Antimicrobial activity of the endophytic fungi isolated from the medicinal plants

A thesis submitted in partial fulfillment of the requirement
for the award of the degree of

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Submitted by
Harpuneet Mandaher
(301601010)

Under the supervision of
Dr. M. Vasundhara
Assistant Professor

DEPARTMENT OF BIOTECHNOLOGY
THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY, 147004, PATIALA

June, 2018

I

CERTIFICATE

This is to certify that the thesis entitled **"Antimicrobial activity of the endophytic fungi isolated from the medicinal plants"** submitted by Harpuneet Mandaher in the partial fulfillment of the requirement of the award for the degree of Masters of science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is a record of the student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Vasundhara

Dr. M. Vasundhara
Supervisor
DBT
Thapar Institute of
Engineering and Technology
Patiala

DECLARATION

I hereby declare that the work that has been presented in the thesis **"Antimicrobial activity of the endophytic fungi isolated from the medicinal plants"** in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is an original record of my own work done during the period from January 2018 to June 2018, carried out under guidance and supervision of **Dr. M. Vasundhara**. This dissertatiom report has not been submitted in part or full to any other university or institute for the award of any degree.

Harpuneet Mandaher

Place- Patiala

(Harpuneet Mandaher)

Date- August 20, 2018

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Harpuneet Mandaher

Harpuneet Mandaher

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LIST OF ABBREVIATIONS

Abbreviation	Name
%	Percentage
˚C	Degree Celsius
L	Litre
ml	Millilitre
Mg	Milligram
M	Molar
mM	MilliMolar
min	Minutes
hr	Hour
O.D	Optical Density
CFU	Colony Forming Units
mg	Milligram
NB	Nutrient Broth
MHB	Muller Hinton Broth
MHA	Muller Hinton Agar
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
v/v/v	Volume by volume by volume
w/v	Weight by Volume
Sp.	Species
pH	Potential of hydrogen ion
Temp	Temperature
Rpm	Rotation Per Minute
H_2SO_4	Sulfuric Acid
MIC	Minimum Inhibitory Concentration
LAF	Laminar air flow
GAE	Gallic acid equivalent
QR	Quercitin equivalent
MTT	3-(4,5-Dimetylthiazol-2-YI)-2,5-Dipehnyltetrazolium Bromide
E.coli	*Escherichia coli*
P.aeruginosa	*Pseudomonas aeruginosa*
S.aureus	*Staphylococcus aureus*
B.megaterium	*Bacillus megaterium*
C.albicans	*Candida albicans*

ABSTRACT

Medicinal plants are used since ancient times to cure various ailments, a lot of therapeutically important drugs have been obtained from plants with medicinal value. A great interest is shown in isolating fungal endophytes from these plants of medicinal value. Endophytic fungi present in these medicinal plants produce numerous novel compounds that have antibacterial, antifungal, anti cancerous, anti inflammatory, antioxidant and antidiabetic properties. Discovery of an important compound taxol, from endophytic fungi of plant *Taxus brevifolia,* encouraged scientists over the globe to explore more about endophytic fungi. The aim of the current research work was to isolate endophytic fungi from medicinal plant, screening of extracts for antimicrobial activity from isolated endophytic fungi and antimicrobial activity study of the fractions.

In disc diffusion preliminary assay EL showed zone of inhibition of 21mm and 11mm against Staphylococcus *aureus* and *Bacillus megaterium* respectively. In MTT assay, EL showed maximum inhibition of 60% against *Staphylococcus aureus* and 41% against *Bacillus megaterium.* EL also had maximum phenolic content (126.8 mg GAE/ g of dried extract). Thus, EL was selected for further analysis. IC_{50} value for EL was found out to be between a concentration 512µg/ml and 1024µg/ml by performing MTT assay. Finally TLC of crude extract EL was run to find out the actual fraction responsible for antimicrobial activity against *Staphylococcus aureus*. Each fraction was tested by disc diffusion assay, in which fraction 1 gave a zone of inhibition of 10mm. EL was viewed morphologically under microscope for fungal identification purpose. When viewed under microscope it consisted of sac like structures containing spores and had septate hyphae.

Thus the results from above studies show that the microorganisms present within the plant could be a source of new bioactive compounds. Identifying and characterizing metabolites produced by endophytes is a promising and current area of research with wide applications in pharmaceutical industry.

INTRODUCTION

There is development of resistance by various existing pathogenic bacteria and fungi to the already existing drugs in the market. This has raised huge concern in the world and an immediate need for new agents against these microorganisms is there. Alexander Fleming anticipated this phenomenon of antibiotic resistance since the discovery of penicillin. If antibiotics are not used correctly, the microorganisms adapt and hence develop resistance. Then the resistance gene is passed by plasmid exchange because of rapid multiplication. But not all microorganisms are same in developing resistance, because development of resistance depends upon the concentration at which an antibiotic is used, bioavailability of the antibiotic in the body (Coates *et al*, 2002). However, there has been a great misuse of antibiotics since they have been employed in various number of practices repetitively which contribute to problem of antibiotic resistance. So, new antimicrobial agents should be developed fast to fight against these resistant microorganisms.

Endophytes often bacterium (actinomycetes or mycoplasma) or fungi are endosymbiotic group of microorganisms. They colonize the intra or intercellular locations of the plant (Gouda *et al*, 2016). Endophyte resides within the plant without any symptoms. All or some part of their life cycle occurs inside the host. Antagonism, mutualism and only sometimes parasitism are different relations endophytes have with plants (Gouda *et al*, 2016).

Endophytes enhance the ability of the plant to tolerate abiotic and biotic stress. The increased plant resistance to various pests and insects and increasing host nutrient gain and growth may be attributed to endophytes.

At first the endophytes were defined as, “any organism growing inside the plant” was given by DeBary (1866). But this definition continues to change with new research. Most suitable definition of endophyte provided is by Petrini (1991) which states that any organism residing inside the plant tissue at any time of its life cycle, without giving any sign of its presence.

Nearly all plants examined till today have been found to inhabit endophytes either bacteria or fungi (Saikkonen *et al*, 1998).

Endophytic fungi are classified broadly into two groups. These two groups are clavipitaceous fungi, that infect grasses limited to cool areas and non clavipitaceous fungi, that are from conifers, angiosperms and ferns, and are confined to basidiomycetes and ascomycota group (Jalgaonwala *et al*, 2011).

Plant endophytes are novel resources for many novel bioactive compounds having important applications in pharma, agricultural and food industry. In last two decades numerous useful compounds have been obtained from endophytic fungi showing antimicrobial, antioxidant, anti-cancerous, anti-diabetic potential. These bioactive compounds could be classified as terpenoids, alkaloids, lignans, steroids, quinones, lactones, and phenols (Zhang *et al*, 2006).

Due to co-evolution for such a long time, there is development of friendly interaction between plant and the endophytic fungi. The host plant provides habitat and nutrition for the endophytic fungi whereas the endophytic fungus produces bioactive compounds helpful to the plant (Rodriguez, 2009).

The fungal endophyte can produce or mimic the phytochemical or medicinal agents produced by the plant which may be due to their co-evolution along with the host plant mediated through horizontal gene transfer (Jia *et al*, 2016).

Endophytic fungi are different from fungi isolated from non plant source because the plant has chemical biology that is responsible for evolution of endophytic fungi to produce compounds that can be exploited for developing new drugs (Jia *et al*, 2016).

Endophytic fungi are replacing the use of plant as a source of bioactive compounds because the bioactive compounds from fungi can be produced in large quantities by fermentation.

All the medicinal plants act as a treasure house of these endophytic fungi. The medicinal plants chosen for the study are *Terminalia arjuna* and *Eucalyptus tereticornis*. *Terminalia arjuna* belongs to family combretaceae and is commonly referred to as Arjuna. It is deciduous tree found all over India. The tree has great Ayurvedic value. The bark of Arjuna has diuretic and cardiotonic effect and the bark powder is seen to be effective against bone fractures, heart problems, skin problems, hypercholesterolemia, polyuria, fever, white discharge and giddiness (Diwedi *et al*, 1989). It has been reported in the literature that extract of Arjuna is effective

against *Staphylococcus* (Thangaraj and Fathima, 2017). The plant is suspected to be useful against cancer based on closeness of different compounds (Thangaraj and Fathima, 2017).

Eucalyptus belongs to family myrtaceae. It is a tall evergreen tree native to Tasmania and Australia and now widely cultivated in many countries including India. Phytochemical studies in *Eucalyptus tereticornis* bark and leaf extract showed that cardiac glycosides are present in bark whereas flavnoids, saponins and tannins are present in both leaf and bark (Jain *et al*, 2010). Studies have shown that the *Eucalyptus* species have antibacterial, antioxidant, antihyperglycemic and anti-inflammatory properties. The decoction of leaves of *Eucalyptus tereticornis* reduces fever and pulmonary problems and the essential oil from leaves shows antimicrobial activities.

Objectives of the project

- Isolation of endophytic fungi from host medicinal plants
- Screening of extracts from isolated endophytic fungi for antimicrobial activity.
- Antimicrobial activity of the fractions

REVIEW OF LITERATURE

2.1 Medicinal plants as a source of natural drugs

Since ages humans depended on nature for their basic needs, similarly in case of diseases humans have relied on nature for the treatment of these diseases. Medicinal plants have been used since ancient times dating from around 2600 BC. Documentation of Indian ayurvedic system is from before 100 B.C. Plant contains a lot of biologically active compounds, and only 10-15% of higher plants have yet been investigated and only 6% out of these plants have been screened for biologically active compounds (Verpoortr, 2000). This has developed interest of scientists worldwide in exploring plants to find various drugs. A lot of research today is going on various medicinal plants.

Medicinal plants contain novel bioactive compounds responsible for the biological activity. The biological activity (antimicrobial, antioxidant, anti-cancerous, anti inflammatory etc) are due to specific chemical compounds like phenols, flavinoids, alkaloids, steroids, terpenoids and others. Some important compounds from plants developed for human benefits include opium alkaloids, tubocurarine and strychnine, cardiac glycosides, quinine and artemisinin, podophyllotoxin, vinblastine and vincristine (Zhao *et al*, 2011).

