CHAPTER 1

Literature review

1. INTRODUCTION

In the 1990s, drug resistance had become an important problem in a variety of serious infectious diseases of humans including human immunodeficiency virus (HIV) infection, tuberculosis, and other bacterial infections. At the same time, there have been dramatic increases in the incidence of fungal infections, which are probably the results of alterations in immune status associated with the acquired immunodeficiency syndrome (AIDS) epidemic, cancer chemotherapy and organ and bone marrow transplantation. The rise in the incidence of fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, are ineffective against new or re-emerging fungi, or lead to the rapid development of resistance. Antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem (Graybill, 1988).

Resistance to antimicrobial agents has important implications for morbidity, mortality and health care costs all over the world. Substantial attention has been focused on developing a more detailed understanding of the mechanism of antimicrobial options, new antimicrobial options for the treatment of infections caused by resistance organisms and methods to prevent the emergence and spread of resistance in the first place. The study of resistance to antifungal agents has lagged behind that of antibacterial resistance for several reasons. Prior to the late 1980's with the rise of AIDS, fungal infections were rare (Wey et al., 1988).

These developments and the associated increase in fungal infections intensified the search for new, safer, and more efficacious agents to combat serious fungal infections. One of the options in tackling this problem is by ethnopharmacological approach.

Ethnopharmacology is the cross-cultural study of how people derive medicines from plants, animals, fungi, or other naturally occurring resources. Up to now, the field has focused mostly on developing drugs based on the medicinal use of plants by indigenous people. The "discovery" that indigenous knowledge about medicinal plants may hold clues for curing "western" diseases has become one of the most widely used arguments for conserving cultural and biological diversity (Farnsworth, 1988). Due to the potential for profit, some drug companies have teamed up with botanists, anthropologists, biochemists, conservation

organizations, and governments of less-developed countries to protect biologically diverse areas and search for new drugs.

Medicinal plant research is urgently needed. The AIDS virus, the crisis of bacterial resistance to antibiotics, and other recent developments have increased the value of indigenous medicinal plant knowledge, which may hold clues for solving these deadly problems. Indigenous medicinal plant knowledge is also critical because synthetic chemical processes have proved inadequate for dealing with the rapid evolution of pathogens. Unfortunately, many opponents of medicinal plant research that involves indigenous people have chosen to ignore the fact that "western" medicine relies on plants and traditional knowledge for clues to cure our worst diseases.

In addition, plant species are disappearing, and many indigenous people have stopped transmitting traditional medicinal knowledge to their children. In many places, the current generation represents our last chance to find ways that indigenous people can benefit from their knowledge instead of simply liquidating their biological resources to join a global economy in which they are at a serious disadvantage, including not being able to afford "western" medicines. New and innovative programs of benefits sharing between indigenous people and biomedical scientists are intended to achieve this goal. (Casagrande, 2000).

Medicinal plant research includes much more than the discovery of new drugs. Recently, the field has been expanding to also include such diverse subjects as negotiation of power based on medicinal plant knowledge (Garro, 1986) and the co-evolution of humans and plants (Alcorn, 1981). The field also provides opportunities to study how human interaction with biological diversity is influenced by human psychology, cognition, and evolution.

1.1. Medicinal plants

According to the World Health Organization (WHO), a medicinal plant is defined as any plant which contains substances that can be used for therapeutic purposes or which contain precursors of chemopharmaceutical semisynthesis (World Health Organization, 1979).

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties (Chopra *et al.*, 1992, Harborne and Baxter, 1995, Ahmad and Beg, 2000). The substances that can either inhibit the growth of pathogens or kill them and have no or low toxicity to host cells are considered candidates for developing new antimicrobial drugs. In recent years, antimicrobial properties of medicinal plants are increasingly reported from different parts of the world (Nimri *et al.*, 1999, Saxena and Sharma, 1999). Higher plants are still regarded as potential sources of new medicinal compounds. Throughout the world,

plants are used traditionally to treat many ailments, particularly infectious diseases, such as diarrhoea, fever and colds, as well as for the purposes of birth control and dental hygiene (Mitscher *et al.*, 1987). In addition, many psychoactive substances used in traditional medicine are of plant origin (Deans and Svodoba, 1990).

More than 80% of the population in developing countries depend on plants for their medical needs (Farnsworth, 1988, Balick *et al.*, 1994). Medicinal and poisonous plants have always played an important role in African society. Traditions of collecting, processing and applying plants and plant-based medications have been handed down from generation to generation (von Maydell, 1996). In South Africa, and also in many other African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers in their homes (Fyhrquist, 2002). Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994).

The value of ethnomedicine and traditional pharmacology is gaining increasing recognition in modern medicine because the search for new, potential medicinal plants is more successful if the plants are chosen on an ethnomedical rather than a random basis. It has been estimated that 74% of pharmacologically active plants-derived components were discovered after the ethnomedical uses of the plants were investigated (Farnsworth and Soejarto, 1991).

1.1.1. Approaches for selecting medicinal plants

Four different approaches of selecting plants for pharmacological screening, are known, and are as follows: (1) 'random approach' which involves the collection of all plants found in that area; (2) 'phytochemical targeting' which entails the collection of all members of a plant family known to be rich in bioactive compounds; the (3) 'ethno-directed' sampling approach, based on traditional medicinal use(s) of the plant; (4) 'chemotaxonomic approach' and a method based on 'specific plant parts' such as seeds (Cotton 1996, Khafagi and Dewedar, 2000).

1.1.2. Importance of medicinal plants

Plants were once a primary source of all the medicine in the world and they still continue to provide mankind with new remedies. Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world (van Wyk *et al.*, 1997). Well-known examples of plants derived medicine include quinine, morphine, codeine, aspirin, atropine,

reserpine and cocaine. Recently, important new drugs such as taxol and vincristine have been developed.   Taxol is a highly effective drug against breast cancer and was recently also approved for the treatment of ovarian cancer. It is a diterpenoid originally extracted from the bark of the pacific yew (*Taxus brevifolius*). Quinine is an alkaloid from the bark of the quinine tree (*Cinchona pubescens*), and is an effective remedy for malaria. Atropine and various tropane alkaloids are extracted from deadly nightshade and other plants for example *Datura stramonium*. Extracted alkaloids are used in eyedrops and in skin patches to treat motion sickness, and are injected to treat Parkinsonism (van Wyk *et al.*, 1997). South Africa's contribution to world medicine includes Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*) (van Wyk *et al.*, 1997) and many more.

1.1.3. Traditional herbal medicine

In Africa, the use of plants to treat various ailments in humans and animals has been extensively documented by scientists. Herbalists use stems, leaves, roots and shoots of plants to prepare extracts, decoctions, concoctions, mixtures, potions, creams, infusions and pastes, which are then used to cure all sorts of afflictions. The variety of plants used in a community reflects the duration of a people's presence in a certain location, their medicinal knowledge, the diversity of plants present and the availability of plants with a possible medicinal use. Unfortunately, discovering the potential of a herb is not easy and can often only be done by careful and time-consuming experimentation. By this process, many people have discovered herbs to be effective against diseases. People in different places have independently discovered some of these remedies.

Many herbal remedies cure disease not understood by 'Western' medicine, i.e. diseases of the spirit, curses and spells. Although many cures are often available against the most common and easily diagnosed illnesses within a community, not all are effective. Some however do contain effective ingredients, which may be applied in Western medicine.

Primarily, healers use herbal medicine to cure diseases of the body and the spirit of their patients. This group of herbal remedy users can be split into subgroups, namely the traditional healer, who is usually a male whose family tradition it is to be the healer or doctor. He can cure diseases both of the body and spirit, using different remedies for children and women. Another subgroup, which usually consists of the wives of the healers, is concerned with the problems of women within the community. Wives of healers can advise about pregnancy and childbirth as well as herbal remedies to fight menstrual pains and the spiritual well-being of the unborn child or the young baby. A last subgroup is the normal person within

the community who has a basic knowledge of the herbs in its vicinity to cure such minor illnesses as colds, fevers, muscle aches, headaches, sore throats and joint pains. He may also be knowledgeable about plants that can be used to cure diseases of cattle or pets.

1.1.4. Ethnobotanical research

Ethnobotanical research is done primarily for three reasons, including an ethnological, developing and pharmaceutical motivation (Portillo et al., 2001).

1.1.4.1. Ethnological

In ethnological research, the investigator records how the plants are used, their use and beliefs. The anthropologist does not test the effectiveness of the plants, nor does he/she devise ways in which the plants can be put in better use (Kårehed, 1997).

1.1.4.2. Developing

The reason for ethnobotanical research is to document the knowledge of the healers in the community to save it for future generations. Many traditional healers are old and have no successors. People tend to think that Western medicine is better, and young people move to the cities where they have easy access to this medicine. Traditional knowledge should be written in a local language. It is most of the times impossible to document all the knowledge of the traditional healer. This makes it necessary to make through observations of the community in order to be able to make a good selection of plants that may be of use for future generations. A common way of selecting plants for documentation is to interview several traditional healers and to search for consensus (Schlage, 2000). This is done from the perspective that it would be more likely that a certain cure actually works, if it is used by more than one traditional healer (Mahunnah, 1996).

Within this motivation for research, one can also include the study of the role of traditional medicine in relation to modern medicine. Many people in developing countries have limited access to health clinics or hospitals, but ready availability of traditional healers. These healers play an important role in these societies as an institution to consult before attending a hospital or clinic, thereby reducing the number of patients going to the hospitals, as well as allowing medical facilities to be shared among a greater number of people.

1.1.4.3. Pharmaceutical

Scientists could chemically screen all possible plants to find new pharmaceuticals to be used in Western medicine. However, the knowledge of the chemical functioning of the human body is by far not extensive enough yet and a lot of possibilities are missed that way.

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Therefore ethnobotanical research is a good way to start. In this kind of research the consensus of healers is also used very often (Schlage, 2000, Leaman, 1995). This might be a good way to find a number of plants that probably contain interesting chemicals, but there is a risk of missing the less commonly-known cures used by the traditional healers. In this research, plant taxonomy also plays an important role. If a plant contains bioactive chemicals it is definitely worthwhile looking at its close relatives. Using this kind of research a lot of important pharmaceuticals are found. Some examples are quinine, aspirin, and several HIV-blockers (Portillo *et al.*, 2001).

