Isolation of bioactive compounds from endophytic fungi of medicinal plants

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Submitted by

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CERTIFICATE

I hereby declare that the work that has been presented in this thesis "**Isolation of bioactive compounds from endophytic fungi of medicinal plants**" submitted by **Ms. Harman Gill** in the partial fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is a record of student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this institute or any other institute.

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DECLARATION

I (**Harman Gill**) declare that the work presented in this thesis **"Isolation of bioactive compounds from endophytic fungi of medicinal plants"** submitted under the supervision of Dr. M. Vasundhara in the partial fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, TIET, Patiala, is my own original research work done during the period of August 2017 to June 2018. This report has not been submitted for the award of any other degree or certificate in this institute or any other institute.

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Harman Gill
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ABBREVATIONS

Abbreviation	Name
%	Percentage
˚C	Degree Celsius
L	Litre
ml	Millilitre
mg	Milligram
µg	Microgram
O.D	Optical Density
MHB	Muller Hinton Broth
MHA	Muller Hinton Agar
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
w/v	Weight by Volume
pH	Potential of hydrogen ion
Temp	Temperature
Rpm	Rotation Per Minute
H_2SO_4	Sulfuric Acid
MIC	Minimum Inhibitory Concentration
EtBr	Ethidium Bromide
DNA	Deoxyribose Nucleic Acid
sp.	Species
EDTA	Ethylenediamine tetraacetic acid
TLC	Thin layer chromatography
Rf	Retention factor
DPPH	2, 2-diphenyl-1-picryl-hydrazyl-hydrate

ABSTRACT

Medicinal plants are the natural and known source of number of therapeutic molecules. Endophytic fungi colonizing the medicinal plants are of great focus of research as they have a potential to produce biologically active compounds that have the antimicrobial, antioxidant, cytotoxic and immunomodulatory properties.

In this study two medicinal plants *Terminalia arjuna* (arjuna) and *Tinospora cordifolia* (Giloy) have been used for the isolation of the endophytic fungi. The total of 21 fungal isolates were obtained from *T.arjuna* (arjuna) and *T. cordifolia* (Giloy), fourteen from *T. arjuna*and seven from *T. cordifolia*. The crude extracts obtained from these isolates weretested for preliminary antimicrobial activity by agar disc diffusion method and MTT assay using test cultures. The maximum inhibition was shown by fungal extract AL2 amongst all the extracts of*T. arjuna*. It showed inhibitory activity of 71% against *P. aeruginosa,* 82.23% against *B. megaterium* and75% against *E.coli.* Amongst the extracts from *T. cordifolia* fungal crude extract GS4 showed the prominent activity of 28%, 60% and 59% against *E.coli*, *P. aeruginosa*, and *B. megaterium* respectively. These extracts were also tested for their potential to scavenge free radicals. GS4 showed maximum radical scavenging activity of 75.65% at 250 µg/ml followed by AL2 which showed maximum radical scavenging activity of 64.34 at 500 µg/ml of concentration.

Extract AL2 showed the presence of alkaloids and triterpenoids and crude extract GS4 indicated the presence of phenols, carbohydrates, alkaloid and steroids in phytochemical analysis. AL2 gave positive results for the lipase, protease and laccase enzyme activity tests whereas GS4 showed positive test for lipase and protease.On the basis of morphological and molecular studies endophytic fungi (AL2) isolate was identified as *Alternaria spp.*Preliminary TLC analysis was done and for fractionation column chromatography was performed. After column chromatography seven fractions were obtained andthese were subjected to antibacterial assay. The fractions 4 and 7 showed maximum bioactivityand hence,further characterized. Fraction 7 was analyzed using UV spectroscopic, HPLC, FTIR and LCMS analyses. The data obtained by analysis indicated the presence of taxol. Results obtained were compared with standard taxol.Endophytic fungi isolated from medicinal plants could be a vital source for obtaining novel and potent molecules targeted against various life threatening diseases.

INTRODUCTION

Nature has legions of life forms on earth. Among these life forms microorganisms are omnipresent in nature.Endophytes are one of the most attractive communities of microorganisms.The word endophyte is a Greek work means 'within the plant' (endon = within; phyton = plant).De Bary (1866) termed endophytes as the organisms that are invade inside the tissue of host plant without causing any adverse effect to the plant.This term was further explained by Bacon and White (2000) they elaborated the endophytes definition and explained these as the organism that colonize inside the internal tissues of plant without showing immediate harmful effect to plants.

They are found virtually in every plant on earth. It is assumed that we are familiar with approx 300,000 plant species that exist on earth and they harbor one to several endophytes. Varieties of endophytes exist inside the single tissues of host plant. Endophytes include fungi, bacteria and actinomycetes. Out of all the endophytes, endophytic fungi have proved to be the most beneficial one. Hawksworth (2001)reported that only one lakh fungal species are known from the estimated 1.5 million species of fungus. The remaining undiscovered fungi may be in the form of unrevealed endophytes. This provides an opportunity for researchers to work in this field.

Endophytic fungi belong to varied polypletic groups of organisms that dwell in coalition with their host plant mainly within leaves, stems, roots (Bacon and White 2000).They exhibit variety of relationship with their host plant ranges from mutualistic to antagonistic relation. Colonization of fungal endophytes is not a fortuitousopportunity because of the chemotaxis that isspecific chemicals produced by the host plants.At the same time, variety of defense secondary metabolites such as oils and saponin are produced by plants, asaresistancemechanism to the foreign plethora of microorganisms including endophytic fungi present around or within the plant. Therefore, these secondary metabolites create a problem in colonization of endophytic fungi. In response of metabolites fungal endophytesactivates their self defense system by releasing detoxifying enzymes such as proteases, laccases, cellulases, lactases and xylanases for the detoxification of secondary metabolites to make their pathway easy for entering and colonizing inside the host plants. After a long term of co- existence a

friendly relationship was built up between each endophytic fungi and its host plant. Both the organism gets benefitted by this relation. Endophyte fungi receive nutrition, shelter and protection from the host plant and in return provide many benefits to the host plant such as enhanced immunity, increased resistance against herbivores, pathogens and also enhance their tolerance towards abiotic and biotic stresses by attaining the metabolic substances of fungi (Saikkonen *et al*., 1998; Tan *et al*2001; Zhang *et al*., 2006).

Endophytic fungi are believed to be source of varied bioactive compounds. Sometimes, endophytic fungi produce similar novel secondary metabolites that are produced by their host plants (Tan and Zou, 2001). Discovery of*Taxomyces andreanae*anendophytic fungus from Pacific yew (*Taxus brevifolia*) can produce taxol, a potent anticancer drug originally derived from its host plant, is an instance of production of novel compounds from fungus (Stierle *et al*., 1993,Wani*et al*., 1971). It has been hypothesized that during the evolution, endophytic fungi might have undergone genetic recombination by inheriting the ability to synthesize the metabolites similar to host plants. It is alsobelieved that the ability to synthesize the host metabolites is might be due to genetic recombination during evolution. Also there are large numbers of endophytic fungi that are produced from single tissue of the plant and require the screening of the endophytic fungi. Screening helps in selection of fungi that have the ability to produce the compounds that might have therapeutic value (Stierle *et al*., 1993; Zhang *et al*., 2006). The bioactive compounds obtained from endophytic fungi could be classified as alkaloids, steroids, tannins, terpenoids, quinones, lignans, phenols and lactones.In literature many treasured secondary metabolites have been tapped from fungal endophytes having potential to use them as antimicrobial, herbicidal, antioxidant, insecticidal, cytotoxic and anticancer compounds (Vasundhara *et al*., 2016).

Medicinal plants having a distinctive invading microbiome that have potential to produce unique and divergent bioactive compounds (Qin *et al*.,2012), they have been recognized as a cache for fungal endophytes with novel metabolites having pharmaceutical importance (Strobel *et al*., 2004; Wiyakrutta *et al*., 2004; Kumar *et al*., 2005; Tejesvi *et al*., 2007). The secondary metabolites produced by endophytic fungi possess great bioactivities, representing a huge reservoir which holds astupendous potential for exploring them in medicinal, agricultural and industrial fields (Tan and Zou, 2001; Zhang *et al*., 2006). Novelantimicrobial,

antimycotics, immuno-suppressants and antineoplastic compounds have been found after the screening of individual fungal endophyte followed by purification and characterization of their natural products (Strobel *et al*., 2004). In this present study, *Terminalia arjuna*and *Tinospora cordifolia*plantswere selected for study. Both of these have medicinal properties. From ancient times they have been used in ayurveda and exhibits potential in treating various diseases. The stem of the *tinospora* plant has been reported with antibacterial activity against some bacterial strains like *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella dysenteria* (Singh *et al*., 2012).

For not making the plants go extinct, interests have been shown to isolate the endophytes that have inhabited plants since long and tend to produce the same compound that have been produced by host plants or sometimes more than its host plant. The endophytes are subjected to exploration at a large level as maximum of them are still not understood completely. Recently in literature it has been cited that 51% of bioactive novel compounds recovered from endophytic fungi had been earlier unknown when in comparison to 38% from soil fungi (Strobel*et al*., 2003).

This provides an opportunity to do research project in this field because it is necessary to develop new drugs which can provide help and relief in all phases such as emerging new human health problems, the microorganisms are evolving at faster rate and getting resistant to the already present drugs. To cope up with this problem, producing the drugs from the endophytic fungi isolated from medicinal plant would be effective.And it is easy to produce them in large quantity by fermentation of fungi (Verma *et al*., 2011).

OBJECTIVES

The objectives of the project are as given below:

1. Isolation and screening of endophytic fungi from *Tinospora cordifolia*and *Terminalia arjuna*for bioactivity.

2. Fractionation, purification and characterization of crude extractfrom endophytic fungi

3. Identification of endophytic fungi showing maximum bioactivity

REVIEW OF LITERATURE

2.1 Endophytes

In recent times, the term endophyte has been defined by different researchers in various ways. Initially, De Barry (1866) coined the term 'endophyte' for the fungi that reside within host tissues and distinguish them from epiphytes. Carroll (1986) explained these as the organisms that reside in plant tissues without showing asymptomatic infection to the host plant except mycorrhizal and pathogenic fungi.

Afterwards, Petrini (1991) elaborated Carroll's definition as all the organisms that colonize within plant tissue without causing any negative effects to the host at any stage of their life cycle. Wilson (1995) classified endophytes into two type's fungi and bacteria,which dwell in plants for all or part of their life cycle and does not cause any infections. This term was further explained by Bacon and White (2000) by defining endophytes as the microbes that colonize the internal tissues of plant without causing immediate harmful effect to plants. Some endophytes mediate interaction between plants and their competitors. Endophytes include fungi (Wilson and Carroll., 1995; Rajagopal *et al*., 2000;Gond *et al*., 2007), bacteria (Hallman *et al*., 1997), and actinomycetes (Verma *et al*., 2009).

2.2 Transmission and classification of endophytic fungi

Endophytes transmit through two modes of transmission, one is horizontal and another is vertical. Horizontal mode of transmission is followed by majority of endophytes to reach their host plants via airborne spores. Other endophytes may follow vertical mode of transmission through seeds. The endophytes which never appear outside their host and are considered as true endophytes and these are vertically transmitted to the host (Hartley and Gange 2009).

Primarily based at the variations in host plant, taxonomy, ecological features, and evolutionary relatedness,Rodriguez (2009)placed endophytic fungi into two groups (Clavicipitaceous endophytes and non clavicipitaceous endophytes) or four classes (class 1, 2, 3, 4).

Class 1 Clavicipitaceous endophytes: Endophytes of this group infects grasses, sedges and rushes. These invade as endophytes, saphrophytes and pathogens believed to produce some bioactive compounds.

Class 2Non-clavicipitaceous endophytes: Majority of them belongs to ascomycetes and minority to basidiomycetes. They inhabit inside the stems, roots, and leaves of host plants.

Class 3Non-clavicipitaceous endophytes: Endophytes of this class belong to dikaryomycota kingdom and invade inside the stems of plant.

Class 4Non-clavicipitaceous endophytes: These endophytes are classified as dark septate endophytes and they reside only inside the roots of plants.

2.3 Endophytic Fungi-Host Interaction

Endophytic fungi, living asymptomatically can be found within the stems, leaves and roots of plants with wide diversity. Endophytic fungi show mutual relationship with their host plant. They receive nutrition, shelter and protection from the host plant in favor of that host plant may take benefit from enhanced tolerance to biotic and abiotic stress, increased resistance to herbivores, pathogens etc. Some studies discovered that endophyte-host plant relationship is irregular and vary from antagonistic to mutualistic relationship (Saikonnen *et al*., 1998). Large number of metabolites areproduced by endophytic fungi that help in promoting the growth of the plant. Some of the fungal endophytes, especially those that follow horizontal mode of transmission invade woody plants and do not show positive impact to the host growth and resistance.

Interaction between host plant and endophyte can be affected by various factors such as pattern of infection, conditions of environment, mode of transmission, genetic background (Aly *et al*., 2011). A complex relationship exists between the endophyte and its host plant. The outcome of the association may be symbiotic, parasitic, commensalism or pathogenic depending on various factors. Mutualism is the result of balanced interaction between host and endophytes under the influence of environmental, physiological and genetic control whereas parasitism/ pathogenesis /antagonism is the result of unbalanced state where one is benefitted and other develop negative effects (Kogel *et al*., 2006). Endophytes that transmit through seeds are usually involved in symbiotic relation whereas those transmitted through vertical mode via sporeslives in antagonistic relation with their host (Aly *et al*., 2011). Another aspect of this interaction is the duration of the time period for which fungi lives inside the host. Endophytic fungi reside inside the plant throughout its life cycle or during particular period of life without ever causing apparent symptoms of disease (Hartley and Gange 2009). According to Schulz and Boyle (2005) disease triangle, consists of three factors

which decides the consequence of the interaction between host and endophytes such as virulence of endophytes, host defense response and environmental conditions.

Endophyte of a certain plant could be a pathogen for other plant. While colonizing different hosts, they can adapt to various life styles ranging from mutualism to pathogenic (Kogel *et al*., 2006). Endophytes establish biotrophic association with the host. During this long term association, depending upon the availability of nutrients to endophytes and metabolic status of the host this interaction may turn towards either mutualism or antagonism (Lahrmann *et al*., 2013). There is a possibility that a fungus isolated as endophyte may become pathogen under stressful conditions. It may behave asan endophyte in a given host under a specific set of conditions and act as pathogen in another host or in a changed environment (Garcia-Guzman and Heil2013). Endophytes can be regarded as incidental opportunists or latent pathogens that cause disease under stress. A balanced antagonism hypothesis was proposed by Kusari *et al.* (2012), according to which mutualistic symptomless colonization is a balance of antagonism between fungal endophyte and host. *Discula quercina* is a symptomless endophyte of *Quercus cerris* and it turns into weak pathogen under stressful climatic factors (Moricca and Ragazzi 2008).

Under stressful conditions various defense chemicals in form of secondary metabolites are released by the fungal endophytes to cope up with the defense system of plants activated against the invading pathogen. During this ability to synthesize novel secondary metabolites also increases. Various environmental conditions effect the host plant which in turn affects the endophytes to modify their metabolite profile (Selim *et al*., 2012).

2.4Fungal endophytes isolated from medicinal plants

Since times large number of medicinal plants have been explored to cure various diseases. In ancient records such as Vedas and Bible it is found that herbal remedies and their preparation are useful in treating various ailments, and these are found to be helpful in curing disease (Kaul *et al*., 2012). Diverse microbiome resides within medicinal plants that produce novel and varied secondary metabolites (Qi *et al*.,2012). The microbiome that resides on plants are called epiphytes and the other class is classified as“endophytes”. These are a microorganism that resides inside the host tissue by following distinct metabolic pathway. The metabolic pathway of host and endophytes is little similar, that increases the probability of production of

secondary metabolites similar to host plant. Rate and production of these compounds increases under stress conditions.

Researchers are trying to explore more undiscovered medicinal plants for the isolation of novel compounds from them and their residing endophytes. This would be helpful to help pharmaceutical industries to fight against diseases. Endophytes are also found to mimic their host, and produce similar kind of bioactive compounds as that of host plant. Therefore it is significant to explore fungal endophytes. This may plants may result in the ecofriendly, inexhaustible and inexpensive production of interested compounds and therefore help in conservation of biodiversity. These also provide chance to isolate the bioactive compounds from endemic plants. These compounds can be produced on large scale by fermentation. Their capability to synthesize bioactive metabolites has created the enthusiasm on bioprospecting them from medicinal plants (Kusari *et al*., 2012).

Bioprospecting of fungal endophytes from different medicinal plants and their activity are given below (Table 1)

Table 1: Endophytic fungi isolated from different medicinal plants

S.No	Endophytic fungi	Medicinal plants	Activity	References
1	*Fusarium sp.*	*Dysosma versipellis*	Antimicrobial Anticancer	(Xiaoming *et al*., 2018)
2	*Alternaria alternate*	*Rhusmysorensis*	Not reported	(Kartikeya *et al*., 2018)
3	*Alternaria* sp. *Preussia minima*	*Eremophila longifoli*	Anti cancer	(Bita *et al*., 2018)
4	Not reported	*Cupressus torulosa*	Antifungal Enzymatic Potential	(Mohd *et al*., 2017)
5	*Trichoderma sp.*	*Centaurea stobe*	Antifungal, Cytotoxic	(Abdou *et al*. ,2015)
6	*Rhizoctonia sp.*	*Annona crassiflora*	Antibacterial	(De Mendonca *et al*., 2015)

7	*Rhizoctonia spp.*	*Annona crassiflora*	Antimicrobial	(De Mendonca *et al*., 2015)
8	*Aspergillus sp.*	*Limonia acidissima*	Cytotoxic	(Siriwardane *et al*., 2015)
9	*Penicillium funiculosum Alternaria solani*	*Gloriosa superba*	Antimicrobial	(Devi *et al*., 2014)
10	*Aspergillus flavus Diapothe arengae, Alternaria sp. Lasiodiploida theobromae*	*Terminalia arjuna*	Antimicrobial Antioxidant	(Patil *et al*., 2014)
11	*Alternaria alternata*	*Tabebula argentea*	Cytotoxic	(Govindappa *et al*.,2014)
12	*Diaporthe phaseolorum Pestalotiopsis sp. Preussia pseudominima*	*Baccharis trimera*	Antimicrobial	(Vieira *et al*., 2014)
13	*Botryosphaeria Fusarium*	*Camptotheca acuminate*	Antimicrobial	(Ding *et al*., 2013)
14	*Aspergillus fumigates Fusarium sp.*	*Gracinia spp.*	Antioxidant, Anti-inflammatory Antimicrobial	(Ruma *et al*., 2013)
15	*Fusarium oxysporum*	*Zingiber zerumbet*	Antioxidant	(Nongalleima *et al*., 2013)
16	*Chaetomium globosum*	*Withania somnifera*	Antifungal	(Kumar *et al*.,2013)
17	*Aspergillus niger Aspergillus flavus*	*Terminalia brownii*	Antimicrobial	(Basha *et al*., 2012)

	Rhizophus oryzae			
18	*Rhizophus oryzae, Aspergillus niger Aspergillus flavus*	*Terminalia brownii*	Antimicrobial	(Basha *et al*., 2012)
19	*Phomopsis spp.*	*Gossypium hirsutum*	Antifungal	(Fu *et al*., 2011)
20	*Periconia spp.*	*Piper longum*	Antimyco-bacterial	(Verma *et al*., 2011)
21	*Fusarium, Phaeoacremonium*	*Aquilaria sinensis*	Antitumor Antimicrobi	(Cui *et al*., 2011)
22	*Pichia guilliermondi*	*Paris polyphylla*	Antibacterial	(Zhao *et al*., 2010)
23	*Cylindrocarpon sp. Phoma sp. Fusarium sp.*	*Saussurea involucra*	Antimicrobial	(Lv *et al*., 2010)
24	*Phyllosticta sp.*	*Cuazuma tomentosa*	Antioxidant	(Srinivasan *et al*.,2010)

2.5 Medicinal Plants: Two medicinal plants - *Terminalia arjuna* and *Tinospora cordifolia*from the campus of Thapar Institute of Engineering and Technology, Punjab (India) were sampled for isolation of endophyte fungi.

2.5.1 *Terminalia arjuna*

Terminalia arjuna (Arjuna) is a deciduous tree found in various regions of India. It is a large tree that attains a height of 20-25 metres. It forms spreading crown with wide canopy with drooping branches. Leaves are elliptical having green upperside having a pinch of brown color. Flowers appear between March and June and areglabrous. The fruits of arjuna appearsbetween september and November. The size of fruit is 2.5 to 5 cm with woody covering and divided into five wings. When different species of *Terminalia*are compared the bark of *Terminalia arjuna* has its own specific features. The bark of *Terminalia*

*arjuna*appearspinkish grey from outside and wafers off in large curved and rather flat pieces and is smooth in texture.

Fig 1: *Terminalia arjuna*

Table 2: Classification of *Terminalia arjuna*

S.No	Classification	*Terminalia arjuna*
1	Class	Magnoliopsida
2	Division	Magnoliophyta
3	Family	Combretaceae
4	Genus	Terminalia
5	Kingdom	Plantae
6	Order	Myrtales
7	Species	Arjuna

2.5.1.1Chemical Constituents:A range of constituents have been isolated from different parts of*Terminalia arjuna*. They belong to different classes as discussed in the following Table 3.

Table 3: List of bioactive compounds isolated from *Terminalia arjuna*

S. No	Compounds	Active component	Biological activity
1	Arjunin, arjunic acid, oleanolic acid , arjunolic acid, arjungenin, terminic acid	Triterpenoids	Antifungal Cardioprotective
2	Arjunetin, Terminoside-A Glucopyranoside arjunaphthanoloside, arjunoside	Glycosides	Cardioprotective
3	Sitosterol	Sitosterol	Anti-inflammatory, Antitussive, Antimutagenic
4	Ethyl gallate, Arjunolone, Arjunone, Bicalein, Luteolin,Quercetin, Gallic acid, Kempferol, Proanthocyanidins, Pelorgonidin	Flavonoids	Antibacterial, Cytotoxic, Antiasthmatic, Antiallergic, Antifungal, Antioxidant
5	Pyrocatechols, Casuarinin, Punicalagin, Castalagin, Casurin, Punicallin, Terchebulin, Terflavin C	Tannins	Astringent, wound healing and antimicrobial
6	Calcium, Aluminium, Zinc Magnesium, Silica, Copper	Trace elements	Fill up ion requirement

(Khan*et al*., 2013)

2.5.1.2 Endophytic fungi isolated from *Terminalia arjuna*:Researchers have been working since many years for exploring endophytic fungi from *Terminalia arjuna*, a number of fungal isolates have been isolated from *Terminalia arjuna* (Table 4).

Table 4: List of Endophytic fungi from *Terminalia arjuna*

S. No	Endophytic fungi	Medicinal Plant	References
1	*Aspergillus flavus* *Diaporthe arengae*	*Terminalia arjuna*	(Maheshwari *et al*., 2014)
1	*Pestalotiopsis clavispora* *Pestalotiopsis theae* *Pestalotiopsis microspora*	*Terminalia arjuna*	(Tejesvi *et al*., 2009)
2	*Chaetomella raphigera*	*Terminalia arjuna*	(Gangadevi *et al*., 2009)
3	*Pestalotiopsis terminaliae*	*Terminalia arjuna*	(Muthumary et al., 2009)
4	*Alternaria alternata* *Epicoccum nigrum* *Alternaria spp.* *Cladosporium elatum* *Gliocladium penicilloides* *Hyphomycetes* *Nigrospora oryzae* *Cocblonema spp.* *Penicillium spp.* *Trichoderma harzianum* *Chaetomium crispatum* *Monocillium spp.* *Peslalotiopsis spp.* *Phialophora spp* *Cladosporium spp..* *Myrolhecium cinctum* *Myrothecium verrucaria* *Botryodiplodia theobromae* *Chaelomium globosum* *Gliocladium delequescens* *Coelomycetes* *Cytosporella spp.* *Chloridium spp.* *Cladosporium* *Stemphylium spp* *Trichoderma spp.* *Trichoderma viridae* *Tubercularia vulgar*	*Terminalia arjuna*	(Tejesvi *et al*., 2005)

2.5.2 *Tinospora cordifolia*

*Tinospora cordifolia*is commonly known with the name of Giloy in india. It is a large, deciduous, glabrous,climbing shrub.The color of bark is creamy white to grey, which rotate spirally towards left side and the stemof Giloy contains rosette like lenticles. The leaves are heart shaped which provides unique identity to this.Small yellow colored flowers are present in auxillary position that grows in summer season, male flowers are clustered andfemale are generally grow individually. Single seeded fleshy fruits are observed during the winter (BV Shetty *et al*., 2010).

Fig 2: *Tinospora cordifolia*

Table 5: Classification of *Tinospora cordifolia*

S. No	Classification	*T. cordifolia*
1	Class	Magnoliopsida
2	Division	Angiosperms
3	Family	Menispermaceae
4	Genus	Tinospora
5	Kingdom	Plantae
6	Order	Ranunculaceae
7	Species	Cordifolia

2.5.2.1 Chemical constituents of *T. cordifolia*: A variety of constituents have been obtained from different parts of *Tinospora cordifolia*. They belong to different classes as discussed in Table 6

Table 6: List of bioactive compounds found in *Tinospora cordifolia*

S.No	Compounds	Active Components	Biological activity
1	Aporphine alkaloids,Berberine, Choline, Isocolubin, Jatrorrhizine, Magnoflorine, Palmetine, Tembetarine, Tinosporin	Alkaloids	Anti-viral infections, Anticancer, anti-diabetes, inflammation, Neurological
2	Clerodane, Columbin, Furanolactone, Jateorine, Tinosporides, Tinosporon	Lactones Diterpenoid	Vasorelaxant
3	18-norclerodane glucoside, Cordifolioside, Cordioside, Furanoid diterpene glucoside, Tinocordiside, Palmatosides, Pregnane glycoside, Syringin, Syringinapiosyl glycoside,	Glycosides	Useful in treatment of neurological disorders, Immunomodulation, Inhibits NF-kBand that act as nitric oxide scavenger to show anticancer activities
4	20 β-hydroxyecdysone, Ecdysterone, Giloinsterol, Makisterone A, β–sitosterol, δ-sitosterol	Steroids	IgA neuropathy, protection against osteoporosis inducedby glucocorticoid
5	Tinocordifolin	Sesquiterpenoid	Antiseptic
6	Octacosanol	Aliphatic	Anti-inflammatory, Antinociceptive, Provides protection against hydroxydopamine induced Parkinson disease in rats.

(Jitendra Mittal, 2014)

2.5.2.2 Endophytic fungi isolated from *Tinospora cordifolia*:*Tinospora cordifolia*, well known with a name of 'Guduchi' in India is a medicinal plant which is used to treat various ailments.Reseachers are working since many years for exploring endophytic fungi from*Tinospora cordifolia*anda variety of fungal species have been isolated (Table 7).

