"Screening of endophytic fungi for antimicrobial activity"

A thesis submitted in partial fulfillment of the requirement

for the award of the degree of

Masters of Science

In

Biotechnology

Submitted By

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DECLARATION

I, hereby declare that the work which is being presented in the dissertation entitled "Screening of endophytic fungi for antimicrobial activity", in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab, is an authentic record of my own work during a period of six months from January 2012 to July 2012, under the supervision of Mrs. M. Vasundhara, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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This is to certify that the above statements made by the student are correct and true to the best of our knowledge and belief.

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This is to certify that the thesis entitled "Screening of endophytic fungi for antimicrobial activity" submitted by Shivani, Roll no. 301001024 in partial fulfillment of the requirement for the award of the Degree of Masters of Sciences in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University.

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LIST OF ABBREVIATIONS

%	Percent
µl	Microlitre
°C	Degree centigrade
hr.	Hour
µg	Microgram
ml	Milliliter
mg	Milligram
CFU	Colony forming unit
EtOAC	Ethyl acetate
SDA	Sabouraurd Dextrose Agar
SDB	Sabouraud Dextrose Broth
LB	Luria Broth
LA	Luria Agar
NB	Nutrient Broth
NA	Nutrient Agar
MHA	Muller Hinton Agar
PDB	Potato Dextrose Broth
MEB	Malt Extract Broth
PDA	Potato Dextrose Agar

w/v	Weight by Volume
Sp.	Species
pH	Potential of hydrogen ion
Temp.	Temperature
rpm	Rotation per minute
E.coli	*Escherichia coli*
P.aeruginosa	*Pseudomonas aeruginosa*
S.aureus	*Staphylococcus aureus*
B.megaterium	*Bacillus megaterium*
C.albicans	*Candida albicans*
A.fumigatus	*Aspergillus fumigatus*
TTC	2,3,5-triphenyltetrazolium chloride
Rf	Retardation Factor
nm	Nanometer
O.D.	Optical Density
MIC	Minimum Inhibitory Concentration
H_2SO_4	Sulphuric acid
$FeCl_3$	Ferric chloride

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INTRODUCTION

CHAPTER-1

INTRODUCTION

Fungi are ubiquitous occurring, eukaryotic, heterotrophic organisms. Fungal life is found worldwide, in soil samples as well as deep sea vents and arctic ice, and often reveals symbiotic traits. Similar to plants, there is a long history of the utilization of fungi by mankind as remedies and in everyday life. Nearly 3000 years ago the Mayans used fungi to treat intestinal ailments (Strobel *et al*. 2004). After the discovery of penicillin isolated from *Penicillium notatum* by Sir Alexander Fleming in 1928 resulted in a breakthrough in the treatment of bacterial infections, that fungi became an important source of drugs for the treatment of a variety of diseases. Since then, especially fungi isolated from soil samples have been identified as a rich source of biologically active secondary metabolites. Besides other well known antimicrobial agents like griseofulvin (Grove *et al*. 1952), novel semi synthetic antifungal drugs like anidulafungin and caspafungin are likewise derived from fungal metabolite. With the discovery of cyclosporine isolated from *Tolypocladium inflatum* in 1971, an important step in immune pharmacology was made because this substance prevents rejection after organ or tissue transplantations. Cyclosporine exhibits, in addition to its potent immunosuppressant activity pronounced antiviral activity. Probably the most economical important fungal metabolites represent anti-lipidemic drugs collectively known as "statins", with their parent compounds mevastatin and lovastatin isolated from *Penicillium citrinum* and *Aspergillus terreus*, respectively.

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, negative effects (Bacon *et al.* 2000). As almost all vascular plant species appear to be inhabited by endophytic bacteria or fungi, these represent important components of microbial diversity. The relationship between the host plant and its endophyte shows symbiotic characteristics as the endophytic fungi usually obtains nutritients and protection from the host plant and in return profoundly enhances the fitness of the host by producing certain functional metabolites. Still, if the host plant is weakened, the endophyte can also become an aggressive saprophyte and thereby reveal the smooth transition between symbiont and opportunistic pathogen. Fungal endophytes are a group of primarily ascomycetous fungi, whereas basidio-

mycetes, deuteromycetes and oomycetes are rarely found (Arnold *et al.* 2007). Although they do not show host specificity, certain fungal lineages appear with greater frequency in plants representing particular families and thus denote host preference (Cannon and Simmons 2002, Arnold *et al.* 2007). Consistent with the tremendous diversity of endophytic fungi and their ecological roles is the astounding chemical variety of their secondary metabolites, which often display promising pharmaceutically or agro chemically exploitable activities when tested in various bioassays. Due to the world's urgent need for new antibiotics, chemotherapeutic agents and agrochemicals to cope with the growing medicinal and environmental problems facing mankind, growing interest is taken into the research on the chemistry of endophytic fungi.

The *Xylariaceae* are family of mostly small ascomycetous fungi. It is one of the most commonly encountered groups of ascomycetes and is found throughout the temperate and tropical regions of the world. They are typically found on wood, seeds, fruits, or plant leaves, some even associated with insect nests. Most decay wood and many are plant pathogens.

Scientific Classification

Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	*Xylariales*
Family	*Xylariaceae*

The Ascomycota are division of the kingdom fungi. Its members are commonly known as the sac fungi. They are the largest phylum of Fungi with over 64,000 species (Kirk *et al*. 2008). The defining feature of this fungal group is the ascus, a microscopic sexual structure in which non motile spores, called ascospores are formed. The Sordariomycetes are a class of fungi in the subdivision ascomycota. Sordariomycetes generally produce their asci in perithecal fruiting bodies. Sordariomycetes are also known as Pyrenomycetes, because of the usually somewhat tough texture of the tissue. Sordariomycetes possesses great variability in morphology, growth form and habitat. Except having perithecial (flask-shaped) fruiting bodies, ascomata can be less frequently cleistothecial fruiting bodies may be solitary or gregarious, superficial or immersed

within stromata or tissues of the substrates and can be light to bright or black. Members of this group can grow in soil, dung, leaf litter, and decaying wood as decomposers, as well as being fungal parasites, and insect, human and plant pathogens (Neuveglise *et al*. 1994, Berbee *et al*. 1992, Spatafora *et al*. 1995).

Objectives of this project

Endophytes are poorly investigated group of microorganisms, but they represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in wide variety of medicine, agriculture and industrial areas (Vanden *et al*. 1991). The objective of this project was to determine the antibacterial, antifungal activity and preliminary phytochemical analysis of fungal culture extract from *Xylaria* species.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER-2

REVIEW OF LITERATURE

2.1 Endophytic fungi as a source of bioactive compounds

Inmaculada *et al*. (2000) assayed 317 isolates of basidiomycetes representing 204 species collected in Spain against a range of human clinical pathogens and laboratory controls. The proportion of extracts from basidiomycetes shown antimicrobial activity (Anke *et al.* 1989) was similar to or above that obtained for representative orders of Ascomycetes, such as Pezizales and Xylariales, but lower than that produced by members of the orders Diaporthales, Eurotiales, Hypocreales, Leotiales & Sordariales. Suprageneric taxa (orders and families) did not show pronounced differences in their antimicrobial activities though such differences were observed at the genus level (Pelaez *et al.* 1998), suggesting that the ability to produce these bioactive compounds is not homogenously distributed amongst the basidiomycetes. From study it was concluded that extracts from 45% of the isolates, representing 109 species, showed antimicrobial activity. Antibacterial activity was more pronounced than antifungal activity. Isolates from some species showed large differences in their ability to produce metabolites with antimicrobial activity, possibly reflecting genetic differences at the infraspecific level.

Between 1987 and 2000 approximately 140 new natural products were isolated from endophytic fungi (Zhang *et al*. 2006).

Jalgaonwala *et al*. (2010) assayed 78 endophytic bacterial isolates and 142 endophytic fungal isolates from various parts of medicinal plants (Fischer 1984) belonging to Jalgaon Maharashtra (India) for evaluation of antimicrobial activity against several pathogenic and opportunistic microorganisms. The results indicated that fifteen bacterial isolates and seventy eight fungal isolates exhibited antimicrobial activity.

Xia Yan *et al.* (2011) the antibiotic-producing potential of endophytic populations from medical plant of *Salvia miltiorrhiza* was examined (Zhang *et al.* 1997). A total of 63 isolates was screened against five fungal and three bacterial species for the production of antimicrobial

compounds. It showed that more isolates was antagonistic to fungi than to bacteria. One bacterial isolate named DX30 showed broad-spectrum of antagonistic activity against all the target organisms in plate assays. Morphology, physiological, chemical characteristics and 16S rRNA gene sequence analysis of strain DX30 demonstrated that it belonged to the genus *Bacillus* (Thompson *et al*. 1997). The strain DX30 had maximum activity at temperature of 25°C and pH of 6.0 respectively and remained stable activity at temperature between 30 and 100°C and pH between 6 and 12. Strain DX30 seems promising in the biological control (Knosel *et al.*1984).

Many of these exhibit interesting activity profiles. Cryptocin , for example, is an tetramic acid isolated from the endophytic fungus *Cryptosporiopsis quercina*, an endophyte of *Tripterigeum wilfordii*, that possesses potent activity against the world's worst plant pests *Pyricularia oryzae* and other plant pathogenic fungi, advocating it for possible agrochemical usage (Li *et al*. 2000). From the medicinal plant *Erythrina crista-galli* the endophyte *Phomopsis* sp. was isolated, which produced the anti-inflammatory as well as antifungal and antibacterial active polyketide lactone, phomol (Weber *et al*. 2004).The well known plant metabolite taxol , the "world's first billion-dollar anticancer compound" (Strobel *et al.* 2004), was originally isolated from the bark of the endemic Pacific yew tree, *Taxus brevifolia*. The taxol affects dividing cells, especially fast dividing ones like cancer cells. For the treatment of one patient suffering from cancer, 2 g taxol are required, which represents an amount equivalent to twelve trees and thereby posing a challenge to the limited natural resources, since the isolation from the inner bark implies the destruction of trees. The demand for taxol greatly exceeds the supply that can be sustained by isolation from its natural source and alternative sources of the drug have been sought for a long time.

Optimization of the fermentation conditions of the endophytic fungus may lead to the development of an economically and eco-friendly process for the production of camptothecin that could overcome the ever demanding supply problem (Puri *et al*. 2005). Thus, the ability to produce pharmacologically important natural products previously only known from plant sources is occasionally also inherent to endophytic fungi.

2.2 Endophytic fungi *Xylaria*- Biological and chemical diversity

Whalley, Edward (1999) demonstrated biological and chemical diversity of *Xylariaceae.* The study revealed that filamentous fungi have an excellent track record regarding their ability to

synthesize a diverse range of metabolites which often possess potent biological activity. The ascomycete family, the *Xylariaceae*, is well known for the wide biological diversity of many of its species and genera (Laessoe 1994). It has also been found to be the source of an impressive array of metabolites of which many have proved to be novel structures. The major metabolites produced by the representatives investigated can be grouped as dihydroisocoumarins and derivatives (Anderson *et al.* 1983), succinic acid and derivatives (Anderson *et al.* 1985), butyrolactones (Edward *et al*. 1989), cytochalasins, sesquiterpene alcohols (punctaporonins), griseofulvin and griseofulvin derivatives (Whalley *et al*. 1995), naphthalene derivatives, and long chain fatty acids (Adeboya *et al*. 1995).

Following a three year survey of the *Xylariaceae* in Thailand Thienhirun (Thienhirun1997) found that over 50% of *Hypoxylon* species were new. The genus *Camillea* has always been considered to be mainly restricted to the forests of South and Central America with *C. tinctor* (Berk.) Laessoe, Rogers, Whalley as the only previously recorded species from Asia (Rogers 1991).

In the study of *Daldinia* 17 species were recognised of which (41%) were cultured and for *Hypoxylon* 67 or 53% of the 126 species accepted have been cultured (Yao *et al.*1996). Recent investigations of the family in different tropical regions all indicate a large number of undescribed species. In an account of *Xylaria* from the Cerro de la Neblinia in Venezuela about 37% of the taxa examined were unknown and roughly half of the taxa described had not been included by Dennis in his account of the family in Venezuela and surrounding countries (Dennis 1970). Likewise in a study of *Xylaria* of Mexico 28% of the taxa found proved to be unknown. Similar indications of considerable numbers of undescribed species are known for *Hypoxylon* in Thailand with approximately 50% believed to be new (Thienhirun1997) and investigations of the *Xylariaceae* from Sulawesi and Papua New Guinea confirm that there are a significant number of new taxa in those regions (Vander1996).

Park *et al.* (2004) identified and isolated griseofulvin-producing endophytic fungus from *Abies holophylla* and evaluated its *in vivo* antifungal activity against plant pathogenic fungi. The fungus was labeled as *Xylaria* sp. F0010. Two antifungal substances were purified from liquid cultures of *Xylaria* sp. F0010, and their chemical identities were determined to be griseofulvin and dechlorogriseofulvin through mass and NMR spectral analyses. On comparison it was

concluded that, griseofulvin as compared to dechlorogriseofulvin showed high *in vivo* and *in vitro* antifungal activity and effectively controlled the development of rice blast (*Magnaporthe grisea*), rice sheath blight (*Corticium sasaki*), wheat leaf rust (*Puccinia recondita*), and barley powdery mildew. The liquid culture of *Xylaria* sp. F0010 exhibited potent and broad antifungal activity against plant pathogenic fungi (Park *et al*.2004).

Liu Xiaoli *et al*. (2007), isolated a bioactive compound P3 from culture extract of endophytic *Xylaria* sp. (Strobel *et al*. 1996, Li and Strobel 2001) having broad antimicrobial activity and identified as 7-amino-4-methylcoumarin by NMR, infrared, and mass spectrometry spectral data. The study revealed that the compound showed strong antibacterial and antifungal activities in vitro against *Staphylococcus aureus* [(MIC) 16 μg/ml], *Escherichia coli* (MIC, 10μg/ml), *Salmonella typhia* (MIC, 20 μg/ml), *Salmonella typhimurium* (MIC, 15 μg/ml), *Salmonella enteritidis* (MIC, 8.5 μg/ml), *Aeromonas hydrophila* (MIC, 4 μg/ml), *Yersinia sp*. (MIC, 12.5 μg/ml), *Vibrio anguillarum* (MIC, 25 μg/ml), *Shigella sp.* (MIC, 6.3 μg/ml), *Vibrio parahaemolyticus* (MIC, 12.5 μg/ml), *Candida albicans* (MIC, 15 μg/ml), *Penicillium expansum* (MIC, 40 μg/ml), and *Aspergillus niger* (MIC, 25 μg/ml).This was the first report of 7-amino-4-methylcoumarin in fungus and of the antimicrobial activity of this metabolite (Zhu *et al*. 1993).

