

Antimicrobial activity of the endophytic fungi isolated from the medicinal plant

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Submitted by

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CERTIFICATE

This is to certify that the thesis report entitled “**Antimicrobial activity of the endophytic fungi isolated from the medicinal plant**” submitted by **Ms. Ria Rawal** in the partial fulfillment of the requirement for the award for the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is a record of the student’s own work carried out under my supervision & guidance. This report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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DECLARATION

I hereby declare that the work that has been presented in this thesis “**Antimicrobial activity of the endophytic fungi isolated from the medicinal plant**” submitted by **Ms. Ria Rawal** in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an original record of my own work done during the period from January 2017 to July 2017, carried under the supervision of **Dr. M. Vasundhara**. This dissertation report has not been submitted in part or full to any other university or institute for the award of any degree.

Place: Patiala

Date: 23/AUGUST /2017


(Ria Rawal)

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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	Microgram
NB	Nutrient Broth
MHB	Muller Hinton Broth
MHA	Muller Hinton Agar
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
w/v	Weight by Volume
pH	Potential of hydrogen ion
Temp	Temperature
Rpm	Rotation Per Minute
H ₂ SO ₄	Sulfuric Acid
MIC	Minimum Inhibitory Constant
<i>E.coli</i>	<i>Escherichia coli</i>
<i>P.aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>S.aureus</i>	<i>Staphylococcus aureus</i>
<i>B.megaterium</i>	<i>Bacillus megaterium</i>
<i>C.albicans</i>	<i>Candida albicans</i>
EtBr	Ethidium Bromide
EtOAc	Ethyl Acetate
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylenediaminetetraacetic acid
sp.	Species
BSA	Bovine Serum Albumin

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ABSTRACT

Medicinal plants are the natural source of therapeutic drugs. There has been great interest shown to isolate the endophytic fungi colonizing the medicinal plant. These endophytes are residing in plants from thousands of years and are believed to be the treasure of structurally and biologically active compounds that have the antimicrobial, cytotoxic, immunomodulatory properties. These endophytic fungi are subjected to great exploration to isolate new species from which novel bioactive compounds can be extracted.

In this study medicinal plants *Tinospora cordifolia* (Giloy) and *Terminalia arjuna* (arjuna) have been used for the isolation of the endophytic fungi. Also, Extracellular Polymeric Substance (EPS 1 and EPS 2) was extracted from the given isolated endophytic fungus T2.

The number of fungal isolates obtained from Giloy were seventeen (G1-G17) and five (A1-A5) from Arjuna. These were tested for preliminary antimicrobial activity by agar diffusion method and final antimicrobial assay by microdilution method using cultures of *E.coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* and *Candida albicans*.

The maximum inhibition was shown by extract A amongst all the extracts of Arjuna. It showed inhibition of 63% against *Pseudomonas aeruginosa*, 62% *Bacillus megaterium*, 57% against *Staphylococcus aureus*, and 58% against *E.coli* and showed 40% against *Candida albicans*.

Amongst the extracts from Giloy G15 showed the best activity of 48%, 61%, 59%, 60%, 45% against *E.coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* and *Candida albicans* respectively.

The isolated endophytic fungi from the bark of *Terminalia arjuna* exhibiting the best antimicrobial activity was further chosen for identification. The culture characteristics and microscopic features revealed that the above isolated endophytic fungus belongs to *Penicillium sp.*

EPS was isolated from the endophytic fungus T2 obtained from Taxol plant and incubated for different time periods i.e. 15 days (EPS 1) and 20 days (EPS 2). These samples were subjected to the antimicrobial activity where EPS 1 exhibited more activity as compared to EPS 2. The reason for such activity could be that EPS are the primary metabolites secreted by the fungi during the growth phase. The metabolites secreted during this phase might have contributed to the activity of EPS1.

Medicinal plants are the natural source for drug molecules as they have bioactive compounds that benefits humans. These have kept on attracting researchers due to their medicinal properties. For example stem of the medicinal plant *Tinospora cordifolia* is used in fevers, urinary diseases and many more. The plant *Tinospora cordifolia* belongs to the family Menispermaceae and is a large deciduous climbing shrub which is found throughout India, Srilanka, Bangladesh and China (Raghu et al. 2006). Another medicinal plant *Terminalia arjuna* has widely been used as ayurvedic medicine for cardiac disorders since very long time. Experimental and clinical studies have shown that bark of the plant is beneficial in congestive heart failure and other cardiovascular complications (Nammi, 2003).

A variety of phytochemicals have been isolated from different parts of these medicinal plants which belong to classes like alkaloids, diterpenoid, steroids, lactones, glycosides, aliphatic compounds and polysaccharides which provide them therapeutic value (Singh et al. 2003).

For not making the plants go extinct, there have been interests shown to isolate the endophytes that have inhabited plants since long and tend to produce the same compound that have been produced by host plants or sometimes more than its host plant.

De Bary (1866) introduced the term endophytes as the organisms that are living inside the tissues of the plants. This term was further explained by Bacon and White (2000) by defining endophytes as the microbes that colonize the internal tissues of plant without causing immediate harmful effect to plants. Endophytes include fungi, bacteria, and also actinomycetes. Out of all the endophytes, endophytic fungi have proved to be the most beneficial one.

The endophytes are subjected to exploration at a large level as maximum of them are still not understood completely. Recently in literature it has been cited that 51% of bioactive novel compounds isolated from endophytic fungi were earlier unknown when compared to 38% from soil fungi (Strobel, 2003).

Colonization of the endophytic fungi in the host plant has contributed to the adaptation to the abiotic and biotic stress factors. Beneficial endophytes help in promoting host plant growth, increase the uptake of nutrients by plants, inhibits pathogenic growth inside the host plant, and

reduces disease severity (Redman et al. 2011; Zhang et al. 2006; Akello et al. 2007; Bae et al. 2009). For example, inoculation of barley roots with endophytic fungi *Fusarium equiseti* and *Pochonia chlamydospora* promoted the plant growth and also suppressed diseases caused by different pathogen (Macia-Vicente et al. 2009).

Fungal endophytes are believed to be treasure of structurally and biologically active compounds. Endophytic fungi produce novel secondary metabolites that are produced by their host plants (Tan and Zou, 2001). It has been hypothesized that during the evolution, endophytic fungi might have undergone genetic recombination there by inheriting the ability to synthesize the metabolites produced by the plants. Also there are large numbers of endophytic fungi that are produced from single part of the plant so this requires the screening of the endophytic fungi. Screening helps selecting fungi that have the potential to produce the compounds that have the therapeutic value (Stierle et al. 1993; Zhang et al. 2006).

Novel bioactive metabolites produced by the endophytic fungi exhibit biological activities like: antimicrobial, antioxidant, immunosuppressive, antineoplastic, cytotoxic activities etc., due to the presence of the compounds in the fungal extract that have therapeutic value, so are replacing plants to be used instead. These are easy to be produced in large quantity by fermentation (Verma et al. 2011).

Secondary metabolites extracted from the endophytic fungi through organic solvent are to be dried and used to perform different assays like the antimicrobial, antioxidant. These assays are done to screen the bioactive potential of the endophytic fungi. The preliminary phytochemical tests are done to know which compounds are present in a particular fungal extract like alkaloids, terpenoids, steroids, quinones, phenols, polysaccharides and more. Crude extract consist of many compounds which are fractionated to isolate the pure compounds and test which compound actually exhibits antimicrobial or antifungal or antioxidant property.

It is required to develop new drugs constantly as the microorganism are evolving at faster rate and getting resistant to the already present drugs. So producing the drugs from the endophytic fungi would be effective and economical at the same time.

Although the great exploration is done on the endophytic fungi for the isolation of the therapeutic compound but very less is known about the Extracellular Polymeric Substances (EPSs).

The endophytic fungi produce EPSs that are high molecular weight polymers with long chains and are secreted into the environment which dissolve and disperse into the growth medium. Fungal exopolysaccharides have emerged to be the important bioactive substances which are highly active, possess low toxicity and will have minor environment impact (Gangadevi et al. 2008). These are produced by the organism in their growth phase. It has wide application in medicine as well as in other industries as thickening and stabilizing agents. With the knowledge of production of EPS from fungi, it is important to know the structural characterization of EPS and also a need to understand the structure-functional relationship. So since few years there has been a focus on these bioactive compounds called exopolysaccharides, as these are also proving to be the boon to pharmaceuticals and other industries (Mahapatra et al. 2016).

Objectives of the project

Endophytic fungi are to be extensively investigated as they represent an abundant and dependable source of novel bioactive compounds with large potential for exploration in the field of medicine, agriculture and industrial areas (Vanden et al. 1991).

The objectives of the project are as given below:

- Isolation of endophytic fungi from the medicinal plants.
- Testing for the bioactivity of the fungal extract obtained from the endophytic fungi.
- Identification of the endophytic fungi possessing the maximum bioactivity.

2.1 Medicinal plants: source of natural drugs

Plants have always been a boon for the humankind whether it is for food products like grains, fruits, vegetables, or for the ornaments like flowers and variety of plants have served as the source of most medicines and drugs. This is the reason why scientists have developed interest in the medicinal plants and till now there are many researches that are done on the medicinal plant.

Medicinal plants contain novel compounds that have therapeutic value and are proving beneficial to humans. Since ancient times till present the medicinal plants have attracted the researchers for identification of novel active molecules that have great biological activities (like antifungal, immunomodulatory, antimicrobial etc.). The medicinal properties of these plants are due to the presence of compounds like alkaloids, terpenoids, steroids, quinones, phenols, polysaccharides and other.

Tinospora cordifolia a medicinal plant also called as Giloy has great medicinal properties. It is the most commercially exploited plants in pharmaceutical industries. Estimated annual demand of this plant species used as crude herbal drug in the Indian system of medicines (ISM) is 10,000 tonnes. The whole plant of Giloy possesses various health benefits and has been used as traditional against various human diseases since long back (Khare, 2008). The stem of the *Tinospora* plant has been reported with antibacterial activity against some bacterial strains like *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella dysenteria* (Singh et al. 2012).

The medicinal plant *Terminalia arjuna* has also been widely been used in Ayurvedic medicines for cardiac disorders since very long time. Experimental and clinical studies have shown that bark of the plant is beneficial in congestive heart failure and other cardiovascular complications as well (Nammi, 2003).

Table 1: Classification of Giloy and Arjuna

CLASSIFICATION	GILOY	ARJUNA
Class	Magnoliopsida	Magnoliopsida
Order	Ranunculaceae	Myrtales
Family	Menispermaceae	Combretaceae
Genus	<i>Tinospora</i>	<i>Terminalia</i>
Species	<i>cordifolia</i>	<i>Arjuna</i>

Classification of *Tinospora cordifolia* and *Terminalia arjuna* has been shown in Table 1. For not making the plants go extinct by using them for our needs there has been interest shown in its endophytes, so that we use endophytes of our interest (that produce the required compound) and not taking the plant itself. It has been studied that endophytes that inhabit the plants possess the ability to produce the same compounds that have been produced by the host plant itself and, sometimes more than its host plant also.

