

Isolation and characterization of bioactive molecules from endophytic fungi

A Thesis

Submitted in fulfillment of the requirement
for the award of the degree of

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

By

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Certificate

This is to certify that the thesis entitled, **“Isolation and characterization of bioactive molecules from endophytic fungi”** submitted by M.Vasundhara in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar University, Patiala, is an authentic record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.



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Candidate's Declaration

I, hereby declare that the work which is being presented in this thesis entitled **“Isolation and characterization of bioactive molecules from endophytic fungi”**, in fulfillment of the requirement for the award of the Doctor of Philosophy in Biotechnology, Department of Biotechnology, Thapar University, Patiala, Punjab, is an authentic record of my own independent and original research work carried out under the supervision of Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree in India or abroad.


M. Vasundhara

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Date: 13/2/2017

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Dedicated to My Parents.....

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The following publications are the outcome of the present research work

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Abbreviations

Abbreviation	Description
µm	Micrometer
Aa	amino acid
AD	Anno Domini
ACN	acetonitrile
Bp	base pair
BLAST	Basic Local Alignment Search Tool
AIDS	acquired immune deficiency syndrome
cDNA	complementary DNA
CHCl ₃	chloroform
Cm	centimeter
CTAB	Cetyl trimethylammonium bromide
DAD	diode array detector
DCM	dichloromethane
Dd	double distillation
DMSO	dimethyl sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
et. al.	et alii
F	forward
FW	fresh weight
G	gram
Inc.	Incorporation
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kg	kilogram
kV	kilo volt
L	Litre
L.	<i>Linnaean</i>
M	molar
m/z	mass-to-charge ratio
MeOH	methanol
Mg	milligram
Min	minute
mM	millimolar
NCBI	National Center for Biotechnology Information

Ng	nanogram
Nm	nanometer
No.	number
°C	degree celsius
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative decimal logarithm of the hydrogen ion activity
R	reverse
RNA	ribonucleic acid
Rpm	revolutions per minute
SD	standard deviation
Sec	second
sp.	specie
spp.	species
T. baccata	<i>Taxus baccata</i>
µg	micro gram
µL	micro liter
Tm	melting temperature
V	volt
V_m/V_f	medium-to-flask volume
v/v	volume per volme
w/v	weight per volume
DPPH	2,2-diphenyl-1-picrylhydrazyl
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PBMC	peripheral blood mononuclear cell
UHPLC–QTOF-MS/MS	ultra-high-performance liquid chromatography quadrupole time of flight mass spectrometry
UHPLC–DAD	ultra-high-performance liquid chromatography diode array detector
HRMS	High resolution mass spectrometry
TNF- α	Tumor necrosis factor alpha
Hela	Cervical cancer cell lines
MCF-7	human breast adenocarcinoma cell line
ESI	Electrospray ionization

LC/ESI-MS	Liquid chromatography electrospray ionization tandem mass spectrometry
FDA	Food and Drug Administration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
ATCC	American Type Culture Collection
SDB	Sabouraud dextrose broth
MHB	Mueller hinton broth
DMEM	Dulbecco's Modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
MIC	Minimum Inhibitory Concentration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
BSA	Bovine Serum Albumin
CMC	Carboxymethyl cellulose
pNPP	para-Nitrophenylphosphate
ITS	Internal transcribed spacer

I. Introduction

Endophytes are ubiquitous organisms present in the tissues of the plants, during a part of their life without technically infecting the host (Bacon and White 2000) and fungi are the dominant endophytes (Strobel and Daisy 2003). Majority of the endophytic fungi belong to Ascomycota and colonize the intercellular spaces of the plant without any visual symptoms of their presence (Corrêa et al. 2014). Endophytic fungi are capable of producing a wide range of bioactive compounds. Through accumulation of secondary metabolites, the endophytic fungi reduce the damage from the pathogens on the hosts (Cabezas et al. 2012). Several of bioactive compounds produced by these fungi have applications in environment, agriculture, food and pharmaceutical industries (Deshmukh et al. 2015; Suryanarayanan et al. 2009; Kharwar et al. 2011).

Endophytic fungal communities mainly belong to Ascomycota, Basidiomycota and Zygomycota. Identification of the fungal isolates heavily depends on the reproductive structures in classical taxonomy. However, the non-sporulating fungi cannot be easily identified (Sun and Guo 2012). The use of molecular tools, such as molecular markers and sequencing methods overcomes the difficulties in conventional taxonomy of culturable fungi. In endophytic fungi, internal transcribed spacers (ITS) region, 18S and 28S rRNA genes are being used in the identification of endophytic fungi. Pandey et al. (2003) identified different isolates of *Phyllosticta* that were isolated from different tropical tree species in India as *P. capitalensis* based on ITS sequence analysis. Different endophytic fungi were isolated from *Phyllostachy* and *Sasa* species and placed into Sordariomycetes and Dothideomycetes based on 18S rRNA sequence analyses (Morakotkarn et al. 2007). Endophytic fungi belonging to Xylariaceae isolated from 22 tree species of a dry thorn forest and 27 tree species of a stunted montane evergreen forest of the Western Ghats in southern India were identified as *Xylaria* or

Nemania species based on their ITS sequence analysis (Govindarajulu et al. 2013). Suryanarayanan et al. (2011) identified different endophytic fungi isolated from the Western Ghats of India based on ITS sequences and phylogenetic analysis. In endophytic fungi, ITS region is considered as the most widely used DNA barcode in molecular identification, despite some limitations in species distinction (Sun et al. 2011).

Endophytic fungi are the source for natural products with diverse variety of biological activities. The extracts of endophytic fungi have been reported to show antimalarial, antimicrobial and cytotoxic activities on human cell lines (Rodrigues et al. 2006). Diverse variety of natural products have recently been identified from endophytic fungi that include substances that have shown promising anti-cancer, antioxidant, anti-viral, immunosuppressing and other bio-activities. (Strobel and Daisy 2003; Nisa et al. 2015).

These natural products include steroids, xanthenes, phenols, isocoumarines, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides, cytochalasins, etc. (Schulz and Boyle, 2005). Number of novel chemical structures produced by endophytes (51%) is significantly higher than the soil fungus (38%), an aspect that has been hitherto overlooked in the search of novel lead molecules and other bioactive substances (Schulz and Boyle. 2005; Nisa et al. 2015). These secondary products or metabolites facilitate effective resistance to plants from nematodes, insects and other pests. Further, these substances that are associated with plants of medicinal value are envisaged to be exploited as antidotes for various diseases (Tejesvi et al., 2007). Recent discoveries in the area of these secondary metabolites from endophytic fungi has risen the promise towards using such organisms for the production of bioactive substances of medicinal value (Priti et al. 2009).

Filamentous fungi are the important source of industrial enzymes due to their ability to produce extracellular proteins. Amylase, cellulase, lipase, pectinase, laccase, phytase and protease are some examples of enzymes produced by these fungi (Dicosimo et al. 2013; Singh et al. 2013).

Industrial processes generally require robust enzymes, able to work in a wide range of conditions such as extreme pH, temperature, osmolarity, pressure, etc. The metabolites have also been found to facilitate resistance to the host plants against herbivores and pathogens (Ganley et al. 2008). They utilize several hydrolytic enzymes for cell wall degradation (Goldbeck et al. 2013). Though endophytic fungi are well known to produce unique bioactive metabolites, they have not yet been extensively exploited as sources of industrial enzymes although it has been well studied that these organisms produce variety of enzymes as a part of their natural processes. These include hydrolytic and oxidative enzymes that facilitate colonization in their hosts (Corrêa et al. 2014).

The main objectives of the present thesis include isolation and identification of effective endophytic fungi from *Taxus baccata*, screening of the fungal extracts for their antimicrobial, anticancer and antioxidant activities and identification of different bioactive compounds from the efficient endophytic fungi. An attempt has been made for the production of different extracellular enzymes by thermotolerant (Agni's fungi) endophytic fungi isolated from different hosts from Western Ghats (Suryanarayanan et al. 2011), India. Some of these enzymes produced by endophytic fungi were tested for their thermal tolerance and thermal stability.

II. Review of Literature

2.0 NATURAL PRODUCTS IN DRUG DISCOVERY AND DEVELOPMENT

Antibiotics first came into market half a century ago and overuse of these drugs led to emergence of human pathogenic bacteria that became multi drug resistant. Many of the antibiotics are losing their therapeutic effectiveness since many Gram positive and Gram negative bacteria have evolved resistance against them. Over the past 25 years, the number of antibacterial drugs clearing all phases of drug development process and reaching the market has decreased. The steady decline in the new chemical entities (NCEs) being brought into the market by pharmaceutical companies has led to interest and appreciation of the value of natural products. To overcome the problem of resistance to drugs there is an urgent need to look for alternatives to the currently available antimicrobial drugs (Cuevas, 2003; Radic et al. 2012). There is a quest to develop new pharmaceuticals for curing infections such as cancer, HIV and other emerging new diseases. Traditional plant based systems, microorganisms, extremophiles, endophytes and more recently marine organisms have provided number of natural products with bioactivity that have wide applications. The novel drugs from these natural sources remains unexplored (Cragg and Newman 2013).

2.1 ENDOPHYTIC FUNGAL DIVERSITY

Endophytic fungi are permeate in nature occurring in majority of plants, playing a significant role in natural existence of plants (Sun and Guo 2012). Endophytic fungi mainly belong to the Ascomycota or their mitosporic fungi, and to the Basidiomycota, Zygomycota and Oomycota (Zheng and Jiang 1995; Sinclair and Cerkaskas 1996). These endophytic fungi reside in the living tissue of host plant (Perotto et al. 2002; Strobel et al. 2004; Verekar et al. 2014). Some types of mycorrhizae and endophytes such as ericoid mycorrhizae and pseudomycorrhizae are indistinct. Therefore, some mutualistic root-inhabited or mycorrhizal fungi associated with

Ericaceae and Orchidaceae plants are termed as endophytes (Bills 1996; Bayman et al. 1997; Stoyke and Currah 1991). These fungi are able to produce various bioactive compounds (Liu et al. 2011; Aly et al. 2010; Deshmukh et al. 2015), that facilitate growth of the host and also its capacity to degrade plant litter (Purahong and Hyde 2011; Sun et al. 2011) and tolerate environmental stress (Ting et al. 2008; Saikkonen et al. 2010); and more importantly in the host's participation in bio-geochemical cycling. The colonization rate and the isolation rate of these fungi from plants vary considerably; some medicinal plants harbor more endophytes than others (Huang et al. 2008).

2.1.1 Traditional techniques used in endophyte studies

Traditionally, fungi living inside the plant tissues can be recognized either by direct observation or cultivation-dependent methods. Fungi that are endophytic can be observed within living plant tissues using tools such as light and electron microscope (Deckert et al. 2001; Lucero et al. 2011). However, most of these fungi have only hyphal structure within their hosts thus resulting in limitations in their taxonomic categorization and therefore, are not commonly used in endophyte diversity studies (Deckert et al. 2001). The culture dependent methods have been routinely used to study the diversity of endophytes (Suryanarayanan et al. 2011; Sun and Guo 2012). It is important to isolate these fungi for their characterization, species diversity, population dynamics and screening for bioactive compounds (Ding et al. 2009; Li et al. 2011; Reddy et al. 2016; Tejesvi et al. 2011).

The primary and important step in the isolation of endophytic fungi involves culture-dependent technique wherein the fungi grow from the plant tissues that are placed in solid growth medium. This technique generally involves i) thorough washing of the plant tissue to remove soil particles and major epiphytes adhering to the tissue, ii) surface sterilisation to kill any microorganisms on the host surface (Hallmann et al. 2006), iii) isolation of endophytic fungi growing out from tissues placed on nutrient agar, iv) purification and sporulation of endophytic

fungi under varied incubation conditions, and v) identification of the endophytic fungi based on their morphological characteristics (Sun and Guo 2012) (Figure 2.1).

To increase fungal diversity, the sterilized plant tissue is cut into small segments (ca. 5 mm diam.), macerated or ground, before transferring onto nutrient agar for incubation. Media such as potato dextrose agar (PDA) and malt extract agar (MEA), as well as minimal media with plant tissue or extract are commonly used for isolation of endophytic fungi. Therefore, methods have been developed to promote sporulation, which includes use of different media and inclusion of sterile host tissue in cultures. Guo et al. (1998) reported the increased sporulation from 48% to 59% by the addition of sterile leaf tissue for isolates obtained from *Livistona chinensis*. The number of identifiable isolates were further increased to 83.5% by inoculating the remaining unidentified isolates onto sterile petiole pieces at room temperature for few months. Some fungi may sometimes get to remain ignored or missed as of limitations or absence of growth, resulting in the domination of fast-growing species. Despite the development of different methods to induce sporulation (Guo et al. 2000), high numbers of isolates do not sporulate in cultures (Guo et al. 2008; Photita et al. 2001; Kumaresan and Suryanarayanan 2002; Sun et al. 2011). Traditional classification of fungi depends dominantly on reproductive structures, and thus, non-sporulating species cannot be identified taxanomically. The potential technical biases in traditional endophyte studies can be overcome by molecular techniques. DNA fingerprinting and sequencing methods; and other related molecular techniques, help in overcoming the limitations of the conventional cultivation-dependent methods (Fig. 2.1). Ercole et al. (2013) reported that cryopreservation of fungi including mycorrhizal fungi at -80°C is an effective method to keep their infective ability intact and store for longer duration.

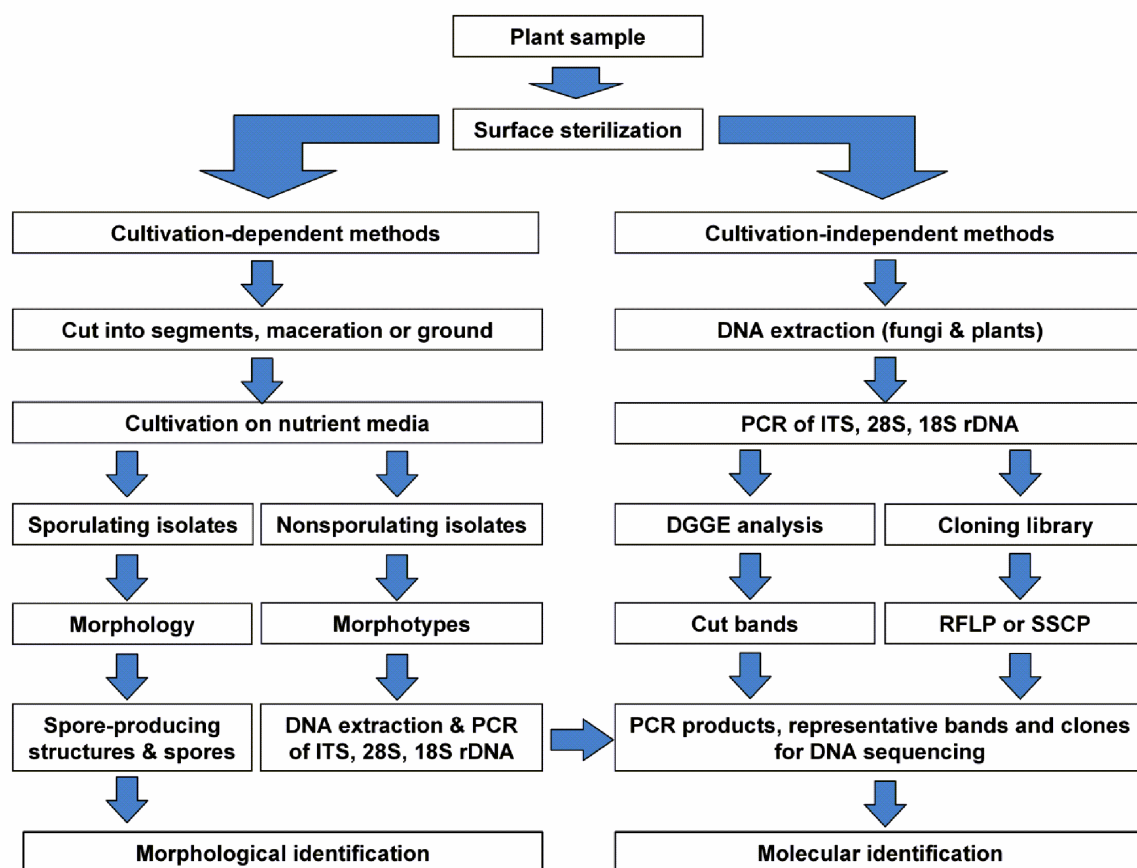


Fig. 2.1. Schematic diagram showing identification of the cultivable and non-cultivable endophytic fungal communities from a plant source (Source: Sun and Guo 2012)

2.1.2 Molecular identification of fungi

In endophyte studies, 5.8S gene and flanking internal transcribed spacers (ITS1 and ITS2) of the rDNA, 18S and 28S rRNA genes have been employed in the identification of endophytic fungi. Pandey et al. (2003) identified different isolates of *Phyllosticta* that were isolated from different tropical tree species in India as *P. capitalensis* based on ITS sequence analysis. Morakotkarn et al. (2007) isolated 71 (of 257 strains) endophytic fungi from *Phyllostachy* and *Sasa* species and placed them into Sordariomycetes and Dothideomycetes based on 18S rRNA sequence analyses and further identified them into lower taxonomic levels based on ITS sequences. Endophytic fungi belong to Xylariaceae isolated from 22 tree species of a dry thorn forest and 27 tree species of a stunted montane evergreen forest of the Western Ghats in

southern India were identified as *Xylaria* or *Nemania* species based on their ITS sequence analysis (Govindarajulu et al. 2013). Sun et al. (2011) clustered 221 non-sporulating endophyte strains into 56 morphotypes, and placed these morphotypes into 37 taxa based on ITS sequence similarity and phylogenetic analysis. Suryanarayanan et al. (2011) identified different endophytic fungi isolated from the Western Ghats of India based on ITS sequences and phylogenetic analysis. DNA sequencing analyses coupled with morphology have been widely used in the investigation of endophyte diversity, particularly for ecology studies. Reddy et al. (2016) studied for the occurrence of *Pestalotiopsis* as foliar endophytes from one hundred tree species from 4 different forest types of the Western Ghats. Morphological and ITS sequence studies showed that species of *Pestalotiopsis* are generalist endophytes infecting taxonomically unrelated tree hosts. Hoff et al. (2004) studied 27 fungal genera belonging to Ascomycota, Zygomycota and Basidiomycota from roots of *Pseudotsuga menziesii* and *Pinus ponderosa* using a combination of morphology and ITS sequence data. Botella and Diez (2011) studied the phylogenic diversity of fungal endophytes of *Pinus halepensis* based on the analyses of ITS and 28S gene sequences, and reported that Dothideomycetes was the dominant class.

Due to the limitations of traditional isolation procedures, it is highly probable that many endophytic fungi cannot be brought onto the culture. To overcome the potential technical bias, molecular approaches have been applied in the identification of endophytic fungi directly within the host tissues. This process involves i) extraction of total genomic DNA from surface-sterilized plant tissues, ii) amplification of DNA fragments (e.g. ITS, 28S and 18S genes) with fungal specific primers, iii) denaturing gradient gel electrophoresis (DGGE) and excision of different bands iv) cloning and sequencing of representative clones and identifying the sequences into various taxonomic levels based on phylogenetic analysis (Figure 2.1). High-throughput sequencing also serves as a powerful alternative to molecular studies of fungal community in natural environments. This technique has been successfully employed to study

the fungal diversity in phyllosphere fungi (Jumpponen and Jones 2009), mycorrhizal fungi (Daghino et al. 2008; 2012) and other natural environments. Broad spectrum of fungal ITS sequences were amplified from genomic DNA isolated from *Heterosmilax japonica* tissues by Gao et al. (2005). Some of them were identified as *Aureobasidium*, *Botryosphaeria*, *Cladosporium*, *Glomerella*, *Mycosphaerella*, *Phomopsis* and *Guignardia* which are commonly isolated fungi while others as uncultured and may represent novel fungal taxa. PCR primers were developed by Barbi et al. (2014) to understand the diversity of genes that encode lignocellulolytic enzymes in soils with use of high-throughput sequencing. The method followed was observably robust and facilitated diversity characterization of different fungal transcripts involved in the degradation of floral organic matter as well as complex patterns in the expression of genes in the communities. Damon et al. (2012) revealed that metatranscriptomics approach plays an important role in the studies related to the genetic diversity of eukaryotes in soils sourced from forests. Compared to traditional Sanger sequencing methods, pyrosequencing enhances the characterization of fungal diversity as reported by Gillevet et al. (2009) wherein this method not only enhances the number of different contigs detected but also in revealing entire clades missed by traditional sequencing by using different fungal ITS contigs from *Spartina alterniflora*. Pyrosequencing technique was applied first time in endophytic fungi of *Atriplex canescens* and *A. torreyi* var. *griffithsii* by Lucero et al. (2011).

DNA barcoding systems is another technique employed to identify fungal species (Hebert et al. 2003). DNA barcode region used should be a single locus for all groups of organisms across all kingdoms. In endophyte studies the most widely used DNA barcode in molecular identification is the ITS region (Guo et al. 2003; Murali et al. 2006; Promptuttha et al. 2007; Sun et al. 2011).

2.1.3 Internal transcribed spacers (ITS): The nucleotide sequence of ribosomal DNA (rDNA) changes very slowly and it is arranged in tandemly repeated units containing the coding regions, in eukaryotes, for highly conserved regions and variable regions such as internal transcribed spacer (ITS) regions and 18S, 5.8S, and 28S ribosomal RNA separated by spacers. Fungal rRNA operons contain two ITS regions (Fig. 2.2). One is located between the 18S and 5.8S rRNA genes (ITS1) and the other exists between the 5.8S and 28S rRNA genes (ITS2). The sequence of the two ITS regions accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes because the two ITS sequences are deleted as they are not required after the transcription of rRNA operon. Hence analysis of ITS regions (variation in the spacers) has proven useful for distinguishing among a wide diversity of taxa that are not identifiable. The ITS region is now conceivably the most widely sequenced DNA region in fungi. Gardes and Bruns (1993) designed two taxon selective primers, ITS1-F and ITS4-R specific for fungi and basidiomycetes, respectively.

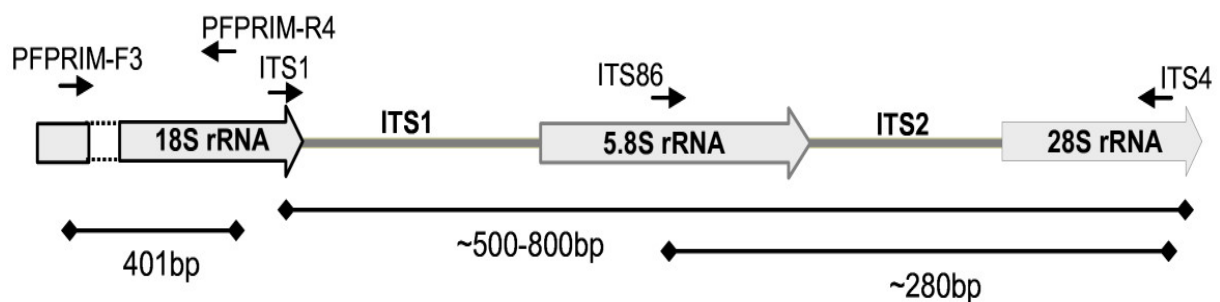


Fig. 2.2. Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations (Embong et al. 2008)

2.2 ANTIBACTERIALS / ANTIMYCOBACTERIALS FROM ENDOPHYTIC FUNGI

ESKAPE organism's, *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species are the main infectious agents

in a majority of US hospitals and are resistant to many of the currently available drugs as reported by Rice (2008). European Federation of Pharmaceutical Industries and Associations (EFPIA) supported the launch of 'Innovative Medicines' to solve antimicrobial resistance and to develop new antibiotics (Radic et al. 2012).

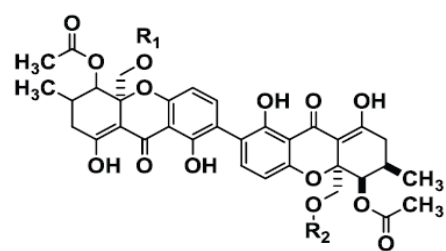
Lim et al. (2010) isolated different antibacterial compounds such as dicerandrol A (1), dicerandrol B (2), dicerandrol C (3), deacetylphomoxanthone B (4) and fusaristatin A (5) (Fig. 2.3) from *Phomopsis longicolla* S1B4 from a plant sample in South Korea. These compounds showed antibacterial activities against *Xanthomonas oryzae* KACC 10331 with minimum inhibitory concentrations (MICs) of 8, 16, >16, 4, and 128 µg/mL, respectively. Dicerandrol A also showed antibacterial activity against *S. aureus* KCTC 1916, *Bacillus subtilis* KCTC 1021, *Clavibacter michiganensis* KACC 20122 and *Erwinia amylovora* KACC 10060, with MIC values of 0.25, 0.125, 1.0, and 32.0 µg/mL, respectively. Phomoxanthone A (6) (Fig. 2.3) isolated from *Phomopsis* species associated with *Costus* sp. showed antibacterial activity against *B. megaterium* (10 mg/mL) with 3-4 cm zone of inhibition (Elsaesser et al. 2005). Many endophytic fungi belonging to Ascomycetes are known to produce antibacterial compounds such as colletotric acid (7) (Fig. 2.3) isolated from endophytic fungus *Colletotrichum gloeosporioides* associated with *Artemisia mongolica*. This compound has shown antibacterial activity against *B. subtilis*, *S. aureus*, and *Sarcina lutea* with MIC values of 25, 50, and 50 µg/mL, respectively (Zou et al. 2000).

Kharwar et al. (2009) isolated javanicin (naphthaquinone), an antibacterial compound (8) (Fig. 2.3), from endophytic fungus *Chloridium* sp., associated with *Azadirachta indica*. The growth of *P. fluorescens* and *P. aeruginosa* was inhibited by this compound with MIC values of 2 µg/mL. Arunpanichlert et al. (2010) isolated (+)-sclerotiorin, (9) (Fig. 2.3) from *Penicillium sclerotiorum* PSU-A13 which showed antibacterial activity against *S. aureus* ATCC 29213 with a MIC value of 128 µg/mL (Lucas et al. 2007). Wang et al. (2014) isolated sanguinarine,

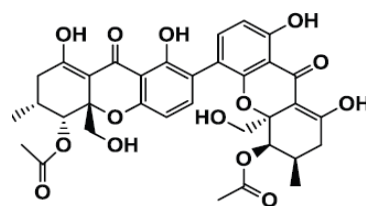
(10) (Fig. 2.3) a benzophenanthridine alkaloid from *Fusarium proliferatum* associated with *Macleaya cordata*. This compound exhibited antibacterial, anti-inflammatory and anthelmintic activities. Antibacterial activity of this compound against fifteen clinical isolates of *S. aureus* (MICs of 3.12–6.25 µg/mL) and two strains used as reference (MIC 3.12 µg/mL for ATCC 25923 and 1.56 µg/mL for ATCC 33591) has also been reported.

Sim et al. (2010) isolated 24 endophytic fungi belonging to *Garcinia mangostana* and *G. parvifolia* and studied the antibacterial activity by using filtered broth suspension. Eleven isolates (about 46%) showed antibacterial activity against at least one test microorganism. *Colletotrichum* sp. isolated from a medicinal plant *Lippia sidoides* showed an antiseptic activity (de Siqueira et al. 2011). More than 200 endophytic fungi were isolated from leaves and stems of *L. sidoides* which represented species belonging to Ascomycota, Coelomycetes and Hyphomycetes. *Colletotrichum gloeosporioides* was the fungus most prominently found, followed by *A. alternata*, *Guignardia bidwelli* and *Phomopsis archeri*. The endophytic fungi, *A. alternata*, *P. archeri*, *C. gloeosporioides* and *Drechslera dematioidea* exhibited antimicrobial activity against *S. aureus* and *B. subtilis* (Ichikawa et al. 1971).

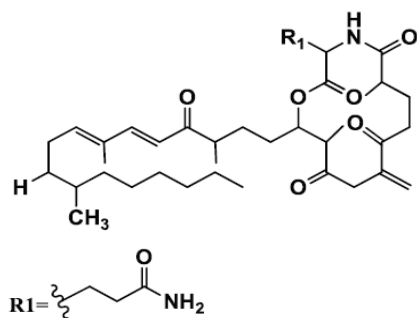
The first cytochalasin-type compound, phomopsichalasin, (11) (Fig. 2.4) was obtained as a metabolite from an endophytic fungus, *Phomopsis* species which exhibited antibacterial activity against *B. subtilis* and *S. aureus* (Horn et al. 1995).



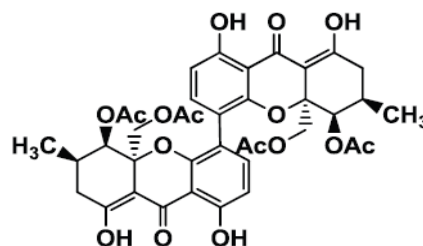
R₁=R₂=H Dicerandrol A (1)
 R₁=Ac, R₂=H Dicerandrol B (2)
 R₁=R₂=Ac Dicerandrol C (3)



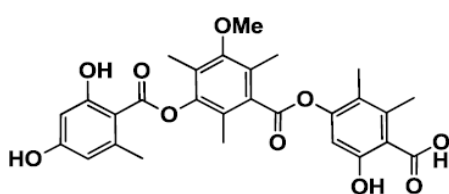
Deacetylphomoxanthone B (4)



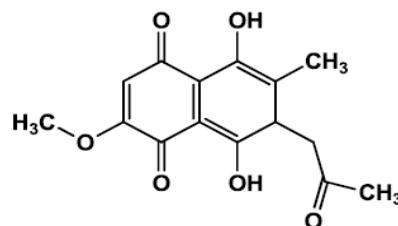
Fusaristatin A (5)



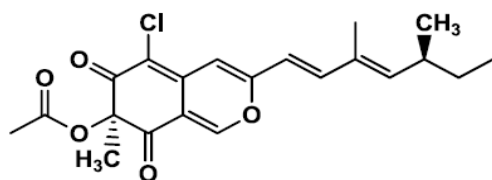
Phomoxanthone A (6)



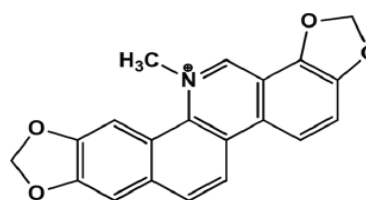
Colletotric acid (7)



Javanicin (8)



Sclerotiorin A (9)



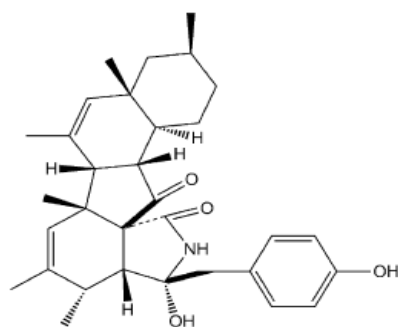
Sanguinarine (10)

Fig. 2.3. Antimicrobial molecules from endophytic fungi

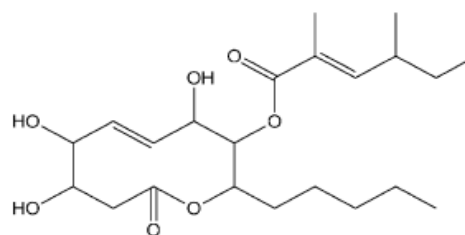
Weber et al. (2004) isolated phomol (12) (Fig. 2.4) from *Phomopsis* species associated with the medicinal plant *Erythrina crista-galli*, an Argentinean leguminous plant. This compound exhibited antimicrobial, anti-inflammatory and moderate cytotoxicity. The anti-inflammatory activity was tested in different reporter gene systems such as tumor necrosis factor (TNF)- α , STAT1/STAT2 and NF- κ B) and in a mice oedema (ear) model. Phomol exhibited no activity

in the reporter gene assay, whereas anti-inflammatory activity was seen in the mouse ear assay. Several new aromatic, hydrogenated, and structurally unique ring-extended xanthenes were reported from different endophytic fungi (Krohn et al. 2009). Three new fungal metabolites, microsphaeropsone A (13), B and C (14) (Fig. 2.4) with a unique oxepino [2,3-b] chromen-6-one (ring-enlarged xanthone) skeleton, citreorosein and an enone (oxidized microsphaeropsone A) were characterized from the endophytic fungus *Microsphaeropsis* sp. Fusidienol A and known aromatic xanthenes were isolated from the endophytic fungus *Microsphaeropsis* sp. A compound, 3,4- dihydroglobosuxanthone A isolated from *Microdiplodia* sp., showed antibacterial activity against *E. coli* (Krohn et al. 2009). The production of hypericin, a naphthodianthrone derivative, along with emodin, its precursor, from endophytic fungi, *Thielavia subthermophila* from *Hypericum perforatum* showed antibacterial activity towards variety of microbes such as bacteria and fungi, including *S. aureus* ssp. *aureus*, *K. pneumoniae* ssp. *ozaenae*, *P. aeruginosa*, *Salmonella enterica* ssp. *enteric*, and *E. coli* (Kusari et al. 2008).

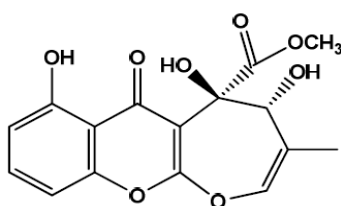
The emergence of antibiotic-resistant *S. aureus* has led to the screening of antimicrobial compounds from endophytes. The extracts obtained from hexane, ethyl acetate and methanol of *C. gloeosporioides* isolated from *Vitex negundo* L exhibited antibacterial activity against bacteria and fungi (Arivudainambi et al. 2011). The fungal extracts were also tested against methicillin, penicillin- and vancomycin-resistant *S. aureus* strains (1–10). In addition, antibacterial activity was repeated with methanol extract, against *S. aureus* strain 9. The concomitant action of the extract along with antibiotics viz., methicillin, penicillin and vancomycin was observed against *S. aureus* strain 6.



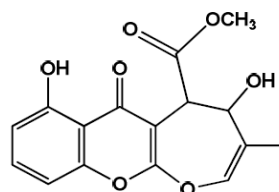
Phomopsichalasin (11)



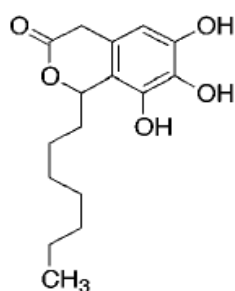
Phomol (12)



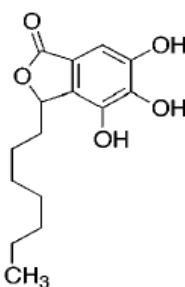
Microsphaeropsone A (13)



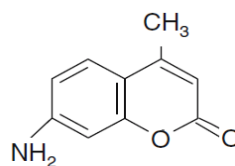
Microsphaeropsone C (14)



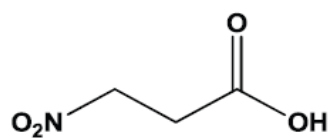
Cytosporone D (15)



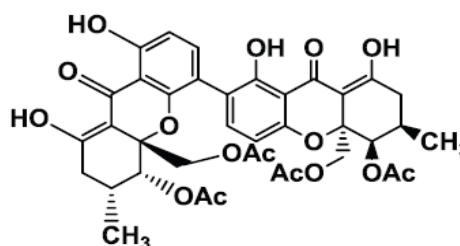
Cytosporone E (16)



7-amino-4-methylcoumarin (17)



3-nitropropionic acid (18)



Phomoxanthone B (19)

Fig. 2.4. Antimicrobial molecules from endophytic fungi.

Casella et al. (2013) isolated 138 endophytes from 24 plant species and tested for their antifungal and cytotoxic activities. They reported that some of endophytes exhibited antifungal activity against *C. albicans* ATCC 10213 and cytotoxic potential on human cell lines such as

KB (uterine cervical carcinoma), MDA-MB-435 (melanoma), and MRC5 (normal human lung fibroblasts). They isolated different antifungal compounds which includes acremonisol A, semiochliodinol A, cochliodinol, griseofulvin, pyrenocin A, novae zelandin A and alterperyleneol A and pyrrocidine C. Antibiotic activity of the extracts obtained from the endophytic fungal strains from Guanacaste Conservation Area of Costa Rica was investigated (Brady et al. 2000). Antibiotic activity was reported in two endophytes CR146 (*Diaporthe* sp.) and CR200 (*Cytospora* sp.). The fractionated extracts resulted in characterization of cytosporones D (15) and E (16) (Fig. 2.4) and antibacterial active trihydroxybenzene lactones. Using X-ray crystallography and NMR five new octaketides were characterized.

Antimicrobial metabolite, 7-amino-4-methylcoumarin (17) (Fig. 2.4) was obtained from the endophytic fungus *Xylaria* sp., isolated from *Ginkgo biloba* L. (Liu et al. 2008). This compound showed strong antibacterial and antifungal activities against various pathogens *in vitro*. Some of these organisms include *S. aureus* (MIC 16 µg/mL), *E. coli* (MIC, 10 µg/mL), *Salmonella typhimurium* (MIC 20 µg/mL), *S. typhimurium* (MIC 15 µg/mL), *Vibrio anguillarum* (MIC 25 µg/mL), *C. albicans* (MIC 15 µg/mL), *Penicillium expansum* (MIC 40 µg/mL), and *Aspergillus niger* (MIC 25 µg/mL). Flores et al. (2013) isolated 3-nitropropionic acid (18) (Fig. 2.4) from the endophytic fungus, *Phomopsis longicolla* associated with *Trichilia elegans* A. JUSS ssp. *Elegans*. 3-nitropropionic acid has been reported as potential neurotoxicant and is a inhibitor of *M. tuberculosis*. The study reported by Rakshith et al. (2013) revealed that endophytic *Phomopsis* species are prolific producers of antimicrobial metabolites with a wide range of antimicrobial activity. An endophytic fungus belonging to the genus *Phomopsis* was isolated from stem of *Ficus pumila* (Moraceae) and identified based on morphological and molecular characteristics. Antimicrobial potential was investigated by agar plug, disc diffusion and TLC bioautography agar overlay methods. Detection of polyketide synthase (PKS) genes was done using DOP-PCR techniques. Diverse antibacterial activities were observed against Gram

positive and Gram negative human and phytopathogenic bacteria and fungi in the extracts. TLC bioautography detects the production of antimicrobial metabolites.

Ola et al. (2013) studied the accumulation of metabolites by co-culturing the fungal endophyte *Fusarium tricinctum* and *B. subtilis* 168 trpC2. Accumulation of secondary metabolites increased 78-fold due to cocultivation. Some of the compounds increased due to cocultivation which includes, lateropyrone, cyclic depsipeptides of the enniatin type, and the lipopeptide fusaristatin A. Four compounds, (-)-citreoisocoumarin in addition to three new novel natural molecules were present only in coculture but not individual growth. 1D, 2D NMR and HRMS analysis revealed the identification of these new compounds as macrocarpon C, 2-(carboxymethylamino) benzoic acid, and (-)-citreoisocoumarinol. Enniatins B1 and A1, production enhanced and inhibited the growth of the *B. subtilis* in cocultivation with MICs of 16 and 8 µg/mL, respectively. These compounds also showed antibacterial activity against *S. aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* (MIC:2-8 µg/mL). Lateropyrone also exhibited antibacterial activity against *S. aureus*, *S. pneumoniae*, *B. subtilis*, and *E. faecalis*, with MIC ranging from 2 - 8 µg/mL.

Fusarium species are a group of commonly known endophytic fungi that produce a group of antibiotics viz., enniatins (Ens). These are six-membered cyclic depsipeptides formed by the union of three molecules of D- α -hydroxyisovaleric acid and three N-methyl-L-amino acids. Zaher et al. (2015) isolated *F. tricinctum corda* from *Hordeum sativum* Jess. fruits and was cultivated on a medium (rice). Identification of the metabolites by liquid chromatography-mass spectrometry revealed the compounds as ENs A, A1, B, B1, B2 and Q. EN Q is a new compound similar to EN A and the presence of EN B2 has been reported for the first time from this endophyte, in addition to four already known ENs (A, A1, B and B1). Methanolic extract from the fermentation broth of *F. tricinctum* indicated mild antibacterial and antileishmanial activities. The extract also displayed inhibitory potential on thioredoxin reductase produced by

Plasmodium falciparum. *Fusarium solani* obtained from the bark of yew from eastern Himalaya showed antimicrobial activity against Gram-positive bacteria (*S. aureus*, *B. subtilis* and *S. epidermidis*), Gram-negative bacteria (*Klebsiella pneumoniae*, *E. coli* and *Shigella flexneri*) and pathogenic fungi (*C. albicans* and *C. tropicalis*) (Tayung et al. 2011).

An endophytic fungus *F. tricinctum* isolated from *Salicornia bigelovii* Torr. by Zhang et al. (2015) was observed to produce three compounds identified on the basis of ESI-MS and NMR analysis. These compounds were fusartricin, a new sesquiterpenoid ether and fusarielin B and enniatin B. Antimicrobial activity of fusartricin against *E. aerogenes*, *M. tetragenu* and *C. albicans* is with MIC values of 19, 19 and 19 μ M, respectively. Fusarielin B showed antimicrobial activity (MIC) against *M. smegmati* (19 μ M), *B. subtilis* (19 μ M), *M. phlei* 10 μ M) and *E.coli* (19 μ M) respectively, and enniatin B exhibited antimicrobial activity against *B. subtilis* (13 μ M), *E. aerogenes* (13 μ M) and *M. tetragenus* (6 μ M). Two compounds phomoxanthon A (6) (Fig. 2.3) and B (19) (Fig. 2.4) from *Phomopsis* sp. BCC 1323 obtained from the *Tectona grandis* L. leaf showed antitubercular activity against *M. tuberculosis* H37Ra strain (Isaka et al. 2001). The MICs were 0.5 and 6.25 μ g/mL respectively for these compounds.

2.3 ANTIFUNGALS FROM ENDOPHYTIC FUNGI

Invasive fungal infections are significantly increasing during organ transplantation, allogeneic bone marrow transplantation and cancer chemotherapy. A limited number of antifungal drugs are available for life-threatening fungal infections. Development of resistance to antifungal drugs are increasing in patients undergoing long term treatment, though new antifungal agents have been introduced in the market. Endophytic fungi are one of the most potential alternative source for the isolation of novel metabolites for treatment of fungal diseases (Deshmukh and Verekar. 2012)

Liu et al. (2001) isolated endophytes from *A. annua* and tested against crop-threatening fungi such as *Gaeumannomyces graminis* var. *tritici*, *R. cerealis*, *Helminthosporium sativum*,

Fusarium graminearum, *Gerlachia nivalis* and *Phytophthora capsici*. Ethyl acetate extracts showed maximum activity. Tejesvi et al. (2007) isolated *Pestalotiopsis* strains from medicinally important plants, *A. indica*, *Holarrhena antidysenterica*, *Terminalia arjuna* and *T. chebula*. Ethyl acetate extracts showed maximum antifungal activity against six test organisms. Six bioactive compounds, cerulenin (20), arundifungin (22), sphaeropsidin A (21), 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5H)-furanone (23), ascosteroside A (24) and ascosteroside B (25) (Fig. 2.5) were isolated from these fungi. Antifungal activity of arundifungin, 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5H)-furanone, ascosteroside A and ascosteroside B was comparable to control drug amphotericin (Weber et al. 2007).

Michelia champaca is a medicinal plant producing number of secondary metabolites having many pharmacological properties. Endophytic fungi isolated from this plant showed inhibitory activities against fungi, cancer and neurotransmission process (AChE) (Chapla et al. 2014). These extracts also exhibited antimicrobial activity against phytopathogenic fungi. Ethyl acetate extracts of the endophytic fungus *C. gloeosporioides* yielded one new compound, 2-phenylethyl 1*H*-indol-3-yl-acetate (26), and seven known compounds: uracil (27), cyclo-(S*-Pro-S*-Tyr) (28), cyclo-(S*-Pro-S*-Val) (29), 2(2-aminophenyl) acetic acid (30), 2(4-hydroxyphenyl) acetic acid (31), 4-hydroxy- benzamide (32) and 2(2-hydroxyphenyl) acetic acid (33) (Fig. 2.6).

Li et al. (2008) isolated endophytic fungus *Pestalotiopsis adusta* (L416) and identified three metabolites that were chlorinated benzophenone derivatives viz., pestalachlorides A–C (34, 35, 36) (Fig. 2.6) using NMR and X-ray crystallography. The organic solvent extract obtained from the fermentation broth exhibited notable antifungal activities against plant pathogenic fungi such as *Fusarium culmorum* (CGMCC 3.4595), *Gibberella zeae* (CGMCC 3.2873), and *Verticillium albo-atrum* (CGMCC 3.4306). Their studies further showed that pestalachloride A displayed good antifungal activity against *F. culmorum*, while pestalachloride B against *G.*

zeae. Shentu et al. (2014) isolated number of endophytic fungi from garlic plant. The isolate showed significant antifungal activity against phytopathogens. The bioactive metabolite produced by *Trichoderma brevicompactum* was identified as trichodermin by spectral and mass data analysis. The antifungal activity of the compound was compared with control carbendazim.

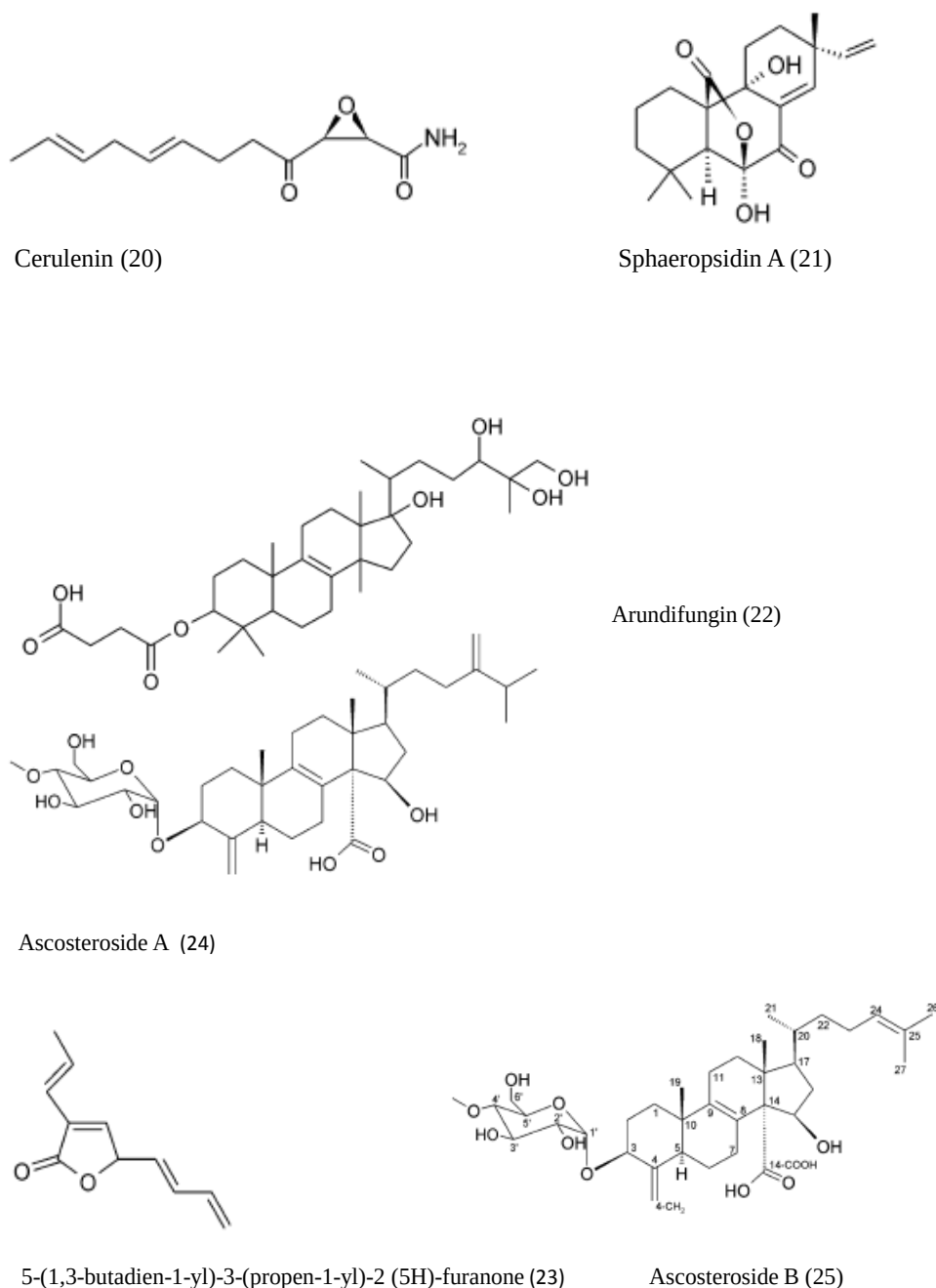
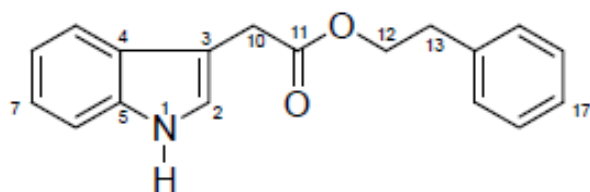
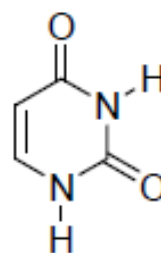


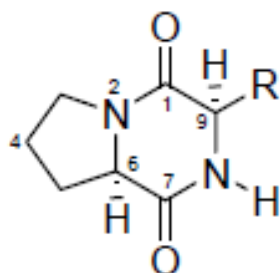
Fig. 2.5. Anticandida molecules from endophytic fungi



2-Phenylethyl 1*H*-indol-3-yl-acetate (26)



Uracil (27)

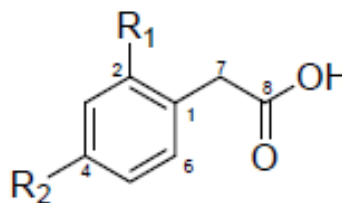


cyclo-(*S**-Pro-*S**-Tyr) (28)

cyclo-(*S**-Pro-*S**-Val) (29)

R=CH₂PhOH (28)

R=CH(CH₃)₂ (29)



2(2-aminophenyl)acetic acid (30)

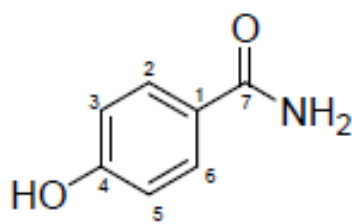
2(4-hydroxyphenyl)acetic acid (31)

2(2-hydroxyphenyl)acetic acid (33)

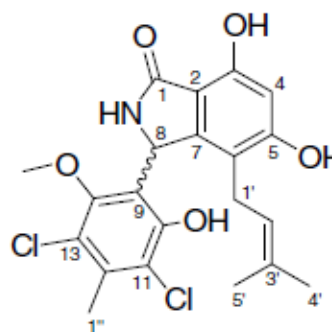
R₁=NH₂, R₂=H (30)

R₁=H, R₂=OH (31)

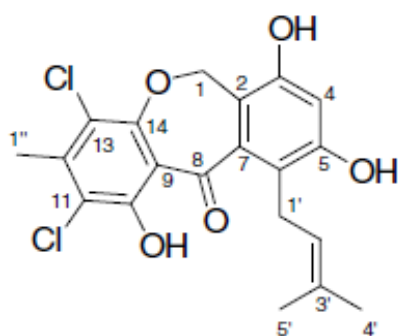
R₁=OH, R₂=H (33)



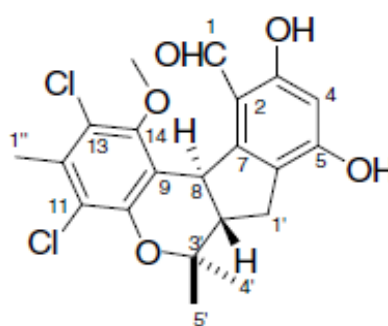
4-hydroxy- benzamide (32)



Pestalochlorides A (34)



Pestalochlorides B (35)



Pestalochlorides C (36)

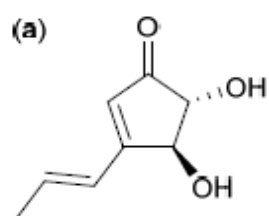
Fig. 2.6. Antifungal molecules from endophytic fungi.

2.4 ANTICANCER, IMMUNOSUPPRESSIVE, ANTIINFLAMMATORY ACTIVITIES OF ENDOPHYTIC FUNGI

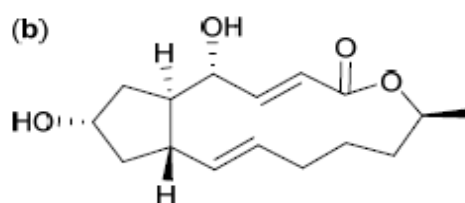
Cancer is characterized by uncontrolled cell growth that leads to metastasis of abnormal cells and associated growth of tissues (American Cancer Society 2009). Reports of endophytic fungi able to synthesize essential plant-derived anticancer drugs has resulted in improved focus of attention on these sources. Some representative examples are paclitaxel (Taxol®) from *Taxomyces* (Stierle et al. 1993) and many *Pestalotiopsis* species (Li et al. 1998), as well as camptothecin (Puri et al. 2005; Amna et al. 2006), podophyllotoxin (Eyberger et al. 2006; Puri et al. 2006) vinblastine (Guo et al. 1998), and vincristine (Zhang et al. 2000; Yang et al. 2004), that are produced by endophytic fungi. It has also been demonstrated that the identification of the gene or gene products regulating metabolite production by these microorganisms provides an understanding of greatly increasing the production of these compounds (Cragg and Newman 2013).

Azila et al. (2014) studied the anti-inflammatory activities of *Trametes lactinea* and suggested that the growth and activities depend on culture conditions such as media composition and pH, inoculum volume, temperature of incubation and incubation time. Polyketide fungal metabolites like terrein (37), brefeldin A (38) and asperlin (39) (Fig. 2.7) are known to possess novel anticancer properties. The antifungal (Ghisalberti et al. 1990) polyketide terrain produced by *A. terreus* is known since long time (Raistrick and Smith 1935). In addition, terrain also inhibits breast cancer. The compound is observed to induce apoptosis (IC₅₀ : 1.1 nM) on MCF-7 cell lines indicating that the compound was many times more potent than taxol. Reports also indicate that terrein is active against other cancer cell lines (Liao et al. 2012.)

