ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS FROM *DIOSPYROS KAKI*

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

SUNITY

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)

JUNE, 2009

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS FROM DIOSPYROS KAKI** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period of January 2004 to June 2009 under the supervision of Dr. R. Prasad, Associate Professor and Dr. G.S. Randhawa, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(SUNITY)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

24.6.2009

(G.S.Randhawa)
Supervisor

(Ramasare Prasad)
Supervisor

Dated: 24.6.2009

The Ph.D. Viva-Voce Examination of **Ms. Sunity**, Research Scholar has been held on Oct 9, 2010, 9.30 A.M.

(R. PRASAD)
Signature of Supervisor

9/10
Signature of External Examiner

24.6.2009

ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge those individuals who inspired and encouraged me to fulfill this herculian task and without their help and co-operation this work would not have been possible, therefore, I take this opportunity to express my feelings and sense of gratitude to all of them.

I express my deepest and profound gratitude to my supervisor Dr. Ramasare Prasad, Department of Biotechnology, Indian Institute of Technology Roorkee, for his miraculous guidance, discreet support and keen interest throughout the course. His unbounded affection, inspirational guidance, unfailing enthusiasm, and tireless interest make me remain indebted to him. His earnest desire for my success will always be my pride and my devotion and respect for him will be forever.

I have deep and sincere sense of gratitude towards Prof. G.S. Randhawa, who was my mentor in the beginning of this study and had provided me constant encouragement and morale support throughout my stay in the department.

My sincere thanks goes to Dr. B.N. Pal, Nursery Incharge, Company Garden, Saharanpur and Mr. Madan, Rajeshwari Nursery, Jwalapur for providing me the authentic plant samples. My thanks also goes to Indian Agriculture Research Institute (I.A.R.I), New Delhi, and Post Graduate Institute of Medical Education and Research, Chandigarh for providing the fungal isolates for the study.

I would also like to express sincere thanks to Mrs. Chanda for Transmission Electron Microscope facility in the Department of Microscopy, All India Institute of Medical Sciences (AIIMS), New Delhi. I would like to acknowledge Professor Ravi Bhushan, Head, Department of Chemistry, I.I.T. Roorkee for his consent to use the analytical facilities in the chemistry department for the characterization of my compounds.

This work would not be possible without the help and support of all the members of the office staff. I appreciate and wish to express my heartfelt thanks to my colleagues Ajeet Singh, Ashish Deep Gupta, Seema Parveen, Nilesh Kumar Sharma, Garima Sharma, Sreela Dey and Alok Jha for their complete support in all possible ways during this period.

Last, but not least I want to thank my mother, brother, sister and my whole family for their blessing and unconditional support, who provided me strength and confidence to attain this academic level.

Finally, I thankfully acknowledge the Ministry of Human Resource Development (MHRD) for providing me financial support during this period.

Sunity

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
Am-B	Amphotericin-B
CFU	Colony Forming Unit
CNS	Central Nervous System
^{13}C NMR	Carbon-13 NMR
D_2O	Deutrium Oxide
DMSO	Dimethyl Sulfoxide
EI-MS	Electron Impact Mass Spectroscopy
5-FC	5-fluorocytosine
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FTIR	Fourier Transform Infra Red Spectroscopy
^{1}H NMR	Proton NMR
HIV	Human Immunodeficiency Virus
IC_{50}	50 % Inhibitory Concentration
ITCC	Indian Type Culture Collection
MFC	Minimum Fungicidal Concentration
MHZ	Megahertz
MIC	Minimum Inhibitory Concentration
MTCC	Microbial Type Culture Collection
MCCL	Mycology Culture Collection

MTT	Methyl Tetrazolium Violet
NCCLS	National Centre of Clinical Laboratory Standards
PBS	Phosphate BufferSaline
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PTLC	Preperative Thin Layer Chromatography
RPMI 1640	Roswell Park Memorial Institute Media
Rf	Retention Factor
S.E	Standard Error
SDA	Sabouraud Dextrose Agar
SDB	Sabourauds Dextrose Broth
SEM	Scanning Electron Microscopy
SiO_2	Silicon Dioxide
TEM	Transmission Electron Microscopy
TLC	Thin layer Chromatography
UV	Ultra Violet
WHO	World Health Organization
Fig	Figure
mg	Milligram
ml	Millilitre
mM	Millimole
°C	Degree Centigrade
µg	Microgram
v/v	Volume/Volume
w/v	Weight/Volume

sec	Second
h	Hour
min	Minute
g	Gram
l	Litre
M	Molarity
α	Alpha
β	Beta

INTRODUCTION

The epidemiology of invasive fungal infection has changed during the past 20 years. The incidence has increased and the population of patients at risk has expanded to include those with a broad list of medical conditions, such as solid organ and hematopoietic stem cell transplantation (HSCT), cancer, receipteints of immunosuppressive therapy, AIDS, premature birth, advanced age and major surgery (Chakrabarti, 2005; Groll and Walsh, 2001). Furthermore the etiology of these infections has changed. In the 1980s, yeasts (particularly *C. albicans*) were the most common causative agents of invasive mycosis. In recent years, infection by molds has become more frequent in certain group of patients (Nucci and Marr, 2005; Krcmery *et al.*, 2005).

Fungal infections are associated with high morbidity and mortality in patients with hematologic malignancies and immunocompromised host defenses (Ascioglu *et al.*, 2002; Fridkin and Jarvis, 1996; Hospenthal and Rinaldi, 2003; Wanke *et al.*, 2000). While *Aspergillus* and *Candida spp.* collectively account for the majority of infections in these settings, recent epidemiological trends indicate a shift towards infections caused by non albicans *Candida spp.* as well as previously uncommon opportunistic fungi including the agents of mucormycosis (Zygomycoses), filamentous fungi (such as *Fusarium* species, *Acremonium* species, *Paecilomyces* species, *Pseudallescheria boydii* and *Scedosporium prolificans*), dematiaceous fungi (such as *Bipolaris* species, *Cladophialophora bantiana*, *Dactylaria gallopava*, *Exophiala* species, and *Alternaria* species) and yeast like pathogens (such as *Trichosporon* species, *Blastoschizomyces capitatus*, *Malassezia* species, *Rhodotorula rubra* and others) are increasingly encountered as causing life threatening invasive infections that are often refractory to conventional therapies. The mortality of fungal infections in solid organ transplant recipients varies from 27-77 % (Fisher-Hosh and Hutwagner, 1995; Patel and Paya, 1997). The clinical agents involved include *Candida albicans, Candida krusei, Candida glabrata, Candida tropicalis, Candida zeylanoides, Candida neoformans*, *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terrus, Pseudallescheria boydii, Scopulariopsis brumptii, Chaetomium globosum, Trichosporon beigelii, Sporothrix schenckii, Dactylaria constricta, Scedosporium inflatum, Trichosporon viride, Phialemonium spp, Cladosporium trichoides, Conidiobolus coranatus, Histoplasma capsulatum, Coccidiodes immitis, Blastomyces dermatitidis, Paracoccidiodes brasiliensis* etc.

Although it appears that many drugs are available for the treatment of systemic and superficial mycoses, their clinical usefulness is hampered by drawbacks associated with safety or efficacy (Graybill, 2000; Mohsen and Hughes, 1998; Nosanchuk, 2006). Many of the drugs currently available have undesirable effects or are very toxic e.g amphotericin B. Some like azoles are fungistatic and not fungicidal and some may lead to the development of resistance, as in flucytosine (Allende *et al.*, 1994; Layman and Walsh, 1992; Rapp, 2004). According to Pollak 1999, ideal drugs to cure fungal infections have not been discovered yet. In the meantime resistance to currently available antifungal agents continues to grow (Ghannoum and Rice, 1999; Kontoyiannis and Lewis, 2002; Moore, 2000; Patterson, 2003; Rex *et al*, 1995; Sanglard, 2002). Therefore there is a real need for a next generation of safer and more potent antifungal agents (Pauw, 2000; Sangamvar, 2008).

Certain conditions are required for a compound to be a good antifungal agent : it must be fungicidal rather than fungistatic and have a broad spectrum of activity, a minimum emergence of resistant strains, and a selective mechanism of action. In addition, the agent should have minor toxic side effects and good availability (Ablordeppey *et al.*, 1999; Pollak, 1999).

Plants have been used as medicine since millennia. Out of estimated 25,000 to 35,000 plant species identified so far, about 35,000 are used worldwide for medicinal purposes (Iwu *et al.*, 1999; Ming *et al.*, 2003). It has been confirmed by WHO that herbal medicines serve the health needs of 80 percent of world population; especially for millions of people in the vast areas of developing countries (Ming, 2003; Pal and Shukla, 2003). Meanwhile consumers in developed countries are becoming disillusioned with modern healthcare and are seeking alternatives (Briskin, 2000; Patwardhan *et al.*, 2004; Balunas and Kinghorn, 2005). Several important drugs used in modern medicine have come from medicinal plant studies eg. taxol, vinblastine, vincristine (Raskin *et al.*, 2002; Raskin and Ripoll, 2004). However studies on plants are very limited (Fabricant and Farnsworth, 2001; Jhachak and Saklani, 2007). Only about a third of the million or so species of higher plants have been identified and named by scientists. The recent resurgence results from several factors :

1. Problems with drug resistant microorganisms, side effects of modern drugs and emerging diseases where no medicines are available.

2. The effectiveness of plant medicines.

3. Improvements in the methods of purification and analysis and powerful new technologies such as automated separation techniques, high throughput screening and combinatorial chemistry (Borris, 1996; Nash and Turner, 1996).

4. Pharmaceutical scientists are experiencing difficulty in identifying new lead structures, templates and scaffolds in the finite worlds of chemical diversity and therefore natural products are preferred due to their high molecular weight and exhibit a different distribution of heteroatoms. They comprise structural elements that are under represented by synthetic compounds and contain significantly more rings and chiral centers (Bindseil *et al.*, 2001).

The role of natural products is two fold in the development of new drugs (Turner, 1996)-

1. They may become base/blueprint for the development of a medicine. This is when the potency, selectivity and specificity of bioactive principle is acceptable, isolation, purification and structural elucidation follows. This bioactive principle is then modified through medicinal chemistry synthesis programme in order to produce a molecule that has both the essential biological and desirable chemical properties to become a drug development candidate.
2. Plant extract to be used directly as phytomedicine.

As India is one of the top megadiverse countries of the world, medicinal plants are widely used by all sections of people as folk remedies in indigineious system of medicine (J achak and Saklani, 2007). Researchers are increasingly turning their attention to folk medicine looking for new leads to combat viral and microbial infections (Borris, 1996; Cowan, 1999). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority have not been adequately evaluated and their active principle are not known yet (Putheti and Okigbo, 2008). Therefore an attempt has been made in this study to search the plant with desired bioactivity and locate its active principle.

The objectives of the present study are :

Objectives :

- ✓ Screening of aqueous and methanolic extract of several plants for presence of antifungal activity.
- ✓ Selection of the plant and isolation/purification of the active compound using various chromatographic techniques.
- ✓ Characterization of active compound using various analytical techniques like EI-MS, FTIR, ^{1}H and $^{13}CNMR$.
- ✓ Evaluation of MIC, MFC, IC_{50} and pharmacodynamics of the pure compounds.
- ✓ Study of induced ultrastructural and morphological changes in fungi on exposure to the pure compounds by scanning and electron microscopy.
- ✓ Evaluation of *in vitro* cytotoxicity of the pure compounds.

LITERATURE REVIEW

2.1 HUMAN MYCOSES

Fungi are ubiquitous eukaryotic plant like organisms living in soil and decaying matter. They are generally classified as either yeasts or molds, although some are also dimorphic, which exists as yeasts within the host but take the form of mold at room temperature *in vitro*. There are more than a million species of fungi but only a minority of them less than 200 are pathogenic. Some mycotic infections are not transmissible amongst humans and do not require special precautions. However some fungi can be highly infectious if inhaled. Fungi have emerged as significant pathogens during the past few decades when they became more and more frequently diagnosed as opportunistic infections in immunocompromised host.

Fungal infections in humans are known as mycoses and are broadly of three types – superficial, subcutaneous and systemic mycoses (Friend, 1999; Anantnarayan, 2000). Superficial infections are by far the common ones and comprise various types of tinea or ringworm affecting the skin, hair and nails. These are mild though chronic disease. Subcutaneous mycoses involves the dermis, subcutaneous tissues, muscles, and fascia. Systemic mycoses occurs in varying degree of severity ranging from asymptomatic infections to fatal diseases. Oppurtunistic infections occur in patients with debilitating diseases such as cancer or diabetes or in whom the physiological state has been altered by immunosuppressive drugs, steroid, X- rays or broad spectrum antibiotics.

The list of documented fungal pathogens is extensive, and one can no longer ignore or dismiss fungi as contaminants or clinically insignificant when they are isolated from clinical material. It is also apparent that the prognosis and response to therapy may vary with the type of fungus causing the infection as well as with the immunological status of the host. Table 2.1 lists the main groups of mycoses, their etiologic agents, clinical manifestations and treatment options.

2.2 CURRENT ANTIFUNGAL ARMENTARIUM AND THEIR DRAWBACKS

Antifungal chemotherapy began in 1903 with the successful use of potassium iodide for the treatment of sporotrichosis. There was little progress in the next 50 years until nystatin was introduced in 1951. This was soon followed by the golden standard of antifungal chemotherapy Am-B in 1956. At present two groups of drugs are used-

Table 2.1 Major groups of mycoses in humans, their etiological agents, clinical manifestations and their treatment

Mycoses	Etiologic agents	Clinical manifestations	Therapy
Cutaneous Mycoses			
Dermatophytoses	*Microsporum, Trichophyton* and *Epidermatophyton*	Tinea corporis, T. cruris, T. barbae, T. pedis and T. capitis	Topical antifungal agents e.g Tolnaftate, Miconazole, Itraconazole and Terbinafine (Cutler,wwwdoctorfungus.org, www.cfsph.iastate.edu, 2005, Sen, 2006)
Subcutaneous Mycoses			
Phaeohyphomycois	*Exophiala, Phialophora, Bipolaris, Cladophialophoa, Cladosporim, Curvularia, Pseudallescheria*	Subcutaneous phaeohyphomycosis, paranasalsinus phaeohyphomycosis and cerebral phaeohyphomycosis	5flurocytosine, Itraconazole, Terbinafine (Mycology online, 2004, Nóbrega *et al.*, 2003, Kumar, 2006)
Sporotrichosis	*Sporothrix schenkii*	Cutaneous and lymphocutaneous sporotrichosis, pulmonary osseous disseminated disease	AmphotericinB,5flurocytosine and Itraconazole (Rippon, 1982, Feeney *et al.*, 2007)
Mucormycosis	*Rhizopus, Mucor* or *Absidia*	Sinusitis, pneumonia, skin infection, and sometimes gastrointestinal infections	Amphotericin B (Randhawa and Chaudhary, 2008; Wingard *et al*, 2006)
Pseudallescheriasis	*Pseudallescheria boydii*	Pulmonary disease involving paranasal sinuses and disseminated infection with involvement of deep viscera including the thyroid kidneys, brain, and heart	Miconazole (Nonaka, 2001, Kowacs, 2004)
Fusariosis	*Fusarium solani, F. oxysporum* and *F. moniliforme*	Single organ involvement, keratitis, endophthalmitis,	High dose Amphotericin B, lipid based amphotericinB formulations, Itraconazole and

		onchomycosis and cutaneous infection	Voriconazole (Ozkaya *et al.*, 2007; Dignani and Annaise, 2000)
Systemic Mycoses			
Cyptococcosis	*Cryptococcus neoformans*	Pulmonary cryptococcosis central nervous system cutaneous and mucocutaneous	AmphotericinB, 5-flurocytosine(Rippon, 1982, Mara *et al.*, 2003, Saif *et al.*, 2006)
Blastomycoses	*Blastomyces dermatitidis*	Acute and chronic pneumonia, skin lesions, subcutaneous nodules, bone and joint infection, central nervous system and genitourinary urinary infections	Amphotericin-B, Itraconazole (www.doctorfungus.com)
Coccidioido mycoses	*Coccidiodes immitis*	Pulmonary syndrome, diffuse pneumonia, skin manifestations, joints and bones and meningeal disease .	Amphotericin-B, Fluconazole (www.doctorfungus.com)
Paracoccidioi mycoses	*Paracoccidioides brasiliensis*	Mucosal lesions, pulmonary, skin and other organ involvement	Itraconazole (www.doctorfungus.com)
Histoplasmosis	*Histoplasma capsulatum*	Acute and pulmonary histoplasmosis, gastrointestinal, central nervous system and cardiac disease and ocular histoplasmosis	Amphotericin-B, Itraconazole (www.doctorfungus.com)

1. Drugs produced by various organisms
2. Drugs made synthetically

In the first group, only amhotericin-B administered systemically is active in numerous deep mycoses. Antifungal agents currently in the market falls into five major groups based on their structure. The antifungal agents, their spectrum, mode of action and resistence mechanism are given in Table 2.2. and are explained below.

2.2.1 Polyene Class

Amphotericin-B

Am-B (Fungizone) is the oldest class of systemic antifungals and polyenes remain the most rapidly acting of the antifungals. It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other. It is active against a wide variety of fungal species of both yeast and filamentous forms. Amphotericin B is not well absorbed orally and must be given intravenously for systemic infections. The drug is fungicidal or fungistatic depending on their concentration in body fluids and on the susceptibility of the causative fungus. It selectively binds to ergosterol, the major fungal sterol in the cell membrane of susceptibile fungi forming channels, which leads to changes in membrane permeability and leakage of cell components and ultimately cell death. Fevers and rigors occurs in more than 50 % of patients upon initial administration of amhotericin-B. Anorexia, vomiting, headache and myalgia are common reactions. Nephrotoxicity occurs early in the course of treatment generally within two weeks and is usually reversible in 80 %. Hemolytic anemia occurs in more than 75 % of patients. Polyene binds to cholesterol in human cell membrane which causes osmotic leak. When amhotericin-B is rapidly infused in patients with preexisting renal failure the release of potassium can cause dangerously high serum potassium concentrations. This can cause cardiac arrhythmias (Mohsen and Hughes, 1997; Nosanchuk, 2006; Bates, 2001, Graybill *et al.*, 2000; Dismukes *et al.*, 2000; Sawaya, 1995). Lipid formulations were developed to decrease adverse effects, especially nephrotoxicity. Although these products cause much less nephrotoxicity, chills and fever they are much more expensive than the deoxycholate formulation. As a result, they are usually recommended for use only in clients who cannot tolerate the older formulation or who are at risk for developing nephrotoxicity (Groll and Walsh, 2002).

2.2.2. Allylamine Class

Terbinafine

Terbinafine (Lamisil) is an allylamine structurally related to naftifine. Like other allylamines, terbinafine inhibits ergosterol biosynthesis via inhibition of squalene epoxidase. This enzyme is part of the fungal sterol synthesis pathway that creates the sterols needed for the fungal cell membrane. It is effective against dermatophytes, *Candida spp.*, *Sporothrix shenkii*, *Aspergillus spp.*, *Cryptococcus neoformans*,

Penicillium marneffei. The typical dose of terbinafine is 250 mg/day and is available for oral and topical administrations (Ingroff *et al.*, 2008). Topically 1 % cream and solution formulations are used. Adverse reactions to terbinafine are in general transient and mild. The incidence of these reactions has been found to be 10.5 % in a large scale study. Most involve the gastrointestinal system and the skin. Reversible agranulocytosis has been reported as a rare side effect.

2.2.3 Pyrimidine Class

Flucytosine (5-fluoro-2-pyrimidone) (Ancotil) is a pyrimidine class of drug and is fluorine analogue of cytosine which is readily absorbed orally. Flucytosine belongs to antimetabolite class of drugs. It is well distributed into tissues. It enters cells via cytosine permease and is deaminated to 5-flurouracil which is incorporated into RNA. Uridine 5-monophosphate phosphorylase then converts 5-flurouracil into flurodeoxyuridine monophosphate which inhibits thymidylate synthetase and interferes with DNA synthesis. It is active against *Candida, Crytococcus* and agents of *Chromoycosis*. Flucytosine is now used in initial therapy of cryptococcal meningitis, usually 100 mg/kg/day combined with amphotericin B at 0.7 mg/kg. It can be effective if used alone, but this is not usually done. Gastrointestinal upset is very common. Myelotoxicity including leucopenia and thrombocytopenia appears to be dose dependent. Hepatic and other adverse effects include increased transaminases, anemia, leucopenia and hematologic thrombocytopenia (Mohsen and Hughes, 1997; Nosanchuk, 2005; Bates *et al.*, 2001; Graybill *et al.*, 2000; Dismukes *et al.*, 2000).

2.2.4 Azoles

The azoles comprise the largest group of commonly used antifungal agents. Many of these are used topically and some are available without a prescription for dermatologic. An azole is a class of five-membered nitrogen heterocyclic ring compounds containing at least one other noncarbon atom, nitrogen, sulfur or oxygen. The parent compounds are aromatic and have two double bonds. In serious invasive fungal infections these drugs are often used long term following initial treatment with amphotericin B (Mohsen and Hughes, 1997; Yamaguchi, 2003). Primary target of azoles is the heme protein, 14-α-demethylase which cocatalyzes cytochrome P-450 dependent 14-α-demethylation of lanosterol. Inhibition of 1,4-α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors including 14-α-methylated sterols resulting in formation of plasma membrane with altered structure and function.

2.2.4.1. Ketoconazole

Nizoral was an important antifungal agent when it was first introduced. It had several advantages over amphotericin B in that it could be given orally, on an outpatient basis, and was somewhat less toxic. Little absorption occurs with topical use. It is active against most pathogenic fungi. Its side effects include nausea, anorexia, vomiting and diarrhea, itching and allergic rash. Hepatotoxicity with mild elevations in liver transaminases occurs in 2 % - 5 % of patients (Fung, 2008; Nosanchuk, 2006). It can interfere with adrenal corticosteroid synthesis although reports of adrenal insufficiency are rare. Also it may cause gynecomastia, oligospermia and decreased libido which are dose dependent symptoms that results from the lowering of serum testosterone levels with ketoconazoles action upon the cytochrome P450 system.

2.2.4.2. Fluconazole

Diflucan is a synthetic, broad-spectrum agent that is effective for *Candida spp.* with the exception of *Candida krusei, Crytococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Sporothrix shenkii* and *Aspergillus* species. Fluconazole can be given orally or intravenously. Fluconazole is usually well tolerated. Adverse effects, include gastrointestinal complaints, headache and skin rash including rare exfoliative skin reactions. Few reports of severe hepatotoxicity and about 1 % show an increase in transaminases (Mohsen and Hughes, 1997; Nosanchuk, 2006, Bates, 2001; Graybill *et al.*, 2000; Dismukes *et al.*, 2000).

2.2.4.3. Itraconazole

Sporanox is a synthetic, broad-spectrum agent covering *Candida, Aspergillus, Blastomyces, Histoplasma, Sporothrix* and *Dermatophytes.* It is available as oral or intravenous formulation. The drug is well tolerated in usual doses but may cause nausea and gastric distress. Higher doses may cause impotency, hypokalemia, hypertension, edema, and congestive heart failure. Itraconazole has significant interactions with several commonly prescribed drugs (aids.info.nih.gov, 2007; Fung, 2008).

2.2.4.4. Voriconazole

Voriconazole (Vfend) was first marketed in 2002. Its spectrum includes *Candida, Aspergillus, Scedosporium apiospermum* and *Fusarium* spp. It is available both in oral and intravenous form and has excellent bioavailability (90 %). As with other azoles, it interacts with drugs that are substrates of cytochrome P450 (terfenadine, cisapride, astemizole etc.), increasing their serum levels. Other adverse effects are mild elevation of transaminases and visual disturbances, rash and gastrointestinal symptoms (Petrikkos and

Skiada, 2007; Groll and Walsh, 2002).

2.2.4.5. Posaconazole

Posaconazole (Noxafil) is a hydroxylated analogue of itraconazole. It first became available in Europe in 2005 and it was approved by the FDA in 2006 for prophylaxis against invasive *Aspergillus* and *Candida* infections. Posaconazole is available only as an oral formulation. It has a bioavailability of 8-47 % on an empty stomach, which increases by 400 % upon ingestion of a high-fat meal. It has fewer side effects which include nausea, vomiting, headache, abdominal pain and diarrhea. Elevated liver enzymes may be observed. The main limitation of posaconazole is the fact that no intravenous formulation is available and therefore it cannot be used in some severely ill patients (Petrikkos and Skiada, 2007).

2.2.5. Echinocandins

The echinocandins are amphiphilic lipopeptides. They are cyclic hexapeptides N-linked to a fatty acyl side chain. They have a mode of action that is different from all the other antifungals. Their mode is non competitive inhibition of (1,3)-β-D-glucan synthase, an enzyme that is absent in mammalian cells which produces an important component of the cell wall of many pathogenic fungi. Inhibition of (1,3) -β-D-glucan synthesis disrupts the structure of the growing cell wall, resulting in osmotic instability and death of susceptible yeast cells (Randhawa and Sharma, 2004, Groll and Walsh, 2002; Petrikkos and Skiada, 2007).

2.2.5.1 Caspofungin

(Cancidas) is the first echinocandin antifungal. These drugs are usually fungicidal but they do not act as rapidly as amphotericin B. They are active against *Candida* including azole resistant strains, *Aspergillus* and the organisms that cause blastomycosis and histoplasmosis. Caspofungin is given intravenously and is highly bound to plasma albumin. It is usually well tolerated with doses of 50 mg/day. Its adverse effects include histamine type reactions like fever and rash, infusion related reactions including phlebitis, transient elevations in liver transaminase levels, headache, nausea and anaemia. Facial flushing has occurred during infusions (Petrikkos and Skiada, 2007).

2.2.5.2 Micafungin

Fungard in general has the same spectrum of antifungal activity as caspofungin including *Aspergillus spp, Cladosporium trichoides, Exophiala spinifera, Fonsecaea pedrosoi* and *Exophiala dermatitidis*. Its mode of administration is intravenous and adverse effects are nausea, vomiting, headache, diarrhoea, phlebitis and leucopenia possible histamine

mediated symptoms such as rash, pruritis, facial swelling, vasodilation and isolated cases of anaphylaxis and hemolysis have been described (Yamaguchi, 2003).

2.2.5.3 Anidulafungin

Anidulafungin (Eraxis) is the newest echinocandin approved for use in the USA and is active against *Bipolaris spicifera, Exophiala jeanselmi, Fonsecaeae pedrosoi, Madurella mycetomatis, Penicillium marneffei, Phialophora verrucosa, Pseudallescheria boydii, Sporothrix shenkii, and Wangiella dermatitidis* (Randhawa and Sharma, 2004; Groll and Walsh, 2002). It is used intravenously and the adverse effects reported from clinical trials were hypotension, vomiting, constipation, nausea, fever, hypokalaemia and elevated hepatic enzymes.

Table 2.2 Antifungal agents: spectrum, principal modes of action and resistance mechanisms of fungal pathogens

Antifungal agent	Spectrum	Mode of action	Mechanism of resistence
Polyenes			
Amphotericin -B	Broad activity against *Candida spp.* (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except, of the *Aspergillus* spp, *A. terreus* and *A. flavus*).	Binding to ergosterol and Alteration in specific steps of destabilization of cell ergosterol biosynthesis	Alteration in specific steps of ergosterol biosynthesis
Pyrimidines analogues			
5-fluorocytosine	Active against *Candida spp.* and *Cryptococcus spp*; however, rapid emergence of resistance can appear when 5-FC is used as monotherapy	Impairment of nucleic acid by formation of toxic fluorinated pyrimidine antimetabolites	Decreased uptake of 5-FC; decreased formation of toxic metabolites
Azoles			
Fluconazole	Active against *Candida spp.* and *Cryptococcus* spp, less active against *C. glabrata* and no activity against *C. krusei*; and filamentous fungi	Inhibition of cytochrome P450 14 α lanosterol demethylase	Enhanced efflux by upregulation of multidrug transporter genes, target alteration by occurrence of mutations and alteration of specific steps in ergosterol biosynthetic pathway

Voriconazole	Like Fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp		
posaconazole	Closely related to Itraconazole, but more active		
Allylamines			
Terbinafine	Active against most dermatophytes, poor activity against *Candida spp.*	Inhibition of sqalene epoxide	Unknown
Echinocandins			
Caspofungin	Active against *Candida spp.* with fungicidal activity, moderately active against *Aspergillus spp.*, poor activity against *C. neoformans*	Inhibition of the cell wall synthesis enzyme $\beta^{1,3}$glucan synthase	Unknown
Micafungin	Same spectrum of antifungal activity as Caspofungin		
Anidulafungin	*Bipolaris Exophiala, Fonsecaeae, Madurella, Fusarium, Pseudallescheria and sporothrix*		

2.3 DRUG RESISTANCE IN FUNGI

Like other living organisms, fungal cells may become resistant to toxic compounds (Anderson, 2005; Yang, 2001). Antifungal resistance may be defined as a stable, inheritable adjustment by a fungal cell to an antifungal agent, resulting in a less than normal sensitivity to that antifungal or the failure to eradicate fungal infections despite the administration of antifungal agent with *in vitro* activity against the organisms. Such failures can be attributed to a combination of factors related to the host, antifungal agent or the pathogen. Second the resistant pathogenic strains may also spread not only within healthcare institutions but in communities as well (Francis *et al.*, 2005; Herald *et al.*,1998). Third the spread of resistant pathogens within the community poses obvious additional problems for infection control. Finally with respect to the cost containtment pressures of todays healthcare environments, antimicrobial drug resistance places on additional burden on healthcare costs (Mcghowan, 2001). Microbiological resistance can be primary (intrinsic) or secondary (extrinsic) (Kanafani *et al.*, 2008).

Although extremely rare 10 years ago, antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem. For

instance, 33 % of late-stage AIDS patients in one study had drug-resistant strains of *C. albicans* in their oral cavities (White *et al.*, 1998; Ghannoum and Rice, 1999). There are no large-scale surveys of the extent of antifungal drug resistance, which has prompted requests for an international epidemiological survey of this problem. The current scenario of drug resistance to the commonly used antifungal agents is discussed below -

In general resistance to amphotericin-B is uncommon but several molds (*Pseudallescheria boydii*, *Scopulariopsis*, *Fusarium*) as well as the yeasts *T. beigelii*, *C. lusitaniae* and *C. guilliermondii*, possess primary resistance to amphotericin-B (White *et al.*, 1998; Bossche, 1997). Secondary resistance to amphotericin B has been found in yeasts causing infections in patients with cancer. According to SENTRY program, the prevalence of polyene resistance among *Aspergills spp.* has increased remarkably with only 11.5 % of *Aspergillus fumigatus* isolates inhibited at less than 1 μg/ml (Kanafani and Perfect, 2008).

Primary resistance to 5-flucytosine is common in certain yeasts and molds (Lupetii *et al.*, 2002). Non albicans *Candida* species as well as well as *Aspergillus spp.*, *Cryptococcus* and the dimorphic fungi, have high rates of 5-FC resistance (Ghannoum, 1999; Dismukes, 2000; Kontoyiannis and Russel, 2002). In addition, secondary resistance is a common development, especially in patients receiving 5-FC monotherapy. The severity of immunosuppression and fungal burden may be important risk factors leading to the development of resistance. Because 5-FC resistance develops frequently, the drug should never be used as a single agent to treat either yeast or mold infections.

As azole antifungal agents have become important in the treatment of mucosal candidiasis in AIDS patients, reports of resistance have increased. Earlier most investigations were focused on fluconazole resistant *C. albicans,* however during 1990s azole resistance in other *candida spp.* such as *C. glabrata* and *C. krusei* has been observed with the use of azoles. *C. krusei* is intrinsically resistant to fluconazoles. There are few reports of resistance developing in *C. albicans, C. tropicalis* or *C. glabrata* during short treatment of candidiasis. In fact, azole resistance has now been found in patients not infected with HIV and, in some situations, in patients not previously exposed to antifungal agents (Rex *et al.*, 1995; Sanglard, 2002). According to data from ARTEMIS, global antifungal surveillance program, the incidence of fluconazole resistance in *C. glabrata* increases from 7 % in 2001 to 12 % in 2004. Although resistance to triazoles is not common among *Aspergillus spp.* it has been reported a few times especially to itraconazole.

In case of echinocandins *in vitro* cross resistance is rare. *C. parapsilopsis* and *C.*

gulliermondii isolates have higher MIC values. There are reports of caspofungin resistence in *C. albicans*, *C. parapsilopsis* and *C. gulliermondii* (Ingroff, 2008; Perkin, 2007). Drug resistance to terbinafine has been reported in clinical isolates of *T. rubrum* and *Aspergillus spp.* (Ingroff, 2008).

So far much emphasis has been put on investigation of multidrug resistance phenomena in human pathogens which is spread through out the evolutionary scale. Studies on azole resistant strains of *C. albicans*, *A. nidulans* and *P. italicum* indicated a decreased accumulation of these compounds in the cells (Stergiopoulos, 2002). Accordingly, a host of responsible genes have been identified in the genetically tractable budding yeast *Saccharomyces cerevisiae*, as well as in a pathogenic yeast *C. albicans*. Studies so far suggest that while antifungal resistance is the culmination of multiple factors there may be unifying mechanism of drug resistance in these pathogens. ABC (ATP binding cassette) and MFS (major facilitator superfamily) drug transporters belonging to two different superfamilies, are the most prominent contributors to MDR in yeast (Prasad *et al.*, 2002; Gulshan *et al.*, 2007). Many of the genes of drug resistant fungi have been studied and are listed in Table 2.3.

2.4 PHYTOMEDICINE AS AN ALTERNATIVE TO SYNTHETIC DRUGS

Plants are the oldest source of pharmacologically active compounds and have provided human kind with many medicinaly useful compounds for centuries (Cordell, 1981; Iwu, 1999; Ming, 2003; Pal and Shukla, 2003). People on all continents have used poultices and imbibed infusions from thousands of indigenous plants for various ailments (Lee, 2005; Akinpelu and onakoya, 2006; Heinrich, 2003).

Indian ayurvedic medicine, unani and chinese system of medicines have well documentations of a large number of medicinal plants and their possible uses (Fabricant and Farnsworth, 2001; Guleria and kumar, 2006; Janovska, 2003). However, in spite of rich diversified antimicrobials and other therapeutics constituents, since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobial have been virtually ignored, as the emphasis was given on search and development of microbial origin drugs. Diversified group of bacteria and fungi from various sources were collected from all over the world and screened for new antibiotics which lead to an impressive arsenal of antimicrobial agents such as cephalosporins, tetracyclines, aminoglycosides, rifamycins, and chloramphenicol by the early 1970s.

Table 2.3. ABC transporter genes from fungi

Subfamily	Gene	Accession No	Size	Protein topology	Function
				Aspergillus flavus	
MDR	*Afimdr 1*	U62931	1307	(TMD-NBF)	Not known
				Aspergillus fumigatus	
MDR	*Afumdr 1*	U62934	1349	(TMD-NBF)	Resistance to cilofungin
MDR	*Afumdr 2*	U62936	791	TMD-NBF	Not involved in MDR
-	*ADR1*	-	-	-	Upregulated by itraconazole
				Aspergillus nidulans	
PDR	*AtrA*	Z68904	1466	(NBF-TMD)	Not known
PDR	*AtrB*	Z68905	1426	(NBF-TMD)	MDR
MDR	*AtrC*	AF071410	1284	(TMD-NBF)	Up-regulated by various toxicants
MDR	*AtrC2*	AF082072	1293	(TMD-NBF)	Up-regulated by cycloheximide
MDR	*AtrD*	AF071411	1348	(TMD-NBF)	MDR
-	*AtrA* *AtrB* *AtrC* *AtrD*			-	Not known
				Botryotina fuckeliana	
PDR	*BcatrA*	Z68906	1562	(NBF-TMD)	Up-regulated by cycloheximide
PDR	*BcatrB*	AJ006217	1439	(NBF-TMD)	Phenylpyroles + stilbene resistance
				Candida albicans	
PDR	*CDR1*	X77589	1501	(NBF-TMD)	MDR
PDR	*CDR1*	U63812	1499	(NBF-TMD)	MDR
PDR	*CDR1*	U89714	1501	(NBF-TMD)	Expressed in opaque phase of growth
PDR	*CDR1*	AF044921	1490	(NBF-TMD)	Not involved in resistance to azole
				Leptospaeria maculans	
-	*LMABC1*	-	-		MDR
-	*LMABC2*	-	-		Not involved in MDR
				Magnoporthe grisea	
PDR	*ABC1*	AF032443	1619	(NBF-TMD)	Essential for pathogenicity
				Mycosphaerella graminicola	

PDR	*Mgatr1*	AJ243112	1562	(NBF-TMD)	Upregulated by cyclo heximide and eugenol
PDR	*Mgatr2*	AJ243113	1499	(NBF-TMD)	Upregulated by eugenol and imaz
PDR	*Mgatr3*				
PDR	*Mgatr4*				
PDR	*Mgatr5*				
				Penicillium digitatum	
PDR	*PMR1*	AB010442	1619	(NBF-TMD)	Resistance to azoles
PDR	*PDR5*	L19922	1511	(NBF-TMD)	MDR
PDR	*PDR12*	U39205	1529	(NBF-TMD)	C1-C7 organic acids resistance
PDR	*PDR15*	U32274	1501	(NBF-TMD)	Inducible upon stress
ALDP	*PXA1*	Z73503	758	(NBF-TMD)	MDR
ALDP	*PXA2*	Z28188	853	(TMD-NBF)	Required for β oxidation of fatty acids
MRP	*BTA1*	Z73153	1661	(TMD-NBF)	Required for β oxidation of fatty acids
MRP	*YCF1*	Z48179	1515	(TMD-NBF)	Bile acid transporter
MRP	*YORI*	Z73066	1477	(TMD-NBF)	Multidrug and heavy metal resistance
MDR	*ATM1*	Z49212	690	(TMD-NBF)	MDR
MDR	*STE6*	Z28209	1290	(TMD-NBF)	Mitochondrial DNA maintenance
YEF3	*YEF3*	U20865	1044	(NBF)	Secretion of the a mating factor
YEF3	*GCN20*	D50617	752	(NBF)	Interaction with aminoacyl-t-RNA
				Schizosaccharomyces pombe	
PDR	*Abc1*	Y09345	1427	(TMD-NBF)	Unknown
MDR	*Mam1*	U66305	1336	(TMD-NBF)	Secretion of mating factor
PDR	*Bfr1*	S76267	1530	(NBF-TMD)	MDR
PDR	*Pmd1*	D10695	1362	(TMD-NBF)	MDR

Indian ayurvedic medicine, unani and chinese system of medicines have well documentations of a large number of medicinal plants and their possible uses (Fabricant and Farnsworth, 2001; Guleria and kumar, 2006; Janovska, 2003). However, in spite of

rich diversified antimicrobials and other therapeutics constituents, since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobial have been virtually ignored, as the emphasis was given on search and development of microbial origin drugs. Diversified group of bacteria and fungi from various sources were collected from all over the world and screened for new antibiotics which lead to an impressive arsenal of antimicrobial agents such as cephalosporins, tetracyclines, aminoglycosides, rifamycins, and chloramphenicol by the early 1970s. It was believed that virtually any microbial infection could be treated by the use of these antibiotics. However the euphoria over the potential conquest of infectious diseases was proven wrong in short time due to following main factors :

1. Prolong and indiscriminate use of these microbial antibiotics gave rise to various drug resistant and more recently multidrug resistant strains worldwide. Hospitals have become breeding grounds for human associated microorganisms.
2. The alarming increase in the incidences of bacterial and fungal diseases caused by these drug resistant strains and also by opportunistic pathogens in the immunocompromised host, patients with organ transplant and AIDS patients.
3. Nonetheless the same time bomb effect is also found to be developing with plant and animal associated pathogens in commercially driven activities, such as aquaculture and confined poultry breeding, where the indiscriminate use of antibiotics is perceived as essential for industries survival.
4. In addition the side effects are associated with these commonly used antibiotics.

The control and treatment of infectious diseases caused by these resistant strains have presently become one of the major health problems globally. Meanwhile consumers in developed countries are becoming disillusioned with modern healthcare and are seeking alternatives (Ahmad *et al.*, 2006). Consequently there is an urgent need to search for safer alternatives to synthetic antibiotics.

Once again the attention has been drawn towards plants, due to its century old established therapeutic potential and thought to be relatively safer. Plants in their natural surroundings have contact with large number of fungi, dermatophytes, yeast, bacteria viruses and parasites. The secondary compounds present in them have a considerable protective role against infections as well as providing their physiological role. These compounds are present up to 10 percent of their dry mass, ranging from small molecules (for example phytoalexins) to proteins (example glucanases, chitinases). This relationship can be seen particularly in essential oils etc (Mongelli, 2003). One of the important reason

for the comeback of phytomedicine has been that despite the remarkable progress made by chemistry, pharmacology, molecular biology, genome research and high throughput screening the NCE (new chemical entity) pipelines of the pharmaceutical companies are at historically low levels (Raskin *et al.*, 2002, 2004).

2.5 MAJOR GROUPS OF PLANT ANTIFUNGAL COMPOUNDS

A wide variety of active phytochemicals with different biological activity have been reported from plants but the present discussion focuses mainly around the antifungal agents. Useful antifungal phytochemicals can be divided into several categories, and are summarized below -

2.5.1 Phenolics and polyphenols

Single substituted ring is present in simple phenols and phenolic acids. Cinnamic and caffeic acids are common examples of phenylpropane derived compounds. Caffeic acid from terragon and thyme is effective against fungi (Duke, 1985). Catechol and pyrogallol both are hydroxylated phenols and are toxic to microorganisms. Eugenol, a phenolic compound from clove oil is having antimicrobial activity (Thomson, 1978; Duke, 1985).

2.5.2 Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes (Stern, 1996). Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined.

2.5.3 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Phloretin, an isoflavone isolated from apples, has antimicrobial activity against a variety of microorganisms (Hunter and Hull, 1993). Galangin (3,5,7 trihydroxyflavone), isolated from *Helichrysum aureonites*, shows antimicrobial activity against fungi (Afolayan and Meyer, 1997).

2.5.4 Tannins

Tannins are polymeric phenolic substances and are divided into two groups, hydrolysable and condensed tannins. Alternatively tannins may be formed by polymerization of quinone units (Geissman, 1963). In 1991, Scalbert reviewed 33 studies on antimicrobial properties of tannins. According to these studies, tannins can be toxic to filamentous fungi and yeasts.

2.5.5 Coumarins

Coumarins are phenolic substances made up of fused benzene and α-pyrone rings (O' kennedy and Thornes, 1997). Phytoalexins, which are hydroxylated derivates of coumarins, isolated from carrots, show antifungal activity (Hoult and Paya, 1996).

2.5.6 Terpenoids and essential oils

The fragnance of plants is due to essential oil fraction. These oils are secondary metabolites that are highly enriched compounds based on isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$ and they occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30} and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}) (Cowan, 1999). When compounds contain additional elements, usually oxygen, they are termed as terpenoids. Terpenes or terpenoids are active against fungi (Rana *et a.l*, 1997; Suresh *et al.*, 1997). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by lipophilic compounds.

2.5.7 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful alkaloid, morphine was isolated in 1805 from opium poppy *Papaver somniferum* in 1805. Diterpenoid alkaloids, commonly isolated from the plants of Ranunculaceae, are commonly found to have antimicrobial properties (Omuloki *et al.*, 1997).

Many researchers, particularly from countries with a rich biodiversity have contributed to the detection of new antifungal compounds from medicinal plants.

Table 2.4 lists some of the major and important antifungal compounds isolated till date.

2.6 DIOSPYROS KAKI

Diospyros kaki is a deciduous tree measuring to 12 m in height and 7 m in diameter. It belongs to family Ebenaceae. Its common name is Japenese persimmon and the country of origin is Japan (Gilman and Watson, 1993).

