

CHARACTERIZATION OF COMPOUNDS FROM *CURTISIA DENTATA* (CORNACEAE) ACTIVE AGAINST *CANDIDA ALBICANS*

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**THESIS SUBMITTED TO THE DEPARTMENT OF PARACLINICAL
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PHILOSOPHY**

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DECLARATION

I declare that the thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor has not previously been submitted by me for a degree at this or any other university, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

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'Moreko ga itekole, ebile motho ke motho ka bangwe batho (Sepedi proverb). In short, no man is an island'. THIS PROJECT IS DEDICATED TO MY FAMILY (My wife Grace, my daughters Pontsho and Bonnie, I love you. This is for you).

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LIST OF ABBREVIATIONS

Af	<i>Aspergillus fumigatus</i>
AMB	Amphotericin B
BEA	Benzene, ethyl acetate, ammonia (90:10:1)
Ca	<i>Candida albicans</i>
ACN	acetone
CD	<i>Curtisia dentata</i>
CEF	Chloroform: ethyl acetate: formic acid (5:4:1)
Cn	<i>Cryptococcus neoformans</i>
CZ	<i>Cussonia zuluensis</i>
DCM	dichloromethane
DE	Dichloromethane: ethyl acetate (4:1)
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethylsulfoxide
Ec	<i>Escherichia coli</i>
Ef	<i>Enterococcus faecalis</i>
EMW	Ethyl acetate: methanol: water (40:5.4:4)
IPIUF	Indigenous Plant Use Forum
KA	<i>Kigelia africana</i>
Mc	<i>Microsporum canis</i>
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
Pa	<i>Pseudomonas aeruginosa</i>
Rf	Retardation factor
Sa	<i>Staphylococcus aureus</i>
Ss	<i>Sporothrix schenckii</i>
TE	<i>Trichilia emetica</i>
TLC	Thin layer chromatography
TP	<i>Terminalia phanerophlebia</i>
TS	<i>Terminalia sambesiaca</i>
UPPP	University of Pretoria's Phytomedicine Programme
UV	Ultraviolet
VR	<i>Vepris reflexa</i>



PAPERS PREPARED FROM THIS THESIS

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Lupeol, ursolic acid, betulinic acid and extracts of *Curtisia dentata* inhibit motility of *Trichostrongylus colubriformis*, *Haemonchus contortus* and *Caenorhabditis elegans* (Manuscript)

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CONFERENCE PRESENTATIONS

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ABSTRACT

The main aim of the study was to isolate compounds active against *Candida albicans* from the most active species from a pool of several trees. Seven tree species with good antifungal activity were selected from the Phytomedicine Programme database. The selected plant species investigated were screened for growth inhibitory activity against *Candida albicans* using bioautography and serial microplate dilution methods. These tree species were: *Cussonia zuluensis*, *Vepris reflexa*, *Curtisia dentata*, *Trichilia emetica*, *Terminalia phanerophlebia*, *Terminalia sambesiaca* and *Kigelia africana*. Using the serial microplate dilution method for the determination of minimal inhibitory concentrations, *Terminalia phanerophlebia* and *T. sambesiaca* were active against *Candida albicans* with MIC values as low 0.02 mg/ml. The acetone and dichloromethane extracts of all plant leaves were active against *C. albicans* with MICs varying from 0.02-2.5 mg/ml. Based on bioautography, the acetone extract of the leaves of *Curtisia dentata* had more active (5) compounds against *C. albicans* than any of the tree species investigated.

The dichloromethane, acetone and hexane extracts of the seven tree species were further screened for antifungal activity using other fungal test organisms. The fungal species used were *Aspergillus fumigatus*, *Microsporum canis*, *Sporothrix schenckii* and *Cryptococcus neoformans*. Extracts of *Curtisia dentata*, *Terminalia sambesiaca* and *Terminalia phanerophlebia* had the highest activities against these fungal test organisms with minimal inhibitory concentration (MIC) values as low as 0.02 mg/ml. *Cussonia zuluensis* was the least active with high MIC values (>250 µg/ml in some cases) and the lowest number (1) of active chemical components on bioautograms. The highest number of active compounds (5) against *C. albicans* on bioautograms was observed in the acetone extracts of *C. dentata*. The plant species were further investigated for presence of antibacterial compounds, using *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* as test bacterial organisms. Compounds with similar R_f values in the acetone extract of *C. dentata* were active against both bacterial and fungal test organisms, suggesting that the growth inhibitory activity of *C. dentata* extracts was non-selective. *C. dentata* was chosen for isolation of compounds due to 1) the highest number of active compounds on bioautogram against *C. albicans*, 2) the MIC values (0.12-0.6 mg/ml) against *C. albicans*.



Acetone extracts of the leaves, stem bark and twigs of *Curtisia dentata* were compared for antibacterial and antifungal activity using the serial microplate dilution and bioautography methods in order to select the plant part to isolate compounds from. The TLC fingerprints of the twigs and leaves were largely similar. A non-polar compound and two medium polarity compounds, present in the leaves and twigs, were missing in the stem bark extract. Bioautography indicated that the leaves contained more antibacterial and antifungal compounds than the stem bark extracts. Extracts of the leaves were 5-fold more active than the stem bark extracts against *Candida albicans*, with total activities of 1072 and 190 ml/g, respectively. Against bacterial test organisms extracts of the leaves, stem bark and twigs resulted in comparable activities. These findings encourage the interchangeable usage of the stem bark, leaves and twigs of this plant, which may lead to sustainable harvesting of the species. This approach may conserve this and other threatened or endangered plant species.

The leaves of *Curtisia dentata* (Cornaceae) were serially extracted with solvents of varying polarities, starting with hexane, then dichloromethane, followed by acetone with methanol completing the fractionation. The dichloromethane (DCM) and acetone bulk fractions of *Curtisia dentata* contained the highest number of active compounds and resulted in low MIC values. The hexane and the methanol bulk fractions were the least active. In the hexane bulk fraction, bioautography revealed the presence of one active compound. The DCM bulk fraction showed cytotoxicity against Vero cells similar to the positive control, berberine with an LC₅₀ value of 10 µg/ml. The acetone and dichloromethane fractions resulted in total activity values of 3312 and 4240 ml, respectively. However, these fractions were cytotoxic to the Vero cells with LC₅₀ values of 24.4 µg/ml for acetone fraction and 6.6 µg/ml for the dichloromethane fraction. The cytotoxicity data may serve to discourage the use of these extracts to treat candidosis. However, preparations of these fractions may be used topically on wounds to combat infections. The application of these extracts on rat wound model did not result in any observable pathologies.

The DCM and acetone bulk fractions each contained 4 compounds active against *Candida albicans*. Only the dichloromethane extract was fractionated as these extracts contained almost similar active compounds. Column chromatography using silica as the stationary phase afforded four compounds from the DCM extract. These compounds were identified using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) as lupeol (**CI**), betulinic acid (**CII**), ursolic acid (**CIII**) and



hydroxyl-ursolic acid (**CIV**). These compounds have been isolated from several plant species and have been found active against several pathogens including the human immunodeficiency virus (HIV). This is the first report of the isolation of these compounds from *Curtisia dentata*. The antibacterial activity of these compounds has been reported. The anti-*Candida* activity of ursolic, oleanolic and ursolic acid has been reported with MIC values exceeding 128 µg/ml (Hiriuchi *et al.*, 2007). However, the anti-*Candida* activity of betulinic acid and lupeol has not been reported.

The four isolated compounds were tested for activity against several fungal (*Candida albicans*, *C. spicata*, *C. guilliermondi*, *Aspergillus fumigatus*, *Sporothrix schenckii*, *Cryptococcus neoformans* and *Microsporum canis*) and bacterial (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*) species. Ursolic acid and hydroxyursolic acid were the most active with MIC values. Hydroxyursolic acid resulted in an MIC value as low as 8 µg/ml against *M. canis*. *A. fumigatus* was the most resistant microorganism while *M. canis* and *S. schenckii* were the most sensitive. *C. albicans* was moderately sensitive to the compounds with MIC values ranging from 16 µg/ml for betulinic acid to over 250 µg/ml for lupeol.

Compounds isolated in sufficient quantities, namely, lupeol and betulinic acid, were investigated for cytotoxicity against Vero cells. It appeared that lupeol was less toxic than betulinic acid, with LC₅₀ values of 89.5 and 10.9 µg/ml, respectively. The cytotoxicity of betulinic acid was comparable to that induced by the positive control, berberine with an LC₅₀ of 10 µg/ml.

Lupeol was the least active of the isolated compounds. Betulinic acid and lupeol, together with the water and acetone extracts were tested in an *in vivo* rat model to determine antifungal and wound healing activities. The rats were immunocompromised prior to the surgical and treatment procedures. Treatments with any of the formulations did not affect wound healing activity. The rate of wound healing was comparable to both the positive (amphotericin B) and negative (cream only) controls. It was however difficult to judge and score antifungal activity. The model developed to evaluate skin infections will have to be improved to allow for testing for anti-*Candida* activity *in vivo*.

Some antifungal compounds, such as azoles, are known to also have anthelmintic activity. The isolated compounds, which had antifungal activity, were tested for anthelmintic activity against both parasitic and free-living nematodes. Furthermore, other publications demonstrated that betulinic acid had anthelmintic activity against



C. elegans. Lupeol, ursolic acid and betulinic acid, together with the DCM and acetone extracts were investigated for anthelmintic activity against both free living and parasitic nematodes. The acetone and dichloromethane extracts were active against all nematodes to concentrations as low as 160 µg/ml. Betulinic acid and lupeol were active against the parasitic nematodes at high concentrations of 1000 and 200 µg/ml. All compounds were active against the free-living *Caenorhabditis elegans* with concentrations as low as 8 µg/ml. Betulinic acid was less active than lupeol and ursolic acid against *C. elegans*. The acetone and dichloromethane extracts were also active against *C. elegans* with a concentration of 0.31 mg/ml resulting in almost 80% inhibition of larval motility. It would appear that the anthelmintic activity against both parasitic and free-living nematodes occurred at high concentrations of the compounds or extracts. Extracts of various medicinal plant species may provide the solutions to ill-health of small ruminants caused by parasitic nematodes in poor communities of southern Africa.

The extracts of *Curtisia dentata* and isolated compounds have anti-*Candida* activity *in vitro*. Their usage is hampered by associated toxicity. The cytotoxicity of the compounds and extracts was only demonstrated with Vero cells (monkey line). Experiments with several human cell lines may indicate the safety of these compound and extracts when used as treatment against *Candida* infections. No toxic effects were noted when extracts and isolated compounds were tested in an animal experiment indicating that extracts may be safe in a topical application. The extract from 1 g of leaf material can be diluted to more than a litre and still inhibit the growth of *C. albicans*.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. THE IMPACT OF INFECTIOUS DISEASES

Infectious diseases are important in public health for communities in Africa and the developing world (Sparg *et al.*, 2000). These diseases, and subsequent deaths, have devastating consequences for developing economies. Meagre health budgets and lack of adequate medical facilities hinder efforts by poor African countries to match the overwhelming treatment and prevention burden presented by these diseases (Louw *et al.*, 2002).

A diverse range of diseases afflicts people and animals worldwide. Over 200 million people in many countries are infected with *Schistosomiasis*, while a further 500-600 million (4.5% of the world's population) are at risk of being infected (Basch, 1991). Sexually transmitted diseases (STD's), including HIV/AIDS are a major public challenge in most African countries (Green, 1992; Ndubani, 1999). In sub-Saharan Africa, communicable diseases account for more than 70% of the burden of ill health as compared to about 10% in developed countries (World Bank, 1997).

In South Africa, outbreaks of cholera and typhoid (Sidley, 2005) have been reported in recent times. In 2005 alone, Sidley (2005) reported that 14 000 people in rural parts of the KwaZulu-Natal province of South Africa contracted cholera and that 50 people died allegedly due to the outbreak. About 18 million South Africans in rural areas do not have access to clean running water, toilets and adequate sanitation, further increasing the risk of contracting cholera. Pneumonia, especially in alcoholics, continues to be an important community-acquired infection (Ko *et al.*, 2002). Some hospitals in South Africa have been ravaged by *Klebsiella*. In Durban's Mahatma Gandhi Memorial Hospital in 2005, 110 babies died allegedly as a result of *Klebsiella* infections between 2004 and 2006 (Cullinan, 2006).

Western or modern medicine has for many years been used, with varying degrees of success, in the treatment of infectious diseases. Furthermore, improved sanitation, clean water, better living conditions and vaccines brought many infectious diseases under control (Wilson, 1995). Despite this, many obligate and opportunistic

pathogens are becoming increasingly resistant to most available drugs at an alarming rate that is unmatched by the development of new drugs (Neu, 1992).

In South Africa HIV/AIDS problems continue to ravage the communities, leading to high levels of opportunistic infections, manifesting as the so-called AIDS-related symptoms. Among the opportunistic infections in immunocompromised patients is candidosis, commonly caused by *Candida albicans*.

1.2. CANDIDOSIS

Candida albicans, which accounts for more than 80% of candidosis cases, is a member of the commensal flora of the gastrointestinal and genitourinary tract of dogs, cats and humans (Greene & Chandler, 1998). This microorganism exists in a variety of morphological forms, ranging from unicellular budding yeast to true hyphae (Sudbery *et al.*, 2004). The morphology is dependent on cell density, nutrient availability and pH of the environment (Sudbery *et al.*, 2004).

Candida albicans is considered to be an opportunistic pathogen in these species, able to access different locations of the body, causing disease when the host defense is compromised (Kobayashi and Cutler, 1998). Candidal urinary infections in dogs are associated with cystic calculus (Tan & Lim, 1977), hypothyroidism and diabetes mellitus (Forward *et al.*, 2002). In cats infections with *Candida* are reported with perineal urethrostomy (Fulton & Walker, 1992), diabetes mellitus and urinary tract infection (Marshall *et al.*, 2002) and hyperadrenocorticism (Gerding *et al.*, 1994). Pressler *et al.* (2003) reported the observation of *C. albicans* and other non-*albicans* strains in 62% of dogs and 43% of the cats in a study to evaluate urinary tract infection in these animals.

C. albicans causes vaginitis in otherwise healthy women, severe surface infections of the mouth and the esophagus in human immunodeficiency virus (HIV) patients and blood stream infections in vulnerable intensive care patients (Kao *et al.*, 1999). Vaginal candidosis, resulting from infection with *C. albicans* and *C. vaginitis*, is a common condition that affects approximately 75% of women at least once in their lifetime. *C. albicans* is the most common cause of vaginal candidosis, accounting for over 80% of infections (Richardson & Warnock, 1993).

The combination of microbial virulence, environmental influences and host defense factors determine the progression of candidosis and various manifestations that accompany the infection.

1.2.1. Predisposing factors to candidosis

Environmental conditions such as pH and temperature changes trigger dimorphism, which allows *C. albicans* to switch from unicellular to hyphal growth. Dimorphism permits the organism to invade tissues and thus, contributes to the virulence of this microorganism (Ryley & Ryley, 1996; Magee, 1998; Corner and Magee, 1997). Reduced salivary flow, epithelial changes, changes in commensal flora, and a high carbohydrate diet have been observed as local factors predisposing humans to oral candidosis (Farah *et al.*, 2000). A variety of nutritional factors that include deficiencies of iron, folic acid and vitamins have also been linked with candidosis (Cawson, 1966; Higgs and Wells, 1972). Diabetes, hypothyroidism, hyperparathyroidism and adrenal suppression resulting from an altered hormone state, have also been implicated in progression of candidosis, provided there is a preceding immune system defect (Kostiala *et al.*, 1979).

Pregnancy, uncontrolled diabetes, oral contraceptives containing high estrogen doses, systemic or local antibiotics and increased frequency of sexual intercourse have been reported as major factors that predispose women to candidal vulvovaginitis (CVV). During pregnancy, *C. albicans* is easily isolated from 30 – 40% of expectant mothers, and the infection shows pronounced virulence during the last trimester (Odds, 1988).

1.2.2. Immunodeficiency and candidosis

Infection with the human immunodeficiency virus (HIV), which leads to acquired immunodeficiency syndrome (AIDS) through the weakening of T-helper lymphocytes, makes human patients more susceptible to secondary infections, particularly opportunistic *C. albicans* infections. *C. albicans* and candidosis are observed and detected in more than 80% of HIV-infected patients (McCarthy *et al.*, 1991). Generally, up to two thirds of HIV infected individuals display signs of oral candidosis (McCarthy *et al.*, 1991).

Candidosis is a common feature in a variety of other immunodeficiencies. In patients suffering from severe combined immunodeficiency syndrome (SCID), a condition characterized by an improperly functioning immune defense system, especially depressed cell-mediated and phagocytotic immunity, chronic mucocutaneous candidosis (CMC) is observed (Porter & Scully, 1990). Again, chronic recalcitrant mucocutaneous candidosis is observed in patients suffering from DiGeorge syndrome, a condition characterized by lowering of T lymphocytes due to thymus hypoplasia (Cleveland *et al.*, 1968).

1.2.3. Pathogenesis

1.2.3.1. Adhesion

Candida follows a three-stage mechanism of invasion that involves 1) adhesion, 2) blastospore germination, mycelium or hyphae development and 3) epithelium invasion. The phospholipid- and fibronectin-containing receptor is the target for binding to epithelial cell surfaces (Krivan, 1989; Bohbot, 1996). Binding is mainly through a mannose-rich glycoprotein in the fungal protein (similar to integrins), which is able to anchor it on epithelial cells. Interestingly, estrogen impregnation in vaginal epithelium facilitates surface exposure of glycoprotein complexes acting as receptors, thereby enhancing blastospore adherence (Powell & Drutz, 1983). It is thus not surprising that oral contraceptives containing high doses of estrogen increase blastospore adhesion and subsequent high symptomatic infection rate.

Lactobacilli, through co-aggregation, hinder spore adhesion to the surface receptors on vaginal epithelium cells. In other words, reduction of the normal lactobacilli populations increases the chances of spore adhesion. Lactobacilli also produce bacteriocins that hinder mycelia germination (Narayanan & Tao, 1976). It has been reported that a low density of lactobacilli is common in vaginal exudate samples from patients with CVV. Exogenous factors promoting spore germination can trigger candidal vaginitis in women.

1.2.3.2. Epithelium invasion

Candida penetrates and invades vaginal epithelium cells upon development of mycelium. The invasion leads to release of prostaglandins and bradykinins that stimulate the inflammatory reactions in the invaded tissues. Edema, erythema,

exudate increase and cell shedding are consequences of the invasions (Ferrer, 2000). Also, elevated levels of spores in the vagina, which is proportional to high frequency of sexual penetration, may stimulate invasion and candidal colpitis (Foxman, 1990).

1.2.4. Treatment

Most patients with *C. albicans* respond to topical treatment with nystatin, polyenes and amphotericin B (Farah *et al.*, 2000). Amphotericin B as the preferred drug to treat fungal infections is complicated by severe side effects such as fevers, chills, headache, anorexia, nausea, vomiting, diarrhea, kidney damage and anemia (Bennett, 1996; Dupont, 1992).

Common methods of drug delivery include solutions, suspensions, gels, foams and tablets (Knuth *et al.*, 1993). These methods, though effective and widely used, are not without their disadvantages. For instance, creams and gels provide sufficient lubrication but tend to be messy and easily removed if water soluble, while suspensions and solutions spread unevenly in the vagina (Knuth *et al.*, 1993).

The drugs that are commonly used and currently available often display unwanted side effects, fail to combat infections by new or re-emerging fungi and may result in rapid development of resistant strains of pathogens. For instance, 33% of late-stage AIDS sufferers harbored resistant strains of *C. albicans* in their oral cavities (Law *et al.* 1994). Furthermore, the escalating costs of antibiotics limit access to treatment to a fraction of the world population, mostly in developed countries. For example, the supply of the UK-recommended daily dose of 200 mg of itraconazole solution to treat oral candidosis in AIDS patients cost around £53 per week in 1999 (Martin, 1999), translating into ZAR370. With the high rate of unemployment and poverty in poor rural communities, this figure makes this and other drugs unavailable to the majority of potential users. Structures of some of the commonly used antifungal drugs are displayed in Fig. 1.1.

Most of the anti-*Candida* drugs commonly used, with the exception of 5-flucytosine, target a major sterol of the fungal plasma membrane, ergosterol, which is vital for the fluidity and integrity of the membrane. Ergosterol is essential for effective activity of membrane-bound enzymes such as chitin synthetase (Joseph-Horne & Hollomon,

1997; Vanden Bossche *et al.*, 1987). The major classes of commercially available anti-*Candida* drugs are polyenes and azoles.

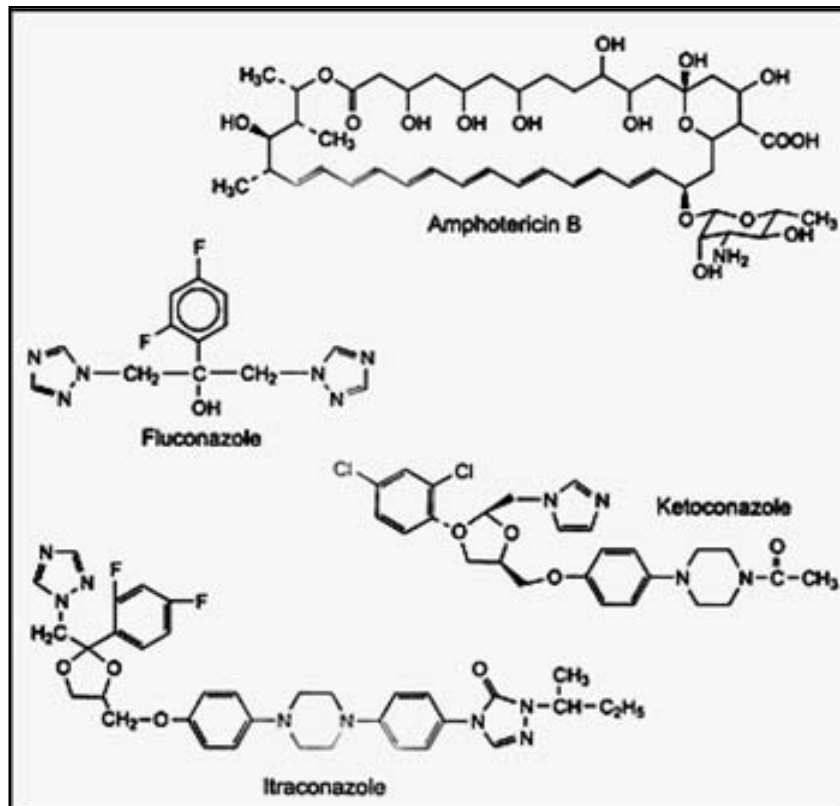


Figure 1.1. Structures of some of the antibiotics used to treat fungal infections in humans and animals (Lupetti *et al.*, 2002).

1.2.4.1. Polyenes

This class of antifungal compounds, which includes amphotericin B and nystatin, targets membranes containing ergosterol by a mechanism thought to involve the integration of the drugs into the membranes followed by formation of ion channels. These channels disturb the proton gradient across the membrane (Vanden Bossche *et al.*, 1994). Unfortunately, several species of yeast and molds possess primary resistance to polyenes (Dick *et al.*, 1985; Walsh *et al.*, 1990). Interestingly, several strains of amphotericin B resistant *C. albicans* have been isolated from HIV-infected patients who received prolonged treatment with azoles (Kelly *et al.*, 1997). Resistance to polyenes may result from reduced ergosterol content in the fungal cell membrane. Furthermore, resistance may occur from replacement, by fungi, of ergosterol with sterols with low affinity for polyenes (Rogers, 2002).

Amphotericin B, in many cases, results in nephrotoxicity which limits its use as an antifungal agent (Rogers, 2002). Nystatin, on the other hand, is restricted to topical administration due to its potential for toxicity.

1.2.4.2. 5-Flucytosine

5-Flucytosine (5-FC) is taken up into the fungal cell by a cytosine permease and deaminated into 5-fluorouracil (FU) by cytosine deaminase (Vanden Bossche *et al.*, 1994). FU is converted by cellular pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP), a specific inhibitor of thymidylate synthetase, and 5-fluoro-UTP (FUTP). Thymidylate synthetase is an essential enzyme for DNA synthesis (Vanden Bossche *et al.*, 1987). FUTP incorporates into RNA leading to disruption of protein synthesis. Primary resistance to 5-FC has been observed in some yeast species and molds. Flucytosine resistance in *C. albicans* and *Cryptococcus neoformans* may arise from mutational changes that lead to structural alterations in cytosine deaminase or uracilphosphoribosyltransferase (Rogers, 2002). Secondary resistance to 5-FC, triggered by resistance to other drugs, is not uncommon in patients on 5-FC therapy. It is for these reasons that 5-FC is not used singly but in combination with other drugs (Francis & Walsh, 1992; Schonebeck & Ansehn, 1973).

1.2.4.3. Azoles

Azoles are the largest class of antifungal agents utilized clinically (Vanden Bossche *et al.*, 1995). Azoles function by inhibiting cytochrome P-450-dependent 14 α -demethylation of lanosterol in the ergosterol biosynthetic pathway (Vanden Bossche *et al.*, 1995, Rogers, 2002). It appears the azole resistance by *Candida*, estimated in 21-32% of symptomatic patients, is enhanced by immunosuppression and previous exposure to oral azoles (White *et al.*, 1998). Azole resistance has been reported as the emerging major problem in patients treated for yeast infection (White *et al.*, 1998). The resistance to azoles by some *Candida* spp. results in part from the point mutations in the *ERG11* gene encoding lanosterol demethylase leading to diminished affinity of the enzyme to azoles (Sanglard *et al.*, 1998). Mutations that lead to overexpression of *ERG 11* and genes encoding enzymes acting both downstream and upstream of demethylase have been reported as contributing to increased resistance to azoles (Henry *et al.*, 2000). In addition, in some fungal species efflux systems that reduce intracellular amounts of azoles have been reported (Sanglard *et al.*, 1995; White, 1997).

Owing to the challenges highlighted in this chapter it would seem that the search for cheap, available and efficacious sources (mainly medicinal plant species) of treatment is becoming increasingly necessary (Ahmad & Beg, 2001). In rural areas of Africa, reliance on plant-derived remedies is high and is attributed to both economic and social factors (Aketch, 1992).

1.3. MEDICINAL PLANTS

1.3.1. Overview

Traditional knowledge to solve health problems of mankind and animals exists in all countries of the world (Rukangira, 2001). The first official recognition of traditional medicine as an important participant in primary health care was expressed in the World Health Organization's (WHO) Primary Health Care Declaration of Alma Ata (WHO, 1978). Traditional medicine, according to the Declaration, is "the sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing". African traditional medicine is one of the oldest health systems in the world, in which the healer treats the psychological basis of the disease before attempting to tackle the symptoms (Gurib-Fakim, 2006). The use of medicinal plants in most African countries is often associated with witchcraft and superstition, because people lacked the scientific insight to explain the curative action of plants (Gurib-Fakim, 2006).

The use of medicinal plants as a source of relief from diseases can be traced back over five millennia to written documents of early civilizations in China, India and the Near East (Solecki & Shanidar, 1975). Approximately 80% of the population in the so-called Third World and developing countries and an estimated 80% of South Africans rely fully or partly on plants for their medicinal needs (Farnsworth, 1988; Balick *et al.*, 1994). About 500 medicinal plant species or their products are commercialized (Hoareau and DaSilva, 1999). Furthermore, in South Africa and most parts of Africa indigenous plants are an integral part of the religious and cultural practices. Developed countries also use traditional medicines that involve the use of herbal drugs and remedies (Hoareau and DaSilva, 1999). Since the early 1980's there has been an increase in consumption of medicinal plants in Western Europe (Hamburger & Hostettmann, 1991).

In South Africa, many ethnic groups use several medicinal plant species for treatment of domestic animal and human diseases (Smith, 1895; Masika & Afolayan, 2002). The widespread usage of plant-based remedies in South Africa is estimated to involve about 147 plant families, and the most prominent in Zulu, Sotho and Xhosa ethnomedicine are the Fabaceae, Asteraceae, Euphorbiaceae, Rubiaceae and Orchidaceae families (Hutchings *et al.*, 1996). Roughly a third of the most frequently used indigenous plants are tree species, another third are herbaceous plants or shrubs whilst rhizomatous, succulent, leafy and bulbous plants complete the last third (van Wyk *et al.*, 1997).

Medicinal plants also play an important role in ethnoveterinary medicine. In many countries medicinal plants are used in the treatment and prevention of livestock diseases. The frequency of usage increases in many countries as a consequence of the escalating cost of livestock maintenance and emerging technologies in vaccine and drug production (Hoareau and DaSilva, 1999). For instance, in Mexico intestinal disorders in cows are treated with extracts of *Polakowskia tocacco* and dietary supplements in poultry feeds in Uganda are supplied through enrichment with *Amaranthus sp.* (Hoareau and DaSilva, 1999).

This reliance on plants as medicines warrants scientific validation of their safety, efficacy and the appropriate dosage of the plant material used (Masika & Afolayan, 2002). The relative appropriate dosage, side effects, toxicity and specific parts of the plant in mixtures or remedies have to be carefully considered, investigated and validated (Halberstein, 2005). For example, some of the plant species used for treating gynaecological complaints are toxic or contain known toxic compounds (Steenkamp, 2003). Large numbers of deaths are associated with toxic effects of extracts of *Erythroxylon coca* leaves (Winslow & Kroll, 1998). Furthermore, the prescription and administration of traditional medicine cocktails is currently not strictly regulated in South Africa (Fennell *et al.*, 2004), further increasing the potential for poisoning and other damages associated with prolonged exposure. For instance, Bodestein (1973) reported that many potentially toxic plants were available over the counter from traders and retailers without regulation. Poisoning usually is a result of misidentification of the plant species by the user or healer, incorrect preparation or over-dosage (Stewart & Steenkamp, 2000). Measures to reduce incidence of toxicity and fatalities resulting from poisoning will further facilitate the incorporation of traditional medicine practices into the formal health care systems.

With the growing acceptance of traditional medicine as an alternative form of health care, the screening of medicinal plants for active compounds is becoming increasingly important (Rabe & van Staden, 1997). The screening of plant extracts for antimicrobial activity has shown that a great number of these plants possess active compounds. The presence of antibacterial, antifungal and other biological activities has been confirmed in extracts of different plant species used in traditional medicine practices (Masoko *et al.*, 2005; Masoko and Eloff, 2005; McGaw *et al.*, 2000; Martini and Eloff, 1998; McGaw *et al.*, 2001). Katerere *et al.* (2003) reported that pentacyclic triterpenes isolated from some members of the African Combretaceae were active against *Staphylococcus aureus*, *C. albicans*, *Mycobacterium fortuitum* and *Escherichia coli*.

The elderly members of the several rural and tribal communities and trained practitioners in South Africa have expertise on the uses and preparations of plant remedies. In urban settlements traditional remedies are accessed from traditional practitioners at market places (Mabogo, 1990). Furthermore, knowledge on traditions of collecting, processing and applying plants and plant-based treatment preparations has been passed on through the generations (von Maydell, 1996). The part of the plant utilized in preparation of the medicines is determined by the nature and state of the disease being treated (Mabogo, 1990). Some herbalists though, claim that underground parts of the plants contain the highest concentration of potent healing agents (Shale *et al.*, 1999). The remedies are prepared in water since the traditional medical practitioners do not usually have access to more lipophilic extractants (Kelmanson *et al.*, 2000).

Medicinal plants are used as complex mixtures containing a broad range of chemical components in the form of infusions, tinctures or extracts (Balick and Cox, 1997). The preparations provide the patients with a complex mixture of natural metabolites that have smoother action, are better tolerated than synthetic drugs and, it is alleged, produce few allergic reactions (Lovkova *et al.*, 2001). Pure active compounds may also be isolated from plants and used for development of commercial drugs (Lovkova *et al.*, 2001).

Pure compounds are generally utilized in commercial pharmaceuticals because the active principles of the medicinal plants display potent specific activity and/or have a small therapeutic index, requiring accurate and reproducible dosage. Safety, toxicity, efficacy and quality are of concern. On the other hand, the chemical compositions of

each traditional preparation remain unknown to the healers and users; hence toxicity of medicinal plant preparations has been reported. For example, Steenkamp *et al.* (2000) reported that a number of poisonings and fatalities in two Johannesburg hospitals were due to patients having taken traditional medicine containing hepatotoxic pyrrolizidine alkaloids. It is clear that information about toxicity and efficacy should be relayed back to traditional healers.

The worrying emergence of antibiotic resistant strains of pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and valinomycin-resistant enterococci (VRE) seeks counter measures such as the development of new antimicrobial formulations (Tally, 1999).

The benefits of medicinal plants are attributed to combinations of secondary metabolic products present in the plants (Wink, 1999). These secondary products play diverse roles in the plants, such as a defensive role against herbivory and pathogen attack (Wink & Schimmer, 1999). They are also important in inter-plant competition and as attractants for beneficial effectors like pollinators and symbionts (Kaufman *et al.*, 1999; Wink and Schimmer, 1999). These secondary products may also have protective actions against deleterious effects of abiotic stresses associated with changes in temperature, water status, light levels, UV exposure and nutrient levels (Kaufman *et al.*, 1999).

Many phytomedicines exert their beneficial effects through additive or synergistic action of several chemical components acting at single or multiple target sites associated with physiological or pharmacological processes (Tyler 1999). The additive or synergistic actions of plant-based medicinal preparations (at multiple sites) not only ensure effectiveness against the invading pathogen, but also decrease the chances of the pathogen developing resistance or adaptive responses (Kaufman *et al.*, 1999; Wink, 1999).

1.3.2. Isolation of compounds from medicinal plant species

The process that is followed to isolate pharmacologically active, pure constituents from a plant involves a number of multidisciplinary steps, including 1) collection, proper botanical identification and drying of the plant material, 2) preparation of extracts and preliminary chromatographic analysis by TLC or HPLC, 3) biological and

pharmacological screening of the crude extracts, 4) chromatographic separation and bioassay-guided fractionation, 5) verification of purity of the isolated compounds, and 6) structure elucidation using spectroscopic and spectrometric techniques (Hamburger and Hostettmann, 1991). Subsequent steps may involve chemical synthesis of the compounds and their derivatives/analogues and large-scale isolation for biological assays (Hamburger and Hostettmann, 1991). *In vitro* activity frequently does not lead to *in vivo* activity for several reasons. Animal experiments to prove effectivity also require that the toxicity of the isolated compounds or extracts should be determined. In many published studies these were not addressed.

The decision to investigate a certain species for activity is not without its difficulties. A researcher usually makes this decision based on one or more of the following criteria; chemotaxonomic criteria, information from traditional medicine, field observations and random collection (Hostettmann *et al.*, 2000). The chemotaxonomic method is based on the observations that some chemical constituents are often specific to a given botanical family, to a genus or species. For instance if one chemical constituent was discovered from one plant species and shown to possess therapeutic properties, it is not surprising that a strong belief about the existence of this constituent or similar constituents in plants of the same genus or belonging to the same family arises (Hostettmann *et al.*, 2000). For example, combretastatins were initially isolated from *Combretum caffrum* and later found in other members of the Combretaceae (Famakin, 2002).

Plant species that grow in hostile environments are exposed to hazards that include fungal infections, parasitic infestations and insect damage. Such plant species frequently produce secondary metabolites that help in defense against these hazards (Hostettmann *et al.*, 2000). A plant species may be selected based on these field observations. For example, if leaves of a particular plant species show no signs of fungal infection in an environment where most species are infected, it may mean that this particular species contains fungicidal constituents. A yellow layer under the bark of a tree is indicative of antifungal polyphenols (Hostettmann *et al.*, 2000). African plant species are good candidates for isolation of fungicides as they thrive under difficult conditions coupled with existence of fungal and parasitic infections (Hostettmann *et al.*, 2000).

Plants used in traditional medicine are likely to yield pharmacologically and biologically active compounds. For instance, in the field of anticancer activity a direct

correlation between biological activity and use in traditional medicine has been proven (Hamburger and Hostettmann, 1991). More than 35 000 plant species were randomly selected and screened *in vitro* and later *in vivo* biological studies were carried out at the National Cancer Institute (NCI) in US from 1960 to 1981. Taxol, camptothecin and calanolide A were discovered in this program, as well as other compounds that were not effective in human studies (Wall & Wani, 1996). It has been estimated that 74% of the known pharmacologically active, plant-derived components were discovered after the ethnomedical uses of the plants started to be investigated (Farnsworth and Soejarto, 1991; Sheldon *et al.*, 1997). These and other literature reports indicate the potential of plants as sources of new efficacious drugs, hence the importance of screening plants for biological activity.

1.3.3. Some compounds isolated from plants

1.3.3.1. Phenolic compounds

Plants contain an abundance of phenolic compounds. These compounds include flavonoids, flavones, catechols, anthocyanins and polymeric phenolic compounds. Phenolic compounds account for pharmacological and therapeutic effects of many medicinal plant species (Lovkova *et al.*, 1990). Flavonoids have antioxidant activity which protects cells and tissue from injurious effects of free radicals (Sigh *et al.*, 2005). The compounds 2,3-digalloyl-4-(E)-caffeoyl-L-threonine and kaempferol 3-O- α -L-rhamnoside have been isolated from the leaves of *Cornus controversa*, a member of the family Cornaceae (Lee *et al.*, 2000).

1.3.3.2. Terpenoids

Terpenoids are a major class of physiologically active substances that includes more than 10 000 compounds. They also contribute tremendously to the pharmacological activity of many medicinal plants. Depending on the number of isoprenoid groups (C_5H_8), monoterpenes, sesquiterpenes, diterpenes, tetraterpenes and polyterpenes are found in plants (Paseshnichenko, 1987; McGarvey & Croteau, 1995). In plants triterpenoids act as attractants for pollinators and seed dispensers, competitive phytotoxins, herbivore repellents and toxins (Harborne, 1991). They also function as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids) and electron carriers (ubiquinone, plastoquinone) (McGarvey & Croteau, 1995).

Katerere *et al.* (2003) isolated pentacyclic triterpenes from members of the African Combretaceae, and demonstrated that these compounds had antimicrobial activity against *Candida albicans*, *Escherichia coli*, *Mycobacterium fortuitum*, *Proteus vulgaris* and *Staphylococcus aureus*. Betulinic acid, platonic acid and oleanolic acid isolated from leaves of *Syzigium claviform* had anti-HIV activity in the H9 lymphocyte cell line (Fujoka & Kashiwada, 1994). Triterpenes and their derivatives display activities such as cytotoxicity to tumor cell lines *in vitro* through the induction of apoptosis (Lee *et al.*, 1989).

1.3.3.3. Essential oils

Essential oils are complex mixtures of fragrant volatile substances, monoterpenes, sesquiterpenes, aromatic compounds and their derivatives. Bactericidal, astringent and anti-inflammatory effects of some plant species have been linked to presence of essential oils (Lovkova *et al.*, 2001).

1.3.4. Ethnobotany and drug discovery

Substances derived from plants or their model derivatives constitute over 25% of all prescribed commercial drugs (Farnsworth, 1988; Fabricant & Fansworth, 2001). Some of the plant-derived commercial drugs are displayed in Table 1.1. Indigenous knowledge systems provide leads that largely guide the discovery of these drugs. Plant-derived drugs are of three types, namely 1) unmodified natural products where the ethnomedical use suggested clinical efficacy, 2) unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use and, 3) synthetic substances based on a natural product from a plant used in traditional medicine (Cox 1994; Balunas & Kinghorn, 2005).

Investigation of *Catharanthus roseus* led to the isolation of the bis-indole alkaloids vincristine and vinblastine, which have been developed as commercial drugs for treatment of cancers (Noble *et al.*, 1958; Neuss *et al.*, 1964). The dimeric sesquiterpene gossypol, which occurs in seeds of *Gossypium* species, has shown contraceptive activity on human males with 99.89% efficacy (Pei-gen and Nai-gong, 1991). Following past success in finding useful therapeutic drugs from plants, it is important to screen medicinal plants for more potentially useful compounds with varying pharmacological activities.

Diseases resulting from infections are probably generally easy to diagnose by traditional healers and herbalists. Healers have thus selected plant species to use as therapies against some of the easily diagnosed infections, including bacterial and fungal infections. Following leads from traditional healers may increase the chances of finding plant species with substantial antimicrobial activities. Furthermore, the number of higher plant species (angiosperms and gymnosperms) on earth is estimated at 215 000 (Ayensu and DeFillipps, 1978; Cronquist, 1981). Only 6% of the species has been screened for biological activity and an estimated 15% investigated phytochemically (Verpoorte, 2000), further justifying intensive screening of plants for pharmacological activity.

1.3.5. Determination of biological activities of medicinal plants

Investigation of antimicrobial activities of plant extracts and isolated compounds involves exposure of a known pathogen to different concentrations of the extracts or isolated compounds in order to observe inhibition of growth. Plants have great potential as sources of future drugs due to their antimicrobial properties (Hostettmann *et al.*, 2000). Some secondary metabolites are produced by the plant in response to and in defense against herbivores and interplant competition, whilst others are produced as attractants for pollinators and symbionts (Kaufmann *et al.*, 1999; Wink and Schimmer, 1999).

Exposure of a known human or animal pathogen to plant extracts or isolated compounds may result in inhibition of growth or death of the target pathogen (Hamburger & Hostettman, 1991; Hamburger & Cordell, 1987). The method of exposure must be rapid, inexpensive, reproducible, simple and sensitive (Do & Barnard, 2004).

The assays used for antifungal activity are quite similar to those used in antibacterial assay techniques. Diffusion of the compound or extract in agar in a Petri dish requires no sophisticated equipment and has been the method of choice in many studies. Though the technique is commonly used for most antibiotics, examining plant extracts containing unknown compounds using this method is associated with problems. Eloff (1998b) writes that the assay is affected by agar type, salt concentration, incubation temperature and the molecular size of the test compound(s). The serial microplate dilution method allows for testing of relatively

large numbers of extracts simultaneously and the determination of the minimal inhibitory concentration (MIC) value (Eloff, 1998b).

Bioautography combines TLC with a bioassay *in situ* and allows the localization of the active compounds in a complex sample (extract). Bioautography is regarded as the most efficient method for activity-guided separation of components (Hostettmann *et al.*, 2000). Cultures of bacteria or fungi are sprayed on developed TLC plates and incubated for a specified duration in humid chambers. Zones of inhibition are visualized by the detection of dehydrogenase activity with a tetrazolium salt. A reference chromatogram stained with a suitable reagent provides information about the nature of the active component, such as relative polarity.

Table 1.1.: Examples of prescription drugs discovered from ethnobotanical leads (Fabricant & Fansworth, 2001).

DRUG	MEDICAL USE	PLANT SOURCE
Aspirin	Analgesic, antiinflammatory	<i>Filipendula ulmara</i>
Cocaine	Ophthalmic, anaesthetic	<i>Erythoxylum coca</i>
Morphine	Analgesic	<i>Papaver somniferum</i>
Quinine	Malaria prophylaxis	<i>Cinchona pubescens</i>
Vinblastine	Hodgkin's disease	<i>Catharanthus roseus</i>
Vincristine	Leukemia	<i>Catharanthus roseus</i>
Colchicine	Gout	<i>Colchicium autumnale</i>
Emetine	Amoebic dysentery	<i>Psychotria ipecacuanha</i>
Eugenol	Toothache	<i>Syzygium aromaticum</i>
Scopolamine	Motion sickness	<i>Datura stramonium</i>

The serial microplate dilution method (Eloff, 1998b) provides information about the efficacy of the extract or compound. The minimal inhibitory concentration (MIC) is taken as the lowest concentration of the extract or compound that results in inhibition of growth of the test microorganism. The activity of the dehydrogenase in viable and non-viable cells is assayed using tetrazolium salts. Some of the recommended approaches to investigating antimicrobial activities in plant extracts are shown in Table 1.2. Though plant-derived remedies may contain compounds of biological importance, the toxicity of these mixtures must be tested to improve biosafety of medicines.

Table 1.2.: Simple bioassays for phytochemical laboratories (Hamburger & Hostettman, 1991).

Activity	Target
Antibacterial activity	Human/animal pathogenic bacteria (e.g. <i>E. coli</i> , <i>S. aureus</i> , <i>E. faecalis</i> , <i>P. aeruginosa</i>) exposed to known concentrations of extract or compound.
Antifungal activity	Human/animal pathogenic fungi and yeasts (e.g. <i>C. albicans</i> , <i>Aspergillus spp</i>) exposed to known concentrations of plant extract or compound.
Brine shrimp toxicity	<i>Artemia salina</i>

Registration of medicines in South Africa is regulated by Act 101 of 1965 (Medicines and Related Substances Act). The Act stipulates that all medicines should be safe (non-toxic), efficacious and of good quality. Some plants used as food or medicines are potentially toxic, mutagenic and carcinogenic (Higashimoto *et al.*, 1993; Kassie *et al.*, 1996; Schimmer *et al.*, 1988). From several investigations it would appear that a clear link between herbal medicines and toxic incidents applied to less than 0.1% toxicity cases (Wolpert, 2001). Misidentification of the plant species, poor quality of the preparations, prolonged usage and addition of toxic substances (from Western pharmaceuticals) to plant derived remedies contribute significantly to the toxicity associated with plant extracts (Wolpert, 2001; Fennell *et al.*, 2004; Stewart & Steenkamp, 2000). The testing of the toxicity of plant extracts and the isolated compounds involves exposure of the cells (human, mammalian) to specified concentrations of the test substance or mixture. After incubation for specific periods the cell viability is determined using various methods (Mosmann, 1983).

1.4. PLANT SPECIES USED IN THE STUDY

During a broad random screening initiated in the Phytomedicine Programme to investigate the biological activity of leaves of a range of tree species (unpublished data), extracts of a number of tree leaves exhibited high antifungal activity. Using criteria such as antifungal activity, recorded medicinal uses, availability of plant material and research previously published on each species, seven species were selected for anti-*Candida* studies. Most of the trees selected are traditionally used to treat ailments associated with microbes.

1.4.1. *Curtisia dentata* (Burm. f) C.A. Sm.

Curtisia dentata (Assegai tree, modula-tswene in Pedi, umLahleni in Zulu and Xhosa, mufhefhera in Venda), is a medium to tall evergreen tree with smooth grey bark and simple egg-shaped leaves with pointed tips and coarsely toothed edges. The flowers are inconspicuous, small, drab, cream coloured and appear in spring-summer (October to March). The flowers are often infested with parasites and thus most do not produce viable seeds. The small round fruits appear 6-10 months after flowering and contain a four-chambered nut, with a seed in each chamber (van Wyk *et al.*, 1997). Fig. 1.2 shows the leaves and stem of *Curtisia dentata*.

Curtisia is a genus of only one species that belongs to the Cornaceae (dogwood family). The Cornaceae is a family of 15 genera of which the most well known is *Cornus* (the decorative dogwoods). Interestingly, *Curtisia* is the only southern African member of the Cornaceae family. *Curtisia* was named in honour of William Curtis (1746-1799) who was the founder of Curtis's Botanical Magazine in 1786. The species name *dentata* is a Latin word for toothed, and refers to the toothed leaves.

The assegai tree is widely distributed in most forests in southern Africa and Swaziland. It is found in forest patches of Western Cape, Knysna, Eastern Cape, KwaZulu-Natal, Mpumalanga and Limpopo provinces of South Africa on grassy mountain slopes and in coastal forests where it appears as a small bushy tree.

The reddish timber of the assegai tree is strong and durable. It has been so severely exploited since colonial years that well-grown mature trees are uncommon. In KwaZulu-Natal *C. dentata* is perceived as vulnerable and declining, hence its classification as a protected species (Cunningham, 1988; Scott-Shaw, 1999). The timber was used by Voortrekkers to make axles for their wagons, tool handles, furniture, rafters and flooring. The bark, twigs and leaves were used for tanning leather. In traditional medicine the bark is used to treat stomach complaints and diarrhea (Pujol, 2000). The bark is also used as an aphrodisiac and blood purifier by local cultures. It is used in the Eastern Cape for the treatment of heartwater in cattle (Dold & Cocks, 2001). Bark infusions are used for treatment of pimples (Grierson & Afolayan, 1999). The traditional medical practitioners use this species in special mixtures because it is scarce (Cunningham, 1988).

Other members of the family Cornaceae have been used in different parts of the world for various purposes. Fruits of *Cornus officinalis* have been used as tonics in traditional medicine in Japan and China (Okuda *et al.*, 1984; Hatano *et al.*, 1989). *Cornus controversa* has been used as an astringent in Korea and China (Lee, 1993)

Publications reporting the phytochemical and pharmacological investigations on

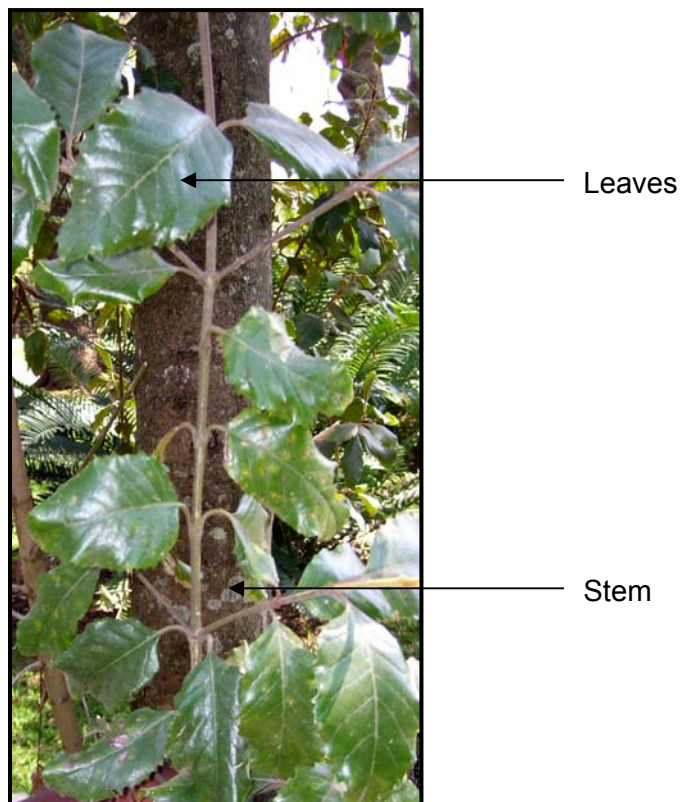


Fig. 1.2. The leaves and stem of *Curtisia dentata* (Photographed at the National Botanical Garden, Mpumalanga, South Africa in June 2007).

Curtisia dentata are scarce. Other members of the family Cornaceae have been subjected to phytochemical and pharmacological studies. Various flavonoids, phenolic compounds and terpenoids have been isolated from *Cornus controversa* (Jang *et al.*, 1998; Nakaoki & Moira, 1958; Lee *et al.*, 2000).

1.4.2. *Trichilia emetica* Vahl

Trichilia emetica or Natal mahogany (also called “umkhuhlu” in Zulu, “mmaba” in Sepedi) belongs to the Meliaceae family which is widespread in tropical and subtropical regions of Africa. *T. emetica* is a poorly developed shrub, or a small tree of about 10 m. The flowers are green, small and 3-8 cm thick. The fruits are velvety

capsules, globular, crimson or red at maturity and contain black seeds (Cronquist, 1981; Burkill, 1997). *T. emetica* is widely used in tropical Africa to treat various diseases. The flowers, leaves and fruits of *Trichilia emetica* are shown in Fig. 1.3.

Malian traditional medical practitioners use this plant species to treat hepatic diseases. It is also used as a purgative, an antiepileptic, antipyretic and antimalarial agent (Iwu, 1993). Other specified conditions treated with extracts of the plant species include colds and bronchial inflammations (Kokwaro, 1976; Malgras, 1992), jaundice and worms in the intestine (Aké Assi and Guinko, 1991) and skin diseases (Oliver-Bever, 1986).