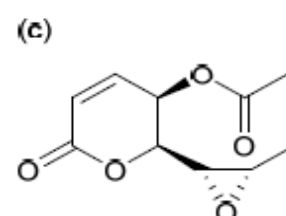
Liu et al. (2009) studied the endophytic fungi that were isolated from bark of *Taxus chinensis* and examined their diversity. They have isolated 115 endophytic fungal isolates and assigned to 31 morphotypes.



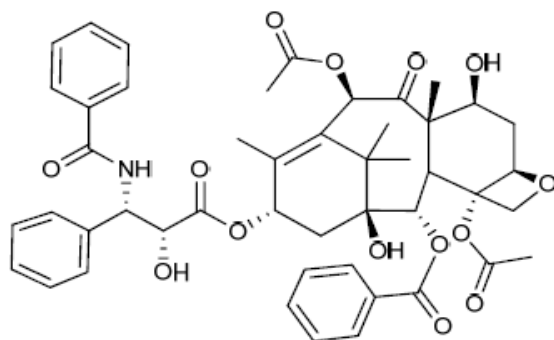
Terrein (37)



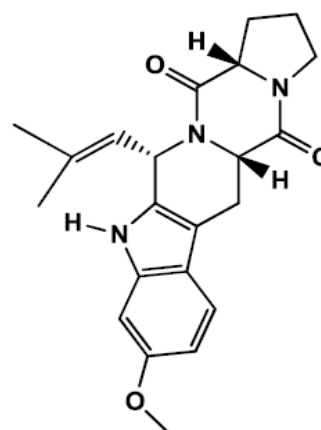
Brefeldin A (38)



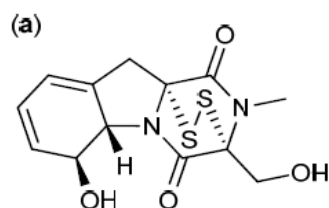
Asperlin (39)



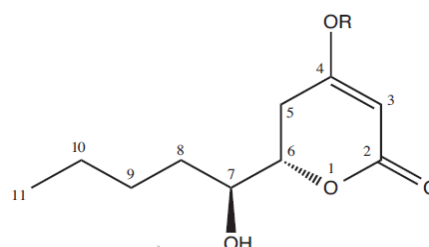
Taxol (40)



Fumitremorgin C (41)



Gliotoxin (42)



(S)-4-butoxy-6-((S)-1-hydroxypentyl)-5,6-dihydro-2H-pyran-2-one (43) R = Methyl 1 R = Butyl 2

Fig. 2.7. Anticancer molecules from endophytic fungi.

Phomopsis, *Diaporthe*, *Acremonium* and *Pezicula* were the dominant genera and *Botryosphaeria obtuse*, *Xylaria*, the fungus H-8, *Pezicula* sp H-33 were among the rare morphotypes. The extracts from these 31 morphotypes were screened for taxol (40) (Fig. 2.7) production. Maximum taxol production was seen in *Metarhizium anisopliae* and the yield obtained was 846.1 µg/l. HPLC-MS analysis confirmed the presence of fungal taxol.

Kumaran et al. (2010) isolated *Pestalotiopsis versicolor* and *P. neglecta* from the bark and leaves of *Taxus cuspidata* and were identified based on their morphological vis-à-vis molecular analysis. Taxol detection was done by HPLC and further confirmed by ¹H NMR, and LC–MS methods of analysis. Endophytic fungus *P. versicolor* produced higher amount of taxol (478 µg/l) compared to *P. neglecta* (375 µg/l). Their findings showed that endophytic fungal taxol exhibited a potent cytotoxic activity on the human cancer cells.

Diketopiperazines are cyclic di-peptides and are known inhibitors of cell cycle of the G2/M phase (Clark et al. 1977). Many compounds belong to this group such as fumitremorgins, stephacidins, notoamides, tryprostatins, etc. produced by variety of *Aspergilli*. *A. fumigatus* and *A. fischeri* are known to produce fumitremorgins (41) (Fig. 2.7) (Frisvad et al. 2013; Wu et al. 2004; Yuan et al. 2010; Qian-Cutrone et al. 2002) Fumitremorgin C has shown cytotoxicity against human leukemia P-388 (ED₅₀ : 3.9 µg/mL); and the related compound 12,13-dihydroxyfumitremorgin C with antiproliferative activity on human leukemia U-937 and human prostate cancer PC-3 (IC₅₀ : 1.8 and 6.6 µM, respectively) (Qian-Cutrone et al. 2002; Finefield et al. 2012). Reports also indicate that fumitremorgin C is a highly potent cytotoxic agent against MDR cancer of breast and colon. (Zhao et al. 2002; Wang et al. 2008).

Gliotoxin (42) (Fig. 2.7) belonging to a group of diketopiperazines possess anticancer activity and constitutes a di-sulfide bridge in the diketopiperazine ring. This compound obtained from *A. fumigatus* and *D. cejpii* has antifungal, immunosuppressive and antimicrobial properties. (Kanoh et al. 1997; Kanoh et al. 1999; Yamazaki, 2012). Gliotoxin was also reported as a

potent inhibitor of six breast cancer cell lines with (IC_{50} values between 38 and 985 nM) (Hayashi et al. 2000, Larsen et al. 1992). Finefield et al. (2012) reported the activity of gliotoxin against human prostate cancer PC-3 and human leukemia U-937 cell lines (IC_{50} : 0.2 and 0.4 μ M, respectively).

Trichothecenes, a group of mycotoxins covering more than 150 analogs are reported to be produced by different *Fusarium* species. These compounds constitutes a sesquiterpenoid ring structure associated with an epoxide. The epoxide binds to the 60S ribosomal subunit of eukaryote cells and inhibits the protein synthesis and thus exhibits cytotoxic activity (Pittayakhajonwut et al. 2011; Hirota et al. 1986). It has been reported that trichothecenes exhibited cytotoxicity against microbial and human cancer cell lines (Wijeratne et al. 2003, Wu et al. 2013, Woloshuk et al. 2013). Sudakin et al. (2003) reported that AETD, one of the trichothecene analogue showed cytotoxic activity against different cell lines such as HL-60, U-937, HeLa, MCF-7 and Hep-G2 cell lines (IC_{50} : 10, 22, 45, 53 and 170 nM, respectively). Shifrin and Anderson (1999) reported the activity of roridins (trichothecenes, where a macrocyclic ring is connected to the sesquiterpenoid unit) against cell lines. One of the compounds, 12'-hydroxyroridin E, inhibited leukemia L-1210 with an IC_{50} value of 0.2 μ M. Anguidine, another trichothecene that had been screened for clinical trials against cancer, did not progress beyond phase II clinical trials due to absence of any therapeutic value. (Pittayakhajonwut et al. 2011, Campos et al. 2011).

Filamentous fungi produce meroterpenoids which are secondary metabolites that comprise of different compounds integrating a polyketide part with a terpenoid part (Yang et al. 2012) and the cytochalasins (Zhang et al. 2012). The cytochalasins contain phenylalanine coupled to the polyketide chain whereas a tryptophan moiety is present in the chaetoglobosins (Zhang et al. 2012; Wang et al. 2013). Cytochalasins and chaetoglobosins are known to exhibit antifungal effect against diverse variety of fungi (Buommino et al. 2007; Li et al. 1995; Krizsán et al.

2010). Many fungal genera including *Aspergillus*, *Metarrhizium*, *Zygosporium*, *Hypocrella*, *Hypoxylon* and *Phoma* are known to produce cytochalasins (Fang et al. 2006; Geris and Simpson 2009; Schümann and Hertweck 2007; Sekita et al. 1973; Fu et al. 2011). Cytochalasins are known to inhibit lung cancer A-549 cell lines. Cytochalasin E has been reported to inhibit human ovarian A-2780S, human colon HCT-116 and SW-620, and lung A-549 cancer and human leukemia P-388 (IC_{100} : 0.02, 1.0, and 0.2 $\mu\text{g/ml}$ and IC_{50} values of 0.006 and 0.09 μM , respectively) (Wicklow et al. 2011, Frisvad et al. 2008). Three new cytochalasins, trichalasin E, F and H (44, 45, 46), and four known analogues, trichalasin C (47), aspochalasin K (48), trichalasin G (49) and aspergillin PZ (50) (Fig. 2.8) are reported to be produced by endophytic fungus *Trichoderma gamsii* isolated from the medicinal plant *Panax notoginseng* (Chen et al. 2014). Liang et al. (2015) isolated fumitremorgin 12-methoxy-13-[5'-hydroxy-2'-(1''-hydroxy-3''-methoxy-5''-methylbenzoyl)-3'-methoxy] benzoic acid methyl ester (fumitremorgin D) and 4,8,10,14-tetramethyl-6-acetoxy-14-[16-acetoxy-19-(20,21-dimethyl)-18-ene]-phenanthrene-1-ene-3,7-dione from the endophytic fungus *Aspergillus fumigatus*. Cytochalasin H2 and cytochalasin H were isolated from endophyte *Xylaria* sp. A23, hosted by *Annona squamosa* and characterized using 1D-, 2D-NMR and HRFTMS (Li et al. 2012). The MTT assay indicated that the former substance exhibits moderate cytotoxicity against HeLa and 293T cell lines.

To obtain bioactive secondary metabolites, Akay et al. (2014) isolated endophytes from different parts of hazelnut tree *Corylus avellana* L. Investigation was also done to search for gene region of taxadiene synthase (Ts), an important enzyme in taxol biosynthesis, on some fungi. Fourteen fungal species were isolated and further fermentation was carried out. The chloroform extracts were tested for cytotoxicity using MTT method. After screening for bioactivity, the fungal isolate *Phomopsis amygdali* was identified by internal transcribed spaces

(ITS) sequence analysis. After fermentation and purification, two major compounds, one of it was a new secondary

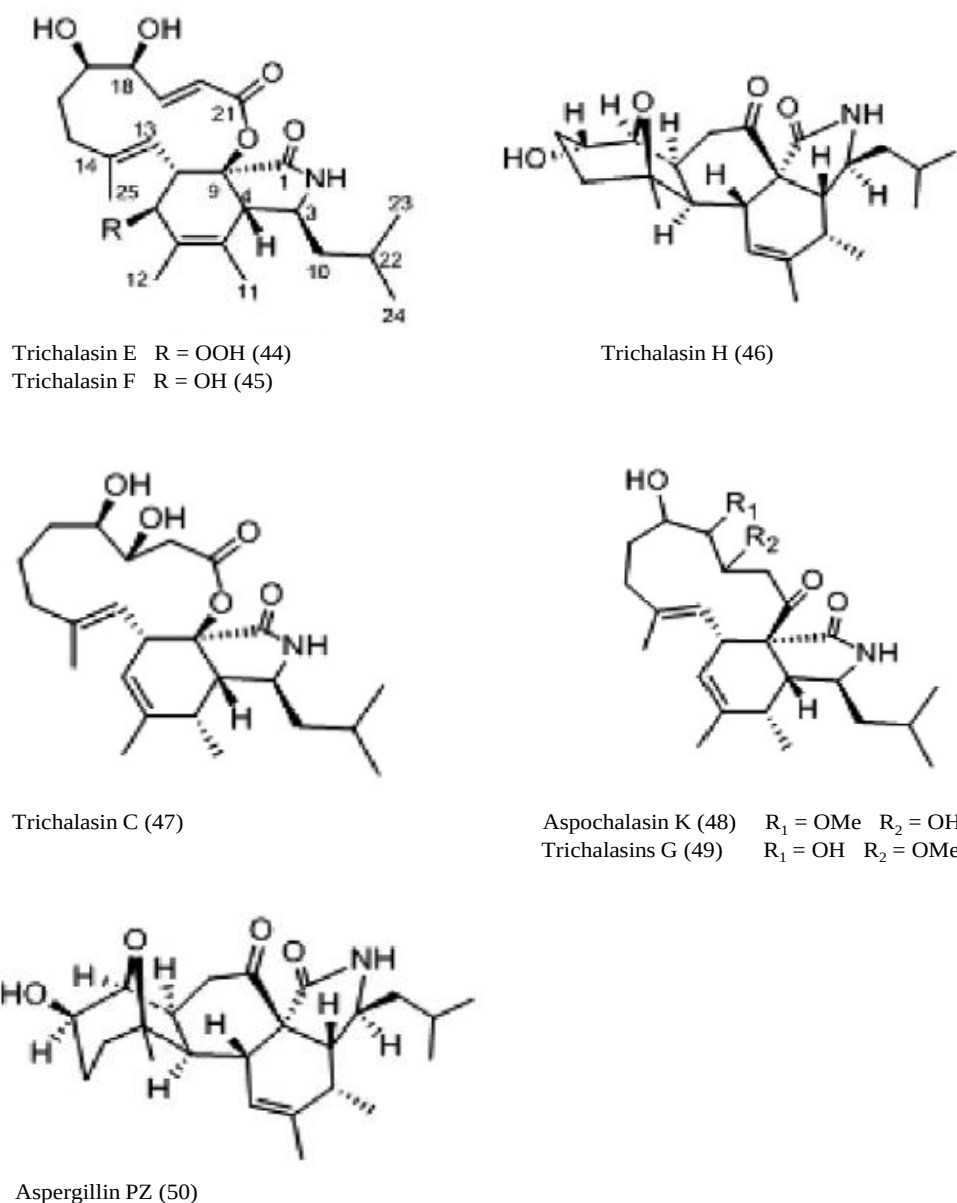


Fig. 2.8. Anticancer molecules from endophytic fungi.

metabolite, (S)-4-butoxy-6-((S)-1-hydroxypentyl)-5,6-dihydro-2H-pyran-2-one (43) (Fig. 2.7) and the other was identified as (-) pestalotin.

Ding et al. (2013) reported that *Phomopsis* sp. HNY29-2B isolated from mangrove plants produced three new phomoxanthone compounds, phomolactonexanthones A (51), B (52) (Fig.

2.9) and deacetylphomoxanthone C. This fungus was also able to produce five other known phomoxanthenes such as dicerandrol A, dicerandrol B, dicerandrol, deacetylphomoxanthone B and penexanthone A. The chemical characterization was carried out based on spectroscopic analysis. Further, these compounds exhibited cytotoxicity against diverse variety of cell systems such as MDA-MB-435, HCT-116, Calu-3 and Huh7.

Phomopsis sp. XZ-01 isolated from *Camptotheca acuminata* produced three oblongolides, C1, P1, and X1 and four new metabolites, 6-hydroxyphomodiol, along with eight known compounds – oblongolides B, C, D, O, P and U, (3*R*,4*aR*,5*S*,6*R*)-6-hydroxy-5-methylramulosin, and (3*R*)-5-methylmellein (53-64) (Fig. 2.9) Mild selective cytotoxic activities were observed with these compounds (Lin et al. 2011).

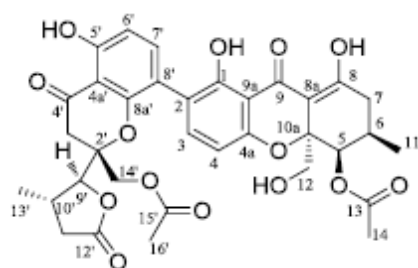
Many investigations have shown that the genera *Phomopsis* can be a rich source for the production of therapeutically important biomolecules like phomopsichalasin, cytochalasin, phomopsin A from *P. leptostromiformis*, convolvulanic acids and other phytotoxic isobenzofuranones from *P. convolvulus*, and oblongolide and phomopsolides (phytotoxic sesquiterpene ζ -lactones) from *P. oblogna*. Two novel xanthone dimers, Phomoxanthenes A and B were isolated by Isaka et al. (2013) from the endophytic fungus *Phomopsis* sp. BCC 1323. These molecules exhibited cytotoxicity as well as antimalarial and antitubercular activities. The studies further resulted in isolating and characterizing two novel compounds viz., phomoxanthenes A and B. Phomoxanthenes A and B showed potent activity against MDR strain, *Plasmodium falciparum* (K1, strain) and against *M. tuberculosis* (H37Ra strain). These compounds also showed cytotoxicity against cancer cell lines viz., KB and BC-1 as well as Vero cells.

Zhang et al. (2014) purified phomopsidone A (65), excelsione (66) and four known isobenzofuranones (67–70) (Fig. 2.10) from the fermentation extracts of the mangrove

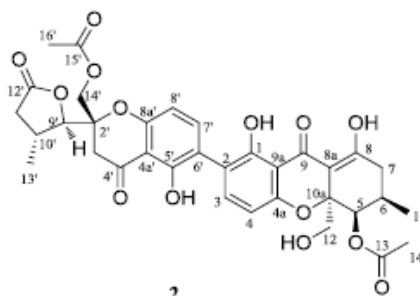
endophytic fungus *Phomopsis* sp.A123. The structures were determined by 1D/2D NMR and HR-MS. These compounds showed cytotoxic, antioxidant, and antifungal activities.

Ngankaranatikarn et al. (2013) isolated the endophytic fungus *Phomopsis* sp. AANN8 from the twigs of the Thai medicinal plant *Artemisia annua* L. The antileukemic activity was reported in the crude extracts and fractions using sulforhodamine B (SRB) colorimetric assay with human acute monocytic leukemia (THP-1) cell line. The crude extract and fractions with the final concentration of 20 µg/ml exhibited antileukemic activity against THP-1 cell lines.

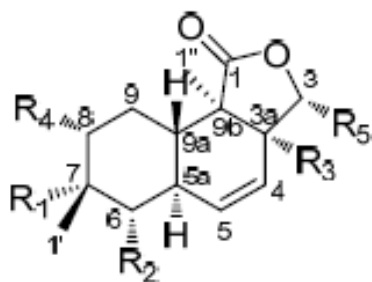
Enniatins (ENs) (71) (Fig. 2.10) are six membered cyclic depsipeptides produced as secondary metabolites by several strains of *Fusarium* spp. ENs A, A1, B, B1 were extracted from *Fusarium tricinctum* by Meca et al. (2010) by low pressure liquid chromatography (LPLC) on reverse phase of Amberlite XAD-7. Further, purification of ENs was done by semi-preparative column LC. Cytotoxicity tests were done by MTT assays. Only EN A1 and B1 exhibited inhibition of cell lines viz., epithelial colorectal adenocarcinoma cells and Caco-2. Enniatins are mycotoxins that can be used as possible anticancer agents. Enniatins A1, B and B1 obtained from the endophytic fungus *F. tricinctum* showed moderate cytotoxic activity against HepG2 and C6 cells (EC_{50} 10–25 µM) and showed high toxicity in H4IIE cells (EC_{50} 1–2.5 µM). (Wätjen et al. 2009). The compounds have been observed to increase nuclear fragmentation as well as caspase 3/7 activity in H4IIE cells. Enniatins A1 and B1 showed moderate TNF- α -induced NF- κ B activation. Their results suggested that enniatins A1 and B1 and, to a certain extent, enniatin B also possesses anticancer properties by inducing disruption of pathway associated with ERK signalling.

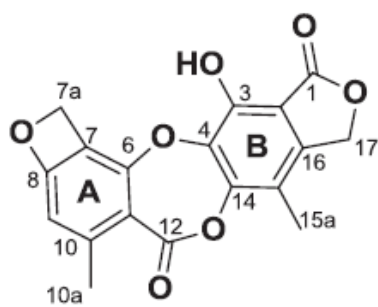


Phomolactonexanthones A (51)

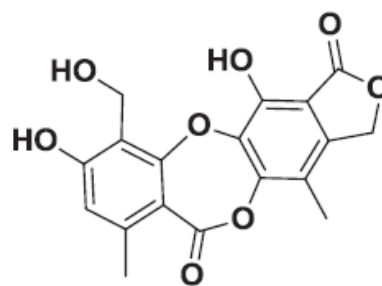


Phomolactonexanthones B (52)

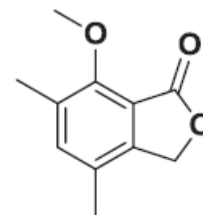
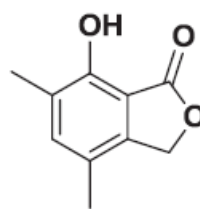
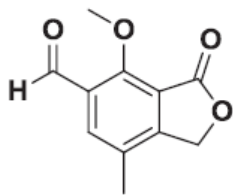
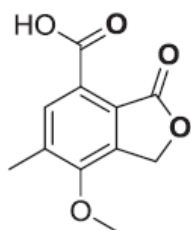




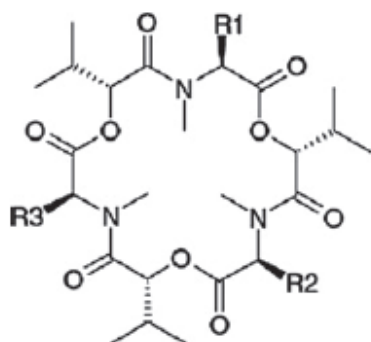
Phomopsidone A (65)



Excelsione (Phomopsidone) (66)



Isobenzofuranones (67–70)



Enniatins (71)

Enniatin	R1	R2	R3
A	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃
A ₁	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃) ₂
A ₂	CH(CH ₃)CH ₂ CH ₃	CH(CH ₃)CH ₂ CH ₃	CH ₂ CH(CH ₃) ₂
B	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃) ₂
B ₁	CH(CH ₃) ₂	CH(CH ₃) ₂	CH(CH ₃)CH ₂ CH ₃
B ₄	CH(CH ₃) ₂	CH ₂ CH(CH ₃) ₂	CH(CH ₃) ₂
J ₃	CH(CH ₃) ₂	CH(CH ₃) ₂	CH ₃

Fig. 2.10. Anticancer molecules from endophytic fungi.

Weber et al. (2004) isolated *Phomopsis* from the *Erythrina crista-galli*, a medicinal plant, and isolated a novel antibiotic compound phomol. This compound showed antimicrobial, anti-inflammatory and cytotoxic activity. Verekar et al. (2014) isolated depsipeptide (PM181110) from fungus *Phomopsis glabrae* associated with the leaves of *Pongamia pinnata*. PM181110 exhibited anticancer activity against 40 human cancer cell lines with a mean IC₅₀ of 0.089 IM *in vitro* and 24 human tumor xenografts with IC₅₀ of 0.245 IM under *ex vitro*.

2.5 ANTIOXIDANTS FROM ENDOPHYTIC FUNGI

Antioxidant compounds are well known to be effective against cellular damage induced by reactive nitrogen and oxygen species (RNOS). The pathological effects caused by ROS include DNA damage, degeneration of cells and carcinogenesis (Huang et al. 2007; Seifried et al. 2007). Antioxidants provide a resolution to ROS-linked diseases such as cancer, atherosclerosis, cardiovascular disease, hyper-tension, and variety of other ailment such as ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, etc. (Valko et al. 2007). Fungal endophytes, thus, have proved to be good source of novel anti-inflammatory substances and antioxidants.

Zeng et al. (2011) examined the antioxidant profile of 49 endophytic fungi isolated from liverwort *Scapania verrucosa* (Xylariaceae). They grouped these fungi into seven genera i.e., *Hypocrea*, *Penicillium*, *Tolypocladium*, *Chaetomium*, *Xylaria*, *Nemania*, and *Creosphaeria*. The extracts of some endophytic fungal isolates were examined for their free-radical scavenging activity using DPPH-free radicals and hydroxyl radicals as indicators. These above investigations indicate that endophytic fungi isolated from *S. verrucosa* can be a potent source of natural antioxidants.

Rhodiola plant species exhibit pharmacological effects like anti-aging and antioxidant properties. Cui et al. (2015) isolated and examined the molecular diversity of 347 endophytic fungi from *R. crenulata*, *R. angusta*, and *R. sachalinensis* and studied the antioxidant activities

of their crude extracts. These fungi were categorized into 180 morphotypes based on their cultural characteristics. ITS sequence analysis revealed that except 12 unidentified fungi other sequences were affiliated to 57 genera belong to Ascomycota, Basidiomycota, Zygomycota and Glomeromycota. As reported by the authors, the radical-scavenging rates of 114 isolates were >50%, and five isolates (Rct45, Rct63, Rct64, Rac76, and Rsc57) showed >90%. The EC₅₀ values of five antioxidant assays showed promising potential of these strains on scavenging free radicals, as well as scavenging RNOS and chelating iron, which indicated preference and selection between endophytes and their hosts. The research findings also indicate that Rac12 could produce salidroside and p-tyrosol.

Liu et al. (2007) isolated endophytic *Xylaria* sp. from the medicinal plant *Ginkgo biloba* and evaluated the antioxidant activity. Strong antioxidant capacity was seen in the methanolic extract due to the presence of “phenolic” and “flavonoid” compounds among 41 identified compounds.

Huang et al. (2007) studied antioxidant potential of endophytic fungi isolated from Chinese medicinal plants and its relation with the total amount of phenolic contents. Their results suggested that the phenolics were the major antioxidant constituents in endophytes. Two compounds, “pestacin” and “isopestacin” (1,3-dihydroisobenzofurans) were obtained from *Pestalotiopsis microspora* associated with *Terminalia morobensis* a plant growing in the Papua New Guinea. These compounds showed antioxidant activity and also antimycotic activity. The antioxidant activity of pestacin (72) (Fig. 2.11) is many times higher than Trolox. Isopestacin, that is structurally closer to flavonoids also exhibits antioxidant activity through scavenging of both superoxide and hydroxy free radicals (Harper et al. 2003, Strobel et al. 2002). Song et al. (2005) isolated graphis lactone A” (73) (Fig. 2.11) from the extracts of endophytic fungus *Cephalosporium* sp. IFB-E001 residing inside *Trachelospermum jasminoides*. This compound

exhibited free radical-scavenging and antioxidant activity more than the standard controls, butylated hydroxytoluene (BHT) and ascorbic acid.

A stilbene phytoalexin, resveratrol (74) (Fig. 2.11), produced by diverse variety of plants as response to biotic and abiotic stresses, is extensively used as nutraceutical due its well known antioxidant properties. Endophytic fungi, *Alternaria*, *Cephalosporium*, *Aspergillus*, *Geotrichum*, *Botryosphaeria*, *Mucor* and *Penicillium* isolated from *Vitis vinifera*, *V. quinquangularis*, and *P. cuspidatum* were able to produce resveratrol. *Alternaria* sp. isolated from leaf and the root strains, *F. solani*, *F. proliferatum* and *F. oxysporum* associated with *C. cajan*, are able to produce cajaninstilbene acid (75) (Fig. 2.11) which showed antioxidant activity (Shi et al. 2012, Zhao et al. 2012)

The ethanolic extract of endophytic fungus *Phyllosticta* sp. exhibited antioxidant activity when ABTS and DPPH radicals were used for assay. The EC₅₀ value of 580 and 2030 µg/ml, were reported for ABTS and DPPH, respectively. They also reported the phenol and flavonoid content as 18.3 gallic acid equivalents per gram and 6.4 µg/mg of quercetin equivalent, respectively to correlate the antioxidant activities of these compounds (Srinivasan et al. 2010) Huang et al. (2007) reported the antimicrobial activity, antioxidant capacity, xanthine oxidase inhibition and total phenolic content from the endophytic fungi isolated from the *Nerium oleander* L., a known medicinal plant. The total phenolic content of the endophytic fungi ranged from 9.6 - 150.8 µmol trolox/100 ml culture, and the anti-oxidant activity was ranging from 0.52 - 13.9 mg gallic acid/100 ml culture, respectively. *Chaetomium* sp. showed highest level of phenolics indicating its strong antioxidant capability and also inhibition of xanthine oxidase activity. Yadav et al. (2014) isolated endophytic fungi from *Eugenia jambolana* Lam., a tree known to possess lot of medicinal properties. Organic solvent extraction of fermentation broth was done to obtain crude extracts. Phytochemical screening, total phenolic content estimation, antioxidant assays by DPPH, hydrogen peroxide scavenging assay and reducing

power assay were performed on twenty one crude extracts. The main phytochemicals detected were phenols, alkaloids, saponins, flavonoids and terpenes. It was seen that there is a direct relationship between the phenolic content and antioxidant activity. *Aspergillus peyronelii*, *A. niger* and *Chaetomium* sp. showed maximum antioxidant activity ranging from 50-80% as compared to ascorbic acid (90%) which was used as positive control.

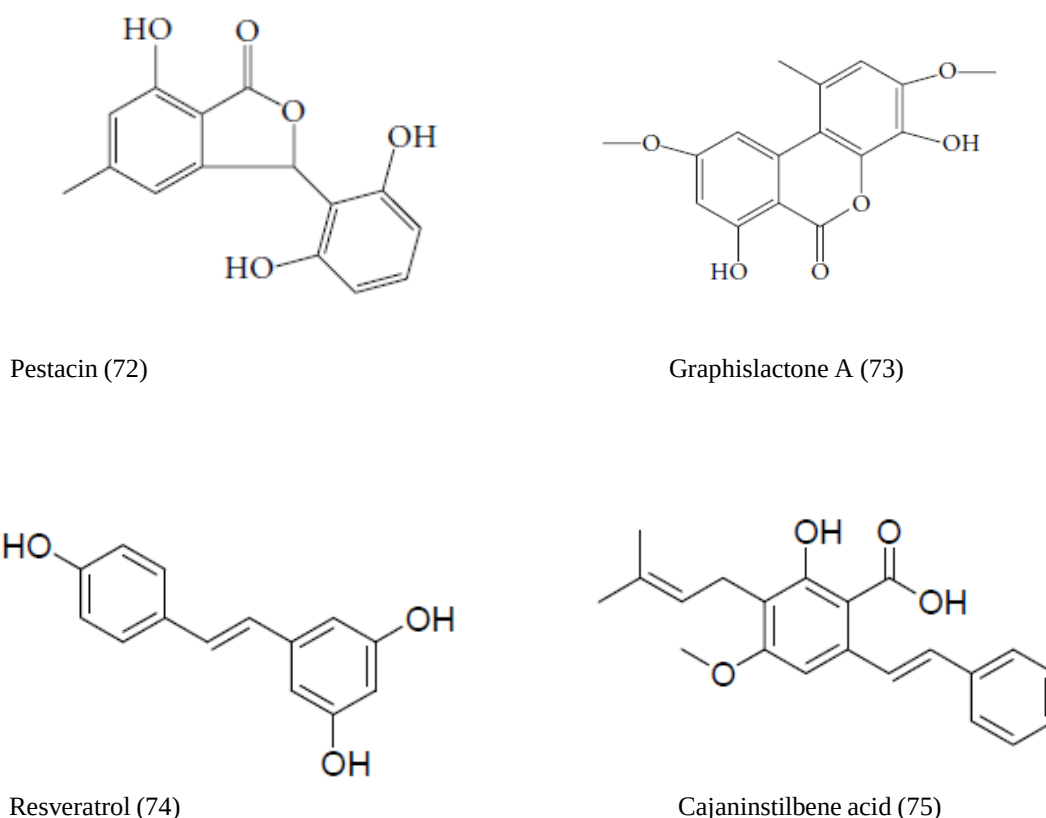


Fig. 2.11. Antioxidant molecules from endophytic fungi.

Elfita et al. (2012) reported the antioxidant activity of endophytic fungi, *Penicillium* sp., *Chrisonilia sitophila*, and *Acremonium* sp. associated with tissues of the kandis gajah stems by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay. They found that *Acremonium* sp. exhibited significant antioxidant activity (IC_{50} value of 10.3 $\mu\text{g/mL}$)

equalling that of ascorbic acid (IC_{50} value of 9.8 $\mu\text{g/mL}$). They identified the bioactive compound as sesquiterpene 3,5-dihydroxy-2,5-dimethyltrideca-2,9,11-triene-4,8-dione based on spectroscopic data.

Sadananda et al. (2014) isolated lectin from the endophytic fungi *Aspergillus flavus*, *Fusarium oxysporum*, *F. moniliforme* and *Trichothecium* sp. associated with *Viscum album*. These lectins were able to agglutinate A+ve erythrocytes. *Fusarium oxysporum* showed higher activity (IC_{50} value 348.54 $\mu\text{g/mg}$) and *A. flavus* showed low IC_{50} value (127.9 $\mu\text{g/ml}$) as compared to *Viscum album* (92.4 $\mu\text{g/ml}$). Concentration dependent scavenging activity was observed when H_2O_2 was used as scavenging agent. *Fusarium moniliforme* showed IC_{50} value of 171.2 $\mu\text{g/ml}$ compared to *Viscum album* (183.4 $\mu\text{g/ml}$). Their results suggested that lectin isolated from endophytic fungi has potential free radical scavenging and antioxidant activity that was comparatively higher in endophytes than the host plant. Madagundi et al. (2013) isolated root fungal endophytic fungi (TRF-3 and TRF-6) from Tulsi (*Ocimum sanctum* Linn.) and studied free radical DPPH, radical scavenging and immunomodulatory activities on the functions of human polymorphonuclear cells such as phagocytosis, chemotaxis and reduction of nitroblue tetrazolium (NBT) dye. They reported the IC_{50} values for TRF-3 and TRF-6 as 271.7 $\mu\text{g/mL}$ and 140.5 $\mu\text{g/mL}$, respectively for DPPH assay and 298.6 $\mu\text{g/mL}$, and 361.7 $\mu\text{g/mL}$ for hydroxyl assay, respectively. Maximum reduction of neutrophils reported was 89.78% and 74.75% for TRF-3 and TRF-6, respectively. The mean number of phagocytosis of killed *C. albicans* was 7 and 8 for both fungi, respectively. Their results confirmed the antioxidant potential and immunostimulatory effects of *O. sanctum* endophytic fungi.

The endophytic fungi. *Penicillium*, *Mucor*, *Fusarium* and *Aspergillus* species were isolated from the medicinal plant *Lobelia nicotianifolia* and the antioxidant activity was studied by phosphomolybdic acid method and total phenolic contents by Folin's Ciocalteu method by Murthy et al. (2011). All the extracts were observed to show significant antioxidant potential.

2.6 ANALYSIS OF BIOACTIVE METABOLITES

Metabolomics, the study of metabolites in a cell or an organism, is fast emerging as an important area (Last et al. 2007). Plants, fungi and bacteria have a capability to synthesize large number of metabolites (Fernie et al. 2004). Metabolites that are synthesized by these organisms serve to perform a broad range of functions including signalling molecules and help the species for its defense against pathogens, protection against stress. A metabolite could be a lead for the discovery of novel drugs and may represent a source for important bioactive molecules. NMR and mass spectrometry are the prominent techniques widely used for the metabolite characterization. NMR although provides structural information, the technique lacks sensitivity and requires large amount of purified sample. Many a times it is seen that the metabolite of interest is produced only in small amounts and difficult to purify. Mass spectrometry (MS) allows for high-throughput analysis with little requirement of sample. It also does not require a purified sample. A small amount of extract or a biofluid can be analysed with a limited sample preparation, by using a chromatographic method before conducting the spectrometric measurement, with LC-MS detecting more metabolites than any other technique (Patti et al. 2012). It has become a major tool in metabolomics and has received significant attention. (Kind and Fiehn 2010; Dunn et al. 2013; Draper et al. 2009; Wishart 2011; Scheubert et al. 2013).

Level I identification: Experimental spectral information is matched with that of authentic compounds. In metabolomics, sometimes because of the lack of authentic compounds that would help in identification of molecules (Wishart 2011) MSI level II identification is used.

Level II identification: In this level of identification, compounds are putatively identified utilizing the information on physicochemical properties and/or based on similarities of the spectra with spectral libraries devoid of chemical standards (Sumner et al. 2007; Zhu et al. 2013).

Level III identification: In this identification, chemical compound classes are putatively characterized based upon characteristic physicochemical properties of a chemical class of compounds.

Level IV identification: Based on the spectral data, these metabolites can be differentiated and quantified even if they are unidentified or unclassified.

In mass spectrometry, the compound is fragmented and masses of the fragments are recorded. Here, metabolites are ionized in the ion source of the mass spectrometer by electrospray ionization (ESI) and then ions are selected automatically for isolation and fragmentation to obtain mass spectra MS² or MS/MS. The approach is based on comparison of a fragmentation spectrum with other such spectra in a database. There exist several metabolite databases, all with a slightly different focus: The Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2006), the METLIN database (Wishart et al. 2009) and the Human Metabolome database (HMDB) (Smith et al. 2005). Many of the secondary metabolites still remain unknown although huge number of structures are stored in such databases.

2.6.1 Mass Spectrometry

A typical mass spectrometer constitutes an ion source that charges the molecules, a mass analyzer that separates the molecules by their mass-to-charge ratio, and a detector that approximately measures the number of incoming ions. Molecules are charged or ionized to respond to the measurement. Several methods to ionize a sample exist and if the analyte molecule is intact it is called soft ionization or if it fragments it then it is called as hard ionization. The resulting ions either carry an additional proton ($[M+H]^+$; M denotes the sample molecule), or have lost one in negative mode ($[M-H]^-$), but sample ions may also form through the addition of other ions, such as sodium ($[M+Na]^+$) or ammonium ($[M+NH_4]^+$). Other soft ionization techniques include the matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988). Here the sample is embedded into a crystalline matrix. A mass analyzer

separates ions by their mass-to-charge ratio. Various methods exist to achieve separation by mass-to-charge namely time-of-flight (TOF), quadrupole, linear ion trap and Orbitrap mass analyzers.

Mass spectrometry combined with liquid chromatography (LCMS) is one of the major analytical technique that may be used in metabolomics. Qualitative analysis of the samples was done and the signals that were detected by LCMS were characterized as peaks, mass over charge ratio (m/z) and retention time.

2.7 INDUSTRIAL ENZYMES FROM ENDOPHYTIC FUNGI

Fungi exhibit absorptive mode of nutrition and fungal endophytes that colonize the plant tissues are able to derive nutrition from plant cells. The diverse nature of substrates allows fungi to produce diverse variety of enzymes such as cellulases, amylases, chitinases, lipases, and proteases (Suryanarayanan et al. 2012). Thermostable amylases are being studied to improve industrial processes for starch degradation. The endophytic actinomycete *Streptosporangium* sp., isolated from the leaves of maize (*Zea mays* L.) showed glucoamylase production which exhibited thermostable properties (Stamford et al. 2002). Endophytic microorganisms are known to produce many enzymes of industrial importance (Firáková et al. 2007).

An endophyte, *Cylindrocephalum* sp. was isolated from *Alpinia calcarata* (Haw.) Roscoe which is capable of producing lipase enzyme. The organism is also able to produce amylase which showed maximum activity at 30°C and at pH 7.0. As a carbon source, maltose was supplemented at a concentration of 1.5% and sodium nitrate (0.3%) was provided as nitrogen resulting in maximum production of amylase (Sunitha et al. 2012). Marlida et al. (2000) compared amylase production by four endophytic fungi, viz., *Gibberella pulicaris*, *Acremonium* sp., *Nodilusporium* sp. and *Synnematous* sp. Their results showed that *Acremonium* sp. uses a broad raw starch degrading activity on raw starch. The hydrolysed end

product with *Gibberella pulicaris*, *Acremonium* sp., *Nodulisporium* sp. was glucose and with *Synnematous* sp. was glucose and maltose. Further, optimization of nitrogen source, pH, incubation temperature on the growth and production of amylase by *Acremonium* sp., was performed and 19-22 fold increase in amylase activity was observed under these conditions. (Marlida et al. 2000)

Panuthai et al. (2012) isolated 65 endophytic fungal isolates and tested for extracellular lipase production. Among 65 isolates, ten fungi produced extracellular lipase. *Fusarium oxysporum* isolated from the leaves of *Croton oblongifolius* Roxb. (Plao yai) showed maximum lipase activity in olive oil, peptone and sodium nitrate as carbon and nitrogen sources. The enzyme was purified and showed reasonable stability at 40°C and exhibited maximum activity at pH 8 and 30°C (Panuthai et al. 2012).

R. oryzae was isolated as endophytic fungi from Mediterranean plants and a mycelium bound lipase that catalysed the fatty acid esterification was obtained. Various process factors influencing the ester synthesis were studied. At a pH range of 3-8 the enzyme was found to be active and the thermostable enzyme had maximum activity at 60°C (Torres et al. 2003). Endophytic fungus *Cercospora kikuchii* showed maximum production of lipase when supplemented with 2% soya bean oil in the medium for 6 days. Stability of the lipase with different adjuvants was studied. The studies showed that the activity is lost in the absence of adjuvants and that these adjuvants help in retaining 50% of the activity at 5°C after 8 months. (Costa-Silva et al. 2011)

In investigations carried out by Wu et al. (2009), a novel fibrinolytic enzyme Fu-P was obtained from *Fusarium* species CPCC 480097 isolated from chrysanthemum stems. This enzyme protease was used in the prevention of thrombosis. The purified enzyme has maximum activity at pH 8.5 at 45°C, and molecular weight of 28 kDa and isoelectric point of 8.1. The activity of enzyme was seen to be inhibited by EDTA and phenylmethanesulfonyl fluoride. Endophytic

fungi from *Opuntia ficus-indica* Mill. a forage cactus found in Brazil were isolated to examine the production of hydrolytic enzymes. More than forty endophytes were isolated wherein *Cladosporium cladosporioides* (20.43%) and *C. sphaerospermum* (15.99%) were commonly found. Many of the fungal isolates showed production of xylanases, pectinases, cellulases and proteases. *Xylaria* species exhibited maximum cellulose activity (Bezerra et al. 2012)

Sunitha et al. (2013) isolated 50 endophytic fungi from medicinal plants *Calophyllum inophyllum*, *Catharanthus roseus*, *Alpinia calcarata* and *Bixa orellana* and were screened for extracellular enzyme production such as amylase, lipase, pectinase, protease, cellulose and laccase on solid media. They reported that these fungi were able to produce cellulase, lipase, amylase, laccase, protease and pectinase. The different enzymes produced varied among fungi and depended on the host and other factors.

Katoch et al. (2014) screened endophytic fungal species of medicinal plant *Bacopa monnieri* (L.) Pennell for antimicrobial activity against a range of microorganisms. The ability of these endophytes to produce enzymes such as protease, amylase, cellulase, and lipase and their ecological role with the host plant was studied. Amylase activity was shown by all endophytes. 98% showed lipolytic activity, 28% cellulolytic and 31% proteolytic activity. All the endophytes exhibited different percentage of antimicrobial activity against various microorganisms. Devi et al. (2012) isolated endophytic fungus *Penicillium* sp. *Centella asiatica* plant capable of producing cellulase enzyme.

III. Materials and Methods

3.1 Collection of bark samples

Bark samples of *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan yew) were collected from Northern Indian Himalayan Region (IHR). This region extends from Kashmir to Uttarakhand with latitudes 30°-38° N and longitudes 78°-89° E and includes parts of Jammu and Kashmir, Himachal Pradesh and Uttarakhand. In the present investigation, bark samples were collected from Bhaderwah (district Doda, Jammu & Kashmir) which is situated 32.98° N 75.72°E Fig. 3.1).

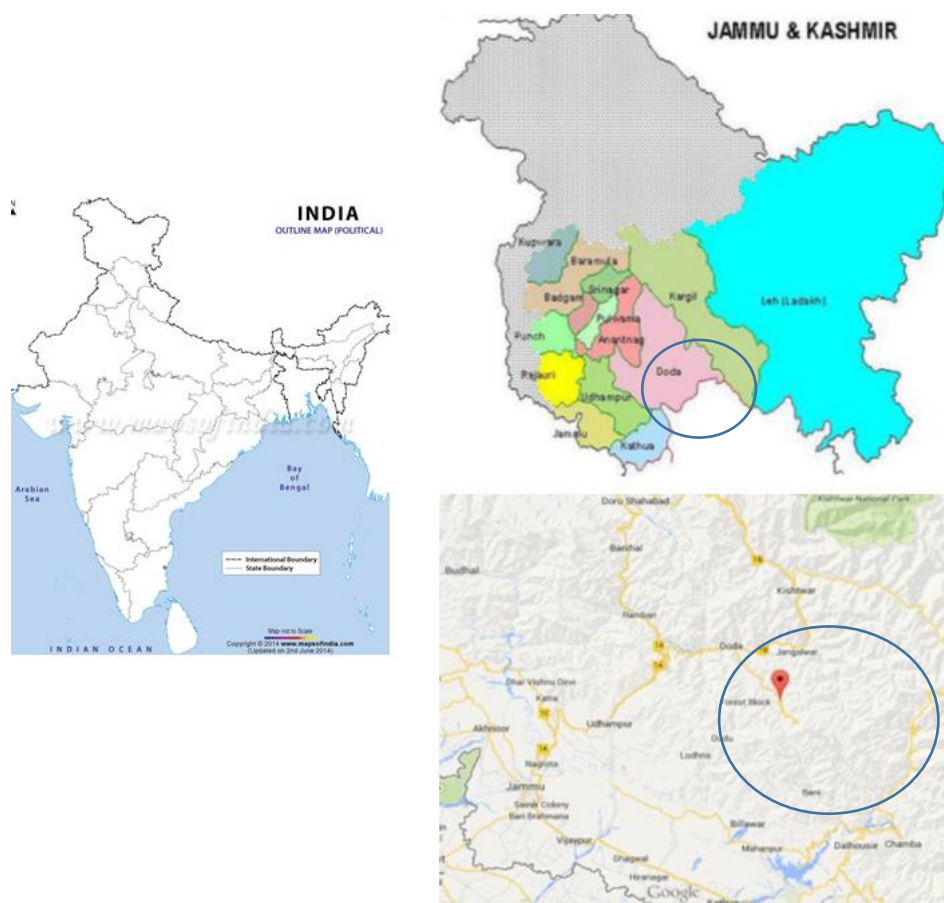


Fig. 3.1. Maps showing the collection site of bark samples of *Taxus baccata* from Bhaderwah (district Doda, Jammu and Kashmir).

3.1.1 Isolation of endophytic fungi from *Taxus baccata* bark samples

Bark samples (1 × 3 cm) from the stem of relatively young *T. baccata* subsp. *wallichiana* growing at different locations of the northern Himalayan region were collected. Bark pieces were placed in a sealed plastic bag, transported to the laboratory, and stored at 4°C. For isolation of endophytic fungi, bark samples were washed under running tap water, followed by final washing with sterile distilled water under aseptic conditions. The bark samples were then surface sterilized by treating with 70% aqueous ethyl alcohol (v/v) for 60 s followed by washing in 4% sodium hypochlorite for 60-90 s and finally rinsing in sterilized distilled water. The excess moisture on the bark pieces was blotted using sterile filter paper. The outer bark was teased apart with the help of flame sterilized sharp blade in order to procure inner bark. Surface disinfected small pieces (~ 0.5 × 0.5 × 0.5 cm) of inner bark were excised and placed on the surface of potato dextrose agar (PDA) medium (Appendix I) supplemented with ampicillin (50 µg/ml) in Petri plates. The plates were incubated at 28°C for 5-10 days to permit the growth of endophytic fungi. The plates were periodically observed for the growth of endophytic fungal colonies and culture purity. Pure fungal cultures of endophytic isolates were acquired by the hyphal tip method (Strobel et al. 1996). All the fungal isolates were coded and stock cultures were preserved by sub-culturing at monthly intervals. For long term preservation, isolates were stored in sterile distilled water as agar plugs. From an actively growing stock culture, sub-cultures were made as fresh plates and incubated at 25°C for 7 days.

3.2 Preparation of fungal extracts for biological assays

Among the different endophytic fungi isolated, 4 isolates designated as T1, T2, T5 and T6 were selected for further studies based on their biological activities from the preliminary screening. Plugs of 5 x 5 mm of the isolated endophytic fungi were cut from the actively growing colony and inoculated into the center of the petri plate (90 mm diameter, 25 ml PDA). The plates were then incubated at 25 ± 2°C for 1 week. Five mycelial (5 x 5 mm) agar plugs were inoculated

into 1000 ml of Erlenmeyer flasks containing 500 ml of potato dextrose broth and incubated at 27°C for three weeks under stationary conditions. After the fermentation process, the culture broths were separated from the mycelia by filtration through sterile muslin cloth. Equal volumes of the culture filtrate and ethyl acetate were taken in a separating funnel and were shaken for 10 min. The solvent phases were then allowed to stand for 5 mins, where the phases got separated and the organic phase (ethyl acetate) so obtained was collected. This way the filtrate was extracted three times with ethyl acetate. Sodium sulphate was added to remove any amount of water in the organic phase. The ethyl acetate fractions were then evaporated under reduced pressure in a rota evaporator to yield crude ethyl acetate extracts. The crude ethyl acetate extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions and stored at 4°C for further antimicrobial, antioxidant and cytotoxic studies.

3.3 Assay for antimicrobial activity

3.3.1 Test microorganisms and growth conditions

The following bacterial strains were used for the antimicrobial screening: Gram-positive: *Staphylococcus aureus* (MRSA, ATCC 33591), and *Bacillus megaterium* FH 1127. Gram-negative: *Escherichia coli* ESS 2231 and *Pseudomonas aeruginosa* M35. For the antifungal screening *Candida albicans* ATCC 10231 was used. These cultures were kindly provided to us by Dr. Sunil Kumar Deshmukh, Natural Products Division, Piramal Enterprises Limited, Mumbai, India. The bacterial parent strains were activated in Mueller-Hinton Broth (Appendix I) at 37°C for 18-24 hours at 120 rpm prior to assays. *Candida albicans* was activated in Sabouraud dextrose broth (SDB) (Appendix I) at 30°C for 48 hours at 120 rpm. The bacterial and fungal cultures were maintained and stored at 4°C. Activation of the bacterial culture was carried out by streaking culture on to a Mueller-Hinton Agar (MHA) (HiMedia) plate and then incubating it overnight at 37°C. A single colony was then picked from this plate and transferred to Mueller Hinton broth and incubated for 18-24 hours at 37°C prior to the test.

3.3.2 Antimicrobial agent (as control)

The antimicrobial agents used as control in the present study were Ampicillin and Streptomycin, a product of Cipla pharmaceutical Pvt. Ltd, India. The drug was stored in sealed containers in the dark at 4°C with a desiccant. Prior to experiments these antibiotic powders/stock solutions were brought to room temperature. Amphotericin-B, a product of Cipla pharmaceutical Pvt. Ltd, India was used as antifungal control. Dimethylsulfoxide was used as a negative control.

3.3.3 Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a Barium sulphate (BaSO_4) turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), was used. A BaSO_4 0.5 McFarland standard was prepared as 0.5 mL aliquot of 0.048 mol/L BaCl_2 (1.175% w/v $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) added to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.144 and 0.146 for the 0.5 McFarland standard (Table 3.1). The Barium sulfate suspension was transferred in 4 to 6 mL aliquots into screw-cap tubes of the same size as those used for growing or diluting the bacterial inoculum (NCCLS, 1997).

Table 3.1 McFarland Standard

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (mL)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (mL)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×10^8 CFU/ml)	1.5	3.0	6.0	9.0	12.0
Absorbance (600 nm)	0.146	0.210	0.449	0.661	0.850

3.3.4. Prescreen assay: Agar-well diffusion assay

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

1. Well-isolated colonies from an agar plate culture were transferred into a tube containing 5 mL of broth medium according to the culture.
2. The broth culture was incubated at 37°C until it achieves the turbidity of the 0.5 McFarland standard (10^8 CFU/mL).
3. The turbidity of growing broth culture was adjusted with sterile saline solution or with the medium itself.
4. Then 100 µl of inoculum was spread on the agar plate.
5. Wells of 6 mm each using a sterile cork-borer under aseptic conditions were prepared.
6. A fixed volume of extract (of different concentrations) was then introduced into the bored agar wells (the antimicrobial compound present in the extract was allowed to diffuse out into the medium and interact in a freshly seeded plate with the test organism) and then incubated at optimum temperature and duration depending upon the test organism.
7. For data analysis, after incubation, the clear zones of inhibition around the fungal extracts were measured in millimeter with a ruler and compared with the positive controls.
8. The test was carried out in triplicates.

3.3.5 Screening: Microplate broth dilution assay

Broth micro-dilution denotes the performance of antimicrobial / antibacterial potential of a dilution in the 96 wells micro-dilution plate (micro-titre plate) with a capacity of 300 µl per well. In the screening of antimicrobial compounds, the microplate method provides a potentially useful technique for determining minimum inhibitory concentration (MICs) of large numbers of test samples, requiring small amounts of substance. This can be particularly important if the antimicrobial agent is scarce as is the case for many natural products. This method can also be used for a wide variety of microorganisms. The MIC values for a drug are expressed as the lowest concentration that inhibits the bacterial growth. The micro dilution method was performed according to the method described in Jorgensen et al. (1999).

A 96-well ELISA tray was filled with 100 µl of the sterile Mueller-Hinton broth (MHB) as media control. For cell control 50 µl MHB and 50 µl of exponentially growing culture (0.5 McFarland) ($1-2 \times 10^8$ bacterial cells/ml and 1×10^6 yeast cells per ml) was added to these wells. Solutions of extract were prepared in MHB at a concentration of 5 mg/ml. The stock solution of extract was dissolved in DMSO. Extracts were tested at different concentrations. Antibiotic stock solutions were also made in the recommended media. 50 µl of the antibiotic control or 50 µl of the extract was added to attain different concentrations in the respective wells. Solvent controls were also made accordingly. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm (Multiskan Spectra Readers, Thermo). The plate was incubated at 37°C for 24 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. Further as an indicator of microorganism growth, after the specified incubation period, MTT assay was performed. 10 µl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT/Sigma-USA) (dissolved

in sterile water at 5mg/ml) was added to each well and incubated at 37 °C for 4 hours. Formazan product formation indicated by purple colour was observed according to the viability of the cells. Medium was removed from each of the wells using pipette and 100 µl of pure DMSO was added. The plate was kept for 15 min so as to solubilize the crystals and then the absorbance of each well was determined using an automatic ELISA tray reader adjusted at 540 nm. The results were expressed as percent inhibition with respect to suitable controls.

