Chapter II
Fungi are amazing organisms, being able to use almost any surface (e.g., bathroom tile, skin, or leaves) for growth. Muller and Loeffler (1976) estimated that the weight of fungi on earth exceeds that of humans; Armillaria bulbosa, a tree root pathogen, is reported to be among the largest and oldest organisms on the planet (Smith et al.1992). They are an extremely diverse group of organisms, with about 250,000 species widely distributed in essentially every ecosystem. Unfortunately, they also are proficient at colonizing and using plants, humans, and animals as substrates. Humans and other animals are exposed to fungi from the moment of birth. During the last two decades, the incidence of human fungal infections, especially involving immunocompromised patients, has dramatically increased (Baddley et al.2001, Lin et al.2001, Wilson et al.2002). This is in part due to the tremendous advances in medicine that permit the saving of patients with neoplastic and immunocompromising diseases who would otherwise not have survived. It is ironic that many of these patients succumb to fungal infections for which there are few or no drugs available for treatment. Immunocompromised patients are at higher risk of fungal infections which can be life threatening; for example, systemic fungal infections of leukemia patients account for 50% of fatalities (Pfaller et al.2006). Nosocomial bloodstream infections have a similar fatality rate (Gudlaugsson et al.2003).

The filamentous fungus Aspergillus fumigatus and yeast Candida albicans are by far the most important causes of life-threatening invasive mycoses. Apart from A. fumigatus, around 10% of the more than 200 species of the genus Aspergillus are regarded as human pathogens or as having other adverse effects, e.g. A. terreus, A. flavus, and A. niger (Brakhage, 2005). The prevalence of C. albicans in clinical Candida samples is 50–70%, followed by infections with Candida glabrata, which is found in 20–25% of clinical Candida samples. Other pathogenic Candida species include C. tropicalis, C. dubliniensis, C. krusei and C. parapsilosis (Pfaller and Diekema, 2007). Another important human-pathogenic fungus of clinical relevance is the fungus Cryptococcus neoformans. The most common fungal infection among
AIDS patients, cryptococcal meningitis, is caused by this basidiomycete. Furthermore, other fungal species, such as *Pneumocystis jiroveci*, *Zygomycetes*, *Fusarium* species, and *Scedosporium* species have emerged as causal agents of invasive mycoses (Pfaller and Diekema, 2007). Despite the different pathogenesis of infections caused by *C. albicans* and *A. fumigatus*, there are several common traits, particularly when the host response is considered: (i) the pathogens must be able to overcome epithelial barriers (ii) innate immunity represents the major defence system (iii) pathogenic fungi possess physiological characteristics, virulence determinants, and capabilities for immune evasion that make them aggressive pathogens and (iv) invasive candidiasis and invasive aspergillosis are mainly found in patients with a weakened immune system either due to reduced activity of immune effector cells or defects in epithelial barriers. However, Invasive aspergillosis has overtaken candidiasis as the most frequent fungal pathogen detected post mortem in tertiary care hospitals in Europe (Kontoyiannis et al. 2005). It was observed on autopsy that 4% of all patients died had invasive aspergillosis, compared to about 2% with invasive candidiasis (Groll et al. 1996).

**MYCOSES:**

Fungal infections or mycoses are classified depending on the degree of tissue involvement and mode of entry into the host. These are:

- **Superficial** - localised to the skin, the hair and the nails.
- **Subcutaneous** - infection confined to the dermis, subcutaneous tissue or adjacent structures.
- **Systemic** - deep infections of the internal organs.
- **Opportunistic** - cause infection only in the immunocompromised.

Human fungal infections in large parts of the world are uncommon in normally healthy persons, being confined to conditions such as candidiasis and dermatophyte skin infections such as athlete's foot. However, in the immunocompromised host, a variety of normally mild or nonpathogenic fungi can cause potentially fatal infections.

**Superficial Mycoses**

In superficial mycoses, infection is localised to the skin, the hair, and the nails. An example is "ringworm" or "tinea", an infection of the skin by a dermatophyte. Ringworm refers to the characteristic central clearing that often occurs in dermatophyte infections of the skin. Dermatophytes are members of the genera *Trycophyton, Microsporum* and *Epidermophyton* are responsible for the disease.
Tinea can infect various sites of the body, including the scalp (tinea capitis), the beard (tinea barbae) the foot (tinea pedis: "athlete's foot") and the groin (tinea cruris). *Candida albicans* is yeast causing candidiasis or "thrush" in humans. As superficial mycoses, candidiasis typically infects the mouth or vagina. *C. albicans* is part of the normal flora of the vagina and gastrointestinal tract and is termed a "commensal". However, during times of ill health or impaired immunity, the balance can alter and the organism multiplies to cause disease. Antibiotic treatment can also alter the normal bacterial flora allowing *C. albicans* to flourish.

**Subcutaneous Mycoses**

These are infections confined to the dermis, subcutaneous tissue or adjacent structures. Infection may arise following the wounding of the skin and the introduction of vegetable matter. These mycoses are rare and confined mainly to tropical regions. They tend to be slow in onset and chronic in duration. An example is sporotrichosis caused by *Sporothrix schenckii*. The fungus is dimorphic, being a mould that can convert to a yeast form at 37°C on rich laboratory media or in infection. Sporotrichosis was once common in Europe but cases are now rare. The disease is most prevalent in the Americas, South Africa and Australia. Infection usually follows an insect bite, thorn prick or scratch from a fish spine. Certain occupation groups appear to have increased risk from infection. These include florists, farm workers and others who handle hay and moss. The most common symptom is an ulcerative lesion that may develop into lymphangitis.

**Systemic Mycoses (primary and opportunistic)**

These are invasive infections of the internal organs with the organism gaining entry by the lungs, gastrointestinal tract or through intravenous lines. They may be caused by: (i) primary pathogenic fungi or (ii) by opportunistic fungi that are of marginal pathogenicity but can infect the immunocompromised host.

**Primary Pathogenic Fungi**

Infection occurs in previously healthy persons and arises through the respiratory route. Examples include histoplasmosis, blastomycosis, coccidiomycosis and paracoccidioidomycosis. The fungi occur throughout the world but not in large parts of Europe.
Histoplasmosis
This is caused by *Histoplasma capsulatum*. The organism is dimorphic (being a mould that can convert to a yeast form). *H. capsulatum* is endemic in many parts of the world including North and South America. It is found in the soil and growth is enhanced by the presence of bird and bat excreta. Environments containing such material are often implicated as sources of human infection. The lungs are the main site of infection but dissemination to the liver, heart and central nervous system can occur. Pulmonary infection can resemble symptoms seen in tuberculosis.

