Chapter III

INTRODUCTION

Need for new and useful compounds that provide relief in all aspects of human ailments is ever growing. Drug resistant pathogenic bacteria, life threatening viruses and tremendous un-identified diseases were the major medical problems. In addition, environmental degradation, loss of biodiversity and spoilage of land and water are also major problems faced by mankind. Therefore, apart from medicinal plants, search for alternative source for production of secondary metabolites of medicinal importance resulted in studies on fungi. They act as an important resource for finding new biotechnological products.

Fungi that inhabit specific habitats produce unique biochemicals and they became an alternative source to obtain such compounds. Endophytic fungi are a best example and excellent source for natural bioactive products (Azevedo et al., 2000). Their natural products become more important in the field of medicine, industry and agriculture. Therefore, it is widely used for novel drug discovery (Strobel, 2003; Strobel et al., 2004). From 2008-2009 more than 100 natural products have been discovered from endophytic fungi. Recent investigations gave clear explanations about chemical nature of compounds produced by endophytes. They are alkaloids, steroids, terpenoids, isocoumarins, quinines, flavonoids, phenylpropanoids, peptides, phenolics and aliphatics (Zhang et al., 2006) with wide properties like antimicrobial, antiparasitic, cytotoxic, neuropropective, antioxidant and as immunosuppressant.

Secondary metabolites produced by microbes are not needed for the growth and other essential processes. Secondary metabolties produced by microbes greatly differ qualitatively and quantitatively depending on the type of strain, their nutrition and culture conditions. Of late biotechnology industries focus on fungal growth and secondary metabolite production as essential features. Various factors like temperature, pH, incubation period, salinity, carbon and nitrogen sources and amino acid plays a major role in the production of secondary metabolites (Adinarayana et al., 2001). Optimization of media/broth is generally carried out at two levels, initially the effects of the ingredients / nutrients on growth are screened and few parameters are selected and optimized. Studies on biosynthesis of secondary metabolites involve an optimization of culture media for their production that can be achieved by a systematic study of the suitability of physiological parameters and supplementary nutrition.

Plant derived secondary metabolites are chemical compounds which are not involved in development, primary growth and reproduction but involved in defense mechanism, communication, attraction of pollinators etc., (Verdine, 1996). On the basis of their structure, these compounds are grouped as alkaloids, terpenoids, phenolics, phytosteroids, flavonoids etc. Usually plant derived phytochemicals are important in the fields of pharmaceutical industries for the preparation of drugs and medicines. Now-a- day's some important plants and their compounds were diminishing due to deforestation and over exploitation. It is very laborious, costly and time consuming to extract chemicals from plants (Staniek et al., 2008). Investigation of the natural product chemistry of endophytes showed their potential to synthesize phytochemicals that are normally produced by the host plant. Endophytes are now well known to possess phytochemicals with various biological activities (Aly et al., 2010).

Endophytic fungi play an important role in protecting the host against various biotic and abiotic stresses. Hallman et al., (1997) stated that enzymes produced by endophytes help to initiate the host symbiosis process. Many researches showed that, endophytes reduce stress through the production of essential biochemicals (Khan et al., 2015). Among all biochemicals, extracellular enzymes have been regarded as important and significant source for endophytes which helps to associate with plants. They are widely used in food, beverage, confectionary, textile and leather industries (Traving et al., 2015). Enzymes obtained from endophytes were more stable than enzymes derived from other sources.

Microbial production of organic acids was considered as promising strategy to obtain building- block chemicals from renewable feedstocks being compatible with the biorefinery concept (Sauer et al., 2008). They are widely used in food industries as additives, preservatives etc. Fungi are particularly useful for industrial applications because of their production of low molecular weight organic acids. The organic acids were fully degradable molecules used in production of biodegradable polymers and replace petroleum based or synthetic chemicals. Organic acids obtained from fungi helps in solubilization of soil minerals and release nutrient ions to plants (Vassilev et al., 2015).

Antimicrobial metabolites (antibiotics) are low molecular weight organic compounds produced by microbes that are active at low concentrations, against another microbe. These chemicals are not required for its growth, but produced as an adaptation for specific

function in nature and are the most bioactive natural products, isolated from endophytes (Guo et al., 2008). Therefore, endophytes were believed to carry out a resistant mechanism against pathogenic invasions by producing secondary metabolites with antimicrobial activity. Screening of antimicrobial compounds from endophytes help to produce important drugs, which are active against human and plant pathogen (Yu et al., 2010).

Discovery of compounds with antioxidant activity is important because they are highly active against damage caused by reactive oxygen species and oxygen derived free radicals, which contribute to a variety of pathological effects (DNA damages, carcinogenesis and cellular degeneration). They also act as a useful therapy for treating diseases like cancer, cardiovascular disease, hypertension etc., (Valko et al., 2007). Fungal endophytes present in higher plants act as good source for novel antioxidants.

Endophytes act as plant growth promoters by secreting different types of phytohormones. Their production is similar to the production of phytohormones by host plant but in higher quantities (Zhao et al., 2010). Production of growth harmones by endophytes depends on conditions like pH, temperature, incubation period, internal physiology of microbes etc., (Khan et al., 2012). Gibbrellins and indole acetic acid (IAA) were important in promoting growth. Gibbrellins enhance crop growth and protects plants from harmful effects of abiotic stresses and also regulate various developmental and physiological processes in plants. Similarly, IAA promotes developmental process in plants like root, axillary bud development and flower formation etc.

Owing to great importance of biological property of endophytic fungi, the present study was undertaken to evaluate the biological properties (Phytochemicals, extracellular enzymes, organic acids, pigments, growth hormones, antimicrobial activity and antioxidant activity) of true endophytes.

REVIEW OF LITERATURE

Recent researches focus on searching different habitats to obtain potential sources of natural bioactive agents which are of pharmaceutical, agricultural and industrial applications (Liu et al., 2001). Natural products are chemical compounds which are usually obtained from different living organisms such as plants, animals, marine macroorganisms (Sponge, corals and algae) and microorganisms (Bacteria, fungi and actinomycetes). Discovery of natural products involve some crucial steps like isolation, structural elucidation and finding the bio-synthetic pathway for the production of secondary metabolites. Studying about different natural sources made considerable interest to researchers due to their variation in habit, habitat, diversity etc., (Suryanarayanan et al., 2009).

More than 20000 bioactive metabolites are produced from microorganisms. Among microbes, fungi play an important role in the production of secondary metabolites. These compounds are sometimes directly used as drugs or used as lead structures for synthetic modifications (Gunatilaka, 2006).

