

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.

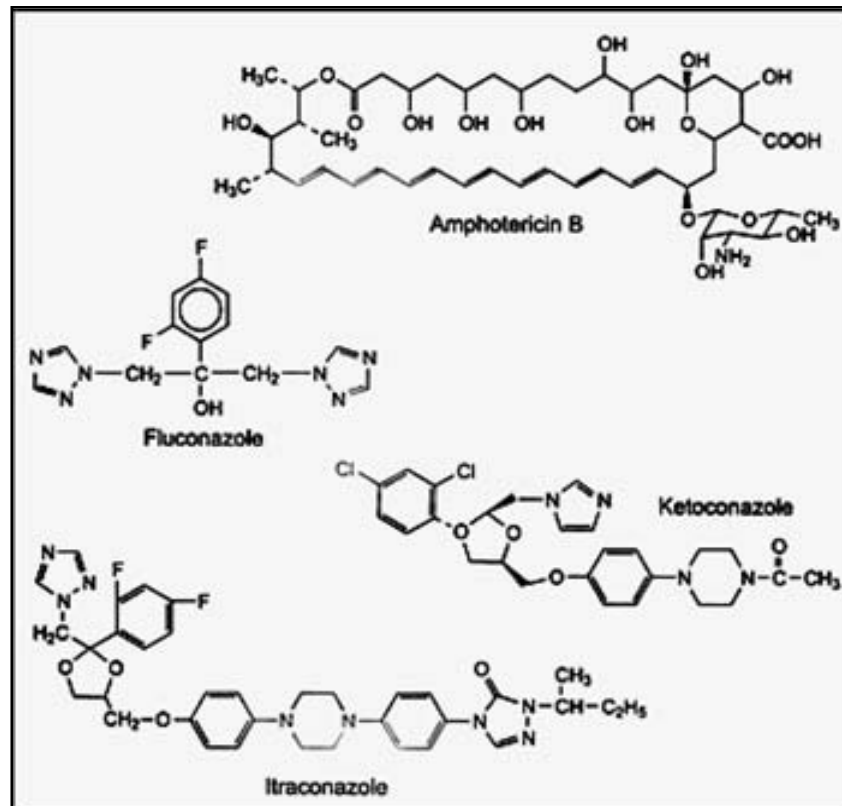


Figure 1.1. Structures of some of the antibiotics used to treat fungal infections in humans and animals (Lupetti *et al.*, 2002).

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

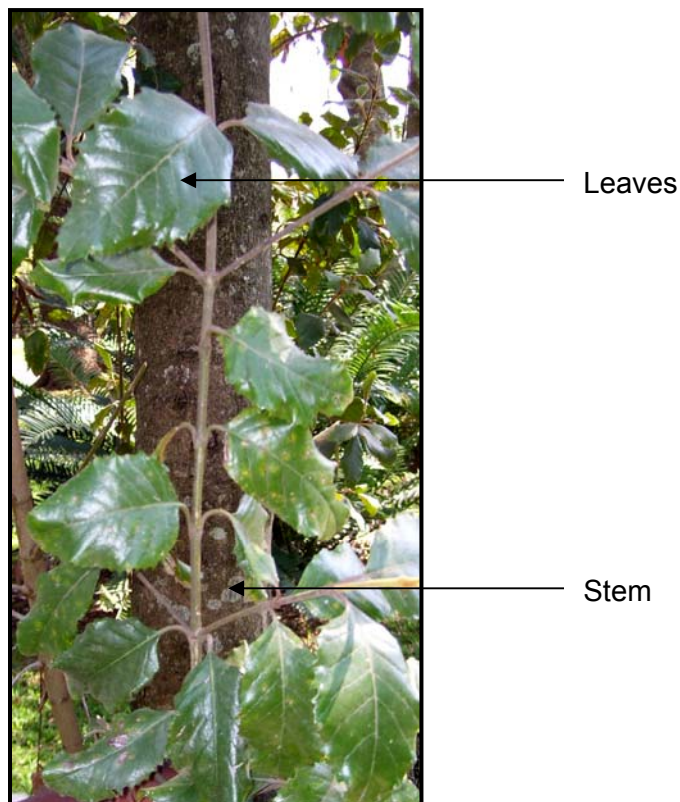


Fig. 1.2. The leaves and stem of *Curtisia dentata* (Photographed at the National Botanical Garden, Mpumalanga, South Africa in June 2007).

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).

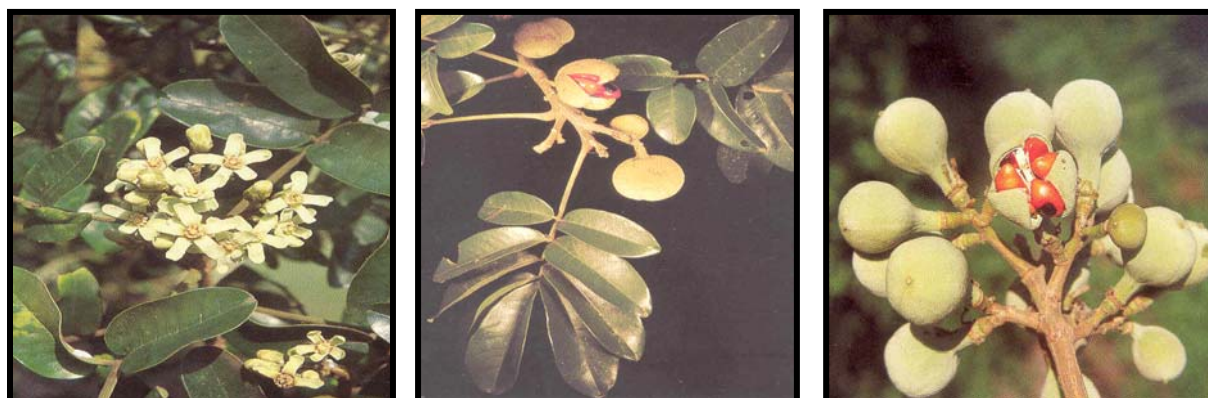


Fig. 1.3. *Trichilia emetica* flowers, leaves and fruits (van Wyk *et al.*, 1997).

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.

