PLANT PATHOGENIC FUNGI AS A SOURCE OF ANTICANDIDAL DRUGS

A

Thesis Presented

In Partial Fulfillment for the

Award of M. Sc. Degree in Biotechnology

By

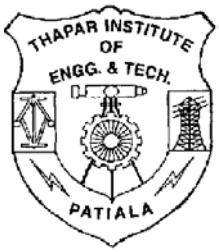
SHIVANI TAYAL

M.Sc. 2nd Year

2005

Under The Guidance

Of
Dr. Sanjai Saxena



DEPARTMENT OF BIOTECHNOLOGY & ENVIRONMENTAL SCIENCES

THAPAR INSTITUTE OF ENGINEERING & TECHNOLOGY
(Deemed University)

PATIALA 147004 (India)

CERTIFICATE

This is to certify that the thesis entitled **"Plant Pathogenic Fungi as a Source of Anticandidal Drugs"** submitted by Ms. Shivani Tayal in partial fulfillment of the requirement for the award of Degree of Master of Science in Biotechnology, to Thapar Institute of Engineering & Technology (Deemed University), Patiala is a record of student's own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

Dr. Sanjai Saxena **Head**
Supervisor

Department of Biotechnology and Environmental Sciences
Thapar Institute of Engineering and Technology
Patiala- 147004

Dean (Academic Affairs)
Thapar Institute of Engineering & Technology,
Patiala

ACKNOWLEDGEMENT

I thank the almighty whose blessings have enabled me to accomplish my dissertation work successfully.

It is my pride privilege to express my sincere thanks and deep sense of gratitude to **Dr. Sanjai Saxena, Lecturer, Department of Biotechnology & Environmental Sciences, TIET, Patiala** for his valuable advice, splendid supervision and constant encouragement in pursuance of this work. His association with this endeavor of mine will remain a beacon light to me throughout my life.

My sincere thanks to **Dr. N.Das, Head, Department of Biotechnology & Environmental Sciences, TIET, Patiala** for providing me an opportunity to do this project work. I am very thankful to **Dr.Sunil Khanna, former Head, Department of Biotechnology & Environmental Science** for his guidance, suggestions & constant support. My gratitude is due to all faculty members for their help, inspiration and moral support, during the course of my study.

I do not find enough words with which I can express my feeling of thanks to **Ms. Charu Gomber** for their invaluable suggestion, constant support and encouragement that went a long way in successfully completion of my work.

I am especially thankful to **Ms.Sunita Bansal, Ms. Vijay Rana, Mr.Anshu Bansal**, for their constant cooperation and timely help.

The cooperation received through my colleagues namely **Ms.Navdeep Kaur, Ms. Shaweta Goyal, Ms.Urvashi Jaiswal, Ms.Jyoti, Mr. Rajneesh, Mr. Gurdeep Singh Sandhu, Ms. Dakshi Taneja, Mr. Jesse S.Sameul, Mr. Nilabh Anand, Mr. Jatinder Singh, Mr. Mukesh Kumar** is thankfully acknowledged. I owe a word of thanks to all the research scholars, lab assistants and those that could not find a separate name but helped me directly or indirectly.

Date:

Place: Patiala **Shivani Tayal**

CONTENTS

ABBREVIATIONS

AWD assay	Agar Well Diffusion Assay
AIDS	Acquired Immune Deficiency Syndrome
CFU	Colony Forming Unit
DMSO	Dimethylsulfoxide
HIV	Human Immunodeficiency Virus
MEB	Malt Extract Broth
MIC	Minimum Inhibitory Concentration
MIZ	Maximum Inhibitory Zone
OPC	Orophyrangeal Candidiasis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Rf	Retention Frequency
RCHDB	Richard Broth
SDA	Saborauds Dextrose Agar
TLC	Thin Layer Chromatography
TTC	2,3,5-TriphenylTetrazolium Chloride
UV	Ultra Violet
VVC	Vulvovaginal candidiasis
YEPDA	Yeast Extract Peptone Dextrose
YEPDB	Yeast Extract Peptone Broth

EXECUTIVE SUMMARY

All higher plants are host to one or more microbes. Only a few these plants have been completely studied relative to their microbial biology. However fungi, which infect plant have mechanism to overcome the plant defense mechanism by the way of production of some secondary metabolites. These metabolites however have not been screened for their ability to be used as new antimycotics. So plant pathogenic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas.

In the present study, screening of pathogenic phyllosphere fungi of plants likes *Lantana camara, Hibiscus rosa-sinensis & Cannabis sativa* was done by production of secondary metabolites by the process of fermentation. Antifungal activity of chloroform, dichloromethane, ethyl acetate, acetone, and methanol extracts of spent broth of pathogenic phyllosphere fungi was evaluated against test microorganisms by using agar well diffusion assay (prescreen). Studies revealed the activity of the different extracts produced by pathogenic phyllosphere fungi against multidrug resistant yeast. Among the extracts tested, chloroform extract was the most active. It exhibited prominent antifungal activity against *Candida albicans* (G1-isolate), *Candida albicans* (G2-isolate) and *Candida albicans* (G4-isolate). Chloroform extract evaluated was validated against standard antifungal antibiotics e.g., Griseofulvin to know the efficacy of the extract.

MIC of the chloroform extract was determined using broth macrodilution assay. Chloroform extract exhibited similar trend of MIC against the test organisms. The MIC of the chloroform extract was ranging between 40 µg/ml to 128 µg/ml for all the test microorganisms. Death pattern of the test organisms in presence of the extracts was determined using macrobroth test tubes and plate count method. t was found that 90-100% reduction in the microorganism count occurred between 8-16 hours after the growth.

Partial purification of the extracts was done using Thin Layer Chromatography (TLC). TLC purified fractions 4,6,7,8,9 exhibited antifungal activity against test organisms. So the present study establishes the role of pathogenic phyllosphere fungi as a potential source of natural products for

exploitation in medicine, agriculture & industry. These could be helpful in overcoming resistant of fungal pathogens.

INTRODUCTION

INTRODUCTION

Like bacteria, fungi are also responsible for a variety of infections in human beings. Fungi are a large group with about 250,00 species which are major pathogens of agricultural plants and important opportunistic pathogens of humans recently ranking as the seventh most common cause of infectious disease–related deaths in the United States (Odds, 2000). More than 300 species have been reported to be potentially pathogenic to humans (Guarro *et al.,* 1997). The infections caused by fungi are generally referred to as Mycoses. These can further be classified into four groups viz. Superficial, Subcutaneous, Systemic and Opportunistic. Fungal infections in humans are generally confined to superficial areas such as skin infections, however in immunocompromised people mild or nonpathogenic fungi can prove to be fatal. Opportunistic fungal pathogens are the causes of variety of invasive or systemic fungal infections in immunocompromised or immunosuppressed individuals.

Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients. Fungi previously thought to be non-pathogenic for humans or sporadically associated with human diseases. Pathogens like, *Candida* (except *albicans*), *Fusarium*, *Trichosporon*, and *Malassezia* species are emerging as important nosocomial pathogens.

Approximately 90% of human fungal infections are caused by *Aspergillus, Candida, Cladosporium, Epidermophyton, Microsporum and Trichophyton species* (Pathak, 1998). Of major concern are the cases of systemic mycoses caused by *Aspergillus fumigatus*, *Candida albicans, Cryptococcus neoformans, Fusarium speceis, Histoplasma capsulatum and Pneumocystis carinii,* which have been increasing over the years (Daniel, 1999)

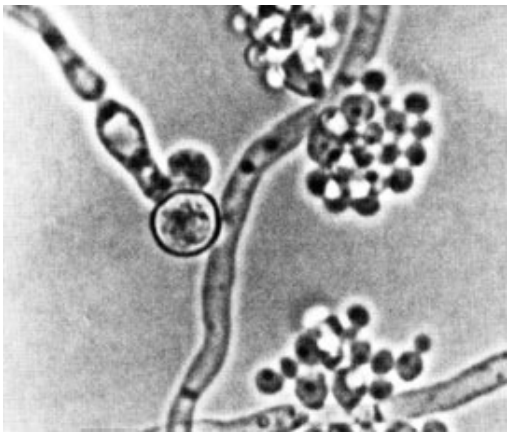
The prevalence of invasive or systemic fungal infections has increased significantly during the past decades (Walsh *et. al.*, 1996). These have become one of leading cause of death among patients due to greater use of broad spectrum antibiotics, immunosuppressive agents, intensive care of low birth weight infants, organ transplantation & the acquired immunodeficiency syndrome (AIDS) epidemic (Graybill, 1996).

Diseases like Candidiasis (*Candida albicans*), Apergillosis (*Aspergillus niger*) and Cryptococcosis (*Cryptococcus* species) are the forerunners in all

major fungal infections. The frequency of candidiasis has increased ten-fold, so that *Candida albicans* has become the fourth most common culture isolate. *Candida* species are the second most frequent isolates from blood cultures in hospitals with large population of immunocompromised patients, second only to coagulase-negative *Staphylococcus aureus* (Banerjee, 1989). Invasive pulmonary aspergillosis is a leading cause of attributable mortality inbone-marrow transplant recipients (Beck-Sague and Jarvis, 1993).

Today fungal infections are a real problem, having doubled in number from the 1980's to the 1990's, and with bloodstream infections increasing five-fold with an observed mortality of 55% (Torres *et al*., 1995). Candidiasis, Cryptococcosis and Aspergillosis have been frequently reported in persons suffering from Acquired Immune Deficiency Syndrome (AIDS) (White, 1997). Systemic candidiasis has been reported to occur in up to 10% of infants weighing less than 1kg; the greatest increases in surgical patients & 78% of fungal infections are due to *Candida* spp. (Beck-Sague, 1993)

Fig.1-***Candida albicans***



Candida albicans is yeast that lives in the mouth, throat, intestine & genitourinary tract of most humans & is usually considered to be normal part of bowel flora (the organisms that coexists with us in our lower digestive tract known as fungi). *Candida* coexists in our bodies with many bacterial species in a competitive balance. Other bacteria act in part to keep *Candida* growth in check in our body ecology unless that balance is upset. When health is present, the immune system keeps *Candida* proliferation under control, but when immune response is weakened, *Candida* growth can precede unhindered. It is an "opportunistic organism" one which when given the opportunity, will attempt to colonize all bodily tissues. The uncontrolled growth of *Candida* is known as **Candida overgrowth**. The major culprits of *Candida* overgrowth are antibiotics & sulfa drugs; they kill the "good" flora, which normally keeps the *Candida* under control. This allows for the unchecked growth of *Candida* in the gastrointestinal tract. *Candida* sp. in particular is the predominant mycotic pathogen whose isolation from the blood is an independent risk factor for

mortality (Pfaller *et. al.*, 1997). A cost of illness study has indicated that the annual cost of treating hospitalized patients suffering from Candidiasis is to the tune of $ 250 millions (Rentz *et.al*, 1998).

Need for New Antifungals

Fungal resistance has received little attention in contrast to the critical importance of bacterial resistance. Clinical resistances to the antifungal agents were rare till 1980's. The incidence of fungal infections including resistant infections has increased during 1990's reflecting increased incidence of Immunodeficiency associated cancer chemotherapy, organ and bone marrow transplantation and HIV epidemic (Iwamoto, *et.al,* 1994; Hosoe *et.al.*, 2000). Antifungal resistance may be defined as a stable inheritable adjustable by a fungal cell to an antifungal agent resulting in less than normal sensitivity to the antifungal agent. There are 3 factors contribute to antifungal drug resistance (White, 1998):

- Many clinical factors that contribute to resistance are associated with the pharmacology of drugs, or type of fungal infections present
- Cellular factors associated with a resistance fungal strain.
- Molecular factors that is ultimately responsible for resistance phenotype in the cell. The molecular mechanism of resistance that have been identified to date in *Candida albicans* include over expression or mutation of target enzyme & alteration of the enzyme in same biosynthetic pathway as target enzyme.

There have been several repots in 1990's, that drug resistance has become an important problem in a variety of infectious diseases including HIV infections, tuberculosis & other fungal or bacterial infections, which have profound effects on human health (Law *et.al.,* 1994)

Pathogenic fungi present a threat not only to immunocompromised patients with immune systems weakened by AIDS, aggressive cancer chemotherapy, or drugs aimed at foiling rejection of transplanted organs but also to others, particularly when microbes are resistant to antifungal agents (McGinnis *et.al.*, 1997; Odds *et.al.*, 2000)

For instance, 33% of late-stage AIDS patients in one study had drug-resistant strains of *Candida albicans* in their oral cavities (Denning, 1997) The rise in the incidence fungal infections has exacerbated the need for next

generation of antifungal agents, since many of currently available drugs have undesirable side effects, are ineffective against new or reemerging fungi or lead to rapid development of resistance (Vanden Bossche *et.al,* 1994; Graybill *et.al.,* 1988**)**. This drug resistance has resulted in drastic increase in incidence of opportunistic & systemic fungal infections. Resistance is considered as primary when an organism is resistant to drug before exposure. Whereas secondary resistant is that which develops in response to the drug. The latter mechanism of resistance accounts for the emergence of resistant fungi to azoles & polyenes seen over last few years (White, 2002).

The prevalence of resistant strains is due to

- An increased reliance on antimicrobial medication, giving resistant strains a selective advantage
- The recent trend towards aggressive resuscitation & invasive surgery, favoring infection.
- The treatment of more immunocompromised patients such as very elderly, the HIV positive & intentionally immunodepressed.

There are only few available drugs for treatment of fungal infections in immunocompromised patients or in severe systemic pathology i.e. therapeutic choices for treatment of fungal infections are limited. So search for new compound with low toxicity & stability is a priority in field of anti-fungal therapy.

Antifungal drugs basically belong to two broad categories:

(a) Those made synthetically.

(b) Those produced by various organisms.

Most people become interested in synthetic drugs because of their quick action as compared to traditional medicines & secondly because of their bulk production in industries. New microbes and their products are discovered for medicinal uses. Their products were extracted & then synthesized in the laboratory since 1970 almost 75% of such medicines are of synthetic origin or products of fermentation (Brewer *et.al.*,2000).

The Antibiotic Era: Microbial Secondary Metabolites

It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive

natural products. Then, scientific serendipity and the power of observation provided the impetus to Fleming to usher in the antibiotic era via the discovery of penicillin from the fungus *Penicillium notatum*. Since then, people have been engaged in the discovery and application of microbial metabolites with activity against both plant and human pathogens (Grabley *et.al.*1999; Concepcion *et.al.*2001). The discovery of antifungal antibiotics started with the discovery of Griseofulvin or the curling factor from the fungus *Penicillium griesofulvum* and *P. janczewskii*. After this Cyclohexamide was isolated from Streptomycin yielding culture of *Streptomyces griesus* (Whifften *et.al.*, 1946). The discovery of antibacterials were pursed more vigorously as compared to antifungals since fungi are eukaryotes and like mammalian cells have the same targets of action and therefore could prove more toxic (Georgopapadakou and Walsh, 1994)

The pace of screening antibiotics for their uses in agrochemical and pharmaceutical industry increased at an amazing rate from 200-300 per year in late 1970's and increased to 500 per year by 1997 (Demain, 1999). Around 90% research organizations across the world screen microroganisms without targeting a taxon. However actinomycetes and fungi are screened by all organizations that are involved in natural product drug discovery programs. The initial trends was screening of microfungi in litter but more recently emphasis is paid on host- microbe interactions of fungi at different environmental settings like plants, insects and the marine organisms for finding out new biologically active structures. For example *Tolypocladium inflatum* is an entomopathogenic fungi from which the first immunomodulatory drug as well as antifungal drug Cyclosporine was isolated for the first time (Borel *et.al,.*1976).