The Combretaceae plant family has been used for medicinal purposes all over South Africa. In the present study, attention will be focused on this plant family.

1.2. Combretaceae

The plants in this family are used for many medicinal purposes by traditional healers. They include treating abdominal disorders, backache, bilharzia, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bites, swelling caused by mumps, syphilis, toothache, gastric ulcers, venereal diseases, heart diseases, cleansing the urinary system, dysentery, gallstones, sore throats, nosebleeds and general weakness (Hutchings *et al.*, 1996, van Wyk *et al.*, 1997, McGaw *et al.*, 2001).

The Combretaceae family belongs to the order Myrtales consisting of 18 genera, the largest of which are *Combretum*, with about 370 species, and *Terminalia*, with about 200 species (Lawrence, 1951). The other genera are smaller; e.g. *Calopyxes* and *Buchenavia* comprise 22 species each, *Quesqualis* 16, *Angioeissis* 14, *Conocarpus* 12 and *Pteleopsis* 10 species (Rogers and Verotta, 1996). The genus *Combretum* has two subgenera, which are subgenus *Combretum* and subgenus *Cacoucia* with several sections in each subgenus (Carr, 1988). (**Table 1.1**).

Species from the genus *Combretum,* and to a lesser extent *Terminalia,* are most widely used for medicinal purposes. These genera are widespread all over Africa including southern Africa and Asia, where some are often the dominant species (Carr, 1988). They are easily characterized by the wing-shaped appendages of the fruits, and are either trees, shrubs or climbers (Rogers and Verotta, 1996). The leaves and the bark of *Combretum* species are predominantly used. Fruits do not feature in medicine owing to their reported toxicity to humans.

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Members of the family are often tanniferous and produce ellagic and gallic acids and frequently also proanthocyanins (Cronquist, 1981). They are sometimes cyanogenic and often accumulate triterpenoids, especially as saponins, but are without iridoid compounds. Mucilaginous secretory cells or canals are often present in the parenchymatous tissues and sometimes even in the wood. Solarity or clustered crystals of calcium oxalate frequently occur in some cells of the parenchymatous tissues, those in leaves often taking the form of stellate idioblasts.

Their leaves are simple, petiolate or sessile, opposite, alternate, verticillate, whorled, without stipules or very small, with margins entire (in one instance sometimes crenulate), with indumentum comprising hairs, stalked glands, and scales. The inflorescences are axillary, terminal, spicate (sometimes panicullate or subcapitulate). The flowers are sessile, or pedicellate, bisexual or sometimes unisexual, usually actinomorphic, and male on the same inflorescence.

Table 1.1. The Combretaceae family (Carr, 1988)

THE COMBRETACEAE FAMILY		
Combretum L	Section *Spathulipetala* Engl. & Diels	Section *Oxystachya* Excell
SUBGENUS *Combretum*	*C. zeyheri* Sond.	*C. oxystachyum* Welw. Ex Laws.
Section *Hypocrateropsis* Engl. & Diels	**Section *Ciliatipetala* Engl. & Diels**	**Section *Poivrea* (Comm. Ex DC)**
C. celastroides Welw. Ex Laws.	*C. albopunctatum* Suesseng.	*C. bracteosum* (Hochst.)
C. imberbe Wawra	*C. apiculatum* Sond.	*C. mossambicense* (Klotzsch)
C. padoides Eng. & Diels.	*C. edwardsii* Exell. (provisional)	**Section M*egalantherum* Excell**
Section *Combretastrum* Eichl	*C. moggii* Excell. (provisional)	*C. wattii* Excell.
C. umbricola Engl	*C. molle* R. Br.	
Section *Angustimarginata* Engl. & Diels	*C. petrophilum* Retief	***Terminalia* L.**
C. caffrum (Eckl. & Zeyh.) Kuntze	*C. psidioides* Welw.	
C. erythrophyllum (Burch.) Sond.	**Section *Fusca* Engl. & Diels**	**Section *Abbreviatae* Excell**
C. kraussii Hochst. (incorporating *C. nelsonii* Duemmer)	*C. coriifolium* Engl. & Diels.	*T. prunioides* Excell.
C. vendae Van Wyk	**Section *Breviramea* Engl. & Diels**	*T. randii* Bak.f.
C. woodii Duemmer	*C. hereroense* Schinz.	*T. stuhlmannii* Engl.
Section *Macrostigmatea*	**Section *Elaegnoida***	**Section *Psidioides***

Engl. & Diels	engl. &Diels	Excell
C. engleri Schinz.	*C. elaeagnoides* Klotzsch.	*T. brachystemma* Welw. Ex Hierr
C. kirkii Laws		*T. sericea* Burch. Ex DC
Combretum sp. nov.(provisional)	**SUBGENUS *Cacoucia* (AUBL.)**	*T. trichopoda* Diels.
Section *Metallicum* Excell & Stace	**Section *Lasiopetala* Engl. & Diels**	**Section *Platycarpae* Engl. & Diels**
C. collinum Fresen	*C. obovatum* F.Hoffm.	*T. gazensis* Bak.f.
Section *Glabripetala* Engl. & Diels	**Section *Conniventia* Engl. Diels**	*T. phanerophlebia* Engl. & Diels
C.	*C. microphyllum* Klotzsch	*T. mollis* Laws
	C. paniculatum Vent.	*T. sambesiaca* Engl. & Diels
	C. platypetalum Welw. Ex Laws	*T. stenostachya* Engl. & Diels

The perianth arises from near the summit of a tubular epigynous zone; calyx of usually four or five distinct to slightly connate sepals; corolla commonly of four or five distinct petals, occasionally absent. The androecium of 4-10 stamens is adnate to the epigynous zone, commonly in two cycles, often strongly exserted. The gynoecium is a single compound pistil of 2-5 carpels; style and stigma 1; ovary inferior, with 1 locule containing 2(-6) apical ovules pendulous on long funiculi. The nectary is usually a disk (often hairy) above the ovary. The fruit is 1-seeded, often a flattened, ribbed, or winged drupe. The receptacles are usually in two parts, the lower containing the ovary, the upper terminating in four or five sepals. The style is centrally situated on a disc (Carr, 1988).

1.2.1. Ethnopharmacology of Combretaceae

There is a large variation in the chemical composition and antibacterial activity among different genera and species in the Combretaceae. Seven species of Combretaceae used in traditional medicine in West Africa have been investigated for their antifungal activity against the pathogenic fungi. Phytochemical screening revealed that these plants are particularly rich in tannins and saponins, which might be responsible for their antifungal activity (Baba-Moussa *et al.*, 1999).

1.2.2. Antimicrobial activity of the Combretaceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytototoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial activity have been isolated from species of

Combretum (Rogers and Verotta, 1996; Eloff *et al*., 2005a). There is a large variation in the chemical composition and antimicrobial activity among different genera and species in the Combretaceae.

Leaf extracts of *Combretum padoides, Combretum celestroides, Combretum hereroense, Combretum obovatum*, *C. zeyheri, C. erythrophyllum, Combretum paniculatum, Combretum edwarsii, C. apiculatum* and *C. imberbe* have been shown to have some activity against *S. aureus, Bacillus subtilis, E. coli, Serratia marcescens*, *Mycobacterium phlei* and *Saccharomyces cerevisiae* (Alexander, 1992).

Eloff (1999) investigated the antibacterial activity 27 southern African members of Combretaceae including *C. woodii*, using minimum inhibitory concentrations (MICs) and total quantities extracted. All the plants tested exhibited antibacterial activity against *S. aureus, E. coli, E. faecalis* and *P. aeruginosa,* while Rogers and Verotta (1996) reported the leaves of *C. molle* and *C. imberbe* to possess anti-inflammatory and molluscicidal activity against *Biomphalaria glabrata*.

1.2.3. Phytochemistry of the Combretaceae

Members of the family are often tanniferous and produce ellagic and gallic acids and frequently proanthocyanins. They are sometimes cyanogenic and often accumulate triterpenoids, especially as saponins (Hutchings *et al.*, 1996).

Chemical studies of the *Combretum* genus have yielded acidic triterpenoids and their glycosides, phenanthrenes, amino acids and stilbenes (Pellizzoni *et al.*, 1993). A series of closely related bibenzyls, stilbenes and phenanthrenes have been isolated from *C. caffrum* (Petit *et al.*, 1995). Some of these stilbenes have been found to be anti-mitotic agents that inhibit both tubulin polymerisation and binding of colchicine to tubulin. Flavonoids have been isolated from *C. micranthum* leaves (Rogers and Verotta, 1996).

The fruits of *Terminalia cheluba* have yielded complex esters of gallic acid e.g. corilagin (Haslam, 1996). The aerial parts and fruits of *C. zeyheri* have been found to contain ursolic acid, and a compound named as CZ 34 and L-3 (3-aminomethylphenyl) alanine (Breytenbach and Malan, 1998). With the exception of the simple indole alkaloids that Harman and Eleagnine isolated from the roots of *Galago senegalensis*, there have been no other reports on the presence of alkaloids contained by Combretaceae (Rogers and Verotta, 1996).

Anti-inflammatory and molluscicidal compounds such as mollic acid –D – glycoside and imberbic acid have been isolated from *C. molle* and *C. imberbe* respectively (Pegel and Rogers, 1985). The saponin, jessic acid linked to α-L-arabinose has been isolated from *Combretum eleagnoides* leaves (Osborne and Pegel, 1984).

1.3. Some of the work done on Combretaceae family in Phytomedicine Programme

Our laboratory has developed methods on screening and activities of Combretaceae. Some of the work was as follows:

(i)   *Selection of plants to investigate*
An analysis was made of approaches to be followed towards selecting plants for research and gene banking. Plants used as phytomedicines in Africa and were also analysed and the Combretaceae made up a major group. (Eloff, 1998a)

(ii)   *Selection of best extraction procedure*
Several extractants were tested and evaluated on many different parameters. Acetone was found to be the best extractant. (Eloff, 1998b)

(iii)   *Selection of best purification procedures*
The solvent solvent fractionation procedure used by the USA National Cancer Institute was tested and refined and several TLC separation procedures were also developed. (Eloff, 1998c)

(iv)   *Developing a novel way of determining antibacterial activity*
It could be shown that the traditional agar diffusion assays for determining activity of plant extracts did not work. A new serial dilution microplate assay using INT was developed. (Eloff, 1998 d)

(v)   *Antibacterial activity of Combretum erythrophyllum*
Using the techniques developed above we could show that *Combretum erythrophyllum* contains at least 14 antibacterial compounds. [Martini and Eloff 1998]. Extracts had MIC values as low as 50 μg/ml.