*Blastomyces dermatitidis*
*B. dermatitidis* is the causative agent of the ‘Chicago disease’. Blastomycosis may be a benign infection or a chronic granulomatous mycosis. Primary infection occurs in the lungs, causes an influenza-like pneumonia, later affecting bones and skin. The virulence is probably caused by a 120 kDa antigen, adhesion WI-1, which is homologous to bacterial invasions from *Yersinia*. The adhesion binds CD11b/CD18 and CD14 on host cells, thereby interacting with macrophages.

*Coccidioides immitis*
*Coccidioides immitis* produces mycoses (Valley fever) that can become acute, chronic, severe or fatal and is manifest in lung, bone and joints, or may disseminate to meningitis. *Paracoccidioides brasiliensis* causes a granulomatous disease that originates as a pulmonary infection. Dissemination occurs resulting in ulcerative granulomatoma in the nasal and buccal, occasionally in the gastrointestinal mucosa or lymph nodes.

**Opportunistic Fungi**
Here patients usually have some serious immune or metabolic defect or have undergone surgery. The diseases include aspergillosis, systemic candidiasis and cryptococcosis. Exceptionally, other fungi that are normally not pathogenic, such as *Trichosporon, Fusarium* or *Penicillium*, may cause systemic infections.

**Candidiasis**
In severely immunocompromised patients (e.g. those receiving chemotherapy) *C. albicans*, that is part of the normal human flora, can proliferate and disseminate throughout the body. The relevance of nosocomial candidiasis has been subject of several studies. According to Trick et al.(2002) an analysis of data collected by the Centers for Diseases Control (CDC) in 115 hospitals in the United States between 1980 and 1990 demonstrated an almost 2-fold increase of the nosocomial fungal
infections. Moreover, 78.3% of these infections were caused by Candida spp. Another study demonstrated a 48.7% increase in the incidence of fungaemia caused by Candida spp. acquired in university hospitals in the United States during the 80’s (Pappas et al.2003). The most important human pathogenic Candida species are C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. guilliermondii, C. lusitaniae and C. krusei. In the meanwhile, the number of new species related to different clinical syndromes is increasing progressively as a consequence of new invasive diagnostic and therapeutic procedures, as well as the increasingly debilitated immunological status of the patients attended at tertiary hospitals facilitates the infection by microorganisms of low pathogenicity. The most important predisposing factors mentioned to be involved in these epidemiological changes are the pathologic and iatrogenic immunosupression and the increase in the use of broad-spectrum antibiotics. The growing number of patients with candidiasis caused by non-C. albicans species was observed worldwide. However, the pattern of its geographic distribution is not uniform. In USA and in some European countries a trend of increasing non-C. albicans infections, mainly the infections caused by C. glabrata and C. krusei, is observed (Kao et al.1999). It has been suggested that the decreased susceptibility of these species to commonly used antifungal drugs, such as fluconazole, may be an important factor to their emergence as opportunistic pathogens (Pfaller and Diekema, 2004).

**Cryptococcosis.**

This is a systemic infection caused by the yeast Cryptococcus neoformans. The commonest manifestation is a subacute or chronic form of meningitis resulting from the inhalation of the organism. Pulmonary infection can also occur. The disease affects both healthy and immunosuppressed individuals and occurs world-wide. C. neoformans can be isolated in large numbers from pigeon droppings in the environment, although such birds do not appear to harbour the yeast.

**Aspergillosis.**

This is the name given to a number of different diseases caused by the mould Aspergillus. In the immunosuppressed host, Aspergillus can disseminate throughout the body. Though it is considered to be opportunistic pathogen, it has recently been found that Aspergillus could be primary pathogen in humans (Vandewoude et al.2006). Aspergillosis encompasses a broad spectrum of diseases caused by members of the genus Aspergillus (Perfect et al.2001). Exposure to Aspergillus species in the
environment may cause allergic reactions in hypersensitized hosts or destructive, invasive pulmonary and disseminated disease in highly immunosuppressed individuals (Patterson et al. 2000). Although 19 species of *Aspergillus* have been documented as agents of human disease, the majority of infections are caused by *A. fumigatus, Aspergillus flavus, Aspergillus niger* and *Aspergillus terreus* (Baddley et al. 2004). *Aspergillus* species are common throughout the world. Their conidia are ubiquitous in air, soil, and decaying matter. Within the hospital environment, *Aspergillus* species may be found in air, showerheads, water storage tanks, and potted plants (Anaissie et al. 2002). As a result, the conidia are constantly being inhaled. The type of host reaction, the associated pathologic findings, and the ultimate outcome of infection depend more on host factors than on the virulence or pathogenicity of the individual *Aspergillus* species (Steinbach et al. 2003). Invasive aspergillosis (IA) affects a more narrow range of patients than does invasive *Candida*. Nearly two-thirds (61%) of patients with IA have underlying hematological diseases (including hematological cancers) or have undergone BMT (Marr et al. 2002). Multiple analyses have examined which patients within these groups face the highest risk of IA. Risk factors include grade III–IV graft-versus-host disease, receipt of steroids, prolonged or repeated episodes of profound neutropenia, age ≥40 years, receipt of BMT from an HLA-mismatched or unrelated donor, and infliximab (monoclonal antibody) therapy (Perfect et al. 2001). Although rare, invasive fungal infections due to *Aspergillus* species and the Zygomycetes may occur in individuals with late-stage AIDS (stage III) (Benson et al. 2005). In high-risk patients, a respiratory tract sample (e.g., sputum or bronchoalveolar lavage) that is culture positive for *Aspergillus* species is associated with invasive disease. A positive culture was associated with IA in nearly two-thirds of allogeneic BMT recipients (64%) and patients with neutropenia (64%) and in half of patients with haematological cancers (50%) (Nucci et al. 2003). IA is associated with a high mortality rate. In one series of 1209 aspergillosis cases in 24 medical centers, 62% of patients with *Aspergillus* species infection had died within 3 months of receiving a positive culture result (Perfect et al. 2001). Mortality rates as high as 85% have been reported for IA (Lin et al. 2001). Specific antifungal therapy for aspergillosis often involves the administration of amphotericin B or one of its lipid-based formulations. However the authors suggested that amphotericin B prophylaxis has led to emergence of resistant organisms (Perfect et al. 2004). Its high
nephrotoxicity has been its major limitation. Therefore, the diagnosis and therapy of invasive aspergillosis remains a clinical challenge.

