0d	0.72	H- 23, H- 24, H- 25 ^{3}J	
6	17.7t	1.47, 1.32		
7	32.5t	1.33	H- 26 ^{3}J	
8	38.5s	-	H- 26 ^{2}J; H- 27 ^{3}J	
9	46.5d	1.49	H- 25, H- 26 ^{3}J	
10	36.2s	-		
11	22.7t	1.77		H-11 and H-12
12	119.7,8d	5.12		
13	145.2s	-	H- 27 ^{3}J	
14	41.3s	-	H- 26 ^{3}J; H- 27 ^{2}J	
15	34.7t	1.79, 1.08	H-27 ^{3}J	
16	73.4d	4.16		H-16 and H-15, H-15'
17	49.6s	-		
18	41.2d	3.14	H-12, H-16 ^{3}J	H-18 and H-19, H-19'
19	46.7t	2.4, 0.98	H- 29, H- 30 ^{3}J	
20	29.8s	-	H- 29, H- 30 ^{2}J	
21	35.2t	1.70, 0.98	H- 29, H- 30 ^{3}J	
22	28.3t	1.20		
23	27.8 q	0.97 s	H- 24 ^{3}J	
24	16.4 q	0.74 s	H- 23 ^{3}J	
25	15.11q	0.85 s		
26	17.1 q	0.70 s		
27	26.9q	1.27 s		
28	181.2s			
29	33.1	0.80 s	H- 30 ^{3}J	
30	26.3q	0.91 s	H- 29 ^{3}J	
C-3-O-sugar GlcNH$_2$				
1'	105.4d	4.11		H-1' and H- 2'
2'	55.8d	2.39		H- 2' and H- 3'
3'	76.2d	3.03	H- 2' ^{2}J	
4'	70.5d	3.01		H- 4' and H- 5'
5'	75.2d	3.28		

6'	68.0t	3.5, 3.85		H-6' and (H- 6") '
NH2				
Ara				
1"	100.9d	4.45		H-1"and H- 2"
2"	77.2d	3.54	H-1" ^{3}J	
3"	70.9d	3.54	H-1"^{3}J, H- 2" ^{2}J	
4"	65.9d	3.67		
5"	63.0t	3.28, 3.66		H- 5" and (H-5") '
Ara				
1""	103.1d	4.40	H- 5"", H- 5"", H- 3"" ^{3}J, H- 2"" ^{2}J	H-1""and H- 2""
2""	70.6d	3.42	H- 3 ^{2}J	
3""	72.2d	3.4	H-1"" ^{3}J, H-5"" ^{3}J, H-2"" ^{2}J	
4""	66.7d	3.63	H- 5"" ^{2}J	
5""	64.4 t	3.32, 3.74	H-1"" ^{3}J	H- 5"" and (H- 5"") '

*Spectra determined in DMSO (D_6) at 300 and 75 MHz for ^{1}H NMR and ^{13}C NMR respectively, DEPT was used to determine the multiplicities

The spectral data (Table 3:8, Figures 3:22- 3:28) of **3.27** showed characteristic features for echinocystic acid as discussed for the last four compounds. The homonuclear ^{1}H-^{1}H COSY and heteronuclear correlation of HMBC of **3.27** (Figure 3:29) confirmed that the aglycone in **3:27**, like compounds **3.6**, **3.22**, **3.23** and **3.26** was also echinocystic acid.

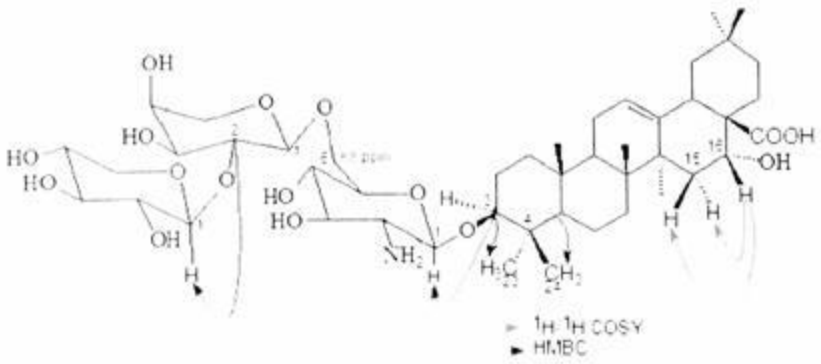


Figure 3:29 Important homonuclear and heteronuclear correlations of Compound **3.27**

The sugar chain present in compound **3.27** was determined on the basis of NMR and ES-MS spectral data. The ^{13}C NMR of **3.27** showed three anomeric carbons at δ 100.9, 103.1 and 105.4 suggesting the presence of at least three sugar molecules in compound **3.27**. From the ES-MS spectral data the molecular weight of **3.27** was deduced as 897. The odd molecular weight required the presence of at least one nitrogen atom in the molecule of **3.27**. Based on this molecular weight and the ^{13}C NMR and DEPT spectral data the molecular formula of **3.27** was deduced as $C_{46}H_{75}NO_{16}$. In comparison with the previously discussed saponins, the ^{1}H NMR and ^{13}C NMR of **3.27** did not show the characteristic signals for the acetyl group which are signals for methyl and HN- C=O groups. This observation and the presence of a signal at δ 55.8 and proton signal at δ 2.39 in the ^{13}C NMR and ^{1}H NMR of **3.27** respectively meant that one of the sugars in **3.27** is glucosamine instead of acetyl glucosamine as seen in saponins **3.6, 3.22, 3.23** and **3.26**. Furthermore TLC of the hydrolysis product of **3.27** confirmed the presence of glucosamine*.

Furthermore a molecular formula $C_{46}H_{75}NO_{16}$ for compound **3.27** led to the prediction that, the remaining two sugars were pentoses and from the ^{13}C NMR and the literature (Caprani et al., 1989) it was evident that both sugars were L-arabinose. The configuration of the anomeric center (δ 4.11) for glucosamine was determined to be β- as it was revealed by $^{3}J_{H-1''H-2''}$ of 6.9 Hz. Furthermore an anomeric proton H-1''' at δ 4.40 had a coupling constant of 4.8 Hz indicating an α- configuration, however the configuration of the other arabinose unit was not evident from the ^{1}H NMR spectrum. From the literature (Melek et al., 2007; Caprani et al., 1989) the other arabinose was assigned a α-configuration.

The connectivity between echinocystic acid and the sugar was confirmed by the HMBC spectrum of **3.27** (Figure 3:27, Table 3:8)). A cross peak was seen due to long- range

* Ten (10) mg of **3.27** was mixed with 4 mls of 10% HCl and refluxed over a water bath for 5 hours, followed by partitioning the aqueous hydrolyzate with ethylacetate, neutralizing the aqueous fraction with sodium carbonate, the mixture was dried under vacuo and redissolved into methanol. The clear methanolic solution was checked for the presence of glucosamine chloride by TLC developed in Butanol: acetic acid: water 4:2:2 and an authentic sample of glucosamine chloride used as a reference. The plates were sprayed with ninhydrin reagent followed by gentle heating in an oven giving orange spots with similar hR_f values.

correlation between C-3 of echinocystic acid and H-1' (δ 4.11) of glucosamine indicating the presence of a glycosidic bond at this carbon atom. Furthermore the resonances of C-3 (δ 87.1d) and C-2 (δ 25.5) were shifted as expected due to glycosylation (Mahato and Kundu, 1994).

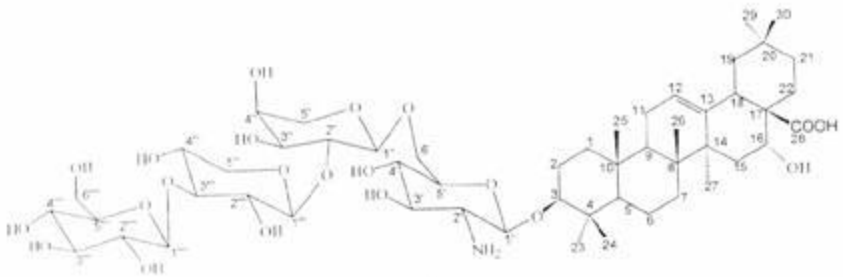
The linkage between glucosamine and one of the pentose sugars was not evident from long- range correlation HMBC experiment, it was however, established through ^{13}C NMR chemical shifts. The chemical shift of δ 68.0, for C-6' clearly indicated that this is the carbon linked to L- arabinose. The linkage between the two arabinose sugars was made clear by the HMBC experiment (Figure 3:27) of **3.27**, which showed a cross peak due to long range correlation between the H-1''' (δ 4.40) and C-2'' (δ 77.2). This linkage was further confirmed by ^{13}C NMR chemical shifts, which showed a chemical shift of δ 77.2 and δ 70.6 for C-2'' and C-2''' respectively. The observed shift of 6.6 ppm for C-2 was attributed to glycosylation.

From the above spectroscopic data compound **3.27** was characterized as 3-*O*-[α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid. This compound is related to compound **3.6** a known compound of *A. anthelmintica* (Caprani et al., 1989), the only difference seen is the absence of an acetyl group in this compound as compared to **3.6**. The isolation of the two compounds related in this manner could mean that this compound may be a precursor of **3.6**, which means that within the plant compound **3.27** is acetylated to **3.6** with acetyl co enzyme A. Furthermore, compound **3.27** is being reported in nature for the first time, hence it is a new compound.

3.3.7 Isolation and characterization of 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β-D-glucopyranosyl echinocystic acid (3.28)

A white amorphous compound **3.28** (9 mg) was isolated along with compound **3.22** from PTLC4 (Figure 3:4) and also a small amount of **3.28** was obtained from PTLC2 along with compound **3.27** (Figure 3:4).

Compound **3.28** in its electro spray mass spectra (ES-MS) showed base peaks at *m/z* 1058 and 1082 in –ve and +ve mode, respectively, the *m/z* 1058 is the molecular ion less H (M^+ -H) while the *m/z* 1082 is M^+ + Na.



3.28

The structure of **3.28** was characterized by analysis of its ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and ES-MS spectroscopic data. The NMR experiments, like the previous compound **3.27** were carried out in DMSO, hence, most of the chemical shifts were upfield, due to solvent effects.

The ^{1}H NMR of **3.28** showed 6 upfield singlets at δ 0.72, 0.78, 0.84, 0.88, 0.94, and 1.22 for 7 tertiary methyl groups, one peak, at δ 0.72 represented two methyl groups. Other characteristic signals in the spectrum were an olefinic proton at δ 5.11, carbinolic methine proton at δ 4.17 and several sugar protons between δ 3.08- 4.41. The ^{13}C NMR of **3.28** (Table 3.9) showed 48 intense signals suggesting presence of at least 48 carbon atoms in the molecule. The DEPT spectrum showed 7 methyl, 12 methylene, 23 methine and 7 quaternary carbons. One peak at δ 65.6 represented two carbons one for a methylene and other for a methine. Seventeen of the methine carbons, appearing downfield at δ 65.6, 66.9, 67.3, 69.8, 69.9, 70.6, 73.7, 75.0, 76.2, 76.9, 78.5, 82.2, 87.6, 100.8, 103.8, 104.0 and 105.8 were oxygenated. Similarly four of the methylene carbons appearing at δ 60.9, 62.6, 65.6 and 68.4 were oxygenated.

Table 3:9 **NMR spectral data for 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid (3.28)**

C	^{13}C NMR*	HSQC (δ Values for attached Hs	HMBC C-H Correlations	^{1}H-^{1}H COSY coupling Hs
1	38.2t	0.94, 1.50		
2	25.02t	1.81, 1.51		
3	87.6d	3.08	H-23, H-24 ^{3}J	H-3 and H-2, H-2'
4	38.7s	-	H-23, H-24 ^{2}J	
5	55.1d	0.71		
6	17.9t	1.40		
7	32.8t	1.32, 1.35		
8	38.7s	-	H-26 ^{2}J	
9	46.6d	1.49	H-25 ^{3}J	
10	36.4s	-	H-25 ^{2}J	H-1 and H-12
11	23.0t	1.75		
12	119.9d	5.11		
13	145.5s	-		
14	41.4s	-		
15	34.9t	1.09, 1.74		
16	73.9d	4.17		H-16 and H-15, H-15
17	48.6s	-		
18	41.2d	3.10		H-18 and H-19, H-19'
19	47.0t	0.98, 2.04	H-30 ^{3}J	H-19 and H-19'
20	30.3s	-	H-29, H-30 ^{2}J	
21	35.6t	1.00	H-29 ^{3}J	
22	-			
23	28.0q	0.94	H-24 ^{3}J	
24	16.7q	0.72	H-23 ^{3}J	
25	15.4q	0.84		
26	17.4q	0.72		
27	26.9q	1.22		
28	181.8s	-		
29	33.4q	0.78	H-30 ^{3}J	
30	25.8q	0.88	H-29 ^{3}J	
C-3 O-sugar	GlcNH$_2$			
1'	105.5d	4.08		
2'	57.9d	2.40		H-2' and H-1', H-3'
3'	73.7d	3.08		
4'	70.6d	3.59		
5'	75.0d	3.27		
6'	68.4t	3.5, 3.82		
NH2				
Ara				
1"	100.8d	4.4		
2"	78.5d	3.60		
3"	70.6d	3.59		
4"	66.9d	3.84		

5'''	62.6t	3.32, 3.65	
Ara			
1''''	104.6d	4.34	H-2'''' ^2J,
2''''	69.8d	3.1	
3''''	82.2d	3.52	H-1'''' ^3J
4''''	67.3d	3.64	
5''''	65.6dt	3.41, 3.75	
Glc			
1'''''	103.8d	4.34	
2'''''	70.6d	3.59	
3'''''	76.9d	3.16	
4'''''	69.9d	3.10	
5'''''	76.2d	3.16	
6'''''	60.9d	3.62, 3.46	

*Experiments determined in DMSO (D_6) at 400 and 100 MHz for ^{1}H NMR and ^{13}C NMR respectively. DEPT was used to determine the multiplicities

Like the other compounds discussed earlier, the spectral data of compound **3.28** indicated that the aglycone was echinocystic acid. The homonuclear ^{1}H-^{1}H COSY and heteronuclear HMBC correlations confirmed that the aglycone in **3.28**, like in compounds **3.6, 3.22, 3.23**, **3.26** and **3.27** was also echinocystic acid.

The sugar chain present in compound **3.28** was determined on the basis of its NMR, ES-MS spectral data and by comparison of ^{13}C NMR chemical shifts of **3.28** to that of related compounds **3.22** and **3.27**. The ^{13}C NMR of **3.28** showed four anomeric carbons at δ 100.8, 103.8, 104.0 and 105.8, suggesting the presence of at least 4 sugar molecules in the compound. From the ES-MS spectral data the molecular weight of **3.28** was deduced as 1059. Based on this molecular weight and the ^{13}C NMR and DEPT spectral data the molecular formula of **3.28** was deduced as $C_{52}H_{85}NO_{21}$. Like compound **3.27**, the ^{1}H NMR and ^{13}C NMR of **3.28** did not show the characteristics signals of the *N*-acetyl group, which meant that like in compound **3.27** one of the sugars in **3.28** was also glucosamine.

Furthermore, in support of the molecular formula $C_{52}H_{85}NO_{21}$ for compound **3.28**, the remaining three sugars had to be, two pentoses and one hexose. During the isolation (Figure 3.4), it was observed that compound **3.28** was related to compound **3.22** in a

similar manner seen between compounds **3.27** and **3.6**. From this observation it was postulated that the remaining sugars in compound **3.28** had to be one D-glucose and two L-arabinose and with similar configurations to those of compound **3.22**. Due to the poor resolution of the peaks in ^{1}H NMR the anomeric configuration of the sugar could not be confirmed.

The connectivity between echinocystic acid and the sugar moieties was not evident from the HMBC spectrum of **3.28** (Table 3:9) but from ^{13}C NMR spectrum of **3.28** the chemical shift of δ 87.6 for C-3 of echinocystic acid clearly indicated that the sugar is attached to this carbon atom. Furthermore the resonances of C-3 (δ 87.6) and C-2 (δ 25.0) were shifted as expected due to glycosylation (Mahato and Kundu, 1994). It was further postulated that similar to compound **3.22** discussed earlier, the glycosidic linkage was between C-3 of echinocystic acid and C-1' of the glucosamine.

The linkage between sugars, were established from the ^{13}C NMR chemical shifts (Table 3:9). The chemical shift of δ 68.4 for C-6' in glucosamine, clearly indicated that this was the carbon linked to the first L-arabinose. Likewise, the chemical shifts of δ 78.5 for C-2'' indicated that this carbon was connected to the second L-arabinose. The chemical shift of δ 82.2 for C-3''' indicated that this carbon was linked to the terminal sugar, D-glucose. This was also supported by the HMBC spectrum of **3.28**, which showed a cross peak due to long- range correlation between the H-1'''' (δ 4.34) and C-3''' (δ 82.2).

From the above spectroscopic data compound **3.28** was characterized as 3-*O*-[β- D-glucopyranosyl (1→3)] [α-L-arabinopyranosyl (1→2)][α-L-arabinopyranosyl (1→6)]-2-amino-2-deoxy -β- D-glucopyranosyl echinocystic acid. This compound was related to compound **3.22** a known compound of *A. anthelmintica* (Caprani et al., 1989), in a similar manner to what was observed for compounds **3.27** and **3.6** and with the same implications as discussed earlier. This is the first report for the occurrence of compound **3.28** in nature.

3.3.8 Minimum Inhibitory Concentration of the compounds isolated from *Albizia anthelmintica* root bark

The Minimum Inhibitory Concentrations (MICs) of the isolated compounds were determined for standard strains *Candida albicans* ATTC 90028 and MTCC 1637 and one clinical isolate (CI). The MICs for the compounds **3.6**, **3.22**, **3.23**, **3.25-3.28** are shown in Table 3:10. Three out of the seven compounds isolated from the root bark of *A. anthelmintica* showed activity against *Candida albicans*. Compound **3.27** was the most active with MIC of 25, 25 and 50 µg/ml for *C. albicans* MTCC1637, clinical isolate and ATCC 90028, respectively. This was followed by compound **3.6**, which had MICs of 50 and 100 µg/ml, for MTCC 1637 and the clinical isolate, respectively and lastly compound **3.28** with MICs of 100 and 125 µg/ml for MTCC 1637 and ATCC 90028, respectively. The other compounds showed no inhibition at the concentrations tested. The lowest MIC for all tested *Candida* strains was shown by FC a chromatographic fraction containing compounds **3.6** and **3.27**. This fraction showed MICs of 15.6, 31.1 and 15.6 µg/ml for MTCC1637, clinical isolate and ATCC 90028 respectively.

Table 3:10 MICs of compounds isolated from *Albizia anthelmintica* root bark

S/N	Compounds	*C. albicans* strains*		
		MTCC1637	ATCC90028	CI
1	**3.6**	50.00	>100.00	100.00
2	**3.22**	>1000.00	>1000.00	>1000.00
3	**3.23**	>500.00	>500.00	>500.00
4	**3.25**	>100.00	>100.00	>100.00
5	**3.26**	>500.00	>500.00	>500.00
6	**3.27**	25.00	50.00	25.00
7	**3.27:3.6** (1:3)	-	12.50	-
8	**3.27:3.6** (1:2)	-	12.50	-
9	**3.27:3.6** (1:1)	-	25.00	-
10	**3.28**	100.00	125.00	>100.00
11	**3.28:3.22** (1:1)	-	62.50	-
12	FC*	15.60	31.30	15. 60
13	A*	3.13	3.13	6.25
14	C*	0. 39	0.78	0. 39

*A: amphotericin B, C: clotrimazole, CI: clinical isolate; FC: chromatographic fraction containing compounds **3.6** and **3.27**, - Not done

The MIC profile shown by the isolated compounds **3.6** and **3.27** when compared with the chromatographic fraction FC meant that these compounds could be potentiating the activity of one another. In order to prove synergism among these compounds, an experiment was carried out in which MICs for several combinations of both compounds were determined using *C. albicans* standard strain ATCC 90028. As anticipated the combinations gave lower MICs of 12.5 –25 µg/ml (Table 3:10) when compared with those of each of the compounds. Also the MICs were lower than those observed for FC but due to limited sample enough data could not be collected so as to find out the best combination. However, from the above results it was observed that increasing the proportion of compound **3.27** increased the MICs. Also MICs for combinations of compounds **3.22** and compound **3.28** in a ratio of 1:1 was determined and found to be 62.5 µg/ml. Again the MIC was decreased by half when compared to that of compound **3.28** alone. However, compound **3.22** alone had shown no activity even at a concentration of 1000 µg/ml. This clearly showed that there was potentiation of the anticandida activity of compound **3.28** by compound **3.22**, a compound probably completely devoid of anticandida activity.