In fungus, secondary metabolite production was regulated by several developmental pathways. Production of secondary metabolites may take place during vegetative growth and sexual/asexual development (Tag et al., 2000) which are mediated by G- protein signaling. Example, promoting vegetative growth by suppressing sexual/asexual development takes place by signaling of α- subunit (FadA) of G-protein (Calvo et al., 2002). Oxylipins, a hormone like signaling molecule mediating the balancing the sexual spore to asexual spore production in some fungal species was found to contribute to the regulation of secondary metabolite production (Tsitsigiannins and Kellet, 2007). Likewise, conidiogenone that induce conidiation also induce secondary metabolite production (Roncal et al., 2002). Certain environmental factors such as carbon, nitrogen sources, temperature, light, pH, host-plant interaction and their communication also influence the fungal secondary metabolite production. Some environmental effects transmitted through proteins may positively or negatively induce secondary metabolite production. Example, Cre A for carbon signaling and Pac C for pH signaling (Tilburn et al., 1995). Competition among different fungi or among endophytic fungi within a plant organ may trigger the natural product accumulation (Ho et al., 2003). Additionally, in nature, when endophyte- plant interaction occurs, it also affects the metabolite profile of endophytes.

Among different types of fungi, endophytic fungi which are found in the intercellular spaces of the plants synthesize different types of bioactive agents that can be used by plants for its growth and defense processes (Zhao et al., 2010). Endophytes are present in almost all plants in the world. So the plants might support certain endophytes to produce secondary metabolites. Thus if a microbial source of the drug is available, then we can eliminate the harvesting of slow growing and rare trees. Since fermentation techniques are used to recover important compounds, supply of some important drugs increased which led to reduction in the price (Strobel, 2003).

Optimization studies

Microbial sources obtained from different environments are main producers of natural compounds with massive usuage as drug, towards pathogenic microorganisms. It is very essential to search for novel secondary metabolites with wide applications in chemotherapy, plant pathology, food preservation, veterinary medicine, biotechnology and molecular biology. Number of regulatory factors, including growth conditions, carbon, nitrogen, mineral salt levels and physical parameters like temperature, pH and agitation helps in biosynthesis of secondary metabolites (Padmavathy et al., 2012). These conditions may vary from species to species. Many of the microbes that live in extreme environments like high temperatures, high salt concentrations, low pH, and high radiation etc, influence fungal growth and metabolite productions. Usually the bioactive metabolite production from microorganisms depends on their special adaptations to their environment. Some of primary metabolic components help in biosynthetic pathways which lead to the secondary metabolite production. Secondary metabolism is regulated by the factors like carbon sources, nitrogen sources, phosphate, NaCl, trace elements and different parameters like temperature, pH and incubating time intervals. The regulatory factors involve in balancing of biosynthesis and yield of secondary metabolites. Conventional practice of single factor optimization maintaining other factors, improved from unspecified level to optimum constant level of production (Anuhya et al., 2017).

Gogoi et al., (2008) optimized culture conditions for improved growth and secondary metabolite production by an endophytic fungus *Fusarium* sp. isolated from *Taxus wallichiana*. Enhanced growth and secondary metabolite production was observed

when media was supplemented with 0.1% dextrose as carbon and yeast extract as nitrogen source with temperature optimum 25 ± 2°C and pH 6. Incubation period of 10 days was observed optimum for growth and production of metabolites.

Bhattacharyya and Jha (2011), studied optimization of culture conditions affecting growth and improved bioactive metabolite production by *Aspergillus* strain. They grew well and produced optimum bioactive metabolites in Potato dextrose broth at 25 °C on the 14^{th} day of incubation. The optimal mycelial growth, however, was obtained at pH 5.5. Sucrose (2.0 g/l) and asparagine (1.0 g/l) were the best carbon and nitrogen sources respectively for optimum growth and production of active metabolites by the isolate.

Jain and Gupta (2012), studied the effect of carbon and nitrogen source on secondary metabolite procuction by endophytic fungus *Penicillium* sp. The Result showed that maximum production of secondary metabolite was obtained when medium was supplemented with dextrose followed by xylose, maltose and lactose. There was a reduction in metabolite production when maltose and lactose were used as carbon source. Culture containing sodium nitrate showed the highest metabolite production followed by ammonium chloride, peptone and yeast extract as nitrogen source.

Mathan et al., (2013) reported that *Aspergillus terreus* produced high bioactive metabolites in potato dextrose broth, compared to the other media. Glucose and yeast extract were found to be the best and most suitable carbon and nitrogen sources respectively, for the optimum growth and production of bioactive metabolites. Maximum bioactive metabolite production occured at pH of 5.5 and temperature of 25°C.

Vandermolen et al., (2013) evaluated different types of culture media for screening the natural products from endophytic fungal strains obtained from *Asimina triloba* L., *(*Annonaceae). Both liquid and solid media were used for optimization process. Results suggested that, fungal strains grown on solid media produced more secondary metabolites than in liquid media.

Goutam et al., (2016) studied the enhanced secretion of active compound by *Aspergillus terreus* isolated from *Achyranthus aspera* by optimizing different parameters of culture conditions. Among different media used, potato dextrose broth (PDB) and

sabouraud's dextrose broth (SDB) proved to be better media for growth of fungus as well as metabolites production. 1% yeast extract and 4% dextrose resulted in higher cell inhibition. Ethyl acetate served as good extracting solvent.

Kalyani et al., (2016) studied *the* influence of cultural conditions and environmental parameters affecting the growth and bioactive metabolite production of the fungi *Aspergillus flavus*. It was found that, metabolite production by this isolate was greatly influenced by various cultural conditions. The optimum carbon source starch, nitrogen source beef extract, pH 6.0, temperature at 40^{o}C, 3 % NaCl concentration and incubation period of 144 h were found for the maximum metabolite production.

Astuti et al., (2017) optimized culture conditions of *Aspergillus fumigatus* isolated from *Piper crocatum* to enhance secondary metabolite production. The optimal total metabolite production was obtained in Czapex Dextrose Broth. The strain produced higher total metabolite when it was grown at pH ranging 6 – 7. Optimum production was achieved in basal media supplemented with glucose as carbon source and beef or peptone as nitrogen source. The optimal mycelial growth was achieved in Sabourod Dextrose Broth, with the maximum growth at 29°C supplemented with glucose and yeast as carbon and nitrogen sources, respectively. Varying pH of basal medium showed no significant differences in biomass production.

Deka and Jha (2018), optimized the culture parameters for improved bioactive secondary metabolite production by *Geosmithia pallida*, a potent endophytic fungi, isolated from endangered medicinal plant *Brucea mollis*. Maximum metabolite production was observed at 25°C incubation temperature and pH -6.5 on 28th day of inoculation, when lactose and yeast extract were added in the basal medium respectively as carbon and nitrogen source along with 3.5 g/l of NaCl.