(vi)   *Antibacterial activity and stability of 27 members of Combretaceae*

Acetone leaf extracts of 27 species of *Combretum, Terminalia, Pteleopsis* and *Quisqualis* all had antibacterial activity ranging from 0.1 –6 mg/ml. Storing extracts for 6 weeks at room temperature did not affect MIC values (Eloff, 1999).

(vii)   *Stability of antibacterial activity in C. erythrophyllum*

Leaves of *C. erythrophyllum* stored in herbaria for up to 92 years did not lose any antibacterial activity (Eloff, 1999).

(viii)   *A proposal for expressing antibacterial activity*

MIC values do not give any indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity extracted from 1 g of plant material in mg by the MIC in mg/ml. The resultant value in ml /g gives the highest dilution to which a plant extract can be diluted and still inhibited the growth of the test organism (Eloff 2000).

(ix)   *Other biological activities of Combretum species*

The anti-inflammatory anthelminthic and antischistosomal activity of 20 *Combretum* species was determined. There was very little antischistosomal activity, low to medium anthelminthic activity and medium to strong anti-inflammatory activity in extracts of the different species (McGaw *et al.* 2001)

(x)   *Antibacterial activity of Marula bark and leaves*

Both leaf and bark extracts had antibacterial activity and there were two main bioactive compounds i.e. a very polar and a very non-polar compound (Eloff, 2001).

(xi)   *The stability and relationship between antibacterial and anti-inflammatory activity of southern African Combretum species*

Both antibacterial and anti-inflammatory activity was stable and there was a reasonable correlation between antibacterial and anti-inflammatory activity indicating that similar compounds may be responsible for the biological activities (Eloff *et al.*, 2001).

(xii)   *Extraction of antibacterial compounds from Combretum microphyllum*

Several extractants were tested to determine if any extractant selectively extracted antibacterial compounds. The three most promising extractants were di-isopropyl ether,

ethanol, ethyl ether, acetone and ethyl acetate. The activity towards Gram negative and Gram-positive bacteria was similar (Kotze and Eloff, 2002)

(xiii)   *Isolation of antibacterial compounds from C. erythrophyllum*
Martini *et al.,* (2004a) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3 -dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone.
All test compounds had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25-50 μg/ml. Rhamnocitrin and quercetin-5,3-dimethylether showed additional good activity (25 μg/ml) against *Micrococcus luteus* and *Shigella sonnei*. Toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7,4-dimethoxyflavone (Martini *et al.*, 2004b). This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds.

(xiv)   *Variation in the chemical composition*
Variation in the chemical composition, antibacterial and anti-oxidant activity of fresh and dried Acacia leaf extracts (Katerere and Eloff, 2004).

(xv)   *Isolation of antibacterial compounds from C. woodii*
The stilbene 2, 3, 4-trihydroxyl, 3, 5, 4-trimethoxybibenzyl (combretastatin B5) from the leaves of *C. woodii* was isolated. It showed significant activity against *S. aureus* with an MIC of 16 μg/ml MIC of 16 μg/ml [*Ps. aeruginosa (125* μg/ml), *E. faecalis* (125 μg/ml) and slight activity against *E. coli.*] (Eloff *et al.*, 2005a,b). This is the first report of the antimicrobial activity of combretastatin B5.

(xvi)   *Isolation of antibacterial compounds from C. apiculatum*
For his M.Sc study Serage (2003) isolated and elucidated the structures of two flavanones alpinetin, pinocembrin, and one chalcone flavokawain-from the leaves of *C. apiculatum subsp apiculatum*. All the compounds had substantial activity against the bacterial pathogens tested.

(xvii)   *Isolation of antibacterial compound from Terminalia sericea*

In his PhD study Kruger (2003) investigated eleven extractants and seven *Terminalia spp* to find the best extractant and species to use for isolating antibacterial compounds. He isolated terminoic acid from *Terminalia sericea* and showed that it could be used as a topical agent.

(xviii)   *Use of Urginea sanguinense in ethnoveterinary medicine*

Pretreatment of bulbs of *Urginea sanguinense* used in ethnoveterinary medicine influences chemical composition and biological activity (Naidoo *et al*., 2004).

(xix)   *Use of Gunnera perpensa extracts in endometriosis*

McGaw *et al*., (2005) checked whether the use of *Gunnera perpensa* extracts in endometriosis were related to antibacterial activity.

(xx)   *Use of Peltephorum africanum extracts in veterinary medicine*

 The rationale for using *Peltephorum africanum* (fabaceae) extracts in veterinary medicine was investigated (Bizimenyera *et al*., 2005).

(xxi)   *Toxic effects of the extracts of Allium sativum bulbs on adults of Hyalomma*
     marginatum rufipes and Rhipicephalus pulchellus.

In vitro investigation of the toxic effects of the extracts of *Allium sativum* bulbs on adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus* (Nchu *et al*., 2005).

(xxii)   *Screening of sixteen poisonous plants*

Sixteen poisonous plants were screened for antibacterial, anthelmintic and cytotoxic activity *in vitro* (MacGaw and Eloff, 2005).

(xxiii)   *Antibacterial and antioxidant activity of Sutherlandia frutescens*

Antibacterial and antioxidant activity of *Sutherlandia frutescens* (Fabaceae) were investigated (Katerere and Eloff, 2005a).

(xxiii)   *Identification of anti-babesial activity*

Anti-babesial activity of four ethnoveterinary plants were identified *in vitro* (Naidoo *et al*., 2005).

(xxiv)   *Management of diabetes in African traditional medicine*

Management of diabetes in African traditional medicine in Soumyanath (Katerere and Eloff, 2005b).

1.4. Existing antifungal drugs

The information in this section was compiled from the following publications: (Wills *et al.*, 2000; White *et al.*, 1998; Ghannoum and Rice, 1999; Tkacz and Didomenico, 2001, Didomenico, 1999).

There has been extensive research on the development of antifungal drugs, but only six of these of these antifungal agents were licensed for use in 1995. These include only polyene amphotericin B, three azoles, miconazole, ketoconazole, fluconazole and itraconazole and one pyrimidine synthesis inhibitor flucytosine (5-FC) (Espinel-Ingroff and Pfaller, 1995).

Polyenes act by binding to ergostel present in the fungal cell membrane, causing osmotic instability and loss of membrane integrity. The azoles on the other hand inhibit fungal cytochrome P450-dependent enzymes, with resulting impairment of ergosterol synthesis and depletion in the fungal cell membrane (Espinel-Ingroff and Pfaller, 1995). Fluconazole is a water-soluble bifluorinated triazole, with low binding affinity for plasma protein. It distributes extensively throughout the body, and readily diffuses into saliva. This drug is highly successful in the treatment of AIDS patients who had relapsed after amphotericin B and flucytosine (5-FC) treatment (Drouhet and Dupont, 1989).

However, it has been found that treatment with these drugs, especially for extended periods, can lead to problems with toxicity to the patients (amphotericin B) or with the development of resistant pathogenic organisms during the course of therapy (5-fluorocystine) (Boonchird and Flegel, 1982). Since the incidence of these opportunistic infections is on the increase, attempts are made to develop new chemotherapeutic agents or a combination of agents to treat the causative fungus.

Due to the sterol-binding action of amphotericin B in the fungal cell membrane, renal damage is found to occur in more than 80% of patients and can be permanent in patients receiving larger doses of the drug (Clark and Hufford, 1993). Flucytosine in combination with amphotericin B is designed to reduce the dosage of amphotericin and to eliminate the development of resistance to flucytosine. However, it has been noted that flucystoine toxicity may increase when it is used in combination with amphotericin B (Clark and Hufford, 1993).

The above-mentioned problems therefore illustrate the need for antifungal compounds with low or no toxicity, and natural products are an important potential source of the compounds.

1.4.1. Novel antifungal medicine

Fungi, like their hosts are eukaryotic organisms, making it more difficult to select intracellular fungal targets whose inhibition would not also be deleterious to the host cell. Of the four classes of antifungal compounds currently in use, three affect ergosterol, namely polyenes, azoles, and allylamines. Fluoropyrimidine 5-fluorocytosine (5-FC) achieves its specificity through a converting enzyme not present in mammalian cells. **Table 1.2** shows general overview of presently used antifungal agents.

Table 1.2. An overview of antifungal agents (Didomenico, 1999)

Compound/Class	Mode of action	Comments
Amphotericin B/polyene	Selective binding to ergosterol, major sterol of fungal membranes	Fungicidal Broad spectrum Intravenous Little resistance observed Significant nephrotoxicity
Abelcet/polyene Ambisome Amphotec	Selective binding to ergosterol, major sterol of fungal membranes	Liposomal formulation of AMB Similar efficacy as AMB Reduced toxicity observed
Nyotran/nystatin	Selective binding to ergosterol major sterol of fungal membranes	Liposomal formulation of nystatin Lowered toxicity compared to nystatin
5- Fluorocytosine (5-FC)/nucleoside analog	Selective conversion to toxic intermediate	Most often given in combination with AMB for: Cryptococcal meningitis Poor activity against filamentous fungi Significant resistance observed
Miconazole/azoles Ketoconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase	Static activity against yeast, dimorphic fungi, dermatophytes General fungistatic activity
Fluconazole/triazoles Itraconazaole Voriconazole Posaconazole UR-9825 SYN-2869 BMS-207147	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase	Broad spectrum including *Aspergillus* spp. FLU-resistant *C. albicans* strains and non-albicans strains increasing Efficacious in immune compromised models
LY303366?candins Caspofungin FK-463	Fungal β-1,3-glucan synthase inhibitors	Partly fungicidal Broad spectrum except for *Cryptococcus, Fusarium, Sporothrix, Trichosporon* Efficacious in immune compromised models
BMS181184/pradimicins	Calcium-dependent binding to mannoproteins in cell wall	Broad spectrum except for *Fusarium* Oral Hepatotoxicity led to

		discontinution
Nikkomycin/nikkomycins	Chitin synthase inhibitors	Liposomal formulation of nikkomycin Limited spectrum for fungi Effective against cells with high chitin content
Terbinafine/allylamines	Squalene epoxidase inhibitors	Fungicidal Active against dermatophytes Topical and oral formulations
Basifungin/aureobasidins	Inositol-P ceramide synthase inhibitor	Fungicidal Broad spectrum
Sordarin/Sordarins	Selectively binds to fungal EF2/ribosomal stalk proteins	Fungicidal Broad spectrum

AMB, amphotericin B

1.4.1.1.   Inhibitors of fungal cell membranes

Polyenes

The only polyene approved for systemic use is Amphotericin B (AMB). Its primary advantages include its fungicidal activity against most clinically relevant pathogens, and the low occurrence of resistance. The primary disadvantage of AMB is its nephrotoxicity. Ambisome, Abelcet and Amphocil/Amphotech all exert relatively similar efficacies with fewer side effect than AMB (Walsh *et al*., 1998). Composition of the lipid bilayer containing the polyenes appears to contribute to slight differences in efficacy as a result of both redistribution of the antifungal drug to tissues and the selective release of active AMB from the complex (Boswell *et al.*, 1998).