**CURRENT ANTIFUNGAL THERAPY:**

An antifungal drug is a medication used to treat fungal infections such as athlete's foot, ringworm and candidiasis, serious systemic infections such as Aspergillosis, Cryptococcal meningitis and others. The number of agents available to treat fungal infections has increased by 30% since the year 2000, yet still only 15 agents are currently approved for clinical use. The greater number of medications now available allows for therapeutic choices; however, differences in antifungal spectrum of activity, bioavailability, formulation, drug interactions, and side effects necessitates a detailed knowledge of each drug class. At present we have four classes of synthetic molecules (azoles, flucytosine, allylamines and phenylmorpholines) and three classes of natural products (griseofulvin, polyenes and echinocandins) which have been found to be of clinical value against fungal infections.

**SYNTHETIC DRUGS:**

**Azoles**

Theazole antifungal agents include imidazoles (e.g. miconazole, ketoconazole and clotrimazole) and triazoles (e.g.itraconazole, fluconazole, voriconazole and Posaconazole).Imidazole and triazole are synthetic antifungal drugs that inhibit the enzyme 14α-demethylase, a cytochrome P450 dependent enzyme system resulting in the blockade of ergosterol synthesis. Thereby the permeability of the cell membrane of sensitive fungi is altered. This enzyme converts lanosterol to ergosterol, and is required in fungal cell membrane synthesis. These drugs also block steroid synthesis in humans. The triazoles also exert their effects within the fungal cell membrane. The inhibition of cytochrome P450 (CYP)-dependent 14-a-demethylase prevents the conversion of lanosterol to ergosterol. This mechanism results in the accumulation of toxic methyl sterols and resultant inhibition of fungal cell growth and replication.

**Imidazoles:** Imidazoles include Miconazole, Ketoconazole and clotrimazloe.

**Miconazole**

Miconazole was introduced for topical therapy in 1969 and its intravenous formulation was subsequently marketed for treatment of systemic mycoses. It has been found to be highly effective for treating pseudoallescheriasis (Stevens,1977). It showed a broad spectrum of activity against *Nocardia, Streptomyces* and yeast forms
of H. capsulatum and B. dermatitidis. The miconazole has been a less tolerated drug and side effects such as phlebitis, rash, fever, hepatic toxicity and thrombocytopenia have been found to be associated with treatment of patients with this drug (Bodey, 1992).

**Ketoconazole**

Ketoconazole was introduced for the treatment of both superficial and deep seated candidal infections (Borelli et al.1979). It was active against Candida and Cryptococcus, however, its activity was less in comparison to that of fluconazole and itraconazole (St-Germain et al.1995). It was shown to be clinically ineffective in meningeal cryptococcosis and not recommended for treating cryptococcal disease (Subramanian and Mathai, 2005). Clinical response in histoplasmosis by the treatment with ketoconazole in immunocompetent hosts has been demonstrated. The toxic effects of ketoconazole are generally characterized by nausea, anorexia, vomiting, diarrhea, itching, allergic rash and hepatotoxicity (Como and Dismukes, 1994). It caused gynecomastia and oligospermia, the symptoms of which were dose-dependent that resulted from the lowering of serum testosterone levels due to ketoconazole's action on the cytochrome P450 system (Hitchcock, 1991).

**Triazoles:**
Triazoles include the new generation azoles i.e Fluconazole, Voriconazole, Itraconazole and Posaconazole.

**Fluconazole:**
Fluconazole (Diflucan) remains one of the most frequently prescribed triazoles because of its excellent bioavailability, tolerability, and side effect profile. It is the safest azole and doses 4–5 times in excess of the recommended daily dose have been
well tolerated. The in vitro activity of fluconazole is generally considered fungistatic and its relatively narrow spectrum of activity is essentially limited to yeasts (Wheat et al. 1997). Specifically, an 8.5-year global surveillance of susceptibilities of Candida species and other yeasts demonstrated that fluconazole is very active against Candida species including C. albicans, C. parapsilosis, C. tropicalis, and C. lusitaniae. (Gonzalez et al. 2005). However, fluconazole is much less active against other Candida spp. C. krusei is inherently resistant, and species such as C. glabrata and C. guilliermondii have reduced susceptibilities to fluconazole (Pfaller et al. 2007, Pfaller et al. 2004a). Fluconazole may also produce transient transaminase abnormalities, but progression to severe drug-induced hepatitis is exceedingly rare. However, there is no appreciable activity against Aspergillus, Fusarium, Pseudoallescheria, or the Zygomycetes. Although fluconazole has substantially fewer drug–drug interactions than other triazole compounds, caution remains necessary because of increases in the serum levels of phenytoin, glipizide, glyburide, warfarin, rifabutin, and cyclosporine (Pappas et al. 1995).
**Itraconazole**

Itraconazole exerts fungicidal activity against filamentous fungi and some strains of *C. neoformans* and is generally fungistatic against many yeasts (Groll et al. 2001). With the exception of *C. glabrata*, itraconazole is moderately to very active against most medically important fluconazole-susceptible and -resistant *Candida* species (Pfaller et al. 2005). Itraconazole has modest activity against *C. neoformans*; however, the poor central nervous system penetration of this drug limits its usefulness for treating cryptococcosis (Almyroudis et al. 2007). It has good activity against many *Aspergillus* spp. But it has variable activity against *Fusarium* spp. and very limited activity against the agents of zygomycosis (Espinel-ingroff. 2001). In addition, itraconazole is unique in that hydroxyitraconazole, its primary metabolite in humans, is bioactive. Data suggest that hydroxyitraconazole activity against *C. pseudotropicalis* was nearly twice that of itraconazole but its activity against *A. fumigatus*, *A. terreus*, *C. neoformans* and *C. immitis* was the same as that of itraconazole (Hostetter et al. 1993). Like all azoles, transient transaminase abnormalities and gastrointestinal adverse effects, particularly nausea, abdominal pain and diarrhoea, are common with itraconazole administration (Willems et al. 2001).