Bioprospecting of endophytic fungi and their natural products

By considering the increased global health convern over the failure of currently used drugs to many uncurable diseases, indiscriminate exploitation of medicinal plants for extraction of important drugs of plant origin and limitations of plant resources due to some factors like requirement of land for cultivation, environmental competence of plants, seasonal specificity etc., the search for new and effective drugs is becoming a necessity.

Throughout the world, searching for novel bioactive compounds from various biological sources was increasing with high effectiveness, low toxicity and negligible environmental impacts. Among all biological sources, microbes generally possess abundant source of novel chemo-types and pharmacophores. Recently, many drug companies focused their research entirely on combinational chemistry involving the automated synthesis of small molecules that are structurally related (Bills et al., 2002). Their loss of interest on natural products is due to the enormous expenses and efforts that are required to identify a source, isolate active natural products, interpretation of their structure and being the long process of product development. Apart from these, extraction of natural products leads to the depletion of plant population which is undesirable. At the same time, production of plant based natural drugs is always not upto the expected level because it depends on specific developmental stage, environment condition, stress, nutrient availability etc. Therefore, endophytic fungi in particular, act as ready renewable and inexhaustible source of novel compounds.

Here, the role of endophytes became important. Endophytic fungal strains were capable to produce biological substances with various properties like pigments, bioactive metabolites, immune-suppressants, anticancer, antimicrobial, antidiabetic, enzyme inhibitor, biocontrol agents and also source of alkaloids, flavonoids, peptides, phenols, quinines, steroids, terpenoids and aliphatic compounds (Gangadevi and Muthumary, 2007). Usually traditionally used medicinal plants are natural choice for the study of endophytes due to their proved medicinal properties. Table-18 shows some examples of bioactive products from endophytic fungi.

Table-18: Natural products of endophytic microorganisms

Microbial strain	Plant host/plant part or tissue	Natural product	Biological activity
Aspergillus clavatus	*Taxus mairei*	Brefeldin A	Antifungal, antiviral, anticancer and weed management
Aspergillus fumigatus	*Cynodon dactylon*	Asperfumoid	Antifungal, mycotoxin
Aspergillus niger	*Cynodon dactylon*	Rubrofusarin	Cytotoxic, Xanthine oxidase inhibitor
Aspergillus sp.	*Cynodon dactylon*	Helvolic acid	Antibacterail; eosinophil inhibitor
Aspergillus terreus	*Hyptis suaveolens*	Terrein	Antioxidant and Antimicrobial
Botrytis sp.	*Taxus brevifolia*	Ramulosin	Antibiotic
Chaetomium globosum	*Ephedra fascicualata*	Globosumone A	Cytotoxic
Diaporthe sp.	*Forsteronia spicata*	Cytosporone A	Antifungal, cytotoxic, antibacterial
Fusarium oxysporum	*Catharanthus roseus*	Vincristine	Anticancer
Mycelia sterilia	*Cirsium arvense*	Perussomerin	Antifungal and Antibacterial
Nodulisporium sp.	*Bontia daphnoides*	Nodulisporic acid	Insecticidal
Paecilomyces	*Taxus mairei*	Brefeldin A	Antifungal, antiviral, anticancer and weed management
Penicillium implicatum	*Diphylleia sinensis*	Phodophyllotoxin	Antifungal, antiviral and weed management

Source (Gunatilaka, 2006)

Phytochemicals

Screening of phytochemicals produced by endophytic fungi helps to determine the chemical components which act as important source for medicinal and industrial uses. Presence of phytochemicals is an indicator that they can be exploited as precursors in the development and advancement of synthetic drugs

Murthy et al., (2011) isolated endophytic fungi from *Lobelia nicotianifolia* and screened methanolic extracts of *Fusarium*, *Aspergillus*, *Penicillium* and *Mucor* for the presence of phytochemicals. All the four isolates showed the presence of glycosides. Similarly, ethyl acetate extract of *Penicillium* also showed the presence of glycosides in addition to phytochemicals like alkaloids, phenols, flavonoids and tannins (Devi et al., 2012) when isolated from *Centella asiatica*.

Different parts of *Plumeria acuminata* and *Plumeria obustifolia* were used for isolation of endophytic fungi. Among twenty four endophytes, ethyl acetate extract of two endophytes, *Colletotrichum gloeosporiorides* and *Fusarium oxysporum* showed the presence of phytochemicals like flavonoids, phenols, alkaloids and steroids (Ramesha and Srinivas, 2014). Likewise, Yadav et al., (2014) isolated twenty one endophytic fungi from *Eugenia jambolana*. Ethyl acetate extracts of *Aspergillus* sp., *Chaetomium* sp., and *A.terreus* showed the presence of phenols, flavonoids, aminoacids, alkaloids and terpenoids.

Bhardwaj et al., (2015) isolated seventeen endophytic fungi from spikes of *Pinus roxburghii*. Four endophytes *Alternaria alternata*, *Thielaviopsis basicola*, *Geotrichium alba* and *Penicillium frequentans* showed the presence of alkaloids, flavonoids, phenols, tannins and terpenoids. Similarly ethyl acetate extract of *Alternaria* sp., isolated from *Tridax procumbens* showed the presence of alkaloids, flavonoids, cardiac glycosides, terpenoids and steroids (Kumar et al., 2015). Ladoh et al., (2015) isolated four genera of endophytic fungi from stems of *Phragmanthera capitata*, namely *Aspergillus*, *Penicillium*, *Trichoderma* and *Fusarium* and showed the presence of Anthraquinone, coumarins, phenols, tannins, flavonoids, steroids and terpenoids.

Methanolic and ethyl acetate extracts of six endophytic fungi isolated from leaves of *Cupressus torulosa* showed the presence of saponins, phenols, tannins, terpenoids, flavonoids, alkaloids and carbohydrates (Sharma et al., 2016). Likewise, Sowparthani (2016)

isolated *Psetalotiopsis* sp., from *Acalypha indica* and tested crude culture filtrates for phytochemicals. Results showed the presence of only terpenoids.

Dinesh et al., (2017), Gayathri and Chandra (2017) and Murugan et al., (2017) studied the production of phytochemicals by *Penicillium* sps., isolated from plants like *Simarouba glauca*, *Helicteres isora* and *Tabebuia argentea*. All the studies inferred that they have the capacity to produce flavonoids, alkaloids, terpenoids and phenols.

Mani et al., (2018) isolated endophytic fungi from *Aegle marmelos*. Most dominant and potent endophytic strains like *Curvularia australiensis* and *Alternaria citrimacularis* were screened for the presence of phytochemicals. Both strains exhibited the presence of flavonoids and phenols in hexane extracts. Satari et al., (2018) isolated various species of *Aspergillus* sp., from *Achilea millefolium*. Among the isolates *A.terreus* showed the presence of tannins, steroids, flavonoids, terpenoids, alkaloids, phenols and saponins.

