Chapter I
CHAPTER – 1

INTRODUCTION

In this century and more increasingly in the past two decades, many remarkable changes occurred in our way of life, producing opportunities for microbes. All these changes are related to the recent emergence of previously unrecognized diseases, or the resurgence of diseases that were thought to be under control. Ubiquitous fungi that previously had merely interfered with the freshness of our bread or simply colonized our skin, gastrointestinal tract, and environment are now known to cause invasive and potentially life-threatening infections. Not only are these new fungal opportunists increasing in frequency, but timely diagnosis and management of the infections they cause became difficult and will require a greater understanding of mycologic aspects of pathogenesis and principles of treatment than is currently accepted as standard.

The outcome of an infection with a human-pathogenic fungus often depends on the immune status of the host organism. Patients suffering from a weakened immune system are at high risk of developing a severe fungal infection. Continuous progress in medicine, e.g. in chemotherapy and organ or bone marrow transplantation, has led to an increasing number of patients with impaired immune status. Therefore incidence of opportunistic mycotic infections has increased significantly over the past two decades (Wisplinghoff et al.2004). This increase in infection is associated with excessive morbidity and mortality and is directly related to the expanding population of patients who are at risk for the development of serious fungal infections, including patients undergoing blood and bone marrow transplantation (BMT), solid-organ transplantation and major surgery (especially gastrointestinal surgery); patients with AIDS, neoplastic disease and advanced age; patients receiving immunosuppressive therapy; and premature infants (Zaoutis et al.2005, Pfaller et al.2004b). Given the complexity of the population of patients who are at risk for infection and the diverse and increasing array of fungal pathogens, opportunistic mycoses pose considerable diagnostic and therapeutic challenges.
Among the opportunistic fungal pathogens the filamentous fungus *Aspergillus fumigatus* is by far the most important cause of life-threatening invasive mycoses. The predominance of *A. fumigatus* in contrast to other *Aspergillus* species may be due to its ability to (1) grow abundantly everywhere; (2) produce tiny conidia, that can easily penetrate deep into the alveolar region; and (3) growth at 37°C (Pitts, 1994). Conidia of this saprophytic fungus can be found almost everywhere, from the winds of the Sahara to the snow of the Antarctic. Because of their ubiquitous presence in the air, each person inhales several hundred *A. fumigatus* conidia daily. In immunocompetent individuals, mucociliary clearance and phagocytic cells normally prevent the disease (Brakhage et al. 2010). The clinical manifestations of the diseases caused by *Aspergillus* depend upon the immunological state of the patient, and range from hypersensitivity reactions (Allergic Bronchopulmonary Aspergillosis) to non invasive colonization of previously damaged tissue (Pulmonary Aspergilloma) to acute or chronic limited invasive disease (Chronic necrotizing pulmonary Aspergillosis) to rapidly progressive invasive disease i.e Invasive Aspergillosis (Fridkin, 2005). In the above mentioned disease spectrum, Invasive Aspergillosis is often a fatal infection that occurs in severely immuosuppressed patients and is characterized by invasion of blood vessels.

(Figure A) Underlying disease in 595 patients with invasive aspergillosis (Patterson et al. 2000). BMT, bone marrow transplant; SOT, solid-organ transplant.

It is most prevalent in patients particularly those with haematological malignancy (Figure A) and patients undergoing haematopoietic stem cell transplantation (HSCT). Epidemiological surveillance demonstrates that invasive disease following HSCT is now often associated with ongoing immunosuppression
beyond the classic neutropenic period, associated with graft-versus-host disease (Pfaller et al.2006). The impact of more novel transplant regimens, such as the use of peripheral blood stem cells, use of antithymocyte globulin, nonmyeloablative conditioning or T cell–depleting therapies, appears to increase likelihood of invasive aspergillosis infection and will likely impact disease incidence (Fukuda et al.2003).

Critically ill patients within the ICU may also be at risk of invasive aspergillosis with corticosteroid usage being an underestimated risk factor in certain patient groups such as those with chronic pulmonary disease (Cornillet et al.2006). However, there is a correlation between the degree of immunosuppression and the risk of contracting IA. Consequently, important risk factors include neutropenia, T cell depletion, CD34 selected stem cell products, corticosteroid therapy and cytomegalovirus infections (Marr et al.2002). Outbreaks of hospital-acquired aspergillosis were attributed to demolitions and re-buildings of old houses, hospitals and other constructions very close to the places where the risk patients were housed. Defective or incorrect handling of air conditioning systems also represents risk for nosocomial Aspergillus outbreaks (Richardson, 1998). The initial and most common site of infection for IA is the respiratory tract (paranasal sinuses, lungs), followed by severely traumatized skin. From these sites, dissemination to other organs by haematogenous spread occurs frequently. Aspergillus species, as well as all the other hyphomycetes (agents of hyalohyphomycosis and phaeohyphomycosis) and the Mucorales, have the tendency to invade the blood vessels causing haemorrhagic infarction and thrombosis in these severely immunosuppressed patients (Patterson et al.2000). Many studies have shown that IA is now more widely recognized, reflecting its main association with severe immunosuppression as a result of both neutropenia and compromised cell-mediated immunity.