McGaw *et al.* (1997) reported that an ethanolic extract of *T. emetica* leaves inhibits cyclooxygenase and thus has anti-inflammatory activity. Antiplasmodial (El-Tahir *et al.*, 1999; Traore-Keita *et al.*, 2000) and antischistosomiasis activities (Sparg *et al.*, 2000) have also been demonstrated in extracts of *T. emetica*. Ethyl ether extracts of the roots showed activity against several bacterial species (Germanò *et al.*, 2005).

Several types of limonoids have been isolated from *T. emetica* (Nakatani *et al.*, 1981). Nymania 1, Tr-B, drageana 4, trichilin A, rohituka 3 and seco-A-protoliminoid were isolated from *T. emetica* stem bark extracts (Gunatilaka *et al.*, 1998). The water infusion of the bark contains a considerable amount of tannins. The bitter taste of the stem and root has been attributed to calicedrin-like compounds (Burkill, 1997).

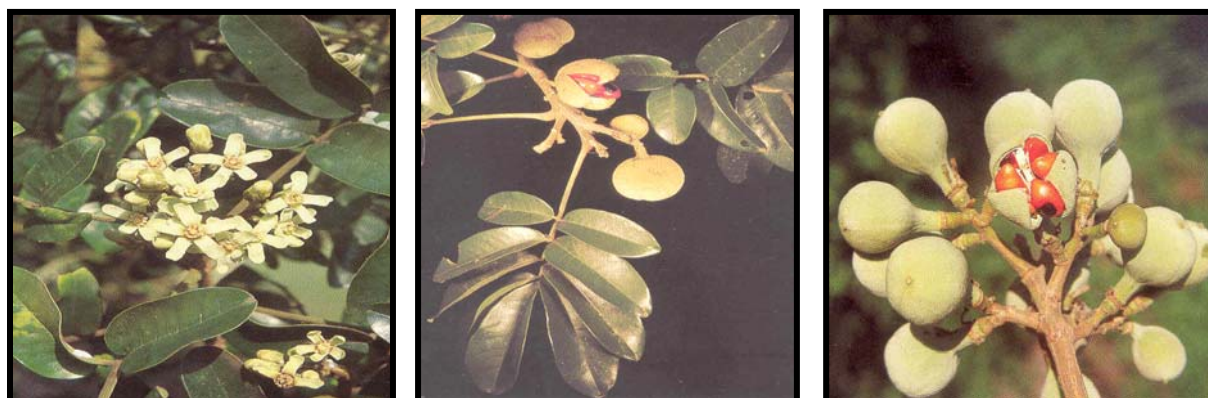


Fig. 1.3. *Trichilia emetica* flowers, leaves and fruits (van Wyk *et al.*, 1997).

1.4.3. *Kigelia africana* (Lam.) Benth

Kigelia africana, also called modukguhlu (Sepedi), muvevha (Venda), sausage tree or worsboom (Afrikaans) is widely distributed in the northern and northeastern parts of South Africa and further north in tropical Africa (Palmer & Pitman, 1972). *K. africana* is a large rounded tree with a thick trunk and smooth grey bark (Palmer & Pitman, 1972; Coates Palgrave, 1977). The flowers, though attractive, possess an unpleasant smell. The greyish-brown sausage-shaped fruits are up to 1 m in length and contain fibrous pulps with numerous seeds (Fig. 1.4).

K. africana is used to treat dysentery, haemorrhoids, constipation, wounds, ulcers, boils, abscesses, rheumatism, syphilis and gonorrhoea (Palmer & Pitman, 1972; Watt & Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). The fruits, and bark to a lesser extent, are used in medicinal preparations (Watt & Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996).

The naphthoquinone lapachol and the dihydroisocoumarin kigelin are major components of both the roots and bark of *K. pinnata* (Purushotaman & Natarajan, 1974; Govindachari *et al.*, 1971). Kigelinone, pinnatal, isopinnatal, stigmaterol and β -sitosterol have been isolated from the bark of *K. pinnata* (Dictionary of Natural Products, 1996). Antimicrobial activity has been demonstrated using bark extracts and kigelin and related compounds are presumed to account for the observed activity (Akunyili *et al.*, 1991).



Fig 1.4. *Kigelia africana* tree, flowers and fruit (van Wyk *et al.*, 1997).

1.4.4. *Cussonia zuluensis* Strey

Cussonia zuluensis, also called Zulu cabbage tree, belongs to the family Araliaceae. *C. zuluensis* is a small, several stemmed plant that grows up to 4 m in height (Coates Palgrave, 1977). The greyish-green bark is smooth to flaking. The leaves are multi-digitated. The flowers are greenish yellow, while the resulting fruits are goblet-shaped, pale purplish and closely crowded along the axes. The fruits appear around November – April period (Coates Palgrave, 1977).

The medicinal uses of the species have not been well documented. Other members of the genus *Cussonia* are used for treatment of various diseases. The root infusions of *C. spicata* are used in Zulu traditional medicine as emetics for fever and nausea, while in Lesotho they are used for treatment of venereal diseases (Hutchings *et al.*, 1996). The vhaVenda tribe of Limpopo province and some Zimbabwean cultures use the bark extracts to treat malaria. The roots are also used as diuretics, laxatives and for weaning of infants (Hutchings *et al.*, 1996). The leaves of *C. umbellifera* are used in traditional medicine to treat rheumatism, colic and insanity in unspecified parts of southern Africa (Watt & Breyer-Brandwijk, 1962).

From some members of the genus *Cussonia* molluscidal active saponins have been observed in stem bark extracts (Gunzinger *et al.*, 1986). Anthocyanins, tannins and alkaloids have been isolated from the root bark of *C. spicata* (Chhabra *et al.*, 1987). Triterpenoid saponins and other triterpenoid compounds are present in the family (Cronquist, 1981).

1.4.5. *Terminalia phanerophlebia* Engl. & Diels.

Terminalia phanerophlebia, also known as umkhonono in Zulu, is a medium size tree that grows up to 6 m in height. It is found in low altitudes in bushvelds, often on rocky hillsides or along rocky watercourses (Coates Palgrave, 1977). It belongs to the family Combretaceae. The bark is dark grey, fissured with ridges splitting and joining. The leaves are light green, broadly obovate with distinct net veins (Fig. 1.5). The flowers are white or cream in colour, sometimes tinged with pink and appear during the October – February period. The fruits are greenish yellow to dull pinkish or reddish brown, appearing during the January – June period (Coates Palgrave, 1977).

In Zulu traditional medicine *T. phanerophlebia* is used to treat diseases collectively called “*amanxeba*”, which refers to diseases associated with witchcraft and manifesting through pain of the chest, neck or shoulders (Gerstner, 1941). These diseases, according to Zulu beliefs, culminate in coughing as in tuberculosis and rheumatism (Watt & Breyer-Brandwijk, 1962). The vhaVenda tribe uses the plant in wound healing, to treat menorrhagia, diarrhea and venereal diseases (Mabogo, 1990). In Botswana and Zimbabwe the roots are used to treat gastric disorders, gynaecological complaints, venereal diseases, general weakness, sore throats and nosebleeds (Gelfand *et al.*, 1985; Hedberg & Staugard, 1989). The root decoctions are used to treat diarrhea, for colic relief and to control schistosomiasis (Kokwaro, 1996). In the Sotho cultures root powders of *T. sericea* are mixed with mealie meal to treat diabetes (Watt & Breyer-Brandwijk, 1962).

The isolation of compounds from *T. phanerophlebia* is not well documented. However, several compounds have been isolated from its close relative, *T. sericea*. Triterpenoids, seriac acid and sericoside and hydroxystilbene glycoside have been isolated from roots of plants from Mozambique (Bombardelli *et al.*, 1974).



Fig. 1.5. *Terminalia phanerophlebia* leaves. Photographed at the Lowveld National Botanical Garden in Nelspruit, Mpumalanga, South Africa, in October 2006.

1.4.6. *Terminalia sambesiaca* Engl. & Diels.

Terminalia sambesiaca, a member of the Combretaceae family, is a small to large tree of about 4-5 m in height found predominantly at low altitudes in riverine fringes and occasionally on rocky hillsides (Coates Palgrave, 1977). The grey bark contains dark patches, giving it a mottled appearance. The stem is roughish in large specimens. The leaves, crowded at the ends of the branches, are elliptic to broadly obovate and have a thin texture. The flowers are creamy white, sometimes tinged with pink and release an unpleasant smell. These flowers occur during the December – January period. The fruits are elliptic, green flushed with pink and are present during the January – May period (Coates Palgrave, 1977).

Information in literature about the pharmacological and phytochemical studies of the species is not well documented. The methanolic extract of *T. sambesiaca* roots showed activity against several test microorganisms including *C. albicans* (Fyhrquist *et al.*, 2002). From other members of the genus *Terminalia*, potential toxins such as hydrocyanic acid, saponins, shikimic acid and tannic acid have been isolated (Duke, 1985). Antibacterial activity, anti-inflammatory activity and reverse transcriptase inhibition have been demonstrated with several other members of the genus (Iwu & Anyanwu, 1982; Kusumoto *et al.*, 1992).



1.4.7. *Vepris reflexa* I. Verd

Vepris reflexa, also called rock white-iron wood, belongs to the family Rutaceae. It is a shrub or medium dense tree up to 6 m in height and occurs in dry deciduous woodlands, forests or on rocky hillsides near rivers (Coates Palgrave, 1977). The bark is grey to dark grey and smooth. The leaves are shiny green, leathery, conspicuously dotted and aromatic when crushed. The flowers, which appear during the July – December period, are small and greenish yellow in colour. The fruits are smooth, fleshy and ellipsoid, appearing during the September – February period (Coates Palgrave, 1977).

Members of the genus *Vepris* are used in the Zulu culture to offer protection against ‘*umkhovu*’, spirits associated with sorcery (Gerstner, 1938). The root powders are used to treat colic and influenza by the Zulu tribe (Watt & Breyer-Brandwijk, 1962). Fruits are used as an adulterant, to treat gonorrhoea and bronchitis (Watt & Breyer-Brandwijk, 1962). Plant decoctions are used as astringents and to treat amenorrhoea in Mauritius (Gurib-Fakim *et al.*, 1993).

Antibacterially active quinolone alkaloids like veprisinium salt are widespread within the genus *Vepris* (Ayafor *et al.*, 1982). The bark of *Vepris* species contains a substantial amount (about 2%) of tannins (Watt & Breyer-Brandwijk, 1962).

The plant species used in the study are summarized in Table 1.3 with their medicinal uses and authorities.



Table 1.3. Summary of medicinal plants used in the study, their medicinal uses and families.

Plant species	Family	Medicinal Uses	Reference
<i>Curtisia dentata</i> (Burm.f) C.A. Sm.	Cornaceae	Stomach ailments including diarrhea, aphrodisiac, blood strengthener and heartwater in cattle.	Pujol, 1990 Dold & Cocks, 2001
<i>Trichilia emetica</i> Vahl	Meliaceae	Wound healing, cirrhosis, ascaris stomachaches, dysentery, kidney problems, indigestion, dysmenorrhoea, gonorrhoea, syphilis, parasites and rheumatism.	Watt & Breyer-Brandwijk, 1962; Hutchings <i>et al.</i> , 1996; Pujol, 1990.
<i>Kigelia africana</i> (Lam.) Benth	Bignoniaceae	Purgative, constipation, dysentery, wounds, ulcers, abscesses, rheumatism, syphilis and gonorrhoea	Watt & Breyer-Brandwijk, 1962
<i>Terminalia sambesiaca</i>	Combretaceae	Combretaceae used to treat backache, bilharzias, dysmenorrhoea, earache, pneumonia, syphilis, hookworm.	Watt & Breyer-Brandwijk, 1962; Gerstner, 1938
<i>Vepris reflexa</i> I. Verd	Rutaceae	No use reported in literature. Members of the genus <i>Vepris</i> used as protective charm against sorcery, to treat colic and influenza, gonorrhoea and bronchitis.	Watt & Breyer-Brandwijk, 1962
<i>Terminalia phanerophlebia</i> Engl. & Diels	Combretaceae	Treatment of schistosomiasis. Also those listed for <i>T. sambesiaca</i>	Sparg <i>et al.</i> , 2000; Watt & Breyer-Brandwijk, 1962
<i>Cussonia zuluensis</i> Strey	Araliaceae	None recorded. <i>C. spicata</i> used for spasm, cramps, malaria.	Roberts, 1990, Mabogo, 1990

1.4.8. Summary and problem statements

- Candidosis in animals and humans is a major health problem. Fungal infections, particularly opportunistic ones accompanying HIV infections, remain of concern in sub-Saharan Africa and the rest of the world.

- Acquired resistance by pathogens to licensed commercial antibiotics makes many infections difficult to treat and almost impossible to eradicate in some patients (Van der Waaij, 1987).
- Most of the registered antibiotics display unwanted side effects such as skin rashes, gastrointestinal symptoms and thrush. These side effects are tolerated because “benefits outweigh toxic effects” (Dancer, 2004)
- Traditional medicine, though fairly popular in South Africa, does not enjoy similar recognition as Western medicine. This is largely due to the practice being perceived as inferior and primitive.
- Data concerning the efficacy, dosage, side effects and toxicity about traditional remedies in South Africa is not well documented.

1.5. HYPOTHESIS

In view of the alarming emergence of drug-resistant pathogens and high cost of medicine, medicinal plants with excellent antifungal activity may be useful alternative sources of new antimicrobial agents in the form of pure compounds or crude extracts. The long-standing use of medicinal plants by local southern African cultures indicates that they (plants) are likely to contain antimicrobial compounds that are effective *in vivo*.

1.6. AIM OF THE STUDY

The aim of this study was to isolate and characterize compounds active against *C. albicans* from leaves of the most promising tree species and investigate the potential use of these compounds or extracts against *C. albicans* infections.

1.7. OBJECTIVES OF THE STUDY

The objectives of the study were to

- Screen extracts of plant species selected from a database for antifungal activity using the serial microplate dilution method and bioautography.
- Select the most promising plant species based on activity against *C. albicans* and number of antifungal compounds present in extracts.



- Isolate compounds active against *Candida albicans* and characterize them using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).
- Investigate the biological activity of extracts and isolated compounds against organisms.
- Investigate the *in vitro* cytotoxicity of isolated antifungal compounds.
- Investigate the efficacy of the isolated compounds or crude extracts in an animal model.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. REVIEW OF GENERAL METHODS

2.1.1. Selection of plant species

The Phytomedicine Programme, University of Pretoria (UPPP) conducts biological activity investigations on plant species collected randomly or through ethnomedical leads. Of the more than 350 species collected thus far, many species have shown promising activity, with MIC values of crude extracts ranging from 0.08 – 1.0 mg/ml against *Candida albicans*. Seven of these species were selected for further focused investigations of antifungal and antibacterial activity based on antifungal activity, traditional use, scientific research carried out to date and availability. After further work on the seven selected species *Curtisia dentata* was selected for in depth investigation.

2.1.2. Extraction

Investigation of the chemistry and activity of medicinal plants requires, among other things, the extraction of plant material with a solvent, testing the activity of the extract against known biological parameters and isolation of active components. The choice of the extractant is dependent on the purpose of the extraction (Eloff, 1998a). In a comparative study of different extractants, Eloff (1998a) concluded that acetone was the best solvent for extraction for screening of plants for activity. Acetone is volatile, miscible with polar and non-polar solvents and is also less toxic to test organisms (Eloff, 1998a). Extraction often involves the soaking of powdered material in a solvent, vigorous shaking, filtering and concentration of the extract by evaporation of the extractant.

2.2. PROCESSING OF PLANT MATERIAL

2.2.1. Plant collection and storage

Leaves and stem bark of *Curtisia dentata* were collected at the University of Pretoria's Botanical Gardens in Hatfield, Pretoria, during February-March 2005. Leaves of all other plant species were collected from the Lowveld Botanical Garden in Nelspruit, Mpumalanga, during November 2004. The leaves were collected in loosely woven orange bags and dried in the dark at room temperature. The dried leaves were ground to powder using a Macasalab mill (Model 200 Lab). The leaf powders were stored in closed bottles at room temperature in the dark until needed.

2.2.2. Preliminary extraction for screening

Powders of plant material (1g) were extracted with 10 ml of acetone or hexane or dichloromethane (using different plant samples) on a shaking machine for 30 min at room temperature. After centrifuging the mixture at 4 000 x g for 10 min the supernatant was dried in a pre-weighed beaker under a stream of air and the mass of the extract determined. Concentrations of 10 mg/ml were prepared in acetone for biological activity assays.

2.2.3. Serial exhaustive extraction

In a bulk extraction, powdered leaves (830 g) of *Curtisia dentata* were serially extracted with hexane, dichloromethane, chloroform, acetone and methanol (in the order mentioned). The dry material was extracted (three times) with 2 L of *n*-hexane on a shaking machine for 2 hours at room temperature. The filtrate was collected by passing the mixture through Whatman No.1 filter paper using a Büchner funnel. The filtrate was concentrated under reduced pressure using a Büchi rotavapor at temperatures not exceeding 50°C. The dried residue was further extracted three times with dichloromethane. The procedure was repeated with acetone and methanol (in the order listed). The dried extract residues were weighed before analysis was carried out on thin layer chromatography (TLC) plates.

2.2.4. Thin layer chromatography (TLC)

After extraction, solutions of 10 mg/ml of each extract were prepared in acetone. A sonicator was used to enhance the solubility of the extracts. For each extract, 10 μ l aliquots (100 μ g) were loaded on aluminium-backed TLC plates (Merck Silica F₂₅₄ plates) and developed in various mobile phases of varying polarities. Benzene: ethanol (9:1) [BEA] was used as a relatively non-polar mobile phase with 1% ammonium chloride added to minimize streaking of basic compounds. A moderate polarity eluent used was chloroform: ethyl acetate: formic acid (5:4:1) [CEF]. Polar/neutral ethyl acetate: methanol: water (40:5.4:5) [EMW] mixture was also used (Kotze & Eloff, 2002). Plates were visualized under ultraviolet (UV) light at 254 nm and 356 nm (CAMAG universal UV lamp) and visible spots were circled. For visualization of non-fluorescing spots plates were sprayed with vanillin-sulphuric acid mixture (0.1 g vanillin dissolved in 28 ml methanol and mixed with 1 ml sulphuric acid) and heated at 100°C for 5 min. Plates were scanned using HP Scanjet 5470c scanner immediately after heating to record the chromatograms.

2.3. BIOLOGICAL ACTIVITY METHODS

The 10 mg/ml preparations were subjected to a series of biological activity assays to investigate the presence of bioactive compounds in the extracts against different fungi and bacteria. The extracts were screened for activity using bioautography on TLC plates and determination of minimal inhibitory concentrations (MIC) was performed using a serial microplate dilution method to determine activity against selected microorganisms (Eloff, 1998b).

2.3.1. Fungal cultures

Fungi were cultured at 30°C in universal bottles as slants in Sabouraud dextrose agar (65 g dissolved in 1 L distilled water and sterilized by autoclaving at 121°C for 30 min) (Sigma, Steinheim, Germany). Using sterile cotton swabs the colonies were inoculated into Sabouraud dextrose broth (Sigma, Germany) (30 g dissolved in 1 L distilled water and sterilized by autoclaving at 121°C for 30 min) prior to bioactivity assay procedures. Some of the fungal cultures were stored in Sabouraud dextrose agar slants at 4°C to prevent overgrowing and morphological changes. Densities of fungal cultures before antimicrobial activity testing were as follows: *Candida albicans*, 2.5×10^6 cfu/ml; *Sporothrix schenckii*, 1.4×10^5 cfu/ml; *Cryptococcus neoformans*,

2.6×10^6 cfu/ml; *Aspergillus fumigatus*, 8.1×10^6 cfu; *Microsporum canis*, 1.5×10^5 cfu/ml.

2.3.2. Bacterial cultures

Bacterial test organisms used in screening tests were *Staphylococcus aureus* (Gram-positive) (American Type Culture Collection [ATCC] number 29213), *Enterococcus faecalis* (Gram-positive) (ATCC 29212), *Pseudomonas aeruginosa* (Gram-negative) (ATCC 27853) and *Escherichia coli* (Gram-negative) (ATCC 25922). These species are considered the most important nosocomial pathogens (NCCLS, 1992). Bacterial cells were maintained at 4°C on Müller-Hilton (MH) agar on slants until needed. Bacterial cells were inoculated and incubated at 37°C in MH broth (Fluka, Switzerland) for 14 hours prior to the screening procedures. Densities of bacterial cultures before antimicrobial activity testing (bioautography and serial microplate dilution method) were approximately as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml.

2.3.3. Bioautography procedure

For bioautography analysis thin layer chromatography (TLC) plates were loaded with 100 µg of each extract, and dried in a stream of air before developing in mobile phases of varying polarities (BEA, EMW, CEF). Plates (solvent evaporated) were then sprayed with concentrated cultures of test microbial organism until completely moist with the aid of a spraying gun enhanced using a vacuum pump. The moist plates were incubated at 37°C in a humidified chamber for 2 hr. The plates were then sprayed with 2 mg/ml of *p*-iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 12 h (Begue and Kline, 1972). The emergence of purple-red colour resulting from the reduction of INT into its respective formazan was a positive indicator of cell viability. Viable bacterial and fungal cells, through active NAD-dependent dehydrogenases such as threonine dehydrogenase, reduce INT into a purple/red-coloured formazan. Clearing zones were indicative of anti-proliferative activity of the extracts.

2.3.4. Minimal inhibitory concentration determination

Minimal inhibitory concentrations (MIC) are regarded as the lowest concentration of extract that inhibits growth of test organisms. The method of Eloff (1998b) was used.

The assay was initiated by pouring sterile water aliquots (100 μ l) into wells of microtitre plates. Exactly 100 μ l of 10 mg/ml extract prepared in acetone was added in row A and mixed using a micropipette. From row A 100 μ l was aspirated and added into row B and mixed. The procedure was repeated until all the wells were filled. An additional 100 μ l in row H was discarded. Two columns were used as sterility control (no cultures were added) and growth control (the extracts were replaced with 100 μ l of acetone). Concentrated suspensions of microorganisms (100 μ l) were added to each well except the sterility controls. The microtitre plates were sealed in a plastic bag with a plastic film sealer (Brother) before incubating at 37°C in a 100% humidified incubator for 18 hours. After incubation 40 μ l of 0.2 mg/ml INT was added to each well and plates incubated for a further 2 hours before observation in antibacterial activity assays. In antifungal assays 40 μ l of 0.2 mg/ml INT was added before the 18 hour incubation. The development of red colour, resulting from the formation of the red/purple formazan, was indicative of growth (positive indicator of cell viability). MIC values were regarded as the lowest concentrations of the compound or extracts that inhibited the growth of the test organisms (decrease in the intensity of the red formazan colour). Amphotericin B was used as a standard in antifungal activity assays, while gentamicin was used in antibacterial tests. The experiments were performed in triplicate and repeated once.

2.3.5. Cytotoxicity

The cytotoxicity of the most active plant species was determined using the MTT assay. The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction assay is widely used for measuring cell proliferation and cytotoxicity. MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer (Mosmann, 1983).

Vero African monkey kidney cells (Vero cells) of a subconfluent culture were harvested and centrifuged at 200 x g for 5 min, and the cell pellet resuspended in growth medium to a density of 2.4×10^3 cells/ml. Minimal Essential Medium (MEM) (Highveld Biologicals, South Africa) supplemented with 0.1% gentamicin (Sigma) and 10% foetal calf serum (Highveld Biologicals, South Africa) was used. Cell suspension (200 μ l) was added into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 μ l) was added into wells of columns 1 and 12

to minimize the “edge effect” and maintain humidity. The plates were incubated for 24 h at 37°C in a 5% CO₂ incubator, until the cells were in the exponential phase growth. The medium was then removed from wells using a thin tube attached to a hypodermic needle and immediately replaced with 200 µl of test compound or plant extract or berberine chloride (Sigma) (positive control) at various known concentrations (quadruplicate dilutions prepared in growth medium). The microtitre plates containing treated and untreated cells were incubated at 37°C in a 5% CO₂ incubator for a defined contact period. MTT (30 µl) (Sigma) (stock solution of 5 mg/ml in phosphate-buffered saline [PBS]) was added to each well and the plates incubated for a further 4 h at 37°C. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl of DMSO to each well, followed by gentle shaking of the MTT solution. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm using a microplate reader (Versamax). The wells in column 1, containing only medium and MTT but no cells, were used to blank the reader. The LC₅₀ values were calculated as the concentration of test compound or plant extract resulting in a 50% reduction of absorbance compared to untreated cells. Selective activity of the most active extracts was calculated as follows:

Selectivity index (SI) = LC₅₀/MIC.

2.4. BIOASSAY-GUIDED FRACTIONATION OF EXTRACTS

Extracts were separated using silica gel packed in columns. The active fractions received preference in bioassay-guided fractionation, though the less active fractions were also further separated to obtain pure compounds. Column chromatography using Silica gel was used to isolate active compounds. The details of the procedures are presented in subsequent chapters. Figure 2.1. represents the scheme of work followed in the isolation of active compounds.

2.5. STRUCTURE ELUCIDATION

The structures of the isolated compounds were analysed using Nuclear Magnetic Resonance spectroscopy (NMR) (University of Limpopo, Medunsa Campus) and Gas Chromatography-Mass Spectroscopy (GC-MS) (University of Johannesburg). Details are presented in subsequent chapters.

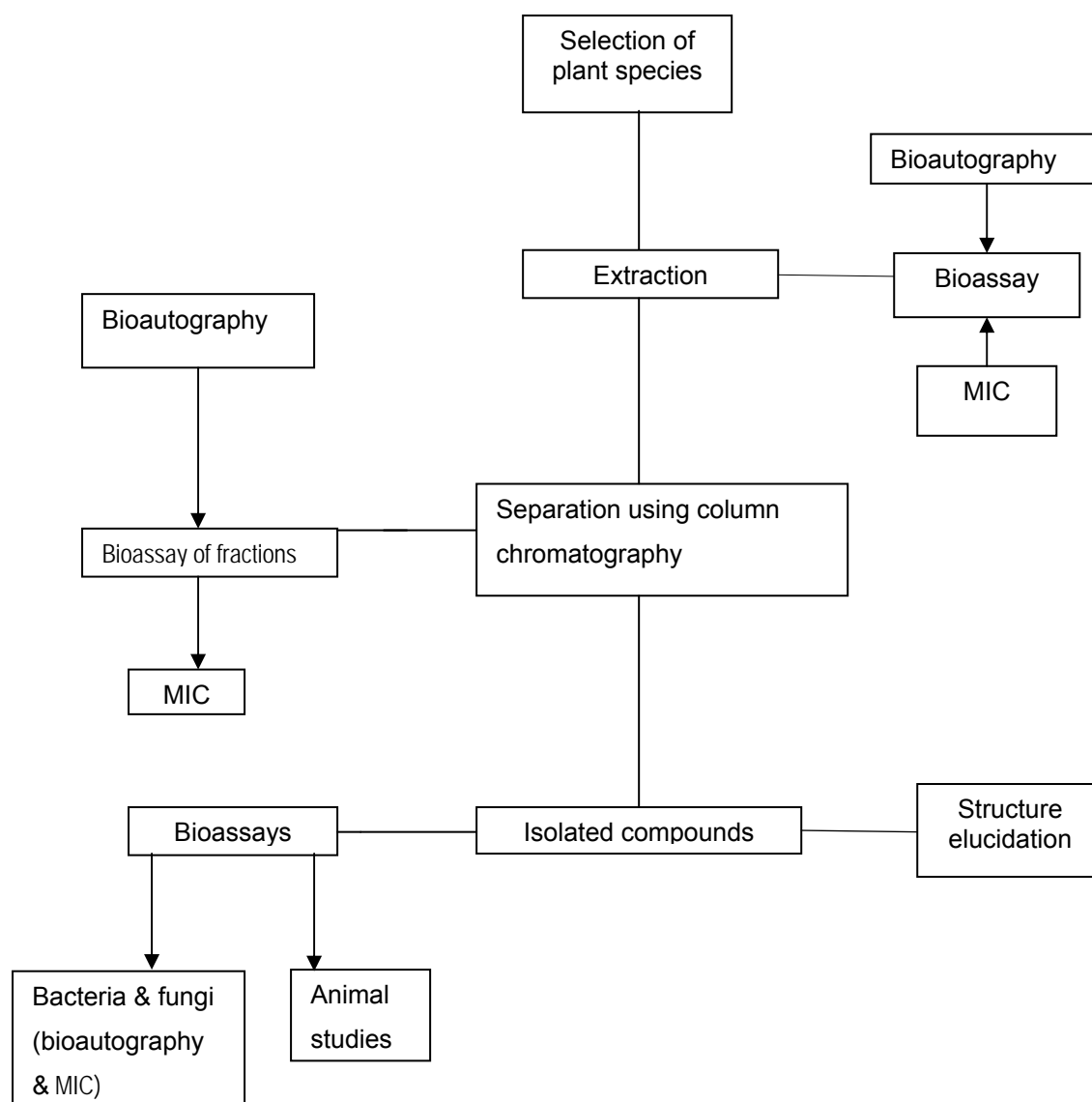


Figure 2.1. Flow diagram showing the layout of the study procedures and methods carried out in this project.

CHAPTER 3

PRELIMINARY SCREENING OF SELECTED SPECIES

The collections made by the University of Pretoria's Phytomedicine Programme (UPPP) in one of its projects is based on random collection of leaves of tree species and then screening the acetone extracts on antibacterial and antifungal activity against 8 important pathogens. Plant material is screened for antibacterial, antifungal and antioxidant activities before active chemical principles are isolated from the most active species. For screening, bioautography and serial microplate dilution methods are used.

From over 350 plant species screened as part of the Tree Screening Project in the UPPP, plant species, which were active against *Candida albicans* at MIC values of 0.5 mg/ml or below, were selected. From this pool 7 plant species were selected for in depth study, using the following parameters:

- 1) number of active compounds on bioautography,
- 2) availability of the species,
- 3) extractability with solvents and
- 4) research reported in literature on this species.

The tree species selected from the UPPP database were *Curtisia dentata*, *Vepris reflexa*, *Terminalia phanerophlebia*, *Terminalia sambesiaca*, *Cussonia zuluensis*, *Kigelia africana* and *Trichilia emetica*. The conservation status of some of these species is of major concern in different locations. For instance, the Ministry for Agriculture and Cooperatives in Swaziland listed different plant species as protected, vulnerable or rare. Among the rare species were *Curtisia dentata*, *Vepris reflexa*, *Terminalia phanerophlebia*, *Kigelia africana* and *Trichilia emetica* (Dlamini, 2000). *Curtisia dentata* is growing at the University of Pretoria's Botanical garden and leaves can be harvested in large quantities. Both the leaves and the stem bark were collected for comparison of their activity.

The choice of the part of the selected plant species to use for isolation of active compounds requires a thorough understanding of traditional uses of the plant species concerned. Tree bark is the most preferred source of herbal remedies in South Africa, constituting approximately 27% of market produce traded annually in KwaZulu-Natal (Mander, 1998). For example, approximately 24 tons of *Curtisia*

dentata bark is harvested and traded annually in KwaZulu-Natal (Mander, 1998), at R2.22/kg or R30/bag (Mander, 1998; Cunningham, 1988).

The harvesting of stem bark endangers the survival of the trees. Harvesting of bark kills trees through effectively terminating downward phloem translocation. This stimulates the diffusion of carbohydrates and hormones from phloem above the wound into the xylem, thereby linking with the upward translocation, resulting in the death of the plant (Grace *et al.*, 2002). *C. dentata* is classified as vulnerable, declining (Cunningham, 1993), conservation-dependent and protected in KwaZulu-Natal (Scott-Shaw, 1999). In the Malowe State Forest in the Transkei district, the level of damage to *Curtisia dentata* trees amount to over 50%, with more than half of the trunk bark removed (Cunningham, 1991).

Grace *et al.* (2002) suggested the replacement of non-sustainable stem bark, roots and bulbs with aerial parts such as leaves and twigs as these inflict little damage on the plants (Zschocke *et al.*, 2000a). Depending on the plant species, phytochemical constituents of the bark and leaves may be similar and have identical biological activities (Zschocke *et al.*, 2000a; Zschocke *et al.*, 2000b). Owing to the scarcity of *C. dentata* trees and their threatened conservation status, as well as the popularity of the bark in traditional remedies, a comparison of the activity of the bark and leaves was conducted. Plant part substitution in traditional medicine is a potential means of conservation of rare trees.

The objectives of this part of the study were to:

- 1) investigate antibacterial and anti-*Candida* activities of the selected seven plant species, with the bioautography and serial microplate dilution (Eloff, 1998b) as the methods of choice for screening and
- 2) compare the activity of extracts of bark and leaves of *Curtisia dentata* against fungal and bacterial test organisms.

3.1. MATERIALS AND METHODS

3.1.1. Extraction

Acetone, hexane and dichloromethane were used as extractants. Leaves of the selected seven plant species and stem bark of *Curtisia dentata* were dried at room temperature and ground to powder using a mill. Powders of leaves of each plant

material (1 g) were extracted with 10 ml of appropriate solvent on a shaking machine for 30 min, and the mixture centrifuged at 4 000 x g for 10 min. The supernatant was retained and allowed to dry in pre-weighed beakers. *Curtisia dentata* stem bark was extracted with acetone and compared with the acetone extracts of the leaves.

3.1.2. Test Organisms

The cultures of microorganisms (Section 2.3.1. and 2.3.2) were obtained from the Microbiology Unit, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science at the University of Pretoria, South Africa.

3.1.3. Bioautography of extracts

TLC plates were loaded with 100 µg of each extract, developed in different mobile phases and used for bioautography as described previously (Section 2.3.3). Duplicate TLC plates were visualized under UV light at 254 and 350 nm and sprayed with vanillin-sulphuric acid and served as reference plates.

3.1.4. Minimal Inhibitory Concentration Determination

To determine the minimal inhibitory concentration (MIC) values, the microplate dilution method developed by Eloff (1998b), with slight modifications for antifungal activity assay by Masoko *et al.* (2005) was used. MIC values were regarded as the lowest concentrations of extract that inhibited growth of test organisms. Total activity values were calculated as previously described (Eloff, 2004). The total activity (ml) of the extracts was calculated as the total mass (mg) of the extract divided by the MIC value (mg/ml). Total activity value indicates the volume to which the extract can be diluted and still inhibit the growth of microbial cells (Eloff, 2004).

3.1.5. Cytotoxicity and Selectivity index

Cytotoxicity of the most active extracts was performed using the MTT assay described in Section 2.3.5, and the selectivity index was calculated for each extract.

3.2. RESULTS

3.2.1. Mass extracted from leaves

Acetone extracted the highest mass of extractible material from plant leaves, while the lowest masses were obtained with hexane as the extractant. The largest mass of extract from 1 g of plant material was 130 mg (13%), obtained from leaves of *Curtisia dentata* with acetone as the extracting solvent. Our results are comparable with data reported by Eloff (1999) in a study involving the biological activity of 27 different members of the Combretaceae family. The amounts of acetone extracts ranged from 2.6 to 22.6% with average value of 10.7% (Eloff, 1999). The lowest amount of extract was 10 mg, (1%) obtained from *Kigelia africana* with hexane as the extracting solvent. Generally, *Kigelia africana* material was the least extractible of all the plant material while *Curtisia dentata* powders gave a high mass of extracts with all the extracting solvents (Fig. 3.1).

3.2.2. Antifungal activity of extracts of the leaves

3.2.2.1. Bioautography

The compounds whose R_f values are listed in Table 3.1 were identified as the active constituents that displayed growth inhibition of fungal organisms. Acetone extracts of *Terminalia sambesiaca* leaves had no visible activity against *Candida albicans* as evidenced by the absence of clear zones on TLC bioautograms. Active compounds in dichloromethane extracts of *Terminalia sambesiaca* were observed against *Candida albicans* (Fig. 3.3). The acetone extracts of *Curtisia dentata* had more (5) chemical constituents than any other plant extract tested with activity against the opportunistic pathogen, *Candida albicans* (Fig. 3.2).

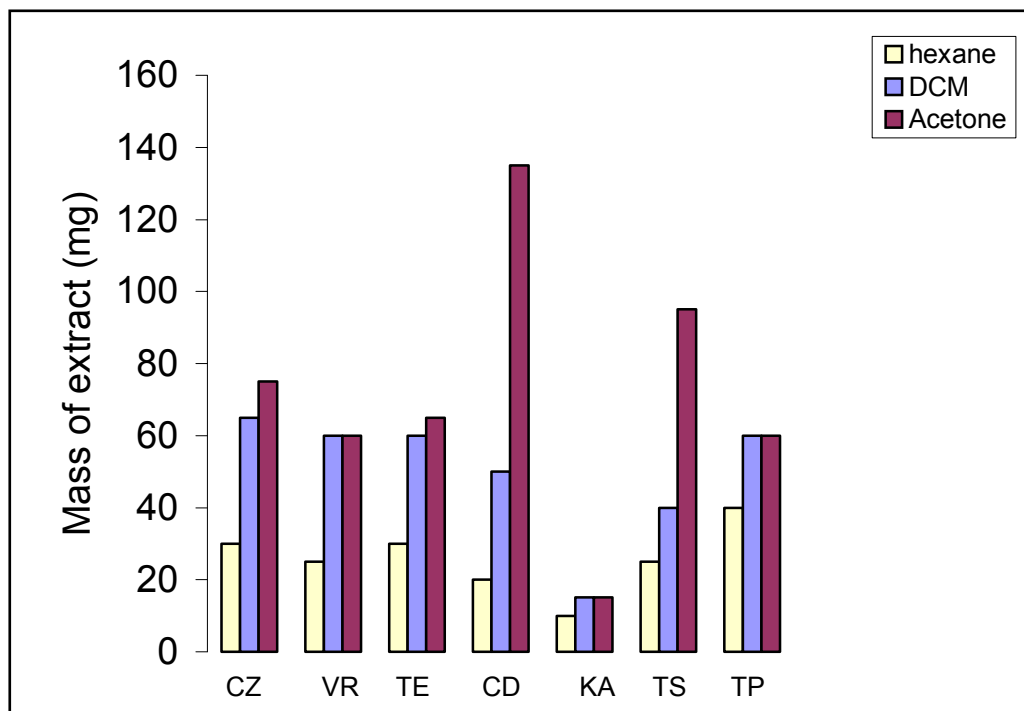


Figure 3.1. Mass extracted (mg/g of dry material) from leaves of different plant species. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *Terminalia phanerophlebia*.

From the TLC fingerprints of all the seven plant species under investigation it would appear that *Curtisia dentata* leaves contain three major compounds that are not present in other plant species investigated. These compounds are probably the active principles of *Curtisia dentata* as the R_f values of these compounds are comparable with the three compounds that showed activity against *Candida albicans* in bioautography.

The highest number of active compounds against other pathogenic fungal species was observed in *Curtisia dentata* leaf extracts, with an average of four compounds active against the fungal species tested (Table 3.1). The calculated R_f values revealed that these compounds were probably active against most of the fungal species tested. *Aspergillus fumigatus* was more resistant to extracts than other fungal species as indicated by fewer growth inhibiting compounds on bioautograms,

while *Candida albicans* was the most sensitive. *Cussonia zuluensis* and *Trichilia emetica* extracts were not active against any of the fungal species on bioautograms.

Against *Cryptococcus neoformans* and *Sporothrix schenckii*, *Curtisia dentata* was the most active with 5 active compounds visible using bioautography. These compounds were the same as those found active against *Candida albicans* as evidenced by the Rf values (Table 3.1). *Kigelia africana*, *Terminalia phanerophlebia* and *Terminalia sambesiaca* contained a few active compounds on TLC bioautograms sprayed with *Cryptococcus neoformans* and *Sporothrix schenckii* (Table 3.1).

3.2.2.2. Minimal Inhibitory Concentrations against fungi

The acetone extract of *Terminalia* resulted in very low MIC values against some of the fungal species investigated. Low MIC values were also realized with all the extracts of *Terminalia phanerophlebia* (Table 3.2). The total activity values against fungi resulting from *Terminalia sambesiaca* and *Terminalia phanerophlebia* were the highest. The lowest total activity was obtained with acetone and hexane extracts of *Kigelia africana* (Table 3.2). *Curtisia dentata*, *Terminalia phanerophlebia* and *Terminalia phanerophlebia* extracts had the best MIC values (0.02 mg/ml) against *Microsporum canis*.

Low MIC values against *Candida albicans* were observed with both the acetone and dichloromethane extracts of *Curtisia dentata*, with MIC values of 0.12 and 0.15 mg/ml, respectively. *Terminalia phanerophlebia*, *Terminalia sambesiaca* and *Kigelia africana* extracts resulted in low MIC values against *Candida albicans*. *Trichilia emetica*, *Vepris reflexa* and *Cussonia zuluensis* resulted in MIC values above 0.5 mg/ml (Table 3.2). Against *Candida albicans*, *Trichilia emetica* and *Vepris reflexa* were the least active. Furthermore, *Curtisia dentata* extracts were the only ones that resulted in high total activity against *Candida albicans*. The acetone extracts of *Curtisia dentata* had the highest total activity (1083 ml/g) against *Candida albicans*

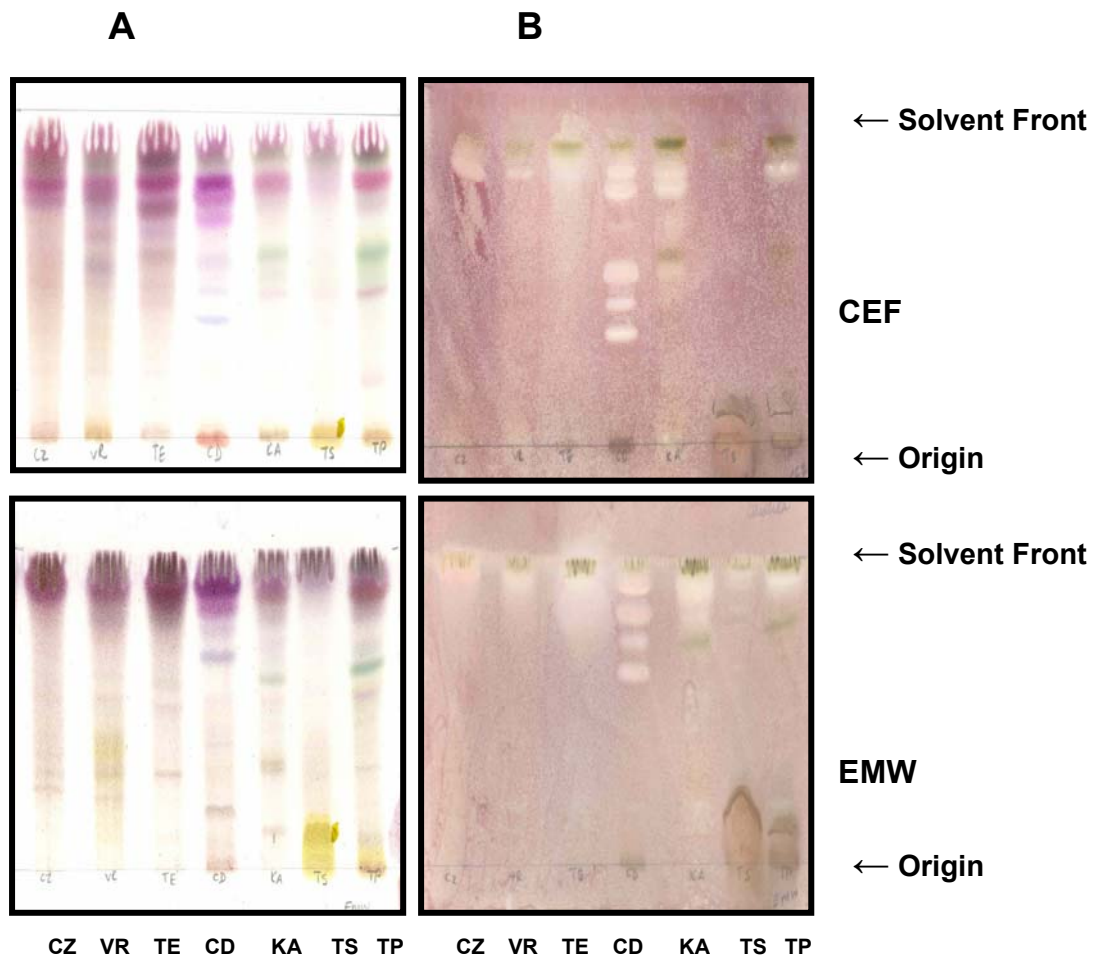


Figure 3.2. TLC plates sprayed with vanillin-sulphuric acid (A) and bioautograms showing growth inhibitory activity (B) of acetone extracts of seven selected plant species against *Candida albicans*. TLC plates were developed in CEF and EMW respectively before spraying with concentrated suspension of the test organism. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *Terminalia phanerophlebia*.

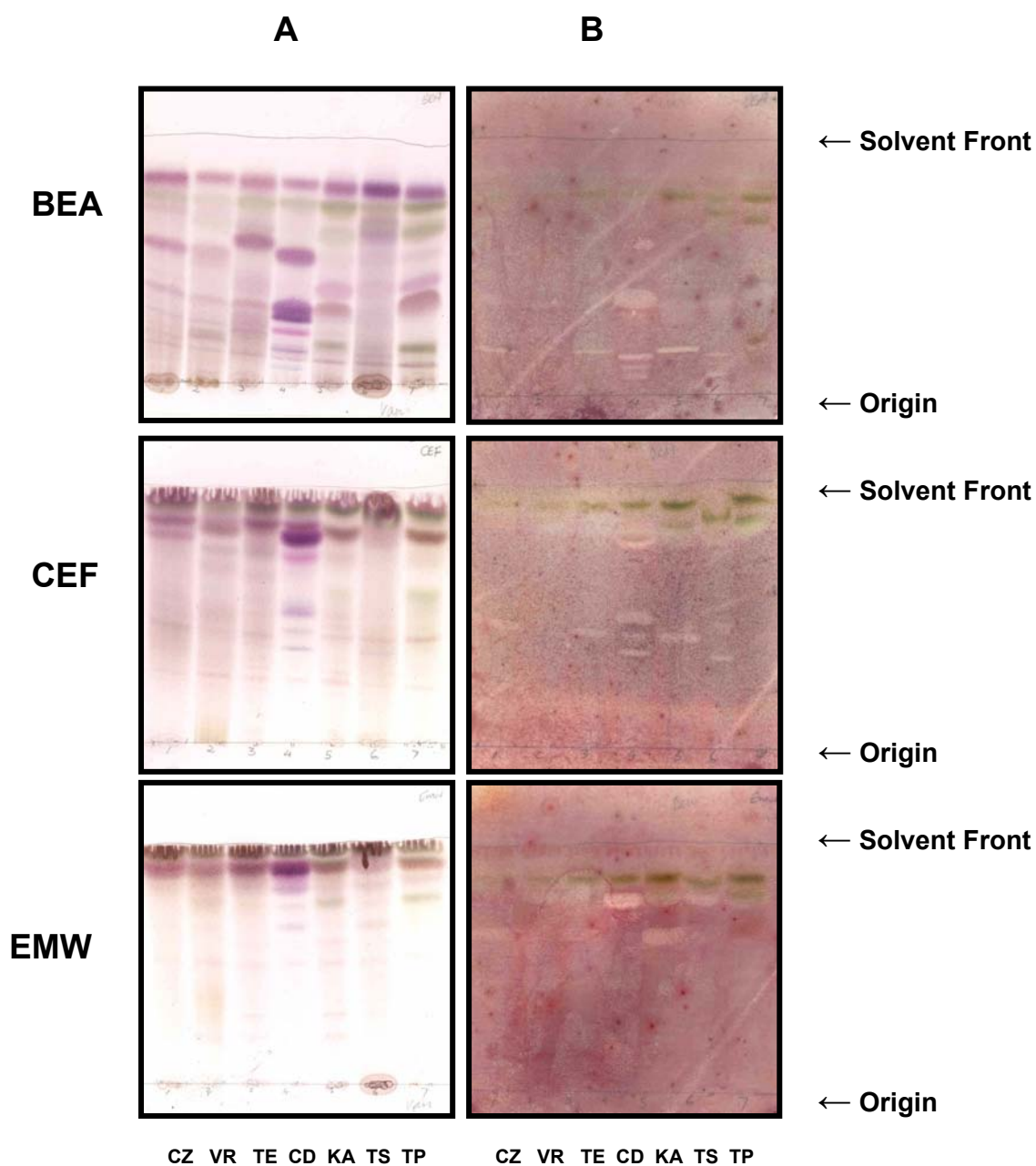


Figure 3.3. TLC fingerprints (A) and anti-*Candida* activity (B) of DCM extracts of seven different plant species. BEA, CEF and EMW, respectively. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *Terminalia phanerophlebia*.

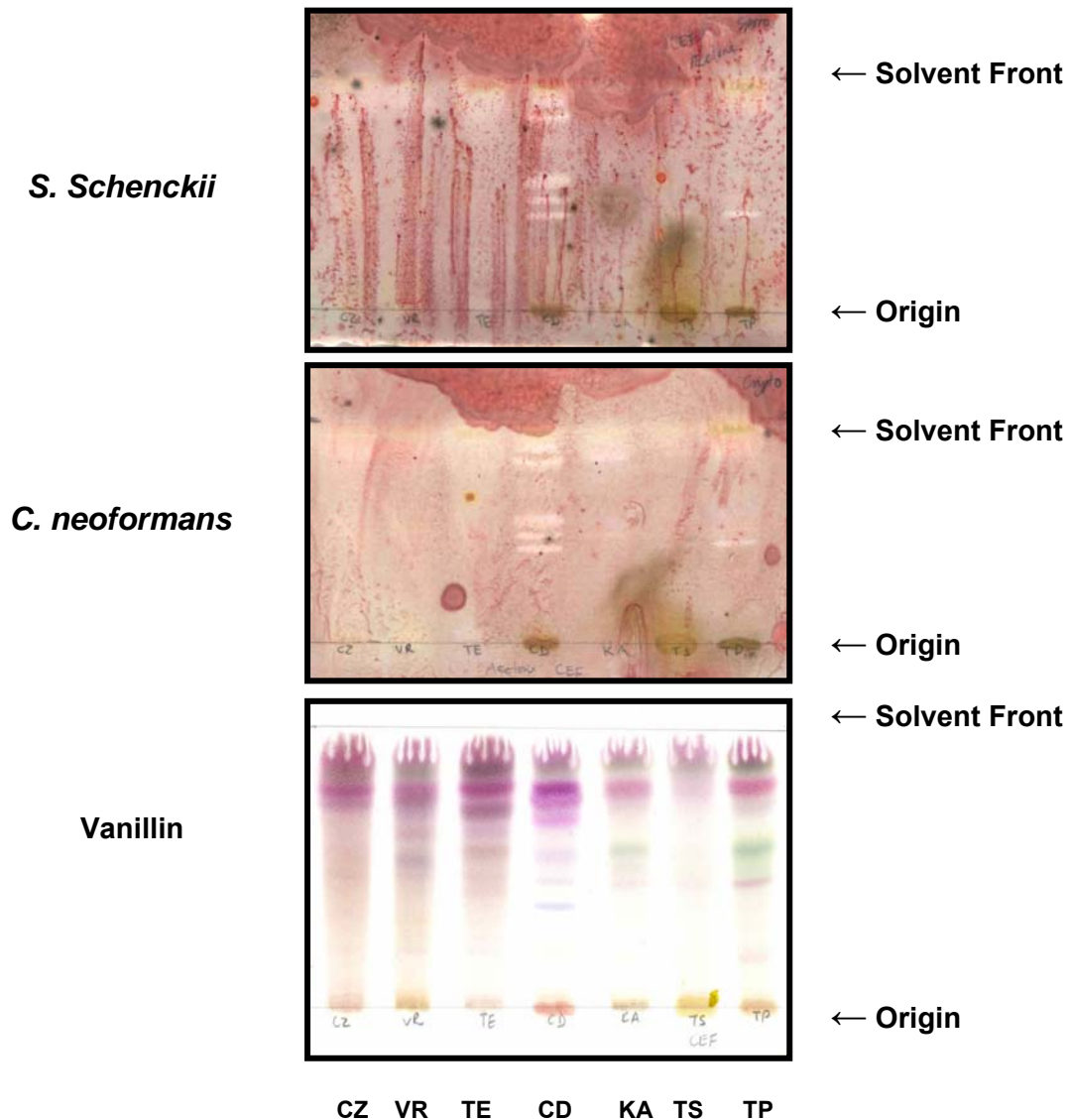


Figure 3.4. TLC bioautograms showing antifungal activity of acetone extracts of leaves of different species against *Cryptococcus neoformans* (CN) and *Sporothrix schenckii* (Ss). The TLC plates were developed in CEF before spraying with cultures of test organisms. A reference plate was sprayed with vanillin-sulphuric acid (bottom). CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *Terminalia phanerophlebia*; Van, vanillin-sulphuric acid sprayed.

