Antimicrobial Activity of Endophytic Fungi from *Xylaria* Species

A

DISSERTATION

Submitted in partial fulfillment of the requirements

for the Award of the Degree of

Masters of Science

(Biotechnology)

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Under the Guidance of
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(July-2012)

Certificate

This is to certify that the dissertation entitled, **"Antimicrobial Activity of Endophytic Fungi from *Xylaria* Species"** submitted by Ranjan Singh in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Thapar University, Patiala, is an authentic record of his own work carried out by him during the period of six months from January 2012 to June 2012, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Candidate's Declaration

I, hereby declare that the work which is being presented in the dissertation entitled **"Antimicrobial Activity of Endophytic Fungi from *Xylaria* Species"**, 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 the period of six months from January 2012 to June 2012, under the supervision of Mrs. M. Vasundhara, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University.

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

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Date: **Ranjan Singh**

Place:

Table of Contents

LIST OF TABLES

LIST OF FIGURES

17.	Effect of different concentrations of extracts on growth of *Pseudomonas aeruginosa* after 24 hours.
18.	Effect of different concentrations of streptomycin (control) on growth of *Pseudomonas aeruginosa* after 24 hours
19.	Effect of different concentration of extract on growth of *Escherichia coli* after 24 hours
20.	Effect of different concentrations of streptomycin (control) on growth of *Escherichia coli* after 24 hours
21.	Effect of different concentrations of extracts on growth of *Staphylococcus aureus* after 24 hours
22.	Effect of different concentrations of streptomycin (control) on growth of *S. aureus* after 24 hours
23.	Effect of different concentrations of extracts on growth of *Candida albicans* after 24 hours
24.	Effect of different concentrations of Fluconazole (control) on growth of *Candida albicans* after 24 hours
25.	Effect of different concentrations of extracts on growth of *Candida albicans* after 48 hours
26.	Effect of different concentrations of Fluconazole (control) on growth of *Candida albicans* after 48 hours
27.	Presence of phytochemical compounds in XF-8 and XF-15 extract
28.	TLC of methanolic extracts, spot (s) = control, (a) = XF-8(P), (b) = XF-8(M), (c) = XF-15(P) and (d) = XF-15(M)

ABBREVIATIONS

AWD assay	**Agar Well Diffusion Assay**
CFU	**Colony Forming Unit**
EtOAc	**Ethyle acetate**
$FeCl_3$	**Ferric chloride**
KI	**Potassium iodide**
MHA	**Mueller-Hinton Agar**
MHB	**Mueller-Hinton Broth**
MIC	**Minimum Inhibitory Concentration**
OD	**Optical Density**
rpm	**Revolutions per minute**
SDA	**Sabouraud Dextrose Agar**
SDB	**Sabouraud Dextrose Broth**
TLC	**Thin Layer Chromatography**
TTC	**2, 3, 5-Triphenyl Tetrazolium Chloride**
WHO	**World Health Organization**

INTRODUCTION

1. Introduction

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Many complementary and alternative medicines have enjoyed increased popularity in recent decades (Joseph and Priya, 2011). Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful especially in the areas of infectious disease and cancer (Cragg and Newman, 2005). More than 90% of the terms recorded in Indian medical literature are derived from plant sources (Joseph and Priya, 2010). For the past two years, there has been an increasing interest in the investigation of different novel natural bioactive products from plants (Joseph *et al*., 2010). Recent trends, however, show that the discovery rate of active novel chemical entities is declining (Lam, 2007). Therefore, there is a need to bio-prospect new sources and if possible from less explored regions and habitats to maximize the discovery of novel bioactive metabolites.

The *Xylariaceae* represent one of the largest and most important families of the *Ascomycotina* and the Fungi. The exact number of currently accepted taxa varies between authors. For instance, sixty genera that potentially belong to the *Xylariaceae* were listed by Lumbsch & Huhndorf (2007). The Dictionary of the Fungi (Kirk *et al.,* 2008) listed 85 accepted genera (including 71 synonyms) and 1343 accepted species. The vast majority of those are associated with plants, but some genera and species are known from herbivore dung or termite nests.

Xylaria species hedge their reproductive bets by engaging in both sexual and asexual reproduction. The spores, asci, and perithecia mentioned above occur when the fungus is mature and reproducing sexually. In immature stages, a *Xylaria* produces asexual spores, officially called "conidia," in a powdery coating (morel hunters frequently encounter the conidial stage of *Xylaria polymorpha* in late spring).

Xylaria is characterized by perithecial ascocarps bearing paraphyses and periphyses that are embedded in stroma. The asci of most species bear a ring at the apex that appears as a characteristic amyloid ascal plug when stained with iodine. Some other studies dealt with the role of *Xylariaceous* and other fungal endophytes in the process of wood decay, in which these fungi turn from endophytes to saprotrophs (Boddy & Griffith, 1989). Visser *et al.,* (2009) revealed an interesting phenomenon of co-speciation between termite-associated *Xylaria* sp. and their invertebrate hosts.

Endophytes are microorganisms that include bacteria and fungi living within plant tissues without causing any immediate overt negative effects have been found in every plant species examined to date and recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms (Bacon and White, 2000; Strobel and Daisy, 2003; Kumar and Sagar, 2007). Endophytes are ubiquitous with rich biodiversity, which have been found in every plant species examined to date. It is noteworthy that, of the nearly 3,00,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes (Strobel and Daisy, 2003). In this view of the special colonization in certain hosts, it is estimated that there may be as many as 1 million different endophyte species. However, only a handful of them have been described (Andrew and Hirano, 1991), which means the opportunity to find new and targeting natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great. Some of the endophytes are the chemical synthesizers inside the plants (Owen and Hundley, 2004).

Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against human pathogens and some of these compounds have been proven useful for novel drug discovery. Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes. Up to now, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents and other bioactive compounds by their different functional roles. Thus far, they have not been widely explored for therapeutic properties. A single endophyte may be able to produce not one but several bioactive metabolites. As a result, the role of endophytes in the production of novel structures for exploitation in medicine is receiving increased attention (Wang *et al*., 2000; Gunatilaka, 2006).

A small amount of endophytes have been studied, recently, several research groups have been motivated to evaluate and elucidate the potential of these microorganisms applied on biotechnological processes focusing on the production of bioactive compounds. The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt to plant host and carry out some functions such as protection from pathogens, insects and grazing animals (Strobel, 2003). Endophytes are chemical synthesizer inside plants (Owen and Hundley, 2004), in other words, they play

a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms (Strobel, 2003).

Bioactive natural compounds produced by endophytes have been promising potential usefulness in safety and human health concerns, although there is still a significant demand of drug industry for synthetic products due to economic and time-consuming reasons (Strobel *et al*., 2004). Problems related to human health such as the development of drug resistance in human pathogenic bacteria**,** fungal infections and life threatening virus claim for new therapeutic agents for effective treatment of diseases in human, plants and animals that are currently unmet (Strobel and Daisy, 2003; Strobel, 2003; Zhang *et al*., 2005). Recent review by Cragg and Newman (2007) presented a list of all approved agents from 1981 to 2006, from which a significant number of natural drugs are produced by microbes and/or endophytes. Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones and others (Tan and Zou, 2001). Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants and anticancer agents (Gunatilaka, 2006).

The objective of this project is to investigate the into antimicrobial activity of compounds produced by *Xylaria* species and preliminary phytochemical investigation of the bioactive compounds produced by these endophytes.

Significance of the project

Studies have been done where it has been shown that endophytic *Xylaria* species produce secondary metabolites that are biologically active to treat certain diseases. As only a small amount of endophytes have been studied for their therapeutic potential, hence more research studies are required to investigate the use of these secondary metabolites against various disease causing microbes.

REVIEW OF LITERATURE

Review of literature

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2.1 Need of new antimicrobials

Antibiotics were treated as miracle drugs when they first became available half a century ago. However, their popularity rapidly led to overuse. Over the last decade, it has become well known that antibiotics are losing their effectiveness as bacteria evolve resistance against them and new drugs only rarely reach the market (Cuevas, 2003). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetic or natural (Nascimento *et al.*, 2000).

The rise of new and more drug resistant infectious diseases as a result of climatic change and deforestation remain one of the biggest killers in developing countries (Stella *et al*., 2010). Additionally, the resistance pattern of pathogens to synthetic drugs often creates a need for stronger and stronger drugs or an increased dosage that could result in severe deterioration of the patient's health and an unexpected growth of drug resistance organisms like NDM-1 bacteria (Kumarasamy *et al*., 2010). So, countries spend billions of dollars to identify new broad spectrum antibiotics (Breman *et al*., 2007). Antibiotics have undoubtedly made major contributions to the improvement of health and welfare in both human and animals. In recent year there has been an alarming rise in the prevalence of resistance to some agents among certain groups of pathogens (Carl and Pascal, 2006). Concern is growing that therapeutic options will become increasingly limited if resistance rates continue to rise as it has in the past few decades. There is widespread agreement that immediate action is required to reverse or at least slow this process. Vector transmitting pathogens are a high level threat to humans and animals. The climatical changes will tremendously influence life cycles of arthropod vectors for the maximum productivity which resulting in changes in both vector and pathogen distribution and changes in the ability of arthropods to transmit pathogens (Tabachnick, 2010). So, there is an urgent need to study the most commonly used drugs for their potency in reducing parasite growths and symptoms of the disease (Talisuna *et al*., 2004).

Traditional approaches from natural sources: The World Health Organization estimates that over 65% of the world's population relies on traditional medicine for their primary health needs (Wang *et al*., 2007; Fabricant and Farnsworth, 2001). The planet has various plant biodiversity. Natural products especially from plants have been used to treat various infectious diseases for thousands of years. Almost all plant varieties have at least some minimal source of medicinal properties. History records the use of natural products (Bhutani and Gohil, 2010) to cure many diseases i.e., TB, Leprosy, Gonorrhoea, various skin diseases and some internal disorders. In the battle field, wounded soldiers were treated with extracts of various plant leaves. Indian Ayurvedic systems, Susruta and Charaka dating back to 1000 BC and still now widely practiced, have used plant extracts as their main source of treatment (Kapoor, 1990). After invention of Penicillin by A. Flemming, the biomedical world has realized importance of microbial sourcing.

Plants based phenols and polyphenols, flavonoids, isoflavones, terpenes and glucosinolates are responsible for bitterness (Drewnowski and Gomez-Carneros, 2000) and these "bitter," chemicals generate signals which affect a cascade of regulatory events resulting in chemical differentiation in Streptomyces genome by activating "cryptic pathways" for secondary metabolite biosynthesis (Rigali *et al*., 2008) and offers new prospects in the fight against emerging diseases. The chemical and biological diversity of nature is immeasurable and provides an extraordinary resource for the discovery of new drugs (Luke *et al*., 2005).

Endophytes are microorganisms that colonize inside plant tissue and are relatively crucial for the medical and agricultural industries (Lodewyckx *et al*., 2002). They habitat in tissues below the plant's epidermal cell layers due to the fact that some host plant's tissues are transiently asymptomatic (Strobel, 2003; Tharek *et al*., 2011). Some plants were studied for endophytes; fortunately, the opportunity to find novel endophytic microorganisms from variety of plants in the ecosystems was great.

2.2 Endophytes as antimicrobial agents

Metabolites bearing antibiotic activity can be defined as low-molecular-weight organic natural substances made by microorganisms that are active at low concentrations against other microorganisms (Guo *et al*., 2008). Endophytes are believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites (Tan and Zou, 2001). So far, studies reported a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes

like alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids (Yu *et al*., 2010). The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens, the insufficient number of effective antibiotics against diverse bacterial species and few new antimicrobial agents in development, probably due to relatively unfavorable returns on investment (Yu *et al*., 2010; Song, 2008). The antimicrobial compounds can be used not only as drugs by humankind but also as food preservatives in the control of food spoilage and food-borne diseases, a serious concern in the world food chain (Liu *et al*., 2008).

The production of Hypericin, a naphthodianthrone derivative and Emodin believed to be the main precursor of hypericin by the endophytic fungus isolated from an Indian medicinal plant was reported. Both compounds demonstrated antimicrobial activity against several bacteria and fungi including *Staphylococcus aureus* sp., *Klebsiella pneumoniae* sp., *Pseudomonas aeruginosa***,** *Salmonella enterica* sp., *Enteric* and *Escherichia coli* and fungal organisms *Aspergillus niger* and *Candida albicans* (Kusari *et al*., 2008).

Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones and others (Tan and Zou *et al.,* 2001). Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants and anticancer agents (Gunatilaka, 2006).

Chlorinated metabolites such as (-) mycorrhizin A, (+)- cryptosporiopsin isolated from endophytic *Pezicula* strains were reported as strongly fungicidal and herbicidal agents and to a lesser extent, as algicidal and antibacterial agents (Schulz *et al.,* 1995). Similarly, two other new chlorinated benzophenone derivatives, Pestalachlorides A and B, from the plant endophytic fungus *Pestalotiopsis adusta,* proven to display significant antifungal activity against three plant pathogenic fungi, *Fusarium culmorum, Gibberella zeae* and *Verticillium albo-atrum* (Li *et al*., 2008).

An endophytic *Streptomyces* sp., from a fern-leaved grevillea (*Grevillea pteridifolia*) in Australia was described as a promising producer of novel antibiotics, kakadumycin A and echinomycin. Kakadumycin A is structurally related to echinomycin, a quinoxaline antibiotic and presents better bioactivity than

echinomycin especially against grampositive bacteria and impressive activity against the malarial parasite *Plasmodium falciparum* (Castillo *et al*., 2003). Another novel endophytic Streptomyces SUK 06 from *Thottea grandiflora* in Malaysia was reported bioactive secondary metabolites with ethyl acetate have killing activity against *Bacillus subtilis***,** *Pseudomonas aeruginosa, Bacillus cereus***,** *Pleisiomonas shigelloides* and MRSA. Nevertheless, there were some antifungal activity measured against *Fusarium solani***,** *Aspergillus fumigatus, Pythium ultimum, Phytophthora erythroseptica* and *Geothrichum candidum* (Ghadin *et al*., 2008). More than 50% of endophytic fungi strains residing in *Quercus variabilis* possessed growth inhibition against at least one pathogenic fungus or bacteria. *Cladosporium* sp., displaying the most active antifungal activity, was investigated and found to produce a secondary metabolite known as brefeldin A, a lactone with antibiotic activity. Results showed brefeldin A to be more potent than the positive control in antifungal activity (Wang *et al.,* 2007).