3.4 Cell growth inhibition assay

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were procured from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Appendix I) (Sigma, USA), containing 10% (v/v) foetal bovine serum (Gibco), 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin. Cells were maintained in a humidified incubator with 5% CO₂ at 37°C. The effect of fungal extract on the growth of cancer cell lines was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. Cells were trypsinised and seeded at a density of $\sim 2 \times 10^4$ cells per well in 96 well cell culture plate and incubated overnight. After 16 h, fungal extracts were added in varying final concentrations to the wells. After 72 h of incubation, 20 µL of MTT reagent (Sigma USA, 5mg ml⁻¹) was added to each well and again incubated for 4 h. The formazan crystals formed were solubilized in 100 µl DMSO (Merck, Germany). Finally, the absorbance of each well was recorded at 570 nm, taking 630 nm as the reference wavelength, using the microplate reader (Tecan infinite, Austria). Paclitaxel was used as a positive control at the concentration of 20 µg ml⁻¹. Percentage of inhibition was calculated as:

$$(\text{Mean OD of untreated cell} - \text{Mean OD of treated cell} / \text{Mean OD of untreated cell}) \times 100$$

3.4.1 Isolation of peripheral blood mononuclear cells:

Peripheral blood mononuclear cells (PBMCs) were isolated based on ficoll density gradient method (Kumar et al 2004). To a sterile 15 mL conical centrifuge tube, 5 ml of Histopaque® - 1077 was added and brought to room temperature. 5 mL of whole blood was carefully layered onto the Histopaque-1077 and centrifuged at 400xg for 30 min at room temperature in swinging bucket rotor (Thermo Scientific Biofuge Stratos). This density based centrifugation technique fractionates blood into plasma, red blood cells and peripheral blood mononuclear cells (PBMCs). After centrifugation, carefully discarded the upper plasma layer with a micropipette and collected opaque interface (buffy coat) containing PBMCs in a sterile 15-mL conical centrifuge tube. The cells were washed twice in 10 mL of isotonic phosphate buffered saline solution and centrifuged at 250x g for 10 minutes. Finally, the cell pellet was re-suspended in 1 mL of complete medium (Appendix I) (RPMI-1640 supplemented with 10% foetal bovine serum, 100 µg/mL streptomycin, 100 I.U. /mL penicillin and 10 mM HEPES).

3.4.2 Counting of cells

Cell viability is calculated by trypan blue exclusion assay on hemocytometer (Strober 2001). Briefly, 10 µL of cell sample was diluted with 0.4% trypan blue solution in the ratio 1:5 or 1:10 in a vial and incubated for 1-2 min. The preparation was loaded on a hemocytometer and examined immediately under a microscope at 40X magnification (Nikon Eclipse E100-LED). The number of unstained viable cells (non-viable cells take up dye and appear blue) were counted in all the four corner squares. Number of viable cells in the original cell suspension was calculated from the following formula

$$\text{Number of viable cells/ml} = \frac{(\text{Total no of unstained cells} \times \text{dilution factor} \times 10^4)}{4}$$

3.4.3 Lymphocyte proliferation assay

Effect of fungal extract on proliferation of PBMCs was measured by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) based colorimetric assay (do

Livramento, et al. 2013). MTT assay measures the cell viability based on reduction of the yellow coloured tetrazolium salt MTT into purple formazan by mitochondrial succinate dehydrogenase of metabolically active cells (Mosmann 1983). PBMCs were seeded at a density of 1×10^5 cells per well and then fungal extracts were added in varying final concentrations. After 2 h, concanavalin A ($5 \mu\text{g ml}^{-1}$), a mitogen was added in order to stimulate the PBMCs. After 72 h of incubation, MTT assay was carried out to measure the cell proliferation. MTT assay and calculation of inhibition in mitogen-induced proliferation were done as described in the case of a cell growth inhibition assay.

3.4.4 Measurement of TNF- α

In order to evaluate the production of TNF- α , MCF-7 and concanavalin A stimulated PBMCs were incubated with varying concentrations of fungal extract for 72 h. Culture supernatant was collected after 72 h incubation and stored at -80°C . Extracellular IFN- γ secretion in the culture supernatant was measured by sandwich ELISA (PeproTech, USA) in 96-well ELISA plate (Nunc MaxiSorp®) as per manufacturer's instruction (Agallou et al. 2014). Briefly, capture antibody was diluted with phosphate buffer saline (PBS) to a concentration of $1 \mu\text{g/ml}$ and added $100 \mu\text{l}$ to each ELISA plate well and incubated overnight at room temperature. The wells were aspirated to remove liquid and the plate was washed 4 times using $300 \mu\text{l}$ wash buffer (0.05% Tween-20 in PBS) per well. After the last wash, the plate was tapped in inverted position to remove residual buffer on paper towel. $300 \mu\text{l}$ of blocking buffer was added to (1% BSA in PBS) to each well. The plate was incubated for 1 h at room temperature. The plate was aspirated and washed 4 times with wash buffer. $100 \mu\text{l}$ of the test samples and the standard (TNF- α , concentration 3000 ng/mL to zero) were added to each well in triplicate. The plate was incubated at room temperature for overnight. The liquid was aspirated plate was washed 4 times. Detection antibody was diluted $1.0 \mu\text{g/ml}$ and added $100 \mu\text{l}$ to each ELISA plate well in the plate and incubated at room temperature for 2 hours. Plate was washed 4 times and

100 µl of diluted avidin-HRP conjugate (1:2000) in sample diluent (0.05% Tween-20, 0.1% BSA in PBS) was added. The plate was aspirated and washed 4 times. Finally, 100 µl of ABTS substrate solution (Thermo Fisher Scientific) was added to each well. The plate was wrapped in a oil and incubated at room temperature for 15 minutes for colour development. Absorbance was recorded at 405 nm with wavelength correction set at 650 nm in ELISA plate reader (Tecan, Austria). Inhibition in TNF- α production was calculated in the same manner as mentioned in the cell growth inhibition assay.

3.4.5 Antioxidant assay

In order to know the antioxidant potential of fungal extract, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed. DPPH assay is used generally for the detection of free radical scavenging potential of an antioxidant compound. DPPH is actually a stable radical in solution. It gives purple color when dissolved in methanol and absorbs at a wavelength of 517 nm. Fungal (50 µl) extract with varying concentration was mixed with 150 µl of DPPH (100 µM) in methanol, added in wells of a 96-well microtiter plate. Ascorbic acid (100 µg ml⁻¹; 50 µl) was used as a positive control. The plate was incubated in the dark for 45 min, after which the absorbance of the solution was measured at 517 nm in ELISA microtitre plate (Tecan infinite, Austria). Free radical scavenging activity was expressed as the inhibition percentage calculated using formula,

$$\text{Free radical scavenging activity} = (A_{\text{Control}} - A_{\text{Sample}} / A_{\text{Control}}) \times 100$$

3.5 Identification of endophytic fungi

3.5.1 Morphological characters

The fungal isolates which showed positive results for antimicrobial and cytotoxicity were characterized based on their morphological characters. For examining the cultural and morphological characters, the fungal isolates were subcultured onto fresh media (PDA) and incubated at 28°C for 2 weeks. Cultural characters such as colour and nature of the growth of

the colony were intended by visual observation. Morphological characteristics of the fungal endophytes like mycelia, conidiophores and conidia were microscopically (Nikon Eclipse E200) examined (Barnett et al. 1998; Wei 1979).

3.5.2 Molecular characterization

3.5.2.1 Isolation of fungal genomic DNA

The fungal isolates were inoculated aseptically and individually in 50 ml of potato dextrose broth in 250 ml Erlenmeyer flasks. Cultures were incubated at 28°C for 7 days and the mycelium of each fungus was harvested by filtering through cheese cloth. Genomic DNA was extracted from the mycelia using the CTAB method as described in Zhang et al. (1996).

3.5.2.2 CTAB method for DNA extraction

1. Mycelium was ground into powder in liquid nitrogen in an autoclaved mortar and immediately transferred to 50 ml oak ridge tube and added preheated CTAB buffer to make slurry along with 200 μ L β -mercaptoethanol. Incubated at 60°C for 1h in water bath with mixing at regular intervals.
2. Equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed well for about 5 min and centrifuged for 10 min at 5000 rpm.
3. Aqueous phase was removed with wide-bore pipette (cut off tip from mouth) to clean oak ridge tube. Repeated chloroform extraction if extract was still colored.
4. DNA was precipitated with 0.66 volumes of cold isopropanol and incubated for 1 h at -20°C.
5. Centrifuged at 10000x g for 15 min.
6. Supernatant was discarded and dissolved the pellet in 1 mL TE buffer (Appendix II) and taken solution in microfuge tube.
7. 2 μ L RNase solution (10 mg/mL stock) was added and incubated at 37°C for 1 h. RNase stock solution was preheated for 5 min at 60°C before use.

8. Re-extracted with equal volume of phenol: chloroform (1:1 v/v). Centrifuged (1000 x g, 10 min) and retained aqueous phase.
9. 0.3 volume of 3M sodium acetate and 0.6 volume of chilled iso-propanol were added. Incubated for 1 h at -20°C.
10. Centrifuged (10000 x g, 8 min) and retained pellet. Washed pellet with 30 µL of 70% EtOH and air-dried pellet.
11. Dissolved pellet in TE buffer and stored at -20°C.

3.5.2.3 Checking of DNA (Agarose Gel Electrophoresis)

1. Agarose gel (0.8 % (w/v)) in TBE buffer (Appendix II) was prepared and ethidium bromide (1.0 µL/30mL) was added after cooling down and poured the gel in mini gel tray with comb. Allowed the gel to solidify.
2. Placed gel tray in electrophoresis unit, filled the unit with 0.5X TBE buffer and removed the comb carefully.
3. Prepared samples by adding tracking dye to DNA samples and mixed.
4. After loading the samples in wells, electrophoresis was carried out at 60 V for 30-45 minutes and visualized on a U.V. transilluminator.

3.5.2.4 Quantification of DNA using Nano drop

The DNA concentration was estimated by using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc. USA). For DNA quantification 1 µl of sample was loaded onto the lower optical surface and then the lever arm was lowered. The upper optical surface engages with the sample, establishing a liquid column with the path length determined by the gap between the two optical surfaces. The sample is analyzed at both 1 mm and 0.2 mm path, allocating a large dynamic range of nucleic acid detection. The quality of the DNA was evaluated by measurement of the A260/A280 and the A230/A260 ratios. Ideally, the A260/A280 ratio should be 1.8-2.0 while

the A230/A260 ratio should be 0.3-0.9. Ratios (A260/A280) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.5.2.5 Amplification of Internal Transcribed Spacer (ITS) region

ITS regions of the rDNA from genomic DNA of selected endophytic fungi together with the 5.8S rRNA gene were amplified using the universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White et al. (1990). Reaction mixture for the PCR contained 1X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 μ M, 1.5 mM MgCl₂, each primer at a concentration of 0.1 μ M, 2.5 units of Taq DNA polymerase (Fermentas, USA) and 50 ng DNA in a final volume of 50 μ l. The amplification of ITS region from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). The PCR programme consisted of an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min (annealing) and 72°C for 1 min with a final extension of 72°C for 10 min. Controls containing no DNA template were included for verifying that there is no contamination of reagents and reaction buffer. Successful amplifications were confirmed by agarose gel (1.5% w/v) electrophoresis and visualization on a U.V. trans- illuminator.

3.5.2.6 Purification of PCR products and sequencing

Amplified ITS products were purified by agarose gel (0.8%) electrophoresis prior to cloning. The DNA fragment was excised from the gel, using the QIAquick gel extraction kit (Qiagen Inc., USA) following the instructions of the manufacturer. Purified PCR products were eluted with 30 μ l TE buffer (pH 8.0). Purified PCR products were then used for sequencing. The sequence was generated by chain termination method (Sanger et al. 1977) using an Applied Biosystems automatic sequencer.

3.5.2.7 Sequence analysis

The ITS nrDNA sequences of the isolates were compared with those available in GenBank databases using BLAST search program (Altschul et al. 1997) to find the possible homologous sequences of the newly sequenced taxa for each fungus. The sequences of closely related fungi retrieved from GenBank were aligned to minimize the number of inferred gaps. The sequences were edited with BioEdit 5.0.6 (Hall 1999) and aligned using multiple alignments ClustalW software (Larkin et al. 2007). The phylogenetic trees were reconstructed by using MEGA5.1 software (Tamura et al. 2011). The bootstrap was 1,000 replications to assess the reliable level for the nodes of the tree. All sequences were generated in this study were submitted to the NCBI GenBank database.

3.6 Extracellular enzymes production by thermotolerant endophytic fungi

3.6.1 Agar Plate Assays

3.6.1.1 Amylase activity (Hankin and Anagnostakis 1975)

Glucose, Yeast, Peptone (GYP) medium (Appendix I) was prepared with 0.2% soluble starch (pH 6.0). Mycelial discs of the endophytic fungi were placed on the agar plates and incubated for 7 days. Then the plates were flooded with iodine solution. A yellow zone around the fungal colony in an otherwise blue medium indicated the presence of amylase activity.

3.6.1.2 Cellulase activity (Rohrmann and Molitoris 1992)

GYP medium without glucose supplemented with 0.5% of Na-carboxy methylcellulose was used to assess the cellulase activity. After 7 days of incubation of the fungi in the medium, the plates were flooded with 0.2% aqueous Congo red solution and de-stained with 1.0 M NaCl for 15 minutes. Appearance of yellow zone around the fungal colony in otherwise red medium indicated cellulase activity.

3.6.1.3 Laccase activity (Rohrmann and Molitoris 1992)

GYP agar medium supplemented with 0.05 g/l 1-naphthol was used to assess the laccase production of endophytic fungi. As the fungus grows, the colourless medium turns into blue due to oxidation of 1-naphthol by the action of laccase enzyme.

3.6.1.4 Lipase activity (Hankin and Anagnostakis 1975)

Endophytic fungi were grown on a medium (g/l: 10.0 g peptone, 5.0 g NaCl, 0.1 g of CaCl₂·2H₂O, 15.0 g agar, pH 6.0) supplemented with sterilized 1 ml of Tween 20 (Appendix I. Precipitation or clearing around the fungal colony indicated the production of lipase.

3.6.1.5 Pectinase activity (Hankin and Anagnostakis 1975)

Endophytic fungi were grown on a medium consisting of 0.5% pectin, 0.1% yeast extract and 1.5% agar. The pH of the medium was adjusted to 7.0 to detect pectate transeliminase production and pH 5.0 was used to detect pectinase activity. After 7 days of fungal growth, the plates were flooded with 1% aqueous solution of hexadecyl-trimethylammonium bromide. A clear zone formed around the fungal colony indicated pectinase activity.

3.6.1.6 Proteinase activity (Hankin and Anagnostakis 1975)

GYP agar medium with 0.4% gelatin (pH 6.0) was used to assess the proteinase activity. After 7 days of incubation, degradation of gelatin was observed as a clearing zone in somewhat opaque agar around the colony. The plates were then flooded with aqueous saturated solution of ammonium sulphate which resulted in the formation of a precipitate. This made the agar plates opaque and enhanced the clear zone around the fungal colony.

3.6.1.7 Tyrosinase activity (Rohrmann and Molitoris 1992)

Endophytic fungi were grown on GYP agar medium. After 7 days of incubation, p-cresol (1.08 g/l with 0.05% glycine) was added. Formation of red brown colour around the fungal colony indicated tyrosinase activity.

3.6.1.8 L-asparaginase activity (Gulati et al. 1997)

A modified Czapek Dox's medium (2.0 g glucose, 10.0 g L-asparagine, 1.52 g of KH_2PO_4 , 0.5 g KCl, 0.52 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 g agar, water 1000 ml) was used for determining the L-asparaginase activity. Stock solution (3% w/v) of phenol red was prepared in ethanol (pH 6.2) and 3 ml of this preparation was added to 1000 ml of modified Czapek Dox's medium. After 5-6 days of incubation, the plates were observed for pink zone around the colony in an otherwise yellow medium indicating the L-asparaginase activity.

3.7 Quantitative Assay

3.7.1 Cellulase Activity

Two endophytic fungi, *Pestalotiopsis microspora* and *Phoma* sp., which showed maximum cellulase activity by plate assay were further selected to test for their ability to produce cellulase.

3.7.2 Culture conditions for cellulase production

The fungi were grown in 250 ml Erlenmeyer flasks containing 100 ml of Czapek Dox's medium (Appendix I) at 28°C under shaking conditions (120 rpm). After 10 days of incubation, the culture was filtered and the filtrate was used (crude extract) to determine the cellulase activity according the method described by Ghose (1987). One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole of glucose from the appropriate substrates per minute under the assay conditions.

3.7.3 Cellulase assay

For cellulase assay different reagents were prepared (Appendix II).

Procedure

1. Crude enzyme extract was prepared from the culture supernatant by filtration with Whatman filter paper 42. Cellulase activity was determined with CMC by assay method developed by Ghose (1987) with slight modification. In this method, reducing

sugar formed by the hydrolysis of cellulosic substrate was estimated by DNS (3, 5-Dinitrosalicylic acid) and the colour formed was measured spectrophotometrically at 540 nm.

2. The substrate for this reaction was made by dissolving 1% CMC in distilled water and kept for overnight at room temperature.
3. The assay mixture contained 1 ml of substrate, 1 ml of buffer (0.1M Citrate buffer), pH 5, (Appendix I) and 1 ml of diluted enzyme (0.5 ml enzyme + 0.5 ml of distilled water).
4. Control sample contained all the above mixture except the substrate CMC.
5. This reaction mixture was incubated at 50° C for 60 min.
6. The enzyme activity was stopped by adding 3 ml of DNS and was again incubated for 15 minutes at 95°-100° C.
7. 1 ml Rochelle salt was added just after 15 minutes of incubation period.
8. After cooling the reaction mixture, the absorbance was measured at 540 nm against blank made up of buffer and distilled water.
9. The standard graph was prepared by using 2 mg/ml glucose stock solution in 0.1 M Citrate buffer of pH 5 and absorbance taken at 540 nm.

For calculating the cellulase activity, the absorbance value of controls were subtracted from their corresponding test. CMCase were calculated by using the formula described by Ghose (1987). Absorbance values of sample was translated into glucose using standard.

CMCase = 0.185/ critical enzyme concentration U/ml

3.7.4 Characterization of enzyme

3.7.4.1 Optimization of time for cellulase production

To study the effect of incubation time on cellulase activity of endophytic fungi, the fungi were grown in submerged condition by incubating at 28° C at different time intervals (5, 10, 15 and

20 days). The cultures were harvested at different time intervals and the filtrate was used to estimate the cellulase by using 1% CMC and reducing sugars were determined using DNS method (Ghose 1987). Citrate buffer (pH 5.0) was used and reaction mixture was incubated at temperature 50° C.

3.7.4.2 pH

The incubation time of 10 days which gave the highest cellulase activity was selected to study the effect of pH on cellulase activity of *Phoma* sp. and *P. microspora*. In order to determine the most effective pH for cellulase activity, the submerged state fermentation was carried out in the Erlenmeyer flask (250 ml) containing Czapek Dox's with different pH (pH 4, 5, 6, 7, 8, 9, and 10). After 10 days of incubation at 28°C in shaking condition (120 rpm), the culture broth was filtered and the crude extract obtained was then used to assay cellulase enzyme with buffers (0.1 M) of different pH (sodium acetate buffer, pH 3-4; citrate buffer, pH 5; potassium phosphate buffer, pH 6-7; Tris HCl, pH 8; glycine-NaOH buffer, pH 9-10) by using DNS method (Ghose 1987). CMCase activity was determined by comparing the O.D with standard curve of glucose. The relative cellulase activity was calculated in percentage by assuming the highest activity as 100%.

3.7.4.3 Temperature and thermostability

To determine the enzyme activity at different temperatures, the reaction mixture containing crude enzyme extract, substrate and MES buffer (pH 5.0) was incubated for 60 min in the temperature range of 30°-100° C (with an interval of 10° C). Thermal stability of the enzyme activity was determined by incubating the enzyme-buffer mix i.e. without substrate at 100°C for different time intervals (24, 48, 72 and 96 hours) as described by Ariffin et al. (2006).

3.7.4.4 Lipase Activity

The endophytic fungus, *Montagnulaceae* sp., which was showing maximum lipase activity by agar plate assay was selected to produce lipase.

3.7.4.5 Culture condition for lipase production

The endophytic fungus *Montagnulaceae* sp. was grown in 250 ml Erlenmeyer flasks containing 100 ml of Basal medium (Appendix I) at 28°C under shaking conditions (120 rpm). After desired times of incubation, the culture was filtered and the filtrate was used (crude extract) to determine the lipase activity according the method described by Tiwari et al. (2011).

3.7.4.6 Lipase assay

1. Crude enzyme extract was prepared from the culture filtrate which was centrifuged at 10,000 rpm for 15 min at 4°C. Lipase activity was determined with p-NPP by the method reported by Licia et al. (2006). In this method p-NPP was hydrolysed by lipase to produce p-NP which gave yellow colour. The absorbance was measured spectrophotometrically at 410 nm.
2. The substrate for this reaction composed of solution A and solution B. Solution A contained 40 mg of p-NPP dissolved in 12 ml isopropanol. Solution B contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water.
3. The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h.
4. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer {Potassium phosphate buffer, pH 7, 0.1 M (Appendix II), 0.1 ml of enzyme (the filtrate) and the volume was made up to 4 ml with distilled water. This mixture was incubated at 50°C for 30 min.
5. The enzyme activity was stopped by adding 0.2 ml of isopropanol and the absorbance was measured at 410 nm against enzyme free blank.
6. The standard graph was prepared by using p-NP (20-100 μ M) in 2 mM CAPS buffer (Appendix II) pH 11 and absorbance taken at 410 nm.

Enzyme activity (U/ml enzyme) was calculated according to equation mentioned below. The highest enzyme activity data was assumed 100% and relative enzyme activity was estimated for each data. To calculate specific enzyme activity (U/mg protein), Bradford assay was applied. Specific enzyme activity was obtained by division of enzyme activity value to protein content (mg/ml). One lipase unit (U) is defined as the amount of enzyme that liberated 1 μ M p-NP per min under the assay conditions (Maia et al.1999). Enzyme activity and specific activities were calculated as per the following formula:

U/ml Enzyme can be calculated as:

$$\mu\text{M of p-NP released} \times \frac{\text{Total reaction volume}}{\text{Vol. used in spectrophotometric determination}} \times \frac{\text{Vol. of enzyme}}{\text{Incubation time}} \quad (1)$$

Where

Total reaction volume = 4 ml

Vol. used in spectrophotometric determination = 3ml

Vol. of enzyme = 0.1 ml

Incubation time = 30 minutes

* μ M of p-NP released were calculated using straight line equation obtained from standard graph of p-NP as:

$$OD_{410} = 0.008 \times \text{Conc. p-NP } (\mu\text{M}) + 0.009$$

Hence

$$\text{Conc. p-NP } (\mu\text{M}) = \frac{OD_{410} - 0.009}{0.008} \quad (2)$$

$$\text{Specific activity (U/mg protein)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein content (mg/ml)}} \quad (3)$$

3.7.4.7 Protein assay

The Bradford assay was used to determine the total protein concentration of respective sample. In this method, Coomassie brilliant blue G-250 dye binds to proteins and changes their colour from green to blue. That colour change is monitored at 595nm in UV-visible

spectrophotometer. As the concentration of protein content increased, the colour gets darker. Coomassie brilliant blue G-250 binds to arginine, lysine, and histidine residues in protein samples (Bradford 1976). A series of BSA standard in different concentrations were prepared. According to this BSA standard, standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation.

Preparation of protein sample:

1. 1 ml of crude extract (protein) was diluted with 1 ml of distilled water.
2. 3 ml of Bradford reagent was added.
3. Mixture was left for 5 minutes at room temperature and the absorbance was measured at 595nm.

3.7.4.8 Characterization of enzyme

3.7.4.8.1 Optimization of time for lipase production

To study the effect of incubation time on lipase activity, the fungus was grown in submerged condition by incubating at 28° C at different time intervals (4, 8, 12, 16 and 20 days). Basal medium supplemented with 1% (v/v) olive oil was used for lipase production. The cultures were harvested at different time intervals and the filtrate was used to estimate the lipase as mentioned above

3.7.4.8.2 pH

The incubation time of 12 days which gave the highest lipase activity was selected to study the effect of pH on lipase activity of *Montagnulaceae* sp. In order to determine the most effective pH for lipase activity, the submerged state fermentation was carried out in the Erlenmeyer flasks (250 ml) containing basal medium with 1% olive oil at different pH (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12). pH was adjusted with 0.1 M of different buffers for different pH (sodium acetate buffer, pH 3-4; citrate buffer, pH 5; potassium phosphate buffer, pH 6-7; Tris HCl, pH

8; glycine-NaOH buffer, pH 9-12). The assay mixture (500 μ l each buffer, 1 ml substrate and 100 μ l enzyme solution) was incubated at 40°C for 30 minutes and enzyme activity was determined by spectrophotometric assay using *p*-NPP as a substrate. The relative lipase activity was measured as a percent (%) in comparison to highest value.

3.7.4.8.3 Temperature and thermostability

To determine the enzyme activity at different temperatures, the reaction mixture containing crude enzyme extract, substrate and 2 mM CAPS buffer (pH 11.0) (Appendix II) was incubated for 60 min in the temperature range of 30°-100° C (with an interval of 10° C). The enzyme activity was determined as described above.

3.8 Analysis of bioactive molecules

3.8.1 Sample preparation

Endophytic fungi (T1 and T6) were grown on potato dextrose broth and after incubation at 28°C for three weeks, the cell suspension cultures were filtered to remove mycelia to obtain filtrate. The filtrate was extracted thrice with ethyl acetate and the organic ethyl acetate phase so obtained was dried using sodium sulphate. The organic ethyl acetate phase was evaporated to get dry residue.

3.8.2 Mass spectrometry

A typical mass spectrometer consists of three parts: An ion source, where the molecules get charged, a mass analyzer that separates the molecules by their mass-to-charge ratio, and a detector, which approximately measures the number of incoming ions. Molecules are charged or ionized to respond to the measurement. Several methods to ionize a sample exist and if the analyte molecule is intact it is called soft ionization or if it fragments it then it is called hard ionization. The resulting ions either carry an additional proton ($[M+H]^+$, M denotes the sample molecule), or have lost one in negative mode ($[M-H]^-$), but sample ions may also form through the addition of other ions, such as sodium ($[M+Na]^+$) or ammonium ($[M+NH_4]^+$). Other soft

ionization techniques include the matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988). Here the sample is embedded into a crystalline matrix. In the ion source, the matrix is then evaporated by laser pulses, releasing charged molecules into the gas phase. A mass analyzer separates ions by their mass-to-charge ratio. The unit Thompson (Th) is sometimes used for this ratio in mass spectrometry, although it is officially dimensionless. Various methods exist to achieve separation by mass-to-charge namely time-of-flight (TOF), quadrupole, linear ion trap and Orbitrap mass analyzers.

3.8.3 UHPLC-QTOF-MS/MS analysis

For UHPLC-QTOF-MS/MS analysis, the residue was dissolved in methanol and used as such for analysis. Chromatographic separation was carried out using Agilent 1200 UHPLC system equipped with reverse phase column (C18 column, 150 mm x 2.1 mm i. d. pore size 5.0 μm). Sample (5 μl) was injected into the column and elution was carried out in a gradient mode with acidified water (0.2 % v/v formic acid) to 90 % methanol over a period of 40 min at a flow rate of 0.3 mL/min. High resolution mass spectroscopic (HRMS) detection was performed using Bruker Impact QTOF mass spectrometer (Bruker, Germany), operating in Top5 data-dependent mode from 100-1500 m/z with electron spray ionization (ESI) and both positive and negative ions were detected. Smart Formula 3D™ and Fragment Explorer part of Data Analysis 4.2 were used for compound formula generation and assignment of fragment structures. Qualitative analysis of the samples was done and the signals that were detected by LCMS were characterized as peaks, mass over charge ratio (m/z) and retention time. The compound spectrum report shows the presence of about 500 compounds in the crude extract. Among these compounds, some of these were putatively identified based on physico-chemical properties. Spectral similarities of the compounds with the public spectral data libraries was done without reference to authentic chemical standards. SmartFormula3D™ and Fragment Explorer are part of Bruker Data Analysis 4.2 and were used for elemental formula generation

and assignment of fragment structures. METLIN and mass bank spectral databases were used for analysis. Positive hits were selected and also based on reported literature and research findings putative compounds were finally selected.

From these compounds, those having anticancer, antioxidant and antimicrobial activity were selected. The nature of the crude extract and the source of extract are compared with that of the selected compounds from the database. The literature that has already been reported was studied and from these research findings the possible or putative compounds as positive hits were finally selected. The high quality data i.e. accurate mass and true isotopic patterns (TIP), in combination with Bruker's unique ab initio formula discovery tools, Smart Formula™ and SmartFormula 3D™, provided accurate molecular formulae with a maximum level of certainty. Further high quality spectra with TIP provides isotopic abundance information which is essential to narrow down potential molecular formulae with confidence. Comparison of the theoretical and observed isotopic patterns resulted in a statistical match factor σ -fit (mSigma value) – increasing certainty in the identified compounds.

3.9 Statistical analysis

The data were expressed as mean values and standard deviation (SD). The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software (GraphPad software, Inc. USA).

IV. Results and Discussion

4.0 Isolation, identification and biological activities of endophytic fungi

4.1 Isolation of endophytic fungi from *Taxus baccata* bark samples

The samples of bark from *T. baccata* collected from Bhaderwah (Dist. Doda, India) (Fig. 4.1) were inoculated on PDA and incubated at 25 ± 2 °C for 5-10 days after surface disinfection. The cultures were checked for their purity and recultured at regular time intervals. A total of 25 endophytic fungi were isolated from bark samples of *Taxus baccata*.



Fig. 4.1. *Taxus baccata* bark samples collected from Bhaderwah (district Doda), Jammu & Kashmir, India.

4.2 Preliminary screening for bioactivity

The mycelial mats (5.0 mm diam) of 7-10 days old endophytic fungi were inoculated into potato dextrose broth (100 ml) and incubated at 25 ± 2 °C on a shaker for 5 days and resulting culture was used as the culture seed. The same was then transferred to 500 ml of potato dextrose broth and incubated at 25 ± 2 °C for 21 days in dark as a stationary culture. The culture was separated from the growth medium and the culture broth were extracted with ethyl acetate. The fractions were combined, and the solvent was then evaporated under reduced pressure at 35 °C. The residue so obtained was re-suspended in DMSO and tested for antimicrobial, cytotoxicity and antioxidant activities. Among the 25 strains tested, four isolates showed higher antimicrobial, cytotoxic and antioxidant activities compared to other isolates. These isolates were designated as T1, T2, T5 and T6, and selected for further studies. These strains were identified based on their phenotypic characteristics and ITS sequence analysis.

4.3 Identification of endophytic fungal isolates

The selected fungal isolates were differentiated based on their morphological characteristics as well as comparison of ITS sequences. The morphological appearances of the selected fungal isolate were characterized based on mycelial colour, growth patterns, and structure of fruiting bodies. Genomic DNA was extracted and PCR-amplified using universal primer pair of ITS1 and ITS4. PCR products were then purified and sequenced. Sequences were compared with NCBI database using BLASTN. NCBI GenBank database was used for retrieving the closely associated sequences. Phylogenetic trees were reconstructed, and the distances between sequences were calculated. Bootstrap analysis was performed with 1000 replications to assess the confidence limits of the branches.

Different size products, ranging from 560 bp to 820 bp, were obtained after amplification of ITS/5.8S rRNA region of fungi using ITS1 and ITS4 primers. Among the four fungal isolates studied, isolate T1 showed ITS size of ~590 bp, T2 ~820 bp, T5 ~610 bp and T6 ~560 bp (Fig.

4.2). Sequences obtained in this study were submitted to GenBank of NCBI under the following accession numbers. Isolate T1: KX355165; Isolate T2: KY411698; Isolate T5: KY421921 and isolate T6: KT779291 (Appendix III).

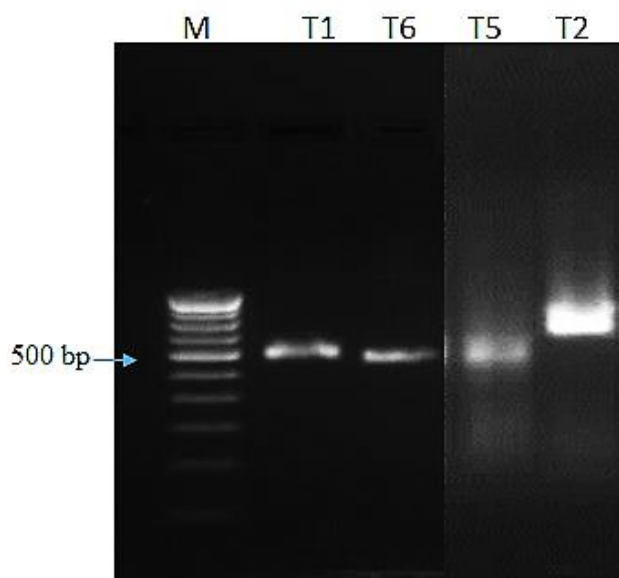


Fig. 4.2. Gel photograph of PCR amplified DNA of endophytic fungal isolates. M – Marker, T1, T6, T5 and T2 isolated from endophytic fungi

4.3.1 T1 isolate

In a pure culture (PDA, 25°C), the fungus formed less dense white mycelium, which spread over the Petri dish (9 cm in diameter) over eight days. The back side of the culture was whitish to beige in colour. Microscopic observations revealed the presence of colorless, elliptical, single α -spores measuring $4.5-7.0 \times 2.0-2.8 \mu\text{m}$ and needle/hair-like, filamentous, colorless β -spores, curved at one end measuring $15-25 \times 0.5-1.5 \mu\text{m}$ in size (Fig. 4.3).

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 showed 588 bp in length. BLAST analysis revealed 98% similarity (query coverage of 100%) with *Diaporthe* sp. (Fig. 4.4) Phylogenetic analysis of ITS sequences of different species of *Diaporthe* yielded a consensus tree and the present isolate (T1) clustered with *Diaporthe* sp. (KC357558 and KC357559) (Fig. 4.5) and thus identified as a *Diaporthe* sp. The ITS sequence obtained for

the present study isolate is submitted to NCBI database under the accession number KX355165.

The anamorphic form (the asexual reproductive morphological stage) of *Diaporthe* is the genus *Phomopsis*. *Diaporthe/Phomopsis* are either plant pathogens or endophytic species (van Niekerk et al. 2005; Murali et al. 2006; Dos Santos et al. 2016), and are associated with dicotyledonous and monocotyledonous plants of tropical and temperate regions (Eriksson and Vuorela 1998; Guo et al. 2000). The *Diaporthe/Phomopsis* complex occurs as endophytes in medicinal plants such as *Taxus chinensis* (Liu et al. 2009), *Taxus globosa* (Soca-Chafre et al. 2011) and some medicinal shrubs of India (Naik et al. 2008). Here, we report an endophytic association of a *Diaporthe* sp. with *Taxus baccata* subsp. *wallichiana* for the first time. *Diaporthe* is one of the most abundant genera of endophytes and is known to synthesize diverse variety of compounds of medical and research interest (Silva et al. 2005; Pompakakul et al. 2007; Rukachaisirikul et al. 2008).

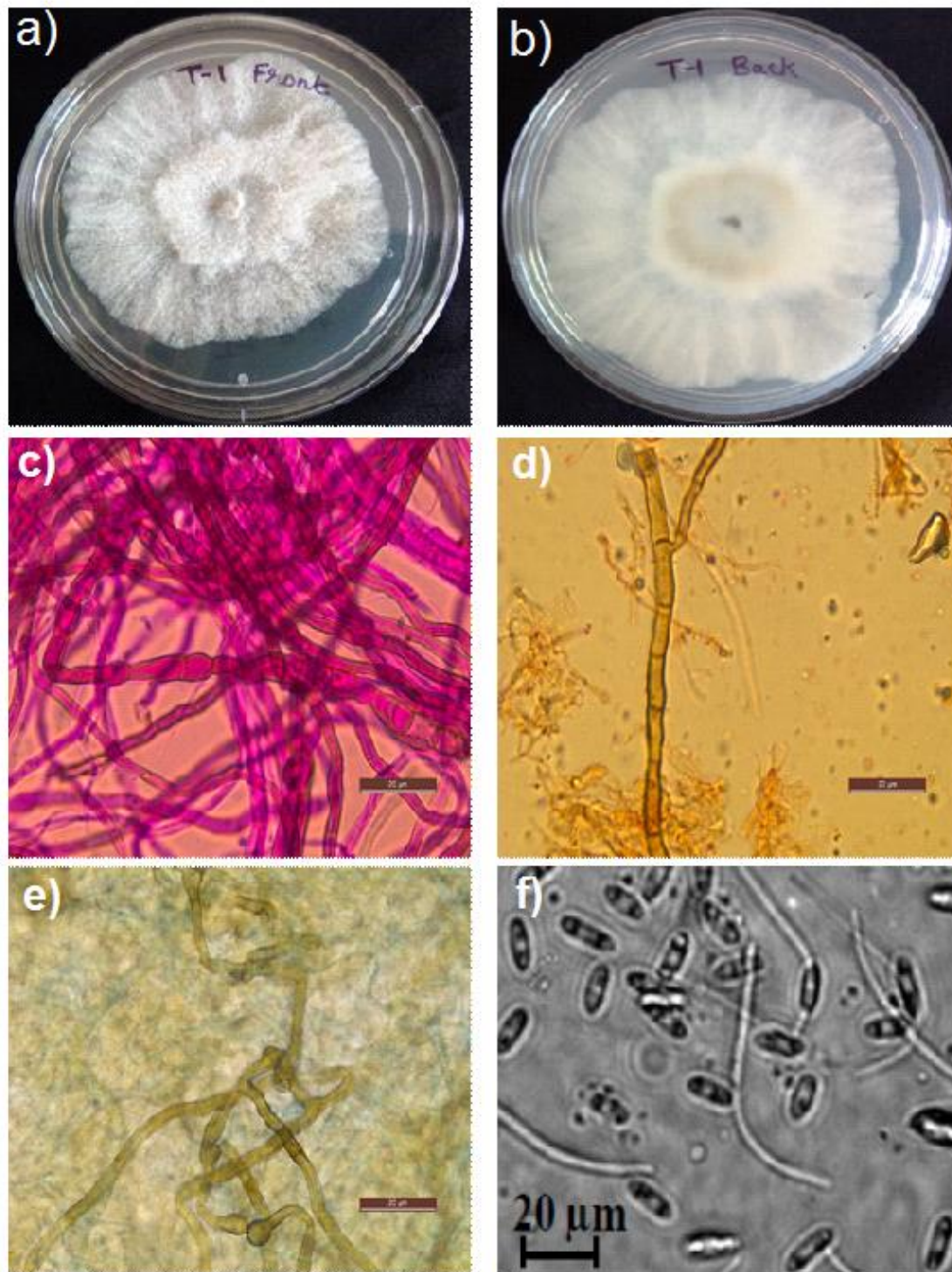


Fig. 4.3. Macroscopic and microscopic characteristics of T1 isolate. a) and b): colony features on PDA upper and lower surface respectively; c, d, and e) hyphal structures and f): α -conidial spores (elliptical) and hair-like filamentous β -conidial spores (needlelike).

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Database Name nr
Description Nucleotide collection (nt)
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	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Phomopsis sp. FH-2012b isolate cgyg1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1026	1026	100%	0.0	98%	JQ954648.1
☐	Diaporthe sp. FH-2013b isolate X41 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1007	1007	100%	0.0	98%	KC357558.1
☐	Phomopsis sp. LH157 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1000	1000	100%	0.0	97%	HQ832815.1
☐	Phomopsis vaccinii isolate Lan1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	981	981	100%	0.0	97%	KC488258.1
☐	Diaporthe vaccinii genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isolate TS08-123-2	981	981	100%	0.0	97%	AB470842.1
☐	Phomopsis sp. FN-1-N1-2-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	976	976	100%	0.0	97%	KJ463517.1
☐	Phomopsis sp. 1 R4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	972	972	95%	0.0	98%	FJ042516.1
☐	Phomopsis sp. FN-1-S1-1-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	970	970	100%	0.0	97%	KJ463519.1
☐	Diaporthe eres isolate SIAP12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	965	965	100%	0.0	96%	KR909214.1
☐	Diaporthe eres strain xsd08090 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	965	965	100%	0.0	96%	FJ478132.1

Fig. 4.4. BLASTN results of T1 isolate.

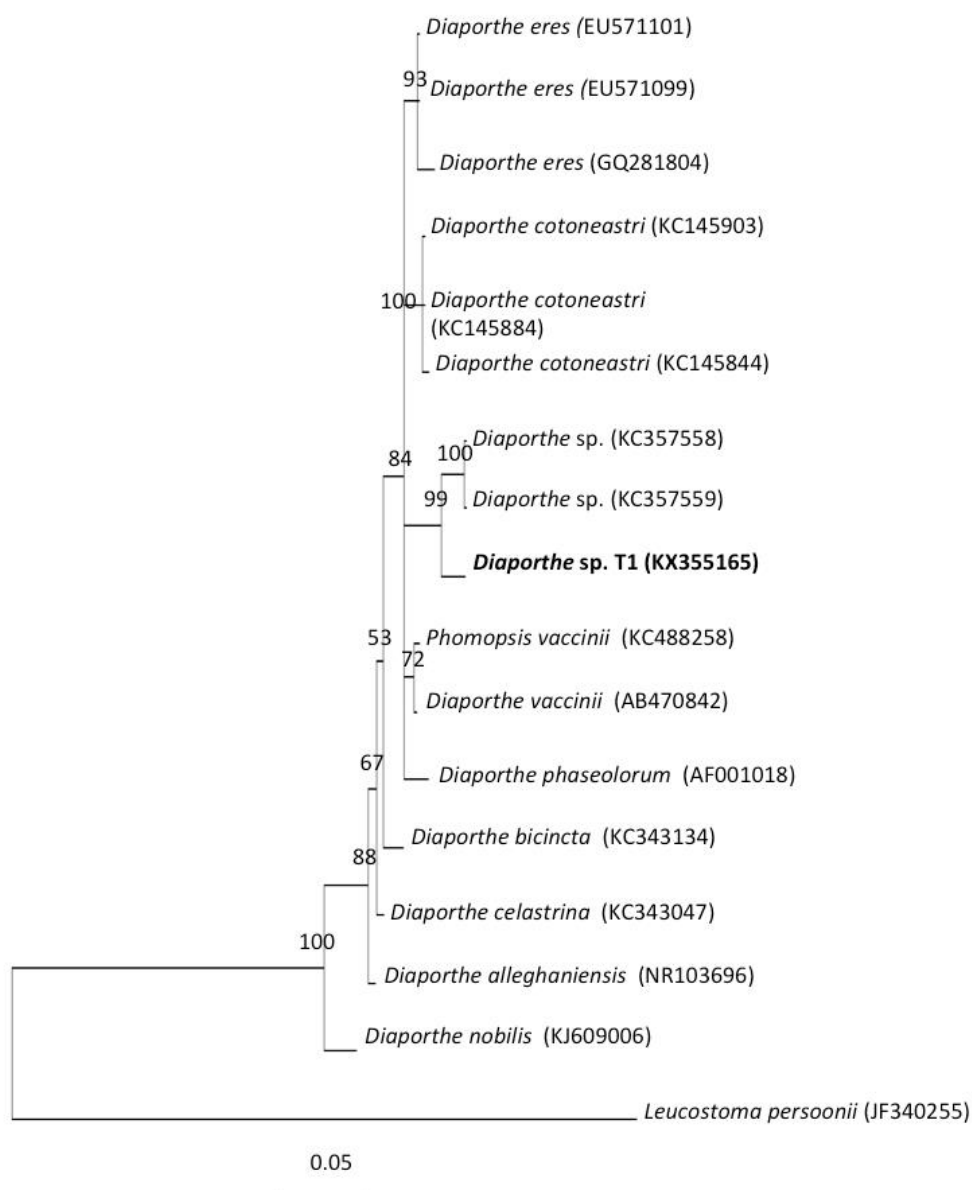


Fig. 4.5. Phylogenetic analysis (Bayesian tree) showing the relationships between the internal transcribed spacer (ITS) sequence *Diaporthe* sp. (shown in bold) and those of related species retrieved from GenBank. Numbers at nodes stand for the posterior probability percentages (>50%) of the Bayesian analysis. *Leucostoma persoonii* (JF340255) was used as an outgroup taxa. Bar = 5 nucleotide substitutions per 100 nucleotides

4.3.2 T2 isolate

Aerial mycelium of T2 was consistently white, flat, and smooth with no pigment production. When observed microscopically, the mycelia had clamp connection, which is a characteristic of basidiomycetous fungi. The isolate however did not produce any spore like structures, which normally provided the basis for fungal identification (Fig. 4.6). Isolation of a mycelial sterilia, fungi that do not sporulate in culture, such as isolate T2, in this case, however is not uncommon during the isolation of endophytic fungi.

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 showed 820 bp in length. Sequence analysis using BLASTN revealed that the sequence of T2 showed homology with *Marasmius scorodonius* (99% similarity with 99% query coverage) (Fig. 4.7). The ITS sequence obtained for the present study isolate is submitted to NCBI database under the accession number KY411698. A phylogenetic tree was constructed to determine the phylogenetic relationship of isolate T2 with other *Marasmius* species using *Crinipellis malesiana* as the outgroup (Fig. 4.8). The short branches and clustering of isolate T2 together with *M. scorodonius* show that isolate T2 was closely related to *M. scorodonius*.

Some of the *Marasmius* species have been reported as endophytes associated with different plants and capable of producing some bioactive compounds (Orlandelli et al. 2012, 2015, 2016; Ngieng et al. 2013; Yuan et al 2010). Wilson and Desjardin (2005) studied the phylogenetic relationships in the gymnopoid and marasmioid fungi. The authors reported one lineage containing the genera *Lentinula*, *Rhodocollybia*, *Tetrapyrgos*, a redefined *Mycetinis*, and two unresolved clades designated /marasmiellus and /gymnopus /marasmiellus includes the type species of *Marasmiellus* and is dominated by members of *Gymnopus* sect. *Vestipedes*. /gymnopus includes the type species of *Gymnopus*, *Micromphale* and *Setulipes*, and members of *Gymnopus* sect. *Levipedes*. According to Wilson and Desjardin (2005), the *Marasmius scorodonius* has been designated as *Mycetinis scorodonius*.

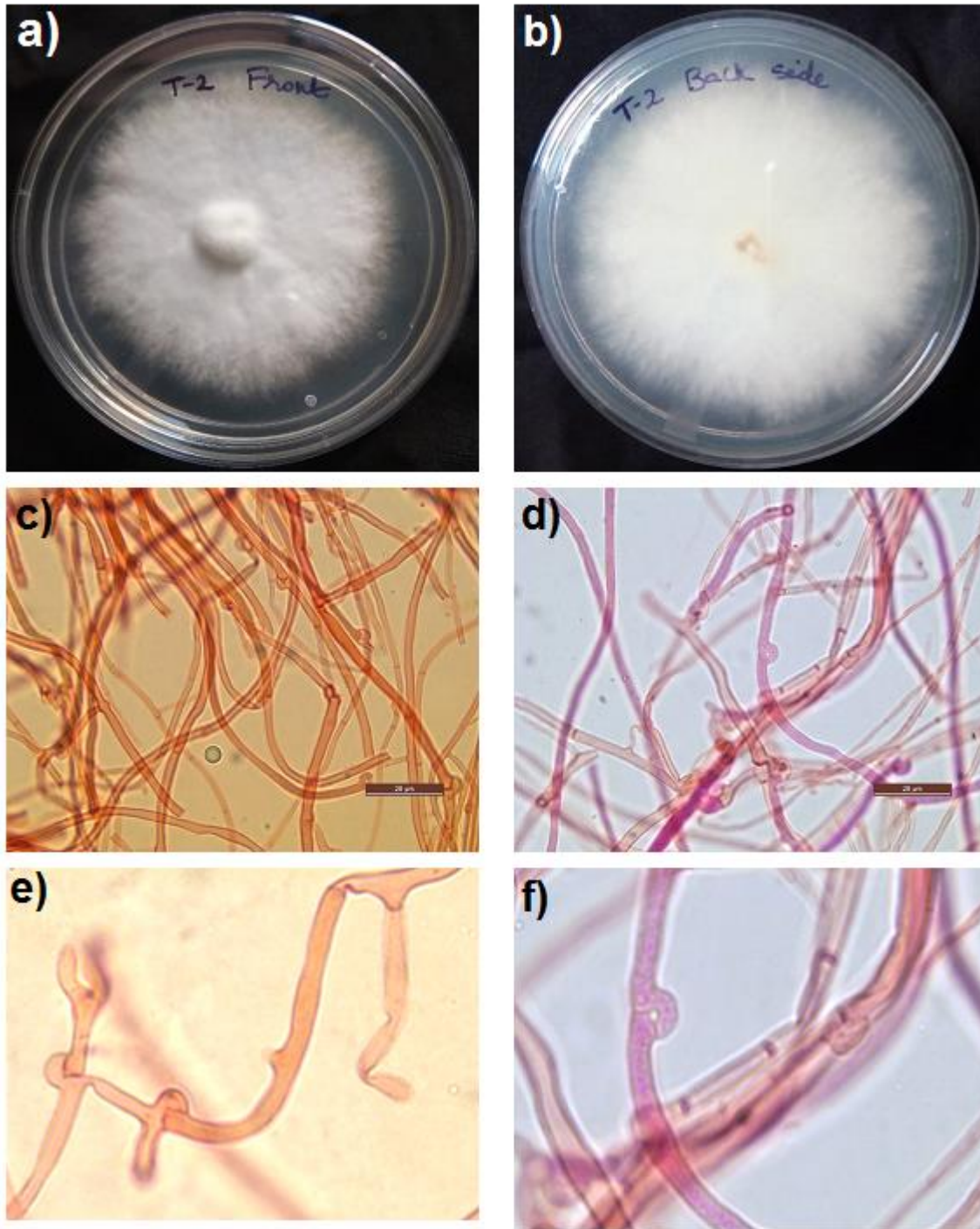


Fig. 4.6. Endophytic isolate T2 a) and b): colony features on PDA upper and lower surface respectively; c, d, hyphal structures and e, f): showing hyphae with production of clamp connections.

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	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Mycetinis scorodoni isolate strain T2 small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1519	1519	100%	0.0	100%	KY411698.1
☐	Marasmius scorodoni voucher LF88B2E7 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1450	1450	99%	0.0	99%	JQ272364.1
☐	Uncultured Marasmius genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, clone H12IK28	1426	1426	96%	0.0	99%	HF675650.1
☐	Uncultured basidiomycete ITS region including 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, clone BF-OTU119	1411	1411	96%	0.0	99%	AM901954.1
☐	Uncultured Marasmiaceae clone Gs1A 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1404	1404	99%	0.0	98%	FJ857921.1
☐	Marasmius scorodoni strain NN009981 internal transcribed spacer 1, partial sequence, 5.8S ribosomal RNA gene, complete sequence, and internal transcribed spacer 2, partial sequence	1354	1354	91%	0.0	99%	JN943593.1
☐	Marasmius scorodoni strain NN055732 internal transcribed spacer 1, partial sequence, 5.8S ribosomal RNA gene, complete sequence, and internal transcribed spacer 2, partial sequence	1332	1332	90%	0.0	99%	JN943594.1
☐	Marasmius scorodoni strain S6 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1330	1330	93%	0.0	98%	KJ817281.1
☐	Marasmius scorodoni strain J4 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1273	1273	95%	0.0	96%	JQ088278.1

Fig. 4.7. BLASTN results of T2 isolate.

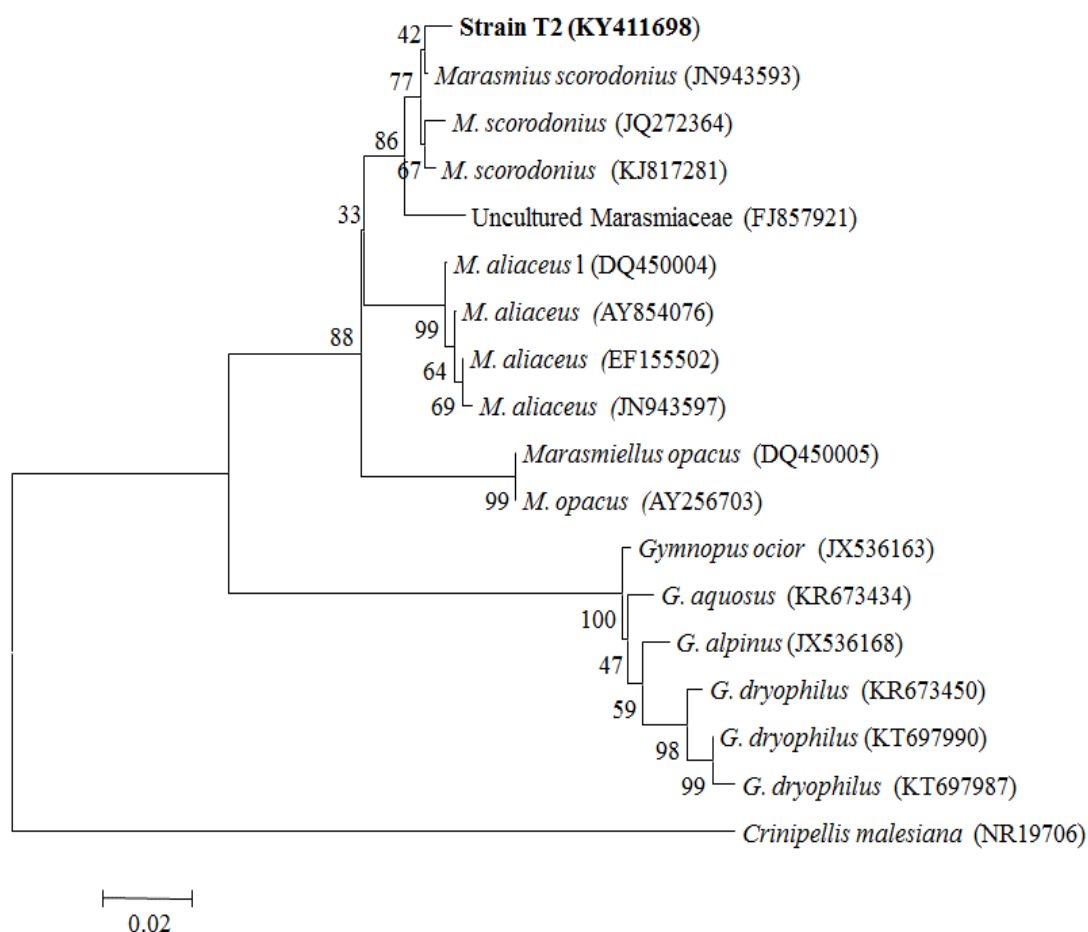


Fig. 4.8. Neighbour-joining tree from ITS sequences showing the relationship between isolate T2 and other closely related *Marasmius* species retrieved from the GenBank. Bootstrap values (1000 replicates) are shown on the branches. *Crinipellis malesiana* was used as an outgroup taxa. Bar = 2 nucleotide substitutions per 100 nucleotides

The other synonyms for *M. scorodonius* are *Agaricus scorodonius*, *Chamaeceras scorodonius* and *Gymnopus scorodonius*. Hence, the isolated *M. scorodonius* of the present study has been identified as *Mycetinis scorodonius* (Fr.) A.W. Wilson & Desjardin, 2005.