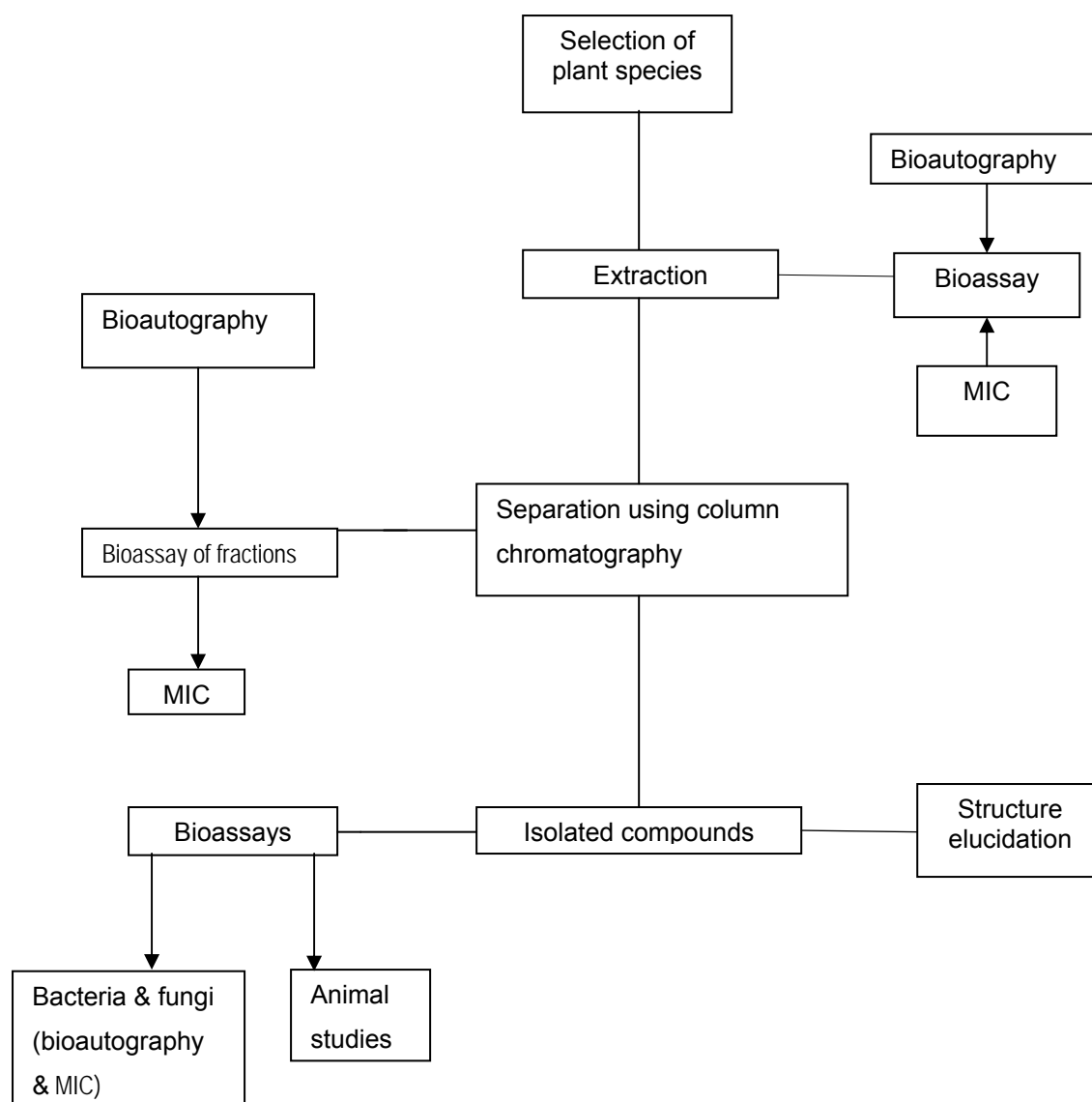


Figure 2.1. Flow diagram showing the layout of the study procedures and methods carried out in this project.

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).

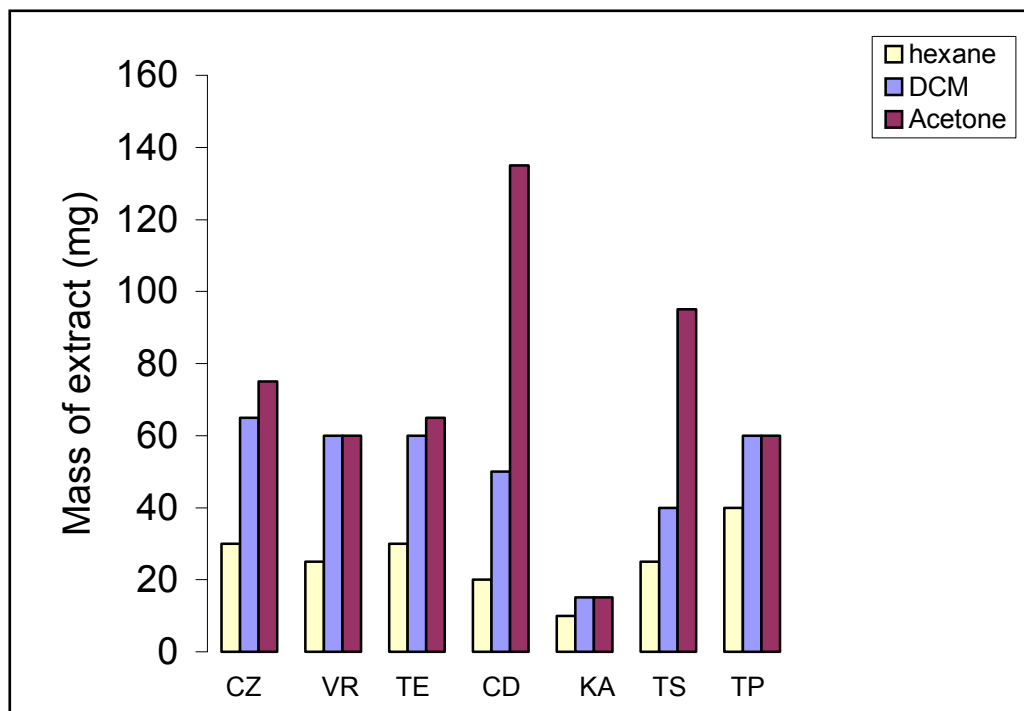


Figure 3.1. Mass extracted (mg/g of dry material) from leaves of different plant species. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP, *Terminalia phanerophlebia*.