2.2 Endophytes as promising organisms

Endophytes are omnipresent in the plant kingdom, many of them are proving to improve the ecological adaptability of plants they are residing in and these are recently getting more explored entity of the microbial world. These colonize the intracellular or intracellular spaces of the plants. De Bary (1866) introduced the endophyte term as the organism that are living inside the healthy tissues of the plants. And this term was further explained by Bacon and White (2000) by defining endophytes as the microbes that colonize the internal tissues of plant without causing immediate harmful effect to plants. Some endophytes mediate interaction between plants and their competitors. Endophytes include fungi (Gond et al. 2007; Rajagopal et al. 2000; Wilson and Carroll, 1995), bacteria (Hallman et al. 1997), and also actinomycetes (Verma et al. 2009). First symptomless endophyte was isolated from seeds of *Lolium temulentum* in 1898 (Wilson, 1995)

2.3 Endophytic fungi source of bioactive novel compounds

Out of all the endophytes, the most trusting and beneficial one is the endophytic fungi. These are the most explored and extensively studied microorganisms.

Occurrences of endophytic fungi is diversified and are known to inhabit plants growing in different environmental regions such as Arctic, Antarctic, deserts, oceans, rainforests, etc. Endophytic fungi distribution is ecologically influenced, colonization patterns of the endophytic fungi have been described at different levels. Fungal endophytes are mainly basidiomycetes, ascomycetes, deuteromycetes and rarest found are oomycetes (Petrini, 1991).

Colonization of the endophytic fungi in the host plants has developed the tolerance of abiotic and biotic stress factors in the host plants. These help in the promotion of the plant growth, increase uptake of the nutrient, inhibition of plant pathogen growth, and also the reduction of disease severity. Discovering a vast number of unexplored endophytic fungi species, some of which have potential to be used as an outstanding source of new bioactive natural products that are useful in agriculture and pharmaceutical industries (Redman et al. 2011; Zhang et al. 2006; Akello et al. 2007; Bae et al. 2009).

Fungal endophytes are believed to be treasure of structurally and biologically active compounds (Tan and Zou, 2001; Strobel, 2003; Strobel et al. 2004; Gunatilaka, 2006; Zhang et al. 2006; Kumar et al. 2008; Aly et al. 2011). These endophytes producing immunologically active compounds are residing in the plants from thousands of years so they synthesize novel bioactive metabolites by being in metabolic aggressive state (Tejesvi et al. 2005).

Recently in literature it has been cited that 51% of bioactive novel compounds isolated from endophytic fungi were earlier unknown when compared to 38% from soil fungi. So, endophytic fungi should be explored more as majority of them are still elusive (Strobel, 2003).

Sometimes, the medicinal properties of plants results from the endophytes present in the host plant and the type of biologically active secondary metabolites that are been produced by that endophytes. Also some endophytic fungi are evolved to allow transfer of genetic information to their host plant. Working hypothesis of the endophytes inside the plants has been described, this hypothesis is based on the balanced antagonism. Triggering of the defense action occurs by both

pathogen and endophyte but pathogen overcomes it which causes disease to develop whereas the endophyte also overcomes the defense reaction of plant so as to infect and colonize the plant but to an extent that there is not the development of the disease. Hence, pathogen-host interactions are imbalanced resulting in disease whereas endophytes maintain balanced antagonism with host without development of any disease (Schulz and Boyle, 2006).

2.4 Metabolites produced by the endophytes

There are two types of metabolites produced namely primary and secondary metabolite. Primary metabolites are mainly produced in the growth phase of the organism, maximum of the primary metabolites produced are utilized by them for the growth process. Secondary metabolites are mainly produced during the stationary phase of the organism when they are in stress conditions. Secondary metabolites of endophytic fungi hold great biological activity, so are referred to as novel metabolites. Both the metabolites have been isolated from the microorganisms.

These novel metabolites exhibit biological activities like: antibacterial (Kharwar et al 2009), antifungal (Ding et al. 2008), antioxidant, immunosuppressive, antineoplastic, cytotoxic (Kharwar et al. 2011) activities and many more of them are already cited in the literature (Rodrigues et al. 2000; Huang et al. 2001; Liu et al., 2001; Wiyakrutta et al. 2004)

Discovering large number of undescribed species of endophytic fungi. Some of which have potential to be used in agriculture and pharmaceutical industries as an outstanding source of production of bioactive compounds (Strobel, 2003).

Secondary metabolites extracted from the different plants have thought to be produced by plants but many a times these metabolites are produced by the endophytic fungi that colonize the specific plant. For example Taxol, a potent anti-breast cancer drug, being produced by the endophytic fungus *Taxomyces andreanae* colonizing the plant *Taxus brevifolia* (Stierle et al. 1993).

During the long period of existence of endophytic fungi inside host plant they start synthesizing the metabolites that are being produced by the host plant. It has been hypothesized that during evolution of the endophytic fungi might have undergone genetic recombination there by inheriting the ability to synthesize the metabolites produced by the plants (Stierle et al. 1993;

Zhang et al. 2006). For example, *Alternaria sp.* inhabiting inside the plant *Podophyllum hexandrum* as the endophytic fungus was reported to produce podophyllotoxin (Yang et al. 2002). Puri et al. (2005) first reported an endophytic fungus *Entrophospora infrequens* that was obtained from plant *Nothapodytes foetida* that had ability to produce Camptothecin. Huperzine A, a cholinesterase inhibitor was synthesized from *Shiraia sp.* residing as an endophyte in its host plant *Huperzia serrata* (Wang et al. 2011). Piperine production by endophytic fungus *Periconia sp.* isolated from *Piper longum* has been recently described (Verma et al. 2011). These examples suggest that there are many important drugs that can be produced by endophytic fungi, and these molecules appear similar to their plant origin.

2.5 Screening of the primary metabolite from endophytic fungi producing Extracellular Polymeric Substances (EPSs)

EPS are the primary metabolites mostly secreted by the endophytes in their growth phase. Although the great exploration is done on the endophytic fungi for the isolation of the therapeutic compound from it but very less is known about the Extracellular Polymeric Substances (EPSs). The endophytic fungi are known to produce exopolysaccharide that are high molecular weight polymers with long chains and are secreted by micro-organisms into the environment which dissolve and disperse into the growth medium. Fungal exopolysaccharides have emerged to be the important bioactive substances that are highly active and possess low toxicity and will have minor environment impact. These exopolysaccharides are primary metabolites that are secreted by the organisms in their growth medium (Lee et al. 2003).

Fungal Polysaccharides (PSs) are classified into different categories based on their structure (branched or linear), composition of sugars (homo- or hetero- polysaccharides), type of bonds that are present between the monomers and their location in cell can be cell wall PSs, endoPSs or exoPSs. Exopolysaccharides are intensively explored by scientists now days, but their definition, classification, and origin are still not properly clear. EPSs play different biological functions, for example they protect against environmental stress factors and in interactions with other harmful organisms. EPSs obtained from Ascomycetes and Basidiomycetes fungal cultures are well known for their immunostimulating, antioxidant, antitumor, and antimicrobial properties (Osinska-Jaroszuk et al. 2014).

Mahapatra et al. (2016) optimized the production of the exopolysaccharide from the endophyte *Pestalotiopsis* sp. To investigate the culture producing maximum exopolysaccharide different methods were applied like one variable at a time, response surface methodology at different media composition were used for it. The culture that produced the maximum of exopolysaccharide was in Potato Dextrose Agar (PDA) with pH of 6.93 and incubated at 24°C for 4 days. Molecular weight of exopolysaccharide was also determined. Structural elucidations were also done after the series of experiments.

Guo et al. (2014) isolated galactomannan an exopolysaccharide with the novel structures that is produced by endophytic fungus *Aspergillus ochraceus*. It was observed by experiments that galactomannan was composed of mannose and galactose of ratio 2.16:1.00 and molecular weight 29 kDa. Structure of it was investigated by chemical and spectroscopic methods, including methylation analysis, one and two dimensional nuclear magnetic resonance (1D, 2D NMR) and electrospray mass spectroscopy. Results showed back bone of the EPS. The whole investigation demonstrated that this EPS is novel galactomannan with different structural characteristics from other fungal galactomannans and possibly can be a potential source of linked β -D-galactofuranose oligosaccharide

2.6 Screening of the secondary metabolites from the endophytic fungi

The metabolites that are extracted from the endophytic fungi have the different biological property that can be checked by performing different assay. Antimicrobial sensitivity test can be performed by taking the test organism for example *E.coli*, *Bacillus megaterium* and more. If the organisms did not grow in the presence of the extract this shows the extract is inhibitory to that particular organism. There are large numbers of endophytic fungi that are produced from single part of the plant so this requires the screening of the endophytic fungi. Screening helps selecting fungi that have the potential to produce the compounds that have the therapeutic value.

De Siqueira et al. (2011) isolated endophytic fungi from healthy tissues of *Lippia sidoides* a medicinal plant used as an antiseptic. It was seen that endophytic colonization was greater in leaves (50.4%) than in stems (35.4%). *Colletotrichum gloeosporioides* had the maximum colonization frequency, it was found that out of total fungi 16 isolates were found to produce antimicrobial metabolites against bacterial and fungi.

Magiorakos in (2012) has reported that ESKAPE organism's *E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species seen in many hospitals are the main causative agents of infections. To overcome all these continuing developments, a search for new and novel drugs should be the priority.

Indian medicinal plants have been reported with the presence of endophytic fungi mainly *Phomopsis*, *Colletotrichum* and *Alternaria* as dominant species. These have been reported as reservoir of unique metabolites. For example, antimicrobial phomosines A-D (Krohn et al. 2011), cytochalasin compounds (Fu et al. 2011), phomochromone A, B and cyclopentenone derivative phomotenone (Ahmad et al. 2011) have been isolated successfully from various sp. of *Phomopsis*. Similarly, various species of *Colletotrichum* are sources of novel bioactive secondary metabolites (Arivudainambi et al. 2011; Inacio et al. 2006). A novel cytotoxic compound isolated from *colletrichum sp.* alterporriol K (Huang et al. 2011), antibiotic Xanalteric acids I and II, (Kjer et al. 2009) alterperyleneol, altertoxin, alternariol, and tenuazonic acid (Qin et al. 2009) are few of the secondary metabolites that are identified from *Alternaria sp.* This proves that endophytic fungi can be used as the outstanding source for production of the therapeutic drugs

New drugs discovered such as Linezolid and Daptomycin have acquired resistance already. It is required to develop new drugs as microorganisms are evolving at faster rate. So there is an extensive need to isolate new sp. of endophytic fungi that have the ability to produce therapeutic drugs.

3.1 Materials

Media: PDA (Potato Dextrose Agar), PDB (Potato Dextrose Broth), NA (Nutrient Agar), NB (Nutrient Broth), MHA (Muller Hinton Agar) Table 11 (Annexure 1).

Instruments: Laminar air flow bench, incubator, Rota-evaporator, centrifuge, vortex, spectrophotometer, ELISA reader, magnetic stirrer, magnetic bead.

Apparatus: Petri plates, flasks, separating funnel, rota flask, test tubes, beaker, microtiterplate (96 well), dialysis membrane, glass vials, microfuge tubes, microfuge tube stand, tips, auto-pipette, motor pestle, filter paper.

Other Chemicals: DMSO (Dimethyl Sulfoxide), ethyl acetate, methanol, distilled water, milli q water.