Terminalia arjuna also called kumbuk or Arjuna is a woody tropical tree grown throughout India. It belongs to family Combretaceace. The bark of Arjuna contains compounds like arjunetin, arjunine etc. The bark also contains calcium carbonate, different salts of calcium, aluminium, tannins, magnesium. The plant shows a mixture of properties like antibacterial, antifungal, antipyretic etc. The powdered bark has been used as general tonic for cirrhosis in liver and as diuretic. The compounds from Arjuna have anticancerous activity against some cell lines like A498, SK-MEL etc (Thangaraj and Fathima, 2017). The leaves of Arjuna contain compounds like glycosides and urosolic acid, earlier research have shown them to be antagonistic to cancer and leishmanial activity (Thangaraj *et al*, 2017).

Eucalyptus belongs to family myrtaceae. *Eucalyptus* is a native of Australia, New Guinea, Phillipines and Indonesia. The most valuable product from *Eucalyptus* is the *Eucalyptus* oil. The essential oil contains 90% cineole, and the *Eucalyptus* oil containing cineole is seen to show

antimicrobial property. *Eucalyptus* oil is widely used in cough drops, decongestants, deodorizing and cleaning products, it also has insect repellent properties.

Table 1: Classification of *Eucalyptus* and Arjuna

CLASSIFICATION	***EUCALYPTUS***	**ARJUNA**
Class	Magnoliopsida	Magnoliopsida
Order	Myrtales	Myrtales
Family	Myrtaceae	Combretaceae
Genus	*Eucalyptus*	*Terminalia*
Species	*teriticornis*	*Arjuna*

Table 1 shows classification of *Eucalyptus* and Arjuna. Using the plant for human needs have raised concern of plants going extinct, so the interest is shifted towards using its endophyte as a source of bioactive compounds showing biological activity. It has been studied that endophyte possess the ability to synthesize the bioactive compounds of the plants and sometimes it can even produce bioactive compounds more than the plant.

2.2 Endophytes as promising organisms

The first definition of endophytes was provided by De Bary (1866) stating that any organism that grows inside the plant tissue is termed as endophyte. But the correct definition of endophytes was given by Petrini (1991) stating that any organism that at any part of its life cycle has lived inside the plant without causing any symptoms is termed as an endophyte.

Endophytes are ubiquitous in nature, presence of one or more type of endophytes in each plant is shown in research. Endophytes are found in leaves, petioles, roots, stems and other parts of the plant. The endophyte population in any plant is highly variable it depends upon many factors like the species of plant, the development stage of plant, the environmental conditions in which the plant is growing (Siakkonen *et al*, 1998).

It is considered that a single plant species could possess thousands of microbes, categorized as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those near or on plant tissue) or endophytes (microbes residing within plant tissues in leaves, roots or stems), depending on their area of colonization in the plant species (Gouda *et al*, 2016).

Endophytes are shown to enhace the host plant ability to tolerate abiotic and biotic stress. Endophytes also increase the plant resistance to pests and insects. Endophytes are producers of many bioactive compounds that have high biotechnological value.

Colonization of endophytes in the host plant increases the nutrition uptake by the plant, enhanced plant growth, and can increase the production of secondary metabolites that are biologically active (Zhang *et al*, 2006) which can be used by humans.

Fungal endophytes belong mainly to group ascomycota. Endophytic fungi range from artic to tropical regions and also occur in wilderness and agricultural areas. The endophytic fungi have wide range of impact on the host plant that varies from completely without symptoms or that can become pathogenic to the host plant in change of environmental conditions. The endophyte can also be in a mutualistic relationship with the host plant. The overall plant and host relation depends upon host plant, environment, age and genotype diversity (Saikkonen, 1998). Endophytic are divided into groups (Jia *et al*, 2016) -

(a) Mycorrizal
(b) Non pasture endophytic fungi
(c) Pasture endophytic fungi

The overall endophyte-plant interaction depends on environment, age and genotype diversity of the host plants (Saikkonen, 1998).

Endophytes living inside the plant tissues produce hydrolases as a mechanism of resistance to overcome host attack for the pathogenic invasion and for getting nutrition from host. Such enzymes includes esterases, pectinases, lipases and cellulases (Petrini et al,1993), phosphatases (Maccheroni *et al*, 1998), proteinase (Reddy *et al*, 1996) and α 1,4 glucan lyase (Nielsen *et al*, 2000).

The evidence taken from microbes associated with fossilized leaves and stems show that endophytes associated with plants may have evolved billions of years ago. In this long period of co-evolution the endophytic fungi have gradually adapted themselves to the environment by genetic variation either by up taking the plant DNA or by inserting their DNA into the host plant. This is how certain plants have the ability to produce same compounds as the host plant (Zhang *et al*, 2006). An example of this is both plant and fungi producing gibberlins.

Endophytes are considered as mine of novel bioactive compounds so they are exploited to find novel metabolites having biological activities.

2.3 Metabolites produced by endophytic fungi

Amongst all the endophytic organisms, endophytic fungi are the most studied group of organisms. A lot of research work is going on endophytic fungi.

The impact of endophytic fungi on our lives is seen in many ways, from production of myco-diesel as an alternate source of fuel from *Gliocladium roseum* (Strobel *et al*, 2008) to insecticide production against potato tuber moth larvae and adult, from *Muscodar albus* (Lacey and Neven, 2006). In these extremes, endophytes are seen producing numerous important pharmacological compounds like antimycotics ambuic acid (Li *et al*, 2001) and cryptocin (Li *et al*, 2000), ergoflavin having anti inflammatory activity (Deshmukh *et al*, 2009), torreyanic acid (Lee *et al*, 1996) acid and taxol (Strobel *et al*, 1996) having anticancerous activity, pestacin having anti oxidant activity (Harper *et al*, 2003), Cytonic acids A and B showing antiviral activity (Guo *et al*, 2000), subglutinols A and B as immunosuppressive agents (Lee *et al*, 1995).

Endophytes are shown to produce various bioactive compounds that are used against various diseases and also used in cosmotology, agriculture and food industry.

Taxol is one of the most promising anti cancerous agent isolated from endophyte, *Metarhizium anisopliae* from bark of Taxus tree (Zhang *et al*, 2009). Many other compounds were isolated from endophytes like camptothecin having antifungal properties from *Nothapodytes foetida* (Joseph and priya, 2011), huperzine A act as cholinesterase inhibitor from *Huperzia serrata*. Anticancerous activity possessed by lignins isolated from *Podophyllum hexandrum* and resins

isolated from *P. emodi* (Konuklugil, 1995). Many compounds like ampicillin, oxacillin possess strong antibacterial properties (Akiyama *et al*, 2001).

The endophytic fungi residing inside the plants produce two types of metabolites, primary metabolites and secondary metabolites. Primary metabolites are used by endophytic fungi for growth and are produced majorly during growth phase. Secondary metabolites are largely produced in stress conditions in the stationary phase of the endophyte. The secondary metabolites are of great biological value as they have great biological activities and are thus called as novel metabolites.

The secondary metabolites produced by the endophytes can be of different classes which are-

1. Alkaloids (amines and amides, indole derivates and pyrrolizidines)
2. Steroids
3. Terpenoids (sesquiterpenes and diterpines)
4. Isocoumarin derivatives
5. Quinines
6. Flavonoids
7. Phenylpropanoids and lignans
8. Peptides
9. Phenol and phenolic acids
10. Aliphatic compounds and others (R.X. Tan et al., 2001)

Endophytes are considered as mine of novel bioactive compounds and using techniques of genetic engineering these can be made more useful for humans. Naturally screened endophytic fungi that have high productivity can be selected for fermentation and can be produced in large quantities. The fermentation of fungal cell culture is far easier than plant cell culture as it is less expensive, simple, the cost of production is low, the time for fermentation is also very less and the parameters of culture can be optimized according to the applications.

Table 2: Some bioactive compounds produced by endophytic fungi

Endophytic fungus	**Host plant**	**Bioactive compound**	**Reference**
Fusarium mairei	*Taxus chinensis*	Paclitaxel	Cheng *et al*, 2007
Aspergillus fumigatus	*Juniperus communis*	Podophyllotoxin	Kusari *et al*, 2009
Entrophospora infrequens	*Nothapodytes foetida*	Camptothecin	Amna *et al*, 2006
Alternaria sp	*Catharanthus roseus*	Vinblastine	Gou *et al*, 1998
Fusarium oxysporum	*Catharanthus roseus*	Vincristine	Zhang *et al*, 2000
Acremonium sp.	*Huperzia serrata*	Huperzine A	Li, W *et al*, 2000
Alternaria tenuissima	*Vaccinium* sp	Flavonoids	Shu-Nou *et al*, 2009
Cephalosporium sp	*Paris polyphylla*	Disogenin	Xian-dong *et al*, 2007
Chaetomium globosum	*Hypericum perforatum*	Hypericin	Kusari *et al*, 2008
Rhizopus oryzae	*Iris germanica*	α-Irone, β-Irone	Zhang *et al*, 1999

2.4 Antimicrobial compounds from endophytic fungi

There is an urgent need for new antimicrobial compounds in the market. New antibacterial compounds are needed due to increased resistance of bacteria to the already existing antibiotics in the market. The microorganisms that are getting resistant at fastest rate are *Staphylococcus aureus* which is methicillin resistant, *Enterococcus faecium* resistant to vanomycin, *Streptococcus pneumonia* resistant to penicillin, tuberculosis bacteria which is resistant to many drugs are just a few examples of such bacteria (Deshmukh *et al*, 2015). There is also increased infections by fungus at the time of cancer, chemo, organ transplant and in allogenic transplantation of bone marrow, but not many antifungal agents are available for treatment of these fungal infections which are life threatening. Similarly, there is resistance development by

the fungus mostly in long term infected patients (Deshmukh *et al*, 2012). So there is an urgent need of finding new ways of dealing with these micro organisms. So endophytes are considered as a treasure of bioactive compounds and are exploited worldwide to find new antimicrobial agents to deal with these problems.