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*

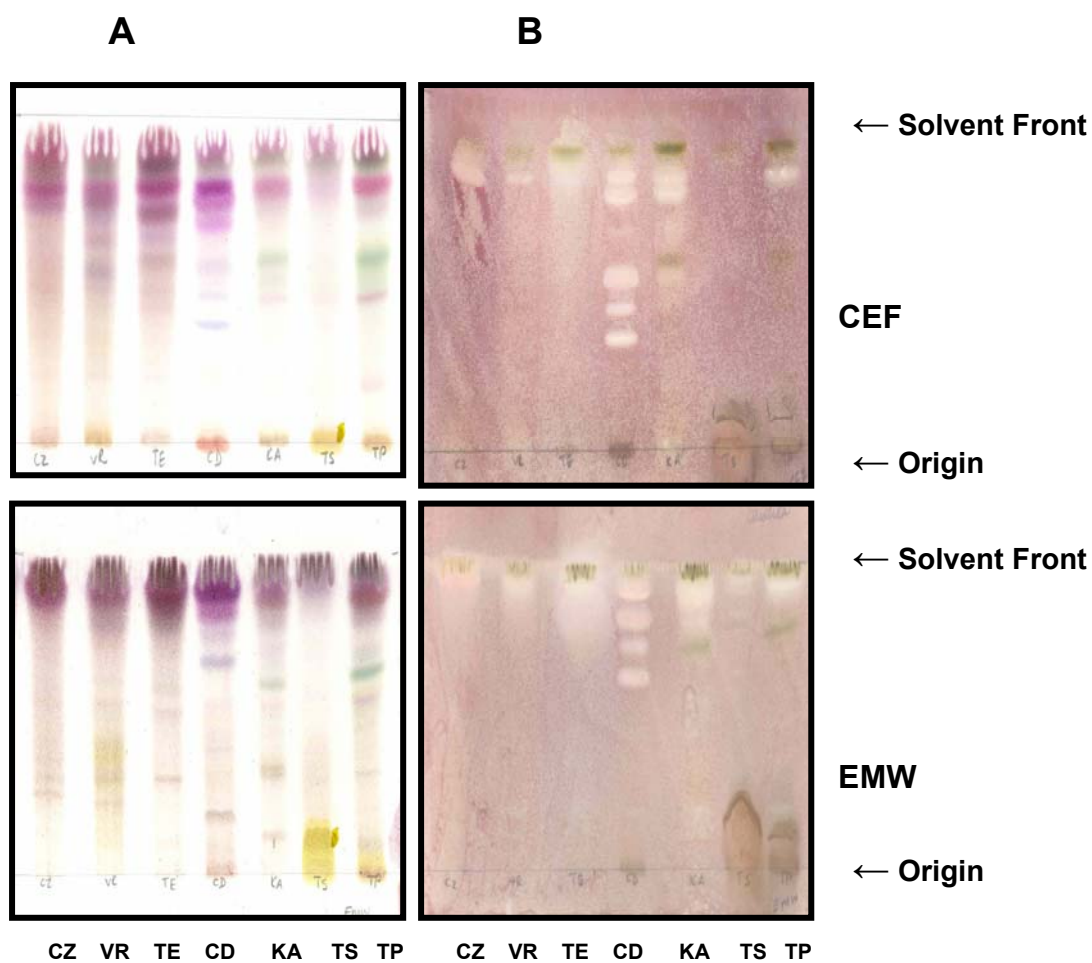


Figure 3.2. TLC plates sprayed with vanillin-sulphuric acid (A) and bioautograms showing growth inhibitory activity (B) of acetone extracts of seven selected plant species against *Candida albicans*. TLC plates were developed in CEF and EMW respectively before spraying with concentrated suspension of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

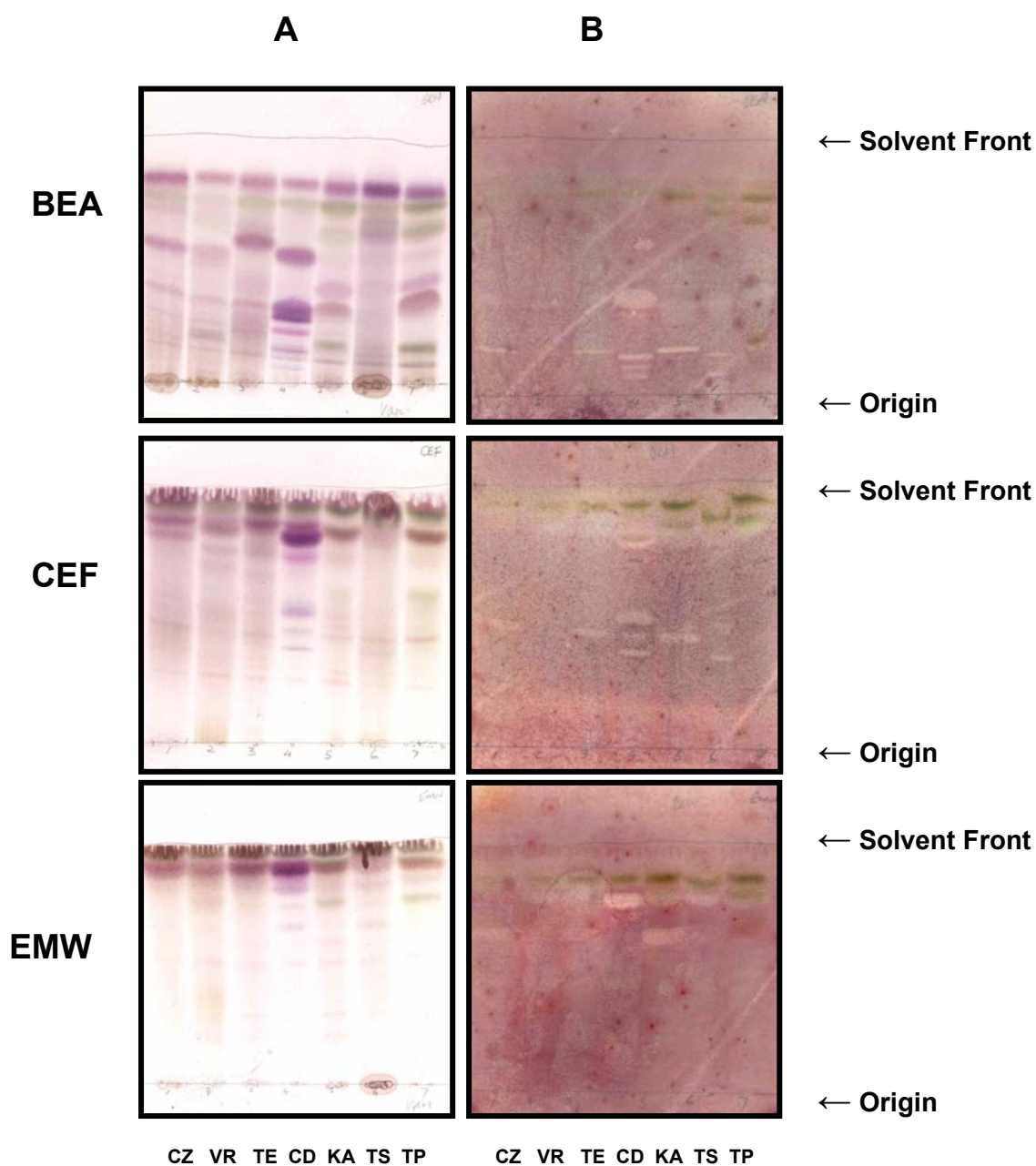


Figure 3.3. TLC fingerprints (A) and anti-*Candida* activity (B) of DCM extracts of seven different plant species. BEA, CEF and EMW, respectively. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia africana*; TS, *Terminalia sambesiaca*; TP,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