Azoles

There is a wide variety of azoles that have *in vitro* efficacy, but only a few have had significant clinical utility. Azoles inhibit cytochrome P450-dependent lanosterol 14-alpha-demethylase, causing accumulation of methylated sterols, depletion of ergosterol, and inhibition of cell growth (Koltin and Hitchcock, 1997). Sensitivity of other P450-dependent enzymes accounts for their primary mode of toxicity. Although azoles demostrate a broad spectrum of activity with less toxicity than AMB, they are not generally fungicidal but rather fungistastic.

Aureobasidins

Basifungin is a cyclic depsipeptide with good *in vitro* and *in vivo* activity against a number of pathogenic fungi including most *Candida* species, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Blastomyces dermatidis*, with poor activity against *Aspergillus* spp. and dermatophytes (Takesako *et al.,* 1993). This compound inhibits phosphatidyl-

inositol:ceramide phospho-inositol transferase (IPC synthase), which is encoded by an essential gene (Nagiec *et al.*, 1997). Other natural products, kafrefungin and rustmicin also inhibit IPC synthase (Mandala *et al.*, 1997).

1.4.1.2. Inhibitors of fungal cell wall

The fungal cell wall is an ideal target for the search for novel, fungicidal compounds. Several of the enzymes involved in the biosynthesis of the cell wall are unique to fungi, including chitin and glucan synthases (Georgopapadakou, 1997)

Echinocandins and pneumocandins
β-1,3-Glucan synthase is the target of both the echinocandins and pneumocandins (Radding *et al.*, 1998). Indianapolis is a derivative of cilofungin, an early echinocandin B analog that has a limited spectrum. LY303366 compound is both orally and parenterally active and more potent. It has *in vitro* and *in vivo* activity against numerous clinical isolates of *C. albicans, B. dermatididis, H. capsulatum, A. fumigatus* and the cystic form of *Pneumocystis carinii* (Espinel-Ingroff, 1998). Caspofungin has partly fungicidal activity *in vitro* against some *Candida* spp. and some dimorphic fungi.

Nikkomycins
Members of this class of compound have been known for many years. They appear to act competitively as substrate analogs of UDP-N-glucosamine in preventing the synthesis of chitin. Although chitin synthesis is an essential function, multiple isozymes present in fungi add a level of complexity. The potency of an inhibitor may depend on the isoform's relative effectiveness in building a cell wall as well as its affinity to a given enzyme. Nikkomycin has a relatively narrow spectrum as a solo agent but has been shown to have either additive or synergistic effects in combination with azoles against a number of human pathogens (Li and Rinaldi, 1999).

Pradimicins
The pradimicin family of antifungals exerts its selectivity by calcium-dependent binding of cell surface mannoproteins leading to cell membrane leakages and loss of viability (Watanable *et al.*, 1996). These compounds exhibit broad *in vitro* and *in vivo* activity (Oki *et al.*, 1992). In a direct comparison with AMB, the compound is 40- to 50-fold less active, but also 130-fold less toxic (Oki *et al.*, 1992). Azole and 5FC-resistant strains remain susceptible. The pradimicins have demonstrated antiviral activities *in vitro*, via a critical interaction with mannose-containing polysaccharides on the viral coat surfaces.

Geranylgeranyltransferase inhibitors

Cell wall integrity requires a functional geranylgeranyltransferase (GGT). A human ortholog has been identified, there is only about 20 % homology between the fungal and mammalian GGT therefore it may be possible to obtain specificity in action. There are number of selective active-site inhibitors targeted specifically against GGT in the micromolar to nanomolar range, and some appear fungicidal.

1.4.1.3. Inhibitors of protein synthesis

Sordarins

The search for suitable, unique targets within the fungal ribosome is challenging, (with the exception of elongation factor (EF3)), due to the structural and sequence similarity between fungal and mammalian ribosomal RNAs, subunits and soluble factors. The EF3 120 kDa soluble factor was originally discovered in *S. cerevisiae* and has subsequently been identified in other fungal pathogens (Uritani *et al.*, 1999). Sordarins are highly specific inhibitors of fungal translation. Several derivatives are active against *C. albicans* (Aviles *et al.*, 1998). The ability of the sordarins to selectively inhibit fungal translation underscores the possibility that other essential proteins, as well as EF2, may be important targets in antifungals.

1.4.1.4. N-myristoyltransferase inhibitors

The transfer of myristate, a 14-carbon fatty acid, from CoA to the terminal glycine of certain proteins has been shown to be essential in *C. albicans, C. neoformans* and other fungi (Weinberg *et al.*, 1995). A number of inhibitors targeted towards N-myristoyltransferase (NMT) are known.

1.5. New potential targets for antifungal development

Information in this section is compiled from several reviews (Wills *et al.*, 2000; White *et al.*, 1998; Ghannoum and Rice, 1999; Tkacz and Didomenico, 2001, Didomenico, 1999).

There is an attempt to find sensitive fungicidal targets with potential for selectivity over mammalian cells. In this section I will attempt to examine in-depth several of these focused strategies on antifungal development (**Figure 1.1.**).

1.5.1. The fungal cell wall

The fungal cell wall acts as the interface between the fungus and its environment. It has several roles, which include providing the fungus with its shape and supporting it against osmotic forces. It acts as a filter, controlling the secretion and uptake of molecules into the cell. Some enzymes are also responsible for enzymatic conversion of nutrients into metabolisable forms, prior to their entry into the protoplast (Pebery, 1990). This structure is not only important to viability of the fungal cell, it is also unique to fungi and not present in mammalian cells. These features make it an ideal antifungal target.
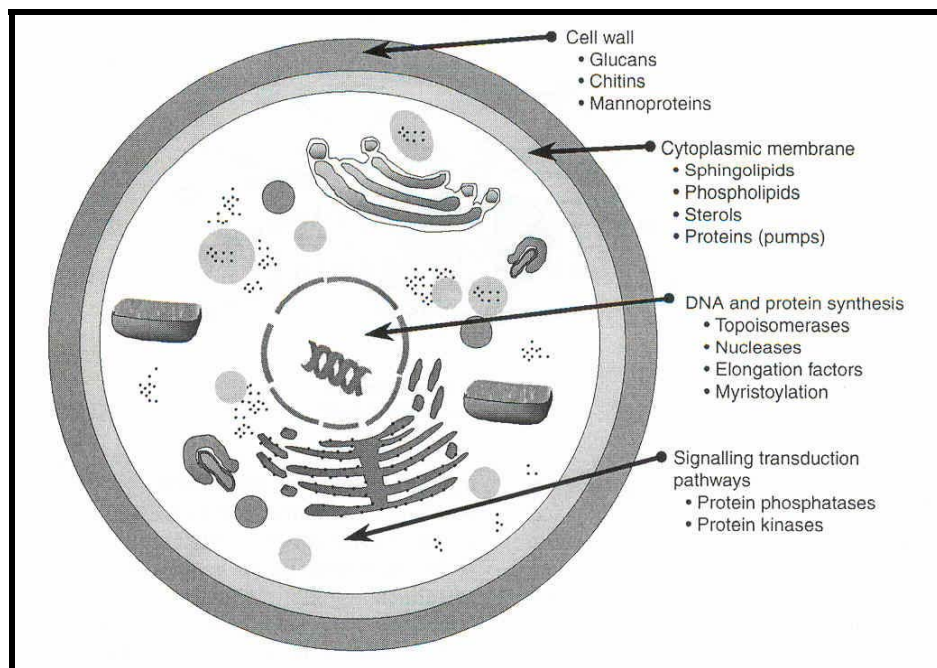

Figure 1.1. Schematic view of emerging targets for antifungal drug development (Wills *et al.*, 2000).

1.5.1.1. (1,3)-β-D-Glucan synthase

The β-Glucans are an abundant class of polysaccharides that are involved in structural, functional and certain morphological roles at the fungal cell surface (Fleet and Phaff, 1981). The membrane bound-enzyme (1,3)-β-D glucan synthase (GS) catalyses the synthesis of (1,3)-β-glucan, an essential glucose polymer found in fungi. It forms a fibril composed of three helically entwined linear polysaccharides, which provide rigidity and integrity to the cell structure. Since the (1,3)-β-glucan structure is not found in mammalian cells, the GS enzyme has become a target for research into antifungal agent development (Inoue *et al.*, 1995). The current proposed model for GS is shown in **Figure 1.2**.