Currently used Antifungal Agents

These antifungal agents have a wide application in human medicine, agriculture and veterinary medicine (Vandamme *et. al.*, 1984). Five major classes of systemic antifungal compounds are currently in clinical use: the polyene antibiotics (Amphotericin B), the azole derivatives (Fluconazole & Itraconazole), the allylamines (Terbinafine) and thiocarbamates, the morpholines and the nucleoside analogs (Georgopapadakou and Walsh, 1996). The first three are targeted against ergosterol, the major fungal sterol present in the plasma membrane. The fourth inhibits sterol biosynthesis and the fifth class targets the DNA synthesis. Griseofulvin, a nuclear division and

membrane tubule inhibitor, and lipopeptides that are known to act on *Pneumocystis carinii* belong to a miscellaneous class of compounds (Morris *et al.* 1994). But these antifungal antibiotics are becoming resistant. More recently new antifungals have been introduced by the Merck R&D, which is known as Echinocandins (Caspofungin).

Today's market for systemic antifungal drugs that can be used to treat invasive fungal infections is estimated to be around $5 billion globally. The most dominated azole the fluonazole {Pfizer's Diflucan} was first launched in the mid-1980 and now enjoys sales of over $1billion per year. Fluconazole succeeded in displacing amphotericinB for the treatment of invasive *Candida* infections; Jhonson & Jhonson's Sporanox (itraconazole) suffered from lack of an intravenous formulation for years. The toxicity problems of amphotericin have been ameliorated to some extent by the development of liposome formulations, the most successful of which is Gilead's Ambisome, though these formulations are very costly.

Plant Pathogenic Fungi as a Source of Antifungal Agents

All higher plants are host to one or more microbes. These are relatively unstudied & potential sources of novel natural products for exploitation in medicine, agriculture & industry. It is noteworthy that of nearly 300,000 plants speceis that exists on earth, each individual plant is host to one or more microbes. Only a few of these plants have ever been completely studied relative to their microbial biology. As a result, opportunity of finding new & interesting microorganism within myriad of world's plant is great (Demain *et. al.*, 2000)

In addition, in case of microbe being symbiotic, new & unusual organic substances may be discovered that contribute to host-microbe relationship while at same time providing new & interesting bioactivating compounds that may find uses in medicine, agriculture & industry. The most frequently isolated microbes are the fungi. It turns out that vast majority of plants have not been studied for their pathogenic fungi. Thus, enormous opportunities exist for recovery of novel fungal form, taxa & biotypes. It was estimated that there might be as many as one million different fungal species, yet only about 100,000 have been described (Hawkswarth & Rossman, 1987)

Natural products often serve as lead molecules whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry.

Natural products have been the traditional pathfinder compounds, offering an untold diversity of chemical structures unparalleled by even the largest combinatorial databases (Thiericke *et. al.*, 1999)

However fungi, which infect plant, have mechanism to overcome the plant defense mechanism by the way of production of some secondary metabolites. These metabolites however have not been screened for their ability to be used as new antimycotics. It is conceivable that these plants have microbes that mimic the chemistry of their respective host plants and make the same bioactive natural products that are more bioactive than those of their respective host (Strobel *et. al.*, 2002) and these could be helpful in overcoming resistance of fungal pathogens.

REVIEW OF LITERATURE

Occurrence of Resistance in Fungi

The incident of fungal infections, including resistant infections, has increased during the last ten years, reflecting increased incidence of immunodeficiency associated with cancer chemotherapy, organ & bone marrow transplantation & HIV epidemic (Mary *et. al.*, 2004). More than 450,000 patients annually suffer from serious, systemic infections caused by fungi & number is expected to increase significantly.

Existing drugs are not adequately addressing the problem & mortality rate remains high. Recently it was reported that 30% of patients with advanced AIDS have developed azole resistance *Candida* infections **(**Revankar *et.al.,* 1996). Nearly 60% of patients infected with candidiasis have developed fluconazole-resistant *Candida* isolates (Cartledge *et.al.*, 1997). Amphotericin B is used in the treatment of serious disseminated dimorphic fungal and yeast infections, caused by *Blastomyces*, *Candida*, *Cryptococcus* and *Histoplasma* spp. However, it causes nephrotoxicity, reduction of renal blood flow, nausea, vomiting and anorexia. Nystatin, although too toxic for systemic use, is mainly applied topically in cases of mucous membrane candidiasis.

Griseofulvin causes hepatotoxicity and gastrointestinal distress but is used for the treatment of certain dermatophyte infections, caused by *Epidermophyton*, and *Trichophyton* species. 5-Fluorocytosine interferes with DNA synthesis and causes bone-marrow toxicity, leukopenia and liver enzyme elevations (Iyer, 1998). The predominant factors that affect and limit the use of some of the existing antifungal antibiotics, are low potency, poor solubility, limited or inconvenient dosage forms, narrow clinical spectrum, rapid emergence of resistant strains and drug toxicity (Shadomy, 1987; Bennett. 1990; McGinnis and Rinaldi, 1991; Odds, 2000).

The possible mechanisms of drug resistance are: (1) changes in membrane permeases (Kurtz, 1998), (2) changes in cellular efflux mechanism (Clark *et. al.*, 1996), (3) changes to a particular fungal “activase”whose action is required before that agent becomes metabolically active (Kurtz, 1998) and (4) mutations that render the target enzyme less sensitive or insensitive to the antimycotics agent. In addition, regulatory mutations that increase cellular levels of an essential enzyme can also provide means of resistance to a particular agent. For instance, increased production of lanosterol demethylase enables *Candida albicans* to withstand antimycotics agents such as amphotericin B (Kurtz, 1998; Wills *et. al.* 2000).

Candida species are now emerging as major agents of hospital-acquired infections; they are ranked as the third most commonly isolated bloodstream pathogens, surpassing gram-negative bacilli in frequency (Ellis *et.al.*, 2002). The genus *Candida* is composed of an extremely heterogeneous group of organisms that grow as yeasts. Most members of the genus also produce a filamentous type of growth (pseudohyphae). In addition to pseudohyphae, *Candida albicans* and *C. dubliniensis* form true hyphae (germ tubes) and thick-walled cells referred to as chlamydospores. Although *C. albicans* is the predominant etiologic agent of candidiasis, other *Candida* species that tend to be less susceptible to the commonly used antifungal drugs such as *C. krusei, C. glabrata, C. lusitaniae,* and the newest *Candida* species, *C. dubliniensis,* have emerged as substantial opportunistic pathogens. *Candida dubliniensis* shares with *C. albicans* many virulence factors, such as germ tube formation, exoenzyme production, and phenotypic switching (Sullivan and Coleman, 1997). This species, however, unlike *C. albicans,* has been shown to readily develop stable resistance to Flucanazole *in vitro* and in infected patients, strongly suggesting that *C. dubliniensis* possesses a readily inducible Flucanazole resistance mechanism (Jabra-Rizk *et.al.,* 2000). In 1990, 16.1 fungal infections per 1,000 discharges were seen in burns and trauma patients, 10.1 per 1,000 in cardiac surgery patients, and 7.3 per 1,000 in general surgery patients; the vast majority (78%) of which were due to *Candida* species (Beck-Sagué and Jarvis, 1990).

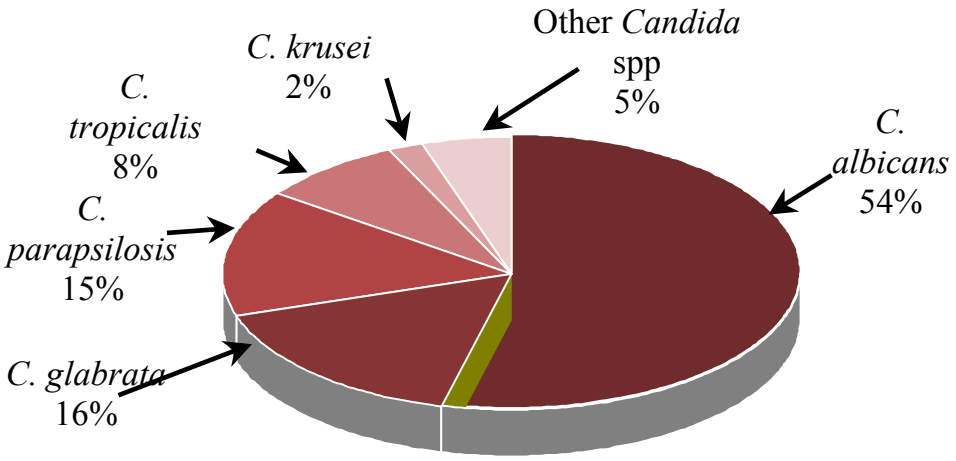


Fig2-Species of *Candida* most commonly isolated in blood stream infections (Source:International surveillance study in USA 1990)

Candida albicans is frequently present as part of the microflora of the gastrointestinal tract or the oropharynx in the normal human host. Between 10 to 40% of healthy people carry yeasts in throat and gut in low concentration of 10^3 cfu/ml of saliva or gram of faeces. Changed host defense can lead to an overgrowth of *C.albicans* (Vincent *et.al.*, 1998).

The European Prevalence of Infections in Intensive Care (EPIC) study collected data on 10,038 patients in 1,417 ICUs in 14 European countries on a single day in 1992. Of the patients, 44.8% were being treated for infection, of which 17.1% were associated with fungi (mainly *Candida* species) (Vincent *et.al.,* 1995). Fungi were the fifth most common pathogens, after *Enterobacteriaceae, Staphylococcus aureus, Pseudomonas aeruginosa* and coagulase-negative *Staphylococci. Candida albicans* used to be the most common pathogen, but in recent years there has been a major shift towards other *Candida* types such as *tropicalis, krusei, glabrata and parapsilosis*. Autopsy data indicate that more than half of the patients who die with malignancies are infected with *Candida* spp., approximately one-third with *Aspergillus* species, and increasing numbers with *Cryptococcus* species or other fungi such as *Fusarium* species (Richardson, 1991; Walsh, 1992). *Fusarium* causes serious morbidity and mortality and may mimic aspergillosis caused by *A.fumigatus, Cryptococcus, Trichosporon beigelii, Penicillium* and *Mucor* spp. are pathogens mainly affecting severely immunocompromised or neutropenic patients, with grave consequences if untreated. *Pneumocystis carinii* is also now classified as a fungus. During the 1980's *Pneumocystis carinii* was the most common cause of severe respiratory failure in AIDS patients (Torres *et al.*, 1995).

An estimated 75% of women will experience at least one episode of vulvovaginal candidiasis (VVC) during their lifetime. VVC is often associated with conditions such as diabetes mellitus, antibiotic therapy, and pregnancy, but many women have no predisposing factors (Edwards,1995.). High degrees of anti-fungal drug resistance have been reported in *Candida* species and these have exhibited primary resistance patterns towards 5- Flucytosine (5-FU), Fluconazole and Ketoconazole (Cuenca-Estrella *et.al.*, 2001). In India the occurrence of *Candida* in vaginal swabs of infertile women is 13% of which 40.7% are isolates of *Candida albicans* (Verghese *et. al.*, 2001).

Need for New Antifungal Agents

An antifungal agent is a drug that selectively eliminates fungal pathogens from a host with minimal toxicity to the host. Although the first agent with antifungal activity, griseofulvin, was isolated in 1939 (Abi-Said *et. al.*, 1997) and the first azole and polyene antifungal agents were reported in 1944 and 1949, respectively (Fromtling, 1988). It was not until 1958 that oral griseofulvin and topical chlormidazole became available for clinical use (Gupta *et.al.,* 1994). The introduction of griseofulvin was followed in 1960 by that of amphotericin B, which is still the "gold standard" for the treatment of severe systemic mycoses (Georgopapadakou *et.al.*, 1996). Two topical azole antifungal agents, miconazole and clotrimazole, were introduced in 1969; this was followed by the introduction of econazole in 1974 and a parenteral formulation of miconazole in the late 1970s (Graybill, 1996). Today, these three agents remain the mainstay of topical therapy for many dermatophytoses.

Progress in the development of both topical and systemic antifungal agents lagged, due in part to the intensive research efforts in the area of antibacterial therapy, which began in the 1940s following the large-scale production of penicillin, and also to the relatively low incidence of serious fungal infections compared with that of bacterial infections. By 1980, members of the four major classes of antifungal agents polyenes, azoles, morpholines, and allylamines had been identified, yet the only new drug introduced for the treatment of systemic fungal infections was oral ketoconazole (Kauffman *et.al.*, 1997). It would be more than 10 years before either Flucanazole or Itraconazole became available for the treatment of systemic mycoses. During the 1980's and 1990's, the marked increase in the population of immunocompromised or severely ill individuals as the result of the spread of human immunodeficiency virus (HIV) infection, the increased use of immunosuppressive agents in association with organ transplants, chemotherapy, and improved life-saving medical techniques necessitating indwelling catheters led to a substantial increase in the occurrence of serious fungal infections (Ghannoum, 1997). Consequently, although it takes 10 to 12 years to bring a new drug to market and the cost of doing so has risen steadily (to $360 million in 1994) (Billstein, 1994). The growing need for antifungal agents, particularly those for the treatment of systemic mycoses, has made this a worthwhile area of pharmaceutical research.

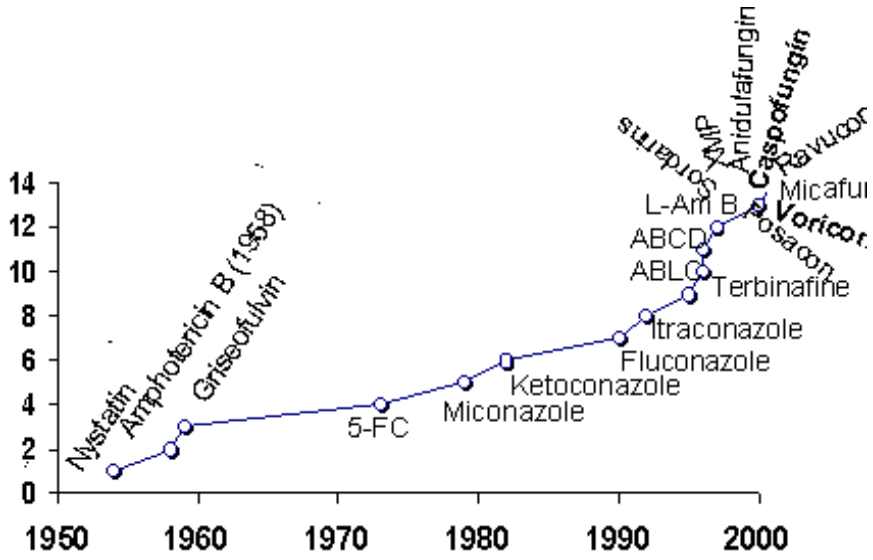


Fig.3- Medical Mycology of the Last 50 Years (Source: **WHO** 2000)

Advances made during the 1990s led to the introduction of a new allylamine, terbinafine, for the treatment of dermatophytoses and new lipid formulations of amphotericin B with improved safety profiles. In addition, new classes of antifungal agents such as the candins (pneumocandins and echinocanidins), the nikkomycins, and the pradamicins-benanomicins are being studied (Kauffman and Carver, 1997). However, with 15 different marketed drugs worldwide, the azoles are currently the most widely used and studied class of antifungal agents (Fromtling, 1998). Increased use of the azoles, coupled with the fact that they are fungistatic drugs, has likely resulted in the emergence of resistance to azoles (White, 2002). Oropharyngeal and esophageal candidiasis is a frequent problem in HIV-infected patients and usually precedes esophageal candidiasis.

Up to 90% of AIDS patients will experience at least one episode of Orophyrangeal candidiasis (OPC), and up to 50% may develop esophageal infection (Powderly, 1994). Although both ketoconazole oral tablets and itraconazole oral capsules are effective in the treatment of oral and esophageal candidiasis in HIV-infected patients, their use in patients with more advanced HIV infection has been limited because of problems with drug absorption (Elewski and Hay, 1996). Fluconazole, which is effective in both OPC and esophageal candidiasis, has become the standard agent for the treatment of these infections (Quart *et. al.*, 1995).