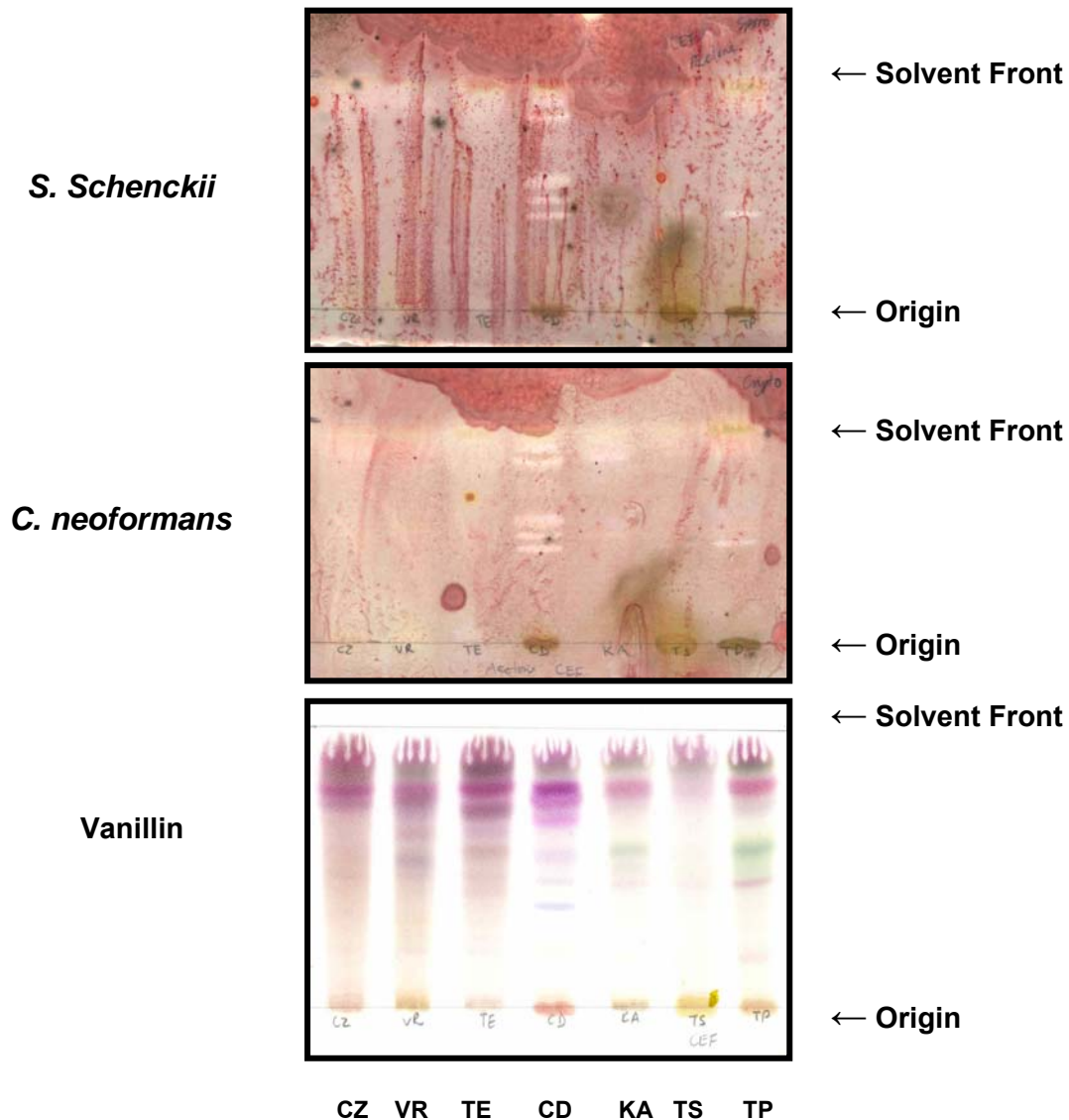


Figure 3.4. TLC bioautograms showing antifungal activity of acetone extracts of leaves of different species against *Cryptococcus neoformans* (CN) and *Sporothrix schenckii* (Ss). The TLC plates were developed in CEF before spraying with cultures of test organisms. A reference plate was sprayed with 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

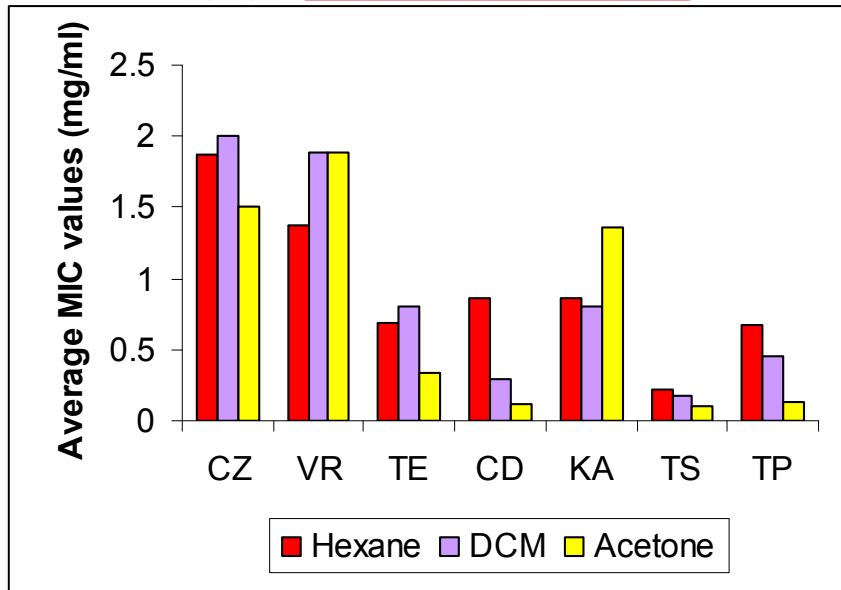


Figure 3.5. Combined average MIC values of each plant extract against all the fungal species. All MIC values from a specific plant extract against all fungal species were combined into an MIC average for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD,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