Kanjana et al., (2019) isolated endophytic fungi from *Justicia adhatoda*, *Memecylon edule* and *Passiflora foetida*. Ethyl acetate extracts of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* were tested for phytochemicals. *C.globosum* showed positive results on carbohydrates, proteins, phenols, flavonoids, alkaloids, tannins and saponins. Likewise, methanol, chloroform and ethyl acetate extracts of *Aspergillus* sp., isolated from *Boerhavia diffusa* showed the presence of alkaloids, flavonoids, phenols, steroids, terpenoids, carbohydrates, tannins, proteins and aminoacids except saponins (Thakur and Sahani, 2019).

Extracellular enzymes

Microbial source play an important role in the production of extracellular enzymes. Fungi and yeast were capable of producing 50% of industrial enzymes. 35% produced by bacteria and remaining 15 % by phytopathogenic fungi. They produce industrially important enzymes like amylase, cellulase, protease, pectinase, laccase and lipase. Like other organisms endophytes also produce extracellular enzymes for colonization in host. Therefore, it is important to study the enzyme activity of endophytes.

Maria et al., (2005) isolated fourteen endophytic fungi from *Acanthus ilicifolius* and *Acrostichum aurem*. Among them, different strains of *Aspergillus* sp., showed the presence of

cellulase, protease and lipase enzymes. Similarly *Aspergillus* sp., and *Penicillium* sp., isolated from oil-rich environment have the capability to synthesize cellulase enzymes (Gopinath et al., 2005).

Amirita et al., (2012) isolated twelve different species of endophytic fungi from *Adhatoda vasica*, *Costus igneus*, *Coleus aromaticus* and *Lawsonia inermis*. Among them, eight strains of *Aspergillus* sp., *Penicillium* sp., and *C.globosum* exhibited the presence of amylase, cellulase, lipase and protease. Likewise, Bhagobaty and Joshi (2012), isolated *Talaromyces* sps., and *Penicillium* sp., as endophytes from five medicinal plants of sacred forests of Meghalaya. Preliminary screening of extracellular ezymes from endophytes showed the presence of cellulase, lipase, protease and xylanase.

Sunitha et al., (2013) isolated fifty endophytic fungi from *Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum* and *Catharanthus roseus*. Species of *Chaetomium*, *Aspergillus*, *Penicillium* and *Talaromyces* showed positive result for production of cellulase, amylase, lipase, laccase, protease and pectinase enzymes.

Azeem et al., (2014) collected *C.globosum* from different habitats of Egypt and screened for extracellular enzymes. Results showed the presence of amylase, cellulase, laccase, protease, lipase, pectinase and chitinase. Similarly, *Aspergillus* sp., and *Chaetomium* sp., exhibited the presence of amylase, cellulase and protease when isolated as endophytes from *Butea monosperma* (Tuppad and Shishupala, 2014). Patil et al., (2015) also reported similar results in *Aspergillus* sp., and *Chaetomium* sp., when isolated from seven different medicinal plants.

Alberto et al., (2016) isolated five hundred endophytic fungi from some important economic and medicinal plants and screened for enzyme activity. 62% of isolates showed positive towards amylase, 93% for cellulase, 50% for pectinase and 64% exhibited protease activity. Similar results were obtained from endophytes isolated from six important medicinal plants. *Aspergillus* sp., *Chaetomium* sp. and *Penicillium* sp., exhibited the presence of amylase, cellulase, pectinase and asperginase. None of the strains showed laccase activity (Uzma et al., 2016).

Fareed et al., (2017) obtained *Aspergillus* sp. and *Penicillium* sp. as endophytes from *Ziziphus nummularia*. Preliminary screening of extracellular enzymes from isolated endophytes resulted in the presence of amylase, lipase and protease. Shubha and Srinivas (2017) also

obtained one hundred and sixty five endophytes from different parts of *Cymbidium aloifolium* and analyzed for extracellular enzymes. *Aspergillus* sp., *Penicillium* sp., *Aspergillus terreus* and *Talaromyces* sp., showed the production of amylase, cellulase, protease and pectinase. Lipase was present in lower concentration and laccase enzyme was completely absent in all isolates.

Apart from terrestrial plants, endophytes obtained from hydrophytes also produced extracellular enzymes. Rajagopal et al., (2018) isolated eighteen different isolates from *Eichhornia crassipes*, *Nymphea nouchali* and *Vallisneria spiralis*. Among all isolates *Chaetomium indicum* showed presence of amylase, protease, cellulase and laccase.

Solanum tuberosum was subjected to endophyte isolation and species like *Aspergillus*, *Penicillium* and *Chaetomium* were analyzed for extracellular enzyme activity. Results inferred the presence of amylase, cellulase and protease (Yasser et al., 2019).

Organic acids

Organic acids produced from natural sources were feasible and economical. Based on their functional groups, organic acids were used as raw materials in many industries which help in potentially replacing petroleum- based or synthetic chemicals (Sauer et al., 2008). Among microbial origin of organic acids, fungi play an important place in production of high amounts of organic acids (Max et al., 2010). Endophytic fungi also represent as an important source of organic acid production.

Sheikh and Qureshi (2013) recovered forty seven fungal cultures from diverse habitats like decaying fruits, vegetables and from soil. Among them seven isolates namely, *Aspergillus* sp., *Rhizopus*, *Mucor* and four unidentified species were reported to have the capacity to produce organic acids.

Dezam et al., (2017) isolated 35 endophytic fungi from leaves and branches of trees inhabiting two mangroves of Brazil. Among them, *Aspergillus* sp. produced high amount of organic acids in acid indicator medium. Solid state fermentation along with agroindustrial wastes resulted in production of organic acids. Similar results were obtained from studies of Khan and Gupta (2017) where they recovered sixty four fungal isolates from eight mines of Chattisgarh region. All species of genera *Aspergillus* and *Penicillium* were positive towards organic acid production.

Pigment

Fungi have the capacity to produce large scale production of stable pigments. Their production takes place naturally under stress condition. Pigments from fungi can be produced at low cost, in large quantities (Huang et al., 2011).

Like other secondary metabolites, pigments of endophytic fungi may have coding sequences similar to that in host plants. Presence of pigments in endophytic fungi helps in preliminary identification of pure strains. They can produce deep and vibrant colours which can be used as alternative source for synthetic dyes or food colourants. They also possess strong bioactivity against human pathogens.

Endophytic fungi belonging to division ascomycota produce food- grade pigments. In industries, pigment production from fungi was carried out using submerged fermentation process with controlled temperature and pH. Submerged fermentation helps in pigment recovery and purifications (Dikshit and Tallapragada, 2013). Pigments obtained from endophytes belongs to azaphilones, anthraquinones, polyketides and other class of chemicals. Sometimes high antibacterial and antifungal activity makes the pigment more important than pharmaceutical products.