Antimicrobials: Ampicillin, streptomycin, amphotericin

3.2 Collection of samples

The samples were collected from the Thapar university campus, Patiala. Stem of the medicinal plant *Tinospora cordifolia* (Giloy) was collected from #C- 10, and the bark of the plant *Terminalia arjuna* was collected from #C- 8 of Thapar University. These were transferred into sterile bottles and were brought to TIFAC- CORE laboratory for further studies.

3.3 Surface disinfection of the samples

The collected samples were firstly rinsed thoroughly in running tap water, followed by rinsing it with double distilled water to remove the dust and debris from the surface. Samples were dipped in 70% ethanol for 1 min, and then immersed in aqueous solution of 4% sodium hypochlorite for 3 min, followed by dipping the samples again in 70% ethanol for 10sec. Further the samples were rinsed in double distilled sterile water and dried under the aseptic conditions in laminar air flow bench (Petrini et al. 1993).

3.4 Isolation of the endophytic fungi from the sample

After drying, the samples were cut from the edges and outer layer was removed by sterile blade. The samples were further cut horizontally into two halves. These were transferred onto the PDA plates, which were incubated for 7- 14 days at 25°C. The plates were observed after every 2-3 days for the growth of endophytic fungi. Pure fungal cultures were obtained by sub-culturing. All the isolated fungi were given code and the master cultures were preserved at 4°C.

3.5 Sub-culturing of the endophytic fungi

Sub-culturing of the endophytic fungi was done periodically by cutting out 1cm² of the mycelia from the plate by sterile blades. These discs were transferred to the center of the fresh PDA plate and incubated for 7 days at 25 °C.

Already maintained culture of endophytic fungi (T2) was sub-cultured on the PDA plates and incubated at 25°C for 7-14 days to allow it to grow over the plate. All the cultures were stored as glycerol stocks.

3.6 Culturing of fungi

The fresh mycelia sub-cultured on PDA were transferred to 250 ml Erlenmeyer flask containing 100 ml of the PDB. The cultured flasks were incubated for 21 days at 25°C at stationary conditions. After the incubation period of 21 days, the culture was filtered with sterile muslin cloth to separate the mycelia from the broth (Prabavathy et al. 2011).

3.7 Extraction of endophytic crude extracts

The fungal metabolites were extracted by solvent extraction procedure using ethyl acetate as solvent. Firstly equal volumes of the culture filtrate (broth) and ethyl acetate were taken in a separating funnel, shaken for 10-15 min. Further it was allowed to stand till the two phases (organic and inorganic phase) got completely separated. Organic phase was collected in a separate flask. Same steps were repeated and the organic phase was again collected. Obtained organic phases were combined in a flask and dried by using the Rota-evaporator at 40°C. The residual crude extract was further dried completely in a vial by keeping at room temperature and weighed. These extracts were further used for testing of biological activities.

3.8 Screening for antimicrobial activity of crude extract

3.8.1 Cultures used for antibacterial and antifungal testing

Test bacterial cultures

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

Test fungal culture

- *Candida albicans*

These cultures are standard cultures those does not possess pathogenicity.

3.8.2 Preparation of McFarland Standard

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

McFarland standard- Appendix 1(Table 13)

Preparation of McFarland standard (NCCLS, 2003)

1. 85 ml of 1% H_2SO_4 was added to a 100 ml volumetric flask.
2. 0.5 ml of 1.175% $BaCl_2 \cdot 2H_2O$ was added dropwise with auto-pipette to H_2SO_4 by constantly swirling the flask.
3. Final volume was made 100 ml with 1% H_2SO_4 .
4. The flask was placed on magnetic stirrer for 4-5 min.
5. The O.D. was recorded at 600nm.

3.8.3 Techniques used for screening of crude fungal extract

3.8.3.1 Agar disc diffusion technique

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

Small molecules such as antibiotics are able to diffuse through agar. Antibiotics are actually applied to the sterile disc and the disc was kept on the agar plate. Antibiotics have the tendency to move from higher concentration to lower concentration. If there is more of the antibiotic on the disc then it will tend to make larger zone of inhibition. Firstly the bacterial colony is spread over the agar, which is then allowed to stand for a while then the discs are kept on to the agar. There can be different concentration of the same antibiotic or many other antibiotics used, following the incubation for allowing the bacteria to grow. After the incubation period agar plates are examined for bacterial growth. If the growth of microorganisms covers the whole plate, it indicates that bacteria are resistant to that particular antibiotic. If there is zone of inhibition formed (clear zone), it indicates that microorganisms are sensitive for that antibiotic. The diameter of zone of inhibition is measured according to standards (generally measured in millimeters) and would suggest that how much the bacteria are sensitive. Zone of inhibition also depends on the concentration of the antibiotic. At more concentrations the zone of inhibition is large and at low concentration the zone of inhibition is less. The agar diffusion allows bacteria to be screened routinely and economically in the microbiological laboratory. Similarly, the fungal extracts are allowed to diffuse into the agar and show its effect over the test microorganisms. The resulting zone of inhibition is uniformly circular as there is always the confluent lawn of growth of the test organism (NCCLS 2000).

Medium used for the antimicrobial testing is: MHA (Muller Hinton Agar) This medium is used as the microbiological growth medium for antimicrobial susceptibility testing. Composition of MHA (Table 12, Annexure 1) (Atlas, 2004)

The preparation of the media was done by dissolving 33.9 g of commercially available Muller Hinton agar media (HiMedia Pvt. Ltd.) in 1000 ml of distilled water. The dissolved media was firstly boiled so that the starch does not settle, further the media was autoclaved at 15 lbs pressure at 121°C for min. The autoclaved media was poured on petri plates and then allowed to solidify.

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

PROCEDURE:

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

For the fungal cultures the PDA plates were used to streak and colony was transferred in PDB and incubated at 30°C for 1-2 hrs and spreading was done on PDA plates. Plates were kept for 30 min.

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

3.8.3.2 Microdilution method- Determination of MIC (Spectrophotometric method)

Material Required: Suspension of test organisms (bacterial and fungal), Microtitre plate, ELISA plate reader, Autopipettes, Standard antimicrobial (Streptomycin, ampicillin and amphotericin), test extracts, MHB.

PROCEDURE

A suspension of test organism was prepared equivalent to 0.5 McFarland standard using isolated colonies. Stock of streptomycin and ampicillin (50 µg/ml) was prepared in distilled water for antibacterial assay. For antifungal assay stock of amphotericin (1 µg/ml) was prepared in pure DMSO and fungal crude extract (1 mg/ml) was prepared in 10% DMSO. Experiment was performed in 96 well plate, with total volume of 100 µl of cell suspension, media and different fungal crude extract in a well. MHB (media control) served as a blank, MHB with bacterial cells suspension (cell control) was used as positive control, MHB with cell suspension and 10% DMSO (solvent control) was also used as a positive control. MHB with cells suspension and antibiotic (streptomycin and ampicillin for bacterial test cultures) and for fungal test cultures (amphotericin) served as the antibiotic control.

ELISA plate reader was set at 600 nm to record the absorbance firstly at 0 hr (initial reading) and then the plate was incubated at 37°C (bacterial test cultures) and 30°C for (*C.albicans*) for 24hrs, absorbance was again recorded after 24 hrs (final reading) at same wavelength. The reading was taken after the 48hrs of incubation at 30°C for *C.albicans*. The entire test was performed in triplicates.

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

Where, SC- solvent control

Sample- fungal crude extracts

3.9 Preliminary phytochemical analysis of bioactive compound

1mg of each crude extract were dissolved in 1ml of methanol for preliminary phytochemical tests (Kokate et al. 2009).

3.9.1 Test for Alkaloid (Wagner's test)

For this test few drops of Wagner's reagent were added and formation of reddish brown precipitates showed presence of alkaloid in fungal extract.

3.9.2 Test for Amino acids (Ninhydrin test)

3 drops of 5% Ninhydrin solution were added to extract and heated in water bath for 10 min, if there were presence amino acids present in extract purple bluish colour will appear.

3.9.3 Test for Carbohydrates (Molisch's test)

α -naphthol solution was added to fungal extract dissolved in methanol, after shaking conc. H_2SO_4 was added from sides of the test. Presence of carbohydrate showed violet ring at the junction of two liquids.

3.9.4 Test for Fats and Oils

0.5N alcoholic KOH was added to fungal extract dissolved in methanol followed by few drops of phenolphthalein and incubated in water bath for 1hr. Presence of fat and oils is shown by the soap formation after 1hr incubation.

3.9.5 Test for Flavonoids

When increasing amount of sodium hydroxide is added to the extract dissolved in methanol and it decolourises by adding acid then this shows the presence of flavonoids by yellow colour.

3.9.6 Test for Glycosides (Keller-Killiani test)

Reddish brown colour is observed at the junction of liquid layer and upper layer appears bluish green when glacial acetic acid and 1 drop of 5% $FeCl_3$ and conc. H_2SO_4 are added to the fungal extract.

3.9.7 Test for Steroid and Terpenoids (Salkowski test)

Addition of conc. H_2SO_4 to the extract, appearing of red colour indicates the presence of the steroids and appearing of the blue colour indicates the presence of terpenoids.

3.9.8 Test for tannins and phenolic compounds (Ferric chloride test)

When drops of FeCl_3 (5%w/v) is added to extract then presence of tannins is indicated by the blue colour whereas presence of phenolic compound is indicated by green colour.

3.10 Identification of the endophytic fungus

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

3.10.1 Morphology based identification:

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

3.10.2 Molecular based identification of endophytic fungus:

Selected endophytic fungi were grown on PDA plates where they were grown on cellophane sheets (treated with EDTA) for 5 days. After 5 days, the mycelia was scrapped from cellophane sheets and crushed with liquid nitrogen. The crushed samples were stored at -80°C , for further isolation of genomic DNA.

3.10.2.1 Genomic DNA Isolation

100mg of liquid nitrogen crushed mycelia was taken in 1.5 ml of microfuge tube. 600 μl of extraction buffer containing 100 mM Tris-HCl pH-8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB was added and vortexed for 1 min. The tubes were than incubated at 65°C for 1 hr followed by

centrifugation at 12000 g for 10 min. The supernatant so obtained was mixed with equal volume of phenol: chloroform: Isoamyl alcohol and incubated at room temperature for 25 min, followed by centrifugation at 12000 g for 15 min. The top aqueous layer was collected in a separate microfuge tube and 800 µl of isopropanol was added. The tubes were then incubated at -20°C for 1 hr, followed by centrifuged at 14,000 g for 10 min. The pellet obtained was mixed in 25 µl of 3M sodium acetate and incubated overnight at 4°C. 600 µl of precooled 95% ethanol was added to the suspension, mixed and incubated at -20°C for 20 min. The mixture was then centrifuged at 14000 g for 10 min. The pellet recovered was washed with 500 µl of cold 70% ethanol. Air dried the pellet and resuspended in 25 to 30 µl Milli Q water (Zhang et al. 2010).

3.10.2.2 Agarose Gel Electrophoresis for DNA

For 0.8% of agarose Gel, 0.4 gm of agarose was mixed in 40 ml of 0.5X TBE buffer. The solution was cooled down and 2µl of ethidium bromide (EtBr) was added and mixed properly. Agarose was poured in casting tray and comb was placed in the tray. The gel was allowed to solidify at room temperature. The comb was carefully removed so that the wells did not damage. The gel was then placed carefully on to electrophoresis chamber. 0.5X TBE buffer was added to the chamber to cover the gel. The DNA sample was mixed with 0.5X loading dye and loaded into the wells. The ladder was added into first well. The gel was allowed to run at 70V for 1hr 40 min and bands were observed in UV transilluminator.