Table 7: List of endophytic fungi from *Tinospora cordifolia*

S. No	Endophytic Fungi	Medicinal Plant	References
1	19 fungal endophytes	*Tinospora cordifolia*	(Kapoor Neha *et al*., 2018)
2	*Penicillium spp.* *Colletotrichum spp.* *Cladosporium spp.* *Alternaria alternata* *Chaetomium globosum* *Curvularia spp.*	*Tinospora cordifolia*	(Mishra *et al*., 2012)
3	*Fusarium culmorum*	*Tinospora cordifolia*	(Sonaimuthu *et al*., 2010)

2.6 Benefits of exploring endophytic fungi

1. Development of resistance in infectious microorganism gives rise to many diseases and limited the available drugs to fight against these diseases. Bioactive compounds obtained from endophytic fungi inherited chemical diversity and biological potency that may help in production of many compounds with therapeutic potential against many diseases.
2. Microbial natural products can be produced by large scale fermentation by optimizing the conditions required for fermentation such as type of components in the nutrient media, pH and temperature.
3. Microorganisms can be genetically modified to overproduce the desired natural products.

2.7 Endophytic fungi as a source of secondary metabolites

Fungal endophytes are believed to be a treasure of structurally and biologically active secondary metabolites (Tan and Zou, 2001; Strobel, 2003; Strobel *et al*. 2004; Zhang *et al*. 2006;Gunatilaka, 2006; Kumar *et al*. 2008; Aly *et al*. 2011).Colonization of the endophytic fungi within host plants has developed the tolerance against abiotic and biotic stress factors in the host plants. These help in increasing nutrient uptake, inhibition of plant pathogen growth,promotion of the plant growth and also in the reduction of disease severity. Sometimes, the medicinal properties of plants are present in the endophytes present in the host plant and the type of biologically active secondary metabolites that are been produced by that endophytes is same as that of host plant.

Literature survey provides a information that 51% ofbioactive compounds isolated from endophytic fungi were novel as compared to 38% compounds recovered from soil fungi. So, endophytic fungi provide an opportunity to researchers to explore this field more because majority of them are still untapped (Strobel, 2003).

Exploring ahuge number of unexplored endophytic fungi species, helps in discovering new bioactive compoundshaving chemical diversity, which provides potential to use them in various fields such asenvironment, agriculture and pharmaceutical industries (Zhang *et al*. 2006; Akello *et al*. 2007; Bae *et al*. 2009; Redman *et al*. 2011).

Few of the secondary metabolites obtained from the fungal endophytes of host medicinal plants are tabulated in Table 8

Table 8: List of bioactive compounds obtained from fungal endophytes

S. No	Endophytic Fungi	Compounds	Plant	References
1.	*Phomopsis sp.*	Phomol	*Erythrina cristagalli*	(Weber *et al*., 2004)
2.	*Polysiphonia urceolata*	Chaetopyranin	*Chaetomium globosum*	(Wang *et al*., 2006)
3.	*Phyllosticta sp.*	Taxol	*Ocimum basilicum*	(Gangadevi ., 2007)
4.	*Fusarium solani*	9-methoxycamptothecin and 10hydroxycamptothecin	*Camptotheca acuminata*	(Shweta *et al*.,2008)
5.	*Alternaria sp.*	Alternariol	*Polygonum senegalense*	(Aly *et al*., 2008)
6.	*Pestalotiopsis photiniae*	Photinides A–F	*Roystonea regia*	(Ding *et al*., 2009)
7.	*Pestalotiopsis terminaliae*	Taxol	*Terminalia arjuna*	(Gangadevi *et al*., 2009)
8.	*Chaetomium sp.*	Cochliodinol	*Salvia officinalis*	(Debbab *et al*., 2009)
9.	*Alternaria sp.*	Berberine	*Gastrodia elata*	(Duan, 2009)
10.	*Chloridium sp.*	Javanicin	*Azadirachita indica*	(Kharwar *et al*., 2009)
11.	*Xylaria sp.*	Eremophilanolides	*Licuala*	(Isaka *et al*., 2010)
12.	*Lasiodiplodia theobromae*	Taxol	*Morinda citrifolia*	(Pandi *et al*.,2011)
13.	*P. archeri*	Phomoarcherins A–C	*V. albindia*	(Hemtasin et al., 2011)
14.	*Bionectria ochroleuca*	Pullularins E and F	*Sonneratia caseolaris*	(Ebrahim *et al*.,2012)

15.	*Fomitopsis sp.*	Camptothecine	*Miquelia dentata*	(Shweta *et al.*, 2013)
16.	*Penicillium sp.*	Arisugacins I and J	*Tamarix chinensis*	(Sun *et al*., 2013)
17.	*Chaetomium globosum*	Chaetoglobosins A, G, V, Vb, and C	*Ginkgo biloba*	(Li *et al*., 2014)
18.	*Lasiodiplodia sp.*	Lasiodiplodins	*Mangrove plant*	(Li *et al*., 2015)
19.	Bipolaris sorokiniana	Sesquiterpenes	*Costus speciosus*	(Qader *et al*., 2017)
20.	*Fusarium* sp.	Podophyllotoxin	*Dysosma versipellis*	(Xiaoming *et al*., 2018)

MATERIAL AND METHODS

3.1 Collection of samples

The samples were collected from the campus of TIET, Patiala. Stem and leaves of the medicinal plant *Tinospora cordifolia* were collected from C#10, and the bark, stem, leaves andfruit of the plant *Terminalia arjuna* were collected from G block area. The collected samples were transferred into sterile bottles and were brought to TIFAC- CORE laboratory for further studies.

3.2 Surface disinfection of the samples

The above collected samples were initially washed in running tap water, followed by rinsing with double distilled water to remove the dust and debris from the surface. Collected samples were immersed in 70% ethanol for 1 min, and then dipped in aqueous solution of 1% sodium hypochlorite for 3 min, followed by rewashing in 70% ethanol for 10sec. Last washing was given with double distilled autoclaved water and dried in laminar air flow bench (Petrini *et al*., 1993).

3.3 Isolation of the endophytic fungi from the sample

After drying, the samples were cut from the edges and outer layer was removed by sterile blade. The samples were further cut horizontally into two halves. These were transferred onto the PDA + chloramphenicol plates, and the plates were incubated for a perioid of 7-14 days at 27±2˚C and observed after every 2-3 days for the growth of endophytic fungi.

3.4 Purification of Fungal Endophytes

The purification of fungal endophytes from a mixed population of fungal endophytes was done so as to get the pure strains. It was done by using sterile blade and inoculum was taken from master plate and placed onto the potato dextrose agar medium. The plates were incubated for 7- 14 days at 27±2˚C. The master cultures were preserved at 4˚C.

3.5 Coding of fungal endophytes

The isolated and purified fungi from different plants and their respective parts were named accordingly in this project.

Following labeling pattern was used: For example : the endophytes isolated and purified from the leaves of the plant *Terminalia arjuna*was labeled as AL (A=Arjuna and L=leaves) followed by the numerals as AL 1, 2 ….etc. for stem as AS 1, 2…….etc. and for the fruits as AF 1, 2 ….etc. Similarly for the endophytes isolated and purified from the plant *Tinospora cordifolia*(G= Giloy and R=Root) as GR 1, 2….etc. for stem GS 1, 2…..etc. for leaves GL 1, 2, 3….etc.

3.6 Sub-culturing of the endophytic fungi

Sub-culturing of the endophytic fungi was done periodically by cutting out 1-2 cm discs of the mycelia from the plate by sterile blades. These discs were transferred to the middle of the fresh PDA plate and incubated at 27±2˚C for 7 days.

3.7 Production of secondary metabolites by fermentation

The fresh mycelia sub-cultured on PDA were transferred to 500 ml Erlenmeyer flask containing 250 ml of the PDB. The cultured flasks were incubated for 21 days at 27±2˚C under stationary conditions. After 21 days of incubation period, the culture was filtered using sterile muslin cloth to remove the mycelia from the broth (Prabavathy *et al*., 2011). Mycelia of each isolated fungi was dried till constant weight and dry weight was determined.

3.8 Solvent extraction to obtain metabolites

Ethyl acetate was used as a solvent to extract the fungal metabolites by solvent extraction procedure. Equal volume of ethyl acetate and filtrate was taken in separating funnel and shaken for 10-15 min. Further it was allowed to stand stillso as to allow the two phases (organic and inorganic phase) to get completely separated. Organic phase was collected in a separate flask. Same was repeated again. Obtained organic phases were combined in a flask and dried by using the Rota-evaporator at 32˚C temperature of water bath and 4˚C of chiller.

The residual crude extract was further dried completely in a vial by keeping at room temperature and weighed. These crude extracts were further used for testing of biological activities.

3.9 Preliminary bioactive screening of the fungal extracts

3.9.1 Agar well diffusion antimicrobial assay of crude extract

Cultures used for antibacterial and antifungal testing

Test bacterial cultures

- Gram positive bacteria:*Bacillus megaterium*, *Staphylococcus aureus*
- Gram negative bacteria: *Pseudomonas aeruginosa , Escherichia coli,*

Test fungal culture

- *Candida albicans*

Materials required: Cultures of *E.coli, B. megaterium, Staphylococcus aureus and Pseudomonas aeruginosa*in MHB and *Candida albicans*in PDB, auto-pipettes, MHA plates, spreader, borer, Ampicillin, Streptomycin, and Amphotericin

Procedure:Agar well diffusion assay was performed to evaluate the antimicrobial (bacterial and fungal) potential of crude extracts against the above test cultures.

3.9.1.1 Antibacterial activity

Preparation of McFarland Standard

The approximate number of bacteria in liquid suspension was standardized by using McFarland standard. This was used by comparing the turbidity of McFarland standardwith the turbidity of test organism's suspension. The standard was prepared by addition of barium chloride into the sulfuric acid for the precipitation barium sulfate. By altering the volume of these two reagents standard of different degree of the turbidity can be prepared to represent the many different concentrations of bacteria. Most commonly used standard in clinical microbiology laboratory for antimicrobial susceptibility testing is 0.5 which represents 1.5×10^8 (these range from 1.0×10^8 to 2.0×10^8 bacteria/ml).

1. 85 ml of 1% H_2SO_4 was added to a 100 ml volumetric flask.
2. Drop wise 0.5 ml of 1.175% $BaCl_2.2H_2O$ was added with auto-pipette to sulphuric acid by continuously swirling the flask.
3. Volume was made up to 100 ml with 1% sulphuric acid.
4. The flask was kept on magnetic stirrer for 4-5 min and the O.D. was recorded at 600 nm.

Well grown colonies of the bacteria on the Muller Hinton agar plate were sub cultured on the MHA plates and incubated overnight at 37˚C. From the MHA plates the colony was picked with a sterile loop and transferred to MHB. This was then placed on shaker at 37˚C for 1-2 hrs, so that the O.D. reaches 0.5 McFarland. 100µl of the test organism was then spread over the MHA plates.

50µl of the crude extract was loaded into the wells and then petri plates were incubated at 37˚C for 24hrs.Then the diameter of zone of inhibition was measured that formed around the wells.

Streptomycin and Ampicillin were used as antibacterial control (Hadacek and Greger, 2000)

3.9.1.2 Antifungal activity

Colonies of the *candida albicans* were streaked on the PDA plates and incubated overnight at 30±2˚C. From the PDA plates the colony was picked with a sterile loop and transferred to PDB. This was then placed on shaker at 30±2˚C for 4-5 hrs, so that the O.D. reaches 0.5 McFarland standards. 100µl of the test organism was then spread over the PDA plates.

Then 50µl of the crude extract was loaded into the wells and then petri plates were incubated at 30±2˚C for 48 hrs. Then the diameter of zone of inhibition was measured that formed around the well.

3.9.2 Antioxidant assay of crude extracts

DPPH assay: DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) is widely applied antioxidant assaybased on transfer of electrons that produces a violet solution in methanol.Free radicals arestable at room temperature and get reduced in the presence of an antioxidant molecule. On scavenging free radicals it changes the solution from violet to colorless.

Materials required: DPPH, ascorbic acid, water, 96 well microtiter plate, methanol, pipette, tips, extracts, aluminium foil and ELISA plate reader.

Procedure: 5µl of each serial dilution of endophytic fungal extracts were mixed with 150µl of DPPH (100 µM) and added in 96 well micro titer plate. Ascorbic acid (100µg) was added as positive control and water as negative. The plate was wrapped in aluminium foil and kept for 45 min in dark. The change in color intensity (from deep violet to light yellow) was recorded at 517 nm using ELISA reader.

Free radical scavenging activity was calculated using formula,

Free radical scavenging activity = (A Control - A Sample / A Control) x100

The entire test was performed in triplicates.

3.10 Selection of crude extract

After preliminary assays, the crude extracts exhibiting maximum antimicrobial potential against test cultures and antioxidant activity were selected for further studies.

3.11Bioactivities of screened extracts

3.11.1 Agar disc diffusion and MTT assay on the screened extracts

Diffusion refers to the movement of molecules in the matrix provided by the agar after gelling. The degree of the movement of the molecules can be related with concentration of the molecule. This is basically used to determine the susceptibility or resistance of the bacterial strain to the particular antibacterial agent. The agar that is used as the matrix is not completely impermeable it has spaces that are present between strands of agar that comprise of the hard polymer.

Small molecules such as antibiotics are able to diffuse through agar. Antibiotics are actually applied to the sterile disc and the disc was kept on the agar plate. Antibiotics have the tendency to move from higher concentration to lower concentration. If there is more of the antibiotic on the disc then it will tend to make larger zone of inhibition. Firstly the bacterial colony is spread over the agar, which is then allowed to stand for a while then the discs are

kept on to the agar.After the incubation period agar plates are examined for bacterial growth. If the growth of microorganisms covers the whole plate, it indicates that bacteria are resistant to that particular antibiotic. If there is zone of inhibition formed (clear zone), it indicates that microorganisms are sensitive for that antibiotic. The diameter of zone of inhibition is measured according to standards (generally measured in millimeters) and would suggest that how much the bacteria are sensitive. Zone of inhibition also depends on the concentration of the antibiotic. At more concentrations, the zone of inhibition is large and at low concentrations the zone of inhibition is less. The agar diffusion allows bacteria to be screened routinely and economically in the microbiological laboratory. Similarly, the fungal extracts are allowed to diffuse into the agar and show its effect over the test microorganisms. The resulting zone of inhibition is uniformly circular as there is always the confluent lawn of growth of the test organism (NCCLS 2000).

Materials required: Cultures of *E.coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus megaterium* in MHB and *Candida albicans* in PDB, auto-pipettes, MHA plates, spreader, punched sterile filter paper (6mm diameter), Ampicillin, Streptomycin, and Amphotericin

Procedure:Well grown colonies of the bacteria on the nutrient agar plate were streaked on the MHA plates and incubated overnight at 37˚C. From the MHA plates the colony was picked with a sterile loop and transferred to MHB. This was then placed on shaker at 37˚C for 1-2 hrs, so that the O.D. reaches 0.5 McFarland. 100µl of the test organism was then spread over the MHA plates. For the fungal cultures the PDA plates were used to streak and colony was transferred in PDB and incubated at 30˚C for 1-2 hrs and spreading was done on PDA plates. Plates were kept for 30 min.

Then 20µl (5mg/ml) of the fungal extract was applied to the sterile disc (punched filter paper) and then after drying the disc, it was placed on the petri plate that was earlier spread with the bacterial test organisms or fungal test organisms. Plates were incubated at 37˚C for 24hrs in case of bacterial test organism and at 30˚C for 48 hrs for fungal test organism. The diameter of zone of inhibition was measured that formed around the disc. Streptomycin and Ampicillin were used as antibacterial control whereas the amphotericin was used as the antifungal control (Hadacek and Greger, 2000).

3.11.2 MTT assay

Soluble tetrazolium saltsare converted to insoluble formazan crystals by metabolically active cellswhich is a base of the assay (Mosmann, 1983). The succinate dehydrogenase enzyme produced by the mitochondria of the live cells is able to convert yellow colored tetrazolium salt to purple colored formazan crystals. The crystals are solubilized in DMSO solution. The purple color developed was read at 600nm in an ELISA reader.

Materials required:

MTT (5mg/ml), extracts, Dimethyl sulfoxide (DMSO), MHB, PDB, ampicillin, amphotericin, 96 well plate, pipette, tips, ELISA reader

Procedure:

A suspension of test organism was prepared equivalent to 0.5 McFarland standard using isolated colonies. Stock of ampicillin (50µg/ml) was prepared in distilled water for antibacterial assay. For antifungal assay stock of amphotericin (1µg/ml) was prepared in pure DMSO and fungal crude extract (5 mg/ml) was prepared in media. Experiment was performed in 96 well plate, with total volume of 100 µl of cell suspension, media and different fungal crude extracts at varying concentration (100µg, 250µg, 500µg and 1000µg) were added into the well. MHB (media control) served as a blank, MHB with bacterial cells suspension (cell control) was used as positive control, MHB with cells suspension and antibiotic (ampicillin for bacterial test cultures) and for fungal test cultures (amphotericin) served as the antibiotic control.

TheELISA plate reader was set at 600 nm to record the absorbance firstly at 0 hr (initial reading) and then the plate was incubated at 37˚C (bacterial test cultures) and 30˚C for (*C.albicans*) for 24hrs, absorbance was again recorded after 24 hrs (final reading) at same wavelength. The reading was taken after 24hrs of incubation at 30˚C for *C.albicans*. After taking reading, 10µl of MTT was added to all the wells and incubated for 4 hrs. Crystals were formed at the bottom of the plate, so DMSO was added to dissolve it. The entire test was performed in triplicates.

3.11.3 Antioxidant activity: DPPH assay was repeated again in 96 well plate, with total volume of 200 µl and in different fungal crude extracts at varying concentration (100µg, 250µg, 500µg and 1000µg) was added to calculate IC_{50} values.

3.12Phytochemical analysis of crude extracts(Hadacek and Greger, 2000)

3.12.1 Test for phenols and tannins (Ferric chloride test):

1ml of the extract(s) was treated with 1 ml of ferric chloride solution (5% w/v), blue color appears if hydroxylate tannins are present and if green colour appears it shows presence of condensed tannins.

3.12.2 Test for amino acids (Ninhydrin test):

To 1 ml of ninhydrin solution added 1 ml of test solution and heated to boil. Violet color indicated the presence of amino acids.

3.12.3 Test for carbohydrates (Molisch's test):

Molisch's reagentwas added drop wise to 1 ml of extracts followed by addition of few drops of concentration sulphuric acid along the sides of the tubes. Purple to violet color ring appears at the junction if carbohydrate were present.

3.12.4 Test for alkaloids (Wagner's reagent test):

Took 1 ml of extract(s) and few drops of Wagner's reagent wereadded. Reddish brown ppt indicated the presence of alkaloids.

3.12.5 Test for fats and fixed oils (Saponification test):

To 1 ml of extract(s) few drops of 0.5 N alcoholic KOH was added along with a drop of phenolphthalein. Then the test tubes wereplaced in pre-heated water bath (55°C) for 1hr. The formation of soap (soapy bubbles) indicated the presence of oils and fats.

3.12.6 Test for steroids and triterpenoides (Salkowoski's test):

1 ml of the extract(s) were treated with few drops of conc. sulphuric acid, formation of yellow colored lower layer showed the presence ofthe triterpenoids and the presence of steroids is indicated by formation of red color at lower layer .

3.12.7 Test for glycosides (Keller-Killiani test for Deoxy sugars):

2 ml of the extractwas taken,1ml glacial acetic acid, one drop 5% $FeCl_3$ and few drops of conc.wereadded into the test tube. Appearance of reddish brown color at the junction of two liquid layers and bluish green color at upper layer confirms the presence of deoxy sugars.

3.13 Screening for fungal extracellular enzymes

Exploring large number of microorganism for production of useful enzymes provides an idea that filamentous fungi are most effective amongst all other microorganism. Easy recovering and high production of extracellular enzymes by filamentous fungi gain interest of many researchers (Bhagobaty and Joshi 2012) to obtain enzymes from these species. These enzymes are morethermo tolerant than the enzymes obtained from other sources, because of that they gain interest in many fields such as in human health, agriculture and industry. Endophytic fungi are known to produce many novel molecules and represent an interesting option to be explored for the production of enzymes. Varied extracellular enzymes such as lipase, pectinases, laccases, cellulases, chitinases etc. are produced by fungal endophytes. These have the potential to use them in various processes such as textile, leather, pulp, paper,food and beverages industries. The production of extracellular enzymes differs among the endophytes.

3.13.1 Amylolytic Activity

Amylase activity of AL2, GS4 was assessed by growing the fungi on Glucose Yeast Extract Peptone Agar (GYP) medium (yeast extract-0.1g,glucose-1g, agar -16g, distilled water-1L) supplemented with 0.2% soluble starch. At the end of incubation iodine treatment was given with 1% iodine in 2% potassium iodide. After the iodine treatment, a visible zone of starch degradation on the plate indicated the presence of amylase.

3.13.2 Lipolytic Activity

Lipase activity was assessed by growing the fungi on Peptone Agar medium with addition of sterilized 1% tween 20 to the medium. After incubation a visible precipitate around the colony was developed due to the formation of calcium salts of the lauric acid and indicated positive lipase activity.

3.13.3 Laccase Activity

Glucose Yeast Extract Peptone Agar medium with 0.05g 1-napthol L, pH 6.0 was used. As the fungus grows the colorless medium turns blue due to oxidation of 1-napthol by laccase.

3.13.4 Proteolytic Activity

Isolated fungal endophytes were grown on Glucose Yeast Extract Peptone Agarmediumsupplementedwith 0.4% gelatin (pH 6.0). (8% of gelatin solution in distilled water was autoclaved separately and added to medium at the rate of 5mL per 100mL). After incubation, clear zone around the colonies indicates the degradation of gelatin. The petri plates were flooded with saturated aqueous ammonium sulphate, which made the agar opaque and a clear zone around the fungal colony could be seen.

3.14Identification of the endophytic fungus

The endophytic fungus that was isolated from the medicinal plant was further identified morphologically by visualizing its colony, growth pattern, spores and hyphae under the microscope and molecularly by isolating the DNA from the fungi. The DNA was amplified and sent for sequencing.

3.14.1 Morphology based identification

Microscopic examination of the selected endophytic fungus was done using the lactophenol cotton blue dye. Firstly, the slides were washed with 70% ethanol, then 2-3 drops of lactophenol cotton blue dye was added on the slide using dropper. Mycelia were picked using the needle from the plate. Spores were spread over the glass slide. A sterile cover slip was carefully placed over the slide. The slide was examined under the microscope.

3.14.2 Molecular based identification of endophytic fungus

Endophytic fungal species were grown in 50 mL conical flasks containing 25 mL of potato dextrose broth (PDB) at 27±2˚C in static condition for 6-8 days. After 8 days, the mycelia were filtered through muslin cloth. The mycelia on the muslin cloth were dried under aseptic

conditions. Then it was scrapped from muslin cloth and crushed with liquid nitrogen in pestle and mortar. The crushed samples were collected as 100 mg in each microfuge tube and stored at -80˚C, for further isolation of genomic DNA.

3.14.2.1Genomic DNA Isolation

1. 100mg of liquid nitrogen crushed mycelia was taken in 2 ml of microfuge tube.
2. 600 µl of preheated extraction buffer (1M Tris-HCl pH-8, 5M NaCl, 0.5M EDTA, 2% CTAB) was added and vortexed for 1 min.
3. The tubes were than incubated at 65˚C for 1 hr in an incubator or water bath, tubes were inverted after every 5-10 min to allow mixing.
4. Tubes were then centrifuged at 13500g for 15 min at 4˚C to remove soluble debris.
5. The supernatant so obtained was mixed with equal volume of phenol: choloform: Isoamyl alcohol and incubated at room temperature for 25 min, followed by centrifugation at 13500 g for 15 min.
6. The top aqueous layer was collected in a separate microfuge tube and 800 µl of chilled isopropanol was added.
7. The tubes were then incubated at -20˚C for 1 hr, followed by centrifugation at 13500 g for 10 min.
8. The pellet obtained was mixed in 25 µl of 3M sodium acetate and incubated overnight at 4˚C. 600 µl of chilled 95% ethanol was added to the suspension, mixed and incubated at -20˚C for 20 min.
9. The mixture was then centrifuged at 14000 g for 10 min and the pellet recovered was washed with 500 µl of cold 70% ethanol.
10. Pellet was air dried and put in 25 to 30 µl Milli Q water (Zhang *et al*. 2010).
11. DNA obtained in the above step was checked by running it in 0.8% agarose

3.14.2.2Agarose Gel Electrophoresis for DNA

1. For 0.8% of agarose Gel, 0.4 gm of agarose was mixed in 40 ml of 0.5X TBE buffer.
2. The solution was cooled down and 2µl of ethidium bromide (EtBr) was added and mixed properly.
3. Agarose was poured in casting tray and comb was placed in tray.

4. The gel was allowed to solidify at room temperature.
5. The gel was then placed carefully on to electrophoresis chamber. 0.5X TBE buffer was added to the chamber to cover the gel.
6. The comb was carefully removed so that the wells did not damage.
7. The DNA sample and ladder was loaded into the wells.
8. The gel was allowed to run at 70V for 1hr 40 min and bands were observed in UV transilluminator.

3.14.2.3Amplification of Gene: To amplify the ITSregion from the DNA, PCR amplification was done and following PCR reagents were used:

i. DNA nucleotides; Used to build the new strands
ii. Template DNA; Provides the sequence we want to amplify
iii. Primers; Bind to the complementary regions in the template DNA and thus provides the site for polymerase binding.
iv. DNA polymerase; A heat stable enzyme that catalyses the DNA synthesis

3.14.2.4 Composition of amplification reaction mixture: For the amplification of ITS regions, PCR was conducted in 25 µL reaction volume containing following components (Table 9) in their respective quantity.

Table 9: Composition of amplification reaction mixture

Components	**Quantity**
2 mM dNTPs	2 µl
Nuclease-Free Water	16.45 µl
10X Buffer	2.5 µl
Taq Polymerase	0.3 µl
Forward Primer	1 µl
Reverse Primer	1 µl
$Mgcl_2$ (50 mM)	0.75 µl
Template DNA	1 µl
Total Volume	25 µl

3.14.6 PCR conditions:PCR was performed using a thermal cycler, and using the conditions described in Table 10.

Table 10: PCR Conditions for amplification of ITS region

Process	Temperature	Time
Initial denaturation	94˚C	3 min
Denaturation	94˚C	1 min
Annealing	55˚C	1min
Elongation	72˚C	1 min
Final extension	72˚C	8 min

×35 cycles

After amplification approx 100µl of amplified product was purified by using QIAquick PCR Purification Kit (purchased from Qiagen India Ltd), following the manufacturer enclosed protocol.

3.14.7Ligation: The purified PCR product was ligated into pMD20-T vector using the reaction mixture (Table 11).