2.3 Screening and characterization techniques of bioactive compounds

Pfaller *et al.* (1993) conducted a comparative evaluation of methods for broth macro- and microdilution susceptibility testing of fluconazol with 119 clinical isolates of *Candida albicans*. The macro and microdilution testing were performed according to National Committee for Clinical Laboratory Standards recommendations. In the study the reference macrodilution testing (Espinel-Ingroff *et al.* 1992) an 80% inhibition endpoint (MIC 80%) was determined after 48 h of incubation in accordance with National Committee for Clinical Laboratory Standards proposed standard M27-P. Microdilution endpoints were scored as the first tube or well in which a prominent reduction in turbidity (score 2 out of a possible 4) was observed compared with the growth control (Micro MIC-2). Alternative endpoint criteria were assessed independently of the reference MIC 80% and Micro MIC-2 values and included a colorimetric microdilution endpoint determined by using an oxidation-reduction indicator (Tellier *et al*.1992) The MICs for the two microdilution test systems were read after 24 and 48 h of incubation. From the study it was

concluded that the percentage of fluconazole MICs within 2 doubling dilutions of the macrodilution reference values was 94% for both microdilution tests read at 24 hr. Agreement was slightly lower at 48 h and ranged from 91 to 93%. Comparison of Micro MIC-2 and colorimetric microdilution MICs resulted in agreements of 97 and 93% at 24 and 48 h, respectively. These results show excellent agreement among alternative methods for fluconazole susceptibility testing.

De Logu *et al*. (2001) investigated two colorimetric methods for the determination of the susceptibility or resistance of *Mycobacterium tuberculosis* to rifampin, streptomycin, and isoniazid in liquid medium based on the reduction of 2,3-bis(2-methoxy-4-nitro- 5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) (Gomez-Flores *et al.* 1995, Mshana *et al.* 1998). In the study the agar proportion method was used as the reference method. It was concluded that the sensitivity of the XTT (Otero *et al*. 1991) reduction assay for the detection of rifampin resistance was comparable to that observed, for the MTT assay. However, the reduction of XTT yields a water-soluble formazan that can be easily quantified without performing additional steps such as addition of lysing buffer and solubilization. Furthermore, the colorimetric assays, based on the reduction of XTT and MTT for the detection of isoniazid and streptomycin resistance in *Mycobacterium tuberculosis*, were standardized. The inhibition of MTT and XTT reduction after treatment with rifampin, streptomycin, or isoniazid was directly proportional to the reduction in the number of viable bacteria, and a strain of *Mycobacterium tuberculosis* could be reported as susceptible or resistant to rifampin, streptomycin, or isoniazid after 3, 6, or 8 days, respectively (Mattila *et al*.1987). The XTT and MTT reduction assays are rapid, reliable, and affordable and do not require the use of radioisotopes. Moreover, they can be performed with common laboratory equipment.

Mohammadzadeh *et al*. (2006) evaluated a colorimetric method using 2,3,5-tri-phenyltetrazolium chloride (TTC) for antibiotic susceptibility testing of M. tuberculosis isolates. (Caviedes *et al*. 2002, Denizot, Lang, 1986. Thom *et al.*1993). In study eleven multidrug-resistant (MDR) isolates of M. tuberculosis and 12 isolates which were susceptible to rifampicin (RIF) and isoniazid (INH) were used. The test was performed with a critical concentration of 0.2 mg/ml for

INH and 2.0 mg /ml for RIF in 7H9GC broth with 0.625 mg TTC per ml (Syre *et al*. 2003). Each isolate was inoculated under these conditions and inspected daily for colour changes, the results were obtained after a mean of 14 days. The sensitivity and specificity of this method were 100% and 92 % respectively, for both antibiotics. It was concluded that TTC assay is a good alternative method for drug susceptibility testing of M. tuberculosis isolates.

Valgas *et al.* (2007) assayed a varied range of natural products of plant, fungi and lichen origin against two bacterial species *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, by two variants of the agar diffusion method (Smânia 1999 *et al*.) (well and disc), bioautographic method (Hamburger *et al*. 1987) and by micro dilution assay (Vanden Berghe 1991) to determine antibacterial activity. It was concluded that the well-variant of the diffusion method was more sensitive than the disc-variant, while the direct-variant of the bioautographic method exhibited a greater sensitivity if compared to indirect variant.

Swarnkar *et al.* (2009) assayed thirteen medicinal plants belonging to nine families from different localities of tribal areas of Rajasthan. The plant tubers were extracted with methanol and cold water to yield 26 extracts. The extracts were tested for their anti microbial activities against *Escherischia coli, Staphylococuus aureus, Klebsiella pnuemoniae, Pseudomonas aeruginosa* and a fungus *Candida albicans* using agar diffusion assay (Bauer *et al*. 1966).

Nurcihan *et al.* (2011) assayed antimicrobial potential of ethanolic extracts (Dulger and Sener,2010) obtained from the macrofungus *Xylaria polymorpha* against *E. coli* ATCC 11230, *S. aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427, *Bacillus cereus* ATCC 7064, *Micrococcus luteus* CCM 169, *Candida albicans* ATCC 10231, *Rhodotorula rubra* DSM 70403, *Geotrichum capitatum* ATCC 28576, *Debaryomyces hansenii* DSM 70238, *Kluyveromyces fragilis* ATCC 8608 & *Cryptococcus neoformans* ATCC 90112. It was revealed that antimicrobial activity against all tested microorganisms especially, *Greotricum capitatum* is more susceptible to the extracts (Girmenia *et al*. 2005, Pfaller and Diekema, 2004).

Hidayathulla *et al.* (2011) extracted phytochemicals from the leaves of *Pterospermum diversifolium* using different solvents like hexane, ethyl acetate, methanol and water. K. R.

Chandrashekar studied the antimicrobial activity (Jain *et al*. 2010) against *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aerugenosa* by Disc diffusion method (Harbone *et al.* 1984) and Minimum Inhibitory Concentration (MIC) by broth dilution method. It was concluded that the water extract was able to inhibit *Staphylococcus aureus* and *Bacillus subtilis* and methanolic extract was most effective against the tested micro organisms. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, glycosides and terpenes (Helen 1999).

Yu Li *et al.* (2011) isolated a new natural product, cytochalasin H2, together with cytochalasin H (Tao *et al.* 2008) from the agar cultures of the strain *Xylaria* sp. A23, which was isolated from *Annona squamosa* (Yang *et al.* 2008, Chavan *et al.* 2010, Liaw *et al*. 2008).The chemical structures of them were elucidated by spectroscopic and mass spectrometric analyses, including 1D-, 2D-NMR (Tao *et al*. 2008) and MS. It was concluded that the Compound 1 showed week cytotoxicity against HeLa and 293T cell lines by MTT assay (Mosmann1983). The antibacterial activities of compound 1 were tested against *Bacillus subtilis* (CMCC (B) 63501) using slip method. Compound 1 exhibited week cytotoxicity against HeLa and 293T cells (1.0 μg/mL, 25.04% and 32.8%, respectively), and induced cell contraction in both cell lines. Compound 1 had no effect on the growth of tested bacteria at 20 μg/disc.

CHAPTER 3

EXPERIMENTAL WORK

CHAPTER-3

EXPERIMENTAL WORK

3.1 Laboratory instruments & chemicals

Various apparatus & instruments used are

Petri plates, Separation funnel, Borer (7mm diameter), Spreader, Microtitre plates (Tarson), Auto pipettes (1ml, 20-200µl), Shaker at 25°C/120rpm (Kuhner Shaker), Laminar air hood, Spectrophotometer (HITACHI,U-2900), cuvettes, Wash bottle, Test tube stand, Test tubes, TLC plates, Chromatographic chamber, TLC applicator, Spray bottle, Autoclave (Equitron), Rota-evaporator (Yamato), Rota flask, Centrifuge (Sigma), ELISA reader (Thermo Scientific MULTISKAN SPECTRUM), Voretx (Genei).

Chemicals used

Ninhydrin, Conc. H_2SO_4, Molisch reagent, Wagner reagent, 5%$FeCl_3$, Ethyl acetate (Merck), Methanol, TTC dye, Distilled water, Muller –Hinton Agar (MHA), Luria broth (LB), Luria agar plates, Nutrient broth (NB), Nutrient agar plates, Antimicrobial (Streptomycin, Fluconazole etc.)

3.2 Source of endophytic fungi

Endophytic fungal cultures were isolated from the different host plants of Western Ghats of India. In this project fungi used were species of XF-2 & XF-4

Code no.	Host	Family
XF-2	Memycylon	*Melastomaceae*
XF-4	Lasianthus venulosus	*Rubiaceae*

These isolated cultures from CORE, Thapar University were further used for investigating antimicrobial activity.

3.3 Subculturing of *Xylaria*

From the master culture 1 piece of fungus was cut with the help of sterile blade and sub cultured on PDA plates and grown for 14 days at 25°C.

3.4 Composition of media used for culturing of *Xylaria*

Medium 1 (M1): Potato Dextrose Broth (PDB) (HiMedia Laboratories Pvt. Ltd)

(Mac Faddin *et al*.1985)

Suspend 24 g of PDB in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure and 121°C temp. for 15 minutes.

Standard Formula

Ingredients	Gram/liter
Potato infusion	200
Dextrose	20
Final pH (at 25°C) 5.1	

Medium 2 (M2): Malt Extract Broth (MEB) (HiMedia Laboratories Pvt.Ltd).

MEB is an aqueous extract of sprouted malt grains and dried at a low temperature to preserve nutrients. 5% concentration was used for the culturing of *Xylaria*.

3.5 Culturing of Fungi

The fresh mycelia (grown on PDA) of representative endophytic fungi (XF-2 & XF-4) were transferred to 500ml Erlenmeyer flask containing 250 ml of the PDB. The culture flasks were incubated for 10 days at 25±1°C and at 120 rpm. After the incubation period, the cultures were taken out and filtered through sterile mesh cloth to separate the mycelia from the culture broth (Prabavathy, *et al.* 2011).

3.6 Extraction Procedure

The fungal metabolites were extracted by solvent extraction procedure using ethyl acetate as solvent. Equal volumes of the culture filtrate/mycelial extract and ethyl acetate were taken in a separating funnel and were shaken vigorously for 15-20 min. The solution was then allowed to stand for 1 hr. wherein the aqueous and organic phase got separated and the organic phase so obtained was collected. Ethyl acetate was then evaporated using rotaevaporator at 40°C for 15-40 minutes and the residual compound was dried and the weight of the residue was estimated. The compound was stored in 5 ml glass tubes. These extracts were further used to check for their antibacterial & antifungal activities.

3.7 Cultures used for antibacterial & antifungal susceptibility testing

1. Bacterial cultures

Gram positive bacteria**:** *Staphylococcus aureus, Bacillus megaterium*

Gram negative bacteria: *E.coli, Pseudomonas aeruginosa*

2. Fungal culture

Candida albicans

Medium used for antibiotic susceptibility testing

MHA (Muller Hinton Agar): It is a microbiological growth medium that is commonly used for antibiotic susceptibility testing.

It contains (w/v) (Atlas, 2004)

- 30.0% beef infusion
- 1.75% casein hydrolysate
- 0.15% starch
- 1.7% agar
- pH adjusted to neutral at 25°C

3.8 Preparation of McFarland standard

McFarland turbidity standard are used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of test suspension with the turbidity of McFarland standard. The McFarland standard is prepared by adding barium chloride to sulfuric acid to obtain a barium sulfate precipitate. By adjusting the volumes of these two reagents standard of varying degree of turbidity can be prepared to represent several different concentrations of bacteria. The standard most commonly used in clinical microbiology laboratory for routine antimicrobial susceptibility testing is 0.5 which represents 1.5 x 10^8 (generally range is 1.0 x 10^8 to 2.0 x 10^8 bacteria/ml).

A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 ml of 1% sulfuric acid (H_2SO_4).

Preparation of McFarland strandard (NCCLS, 2003).

1. Added 85 ml of 1% H_2SO_4 to a 100 ml volumetric flask.
2. Using autopipette added 0.5 ml of 1.175% $BaCl_2.2H_2O$ dropwise to H_2SO_4 while constantly swirling the flask.
3. Final volume was made upto 100 ml with 1% H_2SO_4
4. The flask was placed on magnetic stirrer for 3-5 minutes.
5. The solution was checked for any visible clumps. The O.D. was recorded at 600 nm.

McFarland No.	1.0% $BaCl_2.2H_2O$ (ml)	1.0%H_2SO_4 (ml)	Appox.Cell density (cfu/ml)	Percentage transmittance	Absorbance At 600 nm
0.5	0.05	9.95	$1x10^8$	74.3	0.132

3.9 Screening for antimicrobial activity of crude extract

3.9.1 Well Diffusion Method

Agar diffusion refers to the movement of molecules through the matrix that is formed by the gelling of agar. When performed under controlled conditions, the degree of the molecule's

movement can be related to the concentration of the molecule. This phenomenon forms the basis of the agar diffusion assay that is used to determine the susceptibility or resistance of a bacterial strain to an antibacterial agent, (e.g., including antibiotics). When the seaweed extract known as agar is allowed to harden, the resulting material is not impermeable. Rather, there are spaces present between the myriad of strands of agar that comprise the hardened polymer.

Small molecules such as antibiotics are able to diffuse through the agar. Typically an antibiotic is applied to a well that is cut into the agar. Thus, the antibiotic will tend to move from this region of high concentration to the surrounding regions of lower antibiotic concentration. If more material is present in the well, then the zone of diffusion can be larger. A bacterial suspension is spread onto the surface of the agar. Then, antibiotic is applied to a number of wells in the plate. There can be different concentration of a single antibiotic or a number of different antibiotics present. Following a time to allow for growth of the bacteria then agar is examined. If bacterial growth is right up to the antibiotic containing well, then the bacterial strain is deemed to be resistant to the antibiotic. If there is a clearing around the antibiotic well, then the bacteria have been adversely affected by the antibiotic. The size of the inhibition zone can be measured and related to standards, in order to determine whether the bacterial strain is sensitive to the antibiotic. The agar diffusion assay allows bacteria to be screened in a routine, economical and easy way for the detection of resistance. The antimicrobials present in the fungal extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimetres (NCCLS2009).

Reagents used:

Muller Hinton Agar Medium

The medium was prepared by dissolving 33.9 g of the commercially available Muller Hinton Agar Medium (HiMedia Pvt. Ltd.) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium poured onto100mm petriplates (25-30ml/plate) while still molten.

Materials required Cultures (*E.coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus megaterium, Candida albicans),* Autopipettes**,** MHA plates**,** Spreader**,** Borer (4-6mm diameter)

PROCEDURE

Well grown colonies of test organism from master plate were picked with the help of sterile loop and transferred to test tube containing broth. This test tube was then incubated at 37°C in incubator with continuous shaking until growth reaches 0.5 McFarland standard. The turbidity of culture was adjusted with saline solution. Petriplates containing 30ml Muller Hinton Agar medium were seeded with culture of bacterial strains (0.5 McFarland Standard).100 µl of test organism was spread on MHA plate with the help of glass spreader. Wells were made and 50 µl of the fungal extracts were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin was used as a positive control.