The bioautogram of the crude extract had shown that the plant had at least four active compounds as indicated by the presence of four active spots (Figure 2:4 and Figure 2:6) [Chapter 2]). Two of the active spots were well separated higher up on the bioautogram and from the chromatogram they were for compounds **3.23** and **3.26**. The other active spots on the bioautogram could have been due to any of the compounds **3.6**, **3.22**, **3.27** and **3.28** which were rather down on the chromatogram. The fact that compounds **3.23** and **3.26** were not able to show any inhibition at the maximum concentration of, 500 µg/ml, indicates that they have a very weak activity or the activity observed in bioautography agar overlay was due to other compounds present in very low concentrations which could not be isolated by the isolation methods used.

Another observation on MICs is that the *C. albicans* strain ATCC 90028 was more resistant when compared to the other two *C. albicans* strains used in the study.

CHAPTER 4

ISOLATION, CHARACTERIZATION AND ANTICANDIDA ACTIVITY OF COMPOUNDS OF *COMBRETUM ZEYHERI* LEAVES

4.1 INTRODUCTION

4.1.1 The Genus *Combretum* Loefl

Combretum is the largest genus of the family Combretaceae, a family of the order Myrtales which comprises 20 genera and 600 species widely distributed in the tropics and subtropics including Africa, South America and Asia (Rogers and Verotta, 1996; Eloff et al., 2005). The genus *Combretum* comprises of about 250 species, which are characterized by bearing 4-5 winged, ridged, angled, sessile or stipitate fruits. Three, subgenera are recognized by Excell and Stace, they are *Combretum*, *Cacoucia* and Asian subgenus *Apetalanthum* (Wickens, 1973). The species of *Combretum* are trees, shrubs, shrublets or woody climbers very rarely subherbaceous (Wickens, 1973). In Tanzania there are at least 36 *Combretum* species (Appendix 6) among which four including *C. chionanthoides*, *C. longispicatum*, *C. purpureiflorum* and *C. tenuipetiolatum* are endemic (Selemani, 2007).

The wood of *Combretum* species has a high resistance to borers and termites and it is due to this that several species are used in Africa as a source of wood fuel (Abbot and Lowore, 1999; Tabuti et al., 2003a; Luoga et al., 2004). However, majority of the species are used in traditional medicine in Africa, Asia and other parts of the world.

4.1.1.1 Ethnomedical uses of *Combretum* species

Species of *Combretum* have been used extensively in traditional systems of therapy in Africa and Asia (Katerere et al., 2003), some of the uses are summarized in Table 4:1. Some *Combretum* species, which find use in Tanzanian traditional medicine, include *C. collinum*, *C. fragrans*, *C. molle*, *C. psidioides*, *C. adenogonium* and *C. zeyheri* (Sawhney et al., 1978a, b; Hedberg et al., 1983a, b; Khan et al., 2000; Fyhrquist et al., 2002; Maregesi et al., 2007).

Traditional healers in Africa most frequently use leaves and roots of the plants, whereas the four winged fruits, which are produced in great amounts, are never used or eaten by indigenous people or wild animals because they are considered toxic (Rogers and Verotta, 1996). One of the most important symptoms reported in all poisoning was a violent and prolonged hiccupping; hence, the name 'hiccup nut' given, to many species which suggests that the toxins are common to fruits of all *Combretum* species.

Table 4:1 Ethnomedical uses of some *Combretum* species

Species	Plant parts*	Country	Uses/Used for	References
C. aculeatum	L	Sudan	Tuberculosis	Almagboul et al., 1988
	R		Skin diseases	El-Kheir and Salih, 1980
C. adenogonium	L, Sb	Tanzania	Wounds and skin fungal infections	Maregesi et al., 2007
C. apiculatum	R	India	Snake bite	Kamdem et al., 1986
	L	S. Africa	Enema	Mc Gaw et al., 2000
	-	E. Africa	Dysentery, leprosy	Rogers and Verotta, 1996
		S. Africa	Abdominal problems and conjuctivitis	
		Zimbabwe	Body weakness	
C. bauleranum	-	Uganda	Diarrhoea	Anokbonggo et al., 1990
C. bracteatum	S	Nigeria	Dysentery	Olukoya et al., 1993
C. caffrum	R	S. Africa	Analgesic	Bhat and Jacobs, 1995
	Rb		Anticancer	Pettit et al., 1982
C. collinum	R	India	Antivenin	Selvanayahgam et al., 1994
		Kenya	GIT problems	Johns et al., 1995
		Tanzania	Dysmenorrhea, dysentery	Hedberg et al., 1983a
C. confertum	-	Cameroon	Fractures	Kamdem et al., 1986
C. constrictum	R	E. Africa	Aphrodisiac	Kokwaro, 1976
C. decandrum	L	India	Boils	Girach et al., 1994
			Diarrhoea and GIT problems	Laurens et al., 1985
	So		Eczema and ringworm	Tiwari and Padhye, 1993
C. dolichopetalum	Rb	Nigeria	GIT problems, liver diseases	Udem et al., 1997; Azuzu and Adimorah, 1998
C. erythrophyllum	R	S. Africa	Cough	Arnold and Gulumian, 1984
		Zimbabwe	Aphrodisiac and purgative.	Sohni and Kale, 1997
C. fragrans	-	E. Africa	Cough, syphilis	Rogers and Verotta, 1996
		Zimbabwe	Aphrodisiac	

	R	Tanzania	Diarrhoea	Fyhrquist et al., 2002
	L	Togo	Wounds	Batawila et al., 2005
C. glomeruliflorum	R	S. Africa	Aphrodisiac	Watt and Breyer-Brandwijk, 1962
C. glutinosum	L	Senegal	Antitussive	Tignokpa et al., 1986
	-		Hepatic and bronchial disease, diuretic, antihypertensive	Rogers and Verotta, 1996
C. hereroense		E. Africa	Bilharzia	Kokwaro, 1976
		Somali	Tonsillitis	Samuelsson et al., 1992b
C. imberbe	R	S. Africa	Schistosomiasis	Sparg et al., 2000
	-	S. Africa	Cough, colds	Rogers and Verotta, 1996
		Zimbabwe	Diarrhoea	
C. kraussii	R	S. Africa	Analgesic	Bhat and Jacobs, 1995
C. micranthum	B	Senegal	Wounds, sores, bronchitis and Guinea worms,	Le Grand and Wondergem, 1987
	L		Lipotropic, choleretic, cholagogue and sores	Bassene et al., 1985, 1986
			Cough and bronchitis	Le Grand, 1989
	L & S		Febrifuge	Tignokpa et al., 1986
			Tonic	Laurens et al., 1985
	L	Mali	Antimalarial	Ancolio et al., 2002
	R	Senegal	Blennorrhagia	Le Grand, 1989
		Ghana, Nigeria and Guinea	Guinea worms Parasitic sores	Comley, 1990
	-	Sierra Leone	Guinea worms	
C. microphyllum	-	Zambia	Lunacy	Rogers and Verotta, 1996
C. molle	B	S. Africa	Tuberculosis	Lall and Meyer, 1999
			Anthelmintic	Arnold and Gulumian, 1984
	L	E. Africa	Anthelmintic	Kokwaro, 1976
	-		Stomachache, snake bite, leprosy, fever and dysentery,	Rogers and Verotta, 1996
		Zimbabwe	Diarrhoea, convulsions, wounds and headache	
		Malawi	Anthelmintic, snake bite	
	R, L	Tanzania	GIT problems	Mathias, 1982
	S		Chewing stick	Khan et al., 2000
	R, L		Venereal diseases, wounds, skin infections influenza and aedema	Fyhrquist et al., 2002

	R	India	Snake bite	Selvanayahgam, et al., 1994
		Africa	abortifacient	Kokworo, 1981
		S. Africa	Anthelmintic	Arnold and Gulumian, 1984
		Tanzania	Stomachache and diarrhoea,	Hedberg et al., 1983b
C. mossambicense	R	E. Africa	Bilharzia	Kokwaro, 1976
C. ovalifolium	R	India	Headache, measles, smallpox and snakebite	Sabnis and Bedi, 1983
C. padoides	R	E. Africa	Anthelmintic	Kokwaro, 1976
C. paniculatum	R		Venereal diseases	Arnold and Gulumian, 1984
	L	Togo	Diarrhoea	Batawila et al., 2005
		Ethiopia	Diarrhoea and leprosy	Desta, 1993
	S	Nigeria	Fever	Gill and Akinwumi, 1986
C. pentagonum	R	E. Africa	Aphrodisiac	Kokwaro, 1976
C. psidioides	Rb	Tanzania	Antimalarial	Gessler et al., 1994
	R, L		Muscle pains, aedema and diarrhoea,	Fyhrquist et al., 2002
C. platypetulum	-	Zambia	Swelling due to mumps	Rogers and Verotta, 1996
		Zimbabwe	Earache, diarrhoea, antiemetic and pneumonia	
C. racemosum	R, L	Nigeria	GIT problems, diarrhoea and premature labour	Akubue et al., 1983
C. roxburghii	L	Nepal	Wounds between toes	Manandhar, 1995a
		India	Fever	Mukherjee and Namhata, 1990
C. xanthothyrsum	R	E. Africa	Aphrodisiac and anthelmintic	Kokwaro, 1976
C. zeyheri	B	Mozambique	Gynecological problems	Amico, 1977
	L	Zimbabwe	Diarrhoea, eye infection	Breytenbach and Malan, 1989
	Ap	Tanzania	Diarrhoea,	Sawhney et al., 1978a
	R, L		Diarrhoea and Cancer	Fyhrquist et al., 2002

Key: Ap: aerial parts, B: barks; L: leaves; Hw: heart wood, R: roots; Rb: root bark; S: stem; Sb: stem bark, Sc: seeds, Soc seed oil

4.1.1.2 Biological activities of the genus *Combretum*

The *Combretum* species have been investigated for varied types of biological activities (Appendix 7). Cytotoxic and antimicrobial activities, which include antibacterial, antifungal and antiviral activities, are the most studied. Generally the tested biological activities were found in almost all the plant organs and were depicted by solvents with varied polarity meaning that the activities are not inherent or restricted to a particular class of compounds (Appendix 7).

Combretum caffrum and other species including *C. collinum*, *C. erythrophyllum*, *C. fragrans*, *C. molle*, *C. nigricans* and *C. paniculatum* have been reported to be cytotoxic (Pettit et al., 1982; 1987a; b; 1988a; b; c; 1989; 1995 Wall et al., 1996; Schwikkard et al., 2000; Asres et al., 2001; Simon et al., 2003; Fyhrquist et al., 2006). The South African species have been extensively investigated on this aspect. Cytotoxic activity testing involved both *in vivo* and *in vitro* assays using a number of cell lines. Bioassays such as murine P-388 lymphocytic leukemia (PS) cell growth inhibition (Pettit et al., 1987), agar diffusion yeast bioassay for DNA –damaging agents (Schwikkard et al., 2000; Mc Gaw et al., 2001) and topoisomerase II inhbition (Wall et al., 1996) were performed on a number of extracts and also used in the bioassay- guided isolation of the active compounds. Other bioassays involved inhibition of the growth of several cancer cells such as bladder (J82), small cell lung (A549), colon (HCT-15) and glioblastoma (U-373-MG) (Simon et al., 2003) breast cancer [CA-MDA-MB-231], human melanoma [CA-SK-MEL-28], mammalian HeLa and lung cancer cell lines [A459] (Abreu et al., 1999, Schwikkard et al., 2000). It is interesting to note that some potent anticancer compounds have been isolated from the genus. Also some species including *C. quadrangulare* were found to have hepatoprotective activity on cultured mouse hepatocytes (Adnyana et al., 2000; 2001).

Several species of *Combretum* have been investigated for antimicrobial activity and the most studied ones were *C. erythrophyllum* (Martin and Eloff, 1998; Eloff, 1998, 1999; Lindsey et al., 1999) and *C. micranthum* (Malcolm, and Sofowora, 1969; Laurens et al., 1985; Le Grand et al., 1988; Ferrea et al., 1993; Bassene et al., 1995; Benoit et al., 1996; Adoum et al., 1997; Abreu et al., 1999; Lall and Meyer, 1999). Both Gram- positive and

Gram -negative bacteria, yeast, dermatophytes and viruses were found sensitive. Promising results have been obtained, for instance acetone extract of *C. woodii* leaf was found active against Gram +ve and Gram –ve bacteria. In some cases it displayed a stronger activity than the positive controls, ampicillin and chloramphenicol (Eloff et al., 2005). Several antibacterial compounds have also been isolated from the genus (Katerere et al., 2003; Eloff et al., 2005). The efficient and economical bioautography method has enabled antifungal screening of a number of South African *Combretum* species most of which were found to be active on a number of fungi (Masoko and Eloff, 2006).

The species of the genus *Combretum* have also been evaluated and found to be active against a number of important tropical parasitic diseases including malaria (Gessler et al., 1994; Karou et al., 2003), schistosomiasis (Sparg et al., 2000; Mc Gaw et al., 2001), trypanosomiasis (Udem et al., 1996; Asres and Balcha, 1998; Asres et al., 2001; Kiuchi et al., 2002) and filariasis (Ampofo, 1977). Also some species were found to have molluscicidal activity (Adewunmi, 1984; Kloos et al., 1987), hence, the plants of the genus can as well be used to control schistosomiasis at vector level. Rogers and Verrotta (1996) had suggested and discussed the feasibility of using *C. molle* in Africa for that purpose.

The plant extracts of the genus have been reported to exert various activities on various biological systems. They displayed activity on skeletal and smooth muscles (Akubue et al., 1983; Asuzu and Onu, 1990; Asuzu and Njoku, 1992) and had effects on the cardiac as well as the uterine muscles (Bamgbose and Dramane, 1977; Akubue et al., 1983; Brookes et al., 1999; Lindsey et al., 1999). They also exhibited effects on several enzymes including topoisomerase II (Wall et al., 1996), angiotensin-converting enzyme (Braga et al., 2000), adenyl cyclase (Pousset et al., 1993), cyclooxygenase 1(Lindsey et al., 1999) and HIV-1 integrase (Tewtrakul et al., 2003). Other reported activities include; antiulcer, analgesic, CNS depressant, anthelmintic and antitussive activities.

4.1.1.2.1 Anticandida activity from *Combretum* Species

A number of species in the genus have been shown to have activity against a number of fungi including *Candida albicans*. Chloroform, methanol and water extracts, of the dried

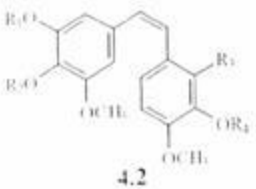
leaves and stem of *C. aculeatum* from Sudan were found to have activity against *Aspergillus niger* and *C. albicans* (Almagboul et al., 1988). The ethanolic extract of the leaves of *C. micranthum* from Guinea Bissau showed a weak activity against *C. albicans* (Abreu et al., 1999) and in Nigeria ethanolic and water extracts of the same plant part were found to have antimicrobial activity on a number of microorganisms including *C. albicans* (Adoum et al., 1997). Also, the decoction and water extract of the roots of the same species from Senegal was reported to have varied degrees of activity on several bacteria and yeasts including *C. albicans* (Bassene et al., 1995). The methanolic extract of the dried bark of *C. molle* from Tanzania was reported to have weak activity against several microorganisms including *C. albicans* (Khan et al., 2000). However, the leaf extract of *C. molle* from Togo was inactive against *C. albicans* but active on *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum* (Baba-Moussa et al., 1999). The acid ethanol and water extracts of the leaves of *C. paniculatum* from Ethiopia were reported to have a strong activity against *C. albicans* (Desta, 1993). The aqueous ethanolic extract of *C. fragrans* leaves from Togo was reported to have MICs in the range of 0.25- 4 mg/ml on various species of *Candida* including *C. albicans* for which the MIC was 1.0 mg/ml. The extracts were also active on a number of other fungi including *Cryptococcus neoformans* and several dermatophytes (Batawila et al., 2005). Masoko and Eloff (2006) reported anticandida activity for most of the 24 *Combretum* species from South Africa, which they investigated for antifungal activity. Methanolic and water extracts of the roots of *C. molle* and *C. paniculatum* from Venda, South Africa were reported to have MICs in the range of 1.00 -14.14 mg/ml for *C albicans* ATCC strain (Steenkamp et al., 2007).

4.1.1.3 Bioactive compounds from *Combretum*

A number for secondary metabolites have been isolated from the genus. These compounds belong to diverse classes of compounds including, stilbenes, triterpenes, diterpenes, flavonoids, phenanthrenes, cyclobutanes, macrocyclic lactones, steroids, tannins, proteins and akaloids. Some classes of compounds including stilbenes, triterpenes, flavonoids, phenanthrenes, tannins and macrocyclic lactones have been found to exhibit a number of biological activities.

into microtubules, leading to inhibition of cancer proliferation through disturbances of mitotic spindle function, which leads to cell apoptosis. In addition to its potent cytotoxic and inhibitory activity on tubulin polymerization, CA-4 is one of the few antitubulin agents reported to have selective vascular targeting activity (Tozer et al., 2002). Its poor solubility in aqueous media, its potential isomerisation to stable but inactive isomer and a desire to obtain more potent and selective compound have prompted a number of groups of scientists to design a more soluble, stable and /or active analogue (Pandit et al., 2006; Kerr et al., 2007). Combretastatin A-4 is currently in phase II and III clinical trials based on vascular shutdown mechanism of action (Chin et al., 2006, Kerr et al., 2007).

Glucosilated stilbene **(4.9)** and its analogue **(4.10)** isolated from *C. erythrophyllum* showed a positive activity in agar diffusion yeast bioassay for DNA – damaging agents (Schwikkard et al., 2000).

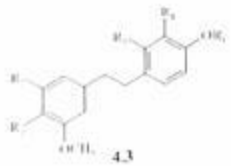


4.2

Cp	R1	R2	R3	R4	NAME
4.4	CH_3	CH_3	OH	H	A-1
4.5	- CH_2-	- CH_2-	OH	H	A-2
4.6	H	CH_3	H	OH	A-3
4.7	CH_3	CH_3	H	OH	A-4
4.8	H	CH_3	H	CH_3	A-5, A-6
4.9	OCH_3	OCH_3	O-Gl	OH	

Of the B series combretastatins B-1, B-2, B-3, B-4 **(4.11-4.14)** and bibenzyl **4.15- 4.18** isolated from the stem wood of *C. caffrum*, were found to inhibit the growth of PS cell lines (Pettit et al., 1987a; b; 1988a). Apart from their anticancer activity, some combretastatins, for example combretastatin B-5 **(4.15)**, a compound isolated the leaves of *C. woodii*, have been reported to have antibacterial activity (Eloff et al., 2005). Combretastatin B-5 was shown to have an activity equal to that of ampicillin and

chloramphenicol on *Pseudomonas aeruginosa* and also to be more active than these positive controls on both *Staphylococcus aureus* and *Enterococcus faecalis*.