Under non-stressful condition, overexpression of certain genes throughout their lifecycle, helps in production of secondary metabolites. This may be the reason why endophytic fungi are often found pigmented. Some other research findings suggested that their pigment production may be due to influence of nutrients to which fungi were exposed, particularly to starch (Radu and Ferders, 2011).

Already different types of pigments recovered from algae and plants were readily available in market and certain groups of consumers are liable towards these products due to their natural origins when compared with completely synthetic ones (Chattopadhay et al., 2008). Fungi are capable of producing wide range of pigments (Table-19) including melanins, carotenoids, quinines and flavins. Pigments like Arpink red from *Penicillium oxalicum* and riboflavin from *Ashbya gossypii* were already present in the market (Dufosse et al., 2014). Likewise, many different types of pigments from endophytic fungi have been converted to useable colourants. There are more advantages of

using fungal pigments as colourants. Fungi are a readily available source of raw material. Also, the pigments produced possess flexibility in their colour and chemical profiles. It is also possible to carry out large scale cultivation using fungal species.

Brewer et al., (1968) isolated purple pigment from two species of *Chaetomium* namely *C.cochliodes* and *C.globosum.* The pigmentation process started by synthesizing the secondary metabolite Cochlioclinol. In contrast, Yang et al., (2012) stated that Chaetoglobosin, a secondary metabolite from *C.globosum* help to secrete pigments.

Muntanola-cvetkovic et al., (2001) screened yellow pigment production from four *Penicillium* sp. like *P. aureocephalum, P.dulaxii, P.dendriticun* and *P.pseudostromaticum*. All these isolates produced yellow pigment on Czapek yeast autolysate agar and in Malt extract agar.

Table-19: Fungal pigments and their sources

Fungi	Colour of the pigment	Reference
Penicillium herquei	Yellow	Takahashi and Carvalho (2010)
Penicillium purpurogenum	Orange to yellow	King et al., (1970)
Penicillium oxalicum	Red	Atalla et al., (2011)
Aspergillus niger	Yellow	Teixeria et al., (2012)
Aspergillus sclerotiorum	Brown	Atalla et al., (2011)
Aspergillus versicolor	Yellow	Miao et al., (2012)

Pornsuriya et al., (2008) reported that *Chaetomium* species from soil taken from various regions of Thailand, namely *C.aureum*, *C.bostrychodes*, *C.cochliodes*, *C.cupreum* and *C.flavigenum* produced red/purple pigments.

Qiu et al., (2010) isolated a endophytic fungus from twigs of *Ginkgo biloba*. Morphological observation of *P.purpurogenum* showed that, during sporulation stage the colour of mycelium changes from greyish olive to anaphalium green, reverse side of the colony with yellow pigment.

Celestino et al., (2014) isolated some *Aspergillus* sp. and *Penicillium* sp. from soils collected from different regions of Amazon. All the pigment producing endophytes were identified using molecular techniques and their production were enhanced using different carbon, nitrogen, pH, temperature sources.

Soumya et al., (2014) reported that *Chaetomium cuprenum* produced dark red pigment in the media. Optimization of pigment production using different light condition resulted that green light incubation induced maximum pigmentation.

Da costa souza et al., (2016) reported that *A.sydowii, A.aureolatus*, *A.keveii*, *P.flavigenum, P.chermesinum, Epicoccum nigrum*, *Lecanicillium aphanocladii* and *Fusarium* sp. produced red/brown pigment. Pigments were produced by secondary metabolites like Oosporein, Orevactaene and dihydrotrichodimerol present in these strains. Results inferred that these strains acted as novel sources of pigments with important industrial applications.

Sreedevi and Pradeep (2016) isolated *A.terreus* that produced yellow pigments initially and on maturation produced brown pigments. Some *Aspergillus* sp., *A.flavus*, *A.terreus* and some *Penicillium* sp. isolated both from plants and soil samples had the capacity to produce brown and yellow pigments. Maximum pigment production was achieved at 27- 30°C with pH 5-6.5 having glucose as carbon source, yeast and peptone as nitrogen source (Akilandeswari and Pradeep, 2017; Gurupavithra et al., 2017; Narendrababu and Shishupala, 2017).

Similarly *Talaromyces* sp. like *T.verruculosus*, *T.pigmentosus, T.mycothecae*, *T.brasiliensis* and *T.purpurogens* produced brown pigment under solid and submerged culture conditions. Pigment production were stable at temperature 60-100°C at pH 9-11. Adequate yield of colour suggested that this fungal pigment could act as potential replacement of synthetic dyes (Barbosa et al., 2018; Ogbonna, 2018; Rajeshkumar et al., 2019). Yilmaz et al., (2014) clearly explained the morphology of *T.radicus* where, they produce brown pigments on reverse side of the colony.

Antimicrobial activity

Drug resistant bacteria and fungi which cause important health problems were increasing. Deep research are needed to find newer and effective antimicrobial agent. Therefore, developing different source to find new antibiotics is now underway. So the isolation of novel secondary metabolites from endophytes is increasing (Hung et al., 2008). Studying about antimicrobials became important because organisms like *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Echerichia coli* were important human pathogens

which developed resistance towards higher level antibiotics. Now-a-days endophyte derived antimicrobials act as an alternative source to fight against infectious diseases. Many endophytic strains produced novel antimicrobial compounds which belong to different classes like peptides, alkaloids, terpenoids and some other derivatives (Zhang et al., 2009). They were active against many human pathogens and highly considered for novel drug discoveries. They help the plants by enhancing the growth and competitiveness of the host. Crude culture filtrates of endophytic fungi have shown antimicrobial activity against pathogenic fungi, bacteria and yeasts, cytotoxic activity on human cell line, anti- Herpes simplex virus type 1 activity (anti-HSV) and antimalarial (Rodrigues et al. 2000; Zabalgogeazcoa, 2008; Lakshmi and Selvi, 2013; Anand et al., 2014; Ibrahim et al., 2018). Some of important antimicrobial compounds from endophytic fungi is shown in Table- 20.

Antimicrobial activities of fifteen endophytic fungi isolated from two mangrove plants, *Acanthus ilicifolius* and *Acrostichum aureum* were (Maria et al., 2005) and reported likely to possess novel metabolites. *Aspergillus* sp., showed strong inhibition towards some pathogens like *Bacillus subtilis*, *K.pneumoniae*, *P.aureginosa*, *S.aureus* and *C.albicans*. Meenupriya and Thangaraj, (2010) screened endophytes like *A.niger*, *A.flavus*, *Hypocrea lixii*, *Trichoderma hypericum* and *Eurotium amstelodam* from Sponge (*Callyspongia* sp.) for their antibacterial activity and concluded that all species of *Aspergillus* possessed bioactive antibacterials for better management of bacterial pathogens.