Table 3.1. The R_f values of active compounds from acetone leaf extracts of different species against 5 test fungal pathogens. Key: Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Cn, *C. neoformans*; Mc, *M. canis*; Ss, *S. schenckii*.

Plant species	Ca	Cn	Mc	Ss	Af
<i>C.zuluensis</i>	0.80	-	-	-	-
<i>Vepris reflexa</i>	0.80	0.80	-	-	-
<i>Trichilia emetica</i>	0.80	-	-	-	-
<i>Curtisia dentata</i>	0.85	0.85	0.85	0.85	-
	0.80	0.80	-	0.80	0.80
	0.54	0.54	0.54	0.54	-
	0.46	0.46	0.46	0.46	-
	0.33	0.33	-	0.33	-
<i>Kigelia africana</i>	-	-	0.88	-	-
	0.80	0.80	-	-	-
	0.73	-	-	-	-
	0.48	0.48	-	0.46	-
<i>Terminalia sambesiaca</i>	-	-	-	0.40	-
<i>Terminalia phanerophlebia</i>	0.80	-	-	-	-
	-	0.47	0.49	0.40	-

The MIC and total activity values from each plant extract recorded against all fungal species were combined into MIC and total activity averages for each plant extract. The averages of each plant extract against all the fungal species revealed that *Terminalia sambesiaca*, *Terminalia phanerophlebia* and *Curtisia dentata* resulted in lowest MIC values against all fungal species (Fig. 3.5). Total activity averages further indicated that the acetone extracts of *Terminalia sambesiaca*, *Terminalia phanerophlebia* and *Curtisia dentata* were the most active against fungal species (Fig. 3.6).

The overall average of all the plant extracts against each of the fungal species indicated that *Candida albicans*, *C. neoformans* and *M. canis* were more sensitive than *S. schenckii* and *A. fumigatus* to plant extracts (Fig. 3.7). The calculated average total activity value for *M. canis* was the highest (879 ml/g), indicating that this organism was the most sensitive to plant extracts. The lowest total activity value was calculated for *A. fumigatus*, further indicating that this organism was the most resistant to plant extracts (Fig. 3.8).

Table 3.2.: MIC values (mg/ml) and total activity values (ml) of extracts of leaves of seven different plant species against five fungal test organisms. The MIC values ($\mu\text{g/ml}$) for Amphotericin B were *Ca*, 0.2; *Cn*, 0.8; *Mc*, 0.8; *Ss*, 0.8; *Af*, 0.2. Key; *Ca*, *Candida albicans*; *Cn*, *C. neoformans*; *Mc*, *M. canis*; *Ss*, *S. schenckii*; *Af*, *Aspergillus fumigatus*; H, hexane extract; D, dichloromethane extract; A, acetone extract; -, not active (MIC value taken as 2.50 mg/ml to allow for calculation of total activity, averages and selectivity index).

Fungal Species	Plant Extracts																				
	<i>Cussonia zuluensis</i>			<i>Vepris reflexa</i>			<i>Trichilia emetica</i>			<i>Curtisia dentata</i>			<i>Kigelia africana</i>			<i>Terminalia sambesiaca</i>			<i>Terminalia phanerophlebia</i>		
	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A
	MIC values mg/ml																				
<i>Ca</i>	-	1.88	1.25	1.25	1.25	1.25	0.30	0.60	0.60	0.60	0.15	0.12	0.45	0.23	0.23	0.23	0.23	0.12	0.30	0.30	0.15
<i>Cn</i>	0.60	0.60	-	0.60	-	-	1.25	0.78	-	0.30	0.30	0.15	0.30	0.38	1.25	0.08	0.04	0.02	0.08	0.08	0.02
<i>Mc</i>	-	-	-	1.25	1.88	1.88	0.15	0.23	1.56	0.30	0.38	0.02	0.08	0.30	-	0.08	0.04	0.02	0.15	0.08	0.02
<i>Ss</i>	-	-	0.93	-	-	0.93	1.25	1.25	-	0.60	0.30	0.08	-	-	-	0.12	0.12	0.03	0.03	0.15	0.03
<i>Af</i>	1.25	-	1.25	1.25	1.25	-	0.45	0.60	0.93	-	0.30	0.20	0.93	0.60	0.30	0.60	0.45	0.30	-	0.45	0.13
Average	1.87	2.00	1.50	1.37	1.88	1.88	0.68	0.81	0.33	0.86	0.29	0.11	0.86	0.80	1.36	0.22	0.18	0.10	0.67	0.45	0.13
	Total Activity in ml/g																				
<i>Ca</i>	8	43	56	16	56	40	67	100	100	33	266	1083	22	87	43	87	174	667	133	200	333
<i>Cn</i>	33	133	28	33	28	20	16	77	24	67	133	867	33	53	8	250	1000	4000	500	3000	2500
<i>Mc</i>	8	32	28	16	37	27	133	261	38	67	105	6500	125	67	4	250	1000	4000	267	3000	2500
<i>Ss</i>	8	32	75	8	28	54	16	48	24	33	133	1625	4	8	4	167	333	2667	133	400	1667
<i>Af</i>	16	32	56	16	56	20	44	100	65	67	133	650	11	33	33	33	89	267	16	32	111
Average	15	54	49	18	41	32	55	117	50	53	154	2145	39	50	18	157	519	2320	210	1326	1422

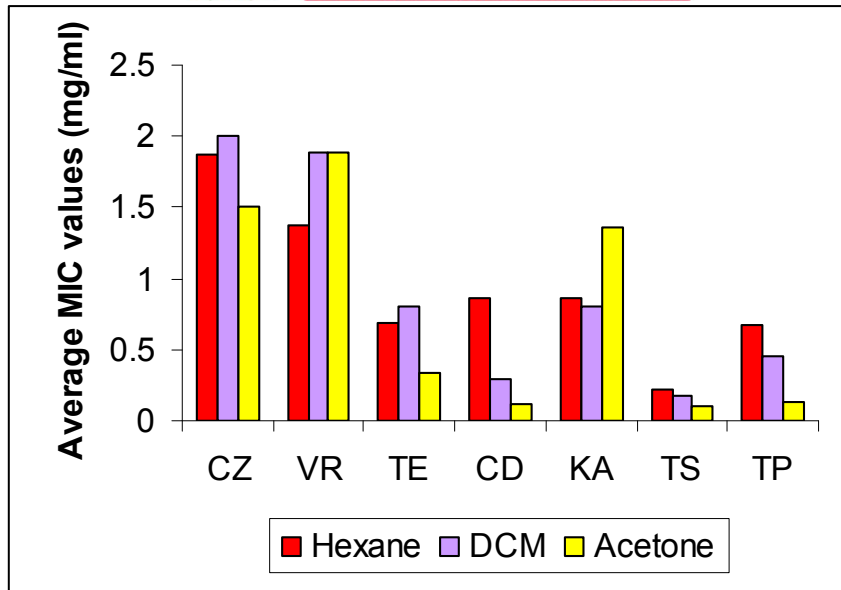


Figure 3.5. Combined average MIC values of each plant extract against all the fungal species. All MIC values from a specific plant extract against all fungal species were combined into an MIC average for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *T. sambiesca*; TP, *T. phanerophlabia*

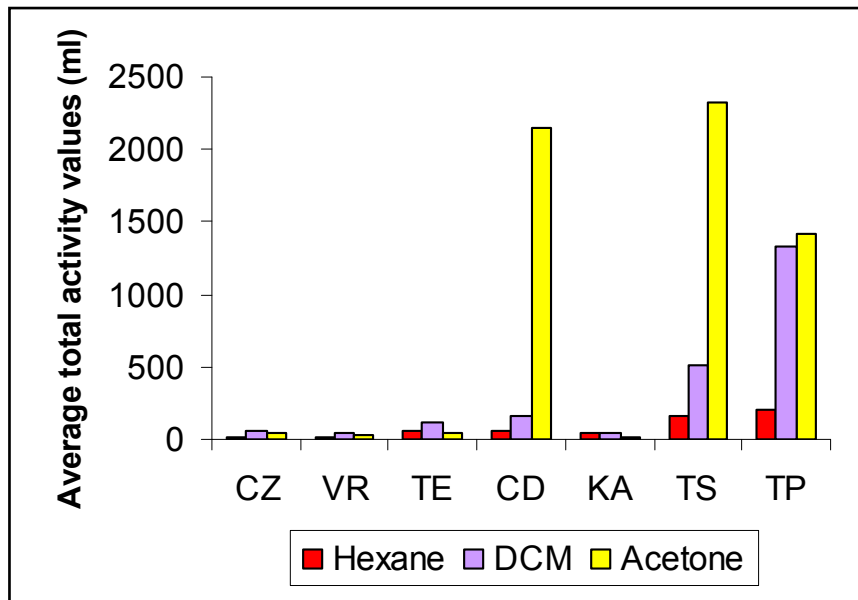


Figure 3.6. Combined average total activity values of each plant extract against all the fungal species. All total activity values from a specific plant extract against all fungal species were combined into an average total activity value for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *T. sambiesca*; TP, *T. phanerophlabia*

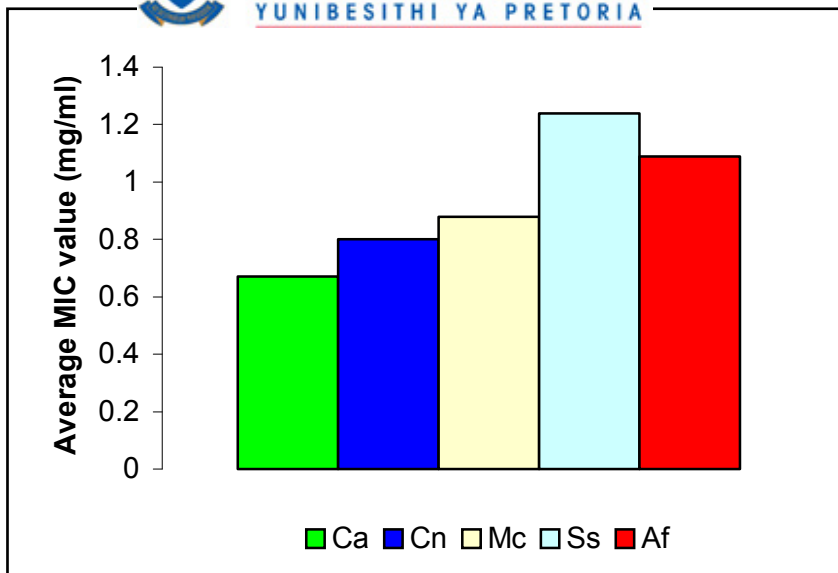


Figure 3.7. Sensitivity of fungal test organisms to all plant extracts (MIC values). Average of MIC values of all the plant extracts against each of the fungal species were calculated. Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Mc, *M. canis*; Ss, *S. schenckii*.

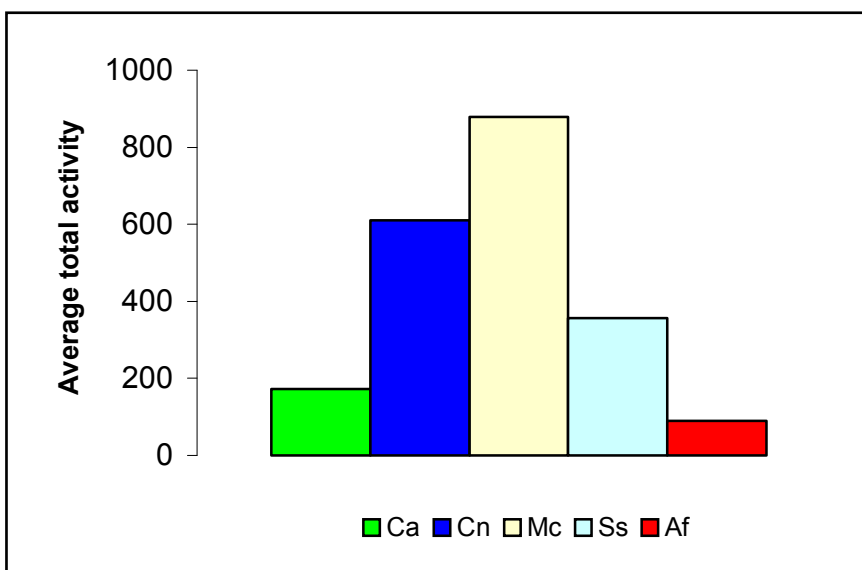


Figure 3.8. Sensitivity of fungal test organisms to all plant extracts (total activity values). Average of total activity values of all the plant extracts against each of the fungal species were calculated. Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Mc, *M. canis*; Ss, *S. schenckii*.

3.2.3. Antibacterial activity of extracts of the leaves

3.2.3.1. Bioautography

The plant species screened were active against both Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*P. aeruginosa* and *E. coli*) bacterial species. The clear zones showing on TLC plates sprayed with bacterial culture suspensions and INT are indicative of growth inhibition due to activity of chemical components present in plant leaves (Fig. 3.9). *Curtisia dentata* had the highest number of active chemical components compared to the rest of the species investigated. Chemical components with similar properties may be responsible for antibacterial activity across all the species investigated, as indicated by the similar retardation factor (R_f) for the components (Table 3.3). Selectivity, according to bioautography results was not observed as both Gram negative and Gram positive bacterial species were probably also affected by the same compounds

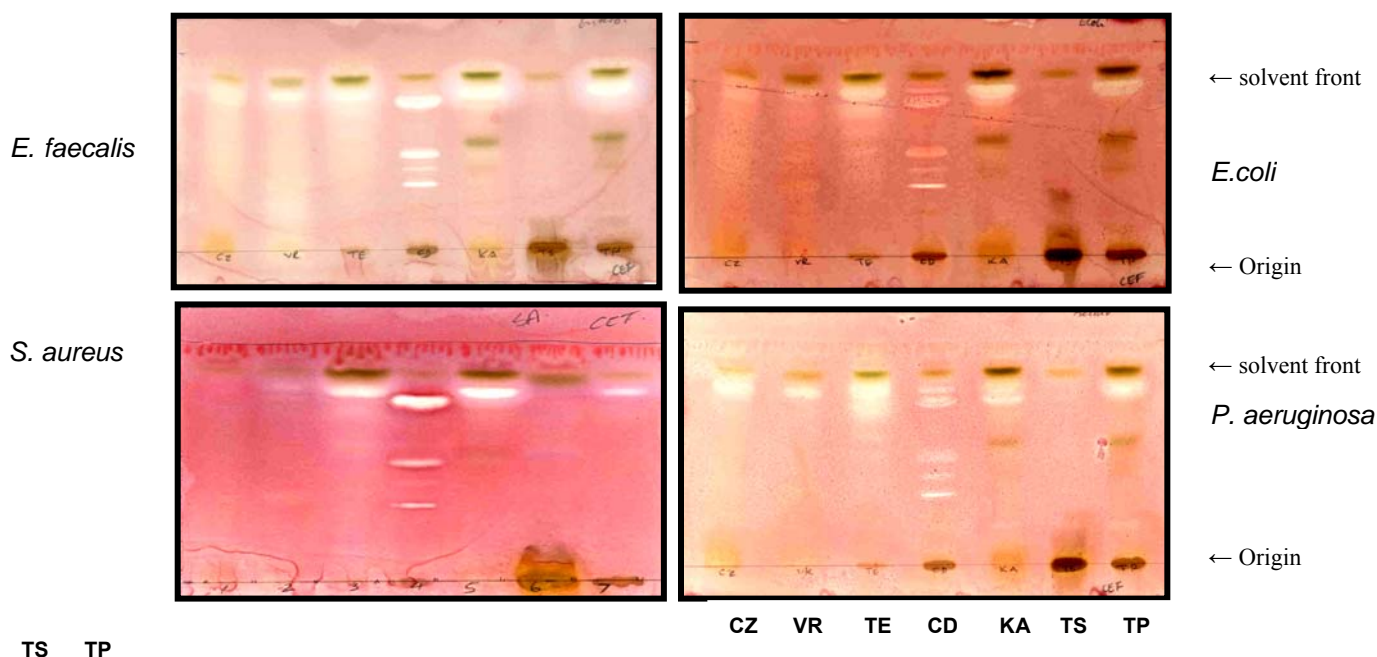


Figure 3.9. TLC bioautograms showing antibacterial activity against four test bacterial suspensions. The leaf powders were extracted with acetone and TLC analysis was performed using CEF as eluent. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *T. sambesiaca*; TP, *T. phanerophlebia*.

The acetone extracts of *Terminalia sambesiaca* had no visible activity against any of the four bacterial test pathogens. The other plant species, with the exception of *Terminalia sambesiaca* and *Curtisia dentata*, had more non-polar compounds as active components, as shown by the mobility of the active constituents on TLC plates developed in CEF.

Table 3.3. Comparison of *Curtisia dentata* chemical constituents showing activity using bioautography against the four bacterial test organisms and *Candida albicans*.
Key: +, less activity observed; +++++, high activity observed by visual comparison of the size of the clearing zones; -, no activity.

Rf Values	<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>	<i>Candida albicans</i>
0.85	-	+	-	+	+++
0.80	+++++	++++	++++	+++	+++++
0.54	+++	++	++	++	+++
0.44	+	+	+	++	+++
0.33	++	++	++	+	++++

3.2.3.2. MIC values against bacteria

MIC values of the seven plants against bacteria were high, though *E. faecalis* was observed, in most cases, to be the most sensitive species of bacteria investigated, with 0.053 mg/ml recorded as the lowest active concentration. The DCM extract of all the plant species investigated did not result in any obvious inhibition of *P. aeruginosa* and *E. faecalis* growth with the highest concentration used, 2.5 mg/ml failing to inhibit growth (Table 3.4).

Extracts were dried and reconstituted to desired concentrations (10 mg/ml) in DMSO. It was observed that when these extracts were mixed with water (in microtiter plates) for determination of MIC values, clumping occurred. Clumping of chemical components may have resulted in poor absorption of the active compounds by the bacterial cells. As was the case with bioautography results, *Curtisia dentata* was the most active against bacterial species investigated as indicated by the calculated total activity values (Table 3.4). The hexane extracts of all the plant species were the least active against bacterial test organisms.

Table 3.4: MIC values (mg/ml) and total activity values (ml) of extracts of leaves of seven different plant species against four bacterial test organisms. MIC values ($\mu\text{g/ml}$) for gentamicin were *Sa*, 0.06; *Ec*, 0.06; *Pa*, 0.08; *Ef*, 0.02. Key; *Sa*, *S. aureus*; *Ec*, *E. coli*; *Pa*; *P. aeruginosa*; *Ef*, *E. Faecalis*; -, not active (MIC value taken as 2.50 mg/ml to allow for calculation of total activity, averages and selectivity index).

Bacterial species	Plant Extracts																				
	<i>Cussonia zuluensis</i>			<i>Vepris reflexa</i>			<i>Trichilia emetica</i>			<i>Curtisia dentata</i>			<i>Kigelia africana</i>			<i>Terminalia sambesiaca</i>			<i>Terminalia phanerophlebia</i>		
	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A	H	D	A
	MIC values mg/ml																				
<i>Sa</i>	-	-	0.60	-	-	1.25	-	1.25	0.60	-	-	1.00	-	0.60	0.60	-	-	0.30	0.60	0.30	0.30
<i>Ec</i>	-	-	1.90	0.60	1.25	0.80	-	0.60	0.40	0.60	0.60	0.80	1.25	0.60	0.60	0.30	0.60	0.20	-	0.30	0.30
<i>Pa</i>	-	-	1.80	1.25	-	0.16	1.25	1.25	0.40	0.60	1.25	0.80	0.60	1.25	0.10	1.25	0.60	0.10	-	0.60	0.16
<i>Ef</i>	-	0.30	-	0.30	0.08	2.00	0.30	0.08	0.26	0.60	0.30	0.05	0.30	0.08	0.42	1.25	0.08	2.50	0.30	0.08	0.30
Average	-	1.95	1.70	1.16	1.58	1.05	1.64	0.80	0.42	1.08	1.16	0.67	1.16	0.63	0.43	1.33	0.95	0.78	1.48	0.32	0.27
	Total Activity in ml																				
<i>Sa</i>	8	32	117	6	28	40	8	40	100	8	16	126	4	33	17	8	16	267	67	200	167
<i>Ec</i>	8	32	37	33	56	63	8	100	150	33	67	163	8	33	17	67	67	400	16	200	167
<i>Pa</i>	8	32	39	16	28	313	16	48	150	33	32	163	17	16	100	16	7	800	16	100	313
<i>Ef</i>	8	267	28	67	875	25	67	750	231	33	67	2600	33	250	4	16	500	32	133	750	167
Average	8	91	55	31	247	110	25	237	158	27	46	763	16	83	35	27	148	375	58	313	203

The MIC and total activity values from each plant extract recorded against all bacterial species were combined into MIC and total activity averages for each plant extract. Acetone extracts of all plant species were the most active with the lowest MIC averages against all the bacterial species tested (Fig. 3.10). Averages of total activities for each extract further confirmed that the acetone extracts of *Curtisia dentata* and *Terminalia sambesiaca* were the most active extracts against all the bacterial species tested (Fig. 3.11).

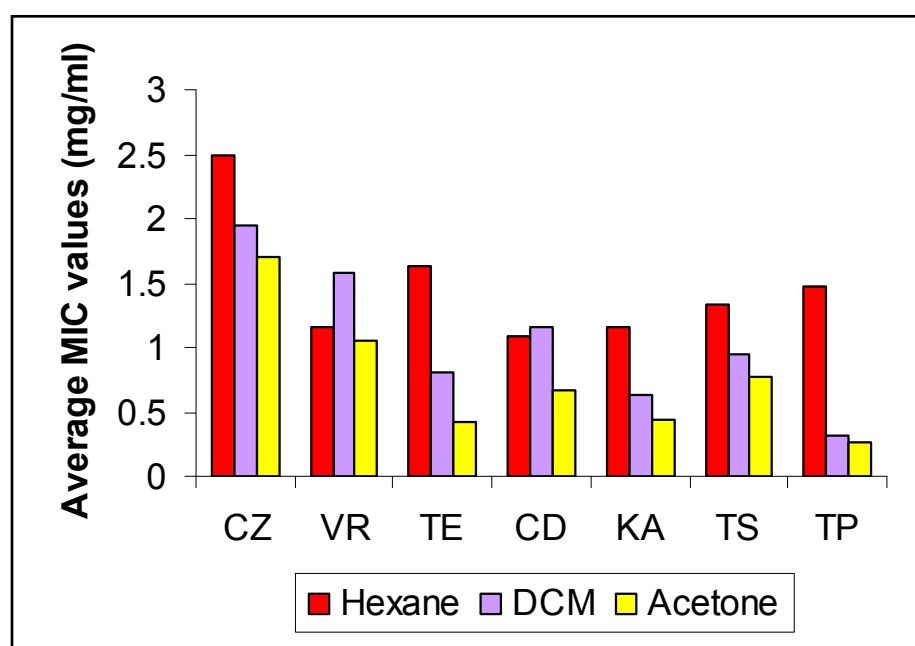


Figure 3.10. Combined average MIC values of each plant extract against all the bacterial species. All MIC values from a specific plant extract against all bacterial species were combined into an MIC average for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *T. phanerophlaba*.

The MIC and total activity values against specific bacterial species by all plant extracts were combined into averages to determine the sensitivity of each bacterial species to plant extracts. The lower the MIC average values against a specific bacterium the more sensitive the bacterial species. The lowest overall average MIC value (0.69 mg/ml) was calculated for *E. faecalis*, thus indicating that this species was the most sensitive to the plant extracts. *S. aureus* was the most resistant as was indicated by the high average MIC calculated for all plant extracts (Fig. 3.12). The highest average total activity (328 ml/g) calculated further indicated that *E. faecalis*

was the most susceptible test bacterial organism to plant extracts (Fig. 3.13). The total activity average value was the lowest against *S. aureus* further confirming that *S. aureus* was the most resistant to plant extracts (Fig. 3.13).

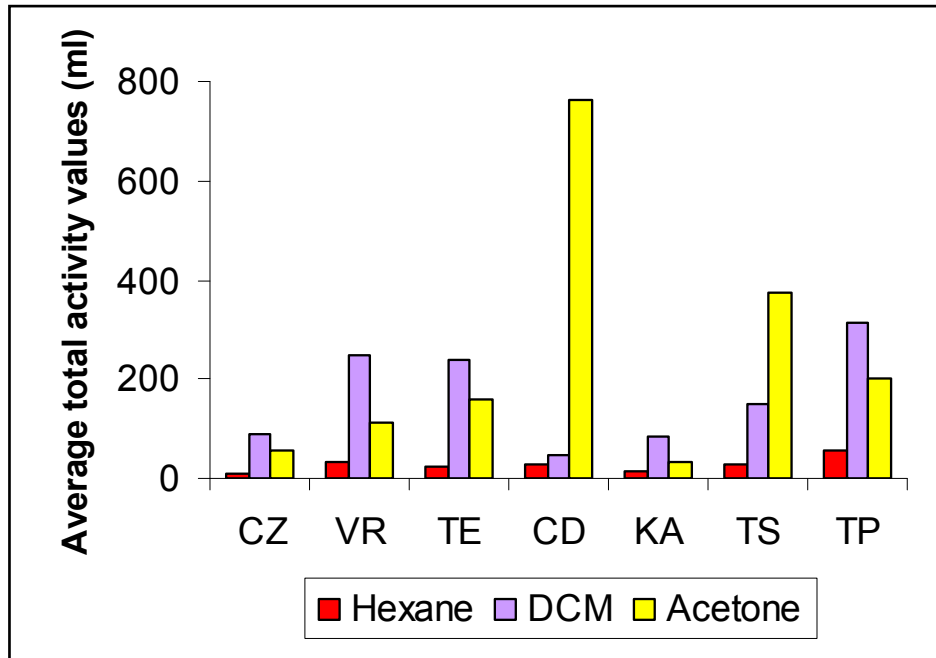


Figure 3.11. Combined average total activity values of each plant extract against all the bacterial species. All total activity values from a specific plant extract against all the tested bacterial species were combined into an average total activity value for that plant extract.

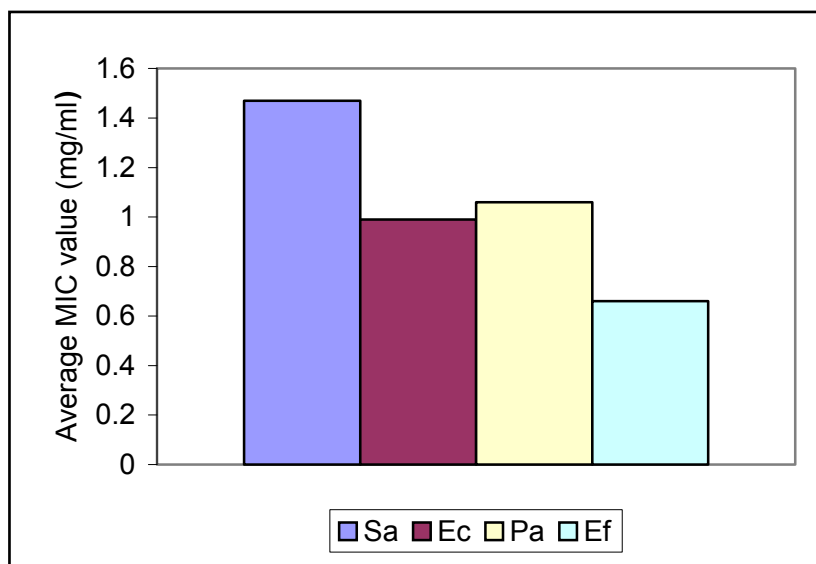


Fig. 3.12. Sensitivity of bacterial test organisms to all plant extracts (MIC values). Averages of MIC values of all the plant extracts against each of the bacterial species were calculated. Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; Ef, *E. faecalis*.

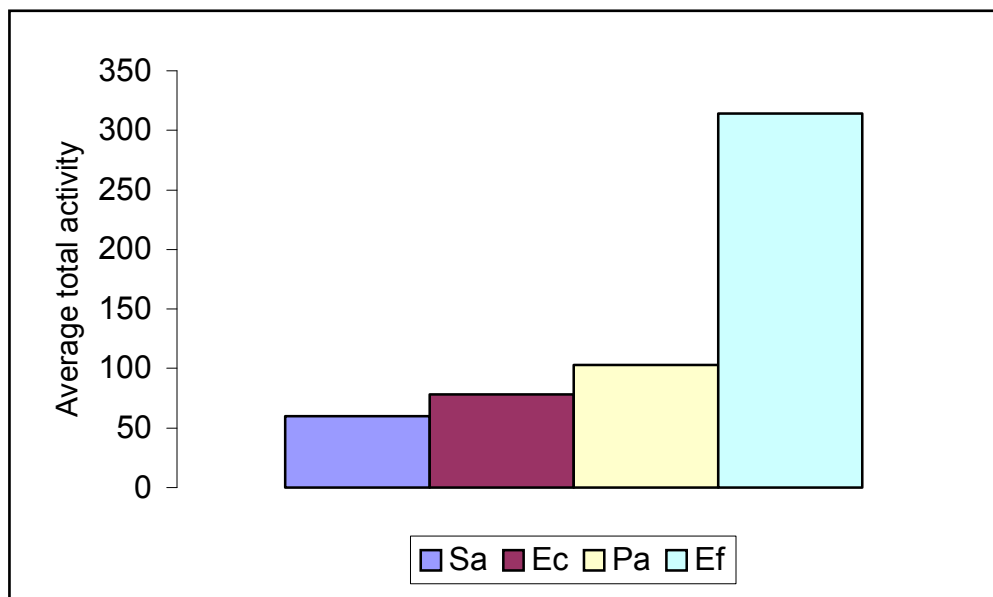


Figure 3.13. Sensitivity of bacterial test organisms to all plant extracts (total activity values). Averages of total activity values of all the plant extracts against each of the bacterial species were calculated. Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; Ef, *E. faecalis*.

3.2.4. Cytotoxicity of acetone and dichloromethane extracts of *Curtisia dentata* leaves

The MTT assay was used to determine the cytotoxicity of the dried acetone and dichloromethane extracts of *Curtisia dentata* reconstituted in DMSO against Vero cells. The acetone extract of *Curtisia dentata* was slightly less toxic to Vero cells, with an LC₅₀ value of 24.4 µg/ml. The dichloromethane extract was more toxic to Vero cells with an LC₅₀ of 6.6 µg/ml (Figs. 3.14 and 3.15). Berberine, the positive control, had an LC₅₀ of 10 µg/ml.

The selectivity index is the ratio of the toxicity to the biological activity (MIC value). It relates to the degree to which the observed activity of a substance can be attributed to the toxicity of that substance. The higher the value, the higher the selectivity of the substance. The selectivity index (Table 3.5) was the highest for amphotericin B, ranging from 35 to 175 against all tested fungal species. The average selectivity index of the acetone extract against all tested fungi was 16 times better than that resulting from the dichloromethane extract (Table 3.5). These data suggest the

antibacterial and antifungal activity of the extracts were as a result of toxicity, hence the low selectivity indices.

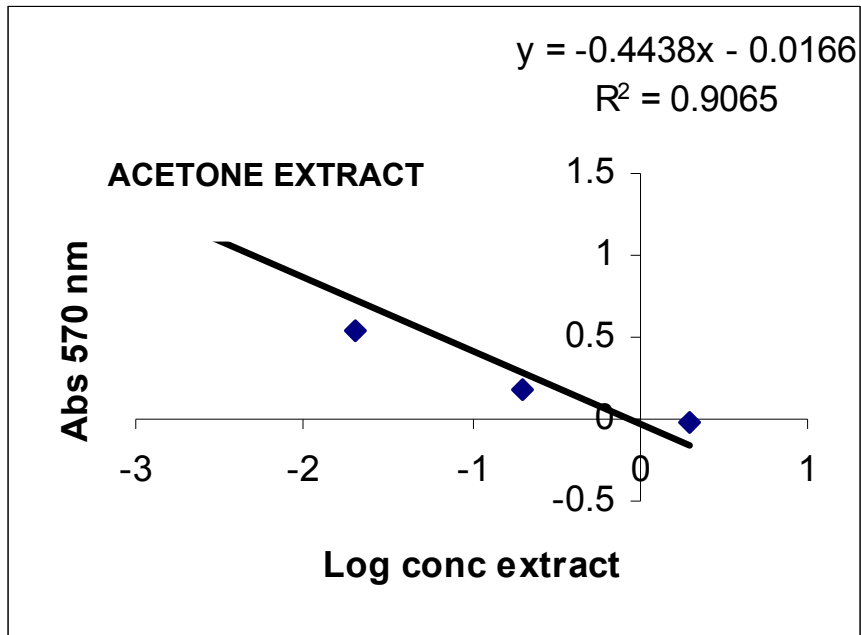


Figure 3.14: Cytotoxicity of acetone extract against Vero cells ($LC_{50} = 24.4 \mu\text{g/ml}$)

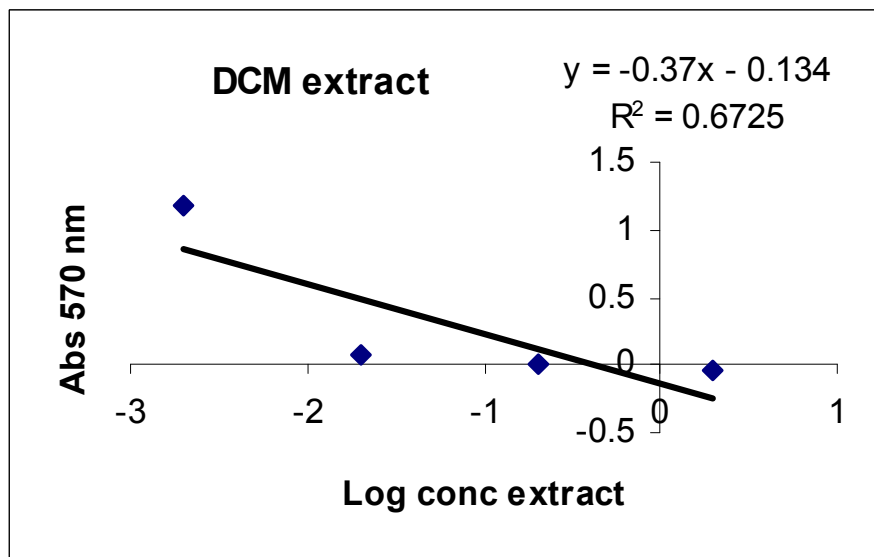


Figure 3.15: Cytotoxicity DCM extract against Vero cells ($LC_{50} = 6.6 \mu\text{g/ml}$).

Table 3.5. Selectivity index (SI) of the dichloromethane and acetone extracts of *Curtisia dentata* against fungal species. Key; Ca, *Candida albicans*; Cn, *C. neoformans*; Mc, *M. canis*; Ss, *S. schenckii*; Af, *Aspergillus fumigatus*;

Extracts	Ca	Cn	Af	Ss	Mc	Average
Acetone	0.200	0.163	0.122	0.305	1.220	0.402
Dichloromethane	0.044	0.022	0.022	0.022	0.017	0.025
Amphotericin B	140	35	175	35	175	112

3.3. Comparison of antifungal and antibacterial activity of stem bark and leaves of *Curtisia dentata*

3.3.1. TLC fingerprints

Leaves, twigs and stem bark of *Curtisia dentata* were extracted with acetone and analysed using TLC to compare their chemical compositions (Fig. 3.16). Extracts of the leaves contained similar chemical components on TLC plates. There were slight differences in chemical composition between leaves and stem bark regarding the levels of specific chemical components as indicated by the intensity of spots on TLC plates. The bark contained several compounds visible under UV light, which were not present in leaf extracts. Some components that were observed in leaves were undetectable in the bark extract. In the bark extract there was a high concentration of a compound that stained orange-red when sprayed with vanillin, while in the leaves there was an accumulation of several components that stained dark blue-purple (Fig. 3.16).

3.3.2. Comparative bioautography

Four compounds in the *C. dentata* leaf extract were active against *Candida albicans* compared with two in the bark extract (Fig. 3.17). The extracts were also tested for activity against bacterial test organisms using the bioautography method. Since the leaves and twigs seemed to contain similar compounds, only the leaves were tested for antibacterial activity using the bioautography method. The leaves contained more (4) antibacterial and anti-*Candida* compounds than the bark (2) (Fig. 3.17). Similar observations were recorded with bacterial species, namely *E. coli* and *S. aureus*, where extracts of the leaves had more active compounds inhibiting the growth of

these microorganisms (Fig. 3.17). The R_f values of active compounds are displayed in Table 3.6.

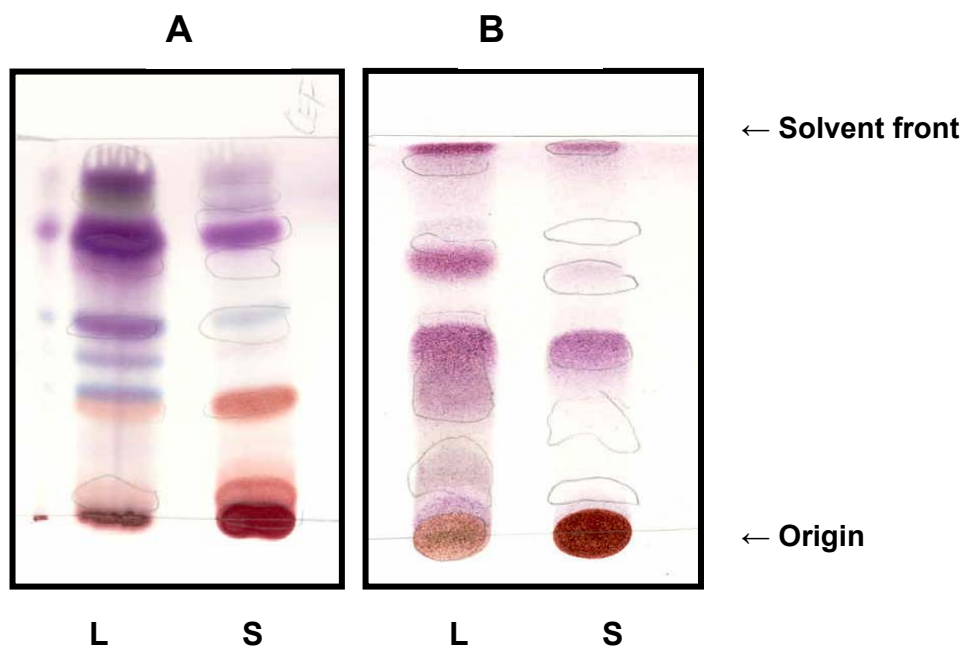


Figure 3.16. Comparison of the chemical components present in leaves (L) and stem bark (S) of *Curtisia dentata*. TLC plates were developed in CEF (A) and DE (4:1) (B) and sprayed with vanillin-sulphuric acid.

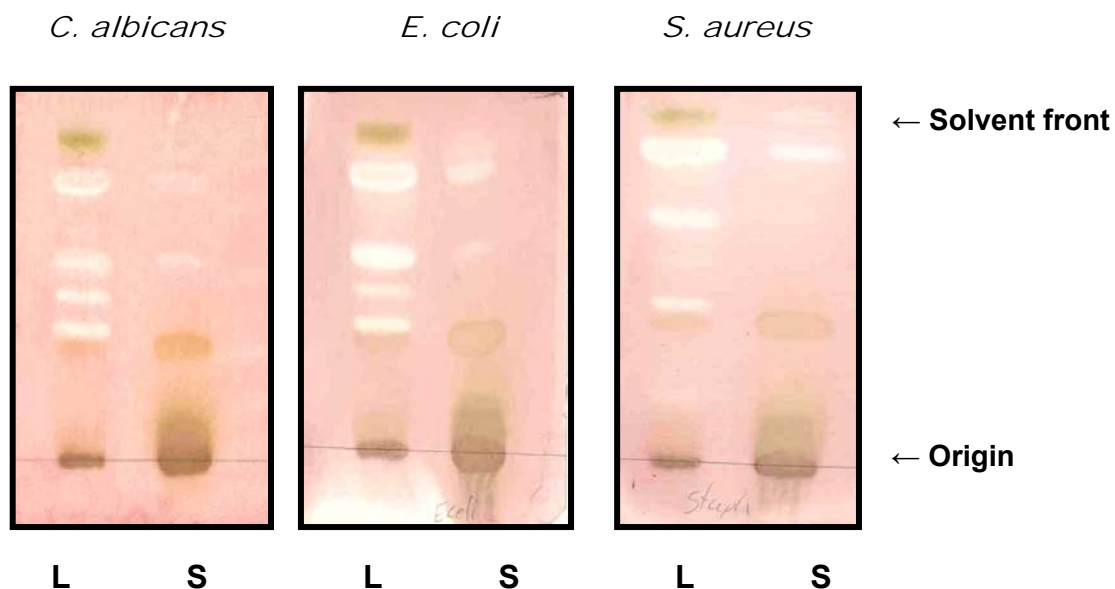


Figure 3.17. Comparison of the antibacterial chemical components present in leaves (L) and stem bark (S) of *Curtisia dentata*. Acetone extracts of leaves and bark were analysed on TLC plates using CEF as eluent, and then sprayed with *C. albicans*, *E. coli* or *S. aureus*.

Table 3.6. R_f values of components active against *Candida albicans* and bacterial test organisms in leaf and stem bark acetone extracts of *Curtisia dentata* using CEF as eluent.

Leaf extract	Stem bark extract
0.88	-
0.85	0.85
0.54	0.54
0.44	-
0.36	-

3.3.3. Minimal inhibitory concentrations

From the results of the microdilution assay the leaf extract was more active than the bark extract (Table 3.7). The MIC recorded against *Candida albicans* for acetone extracts of the leaves of *Curtisia dentata* was 0.11 mg/ml, while that resulting from the bark extract averaged at 0.61 mg/ml. The total activity of the leaf extract was more than five-fold higher than that of the bark extract, at 1072 ml and 190 ml, respectively (Table 3.7). MIC values against bacteria were not determined. Based on the number of antifungal and antibacterial compounds showing on bioautograms and the MIC values, leaves were chosen for isolation of active compounds.

The acetone extracts of the leaves, twigs and stem bark were investigated for antibacterial activity using the serial microplate dilution method to determine the MIC values. All the extracts were active against the four selected bacterial test organisms with average MIC values ranging from 0.16-0.30 mg/ml. The extracts of the leaves and twigs showed similar MIC values against *P. aeruginosa* and *Enterococcus faecalis*. These extracts resulted in lower MIC values compared with those resulting from stem bark extracts. Total activity values further confirmed that the aerial parts were more active than the stem bark extracts. *S. aureus* and *E. coli* were less sensitive to the extracts while *E. faecalis* was the most sensitive bacterial species. The highest total activity (1311 ml) was observed with the acetone extracts of the leaves against *Enterococcus faecalis* (Table 3.8).



Table 3.7. MIC values (mg/ml) of *Curtisia dentata* leaf and stem bark acetone extracts against *Candida albicans*. Amp B, amphotericin B

Plant Part	MIC (mg/ml)	Mass of extract (mg/g)	Total activity (ml/g)
Stem bark	0.61	116	190
Leaves	0.11	118	1072
Amp B	0.02	-	-

Table 3.8. MIC values (mg/ml) of the extracts of leaves, twigs and stem bark of *Curtisia dentata* against bacterial test organisms.

Bacterial species	Plant extracts		
	Leaves	Twigs	Stem bark
MIC values (mg/ml)			
<i>E. coli</i>	0.56	0.32	0.60
<i>S. aureus</i>	0.21	0.12	0.30
<i>P. aeruginosa</i>	0.12	0.11	0.15
<i>E. faecalis</i>	0.09	0.10	0.15
<i>Average MIC values</i>	0.25	0.16	0.30
Total activity (ml)			
<i>E. coli</i>	210	359	387
<i>S. aureus</i>	562	958	387
<i>P. aeruginosa</i>	983	1045	773
<i>E. faecalis</i>	1311	1150	773
<i>Average total activity</i>	767	878	580

3.4. DISCUSSION

The selected plant species, or plant species closely related to them, in the case of *Cussonia zuluensis* and *Vepris reflexa*, have a long history of usage in traditional medicine by different cultures in Africa. These plants are used to treat many diseases arising from diverse origins. The extracts of many plants are administered without

prior knowledge of their chemical compositions, toxicity and efficacy. This study seeks to address these and other concerns.

From the results it would seem that the acetone and dichloromethane extracts of the leaves of the different plant species screened contain antibacterial and antifungal activity. This observation was demonstrated through bioautography and MIC determination. The presence of active chemicals in the extracts supports the continued usage of the plant species under investigation, in traditional medicine.

All the extracts of *Terminalia sambesiaca* and *Terminalia phanerophlebia* showed very high activity against *Cryptococcus neoformans* and *Microsporum canis*, with MIC values ranging from 0.02 to 0.08 mg/ml. *Cussonia zuluensis* and *Vepris reflexa* were the least active. Masoko *et al.* (2005) reported MIC values comparable with the values obtained in this study for *Terminalia sambesiaca* extracts. In this case, as reported by Masoko *et al.* (2005), *Aspergillus fumigatus* was the least sensitive of the test microorganisms while *Cryptococcus neoformans* and *Microsporum canis* were the most sensitive.

Curtisia dentata possessed more active chemical components (five) than any of the species screened, as revealed by bioautography. These compounds were active against all the test organisms used in the investigation, suggesting the non-selective nature of the activity. This may suggest that the target macromolecule for the compound may be generally found across the species investigated or the active compounds are general metabolic toxins. The active components in the acetone extract of the leaves of *Curtisia dentata* range from polar to non-polar in nature. Their R_f values were 0.33, 0.44, 0.54, 0.80 and 0.85 and they all stained purple to red on TLC plates sprayed with vanillin-sulphuric acid. These compounds were also present in the DCM extract of *Curtisia dentata* leaves. Though the other plant species screened showed activity on TLC bioautograms, none of them had more than 2 compounds inhibiting the growth of the test organisms.

The presence of active compounds in *Curtisia dentata* was not unexpected. Other members of the family Cornaceae are active against several test microorganisms. For example, Dulger and Gonuz (2004) showed that ethanol extracts of *Cornus mas* were active against *P. aeruginosa*, *Proteus vulgaris* and *Micrococcus luteus*.

Though *Trichilia emetica* showed minimal antimicrobial activity in our study, other activities of this plant were reported elsewhere. Compounds with larvicidal and insecticidal activities were isolated from this species (Xie *et al.*, 1994; Nakatani *et al.*, 1981; Kubo & Klocke, 1982). Cyclooxygenase inhibitory activity was also reported (McGaw *et al.*, 1997). Sparg *et al.* (2000) reported activity against schistosomula worms. However, Germano *et al.* (2005) reported that ethyl ether extracts of *Trichilia emetica* were active against *S. aureus* and other bacterial species, with MIC of 15.6 – 31.25 µg/ml recorded against *S. aureus*. They attributed the activity to limonoids, which, they claimed, were extracted with ethyl ether.

The acetone extract of *Terminalia sambesiaca* was active against all test organisms except *E. faecalis*. Fyhrquist *et al.* (2002) reported activity of the methanol extract of *Terminalia sambesiaca* roots and stem against *S. aureus* and *Candida albicans* on hot-plate agar diffusion method. The MIC recorded in this study, 0.18 mg/ml against *Candida albicans*, is comparable with published results (Masoko *et al.*, 2005).

Though *Terminalia sambesiaca* showed activity against *Candida albicans* using the microplate dilution method, the separated active compounds in the acetone extract were not visible on bioautography.

The reasons for low activity of some plant species may vary. Most of the traditional preparations are made using water as the extractant. Water extracts should be investigated to fully confirm antimicrobial activity of the traditional preparations of the plants under study. All evidence in the Phytomedicine Programme to date support the results of Kotze and Eloff 2000 that water extracts generally do not have antibacterial activity. Acetone was used in this study since it extracts a wide range of compounds of varying polarities. Eloff (1998a) reported that acetone is the best extractant as it extracts more antimicrobial compounds than other commonly used solvents.

The selectivity index is calculated to determine the relationship of activity of a test product to its cytotoxic concentration. The higher the number the better the product. The selectivity index obtained for amphotericin B was by far the highest compared with acetone and dichloromethane extracts of *Curtisia dentata*. It would mean that amphotericin B results in the highest inhibition of fungal growth with relatively lower toxicity to host cells. Though both extracts of *Curtisia dentata* resulted in relatively low selectivity indexes, the acetone extract was 16 times better than the dichloromethane extract. The acetone extract is therefore more active against fungal

species tested with less toxicity than the dichloromethane extract. Since the same compounds were active against both bacterial and fungal test organisms, it would seem that the activity may be nonspecific and non-selective, hence the low selectivity index values.

The leaf and stem bark acetone extracts of *Curtisia dentata* were also tested for activity against bacterial test organisms using the bioautography method. Both extracts were active against bacterial test organisms (*S. aureus* and *E. coli*). More active compounds were observed in the acetone extracts of leaves (4 compounds) than the bark extracts (2 compounds). The total activity of the leaves was five-fold higher than that of the bark, further pointing to the leaves being more active. Some literature reports suggest that the phytochemical constituents of the bark and leaves are similar for certain species (Zschocke *et al.*, 2000a; Zschocke *et al.*, 2000b). However, Eloff (2001) observed that there were significant differences between the antibacterial activities of leaves and bark of *Sclerocarya birrea*. This finding may suggest that leaves may be used to replace stem bark in traditional medicine preparation for treatment of bacterial and fungal-related infections as the plant species is threatened (Cunningham, 1998; Scott-Shaw, 1999). Based on these findings the leaves of *Curtisia dentata* were chosen for further work.

The bark and leaf extracts of *Curtisia dentata* were also tested for anti-*Candida* activity using the serial microplate dilution method. The MIC values questioned the preference of bark over leaves in preparation of traditional medicine cocktails using *C. dentata*. The MIC values of leaf extracts were five-fold better than those of the extracts of the bark, as were the total activity values. In other words, five times as much of the bark extract is required to achieve similar results as the leaf extract. The comparison of the water extracts of both the leaves and the stem bark may serve to further encourage harvesting of leaves and not the bark for traditional medical purposes thereby conserving *C. dentata* trees in the wild.

3.5. CONCLUSIONS

All the plant species tested showed varying degrees of activity against test organisms in the study. Bioautography results revealed that *Curtisia dentata* contained more antibacterial and antifungal compounds than any of the plant species screened. Similar chemical components from *Curtisia dentata* were active against all the microbial species tested, suggesting the non-specific nature of the activity. MIC data



indicated that *Terminalia sambesiaca* and *Terminalia phanerophlebia* were the most active of the plant species tested against fungi, while *Vepris reflexa* and *Cussonia zuluensis* were the least active. *Aspergillus fumigatus* was the least sensitive fungal test organism to extracts while *M. canis* and *C. neoformans* were the most sensitive. The leaves of *C. dentata* can be used to replace the stem bark in the preparation of traditional herbal medicines, thereby conserving this threatened medicinal plant species. The leaves contained more active compounds and resulted in lower MIC values compared with the stem bark extracts.