Table 2.4. Important antifungal compounds isolated from plant

Plant	Family	Parts	Compound type	Reference
Artemisia giraldi	Asteraceae	Aerial part	Flavones	Zheng *et al.*, 1996
Artemisia absinthium, *A. santonicum* and *A. spicigera*	Asteraceae	Whole plant	Essential oil	Kordali *et al.*, 2005
Alium sativum	Liliaceae	Bulb	Essential oil	Pyan and Shin, 2006
Aniba rosaedora	Lauraceae	Bark	Essential oil	Simic *et al.*, 2004
Ajania fruticulosa	Asteraceae	Aerial parts	Sesquiterpene lactones	Meng *et al.*, 2001
Anomospermum grandifolium	Meninspermaceae	Stems	Saponin	Plaza *et al.*, 2003
Astragalus mongholicus	Fabaceae	Whole plant	Protein	Yan *et al.*, 2005
Aquilegia vulgaris	Ranunculaceae	Leaves and Stems	Flavonoid	Bylka *et al.*, 2004
Bauhinia rutescens	Caesalpineaceae	Root bark	Stilbenes	Maillard *et al.*, 1991
Blumea balsamifera	Asteraceae	Leaves	Flavanoid	Ragasa *et al.*, 2005
Bidens paludosa	Asteraceae	Whole plant	Polyacetylenes	Alvarez *et al.*,1996
Buddleja madagascarensis	Loganiaceae	Leaves	Triterpenoid saponins	Emam *et al.*, 1996
Calycodendron mileni	Rubiaceae	Whole plant	Alkaloids	Saad *et al.*,1995
Cassia tora	Fabaceae	Seeds	Anthraquinone aglycones	Kim *et al.*, 2005
Camptotheca acuminate	Nyssaceae	Aerial parts	Flavonoid	Lee *et al.*, 2005
Calocedrus formosana	Cupressaceae	Leaf	Essential oil	Cheng *et al.*, 2004
Capsicum frutescens	Solanaceae	Whole plant	Triterpene saponin	Renault *et al.*, 2003
Clerodendron wildi	Verbenaceae	Roots	Triterpenoid saponins	Toyota *et al.*, 1990
Citrus sinensis	Rutaceae	Epicarp	Essential oil	Sharma *et al.*, 2006
Chrysanthemum coronarium	Asteraceae	Flower	Essential oil	Alvarez *et al.*, 2001
Coccoloba dugandiana	Polygonaceae	Leaves, Twigs	Flavonoids	Li *et al.*,1999
Centaurea thessala	Asteraceae	Aerial parts	Sesquiterpene lactones	Skaltska *et al.*, 2000
Celastrus hypoleucus	Celastraceae	Roots	Triterpenoid	Luo *et al.*, 2005
Cryptomeria japonica	Taxodiaceae	Leaves	Essential oil	Cheng *et al.*, 2005
Chenopodium botrys	Chenopodiaceae	Aerial parts	Essential oil	Maksimovic *et al.*, 2005
Clematides tangutica	Ranunculaceae	Aerial parts	Triterpene saponins	Du *et al.*, 2003
Cinnamomum zeylanicum	Lauraceae	Bark	Essential oil	Simic *et al.*, 2004
Chrysactinia mexicana	Asteraceae		Essential oil	Cardenas *et al.*, 2005
Drimys Brasiliensis	Winteraceae	Stem bark	Sesquiterpene	Angelina *et al.*, 2005
Datura metel	Solanaeceae	Leaves	alkaloid	Dabur *et al.*, 2005
Daucus carota	Apiaceae	Roots	Sesquiterpene esters	Jasicka *et al.*, 2004
Detarium microcarpum	Fabaceae	Fruit pulp	Diterpenes	Cavin *et al.*, 2006
Erythrina burtii	Fabaceae	flavonoid	Stem bark	Yenesewa *et al.*, 2005
Evonymus europaeus	Celastraceae	Aerial parts	Chitinbinding protein	Vanden *et al.*, 2004
Glehnia littoralis	Apiaceae	Roots	Polyenic alcohols	Matsura *et al.*, 1996
Glycopetalum sclerocarpum	Celastraceae	Stem bark	Sesquiterpenes	Sotonaphun *et al.*, 1999
Glycyrhiza glabra	Fabaceae	Roots	Flavonoids	Lee *et al.*, 1998

Hebe cupressoides	Scrophulariaceae	Aerial parts	Flavonoids	Perry and Foster, 1994
Helichrysum aureonitens	Asteraceae	Shoots	Trihydroxy flavones	Afolayan and Meyer, 1997
Hypericum calcinum	Guttiferae	Aerial parts	Phloroglucinol derivates	Decosterd *et al.*, 1998
Haplophylum sieversii	Rutaceae	Aerial parts	Alkaloids	Cantrell *et al.*, 2005
Inula viscosa	Asteraceae	Leaves	Sesquiterpene lactones	Maoz *et al.*,1999
Juniperus thurifera	Cupressaceae	Wood	Sesquiterpene	Barrero *et al.*, 2005
Juniperus comunis	Cupressaceae	Berry	Essential oil	Cavaleiro *et al*, 2006
Kigelia pinnata	Bignoniaceae	Fruits	Napthaquinones	Binutu *et al.*,1996
Kaempferia marginata	Zingiberaceae	Stem	Diterpenes	Thongnest, 2005
Khaya ivorensis	Meliaceae	Stem bark	Triterpenes	Abdelgaleil , 2005
Laurus nobilis	Lauraceae	Aerial parts	Essential oil	Simic *et al.*, 2004
Lavendula angustifolia and *L .stoechas*	Lamiaceae	Stems,Leaves and Flower	Essential oil	D'auria *et al.*, 2005 and Angioni *et al.*, 2006
Lippia berlandieri and L. javanica	Verbenaceae	Leaf	Essential oil	Portillo *et al.*, 2005 and Vilijoen *et al.*, 2005
Melia azedarach	Meliaceae	Seed kernels	Hydroxy coumarins	Carpinella *et al.*, 2005
Mahonia aquifolium	Berberidaceae	Stem bark	Alkaloids	Slobodnikova *et al.*, 2004
Melochia odorata	Sterculiaceae	Leaves	Alkaloids	Emile *et al.*, 2007
Nepeta crispa	Lamiaceae	Aerial parts	Essential oil	Sonboli *et al.*, 2004
Ocimum gratissimum and O.micranthum	Lamiaceae	Leaves	Essential oil	Nakamura *et al.*, 2004 and Sachetti *et al.*, 2004
Parthenium hysterophorus	Asteraceae	Whole plant	Sesquiterpenoid lactones	Ganeshan and Ganeshan, 1993
Piper angustifolium	Piperaceae	Seed	Camphene	Trillini *et al.*, 1996
Piper betle	Piperaceae	Leaves	Essential oils	Garg and Jain, 1992
Phaseolus vulgaris	Fabaceae	Seeds	Peptide	Wong J.H and Ng T.B, 200
Polygonium hydropiper	Polygonaceae	Whole plant	Sesquiterpene dialdehydes	Lee *et al.*, 1999
Polyalthia longifolia	Annoceae	leaves and berries	Diterpenoid	Marthanda *et al.*, 2005
Prismatomeris fragrans	Rubiaceae	Roots	anthraquinones	Kanokmedhakul *et al.*, 2005
Psiadia lithospermifolia	Asteraceae	Leaves	Essential oil	Govindan, *et al.*, 2004
Raphanus sativus	Brassicaceae	Seeds	Proteins	Bolle *et al.*, 1996
Rubia tictorum	Rhamnaceae	Root	Anthraquinone aglycone	Manojlovic *et al.*, 2005
Rhicanthus nasutus	Acanthaceae	Leaves, Stems	Napthopyran derivates	Kodoma *et al.*, 1993
Serjania salzmanniana	Sapindaceae	Whole plant	Saponins	Ekabo *et al.*,1996
Sassafras albidum	Lauraceae	Bark	Essential oil	Simic *et al.*, 2004
Sium nodiflorum	Apiaceae	Whole plant	Heterosides	Larshini *et al.*,1996
Solanum hispidum	Solanaeceae	Leaves	Saponin	Gonzalez , 2004
Solanum abutiloides	Solanaeceae	Root	Sesquiterpene	Yokose *et al.*, 2004
Strychos usambarensis	Loganiaceae	Seeds	Alkaloids	Leclerreq *et al.*,1995
Solanum tuberosum	Solanaeceae	Potato tubers	Protein	Park *et al.*,2005
Swartzia polyphylla	fabaceae	Bark	Flavonoid	Rojas *et al.*, 2006
Tagetes patula	Asteraceae	Aerial parts	Essential oil	Romagnoli *et al.*, 2005
Terminalia bellerica	Combretaceae	Fruit	Flavans, Lignans	Valsraj *et al.*, 1997
Trachyspermum ammi	Apiaceae	Seed	Essential oil	Singh *et al.*, 2005
Thujopsis dolabrata	Cupressaceae	Seeds	Terpenoids	Keiko *et al.*, 2007
Thymus vulgaris	Lamiaceae	Aerial parts	Essential oil	Giordani *et al.*, 2004
Thymbra capitata	Lamiaceae	Aerial parts	Essential oil	Salgueiro *et al.*, 2004

Teramnus labialis	Fabaceae	Stems	Flavanol glycoside	Yadava and Jain, 2004
Trigonella-foenum-graecum	Fabaceae	Leaves	Cysteine rich peptides	Olli S. and Kirti P.B, 2006
Tithonia diversifolia	Asteraceae	Aerial parts	Isocoumarin dimer	Yemele *et al.*, 2006
Urginea indica	Liliaceae	Bulbs	Protein	Shenoy *et al.*, 2006
Virola surinamensis	Myristiaceae	Leaves, Bark	Neolignans	Zacchino *et al.*, 1998
Vitis vinifera	Vitaceae	Beery	Peptide	Abré de Beer, 2008
Vernonanthura tweedieana	Asteraceae	Roots	Sesquiterpene	Portillo *et al.*, 2005
Zuccagnia puncata	Fabaceae	Aerial parts	Chalcones and Flavanone	Svetaz *et al.*, 2004
Zingiber officinale	Zingiberaceae	Root	Gingerols	Ficker *et al.*, 2003

2.6.1 PHYSICAL CHARACTERISTICS

Sastry (1952) reported some physical characteristics of the genus. The plant prefers light (Sandy), medium (loamy) and heavy (clay) soil and well drained soil. It flowers from July to August and the seeds ripen in November. The flowers are dioecious. It can grow in semishade (light woodland) or no shade. It requires moist soil (plants for a future database). *D. kaki* needs a subtropical to mild-temperate climate. It may not fruit in tropical lowlands. In Brazil, the tree is considered suitable for all zones favourable to citrus, but those zones with the coldest winters induce the highest yields. The atmosphere may range from semi-arid to one of high humidity. Propagation is by seed, root suckers or grafting onto wild rootstocks.

2.6.2 GEOGRAPHIC DISTRIBUTION

D. kaki is native to China, India, Japan and Myanmar. It is exotic to Afghanistan, Algeria, Australia, Brazil, Egypt, France, Indonesia, Israel, Italy, Korea, Republic of, Palestine, Philippines, Russian Federation, Union of Soviet Socialist Republic (Former), United States of America and Vietnam.

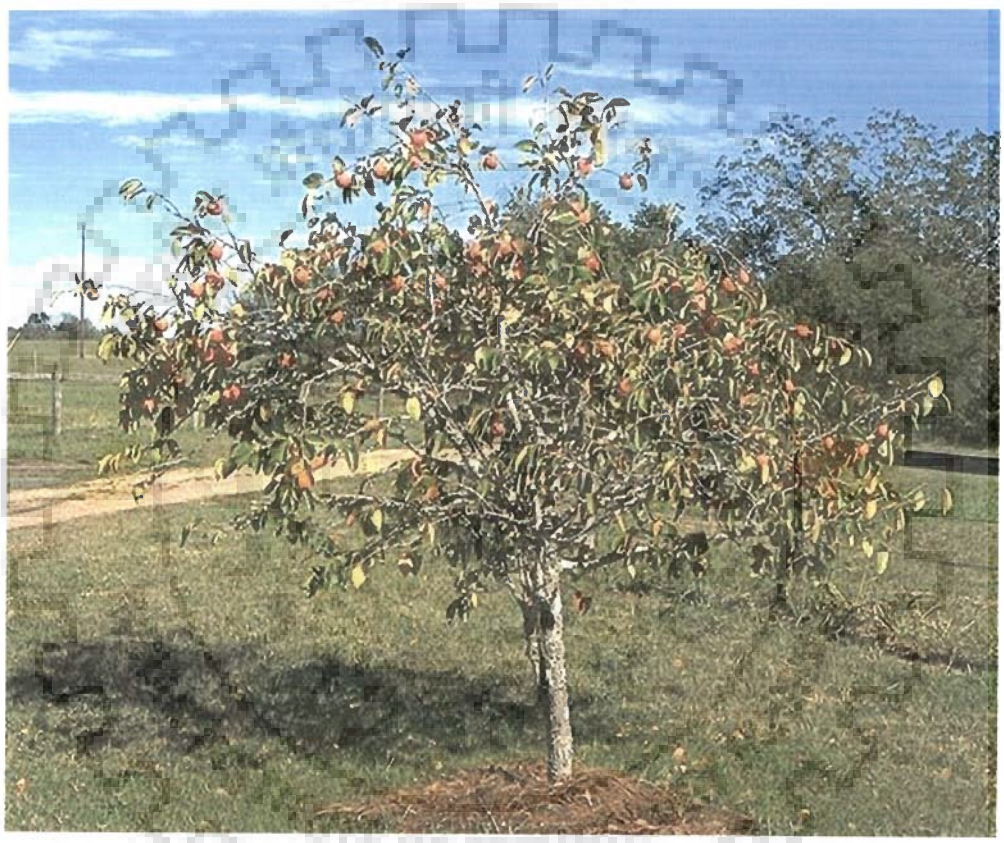
2.6.3 PRODUCTS

Food : Fully ripe fruits are usually eaten out-of-hand. The flesh may be added to salads, blended with ice cream mix or yoghurt, used in pastries, puddings or made into jam or marmalade. Ripe fruits can be frozen whole or pulped. Drying is commonly practised. Roasted seeds have served as a coffee substitute. Tea can also be made from fresh or dried leaves. *D. kaki* is high in vitamin and a moderate source of ascorbic acid.

Timber : Wood fairly hard and heavy, black with streaks of orange-yellow, salmon, brown or grey; close-grained; takes a smooth finish and is prized in Japan for fancy

inlays, though it has an unpleasant odour. Tannin from unripe fruits has been employed in brewing sake, also in dyeing and as a wood preservative.

Other uses : Fruit may be converted into molasses, cider, beer and wine. Juice of small, inedible wild fruits, calyx and seeds is diluted with water and painted on paper or cloth as an insect and moisture-repellent medicine. It is a handsome ornamental tree with drooping leaves and branches that give it a languid, rather tropical appearance. Trees can be planted as a hedge or as a screen if pruned heavily.



2.6.4 MEDICINAL USES

The stem bark is astringent and styptic. The fruit is said to have different properties depending on its stage of ripeness, though it is generally antitussive, astringent, laxative, nutritive and stomachic. The fresh fully ripe fruit is used raw in the treatment of constipation and haemorrhoids and when cooked is used to treat diarrhea. The dried fruit is used in the treatment of bronchial complaints, whilst when ground into powder it is used to treat dry coughs. Juice from the unripe fruit is used in the treatment of hypertension. The fruits are considered to be antifebrile, antivininous and demulcent. The peduncle is used to treat coughs and hiccups and the calyx is used to treat hiccups.

2.6.5 BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

1 Homeopathic medicine 'Creveld' had been developed from *D. kaki* (Creveld 2001-2005). The remedy has been used for patients suffering severe physical, psychological and mental complaints due to post-traumatic stress. *D. kaki* has been much used during chemotherapy and radiotherapy in which it substantially reduced the side effects caused by primary therapy. In some cases, the tumours or metastases disappeared (during or after treatment) sooner than one would normally expect.

2 The tannins present in the fresh unripe fruits of *D. kaki* have very strong detoxifying activity on snake venoms of two snake species i.e. *Laticauda semifasciata* and *Trimeresums flavoviridis* (Houghton, 1993; Okonogi *et al.*, 1979).

3 Fruits are known to inactivate bacterial toxins of *Clostridium tetani, Diptheria, Straphylococcus alpha* and *Bordetella pertussis* (Mallavadhani, 1998).

4 The leaves of Persimmon possess antithrombotic activity. It has long been used for tea in Korea since it was thought to be effective against hypertension. (Sa *et al.*, 2005) reported a 10,000 D anticoagulant fraction has been purified from the leaves of *D. kaki* that inhibited thrombin-catalyzed fibrin formation with a competitive inhibition pattern.

5 *D. kaki* exhibited strong radical scavenging activity which can be attributed to the presence of catechin, epicatechin, epigallocatechin, chlorogenic acid, caffeic acid and gallic acid (Chen *et al.*, 2007).

6 *D. kaki* peels extract possess significant cytotoxic activity against human oral squamous cell carcinoma cells (HSC-2) and human submandibular gland tumor (HSG) cells, multidrug resistance (MDR) reversal activity, anti-human immunodeficiency virus (HIV) activity and anti *Helicobacter pylori* activity (Kawase *et al.*, 2002).

7 (Funayama, 1979) reported that astragalin and isoquercitrin present in the leaves of *D. kaki* are responsible for hypotensive effect as tested on urethane anasthetized rats.

8 Persimmon peel containing high levels of dietary fiber and antioxidants with antidiabetic properties represents a potential dietary supplement for improving hyperglycemia and diabetic complications (Lee, 2006; Li *et al.* 2007; Hu *et al.*, 2006).

9 Polyphenols isolated from the persimmon leaf can be used as natural materials or additives for human skin owing to their beneficial biological functions, including the antiwrinkle effect and the inhibition of skin problems, for food or cosmetic compositions (An *et al.*, 2005).

10 A pharmaceutical composition for preventing and treating liver disease having liver-

protecting activity, anti-oxidizing activity, anti-cancer activity, and anti-allergy contained triterpenoid compound isolated from *D. kaki* folium have been reported by (Kyung.and Young,2005)

11 *D. kaki* extract and related polyphenol compounds such as catechin (C), epicatechin (EC), epicatechingallate (ECG), epigallocatechin (EGC), and epigallocatechingallate (EGCG) strongly inhibited the growth of human lymphoid leukemia Molt 4B cells in a dose-dependent manner, while C and EC inhibited the growth of the cells only moderately (Achiwa *et al.*,1997). Ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, was inhibited by 10-20 % by these polyphenol compounds. The morphology of the Molt 4B cells indicated severe damage 3 days after treatment with PS, ECG, EGC, and EGCG. Irregular shape of the cells and DNA fragmentation were observed in PS, ECG, EGC, or EGCG-treated cells. These results suggest that PS, ECG, EGC, and EGCG induce apoptosis (programmed cell death) of Molt 4B cells.

12 The life span of stroke prone spontaneously hypersensitive rats was significantly prolonged on ingestion of tannins of *D. kaki* (Weijian, 2005). Persimmon tannin was 20 times more effective than tocopherol in terms of the 50 % inhibitory concentrations (Uchida, 1990). NaoXingQing (NXQ), a novel and patented traditional Chinese medicine (TCM) drug made from the flavonoids of the leaves of *D. kaki* has been used for the treatment of stroke or syndrome of apoplexy in clinic to improve the outcome of ischemia stroke for years in China.

13 *Plumbagin* and its derivates isolated from *D. kaki* Thunb.roots possess acaricidal activity and of fuction of mite indicator (Lee and Lee, 2008).

2.6.6 ANTIMICROBIAL PROPERTIES

Fractionated extracts of persimmon peels showed a remarkable MDR reversal activity. The active compounds in persimmon leaves i.e presence of volatile oil, total flavonoid, coumarins, and organic acid are responsible for antimicrobial activities against seven food spoilage and food-borne pathogens (Lilian, 2003). *D. Kaki* tannins with purity of 30 %, and oligosaccharides (non-fermentable tetra- or less- saccharides) are claimed as human intraoral pathogenic bactericidal agents (Kato *et al.*, 2006). The isolation and identification of an antimicrobial compound kaempferol (kaempferol is an aglycon of astragalin (kaempferol 3-O-β-D-glucopyranoside) against *Streptococcus mutans* from the leaves of *D. kaki* has also been reported by (Yasumasa *et al.*, 1999).

2.6.7 PHYTOCHEMISTRY

2.6.7.1 Leaves

Leaves are reported to contain following compounds, 40-dihydroxy-a-truxillic acid tatarine C, myricetin, annulatin, trifolin, astragalin, hyperin, isoquercetin, rutin, quercetin, kampferol and kakispyrone and kaki saponin (Chen *et al.*, 2007).

2.6.7.2 Fruits

Commercially, there are generally two types of persimmon fruit: astringent and non-astringent. The heart-shaped hachiya is the most common variety of astringent persimmon. Astringent persimmons contain very high levels of soluble tannins and are unpalatable if eaten before softening. The non-astringent persimmon is squat like a tomato and is most commonly sold as fuyu. Non-astringent persimmons are not actually free of tannins as the term suggests, but rather are far less astringent before ripening, and lose more of their tannic quality sooner. Non-astringent persimmons may be consumed when still very firm to very soft (Wikipedia). Persimmon contains the following (g/100 g of fresh fruit)- water,80.3 g; protein, 0.58 g; total lipids, 0.19 g; total carbohydrates, 18.6 g; total dietary fiber, 1.48 g; and some minerals i.e., magnesium, iron, zinc, copper and manganese. A high concentration of antioxidants such as ascorbic acid (up to 7.5 mg), carotenoids (particularly cryptoxanthin, zeaxanthin and carotene), polyphenols and a specific group of polyphenols (tannins), catechin and gallocatechin, as well as the anti-tumor compounds betulinic acid and shibuol were found in persimmon. The dry residue of persimmon includes the following (g/100 g): polyphenols, 0.16-0.25; carotenoids, 0.002; and soluble and nonsoluble proteins, 0.64-1.3. Liposoluble compounds in the rind and flesh of persimmon fruit includes monogalactosyldiglycerides, carotenoids, diacylglycerols, ceramideoligosides,digalactosyldiglycerides,phosphatidylglycerols,phosphatidylcholines and free and glycosylated sterols. In the fatty acid composition of the lipids unsaturated fatty acids linolenic, oleic, linoleic and palmitoleic predominated (>70 %). Some components of persimmon show special activities (Uchida *et al.,* 1989). Persimmon tannins prolonged life and reduced the incidence of stroke in hypertensive rats. This effect was attributed to the fact that persimmon tannins are 20 times more potent than antioxidant vitamin E. However, the effect of a persimmon-supplemented diet on lipid metabolism has not been examined (Gorinstein *et al.*, 1998).

2.6.7.3 Roots

Dicoumarol derivatives gerberinol ($C_{21}H_{16}0_6$) and methylgerberinol ($C_{22}H_{18}0_6$) have been isolated from the roots of *D. kaki* Thunb. and *D. kaki* Thunb var. *sylvestris* Makino. Other napthaquinone derivates isolated from the roots of *D. kaki* were 7-methyljuglone, isodiospyrin, mamegakinone, plumbagin, diospyrin and a new 7-methyljuglone dimer named neodiospyrin. Also from the roots and woods of *D. kaki* var. sylvestris shinanolone, the binapthyl-1,1- quinone, lupeol, betulin and betulinic acid were identified (Tezuka *et al.*, 1972; Paknikar *et al.*, 1995).

2.6.7.4 Stems

Three compounds were isolated from the stem of *D. kaki* sylvestris and were identified as 7-methyljuglone, isodiospyrin, and taraxerol (Zhong and Feng, 1987).

2.6.7.5 Seeds

The seeds of persimmon (3 varieties) contained 0.43-0.67 % lipids and complex lipids constituted 11.4-18.3 % of this fraction. The complex lipids consisted of 18.5-22.3 % cerebrosides, 14.1-17.9 % acylsterylglycosides, 8.3-10.3 % phosphatidylinositols, 7.4-7.5% sterylglycosides, 5.2-7.2 % monogalactosyldiglycerides, 3.7-5.3 % sulfoquinovosyldiglycerides, 3.8-4.7 % phosphatidyl-N,N-dimethylethanolamines, 2.5-4.2 % phosphatidyl-N-methylethanolamines, and 27.4-30.7 % unidentified constituents. The main fatty acids of these complex lipids were C18:1 (23.2-37.7), C18 (20.3-52.3) and C18:2 (9.9-33.8 %) (Suzuki *et al.*, 1982).

2.6.7.6 Calyx

Twenty-three compounds were isolated from the calyx of *D. kaki*. Stearic acid, palmitic acid, succinic acid, syringic acid, vanillic acid, gallic acid, kaempferol, quercetin, trifolin, hyperin, β-sitosterol, β-sitosteryl-β-D-glucoside, friedelin, oleanolic acid, ursolic acid, and 19 β -hydroxyursolic acid (Zhong and Feng, 1987).

Although phytochemical constituents of *D. kaki* have been studied earlier, (Tezuka *et al.*, 1972) no studies have been done on antifungal activity of rootbark of *D. kaki* extract. Therefore an attempt have been made in the present study to evaluate its antifungal property *in vitro* against a very broad range of human pathogenic fungi.

MATERIALS & METHODS

3.1 MATERIALS

3.1.1 Chemicals:

All chemicals used were of analytical grades and purchased from Sigma (USA, NewYork) and Merck (Germany, Darmstadt). Culture media, Sabourads dextrose agar (SDA), Potato dextrose agar (PDA) and Rosewell park memorial Institute (RPMI) were purchased from Hi-Media (India).

3.1.2 Plant materials: A total of twenty-five plants were collected from Rajeshwari nursery, Jwalapur and Company garden, Saharanpur. The plants were authenticated by Dr. S.K. Upadhayay, Department of Botany, M.S. College, Saharanpur. Table 3.1 enlists the different parts and the plants used in screening for antifungal activity.

Table 3.1 Plants, their family and part used for screening

Plant	Family	Part used
Jasminum undulatum	*Oleaceae*	Wp
Pongammia pinnata	*Fabaceae*	L & Sd
Carica papaya	*Caricaceae*	R, Sd, L, Sb & Rb
Cannabis sativa	*Cannabaceae*	R, L, Sd & Sb
Thuja occidentalis	*Cuppressaceae*	R, Fr, L & Sb
Bauhenia variegata	*Leguminosae*	Wp
Acyranthus aspera	*Amaranthaceae*	Wp
Oroxylum indicum	*Bignoneaceae*	L, Sb & Rb
Argemone maxicana	*Papaveraceae*	Rb & Fl
Bombax ceiba	*Bombaceae*	R, L & Fl
Tinospo rockordifolia	*Meninspermacae*	F, St & L
Hemidesmus indicus	*Asclepiadiaceae*	R, St & L
Andrographis paniculata	*Acanthaceae*	Wp
Acacia catechu	*Mimosaceae*	R, Sb & L
Cassia occidentalis	*Fabaceae*	R, L & Fl
Calatropis gigentia	*Asclepiadiaceae*	R, L & Fl

Tectona grandis	*Verbenaceae*	L, R, St & Fl
Pyrus communis	*Rosaceae*	L, R, S & Fl
Scyzium cumini	*Myrtaceae*	L, R, S & Fl
Codiaeum variegatum	*Euphorbiaceae*	Sb, L, R & F
Artemisia absinthium	*Asteraceae*	S, L, R & Fl
Plumbago zeylanica	*Plumbaginaceae*	R, Sb & L
Plumbago capensis	*Plumbaginaceae*	R, Sb & L
Plumbago rosea	*Plumbaginaceae*	R, Sb & L
Diospyros kaki	*Ebenaceae*	L, Rb, Sb & Fr

Wp (whole plant), L(leaves), St (stems), Sd (Seed), R (Roots), Sb (Stem bark), Rb (root bark), Fl (flower) and Fr (fruit).

3.1.3 Test fungi :

The fungi used in the study were obtained from Indian Agriculture Research Institute, New Delhi and Postgraduate Institute of Medical Education and Research, Chandigarh. They were *Candida albicans* MTCC 227, *Candida tropicalis* ITCC 6258, *Candida krusei* ITCC 750, *Cryptococcus neoformans* ITCC 1672, *Sporothrix shenkii* ITCC 2317, *Fusarium oxysporum* ITCC 4998, *Trichophyton mentegrophytes* ITCC 3572, *Microsporon gypseum* ITCC 5277, *Aspergillus flavus* ITCC 5290, *Aspergillus fumigatus* ITCC 4880, *Curvularia lunata* ITCC 5248, *Rhizomucor pussilus* ITCC W-14, *Phialophora verrucosa* MCCL 32006 and *Pseudaresheria boydii* MCCL W-14.

3.2 METHODS

3.2.1 Plant extract preparation :

3.2.1.1 Preparation of aqueous extract

The preparation of aqueous extract was done following the method of Xi *et al.* (2003) with little modification. Fresh plant tissue, 10 g was washed with sterile water and treated with 0.2 % mercuric chloride for 10-15 min for surface sterilization. It was then crushed in liquid nitrogen. Powdered sample was extracted in 100 ml 20 mM sodium phosphate buffer (pH 7.2), 0.2 M sodium chloride with gently stirring for about 3 h. Homogenate was centrifuged at 9000 g, 4 °C for 30 minutes. It was then filtered with muslin cloth and used for the disc

diffusion assay.

3.2.1.2 Preparation of methanolic extract

Preperation of the extract was done using the method of Tadhani and Subash (2006) with little modification. Plants (10 g dry weight) were dried in oven at 40 °C for 24 - 48 h and surface sterilized with 0.2 % mercuric chloride. Dry plant powder was dissolved in 100 ml of methanol and extracted using dynamic maceration on shaker for 24 h at room temperature. The procedure was repeated three times. Extract was then filtered and dried under reduced pressure in rotary evaporator. Samples were stored at 4 °C in airtight bottles for further use.

3.2.2 Culture and maintenance of fungal strains :

3.2.2.1 Fungal cultures were maintained by monthly sub culturing on SDA at 4 °C and 15 % glycerol stocks were kept at -70°C for long term storage.

3.2.2.2 Preparation of inocula

Preparation of inocula was done as per the protocol of NCCLS M38-P (1998). Three-four days old PDA slants freshly subcultured were scraped with 0.85 % saline and transferred to 5 ml distilled water after hyphal fragments deposited at the bottom. One-two drop of 1 % Tween 80 was added. The suspension was vortexed for about 5 min and the density of suspension (0.4×10^4 - 5×10^4 cfu/ml) was determined using haemocytometer. In case of yeast overnight cultures in PDB were adjusted to an optical density of ~2 at 600 nm and then diluted to the desired count.

3.2.3 Evaluation of antifungal activity of aqueous and methanolic plant extract :

3.2.3.1 Disc diffusion assay

Preliminary antifungal assay was done as per the technique of Pepeljnjak (2003). Fungal inoculum prepared earlier was spread on SDA plates (100 µl). Discs impregnated with 1, 2.5, 5 and 7.5 mg of extract (in DMSO) were made and transferred to the plates. Amphotericin-B (100 µg) was used as positive control and DMSO as negative control. Plates were kept at 4 °C for 2-3 h and then incubated at 28-30 °C. Zone of inhibition were examined after 48 h. After incubation the zone of inhibition was measured using Hi antibiotic zone scale (HiMedia, Mumbai, India).

3.2.4 Qualitative phytochemical analysis of crude extract for major group of antimicrobial compounds :

3.2.4.1 Phytochemical assay

Qualitative analysis of the crude extract was done using standard procedures to identify the presence of major phytoconstituents. Analysis for the presence of tannins, saponins, flavonoids, steroids, terpenoids and glycosides were carried out by method of Edeoga (2005), phenols by the method of Harborne (1998) and alkaloids by Karumi (2004) respectively.

Test for phenols : On addition of 1 % alcoholic ferric chloride to the five ml extract intense green, purple or blue black colour is produced.

Test for flavonoids : Five ml of dilute ammonia solution was added to five ml of crude extract followed by addition of conc. sulphuric acid. Yellow colour formation (disappears on standing), indicates the presence of flavonoids.

Test for terpenoids : Five ml of each extract was mixed in two ml chloroform and aproximately three ml of conc. sulphuric acid was added over it to form a layer. The formation of a reddish brown colouration is seen at the interface which indicates the presence of terpenoids.

Test for tannins : One g of the dry powdered sample was boiled with twenty ml of distilled water in a test tube and thereafter filtered. Few drops of 0.1 % ferric chloride was added to it. Brownish or blue-black colour indicates the presence of tannins.

Test for steroids : One g crude extract was taken and two ml of acetic acid was added followed by two ml sulphuric acid. The colour changed from violet to blue green in some samples indicating the presence of steroids.

Test for saponins : Two g of the dry powderd sample was boiled in aproximately twenty ml distilled water and filtered. Ten ml of the filtrate was mixed with five ml of distilled water and shaken vigorously for a stable persistent froth. It was mixed with three drops of olive oil and shaken vigorously. The formation of emulsion on vigorous shaking confirms the presence of saponins.

Test for glycosides : Five ml of the extract was mixed with two ml glacial acetic acid. One ml of ferric chloride was underlayered with one ml conc. sulphuric acid. Brown ring at

interface indicates a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring while in acetic acid layer a greenish ring may form.

Test for alkaloids : Two ml of extract was stirred and placed in 1 % aqueous hydrochloride acid (5ml) on a steam bath and then treated with Dragendorff's reagent. Turbidity or precipitation with these reagents was considered as evidence for the presence of alkaloids.

3.2.4.2 TLC analysis of crude extract

Determination of most suitable mobile phase and extensive analysis of the crude extract was done through TLC analysis, using various solvent systems following Harbone (1998). Silica gel plates 60 F254+366, 20 x 20 cm (Merck, Germany) were used for analytical TLC. Five µl of crude extract (0.1 mg) was applied on TLC plate. The extract was applied as separate spots to a TLC plate about 1.3 cm from the edge (spotting line), using 20 µl capillary tubes. Development of the chromatogram was done in closed tank in which atmosphere had been saturated with eluent vapour. After the mobile phase had moved about 80 % from the spotting line, the plate was removed from the developing chamber and dried in a fume hood. Thereafter plates were analysed for the presence or absence of terpenoids, flavonoids, phenols, quinones, alkaloids and tannins by means of various detection reagents as per the method of Wrangler, 1996 and coumarins, glycosides and steroids by instructions given in the website www.emdchemicals.com.

1. Analysis of terpenoids was done using the solvent system benzene-chloroform (1:1), benzene-methanol (9:1), benzene-ether (2:3), toluene-ethylacetate (93:7), toluene-chloroform-ethanol (40:40:10), n-hexane-ethylacetate (17:3), hexane-ethylacetate (1:1). The plates were then sprayed with anisaldehyde-sulphuric acid reagent for the detection of terpenoids. It is prepared by mixing 0.5 ml anisaldehyde and 10 ml glacial acetic acid followed by 85 ml methanol and 5 ml concentrated sulphuric acid. TLC plate was sprayed with 10 ml of reagent, heated at 100 for 5-10 min and evaluated in UV at 365 nm. A range of colours from brown to blue colour were obtained indicating the presence of terpenoids.

2. For the analysis of flavonoids the solvents system used were : ethylacetate-formicacid-water (8:1:1), ethylacetate-formicacid-water (100:11:11:26), ethylacetate-methanol-water (100:13.5:10), toluene-acetic acid (4:1), chloroform-ethylacetate-acetic acid (5:4:1), chloroform-methanol (89:11). Polyethyleneglycol reagent was used as detection reagent. The plate was spread with 1 % methanolic diphenylboric acid -ß-ethylaminoester followed

by 5 % ethanolic polyethylene glycol- 4000 (PEG) 10 ml and 8 ml respectively. Intense fluorescence (yellow, green and orange) was produced in UV 365nm.

3. For the analysis of quinones following different mobile phase were used : ethylacetate-methanol-water (10:16.5:13.5), toluene-ethylformate-formic acid (5:4:1), petroleum ether-ethylacetate (7:3), petroleumether-ethylacetate-formicacid (90:4:1), n-hexane-ethylacetate (17:3). About 10 g solid iodine were sprayed on the bottom of a chromatograph tank. The developed TLC plate was placed into tank and exposed to iodine vapour. Yellow brown zones indicated the presence of quinones.

4. Analysis of phenols was done using the solvent systems : ethylacetate-formicacid-water (3:1:3), toluene-ethylacetate-formicacid (36:12:5), benzene-methanol-acetone (45:8:4), butanol-ethanol-water (5:1:2). Phenols absorb in short UV and can be detected in light of wavelength 253 nm as dark absorbing spots on plates containing fluroscent indicator and the plates were sprayed with Folin-ciocalteu reagent. Blue spots or grey spots appear when plates were fumed with ammonia vapour.

5. For tannins TLC was run in butanol-aceticacid-water (14:1:5). TLC plates were then sprayed with ferric chloride reagent which gives blue-black or green spots.

6. The solvent systems used for the analysis of coumarins were chloroform (100 %), benzene-ethanol (45:5), diethylether-toluene (1:1), toluene-ethylacetate (93:7). The plate was sprayed with 5 % or 10 % ethanolic potassium hydroxide and evaluated in UV at 365 nm with or without warming. Blue fluorescence indicates the presence of coumarins.

7. In case of alkaloids, methanol-conc NH_4OH (200:3), ethylacetate-methanol-water (100:13.5:10), toluene-ethylacetate-diethylamine (70:20:10) were used as seperation solvents. Dragendorff reagent was used to confirm the presence of alkaloids. It consists of Solution A (0.85 g basic bismuth nitrate in 10 ml glacial acetic acid and 40 ml water) and Solution B (8 g potassium iodide in 30 ml water). Stock solution A and B are mixed in the ratio of 1:11 ml, mixed with 2 ml acetic acid and 10 ml water and used as spray reagent for TLC plates. Dark brown or bright orange coloured spots indicated the presence of alkaloids.

8. Analysis of saponins was done using the solvent systems-chloroform-aceticacid-methanol-water (64:32:12:8), n-butanol-water (1:1), hexane-ethylacetate (5:95), benzene-methanol (10:7.5), chloroform-methanol (22:1.5). Anthrone (9-10-dihydroxy-8-oxaanthracene) in concentrated sulphuric acid when sprayed on plates exhibits green or greenish blue spots

which confirms the presence of saponins.

9. TLC plate was developed using the mobile phase ethylacetate-methanol-water (100:13.5:10) for the analysis of glycosides. Phosphoric acid reagent was sprayed heavily until the layer appeared transparent with a solution of 85 % phosphoric acid with water (1:1, v/v) and heated 10-15 minutes at 120°C.

10. For the analysis of steroids, benzene-ethanol (95:5), benzene-ethylacetate (9:1) were used. Plates were sprayed with diphenylamine reagent containing 10 ml of 10 % diphenylamine in ethanol, 100 ml HCl and 80 ml glacial acetic acid. Orange to violet colour is produced indicating the presence of steroids.

3.2.5 TLC bioautography :

The direct TLC bioautography method of Masoka *et al.* (2005) with little modification was performed for the identification of number of active compounds present in the crude extract. *C. albicans, A. flavus* and *T. mentegrophytes* were used.

Preparation of inocula

Cultures were grown on sabouraud dextrose agar for 2-3 days. Sabouraud broth was prepared and autoclaved (50 ml). Actively growing cultures were transferred into the broth with a sterile loop. The density of suspension (0.4×10^4- 5×10^4 cfu/ml) was determined using haemocytometer.

Bioautographic procedure

Five µl of the solution of each extract was applied on Merck TLC F254 plates (20 x 20 cm, 250µm thickness) and was developed to a distance of about 10 cm using the mobile phase benzene-chloroform 1:1. The mobile phase was removed from the plate by air drying for about 15-20 minutes. The plates were then inoculated with a fine spray of the concentrated suspension containing (0.4×10^4- 5×10^4 cfu/ml) of actively growing fungi. The plates were sprayed until they were just wet. They were sealed in the clear plastic envelopes and incubated overnight and then sprayed with a 2 mg/ml solution of methyltetrazolium violet (MTT) and further incubated overnight at 35 °C in a clean chamber at 100 % relative humidity in the dark. This is due to the fact that reduction of MTT to coloured formazan did not take place due to the presence of compounds that inhibited the growth of test fungi.

3.2.6 Purification of active constituents using open column chromatography :

As a result of performing TLC using a number of solvent combinations the benzene-chloroform was chosen as it was giving the best most favourable separation of compounds. The active compounds were purified using gravity column (1m x 40 mm) employing silica gel as matrix following the method of Iroegbu (2005) with some modification. Silica gel (60-120 mesh) was wet packed using chloroform. After settling the residue weighing 10 gm was suspended in minimum amount of particular solvent in which it would dissolve and filtered to remove impurities and any large particles which could cause diffusion problems whilst developing the column. The fraction was applied to the top of the column using a pipette with great care as not to disturb the top of the column. After application the solvent flask was raised to facilitate solvent flow into the column and was run using gravitational force. The column was left to run overnight, connected to a fraction collector, at a flow rate of 0.6 ml/min. Benzene-chloroform step gradient, starting with 100 % benzene to 100 % chloroform was used for elution. The total of 132 fractions of 25 ml were collected. All the eluted fractions were dried in waterbath at 45-50 °C and monitored by using the same TLC system. The fractions with similar TLC profile were pooled. The major fractions so obtained were assayed for the antifungal activity by disc diffusion method against *C. albicans, A. flavus* and *T. mentegrophytes*. The fractions found active were purified further by repeated silica gel column chromatography and by preperative TLC (Silica gel PLC plates 60 F254+366, 20 x 20 cm, 2 mm, Merck, Germany). The active compounds so obtained were identified by comparision of their spectral data with the literature.

3.2.7. Characterization of active compounds using various analytical methods :

As a result of purification of crude extract by silica gel column chromatography, two bioactive compounds A and B were obtained. Both the compounds were dissolved in methanol (HPLC grade) and filtered with syringe filter to facilitate the removal of impurities. The samples were then characterized using various analytical techniques as given below-

3.2.7.1 Melting point determination

The melting point measurement was carried out using glass capillary containing 1 mg sample in Büchi®melting point apparatus, Model B-545, AC input 230V(Z319287Aldrich).

3.2.7.2 EI-MS

Stock was prepared by dissolving sample 0.1 mg/ml in methanol (HPLC grade). EI-MS was measured by direct insertion of sample (5 µl) at ionization voltage of 70 ev using a Micromass autospec Q (Waters Micromass UK ltd.) spectrometer containing HP5-MS column. The programme was set as given- Injector temperature was 270°C and column temperature was 70 °C for 1 min at the beginning and then raised to 120 °C at rate 12 °C/min with hold for 1 min at 200 °C at rate 15 °C/min. 1 min hold was given at 300 °C at rate 20 °C/min and again held 15 min.

3.2.7.3 IR spectroscopy

Five mg sample was crushed with KBr pellets and used for recording the IR spectra. The IR spectra were obtained using a Perkin-Elmer FTIR Spectrophotometre, in the range 4000 - 667 cm-1 using Omniq software.

3.2.7.4 NMR spectroscopy

Samples (5 mg) were cleaned using various solvent systems starting with non polar solvents e.g. hexane and then introducing, methanol, ethylacetate, chloroform and acetone. The clean samples were weighed and dissolved in maximum 2 ml deuterated DMSO-d6. The samples were then pipetted into NMR tubes. The ^{1}H NMR spectra were recorded by using Bruker DRX500 at 500 MHZ and ^{13}C at 250 MHZ.

3.2.8 Evaluation of antifungal potential of *Plumbagin* and *Isodiospyrin* :

3.2.8.1 MIC determination

MIC of the crude extract and pure compounds *Plumbagin* and *Isodiospyrin* were performed using micro broth dilution method of NCCLS M38 P (1998) with some modification. Two fold serial dilution of the extract and compounds was done in RPMI starting with 10 mg/ml - 0.005 mg/ml. Each well of the 96 well plates was inoculated on the day of test with 100 µl of 2 x conidial inoculum suspension (0.5×10^3 - 2.5×10^3 cfu/ml) in case of yeast and (0.4×10^4- 5×10^4 cfu/ml) for filamentous fungi. 100 µl of the test compound was than added in the desired concentration to each well of the microtitre plate. The growth control wells contained 100 µl of the corresponding diluted inoculum suspension and 100 µl of 2 x sterile drug free medium and DMSO. Both quality control isolates *C. tropicalis* and *C. krusei* were also included in the study. The micro dilution trays were incubated at 35 °C and examined after

21-26, 46-50 and 70-74 hours of incubation. The growth in each MIC well was compared with that of growth control with the aid of reading mirror. The MIC was defined as the lowest concentration able to inhibit any visible growth.

3.2.8.2 MFC determination

MFC was determined by the method of Ingroff (2001). Twenty µl aliquots were sub cultured from each well that showed complete inhibition (100 % or an optically clear well) as compared to positive well (growth similar to that for the growth control, drug free medium) onto SDA plates. The plates were incubated at 28-30 °C until growth was seen in the growth control subculture (usually 48 h). The MFC was the lowest drug concentration that resulted in either no growth or fewer than 3-5 colonies.

3.2.8.3 IC_{50} determination

To determine the IC_{50} of *Plumbagin* and *Isodiospyrin* against various fungi SDA plates were used. To the autoclaved SDA which were cooled down to 45 °C different amount of above mentioned pure compounds 2, 4, 6, 8 and 10 µg/ml, *Plumbagin* and 10, 20, 30, 40 and 50 µg/ml, *Isodiospyrin* respectively were added, mixed and poured into petridishes. After solidification of the medium, the inoculum in the range of 1×10^5 cells were added on to the plates. DMSO alone served as control. After incubation at 28 °C for 72 h the area of mycelia was measured and the percentage inhibition of fungal growth was determined by the given formula. In case of *Candida* the number of colonies were counted and the percentage of inhibition calculated as per the formula –

$$\text{For } \textit{Candida} \text{ \% inhibition} = \frac{\text{No of colonies in absence - Presence of compound}}{\text{No of colonies in absence of compound}}$$

$$\text{For molds \% inhibition} = \frac{\text{Area of mycelial colony in absence - Area of colony in presence of compound}}{\text{Area of mycelial colony in absence}}$$

A graph between percentage inhibition of fungal growth and the concentration of *Plumbagin* and *Isodiospyrin* was plotted and used to determine IC_{50} of both the compounds. IC_{50} value was defined as the concentration of compound at which 50 % inhibition was observed.

3.2.9 Time kill study

The study was performed as per the method of Takemoto (2006) with little modification. In this study three groups of fungi were used i.e. *C. albicans*, *T. mentegrophytes* and *A. flavus*.

Fungi were subcultured on PDA plates. The inoculum was prepared in the same way as described above. One ml of adjusted fungal suspension (4 x 10^4 cfu/ml) was than added to solution of RPMI plus appropriate amount of antifungal compound 1, 2, 4 and 8 x MIC. Test solutions were placed on a shaker and incubated with agitation at 35 °C. At predetermined time intervals 100 µl samples were withdrawn from each solution, serially diluted ten fold, and a 50 µl sample was plated on SDA plate for colony counting. Colony counts were determined after incubation of these plates at 35 °C for 24-48 h. The detection limit was 1 $\log_{10}$ cfu/ml.