Table 11: Composition of ligation mixture

Components	**Quantity**
H_2O	2 µl
pMD20-T vector	1 µl
Purified product	3 µl
Ligase	5 µl

Note: The ligated PCR product was kept overnight at 4^0C

3.14.8 Transformation: The ligated PCR product was transformed into competent cells, and the competent cells were prepared as follows.

3.14.8.1 Competent cells were prepared using following protocol

1. *E. coli* DH5α was inoculated in 5 ml LB and was grown overnight at 37˚C in shaker.

2. The cells were reinoculated in 50 ml LB and kept at 37˚C in shaker until OD_{600} reaches 0.6-0.8.
3. The culture was added in tubes (25 mL each) and centrifugation was done at 8000 rpm for 5 min at 4˚C.
4. Supernatant was discarded and 5 ml chilled 0.1 M $CaCl_2$ was added to the pellet.
5. It was mixed gently by tapping and kept on ice for 30 min followed by centrifugation at 6000 rpmfor 5 min.
6. 500 µl $CaCl_2$ was added to the pellet, mixed gently and 6 µl of ligated product was added into it.
7. The contents were mixed gently and eppendorf was stored for 30 min on ice.
8. It was kept at 42˚C in a water bath for 90 sec and again kept at ice for 1-2 min.
9. 900 µl LB medium was added to the eppendorf and the tube was incubated then for 2 hours at 37˚C followed by
10. Centrifugation was done at 6000 rpm for 10 min at 4˚C and the supernatant was discarded and 100 µl LB medium was added to the pellet and mixed well.

3.14.9 Transformation of ligated product

1. 40 µl of cells were added in three centrifuge tubes.
2. 4 µl of ligated mixture was added into it, the tubes were then placed in ice for 30-45 min and in the mean time the temperature of water bath was adjusted to 42^0C.
3. Centrifuge tube were taken out from ice and kept in incubator maintained at 42^0C for 60-90 sec.
4. Centrifuge tube were taken out from the water bath and shifted again into ice for 2 min.
5. 900 µl LB was added into each eppendroff aspectically in laminar air flow and placed in incubator shaker at 37^0C for 1-2 hr for the proper growth.
6. Cells were pellet down by centrifuging at 8000 rpm for 5 min.
7. LB was done and the mixture was gently mixed.
8. Luria Agar Plates were prepared containing ampicillin (100µg/ml).
9. After the media was solidified, 40 µl of 20mM X-gal and 40 µl of 1 M IPTG were spread over the plates and 100 µl of transformed cells were spread over the plates.

10. The plates were incubated overnight at 37°C and then stored at 4°C for several hours. This allows the blue color to develop fully.

3.14.10 Blue White screening of the Transformed Bacterial colonies: Blue-white screening is a molecular technique used for the detection of positive transformants. This was carried out in LB-agar plates containing X-galand IPTG. This method allows the detection of successful transformation of Vector inside the competent cells. Gene is ligated into a vector, followed by transformation of vector into competent cell (bacteria). These cells were grown in the plates containing IPTG and X-gal. If the ligation was positive, the transformed bacterial colony will be white otherwise it will be blue.

After successful transformation, patches of white color colonies were streaked on luria agar plates containing ampicillin (100µg/ml) and plates were incubated overnight at 37°C.

PCR amplification of transformed colony was done to confirm the transformation of gene.

3.14.11 Sequencing of sample: Recombinant clones were sequenced employing M13 F and M13 R universal primers by using chain termination method.One clone was sequenced.

3. 14.12 Sequence analysis of ITS gene: The sequences obtained were then searched for sequence similarity with the non-redundant database maintained by NCBI (National Center for Biotechnology Information). The homology in the sequence was searched by comparing the sequence of interest with already available data using BLAST tool (Basic Local Alignment Search Tool). The similar sequences obtained from NCBI GenBank were aligned with the sequences of interst, using multalign software(Altschul *et al*., 1997).Further, Molecular Evolutionary Genetics Analysis (MEGA version 7) software was used for phylogenetic analyses (Tamura *et al*., 2011).Maximum parsimony method was used to construct the phylogenetic tree.

3.15 Purification of crude extracts:

3.15.1 Thin layer chromatography

Instruments: Glass plates, sample applicator, capillary tube, glass chamber, UV torch

Reagents: All solvents of A.R. grade were employed throughout the determination.

TLC is a simple and quick procedure to find out the number of components present in a crude extract. The ethyl acetate extracts of the fungus were spotted above 2cm from the base over TLC plates (Silica Gel 60 F_{254} – Merck).The spotted TLC plates were run in optimized mobile phase. The mobile phase separated the components into various ranges of R_f values. The developed chromatograms were visualized under UV light at 254 wavelength. Sample components that absorb light in this region appear as a dark spot by quenching the greenish yellow fluorescing background (Fried, 1994).

Various steps were involved:

- Selection of stationary phase
- Selection of mobile phase
- Sample application
- Type and size of developing chamber
- Mode of development
- Visualization and detection

3.15.2 Optimization of mobile phase: The general strategy for optimizing the mobile phase in TLC is to adjust the solvent strength according to polarity index (Table 12) or by replacing a pure solvent by another, so that the Rf values are in the range of 0.15 – 0.85. The extracts in which more than one componentsto be separated are present, the separation may not be achieved using mixture of two solvents and in such cases a combination of more number of solvents may be used.

Table 12: Polarity index for various solvents

Solvent	Solvent Polarity Index, *P*
Hexane	0.1
Carbon tetrachloride	1.56
Isopropyl ether	1.83
Toluene	2.4
Methyl-*t*-butyl ether	2.4
Chloroform	2.7
Diethyl ether	2.8
Dichloromethane	3.1
Isopropanol	3.92
Tetrahydrofuran	4.0
Ethyl Acetate	4.4
Methanol	5.1
Acetone	5.1
Dioxane	5.27
Acetonitrile	5.8
Water	10.2

3.15.3 Separation of active compounds by silica gel column chromatography

Requirements: Glass column, stationary phase – Silica gel G (60-120 mesh), Mobile phase – Hexane, ethyl acetate and methanol, cotton, test tubes, test tube stand, Silica gel G, TLC plates

Sample Preparation: The ethylacetate extract of the fermented culture media was evaporated to dryness, weighed and pulverized. 150 mg of sample was used for the separation of various fractions.

Column preparation: The extract selected for fractionation was adsorbed on stationary phase. Glass column with appropriate dimensions wasselected based on the quantity of the sample to be separated. Selected column was rinsed with solvent to remove impurities. A cotton pad was placed firmly at the bottom to prevent the flow of stationary phase. The column was charged with stationary phase by wet packing method. Second layer of cotton pad

was placed above the mixture to prevent messing of the stationary phase while adding eluting solvents from the top.

Procedure: The slurry was poured gently ontop of column to avoid the production of air bubbles and packed with gentle forced air. The column was then washed with hexane and then prepared by running an adequate amount of the first solvent mixture through. Crude extract was dissolved in an appropriate solvent then loaded onto the column. The column was then eluted with a 100% hexane, gradient of hexanes and ethyl acetate(5% ethyl acetate to 100% ethyl acetate), followed by methanol in ethyl acetate (5% ethyl acetate). Final washing of the column was done with pure methanol.

The solvents eluted from the column were collected in test tubes. Each fraction was concentrated and spotted on a TLC plate. The TLC was developed with appropriate mobile phase and visualized under long UV (365nm) or in Iodine chamber. Based on the TLC profile similar fractions were pooled together and the fractions with more than one compound were again subjected to small sized column chromatography.

Final fractions were evaporated using rotary evaporator.

3.16Screening of bioactive fractions:

The fractions were further tested for final antimicrobial, antioxidant (DPPH),antiproliferattive activities. The fractions showing maximum potential were characterized to identify the bioactive molecules that may be present in the fraction.

3.18Characterization of fractions exhibiting maximum activity

3.17.1 UV-Visible spectroscopic analysis of compound: Ultra violet and visible spectrophotometer techniques contain analytical methods works on the basis of measurement of light absorbed by the compounds lie in the wavelength range of 190 to 900 nm. The region between 190 to 380 nm is called as the UV region and from 380 to 900 nm it is known as visible region of the spectrum. Electronic transitions with the molecules help in absorption of molecules in UV – visible region. Large number of instruments are available for measuring the light absorption of the spectrum. Generally, the UV spectrum serves as a confirmatory proof for the identification of compounds. It is also used as detector in HPLC.

3.17.2 High pressure liquid chromatography (HPLC): This analytical procedure was used for detection of compound from fractions which were earlier separated using column chromatography. The fraction showing maximum activity was further purified by using C18reverse phase column. UV detector was coupled with HPLC instruments for the detection of compound at 232 nm.Thirty microliters of the sample was injected into the column. The mobile phase used for separation was methanol/acetonitrile/water (25:35:40, by v/v/v) over a period of 30 min at flow rate of 1.0 ml min^{-1}. The sample waspassed through 0.2 μm microgene syringe filter before loading into the column.

3.17.3 Fourier-transform infrared spectroscopy (FTIR):FTIR is an analyticaltechnique is works by measuring the concentration of fluorinated molecules by extractive fourier transform infrared spectrometry.

Procedure: Three samples (AL2, AL2-4, and AL2-7) of 10mg/ml concentration were deposited to SAIF, labs, Punjab University, Chandigarh for FTIR characterization.

3.17.4 LC/MS: Liquid chromatography-mass spectrometry (LC-MS) is an analytical purification technique that fused with the physical separation of the compounds (or HPLC) with the mass detection potential of mass spectrometry (MS). For LCMS analysisthree samples (AL2, AL2-4, and AL2-7) were dissolved inmethanol and used as such for analysis. Chromatographic separation was carried out using UHPLC system equipped with C18 column. Sample was injected into the column and separation was carried out using methanol/acetonitrile/water (25:35:40, by v/v/v) over a period of 30 min with flow rate of 1ml/min. The m/z values obtained were further analysed for the identification of compound.

RESULTS AND DISCUSSION

In this present study, two medicinal plants*Terminalia arjuna*and*Tinospora cordifolia* have been selected for the isolation of endophytic fungi. The above two medicinal plants were chosen for study as not much work has been done on isolation of endophytic fungi, characterization of bioactive compounds obtained from these fungi. As these medicinal plants have therapeutic potential, hence they could be explored for endophytic fungi having capability to produce bioactive compounds.

4.1 Collection of samples

The samples were collected from the campus of TIET, Patiala. The bark, stem, leaves and fruit of the plant *Terminalia arjuna* were collected from G block area (Fig 3) and the stem and leaves of the medicinal plant *Tinospora cordifolia* (Fig 4) were collected from C#10. Samples were then transferred into sterile bottles and brought to TIFAC- CORE laboratory for further studies.

Fig 3: Collection of samples from different parts of *Terminalia arjuna*, (A) Tree of *T. arjuna*, (B) Leaves of *T. arjuna*, (C) Stem of *T. arjuna*, (D) Bark of *T. arjuna*, (E) Fruit of *T. arjuna*.

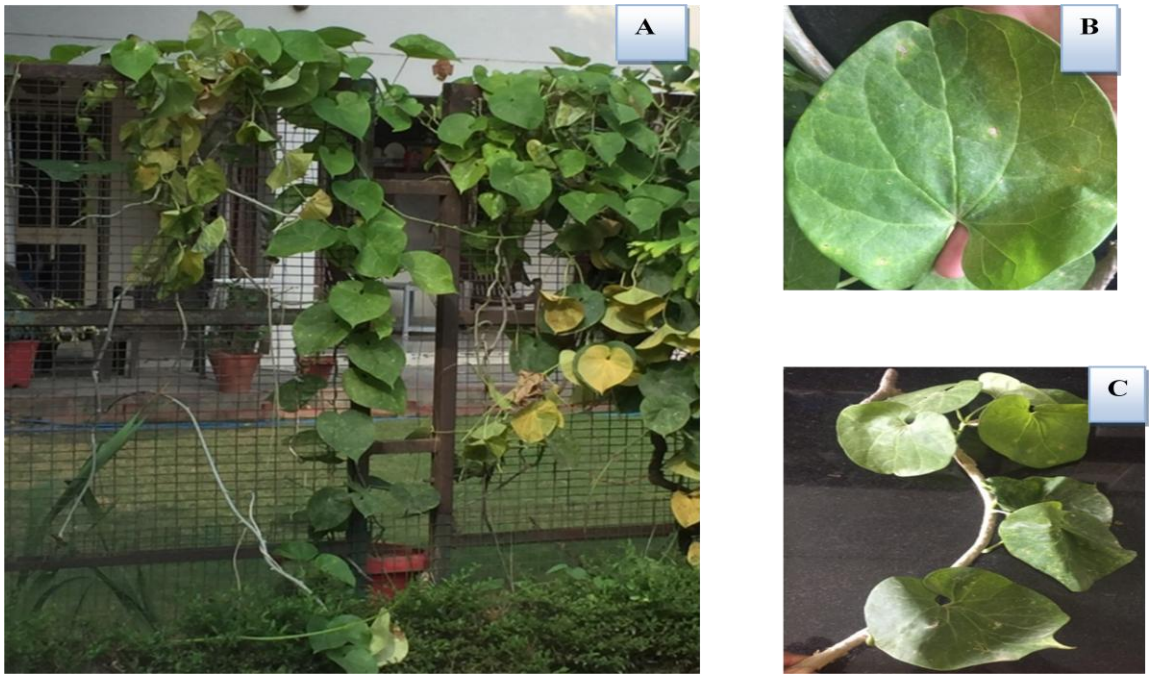


Fig 4: Collection of samples from different parts of *T. cordifolia* (A) Shrub of *T. cordifolia* (B) Leaf of *T. cordifolia*, (C) Stem of *T. cordifolia.*

4.2 Isolation of the endophytic fungi

The tissues of the plant weresurface disinfected and transferred to the PDA plates which were then incubated at 27±2˚Cfor 5-14 days. The plates were observed regularly to check the growth of endophytes (Fig 5). In total14 fungal endophytes were obtained from*Terminalia arjuna* (Fig 6) and 7 from *Tinospora cordifolia* (Fig 7) during a period of 5-14 days.

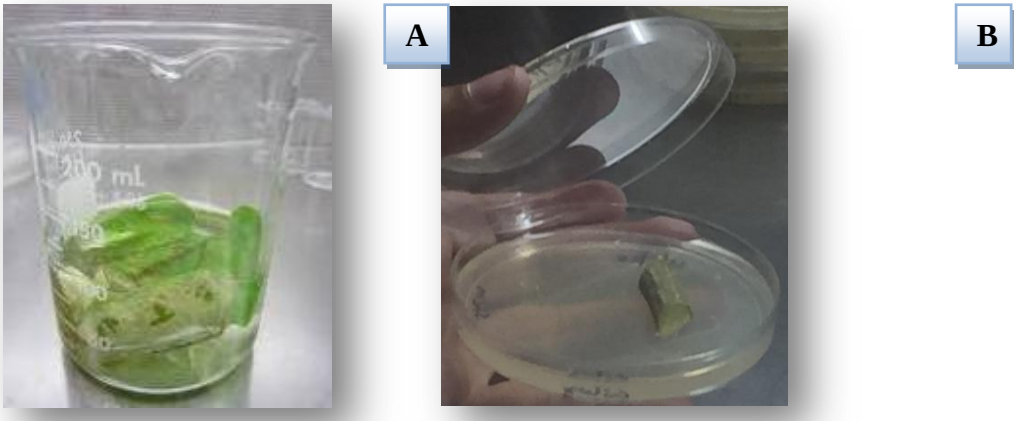
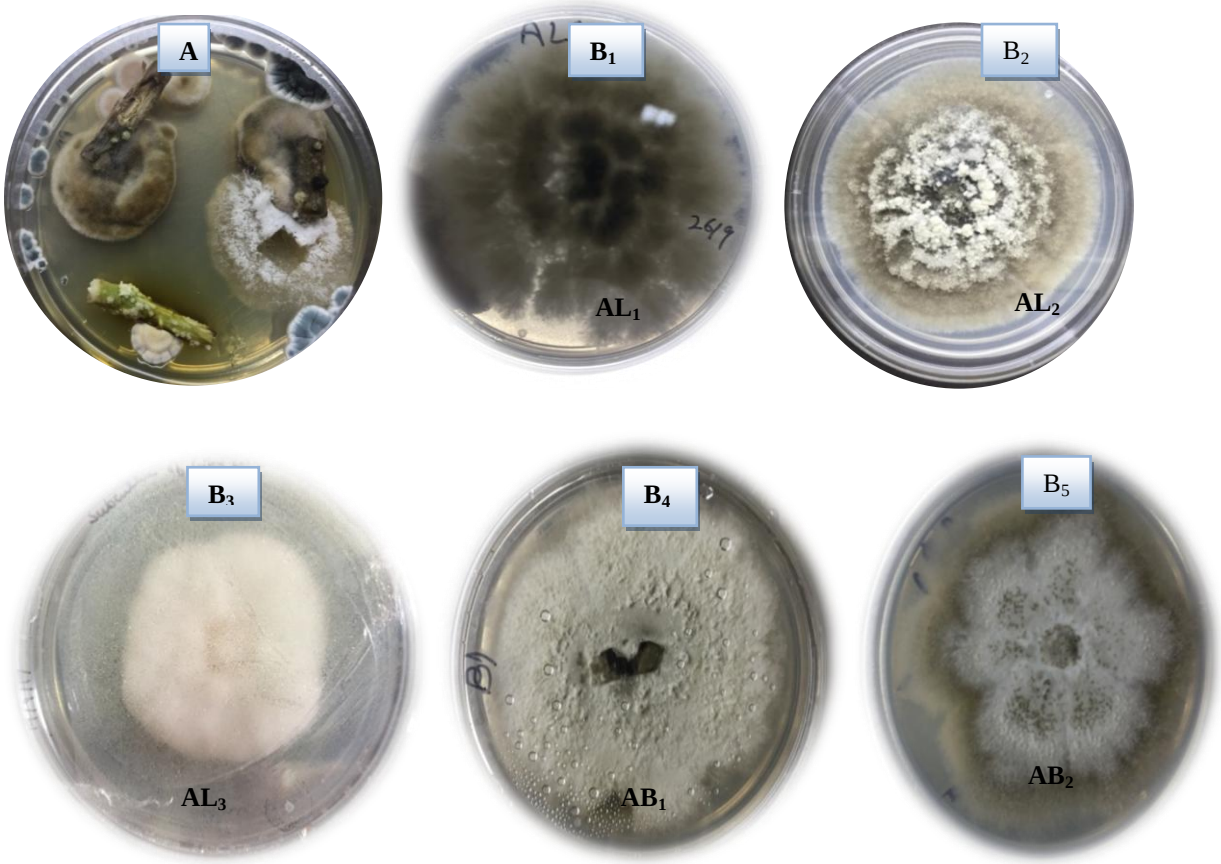


Fig 5: Surface sterilization of tissues, (A)Ethanol washing of tissues, (B) Culturing of sterilized tissues on PDA plates

The purification of the fungal endophytes from a mixed population of fungal endophytes was done so as to get the pure strains (Fig 6). It was done by using sterile blade and inoculum was taken from master plate and placed onto the potato dextrose agar medium. The plates were incubated for 3- 10 days at 27±2˚C. After the growth of the isolated endophytic fungi, the master cultures were given respective codes and preserved at 4˚C for further studies.



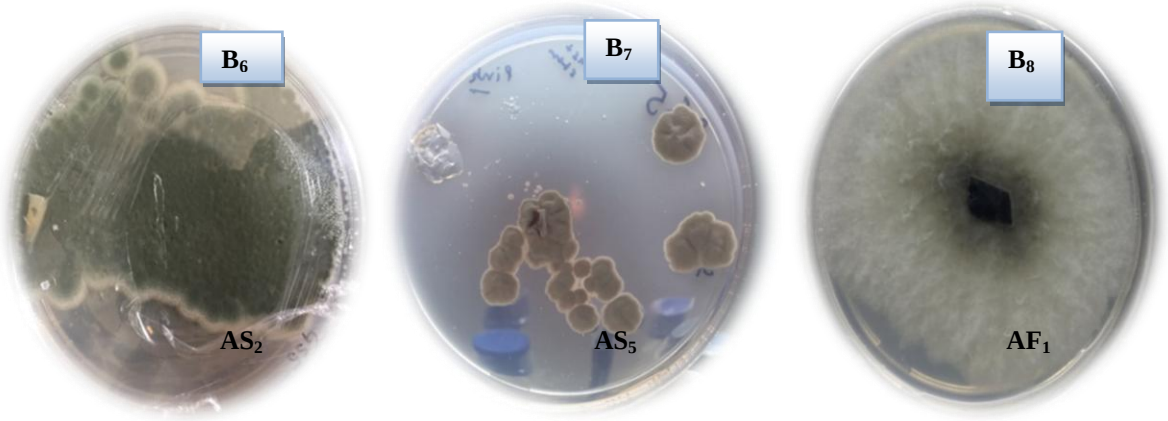
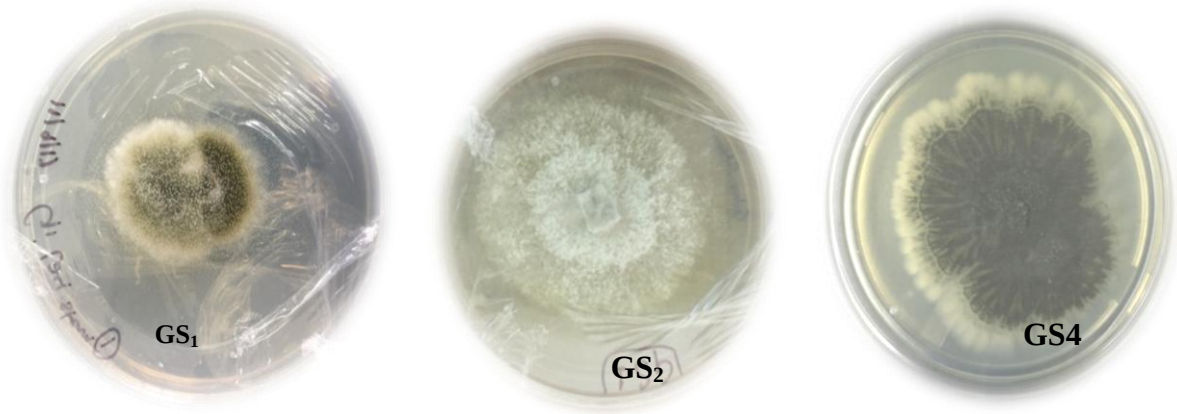


Fig 6: Purification of mixed population of endophytic fungi followed by subculturing for the purification of fungus, (A) Mixed population from stem of *T. arjuna*, (B_1-B_8). Different isolates obtained from *T. arjuna*.



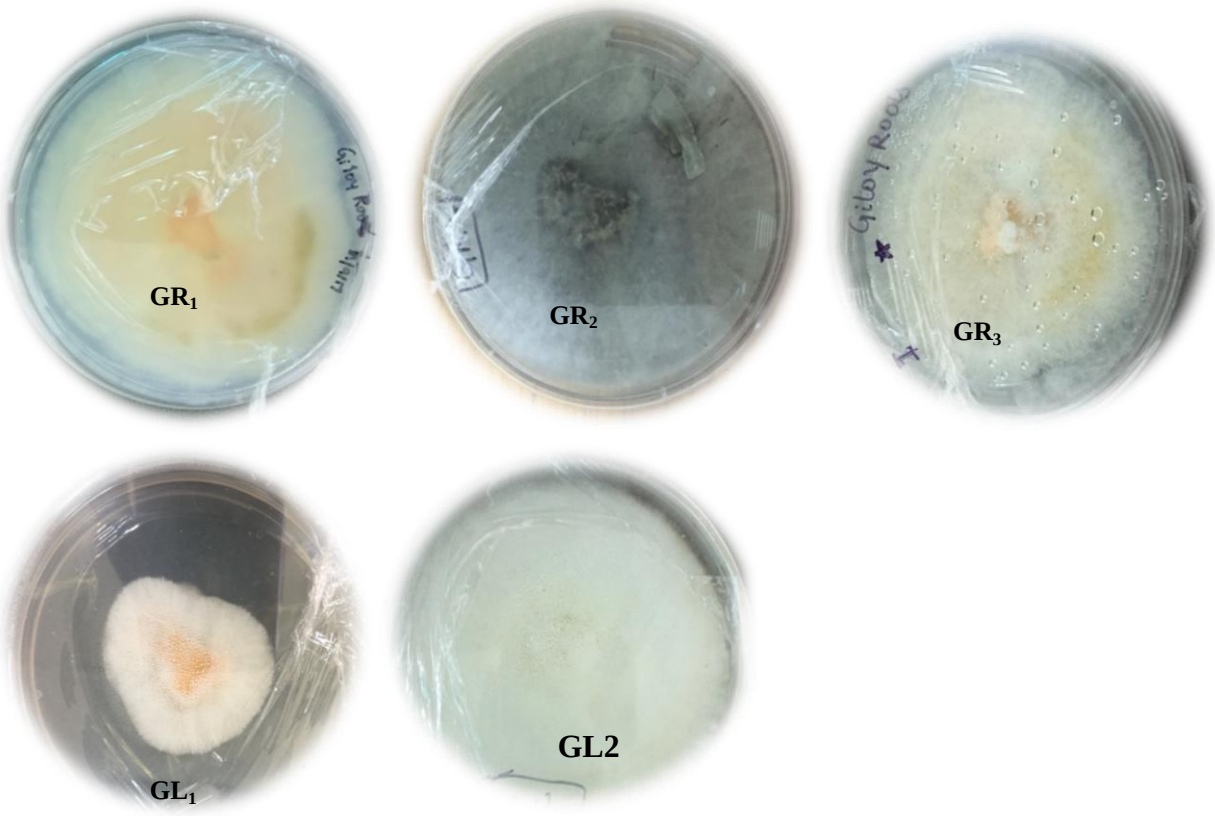


Fig 7: Different fungal isolates obtained from *Tinospora cordifolia*.

4.3 Production of secondary metabolites by fermentation

The isolated endophytic fungi from *T. arjuna* and *T. cordifolia* were inoculated in the PDB for fermentation. This was incubated at 27±2˚C for 21 days under stationary conditions.

4.4 Extraction of the crude extract from the broth

After 21 days of incubation, the broth was separated from the mycelia and extraction was done with ethyl acetate to obtain the organic phase containing metabolites. This was further dried to obtain the fungal crude extract (Fig. 8). The crude fungal extracts so obtained were subjected to preliminary bioactive studies.