**Voriconazole:**

Voriconazole is a low molecular weight water soluble second-generation triazole with a chemical structure similar to fluconazole. It was developed by Pfizer Pharmaceuticals and its clinical use was approved by FDA in 2002. Voriconazole possesses a very broad spectrum of activity against dermatophytes, yeasts, and moulds. This agent is active against all *Candida* spp., including fluconazole-resistant *C. albicans*, *C. glabrata*, and *C. krusei* (Pearson et al. 2003). It exhibits excellent *in vitro* activity against *Aspergillus* spp. and is highly active against *A. fumigatus*, *A. flavus*, and *A. terreus*. (Pfaller et al. 2002). However, over time *A. fumigatus* isolates have become slightly less susceptible to several antifungal agents, including voriconazole (Messer et al. 2003). In addition to the gastrointestinal and dermatologic adverse effects seen with other azoles, voriconazole produces visual disturbances and clinically significant transaminase abnormalities in approximately 30% and 12.7% of patients, respectively (Tan et al. 2006).
**Posaconazole:**

Posaconazole, a highly lipophilic weak base, is chemically similar to itraconazole. It is available only as an oral suspension. Posaconazole exerts fungicidal activity against non-\emph{albicans} \emph{Candida} species including \emph{C. krusei}, \emph{C. inconspicua} and \emph{C. lusitaniae}, but is fungistatic against \emph{C. albicans}, \emph{C. glabrata}, \emph{C. tropicalis}, \emph{C. guilliermondii} and \emph{C. parapsilosis} (Scozo et al. 2007). Like voriconazole, posaconazole demonstrates \textit{in vitro} fungicidal activity against \emph{Aspergillus} spp and \emph{C. neoformans} (Ulmann et al. 2006). \textit{In vitro}, posaconazole is the most active azole against \emph{Aspergillus} spp. and is highly active against \emph{A. fumigatus}, \emph{A. flavus}, and \emph{A. terreus} (Espinell- Ingroff, 2001). Compared to other azoles, there is much less clinical experience with this agent. Consequently its safety profile may not be fully realized. Nonetheless, to date the common adverse effects associated with posaconazole use have been similar to those observed with other agents in the class i.e. gastrointestinal, transient transaminase abnormalities (Gubbins et al. 2006).

**Flucytosine:**

![Flucytosine](image)

Flucytosine is the lone member of the group of fluorinated pyrimidine analog antifungal compounds. To exert its effect, flucytosine is taken up in susceptible fungi by the transport enzyme cytosine permease. Once inside the fungal cell, flucytosine rapidly undergoes intracellular conversion to 5-fluorouracil via cytosine deaminase. (Polak and Scholer, 1975). Fungi lacking cytosine deaminase are intrinsically resistant to flucytosine. Through a series of phosphorylation reactions, 5-fluorouracil is ultimately converted to its triphosphate form, 5-fluorouridine triphosphate. The triphosphate form is incorporated into fungal RNA in place of uridylic acid, which alters the amino acylation of tRNA and ultimately inhibits protein synthesis (Waldorf and Polak, 1983). The antifungal spectrum of flucytosine is extremely narrow and is limited to \emph{Candida} species and \emph{C. neoformans}, although there are some anecdotal
recommendations for aspergillosis and chromoblastomycosis (Polak et al. 1982). Because resistance to flucytosine may occur at multiple steps in its mode of action, including transport into the cell and deamination to the active compound, flucytosine is only used in combination with other agents, including amphotericin B and fluconazole (Rex et al. 2000). The primary toxicities of flucytosine are myelosuppression, gastrointestinal intolerance, and hepatic toxicity.

**Allylamines**

Allylamines such as naftifine and SF 86-327 were developed as antifungal agents for the topical treatment of superficial mycoses which were specific inhibitors of the fungal squalane epoxidase (Ryder, 1985; Ryder, 1992). They have been found to be active against a wide range of pathogenic fungi primarily fungicidal against dermatophytes and *C. parapsilosis* but fungistatic against *C. albicans*. Allylamines have been reported to adversely affect fatty acid and phospholipid biosynthesis in fungi which would influence a large number of membrane-bound enzymes in humans. Ultrastructural studies showed evidences of defective cell wall biosynthesis in naftifine treated cells (Evans, 1997).

**Morpholines**

Amorolfine is a new antifungal drug, belonging to the class of phenylpropylpiperidine and morpholine derivatives. Amorolfine has been found to inhibit the synthesis of membrane sterol which leads to impairment of membrane function (Pommer, 1995). It has been effective against *C. albicans, T. mentagrophytes, H. capsulatum, C. neoformans, Scytalidium* and *Wangiella dermatitidis*. The therapeutic efficacy of amorolfine in animal models has been found to be limited to superficial fungal infections (Polak, 1988). The drug showed no remarkable activity in systemic mycosis after oral administration (Khan and Jain, 2000).

**RECENT ADVANCES:**

There are several groups of researchers around the world who are engaged in synthesizing molecules of different moieties and evaluating them for their antifungal potential (Keasling, 2008). A number of synthetic compounds including pyrazoles, indoles and dihydropyridine derivatives (Chaudhary et al. 2006, Sharshira et al. 2011, Sharshira et al. 2012) have been screened recently. One of the dihydropyridine derivatives named, SyC 18 was found to have strong activity against *Aspergilli* (Chhillar et al. 2006). The MIC of SyC 18 was found to be 15.62, 62.5, 62.5 and 31.2
μg/ml against *A. fumigatus*, *A. flavus*, *A. niger* and *C. albicans* respectively. The results of in vitro cytotoxicity showed that it was nontoxic to human erythrocytes up to a concentration of 625.0 μg/ml. Thus, SyC 18 may be a better lead molecule.

**NATURAL PRODUCT BASED DRUGS:**
Microbial natural products are the origin of most of the antibiotics in the market today. About 100,000 secondary metabolites of molecular weight less than 2500 dalton have been characterized, mainly produced by microbes and plants (Roessner and Scott, 1996). Around 50,000 compounds have been reported from micro-organisms with various biological activities (Fenical, 1997, Berdy, 2005). Of the 12,000 antibiotics known in 1995, 55% were produced by filamentous bacteria (actinomycetes) of the genus Streptomyces, 11% by other actinomycetes, 12% by non filamentous bacteria and 22% by filamentous fungi (Berdy, 1995, Strohl, 1997). New bioactive products from microbes are continued to be discovered at an amazing pace. The number of new identified compounds in late 1970s had been 200 to 300 per year which increased to approximately 500 per year by 1997. Hence the vast number and variety of chemotherapeutic agents isolated from microbial natural products and used to treat fungal infections have greatly contributed to the improvement of human health during the past century. Most effective antifungal molecules (polyenes plus the recently introduced echinocandins) have been reported from natural sources. The natural product based drugs include secondary metabolites (griseofulvin and polyenes) and proteins/peptides (echinocandins and others).