4.3

Cp	R1	R2	R3	R4	R5	Name
4.10	OCH_3	OCH_3	CH_3	OH	O-Gl	
4.11	OCH_3	OCH_3	CH_3	OH	OH	B-1
4.12	O-CH_2-O	O-CH_2-O	CH_3	OH	OH	B-2
4.13	OCH_3	OCH_3	H	OH	H	B-3
4.14	OCH_3	H	H	OH	H	B-4
4.15	OCH_3	OH	CH_3	OH	OH	B-5
4.16	OCH_3	H	CH_3	OH	H	
4.17	OCH_3	OCH_3	H	H	H	
4.18	OCH_3	H	H	H	H	

4.1.1.3.2 Triterpenes

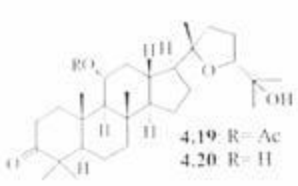
The triterpenes are one of the most commonly occurring groups of compounds in the genus and it is due to this that Carr and Rogers (1987) carried a chemosystematic study on the genus based on triterpenes and flavonoids. The triterpenes are secreted by the glandular hairs found on the leaves and fruits of all species belonging to the subgenus *Combretum* where they act as surface coatings (Rogers and Verotta, 1996). They are variously presented and they include acids, lactones and glycosides, particularly, those of cycloartane and oleanane type (Rogers and Verotta, 1996). Isolation of oleanane type of triterpene containing 29- carboxy-1α- hydroxy group, from *C. molle*, *C. elaeagnoides*, *C. imberbe*, *C. apiculatum*, *C. kraussii*, and *C. padoides* confirmed the taxonomic significance of triterpene synthesis in *Combretum* species (Rogers and Verotta, 1996).

The activities displayed by this group of compounds include cytotoxic, antiinflamatory, molluscicidal and antimicrobial activities. The Compounds, 11α-acetoxy-20, 24 epoxy-25-hydroxy-dammar-3-one **(4.19)** and 20, 24 epoxy-11, 25-dihydroxy-dammar-3-one **(4.20)** isolated from the methanolic extract of the leaves of *C. nigrans* were found to inhibit the proliferation of cultured U-373 MG, HCT-15, A549 and J82 human tumor cell lines (Simon et al., 2003). Several cycloartane triterpenes isolated from the methanolic extract of *C. quadrangulare* leaves had activity against murine colon 26 L5 carcinoma cells (Banskota et al., 1998), the most potent were methyl quandrangularate B **(4.21)** and

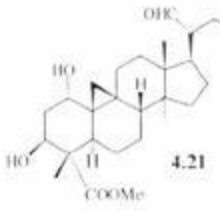
D **(4.22)**. Also a methanolic extract of the seeds of the same species yielded 2α, 6β-dihydroxybetulinic acid **(4.23)**, 6β –hydroxyhovenic acid **(4.24)**, arjunolic acid **(4.25)**, and 2α, 3β, 23 trihydroxyurs-12, 18-dien-28-oic acid **(4.26)**, which were reported to have a strong hepatoprotective activity (Adnyana et al., 2001). Arjunolic acid was also isolated from the ethanolic extract of *C. leprosum* in which it is present to the extent of 65% (Facundo et al., 2005).

Mollic acid β- D-glucoside **(4.27)** and imberbic acid **(4.28)** isolated from *C. mollie* and *C. imberbe*, respectively, were reported to have antiinflamatory and molluscicidal activities (Panzini et al., 1993; Angeh et al., 2007). Imberbic acid and other oleanene pentacylic triterpenes including 1α-hydroxylolean-12-en-30-oic acid **(4.29)**, 3, 30-dihydroxylolean-12-en-22-one **(4.30)**, 1, 3, 24- trihydroxylolean-12-en-29-oic acid **(4.31)** and 3- *O*-[β -2, 4- di- acetyl-L-rhamnopyranosyl-1α, 23- dihydroxylolean-12-en-29-oic acid **(4.32)** isolated from the leaves of *C. imberbe* were reported to have antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Angeh et al., 2007). Also in the same study, imberbic acid and 1α, 23- dihydroxy-12-oleanen-29-oic acid- 3β-*O*-2, 4-di- acetyl-L-rhamnopyranose revealed a strong anti-inflammatory activity, which was determined by inhibition of 3α- hydroxysteroid dehydrogenase. The latter also, had a moderate antiproliferative activity against leukemia [K-562] and L-929 and was cytotoxic against HeLa cell lines. Three glycosides **(4.33-4.35)** based on hydroxyimberbic acid and isolated from the leaves of *C. imberbe* were reported to have antibacterial activity against *Staphylococcus aureus*, *Proteus vulgaris* and *Mycobacterium fortuitum* (Katerere et al., 2003).

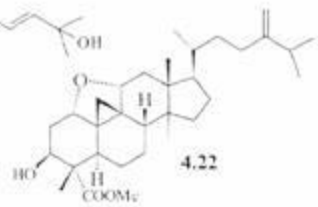
Other pentacyclic triterpenes isolated from the genus included ursolic acid, arjunic acid, betulinic acid, jessic acid and arjunglucoside (Osborne and Pegel, 1984; Rogers and Subramony, 1988; Breytenbach and Malan, 1989; Jossang et al., 1996; Adnyana et al., 2000). In addition to triterpenes, several diterpenes have also, been isolated from the genus (Facundo et al., 1993).



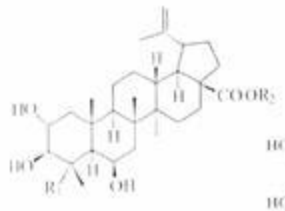
4.19: R= Ac
4.20: R= H



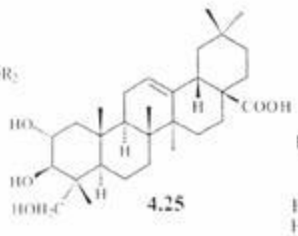
4.21



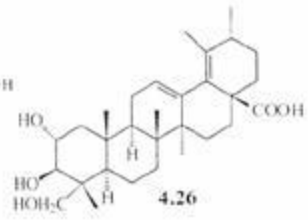
4.22



4.23: R_1: CH_3; R_2: H
4.24: R_1: CH_2OH; R_2: H



4.25



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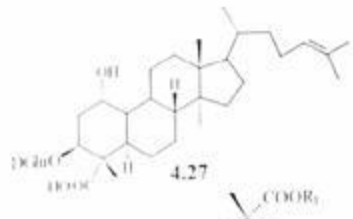
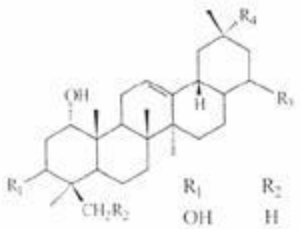
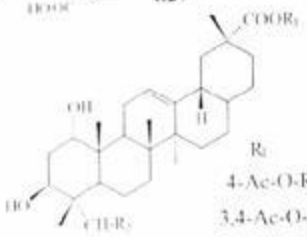


4.27



R_1	R_2	R_3	R_4	
OH	H	H	COOH	**4.28**
H	H	H	COOH	**4.29**
H	H	=O	CH_2OH	**4.30**
H	H	OH	COOH	**4.31**
2,4-Di-Ac-Rh	OH	H	COOH	**4.32**

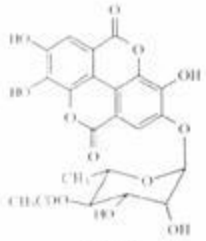


R_1	R_2	
4-Ac-O-Rh	H	**4.33**
3,4-Ac-O-Rh	H	**4.34**
3,4-Ac-O-Rh	Rh	**4.35**

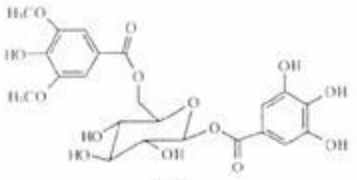
4.1.1.3.3 Tannins

Tannins, in particular hydrolysable tannins, including derivatives of gallic and ellagic acids have been isolated from several species of *Combretum* including *C. glutinosum*, *C. quadrangulare* and *C. yunnanensis* (Jossang et al., 1994; Adnyana et al., 2001; Asami et al., 2003). Some of them exhibited various biological activities, for instance 4-(4'' -*O*-

acetyl-rhamnopyanosyl) ellagic acid **(4.36)** isolated from the branches of *C. yunnanensis*, was reported to have a weak inhibitory activity against the growth of various tumor cells and it also inhibited HIV-1 protease (Asami et al., 2003). Also, a gallic acid derivative, 1-*O*-galloyl-6-*O*- (4-hydroxyl-3, 5-dimethoxy) benzoyl-β-D-glucose **(4.37)** isolated from the polar extract of the seeds of *C. quadrangulare* was reported to exhibit a potent hepatoprotective activity (Adnyana et al., 2001).



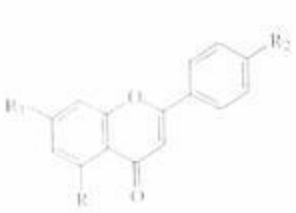
4.36



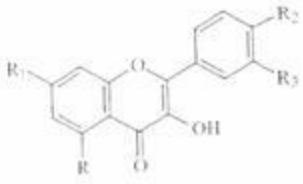
4.37

4.1.1.3.4. Flavonoids

Several species of *Combretum* have been reported to possess flavonoids, which are polyphenolic compounds. These compounds include flavones, flavonones and flavonols which have been isolated mostly from the leaves of *C. albopunctatum*, *C. micranthum*, *C. caffrum*, *C. imberbe*, *C. leprosum*, *C. erythrophyllum* and *C. quadrangulare* (Bassene et al., 1987; Pettit et al., 1987a; Rogers and Subramony, 1988; Facundo et al., 1993; Adnyana et al., 2000; Martin et al., 2004; Katerere et al., 2004). Some of these flavonoids have shown antibacterial activity; for example Martin et al., (2004) isolated seven antibacterial flavonoids from *C. erythrophyllum* leaves which included apigenin **(4.38)**, genkwanin **(4.39)**, 5-hydroxy-7, 4-dimethoxyflavone **(4.40)** rhamnocitrin **(4.41)**, kaempferol **(4.42)**, quercetin-5, 3-dimethylether **(4.43)** and rhamnazin **(4.44)**. All the compounds showed a good activity against *Vibrio cholerae* and *Enterococcus faecalis* with MICs in the range of 25-50 μg/ml. Rhamnocitrin and quercetin-5, 3-dimethylether also inhibited *Micrococcus luteus* and *Shigella sonei* at 25 μg/ml.



R	R_1	R_2	
OH	OH	OH	**4.38**
OH	OCH_3	OH	**4.39**
OH	OCH_3	OCH_3	**4.40**

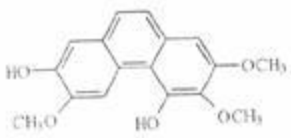


R	R_1	R_2	R_3	
OH	OCH_3	OH	H	**4.41**
OH	OH	OH	H	**4.42**
OCH_3	OH	OH	OCH_3	**4.43**
OH	OCH_3	OH	OCH_3	**4.44**

4.1.1.3.5. Phenanthrenes and dihydrophenathrenes

Occurrence of this group of compounds in various species has also been discussed in depth by Rogers and Verrotta (1996). These compounds have been isolated from the heartwood of some species including *C. apiculatum* (Malan and Swinny, 1993), *C. molle* (Letcher et al., 1972), *C. caffrum* (Pettit et al., 1988c), *C. hereroense* (Letcher and Nhamo, 1973) and *C. psidioides* (Letcher and Nhamo, 1972). The compounds 2, 7 dihydroxy-2, 4, 6-trimethoxy-9, 10-dihydrophenanthrene **(4.45)** and 4, 7- dihydroxy-2, 3, 6-trimethoxyphenantrene **(4.46)** isolated from *C. apiculatum* inhibited the growth of *Penicillium expansum* (Malan and Swinny, 1993).

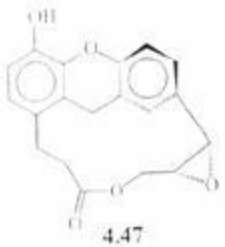
4.45



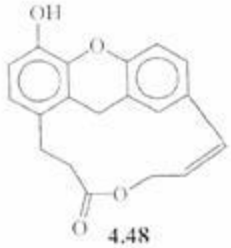
4.46

4.1.1.3.6. Macrocyclic lactones

This is also an interesting group of compounds for instance two cytostatic (P388 lymphocytic leukemia) macrocyclic lactones designated combretastatin D-1 **(4.47)** and D2 **(4.48)** were isolated from *C. caffrum* (Pettit et al., 1988b; Singh and Pettit, 1990). Combretastatin D-1 had an ED_{50} of 3.3 µg against PS cell lines (Pettit et al., 1988b).



4.47



4.48

4.1.1.3.7. Other compounds isolated from the Genus

There are very few reports on the occurrence of steroidal compounds in the genus in comparison to tritepernoids despite the fact that these groups of compounds share the same biosynthetic pathway, having squalene as their precursor. Steroids have been isolated from leaves of *C. padoides* (Rogers, 1989). Nitrogenous compounds including amino acids and alkaloids have been isolated from the seeds and fruits of *C. zeyheri* (Mwauluka et al., 1975a; 1975b; Panzini et al., 1993) and *C. mierathum* (Bassene et al., 1986). Also cyclobutanes have been isolated from the dichromethane extract of the aerial parts of *C. albopunctatum* (Katerere et al., 2004).

4.1.2 *Combretum zeyheri* Sond

4.1.2.1 Morphology

Combretum zeyheri (Figure 4:1) is a shrub or small tree 4-5 m. tall but it can grow taller. The bark is brown or greyish brown, branches are light brown and pubescent. Leaves opposite or 3-verticillate, lamina broadly to narrowly elliptic to obovate-elliptic or oblong elliptic, apex rounded to obtuse, sometimes acute, base usually rounded. Inflorescences usually unbranched, flowers greenish yellow. Fruits are large and winged (Wickens, 1973).

Figure 4:1 *Combretum zeyheri*

4.1.2.2 Ethnomedical uses of *Combretum zeyheri*

Combretum zeyheri is commonly known as, the large fruited bushwillow or '*raaslaar*,' in South Africa, the name referring to the sound of the leaves in the wind (Breytenbach and Malan, 1989). The plant is known as *Mlama mweupe* in Tanzanian national language, *Kiswahili*, where, the plant is used for various medical conditions. The plant is particularly a popular source of medicine in Mbeya region, where it is known as '*Kakati*' meaning 'always used for medicine' (Fyhrquist et al., 2002). The leaves, aerial parts, root bark and stem bark are used in Tanzania for snakebite, diarrhoea, tumours and hypertension (Watt and Beyer Brandwijk, 1962; Sawhney et al., 1978a; Fyhrquist et al., 2002). The leaves of the plant in the form of smoke inhalation are used in Tanzania to relieve cough (Hedberg et al., 1982). Sawhney et al. (1978b) reported that the fresh fruits are used in traditional medicine, however fruits from South and Central Africa are said to be highly poisonous and are never used by traditional healers as medicine (Panzini et al., 1993). In Mozambique the hot water extract of the bark of the plant is drunk to regulate

menstruation (Amico, 1977). In Zimbabwe the plant is used for diarrhoea and eye infections (Breytenbach and Malan, 1989).

4.1.2.3 Previously reported biological activities of *Combretum zeyheri*

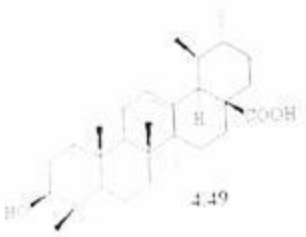
The plant has been investigated for several biological activities. The water and ethanolic extracts of the dried bark of South African plant exhibited cyclooxygenase 1 inhibition on guinea pig uterus (Lindsey et al., 1999). Water, acetone and ethyl acetate extracts of the South African plants exhibited anticrustacean activity against *Artemia salina* (Panzini et al., 1993); however, the dried roots of the Tanzanian plants were devoid of this activity (Massele and Nshimo, 1995). The extract of the twigs prepared using dichloromethane: methanol (1:1) was reported to have a moderate activity with an IC_{50} of 15 µg/ml against *Plasmodium falciparum* (Clarkson et al., 2004). Methanolic extracts of the fruits of the plant from Tanzania showed antiproliferative and cytotoxic effects against three human cancer cell lines including; HeLa, cervical carcinoma; T 24, bladder carcinoma; and MCF 7, breast carcinoma (Fyhrquist et al., 2006).

4.1.2.3.1. Previously reported antifungal studies on *Combretum zeyheri*

Several reports are available regarding the activity of *C. zeyheri* against *Candida albicans*, and other species of *Candida* and fungi. The methanolic extract prepared from different parts of the Tanzanian plant was found to be active against *C. albicans* and *Trichophyton mentagrophytes* (Sawhney et al., 1978b). Using bioautography technique, Masoko and Eloff (2006) found that hexane, acetone, dichloromethane and methanol extracts leaf of the South African plant were active against *C. albicans*, *Cryptococcus neoformans*, *Microsporum canis*, *Sporothrix schenckii* and *Aspergillus fumigatus*. However, this is contrary to what was reported by Breytenbach and Malan (1989) that the petroleum ether, ethyl acetate and methanolic extracts of the leaves of a South African plant had no activity against several fungi including, *C. albicans* and *Aspergillus* species but had antibacterial activity against several bacterial species. The methanolic extract of the leaves of the plant, from Zambezi valley Zimbabwe, was reported to have activity against *C. krusei*, *C. albicans* and *C. parapsilosis* with MICs of 8, 32 and 63 mg/l respectively (Liu et al., 2007).

4.1.2.4 Compounds previously isolated from *Combretum zeyheri*

The only report on the isolation of compounds from the leaves of the plant is by Breytetibach and Malan (1989) who isolated four compounds from the ethyl acetate extract of the leaves including ursolic acid **(4.49)**, other isolated compounds were coded Cz 34 **(4.50)**, Cz 37 **(4.51)** and Cz 39. Furthermore they found that the antibacterial activity of the plant was due to the last three compounds. They also isolated nonacosane ($C_{29}H_{60}$) and inactive compounds Cz 36, Cz 38 as well as the active compound Cz 39 from the benzene extract of the twigs. Due to insufficient material complete structure elucidation of compounds Cz 36, Cz 38 and Cz 39 could not be done. The scanty data could only indicate that the compounds were probably triterpenes. However, Carr and Rogers (1987) carried out a chemosystematic study on the genus *Combretum* and reported the presence of triterpenoids and flavonoids in the leaves of *C. zeyheri*. In another study compounds were isolated from the seeds and fruits of East and South African plants (Mwauluka et al., 1975a; b; Panzini et al., 1993). Panzini et al. isolated two amino acid glucosides, L-*N*-methyltyrosine-4' -*O*-β-D-glucoside **(4.52)**, L-3-(3' hydroxymethylphenylalanine –3'- β-D-glucoside **(4.53)** and its amino acid agylycone **(4.54)** from the fruits of the plant. Mwauluka et al. (1975a; b) reported the occurrence of the amino acid, N-methyl-L-tyrosine **(4.55)** and L-3- (3' aminomethylphenyl) alanine **(4.56)** in the seeds of the East African plant.



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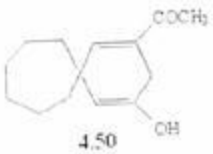
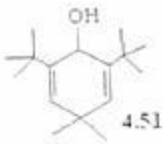


4.50



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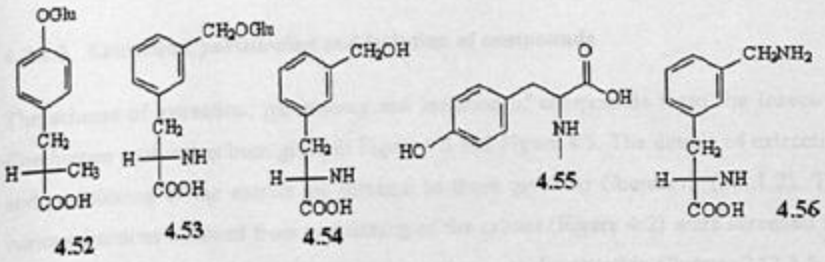


4.52 4.53 4.54 4.55 4.56

4.1.2.5 Prioritization of *Combretum zeyheri* for the isolation of anticandida compounds

As indicated in Chapter 2, *Combretum zeyheri* was among twenty plants, which were collected for study through the literature, where it was reported that the methanolic extract prepared from different parts of the plant was active against *Candida albicans* (Sawhney et al., 1978a). However the compounds responsible for the activity had not been isolated from the plant before. The activity of the plant has been confirmed in this study (Chapter 2; 2.4.3) whereby the aqueous methanolic extract showed two active spots (hR_f 46 and 61) in the agar overlay bioautography, hence, the plant was selected for isolation of the active compounds.