Another fascinating use of antibiotic products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors, cytonic acids A and B have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp., Their structures as p-tridepside isomers were elucidated by mass spectrometry and NMR methods (Guo *et al.,* 2000). An endophytic fungus *Pestalotiopsis theae* of an unidentified tree on Jianfeng Mountain, China, was capable of producing Pestalotheol C with anti-HIV properties (Li *et al*., 2008). It is apparent that the potential for the discovery of compounds, from endophytes, having antiviral activity is in its infancy. The fact, however, that some compounds have been found is promising. The main limitation in compound discovery is probably related to the absence of appropriate antiviral screening systems in most compound discovery programs (Strobel and Daisy, 2003).

Tan and Zou (2001) believe the reason why some endophytes produce certain phytochemicals originally characteristic of the host might be related to a genetic recombination of the endophyte with the host that occurs in evolutionary time. This is a concept that was originally proposed as a mechanism to explain why the endophytic fungus *T. andreanae* may be producing paclitaxel (Stierle *et al*., 1993). Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow growing and possibly rare plants but also preserve the world's ever diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued

product may be easier and more economical to produce, effectively reducing its market price (Strobel and Daisy, 2003).

2.3 The Genus *Xylaria* and antimicrobial activity

Many bioactive compounds, including antifungal agents, have been isolated from the genus *Xylaria* residing in different plant hosts, such as sordaricin with antifungal activity against *Candida albicans* (Pongcharoen *et al*., 2008), mellisol and 1,8- dihydroxynaphthol 1-O-a-glucopyranoside with activity against herpes simplex virus type 1 (Pittayakhajonwut *et al*., 2005), multiplolides A and B with activity against *Candida albicans* (Boonphong *et al*., 2001).

The bioactive compound isolated from the culture extracts of the endophytic fungus *Xylaria* sp. YX-28 isolated from *Ginkgo biloba* L. was identified as 7-amino-4-methylcoumarin (Liu *et al*., 2008). The compound presented broad-spectrum inhibitory activity against several food-borne and food spoilage microorganisms including *S. aureus, E. coli, S. typhia, S. typhimurium, S. enteritidis, A. hydrophila, Yersinia* sp., *V. anguillarum, Shigella* sp., *V. parahaemolyticus, C. albicans, P. expansum* and *A. niger*, especially to *A. hydrophila* and was suggested to be used as natural preservative in food (Liu *et al*., 2008).

Another strain F0010 of the endophytic fungus *Xylaria* sp., from *Abies holophylla* was characterized as a producer of griseofulvin, a spirobenzofuran antifungal antibiotic agent used for the treatment of human and veterinary animals mycotic diseases (Park *et al*., 2005).

Another interesting aspect of earlier Xylaria sp. was that the endophytic *Xylariaceae* species as chemical signals during the establishment of fungus-plant interactions (Chapela *et al.,* 1991).The compounds 2-hexyl-3-methyl-butanodioic acid and cytochalasin D were isolated from the endophytic fungus *Xylaria* sp., isolated from Brazilian Cerrado and presented antifungal activity (Cafeu *et al.,* 2005).

Based on the abundance of secondary metabolites found in Xylariaceae family, Liu *et al.,* (2008), identified and described the biological activity of 7-amino-4-methylcoumarin, a compound extracted from *Xylaria* sp YX-28 endophytic fungus. The chemical investigation of *Xylaria* (Xylariacea) genus

fungus leads to potential sources of natural products, as Xylarenal A, a terpenoid isolated form *Xylaria persicaria* fermentation (Smith *et al.,* 2002) xylactam, a nitrogened compound obtained from *Xylaria eμglossa* ascomycete (Wang *et al.,* 2005).

2.4 Screening techniques for antimicrobial compounds and Bioassays

Bioassays play an important role while evaluating antimicrobial activity of plant fractions and antibiotics. Bioassays are divided into two categories based on their performance: Prescreen assays and Screen assay. Prescreen assay is applied to large number of initial samples to check whether the plant extracted fractions have desired bioactivity or not. While the screen assays are used to select a compound or material for the detailed study for its bioactivity. *In vitro* assessment of anti-microbial activity of any extract or material is done by two methods: Agar well diffusion assay and Broth dilution method.

2.4.1 Prescreen assay: Agar well diffusion method

The agar well diffusion assay is most commonly used prescreen to determine antimicrobial susceptibility (Cowan, 1999). Initial screening of potential antibacterial and antifungal compounds from plants may be performed with pure substances or crude extracts. Agar well diffusion assay (Reddish, 1929) is an advanced version of ditch plate assay initially designed by Alexander Flemming which uses wells prepared in agar to evaluate the antimicrobial qualities in antiseptic solutions (Flemming, 1924).

Similarly, Heatley (1944) used agar diffusion assay as a means of monitoring the extraction and purification of penicillin. In this assay, the test material is placed in the agar well in the centre of agar plate and then seeded with test organism. If on incubation, a circular clear "zone of inhibition" surrounded the reservoir or agar well, that means the organism is sensitive to the test material. Agar well assay is popular prescreen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for the treatment of infectious diseases (Navarro *et al*., 1996).

2.4.2 Screening assay: Micro-broth dilution method

Broth micro-dilution denotes the performance of broth dilution test in micro-dilution plate (microtitre plate) with a capacity of 300 µl per well. This test is useful in the determination of Minimum Inhibitory

Concentration (MIC) using dilution method. The lowest concentration that will inhibit the visible growth of the microorganism is referred is known as the MIC. This test is rapid in the assessment of antimicrobial activity of fungal extracts.

Microtiter plate-based assays have been developed for a wide range of applications including monitoring of bioactive compound production in fermentation samples (Casey *et al*., 2004), determination of antimicrobial susceptibility patterns of microorganisms (Jones & Dudley, 1997), etc. The major problem of this kind of assays is that they use turbidity or absorbance to monitor biomass growth and death rates (Lopez-Garcia, Veynat, Perez-Paya, Gonzalez-Candelas, & Marcos, 2003; Nayak, Khan, Watson, & Cernigila, 2002). In this way, such assays are time consuming because of the need of reaching an enough growth to take the readings.

Minimum inhibitory concentrations (MICs) of crude ethyl acetate extracts were determined by agar microdilution according to NCCLS (National Committee for Clinical Laboratory Standards (NCCLS) 2004) against bacteria and yeasts and a modification of the microbroth dilution NCCLS 38-A (National Committee for Clinical Laboratory Standards (NCCLS) 2002) method against M. *gypseu.*

MATERIAL AND METHODS

3.1 Source of endophytic fungi

Endophytic fungi were isolated from the different host plants of Western Ghats of India. The fungal isolates XF-8 (Host – *Euraya nitida*, Family – Terstroemiaceae) and XF-15 (Host-*Neolitsea scrobiculata*, Family – Lauraceae) were provided by TIFAC-CORE, Thapar University, Patiala, Punjab, which were used in this study.

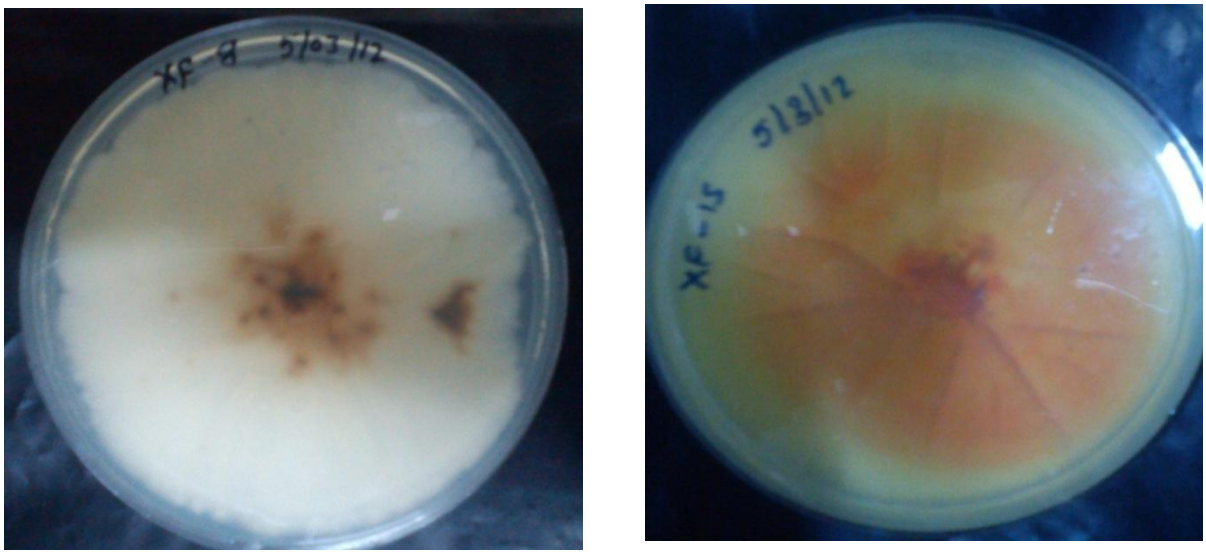
3.2 Materials, chemicals and instruments used

In this project the instruments and apparatus used were: Petri plates, Separation funnel, Borer (7mm diameter), Spreader, Microtitre plates (Tarson), Auto pipettes (1ml, 20-200µl), Shaker at 25°C/120 rpm (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), Vortex (Genei), etc.

And also various chemicals used were TTC dye, Distilled water, Muller –Hinton Agar (MHA), Luria broth (LB), Luria agar plates, Nutrient broth (NB), Nutrient agar plates, Antimicrobial as control (Streptomycin and Fluconazole), Ninhydrin, Conc. H_2SO_4, Molisch reagent, Wagner, Biuret reagent, 5% $FeCl_3$, Ethyl acetate (Merck), Methanol (Merck) etc.

3.3 Fermentation and filtration

Each of the isolated fungi were grown on PDA at 25^oC for 14 days. Three pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300mL potato dextrose broth (PDB) and malt extract broth (MEB), incubated for 9 days under agitation (120 rpm) at 25±1°C. After the fermentation process, the culture broths were separated from the mycelia by filtration through sterile mesh cloth. The filtrate was used for preliminary testing of antimicrobial activity.



(a) **(b)**

Figure 1: Growth of (a) XF-8 and (b) XF-15 on PDA

Table 1: Liquid phase fermentation conditions

Media	Fungal Culture	Temp (°C)	Incubation period (Day)	Conditions (120 rpm)	replicates
PDB	XF-8	25	9	Shaken	4
	XF-15	25	9	shaken	4
MEB	XF-8	25	9	shaken	4
	XF-15	25	9	shaken	4

3.4 Extraction of Bioactive compounds

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 10 min. The solution was then allowed to stand, where the cell mass got separated and the solvent so obtained was collected. Ethyl acetate was then evaporated using rotary evaporator under reduced pressure at 40^{o}C to yield an EtOAc extract and then powdered extract was dissolved in methanol and stored at 4^{0}C in eppendorf tubes. These extracts were used to check for their antimicrobial assay.

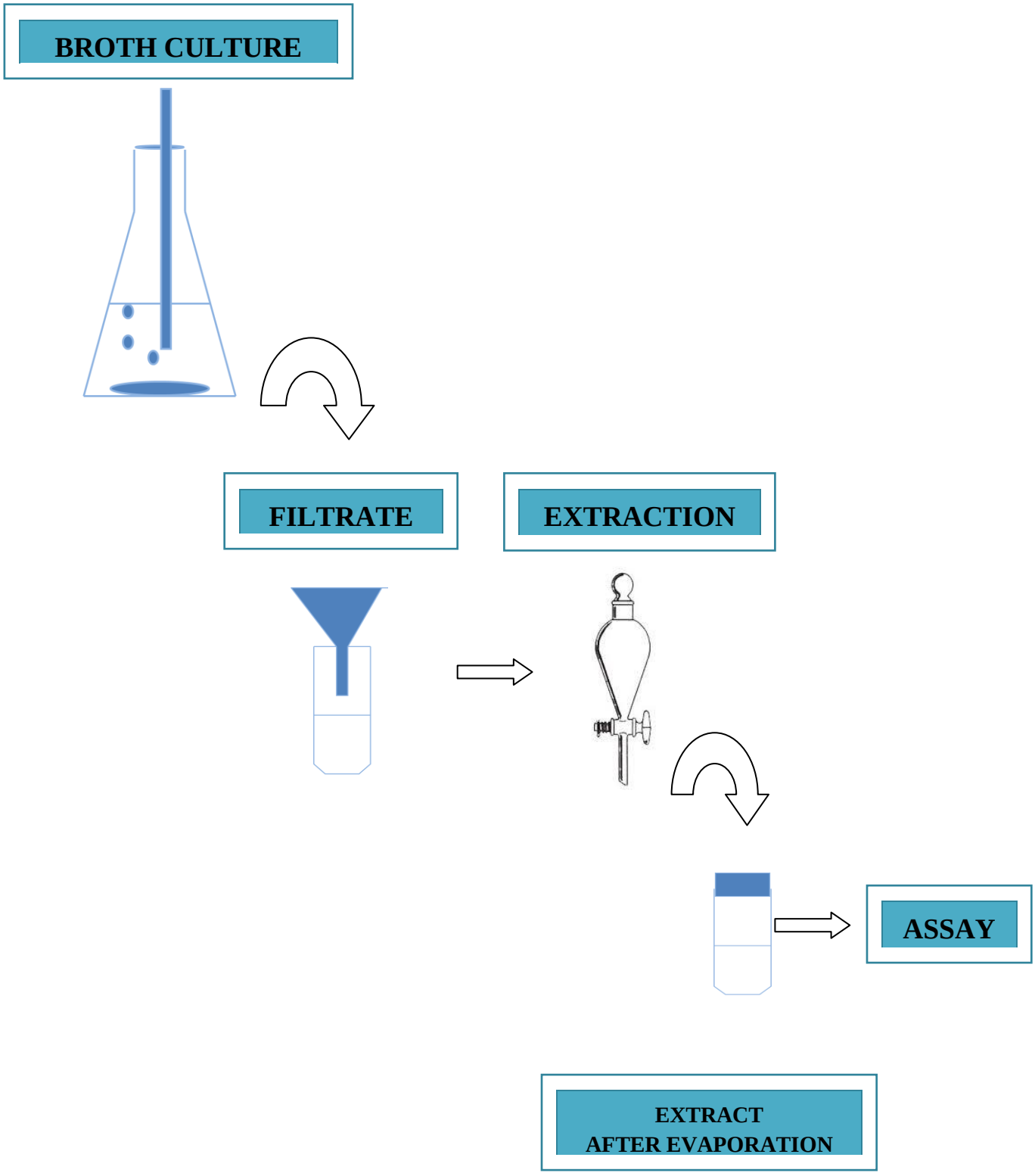


Figure 2: Extraction of Bioactive compounds

3.5 Test microorganisms and growth conditions

The following bacterial strains were employed for the antimicrobial screening: Gram-positive: *Staphylococcus aureus* and *Bacillus megaterium.* Gram-negative: *Escherichia coli* and *Pseudomonas aeruginosa*. For the antifungal screening the following fungi were used: *Aspergillus fumigates* and *Candida albicans*. The bacterial parent strains were activated in Mueller-Hinton Broth at 37^{o}C for 18-24 hours at 120 rpm prior to assays. *Aspergillus fumigates* was activated on PDA at 25^{o}C for 7 to 9 days and *Candida albicans* was in sabouraud dextrose broth (SDB) at 30^{o}C for 48 hours at 120 rpm.