From 1988 to date, more than 20 million patients in the United States, including 500,000 with AIDS, have received Flucanazole. Clinical resistance to Flucanazole has emerged among patients with AIDS and OPC, and it was estimated that approximately 5% of patients with advanced AIDS and OPC or esophageal candidiasis will eventually fail to respond to fluconazole therapy (Rex *et. al.*, 1995). Recent reports suggest that in this clinical setting, approximately 30% of patients with advanced AIDS will develop azole-resistant *Candida* infection (Maenza *et.al.*, 1997). So due to rapid increase of resistance, there is need for antifungal drugs that actually kill the fungus (i.e.fungicidal) rather than only inhibiting its growth (i.e.fungistatic).

Screening Plant Pathogenic Fungi as a source of New Antifungal Agents

Due to the pharmaceutical potential of fungi, secondary metabolites of fungi have been studied for more than 70 years. The search for new drugs from fungi started with the discovery of Penicillin (Fleming, 1929), a potent antibiotic against Gram-positive bacteria, which was produced by *Penicillium notatum*. A further milestone in the history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine, which is produced by *Tolypocladium inflatum and Cylindrocarpon lucidum* (Dreyfuss *et. al.*, 1976). It was first discovered as an antifungal metabolite and later found to be immunosuppressive which made cyclosporine useful for the treatment following organ transplantation. The antifungal agent griseofulvin being isolated from *Penicillium griseofulvum* (Rehm, 1980) and the cholesterol biosynthesis inhibitor lovastatin isolated from *Aspergillus terreus* (Alberts *et. al.*, 1980) are two further examples supporting today's great interest in new secondary metabolites from fungi.

Far more than 4000 fungal metabolites are described (Dreyfuss & Chapela, 1994) and 5000-7000 taxonomic species have been studied with respect to their chemistry (Hawksworth, 1991). In 1995 Hawksworth estimated the probable number of existing fungi to be 1.5 millions with only 71, 000 being described so far. Apparently, the majority of fungi inhabiting the world have not yet been described. This implicates fungi to represent an enormous source for natural product with diverse chemical structures and activities. Of special interest are creative fungal strains. Creativity in this sense is defined as the ability to produce compounds of interest for human activities (Dreyfuss &Chapela, 1994). Even if the natural function of

secondary metabolites often is unknown, it is assumed that they play an important role in chemical defense and communication (Krohn, 1996). Many of them have been suggested to act as pheromones, antifeedants or repellents, and as regulators in the development of organism (Sterner, 1995). It was suggested that the biosynthesis of secondary metabolites does not occur randomly but is correlated with ecological factors (Gloer, 1995)

Most fungi studied to date have been isolated from soil and were proven to have a high creativity index. Genera such as *Aspergillus, Penicillium, Acremonium, Fusarium*, all typical soil isolates, are known for their ability to synthesize diverse chemical structures. Dreyfuss (1986), however, described a problem, which is often encountered during microbiological screening of fungal isolates for their secondary metabolite content. Increasingly, known metabolites are rediscovered making screening programs less efficient. This may be caused partially by using always the same well established isolation methods for fungi. Thus, often the same fungal strains are reisolated when investigating an ecological niche. This indicates that it might be more useful to investigate fungal isolates from other ecological niches than soil in order to take a more direct approach towards creative and novel fungal groups. Some relatively unexplored fungal groups derived from such ecosystems are, e.g., fresh-water fungi, marine fungi and endophytic fungi (Dreyfuss & Chapella, 1994).

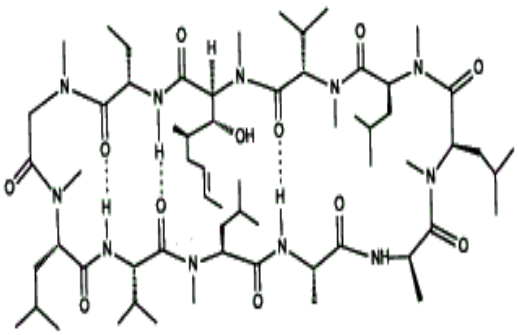
It is now known a untold number of microbes live within the plants and are known as endophytes. Some of these endophytes produce various useful bioactive molecules, which has encouraged worldwide scientific efforts to isolate & study them. Endophytic fungi isolated from medicinal plants are promising and microorganisms particularly are an important source of novel metabolites with antifungal, antibacterial & antiviral activity (Bacon and White 2000; Ellis and Ellis 1985). Eventually, a potent antifungal strain of *Serratia marcescens* was recovered from *Rhyncholacis penicillata* and was shown to produce oocydin A, a novel antioomycetous compound having the properties of a chlorinated macrocyclic lactone (White *et.al.*, 2000). It is conceivable that the production of oocydin A by *S. marcescens* is directly related to the endophyte's relationship with its higher plant host. In 1993, a novel paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized (Strobel *et al.*, 1999). Paclitaxel is a highly functionalized diterpenoid and famed anticancer agent.

Cryptosporiopsis quercina is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia. On petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens—*Candida albicans and Trichophyton species*. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from *C. quercina* (Strobel et.al. 1999). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins. Cryptocin, a unique tetramic acid, is also produced by *C. quercina*. This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant-pathogenic fungi (Li et. al. 2000). Ambuic acid, an antifungal agent that has been recently described from several isolates of *Pestalotiopsis microspora* (Li *et.al.* 2001) A strain of *P. microspora* was also isolated from the endangered tree *Torreya taxifolia* and produces several compounds that have antifungal activity, including pestaloside, an aromatic ß glucoside, and two pyrones: pestalopyrone and hydroxypestalopyrone (Lee *et.al.*, 1995)). These products also possess phytotoxic properties.

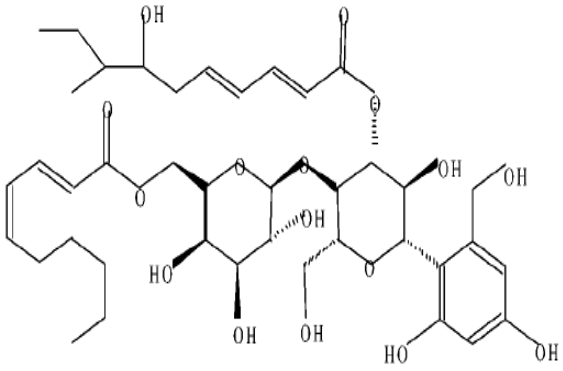
Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. It displays a moderate activity against the yeast *Candida tropicalis*. An endophytic *Fusarium* sp. from the plant *Selaginella pallescens*, collected in the Guanacaste Conservation Area of Costa Rica, was screened for antifungal activity. A new pentaketide antifungal agent, CR377, was isolated from the culture broth of the fungus and showed potent activity against *C. albicans* in agar diffusion assays performed on fungal lawns (Brady *et.al.*, 2000). Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in Artemisia mongolica, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou et.al. 2000). *Muscodor albus* is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong *et.al.*, 2001). This xylariaceaous (non-spore-producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds.

Corynecandin is a novel glycolipid analogous to the structural variant of papulacandin, named chaetiacandin and isolated from *Coryneum modonium* (Gunawardana *et al.*, 1997). Mer-WF3010 is another new member of the papulacandin class, isolated from the culture broth of *Phialophora cyclaminis* and similar to papulacandin B in that it inhibits the growth of *Candida albicans* and *C. kefyr* but not the growth of *A. fumigatus* or *C. neoformans*. Fusacandin, isolated from *Fusarium sambucinum*, is a structural variant of chaetiacandin (Lartey, 1997) and is active against *C. albicans*. Arthrichitin, a cyclic depsipeptide identified through screening for chitin synthesis inhibitors, is produced by *Arthrinium phaeospermum*, whereas LL15G256ϒϒγ is produced by the marine fungus *Hypoxylon oceanicum* and hasa broad-spectrum activity against *Candida, Trychophyton* and several phytopathogens (Fostel and Lartey, 2000). Aureobasidin A produced by *Aureobasidium pullulans,* belongs to the family of potent cyclic depsipeptide antifungals. Rustmicin (galbonolide A), galbonolideB and analogous antifungal macrolides isolated from *Micromonospora sp*. are inhibitors of sphingolipid biosynthesis. Khafrefungin isolated from an unidentified sterile fungus from a Costa Rican plant is active against *C. albicans* and *C. neoformans* (Fostel and Lartey, 2000). Spinofungins, myriocin, lipoxamycin and viridiofunginsare other compounds that have been reported (Zweerink *et al.*, 1992; Horn *et al.,* 1994; Mandala *et al.,* 1997).

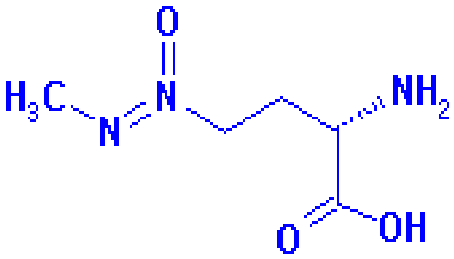
Pneumocandin Do, a new member of the echinocandin class of antifungal agents isolated from the filamentous fungus *Zalerion arboricola*, has been found to be a potent inhibitor of *Pneumocystis carinii* (Morris et al., 1994). Tricholin, a ribosome inactivating protein isolated from the culture broth of *Trichoderma viride*, has been shown to exert fungicidal effects on *Rhizoctonia solani* (Lin *et al.*, 1994). Azoxybacilin, a novel antifungal agent produced by *Bacillus cereus* NR2991 shows potent antifungal activity against mycelial fungi such as *Aspergillus fumigatus* and *Trichophyton mentagrophytes* (Fujiu *et al.*, 1994). Soraphen, known to have inhibitory activity against numerous fungi, was isolated from *Sorangium cellulosum* (Myxococcales) (Gerth *et. al.*, 1994).



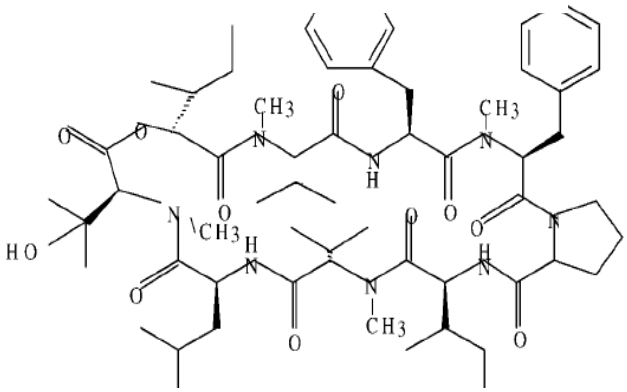
Cyclosporine



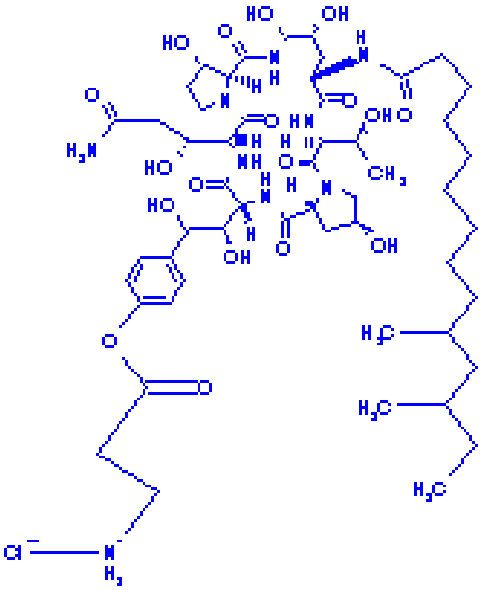
Corynecandin



Azoxybacilin



Aureobasidin



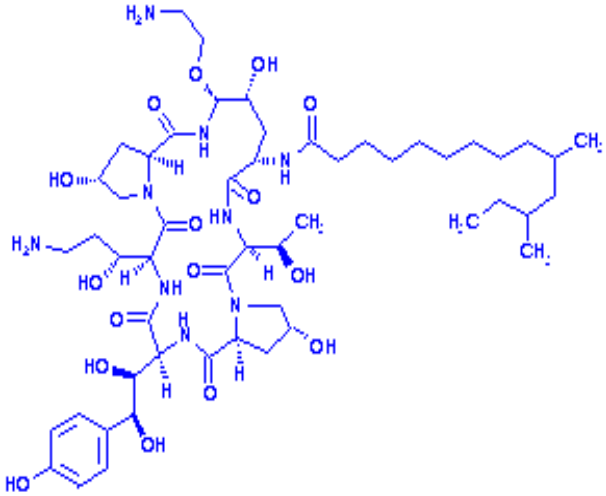


Fig4: Structures of some Commonly used antifungal agents

WF11899A, B, C, the novel antifungal lipopeptide antibiotics belonging to the echinocandin class, possess potent *in vitro* antifungal activities against *Candida* sp. (Iwamoto *et. al.,* 1994a, b).

Ascosteroside, a novel antifungal compound, was isolated from the culture broth of *Ascotricha amphitricha*. It is active against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* and against filamentous fungi but shows no activity against bacteria (Gorman *et.al.*, 1996*)*. *Aspergillus fumigatus* CY018 was recognized as an endophytic fungus for the first time in the leaf of *Cynodon dactylon*. A new nonaride compound, dihydroepiheveadride, was isolated from unidentified fungus IFM 52672 as the most potent antifungal principle from this organism. This compound showed strong antifungal activity against various filamentous fungi including human pathogens *Aspergillus fumigatus, Penicillium marneffei* and *Trichophyton species*. It also showed the growth inhibition activity against certain human pathogenic yeasts such as *Trichosporon* species, while it had weak or no antifungal activity against *Candida* spp and *Cryptococcus neoformans* (Hosoe *et.al.*, 2004). A new antifungal agent, CJ-19, 784, was isolated from the fermentation broth of a fungus, *Acanthostigmella sp*. CL12082. This compound inhibits the growth of pathogenic fungi, *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus* (Watanabe *et.al.*, 2001). Antifungal metabolites with a new carbon skeleton from *Keissleriella sp*. YS4108, a marine filamentous fungus were inhibitory to the growth of the human pathogenic fungi *Candida albicans*, *Tricophyton rubrum* and *Aspergillus niger* (Liu *et.al.*, 2002). A new pentanorlanostane derivative, cladosporide A, as a characteristic antifungal agent against *Aspergillus fumigatus*, isolated from *Cladosporium* sp. (Hosoe *et.al.*, 2000). Fungal strains of *Dichotomomyces Cejpii* isolated from fruit in Moldavia produce exo & endometabolites with antimicrobial activity against yeasts, moulds like *Aspergillus fumigatus, Fusarium moniliforme* etc. (Pieckova and Roeifmans, 1999). Ergokonin A an extracted compound from a *Trichoderma longibrachiatum* shows potent activity against *Candida albicans* & *Aspergillus fumigatus* (Vicente *et.al.*, 2001). *Oudemansiella mucida* and *Oudemansiella radicata* produced strobilurins and oudemansins. These kill oppurtunist pathogens such as *C. albicans* & dermatophytes belonging to genus *Trichophyton, Microsporum* (Anke *et.al.*, 1990; Florianowicz, 1999). Fungus *Guignardia* species, an endophyte of *Spondias mombin* having

activity against *Fusarium oxysporum, Saccharomyces cerevisiae* and *Aspergillus niger* (Rodrigues-Heerklotz *et.al.*, 2001).*Phomopsis* speceis isolated as endophytes from leaves of *Aspidosperma tomentosum* and twigs of *Spondias mombin* having activity against *Fusarium* species & *Candida albicans* (Christa Werner *et.al.*, 2000).

There exist a huge database of antifungals derived from endophytic fungi as well as soil fungi but plant pathogenic fungi particularily in the phyllosphere and rhizosphere have not been investigated for their abilities to produce novel chemical structures under different set of nutritional and environmental conditions.

Plant pathogenic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. They also exibit ample opportuntities of biotransformation of plant bioactives, which act as defence mechanisms and due course produce superior chemical moieities, which can be a part of armamentarium to combat antifungal drug resistance.