4.3.3 T5 isolate

T5 colony gave whitish colour from front and creamish dark brown centered when viewed from reverse of culture plate. Margins were regular, smooth and submerged, and the radial growth on PDA at 25°C under dark conditions. When viewed under microscope, the aerial mycelium was sparse and conidiogenous cells were found lining the inner conidiomatal cavity. Conidiophores of aerial mycelia were mostly monophialidic but occasionally polyphialidic in nature. Septate thick walled conidia, initially hyaline, smooth becoming golden-brown measured $(10-12) \times (4-5) \mu\text{m}$ in size were recorded (Fig. 4.9).

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 showed 610 bp in length. BLAST analysis showed 99% similarity with *Microdiplodia* sp., and *Paraconiothyrium brasiliense* (query coverage of 99%) (Fig. 4.10). Phylogenetic analysis of ITS sequences of different species of *Microdiplodia* and *Paraconiothyrium brasiliense* gave a consensus tree and the present isolate (T5) clustered with *Microdiplodia* sp., and *Paraconiothyrium brasiliense* (Fig. 4.11). Based on the analysis of morphology and ITS region, this isolate has been identified as *Microdiplodia* sp. The ITS sequence obtained for the present study isolate is submitted to NCBI database under the accession number KY421921.

Microdiplodia species have been reported as endophytes with different plant species and are able to produce different bioactive compounds (Hatakeyama et al. 2010; Siddiqui et al. 2011). Siddiqui et al. (2011) isolated *Microdiplodia* sp. from the shrub *Lycium intricatum* capable of producing diversanol and blennolide derivatives which were biologically active, with antibacterial and antifungal activity. Hatakeyama et al. (2010) isolated endophytic fungus *Microdiplodia* sp. KS 75-1, from the stems of conifer trees (*Pinus* sp.) capable of producing sesquiterpenes with antibacterial activities.

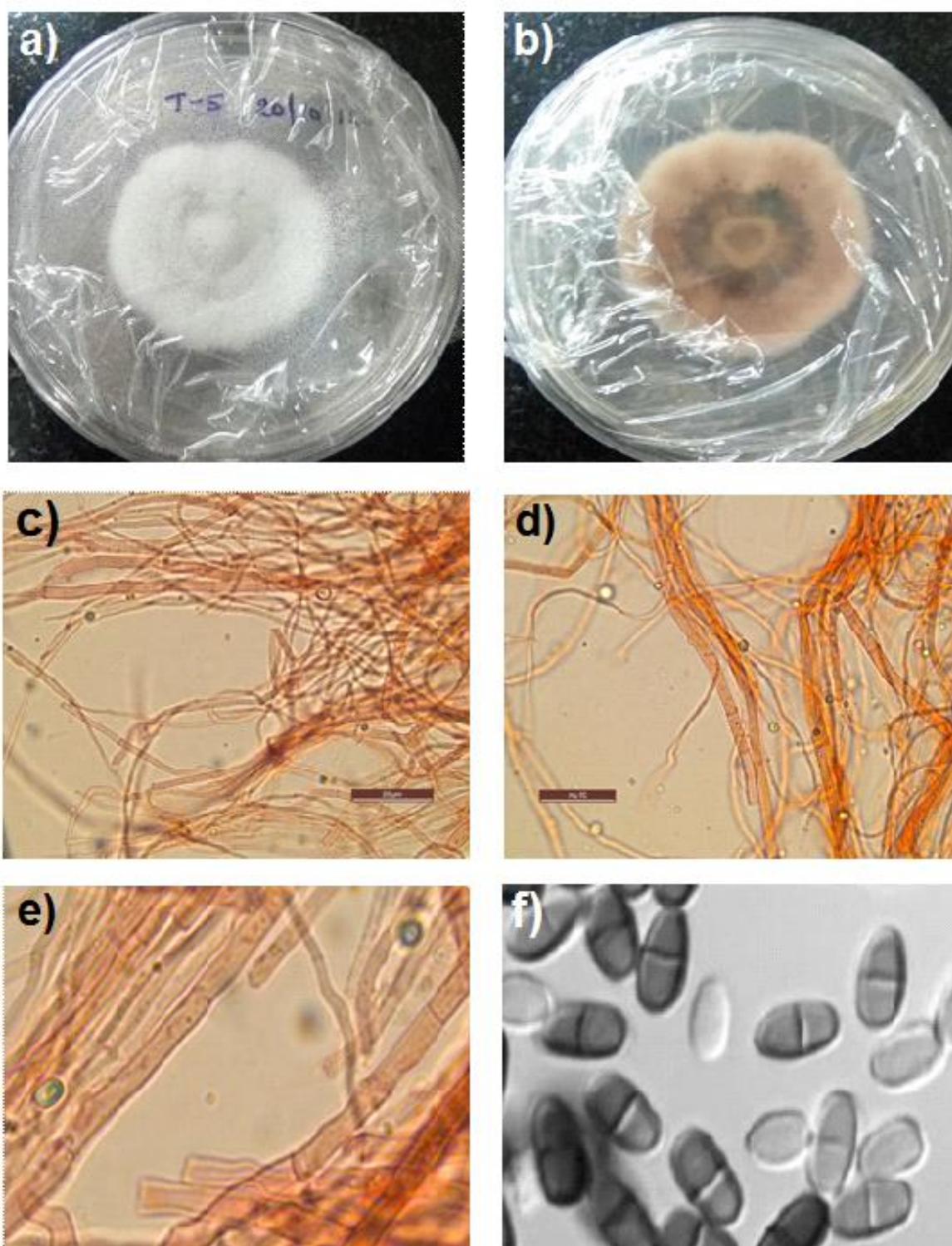


Fig. 4.9. Macroscopic and microscopic characteristics of T5 isolate. a,b): colony features on PDA upper and lower surface respectively; c,d and e): hyphal structures and f): conidiophores (brown, one septate conidia and hyaline, aseptate conidium)

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Molecule type
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Description
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Fig. 4.10. BLASTN results of T5 isolate

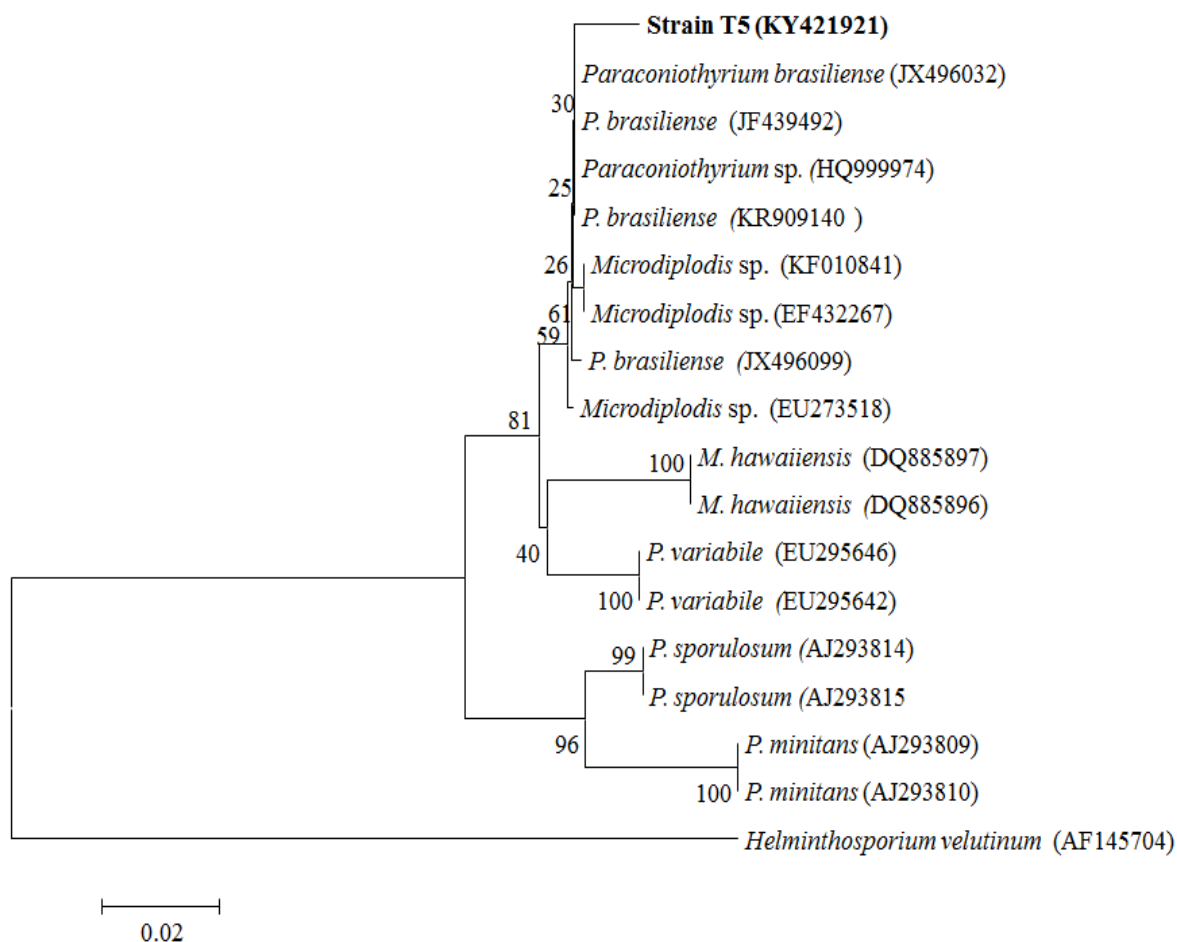


Fig. 4.11. Neighbour-joining tree from ITS sequences showing the relationship between isolate T5 and other closely related *Microdiplodia* and *Paraconiothyrium brasiliense* species retrieved from the GenBank. Bootstrap values (1000 replicates) are shown on the branches. *Helminthosporium velutinum* (AF145704) was used as an outgroup taxa. Bar = 2 nucleotide substitutions per 100 nucleotides

4.3.4 T6 isolate

The strain T6 grew well on PDA media at 28 °C in 7 days. The morphological traits of strain T6 were: pink coloured centre surrounded by white coloured margins, cottony appearance, nearly round margins and broken edges. The back side of culture was of dark brown. Microscopic observations indicated the presence of tubular, thick walled, septate hyphae, simple or branched and sickle-shaped macroconidia and oval microconidia (Fig. 4.12).

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 showed 561 bp in length. Sequence analysis using BLASTN showed that the sequence of T6 showed homology with *Fusarium tricinctum* (99% similarity with 100% query coverage) (Fig. 4.13). The ITS sequence obtained for the present study isolate is submitted to NCBI database under the accession number KT779291. A phylogenetic tree was constructed to determine the phylogenetic relationship of isolate T6 with other *Fusarium* species (Fig. 4.14). The short branches and clustering of isolate T6 together with *F. tricinctum* show that isolate T6 was closely related to *F. tricinctum*.

Many *Fusarium* species, the endophytic fungi, are well known to produce bioactive secondary metabolites with varied activities such as antibacterial (Zaher et al. 2015) and anticancer activity (Garyali et al. 2014; Zhao et al. 2008).

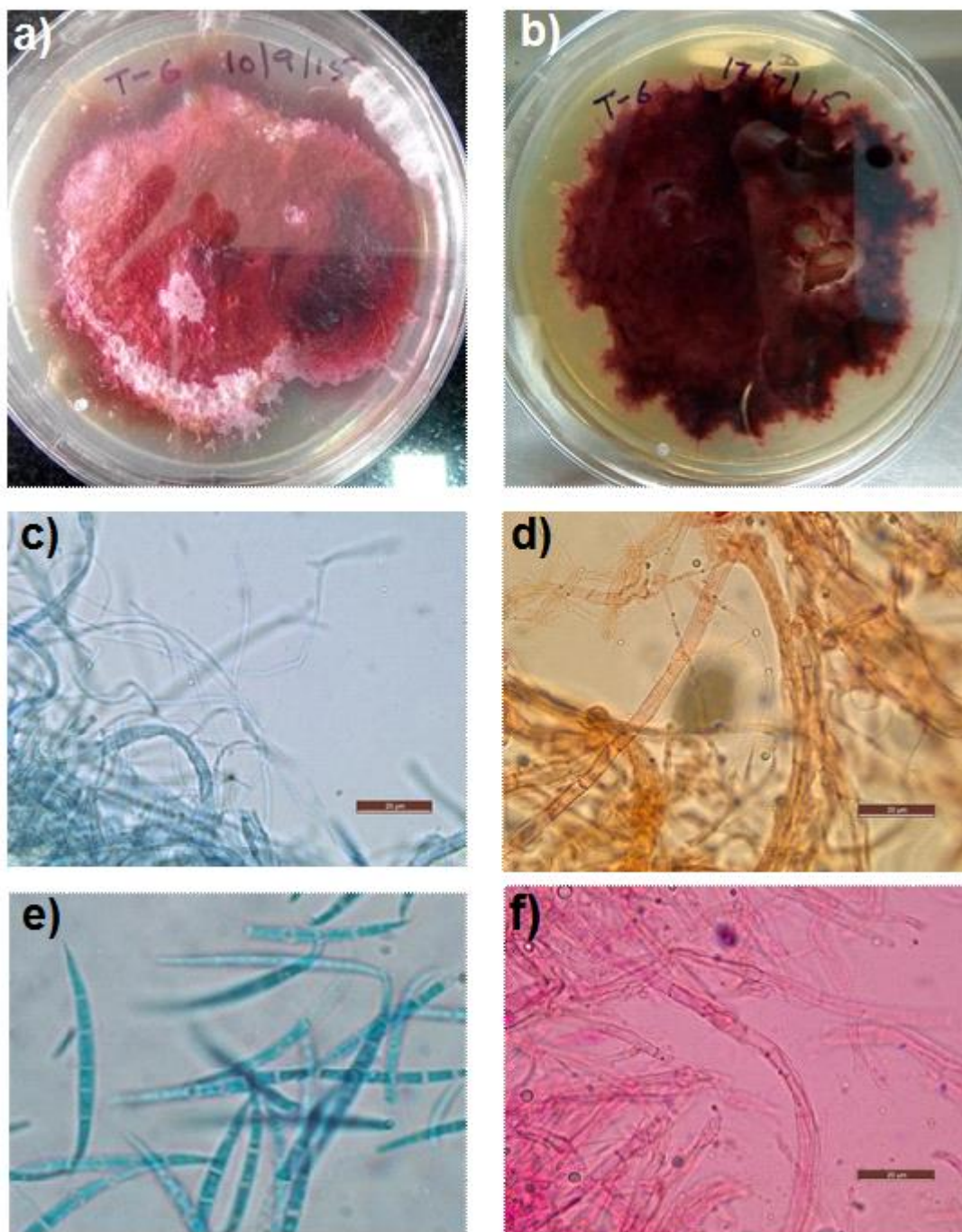


Fig. 4.12. Macroscopic and microscopic characteristics of T6 isolate. a,b): colony features on PDA upper and lower surface respectively; c,d): hyphal structures and e): septate conidia and f): hyphal structures

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Molecule type nucleic acid

Query Length 561

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	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Fusarium tricinctum genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isolate: TS08-58-2	1037	1037	100%	0.0	100%	AB470855.1
☐	Fusarium tricinctum strain 1-00050-1 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1031	1031	100%	0.0	99%	KT192345.1
☐	Fusarium sp. C2 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1031	1031	100%	0.0	99%	JF273502.1
☐	Fusarium tricinctum strain A1 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1031	1031	100%	0.0	99%	JF273496.1
☐	Nectria rigidiuscula genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isolate: TS08-90	1031	1031	100%	0.0	99%	AB470830.1
☐	Fusarium tricinctum isolate T105 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1031	1031	100%	0.0	99%	FJ459979.1
☐	Fusarium sp. Dzf9 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1031	1031	100%	0.0	99%	DQ657854.1
☐	Fusarium tricinctum strain CS15 small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S	1027	1027	100%	0.0	99%	KX015993.1

Fig. 4.13. BLASTN results for T6 isolate.

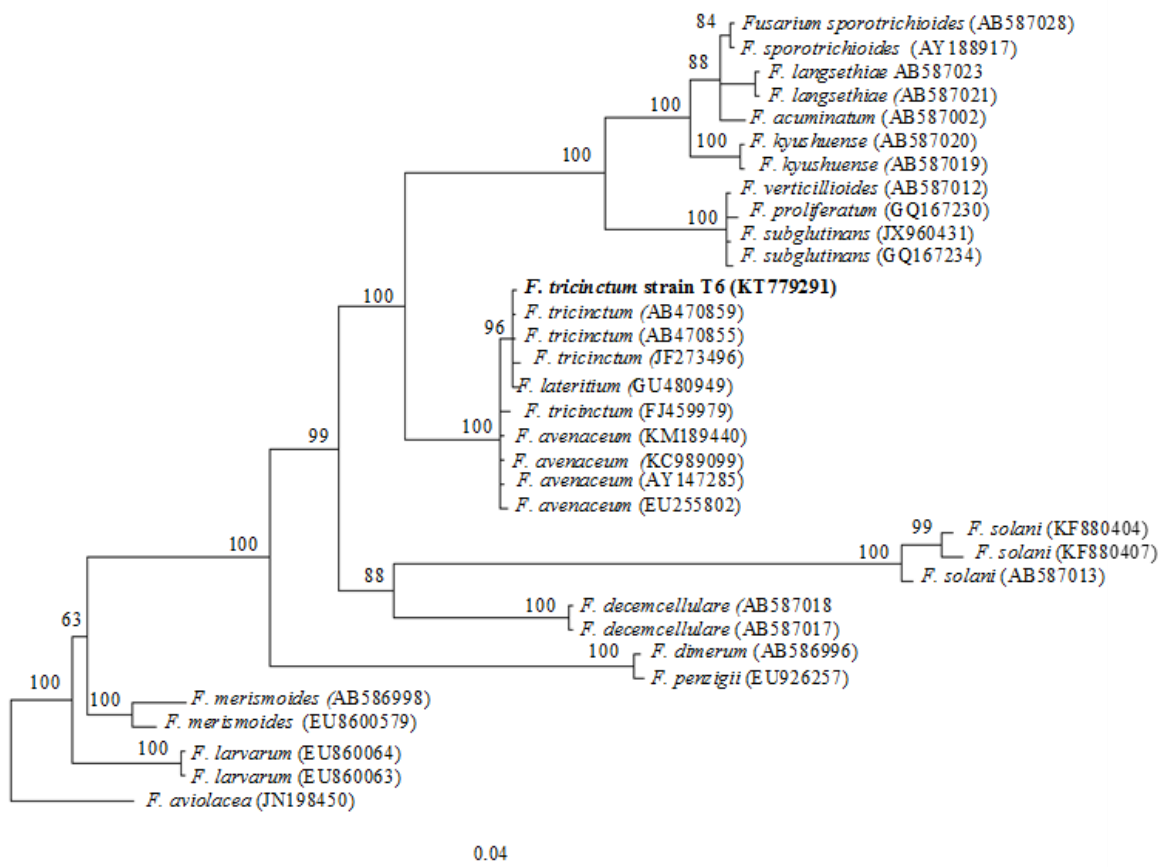


Fig. 4.14. Neighbour-joining tree from ITS sequences showing the relationship between isolate T6 and other closely related *Fusarium* species retrieved from the GenBank. Bootstrap values (1000 replicates) are shown on the branches. Bar = 4 nucleotide substitutions per 100 nucleotides

The scientific classification of four identified endophytic fungi isolated from *T. baccata* are as follows:

A) Scientific classification T1	
Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Diaporthales
Family	Diaporthaceae
Genus	<i>Diaporthe</i>
<i>Diaporthe</i> sp.	

B) Scientific classification T2	
Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Omphalotaceae
Genus	<i>Mycetinis</i>
<i>Marasmius scorodoni</i> / <i>Mycetinis scorodoni</i>	
C) Scientific classification T5	
Kingdom	Fungi
Division	Ascomycota
Class	Dothideomycetes
Order	Botryosphaeriales
Family	Botryosphaeriaceae
Genus	<i>Microdiplodia</i>
<i>Microdiplodia</i> sp.	
D) Scientific classification T6	
Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	<i>Fusarium</i>
<i>Fusarium tricinctum</i>	

In conclusion, twenty five endophytic isolates were obtained from the bark samples of *T. baccata*. Out of these, four (T1, T2, T5 and T6) strains were shortlisted for further characterization based on their preliminary screening for antimicrobial and anticancer activities. These fungi were characterized morphologically as well as on the basis of ITS rDNA sequence analysis. These fungi were identified as *Diaporthe* sp. (T1), *Marasmius scorodonius*/ *Mycetinis scorodonius* (T2) *Microdiplodia* sp. (T5) and *Fusarium tricinctum* (T6).

4.4 Antimicrobial activity

The bacterial strains *Staphylococcus aureus* (MRSA, ATCC 33591), *Bacillus megaterium* FH 1127, *Escherichia coli* ESS 2231 and *Pseudomonas aeruginosa* M35 were activated in Mueller-Hinton agar before the antibacterial test. *Candida albicans* ATCC 10231 was activated on SDB. Ampicillin and streptomycin were used as antibacterial control and amphotericin-B was used as antifungal control. Dimethylsulfoxide (DMSO) was used as a negative control. The inoculum density of the microbial cell suspension was kept equivalent to 0.5 McFarland standard. Initial screening for potential anti-bacterial and anti-fungal activity of the extracts was performed by agar well diffusion method. The crude extracts were dissolved in DMSO. A fixed volume of extract was introduced into the agar wells and the extract was allowed to diffuse into the medium and then incubated at optimum temperature and time duration depending upon the test microorganism. After incubation, zones of inhibition were observed around the fungal extracts. The extract of T1 showed more activity against *S. aureus*, *E. coli* and *C. albicans* while T2 showed more activity against all isolates. The activity was maximum against *E. coli*, *P. aeruginosa* and *C. albicans* (Table 4.1, Fig. 4.15). T5 showed maximum activity against *S. aureus*. Isolate T6 showed maximum activity against *E. coli* followed by *S. aureus*, *P. aeruginosa* and *C. albicans* (Table 4.1, Fig. 4.16). Further,

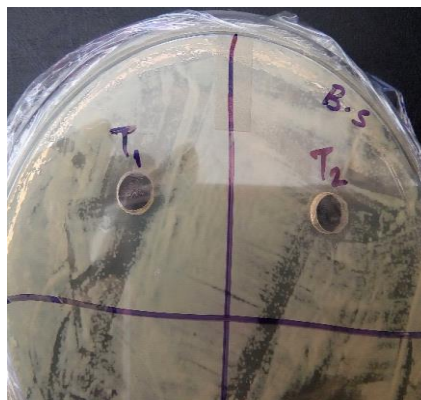
these extracts (500 µg/ml) were tested on the growth inhibition of these microorganisms by MTT assay.

Table 4.1. Antimicrobial activity of fungal extracts of T1, T2, T5 and T6 tested with different microorganisms.

Fungal extracts	<i>Staphylococcus aureus</i>	<i>Bacillus megaterium</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>
T1	++	+	++	+	++
T2	++	++	+++	+++	+++
T5	++	+	+	+	+
T6	++	+	+++	++	++



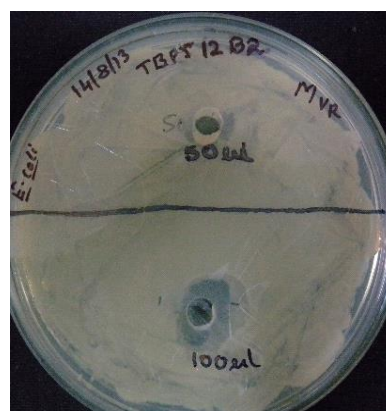
Extract T1-*C. albicans*



Extract T1-*B. megaterium*



Extract T2-*C. albicans*



Extract T2-*E. coli*



Extract T2-*P. aeruginosa*

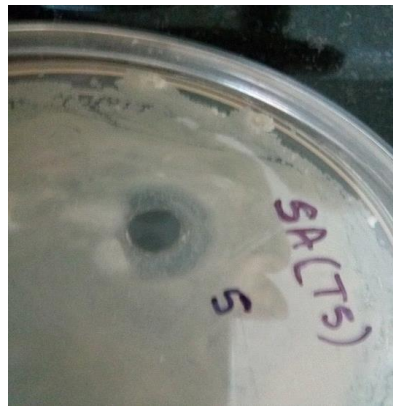


Ampicillin-*E. coli*

Fig. 4.15. Antimicrobial activity of crude extracts of T1 and T2 isolates against bacterial species and *C. albicans* by diffusion method



Extract T5-*E. coli*



Extract T5-*S. aureus*



Extract T6-*C. albicans*



Extract T6-*E. coli*



Extract T6-*B. megaterium*



Ampicillin-*E. coli*

Fig. 4.16. Antimicrobial activity of crude extracts of T5 and T6 isolates against bacterial species and *C. albicans* by diffusion method

The MTT assay results showed that *S. aureus* was significantly inhibited with the extracts of T6 followed by T2 and these results are comparable with the standard antibiotics used in this study. Maximum inhibition of *B. megaterium* was recorded with the extracts of T2 and T6, however these inhibitions are not significant when compared with the standard antibiotics tested. Extract of T6 showed maximum inhibition of *E. coli* and the results are comparable with streptomycin inhibition levels compared to other organisms. The extracts of T6 inhibited the growth of *P. aeruginosa* compared to other extracts and the inhibition levels were comparable with ampicillin used in this study. The growth of *C. albicans* was notably inhibited by the presence of the extract of T2 than other fungal extracts and the inhibition levels are comparable with amphotericin-B used in this study. The extracts of T2 and T6 showed more growth inhibition levels compared to other extracts for different microorganisms tested in this study (Table 4.2).

Table 4.2. Effect of the fungal extracts (500 µg/ml) of T1, T2, T5 and T6 isolates on growth inhibition (%) of different microorganisms by MTT assay

Fungal extract	<i>Staphylococcus aureus</i>	<i>Bacillus megaterium</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>
T1	34 ± 2.2d	11 ± 0.9d	50 ± 2.5c	8.2 ± 0.5d	54 ± 3.5b
T2	71 ± 4.8bc	60 ± 3.8c	20 ± 1.1d	57 ± 3.1c	78 ± 5.6a
T5	5.0 ± 0.7e	5.2 ± 0.4e	6.3 ± 0.3e	6.5 ± 0.3d	24 ± 1.2c
T6	80 ± 6.2ab	60 ± 3.4c	73 ± 4.2b	70 ± 3.8b	15 ± 1.0c
Ampicillin	87 ± 6.8a	71 ± 5.2b	90 ± 6.5a	73 ± 4.2b	86 ± 3.3a
Streptomycin	61 ± 3.5c	81 ± 3.7a	75 ± 4.2b	84 ± 4.5a	(Amphotericin)

Values (Mean ± SD) sharing a common letter within the column are not significant at P<0.05

Endophytes produce secondary metabolites that protect the host from pathogen attack. The metabolites of these organisms are well known as anti-microbial agents (Nisa et al. 2015).

Endophytic fungi develop resistance in host plants by producing secondary metabolites (Tan and Zou 2001). Many endophytic fungi are known to produce antimicrobial substances. Antimicrobial metabolites are low molecular weight substances synthesized by microorganisms that are active at low concentrations against other microorganisms (Wani et al. 1971). The crude extracts obtained from culture broths of endophytic fungi have shown antimicrobial activity against variety of microorganisms as well as cytotoxic activity on human cell line (Carvalho et al. 2012). Antimicrobial activity have been reported from extracts of different endophytic fungi isolated from different geographical locations (Yang et al. 1994; Li et al. 2005; Liu et al. 2001; Phongpaichit et al. 2007; Zhang et al. 2009; Hormazabal et al. 2009; Maria et al. 2005; Raviraj et al. 2006; Tejesvi et al. 2007).

Naphthaquinone Javanicin, an antibacterial compound isolated from the endophytic fungus *Chloridium* sp. associated with neem exhibited activity against *Pseudomonas* sp. (Kharwar et al. 2008). Maria et al. (2005) reported antifungal and antibacterial activities of fourteen endophytic fungi isolated from mangrove plants (*Acanthus ilicifolius* and *Acrostichum aureum*). The antimicrobial potential of 15 endophytic fungi isolated from eight medicinal plants from Western Ghats of India has been reported by Raviraj et al. (2006). They also reported that partially purified extracts of *Alternaria* sp., *Nigrospora oryzae* and *Papulospora* sp. showed considerable antimicrobial activity against selected bacteria and fungi. Tejesvi et al. (2007) screened *Pestalotiopsis* strains isolated from different plants for their antimicrobial activity and reported that *Pestalotiopsis* sp. could be exploited for bioactive antifungals. Zhang et al. (2012) reported antimicrobial activities by three strains of *Aspergillus* associated with *Artemisia annua* against *E. coli*, *S. aureus* and *T. rubrum*. In the present study, among the four endophytic fungi (T1, T2, T5

and T6) tested for their antimicrobial activity against different microorganisms, the extracts of T2 and T6 showed better antimicrobial activities compared to other extracts.

4.5 Cytotoxic activity

The cytotoxic activity test was performed with the extracts (100 µl) of endophytic fungal isolates, T1, T2, T5 and T6. Screening test was done by MTT assay to observe the effect of fungal extracts on the growth of MCF-7 cells. It was found that extracts of T1 and T6 significantly inhibited the growth of MCF-7 cells (Table 4.4a, Fig. 4.17a). Further, to confirm the inhibitory effect, 10 and 20 µl of the extracts of T1 and T6 were tested and it was found that both the extracts inhibited the growth of MCF-7 cell lines (Table 4.4b, Fig. 4.17b,c).

Table 4.4a. Effect of fungal extract of different endophytic fungi on the growth inhibition of MCF cell lines.

Fungal Isolates	Absorbance
Control	0.472 ± 0.01a
T1	0.226 ± 0.03c
T2	0.333 ± 0.02b
T5	0.442 ± 0.1a
T6	0.226 ± 0.03c

Values (Mean ± SD) sharing a common letter are not significant at P<0.05

Table 4.4b. Effect of different concentration of fungal extract of T1 and T6 isolates on the growth inhibition of MCF cell lines

Fungal Isolates	Absorbance	Absorbance
	10 μ l	20 μ l
Control	$0.68 \pm 0.08a$	$0.69 \pm 0.08a$
T1	$0.447 \pm 0.03b$	$0.31 \pm 0.01c$
T6	$0.52 \pm 0.02b$	$0.45 \pm 0.04b$

Values (Mean $\pm$ SD) sharing a common letter within the column are not significant at $P < 0.05$

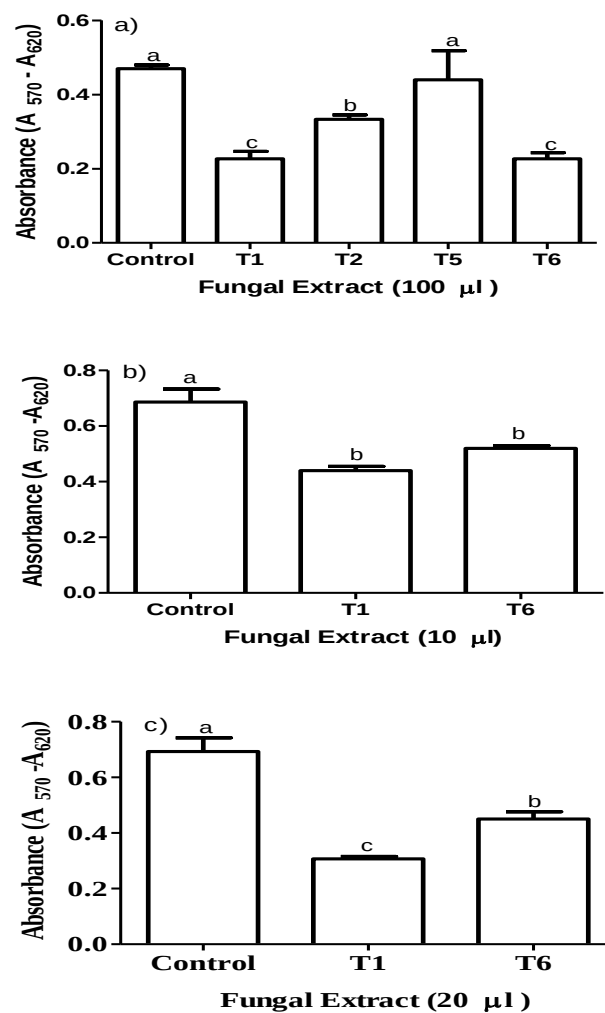


Fig. 4.17. Effect of fungal extract on the growth of MCF-7 cell lines a) 100 μ l of T1, T2, T5 and T6. Control is media + cell only b. 10 μ l of T1 and T6 c. 20 μ l of T1 and T6

4.6 Antioxidant assay

Free radical scavenging activity was performed to evaluate the antioxidant activity of the extracts of T1, T2, T5 and T6. It was observed that the scavenging activity significantly increased with increase in the concentration of fungal extract. Ascorbic acid was used as positive control where it showed 84% antioxidant activity. Among the isolates, T1 and T6 showed more antioxidant activities compared to other isolates (Table 4.5, Fig. 4.18).

Table 4.5. Effect of different concentrations of fungal extracts of T1, T2, T5 and T6 on the antioxidant activity.

Fungal extracts ($\mu\text{g/ml}$)	Antioxidant activity (Scavenging activity %)			
	T1	T2	T5	T6
100	$27 \pm 2.5\text{dA}$	$27 \pm 2.1\text{cA}$	$23 \pm 1.9\text{dA}$	$22 \pm 1.8\text{dA}$
250	$51 \pm 3.5\text{cA}$	$36 \pm 3.0\text{cC}$	$29 \pm 2.1\text{dD}$	$45 \pm 2.5\text{cB}$
500	$71 \pm 5.2\text{bA}$	$52 \pm 4.5\text{bC}$	$48 \pm 2.3\text{cC}$	$60 \pm 3.8\text{bB}$
1000	$73 \pm 4.8\text{abA}$	$62 \pm 4.9\text{bB}$	$60 \pm 3.8\text{bC}$	$70 \pm 5.2\text{bA}$
Ascorbic acid (100 $\mu\text{g/ml}$)	$84 \pm 6.2\text{aA}$	$84 \pm 6.2\text{aA}$	$84 \pm 6.2\text{aA}$	$84 \pm 6.2\text{aA}$

Values (Mean $\pm$ SD) sharing a common lowercase letters within the column and uppercase letters within the row are not significant at $P < 0.05$.

Based on the preliminary screening for anti-cancer and anti-oxidant activity of the selected fungal strains, further experimentation was performed by using the extracts of T1 and T6 for their biological activities.

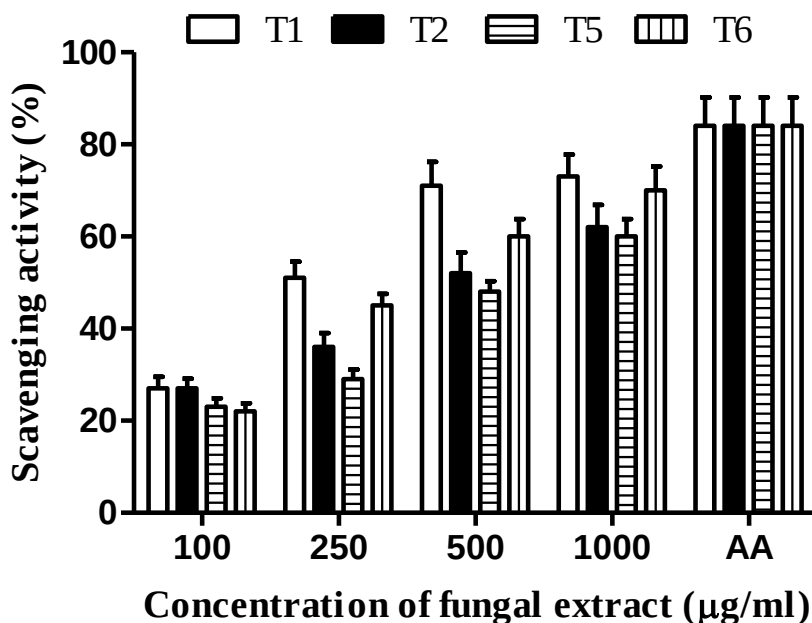


Fig. 4.18. Effect of different concentrations of fungal extracts of T1, T2, T5 and T6 on the antioxidant activity. Error bars represent $\pm$ Standard deviation. AA represents ascorbic acid ($100 \mu\text{g ml}^{-1}$) used as a positive control.

4.7 Cell growth inhibition and antioxidant activity of *Diaporthe* sp. T1

4.7.1 Cytotoxicity

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were used to study the effect of fungal extracts on the cell growth inhibition by using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium- bromide (MTT) assay. Paclitaxel was used as a positive control at the concentration of $20 \mu\text{g/ml}$. Fungal extract showed cytotoxic effect against both human breast cancer cell line (MCF-7) and human cervical cancer cell line (HeLa). The cytotoxic effect became significantly pronounced at higher concentrations of the fungal extract (Table 4.6, Fig. 4.19). The IC_{50} value of the extract was 1058 ± 44 and $1257 \pm 80 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. Paclitaxel showed $79 \pm 6\%$ and $97 \pm 2\%$ growth inhibition for MCF-7 and HeLa cell lines, respectively.

Table 4.6. Cytotoxic effect of *Diaporthe* sp. extract against human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines.

Fungal Extract ($\mu\text{g/ml}$)	Cell Inhibition (%)	
	MCF-7	HeLa
200	$9.4 \pm 2.1\text{c}$	$18.3 \pm 1.3\text{c}$
500	$17.4 \pm 4.0\text{c}$	$31.5 \pm 3.8\text{bc}$
750	$44.9 \pm 7.4\text{bc}$	$41.9 \pm 14.1\text{bc}$
1000	$57.1 \pm 12.2\text{ab}$	$56.5 \pm 7.5\text{b}$
1500	$69.4 \pm 8.6\text{a}$	$61.0 \pm 1.0\text{b}$
Paclitaxel	$79.4 \pm 6.6\text{a}$	$97.8 \pm 2.1\text{a}$

Mean values sharing a common letter within the column are not significant at $P < 0.05$

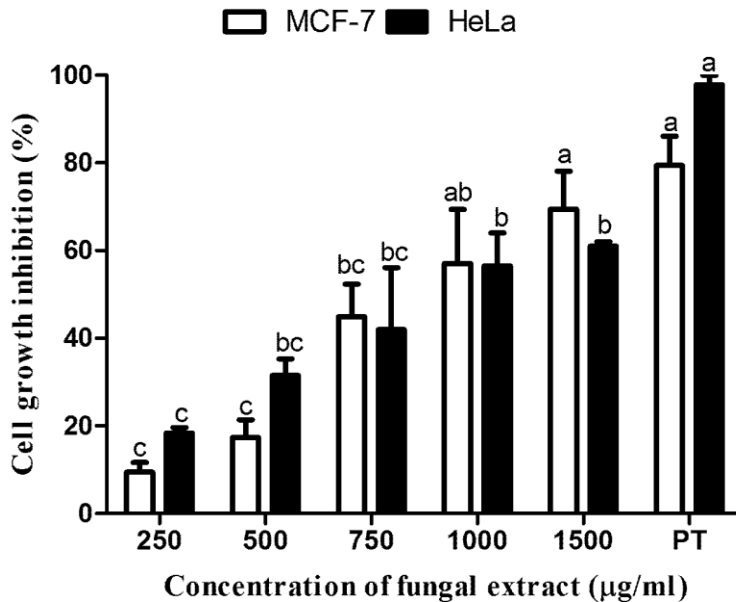


Fig.4.19. Cytotoxic effect of *Diaporthe* sp.extract against human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines. Mean values sharing a common letter within cell type are not significant at $p < 0.05$. Error bars represent $\pm$ Standard deviation. PT represents paclitaxel ($20 \mu\text{g ml}^{-1}$) used as a positive control.

4.7.2 Antioxidant activity

The antioxidant potential of the fungal extract was studied by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Ascorbic acid (100 µg/ml) was used as positive control. The scavenging activity significantly increased with increase in the concentration of fungal extract (Table 4.7, Fig. 4.20). The IC₅₀ value (concentration of sample required to scavenge 50% of free radicals) of extract was found to be 250 ± 3.5 µg ml⁻¹. Ascorbic acid was used as positive control and where it showed 84 ± 3.5 % antioxidant activity.

Table 4.7. Antioxidant effect of *Diaporthe* sp., extracts based on free radical scavenging activity. Ascorbic acid (AA) (100 µg/ml) was used as positive control.

Fungal Extract (µg/ml)	Scavenging activity (%)
100	30.0 ± 2.6 c
250	50.6 ± 3.5 b
500	77.3 ± 3.2 a
AA	84.6 ± 3.5 a

Mean values sharing a common letter within the column are not significant at P < 0.05

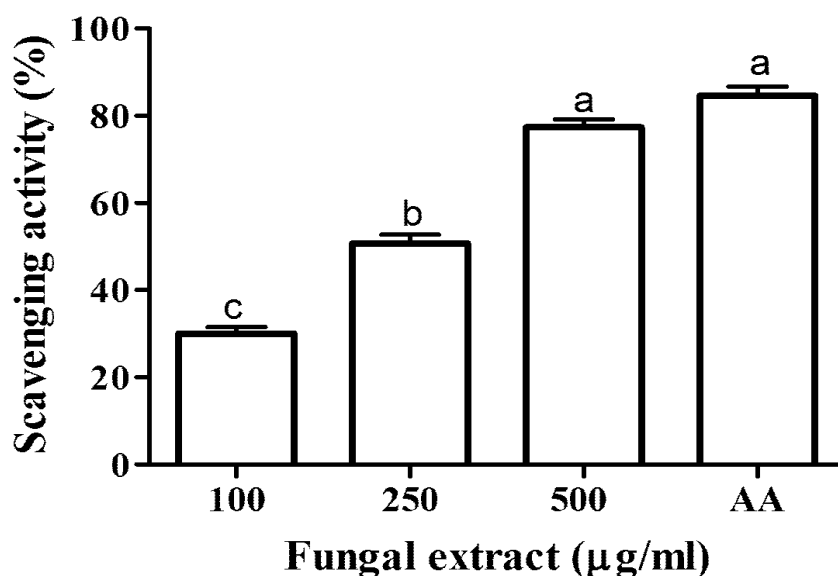


Fig. 4.20. Antioxidant effect of *Diaporthe* sp. extracts based on free radical scavenging activity. Mean values sharing a common letter are not significant at $p < 0.05$. Error bars represent $\pm$ Standard deviation. AA represents ascorbic acid (100 µg/ml) used as a positive control.

The anamorphic form (the asexual reproductive morphological stage) of *Diaporthe* is the genus *Phomopsis*. *Diaporthe/Phomopsis* are either plant pathogens or endophytic in nature (van Niekerk et al. 2005; Murali et al. 2006; Dos Santos et al. 2016), and are associated with dicotyledonous and monocotyledonous plants of tropical and temperate regions (Eriksson and Vue 1998; Guo et al. 2000). The *Diaporthe/Phomopsis* complex occurs as endophytes in medicinal plants such as *Taxus chinensis* (Liu et al. 2009), *Taxus globosa* (Soca-Chafre et al. 2011) and some medicinal shrubs of India (Naik et al. 2008). Here, we report an endophytic association of a *Diaporthe* sp. with *Taxus baccata* subsp. *wallichiana* for the first time.

Medicinal plants are a repository of endophytic fungi that could produce bioactive compounds of biotechnological interest (Vieira et al. 2012). In the present study, a *Diaporthe* sp. recovered from *T. baccata* as an endophyte produced metabolites which displayed cytotoxic activity against MCF-7 and HeLa tumor cells. Agusta et al. (2006) isolated (–)-epicytoskyrin, a compound produced by

Diaporthe sp. isolated from tea plant exhibited moderate cytotoxic activity against KB cells (IC_{50} : $0.5 \mu\text{g ml}^{-1}$). Various researchers have reported the cytotoxic activity of the extracts of *Diaporthe* species against different cell lines (Lin et al. 2005; Carvalho et al. 2012; Casella et al. 2013). The culture filtrate of *Diaporthe* sp. showed antioxidant activity when assayed by DPPH method.

The DPPH assay has been extensively used as a reliable method to measure antioxidant activity of pure compounds (Koleva et al 2002). The present study results suggest that metabolites of *Diaporthe* sp. could serve as potential agents in scavenging free radicals. Antioxidant activity of *Diaporthe/Phomopsis* complex has been reported by Nath et al. (2012) and Ascêncio et al. (2014).

In conclusion, our results showed that the extracts of *Diaporthe* sp. exhibited strong cell growth inhibition and antioxidant properties which indicates the ability of this fungus to produce bioactive compounds which may be useful as sources of novel drugs.

4.8 Cell growth inhibition and antioxidant activity of *Fusarium tricinctum* T6

4.8.1 Cytotoxicity

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were used to determine the effect of extracts on the cell growth inhibition by MTT assay with Paclitaxel used as a positive control at the concentration of $20 \mu\text{g/ml}$. Inhibition in cell growth was observed in both MCF-7 and HeLa. The cytotoxic effect significantly increased with increase in the concentration of the fungal extract (Table 4.8, Fig. 4.21). The IC_{50} value for extract was found to be 225 ± 26 and $220 \pm 18 \mu\text{g/ml}$ for MCF-7 and HeLa cell lines, respectively. Paclitaxel showed 86.4 ± 7.5 and $100 \pm 3.4\%$ growth inhibition for MCF-7 and HeLa cell lines, respectively

Table 4.8. Cytotoxic effect of *F. tricinctum* T6. extract against human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines

Conc. (µg/ml)	MCF-7	HeLa
50	-2.453 ± 16.3e	-2.487 ± 14.6d
100	12.08 ± 20.5d	6.3 ± 18.2c
150	30.0 ± 19.2c	18.8 ± 21.8c
200	49.5 ± 19.1b	40.6 ± 19.3b
250	59.7 ± 9.6b	74.9 ± 1.42ab
300	70.3 ± 5.4ab	80.3 ± 7.4ab
350	76.0 ± 9.9a	84.9 ± 15.3a
Paclitaxel	86.4 ± 7.5a	100 ± 3.4a

Mean values sharing a common letter within the column are not significant at $P < 0.05$

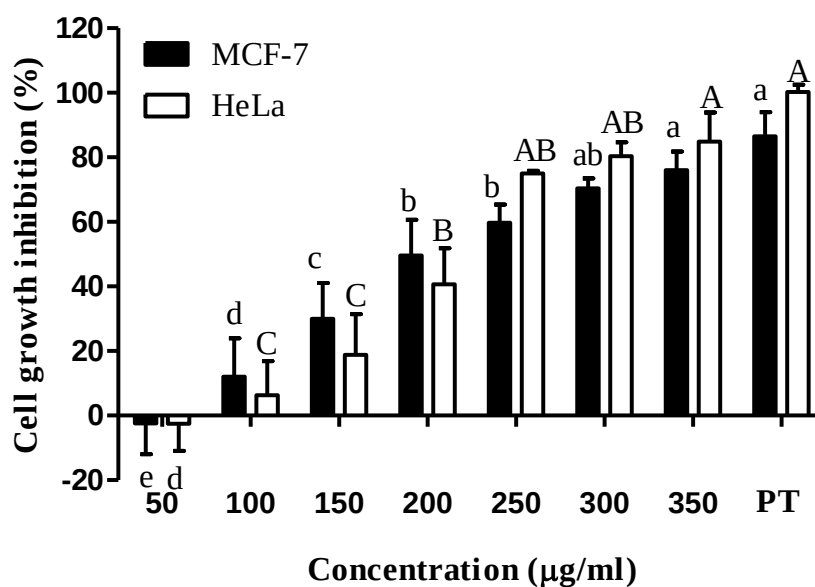


Fig. 4.21. Cytotoxic effect of *Fusarium tricinctum* T6 extract on human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines. Values sharing a common letter within treatment are not significant at $P < 0.05$

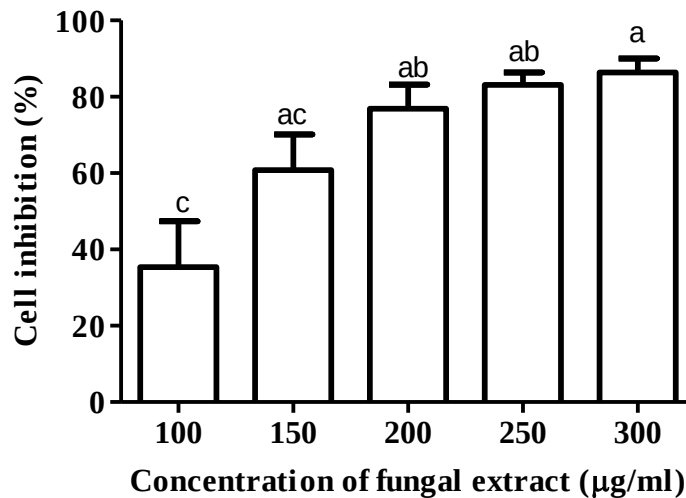
4.8.2 Anti-proliferative activity of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) isolated from heparinized venous blood drawn from healthy volunteers were incubated with fungal extracts. After 2 h, concanavalin A, a mitogen was added in order to stimulate the PBMCs. After 72 h of incubation, MTT assay was carried out to measure the cell proliferation. PBMCs did not show proliferating response against the mitogen concanavalin A in the presence of fungal extracts indicating its immunosuppressive activity. With the increase in the concentration of the fungal extract, the immunosuppressive effect became significantly more pronounced (Table 4.9, Fig. 4.22). The IC₅₀ value for extract was found to be 110 ± 44 µg/ml.

Table 4.9. Immunosuppressive effect of *Fusarium tricinctum* extract on concanavalin stimulated peripheral blood mononuclear cells.

Fungal Extract (µg/ml)	Cell Inhibition (%)
100	35.3 ± 20.7c
150	60.76 ± 16.1ac
200	76.9 ± 10.9ab
250	83.16 ± 5.6ab
300	86.3 ± 6.4a

Mean values sharing a common letter within the column are not significant at P < 0.05



*

Fig. 4.22. Immunosuppressive effect of *Fusarium tricinctum* extract on concanavalin stimulated peripheral blood mononuclear cells (PBMCs). Values sharing a common letter among the treatments are not significant at $P < 0.05$

4.8.3 TNF- α production

In order to evaluate the production of TNF- α , MCF-7 and concanavalin A stimulated PBMCs were incubated with varying concentrations of fungal extract for 72 h. After incubation, supernatants were collected and the secretion of TNF- α was measured. TNF- α is an inflammatory cytokine which plays an important role in cancer progression and metastases. Results of the present study showed inhibition in TNF- α production in MCF-7 cells treated with fungal extract which show that cytotoxic effect may be mediated by inhibition of this cytokine production. Similarly, TNF- α production was found to be inhibited in concanavalin A stimulated PBMCs. Inhibition in cytokines production against MCF-7 and concanavalin A stimulated PBMCs was observed in different concentrations of extract tested in this study, but the inhibition effect appears independent of concentrations (Table 4.10, Fig. 4.23).

Table 4.10. Effect of *Fusarium tricinctum* extract on TNF- α production in MCF-7 concanavalin A stimulated peripheral blood mononuclear cells (PBMCs).

Fungal Extract ($\mu\text{g/ml}$)	TNF- α Inhibition (%)	
	MCF-7	PBMCs
200	68.3 $\pm$ 1.8a	57.9 $\pm$ 1.5b
250	75.3 $\pm$ 2.05a	70.7 $\pm$ 1.0a
300	72.5 $\pm$ 1.7a	49.3 $\pm$ 1.1c

Mean values sharing a common letter within the column are not significant at $P < 0.05$

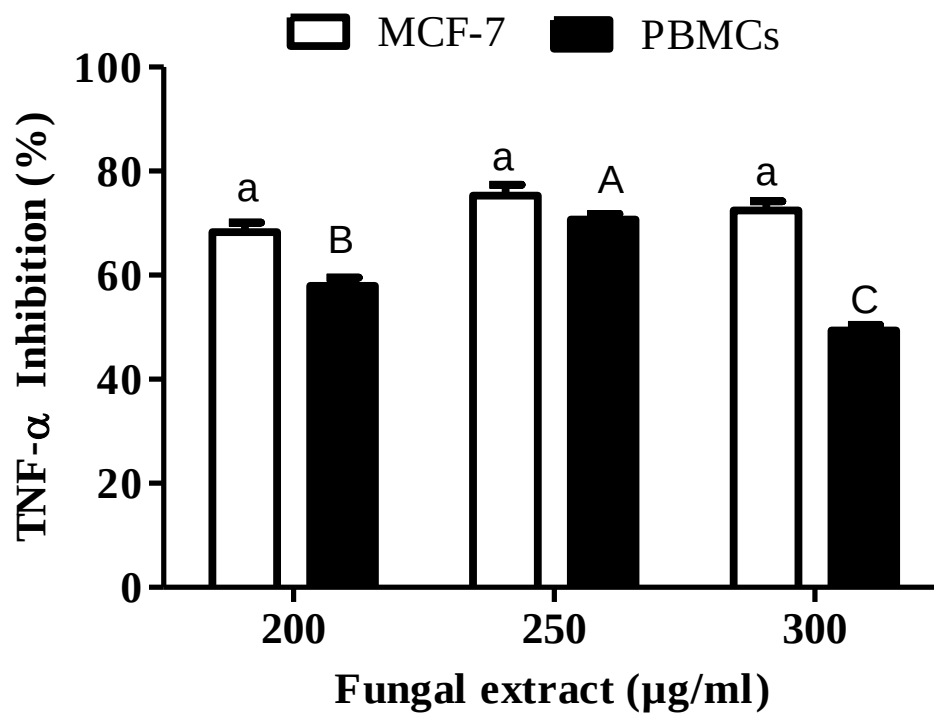


Fig. 4.23. Effect of *Fusarium tricinctum* extract on TNF- α production in MCF-7 concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Values sharing a common letter within the treatment are not significant at $P < 0.05$

4.8.4 Antioxidant activity

Free radical scavenging activity was performed to evaluate the antioxidant activity of *F. tricinctum*. It was observed that the scavenging activity significantly increased with increase in the concentration of fungal extract. The IC₅₀ value of scavenging activity of extract was found to be 482 ± 9 µg/ml. Ascorbic acid was used as positive control and where it shows 80 ± 2 % antioxidant activity. The scavenging activity of the crude fungal extract was found comparable with ascorbic acid (100 µg/ml) when the concentration of the extract was 1000 µg/ml (Table 4.11, Fig. 4.24).