3.6 Maintenance of microorganisms

The cultures were maintained and stored at 4ºC. Activation of the culture was carried out by streaking culture on to a Muller-Hinton Agar (MHA) (HiMedia) plate and then incubating them overnight at 37ºC. A single colony was then picked from this plate and transferred to Muller Hinton broth and incubated for 18-24 hours at 37ºC prior to the test.

3.7 Antimicrobial agent (as control)

The antimicrobial agent used as control in the present study was Streptomycin, a product of Cipla pharmaceutical Pvt. Ltd, India. The Streptomycin used was with an Expiry Date of June 2013 and potency of 500 mg / 704.125 mg of powder. The drug was stored in sealed containers in the dark at 4^{o}C with a desiccant. Prior to experiments these antibiotic powders/stock solutions of Streptomycin were brought to room temperature. And fluconazole, a product of Cipla pharmaceutical Pvt. Ltd, India was used as antifungal control.

3.8 Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), was used. A $BaSO_4$ 0.5 McFarland standard was prepared as 0.5 mL aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) added to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.144 to 0.146 for the 0.5 McFarland standard. The Barium Sulfate

suspension was transferred in 4 to 6 mL aliquots into screw-cap tubes of the same size as those used for growing or diluting the bacterial inoculum (NCCLS, 1997).

Table 2: McFarland Standard

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (mL)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (mL)	9.95	9.9	9.8	9.7	9.6
Approx. cell density ($1x10^8$ CFU/mL)	1.5	3.0	6.0	9.0	12.0
Absorbance*	0.146	0.210	0.449	0.661	0.850

*at wavelength of 600 nm

3.9 Preparation of stock solutions for MIC determination

For the preparation of stock solutions, potency of the drug powder has to be ascertained which can be done by using the formula:

$$\text{Weight of powder (mg)} = \frac{\text{volume of stock (mL) x concentration (mg/L)}}{\text{Potency of powder (mg/g)}}$$

A stock solution of 100 mg/mL of streptomycin was used in the present study. The diluents used was sterile distilled water or 0.90% saline solution. This was then passed through a bacterial filter under aseptic conditions in sterile bottles/vials and stored at -20^{o}C or below until used.

3.10 Preparation of Mueller-Hinton Agar

Mueller-Hinton agar was prepared from a HiMedia dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, it was cooled and dispensed into plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 5 ± 0.5 mm. This corresponds to 25 mL of medium for plates with a diameter of 90 mm. The agar medium was allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C). Plates were used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar. Representative samples of each batch of plates were examined for sterility by incubating at 37°C for 24 hours or longer.

3.11 Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

Agar-well diffusion assay

Minimum Inhibitory Concentration-Broth dilution method

MIC by the spectrophotometric method.

3.11.1 Pre screen assay: Agar-well diffusion assay

Initial screening for potential antibacterial and antifungal compounds from endophytic fungus was performed with crude extracts. Agar well assay is popular prescreen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for the treatment of infectious diseases (Navarro *et al*., 1996).

The growth method was performed as follows:

1. Well-isolated colonies from an agar plate culture were transferred into a tube containing 5 mL of broth medium.
2. The broth culture was incubated at 37°C until it achieves the turbidity of the 0.5 McFarland standard (10^8 CFU/mL).
3. The turbidity of growing broth culture was adjusted with sterile saline solution.
4. Then 100 µl of inoculum was spread on the agar plate.
5. Prepared 4 wells of 7 mm each using a sterile cork borer under aseptic conditions.
6. A fixed volume of extract (of different concentrations) was then introduced into the bored agar wells (The antimicrobial present in the extract was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organism) and then incubated at optimum temperature and duration depending upon the test microorganism.
7. After incubation, zone diameter was measured to the nearest whole millimeter (the resulting zones of inhibition were uniformly circular as there was a confluent lawn of growth).
8. The test was carried out in triplicates.

3.11.2 Screening: Microplate - broth dilution assay

Broth micro-dilution denotes the performance of antimicrobial / antibacterial potential of a dilution at the least in 96 wells micro-dilution plate (microtitre plate) with a capacity of above 300 µl per well. The MIC was determined using MH broth (HiMedia) and using a color indicator, 2,3-5 Triphenyl tetrazolium chloride (TTC), which was reduced to formazan (pink to red color) by living cells. MIC concentration does not exhibit reduction of TTC into formazan.

Steps followed

The Minimum Inhibitory Concentration (MIC) values of extract were determined in triplicate by using the microdilution broth method in 96-well microplates. 100 µl of sterile Mueller-Hinton broth was dispensed in the wells of 96 well sterile microtitre plate and inoculated with 10 µL of 0.5 McFarland adjusted overnight grown culture (18-24 hours old culture) of culture, to each well. Plate was sealed with clean film or covered with microtitre clean film. Incubated the plate at 37^{o} C at 120 rpm for 3-4 hours. After 3-4 hour incubation, added 100 µL of different drµg concentration in decreasing concentrations, as given in Table 3, in the respective wells of microtitre plate, marked properly according to different dilutions and sealed the plate with plate lid. Incubated the microtitre plate again at 37^{o}C at 120 rpm for 12 hours. After 12 hour incubation, added 10 µL of 0.2% TTC (Triphenyl tetrazolium chloride) solution to each well of microtitre plate. Observed red colour formation, which showed the growth of organism. MIC is the minimum inhibitory concentration at which no visible growth of organism appears.

3.11.3 MIC determined by the spectrophotometric method

Due to the development of resistant bacterial strains, the number of publications on antibacterial activity of phytochemicals is increasing. The two most commonly used methods for screening of antimicrobial potential of extracts are the disc diffusion test and the dilution plate assay. These techniques do not distinguish bactericidal and bacteriostact effects and the minimal inhibitory concentration (MIC) can not be determined. In the screening of antimicrobial compounds, the microplate method provides a potentially useful technique for determining MICs of large numbers of test samples, requiring small amounts of substances; this can be particularly important if the antimicrobial is scarce as is the case for many natural products. This method can also be used for a wide variety of microorganisms, is not expensive and presents reproducible results. The purpose of this study was to evaluate the microplate

method for screening for antimicrobial activity of extracts and natural products. The MIC values for a drug are expressed as the lowest concentration that inhibits the bacterial growth. The micro dilution method was performed according to Jorgensen *et al*., (1999).

Procedure

The wells of a 96-well ELISA tray were filled with 100µl of sterile Mueller-Hinton broth with 10 µl of exponentially growing culture (0.5 McFarland) was added to these wells. Then 100 µl of control and 100 µl of extract was added in the respective wells. The absorbances of each were determined using an automatic ELISA tray reader adjusted at 600 nm (Multiskan Spectra Readers, Thermo). The plate was incubated at 37ºC for 24 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. The MICs value for a drug was expressed as the lowest concentration that inhibits the bacterial growth. The macro dilution method was performed according to Jorgensen *et al*. (1999).

Table 3: Preparation of dilutions of antimicrobials for use in micro-broth dilution

Streptomycin(µg/mL)	10	20	30	40	50	60	70	80	90	100
XF-8 P (µg/mL)	1	2	3	4	5	6	7	8	9	10
XF-8 M (µg/mL)	1	2	3	4	5	6	7	8	9	10
XF-15 P (µg/mL)	1	2	3	4	5	6	7	8	9	10
XF-15 M (µg/mL)	1	2	3	4	5	6	7	8	9	10

3.12 Phytochemical analysis

Test for amino acids

To 1 mL of 5% ninhydrin solution, 1 mL of extract was added and heated on a boiling water bath for 10 minutes. Violet colour indicated the presence of amino acids.

Test for alkaloids (Wagner's reagent test)

To 1 mL of extract, few drops of Wagner's reagent (1.25g iodine and 2g of KI in 5 mL water and made up the volume up to 100 mL) was added. Reddish brown ppt. indicated the presence of alkaloids.

Test for carbohydrates (Molisch's test)

To 1 mL of extract, few drops of Molisch's reagent (1 g of α-naphthol dissolved in 60 mL 95% alcohol) was added. And then few drops of concentrated sulfuric acid was slowly added down the sides of the sloping test-tube, without mixing, to form a bottom layer. A positive reaction was indicated by the appearance of a purple ring at the interface between the acid and test layers.

Test for Flavonoids

1 mL of the extract was dissolved in diluted Sodium hydroxide and 1 mL diluted Hydrochloride was added. Yellow solution turns colorless which indicates the presence of flavonoids.

Test for fats and fixed oils (Saponification test)

Few drops of 0.5N alcoholic KOH was added to 1 mL of extract along with a drop of phenolphthalein. Heated on a water bath for 1 hour. The formation of soap indicated the presence of oils and fats.

Test for glycosides (Keller-Killani test)

To 2 mL of the extract, glacial acetic acid was mixed with one drop 5% $FeCl_3$ and 0.5 mL of concentrated sulphuric acid. A brown ring at the interface indicated a deoxysugar characteristic of cardiac glycosides. The acetic acid layer showed bluish green color.

Test for tannins and phenolic compounds (Ferric chloride test)

The 1 mL of extract was treated with ferric chloride (5% w/v) and observed Blue colour appeared if hydrosylate tannins were present and dark green solutions indicates the presence of condensed tannins.

Test for Terpenoids

1 mL of extract was treated with 2 mL of chloroform. Concentrated Sulphuric acid was added carefully to form a layer. Observed for the presence of reddish brown color at the interface which shows positive results for the presence of terpenoids.

Test for Steroids

To 2 mL of extract, 2 mL of acetic anhydride was added and heated on water bath for a minute. Then few drops of concentrated Sulphuric acid was added. The color change from violet to blue or green in samples indicates the presence of steroids.

3.13 Instrumental Analyses:

The crude ethyl acetate extract was subjected to Silica coated TLC plates. Plates were developed in Ethyl acetate-Methanol (7:3). Thin-layer chromatography (TLC) is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction.

Table 4: Stationary phase and mode of separation

Stationary Phase	Mobile Phase	Chromatographic Mechanism	Typical Application
Silica Gel	Ethyl acetate-Methanol (7:3)	adsorption	steroids, amino acids, alcohols, hydrocarbons, lipids, aflaxtoxin, bile, acids, vitamins, alkaloids

Procedure

1. For stationary phase silica matrix was prepared on coated on a glass plate (a thin layer ~ 0.25 mm).
2. Then plate was kept in oven for drying (2-3 hours).
3. After drying, a thin line was drawn with pencil which was 0.5-1 cm from the bottom of plate.
4. Samples were spotted on line with the help of capillary.
5. After that, the plate's end with samples was immersed into closed chromatography chamber filled with solvent (The solvent level was below the starting line of the TLC).
6. Allowed capillary action to draw the solvent up the plate until it was approximately 1 cm from the end.
7. The spots were visualized by spraying with 0.2% ninhydrin and kept in oven for 5-10 minutes to developing the spots (not over heated).
8. The colors of spots was analyzed.

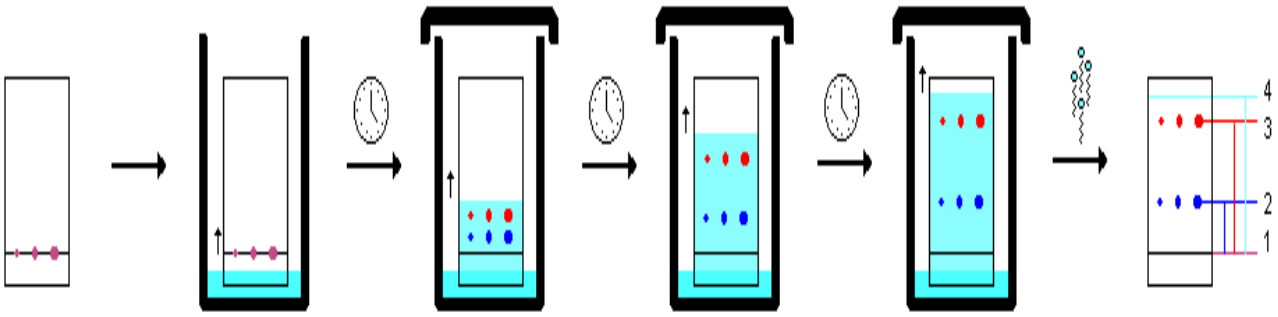


Figure 3: Steps in TLC method

RESULTS AND DISCUSSION

Results and Discussion

In the present study the two different isolates of endophytes from *Xylaria sp.* XF-8 and XF-15 were investigated for their antimicrobial activity. The following bacterial strains were employed for the screening: Gram-positive: *Staphylococcus aureus* and *Bacillus megaterium.* Gram-negative: *Escherichia coli* and *Pseudomonas aeruginosa*. For the antifungal screening the following fungi were used: *Aspergillus fumigates* and *Candida albicans*.