4.2 METHODOLOGY

4.2.1. Isolation of compounds from *Combretum zeyheri* leaves

4.2.1.1 Bulk plant collection

The leaves were collected in August 2004 from plants growing wild in Melela, Morogoro Region, Tanzania. The locality and time of collection was the same as reported in case of earlier collection (Chapter 2:2.4.1). The plant was identified in the field by a botanist and a herbarium specimen was prepared and compared to the herbarium specimen prepared in the initial collection. Prior to extraction the leaves were dried in the shade for four days.

4.2.1.2 Extraction, partitioning and isolation of compounds

The scheme of extraction, partitioning and isolation of compounds from the leaves of *Combretum zeyheri* has been given in Figure 4:2 and Figure 4:3. The details of extraction and partitioning of the extract are thrsame as those given in Chapter 3 (3.2.1.2). The various fractions obtained from partitioning of the extract (Figure 4:2) were screened for anticandida activity using bioautography agar overlay as discussed in Chapter 2 (2.3.5.3), whereby the ethyl acetate extract was found to be active, followed by n-hexane extract while the aqueous extract was not active. The most active ethyl acetate extract was used for isolation of the active compounds (Figure 4:3). Details of instruments, adsorbents and solvents used in the isolation of compounds are as discussed in Chapter 3 (3.2.1.3.2).

During isolation column separations were monitored by thin layer chromatography, developed with chloroform: methanol 9:1, for the first fractions and the polarity was increased as relatively more polar compounds were being eluted. The developed TLC plates were visualized using a UV lamp followed by dipping in 10% aqueous sulphuric acid and heating at 100 °C in the oven where coloured spots developed. The isolated compounds are as indicated in the TLC profile shown in Figure 4:4.

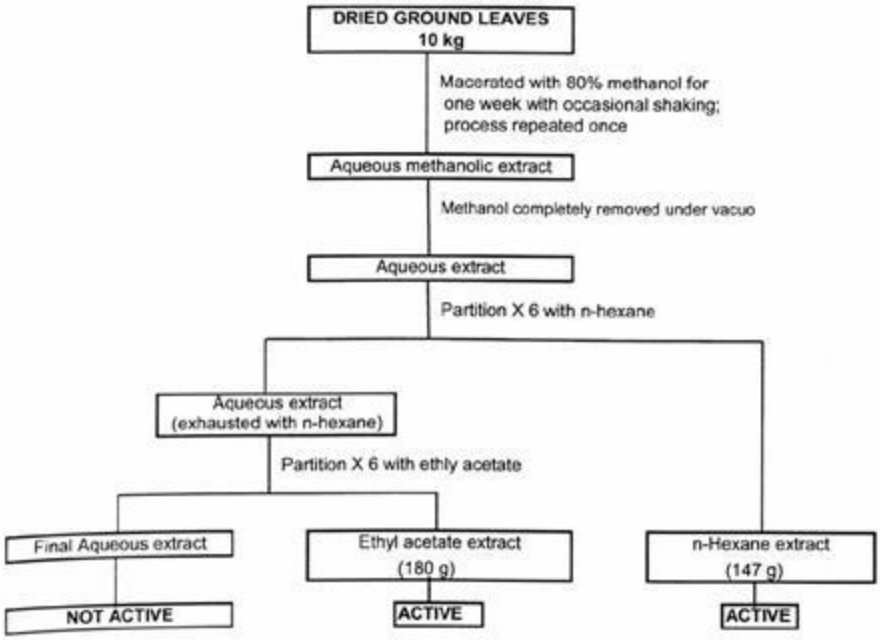
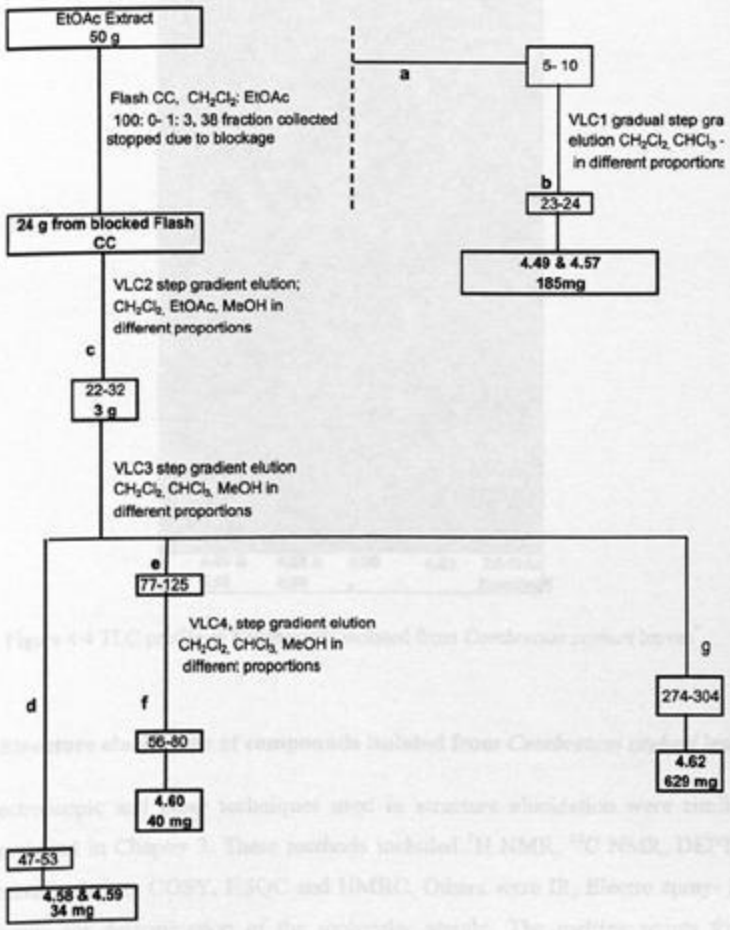


Figure 4:2 Scheme for extraction and partitioning of extract of *Combretum zeyheri* leaves



Eluting solvents: a: EtOAc: CH_2Cl_2 3:1; **b**: $CHCl_3$: CH_2Cl_2 1:1; **c**: EtOAc: CH_2Cl_2 3:1; **d**: $CHCl_3$: CH_2Cl_2 3:1 **e**: $CHCl_3$: MeOH 98.5: 1.5; **f**: $CHCl_3$: MeOH 99: 1; **g**: $CHCl_3$: MeOH 97.5: 2.5

Figure 4:3 Scheme of isolation of compounds **4.49**, **4.57-4.60** and compound **4.62** from ethyl acetate extract of *Combretum zeyheri* leaves

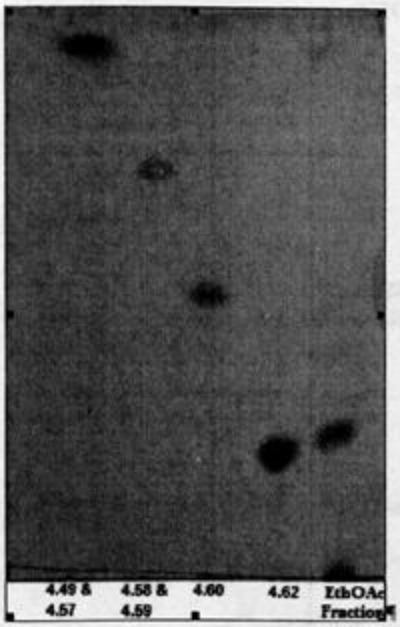


Figure 4:4 TLC profile of Compounds isolated from *Combretum zeyheri* leaves*

4.2.2 Structure elucidation of compounds isolated from *Combretum zeyheri* leaves

The spectroscopic and other techniques used in structure elucidation were similar to those employed in Chapter 3. These methods included ^{1}H NMR, ^{13}C NMR, DEPT, 2D experiments including COSY, HSQC and HMBC. Others were IR, Electro spray- Mass spectroscopy for determination of the molecular weight. The melting points for the isolated compounds were determined also.

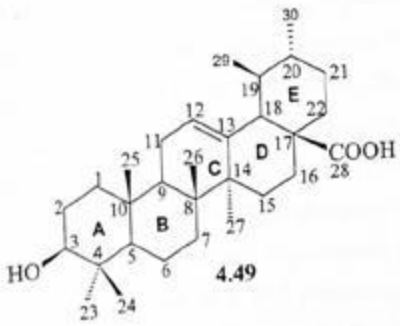
* Mobile Phase Chloroform: Methanol 22:3

4.2.3 Determination of minimum inhibitory concentrations (MICs) for the isolated compounds

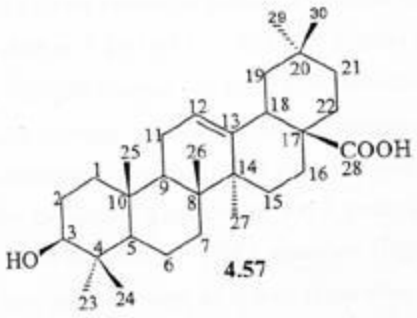
The minimum inhibitory concentrations were determined using broth microdilution method as explained in Chapter 3.

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation and Characterization of 3-β-hydroxyurs-12-en-28-oic [ursolic acid] (4.49) and 3-β-hydroxyl olean-12-en-28-oic (oleanolic acid) (4.57)



4.49



4.57

The active ethyl acetate fraction (Figure 4:3) [50 g] was subjected to flash column chromatography. Stepwise gradient elution was carried out starting with dichloromethane with an elution rate of 20 mls/min. At fraction No. 38 (eluting with solvent: 75% ethyl acetate in dichloromethane), the elution was terminated due to blockage. Fractions 5- 10, which were very rich in **4.49** and **4.57,** were pooled and subjected to vacuum liquid chromatography (VLC1) [Figure 4:3] step gradient eluted with dichloromethane, chloroform and methanol in various proportions. A total of 160 fractions were collected. Fractions 23- 24 eluted with chloroform: dichloromethane 1:1 resulted in the isolation of a white powder (185 mg), which gave one spot on TLC plates developed in several solvent systems. However, its ^{13}C NMR showed it to be an isomeric mixture of compounds **4.49** and **4.57**.

The electro spray mass spectra (ES-MS) of the isomeric mixture showed base peaks at *m/z* 455 and 479 in –ve and +ve mode respectively. The peak at 455 was for $[M^+ - H]$, while the peak at 479 was for $M^+ + Na$. The IR spectrum showed absorption bands at 3430 (O-H), 2932 (C-H), 1693 (COOH), 1459, 1384, 1248, 1186, 1095, 1034, 998 and 760 cm^{-1}.

The structures of compounds **4.49** and **4.57** were determined on the basis of their ^1H NMR, ^{13}C NMR, DEPT, ES-MS and IR spectroscopic data. Proton (^1H) NMR spectrum (Figure 4:5; Table 4:2) of the isomeric mixture showed 7 singlets at δ 0.89, 0.96, 0.97, 1.0, 1.02, 1.04, 1.23 (3H each), a triplet at δ 3.45 for carbinolic methine hydrogen, one singlet, at δ 5.48 for olefinic proton, a doublet at δ 2.62 (*J* 11.1 Hz) and doublet of doublets at δ 3.27 (*J* 13.5 and 3.6 Hz). The ^{13}C NMR (Figure 4:6; Table 4:3) showed 43 signals of which 29 signals were intense while the other 14 were less intense suggesting the presence of an isomeric mixture. The intense and less intense peaks were assigned to compounds **4.49** and **4.57** respectively. The 29 intense signals were for 7 methyl, 9 methylene, 7 methine and 7 quaternary carbons according to DEPT spectrum (Figure 4:7). The one peak at δ 17.7 represented two methyl groups as it was made clear in DEPT, which showed signals at δ 17.6 and δ 17.7. One methine, carbon atom appeared downfield at δ 78.4 suggesting that it was oxygenated.

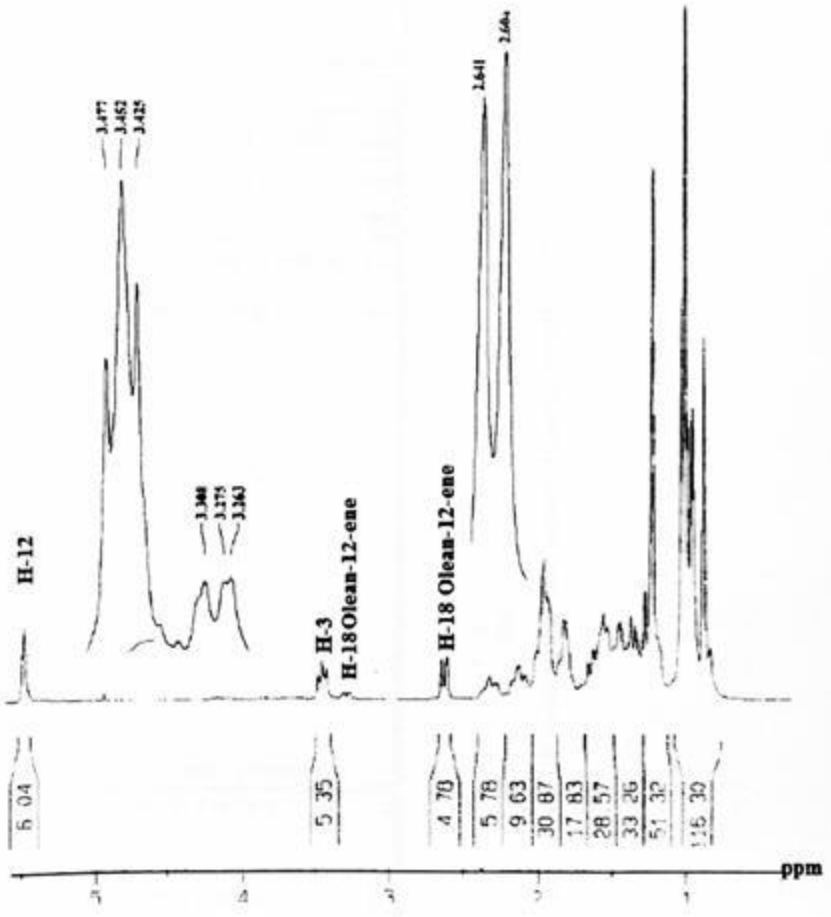


Figure 4:5 ^{1}H NMR of isomeric mixture of Compounds **4.49** & **4.57**

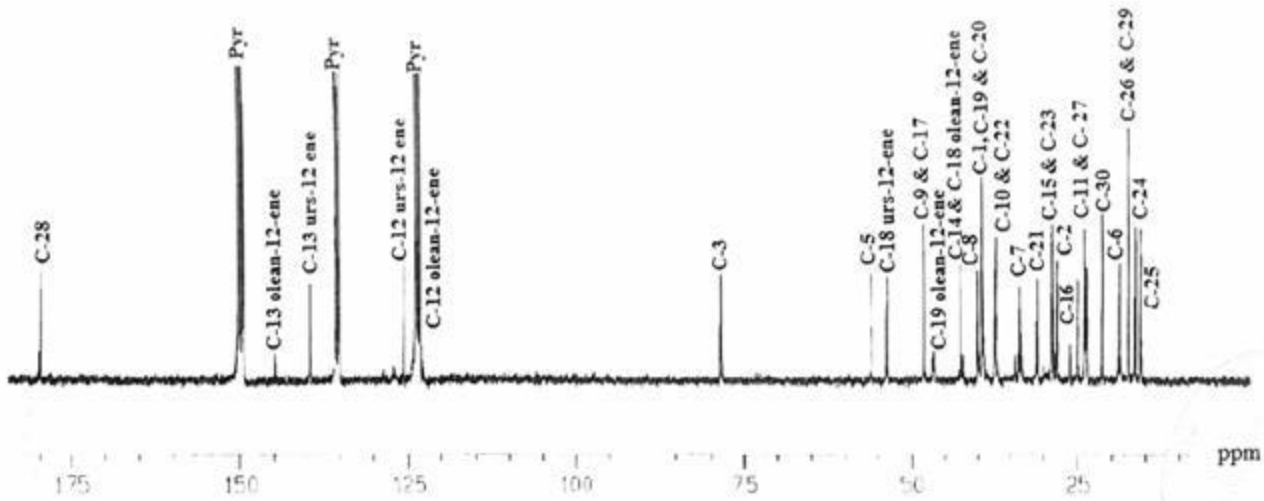


Figure 4:6 ^{13}C NMR Spectrum of isomeric mixture of Compounds **4.49** & **4.57**

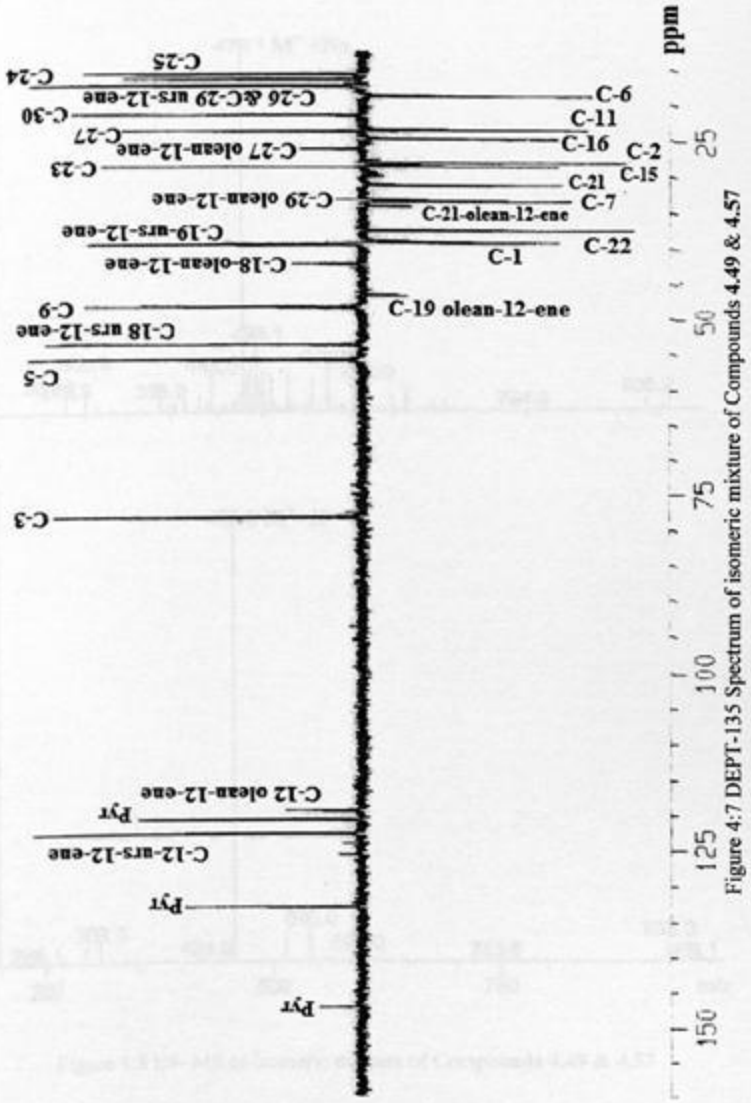


Figure 4:7 DEPT-135 Spectrum of isomeric mixture of Compounds **4.49** & **4.57**