Table 4.11. Antioxidant effect of *Fusarium tricinctum* extracts based on free radical scavenging activity

Fungal Extract (µg/ml)	Scavenging activity (%)
100	$21.5 \pm 0.8d$
200	$44.6 \pm 4.7c$
500	$60.2 \pm 0.6b$
1000	$69.6 \pm 3.2ab$
Ascorbic acid	$80.0 \pm 2.0a$

Mean values sharing a common letter within the column are not significant at $P < 0.05$

Many *Fusarium* species are reported as endophytes and often isolated from different forest plants. These endophytic species are well known to produce bioactive secondary metabolites with varied activities such as antibacterial (Zaher et al. 2015) and anticancer (Garyali et al. 2014; Zhao et al. 2008).

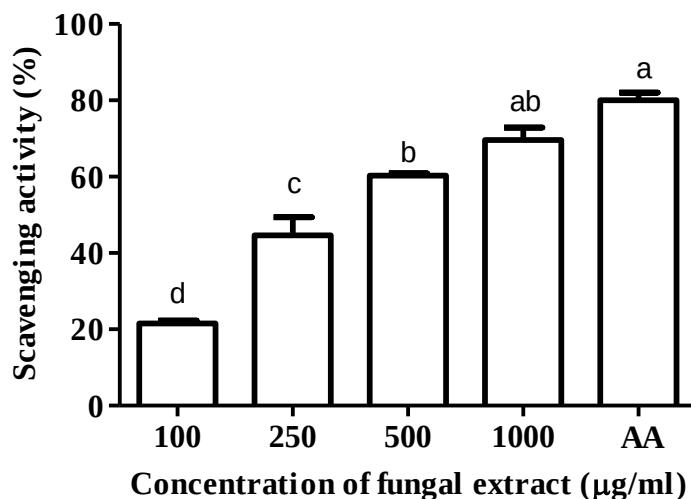


Fig. 4.24. Antioxidant effect of *Fusarium tricinctum* extracts based on free radical scavenging activity. Values sharing a common letter among treatments are not significant at $P < 0.05$

Two human cancer cell lines were used in this study for the investigation of the antiproliferative effect of *F. tricinctum* extracts and the results clearly showed cell growth inhibition (cytotoxicity) against both cancer cell lines. Similar results were reported from *F. oxysporum* which showed cytotoxicity in different human cancer cell lines (Zhan et al. 2007). Puri et al. (2005) reported the cytotoxic activity of endophytic fungus *Entrophospora infrequens* inhabiting *Nothapodytes foetida* against different human cancer cell lines and found activities comparable to the camptothecin. It has been reported that several anticancer drugs have an impact on immune system and possess immunosuppressive properties (Bracci et al. 2014). Immunosuppressive compound such as subglutinol-A and subglutinol-B were isolated from the fungal endophyte *F. subglutinans* inhabiting *Tripterygium wilfordii* (Lee et al. 1995). The present study revealed that PBMCs did not show proliferating response against the mitogen concanavalin A after addition of fungal extract indicating immunosuppressive effect. These findings were in line with the reported anti-

lymphocyte proliferative activities against phyto-hemagglutinin (PHA) stimulated PBMCs by endophytic fungi from *Tripterygium wilfordii* (Kumar et al. 2004). The immunosuppressive effect was further supported by the fact that there is inhibition in the production of inflammatory cytokines TNF- α in stimulated PBMCs culture. TNF- α , a cytokine which mediates the inflammatory response and is also known to influence cancer growth (Balkwill 2006). The present study revealed that *F. tricinctum* extract inhibits the TNF- α production in breast cancer cell lines (MCF-7), which indicate that cytotoxic effect may be mediated by TNF- α inhibition.

Antioxidants protect the cells from the damages caused by free radicals. Free radical mediated reactions are associated with various diseases which include Alzheimer's disease, diabetes, cardiovascular disorder and cancer (Tong et al. 2015). In the present study, the extract of *F. tricinctum* showed antioxidant activity which increased with extract concentration indicating the possibility of exploring this fungus as a source of antioxidant agent. Different fungal compounds with antioxidant activity were isolated from various endophytic fungi from different sources (Harper et al. 2003; Kaul et al. 2012) including phenolics showing potent antioxidant activity (Yadav et al. 2014).

In conclusion, the extracts of *F. tricinctum* exhibited strong cell growth inhibition and antioxidant properties. Further studies focusing on purification and characterization of bioactive compound responsible for these activities may help in isolating some new compounds of pharmaceutical importance.

5.0 Identification and characterization of putative bioactive molecules

The endophytic fungi, *Diaporthe* sp. (T1) and *Fusarium tricinctum* (T6) isolated from *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger were grown on potato dextrose broth at 28°C for three weeks as static culture. The cultures were then filtered and the filtrate was extracted with ethyl acetate (x 3) and the organic phase obtained was dehydrated using anhydrous sodium sulphate. The ethyl acetate fractions were dried *in vacuo* at 35°C to obtain the residue. The residue was dissolved in spectroscopic grade methanol for UHPLC-QTOF-MS/MS analysis. Chromatographic separation was carried out using Agilent 1200 UHPLC system loaded with reverse phase column (C18 column, 150 mm x 2.1 mm i. d. pore size 5.0 µm). Sample (5 µl) was injected into the column through auto-sampler and elution was carried out in a gradient mode with acidified water (0.2 % v/v formic acid) to 90% methanol over a period of 40 min at a flow rate of 0.3 ml/min. High resolution mass spectroscopic (HRMS) detection was performed using Bruker Impact QTOF mass spectrometer (Bruker, Germany), operating in Top5 data-dependent mode from 100-1500 m/z with electron spray ionization (ESI) and both positive and negative ions were monitored. Smart Formula 3D™ -and Fragment Explorer part of Data Analysis 4.2 (Source) was used for compound formula generation and assignment of fragment structures. UHPLC-DAD chromatograms of fungal extracts are presented in Fig. 5.1. The data obtained was analysed and five compounds belonging to two groups were identified in extract of strain T1 and six compounds were identified in the extract of strain T6.

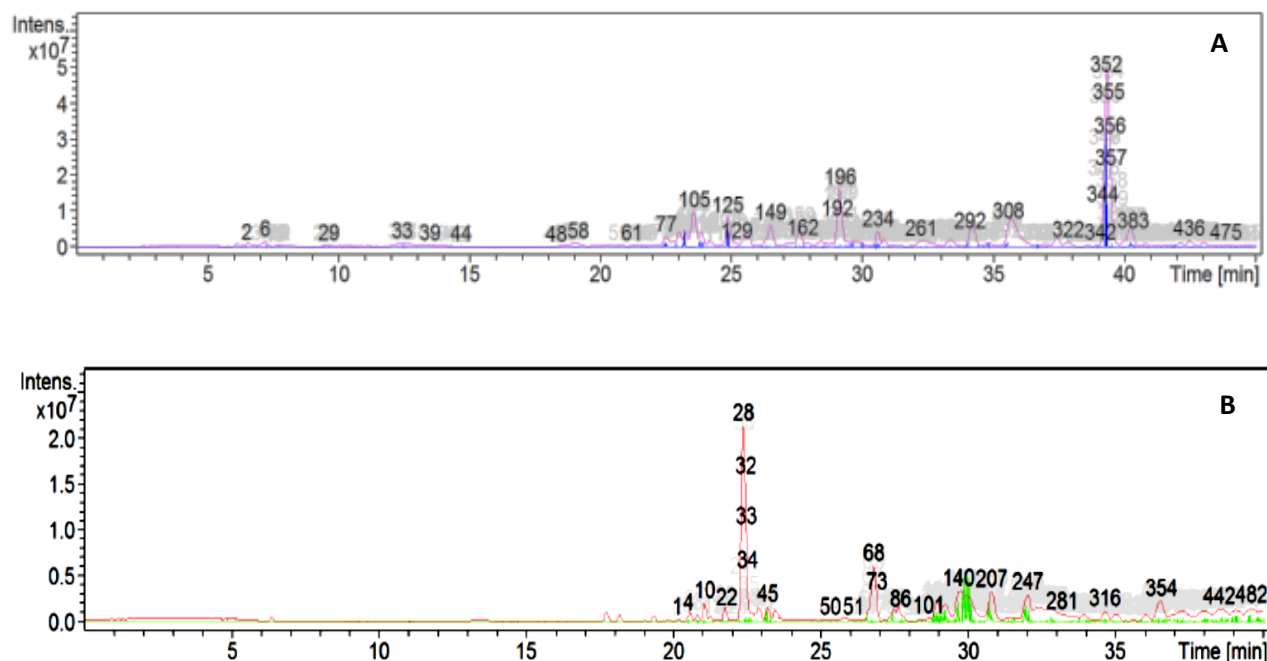


Fig. 5.1 UHPLC-DAD chromatograms of fungal extracts A: *Diaporthe* sp. (T1) and B: *Fusarium tricinctum* (T6) showing the elution of metabolites.

5.1 UHPLC-MS ANALYSIS OF EXTRACT OF T1

5.1.1 CYTOSPORONES C & E

The mass spectral data of extract of the endophytic fungus *Diaporthe* sp. (T1) showed a molecular ion peak at m/z 279.12 $[M+H]^+$ at 23.8 min and another molecular ion peak at m/z 280.09 $[M+H]^+$ at 6.7 min (Table 5.1). These peaks correspond to reported molecular ion peaks of 279.1596 $[M+H]^+$ for cytosporone C and 280.1311 $[M+H]^+$ for cytosporone E (Fig 5.2 & 5.3). Cytosporone C and E are octaketide antibiotics. Brady et al. (2000) identified antibacterial trihydroxybenzene lactones, cytosporones D and E from the cultures of two endophytic fungi *Cytospora* sp. and *Diaporthe* sp. They found that these compounds possessed potent antibacterial activity and characterized those using NMR spectroscopy and X-ray crystallography.

Wu et al. (2013) identified two new octaketides, cytosporones T and U from the culture of *Phomopsis* sp. This endophyte was isolated from the stem of *Scaevola hainansis* Hance. Other

known metabolites cytosporones A, B, C, M, N, pestalotiopsone A, B, and F and aspergillides A and C were also obtained. Aspergillide C and pestalotiopsone F showed *in vitro* neuroaminidase inhibitory activity.

Table 5.1 LC/ESI-MS analysis data of cytosporone C & cytosporone E from *Diaporthe* sp. (T1) and their literature reported data.

Retention time (min)	23.8 and 6.7
Molecular ion (MS1) m/z	m/z 279.12, [M+H] ⁺ 23.8 min
(measured value)	m/z 280.09 [M+H] ⁺ 6.7 min
Ion type	[M+H] ⁺
Molecular ion (MS1) m/z	279.1596 [M+H] ⁺ - Cytosporone C
(reported value)	280.1311 [M+H] ⁺ - Cytosporone E
Molecular weight	278.34 g/mol - Cytosporone C, 279 g/mol - Cytosporone E
Molecular formula	C ₁₆ H ₂₂ O ₄ - Cytosporone C, C ₁₅ H ₁₉ O ₅ - Cytosporone E
Putative compound	Cytosporones C & E
Bioactivity	Octaketide antibiotic
References	Brady et al. (2000), Wu et al. (2013)

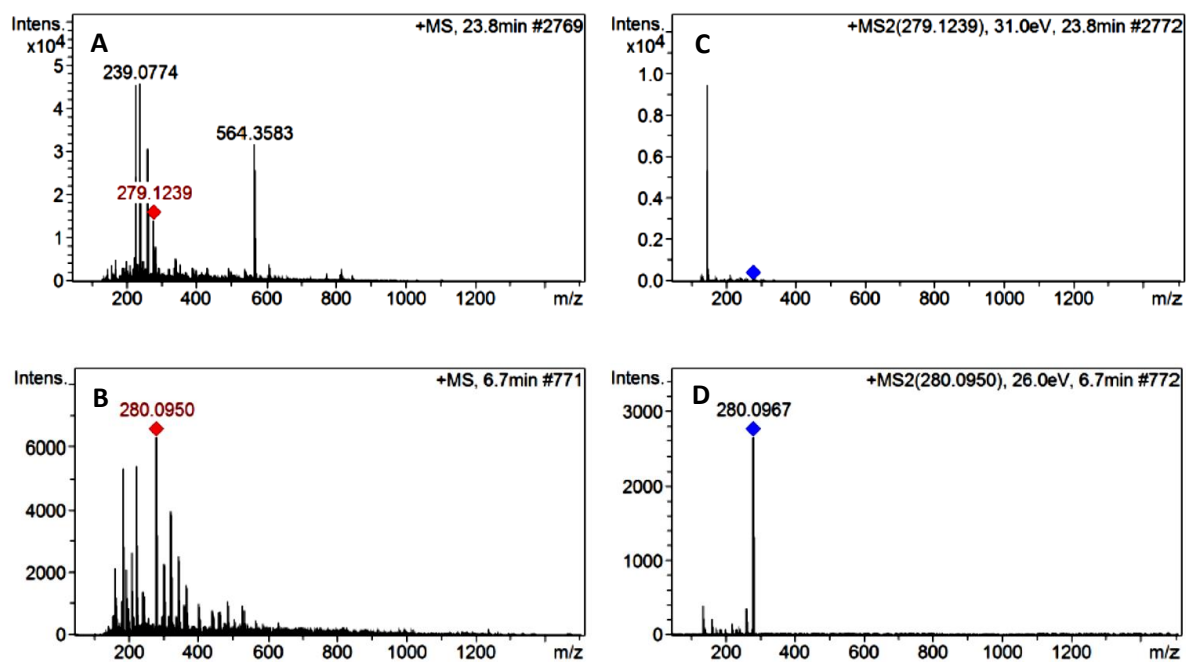
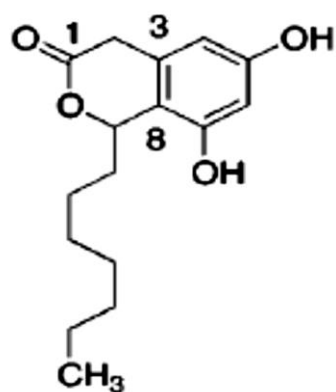
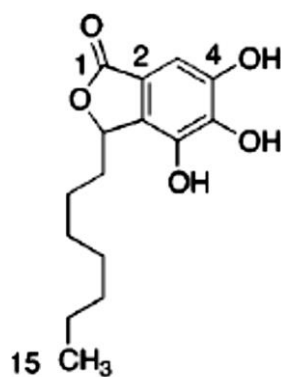


Fig. 5.2 ESI-MS spectra (A & B) and MS/MS spectra (C & D) of cytosporone C & E. MS/MS analyses of the precursor $[M+H]^+$ ion at m/z 279.12 of cytosporone C (A & C) and of the precursor $[M+H]^+$ ion at m/z 280.09 of cytosporone E (B & D).



Cytosporone C



Cytosporone E

Fig. 5.3 Structure of cytosporones C & E.

5.1.2 CYTOCHALASINS

Cytochalasins are a group of cytotoxic fungal metabolites, which have been reported to exhibit varied range of biological activities such as anticancer, antimicrobial, antiparasitic, phytotoxic etc. (Scherlach et al. 2010; Chen et al. 2014). Cytochalasins have also been isolated from a variety of fungal species, including *Helminthosporium* sp., *Phoma* sp., *Xylaria* sp., *Hypoxylon* sp., and *Rhinocladiella* sp. (Scherlach et al. 2010).

Table 5.2 LC/ESI-MS analysis data of trichalasin E, trichalasin F and trichalasin H from *Diaporthe* sp. (T1) and their literature reported data.

	trichalasin E	trichalasin F	trichalasin H
Retention time (min)	39.4	32.5	26.1
Molecular ion (MS1)	472.1511	420.1391	402.1753
m/z (measured value)			
Ion type	[M+Na] ⁺	[M+H] ⁺	[M+H] ⁺
Molecular ion (MS1)	472.2316	420.2706	402.2640
m/z (reported value)			
Molecular formula	C ₂₄ H ₃₅ NO ₇	C ₂₄ H ₃₇ NO ₅	C ₂₄ H ₃₆ NO ₄
Putative Compound	Trichalasin E	Trichalasin F	Trichalasin H
Bioactivity	Anticancer, antimicrobial, antiparasitic,	Anticancer, antimicrobial, antiparasitic,	Anticancer, antimicrobial, antiparasitic,
References	Scherlach et al. (2010)	Schumann and Hertweck (2007)	Ding et al. (2013)

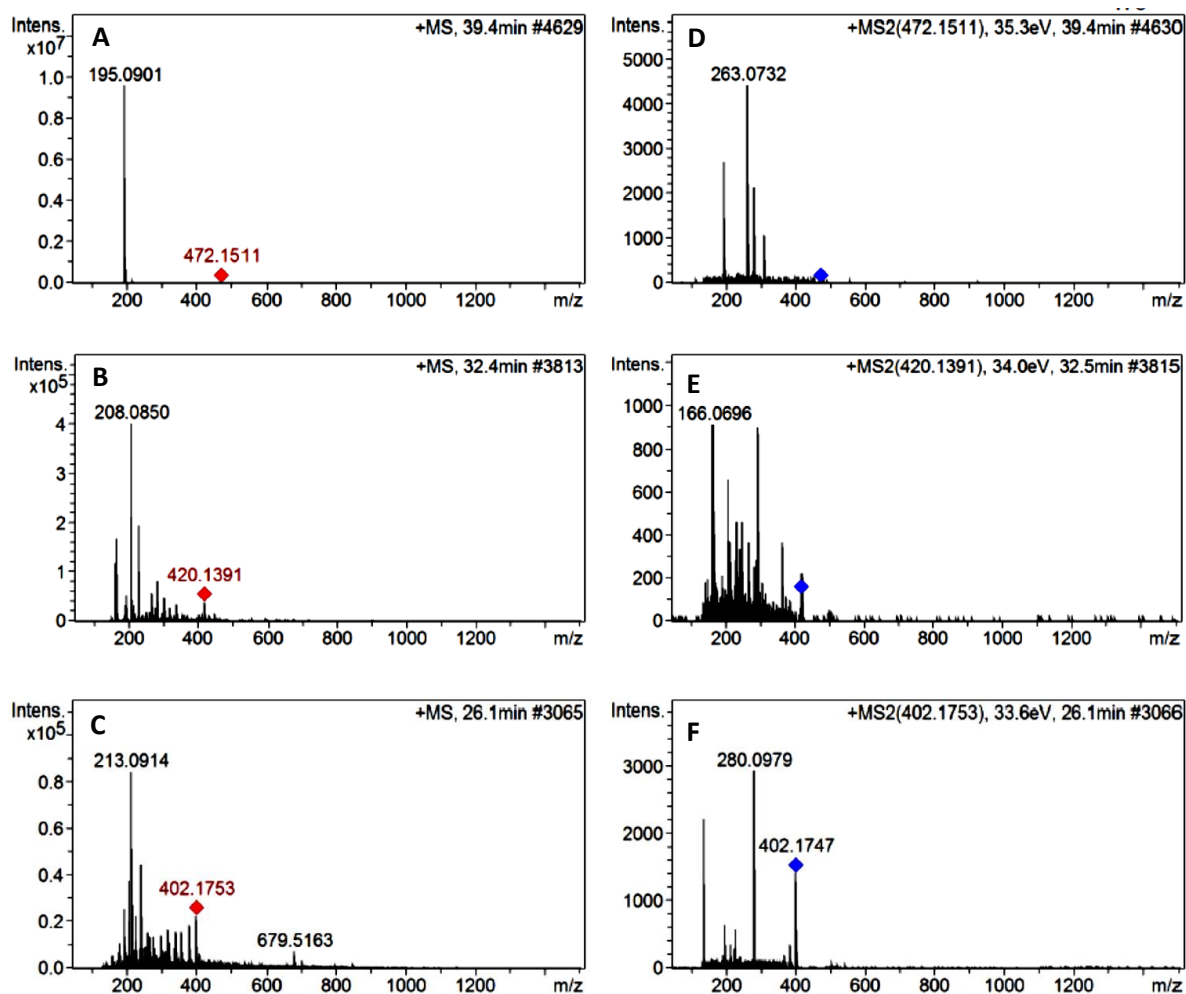
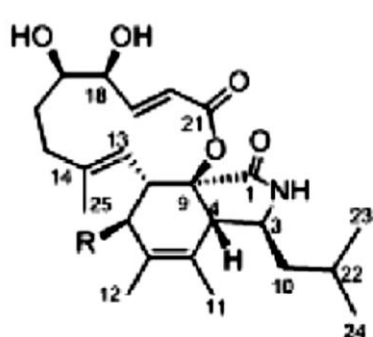
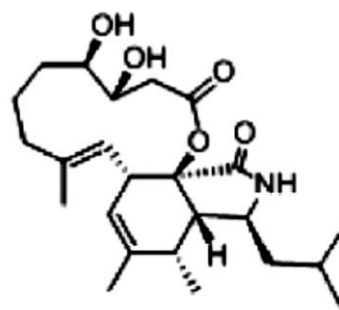


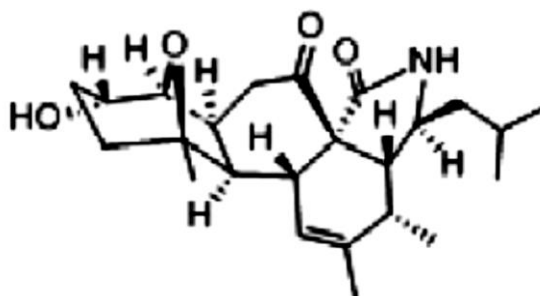
Fig. 5.4 ESI-MS spectra (A, B & C) and MS/MS spectra (D, E & F): A & D- of the precursor $[M+Na]^+$ ion at m/z 472.15 of trichalasin E; B & E- of the precursor $[M+H]^+$ ion at m/z 420.13 of trichalasin F; C & F- of the precursor $[M+H]^+$ ion at m/z 402.17 of trichalasin H



R=OOH
Trichalasin E



Trichalasin F



Trichalasin H

Fig. 5.5 Structure of trichalasin E, trichalasin F and trichalasin H.

Cytochalasins are mold metabolites that exhibit a diverse and distinct biological functions. Several members of this family are categorized under phytotoxins or virulence factors and exhibit antimicrobial or cytotoxic activities (Wagenaar et al. 2000). Cytochalasins have been found from a variety of fungal genera which include *Phomopsis*, *Aspergillus*, *Penicillium* and *Chaetomium* (Yan et al. 2016). Structurally, cytochalasins are comprised of a highly substituted isoindolone ring with a benzyl group at the C-3 position and fused to an 11- to 14-membered macrocyclic ring. Wagenaar et al. (2000) reported four cytotoxic cytochalasins from a culture of the endophytic fungus *Rhinochlaena* sp. Four cytochalasins (Phomopchalasin A, B, C and J) were also reported from the endophytic fungus *Phomopsis* sp. isolated from the stems of *Isodon eriocalyx* var. *laxiflora* (Yan et al. 2016). Pornpakakul et al. (2007) isolated CYP3A4 inhibitor, diaporthichalasin from an endophytic fungus *Diaporthe* sp. from *Croton sublyratus*. Chen et al. (2014) reported the production of 3 new cytochalasins (trichalasin E, F and H) along with four analogues from the endophytic strains sourced from *Trichoderma gamsii* from the Chinese medicinal plant *Panax notoginseng*. In the present study, we have identified three cytochalasins (trichalasin E, F and H) (Table 5.2, Figs. 5.4 & 5.5) from *Diaporthe* sp. and confirmed their identity based on their m/z values. The biological activities exhibited by this fungus may be correlated with the production of

these bioactive compounds (trichalasins). The occurrence of trichalasins (E, F and H) from the endophytic fungus *Diaporthe* sp. isolated from *T. baccata* subsp. *Wallichiana*, is being reported for the first time, through these studies.

5.2 LC-MS ANALYSIS OF EXTRACT T6

5.2.1 GNIDITRIN

The mass spectral data of the extract of endophytic fungus *Fusarium tricinctum* (T6) showed molecular ion peaks at 23.1 min having m/z of 669.26 $[M+Na]^+$ and at 27.7 min having m/z of 647.37 $[M+H]^+$ (Table 5.3; Fig 5.6). These were putatively identified as gniditrin; a diterpenoid with a molecular formula of $C_{37}H_{42}O_{10}$ (Fig 5.7). This compound is reported to have antileukaemic activity and weak carcinogenic antitumor activity (Nyborg and La Cour, 1975). Kupchan et al. (1975) has also reported the antitumor activity in the ethanolic extract of *Gnidia lamprantha* Gilg; a source of gniditrin. The isolation and structure elucidation of gniditrin indicates that the extract had similar bioactive novel components such as gnididin, gniditrin and gnidicin belonging to daphnetoxin class under diterpenoids (Görick and Melzig 2013).

Table 5.3 LC/ESI-MS analysis data of gniditrin from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	23.1, 27.7
Molecular ion (MS1) m/z	647.37, $[M+H]^+$ 27.7 min
(measured value)	669.26 $[M+Na]^+$ 23.1 min
Ion type	$[M+H]^+$, $[M+Na]^+$
Molecular ion (MS1) m/z	669.26 $[M+Na]^+$
(reported value)	
Molecular Weight	646.27
Molecular formula	$C_{37}H_{42}O_{10}$
Putative Compound	Gniditrin
Bioactivity	Diterpenoid tumor inhibitor
References	Kupchan et al. (1975), Görick and Melzig (2013), METLIN database

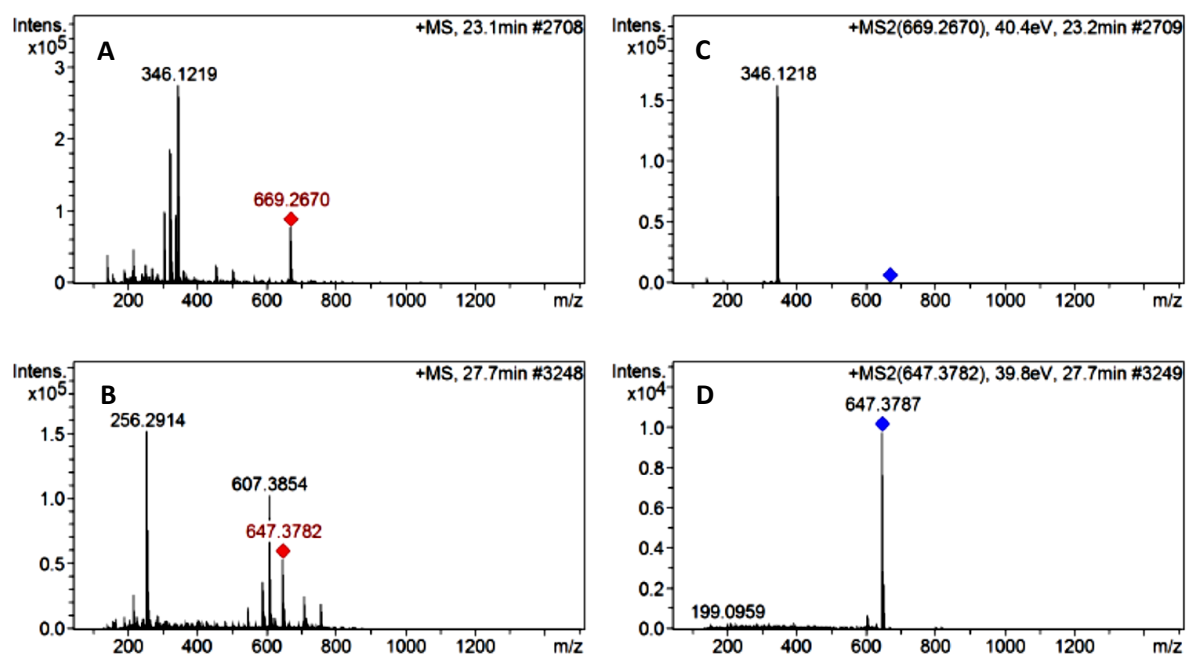


Fig. 5.6 ESI-MS spectra (A & B) and MS/MS spectra (C & D) of gniditrin. MS/MS analyses of the precursor $[M+Na]^+$ ion at m/z 669.26 and of the precursor $[M+H]^+$ ion at m/z 647.37 of gniditrin.

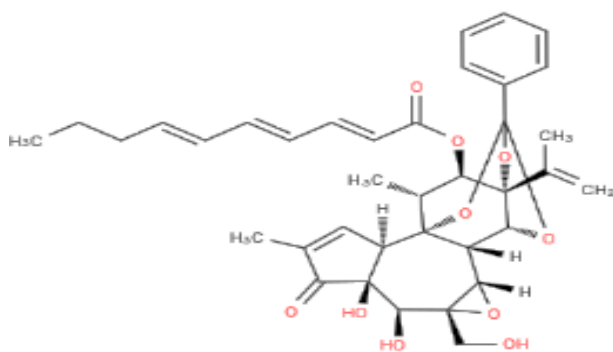


Fig. 5.7 Structure of gniditrin.

5.2.2 7-HYDROXYHEPTAPHYLLINE

The mass spectral data of the extract of the endophytic fungus *Fusarium tricinctum* (T6) also showed a another molecular ion peak of m/z 296.12, $[M+H]^+$ at a retention time of 20.5 min (Table 5.4; Fig 5.8). The peak corresponds to 7- hydroxyheptaphylline with a molecular formula of $C_{18}H_{17}NO_3$

(Fig 5.9). This compound is an alkaloid naturally found in the leaves of *Clausena lansium* and belongs to the family of carbazoles. Genus *Clausena* belongs to family rutaceae having several medicinal properties. Many carbazoles have been isolated from various members of the genus (Songsiang et al. 2012). These authors reported strong cytotoxic and antioxidant activities of these compounds using various bioassays.

Table 5.4 LC/ESI-MS analysis data of 7-hydroxyheptaphylline from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	20.5
Molecular ion (MS1) m/z	296.12
(measured value)	
Ion type	$[M+H]^+$
Molecular ion (MS1) m/z	296.12 , $[M+H]^+$
(reported value)	
Molecular Weight	295.12
Molecular formula	$C_{18}H_{17}NO_3$
Putative Compound	7-Hydroxyheptaphylline
Bioactivity	Antioxidant, cytotoxic activity
References	Songsiang et al. (2012), METLIN database

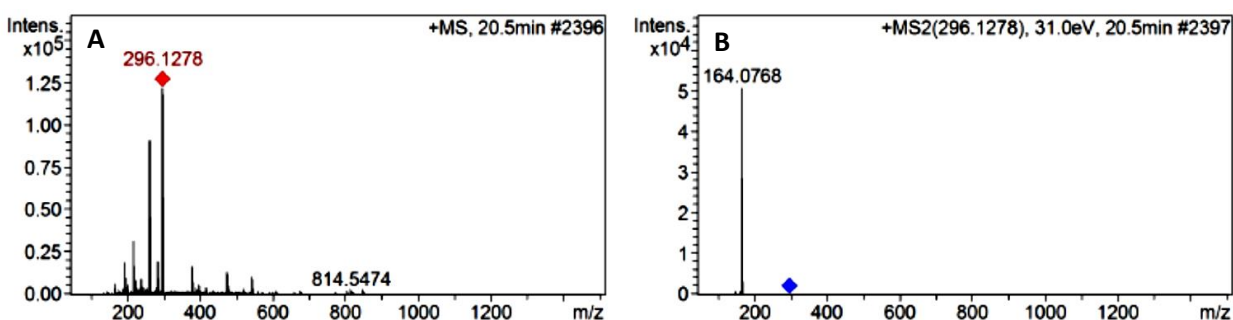


Fig. 5.8 ESI-MS spectra (A) and MS/MS spectra (B) of the precursor $[M+H]^+$ ion at m/z 296.12 of 7-hydroxyheptaphylline.

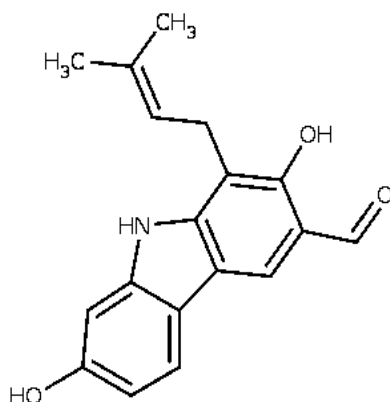


Fig. 5.9 Structure of 7-hydroxyheptaphylline.

5.2.3 TIRANDAMYCIN

A molecular ion peak was observed at 29.8 min retention time with an m/z value of 418.18 (Table 5.5; Fig 5.10). From the search carried out with METLIN database, the putative compound was identified as tirandamycin A (Fig 5.11). The compound has a molecular weight of 417.17 and a molecular formula of $C_{22}H_{27}NO_7$. Trindamycin was also reported by Meyer (1971) and described its spectroscopic properties. Subsequently, many trindamycins were isolated from a marine-derived *Streptomyces* sp., and established their molecular formulae using ESI-HRMS (Carlson et al. 2009). The literature and research reports indicate that the compound possess antioxidant, cytotoxic, antibiotic, antiparasitic and antifungal activity (Carlson et al. 2009).

Table 5.5 LC/ESI-MS analysis data of tirandamycin from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	29.8
Molecular ion (MS1)	418.18
m/z (measured value)	
Ion type	$[M+H]^+$
Molecular ion (MS1)	418.18, $[M+H]^+$
m/z (reported value)	
Molecular Weight	417.17
Molecular formula	$C_{22}H_{27}NO_7$
Putative Compound	Tirandamycin
Bioactivity	Antioxidant, cytotoxic activity, antibiotic, antiparasitic, antifungal and a potent antibacterial.
References	Carlson et al. (2009), METLIN database

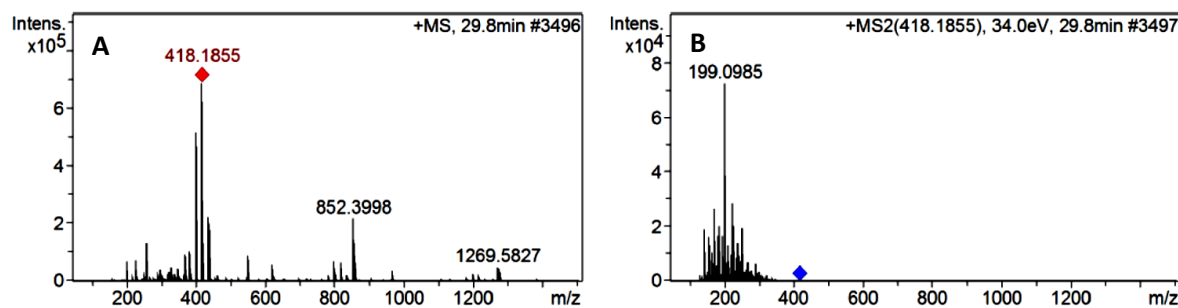


Fig. 5.10 ESI-MS spectra (A) and MS/MS spectra (B) of the precursor $[M+H]^+$ ion at m/z 418.18 of tirandamycin.

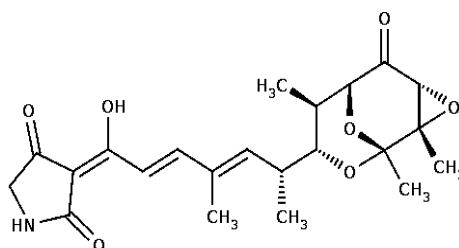


Fig. 5.11 Structure of tirandamycin.

5.2.4 FUMITREMORGIN C

A parent ion of m/z 402.18 was observed at a retention time of 29.0 min. This molecular ion peak corresponds to fumitremorgin C with reported m/z value of 402.18 of $[M+Na]^+$ (Table 5.6; Fig 5.12). The compound has a molecular weight of 379.45 and a molecular formula of $C_{22}H_{25}N_3O_3$ (Fig 5.13). The compound fumitremorgin C has the potential to be used in breast cancer. Liu et al. (2006) studied the combined effect of gefitinib, which is an epidermal growth factor receptor tyrosine kinase inhibitor and fumitremorgin C, which is a specific breast cancer resistant protein inhibitor on the cell growth of fulvestrant resistant MCF-7 cells. In these studies fulvestrant resistant MCF-7 cells were developed and it was reported that the efficiency of drug mitoxantrone was increased by fumitremorgin C. The inhibitory effect of mitoxantrone was also increased by gefitinib. Rabindran et al., (1998) also revealed that fumitremorgin C effectively reverses resistance to multidrugs like mitoxantrone, doxorubicin etc. in human colon carcinoma cells. Rabindran et al. (2000) further reported that multidrug resistance in cells transfected with the breast cancer resistance protein, was reversed by fumitremorgin C.

Table 5.6 LC/ESI-MS analysis data of fumitremorgin C from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	29.0
Molecular ion (MS1) m/z (measured value)	402.18
Ion type	$[M+Na]^+$
Molecular ion (MS1) m/z (reported value)	402.18 $[M+Na]^+$
Molecular Weight	379.45
Molecular formula	$C_{22}H_{25}N_3O_3$
Putative Compound	Fumitremorgin C
Bioactivity	Reverses multi drug resistance in breast cancer
References	METLIN database

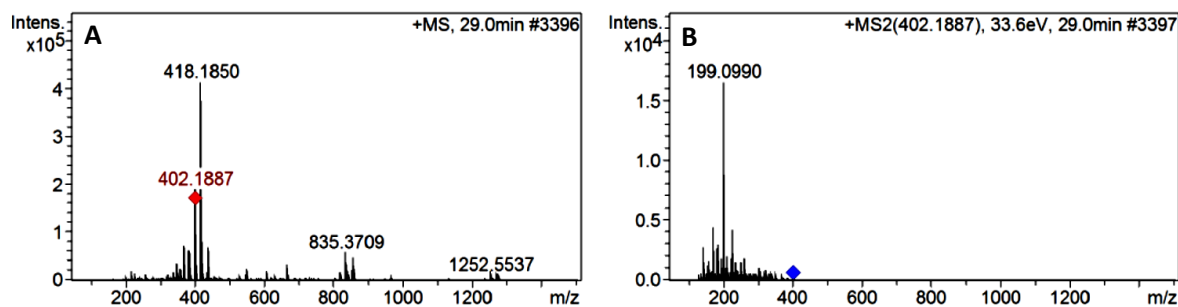


Fig. 5.12 ESI-MS spectra (A) and MS/MS spectra (B) of the precursor $[M+Na]^+$ ion at m/z 402.18 of fumitremorgin C.

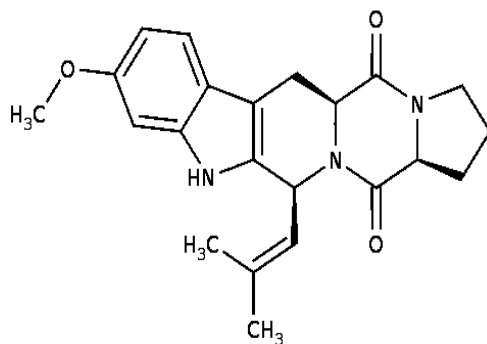


Fig. 5.13 Structure of fumitremorgin C

5.2.5 PACLITAXEL (TAXOL)

A parent ion of m/z 854.59 $[M+H]^+$ was observed after 34.7 min of elution, 892.55 $[M+K]^+$ after 39.4 min of elution (Table 5.7; Fig 5.14). These were identified as H^+ and K^+ (potassium) ions of paclitaxel (Fig 5.15). The reported m/z values of taxol are 854 $[M+H]^+$, 876 $[M+Na]^+$, 892 $[M+K]^+$ (Nadeem et al. 2002). The peak at a value of 854.59 represents $[M+H]^+$ and the peak at a value of 892.55 represents an ion of type $[M+K]^+$. The molecular weight of paclitaxel is 853.90 and molecular formula is $C_{47}H_{51}NO_{14}$. Garyali et al. (2013) also reported the mass spectra with similar molecular ions corresponding to paclitaxel from an endophytic fungi. Taxol is a potent anticancer drug approved by FDA for treatment of various forms of cancer (Kumaran et al. 2010).

Madhusudan et al. (2002) carried out LC-ESI-MS analysis of methanol extracts of the bark of the stem of *Taxus wallichiana*. The bark samples were collected from the various parts of the Himalayas.

Table 5.7 LC/ESI-MS analysis data of paclitaxel from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	34.7
Molecular ion (MS1) m/z (measured value)	854.59- 34.7 min, 892.55- 39.4 min
Ion type	$[M+H]^+$
Molecular ion (MS1) m/z (reported value)	854 $[M+H]^+$, 876 $[M+Na]^+$, 892 $[M+K]^+$
Molecular Weight	853.90 g/mol
Molecular formula	$C_{47}H_{51}NO_{14}$
Putative Compound	Paclitaxel
Bioactivity	Mitotic inhibitor used in cancer chemotherapy
References	METLIN database, mass database

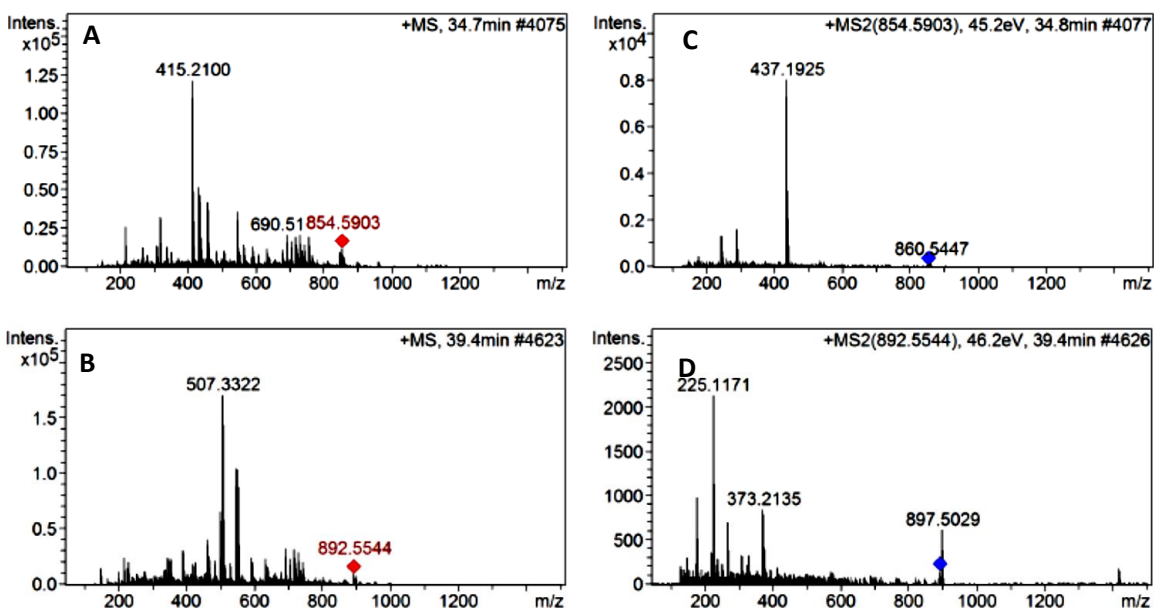


Fig. 5.14 ESI-MS spectra (A & B) and MS/MS spectra (C & D) of paclitaxel. A & C - the precursor $[M+H]^+$ ion at m/z 854.59 and B & D - the precursor $[M+K]^+$ ion at m/z 892.55 of paclitaxel.

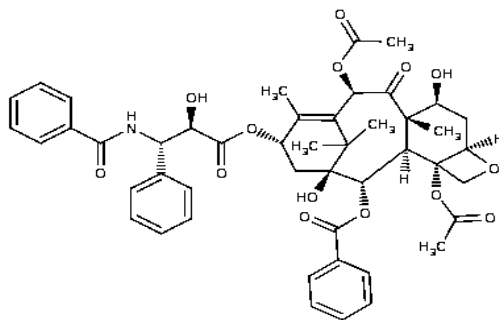


Fig. 5.15 Structure of Paclitaxel

The above studies resulted in identification of 75 taxoids and 36 aminotaxoids from *T. wallichiana* species. Zhao et al. (2006) also used HPLC and LC/ESI-MS/MS methods of analysis to identify the presence of taxoids from the cell cultures of extracts of *Taxus chinensis*. Results were compared with the reference substances and with the literature reports and six taxoids were identified. Quantification of the major taxoids was also done. The investigations revealed that the above methods are rapid as well as reliable techniques to characterize and identify the compounds.

5.2.6 10-DEACETYL BACCATIN III (10-DAB)

The extract showed a peak at 545.24 with a retention time of 29.3 min and a peak at 567.33 with a retention time of 38.9 min (Table 5.8; Fig 5.16). The reported value are 545.23 $[M+H]^+$, 567.23 $[M+Na]^+$ for the compound 10-deacetyl Baccatin III (Fig 5.17). The compound has a molecular weight of 544.6 g/mol and a molecular formula of $C_{29}H_{36}O_{10}$. 10-Deacetyl Baccatin III is a precursor of paclitaxel and inhibits the depolymerization of microtubules leading to inhibition of mitosis. This compound is likely to be present in the extracts of organism capable of producing paclitaxel.

Table 5.8 LC/ESI-MS analysis data of 10- deacetyl baccatin III from *Fusarium tricinctum* (T6) and the literature reported data.

Retention time (min)	29.3, 38.9
Molecular ion (MS1) m/z (measured value)	545.24- 29.3 min, 567.33- 38.9 min
Ion type	$[M+H]^+$
Molecular ion (MS1) m/z (reported value)	545.23 $[M+H]^+$, 567.23 $[M+Na]^+$
Molecular Weight	544.6 g/mol
Molecular formula	$C_{29}H_{36}O_{10}$
Putative Compound	10-Deacetyl baccatin III (10-DAB)
Bioactivity	Precursor of paclitaxel. Inhibits the depolymerization of microtubules leading to inhibition of mitosis.
References	METLIN database, mass database

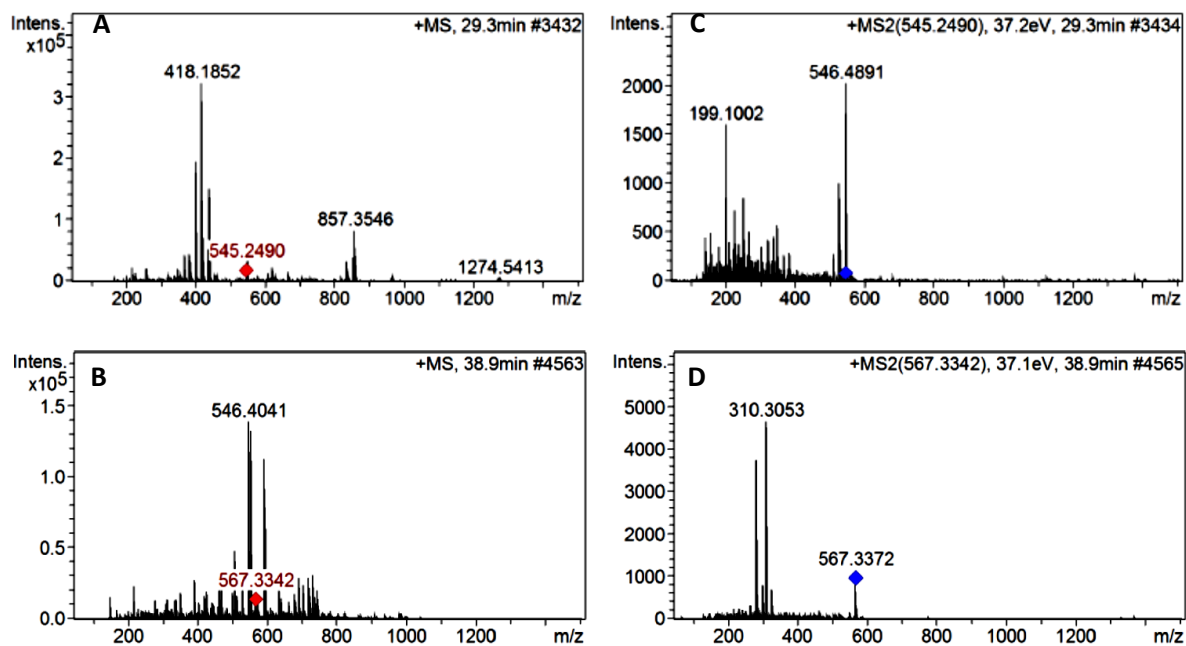


Fig. 5.16 ESI-MS spectra (A & B) and MS/MS spectra (C & D) of the precursor $[M+H]^+$ ion at m/z 545.24 (B & C), $[M+Na]^+$ ion at m/z 567.33 (B & D) of 10-deacetyl baccatin III.

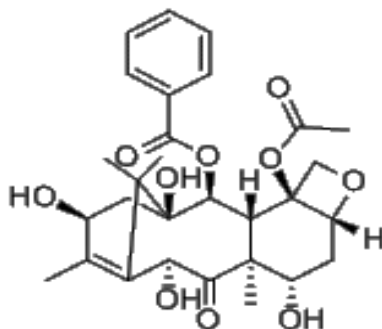


Fig. 5.17 Structure of 10-deacetyl baccatin III.

Paclitaxel is one of the widely used anticancer agent obtained from the bark of yew trees. Baccatin III, 10-deacetyl baccatin III and related compounds are precursors in the synthesis of paclitaxel. Zaiyou et al. (2013) isolated 192 endophytic fungi from the bark of *Taxus wallichiana* var. *mairei*. One endophytic fungal strain *Diaporthe phaseolorum* produced baccatin III in the extracts and was confirmed by LC-MS.

In conclusion, the extracts obtained from two endophytic fungi (*Diaporthe* sp. (T1) and *F. tricinctum* (T6) were subjected to LC-MS analyses. The results showed many compounds and from the data five compounds belonging to two groups were identified from strain T1 and six compounds were identified from strain T6. All these compounds have been reported to show cytotoxic antimicrobial and antioxidant activities. The identified compounds could have contributed significantly to the observed activities of these compounds.

6.0 Extracellular enzymes production by thermotolerant endophytic fungi

Fungal enzymes are gaining importance in agriculture, industry and human health as they are often more stable at extreme conditions than the enzymes derived from plants and animals. Endophytic fungi are known to produce enzymes such as amylases, lipases and proteases as part of their mechanism to overcome the defense of the host during invasion and to obtain nutrients for their development (Sunitha et al. 2012; Costa-Silva et al. 2011). During the past few years, researchers have made significant attempts to document endophytes as enzyme producers with potential for practical applications (Nisa et al. 2015).

In the present study, different thermotolerant endophytic fungi isolated from different tree hosts from Western Ghats (obtained from Dr. T.S. Suryanarayanan, VINSTROM, India) were tested for their ability to produce various extracellular enzymes. Production of extracellular enzymes by nine thermotolerant fungal endophytes such as *Bartalinia* sp., *Chaetomella* sp., *Curvularia* sp., *Exserohilum rostratum*, *Leptosphaerulina* sp., *Montagnulaceae* sp., *Pestalotiopsis microspora* and *Phoma* sp., were determined by digestion of suspended or dissolved substrates in agar plates after inoculation with the mycelial plugs and incubating the cultures for 8-10 days at 37°C. The fungal isolates and their hosts and the GenBank accession numbers used in this study are presented in Table 6.1.

6.1 Screening for extracellular enzymes

Screening of enzymes produced by endophytic fungi was performed by agar diffusion assay. The enzymes tested in this study includes cellulase, lipase, amylase, lipase, pectinase, protease, L-asparaginase and tyrosinase. The results showed that each isolate was able to produce one or the other extracellular enzymes (Table 6.2). All isolates except *Curvularia* sp., were able to produce cellulase when CMC was amended in the medium. Maximum cellulase was produced by

Pestalotiopsis microspora (D) and *Phoma* sp (Fig. 6.1). Maximum laccase activity was observed in *Phoma* sp., followed by *Curvularia* sp., and *Montagnulaceae* sp., which showed moderate activity and least activity was observed in *Chaetomella* sp. Other isolates failed to produce the laccase enzyme activity (Fig. 6.2).

Table 6.1. The fungal isolates tested for the production of extracellular enzymes

Fungal isolates	Host	GenBank Accession No.
<i>Bartalinia</i> sp.	<i>Bridelia retusa</i>	HQ909075
<i>Chaetomella</i> sp.	<i>Pterocarpus marsupium</i>	HQ909076
<i>Curvularia</i> sp.	<i>Holoptelia integrifolia</i>	HQ909079
<i>Exserohilum rostratum</i>	<i>Bridelia retusa</i>	HQ909080
<i>Leptosphaerulina</i> sp.	<i>Voluptea</i> sp.	HQ909074
<i>Montagnulaceae</i> sp.	<i>Sapindus emarginatus</i>	HQ909081
<i>Peatalotiopsis microspora</i> (D)	<i>Maytenus emarginatus</i>	HQ909077
<i>P. microspora</i> (J)	<i>Syzygium cumini</i>	HQ909082
<i>Phoma</i> sp.	<i>Butea monosperma</i>	HQ909078

Table 6.2. Production of different enzymes by endophytic fungi isolated from Western Ghats from different tree species.