A study using the National Hospital Discharge Data from the 1990s estimated that 10,190 aspergillosis-related hospitalizations occurred annually in the United States, resulting in 1970 deaths and $633.1 million in costs (Dasbach et al.2000). The aspergillosis related mortality in acute myeloid leukemia is 30-40% (Pagano et al.2010) and number of patients with aspergillosis in the US ranges from 0.5% (after autologous hematopoietic stem cell transplantation) to 3.9% (after transplantation from an unrelated donor). In these patients after diagnosis of aspergillosis within three months mortality was 53.8% in autologous transplant recipients and 84.6% in those with unrelated donor transplants (Morgan et al.2005) but in the most recent data from
the US, 12 month mortality from IA (invasive aspergillosis) was 25% (Kontoyiannis et al. 2010). This shows that there is improvement but the risk of fungal infection after transplantation is multifactorial.

There is currently a lack of reliable diagnostic tools and effective treatment options for invasive aspergillosis, resulting in a high mortality rate despite therapy. Remarkably, A. fumigatus causes 90% of all systemic Aspergillus infections (Horn et al. 2012). This indicates that A. fumigatus possesses certain virulence determinants that favour this species becoming an opportunistic human pathogen. But unfortunately these fungi are one of the most neglected pathogens as demonstrated by the fact that the Amphotericin B (Ampho.B), a polyene antibiotic discovered as long ago as 1956, is still used as a gold standard for antifungal therapy.

Until the 1970s, fungal infections were considered largely treatable and the demand for new medicines to treat them was very small. Before this period, antifungal chemotherapy included only two kinds of compounds: potassium iodide, effective in the treatment of sporotrichosis; and two useful polyenes, nystatin and Ampho.B, which were introduced in the 1950s. Except for the development of flucytosine (1964), there was little progress until the development of theazole drugs in the early 1970s. Therefore, only a limited number of antifungal agents (polyenes and azoles plus the recently introduced Cancidas; Caspofungin acetate) are available for the treatment of life-threatening fungal infections. This shows that our current antifungal armamentarium is not adequate. Moreover, the development of antifungal drugs has lagged far behind that of antibacterial agents. Fungi are eukaryotes and despite the presence of a cell wall, fungi are more similar to mammalian cells on a cellular level than to bacteria. Additionally, fungi replicate more slowly than bacteria and are often difficult to quantify, particularly for moulds, which complicates efficacy assessments.

Currently drugs used for systemic therapy of invasive mycoses have three main targets: the polyenes and azoles target the cell membrane, the antimetabolite 5-fluorocytosine interferes with DNA and RNA synthesis and echinocandins affect the cell wall. In January 2001, the echinocandins (Caspofungin) became the first semisynthetic antifungal natural product to be approved since polyene was approved forty years earlier, and in 2005 micafungin (Mycamine, Astellas) became second and in 2006 Anidulafungin (VER-002, V-echinocandin, LY303366, Vicuron
Pharmaceuticals) became third FDA approved echinocandin. The other class azole except Voriconazole (2002) and Posaconazole (2006), allylamine and antimetabolite were present prior to 2001. Although the new generation azoles have been much improved as compared to previous compounds of this class; the concerns with respect to limited efficacy could not be resolved. The fungistatic character has been the main drawback of these compounds. Ampho.B showed strong antifungal potential, but extremely high toxicity was undesirable. New liposomal formulations of Ampho.B were found to have reduced toxicity, but cure rate did not improve much. A new antifungal agent called caspofungin was launched in 2002 and included in the existing therapeutic options for candidiasis and invasive aspergillosis in patients refractory to, or intolerant of, treatment with other drugs. Caspofungin showed good activity against Aspergillus and Candida, however, its clinical efficacy guided market is yet to be established. Other major concerns associated with available antifungal drugs have been the drug induced immunosuppression and development of resistance in pathogens against almost all the chemotherapeutic agents. It is thus evident that we do not have any useful drug with desirable cure rate and acceptable levels of adverse effects. Therefore, the identification of new antifungal agents, preferably from natural sources with novel mechanisms of action, is an urgent medical need.

