

Chapter 1

Introduction

Long before mankind discovered the existence of microbes as the major cause of disease, the idea that certain plants had healing potential was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Buwa, and van Staden, 2006). For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich et al., 2004). Archaeological records suggest that the earliest human use of plants for medicinal purposes date back to about 60,000 years ago (Fabricant and Farnsworth, 2001). The famous physician of the middle ages, Philippus Aureolus Theophrastus Bombast von Hohenheim, commonly known as Paracelsus, who lived from 1490 to 1540 probably applied the term “laudanum” (something to be praised) to several different preparations, all of which contained opium as the basic constituent. The laudanum of the early London pharmacopoeias contained opium, wine, and other ingredients. One of first documented evidence of medicinal plant use was that of a French surgeon, Ambrose (1517-1590), who treated gunshot wounds with a mixture of chamomile, melilot flowers, lavender, rosemary, sage, thyme and the extract from red roses boiled in white wine (Macht, 1915).

For centuries people have used plants for healing purposes (Raskin et al., 2002). Plant products, as parts of foods or botanical potions and powders have been used with varying success to cure and prevent diseases throughout history. The strong historic bond between plants and human health began to unwind in 1897, when Friedrich Bayer and his co-workers introduced synthetic acetyl salicylic acid (aspirin) to the world. Aspirin is a safer synthetic analogue of salicylic acid, an active ingredient of willow bark, and was discovered independently by residents of both the New and Old worlds as a remedy for aches and fevers (Pierpoint, 1994). This was the start of the modern pharmaceutical industry that made medicines much more accessible to the general public. This wide scale of use came with the major pitfall of drug resistance. Nonetheless, in the global context, natural products and their derivatives form about 50% of drugs in clinical use with about 25% derived from higher plants (Farnsworth, 1984; O'Neill and Lewis, 1993; Harborne, 1998). Many conventional therapeutic agents are obtained from plant sources. Examples include salicylic acid (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Vickers and Zollman, 1999). With the emergence of extreme drug resistance from the modern pharmaceutical industry new drugs molecules need to be found.

The plant kingdom is a reservoir of varied chemicals and so far only a small fraction of plants have been assayed for medicinal activity. It has been variously estimated that there are about 250,000 species of plants on Earth (Cowan, 1999). More importantly, with herbal remedies continuing to be widely used in management of human disease, this represents major ethnobotanical leads in the discovery of new medicines. In South Africa and in many African homes, medicinal plants are sold or prescribed by traditional medical practitioners (Fyhrquist et al., 2002). Southern Africa is exceptionally rich in plant diversity with some 24,000 species of flowering plants almost one tenth of the worlds higher plants of which 80% are endemic. This includes 10 plant families and 29% of the total genera of the world (Goldblatt, 1978). It is estimated that 27 million South Africans depend on traditional medicines from as many as 1020 plant species (Dauskardt, 1990; Mander, 1998). The trade equates to approximately 20,000 tonnes of plant material sold annually as drugs in South Africa (Mander, 1998).

The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s (Martindale 1910; Hoffman and Evans 1911). Plant volatile oils are generally isolated from non-woody plant material by distillation methods, usually steam or hydrodistillation, and are variable mixtures of principally terpenoids, and a variety of low molecular weight aliphatic hydrocarbons, coumarins and homologues of phenylpropanoids. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants (Dorman and Deans, 2000). Although plant secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has shown a pivotal role of many of these chemicals in ecophysiology of plants (Briskin, 2000). Accordingly secondary products have both a defensive role against herbivory, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999).

Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals, but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man (Wallace, 2004).

The diversity of plants growing worldwide, along with their known ethnopharmacological uses offer an enormous possibility of finding novel chemical agents with efficacious antifungal properties. These plants include amongst others *Terminalia australis* which is used to treat aspergillosis and candidosis (Carparo et al., 2003), and *Acacia caven* for treating vaginal mycoses (Hilgert, 2001). The aqueous and alcoholic

extracts of *Sebastiania commersoniana* have shown antibacterial and antifungal activities (Penna et al., 2001). Tannins isolated from *Terminalia trifolora* demonstrated a powerful antifungal action in clinical studies (Latte Kolodziej, 2000). In a similar study, Masoko et al (2005), showed the efficacy of six *Terminalia* species found in South Africa against *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*. Quite a number of plants in Tanzania are used in traditional medicine and have shown good results when tested against *Candida* infections of human (Runkoro et al., 2006). The volatile oils of black pepper *Piper nigrum* L. (Piperaceae), clove [*Syzygium aromaticum* (L.) Merr. & Perry (Myrtaceae)], geranium [*Pelargonium graveolens* L'Herit (Geraniaceae)], nutmeg [*Myristica fragrans* Houtt. (Myristicaceae)], oregano [*Origanum vulgare* ssp. *hirtum* (Link) Letsw. (Lamiaceae)] and thyme [*Thymus vulgaris* L. (Lamiaceae)] were assessed for antibacterial activity against 25 different genera of bacteria. These included animal and plant pathogens, food poisoning and spoilage bacteria. The volatile oils exhibited varying levels of inhibitory effects against all the organisms (Dorman and Deans, 2000).

1.1. Hypothesis

Plants contain antimicrobial agents which are active against animal and human pathogenic bacteria and fungi including *Aspergillus fumigatus*. They also contain antioxidant and anti-inflammatory compounds that could be used to ameliorate or abolish the severity of diseases caused by antimicrobial agents. These active compounds can be isolated and characterized to yield potential drugs with novel structures or activities for use in treating and protecting humans and animals against pathogenic bacteria and fungi, and also against certain inflammatory diseases. Moreover, the plant extracts or compounds can also be used to enhance animal productivity.

1.2. Justification of the study

Results generated from this study will add to the ever increasing database knowledge on plant medicines and contribute to the safe and efficacious use of plant drugs in both rural and urban set ups. Natural plants that are not effective will not form part of our disease management system in future. Because aspergillosis is an important poultry disease searching for plant extracts with good activity against *Aspergillus fumigatus* may lead to a commercially useful therapeutic agent.

Organised ethnomedical research and development through cultivation of medicinal herbs and the creation of local trade and industry can bring more jobs and income to rural inhabitants (McCorkle, 1995). The use of alternative drugs has been recommended as a measure to avoid the development of resistant strains of

microorganisms and to increase the possibility of reducing the cost of controlling the disease (Cowan, 1999).

The high incidence of fatal fungal and bacterial infections occurring in association with increased use of immunosuppressive drugs stimulated the desire to explore an additional chemotherapeutic weapon in the fight against microbial diseases (Frank et al., 2003). A practical solution to this problem is to develop acceptably effective drugs from reasonably inexpensive and locally available raw materials. An obvious way of achieving this goal is through the study of traditional medical herbs, selecting from those that show promising results for development into effective and safer drugs (Ibrahim et al., 1983). Another approach is to use data for plant species randomly screened for biological activity such as the Phytomedicine Programme database of activities against leaf extracts of close to 500 tree species. (Phytomedicine Programme website www.up.ac.za/phyto).

1.3. Summary and problem statements

- Aspergillosis in poultry and domestic animals is of economic importance, while it is a major health problem in humans. Diseases caused by fungal organisms particularly in immunocompromised patients remain a major cause of concern in Africa and the rest of the world.
- Resistance developed by microbial organisms against commonly and licensed commercial antimicrobials render many infectious diseases difficult to treat and control (Van der Waaij, 1987).
- Although there appear to be an array of drugs for the treatment of systemic and superficial mycoses, none of them is ideal in terms of efficacy, safety or antifungal spectrum (Di Domenico 1998; Ablordeppey et al., 1999).

1.4. Aim and objectives

The aim of this study is to find a plant extract or isolated compound that could be used to combat aspergillosis in poultry. The following objectives can be identified to attain this aim:

- Evaluate the antibacterial and antifungal activity selected South African plant species against a range of pathogenic bacterial and fungal species, in order to select the species with the best antimicrobial activity for further investigation.
- Isolate and characterize the compounds active against *Aspergillus fumigatus* from extracts of selected plant species.
- Determine the *in vitro* antimicrobial, antioxidant and anti-inflammatory activities and cytotoxicity of the extracts and isolated compound(s) of selected plant species.
- Evaluate the efficacy of the isolated compound(s) or crude extracts *in vivo* in a poultry model.

Chapter 2

Literature Review

2.1. Importance of poultry

Poultry is by far the largest livestock group and is estimated to consist of about 14 000 million birds, consisting mainly chickens, ducks and turkeys (FAO 1999). In total, poultry products (egg and meat) constitute 30% of all animal protein consumed worldwide. Within the last 10 years, this proportion has increased from 20% to 30% of all animal protein and is predicted to increase to 40% before the year 2015 (FAO 1999). Poultry provides a vital supply of food for the world's population. All over the globe, poultry meat and eggs are preferred to other kinds of animal food products for a variety of reasons. Mack et al., (2005) estimated that 25 percent of the world's meat supply is derived from poultry, i.e. chicken, turkey, duck, geese, domesticated quail, etc. and the proportion is increasing steadily. The trend has been more noticeable in developing countries in recent years. World poultry meat output increased nearly eight-fold in 1961-2001, while the output in middle-income countries even rose more than twelve-fold. The biggest global poultry meat producers are the United States, the EU, China, Brazil, Mexico, Canada and Japan. Among middle-income countries, China was the major producer in 2001, followed by Brazil, Mexico, Argentina, Iran, Russia, Egypt and Poland. In 1961, middle-income countries produced 34 percent of world poultry meat, high-income countries 61 percent, and low-income countries the remaining 5 percent. By the mid-1990s, middle-income country production had reached a level of 47 percent of the output of high-income countries. By 2001, middle-income countries accounted for the major share of world poultry production (52 percent) compared with 42 percent in high-income countries and less than 6 percent in low-income countries (Regmi, 2001).