3.2.10 Ultrastructure studies

3.2.10.1 SEM

The measured amount of pure antifungal compound 4 x MIC i.e. 5 µg/ml, 10 µg/ml and 40 µg/ml of *Plumbagin* and 8 x MIC *Isodiospyrin* i.e. 40 µg/ml, 40 µg/ml and 320 µg/ml, were poured into sterilized petriplates. For control sets, SDA medium was supplemented with the same amount of DMSO. The pathogenic fungi was inoculated by placing a disk of mycelium (0.5 in diameter) in the centre of petriplate. Plates were incubated at 28 ± 1 °C. The growth of test fungi were recorded for 48 h. The processing of samples were done by the method of Sharma and Tripathi (2006). Two days old fungal cultures treated with the above mentioned concentration of *Plumbagin* and *Isodiospyrin* were collected from the rim and from the centre of the fungal colonies of the same age in control petriplate and in those treated with antifungal compound. For fixation, samples were promptly placed in vials containing 3 % glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at 4 °C for 48 h. Samples were then washed with distilled water three times for 20 min each following which they were dehydrated in ethanol series (30 %,50 %,70 % and 95 %) for 20 min in each alcohol dilution and finally with absolute ethanol for 45 min. Samples were then critical point dried in liquid carbondioxide. Following drying samples were gold plated and then viewed in a Cambridge LeoS-430 SEM operating at 15 kv at various levels of magnification. Cells of *C. albicans* were harvested by centrifugation at 2000 x g for 5 min, washed three times with phosphate buffer saline and resuspended in the same buffer. Controls were run in the presence of DMSO. Each sample was spread on a poly (L-lysine) coated glass slide (18 x 18 mm) to immobilize yeast cells. Glass slides were incubated at 30 °C for 90 min. Slide immobilized cells were fixed with 3 % (w/v) glutaraldehyde in 0.05 M sodium phosphate buffer, extensively washed with the same buffer and dehydrated with a graded ethanol series (30 %,

50 %, 70 % and 95 %) for 20 min each. They were finally dried under vaccum. Samples were then mounted, sputter coated with gold and examined under scanning electron microscope (LEO, model 435 VF, England).

3.2.10.2 TEM

Fresh fungal culture of *C. albicans*, *T. mentegrophytes*, *A. flavus* was treated with 4 x MIC of *Plumbagin* i.e. 5 µg/ml, 10 µg/ml and 40 µg/ml and 8 x MIC of *Isodiospyrin* i.e. 40 µg/ml, 40 µg/ml and 320 µg/ml by the same procedure as used for SEM. Processing was done using the method of Mares *et al.* (2004). Fungus was fixed with 6 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 3 h at 4 °C. After having been rinsed in the same buffer, the fungi which were to undergo TEM were post fixed for 20 h at 4 °C in 1 % osmium tetraoxide in the same buffer. They were then dehydrated in a graded series of ethanol solutions and embedded in epon araldite resin. Sections were cut with an l kb ultratome III, stained with uranyl acetate and lead citrate and observed with TEM at 100 KV. Samples containing *C. albicans* cells (1 x 10^6 cells/ ml) were centrifuged at 2000 x g. Control were run in the presence of DMSO. The cells were fixed by 6 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 3 h at 4 °C. Cells were then post fixed for 20 h at 4 °C in 1 % osmiumtetraoxide in the same buffer at room temperature. They were washed three times with buffer and dehydrated in graded series of ethanol solutions and embedded in epon resin. Ultrathin sections were mounted on copper grid and stained with uranylacetate and lead acetate. The grids were examined using a transmission electron microscope (Phillips, model CM10, Holland).

3.2.11 Cytotoxicity studies

3.2.11.1 RBC hemolysis assay

Hemolysis assay was performed as per the method of Iwamoto *et al.* (1994) with some modification. Fresh red blood cells prepared from ICR mouse (female, four weeks old) were used in the hemolytic assay. The whole blood cells were collected under the heparinized condition, and washed with saline three times. The resultant red blood cells (RBC) were suspended in saline to yield 2 % (v/v) suspension. Fifty µl of RBC suspension was added to 50 µl of the compound to yield final concentration of *Plumbagin* and *Isodiospyrin* (100-10 µg/ml) in U-bottom microtiter plates. The plate was incubated for two hours at room temperature with gentle shaking, and then allowed to stand for a while to sediment RBC. The

percent hemolysis was calculated by the following formula: A_{540} for the sample with hemolysin - A_{540} for the control without hemolysin]/A_{540} for the complete lysis caused by mixing ultrapure grade water] x 100.

3.2.11.2 Preparation of peritoneal mouse macrophages

The process of preparing peritoneal macrophages was done as per given by Manosroi *et al.* (2003) with little modification. Mice was injected with fetal calf serum intraperitoneally as a stimulant to elicit peritoneal macrophages. After three days of injection, the resulting peritoneal cells were harvested by peritoneal lavage with RPMI-1640 supplemented with 10 % heat-inactivated FCS, 50 µM 2-mercaptoethanol, 100 I.E. penicillin, 100 µg streptomycin and 0.25 µg/ml amphotericin-B. The exudate was centrifuged at 1000 rpm, 25 °C for 20 min. The erythrocytes in the cell pellets were lysed by hypotonic solution (0.2 % NaCl). Isotonicity was restored with 1.6 % NaCl solution. Cell suspension was centrifuged and the cells were washed twice and re-suspended in complete RPMI-1640. The cell number was adjusted to 4×10^6 cells/ml. The cells were incubated in 96 well culture plate for 2 h at 37 °C in 5 % CO_2 in an incubator. The nonadherent cells were removed by repeated washing with ice cold serum RPMI 1640 medium while the adherent cells were cultured for further studies.

3.2.11.3 Preperation of splenic lymphocytes

Splenocytes were isolated by the method of Tanaka (1999) with some modification. Mice were killed with cervical dislocation under ether anesthesia and a single cell suspension were prepared by pressing the spleen between two slide glasses. The cell suspensions were passed through a 200 gauge stainless sieve and then left to stand to remove tissue fragments. The cell suspensions were centrifuged (600 g for 10 min), resuspended gently in RPMI1640. The cell suspensions were adjusted to 4×10^6 cells/ml and the cells were seeded in flat bottom 96-well polysterene microtitre plate and cultured for further studies.

3.2.11.4 : In vitro cytotoxicity assay

Viable counts of macrophages and splenocytes were measured by tryptan blue dye exclusion method described by Ignacio *et al.* (2001) with little modification. Adherent peritoneal macrophages (4×10^6 cells/well) and splenocytes (4×10^6 cells/well) were placed in microtiter plates with medium supplemented with 2 % FCS, containing different concentrations of compound (10-100 µg/ml) and incubated up to 24 h at 37 °C. The cell suspensions were mixed with equal volume of dye (0.1 % in PBS) and incubated at room

temperature for 5 min. The cells were washed to remove the free dye. Viable cell counts were taken by using a haemocytometer under 40 x magnification of the phase contrast microscope. Cells with dye were excluded and only transparent cells were taken and counted as viable cells. Triplicate wells were set for each concentration.

RESULTS

4.1 SELECTION OF PLANTS BY SCREENING OF AQUEOUS AND ORGANIC EXTRACT :

Aqueous and methanolic extract of twenty plants used in the present study were screened for antifungal activity by disc diffusion assay against fourteen human pathogenic fungi. The result of screening of aqueous and methanolic extracts are shown in Table 1 and 2 respectively. It is clear from the table that aqueous extract of most of the plants did not exhibit any promising antifungal activity even at the highest concentration tested and only two plants were found to be active. *Hemidesmus indicus* showed moderate activity against *F. oxysporum*, *C. krusei*, *C. albicans* and *C. tropicalis* while *Plumbago capensis* was effective only on *Cryptococcus*. On the other hand methanolic extract of eleven plants were found to be active against one or more fungi (Table 2). *Pongammia pinnata* showed quite good activity against *T. mentegrophytes* and *M. gypseum*, moderate activity against *Candida spp.* and only slight activity on *A. fumigatus*. *Carica papaya* was found active against *S. shenkii* and *Carica sativa* against *S. shenkii*. *Oroxylum indicum* was effective on *A. fumigatus* and *Candida spp* while *Hemidesmus indicus* and *Andrographis paniculata* were slightly active on dermatophytes and showed moderate to good activity against *Candida spp*. *Cassia occidentalis* exibited slight activity against *Sporothrix*, *dermatophytes* and *Candidia*. All the three *spp.* of *Plumbago* were found moderately effective in reducing the growth of dimorphic fungi *Sporothrix*, *Fusarium* , *Aspergillus* and *Candida*.

Amongst all the plants *D. kaki* exhibited considerably good activity against *A. flavus*, *A. fumigatus*, *F. oxysporum*, *C. neoformans*, *S. shenkii* , *T. mentegrophytes*, *M. gypseum*, *C. albicans*, *C. tropicalis* and *C. krusei* at 5 and 7.5 mg/disc. Therefore *D. kaki* was found to have broad spectrum antifungal potential and was selected for further studies.

4.2 EXTRACTION EFFICIENCY OF DIFFERENT SOLVENTS ON *D. KAKI* ROOT BARK :

After selection of the plant as a result of screening the best extraction solvent need to be selected for the preparation of plant extract. Four different extraction solvents covering the wide polarity range from nonpolar to polar i.e. petroleum ether to chloroform, ethylacetate and methanol were used. The results are shown in Fig. 1. Methanol showed maximum yield i.e. 2.43 % followed by choloroform, 1.82 %, ethylacetate, 1.68 % and petroleum ether, 0.95 % of dry weight of root bark respectively.

Table 1. Screening of aqueous extract for antifungal activity

Plant name	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Jasminum undulatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pongammia pinnata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carica papaya	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cannabis sativa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thuja occidentalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bauhenia variegata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acyranthus aspera	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oroxylum indicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Argemone maxicana	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bombax ceiba	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tinospo rockordifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemidesmus indicus	-	-	-	++	-	-	-	-	-	-	-	+	+	++
Andrographis paniculata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cassia occidentalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Calatropis gigentia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tectona grandis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyrus communis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scyzium cumini	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Codiaeum variegatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Artemisia absinthium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plumbago zeylanica	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plumbago capensis	-	-	-	++	-	-	-	-	-	-	-	-	-	-
Acacia catechu	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plumbago rosea	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diospyros kaki	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1. A. flavus 2. *A. fumigatus* 3. *F. oxysporum* 4. *C. neoformans* 5. *S. shenkii* 6. *T. mentegrophytes* 7. *M. gypseum* 8. C. *lunata* 9. *R. pussilus* 10. *P. boydii* 11. *P. verrucosa* 12. *C. albicans* 13. *C . tropicalis* 14. *C. krusei.* (-) No activity & (+) indicates antifungal activity

Table 2. Screening of methanolic extract for antifungal activity

Plant name	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Jasminum undulatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pongammia pinnata	-	+	-	-		+++	+++	-	-	-	-	++	++	++
Carica papaya	-	-	-	-	++	-	-	-	-	-	-	-	-	-
Cannabis sativa	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Thuja occidentalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bauhenia variegata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acyranthus aspera	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oroxylum indicum	-	++	-	-	-	-	-	-	-	-	-	+	+	+
Argemone maxicana	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bombax ceiba	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tinospo rockordifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemidesmus indicus	-	-	-	-	-	+	+	-	-	-	-	++	++	++
Andrographis paniculata	-	-	-	-	-	+	+	-	-	-	-	+++	+++	+++
Cassia occidentalis	-	-	-	-	+	+	+	-	-	-	-	+	+	+
Calatropis gigentia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tectona grandis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyrus communis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scyzium cumini	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Codiaeum variegatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Artemisia absinthium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plumbago zeylanica	-	-	-	+	+	+	+		-	-	-	++	++	++
Plumbago capensis	-	-	-	+	+	+	+	-	-	-	-	+	+	+
Plumbago rosea	-	-	-	+	+	++	++	-	-	-	-	++	++	++
Acacia catechu	-	-	-	-	-	++	++	-	-	-	-	+++	+++	+++
Diospyros kaki	++	++	++	++	+++	+++	+++	-	-	-	-	+++	+++	+++

1. A. flavus 2. *A. fumigatus* 3. *F. oxysporum* 4. *C. neoformans* 5. *S. shenkii* 6. *T. mentegrophytes* 7. *M. gypseum* 8. C. *lunata* 9. *R. pussilus* 10. *P. boydii* 11. *P. verrucosa* 12. *C. albicans* 13. *C . tropicalis* 14. *C. krusei.* (-) No activity & (+) indicates antifungal activity

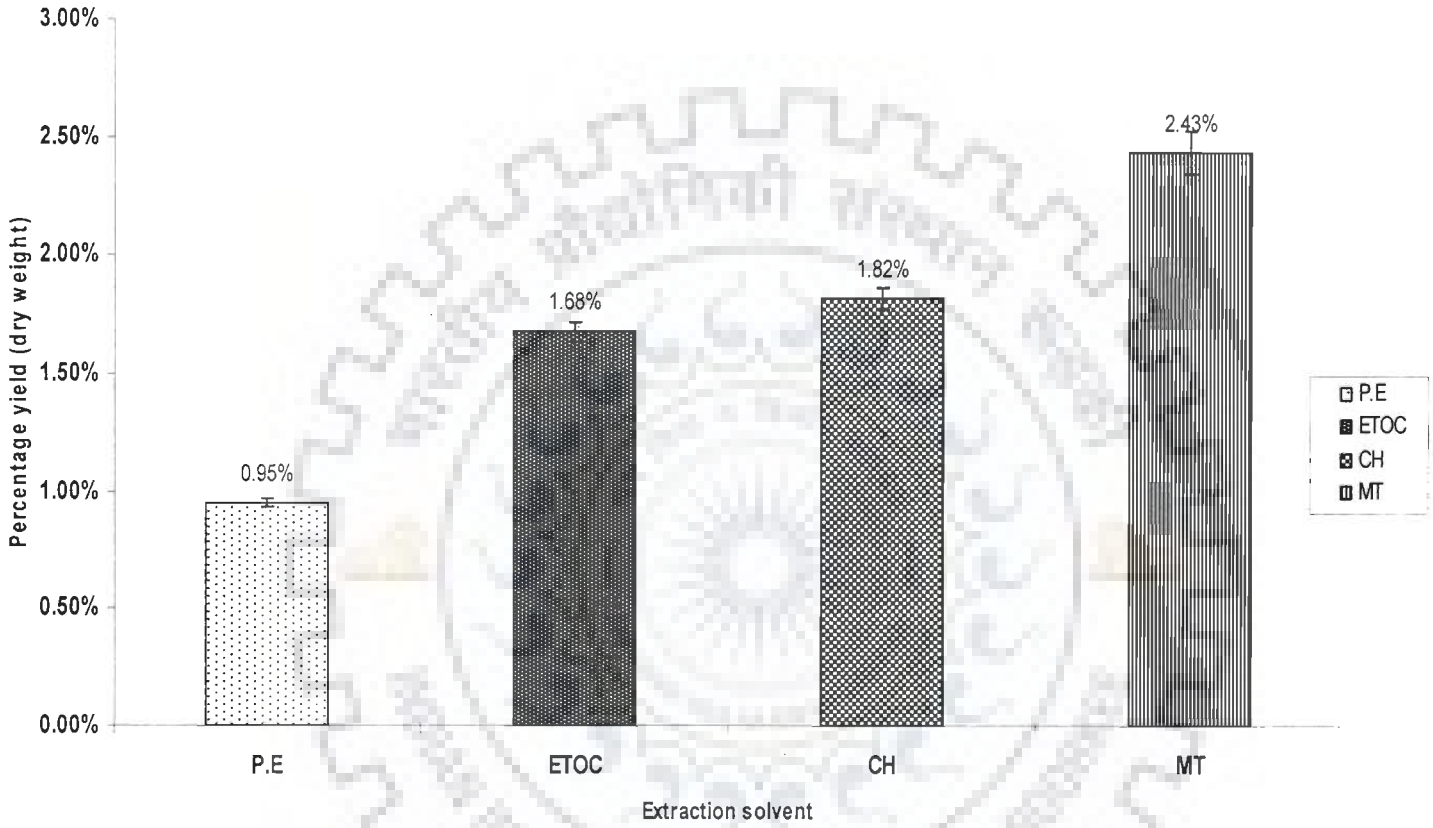


Fig 1: Extraction efficiency of different organic solvents on *D. kaki* root bark. The % yield of petroleum ether (PE), ethylacetate (ET), chloroform (CH) and methanol extract (MT) is 0.95 %, 1.68 %, 1.82 % and 2.43 % dry weight of root bark. Values are mean of triplicate ± SE

4.3 EVALUATION OF ANTIFUNGAL ACTIVITY OF CRUDE EXTRACT :

Since in the present study our emphasis was to look for a potential antifungal product, the percent dry weight yield obtained for different solvents could not be correlated with activity. Therfore in order to find the suitable solvent the antifungal activity of various extraction solvents were evaluated. The antifungal activity of different extracts against various fungi are shown in Table 3. Chloroform extract was found to have significant antifungal activity with maximum diameter range (5mm-25mm) and is active against all the fungi used at 5 mg/ml while at lower concentrations it showed no effect on few dematacieous fungi. The result of antifungal assay of chloroform extract are shown in Fig 2 - Fig 5. *Candida spp* along with dermatophytes were more susceptible to the extract followed by other hyaline fungi. *Candida spp* along with dermatophytes were more susceptible to the extract followed by other hyaline fungi. Inhibition zone diameter of petroleum ether varies from 10.67 - 23 mm at 7.5 mg but it showed no activity against *C. lunata*, *R. pussilus*, *P. verrucosa and P. boydii* at this concentration. Inhibition zone diameter range of ethylacetate and methanol extracts were relatively low i.e. 9.34 - 18.34 mm and 8.67-18 mm respectively at the highest concentration used in the study i.e. 7.5 mg. Both these extracts were inactive against *C. lunata*, *R. pussilus*, *P. verrucosa* and *P. boydii* at 7.5 mg. Therefore chloroform was chosen as extraction solvent for preparation of plant extract.

Results are indicated in Table 4. Activity index increases with increasing amount of plant extract. Thus the chloroform extract was positively effective in reducing the growth of fungi in the petriplates. Highest activity index was exhibited by the yeast followed by dermatophytes and least by the dematacieous fungi.

4.4 PHYTOCHEMICAL ANALYSIS :

Phytochemical analysis of chloroform extract of root bark was performed by means of standard qualitative tests for the major class of compounds present. Result of the phytochemical analysis is shown in Table 5. It shows positive test for four groups i.e. phenolics, flavonoid, terpenoid and tannins while negative for alkaloids, steroids, saponins, glycosides and proteins. The results rules out the possibility of the presence of any major compound belonging to the polar class in the root bark. Therefore the active compound present in chloroform extract are probably of non polar nature.

Table 3. Inhibition zone diameter (mm) of crude extract of *D. kaki* root bark using different solvents

Fungi	Petroleum ether extract (mg/disc)				Methanol extract (mg/disc)				Ethylacetate extract (mg/disc)				Chloroform extract (mg/disc)			
	1	2.5	5	7.5	1	2.5	5	7.5	1	2.5	5	7.5	1	2.5	5	7.5
1	12.67± 0.34	15.34 ±0.34	18.67± 0.34	23.34± 0.34	8.67± 0.34	12± 0.34	15± 0.58	18±0	8.67± 0.34	11.67± 0.34	15±0	18.34± 0.34	11.67 ± 0.34	15.34± 0.34	19.67± 0.34	25± 0
2	9±0.58	13±0	17±0	20.34± 0.34	8.34± 0.34	12±0	14.67± 0.34	18.67± 0.34	8.34± 0.34	12±0	14.67± 0.34	19±0	13±0	16.34± 0.34	20.34± 0.34	24.67± 0.34
3	8.67± 0.34	11.67 ±0.34	16.67± 0.34	21± 0.34	8±0	11.67± 0.34	14.67± 0.34	18.34± 0.34	8.34± 0.34	11.67± 0.34	14.67± 0.34	18± 0.58	11.67± 0.34	16±0	20±0	23.67± 0.67
4	8±0	11±0	15.67± 0.34	18.67± 0.34	6.34± 0.67	8.67± 0.34	11.34± 0.34	16± 0.58	7±0	8± 0.58	11.34± 0.34	17±0	9.0± 0.58	14.34±0 .58	17.34± 0.34	20.34± 0.67
5	5.34± 0.34	6.34± 0.89	10.67± 0.34	14.34± 0.67	4.67± 0.58	6.0± 0.58	7.61± 0.67	12± 0.58	4.67± 0.34	6±0	8± 0.34	12.34± 0.34	9.34± 0.67	14±0	17±0	21.67± 0.67
6	5.67± 0.34	9.67± 0.34	12.67± 0.89	16± 0.58	5.34± 0.34	9±0	12.34± 0.34	16.67± 0.34	5.0± 0.58	8.34± 0.34	11± 0.34	11± 0.58	7.67± 0.34	11.34±0 .34	14.34± 0.34	17± 0
7	8±0	11.67 ±0.34	14.67± 0.34	18.67± 0.34	-	5.0± 0.58	9.34± 0.58	13.34± 0.34	-	5.67± 0.34	9.34± 0.34	14.67± 0.34	7±0	11.67± 0.67	17± 0.67	20.34± 0.34
8	4.67± 0.34	8±0	11± 0.58	17.34± 0.89	-	4.67± 0.67	9±0	14±0	-	4.67± 0.67	8.67± 0.58	14.67± 0.34	9±0.58	14.34± 0.34	16.34± 0.34	20.67± 0.67
9	-	4.67± 0.34	9±0	12± 0.58	-	-	6.0± 0.58	10.67± 0.34	-	-	6±0.58	11±0	7.67± 0.34	13.34± 0.34	13.34± 0.58	21.67± 0.34
10	-	-	6.67± 0.34	13.34± 0.58	-	-	5.34± 0.34	8.0± 0.67	-	-	5±0	9.34± 0.34	5±0.58	10± 0.58	12.67± 0.34	16± 0
11	-	-	5.67± 0.34	11±0	-	-	-	-	-	-	6±0	-	-	5.67± 0.34	8±0.58	13.34± 0.58
12	-	-	-	-	-	-	-	-	-	-	5±0	-	-	-	7.34± 0.34	11.67± 0.34
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.34± 0.67	9.34± 0.34
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5±0.58	11±0

DMSO was used as negative control & Am-B as positive control. Values are mean of triplicate ± SE *1. C. albicans 2. C. tropicalis 3. C. krusei , 4. C. neoformans, 5. S. shenkii 6. F. oxysporum, 7. T. mentegrophytes, 8. M. gypseum, 9. A. flavus, 10. A. fumigatus,11. R. pussilus, 12. P. verrucosa, 13. C. lunata, 14. P. boydii*

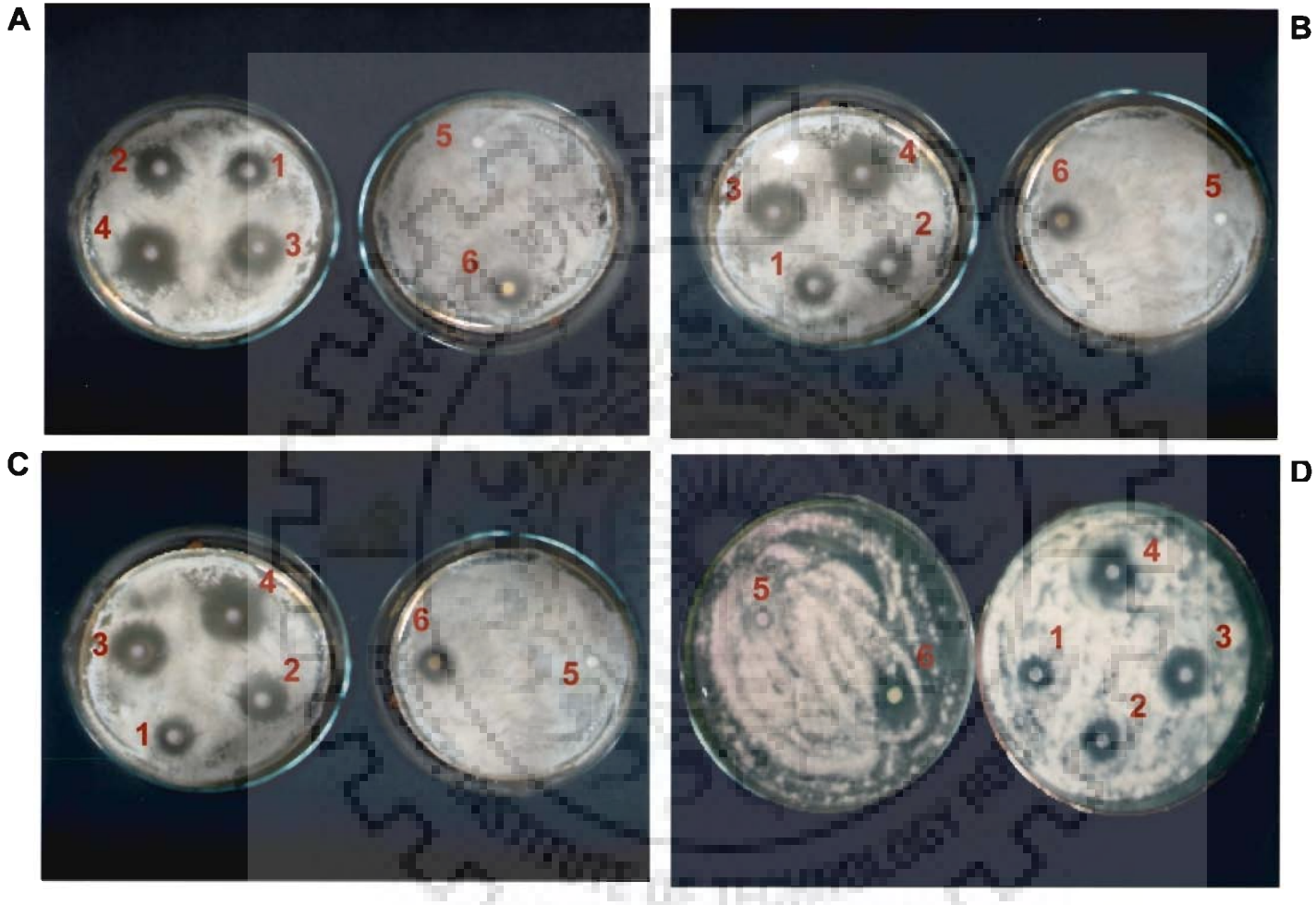


Figure 2. Disc diffusion assay of chloroform extract of *D. kaki* root bark A) *C. albicans* ; B) *C. tropicalis* ; C) *C. krusei* ; D) *C. neoformans* 1, 2, 3 & 4 are 1, 2.5, 5 & 7.5 mg/ disc of extract ; 5) DMSO ; 6) 100 µg Am-B.

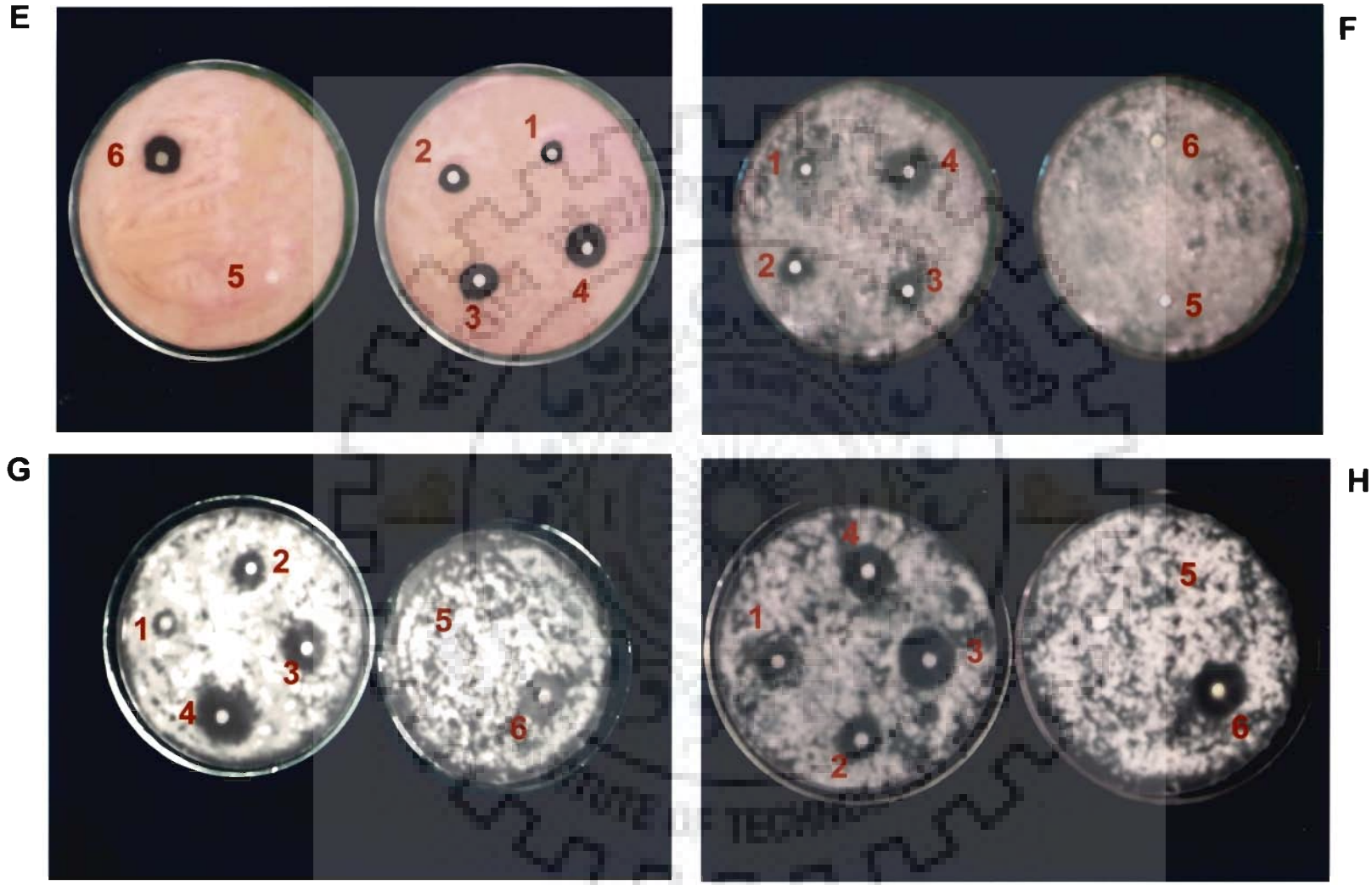
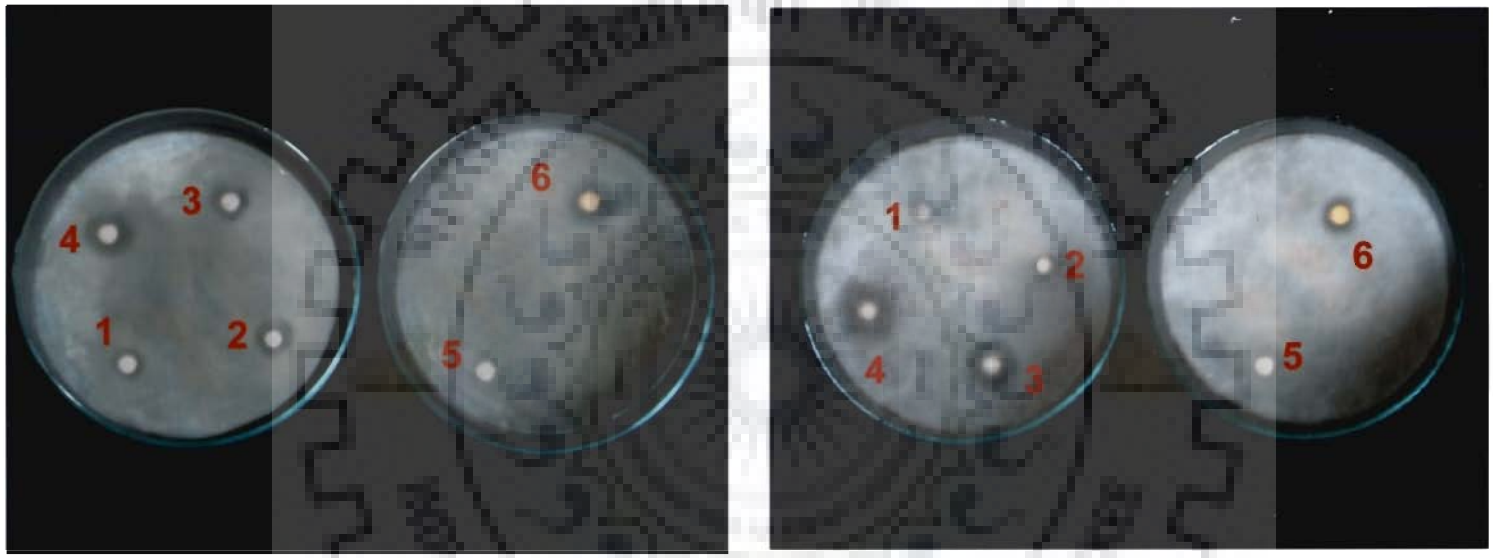


Figure 3. Disc diffusion assay of chloroform extract of *D. kaki* root bark E) *S. Shenkii* ; F) *F. oxysporum* ; G) *T. mentegrophytes* ; H) *M. gypseum.* 1, 2, 3 & 4 are 1, 2.5, 5 & 7.5 mg/ disc of extract ; 5) DMSO ; 6) 100 µg Am-B

Figure 4. Disc diffusion assay of chloroform extract of *D. kaki* root bark I) *A. fumigatus* ; J) *A. flavus* ; K) *C. lunata* ; L) *P. boydii.* 1, 2, 3 & 4 are 1, 2.5 ,5 & 7.5 mg/ disc ofextract ; 5) DMSO; 6) 100 µg Am-B

M



N

Figure 5. M) Disc diffusion assay of chloroform extract of *D. kaki* root bark *R. pussilus* ; N) *P.verrucosa* 1, 2, 3 & 4 are 1, 2.5 ,5 & 7.5 mg/ disc of extract ; 5) DMSO; 6) 100 µg Am-B

Table 4. Activity index of crude chloroform extract of *D. kaki* root bark

Fungus	1mg/disc	2.5mg/disc	5 mg/disc	7.5mg/disc
Candida albicans (MTCC 227)	0.82	1.07	1.38	1.75
Candida tropicalis (ATCC 6258)	0.93	1.17	1.46	1.77
Candida krusei (ATCC 750)	0.88	1.2	1.5	1.78
Cryptococcus neoformans (ITCC 1672)	0.78	1.23	1.49	1.75
Soprothrix shenkii (ITCC 2317)	0.7	1.04	1.49	1.55
Fusarium oxysporum (ITCC 4998)	-	-	-	-
Trichophyton mentegrophytes (ITCC 3572)	0.64	1.07	1.55	1.85
Microsporon gypseum (ITCC 5277)	0.66	1.05	1.2	1.52
Aspergillus flavus (ITCC 5290)	0.59	1.03	1.03	1.67
Aspergillus fumigatus (ITCC 4880)	0.49	0.97	1.17	1.55
Rhizomucor pussilus (ITCC W-14)	-	0.52	0.73	1.22
Phialophora verrucosa (MCCL 32006)	-	-	0.71	1.13
Curvularia lunata (ITCC 5248)	-	-	0.52	0.91
Pseudaresheria boydii (MCCL W-48)	-	-	-	-

Calculated by the formula -inhibition zone diametre of sample / inhibition zone diametre of standard. Am-B 100 µg/disc was used as reference

Table5. Qualitative analysis of the phytochemical constituents of chloroform crude extract of *D. kaki* rootbark

Class of compound	Presence/absence
Phenolics	+
Flavonoids	+
Tannins	+
Terpenoids	+
Alkaloids	–
Steroids	–
Saponins	–
Glycosides	–
proteins	–

4.5 SELECTION OF SOLVENT SYSTEM FOR PURIFICATION BY TLC :

The qualitative analysis of crude extract showed the presence of phenolics, flavonoid, terpenoid and tannins. To further confirm the presence of these group of compounds and to choose the appropriate elution solvents for performing purification using column chromatography, TLC analysis of the crude extract was performed. The result of TLC studies are shown in Table 6. Similar to qualitative tests TLC also confirmed that indicates that phenolics, terpenes, flavonoids and quinines are major constituents as more number of clearly separated bands could be seen for these group of compounds. The various solvent system used, number of bands observed and Rf in particular group of compounds are shown. Maximum number of bands were observed in benzene-chloroform (1:1). This study further indicates that nonpolar compound are the major constituents and most probably may be active principle. The result clearly indicates that which type of solvent is suitable for a particular group of compounds and how many compounds are present in particular group, but it is not clear from this experiment that which fraction contains the active constituents.

4.6 TLC BIOAUTOGRAPHY :

Before performing purification by column chromatography the simple TLC bioauatography of all the three fungi i.e. *C. albicans, T. mentegrophytes and A. flavus* were performed to locate the active principle on TLC plate itself. The result is shown in Fig 6. As clear from the figure that white zones on a purple background were observed. Spot 1 and 2 indicates the presence of two active compounds in the crude extract. These white areas or zone indicates that reduction of MTT to the coloured formazan did not take place in this region due to the presence of compounds inhibiting the growth of tested fungi.

4.7 PURIFICATION OF ACTIVE COMPOUNDS BY OPEN COLUMN CHROMATOGRAPHY :

The benzene-chloroform was found to be the best solvent system for resolution of active constituents as observed in TLC. Therefore open column chromatography was done as described in material and methods. Column was eluted with benzene-chloroform step gradient. The scheme of purification of chloroform extract as given in Fig 7, shows that first active fraction was obtained on elution with 50:50 benzene-chloroform. Fraction no F3-F9 was found to be active and this fraction was named A1. A1 was further purified using

Table 6. TLC analysis of chloroform extract using various solvents and detection system. The number of spots and their Rf value are indicated

Class of compound	Solvent system	Detection method	No of spots	Rf value
Phenolics	-Ethylacetate-formic acid-water (3:1:3)	Folin -ciocalteu reagent	–	–
	-Toluene-Ethylacetate-formic acid (36:12:5)		3	0.74, 0.53, 0.49
	-Benzene-methanol-acetone (45:8:4)			
	-Butanol-ethanol-water (5:1:2)		4	0.81, 0.61, 0.58, 0.54
			–	–
Coumarins	-Chloroform (100%)	Potassium hydroxide reagent & UV-365nm	3	0.86, 0.45, 0.48
	-Benzene-ethanol (45:5)		2	0.73, 0.64
	-Diethylether-Toluene (1:1)		–	–
Terpenoids	-Benzene-chloroform (1:1)	Anisaldehyde sulphuricacid reagent	9	0.92, 0.88, 0.81,0.77, 0.72, 0.68, 0.65, 0.46, 0.42
	-Benzene-methanol (9:1)		4	0.86, 0.72, 0.68, 0.64
	-Benzene-ether (2:3)		4	0.91, 0.87, 0.67, 0.51, 0.48
	-Toluene-ethylacetate (93:7)		4	0.71, 0.63, 0.57, 0.53
	-Toluene-chloroform-ethanol (40:40:10)		3	0.81, 0.56, 0.59
	-nHexane-ethylacetate (17:3)		3	0.74, 0.61 , 0.58

Flavonoids	-Ethylacetate-formic acid-water (8:1:1) -Ethylacetate-formicacid-aceticacid-water (100:11:11:26) -Ethylacetate-ethanol-water (11:13.5:10) -Toluene-aceticacid (4:1) -Chloroform-ethylacetate-aceticacid (5:4:1) -Chloroform-methanol (89:11)	Natural products-polyethylene reagent & UV-365nm	– 1 – 3 2 1	– 0.68 – 0.79, 0.53, 056 0.63, 0.42 0.48
Quinones	-Benzene-chloroform (1:1) -Ethylacetate-methanol –water (10:16.5:13.5) -Toluene-Ethylformate-formic acid (5:4:1) -Petroleum ether-ethylacetate (90:4:1)	Iodine reagent	9 – 2 5	0.92, 0.88, 0.81, 0.77,0.72, 0.68, 0.65, 0.46, 0.43 – 0.81, 0.51 0.86, 0.71, 0.63, 0.54, 0.58
Tannins	-Butanol-aceticacid-water (14:1:5)	Iron(III) Chloride solution	–	–
Saponins	-Chloroform-aceticacid-methanol-water (64:32:12:8) -N-butanol-water (1:1) -Hexane-ethylacetate (5:95) -Benzene-methanol (10:7.5) -Chloroform-methanol (22:1.5)	Anisaldehyde-sulphuricacid reagent	– – – 2 2	– – – 0.83, 0.79 0.62, 0.58
Glycosides	-Ethylacetate-methanol-water (100:13.5:10)	Diphenylamine	–	–

Alkaloids	-Methanol-conc NH4OH (200:3) -Ethyacetate-methanol-water (100:13.5:10) -Toluene-ethylacetate-diethylamine (70:20:10) -Choloroform-ethylacetate-formic acid (5:4:1)	Dragendorff reagent	– – – 2	– – – 0.64, 0.44
Steroids	-Benzene-Ethanol (95:5) -Benzene-ethylacetate (9:1)	Phosphoric acid	3 3	0.72, 0.58, 0.55 0.86, 0.72, 0.68, 0.64

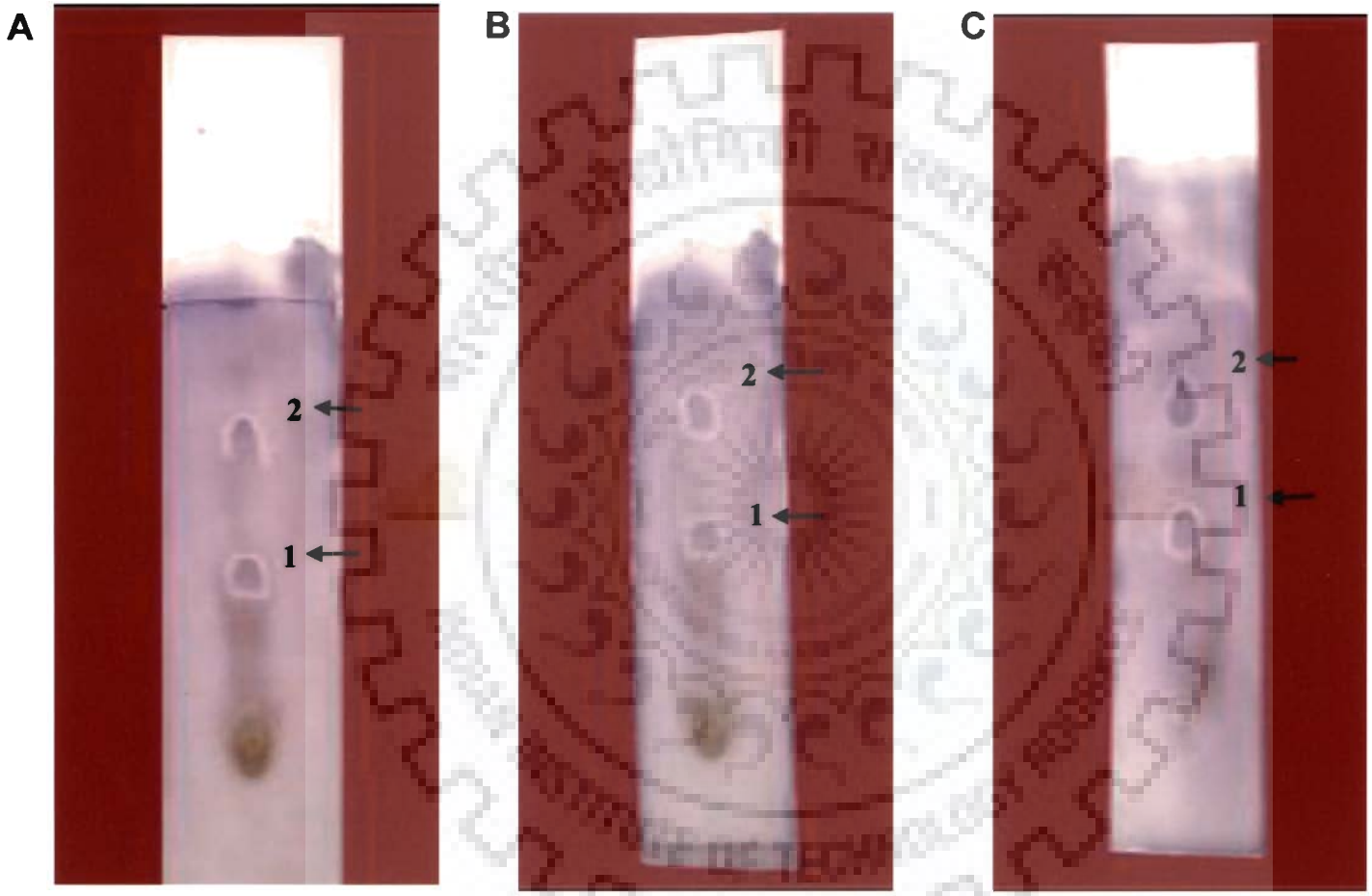


Figure 6. TLC Bioautogram of chloroform extract using Benzene-Chloroform 1:1 system against A) *C. albicans;* B) *T. mentegrophytes*; C) *A. flavus*. Two white spots, 1 & 2 as indicated by arrow on TLC plates after spraying with MTT, indicates the presence of active compound