1.5.1.2. Chitin synthase

Chitin is a major structural component of the cell walls of many fungi. It is a (1-4)-β-linked homopolymer of *N*-acetyl-D-glucosamine, and is produced by chitin synthase from the nucleotide UDP-GlcNAc and follows the reaction (Cabib, 1987):

$2n$ UDP-GlcNAc $\rightarrow$ (GlcNAc-β-(1-4)-GlcNAc)$_n$ + $2n$ UDP
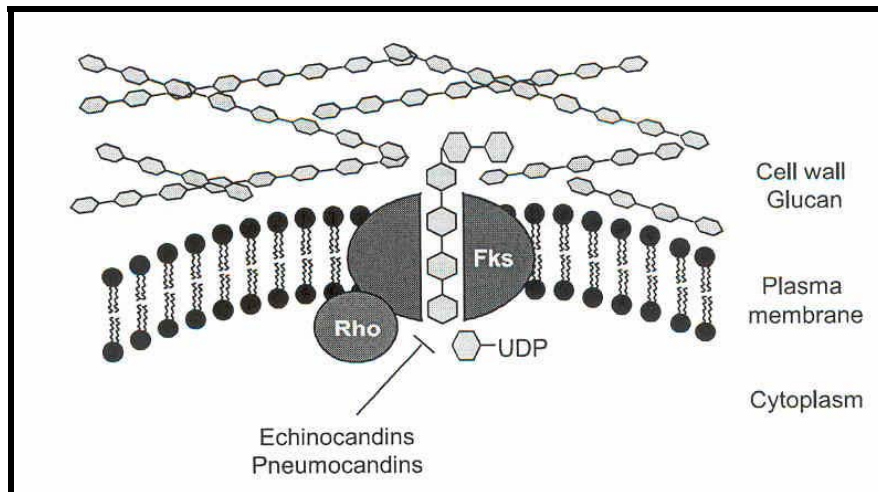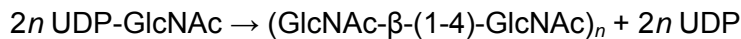
Fks: Glucan sunthase complex; Rho: GTP-binding regulatory subunit; UDP: Uridine diphosphate

Figure 1.2. Working model of glucan synthase (Wills *et al.*, 2000)

In *S. cerevisiae*, the cell wall is relatively poor in chitin, but the primary septum, that separates the mother and daughter cells, and bud scars are mostly composed of chitin (Cabib *et al.*, 1997). It is also found in the cell wall and plays a role in cell wall integrity. Chitin synthesis is cell cycle regulated, and the amount and distribution of chitin in the cell wall changes as the cell proceeds from vegetative growth to diploid formation and then sporulation. Since chitin is not present in mammalian cells, it has the potential to be a highly selective target for therapeutic use.

1.5.1.3. Mannoproteins

Mannose constitutes a major portion of the cell wall of many fungi, as well as the glycoproteins that form the protective capsule in *C. neoformans.* The biosynthetic pathway of this polysaccharide may be important to its survival in the host. Mannoproteins are formed by *O*-linkages joining mannose and small oligosaccharides to the hydroxyl groups of the amino acids serine or threonine. A second type of linkage connects high molecular weight and highly branched mannoproteins to the protein moiety via an N-acetylglucosamine and asparagines (Ballou, 1990). Once mannose has been synthesised, dolichol phosphate mannose synthase transfers mannose from GDP-mannose to dolichol phosphate, forming Dol-P-mannose, a key intermediate in protein glycosylation (Herscovics and Orlean, 1993).

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The glycosylation of proteins occurs in the rough endoplasmic reticulum, after which they are transported to the cell wall. All these steps might become antifungal drug targets.

1.5.2. The fungal cytoplasmic membrane

The fungal plasma membrane is similar to its mammalian counterpart. It contains phospholipids, sphingolipids, sterols and proteins. The key factors for the plasma membrane to function are its fluidity, its rigidity and its transport mechanisms, determined by lipid composition, sterol composition and protein composition, respectively.

1.5.2.1. Sphingolipids

Sphingolipids are essential components of all eukaryotic plasma membranes and modulation of them exerts a deep impact on cell viability (Hannun and Luberto, 2000). Although the presence and role of sphingolipids are common to these two organisms, their biosynthetic pathways differ. These differences may represent a new suitable target for the development of antifungal agents. Sphingolipid synthesis and metabolism appear to be conserved among non-pathogenic and pathogenic fungi (Zhong *et al.*, 2000).

1.5.2.2. Phospholipids

The fungal phospholipid pathway is structurally similar to the mammalian counterpart (Daum *et al.*, 1998). The only difference is the synthesis of phosphatidylserine, which is synthesised from CDP-diacylglycerol in fungi, but from phosphotidylethanolamine and serine in mammalian cells (Klig *et al.*, 1988). Presently there is no specific target or compound reported that inhibits fungal phospholipid biosynthesis.

1.5.2.3. Ergosterol synthesis

The ergosterol biosynthesis pathway and its target sites for antifungal agents are known. Azole antifungal agents prevent the synthesis of ergosterol by inhibition of the cytochrome P450-dependent enzyme, lanosterol demethylase (also referred to as 14α-sterol demethylase or $P450_{DM}$) (Ghannoum and Rice, 1999). This enzyme is also found in mammalian cells where it plays an important role in cholesterol synthesis (Koltin and Hitchcock, 1997). However, azoles possess a much greater affinity for the fungal enzyme than their mammalian counterparts, and as such are currently the most widely used antifungal agents.

1.5.2.4. Plasma membrane ATPase

The plasma membrane ATPase (P-ATPase) is encoded by the PMA1 gene and controls both efflux and influx of cations (H^+, Ca^+, Na^+, and K^+) across the plasma membrane. The fungal Pmal enzyme differs considerably from the mammalian and plant enzymes, especially in transmembrane segments 1, 2, 3, and 4 (Monk *et al.*, 1995). Site-directed mutagenesis of these regions frequently results in lethal mutations in *S. cerevisiae*. These observations suggest that the P-ATPase pumps can be considered potential targets for the development of new antifungal agents.

1.5.2.5. Antifungal peptide

Antifungal peptide molecules appear to act mainly on plasma membrane synthesis. A different class of peptides, lipopepetides, affect mainly cell wall synthesis (Balkovec, 1994). These peptides may help both dissect important targets in the plasma membrane and themselves become antifungal agents.

1.5.3. DNA and protein synthesis

1.5.3.1. Topoisomerases

Topoisomerases control the topological state of DNA by introducing transient DNA breaks (single-strand DNA for Type I and double-strand DNA for Type II) that allow for the manipulation of DNA strands (Wang, 1971). Topoisomerases stabilise the nicked DNA strands by forming a covalent phosphate-tyrosine linkage with either the 3'- or 5'- end of the DNA. Topoisomerase-specific inhibitors stabilise this covalent protein-DNA linkage, effectively slowing the religation of catalysis and ultimately leading to DNA damage and cell death (Lima and Mondragon, 1994). Studies on *C. albicans* and *C. neoformans* have revealed that topoisomerase I (*TOP1*) is essential for viability (Del-Poeta *et al.*, 1999; Jiang *et al.*, 1997), so *TOP1* appears critical for viability. Fungal TOP1 enzymes contain an amino acid insertion, located in the linker domain region, not found in the mammalian enzyme.

1.5.3.2. Nucleases

The dicationic aromatic compounds (DACs) are pentamidine derivatives that have been shown to posess excellent *in vitro* and *in vivo* activity against pathogenic microorganisms (Tidwell *et al.*, 1993). These compounds have *in vitro* antifungal activity against *C. neoformans* and *C. albicans.* Several of these agents exhibited excellent *in vitro* fungicidal activity against a *C. albicans* mutant strain containing a fluconazole-resistant mechanism (Del-Poeta *et al.*, 1998). Since these compounds have been administered safely to animals,

they have the potential of being developed into potent antifungal agents for general use in humans.

1.5.3.3. Protein synthesis

Several well-characterised compounds are known to inhibit the RNA polmerases and elongation factors required for transcription and protein synthesis. The evaluation of the degree to which these compounds are selective to fungi will determine whether this class of compounds has the potential of becoming novel antifungal agents. Elongation factor 3 (EF-3) is a unique and essential requirement of the fungal translation machinery. Non-fungal organisms do not have and do not require a soluble form of the EF-3 for translation (Kovalchuke and Chakraburtty, 1994), therefore, it is an ideal antifungal target (Kovalchuke *et al.*, 1998). No inhibitors of EF-3 have been identified (Wills *et al.*, 2000).

1.5.3. Signal transduction pathways

The signal transduction cascades in fungi have become very attractive since their components are now emerging as targets for new natural antifungals. Cardenas *et al* (1998) studied the mechanism of action of five natural products, cycosporin A (CsA), FK506, rapamycin, wortmannin and genldanamycin on signalling and found that they targeted calcineurin-mediated signal transduction.

1.5.4.1. Calcineurin

Calcineurin is a serine/threonine-specific Ca^{2+}-calmodulin-activated protein phosphatase that is conserved from yeast to man (Hemenway and Heitman, 1999). Calcineurin is the target of CsA and FK506 in T-cells, *C. albicans*, *C. neoformans* and *A. fumigatus* (Odom *et al.*, 1997). A number of non-immunosuppressive FK506 and CsA analogues have been described, including L-685, 818 (18-OH, 21-ethyl-FK506), which retain antifungal activity *in vitro* via inhibition of calcineurin (Odom *et al.*, 1997). If these non-immunosuppressive CsA analogues have antifungal activity they will need to be tested in animal models for antifungal efficacy.

1.5.5. Virulence factors
1.5.5.1. Melanin

Melanin is produced by the enzyme laccase and has been thought to be major virulence factor in the pathogenic fungus *C. neoformans* (Liu *et al.*, 1999). Melanin production has also been discovered in other pathogenic fungi, including the dematiaceous fungi, which produce compounds classified as phaeohyphomycoses (Fothergill, 1996). The focus on *C.*

neoformans and its melanin production has two potential benefits, firstly it facilitates understanding of the function of melanin in yeast cells within the host, and secondly with further understanding of biochemistry and molecular biology of melanin, it could become a unique target for antifungal drugs against *C. neoformans* and other dematiaceous fungi.

1.5.5.2. Mannitol

Other than mannose, another possible metabolic target associated with virulence in *C. neoformans* is the mannitol pathway. Chaturvedi *et al.* (1996) isolated one mutant with decreased mannitol production and found it to be more susceptible to polymorphonuclear leukocyte killing. Further studies are needed to understand and validate the role of the mannitol pathway in fungal virulence.

1.5.5.3. Phospholipases

Phospholipases are a group of enzymes that hydrolyse specific ester linkages in glycerophospholipids. Invasion of the host cells by microbes involves penetration and damage of the outer cell envelope. This happens by enzymatic or physical means, and phospholipases are involved in the cell disruption process that occurs during infection. The enzyme could promote the pathogen entering into the host cell (Ibrahim *et al.*, 1995). Extracellular phospholipases have been found to be implicated with pathogenecity in fungi including *C. albicans, C. glabrata, Penicillum notatum, A. fumigatus and C. neoformans*. The potential of these enzymes as targets for drug design is still under development.