Fermentation

Fermentation is a process very much similar to anaerobic respiration and is carried out in a mixture of nutrients and metabolites essential for the growth and reproduction of the microbe. It is a metabolic process in many microorganisms and involves oxido-reduction reactions resulting in the breakdown of complex organic molecules into various end products with the release of energy. Fermentation is mostly extracellular and is brought about with the help of enzymes released by the microorganisms. The end products or the various intermediate products (primary and secondary metabolites) of the fermentation activities of many microorganisms are highly useful. Hence, microorganisms have been commercially exploited by the fermentation industry. Thus, with reference to industrial microbiology, fermentation may be defined as "a process for the production of useful products through mass culture of micro-organisms." The various intermediate compounds produced during fermentation activity are classified as primary and secondary metabolites. Some of the commercially important metabolites are Secondary metabolites such as antibiotics, toxins, alkaloids, gibberellins, etc. Various fungi like *Aspergillus niger*, *Penicillium notatum*, *P. chrysogenum*,

Gibberella fujikuroi, *Fusarium moniliforme*, produce different type of secondary metabolites by the process of fermentation. Ergokonin A, Lovastatin & Guignardic acid are having potential antimicrobial activity and all these are secondary metabolites produced by fermentation of *Trichoderma longibrachiatum*, *Aspergillus terreus* and *Guignardic* speceis respectively (Samiee *et.al.*, 2003; Vicente *et.al.*, 2001; Rodrigues-Heerklotz *et.al.,* 2001). The advantage of liquid fermentation over solid is that it can be scaled up easily for commercial purposes. The qualilty and quantity of metabolites produced also depends on the duration of fermentation. It is difficult to measure the real activities of the mixture even with modern *in vitro* or cell-based assays, as there is lot of interference by other intermediates or byproducts of the fermentation process. The three worst interfering substances are: known compounds or non-specific inhibitors (67%), fatty acids (52%), high molecular substances (48%). In order to decrease or eliminate these interfering substances, extraction with organic solvents is widely applied (85%), and some organizations (15%) use fractionated samples.

Isolation of Pathogenic Phyllosphere Fungi

Pathogenic phyllosphere fungi can be isolated by employing the **dilution plate-method**. Small pieces of the leaves were cut with sterile scissor. Leaf segments were used for fungal isolation, One ml of the dilution was transferred into sterile Petri dishes, which were then poured with melted but cooled agar medium and incubated at25^{o}C for 7-10 days. (Johnson and Curl, 1972). Phyllosphere fungi can also be isolated by growing mycelia on culture media with two approaches: in the first one, **surface sterilization** of leaf samples was performed by dipping in ethanol (70% v/v) before soaking in (2% w/v) sodium hypochlorite solution for 5 min. and washed three times insterile distilled water, with the subsequent plating of the sterilized fragments on Petridishes containing potato dextrose agar (PDA) medium. The plates were sealed with parafilm and incubated at 28^{o}C for 7 days. The outgrowing colonies were counted and transferred to fresh PDA and cultivated in pure culture until sporulation. The second approach was the same as the previous one but samples were not surface-sterilized before plating (Santamar and Diez, 2005).

To confirm whether isolated phyllosphere fungi is pathogenic or not, Koch's Postulates can be followed which is an important component of the Germ Theory of Disease. The following rules of experimental proof were developed by Koch (1882) to verify the causal agent of disease: The pathogen must be found in association with disease in all plants examined, isolated, grown and characterized in pure culture, inoculated from pure culture onto healthy plants of the same species or cultivar on which the disease occurs and it must produce the same disease on the inoculated plants and isolated in pure culture from the inoculated plants and its characteristics must be exactly like those from the original culture. Outbreak of Leaf Spot of Saponaria Caused by *Alternaria saponariae* in California was checked by following Koch's Postulates (Ebenebe, 1980)

Plant Pathogenic Fungi

The present study is oriented to evaluate the antifungal activity of pathogenic fungi of *Lantana Camara*, *Hibiscus Rosa Sinensis* & *Cannabis Sativa*. These plants are selected on basis that extracts of these plant leaves have shown antimicrobial activity (Oyedapo *et.al.,* 1999; Joy et al., 1999). So it is possible that plant provides to the pathogenic fungi, compounds critical for the completion of its life cycle or essential for growth or self-defense. In addition, one of least studied, yet imaginable roles of pathogenic fungi is to initiate the biological degradation of dead or dying host plant, which begins the process of nutrient recycling. On still another, more molecular biological front, it is likely that mechanisms exists for transfer of nucleic acid from plants to these microbes or vice versa. Since some of same relatively rare bioorganic molecules made by specific higher plants can be produced by certain microbes as well. So it is conceivable that these plants have microbes that mimic the chemistry of their respective host plants and make the same bioactive natural products or derivatives that are more bioactive than those of their respective hosts (Strobel, 2001)

Extraction

The precise mode of extraction depends upon the nature of different type of secondary metabolites produced by fungi. Initial screening of different type of fungi for the assessment of bioactivity (prescreen) and purification of the bioactive compounds produced by them is done by using a variety of methods like Soxhlet Extraction, Cold extraction (solvent extraction), Distillation and

Acid & Alkaline extraction for obtaining a crude extract (Katarina Jernejc, 2004). As separation by distillation is ineffective or difficult and the solvent extraction (liquid-liquid extraction) is method of choice since it has an advantage of being carried out at room temperature thereby preventing the loss of heat labile components, which are lost at high temperature.

Liquid - Liquid Extraction is a separation process that takes advantage of the relative solubilities of solutes in immiscible solvents. The solute dissolves more readily and becomes more concentrated in the solvent in which it has a higher solubility. A partial separation occurs when a number of solutes have different relative solubilities in the two solvents used. These are separated on the basis of relative solubility. The Distribution Coefficient determines the ratio of the concentration of the solute in each solvent. Two immiscible solvents flow in opposite directions. The lighter solvent flows upward while the heavier solvent flows downward. The substance to be separated is in contact with both solvents and is dissolved in each stream according to a ratio determined by the distribution coefficient (Smith *et.al.*, 1993). A variety of compounds like the Aureobasidin A, Corynecandin, Arthrichitin, Cispentacin, Lovastatin & Khafrefungin having potential antimicrobial activity have been obtained by solvent extraction (Luiz Henrique Rosa *et.al.*, 2003, Gupte *et.al.*, 2002, and Samiee *et.al.*,2003)

Bioassay

Bioassays play an important role in evaluation of a particular bioactivity. A bioassay which is applied to large numbers of initial samples to determine whether or not they have any bioactivity of the desired type is referred to as a prescreen assay. A bioassay used to select materials for detailed individual study is referred to as screen assay. Bioassays are also used to guide fractionation of a crude material towards isolation of the pure bioactive compounds (monitor), which is referred to as bioassay guided fractionation (Hostettmanns and Marston 1994; Pieckova *et.al.* 1999; Silva *et.al.*,2004)

For these purposes, bioassay tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive. The *in vitro* assessment of antimicrobial susceptibility is done by two methods: Diffusion Assay and Broth Assay.

Pre Screen Assay: -Agar Well Diffusion Assay

The agar diffusion assay was devised by Heatley (1944) as a means of monitoring the extraction and purification of penicillin. Graded doses of solutions of reference standard and 'unknown' were applied to reservoirs [holes cut by a sterile cork-borer] in a layer of agar seeded with an organism sensitive to penicillin. On incubation, a circular clear zone of inhibition surrounded the reservoir in contrast to the turbidity where the organism had multiplied. Heatley showed that the diameter of the circular zone of inhibition was directly proportional to the logarithm of concentration of the penicillin. The principles involved in the formation of inhibition zones were studied by several workers during the years 1946 to 1952. They include Cooper and Woodman (1946), Mitchison and Spicer (1949), Cooper and Gillespie (1952) and Cooper and Linton (1952). The zone width was defined as the distance between the edge of the reservoir and the outer edge of the inhibition zone This technique is popularly referred as Agar Well Diffusion technique, Radial Diffusion Assay and Cylinder plate Assay (Lehrer, 1991). Agar well diffusion assay is a popular prescreen employed by clinical microbiologists working on the antimicrobial drug development from plant pathogenic fungi (Bonjar, 2004)

Screening: - Broth Macrodilution Method

Broth Macrodilution method is a rapid assay for ascertaining the antimicrobial susceptibility of fungal extract/partially purified fraction. The method also helps in determination of the Minimal Inhibitory Concentration (MIC) using this dilution method. The lowest concentration that will inhibit the visible growth of the microorganism is referred is known as the MIC. The test also provides an opportunity to actually monitor the killing efficacy of the fungal extract by using a plate count method (Vazquez *et.al.,* 1995, Ken Bartizal *et.al.,* 1997; EUCAST, 2000)

Analytical Methods

Isolation of pharmacologically active constituents from fungal extracts remains a long and tedious process. For this reason, it is necessary to have methods available, which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful types of constituents with potential activities. This procedure enables recognition of known metabolites in extracts or at the

earliest stages of separation and is thus economically very important. Analytical methods like Thin Layer Chromatography and Bioautography are the economical methods. Screening of Lovastatin production and secondary metabolites produced by *Guignardia* sp.*, Phomopsis* sp. were separated by TLC (Christa Werner *et.al.*,2000;Siamak *et.al.*,2003)

Thin Layer Chromatography

Thin-layer chromatography (TLC) is the simplest and cheapest method of detecting components of microbial extracts because the method is easy to run, reproducible and requires little equipment i.e. does not require expensive instrumentation, nor do samples generally require extensive cleanup prior to analysis. Compounds can be separated with good resolution, and methods are readily adaptable for applications ranging from high throughput to preparative-scale work. Microbial extracts do not require extensive purification and many samples can be run simultaneously. Both normal and reversed-phase adsorbents have been used with a variety of mobile-phase solvent systems. Substances are visualized by UV absorption, chromogenic reaction with spray reagents, or bioautography, in which suspensions of indicator organisms in agar or broth are overlaid on chromatograms to detect bioactive spots (Homans *et.al.*, 1997). Crude fungal extracts produced by endophytic strains of *Phomopsis* species and *Dichotomomyces cejpii* are separated by thin layer chromatography (Pieckova and Roeijmans, 1999; Corrado and Rodrigues, 2004)

AIM OF THE STUDY

AIM OF STUDY

The current study was oriented towards screening of extracts of plant pathogenic fungi for their antimicrobial activity against clinical isolates of *Candida albicans*. These plant pathogenic fungi were selected on the basis that Fungi, which infect plant, have mechanism to overcome plant defense mechanism by production of secondary metabolites.

The objectives of the present study were:

- ✓ Screening of fermentation cocktails of selected pathogenic fungi for their antifungal activity against *Candida albicans*
- ✓ Isolation of bioactive compounds exhibiting a potential antimycotic activity
- ✓ Determination of the range of MIC of the fungal extracts exhibiting a potential antimycotic activity and their kill kinetics (Death Pattern).
- ✓ Bioassay guided isolation and partial purification of fungal extracts exhibiting prominent in vitro antifungal activity.

MATERIALS AND METHODS

MATERIALS AND METHODS

Isolation of Fungi -Fungi was isolated from infected leaves of *Lantana camara*, *Hibiscus rosa-sinensis* and *Cannabis sativa* from Thapar Institute of Engineering & Technology Campus, Patiala. The infected leaves of these plants were first washed with distilled water and then sterilized the surface of leaves by swabbing with 70% alcohol thoroughly under a laminar flow hood until dried (Strobel, 2001;Cabello *et.al.*, 2001;Rosa *et.al.,* 2003). Then leaf discs of 3mm size were cut and placed in petridishes containing potato dextrose agar and incubated at 28°C and these were observed for growth regularily. Once adequate growth was observed, these were maintained as pure cultures in petridishes containing PDA (Rodrigues and Samuels, 1999). Then slants of potato dextrose agar were prepared & all the fungal isolates were preserved at 4°C. Kochs postulates was confirmed by sparying these organisms on the host leaves to observe the development of infection symptoms (Belisario *et.al.*,1996; Ebenebe,1980). These isolated fungi were coded by naming *Hibiscus rosa-sinensis*as P1, *Lantana camara* as P2, *Cannabis sativa* P3 and location TIET is named as L1 and respective leaves are represented as l.

Fermentation or Growth Conditions

All isolates were grown on PDA [300g/l Potatoes infusion, 20g/l Dextrose, 15g/l Agar and Final pH at 25°c was 5.6±0.2] and recultured several times until a pure culture was obtained. 5mm diameter mycelial plugs of 7-day old culture grown on fungal PDA plates were used to inoculate 100 ml presterilized fermentative media in Erlenmeyer flasks of 250 ml. The flasks were kept at shaker at 120 rpm and 28°c for 14 days for production of secondary metabolites (Rodrigues, 2000; Santamarina *et.al.,* 2002; Rosa *et.al.,* 2003). Media used for fermentation were

1. Richards Broth (RCHDB) [10 g/l KNo_3, 5 g/l KH_2PO_4, 2.5 g/l $MgSo_4$, 0.02 g/l $FeCl_3$, 35 g/l Sucrose and Final pH at 25°c was 7.0]{Corrado *et.al.,*2004]
2. Potato Dextrose Broth (PDB) [300 g/l Potatoes infusion, 20 g/l Dextrose and Final pH at 25°c was 5.6±0.2]{Cabello *et.al.,*2001}

3. Malt Extract Broth (MEB) [20 g/l Malt extract, 20 g/l Glucose, 1.0 g/l Peptone and Final pH at 25°c was 7.0]{Rosa *et.al.*,2003].

After 14 days, spent broth or culture supernatants are isolated from mycelia by filtration. Filtration is carried out by firstly through muslin cloth & then through whatman paper 4 (Rodrigues *et.al.,* 2000)

Extraction

Liquid-liquid extraction procedure was adopted. The extraction of culture supernatants was carried by different solvents like Chloroform, Dichloromethane, Ethyl acetate, Acetone, and Methanol.

Extraction of culture supernatants was carried three times with each solvent and the solvent layers were pooled. Then organic layer containing compounds of interest was collected and remaining solvent was evaporated by putting in water bath maintained at 60°c for the dehydration of extract. After removal of solvent, stock solutions of extracts (20mg/ml) were prepared in 80% DMSO (dimethylsulfoxide) & stored at –20°C till use (Cabello *et.al.,* 2001; Corrado and Rodrigues, 2004).

Test Microorganisms

The test microorganisms included fungi (budding yeast) and were broadly grouped as Clinical isolates. The clinical isolates included the following organisms: *Candida albicans* (G1-isolate), *Candida albicans* (G2-isolate), *Candida albicans* (G3-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (C1-isolate), *Candida albicans* (C2-isolate)

Maintenance of Microorganisms

The yeasts were maintained on the Sabouraud Dextrose Agar (SDA) Slants and Yeast Extract Peptone Dextrose Agar slants & stored at 4°C. Cultures were reactivated before every test. In case of fungi, Yeast extract peptone dextrose (YEPD) (Hi Media) agar was used for the activation and a single colony was transferred to YEPD broth and incubated for 16-18 hours at 37°C (Bonjar, 2004)

Preparation of 0.5 Mcfarland Standard: -

Added 0.5 ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2$ .2H20) to 99.5 ml of 0.18M H_2So_4 (1% w/v) with constant stirring. Recorded the O.D. of the solution; it should be in the range of 0.08-0.1 at 625 nm ($1.5x10^8$ cells/ml for yeasts). Stored the standard in Amber colored bottle to prevent it from light at room temperature. Vigorously vortex the standard on a vortex mixer prior to use. (NCCLS, 1997)

Pre Screen Assay- Agar Well Diffusion Method (Heatley, 1944)

Preparation of Stock Solution of test material: The fungal extracts so obtained were dissolved in Dimethylsulfoxide (DMSO) to give a concentration of 20mg/ml. These were stored at -20°C till further use.