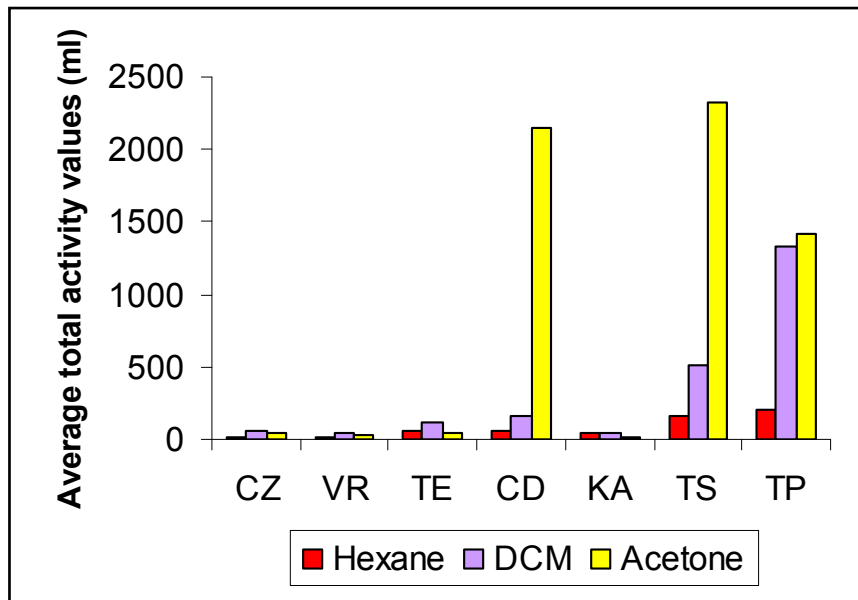


Figure 3.6. Combined average total activity values of each plant extract against all the fungal species. All total activity values from a specific plant extract against all fungal species were combined into an average total activity value for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris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

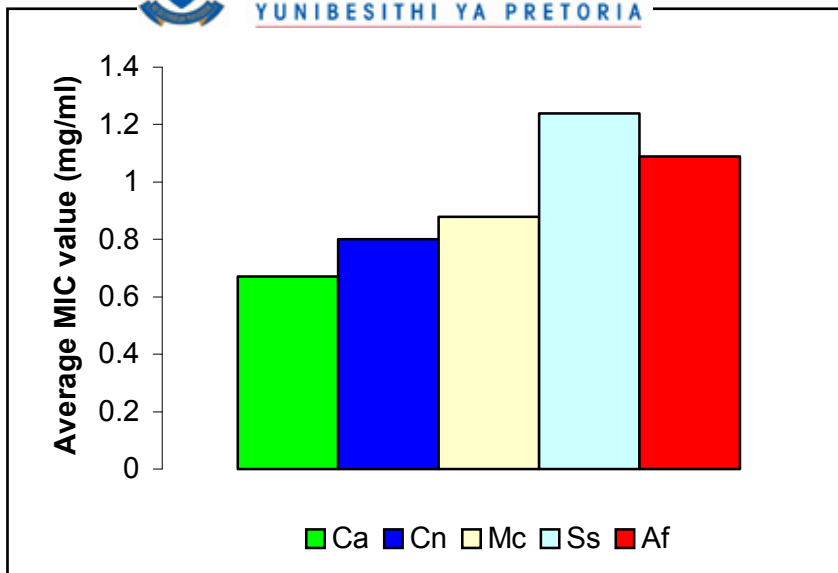


Figure 3.7. Sensitivity of fungal test organisms to all plant extracts (MIC values). Average of MIC values of all the plant extracts against each of the fungal species were calculated. Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Mc, *M. canis*; Ss, *S. schenckii*.

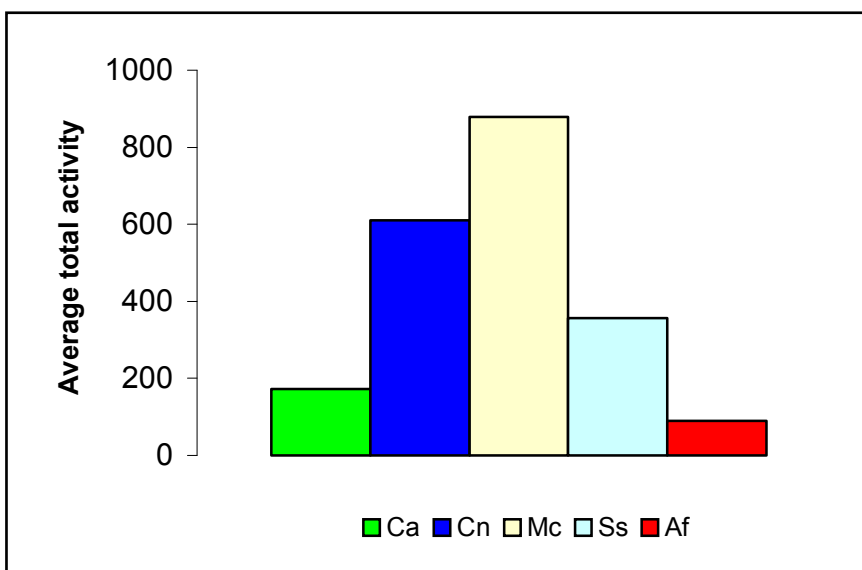


Figure 3.8. Sensitivity of fungal test organisms to all plant extracts (total activity values). Average of total activity values of all the plant extracts against each of the fungal species were calculated. Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Mc, *M. canis*;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