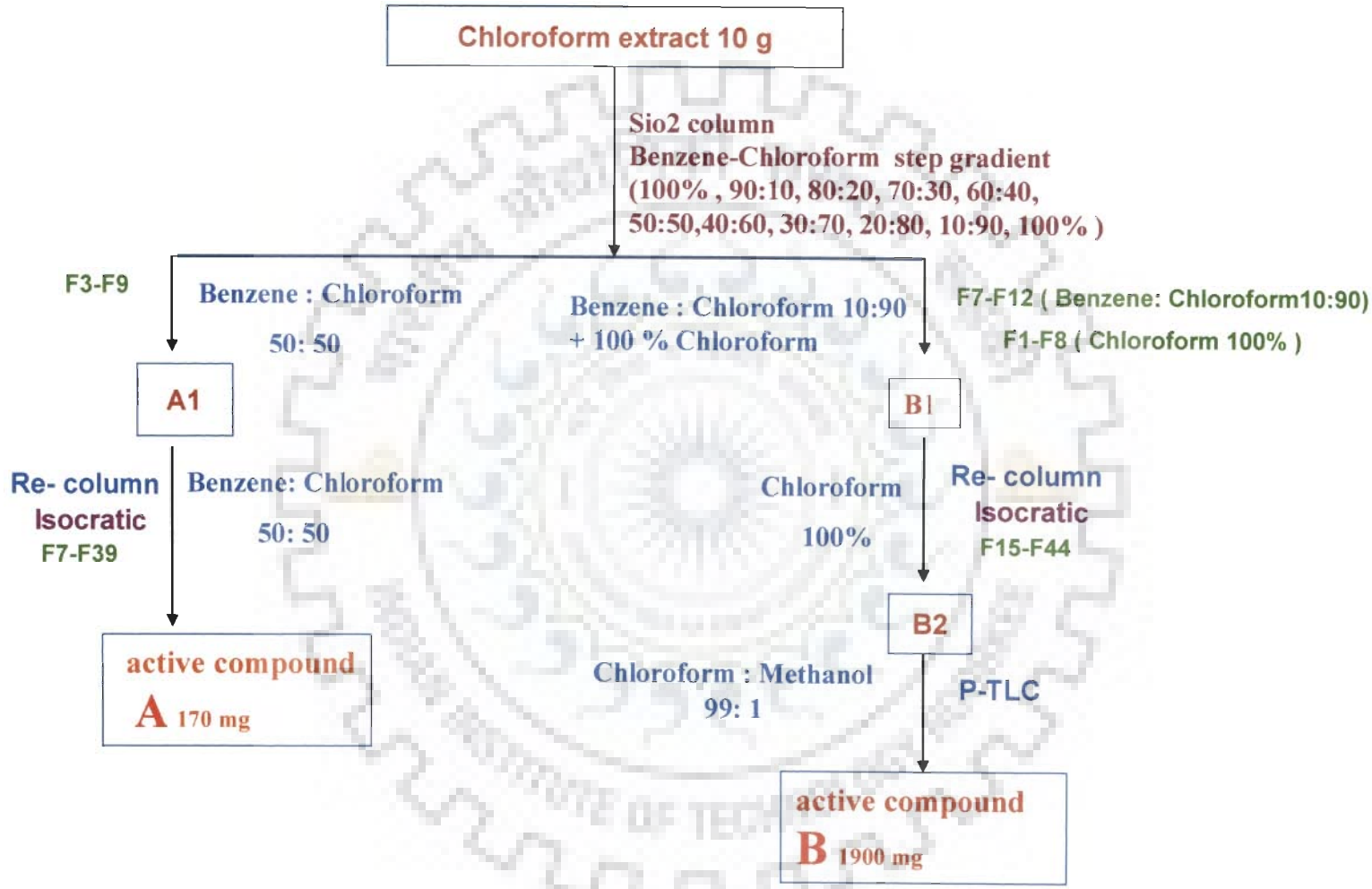


Figure 7. Flowchart of purification of active compounds by open column chromatography of crude chloroform extract of *D. kaki* root bark

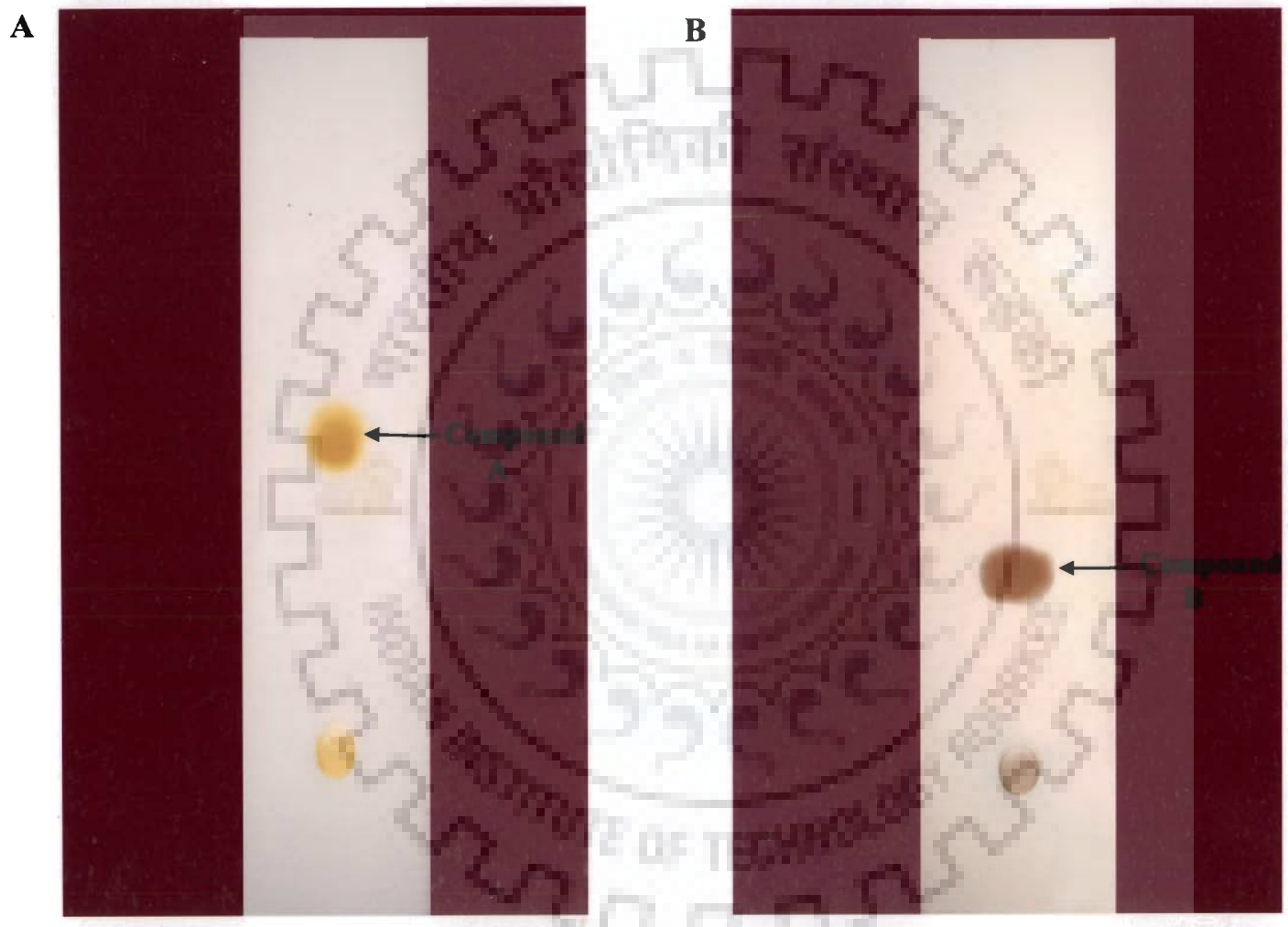


Fig 8. TLC plates showing Rf value and bioutogram of pure compounds A & B in benzene-chloroform 1:1. Rf value of compound A is 0.72 and B is 0.42

isocratic elution with 50:50 benzene-chloroform on a smaller column and 50 fractions of 5 ml were collected. All the fractions were monitored by TLC. The fractions with similar TLC profile were pooled and checked for presence of antifungal activity. Fraction no F7- F39 was obtained as pure compound in the form of orange needles and was named A. The yield of A was 170 mg. Another active fraction was obtained with 10:90 benzene-chloroform and 100 % chloroform. As fraction no F7- F8 obtained on elution with 10:90 benzene-chloroform and fraction F1-F8 with 100 % chloroform had similar TLC profile they were pooled and named B1. B1 was further purified on smaller column using isocratic elution with 100 % chloroform. Again 50 fractions of 5 ml were collected and evaluated by TLC. The resulting fraction F15-F44 named B2 was found to be active and it showed two very close bands on TLC. These bands were separated using P-TLC with Chloroform-methanol 99:1. Both the bands was scraped from P-TLC plate and the compound showing lower band in the TLC was found to be active. The active compound B was obtained as red needles and the yield was 1900 mg. The TLC of A and B as single band and their Rf values are shown in Fig 8.

4.8 CHARACTERIZATION OF ACTIVE COMPOUNDS :

4.8.1 Physical characteristics of compounds A and B

Compound A was obtained as orangish yellow needles (daylight), reddish (UV 254nm), M.P 76°-79°C. It is Soluble in alcohol, acetone, chloroform and benzene.

Compound B was obtained as red needles (day light), dark brown colour (UV 254 nm), M.P. 231-234°c. It is Soluble in alcohol, acetone, chloroform and benzene.

4 8.2 Analytical studies of compounds A and B

EI-MS spectrum of compound A is shown in Fig. 9. EI-MS *m/z* (intensity %)- 188 (100), 173 (26), 160(27), 131 (44), 120 (30), 92 (39), and 63 (43). The FTIR spectra of A is shown in Fig. 11. The IR spectrum (KBr) – 2925, 1735, 1650, 1610 and 1450. ^{1}H and ^{13}C NMR (DMSO) spectra are shown in Fig. 13-15. Spectra showed the following signals- δ 2.19(Me-2), 7.06(H-3), 7.40(H-6), 7.42(H-7), 7.82(H-8), 11.97(-OH). 114.9(C-10), 119.3(C-8), 124.23(C-6), 132.0(C-9), 135.66(C-3), 136.98(C-7), 149.8 (C-2), 161.2(C-5), 184.8(C1), 191.0(C-4), 16.9(C-11). This spectral data matches with the standard of compound 1,4-napthoquinone, *Plumbagin*.

EI-MS spectrum of compound B is shown in Fig. 10. EI-MS *m/z* (intensity, %) 374(100),

Characterization of Active compounds

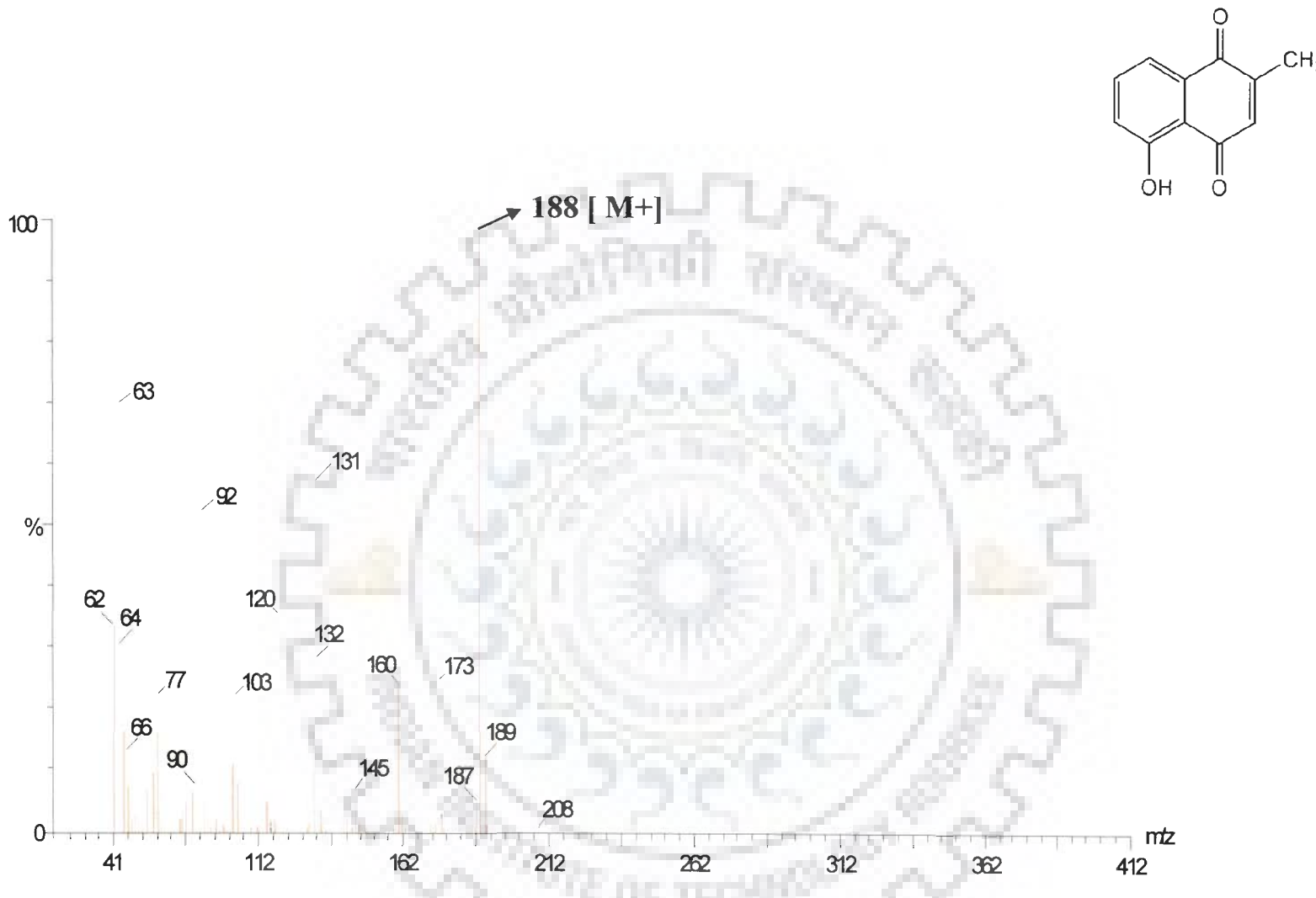


Figure 9. EI-MS spectrum of compound A. The molecular ion base [M+] peak is at m/e 188.18

Figure 10. EI-MS spectrum of compound B. The molecular ion base peak is at m/e [M+] at 374.34

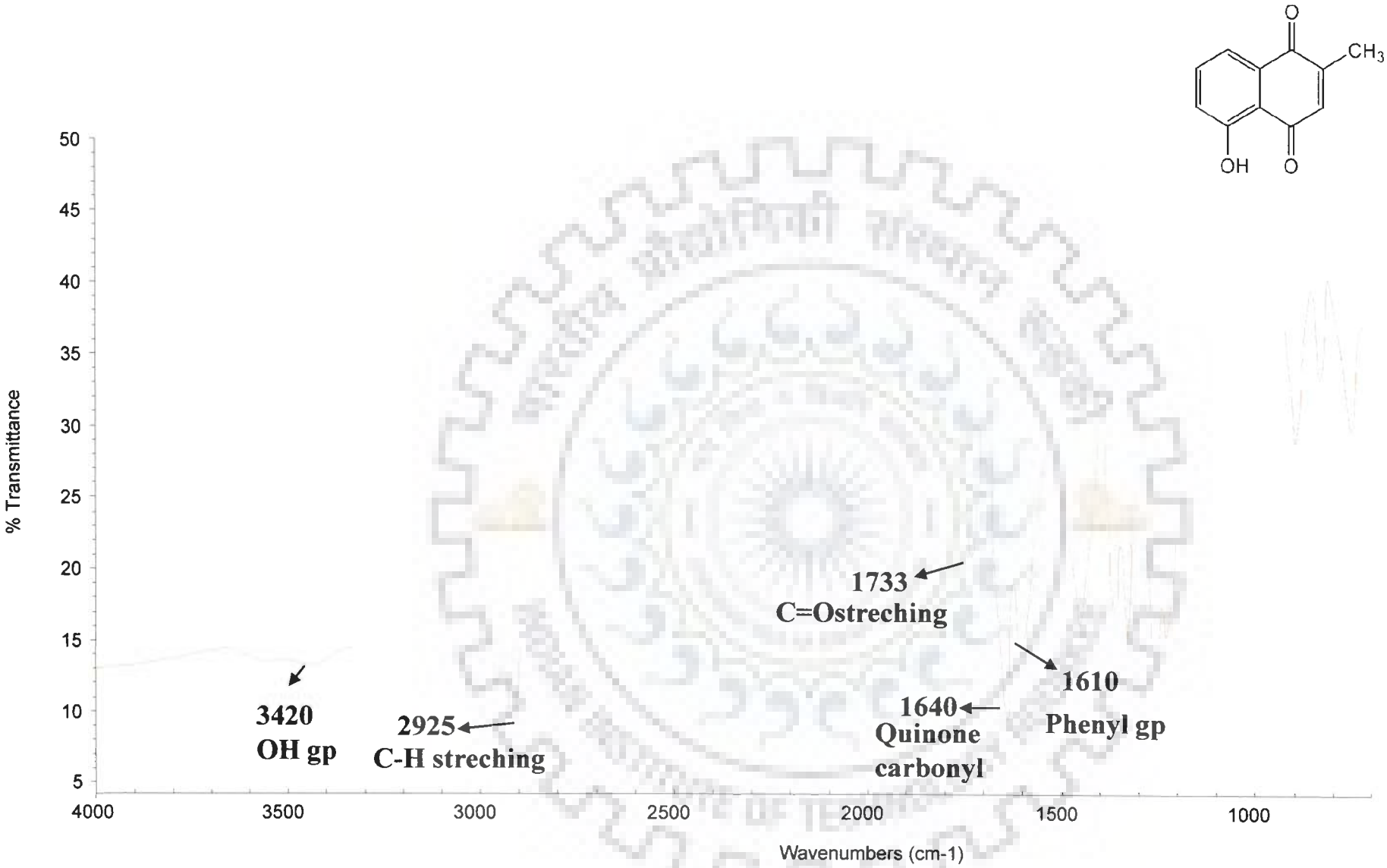


Figure 11. FTIR spectra of compound A. The absorption bands in the functional group region, 4000 to 1300 cm^{-1} indicates the presence of functional groups shown in spectra

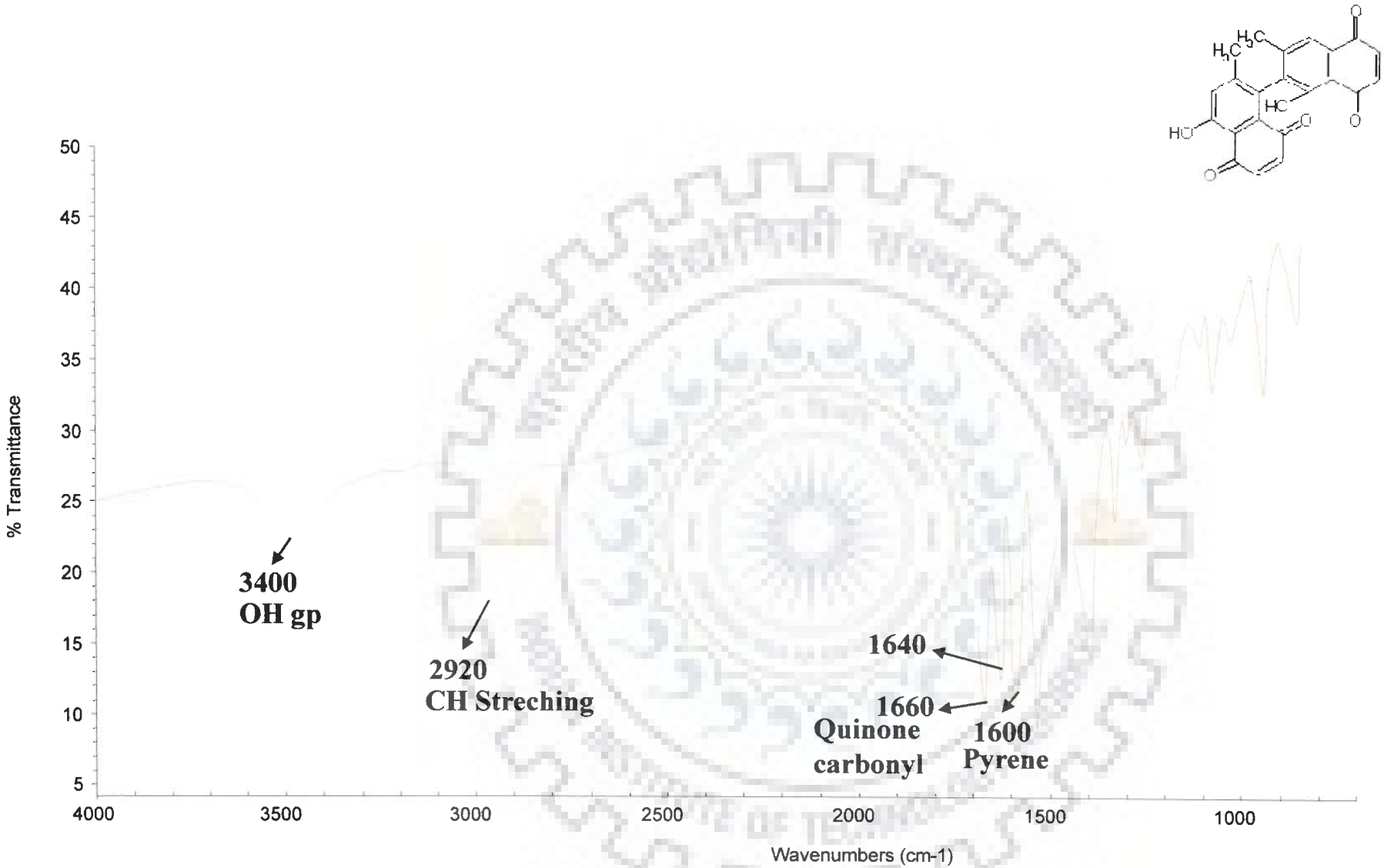


Figure 12. FTIR spectra of compound B. The absorption bands in the functional group region, 4000 to 1300 cm^{-1} indicates the presence of functional groups shown in spectra

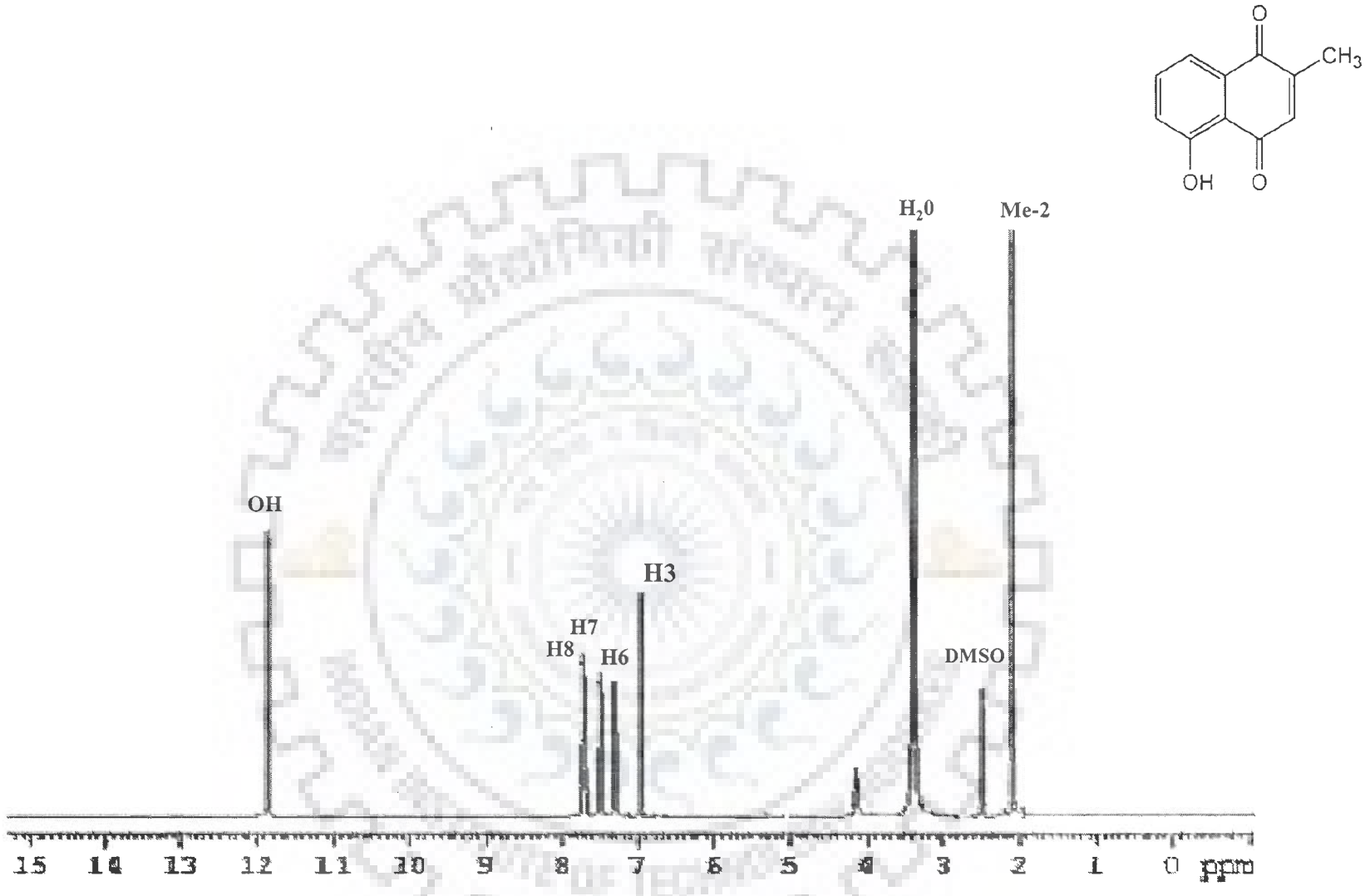


Figure 13. ^{1}H spectra NMR of compound A as recorded in Bruker DRX500, at 500 MHZ

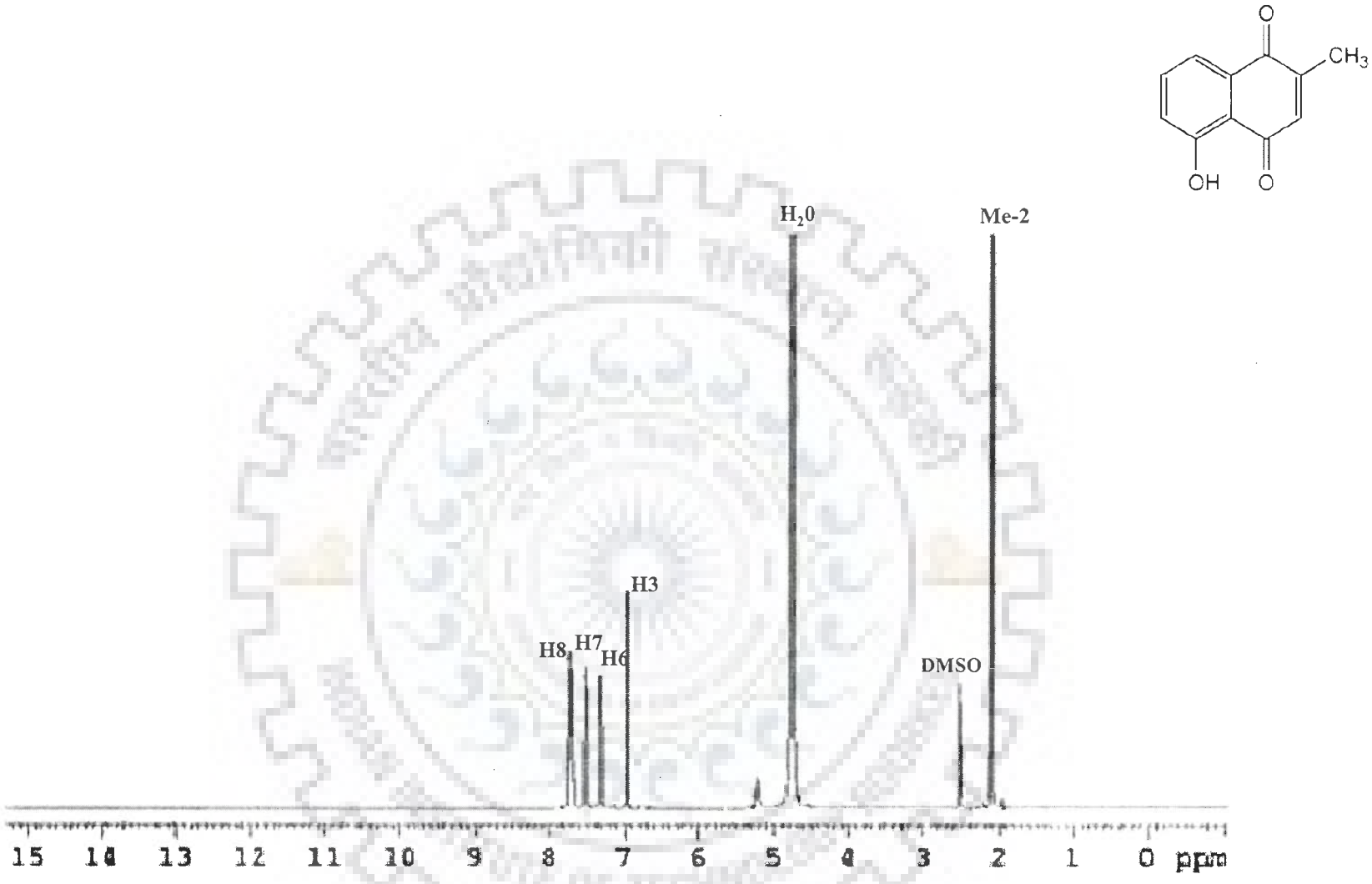


Figure 14. ^1H spectra NMR of compound A after D_2O shake as recorded in Bruker DRX500. δ 11.97 was absent and δ of H_20 was shifted to 4.7

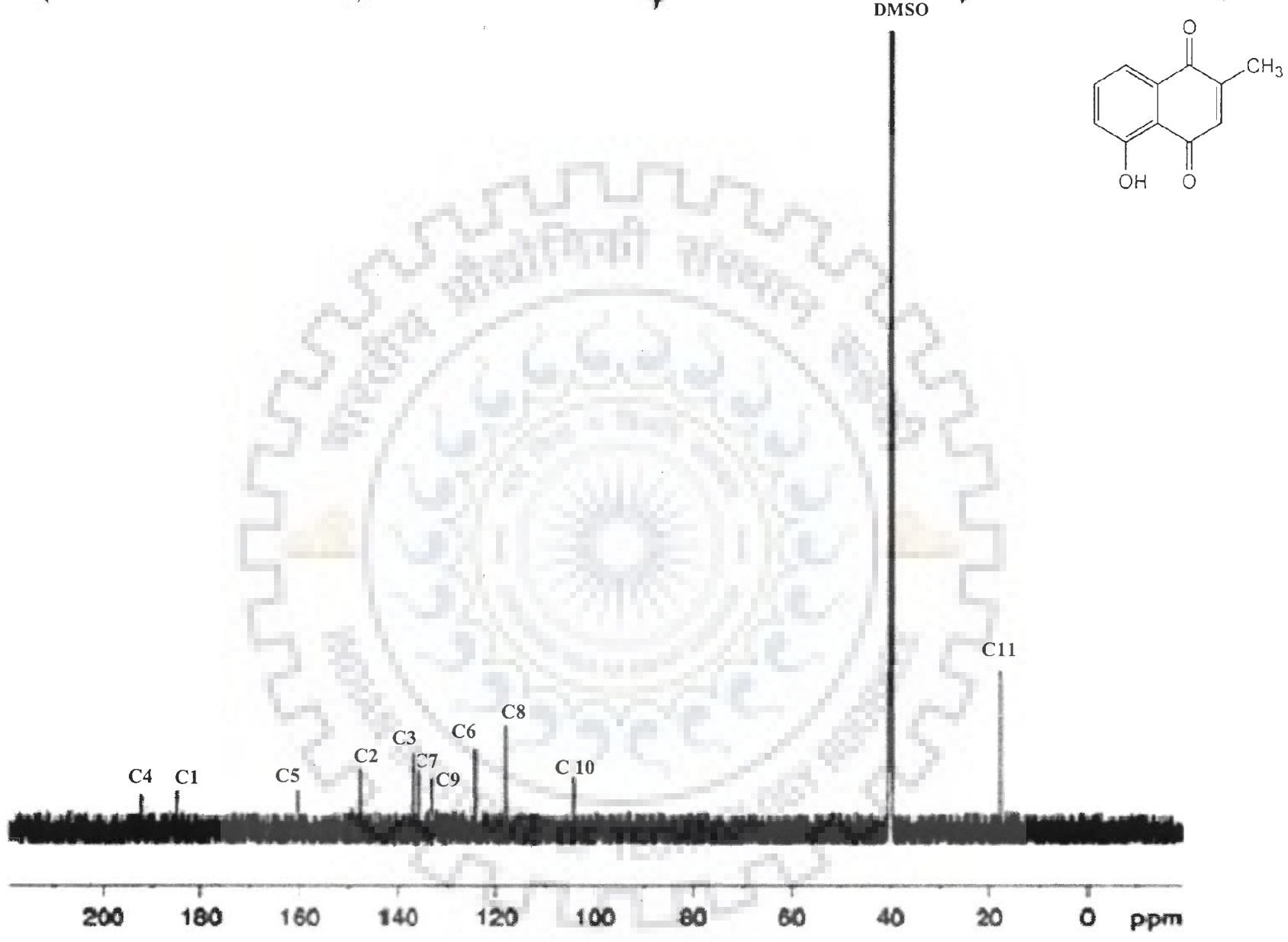


Figure 15. ^{13}C spectra of compound A as recorded in Bruker DRX 500 at 250 MHZ

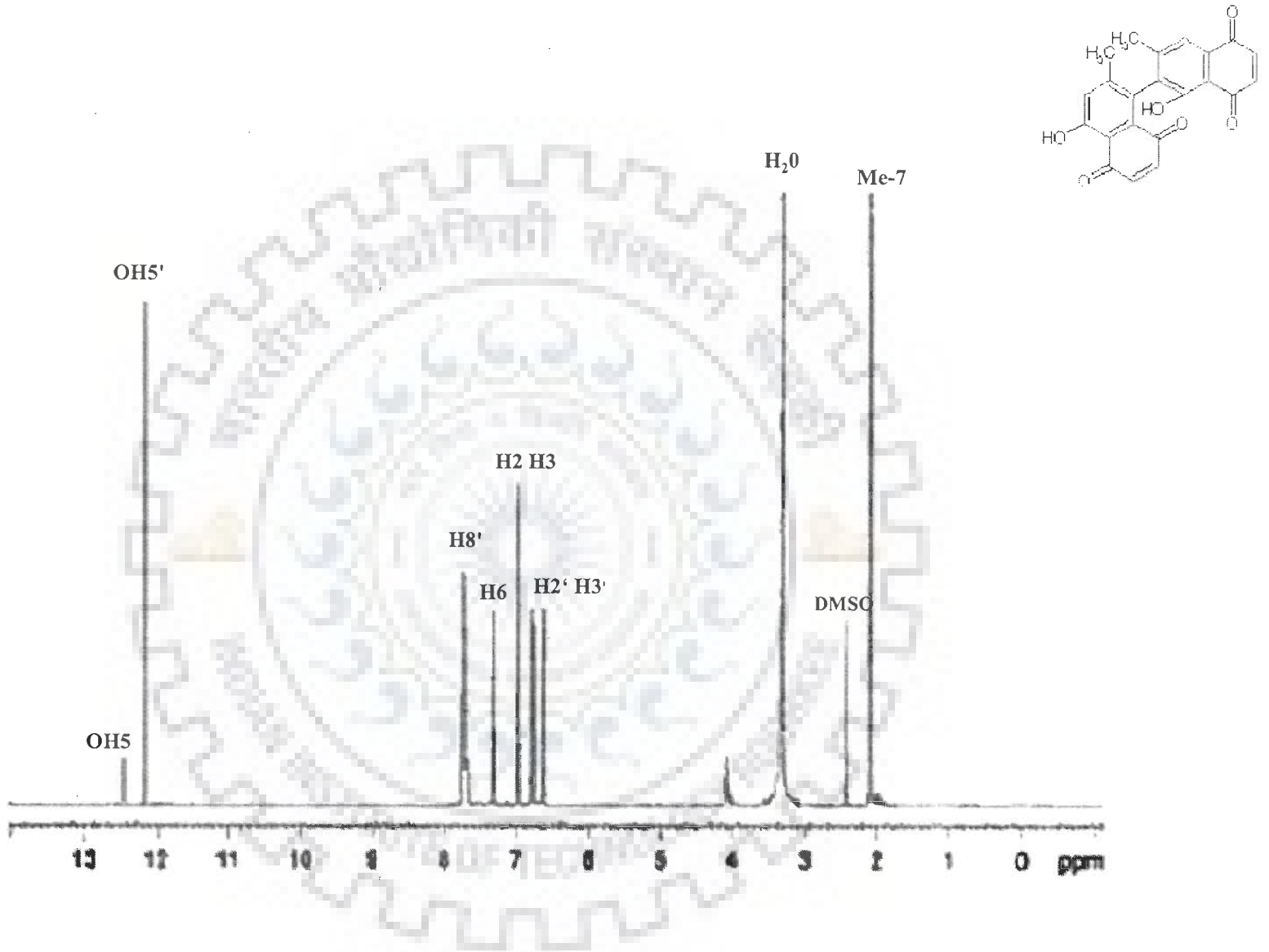
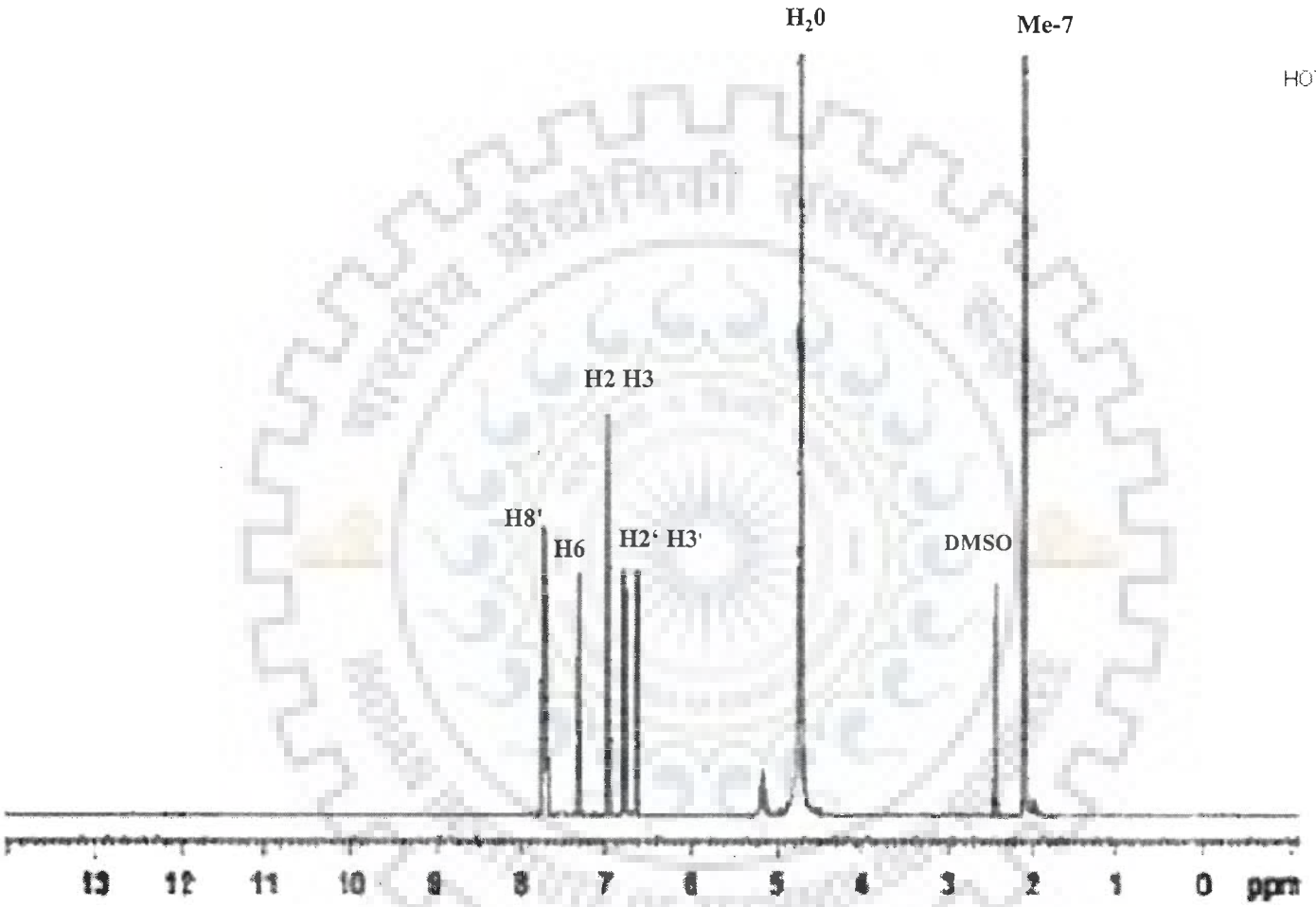


Figure 16. ^{1}H NMR spectra of compound B as in Bruker DRX500 at 500 MHZ



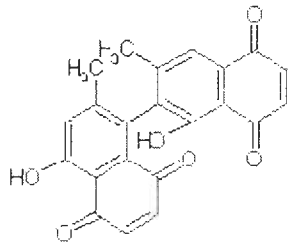


Figure 17. ^{1}H spectra NMR of compound B after D_2O shake as recorded in Bruker DRX500. δ 12.02 and 12.40 were absent and δ of H_20 was shifted to 4.7

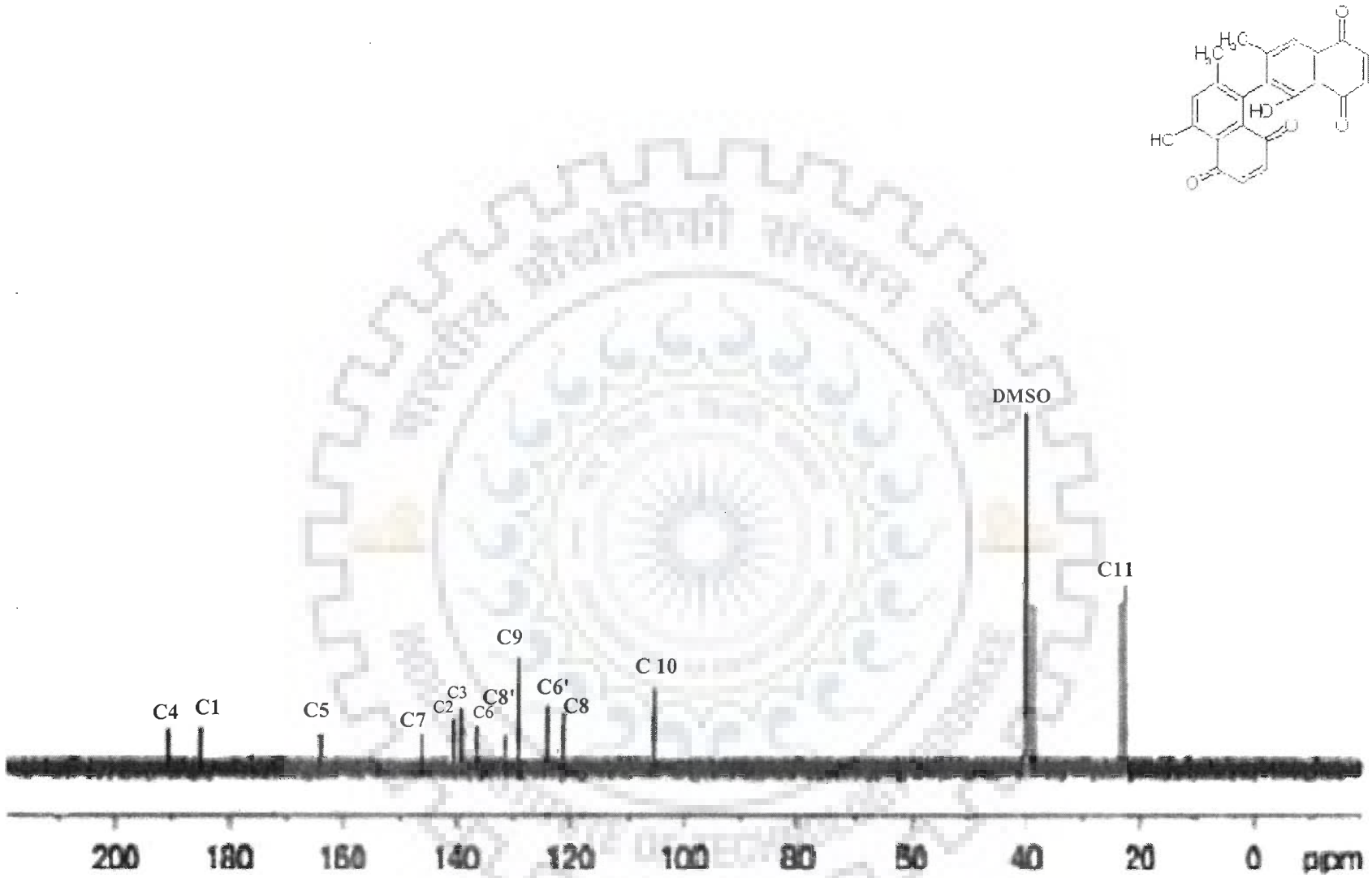


Figure 18. ^{13}C NMR spectra of compound B as recorded in Bruker DRX 500 at 250 MHZ

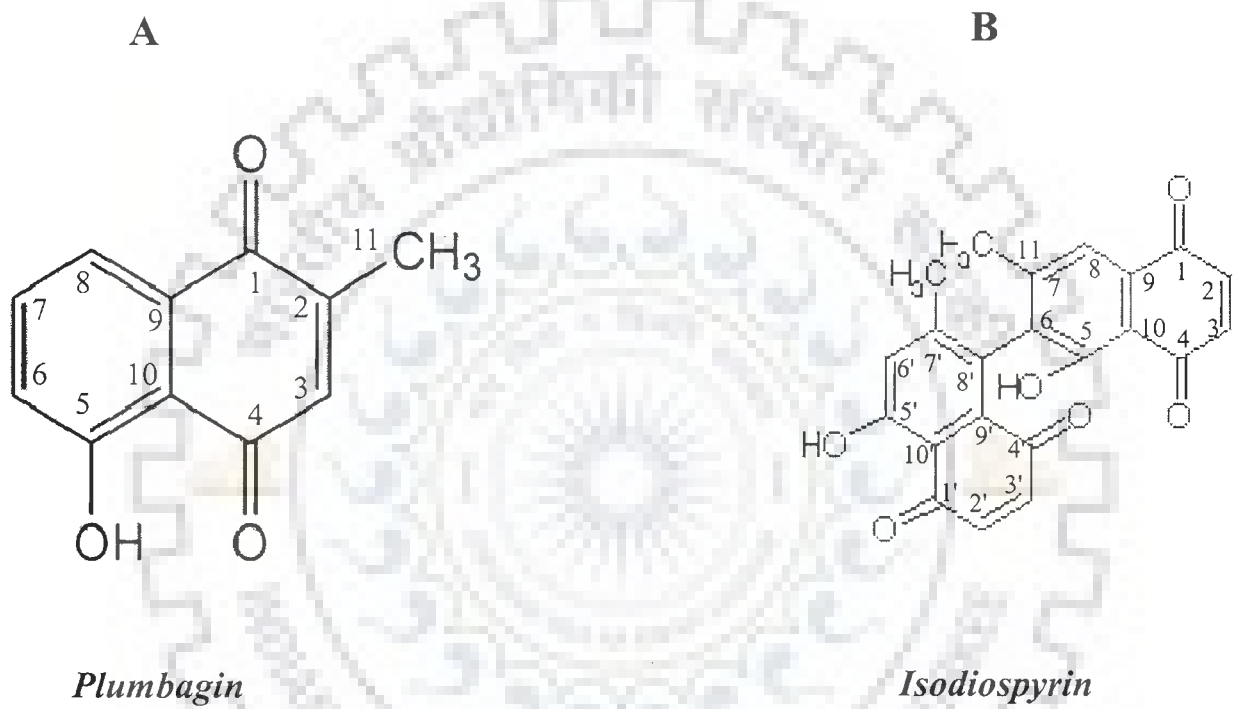


Fig. 19. Structure of pure compounds A & B

345(10), 187(7.5), 189(5), 383(4.5), 152(4). The FTIR spectra of B is shown in Fig. 12. The IR spectrum (KBr) – 3405, 2920, 1660, 1640, 1600 and 1585. ^{1}H and ^{13}C NMR (DMSO) are shown in Fig. 16-18. Spectra showed the following signals : δ 2.09(7-Me), 6.77(H-2), 6.89(H-3), 6.90(H-2, H-3), 7.30(H-6), 7.60(H-8), 12.02(OH-5'), 12.40(OH-5) δ 20.40(C-11), 113.31(C-10), 128.65(C-9), 121.37(C-8), 125.71(C6'), 135.22(C-6), 137.56(C-3), 130.40(C-8'), 140.14(C-2), 158.79(C-5), 184.54(C-1), 190.16(C-4). This spectral data matches with the binapthoquinone *Isodiospyrin.*

4.9 MIC AND MFC OF *PLUMBAGIN* AND *ISODIOSPYRIN* :

MIC and MFC of *Plumbagin* and *Isodiospyrin* were determined using broth microdilution method. MIC and MFC of the crude extract and active compounds are shown in Table 7 and 8. The trend of susceptibility was similar to that of crude extract as shown by inhibition zone diameter. However there was significant difference in the MIC values of crude extract (160-2500 µg/ml) and active compounds, *Plumbagin* (1.25 µg-20 µg/ml) and *Isodiospyrin* (5-160 µg/ml). *Candida spp.* exhibited the lowest MIC followed by dermatophytes, *Sporothrix* and *Fusarium* while the susceptibility of dematacеious fungi to the above mentioned compounds were lowest. In general the MIC values of *Plumbagin* were higher then that of *Isodiospyrin* and were close to that of amphotericin-B in case of *Candida.* MFC value of crude extract, *Plumbagin* and *Isodiospyrin* was found to be two to three dilutions higher than MIC in most of the cases. MFC value of the crude extract, *Plumbagin* and *Isodiospyrin* is (630- 5000 µg/ml), (5-160 µg/ml) and (40-160 µg/ml) respectively. IC_{50} of crude extract, *Plumbagin* and *Isodiospyrin* was determined against *C. albicans* (yeast), *T. mentegro*phytes (dermatophytes) and *A. flavus* (filamentous fungi) and results are shown in Fig 20 and 21. IC_{50} of both the pure compounds are given in Fig 20 and 21. IC_{50} of *Plumbagin* was 1.2, 1.4 and 8.8 µg/ml and of *Isodiospyrin* was 5.1, 5.4 and 31.5 µg/ml for *C. albicans* (yeast), *T. mentegro*phytes (dermatophytes) and *A. flavus* (filamentous fungi) respectively.