3.11 EPS

EPS are the primary metabolites mostly secreted by the endophytes in their growth phase. They are high molecular weight polymers with long chains. These are secreted into the environment and get dispersed in the growth medium. They have emerged to be the important bioactive substances which are highly active, possess low toxicity and will have minor environment impact.

3.11.1 Extraction of exopolysaccharides from endophytic fungi

The culture of the endophytic fungi (T2) obtained from TIFAC-CORE Laboratories were sub-cultured on fresh PDA plates. These were incubated for 7-14 days at 25°C till the endophytic fungi was grown all over the plate. The discs of 1cm² were cut from these PDA plates with the

help of sterile blade and transferred in two Erlenmeyer flask containing 250 ml of PDB in each. It was incubated at 25°C at 120 rpm. One flask was removed after 15 days of incubation period. The culture was filtered with sterile muslin cloth and centrifuged at 14,000 g for 20 min to remove remaining mycelia present in the broth. The broth was further concentrated by precipitating it using absolute ethanol and incubated overnight at 4°C for 16 hrs. The precipitates were obtained by centrifuging at 12,000 g for 15 min. Further purification of the sample was done by dialysis (to remove the salts from the samples) against distilled water for 48 hrs. Another flask was removed after the incubation period of 20 days and processed similarly.

3.11.2 Dialysis

Dialysis is done to separate the colloidal particles from the dissolved substances via diffusion. The dialysis was done using semi permeable dialysis membrane of 12 kDa pore size which was activated by boiling firstly into 2% sodium bicarbonate solution and then in double distilled water for 10 min each. After the activation of membrane, the extract was added to it and tied with thread to the flask containing distilled water. It was stirred for 48 h using magnetic bead.

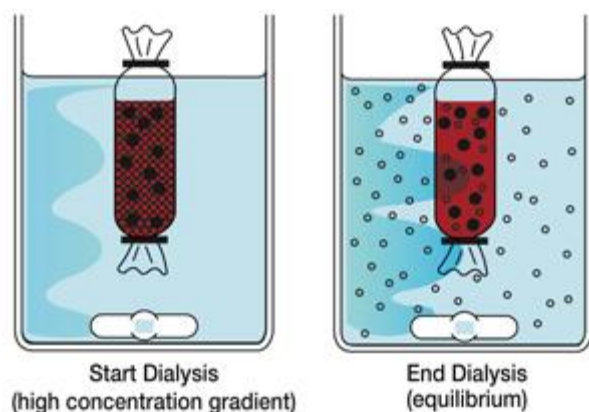


Fig 1. Dialysis set up

3.11.3 Bradford assay

The Bradford's assay used for the estimation of protein content is one of the most commonly used assays. The principle of this assay involves binding of Coomassie blue dye to certain amino acids present in the sample and forming a complex with it which can be measured at an absorbance of 595nm. It uses BSA as a standard (Bradford, 1976).

The assay was performed in 96 well micro titer plate. Different concentrations (10, 20, 40, 60, 100 µg/mL) of BSA standard were prepared. The volume was made up to 50µl using distilled water. Finally, 100µl of Bradford reagent was added to it and reading was taken in ELISA plate reader at 592nm.

3.11.4 Phenol sulfuric assay

Phenol sulfuric acid method is used for the analysis of total carbohydrates present in the sample. In this method, the concentrated sulfuric acid breaks down complex sugar molecules to simpler sugar molecules. Pentose and hexose are dehydrated to furfural and hydroxymethyl furfural respectively which react with phenol and form a yellow-gold colour (Dubis et al. 1962).

For the analysis of sugar in endophytic fungi, different concentrations (10, 20, 40, 60, 100µg/mL) of glucose standard were prepared. 50 µl of the standards and samples were prepared using distilled water in the 96 well plate. Then 150 µl of sulfuric acid was immediately added followed by addition of 30µl of phenol to it. It was then incubated in water bath for 5 min at 35°C. Finally, absorbance was taken at 492nm in the ELISA plate reader.

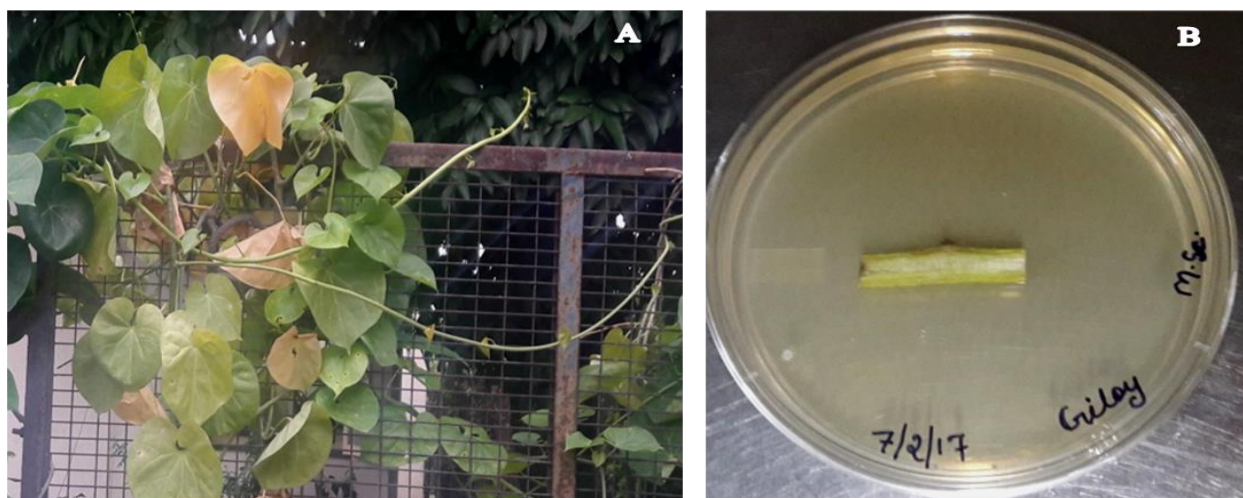
4.1 Isolation of the endophytic fungi from medicinal plant

Medicinal plants have therapeutic value that has ruled Indian Ayurveda system to a great extent. Endophytic fungi colonizing the medicinal plants inherit the ability to produce therapeutically important compounds that are produced by its host plant. For discovering new drugs there is a need to isolate new sp. of endophytic fungi.

In this study endophytic fungus has been isolated from two medicinal plant *Tinospora cordifolia* and *Terminalia arjuna*. Isolated endophytic fungi have been further characterized by their antimicrobial activity by agar disc diffusion method and micro-dilution method. The main aim of this study was to isolate the endophytic sp. that produces certain compounds, which have medicinal properties.

- **Isolation of the endophytic fungi from Giloy (*Tinospora cordifolia*)**

The collected sample of stem from the plant Giloy were surface sterilized, cultured on the PDA plate and endophytic fungi was obtained (as shown in fig. 2) after incubation of 7 days at 25°C. A total of 17 endophytic fungi were obtained from the stem of the *Tinospora cordifolia* and were designated as G1- G17 for further reference in studies.



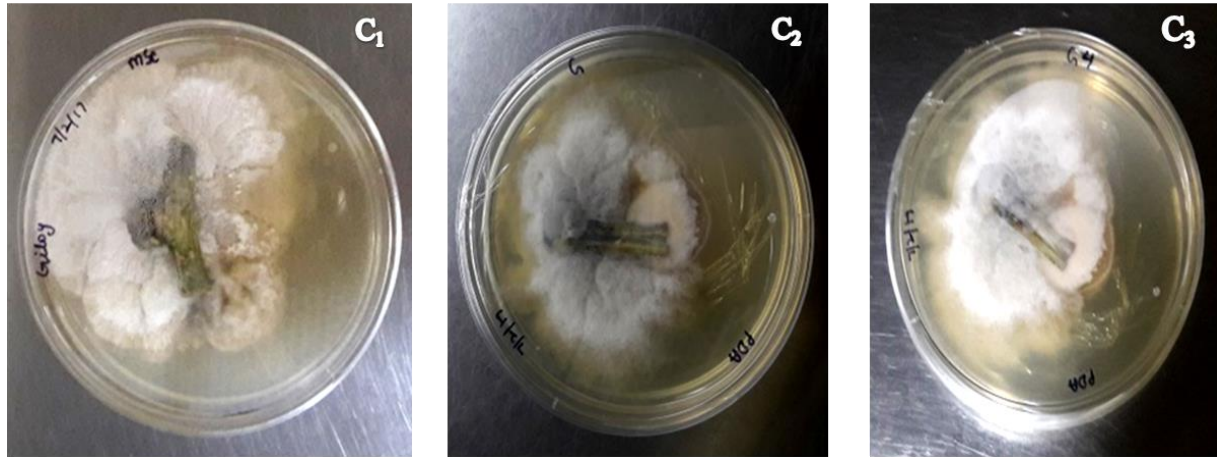


Fig. 2: Isolation of endophytic fungi from *Tinospora cordifolia*: (A) Plant of *Tinospora cordifolia* (B) Surface sterilized stem of *T. cordifolia* on PDA plate (C₁, C₂, C₃) Endophytic fungi obtained from stem on PDA plates.

- **Isolation of the endophytic fungi from bark of Arjuna (*Terminalia arjuna*)**

The bark of the plant was sterilized and cultured on the PDA which was then incubated at 25°C for 7 days to obtain the endophytic fungi as shown in fig. 3. A total of five endophytic fungi were obtained from the bark of *Terminalia arjuna* and were designated as A1- A5 for further reference in studies.

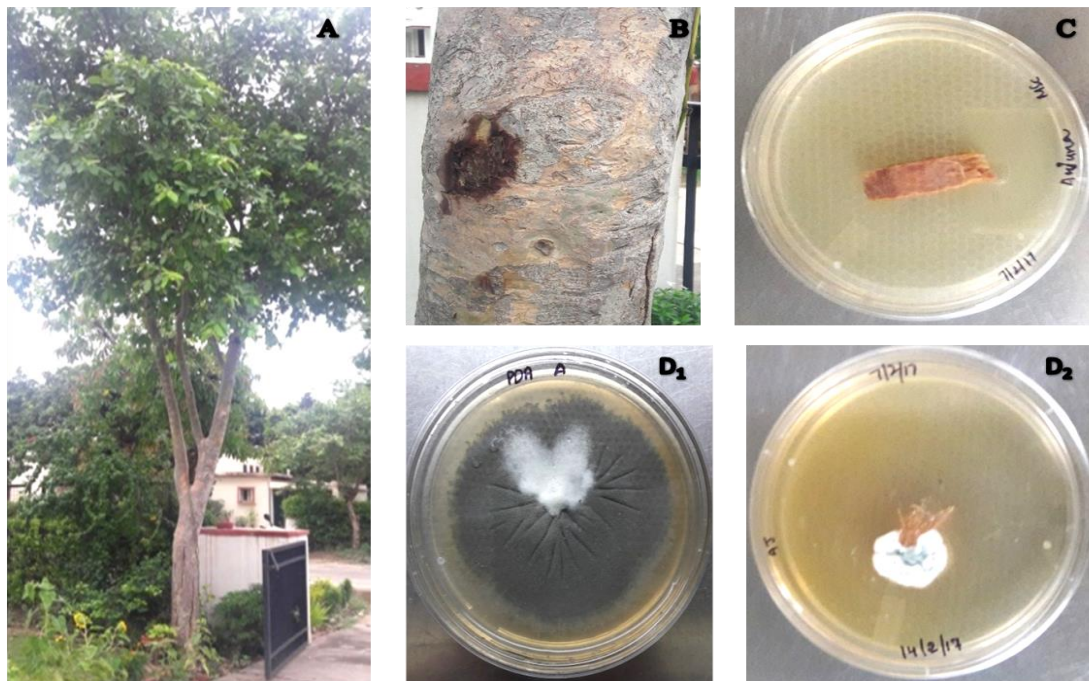


Fig. 3: Isolation of endophytic fungi from *Terminalia arjuna* (A) tree of *T. arjuna*, (B) bark of tree from where sample is collected, (C) surface sterilized stem on PDA, (D₁,D₂) endophytic fungi grown on PDA plate

4.3 Extraction of the crude extract from the broth

The isolated endophytic fungi from the *T.cordifolia* and *T.arjuna* were subcultured to obtain pure cultures and further inoculated in the PDB. This was incubated at 25°C for 21 days under stationary conditions. Broth was separated from mycelia and further extractions were done with ethyl acetate to obtain the organic phase. This was further dried to obtain the fungal crude extract as shown in fig. 4. The crude fungal extracts so obtained were subjected to further studies.