1.6. Major groups of antimicrobial compounds from plants

The information in this section is summarised from Cowan (1999).

1.6.1. Phenolics and Polyphenols
Simple phenols and phenolic acids.

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds that are in the highest oxidation state (**Figure 1.3**). The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner *et al.*, 1996), and fungi (Duke, 1985). Catechol and pyrogallol are both hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two 2-OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased

hydroxylation results in increased toxicity (Geissman, 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in clove oil (**Figure 1.4**). Eugenol is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Thomson, 1978).
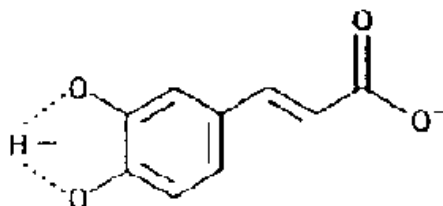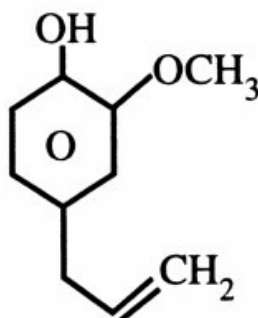
Figure 1.3. Caffeic acid

Figure 1.4. Eugenol

1.6.2. Quinones.

Quinones are aromatic rings with two ketone substitutions (**Figure 1.5**). They are ubiquitous in nature and are characteristically highly reactive. These compounds,

Figure 1.5. Quinone

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being coloured, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone-hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems. Vitamin K is a complex naphthoquinone. Its antihaemorrhagic activity may be related to its ease of oxidation in body tissues (Harris, 1963). Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase (Vamos-Vigyazo, 1981).

In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al.*, 1996), often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes.

1.6.3. Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones) (**Figure 1.6**). The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C6 -C3 unit linked to an aromatic ring. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996).
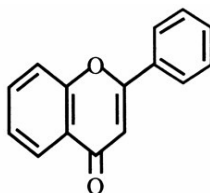

Figure 1.6. Flavone

Catechins are the most reduced form of the C3 unit in flavonoid compounds, and these flavonoids have been extensively researched due to their occurrence in oolong green teas. Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin against HIV (Pengsuparp *et al.*, 1995). More than one study has

found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV) (Kaul et al., 1985). Kaul et al. (1985) provide a summary of the activities and modes of action of quercetin, naringin, hesperetin, and catechin in *in vitro* cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3, or RSV, the other three flavonoids were effective in various ways.

1.6.4. Tannins

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin and other proteins from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers (**Figure 1.7**). Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units (Geissman, 1963). This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al.*, 1994).
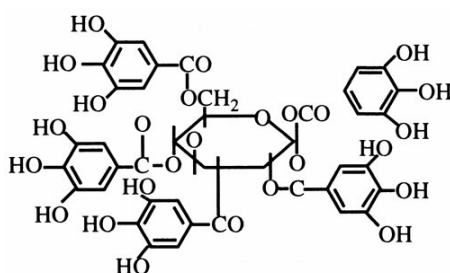

Figure 1.7. Tannins

1.6.5. Coumarins

Coumarins (**Figure 1.8**) are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy and Thorne, 1997). They are responsible for the characteristic odour of hay. As of 1996, at least 1,300 had been identified (Hoult and Paya, 1996). Their fame has come mainly from their antithrombotic, anti-inflammatory, and vasodilatory activities (Namba, 1988). Warfarin is a particularly well-known coumarin which is used both as an oral anticoagulant and, interestingly, as a rodenticide (Keating and O'Kennedy, 1997). It may also

have antiviral effects (Berkada, 1978). Coumarins are known to be highly toxic in rodents and mammals, therefore are treated with caution by the medical community.
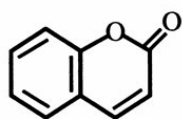

Figure 1.8. Coumarins

Coumarin was found *in vitro* to inhibit *Candida albican*s. As a group, coumarins have been found to stimulate macrophages (Casley-Smith and Casley-Smith, 1997), which could have an indirect negative effect on infections. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978) but was found ineffective against leprosy. Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to Gram-positive bacteria (Fernandez *et al.*, 1996). Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity (Hoult and Paya, 1996).

1.6.6. Terpenoids and Essential Oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (**Figure 1.9**). They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as monoterpenes, diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized.
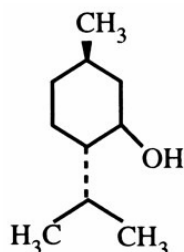

Figure 1.9. Terpenoids

Examples of common terpenoids are menthol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Artemisin and its derivative a-arteether, also known by the

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name qinghaosu, find current use as antimalarials (Vishwakarma, 1990). Terpenenes or terpenoids are active against bacteria (Amaral *et al.*, 1998, and Barre *et al.*, 1997), fungi (Ayafor *et al.*, 1994), viruses (Fujioka and Kashiwada, 1994), and protozoa (Ghoshal *et al.*, 1996). In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1977). The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Accordingly, Mendoza *et al.* (1997) found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity.

1.6.7. Alkaloids

Heterocyclic nitrogen compounds are called alkaloids (**Figure 1.10**). The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden, 1982); the name morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family, are commonly found to have
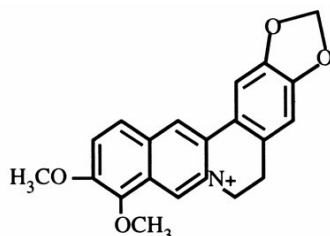

Figure 1.10. Berberine

antimicrobial properties (Omulokoli *et al.*, 1997). Solamargine, a glycoalkaloid from the berries of *Solanum khasianu*m, and other alkaloids may be useful against HIV infection (McMahon *et al.*, 1995) as well as intestinal infections associated with AIDS (McMahon *et al.*, 1995). While alkaloids have been found to have microbicidal effects (including against *Giardia* and *Entamoeba* species), the major antidiarrheal effect is probably due to their effects on transit time in the small intestine.

Berberine (**Figure 1.10**) is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia (Omulokoli *et al.*, 1997). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1987).

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1.6.8. Lectins and Polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 by Balls and colleagues. They are often positively charged and contain disulfide bonds (Zhang and Lewis, 1997). Their mechanism of action may be the formation of ion channels in the microbial membrane (Zhang and Lewis, 1997) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors. Recent interest has been focused mostly on studying anti-HIV peptides and lectins, but the inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthu*s, has long been known (De Bolle, 1996). Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues. They are toxic to yeasts and Gram-negative and Gram-positive bacteria (Fernande de Caleya *et al.*, 1972). Thionins AX1 and AX2 from sugar beet are active against fungi but not bacteria (Kragh *et al.*, 1995). Fabatin, a newly identified 47-residue peptide from fava beans, appears to be structurally related to g-thionins from grains and inhibits *E. coli*, *P. aeruginos*a, and *Enterococcus hirae* but not *Candida* or *Saccharomyces* (Zhang and Lewis, 1997). The larger lectin molecules, which include mannose-specific lectins from several plants, MAP30 from bitter melon, GAP31 from *Gelonium multiflorum*, and jacalin (Lee-Huang et al., 1995), are inhibitory to viral proliferation (HIV, cytomegalovirus), probably by inhibiting viral interaction with critical host cell components.

1.7.   FUNGI

Fungi are eukaryotic microorganisms, which are heterotrophic and essentially aerobic with limited anaerobic capabilities. Fungi synthesize lysine by the L-aadipic acid biosynthetic pathway. They possess chitinous cell walls, plasma membranes containing ergosterol, 80SrRNA and microtubules composed of tubulin. Fungi grow as yeasts, moulds or a combination of both (i.e. dimorphism). They lack chlorophyll and are classified into a separate kingdom.

1.7.1. Structure

Fungi can grow as yeasts and/or as moulds or both. The latter is known as dimorphism. Yeasts are single-celled forms that reproduce by budding, whereas moulds form multicellular hyphae. Many human and animal fungal pathogens exhibit thermal dimorphism in that they exist as yeast cells at 37 °C and as moulds at 25°C. Dimorphism is regulated by factors such as temperature, CO_2 concentration, pH, and the levels of cysteine or other sulfhydryl-containing compounds, depending upon the dimorphic fungus.

1.7.1.1. Yeast

Yeasts are unicellular fungi. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well-known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Hemiascomycetes*. The true yeasts belong to one main order *Saccharomycetales*.

Yeasts are characterized by a wide dispersion of natural habitats, and are common on plant leaves and flowers, in soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. In humans, *Candida albicans* causes vaginal infections, diaper rash and thrush of the mouth and throat.

Yeasts multiply as single cells that divide by budding (e.g. *Saccharomyces*) or direct division (fission, e.g. *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium). In sexual reproduction most yeasts form asci, which contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores.

1.7.1.2. Moulds

Moulds are microscopic, plant-like organisms, composed of long filaments called hyphae. Mould hyphae grow over the surface and inside nearly all substances of plant or animal origin. Included in this group are the familiar mushrooms and toadstools. When mould hyphae are numerous enough to be seen by the naked eye they form a cottony mass called a mycelium.

Moulds reproduce sexually by spores and asexually by conidia. Spores are in certain aspects like seeds; they germinate to produce a new mould colony when they land in a suitable place. Unlike seeds, they are very simple in structure and never contain an embryo. Spores are produced in a variety of ways and occur in a bewildering array of shapes and sizes. In spite of this diversity, spores are quite constant in shape, size, colour and form for any given mould, and are thus very useful for mould identification.

1.7.1.3. Dimorphic fungi

The dimorphic fungi have two forms, which are: (1) Yeast - (parasitic or pathogenic form). This is the form usually seen in tissue, in exudates, or if cultured in an incubator at 37 ^{o}C. (2) Mycelium - (saprophytic form). The form observed in nature or when cultured at 25 ^{o}C. Conversion to the yeast form appears to be essential for pathogenicity in dimorphic fungi. Fungi are identified by several morphological or biochemical characteristics, including the appearance of their fruiting bodies. The asexual spores may be large (macroconidia, chlamydospores) or small (microconidia, blastospores, arthroconidia).