The agar well diffusion assay was carried out by preparation of 4 wells of 5mm diameter using a sterile cork borer per 90 mm YEPD agar plate aseptically to give a mean depth of 4.00 mm ± 0.5 mm. The agar cylinders were removed using a sterile loop. The wells were grouped as the test well and the control wells. The test well was filled with different volume of the stock solution of the test material and the control well was filled with the same amount of the solvent i.e. DMSO or unspent broth. Flucanazole was used as the positive control to compare antimicrobial activity. The range of volume of test solution was 20µl. When diffusion of extract has occurred, then wells were then sealed with molten YEPD agar and kept for 10 minutes for solidification. These plates were then swabbed with 0.5 McFarland adjusted 16-18 hour old culture of the test organisms, swabbing was done in 3 directions by rotating plates at 60° angle and plates were wrapped with parafilm & incubated at 37°C overnight. After 18 to 24 hours of incubation, each plate was examined. The inhibition zones were recorded in the test well as well as the control well within 24 to 48 h. Anti-yeast activities were determined by measuring Diameter of inhibition zones (DIZ) in mm (Cooper and Linton, 1952). Each experiment was repeated thrice and the average values of antimicrobial activity were calculated.

Screen Assay: -Broth Macrodilution Assay (NCCLS, 1992; EUCAST 2000)

Antifungal drug dilutions are used to determine the MIC for an isolate when grown in the presence of an antifungal agent. Serial dilutions of antifungal agents are dispensed into appropriately labeled tubes. Each tube is then inoculated with a standardized YEPD broth suspension of the yeast being tested and tetrazolium salt, 2, 3,5 Triphenyltetrazolium Chloride (TTC) was used to assess the yeast growth, determine the Minimal inhibitory Concentration and determination of the death pattern (death kinetics). The primary advantage of the broth dilution test is that it permits a quantitative estimate of both the inhibitory and fungicidal activities of the antifungal agent.

For making stock of chloroform fungal extract, weighed 5mg of chloroform fungal extract and dissolved it in DMSO to make stock of 5mg/ml. Then diluted it to 30.25 ml of YEPDB to make an initial conc. of 160 μg/ml. One test tube was taken as blank containing only YEPDB. In control test tube, no extract was added and control over control contains only DMSO in which extract was dissolved. Firstly poured different volume of YEPDB in test tubes under aseptic conditions and inoculated 100 μl of phosphate buffer saline adjusted activated culture in test tubes except blank & incubated at shaker at 120rpm at 37°C (Culture inoculum is prepared by centrifuging overnight grown culture in 25 ml of YEPDB.Centifugation is done at 5000 rpm for 10 min. Discarded the supernatant & dissolved the pellet in 5 ml of sterile phosphate buffer saline, vortexed it for making suspension & checked the optical density at 530 nm .It should have transmittance of 85%(1-5x10^6 cfu/ml)and if not, further diluted using phosphate buffer saline).then after 5 hrs ,poured different volume of chloroform extract from stock solution in different test tubes to make varying conc. of (0.0125, 0.25, 0.5, 1,2,4 ,8, 16 ,32 ,40 .64, 80 ,96, 112,128,144) μg/ml as shown in Table-1. After 24 hrs, 500μl of 0.2 % TTC was further added to all the dilutions, blank, control and control over control. The final volumes of all the test tubes were 10000μl. All the test tubes were incubated at 37°C at 120 rpm. Each assay was repeated twice.

Prepartion of Phosphate Buffer Saline

To make 100 ml of phosphate buffer added 6.177g of Na_2HPo_4 & 1.014 g of NaH_2Po_4 & made up to 100 ml with distilled water. Took 20 ml of phosphate buffer and poured 8.7 g of NaCl & made up to 1 liter with distilled water and adjusted the pH to 7.4.

TABLE 1: DIFFERENT CONCENTRATIONS OF FUNGAL CHLOROFORM EXTRACT USED TO DETERMINE MIC BY BROTH MACRODILUTION ASSAY

Sr. No.	Vol of YEPDB (µl)	Vol Of PBS adjusted Culture (µl)	Vol. Of Stock solution (µl)	Antimicrobial conc. obtained (mg/µl)	Final Conc. in 10 ml of YEPDB (µg/ml)
1	9837	100	62.5	0.000001	1
2	9775	100	125	0.000002	2
3	9650	100	250	0.000004	4
4	9400	100	500	0.000008	8
5	8900	100	1000	0.000016	16
6	7900	100	2000	0.000032	32
7	6900	100	3000	0.00004	40
8	5900	100	4000	0.000064	64
9	4900	100	5000	0.00008	80
10	3900	100	6000	0.000096	96
11	2900	100	7000	0.000112	112
12	1900	100	8000	0.000128	128
13	9000	100	9000	0.000144	144
Control	9900	100	0	0	0

Plate Count Method to Estimate the MIC & Death Pattern of Test Oganisms at MIC (Cuenca-Estrella *et.al.*, 2002; Bartizal *et.al.*, 1995)

To corroborate our findings obtained visually by broth macrodilution assay, 100µl of the broth from test tube exhibiting MIC and control tube were taken aseptically and diluted serially. 10 µl of the 10^{-6} concentration of the control and test extract were plated on one-day-old YEPD agar plate as a point inoculum and allowed to dry for 10 min. under the laminar air hood. These were then sealed and incubated at 37°C for 24 hours (EUCAST, 2000). The number of colonies that appeared was counted. The growth pattern in the control test tube and the death pattern of test extracts in the test tube were estimated by taking aliquots from the test and control tubes at regular intervals i.e., 0, 6, 12,and 24 hours after incubation. The Colony forming units (CFU's) of the Control and the test extracts were estimated.

Fractionation of Fungal Extract

TLC plates (20X20cm) coated with Silica gel GF 254 with Fluorescent Indicator (0.25mm thick) were used for separation of the extracts showing predominant antimicrobial activity in the prescreen and screen assays by using different combinations of mobile phases like Chloroform: Acetone: Ethyl Acetate (1:1:2 or 1:2:3) at 28°C. The TLC chamber was conditioned for a period of 30 minutes prior to the run. 10µl of the fungal extract from the stock solution were loaded 1 cm above the TLC plate. The resolution of the extracts was recorded by their Rf values in visible and long UV (Pieckova and Roeijmans, 1999; Siamak *et.al.*,2003;Rodrigues *et.al.*,2000)

AWD assay of the TLC Fractions

Separated lines with right Rf values were scraped off the air-dried plates, the active substances then were eluted 3-times from the silica gel plates with ~2 ml of chloroform and evaporated for dehydration of extract. Residues btained by evaporating TLC fractions were dissolved in DMSO, to make a working solution. Fractions were analyzed for their antimicrobial activity by well diffusion assay (Pieckova and Roeijmans, 1999)

RESULTS AND DISCUSSION

Isolation of Pathogenic Phyllosphere Fungi

Pathogenic phyllosphere fungi were isolated by surface sterilization of leaves of plants like *Lantana camara, Hibiscus rosa-sinensis & Cannabis sativa* and these shown adequate growth on PDA plates. Table-2 represents the 16 isolated phyllosphere fungi of which maximum of *Alternaria* species, *Fusarium* species, *Aspergillus* species, *Penicillium* species and *Mucor* species.

Table-2: Fungi Isolated from Phyllosphere

S.NO.	ISOLATE CODE NO.	TENTATIVE IDENTIFICATION
1	$P_1L_1l_2Sa$	*Alternaria* species
2	$P_1L_1l_1Sb$	*Aspergillus* species
3	$P_2L_1l_2Sc$	*Alternaria* species
4	$P_1L_1l_2Sd$	*Mucor* species
5	$P_3L_1l_6Se$	*Penicillium* species
6	$P_1L_1l_3Sf$	*Alternaria* species
7	$P_1L_1l_4Sg$	*Alternaria* species
8	$P_3L_1l_7Sh$	*Fusarium* speceis
9	$P_3L_1l_1Si$	*Mucor* species
10	$P_1L_1l_{14}Sj$	*Penicillum* speceis
11	$P_3L_1l_8Sk$	*Fusarium* species
12	$P_1L_1l_3Sl$	*Fusarium* species
13	$P_2L_1l_{15}Sn$	*Aspergillus species*
14	$P_3L_115_So$	*Penicillium* species
15	$P_2L_1l_{16}Sx$	*Fusarium* species
16	$P_2L_1l_{14}Sy$	*Aspergillus* species

Fermentation

Different fermentative media were investigated to determine in which broth the antifungal activity was produced. The highest level of antifungal activity was observed in the fungi $P_2L_1l_{14}Sy$ identified as *Aspergillus* speceis in PDB media when the spent broth of fungal isolates were screened for their antimicrobial activity using standard agar well diffusion assay. It was found that highest antimicrobial activity was exhibited by the fermented broth of fungal isolate $P_2L_1l_{14}Sy$ identified as *Aspergillus* speceis (Table no.6). Similarly culture broth extracts produced by fermentation of *Guignardia* speceis and *Phomopsis* speceis in PDB which were isolated from leaves of *Aspidosperma tomentosum* and *Spondias mombin,* were evaluated for antimicrobial activity by using agar well diffusion assay (Corrado and Rodrigues, 2004). Spent broth of *Oudemansiella mucida, Oudemansiella radicata* produced by fermentation in PDB also exhibited antimicrobial activity (Anke *et.al.*,1990)

Extraction

Liquid-liquid extraction was used for extraction of culture supernatants. This was carried by solvents like chloroform followed by dichloromethane, ethylacetate, acetone and methanol. The yield of extract was maximum in methanol followed by chloroform, ethyl acetate, acetone and dichloromethane. However it was found that antimicrobial activity was maximum in chloroform extract. Fungi produce variety of aromatic or saturated organic compounds. Hence, they are often extracted using a particular solvent only, for e.g. Lovastatin and Ergokonin are specifically isolated from *Aspergillus terreus* and *Trichoderma longibrachiatum* using chloroform and ethyl acetate as a solvent and these shown potent antifungal activity (Vicente *et.al.,* 2001;Siamak *et.al.*,2003). Methanol is used for isolation of Arundifungin from *Arthrinium arundinis* which exhibited antifungal activity (Angeles, *et.al.*,2001) and variety of antimicrobial compounds have been isolated from the chloroform extract of basidiomes of *Phellinus* speceis,*Gloeoporus thelephoroides* and *Keissleriella* speceis YS4108 (Rosa *et.al.*, 2003;Liu *et.al.*,2002).Table –3 represents the yield of extracts with different solvents in different medias.

TABLE-3: YIELD OF EXTRACTS WITH DIFFERENT SOLVENTS IN DIFFERENT MEDIAS

ISOLATE CODE NO.	YIELD OF EXTRACTS WITH DIFFERENT SOLVENTS IN RCHDB					YIELD OF EXTRACTS WITH DIFFERENT SOLVENTS IN MEB					YIELD OF EXTRACTS WITH DIFFERENT SOLVENTS IN PDB				
	CHL (mg)	DCM (mg)	EA (mg)	AC (mg)	ME (mg)	CHL (mg)	DCM (mg)	EA (mg)	AC (mg)	ME (mg)	CHL (mg)	DCM (mg)	EA (mg)	AC (mg)	ME (mg)
$P_1L_1l_2Sa$	36.19	2.65	4.44	5.25	112.25	3.75	3.73	3.57	2.26	1.2	6.98	5.81	1.79	1.67	64.89
$P_1L_1l_1Sb$	37.79	1.38	3.1	6.12	90.21	17.58	17.8	1.62	1.95	22.9	12.96	5.85	2.85	1.09	20.35
$P_2L_1l_2Sc$	41.14	3.92	5.16	0.96	76.51	13.84	16.49	6.15	2.18	1.28	79.21	10.89	14.49	3.1	144.85
$P_1L_1l_2Sd$	13.18	13.18	4.82	56.96	129.9	16.78	6.11	32.9	14.12	110.48	59.61	6.79	12.12	36.11	27.51
$P_3L_1l_6Se$	11.35	4.48	4.14	18.18	116.72	16.15	3.24	14.38	1.82	44.99	44.07	3.39	7.95	5.36	20.31
$P_1L_1l_3Sf$	38.88	1.33	1.96	15.12	126	19.57	7.74	4.98	1.7	9.22	10.76	3.97	2.71	1.06	56.58
$P_1L_1l_4Sg$	37.82	1.35	2.13	11.67	136.52	16.55	7.89	5.2	0.86	1.56	6.85	2	3.21	4.2	22.49
$P_3L_1l_7Sh$	5.3	4.28	3.51	11.61	48.2	16.24	12.46	1.13	6.38	76.11	54.32	3.51	8.06	3.89	39.7
$P_3L_1l_1Si$	13.93	1.29	11	21	145.47	8.09	4.53	24.74	190.17	110.48	122.42	11	38.15	50.73	213.61
$P_1L_1l_{14}Sj$	12	2	2.58	14.29	121.37	18.07	10.33	5	12	24.66	10.8	5.25	7.2	40.2	60.27
$P_3L_1l_8Sk$	9.25	2.19	3.14	8.17	115.2	18.1	12.16	2.54	0.9	12.36	11.05	6	4.73	2.79	116.13
$P_1L_1l_3Sl$	47.86	5.57	4.48	7.28	58.26	46.55	10.11	1.72	1.05	13.18	44.07	3.39	7.95	5.36	20.13
$P_2L_1l_{15}Sn$	5.27	3.51	3.81	26.2	110.12	18.93	6.31	3.35	17.65	87.81	6.82	8.53	23.5	40.41	63.57
P_3L_115So	16.61	6.3	6.3	9.65	76.2	7.55	9.39	1.76	1.46	36.74	9.45	5.69	2.2	2.16	223.22
$P_2L_1l_{16}Sx$	8.26	6.25	10	4.36	20.16	11	7.8	3.25	0.98	1.8	61.84	5.95	18.51	12.55	115.1
$P_2L_1l_{14}Sy$	20.7	10.81	10.81	7.7	179.2	12.48	9.43	2.2	24.11	78.89	50.3	4.26	16.9	152.96	268.42

CHL-Chloroform, DCM-Dichloromethane, EA-Ethyl acetate, AC-Acetone, Me- Methanol

Agar Well Diffusion –Prescreen assay

As presented in tables-4, 5,6, maximum zone of inhibition was observed in extracts of PDB as compared to RCHDB and MEB.

As shown in Table-4 for antifungal activity of different fungal extracts in Richard broth, the chloroform extracts of fungi from $P_1L_1l_2Sa$ to $P_2L_1l_{14}Sy$ did not show any zone of inhibition against test microorganisms except in case of fungi $P_3L_1l_6Se$ where 16 mm of zone of inhibition was observed against *Candida albicans* (G4-isolate). Dichloromethane, ethyl acetate, acetone and methanol extracts of fungi from $P_1L_1l_2Sa$ to $P_2L_1l_{14}Sy$ also did not show any zone of inhibition against test microorganisms *like Candida albicans* (G1-isolate), *Candida albicans* (G2-isolate), *Candida albicans* (G3-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (C2-isolate), *Candida albicans* (C-isolate).

As evident by Table-5 for antifungal activity of fungal extracts in malt extract broth, chloroform extracts of fungi from $P_1L_1l_2Sa$ to $P_3L_1l_7Sh$ and from $P_1L_1l_{14}Sj$ to $P_1L_1l_3Sl$ did not show any zone of inhibition against test microorganisms except in case of fungi $P_3L_1l_1Si$, $P_2L_1l_{15}Sn$, P_3L_115So, $P_2L_1l_{16}Sx$ and $P_2L_1l_{14}Sy$ where 10mm, 6mm, 8 mm, 15mm and 13mm, 20mm zone of inhibition were observed against *Candida albicans* (C2-isolate), *Candida albicans* (G1-isolate), *Candida albicans* (G4-isolate).Dichloromethane extracts of fungi from $P_1L_1l_2Sa$ to $P_2L_1l_{14}Sy$ did not show any zone of inhibition against test microorganisms except in case of fungi $P_1L_1l_4Sg$, $P_2L_1l_{15}Sn$ where 10mm ,11mm zone of inhibition were observed against *Candida albicans* (G1-isolate). Ethyl acetate extracts of fungi $P_1L_1l_2Sa$, $P_1L_1l_2Sd$, $P_2L_1l_{15}Sn$ showed zone of inhibition of 8 mm, 11mm and 17mm, 12mm against *Candida albicans* (G1-isolate), Candida *albicans* (C2-isolate), *Candida albicans* (G4-isolate) while remaining isolated fungi did not show any zone of inhibition against test microorganisms. Acetone extracts of fungi $P_1L_1l_2Sd$, $P_3L_1l_1Si$, $P_2L_1l_{14}Sy$ showed zone of inhibition of 20mm and 16mm, 16mm and 15mm, 10mm against *Candida albicans* (G1-isolate), *Candida albicans* (G4-isolate) while dichloromethane extracts of remaining isolated fungi did not show any zone of inhibition against test microorganisms. Methanol extracts of fungi $P_3L_1l_1Si$ showed zone of inhibition of 10mm and 13mm against *Candida albicans* (G1-isolate), *Candida albicans* (G4-isolate)

while methanol extracts of remaining isolated fungi did not show any zone of inhibition against test microorganisms.