Table 5: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Bacillus megaterium*

Concentration (µg/mL)	Zone of inhibition*				
	Streptomycin	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
10	9.5±0.7	10.5±0.7	10.5±0.7	10.5±0.7	10.5±0.7
20	11.5±0.7	11.5±0.7	13.5±2.1	20.5±6.3	15.5±0.7
30	12.5±0.7	14.5±2.1	15.0±1.4	18.5±0.7	19.5±0.7
40	13.5±0.7	19.5±0.7	17.5±0.7	20.5±0.7	21.0±1.4
50	13.5±0.7	22.5±2.1	22.5±2.1	22.0±1.4	24.0±1.4
60	15.5±0.7	25.5±0.70	24.0±1.4	25.0±1.4	25.5±0.7

*Each value represents mean of three replicates ± standard deviation.

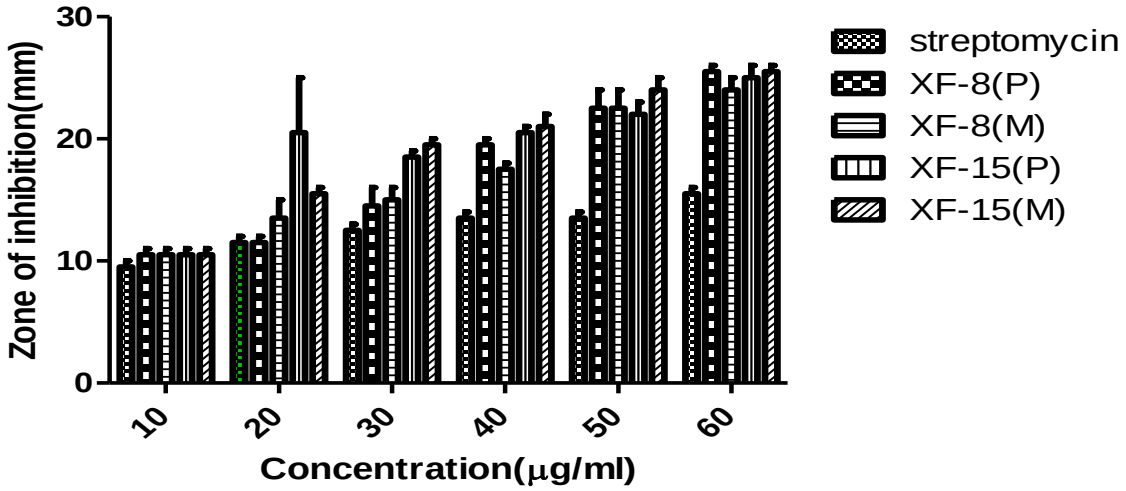


Figure 4 Zone of inhibition (diameter, mm) of different concentrations of extracts on *Bacillus megaterium*

Table 6: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Escherichia coli*

Concentration (µg/mL)	Zone of inhibition*				
	Streptomycin	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
10	9.5±0.7	9.5±0.7	10.0±1.4	10.0±1.4	10.5±0.7
20	11.5±1.4	10.5±0.7	14.5±0.7	15.0±1.4	15.0±1.4
30	12.0±0.7	15.0±1.4	15.5±0.7	17.0±2.8	17.5±2.1
40	13.5±0.7	19.0±1.4	17.5±0.7	20.5±0.7	19.5±0.7
50	13.5±0.7	22.5±2.1	22.5±2.1	22.0±1.4	22.0±1.4
60	14.5±0.7	24.0±1.4	24.0±1.4	24.5±2.1	24.0±1.4

*Each value represents mean of three replicates ± standard deviation.

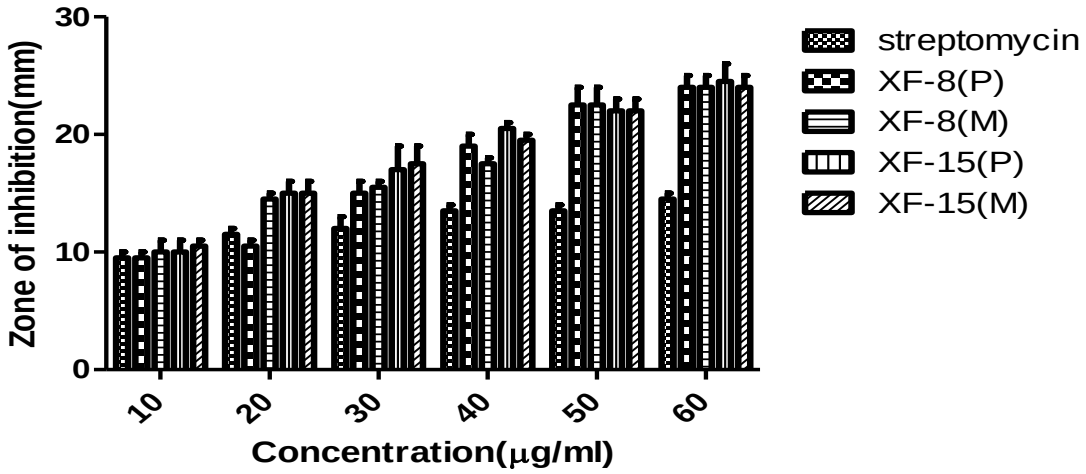
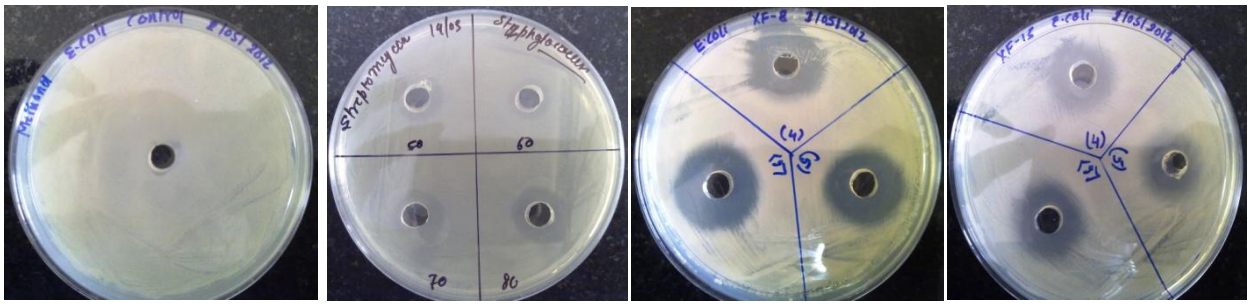


Figure 5: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Escherichia coli*.



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

Figure 6: Zone of inhibition (diameter, mm) of different concentrations of Streptomycin (b) and extracts (c and d) with methanol as control (a) on *Escherichia coli*.

Table 7: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Pseudomonas aeruginosa*.

Concentration (µg/mL)	Zone of inhibition*				
	Streptomycin	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
10	0.0	11.0±1.4	9.0±1.4	10.5±0.7	0.0
20	9.5±0.7	13.0±1.4	13.0±1.4	12.5±0.7	10.5±0.7
30	10.5±0.7	14.0±1.4	19.5±0.7	14.5±0.7	11.5±0.7
40	12.5±0.7	15.0±1.4	20.5±0.7	18.0±1.4	12.5±2.1
50	13.5±0.7	19.5±0.7	20.5±0.7	20.5±0.7	20.5±0.7
60	13.5±0.7	22.0±1.4	21.5±0.7	25.0±1.4	23.5±0.7

*Each value represents mean of three replicates ± standard deviation.

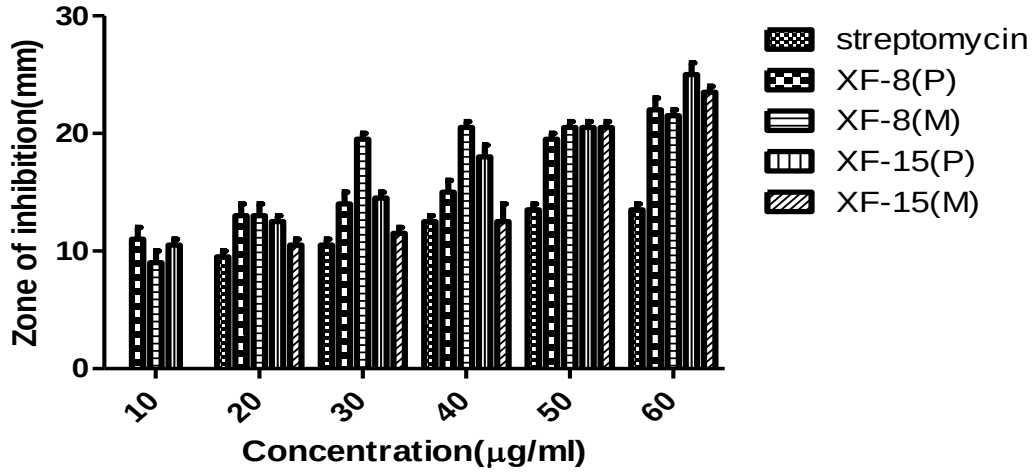
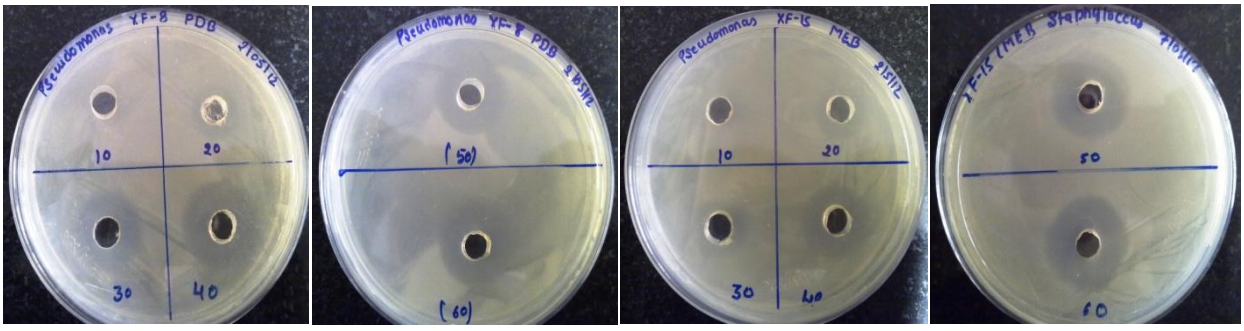


Figure 7: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Pseudomonas aeruginosa*.



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

Figure 8: Zone of inhibition (diameter, mm) of different concentrations of XF-8(P) (a and b) and XF-15(P) (c and d) on *Pseudomonas aeruginosa*.

Table 8: Zone of inhibition (diameter, mm) of different concentrations of extracts on *S. aureus.*

Concentration (µg/mL)	Zone of inhibition*				
	Streptomycin	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
10	0.0	11.5±0.7	11.0±1.4	13.0±1.4	12.5±0.7
20	11.5±0.7	15.5±0.7	16.5±2.1	17.0±1.4	17.5±0.7
30	13.0±1.4	21.5±0.7	19.5±0.7	19.5±0.7	20.0±1.4
40	15.0±1.4	24.5±0.7	22.0±1.4	22.5±0.7	21.5±2.1
50	14.0±1.4	26.0±1.4	23.5±0.7	24.5±0.7	25.0±1.4
60	17.5±0.7	28.5±0.7	26.5±0.7	27.0±1.4	27.5±2.1

*Each value represents mean of three replicates ± standard deviation.

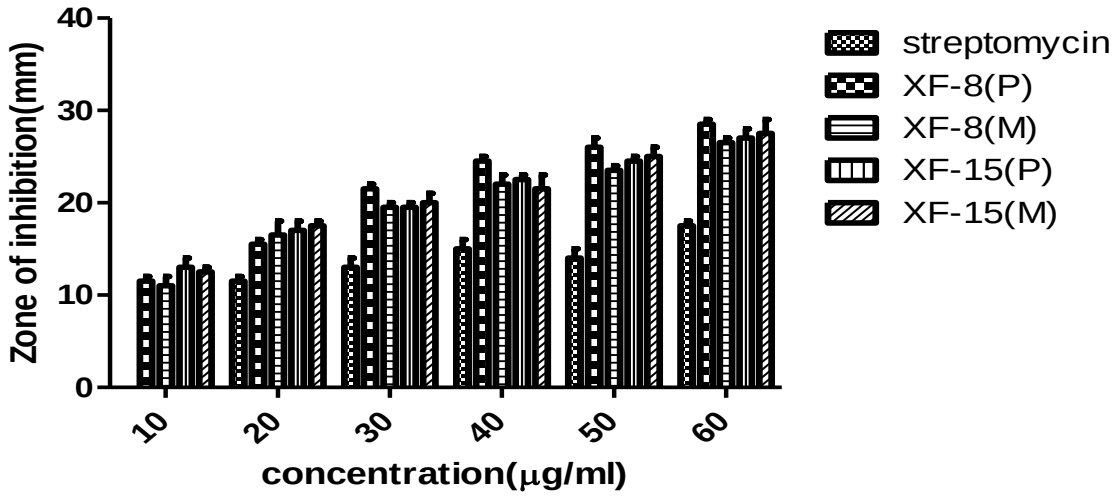
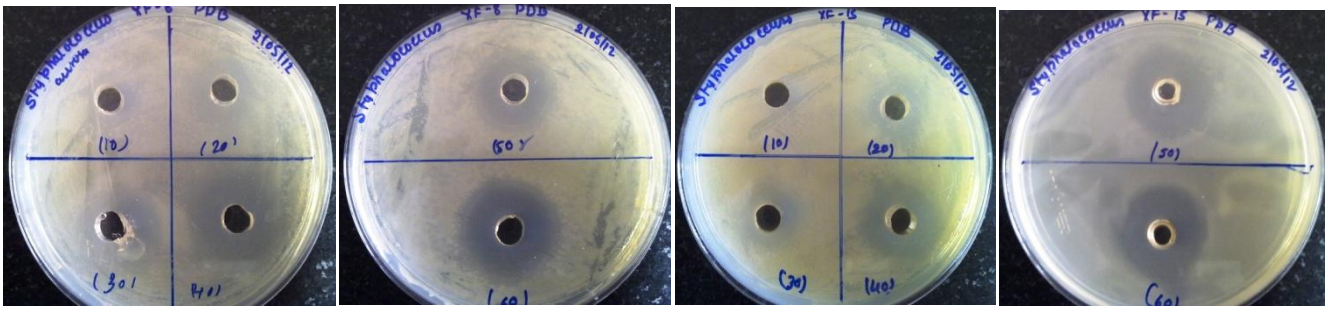


Figure 9: Zone of inhibition (diameter, mm) of streptomycin and extracts on *S. aureus.*



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

Figure 10: Zone of inhibition (diameter, mm) of different concentrations of XF-8(P) (a and b) and XF-15(P) (c and d) on *Staphylococcus aureus*.