Fungal isolate	Cellulase	Laccase	Amylase	Lipase	Pectinase	Protease	L-asparaginase	Tyrosinase
<i>Bartalinia sp.</i>	++	-	+++	++	-	-	+	-
<i>Chaetomella sp.</i>	+	+	-	++	-	-	+	-
<i>Curvularia sp.</i>	-	++	-	-	-	-	++	-
<i>Exserohilum rostratum</i>	+	-	-	-	-	+	-	-
<i>Leptosphaerulina sp.</i>	+	-	++	+++	-	-	++	-
<i>Montagnulaceae sp.</i>	++	++	++	+++	-	+	-	-
<i>Peatalotiopsis microspora</i> (D)	+++	-	-	-	-	-	++	-
<i>P. microspora</i> (J)	++	-	-	+	-	+	+++	-
<i>Phoma sp.</i>	+++	+++	-	+	-	-	+++	-

+++; Maximum activity; ++: Moderate activity; +: Low activity; -: No activity

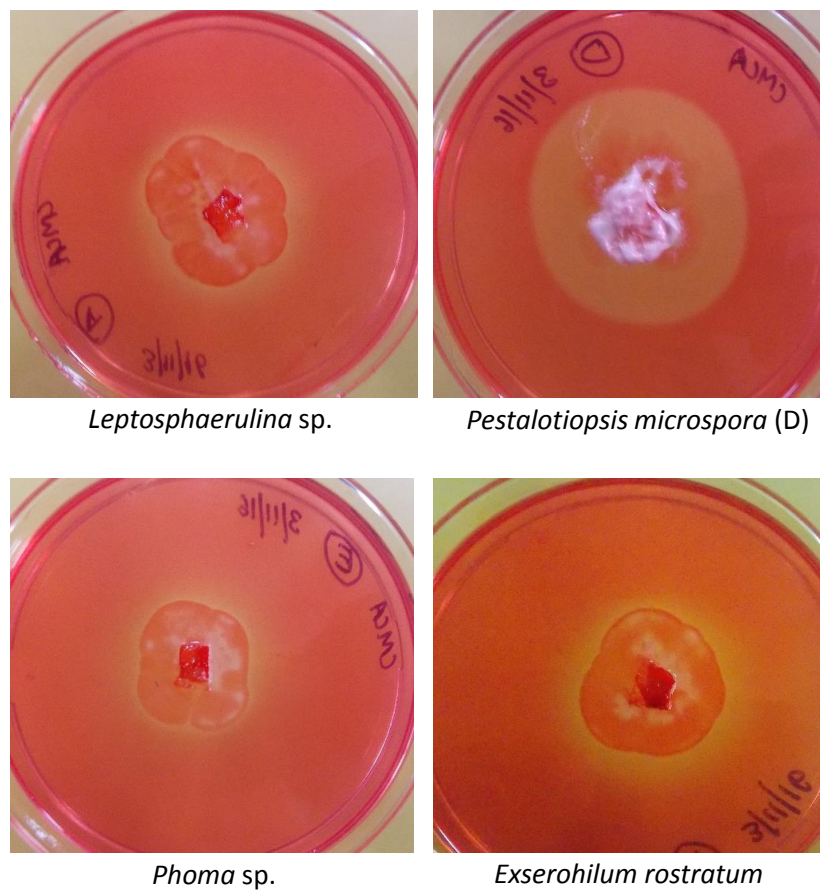


Fig. 6.1. Production of cellulase enzyme by some of the thermotolerant fungi with plate assay.

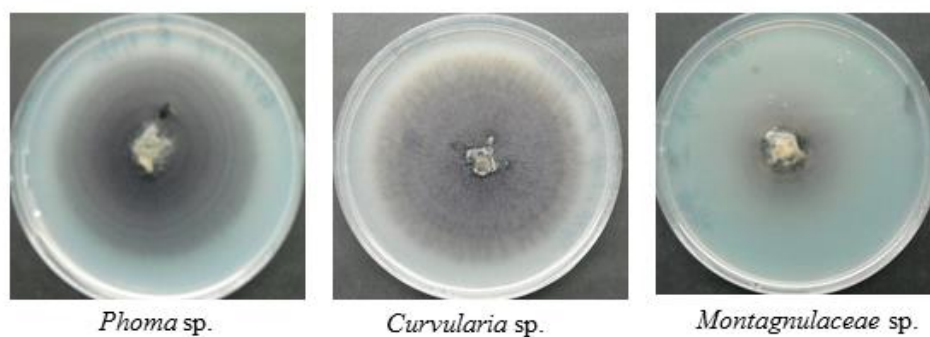


Fig. 6.2. Production of laccase enzyme by some of the thermotolerant fungi with plate assay.

Only three isolates, *Bartalinia* sp., *Leptosphaerulina* sp., and *Montagnulaceae* sp., showed positive to amylase production and the maximum activity was observed in *Bartalinia* sp (Fig. 6.3).

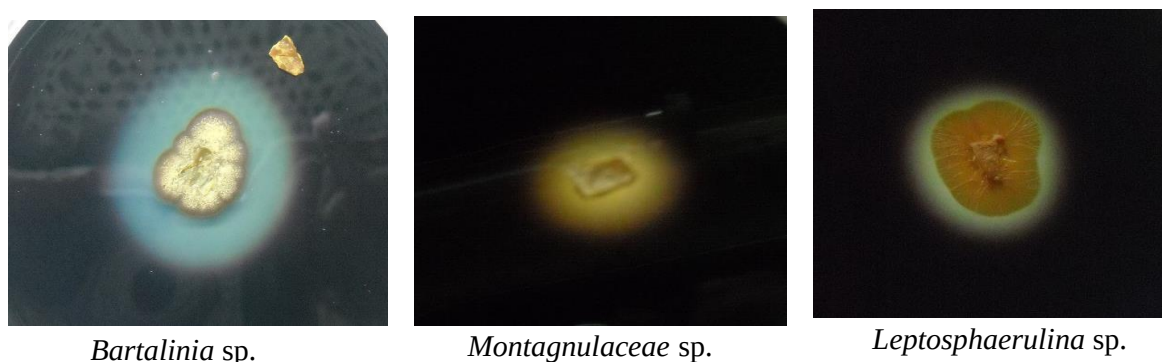


Fig. 6.3. Production of amylase enzyme by some of the thermotolerant fungi tested through agar plate assay

Most of the fungi were able to produce lipase activity and the maximum lipase activity was recorded with *Leptosphaerulina* sp., and *Montagnulaceae* sp. (Fig. 6.4). *Exserohilum rostratum*, *Montagnulaceae* sp., and *P. microspora* (J) showed little protease activity while other isolates failed to produce protease enzyme (Fig. 6.5).



Fig. 6.4. Production of lipase enzyme by some of the thermotolerant fungi tested through agar plate assay

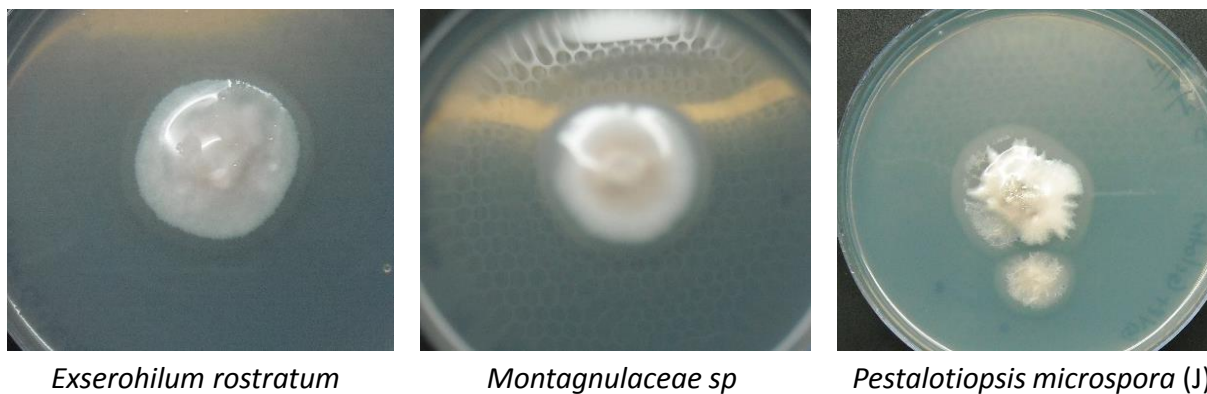


Fig. 6.5. Production of protease enzyme by some of the thermotolerant fungi tested through agar plate assay

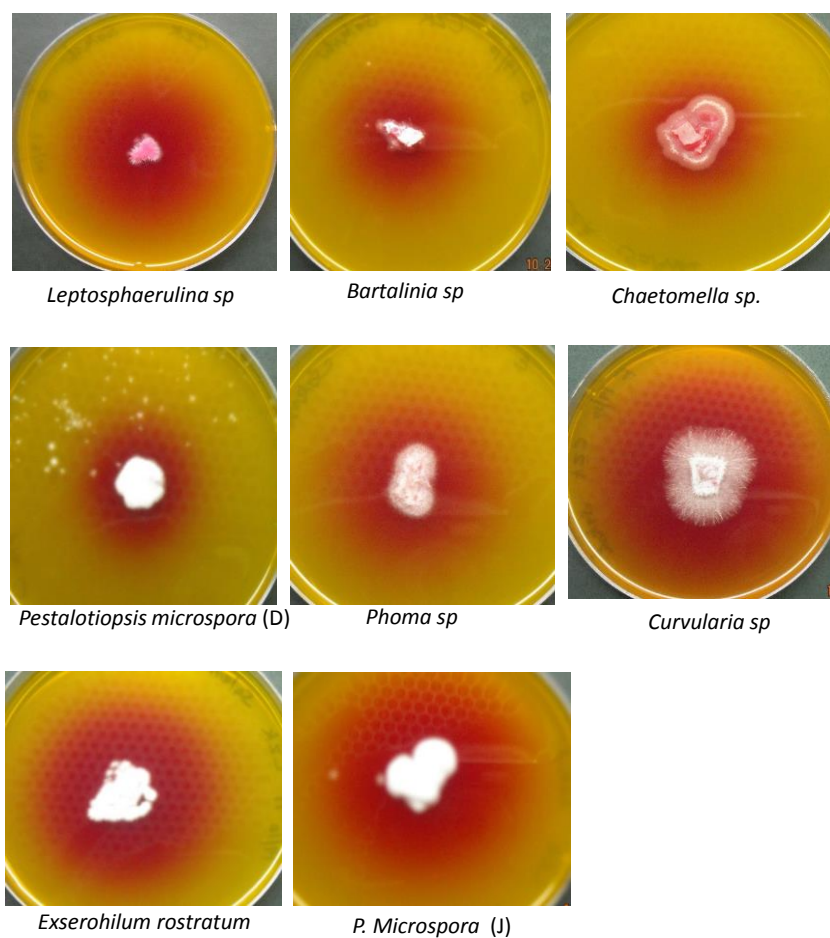


Fig. 6.6. Production of L-asparaginase enzyme by thermotolerant fungi tested through agar plate assay

All isolates except *Montagnulaceae* sp., showed positive for L-asparaginase production. Maximum L-asparaginase activity was recorded in *Curvularia* sp., followed by *E. rostratum*, *P. microspora* (J), *Phoma* sp., *P. microspora* (D) and *Leptosphaerulina* sp., showed moderate activity (Fig. 6.6). None of the isolates showed positive to pectinase and tyrosinase activities. (Table 6.2).

6.2 Quantitative studies on extracellular enzymes

6.2.1 Cellulase

Cellulase producing endophytic fungi *Pestalotiopsis microspora* (D) and *Phoma* sp. and lipase producing *Montagnulaceae* sp. were further tested for their ability to produce these enzymes in liquid culture. The enzyme activities of crude extracts of these fungi were further characterized for their stability at extreme pH and temperature. Cellulase activity was determined at different time intervals (5, 10, 15 and 20 days) by growing the fungi in CMC amended medium. Cellulase activities were calculated by comparing the absorbance with standard of glucose. A standard was made from stock solution of 2 mg/ml of glucose as shown in figure 6.7. Cellulase activity increased up to 10 days and decreased thereafter. In *P. microspora* and *Phoma* sp., maximum cellulase activity was observed at 10 days of incubation time (Table 6.3, Fig. 6.8). Increase in pH slightly reduced the enzyme activity. At pH 4.0, both the isolates showed lower enzyme activity. There was no significant difference between the two isolates with change of pH. The enzyme activity was stable up to pH 7.0 and decreased thereafter (Table 6.4, Fig. 6.9).

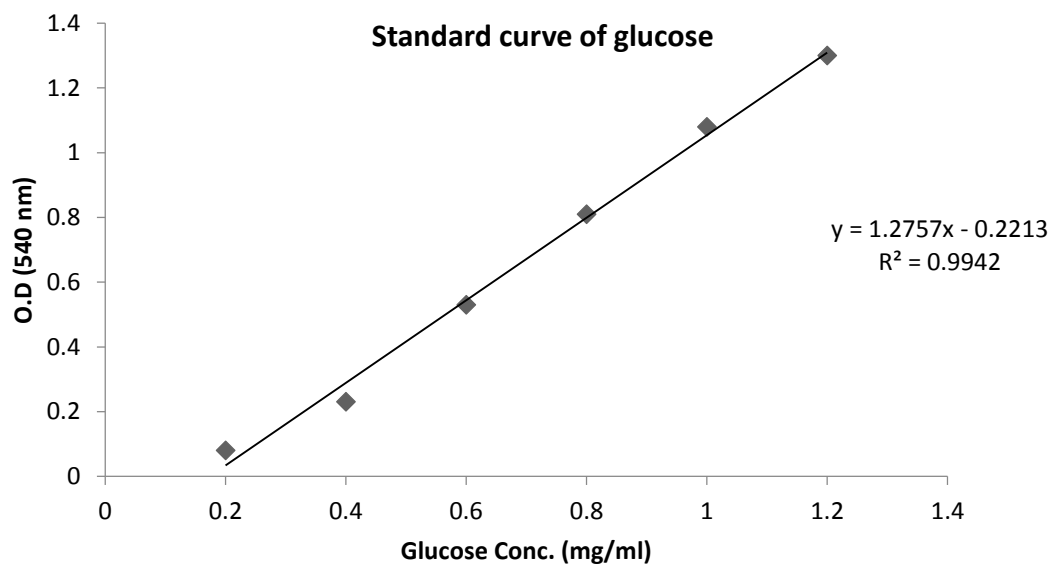


Fig. 6.7. Standard curve of D- glucose at 540 nm

Table 6.3. Effect of incubation time on the cellulase activity (U/ml) of *P. microspora* (D) and *Phoma* sp., grown in presence of carboxy methyl cellulose (CMC)

Days	<i>P. microspora</i> (D)	<i>Phoma</i> sp.
5	0.09 ± 0.001b	0.08 ± 0.002b
10	0.132 ± 0.001a	0.148 ± 0.001a
15	0.105 ± 0.005b	0.131 ± 0.003a
20	0.098 ± 0.001b	0.092 ± 0.001b

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

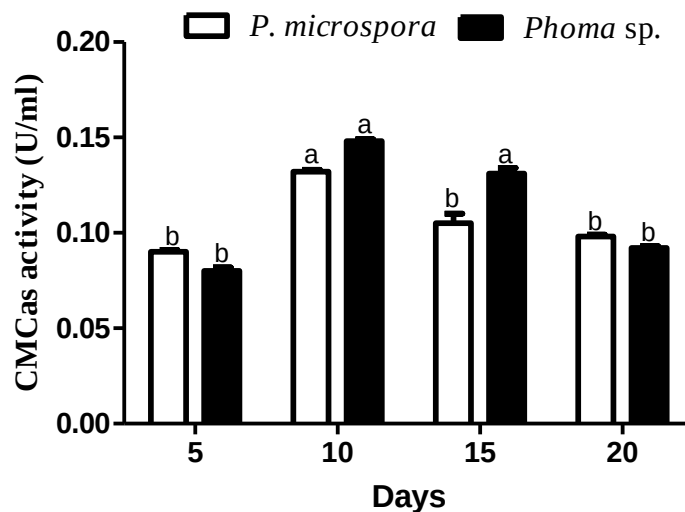


Fig. 6.8. Effect of incubation time on the cellulase activity (U/ml) of *P. microspora* (D) and *Phoma* sp. grown in presence of carboxy methyl cellulose (CMC). Bars showing a common letter within the fungus are not significant at $P < 0.05$. Values are mean $\pm$ SD (n=3).

Table 6.4. Effect of pH on the relative CMCase activity (%) of *P. microspora* (D) and *Phoma* sp. grown in presence of carboxy methyl cellulose (CMC)

pH	<i>P. microspora</i> (D)	<i>Phoma</i> sp.
4	67.5 $\pm$ 5.0d	62.5 $\pm$ 5.0d
5	98.3 $\pm$ 3.0a	98.3 $\pm$ 3.9a
6	95.6 $\pm$ 2.8a	95.3 $\pm$ 4.2a
7	91.3 $\pm$ 3.2a	92.5 $\pm$ 4.9a
8	83.5 $\pm$ 3.5b	87.5 $\pm$ 5.2b
9	78.9 $\pm$ 2.1c	83.5 $\pm$ 3.8c
10	73.5 $\pm$ 3.1d	79.2 $\pm$ 2.1c

Values (mean $\pm$ SD) sharing a common letter within the column are not significant at $P < 0.05$

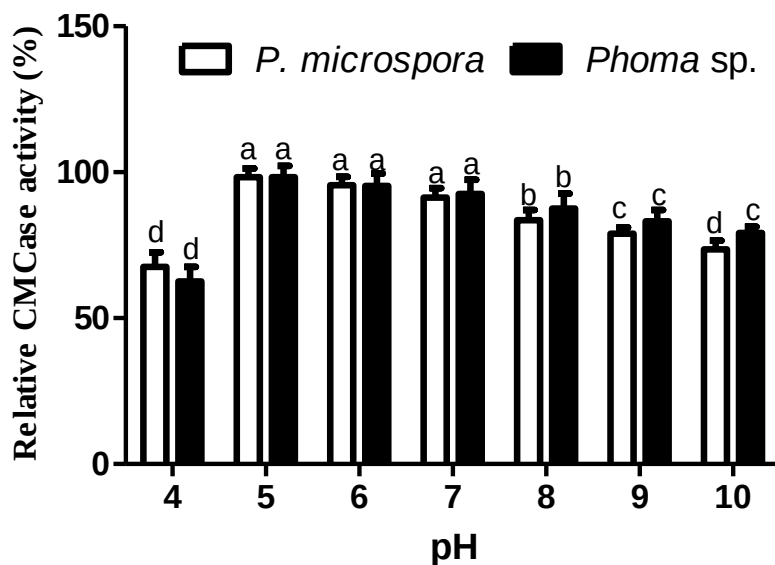


Fig. 6.9. Effect of pH on the relative CMCase activity (%) of *P. microspora* (D) and *Phoma* sp. grown in presence of carboxy methyl cellulose (CMC). Bars showing a common letter within the fungus are not significant at $P < 0.05$. Values are mean $\pm$ SD (n=3).

The enzyme activity was tested at different temperatures ranging from 30-100°C. The enzyme activity enhanced with increase of temperature in both the isolates and the maximum activity was observed at higher temperature. Up to 80°C, *Phoma* sp. showed maximum activity compared to *P. microspora* whereas at higher temperatures *P. microspora* showed the highest activity (Table 6.5, Fig. 10). The stability of the enzyme was tested by incubating the enzyme at 100°C for different time intervals (60, 120, 180, 240 min). Maximum stability was recorded at 60 min when incubated at 100°C and the thermal stability decreased with increase of time (Table 6.6, Fig. 11).

Table 6.5. Effect of temperature on the CMCase activity (U/ml) of *P. microspora* (D) and *Phoma* sp. grown in presence of carboxy methyl cellulose (CMC)

Temperature (°C)	<i>P. microspora</i>	<i>Phoma</i> sp.
30	0.089 ± 0.001d	0.090 ± 0.001c
40	0.123 ± 0.001c	0.135 ± 0.05b
50	0.135 ± 0.001b	0.143 ± 0.003b
60	0.140 ± 0.002b	0.148 ± 0.001b
70	0.148 ± 0.003b	0.153 ± 0.005b
80	0.153 ± 0.001b	0.167 ± 0.005b
90	0.189 ± 0.001a	0.173 ± 0.003b
100	0.203 ± 0.002a	0.192 ± 0.005a

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

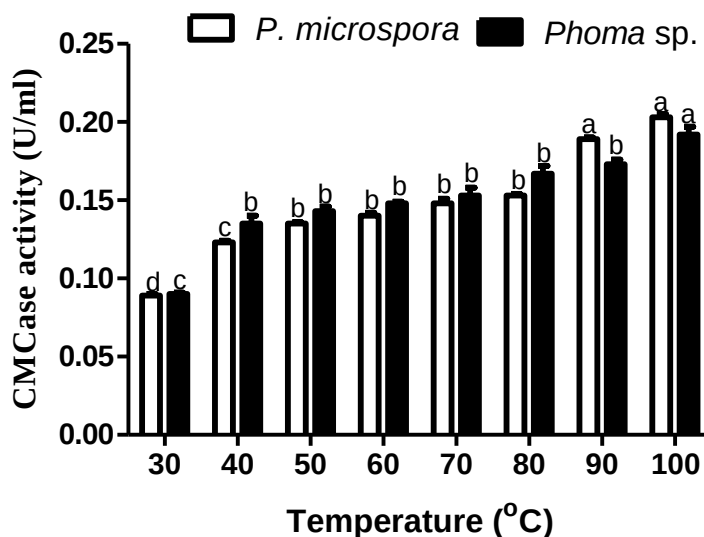


Fig. 6.10. Effect of temperature on the CMCase activity (U/ml) of *P. microspora* (D) and *Phoma* sp. grown in presence of carboxy methyl cellulose (CMC). Bars showing a common letter within the fungus are not significant at $P < 0.05$. Values are mean ± SD (n=3).

Table 6.6. The thermal stability of CMCase activity (U/ml) of *P. microspora* (D) and *Phoma* sp. incubated at 100°C for different time intervals.

Time (hrs)	<i>P. microspora</i>	<i>Phoma</i> sp.
60	0.203 ± 0.001a	0.192 ± 0.005a
120	0.113 ± 0.003b	0.123 ± 0.001b
180	0.082 ± 0.006c	0.079 ± 0.001c
240	0.049 ± 0.001d	0.051 ± 0.005d

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

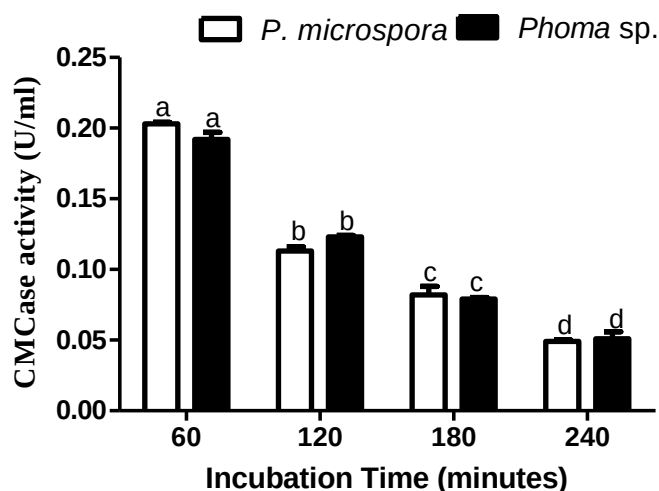


Fig. 6.11. The thermal stability of CMCase activity (U/ml) of *P. microspora* (D) and *Phoma* sp. incubated at 100°C for different time intervals. Bars showing a common letter within the fungus are not significant at $P < 0.05$. Values are mean ± SD (n=3).

6.2.2 Lipase

The lipase activity was determined in endophytic fungus *Montagnulaceae* sp. by incubating the culture at different time intervals. In order to test the action of lipase on p-nitrophenyl phosphate (p-NPP) initially, crude extract was taken after 4th day of incubation and assay was performed as

described in materials and methods section. After 30 minutes of incubation, substrate i.e. p-NPP hydrolyzed to release p-nitrophenol which gave yellow colour. The intensity of yellow colour which was measured spectrophotometrically at 410 nm against enzyme free blank. The standard curves of p-nitrophenol and BSA are presented in figure 6.12 and 6.13, respectively. The enzyme activity increased up to 12 days and decreased thereafter. The maximum activity of lipase was observed at day 12, whereas, the enzyme activity was not significant among 8, 12 and 16 days (Table 6.7, Fig. 14).

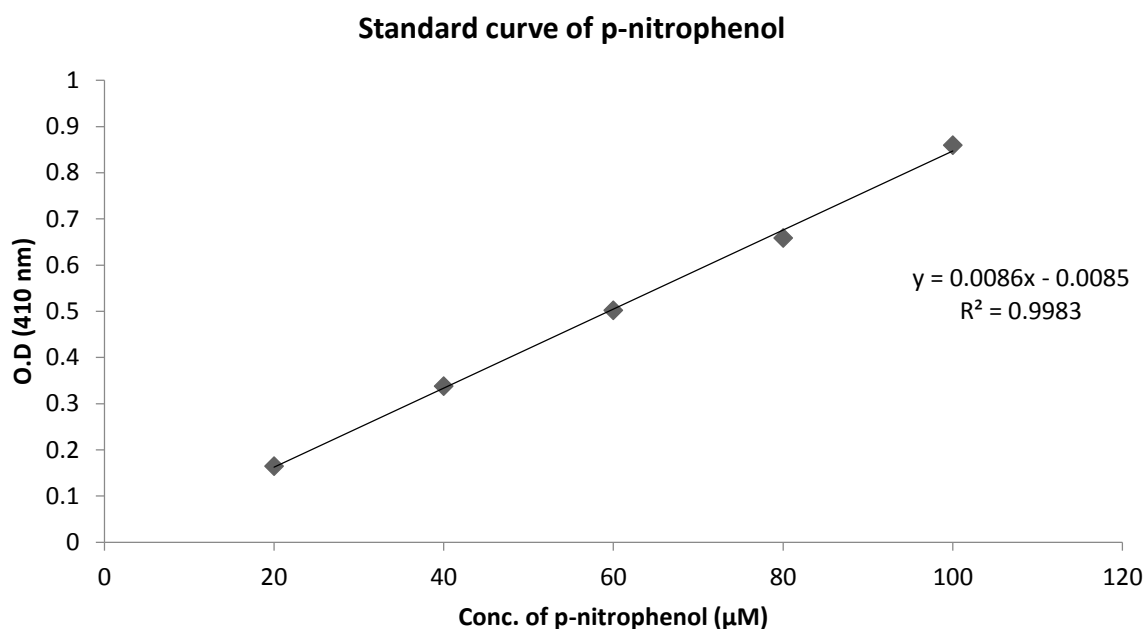


Fig. 6.12. The standard curve of p-nitrophenol.

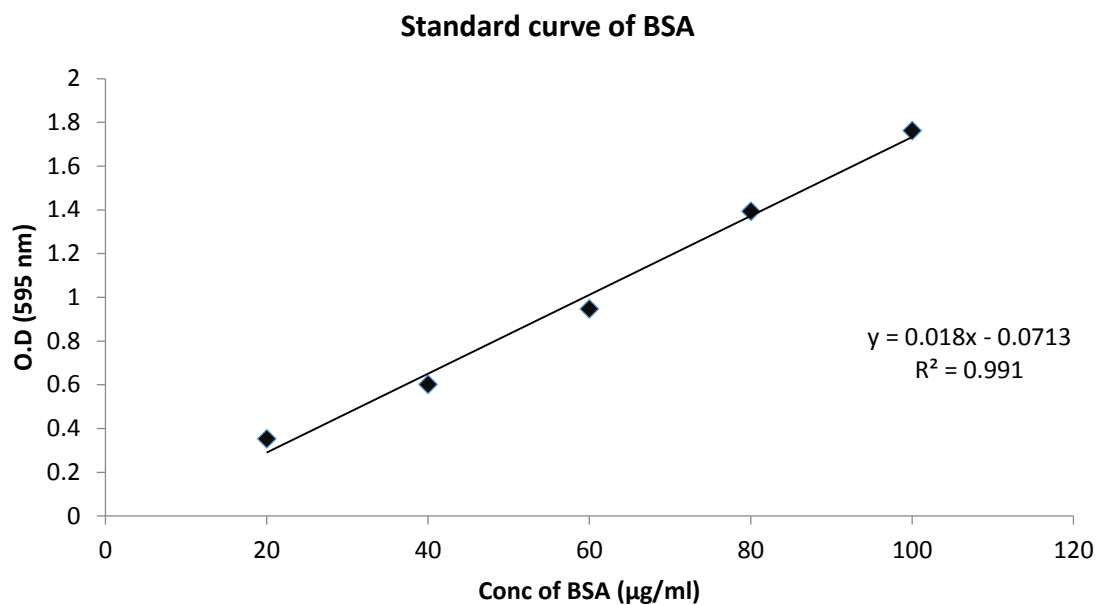


Fig. 6.13. The standard curve of BSA.

Table 6.7. Effect of incubation time on the lipase activity (U/mg) of *Montagnulaceae* sp.

Days	<i>Montagnulaceae</i> sp.
4	98 ± 3.2b
8	143 ± 4.5a
12	152 ± 4.9a
16	148 ± 3.8a
20	110 ± 3.1b

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

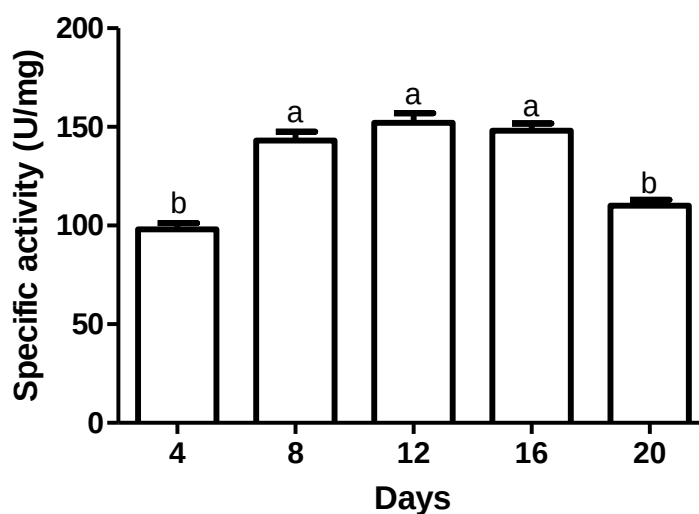


Fig. 6.14. . Effect of incubation time on the lipase activity (U/mg) of *Montagnulaceae* sp. Bars showing a common letter are not significant at $P < 0.05$. Values are mean $\pm$ SD (n=3).

Effect of pH on the relative activity of lipase was studied by incubating the samples at different pH. The enzyme activity significantly increased with increase of pH up to 11.0 and drastically decreased at pH 12.0. The enzyme activity was highest at pH 11.0 followed by pH 10.0 (Table 6.8, Fig. 6.15).

The lipase activity increased significantly with increase in temperature. Among the different temperatures studied, maximum lipase activity was recorded at 100°C (Table 6.9, Fig. 6.16).

These results suggested that the thermotolerant endophytic fungi, used in this study, showed a potential source for different enzyme activities and were able to tolerate extreme pH and temperature conditions.

Table 6.8. Effect of pH on the relative lipase activity (%) of *Montagnulaceae* sp.

pH	<i>Montagnulaceae</i> sp.
4	4.5 ± 0.05h
5	5.3 ± 0.05h
6	10.8 ± 0.92g
7	39.5 ± 2.5f
8	48.5 ± 3.5e
9	68.3 ± 5.0c
10	87.2 ± 6.2b
11	95.3 ± 6.1a
12	59.8 ± 3.2b

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

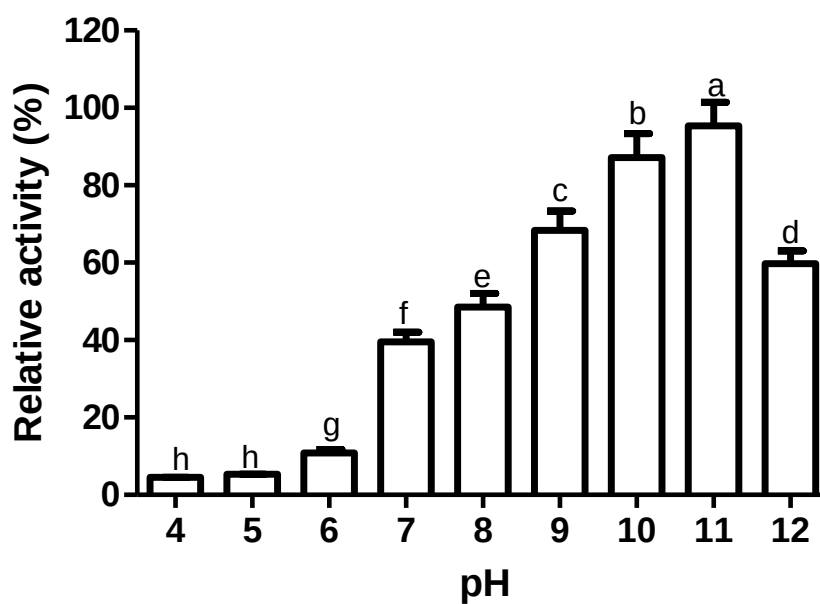


Fig. 6.15. Effect of pH on the relative lipase activity (%) of *Montagnulace* sp. Bars showing a common letter are not significant at $P < 0.05$. Values are mean ± SD (n=3).

Table 6.9. Effect of temperature on the lipase activity (U/mg) of *Montagnulaceae* sp.

Temperature (°C)	<i>Montagnulaceae</i> sp..
30	273 ± 4.8e
40	324 ± 5.8e
50	333 ± 9.2e
60	438 ± 9.7d
70	553 ± 9.8c
80	642 ± 3.5b
90	705 ± 6.5ab
100	754 ± 4.2a

Values (mean ± SD) sharing a common letter within the column are not significant at $P < 0.05$

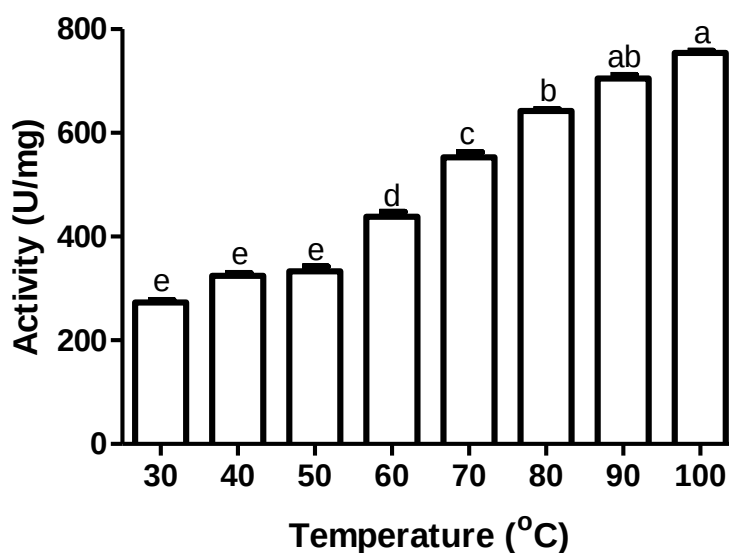


Fig. 6.16. Effect of temperature on the lipase activity (U/mg) of *Montagnulaceae* sp. Bars showing a common letter are not significant at $P < 0.05$. Values are mean ± SD (n=3).

Endophytic fungi seems to have a capacity to produce a wide range of enzymes and secondary metabolites exhibiting a variety of biological activities. However, they have been rarely exploited as sources of enzymes in spite of the fact that they have been recognized as enzyme producers for their natural needs, more specifically as producers of a series of enzymes necessary for penetrating and colonizing their plant hosts, including hydrolytic and oxidative enzymes (Azevedo et al. 2007; Champreda et al. 2007; Suryanarayanan et al. 2009; 2012). Normally endophytic fungi possess two types of extracellular enzymatic systems required to degrade the vegetative biomass: i) the hydrolytic system responsible for polysaccharide degradation consisting mainly in xylanases and cellulases; and ii) the unique oxidative ligninolytic system, which degrades lignin by laccases, ligninases and peroxidases. Endophytic fungi are also capable of producing industrially important enzymes such as lipases, amylases and proteases (Corrêa et al. 2014). Apart from these, endophytic fungi produce enzymes like amylases, lipases and proteases, as part of their mechanism to overcome the defense of the host against microbial invasion and to obtain nutrients for their development (Sunitha et al. 2012; Costa-Silva et al. 2011; Torres et al. 2003). For the past few years researchers have reported endophytes as enzyme producers with potential industrial applications (Corrêa et al. 2014).

In the present study, nine thermotolerant fungal isolates were tested for their ability to produce different extracellular enzymes. Suryanarayanan et al. (2011) isolated these fungi and as their spores are resistant to heat, they termed these fungi as Agni's fungi. These fungi were able to grow after incubation in a drying oven for 2 h at 100°C and the spores of some of these species survived 2 h incubation at 110 – 115°C for 2 h. They suggested that the constitutive heat tolerance expressed by these fungi might be a specific adaptation to prolonged drought periods and the prevalence of fire in their dry habitat. Interestingly, these fungi grow on the leaf litter of a wide range of tree

species and also occur as foliar endophytes in taxonomically unrelated trees in the same vegetation type (Suryanarayanan et al. 2011). Hence, it is interesting to check and explore these fungi for the production of various enzymes and study their thermal stability.

To find new enzyme producers one need to start by screening endophytic fungi for the desired activity using appropriate selection procedures. After isolation and identification, the fungi are transferred to Petri dishes containing solid medium with specific substrates for each enzyme (Uenojo and Pastore 2006). The cultures are then evaluated for the zone of enzyme activity. However, to produce higher quantities of enzymes, cultivation techniques of fungi such as submerged cultivation and solid-state cultivation have been widely used (Li et al. 2012). The qualitative analysis for enzyme production showed that each isolate was able to produce one or the other extracellular enzymes tested in this study.

6.3 Cellulase

Cellulase is a complex enzyme system comprising of endo-1,4- β -D-glucanase (endoglucanase, EC 3.2.1.4), exo-1,4- β -D-glucanase (exoglucanase, EC 3.2.1.91) and β -D-glucosidase (β -D-glucoside glucanhydrolase, EC 3.2.1.21) (Joshi and Pandey1999). Endoglucanase (endo- β -1,4-D-glucanase, endo- β -1,4-D-glucan-4-glucano-hydrolase) - often called as CMCase, hydrolyses carboxymethyl cellulose (CMC) or swollen cellulose in a random manner. Because of this, the length of the polymer decreases, yielding the increase of reducing sugar concentration. Exoglucanase (exo- β -1,4-D glucanase, cellobiohydrolase) hydrolyses cellulose by splitting-off the cellobiose units from the non-reducing end of the chain. β - glucosidase completes the process of hydrolysis of cellulose by cleaving cellobiose and removing glucose from the non-reducing end of oligosaccharides (Sajith et al. 2016). Among the microorganisms, fungi are considered as efficient decomposers of cellulose and responsible for 80% of the cellulose breakdown on earth. This is particularly true in

forest ecosystem, where fungi are the principal agents decomposing cellulose and lignin (Moore Landecker 1996).

Endophytic fungi also produce ligninocellulolytic enzymes and can be regarded as potential alternative sources (Suryanarayanan et al. 2012). In the present study, all isolates except *Curvularia* sp., were able to produce cellulase when CMC was amended in the medium with maximum production by *P. microspora* (D) and *Phoma* sp, Robl et al. (2013) reported best six endophytic strains, *Aspergillus niger* DR02, *Trichoderma atroviride* DR17 and DR19, *Alternaria* sp. DR45, *Annulohyphoxylon stigyum* DR47 and *Talaromyces wortmannii* DR49 capable of producing hemicellulases and related enzymes, suitable for lignocellulosic biomass deconstruction. Two endophytes, *Colletotrichum* sp. and *Alternaria* sp. were reported as cellulase producers when cultured on rice straw and wheat bran in solid-state fermentation (Dey et al 2011; Devi et al. 2012).

Bezerra et al. (2012) reported different endophytic fungi *Fusarium lateritium*, *Nigrospora sphaerica*, *A. japonicas*, *Xylaria* sp., and *Cladosporium cladosporioides* isolated from *Opuntia ficus-indica* capable of producing cellulase with *Xylaria* sp. as the maximum cellulase producer. Grandi and Silva (2006) observed the cellulolytic capability of *Cladosporium oxysporum* associated with *C. echinata*.

Cellulases which are active and stable under extreme conditions have attracted considerable attention because of their potential industrial applications. During the process of industrial bioconversion of lignocellulose, high temperature and acidic/alkaline conditions are needed to get rid of lignin and hemicellulose, reduce the crystallinity of cellulose, and solubilise cellulose and hemicelluloses (Maki et al. 2009; Zhao et al. 2012). In the present study, two endophytic fungi, *P. microspora* and *Phoma* sp., which showed maximum cellulase activity were further tested for their

enzyme activity in liquid culture and also for the enzyme stability at different pH and temperatures. In both fungi, increase in pH slightly reduced the enzyme activity. At pH 4.0 both the isolates showed lower enzyme activity. There was no significant difference between the two fungi with regards to pH. The enzyme activity was stable up to pH 7.0 and decreased thereafter. The enzyme activity increased with increase of temperature in both the isolates and the maximum activity was observed at higher temperature. Up to 80°C, *Phoma* sp. showed maximum activity compared to *P. microspora* (D), whereas at higher temperatures *P. microspora* (D) showed the highest activity. The stability of the enzyme was tested by incubating the enzyme at 100°C for different time intervals (60, 120, 180, 240 min). The activity was more stable at 60 min when incubated at 100°C and the thermal stability decreased with increase of time. Though thermal stability and alkali stability of cellulose has been reported with many thermophilic fungi, no reports are available regarding the thermal stability of cellulase produced by endophytic fungi. This is the first report where the cellulase produced by two thermotolerant endophytic fungi was stable at high temperatures.

Several reports have indicated about fungal succession in which the early colonisers probably originated from host-specific endophytes (Kumaresan and Suryanarayanan 2002; Osono 2002; Hyde et al. 2007). These endophytic fungi might have evolved to colonize plant material before senescence (Wilson 2000). They remain dormant until triggered to grow and sporulate by natural leaf senescence, abscission, or damage. The sequential change of saprobic fungi during succession is one way to verify that endophytes become saprobes in decomposing tissue. The relationships among endophytes and pathogens (Hyde et al. 2009) and among endophytes and saprobes (Promputtha et al. 2007) have been studied. It has been hypothesized that fungal endophytes become saprobes following the senescence of host tissue (Wong and Hyde 2001; Ghimire and

Hyde 2004; Photita et al. 2004; Hyde et al. 2007; Promputtha et al. 2007; Hyde and Soyong 2008). This has been explained that due to the modification of host tissue during senescence, it allows the mycelium to penetrate the epidermis and colonize the host substrates (Dickinson 1976). Therefore, the endophytic fungi produce enzymes to degrade cellulose and lignin. It is also a possible strategy which allows some endophytic fungi to decay host tissue and persist as saprobes after host senescence (Lumyong et al. 2002; Ghimire and Hyde 2004; Oses et al. 2008). This will explain how the endophytic fungi become saprotrophs at leaf senescence with high diversity of saprobic fungi in leaf litter (Wong and Hyde 2001; Parungao et al. 2002; Hyde et al. 2007). Promputtha et al. (2010) reported the correlation between fungal succession and enzyme production patterns during leaf decomposition. The occurrence of saprobes was found to be related to the enzymes that the fungi produce. Their studies further proved the evidence that endophytes can switch lifestyle to saprobes. The endophytic fungi capable of producing degrading enzymes have an important role in litter decomposition, but they do not decompose host tissue in the living host (Promputtha et al. 2010).

6.4 Laccase

Laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2.) are glycosylated polyphenol oxidases and play an important commercial applications in the pulp and paper industry, animal biotechnology, biotransformation and detoxification of phenolic pollutants (Brenna and Bianchi, 1994; Breen and Singleton, 1999; Varshney et al. 2014). Enzymes such as pectinases, xylanase, cellulases and lipases, proteinase and phenol oxidase have been reported with some of the endophytes (Tan and Zou, 2001), but little has been reported about laccase production for degrading lignin by endophytic fungi. In the first study, the endophytic fungus *Monostopora* sp. associated with *Cynodon dactylon* was reported to produce laccase (Wang et al. 2006).

Pestalotiopsis sp. J63, isolated from sea mud samples collected in the East China Sea, was able to utilize various lignocellulosic by-products as substrates (Chen et al. 2011). The endophytic fungus *Phomopsis liquidambari* produced the ligninolytic enzymes laccase and lignin peroxidase when grows on phenolic 4-hydroxybenzoic acid as the sole carbon and energy source (Chen et al. 2013). In the present study, among the nine endophytic fungi tested, the maximum laccase activity was observed in *Phoma* sp., followed by *Curvularia* sp., and *Montagnulaceae* sp., which showed moderate activity and least activity was observed in *Chaetomella* sp. Further, the potential for screening and optimizing more endophytic fungi for laccase production may be exploited in lignin degradation.

6.5 Amylase

Amylases (α -amylases (1,4 α -glucan glucanohydrolase, EC 3.2.1.1); β -amylases (1,4 α -glucan maltohydrolase, EC 3.2.1.2); glucoamylases (1,4 α -glucan glucohydrolase, EC 3.2.1.3)) represent one of the most important enzyme groups which convert starch into different sugar solutions and have important role in food, chemical, detergent, textile and other industries (Pandey et al. 2000). Among the endophytic fungi tested in this study, only three isolates, *Bartalinia* sp., *Leptosphaerulina* sp., and *Montagnulaceae* sp., showed amylase production with maximum activity by *Bartalinia* sp. Several studies have shown that the endophytic fungi are able to produce amylase enzyme (Corrêa et al. 2014). Marlida et al. (2000) isolated four strains of endophytic fungi, *Gibberella pulicaris*, *Acremonium* sp., *Synnematous* sp. and *Nodilusporium* sp., capable of producing amylase. Champreda et al. (2007) reported the amylase production by *Fusicoccum* sp. BCC4124 among the several other endophytic fungi. *Cylindrocephalum* sp. isolated from the medicinal plant *Alpinia calcarata* (Haw.) Roscoe showed amylase activity (Sunitha et al. 2012). The endophytic fungus, *G. pulicaris* was also described as a producer of an amylase capable of

degrading starches from various sources such as cereals and other crops including raw potato, sago, tapioca, corn, wheat and rice starch (Marlida et al. 2000). The α -amylase produced by the endophytic fungus *Preussia minima*, isolated from the Australian native plant, *Eremophila longifolia* was purified by Zaferanloo et al. (2014). In the present study, an attempt has been made to test some of the endophytic fungi as source of enzymes for industrial applications. The *Bartalinia* sp., used in this study showed higher enzyme production and this fungus can be exploited further to test its efficacy to produce the enzyme with different substrates and also for its thermal stability.

6.6 Lipase

Lipases (EC 3.1.1.3) (a special class of esterases) are hydrolytic enzymes which break the ester bond of triacylglycerol, releasing free fatty acids and glycerol (Jaeger and Reetz 1998). Apart from hydrolysis reaction, these enzymes are also involved in catalyzing interesterification, alcoholysis, acidolysis, esterification and aminolysis reactions (Diaz et al. 2006; Damasso et al. 2008). Microbial lipases are highly stable in organic solvents, do not require cofactors and can act on different substrates (Hasan et al. 2006). Production of lipases by endophytic fungi has been the target of research in the last few years. In the present study, most of the endophytic fungi were able to produce lipase activity except one isolate of *P. microspora* (D), *Curvularia* sp., and *E. rostratum*. The maximum lipase activity was recorded with *Montagnulaceae* sp. A mycelium-bound lipase was isolated from the endophytic fungal strain *Rhizopus oryzae* associated with Mediterranean plants that catalyzes the esterification of fatty acids in isooctane (Torres et al. 2003). They also reported the optimal enzyme activity at pH 4–7 and 60 °C. Panuthai et al. (2012) reported the production of lipase by 10 isolates, out of 65 endophytic fungal isolates identified. *Fusarium oxysporum* PTM7 isolated from the leaves of *Croton oblongifolius* showed highest lipase activity

in the basal culture medium with 1 % (v/v) olive oil, 1 % (w/v) peptone and 0.5 % (w/v) sodium nitrate as the carbon, organic and inorganic nitrogen sources, respectively. The purified enzyme showed optimal activity at pH 8 and 30 °C, with reasonable stability at 40 °C and a wide range of pH 8.0 to 12. Costa-Silva et al. (2011) studied the lipase production from the endophyte *Cercospora kikuchii* with maximum enzyme production in a medium supplemented with 2% soybean oil. In the present study, the crude extract of lipase showed its maximum activity at pH 11.0 followed by pH 10.0 and temperature range of 70 - 100°C. Hence, this enzyme could be exploited for industrial applications because of its wide pH and temperature range. Further, studies are required to purify the enzyme and study their characteristics and stability at high temperatures.

6.7 Protease

Protease are a group of enzymes which hydrolyze peptide bonds of proteins and they form a large group of enzymes belonging to the class of hydrolases. They are widely employed for commercial, industrial and health purposes including in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Mahajan and Badgujar 2010). From the last few years, attention has been focused on fibrinolytic enzymes from microorganisms because of their potential use in thrombosis therapy. Wu et al. (2009) reported the production of fibrinolytic enzyme from the endophytic fungus *Fusarium* sp. CPCC 480097 isolated from chrysanthemum stems. Russell et al. (2011) reported the protease activity (serine proteases) from two *Pestalotiopsis microspora* isolates responsible for degradation of polyester polyurethane. In the present study also, *P. microspora* (J), *Montagnulaceae* sp. and *E. rostratum* showed protease activity compared to other isolates. Zaferanloo et al. (2014) also tested three endophytic fungi, *Alternaria alternata*, *Phoma herbarum* and an unclassified fungus isolated from *Eremophila longifolia* for protease

activity. *A. alternata* showed the highest protease activity in a wide range of pH (3–9) and temperature (9 – 50 °C).

6.8 L-asparaginase

L-asparaginase (L-Asparagine amidohydrolase; EC 3.5.1.1) belongs to an amidase group catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia (Theantana et al. 2009). In cancer treatment, L-asparaginase removes L-asparagine in the serum, depriving tumor cells from large amounts of asparagine required for growth, thus, controlling tumor growth effectively (Verma et al. 2007). In fact, L-asparaginase is a clinically acceptable anticancer agent for the treatment of acute lymphoblastic leukemia (Verma et al. 2007). Asparaginase has been investigated since it was found that the enzyme from definite microorganisms has antitumor activity. Fungi are reported as potential producer of asparaginase in comparison to bacteria. *Aspergillus*, *Penicillium* and *Fusarium* are known to produce asparaginase (Jalgaonwala and Mahajan 2014). Endophytic fungi producing L-asparaginase activity predominantly belong to the genus *Colletotrichum*, followed by species of *Fusarium*, *Penicillium* and *Phoma* (Thirunavukkarasu et al. 2011). Although literature on diversity of endophytic fungi are available, documentation of their L-asparaginase production is lacking. In the present study, except *Montagnulaceae* sp., all other isolates tested were capable of producing L-asparaginase. Chow and Ting (2015) reported the L-asparaginase producing endophytic fungi *Fusarium oxysporum* (MKS1) and *Penicillium simplicissimum* (PBL13) from the Malaysian medicinal plants. Jalgaonwala and Mahajan (2014) reported the L-asparaginase activity of endophytic fungus *Eurotium* sp., isolated from rhizomes of *Curcuma longa*. The results of the present study, showed that these fungi could further be extensively exploited for L-asparaginase production.

In Conclusion, these endophytic fungi seems to have a capacity to produce a wide range of enzymes and secondary metabolites exhibiting a variety of biological activities. However, they have been rarely exploited as sources of enzymes in spite of the fact that they have been recognized as enzyme producers as a part of their natural existence. However, further studies are necessary using established methods of fungal cultivation so as to optimize the production of enzymes. This would need to be followed by the characterization of the physicochemical properties of these enzymes so as to exploit the real potential of endophytic fungi as sources of industrial biocatalysts.

Summary

Endophytes are ubiquitous organisms residing in the internal tissues of the plants, at least for a portion of their lives without causing apparent symptoms of infection and the majority of these endophytes are fungi. These fungi appear to have a capacity to produce a wide range of secondary metabolites and enzymes. Several of these compounds have the biological activities of interest for application in environment, agriculture, medicine and food industry. Many endophytic fungi are known to produce secondary metabolites which have shown antimicrobial activity against pathogenic fungi, bacteria and yeasts, cytotoxic activity on human cell lines. An array of natural products such as steroids, xanthenes, phenols, isocoumarines, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides and cytochalasins have been characterized from endophytic fungi, which showed anti-cancerous, antioxidants, anti-viral, anti-insecticidal, immuno-suppressant, anti-microbial, anti-malarial and anti-mycobacterial activities. The secondary metabolites produced by endophytes associated with medicinal plants can be exploited for curing many diseases. Filamentous fungi are the important source of industrial enzymes due to their ability to produce extracellular proteins. Though endophytic fungi are well known to produce unique bioactive metabolites, they have not yet been extensively exploited as sources of industrial enzymes, in spite of the fact that they have since long been recognized as enzyme producers for their natural needs. In the present study, endophytic fungi were isolated from *Taxus baccata* and were identified based on their morphological characteristics and internal transcribed spacer of rDNA sequence analysis. The extracts of these fungi were tested for anticancer and antioxidant activities. Some of the putative compounds involved in these biological activities were identified. An attempt has also been made to examine the potential of thermotolerant endophytic fungi, isolated from the plants growing in the Western Ghats of India, to produce extracellular enzymes.