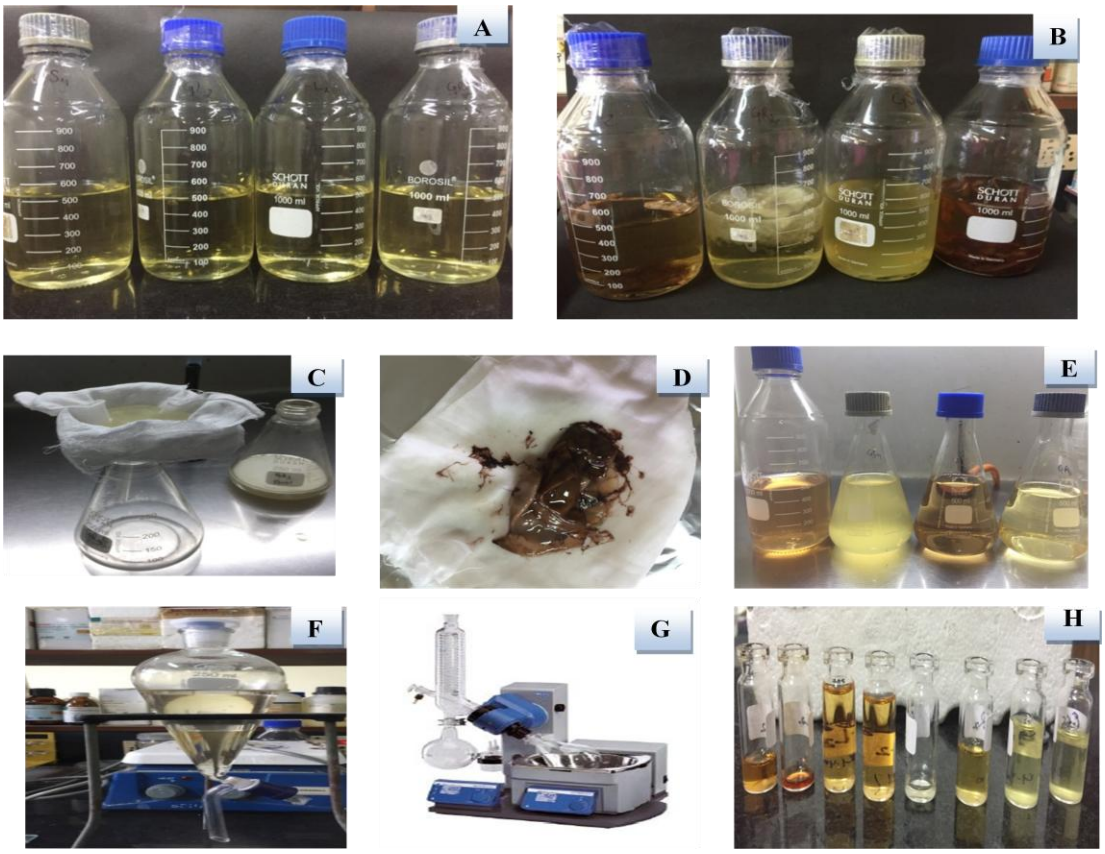


Fig 8: Extraction of the crude extract after fermentation, (A) Broth having inoculum of different fungus before fermentation, (B) Production of metabolites after 21 days, (C) Filtration of broth, (D) Mycelia after filtration, (E) Collection of broth after filtration, (F) Extraction of broth with organic solvent in separating funnel, (G) Evaporation of organic solvent using rotaevaporator, (H) Collection of crude extract.

4.4.1 Dry weight of mycelia: The discs of fresh mycelia from PDA plates were transferred to 500 ml Erlenmeyer flask containing 250 ml of the PDB. The cultured flasks were incubated for 21 days at 27±2˚C under stationary conditions. After the incubation period of 21 days, the culture was filtered with sterile muslin cloth to separate the mycelia from the broth. The sterile muslin cloth containing mycelia was dried in an oven till constant weight was achieved. Dry weight of mycelia was determined for all the isolated fungi (Table 13).

Table 13:The dried mycelial weight of isolated fungi

S. No	Extracts	Dried mycelial weight (mg)
1	AL1	250
2	AL2	210

3	AL3	190
4	AL4	220
5	AB1	240
6	AB2	360
7	AF1	380
8	AF2	370
9	AF3	290
10	AF4	240
11	AS1	310
12	AS2	500
13	AS5 Red	360
14	AS5 White	375
15	GL1	290
16	GL2	450
17	GR1	310
18	GR2	280
19	GS1	310
20	GS2	430
21	GS4	370

4.5 Preliminary bioactivities for screening of the crude extracts

4.5.1Antimicrobial activity of the crude fungal extracts

The activities of the crude fungal extracts were tested on test microorganisms (bacterial and fungal) by agar well diffusion method. The zone of inhibition (clear zone where bacteria did not grow) formed was measured in millimeter (mm). Fourteen crude fungal extracts of *Terminalia arjuna*, seven crude fungal extracts of *T. cordifolia*fungi were tested for preliminary antimicrobial activity with the bacterial test cultures of (*E.coli*,*P.aeruginosa*,*S.aureus*, *B.megaterium*) and fungal test culture (*C. albicans*). Comparing the activity of crude extracts against these test cultures 7 isolates (GL2, GS4, GR_2, AL2, AF2, AS_5 Red and AS_5 white)showed maximum zone of inhibition. Hence thesewere further selected for further studies.

4.5.2 Antioxidant activity of the crude fungal extracts

A total of twenty one crude extracts were screened for their antioxidant activity. Out of 21 extracts, 14 were extracted from *Terminalia arjuna* and 7 from *T. cordifolia*. Comparing the % scavenging activityof all extracts, 7 isolates (GL2, GS4, GR_2, AL2, AF2, AS5 Red and AS5white) were selected for further studies.

4.6Bioactivities of the selected isolates

4.6.1 Antimicrobial activity of selected isolates

4.6.1.1 Agar disc diffusion method: The activities of the crude fungal extracts were tested on test microorganisms (bacterial and fungal) by agar disc diffusion method and MTT assay. The zone of inhibition (clear zone where bacteria did not grow) formed was measured in millimeter (mm). Seven crude fungal extracts, four crude extracts of *T. arjuna* and three crude extracts of *T. cordifolia*were tested for antimicrobial activity with the bacterial test cultures and fungal test culture. On the basis of the activity of the crude extracts against these micro-organisms further selection was done (Table 14).

Table 14: Antimicrobial activity (zone of inhibition-mm) of screened crude fungal extract

Crude extracts	**Test Cultures (bacterial and fungal)**				
	***E. coli* (EC)**	***P. aeruginosa* (PA)**	***S. aureus* (SA)**	***B. megaterium* (BM)**	***C.albicans* (CA)**
AL2	19	0	9	11	12
AF2	7	10	0	7	-
AS5 Red	0	0	0	9	-
AS5 White	10	0	0	0	-
GL2	0	7	8	0	0
GS4	14	8	0	13	0
GR2	13	0	7	10	0
Ampicillin (50µg/ml)	30	23	33	41	-
Methanol	0	0	0	0	-

Amphotericin(1µg/ml)	-	-	-	-	25

The isolated endophytic crude extract AL2showed zone of inhibition ranging from 9-19 mm against *E. coli, B. megaterium, S. aureus*. AL2was the only extract which showed 12 mm zone of inhibition against *C. albicans*. The isolated endophytic crude extract GS4 showed zone of inhibition ranging from 8-14 mm against*E. coli,P.aeruginosa, B. megaterium*. Fungal crude extract GR2showed zone of inhibition ranging from 7-14 mm against *E. coli, S. aureus* and*B. megaterium* and the isolated crude extract GL2showed zone of inhibition only against *P.aeruginosa*and *S.aureus* ranging from 7-8 mm. From the above table it maybe concluded that the maximum zone of inhibition (19mm) was shown by AL2 extract followed by GS4 showing inhibition zone of 14 mm against *E.coli.* AL2 also showed 12 mm zone of inhibition against *C. albicans*(Fig 10).

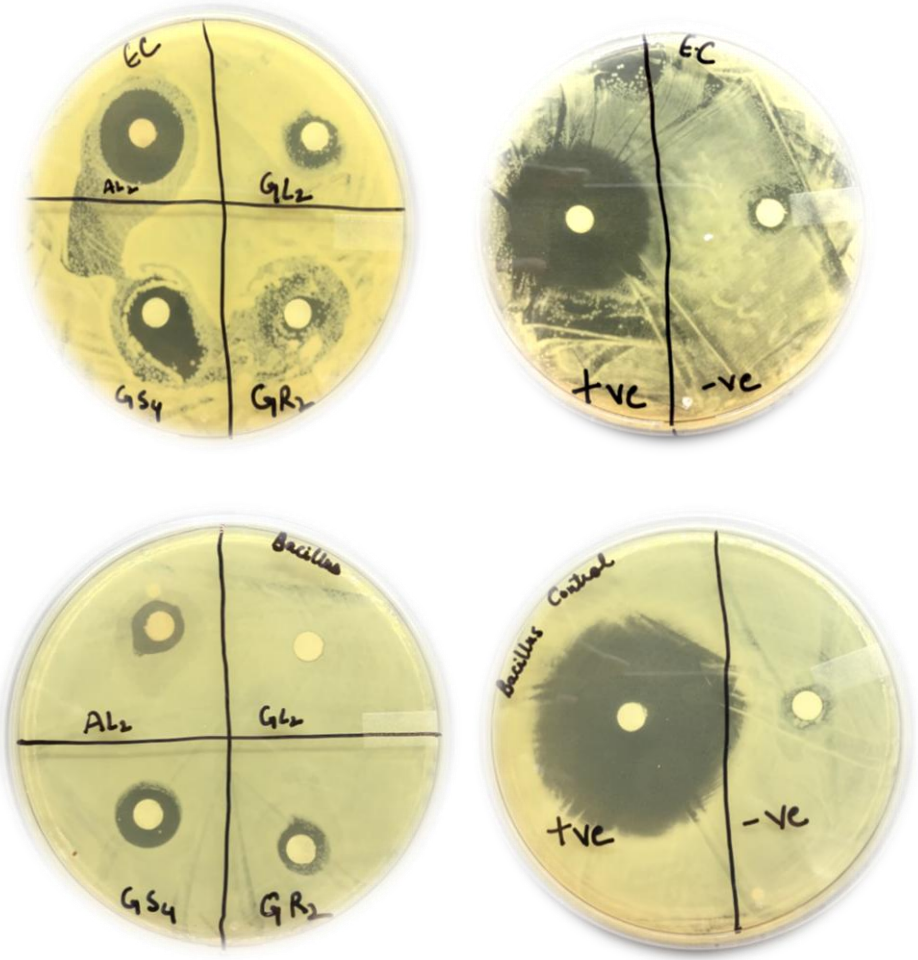


Fig 9: Antibacterial activity of fungal crude extracts against test cultures. The positive control is ampicillin and negative is methanol.

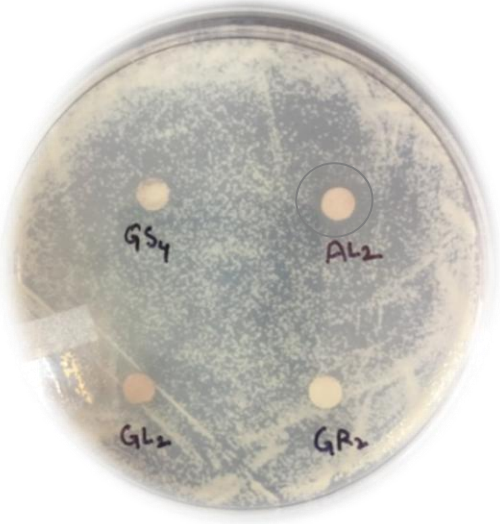


Fig 10: Antifungal activity of fungal crude extracts. AL2 shows maximum activity by showing 12 mm zone of inhibition.

4.6.1.2 Antimicrobial activity by MTT assay: The antimicrobial activity was determined by using a color indicator, MTT (yellow color) which was reduced to formazan (purple color) by living cells. In the screening of antimicrobial compounds microplate method provides a potentially useful technique for determining antimicrobial activity of number of test samples. The antimicrobial activity of 7 testsamples(GL2, GS4, GR_2, AL2, AF2, AS5 Red and AS5 white) atdifferent concentrations (100µg, 250µg, 500µg and 1000µg)was tested against bacterial test cultures of (*E.coli*,*P.aeruginosa*,*S.aureus*, *B.megaterium*).

4.6.1.2.1 Antibacterial activity against *P.aeruginosa*

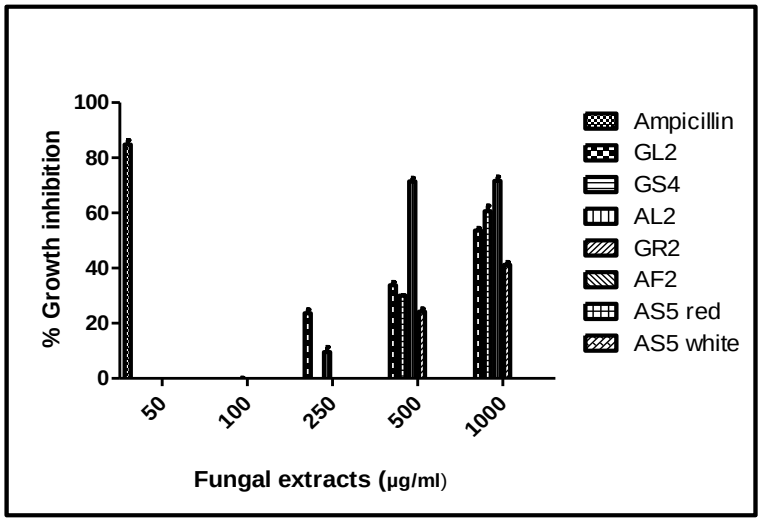


Fig 11: Antibacterial activity of fungal extracts against *P.aeruginosa*

From the above (Fig 11) it may be concluded that none of the samples show growth inhibition against *P. aeruginosa* at 100µg/ml concentration whereas control antibiotic ampicillin exhibits 84.71% growth inhibition at 50µg/ml concentration. GL2 sample shows 23% inhibitory activity at 250µg/ml and shows maximum inhibition of 53.60% at 1000µg/ml of concentration. AL2 shows minimum inhibition of 9.23% at 250µg/ml concentration whereas shows maximum inhibition of 71.66% at 1000µg/ml of concentration. Fungal crude extract GS4 inhibits 30% of the cell growth at 500µg/ml and shows maximum inhibition of 60% at 1000µg/ml. Other samples AF2, AS5 red, AS5 white do not show any inhibition against *P. aeruginosa* cells. From above results it may be concluded that of all the samples AL2 shows maximum inhibitory activity of 71.66%followed by GS4 which shows 60% inhibition at 1000 µg/ml.

4.6.1.2.2Antibacterial activity of crude extracts against *B. megaterium*:

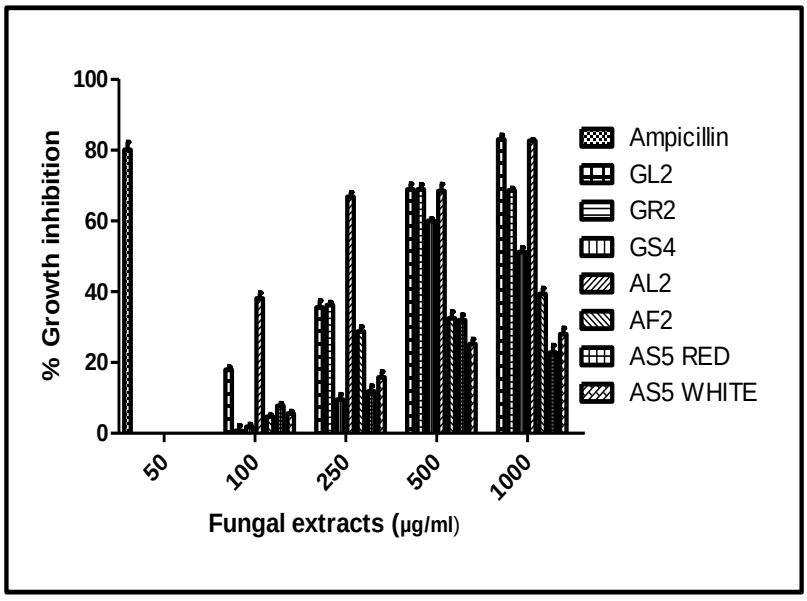


Fig 12: Antibacterial activity of fungal extracts against *B. megaterium*

From the above(Fig 12) it is observed that GL2 shows minimum inhibition of 17.95% at 100µg/ml of concentrationand maximum of 82.94 % at 1000µg/ml of concentration The fungal extract GR2 inhibits 68.58% of cell growth at 1000µg/ml concentration whereas shows minimum of 0.7% inhibition at 100µg/ml of concentration. Crude extract GS4 shows maximum activity at 500µg/ml sample concentration by inhibiting 59.96 %of cells. Fungal crude extract AL2 inhibits 38% of cell growth at100µg/ml sample concentration and 82.32% of cells at

1000µg/ml concentration.AF2, AS5 red, AS5 white also shows inhibitory activity against*B. megaterium.*Amongst these, maximum activity was seen in AF2with 39.8% of inhibition followed by AS5 white showing 28% inhibitory activity.

4.6.1.2.3 Antibacterial activity of fungal extracts against *E.coli*

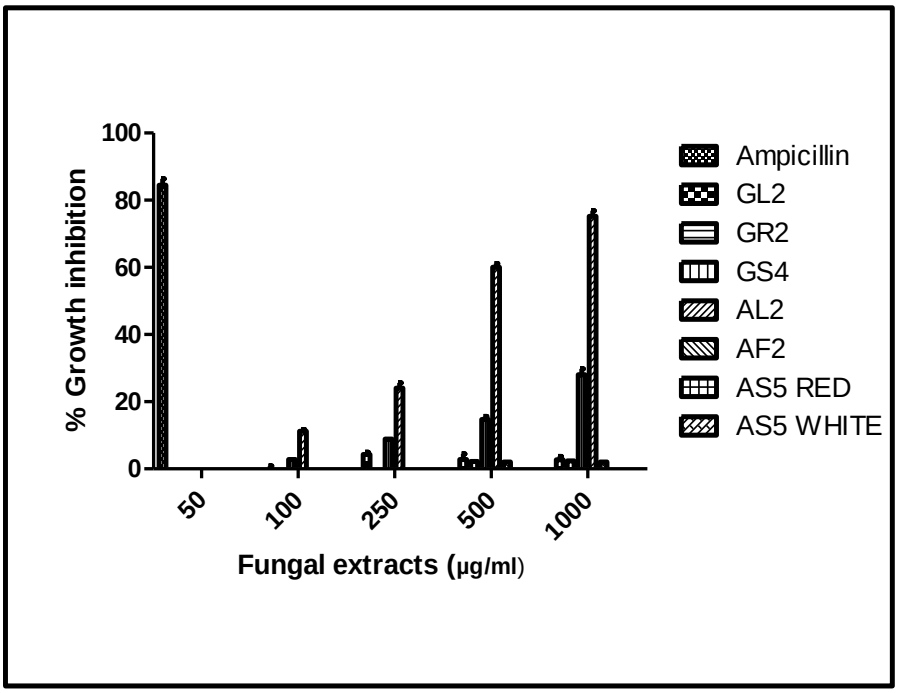


Fig 13: Antibacterial activity of fungal extracts against *E. coli*

AL2 and GS4 are the only samples which showed prominent activity against *E.coli*, AL2 shows maximum inhibition of 75% at 1000µg/ml sample concentration whereas GS4 shows 28% of inhibition at same sample concentration (Fig 13). AL2 and GS4 showed maximum zone of inhibition when tested by agar disc diffusion method.

4.6.1.2.4 Antibacterial activity of fungal extracts against *S. aureus*

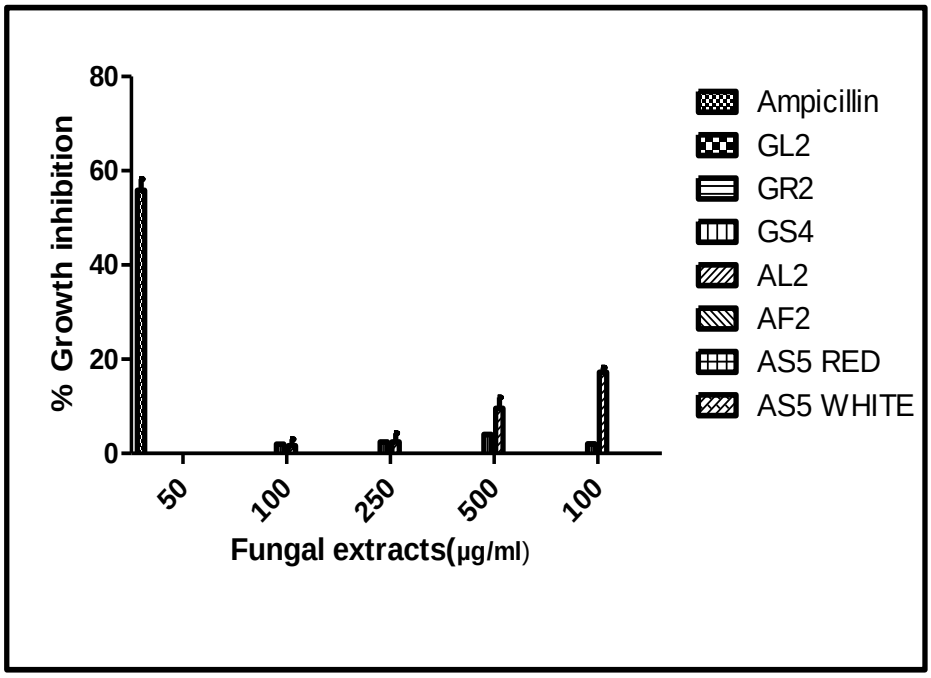


Fig 14: Antibacterial activity of fungal extracts against *S. aureus*

Ampicillin (50µg/ml) inhibits 52% of cells, whereas only two fungal extracts AL2 and GS4 shows inhibition against *S. aureus* (Fig 14). AL2 shows maximum inhibition of 17.25% at 1000µg/ml concentration.

Cui *et al*., 2011isolated endophytic fungi from *Aquilaria sinesis*, a medicinal plantwhich produces fragrant compounds. They reported that out of 28 isolates obtained 13 exhibited antibacterial activity against test strains when agar well diffusion assay was done.

4.6.2 DPPH scavenging Activity:7 different crude fungal extracts at different concentrations (100µg, 250µg, 500µg and 1000µg)were tested for their potential to scavenge free radicals (Fig 15). Ascorbic acid was used as positive control and methanol as a negative control. Ascorbic acid (100 µg/ml)shows 77.5 % radical scavenging activity.

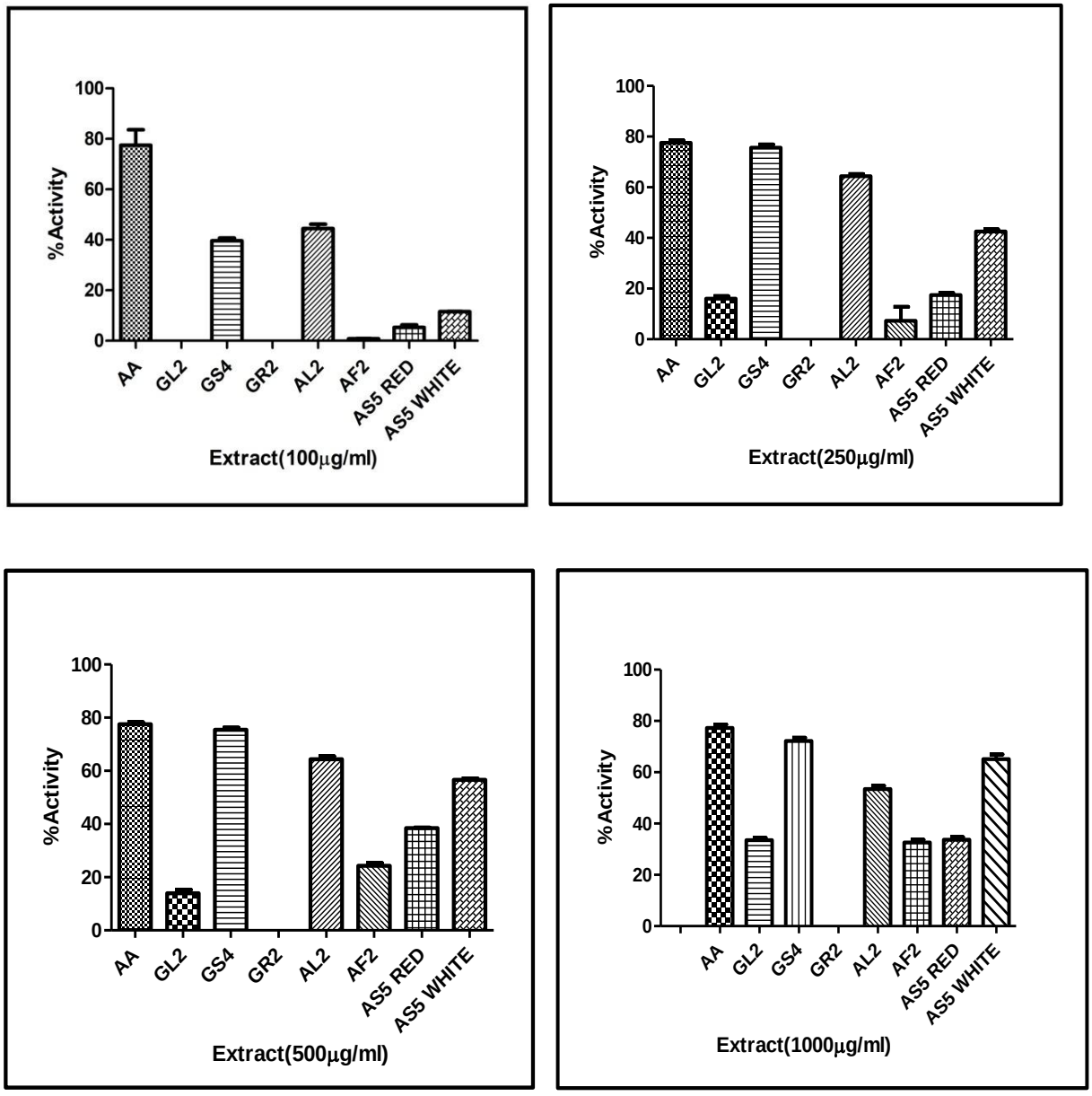


Fig 15: Percentagescavenging activity of fungal crude extracts at different concentrations

From the above (Fig 15) results it is concluded that GS4 showed maximum radical scavenging activity of 75.65% at 250µg/ml concentration followed by AL2 which shows maximum radical scavenging activity of 64.34% at 500µg/ml concentration, and minimum 44.21% at 100µg/ml concentration. The radical scavenging activity of AL2 and GS4 at different concentrations is as given in (Fig 16).