**SECONDARY METABOLITES:**
Secondary metabolites produced by microbes act as antibiotics including polyenes and griseofulvin.

**Amphotericin B**
Amphotericin B is a polyene antibiotic and acts by binding to ergosterol in fungal cell membranes. It has a greater affinity to bind ergosterol and ergosterol-containing membranes than cholesterol or cholesterol-containing membranes (Brajtburg et al. 1990). The binding occurs within minutes of exposure and is followed by increasing leakage of intracellular ions out of fungal cells (i.e., potassium) and extracellular ions into cells, which leads to depolarization of the membrane and increased permeability to protons and monovalent cations, causing lethality to fungal
cells. The antifungal spectrum of Ampho.B is extremely broad, it being easier to list the few exceptions than the targeted species. However, growing evidence suggests less than optimal activity against a number of Candida species (Spellberg et al. 2006). The diminishing susceptibility to Ampho.B among more common Candida spp., including C. glabrata and C. krusei, is a growing concern. Moreover, Ampho.B also exhibits delayed killing against these species when compared to its activity against C. albicans (Canton et al. 2004). Although Ampho.B is active against most moulds, there is interspecies variability with respect to amphotericin MICs. Among Aspergillus spp., A. terreus, A. flavus and A. nidulans typically are less susceptible to Ampho.B than other species. (Sabatelli et al. 2006). Nephrotoxicity is the major adverse effect limiting the use of Ampho.B. The manifestations of nephrotoxicity have been azotemia, decreased glomerular filtration, loss of urinary concentrating ability, and renal tubular acidosis and renal loss of sodium and potassium ions (Gubbins et al. 2002). The renal injury reduced erythropoietin production and led to a normochromic normocytic anemia. Thrombophlebitis may occur at the site of infusion and thrombocytopenia may also be observed (Goodwin et al. 1995). In attempts to avoid the nephrotoxicity seen with amphotericin B deoxycholate (AmBd; Fungizone) several other formulations have been developed. The lipid preparations include: liposomal amphotericin B (L-AMB; Ambisome), amphotericin B lipid complex, (ABLC; Abelcet) and amphotericin B colloidal dispersion (ABCD; Amphotec, Amphocil). However cure rate has not been seen to improve (Hiemenz and Walsh 1996).

Nystatins:
Nystatin was introduced in 1954 for the treatment of thrush. Oral nystatin is also used for treatment of intestinal candidiasis or to prepare the bowel for gastrointestinal
surgery. Nystatin may affect adversely the sense of taste leading to decreased appetite. Other side effects of nystatin could be nausea, vomiting, diarrhea and stomach pain. Sometimes the products may contain propylene glycol, alcohol, parabens or benzyl alcohol which might add to the toxicity of the drug (Wasan, 1997).

**Griseofulvin:**
It was first tested as an antifungal agent in humans in 1950s and was the earliest chemical that could be claimed to have a selective inhibitory activity against fungi. It was isolated from the fungus *Penicillium griseofulvum* and thus was the first example of a product from one fungus acting against another. It was found to block the assembly of microtubules within susceptible fungal cells, without exerting similar effects on mammalian cells (Gull and Trinci, 1973). It has been effective against a number of pathogenic fungi, but showed limited activity against dermatophytes (Odds et al. 2003). The drug is administered orally and has been found to be useful in countries where poverty and distances to the hospitals reduce patients compliance with a complex regime, in such situations a single dose treatment of 2-3 gm of griseofulvin is prescribed, although this approach has been of limited value. The drug has proved its worth over the years, particularly in the successful treatment of scalp ringworms.

**ANTIFUNGAL PROTEINS/PEPTIDES:**
These include the primary metabolites like proteins or peptides with antimicrobial potential.

**Echinocandins:**
The echinocandins represents an important class of biologically active lipopeptides which were prepared by modifying the native molecule derived from fermentation broth of various fungal species. The increased incidence of triazole-resistant *Candida* spp and the fungicidal activity of the echinocandins (caspofungin, micafungin, anidulafungin) have prompted some authorities to recommend these agents as first-line therapy for invasive candidiasis. Additionally, their proven efficacy, infrequency of side effects, and favourable drug interaction profiles make them attractive options over other available antifungals. The echinocandins exert their antifungal effect through noncompetitive inhibition of (1, 3)-β-D-glucan biosynthesis, which causes destabilization of the fungal cell wall, cell lysis, and death. (Denning, 2003). Their
clinical use is primarily limited to Candida spp and Aspergillus spp. and they lack activity against the Zygomycetes, Cryptococcus spp, and other clinically important molds. Echinocandins also have immunomodulatory effects. By exposing Beta-glucan by the disruption of fungal cell wall mannoproteins, additional antigens are exposed for antibody deposition and fungal recognition by the host immune system (Cappelletty, 2007). The echinocandins possess a narrow antifungal spectrum that is restricted to Candida spp. and Aspergillus spp. and there is little difference among the individual drugs. All the echinocandins exert fungicidal activity against Candida spp.; however, in Aspergillus spp. these compounds do not usually cause complete inhibition of growth but instead induce abnormal morphologic hyphal growth (Kurtz et al. 1994). Therefore these agents are considered to be fungistatic against Aspergillus spp.

![Caspofungin](image1.png) ![Anidulafungin](image2.png) ![Micafungin](image3.png)
**Pneumocandin B0**

Pneumocandin B0 was isolated from *Glarea lozoyensis* (Schwartz et al. 1992, Bills et al. 1999). It was found to be effective in animal models of disseminated candidiasis, aspergillosis, coccidiomycosis and pneumonia caused by *Pneumocystis carinii* (Abruzzo et al. 1997). The efficacy of the drug in the treatment of oropharyngeal and oesophageal candidiasis as well as invasive aspergillosis has been demonstrated (Powles et al. 1998) and it has been found to be tolerated well, however, the drug is still under clinical trials. In the meantime pneumocandin B0 was exploited for preparing new antifungal drugs. The introduction of additional amino groups in the peptide ring of pneumocandin B0 increased the solubility of the molecule and the potency against fungal pathogens by two orders of magnitude (Bouffard et al. 1994) and the molecule was named as caspofungin.