Fig 4: Endophytic fungi grown in PDB further filtered and extracted to obtain crude extract

4.4 Preliminary antimicrobial activity of the crude fungal extract

The activities of the crude fungal extracts were tested on test microorganisms (bacterial and fungal) by agar disc diffusion method. The zone of inhibition (clear zone where bacteria did not grow) formed was measured in millimeter (mm). Seventeen crude fungal extracts of *T.cordifolia*, five crude fungal extracts of *T. arjuna* and two crude EPS from T2 endophytic fungi were tested for preliminary antimicrobial activity with the bacterial test cultures of (*E.coli*, *P.aeruginosa*, *S.aureus*, *B.megaterium*) and fungal test culture (*C. albicans*). On the basis of the activity of the crude extracts against these microorganisms further selections were done.

Table 2: Antimicrobial activity (zone of inhibition-mm) of crude fungal extract and EPS obtained from isolated endophytic fungi

Endophytic Fungi (isolate no.)	Test cultures (bacterial and fungal)				
	<i>E. coli</i> (EC)	<i>P. aeruginosa</i> (PA)	<i>S. aureus</i> (SA)	<i>B. megaterium</i> (BM)	<i>C. albicans</i> (24hrs)
G1	0	0	0	0	0
G2	9	10	12	13	0
G3	0	0	0	0	0
G4	0	0	0	0	0
G5	11	0	3	0	0
G6	0	9	0	0	0
G7	0	12	11	10	0
G8	0	0	0	17	0
G9	0	0	9	0	0
G10	0	0	0	7	0
G11	0	0	0	0	0
G12	0	0	0	0	0
G13	0	0	8	0	0
G14	0	0	0	0	0

G15	10	15	16	15	7
G16	0	0	0	0	0
G17	5	0	0	0	0
A	22	14	18	24	17
A1	0	0	8	0	0
A2	0	0	0	0	0
A3	10	0	0	0	0
A4	0	8	7	0	0
EPS 1	16	13	18	11	9
EPS 2	10	9	12	9	8
Ampicillin	18	20	26	18	×
Streptomycin	13	18	22	14	×
Amphotericin	×	×	×	×	16

Total of seventeen endophytic fungal crude extracts from *T. cordifolia* were tested for the preliminary antimicrobial activity. Among these seventeen isolated endophytic fungal extracts nine showed the antimicrobial activity against one or more bacterial cultures, the antimicrobial activity of three fungal crude extracts were more prominent (Table 2).

The isolated endophytic crude extract G2 showed zone of inhibition ranging from 9-16 mm against *E. coli*, *P. aeruginosa*, *S. aureus*.

The isolated endophytic crude extract G7 showed zone of inhibition ranging from 10-12 mm against *P. aeruginosa*, *S. aureus*, *B. megaterium*.

The isolated endophytic crude extract G15 showed zone of inhibition ranging from 10-16 mm against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. megaterium* and *C. albicans*. Also amongst the three crude extracts G15 showed the maximum activity by forming zone of inhibition of 10 mm against *E. coli*, 15 mm against *P. aeruginosa*, 16 mm against *S. aureus*, 14 mm against *B. megaterium* and 9 mm against *C. albicans*.

And total of five endophytic fungal crude extracts from *T. arjuna* were tested for the preliminary antimicrobial testing. Amongst these five extracts three showed the antimicrobial activity against the microorganisms (*E. coli*, *P. aeruginosa*, *S. aureus*, *B. megaterium* and *C. albicans*). The isolated fungal crude extract A showed the maximum activity amongst all the other extracts of endophytic fungi from Arjuna. The zone of inhibition formed was ranging from 17-24 mm. It formed 22 mm of zone of inhibition against *E. coli*, 17 mm against *P.aeruginosa*, 18 mm against *S. aureus*, 24 against *B. megaterium* and 17 mm against *C. albicans*. The other extracts A1 formed 8 mm of zone of inhibition only against *S. aureus*, whereas A3 formed 10 mm of zone of inhibition against *E. coli* and 8 mm against *P. aeruginosa* and A4 extract formed 8 mm of zone of inhibition against *P. aeruginosa* and 7 mm against *S. aureus*.

With exopolysaccharide extracts

EPS (Extracellular Polymeric Substances) was extracted from the endophytic fungi (T2) obtained from taxol plant as described in section 3.11. The endophytic fungus was grown in PDB one with incubation period of 15 days (EPS 1) and other with the incubation period of 20 days (EPS 2) and processed further similarly to obtain the EPS.

EPS extracted from the above endophytes exhibited broad spectrum of activity against microorganisms(*E. coli*, *P. aeruginosa*, *S. aureus*, *B. megaterium* and *C.albicans*). The antimicrobial activity of EPS 1 was more than the EPS 2. The zone of inhibition formed by the EPS 1 was larger than EPS 2.

As EPS is secreted by organism in its growth phase so it is active in that period so EPS 1 (incubation period 15 days) is active and exhibiting good antimicrobial activity. In case of EPS 2 the activity of the organism is lowered as they enter stationary phase and also some other secondary metabolites cause the hindrance.

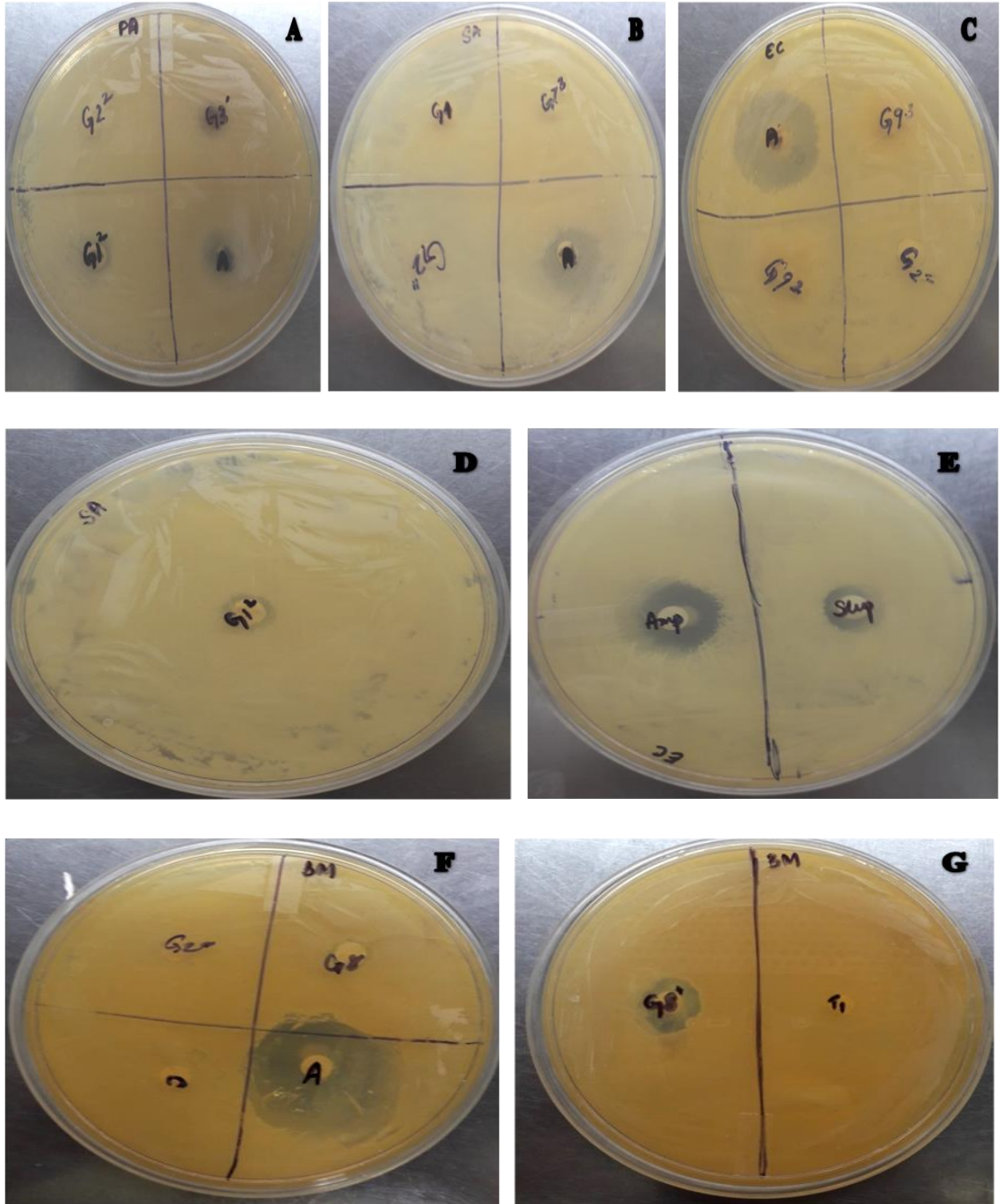


Fig. 5: Zone of inhibition by fungal crude extracts on 4 bacterial test cultures *S. aureus*, *P. aeruginosa*, *E. coli*, *B. megaterium*. (A) activity of extract G1, G7 and A against *P. aeruginosa*, (B) activity of extract A against *S. aureus*, (C) activity of extract A against *E. coli*, (D) activity of extract G1 against *S. aureus*, (E) activity of extract antibiotics against *E. coli*, (F) and (G) activity of extract A and G7 against *B. megaterium*.