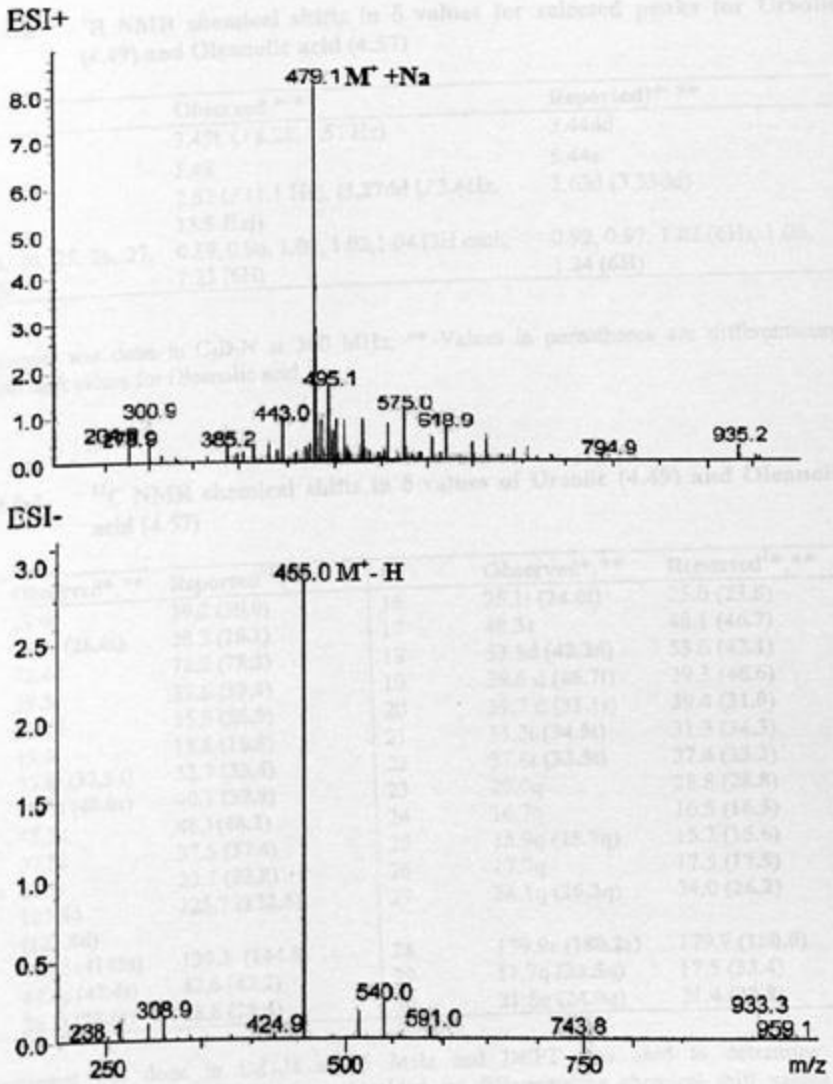


Figure 4:8 ES- MS of isomeric mixture of Compounds **4.49** & **4.57**

Table 4:2 **^{1}H NMR chemical shifts in δ values for selected peaks for Ursolic (4.49) and Oleanolic acid (4.57)**

H	Observed *,**	Reported)*,**
3α	3.45t (*J* 8.28, 7.51 Hz)	3.44dd
12	5.48	5.44s
18	2.62 (*J* 11.1 Hz); **(3.27dd [*J* 3.6Hz, 13.5 Hz])**	2.63d (3.33dd)
H-23, 24, 25, 26, 27, 29, 30	0.89, 0.96, 1.00, 1.02,1.04 (3H each, 1.23 (6H)	0.92, 0.97, 1.02 (6H), 1.06, 1.24 (6H)

*Experiment was done in C_5D_5N at 300 MHz; ** Values in parentheses are differentiating chemical shift values for Oleanolic acid

Table 4:3 **^{13}C NMR chemical shifts in δ values of Ursolic (4.49) and Oleanolic acid (4.57)**

C	Observed*,**	Reported[1]*,**	C	Observed*,**	Reported[1]*,**
1	39.4t	39.2 **(39.0)**	16	25.1t **(24.0t)**	25.0 **(23.8)**
2	28.3t **(28.6t)**	28.2 **(28.1)**	17	48.3s	48.1 **(46.7)**
3	78.4d	78.2 **(78.2)**	18	53.8d **(42.2d)**	53.6 **(42.1)**
4	39.5s	39.6 **(39.4)**	19	39.6 d **(46.7t)**	39.3 **(46.6)**
5	56.1d	55.9 **(55.9)**	20	39.7 d **(31.1s)**	39.4 **(31.0)**
6	19.0t	18.8 **(18.8)**	21	31.3t **(34.5t)**	31.3 **(34.3)**
7	33.8t **(33.5 t)**	33.7 **(33.4)**	22	37.6t **(33.5t)**	37.4 **(33.2)**
8	40.3s **(40.0s)**	40.1 **(39.8)**	23	29.0q	28.8 **(28.8)**
9	48.3d	48.1**(48.2)**	24	16.7q	16.5 **(16.5)**
10	37.5s	37.5 **(37.4)**	25	15.9q **(15.7q)**	15.7 **(15.6)**
11	23.9t	23.7 **(23.8)**	26	17.7q	17.5 **(17.5)**
12	125.8d **(122.8d)**	125.7 **(122.6)**	27	24.1q **(26.3q)**	24.0 **(26.2)**
13	139.5s **(145s)**	139.3 **(144.8)**	28	179.9s **(180.2s)**	179.7 **(180.0)**
14	42.8s **(42.4s)**	42.6 **(42.2)**	29	17.7q **(33.5q)**	17.5 **(33.4)**
15	28.9t **(28.6t)**	28.8 **(28.4)**	30	21.6q **(24.0q)**	21.4 **(23.8)**

*Experiment was done in C_5D_5N at 75 MHz and DEPT was used to determine the multiplicities**Values in parentheses and bolded are differentiating chemical shift values for oleanolic acid [1]Seebacher et al., 2003

The presence of a triplet at δ 3.45 in ^{1}H NMR spectrum (Figure 4:5) and a downfield carbon at δ 78.4 in ^{13}C NMR (Figure 4:6) suggested the presence of a secondary hydroxyl group in the molecule which was also supported by the presence of a broad

absorption band at 3430 cm^{-1} in the IR spectrum. Furthermore the presence of a downfield signal at δ 179.9 in ^{13}C NMR suggested the presence of a free carboxylic acid group in the molecule, which was also supported by the presence of a band at 1693 cm^{-1} in the IR spectrum. The presence of a double bond in the molecule was indicated by the presence of a downfield olefinic proton at δ 5.48 in ^{1}H NMR spectrum suggesting the presence of a trisubstituted double bond in the molecule. This was further supported by the presence of signals at δ 125.8d and δ 139.4s in the ^{13}C NMR (Figure 4:6). From the literature the set of downfield signals at δ 125 and δ 139 are characteristic olefinic carbons C-12 and C-13 in urs-12-ene (Mahato and Kundu, 1994). The presence of urs-12-ene was also supported by presence of a doublet centered at δ 2.62 in ^{1}H NMR spectrum (Figure 4:5) and signal at δ 53.8 in ^{13}C NMR (Figure 4:6) corresponding to H-18 and C-18 respectively, in urs-12-ene (Khare et al., 2002, Seebacher et al., 2003).

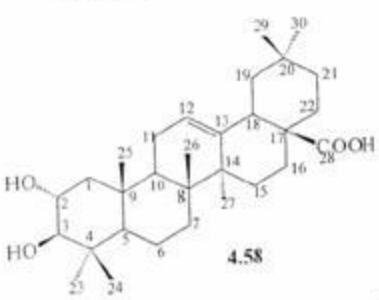
From the ^{13}C NMR (DEPT) (Figure 4:7) spectral data above, the molecular formula of the compound was deduced as $C_{30}H_{48}O_3$ this was supported by its ES-MS (Figure 4:8), which gave the molecular weight 456 for the compound. From the ^{13}C NMR and ^{1}H NMR spectral data, it was deduced that compound **4.49** was a monohydroxyl urs-12-enoic acid. The downfield triplet at δ 3.45 in the ^{1}H NMR spectrum (Figure 4:5) of **4.49** indicated the presence of a hydroxyl group at the C-3 position, which is placed between a tetrasubstituted sp^3 carbon atom (C-4) and a methylene group at C-2. The stereochemistry of C-3 was deduced from ^{13}C NMR data which indicates that introduction of a hydroxyl group at C-3 strongly influences the resonances of C-1, C-5 and C-24 due to γ- effects. The conversion of C-3 hydroxyl group from axial to equatorial stereochemistry results in an downfield shift of C-1 and C-5 and C-24 shifts upfield by approximately 6 ppm leaving C-23 almost unaffected (Srivastava, 1992). On comparing the ^{13}C NMR signals of **4.49** with those expected for C-3 equatorial and axial hydroxyl isomers it was evident that compound **4.49** had an equatorial oriented hydroxyl group at C-3. The location of the carboxyl group at C-17 was established by comparison of the ^{13}C NMR spectrum of rings D and E of compound **4.49** with those of analogous triterpenes (Tkachev et al., 1994).

The ^{13}C NMR and ^{1}H NMR spectral data of **4.49** were comparable to those reported for 3β-hydroxyl urs-12-en-28-oic acids [Table 4:2 and Table 4:3] (Tkachev et al., 1994, Seebacher et al., 2003, Taketa et al., 2004), hence compound **4.49** which showed 29 intense carbon signals was characterized as 3-β-hydroxyurs-12-en-28-oic [ursolic acid]. Further analysis of ^{13}C NMR and DEPT spectra (Figure 4:6 and Figure 4:7) there were 14 signals with low intensity suggesting the presence of an isomer of **4.49**. This prompted proper characterization of this compound. The downfield signals at δ 122.8 and δ 145 are characteristic of C-12 and C-13 resonances for olean-12-ene (Mahato and Kundu, 1994). This suggested that the isomer was an olean-12-ene triterpene. The presence of olean-12-ene is also supported by the presence of doublet of doublets centered at δ 3.27 in ^{1}H NMR spectrum (Figure 4:5) corresponding to H-18, and a signal at δ 42.2 in the ^{13}C NMR (Figure 4:6) corresponding to C-18 which is about 11.5 ppm less than the resonance observed for its isomer **4.49.** This difference is attributable to the shielding effect of 20β methyl group, which is γ-gauche disposed to C-18 in olean-12-enes (Mahato and Kundu, 1994). Further, a signal at δ 180.1 indicated the presence of a free carboxylic acid group in the molecule. Hence on the basis of above spectroscopic data, the other isomer **4.57** was identified as 3-β-hydroxyl olean-12-en-28-oic (oleanolic acid). The ^{13}C NMR spectral data of the 14 signals with low intensity were comparable to those reported in the literature for oleanolic acid [Table 4:3] (Tkachev et al., 1994; Seebacher et al., 2003; Taketa et al., 2004).

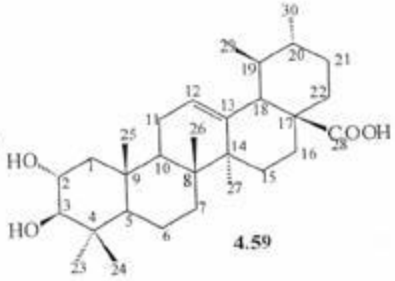
Compounds **4.49** and **4.57** could not be separated on normal silica gel TLC using several mobile phases, as it was an isomeric mixture of ursolic and oleanolic acid.

Although ursolic acid has been reported from the aerial parts and fruits of the South African plant, *C. zeyheri* (Breytenbach and Malan, 1989) the presence of oleanolic acid in this plant is being reported for the first time. Oleanolic acid is a compound which is used clinically in China for treating hepatitis B (Tong et al., 2008) and it has also been reported to have several biological activities including hepatoprotective activity (Liu et al., 2008).

4.3.2 Isolation and Characterization of Compounds, 2α- 3-β-dihydroxyolean-12-en-28-oic acid (Maslinic acid) (4.58) and 2α- 3-β-dihydroxyurs-12-en-28-oic acid (4.59)



4.58



4.59

The ethyl acetate extract (24 g) retrieved from the flash column and subjected to VLC2 [Figure 4:3], was gradient eluted with dichloromethane, ethyl acetate and methanol in various proportions. A total of 96 fractions were collected. Fractions 22- 32 (3 g) eluted with ethyl acetate: dichloromethane 3:1 and containing several compounds was further subjected to VLC3 [Figure 4:3] and gradient eluted with dichloromethane, chloroform and methanol in various proportions. A total of 322 fractions were collected. Fractions 47- 53, eluted with chloroform: dichloromethane 3:1, afforded 34 mg of a white powder, which gave one spot on TLC plates developed in several solvent systems but its ^{13}C NMR showed it to be an isomeric mixture containing compounds **4.58** and **4.59**.

The Electro spray mass spectrum (ES-MS) of the isomeric mixture showed *m/z* at 471 and 495 in –ve and +ve mode, respectively. The peak at *m/z* 471 was due M^+-H while that at *m/z* at 495 was due to M^+ + Na. The IR spectrum showed absorption bands at 3449 and 3424 (O-H), 2931 (C-H), 1686 (COOH), 2371, 2344, 2278, 1560, 1544, 1459, 1398, 1388, 1377, 1248, 1051, 1095, 1031, 998 and 784 cm^{-1}.

The structures of compounds **4.58** and **4.59** were determined on the basis of their ^1H NMR, ^{13}C NMR, DEPT, HSQC, ES-MS and IR spectroscopic data. Proton (^1H) NMR (Table 4:4) of isomeric mixture showed seven singlets at δ 0.93, 0.96, 0.98, 0.99, 1.05, 1.24, 1.25 (3H each), a multiplet proton at δ 4.08, one olefinic proton at δ 5.44s, doublets centered at δ 2.60 (*J* 11.1 Hz) and 3.38 (*J* 9.22 Hz), a doublet of doublets centered at δ 3.29. The ^{13}C NMR (Table 4:5) showed 42 signals of which 28 were intense and 14 were

of low intensity suggesting the presence of an isomeric mixture. The intense and less intense signals were assigned to compound **4.58** and **4.59** respectively. The intense signals were 7 methyl, 9 methylene 6 methine and 8 quaternary carbons according to the DEPT spectrum. The two peaks at 24.1 and 33.4 each represented a methyl and methylene carbon. Two of the methine carbon atoms appearing downfield at δ 68.8 and 84.1 suggested that they were oxygenated.

Table 4:4 ^{1}H NMR chemical shifts in δ values (for selected peaks) of 2α- 3-β-dihydroxyolean-12-en-28-oic acid [Maslinic acid] (4.58) and 2α- 3-β-dihydroxyurs-12-en-28-oic acid (4.59)

H	Observed*, **	Reported [1,2]*, **
2β	4.08m	4.09 ddd, (*J* 4.5, 9.5, 11.5 Hz)
3α	3.38 d (*J* 9.22)	3.38 d (*J* 9.5 Hz)
18	3.29dd, **(2.60 [*J* 11.1Hz])**	3.28 dd (*J* 4.5, 14.5 Hz); **(2.62 *d*, [*J* 11.5 Hz]**
12	5.44s br	5.45 s br
H-23, 24, 25, 26, 27, 29, 30	0.93, 0.96, 0.98, 0.99, 1.05, 1.24, 1.25	0.92, 0.97, 0.98, 1.00, 1.06, 1.25, 1.26

*Experiment was done in C_5D_5N at 300 MHz; **Values in parentheses are differentiating chemical shift values for 2α- 3-β-dihydroxyurs-12-en-28oic acid;
[1]Zucaro et al., 2000; [2] Taniguchi et al., 2002

Table 4:5 ^{13}C NMR chemical shifts in δ values of 2α- 3-β-dihydroxyolean-12-en-28-oic acid [Maslinic acid] (4.58) and 2α - 3-β-dihydroxyurs-12-en-28-oic acid (4.59)

C	Observed*, **	Reported [1,2]*, **	C	Obseved *, **	Reported*, **
1	47.9t (**48.1t**)	47.8 (**48.0**)	16	24.1t (**25.1t**)	23.6 (**24.9**)
2	68.8d	68.6 (**68.6**)	17	46.9s	46.7 (48.1)
3	84.1d	83.8 (**83.7**)	18	42.3d (**53.7d**)	42.6 (**53.6**)
4	39.9s	39.8 (**39.9**)	19	46.7t (**39.6d**)	46.6 (**39.5**)
5	56.1d	55.9 (**55.9**)	20	31.1s (**39.6d**)	30.9 (**39.5**)
6	19.1t	18.9 (**18.9**)	21	34.5t (**31.3t**)	34.2 (**31.0**)
7	33.7t	33.2 (**33.5**)	22	33.4t (**37.5t**)	33.2 (**37.5**)
8	40.1s (**40.3s**)	40.2 (**40.1**)	23	29.5q	29.3 (**29.4**)
9	48.4d	48.2 (	24	16.9q	16.9 (**17.7**)
10	38.8s	38.5 (**38.5**)	25	17.6q (17.1q)	17.5 (**17.0**)
11	24t	23.9 (**23.8**)	26	17.7q	17.7 (**17.5**)
12	122.9d (**125.7d**)	122.5 (**125.**)	27	26.3q (**23.9**)	26.2 (**23.9**)
13	145.1s (**139s**)	144.9 (**139.3**)	28	180.2s (**179.9 s**)	180.2 (**179.9**)

14	42.5s **(42.8s)**	42.2 **(42.6)**	29	33.4q **(21.5q)**	33.2 **(21.4)**
15	28.5t **(28.9t)**	28.2 **(28.7)**	30	24.1q **(17.6q)**	23.1 **(17.5)**

*Experiment was done in C_5D_5N at 75 MHz and DEPT was used to determine the multiplicities
**Values in parentheses and bolded are chemical shift values for 2α- 3-β-dihydroxyurs-12-en-28oic acid,[1]Zucaro et al., 2000; [2]Taniguchi et al., 2002

The presence of a doublet and triplet δ 4.08 and δ 3.38, respectively, in the ^{1}H NMR spectrum and downfield carbons at δ 68.8 and 84.1 in ^{13}C NMR spectrum suggested the presence of 2 secondary hydroxyl groups in the molecule which was also supported by the presence of absorption bands at 3449 and 3424 cm^{-1} in the IR spectrum. A detailed study of ^{13}C NMR of dihydroxyl pentacyclic triterpenes led to the suggestion that carbinolic methines at δ 68.8 and 84.1 ppm could be due to the presence of the 2 hydroxyl groups in **4.58** at C-2 and C-3 with α- and β- configurations, respectively (Mahato and Kundu, 1994). Furthermore the presence of a downfield signal at δ 180.2 in ^{13}C NMR spectrum suggested the presence of free carboxylic acid group in the compound, which was also supported by the presence of a band at 1686 cm^{-1} in the IR spectrum. Further, the presence of a double bond in the molecule was indicated by the presence of a downfield olefinic proton at δ 5.45 in the ^{1}H NMR spectrum; suggesting the presence of a trisubstituted double bond in the molecule. Like the previously discussed compound **4.57**, the ^{13}C NMR of **4.58** showed signals at δ 42.3, 122.9, 145.1 which are characteristic features of olean-12-ene (Mahato and Kundu, 1994). The presence of olean-12-ene is further supported by the presence of a doublet of doublets centered at δ 3.29 in ^{1}H NMR spectrum corresponding to H-18.

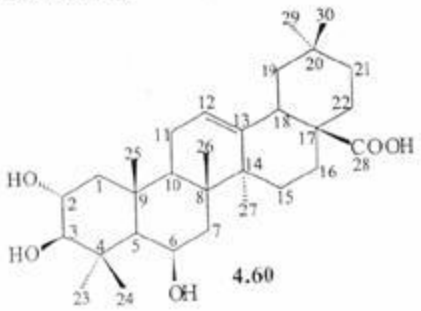
From the ^{13}C NMR and DEPT spectral data of **4.58** the molecular formula of the compound was deduced as $C_{30}H_{48}O_4$; this was supported by its ES-MS, which gave a molecular weight of 472. The location of the carboxyl group at C-17 was established by comparison of the ^{13}C NMR spectrum of ring D and E of compound **4.58** with those of analogous triterpenes (Zucaro et al., 2000). Both ^{13}C NMR and ^{1}H NMR spectral data for **4.58** correlated well with those reported for 2α- 3β-dihydroxyolean-12-en – 28-oic

acid [Table 4:4 and Table 4:5] (Zucaro et al., 2000; Taniguchi et al., 2002), hence, compound **4.58** was characterized as 2α- 3-β-dihydroxyolean-12-en-28-oic acid [maslinic acid].