**Caspofungin**

The caspofungin, formerly known as L-743,872 and MK-0991, is a polypeptide prepared on the basis of pneumocandin B0. It has been manufactured by Merck Research Laboratories, USA and sold under trade name Cancidas. It has recently been approved by the FDA for use intravenously against candidiasis and invasive aspergillosis, refractory to, or intolerant of other therapies. It was found to inhibit the synthesis of a major fungal cell wall component, 1-3-beta-D-glucan (Morrison, 2006). Although caspofungin has been active against *Candida* and *Aspergillus* species (Mora et al. 2002, Maertens et al. 2004), the response of *Trichophyton beigeli*, *Fusarium* and *Rhizopus arrhizus* to treatment with caspofungin was not satisfactory. The side effects due to caspofungin though have been reported to be of no significance, however, liver related problems may be of much clinical significance.

**Micafungin (FK-463)**

Micafungin (MYCAMINE®) was approved in the United States in 2005 for the treatment of esophageal candidiasis and for the prevention of *Candida* infections in patients undergoing HSCT (Haematopoietic Stem Cell Transfer). It is a new lipopeptide echinocandin with a broad-spectrum *in vitro* and *in vivo* antifungal activity, against both *Aspergillus* and *Candida* species (Denning et al. 2006, Pappas et al. 2007). The mechanism of action is similar to caspofungin. Micafungin was also been shown to be effective as prophylaxis during the pre-engraftment period of neutropenia when compared with fluconazole (80% versus 73.5%) in 882 patients.
undergoing HSCT (Vanburik et al. 2004). Micafungin is not yet approved for the treatment of Invasive Candidiasis or Invasive Aspergillosis. Micafungin appears to be a promising agent for invasive fungal infections but requires further clinical evaluation. It is in phase III clinical trials.

**Anidulafungin (LY303366)**

It is a new echinocandin with promising broad-spectrum, antifungal activity *in vitro* and *in vivo* with a mechanism of action similar to caspofungin. Anidulafungin (ERAXIS®) was approved in the United States in 2006 for the treatment of EC, candidemia, and other *Candida* infections (intra-abdominal abscess and peritonitis). Anidulafungin was compared with fluconazole in a randomized double-blind phase III study of 504 patients with EC (Krause et al. 2004). The endoscopic success rate at the end of therapy was similar for anidulafungin and fluconazole (97.2% versus 98.8%), but the relapse rate at the two-week followup was higher for anidulafungin (36% versus 11%, <0.001). Overall, the echinocandins are very well tolerated and a favorable safety profile is one of the key attributes of this class. Similar types of adverse events and laboratory abnormalities are seen across the class. The most common adverse events reported are phlebitis, rash, flushing, fever, chills, headache, nausea, vomiting, diarrhea, and abdominal pain; the most common laboratory abnormalities include elevated transaminases, alkaline phosphatase, and bilirubin (Wagner et al. 2006).

**Nikkomycins**

The nikkomycins, non-toxic to mammalian cells, are a family of potent antifungal peptidyl nucleoside antifungals produced by *Streptomyces tendae* which inhibit chitin biosynthesis (Chapman et al. 1992). It had ability to enter target cells via dipeptide permeases and inhibited chitin biosynthesis in *C. albicans* both *in vitro* and *in vivo* (Mccarthy et al. 1985). Nikkomycin provided antifungal protection to infected kidneys, while other organs were unprotected. Its two forms nikkomycin X and Z were active against pathogenic dimorphic fungi but showed only moderate to poor activity against yeast and filamentous fungi (Tariq et al. 1996). However, they were highly efficacious in murine models of coccidioidomycosis and blastomycosis with moderate efficacy against histoplasmosis (Hector et al. 1990). The nikkomycins afforded protection against deaths of mice infected with a 100 % lethal challenge of *C. immitis* (De Lucca and Walsh, 1999).
**Iturins**

Various strains of *Bacillus subtilis* produced iturin peptides. They are small cyclic peptidolipids characterized by a lipid-soluble β-amino acid linked to a peptide containing D and L amino acids (Bloquiaux and Delcambre, 1956). Iturins were found to affect membrane surface tension which caused pore formation resulting in the leakage of K⁺ and other vital ions paralleling cell death (Peypoux et al., 1973). Another family member, bacillomycin F, also inhibited the growth of fungi including *A. niger*, *C. albicans* and *F. oxysporum* (Landy et al., 1948). The experiments in animals and initial clinical trials involving humans showed that iturin A was effective against dermatomycoses and had a wide spectrum of antifungal properties with low allergenic effects (Clairbois and Delcambre, 1958). Unfortunately, bacillomycin L and iturin A have been found to be hemolytic which may reduce their potential use as antifungal drugs (Latoud et al., 1986).

**Syringomycins**

Members of the *Pseudomonas syringae* pv. *syringae* group produced small cyclic lipodepsipeptides known as syringomycins (Segre et al., 1989), the major form being syringomycin E. This molecule increased transmembrane K⁺, H⁺ and Ca⁺ ion-fluxes and the membrane potential in plasma membranes of plants and yeasts (Reidl and Takemoto, 1987). Sorenson et al. (1996) studied the fungicidal properties of several compounds produced by *P. syringae*, including syringomycin E, syringotoxin B and syringostatin A. These compounds were fungicidal for *Candida*, *Cryptococcus* and *Aspergillus* isolates. *P. syringae* also produced the pseudomycins, another family of peptides with broad-spectrum antifungal activity, but none of these polypeptides has been recommended yet as useful drug (Harrison et al., 1991).