4.10 TIME KILL CURVE STUDY :

To study the pharmacodynamics of the pure compounds, the concentration dependent time killing of *Plumbagin* and *Isodiospyrin* against *C. albicans* (yeast), *T. mentegro*phytes (dermatophytes) and *A. flavus* (filamentous fungi) were studied. The result of time killing is

Table 7: MIC values of crude extract, *Plumbagin, Isodiospyrin* & Am-B against various fungi.

Fungus	MIC µg/ml crude extract	*Plumbagin* µg/ml	*Isodiospyrin* µg/ml	Am-B µg/ml
Candida albicans (MTCC 227)	160	1.25	5	0.5
Candida tropicalis (ATCC 6258)	160	1.25	5	0.5
Candida krusei (ATCC 750)	160	1.25	5	0.5
Cryptococcus neoformans (ITCC 1672)	320	2.5	10	1
Soprothrix shenkii (ITCC 2317)	630	5	10	2
Fusarium oxysporum (ITCC 4998)	630	5	20	-
Trichophyton mentegrophytes (ITCC 3572)	320	2.5	5	0.5
Microsporon gypseum (ITCC 5277)	320	2.5	5	2
Aspergillus flavus (ITCC 5290)	630	10	40	1
Aspergillus fumigatus (ITCC 4880)	630	10	40	1
Rhizomucor pussilus (ITCC W-14)	1250	10	40	4
Phialophora verrucosa (MCCL 32006)	1250	20	80	8
Curvularia lunata (ITCC 5248)	1250	20	80	4
Pseudaresheria.boydii MCCL W-48)	2500	20	160	-

Table 8: MFC values of crude extract, *Plumbagin, Isodiospyrin* & Am-B

Fungus	MFC µg/ml crude extract	*Plumbagin* µg/ml	*Isodiospyrin* µg/ml	Am-B µg/ml
Candida albicans (MTCC 227)	630	5	40	1
Candida tropicalis (ATCC 6258)	630	5	40	1
Candida krusei (ATCC 750)	630	5	40	2
Cryptococcus neoformans (ITCC 1672)	1250	10	40	2
Soprothrix shenkii (ITCC 2317)	1250	10	60	4
Fusarium oxysporum (ITCC 4998)	2500	20	160	-
Trichophyton mentegrophytes (ITCC 3572)	1250	5	60	1
Microsporon gypseum (ITCC 5277)	1250	5	60	4
Aspergillus flavus (ITCC 5290)	2500	40	160	2
Aspergillus fumigatus (ITCC 4880)	2500	40	160	2
Rhizomucor pussilus (ITCC W-14)	5000	40	320	16
Phialophora verrucosa (MCCL 32006)	5000	80	630	16
Curvularia lunata (ITCC 5248)	5000	80	320	16
Pseudaresheria.boydii (MCCL W-48)	5000	160	630	-

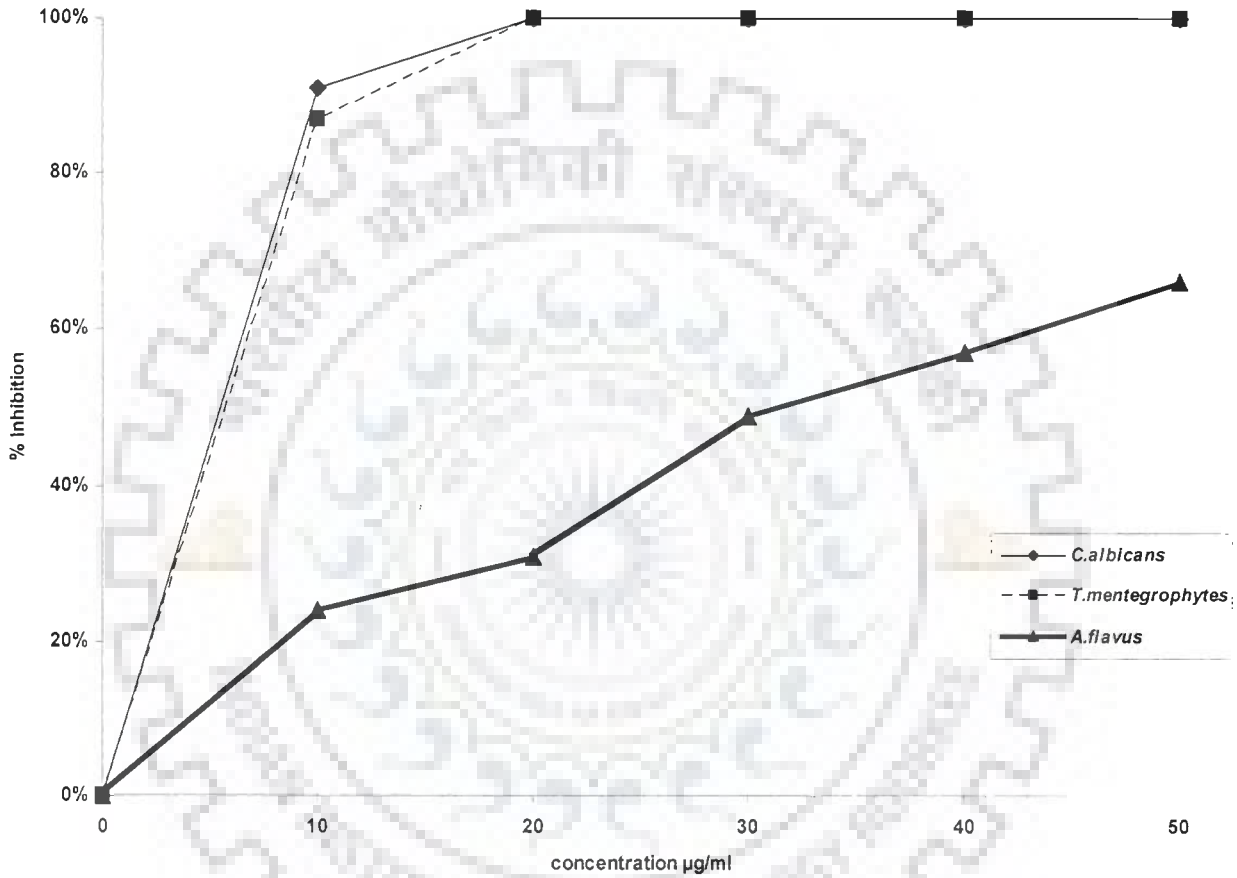


Figure 21. Determination of the IC_{50} value of the antifungal activity of *Isodiospyrin* towards *C. albicans*, *T. mentegrophytes* & *A. flavus*. IC_{50} was calculated to be 5.1, 5.4 & 31.5 µg/ml respectively

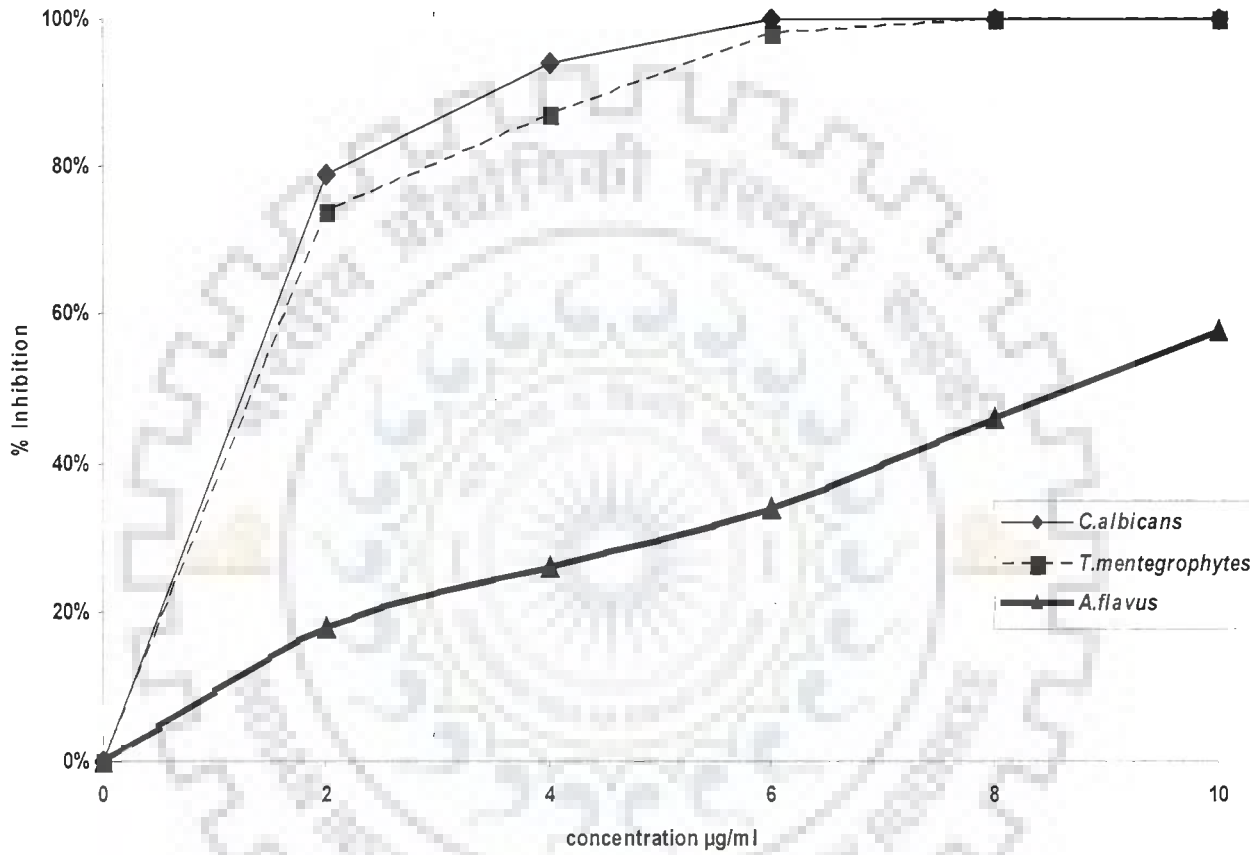


Figure 20. Determination of the IC_{50} value of the antifungal activity of *Plumbagin* towards *C. albicans*, *T. mentegrophytes* & *A. flavus*. IC_{50} was calculated to be 1.2, 1.4 & 8.8 µg/ml respectively

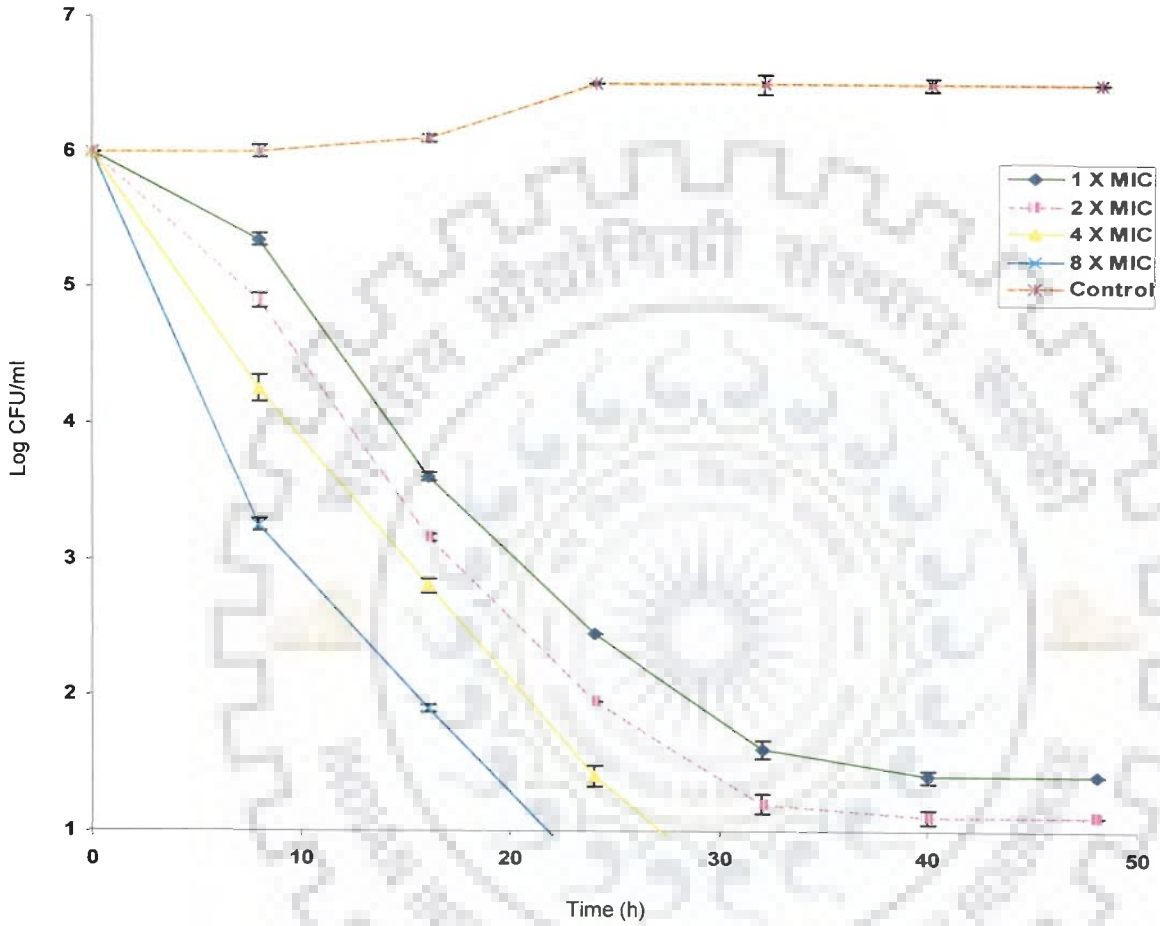


Figure 22. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MICi.e. 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml and 10 µg/ml *of Plumbagin* on *C. albicans.* Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

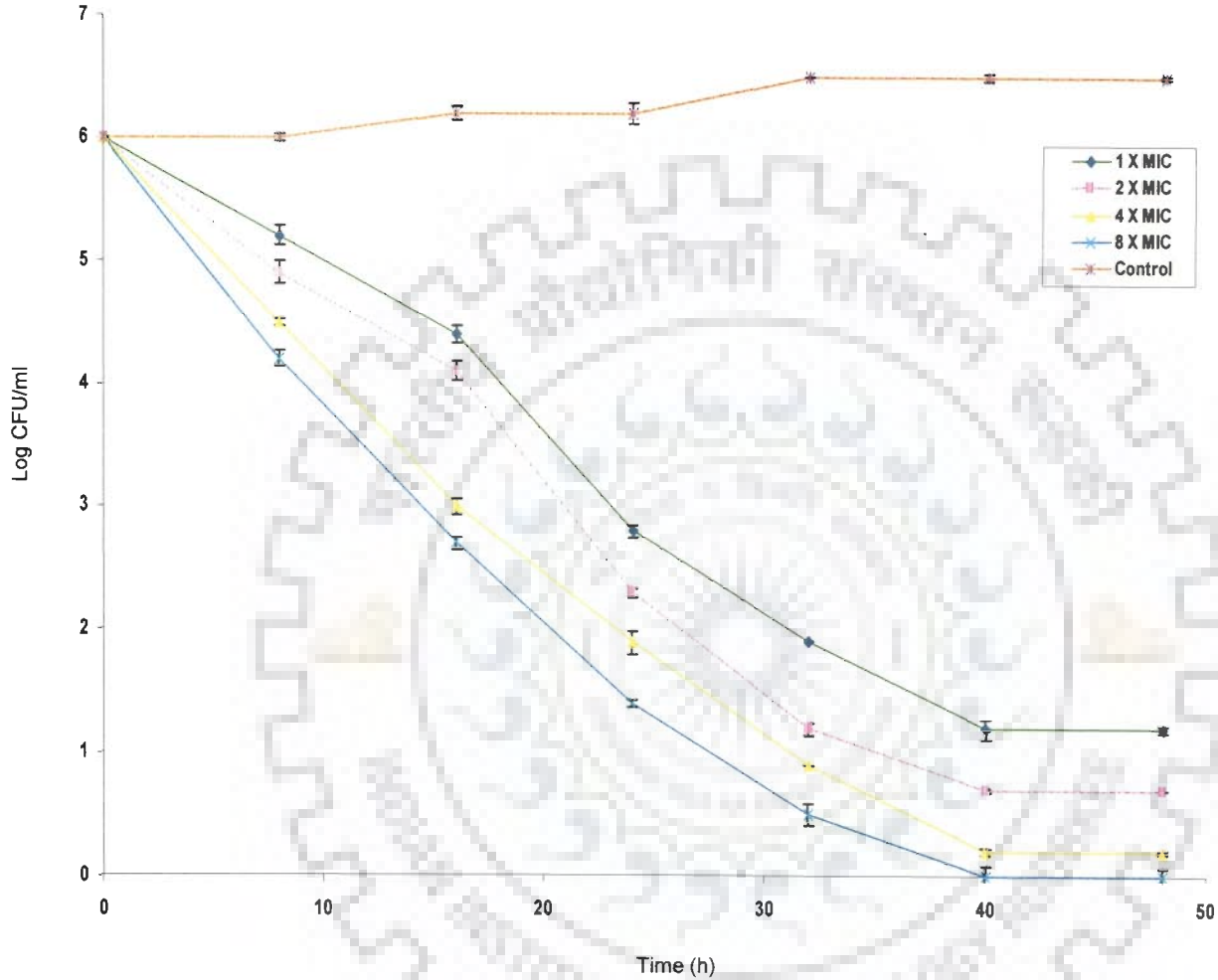


Figure 23. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MIC i.e. 2.5 µg/ml, 5 µg/ml, 10 µg/ml & 20 µg/ml *of Plumbagin* on *T. mentegrophytes.* Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

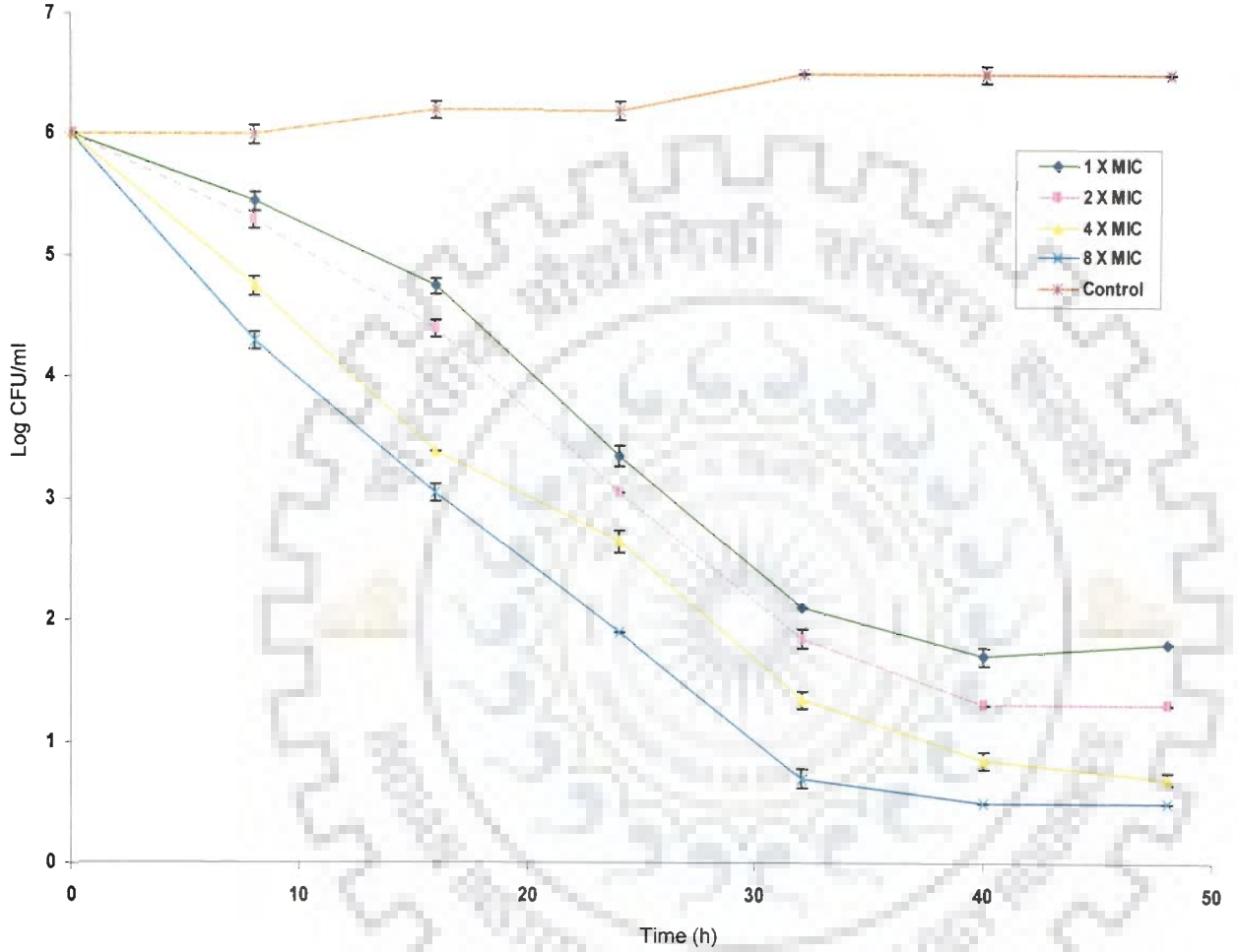


Figure 24. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MIC i.e. 10 µg/ml, 20 µg/ml, 40 µg/ml & 80 µg/ml *of Plumbagin* on *A. flavus*. Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

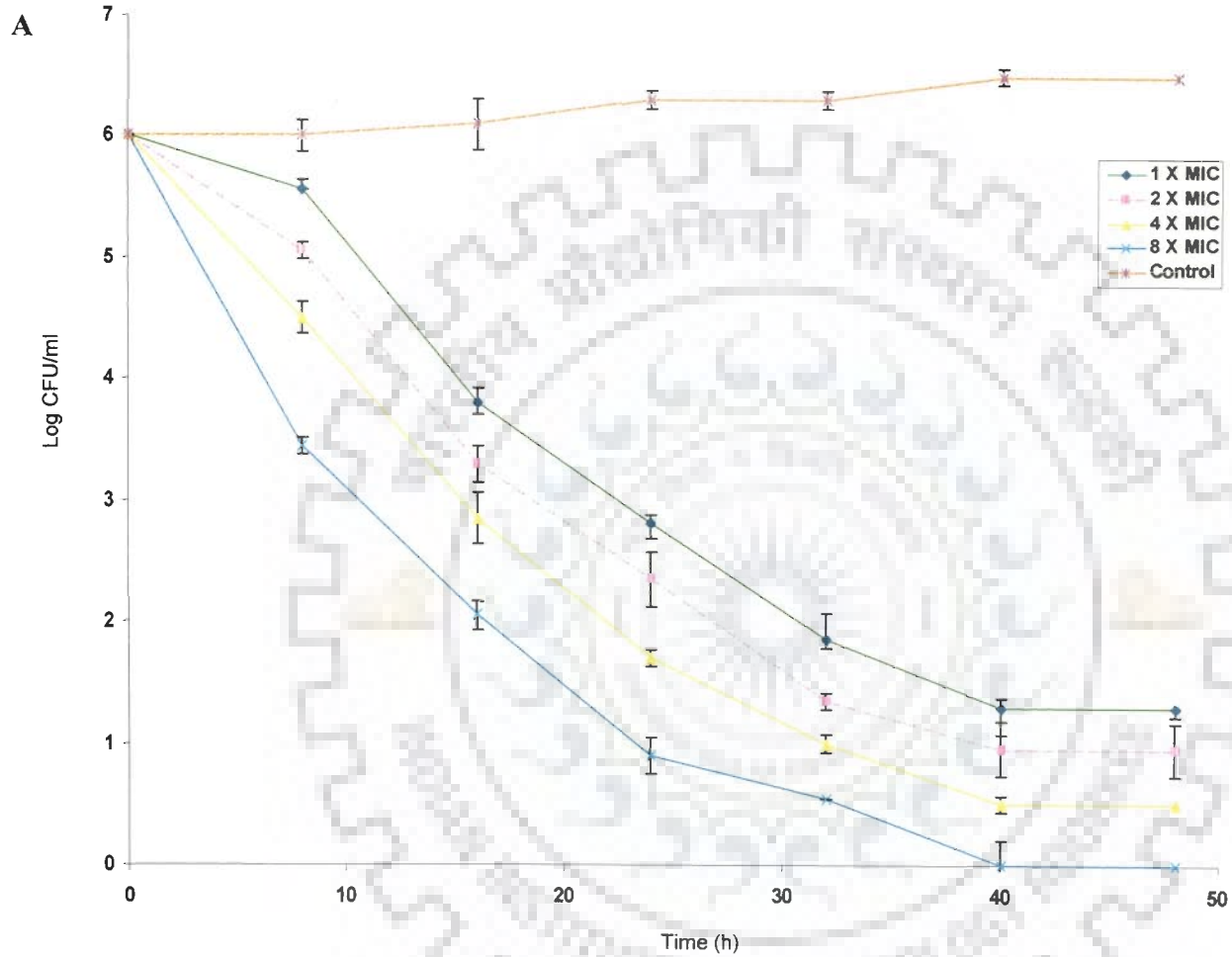


Figure 25. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MIC i.e. 5 µg/ml, 10 µg/ml, 20 µg/ml & 40 µg/ml *of Isodiospyrin* on *C. albicans*. Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

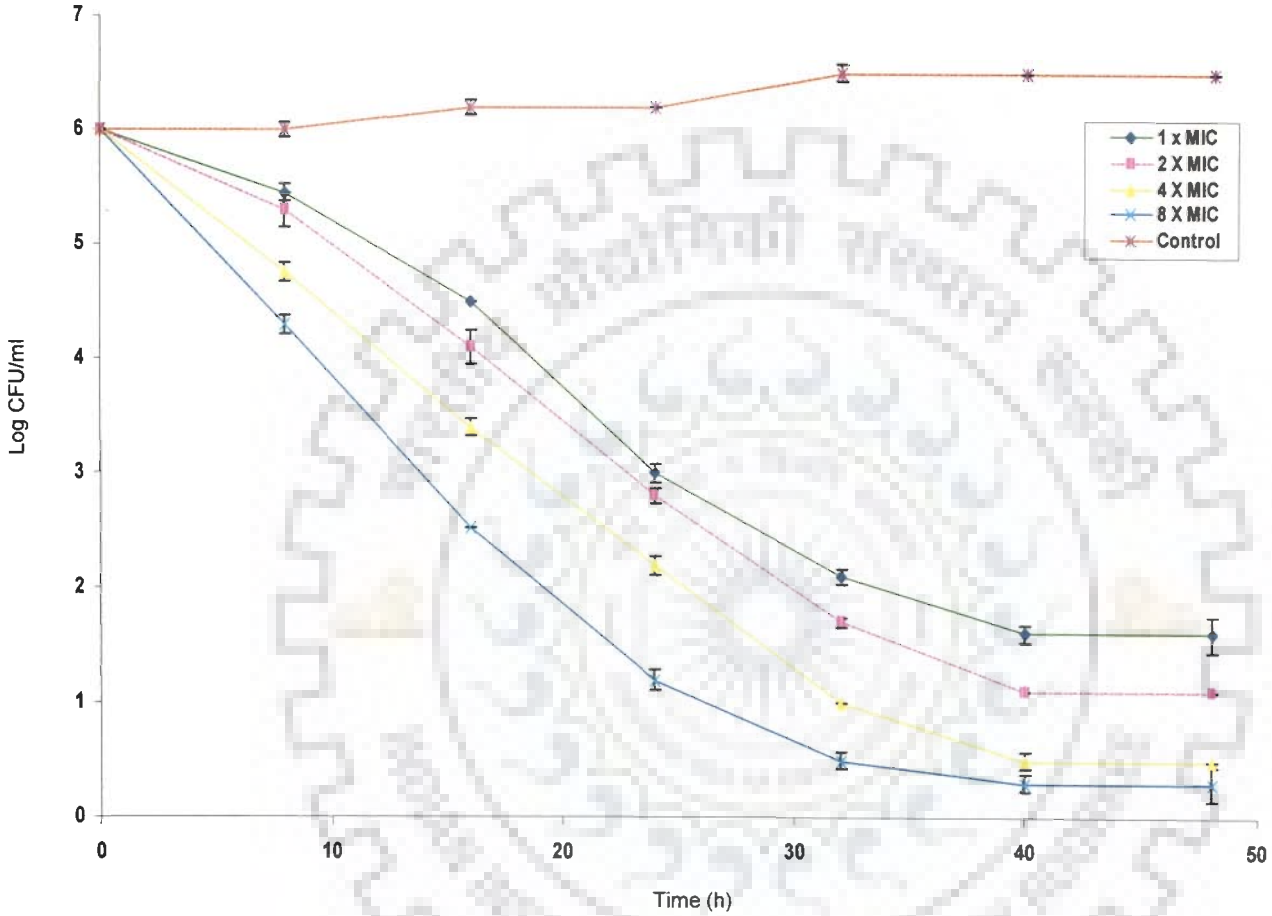


Figure 26. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MIC i.e. 5 µg/ml, 10 µg/ml, 20 µg/ml & 40 µg/ml *of Isodiospyrin* on *T. mentegrophytes*. Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

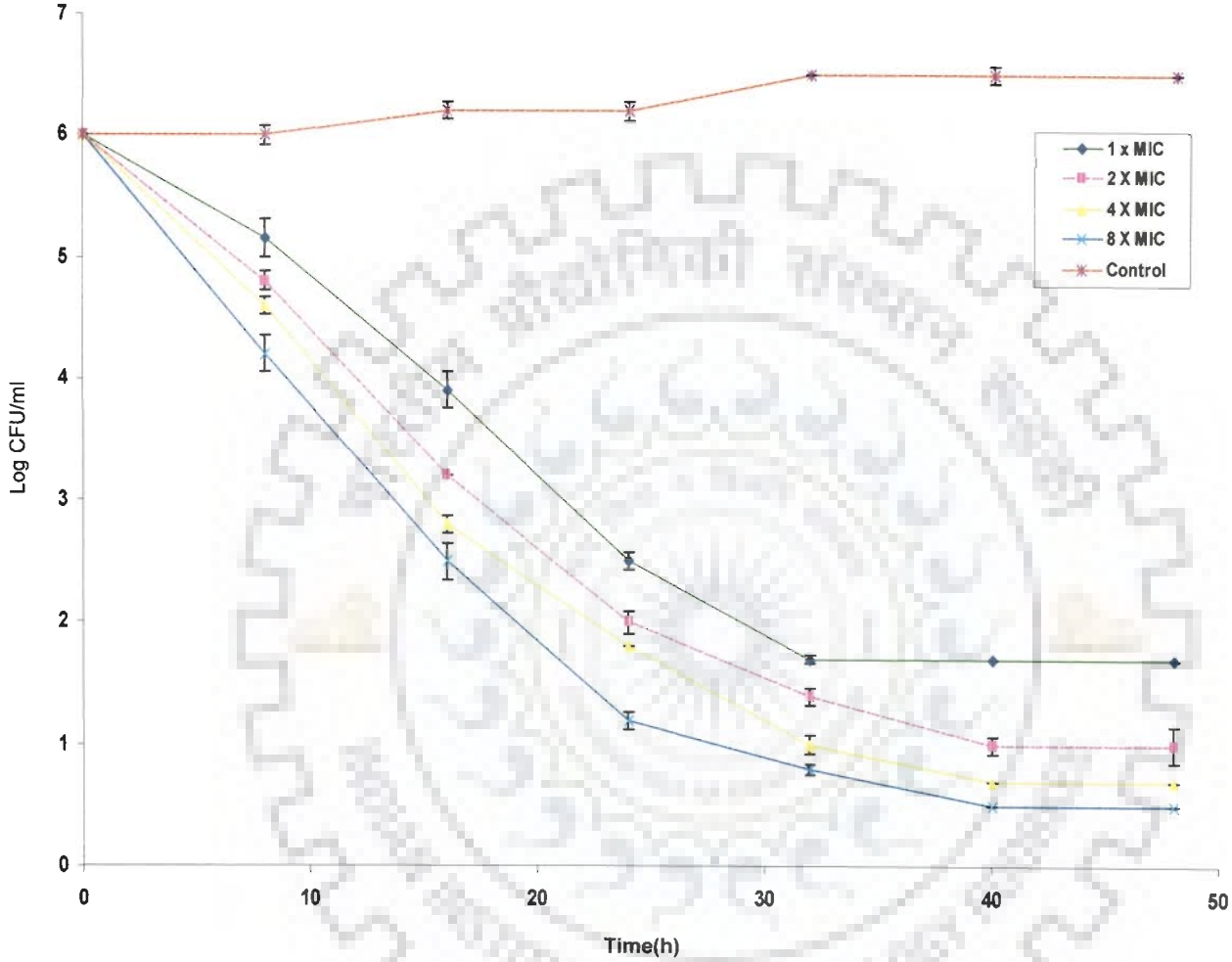


Figure 27. Time kill curve studies of fungi on exposure to MIC concentration 1, 2, 4 & 8 x MIC i.e. 40 µg/ml, 80 µg/ml,160 µg/ml & 320 µg/ml *of Isodiospyrin* on *A. flavus.* Control is fungi grown in absence of pure compound. Values are mean of triplicate ± SE

represented in Figure 22 and 27. The killing was found to be species and concentration dependent, showing rise in killing with rise in concentration. The study once again confirms the susceptibility trend obtained earlier in disc diffusion assay and MIC-MFC. On treatment with *Plumbagin*, *C. albicans* showed fastest rate of killing showing no viable count in less than 30 h at the (5 µg/ml). *T. mentegrophytes* exhibited log 0.2 cfu/ml at 10 µg/ml while in case of *A. flavus* log 0.5 cfu/ml was observed after 48 h at 40 µg/ml also. Similarly on treatment with *Isodiospyrin C. albicans* showed complete inhibition after 40 h at 40 µg/ml, log 0.2 cfu/ml was observed in case of *Trichophyton* at 40 µg/ml while log 0.5 cfu/ml were observed for *Aspergillus* at 320 µg/ml. The fungicidal action of *Plumbagin* was observed at 4 x MIC while 8 x MIC was found more appropriate in case of *Isodiospyrin*. Therefore the concentration dependent time killing curve study was helpful to choose the optimum concentration and the time to observe the morphological changes occurred in the fungal cells after treatment with *Plumbagin* and *Isodiospyrin* using SEM and TEM.

4.11 EFFECT OF ANTIFUNGAL COMPOUNDS ON ULTRASTRUCTURE OF FUNGI :

4.11. 1. SEM

The effect of both *Plumbagin* and *Isodiospyrin* on ultrastructure of fungi are shown in Fig 29 and 28. Significant changes in cell morphology was observed in case of all the three fungi. The hyphae of the treated fungi were severly damaged at the tested concentration of the pure compounds. Cells of *C. albicans* appeared shrunken and highly distorted. Samples of *Trichophyton* and *Aspergillus* treated with pure compounds showed distorted mycelium, squashed and malformed hyphae altering their normal morphology leading to their collapse and reduced diameter as compared to control. On the other hand the control hyphae showed lengthened, regular, homogeneous hyphae of constant diameter with smooth external surface and rounded apex. In case of *Aspergillus* no spores are attached to the vesicles which appeared severly collapsed and shrunken.

4.11. 2. TEM

The effect of both *Plumbagin* and *Isodiospyrin* on ultrastructure of fungi were shown in Fig 31 and 30. The ultrastructural examination of control hyphae did not show any evident alterations i.e the organules are integral and endomembrane system is well developed. In the treated hyphae the most common modification observed was increase in the number and size

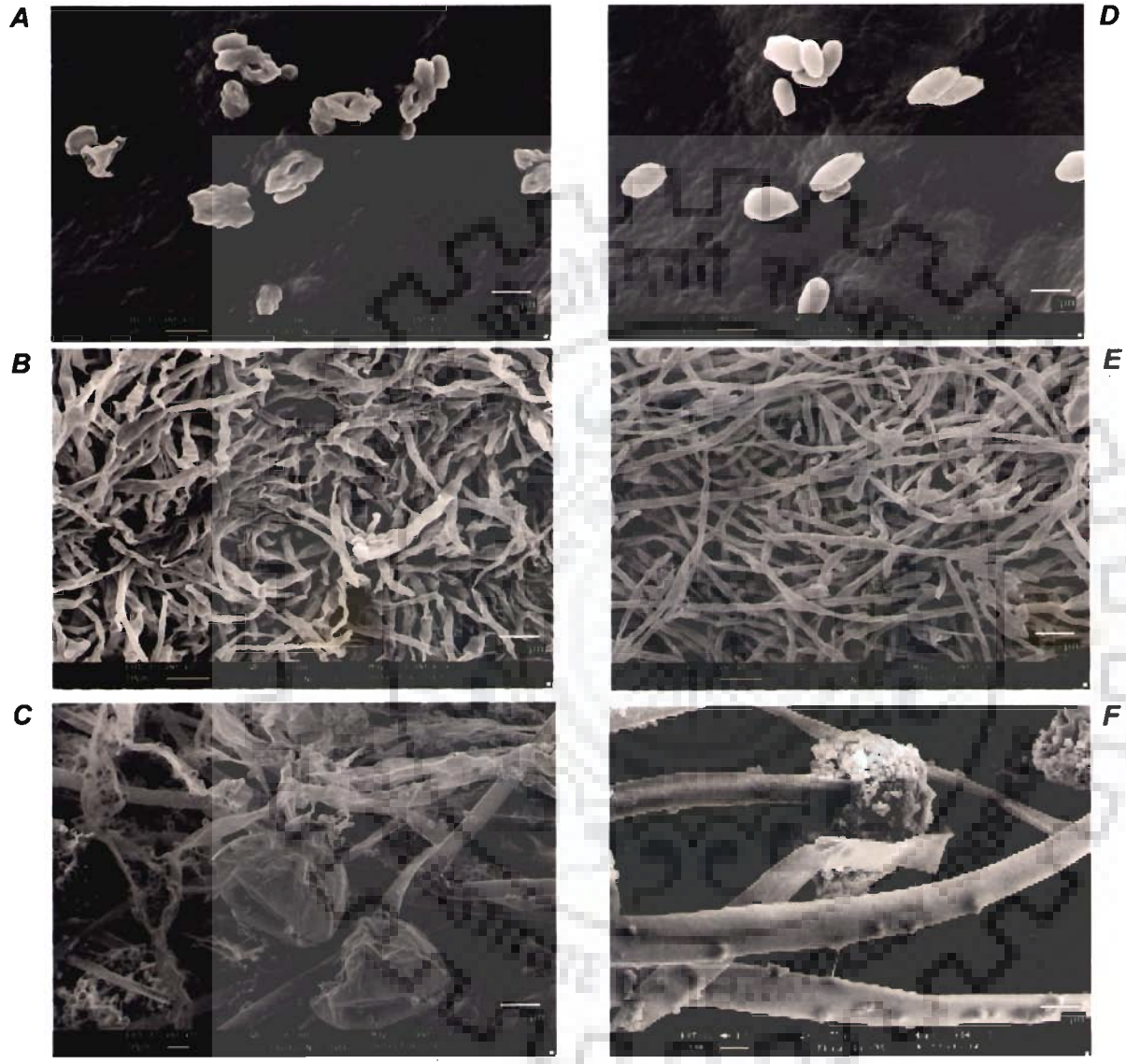


Figure 28. Scanning electron micrograph showing the effect of *Plumbagin* on *A) C.albicans* ; B) *T.mentegrophytes* & C) *A..flavus*. D, E & F are control. Magnification : 2000 X, Bar 2µm.

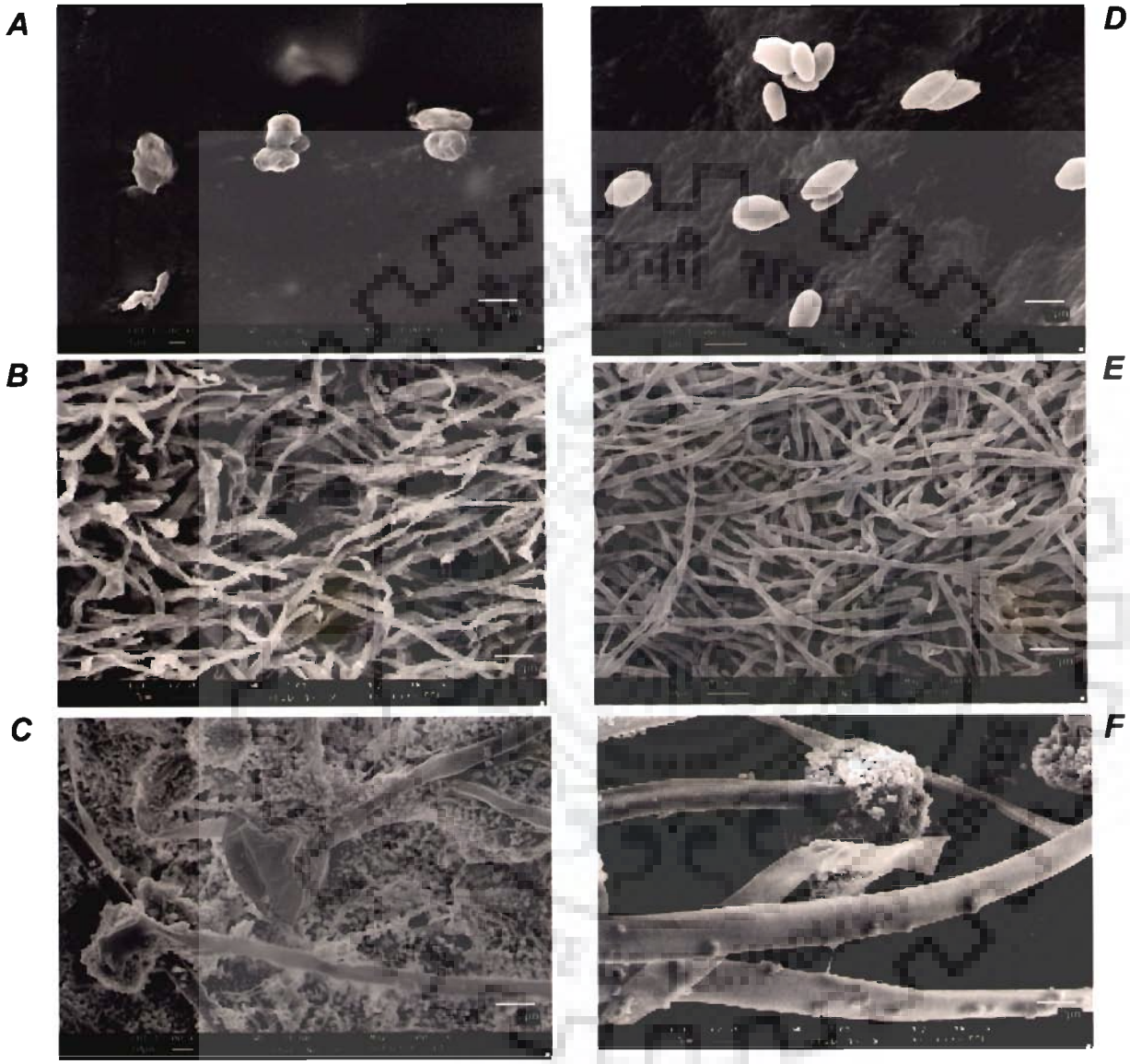


Figure 29. Scanning electron micrograph showing the effect of *Isodiospyrin* on *A) C. albicans* ; B) *T. mentegrophytes* & C) *A. flavus* . D, E & F are control. Magnification: 2000 X, Bar 2µm.