In 1995, African livestock population poultry was the most numerous species of farm animal (Anonymous, 1996). More than 80% of poultry are kept in rural areas (Sonaiya and Olori, 1997). Throughout the African continent all ethnic groups are involved in poultry production (Guéye, 1998). In addition to providing farmers with eggs and meat for their home consumption, poultry or their products are kept for sale (or barter) thereby generating a source of income for the family. Some communities, indigenous fowl have a symbolic importance within many social activities (e.g. special banquets for distinguished guests) and/or religious ceremonies. For example cocks are offered to the deities (Bell and Abdou, 1995). In the Western Middle-Belt region of Nigeria, Atteh (1989) reported the reasons for keeping village fowls as being 11% for income alone, 28% for consumption alone, 45% for income and consumption, 3% for ceremonies, 11% for income and ceremonies, 3% for consumption and ceremonies and 1% for recreational purposes. In the Keita

region of Niger home consumption accounted for 47%, sales for 38% and gifts for 16% (Bell and Abdou, 1995). In the traditional society of the Mamprusi tribe in Northern Ghana, the uses of poultry were 35% for sacrifice, 28% for sale, 15% for consumption, 13% for gifts and 10% for breeding stock, while 71% of the eggs were set aside for hatching, 18% for sale, 5% for consumption and 5% for gifts. (Veluw, 1987) Additionally, indigenous fowl play an important role in traditional ritual prayers to appease the gods (Lul, 1990; Ngou Ngoupayou, 1995).

2.2. Poultry production

World production of poultry meat (all domestic birds) and eggs was 28.0 and 26.5 million metric tonnes respectively in 1979. On a continent basis, Africa, Oceania and South America contribute proportionally considerably less to world production than the other continents (Biggs, 1982). Africa, Asia and South America produce considerably less per person than Europe, North and Central America and Oceania. With the exception of Europe the major part of both poultry meat and egg production for each continent is provided by one to three countries. The difference in production *per capita* reflects each continent's development stage. Production *per capita* 6.5 times greater for poultry meat in developed countries compared with developing countries and 5.4 times greater for egg production (FAO, 1999).

In South Africa, the poultry industry contributes greatly to the Agricultural sector. It is estimated that the industry contributes approximately 16% of the total gross value of agriculture in South Africa. The broiler industry in South Africa currently produces on average 13.8 million broilers per week, growing steadily from 1990 when only 7.6 million broilers per week were produced (Global Agricultural Information Network report SF7042, 2007). In 2007, about 950 million and 23 million broilers and layer chickens, respectively were produced in South Africa (SAPA, 2008). In addition, SAPA (2008) reported the production of 360,000 eggs per week in the year 2007. Latest estimates suggest that up to 7,000 people are employed by the poultry industry in South Africa, making the industry and important rural employer (SAPA, 2008).

2.3. Constrains to poultry production

Some problems associated with poultry production include disease control, protection against various predators, better feeding, genetic improvements, marketing, training and management, access to production inputs, infrastructure and capital, farmer organization, and, foremost, conducive institutions and governmental policies (Mack et al., 2005). The introduction of modern intensive production methods, new breeds and improved preventive disease control and bio-security measures has rapidly change poultry production in the past few decades. The progress in industrial poultry production methods has however had

little effect on subsistence poultry production methods in rural and peri-urban areas, where inputs into disease control remain minimal especially in developing countries (Hoffmann, 1998). There is therefore the need to find more alternative approaches towards disease control and prevention. Important infectious poultry diseases are caused by bacteria, viruses, parasites and fungi. Advances and discoveries in the control and treatment of other infectious poultry diseases have been outstanding in recent years. However, less progress has been made in the control of fungal infections in poultry (Chute and Richard, 1997). Fungal diseases especially aspergillosis caused by *Aspergillus fumigatus* has an impact on health of birds and hence lowers their ability to produce enough meat and egg for the populace. Because of negative associations with the use of antimicrobial feed additives in animal production, there is an incentive to develop plant product based products that can replace antibiotic feed additives.

2.4. Aspergillosis in humans and animals

Denning, et al., 1991 and Latgé, 2001 are excellent references for this topic and some of the material in this section were found there.

Aspergillus fumigatus has become the most prevalent airborne fungal pathogen, causing severe and usually fatal infections in immunocompromised hosts in developed countries (Andriole, 1993). In addition the prevalence of the disease has increased fourfold in the last 12 years. In 1992, invasive *Aspergillus* (IA) was responsible for approximately 30% of fungal infections in patients dying of cancer, and it is estimated that IA occurs in 10 to 25% of all leukaemia patients, in whom the mortality rate is 80 to 90%, even when treated (Bodey et al., 1992). IA is now a major cause of death at leukaemia treatment centres and bone marrow transplantation (BMT) and solid-organ transplantation units (Patel and Paya, 1997).

While in the immunocompetent host, *A. fumigatus* is seldom pathogenic, the downregulation of the immune system induced by immunosuppressive therapies and congenital defects triggers the development of aspergillosis in most human infections (Latgé, 2001). In recent years, aspergillosis has emerged as a significant disease in humans that are immunocompromised by acquired immunodeficiency syndrome, neoplasia, or chemotherapy (Denning, et al., 1991). In humans, aspergillosis resulting from exposure to *A. fumigatus* can be primarily a manifestation of hypersensitivity responses (Disch et al., 1995), damage to host tissues by invasive colonization (Denning, et al., 1991), or a complexity of allergy and infection (Awadhiya et al., 1981).

For most patients, the main portal of entry and site of infection for *A. fumigatus* is the respiratory tract, although other sites of infections have been described in the normal or immunocompromised host, such as

the skin, peritoneum, kidneys, bones, eyes, and gastrointestinal tract, nonrespiratory infections are infrequent (Prescott et al., 1992). Pulmonary diseases caused by *A. fumigatus* can be classified according to the site of the disease within the respiratory tract and the extent of mycelial colonization or invasion, both of which are influenced by the immunological status of the host (Dixon, and Walsh, 1992). Allergic diseases, including asthma, allergic sinusitis, and alveolitis, are also common with infection due to *A. fumigatus*. They occur following repeated exposure to conidia or antigens of *Aspergillus* in the absence of mycelial colonization, and in most cases, removal of the patient from the environmental source results in clinical improvement. In contrast, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, and IA, syndromes involving mycelial growth of *A. fumigatus* in the body, usually require therapeutic intervention (Latge, 1999).

Sinusitis and orbital cellulitis caused by *Aspergillus* species is known to exhibit a granulomatous response in cats (Wilkinson, et al., 1982). This reported case was similar to that observed in dogs (Lane et al., 1974), as a destructive lesion with lysis of the turbinate tissues in the absence of significant soft tissue proliferation. Immuno-incompetence, tissue damage by other microbial infections, trauma or neoplasia have been cited as predisposing factors in the initiation of aspergillosis in the dog (Lane, 1982), dolichocephalic breeds being predominantly affected.

It was also proposed that infection with feline leukaemia virus in cat, combined with prolonged antibiotic treatment regimes allowed aspergillosis to become established in the nasal chamber. Another animal that is affected by aspergillosis is the horse, specifically in the head region. Adult immunocompetent horses are usually affected as a result of epistaxis or neurologic deficits. *A. fumigatus* is the organism most commonly associated with this disease. Guttural pouch mycosis is predisposed by factors that include soft tissue trauma and environmental conditions that encourage conidial germination such as poor ventilation, high humidity, and warm temperatures. The mode of entry is presumed to be oropharyngeal during expiration and deglutination. The guttural pouch is a large air filled extension of the Eustachian tube that has openings into both the middle ear and oropharynx. This anatomic structure is unique to the order Perissodactyl. There are numerous arteries, veins and nerves that pass through the guttural pouch. The guttural pouch helps to cool internal carotid blood before perfusing the brain (Tell, 2005). *A. fumigatus* has been recovered from the faeces of horses with a history of persistent diarrhoea and given aetiological significance (Lundvall and Romberg, 1960).

Aspergillus fumigatus is associated with sporadic bovine mycotic abortion in the northern hemisphere especially in cows that are in their second or third trimester of pregnancy. The incidence of this disease is

highest during the winter months when gravid cows are confined to sheds and fed hay or silage that are heavily contaminated with *Aspergillus* spores. (Tell, 2005).

2.5. Aspergillosis in poultry

The poultry industry faces heavy economic losses (mortality and morbidity) due to *Aspergillus fumigatus* infection and its mycotoxins. The financial losses due to aspergillosis are enormous with about US\$11 million reported as an average annual loss in the United States of America (Kunkle, 2003). *Aspergillus fumigatus* has been recognized as pathogenic to birds for more than a century (Wright et al., 1962) and in addition to having a worldwide distribution it has been reported to be pathogenic in almost all farm birds and in numerous other wild species of birds (Akan et al., 2002; Chang Reissig et al., 2002).