Table 9: Zone of inhibition (diameter, mm) of different concentrations of extracts on *Candida albicans*

Concentration (µg/mL)	Zone of inhibition*				
	Fluconazole	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
10	0.0	0.0	0.0	0.0	0.0
20	13.5±0.7	0.0	0.0	0.0	0.0
30	16.5±0.7	0.0	0.0	0.0	0.0
40	21.5±0.7	0.0	13.0±1.4	10.5±0.7	10.5±0.7
50	21.0±1.4	10.5±0.7	12.0±1.4	13.0±1.4	13.5±0.7
60	21.5±0.7	11.5±0.7	15.0±1.4	14.0±1.4	15.0±1.4
70	24.5±0.7	13.5±0.7	14.0±0.1	15.5±0.7	16.0±1.4
80	26.0±1.4	16.5±2.1	15.5±0.7	17.5±0.7	15.0±1.4
90	27.0±1.4	17.0±1.4	16.5±0.7	17.0±1.4	15.5±0.7
100	26.0±1.4	19.0±1.4	18.5±0.7	20.0±1.4	17.5±0.7

*Each value represents mean of three replicates ± standard deviation.

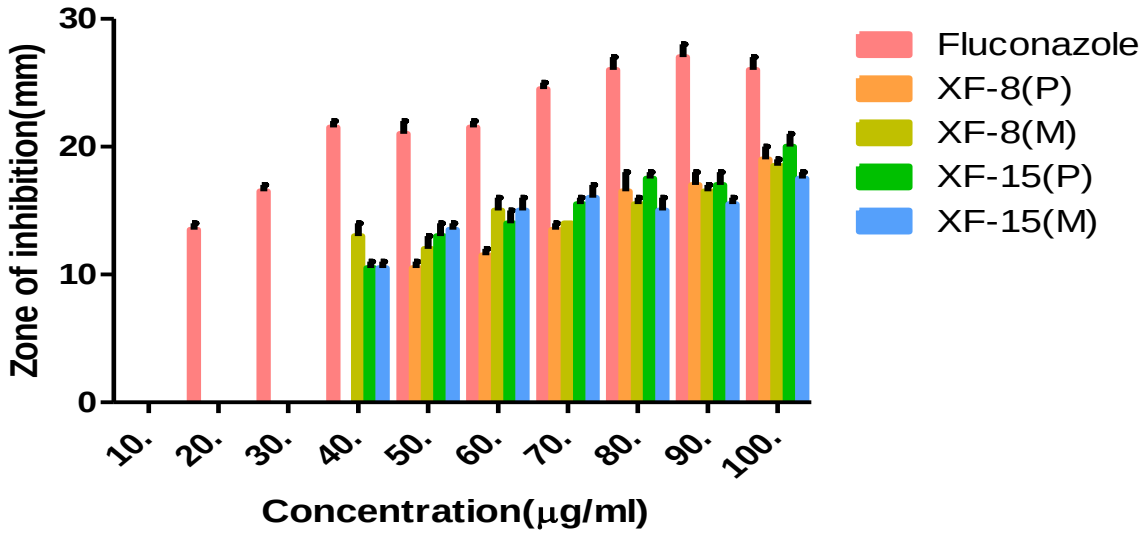


Figure 11: Zone of inhibition (diameter, mm) against *Candida albicans* at different concentrations of extracts.

Agar well diffusion method

Agar well assay is a popular prescreen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for the treatment of infectious diseases (Navarro *et al*., 1996).

Table 5 shows the zone of inhibition of different concentrations of extracts against *Bacillus megaterium*. The range of zone of inhibition of streptomycin was from 9.5-15.5 mm at concentration of 10-60 µg/ml whereas the range in case of extracts was from 10.5-25 mm. Out of these XF-8 PDB and XF-15 PDB extracts showed more antimicrobial activity with higher range of zone of inhibition. Both XF-8 and XF-15 showed better response against *Bacillus megaterium* as compared to control. Table 6 shows the zone of inhibition of extracts against *E. coli.* Here the zone of inhibition of control was from 9.5-14.5 mm whereas extracts showed range of XF-8 PDB 9.5-24 mm, XF-15 PDB 10-24.5 mm, XF-8 MEB 10-24 mm and XF-15 MEB 10.5-24 mm.

From table 7 which shows zone of inhibition on *P. aeruginosa* at different concentration of extracts, the range of zone of inhibition was streptomycin 9.5-13.5 mm, XF-8 PDB 11-22 mm, XF-15 PDB 10.5-25mm, XF-15 MEB 10.5-23.5 mm, XF-8 MEB 9-21.5 mm. XF- 15 PDB shows higher antimicrobial activity. The Table 8 shows the result of zone of inhibition against *S. aureus.* The range of zone of inhibition of streptomycin was from 11.5-17 mm at concentration of 20-60 µg/ml. the zone inhibition of various extracts was XF-8 PDB 11.5-28.5 mm, XF-8 MEB 11-26.5 mm, XF-15 PDB 13-27 mm, XF-15 MEB 12.5-27.5 mm. XF-8 PDB has more effective antibacterial activity as compared to control.

Table 9 shows zone of inhibition of *C .albicans* by different concentrations extracts. From this it can be concluded that the range of zone of inhibition of Fluconazole was 13.5-26 mm at concentrations of 20-100 µg/ml whereas extracts shows a range of 13-20 mm at concentrations of 40-100 µg/ml. Except XF-8 PDB which shows range from 10.5-20 mm at a concentrations of 50-100 µg/ml. Figure 13 shows zone of inhibition of XF-8 and XF-15 extracts against *Aspergillus fumigates.*

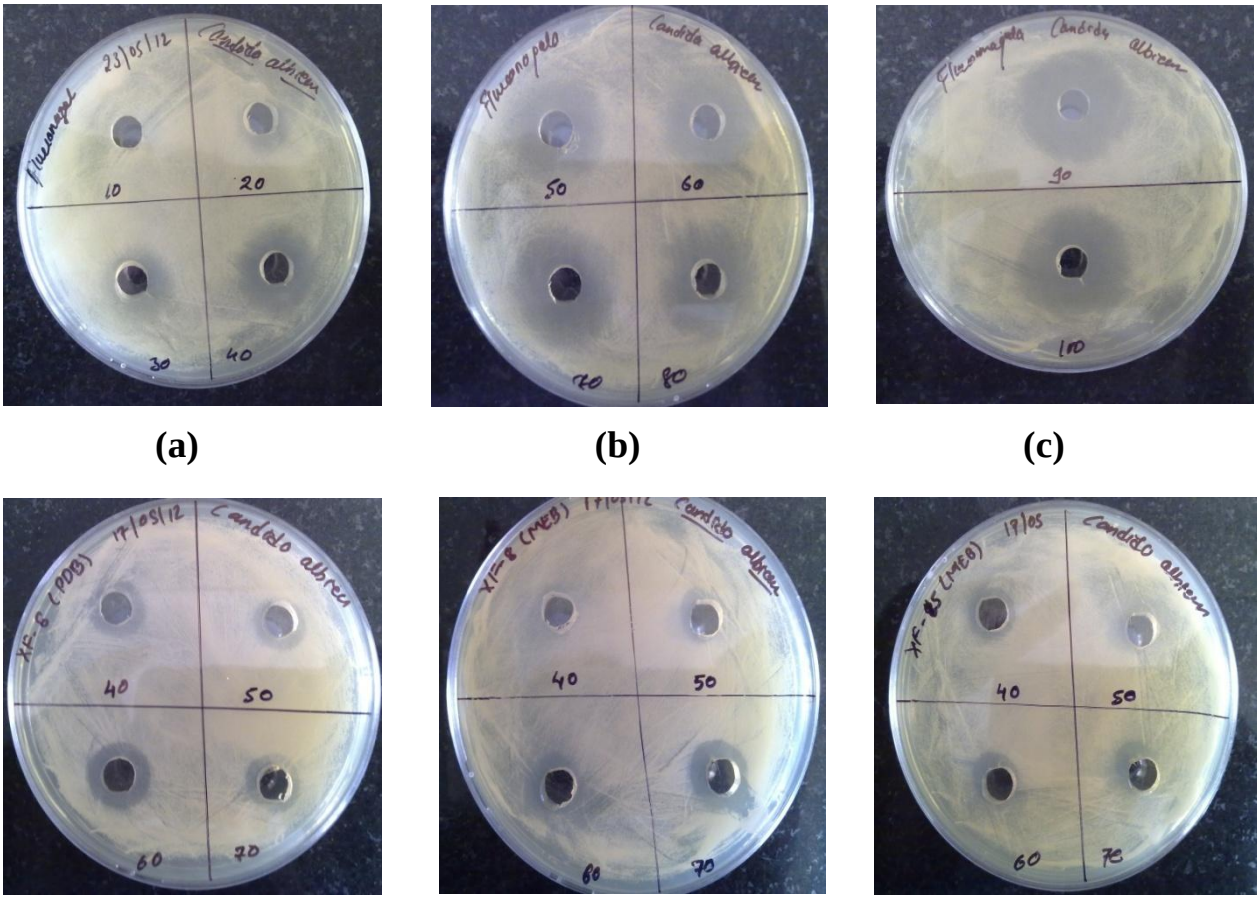


Figure 12: Zone of inhibition (diameter, mm) of different concentrations of fluconazole (a, b and c) and extracts (d, e, and f) against *Candida albicans.*

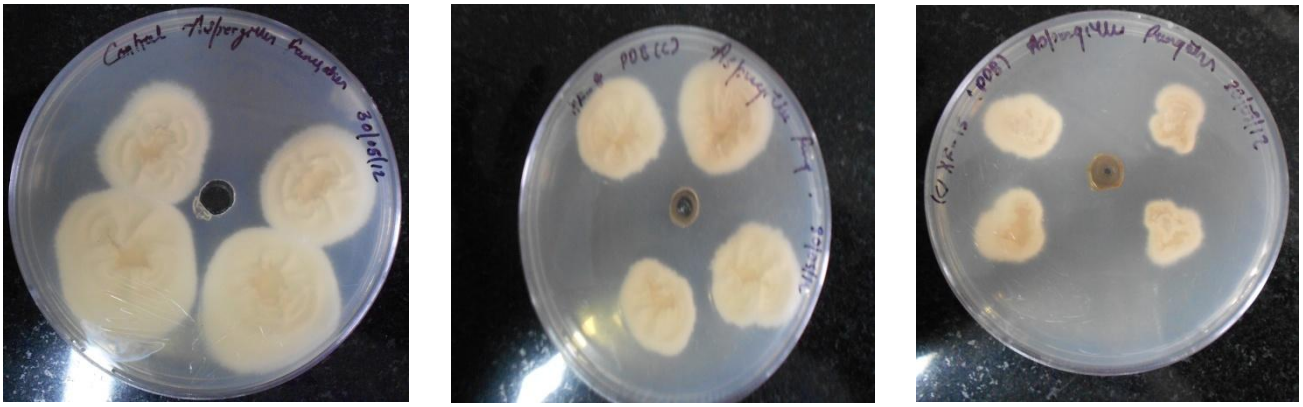


Figure 13: Zone of inhibition (diameter, mm) of (b) XF-8 and (c) and (a) control (without antifungal) against *Aspergillus fumigatus*.