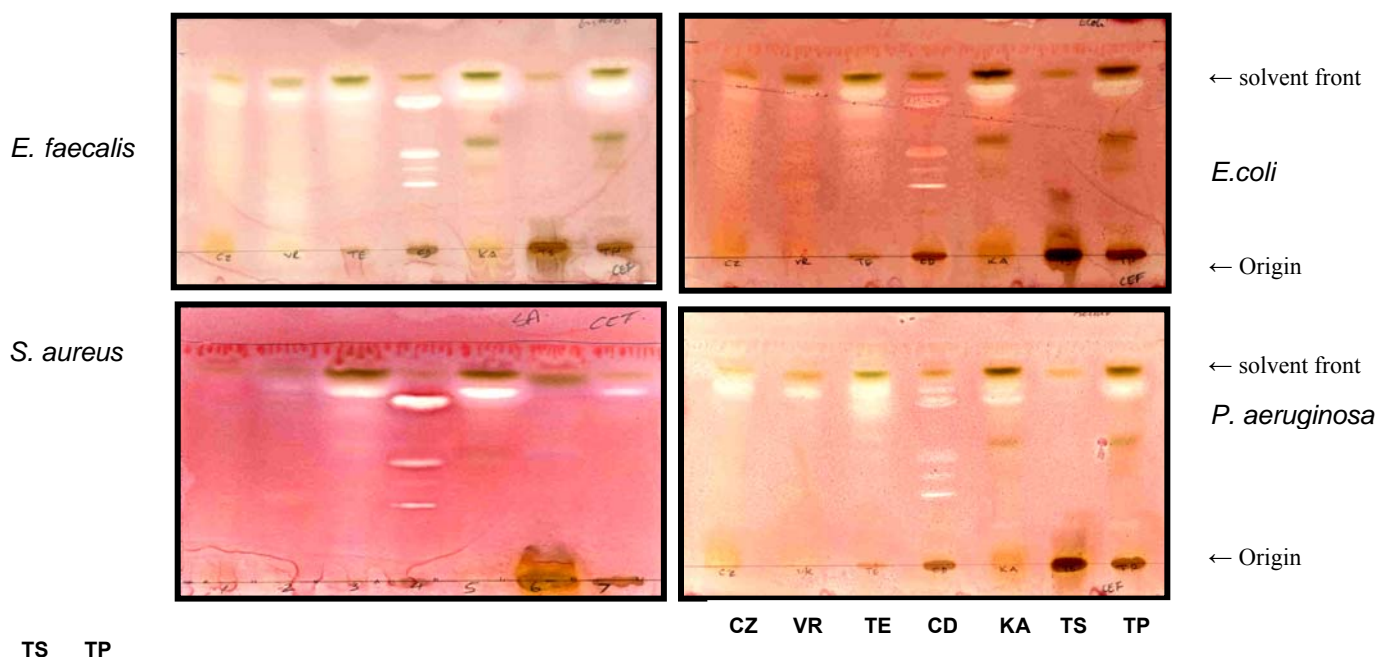


Figure 3.9. TLC bioautograms showing antibacterial activity against four test bacterial suspensions. The leaf powders were extracted with acetone and TLC analysis was performed using CEF as eluent. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; CD, *Curtisia dentata*; KA, *Kigelia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

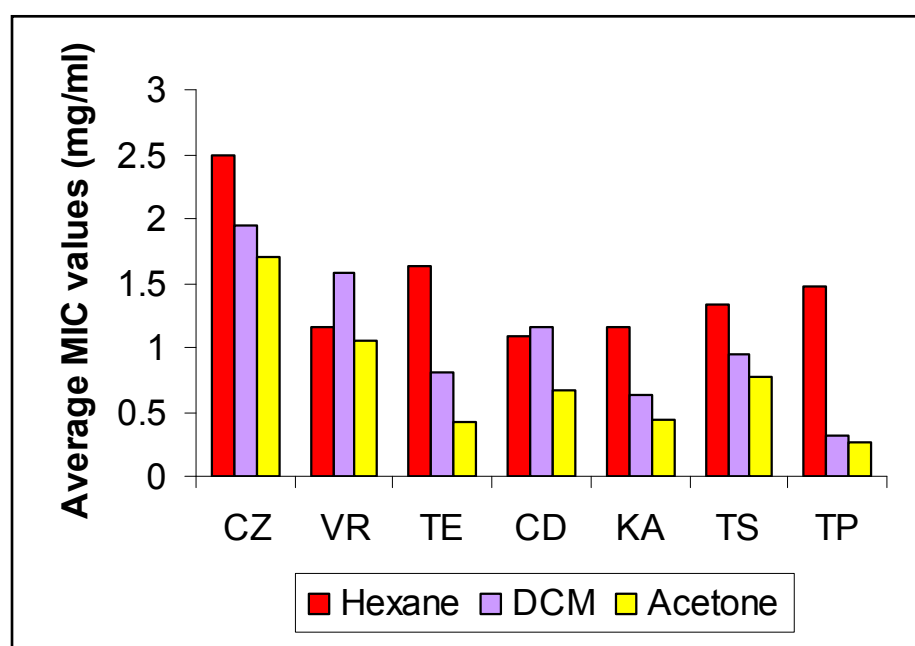


Figure 3.10. Combined average MIC values of each plant extract against all the bacterial species. All MIC values from a specific plant extract against all bacterial species were combined into an MIC average for that plant extract. CZ, *Cussonia zuluensis*; VR, *Vepris reflexa*; TE, *Trichilia emetica*; 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).

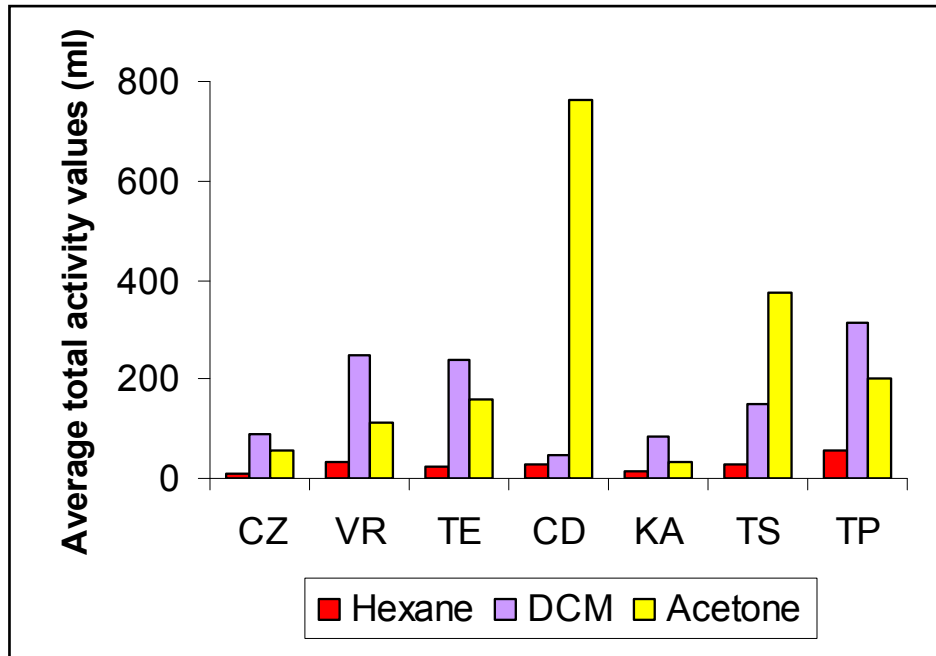


Figure 3.11. Combined average total activity values of each plant extract against all the bacterial species. All total activity values from a specific plant extract against all the tested bacterial species were combined into an average total activity value for that plant extract.