3.9.2 Broth microdilution method (Colorimetric method)

The broth microdilution MIC dilution is used to measure the in vitro activity of antimicrobial agents against a bacterial isolate. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in two fold serial dilutions. A polystyrene tray containing 96 wells is filled with small volumes of serial two-fold dilutions of different antibiotics. The inoculum suspension and standardization is done according to McFarland standard. The bacterial inoculum is then inoculated into the wells and incubated at 37°C overnight. MIC is determined by observing the lowest concentration of an antimicrobial agent which will inhibit visible growth of the bacterium. The NCCLS describes a standard method for broth microdilution MIC testing of bacteria (NCCLS, 2000).

Materials required- Suspension of test organism, Microtitre plate, Autopipettes, Streptomycin, Methanol extracts, NB for dilution, TTC dye, incubator at 37°c.

Principle behind TTC Assay

Triphenyl tetrazolium chloride, TTC, is a redox indicator commonly used in biochemical experiments especially to indicate cellular respiration. It is a white crystalline powder, soluble in water, ethanol and acetone but insoluble in ether. TTC (2,3,5-triphenyltetrazolium chloride) is a redox indicator used to differentiate between metabolically active and inactive tissues. The white

compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism).

PROCEDURE

The minimum inhibitory concentration (MIC) was determined by micro dilution method using serially diluted fungal extracts according to the NCCLS protocol (NCCLS, 2000). In microtitre plate 100 µl of methanolic extract was added in each well and labelled as 1 to 10. To these wells 100µl of NB (in case of *S.aureus, P.aeruginosa*) and LB (in case of *E.coli*) was added for extract dilution. The microorganism suspension of 10µl was added to the broth dilutions. These were incubated for 24 hours at 37ºC. MIC of each extract was taken as the lowest concentration that did not give any visible bacterial growth. After 24 hr incubation 10µl dye TTC was added and MIC was recorded after 1 hr. of incubation.

3.9.3 Microdilution method- Determination of MIC (Spectrophotometric method)

Dilution susceptibility testing methods are used to determine the minimal concentration of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are usually tested at two fold serial dilutions, and the lowest concentration that inhibits visible growth of an organism is regarded as the MIC. The concentration range used may vary with the drug, the organism tested, and the site of the infection. The method and principles of the microdilution method is essentially the same as the macrodilution method except that the antimicrobial dilutions are in 0.1 ml volumes contained in wells of a microdilution tray (usually 96 well trays). Results obtained may be reported as the actual MIC.

The miniaturization and mechanization of the test by use of small, disposable, plastic "microdilution" trays has made broth dilution testing practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 ml that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray (Jorgensen *et al.* 2007 and Clinical and Laboratory Standard 2009). Microdilution panels are typically prepared using dispensing instruments that aliquot precise volumes of pre weighed and diluted antibiotics in broth into the individual wells of trays from large volume vessels. Hundreds of identical trays can be prepared from a single master set of dilutions in a relatively brief period. Inoculation of panels with the

standard 1x 10^8 CFU/ml is accomplished using a micropipette transfers 0.01 to 0.05 ml of standardized bacterial suspension into each well of the microdilution tray. Following incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth. The advantages of the microdilution procedure include the generation of MICs, the reproducibility and convenience of having prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. The main disadvantage of the microdilution method is some inflexibility of drug selections available in standard commercial panels.

Material Required - Suspension of test organism (Bacterial as well as fungal), Microtitre plate, ELISA plate reader, Autopipettes, Standard antimicrobial (Streptomycin), Test extracts, Nutrient broth and Luria broth for dilution.

PROCEDURE

A suspension of test organism was prepared equivalent to a 0.5 McFarland standard using isolated colonies. Streptomycin was prepared as stock solutions of 1 mg/ml. The wells of a 96-well ELISA tray were filled with 100 µl of standard antibiotic and added with 100 µl of diluted extract. Similarly in 100 µl of nutrient broth 100 µl of extracts were added in other wells. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm.10 µl of bacterial suspension was added to each well. And again absorbance of each well was recorded at same wavelength this was considered as 0 hour reading. The plate was incubated at 37ºC for 1 hr, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. Next absorbance was recorded after 2 hr, 3hr, 24 hrs (in case of *Candida albicans* absorbance was also taken after 48 hr). This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. The MICs value for a test extract was expressed as the lowest concentration that inhibits the bacterial growth.

3.10 Preliminary phytochemical analysis of bioactive compound.

(Kokate *et al.* 2009)

3.10.1 Test for Alkaloids (Wagner's test)

Test	Observation	Inference
To the fungal extract few drops of Wagner's reagent was added.	Reddish brown precipitate	Alkaloid present

3.10.2 Test for Amino acids (Ninhydrin test)

Test	Observation	Inference
Fungal extract was heated with 3 drops of 5% Ninhydrin solution in boiling water bath for 10 min.	Purple or Bluish colour	Amino acids present

3.10.3 Test for Carbohydrate (Molisch's test)

Test	Observation	Inference
To fungal extract added few drops of α-naphthol solution in alcohol, shaken and added conc.H_2SO_4 from sides of the test tube	Violet ring at the junction of two liquids.	Carbohydrate is present

3.10.4 Test for Fats & Oils

Test	Observation	Inference
Fungal extract was added to 0.5 N alcoholic KOH followed by few drops of phenolphthalein and incubated in boiling water bath for 1 hr.	Soap formation after 1 hr. incubation.	Fats & oils present

3.10.5 Test for Flavonoids

Test	Observation	Inference
Increasing amount of sodium hydroxide was added to the residue.	Shows yellow colouration, which decolourises after addition of acid.	Flavonoid present

3.10.6 Test for Glycosides (Keller- Killiani test)

Test	Observation	Inference
To fungal extract, added glacial acetic acid, one drop of 5% $FeCl_3$ & conc. H_2SO_4.	Reddish brown colour appears at junction of the liquid layers and upper layer appears bluish green.	Glycoside present

3.10.7 Test for Steroid & Triterpenoids (Salkowoski's)

Test	Observation	Inference
Few drops of conc. H_2SO_4 was added to extract	Red colour indicates presence of Steroid & Yellow colour indicates Triterpenoids	Steroid & Triterpenoids present

3.10.8 Test for Tannins & Phenolic Compounds (Ferric chloride test)

Test	Observation	Inference
1ml of extract was treated with $FeCl_3$ (5%w/v)	Blue colour appears if tannin present & green colour if phenolic compound is present	Tannins & Phenolic compounds present

CHAPTER 4

RESULTS & DISCUSSION

CHAPTER-4

RESULTS AND DISCUSSION

In the present study endophytic fungi isolated from different host plants were characterized for their antimicrobial activity using various methods like Agar well diffusion method, Broth microdilution method (colorimetric) & microdilution (spectrophotometric method). The main purpose of this study was to evaluate these endophytic fungi for the production of bioactive compounds. Methanolic extracts of the culture filterate were checked for their antibacterial activity using four bacterial cultures *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus megaterium* and one fungal culture *Candida albicans.* The code number, host and family of the endophytic fungi is as given below:

Code no.	Host	Family
XF-2	Memycylon	*Melastomaceae*
XF-4	Lasianthus venulosus	*Rubiaceae*

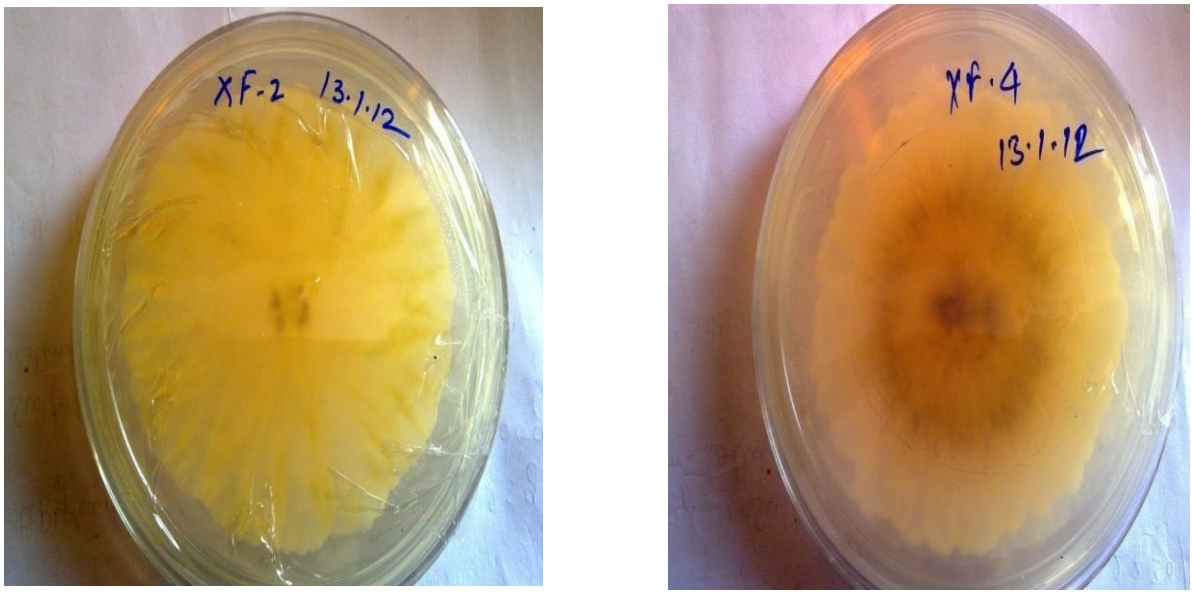

Figure 1 Culture morphology of XF-2 & XF-4 on PDA

Figure 2 Cultivation of XF-2 & XF-4 in PDB

Figure 3 Cultivation of XF-2 & XF-4 in MEB

Results of antimicrobial activity of fungal extract by Agar Well Diffusion method.

Table 1 Zone of inhibition in millimeter (mm) of XF-2 PDB extract against bacterial cultures*

Conc.(µg/ml)	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.megaterium*
10	10.5±0.70	0	0	0
20	12±1.41	0	10.5±0.70	10.5±0.70
30	14.5±0.70	13.5±0.70	12.5±0.70	13.5±0.70
40	15.5±0.70	15.5±0.70	16.5±0.70	15.5±0.70
50	17.5±0.70	20.5±0.70	18±1.41	16.5±0.70
60	19±1.41	23.5±0.70	20	22±1.41

*Bacterial cultures *E.coli = Escherichia coli, S.aureus = Staphylococcus aureus, P.aeruginosa = Pseudomonas aeruginosa and B.megaterium = Bacillus megaterium*

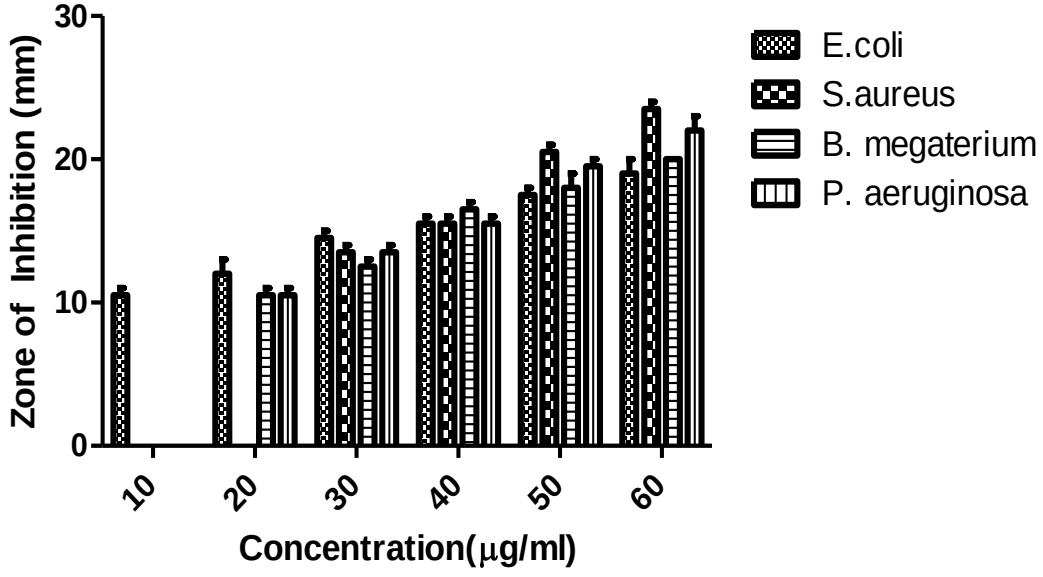

Figure 4 Comparison of zone of inhibition of XF-2 PDB against four different bacterial cultures

Table 2 Zone of inhibition in millimeter (mm) of XF-4 PDB extract against bacterial cultures

Conc. (µg/ml)	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.megaterium*
10	10.5±0.70	0	0	0
20	12.5±0.70	12.5±0.70	10.5±0.70	11
30	14±1.41	14.5±0.70	12.5±0.70	13±1.41
40	16.5±0.70	20±1.41	16.5±0.70	14.5±2.21
50	19±1.41	21.5±0.70	22±1.41	16.5±0.70
60	20±1.41	23.5±0.70	23±1.41	17±1.41

Each value represents mean of 3 replicates± S.D

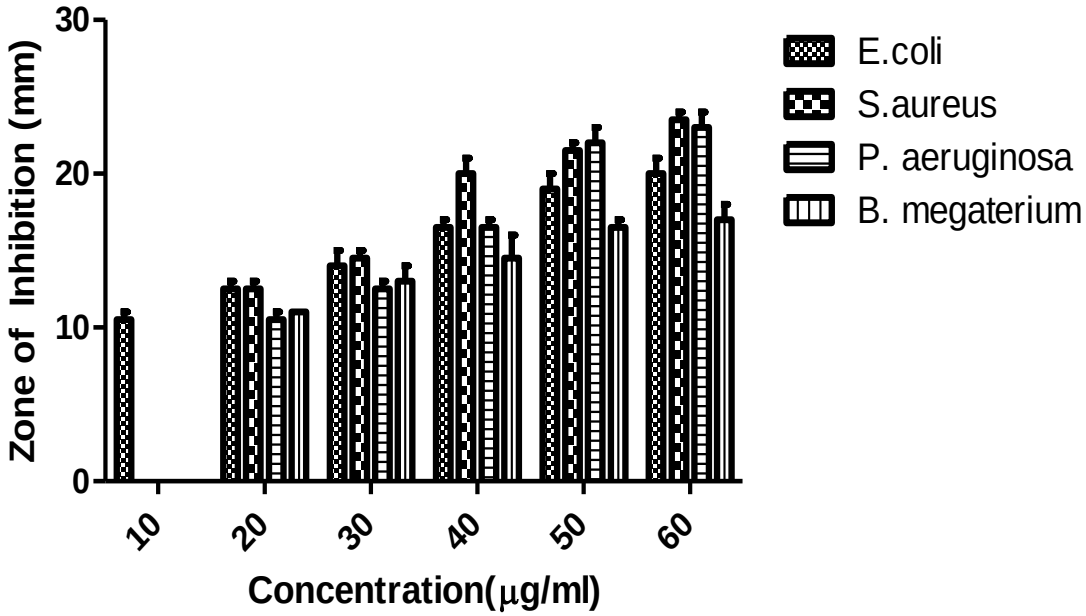

Figure 5 Comparison of zone of inhibition of XF-4 PDB extract against bacterial cultures