Aspergillosis is a fungal disease mainly located in the respiratory system and characterized with lesions in the internal organs, eyes and, in certain cases, the brain in poultry. *Aspergillus fumigatus* has been determined as the most pathogenic among the causative agents of aspergillosis with widespread resistant spores present in the natural environment (Richard, 1997). Poultry aspergillosis, mainly observed in the young of turkeys and chicken, is also reported in other poultry species, including ducks, geese, quails, ostriches, parrots, canaries, pigeons, flamingos and penguins (Richard, 1997). In the acute form of the disease, which develops within 24-48 h in young animals, the rate of mortality may vary from 70% to 90%, whereas in the sub-acute form of the disease which develops within 8-10 days in animals up to the age of 2 weeks various clinical phases are reported (Roy et al 1991). Pathological observations in all poultry species are essentially the same (Richard, 1997). Macroscopic findings differ with regard to the location of the disease. Lungs are the internal organs reported to be most affected. Lesions may range from miliary to larger granulomatous foci (Richard, 1997) which are grey-yellow-white in colour, dry in consistency and protrusive with regard to the surface of the internal organ in which they are located (Richard, 1997). Thickening and dullness of the walls of the air sacs have been reported to develop because of the infection (Richard, 1997). Microscopic examination has revealed the presence of granulomatous foci and caseous necrosis with a surrounding region of proliferation including giant cells, macrophages, hetereophils and lymphocytes and an outer capsule of connective tissue. Fungal hyphae with or without septa and fungal spores located within regions of necrosis may easily be observed microscopically by using special staining methods (Richard, 1997).

As a disease, aspergillosis affects birds in captive or free-ranging environments, young and mature, immunocompetent or immunosuppressed. Predisposing factors include species predilection, environmental

conditions (limited air exchange, exposure to allergens resulting in mucosal irritation, and extreme temperature and humidity), immunosuppression secondary to intensive production, physical exertion (migration), and administration of exogenous corticosteroids. Aspergillosis leads to consequential economic losses related to low productivity, mortality and carcass condemnations at slaughter inspection (Morris & Fletcher, 1988; Richard, 1997). Two forms of the disease are regularly reported in turkey poults. The first form is an acute aspergillosis leading to severe outbreaks in very young birds with clinical signs of dyspnoea, gasping and inappetence. The chronic form of aspergillosis most commonly occurs in 13-week-old to 18-week-old turkeys, late in the growing cycle and associated with respiratory distress as clinical signs.

Aspergillus fumigatus is also considered an opportunistic pathogen that causes disease in immunocompromised birds or in birds exposed to overwhelming numbers of fungal spores. While in most cases, the primary site of development is the respiratory tract (air sacs and lungs) blood dissemination frequently occurs, leading to macroscopic lesions in a wide range of organs or tissues. In spontaneous cases, lesions range from miliary to larger granulomatous foci (Singh et al., 1994), which are white in colour and protrusive to the surface of the internal organ. Thickening of the walls of the air sacs is frequently reported (Perelman and Kuttin, 1992; Richard, 1997). Lesions in avian species are commonly confined to the lungs (Figure 2.1.) and air sacs, although infections of oral mucosa, trachea, brain, eye, skin, bone, liver, kidney (Richard, 1991), and nasal passages (Fitzgerald and Moisan 1995) have also been described. Typical lesions are characterized by granulomatous inflammation with necrosis, haemorrhage, and intralesional fungal elements that are locally invasive.

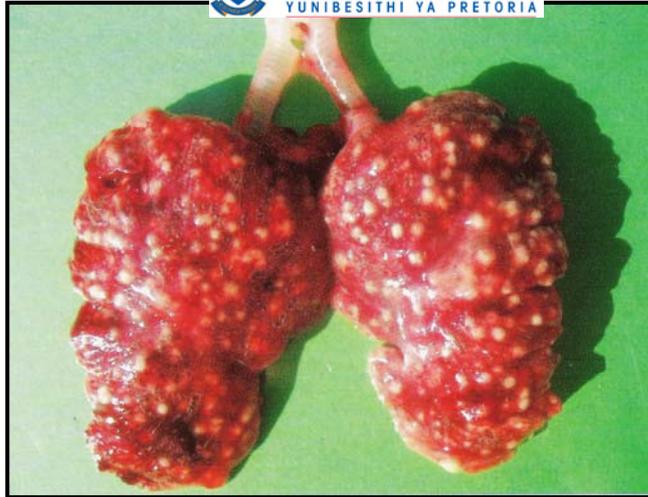


Figure 2.1. Nodular form of aspergillosis in the lungs. Note the presence of yellowish dense nodules on the lungs.

2.6. Mycotoxicoses

Mycotoxicoses is a toxic syndrome resulting from the intake of mould contaminated feed, which has toxic metabolites of mould called mycotoxins. *Aspergillus fumigatus* is one species that produces potent toxic metabolites like aflatoxin, gliotoxin and ochratoxin. Mycotoxicoses have interactions with bacterial infections i.e. the increased intake of aflatoxin in feed will increase susceptibility of chicken to bacteria due to immune suppression in the host animal by the toxin infection. Likewise there will be synergistic effects when more than one type of toxin is present in the feed. Mycotoxicoses are of public health importance since they and their metabolites are present in poultry fed with contaminated feed and are harmful to human beings (Raja and Lakshmana, 1991). Consumption of aflatoxin by poultry can interfere with resistance to various infections (Chute and Richard, 1997).

Gliotoxin is one of the several toxins produced by various isolates involved in an outbreak of aspergillosis of turkeys (Richard, 1990). Gliotoxin is immunosuppressive, cytotoxic and inhibits transformation of turkey peripheral blood lymphocytes (Richard et al., 1994). Ochratoxins are metabolites of both *Aspergillus* and *Fusarium* species which are chemically described as 3,4-dihydromethylisocoumarin derivatives (Cole and Cox, 1981). These compounds are known for their nephrotoxic effects (renal damage) in poultry (Lanza et al., 1980; Manning and Wyatt, 1984).

2.7. Treatment of aspergillosis in birds

The two most important antifungal drugs currently available for the treatment of aspergillosis in birds are amphotericin B and itraconazole. Amphotericin B is the only fungicidal drug currently available and remains the gold standard with which other drugs are compared (Lyman and Walsh, 1992). Amphotericin B has

been used to treat birds both topically and systemically (Orosz, 2000). Itraconazole is a lipophilic triazole that is used for the treatment of aspergillosis. Few, if any, drugs have proven to be effective against *A. fumigatus*. Drugs such as nystatin and amphotericin B have been used in poultry without reproducible results (Chute, 1984).

Although there appear to be an array of drugs for the treatment of systemic and superficial mycoses, none of them is ideal in terms of efficacy, safety or antifungal spectrum (Di Domenico, 1998; Ablordeppey et al., 1999). Many of the drugs have undesirable effects or are very toxic (amphotericin B), produce recurrence, have drug–drug interactions (azoles) or lead to the development of resistance (fluconazole, 5-flucytosine) (White et al., 1998). Numerous useful drugs from higher plants have been discovered by random selection followed by chemical screening or through ethnobotanical (Fabricant and Farnsworth, 2001). The diversity of plants growing in South Africa, offer an enormous possibility of finding novel structures or extracts with antifungal properties.

2.8. Antifungal drugs in common use and their mode of actions

Reviews by Ghannoum and Rice, 1999, Odds et al., 2003, and Parks and Casey, 1996 on the mechanism of actions of antifungal drugs are excellent and some of the materials presented under this heading are obtained from those articles.

2.8.1. Drugs interfering with microtubular function

The earliest inhibitory agent specific to fungal species was griseofulvin (Odds et al., 2003). Griseofulvin is an antifungal agent isolated from the fungus *Penicillium griseofulvum*. The drug is believed to interfere with microtubule assembly and mitosis in the fungal cell. The selective toxicity of griseofulvin for fungi is only moderate (liver toxicity is recognized as an occasional hazard) and its spectrum of action is restricted mainly to the dermatophyte fungi – causes of ringworm and athlete's foot. It is taken up selectively by newly formed skin and concentrated in the keratin (Rang et al., 2003).

2.8.2. Agents affecting fungal sterols

The three major groups of antifungal agents in clinical use, azoles, polyenes, and allylamine/thiocarbamates, all owe their antifungal activities to inhibition of synthesis of or direct interaction with ergosterol. Ergosterol is the predominant component of the fungal cell membrane (Figure 2.2.) (Parks and Casey, 1996).