Microbial natural products are the origin of most of the antibiotics in the market today. However, research in antibiotics and natural products has declined significantly during the last decade as a consequence of diverse factors, among which the lack of interest in screening approaches and the strong competition from collections of synthetic compounds as a source of drug leads. Still, microbial natural products remain the most promising source of novel antibiotics owing to their much diversity and less toxicity. Most of the antibiotics and antifungals present in market today are of bacterial origin as demonstrated by the fact that the gold standard drug Ampho.B and Nystatin are isolated from Streptomyces nodosus and Streptomyces noursei respectively. The exploration of natural bio-resources for treating fungal infections has been emphasized in recent years (Roessner et al.1996, Dabur et al.2005, Valerio et al.2009, Alvarez et al.2011). Antifungal proteins such as thaumatin like proteins, glucanases, chitinases, and ribosome inactivating proteins, cyclophilin like proteins, miraculin like proteins, cysteine rich peptides like thionins and lipid transfer proteins have been reported from a variety of sources including bacteria, mammals.
insects and plants for treating fungal infections (Selitrennikoff,2001, Ye et al.1999). Although these proteins have been shown to inhibit the growth of pathogenic fungi, many of them were found to be highly toxic. However less toxic antifungal proteins have been described from bacterial sources (Sheikh et al.2012). Hence one approach leading to a novel class of antifungal compounds might be testing of non pathogenic bacterial species for their antifungal activities. In recent studies antimicrobial activity was found to be enhanced by a pool of bacteria (Furtado et al.2002). The Nikkomycins, nontoxic to mammalian cells were isolated from *Streptomyces tendae* and studied for their antifungal activities (Bormann et al.1999). The antifungal activity spectrum of *Lactobacillus coryniformis* (sub sp. Coryniformis strain Si3) was investigated for activity against *A. fumigatus* (Magnusson et al.2001). *Bacillus amyloliquefaciens* is widely recognized as a powerful bio-control agent. It has been found to have antifungal activity against many phytopathogenic fungi (Balhara et al.2011). While the main emphasis has been on *B. amyloliquefaciens* metabolites for biocontrol of plant pathogens but they also have potential against human pathogens. One of its metabolites, Macrolactin A displays antimicrobial activity toward human pathogens such as *Klebsiella pneumoniae* and *Staphylococcus aureus* (Patel et al.1995). *B. amyloliquefaciens* LBM 5006 showed significant activity against phytopathogenic fungi, showing potential for use as a biocontrol agent or production of antifungal preparations (Benitez et al.2010). Various products of *Pseudomonas* species were also reported to have activity against different types of fungi i.e. *Pythium ultimum, Rhizoctonia solani, Phytophthora capsici, Fusarium oxysporium* (Ligon et al.2000), *Aspergillus niger* and *Candida* spp (Sorensen et al.1996). A protein called SAP obtained from marine bacteria *Streptomyces* was found to be effective against a fungal species *Pythium porphyra*, the causative agent of Red Rot disease in *Porphyra* species. The SAP protein was found to be non toxic to *Porphyra yezoensis* cells, human dermal fibroblasts and mice (Woo et al.2002). Similarly other bacterial species like *Streptomyces hygroscopicus* strain BS-112 has been reported to possess antifungal activity against *Aspergillus flavus* (Zhang et al.2011).

Another approach leading to non toxic antifungal formulations might be screening of bacterial species belonging to human microbiome for their antifungal potential. The normal resident intestinal microflora is the major factor protecting animals and humans against intestinal colonisation by pathogenic bacteria (Raibaud, 1996). *E. coli* is one of the first bacterial genera, along with *Streptococcus*, to colonise
the intestine of human (Benno et al.1984). It has been reported that several E. coli strains develop a protective effect against antibiotic resistant, colicin sensitive, and enterotoxigenic E. coli (Barrow et al.1996). Different strains of E. coli have been used as probiotics (Matricardi et al.2003, Goerg and Schlorer, 1998). Most of the work of E.coli was concentrated on probiotic strain known as Nissle 1917(discovered by Alfred Nissle). It was isolated during World War I from a soldier who survived a severe outbreak of diarrhoea. The use of E. coli as probiotic under trade name “Mutaflor®” improved symptoms in patients with non-infectious bowel disorders and reduced significantly the incidence of allergies in children. Its usefulness in treating ulcerative colitis (Fric and Zavoral, 2003, Kruis et al.2004) and colonic Crohn’s disease has been reported recently (Malchow, 1997). The replacement of natural pathogenic flora from the gut by useful strains of E. coli after birth has been proved to prevent nosocomial infections and allergies particularly in formula-fed and high-risk infants of allergic mothers (Lodinova-Zadnikova et al.2003). There are also bacterial products, which have been recommended for treatment of fungal diseases but their clinical efficacy is not well documented (Matricardi et al.2003). But supplements of non pathogenic, commensal bacteria, probiotics, can be taken orally to refresh and complement the bacteria present in the gut. Therefore the present study was aimed to screen the antifungal potential of non-pathogenic bacterial strains and to isolate and characterize the active antifungal molecule from the chosen bacteria so that a novel and safer antifungal agent can be developed for potential use in future antifungal therapy.
The aims and objectives of the present study were as follows:

1. Preparation of bacterial supernatant and lysate for antifungal activity against pathogenic fungi.
2. Purification of antifungal protein.
3. To study the in vitro antifungal and fungicidal activity of the purified protein.
4. Studies on the toxicity of the purified molecule.
5. Elucidation of molecular mass.
6. Characterization of purified molecule.