Following the finding that *Curtisia dentata* possessed more active compounds against *Candida albicans* with low MIC values, the next step involved isolation of the active compounds from the leaves of this species.

CHAPTER 4

ISOLATION OF ANTIFUNGAL COMPOUNDS FROM *C. dentata* (Burm.f) C.A. Sm.

4.1. INTRODUCTION

4.1.1. Compounds isolated from Cornaceae family

Reports concerning isolation of compounds from *Curtisia dentata* are scanty, if not unpublished. From other members of the family Cornaceae several phenolic compounds, flavonoids and terpenoids have been isolated (Jang *et al.*, 1998; Nakaoki & Moira, 1958; Lee *et al.*, 2000). The genus *Cornus* is well known to contain large amounts of polyphenols such as tannins (Hatano, 1989). Several iridoid compounds were isolated from the roots of *Cornus capitata* (Tanaka *et al.*, 2001). Lee *et al.* (2000) reported the isolation of two phenolic compounds, namely, (-)-2,3-digalloyl-4-(*E*)-caffeoyl-L-threonic acid and (-)-2-galloyl-4-(*E*)-caffeoyl-L-threonic acid from fresh leaves of *Cornus controversa* Hemsl (Cornaceae). Several anthocyanins are also present in *Cornus florida*, *C. controversa*, *C. kousa* and *C. florida* (Vareed *et al.*, 2006).

The broad aim of this study was to isolate compounds active against *Candida albicans* from extracts of *Curtisia dentata*.

The objectives of this section were to:

- 1) Compare the activity the extracts of the leaves and stem bark of *C. dentata* in order to select the plant part to isolate compounds from.
- 2) Isolate active compounds from the leaves through bioassay-guided fractionation.
- 3) Identify the isolated compounds using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS).

4.2. MATERIALS AND METHODS

4.2.1. General methods

4.2.1.1. Plant part collection

Leaves and stem bark of *C. dentata* were harvested from the University of Pretoria's Botanical Gardens in March 2005, dried at room temperature and ground to powder using a Macsalab mill (Model 200 Lab). The powders were stored in sealed glass containers at room temperature, in the dark until needed.

4.2.1.2. Serial exhaustive fractionation

The leaves and stem bark extracts of *C. dentata* were compared to select the best plant part for isolation of compounds. The leaves contained more active compounds and resulted in better MIC values than the stem bark extracts. For isolation of active compounds, serial exhaustive extraction of the leaves of *C. dentata* leaves (830 g) was performed as outlined in Chapter 2.

4.2.2. Overview of approach followed

The procedure used to isolate active compounds from the leaves of *Curtisia dentata* is presented schematically in Figure 4.1.

4.2.2.1. Selection of stationary phase

Column chromatography using silica gel as the stationary phase was chosen for separation of compounds for the following reasons: 1) silica gel was previously used in the Phytomedicine laboratory for isolation of compounds with a high degree of success (Martini and Eloff, 1998), 2) separation on silica gel represents one of the cheapest methods for isolation of compounds, 3) silica gel is readily accessible and preparation of separating systems is simple and quick, and 4) eluent system polarity can be varied to adjust elution of active compounds.

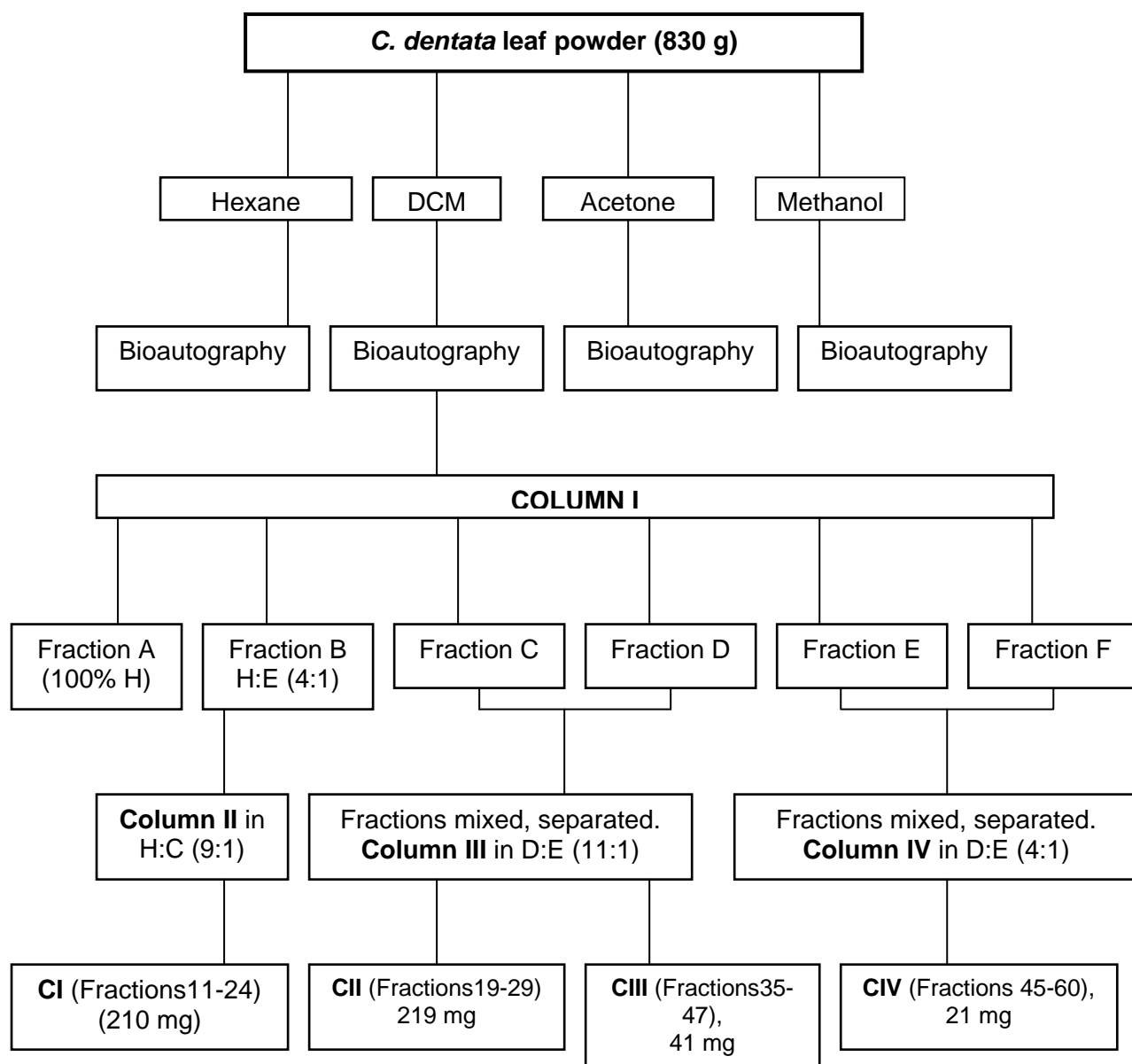


Figure. 4.1. Schematic diagram showing the isolation of active compounds from *Curtisia dentata* leaves using column chromatography with silica gel 60 as the stationary phase.

4.2.2.2. Group separation (Column I)

Silica gel (800 g) was mixed with hexane to form a homogenous suspension/slurry and stirred using a stirring rod to remove bubbles. The silica gel slurry was poured into a glass column (10 cm diameter and 50 cm length) whose outlet was plugged with cotton wool to retain the gel in the column. The solvent was allowed to flow out of the column opening to allow the gel to settle.

The plant extract sample was prepared by dissolving 15 g of the DCM extract in 100 ml of ethyl acetate. To the solution 30 g of silica was added and mixed by stirring with a glass rod. The mixture was allowed to dry at room temperature under a stream of air for approximately 5 hours. The dried silica gel-extract mixture was carefully layered on the column gel bed. For elution hexane was used as the mobile phase with the polarity increasing by 10% increments of ethyl acetate. For each eluent mixture 1.5 L volumes were used and 500 ml fractions collected in glass beakers. Collected fractions were concentrated using a Büchi R-114 Rotavapor. TLC was used to analyze fractions, and those with similar chemical components were combined.

Thirty three (33) fractions were collected and concentrated using a Rotavapor. These fractions were again analyzed by TLC, and those containing similar components were mixed, resulting in 6 major fractions (A – F). These major fractions were dried in pre-weighed beakers and their masses determined.

4.2.2.3. Combination of fractions from column I (DCM extract)

Fractions 1-3 were combined to form major fraction A, as they contained similar components when analyzed using TLC. Fractions 4 & 5 were combined to result in major fraction B. Fractions 6 & 7 were also combined to yield major fraction C. Fraction 8 was not combined with any fraction, and it was designated as fraction D. Fractions 9-14 were combined and the resultant mixture was called fraction E. Fractions 15-33 were combined and to result in fraction F. The antimicrobial activities of the fractions were assayed as described in chapter 2, using bioautography as the method of choice with *Candida albicans* as the test fungal organism. In most instances active compounds were found in more than one fraction. These fractions were combined to maximize the level of active compounds in order to obtain a high yield of the compounds.

4.2.2.4. Isolation of compound CI (Column II)

A glass column with a length of 50 cm and diameter of 2 cm was used for fractionation of fraction B. Silica gel 60 (150 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out for the silica gel to settle and establish a column bed. The sample was prepared by mixing 2 g of fraction B with 4 g silica gel

in 60 ml of ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was loaded onto the gel in the column. After a series of separations of the fraction on TLC plates in different solvent mixtures to obtain a system resulting in good resolution, hexane: chloroform (HC) (9:1) was selected as the solvent system. The loaded column was eluted using HC (9:1) mixture with 50 ml fraction volumes collected at 5 ml/min. Fractions 11-24 contained pure compound **C1**.

4.2.2.5. Isolation of compound CII and CIII (column III)

Fractions C and D were mixed and from this mixture compounds CII and CIII were isolated. A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of the mixture of fractions C and D. Silica gel (200 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out to settle the gel and establish a column bed. The sample was prepared by mixing 3 g of fractions C and D mixture with 6 g silica gel in 90 ml ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was then loaded onto the gel bed and eluted with dichloromethane:ethyl acetate (11:1) mixture at 5 ml/min. Fifty ml fractions were collected. Fractions 19-29 contained pure compound **CII** and fractions 35-47 contained pure compound **CIII**.

4.2.2.6. Isolation of compound CIV (column IV)

Fraction E – F were combined and the mixture used for isolation of compound CIV. A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of the mixture. The column was prepared as in **section 4.2.2.5**. The sample was prepared by mixing 3 g of fractions E and F mixture with 6 g silica gel in 90 ml ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was then loaded onto the gel bed and eluted with dichloromethane:ethyl acetate (4:1) mixture with polarity increased by 10% increments of ethyl acetate. Fifty ml fractions were collected at 5 ml/min.

4.2.2.7. Isolation of compound HI (Column V)

The hexane fraction (2 g) was subjected to column chromatography using toluene as a solvent (mobile phase). A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of a mixture. About 200 g of silica gel was mixed with

toluene and slurry poured into the column whose bottom end opening was plugged with cotton wool. The sample was prepared by dissolving 2 g of hexane extract in 10 ml hexane and mixed with 4 g silica gel. The mixture was dried under a stream of air at room temperature. The dry mixture was carefully layered onto the column and eluted with toluene. Fifty-milliliter (50 ml) fractions were collected in glass test tubes. Similar fractions were combined. Fractions 14-19 contained pure compound **HI**.

4.3. RESULTS

4.3.1. Serial exhaustive fractionation

The serial exhaustive extraction of *C. dentata* leaves is described in section 2.2.3. The same plant material was extracted with different solvents, starting with the least polar, hexane (H), with methanol (M) completing the extraction. The masses of extracts obtained with each solvent are displayed in Fig. 4.1. Acetone and methanol resulted in the largest mass (24 and 21 g, respectively) of extracted chemical components and the least amount (13 g) was obtained with hexane.

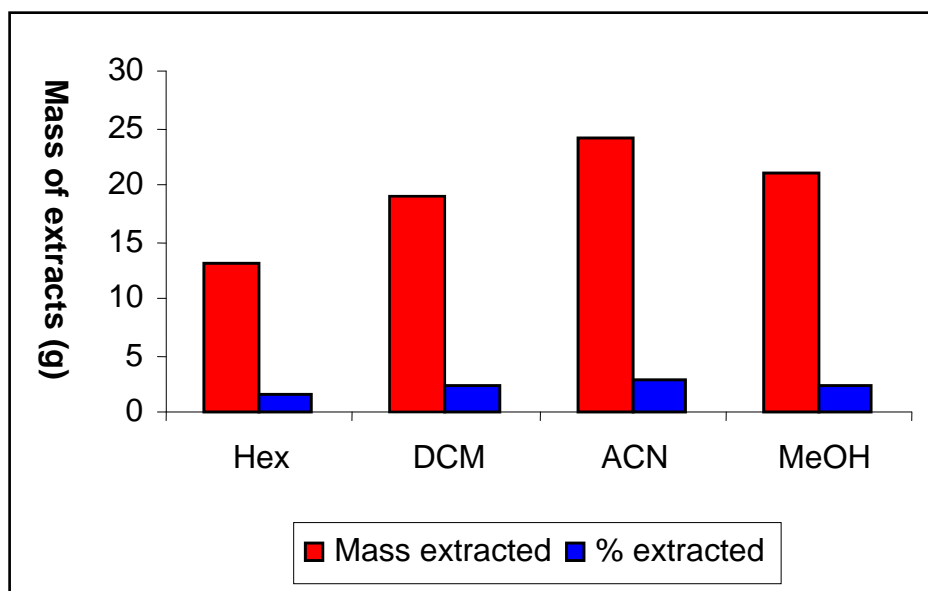


Figure 4.1. Amount extracted from *Curtisia dentata* leaves (837 g) using solvents of varying polarities. The dried leaf material was extracted serially with hexane (HEX), dichloromethane (DCM), acetone (ACN) and methanol (MeOH) in the order listed.

4.3.2. Activity of serial exhaustive extraction samples

4.3.2.1. Anti-*Candida* activity

Extracts acquired from serial exhaustive extraction of *Curtisia dentata* leaves were investigated to determine the presence of active compounds using bioautography and serial microplate dilution. Investigation of activity against *C. albicans* revealed that the acetone and dichloromethane extracts of the leaves of *Curtisia dentata* contained the highest number of active compounds (Fig. 4.2). The active compound in the hexane fraction was also observed in the dichloromethane extract. The methanol extract was not active against *Candida albicans* in bioautography.

The lowest MIC value recorded (0.107 mg/ml) against *C. albicans* was observed with the dichloromethane extract of *C. dentata* leaves (Table 4.1). The hexane extract did not show any observable activity against *C. albicans*, with an MIC value of over 2.5 mg/ml. Acetone and methanol extracts resulted in MIC values of 0.207 and 0.50 mg/ml, respectively. The highest total activity (4240 ml) recorded per bulk fraction resulted from the dichloromethane fraction, whilst the hexane extract resulted in the least total activity (79.3 ml) (Table 4.1). The methanol and the acetone fractions resulted in total activity values of 1050 and 3312 ml, respectively.

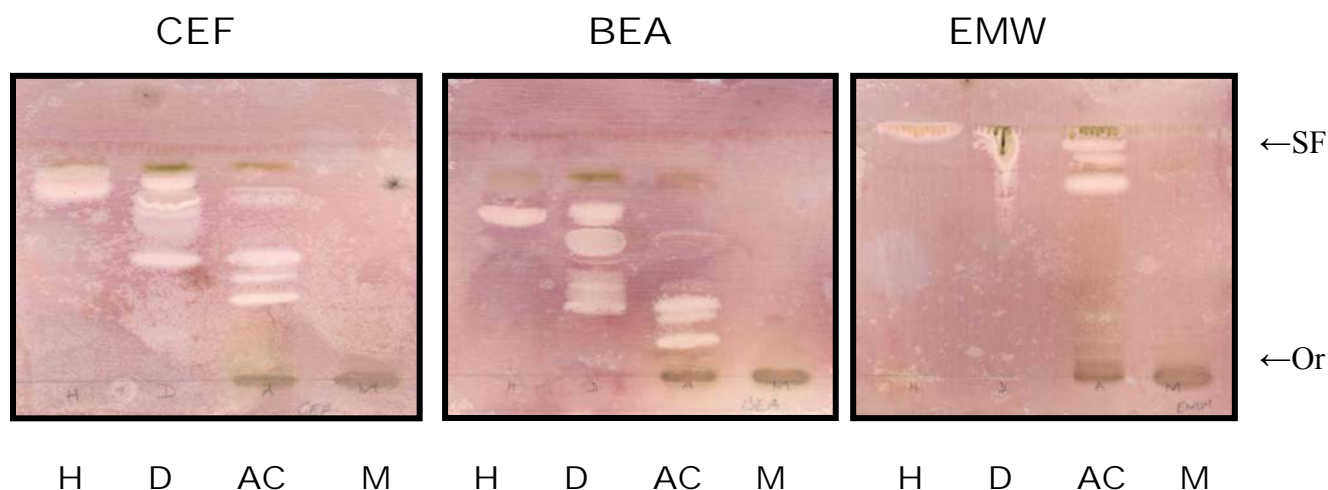


Figure 4.2. TLC plates showing growth inhibiting activity of *Curtisia dentata* leaf extracts against *Candida albicans*. Plant material was extracted with hexane (H), dichloromethane (D), acetone (AC) and methanol (M). SF, solvent front; Or, origin

Table 4.1. Minimal inhibitory concentrations and total activity of various bulk fractions of *C. dentata* against *C. albicans*

Fraction	MIC (mg/ml)	Total Activity (ml)/fraction
Hexane	>2.5	79.3
Dichloromethane	0.107	4240
Acetone	0.207	3312
Methanol	0.50	1050

4.3.2.2. Antibacterial activity

Bioautography is generally much more difficult with fungi than with bacteria. To facilitate the bioassay guided fractionation, bioautography was carried out with four important nosocomial bacteria to determine if the same compounds are active against *C. albicans*. If this is the case, it is easier to use bacterial cultures in the isolation work.

The serial exhaustive extracts of *Curtisia dentata* leaves were also tested for activity against bacterial test organisms using bioautography resulting from serial exhaustive extraction. Dichloromethane and acetone extracted more chemical components active against all test bacterial organisms. The methanol extract had the lowest number of antibacterial compounds against the four bacterial organisms under investigation (Fig. 4.3). Based on these findings, and that the dichloromethane extract contained high numbers of active compounds, the dichloromethane extract was chosen for further fractionation to isolate active compounds.

In the previous Chapter it was shown that the same compounds that were active against *C. albicans* on bioautograms had activity against bacterial species, hence in bioassay-guided fractionation bacteria may be used to follow the active compounds. The TLC plates of the extracts sprayed with vanillin-sulphuric acid are shown on Figure 4.4, revealing the different compounds extracted with each of the four extractants.

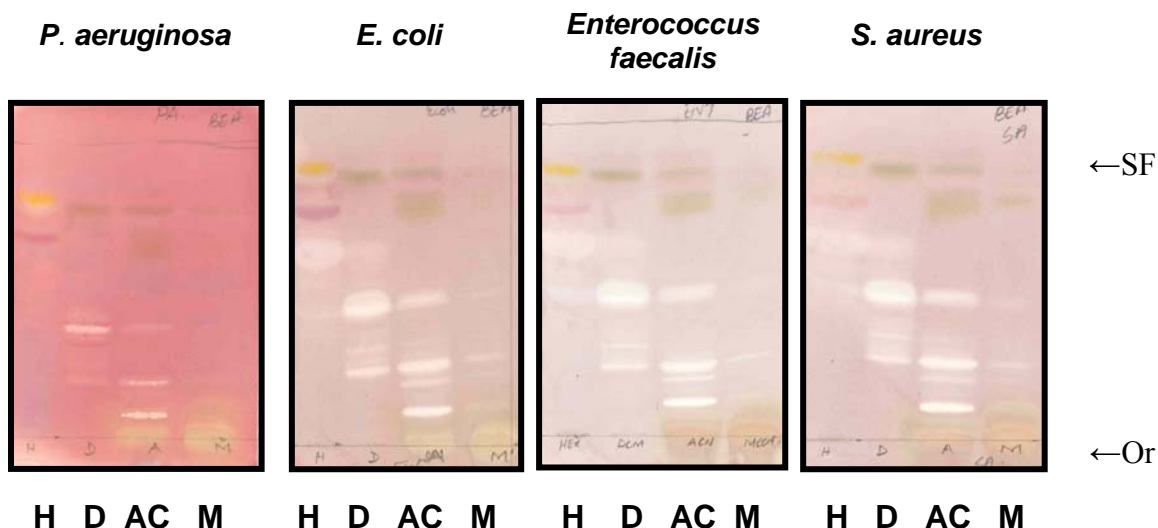


Figure 4.3. TLC bioautograms (BEA as eluent) showing activity of hexane (H), dichloromethane (D), acetone (AC) and methanol (M) extracts of *Curtisia dentata* against *P. aeruginosa*, *E. coli*, *Enterococcus faecalis* and *S. aureus*. SF, solvent front; Or, origin

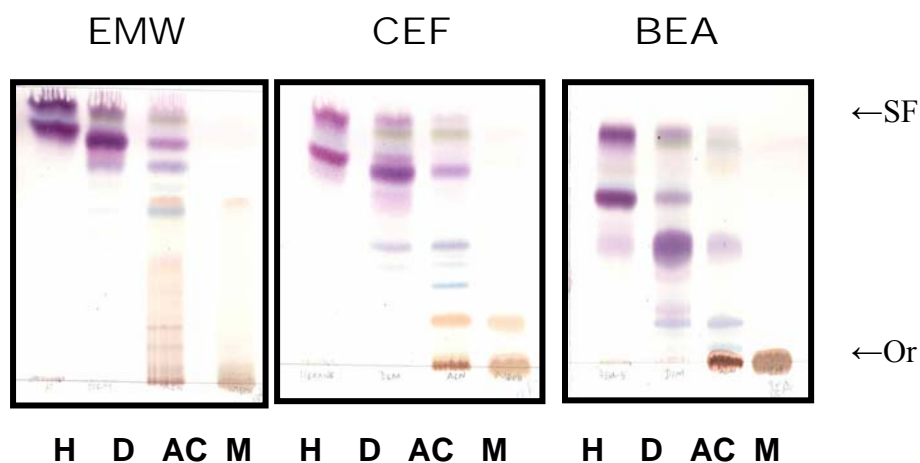


Figure 4.4. TLC chromatograms of serially extracted *C. dentata* leaves developed in EMW, CEF and BEA. Plant material was extracted with hexane (H), dichloromethane (D), acetone (AC) and methanol (M). SF, solvent front; Or, origin.

4.3.3. Isolation of compounds

From column I several fractions were collected and analyzed on TLC plates (Figs. 4.5 & 4.6). Similar fractions were combined into six major fractions A-F. The masses

of the different major fractions are displayed in Table 4.2. These fractions were tested for antifungal activity (Fig. 4.7 and Table 4.3). Active fractions were identified for isolation of anti-*Candida* compounds. Fractions C-F were active against *Candida albicans* on bioautograms (Fig. 4.7).

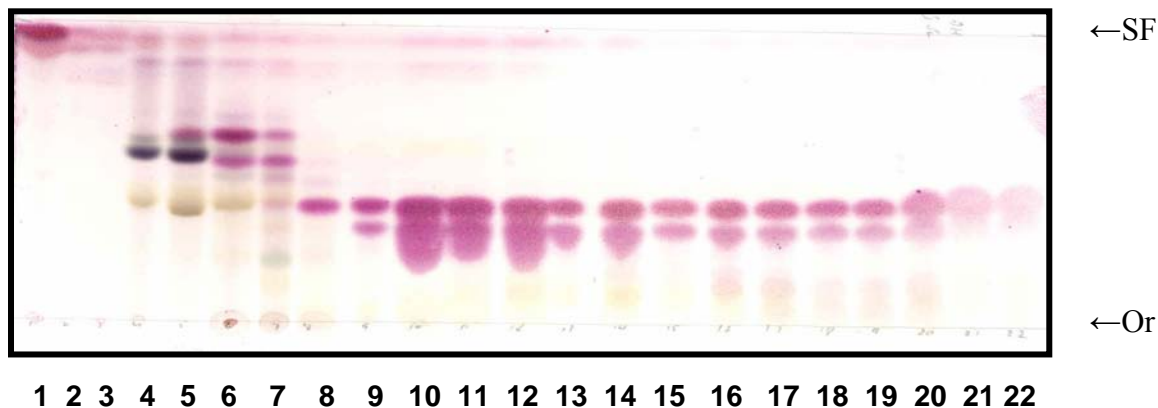


Figure 4.5. Fractions collected from separation of dichloromethane extract of *Curtisia dentata* leaves (fraction 1-22). The sample was first eluted with hexane and polarity was increased with 20% increments of ethyl acetate and then methanol. TLC plates were developed in hexane : ethyl acetate (7:3) and visualized using vanillin-sulphuric acid spray. SF, solvent front; Or, origin.

Fraction 11-24 from column II was a yellow solution, and a white precipitate formed. The precipitate was collected and washed several times with hexane. The dry compound **CI**, white in colour had a mass of 210 mg. The TLC chromatogram of the isolated compound CI is displayed in Fig. 4.8.

Fractions 19 – 29 from column III contained a single spot on TLC plates sprayed with vanillin-sulphuric acid and were pooled together and dried using a Rotavapor (fig. 4.9). The mass of the compound **CII** was recorded as 219 mg. Fractions 35 – 47 contained a single compound, which stained purple-red on TLC plates sprayed with vanillin-sulphuric acid. These fractions were combined and dried using a Rotavapor. The resultant compound **CIII**, creamy-white in colour had a mass of 41 mg (Fig. 4.9).

Fractions 45 – 60 from column IV contained a compound with some minor impurities on TLC chromatograms. These fractions were combined and solvent evaporated using a Rotavapor. The white powder was washed several times with hexane and then dried. The recorded mass of the compound **CIV** was 21 mg.

From column V, 36 fractions were collected. Fractions 14 -19 contained a single compound after separation on TLC plates (Fig. 4.10). These fractions were combined and dried using evaporation under a stream of air. The resultant compound was washed several times in methanol, dried and weighed. The resulting compound **HI**, 86 mg, was a white powder that stained purple upon spraying with vanillin-sulphuric acid.

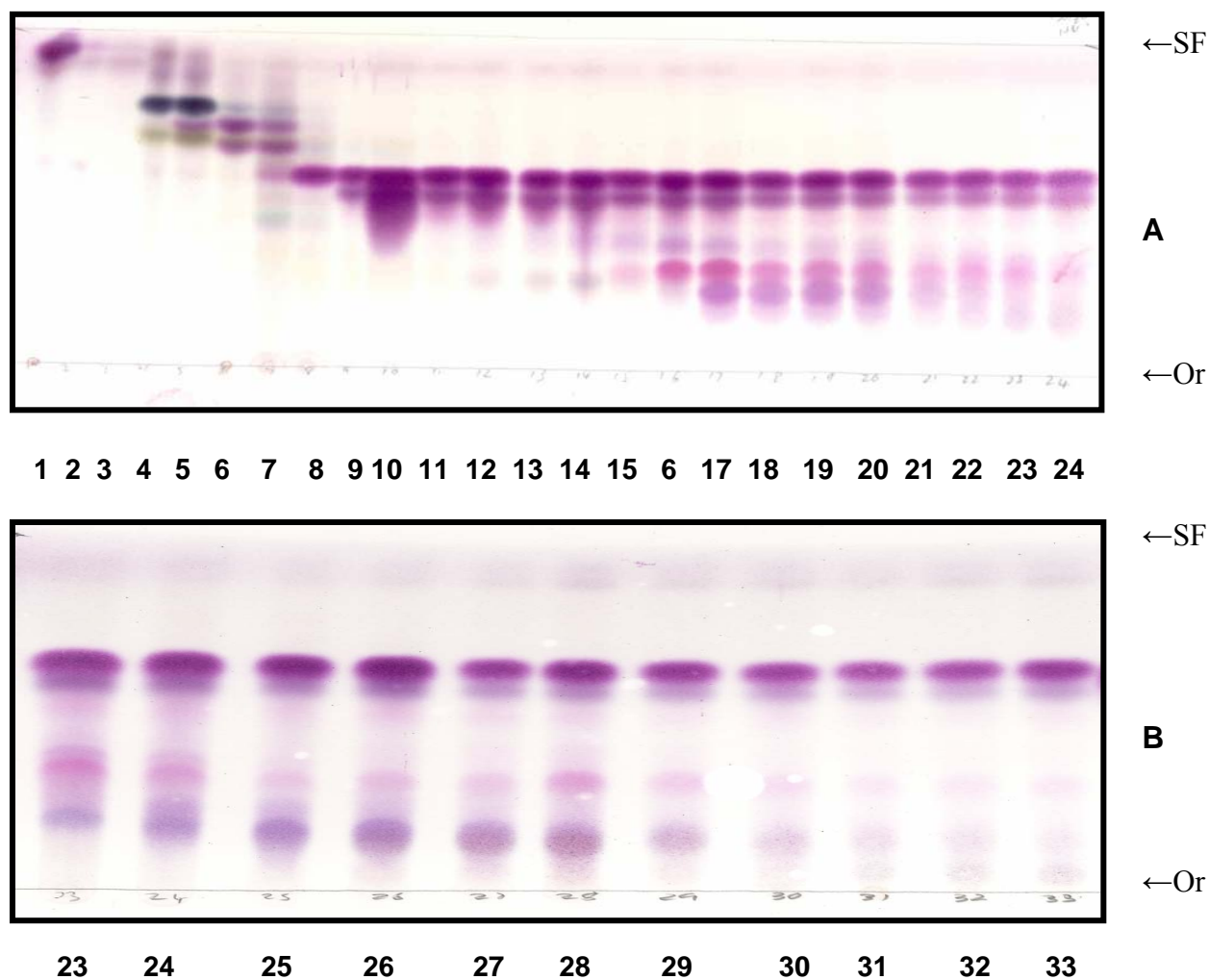


Figure 4.6. Fractions collected from separation of dichloromethane extract of *C. dentata* leaves (fraction 1-24 [A] and 23-33 [B]). TLC plates were developed in hexane: ethyl acetate (1:1) and visualized using vanillin-sulphuric acid. SF, solvent front; Or, origin.

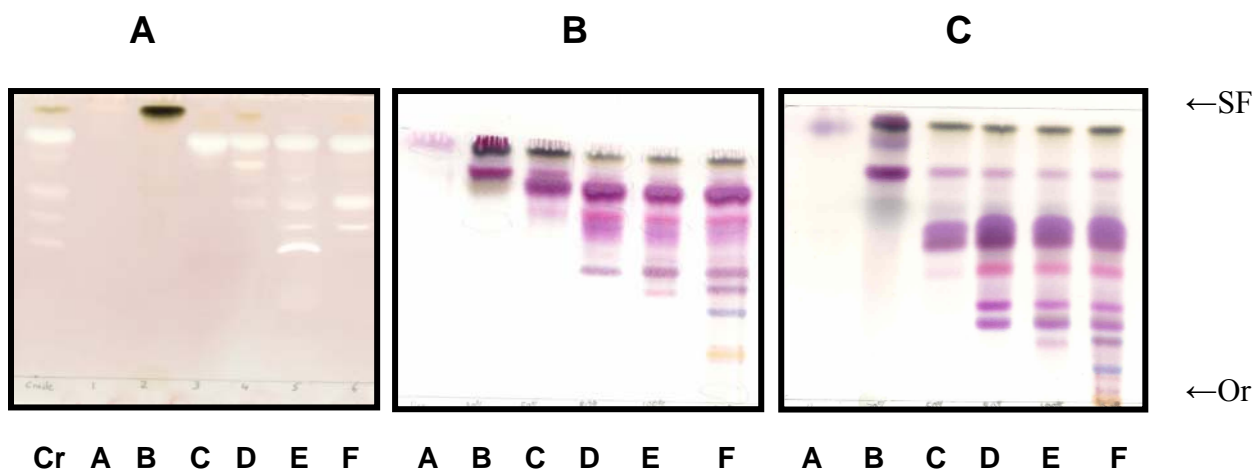


Figure 4.7. TLC bioautograms (A) (CEF) showing activity of fractions A-F against *Candida albicans*, and vanillin-sprayed TLC plates developed in CEF (B) and BEA (C). The dichloromethane crude extract (Cr) was tested alongside various fractions using the TLC bioautography method. SF, solvent front; Or, origin.

Table 4.2. Mass (g) of the major fractions from column I

Column I Fraction	No. of active compounds	Mass (g)
A	0	1.45
B	1	2.15
C	1	2.50
D	4	1.68
E	5	2.09
F	5	2.22

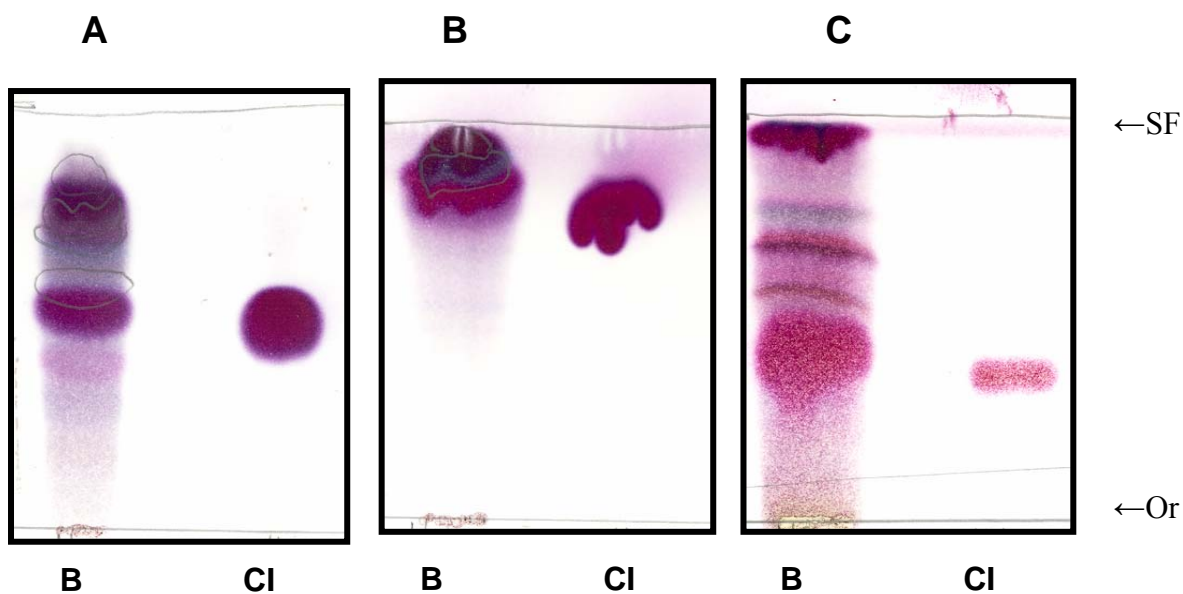


Figure 4.8. TLC chromatograms showing compound CI analysed side-by-side with fraction B from column I. The TLC plates were developed in BEA (A), CEF (B) and dichloromethane (C), and sprayed with reagent vanillin-sulphuric acid reagent. SF, solvent front; Or, origin.

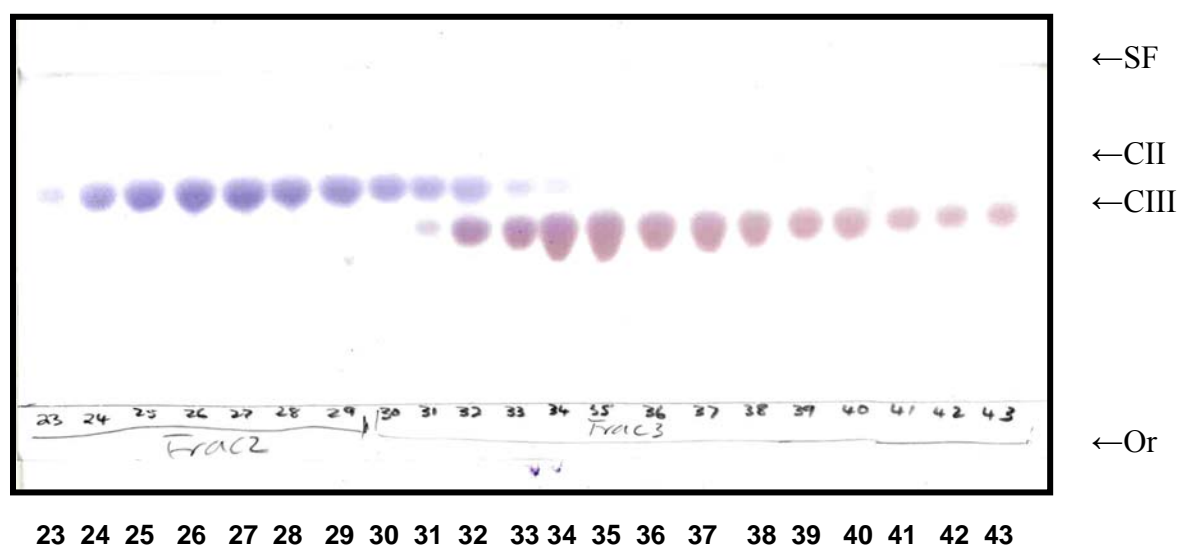


Figure 4.9: TLC chromatogram showing separation of CIII from CII in column III. The TLC plates were developed in HE (1:1) and then sprayed with vanillin sulphuric acid. Only fractions 23 – 43 are shown on the chromatogram. SF, solvent front; Or, origin.

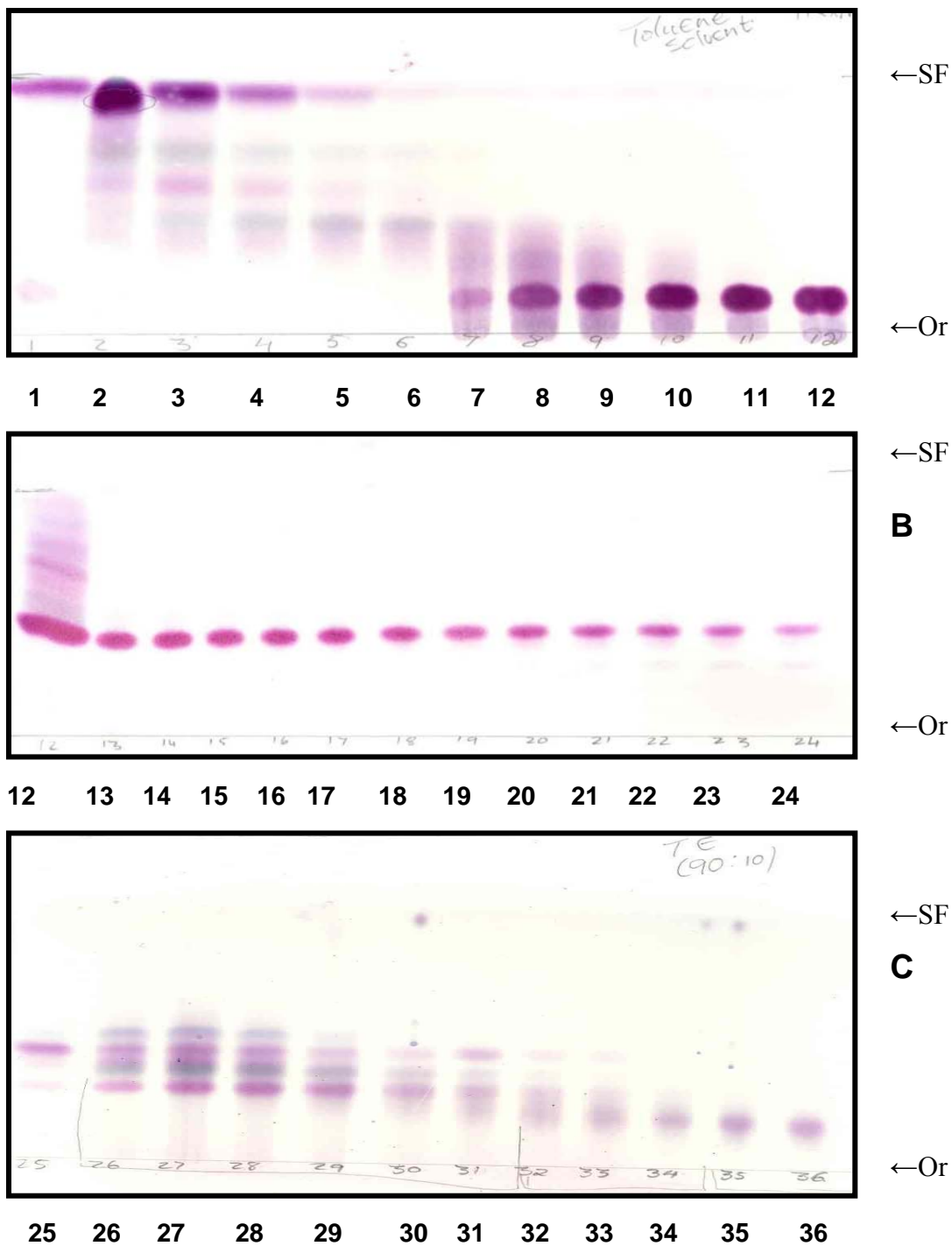


Figure 4.10. Fractions 1 – 36 collected from separation of hexane extract of *Curtisia dentata*. The sample was first eluted with toluene. TLC plates were developed in toluene (A) and toluene: ethyl acetate (9:1) mixture (B, C) and visualized using vanillin-sulphuric acid. SF, solvent front; Or, origin.

4.4. DISCUSSION

The plant species used, *Curtisia dentata*, is utilized for treatment of different health problems by traditional cultures in South Africa. The stem bark is the preferred plant part for preparation of medicines.

4.4.1. Serial exhaustive extraction of *Curtisia dentata* leaves

A higher number of active compounds was present in the acetone extracts of the leaves of *Curtisia dentata* compared to that the bark. The same powdered leaf material was serially extracted with solvents of varying polarities, starting with the least polar (hexane) and ending with methanol. The extracted material was subjected to bioautography experiments with bacterial and fungal species as test organisms. Bioautography results revealed that the highest number of active compounds was present in the dichloromethane and acetone extracts. The bulk methanol fraction showed no visible presence of antimicrobial compounds on bioautograms against *Candida albicans* and bacterial test organisms. The absence of active compounds in the methanol extract is not clear, though it may seem that active compounds were removed by acetone before methanol could be used as the extractant since serial exhaustive extraction was used.

Though the presence of active compounds was observed in the hexane extract against the five test organisms in bioautography, the MIC value recorded against *Candida albicans* was above 2.5 mg/ml. The antifungal compounds extracted by hexane may have been volatile and were lost during evaporation of the TLC solvents from the chromatograms. The lowest MIC values (0.11 and 0.21 mg/ml) resulted from the bulk DCM and acetone fractions against *Candida albicans*, further correlating the MIC data with bioautography results. Furthermore, the bulk DCM fraction resulted in the highest total activity calculated as 4240 ml/fraction. The total activity values for the bulk methanol, hexane and acetone fractions were 1050, 79.3 and 3312, respectively. This finding further emphasized that the hexane fraction was the least active while the acetone and the DCM fractions, with good MIC and total activity values were the most active fractions.

The methanol extract failed to reveal active compounds on bioautograms but was active in the serial microplate method with a MIC value of 0.5 mg/ml. The reasons for the discrepancy are unclear. Synergistic effects may be necessary for the activity of

some of the compounds, and thus serial exhaustive extraction may separate compounds that act together synergistically. Furthermore, some compounds may be volatile, resulting in their loss from TLC plates during drying for bioautography assay.

4.4.2. Isolation of compounds

Four compounds were isolated using column chromatography with silica gel as the stationary phase. The isolated compounds may belong to the same family of compounds as they stained similarly when sprayed with vanillin-sulphuric acid (purple-red colour). Compound **CI**, whose mobility on TLC was closer to the solvent front was the most non-polar whilst compound **CIV** was the most polar of the isolated compounds judging by its mobility in all the mobile phases used. This is the first report of isolation and identification of compounds with antifungal and antibacterial activity from *Curtisia dentata* leaves. Compound **HI** was later found to be the same as compound **CI**, as revealed by analysis on TLC plates.

4.5. CONCLUSIONS

Curtisia dentata has been used for many decades to treat different ailments. The findings of this study reveal that there are several antimicrobial compounds in the leaves of this plant species. Four compounds were isolated using silica gel as the stationary phase in column chromatography, following the principles of bioassay-guided fractionation. This is the first report of isolation of compounds from *Curtisia dentata*.

CHAPTER 5

STRUCTURE ELUCIDATION

5.1. INTRODUCTION

In the available literature, no information about compounds isolated from any *Curtisia* species was found. *Curtisia dentata* is the only species of the genus occurring in Africa. There is only one other member of the Cornaceae family occurring in Africa i.e. *Afrocrania volkensis* (Klopper *et al.*, 2006). Furthermore, there are few reports on chemical constituents of other genera within the family Cornaceae. Betulinic acid was isolated from the bark of *Cornus florida* Hook at a yield of 2% (Robertson *et al.*, 1939). Polyphenolic compounds such as tannins (Okuda *et al.*, 1984; Lee *et al.*, 1989) and iridoid compounds (Stermitz and Krull, 1998; Tanaka *et al.*, 2001) were isolated from some members of the genus *Cornus*. Different anthocyanins are present in extracts of *Cornus alterifolia*, *C. controversa*, *C. kousa* and *C. florida* (Vareed *et al.*, 2006). Triterpenoids such as A1 barringenol, oleanic acid (Silva *et al.*, 1968), 21,23-epoxytirucalla-7,24-diene-3-one and 3 β -acetoxy-21,23-epoxytirucalla-7,24-diene (Bhakuni *et al.*, 1987) are common in the genus *Cornus*. There are no literature records concerning isolation and characterization of chemical components from any *Curtisia* species. The aim of this chapter was to elucidate the structures of the isolated compounds and compare them with those isolated from other members of the Cornaceae.

5.2. MATERIALS AND METHODS

¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry were used to determine the structures of isolated compounds. In some instances DEPT (distortionless enhancement by polarization transfer) NMR was used to further confirm the structures of the compounds. The spectral data was first compared with data recorded in literature to determine whether the compounds were known or novel. The comparison included a search of compounds isolated from the Cornaceae family in which *Curtisia dentata* belongs.

5.2.1. Thin layer chromatography

Isolated compounds were analyzed by TLC separation using different solvent systems to ascertain their purity and determine R_f values. The compounds (10 μg) were loaded on aluminum-backed TLC plates and developed using CEF, BEA and DE (4:1) as mobile phases. The developed plates were visualized under UV light, sprayed with vanillin-sulphuric acid and then scanned.

5.2.2. ^{13}C and ^1H NMR spectroscopic analysis

^1H and ^{13}C NMR experiments were performed on a Varian 300 MHz (University of Limpopo, Medunsa Campus) spectrometer operating at 300.0 MHz for hydrogen and 75.4 for ^{13}C , using DMSO_{d6} or CDCl_3 as solvents. Solutions were prepared with between 18 – 30 mg of pure compounds in 0.5 ml of CDCl_3 for **CI** and **HI** or DMSO_{d6} for **CII**, **CIII** and **CIV**.

5.2.3. Mass Spectrometry

About 1 mg of each compound was submitted for mass spectrometric analysis at the University of Johannesburg. Electron impact mass spectrometry (EIMS) was used to analyze the isolated compounds.

5.3. RESULTS AND DISCUSSION

5.3.1. Thin Layer Chromatographic Analysis of Isolated Compounds

When BEA and CEF were used as mobile phases compounds **CII** and **CIII** migrated similar distances on TLC chromatograms, as confirmed by their R_f values (Table 5.1). Equal quantities (10 μg) were loaded for each compound on TLC plates. DE (4:1) resulted in effective separation of the two compounds (Fig. 5.1). This observation suggests that these two compounds fall within the same solubility range, are related and belong to probably the same family of compounds. Compound **CI**, which seemed non-polar as it migrated a similar distance with the solvent front, may also be related to **CII** and **CIII**, since they all stained red-purple upon spraying with vanillin-sulphuric acid. It is likely that they all belong to the same family and that the differences between them may be attributed to different chemical groups on the parent structure. Compound **CIV** is the most polar of the isolated compounds since it migrated the

shortest distance with the lowest R_f value (Fig. 5.1). From comparing concentration in the crude fractions, it appears that CII is present in the highest concentration in the crude fractions.

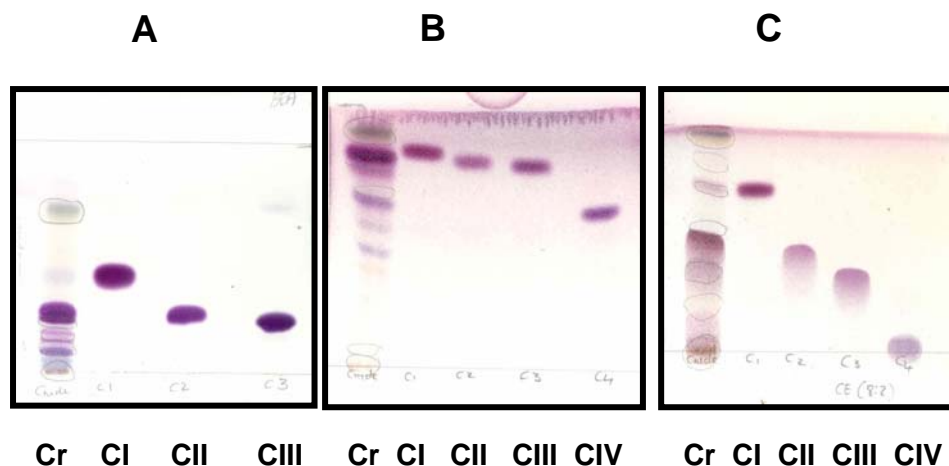


Figure 5.1. TLC analysis of the four isolated compounds (**CI**, **CII**, **CIII** and **CIV**) and dichloromethane crude fraction (Cr). BEA (A), CEF (B) and DE (4:1) (C) were used as mobile phases during development of chromatograms. TLC chromatograms were visualized under UV light and then sprayed with vanillin-sulphuric acid. Exactly 10 μ g of each of the isolated compounds applied

Table 5.1. R_f values of compounds isolated from *C. dentata* and quantity obtained.

Compounds	CEF	BEA	DE (4:1)	Mass (mg)
CI	0.84	0.41	0.79	210
CII	0.76	0.24	0.58	219
CIII	0.76	0.20	0.44	41
CIV	0.54	ND	0.00	21

Key: ND, Not done

5.3.2. Characterization of compound **CI**

Compound **CI** was isolated as an amorphous colourless powder (210 mg). The ^1H NMR showed the presence of seven tertiary methyl groups at δ 0.98, 0.78, 0.84, 1.06, 0.96, 0.78 and 1.67. The ^{13}C NMR assignments of **CI** revealed the presence of seven methyl, 11 methylene and six methane carbons. The chemical shifts observed through ^{13}C NMR experiment were identical to those of lup-20(29)-en-3 β -ol (lupeol) (Burns *et al.*, 2000) (Table 5.2, Fig. 5.2). The mass spectrum of **CI** revealed a

molecular ion peak at m/z 426 corresponding to the molecular formula $C_{30}H_{50}O$. The other fragment ion peak was at m/z 411 $[M-CH_3]^+$, which is characteristic for pentacyclic triterpene with an isopropenyl group (Saied and Begum, 2004).