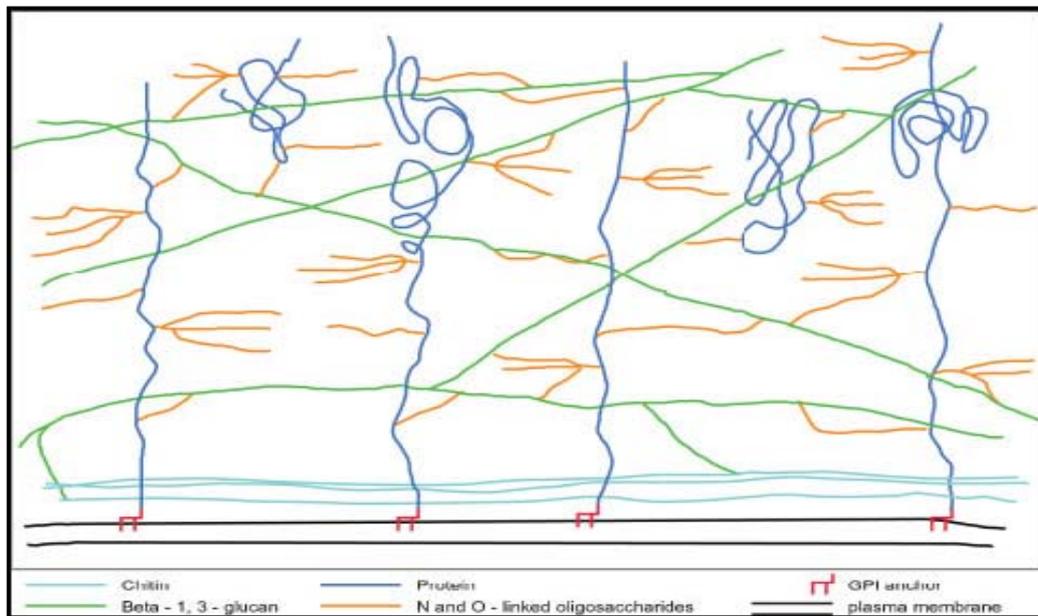


Figure 2.2. Representation of the fungal cell wall. The major components of the fungal cell wall are chitin, glucans and glycoproteins. Most of the chitin is considered to be located near to the plasma membrane. The beta-1, 3-glucan extends throughout the cell wall. The glycoproteins are extensively modified with N- and O-linked oligosaccharides. Many of the glycoproteins have GPI anchors, which tether them to the plasma membrane while other glycoproteins are secreted into the cell wall space. The proteins, glucans and chitin components are integrated into the wall by cross linking the chitin, glucans, protein-associated oligosaccharides and GPI anchors together. (Adapted from Bowman and Free, 2006).

2.8.2.1. Azoles

The first reports of the antifungal properties of azoles were published in the late 1960s (Holt, 1980; Sheehan et al., 1999) These original compounds, such as miconazole and econazole, and those that followed, such as ketoconazole, fluconazole, and itraconazole, proved to be important drugs for combating human fungal infections. The clinical efficacy and safety of fluconazole in particular has resulted in widespread use.

Ergosterol serves as a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells (Nozawa and Morita, 1986). Integrity of the cell membrane requires that inserted sterols lack C-4 methyl groups. The primary target of azoles is the heme protein, which co-catalyzes cytochrome P-450-dependent 14 α -demethylation of lanosterol (Hitchcock et al., 1990). Inhibition of 14 α - demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14 α -

methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of a plasma membrane with altered structure and function. The more recent triazole derivatives, such as fluconazole, itraconazole, and voriconazole (a triazole in development), owe their antifungal activity at least in part to inhibition of cytochrome P-450-dependent 14 α -sterol demethylase (Sanati et al 1997).

Mammalian cholesterol synthesis is also blocked by azoles at the stage of 14 α -demethylation; however, the dose required to effect the same degree of inhibition is much higher than that required for fungi (Hitchcock, 1990). The main hazard of ketaconazole is liver toxicity, which is rare but can prove fatal. Other azoles derivatives produce unwanted effects that are mild and these include gastrointestinal disturbances, nausea, allergic skin reactions and dizziness (Rang et al., 2003).

2.8.2.2. Polyenes

Amphotericin B was licensed in 1959, and is licensed for the treatment of “progressive and potentially life threatening fungal infections: aspergillosis, cryptococcosis (torulosis), North American blastomycosis, systemic candidiasis, histoplasmosis, zygomycosis including mucormycosis due to susceptible species of the genera *Absidia*, *Mucor* and *Rhizopus*, and infections due to related susceptible species of *Conidiobolus* and *Basidiobolus*, and sporotrichosis (Dismukes, 2000.). Amphotericin B has for many years been the only antifungal polyene that can be administered systemically to treat a visceral infection (Odds et al., 2003). The polyene antifungal agents such as amphotericin B represented the standard of therapy for systemic fungal infections (Sugar, 1986). There is an association between polyene susceptibility and the presence of sterols in the plasma membrane of the cells. All organisms susceptible to polyenes, contain sterols in their outer membrane, while resistant organisms do not (Norman et al., 1972). It was suggested that this effect is due to a physicochemical interaction between added sterols and the polyenes, which prevents the drug from binding with the cellular sterols. It has been proposed that the interaction of the polyene antifungal with membrane sterol results in the production of aqueous pores leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism (Kerridge, 1985). The fatty acyl composition of the phospholipids has also been implicated in polyene susceptibility of yeast (Rao et al., 1985). In addition, killing of *C. albicans* has been attributed to oxidative damage caused by polyenes (Titsworth and Grunberg, 1973). Amphotericin B incorporated into liposomes, may participate in a selective transfer mechanism, which involves its transfer from the “donor” liposome to the ergosterol-containing “target” in the fungal cell membrane aided by the fungal and/or host phospholipases (Juliano et al., 1987). The precise way in which this fungicidal effect occurs still remains unclear. The ergosterol molecule of fungi has a cylindrical three-dimensional structure, unlike cholesterol, the major sterol in mammalian

membranes, which has a sigmoid shape. This conformational difference is probably sufficient to explain the greater binding affinity of amphotericin B for ergosterol over cholesterol (Figure 2.3.). This difference and the higher ratio of ergosterol:phospholipid in fungi is the basis for the antifungal selectivity of amphotericin B (Odds et al., 2003). The commonest and most serious unwanted effect of Amphotericin B is renal toxicity. Other effects that are not desirable produce by the drug include impaired hepatic function, thrombocytopenia, and anaphylactic reactions (Rang et al., 2003)

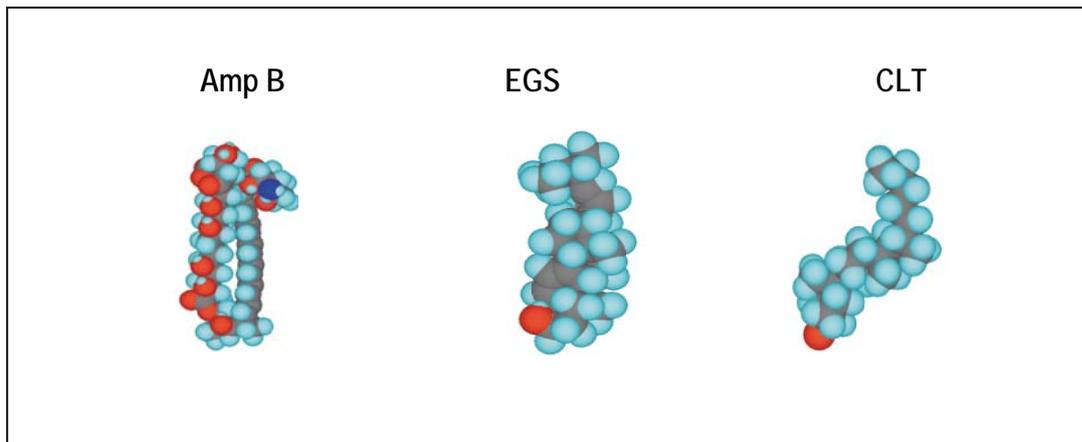


Figure 2.3. The polyene antifungal agent, amphotericin B (Amp B), ergosterol (EGS) and cholesterol (CLT) visualised in three dimensions. Ergosterol, the sterol found in fungal cell membranes, retains a cylindrical shape in all rotations and binds better to the hydrophobic (right-hand) side of the amphotericin B molecule than does cholesterol, with its sigmoid structure. Cholesterol is the membrane sterol found in mammalian cells; the differential binding affinity of amphotericin B for the two sterols is the basis of its selective antifungal action.

2.8.2.3. Allylamines

Terbinafine and naftifine are example of this class of antifungals. They have been developed as a new class of ergosterol biosynthetic inhibitors that are functionally as well as chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents (Ryder et al., 1984; Ryder and Favre, 1997).

Terbinafine is highly effective against dermatophytes *in vivo* and *in vitro*. Ryder and Favre, (1997), reported further that terbinafine has good activity against at least some azole-resistant *C. albicans* strains. It also had activity high activity against *Cryptococcus neoformans*.

Allylamines act by inhibiting early steps of ergosterol biosynthesis. This inhibition coincides with accumulation of the sterol precursor squalene and the absence of any other sterol intermediate (Kerridge, 1980), suggesting that allylamine inhibition of sterol synthesis occurs at the point of squalene epoxidation, a reaction catalyzed by squalene epoxidase. Studies with isolated squalene epoxidase indicate that it is the target for allylamine activity (Ryder and Favre, 1997). Fungal cell death is related primarily to the accumulation of squalene rather than to ergosterol deficiency (Ryder and Favre, 1997). High levels of

squalene may increase membrane permeability (Lanyi et al., 1974), leading to disruption of cellular organization.

2.8.3. Compounds active against fungal cell walls

The fungal cell wall contains compounds, such as mannan, chitin, and α - and β -glucans, which are unique to the fungal kingdom. Since these components are not found elsewhere in nature, they have been identified as possible targets that provide selective toxicity advantages (Hector, 1993). The cell wall of *Candida albicans* is a multilayered structure composed of chitin, β -glucan and mannoprotein, with the last two constituents making up to 80% of the wall mass (Poulain et al., 1978; Cassone et al., 1979). The outer layers are composed of mannan, mannoprotein, and β -(1,6)-glucan, while the inner layers are predominantly β -(1,3)-glucan and chitin with some mannoprotein (Surarit et al., 1988.)