Further analysis of ^{13}C NMR and DEPT spectra (Table 4:5) of 14 signals with very low intensity showed characteristic features for urs-12-ene as discussed for compound **4.49** and they included signals at δ 53.7 (C-18), 125.7, (C-12) and 139 (C-13), also ^{1}H NMR spectrum showed a doublet at δ 2.60 in for H-18. Hence on basis of the above spectroscopic data the other isomer **4.59** was identified as 2α- 3-β-dihydroxyurs-12-en-28-oic acid. Further, the ^{1}H NMR and ^{3}C NMR spectra data of 14 less intense signals were comparable to those reported in literature for 2α- 3-β-dihydroxyurs-12-en-28-oic acid. [Table 4:4 and Table 4:5] (Zucaro et al., 2000; Taniguchi et al., 2002).

4.3.3 Isolation and Characterization of 2α-, 3β-, 6β- trihydroxylolean-12-en-28-oic acid [6β-hydroxyl maslinic acid] (4.60)

Fractions 77- 125 obtained from VLC 3 (Figure 4:3) were rich in compound **4.60** hence were pooled and subjected to VLC4 (Figure 4:3). Step gradient elution of VLC4 was carried out with dichloromethane, chloroform and methanol in various proportions. Fractions 56- 80 eluted with 1% methanol in chloroform gave a single spot on TLC in several solvent systems hence fractions 56- 80 were pooled together, dried under vacuum to yield a white powder **4.60** (40 mg).



4.60

Compound **4.60** had a melting point of 230 °C and its electro spray mass spectrum (ES-MS) gave *m/z* at 487 and 511 in –ve and +ve mode, respectively. The *m/z* 487 was

for M^+ - H while *m/z* 511 was due to M^+ +Na. The IR spectrum showed bands at 3427 (O-H), 2929 (C-H), 1696 (COOH), 1461 and 1050 cm^{-1}.

Compound **4.60** was characterized on the basis of its ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC, ES-MS and IR spectroscopic data. The ^1H NMR (Table 4:6) showed seven upfield singlets due to tertiary methyl groups (δ 0.94, 1.0, 1.26, 1.42, 1.55, 1.68 and 1.75, 3H each). Other characteristic signals in the spectra were for carbinolic methine protons at δ 4.84s, 4.25dt and a doublet at 3.41 (*J* 9.3 Hz), olefinic proton at δ 5.55s and doublet of doublets centred at δ 3.32. The ^{13}C NMR (Table 4:7) spectrum of **4.60** showed the presence of 28 intense signals indicating the presence of at least 28 carbon atoms in **4.60**. From the Proton noise decoupled ^{13}C NMR (PNDC) and DEPT spectra of **4.60**, the presence of 7 methyl, 7 methylene, 7 methine and 8 quaternary carbons in the molecule were confirmed. A peak at δ 33.6 represented methyl and methylene carbons. The presence of three downfield methine carbons, at δ 68.1, 69.2 and 84.6 confirmed that they were oxygenated which was also proved by the presence of signals at δ 4.84, 4.25 and 3.41 in the ^1H NMR spectrum for three secondary hydroxyl groups in the molecule. An IR band at 3427cm^{-1}, for a hydroxyl group, further supported this. A detailed study of ^{13}C NMR of di- and trihydroxyl pentacyclic triterpenes led to conclusion that carbinolic methines at 69.2 and 84.6 ppm were suggestive of the presence of the 2 hydroxyl groups in **4.60** at carbon 2 and 3 with α- and β-configurations respectively (Mahato and Kundu, 1994).

Table 4:6 ^1H NMR chemical shifts in δ values for selected peaks of 2α-, 3β-, 6β-trihydroxylolean-12-en-28-oic acid (6β- hydroxyl maslinic acid) (4.60)

H	Observed*	Reported[1]
2β	4.26dt	4.28dt (*J* 9.5 and 4.5 Hz)
3α	3.41d (*J* 9.3 Hz)	3.42d (*J* 9.5 Hz)
6 α	4.84s	4.85s
18	3.32dd	3.33dd (*J* 14, 4 Hz)
12	5.55s	5.57 br t
H-23, 24, 25, 26, 27, 29, 30	1.44,1.75, 1.68,1.55, 1.26, 0.94, 1.00	1.46, 1.78, 1.71, 1.61, 1.30, 0.96, 1.02

*Experiment was done in C_5D_5N at 300 MHz; [1]Zucaro et al., 2000;

Table 4:7 NMR spectral data of 2α-, 3β-, 6β- trihydroxyolean-12-en-28-oic acid (6β- hydroxyl maslinic acid) (4.60

C	^{13}C NMR *, **, [1]	HSQC (in δ Values for attached Hs)	HMBC C-H correlations	^{1}H-^{1}H COSY Coupling Hs
1	50.5 t (**49.9**)	2.3, 1.4 (2 H)	H-25	
2	69.2 d (**68.2**)	4.25 dt (1 H)	H-3, H-1 ^{2}J	H-2 and H-1, H-1' H-3
3	84.6 d (**83.9**)	3.41d (1 H)	H-23, H-24, H-1, H-5 ^{3}J	
4	41.0s (**39.2**)	-	H-23, H-24, H-3, H-5 ^{2}J	
5	57.1d (**56.4**)	1.16 (1H)	H-23, H-24, H-25, H-7 ^{3}J; H-6 ^{2}J	H-5 and H-6
6	68.1 d (**67.4**)	4.84 s (1H)	H-5, H-7 ^{2}J	
7	41.7 t (**41.1**)	1.85 (2 H)	H-26 ^{3}J	H-7 and H-6
8	39.9 s (**40.6**)	-	H-7, H-26 ^{2}J; H-27 ^{3}J	
9	49.2 d (**48.6**)	1.94 (1 H)	H-25, H-26 ^{3}J	
10	38.9 s (**38.3**)	-	H-25, H-5 ^{2}J	
11	24.4 t (**23.9**)	2.14 (2H)	H-12 ^{2}J	H-11 and H-12
12	123.2 d (**122.7**)	5.55 s (1H)	H-18 ^{3}J	
13	144.6 s (**144.0**)	-	H-18 ^{2}J; H-27 ^{3}J	
14	43.3 s (**42.6**)	-	H-27 ^{2}J; H-26 ^{3}J	
15	28.7 t (**28.1**)	2.27 (2 H)	H-27 ^{3}J	
16	24.4 t (**23.6**)	1.96 (2)		
17	47.0 s (**43.3**)	-		
18	42.6 d (**41.9**)	3.32 (1H)	H-12 ^{3}J	H-18 and H-19, 19'
19	47.2 t (**46.5**)	1.81, 1.3 (2 H)	H-29, H-30 ^{3}J	
20	31.3 s (**30.8**)	-	H-29, H-30 ^{2}J	
21	34.8 t (**34.1**)	1.8, 2.03 (2 H)	H-29 ^{3}J	
22	33.6 t (**33.1**)	2.03 &1.8 (2 H)		
23	29.5 q (**29.0**)	1.42 (3 H)	H-3, H-24 ^{3}J	
24	19.5 q (**19.1**)	1.75 (3 H)	H-3, H-5, H-23 ^{3}J	
25	18.9 q (**18.4**)	1.68 (3 H)	H-1 ^{3}J	
26	19.0 q (**18.3**)	1.55(3 H)		
27	26.7 q (**26.2**)	1.26 (3 H)		
28	180.4 s (**180.0**)	-		
29	33.6 q (**33.2**)	0.94s (3)	H-30 ^{3}J	
30	24.2 q (**23.6**)	1.0 (3 H)	H-29 ^{3}J	

*Experiment was done in C_5D_5N at 75 MHz and DEPT was used to determine the multiplicities;** Values in parentheses and bolded are the reported chemical shift values
[1]Zucaro et al., 2000

Furthermore the presence of a downfield signal at δ 180s in ^{13}C NMR of **4.60** suggested the presence of a free carboxylic acid group, which was also confirmed by presence of an absorption band at 1696 cm^{-1} in the IR spectrum. Compound **4.60** like compounds **4.57**

and **4.58**, showed diagnostic features for olean-12-ene, which were a signal for a downfield proton at δ 5.55 in ^{1}H NMR and signals at δ 42.6 (C-18), δ 123.2 (C-12) and δ 144.6s (C-13) in the ^{13}C NMR spectrum of **4.60.**

From the ^{13}C NMR PNDC and DEPT spectral data the molecular formula of the compound was deduced as $C_{30}H_{48}O_5$, which was supported by its ES-MS, [M^{+}-H] 487, hence for **4.60** M^{+} was 488. From the above ^{13}C NMR and ^{1}H NMR the spectroscopic data it was deduced that compound **4.60** was a trihydroxyolean-12-enoic acid.

As discussed earlier, the two hydroxyl groups had been assigned to positions 2α and 3β, which was further confirmed by ^{1}H-^{1}H COSY, HSQC and HMBC correlation spectroscopic data of **4.60** (Table 4:7). In COSY the carbinolic proton at C-2 (δ 4.25) coupled with the carbinolic proton at C-3 (δ 3.41) and diastereotopic methylene protons at C-1 (δ 2.3 and 1.4). This observation meant that the two hydroxyl groups are present on neighbouring carbon atoms. Further, an important correlation was seen among the carbinolic proton at C-6 (δ 4.84), methine proton at C-5 (δ 1.16) and methylene protons at C-7 (δ 1.85), this suggested that the third hydroxyl is placed at C-6, which lies between C-5 and C-7. The positions of hydroxyl groups and the carbon- carbon connectivity were supported by the HMBC spectrum of **4.60** (Table 4:7, Figure 4:9). The important C-H ^{3}J correlation to confirm the assignment for the hydroxyl group at C-6 was a three-bond correlation between the neighbouring carbon C-5 and the methyl protons of C-23 (δ 1.42) and C-24 (δ 1.75). Another C-H ^{3}J correlation was seen between the C-7 and the methyl protons at C-26 (δ 1.68). Similarly placement of hydroxyl groups at position C-2 and C-3 was also supported by the C-H ^{3}J, correlation between C-3 and methyl protons of C-23 (δ 1.42) and C-24 (δ 1.75) which also proves that the third hydroxyl group was placed on C-2 and its carbinolic proton was seen to be coupled with proton, H-3 in COSY (Figure 4:9).

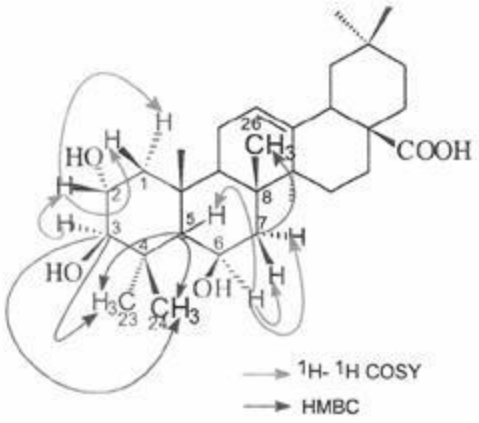
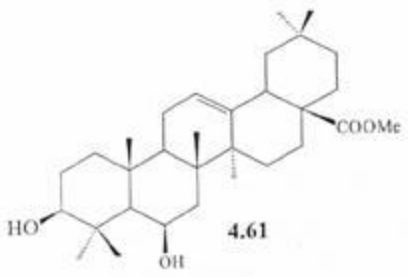


Figure 4:9: Important homonuclear and heteronuclear correlations of Compound **4.60**

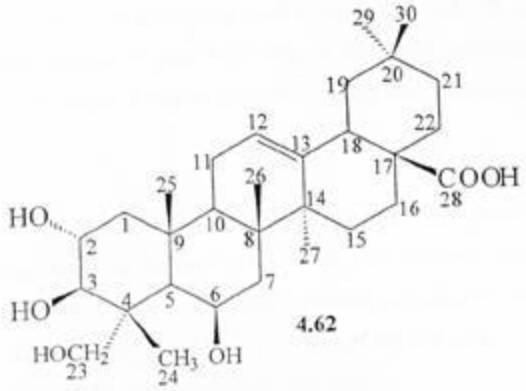
The stereochemistry of the hydroxyl group at C-6 was determined by comparing the ^{13}C NMR spectral data of **4.60** with those reported for closely related compounds with hydroxyl groups at position C-6 in equatorial [α] (Liang et al., 1990) and axial [β] (Chan et al., 1992) orientations. The spectral data for compound **4.60** matched well with those for the axial (β) oriented hydroxyl group. The presence of the carboxyl group at C-17 was established by comparing ^{13}C NMR spectrum of rings D and E for compound **4.60** with those of analogous triterpenes (Zucaro et al., 2000). On the basis of above spectroscopic discussion and data, compound **4.60** was characterized as 2α-, 3β-, 6β-trihydroxylolean-12en-28-oic acid (6β- hydroxyl maslinic) which was in close agreement with the previously reported ^1H NMR and ^{13}C NMR spectroscopic data for this compound [Table 4:7] (Zucaro et al., 2000), except for the difference in the ^{13}C NMR chemical shift assignment of C-17. Previously, Zucaro et al. (2000) assigned δ 43.3 to C-17 but in this study C-17 has been assigned δ 47.1. This is supported by spectral data of a closely related compound, methylsumaresinolate (**4.61**), for which δ 46.7 was assigned to C-17 (Chan et al., 1992). The assignment for C-17 at δ 47.1 was further supported by the 2D NMR experiments (Figure 4:9; Table 4:7). Hence on the basis of above spectroscopic data, compound **4.60** was confirmed as 6β-hydroxyl maslinic acid, which is being reported in the plant, genus and family for the first time.



4.61

4.3.4 Isolation and characterization of 2α, 3β, 6β, 23- tetrahydroxyolean-12-en-28-oic acid [Terminolic acid] (4.62)

Fractions 274- 304 eluted with 2.5 % methanol in chloroform (VLC3 (Figure 4:3) showed a homogenous spot on TLC in several solvent systems. Hence the fractions were pooled and dried under vacuum, which afforded 627 mg of compound **4.62**. The compound was a buff coloured powder with a melting point of 270 °C. Its electro spray mass spectra (ES-MS) showed *m/z* at 503 and 527 in -ve and +ve mode, respectively. The *m/z* at 503 is a molecular ion minus H [M^+ -H], while *m/z* at 527 was due to M^+ +Na. The IR spectrum showed bands at 3426 (O-H), 2943 (C-H), 1698 (COOH), 1462, 1380, 1265, 1183, 1043 and 974 cm^{-1}.



4.62

Compound **4.62** was characterized on basis of its ^1H NMR, ^{13}C NMR, DEPT, HSQC, COSY, HMBC, ES-MS and IR spectroscopic data (Figure 4:10- Figure 4:16). The ^1H

NMR (Figure 4:10; Table 4:8) showed the presence of 6 tertiary methyl singlets at δ 0.89, 0.96, 1.18, 1.55, 1.65, 1.70, (3H each). Other signals were for three carbinolic methine protons [δ 4.16d (1H), 4.32d (2H), 5.02s (1H)] and two doublets for diastereotopic carbinolic methlylene protons [δ 3.98d ([1H), 4.32d (2H)], a doublet at δ 3.25 and an olefinic proton (δ 5.50s). The ^{13}C NMR (Figure 4:11; Table 4:9) spectrum of **4.62** showed the presence of 28 intense peaks indicating the presence of a least 28, carbon atoms. Data from DEPT spectrum (Figure 4:12; Table 4:9) showed the presence of 6 methyl, 9 methylene, 7 methine and 8 quaternary carbons. Two peaks at δ 24.0 and δ 33.4, each represented a methyl and a methylene carbon. Out of seven methines, three, which appeared downfield at δ 67.9, 69.2, 78.8, were oxygenated. Similarly one methylene carbon atom at δ 66.8 was also oxygenated. Hence, the presence of downfield protons at δ 3.98, 4.16, 4.32 and 5.02 in the ^{1}H NMR spectrum (Figure 4:10) of **4.62** and downfield methine carbons at δ 67.9, 69.2, 78.8 and a methylene carbon signal at δ 66.8 clearly indicated the presence of three secondary hydroxyl groups and one primary hydroxyl group in **4.62**. Further, the presence of these hydroxyl groups in **4.62** was supported by the presence of an absorption band, at 3426 cm^{-1} in the IR spectrum. Furthermore, the presence of a downfield signal at δ 180.2 in the ^{13}C NMR spectrum suggested the presence of a free carboxylic group in the molecule, which was also supported by the presence of an absorption band at 1693 cm^{-1} in the IR spectrum. The ^{1}H NMR and ^{13}C NMR spectra (Figure 4:10; Figure 4:11) for compound **4.62** showed diagnostic features for olean-12-ene as discussed earlier for compounds **4.57**, **4.58** and **4.60**.

From the above ^{1}H NMR, ^{13}C NMR and DEPT spectroscopic details, the molecular formula of compound **4.62** was deduced as $C_{30}H_{48}O_6$. This molecular formula was further supported by the ES-MS (Figure 4:15), which showed a molecular weight, of 504 for compound **4.62**. Hence, from ^{13}C NMR, ^{1}H NMR and ES-MS data, it was deduced that compound **4.62** was a tetrahydroxylolean-12-enoic acid.