Table 3: Some compounds isolated from endophytic fungi showing antibacterial activity

Endophytic fungi	**Plant source**	**Antimicrobial Compounds**	**Reference**
Phomopsis longicolla	*Dicerandra frutescens*	Dicerandrols A and B	Wagenar and Clardy, 2001
Pestalatiopsis sp.	*Lichen Clavaroid sp.*	Ambuic acid	Ding *et al*, 2008
Guignardia sp.	*Hopea hainanensis*	Rhizoctonic acid Monomethylsulochrin	Wang *et al*, 2010
Penicillium citrinumstrain	*Bruguiera gymnorrhiza*	Emodin and Erythritol	Li *et al*, 2010
Fusarium sp.	Mangroove plant	Metal complexes of Fusaric acid	Pan *et al*, 2011
Trichoderma sp.	*Paeonia delavayi*	Trichoderic acid, Cyclonerodiol, Cyclonerodiol oxide, hydroxytrichoacorenol and sorbicillin	Wu *et al*, 2011
Phomopsis sp.	*Cistus salvifolius*	Pyrenocines J-M	Hussain *et al*, 2012
Aspergillus sp.	*Bauhinia guianensis*	Fumigaclavine C	Pinheiro *et al*, 2013
Phoma sp. *NRRL46751*	*Saurauia scaberrinae*	Phomapyrrolidone B and C	Wijeratne *et al*, 2013
Fusarium proliferatum	*Macleaya cordata*	Sanguinarine	Wang *et al*, 2014

Table 4: Some antifungal compounds isolated from endophytic fungi

Fungus	**Plant source**	**Antifungal compound**	**Reference**
Dothiorella sp.	*Avicennia marina*	Cytosporone B	Newman *et al*, 2007
Phomopsis sp.	*Laurus azorica*	Cycloepoxylactone	Harper *et al*, 2003
Epichloe typhina	*Phleum pratense*	Epichlicin	Krohn *et al*, 2008
Hormonema sp.	*Juniperus communis*	Enfumafungin	Abdou *et al*, 2010
Trichoderma harzianum	*Llexcornuta Lindl*	Trichodermin	Liu *et al*, 2004
Botrysphaeria rhodina	*Bidens pilosa*	Botryorhodine A	Cafeu *et al*, 2005
Pezicula sp.	*Fagus Sylvatica*	Echinocandins D	Seto *et al*, 2007

2.5 Analysis of endophytic fungal extracts

TLC of the fungal extracts is done to fractionate the various compounds of the fungal extract. It is a convenient method as all the compounds can be separated on a plain surface of TLC plate and gives good resolution. The TLC plate is made by applying polar adsorbant like silica, and the separation in TLC is done on polarity basis of the solvent system. The compounds that are non polar and have least affinity towards the polar adsorbant (silica) travel towards the end of the plate, whereas polar compounds stay near to the origin of the plate due to their affinity towards the silica (Kagan *et al*, 2014). With the help of TLC different compounds present in the extract are separated out and all the different fractions are then tested to find out the fraction responsible for the activity of the extract.

MATERIAL AND METHODS

3.1 Laboratory instruments and chemicals

Apparatus and instruments used-

Petri plates, forceps, blades, flasks, kuhner shaker, funnel, separation funnel, rota flask, pipettes, tips, spreader, paper discs, laminar, test tubes, test tube stand, centrifuge, rota evaporator, spectrophotometer, cuvettes, vortex, TLC plates and applicator, TLC chamber, UV reader for TLC plates, 96 well titre palte, ELISA reader

Chemicals used-

Muller Hinton agar, Muller Hinton broth, ethyl acetate, methanol, potato dextrose agar, DMSO, MTT (3-(4,5-Dimetylthiazol-2-YI)-2,5-Dipehnyltetrazolium Bromide), nutrient agar plates, n-hexane, toluene, chloroform, iodine, sterile water, antimicrobial agents (ampicillin and amphotericin at a concentration of 50µg/ml and 5µg/ml respectively).

3.2 Source of endophytic fungi

3.2.1 Pre isolated fungi from *Terminalia arjuna*

The pre isolated endophytic fungi cultures, two from stem (AS2(ii) and AS1) and one from leaves (AL4) of *Terminalia arjuna* were provided by TIFAC CORE, Thapar University.

3.2.2 Isolation of endophytic fungi (Jariwala and Desai, 2018)

To isolate endophytic fungi, first the plant samples (leaves and bark) from *Eucalyptus teriticornis* were collected from CORE, Thapar Institute of Engineering and technology. The isolated plant samples were then surface sterilized. For surface sterilization, the leaves were cut into pieces and put into two autoclaved beakers. Net was tightly tied to the rim of the beakers and were then kept under running tap water for 10min. Plant samples were then washed with detergent or soap for 5min with continuous shaking. The beakers were again kept under running tap water to completely take off the detergent. The plant samples in the beakers were then dipped in 70% ethanol for 1min followed by dipping in 1% sodium hypochlorite for 5min. Each beaker

was then covered with a lid after dipping the plant samples in ethanol. Lid of the beakers were opened in LAF (Laminar air flow) and the plant samples were given three consecutive washings with sterile water. Moisture was removed from the plant samples by keeping in between layers of filter paper. The leaves after drying were cut into pieces and transferred on to PDA plates containing antibiotic chloramphenicol.

The plates were incubated at 27±2˚C, and observed for growth of endophytic fungi without any contamination from all plant samples. The plates were observed for 14 days. Growth of endophytic fungi was seen in four samples, these were then transferred into new plates that were incubated at 27±2˚C till complete growth of fungus occur, after complete growth the plates are stored at 4˚C. The four endophytic fungi isolated were named as EL, EL1, EL2 and EL3.

3.3 Sub-culturing of endophytic fungi

From the master plate of the endophytic fungi the culture was transferred to new PDA plate with the help of sterile blade. Then this new plate was kept for 14 days at 25˚C for proper growth of the fungus.

3.4 Culturing of fungus

Medium used for culturing the fungus was PDB. It was prepared in the laboratory by boiling fresh raw potatoes and pressing them through muslin cloth and then adding dextrose sugar to it. Then the media was autoclaved before using to culture the fungus. PDB from HiMedia was also used. 24gm of PDB was suspended in 1000ml of distilled water and then autoclaved the media before culturing the fungus.

The medium was autoclaved at 121˚C under 15psi pressure for 15min.

Table 5: Composition of PDB

Potato infusion	200 grams/litre
Dextrose	20 grams/litre

After complete growth of mycelia on the PDA plates, the freshly grown mycelia were transferred by sterile blade and forceps to flask containing 250ml of Potato Dextrose broth. Flasks were then kept in incubator for 21 days at 27±2˚C at stationary conditions.

After completion of the incubation period, the PDB containing fully grown culture of fungus was filtered using sterile muslin cloth for separating the culture broth from mycelia (Nath *et al*, 2016).

3.5 Extraction

Fungal metabolites produced in the broth during incubation period are extracted from the culture broth by solvent extraction using ethyl acetate as solvent. 250ml of ethyl acetate was added to the culture broth in the separating funnel and shaken for 15-20min continuously. Then the separating funnel is made to stand for some time till the organic and aqueous phase separate. The aqueous phase at the bottom is discarded and the organic phase at the top containing fungal metabolites was collected.

The organic phase was then evaporated using rota evaporator at 35˚C for 10-15min. The ethyl acetate from the organic phase evaporates leaving behind the compounds, the compounds containing minimal amount of ethyl acetate were then transferred into a small tube of known weight and kept for drying. After complete evaporation of ethyl acetate the weight of the extracts was found out. These extracts were then used for checking the antimicrobial activities by dissolving in methanol (antibacterial and the antifungal).

3.6 Screening for antimicrobial activity of crude extracts

3.6.1 Cultures for testing antimicrobial susceptibility

The test cultures used for antimicrobial susceptibility testing were taken from Nicholas Piramal ltd. For testing bacterial susceptibility 4 cultures of bacteria were used

1. *Escherichia coli* (gram negative bacteria)
2. *Pseudomonas aeruginosa* (gram negative bacteria)

3. *Staphylococcus aureus* (gram positive bacteria)

4. *Bacillus megaterium* (gram positive bacteria)

For testing fungal susceptibility fungal culture used was

1. *Candida albicans*

Medium for antifungal susceptibility test was potato dextrose agar (PDA) (Table 6), which is infusion of dextrose and potato.

Table 6: Composition of PDA (pH 5.6±0.2)

Components	**Value per litre**
Potato	4g
Dextrose	20g
Agar	15g

Medium used for antibacterial susceptibility test was Muller Hinton agar (MHA) (Table 7).

Table 7: MHA has pH 7.3±0.1 with composition

Components	**Value per litre**
Beef extract	2g
Starch	1.5g
Acid hydrolases	17.5g
Agar	17g

3.6.2 Preparing McFarland standard

McFarland's standards are used to approximately standardize the bacterial number in a liquid suspension by comparison of turbidity of the liquid suspension to the standards of McFarland (Table 8). McFarland standard contains sulphuric acid and barium chloride chemical solution. There is formation of barium sulphate fine precipitates, by reaction of barium chloride and sulphuric acid. After shaking it well the bacterial suspension of known concentrations can be compared to turbidity of McFarlands standard visually as indicated below.

Table 8- Mc Farland Standard

McFarland Standard	**1% H_2SO_4 (ml)**	**1% $BaCl_2$ (ml)**	**CFU/ml**
0.5	9.95	0.05	1.5×10^8
1.0	9.9	0.1	3.0×10^8
2.0	9.8	0.2	6.0×10^8
3.0	9.7	0.3	9.0×10^8
4.0	9.6	0.2	1.2×10^9
5.0	9.5	0.4	1.5×10^9
6.0	9.4	0.5	1.8×10^9
7.0	9.3	0.6	2.1×10^9
8.0	9.2	0.7	2.4×10^9
9.0	9.1	0.8	2.7×10^9
10.0	9.0	0.9	3.0×10^9

3.6.3 Techniques for screening crude fungal extracts

3.6.3.1 Agar disc diffusion method (Bauer, 1966)

Most significant research on agar disc diffusion was done at University of Washington by Kirby and Bauer (Bauer, 1966). It is one of the most widely used tests to check the antimicrobial susceptibility of an antibiotic. Bacterial culture of standard turbidity (0.5 McFarland standard) is used and a lawn of bacterial culture is spread on the plates.

Diffusion refers to the movement of the antimicrobial agent through the agar, which tells about the antibacterial susceptibility of the compound. A disc containing the compound impregnated on it is kept in the middle of the plate containing the lawn of bacterial culture. The plate is then incubated and if the plate after incubation contains complete bacterial lawn over the plate then the compound has no antibacterial activity against that particular bacterium. But if there is a zone around the disc which shows no bacterial growth, it indicates that the compound has antibacterial properties against that bacterium. The more the diameter of the zone, more is antimicrobial activity of that compound. This zone around the disc is called as the zone of inhibition. This zone of inhibition is also dependent upon the concentration of the compound, zone will have larger diameter if the concentration of the compound is more and similarly the zone will be smaller if the concentration of the compound is less. There is always circular zone of inhibition as the growth of the test organism is always confluent.