2.8.3.1. Inhibitors of glucan synthesis

The echinocandins are specific inhibitors of fungal β -glucan synthase. They are natural products discovered in the 1970s (Tkacz, 1992). fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity Echinocandins, which are lipopeptides, have fungicidal activity both *in vitro* and *in vivo* against *Candida* and *Aspergillus* species (Walsh, et al., 1991). β -Glucan inhibitors act as specific noncompetitive inhibitors of β -(1,3)-glucan synthetase, a large (210-kDa) integral membrane heterodimeric protein (Hector, 1993). Treatment of fungi with these compounds inhibits the synthesis of the structural glucan component without affecting nucleic acid or mannan synthesis (Mizoguchi et al., 1977). Inhibitors of glucan synthesis also have secondary effects on other components of intact cells including a reduction in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall (Pfaller et al., 1989). Inhibition of β -(1,3)-glucan synthetase results in cytological and ultrastructural changes in fungi characterized by growth as pseudohyphae, thickened cell wall, and buds failing to separate from mother cells. Cells also become osmotically sensitive (Traxler et al., 1977), with lysis being restricted largely to the growing tips of budding cells (Bozzola et al., 1984).

2.8.3.2. Chitin synthesis inhibitors

Chitin is a linear homopolymer of β -(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues. It is synthesized on the cytoplasmic surface of the plasma membrane, extruded perpendicularly to the cell surface as microfibrils, and crystallized outside the cell through extensive hydrogen bonding as α -chitin (the poly GlcNAc chains run antiparallel). The polymerization of GlcNAc is catalyzed by chitin synthases, membrane-

bound enzymes found in cell homogenates largely as zymogens (Bulawa, 1993). Chitin synthesis is inhibited competitively by polyoxins and nikkomycins. They are nucleoside-peptide antibiotics and were isolated from two different *Streptomyces* species: *S. tendae* (nikkomycin) and *S. cacaoi* var. *asoensis* (polyoxin). They act as analogs of the substrate UDP-GlcNAc, inhibiting chitin synthase. The effect on the fungus is inhibition of septation and osmotic lysis (Becker et al., 1983). The nucleoside-peptide inhibitors are taken up by a dipeptide permease, and thus, peptides in body fluids antagonize their transport. *C. albicans* and other medically important fungi are resistant to polyoxins owing to their poor transport across the cell membrane (Yadan et al., 1984).

2.8.3.3. Compounds inhibiting nucleic acids

2.8.3.3.1. 5-Fluorocytosine

5-Fluorocytosine is a fluorinated pyrimidine with inhibitory activity against many types of yeast, including *Candida* and *Cryptococcus neoformans*. The majority of the candidal isolates studied were susceptible to 5-Fluorocytosine. 5-Fluorocytosine enters fungal cells aided by a permease enzyme. Once inside, it is converted to 5-fluorouracil (5FU) by the enzyme cytosine deaminase. Subsequently, 5FU is converted by UMP pyrophosphorylase into 5-fluorouridylic acid (FUMP), which is phosphorylated further and incorporated into RNA, resulting in disruption of protein synthesis (Polak and Scholer, 1975). 5FU also is converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division (Diasio et al., 1978). Thus, 5FC acts by interfering with pyrimidine metabolism, as well as RNA, DNA, and protein synthesis in the fungal cell. The summary of the mechanism of action of commonly used antifungal agents is presented on figure 2.4.

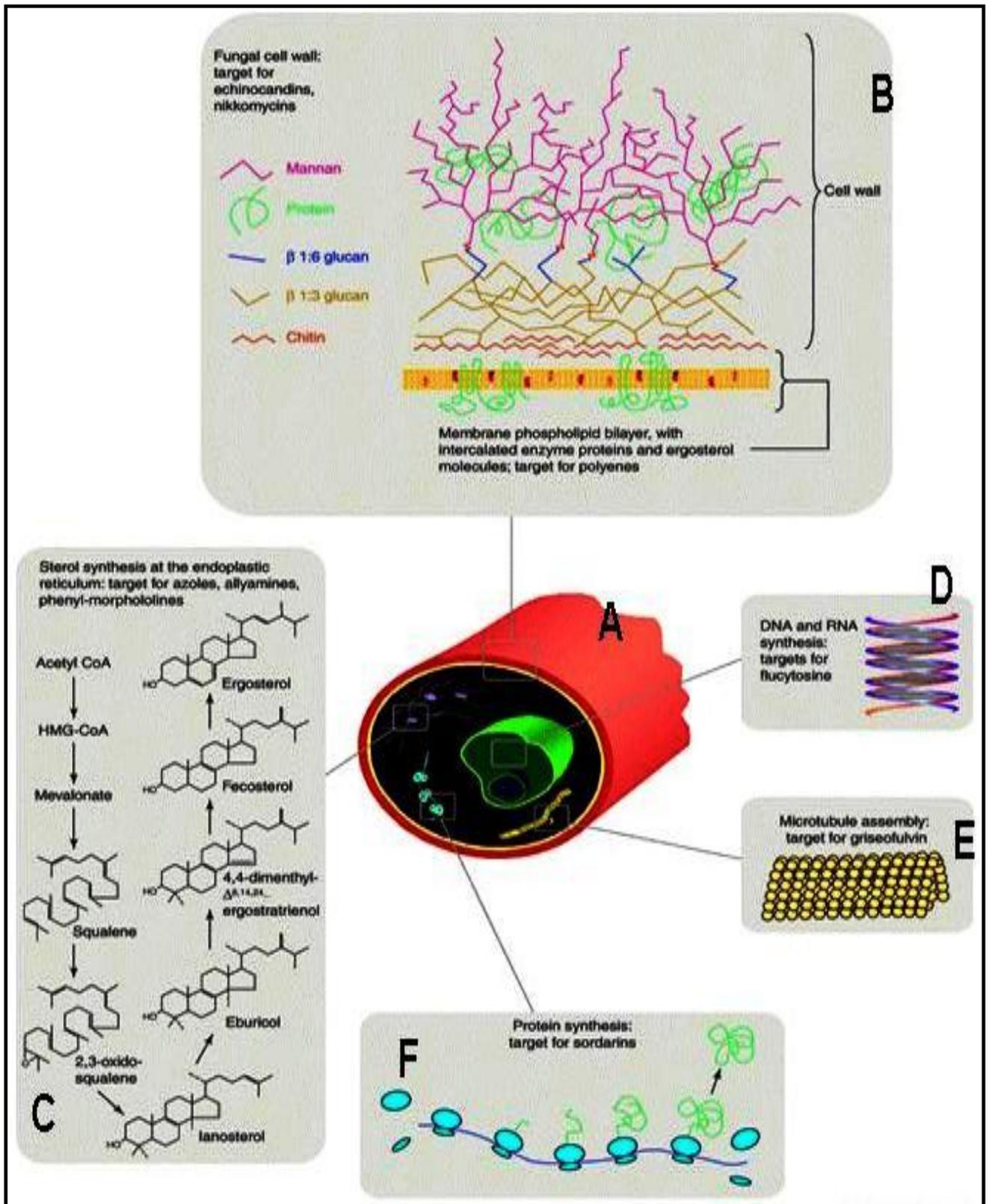


Figure 2.4. Diagrammatic representations showing the mechanism of action of commonly used antifungal agents. Adapted from Odds et al (2003). A= cross section of the fungal cell; B= membrane phospholipids bilayer: a target for polyenes antifungals, while the cell wall serve as target for echinocandins and nikkomycins; C= biochemical pathway for sterol synthesis at the endoplasmic reticulum: a target for azoles and allyamines; D= licytosine inhibits the synthesis of nuclear membrane (DNA and RNA); E= microtubular assembly in the fungal cell is inhibited by griseofulvin; F= The sodarins target and inhibit protein synthesis in the fungal cell.

2.9. Plants as antimicrobial agents

The antimicrobial qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s (Martindale 1910; Hoffman and Evans 1911). Plant volatile oils are generally isolated from non-woody plant material by distillation methods, usually steam or hydro distillation, and are variable mixtures of principally terpenoids, and a variety of low molecular weight aliphatic hydrocarbons, coumarins and homologues of phenylpropanoids. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants (Dorman and Deans, 2000). Although plant secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has revealed a pivotal role of many of these chemicals in ecophysiology of plants (Briskin, 2000). Accordingly secondary products may have both a defensive role against herbivory, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999).

Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals, but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man (Wallace, 2004). Plant secondary products can have a variety of functions in plants. For example, certain plant secondary products are produced to defend the plant against microbial attacks by destroying microbial cells. Those compounds if not toxic to mammalian cells could prove useful as antimicrobial medicines in animals and humans (Briskin, 2000).

The diversity of plants growing in South Africa, along with their known ethnopharmacological uses offer a good possibility of finding novel chemical agents with efficacious antifungal properties. Tannins isolated from *Terminalia triflora* demonstrated a powerful antifungal action in clinical studies (Latte and Kolodziej, 2000). In a similar study, six *Terminalia* species found in South Africa had activity against *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporium canis* and *Sporothrix schenckii* (Masoko et al., 2005). Quite a number of plants in Tanzania are used in traditional medicine and had good results when tested against *Candida* infections of human (Runkoro et al., 2006).