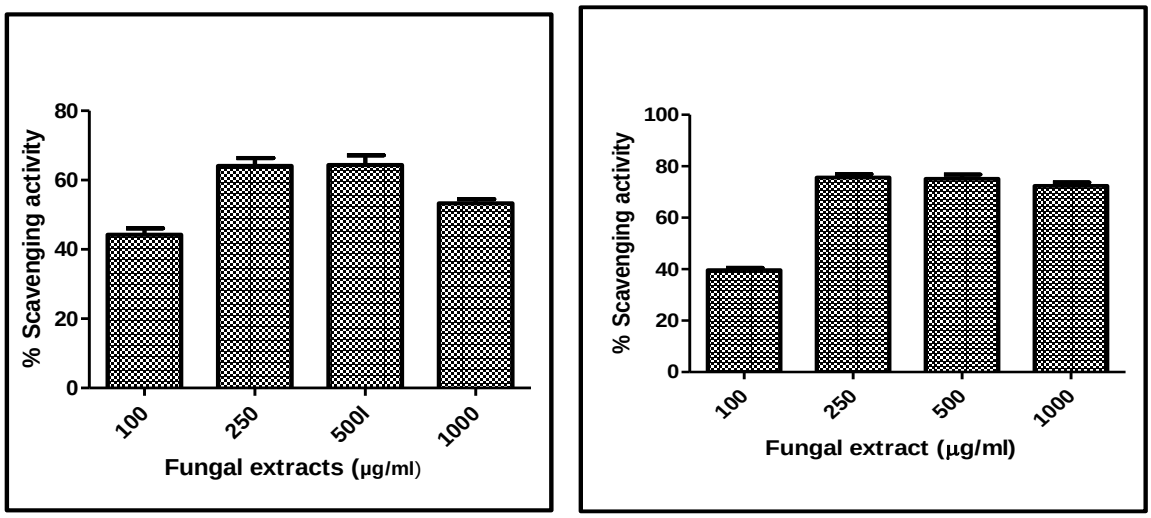


Fig 16: Percentagescavenging activity of AL2 and GS4 at different concentrations

Fungal crude extract AL2and GS4 showed a broad spectrum of activity against test microorganisms with considerably good inhibition against all the test cultures. They also showed prominent antioxidant activity hence, these were selected for further studies.

Phongpaichit *et al*., 2007 studied endophytic fungi isolated from *Garcinia* plants and reported that 22.2% of the isolates exhibited radical scavenging activity.

4.7 Phytochemical analysis of crude extracts

Table 15: Preliminary phytochemical analysis for fungalcrude extracts

S. no	Tests	AL2	GS4
1	Test for Tannins	-	-
2	Test for phenolic compounds	-	++
3	Test for carbohydrates	-	+++
4	Test for alkaloids	+++	-
5	Test for fats and fixed oils	-	+++
6	Test for steroids	-	+++
7	Test for triterpenoids	+++	-
8	Test for Glycosides	-	-

(-) Negative test, (+) Positive test

Extract AL2 shows the presence of alkaloids and triterpenoidswhereas it showed absence of steroids, glycosides, fats and oils. The presence of alkaloids is confirmed by the precipitation of reddish brown color precipitation at the bottom of test tube after addition of Wagner's reagent (Fig17B). During Salkowoski's test yellow colored lower layer indicates presence of triterpenoids (Fig 17A)

Crude extract GS4 indicates the presence of phenols, carbohydrates, alkaloid and steroids whereas tannins, amino acid, glycosides were absent in the extract. The presence of phenol was confirmed by the development of green color when ferric chloride was added to the extract (Fig 17D) Purple to violet color at junction after addition of Molisch's reagentindicates the presence of carbohydrates(Fig 17C).Whereas presence of steroids is confirmed by formation of red color during Salkowoski's test (Fig 17A).

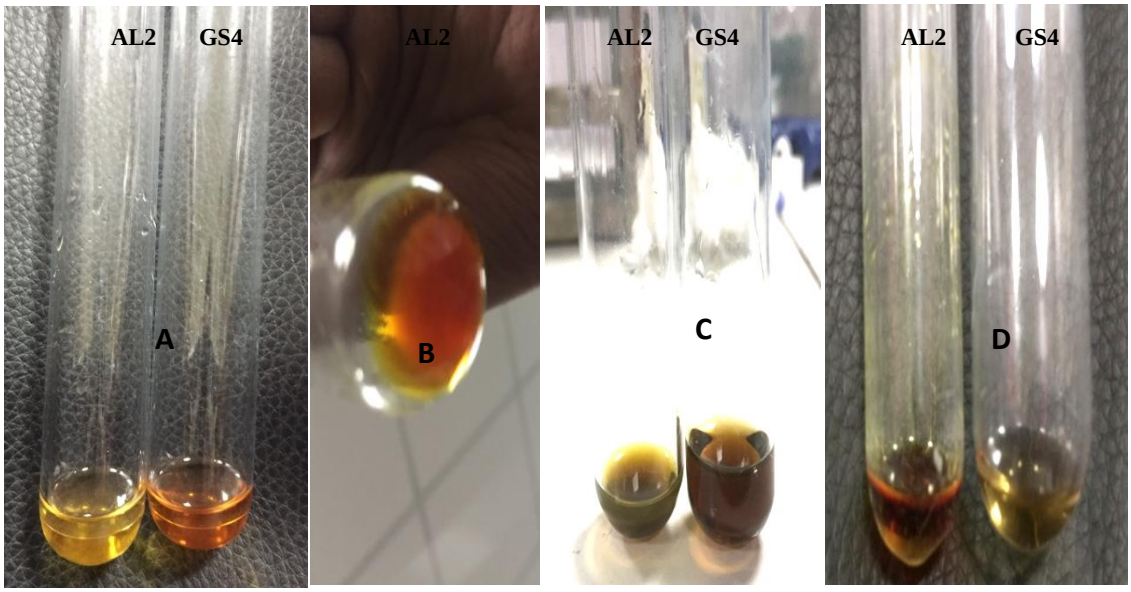


Fig 17: Phytochemical analysis of AL2 and GS4 crude extract.

(A) Yellow color at lower layer indicates the presence of triterpenoid in AL2, formation of red color layer indicates the presence of steroid in GS4.
(B) Reddish brown ppt indicates the presence of alkaloid in AL2.
(C) Purple to violet junction in test tube of GS4 indicates the presence of carbohydrates.
(D) Green color in GS4 indicates the presence of phenols.

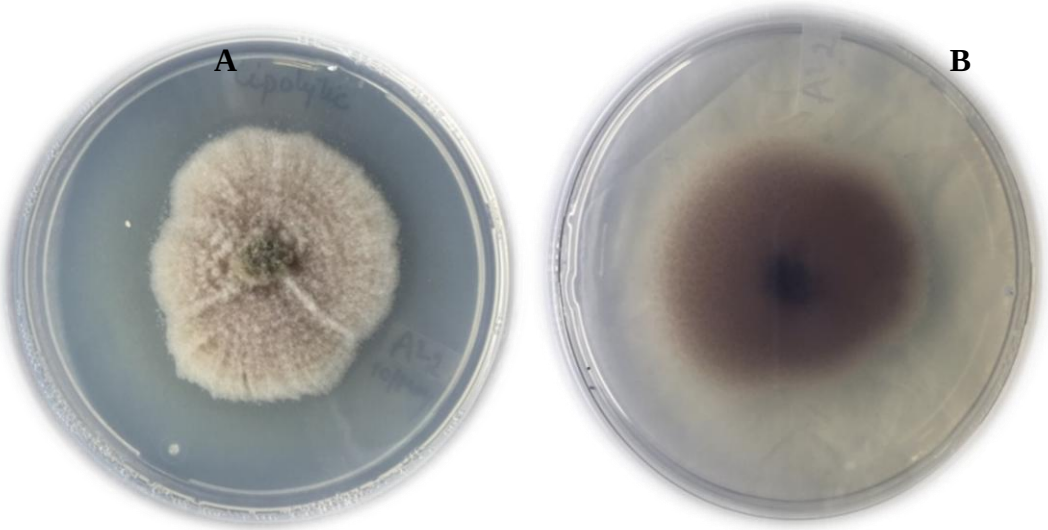
4.8 Extracellular enzymatic activities of selected fungal isolates AL2 and GS4

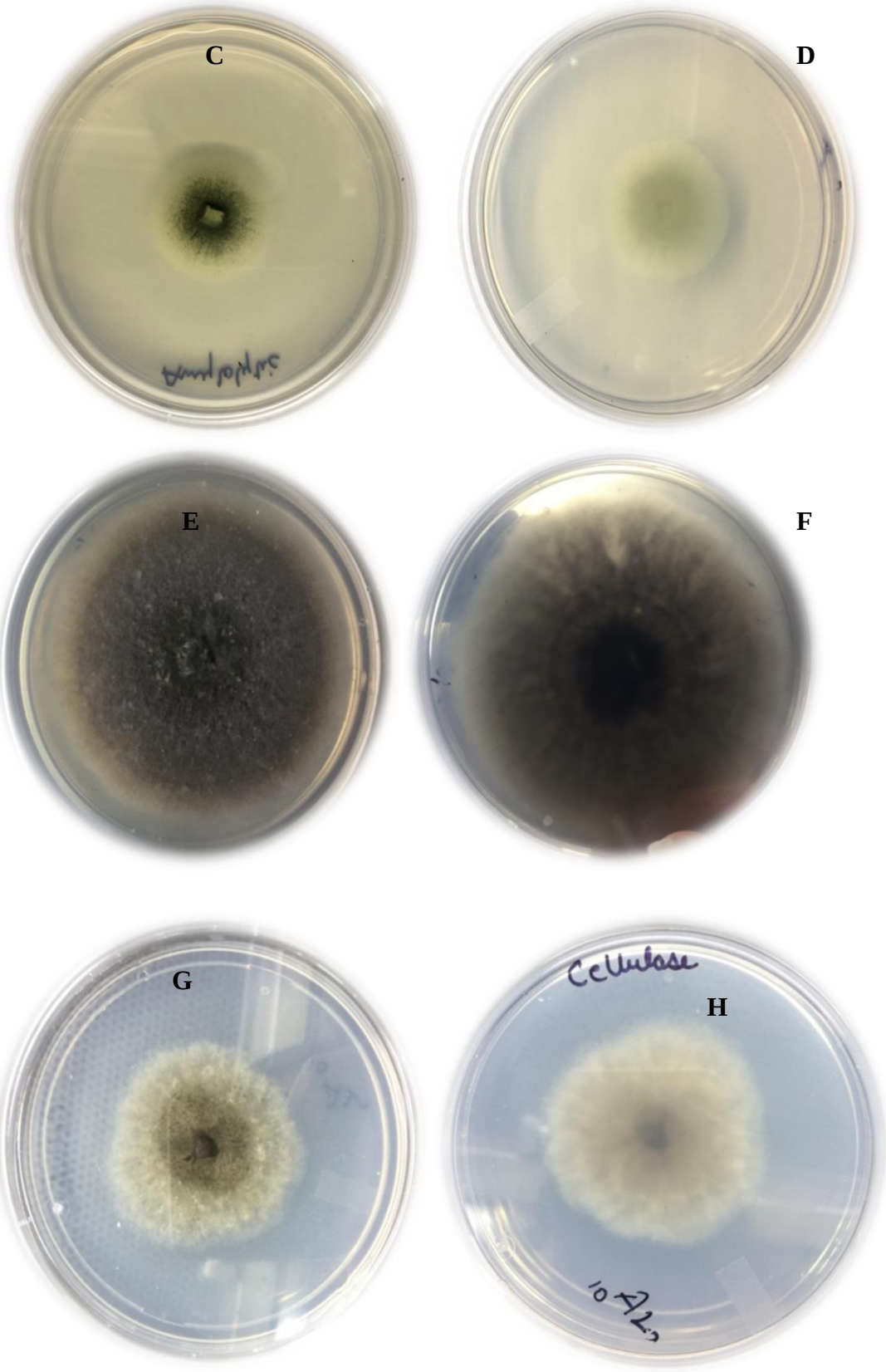
The isolated endophytic fungi AL2 and GS4 were subcultured regularly and maintained in the lab. Both isolates were tested to check whether they were able to produce one or the more of extracellularenzymes.Both of them were screened for production of enzymes amylase, lipase, protease and laccase.

Table 16: Extracellular enzymatic activities of selected fungal isolates AL2 and GS4

S.No	Enzyme	AL2	GS4
1	Amylase	-	-
2	Lipase	+	+
3	Protease	+	+
4	Laccase	+	-

(-) Absence of enzyme activity, (+) Presence of enzyme activity

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F
G
Cellulose
H

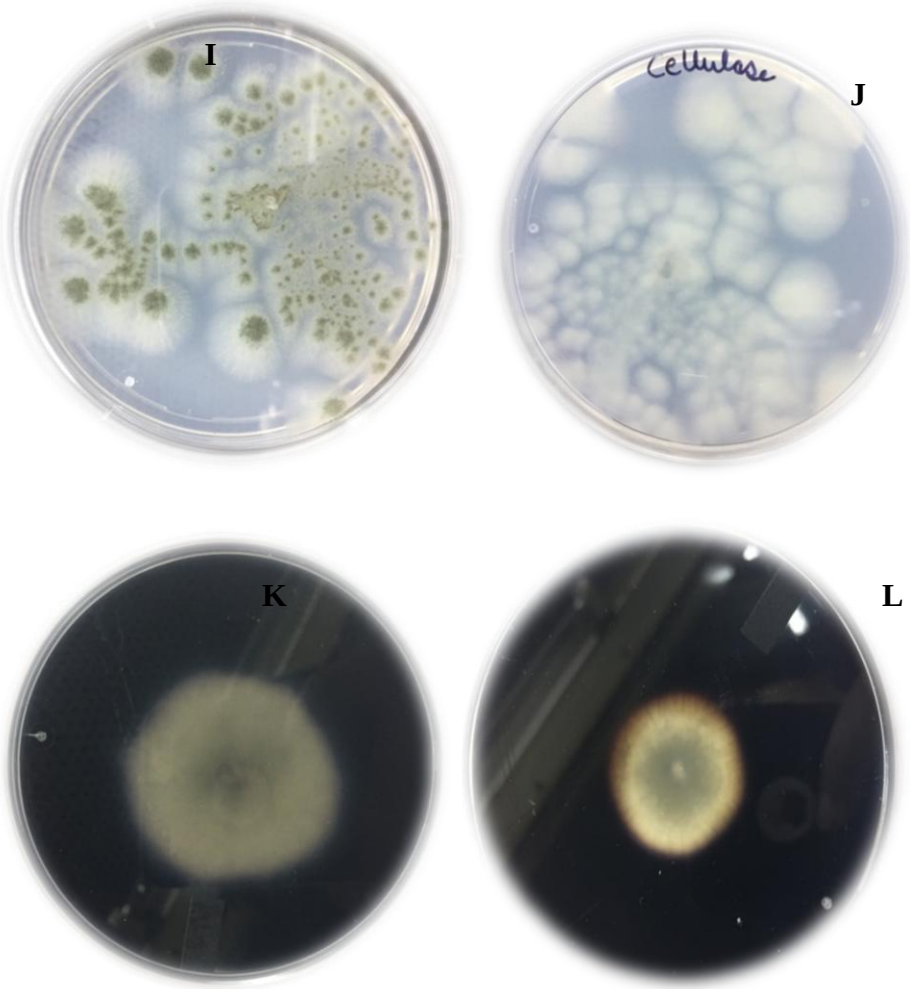


Fig 18: Extracellular enzyme activity by fungal endophytes AL2 and GS4

Fig 18 (A and B) is the front and reverse view ofAL2 culture grown on peptone agar medium. After incubation visible precipitationzone was seen around the colony which indicates the presence of lipase. Front and reverse view of fungal isolate GS4 (Fig 18C, D) on Peptone agar medium also showed visible precipitate around the mycelium and indicates positive response for lipase production. When AL2culture isolate was grown on glucose yeast extract peptone agar, as the fungus grows, because of presence of laccase it turns colorless media to blue color by carrying oxidation of 1-napthol indicating presence of laccase(Fig 18 E, F). Protease activity was confirmed after incubation of glucose yeast extract agar peptone with 0.4 % gelatin agar plateswhich were further flooded with ammonium sulphate that results in opaque media formation around colony indicating presence of protease (Fig 18 G, H) and (Fig 18 I, J). Protease

activity was observed in both cultures. Amylase are negative in both cultures (Fig 18 K, L) AL2 and GS4.

4.9 Identification of endophytic fungi

4.9.1 Identification based on morphology:

The slides of fungi were prepared with lactophenol dye and observed under the microscope. Under the microscope, AL2 showed presence of long chain, branched conidiophores. The surface of fungus on PDA plate was whitish brown and shows velvety growth Fig 19(A); this kind of morphological characteristics are quite similar to *Alternaria spp*. After visualizing the surface of the isolated fungi GS4 on the PDA plate it was observed that it is powdery and green.Microscopical examination of the isolates shows many-branched conidiophores sprouting on the mycelia (Fig20 C, D). These all characteristic featuresare quite similar to *Penicillium sp*.

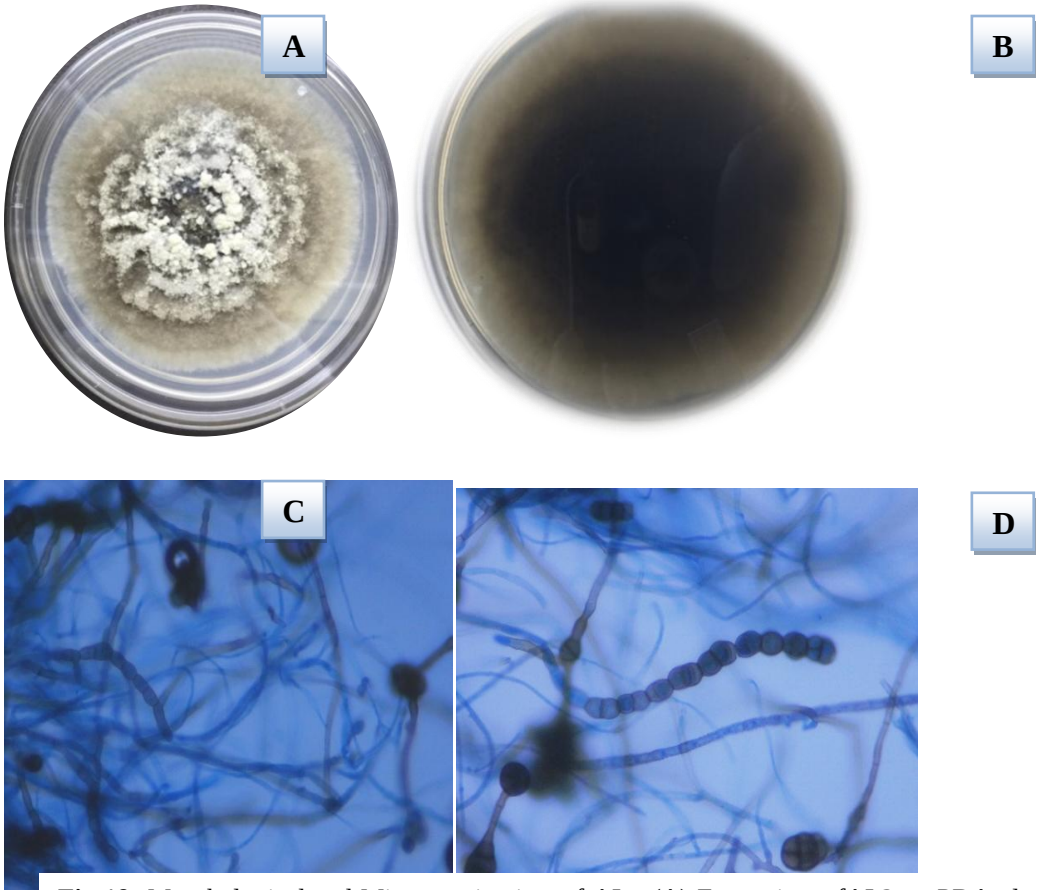


Fig 19: Morphological and Microscopic view of AL_2, (A) Front view ofAL2 on PDA plate, (B) Back view of AL2 on PDA plate, (C) Hyphae of AL2 under 40X microscope, (D) Conidiophores under 40X microscope.

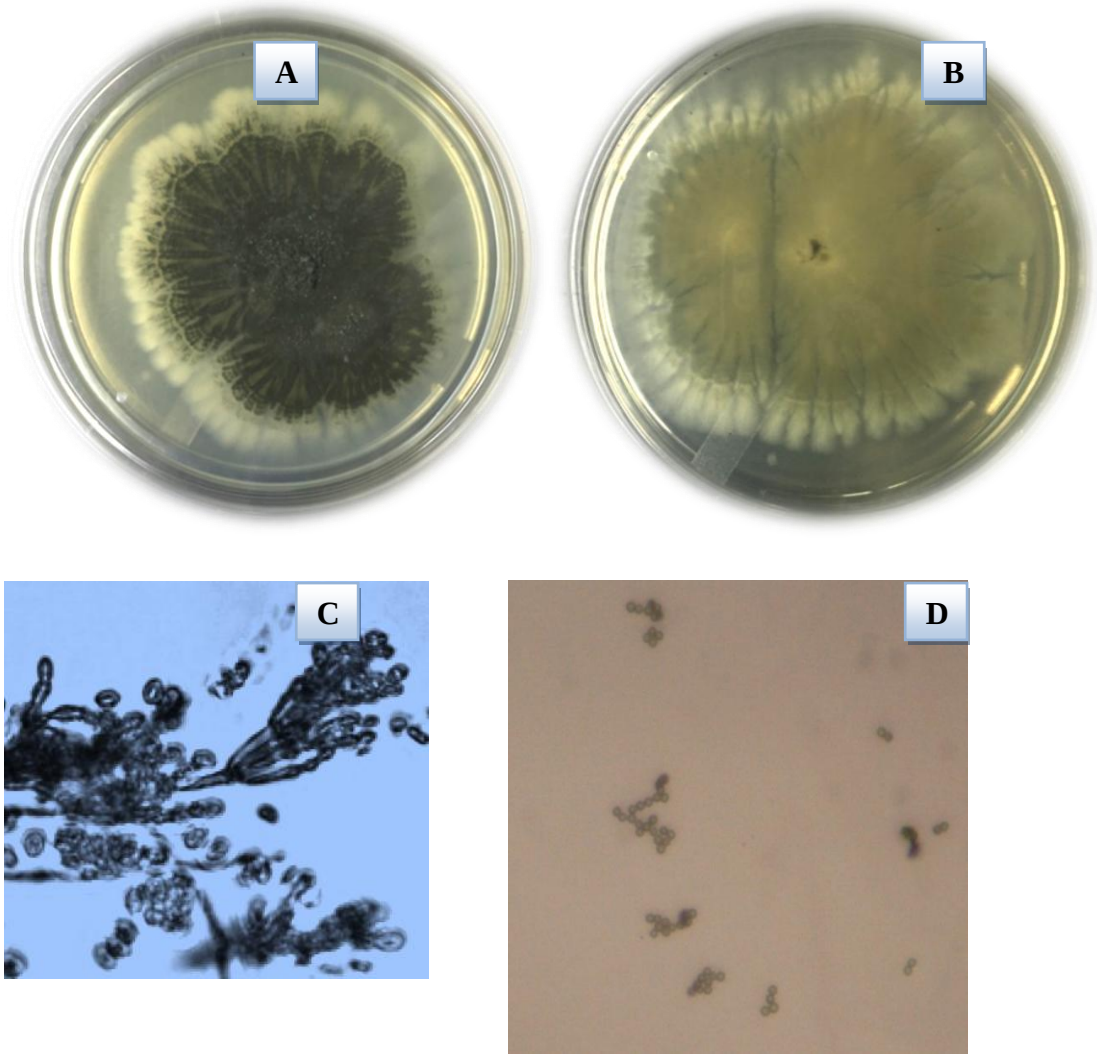


Fig 20: Morphological and microscopic view of GS4, (A) Front view of GS4 on PDA plate, (B) Back view of GS4 on PDA plate, (C) Hyphae of GS4 under 40X microscope, (D) Spores under 40X microscope.

DNA of AL2 fungal isolate from *T. arjuna* was selected for further molecular identification as it exhibited maximum bioactivity as compared to other fungal isolates.

4.9.2 Molecular based identification: Genomic DNA was isolated from endophytic fungi AL2 obtained from *T. arjuna*(Fig 21A).Isolated DNA was PCR amplified with the ITS1 and ITS4 primers (Fig 21B). Transformation of ligated product was done in competent cells of DH5α followed by the screening by IPTG and Xgal (blue white screening) (Fig 22). The positive transformants were sequenced from DNA sequencing facility at UDSC, University of Delhi south campus, New Delhi.

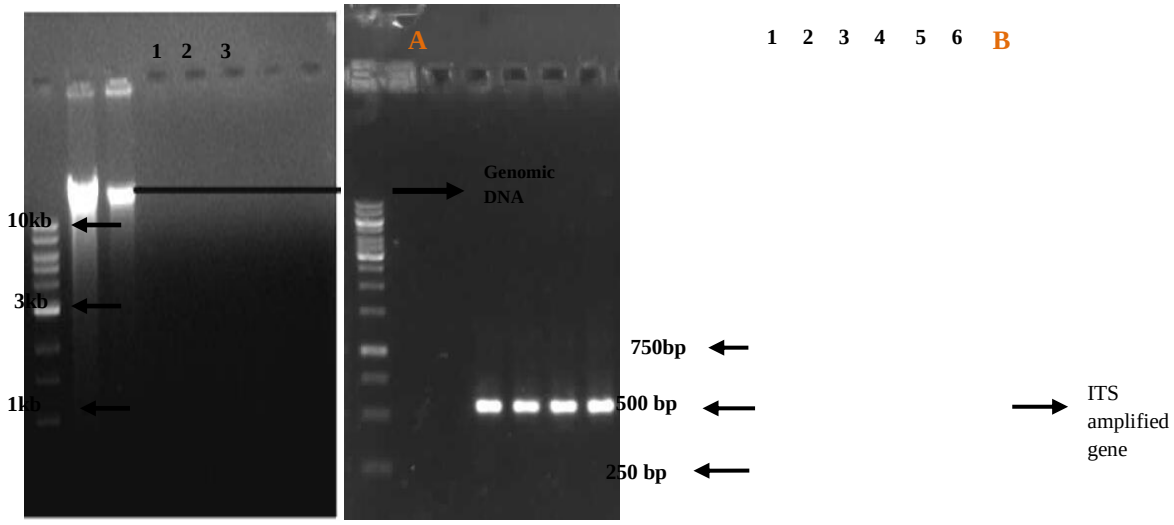


Fig 21: (A) Genomic DNA isolation from AL2 fungal isolate of *Terminalia arjuna*. (B) Amplification of ITS region of AL2using ITS1 and ITS4 primers.