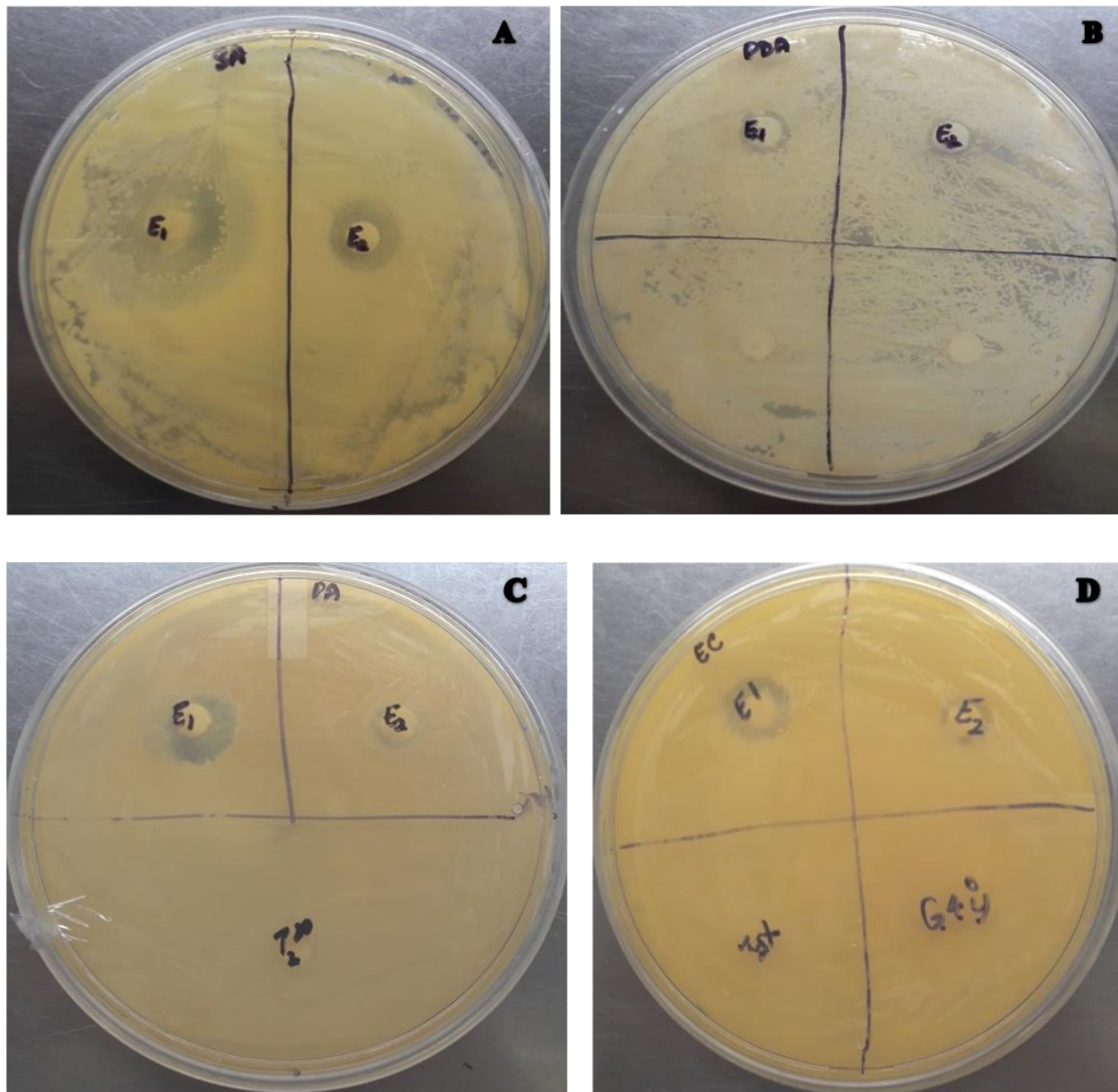


Fig 6: Zone of inhibition formed by EPS 1 and EPS 2 with different microorganisms (A) with *S. aureus* (B) with *C. albicans*, (C) with *P. aeruginosa*, (D) with *E. coli*.

4.5 Microdilution method- Spectrophotometric method

Based on the preliminary antimicrobial activity by agar disc diffusion method, out of all samples four fungal crude extracts three obtained from endophytic fungi of *T. cordifolia* and one from the endophytic fungi of *T. arjuna* and two EPS were selected for further analysis.

Stock (5mg/ml) of the samples was prepared in 10% DMSO for the fungal crude extract and in Milli Q water for EPS. The working concentration used was 1mg/ml.

Antibiotics (Streptomycin and Ampicillin) were used at the concentration of 50µg/ml for all the test bacterial cultures but for fungal test cultures the concentration of 1µg/ml of amphotericin used.

Antimicrobial activity of crude samples for different test culture was studied by taking the O.D at 600 nm.

Table 3: Antimicrobial activity of sample against *P. aeruginosa*

Crude extracts	Percent growth inhibition
A	63.10±0.31
G15	59.80±0.30
G2	62.50±0.01
G7	15.54±0.27
EPS 1	26.38±0.58
EPS 2	17.40±0.48
Antibiotic (streptomycin)	87.44±0.31
Antibiotic (ampicillin)	92.37±0.09

The maximum inhibition of 63% is exhibited by extract A followed by 62% by G2 and further 59% by G15. G7 showed very less of the inhibition (15%). EPS 1 showed inhibition of 26% and EPS 2 showed 17%.

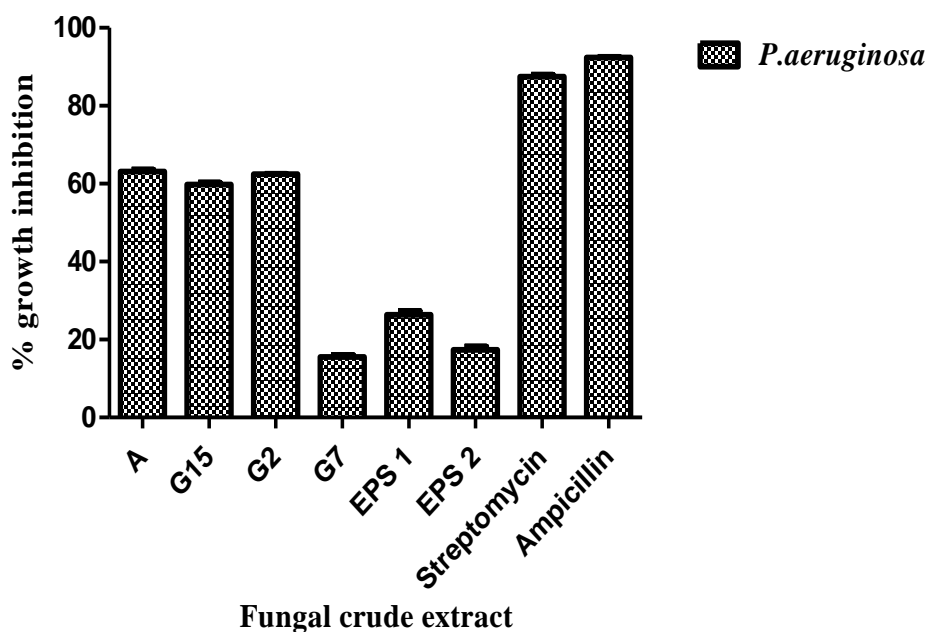


Fig7. Percent growth inhibition against *P.aeruginosa* by different extracts

Table 4: Percent growth inhibition of fungal crude extracts against the *B. megaterium*

Crude extracts	Percent growth inhibition
A	62.38±0.63
G15	60.94±0.22
G2	54.43±1.15
G7	28.37±0.55
EPS 1	39.66±0.65
EPS 2	27.99±0.50
Antibiotic (streptomycin)	87.08±0.38
Antibiotic (ampicillin)	89.22±0.62

The maximum growth inhibition for *B. megaterium* was shown by crude extract A (62%) and almost similar was shown by G15 (60%) and followed by G2 (54%) where as G7 showed least

inhibition among the fungal extracts and same pattern with EPS was seen that, EPS 1 showed more inhibition than EPS 2.

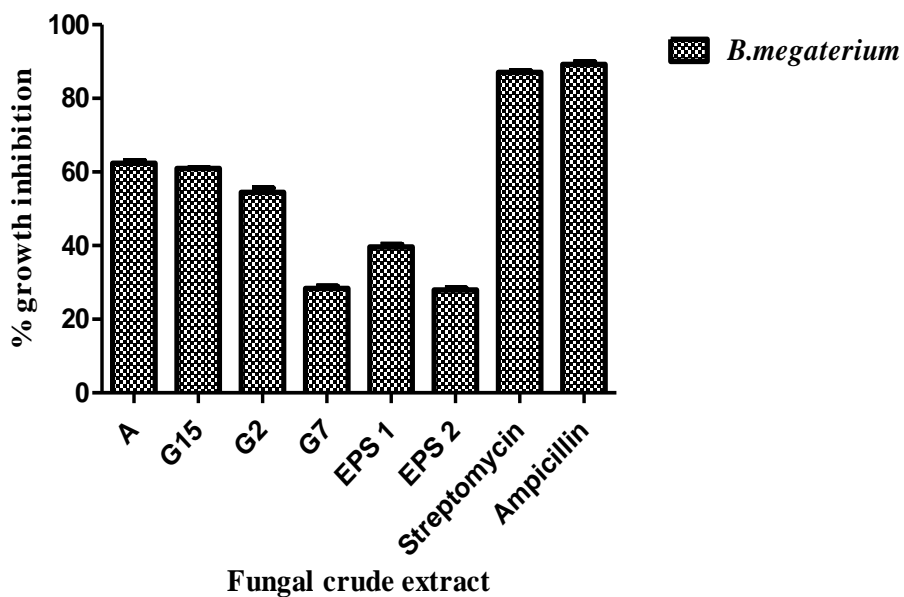


Fig 8: Percent growth inhibition against *B. megaterium* by different extracts

Table 5: Percent growth inhibition of fungal crude extracts against the *E. coli*

Crude extracts	Percent growth inhibition
A	58.02±1.32
G15	48.53±1.04
G2	19.66±0.37
G7	16.57±0.43
EPS 1	55.84±0.28
EPS 2	28.34±0.69
Antibiotic (streptomycin)	82.56±0.65
Antibiotic (ampicillin)	81.22±1.08

The maximum growth inhibition for *E. coli* was shown by crude extract A (58%) followed by G15 (48%) where as G2 and G7 showed least inhibition among the fungal extracts and in case of EPS, EPS 1 showed 55% of the inhibition which is more than EPS 2.