2.9.1. Secondary plant compounds with antimicrobial properties

2.9.1.1. Phenols and phenolic acids

Simple substituted phenolic ring compounds like Cinnamic and caffeic acids are bioactive phytochemicals and represent wide range of phenylpropane-derived compounds (Figure 2.5) which are in the highest oxidation state. Herbs such as tarragon and thyme both contain caffeic acid, which is effective against fungi (Duke, 1985). Hydroxylated phenols like catechol and pyrogallol possessed activity against microorganisms. Chemical hydroxylation of this compounds results in increase in their relative toxicities to microorganisms (Geissman, 1963). Enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins is believed to be responsible for their toxicities to microorganism (Mason and Wasserman, 1987). Eugenol, found in clove oil is a phenolic compound possessing a C3 side chain at a lower level of oxidation and contains no oxygen, this property, to a large extent imparts on the compound bacteriostatic activity against both bacteria (Thomson, 1978) and fungi (Duke, 1985).

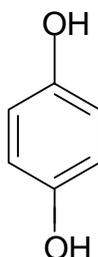


Figure 2.5. Hydroquinone (the most widely distributed phenol in plants)

2.9.1.2. Quinones

Quinones are found abundantly in nature and are highly reactive (Figure 2.6). Being coloured they are responsible for the browning colour in cut or injured fruits and vegetables and serve as intermediaries in the synthesis of melanin in the human skin (Schmidt, 1988). Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. Kazmi et al (1994) described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*.

Hypericin, an anthraquinone from St. John's wort (*Hypericum perforatum*) was reported to possess general antimicrobial properties (Duke, 1985).

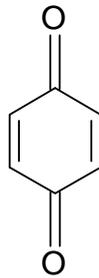


Figure 2.6. Quinones

2.9.1.3. Flavones, flavonoids, and flavonols

Flavones (Figure 2.7.) are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). 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. Since they are known to be synthesized by plants in response to microbial infection (Dixon et al., 1983), it is not surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al., 1996). Catechins, the most reduced form of the C3 unit in flavonoids compounds, exerted antimicrobial activity (Toda, 1989) and that they contain a mixture of catechin compounds. These compounds inhibited *in vitro* *Vibrio cholerae* O1 (Borris, 1996), *Streptococcus mutans* (Batista et al., 1994.), *Shigella* (Vijaya et al., 1995, and other bacteria and microorganisms (Thomson, 1978).

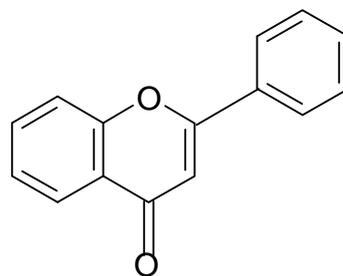


Figure 2.7. Flavone

2.9.1.4. Tannins

“Tannin” is a general descriptive name for a group of polymeric phenolic substances (Figure 2.8) capable of tanning leather or precipitating gelatin 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. One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996). Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones et al., 1994). Tannins are considered for the partial antibiotic activity of methanol extracts of the bark of *Terminalia alata* found in Nepal (Taylor et al., 1996).

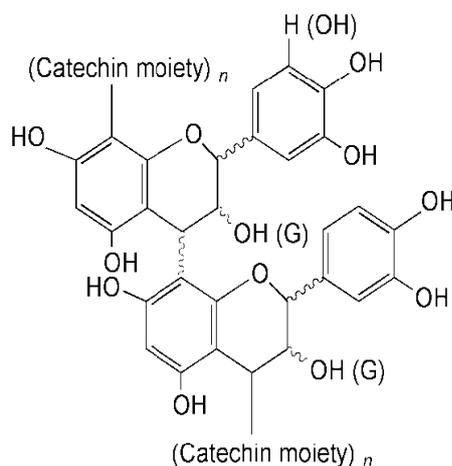


Figure 2.8. Tannins

2.9.1.5. Coumarins

Coumarins are phenolic substances made of fused benzene and α -pyrone rings (Figure 2.9). Coumarin was found *in vitro* to inhibit *Candida albicans*. 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. 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).

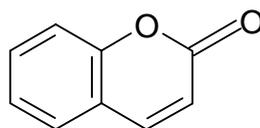


Figure 2.9. Coumarins

2.9.1.6. Terpenoids and Essential Oils

Terpenes are the secondary metabolites that impart the fragrance of plants (Figure 2.10). They are also referred to as essential oils. They occur as monoterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (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 mevalonate units. Examples of common terpenoids are mentol and camphor (monoterpenes), farnesol and artemisinin (sesquiterpenoids). Artemisinin and its derivative α -arteether, also known by the name qinghaosu, find current use as antimalarials (Vishwakarma, 1990). Terpenes or terpenoids are active against bacteria (Ahmed et al., 1993), fungi (Ayafor et al., 1994), viruses (Fujioka and Kashiwada, 1994), and protozoa (Ghoshal et al., 1996). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Two diterpenes isolated by Batista et al (1994) were found to be active against *Staphylococcus aureus*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Candida* spp.

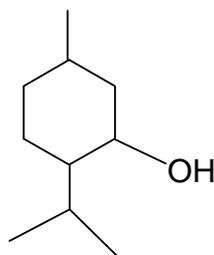


Figure 2.10. Terpenoids

2.9.1.7. Alkaloids

Alkaloids are heterocyclic nitrogen compounds. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the unripe seed capsule of the oriental poppy plant (*Papaver somniferum*) (Fessenden and Fessenden, 1982); the name morphine comes from the Greek Morpheus, god of dreams. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae are commonly found to have antimicrobial properties (Omukoli et al., 1997). Alkaloids have also been found to have microbiocidal

effects (Ghoshal et al., 1996). Berberine (Figure 2.11) is an important representative of the alkaloid group and is potentially effective against some haemoprotozoan parasites (Freiburghaus et al., 1996, Omulokoli et al., 1997). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmaine is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1987). Zhao et al (1998) reported the isolation of 6 alkaloids that are active against the plant pathogenic fungi *Cladosporium cucumerinum*.

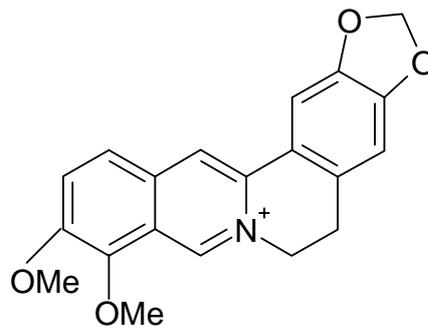


Figure 2.11. Alkaloid (berberine)

2.9.1.8. Lectins and Polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 (Balls et al., 1942). 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 (Shah et al., 1997). Inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthus*, has long been known (De Bolle, 1996). Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues (Colilla et al., 1990). They are toxic to yeasts and gram-negative and gram-positive bacteria (Fernandes de Caley et al., 1972). Thionins AX1 and AX2 from sugar beet are active against fungi but not bacteria (Kragh et al., 1995).

2.10. Fungal diseases and Inflammation

Pathogenic fungi (dermatophytic, subcutaneous, and systemic) have the ability to produce eicosanoids (prostaglandins and leukotrienes) both from host derived arachidonic acid. Host-derived eicosanoids have been previously demonstrated to enhance fungal colonization and the development of inflammation during fungal infections (Noverr et al., 2002). Eicosanoids are potent regulators of host immune responses (Peters-Golden, 1997); they inhibit Th1 type immune responses, chemokine production, phagocytosis, and lymphocyte proliferation (Kunkel et al., 1988; Betz and Fox, 1991; Matsuoka et al., 2000). Leukotrienes are potent leukocyte chemotactic factors (Jonsson, and Dahlen 1999). Moreover, eicosanoids can also

promote Th2 type responses and tissue eosinophilia (Demeure et al., 1997). Fungal diseases cause a shift from Th1 toward Th2 type responses (Romani and Kaufmann, 1998). Host cells are one source of eicosanoids during fungal infection; however, another potential source of eicosanoids is the fungal pathogen itself. The pathogenic fungi *Cryptococcus neoformans* and *Candida albicans* produce prostaglandins de novo from simple exogenous arachidonic acid (Noverr et al., 2002). Phospholipase A₂ and phospholipase B have been identified in a large number of eukaryotic microbes including *A. fumigatus* (Ghannoum, 2000). Phospholipases A₂ and B in fungi cleave the fatty acid side chains of phospholipids and have been implicated in virulence in a number of parasitic and fungal species, presumably via destruction of host cell membranes and subsequent lysis (Noverr et al., 2003). It therefore clearly indicates that enhanced prostaglandin production during fungal infection could be an important factor in promoting fungal colonization and chronic infection.

2.11. Resistance to antifungal agents

Antifungal resistance is a broad concept describing failure of a fungal infection to respond to antifungal therapy (Alexander and Perfect, 1997). Antifungal resistance has been traditionally classified as either primary (intrinsic) i.e., present before exposure to antifungals-or secondary (acquired) i.e., that which develops after exposure to antifungals owing to stable or transient genotypic alterations. A schematic representation of resistance to antifungal agents is presented in Figure 2.12. A third type of antifungal resistance could be described as “clinical resistance”, which encompasses progression or relapse of an infection by a fungal isolate that seems, in laboratory testing, to be fully susceptible to the antifungal used for the treatment of infection. Clinical resistance of fungi is typically seen in patients with persistent or profound immune defects (e.g., AIDS). In some cases, suboptimum drug concentrations in the blood caused by drug interactions might contribute to clinical resistance (Kontoyiannis and Lewis, 2002).