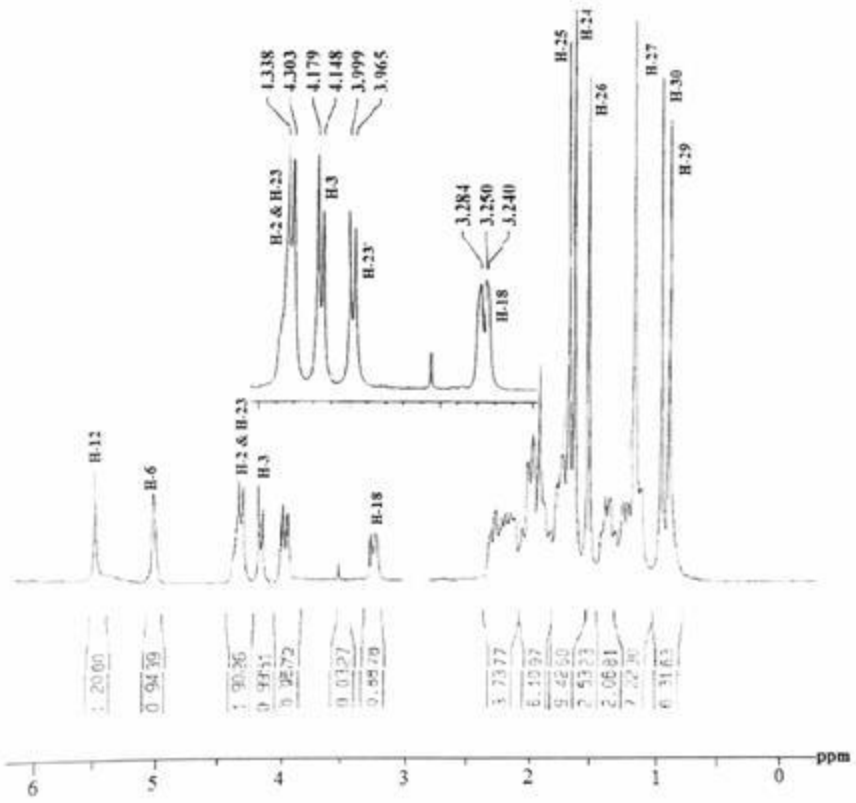


Figure 4:10 ^{1}H NMR Spectrum of Compound **4.62**

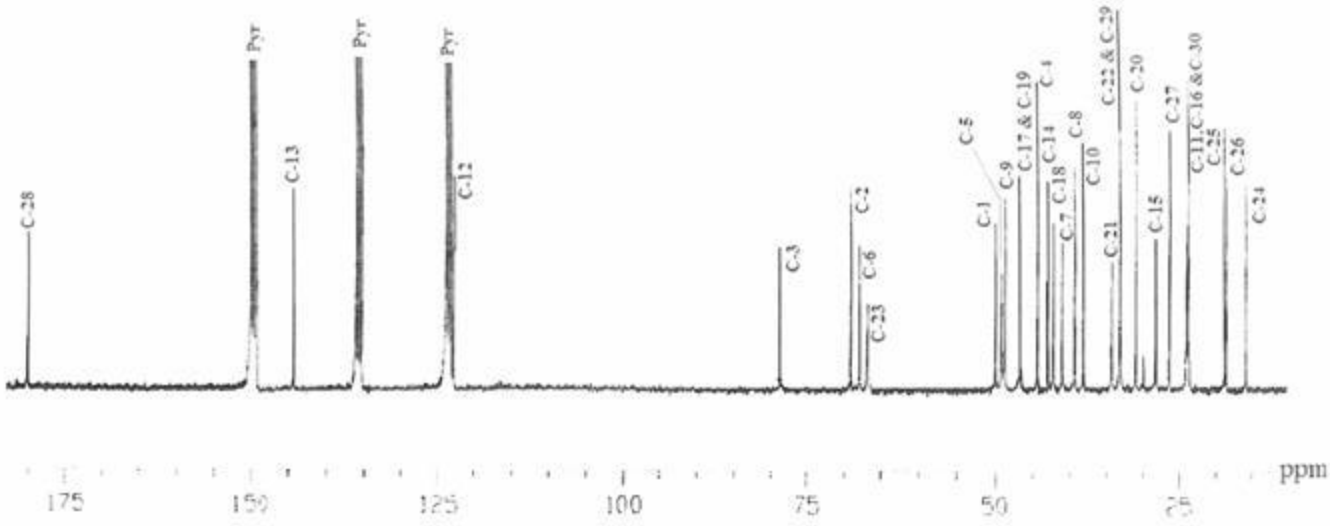


Figure 4:11 ^{13}C NMR Spectrum of Compound **4.62**

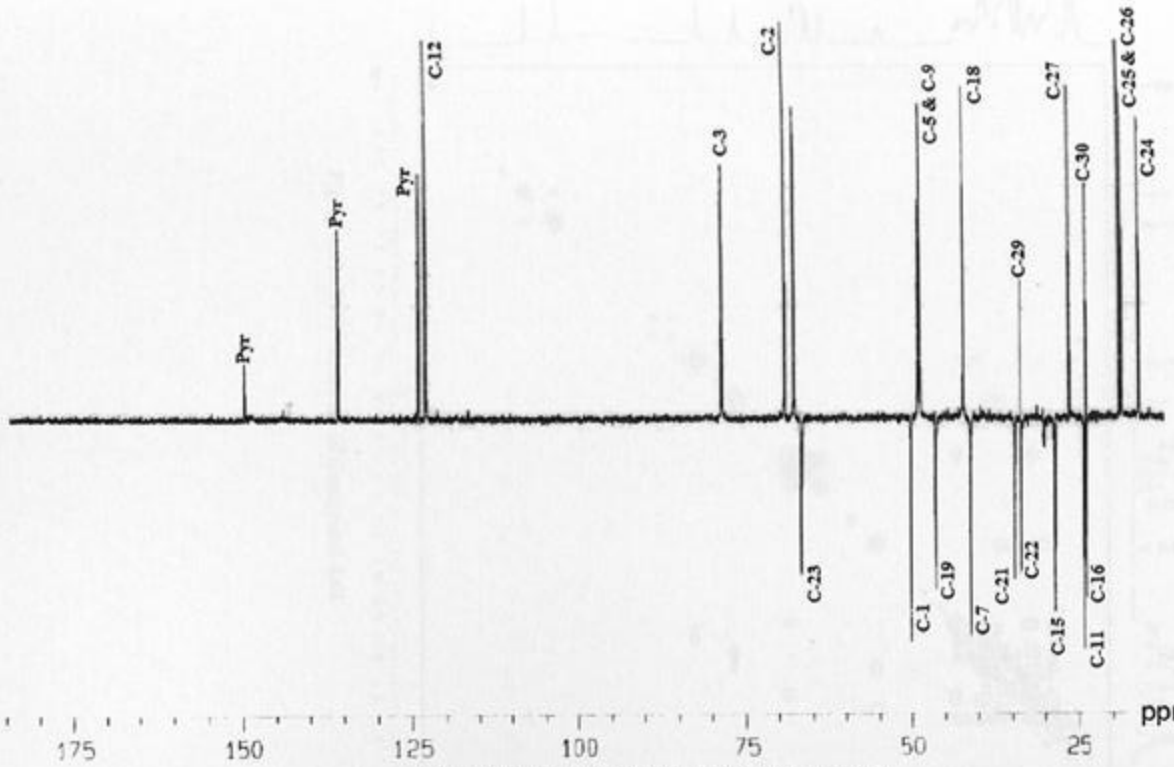


Figure 4:12 DEPT-135 Spectrum of Compound **4.62**

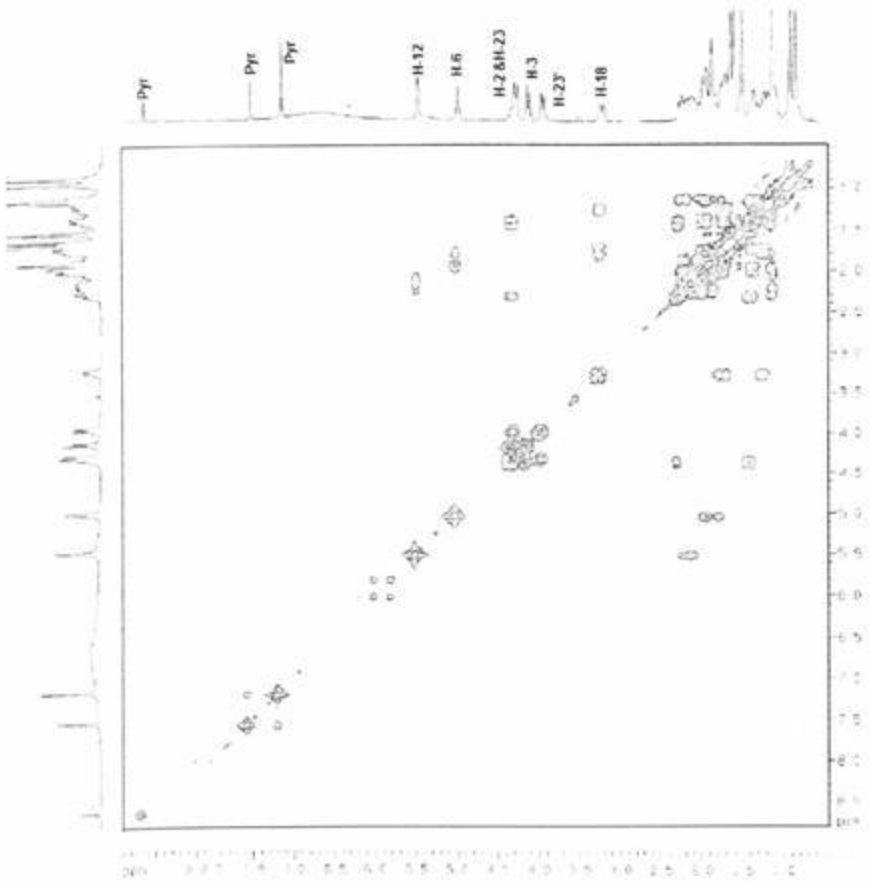


Figure 4:13 COSY Spectrum of Compound **4.62**

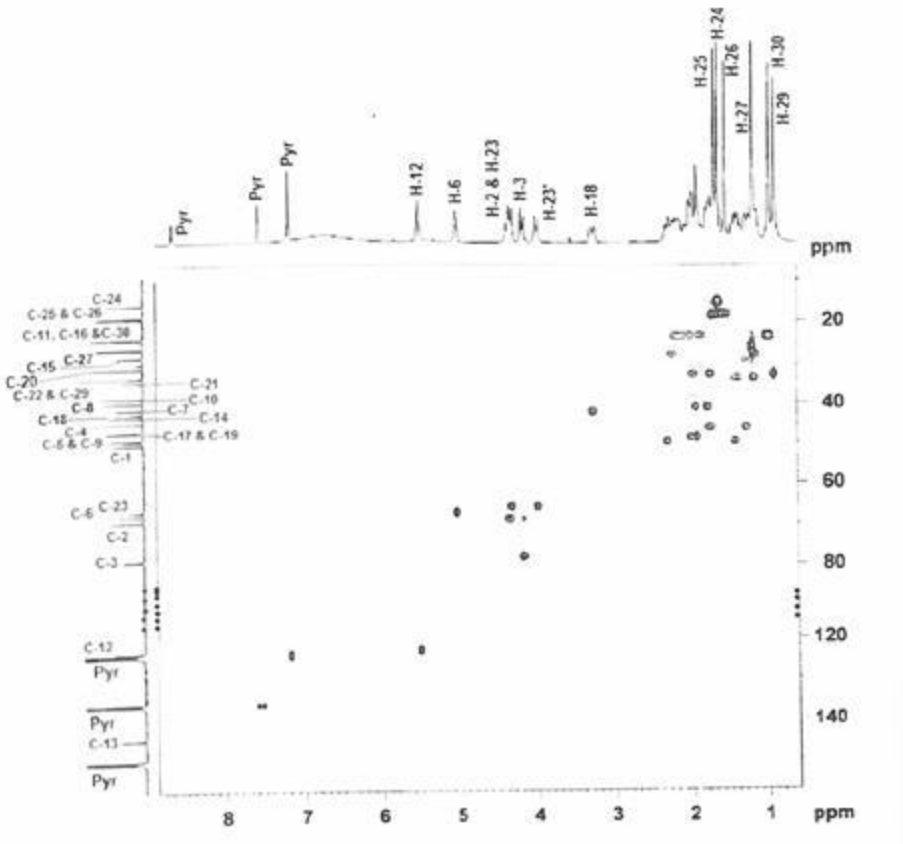


Figure 4:14 HSQC Spectrum of Compound **4.62**

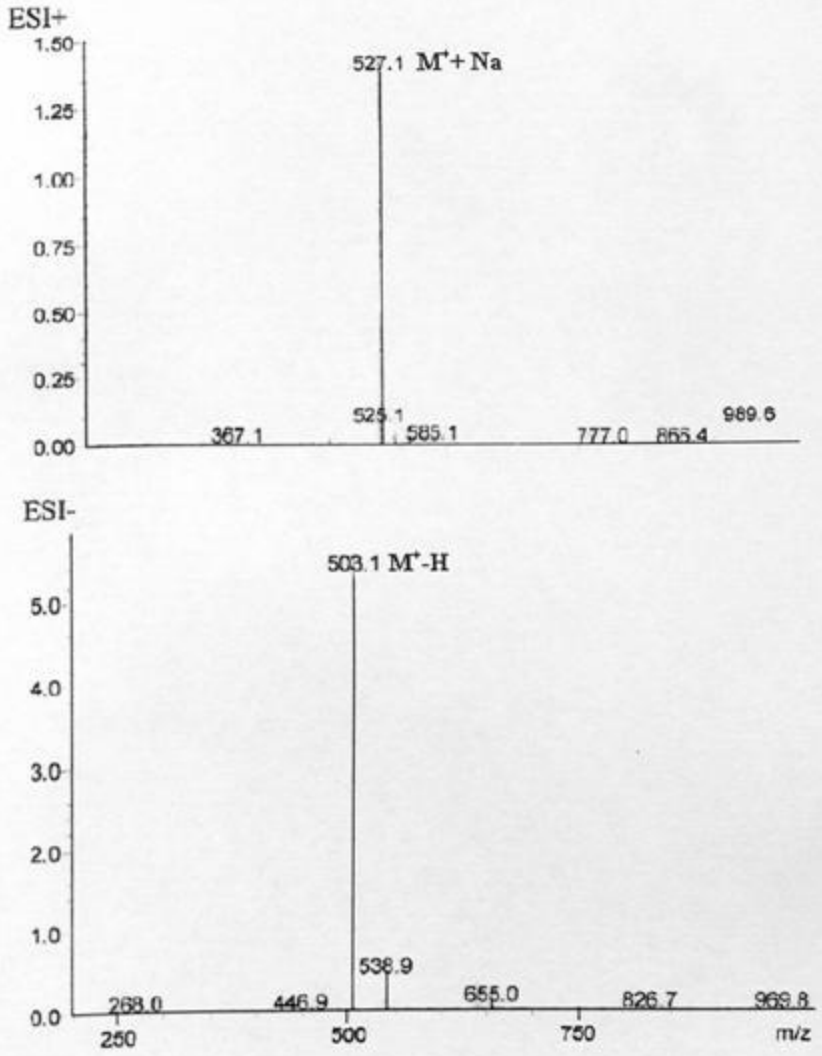


Figure 4:15 ES-MS of Compound **4.62**

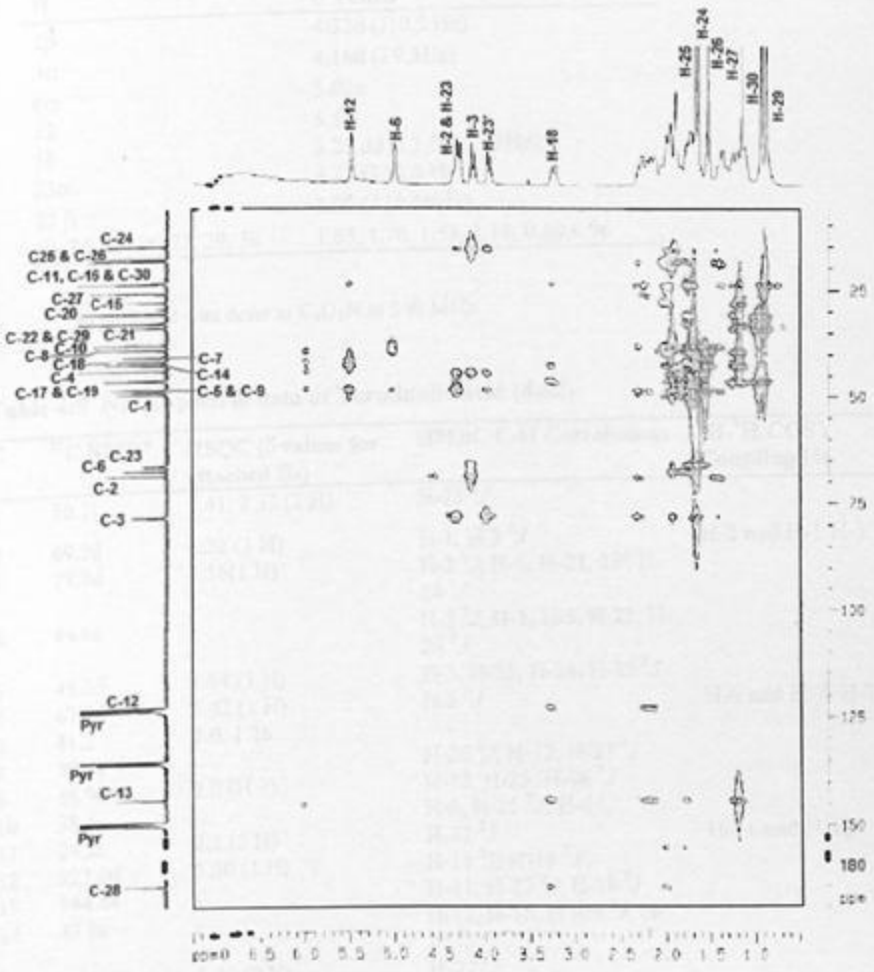


Figure 4:16 HMBC Spectrum of Compound **4.62**

Table 4:8 ^{1}H NMR chemical shifts (δ) for selected peaks of Terminolic acid (4.62)

H	δ Values*
2β	4.32d (J10.5 Hz)
3α	4.16d (J 9.3Hz)
6α	5.02s
12	5.50
18	3.25 dd (J 3.00, 13.2Hz)
23α	4.32 (J 10.5 Hz)
23 β	3.98 (J 10.39Hz)
H –24, 25, 26, 27, 29, 30	1.65, 1.70, 1.55, 1.18, 0.89,0.96

*Experiment was done in C_5D_5N at 300 MHz

Table 4:9 NMR spectral data of Terminolic acid (4.62)

C	^{13}C NMR*	HSQC (δ values for attached Hs)	HMBC C-H Correlations	^{1}H-^{1}H COSY Coupling Hs
1	50.1t	1.41, 2.32 (2 H)	H-25 ^{3}J	
2	69.2d	4.32 (1 H)	H-1, H-3 ^{2}J	H-2 and H-1 H-1'
3	78.8d	4.16(1 H)	H-2 ^{2}J; H-5, H-23, 23', H-24 ^{3}J	
4	44.6s	-	H-2 ^{3}J; H-3, H-5, H-23, H-24 ^{2}J	
5	49.3d	1.94 (1 H)	H-3, H-23, H-24, H-25 ^{3}J	
6	67.9d	5.02 (1 H)	H-5 ^{2}J	H-6 and H-7, H-7'
7	41.2	2.0, 1.76		
8	39.6s	-	H-26 ^{2}J; H-12, H-27 ^{3}J	
9	48.9d	2.0 (H 1)	H-12, H-25, H-26 ^{3}J	
10	38.4s	-	H-5, H-25 ^{2}J; H-6 ^{3}J	
11	24.2t	2.2 (2 H)	H-12 ^{2}J	H-11 and H-12
12	123.0d	5.50 (1 H)	H-11 ^{2}J; H-18 ^{3}J	
13	144.4s	-	H-11, H-27 ^{3}J; H-18 ^{2}J	
14	43.0s	-	H-12, H-18, H –26 ^{3}J; H-27 ^{2}J	
15	28.5t	2.28 (2 H)	H-27 ^{3}J	
16	24.0t	2.0, 1.85 (2 H)	H-18 ^{3}J	
17	46.9s	-		
18	42.3d	3.25 (1H)	H-12 ^{3}J	H-18 and H-19, H-19'
19	46.8t	1.76, 1.27 (2 H)	H-18 ^{2}J; H-29, H-30 ^{3}J	
20	31.1s	-	H-29, H-30 ^{2}J	
21	34.6t	1.38, 1.18 (2 H)	H-29, H-30 ^{3}J	
22	33.4t	2.0, 1.75 (2 H)		
23	66.8t	4.32, 3.98 (2H)	H-3, H-5, H-24 ^{3}J	H-23 and H-23'
24	15.8q	1.65s (3H)	H-3, H-5, H-23, ^{3}J	
25	19.1q	1.70s (3H)	H-5 ^{3}J	
26	18.8q	1.55s (3H)	-	

27	26.5q	1.18 (3H)	-
28	180.3s	-	H-18 ^3J
29	33.4q	0.89s (3H)	H-30 ^3J
30	24.0q	0.97s (3H)	H-29 ^3J

*Experiment was done in C_5D_5N at 75 MHz and DEPT was used to determine the multiplicities

The placement of hydroxyl groups in compound **4.62** was done by first making an assumption that out of four hydroxyl groups in the compound, two had similar configurations as those in compounds **4.58** and **4.60,** that is 2α, 3β dihydroxyl groups. This combination requires resonance around 83- 84 ppm for C-3, but the most downfield resonance for carbinolic carbon in ^{13}C NMR of compound **4.62** was at 78.7 ppm. If the assumption was correct, factors, had to be looked into, that caused an up field shift of C-3 bearing an equatorial hydroxyl group by about 5 ppm. The literature (Mahato and Kundu, 1994) showed that C-3 bearing an equatorial hydroxyl is shielded by about 5 ppm if there is a hydroxyl group on C-23. This confirms the upfield shift for C-3 in **4.62** and placement of the third hydroxyl group at C-23.