Reagents used-

MHA (HiMedia Pvt Ltd) medium is used for antibacterial testing. The composition of MHA is given in table 7. 33.9gm of MHA is dissolved in 1000 ml of distilled water in flask.

PDA is used for antifungal testing. The composition of PDA (HiMedia Pvt Ltd) is given in table 6. 39gm of PDA is dissolved in 1000ml of distilled water in a flask.

Then the medium is autoclaved for 15min at 15psi pressure at 121˚C. 25-30ml of the autoclaved media is then poured into each petriplate before solidification of media.

Materials required-

Autoclaved tips, autoclaved paper disc, spreader, autoclaved forceps, ethanol, petriplates, cultures (*Candida albicans* and *E.coli*, *Pseudomonas aeruginosa*, *Bacillus megaterium*, *Staphylococcus aureus*)

Procedure-

Firstly, all the stock bacterial and fungal cultures were revived, the revived culture was then inoculated into the broth and kept at 37˚C in a shaker till the turbidity reaches similar to 0.5 McFarland standard. The bacterial and fungal cultures were then spread on MHB and PDA

plates respectively and kept for 5min. Paper discs impregnated with fungal extracts (20µl) were then placed on the plates with culture lawns. The plates containing bacterial and fungal cultures were then overnight incubated at 37˚C and 28˚C respectively. In fungal culture it sometimes takes more than 24 hrs to show zone of inhibition. Positive controls used were ampicillin (50µg/ml) and amphotericin (5µg/ml).

3.6.3.2 Determination of percentage inhibition by MTT assay (Malekinejad *et al*, 2012)

MTT assay is used to tell the no. of living cells present. MTT is a yellow colored tetrazole dye which in presence of living cells is reduced to formazan (purple colored crystals). The reduction of tertrazolium to formazan is due to cellular oxidoreductase enzymes which are NADPH dependant. The more the no. of living cells present larger will be the purple formazan crystal formed (Tim, 1983).

Reagents required-

MHB, MTT(5mg/ml) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, pure DMSO (dimethyl sulphoxide), bacterial culture *(E.coli, Pseudomonas aeruginosa, Bacillus megaterium, Staphyllococcus aureus*), ampicillin as positive control.

Materials required-

96 well titre plate, autoclaved tips, pipettes, ELISA reader, test tubes

Procedure-

The bacterial culture is first inoculated into MHB and incubated at 37˚C on a shaker till it reaches the turbidity of 0.5 McFarland standard. Open the titre plate in laminar. Keeping the total volume of each cell 100µl, 50µl bacterial suspension is added into each cell, rest 50 µl MHB and extract dissolved in MHB are added according to the concentration at which the extract it to be tested. After taking initial OD at 540nm the plate is kept for 24hrs for incubation at 37˚C. After overnight incubation 10µl of 5mg/ml MTT is added into each of the well. After incubating it for 4hrs at 37˚C, we see formation of purple colored formazan crystals in some wells and no or less crystals in some wells. 80µl is picked up from each well without disturbing the crystals in any

well. Then pure DMSO was added in each well to completely dissolve the crystals. Then after keeping for 15min, optical density was finally taken at 540nm.

Three controls are used in the experiment. First is media control containing media only, second is culture control containing media and culture without extract, third is antibiotic control containing media and culture with ampicillin at a concentration of 50µg/ml.

IC_{50} is also determined by MTT assay by serially diluting the extract two fold, and then determining the minimum concentration at which the extract shows inhibition of the cell growth.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of solvent control- Absorbance of sample}}{\text{Absorbance of solvent control}} \times 100$$

3.7 Phytochemical analysis of the fungal crude extract (Devi *et al*, 2012 and Bhardwaj *et al*, 2015)

3.7.1 Test for alkaloids

Test	Inference
Wagner's reagent (few drops) added to fungal extract.	Alkaloids present if reddish brown precipitates are seen.

3.7.2 Test for fats and oil

Test	Inference
0.5N KOH was added to the fungal extract followed by addition of 2-3 drops of phenolphthalein and then incubated for 1hr in water bath.	Fats and oils presence is confirmed if soap formation is seen after 1hr.

3.7.3 Tests for flavonoids

Test	Inference
To the fungal extract sodium hydroxide is added followed by addition of hydrochloric acid.	Flavonoids present if after addition of hydrochloric acid decolorisation is seen.

3.7.4 Test for tannins and phenolics

Test	Inference
Ferric chloride is added to the fungal extract.	Tannins present if blue color is seen. Phenolics present if green color is seen.

3.7.5 Test for glycosides

Test	Inference
Glacial acetic acid was added to the fungal extract followed by ferric chloride 5% and conc. sulphuric acid.	Glycosides present if at the interface of liquid layers red brownish color is seen.

3.7.6 Test for carbohydrates

Test	Inference
To the fungal extract Molisch's reagent was added followed by addition of conc. HCL from the side of the test tube.	Carbohydrates present if purple colored ring is seen.

3.7.7 Test for amino acids

Test	Inference
5% Ninhydrin is added to the fungal extract and kept for 10 min in boiling bath.	Amino acids present if violet color is present.

3.8 Estimation of phenolic content (Shah *et al*, 2015)

Reagents required-

Gallic acid (1mg/ml), deionized water, Folin-Ciocalteu reagent and 20% sodium carbonate

Materials required-

Test tubes, test tube stand, pipette, tips and cuvettes

Procedure-

To estimate the phenolic content first, 1ml of the fungal extract was added to 10ml of deionised water. Then 1ml of Folin-Ciocalteu reagent was added, after 5min 20% sodium carbonate was added to the mixture. Test tubes were incubated in the dark for 15min. Took absorbance of each sample at 750 nm.1mg/ml concentration of gallic acid was used as standard and later a graph was plotted so as to estimate the phenolic content in fungal extracts.

3.9 Estimation of flavonoid content (Shah *et al*, 2015)

Reagents required-

5% sodium nitrite, 10% aluminium chloride, 1.0M sodium hydroxide and quercetin

Material required-

Test tubes, test tube stand, pipettes, tips and spectrophotometer

Procedure-

To estimate flavonoid content first 1ml of fungal extract was added to 4ml water. Then 5% sodium nitrite was added to each test tube and after 5min 10% aluminium chloride was added followed by addition of 2ml of 1.0M sodium hydroxide. Took absorbance of each sample at 430 nm. Standard curve using Quercetin was made.

3.11 Chromatographic analysis of the crude fungal extract *(Amakura et al, 2009)*

Thin layer chromatography was used for qualitative analysis of the fungal extract. A fast simple and easy thin layer chromatographic technique was used for giving qualitative assurance of the fungal extract's secondary metabolites. The compounds were separated on TLC plates containing silica using mobile solvent of n-hexane and ethyl acetate (3:1) for *Eucalyptus tereticornis*. The plates after separation of the compound were then viewed under UV light and were also seen by placing TLC plates in chamber containing iodine. The solvent system was optimized for sample EL from *Eucalyptus tereticornis* using solvents of varying polarity and the one showing best results was selected for final TLC run.

Optimization of the solvent for TLC was done by running in pure solvents and then varying the ratio of polar and non polar solvents.

The different fractions obtained after the run of crude fungal extract were separately scratched off with silica from the TLC plates. Then all the different fractions are put in different tubes and dissolved in small amount of ethyl acetate. All the tubes were vortexed for 30min till the fractions dissolve in ethyl acetate. After vortexing, the tubes are centrifuged to settle down the silica and get the extract dissolved in ethyl acetate as supernatant. The supernatant was then transferred to another tube and kept at 37˚C to evaporate ethyl acetate and get the extract. Then antimicrobial test against *Staphylococcus aureus* was performed for each fraction of sample EL.

3.11 Morphological identification of fungus

The fungus isolated from the plant was then examined morphologically using lactophenol cotton blue dye. Firstly glass slides were taken and cleaned with ethanol, and then a drop of the dye was put over the slide. Mycelia from the fungus plate were put over the slide using a needle. The

spores from the mycelia were spread on the plate using the needle. A clean cover slip was then placed on the slide and the fungus was observed under microscope. Morphological identification of fungi can be done by observing the mycelia under microscope.

The morphological identification (Mishra, 2005) of fungus was done on the basis of the kind of hypha and spores. Hypha can be septate, pseudoseptate or aseptate. Spores can be sexual (basidiospore, zygospore or ascospore) or asexual (sporangiospore, macro or micro conidium, blastospore, chlamydospore or arthrospore). Colony morphology of the fungus is seen on the basis of size and basic shape of the colony, elevation of the colony from the side of the plate, color of the plate and surface of the plate (smooth, cottony, rough etc).

RESULTS AND DISCUSSION

4.1 Source of endophytic fungi from *Terminalia arjuna* and *Eucalyptus tereticornis*

As there is an urgent need of new antimicrobial agents so endophytic fungi are isolated from medicinal plants and are screened for production of compounds that have capability to act as antimicrobial agents against different microorganisms.

4.1.1 Isolation of endophytic fungi from *Eucalyptus tereticornis*

Eucalyptus tereticornis present in CORE, Thapar Institute of Engg. and technology were selected (Fig. 1A) and many leaf samples were taken. Then after surface sterilization process the leaves were kept on PDA plates (Fig. 1B) and incubated for 14 days at 28˚C. The plates were checked regularly for growth of endophytic fungi. A total of four endophytic fungi were obtained and named as EL (Fig. 1C), EL1 EL2 and EL3. Out of these four extracts only EL was selected based on preliminary tests performed only on extracts obtained from *Eucalyptus*.

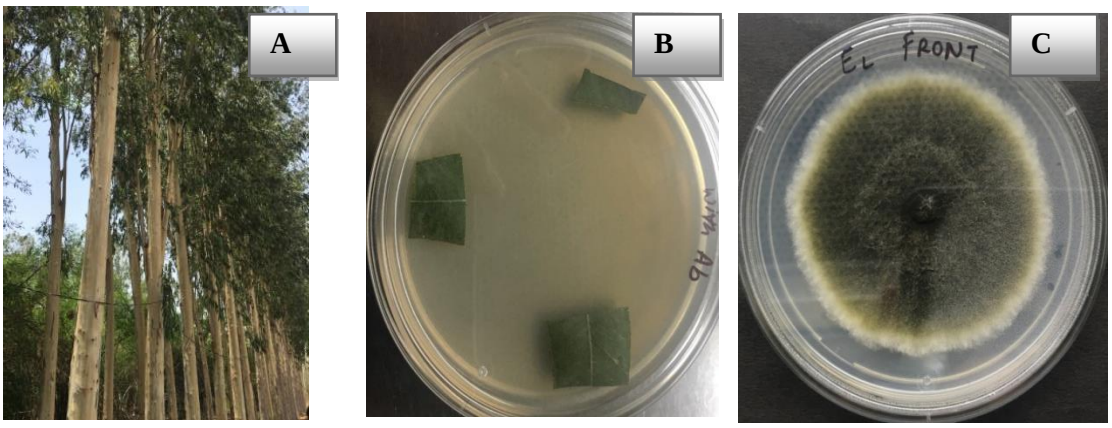


Fig. 1: A) *Eucalyptus tereticornis* selected for isolation **B)** leaf segment kept on PDA plate **C)** Fungal endophyte culture isolated from *Eucalyptus* leaf.