Isolation, identification and screening of endophytic fungi for biological activities

A total of 25 endophytic fungi were isolated from the bark of *T. baccata*. Fungal extract were obtained after culturing for 21 days. Preliminary screening results revealed that among the 25 isolates, four isolates designated as T1, T2, T4 and T6 showed more antimicrobial and cytotoxic activities compared to other isolates. These four isolates were selected for further studies. Further, these isolates were identified based on their morphological characteristics and internal transcribed spacer (ITS) sequence analyses. Based on the morphological and ITS sequence analysis, isolate T1 was identified as *Diaporthe* sp., isolate T2 as *Marasmius scorodonius*, isolate T5 as *Microdiplodia* sp. and isolate T6 as *Fusarium tricinctum*. The effect of these fungal crude extracts were tested on the growth of bacteria such as *Staphylococcus aureus*, *Bacillus megaterium*, *Escherichia coli* and *Pseudomonas aeruginosa* and *Candida albicans*. These isolates showed antibacterial and antifungal activities. The effect of crude fungal extracts on the growth of cancer cell lines was evaluated by MTT assay. Out of the four endophytes, *Diaporthe* sp. (T1) and *F. tricinctum* (T6) have shown pronounced cytotoxic effect. T1 and T6 fungal isolates have shown cytotoxic effect against Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) and the cytotoxic effect significantly increased with increase in the concentration of the fungal extract. The IC_{50} value for T1 extract was found to be 1058 ± 44 and $1257 \pm 80 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. The IC_{50} value for T6 extract was found to be 225 ± 26 and $220 \pm 18 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. The antioxidant potential of fungal extract (T1 and T6) was studied by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The antioxidant effect of T1 and T6 significantly increased with increase in concentration. The IC_{50} value (concentration of sample required to scavenge 50% of free radicals) for T1 and T6 extract was found to be 250 ± 3.5 and $482 \pm 9 \mu\text{g ml}^{-1}$.

Identification and characterization of putative bioactive compounds produced by *Diaporthe* sp. (T1) and *F. tricinctum* (T6)

The endophytic fungi, *Diaporthe* sp. (T1) and *Fusarium tricinctum* (T6) were used in this study for the production of bioactive compounds. The culture filtrates were extracted. The residue was dissolved in methanol and separated using HPLC system. High resolution mass spectroscopic detection was performed using mass spectrometer operating with electron spray ionization (ESI). Both positive and negative ions were monitored. Smart Formula 3D™ -and Fragment Explorer part of Data Analysis 4.2 was used for compound formula generation and assignment of fragment structures. The data obtained was analyzed for identification of the compounds produced by these fungi. Some of the putative compounds identified from the crude extracts of *Diaporthe* sp. (T1) are cytosporones, cytochalasins and docetaxel. Three cytochalasins such as trichalasin E, F and H were identified based on their m/z values of eluting peaks. These compounds are being reported for the first time from the endophytic fungus *Diaporthe* sp (T1). Some of the important compounds produced by *F. tricinctum* (T6) includes, gniditrin, 7-hydroxy heptaphylline, tirandamycin A, fumitremorgin C, paclitaxel and 10-deacetyl baccatin III. These compounds have been reported to have the cytotoxic, antimicrobial and antioxidant activities.

Extracellular enzymes production by thermotolerant endophytic fungi

In the present study, different thermotolerant endophytic fungi isolated from different tree hosts from Western Ghats (Suryanarayanan et al. 2011) were tested for their ability to produce various extracellular enzymes. Production of extracellular enzymes by nine thermotolerant fungal endophytes such as *Bartalinia* sp., *Chaetomella* sp., *Curvularia* sp., *Exserohilum rostratum*, *Leptosphaerulina* sp., *Montagnulaceae* sp., *Pestalotiopsis microspora* and *Phoma* sp., were determined by digestion of suspended or dissolved substrates in agar plates. The results showed

that each isolate was able to produce one or the other extracellular enzymes. All isolates except *Curvularia* sp., produced cellulase when CMC was amended in the medium. Maximum cellulase was produced by *P. microspora* and *Phoma* sp. Maximum laccase activity was observed in *Phoma* sp., followed by *Curvularia* sp and *Montagnulaceae* sp., which showed moderate activity. Only three isolates, *Bartalinia* sp., *Leptosphaerulina* sp., and *Montagnulaceae* sp., showed positive to amylase production and the maximum activity was observed in *Bartalinia* sp. Most of the organisms were able to produce lipase and the maximum lipase activity was recorded with *Montagnulaceae* sp., and *Leptosphaerulina* sp. None of the isolates showed positive to pectinase and tyrosinase activities. *Exserohilum rostratum*, *Montagnulaceae* sp., and *P. microspora* showed little protease activity while other isolates failed to produce protease enzyme. Maximum L-asparaginase activity was recorded in *Curvularia* sp. and moderate levels in *E. rostratum*, *Leptosphaerulina* sp., and *P. microspora*.

Cellulase producing endophytic fungi *P. microspora* and *Phoma* sp. and lipase producing *Montagnulaceae* sp. were further tested for their ability to produce these enzymes in liquid culture. Cellulase activity was determined at different time intervals by growing the fungi in CMC amended medium. Maximum cellulase activity was observed in *P. microspora* and *Phoma* sp., at pH range of 5-10. The enzyme activity increased with increase in temperature with both the isolates. The enzyme was more stable for 60 min when incubated at 100°C and the thermal stability decreased with increase in time. The lipase activity was determined in endophytic fungus *Montagnulaceae* sp. by incubating the culture at different time intervals. The enzyme activity increased up to 16 days and decreased thereafter. The maximum activity of lipase was observed at pH of 11.0. The enzyme activity was lower at pH range of 4.0 to 9.0 and at higher pH 12.0. The lipase activity increased with increase in temperature and the maximum activity was determined

at 100°C. These results suggested that the thermotolerant endophytic fungi can be potential source for different enzyme activities with capacities to tolerate extreme pH and temperature conditions. From these results, it can be concluded that endophytic fungi are novel and important microbial resource for producing bioactive compounds. The endophytic fungi *Diaaporthe* sp. (T1) and *F. tricinctum* (T6) used in this study were able to produce different bioactive compounds which have cytotoxic, antioxidant and immunomodulatory activities. Some of the enzymes such as cellulose and lipase produced by the endophytic fungal isolates showed high tolerance to different pH and temperature which can be used as a source of industrial biocatalysts. Further, studies are required to isolate and purify these bioactive compounds and test their ability towards different biological activities.

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Media Composition

Ingredients	Quantity (Gms/Lit)
Infusion from potato	200 g
Dextrose	20 g
Agar	20 g
Final pH (at 25 ° C)	6.5
Volume made upto 1000 ml with Distilled water	
Sterilized by Autoclaving at 15 lbs pressure (121 °C) for 15 minutes	

Ingredients	Quantity (Gms/Lit)
Infusion from Beef	300 g
Casein acid hydrolysate	17 g
Starch	1 g
Final pH (at 25 °C)	7.3
Volume made upto 1000 ml with distilled water	
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes	

Media	Media
Mueller Hinton Agar	Mueller Hinton Broth containing 20 g/l Agar

Ingredients	Quantity (Gms/Lit)
Mixture of peptic digest of animal tissue & pancreatic digest of casein (1:1)	10 g
Dextrose	20 g
Final pH (at 25 °C)	5.6

Volume made up to 1000 ml with distilled water
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes

1.3 Dulbecco's Modified Eagle Medium (DMEM)

Ingredients	Quantity (Gms/Lit)
Inorganic Salts	
Calcium Chloride	0.2
Ferric Nitrate • 9H ₂ O	0.0001
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Sodium Bicarbonate	3.7
Sodium Chloride	6.4
Sodium Phosphate Monobasic (anhydrous)	0.109
Amino Acids	
L-Arginine • HCl	0.084
Glycine	0.03
L-Histidine • HCl • H ₂ O	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine • HCl	1.46
L-Phenylalanine	0.066
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine • 2Na • 2H ₂ O	0.12037
L-Valine	0.094
Vitamins	
Choline Chloride	0.004
Folic Acid	0.004
<i>myo</i> -Inositol	0.0072
Niacinamide	0.004
D-Pantothenic Acid (hemicalcium)	0.004
Pyridoxine • HCl	0.004
Riboflavin	0.0004
Thiamine • HCl	0.004
Others	
D-Glucose	4.5
Phenol Red • Na	0.0159
Pyruvic Acid • Na	0.11
Additional	
L-Glutamine	0.584

Volume made upto 1000 ml with Distilled Water
Filter Sterillized

1.4 Complete Medium (CM) for growing endophytes

Ingredients	Quantity (Gms/Lit)
Salts stock solution	50 ml
• KCl	10.4 g
• $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	10.4 g
• KH_2PO_4	30.4 g
Vitamins stock solution	1 ml
• Biotin	0.5 g
• Para-amino benzo acid	16 g
• Pyridoxinhydrochloride	20 g
• Nicotinic acid	50 g
Trace elements stock solution	2 ml
• $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1 g
• $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.15 g
• $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.61 g
• $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g
• $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.1 g
Yeast extract	1 g
Casein	1g
Sucrose	10 g
Peptone	2 g
Final pH	6.5

Volume made upto 1000 ml with Distilled Water

1.5 Czapek Dox Agar (CDA)

Ingredients	Quantity (Gms/Lit)
NaNO_3	2.0 g
KH_2PO_4	1.0 g
MgSO_4	0.5 g
KCl	0.5 g
FeSO_4	0.01 g
Sucrose	30 g
Agar	20 g
pH	6.5

Volume made upto 1000 ml with Distilled Water

Sterilized by Autoclaving at 15 lbs pressure (121 °C) for 15 minutes

1.6 Glucose Yeast extract Peptone Agar (GYP)

Ingredients	Quantity (Gms/Lit)
Glucose	1.0 g
Yeast extract	0.1 g
Peptone	0.5 g
Agar	16 g
pH	6.0

Volume made upto 1000 ml with Distilled Water

Sterilized by Autoclaving at 15 lbs pressure (121 °C) for 15 minutes

1.7 Tween 20 Agar

Ingredients	Quantity (Gms/Lit)
Peptone	10 g
NaCl	5 g
CaCl ₂ .2H ₂ O	0.1 g
Agar	15 g
Tween 20 (sterilized)	1 ml

Volume made upto 1000 ml with Distilled Water

Sterilized by Autoclaving at 15 lbs pressure (121 °C) for 15 minutes

1.8 Basal Media

Ingredients	Quantity (Gms/Lit)
Peptone	5 g
MgSO ₄ .7H ₂ O	0.5 g
KCl	0.5 g
KH ₂ PO ₄	2 g
NaNO ₃	0.5 g

Volume made upto 1000 ml with Distilled Water

Sterilized by Autoclaving at 15 lbs pressure (121 °C) for 15 minutes

Appendix II

Buffers and Solutions

1. TBE buffer (10X)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

2. 0.1M Phosphate buffer

Monobasic sodium phosphate, monohydrate (1 M)	61.5 mL
Dibasic sodium phosphate, monohydrate (1 M)	38.5 mL
Dilute to 1 L with distilled water	

3. Phosphate Buffered Saline

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g
Volume made upto 1000 ml	
pH adjusted to 7.4 with HCl	

4) Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol in water	30.0%

5) Ethidium Bromide

0.5µg/mL

6) Loading buffer

Bromophenol blue dye	2.5 mg/ml
Glycerol in 1 x TE (pH 8.9)	50 % (v/v)

7) 0.4% Trypan Blue Solution

0.4 gms Trypan Blue dissolved in 100 ml PBS

8) CTAB (cetyltrimethylammonium bromide)buffer for DNA extraction

2% CTAB	20 gm
20 mM EDTA	40 ml 0.5 M EDTA
100 mM Tris-HCl (pH 8.0)	100 ml 1M Tris-HCl
1.4M NaCl	280 ml 5M NaCl
Volume made upto 1 lit with distilled water	

8) TE buffer 10 ml
 1M Tris HCl pH 8 2 ml
 0.5M EDTA
 Volume made upto 1 lit with distilled water

9) CAPS buffer (100 ml)
 2 mM CAPS 44.2 ml
 10 mM Na₂HPO₄ 0.141 ml
 10 mM Tris-HCl 0.157 ml

10) 0.1M Sodium Acetate (100 ml)

pH	0.1M Acetic acid	0.1M Sodium Acetate
3	98.23 ml	1.77 ml
4	84.7 ml	15.3 ml
5	35.7 ml	64.3 ml

11) 0.1M Potassium Phosphate (Final vol.100 ml using Distilled water)

pH	1M KH ₂ PO ₄	1 KH ₂ PO ₄
6	1.32 ml	8.68 ml
7	6.15 ml	3.85 ml

12) 0.1 M Tris Hcl (100 ml)

2.42 g Tris base + 1.5 ml of 1N HCl in 100 ml distilled water.

13) 0.1 M Glycine – NaOH (100 ml)

Solution (a): Dissolve .750g of Glycine and .585g of Sodium Chloride in water and make up to 100ml

Solution (b): 0.1M Sodium Hydroxide

pH	<i>Solution(a)</i> ml	<i>Solution (b)</i> ml
9.0	8.8	1.2
10.0	6.0	4.0
11.0	5.1	4.9
12.0	4.5	5.5

13) MES buffer 2-(N-morpholino)ethanesulfonic acid (100 ml, pH-5)

25 mM MES	0.082 ml
10 mM Sodium Acetate	0.488 ml

14) Citrate buffer (100 ml, pH-5)

0.1M sodium bicarbonate	35 ml
0.1M sodium carbonate, decahydrate	65 ml

15) Bradford Reagent (5X concentrate)

Coomassie Brilliant Blue G-250	100 mg
Methanol (100 %)	47 ml
Phosphoric Acid (85 %)	100 ml
Volume made upto 200 ml	

NOTE: Coomassie must be dissolved in the methanol FIRST before the other ingredients are added

Appendix III

GenBank details

Diaporthe sp. isolate strain T1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: KX355165.1

FASTA Graphics

Go to:

LOCUS	KX355165	588 bp	DNA	linear	PLN 29-JUN-2016
DEFINITION	Diaporthe sp. T1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.				
ACCESSION	KX355165				
VERSION	KX355165				
KEYWORDS	.				
SOURCE	Diaporthe sp. T1				
ORGANISM	Diaporthe sp. T1 Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Diaporthales; Diaporthaceae; Diaporthe.				
REFERENCE	1 (bases 1 to 588)				
AUTHORS	Vasundhara,M., Kumar,A. and Baranwal,M.				
TITLE	Isolation and characterization of cytochalasin producing endophytic fungus from Himalayan yew				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 588)				
AUTHORS	Vasundhara,M., Kumar,A. and Baranwal,M.				
TITLE	Direct Submission				
JOURNAL	Submitted (03-JUN-2016) Department of Biotechnology, Thapar University, Bhadson Road, Patiala, Punjab 147004, India				
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##				
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/ note="contains 18S ribosomal RNA, internal transcribed
spacer 1, 5.8S ribosomal RNA, internal transcribed
spacer 2, and 28S ribosomal RNA"
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  241 ctggcatcga tgaagaacgc agcgaaatgc gataagtaat gtgaattgca gaattcagtg
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  481 tctggaaggc cctggcggtg ccctgccgtt aaacccccaa cttctgaaaa tttgacctcg
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Mycetinis scorodonius isolate strain T2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: KY411698.1

FASTA Graphics

Go to:

LOCUS KY411698 822 bp DNA linear PLN 08-JAN-2017

DEFINITION Mycetinis scorodonius isolate strain T2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION KY411698

VERSION KY411698.1

KEYWORDS .

SOURCE Mycetinis scorodonius

ORGANISM Mycetinis scorodonius
Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Omphalotaceae; Mycetinis.

REFERENCE 1 (bases 1 to 822)

AUTHORS Vasundhara,M. and Kumar,A.

TITLE Isolation and characterization of endophytic fungus Marasmius sp. from Taxus baccata from Himalayan region

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 822)

AUTHORS Vasundhara,M. and Kumar,A.

TITLE Direct Submission

JOURNAL Submitted (02-JAN-2017) Biotechnology, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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    transcribed spacer 2, and large subunit ribosomal RNA"
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Microdiplodia sp. strain T5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: KY421921.1

FASTA Graphics

Go to:

LOCUS KY421921 611 bp DNA linear PLN 11-JAN-2017

DEFINITION Microdiplodia sp. strain T5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION KY421921

VERSION KY421921.1

KEYWORDS .

SOURCE Microdiplodia sp.

ORGANISM Microdiplodia sp.
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales; Botryosphaeriaceae; Microdiplodia.

REFERENCE 1 (bases 1 to 611)

AUTHORS Vasundhara,M. and Kumar,A.

TITLE Isolation and characterization of endophytic fungus Microdiplodia sp. from Taxus baccata from Himalayan region of India

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 611)

AUTHORS Vasundhara,M. and Kumar,A.

TITLE Direct Submission

JOURNAL Submitted (02-JAN-2017) Biotechnology, Thapar University, Bhadson Road, Patiala, Punjab 147004, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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    transcribed spacer 1, 5.8S ribosomal RNA, internal
    transcribed spacer 2, and large subunit ribosomal RNA"

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Fusarium tricinctum isolate T6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KT779291.1

FASTA Graphics

Go to:

LOCUS KT779291 561 bp DNA linear PLN 06-JUN-2016

DEFINITION Fusarium tricinctum isolate T6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION KT779291

VERSION KT779291.1

KEYWORDS .

SOURCE Fusarium tricinctum

ORGANISM Fusarium tricinctum
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
Fusarium; Fusarium tricinctum species complex.

REFERENCE 1 (bases 1 to 561)

AUTHORS Vasundhara,M., Baranwal,M. and Kumar,A.

TITLE , An Endophytic Fungus Exhibits Cell Growth Inhibition and Antioxidant Activity

JOURNAL Indian J. Microbiol. 56 (4), 433-438 (2016)

PUBMED 27784939

REFERENCE 2 (bases 1 to 561)

AUTHORS Vasundhara,M., Baranwal,M. and Kumar,A.

TITLE Direct Submission

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Isolation and characterization of trichalasin-producing endophytic fungus from *Taxus baccata*

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Abstract An endophytic fungus (strain T1) isolated from *Taxus baccata* was studied for the production of metabolites with anticancer and antioxidant activities. This fungus was identified as *Diaporthe* sp. based on rDNA-internal transcribed spacer (ITS) sequence analysis. The crude extract showed cytotoxic activity against MCF-7 and HeLa cancer cell lines, with IC₅₀ (concentration inhibiting 50% of growth rate) values of 1058 ± 44 and 1257 ± 80 µg ml⁻¹, respectively. The scavenging activity of fungal extract increased significantly with increasing concentration [IC₅₀ (concentration required to scavenge 50% of free radicals) 482 ± 9 µg ml⁻¹]. Ultra-high-performance liquid chromatography-quadrupole-time of flight analysis revealed the presence of three trichalasins (trichalasin E, F and H) in the crude extract of T1 which are known to have antitumour and antioxidant activities. These results suggest that *Diaporthe* sp. has the potential to be used for therapeutic purposes because of its anti-proliferative and antioxidant potential and also for the production of cytochalasins.

Keywords Endophytic fungi · *Diaporthe* · Cytochalasins · Trichalasins · Antioxidant activity

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Introduction

Endophytes are ubiquitous organisms which reside in the tissues of plants without causing apparent symptoms of disease (Bacon and White 2000). The majority of endophytes are fungi (Strobel and Daisy 2003), and they are present in the intercellular spaces of various plant parts, such as the leaf and root (Corrêa et al. 2014). Endophytes reduce damage to their host plants by producing many secondary metabolites (Cabezas et al. 2012). They are also able to inhibit infection and proliferation of pathogens in the host plant directly or indirectly by inducing resistance responses intrinsic to the host defence (Eaton et al. 2011). Currently, endophytic fungal research is focused on the ability of these fungi to produce and accumulate secondary metabolites. Several of these compounds have biological activities of interest for application in environmental, agriculture, pharmaceutical and healthcare and food industries (Suryanarayanan et al. 2009; Kharwar et al. 2011; Deshmukh et al. 2015).

Diverse classes of chemical substances, such as steroids, xanthenes, phenols, isocoumarines, perylene derivatives, quinones, furanones, terpenoids, depsipeptides and cytochalasins, have been isolated from endophytic fungi (Schulz and Boyle 2005). Endophytes produce a significantly higher number of novel chemical structures than soil fungi, indicating that endophytes are a novel source of bioactive secondary metabolites (Nisa et al. 2015). In this context, the secondary metabolites produced by endophytes associated with medicinal plants are important as they could be exploited for the treatment of many diseases (Tejesvi et al. 2007). Several studies have shown that endophytes produce secondary metabolites of their host plants, thus raising the prospect of using them as alternative sources of these metabolites (Priti et al. 2009).

Endophytic fungi of the genus *Diaporthe* have been isolated from a wide variety of plant hosts from both temperate and

tropical regions (Dos Santos et al. 2016). *Diaporthe* is one of the most abundant genera of endophytic fungi and has been reported to produce various compounds of biotechnological interest (Silva et al. 2005; Pornpakakul et al. 2007; Rukachaisirikul et al. 2008). Cytochalasins are a group of cytotoxic fungal metabolites showing a wide range of biological activities, such as anticancer, antimicrobial, antiparasitic, phytotoxic activities, among others (Scherlach et al. 2010; Chen et al. 2014). These metabolites have been isolated from many fungal species, including *Helminthosporium* sp., *Phoma* sp., *Xylaria* sp., *Hypoxylon* sp. and *Rhinochadiella* sp. (Scherlach et al. 2010).

In the investigation reported here, we isolated an endophytic fungus from the bark of the *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan Yew) and subsequently identified it as *Diaporthe* sp. strain T1 based on its internal transcribed spacer (ITS) regions of rDNA. The biological activities, such as anticancer and antioxidant activities, of ethyl acetate extracts of its culture filtrate were studied using various bioassays. These extracts were also analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) to identify some key compounds belonging to the cytochalasin group of fungal metabolites which could be responsible for these activities.

Material and methods

Isolation and identification of endophytic fungus strain T1

Endophytic fungus T1 was isolated from the bark samples of *T. baccata* L. subsp. *wallichiana* (Zucc.) Pilger collected from Bhadrewah (Doda district, India) according to the method described by Garyali et al. (2013). The fungal culture was maintained on potato dextrose agar (HiMedia Laboratories Ltd. Mumbai, India) medium. Molecular characterization was performed by ITS sequence analysis. Specifically, mycelia were harvested from actively growing colonies and ground in liquid nitrogen. Genomic DNA was extracted from the finely ground fungal material by the CTAB method (Zhang et al. 2008). The quality and quantity of DNA was checked with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and samples were stored at -20°C until use. The ITS region of nuclear ribosomal DNA was amplified with the universal primers ITS1 and ITS4 (White et al. 1990) in a thermal cycler (Applied Biosystems, Foster City, CA). The thermal cycling conditions applied for the ITS region consisted of an initial denaturation for 5 min at 94°C followed by 34 cycles of 1 min at 94°C , 1 min at 50°C and 1.5 min at 72°C and a final extension of 7 min at 72°C . PCR products were purified using QIAquick spin columns (Qiagen, Hilden, Germany) following the manufacturer's

instructions and then sequenced. The ITS sequence obtained from this isolate has been deposited in the GenBank under accession number KX355165.

The ITS sequence of T1 was compared to those available in the GenBank database using the BLASTN algorithm. Alignment of the sequences was constructed using MAFFT version 7.0 (Katoh and Standley 2013) and edited with BioEdit version 5.0.6 (Hall 1999). Phylogenetic analysis on the resulting alignment was performed using Bayesian Inference. A Bayesian analysis was implemented in MrBayes v.3.2.2 with two parallel runs, each consisting of four incrementally heated Monte Carlo Markov Chains. The analysis was run using Metropolis-coupled a Markov Chain Monte Carlo search algorithm over 1,000,000 generations, and the convergence of Bayesian analysis was observed by examination of the standard deviation of split frequencies of <0.01 . Trees were sampled every 100th generations resulting in total of 10,000 trees. The first 2500 trees, representing the burn-in phase of the analysis, were discarded, and the remaining 7500 trees were used to calculate posterior probabilities from the 50% majority rule consensus trees.

Fungal extract preparation

Mycelial discs (diameter 5.0 mm) of actively growing culture (7 days old) were inoculated into potato dextrose broth (500 ml) and incubated at $25 \pm 2^{\circ}\text{C}$ for 21 days in the dark as a static culture. The cultures were harvested by filtration through four layers of cheesecloth to remove the mycelia, and the mycelial biomass thus obtained was then dried overnight ($35\text{--}40^{\circ}\text{C}$) and extracted with ethyl acetate for 12 h. The culture broth was also extracted three times with an equal volume of ethyl acetate; the ethyl acetate fractions were then pooled and dried in vacuo at 35°C . Part of the residue was dissolved in dimethyl sulfoxide and used for the bioassays. The other part of the residue was dissolved in methanol and partitioned three times with an equal volume of dichloromethane; the dichloromethane fractions were pooled and dried in vacuo. The residue was used for the LC–two-dimension-MS [ultra-high-performance LC–quadrupole-time of flight analysis (UHPLC-QTOF-MS/MS)] to analyse the bioactive compounds.

Cell growth inhibition assay

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were procured from National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO) containing 10% (v/v) foetal bovine serum (Gibco, Thermo Fisher Scientific), 100 IU ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, and 2.5 $\mu\text{g ml}^{-1}$ amphotericin, in a humidified incubator with 5% CO_2 at 37°C . A well-known 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-

bromide (MTT) assay was carried out to assess the effect of fungal extract on the growth of the cancer cell lines. In brief, approximately 2×10^4 cells per well were seeded in 96 well culture plate and incubated overnight. After 16 h, varying concentrations ($250\text{--}1500 \mu\text{g ml}^{-1}$) of fungal extracts were

added to the wells. After 72 h of incubation, the MTT assay was carried out as described by Denizot and Lang (1986). Paclitaxel was used as the positive control at the concentration of $20 \mu\text{g ml}^{-1}$. The growth inhibition rate was calculated using the formula (OD is optical density):

$$\text{Inhibition rate} = (\text{Mean OD of control well} - \text{Mean OD of treated cell} / \text{Mean OD of untreated cell}) \times 100$$

The IC_{50} in this assay was defined as the concentration of compounds that resulted in 50% inhibition of growth rate.

Antioxidant assay

The antioxidant potential of the fungal extract was studied by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Fungal extract (50- μl samples) of different concentrations were mixed with 150 μl of DPPH (100 μM) in methanol in the wells of a 96-well microtiter plate. Ascorbic acid ($100 \mu\text{g ml}^{-1}$; 50 μl) was used as the positive control. The plate was incubated in the dark (45 min), and absorbance of the reaction mixture was measured at 517 nm using an enzyme-linked immunosorbent assay reader (Infinite microplate reader; Tecan Austria GmbH, Grödig, Austria). The percentage inhibition of DPPH radical by the fungal extract was expressed as the inhibition concentration (IC_{50}) and was calculated using the formula:

$$\text{DPPH scavenging activity (\%)} = (A_{\text{Control}} - A_{\text{Sample}} / A_{\text{Control}}) \times 100$$

Identification of bioactive compounds by UHPLC-QTOF analysis

For the UHPLC-QTOF-MS/MS analysis, the residue was first dissolved in methanol. Chromatographic separation was carried out using the Agilent 1200 UHPLC system equipped with C18 column (Agilent Technologies, Santa Clara, CA). Samples (5 μl) were injected into the column, and elution was carried out in a gradient mode with acidified water (0.2% v/v formic acid) to 90% methanol over a period of 40 min at a flow rate of 0.3 ml min^{-1} . High-resolution mass spectroscopic detection was performed using Bruker Impact QTOF mass spectrometer (Bruker Corp., Billerica, MA, operating in Top5 data-dependent mode from 100–1500 m/z with electron spray ionization, and both positive and negative ions were detected. Smart Formula 3D™ and Fragment Explorer part of Data Analysis 4.2 (Bruker Corp.) were used to generate compound formulae.

Statistical analysis

All the experiments were performed in triplicate. The results were expressed as mean $\pm$ standard deviation. The data were

analysed by analysis of variance, and the means were compared by Tukey's test at $p < 0.05$.

Results

Isolation and identification of fungal strain T1

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 was 588 bp. BLAST analysis revealed 98% similarity (query coverage of 100%) with *Diaporthe* sp. Bayesian analysis of ITS sequences of different species of *Diaporthe* yielded a consensus tree (Fig. 1), and the present isolate was clustered with *Diaporthe* sp. (KC357558 and KC357559).

Cytotoxic effect in cancer cell lines

The fungal extract showed a cytotoxic effect against both the human breast cancer cell line (MCF-7) and the human cervical cancer cell line (HeLa). The cytotoxic effect become significantly pronounced at higher concentrations of the fungal extract (Fig. 2). The IC_{50} value of the extract was 1058 ± 44 and $1257 \pm 80 \mu\text{g ml}^{-1}$ for the MCF-7 and HeLa cell lines, respectively. Paclitaxel ($20 \mu\text{g ml}^{-1}$), an anticancer drug, was used as a positive control. It inhibited the growth of the MCF-7 and HeLa cell lines by 79 ± 6 and $97 \pm 2\%$, respectively.

Antioxidant activity

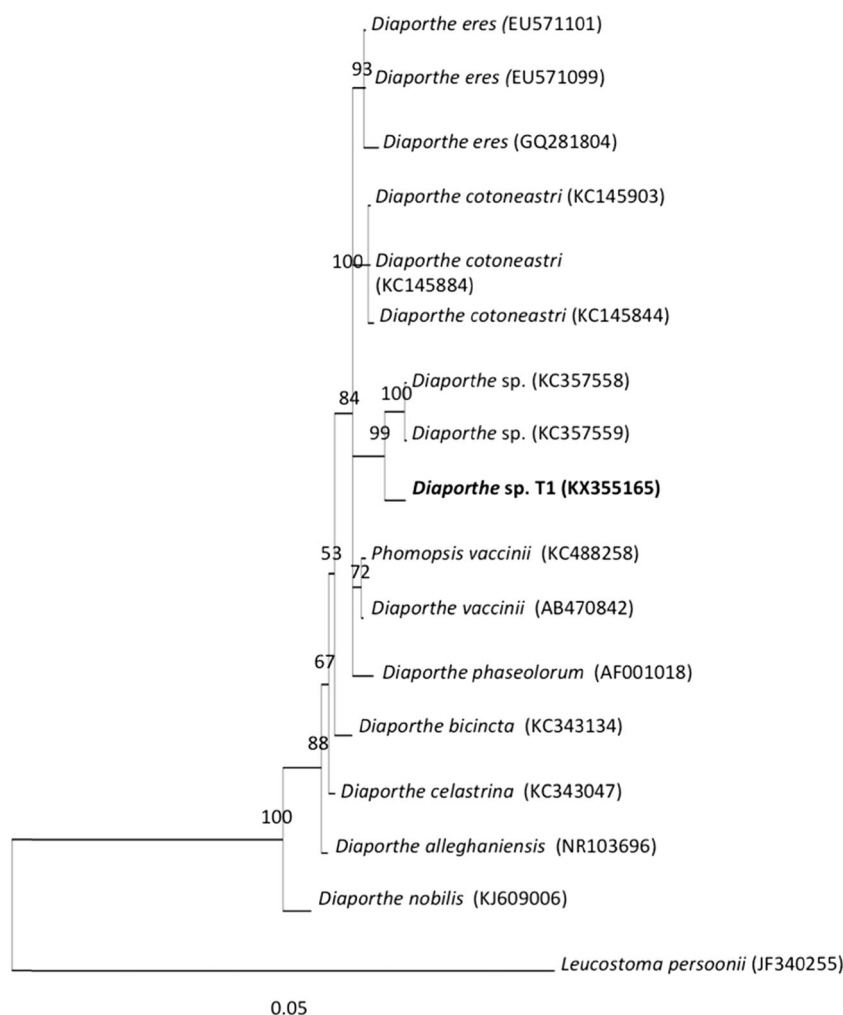
Free radical scavenging activity was performed to determine the antioxidant activity of *Diaporthe* sp. The scavenging activity significantly increased with increasing concentration of fungal extract (Fig. 3). The IC_{50} value (concentration of sample required to scavenge 50% of free radicals) of the crude extract was $250 \pm 3.5 \mu\text{g ml}^{-1}$. Ascorbic acid, the positive control, showed $84 \pm 3.5\%$ antioxidant activity.

Identification of compounds by UHPLQTOF analysis

The UHPLC-QTOF-MS/MS analysis of the crude extract of *Diaporthe* sp. (T1) showed a signal (m/z) corresponding to three trichalasin. Figure 4 shows the mass data of these three compounds that eluted from the column at different times.

Fig. 1 Bayesian tree showing the relationships between the internal transcribed spacer (ITS) sequence of *Diaporthe* sp. (shown in **bold**) and those of related species retrieved from GenBank.

Numbers at nodes Posterior probability percentages (>50%) of the Bayesian analysis



Peaks at m/z ratios of 402.1753, 420.1391 and 472.1511 were observed and identified as Trichalasin H, Trichalasin F) and Trichalasin E, respectively, based on the reported values of these compounds (Electronic Supplementary Material Table).

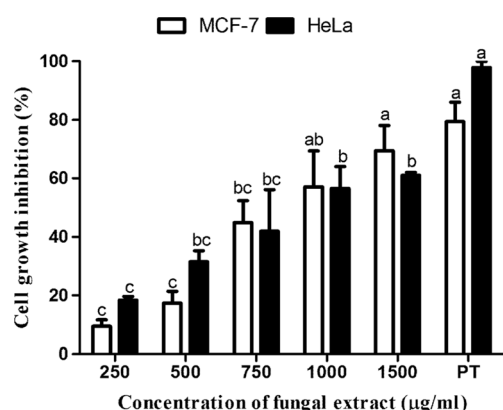


Fig. 2 Cytotoxic effect of *Diaporthe* sp. extract against human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines. Bars Mean values, error bars standard deviation (SD). Bars with the same lowercase letter within the same cell type are not significantly different at $p < 0.05$. PT Paclitaxel ($20 \mu\text{g ml}^{-1}$) used as the positive control

Discussion

The anamorphic form (the asexual reproductive morphological stage) of *Diaporthe* is the genus *Phomopsis*. Members of *Diaporthe/Phomopsis* are either plant pathogens or endophytic species (van Niekerk et al. 2005; Dos

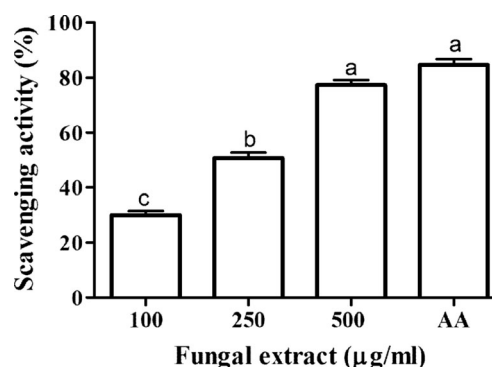
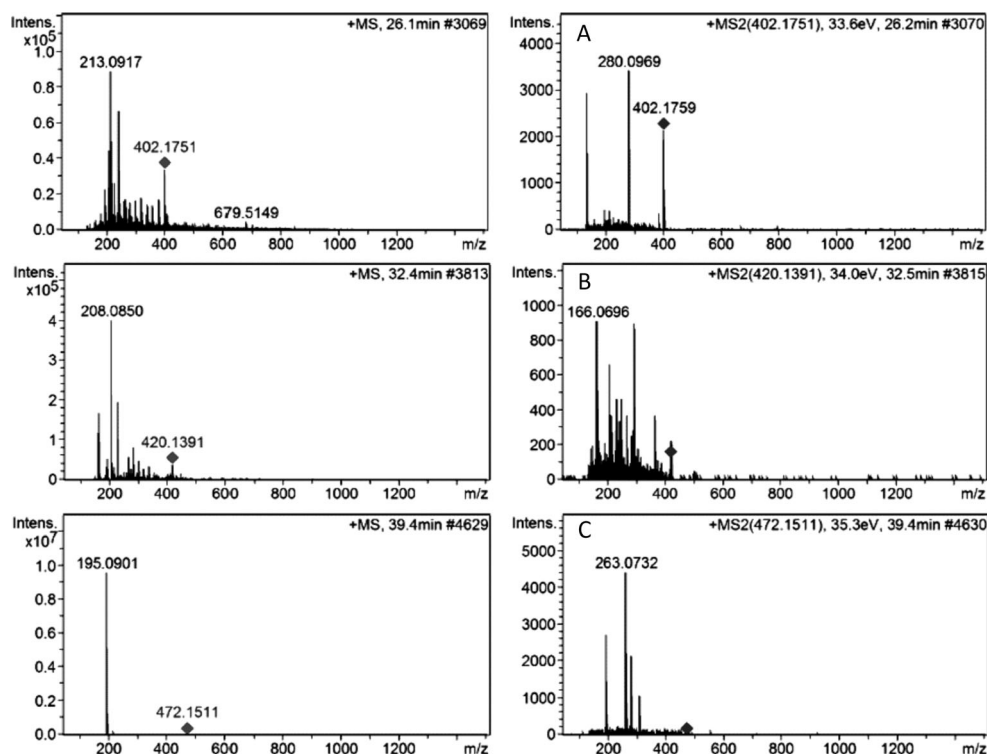


Fig. 3 Antioxidant effect of *Diaporthe* sp. extracts based on free radical scavenging activity. Bars Mean values, error bars standard deviation (SD). Bars with the same lowercase letter are not significantly different at $p < 0.05$. AA Ascorbic acid ($100 \mu\text{g ml}^{-1}$) used as the positive control

Fig. 4 The tandem mass spectrometry profile of selected ions in the extracts of *Diaporthe* sp. T1 corresponding to three trichalasin: **a** Trichalasin H (m/z 402.1753), **b** Trichalasin F (m/z 420.1391), **c** trichalasin E (m/z 472.1511)



Santos et al. 2016) and are associated with dicotyledonous and monocotyledonous plants of tropical and temperate regions (Eriksson and Vue 1998; Guo et al. 2000). The *Diaporthe/Phomopsis* complex occurs as endophytes with medicinal plants such as *Taxus chinensis* (Liu et al. 2009), *Taxus globosa* (Soca-Chafre et al. 2011) and some medicinal shrubs of India (Naik et al. 2008). Here, we report for the first time an endophytic association of a *Diaporthe* sp. with *Taxus baccata* subsp. *wallichiana*.

Medicinal plants are a repository of endophytic fungi that are able to produce bioactive compounds of biotechnological and medicinal interest (Vieira et al. 2012). We recovered a *Diaporthe* species from *T. baccata* as an endophyte that produced metabolites displaying cytotoxic activity against MCF-7 and HeLa tumour cells. Augusta et al. (2006) isolated (–)-epicytoskyrin from a *Diaporthe* species isolated from the tea plant and found that this compound exhibited moderate cytotoxic activity against KB cells, a subline of HeLa cells, with an IC_{50} value of $0.5 \mu\text{g ml}^{-1}$. Various researchers have reported the cytotoxic activity of the extracts of *Diaporthe* species against different cell lines (Lin et al. 2005; Carvalho et al. 2012; Casella et al. 2013). In our study, the culture filtrate of *Diaporthe* sp. showed antioxidant activity when assayed using the DPPH method. The DPPH assay has been extensively used as a reliable method to measure antioxidant activity of pure compounds (Koleva et al. 2002). Our results suggest that metabolites of *Diaporthe* sp. could serve as potential agents in scavenging free radicals. Antioxidant activity of the

Diaporthe/Phomopsis complex has been reported by Nath et al. (2012) and Ascêncio et al. (2014).

Cytochalasins are a known class of mould metabolites that exhibit a wide range of distinctive biological activities. They are implicated as phytotoxins or virulence factors and exhibit antimicrobial and cytotoxic activities (Wagenaar et al. 2000). Cytochalasins are produced by a variety of fungal genera, including *Phomopsis*, *Aspergillus*, *Penicillium* and *Chaetomium* (Yan et al. 2016). Structurally, cytochalasins are comprised of a highly substituted isoindolone ring with a benzyl group at the C-3 position and fused to an 11- to 14-member macrocyclic ring. Wagenaar et al. (2000) reported the isolation of four cytotoxic cytochalasins from a culture of the endophytic fungus *Rhinocladiella* sp., and Yan et al. (2016) identified four cytochalasins (phomopchalsin A, B, C and J) from the endophytic fungus *Phomopsis* sp. isolated from the stem of *Isodon eriocalyx* var. *laxiflora*. Pornpakakul et al. (2007) isolated diaphorthichalsin, a cytochrome P450 3A4 inhibitor, from an endophyte *Diaporthe* species isolated from *Croton sublyratus*. Chen et al. (2014) reported the production of three new cytochalasins (trichalasin E, F and H) along with four analogues from the endophytic fungus *Trichoderma gamsii* residing in the Chinese medicinal plant *Panax notoginseng*. In the present study, we identified three cytochalasins (trichalasin E, F and H) from *Diaporthe* sp. and confirmed their identity based on their m/z values. The biological activities exhibited by this fungus may be correlated with the production of these bioactive compounds (trichalasins). This is the first report of the

occurrence of trichalasin (E, F and H) from an endophyte *Diaporthe* sp. isolated from *T. baccata* subsp. *wallichiana*.

Our results show that the culture extracts of *Diaporthe* sp. exhibited strong cell growth inhibition and antioxidant properties, indicating the ability of this fungus to produce bioactive compounds that may be useful as sources of novel drugs. Our work also confirms the production of trichalasin, which provides an insight into understanding some basis of therapeutic properties of fungal endophyte *Diaporthe* sp. Further studies on the isolation and characterization of these trichalasin and their in vivo assays will be required to assess the biological function of these compounds.

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Molecular Approaches to Screen Bioactive Compounds from Endophytic Fungi

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Endophytic fungi are capable of producing plant associated metabolites and their analogs with therapeutic value. In order to identify the potential endophytic isolates producing bioactive compounds, one need to screen all isolated endophytes, which may run into hundreds. Isolation of endophytic fungi is relatively a simple process; but screening of the isolated fungi for required metabolite production is a cumbersome process. Endophytic fungi producing plant associated metabolites may contain genes involved in the entire biosynthetic pathway(s). Therefore, ascertaining the presence of key enzymes of a particular biosynthetic pathway could serve as a molecular marker for screening of these endophytes to produce that metabolite. In absence of entire biosynthetic pathways in endophytic fungi, plant genes associated with that metabolic pathway could serve as markers. This review focuses on the impact of molecular approaches to screen the endophytic fungi for the production of bioactive compounds. An attempt has been made on screening of anticancer compounds like taxol (paclitaxel), podophyllotoxin, and camptothecin using molecular markers. The advantages of molecular approaches over conventional methods to screen endophytic fungi and also identification of endophytic fungi are discussed.

Keywords: endophytic fungi, secondary metabolites, bioactive compounds, paclitaxel, podophyllotoxin, camptothecin, molecular markers

INTRODUCTION

Endophytic fungi live in the tissues of plants without causing any symptoms of disease (Bacon and White, 2000) and the majority of these endophytes are fungi (Strobel and Daisy, 2003). Endophytic fungi are associated with the host plants, protect the host from pathogens, and at times may become opportunistic pathogens. Majority of endophytic fungi possess biosynthetic capabilities more than the associated host because of their long co-evolution and genetic recombination. Endophytic fungi have been considered as a source for novel natural bioactive compounds with potential application in medicine, agriculture, and food industry (Strobel et al., 1993; Suryanarayanan et al., 2009; Kharwar et al., 2011). Many endophytic fungi are capable of synthesizing various bioactive compounds that are used as therapeutic agents against numerous diseases (Suryanarayanan et al., 2009; Deshmukh et al., 2015).

The production of novel metabolites by fungal endophytes raises questions about the acquisition of the capacity to produce such complex metabolites by these organisms. The plant-endophyte co-evolution hypothesis suggests that it might be possible for endophytic fungi to assist the plant

in chemical defense by producing these secondary metabolites (Ji et al., 2009). The possibility of acquisition of the capacity to produce these metabolites by endophytes from the host is supported by the fact that these endophytes harbor similar biosynthetic pathway and genes encoding enzymes catalyzing various steps as that of the host (Chandra, 2012). There are a few studies showing that the fungi isolated from the particular host produce active principle produced by that host (Stierle et al., 1993; Puri et al., 2005, 2006; Kusari et al., 2009, 2011; Nadeem et al., 2012; Garyali et al., 2013). The endophytic fungi producing such compounds have also shown the presence and expression of the similar homologous genes involved the biosynthesis of respective compounds in their host (Jennewein et al., 2001).

Although, each plant species is known to harbor many endophytic fungi, only a very minor fraction of them are able to produce important metabolites. This fraction has also been reported to belong to different taxa and is not confined to a particular taxonomic group. Therefore, in order to identify the potential isolates capable of producing a particular compound one needs to screen all isolated endophytes that usually runs into hundreds. Although isolation of endophytic fungi is relatively a simple process, screening of the isolated fungi for required metabolites is, however, a cumbersome process (Zhou et al., 2007; Xiong et al., 2013). Under such circumstances there is a possibility of missing some strains that are capable of producing these metabolites. Various procedures used for screening of this diverse group of organisms for the production of bioactive metabolites have been reviewed.

The present work provides an overview on the screening of endophytic fungi for production of the bioactive metabolites. The impacts of molecular approaches to screen the bioactive compounds from endophytic fungi are elaborated by including some of the anticancer compounds. The merits of molecular approaches over conventional methods to screen endophytic fungi, identify those using molecular tools and future perspectives are also discussed.

METHODS OF SCREENING ENDOPHYTIC FUNGI

Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthenes, and others compounds (Tan and Zou, 2001). These bioactive metabolites have pharmacological activity with wide-ranging applications such as antibacterials, antifungal, antiviral, immunomodulators, antiparasitics, antioxidants, and anticancer agents (Wang et al., 2011; Zhao et al., 2011; Deshmukh et al., 2015; Vasundhara et al., 2016). Traditionally, various tools have been used for the screening of endophytic fungi starting from the testing of biological activities through bioassays leading to the purification, identification, and characterization of the bioactive molecules. Each of the isolated endophytic fungus is required to be cultured and then extracted with different organic solvents for the isolation of the metabolites. All the extracts are then

processed through various stages such as testing for the biological activity using various activity specific bioassays, purification of molecules responsible for the tested biological activity (Stierle et al., 1993; Zhou et al., 2007; Bedair and Sumner, 2008; Aly et al., 2010; Garyali et al., 2013; Roopa et al., 2015). These bioactive compounds can be identified at the molecular levels by using various spectroscopic techniques coupled with precision chromatographic equipment. Chemical structure and related spectroscopic data regarding several secondary metabolites including bioactive compounds is available in various databases such as Human Metabolome database (HMDB) (Wishart et al., 2009), the METLIN database (Smith et al., 2005), and the Madison Metabolomics Consortium Database (MMDB) (Cui et al., 2008). These compounds can be identified by comparing their spectroscopic data with the available spectroscopic data in databases. Although, data and structure of large number of compounds is stored in these databases, yet for many unreported compounds, the data is not available in these databases and are identified using various spectroscopic techniques such as molecular ion mass spectrometry and fragmentation pattern (Kind and Fiehn, 2007; Böcker et al., 2009). Recently, tandem mass spectrometry coupled with precision liquid chromatography systems are also used to generate such data from relatively less purified extracts (Sawada and Hirai, 2013). Further, data generated using nuclear magnetic resonance and infra-red spectroscopies also provide vital information about the structure of the unknown compounds, which help in elucidating the molecular structure of these compounds (Castro et al., 2010; van der Hooft et al., 2011). Although, such techniques serve as powerful tools for the identification of molecules in the extract and also for the characterization of new molecules, the screening of large number of isolated endophytic fungi through these processes is laborious and there is always a possibility of missing isolates with a capacity to produce novel metabolites. Thus, there is a need for developing an efficient procedure for the screening of large number of isolated endophytic fungi to identify the strains capable of producing specific novel pharmaceutically important compounds.

GENOME MINING

The quest for the discovery of novel bioactive compounds has opened a new chapter with the availability of enormous genetic data. This information has been explored by mining the whole-genome sequence to identify biosynthetic pathways for undetected metabolites. To discover the new natural products and gene clusters for known metabolites, this information has been “fished out” of DNA libraries (Van and Shen, 2006). Genomes of filamentous fungi reveal that they contain far more gene clusters for secondary metabolite biosynthesis than estimated from the previously identified metabolites. These gene clusters encode enzymes for different classes of secondary metabolites such as non-ribosomal peptide synthetases, terpene synthases and polyketide synthases, known as “signature” genes/enzymes. These genes are presumed to be the founders of secondary metabolic gene clusters. They

also contain genes for tailoring enzymes which modify the skeleton of secondary metabolites (For e.g., oxidoreductases, acyltransferases, glycosyltransferases, and methyltransferases) (Osborn, 2010). Mining of genomes of *Aspergillus* spp., revealed the existence of about 40 cryptic biosynthetic gene clusters for secondary metabolites per genome. It has been reported that *A. nidulans* is capable of generating 32 polyketides, 14 non-ribosomal peptides and two indole alkaloids (Brakhage et al., 2008; Rank et al., 2010). The secondary metabolic gene clusters are silent under standard laboratory conditions in filamentous fungi, due to which no product can be formed. Brakhage and Schroeckh (2011) reviewed the strategies to activate silent gene clusters of fungal secondary metabolites. They showed that the majority of successful approaches to activate the gene clusters are based on the generation of gene “knock outs,” over expression of transcriptional factors, promoter exchange and other pleiotropic regulators. Other strategies such as epigenetics and simulation of the natural habitat of the same ecosystem will promote the activation of silent gene clusters and the production of novel metabolites in *A. nidulans*. They suggested that the simulation strategies play an important role to discover new bioactive compounds. Although the secondary metabolic pathways of many compounds are not well understood, the advancements in the areas of functional genomics and metabolomics, many genes of biosynthetic pathways of various secondary metabolites have been identified and characterized (Wankhede et al., 2013; Lau and Sattely, 2015).

Manipulation of the synthesis of bioactive compounds by endophytic fungi by genome mining will increase the yield and new derivatives with possible superior qualities. For the metabolites where the biosynthetic pathways are partly or fully known in one or more plant taxa, application of polymerase chain reaction help in elucidating the biosynthetic genes in the endophyte producing the same compound (Van and Shen, 2006; Kusari and Spiteller, 2011).

SCREENING OF ENDOPHYTIC FUNGI USING MOLECULAR MARKERS

Each step in a biosynthetic pathway is catalyzed by specific enzyme encoded by respective gene. Thus, for an organism to attain the capacity to produce a particular metabolite, it is necessary to acquire all the genes for encoding various enzymes catalyzing the production of that metabolite. Therefore, ascertaining the presence of key genes encoding important enzymes of that particular biosynthetic pathway could serve as a marker for the potential of these endophytes to produce those metabolites (Kusari and Spiteller, 2011). Various enzymes of biosynthetic pathway of paclitaxel (Taxol) and related taxanes are well characterized and genes encoding these enzymes are cloned (Kusari et al., 2013). It has been reported that fungi showing amplification of DNA fragments specific to genes involved in taxol biosynthesis namely, taxa-4(5),11(12)-diene synthase (*ts*), debenzoyltaxane-2'- α -O-benzoyl transferase (*dbat*) and the gene encoding final step in taxol biosynthetic pathway i.e., baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase (*bapt*) were

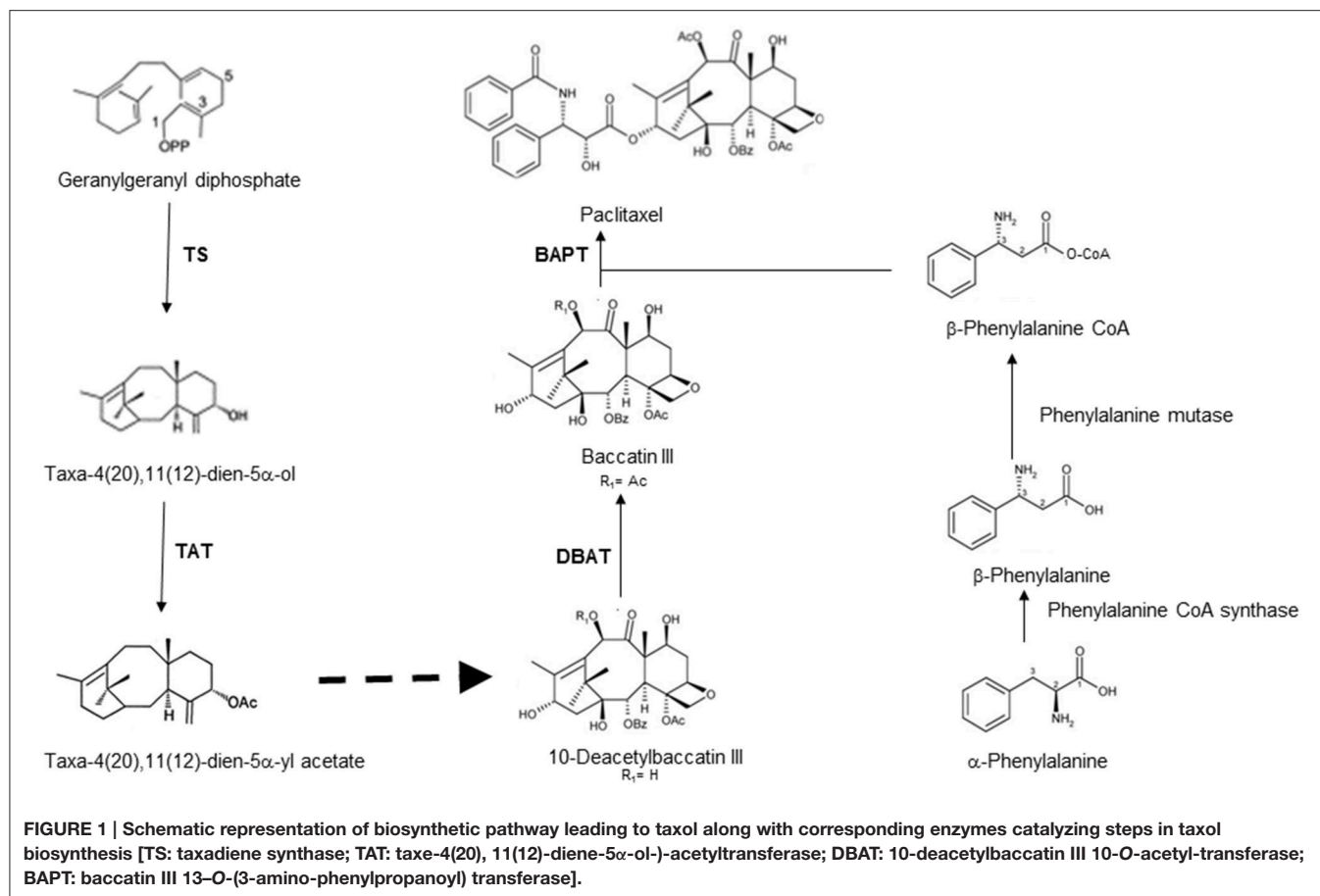
able to produce taxol (Zhou et al., 2007; Zhang et al., 2008; Xiong et al., 2013). However, Garyali et al. (2013) reported that the fungal strains showing the amplification of only *ts* and *dbat* were found to be negative for taxol production. Hence, it is essential to select the appropriate genes/enzymes as markers for screening of endophytic fungi for production of bioactive compounds. Use of such initial screening is likely to reduce the time and improve the efficiency of screening. With less screening time and efforts, one may be able to quickly identify the important isolate from huge diversity of isolated fungal endophytes.