As shown in Table-6, best zone of inhibition were observed in potato dextrose broth. Chloroform extract of fungi $P_1L_1l_1Sb$, $P_1L_1l_2Sd$ to $P_1L_1l_3Sf$, $P_3L_1l_8Sk$, $P_2L_1l_{15}Sn$ to $P_2L_1l_{14}Sy$ showed zone of inhibition of 6mm, 14mm, 20mm, 10mm, 17mm and 15mm and 12mm, 11mm, 10mm and 19mm, and 23mm, 21mm, 24mm against *Candida albicans* (G1-isolate), *Candida albicans* (C2-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (G2-isolate). While chloroform extracts of remaining isolated fungi did not show any zone of inhibition against test microorganisms. Dichloromethane extracts of fungi from $P_1L_1l_2Sa$ to $P_2L_1l_{14}Sy$ did not show any zone of inhibition against test microorganisms except in case of fungi $P_1L_1l_1Sb$, $P_3L_1l_8Sk$, $P_3L_11_5So$, $P_2L_1l_{14}Sy$ where 10mm, 8mm and 12mm, 11mm zone of inhibition were observed against *Candida albicans* (G1-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (G2-isolate). Ethyl acetate extract of fungi $P_1L_1l_2Sa$, $P_3L_1l_7Sh$, $P_3L_1l_1Si$, $P_3L_1l_8Sk$, $P_3L_11_5So$, $P_2L_1l_{16}Sx$, $P_2L_1l_{14}Sy$ showed zone of inhibition of 6mm, 12mm, and 15mm, 11mm and 6mm, 10mm, 24mm and 10mm and 7 mm against *Candida albicans* (G1-isolate), *Candida albicans* (C2-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (G2-isolate).Acetone extracts of $P_1L_1l_2Sd$, $P_3L_1l_1Si$, $P_3L_11_5So$ showed zone of inhibition of 14mm, 20mm and 16mm and 15 mm against *Candida albicans* (G1-isolate), *Candida albicans* (G4-isolate). While acetone extracts of remaining isolated fungi did not show any zone of inhibition against test microorganisms. Methanol extracts of $P_2L_1l_2Sc$, $P_3L_1l_1Si$, $P_2L_1l_{16}Sx$ showed zone of inhibition of 11mm, 13mm and 17mm, 10mm and 17mm against *Candida albicans* (G1-isolate), *Candida albicans* (C2-isolate), *Candida albicans* (G4-isolate), *Candida albicans* (G2-isolate).

Maximum zone of inhibition were observed in chloroform extract of fungi $P_2L_1l_{14}Sy$ identified as *Aspergillus* speceis (Fig. 5). Ergokonin A and Arundifungin which were isolated from *Trichoderma longibrachiatum* and *Arthrinium arundinis* have shown antifungal activity against *Candida* species by using agar well diffusion assay (Angeles *et.al.*,2001;Vicente *et.al.*,2001).Secondary metabolites produced by *Dichotomomyces cejpii* have exhibited antifungal activiy against *Candida* speceis.(Pieckova and Roeijmans,1999).

TABLE -4 ANTIFUNGAL ACTIVITY OF DIFFERENT FUNGAL EXTRACTS IN RICHARD BROTH

Isolate Code No.	Zone of Inhibition (diameter in mm)																													
	Candida albicans (G1- Isolate)					Candida albicans (G-2 Isolate)					Candida albicans (G3- isolate)					Candida albicans (G4- isolate)					Candida albicans (C2- isolate)					Candida albicans (C- isolate)				
	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME
$P_1L_1I_2Sa$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_1Sb$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_2Sc$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_2Sd$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_6Se$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sf$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_4Sg$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_7Sh$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_1Si$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_{14}Sj$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_8Sk$	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sl$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{15}Sn$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_11_5Sc$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{16}Sx$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{14}Sy$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CHL-Chloroform, DCM-Dichloromethane, EA-Ethyl acetate, AC-Acetone, Me- Methanol and (r)-resistant colonies

TABLE – 5: ANTIFUNGAL ACTIVITY OF DIFFERENT FUNGAL EXTRACTS IN MALT EXTRACT BROTH

Isolate Code No.	Zone of Inhibition (diameter in mm)																													
	Candida albicans (G1- Isolate)					*Candida albicans* (G-2 Isolate)					*Candida albicans* (G3- isolate)					*Candida albicans* (G4- isolate)					*Candida albicans* (C2- isolate)					*Candida albicans* (C- isolate)				
	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME
$P_1L_1I_2Sa$	-	6	8(r)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_1Sb$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_2Sc$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_2Sd$	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	-	17	16	-	-	-	-	-	-	-	-	11	-	-
$P_3L_1I_6Se$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sf$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_4Sg$	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_7Sh$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_1Si$	-	-	-	16	10	-	-	-	-	-	-	-	-	-	-	-	-	-	15	13	-	-	-	-	-	10	-	-	-	-
$P_1L_1I_{14}Sj$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_8Sk$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sl$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{15}Sn$	6(r)	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	-	-
$P_3L_11_5So$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{16}Sx$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-	-
$P_2L_1I_{14}Sy$	13	-	-	10		20(r)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CHL-Chloroform, DCM-Dichloromethane, EA-Ethyl acetate, AC-Acetone, Me- Methanol and (r)-resistant colonies

TABLE – 6 ANTIFUNGAL ACTIVITY OF DIFFERENT FUNGAL EXTRACTS IN POTATO DEXTROSE BROTH

Isolate Code No.	Zone of Inhibition (diameter in mm)																													
	Candida albicans(G1-Isolate)					*Candida albicans*(G-2 Isolate)					*Candida albicans* (G3- isolate)					*Candida albicans* (G4- isolate)					*Candida albicans* (C2- isolate)					*Candida albicans* (C- isolate)				
	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME	CHL	DCM	EA	AC	ME
$P_1L_1I_2Sa$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6(r)	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_1Sb$	6(r)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10(r)	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_2Sc$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_2Sd$	14	-	-	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_6Se$	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sf$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10(r)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_4Sg$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_7Sh$	-	-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_1Si$	-	-	12	20	13	-	-	15	-	20	-	-	-	-	-	-	-	11	16	-	-	-	-	-	-	-	-	-	-	13
$P_1L_1I_{14}Sj$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_1I_8Sk$	14	-	-	-	-	10	-	14	-	-	-	-	-	-	-	17(r)	10(r)	6(r)	-	-	-	-	-	-	-	-	-	-	-	-
$P_1L_1I_3Sl$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_2L_1I_{15}Sn$	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$P_3L_11_5So$	12(r)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10(r)	10(r)	10(r)	-	-	-	-	-	-	-	11	-	8	-	-
$P_2L_1I_{16}Sx$	-	-	24	-	17	-	-	-	-	-	-	-	-	-	-	-	-	-	15	10	-	-	-	-	-	19	-	-	-	13
$P_2L_1I_{14}Sy$	23	8(r)	10	-	-	21	12(r)	7(r)	-	-	-	-	-	-	-	24	-	-	-	-	-	-	-	-	-	-		-	-	-

CHL-Chloroform, DCM-Dichloromethane, EA-Ethyl acetate, AC-Acetone, Me- Methanol and (r)-resistant colonies

Griseofulvin, Flucanazole and Ketaconazole –the Positive Controls

Griseofulvin is a Polyenes class and Flucanazole and Ketaconazole are azoles class of antifungal drugs, which were evaluated as positive controls to assess the antimicrobial activity of the fungal extracts. As evident from results of Agar Well Diffusion Assay of extracts, Griseofulvin showed 6mm, 10mm zone of inhibition against *Candida albicans* (G4-isolate), *Candida albicans* (G1-isolate) and Flucanazole and Ketaconazole showed no zone of inhibition against any of test microorganism as compared to extracts of isolated coded fungi as shown in table-7. The chloroform extract of isolated coded fungi $P_2L_1l_{14}Sy$ was selected for detailed studies in the Screen Assay i.e. Broth macrodilution assay.

TABLE-7: AGAR WELL DIFFUSION ASSAY OF POSITIVE CONTROLS

Positive Control	Zone Of Inhibition (mm)					
	A	**B**	**C**	**D**	**E**	**F**
Flucanazole	-	-	-	-	-	-
Griseofulvin	10	-	-	6	-	-
Ketaconazole	-	-	-	-	-	-

A- *Candida albicans* (G1-isolate), **B**-*Candida albicans* (G2-isolate), **C**-*Candida albicans* (G3-isolate), **D**-*Candida albicans* (G4-isolate), **E**-*Candida albicans* (C-isolate), **F**-*Candida albicans* (C2-isolate)

Broth macrodilution Assay – Determination of MIC

The MIC is the lowest concentration at which the fungal growth was inhibited by the fungal extract and it was judged visually by clear test tube showing the non-reduction of TTC in the dilution series (Fig. 6). The chloroform extract of isolated coded fungi $P_2L_1l_{14}Sy$ exhibited MIC of 64 µg/ml against *Candida albicans* (G1-isolate), MIC of 78.12 µg/ml against *Candida albicans* (G4-isolate) and MIC of 128µg/ml against *Candida albicans* (G2-isolate).

MIC has been often used by diagnostic laboratories to confirm resistance but most often as a research tool to determine the *in vitro* activity of new antimicrobial and data of such studies have been taken to study the MIC breakpoints. *In vitro* antifungal activity of Arundifungin,a pure compound isolated from *Arthrinium arundinis* which was extracted in methanol was evaluated in broth macrodilution assay and it exhibited MIC of 4mg/ml against *Candida albicans* (MY 1055), 2mg/ml against *Candida albicans* (CLY539) and 8mg/ml against *Candida tropicalis* (G2-isolate)[Angeles *et.al.*, 2003;Mcginnis *et.al.*,1998].Lovastatin isolated from *Aspergillus terreus* exhibited a MIC of 1 mg/ml against *Candida* speceis(Siamak *et.al.*;2003)Thus, the methanolic and the chloroform extracts exhibit a potential to be investigated for leads for drug resistant *Candida albicans*.

Death pattern or Time Kill of the Microorganisms

As evident from Fig. 6, 7 and 8, chloroform extract of fungal isolate $P_2L_1l_{14}Sy$ identified as *Aspergillus* speceis exhibited maximum growth inhibition. In case of *Candida albicans (*G1-isolate), at 0 hr there is negligible log reduction observed in test extract as compared to control. But at 12 hr there is 4 log reductions observed in test extract and after 24 hrs there is 10-log reduction i.e.100% killing was observed in test extract as compared to control. Similar results obtained for *Candida albicans (*G2-isolate), Candida *albicans (*G4-isolate). In case of *Candida albicans (*G2-isolate), at 0 hr there is negligible log reduction observed in test extract as compared to control. But at 12 hr there is 2 log reductions observed in test extract and after 24 hrs there is 10-log reduction was observed in test extract as compared to control. A fungicidal effect is defined as a 3-log decrease in the CFU/ml or a 99.9% kill over a specified time. A kill can also be determined at 9 or 12 hours (Pfaller *et al.*, 1997, Odds, *et.al.*, 2000). In this study the kill measurement was determined by the actual reduction in viable counts at 6 and 9 hour for each isolate (NCCLS, 1992). Ergokonin A which is isolated from *Trichoderma longibrachiatum* using chloroform as a solvent exhibited growth inhibition of 85% of *Candida tropicalis* after 9hrs (Vicente *et.al.*2001).

Partial Purification by TLC

Chloroform extract exhibited maximum antimicrobial activity and therefore subjected to Thin Layer chromatography for narrowing down the bioactive fractions. As evident from Table-6, fractions in all were isolated from chloroform extract by TLC. The method is easy to run, reproducible and requires a little equipment (Marston *et, al.*, 1997). Fungal chloroform extracts produced by endophytic strains of *Phomopsis* species were fractionated by TLC (Corrado and Rodrigues, 2004). Production of Lovastatin was screened by using TLC and secondary metabolites of *Dichotomomyces cejpii* were fractionated by using Thin Layer Chromatography and fungitoxic substances were separated from the crude extracts of *Dichotomomyces cejpii* have shown Rf values of 0.19-0.22.(Samiee *et.al.*, 2003, Pieckova and Roeijmans,1999)

TABLE 8: TLC SEPARATION OF CRUDE CHLOROFORM EXTRACT

TLC Fractions	Rf (Solvent Front—15.2 cm)	
	UV	VISIBLE
1		0.06
2		0.17
3		0.29
4		0.4
5	0.51	
6	0.66	
7	0.76	
8		0.82
9		0.96

AWD assay of the TLC Fractions

Residues obtained by evaporating TLC fractions were dissolved in DMSO, to make a working solution. Fractions were analyzed for their antimicrobial activity by well diffusion assay. As evident from Table 9, Antifungal activity was observed in fractions 4,6,7,8 and 9. Maximum zone of inhibition i.e., 16mm was observed in case of fraction 6 and 9.TLC fractions of crude extract of *Guignardia* speceis were tested against *Penicillium canadensis*,*Candida krusei* by using agar well diffusion assay(Rodrigues,*et.al.*,2000). Fractions of crude extracts of *Phomopsis* species, which were isolated by TLC, were very potent against *Sacharomyces cerevisiae, Candida tropicalis and Fusarium oxysporum*. All these fractions were tested by agar well diffusion assay (Concepcion, *et.al.*, 2001; Katia, F.R.; *et.al.,* 1999)

TABLE-9: ACTIVITY OF VARIOUS FRACTIONS AGAINST TEST MICROORGANISMS

TLC Fractions	ZONE OF INHIBITION (mm)	
	Candida albicans **(G1-isolate)**	***Candida albicans*** **(G4-isolate)**
1	-	-
2	-	-
3	-	-
4	13	-
5	-	-
6	16	6
7	14	6
8	13	-
9	16	10

Fig 5-Time kill kinetics of MIC (64ug/ml)of chloroform extract of P2L1l14Sy against *Candida albicans* (G1)

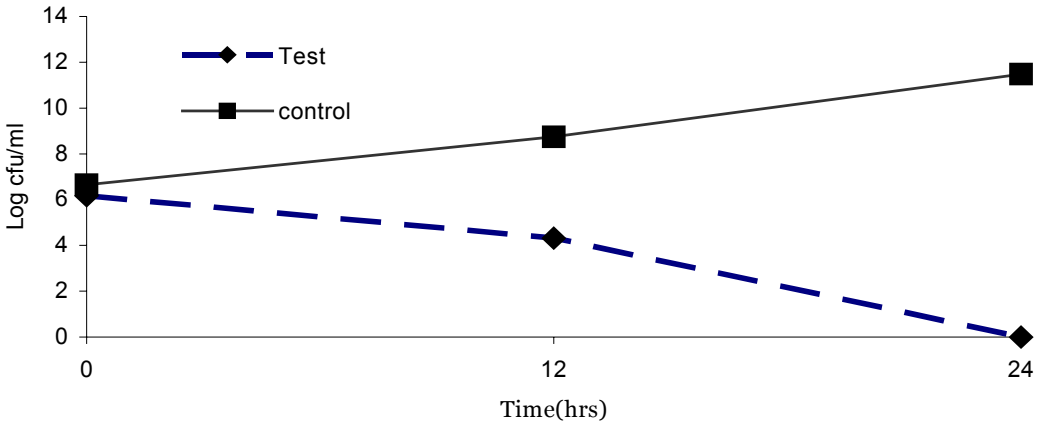


Fig 6-Time kill kinetics of MIC (128 ug/ml) of chloroform extract of P2L1l14Sy against *Candida albicans(* G2)