4.1.2 Endophytic fungi from *Terminalia arjuna*

Pre isolated endophytic fungi from the stem and leaves of Arjuna were given. The endophytic fungi isolated from the leaf was named as AL4 (Fig. 2A) and the isolates from stem were named as AS2(ii) (Fig. 2B) and AS1 for reference in further studies.

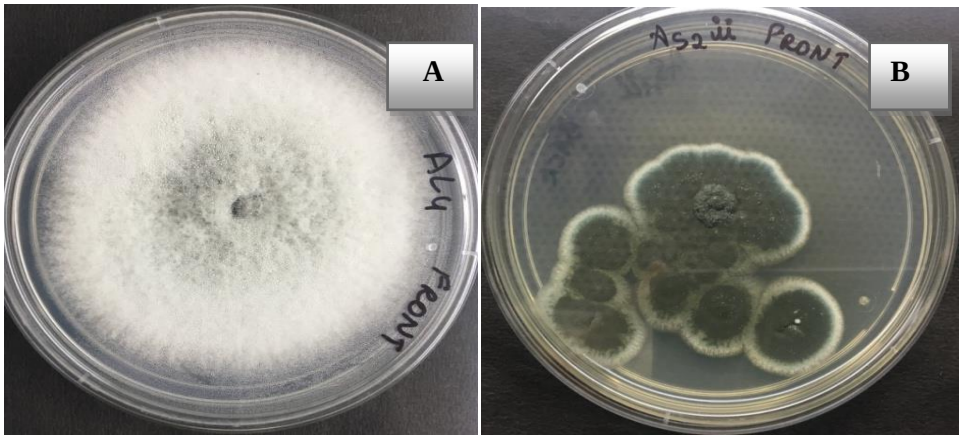
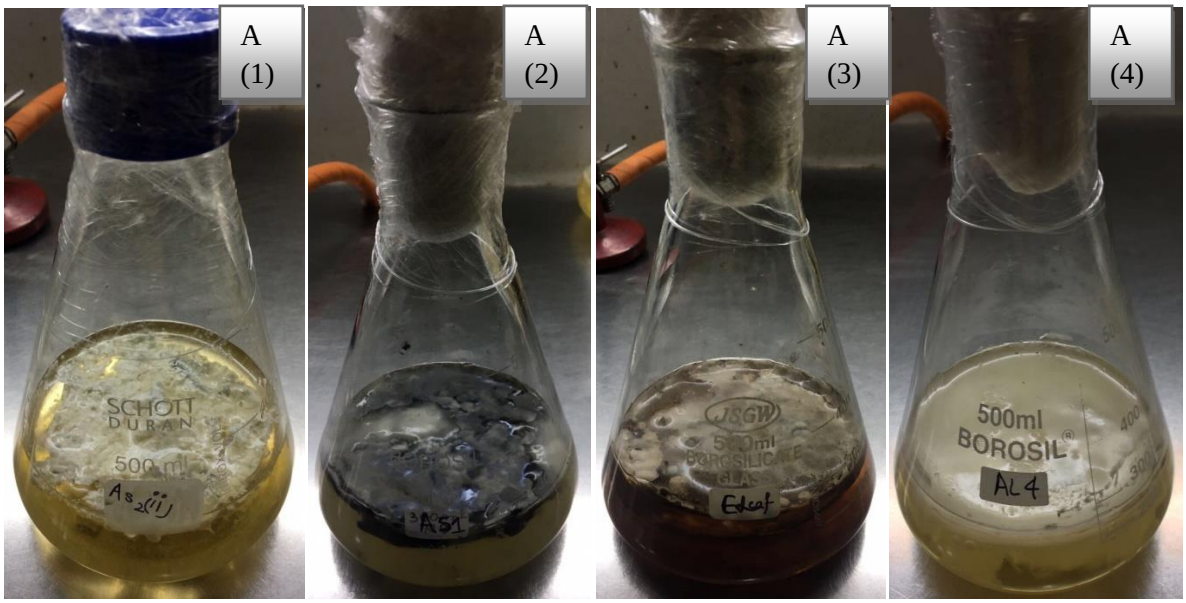


Fig. 2: A) Endophytic fungus AL4 **B)** Endophytic fungus AS2(ii).

Each isolated fungus was then inoculated in PDB and incubated for 21 days at 28˚C at stationary condition. After incubation complete growth of mycelia takes place (Fig. 3: A(1)- A(4)) and the broth is filtered (Fig. 3B). The fungal mycelia are separated from the broth using sterile muslin cloth. Ethyl acetate extraction (Fig. 3C) was then done of the fungal broth and the organic layer after extraction was taken out and rota evaporated (Fig. 3D) to get the final extracts (Fig. 3E) for further studies. The extracts so obtained were kept at 4˚C and were used as and when required.



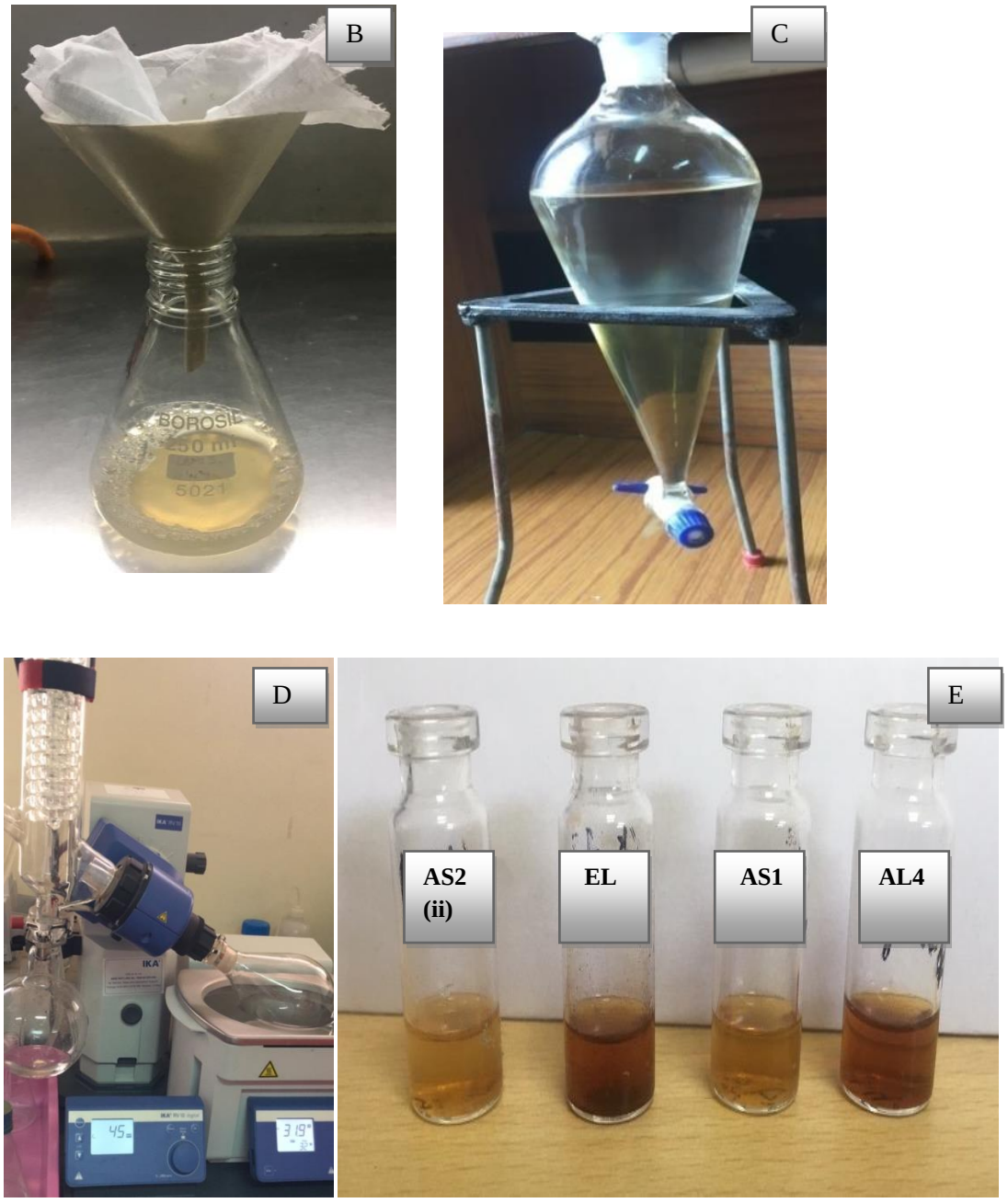


Fig 3: A) Fermented fungal cultures (1)- AS2(ii) (2)- AS1 (3)- EL (4)- AL4 **B)** Filtration of the fungal culture **C)** Solvent extraction of the filtrate **D)** Rota evaporator **E)** Fungal extracts.

4.2 Preliminary antimicrobial activity

Preliminary antimicrobial activity of each extract was tested by paper disc diffusion method. Each extract was tested against five test cultures, *Pseudomonas aeruginosa*, *Bacillus megaterium*, E.*coli*, *Staphylococcus aureus* and *Candida albicans.* Zone of inhibitions were measured in millimeters to find out the antimicrobial activity of each extract and zone of inhibition of ampicillin (50µg/ml) and amphotericin (5µg/ml) were also measured. Methanolic fungal extracts at a conc. of 5mg/ml were used and 20µl of each extract was put on each paper disc. On the basis of these preliminary tests (Table 9) further selection was done.

Table 9: Preliminary antimicrobial activity of fungal extracts.