Table 3 Zone of inhibition in millimeter (mm) of XF-2 MEB extract against test bacterial cultures

Conc. (µg/ml)	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.megaterium*
10	11	0	10.5±0.70	0
20	13	12	13±1.41	11.5±0.70
30	16±1.41	14.5±0.70	18±1.41	14.5±0.70
40	19±1.41	19.5±0.70	20.5±0.70	17±1.41
50	20±1.41	23±1.41	21.5±0.70	19±1.41
60	20±1.41	24.5±2.12	23±1.41	20±1.41

Each value represents mean of 3 replicated± S.D

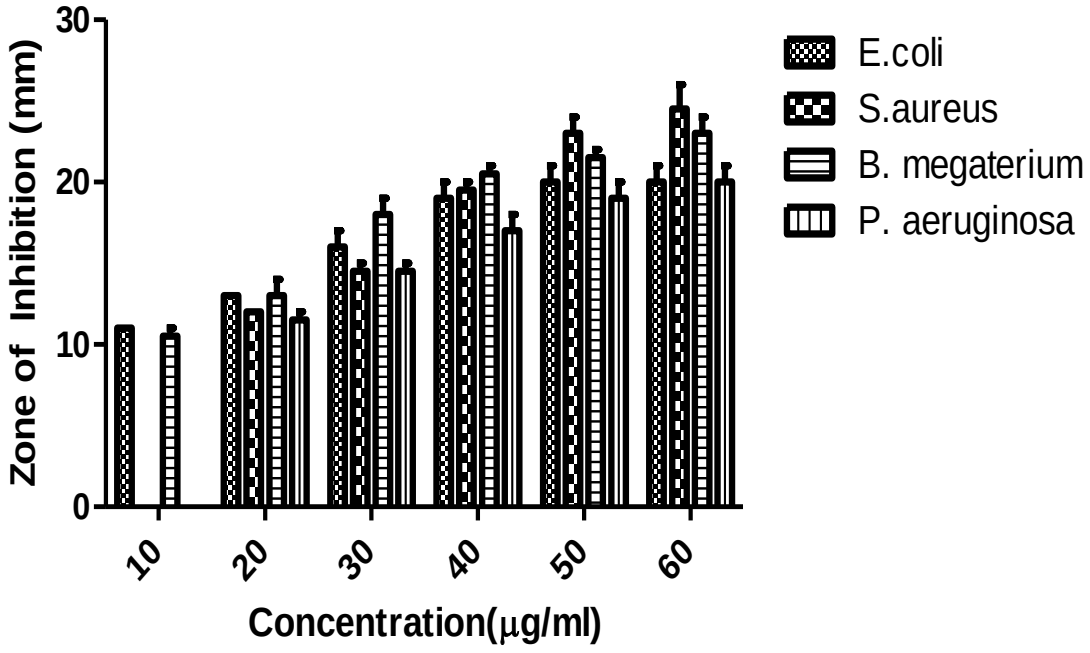

Figure 6 Comparison of zone of inhibition of XF-2 MEB extract against four bacterial cultures

Table 4 Zone of inhibition in millimeter (mm) of XF-4 MEB extract against test bacterial cultures

Conc. (µg/ml)	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.megaterium*
10	11	0	0	0
20	12.5±0.70	12	11±1.41	13±1.41
30	14.5±0.70	15±1.41	13±1.41	18
40	15.5±2.12	19.5±0.70	18.5±0.70	20.5±2.12
50	19.5±0.70	21.5±0.70	20.5±0.70	23.5±0.70
60	20±1.41	23	21.5±0.70	25±1.41

where *E.coli = Escherichia coli, S.aureus = Staphylococcus aureus , P.aeruginosa = Pseudomonas aeruginosa and B.megaterium = Bacillus megaterium*

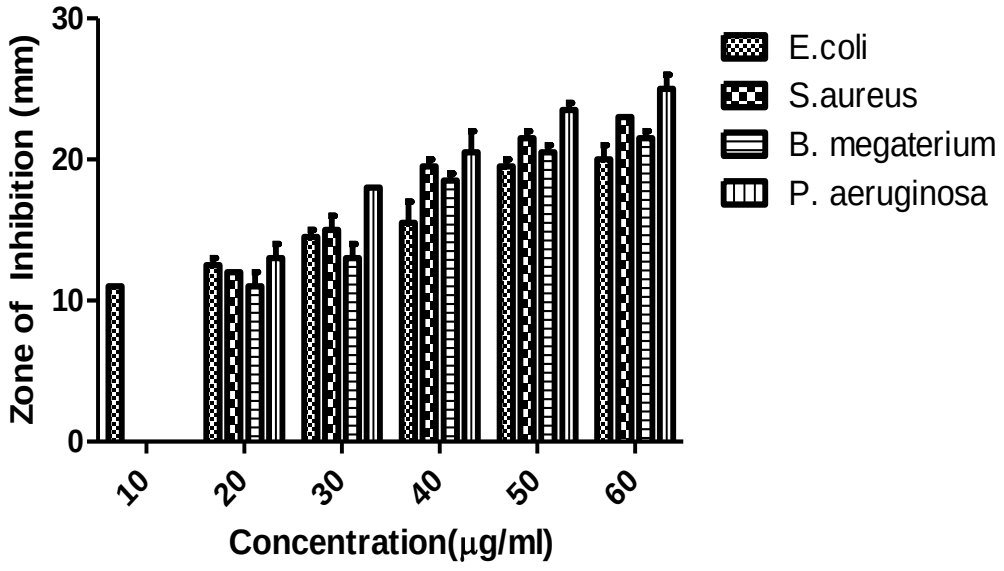

Figure 7 Comparison of zone of inhibition of XF-4 MEB extract against bacterial cultures

Table 5 Zone of inhibition in millimeter (mm) of XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Candida albicans*

Conc. (µg/ml)	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	0	0	0	0
20	12±1.41	12	12	12.5±0.70
30	14.5±0.70	13.5±0.70	14.5±0.70	16.5±2.12
40	16.5±0.70	15±1.41	16±1.41	17.5±2.12
50	19	16.5±0.70	17±1.41	19.5±2.12
60	19.5±0.70	19±1.41	17.5±2.12	21.5±0.70

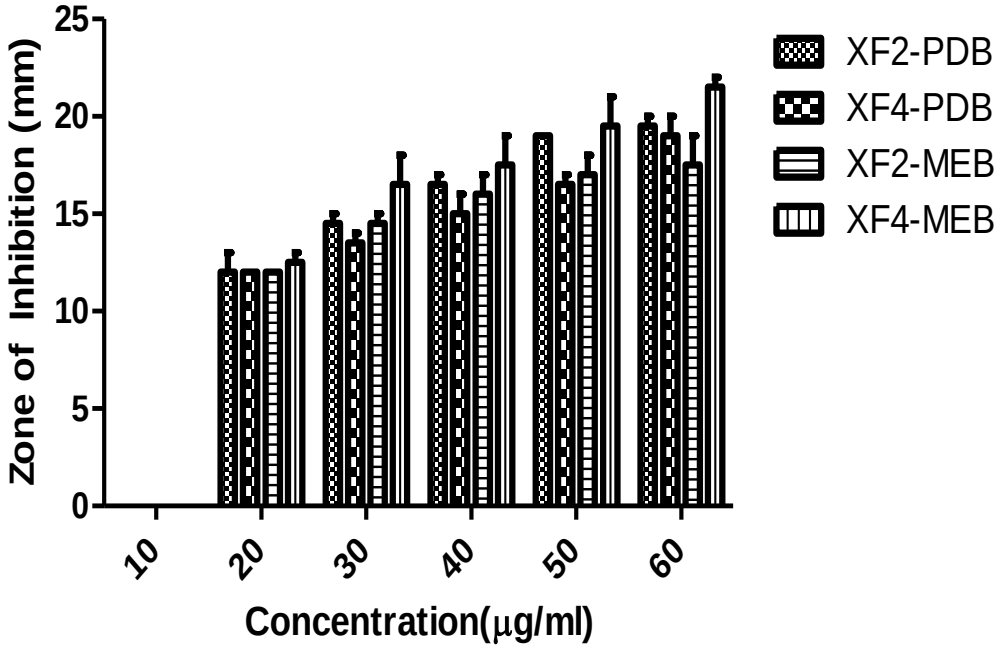

Figure 8 Comparison of Zone of inhibition of XF-2 PDB, XF-4 PDB, XF-2 MEB, and XF-4 MEB extracts against *Candida albicans*

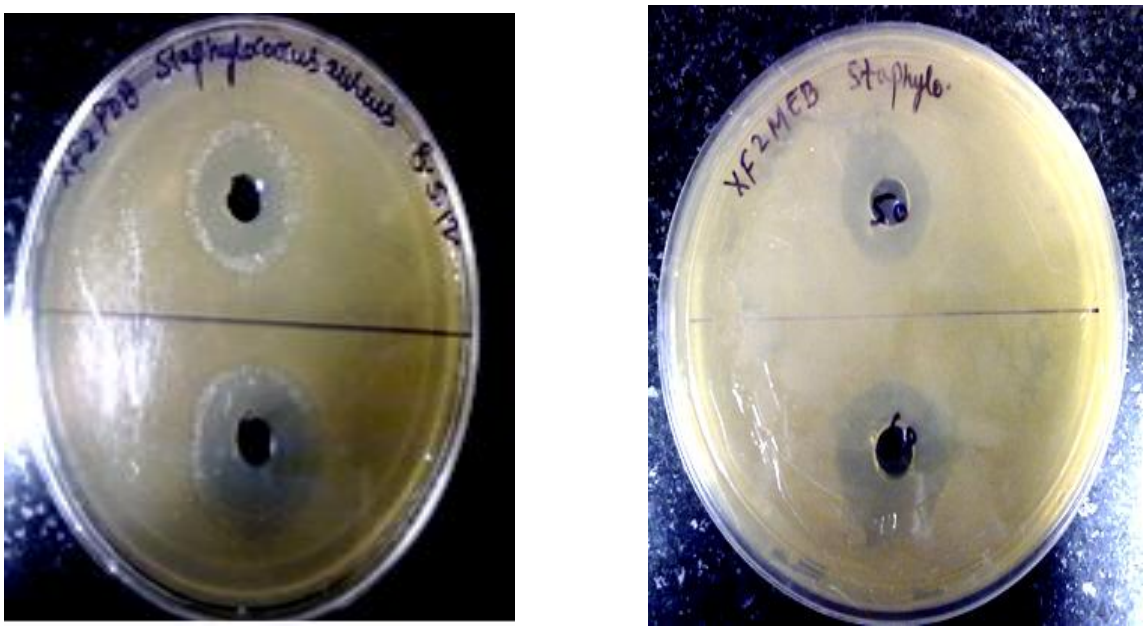

Figure 9 Zone of inhibition of XF-2 PDB & XF-2 MEB against *Staphylococcus aureus* (Conc. 50-60 µg/ml)

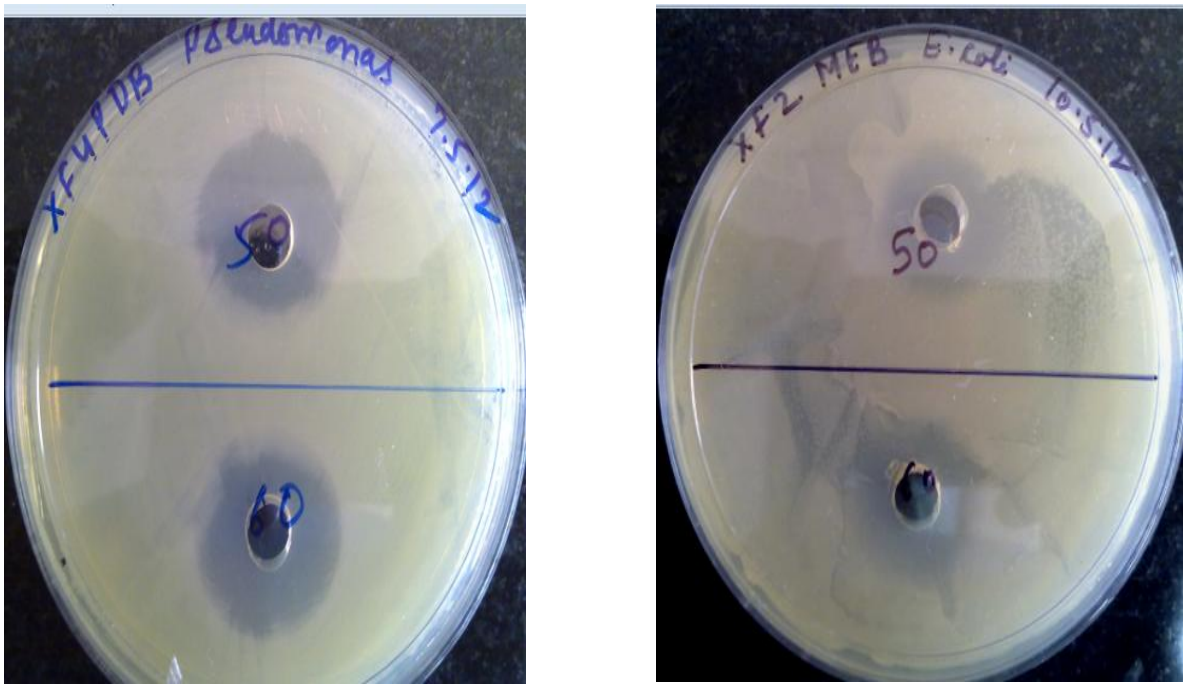

Figure 10 Zone of inhibition of XF-4 PDB against *P.aeruginosa* & XF-2 MEB *against E.coli*