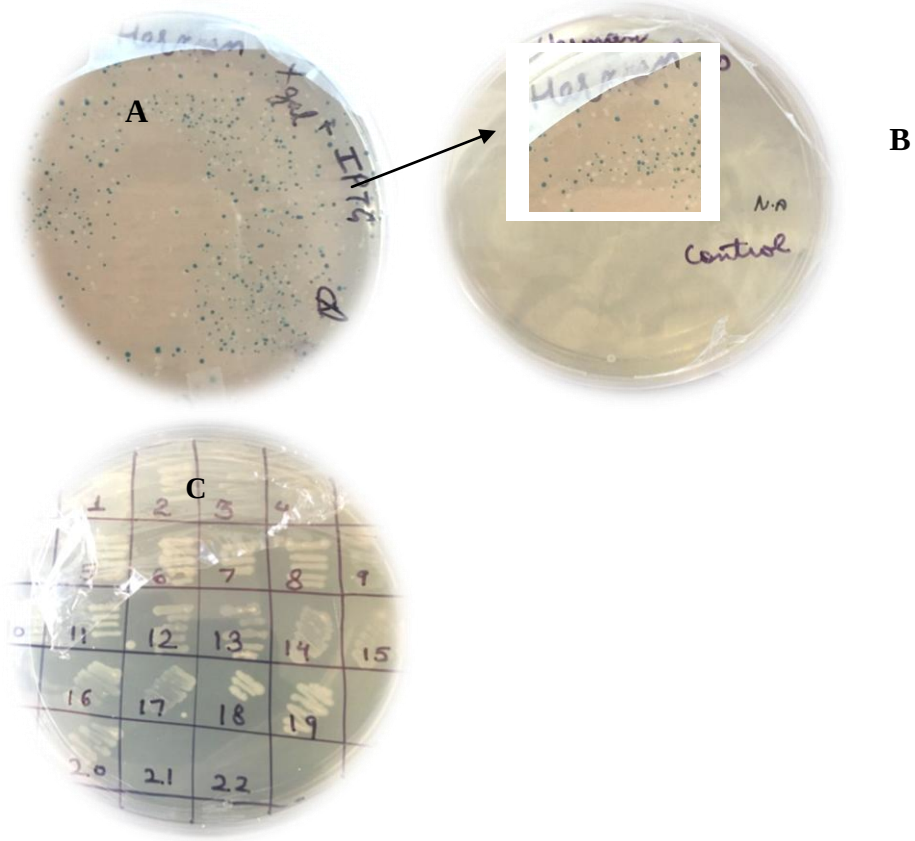


Fig 22: Blue white screening of transformants

(A) Blue white colonies were observed on plate, where white colonies indicates the positive transformants

(B) –ve control where no colonies were observed.

(C) Patches of positive transformants were made on ampicillin luria agar plate.

4.9.2.1 Bioinformatics analysis for identification of AL2: The ITS amplified sequence of 531bp (Fig23) obtained was run through BLASTn (Fig24) and the homologous sequences of different strains were recorded in FASTA format. Different sequences obtained in FASTA format were aligned using multiple alignment tool Multalign (Fig 25) and the alignment was analyzed. The multiple sequence alignment show conserved regions of ribosomal RNA with various endophytic fungi. Further the evolutionary relationship between homologous sequences was found by maximum parsimony phylogenetic tree constructed by MEGA 7 software with 1000 bootstraps (Fig 27).

ITS1: TTCCGTAGGTGAACCTGCGG
ITS4: GCATATCAATAAGCGGAGGA

Forward sequence:

TTCGAGCTCGGTACCCGGGGATCCGATTTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAATATGAAGGC
GGGCTGGAATCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTT
GGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAA
TTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCT
GTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCG
CCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTC
TAGCATCCATTAAGCCTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATC
AATAAGCGGAGGAAATCCATATGACTAGTAGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTTCCCCTAT
AGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTA
TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGC

Reverse Complimentary sequence:

GGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT
TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTAC
CCGGGGATCCGATTTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAATATGAAGGCGGGCTGGAATCTCT
CGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCA
CCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAAC
AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATT
TGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTG
GCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAG
CCTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA
ATCCATATGACTAGTAGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTTCCCCTATAGTGTCACCTAAAT
AGCTTG

Common sequence among both:

GAGGGATCATTACACAAATATGAAGGCGGGCTGGAATCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCT
TGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTG
CAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGT
CTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCAC
AAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTCAACTTTTGACCTCGGATCAGGTA
GGGATACCCGCTGAACTTAA

Fig 23: Forward and reverse sequences obtained after sequencing. ITS1(Red) and ITS4(Green) region were marked in sequences. Common sequences of 531bp among both sequences were used for BLAST.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Alternaria sp. isolate OLS15 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	981	981	100%	0.0	100%	MF380509.1
Alternaria destruens strain S4S201 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large	981	981	100%	0.0	100%	MH037553.1
Alternaria alternata strain SOK3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large su	981	981	100%	0.0	100%	KY484861.1
Alternaria burnsii strain WBR6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sub	981	981	100%	0.0	100%	KY484860.1
Alternaria burnsii strain DPM35 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484859.1
Alternaria alternata strain DPM27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large s	981	981	100%	0.0	100%	KY484858.1
Alternaria tomato strain DPM26 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484857.1
Alternaria jacinthicola strain WBR4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large	981	981	100%	0.0	100%	KY484856.1
Alternaria burnsii strain SOK9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subu	981	981	100%	0.0	100%	KY484855.1
Alternaria burnsii strain DPM18 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484854.1
Alternaria burnsii strain DPM10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484853.1
Alternaria burnsii strain DPM2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subu	981	981	100%	0.0	100%	KY484852.1
Alternaria tomato strain DPM25 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484851.1
Alternaria alternata strain WBR5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large su	981	981	100%	0.0	100%	KY484850.1
Alternaria burnsii strain DPM15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484849.1
Alternaria burnsii strain DPM7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subu	981	981	100%	0.0	100%	KY484848.1
Alternaria tomato strain DPM22 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484847.1
Alternaria burnsii strain DPM6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subu	981	981	100%	0.0	100%	KY484846.1
Alternaria burnsii strain WDK11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sul	981	981	100%	0.0	100%	KY484845.1
Alternaria burnsii strain WBR2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sub	981	981	100%	0.0	100%	KY484844.1
Alternaria burnsii strain WDK2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large sub	981	981	100%	0		
Alternaria tomato strain WB3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subu	981	981	100%	0		

Fig 24: The ITS amplified sequence obtained was run through BLASTn and the homologous sequences of different strains were recorded in FASTA format.

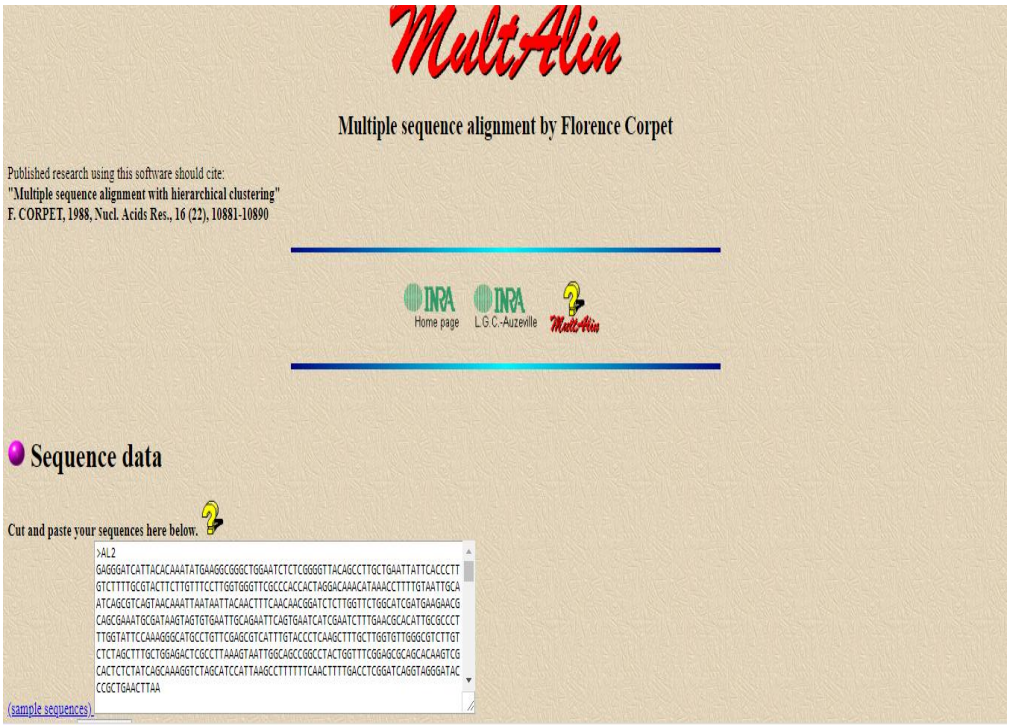


Fig 25: The window of Multalin software used for multiple alignment of sequences.

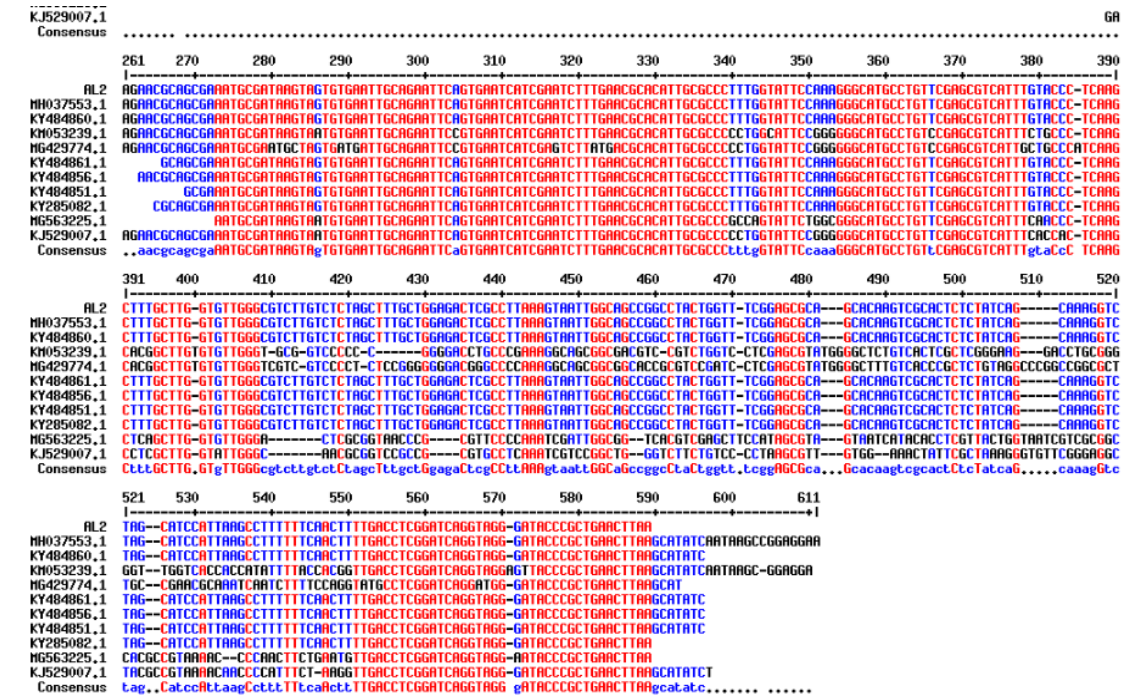


Fig 26: Multiple sequence alignment after using multalin software and the obtained data were saved in fasta format

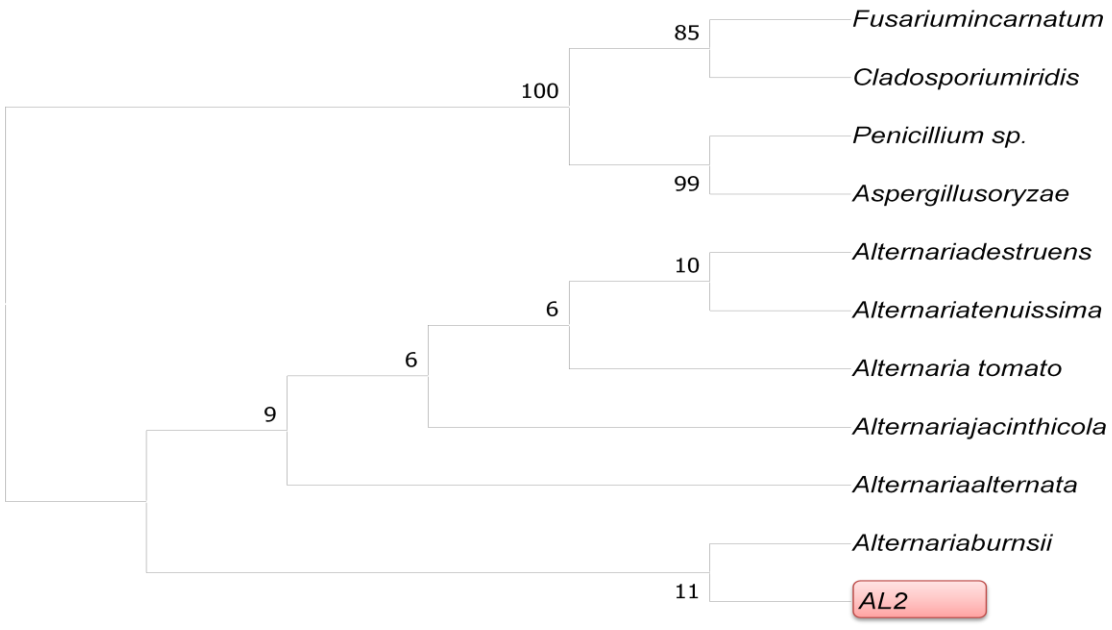


Fig 27: Phylogenetic tree constructed by using maximum parsimony method.

The evolutionary studies using maximum parsimony phylogenetic tree clearly depicts the evolutionary relationship of AL2with *Alternaria spp.*(Fig 27).

Hence, the isolated endophytic fungi (AL2) isolate is identified as *Alternaria spp.*based on morphological and molecular studies.

Tejesvi *et al*., 2005 isolated 278 fungal species from *T. arjuna* and reported the presence of *Alternaria spp.*&*Alternaria alternate.*

Patil *et al.,* 2014 also reported 4 fungal isolates out of which one was *Alternaria spp*. obtained from *Terminalia arjuna*

4.10Fractionation of crude extracts

4.10.1 Thin layer chromatography: Thin Layer Chromatography was performed for the methanolic extract of AL2.Different solvent systems were applied according to the polarity of solvents for the separation of fractions from crude extract. These solvents system were initially applied in nuclear, then in binary combination.

Table 17: Different solvent systems used for TLCof AL2 and GS4

I. Solvent system used for separation of fungal crude extract of AL2

S. No	**Sample**	**Solvent System**	**Ratio of solvents**	**No. of fractions**	**R_f values**
1	AL2	Hexane	100%	0	-
2	AL2	Toluene	100%	0	-
3	AL2	Chloroform	100%	0	-
4	AL2	Methanol	100%	1	0.14
5	AL2	Dichloromethane	100%	4	0.134,0.25,0.625,0.98
6	AL2	Diethylether	100%	4	0.37,0.59,0.72,0.81
7	AL2	Ethyl Acetate	100%	0	-
8	AL2	Toluene: ethyl acetate	93:7	0	-
9	AL2	Toluene: Hexane	20: 80	0	-
10	AL2	Dichloromethane: Methanol	90:10	5	0.17,0.27,0.53,0.69,0.74

After optimization of the solvent system (Table 28) for AL2crude extract it was observed that it best separated in dichloromethane: methanol (90:10). This solvent system separated the crude extract into five fractions (Fig 28). These results also depicts that the non polar compounds move faster than polar compounds. The standard reported range of Rf lies between 0.15-0.85. DCM: Methanol fractions fall under this range, indicating best separation of the crude extract. Same solvent in same ratio was used for GS4 (Table 17 II). These plates were observed under UV 254 and 365. The developed chromatograms were visualized under UV light at 254 wavelength.Sample components that absorb light in this region appear as a dark spot by quenching the greenish yellow fluorescing background (Fig 29).

II. Solvent system used for separation of fungal crude extract of GS4

S. no	**Sample**	**Solvent System**	**Ratio of solvents**	**No. of fractions**	**Rf values**
1	GS4	Hexane	100%	-	0
2	GS4	Methanol	100%	-	0
3	GS4	Diethyl ether	100%	2	0.12, 0.629
4	GS4	Dichloromethane: methanol	90:10	7	0.23,0.41, 0.52, 0.57,0.63, 0.71, 0.72

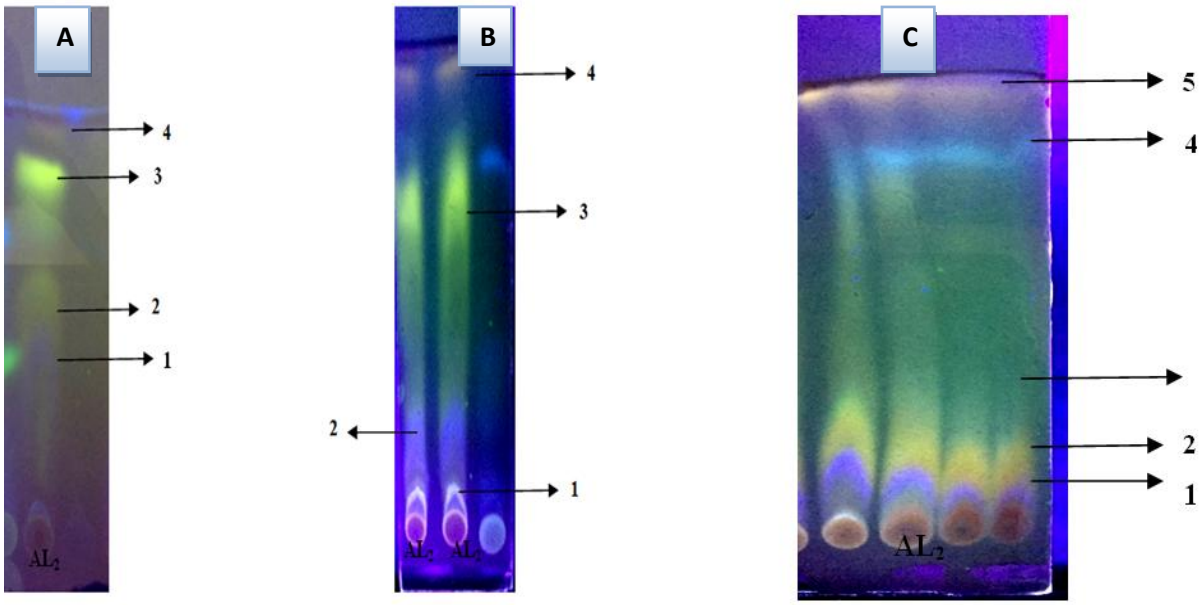


Fig 28: Separation of AL2crude extract into different fractions. (A) Chromatogram obtained by using Dichloromethane and observed under 365nm (B) Separation by using Diethyl ether and observed under 365nm (C) Chromatogram obtained by using Dichloromethane: methanol (90:10) and observed under 365nm.

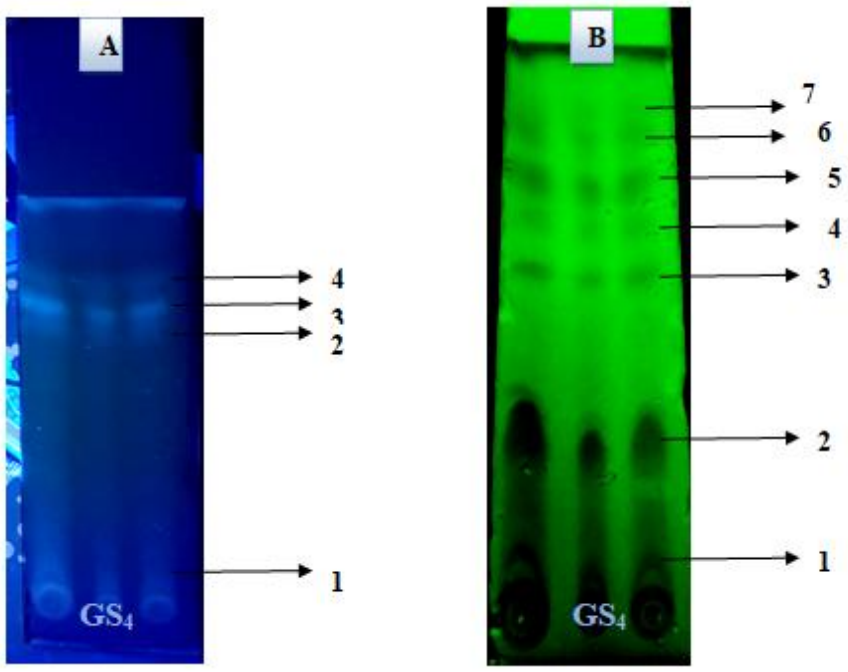


Fig 29: Separation of GS4 crude extract into different fractions (A)Chromatogram obtained by using Dichloromethane: methanol (90:10) and observed under 365nm (B) Observed under 365nm.

4.10.2 Column chromatography: The column preparation included adsorption of the extract, charging and saturation of the column. The extract selected for fractionation was adsorbed on stationary phase. Crude extract was dissolved in an appropriate solvent then loaded onto the column. The column was then eluted with a 100% hexane, gradient of hexanes and ethyl acetate (5% ethyl acetate to 100% ethyl acetate), followed by methanol in ethyl acetate (5% ethyl acetate).Seven fractions of endophytic crude extract AL2 were obtained after silica gel column chromatography. In AL2 mixture, colored compounds, were present this made monitoring of column easy. The colored bands were moved down the column according to polarity of the solvent and as they move towards the end of the column they were collected in test tubes (Fig 28). However, some of organic molecules are colorless. In this case, the reaction was monitored by placing a spot on TLC plate and chromatogram was developed by placing the plate in iodine chamber so as to determine no of spots in each of the collected test tubes. The test tubes having the same spot were combined and further the solvent was evaporated by using rotaevaporator.

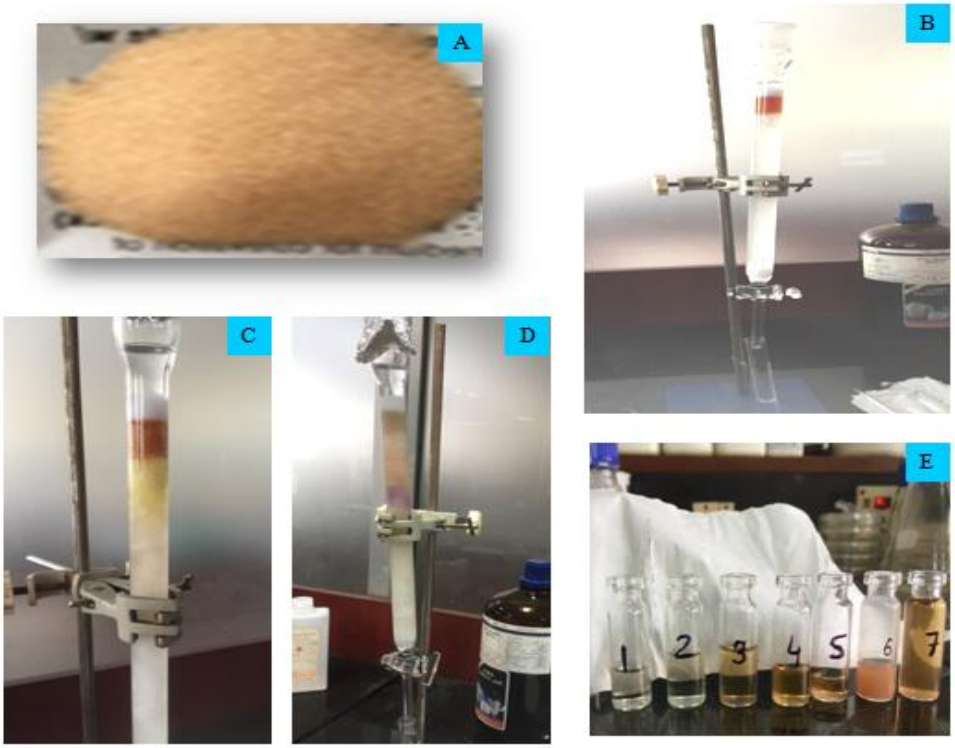


Fig 30: Fractionation of crude extract through column chromatography. (A) Adsorption of extract on silica surface, (B) Extract loading in column, (C) Separation of crude with 100% Hexane, (D) Separation of crude into no. of fractions by increasing polarity, (E) Collection of fractions after rotaevaporation.

4.11 Antibacterial activity of fractions obtained after column chromatography

4.11.1 Antibacterial activityof fractions:

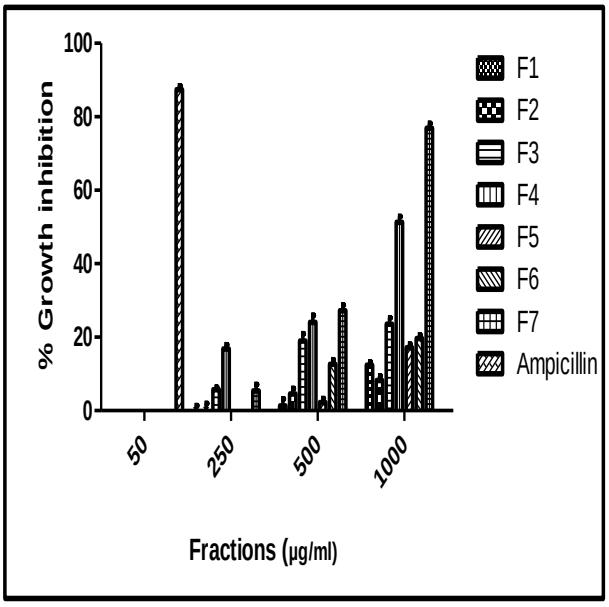


Fig 31 (A) Antibacterial of activity fractions against *P. aeruginosa*

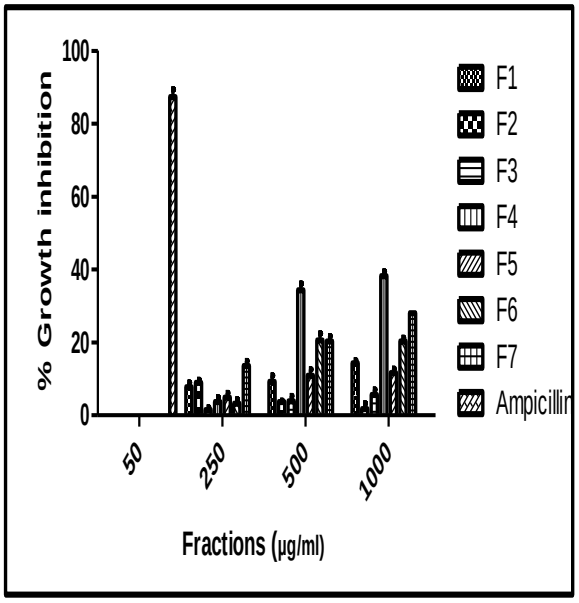


Fig 31 (B) Antibacterial activity of fractions against *S. aureus*

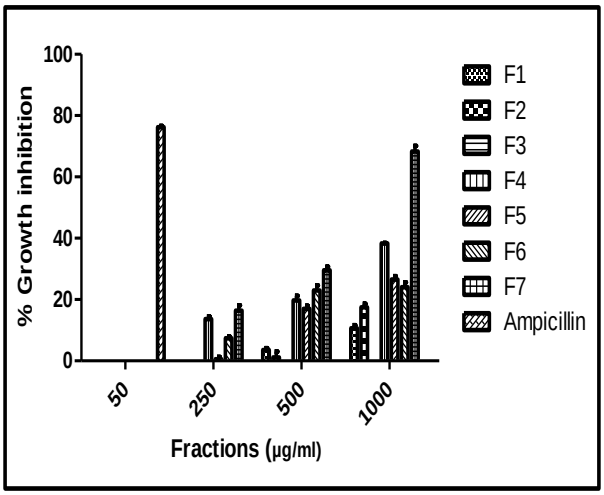


Fig 31 (C) Antibacterial activity of fractions against *E. coli*

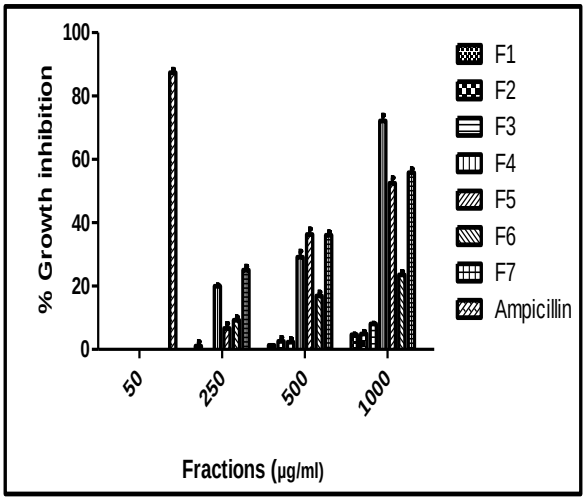


Fig 31 (D) Antibacterial activity of fractions against *B. megaterium*

The obtained seven fractions were tested against different bacterial cultures (Fig 31). The fractions 4 and 7 exhibiting maximum activities were further characterized. Fraction 4 showed

maximum inhibition against gram positive bacteria whereas fraction 7 inhibits the growth of gram negative bacteria.