Endophytic fungal extract	**Test cultures**				
	Pseudomonas aeruginosa	***Bacillus megaterium***	***E. coli***	***Staphylococcus aureus***	***Candida albicans***
AS1	0	0	0	0	0
AS2(ii)	0	0	0	0	0
AL4	0	12mm	0	0	0
EL	0	11mm	0	21mm	0
Ampicillin	29mm	25mm	26mm	28mm	×
Amphotericin	×	×	×	×	24mm

Three endophytic fungal extracts from *Terminalia arjuna* and one from *Eucalyptus teriticornis* were tested against the four bacterial and one fungal culture.

The zone of inhibition was shown by two of the four cultures against two bacterial test organisms.

AL4 gave a zone of inhibition of 12mm against *Bacillus megaterium.*

EL gave a zone of inhibition of 11mm (Fig. 4B) against *Bacillus megaterium* and a zone of 21mm (Fig. 5A) against *Staphylococcus aureus*.

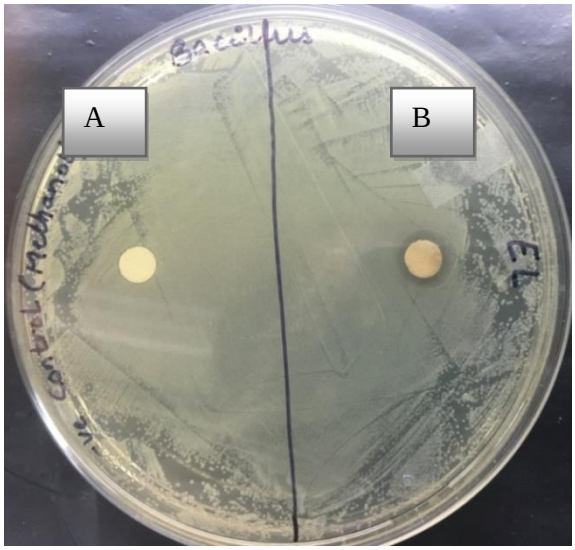


Fig. 4: Zone of inhibition of **(A**) -ve control and **(B**) EL (11mm) against *Bacillus megaterium.*

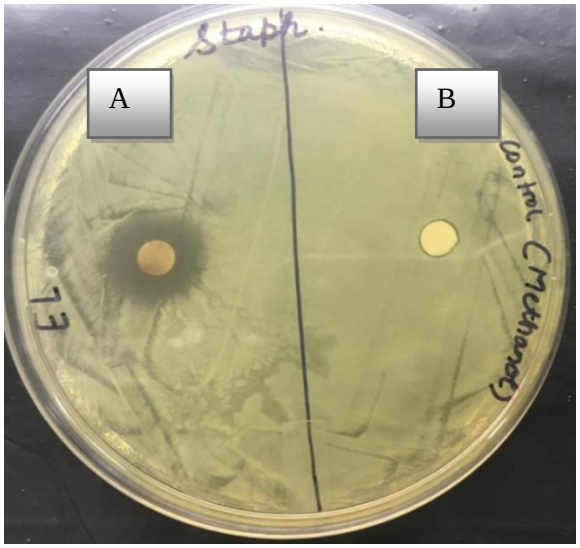


Fig. 5: Zone of inhibition of **(A)** EL(21mm) **(B) -**ve control against *Staphylococcus aureus.*

EL gave maximum zone of inhibition out of the four fungal extracts, but before selecting EL for further analysis, a confirmatory MTT test was done which gave the percentage inhibition of each extract at different concentrations.

4.3 Antimicrobial test by MTT assay

Crude extracts from endophytic fungi dissolved in MHB at concentrations of 100µg/ ml, 250µg/ml, 500µg/ml and 1000µg/ml were tested against *Pseudomonas aeruginosa* (Table 10), *Bacillus megaterium* (Table 11), *E. coli* (Table 12) and *Staphylococcus aureus* (Table 13).

Ampicillin was used as positive control at a concentration of 50µg/ml.

The MTT assay was carried out in 96 well titer plate (Fig. 6) and the final results were measured by taking O.D. at 540nm. After incubating plates for 4hours after addition of MTT in each well, the control containing ampicillin was colorless and had no crystals but there was appearance of purple colored formazan crystals in wells that showed low or less inhibition. More the number of crystals lesser is the inhibition by the fungal extracts.

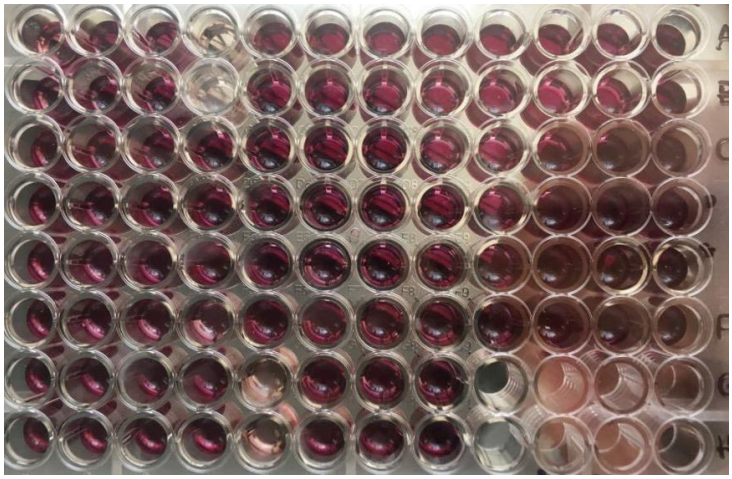


Fig. 6: MTT assay plate

Table 10: Percentage inhibition shown by extracts against *Pseudomonas aeruginosa*

Percentage inhibition exhibited by ampicillin- 86.14%

Concentration	Extracts			
	AS1	AS2(ii)	AL4	EL
100µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
250µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
500µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
1000µg/ml	No inhibition	No inhibition	No inhibition	No inhibition

Table 11: Percentage inhibition shown by extracts against *Bacillus megaterium*

Percentage inhibition exhibited by ampicillin- 74.31%

Concentration	Extracts			
	AS1	AS2(ii)	AL4	EL
100µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
250µg/ml	No inhibition	No inhibition	No inhibition	10.2%
500µg/ml	No inhibition	No inhibition	No inhibition	27.7%
1000µg/ml	No inhibition	No inhibition	39%	42%

Table 12: Percentage inhibition shown by extracts against *E. coli*

Percentage inhibition exhibited by ampicillin- 68.99%

Concentration	Extracts			
	AS1	AS2(ii)	AL4	EL
100µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
250µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
500µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
1000µg/ml	No inhibition	No inhibition	No inhibition	No inhibition

Table 13: Percentage inhibition shown by extracts against *Staphylococcus aureus*

Percentage inhibition exhibited by ampicillin- 82.34%

Concentration	Extracts			
	AS1	AS2(ii)	AL4	EL
100µg/ml	No inhibition	No inhibition	No inhibition	No inhibition
250µg/ml	No inhibition	No inhibition	No inhibition	7.2%
500µg/ml	No inhibition	No inhibition	No inhibition	22.5%
1000µg/ml	No inhibition	No inhibition	No inhibition	60%

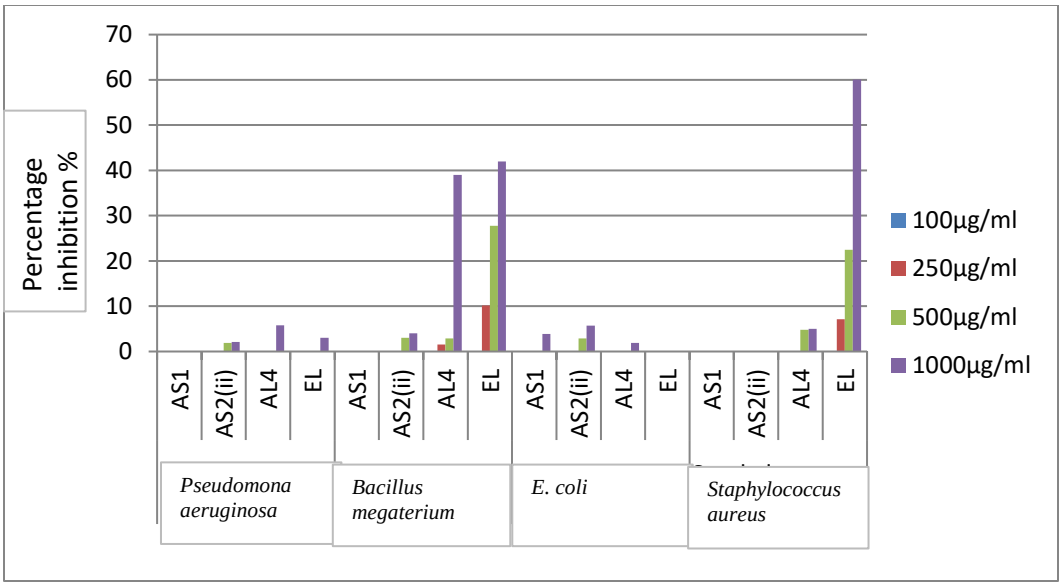


Fig. 7: Percentage inhibition shown by the extracts against test bacterial strains.

Crude extracts of endophytic fungi showed prominent inhibition only against gram positive bacteria, *Staphylococcus aureus* and *Bacillus megaterium.* Fungal extracts EL and AL4 showed inhibitions against these above test cultures (Figure 7, Table 13, Table 11).

Crude extract of endophytic fungi EL showed inhibition of 7.2% at concentration of 250µg/ml, 22.5% at concentration of 500µg/ml and 60% at concentration of 1000µg/ml against *Staphylococcus aureus* (Table 13, Fig. 7). It also showed inhibition of 10.2% at concentration of 250µg/ml, 27.7% at concentration of 500µg/ml and 42% at concentration of 1000µg/ml against *Bacillus megaterium* (Table 11, Fig. 7).

Crude extract of endophytic fungi AL4 showed inhibition of 38.987% at concentration of 1000µg/ml against *Bacillus megaterium* (Table 11, Fig. 7).

The extract EL isolated from the leaves of *Eucalyptus tereticornis* showed maximum antibacterial activity against *Staphylococcus aureus* and *Bacillus megaterium*. This indicates that the isolated fungi produced metabolites that are effective against gram positive bacteria.