2.11.1. Resistance to azoles

Resistant fungal strains either exhibit a modification in the quality or quantity of target enzyme, reduced access to the target, or some combination of these mechanisms. Modification in the quantity or quality of 14 α -demethylase expression is responsible for resistance to azole antifungal agents. Resistance to azoles can also occur by mutations that modify the target molecule or by over expression of membrane efflux pumps that export the antifungals from the cell. Combinations of both mechanisms have been detected in some *C. albicans* isolates.

2.11.2. Resistance to Polyenes

Alterations in the membrane ergosterol content secondary to mutations in the ergosterol biosynthetic pathway seem to be the most characterized mechanism of polyene resistance of *Candida* and the genetically similar non-pathogenic model yeast *Saccharomyces cerevisiae* (Woods and Ahmed, 1968). Alterations in the cell wall composition have also been described as a mechanism of broad-spectrum polyene resistance in a laboratory strain of *A. flavus* (Kontoyiannis and Lewis, 2002). Resistance to polyenes may result from reduced ergosterol content in the fungal cell membrane. Furthermore, resistance may occur from replacement, by fungi, of ergosterol with sterols with low affinity for polyenes (Rogers, 2002).

2.11.3. Resistance to Allylamines

Allylamine resistant fungi are as yet not well described; comparisons of resistance mechanisms are rare (Ghannoum and Rice, 1999). However, the different sites of action of the azoles, polyenes, and allylamine resemble the sequential actions on cell wall synthesis exhibited by different antibacterial agents (Kahan et al., 1974), penicillin (which acts at an intermediate step), and vancomycin (which acts at the final step in cross-linking). As in the study of cell wall synthesis in bacteria, some of the mechanisms of action of antifungal agents have been elucidated by analyzing the accumulation of specific precursors after exposure to the antibiotic. Since all of the antibiotics act at different steps of the same process, it is perhaps not surprising that specific mutations will result in cross-resistance to several of the compounds. One striking example is depicted by *Candida glabrata* isolate, which was initially susceptible *in vitro* to fluconazole, voriconazole and posaconazole, developed cross-resistance to all currently available triazole antifungals after a course of fluconazole therapy and with no known prior exposure to expanded-spectrum triazoles (Magill et al., 2006).

2.11.4. Resistance to echinocandins (Inhibitors of glucan synthesis)

Kurtz and Douglas 1997 reported the isolation of resistant mutants of *Saccharomyces cerevisiae*. The target of lipopeptides, including echinocandins, is glucan synthase (a heterodimeric enzyme), which in *S. cerevisiae* is encoded by *FKS1* and *RHO1*. *S. cerevisiae* also contains another gene, *FKS2*, which is highly homologous to *FKS1*. Mutations in the *FKS1* gene confer high-level *in vitro* resistance to lipopeptides. Low-level resistance is associated with mutations in another cell wall synthesis gene, *GNS1* that encodes an enzyme involved in fatty acid elongation. Mutations in *FKS2* gene do not confer resistance. Studies with *C. albicans* mutants indicated that resistance to pneumocandin in *S. cerevisiae* and *C. albicans* is very much alike.

2.11.5. Resistance to 5-fluorocytosine (Inhibitor of nucleic acid synthesis)

Resistance to 5-fluorocytosine may result from decreased uptake (loss of permease activity) or loss of enzymatic activity responsible for conversion to 5-fluorouridylic acid (FUMP). FUMP is phosphorylated in fungi and further incorporated into RNA, resulting in disruption of protein synthesis. Blocking the formation of FUMP by loss of cytosine deaminase activity or by loss of uracil phosphoribosyltransferase (UPRTase) activity is sufficient to confer 5-fluorocytosine resistance. Resistance in the large majority of both clinical and laboratory strains of 5-fluorocytosine-resistant *C. albicans* and *Cryptococcus neoformans* is attributable to mutational loss of one of the pyrimidine salvage enzymes (Normark and Schonebeck, 1972). Decreased UPRTase activity was associated with resistance in a gene dosage-dependent manner in *C. albicans* (Whelan and Kerridge, 1984).

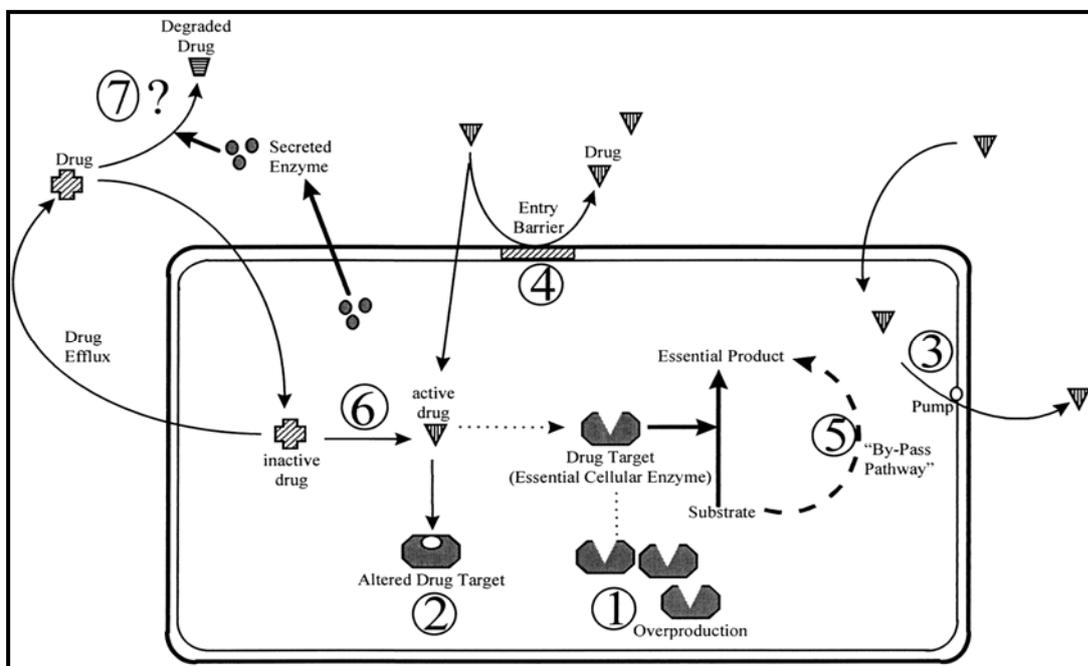


Figure 2.12. Mechanisms by which microbial cells might develop resistance. 1. The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. 2. The drug target is altered so that the drug cannot bind to the target. 3. The drug is pumped out by an efflux pump. 4. The entry of the drug is prevented at the cell membrane/cell wall level. 5. The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity. 6. Some fungal “enzymes” that convert an inactive drug to its active form are inhibited. 7. The cell secretes some enzymes to the extracellular medium, which degrade the drug (Ghannoum, and Rice, 1999).

2.12. Plant species used in the study

Seven plant species that had good activity against the pathogenic fungus *Cryptococcus neoformans* in the tree screening database of the Phytomedicine Programme were selected for further evaluation of their potential value against *Aspergillus fumigatus* and other economically important animal fungi. The species selected are: *Loxostylis alata*, *Protorhus longifolia*, *Kirkia wilmsii*, *Khaya anthotheca*, *Commiphora harveyi*,

Combretum vendae and *Ochna natalitia* (Table 2.1). Most of these species have also been used traditionally to treat diseases associated with infectious and non-infectious agents (Coates-Palgrave, 2002).

2.13. *Loxostylis alata* A. Spreng. ex Rchb. Anacardiaceae

The name *Loxostylis* is derived from the Greek *loxos* meaning 'crooked' or 'oblique', and the Latin *stylis* for style, a reference to the lateral attachment of the style to the ovary. The common name tarwood presumably refers to the oily residue from the fruits. Tarwood is an evergreen, ornamental tree that grows to a height of 5 metres which is grown in cultivation in a wide range of ecological habitats, while naturally it occurs in rocky and forest areas along river banks. The leaves are alternate and compound with 2 to 5 pairs of leaflets, including a terminal leaflet. Typical of the species is the conspicuous winged rachis (midrib). The specific name is based on the Latin *alatus* meaning 'winged'. Young leaves are red. The bark is pale grey and has vertical shallow fissures with latex present. The flowers are male or female on different trees and produced from November to February. The male flowers are white and pleasantly scented and the female flowers are greenish white. The petals of the female flowers fall soon, but their sepals enlarge substantially and turn pink-red, covering the developing fruit and creating a very attractive display. The fruits of *L. alata* are small, fleshy and measure about 4 mm in diameter, usually they are found embedded in the brightly coloured sepals. The seed skin contains a black sticky substance like tar; it is difficult to wash when touched (Coates-Palgrave, 2002).

The bark and leaves are widely used in South Africa as traditional medicine to relieve pain during childbirth particularly among the Zulu tribe (Pooley, 1993). Figure 2.13 shows the leaves and stem of *Loxostylis alata*.



Figure 2.13. The leaves of *L. alata* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, June 2008).