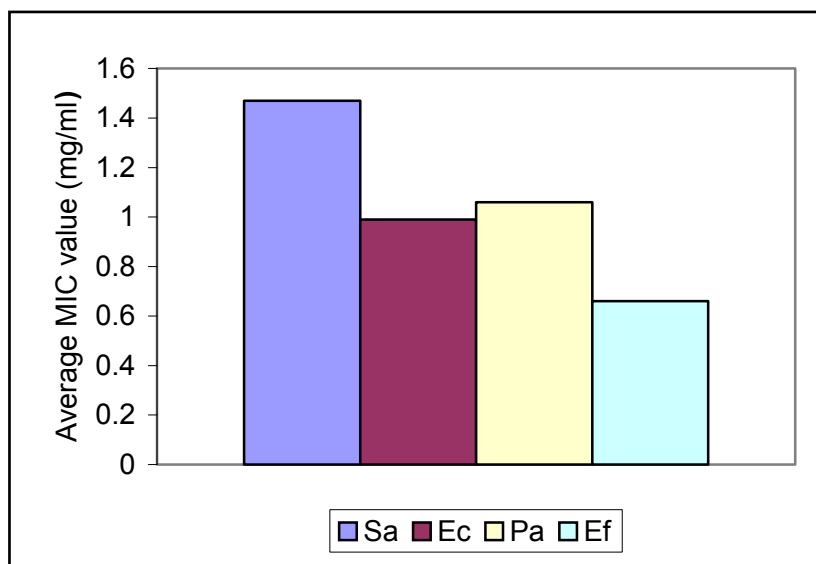


Fig. 3.12. Sensitivity of bacterial test organisms to all plant extracts (MIC values). Averages of MIC values of all the plant extracts against each of the bacterial species were calculated. Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; Ef, *E. faecalis*.

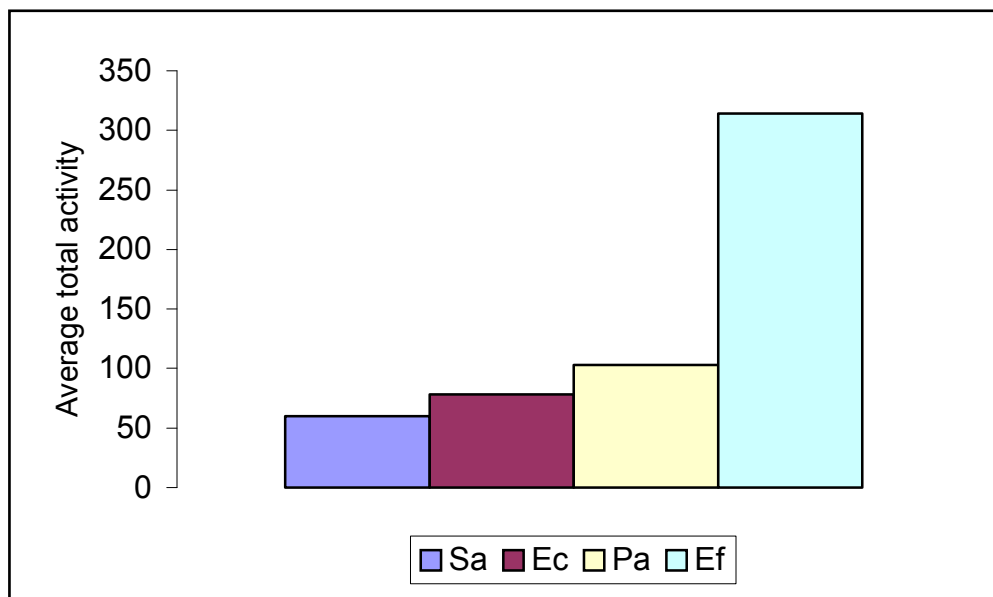


Figure 3.13. Sensitivity of bacterial test organisms to all plant extracts (total activity values). Averages of total activity values of all the plant extracts against each of the bacterial species were calculated. Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; Ef, *E. faecalis*.

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.

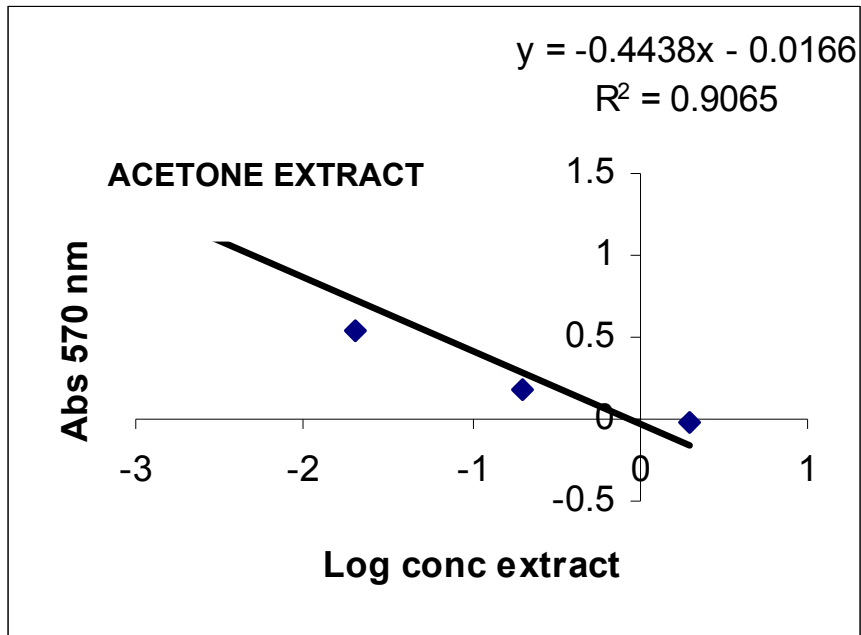


Figure 3.14: Cytotoxicity of acetone extract against Vero cells ($LC_{50} = 24.4 \mu\text{g/ml}$)