4.12 Characterization of fractions

4.12.1 Ultraviolet spectroscopic Analysis: After screening of fractions, purified fraction 7 was analysed by UV absorption by dissolving in 100% methanol. The fraction 7 shows λ max at 230 nm whereas authentic taxol (Paclitaxel) also showed λ max at 230.20 nm (Fig 32). This provides the idea that taxol might be present in the purified fraction.

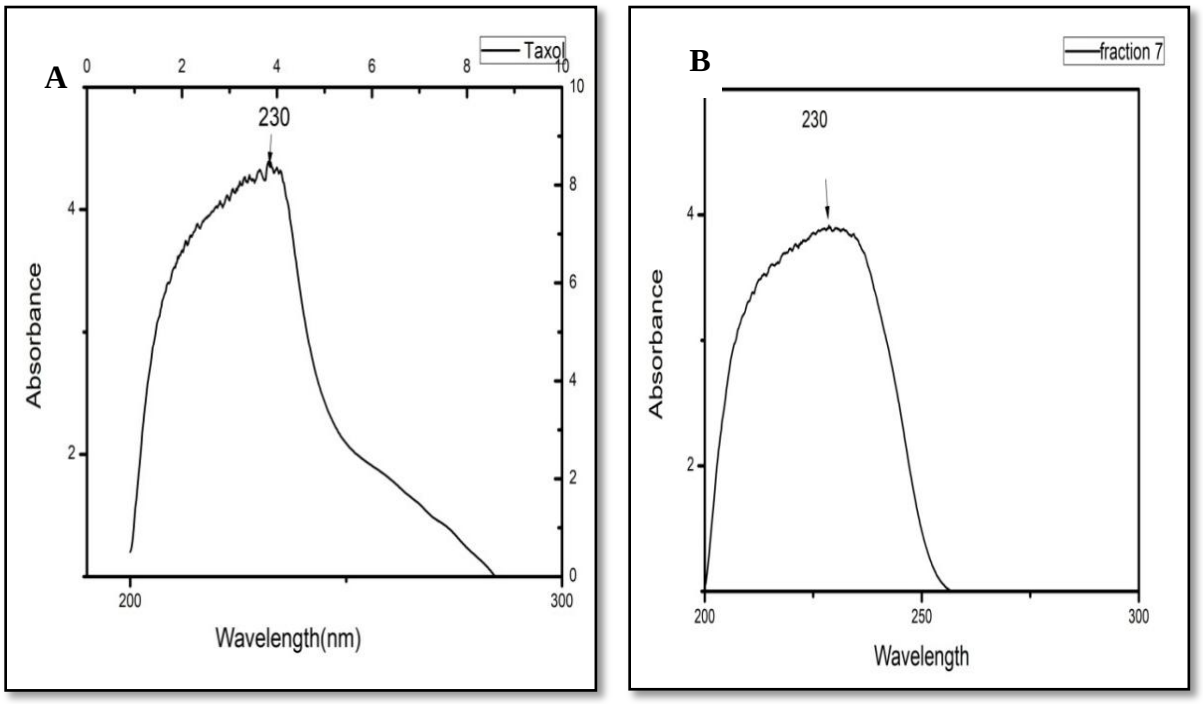


Fig 32: (A) UV absorption spectra of authentic taxol (B) fungal fraction 7 from *Alternaria spp.*, Spectra were recorded in methanol over the wavelength range 200–300 nm

4.12.2 High pressure liquid chromatography (HPLC): This process was used for detection of compounds present in the fractions previously separated using column chromatography. The fraction showing maximum activity was detected by using a reverse phase C18 column with an ultraviolet (UV) detector. Thirty microliters of the sample was injected and detected at 232 nm. The mobile phase was methanol/acetonitrile/water (25:35:40 v/v/v) at a flow rate of 1.0 ml min^{-1}. The fraction 7 was filtered through 0.2 μm sterile minigen syringe filter before loading

into the column. Fraction 7 shows the retention time at 3.91whereas retention time of authentic taxol is 4.12 (Fig 33). Hence the peaks obtained in UV spectroscopic and HPLC analyses are quite similar to that obtained with standard taxol which indicates the presence of taxol.

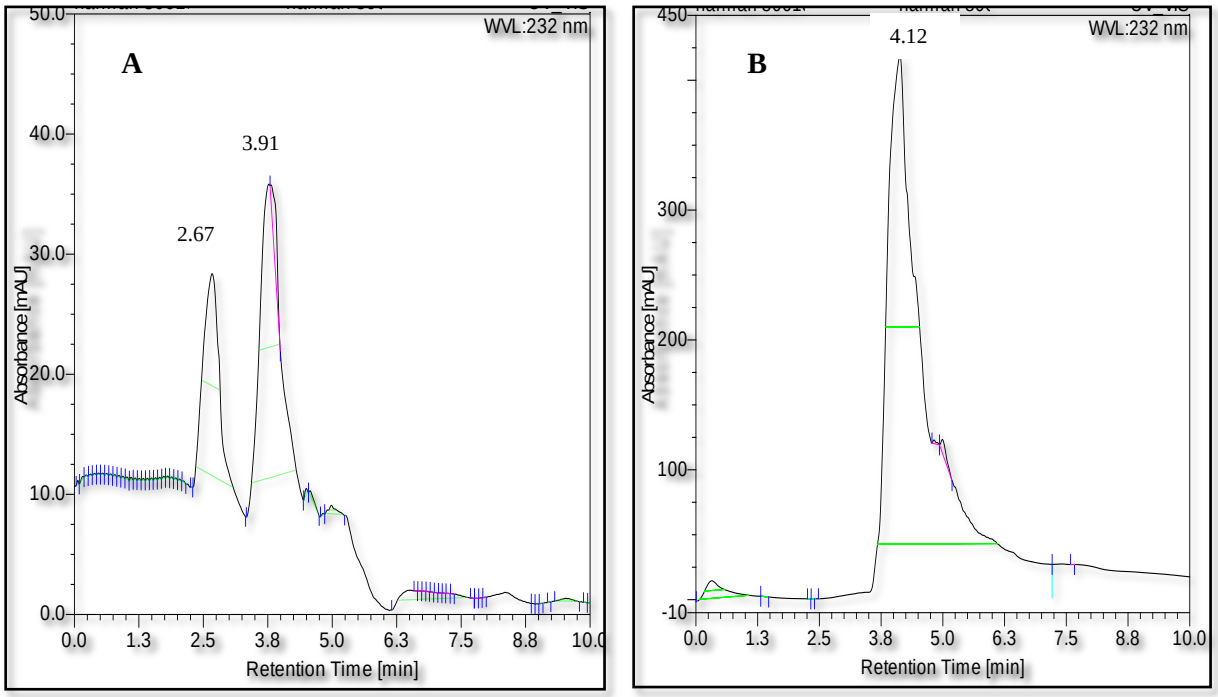
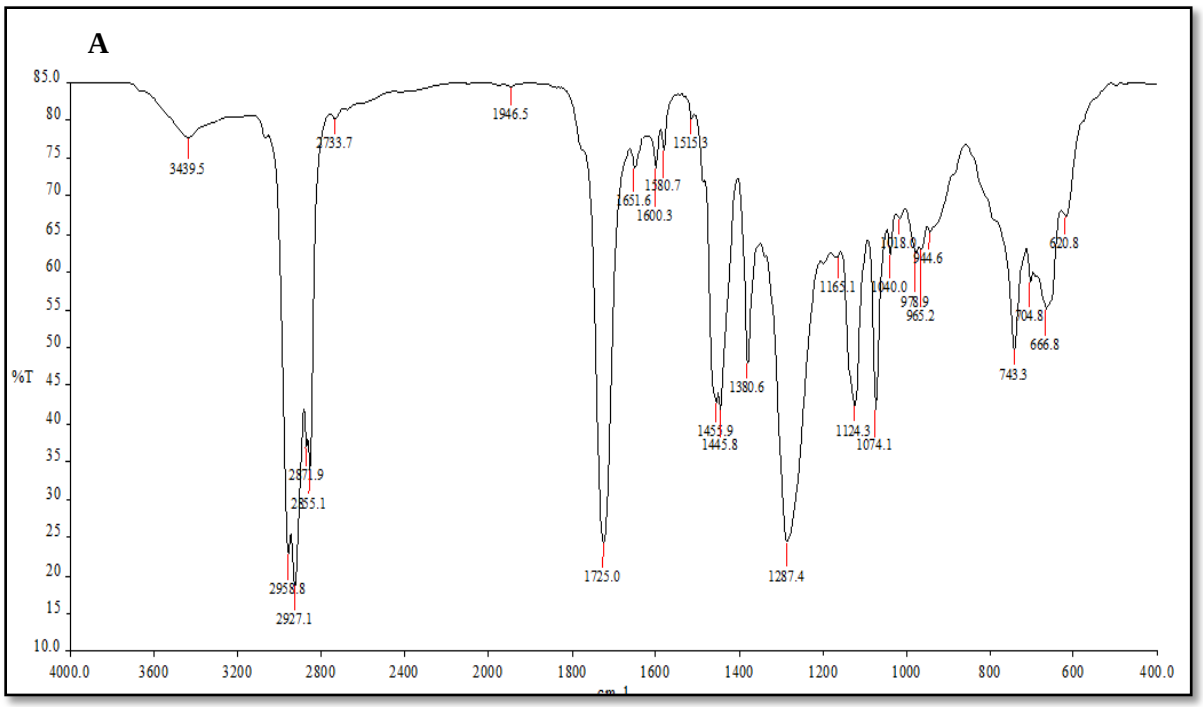
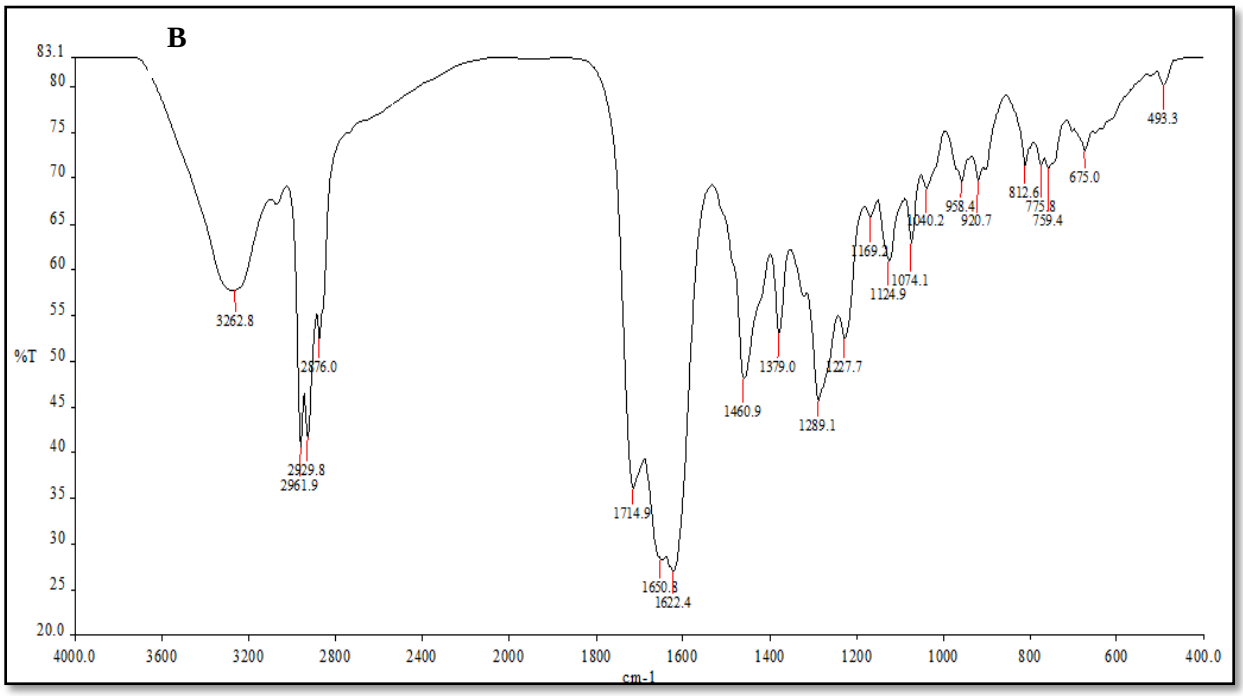


Fig 33: (A)Taxol from *Alternaria spp.*, (B) High performance liquid chromatogram of authentic taxol.

4.12.3 Fourier transmittance Infra Red: FTIR spectrum of AL2, AL2-4, AL2-7 fraction were obtained. These spectrums were analyzed by comparing wavelength of spectrum with that of functional groups. The IR spectra were recorded in the region 4000–500 cm^{-1}.The IR spectral data of fungal taxol from *Alternaria spp*. showed a broad peak in the region of 3418.3 which is due to presence of (-NH) amine group, the peak at 1574.8 region is due to presence of (-C=C) indicated presence of cyclic alkene. The alcohol (-OH) and ether groups (C-O) were observed was in the range of 1416.5 and 1022.2 region respectively (Fig 34). The obtained spectrum was compared with the spectra of authentic taxol and the spectra of both were quite similar. This confirms the presence of taxol in fraction 7.

(Gangadevi 2009) also reported taxol from*chaetomella raphigera*an endophytic fungi isolated from *T. arjuna.* They confirmed the presence of taxol by using UV, IR, mass spectrophotometry.


A
%T
cm-1
3439.5
2733.7
1946.5
1651.6
1600.3
1580.7
1513
2871.9
2855.1
2958.8
2927.1
1725.0
1453.9
1445.8
1380.6
1287.4
1165.1
1124.3
1074.1
1040.0
978.9
965.2
944.6
743.3
704.8
666.8
620.8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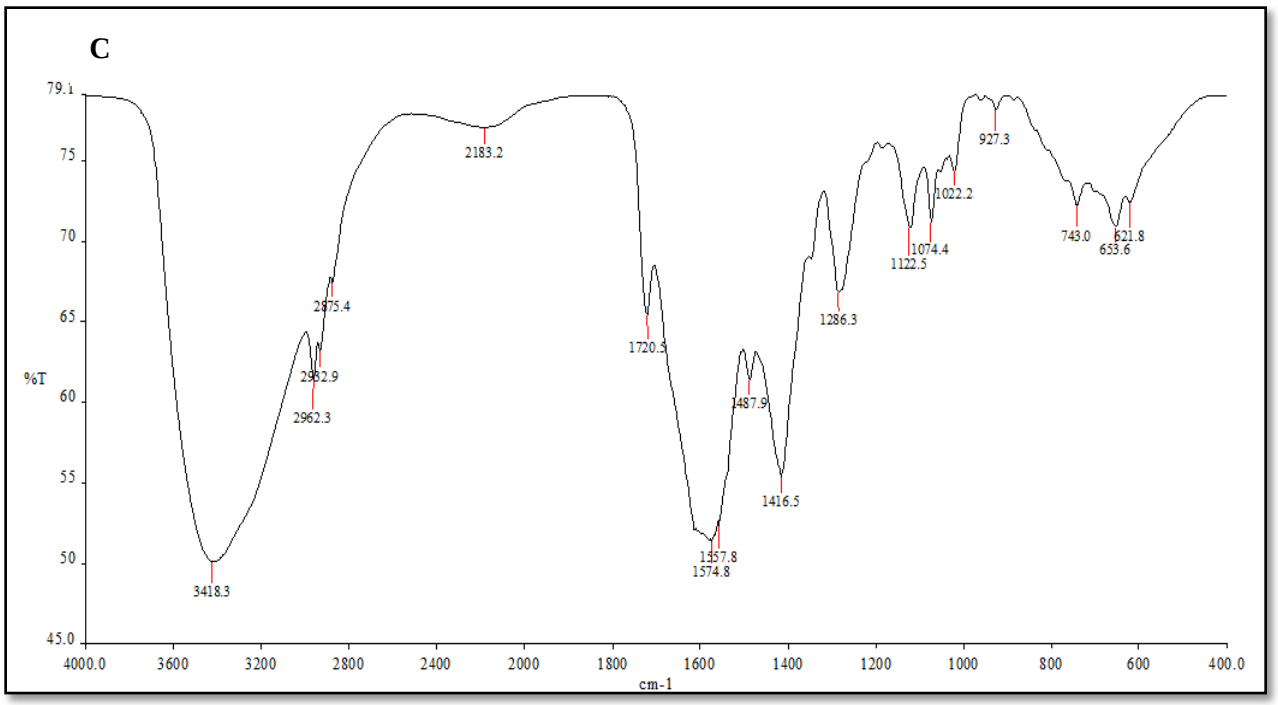

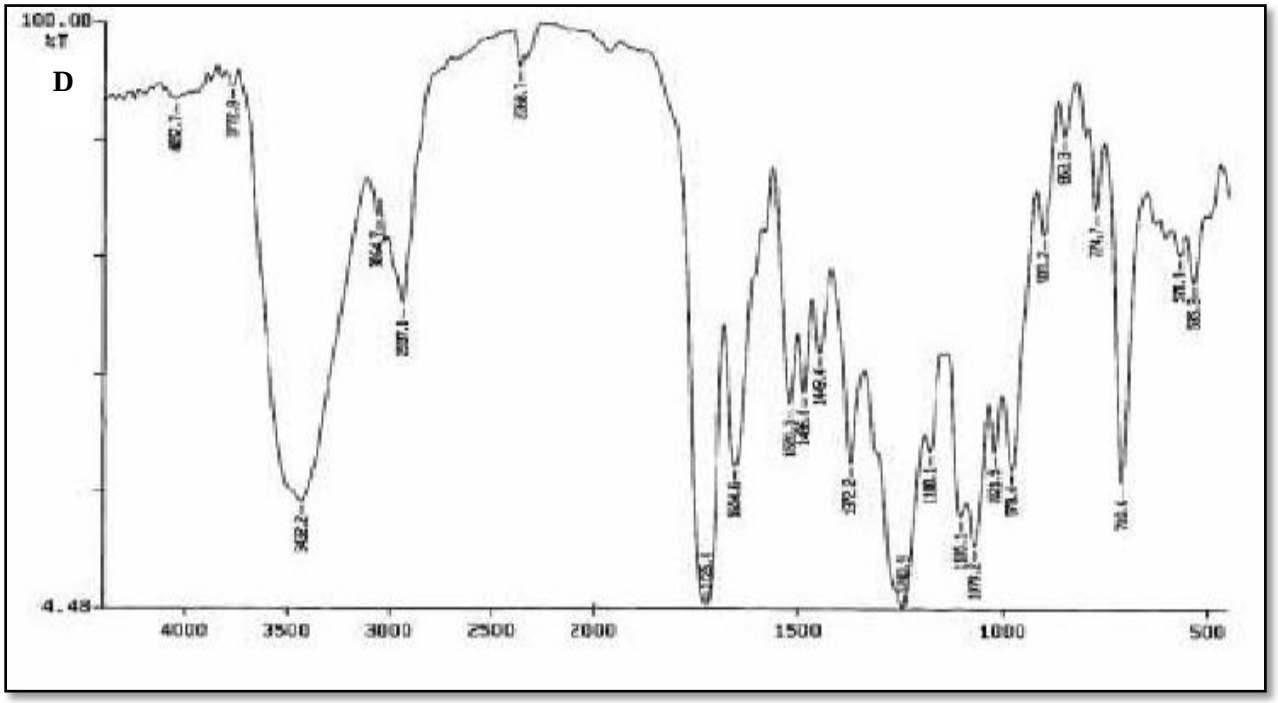

Fig 34: FTIR spectra (A): FTIR spectrum of crude extract AL2, (B) FTIR spectrum of fraction 4, (C) FTIR Spectrum of fraction 7, (D)FTIR spectrum of authentic taxol.

Table 18: Analysis of FTIR results:

(A) Spectra of crude extract of AL2

S.no	Frequency (cm^{-1})	Bond	Group	
1	3262.8	Strong	OH	Carboxylic acid
2	2961.9	Strong	N-H	Amine salt
3	2929.8	Strong	N-H	Amine salt
4	2876.8	Strong	N-H	Amine salt
5	1714.9	Strong	C=0	Cyclohexane
6	1650.8	Strong	C=O	δ lactam
7	1622.4	Medium	C=C	Conjugate alkene
8	1460.9	Medium	C-H	alkane
9	1379.0	Medium	O-H	Phenol
10	1289.1	Strong	C-N	Flouro compound
11	1227.1	Strong	C-O	Aromatic amine
12	1169.2	Strong	C-O	Alkyl aryl ether
13	1124.9	Strong	C-O	Ester
14	1074.1	Strong	C-O	Tertiary alcohol
15	1040.1	Strong	C-O	Primary alcohol
16	1040.2	Strong	CO-O-CO	Anhydride
17	958.4	Strong	CO-O-CO	Anhydride
18	920.7	Strong	CO-O-CO	Anhydride
19	812.6	Strong	C-H	1,2,4 trisubsitute
20	775.6	Strong	C-H	1,2,4 trisubsitute
21	759.4	Strong	C-H	1,2,4 trisubsitute
22	675.0	Strong	C=C	alkene

(B) Spectra of 4 fraction of AL2,

S.NO	**Frequency (cm^{-1})**	**Bond**	**Group**	
1	3439.5	Strong	-NH	Primary amine
2	2958.8	Strong Medium	-NH C-H	Amine salt Alkane
3	2927.1	Strong Medium	N-H C-H	Amine salt Alkane
4	2871.9	Strong	C-H	Alkane
5	2855.1	Strong	C-H	Alkane
6	2733.7	Weak	O-H	Aldehyde
7	1946.5	Medium	C=C=C	Allelene
8	1725.0	Strong	C=O	Cyclopentone
9	1651.6	Medium	C=C	Vinylidene
10	1600.3	Medium	C=C	Conjugate alkene
11	1580.7	Medium	N-H	Amine
12	1515.3	Medium	N-O	Nitro compound
13	1455.9	Strong	C-H	Alkane methyl group
14	1445.8	Medium	O-H	Carboxylic acid
15	1380.6	Strong	S=O	Sulfate
16	1287.4	Strong	C-F	Flouro compound
17	1165.1	Strong	S=O	Sulfonic acid
18	1124.3	Strong	S=O	Sulfone
19	1074.1	Strong	C-O	Alkyl aryl ether
20	1040.1	Strong	CO-O-CO	Anhydride
21	1018.0	Strong	C-O	Vinyl ether
22	978.9	Strong	C-O	Alkene

23	965.2	Strong	C=C	Alkene
24	743.3	Strong	C-Cl	Halo compound
25	704.8	Strong	C=C	Alkene
26	666.8	Strong	C-Br	Halo compound
27	620.8	Strong	C-Br	Halo compound

(C) FTIR results of fraction 7

S.NO	**Frequency of (cm^{-1})**	Bond	Group	
1	3418.3	Medium	N-H	Primary amine
2	2962.3	Strong	N-H	Amine salt
3	2932.9	Strong	N-H	Amine salt
4	2875.4	Medium	C-H	Alkane
5	2183.2	Weak	C= C	Alkyne
6	1720.5	Strong	C=O	Conjugate anhydride
7	1574.8	Medium	C=C	Cyclic alkene
8	1557.8	Strong	N-O	Nitro compound
9	1487.9	Strong	N-O	Nitro compound
10	1416.5	medium	O-H	Alcohol
11	1286.3	Strong	C-N	Aromatic amine
12	1122.5	Strong	C-O	Secondary alcohol
13	1074.4	Strong	C-O	Primary alcohol
14	1022.2	Strong	C-O	Alkyl aryl ether
15	927.2	Strong	C-O	Alkene
16	743.0	Strong	C=O	Alkene
17	653.6	Strong	C=O	Alkene
18	621.8	Strong	C=O	Alkene

4.12.4 LCMS: Fraction 7 was dissolved inmethanol and used as such for analysis. Chromatographic separation was carried out using UHPLC system equipped with C18 column. Sample was injected into the column and elution was carried out using methanol/acetonitrile/water (25:35:40, by v/v/v) over a period of 30 min with flow rate of 1ml/min. A parent ion peak of m/z 854.95 was observed in the the spectrum. The reported m/z value of paclitaxel is at 854$[M+H]^+$ion. Paclitaxel has a weight 853.90 g/mol and the molecular formula of $C_{47}H_{51}NO_{14}$. The spectral results further confirmed the presence of taxol in fraction 7 of AL2. LC-ESI-MS analysis of methanolic extracts of the bark of *Taxus wallichianca*showed the presence of 75 taxoids and 36 aminotaxoids (Madhusudan *et al*., 2012). Paclitaxel from endophytic fungi has also been reported by (Garyali *et al*., 2003) with similar kind of spectra.