Baral et al., (2011) and Selim et al., (2011) studied antimicrobial potential of *Aspergillus* sp. and *Penicillium* sp. from eighteen medicinal plants. Partially purified extracts of these endophytes showed strong inhibition towards *Bacillus subtilis*, *K.pneumoniae*, *E.coli*, *Enterococcus* sp, *A. fumigatus* and *A.niger.*

Deepake et al., (2012); Prabavathy and Vallinachiyar (2012) studied antimicrobial potential of endophytic fungi inhabiting *Enicostemma axillare* and *Justicia adathoda* and reported some *Aspergillus* sp., and *Penicillium* sp., to have excellent inhibitory effect on *E.coli*, *Bacillus* sp., *Proteus vulgaris*, *Staphylococcus aureus* and *Proteus morgani*.

Table-20: Antibiotics from endophytic fungi

Host plant	Endophyte	Antimicrobial compound	Reference
Salix gracilostyla var. *melanostachys*	*Phomopsis*	Phomopsichalasin	Horn et al., 1995
Artemissia annua	*Colletotrichum* sp.	6-isoprenylindole-3-carboxylic acid, Steriods	Lu et al., 2000
Cynodon dactylon	*Rhizoctonia* sp.	Monomethylsulochrin	Ma et al., 2001
Terminalia morobensis	*Pestalotiopsis. microspora*	Pestacin	Harper et al., 2003
T. prostrate	*Muscudor albus*	Naphthalene	Eizra et al., 2004
Taxus cuspidate	*Periconia* sp.	Terpenoids	Kim et al., 2004
Quercus variabilis	*Cladosporium* sp.	Aliphatic compounds	Wang et al., 2006
Ginkgo biloba	*Xylaria* sp.	7-amino-4-methylcoumarin	Liu et al., 2008
Garcinia dulcis	*Phomopsis* sp.	Alkaloids	Rukachaisirikul et al., 2008
Taraxacum mongolicum	*Phoma* sp.	2-hydroxy-6-methylbenzoic acid	Zhang et al., 2013
Mirabilis jalapa L.,	*Aspergillus clavatonanicus*	Miconazole, ketoconazole, fluconazole, ampicillin, streptomycin, chloramphenicol, and rifampicin	Mishra et al., 2017

Lu et al., (2013) and Mathan et al., (2013) isolated endophytic fungi from *Sophora flavescens* and seaweeds. Among various isolates, *Aspergillus terreus* exhibited a broad spectrum of antimicrobial activity against pathogenic bacteria like *E.coli*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Klebsilla oxytoca* and *Vibrio cholerae*. They also extracted Anshanmycin- an antibacterial compound from *A.terreus*.

Rekha and Shivanna (2014), screened thirty endophytic fungi from *Cynodon dactylon* and *Dcatyloctenium aegyptium* among which *Chaetomium globosum* exhibited strong antimicrobial activity. But control like Chloramphenicol and Flucanozole showed more activity.

Sathish et al., (2014) isolated nineteen endophytic fungi from *Eucalyptus citridora*, among which *Aspergillus* sp., showed inhibition towards *P.aeroginosa* and *C.albicans*. Their inhibition was less compared to control - Flucanozole.

Yadav et al., (2014) checked antimicrobial activity of *A.flavus*, *A.niger*, *A.tubingensis*, *A.japonicus*, *A.terreus* and *Chaetomium* sp. They exhibited strong antimicrobial activity against *Candida albicans*, *Aspergillus niger*, *Klebsilla pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Shivakoti et al., (2015) determined the antimicrobial activity of ethanolic extract of *Cucumis dipsaceus*. They were screened against gram positive bacteria (*S.aureus* and *B.subtilis*) and gram negative bacteria (*E.coli*). Results revealed that, extracts showed highest inhibition towards *E.coli*, *P.aeruginosa* followed by *S.aureus* and *P.vulgaris*. Likewise, *A. niger* and *Penicillium* sp. showed strong inhibition against *E.coli* followed by *P.aeruginosa*, *Enterococcus faecalis*, *S.aureus*, *S.typhi* and *C.albicans* (Tolulope et al., 2015). Dissanayake et al., (2016) reported *Chaetomium globosum*, exhibited strongest antimicrobial activity towards *S.aureus*, *B.cereus*, *P.aeruginosa* and *E.coli*. Smiliar results were obtained when fungal endophytes isolated from *Carica papaya*, *Aloe barbadensis* and *Ocimum gratissimum* were subjected to antimicrobial studies (Akinyemi, 2017). Endophytic fungi like *Aspergillus* sp. showed strongest activity towards pathogenic fungi, this is due to production of an antimicrobial compound named octadecane (Rajeswari et al., 2016).

Chagas et al., (2017) and Mishra et al., (2017) isolated some *Aspergillus* sp. as endophyte from *Hancornia speciosa* and *Mirabilis jalapa* and checked for their antimicrobial activity against pathogens like *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas*

aeruginosa, *Echerichia coli*, *Klebselia pneumoniae*, three strains of *Candida* and *Micrococcus furfur*. All *Aspergillus* sp. showed inhibition towards pathogens and they identified seven compounds, responsible for their antimicrobial activity.

Abonyi et al., (2018); Aharwal et al., (2018); Basheer et al., (2018); Handayani et al., (2018); Leong et al., (2018) and Susilwatt et al., (2018) screened *Aspergillus* sp. for their antimicrobial activity against test pathogens like *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Echerichia coli*, *Klebselia pneumoniae* and *Candida albicans*. Two compounds namely p-hydroxyphenylacetic acid and Ferulic acid were extracted from *Aspergillus* sp. which had the capacity to act as an antimicrobial compounds.

Wu et al., (2019) isolated endophytic fungi from *Litsea cubeba* (Lauraceae). Isolates like *Chaetomium globosum*, *Talaromyces* sp., *T. amestolkiae* and *Penicillium* sp. were effective against all pathogens and they were explored as potential helpers in the field of biotechnology, medicine and agriculture.

Antioxidant activity

Many evidences were available indicating that reactive oxygen species and free radical mediated reactions can cause oxidative damage to biomolecules which lead to problems like aging, cancer, artherosclerosis, coronary heart ailment, diabetes, Alzheimer's disease and other neurodegenerative disorders (Finkel and Holbrook, 2000). Therefore, antioxidants are effective in treating these diseases. Antioxidants may be vitamins, minerals and enzymes that protect the cells from damage and they are said to be free radical scavengers. Although, synthetic antioxidant compounds are available, naturally available compounds received much attention in recent days (Schulz et al. 2002). Therefore, search for new and alternative sources for production of effective antioxidants is needed. One such type of source is endophytes. Endophytes possess unique bioactive compounds with different chemical structures.