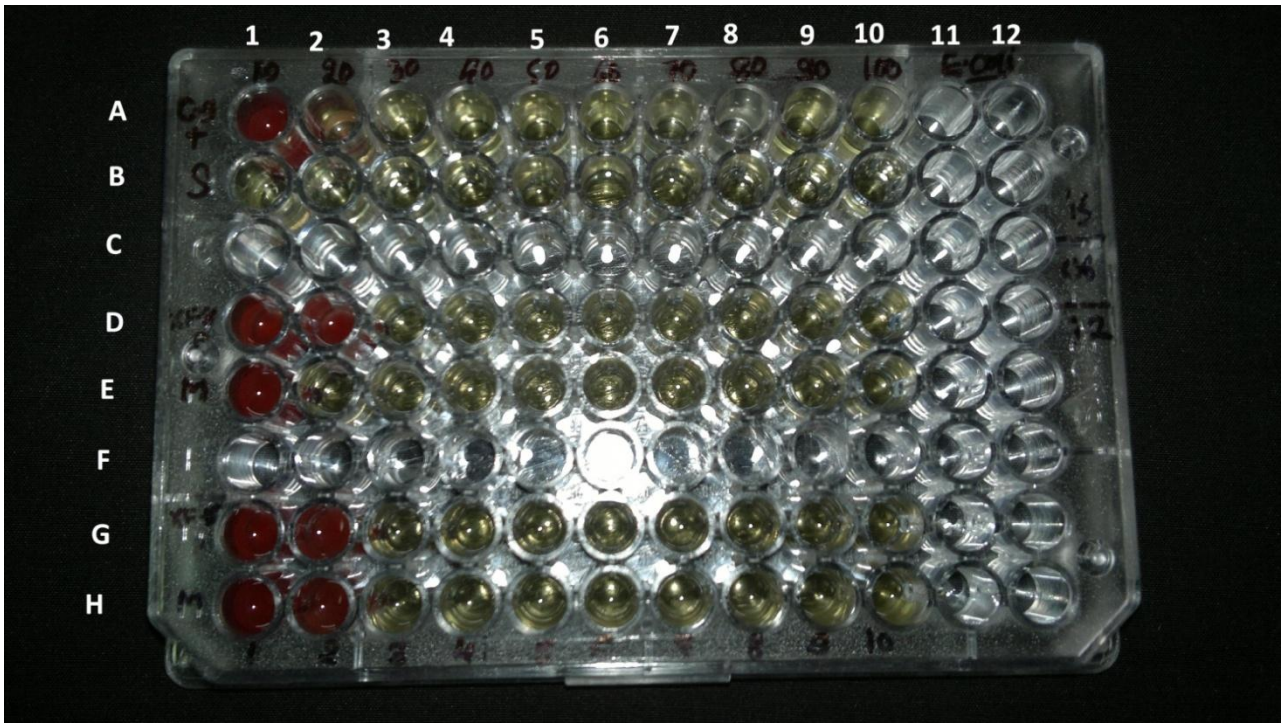


Figure 14: Determination of minimum inhibitory concentration. Representation of serial dilution of methanolic extracts against *Escherichia coli*. Columns 1-10: Different concentrations of antibiotic and extracts. Row (A): Positive control (Streptomycin in serial dilution + broth + indicator + bacterial culture. Row (B): Control of the sterility of the culture medium (MHB). Row (D): Different concentrations of XF-8(P), MIC>2 µg/mL. Row (E): Different concentrations of XF-8(M), MIC>1 µg/mL. Row (G): Different concentrations of XF-15(P), MIC>2 µg/mL and Row (D): Different concentrations of XF-15(M), MIC>2 µg/mL.

Table 10: MIC values of extracts by TTC method

Bacterial and Fungal Srains	MIC values (µg/mL)			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
Bacillus megaterium	>2	>2.5	>2	>1
Escherichia coli	>2	>1	>2	>2
Pseudomonas aeruginosa	>1	>1.5	>1	>1
Staphylococcus aureus	>1.5	>2	>2	>2
Candida albicans	>4	>3	>6	>5

Colorimetric spectrophotometric method

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), while it remains as white TTC in areas of necrosis since these enzymes have been either denatured or degraded.

Table 10 shows the MIC values of extract by TTC method. From the Table it can be concluded that against *B. megaterium* XF-8 PDB has value >2 µg/ml, XF-15 PDB >2 µg/ml, XF-8 MEB >2.5 µg/ml and XF-15 MEB >1 µg/ml. Also the MIC range against *E. coli* was XF-8 PDB >2 µg/ml, XF-15 PDB >2 µg/ml, XF-8 MEB >1 µg/ml and XF-15 MEB >2 µg/ml. Against *P. aeruginosa* the range was XF-8 PDB>1µg/ml, XF-15 PDB >1µg/ml, XF-8 MEB >1.5 µg/ml & XF-15 MEB >1µg/ml. For *S. aureus* the range was XF-8 PDB >1.5 µg/ml, XF-15PDB>2 µg/ml, XF-8 MEB and XF-15 MEB >2 µg/ml. Against *C. albicans* XF-8 PDB MIC was >4 µg/ml, XF-15 PDB >6 µg/ml, XF-8 MEB >3 µg/ml and XF-15 MEB>5 µg/ml. From this table it can be concluded that extracts show more antimicrobial activity.

Table 11: Effect of different concentrations of extracts on the growth of *Bacillus megaterium* after 24 hours.

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	0.060±0.001	0.052±0.001	0.044±0.001	0.065±0.000
2	**0.034±0.005**	0.051±0.001	0.041±0.001	0.063±0.001
3	0.036±0.000	0.042±0.001	0.037±0.001	0.056±0.002
4	0.034±0.001	**0.039±0.001**	0.035±0.000	0.048±0.001
5	0.033±0.001	0.038±0.001	0.031±0.001	0.035±0.001
6	0.032±0.001	0.036±0.002	**0.029±0.001**	0.031±0.001
7	0.029±0.001	0.025±0.001	0.029±0.002	0.030±0.001
8	0.028±0.001	0.014±0.001	0.025±0.001	0.026±0.001
9	0.028±0.001	0.011±0.001	0.024±0.001	**0.025±0.001**
10	0.020±0.001	0.011±0.001	0.011±0.001	0.023±0.001

*Each value represents mean of three replicates ± standard deviation

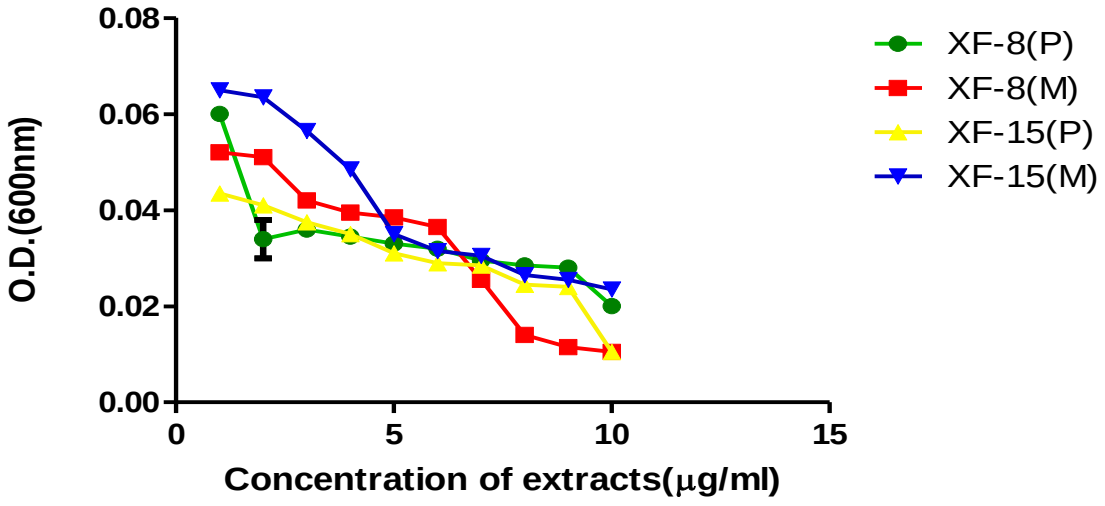


Figure 15: Effect of different concentrations of extracts on growth of *Bacillus megaterium* after 24 hours

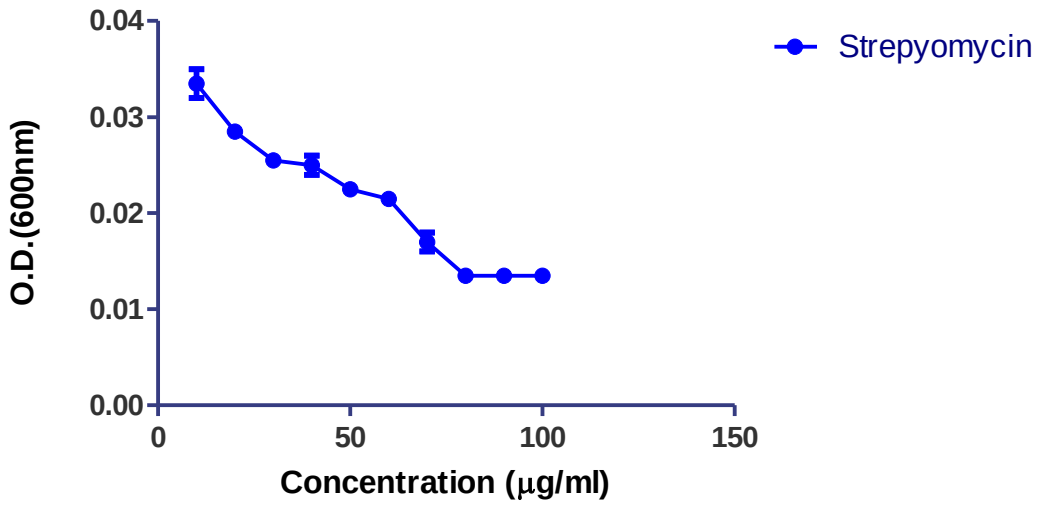


Figure 16: Effect of different concentrations of streptomycin (control) on growth of *Bacillus megaterium* after 24 hours

Table 12: Effect of different concentrations of extracts on growth of *Pseudomonas aeruginosa* after 24 hours.

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	0.100±0.003	0.113±0.003	0.081±0.001	0.113±0.003
2	0.087±0.002	0.084±0.006	0.077±0.001	0.096±0.003
3	0.072±0.001	0.079±0.002	0.072±0.002	0.091±0.001
4	0.060±0.001	0.067±0.002	0.063±0.002	0.083±0.004
5	0.061±0.001	**0.056±0.002**	0.055±0.001	**0.079±0.001**
6	0.058±0.001	0.052±0.001	0.055±0.001	0.077±0.001
7	**0.043±0.001**	0.050±0.001	**0.049±0.001**	0.075±0.001
8	0.035±0.001	0.048±0.002	0.045±0.003	0.070±0.001
9	0.035±0.001	0.044±0.002	0.043±0.001	0.070±0.002
10	0.034±0.003	0.039±0.001	0.026±0.003	0.055±0.002

*Each value represents mean of three replicates ± standard deviation

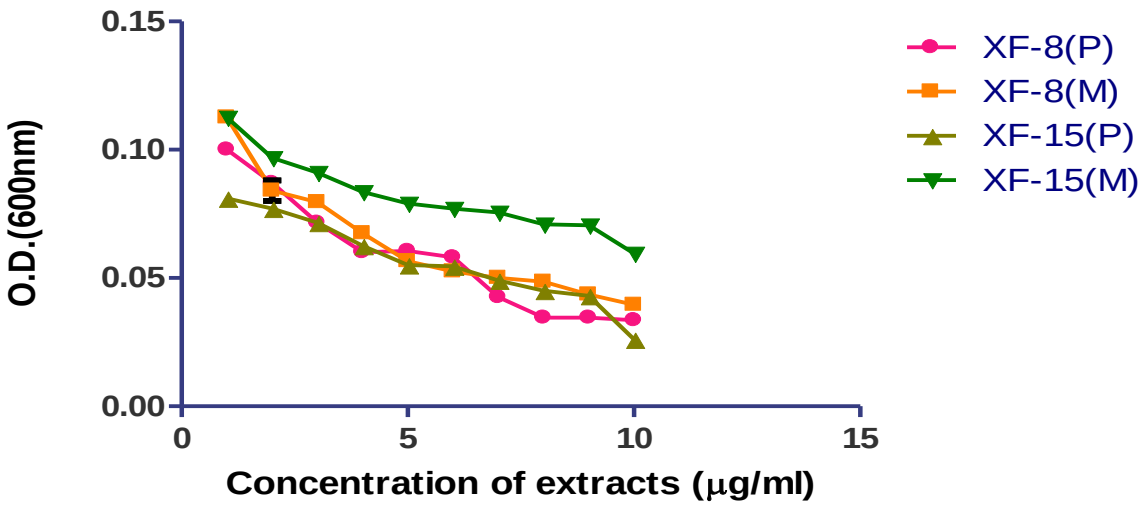


Figure 17: Effect of different concentrations of extracts on growth of *Pseudomonas aeruginosa* after 24 hours

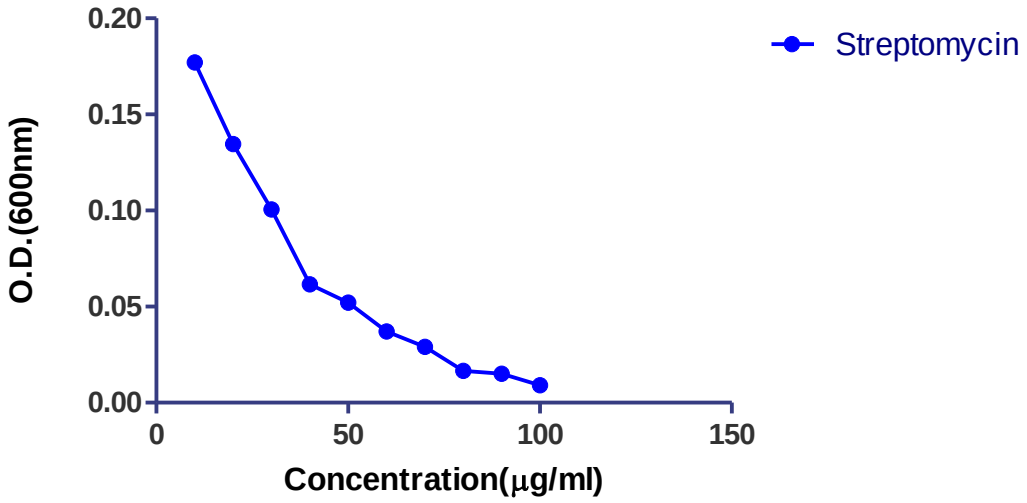


Figure 18: Effect of different concentrations of streptomycin (control) on growth of *Pseudomonas aeruginosa* after 24 hours

Table 13: Effect of different concentrations of extracts on growth of *Escherichia coli* after 24 hours.