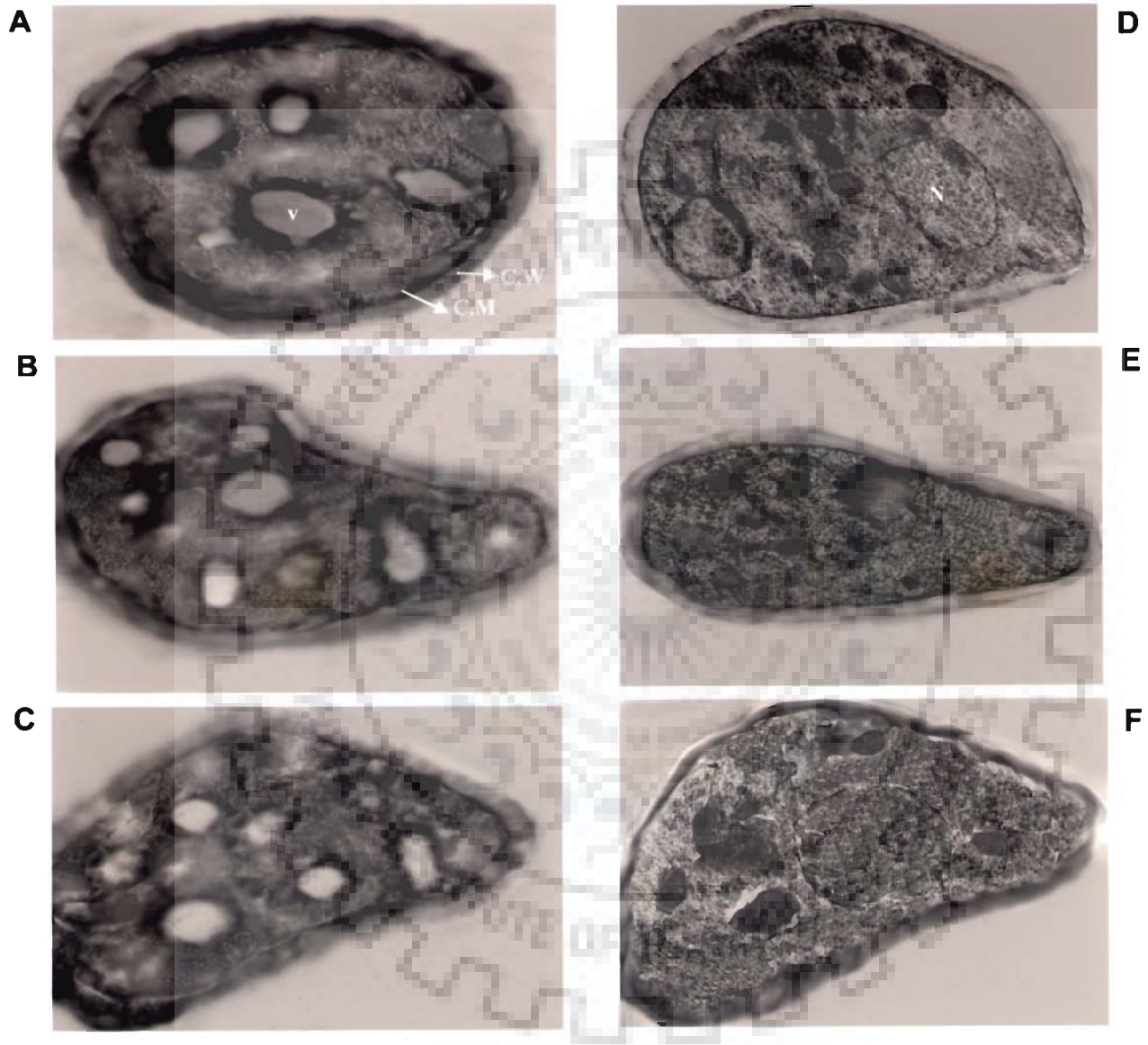


Figure 30. Transmission electron micrograph showing the effect of *Plumbagin* on A) *C. albicans*, B) *T. mentegrophytes* & C) *A. flavus*. D, E & F are control cells. Bar 1µm.

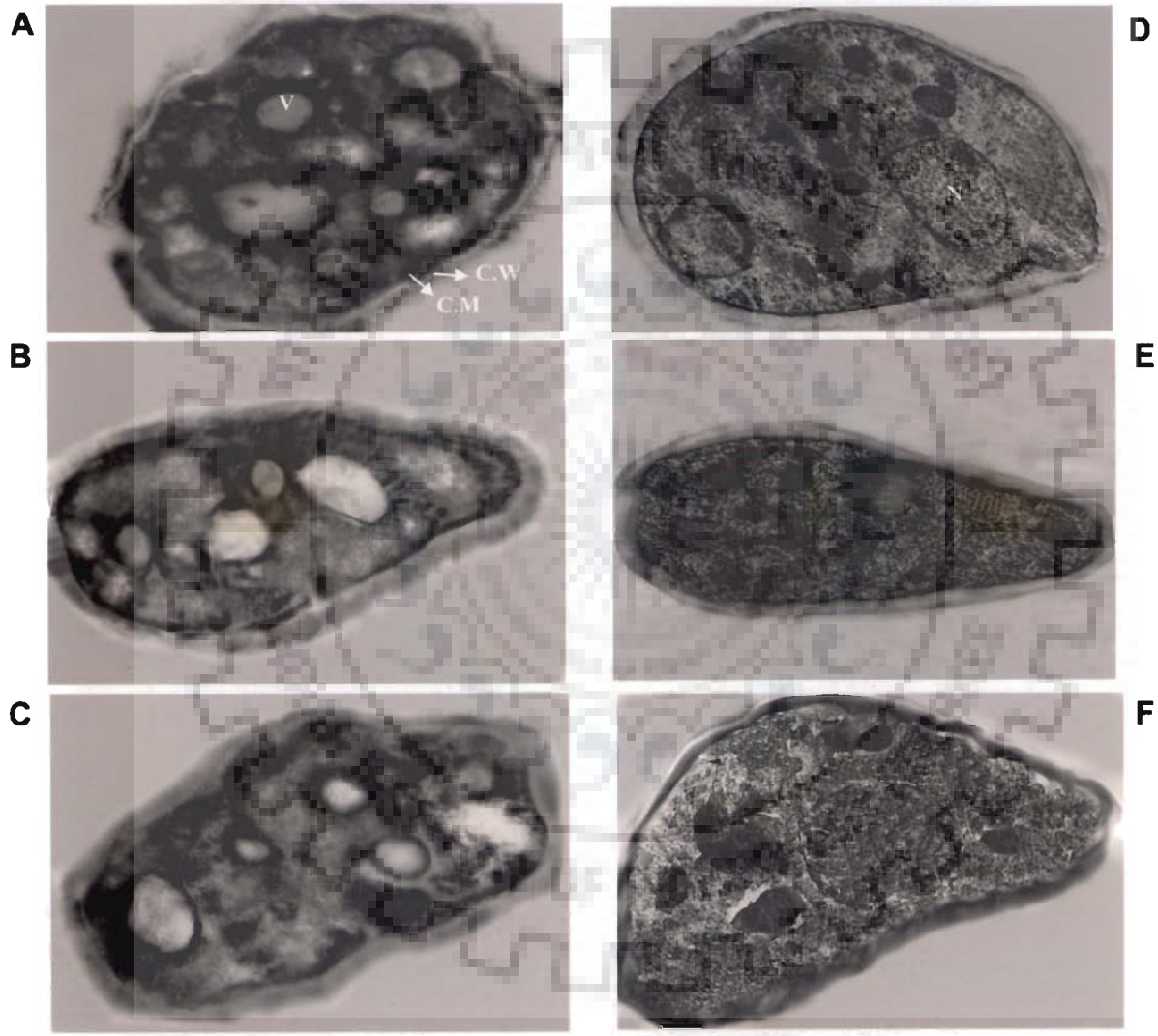


Figure 31.Transmission electron micrograph showing the effect of *Isodiospyrin* on A) *C. albicans*, B) *T. mentegrophytes* & C) *A. flavus* D, E & F are control cells. Bar 1µm

of vacuoles in the cells. Cell wall also appeared distorted and thickened with fragmentations or gaps at some places and there is deleterious effect on cell membrane also which looks irregular at several places. Interior of the cells showed complete cell necrosis accompanied with cytoplasmic deterioration giving rise to electron dense and electron thin areas.

4.12 RBC HEMOLYTIC ACTIVITY :

The results of *in vitro* RBC hemolysis assay is given in Fig 32. The cytotoxicity of both the compounds *Plumbagin* and *Isodiospyrin* were found to be much less in comparision to amhotericin-B which induces hemolysis at relatively lower concentrations (MLC 8 µg/ml) as reported in previous studies. *Plumbagin* induce about 7 % hemolysis at 20 µg/ml and 35 % hemolysis at 100 µg/ml. In case of *Isodiospyrin* no hemolysis was observed up to 40 µg/ml and at 100 µg there is only about 8 % hemolysis.

4.13 *IN VITRO* CYTOTOXICITY ASSAY :

Mouse peritoneal macrophages and splenocytes cultures were incubated with increasing concentration (10-100 µg/ml) of both the compounds for 24 h. The result of *in vitro* cytotoxicity assay are given in Fig 33 and 34. It is clear from the results that both the compounds were found to be less toxic as a little cytotoxicity was observed only at very high concentration. No significant cytotoxicity was observed up to 40 µg/ml of *Isodiospyrin* and 20 µg/ml of *Plumbagin* in both the cell cultures used. In case of splenocytes cell culture, 97 % cells were viable at 20 µg/ml of *Plumbagin* but at 40 µg/ml it showed 89 % viability and less then 70 % viability at 100 µg/ml while for *Isodiospyrin* viability is 96 % up to 60 µg/ml and 89 % at 100 µg/ml. Macrophage cell culture showed 99 % viability at 20 µg/ml, 93 % viability at 40 µg/ml and 68 % at 100 µg/ml in case of *Plumbagin* while 95 % viability was observed at 40 µg/ml and at 100 µg/ml 86 % cells were viable for *Isodiospyrin*.

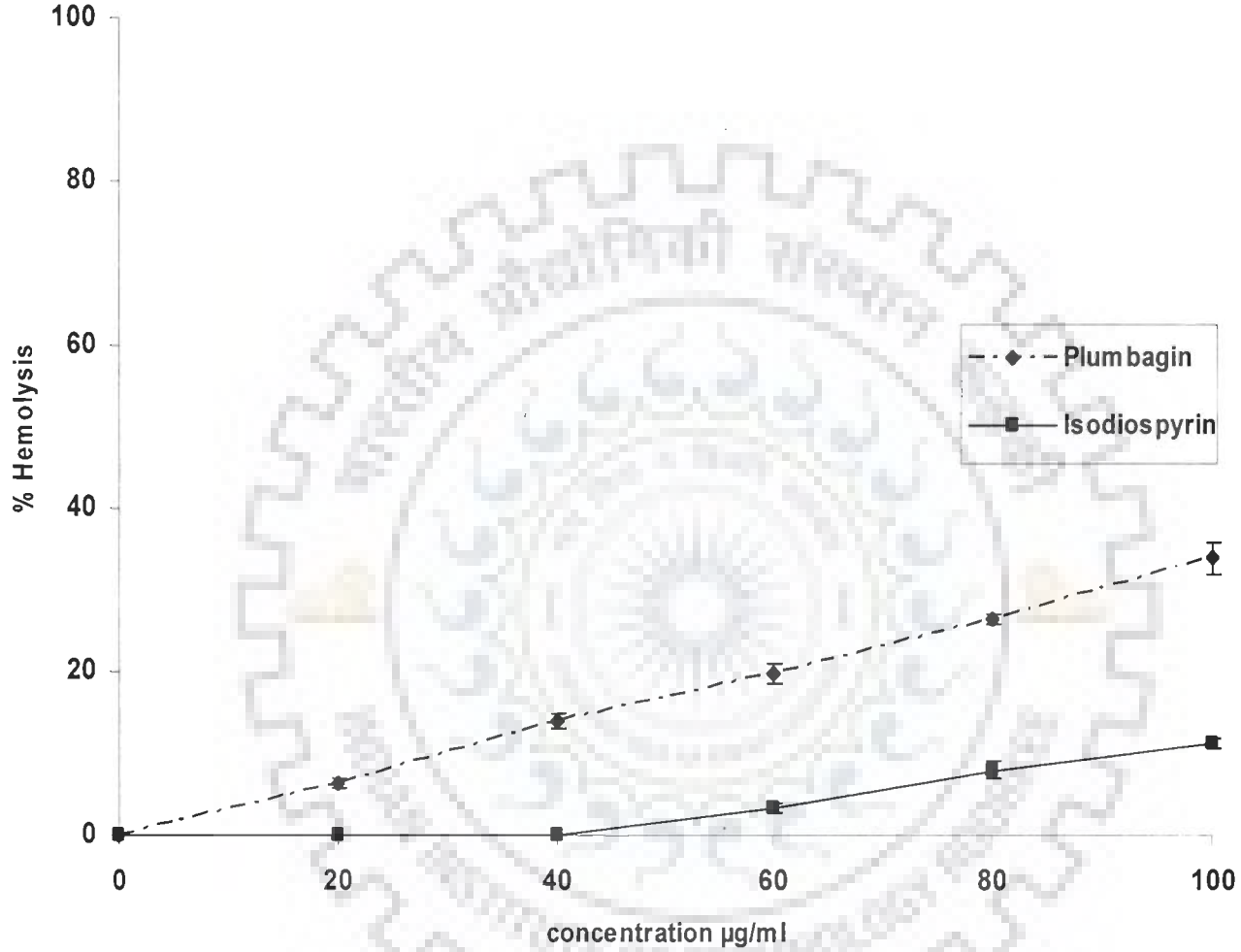


Figure 32. Dose-dependent hemolysis of RBCs by *Plumbagin* & *Isodiospyrin*(25-100µg/ml) *in vitro*. The extent of RBC lysis was determined by spectrophotometer at 540 nm. Values are mean of triplicate ± SE

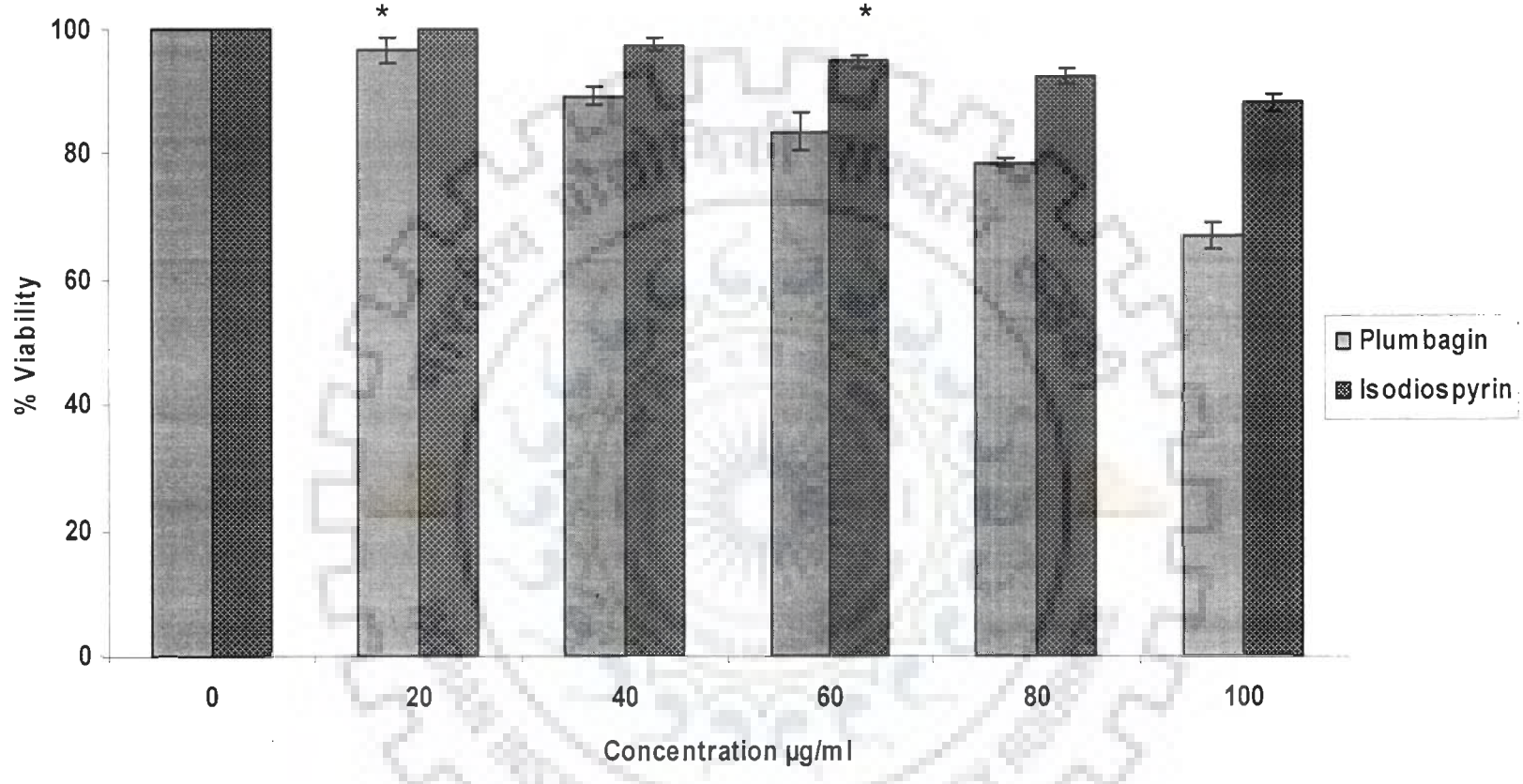


Figure 33. In vitro cytotoxicity assay. Mouse splenocytes were incubated with increasing concentration (10µg/ml-100µg/ml) of *Plumbagin & Isodiospyrin* for 24 h. Viable cells were counted by the tryptan blue exclusion method. Values are mean of triplicate ± SE comparing to control; * $p < 0.05$

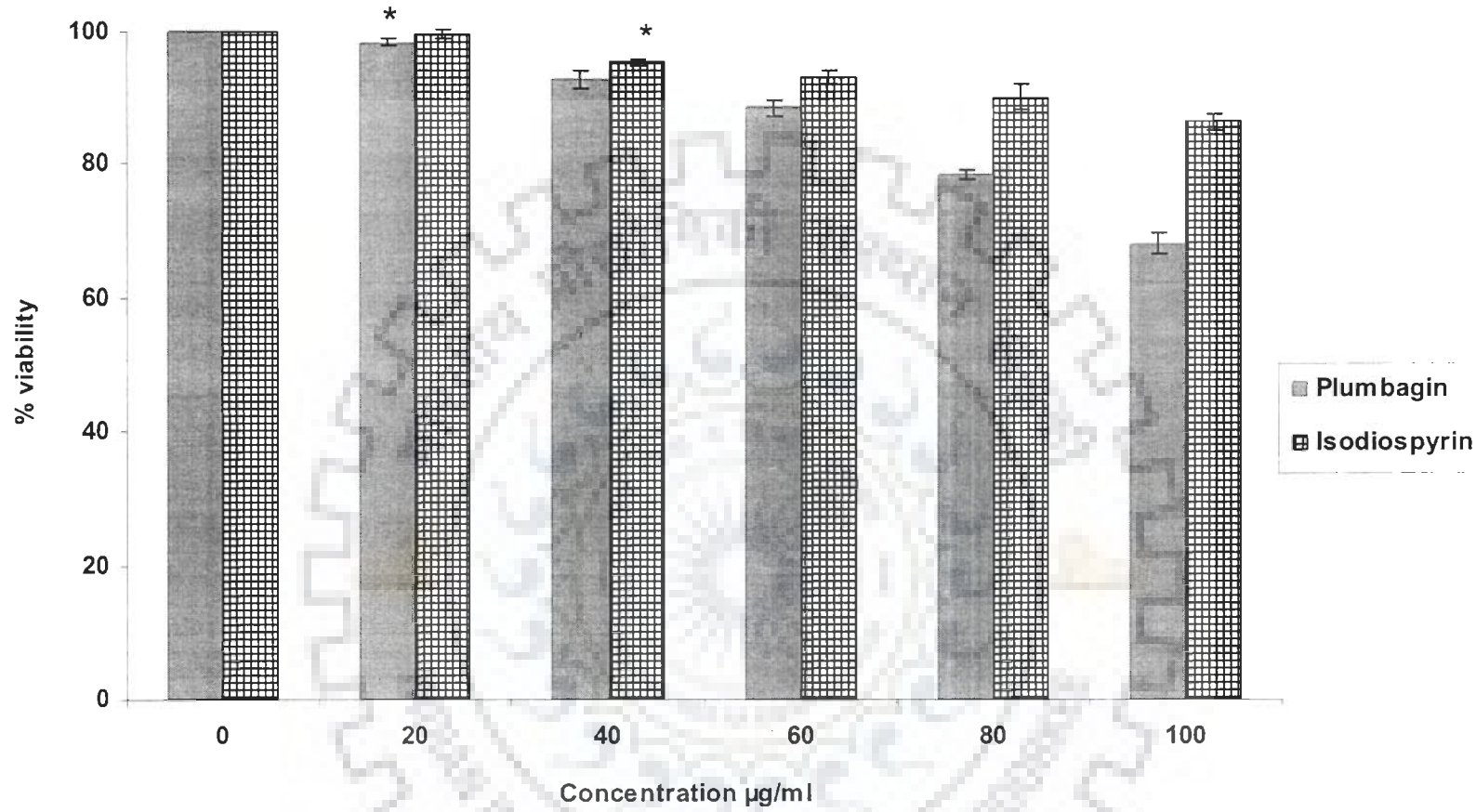


Figure 34. In vitro cytotoxicity assay. Mouse peritoneal macrophages were incubated with increasing concentration (10µg/ml-100µg/ml) of *Plumbagin* & *Isodiospyrin* for 24 h. Viable cells were counted by the tryptan blue exclusion method. Values are mean of triplicate ± SE comparing to control ; *p< 0.05

DISCUSSION

Invasive fungal infections have recently emerged as a severe and complicated problem in immunocompromised hosts as they are more frequent now than during the first half of the century (Ascioglu *et al.*, 2002; Fridkin and Jarvis, 1996; Hospenthal and Rinaldi, 2003; Wanke *et al.*, 2000). Chemotherapy has been one of the most common strategy to treat fungal infections. A number of synthetic antifungal drugs are being used frequently. However their toxicity and the emergence of drug resistence against these drugs emphasize the need of newer, safer and effective antifungal agents (Graybill, 2000; Mohsen and Hughes, 1998; Nosanchuk, 2006, Ghannoum and Rice, 1999; Kontoyiannis and Lewis, 2002; Moore, 2000; Patterson, 2003; Rex *et al.*, 1995; Sanglard, 2002). One of the trends in antifungal drug discovery program is an ample screening of natural products from different sources (Mares and Rai, 2003). Plants due to their diversity, vast array of chemical constituents and traditional use in various kind of ailments in general and skin diseases in particular could be the most viable source of new potent antifungal agents. Also the phenomenon of drug resistance is less common in case of medicines derived from plants than from chemotherapeuticals and antimycotic antibiotics (Ahmad *et al.*, 2006). Since the success rates of the ethnobotanical approaches are substantially higher than those of random screening, in this study an endevour has been made to screen a number of medicinal plants for antifungal activity. Plants are selected based on their known background of ethnobotanical uses in treatment of various infections and skin diseases.

Methanolic extract of root bark of *Diospyros kaki* was found to be most promising amongst aqueous and methanolic extract of various plants in this study. *D. kaki* is chosen for the present study because *Diospyros spp.* have been reported in the literature to exhibit intresting biological and pharmacological properties i.e. treatment of whooping cough, leprosy, snakebite, scabies, skin eruptions, dysentery, eye infection, menstrual troubles etc (Malvadhani *et al.*, 1998). Although there are reports of antimicrobial activity in several species of *Diospyros* genus including *D. crassiflora*, *D. anisandra*, *D. pregrina*, *D. maritima*, *D. novoguinensis*, *D. tricolor, D. nigra* and *D. rubra* (Adeniyi *et al.*, 2000; Dzoyen *et al.*, 2007; Dewanjee *et al.*, 2007; Khan and Timi, 1999; Dinda *et al.*, 2006) this is the first report of antifungal activity from the root bark of *D. kaki*. Moreover in this study a very broad range of potentially human pathogenic fungi have been tested. Therefore this study further confirms the traditional use of *D. kaki* in the treatment of various infections and skin diseases.

Traditional medicinal plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Many of these substances serve as plant defense mechanisms against invasion by micro-organisms, insects and herbivores (Samy *et al.*, 2007; Dixon, 2001). Based on the above fact the focus of the present study was to locate some important group of secondary compounds responsible for bioactivities in the *D. kaki.* Although a number of organic solvents have been used for extraction of antimicrobial compounds (Eloff, 1998), chloroform was chosen as extraction solvent in this study because it is clear from the results that the major bioactive compounds of *D. kaki* were extracted better in this solvent.

In the present study isolation and purification of the antifungal compounds was done from the chloroform extract of *D. kaki* root-bark using silica gel column employing step gradient of benzene-chloroform. Bioassay guided purification of the crude extract using silica gel column chromatography led to the isolation of two active principles A and B.

Compound A and B were characterized by means of various analytical instruments. EI-MS spectra of compound A shows peaks at 131 and 103 which are characteristic of napthoquinones. Peaks at 120, 92 and 63 indicated the presence of hydroxyl groups in benzene rings. The molecular ion base [M^+] peak is at m/e 188.18 (molecular weight 188.18 g/mol) and molecular formula calculated as $C_{11}H_8O_3$. ^1H NMR revealed the presence of 1 quinoid methyl, 4 aromatic protons and 1 hydrogen bonded hydroxyls. 11 lines are seen in the ^{13}C spectrum of A. δ 16.9 indicates carbon in CH_3 group and δ 184.8(C1), 191.0(C-4) shows carbon-oxygen double bonds and δ 114.9(C-10), 119.3(C-8), 124.23(C-6), 132.0(C-9), 135.66(C-3), 136.98(C-7),149.8(C-2), 161.2(C-5) shows carbon C=C in the aromatic region. Therefore on comaprision of the spectral data with the literature A was identified as napthaquinones, *Plumbagin* (5-hydroxyl-2-methyl-1,4 napthoquinone (Hsieh *et al.*, 2005; Shin *et al.*, 2007). *Plumbagin* (methyl juglone) the yellow naphthoquinone pigment, occurs in a colourless combined form and is liberated from root tissue by acid treatment. It had earlier been reported in few species of *Diospyros* (*D. canaliculata*, *D. ebenum*, *D. elliptifolia*, *D. gracilipses*, *D. hebecarpa, D. maritima*, *D. siamang*, *D. siderophylla*, *D. walkeri*, *D. wallichi*) (Tezuka, 1972; Mallavadhani, 1998). The yield of *Plumbagin* obtained in present study (0.04 %) is several folds higher then yield of other *spp.* of *Diospyros* i.e. (0.01 %) for *D. crassiflora* (Dzoyen *et al.*, 2007) and *D. elliptifolia* (Fallas and Thomson *et al.*, 1968) and is much

higher than (0.0000002 % and 0.017 %) for *D. kaki* (Tezuka *et al.*, 1972; Lee and Lee, 2008), (0.002 %) for *D. assimilis* (Fallas and Thomson *et al.*, 1968) and (0.002 %) for *D. sylvatica* (Ganapaty *et al.*, 2004) while in case of *D. canaliculata* it is higher (0.1 %) (Zhong *et al.*,1984).

Plumbagin appears to be characteristic of plants of the family *Plumbagineae* (Harborne 1966) and is known to have antitumoral (Lin *et al.*, 2003), leshmanicidal (Sepulveda and Cassels, 1996), trypanocidal, (Paiva *et al.*, 1996), antimalarial (Suraveratum *et al.*, 2000), acaricidal (Lee and Lee, 2008), antifilarial (Mathew *et al.*, 2002) and antimicrobial properties (Poul *et al.*, 1999. It has antifungal activity against *Rhizopus nigricans*, *Epidermatophyton floccosum*, *Microsporon nanum*, *Penicillium notatum*, *Penicillium funiculosum*, *Penicillium canadense*, *Candida albicans*, *Aspergillus niger* and *Colletotrichum gloesporioides*, *Alternaria alternata*, *Aspergillus niger*, *Bipolaris oryzae*, *Fusarium oxysporum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Rhizopus stolonifer* var. stolonifer and *Sclerotinia sclerotiorum* (Dzoyen *et al.*, 2007; Shin *et al.*, 2007, Paiva *et al.*, 2003; Krishnaswamy and Purushottam, 1980). In the present study the MIC values of *Plumbagin* against *Fusarium oxysporum* (5 µg/ml) and *Aspergillus spp.* (10 µg/ml) were higher then reported by Shin *et al.*, 2007 i.e. MIC of *Fusarium oxysporum* (21.1 µg/ml) and *Aspergillus spp.* (13.5 µg/ml). Also the MIC value of *Plumbagin* 2.5 µg/ml was much higher than 10 µg/ml in case of *Microsporon* as reported by Krishnaswamy and Purushottam, 1980. In the study by Paiva *et al.*, 2003 and Dzoyen *et al.*, 2007, the MIC of *C. albicans*, *C. krusei*, *C. neoformans* and *A. flavus* was found as 0.78, 1.56, 1.56 and 0.78 µg/ml respectively. These values nearly coincide with our results i.e 1.25, 1.25, 2.5 and 10 µg/ml except relatively low MIC for *A. flavus* in the above reported study.

EI-MS of active compound B shows a complex pattern of low intensity peaks. Initial loss of methyl radical is dominant followed by CO. It shows molecular ion base peak at m/e [M^+] at 374.34 (molecular weight is 374.34 g/mol) and its molecular formula calculated as $C_{22}H_{14}O_6$. ^{1}HNMR of B showed the presence of one quinoid methyl, 2 quinoid protons, 3 aromatic protons and 2 hydrogen bonded hydroxyls. 22 lines are seen in the $^{13}\{^1H\}$NMR spectrum of B and the molecular formula supports the unsymmetrical structure. The presence of single intense lines at 125.71 (C-6') and 121.37 (C-8) confirms the presence of 6-8' linkage between the two monomeric moieties. Hence B was identified as another napthoquinone *Isodiospyrin* (5-hydroxy-6-(1-hydroxy-6-methyl-5,8-dioxo-naphthalen-2-yl)-2-methyl-naphthalene-1,4-dione) on comaprision of the spectral data with the literature

(Alves *et al.*, 1980; Fallas and Thomsan, 1968; Akella *et al.*, 1986; Yoshihara *et al.*,1971) *Isodiospyrin* is characteristic of family *Ebenaecae* and is found in many other species of *Diospyros* including *D. abyssinica*, *D. alboflavescena*, *D. bipindensis*, *D. chloroxylon*, *D. dendo*, *D. ebenaster*, *D. ferrea*, *D. gilleli*, *D. gracilescens*, *D. hoyleana*, *D. kakisylvestris*, *D. lotus*, *D. maaingayi*, *D. mespiliformis*, *D. montana*, *D. morrisiana*, *D. nicaraguensis*, *D. texana*, *D. usambarensis*, *D. verrucosa*, *D. virginiana*, *D. whyteana* and *D. zombensis* (Tezuka *et al.*, 1972; Mallavadhani *et al.*, 1998). In the present study the yield of *Isodiospyrin* obtained (0.35 %) is again much higher than reported by Tezuka *et al.*,1972 (0.003 % and 0.0004 %) and also is higher than the earlier reported in other *spp.* i.e. (0.002 %) for *D. piscatorial*, (0.008 %) (Adeniyi *et al.*, 2000), *D. mespiliformis*, (0.002 %) (Fallas and Thomsan, 1968) *D. sylvatica*, (0.0024 %) (Ganapaty *et al.*, 2004), *D. morrisiana*, (0.004 %) (Yan *et al.*, 1989) *D. abyssinica*, (0.021 %) (Zhong *et al.*, 1984), *D. virginiana*, (0.09 %) (Fallas and Thomsan, 1968) *D. zombensis* (Gafner and Rodriguez, 1987). Only one *spp. D. lotus* showed higher yield (0.41 %) than that obtained in the present study (Yoshihira *et al.*, 1971). Therefore present study clearly suggest *D. kaki* to be the better source of *Isodiospyrin*. The major biological activities of *Isodiospyrin* reported in the literature are tumor inhibitory (Wube *et al.*, 2005), topoisomerase inhibitor (Ting *et al.*, 2003), anti-inflammatory (Kuke *et al.*, 1998), antimalarial (Kapadia *et al.*, 2001), antifungal (Marston *et al.*,1984), antimycobacterial (Kooy, 2005), molluscicidal (Gafner and Rodriguiez, 1998), termicidal (Carter *et al.*, 1978) and antibacterial (Adeniyi *et al.*, 2000) but not much is known about its antifungal property. Marston *et al.*, 1984, reported antifungal activity of *Isodiospyrin* against *Cladosporium cucumerinum*.

The susceptibility of different groups of fungi is varied towards *Plumbagin* and *Isodiospyrin*. The activity is more pronounced on yeast and dermatophytes followed by the hyaline and dematacеious fungi i.e. *Rhizomucor, Phialophora, Curvularia* and *Pseudaresheria* being least susceptible. The MIC values of *Plumbagin* and *Isodiospyrin* towards *Candida spp.* was significantly low (1.25 µg/ml and 5 µg/ml respectively). So these results are relevant since *C. albicans* is the leading primary agent causing superficial and often disseminated infection in immunocompromised patients (Powderly *et al.*, 1999; Del, 2009; Eggimann, 2003; Ruhnke, 2006) while in some patients (neutropenic patients) non albicans *spp.* are detected more frequently (Ruhnke, 2006). The MIC values of both the compounds were also appreciably low for *C. neoformans* and dermatophytes, *T. mentegrophytes* and *M. gypseum*. These results hold significance as Cryptococcus is the

cause of most common life threatening meningitis in HIV positive patients (Michael *et al.*, 1999) and infection caused by dermatophytes affects 2-13 % of population worldwide and upto 30 % of groups at high risk such as elderly and the people with diabetes (Levy, 1997; Gupta *et al.*, 1998). *Plumbagin* also exhibited substantial activity against *Aspergillus spp.* (10 µg/ml) while *Isodiospyrin* showed a little higher MIC value (40 µg/ml). As majority (approximately 80 %) of invasive *Aspergillus* infections is caused by *A. fumigatus* and (approximately 15-20 %) by *A. flavus* (Krishnan *et al.*, 2009; Nivoyx, 2008) the above mentioned compounds could be of immense value against these fungi. The MIC values of *Plumbagin* was found to be excellent and quite comparable to amphotericin-B in some cases while the MIC value of *Isodiospyrin* is higher than that of *Plumbagin*. MFC values of both the compounds were one to three dilutions higher then MIC in most of the cases. Spectrum of activity of both the compounds and their MIC values confirms their broad spectrum and potency. Moreover these compounds were found to be active against the known am-B resistant fungi i.e *F. oxysporum* and *P. boydii.* Although their MIC values are high as compared to the standard but considering the high toxicity and rise in resistance of some fungal species to amhotericin-B (Meunier, 1994, Mohsen and Hughes, 1998, Pauw, 2000; Groll and Walsh, 2002) these compounds could be considered worthy of further investigation as potential leads for the development of antifungal agents.

Based on the MIC for each fungi time killing curves were constructed in order to evaluate the effect of different concentration of *Plumbagin* and *Isodiospyrin*. Colony count assays were performed to determine the time required for the pure compounds to kill various fungi. Both the compounds exhibit inhibitory and fungicidal activity, being inhibitory at lower concentration and fungicidal at higher concentration. The rate and extent of fungicidal activity improved as the concentration of drug in solution increases. Also it confirms the susceptibility pattern shown in disc diffusion assay and MIC-MFC i.e *C. albicans* showing highest killing followed by *Trichophyton* and *Aspergillus* respectively. Despite the relatively low MIC seen in the broth microdilution assays, fungicidal effects in the time kill assays require concentrations which are several times higher than MIC. This may be due to differences between the ways these assays measure antifungal activity. In broth dilution method inocula are predominantly conidia and during incubation of the assay these conidia will germinate and grow into hyphae. In the time kill assay again the inocula are conidia; however in contrast this assay asesses the ability of conidia to be penetrated and killed by the antifungal compounds. Since the time kill assays showed that

fungi are not killed rapidly even at concentrations several times the MIC, the time of exposure may play a significant role and is a critical parameter for the outcome of these assays. The lower susceptibility of *Aspergillus* to the compounds may be due to the thickness, density and composition of cell wall (Hammer *et al.*, 2002). The presence of melanin in the pigmented fungi has been associated with virulence by reducing pathogens susceptibility to kill by host antimicrobial mechanism (Nosanchuk, 2006; Mednick *et al.*, 2005; Wang *et al.*, 1995; Mironenko, 2000). Our observation is well in agreement with observation of time killing by amphotericin-B which shows fungicidal activity at very low concentration within 24 h (Fong *et al.*, 1991; Manavathu *et al.*, 1998; Kelspar *et al.*, 1998; Canton *et al.*, 2004).

To get insight into the possible mechanism of antifungal action the effects of *Plumbagin* and *Isodiospyrin* on fungal surface morphology and anatomical changes were investigated using SEM and TEM. The SEM and TEM observation of the treated fungi clearly confirms its potent fungicidal effects. Both the compounds were found to have remarkable changes in cell surface morphology. Cytomorphological alterations like completely squashed and severly collapsed hyphae showing lack of cytoplasm, damage and loss of integrity of cell wall observed. Similar observation have been also observed in case of treatment of *Aspergillus niger* with *Citrus sinensis* essential oil and treatment of citral, eugenol, nerolidol and α-terpineol on the ultrastructural changes of *Trichophyton mentagrophytes* (Sharma and Tripathi, 2006; Park *et al.*, 2009). Also the finding that the vesicles of *Aspergillus flavus* were in a collapsed state and most often devoid of spores is supported by the study on the effect of itraconazole on *Aspergillus* (Cutsem *et al.*, 1984; Shenoy *et al.*, 2005; Nishiyama *et al.*, 2005; Zhang *et al.*, 2008). The surface alterations must be due to changes in cell permeability which provokes an osmotic imbalance (Nollin and Borgez, 1975; Senoy *et al.*, 2005; Phongpaichit, 2005).

TEM micrographs showed complete cell necrosis accompanied with cytoplasmic deterioration and thickened layered and distorted walls. Also accumulation of lipid bodies and abundant and widespread vacuolization have been observed. The reason for abundant vacuolization could be that these are derived from the broken cytoplasmic remnants (Nollin and Borgess, 1975). Similar modifications were also observed earlier in TEM analysis of *Epidermophyton floccosum and Trichophyton rubrum* on treatment with pyrazole-thiocyanates and *Trichophyton mentagrophytes* on treatment with various terpenes (Romagnoli, 2001; Park *et al.*, 2009). Thickening of cell wall induced by these

compounds are similar to those produced by synthetic compounds and growth inhibition of *Trichophyton mentagrophytes* by amorolfine (Sancholle, 1988; Nishiyama *et al.*, 2006; Escalente *et al.*, 2008). Bleomycin family of anticancer antibiotics are also known to induce abnormal thickening in the cell wall at several places. The drug causes the destruction of cell wall components. The damage is accompanied by changes in anchorage of cell wall mannoproteins which leads to increase in permeability (Beaudouin, 1993 and Moore *et al.*, 2003). The overall injury to cell wall is sometimes extended into the cell membrane leading to an overall loss of integrity of the entire cell (Moore, 1992; Arroyo *et al.*, 2007). Ultrastructural alterations of cytoplasmic membrane on treatment with ajoene and azoles, voriconazole and fluconazole have also been reported earlier in some dematiaceous fungi (Binachi *et al.*, 1997; Koul, 1999). The present study therefore confirmed the fungicidal activity of *Plumbagin and Isodiospyrin* and more likely the damage of cell wall and membrane however the exact mechanism of action is yet to be investigated.

Castro *et al.*, 2008 reported that the antifungal mechanism of *Plumbagin* seems to be involved in reacting with glutathione transferases (GSH) as an electrophile. The generation of a complex between *Plumbagin* and GSH could also be associated with oxidative stress due to GSH mobilization. This oxidative stress then cause damage to membranes, proteins and DNA which may induce apoptosis. Its anticancer property is attributed to the generation of ROS and has been found to inhibit the activity of topoisomerase II through the stabilization of Topo II DNA cleavage complex (Fuji *et al.*, 1992; Sandur *et al.*, 2006; Kawaiak *et al.*, 2007; Wang, 2008). Induction of DNA Topoisomerase II mediated DNA cleavage have also been observed in β-Lapachone and related naphthoquinones regarding their anticancer activity (Frydman *et al.*, 1997).

Although the mechanism of antifungal action of *Isodiospyrin* is still not known, its cytotoxicity on tumor cell lines is mediated by binding to topoisomerase I preventing it from binding to DNA (Ting *et al.*, 2003). This can prevent both DNA relaxation and kinase activities of htop I. These findings have important implications on napthoquinones and their cellular mode of action.

This is in aggrement with other studies involving mode of action of napthoquinones which confirms the fact that the toxicity and therapeutic activities of these quinones involve the formation of reactive oxygen species (Rodriguez *et al.*, 2004; Kumagai *et al.*, 2000). The biological redox cycle of quinones can be initiated by one electron reduction leading to the

formation of semiquinones. Under aerobic conditions, the semiquinone radical participates in redox cycling to generate reactive oxygen species (ROS) like superoxide anion (O_2) and hydrogen peroxide (H_2O_2) which causes damage to biological membranes and interferes with biosynthetic pathways (Kelly *et al.*, 2001; Riffel *et al.*, 2002). The toxicity of quinones by catalytic reduction of oxygen to superoxide and other reactive oxygen species (ROS) (redox cycling) is also confirmed by their ability to reduce growth rate on *Sacchromyces cerevisiae* model system (Rodriguez *et al.*, 2004; Castro *et al.*, 2008). Atovaquone, an analog of ubiquinone, acts by interfering with the electron transport chain of mitochondria at site bcI in plasmodium species. This consequently inhibits nucleic acid and ATP synthesis. The study by Foote *et al.*, 1949 shows that certain napthoquinones, which are not substituted by hydroxyl in position 2 and 3, are potent inhibitors of the carboxylase system. Another study by Ambrogi *et al.*, 1970, hypothesised that the microbiologically active 1,4 napthoquinones are substituted in the quinone moiety, with electron attracting groups such as OH or CL. Structure activity relationship studies showed that presence of free keto group at position 1 is required for activity and the most effective ones do not have a hydroxyl group at position 2. Another study of the antimicrobial activity of 1,4 napthoquinones indicated that active compound must possess at least a substitution at position 2 or 3 which is either an electron releasing or weaker electron withdrawing group (Riffel *et al.*, 2002; Weissenberg *et al.*, 1997).

To evaluate the cytotoxicity of the active compounds various *in vitro* assays were done. One of them is RBC hemolysis assay as the synthetic drugs including antifungals are known to possess hematologic malignancies (Dhaliwal *et al.*, 2004; Garratty, 2007; Lightfoot, 2002; Perkins, 2008; Salama *et al.*, 1989; Sinyoung *et al.*, 2002). Both the compounds induced hemolysis of RBCs only at very higher concentrations and these values are much higher than their MIC values. On the other hand amphotericin-B causes complete hemolysis at 6-8 µg/ml (Iwamoto, 1994; Oda *et al.*, 2006).

To further confirm the safety of the antifungal compounds they were tested for their effects on splenocytes and normal mouse peritoneal macrophages. The results showed that both the compounds were slightly cytotoxic only at very high concentrations. There is decrease in the number of viable cells with rise in concentration. Again the values at which the viability becomes less then 95 % are much above their MIC range in most of the cases. From the earlier studies it is already well known that both these compounds are quite active on numerous cancerous cell lines, *Plumbagin* being cytotoxic on Raji, Calu-1,

HeLa and wish tumor cell lines, MCF7, Bowes cancer cell lines, HaCaT keratinocytes and human cervical cancer cells with very low IC_{50} values (1.28 and 1.38 μM). *Isodiospyrin* is toxic to HCT-8 colon tumour, P-388 lymphocytic leukemia and human colon carcinoma cells with ED_{50} values of 4.9, 0.59 pg/ml and 3.8×10^{-2} respectively (Lin *et al.*, 2003; Nguyen *et al.*, 2004; Yan *et al.*, 1989; Gafner and Rodriguez, 1987). *Isodiospyrin* was also reported as nontoxic towards BC cells, KB and Vero cell lines (Prajoubklang *et al.*, 2005). This selective toxicity has also being observed in case of *Diospyrin*, a bisnapthoquinoid natural product showed significantly higher cytotoxicity against the tumor cells including acute myeloblastic leukemia (HL-60), chronic myelogenic leukemia, (k-562), breast adenocarcinoma, (MCF-7) and cervical epithelial carcinoma (HeLa) but spared normal human lymphocytes, IC50 > 70μM suggesting its action is specific for tumor cells (Chakrabarty *et al.*, 2003; Sarma *et al.*, 2008). Therefore the lower cytotoxicity of both *Plumbagin* and *Isodiospyrin* in the present study suggest that both the compounds are selectively noncytotoxic towards normal cells.