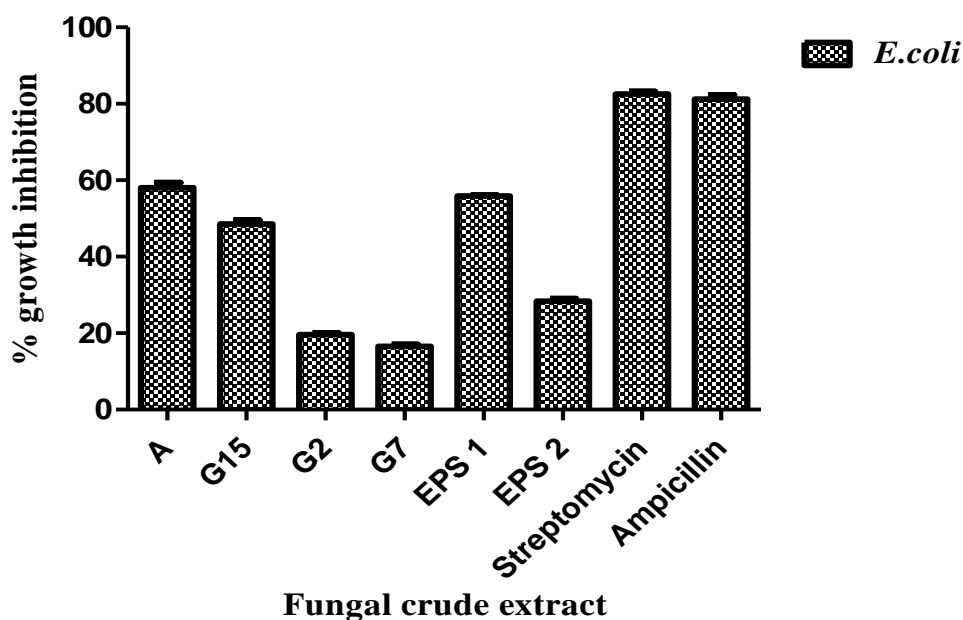


Fig 9 Percent growth inhibition against *E. coli* by different extracts

Table 6: Percent growth inhibition of fungal crude extracts against the *S. aureus*

Crude extracts	Percent growth inhibition
A	57.55±0.25
G15	61.72±0.44
G2	50.5±0.11
G7	22.88±0.33
EPS 1	51.38±0.54
EPS 2	43.09±0.31
Antibiotic (streptomycin)	86.11±0.23
Antibiotic (ampicillin)	89.62±0.35

The maximum growth inhibition for *S. aureus* was shown by crude extract G15 and followed by A that showed almost similar inhibition of approximately 58% where as G2 showed the inhibition of 50% and G7 showed least inhibition among the fungal extracts. In case of EPS, EPS 1 showed more inhibition of 51% than EPS 2 with 43%.

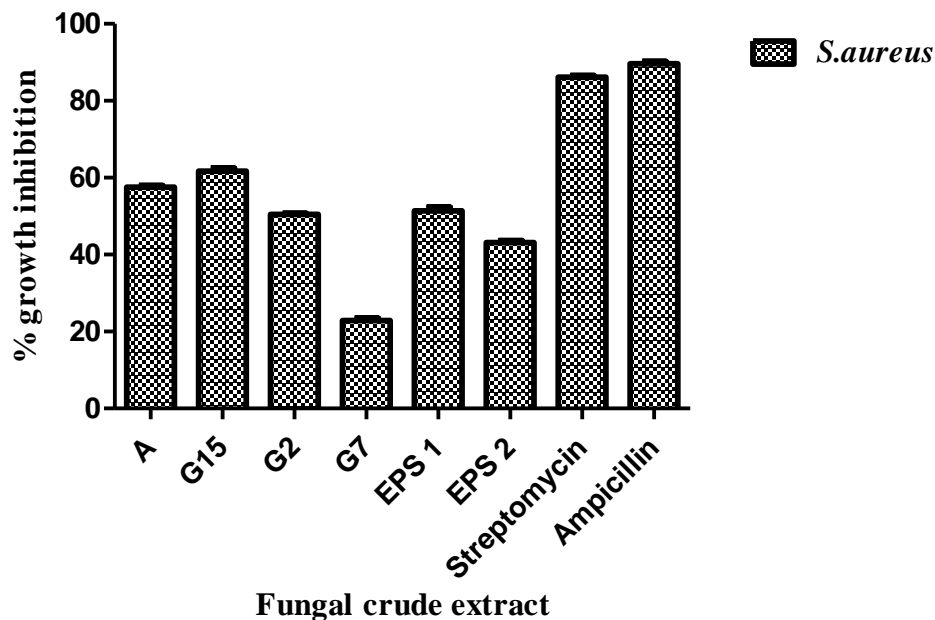


Fig. 10: Percent growth inhibition against *S. aureus* by different extracts

Table 7: Percent inhibition by fungal crude extracts on *C. albicans*

Crude extracts	Percent growth inhibition
A	40.7±1.6
G15	45.6±1.0
G2	47.3±1.0
G7	19.3±1.1
EPS 1	21.2±0.9
EPS 2	20.6±1.3
Amphotericin	43±0.3

For the *C. albicans* the maximum inhibition was shown by G2 of 47% followed by G15 of 45% followed by A 40% and G7 showed the least then EPS 1 and EPS 2 almost same inhibition of 21% and 20% respectively. But none of the extract showed more than 50% of the inhibition for *C. albicans*.

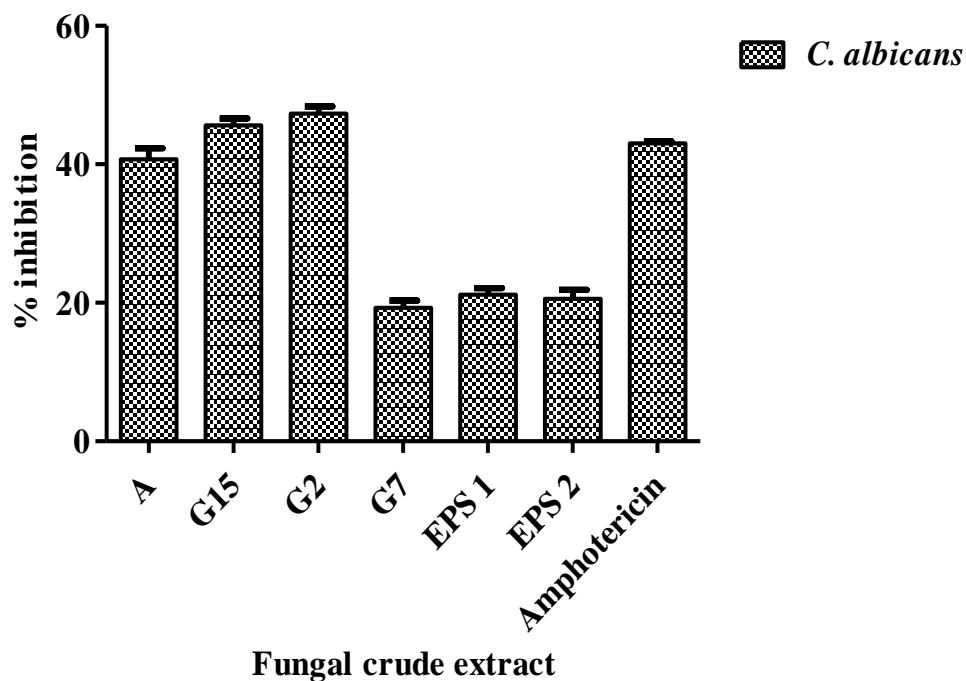


Fig. 11: Percent growth inhibition against *C.albicans* by different extracts

4.6 Preliminary phytochemical analysis of fungal crude extracts

Extracts of stock 1mg/ml were preped in methanol for this analysis and was done to know qualitatively which type of phytochemicals are present in selected crude extracts that were exhibiting good antimicrobial activities

Table 8: Preliminary phytochemical analysis for crude fungal extracts

Tests	G2	G7	A
For Alkaloids	+	+	++
For Amino acid	+	+	+
For Carbohydrate	++	++	+
For Fats and Oils	-	-	-
For Flavonoids	++	+	+++
For Glycosides	-	-	-
For Steroids and Terpenoids	-	+	+
For Tannins and Phenolics	+	-	+

(-) Negative test (no appearance of the required colour, any flocculation or any precipitation)

(+) Weak positive test

(++) Positive test not turbidity and flocculation

(+++) Test firmly positive

The fungal crude extract G2 indicated the presence of alkaloids, amino acids, tannins and phenolics. Presence of carbohydrates and flavonoids was prominent in this extract whereas it showed absence of steroids, glycosides, terpenoids, fats and oils.

Crude extract G7 indicated the positive results for alkaloids, aminoacids, flavonoids, steroids, carbohydrates and terpenoids whereas negative results were seen for fats, oils, glycosides, tannins and phenolics.

In case of crude extract A presence of alkaloids and flavonoids was indicated. It also showed the presence of amino acids, carbohydrates, steroids, terpenoids, tannins and phenolics to an extent but showed absence of glycosides, fats and oils.

4.7 Identification of the endophytic fungi

4.7.1 Morphology based

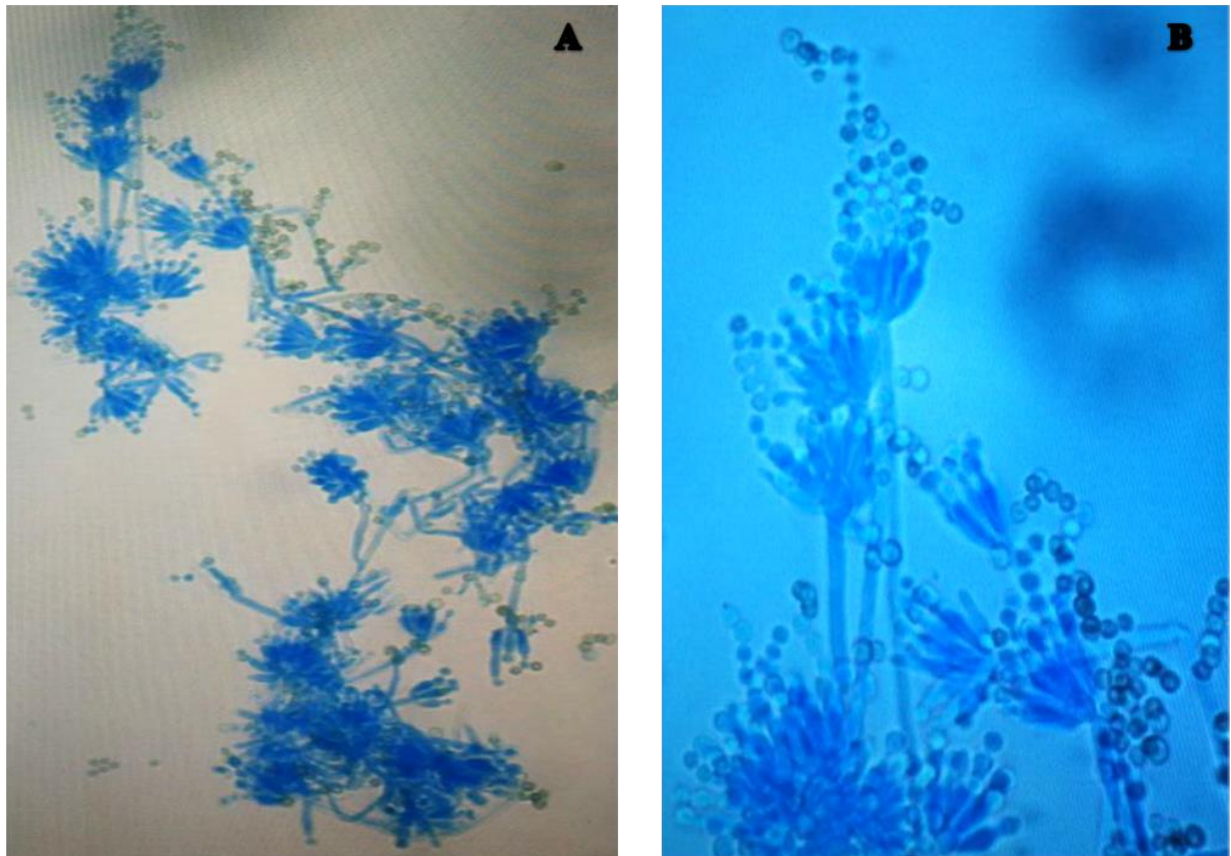


Fig. 12: Microscopic view of endophytic fungi isolated from Arjuna

Fungal crude extract A showed a broad spectrum against all the microorganisms with % inhibition more than 50 against all the bacteria. It also showed the activity against the fungal test culture so this was identified morphologically and molecularly.