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	1.232±0.028	1.227±0.007	1.246±0.011	1.345±0.006
2	1.045±0.064	**0.947±0.052**	1.054±0.076	**0.969±0.001**
3	**0.969±0.009**	0.834±0.000	**0.967±0.001**	0.855±0.013
4	0.834±0.003	0.789±0.012	0.836±0.012	0.741±0.007
5	0.712±0.032	0.677±0.045	0.775±0.019	0.668±0.029
6	0.627±0.025	0.603±0.023	0.678±0.016	0.524±0.019
7	0.595±0.008	0.435±0.063	0.561±0.038	0.459±0.041
8	0.525±0.021	0.416±0.005	0.424±0.008	0.355±0.061
9	0.342±0.062	0.319±0.013	0.380±0.012	0.249±0.042
10	0.238±0.040	0.220±0.014	0.263±0.025	0.166±0.014

*Each value represents mean of three replicates ± standard deviation

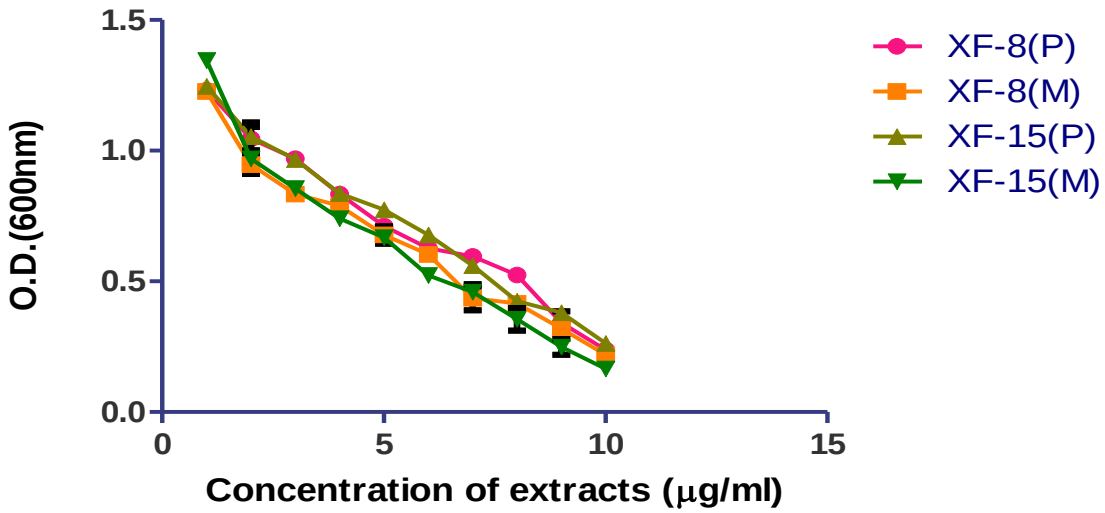


Figure 19: Effect of different concentrations of extracts on growth of *Escherichia coli* after 24 hours

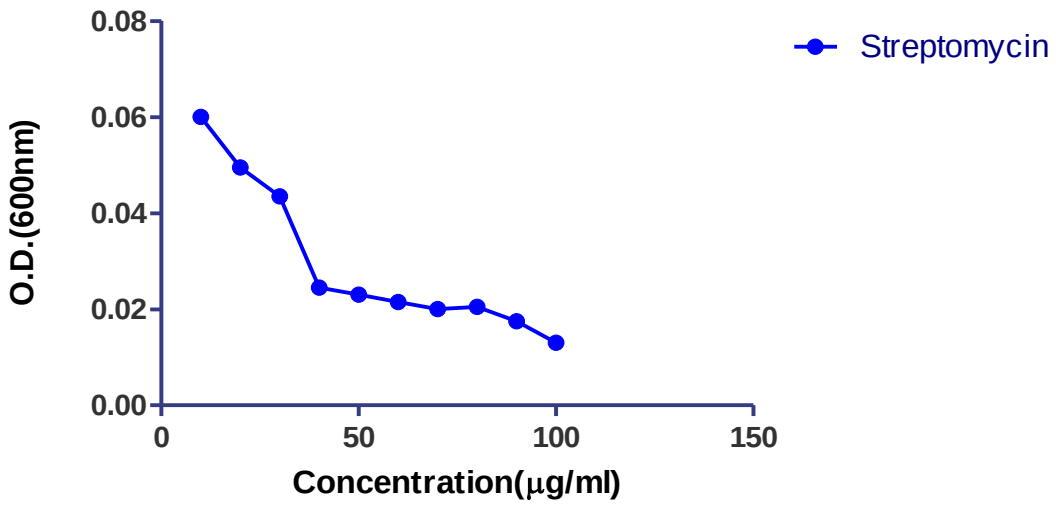


Figure 20: Effect of different concentrations of streptomycin (control) on growth of *Escherichia coli* after 24 hours

Table 14: Effect of different concentrations of extracts on growth of *Staphylococcus aureus* after 24 hours

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	0.105±0.004	0.112±0.005	0.085±0.004	0.113±0.004
2	**0.084±0.007**	**0.094±0.006**	**0.079±0.001**	**0.094±0.001**
3	0.074±0.002	0.083±0.002	0.074±0.003	0.092±0.001
4	0.069±0.001	0.067±0.003	0.061±0.001	0.090±0.001
5	0.063±0.002	0.064±0.001	0.054±0.001	0.088±0.002
6	0.057±0.003	0.061±0.002	0.051±0.001	0.086±0.001
7	0.045±0.004	0.050±0.001	0.046±0.001	0.082±0.002
8	0.043±0.003	0.048±0.004	0.040±0.001	0.079±0.000
9	0.040±0.001	0.045±0.004	0.038±0.001	0.067±0.001
10	0.033±0.001	0.043±0.004	0.031±0.002	0.063±0.002

*Each value represents mean of three replicates ± standard deviation

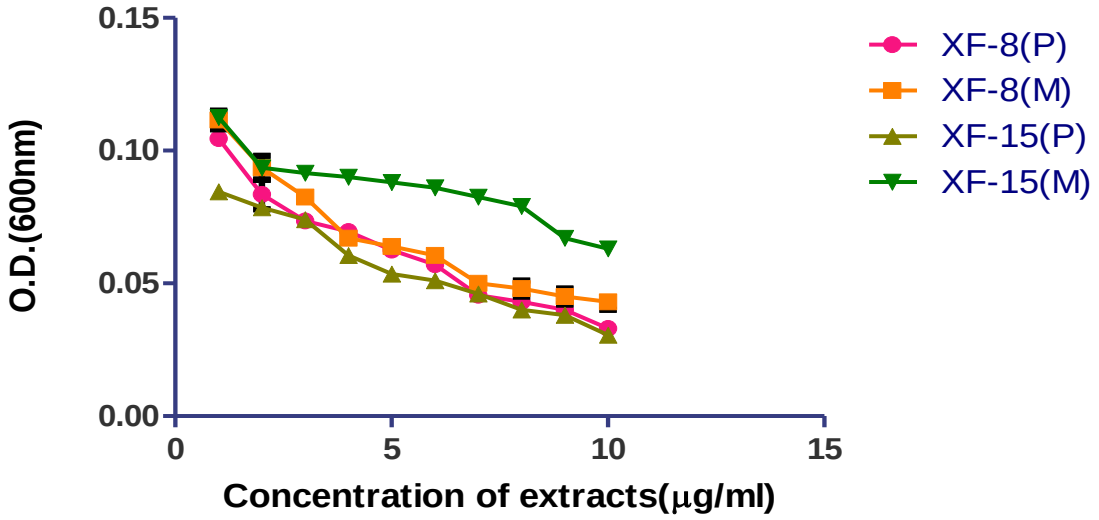


Figure 21: Effect of different concentrations of extracts on growth of *Staphylococcus aureus* after 24 hours

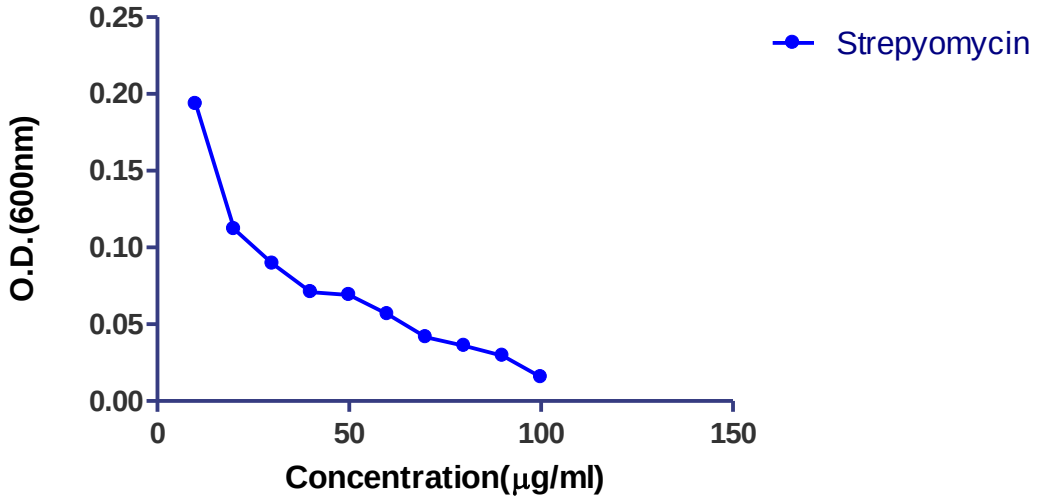


Figure 22: Effect of different concentrations of streptomycin (control) on growth of *S. aureus* after 24 hours

Table 15: Effect of different concentrations of extracts on growth of *Candida albicans* after 24 hours

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	1.372±0.008	1.457±0.058	1.489±0.016	1.248±0.057
2	1.244±0.077	1.169±0.007	1.254±0.049	1.198±0.016
3	0.928±0.070	0.849±0.028	1.151±0.023	0.803±0.023
4	0.645±0.046	0.743±0.017	0.931±0.055	0.729±0.084
5	0.525±0.008	0.683±0.007	0.676±0.034	0.528±0.003
6	0.551±0.118	0.537±0.008	0.536±0.009	0.411±0.017
7	0.385±0.009	**0.421±0.012**	0.419±0.043	0.377±0.004
8	**0.271±0.006**	0.354±0.049	**0.309±0.004**	**0.264±0.050**
9	0.211±0.001	0.249±0.054	0.219±0.002	0.238±0.040
10	0.195±0.006	0.183±0.038	0.211±0.003	0.185±0.0135

*Each value represents mean of three replicates ± standard deviation

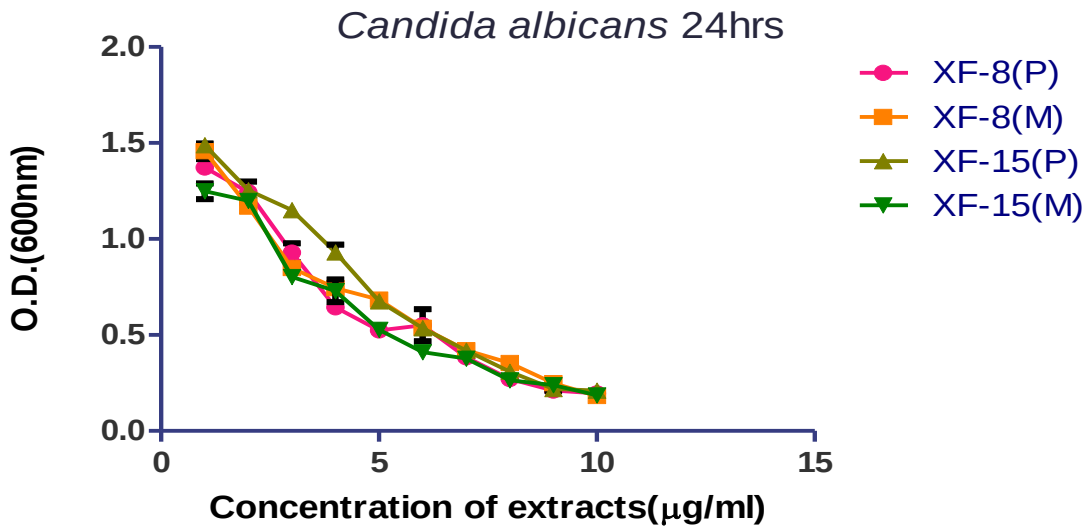


Figure 23: Effect of different concentrations of extracts on growth of *Candida albicans* after 24 hours

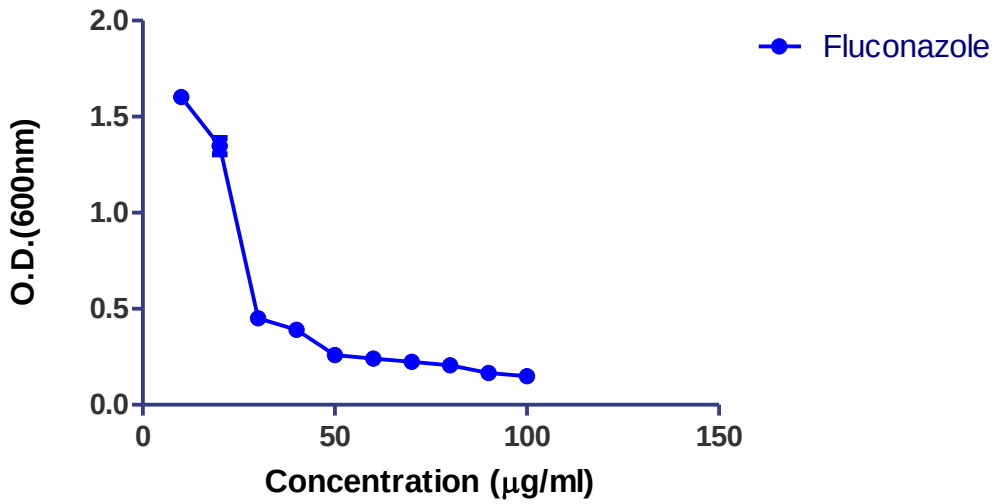


Figure 24: Effect of different concentrations of Fluconazole (control) on growth of *Candida albicans* after 24 hours