Antioxidants from endophytes particularly from fungi endophytes were explored after the discovery of Pestacin and isopestacin as antimicrobial compounds from endophyte *Pestalotiopsis microspora* from host *Terminalia morobensis* (Strobel and Daisy, 2003). Hung et al., (2007) isolated, characterized and screened endophytes associated with leaves

and stem of *Nerium oleander* for their antioxidant potential. The results obtained indicated that *C.globosum* showed significant positive correlation between antioxidant capacity and total phenolic content.

Murthy et al., (2011) isolated endophytic fungi from medicinal plant *Lobelia nicotianifolia* (Lobeliaceae) and screened *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor* for antioxidant potential using DPPH method. All extracts showed highest activity ranging from 320-160µg/ml and there was positive correlation between total phenolic content and antioxidant activity. Bhagobaty and Joshi (2012) reported *Talaromyces flavus* from *Potentilla fulgens* showed 76% of free radical scavenging activity.

Govindappa et al., (2013) screened endophytes like *A.niger*, *A.flavus*, *Rhizophus*, *Penicillium* sp., *Trichoderma*, *Fusarium* and *Alternaria* isolated from plant *Tabebuia argentea* (Bignoniacae) for their antioxidant activity using DPPH, FRAP, TBA, superoxide radical, FTC and iron chelating methods. Among isolates, *A.niger* exhibited the strongest antioxidant activity in all the six methods.

Yadav et al., (2014) reported the antioxidant capacity of endophytic fungi *Chaetomium* sp., *Aspergillus* sp., *A. peyronelii* and *A.niger isolated from the plant Eugenia jambolana. A.flavus* and *A.niger* isolated from *Lannea coromendalica* exhibited 68 and 69% of antioxidant activity (Premjanu and Jayanthy, 2014).

Cui et al., (2015) investigated the antioxidant capacities of endophytic fungal cultures belonging to different genera like *Fusarium*, *Beauveria*, *Trichoderma*, *Phomopsis*, *Pestalotiopsis*, *Chaetomium*, *Alternaria*, *Phoma*, *Cladosporium*, *Penicillium*, *Aspergillus* and *Mucor*. 90% of isolates of different genera showed significant antioxidant activity. This might be due to the presence of salidrosides, *p*-tyrosol and rosavins as antioxidant compounds in the isolates.

Abirami and Boominathan (2016), screened antioxidant potential of endophytes *Aspergillus* sp., *Chaetomium* sp., *Curvularia* sp., *Dreschelara* sp., *Fusarium* sp., *Penicillum* sp., *Colletotrichum* sp., *Nigrospora* sp., *Pestalotiopsis* sp. and *Phyllosticta* sp. from *Hugonia mystax* using DPPH assay. 88% of inhibition percentage was recorded from *Penicillium* sp. followed by 36.5% by *Aspergillus* sp.

Nagda et al., (2017) reported that methanolic extract of *Aspergillus* and *Penicillium* showed potent antioxidant activity against DPPH radicals.

Ali et al., (2018) investigated antioxidant activity of *Aspergillus japonicas*, *A.terreus*, Mycelia sterilia and *Penicillium chrysogenum* from *Thymus vulgaris* using DPPH assay. *A.terreus* exhibited 85% of inhibition percentage. Kumari et al., (2018) screened *Talaromyces purpurogenus* for antioxidant activity using DPPH assay, which showed 80% inhibition.

Satari et al., (2018) evaluated the antioxidant potential of *A.niger*, *A.flavus*, *A.terreus* and *Rhizopus oryzae* isolated from *Achillea millefolium* using DPPH assay. Three isolates of *Aspergillus* showed inhibition percentage ranging from 70-88%. This investigation suggested that they possessed good antioxidant properties. Selim et al., (2018) reported that ethylacetate extracts of endophyte *C.globosum* from *Adiantum capillus* showed a promising DPPH radical scavenging activity. Likewise, ethyl acetate extract of endophyte *Chaetomium nigricolor* from *Catharanthus roseus* possessed significant DPPH radical scavenging activity (Dhayanithy et al., 2019). Some important antioxidant compounds were listed in Table-21.

Production of Phyto hormones

Endophytic fungi may promote plant growth by producing phyto hormones. Varma et al., (1999) reported IAA production by endophytic fungi *Piriformospora indica*. Likewise, Rommert et al., (2002) reported the production of IAA by the endophytic fungi *Phialocephala fortinii*.

Nadeem et al., (2010) reported that *Aspergillus* and *Penicillium* sp. produced considerable amount of phytohormone.

Waqas et al., (2012) reported two endophytes *Phoma glomerata* and *Penicillium* produced phytohormone (IAA and Gibberellins) to overcome abiotic stress like salinity and drought in cucumber plants. Khan et al., (2012) reported that endophyte *Paecilomyces formosus* produced phytohormones under abiotic stress.

Syamsia et al., (2015) isolated 16 fungi from leaves, stem and root tissues of aromatic rice variety Pulu Mandoti. Ability of IAA production varied between isolates.

Table- 21 : Antioxidant compounds from endophytic fungi

S.No.	Host plant	Endophytic fungi	Metabolite	Reference
1.	*Terminalia morobensis*	*Pestalotiopsis microspora*	Pestacin and Isopestacin	Strobel et al., 2002
2.	*Trachelospermum jasminoides*	*Cephalosporium* sp.	Graphislactone A	Song et al., 2005
3.	*Lolium perenne*	*Neotyphodium lolii*	Chlorogenic acid	Qawasmeh et al., 2007
4.	*Tabebuia argentea*	*A.niger*, *Alternaria alternate*	Lapachol	Sadananda et al., 2011
5.	*Ginkgo biloba*	*C.globosum*	Flavipin	Ye et al., 2013
6.	*Crotalaria pallid*	*Aspergillus flavus, A. niger*	Coumarin and Ortho-coumaric acid	Umashankar et al., 2012
7.	*Garcinia gummigutta*, *G. indica*, *G. morella* and *G. xanthochymus*	*Aspergillus fumigatus*	Phloroglucinol	Ruma et al., 2013
8.	*Viscum album*	*Aspergillus flavus*	Lectin	Sadananda et al., 2014

Hamayun et al., (2017) reported that *Porostereum spadiceum* produced gibberellins which helped in alleviating salt stress and promoted plant growth in soybean. Lubna et al., (2018) reported that *A.niger* produced considerable amount of IAA and Gibberellins.

Salazar-cerezo et al., (2018) reported 634 isolates belonging to different genera like *Acremonium*, *Alternaria*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Talaromyces*, *Trichoderma* and *Xylariales*. Among various genera, isolates of *Penicillium* exhibited more gibberellins production.