2.14. *Protorhus longifolia* (Bernh.) Engl. Anacardiaceae

Protorhus longifolia has the common names harpuisboom, red beech, rooiblaar. The plant grows up to a height of 15 metres and occurs in forest areas, open woodland and on river banks. The bark is brown, smooth to rough and has milky latex on it. Domestically, the wood provides general purpose timber, which is used for making furniture. Exudate from the bark is used as gum to fix blades of assegais into their handles, and also as a depilatory (Coates-Palgrave, 2002).

Traditionally, in South Africa the bark is injected to cure hemiplegic paralysis believed to be caused by witchcraft (Gerstner, 1941). Decoctions prepared from the plant are used as emetics to relieve heartburn and bleeding from the stomach (Watt and Breyer-Brandwijk, 1962; Pujol, 1990). The bark is also used traditionally to treat diarrhoea and heartwater (Donald and Cocks, 2001). Figure 2.14 shows the leaves of *Protorhus longifolia*.



Figure 2.14. The leaves and stem of *P. longifolia* in their natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.15. *Kirkia wilmsii* (Engl.) Kirkiaceae

Kirkia wilmsii occurs in mountain slope and rocky hills. The bark is grey in colour and smooth, the branchlets are marked with leaf scars. The plant is deciduous, medium to large tree with a rounded crown with beautiful autumn colours from April to May. The trunk is known to branch near the base. Smooth grey bark, often having irregular patching. The flowers appear from spring to summer in sprays of greenish white to greenish cream. The leaves have brilliant autumn colours and are crowded at the end of the branchlets. The flowers are small (about 4-7 cm long) and are greenish white in colour. Fruits formed small capsule, narrowly ovoid and split into 4 valves which remain joined at the apex. The roots of this plant sometimes

produce shoots as the sprawl among rocks. The wood is light, coarse and provides a good strong fibre (Coates-Palgrave, 2002).

The leaves are used traditionally to treat malaria and feverish conditions (Clarkson et al., 2004). Figure 2.15 shows the leaves and stem of *Kirkia wilmsii*.



Figure 2.15. The leaves and stem of *K. wilmsii* in their natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in October 2008).

2.16. *Khaya anthotheca* (Welm.) C.D.C. Meliaceae

The plant is commonly called east African mahogany. *Khaya anthotheca* is a large evergreen tree up to 60 m tall (up to 30 m in the garden) with an elongated or rounded, much-branched crown; the trunk is buttressed in old specimens. The bark on the young branches is smooth and greyish brown but smooth to sometimes mottled grey and brown, flaking on the older branches and stems. Leaves are alternate, evenly compound with 3–7 pairs of leaflets, 150–300 mm long and dark glossy green, base broadly tapering to round and slightly asymmetric, smooth and glossy, veins distinct on the lower surface, margin smooth. Flowers appear in branched sprays at the tips of branches, are white and sweetly scented, up to 10 mm in diameter, the male and female flowers are separate but on the same tree, and the stamens join to form a tube up to 6 mm long. The flowering period is from September to December. The fruit is a hard, woody, oval, splitting capsule up to 60 mm in diameter, with 4 or 5 valves. Fruiting occurs from March to September (Estherhuysen et al., 2001).

The wood weathers well and is resistant to borers and termites. It is moderately resistant to fungal decay. The timber saws well but is inclined to be tough, and sharp equipment is therefore needed. The wood is dark, hard, reddish brown and durable, suitable for furniture, flooring, panelling, and excellent for boat

building, moderately heavy the bark is bitter, similar to quinine, and is used for colds. Oil from the seed is rubbed into the scalp to kill insects (Estherhuyse et al., 2001). Figure 2.16 shows the leaves and stem of *K. anthotheca*.



Figure 2.16. The leaves of *K. anthotheca* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.17. *Commiphora harveyi* (Engl.) Engl. Burseraceae

The tree is commonly known as copper-stem corkwood. It is a small squat deciduous tree measuring about 4-18 metres in height and it occurs on stony hill slopes and rocky river valleys of coastal forest and bushveld. The leaves are usually pinnate with two to three pairs of opposite leaflets. The bark is bronze papery pieces or discs and dark green underneath. Fruits are ovoid to spherical and measure about 10 mm in diameter, becoming red, pseudo-aril orange or red, 4-lobed, with 2 lobes partially covering the stone and 2 longer lobes almost reaching its apex in the months of January to March (Coates-Palgrave, 2002). The soft, white wood has been used in making spoons and small stools which are often sold to tourist. Traditionally the bark is used as disinfectant for wounds, anthelmintic and treatment of snake bite (Watt and Breyer-Brandwijk, 1962). Figure 2.17 shows the leaves and stem of *Commiphora harveyi*.



Figure 2.17. The leaves of *C. harveyi* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.18. *Combretum vendae* (A.E. van Wyk) Combretaceae

The plant is commonly referred to as the Venda bushwillow. It is usually a shrub growing up to 1.5-3 metres high, occurring on the higher slopes of the Soutpansberg on rocky and deep soils. The bark is grey, smooth and densely covered with white or greyish hairs. Leaves are broadly elliptic to obovate and are medium green above. Flowers are light yellow or cream colour. The fruits are 4-winged, occasionally 3-winged and ellipsoidal, green flushed pink or red, becoming dark wine-red, on a stalk 4-7 mm long around the month of March to August.

The leaves, bark, root are used for treating leprosy and as ophthalmic remedy, and blood purification (Watt and Breyer-Brandwijk, 1962). Figure 2.18 shows the leaves and stem of *Combretum vendae*.



Figure 2.18. The leaves of *C. vendae* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.19. *Ochna natalitia* (Meisn.) (Walp.) Ochnaceae

The common names for this plant are Cape plane, Transvaal boxwood, Rooihout and Ysterhout.

The plant is a small to medium sized shrub measuring up to 10 meters in height, it occurs in bushveld and grassland, frequently in shallow soil among rocks. The bark is grey-brown or brown, finely fissured to rough. Young leaves have attractive coppery red colour and green when matured with numerous lateral veins that are close together. Flowers are yellow to golden yellow and have a diameter of 1.5-3 cm. The fruits occur in 2-3 drupelets, ovoid and 5-10 mm long. The fruits are attached near base and are black in colour becoming red or spreading on recurved stalks (Coates-Palgrave, 2002).

Infusions of the root are taken traditionally to cure barrenness (Palmer and Pitman, 1972). The name *isithundu* refers to medicine used to bring prosperity (Doke and Vilakazi, 1972). Figure 2.19 shows the leaves and stem of *Ochna natalitia*.



Figure 2.19. The leaves of *O. natalitia* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in October 2008).

Table 2.1. Summary on the data and traditional uses of the plants selected for this study.

Botanical name	Voucher specimen number	Family	Part used	Claimed medicinal uses	Reference
<i>Combretum vendae</i> (A.E. van Wyk)	PRU96507	Combretaceae	leaves, bark, root	Leprosy, ophthalmic remedy, and blood purification	Watt and Breyer-Brandwijk, 1962
<i>Commiphora harveyi</i> (Engl.) Engl.	PRU96506	Burseraceae	Bark	Used as disinfectant for wounds, anthelmintic and treatment of snake bite	Watt and Breyer-Brandwijk, 1962
<i>Khaya anthotheca</i> (Welm.)	PRU96509	Meliaceae	Bark	Skin diseases, black quarter, helminthiasis	Watt and Breyer-Brandwijk, 1962, Nfi et al., 2001
<i>Kirkia wilmsii</i> (Engl.)	PRU96503	Kirkiaceae	Leaves	Treatment of malaria and feverish conditions.	Clarkson et al., 2004
<i>Loxostylis alata</i> A.Spreng. ex Rchb.	PRU96508	Anacardiaceae	leaves, bark	Stimulation of immune system, relieve of pain during childbirth	Pooley, 1993; Pell, 2004
<i>Ochna natalitia</i> (Meisn.) (Walp.)	PRU96504	Ochnaceae	leaves, root	Infusions and decoctions for headache, and respiratory diseases	Watt and Breyer-Brandwijk, 1962
<i>Protorhus longifolia</i> (Bernh.) Engl.	PRU96505	Anacardiaceae	Bark	Treatment of diarrhoea and heartwater	Donald and Cocks, 2001

2.20. Conclusion

The importance of poultry as a source of protein and employment of the rural populations in Africa warrants serious research into factors that could jeopardize production. The role *Aspergillus fumigatus* plays in the production process cannot be underestimated. Aspergillosis poses a great health risk to the poultry industry. Currently used antifungals against aspergillosis either proved inefficient or are not safe for use. There is therefore the need to find effective, safer and cheaper remedy against aspergillosis in poultry. A practical solution to this problem is to develop effective drugs or extracts from reasonably inexpensive and locally available raw materials. The most obvious way of achieving this goal is through the study of plants, selecting from those that have promising *in vitro* activity for development into effective and safer drugs.

Plant extracts with antioxidant activity may enhance the immune status of target animals and hence decrease their susceptibility to aspergillosis, which can lead to increased production. Inflammation provides an enabling condition for fungi to thrive well, the use of alternative anti-inflammatory compounds from plants that are more effective and cheaper could arrest the development of aspergillosis in infected poultry. It is with this hypothesis that the extract of *L. alata* that had good activity against *Aspergillus fumigatus in vitro* and also had antioxidant and anti-inflammatory properties both in an *in vitro* study was selected for in depth study. This could eventually lead to an *in vivo* trial so as to find safer and more efficacious antifungal, antioxidant and anti-inflammatory agent for use in target animal species.