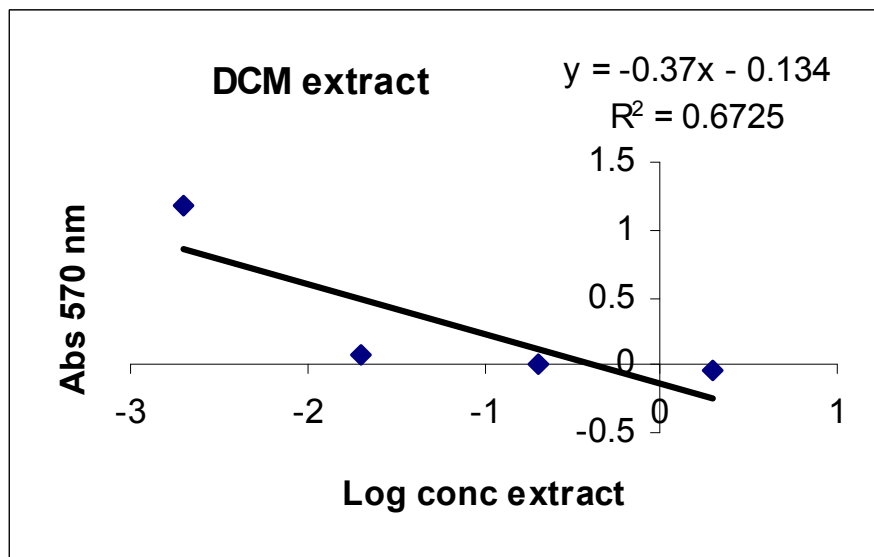


Figure 3.15: Cytotoxicity DCM extract against Vero cells ($LC_{50} = 6.6 \mu\text{g/ml}$).

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.

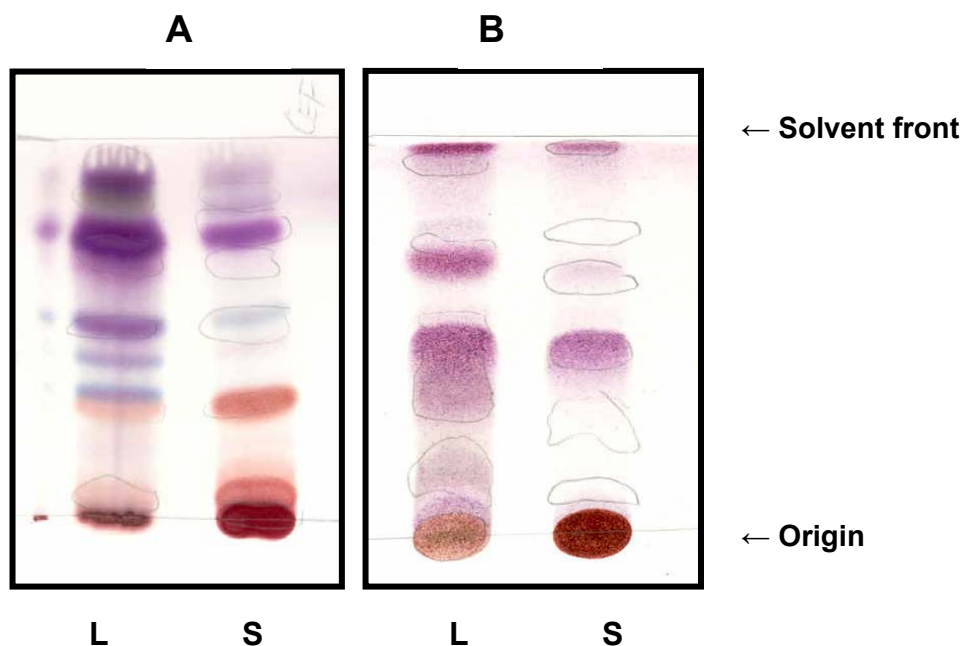


Figure 3.16. Comparison of the chemical components present in leaves (L) and stem bark (S) of *Curtisia dentata*. TLC plates were developed in CEF (A) and DE (4:1) (B) and sprayed with vanillin-sulphuric acid.

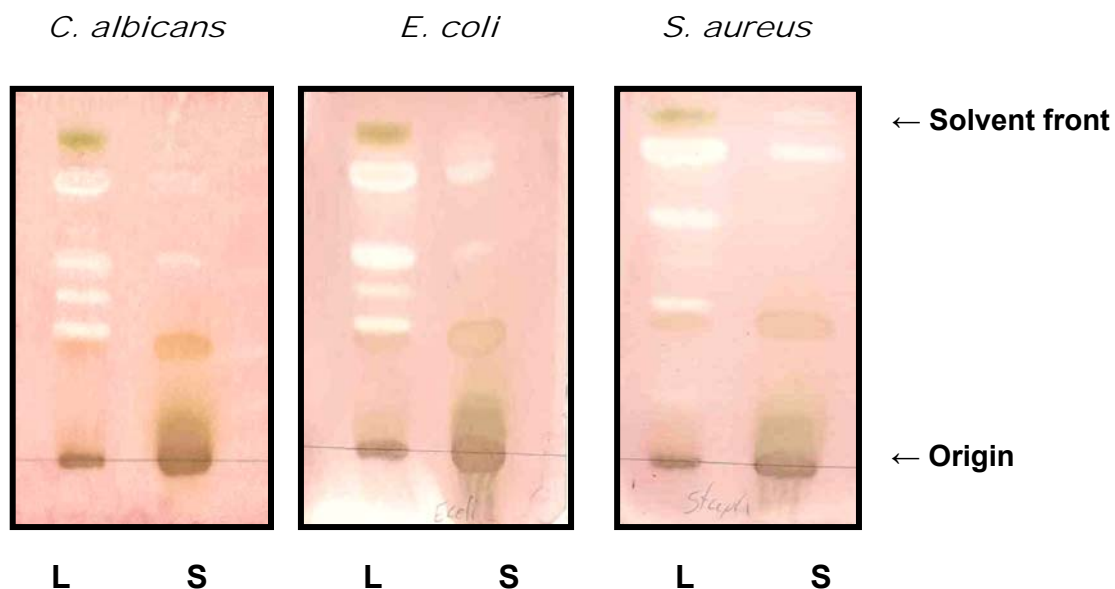


Figure 3.17. Comparison of the antibacterial chemical components present in leaves (L) and stem bark (S) of *Curtisia dentata*. Acetone extracts of leaves and bark were analysed on TLC plates using CEF as eluent, and then sprayed with *C. albicans*, *E. coli* or *S. aureus*.

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.