Morphologically the colony patterns and conidia were seen under the microscope of the endophytic fungi that was grown on PDA plate with the help of dye. Under the microscope, the isolated fungi appeared as *Penicillium* sp. by showing the presence of conidia and hyphae as shown above in the fig 12. The surface of the fungus as observed on the PDA plate can be described as velvety and green, the same was observed in the plate that had the endophytic fungi as well. Many-branched conidiophores sprout on the mycelia as shown in the figure 12. These all characteristic features match to *Penicillium* sp. suggesting it to be the sp. of *Penicillium*.

4.7.2 Molecular based

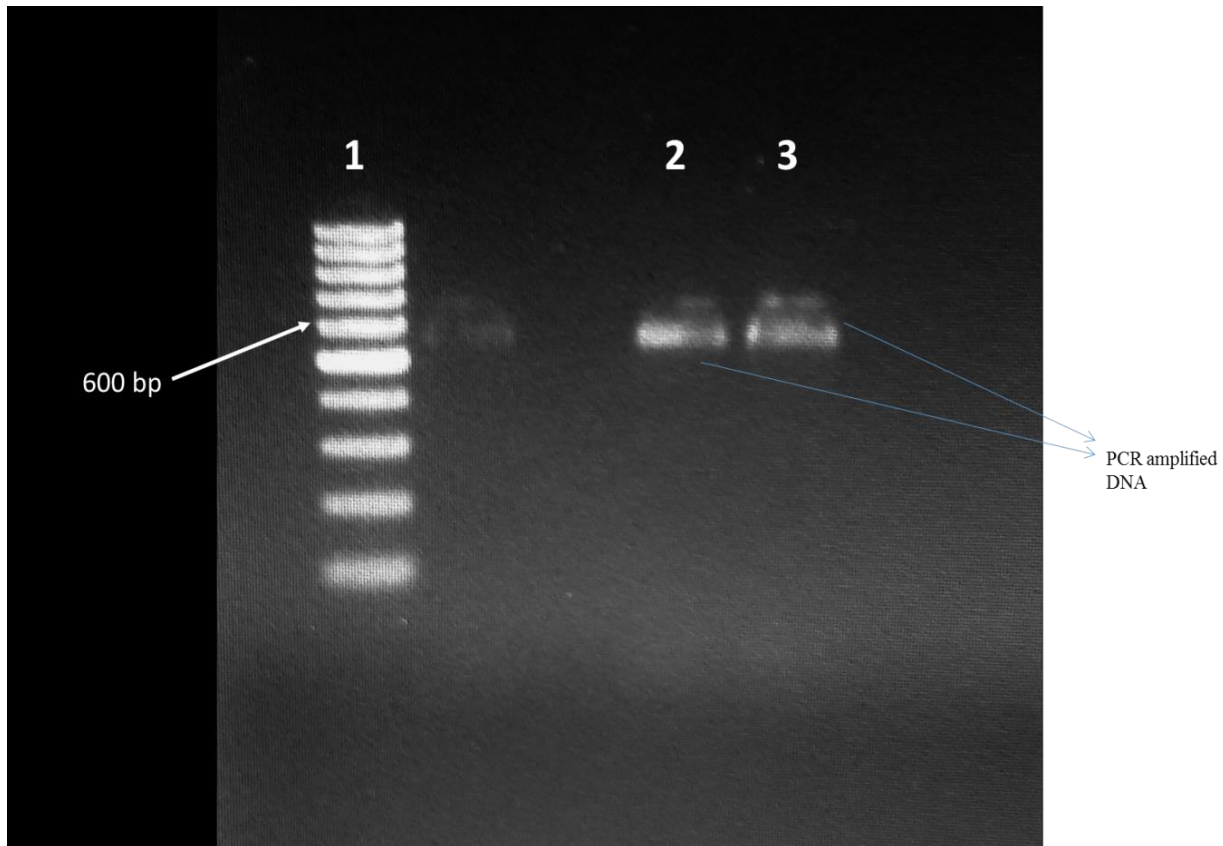


Fig. 13: Electrophoresis of the PCR amplified DNA from the fungus of the Arjuna

After the microscopic analysis the DNA was isolated from the endophytic fungi obtained from Arjuna and its electrophoresis was done to know the presence of genomic DNA. Isolated DNA was PCR amplified with the ITS1 and ITS4 primers. Transformation was done for the DNA followed by the screening by IPTG and Xgal (blue white screening). The positive transformants were further sent for the sequencing.

4.8 EPS

There were two samples of EPS that were obtained based on the period of incubation given to each of them:

- One with 15 days of incubation period
- Second with 20 days of incubation period

Both were processed similarly to obtain the EPS and further given the treatment of Tri-chloroacetic acid (TCA) with which we obtained the crude exopolysaccharide. Further protein estimation and carbohydrate estimation was done of the obtained exopolysaccharide to know the amount of the carbohydrate present in it and to know whether the proteins have been removed from the sample or not.

4.8.1 Estimation of proteins

Bradford assay is a widely used colorimetric assay developed by Marion Bradford for the estimation of the proteins. Total protein content was estimated by Bradford assay. BSA (10-60 µg/mL) was used as standard and O.D. was taken 595 nm (Table 5).

Table 5: Absorbance (595nm) of extract and standard

Concentration (µg/ml)	O.D (592nm)
0	0.09
10	0.13
20	0.15
40	0.19
60	0.2
Sample 1	0.08(-10.3)
Sample 2	0.1(-3.63)

The EPS sample were given the treatment of tri-chloroacetic acid for precipitating the protein so that we remain with crude exopolysaccharide which was characterized for its antimicrobial activity as seen in section 4.4 and 4.5

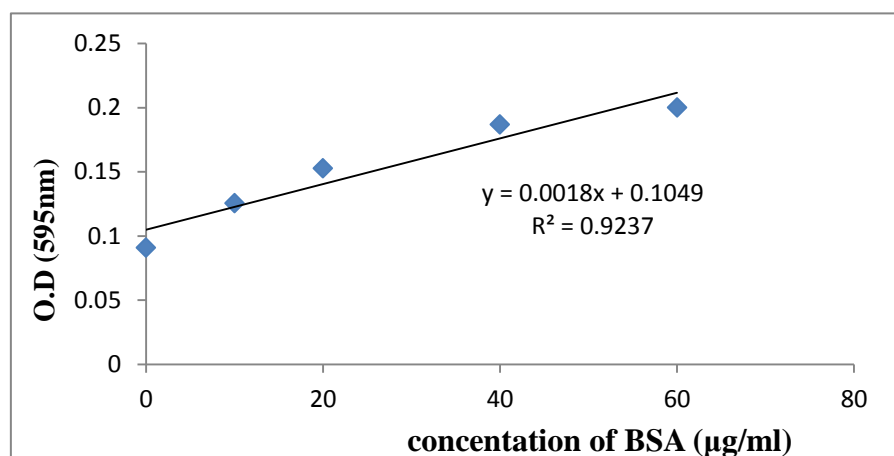


Fig. 6: Standard curve of BSA

4.8.2 Phenol sulfuric test

This colorimetric assay is used for the estimation of the carbohydrate content in the extract. This test was done to estimate the carbohydrate content in the crude EPS. Glucose served the purpose of standard from concentration 10-150µg/ml.

Table 6: Absorbance (492nm) of extract and standard

Concentrations (µg/ml)	O.D. (492nm)
0	0.05
10	0.16
20	0.46
40	0.83
60	0.92
80	1.25
100	1.98
120	2.319
150	3.209
Sample1 (15 days)	0.55(30.18)
Sample2 (20 days)	0.78(41.38)

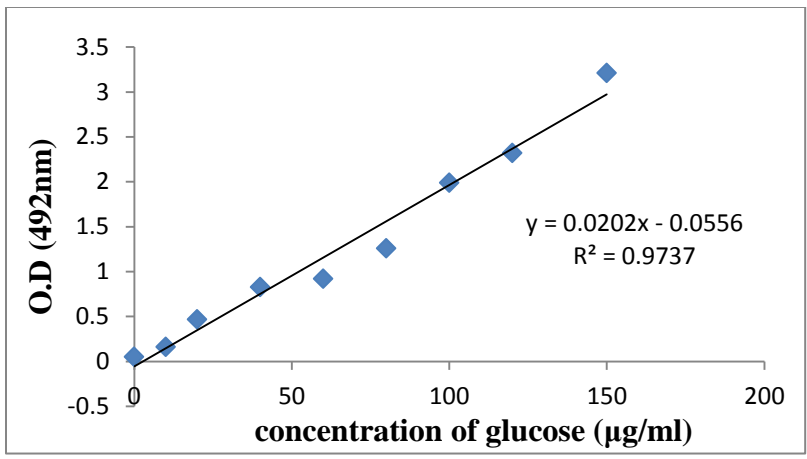


Fig.7: Standard curve of glucose

EPS concentration of sample 1 was found to be 30 µg/ml and of sample 2 was found to be 41µg/ml.

CONCLUSION

In this study medicinal plants *Tinospora cordifolia* (Giloy) and *Terminalia arjuna* (arjuna) have been used for the isolation of the endophytic fungi. Also, Extracellular Polymeric Substance (EPS 1 and EPS 2) was extracted from the given isolated endophytic fungi T2.

The number of fungal isolates obtained from Giloy were seventeen (G1-G17) and five (A1-A5) from Arjuna. These were tested for preliminary antimicrobial activity by agar diffusion method and final antimicrobial assay by microdilution method using cultures of *E.coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* and *Candida albicans*.

The maximum inhibition was shown by extract A amongst all the extracts of Arjuna. It showed inhibition of 63% against *Pseudomonas aeruginosa*, 62% *Bacillus megaterium*, 57% against *Staphylococcus aureus*, and 58% against *E.coli* and showed 40% against *Candida albicans*.

Amongst the extracts from Giloy G15 showed the best activity of 48%, 61%, 59%, 60%, 45% against *E.coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* and *Candida albicans* respectively.

The isolated endophytic fungi from the bark of *Terminalia arjuna* exhibiting the best antimicrobial activity was further chosen for identification. The culture characteristics and microscopic features revealed that the above isolated endophytic fungus belongs to *Penicillium sp.* Further fractionation and purification of the crude fungal extracts is required to determine the exact compound responsible for antimicrobial activity. Further studies are to be done to isolate compounds from this endophytic fungus so as to obtain novel bioactive compounds. To meet the ever increasing demand of new drugs one can explore these endophytic fungi for obtaining novel bioactive compounds.

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ANNEXURE 1

Table 11: Composition of MHA

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

pH adjusted neutral at 25°C

Table 12: Composition of PDA

Ingredients	Gram/liter
Potato infusion	200
Dextrose	20

Final pH (25°C) 5:1

Table 13: McFarland standard

McFarland No.	0.5
1.0% BaCl ₂ .2H ₂ O (ml)	0.05
1.0% H ₂ SO ₄ (ml)	9.95
Approx. Cell density (cfu/ml)	1×10 ⁸
Percentage transmittance	74.3
Absorbance at 600nm	0.132