Fungal infections appear as systemic mycoses with the exception of *S. schenckii* and usually begin by inhaling spores from the mould form. After germination in the tissues, the fungus grows in a non-mycelial form. For example, *Coccidioides immitis* (cause of coccidiodomycosis) produces hyphae and arthrospores when it grows in arid soil but grows as endosporulating spherules (a spherule filled with yeast-like spores) in the lung. *Histoplasma capsulatum*, the cause of histoplasmosis on the other hand, produces hyphae and tuberculate macroconidia in soil contaminated with bird or bat droppings but grows as an encapsulated yeast in the lungs. *Blastomyces dermatitidis* the cause of blastomycosis produces hyphae and conidiospores in soil contaminated with bird droppings but grows as a thick-walled yeast in the body.

1.7.2. Classification

Classification of fungi are mainly based on reproductive structures. Asexual structures are referred to as anamorphs; sexual structures are known as teleomorphs; and the whole fungus is known as the holomorph. Two independent, coexisting classification systems, one based on anamorphs and the other on teleomorphs are used to classify fungi. Fungal infections are usually classified according to the type and degree of tissue involvement and the host response to the pathogen. Fungi can also be classified as exogenous or endogenous depending on the route of infection. Endogenous fungi can cause infections if the host immune system is depressed. Such endogenous infections may originate from normal flora or via reactivation of a previous infection. Classification may be based on the interaction of the organism and the host immune response. Primary pathogens can cause disease even if the host immune system is intact while opportunistic pathogens generally cause disease only in immunocompromised persons.

1.7.2.1 Clinical classification of the mycoses

Fungal diseases may be discussed in a variety of ways. They can be divided into the clinical taxonomy: superficial mycoses, subcutaneous mycoses, systemic mycoses and opportunistic mycoses.

The superficial mycoses (or cutaneous mycoses) are fungal diseases that are confined to the outer layers of the skin, nail, or hair (keratinized layers), rarely invading the deeper tissue or viscera. The fungi involved are called dermatophytes. The subcutaneous mycoses are confined to the subcutaneous tissue and only rarely spread systemically. They usually form deep, ulcerated skin lesions or fungating masses, most commonly involving the lower extremities. The causative organisms are soil saprophytes, which are introduced through trauma to the feet or legs. The systemic mycoses may involve deep viscera and become widely disseminated. Each fungus type has its own predilection for various organs, which will be described as individual diseases are discussed. The opportunistic mycoses are infections due to fungi with low inherent virulence. The etiologic agents are organisms, which are common in all environments.

1.7.3. Multiplication

Fungi may reproduce sexually or asexually. Spores may be either sexual or asexual in origin. Sexual spores include ascospores, basidiospores, oospores and zygospores, which are used to determine phylogenetic relationships. Sexual reproduction occurs by the fusion of two haploid nuclei (karyogamy), followed by meiotic division of the diploid nucleus. Asexual spores are produced in sac-like cells called sporangia and are called sporangiospores. Asexual reproduction results from division of nuclei by mitosis.

1.7.4. Pathogenesis

Fungi have developed many mechanisms to colonize human hosts. The ability to grow at 37°C is one of the most important. Production of keratinase allows dermatophytes to digest keratin in skin, hair and nails. Dimorphism allows many fungi that exist in nature as moulds to change to a yeast form in the host and thus become pathogenic. In contrast, *Candida albicans* exists in the yeast form as normal flora but becomes invasive in the filamentous form. In addition, the antiphagocytic properties of the *Cryptococcus neoformans* capsule and the adherence abilities of *C. albicans* allow pathogenic potential for these fungi.

 Fungi may spread locally, such as dermatophytes on the skin or eumycotic mycetomas in subcutaneous tissue. *Sporothix schenckii*, another subcutaneous pathogen, spreads via local

lymphatics. The fungi-producing systemic mycoses mainly cause pulmonary infections. These fungi are phagocytosed by alveolar macrophages but are not destroyed. Instead the fungi are spread hematogenously to distant sites in the body. An exception is *Cryptococcus neoformans*, which disseminates without being phagocytosed. The pathogenesis of some fungi may be at least partly due to the host's reaction to the organism such as the allergic reactions elicited by some fungi.

1.7.5. Host Defenses

While some fungi have more pathogenic potential than others, the immunologic status of the host is of paramount importance in determining whether an organism will cause disease and will help determine the severity of the infection. Both humoral and cell mediated immunity (CMI) are important in control of fungal infections, but CMI appears to be more important since patients with defects in CMI usually suffer more severe fungal infections than do persons with depressed humoral immunity. Nonspecific barriers to fungal infection must be crossed, however, before specific immune responses to fungi are elicited. These primary barriers to fungal infection include intact skin, naturally occurring long-chain unsaturated fatty acids, competition with normal bacterial flora and epithelial turnover rate. In addition the mucous membranes are covered with fluids containing antifungal substances. Furthermore, many epithelial cells of the mucous membranes contain cilia that actively remove microorganisms.

1.7.6. Epidemiology

Whereas some fungi such as *Sporothrix schenckii* are found worldwide, it is most commonly encountered in persons engaged in professions or hobbies where the organism might gain entry into subcutaneous tissues via trauma (e.g. gardeners). Other fungi would be most commonly seen in persons living in or visiting specific geographic regions (e.g. *Coccidioides immitis* in the desert southwestern United States). More specific examples of the role of the environment in fungal infections include the increased rate of candidal vaginitis in women taking systemic antibacterial drugs and increased prevalence of mycotic mycetomas in barefoot persons living in tropical countries. While immunocompromising conditions result in increases in opportunistic fungal infections, the specific underlying disease partially determines the prevalence of such infections. For example, the rhinocerebral syndrome (a deeply invading, life threatening form of zygomycosis, also known as mucormycosis) might be seen in persons suffering from diabetic ketoacidosis while histoplasmosis would be more common in AIDS patients.

1.7.7. Diagnosis

1. Skin scrapings suspected to contain dermatophytes or pus from a lesion can be mounted in 20% KOH on a slide and examined directly under the microscope.

2. Skin testing (dermal hypersensitivity) used to be popular as a diagnostic tool, but this use is now discouraged because the skin test may interfere with serological studies, by causing false positive results. It may still be used to evaluate the patient's immunity, as well as a population exposure index in epidemiological studies.

3. Serology may be helpful when it is applied to a specific fungal disease; there are no screening antigens for 'fungi' in general. Because fungi are poor antigens, the efficacy of serology varies with different fungal infections. The most common serological tests for fungi are based on latex agglutination, double immunodiffusion, complement fixation and enzyme-linked immunoassays (ELISA). While latex agglutination may favor the detection of IgM antibodies, double immunodiffusion and complement fixation tests usually detect IgG antibodies. Some ELISA tests are being developed to detect both IgG and IgM antibodies. There are some tests, which can detect specific fungal antigens, but they are just coming into general use.

4. Fungi can be identified in tissue or exudate smears by using fluorescing stain such as cocalcifluor white or specifically with direct immunoflorescent staining methods

5. Biopsy and histopathology. A biopsy may be very useful for the identification and as a source of the tissue-invading fungi. Either the Gomori methenamine silver (GMS) stain is used to reveal the organisms, which stain black against a green background or Periodic Acid Schiff (PAS) fungi stain a dark pink against blue background.

6. Culture. A definitive diagnosis requires a culture and identification. Pathogenic fungi are usually grown on Sabouraud dextrose agar (Difco). It has a slightly acidic pH (~5.6); cyclohexamide, penicillin, streptomycin or other inhibitory antibiotics are often added to prevent bacterial contamination and saprophytic fungal overgrowth. Two cultures are inoculated and incubated separately at 25 ^{o}C and 37 ^{o}C to reveal dimorphism. The cultures are examined macroscopically and microscopically. They are not considered negative for growth until after 4 weeks of incubation.

1.7.8. Treatment

Mammalian cells do not contain the enzymes that will degrade the cell wall polysaccharides of fungi. Therefore, these pathogens are difficult to eradicate by the animal host defense mechanisms. Because mammals and fungi are both eukaryotic, the cellular milieu is

biochemically similar in both. The cell membranes of all eukaryotic cells contain sterols; ergosterol in the fungal cell membrane and cholesterol in the mammalian cell membrane. Although one of the first antimycotic agents (oral iodides) were used in 1903, the further development of such agents has been left far behind the development of anti-bacterial agents. The selective toxicity necessary to inhibit the invading organism with minimal damage to the host has been difficult to establish within eukaryotic cells. The primary antifungal agents are:

Amphotericin B.

A polyene antimycotic. It is usually the drug of choice for most systemic fungal infections. It has a greater affinity for ergosterol in the cell membranes of fungi than for the cholesterol in the host's cells. Once bound to ergosterol, it causes disruption of the cell membrane and death of the fungal cell. Amphotericin B is usually administered intravenously (patient usually needs to be hospitalized), often for 2-3 months or as a slow release lipid-bond compound subcutaneously. As it is often toxic it is nowadays used together with other antifungals. The drug is rather toxic; thrombo-phlebitis, nephrotoxicity, fever, chills and anemia frequently occur during administration.

Azoles

The azoles (imidazoles and triazoles), including ketoconazole, fluconazole, and itraconozole, are being used for muco-cutaneous candidiasis, dermatophytosis, and for some systemic fungal infections. Fluconazole is presently essential for the treatment of AIDS patients with cryptococcosis. The general mechanism of action of the azoles is the inhibition of ergosterol synthesis. Oral administration and reduced toxicity are distinct advantages.

Griseofulvin

Griseofulvin is a very slow-acting drug, which is used for severe skin and nail infections. Its effect depends on its accumulation in the stratum corneum where it is incorporated into the tissue and forms a barrier, which stops further fungal penetration and growth. It is administered orally. The exact mechanism of action is unknown.

5-fluorocytosine

5-fluorocytosine (Flucytosine or 5-FC) inhibits RNA synthesis and has found its main application in cryptococcosis. It is administered once daily.

1.8. Fungal pathogens used in this study

1.8.1*. Candida albicans*

Candida is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and stool. As well as being a pathogen and a colonizer, it is found in the environment, particularly on leaves, flowers, water, and soil.