Compound **CI** was identified as lup-20(29)-en-3 β -ol (lupeol) following comparison of our spectral data with published data (Fig. 5.2). The spectral data of **HI** was comparable to that of **CI**, suggesting that **HI** is also lupeol.

This triterpene, a common secondary metabolite of plants, has been isolated from many plant species. It is present in *Salvia horminum* (Ulubelen *et al.*, 1977), *Berberis vulgaris* (Saied and Begum, 2004) and other species (Das & Mahato, 1983; Jassbi, 2006). This appears to be the first report of its presence and isolation from *Curtisia spp.*

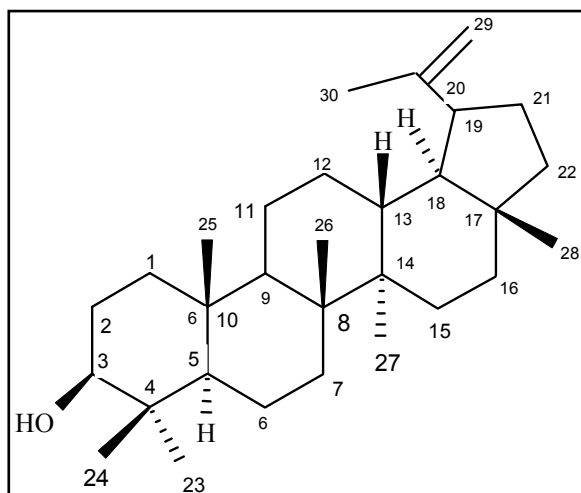


Figure 5.2. The structure of **CI** (lupeol) isolated from the leaves of *Curtisia dentata*.

5.3.3. Characterization of compound **CII**

Compound **CII** was isolated as a colourless amorphous powder (219 mg). The ^{13}C NMR spectra revealed 30 carbon signals (Table 5.3.) which were shown by DEPT experiments to be five methyls, five quaternary carbons, one carboxylic acid, and two olefinic carbons suggesting that compound **CII** is a triterpenic acid having five rings (Siddiqui *et al.*, 1988). The 1H NMR spectrum of compound **CII** showed the signals for five tertiary methyl groups at δ 0.74, 0.85, 0.85, 0.63 and 0.91, one isopropenyl moiety at δ 1.77, 4.55 and 4.67, indicating a lupane-type skeleton (Fig. 5.3). The mass spectrum of **CII** displayed a molecular ion peak (M^+) at m/z 456 corresponding to the formula $C_{30}H_{48}O_3$. Compound **CII** was identified as 3 β -hydroxy-lup-20(29)-en-

28-oic acid, betulinic acid by direct comparison of its spectral data with reported values (Table 5.3.) (Siddiqui *et al.*, 1988).

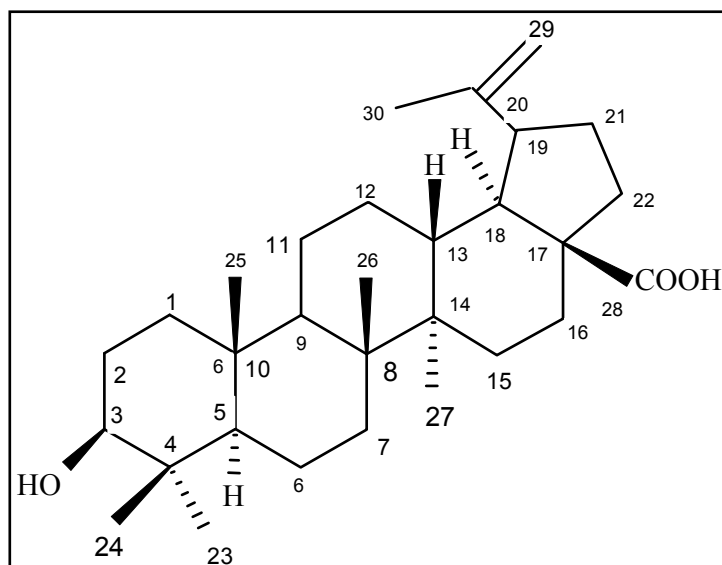


Figure 5.3. The structure of **CII** (betulinic acid) isolated from the leaves of *Curtisia dentata*.

This pentacyclic triterpene (betulinic acid) is a common secondary metabolite of plants. It is very common and abundant (up to 22% yield) in the *Betula* species (Betulaceae) used by Native Americans as folk remedy to treat intestinal problems such as diarrhoea and dysentery (Yogeeswari and Sriram, 2005). It was isolated from species such as *Ziziphus mauritiana* Lam (Rhamnaceae) collected in Zimbabwe (Pisha *et al.*, 1995), *Arbutus menziesii* (Robinson & Martel, 1969) and many other tree species that are valuable for timber purposes (Pavanasasivan & Sultanbawa, 1974). In the Cornaceae family, betulinic acid was isolated from *Cornus florida* Hook (Robertson *et al.*, 1939). The bark of *Cornus florida* is reported to contain about 2% betulinic acid (Robertson *et al.*, 1939). Bhakuni *et al.* (1987) reported the presence of triterpenes from *Cornus capitata*, some of which were of the lupane family. This appears to be the first report of the presence of betulinic acid in *Curtisia dentata*.

5.3.4. Compound CIII

The mass spectral data of compound **CIII** showed the molecular ion peak at m/z 456 and a base peak at m/z 248 along with a strong peak at m/z 203 due to retro-Diels-Alder fragmentation, typical of Δ^{12} -oleanene or ursine triterpene with the molecular formula $C_{30}H_{48}O_3$ (Mahato and Kundu, 1994). The structure of **CIII** is shown in Fig. 5.4 while the retro-Diels-Alders fragmentation is displayed in Fig. 5.5. The 1H NMR

spectrum of **CIII** exhibited signals of an olefinic proton at δ 5.11 and seven methyls characteristic of the oleanene skeleton. This structural type was further supported by the ^{13}C NMR spectrum, which contained resonance for olefinic carbons (δ_{c} 125.2 [C-12], 138.8 [C-13]) and a carboxylic acid at δ_{c} 178.9. The complete ^{13}C NMR data is given in Table 5.4. and Appendix 1. Full characterization of **CIII** was done by comparison with reported data for 3β -hydroxy-urs-12-en-28-oic acid (ursolic acid) (Hamzah & Lajis, 1998).

Ursolic acid has been isolated as the bioactive chemical entity from several plant species of importance in traditional and folk medicine (Mallavadhani *et al.*, 1998). It was isolated from *Hedyotis berbacea* (Hamzah & Lajis, 1998), *Rosmarinus officinalis* (Collins & Charles, 1987) and others (Mahato *et al.*, 1988). However, this appears to be the first report of its isolation from *Curtisia dentata*.

5.3.5. Compound CIV

The spectral data of **CIV** (21 mg) were very similar to that of compound **CIII**. The ^{13}C NMR spectrum displayed signals for 30 carbon atoms, which were one carbonyl (δ_{c} 178.4), two ethylenic carbon atoms, two oxymethines, seven methyls, eight methylene, five methines and six quaternary carbons (Table 5.5, Fig. 5.5 and appendix 1). The ^1H NMR spectrum showed an olefinic proton signal at δ 5.01. The presence of a double bond at C-12 was confirmed by the chemical shifts of C-12 (δ_{c} 124.56) and C-13 (δ_{c} 138.32) characteristic of triterpene of Δ^{12} -ursene type (Mahato & Kundu, 1994). Compound CIV was identified as 2α -hydroxy ursolic acid by comparison of ^1H and ^{13}C NMR spectra with those of CIII and spectral data in literature (Bandaranayake *et al.*, 1975; Kojima & Ogura, 1989).

The compound 2α -hydroxyursolic acid has been isolated from many plant species (Olafsdottir *et al.*, 2001; Begum *et al.*, 2002; Yamagishi *et al.*, 1988). Its isolation from *C. dentata* has not been reported in literature.

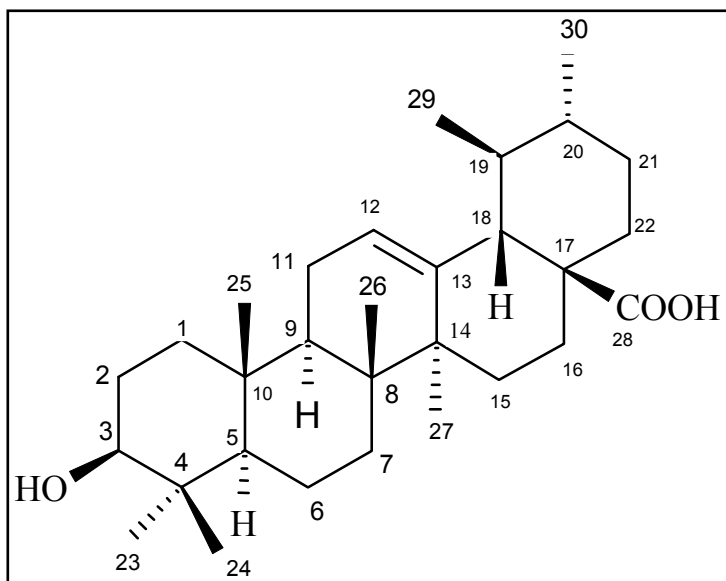


Figure 5.4. The structure of **CIII** (ursolic acid) isolated from the leaves of *Curtisia dentata*.

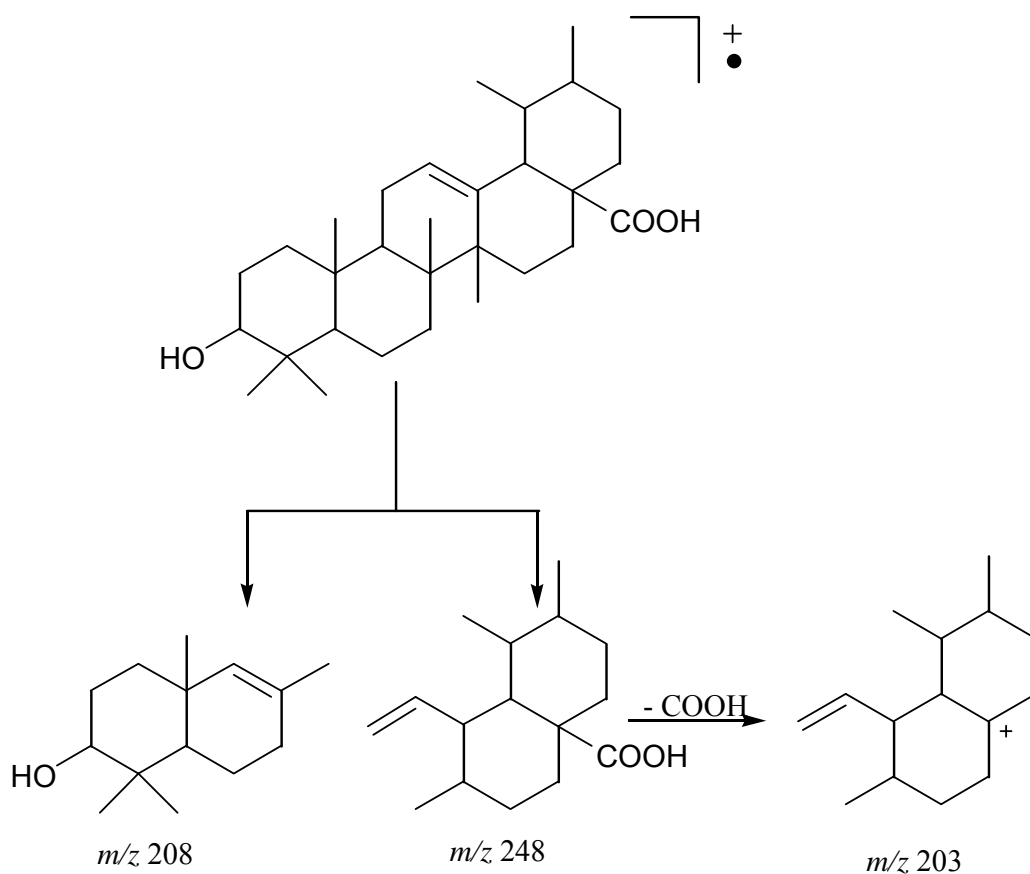


Figure 5.5. Retro-Diels-Alders fragmentation pattern of ursolic acid (**CIII**) isolated from the leaves of *Curtisia dentata*.

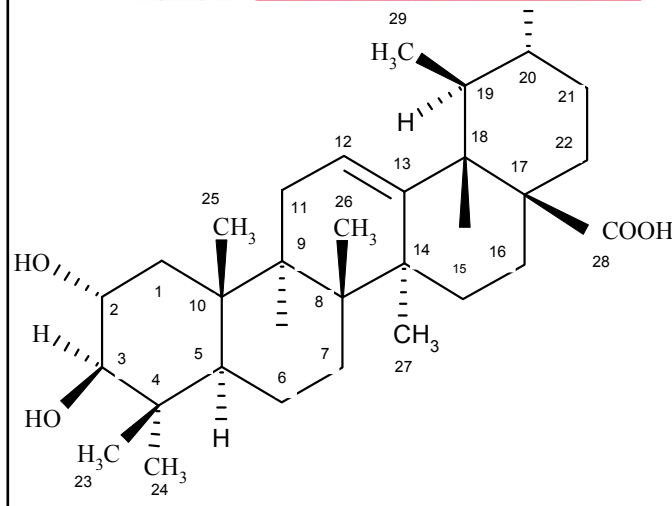


Figure 5.6. The structure of **CIV** isolated from leaves of *Curtisia dentata*.

5.3.6. Estimation of betulinic content of *C. dentata* leaves

Betulinic acid was the most abundant of the isolated compounds, present in most of the bulk fractions of *C. dentata*. The quantity of betulinic acid was estimated by analyzing known concentrations alongside crude acetone and dichloromethane fractions on TLC plates. The amounts of betulinic acid ranged from 0.8-50 μg . For both DCM and acetone crude fractions 100 μg was loaded on TLC plates. The plates were sprayed with vanillin-sulphuric acid spraying reagent and heated for 5 min at 100°C to visualize the compounds. The intensity of betulinic acid in the bulk dichloromethane fraction was comparable to the intensity in the lane loaded with 25 μg of betulinic acid. In 100 μg of the bulk dichloromethane fraction about 25% (25 μg) is the betulinic acid content. The same method was used to estimate the concentration of betulinic acid in the bulk acetone fraction. The quantity of betulinic acid was estimated at 6% (about 6 μg) in the acetone fraction of *C. dentata* leaves (Fig. 5.7). The estimated quantity of betulinic acid in the dichloromethane is lower than to the quantity found in *Betula* species (Betulaceae), at approximately 22% (Yogeeswari and Sriram, 2005).



Table 5.2. ^{13}C NMR data of compound **CI** compared with literature data (Burns *et al.*, 2000). Both experiments were conducted in CDCl_3 as the solvent.

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



Table 5.3. ^{13}C NMR chemical shift assignments for betulinic acid (Siddiqui *et al.*, 1988) and compound **CII**

Carbon	Betulinic Acid (Solichin <i>et al.</i> , 1980;Siddiqui <i>et al.</i> , 1988)	Compound CII
1	39.1	37.6
2	28.1	27.2
3	78.1	76.8
4	39.4	38.5
5	55.7	55.4
6	18.6	18.0
7	34.7	33.9
8	40.9	40.3
9	50.8	49.9
10	37.3	38.3
11	21.1	20.5
12	25.9	25.1
13	38.4	36.7
14	42.4	42.0
15	31.1	31.7
16	32.7	33.9
17	56.3	54.9
18	47.6	46.6
19	49.5	48.5
20	150.7	150.3
21	30.1	30.1
22	37.5	36.4
23	28.5	28.1
24	16.3	15.9
25	16.3	15.8
26	16.2	15.7
27	14.8	14.4
28	178.7	177.3
29	110.3	109.7
30	19.4	18.9



Table 5.4. Ursolic acid (CIII) ^{13}C NMR assignments compared with literature data (Seebacher *et al.*, 2003).

Carbon	Ursolic acid (Seebacher <i>et al.</i> , 2003)	Compound CIII
1	39.2	39.1
2	28.2	28.2
3	78.2	77.5
4	39.6	39.8
5	55.9	55.5
6	18.8	18.7
7	33.7	33.4
8	40.1	41.1
9	48.1	47.5
10	37.5	38.9
11	23.7	23.5
12	125.7	125.2
13	139.3	138.9
14	42.2	42.3
15	28.8	27.7
16	25.0	24.5
17	48.1	47.5
18	53.6	53.0
19	39.5	37.0
20	39.4	37.2
21	31.1	30.9
22	37.4	37.2
23	28.8	28.9
24	16.5	16.8
25	15.7	15.9
26	17.5	17.6
27	24.0	23.9
28	179.7	179.0
29	17.5	17.7
30	21.4	21.8



Table 5.5. 2 α -hydroxyursolic acid ¹³C NMR spectral data (Olafsdottir *et al.*, 2001) compared with NMR data obtained for compound CIV.

Carbon	hydroxyursolic acid (Olafsdottir <i>et al.</i> , 2001)	Compound CIV
1	46.9	47.0
2	67.0	67.2
3	82.1	82.3
5	54.6	54.8
6	17.9	18.1
7	32.5	32.7
9	46.7	46.9
10	38.3	38.5
11	22.8	23.0 ^b
12	124.4	124.6
13	138.1	138.3
14	41.6	41.8
15	27.4	27.5
16	23.7	23.9
17	47.0	47.1
18	52.3	52.4
19	37.5	37.6
20	38.4	38.7
21	30.1	30.2
22	36.2	36.4
23	28.7	28.9
24	17.0	17.3
25	16.3	16.5
26	16.8	17.0 ^a
27	22.8	23.0 ^b
28	178.2	178.4
29	21.0	21.2
30	16.8	17.0 ^a
	C4 & C8 about 40.0 hidden under solvent peak.	

CHAPTER 6

BIOLOGICAL ACTIVITY OF ISOLATED COMPOUNDS

6.1. INTRODUCTION

Four pentacyclic triterpenoids, isolated from *Curtisia dentata* using column chromatography, were identified using NMR spectroscopy and mass spectrometry as lupeol (**CI**), betulinic acid (**CII**), ursolic acid (**CIII**) and 2 α -hydroxy ursolic acid (**CIV**). Various biological activities of these compounds have been reported.

The activities of pentacyclic triterpenoids of ursane, lupane and oleanone skeleton, which accumulate in a number of terrestrial plants, free or linked to carbohydrate moieties (Liu, 1995), have been extensively reported (Ringbom *et al.*, 1998; Saraswat *et al.*, 2000). Ursolic acid demonstrates activities such as hepatoprotective, anti-inflammatory, antiviral and antitumor activities (Shishodia *et al.*, 2003), as well as growth inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* and with MIC values of 50 $\mu\text{g/ml}$ (Mallavadhani *et al.*, 2004). Zeletova *et al.* (1986) also reported activity of ursolic acid against *S. aureus*. Ursolic acid is also identified as one of the active components of *Rosmarinus officinalis* inhibiting growth of food-associated bacteria and yeast (Collins & Charles, 1987). Ursolic acid isolated from *Crataegus pinatifida* is active against HIV-1 protease at a concentration of 100 $\mu\text{g/ml}$ (Pengsuparp *et al.*, 1994). The C-28 carboxylic acid moiety was suggested and proven as the chemical group responsible for enhanced pharmacological activity of ursolic acid (Kashiwada *et al.*, 2000; Ma *et al.*, 2000). Corosolic acid (2 α -hydroxyursolic acid), also called colosolic acid or botanical insulin, enhances uptake of glucose by Ehrlich ascites tumor cells and induced hypoglycemic effects (Murakami *et al.*, 1993).

A variety of biological activities resulting from betulinic acid and other lupane triterpenoids have been published extensively. Betulinic acid is active against *Mycobacterium tuberculosis* with an MIC value of 50 $\mu\text{g/ml}$ (Chaiyadej *et al.*, 2004) and *E. coli* with an MIC value of 64 $\mu\text{g/ml}$ (Rahaman *et al.*, 2002). Betulinic acid is also active against *Bacillus subtilis* at a concentration of 1 000 $\mu\text{g/disc}$, resulting in a zone of inhibition of 18.8 mm² (Chandramu *et al.*, 2003). Betulinic acid isolated from *Syzygium claviflorum* is active against HIV with an inhibitory concentration (IC₅₀ value) of 13 μM (Fujoka and Kshiwada, 1994). Betulinic acid derivatives inhibit HIV-1 entry

(Mayaux *et al.*, 1994), HIV-protease (Xu *et al.*, 1996) and reverse transcriptase (Pengsuparp *et al.*, 1994). Esterification of betulinic acid at C-3 results in increased inhibitory potency against HIV protease activity (Ma *et al.*, 1999). Preclinical studies of this modified betulinic acid (PA-457) indicates significant activity against drug-resistant virus, activity in an animal model of HIV and that the potential drug substance is suitable for combination therapy with other anti-HIV drugs (Sigh *et al.*, 2005). Isolation, characterization and determination of biological activity of betulinic acid from *C. dentata* have not been reported.

Diverse ranges of biological activities triggered by lupeol have been reported. The stimulation of programmed cell death in human leukemia cell line (HL-60) by lupeol was observed and reported (Aratanechemuge *et al.*, 2003). Lupeol triggered a 33% reduction in granuloma weight in a chronic granuloma rat model (Agarwal & Rangari, 2003). Geetha and Varalakshmi (2001) also showed that lupeol reduced paw swelling by 39% in an adjuvant arthritis rat model. Lupeol is further reported to possess antimicrobial activity (Ragasa *et al.*, 2005). However, Chaiyadej *et al.* (2004) published that lupeol, in a study to evaluate biological activities of compounds isolated from *Sonneratia alba*, failed to show any activity against *Mycobacterium tuberculosis* and *Plasmodium falciparum*.

6.2. MATERIALS AND METHODS

6.2.1. Bioautography

Bioautography was performed as described in chapter 2. The isolated compounds (**CI – CIII**) were dissolved in acetone at a concentration of 1 mg/ml and 10 µg was loaded on TLC plates. The amount of **CIV** (21 mg) was not enough for bioautographic investigation. Duplicate TLC plates were developed in CEF and dichloromethane: ethyl acetate mixture (DE) (4:1) before spraying with overnight culture suspensions of the test organisms. *Candida albicans* was used as a test fungal organism while *E. coli*, *Enterococcus faecalis* and *S. aureus* were the selected bacterial organisms. Clear zones on TLC plates indicated inhibition of growth of the test microorganism by a specific compound.

6.2.2. Minimal inhibitory concentration (MIC) determination

The determination of MIC values was performed as outlined in Section 2.3.4. *Candida albicans* was tested together with *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporum canis*, *Cryptococcus neoformans*, *Candida guilliermondii* and *Candida spicata*. Amphotericin B was used as a positive control against fungal test organisms. *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as bacterial test organisms. Gentamicin was used as a positive control against bacterial test organisms.

6.2.3. Cytotoxicity

Cytotoxicity assay was performed as described in Section 2.3.5. With the exception of lupeol and betulinic acid, the compounds were not isolated in sufficient quantities to allow cytotoxicity testing. As a result, only lupeol and betulinic acid were tested against Vero cells.

6.3. RESULTS

6.3.1. Antifungal activity

Bioautography was used in antibacterial and antifungal activity assays to 1) indicate the purity of the isolated compounds and 2) to ascertain that the isolated compounds are similar to those observed in crude extracts. The three compounds investigated using bioautographic experiments were active against *C. albicans*. Ursolic acid showed the most pronounced zones of inhibition against *C. albicans*. Lupeol and betulinic acid were active against *C. albicans* on bioautograms though not to the degree shown by ursolic acid (Fig. 6.1).

The MIC values resulting from each compound against fungal test organisms were recorded (Table 6.1). All the compounds were active against several of the fungal species investigated. Lupeol failed to display appreciable activity against *C. albicans* and *C. spicata* with the highest concentration used (250 µg/ml) failing to inhibit fungal growth. However, lupeol displayed high activity against *S. schenckii* and *M. canis* with MIC values calculated as 12 and 16 µg/ml, respectively. The average MIC values of each compound calculated against all the fungal species tested are displayed in Fig. 6.2. The amphotericin B MIC average was 0.27 µg/ml. The lowest

MIC average resulted from betulinic acid. The highest MIC average was calculated for lupeol to be 128 $\mu\text{g/ml}$. The average MIC values against each fungal test organism indicated that *S. schenckii* and *M. canis* were the most sensitive to the isolated compounds (Fig. 6.3).

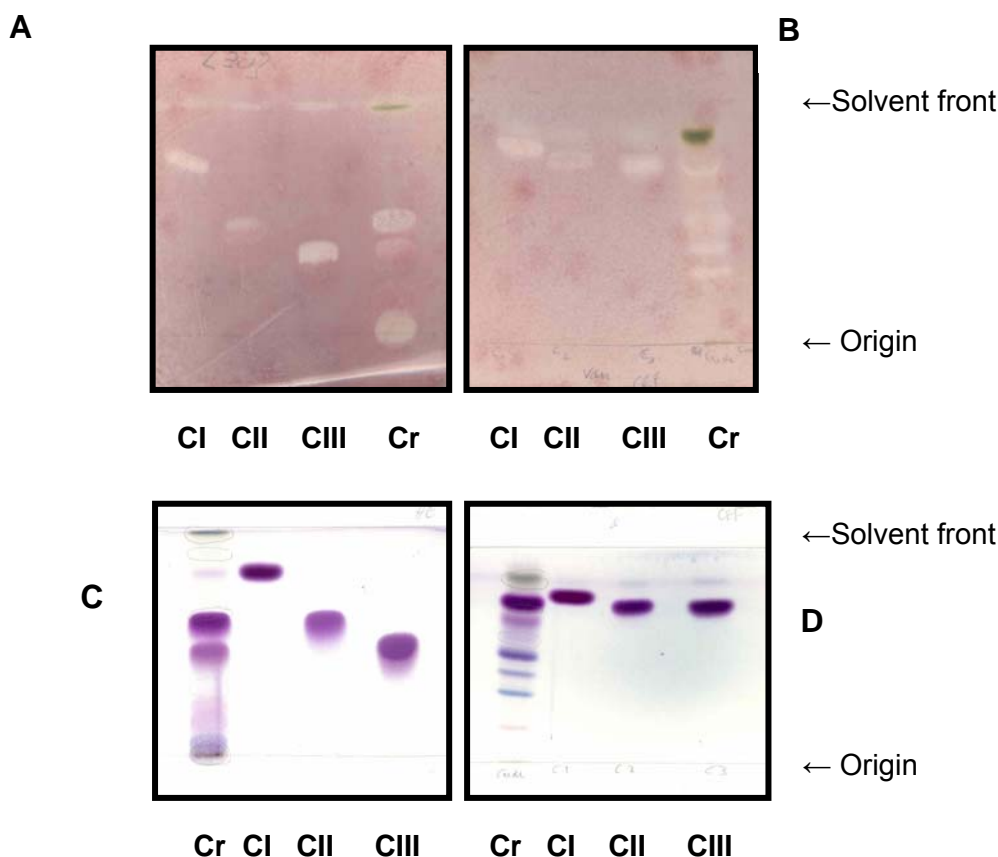


Figure 6.1. TLC bioautograms showing activity of three isolated compounds against *Candida albicans*. TLC plate was developed in DE (4:1) (A & C) & CEF (B & D). CI, lupeol; CII, betulinic acid; CIII, ursolic acid; CIV, hydroxyursolic acid; Cr, crude DCM extract.

Betulinic acid, ursolic acid and 2 α -hydroxyursolic acid resulted in appreciable inhibition of fungal growth with MIC values ranging from 8 to 63 $\mu\text{g/ml}$ (Table 6.1). The lowest MIC recorded was 8 $\mu\text{g/ml}$ resulting from hydroxyursolic acid against *M. canis*. The crude extract was active against all species tested with MIC values ranging from 30 $\mu\text{g/ml}$ against *S. schenckii* to 200 $\mu\text{g/ml}$ against *A. fumigatus*. The overall average MIC values for each of the compounds tested against all fungal species investigated was calculated as 56.9 $\mu\text{g/ml}$ (Fig. 6.2). With an average MIC value of 128 $\mu\text{g/ml}$ lupeol was the least active of the compounds whereas the

average MIC values (overall) for betulinic acid, ursolic acid and hydroxyursolic acid ranged between 23.2 and 38.9 µg/ml. Comparisons of the MIC averages calculated for each fungal species revealed that *S. schenckii* (MIC average of 19.3 µg/ml) was the most sensitive (Fig. 6.3).

Table 6.1: The minimal inhibitory concentration (µg/ml) of isolated compounds against fungal test organisms.

Compound	MIC (µg/ml) and test organism						
	SS	MC	AF	CA	CN	CG	CSP
Lupeol	12	16	93.5	250	180	94	>250
Betulinic acid	16	12	24	16	32	15.6	47
Ursolic acid	32	12	24	63	63	15.6	63
Hydroxyursolic acid	24	8	32	32	32	11.7	63
DCM crude extract	30	120	200	60	160	78	160
Amphotericin B	0.2	0.8	0.2	0.16	0.16	0.2	0.2

Key: SS, *Sporothrix schenckii*; MC, *Microsporium canis*; AF, *Aspergillus fumigatus*; CA, *Candida albicans*; CN, *Cryptococcus neoformans*; CG, *Candida guilliermondii*, CSP; *Candida spicata*.

6.3.2. Antibacterial activity

The compounds lupeol, betulinic acid and ursolic acid were further investigated to determine their antibacterial activity using the bioautography method. Test bacterial species selected for the investigation were *E. coli*, *Enterococcus faecalis* and *S. aureus*. All the compounds tested were active against bacterial test organisms. The most intense zones of inhibition against bacterial growth resulted from betulinic acid and ursolic acid. Though lupeol was active against all the bacterial species tested, the intensity of the zones of inhibition was not comparable to those resulting from ursolic and betulinic acid. Against *E. faecalis*, ursolic acid led to the most intense zones of inhibition compared to lupeol and betulinic acid (Fig. 6.4).

MIC values calculated indicated that ursolic acid and hydroxyursolic acid were active against all bacterial test organisms with the exception of *E. coli* (Table 6.2). The ursane triterpenes ursolic acid and colosolic acid were more active than the lupane triterpenes, betulinic acid and lupeol. MIC averages of each compound against all the

bacterial species indicated that the compounds ursolic acid and betulinic acid were more active than lupeol and betulinic acid (Fig. 6.5). The average MIC values calculated for each the test organisms indicated that *E. coli* was most resistant to the test compounds while *E. faecalis* was most sensitive (Fig. 6.6). In other studies ursolic acid markedly inhibited the growth of *S. aureus* but failed to inhibit Gram-negative bacteria and yeasts (Braghitori *et al.*, 1996).

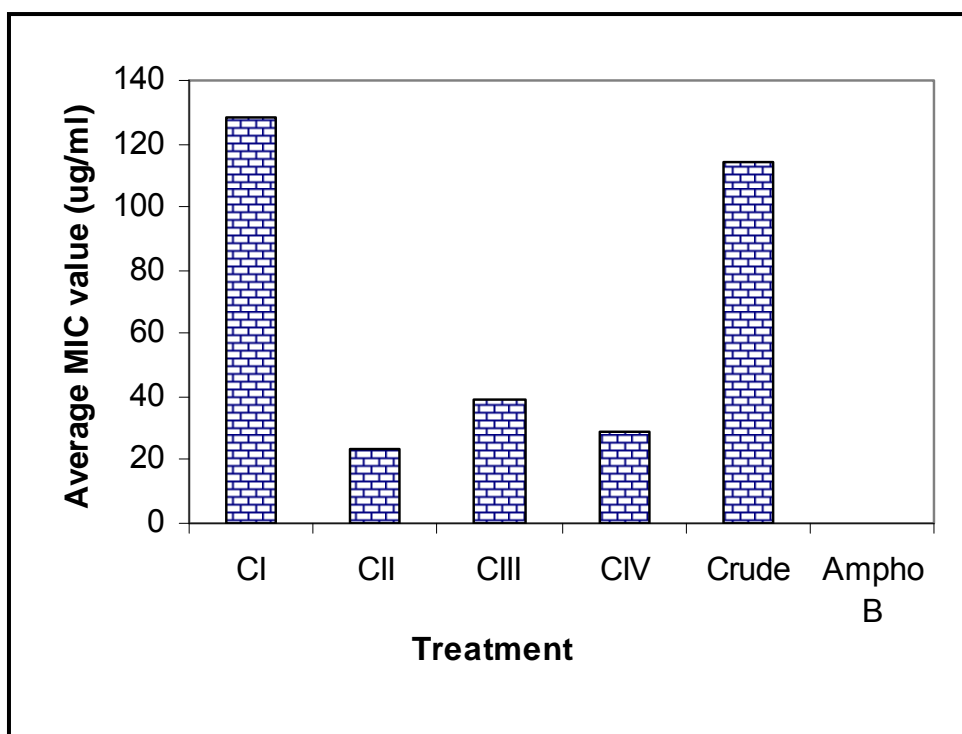


Figure 6.2. Average MIC values of each compound against all the fungal test organisms.

6.3.3. Cytotoxicity

The cytotoxicity of lupeol and betulinic acid was determined against Vero cells using the MTT assay. The results are presented in Figs. 6.7 and 6.8. Berberine was used as a positive control and was found to be toxic with an LC_{50} of about 10 $\mu\text{g/ml}$. Lupeol, with an LC_{50} of about 90 $\mu\text{g/ml}$, was less toxic than berberine and betulinic acid. The latter was comparable to berberine with an LC_{50} of about 11 $\mu\text{g/ml}$. The toxicity of betulinic acid may account for its broad-spectrum activity against both bacteria (both Gram-negative and Gram-positive) and fungi.

The selectivity index for betulinic acid and lupeol was calculated using the cytotoxic concentrations of these compounds. A high number indicates selective

activity against pathogens whilst a low number is indicative of high toxicity. The selectivity index (SI) was calculated as:

Selectivity index (SI) = LC_{50} against Vero cells/MIC values.

Lupeol had high selectivity index values against both *Sporothrix schenkkii* (7.4) and *Microsporium canis* (5.5), while against other fungal species the values remained below 1. The average selectivity index calculated for betulinic acid was 0.58.

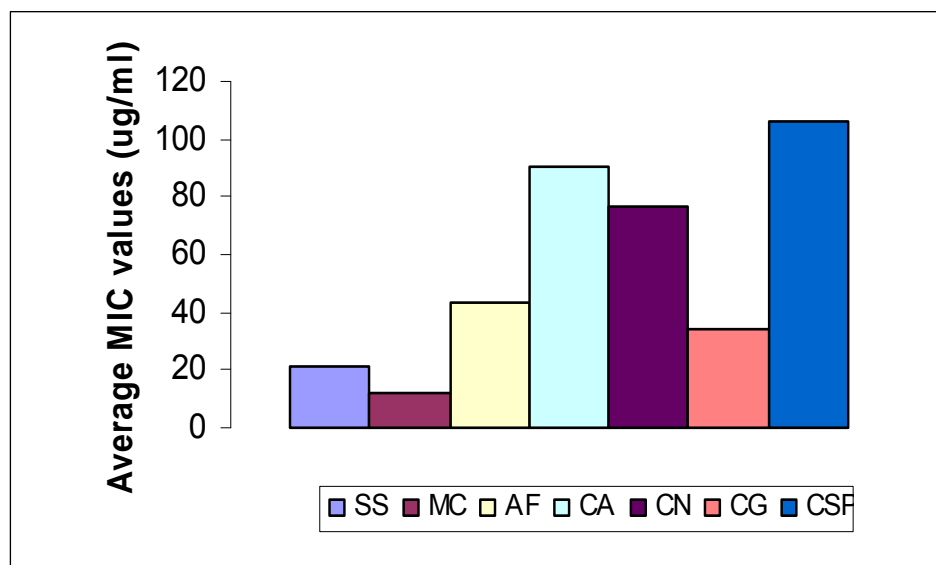


Figure 6.3. Sensitivity of each tested fungal species to all the isolated compounds. MIC averages were calculated from MIC values of all the isolated compounds against each of the fungal species.

Betulinic acid inhibited the growth of bacteria and fungi as evidenced by zones of inhibition and low MIC values. Literature reports outlining the activities of betulinic acid against growth of *C. albicans* and other fungal species are scarce. The antibacterial activity of betulinic acid has been reported. For instance, Chandramu *et al.* (2003) reported that betulinic acid isolated from the leaves of *Vitex negundo* had growth inhibitory activity against *Bacillus subtilis* at a concentration of 1000 $\mu\text{g/ml}$, resulting in a zone of inhibition of 18.8 mm^2 . Other activities of betulinic acid have been reported extensively in literature.

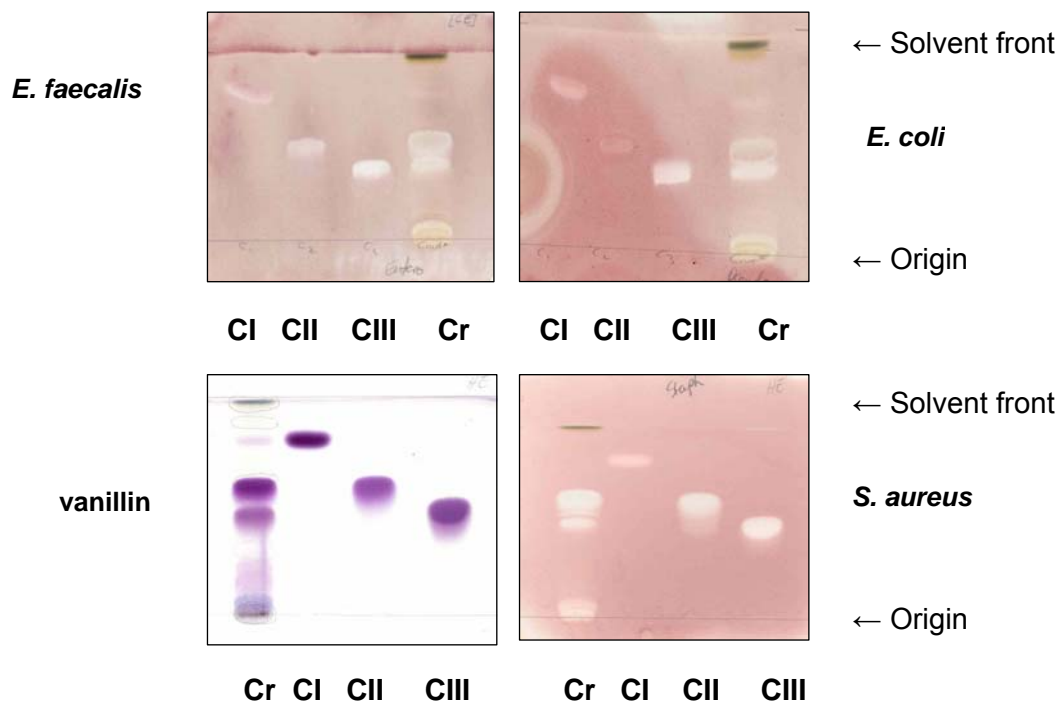


Figure 6.4. Antibacterial activity of isolated compounds against three bacterial test organisms. TLC plates were developed in DE (4:1) before spraying with concentrated suspensions of culture of test organism cultures. CI, lupeol; CII, betulinic acid; CIII, ursolic acid; CIV, hydroxyursolic acid; Cr, crude DCM extract.

Table 6.2: MIC ($\mu\text{g/ml}$) of compounds isolated from *C. dentata* against bacterial test organisms

Compound	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>
Lupeol	250	250	250	63.0
Betulinic acid	250	250	250	16.0
Ursolic acid	62.5	250	4.0	47.0
Hydroxyursolic acid	32.0	250	7.8	38.0
Gentamicin	0.2	8.0	0.2	1.6

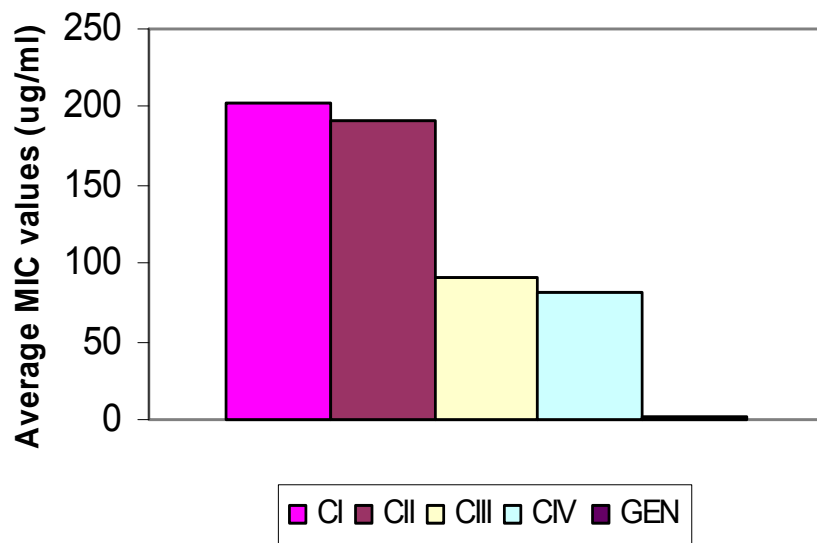


Figure 6.5. Averages of MIC values of each compound against all bacterial test organisms.

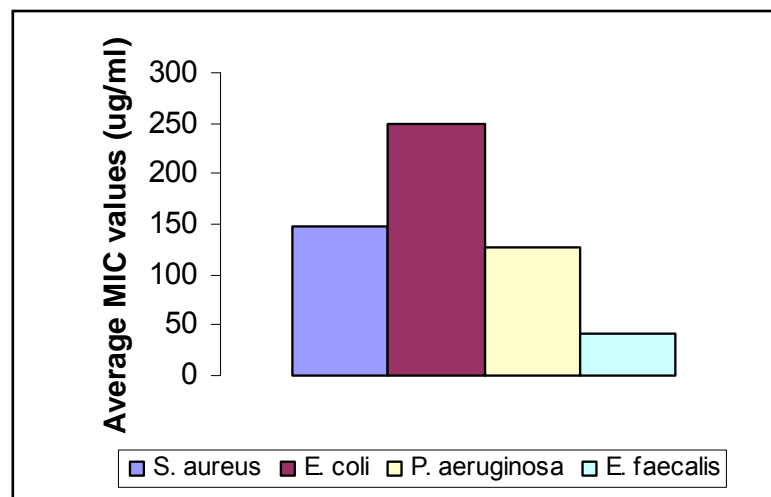


Figure 6.6. Average MIC values against each bacterial test organisms by all the isolated compounds.

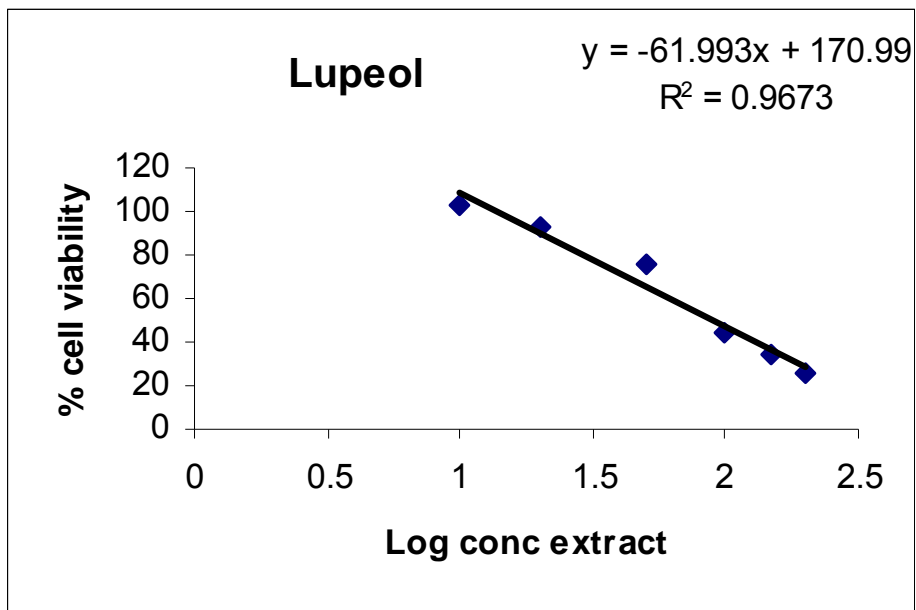


Figure 6.7: Cytotoxicity of lupeol ($LC_{50} = 89.4688722$ ug/ml) against Vero cells

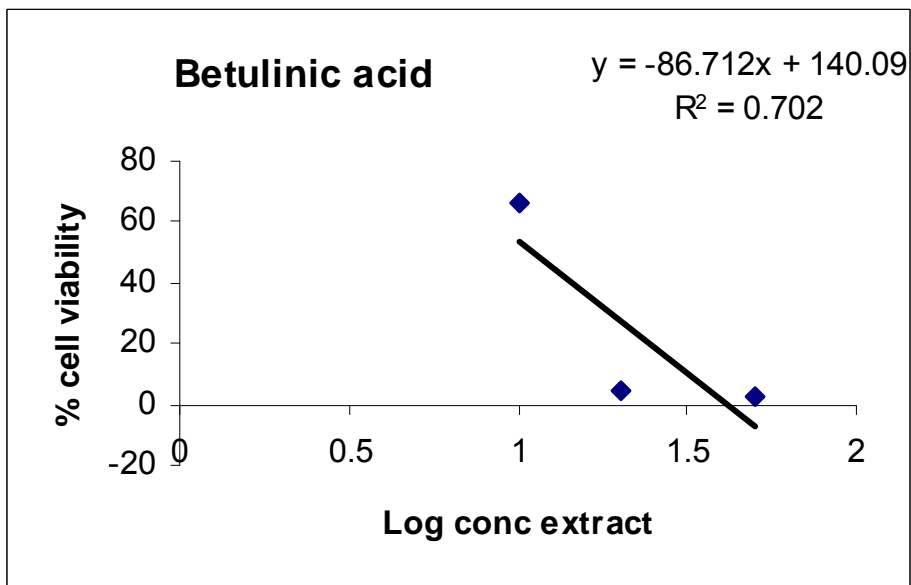


Figure 6.8. Cytotoxicity of betulinic acid ($LC_{50} = 10.93846914$ ug/ml) against Vero cells

Table 6.3. Selectivity index of lupeol and betulinic acid isolated from extracts of *Curtisia dentata* leaves. Key; Ca, *Candida albicans*; Cn, *C. neoformans*; Mc, *M. canis*; Ss, *S. schenckii*; Af, *Aspergillus fumigatus*.

Compounds	Ss	Mc	Af	Ca	Cn	Cg	Csp	average
Lupeol	7.4	5.6	0.95	0.35	0.49	0.95	0.35	2.30
Betulinic acid	0.69	0.92	0.46	0.69	0.34	0.71	0.23	0.58
Amphotericin B	140	35	140	175	175	140	140	135
Average	49.36	13.84	47.14	58.68	58.61	47.22	46.86	45.96

6.4. DISCUSSION

The triterpenic family of compounds to which the four isolated compounds belong, has been reported to possess antifungal and antibacterial activity. For instance, Collins and Charles (1987) identified ursolic acid as one of the active components in rosemary claimed to inhibit growth of some food-associated bacteria and yeasts (Becker *et al.*, 2005). Ursolic acid and its derivatives inhibited the growth of *S. aureus* and *Microsporium lenosum* (Zeletova, 1986). Ursolic acid has been reported as the active constituent from a number of plant species used in traditional and folklore medicine (Ringbon *et al.*, 1988; Saraswat *et al.*, 2000).

6.4.1. Lupeol and betulinic acid

Literature reports that 28-COOH and ester functionality at C-3 are important in determining the pharmacological activities of pentacyclic triterpenes (Mallavadhani *et al.*, 2004). Betulinic acid and lupeol, both of which are lupane pentacyclic triterpenes, slightly differ structurally. Betulinic acid possesses a carboxylic (-COOH) function at C-28 whereas lupeol contains a methyl (-CH₃) group at position 28, making lupeol more hydrophobic than betulinic acid. Since betulinic acid was more active against microbial growth (in this study) than lupeol it would seem that the carboxylic acid moiety at C-28 plays an important role in activity of these triterpenes. In another study Chaiyadej *et al.* (2004) reported that lupeol was not active against *Mycobacterium tuberculosis* H37Ra. Betulinic acid was active (with an IC₅₀ value of 50 µg/ml) against *M. tuberculosis* H37Ra.

Triterpenes possessing a carboxyl group at C-28 show more cytotoxic activity against several cancer cell lines (Chiang *et al.*, 2005; Baglin *et al.*, 2003; Sakai *et al.*, 2004) and induction of apoptosis (Hata *et al.*, 2002). Hiroya *et al.* (2002) reported that the protective activity of betulinic acid derivatives against cadmium toxicity in hepatocytes is closely related to polarity at C-3 and C-28. In another study it was shown that substitution of COOH at C-28 by CONH₂ led to improved antiviral activity of lupane triterpenes (Flekhter *et al.*, 2003). In addition, the hydroxyl function on C-3 is alleged to play a role in the activity of these triterpenes. For instance, changing lupeol to lupeol acetate reduces antimutagenic activity (Guevara *et al.*, 1996).

6.4.2. Ursolic acid and hydroxyursolic acid

Ursolic acid and colosolic acid differ structurally due to the presence of an additional hydroxyl group (-OH), in the α -configuration, at C-2 in the latter compound. Some literature publications indicate that the presence of a hydroxyl group at C-3 is important for activity of ursolic acid. For instance, esterification of 3-OH and 28-COOH results in loss of cytotoxic activity of ursolic acid (Ma *et al.*, 2005). This evidence suggests that a hydrogen donor group at either C-3 or C-28 is essential for activity of triterpenoids. In this study, the antifungal activities of ursolic acid and colosolic acid were comparable as indicated by the MIC values (averaging at 38.9 and 29.0 $\mu\text{g/ml}$, respectively). It would seem that the additional hydroxyl group on C-2 of colosolic acid did not increase its activity. Ma *et al.* (2005) declared that the configuration at C-3 was important for activity of ursolic acid. They arrived at this conclusion after observing that β -orientated hydrogen bond-forming group at C-3 displayed more pronounced (20-fold increase) cytotoxic activity than α -orientated counterpart.

One of the problems associated with triterpenic compounds is poor solubility in aqueous media. For instance, betulinic acid and ursolic acid suffer from low water-solubility, resulting in low biological efficacy (Baglin *et al.*, 2003). This poor solubility behavior was also displayed in this study in acetone by lupeol, betulinic acid and to a slight extent by ursolic acid.

6.4.3. Cytotoxicity

Betulinic acid and berberine were cytotoxic to Vero cells with LC₅₀ values of 10 and 11 $\mu\text{g/ml}$. In other studies, the anticancer activity of betulinic acid was associated

with little toxicity (Pisha *et al.*, 1995). Vero cells may be relatively sensitive to betulinic acid in comparison with other cell types. It would be worthwhile to investigate the toxicity of betulinic acid using other human and animal cell lines, including primary liver cells.

The amount of ursolic and hydroxyursolic acid isolated were not adequate for cytotoxicity testing. Some literature reports suggest that these ursane triterpenes are cytotoxic to tumour cell lines. Ma *et al.* (2005) reported that ursolic acid was cytotoxic to HL-60, BGC, Bel-7402 and Hela cells with ED₅₀ values of 72.0, 53.7, 45.0 and 49.4 µg/ml, respectively. The cytotoxicity of 2α-hydroxyursolic acid on these cells was comparable with that resulting from ursolic acid (Ma *et al.*, 2005).

Lupeol, though not as toxic as berberine, was a non-selective mild inhibitor of both fungal and bacterial growth. The calculated toxicity indexes indicate that amphotericin B has very pronounced antifungal activity associated with little cytotoxicity to the Vero cells. The low SI value (0.58) calculated for betulinic acid suggests that betulinic acid has antifungal activity accompanied by high toxicity. The selectivity index values of betulinic acid against all the fungal species tested suggested that toxicity was accountable for the low MIC values of this compound. The SI values of betulinic acid were lower than 1 against all fungal species tested.