SCREENING FOR BIOACTIVE COMPOUNDS

Paclitaxel

Paclitaxel (taxol) is a diterpenoid and extensively used against a range of cancer types. This compound is the world's first anticancer drug originally isolated from *Taxus brevifolia* (Pacific yew), and known to be present in all *Taxus* species (Strobel et al., 1996). Although, a complete chemical synthesis of taxol has been reported (Holton et al., 1994; Nicolaou et al., 1994), yet the method is highly uneconomical. At present, most taxol is prepared by semi-synthesis from baccatin III or 10-deacetyl baccatin, precursors of taxol, and these compounds also are isolated from yew trees (Collin, 2001). With all these efforts, the demand for the taxol far exceeds the supply. This gap in the demand and supply of taxol has sparked the efforts for the search of alternate sources for taxol. A ray of hope in this direction came with the discovery of endophytic fungi capable of producing taxol, which was isolated from the *Taxus* species (Stierle et al., 1993). Subsequently many workers reported the isolation of huge number of endophytic fungi from various plants and screened this large number of fungi to identify a few capable of producing taxol from yews. These endophytic fungi mostly belonging to ascomycetes and imperfect fungi that includes the genera *Pestalotia*, *Pestalotiopsis*, *Alternaria*, *Seimatoantlerium*, *Sporormia*, *Fusarium*, *Trichothecium*, *Tubercularia*, *Pithomyces*, *Monochaetia*, *Penicillium*, and *Truncatella* amongst others (Flores-Bustamante et al., 2010). Biosynthetic pathway of paclitaxel requires about 19 enzymatic steps with diterpenoid precursor geranylgeranyl diphosphate (GGPP) in yew trees (Hezari and Croteau, 1997). The first step involves the cyclization of GGPP to taxa-4(5),11(12)-diene by taxadiene synthase. The biosynthetic pathway of paclitaxel is represented in **Figure 1**.

The molecular basis of taxol biosynthesis in endophytic fungi is still not known. The screening of endophytic fungi for the production of paclitaxel using some of the biosynthetic genes as molecular markers are reported in **Table 1**. The nucleotide sequence of these genes and amino acid sequence of encoded enzymes of endophytic fungi showed high homology with related genes of *Taxus* species (**Figure 2**). The PCR amplification and cloning of genes of taxol biosynthetic pathway from different taxol producing endophytic fungi facilitated the potential alternative and sustainable source of taxol (Kusari et al.,

**TABLE 1 |** Taxol synthesizing genes reported from endophytic fungi.

Fungus	Host	Accession No.	References
TAXADIENE SYNTHASE (TS)			
<i>Mucor rouxianus</i>	<i>Taxus chinensis</i>	–	Zhou et al., 2007; Miao et al., 2009
<i>Fusarium solani</i>	<i>T. celebica</i>	HM113487	–
<i>Taxomyces andreanae</i>	<i>T. brevifolia</i>	–	Staniek et al., 2009
<i>Gibberella intermedia</i>	<i>Taxus x media</i>	KC337345	Xiong et al., 2013
10-DEACETYL BACCATIN III-10-O-ACETYL TRANSFERASE (DBAT)			
<i>Cladosporium cladosporioides</i>	<i>Taxus x media</i>	EU375527	Zhang et al., 2009a
<i>Fusarium solani</i>	<i>Taxus celebica</i>	EF626531	–
<i>Aspergillus candidus</i>	<i>Taxus x media</i>	EU883596	Zhang et al., 2009b
<i>Lasiodiplodia theobromae</i>	<i>Sara caasoca</i>	KP136287	–
BACCATIN III AMINOPHENYLPROPANOYL-13-O-TRANSFERASE (BAPT)			
<i>Taxomyces andreanae</i>	<i>T. brevifolia</i>	–	Staniek et al., 2009
<i>Colletotrichum gloeosporioides</i>	<i>Taxus x media</i>	KC337344	Xiong et al., 2013
<i>Guignardia mangifera</i>	<i>Taxus x media</i>	KC337343	Xiong et al., 2013
<i>Fusarium redolens</i>	<i>T. baccata sub sp. wallichiana</i>	KC924919	Garyali et al., 2013
<i>Fusarium tricinctum</i>	<i>T. baccata sub sp. wallichiana</i>	KF010842	Garyali et al., 2014
<i>Fusarium avenaceum</i>	<i>T. baccata sub sp. wallichiana</i>	KF010843	Garyali et al., 2014
<i>Paraconiothyrium brasiliense</i>	<i>T. baccata sub sp. wallichiana</i>	KF010844	Garyali et al., 2014
<i>Microdiploidia sp.</i>	<i>T. baccata sub sp. wallichiana</i>	KF010845	Garyali et al., 2014
<i>Alternaria sp.</i>	<i>T. cuspidate</i>	GU323557	–

<i>Taxus x media</i> (AAT73200)	NVLLVYAANMDRV SADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFLEAMADS
<i>T.mairei</i> (AEJ84001)	NVLLVYAANMDRV SADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFLEAMADS
<i>T.cuspidate</i> (AAL92459)	NVLLVYAANMDRV SADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFLEAMADS
<i>T.sumatrana</i> (ACN62085)	NVLLVYTADMDRV SADPAKVI REALSKVLVYYYPFAGRLRNKEDGELEVECTGQGVLFLEAMADS
<i>G.avenacea</i> (KF010838)	NILLVYSA-SHRVSADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFVEAMADS
<i>P.brasiliense</i> (KF010840)	NILLVYSA-SHRVSADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFVEAMADS
<i>F.tricinatum</i> (KF010839)	NILLVYSA-SHRVSADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFVEAMADS
<i>Microdiplodia</i> sp (KF010841)	NILLVYSA-SHRVSADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFVEAMADS
<i>F.redolens</i> (KC924920)	NILLVYSA-SHRVSADPAKVI REALSKVLVYYYPFAGRLRNKENGELVECTGQGVLFVEAMADS
<i>T.baccata</i> (AGJ71919)	NILLVYNA-SDRV SADPAKVI RQALSKVLVYYYSPFAGRLRNKENGDELVECTGEGALFVEAMADT
<i>T.canadensis</i> (ABW84235)	NILLVYNA-SDRV SADPAKVI RQALSKVLVYYYSPFAGRLRNKENGDELVECTGEGALFVEAMADT
<i>T.globosa</i> (ABW84246)	NILLVYNA-SDRV SADPAKVI RQALSKVLVYYYSPFAGRLRNKENGDELVECTGEGALFVEAMADT
	* * * * *
<i>Taxus x media</i> (AAT73200)	DLSVLTDLDDYNP SFQQLIFSLPQDIEDLHLLIVQ---VTRFTCGGFVVG
<i>T.mairei</i> (AEJ84001)	DLSVLADLDYNP SFQQLIFSLPQDIEDLHLLIVQ---VTRFTCGGFVVG
<i>T.cuspidate</i> (AAL92459)	DLSVLTDLDDYNP SFQQLIFSLPQDIEDLHLLIVQ---VTRFTCGGFVVG
<i>T.sumatrana</i> (ACN62085)	DLSVLTDLDDYKPSFQQLIFSLPQDIEDLHLLIVQ---VTRFTCGGFVVG
<i>G.avenacea</i> (KF010838)	DLSVLTDLDDYNP SFQQLLFSVPQDADIEDLHLLFIVQVFMQVTRFTCGGFVVG
<i>P.brasiliense</i> (KF010840)	DLSVLTDLDDYNP SFQQLLFSVPQDADIEDLHLLFIVQVFMQVTRFTCGGFVVG
<i>F.tricinatum</i> (KF010839)	DLSVLTDLDDYNP SFQQLLFSVPQDADIEDLHLLFIVQVFMQVTRFTCGGFVVG
<i>Microdiplodia</i> sp (KF010841)	DLSVLTDLDDYNP SFQQLLFSVPQDADIEDLHLLFIVQVFMQVTRFTCGGFVVG
<i>F.redolens</i> (KC924920)	DLSVLTDLDDYNP SFQQLLFSVPQDADIEDLHLLFIVQVFMQVTRFTCGGFVVG
<i>T.baccata</i> (AGJ71919)	DLSVLGDLDDYSPSLEQLLFLCLPDDIEDIHPLVVQ---VTRFTCGGFVVG
<i>T.canadensis</i> (ABW84235)	DLSVLGDLDDYSPSLEQLLFLCLPDDIEDIHPLVVQ---VTRFTCGGFVVG
<i>T.globosa</i> (ABW84246)	DLSVLGDLDDYSPSLEQLLFLCLPDDIEDIHPLVVQ---VTRFTCGGFVVG
	***** * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *

FIGURE 2 | Alignment of deduced amino acid sequences of BAPT from various species of *Taxus* and endophytic fungal isolates showing high level of sequence homology.

2014). Therefore, PCR based molecular markers specific to taxol biosynthetic pathway genes could be effectively used for the screening of large number of isolated endophytic fungi.

Genes of taxol biosynthetic pathway namely 10-deacetylbccatin-III-10-O-acetyl transferase (*dbat*) from *Cladosporium cladosporioides* showed 99% sequence similarity with the host plant *Taxus media* and 97% with same gene isolated from *T. wallichiana* (Yang et al., 2014). These findings point toward the possibility that the endophytic fungi have acquired the gene(s) for the synthesis of these novel compounds from their host by the process of horizontal gene transfer. On the other hand, Xiong et al. (2013) isolated three endophytic fungi from *T. media*, which are capable of producing taxol. The taxadiene synthase (*ts*) and *bapt* genes isolated from these fungi show very low levels of similarity with the corresponding genes from host i.e., *T. media* indicating that these fungi evolved these genes independently and have not acquired through horizontal gene transfer from the host.

Podophyllotoxin

Podophyllotoxin is a lignan and is chemically converted to pharmaceutically important compounds namely teniposide, etoposide, etopophos, and other compounds. These compounds have high anticancer activity and are prescribed for the treatment of many types of cancers (Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999). These compounds are known to interact with topoisomerase II and inhibit the activity of this enzyme which is vital for DNA replication and cell division (Loike and Horwitz,

1976; Horwitz and Loike, 1977; Minocha and Long, 1984). The presence of podophyllotoxin and its various precursors have been reported from all the species of *Podophyllum*, a herbaceous perennial alpine rosette belonging to family berberidaceae (Haijun et al., 2004). Some of the other plant species which produce podophyllotoxin include *Linum flavum* (Broomhead and Dewick, 1990), *Juniperus virginiana* (Kupchan et al., 1965), *Hyptis verticillata* (Kuhnt et al., 1994). Due to over extraction and slow growing nature, *Podophyllum* plants have been listed among the endangered plant species (Chaurasia et al., 2012). Therefore, to maintain the sustained supply of podophyllotoxin for the preparation of anticancer molecules, there is an urgent need to search for the alternative sources. Among other alternative sources, endophytic fungi have been screened for their potential for the production of podophyllotoxin (Puri et al., 2006). Eyberger et al. (2006) reported the production of podophyllotoxin by two endophytic strains of *Phialocephala fortinii* isolated from *Podophyllum peltatum*. Kour et al. (2008) reported the production of podophyllotoxin by *Fusarium oxysporum* isolated from *Juniperus recurva*.

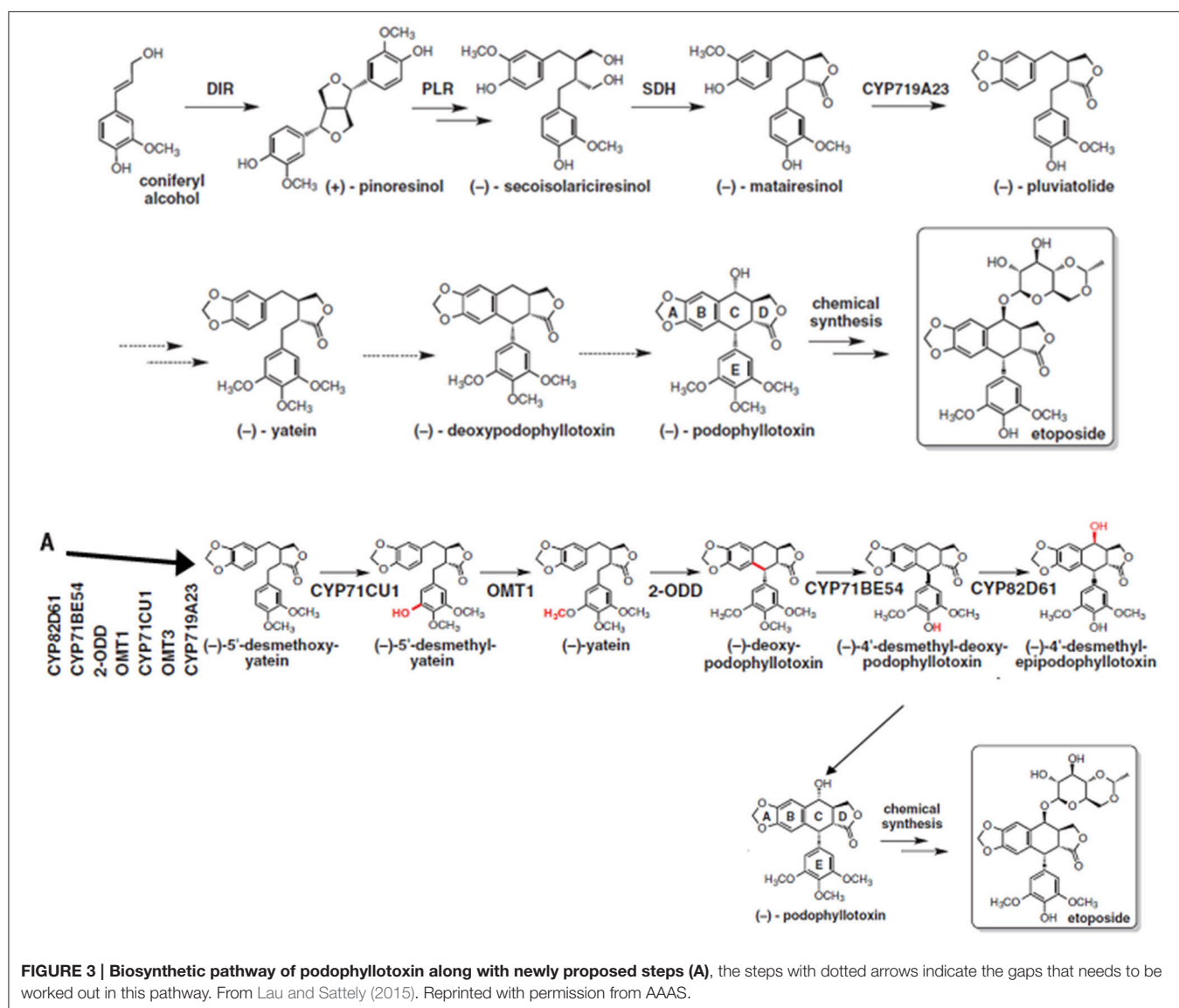
Podophyllotoxin is synthesized through phenylpropanoid pathways; which is ubiquitously distributed among plant species and play important role in plant defense (Fukuda et al., 1985; Figgitt et al., 1989). Until recently, the knowledge about its biosynthetic pathway was fragmented and key enzymes and corresponding genes were not known (Kumar et al., 2015). A thorough knowledge of complete biosynthetic pathway would ease the access to podophyllotoxin and its natural derivatives

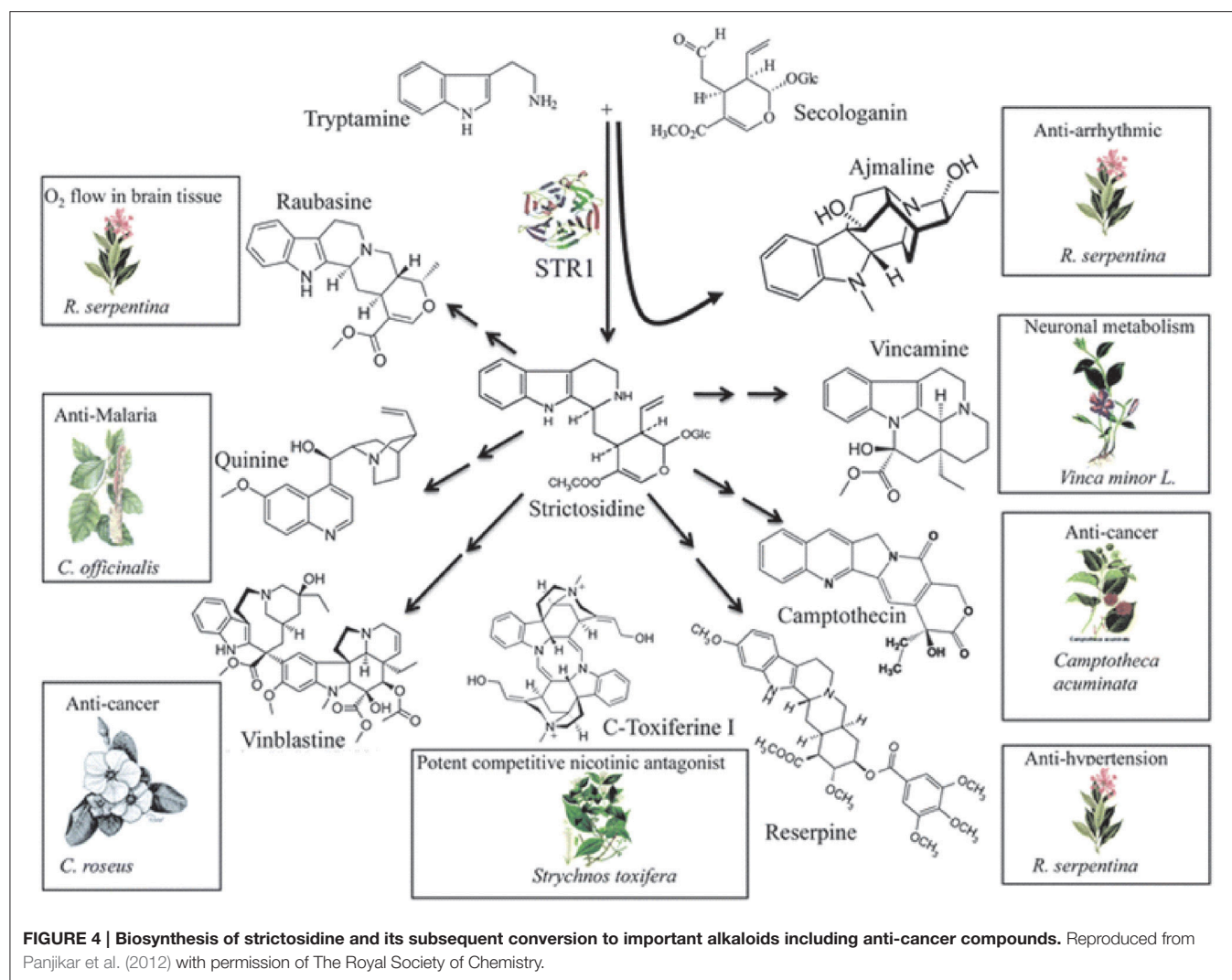
which are difficult to produce synthetically (Kamal et al., 2015). Lau and Sattely (2015) have been able to fill the major gaps in the podophyllotoxin biosynthetic pathway with the identification of six new enzymes catalyzing the key steps of podophyllotoxin production (**Figure 3**). A sequence of enzymes involved in podophyllotoxin biosynthetic pathway are dirigent protein (DIR), to coniferyl alcohol to (+)-pinoresinol, which is converted by pinoresinol-lariciresinol reductase (PLR). PLR is converted to (–)-secoisolariciresinol, which is further converted to (–)-matairesinol by sericoisolariciresinol dehydrogenase (SDH). This will further converted by CYP719A23 to (–)-pluviatolide. This is likely converted by Phex13114 (OMT1) to (–)-yatein, which is converted by Phex30848 (2-ODD) to (–)-deoxypodophyllotoxin (Lau and Sattely, 2015). The PCR based markers can be developed for some of the genes encoding these enzymes and used for the screening of large number of isolated fungal endophytes for podophyllotoxin. Such screening procedure will serve as an aid

to screen the isolated endophytic fungi and also other organisms capable of producing podophyllotoxin.

Camptothecin and Related Molecules

Camptothecin is a plant based alkaloid, which exhibits antitumor activity due to the inhibition of DNA topoisomerase I (Hsiang et al., 1985). Semi-synthetic water soluble analogs of camptothecin (Topotecan and Irinotecan) are prescribed for the treatment of tumors world over. The main precursor for the biosynthesis of camptothecin and many other important alkaloids including some important anti-cancer molecules is strictosidine, which is synthesized by the action of strictosidine synthase from tryptamine and secologanin (STR1) (**Figure 4**) (Panjikar et al., 2012). **Figure 4** depicts the biosynthesis of strictosidine and its subsequent conversion to various important alkaloids with varied medicinal properties. Strictosidine is also a precursor for the synthesis of vinblastine, another important





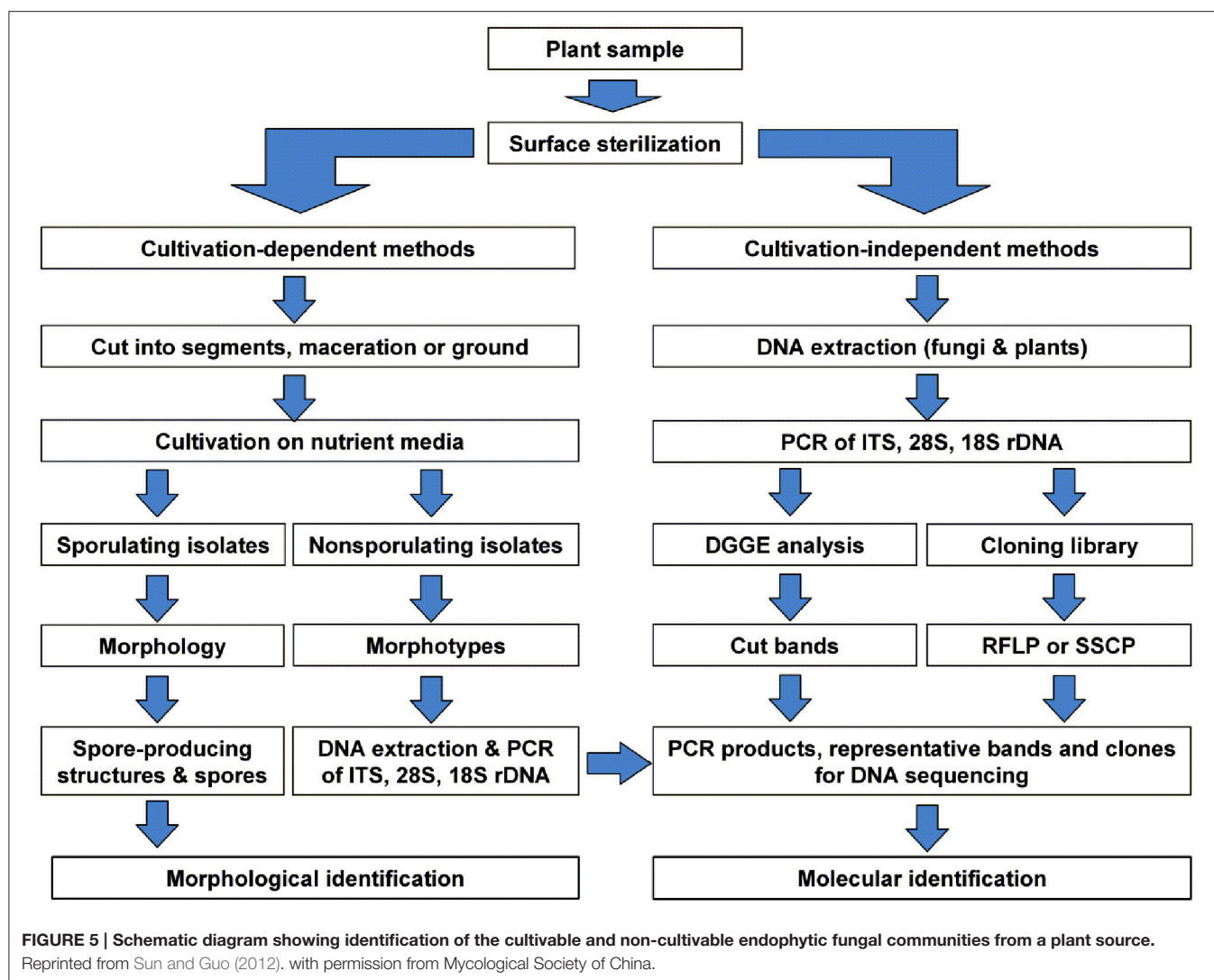
anticancer drug used for the treatment of different cancers. As strictosidine acts as a precursor for variety of alkaloids and detection of the gene encoding enzyme STR1 catalyzing its synthesis could provide significant information about this pathway. Gene encoding STR has been cloned and characterized from hairy root cultures of *Ophiorrhiza pumila* (Yamazaki et al., 2003). Although much of the information regarding the steps in-between strictosidine and camptothecin is not available, Cui et al. (2015) reported that the co-overexpression of geraniol-10-hydroxylase and strictosidine synthase increased camptothecin accumulation in *O. pumila* hairy root cultures.

Fusarium solani isolated from *Apodytes dimidiata* showed the production of 10-hydroxycamptothecin, 9-methoxycamptothecin, and camptothecin (Shweta et al., 2010). Kusari et al. (2009) reported the production of camptothecin and its analogs by an endophytic fungus from *Camptotheca acuminata*. Rehman et al. (2008) reported the production of camptothecin by an endophytic fungus *Neurospora* sp., isolated from *Nothapodytes foetida*. Camptothecin producing endophytic fungi, *Trichoderma atroviride* LY357, *Aspergillus* sp. LY355,

and *Aspergillus* sp. LY341 were isolated from *C. acuminata* by Pu et al. (2013). Therefore, markers specific to geraniol-10-hydroxylase and strictosidine synthase can provide important information.

MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI

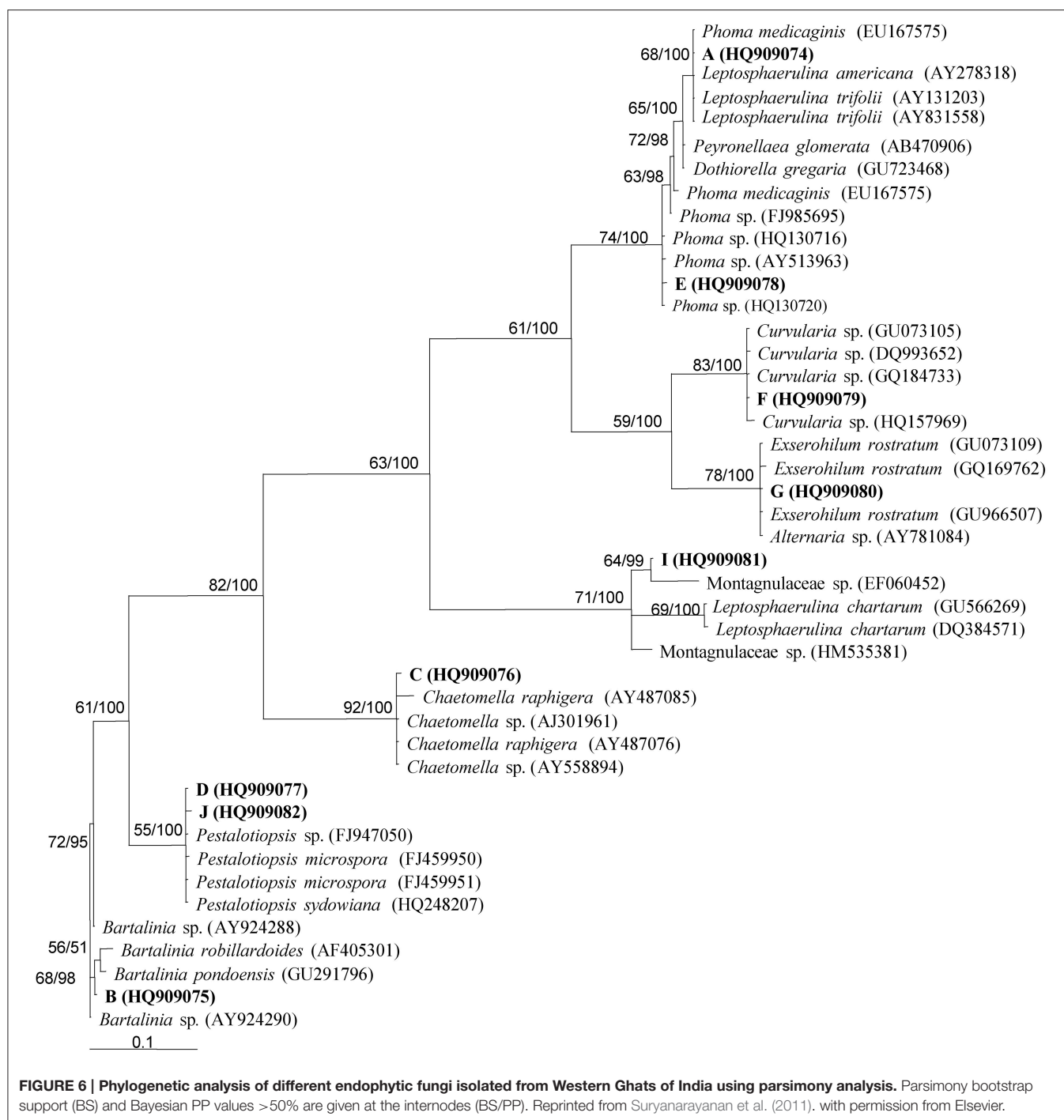
Endophytic fungal communities comes from a broad range of fungal origins, which include Ascomycota, Basidiomycota, and Zygomycota. These fungal isolates can be identified based on their morphological characteristics if they sporulate on the media. Traditional classification of fungi heavily relies on reproductive structures, the non-sporulating fungi cannot be provided with taxonomic names (Sun and Guo, 2012). Application of molecular tools, such as DNA fingerprinting and sequencing methods (Figure 5), showed the potential to overcome the difficulties in traditional taxonomy for cultivable fungi. In endophytic fungi, 5.8S gene and flanking internal



transcribed spacers (ITS1 and ITS2) of the rDNA, 18S and 28S rRNA genes have been employed in the identification of endophytic fungi. Pandey et al. (2003) identified different isolates of *Phyllosticta* that were isolated from different tropical tree species in India as *P. capitalensis* based on ITS sequence analysis. Morakotkarn et al. (2007) isolated 71 (of 257 strains) endophytic fungi from *Phyllostachy* and *Sasa* species and placed them into Sordariomycetes and Dothideomycetes based on 18S rRNA sequence analyses and further identified them into lower taxonomic levels based on ITS sequences. Endophytic fungi belong to Xylariaceae isolated from 22 tree species of a dry thorn forest and 27 tree species of a stunted montane evergreen forest of the Western Ghats in southern India were identified as *Xylaria* or *Nemania* species based on their ITS sequence analysis (Govindarajulu et al., 2013). Sun et al. (2011) clustered 221 non-sporulating endophyte strains into 56 morphotypes, and placed these morphotypes into 37 taxa based on ITS sequence similarity and phylogenetic analyses. Suryanarayanan et al. (2011) identified different endophytic fungi isolated from the

Western Ghats of India based on ITS sequences and phylogenetic analysis (Figure 6).

Due to the limitations of traditional isolation procedures, it is highly probable that many endophytic fungi cannot be brought onto the culture. To overcome the potential technical bias, molecular approaches have been applied in the identification of endophytic fungi directly within the host tissues. This process involves (i) extraction of total genomic DNA from surface-sterilized plant tissues, (ii) amplification of DNA fragments (e.g., ITS, 28S and 18S genes) with fungal specific primers, (iii) denaturing gradient gel electrophoresis (DGGE) and excision of different bands (iv) cloning and sequencing of representative clones and identifying the sequences into various taxonomic levels based on phylogenetic analysis (Figure 5). High-throughput sequencing also serves as a powerful alternative to molecular studies of fungal community in natural environments. This technique has been successfully employed to study the fungal diversity in phyllosphere fungi (Jumpponen and Jones, 2009), mycorrhizal fungi (Dumbrell



et al., 2011), and other natural environments. DNA barcoding systems is another technique employed to identify fungal species (Hebert et al., 2003). DNA barcode region used should be a single locus for all groups of organisms across all kingdoms. In endophytic fungi, ITS region is considered as the most widely used DNA barcode in molecular identification, despite some limitations in species distinction (Sun et al., 2011).

CONSIDERATIONS

It has been reported that fungi showing amplification of DNA fragments specific to genes involved in taxol biosynthesis namely, *ts*, *dbat*, and *bapt* were able to produce taxol. However, it has been reported that the fungal strains showing the amplification of only *ts* and *dbat* were found to be negative for taxol production (Garyali et al. (2013). Hence, it is essential to

select the appropriate genes/enzymes as markers for screening of endophytic fungi for production of bioactive compounds. Further, there are reports regarding the endophytic fungi producing important bioactive compounds which are not specific to host plants. Gangadevi and Muthumary (2008) reported the isolation of endophytic fungi *Colletotrichum gloeosporioides* capable of producing taxol from *Justicia gendarussa*, a plant not known for taxol production. Such exceptional cases need to be studied in detail to clarify the possibilities of acquiring these genes of biosynthetic pathways by endophytic fungi. Also use of gene specific primers will help in screening and identifying the bioactive compounds produced by endophytic fungi which are not specific to host plant.

CONCLUSIONS

Endophytic fungi are important sources of therapeutically active compounds. Driven by the success of molecular screening

of endophytic fungi capable of producing taxol, it seems rational to apply this technology for the compound specific screening of large number of isolated endophytic fungi. The possibility of using such procedures is high in case of novel compounds including anticancer compounds for which either complete or partial biosynthetic pathways are known. If the biosynthetic pathways are not known for some of the metabolites, plant genes associated with that metabolic pathway could be used as markers to screen the endophytic fungi.

AUTHOR CONTRIBUTIONS

MV: Contributed in writing the manuscript (except podophyllotoxin and taxol portion). AK: Contributed writing the podophyllotoxin and taxol portion of the manuscript. MR: Contributed overall compilation and editing of the manuscript.

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Fusarium tricinctum, An Endophytic Fungus Exhibits Cell Growth Inhibition and Antioxidant Activity

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Abstract An endophytic fungus (strain T6) isolated from *Taxus baccata* was studied for its effect on the growth of human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and peripheral blood mononuclear cells (PBMCs) as well as for its antioxidant activity. Based on morphological characters and internal transcribed spacer (ITS) sequence analysis, this fungus (strain T6) was identified as *Fusarium tricinctum*. This fungus has shown inhibition in the growth of the MCF-7 and HeLa cancer cell lines. IC₅₀ values of the fungal extract were 225 ± 26 and $220 \pm 18 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. Further, *F. tricinctum* showed inhibition in the proliferation of concanavalin A stimulated PBMCs indicating its immunosuppressive potential (IC₅₀ value $110 \pm 44 \mu\text{g ml}^{-1}$). Tumour necrosis factor (TNF)- α production in concanavalin A stimulated PBMCs and MCF-7 were found to be inhibited which indicates that the antiproliferative effect may be associated with TNF- α . Free radical scavenging results revealed that this fungus also exhibited antioxidant activity (IC₅₀ value $482 \pm 9 \mu\text{g ml}^{-1}$). Present study results suggested that *F. tricinctum* has the potential to be used for therapeutic purposes because of its antiproliferative and antioxidant potential.

Keywords *Taxus baccata* · Cancer cell lines · Tumor necrosis factor- α · Antioxidant

Introduction

Endophytic fungi are reported to colonize internal plant tissues without causing any adverse effect to the host [1] and are known to protect the host against biotic and abiotic stresses [2, 3]. Endophytes are known to secrete secondary metabolites with potential use for therapeutic purposes [4, 5]. Screening of these diverse fungi for their ability to produce important bioactive molecules is a promising approach to obtain rare drugs. Endophytic fungi are also known to produce complex bioactive molecules produced by the host and this was proved with the isolation of an endophytic fungus from *Taxus brevifolia* capable of producing taxol (anticancer compound) [6]. Since then, extensive research on isolation of endophytic fungi producing important bioactive compounds has been taken up by many research groups [7–9].

Taxus baccata L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan Yew) is the only species of *Taxus* reported from the temperate Himalayas at an altitude of 1800–3300 m amsl [10]. It is a very slow growing, ever-green conifer that has been reported to grow in sub-tropical climates. Some of the endophytic fungi isolated from *T. baccata* growing in this region are reported to produce taxol [11]. Many *Fusarium* spp., are reported as endophytes and often isolated from different forest plants. These endophytic species are well known to produce bioactive secondary metabolites with varied activities such as antibacterial [11] and anticancer [12, 13]. In the present investigation, an endophytic fungus *F. tricinctum* was isolated from the bark of the Himalayan yew and assessed

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for its effect on the growth of human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and peripheral blood mononuclear cells as well as for its antioxidant activity.

Materials and Methods

Isolation of Endophytic Fungi

Endophytic fungi were isolated from the bark samples of *T. baccata* L. subsp. *wallichiana* (Zucc.) Pilger collected from Bhadrehwah (district Doda, India) according to the method described earlier [11]. The plates were incubated at 25 ± 2 °C for 5–10 days and were regularly observed for the growth of endophytic fungi. Each fungal culture was checked for its purity and subcultured to fresh potato dextrose agar (PDA) (HiMedia Laboratories Ltd. Mumbai, India) medium by the hyphal tip method [6]. In the present study, one of the endophytic fungal isolates (strain T6) was selected for its antiproliferative and antioxidant activities based on preliminary investigations.

Identification of Endophytic Fungus Strain T6

The fungal strain T6 was characterized based on its morphological characteristics such as colony, spores and the reproductive structures [14]. Molecular characterization was performed by ITS sequence analysis. For this purpose, mycelia harvested from actively growing strain T6 were ground into a fine powder in liquid nitrogen. Genomic DNA was extracted by CTAB method [15]. Quality and quantity of DNA was checked with Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) and samples were stored at -20 °C until use. The ITS region of nrDNA was amplified with the universal primers ITS1 and ITS4 [16] using Thermal Cycler (Applied Biosystems, California, USA). The PCR programme for amplification of the ITS region was used as described earlier [17]. The PCR amplified fragments were gel purified using Qiaquick columns (Qiagen) as per the manufacturer's instructions and sequenced. The ITS sequence of strain T6 was deposited in the GenBank under the accession numbers KT779291. This ITS sequence was compared to those available in the GenBank database using the BLASTn algorithm. Alignment of the sequences was constructed using MAFFT ver. 7.0 [18] and edited with BioEdit 5.0.6 [19]. The phylogenetic tree was reconstructed using maximum parsimony method and the Kimura two-parameter distance calculation by MEGA5 software [20]. The bootstrap was 1000 replications to assess the reliable level to the nodes of the tree.

Fungal Extract Preparation

The mycelial mats (5.0 mm diam) of 7–10 days were inoculated into potato dextrose broth (100 ml) and incubated at 25 ± 2 °C on a shaker for 5 days and resulting culture was used as the seed culture. Seed culture (15 ml; 3 % v/v) was transferred into 500 ml of potato dextrose broth and incubated at 25 ± 2 °C for 21 days in dark as a stationary culture. The culture was then filtered through four layers of cheesecloth to remove the mycelia. The harvested mycelia were dried overnight at 35–40 °C and extracted with ethyl acetate for 12 h. The culture broth was also extracted with three equal volumes of ethyl acetate; the fractions were collected and combined, and the solvent was then evaporated under reduced pressure at 35 °C. The residue was re-dissolved in dimethyl sulfoxide (DMSO) and used for the assay.

Cell Growth Inhibition Assay

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were procured from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA), containing 10 % (v/v) foetal bovine serum (Gibco), 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin. Cells were maintained in a humidified incubator with 5 % CO₂ at 37 °C. The effect of fungal extract on the growth of cancer cell lines was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay [21]. Cells were trypsinised and seeded at a density of $\sim 2 \times 10^4$ cells per well in 96 well cell culture plate and incubated overnight. After 16 h, fungal extracts were added in varying final concentrations (50, 100, 150, 200, 250, 300 and 350 µg ml⁻¹) to the wells. After 72 h of incubation, 20 µl of MTT reagent (Sigma USA, 5 mg ml⁻¹) was added to each well and again incubated for 4 h. The formazan crystals formed were solubilized in 100 µl DMSO (Merck, Germany). Finally, the absorbance of each well was recorded at 570 nm, taking 630 nm as the reference wavelength, using the microplate reader (Tecan infinite, Austria). Paclitaxel was used as a positive control at the concentration of 20 µg ml⁻¹. Percentage of inhibition was calculated as:

$$\left(\frac{\text{Mean OD of untreated cell} - \text{Mean OD of treated cell}}{\text{Mean OD of untreated cell}} \right) \times 100.$$

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood (5 ml) which was drawn from healthy volunteers with their consent prior to the study. Blood was carefully layered onto the ficoll (Sigma, USA) and centrifuged at $400\times g$ for 30 min at room temperature. After centrifugation, top plasma layer was carefully removed with a pipette and collected opaque interface containing PBMCs in a clean conical centrifuge tube. The cells were washed by adding PBS and centrifuged at $250\times g$ for 10 min.

Lymphocyte Proliferation Assay

PBMCs were seeded at a density of 1×10^5 cells per well and then fungal extracts were added in varying final concentrations. After 2 h, concanavalin A ($5 \mu\text{g ml}^{-1}$), a mitogen was added in order to stimulate the PBMCs. After 72 h of incubation, MTT assay was carried out to measure the cell proliferation. MTT assay and calculation of inhibition in mitogen-induced proliferation were done as described in the case of a cell growth inhibition assay.

TNF- α Measurement

In order to evaluate the production of TNF- α , MCF-7 and concanavalin A stimulated PBMCs were incubated with varying concentrations of fungal extract for 72 h. After incubation, supernatants were collected and the secretion of TNF- α was measured using ELISA kit (Preprotech, USA) as per manufacturer's instruction. Inhibition in TNF- α production was calculated in the same manner as mentioned in the cell growth inhibition assay.

Antioxidant Assay

In order to know the antioxidant potential of fungal extract, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed. Fungal extract (50 μl) with varying concentration was mixed with 150 μl of DPPH (100 μM) in methanol, added in wells of a 96-well microtiter plate. Ascorbic acid ($100 \mu\text{g ml}^{-1}$; 50 μl) was used as positive control. The plate was incubated in dark for 45 min, after which the absorbance of the solution was measured at 517 nm in ELISA microtitre plate (Tecan infinite, Austria). Free radical scavenging activity was expressed as the inhibition percentage calculated using formula,

$$\text{Free radical scavenging activity} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100.$$

Statistical Analysis

All assays were carried out in triplicates. Data were analysed using analysis of variance (ANOVA) and the means were compared by using Tukey's test at $p < 0.05$.

Results

Isolation and Identification of Endophytic Fungus Strain T6

A total of 25 endophytic fungi were isolated from the bark of *T. baccata*. One of the fungal isolates (strain T6) was selected for this investigation. The strain T6 grew well on PDA media at 28 °C in 7 days. The morphological traits of strain T6 were: pink coloured centre surrounded by white coloured margins, cottony appearance, nearly round margins and broken edges. Microscopic observations indicated the presence of tubular, thick walled, septate hyphae, simple or branched and sickle-shaped macroconidia and oval microconidia. Microconidia are sparse, one-celled, smooth, ovoid, non-septate, present solitary and measure 1.6–2.6 μm in length and 1–1.5 μm in width. The apical cells were elongated, blunt to conical, and basal cells were blunt to non-notched. Based on the morphology of the fungal mycelia and characteristics of the spores, the endophytic fungus T6 seemed to be *Fusarium* sp. ITS sequence analysis of T6 showed 100 % similarity with *Fusarium tricinctum* strain A1 (JF273496). Phylogenetic analysis also clustered T6 with *Fusarium tricinctum* species (Supplementary Fig. 1).

Cell Growth Inhibition Against Cancer Cell Lines

Inhibition in cell growth was observed in both human breast cancer cell line (MCF-7) and human cervical cancer cell line (HeLa). The cytotoxic effect significantly increased with increase in the concentration of the fungal extract (Fig. 1). The IC_{50} value for extract was found to be 225 ± 26 and $220 \pm 18 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. Paclitaxel, ($20 \mu\text{g ml}^{-1}$) an anti-cancer drug was used as a positive control which showed $93 \pm 5 \%$ inhibition in this experiment. The inhibition level of the crude fungal extract was comparable with paclitaxel ($20 \mu\text{g ml}^{-1}$) when the concentration of the extract was above $250 \mu\text{g ml}^{-1}$ (Fig. 1).

Anti-proliferative Activity Against Peripheral Blood Mononuclear Cells

Fusarium tricinctum extract was also looked for their effect on the proliferation of mitogenic (concanavalin A) stimu-

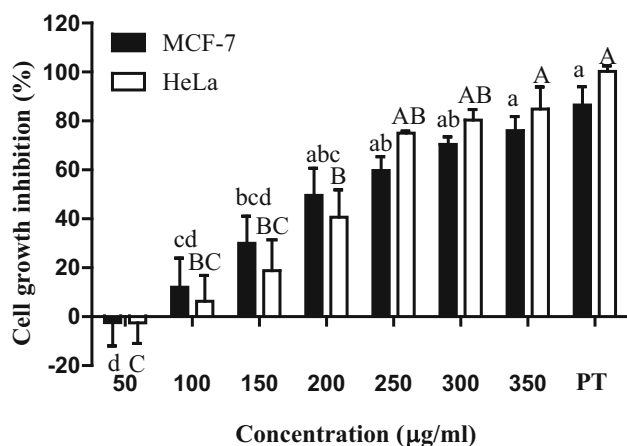


Fig. 1 Effect of *Fusarium tricinctum* extract on the growth of human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines. Means followed by the same letter are not significant at $p < 0.05$. PT represents paclitaxel ($20 \mu\text{g ml}^{-1}$) as a positive control

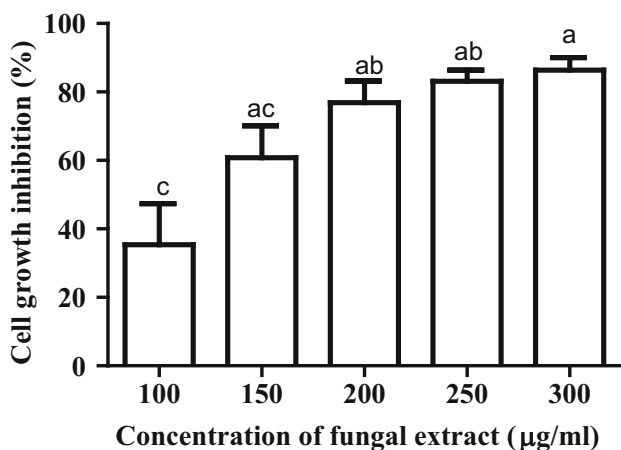


Fig. 2 Immunosuppressive effect of *Fusarium tricinctum* extract on concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Means followed by the same letter are not significant at $p < 0.05$

lated human PBMCs. PBMCs have not shown proliferating response against the mitogen concanavalin A in the presence of fungal extracts indicating its immunosuppressive activity. With the increase in the concentration of the fungal extract, the immunosuppressive effect became significantly more pronounced in PBMCs (Fig. 2). The IC_{50} value for extract was found to be $110 \pm 44 \mu\text{g ml}^{-1}$.

TNF- α Production

TNF- α is an inflammatory cytokine which plays an important role in cancer progression and metastases. Hence, effect of the fungal extract on TNF- α production in MCF-7 was estimated using ELISA. Interestingly,

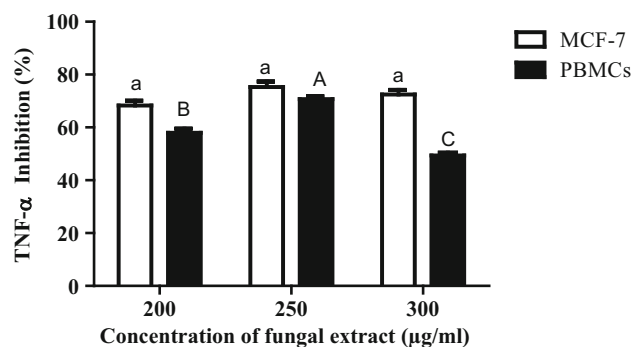


Fig. 3 Effect of *Fusarium tricinctum* extract on TNF- α production in MCF-7 and concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Means followed by the same letter are not significant at $p < 0.05$

inhibition in TNF- α production was observed in MCF-7 cells treated with fungal extract which show that cytotoxic effect may be mediated by inhibition of this cytokine production (Fig. 3). Similarly, TNF- α production was found to be inhibited in concanavalin A stimulated PBMCs. Inhibition in cytokines production against MCF-7 and concanavalin A stimulated PBMCs was observed in different concentrations of extract tested in this study, but the inhibition effect appears independent of concentrations.

Antioxidant Activity

Free radical scavenging activity was performed to evaluate the antioxidant activity of *F. tricinctum*. It was observed that the scavenging activity significantly increased with increase in the concentration of fungal extract (Fig. 4). The IC_{50} value of scavenging activity of extract was found to be $482 \pm 9 \mu\text{g ml}^{-1}$. Ascorbic acid was used as positive control and where it shows $80 \pm 2\%$ antioxidant activity. The scavenging activity of the extract was comparable with ascorbic acid ($100 \mu\text{g ml}^{-1}$) when the concentration of the extract was 1 mg ml^{-1} .

Discussion

Endophytic fungi have been studied as a source of anti-cancer agents ever since the million dollar drug Taxol was isolated from the endophytic fungus *Taxomyces andreanae* [22]. Two human cancer cell lines were used in this study for the investigation of the antiproliferative effect of *F. tricinctum* extracts and the results clearly showed cell growth inhibition (cytotoxicity) against both cancer cell lines. Similar results were reported from *F. oxysporum* which showed cytotoxicity in different human cancer cell lines [23]. Puri et al. [9] reported the cytotoxic activity of

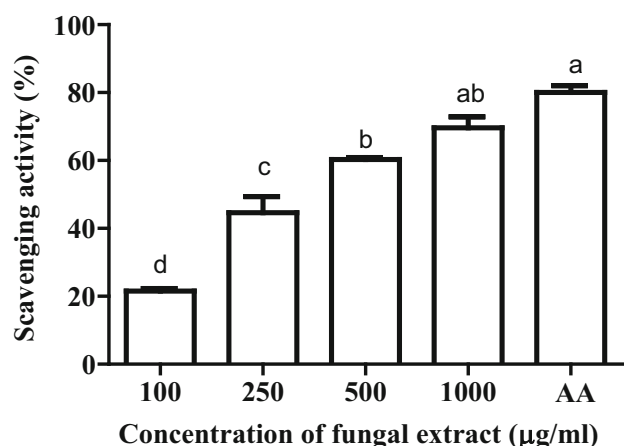


Fig. 4 Antioxidant effect of *Fusarium tricinctum* extracts based on free radical scavenging activity. Means followed by the same letter are not significant at $p < 0.05$. AA represents ascorbic acid ($100 \mu\text{g ml}^{-1}$) as positive control

endophytic fungus *Entrophospora infrequens* inhabiting *Nothapodytes foetida* against different human cancer cell lines and found activities comparable to the camptothecin.

It has been reported that several anticancer drugs have an impact on immune system and possess immunosuppressive properties [24]. Immunosuppressive compound such as subglutinol-A and subglutinol-B were isolated from the fungal endophyte *F. subglutinans* inhabiting *Tripterygium wilfordii* [25]. The present study revealed that PBMCs did not show proliferating response against the mitogen concanavalin A after addition of fungal extract indicating immunosuppressive effect. These findings were inline with the reported anti-lymphocyte proliferative activities against phytohemagglutinin (PHA) stimulated PBMCs by endophytic fungi from *Tripterygium wilfordii* [26]. The immunosuppressive effect was further supported by the fact that there is inhibition in the production of inflammatory cytokines TNF- α in stimulated PBMCs culture. TNF- α , a cytokine which mediates the inflammatory response and is also known to influence cancer growth [27]. The present study revealed that *F. tricinctum* extract inhibits the TNF- α production in breast cancer cell lines (MCF-7), which indicate that cytotoxic effect may be mediated by TNF- α inhibition.

Antioxidants protect the cells from the damages caused by free radicals. Free radical mediated reactions are associated with various diseases which include alzheimer's disease, diabetes, cardiovascular disorder and cancer [28]. In the present study, the extract of *F. tricinctum* showed antioxidant activity which increased with extract concentration indicating the possibility of exploring this fungus as a source of antioxidant agent. Different fungal compounds with antioxidant activity were isolated from various

endophytic fungi from different sources [29, 30] including phenolics showing potent antioxidant activity [31].

In conclusion, the extracts of *F. tricinctum* exhibited strong cell growth inhibition and antioxidant properties. Further studies focussing on purification and characterization of bioactive compound responsible for these activities may help in isolating some new compounds of pharmaceutical importance.

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