Thus in this study two active principles were obtained as a result of bioassay guided purification using silica gel column chromatography. They were identified as napthoquinones *Plumbagin* and *Isodiospyrin* on comparision with literature. Both the compounds possess significantly high antifungal activity and very low MIC as compared to crude extract. Also both the compounds were found to be broad spectrum and fungicidal which is very important for an antifungal to be considered as potent. Therefore *Plumbagin* and *Isodiospyrin* could be considered as promising lead that warrants further investigation regarding the mechanism of action and pharmacological studies.

SUMMARY

The incidence of invasive fungal infections have dramatically increased over the past few decades paralleling the rising number of immunocompromised patients. Despite the increase in fungal infections, therapeutic options are very limited and are often unsatisfactory because of toxicity and drug resistance phenomena. One obvious alternative is plant kingdom which is a great source of bioactive compounds. Therefore in the present study an attempt has been made to search a potent antifungal compound from plants.

Twenty five plants were selected on the basis of their ethanobotanical importance and their different parts were tested against fourteen human pathogenic fungi. Aqueous and methanolic extract of all the different parts of the above mentioned plants were prepared and preliminary antifungal activity was checked by disc diffusion assay on SDA plates and zones of inhibition measured. Aqueous extract did not show any promising result as only two plants were found active. In case of methanolic extract eleven plants were found active against one or more fungi. The methanolic extract of root bark of *D. kaki* was chosen for further studies as it exhibited remarkably good antifungal activity at 5 and 7.5 mg against all the fungi except some dematacеious fungi. A number of extraction solvents covering the wide polarity range from non polar to polar were used to select the best extraction solvent. Chloroform was found to be best extraction solvent as it shows activity against all the strains at 5 mg/ml. Activity index for the chloroform extract is also reasonably high (0.91-1.85) which proves effectiveness of plant extract.

Crude extract was evaluated by standard chemical tests and it showed the presence of phenolics, terpenoids, flavonoids and tannins. Then the TLC analysis of the crude extract was done for these group of compounds using a number of different solvent combinations and the solvent system showing best resolution was chosen for purification by column chromatography. Before it TLC bioautography was done showing two white zones on TLC plate which indicated the presence of two active compounds. The bioassay guided fractionation of the crude extract was done using benzene-chloroform step gradient by silica gel column chromatography. All the major fractions were checked for desired activity. Impure fractions were further purified by re-column of the active fractions and P-TLC. The active compounds obtained were analysed by EI-MS, FTIR and ^{1}H and ^{13}C NMR spectroscopy. As a result, two active compounds (A,170 mg) and (B,1900 mg) were obtained and on comparision of their spectra with literature they were

identified as napthoquinones, *Plumbagin* (5-hydroxyl, 2-methyl 1,4 -Napthoquinone) and *Isodiospyrin* (5-hydroxy-6-(1-hydroxy-6-methyl-5,8-dioxo-naphthalen-2-yl)-2-methyl-naphthalene-1,4-dione).

MIC and MFC of the crude extract as well as *Plumbagin* and *Isodiospyrin* were analysed using micro broth dilution. The MIC values of the crude extract and compounds were (160-2500 µg/ml), (1.25-20 µg/ml) and (5-160 µg/ml). The MFC values were (630-5000), (5-160 µg/ml) and (40-160 µg/ml) respectively. IC_{50} of *Plumbagin* for *C. albicans*, *T. mentegrophytes* and *A. flavus* was calculated as 1.2, 1.4 and 8.8 µg/ml respectively while the IC_{50} of *Isodiospyrin* was 5.1, 5.4 and 31.5 µg/ml respectively. The trend of susceptibility was same as shown in disc diffusion assay. Time kill study was then performed using 1, 2, 4 and 8 x MIC concentration of pure compounds against three different groups of fungi i.e *C. albicans*, *T. mentegrophytes* and *A. flavus*. The concentration dependent time killing of *Plumbagin* and *Isodiospyrin* was found to be species and concentration dependent with *C. albicans* showing fastest rate of killing followed by *T. mentegrophytes* and *A. flavus* in both cases. The rate of killing rises with rise in concentration.

To study the effect of these compounds on fungal morphology and ultrastructure scanning electron microscopy and transmission electron microscopy was performed. Fungi was treated with the 4 x MIC concentration of *Plumbagin* and 8 x MIC *Isodiospyrin* for 48 h and then processing of samples were done. The hyphae of all the treated fungi were severly damaged at the tested concentration of the pure compounds. Cells of *C. albicans* appeared shrunken and collapsed and the hyphae of *T. mentegrophytes* and *A. flavus* were squashed and malformed. In TEM micrographs the most common modification was increase in the number and size of vacuoles in the cells. Cytoplasmic deterioration indicating cell necrosis and thickened and layered cell wall with gaps at some places was also observed. Cell membrane was also affected and showing undulations and irregularity at several places. Therefore this study shows that cell wall and cell membrane are more likely to be the target of these active compounds.

Finally the evaluation of *in vitro* cytotoxic potential of the compounds was done by RBC hemolysis assay to see the effect of compound on mouse RBCs and the percentage hemolysis was calculated. The percent viability of peritoneal mouse macrophages and splenocytes was done and the number of viable cells were determined by tryptan blue dye exclusion assay. The results of *in vitro* RBC hemolysis assay of both the compounds

showed that the compounds were slightly hemolytic only at higher concentration. *Plumbagin* induced 7 % hemolysis at 20 µg/ml and 35 % hemolysis at 100 µg/ml. In case of *Isodiospyrin* no hemolysis was observed up to 40 µg/ml and at 100 µg there was only 8 % hemolysis. On treatment of peritoneal macrophages and splenocytes cultures with different concentrations of both the compounds (10-100 µg/ml), little cytotoxicity was observed only at very high concentrations. No significant cytotoxicity was observed up to 40 µg/ml of *Isodiospyrin* and 20 µg/ml of *Plumbagin* in both the cell cultures used.

CONCLUSION

As a result of screening of aqueous and methanolic extract for antifungal activity, *D. kaki* was chosen as it was showing remarkably good activity against maximum number of fungi used in case of methanolic extract. On bioassay guided purification of the crude chloroform extract using silica gel column chromatography, two active principles were obtained. They were identified as known napthoquinones, *Plumbagin* (5-hydroxyl,2-methyl1,4-Napthoquinone) and *Isodiospyrin* (5-hydroxy-6-(1-hydroxy-6-methyl-5,8-dioxo-naphthalen-2-yl)-2-methyl-naphthalene-1,4-dione) on comparision of the spectral data with literature. Both the compounds possess significantly high antifungal activity and low MIC in most of the case. MIC of *Plumbagin* was higher then that of *Isodiospyrin*. *Candida* and *Cryptococcus* were found to be most susceptible followed by dermatophytes and the dematacеious fungi which were least susceptible to the above mentioned compounds. Pharmacodynamics study of both the compounds proved that the compounds are fungicidal. Ultrastructure and morphology studies were shown to have deletrious effect on the fungi as the surface as well as interior of the cells looked necrotic. *In vitro* studies showed that *Plumbagin* was cytotoxic at lower concentration than *Isodiospyrin* but both the compounds show slight cytotoxicity only at very high concentration. Although detailed followup studies regarding various aspects are needed but it could surely be said that the active compounds obtained in the study were broad spectrum, fungicidal and nontoxic to normal cells. Thus these compounds could be considered as important candidates for the antifungal drug development programme.

BIBLIOGRAPHY

1. Abdelgaleil S.A., Hashinaga F., Nakatani M. 2005. Antifungal activity of limonoids from *Khaya ivorensis*. *Pest. Manag. Sci.* **61** : 186.
2. Ablordeppey S., Fan P., Ablordeppy I. and Mardenborough L. 1999. Systemic antifungal agents against AIDS related opportunistic infections : Current status and emerging drugs in development. *Curr. Med. Chem.* **6(11)** : 51-1195.
3. Achiwa Y., Hibasami H., Katasuzaki H., Imai K. and Komia T. 1997. Inhibitory effects of persimmon (*Diospyros kaki*) extract and related polyphenol compounds on growth of human lymphoid leukemia cells. *Biosc. Biotechnol. Biochem.* **61(7)** : 1099-101.
4. Adeniya B.A., Fong H.H.S, Pezzuto J.M., Luyengi L. and Odeloia H.A. 2000. Antibacterial activity of diospyrin, isodiospyrin and bisisodiospyrin from the root of *Diospyros piscatoria* (gruke) (ebenaceae). *Phytother. Res.* **14** : 112-117.
5. Afolayan A.J. and Meyer J.M. 1997. The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. *J. Ethnopharmacol.* **57** : 177–181.
6. Agroforrestry tree database. A tree species reference and selection guide. *Diospyros kaki*. www.world agroforestry.org.
7. Ahmad I., Aqil F. and Owais M. 2006. Modern phytomedicine. Turning medicinal plants into drugs. Wiley V.C.H Verlag GMbH and Co KGaA, Weinheim.
8. Akella S., Reddy V.B., Narayana V.V., Madugula M. 1986. ^{13}C NMR Spectra of some naturally occurring binapthoquinones and related compounds. *Phytochemistry*. **25(12)** : 2867-2871.
9. Akinpelu D.A. and Onakoya T.M. 2006. Antimicrobial activities of medicinal plants used in folklore remedies in south western. *African. J. Biotech.* **5(11)** : 1078-1081.
10. Alake L.B. 1994. Antibacterial activity of diosquinone isolated from *Diospyros tricolor. Planta. Med.* **60** : 477.
11. Allende C.M., Lee W.J., Francis P., Garrett K., Dollenberg H., Berenguer J., Lyman A.C., Philip A.P. and Thomas J.W. 1994. Dose-dependent antifungal

activity and nephrotoxicity of amphotericin B colloidal dispersion in experimental pulmonary aspergillosis. *Antimicrob. Agents. Chemother.* **38** : 518-522 .

12. Alvarez L., Marguina S., Villard M.L., Alonso D., Aranar E. and Delgado G. 1996. Bioactive polyacetylenes from *Bidens paludosa. Planta. Med.* **62** : 355-356.
13. Alvarez P.P., Bishop C.D. and Pascual M.J. 2001. Antifungal activity of the essential oil of flowerheads of garland chrysanthemum (*Chrysanthemum coronarium*) against agricultural pathogens. *Phytochemistry.* **57** : 99.
14. Alves B.A., Chay C.R., Pena R.C.I., Fernandez L.M.S., Molin S. and G.M.S. 2007. Antimicrobial activity of *Diospyros anisandra. Fitoterapia.* **78** : 370-372.
15. Alves C.A. and Costa C.M.A. 1980. Botacanone, a new napthaquinonylnapthaquinone epoxide. *Tetrahed. Lett.* **21** : 2459-2460.
16. Amaral J.A., Ekins A., Richards S.R., and Knowles R. 1998. Effect of selected monoterpenes on methane oxidation, denitrification, and aerobic metabolism by bacteria in pure culture. *Appl. Environ. Microbiol.* **64** : 520-525.
17. Ambrogi V., Artini D., Carneri I.D., Castellino S., Dradi E., Logemann W., Meinardi G., Di S.M. and Tosolini G. 1970. Studies on the antibacterial and antifungal properties of 1,4 napthoquines. *Br. J. Pharmac.* **40** : 871-880.
18. Ambrogi V., Artini I.D., De C., Castellino S., Dradi E., Logemann W., Meinardi G., Isomna M.D., Tosolini G., Wang J., Burger R.M. and Drlica K. 1998. Role of superoxide in catalase peroxidase mediated isoniazid action against mycobacteria. *Antimicrob. Agents. Chemother.* **42(3)** : 709-711.
19. An B.J., Kwak J.H., Park J.M., Lee J.Y., Park T.S., Lee J.T., Son J.H., Jo C. and Byun M.W. 2005. Inhibition of enzyme activities and the antiwrinkle effect of polyphenol isolated from the persimmon leaf (*Diospyros kaki* folium) on human skin. *Dermatol. Surg.* **31** : 848-54 .
20. Ananthanarayan and Paniker. J. 2000. Textbook of Microbiology, 4th ed. Orient Longman.
21. Anderson J.B. 2005. Evolution of antifungal drug resistance : mechanisms and pathogen fitness. *Nat. Rev. Microbiol.* **3** : 547-556.

22. Angioni A., Barra A., Cereti E., Barile D., Coisson J.D., Arlorio M., Dessi S., Coroneo V. and Cabras P.J. 2004. Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and flowers. *J. Agric. Food. Chem.* **52** : 3530.
23. Apisariyakul A., Vanittanakom N. and Buddhasukh D. 1995. Antifungal activity of turmeric oil isolated from *Curcuma longa* (zingiberaceae). *J. Ethnopharmacol.* **49** : 163-169.
24. Argaez R.B., Chay C.I.C., Rodriguez L.M.P., Fernandez S.S. and Salinas G.M.M. 2007. Antimicrobial activity of *Diospyros anisandra*. *Fitoterapia*. **78** : 370-372.
25. Arroyo F.T., Moreno J., Daza P., Boianova L. and Fernando R. 2007. Antifungal activity of strawberry fruit volatile compounds against *Colletotrichum acutatum*. *J. Agric. Food. Chem.* **55** : 5701-5707.
26. Ascioglu S., Rex J.H., Pauw B.de, Bennett J.E., Bille J., Crokaert F., Denning D.W., Donnelly J.P., Edwards J.E, Erjavec Z., Fiere D., Lortholary O., Maertens J., Meis J.F., Patterson T.F., Ritter J., Selleslag D., Shah P.M., Stevens D.A. and Walsh T.J. 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants : an international consensus. *Clin. Infec. Dis.* **34** : 7-14.
27. Aureli P.A.C. and Zolea S. 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *J. Food. Prod.* **55** : 344–348.
28. Balunas M.J. and Kinghorn A.D. 2005. Drug discovery from medicinal plants. *Life. Sci.* **78(5)** : 431-41.
29. Bandara B.M.R. and Wimalasiri W.R. 1988. Diterpene alcohols from *Croton lactiferous*. *Phytochemistry*. **27** : 225-226.
30. Bandara B.M.R., Gunatilaka A.A.L., Wijerethne E.M.K. and Adikaram N.K.B. 1988. Antifungal constituents of *Limonia acidissima*. *Planta. Med.* **54** : 374-375.
31. Barkada B. 1978. Preliminary report on warfarin for the treatment of herpes simplex. *J. Irish. Coll. Phys. Surg.* **22(l)** : 56-63.
32. Barre J.T., Bowden B.F., Coll J.C., Jesus J. and Simones M.F. 1994. Structure and antimicrobial activity of diterpenes from roots of *Plectranthus hereoensisss*.

J. Nat. Prod. **57** : 858-861.

33. Barre J.T., Bowden B.F., Coll J.C., Jesus J., Fuente V.E., Janairo G.C. and Ragasa C.Y. 1997. A bioactive triterpene from *Lantana camara. Phytochemistry.* **45** : 321–324.
34. Barrero A.F., Quilez J.F., Lara A. and Herrador M.M. 2005. Antimicrobial activity of sesquiterpenes from the essential oil of *Juniperus thurifera. Planta. Med.* **71** : 67.
35. Bates D.W., Su L., Yu D.T., Chertow G.M., Seger D.l., Gomes D.R.J., Dasbatch E.J. and Platt R. 2001. Mortality and costs of acute renal failure associated with amphotericin B therapy. *Clin. Infec. Dis.* **32(1)** : 686-693.
36. Batisa O.D.A., Nascimento J. and Simons M.F. 1994. Structure and antimicrobial activity by multidimensional solid state NMR spectroscopy. *Biochim. Biophys. Acta.* **1462** : 157-183.
37. Batista O.D.A., Nascimento J. and Simones M.F. 1994. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis. J. Nat. Prod.* **57** : 858–861.
38. Baumgartner B., Erdelmeier C.A.J., Wright A.D., Rali T. and Sticher O. 1989. Autofine, a strong antifungal alkaloid from *Ficus septica* leaves. *Planta. Med.* **55** : 652-653.
39. Beaudouin R., Lim S.T., Steid J.A., Powell M., Mc.koy J., Pramanik A.J., Johnson E., Moore C.W. and Lipkel P.N. 1993. Bleomycin affects cell wall anchorage of mannoproteins in *Saccharomyces cerevisiae. Antimicrob. Agents. Chemother.* **37(6)** : 1264-1269.
40. Beek T.A.V., Deelder A.M. and Svendson A.B. 1984. Antimicrobial, antiamoebic, antiviral screening of some *Tabernaemonatum* species. *Planta. Med.* **50** : 181.
41. Beer A.D. and Vivier M.A. 2008. Vv-AMP1, a ripening induced peptide from *Vitis vinifera* shows strong antifungal activity. *BMC. Plant. Biol.* **8** : 8-75.
42. Bei W., Ma W.P.Y. and Xu A. 2005. Flavonoids from the leaves of *Diospyros kaki* reduce hydrogen peroxide-induced injury of NG108-15 cells. *Life. Sci.* **76(17)** : 1975-1988.

43. Benjamin F., Laurence J.M., Jerry S. S., Karen N., Donald T. W., Angela A. L., Hui-M. W., Yong M., Hong Y.W., Marilyn M. S. and Leroy F. L.1997. Induction of DNA topoisomerase II-mediated DNA cleavage by ß-lapachone and related naphthoquinones. *Can. Res.* **57** : 620-627.

44. Berkada N.Y. 1978. Preliminary report on warfarin for the treatment of herpes simplex. *J. Irish. Coll. Phys. Surg.* **22(1)** : 56.

45. Binachi A., Zambonelli A. and D'aulerio A.Z. 1997. Ultrastructural studies of the effects of *Allium sativum* on phytopathogenic fungi *in vitro*. *Plant. Dis.* **81** : 1241-1246.

46. Bindseil K.U., Jakupovic J., Wolf D., Lavayre J., Leboul J. and Pyl D.V. 2001. Pure compound libraries; a new perspective for natural product based drug discovery. *Drug. Discov. Today.* **6(16)** : 840-847.

47. Binutu O.A., Kayode G. and Okugum J.I. 1996. Antibacterial and antifungal compounds from *Kigelia pinnata*. *Planta. Med.* **62** : 352-354.

48. Bolle M.F.C., Terras F.R.G., Cammue B.P.A., Rees S.B., Broekaert W.F. and Fritig B. 1992. Mechanism of plant defense responses. In proceedings of the second international conference of European foundation for plant pathology.legrand,m.(ed).the Netherlands: kluwer academic publishers , 433-466.

49. Borris R.P. 1996. Natural products research: perspectives from a major pharmaceutical company. *J. Ethnopharmacol.* **51** : 29–38..

50. Bossche H.V. 1997. Mechanism of antifungal resistance. *Rev. Iberoam. Micol.* **14** : 44-49.

51. Botha S., Gerritsma L.M. and Vander N.D.G. 1989. Primary biological investigations of *Gladiolus dalenii*. *Planta. Med.* **55** : 115.

52. Brillard J., Ribeiro C., Boemare N., Brehelinand M. and Givaudan A. 2001. Two distinct hemolytic activities in *Xenorhabdus nematophila* are active against immunocompetent insect cells. *Appl. Environ. Micribiol.* **16** : 2515–2525.

53. Briskin D.P. 2000. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant. Physiol.* **124** : 507-514.

54. Bylka W., Szaufer M., Matlawska J. and Goslinska O. 2004. Antimicrobial

activity of isocytisoside and extracts of *Aquilegia vulgaris* L. *Lett. Appl. Microbiol.* **39** : 93.

55. Canton E., Peman J., Gobernado M., Viudes A. and Espinel I.A. 2004. Patterns of amphotericin B killing against seven *Candida* species. *Antimicrob. Agents. Chemother.* **48(7)** : 2477-2482.
56. Cantrell C.L., Schrader K.K., Mamonov L.K., Sitpaeva G.T, Kustova T.S., Dunbar C. and Wedge D.E. 2005. Isolation and identification of antifungal and antialgal alkaloids from *Haplophyllum sieversii*. *J. Agric. Food. Chem.* **53** : 7741.
57. Cardenas O. N.C., Zavala S.M.A., Aguirre R.J.R., Perez G.C. and Perez-G.S. 2005. Chemical composition and antifungal activity of essential oil of *Chrysactinia mexicana* gray. *J. Agric. Food. Chem.* **53(11)** : 4347-4349.
58. Carpinella M.C., Ferravoli C.G. and Palacios S.M. 2005. Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L. fruits. *J. Agric. Food. Chem.* **53** : 2922.
59. Carter F.L., Garlo A.M and Stanley J.B. 1978. Termicidal components of wood extracts : 7 methyljuglone from *Diospyros virginiana*. *J. Agr. Food. Chem.* **26(4)** : 869-73.
60. Castro F.A.V., Mariana D., Panek A.D., Eleutherio E.C.A. and Pereira M.D. 2008. Cytotoxicity mechanism of two napthoquinones (Menadione and Plumbagin) in *Saccharomyces cerevisiae.PLOSONE*.www.plosone.org, **3(12)** : 1-6.
61. Cavaleiro C., Pinto E., Goncalves M.J. and Salgueiro L. 2006. Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J. Appl. Microbiol.* **100** : 1333.
62. Cavin A.L., Hay A.E., Marston A., Stoecki H., Scopelliti R., Diallo D. and Hostettman K. 2006. Bioactive diterpenes from the fruits of *Detarium microcarpum*. *J. Nat. Prod.* **69** : 768.
63. Chakrabarti A. 2000. Microbiology of systemic fungal infections. *J. Postgrad. Med.* **51** : 16-20.
64. Chakrabarty S., Roy M.H. and Bhattacharya R.K. 2002. Induction of apoptosis in

human cancer cell lines by diospyrin, a plant derived bisnapthoquinoid, and its synthetic derivaties. *Cancer. Lett.* **188** : 85-93.

65. Chen G.X.J., Xu S.X. and Zhang R.Q. 2007. Chemical constituents of the leaves of *Diospyros kaki* and their cytotoxic effects. *J. Asian. Nat. Prod. Res.* **9(4)** : 347–353.
66. Cheng S.S., Lin H.Y. and Chang S.T.J. 2005. Chemical composition and antifungal activity of essential oils from different tissues of Japanese cedar (*Cryptomeria japonica*). *J. Agric. Food. Chem.* **53** : 614.
67. Cheng S.S., Wu C.L., Chang H.T., Kao Y.T. and Chang S.T. 2004. Antitermitic and antifungal activities of essential oil of *Calocedrus formosana* leaf and its composition. *J. Chem. Ecol.* **30** : 1957.
68. Cordell G.A. 1981. Introduction to alkaloids : Biogenetic approach. John willey and sons. New York. 1055 P.
69. Cordell G.A. and Shin Y.G. 1999. Finding the needle in the haystack. The dereplication of natural product extracts. *Pure. Appl. Chem.* **71(6)** : 1089-1094.
70. Cowan M.M. 1999. Plants products as antimicrobial agents. *Clin. Microbiol. Rev.* **12(4)** : 564-582.
71. Creveld M. 2001. *Diospyros kaki*, de Wereldboom;droomproving. Dynamis : **25** (in English in LINKS 3/02).
72. Cutler. Medical Mycology-Lec notes .www.doctorfungus.org.
73. Cutsem J.V., Gerven F.V., Marie A.V.V., Borgers M. and Janssen A.J.P. 1984. Itraconazole, a new triazole that is orally active in aspergillosis. *Antimicrob. Agents. Chemother.* **26(4)** : 527-534.
74. D' auria, F.D., Tecca M., Strippoli V., Salvatore G., Battinelli L. and Mazzanti G. 2005. Antifungal activity of *Lavandula angustifolia* essential oil against *Candida albicans* yeast and mycelial form. *Med. Mycol.* **43**. 391.
75. Dahot M. 1999. Antibacterial and antifungal activity of small proteins of *Indigofera oblongifolia*. *J. Ethanopharmacol.* **64** : 277-282.
76. Daliwal G., Cornett P.A. and Tierney L.M. 2004. Hemolytic anemia. *Am. Fam. Phys.* **69(11)** : 2599-2606.
77. De P., Paiva S.R, Figueiredo M.R., Aragao T.V. and Kaplan M.A.C. 2003.

Antimicrobial activity *in vitro* of Plumbagin isolated from *Plumbago* species. *Mem. Inst. Oswaldo Cruz. Rio de. Janeiro.* **98(7)** : 959-961.

78. Decosterd L.A., Stoeckli H.E., Chapnis J.C., Sordat B. and Hostettmann K. 1989. Novel prenylated xanthones from *Garcinia gerardi. Planta. Med.* **55** : 584-589.
79. Del P.A, Villar J, Alhambra A. 2009. Epidemiology of invasive Candidiasis in pediatric and adult populations. *Rev. Iberoam. Mycol.* **26(1)** : 2-7.
80. Dewanjee S., Kundu M., Maiti A., Majumdar R., Majumdar A. and Mandal S. 2007. *In vitro* evaluation of antimicrobial activity of crude extract from plants *Diospyros peregrine, Coccinia grandis* and *Swietenia macrophylla. Trop. J. Pharm. Res.* **6(3)** : 773-77.
81. Dignani M.C. and Anaissie E. 2004. Human fusariosis. *Clin. Microbiol. Infec.* **10(1)** : 67-75.
82. Dinda B., Bhattacharya A., De C.U., Arima S., Takayanag H. and Harigayab Y. 2006. Antimicrobial *C*-Glucoside from aerial parts of *Diospyros nigra. Chem. Pharm. Bull.* **54(5)** : 679-681.
83. Dissrens I.S., Marston A., Hamburger M., Rogers B.C. and Hostettmann K. 1989. Novel prenylated xanthones from *Garcinia gerardii. Planta. Med.* **55** : 584-589.
84. Dismukes W.E. 2000. Introduction to antifungal drugs. *Clinical. Infec. Dis.* **30** : 653–657.
85. Dixon R.A. 2000. Natural products and plant disease resistance. *Nature.* **411** : 843-847.
86. Du Z., Zhu N., Ze N. and Shen Y. 2003. Two new antifungal saponins from the Tibetan herbal medicine *Clematis tangutica. Planta. Med.* **69** : 547.
87. Duke J.A. 1985. Handbook of medicinal herbs. CRC Press, Inc,Boca Raton,Fla. 696.
88. Dzoyen J.P., Tangmouo J.G., Lontsi D., Etoa F.X. and Lohoue P.J. 2007. *In vitro* antifungal activity of extract and Plumbagin from the stembark of *Diospyros crassiflora*. Hiern. (Ebenaceae). *Phytother. Res* . **21** : 671-674.
89. Edeoga H.O., Okwu D.E. and Mbaebie B.O. 2005. Phytochemical constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol.* **4(7)** : 685-688.

90. Eggimann P., Garbino J, Pittet D. 2003. Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. *Lancet. Infec. Dis.* **3(11)** : 685-702.

91. Ekabo D.A., Farnsworth N.R., Henderson T.O., Guohua M., Mukherjee R. and Mao G.H. 1996. Isolation and biological study of triterpenoid saponin from leaves of *Buddleja madagascariensis*. *Planta. Med.* **62** : 92-93.

92. Eloff J.N. 1998.Which extractant should be used for the screening and isolation of antimicrobial components from plants? *J. Ethanopharmacol.* **60(1)** : 1-8.

93. Elswijk D.V. and Irth H. 2002. Back to the roots : nature as source of novel drug structures, *Eur. Biopharm. Rev.* : 1-12.

94. Emam A.M., Moussa A.M., Faure R., Farvel A., Delmas F., Elias R. and Balansard G. 1996. Isolation and biological study of triterpenoid saponin from leaves of *Buddleja madagascariensis*. *Planta. Med.* **62** : 92-93.

95. Emile A., Waikedre J. , Herrenknecht C. , Fourneau C., Gantier J.C., Hnawia E., Cabalion P., Hocquemiller R. and Fournet A. 2007. Bioassay-guided isolation of antifungal alkaloids from *Melochia odorata*. *Phytother.Res.* **21(4)** : 396-400.

96. Escalante A., Gattuso M., Perez P., and Zacchino S. 2008. Evidence for the mechanism of action of the antifungal phytolaccoside B isolated from *Phytolacca tetramera* Hauman. *J. Nat. Prod.* **71** : 1720–1725.

97. Fabricant D.S. and Farnsworth N.R. 2001. The value of plants used in traditional medicine for drug discovery. *Environ. Health. Persp.* **109(1)** : 69-75.

98. Fagboun D.E., Ogundana S.K., Adesanya S.A. and Roberts M.F. 1987. Dihydrostilbene phytoalexins from *Dioscorea rotunda*. *Phytochemistry*. **26** : 3187-3189.

99. Fallas A.L and Thomson R.H. 1968. Ebenaceae extractives. Part III.Binapthaquinones from *Diospyros* species. *J. Chem. Soc.* **18** : 2279-2282.

100. Feeney K.T., Arthur K.T., Whittle A.J., Altman S.A. and Speers D.J. 2007. Outbreak of Sporotrichosis, Western Australia. *Emer. Infec. Dis.* **13(8)** : 1228-1321.

101. Fernandes D.C.R. Gonzalez P.B., Garcia O.F. and.Carbonero P. 1972. Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*. *Appl.*

Microbiol. **23** : 998–1000.

102.Fernandez M.A., Garcia M.D., and Saenz M.T. 1996. Antibacterial activity of the phenolic acids fraction of *Scrophularia frutescens* and *Scrophularia sambucifolia*. *J. Ethnopharmacol.* **53** : 11–14.

103.Fessenden R.J. and Fessenden J.S. 1982. Organic chemistry, 2nd ed.Willard Grant Press, Boston, Mass.

104.Ficker C., Smith M.L., Akpagana K., Gbeassor M., Zhang J., Durst T., Assabqui R. and Arnason J.T. 2003. Bioassay-guided isolation and identification of antifungal compounds from ginger. *Phytother. Res.* **17(8)** : 897-902.

105.Fong I.W., Cheng P.C. and Hinton N.A. 1991. Fungicidal effect of amphotericin B in urine : *in vitro* study to assess feasibility of bladder washout for localization of site of candiduria. *Antimicrob. Agents and Chemother.* **35(9)** : 1856-1859.

106.Foote M.W., Little J.E and Sproston T.J. 1949. On napthoquinones as inhibitors of spore germination of fungi. *J. Biol.Chem* : 481-487.

107.Fung S.L., Chau C.H. and Yew W.W. 2008. Cardiovascular adverse effects during itraconazole therapy. *Eur. Respir. J.* **32(1)** : 240-246.

108.Funayama S. and Hikino H. 1979. Hypotensive principles of *Diospyros kaki* leaves. *Chem. Pharm. Bull.* **27(11)** : 2865-2867.

109.Francis J.S., Doherty M.C. and Lpatin U. 2005. Severe community onset pneumonia in healthy adults caused by methicillin resistant *staphylococcus aureus* carrying the panton valentine leukocidin genes. *Clin. Infec. Dis.* **40** : 100-107.

110.Friend M. and Franson J.C. 1999. Field manual of wildlife diseases. General field procedures and diseases of birds. UCGS, Washington.

111.Frederico A.V.S, Diana M., Anita D.P., Elis C.A.E, Marco D.P. 2008. Cytotoxicity mechanism of two naphthoquinones (Menadione and Plumbagin) in *Saccharomyces cerevisiae*. *Plosone.* **3(1)** : 1-6.

112.Freiburghas F., Kaminsky R., Nkunya M.H.H and Brun R. 1996. Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. *J. Ethnopharmacol.* **55** : 1-11.

113.Fridkin K.S. and Jarvis. R.W. 1996. Epidemiology of nosocomial fungal

infections. *Clin. Microbiol. Rev.* **51** : 499–511.

114. Frydman B., Marton L.J. , Sun J.S., Neder K., Witiak D.T., Liu A.A., Wang H.M., Mao Y., Wu H.Y., Sanders M.M. and Liu L.F. 1997. Induction of DNA topoisomerase II-mediated DNA cleavage by β-lapachone and related naphthoquinones. *Cancer Res.* **57** : 620–627.

115. Fuji N., Yamashita Y., Arima Y., Nagashima M. and Nakano H. 1992. Induction of topoisomerase II mediated DNA cleavage by the plant napthaquinones Plumbagin and Shikonin . *Antimicrob. Agents and Chemother.* **36(12)** : 2589-2594.

116. Fujioka T. and Kashiwada. Y. 1994. Anti-AIDS agents. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* **57** : 243–247.

117. Gafner F. and Rodriguez. E. 1984. Biological chemistry of molluscidal and cytotoxic plant constituents. *Phytochemistry.* **20(1)** : 30-1.

118. Ganapaty S., Thomas P.S., Fotso S. and Laatsh H. 2004. Antitermitic quinone from *Diospyros sylvatica*. *Phytochemistry.* **65** : 1265-1271.

119. Ganeshan G. and Ganeshan J. 1993. Antifungal activity of Parthenin. *Ind. Phytopathol.* **46** : 193-194.

120. Garg S.C. and Jain R. 1992. Biological activity of essential oil of *Piper betle*. *J. Essent. Oil. Res.* **4** : 601-606.

121. Geissman T.A. 1963. Flavonoid compounds, tannins, lignins and related compounds, 265 P. In: Florkin and E.H.stotz (ed) , pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol **9**. Elsevier, New York.

122. Ghannoum A.M. and Rice B.L. 1999. Antifungal agents : Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **12(4)** : 501–517.

123. Ghosal S., Prasad K.B.N. and Lakshmi V. 1996. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica in vitro* and *in vivo*. *J. Ethnopharmacol.* **50** : 167-170.

124. Gilman E.G. and Watson D.G. 1993. *Diospyros kaki* Japenese Persimmon. Fact Sheet ST-229, a series of the environmental horticulture department, Florida

cooperative extension service, Institute of food and agricultural sciences, University of Florida.

125.Gonzalez M., Zamipa A., Marquina S., Navarro V. and Aivarez L. 2004. Antimycotic spirostanol saponins from *Solanum hispidum* leaves and their structure-activity relationships. *J. Nat. Prod.* **67** : 938.

126.Gorinstein S., Bartikowska E., Kulasek G., Zemser M. and Trakhtenberg S. 1998. Dietary Persimmon improves lipid metabolism in rats fed diets containing cholesterol. *J. Nut.* 2023-2027.

127.Gorinstein S., Bartnikowska E., Kulasek G., Marina Z.M. and Soo S.K., Samkeun L. and Byeongjin C. 2007. Antifungal activity of Plumbagin purified from the leaves of *Nepenthes Ventricosa* X maxima against phytopathogenic fungi. *Plant. Pathol. J.* **23(2)** : 113-115.

128.Govinden J., Magan N., Gurib A., Gauvin A., Smadja J. and Kodja H. 2004. Chemical composition and *in vitro* antimicrobial activities of the essential oils from endemic *Psiadia* species growing in Mauritius. *Biol. Pharm. Bull.* **27** : 1814.

129.Graybill J.R. 2000. Systemic antifungal drugs. *Systemic. Antifungals*. 168-173.

130.Groll A.H. and Walsh T.J. 2001. Uncommon opportunistic fungi: new nosocomial threats. *Clin. Microbiol and Infec. Dis.* **7** : 8-24.

131.Grratty G. and Arndt P.A. 2007. An update on drug-induced immune hemolytic anemia. *Curr. Opinion Hematol.* **10(2)** : 159-194.

132.Gupta A.K., Konnikov N., Mac Donald P., Rich P., Rodger N.V.V., Edmonds M.W., 1998. Prevalence and epidemiology of toenail onychomycosis in diabetic subjects: a multicenter survey. *British J. Dermatol.* **139** : 665–671.

133.Guleria S. and Kumar A. 2006. Antifungal activity of some Himalayan medicinal plants using direct bioautography. *J. Cell* and *Mol. Biol.* **5** : 95-98.

134.Gulshan K. and Rowly S.M. 2007. Multidrug resistance in fungi. *Eukary. Cell.* **6(11)** : 1933-1942.

135.Hammer K.A., Carson C.A. and Riley T.V. 2002. *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. *J. Antib. Chemother.* **50** : 195-199.

136. Harborne J.B. 1998. Phytochemical Methods. A guide to modern techniques of plant analysis. Chapmam and Hall, London.

137. Hashimoto S. 2009. Micafungin : a sulfated Echinocandin. *J. Antib.* **62** : 27-35.

138. Heinrich M. 2003. Ethnobotany and natural products : The search for new molecules, new treatments of old diseases or a better understanding of indigenous cultures? *Curr. Topics in Med. Chem.* **3** : 29-42.

139. Herald B.C., Immerguck L.C. and Maranan M.C. 1998. Community acquired methicillin resistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA.* **279** : 593- 598.

140. Honda G. and Tabata M. 1982. Antidermatophytic substances from *Sophora angustifolia*. *Planta. Med.* **46** : 122-133.

141. Hospenthal R.D. and Rinaldi G.M. 2003. Approach to rare and emerging mycoses in patients with hematologic malignancies. *Suppl. Rev. Clin. Exp. Hematol.* **1** : 49-64.

142. Hostettmann K. 1999. Strategy for the biological and chemical evaluationofplant extract. www.iupac.org/symposia/proceedings/phuket 97/hostettmann.html.

143. Houghton P.J. 1993. *In vitro* testing of some West African and Indian plants used to treat snakebites. Médicament Et Nutrition : *L'Approche Ethnopharmacologique.* **1** : 263-274.

144. Hoult J.R.S. and Paya M. 1996. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *Gen. Pharmacol.* **27** : 713–722.

145. Hsieh J.Y., Lin C.L. and Tsai H.T. 2005. Determination and identification of Plumbagin from the roots of *Plumbago zeylanica* L. by liquid chromatography with tandem mass spectroscopy. *J. Chromatogr.* **1083** : 141-145.

146. Hu Z.Z., Yang J.F. and Tan Z.J. 2006. The hypoglycemic effect of flavone from leaves of *Diospyros kaki* in streptozocin induced diabetic mice and its mechanism. *Yingyang. Xuebao.* **28(6)** : 540-541.

147. Hunter M.D. and Hull L.A. 1993. Variation in concentrations of phloridzin and phloretin in apple foliage. *Phytochemistry.* **34** : 1251-1254.

148. Ibrahim E.H., Sherman G., Ward S., Fraser V.J. and Kollef M.H. 2000. The

influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU settings. *Immunohematol.* **23(3)** : 105-119.

149. Inbaraj J.J. and Chignell C.F. 2004. Cytotoxic action of juglone and plumbagin : A mechanistic study using HaCaT keratinocytes. *Chem. Res. Toxicol.* **17(1)** : 55-62.

150. Ingroff A.E. 2008. Mechanism of resistance to antifungal agents: yeasts and filamentous fungi. *Rev. Iberoam. Micol.* **25** : 101-106.

151. Ingroff E.A. 2001. *In vitro* fungicidal activities of voriconazole, itraconazole, and amphotericin B against opportunistic monilaceous and dematiaceous fungi. *J. Clin. Microbiol.* **39(3)** : 954-958.

152. Iroegbu C.U. and Nkere C.K. 2005. Evaluation of the antibacterial properties of *Picralima nitida* stembark extracts. *Int. J. Mol. Med. and Adv. Sc.***1(2)** : 182-189.

153. Irth H., Long S. and Schenk T. 2004. High resolution screening in an expanded chemical space. *www.Current drug discovery.com*

154. Iwamoto A.F., Kumiko N., Seiji H., Masakuni O. and Masanobu K. 1994. WF 1899A, B and C, novel antifungal lipopeptides ii. *J. of Antib. Biol. Prop.* **47(10)** : 1092-1097.

155. Iwu M.M., Duncan A.R. and Okunj C.O. 1999. New antimicrobials of plant origin. Perspectives on new crops and new uses. J. Janick. ASHS Press, Alexandria, VA.

156. Jain S.C. and Sharma K. 1987. Antimicrobial activity of pyrrolidone alkaloids of *heliotropium ellipticum*. *Chem and Pharm. Bull.* **35** : 3487-3489.

157. Janovska D., Kkubikova K. and Kokoska L. 2003. Screening for antimicrobial activity of some medicinal plants species of traditional Chinese medicine. *Czeczh. J. Food. Sci.* **21** : 107-110.

158. Jasicka I., Lipok J., Nowakowska E.M., Wieczorek P.P., Mylnaz P. and Kafarsli P.Z. 2004. Rare trisubstituted sesquiterpenes daucanes from the wild *Daucus carota*. *Naturforsch.* **59** : 791.

159. Ji L., Zhang Q.C., Guiyou J., Lilian Z. and Quianghua C. 2003. Study on antimicrobial activities of persimmon leaves and isolation and elucidation of the active compounds. *Shipin. Kexue.* **24(3)** : 129-131.

160. Jones G.A., McAllister T.A., Muir A.D., and Cheng K.J. 1994. Effects of sainfoin (*Onobrychis viciifolia* scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Appl. Environ. Microbiol.* **60** : 1374–1378.

161. Jones S.B. and Luchsinger A.E. 1986. Plant systematics. McGraw-Hill Book Co., New York, N.Y.

162. Jones, N.L., Shabib S. and Sherman P.M. 1997. Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori. FEMS. Microbiol. Lett.* **146** : 223–227.

163. Kanafani Z.A. and Perfect J.R. 2008. Resistance to antifungal agents : mechanisms and clinical impact. *Clin. Infec. Dis.* **46** : 120-128.

164. Kanokmedhakul K., Kanokmedhakul S. and Phatchana R. 2005. Biological activity of anthraquinones and triterpenoids from *Prismatomeris fragrans. J. Ethanopharmacol.* **100** : 284.

165. Kapadia G.J., Fzuine M.A., Balasubramanium V. and Sridhar R. 2001. Aminonaphoquinones a novel class of compounds with potent antimalarial activity against *Plasmodium falciparum. Pharmacol. Res.* **43(4)** : 363-362.

166. Karumi Y., Onyeyili P.A. and Ogugbuaja V.O. 2004. Identification of active principle of *M. balsamina* (Balsam Apple) leaf extract. *J. Med. Sci.* **4(3)** : 179-182.

167. Kato H., Sakagami R., Harada M., Sakurai J.U.T. and Yamanaka S. *Kaki* tannins and oligosaacharides as intraoral pathogenic bacterial agents. Jpn.Kokai. Tokkyo Koho. 2006. 7pp.coden jkxxxaf jp 2006298827 a 20061102. *patent written in Japanese.*Application JP 2005-123412 20050421.Priority: can 145 : 432248 AN2006: 1147642 caplus.

168. Kawase M., Motohashi N., Satoh K., Sakagami H, Nakashima H., Tani S., Shirataki Y., Kurihara T., Spengler G., Wolfard K. and Molnár J. 2003. Biological activity of persimmon (*Diospyros kaki*) peel extracts. *Phytother. Res.* **17(5)** : 495-500.

169. Kawiak A., Piosik J., Stasilojc W.A.G., Marczak. l., Stobiecki M., Bigda. J. and Lojkowska E. 2007. Induction of apotosis by Plumbagin through reactive

oxygen species mediated inhibition of topoisomerase II. *Toxicol. App. Pharmacol.* **223(3)** : 267-276.

170. Kazmi M.H., Malik A., Hameed S., Akhtar N. and Ali S.N. 1994. An antraquinone derivative from *Cassia italica*. *Phytochemistry*. **36** : 761-763.

171. Keiko Y., Shigeta M., Masaru A., Atsushi K. and Tadakazu N. 2007. The antifungal compound totarol of *Thujopsis dolabrata* var. *hondai* seeds selects for fungi on seedling root surfaces. *J. Chem. Ecol.* **33(12)** : 2254-2265.

172. Kennedy O. R. and Thornes R.D. 1997. Coumarins; biology, applicationa and mode of action. John wiley and sons, Inc, New York.

173. Kelly C.G.D.M., Flavio S.E., Cleverson N.P., Maria D.C., Pintoa F.R., Andrea P.D., Kelly S., Solange L.D.C. and Antonio V.P. 2001. Trypanocidal activity of isolated naphthoquinones from *Tabebuia* and some heterocyclic derivatives : A review from an interdisciplinary study. *J. Braz. Chem. Soc.* **12(3)** : 325-338.

174. Khan M.R. and Timi D. 1999. Constituents of *Diospyros lolin*, *D. maritima* and *D. novoguinensis*. *Fitoterapia*. **70** : 194-196.

175. Kim Y.M., Lee C.H., Kim H.G., Lee H.S. 2004. Anthraquinones isolated from *Cassia tora* (Leguminosae) seed show an antifungal property against phytopathogenic fungi. *Agric. Food. Chem.* **52** : 6096-6100.

176. Klepser E.M., Wolfe J.E. and Michael A.P. 1998. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B against *Cryptococcus neoformans*. *J. Antimicrob. Chemother.* **41** : 397–401.

177. Kooy F.V , Meyer J.J.M. and N. L. 2006. Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from *Euclea natalensis*. *South. Af. J. Bot.* **72(3)** : 349-352.

178. Koul A., Vittulo J., Reyes G. and Ghannoum M. 1999. Effects of voriconazole on *Candida glabrata in vitro*. *J. Antimicrob. Chemother.* **44** : 109-112.

179. Kong J.M., Goh N.K., Chia L.S. and Chiatet F. 2003. Recent advances in traditional plant drugs and orchids. *Acta. Pharmacol. Sin.* **24(1)** : 7-21.