Table 19: LC-ESI-MS analysis of fraction 7

Reported m/z	854$[M+H]^+$
Measured m/z	854.95
Molecular weight	853.90 g/mol
Molecular formula	$C_{47}H_{51}NO_{14}$
Compound	Paclitaxel
Reported bioctivity	Anticancer activity

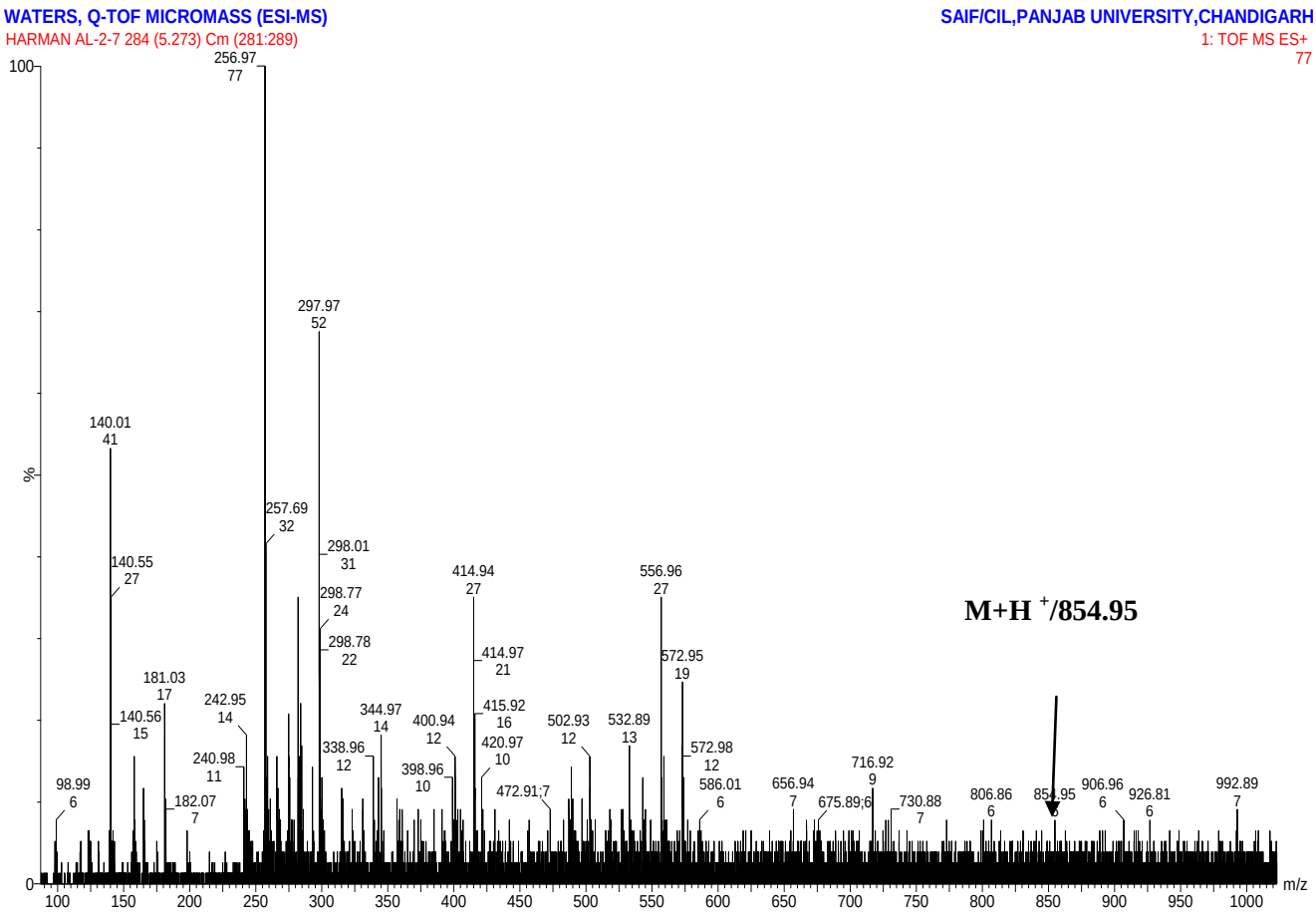

Fig 35: LCMS results showing peak at 854.95$[M+H]^+$

CONCLUSIONS

In this present study, two medicinal plants*Terminalia arjuna*and*Tinospora cordifolia* have been selected for the isolation of endophytic fungi. The above two medicinal plants were chosen for study as not much work has been done on isolation of endophytic fungi, characterization of bioactive compounds from these fungi. As these above medicinal plants have therapeutic potential, hence they could be explored for endophytic fungi having capability to produce bioactive compounds.The samples were collected from the campus of TIET, Patiala.A total of 14 endophytic fungi were obtained from*Terminalia arjuna* and 7 from *Tinospora cordifolia* during a period of 5-14 days.The isolated endophytic fungi from *T. arjuna* and *T. cordifolia* were inoculated in the PDB for fermentation.

The crude extract obtained from these isolates were tested for preliminary antimicrobial activity by agar disc diffusion method and MTT assay using cultures of *E.coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* and *Candida albicans*. The maximum inhibition was shown by fungal extract AL2 amongst all the extracts of*T. arjuna*. It showed inhibitory activity of 71% against *Pseudomonas aeruginosa*, 82.23% against *Bacillus megaterium*, 17% against *Staphylococcus aureus*, and 75% against *E.coli*and showed 17.25% against *Candida albicans*. Amongst the extracts from *T. cordifolia*fungal crude extract GS4 showed the prominent activity of 28%, 60% and 59% against *E.coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* respectively. These extracts were also tested for their potential to scavenge free radicals. GS4 showed maximum radical scavenging activity of 75.65% at 250 µg/ml followed by AL2 which showed maximum radical scavenging activity of 64.34%at 500 µg/mlof concentration. Extract AL2 shows the presence of alkaloids and triterpenoidswhereas it showed absence of steroids, glycosides, fats and oils. Crude extract GS4 indicates the presence of phenols, carbohydrates, alkaloids and steroids whereas tannins, amino acids, glycosides were absent in the extract. AL2 gave positive results for the lipase, protease and laccase enzyme activity test whereas GS4 showed positive test for lipase and protease.The evolutionary studies using maximum likelihood phylogenetic tree clearly depicts the evolutionary relationship of AL2with *Alternaria spp.*Hence, the isolated endophytic fungi (AL2) isolate is identified as *Alternaria spp.*based on morphological and molecular studies. For purification preliminary TLC analysis was done and for fractionation column chromatography was done. The obtained seven

fractions were tested against different bacterial cultures. The fractions 4 and 7 exhibiting maximum bioactivities were further characterized. Fraction 4 shows maximum inhibition against gram positive bacteria whereas fraction 7 inhibits the growth of gram negative bacteria. Fraction 7 shows the retention time at 3.91 min whereas retention time of authentic taxol is 4.12. Hence the peaks obtained in UV spectroscopic and HPLC analyses are quite similar to that obtained with standard taxol which indicates the presence of taxol.Fraction 7 was dissolved inmethanol and used as such for analysis. Chromatographic separation was carried out using UHPLC system equipped with C18 column. Sample was injected into the column and elution was carried out using methanol/acetonitrile/water (25:35:40, by v/v/v) over a period of 30 min with flow rate of 1ml/min. A parent ion peak of m/z 854.95 was observed in the the spectrum. The reported m/z value of paclitaxel is at 854$[M+H]^+$ion. Paclitaxel has a weight 853.90 g/mol and the molecular formula of $C_{47}H_{51}NO_{14}$. The spectral results further confirmed the presence of taxol in fraction 7 of AL2.

REFERENCES

1. Akello J, Dubois T, Gold CS, Coyne D, Nakavuma J and Paparu P. 2007. *Beauveria bassiana* (Balsamo) as an endophyte in tissue culture banana (*Musa spp*.). Journal of invertebrate pathology. 96(1): 34-42.
2. Altschul S F, Madden T L., Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research. 25(17): 3389-3402.
3. Aly AH, Debbab A and Proksch P. 2011. Fifty years of drug discovery from fungi. Fungal Diversity. 50: 3–19.
4. Aly AH, Edrada-Ebel R, Indriani ID, Wray V and Proksch P. 2008. Cytotoxic metabolites from the fungal endophyte *Alternaria sp* and their subsequent detection in its host plant *Polygonum senegalense*. Journal of natural products. 71(6): 972-980.
5. Bacon CW and White JF. 2000. Physiological adaptations in the evolution of endophytism in *Clavicipi taceae*. In: Microbial Endophytes. 29: 237- 262.
6. Bae H, Sicher RC, Kim MS, Kim SH, Strem MD, Melnick R and Bailey BA. 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. Journal of Experimental Botany. 60(11): 3279-3295.
7. Bhagobaty RK and Joshi SR. 2012. Enzymatic activity of fungi endophytic on five medicinal plant species of the pristine sacred forests of Meghalaya, India. Biotechnology and bioprocess engineering. 17(1): 33-40.
8. Bita Zaferanloo, Stephanie A Pepper, Sally A Coulthard, Christopher P F Redfern, Enzo A Palombo. 2018. Metabolites of endophytic fungi from Australian native plants as potential anticancer agents. FEMS Microbiology Letters. 2: 365-371.
9. Bustanussalam, Fauzy Rachman, Eris Septiana, Sylvia JR, Lekatompessy, Tiwit Widowati, Harmastini I and Partomuan Simanjuntak. 2015. Screening for Endophytic Fungi from Turmeric Plant (*Curcuma longa L*.) of Sukabumi and Cibinong with Potency as Antioxidant Compounds Producer. Pak J Biol Sci. 18(1): 42-45.
10. Carrol GC. 1986. The biology of endophytism in plants with particular reference to woody perennials. University Press Cambridge. 5: 205-220.

11. Clay K and Holah J. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. Science. 285(5434): 1742-1744.
12. Cui JL, Guo S X and Xiao PG. 2011. Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. Journal of Zhejiang University Science B. 12(5): 385-392.
13. Cui JL., Guo SX and Xiao PG. (2011). Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. Journal of Zhejiang University Science *B.* 12(5): 385-392.
14. De Bary A, Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten. 1866. Hofmeister's Handbook of Physiological Botany. 2.
15. De Mendonca, and Lemes B. 2015. Potential antimicrobial and antiproferative activity of the crude extract of the endophytic fungus *Rhizoctonia sp.* from *Annona crassifolia*. African Journal of Pharmacy and Pharmacology. 9(10): 327-332.
16. Debbab A, Aly HA, Edrada-Ebel RA and Proksch P. 2009. Bioactive secondary metabolites from the endophytic fungus *Chaetomium sp* isolated from *Salvia officinalis* growing in Morocco. Biotechnologie Agronomie Society. 13(2): 229-234.
17. Devi LS, Bareh DA and Joshi SR. 2014. Studies on biosynthesis of antimicrobial silver nanoparticles using endophytic fungi isolated from the ethno-medicinal plant *Gloriosa superba L*. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. 84(4): 1091-1099.
18. Ding G, Zheng Z, Liu S,Zhang H, Guo L and Che Y.2009. Photinides A− F Cytotoxic Benzofuranone-Derived γ-Lactones from the Plant Endophytic Fungus *Pestalotiopsis photiniae*. Journal of natural products. 72(5): 942-945.
19. Ebrahim W, Kjer J, El Amrani, M Wray, Lin W and Proksch P. 2012. Pullularins E and F two new peptides from the endophytic fungus *Bionectria ochroleuca* isolated from the mangrove plant *Sonneratia caseolaris.* Marine drugs. 10(5): 1081-1091.
20. Fu J, Zhou Y, Li HF, Ye YH and Guo JH. 2011. Antifungal metabolites from *Phomopsis sp*., an endophytic fungus in *Gossypium hirustum.* Afr J Microbiol Res. 5(10):1231–1236.
21. Gangadevi V and Muthumary J .2007. Endophytic fungal diversity from young mature and senescent leaves of *Ocimum basilicum* L with special reference to Taxol production Indian. Journal of Science and Technology. 1(1): 1-12.

22. Gangadevi V and Muthumary J. 2009. A novel endophytic taxol-producing fungus *Chaetomella raphigera* isolated from a medicinal plant *Terminalia arjuna*. Applied biochemistry and biotechnology.158(3): 675-684.
23. García-Guzmán G and Heil M. 2013. Endophytes versus biotrophic and necrotrophic pathogens—are fungal lifestyles evolutionarily stable traits. Fungal Diversity. 60(1): 125-135.
24. Gond S K, Verma V C, Kumar A, Kumar V and Kharwar R N. 2007. Study of endophytic fungal community from different parts of Aegle marmelos Correae (Rutaceae) from Varanasi (India). World Journal of Microbiology and Biotechnology, 23: 1371- 1375.
25. Gond SK, Verma VC, Kumar A, Kumar V and RN Kharwar. 2007. Study of endophytic fungal community from different parts of *Aegle marmelos*. Correae (Rutaceae) from Varanasi (India). World J Microb Biot. 23: 1371-1375.
26. Gunatilaka A. 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity and implications of their occurrence. Journal of Natural Products. 69 (3): 509–526.
27. Hadacek F and Greger H. 2000. Testing of antifungal natural products: methodologies, comparability of results and assay choice. Phytochemical analysis. 11(3): 137-147.
28. Hallmann J, Quadt-Hallmann A, Mahaffee WF and Kloepper JW. 1997. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology. 43(10): 895-914.
29. Hammerschmidt L, Ola A, Muller WE, Lin W, Mándi A, Kurtán T and Aly AH. 2015. Two new metabolites from the endophytic fungus *Xylaria sp.* isolated from the medicinal plant *Curcuma xanthorrhiza*. Tetrahedron Letters. *56*(10): 1193-1197.
30. Hartley, S. E., and Gange, AC. 2009. Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. Annu. Rev. Entomol. 54: 323–342.
31. Hawksworth and DL. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol. Res. 105: 1422-1432.
32. Hemtasin C, Kanokmedhakul S, Kanokmedhakul K, Hahnvajanawong C, Soytong K, Prabpai S and Kongsaeree P. 2011. Cytotoxic pentacyclic and tetracyclic aromatic sesquiterpenes from *Phomopsis archeri*. Journal of natural products. 74(4): 609-613.
33. Kapoor N and Saxena S. 2018. Endophytic fungi of *Tinospora cordifolia* with anti-gout properties. 3Biotech. 8(6): 264.

34. Kaul S, Gupta S, Ahmed M and Dhar M K. 2012. Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. Phytochemistry Reviews. 11: 487–505.
35. Khan AL, Kang SM, Dhakal KH, Hussain J, Adnan M and Kim JG. 2013. Flavonoids and amino acid regulation in Capsicum annuum L. by endophytic fungi under diff erent heat stress regimes. Sci. Hortic. 155: 1–7.
36. Kharwar RN, Verma VC, Kumar A, Gond SK, Harper JK, Hess WM and Strobel GA. 2009. Javanicin an antibacterial naphthaquinone from an endophytic fungus of neem *Chloridium sp*. Current microbiology. 58(3): 233-238.
37. Kumar D SS, Lau C, Wan J M, Yang D and Hyde KD. 2005. Immunomodulatory compounds from *Pestalotiopsis leucothes*, an endophytic fungus from *Tripterygium wilfordii*. Life sciences. 78(2): 147-156.
38. Kumar S, Kaushik N, Edrada, Ebel R and Proksch P. 2008. Endophytic fungi for pest and disease management, Integrated management of diseases caused by fungi, phytoplasma and bacteria. Springer. 3: 365-387.
39. Kumar S, Kaushik, N and Proksch P. 2013. Identification of antifungal principle in the solvent extract of an endophytic fungus *Chaetomium globosum* from *Withania somnifera.* SpringerPlus. 2(1): 37.
40. Kusari S, Hertweck C and Spiteller M. 2012. Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol. 19: 792-798.
41. Lahrmann U and Zuccaro A. 2012. Opprimo ergo sum- evasion and suppression in the root endophytic fungus *Piriformospora indica*. Molecular plant-microbe interactions. *25*(6): 727-737.
42. Li H, Xiao J, Gao YQ, Tang JJ, Zhang AL and Gao JM. 2014. Chaetoglobosins from *Chaetomium globosum* an endophytic fungus in Ginkgo biloba and their phytotoxic and cytotoxic activities. Journal of agricultural and food chemistry. 62(17): 3734-3741.
43. Lv YL, Meng ZX, Chen J and Guo SX. 2010. The promoting role of an isolate of dark-septate fungus on its host plant *Saussurea involucrat*a. Mycorrhiza. 20(2): 127-135.
44. Madhusudanan K P, Chattopadhyay, S K, Tripathi V K, Sashidhara KV, Kukreja, A K and Jain SP. 2002. LC- ESI- MS analysis of taxoids from the bark of *Taxus wallichiana*. Biomedical Chromatography. 16(5): 343-355.

45. Maheshwari VL, Patil MP, Patil RH and Patil SG.2014. Endophytic mycoflora of Indian medicinal plant *Terminalia arjuna* and their biological activities. International Journal of Biotechnology for Wellness Industries. 3(2): 53-61.
46. Mishra A, Gond SK, Kumar A, Sharma VK, Verma SK, Kharwar RN and Sieber TN. 2012. Season and tissue type affect fungal endophyte communities of the Indian medicinal plant *Tinospora cordifolia* more strongly than geographic location Microbial ecology. 64(2): 388-398.
47. Mittal Jitendra, Sharma MM and Amla B. 2014. *Tinospora cordifolia*: a multipurpose medicinal plant. A review. Journal of Medicinal Plants Studies. 2: 32-47.
48. Mohd Mallique Qader, Kumar Savitri, Jayasinghe Lalith and Fujimoto Y. 2017. Bioactive sesquiterpenes from an endophytic fungus *Bipolaris sorokiniana* isolated from a popular medicinal plant *Costus speciosus*. Taylor and Francis. 8(1): 17-20.
49. Mossa-Basha, M Ilica AT, Maluf F, Karakoç O, İzbudak I and Aygün N. 2013. The many faces of fungal disease of the *paranasal sinuses*: CT and MRI findings. Diagnostic and Interventional Radiology. 19(3): 195.
50. Muthumary J and Gangadevi V. 2009. Taxol production by *Pestalotiopsis terminaliae* an endophytic fungus of *Terminalia arjuna* (arjun tree). Biotechnology and applied biochemistry. 52(1): 9-15.
51. Nath K, Gond S K, Mishra A, Sharma VK, Kumar J, Singh DK and Kharwar RN. 2012. Impact of environmental variables on the isolation, diversity and antibacterial activity of endophytic fungal communities from *Madhuca indica Gmel*. at different locations in India. Annals of microbiology. 64(2): 721-734.
52. Pandi M, Kumaran RS, Choi YK, Kim HJ and Muthumary J. 2011. Isolation and detection of taxol an anticancer drug produced from *Lasiodiplodia theobromae* an endophytic fungus of the medicinal plant *Morinda citrifolia*. African Journal of Biotechnology. 10(8): 1428-1435.
53. Patil MP, Patil RH, and Maheshwari VL. 2015. Biological activities and identification of bioactive metabolite from endophytic *Aspergillus flavus* L7 isolated from *Aegle marmelos*. Current microbiology. 71(1): 39-48.
54. Petrini O. 1991. Fungal endophytes of tree leave. Springer-Verlag. 3: 179-197.

55. Phongpaichit S, Rungjindamai N, Rukachaisirikul V and Sakayaroj J. 2006. Antimicrobial activity in cultures of endophytic fungi isolated from Garcinia species. FEMS Immunology & Medical Microbiology. 48(3): 367-372.
56. Pinheiro EA, A Carvalho, JM dos Santos, DCP Feitosa, ADO Marinho and Marinho A. M DR. 2013. Antibacterial activity of alkaloids produced by endophytic fungus *Aspergillus sp*. EJC08 isolated from medical plant *Bauhinia guianensis*. Natural product research, 27(18): 1633-1638.
57. Qin G and Xu R. 2012. Recent advances on bioactive natural products from Chinese medicinal plants. Medical Research Reviews. 18(6): 375–382.
58. Rabha AJ, Naglot A, Sharma GD, Gogoi HK and Veer V. 2014. In vitro evaluation of antagonism of endophytic *Colletotrichum gloeosporioides* against potent fungal pathogens of *Camellia sinensis*. Indian journal of microbiology. 54(3): 302-309.
59. Rajagopal K and Suryanarayanan TS. 2000. Isolation of endophytic fungi from leaves of neem (*Azadirachta indica A. Juss*.). Current Science. 6: 1375-1378.
60. Redman RS, Kim YO, Woodward CJ, Greer C, Espino L, Doty SL and Rodriguez RJ. 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLOS one. 6(7): 128- 148.
61. Rodriguez R J, White Jr J F, Arnold AE and Redman R S. 2009. Fungal endophytes: diversity and functional roles. New Phytologist, 182(2): 314– 330.
62. Ruma K, Kumar S and Prakash HS. 2013. Antioxidant, anti-inflammatory, antimicrobial and cytotoxic properties of fungal endophytes from *Garcinia species*. International Journal of Pharmacy and Pharmaceutical Sciences, 5(3): 889-897.
63. Saikonen K, Faeth SH, Helander M and Sullivan TJ. 1998. A continuum of interactions with host plants. Annu Rev Ecol Syst. 29: 319-343.
64. Schulz B, Boyle C, Draeger S, Römmert A K and Krohn K. 2005. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological research. 106: 996–1004.
65. Selim K, El-Beih A, Abdel-Rahman T and El-Diwany A. 2012. Biology of endophytic fungi. CREAM. 2: 31-82.
66. Sharma R and Kumar BS. 2013. Isolation characterization and antioxidant potential of endophytic fungi of *Ocimum sanctum Linn*.(Lamiaceae). Indian J Appl Res, 3(7): 5-10.

67. Shweta S, Zuehlke S, Ramesha BT, Priti V, Kumar PM, Ravikanth G and Shaanker RU. 2010. Endophytic fungal strains of *Fusarium solani* from *Apodytes dimidiata* E Mey ex Arn (Icacinaceae) produce camptothecin 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry .71(1): 117-122.
68. Singh S and Singh P. 2012. Effectiveness of *Tinospora cordifolia*stem extract on bacteria *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella dysenteriae*. International Journal of Pharmacy & Life Sciences. 3(8): 34-42.
69. Sonaimuthu V, Krishnamoorthy S and Johnpaul M. 2010. Taxol producing endophytic fungus *Fusarium culmorum* SVJM072 from medicinal plant of *Tinospora cordifolia*-a first report. Journal of Biotechnology. 150: 425.
70. Srinivasan K, Jagadish LK, Shenbhagaraman R and Muthumary J. 2010. Antioxidant activity of endophytic fungus *Phyllosticta sp*. isolated from *Guazuma tomentosa*. Journal of phytology. 2(6).
71. Stierle A, Strobel GA and Stierle D. 1993. Taxol and taxane production by *Taxomyces andreanae,* an endophytic fungus of Pacific Yew. Sci. 9: 214-216.
72. Strobel G, Daisy B and Castillo. 2004. Natural products from endophytic microorganisms. Journal of Natural Products. 67: 257-268.
73. Strobel GA. 2003. Endophytes as source of bioactive products. Microbiol Infect. 5: 533-544.
74. Sun H, Xiao Y and Tian Y. 2013. Isolation, characterization, and antimicrobial activity of endophytic fungi from *Tamarix chinesis*. Afr.J.Microbiol. 7: 1496–1504.
75. Sundram K, Sambanthamurthi R, and Tan YA. 2003. Palm fruit chemistry and nutrition. Asia Pacific journal of clinical nutrition. 12(3).
76. Tamura K, Peterson D, Peterson N, Stechel, G, Nei, M and Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution. 28(10): 2731-2739.
77. Tan RX and Zou WX. 2001. Endophytes: a rich source of functional metabolites. Nat Prod Rep. 18: 448-459.

78. Tejesvi MV, Kini KR, Prakash HS, Subbiah V and Shetty HS. 2007. Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants. Fungal Diversity. 24: 37-54.
79. Tejesvi MV, Mahesh B ,Nalini MS, Prakash HS, Kini KR, Subbiah V and Shetty HS . 2005. Endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* W and A(Combretaceae). World Journal of Microbiology and Biotechnology. 21(8-9): 1535-1540.
80. Tejesvi MV, Tamhankar SA, Kini KR, Rao VS and Prakash H S. 2009. Phylogenetic analysis of endophytic *Pestalotiopsis species* from ethnopharmaceutically important medicinal trees. Fungal Diversity. 38: 167.
81. Vasundhara M, Baranwal M, and Kumar A. 2016. *Fusarium tricinctum*, an endophytic fungus exhibits cell growth inhibition and antioxidant activity. Indian J. Microbiol. 56: 433–438.
82. Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN and Gange AC. 2009. Endophytic actinomycetes from *Azadirachta indica* A. Juss.: isolation, diversity, and antimicrobial activity. Microbial ecology. 57(4): 749-756.
83. Verma VC, Lobkovsky E, Gange AC, Singh SK and Prakash S. 2011. Piperine production by endophytic fungus *Periconia sp*. isolated from *Piper longum* L. Journal of Antibiotics. 64(6): 427.
84. Vieira M L, Johann S, Hughes FM, Rosa CA and Rosa LH. 2014. The diversity and antimicrobial activity of endophytic fungi associated with medicinal plant *Baccharis trimera* (Asteraceae) from the Brazilian savannah. Canadian journal of microbiology, 60(12), 847-856
85. Wani M C, Taylor H L, Wall M E, Coggon P and McPhail AT. 1971. Plant antitumor agents VI: the isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. Journal of American Chemical Society. 93: 2325-2327.
86. Weber D, Sterner O, Anke T, Gorzalczancy S, Martino V and Acevedo C. 2004. Phomol a new antiinflammatory metabolite from an endophyte of the medicinal plant *Erythrina crista-galli*. The Journal of Antibiotics. 57(9): 559-563.
87. Wilson D and Carroll GC. 1995. Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*. Mycologia. 86: 635-647.

88. Wilson D. 1995. Endophyte-the evolution of a term, a clarification of its use and definition. Oikos. 73:274–276.
89. Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwiset K and Meevootisom V. 2004. Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. World J Microb Biot. 20: 256-272.
90. Xiaoming Tan, Yaqin Zhou, Xiaolei Zhou, Xianghua Xia, Ying Wei, Lili He, Hongzhen Tang and Liyin Yu. 2018. Diversity and bioactive potential of culturable fungal endophytes of *Dysosma versipellis*; a rare medicinal plant endemic to China. Nature. 8: 5929-5932.
91. Zhang HW, Song YC and Tan RX. 2006. Biology and chemistry of endophytes. Nat Prod Rep. 23: 753-771.
92. Zhao J, Mou Y, Shan T, Li Y, Zhou L, Wang M and Wang J. 2010. Antimicrobial metabolites from the endophytic fungus *Pichia guilliermondii* isolated from Paris *polyphylla* var yunnanensis. Molecules. 15(11): 7961-7970.

APPENDIX

Table 20: Composition of MHA

Ingredients	Amount
Amount Beef infusion	30%
Starch	0.15%
Casein hydrolysate	1.75%
Agar	% 1.7%

Table 21: Composition of PDA

Ingredients	Gram/liter
Potato infusion	200
Dextrose	20

Table 22: McFarland standard

McFarland No.	0.5
1.0% $BaCl_2.2H_2O$ (ml)	0.05
1.0% H_2SO_4(ml)	9.95
Approx. Cell density (cfu/ml)	1×10^8
Percentage transmittance	74.3
Absorbance at 600nm	0.132