Betulinic acid was selectively cytotoxic against several human melanoma cancer cell lines. It was also found to be active *in vivo* against athymic mice carrying human melanoma with little toxicity (Pisha *et al.*, 1995). The mechanism of action of betulinic acid on mammalian cells is thought to involve the induction of apoptosis (Pisha *et al.*, 1995). Preclinical developments towards topical formulations including betulinic acid and its synthetic derivatives as the active pharmaceutical ingredients are underway at the University of Illinois at Chicago (Balunas & Kinghorn, 2005).

6.5. CONCLUSIONS

In this study all the triterpenic compounds tested inhibited the growth of several fungal species with low MIC values. Using bioautography the inhibition of growth of fungal and bacterial species was clearly demonstrated, as indicated by clear zones on TLC chromatograms. The MIC values calculated suggested that lupeol was the least active against test fungi. The COOH group at C-28 and the C-3 hydroxyl group of triterpenoids are important for antibacterial and antifungal activity. Lupeol has –

CH₃ at C-28, while betulinic acid has –COOH at the same position. The activity observed with the isolated compounds further validates the traditional uses of *Curtisia dentata* for treating fungal and bacterial infections. Investigation of efficacy of the isolated compounds on *Candida albicans* infected wounds in animal models could indicate the potential uses of these triterpenes for topical treatment and prevention of infections in wounds. Based on the calculated activity indexes betulinic acid and lupeol, though active against some fungi, are not comparable with amphotericin B. The value of the antifungal activity of betulinic acid is outweighed by its associated cytotoxicity. Lupeol, though less toxic, has relatively low antifungal activity, leading to a low selectivity index.



CHAPTER 7

***IN VIVO* ANTIFUNGAL AND WOUND HEALING ACTIVITY OF *CURTISIA DENTATA* EXTRACTS, LUPEOL AND BETULINIC ACID**

7.1. BACKGROUND

Recently, fungal infections, including *Candida albicans*, have increased owing to immunosuppressive therapy, chemotherapeutic treatments to cancer patients and an increase in HIV/AIDS cases. (Denning *et al.*, 1994). *C. albicans* is the most commonly detected and isolated fungal species in AIDS patients (Fisher-Hoch & Hutwanger, 1995). Non-*Candida albicans* candidiasis, which occurs at increasing frequencies, further compromises the health of the effected patients (Parkinson, 1995).

Amphotericin B (AMB) is used to treat fungal infections with some degree of success. Treatment with AMB is, however, associated with a number of severe side effects. Recently, various new formulations of AMB have been introduced into clinical use. The ability of these new formulations to decrease these side effects at therapeutic doses is still a subject of intense scrutiny. Furthermore, the cost of treatment with such formulations is a major drawback especially in poor communities in the Third World countries (Tang & Bowler, 1997). Fluconazole (FLC) resistance has also been reported. The emergence of resistance to FLC (Nolte *et al.*, 1997), the lack of efficacy of FLC and the limited efficacy of intraconazole (ITC) against fungal infections have highlighted the need for new broad-spectrum antifungal agents.

Resistance to azoles, especially among the *Candida* species, has been the subject of intensive research investigations (Tritz & Woods, 1993). As a consequence of the increasing AIDS epidemic, the past decade has seen an overwhelming increase in mucosal infections caused by *Candida* species associated with emergence of resistance to azoles (Denning *et al.*, 1994). Resistance to AMB emerges associated with an increase in the number of opportunistic and invasive infections due to the so-called emerging fungi. Many of these fungi show resistance to AMB and other antifungal drugs, and may cause invasive infections, usually accompanied by a high mortality rate (Tritz & Woods, 1993). Other *Candida* species have also shown resistance

to AMB (Rex, *et al.*, 2000). In light of these and other problems, an alternative source of cheap yet effective antifungals may provide relief in poor communities.

Many plants species are known to produce a variety of secondary metabolites with known therapeutic properties. Compounds that exhibit either fungistatic or fungicidal activity with low toxicity to host cells are considered good candidates for developing new antimicrobial drugs. In recent years, antimicrobial properties of medicinal plants have been extensively published from different continents of the world (Cowan, 1999). Higher plants are still regarded as potential sources of new medicinal compounds or lead compounds for synthesis of drugs. Throughout the world, plants are used traditionally to treat or control many ailments, particularly infectious diseases (Mitscher *et al.*, 1987). Traditional preparations of some medicinal plants are used in wound healing. It is an entirely exhaustive process to determine if the administration of a plant preparation to a fungal infection site works through antifungal activity and/or wound healing (Masoko, 2006).

Wound healing is a well known physiological process that consists of a cascade of events that re-establish the integrity of the damaged tissue. This process protects damaged tissues from infection with pathogens, especially bacteria and fungi, and promotes the sealing of the damaged tissue (Sumitra *et al.*, 2005). Wound healing is promoted by several plant products (Suguma *et al.*, 1999), which contain several different active compounds (Sharma *et al.*, 1990) and biomolecules (Chithra *et al.*, 1995).

In our previous studies, *Curtisia dentata* extracts had remarkable activity against *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis*, *Sporothrix schenckii* and *Aspergillus fumigatus*, with MIC values as low as 0.02 and 0.04 mg/ml (**Chapter 3**). The next step in the investigation was to evaluate the antifungal activity of extracts of *Curtisia dentata* against fungal infections in an animal model. It was assumed that the extract applied topically would not result in systemic effects. An ethnobotanical study by Grierson and Afolayan (1999) indicated that, in the Eastern Cape, infusions of *Curtisia dentata* bark are used to treat pimples which are characterized by lesions on the skin, suggesting a role for this plant species in the treatment of wounds in this region.

Masoko (2006), a member of the Phytomedicine Programme developed a method to test crude extracts and isolated compounds on rats infected with different fungal pathogens, including *Candida albicans*. Several modifications were introduced and adapted into this method by

Masoko (2006) (Protocol number 1/010/05). The method of Masoko was followed using extracts and isolated compounds from *Curtisia dentata*.

Previous experiments on extracts of *Curtisia dentata* (**Chapter 3**) indicated excellent *in vitro* activity against *Candida albicans*. The aim of the study was to determine *in vivo* activity of the crude extract and compounds isolated in sufficient mass from the leaves of *Curtisia dentata* in a non-infected and *Candida* infected skin wound model in rats.

The objective of the study was to investigate the wound irritancy and efficacy of *C. dentata* extracts and pure compounds applied topically to skin wounds in a *Candida albicans* infected rat model. Wound irritancy and wound healing was evaluated using microscopic, physical and histological methods. Evaluated parameters included wound healing, erythrema, exudate formation and possible toxic effects of the extracts.

7.2. MATERIALS AND METHODS

Ethical approval was obtained from the Animal Use and Care Committee (AUCC), University of Pretoria as well as the Research Committee of the Faculty of Veterinary Science, University of Pretoria. The health care of all rats was under the supervision of a veterinarian and trained laboratory technologist throughout the trial period

The study was conducted in a separate room in a Biosafety II cabinet with restricted access. Only people involved in the study were allowed access to the study site. Protective clothing (coats, gloves and mask) were worn at all times and kept on site. All animal waste and disposable materials were incinerated. All non-disposable clothing was autoclaved after every experiment.

7.2.1. Rats

Healthy female Wistar rats weighing 150-200 g were purchased from the National Health Laboratory Services in Edenvale. The test was conducted using a single gender (female) to minimize variability and the numbers required (OECD, 2000). At the commencement of the study, each rat was 8 – 12 weeks old and the weight variation of animals used did not exceed $\pm 20\%$ of the mean weight of all previously dosed animals (National Institute of Environmental Health Sciences, 2001).

7.2.2. Housing and feeding conditions

Rats were kept at the University of Pretoria's Biomedical Research Centre (UPBRC) in cages at a temperature of 22 °C ($\pm 2^\circ\text{C}$) and controlled relative humidity (50 % - 60 %) in a light/dark cycle of 12 hours. The rats were fed conventional rodent diets with an unlimited supply of drinking water (National Institute of Environmental Health Sciences, 2001). Pieces of paper towel were provided to keep rats busy. Previous work suggests that the provision of enrichment items, which give laboratory rats the opportunity to perform exploratory and gnawing activities, improves their welfare and distracts them from tampering with dressings.

7.2.3. Preparation of animals

Rats were moved into single cages marked rat 1 – 12 for at least 5 days prior to treatment to allow for acclimatization to the laboratory conditions (Spielmann *et al.*, 1999). The rats were immunosuppressed, 4 days before challenge with *Candida albicans*, by subcutaneous injection of 500 μg of estradiol valerate (50 μl from 10 mg/ml ampoule). Estradiol pretreatment inhibits innate and acquired immune defenses (Carlsten *et al.*, 1991).

7.2.4. Wound creation

The hair on the back area of each rat was shaved using an electrical clipper. The area was disinfected using 70% alcohol. Rats were anaesthetized with isoflurane (0.01- 0.05 $\mu\text{g}/\text{kg}$) in a closed chamber. Eight evenly spaced wounds were made on each rat. The outline of the wound was marked using 6 mm diameter biopsy punch (Simosen *et al.*, 1992) and the skin removed from the marked area using surgical scissors. The procedure was conducted in a biosafety class II cabinet to limit infection. The lesions were traced onto a transparent paper using a black permanent marker. The area of the circular lesions was calculated as follows:

$A = \frac{\pi D_1 D_2}{4}$, where A is the area of the lesion, D_1 and D_2 were the horizontal and vertical diameters of the lesion, respectively, and $\pi = 3.142$

7.2.5. Infection with *C. albicans*

Candida albicans was cultured for 24 hours prior to infection on Sabouraud agar in Petri dishes at 30°C. The fungal cultures were scraped aseptically from agar, pooled into 30 ml of sterile water and briefly homogenized. Small pieces (5 mm x 5 mm) of autoclaved dressing were suspended into *Candida albicans* culture in sterile water and placed in the wound area. The area was covered with an occlusive wrapping (Transpore^R) and left to incubate for 48 hours. After 48 hours the test products were introduced and the resultant inhibition of growth or healing quantified on the basis of erythema, exudates and physical size of the lesion on a daily basis for 2 weeks. Infection by fungi was clinically detected by the presence or absence of swelling, erythema, pain and ulceration of the inoculation sites. Clinical signs that were also observed were a rise in body temperature, loss of appetite for 24 hours and weight loss.

7.2.6. Preparation of extracts

Curtisia dentata was extracted with water and acetone as described in Chapter 2. Acetone and water were selected as the extracting solvents to allow *in vivo* testing of an organic and an aqueous extract. The acetone extract had excellent antifungal activity in previous studies (Chapter 3). Extracts were dried at room temperature under a stream of air and ground to powder using mortar and pestle. The extracts were mixed with aqueous cream composed of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol to concentrations of 10 % (1 g/10 g cream) and 20 % (2 g/ 10 g), and kept at 4°C until use. The isolated compound preparations were adjusted to concentrations of 1% (0.1 g/10 g cream) and 2% (0.2 g/g cream). Amphotericin B (0.1%) was used as a positive control. Water extracts of *Buddleja* spp. were used as positive controls as these are known to have wound healing properties (Eloff and Houghton, personal communication). An outline of the treatments applied to each rat was as follows:

Rats 1 – 6:

Site A: treated with aqueous cream only (negative control),

Site B: treated with amphotericin B mixed with aqueous cream (positive control),

Site C: 1% lupeol in aqueous cream,

Site D: 2% lupeol in aqueous cream,

Site E: 1% betulinic acid in aqueous cream,

Site F: 2% betulinic acid in aqueous cream,

Site G: 10% water extract of *Buddleja* spp. aqueous cream and

Site H: 20% water extract of *Buddleja* spp. aqueous cream (positive control).

Rats 7 – 12:

Site A: treated with aqueous cream only (negative control),

Site B: treated with amphotericin B mixed with aqueous cream (positive control),

Site C: 10% acetone extract of *C. dentata* in aqueous cream,

Site D: 20% acetone extract of *C. dentata* in aqueous cream,

Site E: 10% water extract of *C. dentata* in aqueous cream,

Site F: 20% water extract of *C. dentata* in aqueous cream,

Site G: 10% water extract of *Buddleja* spp. aqueous cream and

Site H: 20% water extract of *Buddleja* spp. aqueous cream (positive control).

The diagrammatic representation of wound sites is displayed in Fig. 7.1.

7.2.7. Observations

The rats were inspected three times a week for signs of systemic infection by determining mass and food intake of each rat, as well as temperature using a microchip inserted subcutaneously above the tail region (Fig. 7.1). At the completion of the experiment, *post mortem* and pathological investigations were conducted to determine the emergence of systemic infection with *Candida albicans*.

Each animal served as its own control with four test sites for the crude extracts (acetone and water extracts) and isolated compounds, one site as a positive control with Amphotericin B, one site as a negative control and two sites treated with 10 and 20% water extract of *Buddleja* spp. The presence of factors such as muscle necrosis, foreign bodies and skin contamination were recorded. The measuring of the sizes of the lesions relative to the negative and positive controls or the complete healing of the lesion served as the means of measurement of the antimicrobial activity. Every Monday, Wednesday and Friday at the same time, the dressings on each rat were removed and the different parameters measured. Treatments were reapplied to wounds and fresh dressing used to cover the wounds.

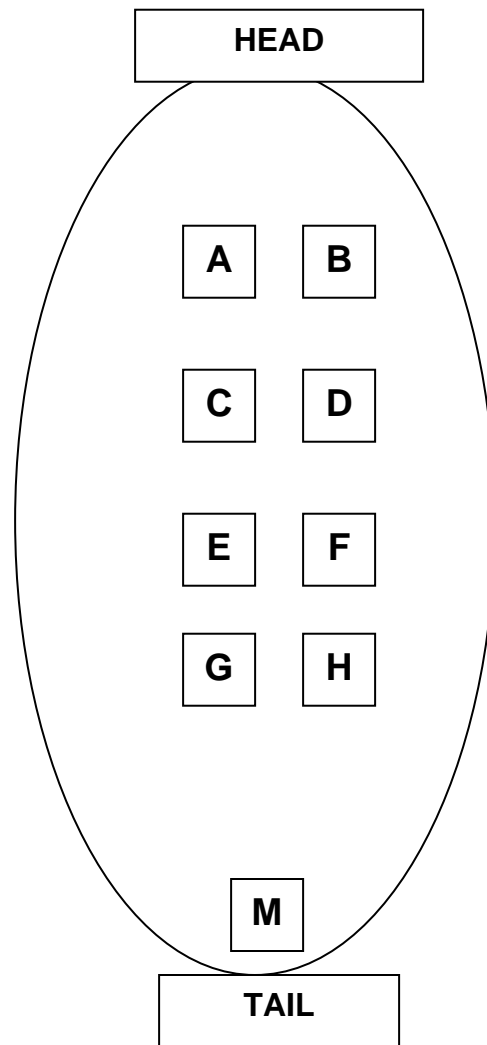


Figure 7.1. A diagrammatic representation of wound positions on the back of each rat. Samples as outlined in **Section 7.2.6.** were applied on the specific wound sites. Site M represents the position where the temperature microchip was inserted.

A photographic record of the overall procedure performed on rats is outlined in Figs. 7.2. and 7.3. The rats were observed daily for two weeks until wounds were about 80% healed. Observations noted included changes to skin and fur, diarrhoea, lethargy, sleep, weight loss and coma. After the completion of the experiment, rats were euthanized using increased doses of isoflurane in closed glass chambers and the *post mortem* performed. Liver, heart and kidney were analysed by the pathologist for different parameters. Sterile swabs were used to collect inocula from internal organs to allow culturing on SB agar (Fig. 7.4).

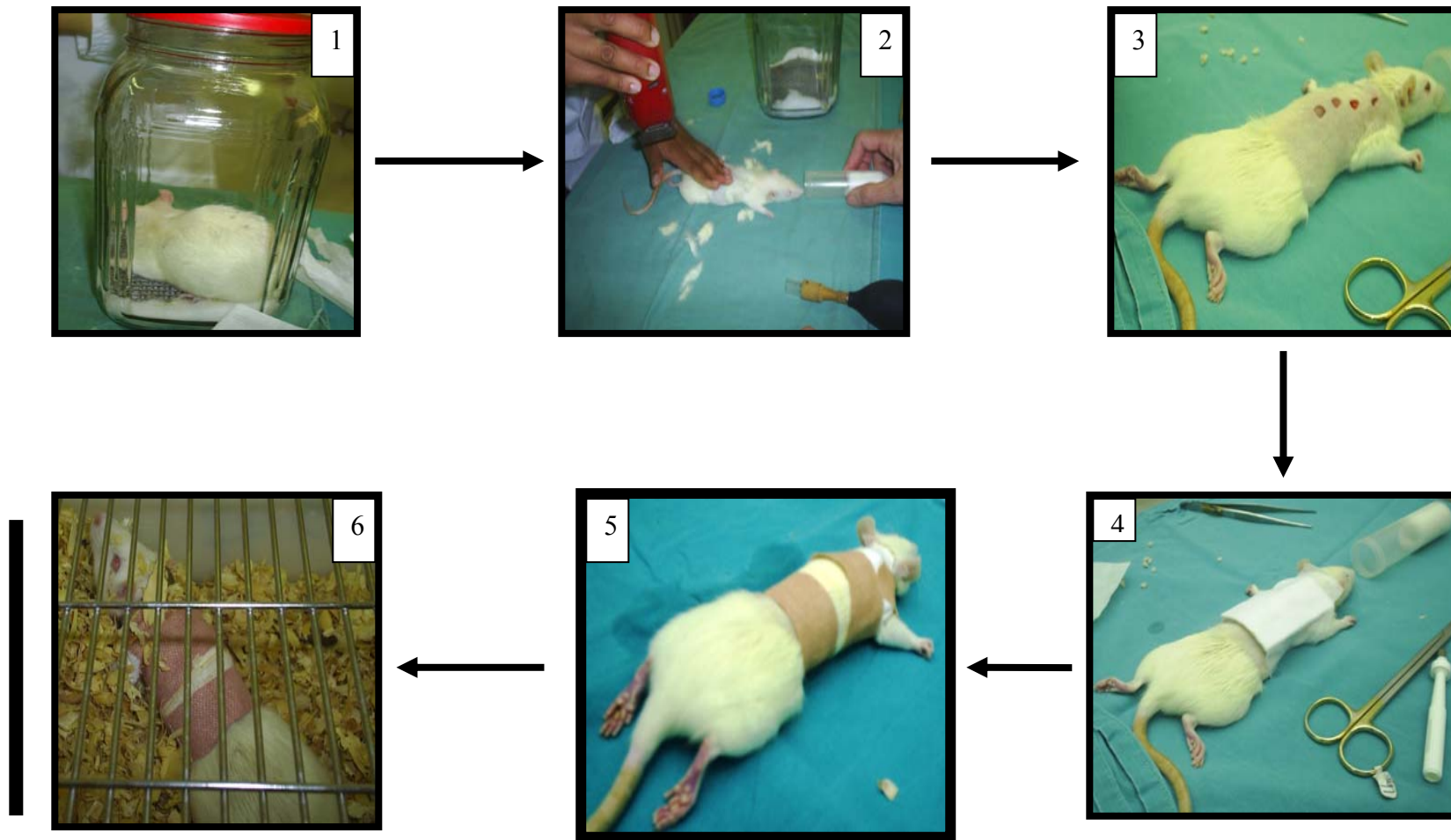


Figure 7.2. The diagrammatic representation of surgical wound creation and dressing of excisional wounds on rats. 1, rat subjected to anaesthesia in a closed glass chamber; 2, shaving of the back with an electric clipper; 3, creation of wounds using scissors and infection with a concentrated overnight culture of *Candida albicans*; 4,5,6, dressing of wounds and housing in separate cages.

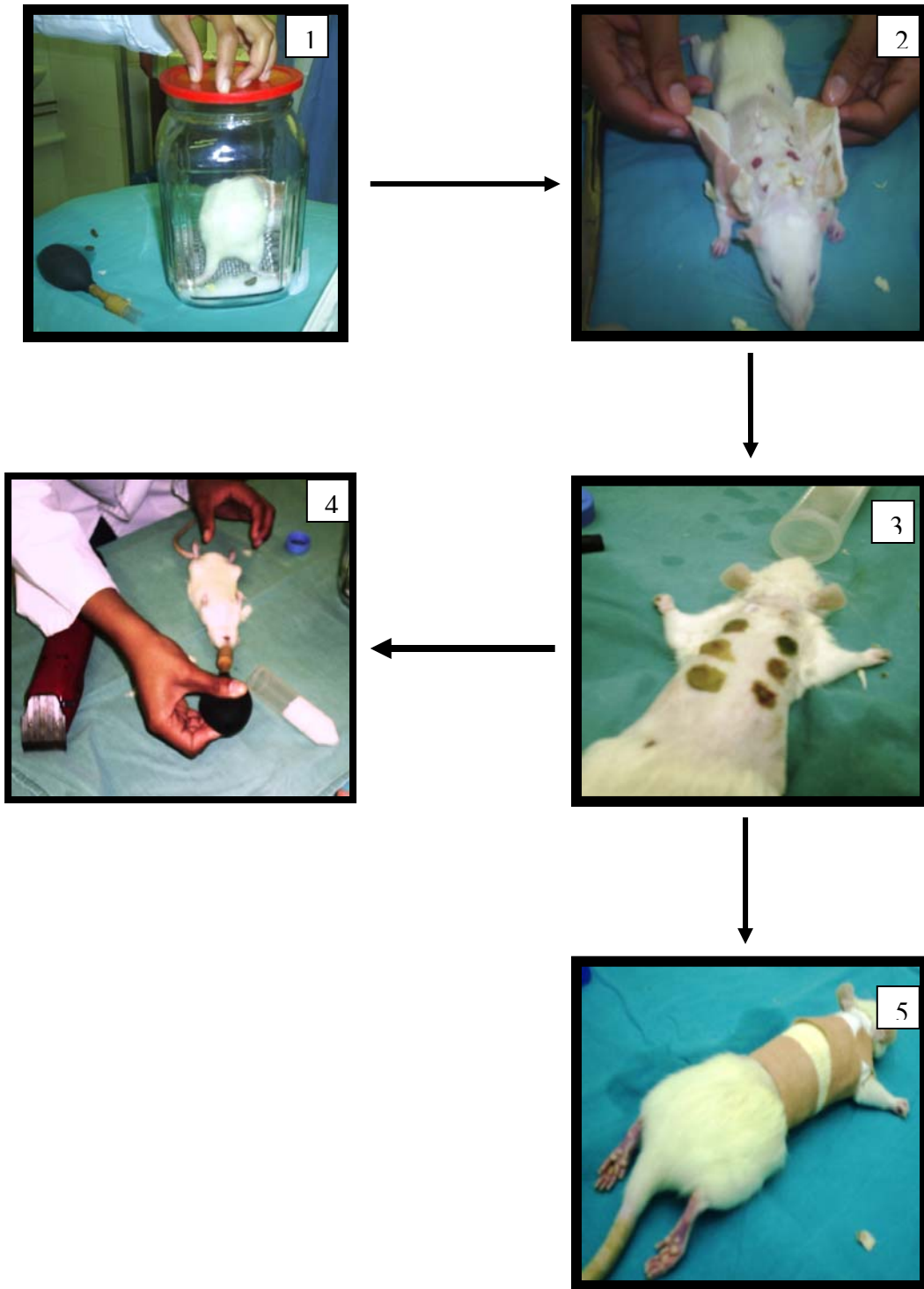


Figure 7.3. Representation of changing of wound dressing and treatment with varying products. 1, rat subjected to anaesthesia; 2, removal of bandages; 3, application of treatment on wounds using sterilized spatulas; 4, resuscitation of non-breathing rat; 5, redressing of wounds.

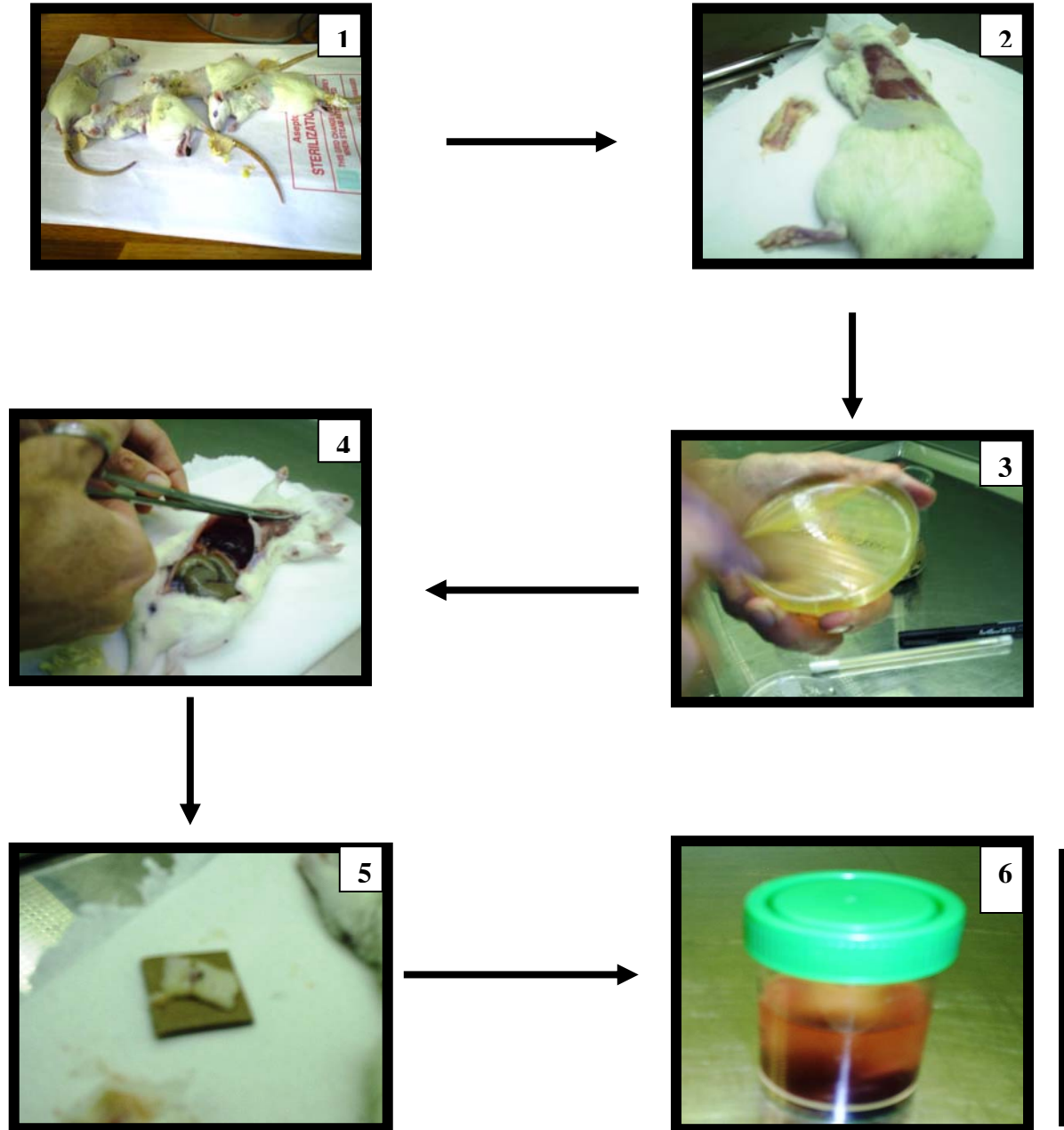


Figure 7.4: Preparation of tissue specimens for histopathological examinations. 1, rats eutheniated in excess isoflurane; wound areas removed from skin; 3, inocula collected from underskin surfaces using sterile swabs and streaked on SD agar; 4, liver, kidneys, lungs and spleen removed and preserved in buffered formalin, and swabs used to collect fungi from organs; 5, 6, wound areas anchored to pieces of cardboard and preserved in formalin.

7.2.8. Evaluation of lesions

The mass of each rat and lesion characteristics, including lesion severity scores, were recorded three times a week and general observations noted. The study was blinded (the person doing evaluation did not know the treatment). The lesion sizes were measured using a caliper on each routine observation day. Erythema and exudate formation were evaluated during routine observation and scored as in Table 7.1.

Table 7.1. Scoring used in the evaluation of erythema and exudate

Score	Erythema	Exudate
0	No red colour at all	No exudate
1	Light red just visible	Exudate just visible
2	Clearly red	Easily visible
3	Dark red, not whole area	Substantial quantity
4	Dark red wide spread	Large quantity

7.2.9. Recording of data

The data for each rat was recorded on a single form for the 2 week period (Table 7.2.). Day 0 data was also collected as for the other days in Table 7.2.

7.2.10. Statistical analysis

Results were represented as general linear model (GLM) with repeated measure ANOVA. One way ANOVA was used to identify differences between groups and $P < 0.05$ was considered significant. Student t-test was used to determine differences between control and treatments.

7.2.11. Pathological and histopathological studies

Histopathological studies were performed with the help of a pathologist at the end of the experiment. Wound tissue specimens from treated and non-treated rats were collected in 10 % buffered formalin and after processing 6 μ m thick sections were cut and stained with haematoxylin and eosin (McManus & Mowry, 1965). Sections were qualitatively assessed under the light

microscope and graded in respect of congestion, oedema, infiltration of polymorphonuclear leukocytes (PMNLs) and monocytes, necrosis, fibroblast proliferation, collagen formation, angiogenesis and epithelisation (Shukla *et al.*, 1999). Table 7.3. was used as a template for recording histopathological results.

7.3. RESULTS

The rats lost weight after the surgical procedure until day 6. During the next routine observation on day 10 the weight of the rats increased substantially through day 11. The rats acclimatized to the handling in the study as indicated by the overall weight gain after day 9 (Table 7.4. and Fig. 7.5.). The average weight of the rats, for reasons not yet understood, dropped on observation day 13. A similar trend also occurred with the temperatures of the rats. Following initial anaesthesia the temperatures of the rats dropped to between 31.8 and 35.5 °C. The temperature only increased with the days of treatment, peaking at around 38°C (Table 7.5 and Fig. 7.6).

The area of the lesions was measured by tracing the boundary of open wounds on a semi-transparent plastic sheet and the sizes measured using an electronic calliper. Statistical calculations using ANOVA single factor revealed statistically significant differences in lesion sizes between groups (treatments) on day 6 with a P-value of 0.0054 at 95% confidence interval. T-tests were calculated for each treatment and compared with cream only control and amphotericin B control. The lesions treated with 20% acetone averaged about 76% of the original lesion sizes and were significantly larger than lesions treated with the cream only and AMB (*P*-value less than 0.05) on day 6.

Table 7.2. Form used for measuring different parameters on the 6 rats (in a group) on the different sites. The exudates and erythema were measured on an arbitrary scale 0-5 with one being the best rate of healing and five the worst. Lesion size was be measured in mm² and mass in grams (g), temperature in °C. Swelling and ulcerations were marked as follows: 1, slight; 2, moderate; 3, marked; 4, extensive; 0, absent. A-H represent wound sites on each rat.

Day	Rat No.	Exudate								Erythema								Lesion size (mm ²)								Weight (g)	Temp. (°C)	Swelling	Ulceration
		A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H				
M1																													
W1																													
F1																													
M2																													
W2																													
F2																													

Table 7.3. Form used for quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams. *Neut's= Neutrophils; Mac's= Macrophages

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
	A											
	B											
	C											
	D											
	E											
	F											
	G											
	H											

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

Table 7.4: Mass of rats 1 – 12 measured during specified time periods.

DAYS	RAT NUMBERS AND MASS IN GRAMS											
	1	2	3	4	5	6	7	8	9	10	11	12
0	169.1	183.7	200.2	200.1	190.1	211.7	205.9	184.1	185.0	199.7	177.6	185.3
2	168.5	182.7	200.3	196.0	187.9	214.2	206.7	182.0	182.4	198.2	177.5	183.6
4	170.0	181.2	201.2	199.1	187.3	208.4	208.8	182.7	188.4	198.9	185.7	181.5
6	169.9	179.3	203.3	194.8	187.3	207.0	210.9	167.3	182.7	201.9	187.7	169.8
9	175.4	182.5	206.8	196.4	190.2	216.1	208.6	180.3	191.2	208.3	185.3	181.2
11	174.0	177.9	208.8	200.0	190.7	212.4	212.2	185.8	194.0	213.4	189.9	178.1
13	174.2	181.6	193.2	184.4	193.4	212.2	209.0	188.8	194.2	210.8	187.1	179.8
Average	171.6	181.3	202.0	195.8	189.6	211.7	208.9	181.6	188.3	204.5	184.4	179.9

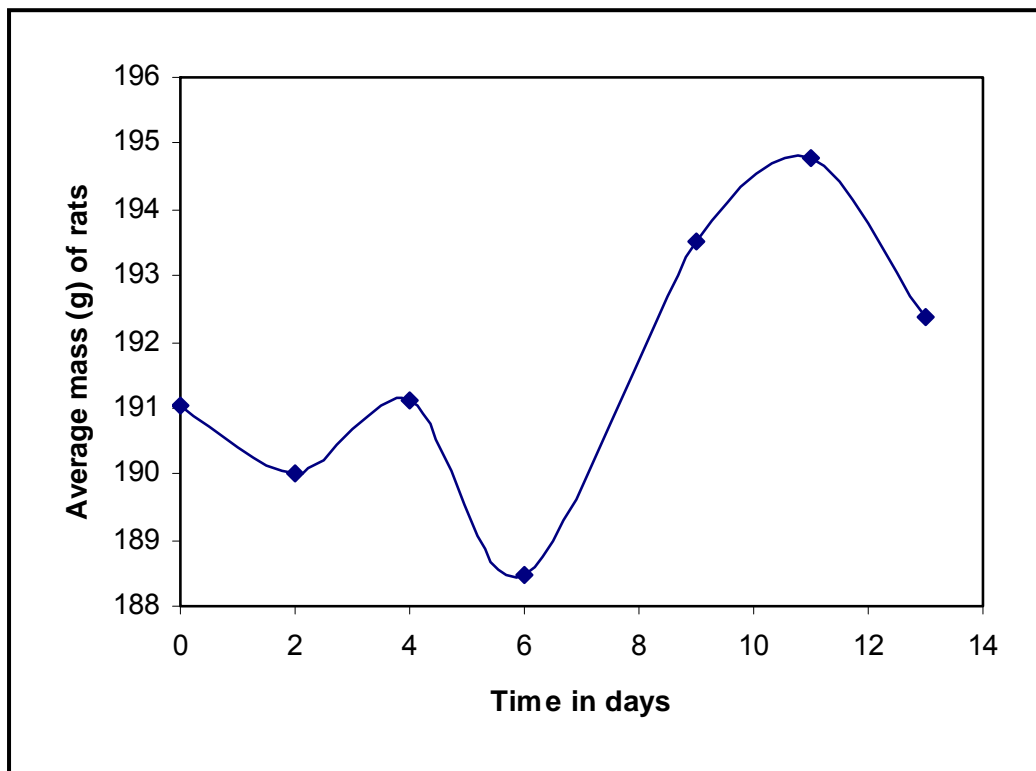


Figure 7.5: Average mass of the rats on different treatment days.

Table 7.5: Temperatures (°C) of rats from day 0 to day 13. **CF**, microchip missing.

DAYS	RAT NUMBERS and temperature in °C											
	1	2	3	4	5	6	7	8	9	10	11	12
0	33.6	34.4	35.5	33.6	34.9	34.6	35.4	34.6	33.7	34.4	33.3	31.8
2	35.1	36.2	38.0	36.5	36.1	36.5	35.7	36.7	38.0	37.1	36.1	39.1
4	37.7	37.0	37.1	36.7	36.1	37.4	35.8	36.5	35.8	37.1	35.8	36.7
6	36.7	35.6	36.3	36.9	36.9	38.3	35.6	36.2	36.7	37.0	37.7	36.1
9	37.3	36.0	37.6	37.3	36.6	36.3	35.3	37.2	35.6	37.3	35.8	37.9
11	35.7	36.3	37.0	37.0	36.0	35.1	35.2	35.7	36.4	37.9	37.2	36.2
13	36.0	35.8	36.2	36.3	37.9	CF	CF	36.6	35.4	38.2	36.5	35.5

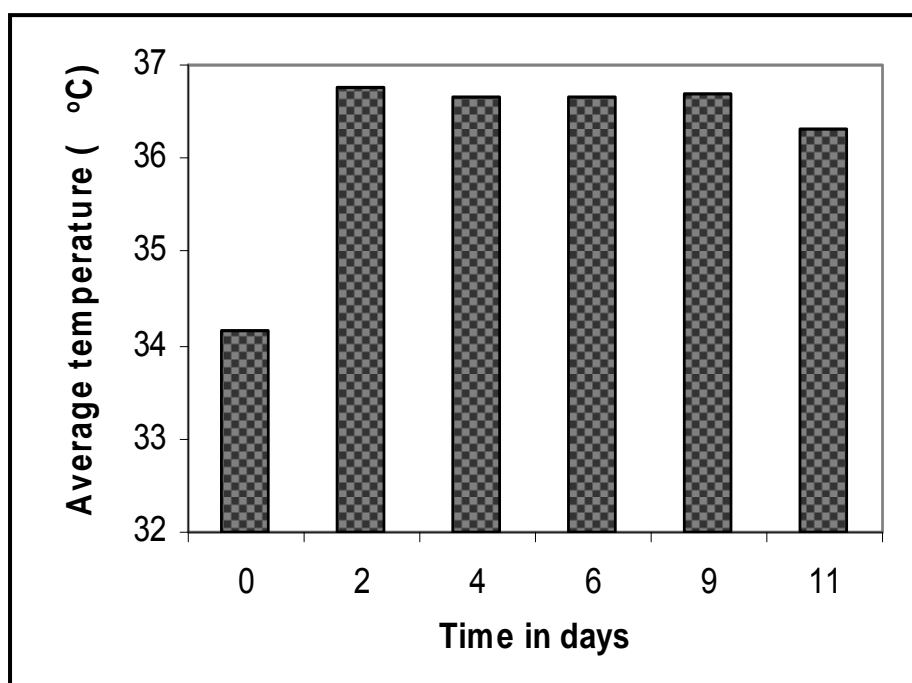


Figure 7.6. Average temperature (°C) of rats up until 11 days of treatment

The wounds treated with 20% acetone extract of *Curtisia dentata* had significantly higher lesion sizes than both the cream only control and amphotericin B-treated control on treatment day 4, averaging at 120% of the original lesion sizes (P -value less than 0.05 at 95% confidence interval). This suggested that the acetone extracts did not result in any observable decrease in lesion sizes compared with the cream only controls and amphotericin B controls. Generally, all lesions

decreased in size with the days of treatment. There was no marked acceleration of reduction of lesion sizes with any single treatment (Figs. 7.7 and 7.8). Statistical data analysis is shown on Tables 7.6 and 7.7).

No swelling was observed in any of the wounds. The excision wounds were initially round or almost round. As they healed all the wounds contracted into an oval shape and gradually healed in a straight line. In literature, it is reported that open wounds contract as if margins are pulled into the centre. Though round wounds are slower to heal than square wounds, the healed scar is linear (Majno, 1975).

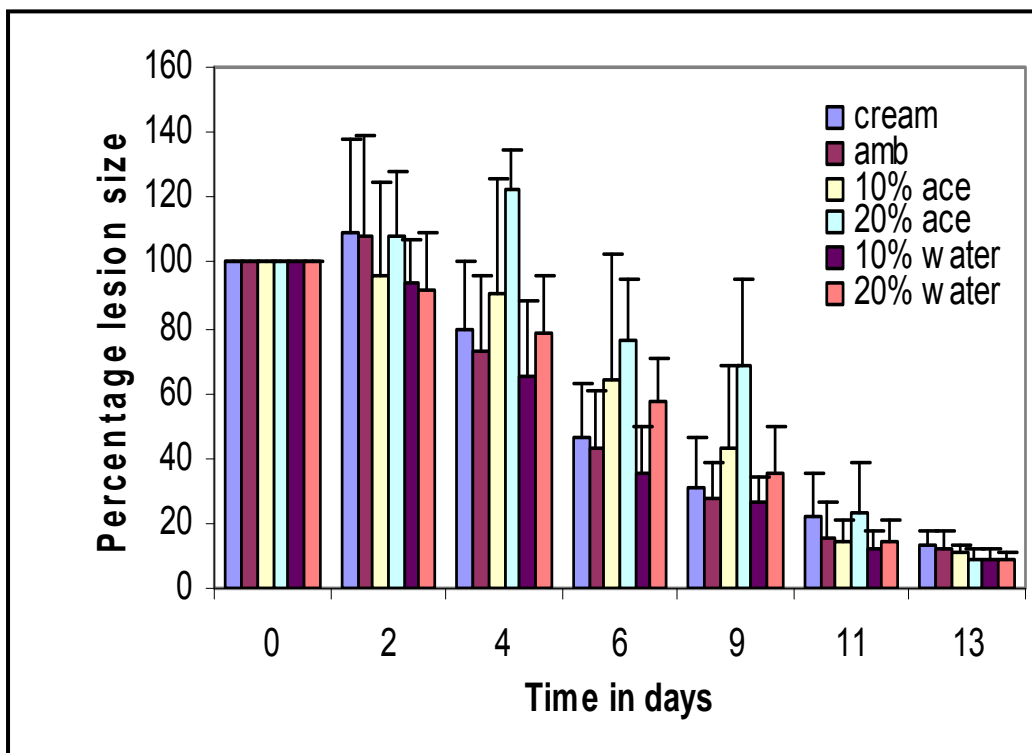


Figure 7.7. Average sizes of lesions treated with different extracts of *C. dentata*. Lesions were calculated as percentages of the original lesion sizes. Amb, amphotericin B; ace, acetone extracts.

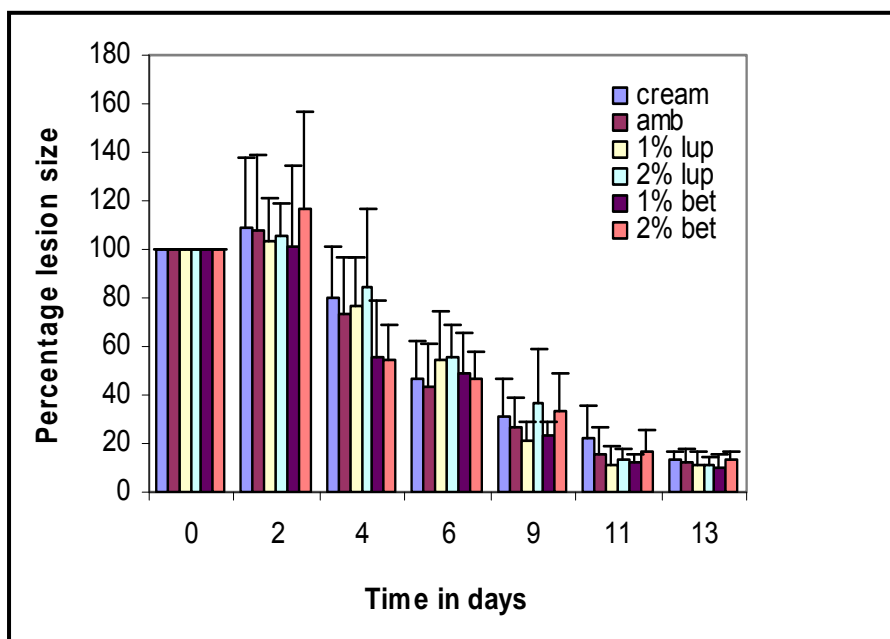


figure 7.8. Average sizes of lesions treated with different compounds isolated from *C. dentata*. Lesions were calculated as percentages of the original lesion sizes. Amb, amphotericin B; lup, lupeol; bet, betulinic acid.

Table 7.6. Treatment day 4 statistical analysis of lesion sizes

Treatments	Count	Sum	Average	Variance
Cream only	12	955.3	79.61	437.08
Amphotericin B	12	878.6	73.22	536.22
1% lupeol	6	461.9	76.98	390.59
2% lupeol	6	504.5	84.08	1071.94
1% betulinic acid	6	331.6	55.27	567.72
2% betulinic acid	6	325.2	54.20	218.51
10% acetone	6	540.8	90.13	1234.16
20% acetone	6	732.6	122.10	149.80
10% water	6	392.0	65.33	510.01
20% water	6	468.4	78.07	335.29
10% Buddleja	12	937.0	78.08	697.04
20% Buddleja	12	1108.9	92.41	645.38

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	22808.82	11	2073.529	3.639065	0.00031	1.904539
Within Groups	47862.96	84	569.7972			
Total	70671.78	95				

Table 7.7. Treatment day 6 statistical analysis of lesion sizes

<i>Treatment</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Cream only	12	560.4	46.70	256.55
Amphotericin B	12	522.1	43.51	292.01
1% Lupeol	6	328.5	54.75	397.36
2% Lupeol	6	335.3	55.88	165.95
1% betulinic acid	6	291.6	48.60	294.86
2% betulinic acid	6	280.0	46.67	121.18
10% acetone	6	387.1	64.52	1475.54
20% acetone	6	456.2	76.03	366.87
10% water	6	213.2	35.53	213.02
20% water	6	342.3	57.05	187.10
10% Buddleja	12	597.8	49.82	499.07
20% Buddleja	12	812.2	67.68	233.30

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	10583.60	11	962.15	2.676194	0.005392	1.904539
Within Groups	30199.69	84	359.52			
Total	40783.29	95				

The average exudate release on wounds was less in wounds treated with 2% betulinic acid than the cream only-treated controls. Treatment with 1% and 2% lupeol resulted in the highest exudate formation (Fig. 7.9). Treatment with 20% water extract formulation resulted in more exudate release than the control wounds treated with cream only, whereas treatment with amphotericin B inhibited exudate release from wounds (Fig. 7.10).

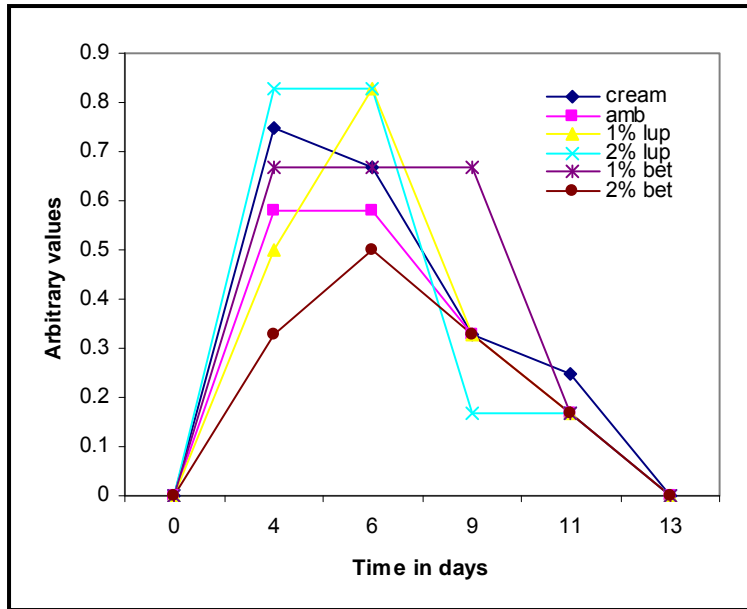


Figure 7.9. Exudate release from lesions treated with different *C. dentata*-derived compounds

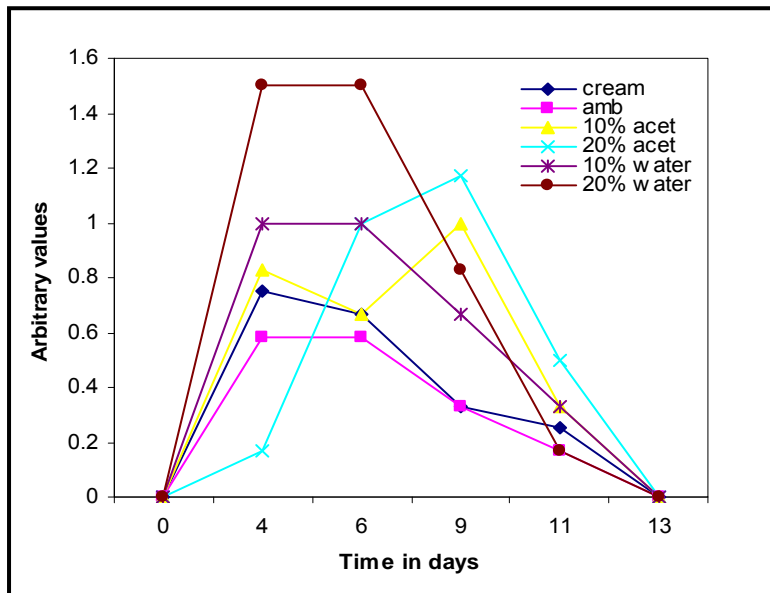


Figure 7.10. Exudate release from lesions treated with different *C. dentata*-derived extracts (acetone and water extracts).

Erythema was consistently high in wounds treated with 10% and 20% acetone extracts. Wounds treated with 10% and 20% acetone extracts had a larger surface area and lower wound contraction capacity. Perhaps there is a correlation between pronounced erythema and wound contraction capacity (Fig. 7.11 & 7.12).

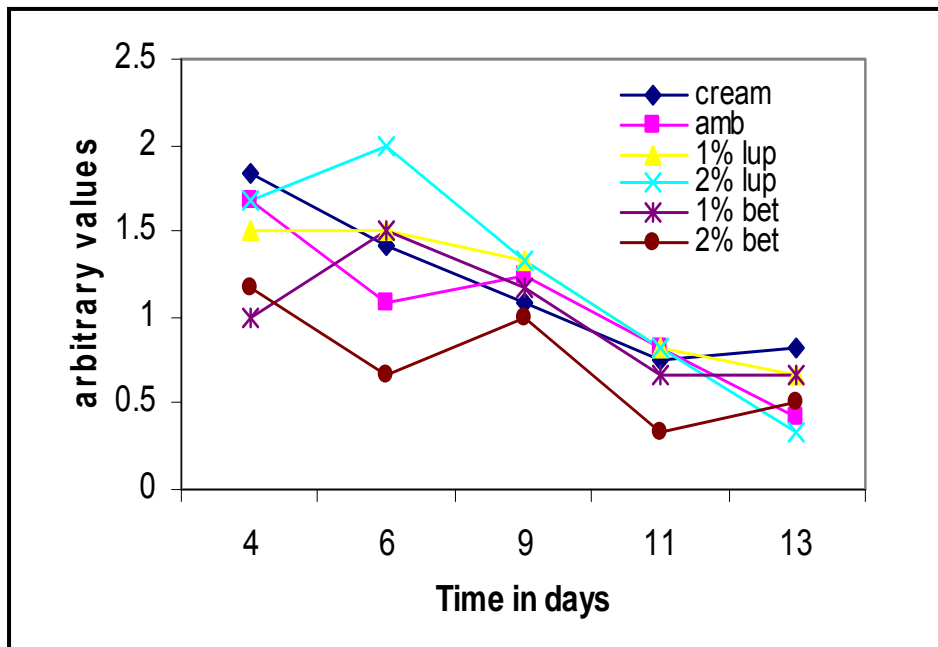


Figure 7.11. Erythema (arbitrary values) in wounds treated with compounds isolated from *C. dentata*.