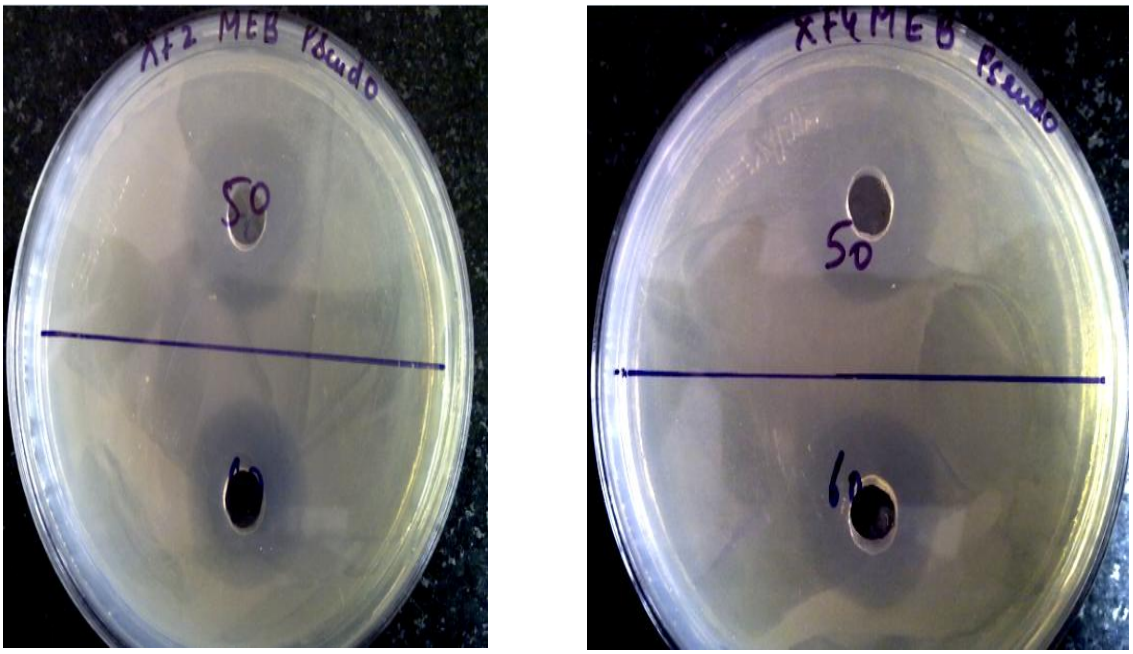

Figure 11 Zone of inhibition of XF-2 MEB & XF-4 MEB against *Pseudomonas aeruginosa*

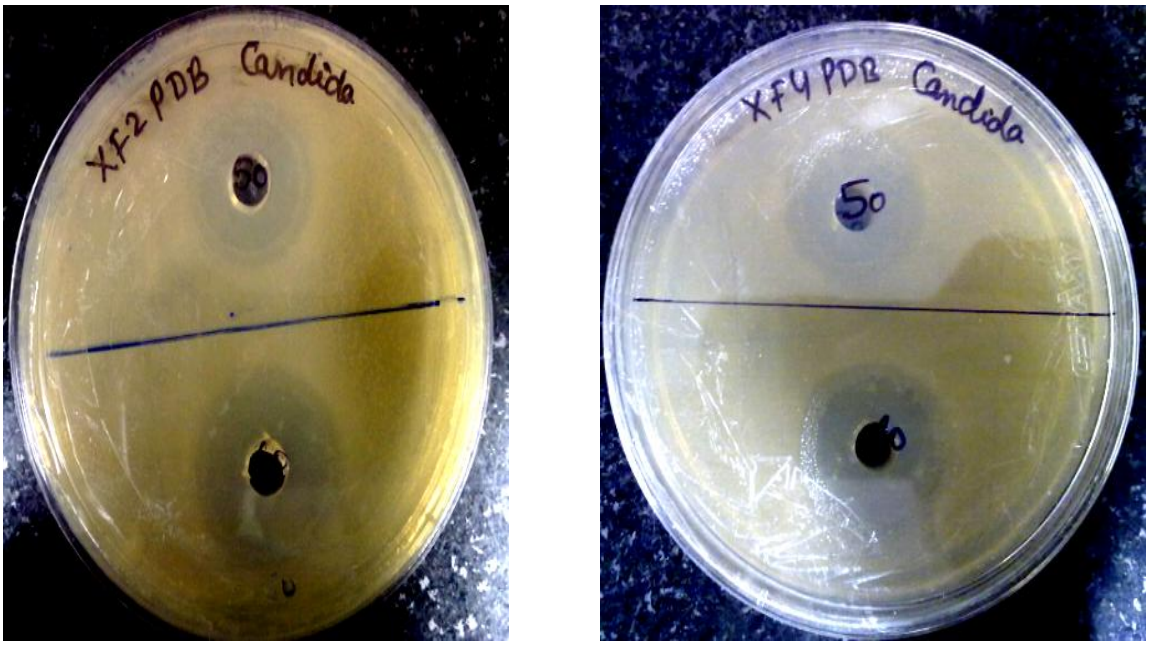

Figure 12 Zone of inhibition of XF-2 PDB & XF-4 PDB against *Candida albicans*

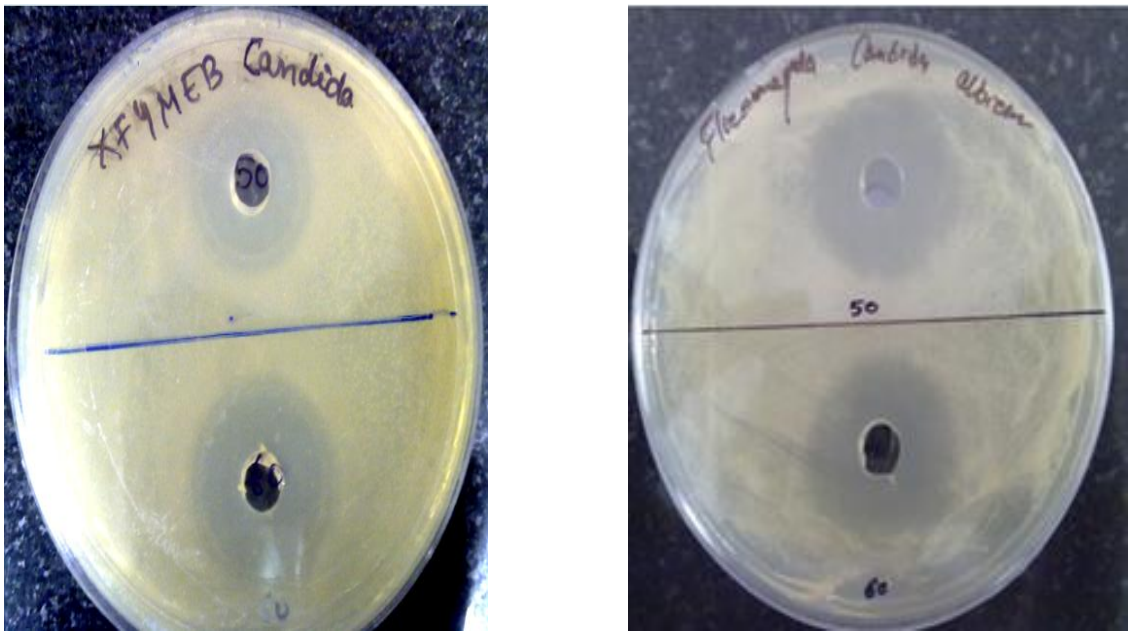

Figure 13 Zone of inhibition of XF-4 MEB against *Candida albicans* & Fluconazole against *Candida albicans*

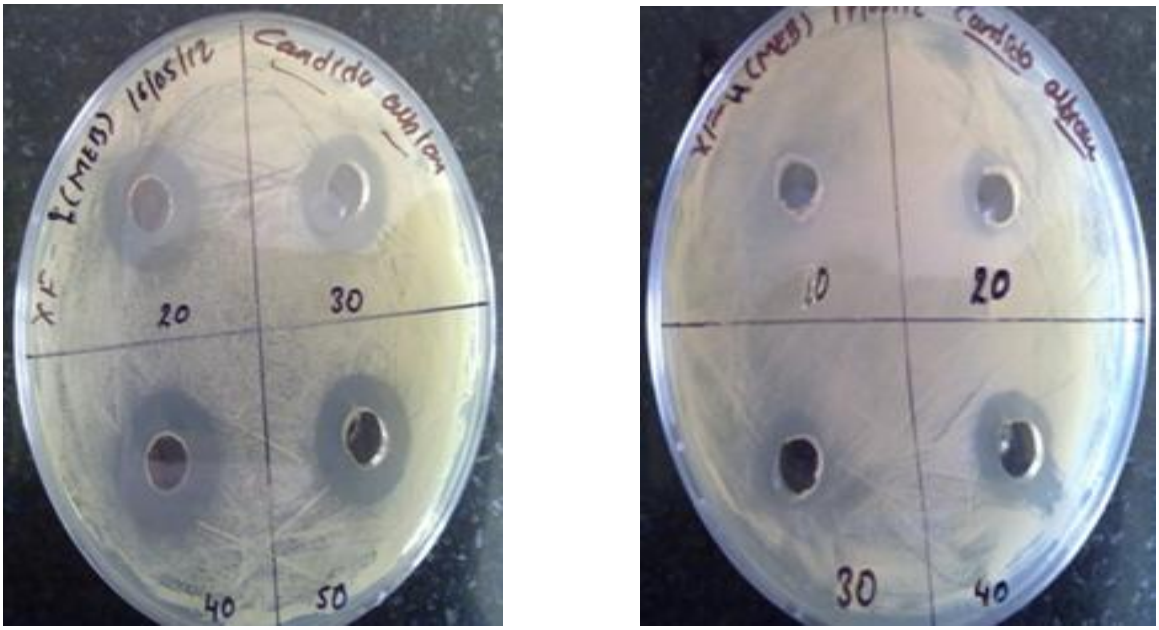

Figure 14 Zone of inhibition of XF-2 MEB & XF-4 MEB against *Candida albicans*

Table 6 Zone of inhibition in millimeter (mm) of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB and XF-4 MEB against *Escherichia coli*

Conc.(µg/ml)	Streptomycin	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	0	10.5±0.70	10.5±0.70	11	11
20	11±1.41	12±1.41	12.5±0.70	13	12.5±0.70
30	12.5±2.12	14.5±0.70	14±1.41	16±1.41	14.5±0.70
40	14±1.41	15.5±0.70	16.5±0.70	19±1.41	15.5±2.12
50	15±1.41	17.5±0.70	19±1.41	20±1.41	19.5±0.70
60	15.5±2.12	19±1.41	20±1.41	20±1.41	20±1.41

Each value represents mean of 3 replicates± S.D

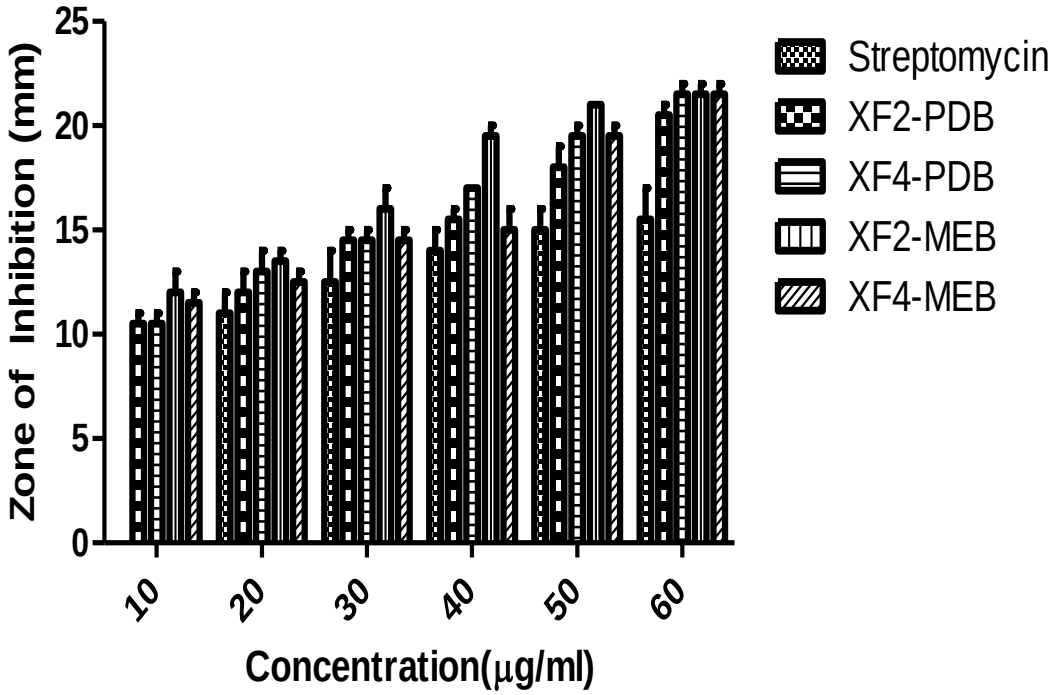

Figure 15 Comparison of Zone of inhibition of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Escherichia coli*

Table 7 Zone of inhibition of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Staphylococcus aureus*

Conc. (µg/ml)	Streptomycin	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	0	0	0	0	0
20	12±1.41	0	12.5±0.70	12	12
30	13±1.41	13.5±0.70	14.5±0.70	14.5±0.70	15.5±1.41
40	14.5±0.70	15.5±0.70	20±1.41	19.5±0.70	19.5±0.70
50	16±1.41	20.5±0.70	21.5±0.70	23±1.41	21.5±0.70
60	17.5±0.70	23.5±0.70	23.5±0.70	24.5±2.12	23

Each value represents mean of 3 replicates± S.D.