180. Kontoyiannis D.P. and Lewis R.E. 2002. Antifungal drug resistance of pathogenic fungi *The. Lancet.* **359** : 1135-44.

181. Kordali S., Cakir A., Mavi A., Kilic H. and Yildrim. 2005. Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole (preparation). A. *J. Agric. Food. Chem.* **53** : 1408.

182. Kowacs P.A., Silvado C.E., Almeida D., Monteiro S., Ramos M., Madaloso L.E., Peinheiro R., Werneck L.C. 2004. Infection of CNS by *Scedosporium apiospermum* after near drowning. Reports of fatal case and analysis of its cofounding factors. *J. Clin. Pathol.* **57(2)** : 205-207.

183. Krishnaswamy M. and Purushothaman K.K. 1980. *Plumbagin* : A study of its anticancer, antibacterial and antifungal properties. *J. Exp. Biol.* **18** : 876-879.

184. Krishnan S., Manavathu E.K., Chandrasekar P.H. 2009. *Aspergillus flavus* : an emerging non-fumigatus *Aspergillus* species of significance. *Mycoses.* **52(3)** : 206-22.

185. Krcmery V., Kunova E. , Jesenska Z., Trupl J. ,Spanik S., Mardiak J., Studena M. and Kukuckova E. 2005. Invasive mold infections in cancer patients : 5 years experience with *Aspergillus, Mucor, Fusarium* and *Acremonium* infections. **20** : 39-45.

186. Kubo I., Muroi H. and Himejima M. 1992. Antibacterial activity of totarol and its potentiation. *J. Nat. Prod.* **55** : 1436–1440.

187. Kuke C.W., Roberts E.M., Watt M.F., Hazra R., Lajubutu B.P. and Yang S. 1998. Antiinflammatory activities of binapthoquinones from *Diospyros* species. *Phytother. Res.* **12(3)** : 155-158.

188. Kumagai Y., Koide S., Taguchi K., Endo A., Nakai, Y. Yoshikawa T., Shimojo N. 2002. Oxidation of proximal protein sulfhydryl by phenanthraquinone, a component of diseal exhaust particles. *Chem. Res. Toxicol.* **15** : 483-489.

189. Kumar R. 2000. Allergic bronchopulmonary aspergillosis review of 29 cases. *Ind. J. Tub.* **47** : 237-239.

190. Kuo Y.H., Chang C.I, Li S.Y., Chou C.J., Chen C.F, Kuo Y.H. and Lee K.H. 1997. Cytotoxic constituents from the stems of *Diospyros maritima. Planta. Med.* **63(4)** : 363-365.

191. Kyung K.O and Kyoon Young. 2005. Pharmaceutical composition for protecting liver containing triterpenoid compound separated from *Diospyros kaki* folium.

No pp. given. CODEN: KRXXA7 KR 2005043130 A 20050511 Patent Kongbo (2005), No pp. given. CODEN2003-77918 20031105. Priority: CAN 145: 278136 AN 2006: 906883 CAPLUS (copyright (c) 2008 ACS.

192. Larshini M., Lazrek H.B., Amarouch H. and Jana M. 1996. Investigation of antifungal and analgesise activities of extract of *Sium nodiflorum*. *J. Ethanopharmacol.* **53** : 105-110.

193. Latha P.G., Abraham A., Suja S.R. and Rajshekharan S. 2003. Current state and future directions in plant derived antimycotics. Plant derived antimycotics : current trends and future prospects. Food products press : 203-227.

194. Leclereq J.Q., Fowel A., Balansard G, Regdi P. and Angenot L. 1995. Screening of in vitro antifungal activities of indole alkaloids. *Planta. Med.* **61** : 475-480.

195. Lee S.H., Lee J.R., Lunde C.S. and Kubo I. 1999. In vitro antifungal susceptibility of *Candida albicans* and other fungal pathogens to polygodial, a sesquiterpene dialdehyde. *Planta. Med.* **65** : 204-208.

196. Lee S.K., Lee H.J., Min H.Y., Park E.J., Lee K.M., Ahn Y.H., Cho Y.J. and Pyee J.H. 2005. Antifungal activity of camptothecin, trifolin, and hyperoside isolated from *Camptotheca acuminate*. Fitoterapia. **76** : 258.

197. Lee, H.C. and Lee H.S. 2008. Acaricidal activity and fuction of mite indicator using Plumbagin and its derivates isolated from *Diospyros Kaki* Thunb.roots (ebenaceae) *J. Microbiol. Biotechnol.* **18 (2)** :189- 193.

198. Levy L.A. 1997. Epidemiology of onychomycosis in special risk populations. *Am. Ped. Med. Ass.* **87** : 546–550.

199. Li C., Bei W., Li Y.and Lou J. 2007. Ethylacetate extract of *Diospyros kaki* leaf for preventing and treating hyperglycemic, diabetes and metabolic syndromes. *Faming zhuanli. Shenqing. Gongkai. shuomingshu.* **32** : 21 -23.

200. Lilian J., Qianghua Z., Guiyou C. 2003. Study on antimicrobial activities of leaves and isolation and elucidation of the active compounds. *Shipin. Kexue.* **24(3)** : 129-131.

201. Li X.C., Elsohy H.N., Nimrod A.C. and Clark A.M. 1999. Antifungal activity of epigallocatechin gallate from *Coccoloba dugandiana*. *Planta. Med.* **65** : 780-782.

202. Lightfoot T. Drug induced hemolytic anemia. 2002. *Transfusion Med.* **4**: 1-4.

203. Lin C.L., Yang L.L. and Chou C.J. 2003. Cytotoxic napthoquinones and plumbagic acid glucosides from *Plumbago zeylanica*. *Phytochemistry*. **62** : 619-622.

204. Lin L., Kuo Y., Chou C., Mathew P.N., Abidha K.P., Kaiyanasundaram V.P., Balaramaram M.K. 2002. Macrofilaricidal activity of the plant *Plumbago indica/rosea in vitro*. *Drug. Dev. Res*. **56(1)** : 33-39.

205. Luo D.Q., Wang H., Tian X., Shao N.J., Liu J.K. 2005. Antifungal properties of pristimerin and celastrol isolated from *Celastrus hypoleucus Pest. Manag. Sci*. **61** : 85.

206. Lupetti A., Danesi R., Campa M., Tacca M.D. And Kelly S. 2002. Molecular basis of resistance to azole antifungals. *Trends in Mol. Med*. **8(2)** : 76-81.

207. Maillard M., Gupta M.P. and Hostettmann K. 1987. A new antifungal prenylated flavone from *Erythrina berteroana*. *Planta. Med.* **53** : 563-565.

208. Maksimovic Z.A., Dordevic S., Mraovic M. 2005. Antimicrobial activity of *Chenopodium botrys* essential oil. *Fitoterapia*. **76**. 112.

209. Mallavadhani U.V., Panda A.K. and Rao Y.R. 1998. Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry*. **49(4)** : 901-905.

210. Manavathu K.E., Curright I.J. and. Chandrasekar H.P. 1998 organism dependent fungicidal activities of azoles. *Antimicrob. Agents and Chemother*. **42(11)** : 3018-3021.

211. Manojlovic N.T., Solujic S., Sukdolak S., Milosev M. 2005. Antifungal activity of *Rubia tinctorum*, *Rhamnus frangula* and *Caloplaca cerina*. *Fitoterapia*. **76** : 244.

212. Maoz M., Kashmau Y. and Neeman I. 1999. Isolation and identification of a new antifungal sesquiterpene lactone from *Inula viscosa*. *Planta. Med.* **65** : 281-282.

213. Marston A., Msonthi J.D. and Hostettmann K. 1984. Naphthoquinones of *Diospyros usambarensis*; their molluscicidal and fungicidal activities. *Planta. Med*: 279-280.

214. Mara C.S.M.P. and Márcia S.C.M. 2003. Cryptococcosis: a review of the brazilian experience for the disease. *Rev. Inst. Med. Trop. S. Paulo.* **45(6)** : 299-

305.

215. Mares D., Romagnoli C., Sacchett G., Vicentini C.B. and Bruni A. 2001. Antifungal activity of 5 new synthetic compounds vs *Trichophyton rubrum* and *Epidermophyton floccosum*. *Mycopathologia*. **153** : 129-132.

216. Marthanda M., Subramanyam M., Hima M and Annapurna J. 2005. Antimicrobial activity of derodane diterpenoids from *Polyalthia longifolia* seeds. *Fitoterapia*. **76** : 336.

217. Masoka P. and Eloff J.N. 2005. The diversity of antifungal compounds of six south African *Terminalia* species (Combretaceae) determined by bioautography. *Af. J. Biotechol.* **4(12)** : 1425-1431.

218. Mathew N., Paily K.P., Abidha V.P., Kalyanasundaram M. and Balaram K. 2002. Macrofilaricidal activity of the plant *Plumbago indica/rosea in vitro*. *Drug. Dev. Res*. **56(1)** : 33-39.

219. Matsura H., Saxena G., Farmer G.W., Hancock R.E.W. and Towers G.H.N. 1996. Antibacterial and antifungal polyine compounds from *Glehnia littoralis* spp.Leicarpa. *Planta. Med.* **62** : 256-259.

220. McDevitt J.T., Schneider D.M., Katiyar S.K. and Edlind T.D. 1996. Berberine: a candidate for the treatment of diarrhea in AIDS patients, abstr. 175. In Program and Abstracts of the 36th Interscience Conference on antimicrobial agents and chemotherapy. American Society for Microbiology, Washington, D.C.

221. McGowan J.E. 2001. Economic impact of antimicrobial resistance. *Emerg. Infec.* **7(2)** : 286-292.

222. Mednick A.J., Nosanchuk J.D. and Casadevall A. 2005. Melanization of *Cryptococcus neoformans* affects lung inflammatory responses during *Crptococcal* infection. *Infec. Immun.* **73** : 2012-2019.

223. Mendoza L., Wilkens M. and Urzua A. 1997. Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids isolated from some Chilean *Pseudognaphalium* (Asteraceae). *J. Ethnopharmacol.* **58** : 85–88.

224. Meng J.C., Hu Y.F., Chen J.H. and Tan R.X. 2001. Antifungal highly oxygenated guaianolides and other constituents from *Ajania fruticulosa*. *Phytochemistry*. **58** : 1141.

225. Meunier F. 1994. Future directions of antimycotic therapy. *Am. J. Med.* **37(2)** : 77-82.

226. Michaels S.H., Clark R., Kissinger P. 1999. Incidence and spectrum of AIDs defining illnesses among persons treated with antiretroviral drugs. *Clin. Infec. Dis.* **29** : 468–469.

227. Miles D.H., Madeiros D.J.M.R., Chittawong V., Swithenbank C., Lidert Z., Weeks J.A. Atwood J.L. and Hedin P.A. 1990. 3-formyl-2,4,6-trihydroxy-5 methyl dihydrochalcone a prospective new agrochemical from *Psidium acutangulum*. *J. Nat. Prod.* **53** : 1548-1551.

228. Ming K.J., Khang G.N., Sai C.l. and Fatt C.T. 2003. Recent advances in traditional plant drugs and orchids. *Acta. Pharmacol. Sin.* **24(1)** : 7-21.

229. Mironenko N.V., Alekhina I.A., Zhdanova N.N. and Bulat S.A. 2000. Intraspecific variation in gamma radiations resistence and genomic structure in filamentous fungus *Alternaria alternate* : a case study of strains inhabiting chemmobyl reactor No 4. *Ecotoxicol. Environ. Safety.* **45** : 177-187.

230. Mohsen I. and Hughes W.T. 1998. Systemic antifungal therapy: past, present and future. *Annals. Saud. Med.* **18(1)** : 28-38 .

231. Mongelli E. 2003. Recent advances in the search for antimycotic activity in South America. Plant derived antimycotics : Current trends and future prospects. Food products press : 192-211.

232. Moore C.B. 2000. Antifungal drug resistance in *Aspergillus*. *J. Infection.* **41** : 203-220

233. Moore C.W., McKoy J., Valle R.D., Armstrong D.B., Edward M., Katz N. and Gordon R.E. 2003. Fungal cell wall septation and cytokinesis are inhibited by bleomycins. *Antimicrob. Agents. Chemother.* **47(10)** : 3281–3289.

234. Moore C.W., Valle R.D., Mc.Koy J., Pramanik A. and Gordon R.E. 1992. Lesions and preferential initial localization of [S-methyl3H] Bleomycin A2 on *Saccharomyces cerevisiae* cell walls and membranes. *Antimicrob. Agents. Chemother.* **36(11)** : 2497-2505.

235. Morita H. and Itokawa H. 1998. Cytotoxic and antifungal diterpenes from seeds of *Alpinia galangal*. *Planta. Med.* **54** : 117-119.

236. Murray W.F., John E.L. and Thomas J.S. 1949. On napthoquinones as inhibitors of spore germination of fungi. *J. Biol. Chem.* 481-487.

237. Musial C.E, Cockerill F.R. and Roberts G.D. 1988. Fungal infections of the immunocompromised host: Clinical and laboratory aspects. *Clin. Microbiol. Rev.* **1(4)** : 349-364.

238. Nakamura C.V., Ishida K., Faccin L.C., Filho B.P., Cortez D.A., Rozental E., De Souza, W. and Ueda T. 2004. *In vitro* activity of essential oil from *Ocimum gratissimum* L. against four *Candida* species. *Res. Microbiol.* **52** : 3486.

239. Nash R. 2000. Plant extracts as medicines and sources of new therapeutic agents www.samedanltd.com. 8-12.

240. National committee for clinical laboratory standards. 1998. Reference method for broth dilution antifungal susceptibility testing of conidium forming filamentous fungi. Proposed standard M38-P.

241. Nguyen A.T., Malonne. H, Duez P., Vanhaelen F.R., Vanhaelen M. and Fontaine J. 2004. Cytotoxic constituents from *Plumbago zeylanica. Fitoterapia.* **75** : 500-504.

242. Nivoix Y., Velten M., Letsher B.V., Moghaddam A., Natrajan A.S., Fohrer C., Lioure B., Bilger K., Lutun P., Marcellin L., Launoy A., Freys G., Bergerat J.P., Herbrenct R. 2008. Factors associated with overall and attributable mortality in invasive aspergillosis. *Clin. Infec. Dis.* **47(9)** : 1167-84.

243. Nishiyama Y., Asagi Y., Hiratani T., Yamaguchi H., Yamada N. and Osumi M. 1992. Morphological changes associated with growth inhibition of *Trichophyton mentegrophytes* by amorolfine. *Clin. Exp. Dermatol.* **17(1)** : 13-17.

244. Nollin S.D. and Borgers D. 1975. Scanning electron microscopy of *Candida albicans* after *in vitro* treatment with Miconazole. *Antimicrob. Agents. Chemother.* **7(5)** : 704-711.

245. Nonaka D., Yfantis M.D.H, Southall P., Sun C.C. 2001. Pseudallescheriasis as an Aggressive Opportunistic Infection in a Bone Marrow Transplant Recipient. *Archives Pathol. and Lab. Med.* **126(2)** : 207–209.

246. Nosanchuk J.D. 2006. Current status and future therapy for systemic mycoses. *Recent Patents on Antiinfective Drug Discovery.* **1** : 75-84.

247. Nucci M. and Marr K.A. 2005. Emerging fungal diseases. *Clin. Infect. Dis.* **41** : 521-6.

248. O'Kennedy R. and Thornes R.D. 1997. Coumarins : biology, applications and mode of action. John wiley and sons, Inc, New York.358 p.

249. Oda N.M., Hargreaves L.P., Beckstead A.J, Redmond A.K., Antwerpen V.R. and Ryan O.R. 2006. Reconstituted high density lipoprotein enriched with the polyene antibiotic Amphotericin-B. *J. Lipid. Res.* **47** : 260-267.

250. Okonogi T., Hattori Z., Ogiso A. and Mitsui S. 1979. Detoxification by persimmon tannin of snake venoms and bacterial toxins. *Toxicon.* **75(5)** : 524-7.

251. Olli S. and Kirti P.B. 2007. Characterization of defensin (*Tfgd2*) from *Trigonella foenum-graecum*. *J. Biochem. Mol. Biol.* **39** : 278.

252. Oluwatoyin A., Binutu Kayoed, Adesogan E. and Joseph. I.O. 1996. Antibacterial and antifungal compounds from *Kigelia pinnata*. *Planta. Med.* **62** : 352-353.

253. Omulokoli E., Khan B. and Chhabra S.C. 1997. Antiplasmodial activity of four Kenyan medicinal plants. *J. Ethnopharmacol.* **56** : 133–137.

254. Orabi K.Y., Mossa J.S. and Elferaly F.S. 1996. Isolation and characterization of two antimicrobial agents from mace (*M.fragrans*). *J. Nat. Prod.* **54** : 856-859.

255. Ozkaya G., Shorbagi A. and Akova M. 2007. Fusarium species as an emerging and life threatening fungal pathogen in immunocompromised host. *Eur. Infec. Dis.* 63-64.

256. Paiva D., Paiva S.R, Figueiredo M.R., Aragao T.V. and Kaplan M.A.C. 2003. Antimicrobial activity *in vitro* of Plumbagin isolated from *Plumbago* species. *Mem. Inst Oswaldo. Cruz. Rio de. Janeiro.* **98(7)** : 959-961.

257. Paknikar S.K., Fondekar K.P.P., Kirtany J.K. and Natori S. 1996. 4-hydroxy-5-methylcoumarin derivates from *Diospyros kaki* Thunb and *D.kaki* var.sylvestris makino; structure and synthesis of 11-methylgerberinol. *Phytochemistry.* **41(3)** : 931-933 .

258. Pal S.K. and Shukla Y. 2003. Herbal medicine : Current status and the future. *Asia. Pac. J. Can. Prev.* **4** : 281-287 .

259. Park M.J., Gwak K.S., Yang I., Kim K.W., Jeung E.B., Chang J.W., Choi I.G. 2009. Effect of citral, eugenol, nerolidol and α-terpineol on the ultrastructural changes of *Trichophyton mentagrophytes*. *Fitoterapia*. 01805 : 1-7.

260. Park Y., Choi B.H., Kwak J.S., Kang C.W., Lim H.T., Cheong H.S. and Hahm K.S. 2005. Kunitz-Type Serine Protease Inhibitor from Potato *Solanum tuberosum* L. cv. Jopung . *Agric. Food Chem.* **53** : 6491.

261. Patel R. and Paya V.C. 1997. Infections in solid-organ transplant recipient. *Clin. Microbiol. Rev.* **10** : 86–124.

262. Patterson T. 2003. Antifungal resistance how big is the problem. *ISAAR* : 78-80.

263. Patwardhan B., Vaidya A.D.B. and Chorghade M. 2004. Ayurveda and natural products drug discovery. *Curr. Sci.* **86(6)** : 789-799 .

264. Pauw B.D. 2000. Is there a need for new antifungal agents? *Clin. Microbiol and Infect. Dis.* **6(2)** : 23-28.

265. Pepeljnjak S., Kosalec I., Kalodera Z. and Kustrak D. 2003. Natural antimycotics from Crotian plants. Plant derived antimycotics current trends and future prospects. Plant derived antimycotics : current trends and future prospects. Food products press .49-79.

266. Pereira M.E.A. 1983. A developmentally regulated neuraminidase activity in *Trypanosoma Cruzi*. *Science*. **219** : 1444-1446.

267. Perkin D.S. 2007. Resistance to Echinocandin class antifungal drugs. Drug Resistance updates. **10** : 121-130.

268. Perkins J. 2008. Fatal drug induced immune hemolytic anemia due to cefotetan : a case study. *Asian* an examination of quinone toxicity using the yeast *Saccharomyces cerevisiae* model system. *J. Transf. Sci.* **2(1)** : 20-23.

269. Perry N.B., and Foster L.M. 1994. Antiviral and antifungal flavonoids plus a triterpene from *Hebe cupressoides*. *Planta. Med.* **60** : 491-494.

270. Petrikkos G. and Skiada A. Recent advances in antifungal chemotherapy. *Antimicrob. Agents. Chemother.* **30** : 108–117

271. Pfaller M.A. and Diekema D.J. 2004. Rare and emerging oppurtunistic fungal pathogens: concerns for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.* **42(10)**, 4419-4431.

272. Phongpaichit S., Pujenjob N., Rukachaisirikul V. and Ongsakul M. 2005. Antimicrobial activities of the crude methanol extract of *Acorus calamus* Linn. *J. Sci. Technol.* **27(2)** : 518-522.

273. Plaza A., Cinco M., Tubaro A., Pizza C. and Piacente S. 2003. New triterpene glycosides from the stems of *Anomospermum grandifolium* . *J. Nat. Prod.* **66** : 1606.

274. Polak A. 1999. Past, present and future of antimycotic combination therapy. *Mycoses.* **42** : 355-370.

275. Portillo M.C., Viramontes S., Munoz L.N., Gastelum M.G. and Nevarez G.V.J. 2005. Antifungal activity of Mexican oregano (*Lippia berlandieri shauer*) Food Prot, **68**. 2713.

276. Poul, B.N., Mukadam D.S., Dama L.B. and Jadhav B.V. 1999. Bioactive spectra of Plumbagin. *Asian. J. Chem.* **11(1)** : 144-148.

277. Powderly W.G., Mayer K.H., Perfect J.R. 1999. Diagnosis and treatment of oropharingeal candidiasis in patients infected with HIV: a critical reassessment. *AIDS Research and Human Retroviruses.* **15** : 1405–1412.

278. Putheti R. and Okigbo R.N. 2008. Effects of plants and medicinal plant combinations as anti-infectives. *African J. Pharm. Pharmacol.* **2(7)** : 130-135.

279. Prasad R., Panwar S.L. and Smriti. 2002. Drug reistance in yeasts- an emerging scenario. *Adv. Microbial. Physiol.* **46** : 155-201.

280. Prajoubklang A., Sirithunyalug B., Charoenchai P., Suvannakad R., Sriubolmas N., Piyamongkol S., Kongsaeree p., Kittakoo P. 2005. Bioactive deoxypreussomerins and dimeric napthoquinones from Diospyros ethretioides fruits : eoxypreussomerins may not be plant metabolits but may be from fungal epiphytes. *Chem. Divers.* **2(10)** : 1358-67.

281. Pyun M.S. and Shin S. 2006. Antifungal effects of the volatile oils from allium plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytochemistry.* **13** : 394.

282. Ragasa C.Y., Co A.L. and Rideout J.A. 2005. Antifungal metabolites from *Blumea balsamifera. Nat. Prod. Res.* **19** : 231.

283. Rana B.K., Singh U.P. and Taneja V. 1997. Antifungal activity and kinetics of

inhibition by essential oil isolated from leaves of *Aegle marmelos*. *J. Ethnopharmacol.* **57(1)** : 29-34.

284. Randhawa H.S. and Chaowdhary. 2008. Medical mycology in India. *Ind. J. Chest. Dis. All. Sci.* **50** : 25-32.

285. Randhawa G.K. and Sharma G. 2004. Echinocandins : A promising new antifungal group. *Indian. J. Pharmacol.* **36(2)** : 65-71.

286. Rapp R.P. 2004. Changing strategies for the management of invasive fungal infections. *Pharmacother.* **24(2)** : 4-28.

287. Raskin I. and Ripoll C. 2004. Can an apple a day keep the doctor away? *Curr. Pharm. Des.* **10** : 1-9.

288. Raskin I., Ribnicky D.M.K., Llic N., Poulev A., Borisjuk N., Brinker A., Moreno D.A Ripoll C., Yakoby N., O'Neal J., Cornwell T., Pastor I. and Fridlender B. 2002. Plants and human health in the twenty first century. *Trends. in. Biotechnol.* **20(12)** : 522-530.

289. Ratnaraja N. 2006. Fungal infections causes and diagnosis. *Hospital pharmacist.* 3313-319.

290. Renault S., DeLucca A.J., Bove S., Bland J.M, Vigo C.B. and Selitrenikoff C.P. 2003. CAY-1, a novel antifungal compound from cayenne pepper. *Med. Mycol.* **4** : 75.

291. Rex J.H., Rinaldi G.M. and Pfaller A.M. 1995. Resistance of *Candida* species to fluconazole. *Antimicrob. Agents and Chemother.* **391** : 1-8.

292. Riffel A., Medina L.F., Stefani V., Santos R.C., Bizani D. and Brandelli A. 2002. *In vitro* antimicrobial activity of a new series of 1,4 napthoquinones. *Braz. J. Med. Biol. Res.* **35** : 811-818.

293. Rippon J.W. 1982. Medical Mycology : The pathogenic fungi and the pathogenic actinomycetes. Elseivier health sciences.

294. Rippon J.W. and Fromtling A.R. 1993. Cutaneous antifungal agents : selected compounds in clinical practice and development. Cutaneous antifungal agents: *Informa Health Care.*

295. Rodriguez C.E., Shinyashiki M., Froines J., Yu R.F.C, Fukuto J.M, and Cho A.K. 2004. An examination of quinone toxicity using the yeast *Saccharomyces*

cerevisiae model system. *Toxicology*. **201(1-3)** : 185-196.

296. Rojas R., Bustamante B., Ventosilla P., Fernandez I., Caviedes L., Gilman R.M., Lock O. and Hammond G.B. 2006. Larvicidal, antimycobacterial and antifungal compounds from the bark of the peruvian plant *Swartzia polyphylla* DC. *Chem. Pharm. Bull.* **54** : 278.

297. Romagnoli C., Bruni R., Andreotti E., Rai M.K., Vicentini C.B. and Mares D. 2005. Chemical characterization and antifungal activity of essential oil of capitula from wild Indian *Tagetes patula* L. *Protoplasma*. **225** : 57.

298. Ruhnke M. 2006. Epidemiology of Candida albicans infections and role of non-candida-albicans yeasts. *Curr. Drug. Targets*. **7(4)** : 495- 504.

299. Sa Y.S., Kim S.J. and Choi H.S. 2005. The anticoagulant fraction from the leaves of *Diospyros kaki* L. has an antithrombotic activity. *Arch. Pharm. Res.* **28(6)** : 667-74.

300. Saad H.E.A., Sharkaroy S.H.E and Shier W.T. 1995. Biological activity of pyrrolidinoindolin alkaloids from *Calycodendron milnei. Planta. Med.* **61** : 313-316.

301. Sachetti G., Medici A., Maietti S., Radice M., Muzzoli M., Manfredini S., Braccioli E. and Bruni R. 2004. Composition and functional properties of the essential oil of amazonian basil, *Ocimum micranthum* Willd., Labiatae in comparison with commercial essential oils. *J. Agric. Food. Chem.* **52** : 3530.

302. Saif M.W. and Raj M. 2006. Cryptococcal peritonitis complicating hepatic failure : Case report and review of the literature. **6(1)** : 43-50.

303. Sakanaka S., Kim M., Taniguchi M. and Yamamoto T. 1989. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans,* a cariogenic bacterium. *Agric. Biol. Chem.* **53** : 2307–2311.

304. Sakanaka S., Shimura N., Aizawa M., Kim M. and Yamamoto T. 1992. Preventive effect of green tea polyphenols against dental caries in conventional rats. *Biosci. Biotechnol. Biochem.* **56** : 592–594.

305. Salama A., Burger M., Eckhardt C. and Mueller. 1989. Acute immune hemolysis induced by a degradation product of amphotericin B. *Blut.* **58** : 59-61.

306. Salgueiro L.R., Piato E., Goncalves M.J., Pina C., Cavaleiro C, Rodrigues A.G.,

Palmeira A., Tavares C., Costa S. and Martinez. 2004. Chemical composition and antifungal activity of the essential oil of *Thymbra capitata*. *Planta. Med.* **70** : 572.

307. Samy R.P. and Gopalakrishnakone P. 2007. Current status of herbal and their future perspectives. Nature proceedings. **21** :1176-1179.

308. Samy R.P. and Gopalakrishnakone P. 2008. Therapeutic potential of plants as antimicrobials for drug discovery. eCAM Advance Access doi:10.1093/ecam/nen036. **5** : 1-12.

309. Sanchelle M., Dargent R., Weele J.D., Rushing A.E., Miller K.S. and Montant C. 1988. Effects of triazoles on fungi VI. Ultrastructure of *Taphrina deformans*. *Mycologia*. **80(2)** : 162-175.

310. Sandur S.K., Ichikawa H., Sethi G., Anh K.S. and Aggarwal B.B. 2006. *Plumbagin*(5-hydroxy-2-methyl-1,4-naphthoquinone) suppressesNF-B activation and NF-B-regulated gene products through modulation of p65 and kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agent. *J. Biol. Chem.* **281(25)** : 17023–17033.

311. Sangamwar A.T. , Deshpande U.D., Pekamwar S.S. 2008. Antifungals : Need to search for a new molecular target. *Indian. J. Pharm. Sci.* **70(4)** : 423-430.

312. Sanglard D. 2002. Resistance of human fungal pathogens to antifungal drugs. *Curr. Opinion. in Microbiol.* **5** : 379-385.

313. Sanjay M.J. and Arvind S. 2007. Challenges and opportunities in drug discovery from plants. *Curr. Sci.* **92(9)** : 1251-1257.

314. Sarma M.D., Ghosh R., Patra A. and Hazra B. 2008. Synthesis of novel aminoquinoid analogues of diospyrin and evaluation of their inhibitory activity against murine and human cancer cell. *Eur. J. Med. Chem.* **43** : 1878-1888.

315. Sastry B.N. 1952. The wealth of India, Raw materials, vol III. CSIR, New Delhi, p136.

316. Sawaya B.P., Briggs J.P. and Schnermann J. 1995. Amphotercin B nephrotoxicity: The adverse consequences of altered membrane properties. *J. Am. Soc. Nephrol.* **6(2)** : 154-162.

317. Scalbert A. 1991. Antimicrobial properties of tannins. *Phytochemistry*. **30** :

3875–3883.

318. Sepulveda B.S. and Cassels B.K. 1996. Plant metabolites active against *Trypanosoma cruzi*. *Planta. Med.* **62** : 98-105.

319. Sharma N. and Tripathi A. 2006. Effects of *Citrus sinensis* (L) *Osbeck epicarp* essential oil on growth and morphogenesis of *Aspergillus niger* (L) van Tieghem. *Microbiol. Res.* **163(3)** : 1-8.

320. Senoy S.R., Kameshwari M.N., Swaminathan S. and Gupta M.N. 2006. Major antifungal activity from the bulbs of Indian squill *Urginea indica* is a chitinase. *Biotehnol. Prog.* **22** : 631.

321. Shin K.S., Samkeunlee and Cha B. 2007. Antifungal activity of *Plumbagin* purified from leaves of *Nepenthes ventricosa* x maxima against phytopathogenic fungi. *Plant. Pathol. J.* **23(2)** : 113-115.

322. Shinji F. and Hiroshi H. 1979. Hypotensive principles of *Diospyros kaki* leaves. *Chem. Pharm. Bull.* **27(11)** : 2865-2868 .

323. Sidhu G.S. and Prasad K.K. 1967. (-) Isodiospyrin a novel binapthaquinone showing atropisomerism and other extractives from *D. Chloroxylon*. *Tetrahedron. Lett.* **30** : 2905- 2910.

324. Simic A., Sokovic M.D., Ristic M., Grujic S., Vukojevic J. and Marin P.D. 2004. The chemical composition of some Lauraceae essential oils and their antifungal activities. *Phytother. Res.* **18** : 713.

325. Simon T. 1998. Dietary Persimmon improves lipid metabolism in rats fed diets containing Cholesterol. *J. Nut.* **9** : 2023-2027.

326. Simonsen H.T., Nordskjold J.B., Smitt U.W., Nyman U., Palpu P., Joshi P. and Varughese G. 2001. *In vitro* screening of Indian medicinal plants for antispasmodial activity. *J. Ethnopharmacol.* **74(2)** : 195-204.

327. Sinyoung K.D., Kyung S.S, Lim H. and Mo L.H. 2002. Ceftrioxone induced immune hemolytic anemia of drug dependent antibody by *ex vivo* antigen in urine. *Yonsei. Med. J.* **43(3)** : 391-394.

328. Sionova E., Rotha D., Sandovsky L.H., Kashmanb Y., Rudib A., Chillb L., Berdicevskyc I., Segala E. 2005. Antifungal effect and possible mode of activity

of a compound from the marine sponge *Dysidea herbacea*. *J. Infec.* **50** : 453–460.

329. Skaltsa H., Lazari D., Panagouleas C., Georgiadov E., Garcia B. and Sokovic M. Sesquiterpene lactones from *Centaurea thessala* and *Centaurea attica* antifungal activity. 2000. *Phytochemistry*. **55** : 903.

330. Slobodnikova L., Kost'alova D., Labudova D., Kotulova D. and Kettmann V. 2004. Antimicrobial activity of *Mahonia aquifolium* extract examined. *Phytother. Res*. **18** : 674.

331. Sonboli A., Salehi P. and Yousefzadi M.Z. 2004. Antimicrobial activity and chemical composition of the essential oil of *Nepeta crispa* Willd. from Iran. *Naturforsch*. **59** : 653.

332. Sotonaphun U., Lipipun V., Suttisri R. and Bavovada R. 1999. A new antiviral and antimicrobial sesquiterpene from *Glycopetalum sclerocarpum*. *Planta. Med.* **65** : 257-258.

333. Srinivas P., Gopinath G., Banerji A., Dinakar A. and Srinivas G. 2004. Plumbagin induces reactive oxygen species which mediate apoptosis in human cervical cancer cells. *Mol. Carcinog*. **40(4)** : 201-211.

334. Stern, J.L., Hagerman A.E., Steinberg P.D. and Mason P. K. 1996. Phlorotannin-protein interactions. *J. Chem. Ecol*. **22** : 1887–1899.

335. Steriopoulos L., Zwiers L.H. and Waard M.A. 2002. Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP binding cassette and major facilitator superfamily. *Eur. J. Plant. Pathol.* **108** : 719-734.

336. Stuart A.E. 1998. Antifungal effect of oil from leaves of *Myrica gale*. *Planta. Med*. **55** : 584.

337. Sun H.D., Qiu S.X., Lin L.Z., Wang Z.W., Lin Z.W., Pengsuparp T., Pezzuto J.M., Fong H.H., Cordell G.A. and Farnsworth N.R. 1996. Nigranoic acid, a triterpenoid from *Schisandra sphaerandra* that inhibits HIV-1 reverse transcriptase. *J. Nat. Prod*. **59** : 525–527.

338. Suraveratum N., Krungkrai S.R., Leangaramgul P., Prapunwattana P. and Krungkai J. 2000. Purification and characterization of *Plasmodium falciparum*

succinate dehydrogenase. *Mol. Biochem. Parasitol.* **105(2)** : 215-222.

339. Suresh B., Sriram S., Dhanaraj S.A, Elango K., and Chinnaswamy K. 1997. Anticandidal activity of *Santolina chamaecyparissus* volatile oil. *J. Ethnopharmacol.* **55** : 151–159.

340. Suzuki I.K., Itoh S. and Tsuyuki H. 1982. Lipids in persimmon.II. Complex lipids of the seeds of persimmon. *Nippon. Shokuhin. Kongo. Gakkaishi.* **29(5)** : 310-15.

341. Svetaz L.,Tapia A.,Lopez S.N., Furlan R.L., Petenatti E., Pioli R., Schmeda G. and Zacchino S.A. 2004. Antifungal chalcones and new caffeic acid esters from *Zuccagnia punctata* acting against soybean infecting fungi . *J. Agric. Food. Chem.* **52** : 3297.

342. Tadhani M.B. and Subhash. R. 2006. *In vitro* antimicrobial activity of Stevia *rebaudiana bertoni* leaves. *Trop. J. of Pharm. Res.* **5(1)** : 557-560.

343. Taira T., Ohdomari. A., Nakama N. and Shimoji M.A. 2004. Purification, characterization, and antifungal activity of chitinases from pineapple (*Ananas comosus*) leaf. *Lett. Appl. Microbiol.* **39** : 7.

344. Takemoto K., Yamamoto Y. and Ueda Y. 2006. Evaluation of antifungal pharmacodynamic characteristics of Ambiosome against *Candida albicans*. *Microbiol. Immunol.* **50(8)** : 579-586.

345. Takuji T., Haruo S., Ryoichi N.A., Akira N.M., Koichi K. and Hajime O. 1999. Immunomodulatory actions of citrus auraptene and cytokine production of lymphocyte in female BALB/cmice. *Carcinogenesis*. **20** : 1471-1476.

346. Taylor R.S.L., Edel F., Manandhar N.P. and Towers G.H.N. 1996. Antimicrobial activities of southern Nepalese medicinal plants. *J. Ethnopharmacol.* **50** : 97-102.

347. Tezuka M., Kuroyanagi M., Yoshihira K. and Natori S. 1972. Napthaquinone derivatives from *Diospyros kaki* THUNB and *D. kaki* THUNB. var. *sylvestris* MAKINO. *Chem. Pharm. Bull.* **20(9)** : 2029-2035.

348. ThomsonW.A.R.(ed.). 1978. Medicines from the earth. McGraw-Hill Book Co., Maidenhead, United Kingdom. P 669.

349. Thongnest S., Mahidol C., Sutthivaivakit S. and Ruchirawat S. 2005.

Oxygenated pimarane diterpenes from *Kaempferia marginata*. *J. Nat. Prod.* **68** : 1632.

350. Ting C., Hsu C., Hsu H., Su J., Chen T., TarnW., Kuo Y., Whang P., Liu L.F. and Hwang 2003. Isodiospyrin as a novel human topoisomerase inhibitor. *Biochem. Pharmacol.* **66(10)** : 1981-1991.

351. Tirillini B., Velasquez E.R. and Pellegrim R. 1996. Chemical composition and antimicrobial activity of essential oil from *Piper angustifolium. Planta. Med.* **62** : 372-374.

352. Turner D.M. 1996. Natural product source material use in the pharmaceutical industry: the glaxo experience. *J. Ethanopharmacol.* **51**: 39-44

353. TLC Visualization reagents. www.emdchemicals.com. Page 1-14.

354. Toyota M., Msonthi J.D. and Hostettmann K. 1990. A molluscidal and antifungal triterpenoid saponin from the roots of *Clerodendron wildii*. *Phytochemistry*. **29** : 2849- 2851.

355. Tripathi R.D., Srivastava H.S. and Dixit S.N. 1978. A fungitoxic principle from the leaves of *Lawsonia inermis*. *Cell. Mol. Sci.* **34(1)** : 51-52.

356. Uchida S., Ohta H., Niwa M., Mori A., Nonaka G. and Ozaki M. 1990. Prolongation of life span of stroke prone spontaneously hypersensitive rats (SHRSP) ingesting persimmon tannin. *Chem. Pharm. Bull.* **38(4)** : 1049-1052.

357. Vaisaraj R., Pushpangadan P., Smitt U.W., Adsersen A. and Nyman U. 1997. Antimicrobial screening of selected medicinal plants from India. *J. Ethnopharmacol.* **58** : 75-83.

358. Vandenberg K.P., Rouge P., Proast P., Coosemans J., Krouglova T., Engelborghs Y., Perumans W.J. and Van Damme E.J. 2004. Synergistic antifungal activity of two chitin-binding proteins from spindle tree *Euonymus Europaeus* L. *Planta.* **219** : 221.

359. Verpoorte R. 1998. Exploration of natures chemodiversity: the role of secondary metabolites as leads in drug development., *Drug. Discov. Today.* **3(5)** : 232-238.

360. Vila R., Valenzuela L., Bello H., Canigneria S., Montes M. and Adset T. 1999. Composition of antimicrobial activity of essential oil of *Pneumus boldus* leaves. *Planta. Med.* **65** : 178-181.

361. Viljoen A.M., Subramoney S., Van V.S.F., Baser K.H. and Demirci B.J. 2005. The composition, geographical variation and antimicrobial activity of *Lippia Javanica* (*Verbenaceae*) leaf essential oils. *J. Ethanopharmacol.* **96** : 271.

362. Wahab S.M.A., Aboutabe E.A., Zalabani S.M.E., Fouad H.A., Pooter H.L.D. and Fallaha B.E. 1987. The essential oil of *Olibanum. Planta. Med.* **53** : 382-384.

363. Wang Y., Aisen P. and Casedevall A. 1995. *Cryptococcus neoformans* melanin and virulence : mechanism of action. *Infec. Immun.* **63** : 3131-3136.

364. Wanke B., Lazéra D.M.S. and Nucci M. 2000. Fungal infections in the immunocompromised host. *Mem. Inst .Oswaldo. Cruz, Rio de. Janeiro.* **95** : 153-158.

365. Weissenberg M., Meisner J., Klein M., Schaeffler I., Eliyahu M., Schmutterer H., and Ascher K.R.S. 1997. Effect of substituent and ring changes in naturally occurring naphthoquinones on the feeding response of larvae of themexican bean beetle, *Epilachna varivestis. J. Chem. Ecol.* **23(1)** : 3-18.

366. White T.C., Marr K.A. and Bowden R.A. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11(2)** : 382-402.

367. Wild R. 1994. The complete book of natural and medicinal cures. Rodale Press, Inc, Emmans, Pa. 784.

368. Wingard J. 2006. Zygomycosis: epidemiology and treatment options. *J. Infec. Dis.* **6** : 526-530.

369. Wong J.H. and Ng T.B. 2005. Vulgarinin, a broad-spectrum antifungal peptide from haricot beans *Phaseolus vulgaris. Int. J. Biochem. Cell. Biol.* **37(8)** : 1626-1632.

370. Wube A.A., Streit. B., Gibbons S., Asres K. and Bucar F. 2005. In vitro 12(s) HETE inhibitory activities of napthoquinnes isolated from the root bark of *Euclea racemosa* spp. schimperi. *J. Ethanopharmacol.* **02(2)** : 191-196.

371. www. cfsph.iastate.edu. 2005. Dermatophytosis. 12-16.

372. Xi. H.C., Feng L., Zhen L. and Zu C.Z. 2003. Purification and characterization of moschatin, a novel type I ribosome-inactivating protein from the mature seeds of pumpkin (*Cucurbita moschata*), and preparationof its immunotoxin against

human melanoma cells. *Cell. Res.* **13(5)** : 369-374.

373. Xu H.X., Zeng F.Q., Wan M. and Sim K.Y. 1996. Anti-HIV triterpene acids from *Geum Japonicum*. *J. Nat. Prod.* **59** : 643–645.

374. Yadava R.N., Jain S. 2004. A novel bioactive flavonol glycoside from *Teramnus labialis* spreng. *Nat. Prod. Res.* **54** : 278.

375. Yamada Y., Yamamato A., Yoneda N. and Nakatani N. 1999. Identification of kaempferol from leaves of *Diospyros kaki* and its antimicrobial activity against *Streptococcus mutants*. *Biocontrol. Sci.* **4(2)** : 97-100.

376. Yan Q., Jiang Z., Yang S., Deng W. and Han L. 2005. Novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity. *Arch. Biochem. Biophys.* **442** : 72.

377. Yan X.Z., Kuo Y.H., Lee T.J., Shih T.S., Chen C.H., Mcphail D.R., Mcphail A.T. and Lees K.H. 1989. A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity. Cytotoxic components of *Diospyros morrisiana*. *Phytochemistry*. **28(5)** : 1541-1543.

378. Yang Y.L., Lo H.J. 2001. Mechanism of antifungal drug resistance. *J. Microbiol. Immunol. Infec.* **34** : 79-86.

379. Yasumasa Y., Aya Y., Yoneda A., Noriko Y., Nobuji N. 1999. Identification of kaempferol from the leaves of Diospyros kaki and its antimicrobial activity against streptococcus mutans. *Biocontrol. Sci.* **4(2)** : 97-100.

380. Yamaguchi H. 2003. Current and emerging antifungal agents in Japan. *Kor. J. Med. Mycol.* **8(2)** : 35-42.

381. Yemele M., Krohn K., Hussain H., Dongo E., Schulz B and Hu Q. 2006. Tithoniamarin and tithoniamide: a structurally unique isocoumarin dimer and a new ceramide from *Tithonia diversifolia*. *Nat. Prod. Res.* **20** : 842.

382. Yenesew A., Derese S., Midiwo J.O., Bii C.C., Heydenreich M. and Peter M.G. 2005. Antimicrobial flavonoids from the stem bark of *Erythrina burttii*. *Fitoterapia*. **76** : 469.

383. Yokose T., Katamoto K., Park S., Matsuura H., Yoshihira T. 2004. Anti-fungal sesquiterpenoid from the root exudate of *Solanum abutiloides*. *Biosci. Biotechnol. Biochem.* **68** : 2640.

384. Yoshihara K., Tezuka M. and Natori S. 1971. Napthoquinone derivates from ebenaceae. isodiospyrin, bisisodiospyrin and mamegakinone from *Diospyros lotus* and *D. morrisiana*. *Chem. Pharm. Bull.* **19(11)** : 2308-2313.

385. Zacchino S., Rodriguez G., Santecchia C., Pezzanati G., Giannini F. and Euriz R. 1998. *In vitro* studies on mode of action of antifungal 8-0-4 neolignans occurring in certain species of varola and related genera of Myristicaceae. *J. Ethnopharmacol.* **62** : 35-41.

386. Zheng W.F., Tan R.X., Yang L. and Liu Z.I. 1996. Two flavones from *Artemisia giraldii* and their antimicrobial activity. *Planta. Med.* **62** : 160-162.

387. Zhong S. and Feng S. 1987. Napthoquinones and a triterpene from stem of Diospyros kaki var sylvestris. *Zhongguo. Yaoke. xuebao.* **18(4)** : 279-80.

388. Jachak S.M. and Saklani A. 2007. Challenges and oppurtunities in drug discovery from plants. *Curr. Sci.* **92(9)** : 1251-1257.

389. Mallavadhani U.V., Panda A.K. and Rao Y.R. 1998. Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry.* **49(4)** : 901-951.

390. Weijian B., Wenlie P. and Yan M. and Anlong X. 2005. Flavonoids from the leaves of *Diospyros kaki* reduce hydrogen peroxide induced injury of NG108-15 cells. *Life. Sci.* **38(4)** :1049-1052.

391. Wrangler,1996. TLC visualization reagents. www.emd chemicals.com : 1-14.