Table 16: Effect of different concentrations of extracts on growth of *Candida albicans* after 48 hours

Concentration (µg/mL)	Absorbance (600nm)*			
	XF-8(P)	XF-8(M)	XF-15(P)	XF-15(M)
1	1.305±0.037	1.440±0.007	1.456±0.015	1.237±0.004
2	1.235±0.005	1.245±0.012	1.274±0.018	1.181±0.011
3	0.765±0.021	0.786±0.008	1.201±0.067	0.878±0.003
4	0.559±0.028	0.649±0.028	1.015±0.003	0.315±0.006
5	0.370±0.014	**0.478±0.012**	0.622±0.007	0.235±0.011
6	**0.252±0.004**	0.356±0.005	**0.507±0.004**	0.219±0.002
7	0.233±0.015	0.273±0.011	0.385±0.011	**0.195±0.001**
8	0.214±0.003	0.216±0.005	0.256±0.015	0.175±0.004
9	0.209±0.001	0.179±0.001	0.211±0.001	0.153±0.006
10	0.203±0.004	0.167±0.004	0.204±0.004	0.124±0.003

*Each value represents mean of three replicates ± standard deviation

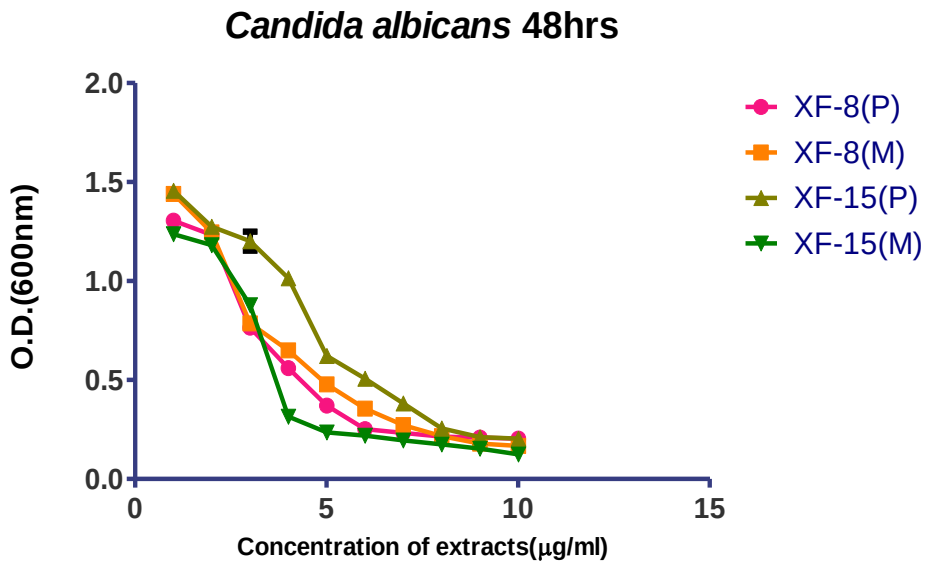


Figure 25: Effect of different concentrations of extracts on growth of *Candida albicans* after 48 hours

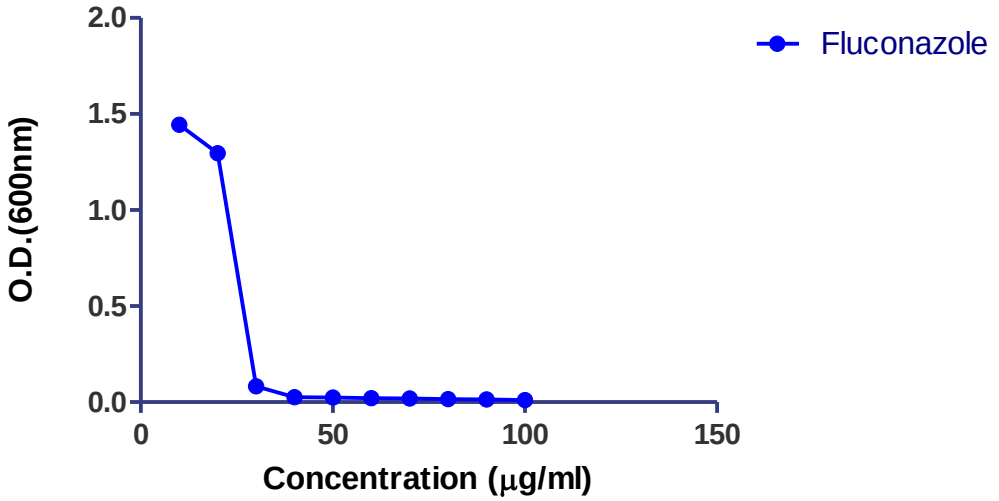


Figure 26: Effect of different concentrations of Fluconazole (control) on growth of *Candida albicans* after 48 hours

The spectrophotometric method for determining the MIC value showed the decrease in absorbance with increase in the concentration value which indicated that was the MIC of the extract. From table 11 which shows absorbance of extracts against *Bacillus megaterium* after 24 hrs. From the Table 11 it may be concluded that XF-8 PDB has MIC 2 µg/ml, XF-15 PDB 6 µg/ml, XF-8 MEB 4 µg/ml & XF-15 MEB 9 µg/ml. It may be concluded that XF-8 PDB was more effective against *Bacillus megaterium*. Table 12 shows MIC of extracts against *Pseudomonas aeruginosa*, the range of MIC was XF-8 PDB 7 µg/ml, XF-15 PDB 7 µg/ml, XF-8 MEB 5 µg/ml and XF-15 MEB 5 µg/ml. Here XF-8 MEB and XF-15 MEB were equally effective against *Pseudomonas aeruginosa.*

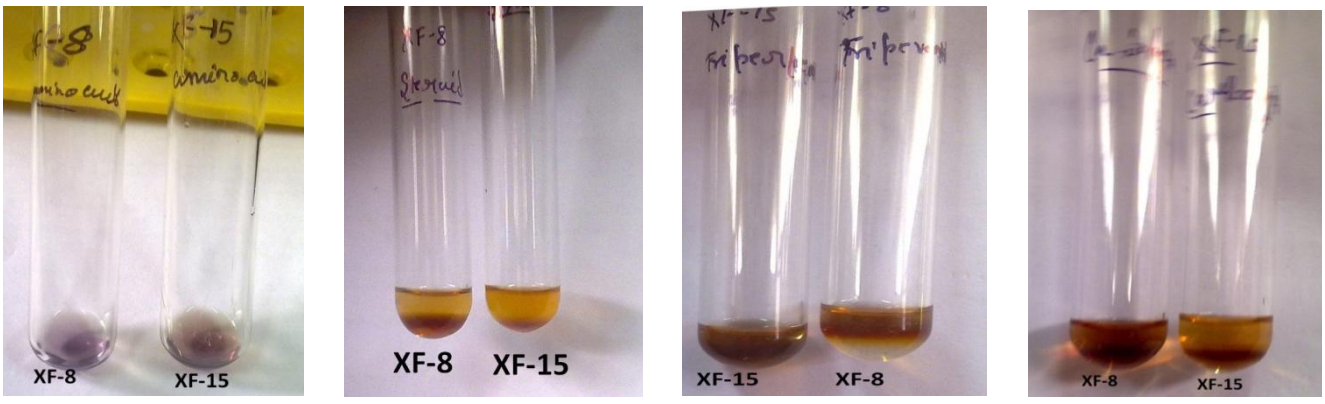
Table 13 shows the absorbance against *Escherichia coli*, the MIC range was XF-8 PDB 3 µg/ml, XF-15 PDB 3 µg/ml, XF-8 MEB 2 µg/ml & XF-15 MEB 2 µg/ml. So in this case XF-8 MEB & XF-15 MEB were equally effective against *Escherichia coli.* From table 14 it may be concluded that MIC of extracts XF-8 PDB, XF-15 PDB, XF-8 MEB & XF-15 MEB were 2 µg/ml against *Staphylococcus aureus.* Hence against *Staphylococcus aureus* all extracts were showed effective antibacterial activity.

Table 15 shows MIC of extracts against *Candida albicans* after 24 hr, the range was XF-8 PDB 8µg/ml, XF-15 PDB 8 µg/ml, XF-8 MEB 7 µg/ml and XF-15 MEB 8 µg/ml. Hence against *Candida albicans* XF-8 MEB was more effective. Table 16 shows MIC against *Candida albicans* after 48 hours extracts has range of MIC XF-8 PDB 6 µg/ml, XF-15 PDB 6 µg/ml, XF-8 MEB 5 µg/ml and XF-15 MEB 7 µg/ml. Out of all the extracts XF-8 MEB showed good antifungal activity.

Table 17: Phytochemical test of fungal extracts

Biochemical compounds Present in extract	**XF-8**	**XF-15**
Tannins & Phenolics	-	-
Amino acids	+	++
Carbohydrates	+++	+++
Alkaloids	+++	+++
Fats & Fixed Oils	-	-
Steroids and Triterpenoides	++	++
Flavonoids	+++	+++
Glycosides	-	-

+ indicate intensity of colour, (+++) High intensity, (++) Low intensity, (+) very low intensity



Amino acids Steroid Terpenoids Carbohydrates

Figure 27: Presence of phytochemical compounds in XF-8 and XF-15 extract.

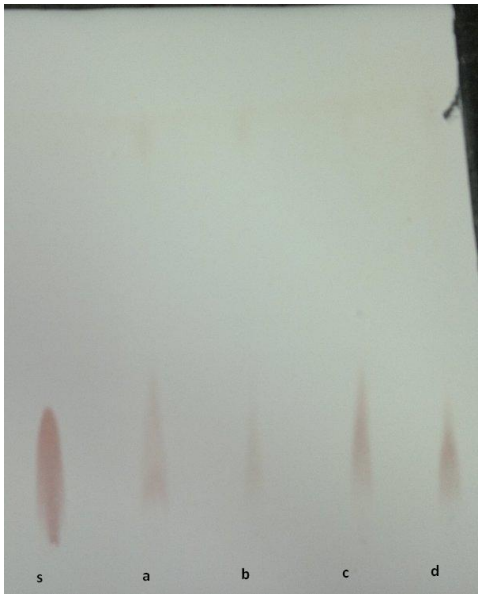


Figure 28: TLC of methanolic extracts, spot (s) = control, (a) = XF-8(P), (b) = XF-8(M), (c) = XF-15(P) and (d) = XF-15(M).

Table 17 shows phytochemical investigation test of fungal extracts from where it may concluded that Tannins & phenolics, amino acids, carbohydrates, alkaloids, steroids and flavonoids were present. Thin Layer Chromatography (TLC) is a commonly used analytical technique that allows for rapid and inexpensive analysis of various mixtures. Figure 28 shows TLC spots of methanolic extract that confirms the presence of amino acids in fungal extract.

Conclusion

From these results it can be concluded that extract of *Xylaria* species XF-8 and XF-15 showed significant antimicrobial activity against the test organisms. The data also showed that these extracts have a broad spectrum antimicrobial activity. Instrumental analysis like TLC which indicated the presence of amine group in the bioactive compound. Preliminary phytochemical investigation of fungal extracts showed the presence of secondary metabolites like alkaloids, amino acids, carbohydrates, steroids & terpenoids. From these crude endophytic extracts the pure compound may be further separated by using various techniques. The pure compounds was be characterized for its exact structures and the nature of the compounds.

Summary

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Fungi are prolific sources of structurally novel and biologically active compounds. Over the past decades terrestrial fungi have been a major source of bioactive metabolites. But often these have been obtained from easily accessible and culturable organisms while the apparently rare and non-culturable groups remain an untapped resource. With the need for new bioactive compounds increasing, the yield of potentially useful new bioactive natural products from microbial sources is declining. Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial areas.

In this present study the isolated endophytic fungi were cultured on PDB and MEB, than the mycelia was separated by filtration after 9 days. It also discusses the antibacterial and antifungal activity of *Xylaria* species on a group of bacterial and fungal organisms by agar well diffusion method, broth microdilution method and spectrophotometric method.

Against *E. coli* XF-8 PDB, XF-8 MEB, XF-15 PDB & XF-15 MEB was effective than streptomycin. Against *B. megaterium* almost all extracts were equally effective. XF-15 PDB was more effective against *P. aeruginosa.* XF-8 PDB showed pronounced effect against *S. aureus* as compared with control & other extracts. However in case of fungal culture *C. albicans* XF-15 PDB has more efficient response. From the result it may concluded that culture grown on PDB show more antimicrobial activity than fungal cultures grown on MEB.

The MIC values obtained from TTC method against *B. megaterium* was XF-8 PDB, XF-15 PDB >2µg/ml, XF-8 MEB >2.5µg/ml, XF-15 MEB >1µg/ml. MIC value against *E. coli* was XF-8 PDB, XF-15 PDB & XF-15 MEB >2µg/ml, whereas XF-8 MEB has MIC >1µg/ml. In case of *P. aeruginosa* the MIC value was XF -8 PDB, XF-15 PDB, XF-15 MEB >1µg/ml & that of XF-8 MEB >1.5µg/ml. MIC value against *S. aureus* was XF-8 MEB, XF-15 PDB & XF-15 MEB >2µg/ml whereas XF-8 PDB has MIC >1.5µg/ml. The MIC value against *C. albicans* varied from XF-8 PDB >4 µg/ml, XF-8 MEB >3 µg/ml, XF-15 PDB>6 µg/ml., XF-15 MEB >5µg/ml.

From all these results it is observe that the crude extract of *Xylaria* species XF-8 & XF-15 showed significant antimicrobial activity against the bacterial cultures *E. coli, B. megaterium. S. aureu, P. aeruginosa* and also fungal cultures like *C. albicans, Aspergillus fumigatus*. The instrumental analysis like TLC indicated the presence of amino group in the extracts. Preliminary phytochemical investigation of fungal extracts shows the presence of bioactive metabolite including alkaloids, amino acid, carbohydrates, flavonoids and steroids & terpenoids. Further studies may be carried out to determine the nature of pure compound responsible for antimicrobial activity.

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