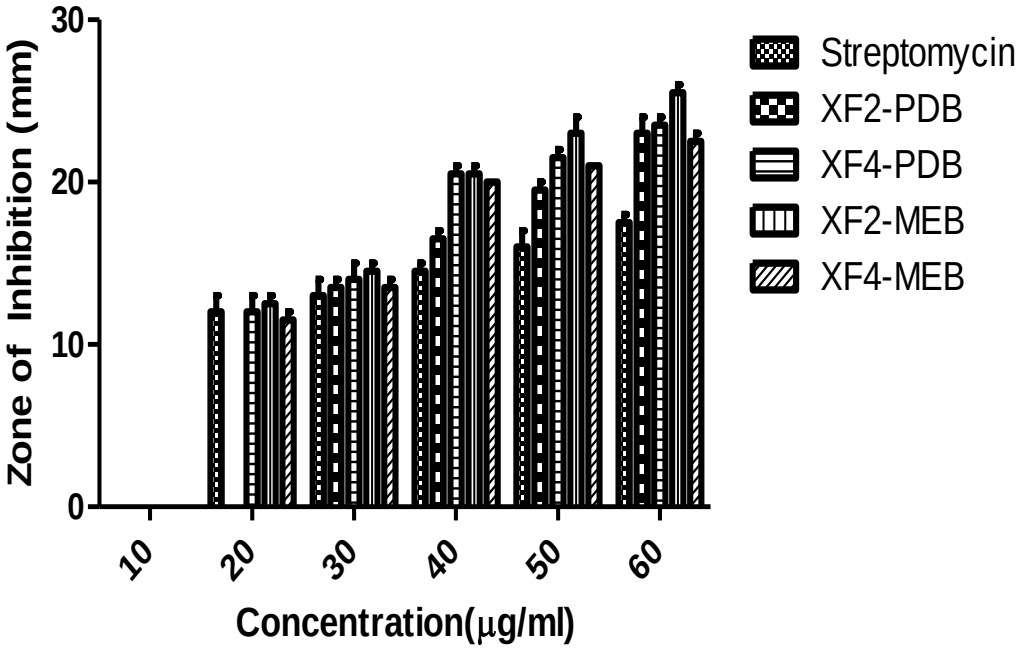

Figure 16 Comparison of Zone of inhibition of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against Staph*ylococcus aureus*

Table 8 Zone of inhibition in millimeter (mm) of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Pseudomonas aeruginosa*

Conc. (µg/ml)	Streptomycin	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	0	0	0	10.5±0.70	0
20	12.5±0.70	10.5±0.70	10.5±0.70	13±1.41	11±1.41
30	15.5±0.70	12.5±0.70	12.5±0.70	18±1.41	13±1.41
40	16.5±0.70	16.5±0.70	16.5±0.70	20.5±0.70	18.5±0.70
50	16.5±0.70	18±1.41	22±1.41	21.5±0.70	20.5±0.70
60	17.5±0.70	20	23±1.41	23±1.41	21.5±0.70

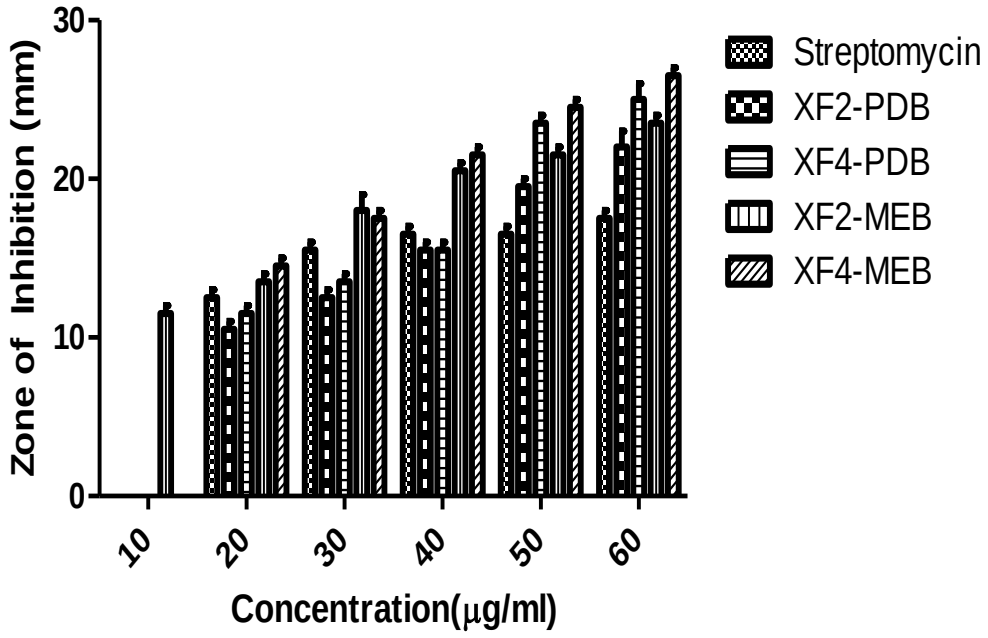

Figure 17 Comparison of Zone of inhibition of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Pseudomonas aeruginosa*

Table 9 Zone of inhibition in millimeter (mm) of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Bacillus megaterium*

Conc. (µg/ml)	Streptomycin	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	10.5±7.07	0	0	0	0
20	12.5±0.70	10.5±0.70	11	11.5±0.70	13±1.41
30	14±1.41	13.5±0.70	13±1.41	14.5±0.70	18
40	15±1.41	15.5±0.70	14.5±2.21	17±1.41	20.5±2.12
50	15.5±2.12	16.5±0.70	16.5±0.70	19±1.41	23.5±0.70
60	16.5±2.12	22±1.41	17±1.41	20±1.41	25±1.41

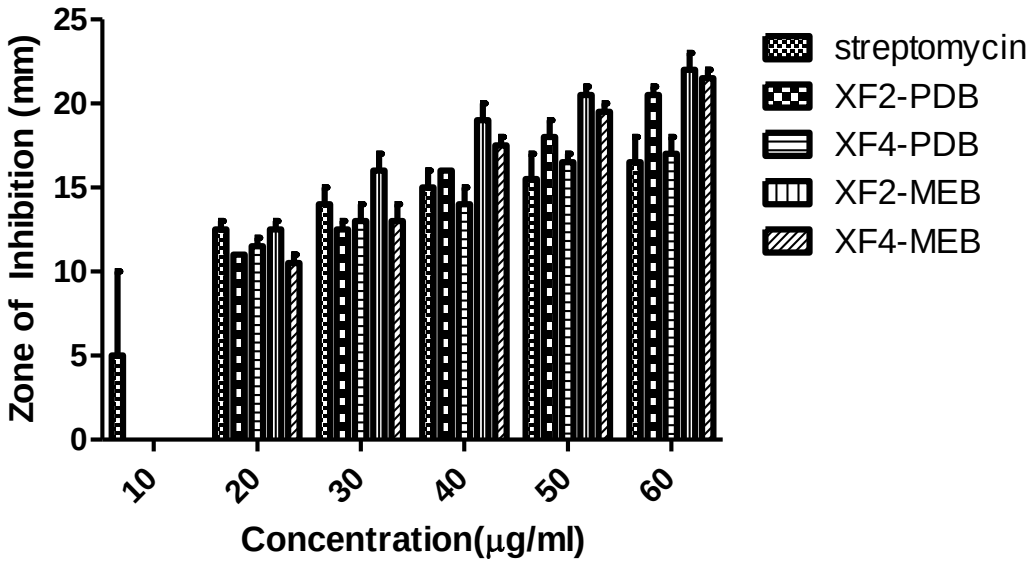

Figure 18 Comparison of Zone of inhibition of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Bacillus megaterium*

Table 10 Zone of inhibition in millimeter (mm) of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Candida albicans*

Conc.(µg/ml)	Fluconazole	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
10	0	0	0	0	0
20	13.5±0.70	12±1.41	12	12	12.5±0.70
30	15.5±0.70	14.5±0.70	13.5±0.70	14.5±0.70	16.5±2.12
40	20.5±0.70	16.5±0.70	15±1.41	16±1.41	17.5±2.12
50	21.5±0.70	19	16.5±0.70	17±1.41	19.5±2.12
60	22	19.5±0.70	19±1.41	17.5±2.12	21.5±0.70

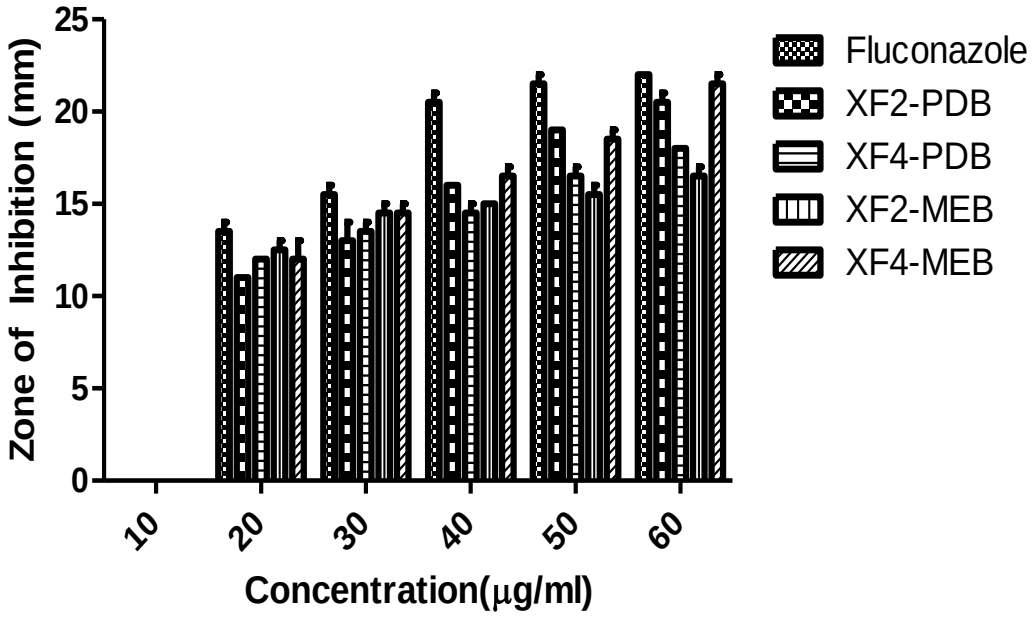

Figure 19 Comparison of Zone of inhibition of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Candida albicans*

Results of Antimicrobial activity of fungal extract by Broth microdilution method (TTC method)

Table 11 The MIC of the extracts of XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Escherichia coli*.

Strain	MIC (µg/ml)
Streptomycin	>3
XF-2 PDB	>5
XF-4 PDB	5
XF-2 MEB	7
XF-4 MEB	>2

Table 12 The MIC of the extracts of XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Staphylococcus aureus.*

Strain	MIC (µg/ml)
Streptomycin	>5
XF-2 PDB	3
XF-4 PDB	>5
XF-2 MEB	6
XF-4 MEB	>6

Table 13 The MIC of the extracts of XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Pseudomonas aeruginosa.*

Strain	MIC (µg/ml)
Streptomycin	>8
XF-2 PDB	3
XF-4 PDB	5
XF-2 MEB	>3
XF-4 MEB	7

Table 14 The MIC of the extracts of XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Bacillus megaterium*.

Strain	MIC (µg/ml)
Streptomycin	2
XF-2 PDB	2
XF-4 PDB	1
XF-2 MEB	1
XF-4 MEB	1

Table 15 The MIC of the extracts of XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Candida albicans.*

Strain	MIC (µg/ml)
Fluconazole	2
XF-2 PDB	2
XF-4 PDB	>3
XF-2 MEB	>2
XF-4 MEB	>2

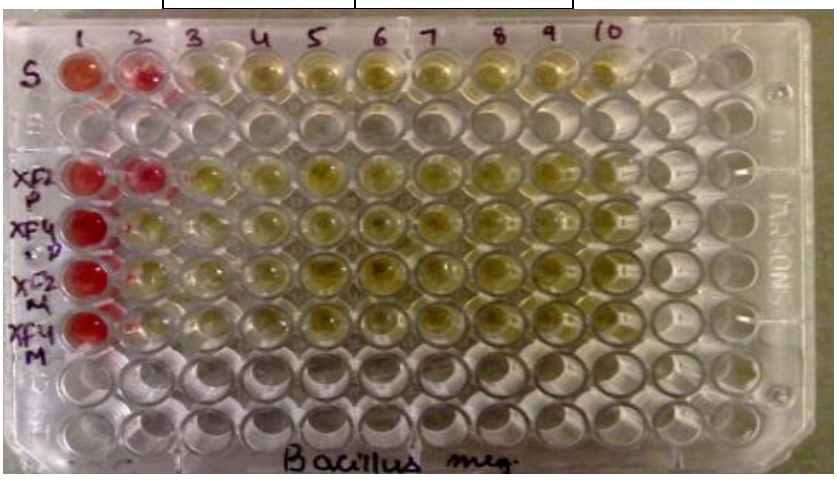

Figure 20 MIC of Streptomycin, XF-2 PDB, XF-4 PDB , XF-2 MEB & XF-4 MEB against *Bacillus megaterium.* Here S represents streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB (Conc. 1-10 µg/ml).

Results of Antimicrobial activity of fungal extract by Broth microdilution method (Spectrophotometric method)

Table 16 O.D. at 600 nm detected by ELISA plate reader of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Bacillus megaterium* after 24 hr

Conc. (µg/ml)	ST	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
1	***0.035±0.007***	0.029±0.007	0.026±0.017	0.235±0.007	0.219±0.007
2	0.014±0.007	0.093±0.004	0.039±0.002	0.148±0.002	0.177±0.003
3	0.015±0.001	0.078±0.001	0.032±0.001	0.105±0.001	0.163±0.002
4	0.026±0.001	0.081±0.001	0.025±0.001	0.076±0.004	0.129±0.002
5	0.027±0.007	0.047±0.003	0.023±0.007	0.069±0.002	0.039±0.001
6	0.022±0.007	0.033±0.002	***0.012±0.001***	0.068±0.001	***0.039±0.002***
7	0.016±0.002	0.038±0.007	0.017±0.001	0.064±0.001	0.030±0.001
8	0.014±0.001	0.018±0.007	0.016±0.007	0.049±0.001	0.027±0.001
9	0.014±0.003	***0.015±0.001***	0.011±0.007	0.032±0.002	0.019±0.001
10	0.014±0.002	0.017±0.001	0.012±0.007	***0.012±0.001***	0.017±0.001

where ST indicates Streptomycin, bold figures represents MIC value.

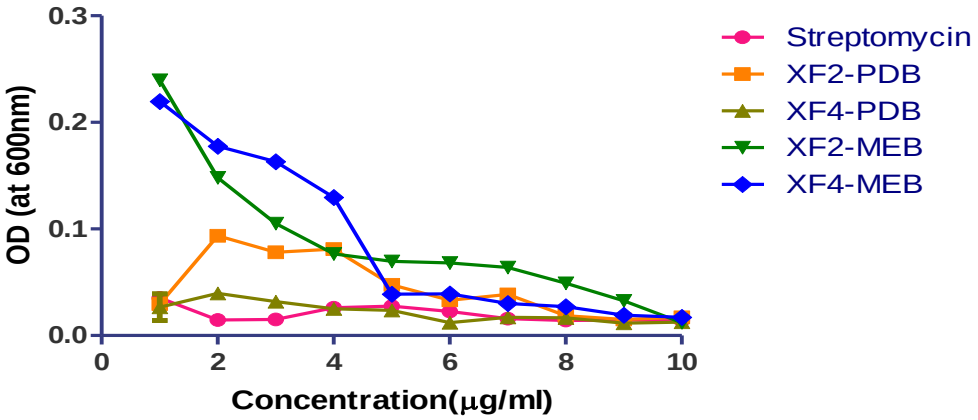

Figure 21 Comparison of MIC at 600 nm of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Bacillus megaterium.*

Table 17 O.D. at 600 nm detected by ELISA plate reader of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Pseudomonas aeruginosa* after 24 hr

Conc. (µg/ml)	ST	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
1	0.389±0.005	0.301±0.005	0.360±0.002	0.242±0.004	0.178±0.004
2	0.243±0.004	0.093±0.004	0.262±0.007	***0.061±0.005***	0.128±0.002
3	0.152±0.005	0.099±0.007	0.019±0.007	0.058±0.004	0.101±0.002
4	0.092±0.007	***0.078±0.005***	0.093±0.004	0.059±0.005	0.092±0.004
5	0.053±0.421	0.064±0.006	***0.078±0.004***	0.048±0.005	0.071±0.002
6	***0.055±0.004***	0.062±0.004	0.035±0.004	0.038±0.004	0.052±0.004
7	0.037±0.005	0.035±0.004	0.034±0.004	0.042±0.007	0.039±0.003
8	0.025±0.002	0.034±0.004	0.027±0.004	0.024±0.004	***0.014±0.003***
9	0.014±0.004	0.035±0.005	0.021±0.005	0.023±0.004	0.013±0.002
10	0.015±0.005	0.028±0.002	0.018±0.004	0.032±0.004	0.010±0.007

Each value represents mean of 2 replicates± S.D.