Since the fourth hydroxyl group is also secondary the possible positions in **4.62** were C-1, C-6, C-7, C-11, C-19, C-15, C-16, C-21 and C-22. The presence of a hydroxyl at position C-11, C-16, C-19, C-21 and C-22 was ruled out as the placement of a hydroxyl group at C-11 would have caused a downfield and upfield shift of the resonances of the olefinic carbons C-12 and C-13 due to β- and γ- effects, respectively. Further, placement of the hydroxyl group on C-16, C-21 and C-22 was ruled out as it would cause the carbinolic carbon to resonate downfield at ~ δ 73-78. Also position C-19 was ruled out by comparing the ^{13}C NMR data of **4.62** with those of arjungenin [19α] (Nandy et al., 1989) and tomentosic acid [19β] (Mahato et al., 1990) where the resonances of most carbons for these isomeric compounds were markedly different from those of compound **4.62**. Thus it left four probable positions for the fourth hydroxyl group, which were C-1, C-6, C-7 and C-15.

Detailed analysis of ^1H-^1H COSY, HSQC and HMBC spectra of compound **4.62** (Figure 4:13; Figure 4:14; Figure 4:16) confirmed the assignment of the first three hydroxyl groups and placement of the fourth hydroxyl group. In the heteronuclear correlation

HMBC (Figure 4:16 and Figure 4:17), the carbinolic carbon atom C-2 (δ 69.2) was observed to have a C-H ^{2}J correlation with the diastereotopic methylene protons at C-1 (δ 1.41, 2.32) and a carbinolic proton at C-3 (δ 4.16) indicating that this carbinolic carbon atom lies between C-1 and C-3. To confirm this further the homonuclear correlation ^{1}H-^{1}H COSY (Figure 4:13 and Figure 4:17), showed the carbinolic proton at C-2 to be coupled with both diastereotopic methylenic protons at C-1. The C- H correlations for carbinolic carbon atom C-3 (δ 78.7) include the C-H ^{2}J correlations with carbinolic proton at C-2 and the C-H ^{3}J correlations with the diastereotopic methylenic protons at C-1, methine proton at C-5 (δ 1.94) and the methyl protons at C-24 (δ 1.65) and diastereotopic methylenic protons at C-23 (δ 3.98, 4.32). Further the carbinolic carbon at C-23 (δ 66.8) showed C-H ^{3}J correlations with H-3, H-5 and methyl protons at C-24. These observations supported the placement of the three hydroxyl groups at C-2, C-3 and C-23.

The important C-H correlation involving the fourth hydroxyl group was the C-H ^{2}J correlation of the carbinolic carbon atom appearing at δ 67.9 with H-5. Similarly a C-H ^{3}J correlation was observed between C-10 at δ 38.4 and the carbinolic proton at δ 5.02. In COSY (Figure 4:13) the only important spin systems were seen between the carbinolic proton (δ 5.02) and the diastereotopic protons at C-7 (δ 2.0, 1.76). These observations led to the placement of the fourth hydroxyl group at position C-6. The configuration of the hydroxyl group was determined by comparing the carbon chemical shifts for ring B for compounds **4.62** with those of analogues having the axial (β) and equatorial (α) oriented hydroxyl groups at C-6. The ^{13}C NMR spectral data of compound **4.62** for rings B, C, D and E were very well correlated with those of protobassic acid (Toyota et al., 1990), which had an axial oriented hydroxyl group, at C-6 therefore compound **4.62** was characterized as 2α, 3β, 6β, 23- tetrahydroxyolean-12-en-28-oic acid (terminolic acid). The reported ^{13}C NMR data (Kundu and Mahato, 1993) for terminolic acid show an agreement with the present data even though use of DMSO caused the reported chemical shifts to be slightly upfield due to solvent effects.

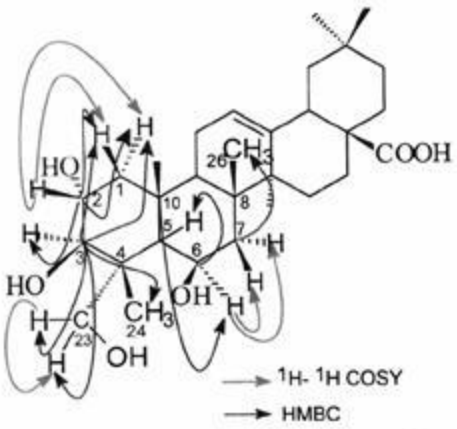


Figure 4:17 Important homonuclear and heteronuclear correlations of Compound **4.62**

Terminolic acid is reported in *C. zeyheri* for the first time and probably in the genus too. However, the compound has been reported to be present in several species of *Terminalia* within the Combretaceae family (Kundu and Mahato, 1993; Conrad et al., 1998).

4.3.5 Minimum inhibitory concentrations for compounds isolated from *C. zeyheri* leaves

The MICs) of the isolated compounds were determined for two standard strains of *Candida albicans* (ATCC 90028 and MTCC 1637) and a clinical isolate (CI) of *C. albicans*. The MICs for the compounds **4.49** and **4.57-4.60** and **4.62** for the various *C. albicans* strains are as given in Table 4:10. It can be seen that compound **4.62** (terminolic acid) had some inhibitory activity on all the tested *C. albicans* strains. The bioautogram of the crude extract of *C. zeyheri* leaves had indicated the presence of at least 2 active compounds, which, were well separated (Chapter 2, Figure 2.5). This means that the other active compounds might have not been isolated or it had a very weak activity. Terminolic acid is present in high concentration and it is the major compound in the fraction of the extract studied and this is the first report on its anticandida activity.

Table 4:10 MICs of compounds isolated from *Combretum zeyheri* leaves in µ g/ml

S/N	Compounds	*Candida albicans* strains		
		MTCC1637	ATCC90028	CI
1	**4.49& 4.57**	> 250	> 250.00	> 250.00
2	**4.58 & 4.59**	>100	>100.00	>100.00
3	**4.60**	> 250	> 250.00	> 250.00
4	**4.62**	125	62.50	125.00
5	A*	3.13	3.13	6.25
6	C*	0.39	0.78	0.39

Key: *A: Amphotericin B; C: Clotrimazole

CHAPTER 5

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Candidiasis is one of the major and most frequent opportunistic infections in individuals living with HIV infection, especially those with severe immunosupression. Management of candidiasis is associated with many problems including development of resistant strains to the available antifungal agents and selection of *Candida* species that are more refractory to the commonly used antifungal agents, thus making it essential to develop new anti-fungal agents. In this study 56 medicinal plants growing in Tanzania were investigated on their potential activity against *C. albicans* standard strain ATCC 90028.

5.1.1 Anticandida activity of plants

Sixty-three aqueous methanolic extracts were screened against the *C. albicans* standard strain using bioautography agar overlay (Rahalison et al., 1991). Bioautography agar overlay assay, a modified method of bioautography is regarded as the method of choice for screening antifungal compounds from plants (Hostettmann and Marston, 1994). The technique allows a combination of a bioassay in *situ* and the localization of the active constituents on TLC plate enabling a large number of extracts to be screened within a short time. The bioautography technique relies on the transfer of the active compounds by diffusion process from the stationary phase into the agar layer containing the microorganisms. When the plate is sprayed with methyl thiozolyl tetrazolium bromide (MTT), a MTT formazan is produced and growth inhibition can be visualized against a purple background.

Twenty-seven out of 56 plant species, constituting 48% of the collected plants were found to be active against *C. albicans* ATCC 90028. Twenty out of 36 plants (55.6%), which were obtained through interviewing traditional healers, were found active. However, several factors could be responsible for the inactivity of some of these plants. The extracts were prepared using aqueous methanol (MeOH: H_2O [4:1]), which was selected as an extracting solvent because it could extract polar and non- polar

constituents and also to some extent mimics the aqueous solvent commonly used by traditional healers in the preparation of the herbal medicines. However, despite the use of aqueous methanol in extracting the plant materials, most of the plant materials collected from traditional healers, were not prepared in a manner similar to how they are prescribed by the traditional healers.

For instance, 10 out of the 36 plants collected from traditional healers (Table 2:2) are normally used in a fresh state while in this study all the collected plants were dried prior to extraction, so as to reduce bulkiness and most importantly remove moisture which could have facilitated deterioration due to rotting and enzymic reactions. The possibility of losing the volatile constituents upon drying is also high. However, the ultimate effect of drying on the activity of the extract depends on the nature of the active constituents. In addition, some of the traditional healers usually prepare the drug from the juice of plants, which does not require any solvent in drug preparation. For example, in the case of *Cajanus cajan*, *Clutia abyssinica* and *Pseudovigna argentea*, fresh leaves are normally crushed and applied directly to the affected area (Table 2:3). Also for *Plectranthus barbatus* and *Physalis peruviana*, the leaves are squeezed to obtain the juices, which are then used as prescribed.

The efficacy of plants collected from traditional healers should be studied by screening combinations of plants as prescribed by the traditional healers to allow a correct conclusion to be drawn with regard to their efficacy. In this regard, a pharmacological study of a Japanese Kampo medicine (Sho-saiko-to-go-keishi-ka-shakuyaku-to - TJ-960) showed enhancement of anticonvulsant activity because it consists of 10 kinds of herbs decocted into the formula (Hosoya, 1988). However, if any single herb except *Zingiberis rhizoma* from the multi-herb formula of TJ-960 is omitted, the anticonvulsant activity disappears. This suggests that the combined decoction of nine herbs in the formula of TJ-960 is necessary for expression of the anticonvulsant activity (Hosoya, 1988). Similarly a combination of herbs including *Boscia salicifolia* and *Sclerocarya birrea*, *Turreae robusta* and *Sclerocarya birrea*, *Rhus natalensis*, *Boscia salicifolia* and *Turreae robusta*, *Lannea schweinfurthii* and *Boscia salicifolia* used traditionally as antimalarial in the treatment of malaria showed enhanced antimalarial activity compared to an extract from

a single plant (Gathirwa et al., 2008). Notably, several plants employed in this study are also used traditionally in combination forms. For instance, the fresh roots of male plants of *Carica papaya* are mixed with fresh roots of *Ocimum suave* and boiled with water. Similarly, the root bark of *Cassia abbreviata* is boiled with a certain gum, the root bark of *Acacia zanzibarica* is mixed with that of *Lannea stuhlmannii* and boiled with water; the peeled roots of *Synaptolepis kirkii* are mixed with castor seeds, crushed and boiled with water. But when these plants were extracted and screened separately for activity, they were found to be inactive (Table 2:5). However, what constitutes, the chemical, pharmacological and pharmaceutical basis for such phenomena of combinations remains obscure. Nevertheless, the plant materials could be re-evaluated in combination forms as prescribed by the traditional healers.

Eight (40 %) of the 20 plant materials collected on the basis of literature search (Sawhney *et al.*, 1978b, Khan *et al.*, 2000) were found active by this bioassay. There may be many factors, which could have led to the inactivity of the remaining 12 (60%) plants. Factors such as age of the plant, season of collection, development stage of the plant and the time of collection, day or night, may influence the type and amount of the chemical constituents in the plant and hence their activity. These factors need to be taken into account when results obtained in this study are compared to those previously reported by others.

5.1.2 Isolation of active compounds

The active compounds from the selected plants, *Albizia anthelmintica* and *Combretum zeyheri* were isolated from the extracts based on the bioautography agar overlay results (Table 2:5). The normal bio-guided isolation procedure in which at every stage of isolation the resulting pooled fractions have to be screened for activity was not followed instead the h_{RF} values of the active spots obtained in Chapter 2 were used to locate and isolate the active compounds. This was found to be economical in terms of both time and consumable materials.

Compounds from *A. anthelmintica* root bark were extremely difficult to separate using the available separation techniques. Various chromatographic methods were employed as

discussed in Chapter 3. All compounds isolated were polar, had close hR_f values and some compounds (e.g. **3.6** and **3.22**) gave a single spot on TLC developed with some solvent systems such as Butanol: Acetic acid: water (BAW) 4:1:2. For some saponins (e.g. **3.22** and **3.27**) it was difficult to use their TLC profile in order to extrapolate their order of elution from VLC or CPTLC. This is because they appeared to possess similar polarity as was indicated by the order in which they eluted from VLC or CPTLC. In Figure 3:4 (Chapter 3; 3.2.1.3.2) when 500 mg of pooled fractions 190- 192 were directly subjected to CPTLC compound **3.6** was separated from **3.27** but it eluted with compound **3.22** and the fractions eluted thereafter contained substantial amounts of compound **3.22** with small quantities of **3.27** and **3.28**, which were successfully separated by CPTLC. In this CPTLC separation process, compound **3.22** eluted before compound **3.27.** However, if a sample from the same fractions 190-192 was first subjected to VLC prior to CPTLC, compounds **3.6** and **3.27** also compounds **3.22** and **3.28** were eluted from the column together and in this order also they could not be separated on CPTLC. Unlike in CPTLC compound **3.27** eluted from the VLC ahead of compound **3.22**.

The separation of *A. anthelmintica* saponins was achieved through planar chromatography, CPTLC and PTLC. In a previous study Caprani et al. (1989) reported on the isolation of three saponins, **3.6**, **3.22** and **3.23**, using gel filtration, reversed phase CC on a lobar RP-18 column and droplet counter- current chromatography (DCCC). In this study a small reversed phase column on RP-18 was attempted on the fraction containing these compounds, but separation of any of these compounds could not be achieved. In addition Fast Centrifugal Partition Chromatography (FCPC), a technique closely related to and more advanced than DCCC, was to be used in this study. However, the critical factor seen in this technique was the determination of an appropriate immiscible solvent system to serve as a mobile and stationary phase. This solvent system was supposed to have a partition coefficient of between 0.5 and 1 for the compounds being separated and a settling time of less than 20 seconds. The only solvent system, which met these criteria, included a small amount of trifluroacetic acid (TFA) in order to improve its settling time. This method was not used due to the possibility of foreseen problems such as hydrolysis of the saponins by TFA upon concentration and drying of the fractions.

The TLC profiles of the various fractions obtained during the course of isolation of the compounds from *Combretum zeyheri* leaves showed the presence of many compounds in very low concentrations and very close hR_f values, making the isolation and purification of these compounds very difficult. However, VLC was found to be very useful in the isolation of major compounds from *Combretum zeyheri* leaves.

5.1.3 Characterization of the isolated compounds

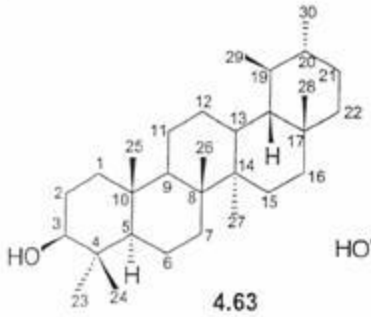
NMR spectroscopy was successfully used in the characterization of isolated pure compounds. The ES-MS and Fourier transform infrared (FT-IR) spectroscopy provided the molecular weights and functional groups, respectively, so as to confirm the structure of compounds, which were already deduced from the NMR spectral data. Most of the isolated compounds were known and hence the previously reported data for these compounds supported further their identity. In the case of unknown compounds and in cases where there was ambiquity in the structure, 2D NMR experiments were used to find out connectivity of atoms within the molecule.

5.1.3.1 Compounds isolated from *Albizia anthelmintica* root bark and *Combretum zeyheri* leaves

With the exception of compound **3.25**, all the compounds isolated from *Albizia anthelmintica* were saponins of echinocystic acid. The similarities of the chemical shifts in ^{13}C NMR and ^{1}H NMR for compounds **3.6**, **3.22** **3.23** and **3.26** (Table 3:3- Table 3:7) whose spectra were acquired in pyridine-D_5 clearly prove that these compounds were closely related. The ^{1}H NMR (Appendix 8) of these compounds indicated that they were very similar; their spectra differed only in the sugar region. Further, the presence of a signal around δ 58 in ^{13}C NMR spectrum of all the saponins isolated clearly indicated that glucosamine is a common sugar in all the saponins isolated from *Albizia anthelmintica*.

Saponins bearing 2-acetamido-2-deoxy-β-D-glucopyranose (acetyl glucosamine) moiety have been previously reported in other species within the genus, including *A. lucida* (Orsini et al., 1991), *A. subdimiata* (Abdel- Kader et al., 2001) and *A. procera* (Melek et al., 2007) and have also been reported in other plants such as *Pithecellobium cubbense*, *P. arboretum* within the family (Ripperger et al., 1981). However, the presence of a deacetylated glucosamine in saponins is being reported for the first time and hence the occurrence of compounds **3.27** and **3.28** in nature is being reported for the first time.

The compounds isolated from *Combretum zeyheri* had their structure based on α- and β-amyrin (**4.63** and **4.64**).



Like the compounds isolated from *A. anthelmintica* the ^{13}C NMR and ^{1}H NMR showed similar features (Table 4:2 - Table 4:9) suggesting that these compounds were related. All the compounds isolated are known and compound **4.49** has been isolated from the plant before.

5.1.4 Anticandida activity of the isolated compounds

The activity of the isolated compounds was determined through their MICs using broth microdilution method. The reference compounds used were amphotericin B and clotrimazole. The MICs for the amphotericin and clotrimazole were 3.13 and 0.78 μg/ml respectively for *Candida albicans* ATCC 90028 strain. The National Committee for Clinical Laboratory Standards (NCCLS) recommend MIC range of 0.25- 2μ g/ml for Amphotericin B when using *Candida albicans* ATCC 90028 (Ambler et al., 2001). The

NCCLS susceptibility test is usually carried in RPMI 1640, a medium, which gives MICs 15 times lower when compared to Sabouraud dextrose broth (Scorzoni et al., 2007), a medium which was used in this study. In view of this, the MICs of 3.13 μ g/ml obtained for amphotericin B in this study was within the required range. It should also be noted that apart from the type of medium used, the MICs are reported to vary depending on a number of technical variables such as inoculum size and preparation, duration and temperature of incubation and the criterion used for MICs end point determination (Epspinel- Ingroff and Pfaller, 2007). In addition, other factors, which might affect the MICs are those problems unique to fungi such as slow growth and ability of certain fungi to grow as unicellular yeast or as hyphal or filamentous forms, depending on pH, temperature or medium composition. Finally the basic properties of antifungal agents themselves, such as solubility, mode of action, and the tendency to produce partial inhibition of growth over a wide range of concentrations are important and have to be taken into consideration when MICs are determined (Epspinel- Ingroff and Pfaller, 2007).

The MICs for the active compounds from *A. anthelmintica* root bark ranged from 25-125 μg/ml. Also various combinations of compounds **3.6** and **3.27** were observed to be more active, reducing MICs to 12.5 μg/ml. The anticandida activity for these compounds is being reported for the first time. For *C. zeyheri*, terminolic acid the only active compound isolated from this plant had a MIC of 62.5 μg/ml. Despite the fact that the MICs of the isolated compounds are markedly high when compared to those of the standard drugs, these active compounds can be studied further for possible development into new anticandida drugs through structural modifications in order to obtain various analogues and determine their structure-activity relationships (SAR). This could be useful, especially, for a less active compound like terminolic acid from *C. zeyheri* leaves, which constituted more than 50% of all the chemical constituents in the studied extract. In view of the abundance of this compound in the plant, many analogues can be synthesized and studied, and thus, increasing the possibility of obtaining more active compounds.

5.2 CONCLUSION AND RECOMMENDATIONS

From this study it can be concluded that 48% of the medicinal plants screened in this study have shown some activity against *Candida albicans* standard strain ATTC 90028. The study emphasizes the importance of ethnomedical knowledge and literature in searching for bioactive compounds from plants. New bioactive compounds **3.27** and **3.28** were isolated from *Albizia anthelmintica* root bark. Furthermore, the MICs obtained for compounds **3.6**, **3.27** and **3.28** and combinations (Table 3:10) of these compounds isolated from *Albizia anthelmintica*, a plant used in the treatment of vulvovaginal candidiasis meant that these compounds, in the herbal drug, act with synergism. With the overall results obtained from this study it is anticipated that even better results could be obtained if i) the plant sample size is increased, based on the fact that Tanzania has approximately 10,000 plant species (Mahunnah and Mshiu, 1991) and ii) A detailed study is carried out on plants collected from different parts of Tanzania with climatic and seasonal variations. Furthermore, future studies could involve synthesis of analogues based on the active compounds isolated from *A. anthelmintica* root bark and *C. zeyheri* leaves and evaluate their SAR. Also, *in vivo* activity could be carried out which will enable the drug to be prepared and administered in the way used by traditional healers.

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