Jain *et al*, 2010 reported antimicrobial activity studies on *Eucalyptus* leaf extract against *Staphylococcus aureus, Streptococcus mutans*, *Escherichia coli* and *Candida albicans*

4.4 Estimation of flavonoid and phenolic content of crude endophytic fungal extracts

For finding phenolic content of the extracts first standard curve of gallic acid was made (Fig. 8).

Methanolic extracts at a concentration of 2.5mg/ml were used for the estimation of phenolic content. Absorbance for each extract was measured at 750nm.

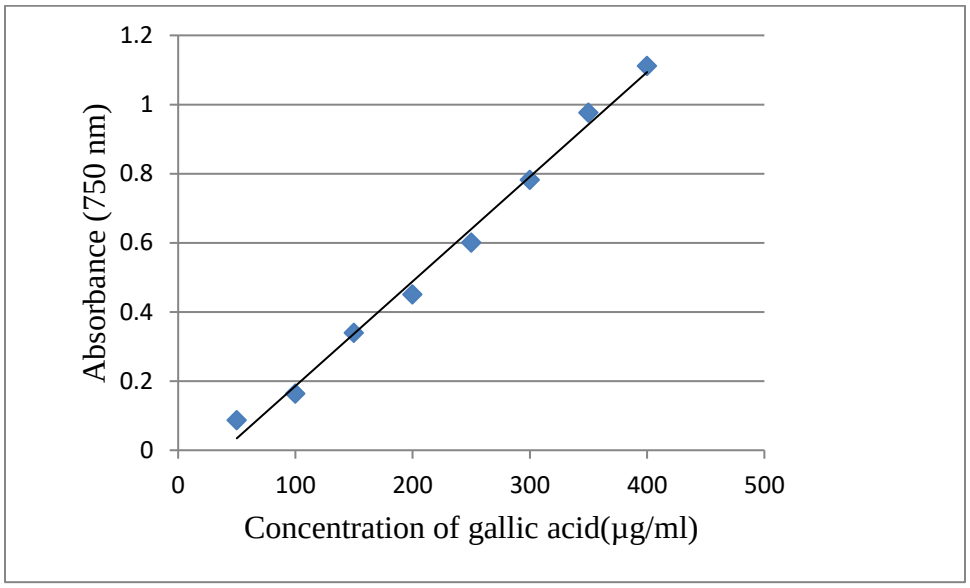


Fig. 8: Standard curve of gallic acid.

Table 14: Phenolic content of extracts.

Extract	**Total phenolic content (mg / g of gallic acid)**
AS1	76.8
AS2(ii)	122.4
AL4	98
EL	126.8

Maximum amount of phenolic content, 126.8 mg GAE/ g of dried extract is in fungal extract EL followed by AS2(ii), AL4 and the least amount of phenolics were present in AS1 (Table 14).

To find out the amount of flavonoids present first standard curve of quercitin was made (Fig. 9).

Extracts dissolved in methanol at a concentration of 2.5mg/ml were used to estimate the flavonoid content and the absorbance for each extract was measured at 510nm.

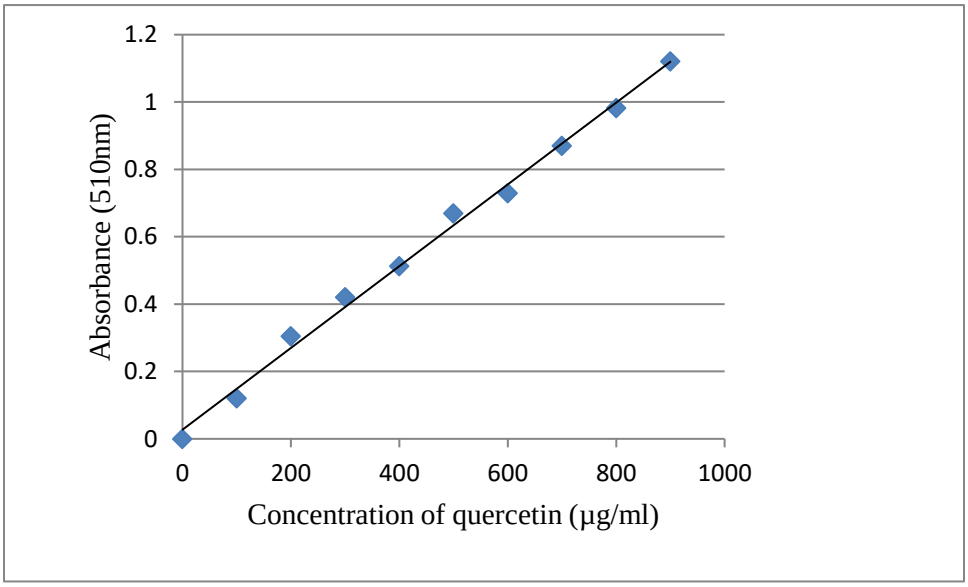


Fig. 9: Standard curve of quercetin.

Table 15: Flavonoid content of extracts.

Extract	**Total flavonoid content (mg/ g quercitin)**
AS1	74.4
AS2(ii)	43.2
AL4	232.4
EL	84.4

Maximum flavonoid content, 232.4 mg QE/ g of dried extract was present in AL4, followed by EL and AS1. Least amount of flavonoids were present in AS2(ii) (Table 15).

The high amount of phenolics seen in the extract EL (126.8 mg GAE/ g of dried extract) may be related to the maximum antimicrobial activity (Stankovic *et al*, 2012). On the basis of highest phenolic content and preliminary antimicrobial activity EL was selected for further analysis.

Stankovic *et al*, 2012 reported a study which indicated a relationship between phenolic content and antimicrobial activity. The extracts having higher phenol content were reported to have high antimicrobial activity.

4.5 Determination of IC_{50} of the selected fungal extract EL

IC_{50} of selected crude extract of endophytic fungi EL was performed using extract of stock concentration of 10mg/ml dissolved in MHB and ampicillin was used at concentration of 50µg/ml. The extract was tested at concentrations of 2µg/ml, 4µg/ml, 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml, 512µg/ml, 1024µg/ml, 2048µg/ml, 4096µg/ml and 8192µg/ml, to find out IC_{50} of the EL extract.

Since EL gave maximum inhibitory activity of 60% against *Staphylococcus aureus* at concentration of 1000µg/ml in MTT assay so it was further tested for IC_{50} values against *Staphylococcus aureus* only.

IC_{50} was determined by MTT assay in 96 well titer plate (Fig. 10) and the OD was taken at 540nm.

Table 16: Percentage inhibition shown by EL against *Staphylococcus aureus.*

Concentration (µg/ml)	**% Inhibition**
2	No inhibition
4	No inhibition
16	No inhibition
32	No inhibition
64	No inhibition
128	3%
256	8.2%
512	20.5%
1024	61.5%
2048	71%
4096	71.7%
8192	72.1%

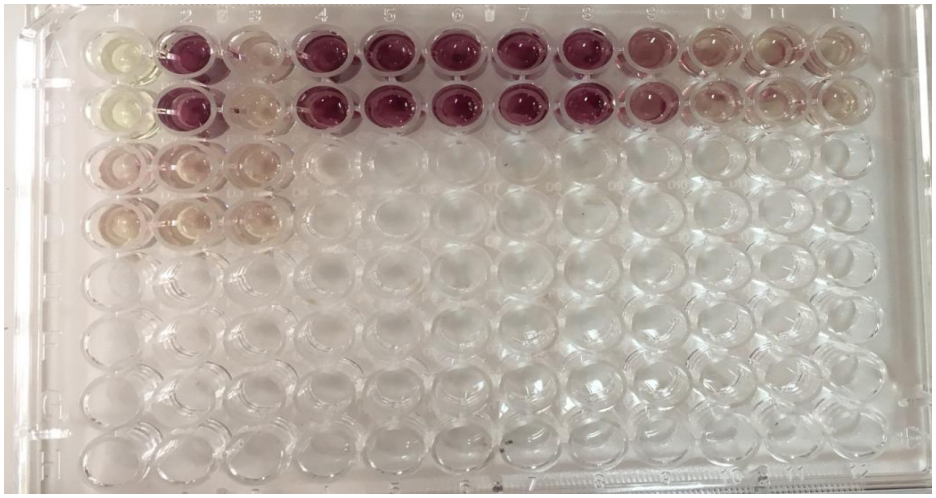


Fig. 10: Antimicrobial activity of EL by MTT Assay.

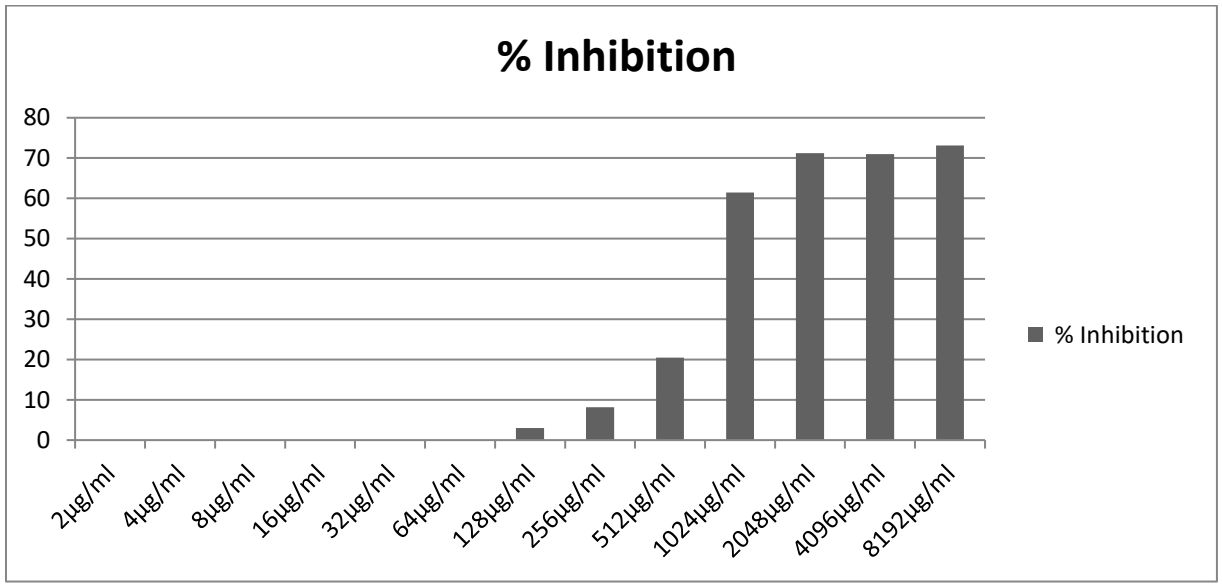


Fig. 11: Percentage inhibition against *Staphylococcus aureus* of extract EL

EL showed no inhibition from a concentration of 2µg/ml to a concentration of 64µg/ml. Then there was a gradual increase of inhibition by extract EL against *Staphylococcus aureus* from a concentration of 128µg/ml to a concentration of 2048µg/ml. It showed inhibition of 3% at a concentration of 128µg/ml, 8.2% at a concentration of 256µg/ml, 20.5% at a concentration of 512µg/ml, 61.5% at a concentration of 1024µg/ml and 71% at a concentration of 2048µg/ml (Table 16, Fig. 11). But after a concentration of 2048µg/ml there was no gradual and significant

increase in the inhibition by EL. Thus IC_{50} value for EL was seen at a concentration between 512µg/ml and 1024µg/ml.