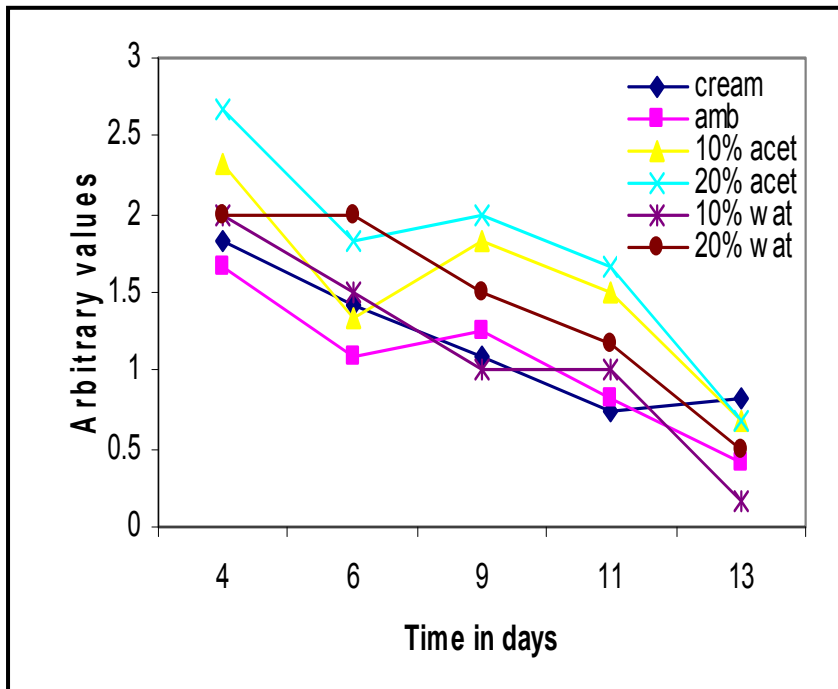


Figure 7.12. Erythema (arbitrary values) in wounds treated with extracts (acetone and water extracts) derived from *C. dentata*.

Crust formation was also determined on all wounds treated with various *Curtisia dentata*-derived products. Presence of crust on wounds was scored as 1 (one) while absence thereof was represented by zero (0). In all cases on day 4 minimal crust formation was observed, and it peaked on day 9 for most treatments. The maximal crust formation on wounds treated with 2% betulinic acid was the highest together with 10 and 20% acetone extract and 20% water extract treatments. Wounds treated with 1% lupeol displayed the least overall crust formation with just over 60% of the wounds positive for crust formation on day 9. On day 13, crust formation dropped in all wounds to levels as low as 0 (arbitrary values) (Figs. 7.13 and 7.14).

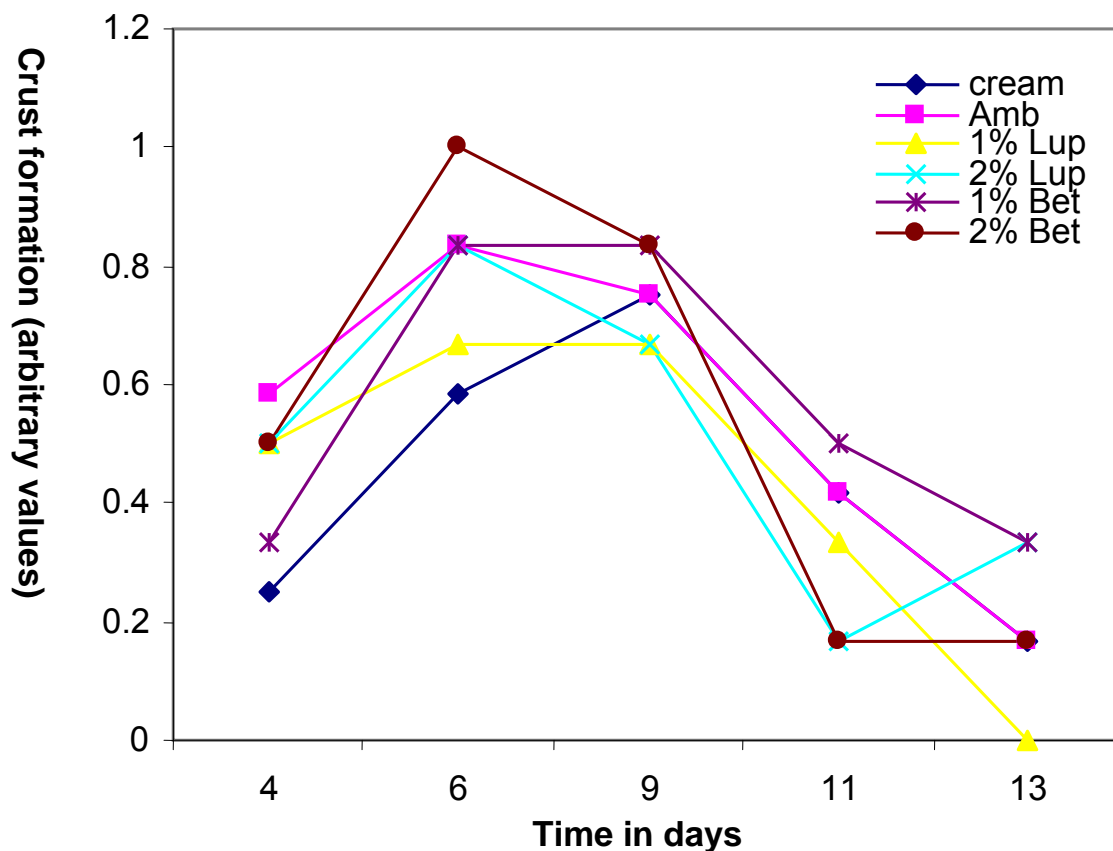


Figure 7.13: Crust formation in wounds treated with various concentrations of compounds during specified days of treatment. Amb, amphotericin B; Lup, lupeol; Bet, betulinic acid.

After 13 days swabs of the subcutaneous skin surfaces of rats 1, 5 and 11 produced viable colonies of *Candida albicans* on SD agar. The skin surface of all the other rats did not have any viable colonies as shown by absence of fungal growth on agar plates. The organs of rats 1, 4, 6, 7 and 11 were infected as indicated by the presence of colonies on agar plates (inoculated with samples of the internal organs). The identity of the infecting microbial culture was not confirmed, though *Candida albicans*, the organism used to infect the rats during the initial stages of the study remains the likeliest candidate (Table 7.8).

Fungal organisms or any fungal fragment were not detected in all haematoxylin-eosin stained skin sections. These skin sections were originally treated with various formulations containing either plant extracts or isolated compounds. The skin lesions were completely healed. Furthermore, no specific pathology could be demonstrated in the parenchymal organs examined.

In most of the kidney specimens, mild or moderate cortical or medullary congestion were present. About half of the liver specimens showed no signs of abnormality, while the rest had mild sinusoidal dilation and congestion. In general, the summary histopathological report suggest that there were no specific pathological abnormalities observed in the skin and parenchymal organ specimens.

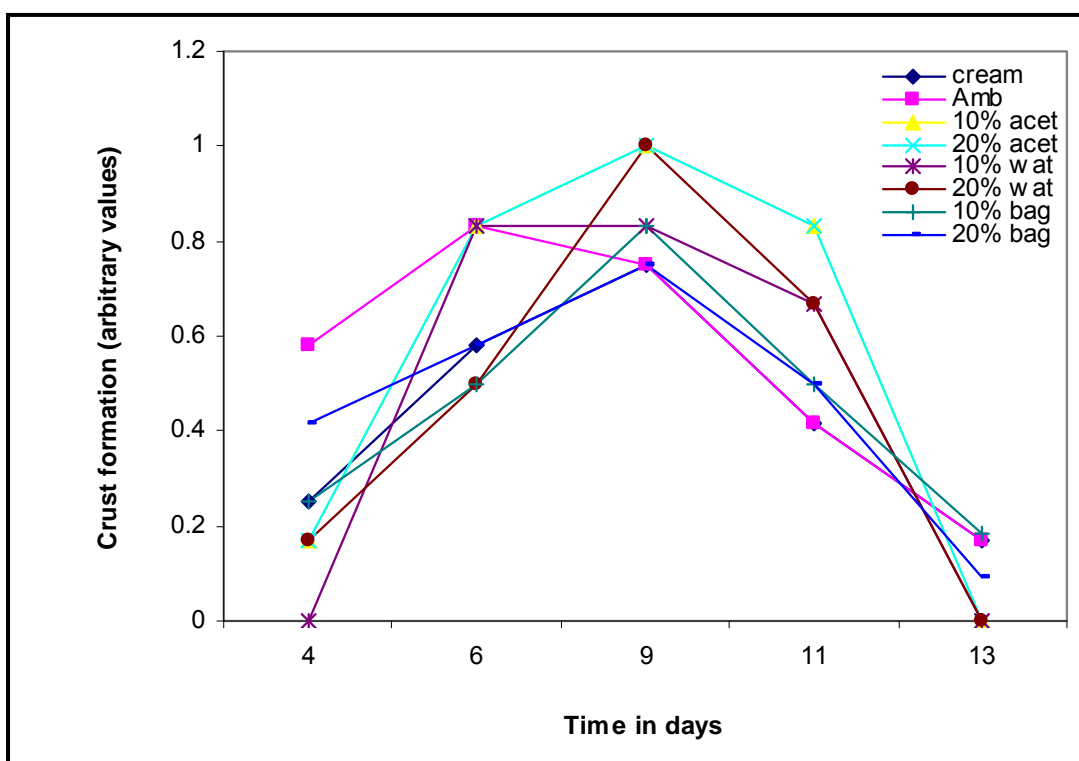


Figure 7.14: Crust formation in wounds treated with various concentrations of compounds during specified days of treatment. Amb, amphotericin B; ace, acetone extract; wat, water extract; bag, *Buddleja*.

Table 7.8. Number of colonies derived from inoculate of the subcutaneous tissue (skin under the wounds) and the internal organs. Key: - no colonies observed

Rat	Skin tissue	Internal organs
1	100	20
2	-	-
3	-	-
4	-	115
5	7	-
6	-	3
7	-	5
8	-	-
9	-	-
10	-	-
11	30	24
12	-	-

Granulomatous dermatitis was less in both amphotericin and cream treated controls. In all the other wounds granulomatous dermatitis was pronounced, recorded above 0.6 (arbitrary values). Wounds treated with 10% acetone extract of *Curtisia dentata* had the highest granulomatous dermatitis (Fig. 7.15).

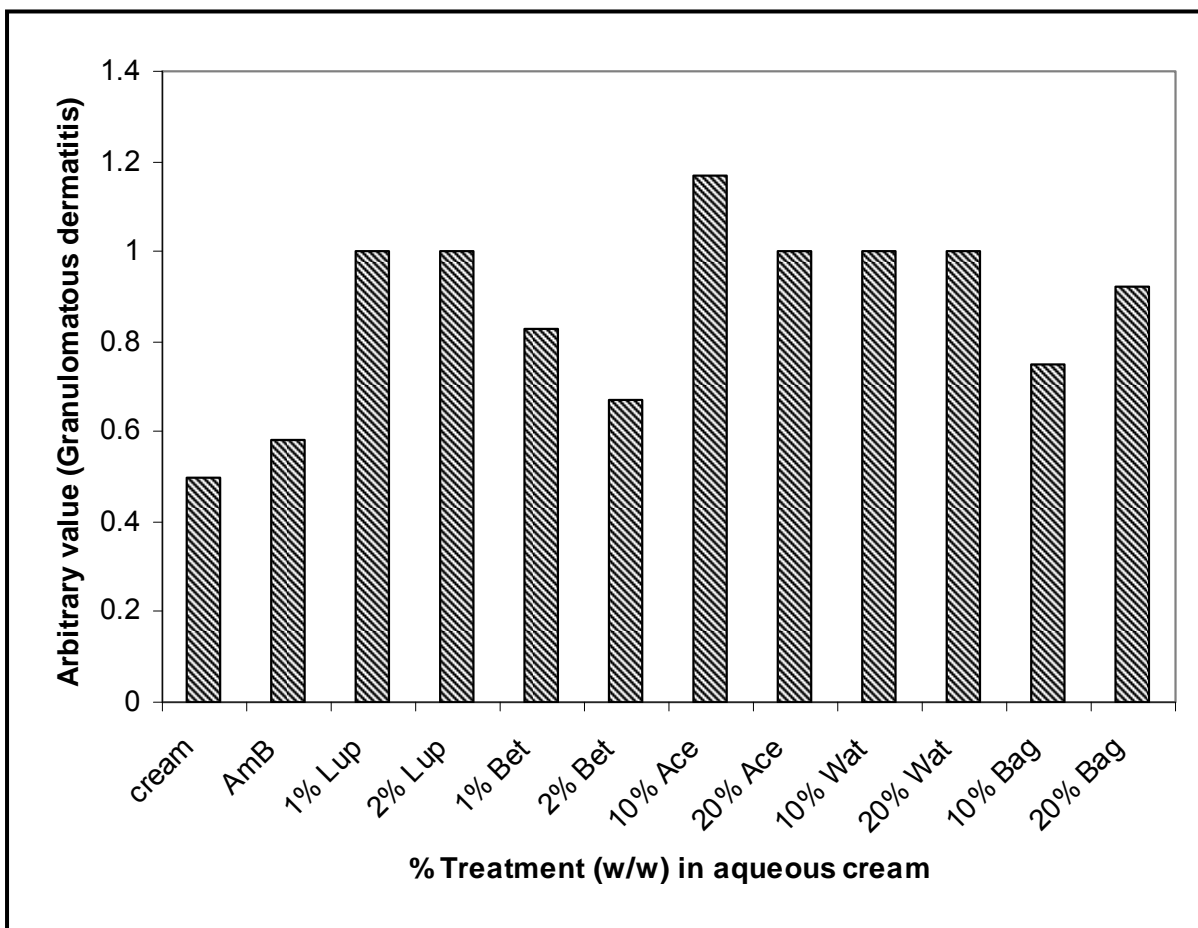


Figure 7.15: Histopathology for granulomatous dermatitis. Amb, amphotericin B; ace, acetone extract; wat, water extract; bag, *Buddleja* spp.; Lup, lupeol; Bet, betulinic acid.

7.4. DISCUSSION AND CONCLUSIONS

7.4.1. Weight loss and temperature

The loss of weight may have been due to the administered anaesthetic and shock from handling, including shaving and surgical creation of wounds. Furthermore, the infection of wounds with *Candida albicans* could have contributed to the overall weight loss. On observation day 9 the average weight of the rats increased, possibly resulting from the animals regaining functioning of their immune system following immunosuppression with estradiol valerate. Perhaps this allowed the animals to fight the infections, hence the increase in weight.

7.4.2. Lesion sizes

Lesions treated with 10 and 20% acetone had significantly larger sizes than the controls at the completion of the experiment. This may emanate from inhibition of tissue regeneration by these extracts. Since it was shown in the earlier chapters that the acetone extracts had *in vitro* antifungal activity against *Candida albicans* it is unlikely that the larger lesion sizes after 6 days resulted from increased infection of the wounds with the pathogen.

Wound healing involves an ordered series of events, namely, coagulation to prevent blood loss, inflammation of wound area, epithelial repair that includes proliferation, mobilization, migration and differentiation, and tissue remodelling and collagen deposition (Rashed *et al.*, 2003). Wound contraction was not markedly improved by any of the treatments applied to wounds since the lesion sizes remained comparable throughout the treatments. However, these extracts, due to their antimicrobial activity, may be used in wound healing mixtures to ward off microbial infection.

This study may be repeated with one excision wound per rat and one plant product applied to minimize interference resulting from systemic absorption of active constituents when more than one extract is applied on the same rat. In the present study, the assumption was made that systemic absorption of the applied extract, compound or commercial antifungal drug did not occur. This was necessary to limit as much as possible the number of rats needed in the study.

7.4.3. Exudate and erythema

Pronounced erythema was observed on wounds treated with 10 and 20% acetone extracts. The lesions had bigger surface areas than other treated wounds, suggesting that a relationship exists between wound contraction and erythema. These same wounds, together with those treated with 10 and 20% water extracts had higher exudation than other treated wounds, further correlating wound contraction to parameters such as exudation and erythema. Exudation is a direct result of the inflammatory response to injury, and includes recruitment of leukocytes to the site of injury (Majno & Joris, 1996). The presence of infection in the wound recruits more white blood cells resulting in pus (Majno & Joris, 1996). Treatment of wounds with amphotericin B and 2% betulinic acid resulted in the slightest release of exudates, suggesting that in these wounds *Candida albicans* load was kept at a minimum. Betulinic acid was shown in earlier chapters to have good *in vitro* antifungal activity.

Lupeol, at concentrations of 1 and 2% led to a pronounced release of exudate. It would seem that the increased exudate was a direct result of accumulation of leukocytes at the infected wound. In earlier chapters it was shown that lupeol did not greatly inhibit the growth of *Candida albicans* and other fungal species. Resulting from the failure of lupeol to sufficiently deal with *Candida albicans* in wounds, inflammation may have increased to combat the infection, hence the explicit exudation.

As the exudate seeps out onto the surface of the wound, it clots and eventually dries out into a scab or crust (Majno & Joris, 1996). The scab's function is to seal the wound from the environment, hence it is sometimes referred to as natural dressing (Grillo, 1964). It was also observed that crust formation on wounds treated with 2% betulinic acid formulation, 10 and 20% acetone and 20% water extracts had pronounced crust formation. Wounds treated with 2% betulinic acid still healed like those treated with amphotericin B if lesion sizes are considered. Wounds treated with both acetone extracts and 20% water extract had higher lesion sizes, indicating decreased wound contraction. However, these wounds still showed signs of healing, hence the pronounced crust formation. Treatment of wounds led to less scab formation for unknown reasons. However, the failure of lupeol to adequately inhibit *Candida albicans* infections might be responsible.

7.4.4. Presence of infection on skin tissue and internal organs

Skin tissues and internal organs of some of the rats harboured some fungal microorganism(s) as shown by the presence of colonies on agar plates inoculated with the respective samples. Though the identity of the infecting microorganism(s) was not confirmed or elucidated, *Candida albicans* remains the likeliest candidate. The masses and the temperatures of the rats that showed infection on the different sites remained unaffected. Furthermore, the organs did not show any abnormalities, whether or not the presence of microorganisms was detected. The wounds on these rats still healed similarly to those on the other rats, suggesting that the presence of infection on skin tissue or internal organs did little to hinder the healing process.

In evaluating the lack of correlation between *in vitro* and *in vivo* results one should keep in mind that *C. albicans* usually infects moist membranes or moist areas and that the model used may not be applicable to test the efficacy of compounds against *C. albicans* infections.

CHAPTER 8

ANTHELMINTHIC ACTIVITY OF *CURTISIA DENTATA* EXTRACTS, LUPEOL, URSOLIC ACID AND BETULINIC ACID

8.1. INTRODUCTION

The increasing emergence of resistance of gastrointestinal nematodes to conventional anthelmintics and resulting economic losses demonstrate an urgent need for intervention (Hertzberg & Bauer, 2000). *Trichostrongylus colubriformis* causes parasitic enteritis that predisposes sheep to diarrhoea, weakness and death. This parasite is frequently found in cattle and sheep in South Africa causing loss in production (Horak, 2003; Horak *et al.*, 2004). *Haemonchus contortus* is one of the most important nematodes due to its high prevalence and pathogenicity (Hounzagbe-Adote *et al.*, 2005). For instance, in southern Benin, the prevalence of *H. contortus* was estimated at 92.5% in goats and sheep (Salifou, 1996).

Control of infection with parasitic nematodes such as *T. colubriformis* and *H. contortus* involves the utilization of commercial anthelmintics. However, resistance to most of the commercialized anthelmintics is threatening productivity (van Wyk *et al.*, 1997). Furthermore, these chemical products are often highly priced so that subsistence and small-scale livestock farmers in developing countries cannot access them (Hounzagbe-Adote *et al.*, 2005). In these countries small-scale and subsistence farmers rely on traditional methods of deworming that include preparation of remedies from plants (Hammond *et al.*, 1997; Waller *et al.*, 2001; Akhtar *et al.*, 2000). The scientific evidence to support employing plant-based remedies as anthelmintics is lacking (Hammond *et al.*, 1997).

Extracts of several African plant species are active against parasitic and free-living nematodes (Enwerem *et al.*, 2001, Hammond *et al.*, 1997; McGaw *et al.*, 2000; Bizimenyera *et al.*, 2006). Extracts of *Artemisia* species have shown activity against *Haemonchus* species (Idris *et al.*, 1982; Iqbal *et al.*, 2004), *Trichostrongylus* species (Sharma, 1993) and other parasitic nematodes. Furthermore, Hördegen *et al.* (2003) demonstrated anthelmintic activity of ethanol extracts of several plant species in an *in vivo* sheep model.

The motivation for investigating antihelmintic activity is based on the activity of betulinic acid against *Caenorhabditis elegans* at 500 µg/ml after seven days of incubation (Enwerem *et al.*, 2001). Betulinic acid is one of the compounds isolated from *Curtisia dentata* leaves. The dichloromethane and acetone extracts of *Curtisia dentata* leaves, which are enriched with betulinic acid, were selected for investigation of anthelmintic activity as they were the most active against *Candida albicans*. Furthermore the azole antifungal agents also have anthelmintic activity. If the extracts with antifungal activity have the same target as the azole agents, they may also have anthelmintic activity.

In this study, extracts of *Curtisia dentata* were tested for *in vitro* anthelmintic activity against *Caenorhabditis elegans*, *Trichostrongylus colubriformis* and *Haemonchus contortus*. In the previous chapters the acetone and dichloromethane extracts of *Curtisia dentata* leaves were active *in vitro* against *Candida albicans* and several other fungal species. Betulinic acid, ursolic acid and lupeol, isolated from the leaves of *Curtisia dentata*, were also tested for anthelmintic activity.

8.2. MATERIALS AND METHODS

8.2.1. Plant extracts and compounds

Powdered leaves of *Curtisia dentata* were serially extracted as described in Chapter 2. Concentrations of 100 mg/ml of the dichloromethane and acetone extracts were dried and then dissolved in DMSO. Stock solutions of 2 mg/ml of betulinic acid, lupeol and ursolic acid were prepared in DMSO.

8.2.2. Anthelmintic activity

8.2.2.1. Recovery and preparation of eggs

The parasite eggs were prepared using guidelines by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles *et al.*, 1992). Faecal pellets were collected from lambs, infected with either *T. colubriformis* or *H. contortus* or both, using sterilized harnesses and collecting bags. Water was added to the faecal pellets to facilitate homogenization in a blender. The liquid slurry was filtered through a sieve of 400 µm mesh size to remove coarse plant debris. The suspension was serially filtered through sieves of 250, 150, 90 and 63 µm mesh sizes. The eggs

were then trapped on a sieve of 38 µm mesh size and washed into 50 ml centrifuge tubes that were filled with distilled water. These tubes were centrifuged at 3 000 x g for 5 min. The supernatant was collected into a 1 L conical cylinder filled with distilled water to allow the eggs to sediment for 2 hours. The eggs were siphoned from the bottom of the conical flask into a beaker subjected to electromagnetic stirring. The suspension was adjusted to a final concentration of 100 eggs per 10 µl. The suspension was used within 1 hr (Bizimenyera *et al.*, 2006).

8.2.2.2. Egg hatch inhibition assay

Approximately 100 eggs in 200 µl aliquots were pipetted into each well of a 48-well microtitre plate. In the test wells, 200 µl of the appropriate plant extract in concentrations of 16.7, 8.3, 4.17, 2.1, 1.03, 0.53 and 0.27 mg/ml or isolated compounds at 333, 167, 83, 42 and 21 µg/ml were added. Triplicates were prepared for each concentration. The eggs were incubated at room temperature in a humidified chamber for 48 hours prior to counting of eggs that failed to hatch to L1 stage of larval development (Bizimenyera *et al.*, 2006).

8.2.2.3. Anthelmintic activity against L3 stage larvae

The eggs (1 ml) were added into Petri dishes in the presence of 50 µl of a suspension of lyophilised *E. coli* (ATCC 9637) for the development of nematode larvae (Hubert & Kerboeuf, 1992) and 10 µl of amphotericin B (Sigma®) to control fungal growth. The dishes were incubated for five days to develop larvae into L3 stage of development. The L3 larvae were frozen until needed. For the anthelmintic assay, the larvae were washed off the base of dish with M9 buffer and treated with various concentrations of compounds and extracts of *Curtisia dentata* in wells and incubated for 48 hrs. Motile and non-motile larvae were counted to obtain percentage inhibition of motility. Larvae that did not display signs of motility were regarded as dead larvae.

8.2.2.4. Anthelmintic activity against developing larvae

The eggs hatched in **8.2.2.2.** above were used in this assay procedure. The emerging larvae were incubated under humidified conditions for 24 hours prior to treatment with various concentrations of isolated compounds and extracts of *Curtisia dentata* leaves. The larval suspensions were incubated for a further 5 days before

counting of motile and immotile larvae. The experiments and controls were performed in triplicate.

8.2.2.5. *C. elegans*

Caenorhabditis elegans was maintained in Petri-dishes on Nematode Growth agar (composition/litre: 3 g NaCl, 2.5 g peptone and 17 g agar, 5 mg cholesterol, 0.246 g MgSO₄.7H₂O, 0.147 g CaCl₂.2H₂O, 2.7 g KH₂PO₄, 0.9 g K₂HPO₄) (Brenner, 1974) seeded with *E. coli*. The dishes were incubated at 20°C in the dark for 6 days prior to anthelmintic activity assay procedures. The plates were washed with 1 ml M9 buffer (composition/litre: 6 g Na₂HPO₄, 3 g KH₂PO₄, 5 g NaCl, 0.25 g MgSO₄.7H₂O) (Brenner, 1974). Several plates were washed into a beaker to establish a density of 100 nematode/10 µl. Into 25-well plates 2 ml of plant extracts and compounds in M9 buffer were added. Nematodes (10 µl) were added into the wells and plates incubated for two hours in the dark. Larvae that did not display signs of motility were regarded as dead larvae. Numbers of dead and viable larvae were counted and percentage of dead cells calculated using the formula:

$$\% \text{ immotile larvae} = \frac{(\text{number of immotile larvae})}{(\text{Number of immotile} + \text{motile larvae})} \times 100 \%$$

Levamisole (5 and 10 µg/ml) was used as a positive control. Untreated larvae were used as negative controls.

8.2.2.6. Calculation of LC₅₀ values

The LC₅₀ (lethal concentration that resulted in 50% inhibition of larval motility) values were estimated from direct extrapolation of a plot of concentration of either compound or extract (X-axis) against the inhibition of larval motility (% of the control). An example of the type of graph drawn is displayed in Fig. 8.1.

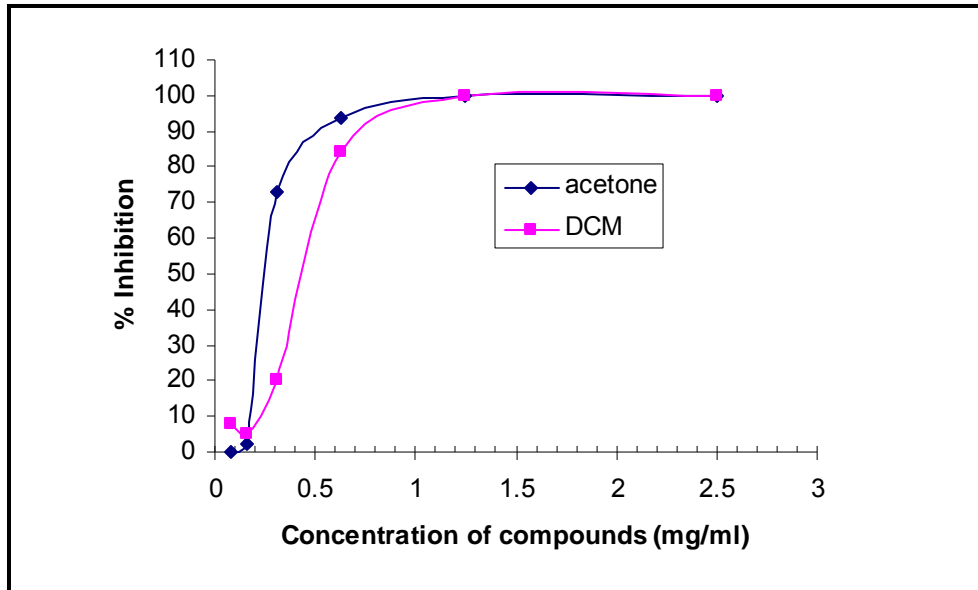


Figure 8.1. An example of a graph drawn to estimate the LC₅₀ values of compounds and bulk fractions against parasitic and free-living nematodes.

8.3. RESULTS

8.3.1. Egg hatching

T. colubriformis and *H. contortus* were treated with various concentrations of isolated compounds and extracts of *C. dentata* and incubated for five days. The larvae were treated a day after egg hatching. The percentages of motile larvae in high concentrations of compounds (333 and 167 $\mu\text{g/ml}$) were low. At these concentrations there were virtually no motile larvae. The number of motile larvae only increased as low concentrations of isolated compounds (Fig. 8.2). Similar results were obtained with crude extracts of *C. dentata* leaves, where high concentrations led to little or no observable evidence of motile larvae after 5 days of incubation. At low concentrations the number of motile larvae increased (Fig. 8.3). In solvent-treated controls about 91% of larvae were motile.

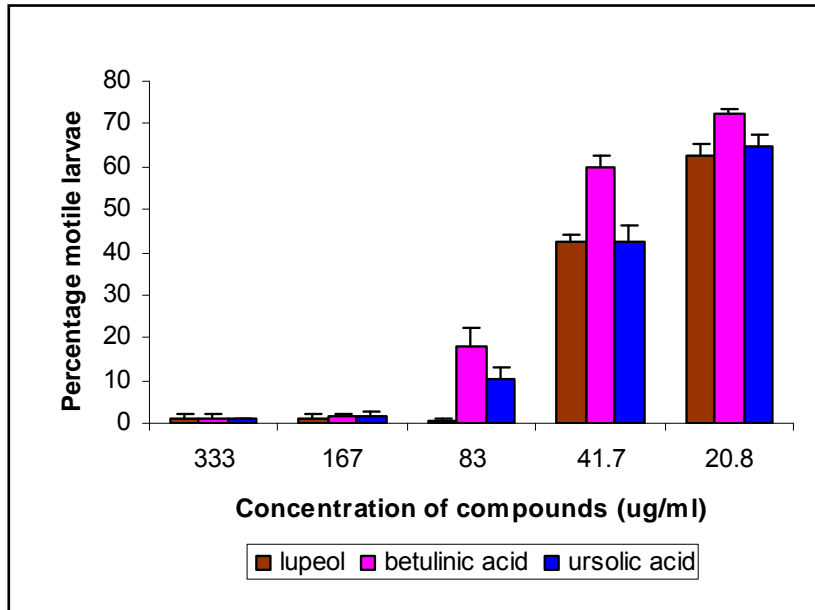


Figure 8.2. Percentage of motile larvae (mixture of *Haemonchus contortus* and *Trichostrongylus colubriformis*) treated with isolated compounds after five days of incubation. The LC₅₀ values were 35 µg/ml for lupeol, 65 µg/ml for betulinic acid and 35 µg/ml for Ursolic acid.

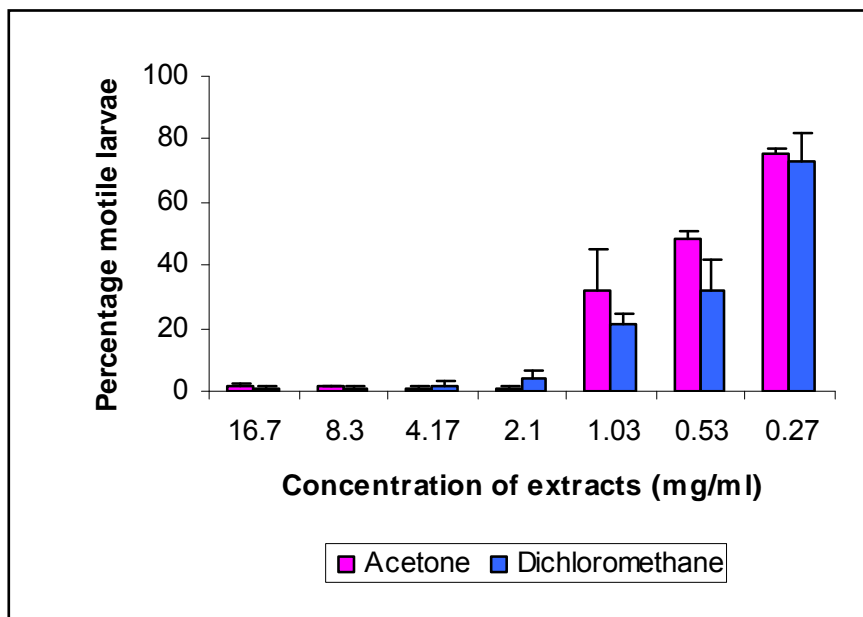


Figure 8.3. Percentage of motile larvae (mixture of *Haemonchus contortus* and *Trichostrongylus colubriformis*) treated with acetone and dichloromethane extracts of *Curtisia dentata* after five days of incubation. The LC₅₀ values were estimated at 0.4 mg/ml for both fractions.

The compounds and extracts did not affect egg hatching of *H. contortus* and *T. colubriformis*. In the controls the number of unhatched eggs was comparable to eggs treated with plant extracts and compounds. It was observed that at high concentrations (16.7, 8.3, 4.17 and 2.1 mg/ml) of plant extracts no eggs or larvae were present, suggesting that lysis occurred. In concentrations where the numbers of motile larvae were low the eggs still hatched prior to paralysis.

8.3.2. Inhibition of motility of adult parasitic nematodes

The acetone and dichloromethane extracts of *Curtisia dentata* inhibited motility of *Trichostrongylus colubriformis* at a concentration range of 0.16 – 2.5 mg/ml. The lowest toxic concentration of both the acetone and dichloromethane extracts, 0.16 mg/ml, led to over 60% paralysis of the larvae. The lowest tested concentration of both extracts, 0.08 mg/ml resulted in less than 10% of non-motile larvae. The larval motility percentages resulting from acetone extracts were very similar to those due to the dichloromethane extracts. The average survival in controls was 99% (Fig. 8.4).

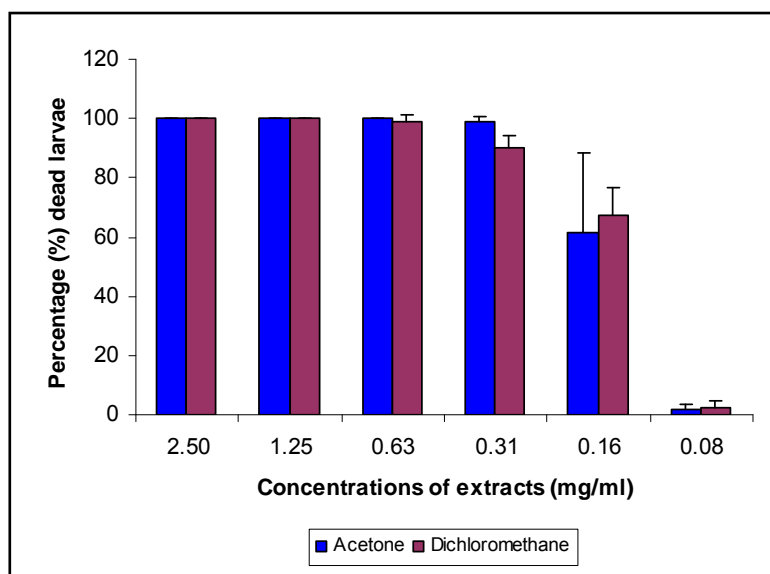


Figure 8.4. Percentage of dead *Trichostrongylus colubriformis* larvae after 48 hours of incubation in the presence of varying concentrations of the acetone and dichloromethane extracts of *Curtisia dentata* leaves. The estimated LC₅₀ value was calculated as 0.15 mg/ml for both acetone and DCM bulk fractions.

The dichloromethane extract was active against *Haemonchus contortus* at a concentration range of 0.63 – 2.5 mg/ml. About 20% average inhibition of motility of

larvae resulted from a concentration of 0.63 mg/ml. The acetone extract was active at concentration range 0.31 to 2.5 mg/ml. Over 70% of the larvae were not motile after 48 hours of incubation in the presence of 0.31 mg/ml of the acetone extract. At this concentration (0.31 mg/ml) the percentage of immotile larvae resulting from the acetone extract was significantly higher than that resulting from the dichloromethane extract. Concentrations of 2.5 mg/ml and 1.25 mg/ml of both acetone and dichloromethane resulted in no motile larvae after 48 hours of incubation. Concentrations of 0.16 and 0.08 mg/ml of both extracts only managed to inhibit motion of less than 10% of the larvae (Fig. 8.5).

Two compounds isolated from *Curtisia dentata* leaves, namely, betulinic acid and lupeol were tested to evaluate their ability to paralyse *Trichostrongylus colubriformis* and *Haemonchus contortus* larvae. The compounds were tested at the concentration range 0.32 – 1000 µg/ml. Both compounds inhibited larval motility at high concentrations. Low concentrations of the compounds did not result in appreciable numbers of motionless larvae. In the presence of compounds at the tested concentration range only less than 80% of the larvae of both nematode species were motionless after 48 hours of incubation. Lupeol and betulinic acid suffer from poor solubility in aqueous media (Figs. 8.6 & 8.7). Upon mixing with water during dilution the precipitation resulted in a milky suspension, making these compounds possibly unavailable to act on the test nematode larvae. Modifications of the structures may result in more soluble derivatives of these lupane triterpenes.

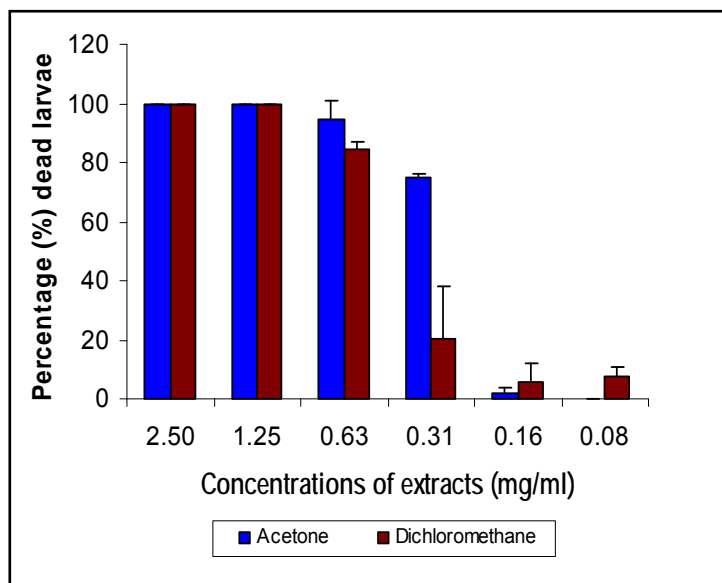


Figure 8.5. Percentage of dead *Haemonchus contortus* larvae after 48 hours of incubation in the presence of varying concentrations of the acetone and dichloromethane extracts of *Curtisia dentata* leaves. The estimated LC_{50} values were 0.2 mg/ml for acetone bulk fraction and 0.45 mg/ml for the DCM fraction.

The extracts showed more activity against the larvae than the isolated compounds. It would seem that, apart from lupeol and betulinic acid, other components in the extract contribute to the activity against larval motility. This emanates from the fact that betulinic acid and lupeol were, by themselves not very active against the tested nematodes. The compounds only managed less than 80% inhibition of larval motility at all tested concentrations.

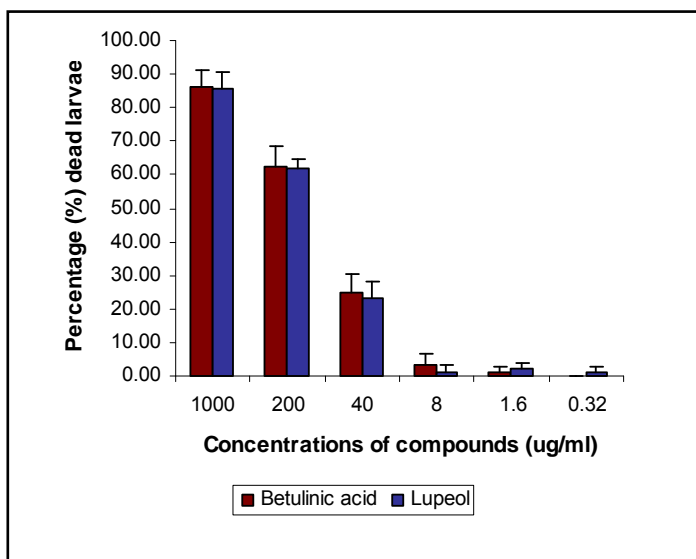


Figure 8.6. Percentage of dead *Trichostrongylus colubriformis* larvae after 48 hours of incubation in the presence of varying concentrations of lupeol and betulinic acid. The estimated LC₅₀ value was 80 µg/ml for both lupeol and betulinic acid. Nonmotile larvae were considered dead.

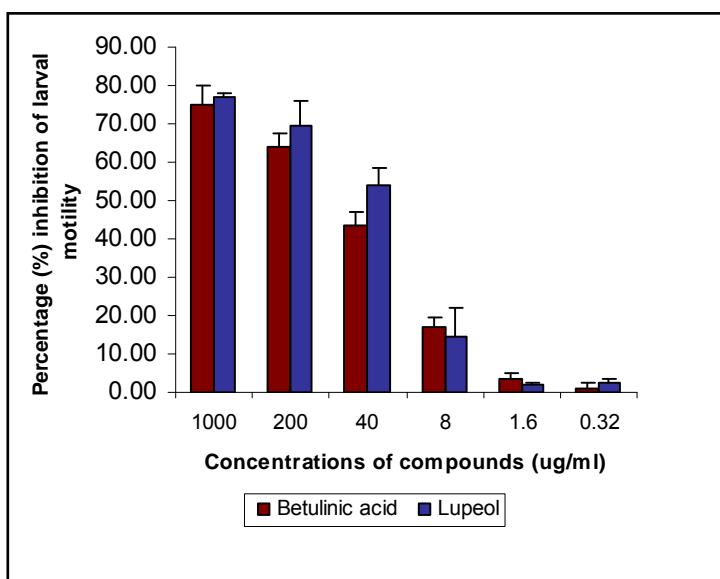


Figure 8.7. Percentage of dead *H. contortus* larvae after 48 hours of incubation in the presence of varying concentrations of lupeol and betulinic acid. LC₅₀ values were 20 µg/ml for lupeol and about 50 µg/ml for betulinic acid.

8.3.3. Inhibition of motility of free-living nematodes

Three compounds isolated from the dichloromethane extract of *C. dentata*, namely, lupeol, betulinic acid and ursolic acid, together with the acetone and dichloromethane extracts were assessed for ability to inhibit the motility of the free-living nematode *Caenorhabditis elegans*. After 2 hours of incubation, the highest concentrations of isolated compounds (200 µg/ml) inhibited up to 90% of larval motility. Concentrations ranging from 0.16 to 40 µg/ml resulted in about 20% inhibition of larval motility after 2 hours of incubation at 25 °C (Fig. 8.8). The highest concentrations of extracts (2.5 mg/ml) resulted in about 35% inhibition of larval motility whereas lower concentration ranging from 1.25 to 0.08 mg/ml resulted in less than 20% inhibition of larval motility (Fig. 8.9). In the untreated control 4% motionless larvae were observed after 2 hrs of incubation. In the levamisole-treated (10 µg/ml) controls average inhibition of larval motility was at 25%.

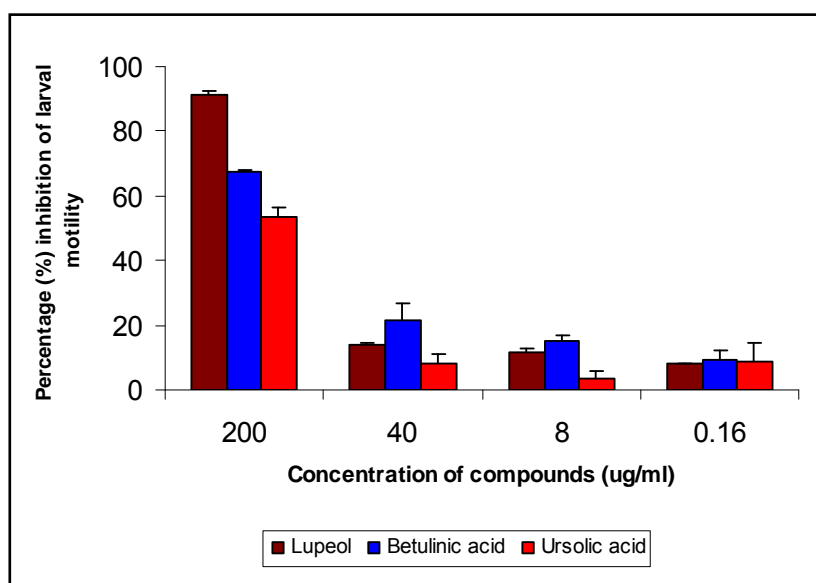


Figure 8.8. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with compounds isolated from *Curtisia dentata* leaves. After treatment with compounds the nematodes were incubated for 2 hrs before counting motionless worms. The LC₅₀ values were estimated at 120 µg/ml for lupeol, 140 µg/ml for betulinic acid and 180 µg/ml for Ursolic acid.

After 7 days of incubation the highest concentrations of all tested compounds resulted in 100% inhibition of larval motility. Lupeol was observed as the most active of the isolated compounds. A concentration of 40 µg/ml of lupeol resulted in over 80% inhibition of larval motility while betulinic acid led to 40% inhibition. Ursolic acid,

though less active than lupeol led to higher inhibition of motility than betulinic acid at most concentrations tested (Fig. 8.10). After 7 days of incubation in the untreated controls approximately 85% motility was observed. In 5 and 10 µg/ml levamisole-treated controls 65% and 48% motility were observed, respectively.

Both the acetone and dichloromethane extracts of *Curtisia dentata* leaves were similar in their inhibition of larval motility after 7 days of incubation, displaying a concentration-dependent inhibition of larval motility. Concentrations ranging from 2.5 to 0.63 mg/ml led to over 80% inhibition of larval motility after 7 days of incubation. At 0.31 mg/ml, both extracts caused over 60% inhibition of larval motility while lower concentrations were less effective (Fig.8.11).

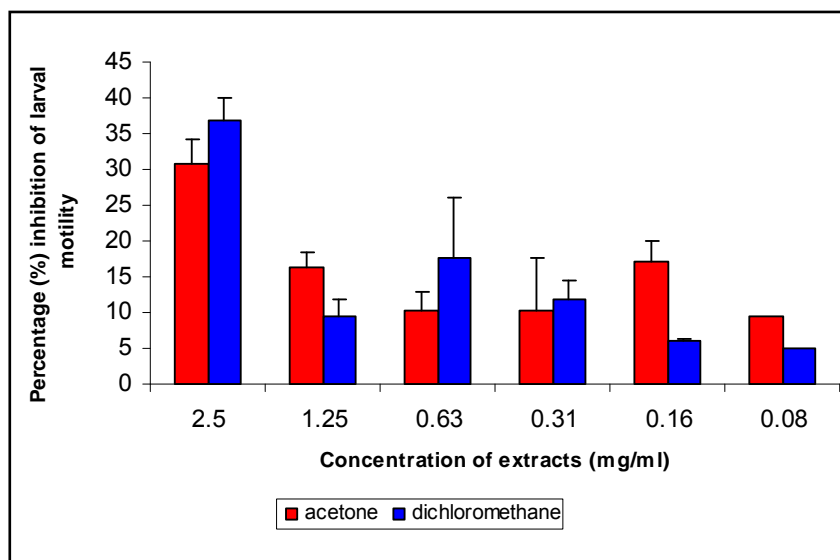


Figure 8.9. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with acetone and dichloromethane of *Curtisia dentata* leaves. After treatment with plant extracts the nematodes were incubated for 2 hrs before counting motionless nematodes.

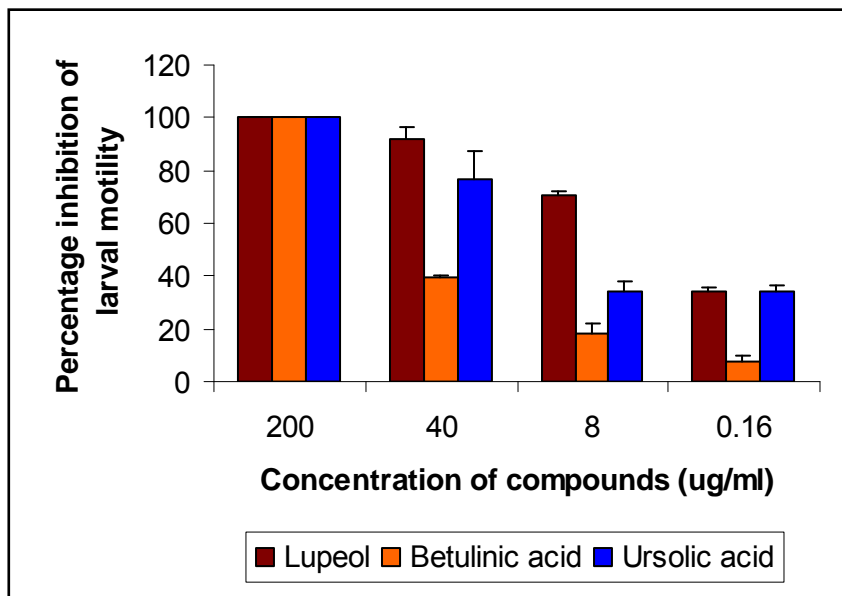


Figure 8.10. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with isolated compounds from leaves of *Curtisia dentata*. After treatment with the pure compounds, the nematodes were incubated for 7 days before counting motionless and motile worms. The LC₅₀ values were 2 µg/ml for lupeol, 70 µg/ml for betulinic acid and about 12 µg/ml for Ursolic acid.

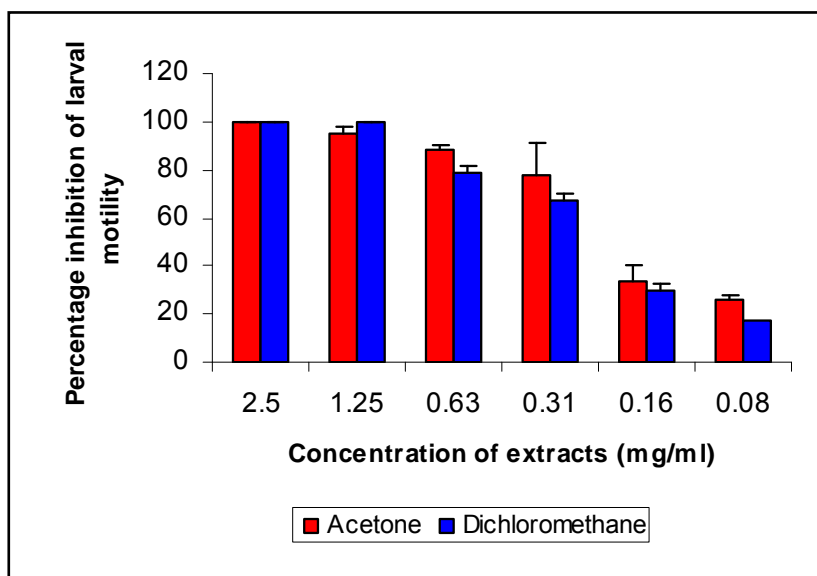


Figure 8.11. Inhibition of motility of free-living nematode, *Caenorhabditis elegans* with the acetone and dichloromethane extracts of the leaves of *Curtisia dentata*. After treatment with the pure compounds, the nematodes were incubated for 7 days before counting of motionless and motile worms. The calculated LC₅₀ values were 0.2 mg/ml for both fractions.

8.4. DISCUSSION AND CONCLUSION

Several extracts of different medicinal plants show anthelmintic activity (Raj, 1975; Enwerem *et al.*, 2001; Ademola *et al.*, 2004). Hounzangbe-Adote *et al.* (2005) tested the *in vitro* effects of four tropical plants on activity against parasitic nematode. They reported that concentrations of between 300 and 2400 µg/ml of ethanolic extracts of Fagara, Papaya, Morinda and Newbouldia are active against *H. contortus*. In this study we report that the anthelmintic activity of our extracts were comparable with reported literature (Hounzagbe-Adote *et al.*, 2005). Acetone extracts of *Curtisia dentata* at concentrations ranging from 310-2500 µg/ml were effective against *H. contortus*.