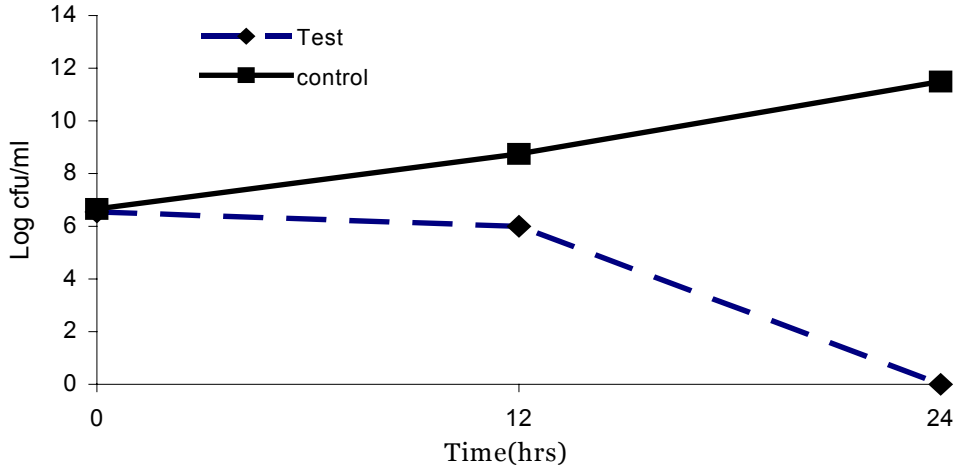


Fig 7-Time kill kinetics of MIC (78.12 ug/ ml) of chloroform extract of P1L2l14Sy against *Candida albicans*(G4)

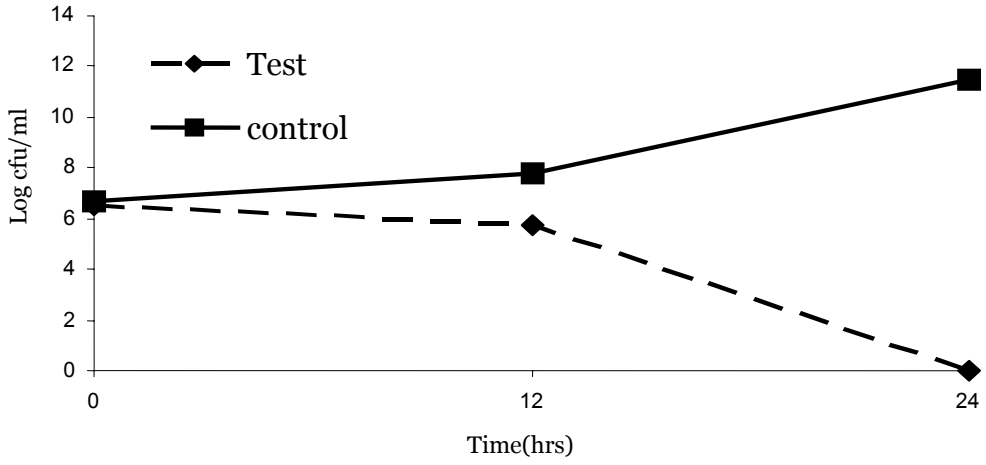


Fig.8: Agar Well Diffusion Assay of Chloroform Extract of P2L1L14Sy against *Candida albicans* (G1, G2 and G4)

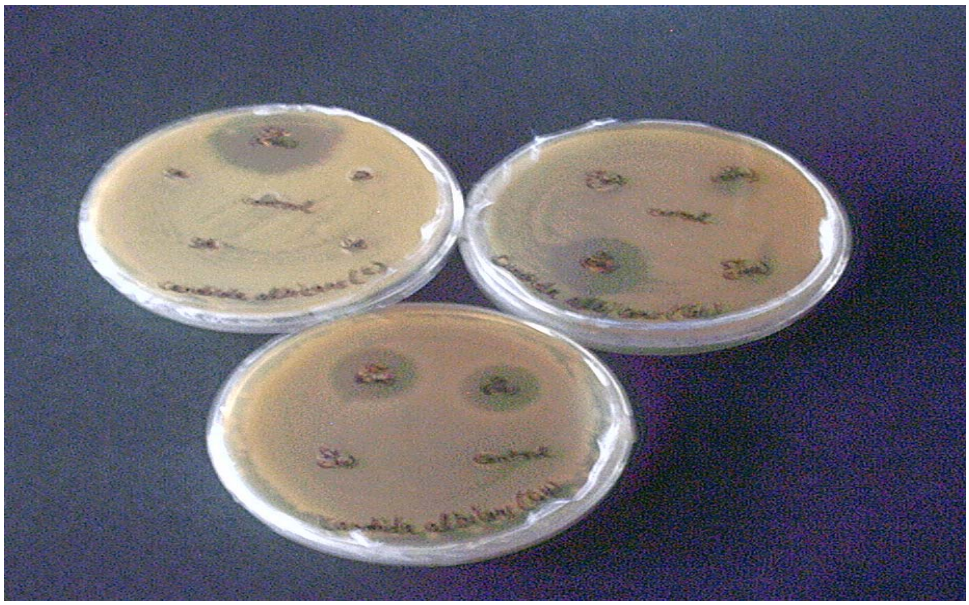


Fig.9: MIC Determination of Chloroform Extract of P2L1L14Sy against *Candida albicans* G1

CONCLUSIONS

The present study revealed potential antifungal activity of the fungal extracts obtained from the isolated phyllosphere pathogenic fungi. The validation of potential antifungal activity has been validated against known pathogenic multidrug resistant organisms, such as *Candida albicans*. The potential of the fungal extract was tested against some standard antifungal antibiotics of different classes like griseofulvin, ketaconazole and Flucanazole.The study highlighted the prevalence of resistance in *Candida albicans* to these antifungal antibiotics and these pose serious threat to society.

Among the different extracts evaluated, chloroform extract of isolated coded fungi $P_2L_1l_{14}Sy$ exhibited higher potency i.e., had MIZ as compared to other extracts against the test microorganisms. It exhibited fungicidal activity against *Candida albicans* (G1-isolate) and *Candida albicans* (G4-isolate). The extract is having potent antifungal activity, so the bioactive compounds present in the fungal extract can be served as broad-spectrum antifungal antibiotic. Its antifungal activity makes it very useful anti-infective agent.

Similar studies on other isolated compound like Arundifungin from *Arthrinium arundinis* shows higher MIC values as compared to our chloroform extract of isolated coded fungi $P_2L_1l_{14}Sy$. The MIC of Arundifungin against all test organisms is being reported as 4mg/ml as against 78.15 µg/ml in our case.

The isolated fractions can be used for treatments against various community and hospital acquired infections. More clinical and pharmacological studies shall be undertaken to establish the dose pattern. The potential drug molecules can be Isolated and can be further chemically characterized.

Overall, the study revealed that bioactive fungal constituents produced by phyllosphere pathogenic fungi are a rich source of novel metabolites exhibiting a wide range of important antifungal activity against yeast. So they have potential for exploitation in a wide variety of medical areas.

BIBLIOGRAPHY

A

1. Abi-Said, D., Anaissie E., Uzun, O.; Raad, I.; Pinzcowski, H.; and Vartivarian, S.; (1997): The epidemiology of hematogenous candidiasis caused by different *Candida* species. ***J Clin. Infect. Dis***; **24:** 1122-1128.

2. Andriole, V.T.; (1996): current and future antifungal therapy: new targets for antifungal agents. *J* ***Antimicrob. Chemother;*** **44:**151–61.

3. Angeles, C.M.; Platas, G.; Collado J.; Abruzzo, G.K.; Douglas, C.; Thompson, G.; Li, K.; and Pelaez, F.; (2001): Arundifungin, a novel antifungal compound produced by fungi: biological activity and taxonomy of the producing organisms**.** ***J Int. Microbiol;*** **4:**93-102.

4. Anke, T.; Werle, A.; Bross, M.; and Steglich, W. (1990): Antibiotics from basidiomycetes. Oudemansin X, a new antifungal (E)-β-methoxyacrylate from Oudemansiella radicata. ***J Antibio;*** **43:**1010-1011.

B

5. Bacon, C. W., and White, J.F.; (2000): Microbial endophytes; *Mycotaxon;* **72:**33-42.

6.Beck-Sagué, C.; Jarvis, W.R.; (1993): Secular trends in the epidemiology of nosocomial fungal infections in the United States 1980-1990**.** ***J Infect Dis*** **;167:**1247-1251.

7. Billstein, S. A.**;** (1994): How the pharmaceutical industry brings an antibiotic drug to market in the United States. ***J Antimicrob. Agents Chemother*****;** **38:**2679-2682**.**

8. Bodey, G. P.; (1992): Azole antifungal agents. ***J Clin. Infect. Dis.*** **14(Suppl. 1);** ***S161-S169.***

9. Brady, S. F.; and Clardy J.; (2000): CR377, a new pentaketide antifungal agent isolated from an endophytic fungus. ***J. Nat. Prod*****;** **63:**1447-1448.

10. Brewer, S.; (2000): The relationship between natural products and synthetic chemistry in the discovery process. ***Mycotaxon;*** **84 :** 39-50.

C

11. Cartledge, J. D.; Midgley, J.; and Gazzard, B.G.; (1997):Itraconazole cyclodextrin solution: the role of *in vitro* susceptibility testing in predicting

successful treatment of HIV-related fluconazole-resistant and fluconazole-susceptible oral candidosis. ***AIDS;* 11:**163-168.

12. Clark, F.S.; Parkinson, T.; Hitchcock, C.A., and Gow, N.A.; (1996): Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species: possible role for drug efflux in drug resistance. ***J Antimicrob Agents Chemother*; 40:**419–425.

13.Corrado, M.; and Rodrigues, K.F.; (2004): antimicrobial evalution of fungal extracts produced by endophytic strains of Phomopsis speceis.***J.Basic.Microbiol*; 44 (2**): 157-160.

14**.** Concepcion, G. P., Lazaro J.E.; and Hyde K.D.; (2001): Screening for bioactive novel compounds, bio-exploitation of filamentous fungi. *Microbiology*; 148:2675-2685.

D

15. Daniel J.S.; Christopher, A.; and Sibley, C.M.; (1999): Current and Emerging Azole Antifungal Agents. ***Clinical Microbiology Reviews*; 12**: 40-79.

16. Demain, A.L.;(1999): Pharmaceutically active secondary metabolites of microorganism**.** ***J Appl Microbial Biotechnol*.52:** 455-463.

17. Demain, A. L.; (2000): Microbial natural products: a past with a future. *Industrial microbiology*. *Science*; **214:**987-994.

18. Denning**,** A.L.; (1994): High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS**.** ***J. Antimicrob. Chemother*; 34:**659-668.

19. Denning, D. W., Venkateswarlu, K.; Manning, N.J.; Stevens, D.A.; Warnock, D.W.; and Kelly, S.L.; (1997):Itraconazole resistance in *Aspergillus fumigatus*. ***J Antimicrob. Agents Chemother*; 41:**1364-1368.

20. Dreyfuss, M. M., and Chapela, I.H.; (1994). Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals, the discovery of natural products with therapeutic potential. ***J. Nat. Prod;* 60:**1214-1215.

E

21. Ebenebe, A.C.; (1980): The effects of fungicidal agrochemicals on heat-resistant fungi *in vitro*. ***J Plant Dis.;* 64:**1030.

22. Edwards, J. E.;(1995): Principles and practice of infectious diseases. *Reviews of Infectious Diseases;* **13**: 480–486.

23. Elewski, B. E., and Hay, R.J.; (1996): Cutaneous antifungal therapy in AIDS: summary of an international summit**.** ***J Infect. Dis. Clin. Pract*****;** **5**:359-370.

24. Ellis, M.; (2002): Invasive fungal infections: evolving challenges for diagnosis and therapeutics**.** ***J Mol Immunol*****; 38:**947–957**.**

F

25. Fostel, J.M.; Lartey, P.A.; (2000): Emerging novel antifungal agents. ***Curr Trends Drug Discov Today*****; 5:25**–32.

26.Francis, P., and Walsh, T.J.; (1992): Evolving role of flucytosine in immunocompromised patients: new insights into safety, pharmacokinetics, and antifungal therapy. ***J Clin. Infect. Dis.*****; 15:**1003-1018**.**

27. Fromtling, R. A.; (1988): Overview of medically important antifungal azole derivatives. ***J Clin. Microbiol. Rev.*****; 1:**187-219.

28. Fujiu, M.; Sawairi, S.; Shimada, H.; Takaya, H.; and Yokose, K.; (1994): Azoxybacilin, a novel antifungal agent produced by *Bacillus cereus* NR2991, production, isolation and structure elucidation**.** ***J Antibiot;*** **47:**833–835.

G

29. Gary.S.; and Daisy, B.;(2003): Bioprospecting for microbial endophytes their natural products. ***Microbiology & molecular biology reviews*****; 67:**491-502.

30. Georgopapadakou, N.H.; Walsh, T.J.; (1996): Antifungal agents: chemotherapeutic targets and immunologic strategies. *J.* ***AntimicrobAgents Chemother;*** **40:**279–291**.**

31. Georgopapadakou, N.H.; Walsh, T.J.; (1994): Human mycoses: drugs and targets for emerging pathogens**.** ***Science*****; 264:**371–373.

32. Gerth, K.; Bedorf, N., Irschik, H., Hofle, G.; Reichenbach, H.; (1994): The soraphens: a family of novel antifungal compounds from *Sorangium cellulosum* (Myxobacteria) fermentation, isolation, biological properties. ***J Antibiot*****; 47:**23–31.

33. Ghannoum, M. A.;(1997): Future of antimycotic therapy. ***Dermatol. Ther.;*** **3:**104-111.

34. Guarro, K. L., and Barradell, L.B.; (1997): Fluconazole. An update of its pharmacodynamic and pharmacokinetic properties and therapeutic use in major superficial and systemic mycoses in immunocompromised patients. ***Drugs;*** **50:**658-690.

35. Graybill, J. R.; (1996): The future of antifungal therapy. ***J Clin. Infect. Dis.*****; 22(Suppl. 2):** S166-S178.

36. Graybill, J. R**.;** (1988): The long and the short of antifungal therapy. ***Infect. Dis. Clin. North Am.*****; 2:**805-825**.**

37. Grabley, S., and Thiericke, E.; (1999): Drug discovery from nature. *J. Am. Chem. Soc.;* **123:**9837-9842.

38. Grant, S. M., and Clissold S.P.; (1990): Fluconazole: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. ***Drugs*****; 39:**877-916.

39. Gupta, A. K., Sauder, D.N.; and Shea, N.H.; (1994): Antifungal agents: an overview**.** ***I. J. Am. Acad. Dermatol;*** **30:**677-698.

40. Gupte, M., Kulkarni, P., Ganguli, B.SN.; (2002): antifungal antibiotics ***.J Appl Microbial Biotechnol;*** **58:** 46-57.

H

41. Hawksworth, D. C., and Rossman, A.Y.; (1987). Where are the undescribed fungi? ***Phytopathology;*** **87:**8.

42. Horn, W.S.; Smith, J.L., and Bills, G.F.;(1994): Sphingofungins E and F: novel serine palmitoyltransferase inhibitors from *Paecilomycesvariotii*. ***J Antibiot;*** **47:**376–379.