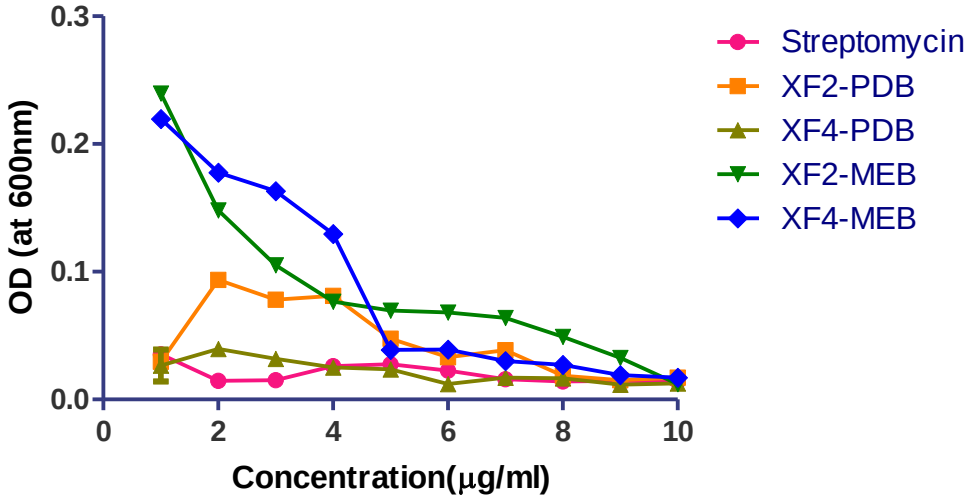

Figure 22 Comparison of MIC at 600 nm of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Pseudomonas aeruginosa*

Table 18 O.D. at 600 nm detected by ELISA plate reader of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Staphylococcus aureus* after 24 hr

Conc. (µg/ml)	ST	XF-2 PDB	XF-4 PDB	XF-2MEB	XF-4 MEB
1	0.192±0.004	0.173±0.003	0.116±0.002	0.196±0.004	0.190±0.002
2	0.112±0.005	0.123±0.002	0.110±0.002	0.159±0.003	0.164±0.004
3	0.092±0.004	***0.083±0.003***	***0.097±0.002***	0.147±0.003	0.075±0.004
4	0.071±0.003	0.068±0.003	0.072±0.005	0.132±0.003	0.073±0.004
5	***0.072±0.006***	0.065±0.001	0.066±0.004	0.133±0.003	0.060±0.002
6	0.059±0.003	0.062±0.004	0.060±0.002	***0.107±0.003***	0.061±0.004
7	0.040±0.003	0.063±0.003	0.050±0.004	0.099±0.003	***0.038±0.005***
8	0.037±0.004	0.056±0.002	0.037±0.003	0.035±0.001	0.027±0.002
9	0.030±0.002	0.034±0.003	0.036±0.004	0.027±0.002	0.017±0.002
10	0.015±0.004	0.027±0.004	0.036±0.004	0.026±0.002	0.015±0.002

Each value represents mean of 2 replicates± S.D.

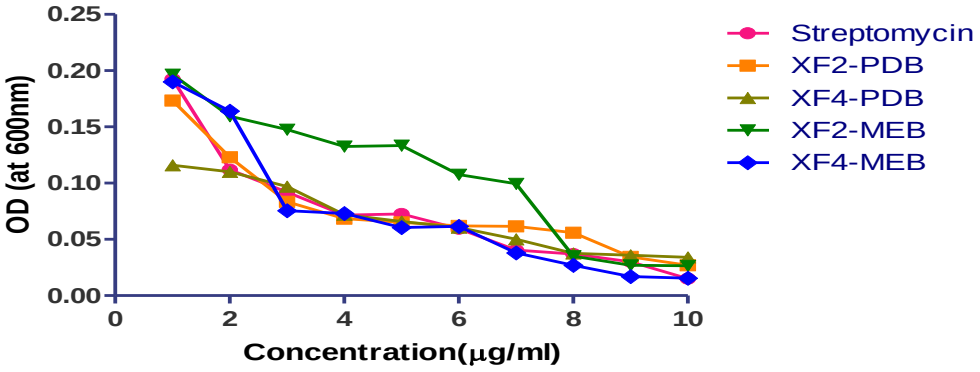

Figure 23 Comparison of MIC at 600 nm of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Staphylococcus aureus.*

Table 19 O.D. at 600 nm detected by ELISA plate reader of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Candida albicans* after 24 hr

Conc. (µg/ml)	Fluconazole	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
1	1.604±0.024	1.393±0.038	1.499±0.031	1.501±0.015	1.251±0.015
2	1.393±0.034	1.330±0.044	1.219±0.001	1.183±0.011	1.815±0.013
3	0.449±0.003	0.555±0.035	1.138±0.005	0.451±0.013	0.843±0.008
4	0.441±0.004	0.309±0.049	0.903±0.020	0.453±0.009	***0.316±0.007***
5	0.260±0.002	0.260±0.002	***0.355±0.009***	0.296±0.012	0.231±0.013
6	***0.256±0.007***	***0.236±0.008***	0.264±0.026	0.275±0.013	0.223±0.007
7	0.149±0.003	0.236±0.004	0.264±0.027	0.253±0.012	0.213±0.005
8	0.249±0.003	0.224±0.006	0.241±0.029	***0.243±0.012***	0.200±0.007
9	0.237±0.003	0.229±0.007	0.231±0.015	0.228±0.005	0.186±0.007
10	0.203±0.003	0.215±0.006	0.217±0.012	0.216±0.009	0.185±0.007

Each value represents mean of 2 replicates± S.D.

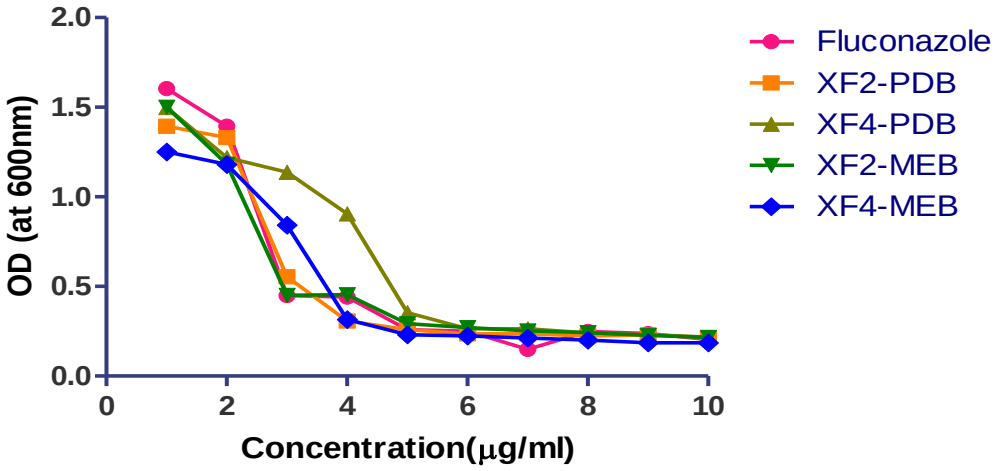

Figure 24 Comparison of MIC at 600 nm of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Candida albicans* at 24 hr

Table 20 O.D. at 600 nm detected by ELISA plate reader of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Candida albicans* after 48 hr

Conc. (µg/ml)	Fluconazole	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
1	1.440±0.041	1.340±0.014	1.448±0.004	1.450±0.014	1.250±0.014
2	1.296±0.005	1.220±0.014	1.138±0.003	1.267±0.010	1.181±0.013
3	0.081±0.002	0.758±0.004	0.963±0.012	1.157±0.006	0.844±0.010
4	0.026±0.006	0.271±0.004	0.449±0.017	1.017±0.005	***0.317±0.008***
5	***0.023±0.002***	0.267±0.006	***0.284±0.007***	0.624±0.010	0.225±0.005
6	0.024±0.007	***0.235±0.007***	0.237±0.004	0.246±0.008	0.219±0.002
7	0.020±0.001	0.230±0.004	0.248±0.007	0.180±0.008	0.211±0.006
8	0.017±0.004	0.227±0.007	0.219±0.003	0.219±0.007	0.199±0.006
9	0.016±0.004	0.211±0.002	0.217±0.009	***0.251±0.008***	0.189±0.113
10	0.014±0.003	0.196±0.005	0.201±0.006	0.249±0.008	0.186±0.008

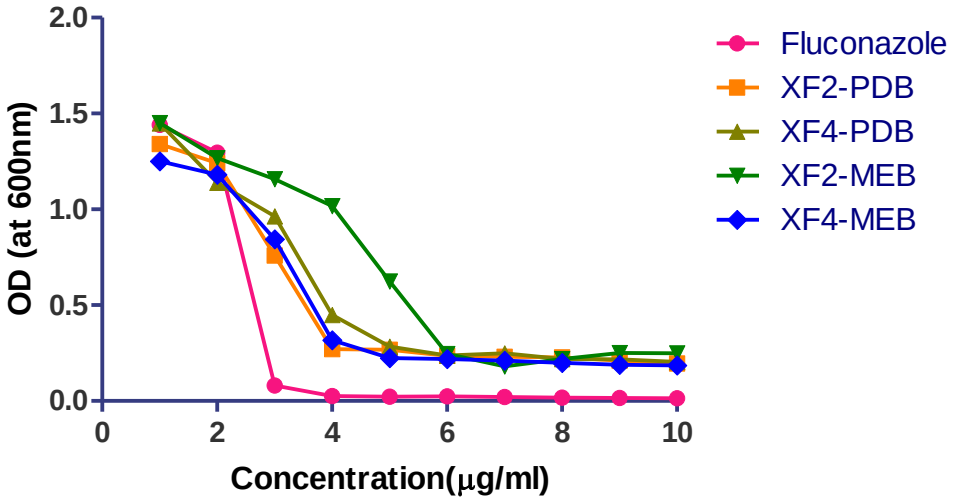

Figure 25 Comparison of MIC at 600 nm of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *Candida albicans* 48 hr

Table 21 O.D. at 600 nm detected by ELISA plate reader of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *E.coli* after 24 hr

Conc. µg/ml	Streptomycin	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
1	0.048±0.002	0.067±0.002	0.155±0.004	0.167±0.003	0.058±0.002
2	0.059±0.002	0.066±0.002	***0.079±0.002***	***0.067±0.003***	0.049±0.002
3	***0.043±0.002***	0.055±0.002	0.055±0.004	0.055±0.006	0.038±0.001
4	0.023±0.002	0.050±0.002	0.052±0.001	0.049±0.005	0.037±0.003
5	0.022±0.002	0.044±0.002	0.042±0.004	0.041±0.002	0.030±0.001
6	0.020±0.003	***0.037±0.002***	0.037±0.001	0.046±0.001	0.028±0.005
7	0.016±0.002	0.033±0.002	0.038±0.001	0.043±0.003	0.027±0.004
8	0.021±0.003	0.029±0.003	0.034±0.002	0.042±0.001	***0.020±0.003***
9	0.018±0.002	0.032±0.004	0.029±0.001	0.039±0.002	0.021±0.002
10	0.017±0.001	0.021±0.002	0.030±0.001	0.037±0.003	0.022±0.001

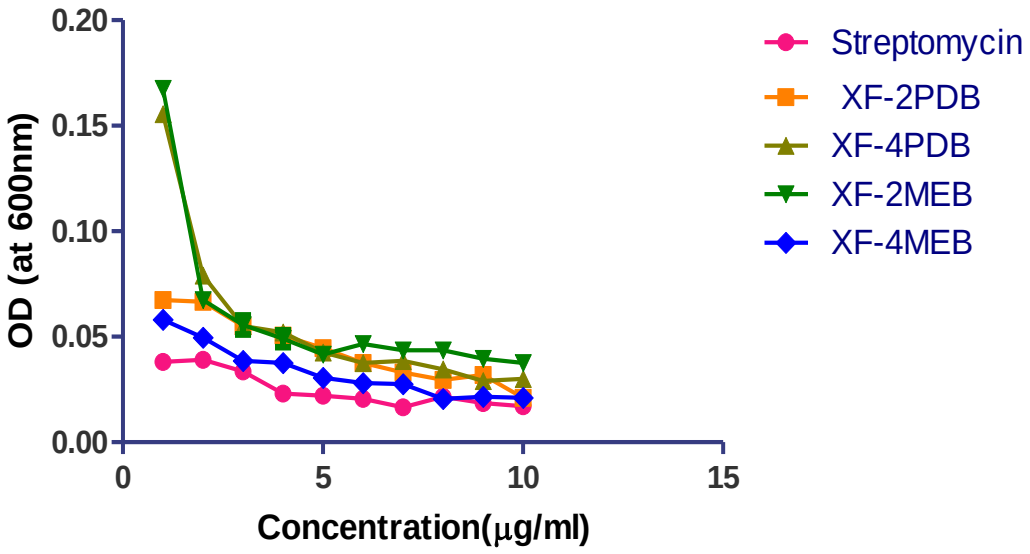

Figure 26 Comparison of MIC at 600 nm of Streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB & XF-4 MEB against *E.coli* after 24 hr

Results of preliminary phytochemical analysis of fungal extracts

Table 22 Preliminary phytochemical analysis of extracts

Tests	XF-2 PDB	XF-4 PDB	XF-2 MEB	XF-4 MEB
Test for Alkaloids	+	+	+	+
Test for amino acid	+	+	+	+
Test for Carbohydrate	+	+	+	+
Test for Fats & oils	-	-	-	-
Test for Flavonoids	+	+	+	+
Test for Glycosides	-	-	-	-
Test for Steroids & Triterpenoids	+	+	+	+
Test for Tannins & Phenolic	-	-	-	-

where – sign indicates absence and + sign indicates presence of a test compound

(a) **(b)** **(c)** **(d)**

Figure 27 Preliminary phytochemical tests for (a) Alkaloids (b) Flavonoids (c) Steriods and (d) Carbohydrate

DISCUSSION:

Fungal extract was assayed by agar well diffusion method. In this method an antibiotic was applied to a well that was cut into agar. The antibiotic moved from its region of higher concentration to the region of lower concentration. If there was a clearing around the antibiotic well then the bacteria has adversely been affected by the antibiotic. The zone of inhibition was measured and expressed in millimeters (mm).