4.6 Phytochemical analysis of selected crude fungal extract EL

Table 17: Phytochemical analysis results

Test	EL
For Alkaloids	**+ + +**
For fats and oils	**+**
For flavonoids	**+ +**
For tannins and phenolics	**+**
For glycosides	**+ +**
For carbohydrates	**-**
For amino acids	**+ +**

+ Positive for the test

- Negative for the test

EL showed presence of fats and oils, flavonoids, tannins and phenolics, glycosides, amino acids and strong presence of alkaloids, whereas it showed absence of carbohydrates.

4.7 Thin layer chromatography of crude extract EL

Solvent optimization was done using solvents of different polarity from polarity index for the crude extract EL (Table 18).

Table 18: Optimization of solvent for EL

S. No.	Solvent System	No. of fractions
1.	Pure n-hexane	5
2.	n-hexane and ethyl acetate (3:1)	7
3.	Pure ethyl acetate	0

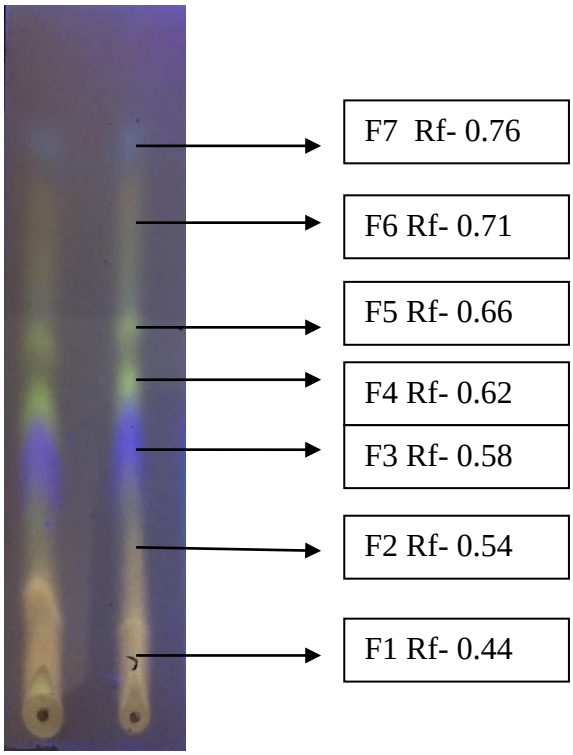


Fig. 12: TLC plate under UV light showing different fractions

Seven fractions were obtained by running the crude extract in the optimized solvent, n-hexane and ethyl acetate (3:1) v/v (Fig. 12, Table 18)

4.8 Antimicrobial activity of the fractions

All the seven obtained fractions were scratched off from the silica plate and each fraction of TLC was then tested against *Staphylococcus aureus* by paper disc diffusion method. All the fractions were used at a concentration of 1mg/ml and 20µl of the extract was impregnated on paper disc.

Fraction 1 gave zone of inhibition of 10mm against *Staphylococcus aureus* (Fig. 13), indicating that the compound responsible for the antimicrobial activity of EL is present in fraction 1.

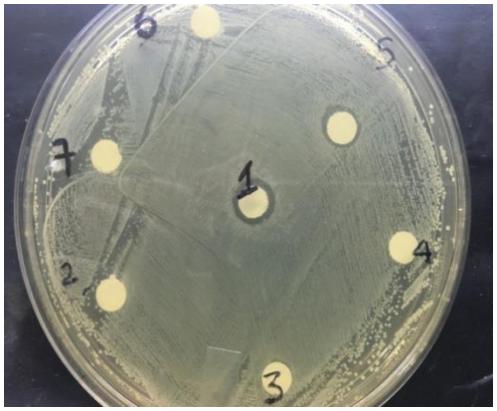


Fig. 13: Antimicrobial activity of different fractions (F1- F7).

4.9 Morphological identification of EL

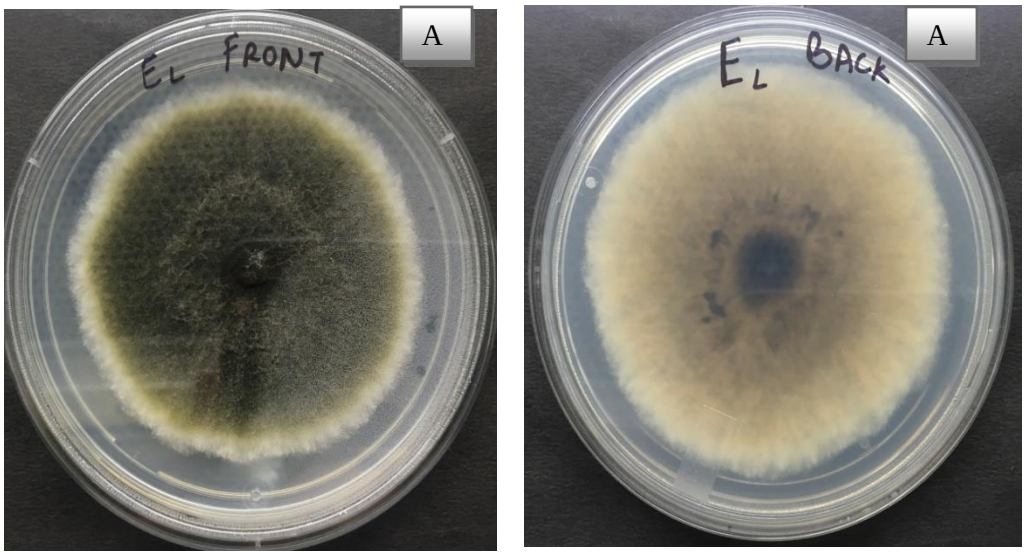


Fig. 14: Morphology of EL **A)** Front **B)** Back

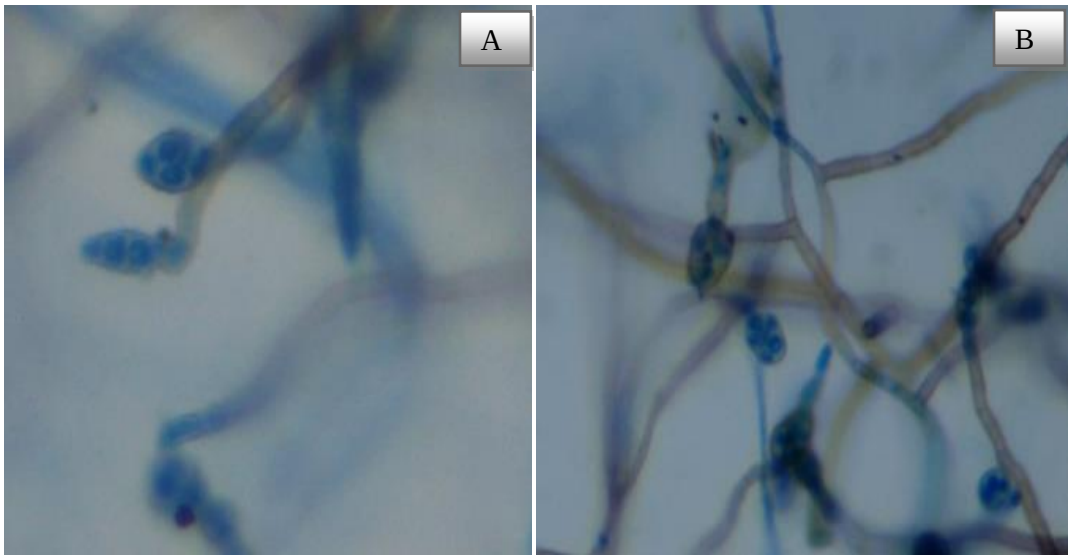


Fig. 15: Microscopic view of EL

Fungal endophyte as seen on PDA plate appeared green and cottony. The plate appeared white in the beginning and then turns green. It is a fast growing fungus and has cottony growth.

When viewed under microscope it consisted of sac like structures containing spores and had septate hyphae.

CONCLUSIONS

Medicinal plants *Terminalia arjuna* and *Eucalyptus tereticornis* have been chosen for study to isolate endophytic fungi producing novel bioactive compounds. From both the plants, 4 fungal isolates 3 from Arjuna stem and leaf and 1 from *Eucalyptus* leaf were taken and preliminary antimicrobial testing was done by paper disc diffusion method. MTT assay was also performed to find out percent inhibition of each extract at different concentrations.

In disc diffusion preliminary assay EL showed zone of inhibition of 21mm and 11mm against *Staphylococcus aureus* and *Bacillus megaterium* respectively. In MTT assay EL showed maximum inhibition of 60% against *Staphylococcus aureus* and 41% against *Bacillus megaterium*. EL also had maximum phenolic content (126.8 mg GAE/ g of dried extract). Thus, EL was selected for further analysis. IC_{50} value for EL was found out to be between a concentration 512µg/ml and 1024µg/ml by performing MTT assay. Finally TLC of crude extract EL was run to find out the actual fraction responsible for antimicrobial activity of EL against *Staphylococcus aureus*. Each fraction was tested by disc diffusion assay, in which fraction 1 gave a zone of inhibition of 10mm. EL was viewed morphologically under microscope for fungal identification purpose. When viewed under microscope it consisted of sac like structures containing spores and had septate hyphae.

Further molecular based identification can be done to find out the fungal species responsible for producing the novel antimicrobial activity. Chemical characterization of the compound can be done to find out the exact compounds responsible for the activity.

Thus the results from above studies show that the microorganisms present within the plant could be a source of new bioactive compounds. Identifying and characterizing metabolites produced by endophytes is a promising and current area of research with wide applications in pharmaceutical industry.

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