Bizimenyera *et al.* (2006) reported that extracts of *Peltophorum africanum* had *in vitro* activity against egg hatching and larval development of *T. colubriformis*. The ED₅₀ values for leaf, bark and root extracts against egg hatching were 0.619, 0.383 and 0.280 mg/ml, respectively. In this study the *in vitro* activity of extracts of *C. dentata* leaves was demonstrated against parasitic and free-living nematodes. These results indicate that the leaves of *C. dentata* may be useful to treat helminthiasis in South African folk medicine.

Despite the indication that some plant extracts are active against several nematodes, the exact mechanism remains unclear. Some compounds such as palasonin, the active principle of *Butea frondosa* inhibit glucose uptake and accelerate glycogen depletion in target nematodes (Kumar *et al.*, 1995). The mechanism of action of many anthelmintic plant extracts may involve inhibition of energy metabolism (Dahanukar *et al.*, 2000).

In this study, betulinic acid induced paralysis of the tested nematodes, *C. elegans*, *H. contortus* and *T. colubriformis* at concentrations of between 200 and 1000 µg/ml. Against *C. elegans* betulinic acid induced 100% paralysis at a concentration of 200 µg/ml. These results confirm the study by Enwerem *et al.* (2001) in which it was reported that betulinic acid isolated from *Berlina grandiflora* had strong activity against *C. elegans* at concentrations of 100 and 500 µg/ml. The mechanism involved in the activity of betulinic acid against parasitic and free-living nematodes is yet to be described. However, its activity against parasitic nematodes *in vivo* and *in vitro* has not been demonstrated. This appears to be the first report of the effects of betulinic acid against parasitic nematodes, *T. colubriformis* and *H. contortus*. Investigations of



the *in vivo* activity of both the extracts of *C. dentata* and the isolated compounds may conclusively indicate their potential as anthelmintics.

CHAPTER 9

GENERAL DISCUSSIONS AND CONCLUSIONS

The aim of this study was to isolate and characterize compounds active against *C. albicans* from leaves of the most promising tree species and investigate the potential use of these compounds or extracts against *C. albicans* infections. The results obtained in addressing the different objectives of the study are discussed below.

9.1. ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF SELECTED PLANT SPECIES

The antifungal and antibacterial activity of acetone extracts of leaves of seven selected trees were determined. Extracts of *Curtisia dentata* had more active compounds on bioautograms against all the bacterial (*Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis*, *Sporothrix schenckii* and *Aspergillus fumigatus*) test organisms. Antifungal compounds with similar R_f values also inhibited bacterial growth, suggesting non-specificity of the antimicrobial activity. The acetone and dichloromethane extracts of *C. dentata* contained more antibacterial and antifungal compounds (4) than the hexane extract. This suggests that the active compounds are of intermediate polarity. The dichloromethane and hexane extracts of six *Terminalia* species had more antifungal compounds (total of 59 and 50) than the acetone extracts (18) (Masoko *et al.* 2005)..

The serial microplate dilution method results showed that the acetone and dichloromethane extracts of most plant species had both antibacterial and antifungal activity. The lowest minimal inhibitory concentration (MIC) recorded was 0.05 mg/ml resulting from the acetone extract of *Curtisia dentata* against *Enterococcus faecalis*, further suggesting that *Curtisia dentata* was the most active species. The acetone extracts of *C. dentata*, *Terminalia sambesiaca* and *T. phanerophlebia* yielded the lowest MIC values (0.12 – 0.15 mg/ml) against *C. albicans*. This observation further confirmed the antifungal activity of *C. dentata*. However, the low MIC values of the *Terminalia* species were surprising considering that active compounds were not observed on TLC bioautograms against *C. albicans*. Masoko *et al.* (2005) reported low MIC values for both *Terminalia* species with few compounds observed on TLC bioautograms against most fungal species tested. Furthermore, all extracts of both

Terminalia species and acetone extract of *C. dentata* resulted in low MIC values (0.02 – 0.08 mg/ml) against *Cryptococcus neoformans* and *Microsporium canis*. The acetone extracts of the three plant species had low MIC values (0.03 – 0.08 mg/ml) against *Sporothrix schenckii*. These observations, supported by the MIC averages, indicated that these three plant species were the most active against fungal test organisms.

9.2. SELECTION OF THE MOST PROMISING TREE SPECIES FOR ISOLATION OF ACTIVE COMPOUNDS

From a pool of seven plant species, *Curtisia dentata* was selected for further work based on the following reasons, 1) the leaf extracts of the plant species possessed more compounds showing activity against *C. albicans* and other tested microbial organisms on bioautograms, 2) extracts of the leaves resulted in low MIC values against *C. albicans* and 3) its leaf powder was more extractible resulting in higher masses of extracts. *Curtisia dentata* was therefore chosen for isolation of active compounds using bioassay-guided fractionation. The leaves of *C. dentata* were preferred to the stem bark because 1) the leaves were more active than the stem bark and 2) the harvesting of the stem bark may kill the plant and *C. dentata* is a threatened species.

9.3. ISOLATED COMPOUNDS

Using bioassay-guided fractionation four (4) pentacyclic triterpenoids were isolated from the leaves of *Curtisia dentata*. ¹³C and ¹H NMR spectroscopic and mass spectrometric data led to identification of the compounds as the lupane triterpenoids lupeol and betulinic acid, and the ursane triterpenes ursolic acid and colosolic (hydroxyursolic) acid. These compounds are common secondary metabolites present in many plant species (Das and Mahato, 1983). However, these compounds have not been isolated from extracts of *Curtisia dentata*, the only southern African member of the Cornaceae previously. Activities of some of these compounds against infections and cancer progression have however been reported.

Reports concerned with antifungal, antibacterial and anthelmintic activities of betulinic acid and other pentacyclic triterpenoids are scanty. Betulinic acid and its derivatives have potent anti-HIV activity (Aiken & Chen, 2005). Ursolic acid has hepatoprotective, anti-inflammatory, antiviral and antitumor activities (Rao *et al.*,

2001), as well as growth inhibitory activity against *Staphylococcus aureus* (Zeletova *et al.*, 1987) and *Escherichia coli* (Malladavhani *et al.*, 2004). Ursolic acid isolated from *Crataegus pinatifida* is active against HIV-1 protease at 100 µg/ml (Pengsuparp *et al.*, 1994). *Curtisia dentata* is an excellent source of betulinic acid, estimated at approximately 25% in the dichloromethane extract of the leaves. This level of betulinic acid has not been reported anywhere in the Cornaceae family. In *Betula* species the betulinic acid content is estimated at 22% (Yogeeswari and Sriram, 2005).

Betulinic acid, lupeol and ursolic acid resulted in clear zones on TLC bioautograms sprayed with *C. albicans*, *S. aureus*, *E. coli* and *Enterococcus faecalis*. Using the serial microplate dilution method, betulinic acid, ursolic acid and colosolic acid resulted in appreciable inhibition of fungal growth with MIC values ranging from 8 to 63 µg/ml. A similar trend was observed with the bacterial test organisms. The extracts were more active than isolated compounds against bacterial test organisms, suggesting that perhaps synergism plays an important role in the activity of the plant species. All the three compounds possess a carboxyl (-COOH) group at position 28. It has been reported that the 28-COOH and the ester functionality at C-3 may be involved in the pharmacological actions of some triterpenoids (Mallavadhan *et al.*, 2004). This suggestion may account for the low antifungal activity of lupeol which lacks the 28-COOH group. This may further suggest that 28-COOH may be important for pharmacological actions of these and other triterpenoids.

9.4. ANTHELMINTHIC ACTIVITY

Some antifungal compounds are also active against nematodes, therefore the anthelmintic activity of the extracts and isolated compounds were investigated. Extracts of different medicinal plants show anthelmintic activity against both parasitic and free-living nematodes in both *in vivo* and *in vitro* experiments (Raj, 1975; Enwerem *et al.*, 2001; Ademola *et al.*, 2004). Recently, Hounzangbe-Adote *et al.* (2005) tested the *in vitro* effects of extracts of four tropical plants on activity against parasitic nematodes. However, there exist no reports regarding anthelmintic activity of *C. dentata* extracts against both parasitic and free-living nematodes.

Lupeol and betulinic acid together with the acetone and dichloromethane extracts of *Curtisia dentata* were investigated for the presence of anthelmintic activity. This appears to be the first report of the anthelmintic activity of extracts of *Curtisia*

dentata. In this study betulinic acid inhibited the motility of *Trichostrongylus colubriformis*, *Haemonchus contortus* and *Caenorhabditis elegans*. Enwerem *et al.* (2001) reported that betulinic acid inhibited the motility of *C. elegans* at 500 µg/ml (100 µg/ml achieved about 76% inhibition of motility). In our study betulinic acid achieved 100% inhibition of motility at 200 µg/ml. Our results are comparable with those reported by Enwerem *et al.* (2001). These findings further suggest that the activity against *C. elegans* was only observable at high concentrations of betulinic acid. Though *C. elegans* is a valid model organism for the discovery of broad-spectrum anthelmintics, the efficacy of betulinic acid as an anthelmintic has not previously been elevated to include studies on parasitic nematodes. This study demonstrated that betulinic acid has anthelmintic activity against parasitic nematodes at high concentrations (40-200 µg/ml). Ursolic acid and hydroxyursolic acid were isolated in inadequate quantities and these compounds could not be tested for anthelmintic activity.

9.5. CYTOTOXICITY

The cytotoxicity of lupeol and betulinic acid was determined against Vero cells. Toxicity of betulinic acid was comparable to that of berberine with an LC₅₀ value of about 10 µg/ml. Lupeol was less toxic to the cells with an LC₅₀ value of 90 µg/ml. Some researchers reported that the pharmacological actions of betulinic acid are associated with little cytotoxic activity.

The selectivity index values calculated for betulinic acid were below 1 for all the microorganisms. This could mean that some of the observed activity of betulinic acid against bacteria and fungi emanated from toxicity. Bacteria and fungi were all susceptible to betulinic acid, further suggesting that this compound is non-specific in its antimicrobial activity.

9.6. IN VIVO STUDIES

The isolated compounds and extracts were investigated for their *in vivo* wound healing activity and antifungal activity against *C. albicans*. Progression of wound healing in a topical rat model (either enhanced or at a normal rate) was used as measure of antifungal activity. The rate of wound healing of lesions treated with different plant-derived formulations remained comparable with the positive (amphotericin B) and negative (cream only) controls. This model needs to be

validated and optimized. It may require the use of known wound-invading microorganisms in the tests. This model did not yield any evidence of *in vivo* antifungal activity and wound healing activity of the acetone and water extracts of *C. dentata* and isolated compounds (lupeol and betulinic acid).

9.7. CONCLUSIONS

From a pool of seven plant species *Curtisia dentata* contained more antibacterial and antifungal compounds than any of the six species investigated. *C. dentata*, *Terminalia sambesiaca* and *T. phanerophlebia* were the most active against fungal pathogens based on MIC values. Part of the activity of *C. dentata* could be attributed to the triterpenoids that were isolated. Ursolic acid, betulinic acid and colosolic acid were active against bacterial and fungal test organisms. Surprisingly the extracts were more active against bacterial organisms than the isolated compounds indicating possible synergistic effects.

Lupeol was more active against nematodes. Therefore, this project is further proof that plants contain biologically active compounds. Synthetic chemistry techniques to make derivatives of the isolated compounds may enhance their solubility in water, increase their efficacy and even improve specificity of the compounds. Betulinic acid was isolated in large amounts from the leaves of *Curtisia dentata*. Derivatives of betulinic acid were shown to be more active inhibitors of HIV-1 reverse transcriptase than the parent compound (betulinic acid) (Pengsuparp *et al.*, 1994).

Based on the cytotoxicity data of betulinic acid and the crude extracts, the therapeutic effects of this compound or extracts may be limited only to low concentrations. Further investigation to ascertain the safety of the compounds and extracts to treat candidosis are required. The cytotoxicity data in this study was only obtained with Vero cells (monkey kidney line), and thus studies with several human cell lines may further indicate the safety of the compounds and extracts of *Curtisia dentata* as treatment regimens against candidosis. Furthermore if candidosis is treated by topical application and the toxic components are not absorbed, a useful product may still be developed from *Curtisia dentata* leaves. Because preparations of the extracts, betulinic acid and lupeol in aqueous cream did not result in any observable pathology on rats, topical application may be a viable route to combat *Candida albicans* infections. A total activity of 1072 ml/g for leaf extracts means that the quantity extracted from 1 g can be diluted to more than a litre and still inhibit the

growth of *C. albicans*. Another approach is that derivatization of this compound may provide less toxic and still efficacious derivatives.

It would therefore be worthwhile to invest human and financial resources for intensified screening of medicinal plants with the realistic hope of finding potential antibiotic drugs and cheap alternative medicines. The focus must not only be to isolate compounds, but also to investigate the efficacy of the extracts as these would be cheaper and more freely available.



CHAPTER 10

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APPENDIX

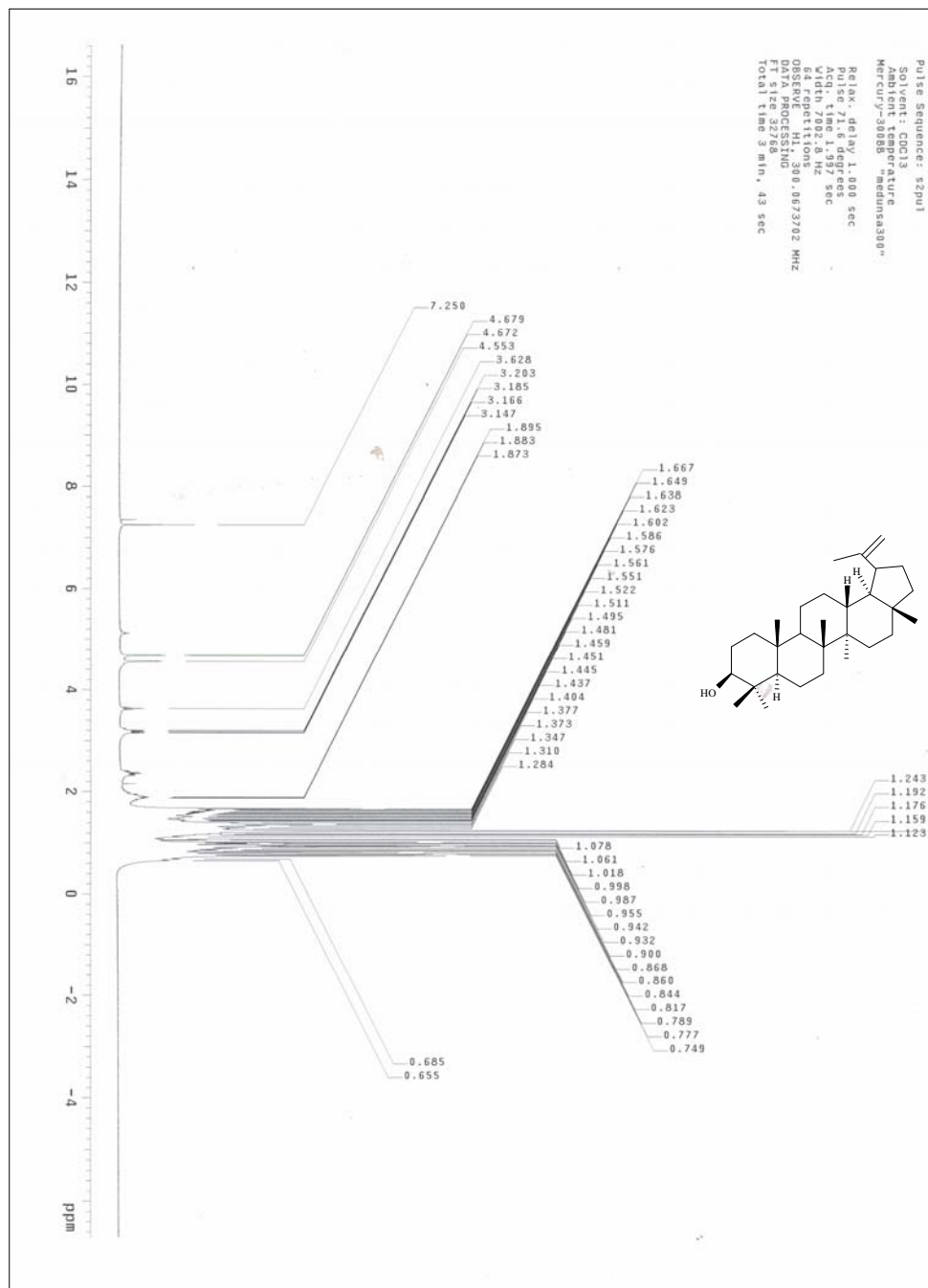


Figure A.1. The ¹H NMR spectrum of lupeol (CI) isolated from the leaves of *C. dentata*.

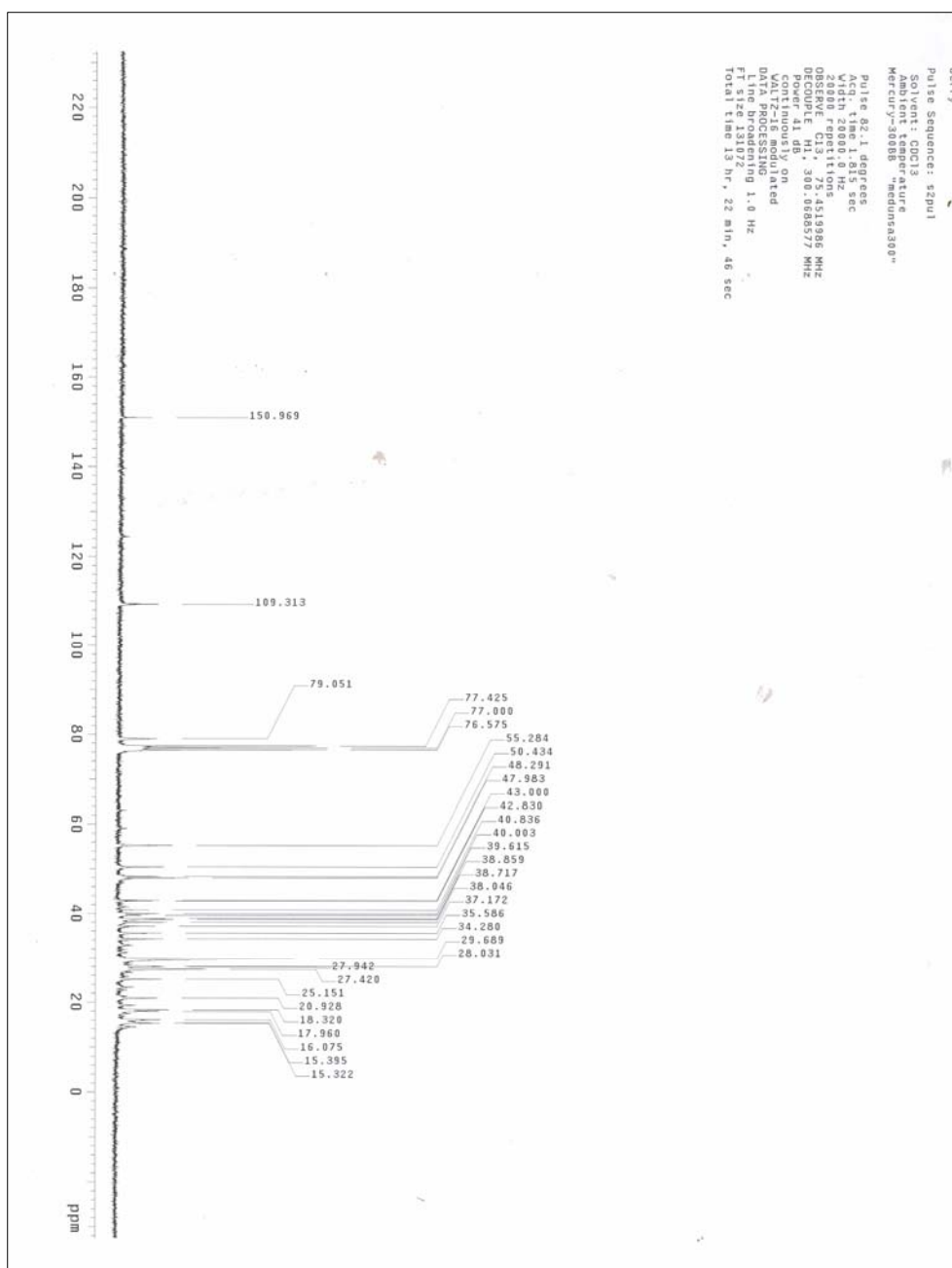


Figure A.2. ^{13}C NMR spectrum of lupeol (CI) isolated from leaves of *C. dentata*

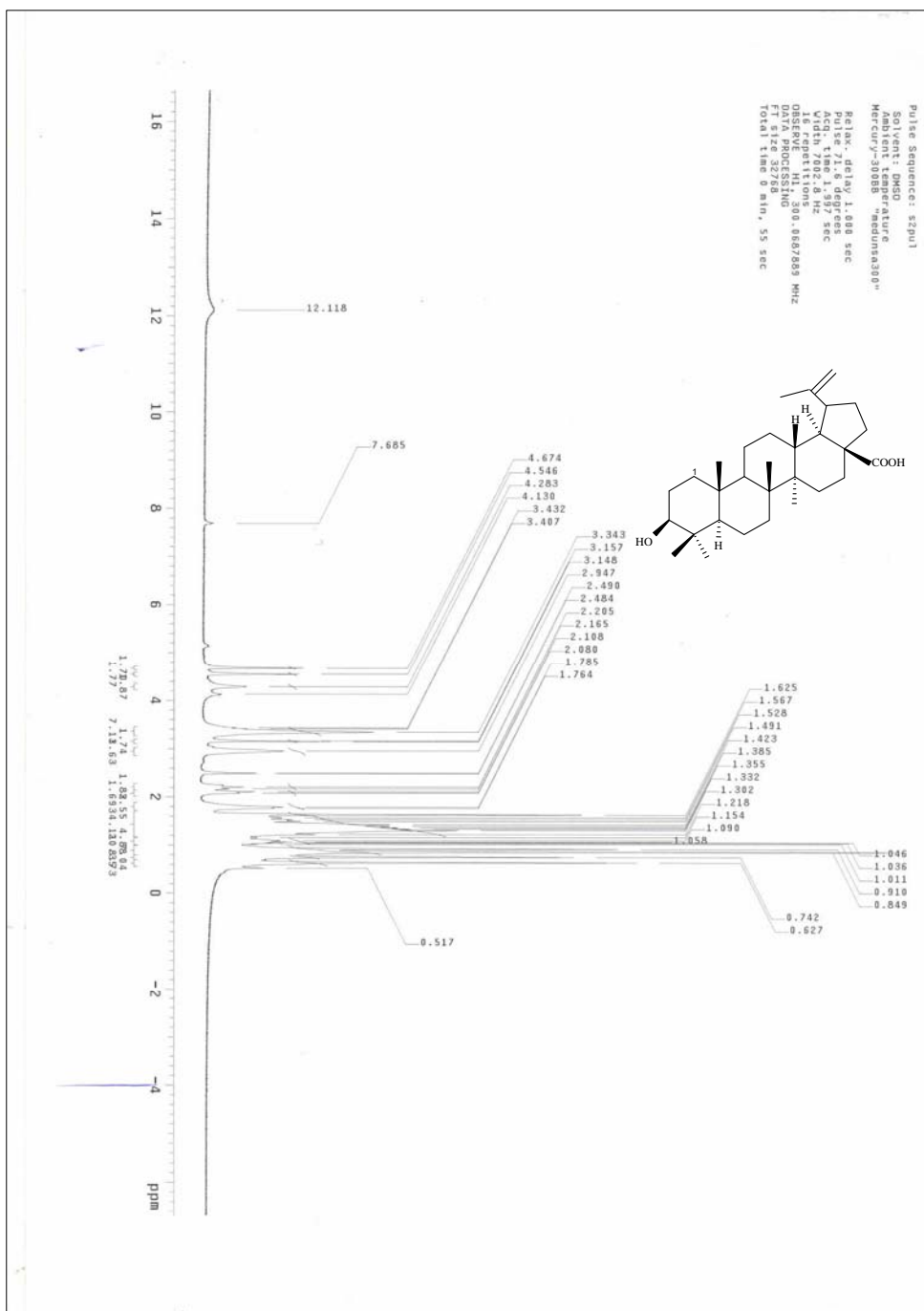


Figure A.3. ^1H NMR spectrum of betulinic acid (CII) isolated from leaves of *C. dentata*.

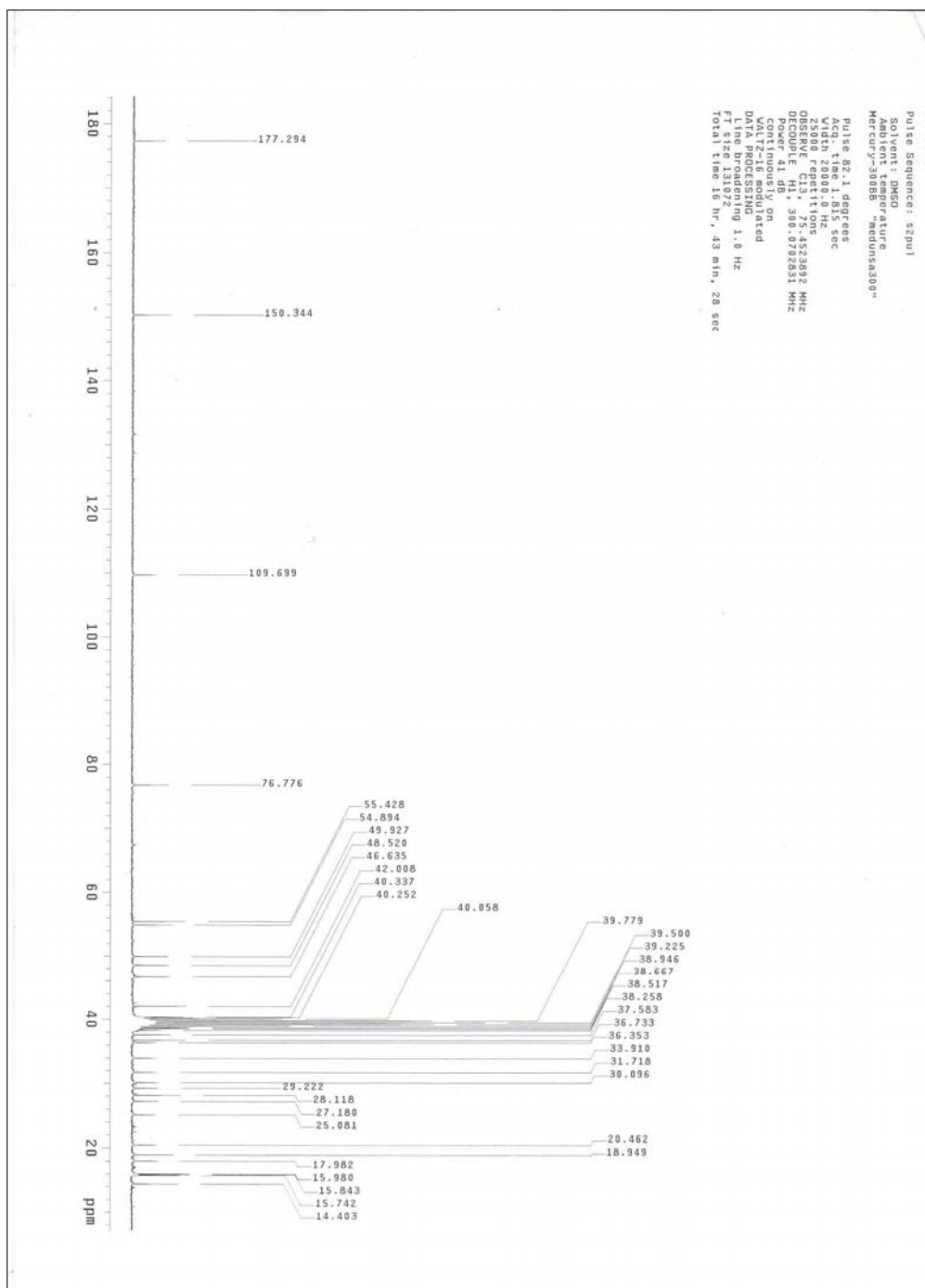


Figure A.4. ^{13}C NMR spectrum of betulinic acid (CII) isolated from leaves of *C. dentata*.

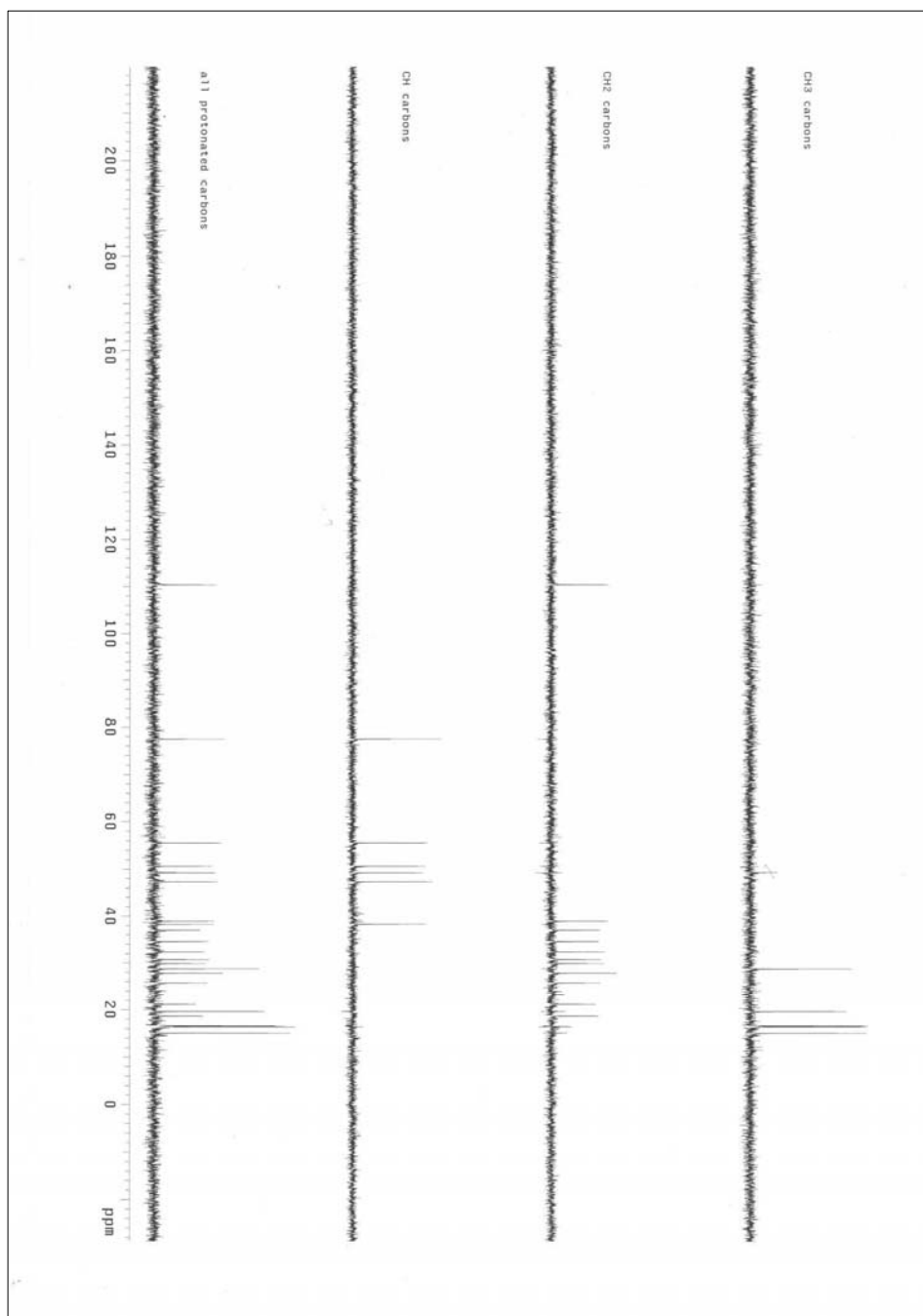


Figure A.5. DEPT experimental data of betulinic acid isolated from the leaves of *C. dentata*.

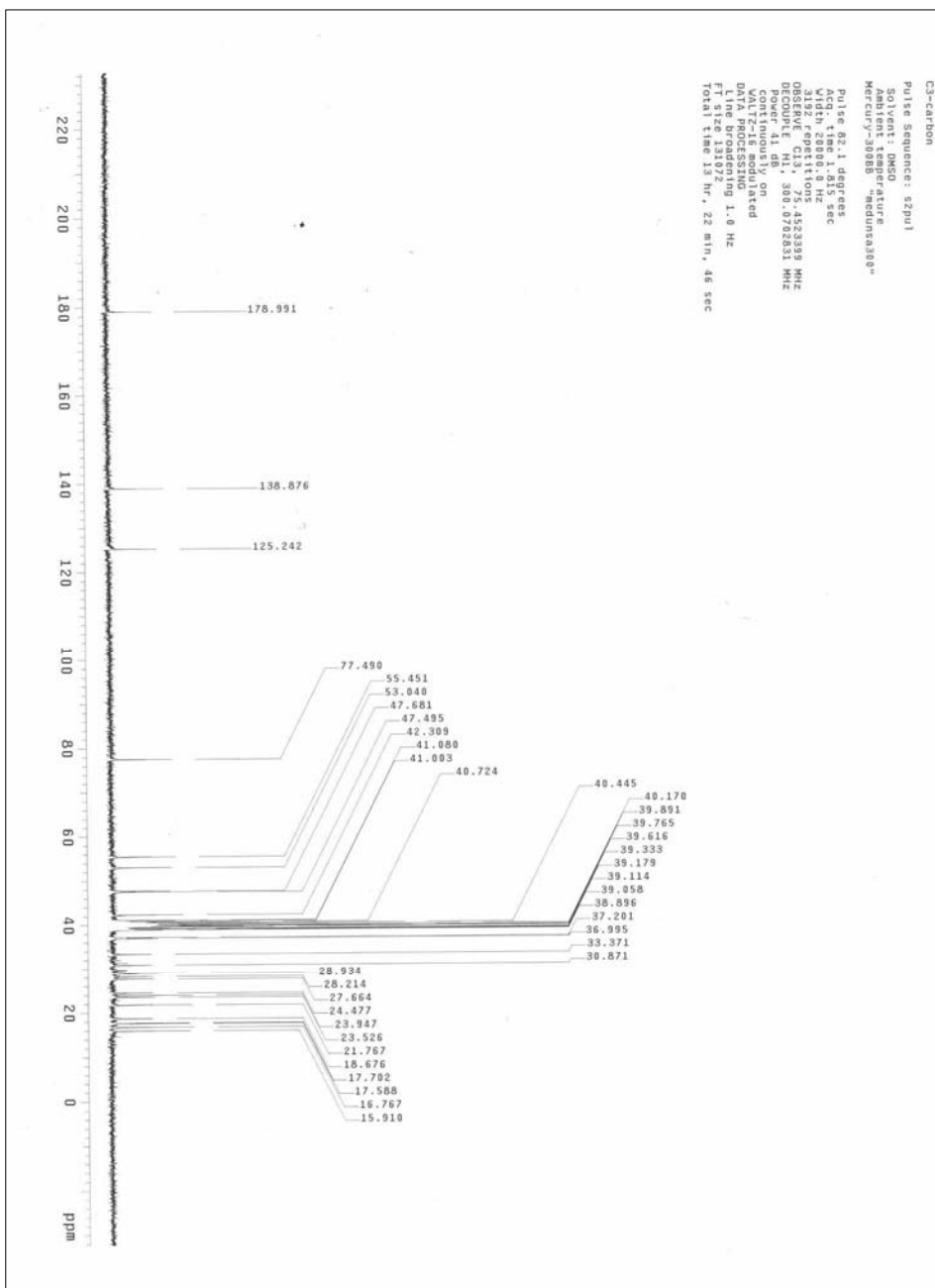


Figure A.7. The ¹³C NMR spectrum of ursolic acid (**CIII**) isolated from the leaves of *C. dentata*.

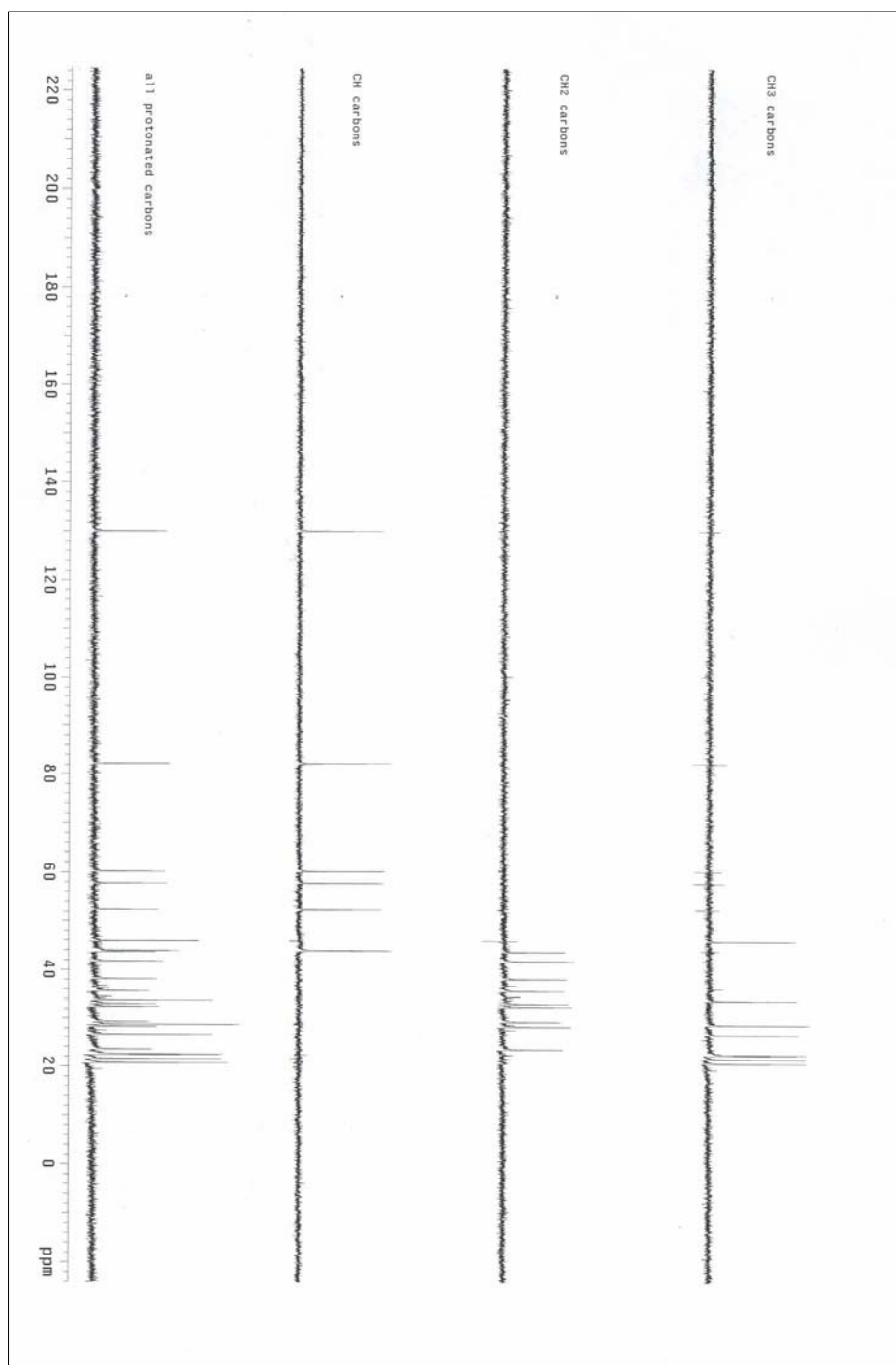


Figure A.8. DEPT experiment data of ursolic acid isolated from *C. dentata*. The DEPT experiment was conducted using CDCl_3 as a solvent instead of DMSO.

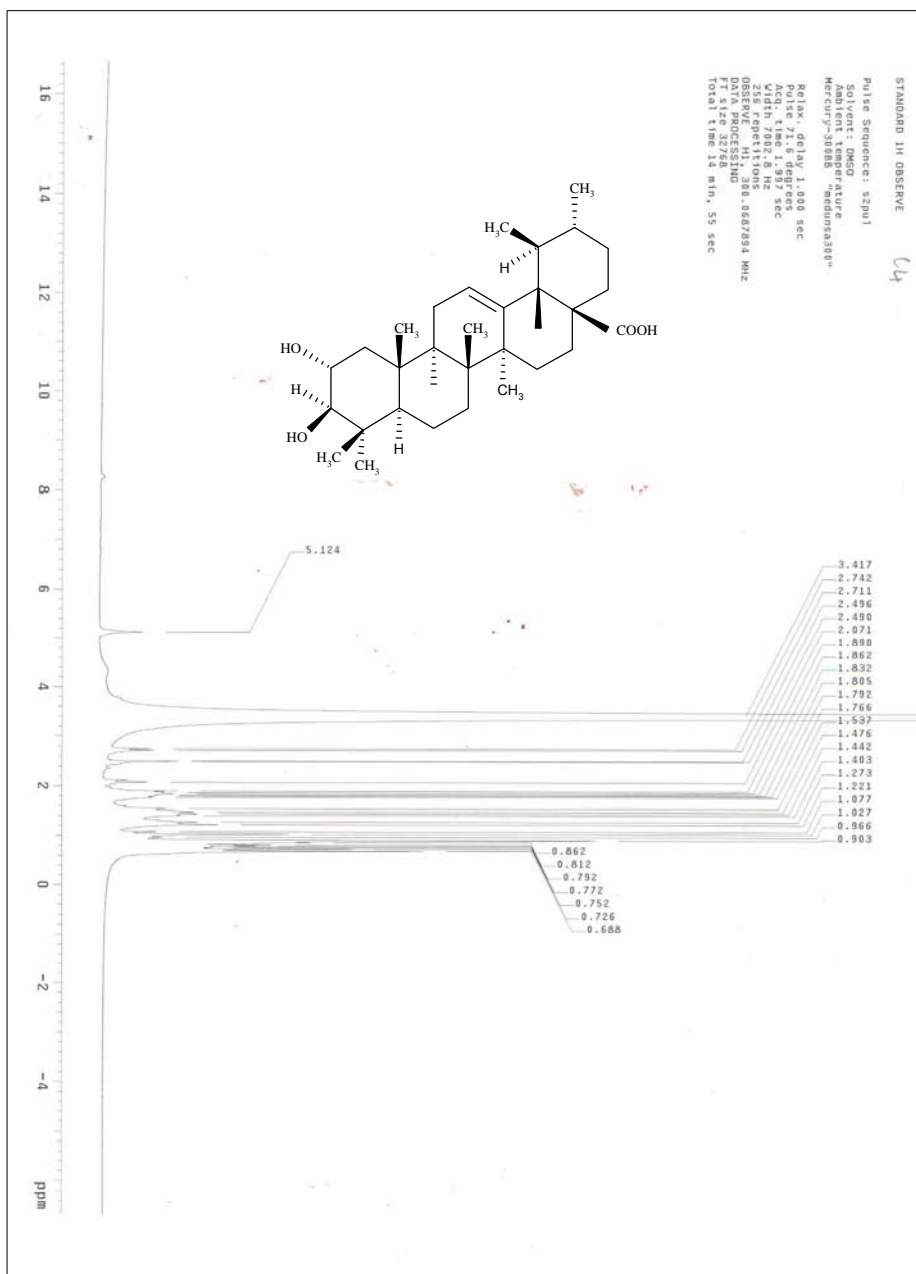


Figure A.9. ¹H NMR spectrum of 2 α -hydroxy-ursolic acid (CIV) isolated from the leaves of *C. dentata*.

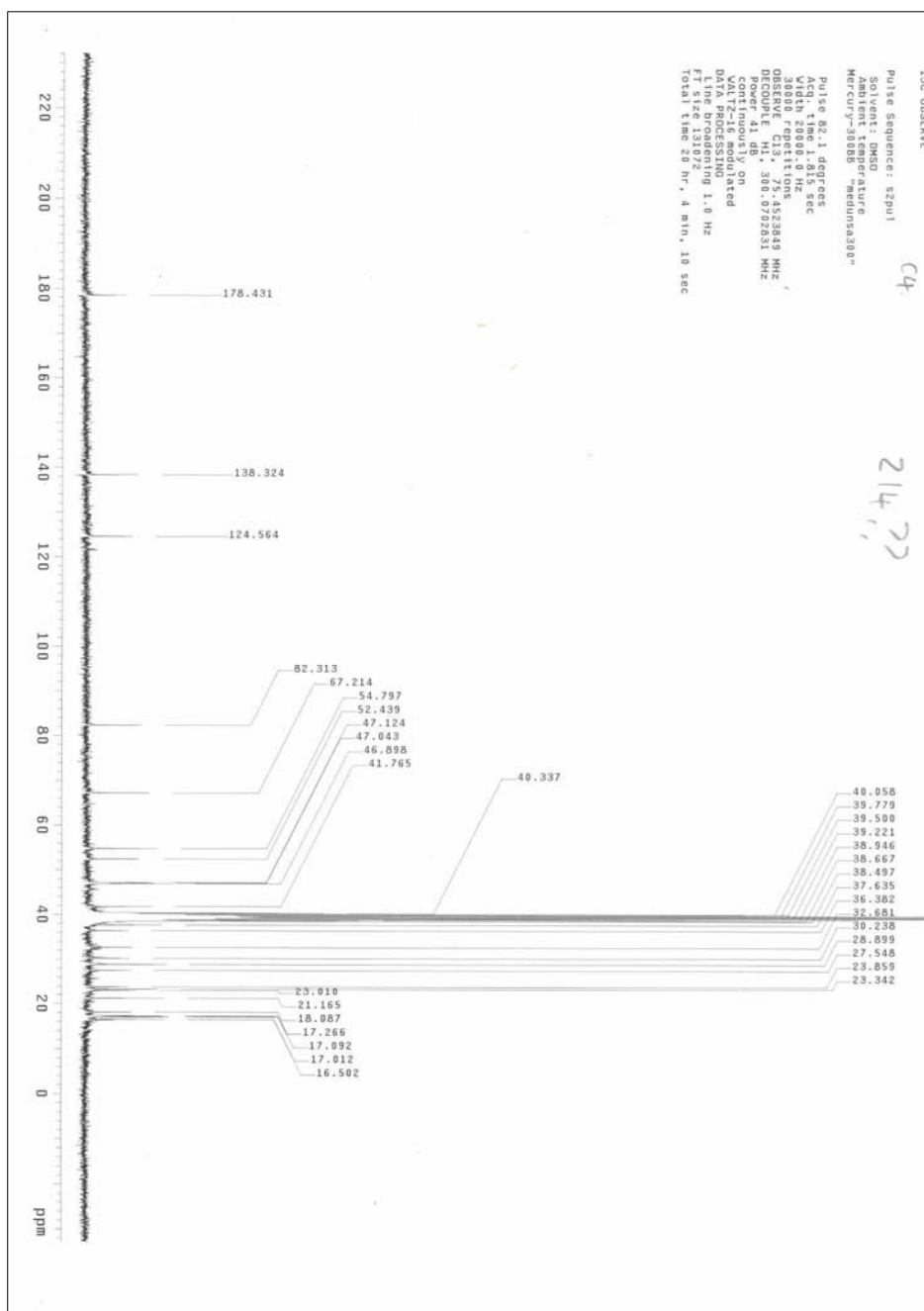


Figure A.11. ¹³C NMR spectrum of hydroxyl-ursolic acid isolated from the leaves of *C. dentata*.

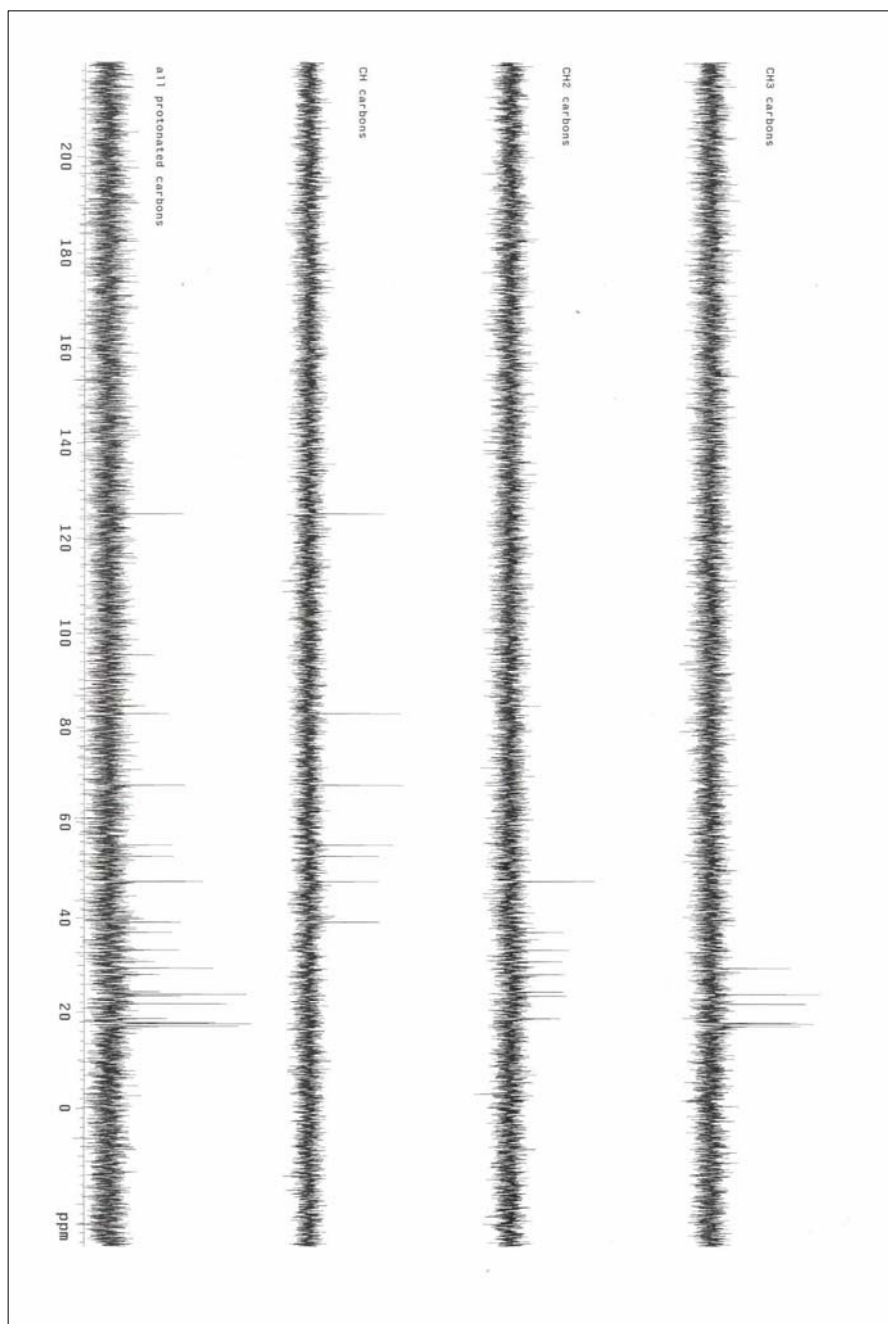


Figure A.12. DEPT experiment data of CIV isolated from the leaves of *C. dentata*.