43. Hosoe, T.; Okada, H.; Itabashi, T.; Nozawna, K.; Okada, K.; Takaki, G.M.; Fukushima, K.; Miyaji, M.; and Kawai, K.; (2000): A new pentanorlanostane derivative, cladosporide A, as a characteristic antifungal agent against Aspergillus fumigatus, isolated from Cladosporium sp. ***Chem Pharm Bull (Tokyo)*****; 48(10):** 1422-6.

I

44. Inmaculada, S.; Francisco, A.; Angela, B.; Angeles C.M.; Teresa, D.M.; and Francisca, V.; (2000): Screening of basidiomycetes for antimicrobial activities**.** ***Antonie van Leeuwenhoek;*** **78:** 129–139.

45. Iwamoto, T., Fujie, A.; Sakamoto, K.; Tsurumi, Y.; Shigematsu, N.; Yamashita, M.; Hashimoto, S.; Okuhara, M.; and Kohsaka, M.; (1994a): WF11899A, B and C, novel antifungal lipopeptides I. Taxonomy, fermentation, isolation and physico-chemical properties. ***J Antibiot;*** **47:**1084–1091.

46. Iwamoto, T.; Fujie, A.; Nitta, K.; Hashimoto, S.; Okuhara, M.; and Kohsaka, M.; (1994b): WF11899A, B and C, novel antifungal lipopeptidesII. Biological properties**.** ***J Antibiot*****; 47:**1092–1097.

J

47. Jabra-Rizk, M.A.; Falkler, W.A.; Merz, W.G.; Kelley J.I.; and Millar, T.F.; (2000): Retrospective identification of *Candida dubliniensis* among *Candida albicans* clinical laboratory isolates from HIV and non-HIV individuals. ***J Clin Microbiol; 38*****: 688-891.**

K

48. Katia F.R.; and Gary J.S.; (1999): Fungal endophytes of *Spondias mombin* leaves in Brazil.***J. Basic Microbiol*****; 39 (2):** 131-135.

49. Kauffman, C. A., and Carver, P.L.; (1997): Antifungal agents in the 1990s. Current status and future developments; ***Drugs*** **53:**539-549.

50. Kelly, S. L., Lamb, D.C.; Kelly, D.E.; Loeffler, J.; and Einsele**,** H.; (1996): Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. ***Lancet; 348:1523***-1524.

51. Kurtz, M.B.; (1998): New antifungal drug targets: a vision for the future. ***ASM News*****; 64:31**–35.

52. Kurtz, M.B.; Douglas, C.M.; (1997): Lipopeptide inhibitors of fungal glucan synthase. ***J Med Vet Mycol;*** **35:**79–86.

L

53. Law, D., Moore, C.B.; Wardle, H.M.; Ganguli, A.M.; Keaney, M.G.; and Denning, D.W.; (1994): High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS**.** ***J. Antimicrob. Chemother.*****; 34:**659-668.

54. Lee, J., E. Lobkovsky, N. B. Pliam, G. A. Strobel, and J. Clardy. (1995): Subglutinols A and B: immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*. **J. *Org. Chem;*. 60:**7076-7077

55. Li, J. Y., Strobel, G.A.; Harper, J.K.; Lobkovsky, E., and Clardy; J.; (2000): Cryptocin, a potent tetramic acid antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina*. ***Org. Lett*, 2:**767-770**.**

56. Li, J. Y., Harper, J.K.; Grant, D.M.; Tombe, B.O.; Bashyal, B.; Hess, W.M., and Strobel, G.A.; (2001): Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaeti*a sp. ***Phytochemistry*; 56:**463-468.

57. Liu, C.H.; Meng, J.C.; Zou, W.X.; Huang, L.; Tang, H.Q.; and Tan, R.X.; (2002): Antifungal metabolite with a new carbon skeleton from Keissleriella sp. YS4108, a marine filamentous fungus. ***J Planta Med.;*68 (4)**: 363-5.

58. Lu, H.W.; Zou, X.; Meng, J.C.; and Tan, R, X.; (2000): New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. ***Plant Sci.;* 151:***67-73*.

M

59. Maesaki, S.; Iwakawa, J.; Higashiyama, Y.; Miyazaki, Y.; Yanagihara, K.; Tomono, K.; and Kohno, S.; (2000): Antifungal activity of a new triazole, voriconazole (UK-109496), against clinical isolates of Aspergillus spp. ***J Infect Chemother*; 6(2):** 101-3.

60. Mary, A.J.; William A. F.; and Timothy F. M.; (2004). Fungal Biofilms and Drug Resistance**.** ***J Emerg Infect Dis;* 10:** 98-105

61. McGinnis, M.R., and Rinaldi, M.G.; **(**1998): Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. *Antibiotics in Laboratory Medicine*; **56**:223-281.

62. McGinnis, M.R.;(1980): Susceptibility testing and bioassay procedures.*Laboratory Handbook of Medical Mycology;* **83**:412-446

63. McGinnis, M. R., Pasarell, L.; Sutton, D.A.; Fothergill, A.W.; and Rinaldi, M.G. (1997): In vitro evaluation of voriconazole against some clinically important fungi. ***J Antimicrob. Agents Chemother*; 41:**1832-1834.

64. McGinnis, M.R.; and Rinaldi, M.G. (1991): Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing and assays of activity in biological fluids. *Antibiotics in laboratory medicine*; **87**: 198–257.

65. Minamoto, G. Y., and Rosenberg, A.S.; (1997): Fungal infections in patients with acquired immunodeficiency syndrome. ***Med. Clin. North Am.;* 81:**381-409**.**

66. Mohamed, A.; and Abdel-Sater, T.; (2001): Antagonistic Interactions Between Fungal Pathogen and Leaf Surface Fungi of Onion (*Allium cepa* L.). *Pakistan Journal of Biological Sciences*; **4 (7):** 838-842.

67. Morris, S.A.; Schwartz, R.E.; Sesin, D.F.; Masurekar, P.; Hallada, T.C., Schmatz, D.M.; Bartizal, K.; Hensens, O.D.; Deborah, Z.L. (1994): Pneumocandin Do, a new antifungal agent and potent inhibitor of *Pneumocystis carinii*. ***J Antibiot;*** **47:**755–764.

O

68. Odds, F. C.; (2000): Resistance of yeasts to azole-derivative antifungal**.** ***J. Antimicrob. Chemother*****; 31:**463-471.

P

69. Pfaller, M. A., Messer, S.; and Jones, R.NS.;(1997): Activity of a new triazole, Sch56592, compared with those of four other antifungal agents tested against clinical isolates of *Candida* spp. and *Saccharomyces cerevisiae***.** ***J Antimicrob. Agents Chemother*****; 41:**233-235**.**

70. Pfaller, M.A., Buschelman, M.B.; Lancaster, M.; Rex, J.H.; Rinaldi, M.G.; Cooper, C.R. and McGinnis, M.R. (1995): Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. ***J. Clin. Microbiol*****; 33:**1104-1107**.**

71. Pieckova, E.; and Roeijmans, H.;(1999): Antibiotics secondary metabolites of *Dichotomomyces cejpii*. . *Mycopathilogia; 146: 121*-126.

72. Pittet, D.; (1997): Microbiological factors influencing the outcome of nosocomial bloodstream infections: a 6-year validated, population-based model. ***Clin Infect Dis*****; 24:**1068-1078.

73. Powderly, W. G.; (1994): Resistant candidiasis. ***AIDS Res. Hum. Retroviruses;*** **10:**925-929.

74. Pulici, M., Yoshida, S.; Lobkovsky, E.; and Clardy, J.;(1996): Metabolites of endophytic fungi of *Taxus brevifolia*-the first highly functionalized humulane of fungal origin. ***J. Chem. Res. N.; 8:*** 378-379.

75. Pulici, M.; Koshino, H.; Uzawa, J.; Yoshida, S.; Lobkovsky, E.; and Clardy, J.;(1996): Pestalotiopsin-A and pestalotiopsin-B: new caryophyllenes from an endophytic fungus of *Taxus brevifolia*. ***J. Org. Chem.*****; 61:**2122-2124.

76. Pulici, M., Sugawara, F.; Koshino, H.; Uzawa, J.; Yoshida, S.; Lobkovsky, E.; and Clardy, J.; (1996): A new isodrimeninol from *Pestalotiopsis* sp. ***J. Nat. Prod*; 59:**47-48.

Q

77. Quart, A., Espinel-Ingroff, A.; and Reich, D.; **(**1995); Fluconazole for refractory oropharyngeal candidaisis in AIDS patients. ***AIDS Patient Care*; 9:56**-59.

R

78. Rentz, A.M.; (1998)**:** The impact of candidemia on length of hospital stay, outcome, and overall cost of illness**.** ***Clin Infect Dis;* 27:**781-788.

79. Revankar, S. G., Redding, S.W.; Rinaldi, M.G.; and Patterson, T.F.; **(**1996): Detection and significance of fluconazole resistance in oropharyngeal candidiasis in human immunodeficiency virus-infected patients**.** ***J. Infect. Dis.;* 174:** 821-827.

80. Rex, J. H., Galgiani, J.N.; and Anaissie E.J.; (1995): Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. ***J Antimicrob. Agents Chemother;* 39:**906-909.

81. Richardson, M. D.;(1991):Opportunistic and pathogenic fungi. ***Journal of Antimicrobial Chemotherapy;* 28, *Suppl. A,*** 1–11.

82. Rodrigues, K.F.; Manfred, H.; and Christa, W.; (2000): Antimicrobial activity of secondary metabolites produced by endophytic fungi from *Spondias mombin***.** ***J. Basic Microbiol;* 40(4):** 261-267.

83. Rosa, L.H., Jacob, C.C., Capelari, M.; and Zani, C.L.; (2003): Screening of brazilian basidiomycetes for antimicrobial activity. ***J Antimicrob. Chemother;* 98 (7):** 967-974.

S

84. Santamarı, O.; and Diez, J.J.; (2005): Fungi in leaves, twigs and stem bark of Populus tremula from northern Spain. ***J For. Path*; 35:** 95–104.

85. Sheehan, D. J., Espinel-Ingroff, A.; Moore, L.S.; and Webb, C.D.;(1993): Antifungal susceptibility testing of yeasts: a brief overview. *Clin. Infect. Dis. 17(Suppl. 2)***:** S494-S500.

86. Shadomy, S.; Espinel-Ingroff, A.; Cartwright, R.Y.; (1985): Laboratory studies with antifungal agents: susceptibility tests and bioassays. *Manual of clinical microbiology*; **146**: 991–999.

87. Siamak M.Samiee, Nasrin Moazami, Saeid Haghighi, Farzaneh Aziz Mohseni, Saeid Mirdamadi and Mohammad Reza Bakhtiari. (2003) Screening of lovastatin production by filamentous fungi. *J Iran. Biomed;* **7 (1):** 29-33.

88. Strobel, G. A.; (2002): Rainforest endophytes and bioactive products. ***Crit. Rev. Biotechnol; 22:***3SS15-333.

89. Strobel, G., Sidhu, R.S.; and Hess, W.M.; (1996): Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallichiana*. ***Microbiology;*** **142:**435-440.

90. Strobel, G. A., Sugawara, F.; Harper, J.; and Hess W.M.; (1999): Oocydin A, a chlorinated macrocyclic lactone with potent anti-oomycete activity from *Serratia marcescens*. ***Microbiology;*** **145:**3557-3564.

91. Strobel, G. A., Miller, R.V.; Teplow, D.W.; and Hess, W.M.; (1999): Cryptocandins, a potent antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina*. ***Microbiology;*** **145:**1919-1926.

92. Sullivan, D.; Coleman, D.; (1997): *Candida dubliniensis*: an emerging opportunistic pathogen. ***Curr Top Med Mycol;*** **8: 15**–25.

T

93. Torres, A.; El-Ebiary, M.; and Marrades, R.;(1995): Aetiology and prognostic factors of patients with AIDS presenting life-threatening acute respiratory failure. ***J Eur Respir;*** **8:**1922-1928.

V

94. Vanden Bossche, H., Marichal, P.; Odds, F.C.; Provost, F.; and Ronin O.; (1994): Mechanisms and clinical impact of antifungal drug resistance. ***J. Med. Vet. Mycol;*** **32:**189-202.

95. Vicente, M.F.; Cabello, A., Platas, G., Basillo A., Diaz, M.T., Thompson, J.; Pelaez F., and Wilson K.E.;(2001): Antimicrobial activity of ergokonin A from *Trichoderma longibrachiatum****.J applied Microbiology ;*****91**:806-813.

96. Vincent, J.L.; Bihari, R.S.; and Suter, P.M.; (1995): The prevalence of nosocomial infection in intensive care units in Europe. Results of the

European Prevalence of Infection in Intensive Care (EPIC) Study. ***EPIC International Advisory Committee; 274***: 639-644.

97. Vincent, T. A.; (1999): Current and future antifungal therapy: new targets for antifungal agents. ***Journal of Antimicrobial Chemotherapy*; 44:** 151-162.

98. Vincent, J.L.; Anaissie, E.; and Bruining, H.; (1998): Epidemiology, diagnosis and treatment of systemic Candida infection in surgical patients under intensive care. ***Intens Care Med;* 24: 206**-216.

W

99. Walsh, T. J.; Gonzalez, C.; Chanock, S.J.; and P. A. Pizzo. (1996): Invasive fungal infectious in children: recent advances in diagnosis and treatment. ***Adv. Pediatr. Infect. Dis*; 11:**187-290.

100. Walsh, T. J. (1992): Invasive fungal infections: problems and challenges in developing new antifungal compounds. ***Emerging Targets in Antibacterial and Antifungal Chemotherapy*; 45:** 349-373

101. Wanger, A., Mills, K.; Nelson, P.W.; and Rex, J.H.; (1995): Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant *Candida* isolates. ***Antimicrob. Agents Chemother*; 39:** 2520-2522.

102. Watanabe, S.; Hirai, H.; Kato, Y.; Nishida, H.; Saito, T.; Yoshikawa, N.; Parkinson, T.; and Kojima, Y.; (2001): CJ-19, 784, a new antifungal agent from a fungus, Acanthostigmella sp. ***J Antibiot (Tokyo); 54(12)*:** 1031-1035.

103. Whiffen, A.J.; Bohonos, N.; and Emerson, R.L.; (1946): The production of an antifungal antibiotic by *Streptomyces griseus*. ***J Bacteriol; 56*:** 283–291.

104. White, M. H.; (1996): Is vulvovaginal candidiasis an AIDS-related illness*?* ***Clin. Infect. Dis*. 22(Suppl. 2):** S124-S127.

105. White, T. C.; (1997): Antifungal drug resistance in *Candida albicans*. *ASM News; 63*:427-433.

106. White, T. C., Marr, K.A.; and R. A. Bowden, R.A.; (1998): Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. ***Clin. Microbiol. Rev.*; 11:**382-402.

107. Wills, E.A.; Redinbo, M.R.; and Perfect, J.R. (2000): New potential targets for antifungal development. ***Emerg Ther Targets;* 4:**265–296.

108. Worapong, J., Strobel, G.A.; Ford, E. J.; and W. M. Hess**.** 2001. *Muscodor albus* gen. sp., an endophyte from *Cinnamomum zeylanicum*. ***Mycotaxon;* 79:** 67-79.

Y

109. Yotsuji, A., Shimizu, K.; Araki, H.; Fujimaki, K.; Nishida, N.; Yamamoto, S.; Hayakawa, H.; and Imaizumi H.; (1997): T-8581, a new orally and parenterally active triazole antifungal agent: in *vitro* and in *vivo* evaluations. ***Antimicrob. Agents Chemother*; 41:**30-34.

Z

110. Zou, W. X.; Meng, J. C.; Lu, H.; Chen, G.X.; Shi, G.X.; Zhang, T.Y.; and Tan, R.X.; (2000): Metabolites of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*. ***J. Nat. Prod*; 63:** 1529-1530.

111. Zweerin, M.M.; Edison, A.M.; Wells, G.B.; Pinto, W.; Lester, R.L.; (1992): Characterization of a novel, potent and specific inhibitor of serine palmitoyltransferase**.** ***J Biol Chem*; 267**: 25032–25038.