**Polyoxins**

Polyoxins, which are produced by *Streptomyces cacaoi*, were active against isolated chitin synthases but had variable activity against intact organisms (Hori et al., 1974). Polyoxin D was fungistatic for several isolates of *C. albicans* and also inhibited the growth of *C. neoformans* (Becker et al., 1983). The polyoxin D reduced the ability of *C. albicans* to bind to buccal epithelial cells by as much as 58% as compared to the binding ability of controls (Gottlieb et al., 1991).
FR-900403
FR-900403 differs in structure from the polyoxins. CB-1 is a chitin-binding peptide containing fatty acids bound to amino acids which showed activity against *F. oxysporum* (Oita et al.1996). The fungicin M-4 was isolated from *B. licheniformis* isolate M-4 (Lebbadi et al.1994). It is a hydrophilic, narrow-spectrum antifungal peptide resistant to proteolytic enzymes and lipase that inhibited the growth of *Microsporum canis, Mucor* species, and *S. schenckii*. However, fungicin M-4 was ineffective against *C. albicans, C. neoformans, A. niger*, and *Trichophyton mentagrophytes*. *B. licheniformis* also produces A12-C, an inhibitor of fungal cell growth and hyphal proliferation. A12-C inhibited the growth of *S. schenckii, T. mentagrophytes* and *M. canis*, as observed in zone-of-inhibition studies (Gàlvez et al.1993).

**Schizotrin A**
A cyanobacterium, *Schizotrix* (TAU strain IL- 89-2), produced schizotrin A, a cyclic undecapeptide (Pergament and Carmelli, 1994). Zone-of-inhibition assays demonstrated that it had activity against *C. albicans* and *C. tropicalis*. It also inhibited *in vitro* the radial growth of *F. oxysporum*.

**Cepacidines**
Cepacidines A1 and A2 are glycopeptides that have similar structures and that are produced by *Burkholderia cepacia* (Lee et al.1994). They displayed potent antifungal properties even superior to those of amphotericin B (De Lucca and Walsh,1999) and was effective against *C. neoformans, A. niger, T. mentagrophytes, T. rubrum, M. canis* and *F. oxysporum*. Its activity against *C. albicans* and *C. neoformans* in the presence of 50% human serum was found to be diminished significantly. These observations may limit its clinical usefulness.

**RECENT ADVANCES IN ANTIFUNGAL PROTEINS:**
The antifungal protein AFP from *Aspergillus giganteus* is found to be highly effective in restricting the growth of major human- and plant-pathogenic filamentous fungi (Hagen et al.2007). AFP is abundantly secreted by *A. giganteus* and exhibits high levels of antifungal activity against species of the genera *Aspergillus* and *Fusarium*, with minimal protein concentrations necessary for total inhibition ranging from 1 to 20 µM, but it does not affect the growth of yeasts or bacteria (Theis et al.2003). In addition, AFP has been demonstrated to have neither cytotoxic nor immunogenic
effects on different types of mammalian cells (Szappanos et al.2006), which is indicative of its excellent potential for medical applications. However, a fundamental prerequisite for the use of AFP as an antifungal drug is a complete understanding of its mode of action. A similar protein PAF has been isolated from *Penicillium chrysogenum* (Galgoczy et al.2005). *Penicillium* antifungal protein (PAF) is a promising antimycotic without toxic effects on mammalian cells and therefore may represent a drug candidate against the often lethal *Aspergillus* infections that occur in humans. However their safety profiles are not fully realized yet.

**COMBINATION THERAPY**

The toxicity of amphotericin B, the fungistatic nature of the azoles and the emerging resistance issues have led to the investigations on combination therapy which may improve efficacy, reduce side effects and decrease duration of therapy (Kontoyiannis et al.2003). Based on limited studies in animal models and with *in vitro* data, both tetracycline and rifampicin showed synergy with amphotericin B against selected organisms (Arroyo et al.1977, Clancy et al.1998) however, evidences for clinical benefits in humans of these combinations against fungal infections are still to be generated. Studies based on animal models of disseminated candidiasis suggested that amphotericin B combined with 5-flucytosine was more effective than amphotericin B alone against most deep-seated *Candida* infections (Louie et al.1999). They were synergistic for cryptococcal meningitis in non-AIDS patients (Larsen et al.1990). Francis and Walsh (1992) investigated the safety and tolerability of flucytosine in myelosuppressed patients and concluded that flucytosine in combination with amphotericin B was well tolerated. The combination of amphotericin B and fluconazole has been reviewed recently by Larsen et al. (2004). Rex et al. (2003) found that no antagonism between amphotericin B and fluconazole occurred using 50% inhibitory endpoints with *C. albicans*. In another study investigating the antifungal activity of amphotericin B plus fluconazole against three strains of *C. albicans*, Rex et al. (2003) found that the combination yielded a slightly greater reduction in colony-forming units compared to fluconazole alone. Sugar et al.(1995) found the combination of fluconazole and amphotericin B was not antagonistic *in vivo* in mice with invasive candidiasis and suggested combination therapy be considered in management of clinical candidiasis. Combinations of antifungal drugs have proven to be the most effective approach for treating cryptococcal meningitis.
A common strategy for selected fungal infections has been combination therapy with agents that exhibit either an additive or synergistic effect when given concomitantly. Studies of antifungal combinations for managing resistant fungal infection and infections associated with high mortality rates focused on invasive infections caused by *Candida*, *Cryptococcus* and *Aspergillus* which generally included combination of polyene, flucytosine, or triazole. *In vitro* studies suggested that echinocandins might have positive interactions with other antifungals such as amphotericin B (Arikan et al.2002). However, attempts to define the best combination therapy have been limited largely due to the lack of standards for *in vitro* susceptibility testing methods, combined with lack of controlled clinical trials (Johnson et al.2004).

Barchiesi et al. (1997) tested the terbinafine, which has been currently used to treat dermatophytic infections in combination with fluconazole and itraconazole against 70 yeast isolates displaying varying azole-susceptibility patterns. They found that there was a dramatic synergism, suggesting exciting treatment possibilities. Further, clinical investigations are needed to determine whether the *in vivo* activity of terbinafine-azole combinations correlated with these *in vitro* data. Another study investigated *in vitro* interactions between terbinafine and amphotericin B and itraconazole and fluconazole using four isolates of *A. fumigatus*. Mosquera et al. (2002) found that there was antifungal synergy between terbinafine and triazoles against *A. fumigatus* as was expected from drugs acting on two different stages of ergosterol biosynthesis. The combination of terbinafine and amphotericin B ranged from variable synergistic effects to possible antagonism. It was, therefore, concluded that terbinafine alone or in combination with amphotericin B or triazoles may have potential in therapy of aspergillosis. Further, clinical data are necessary to substantiate these reports which are limited by small numbers of patients and problematic administration of flucytosine in patients who have bone marrow suppression from underlying disease or secondary to chemotherapy.