It is a dimorphic fungus, most of the time it exists as oval, single yeast cells (10 – 12 μm in diameter), which reproduce by budding. Most yeasts do not produce mycelia but *Candida* has a trick up its sleeve. Normal room temperatures favour the yeast form of the organism, but under physiological conditions (body temperature, pH, and the presence of serum) it may develop into a hyphal form. Pseudohyphae**,** composed of chains of cells, are also common. Chlamydospores may be formed on the pseudomycelium.

Although *Candida* most frequently infects the skin and mucosal surfaces, it can cause systemic infections manifesting as pneumonia, septicaemia or endocarditis in severely immunocompromised patients. There does not appear to be a significant difference in the pathogenic potential of different *Candida* strains, therefore establishment of infection appears to be determined by host factors and not the organism itself. However, the ability to assume various forms may be related to the pathogenicity of the organism. Fortunately, several drugs are available to treat serious systemic infections, e.g. itraconazole and fluconazole.

1.8.1.a. Pathogenicity and Clinical Significance

Infections caused by *Candida* spp. are in general referred to as candidiasis. The clinical spectrum of candidiasis is extremely diverse. Almost any organ or system in the body can be affected. Candidiasis may be superficial and local or deep-seated and disseminated (Beilsa *et al*., 1987). Disseminated infections arise from hematogenous spread from the primarily infected locus. *C. albicans* is the most pathogenic and most commonly encountered species among all (Bodey, 1996). Its ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from yeast to hyphal phase are the major determinants of its pathogenicity. Several host factors predispose to candidiasis (Bodey *et al*.,1992).

Candidiasis is mostly an endogenous infection, arising from overgrowth of the fungus inhabiting in the normal flora. However, it may occasionally be acquired from exogenous sources (such as catheters or prosthetic devices) (Band and Maki, 1979) or by person-to-person transmission (such as oral candidiasis in neonates of mothers with vaginal

candidiasis or endophthalmitis following corneal transplantation from an infected donor) (Behrens-Baumann, 1991).

1.8.2. *Aspergillus fumigatus*

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. *Aspergillus* colonies are downy to powdery in texture. The surface colour may vary depending on the species.

A. fumigatus is a thermotolerant fungus and grows well at temperatures over 40°C. This property is unique to *Aspergillus fumigatus* among the *Aspergillus* species.

1.8.2.a. Pathogenicity and Clinical Significance

Aspergillus spp. are well-known to play a role in three different clinical settings in man: (i) opportunistic infections; (ii) allergic states; and (iii) toxicoses. Immunosuppression is the major factor predisposing to development of opportunistic infections (Ho and Yuen, 2000). These infections may present in a wide spectrum, varying from local involvement to dissemination and as a whole called aspergillosis. Among all filamentous fungi, *Aspergillus* is in general the most commonly isolated one in invasive infections. It is the second most commonly recovered fungus in opportunistic mycoses following *Candida*.

Almost any organ or system in the human body may be involved. Onychomycosis, sinusitis, cerebral aspergillosis, meningitis, endocarditis, myocarditis, pulmonary aspergillosis, osteomyelitis, otomycosis, endophthalmitis, cutaneous aspergillosis, hepatosplenic aspergillosis, as well as *Aspergillus* fungaemia, and disseminated aspergillosis may develop (Denning, 1998 and Arikans *et al*., 1998). Nosocomial occurrence of aspergillosis due to catheters and other devices is also likely (Lucas *et al*., 1999). Construction in hospital environments constitutes a major risk for development of aspergillosis particularly in neutropaenic patients (Loo et al., 1996).

Aspergillus spp. may also be local colonizers in previously developed lung cavities due to tuberculosis, sarcoidosis, bronchiectasis, pneumoconiosis, ankylosing spondylitis or neoplasms, presenting as a distinct clinical entity, called aspergilloma (Hohler *et al*., 1995). Aspergilloma may also occur in kidneys (Halpern et al., 1992).
Some *Aspergillus* antigens are fungal allergens and may initiate allergic bronchopulmonary aspergillosis particularly in atopic host (Germand and Tuchais, 1995). Some *Aspergillus* spp. produces various mycotoxins. These mycotoxins, by chronic ingestion, have proven to

possess carcinogenic potential particularly in animals. Among these mycotoxins, aflatoxin is well-known and may induce hepatocellular carcinoma. It is mostly produced by *Aspergillus flavus* and contaminates foodstuff, such as peanuts (Mori *et al.*, 1998).

In birds, respiratory infections may develop due to *Aspergillus*. It may induce mycotic abortion in the cattle and the sheep (St-Germain and Summerbell, 1996). Ingestion of high amounts of aflatoxin may induce lethal effects in poultry animals fed with grain contaminated with the toxin. Since *Aspergillus* spp. are found in nature, they are also common laboratory contaminants.

1.8.3. *Sporothrix schenckii*

Sporothrix schenckii is a thermally dimorphic fungus, which is distributed worldwide and isolated from soil, living and decomposing plants, woods, and peat moss. *S. schenckii* is an occasional cause of human infections. Despite the existence of the fungus worldwide, infections due to *S. schenckii* are more common in certain geographical areas. Peru is an area of hyperendemicity for *S. schenckii* infections (Pappas *et al.*, 2000).

At 25°C, colonies grow moderately rapidly. They are moist, leathery to velvety, and have a finely wrinkled surface. From the front and the reverse, the colour is white initially and becomes cream to dark brown in time ("dirty candle-wax" color). At 37°C, colonies grow moderately rapidly. They are yeast-like and creamy. The color is cream to beige. The conversion of the mould form to the yeast form is required for definitive identification of *S. schenckii* (Larone, 1995; and Sutton 1998). *Ophiostoma stenoceras* is the teleomorph of *Sporothrix* sp.

1.8.3.a. Pathogenicity and Clinical Significance

S. schenckii is the causative agent of sporotrichosis ("rose handler's disease") (Rex and Okhuysen, 2000). Sporotrichosis is a subcutaneous infection with a common chronic and a rare progressive course. The infection starts following entry of the infecting fungus through the skin via a minor wound and may affect an otherwise healthy individual. Following entry, the infection may spread via the lymphatic route. Nodular lymphangitis may develop (Kostman and DiNubile, 1993). Interestingly, an epidemic of sporotrichosis after sleeping in a rust-stained camping tent has been reported and the tent was identified as the source of infection (Campos et al., 1994). Patients infected with *S. schenckii* may be misdiagnosed as pyoderma gangrenosum due to the large ulcerations observed during the course of sporotrichosis (Byrd et al., 2001).

1.8.4. *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated yeast that can cause disease in apparently immunocompetent, as well as immunocompromised, hosts. Most susceptible to infection are patients with T-cell deficiencies (Kwong-chung, 1992). *C. neoformans* var. *neoformans* causes most cryptococcal infections in humans. *C. neoformans* var. *neoformans* is found worldwide; its main habitats are debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings. Not part of the normal microbial flora of humans, *C. neoformans* is only transiently isolated from persons with no pathologic features (Mitchell and Perfect, 1995). *C. neoformans* var *gitii* is found in the subtropics in decaying bark and affects both immunocompetent and immunocompromised persons.

Colonies of *C. neoformans* are fast growing, soft, glistening to dull, smooth, usually mucoid, and cream to slightly pink or yellowish brown in colour. The growth rate is somewhat slower than *Candida* and usually takes 48 to 72 h. It grows well at 25°C as well as 37°C. Ability to grow at 37°C is one of the features that differentiates *C. neoformans* from other *Cryptococcus* spp. However, temperature-sensitive mutants that fail to grow at 37°C *in vitro* may also be observed. At 39-40°C, the growth of *Cryptococcus neoformans* starts to slow down (Larone, 1995).

1.8.4.a. Pathogenicity and Clinical Significance

C. neoformans is the causative agent of cryptococcosis. Given the neurotropic nature of the fungus, the most common clinical form of cryptococcosis is meningoencephalitis. The course of the infection is usually subacute or chronic. Cryptococcosis may also involve the skin, lungs, prostate gland, urinary tract, eyes, myocardium, bones, and joints (Durden *et al.*, 1994).

The most commonly encountered predisposing factor for development of cryptococcosis is AIDS (Abadi *et al.*, 1999). Less commonly, organ transplant recipients or cancer patients receiving chemotherapeutics or long-term corticosteroid treatment may develop cryptococcosis (Urbini et al., 2000).

1.8.6. *Microsporum canis*

Microsporum canis grows rapidly and the diameter of the colony reaches 3 to 9 cm following incubation at 25°C for 7 days on Sabouraud dextrose agar. The texture is woolly to cottony

and flat to sparsely grooved. The color is white to yellowish from the front and deep yellow to yellow-orange from the reverse.

1.8.6.a. Pathogenicity and Clinical Significance

M. canis is a zoophilic dermatophyte of world-wide distribution which is a frequent cause of ringworm in humans, especially children. Invades hair, skin and rarely nails. Cats and dogs are the main sources of infection. Invaded hairs show an ectothrix infection and usually fluoresce a bright greenish-yellow under Wood's ultra-violet light.

1.9.   Aim and Objectives

Several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years. Although the antibacterial properties of various species of *Combretum, Terminalia* and *Pteleopsis* (Basséne *et al.*, 1995, Silva *et al.*, 1996, Baba-Moussa *et al.*, 1998) have been investigated in depth, this is not the case for their antifungal properties (Bhatt and Saxena, 1979, Baba-Moussa et al., 1998). Due to the increasing importance of fungal infections the aim is to fill this gap to a degree by focusing on antifungal activities of Combretaceae species.

Objectives

1. Developing minimum inhibitory concentration (MIC) and bioautographic procedures for fungi to be used in the laboratory in order to screen *Combretum* and *Terminalia* species for antifungal activity.
2. Selecting three or four species for further investigation based on antifungal activity and availability.
3. Isolating the antifungal compounds from one or more of the selected species.
4. Determining the chemical structure and *in vitro* biological activity of the antifungal compound.
5. Developing and applying a protocol and determining *in vivo* antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats

1.9.1. Hypothesis

The genera *Combretum* and *Terminalia* contain antifungal compounds that can be isolated by bioassay guided fractionation. The chemical structures can be determined and these compounds will have antifungal activity that may be useful in human or animal medicine.