The zone of inhibition in millimeter of XF-2 PDB extract is shown in Table 1. XF-2 PDB shows zone of inhibition in a range of 10.5-19mm against *Escherichia coli*, 13.5-23.5mm against *Staphylococcus aureus*, 10.5-20mm against *Pseudomonas aeruginosa* & 10.5-22mm against *Bacillus megaterium.* However in case of *S.aureus, P. aeruginosa* & *B.megaterium* there was no zone of inhibition at 10µg/ml. Also there was no zone of inhibition at 20µg/ml against *S.aureus*. From the figure 1 it may be concluded that against *S.aureus* and *Bacillus megaterium* XF-2 PDB extract is more effective. Table 2 shows that XF4 PDB has more pronounced inhibition against *S.aureus* with range (12.5-23.5mm) followed by *P.aeruginosa* with zone of inhibition ranges from (10.5-23 mm), *E.coli* (10.5-20mm), *Bacillus megaterium* range (11-17mm). At 10µg/ml *S.aureus*, *P.aeruginosa* and *B.megaterium* showed no zone of inhibition. Similarly XF-2 MEB was effective against *S.aureus* with zone of inhibition range (12-24mm) followed by *P.aeruginosa* with range (10.5-23mm), *E.coli* (11-20mm) and *B.megaterium* (11.5-20mm). However *S.aureus* and *B.megaterium* showed no zone of inhibition at conc. 10µg/ml. While on the other hand *E.coli* and *P.aeruginosa* were effectively inhibited by XF-2 MEB from conc.10µg/ml (Table 3). From Table 4 it may be concluded that XF-4 MEB is more effective against *B.megaterium* with range (13-25mm), followed by *S.aureus* (12-23mm), *P.aeruginosa* (11-21.5mm) and *E.coli* (12.5-20mm). At conc. 10µg/ml *S.aureus*, *P.aeruginosa* and *B.megaterium* showed no zone of inhibition. XF-4 MEB has more effective antifungal activity against *Candida albicans* with zone of inhibition range (12.5-21.5mm) as compared with XF-2 PDB with range (12-19.5mm) followed by XF-4 PDB range (12-19mm) and XF-2 MEB range (12-17.5mm) (Table5). From Table 6 it can be concluded that XF-2 PDB has zone of inhibition range (10.5-19mm), XF-4 PDB range (10.5-20mm), XF-2 MEB range (11-20mm) and XF-4 MEB range (11-20mm) against *Escherichia coli*. Against *Staphylococcus aureus* XF-2 PDB has range (13-23.5mm), XF-4 PDB range (12.5-24.5), XF-2 MEB (12-24.5mm) and XF-4 MEB

range (12-23mm). However XF-2 PDB showed no zone of inhibition at 10-20µg/ml also XF-4 PDB, XF-2 MEB and XF-4 MEB had no zone of inhibition at 10µg/ml (Table 7). Against *Pseudomonas aeruginosa* zone of inhibition XF-2 PDB has range (10.5-20mm) at (conc. 20-60µg/ml), XF-4 PDB range (10.5-23mm) at (conc. 20-60µg/ml), XF-2 MEB range (10.5-23mm) (conc. 10-60µg/ml) and XF-4 MEB range (11-21.5mm) at (conc. 20-60µg/ml) (Table 8). Table 9 revealed that XF-2 PDB has zone of inhibition range 10.5-22mm (conc. 20-60µg/ml), XF-4 PDB range (11-17mm) at (conc. 20-60µg/ml, XF-2 MEB range (11.5-20mm) at (conc. 20-60µg/ml) and XF-4 MEB range (13-25mm) at (conc. 20-60µg/ml) (Table 9). Table 10 showed zone of inhibition of Fluconazole, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *Candida albicans*. The range of inhibition zone for fluconazole is 13.5-22mm (conc. 20-60µg/ml), XF-2 PDB (12-19.5 mm) at (conc. 20-60µg/ml), XF-4 PDB range (12-19mm) at (conc. 20-60µg/ml), XF-2 MEB (12-17.5mm) at (conc. 20-60µg/ml) and XF-4 MEB range (12.5-21.5mm) at (conc. 20-60µg/ml).

From the data of agar well diffusion studies using various culture media, it has been found that the antimicrobial response of the extracts from XF-2 MEB media was more than the extract obtained from PDB. Table 11-15 shows results of Broth microdilution method. From Table 11 it may be concluded that Streptomycin has MIC>3µg/ml, XF-2 PDB >5µg/ml, XF-4 PDB 5µg/ml, XF-2 MEB 7µg/ml & XF-4 MEB >2µg/ml against *E.coli*. Table12 shows MIC range for streptomycin >5µg/ml, XF-2 PDB 3µg/ml, XF-4 PDB >5µg/ml, XF-2 MEB 6µg/ml & XF-4 MEB >6µg/ml against *S.aureus*. Against *P.aeruginosa* MIC range for streptomycin is >8µg/ml, XF-2 PDB 3µg/ml, XF-4 PDB 5µg/ml, XF-2 MEB >3µg/ml & XF-4 MEB 7µg/ml (Table 13). MIC range against *B. megaterium* for streptomycin was 2µg/ml, XF-2 PDB 2µg/ml, XF-4 PDB 1µg/ml, XF-2 MEB 1µg/ml& XF-4 MEB 1µg/ml (Table 14). Table 15 shows MIC range against *C.albicans*. For Fluconazole MIC was 2µg/ml, XF-2 PDB 2µg/ml, XF-4 PDB >3µg/ml, XF-2 MEB >2µg/ml & XF-4 MEB >2µg/ml.

Results of spectrophotometric microdilution method are shown in Tables 16-20. Table 16 shows O.D. at 600 nm of streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *B.megaterium* after 24 hr. The MIC for streptomycin was 1µg/ml, XF-2 PDB 9µg/ml, XF-4 PDB 6µg/ml, XF-2 MEB 10µg/ml, XF-4 MEB 6µg/ml. Similarly from Table 17 MIC of streptomycin, XF-2 PDB, XF-4 PDB, XF-2 MEB, XF-4 MEB against *P.aeruginosa* at 600nm after 24 hr. was

6µg/ml,4µg/ml,5µg/ml,2µg/ml,8µg/ml respectively. Against *S.aureus* MIC range was streptomycin 5µg/ml, XF-2 PDB 3µg/ml, XF-4 PDB 3µg/ml, XF-2 MEB 6µg/ml & XF-4 MEB7µg/ml (Table 18). MIC against *C.albicans* after 24 hr. at 600 nm was Fluconazole 6µg/ml, XF-2 PDB 6µg/ml, XF-4 PDB 5µg/ml, XF-2 MEB 8µg/ml, XF-4 MEB 4µg/ml (Table 19). From Table 20 the MIC range for Fluconazole is 5µg/ml, XF-2 PDB 6µg/ml, XF-4 PDB 5µg/ml, XF-2 MEB 9µg/ml, XF-4 MEB 4µg/ml against *C.albicans* after 48 hr. The range of MIC against *Escherichia coli* was, streptomycin (>8µg/ml), XF-2PDB (>3µg/ml),XF-4 PDB (5µg/ml), XF-2 MEB(7µg/ml) & XF-4 MEB (>2µg/ml) (from Table 11), MIC against *Staphylococcus aureus* was strepto-mycin(>5µg/ml),XF-2 PDB (3µg/ml), XF-4 PDB (>5µg/ml), XF-2 MEB (6µg/ml) & XF-4 MEB (>6µg/ml) (From Table 12). Against *Pseudomonas aeruginosa* the range of MIC was streptomycin (>8µg/ml), XF-2 PDB (3µg/ml), XF-4 PDB (5 µg/ml), XF-2 MEB (>3µg/ml), XF-4 MEB (7µg/ml) (From Table 13). MIC range against *Bacillus megaterium* was streptomycin (2µg/ml), XF-2 PDB (2µg/ml), XF-4 PDB, XF-2 MEB & XF-4 MEB (1µg/ml) (From Table 14).Against fungal isolate *Candida albicans* the MIC range was fluconazole (2 µg/ml),XF-2 PDB (2 µg/ml), XF-4 PDB (>3 µg/ml), XF-2 MEB (>2 µg/ml) & XF-4 MEB (>2 µg/ml) (From Table 15). The MIC range for microdilution method was Streptomycin (1µg/ml), XF-2 PDB (9µg/ml), XF-4 PDB (6µg/ml), XF-2 MEB (10µg/ml) & XF-4 MEB (6µg/ml) against *B.megaterium* (Table 16). Against *P.aeruginosa* the MIC range was Streptomycin (6µg/ml), XF-2 PDB (4µg/ml), XF-4 PDB (5µg/ml), XF-2 MEB (2µg/ml), XF-4 MEB (7µg/ml) (Table 17). MIC against *S.aureus* was Streptomycin (5µg/ml), XF-2 PDB (3µg/ml), XF-4 PDB (3µg/ml), XF-2 MEB (6µg/ml) & XF-4 MEB (7µg/ml) (Table 18). Against *Candida albicans* after 24 hr. MIC of Fluconazole (6µg/ml), XF-2PDB (6µg/ml), XF-4 PDB (5µg/ml), XF-2 MEB (8µg/ml) & XF-4 MEB (4µg/ml).(Table 19). After 48 hr. MIC against *Candida albicans* was in the range like fluconazole (5µg/ml), XF-2 PDB (6µg/ml), XF-4 PDB (5µg/ml), XF-2 MEB (9µg/ml) & XF-4 MEB (4µg/ml) (Table 20).Table 21 shows O.D. at 600 nm of extracts & streptomycin against *E.coli*. The MIC range of streptomycin was3µg/ml, XF-2 PDB 6µg/ml, XF-4 PDB 2µg/ml, XF-2 MEB 2µg/ml, XF-4 MEB 8µg/ml. Endophytic fungi from plants are known to produce various secondary metabolites having antibacterial and antifungal activity. A preliminary phytochemical investigation studies done to detect the presence of these secondary metabolites in fungal extracts. Table 22 shows presence of alkaloids, amino acids, carbohydrates, flavonoids & steroids.

CHAPTER 5

CONCLUSION

CHAPTER-5

CONCLUSION

Synthetic chemicals have since long been used as therapeutic agents in the control of plant, animal and human diseases. The main disadvantage with these chemicals is their cost, harmful effects on the environment and the emergence of pathogenic resistance. There are several studies on the antimicrobial activity of endophytic fungi isolated from plants. As a part of the current studies towards finding novel therapeutic agent an attempt has been made in this study to screen endophytic fungi belonging to *Xylariaceae* species for antimicrobial activity.

From the studies, it may be concluded that fungal culture extract XF-4 PDB, XF-2 MEB & XF-4 MEB (11-20mm) was more effective against *Escherichia coli*. Against *Staphylococcus aureus* XF-2 MEB (12-24.5mm) was effective. XF-4 MEB (13-25mm) has more pronounced effect against *Bacillus megaterium*. Against *Pseudomonas aeruginosa* fungal extracts XF-2 MEB, XF-4 PDB (10.5-23mm) were more effective. While on the other hand for *Candida albicans* XF-4 MEB (12.5-21.5mm) had pronounced effect as compared with fluconazole.

The spectrophotometric microdilution studies revealed that the fungal extracts XF-4 PDB & XF-4 MEB showed strong antimicrobial activity against *Bacillus megaterium* with MIC 6µg/ml each. However against *Pseudomonas aeruginosa* XF-2 MEB had MIC 2µg/ml. XF-2 PDB & XF-4 PDB were effective against *Staphylococcus aureus* with MIC 3µg/ml each.

Also MIC of XF-4 MEB was 4µg/ml against *Candida albicans*. And MIC of extracts XF-4 PDB, XF-2 MEB was 2µg/ml. Further chromatographic methods may be used to separate the active and pure compound from the crude extracts. The structure of pure compound having potential antimicrobial activity can be determine using various techniques.

SUMMARY

Endophytes are poorly investigated group of microorganisms, but they represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in wide variety of medicine, agriculture and industrial areas (Vanden *et al*. 1991). The objective of this project was to determine the antibacterial, antifungal activity and preliminary phytochemical analysis of fungal culture extract from *Xylaria* species. In the present study endophytic fungi isolated from different host plants were characterized for their antimicrobial activity using various methods like Agar well diffusion method, Broth microdilution method (colorimetric) & microdilution (spectrophotometric method). The main purpose of this study was to evaluate these endophytic fungi for the production of bioactive compounds. Methanolic extracts of the culture filterate were checked for their antibacterial activity using four bacterial cultures *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus megaterium* and a fungal culture *Candida albicans.* From the studies, it may be concluded that fungal culture extract XF-4 PDB, XF-2 MEB & XF-4 MEB (11-20mm) was more effective against *Escherichia coli*. Against *Staphylococcus aureus* XF-2 MEB (12-24.5mm) was effective. XF-4 MEB (13-25mm) has more pronounced effect against *Bacillus megaterium*. Against *Pseudomonas aeruginosa* fungal extracts XF-2 MEB, XF-4 PDB (10.5-23mm) were more effective. While on the other hand for *Candida albicans* XF-4 MEB (12.5-21.5mm) had pronounced effect as compared with fluconazole standard antibiotic.The spectrophotometric microdilution studies revealed that the fungal extracts XF-4 PDB & XF-4 MEB showed strong antimicrobial activity against *Bacillus megaterium* with MIC 6µg/ml each. However against *Pseudomonas aeruginosa* XF-2 MEB had MIC 2µg/ml. XF-2 PDB & XF-4 PDB were effective against *Staphylococcus aureus* with MIC 3µg/ml each. Also MIC of XF-4 MEB was 4µg/ml against *Candida albicans* and MIC of extracts XF-4 PDB, XF-2 MEB was 2µg/ml. The results of this study indicate that *Xylaria* species can be explored further for a possible source of useful antimicrobial agents against pathogenic microorganisms which include Gram positive, Gram negative, fungi & yeasts. In the present study phytochemical investigation revealed that oth fungal extracts (XF-2 & XF-4) contain phytoconstituents like alkaloids, steroids, flavonoids, amino acids and carbohydrates.

CHAPTER 7

REFERENCES

CHAPTER-7

REFERENCES

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ANNEXURE-I

1. Composition of Luria Broth

Ingredients	Gram/litre
Tryptone	10
Yeast extract	5
Sodium chloride	10

Final pH 7.0 ± 0.2 at 25ºC

2. Nutrient broth

Ingredients	Gram/litre
Peptone	10
Meat extract	10
Sodium chloride	5

Final pH 7.2±0.2

3. Sabouraud dextrose broth

Ingredients	Gram/litre
Dextrose	40
Peptone	10

Final p H 5.6

4. Potato dextrose broth

Ingredients	Gram/litre
Potato infusion	200
Dextrose	20

Final p H 5.1 at 25°C

5. Malt extract broth

Ingredients	Gram/litre
Malt extract	17

Final pH: 4.8 ± 0.2 at 25 °C.

6. Wagner reagent- Dissolve 1.27 g iodine and 2 g Potassium iodide (KI) in 5 ml of distilled water and make final volume upto 100 ml.

7. Ninhydrin solution - 2g of ninhydrin dissolved in 100 ml of acetone.

8. Molisch reagent - Dissolve 1 g of alpha-naphthol in 60 ml of 95% alcohol.