**SEQUENTIAL THERAPY**

The cure of fungal infections by usual regimen of even most effective drugs has been generally not possible (Groll et al.1996). Therefore, the concept of sequential therapy has emerged to treat persistently immunocompromised patients with invasive fungal infections. This involved the induction, consolidation and maintenance therapies in selected cases of malignancies (Steinbach et al.2003).
Initiation of aggressive therapy with potent broad spectrum antifungal drugs, mostly amphotericin B, may be employed to check the dissemination. The data from clinical trials has revealed the superiority of amphotericin B and flucytosine combination over amphotericin B alone as initial therapy in the management of cryptococcal meningitis in patients with HIV infection (Saag et al.2000). Discontinuation of such long term antifungal therapies resulted in severe complications. Limited in vitro data suggested that administering itraconazole before amphotericin B might inhibit activity of the latter in aspergillosis (Nucci et al.1994, Abuhammour and Hasan, 2004). The issue of antagonism, therefore, is of particular concern when members of the azole class are administered for prophylaxis. Populations largely at risk for antagonism associated difficulties are those infected with HIV or the recipients of bone marrow transplants who receive fluconazole prophylactically for recurrent oral, esophageal and deep-seated candidiasis. The continuation of treatment with potential compound alone or in combination with other drugs has been considered to be useful in maintenance of effects of therapy. Treatment with an azole antifungal has been commonly prescribed after a 2 week induction therapy with amphotericin B and flucytosine. Although fluconazole has been administered most frequently, studies supported itraconazole as an alternative (Saag et al.1999). The duration of maintenance therapy with an azole varies. It is typically life-long in certain high-risk patients with HIV infection. However, in those without persistent immune suppression, therapy may last for 6 months to one year. Although the long term sequential treatment with potential drugs like amphotericin B with or without other drugs has not been feasible due to their administration related inconvenience and the accumulative toxicity, the less toxic drugs like fluconazole may provide a viable option for long term treatment. However, the sequential use of drugs for very long time is still not very frequent. There are few reports which have described the advantages of sequential therapy in treating aspergillosis (Steinbach et al.2003). Amphotericin B or liposomal-based formulations of amphotericin B typically are given as induction therapy, followed by itraconazole for consolidation and maintenance (Patterson et al.2000). The role of the newer azoles such as voriconazole and posaconazole is not defined in Aspergillus infection, but initial studies are encouraging. Although more information is required to establish the usefulness of sequential therapy in human aspergillosis, it could be used as prophylactic therapies.
GENERAL CONSIDERATIONS ON ANTIFUNGAL DRUG DISCOVERY:

The production of antifungal agents is not at all infrequent in microbes. A huge number of molecules obtained from micro-organisms with reported *in vitro / in vivo* antifungal activity are being investigated at different centres to meet the common goal of developing new therapies. Only a small percentage of the micro-organisms living in the biosphere have been investigated till date for biologically active molecules and there has been still a huge unexplored reservoir of microorganisms which could be important source of natural compounds with enormous structural diversity that could be used for the development of novel antifungals. However fungi and humans are eukaryotes and at the molecular level, their cells are similar. This makes it more difficult to find or design drugs that target fungi without affecting human cells. Consequently many antifungal drugs cause side effects. Some of these side effects can be life threatening if the drugs are not used properly. Despite chemical therapies, serious fungal infections remain difficult to treat, and resistance to the available drugs is emerging (Loeffler and Stevens, 2003).

The non pathogenic bacterial species which are part of human gut flora can be tested for their antimycotic potential as they can be used either in the form of probiotics or may lead to non toxic antifungal formulations. In recent studies various lactic acid bacteria isolates including *Lactobacillus cornyiformis*, *Lactobacillus plantarum* has been found to have antagonistic potential against *Aspergillus fumigatus*, *Aspergillus nidulans* etc (Magnusson et al. 2003). An antifungal compound $\delta$-dodecalactone from *Lactobacillus plantarum* AFI1 isolated from kimchi showed strong inhibitory effect against *Aspergillus flavus*, *A. fumigatus*, *A. petrakii*, *A. ochraceus*, *A. nidulans*, and *Penicillium roqueforti* (Yang et al.2011). A lipopeptide that exhibited fungicidal, bactericidal, and insecticidal activity was isolated from *Bacillus thuringiensis* CMB26 (Kim et al.2004). A novel antifungal peptide produced by a *Bacillus* strain B-TL2 was isolated from tobacco stems (Zhang et al.2008). *Bacillus amyloliquefaciens* produces a broad-spectrum antifungal protein, baciamin which induces membrane permeabilization in fungi but not in rabbit erythrocytes (Wong et al.2008). An antifungal compound that is active against Mucoraceae and *Aspergillus* species has been reported from *Bacillus pumilus* (Bottone and Peluso,2003). A promising non-haemolytic anti- *Candida* protein from *Enterococcus faecalis* that might be used to treat candidiasis especially in immunocompromised patients has been characterized recently (Shiekh et al.2012). Since the literature on bacterial antifungal proteins is
rather scanty compared with that on bacterial bacteriocins, there is a pressing need to explore and isolate from new sources potential bacteria capable of producing novel antimicrobial proteins and to characterise them for further applications. In the microbial world, the *Escherichia coli* has been a very important member of great medical, biological, biotechnological and industrial significance. It was first described by Theodor Escherich in 1885 as *Bacillus coli*, inhabiting human intestine as a commensal. Subsequently *B. coli* was named as *Escherichia coli* after the name of inventor and some of its strains were found to be pathogenic also (Su and Brandt, 1995). As part of the normal flora of humans, it plays a crucial role in food digestion by producing vitamin K and creates barrier effect against enteropathogens (Portrait et al. 1999, Hudault et al.2001).

The EM0 and JM105 K-12 strains of *E. coli* isolated from human faecal sample and soil respectively were found to have ability of preventing *in vivo* and *in vitro* infection by *Salmonella typhimurium C5* and allergies (Barrow et al.1987, Barrow and Tucker,1986). However, it is not known if *E. coli* interacts with mycotic pathogens or it could be used as a resource of antinocytic molecules. Therefore, the present study was undertaken to identify, isolate and characterize the potential antifungal molecule (s) from non pathogenic bacterial strains mostly belonging to human microbiome and some natural antagonist species.