Shah et al., (2019) screened nine endophytes from roots of *Dendrobium moniliforme* and checked for production of IAA. Among isolates, *Colletotrichum alatae* showed maximum production of IAA.

MATERIALS AND METHODS

Endophytic fungi which are confirmed as true endophytes were subjected to various tests to assess their biological properties.

Optimization of media to enhance growth and metabolite production of endophytes

Standardization of basal medium

Standardization of media for increased growth and bioactive metabolite production by endophytic fungi was done as per the procedure described by Gogoi et al., (2008). Initially endophytic isolates was selected to grow in various broth such as Czapek Dox broth, Potato Dextrose broth, Malt extract broth and Sabouraud dextrose broth. After incubation, biomass accumulation and secondary metabolite production was recorded.

Composition of broth

Potato dextrose broth (PDB)

To prepare PDB, 200g of potato was cut into pieces and boiled in distilled water to obtain potato extract. The infusion was filtered, 20g of dextrose was added and the volume of media was made up to 1000ml using distilled water. pH was adjusted to 6.5. Broth was autoclaved for 15minutes at 121˚C.

Czapeks dox Broth (CDB)

To prepare CDB, Sucrose – 30g, Sodium nitrite -2.0g, Dipotassium hydrogen phosphate - 1.0g, Magnesium sulphate - 0.5g, Potassium chloride - 0.5g and Ferrous sulphate - 0.01g were added to distilled water and boiled for few minutes to dissolve the ingredients completely. The volume of broth was made up to 1000ml using distilled water. pH was adjusted to 7.3±0.2. Broth was autoclaved for 15minutes at 121˚C.

Malt extract Broth (MEB)

To prepare MEB, Malt extract -30g and Mycological peptone - 5.0g was added to distilled water. After dissolving the chemicals volume of media was made upto 1000ml with distilled water. pH was adjusted to 5.4±0.2. Broth was autoclaved for 15minutes at 121˚C.

Sabouraud Dextrose Broth (SDB)

To prepare SDB, Glucose – 20g and Peptone -10g was added to distilled water. After dissolving the chemicals volume of media was made upto 1000ml with distilled water. pH was adjusted to 5.6±0.2. Broth was autoclaved for 15minutes at 121˚C.

Effect of pH variations

Effect of initial pH on growth and secondary metabolite production of the endophytic fungi was determined by adjusting the pH of production medium ranging from 3-9. The pH of the basal medium was adjusted by addition of 1N HCl or NaOH before autoclaving and pH was measured. The pH exhibiting highest growth and secondary metabolite production was selected as optimum pH for further studies.

Effect of Temperature variations

Optimum temperature for growth and secondary metabolite production was determined by incubating the culture at different temperatures varying from 10°C to 35°C maintaining all other conditions at optimum levels. Temperature showing highest growth and metabolite production was taken as the optimum.

Effect of supplementary Carbon and Nitrogen source

Various carbon sources such as sucrose, fructose, dextrose, maltose and nitrogen sources such as yeast extract, sodium nitrate and ammonium chloride respectively were amended separately into the basal medium at a concentration of 1%. Endophytic fungal cultures was inoculated to the respective media and incubated at 25°C for 10 days and their respective biomass and metabolite production were recorded.

Quantification of Biomass of endophytic fungal cultures

Biomass accumulation was determined by drying the mycelial mat at 70°C until a constant weight was obtained and expressed as mg/ ml.

Extraction of secondary metabolite of of endophytic fungal cultures

The broth culture was filtered to separate the mycelia and filtrate. To the filtrate equal volume of solvents was added, mixed well for 10 minutes in a separating funnel and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of solvent

containing the extracted compounds was separated. The mycelium was ground properly in a pestle and mortar using solvent and then it was filtered using cheesecloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in vacuum evaporator. The extract residue was stored and used to evaluate biological properties.

Characterization of culture filtrate using FTIR (Sharma et al., 2016)

FTIR analysis was performed using Shimadzu FT-IR 8300 instrument. KBr pellet was prepared by mixing 1mg of pigmented endophytic fungal extract with 10mg of anhydrous potassium bromide. The spectra were recorded from 500 to 4500 cm^{-1}.

GC-MS analysis of endophytic fungal filtrate

The crude extract of the fungus was subjected to GC-MS analysis for further purification and chemical characterization. GC-MS analysis was performed using an Agilent 240 MS series chromatograph equipped with an Agilent ion trap mass-spectrometer. Samples were separated on a column of Agilent QP2010S model and the size of the column is 30mx 0.25µm x 0.25mm. The mobile phase was methanol water (linear gradient from 30% to 100% methanol over 30 min) at a flow rate of 1ml min^{-1} at 30 to 32°C. The volume of the injected extract was 50µl and Helium was used as carrier gas. An ion mass spectrometer and PDA detector were used to monitor the eluted compounds. Compounds were identified by absorbance at nm over 10 to 25 min (total analysis time 35 min). Structure of the compounds was putatively identified and evaluated by comparing the molecular masses (m/z values) of the eluted compounds with literature data and standards.

Detection of biological properties of endophytic fungi

Qualitative analysis of phytochemicals (Devi et al., 2012)

The solvent extracts of true endophytes were checked for the presence of secondary metabolites like proteins, flavonoids, phenols, saponins, alkaloids, terpenoids and tannins by standard procedures.

Proteins

Solvent extracts of the culture filtrate were treated with 10% sodium hydroxide solution and two drops of 0.1% of copper sulphate solution. Formation of violet- pink colour indicated the presence of proteins.

Tannins

In a test tube containing 1ml of endophytic fungal extract few drops of 10% alcoholic ferric chloride solution was added. Formation of yellowish brown by adding little sulphuric acid precipitate indicated the presence of tannin.

Alkaloids

Solvent extract of endophytes were evaporated to dryness and residue was collected. Residue was dissolved in 2N HCl. The mixture was filtered and filtrate was obtained. 1ml of filtrate was treated with equal volume of Mayers reagent. Appearance of creamish precipitate indicated the presence of alkaloids.

Flavonoids

To the 1ml of solvent extract, 5-10 drops of diluted HCl and small amount of magnesium was added and boiled for few minutes. Appearance of dirty brown indicated the presence of flavonoids.

Phenols

A small amount of solvent extract of endophytes was mixed with 5ml of distilled water and few drops of 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

Saponins

The residue obtained by evaporating solvents was vigorously shaken with distilled water and allowed to stand for 10 minutes. No froth indicated the absence of saponin and stable froth indicated the presence of saponins.

Terpenoids

1ml of culture filtrate was mixed with 2ml of chloroform and 3ml of concentrated sulphuric acid was added which form layers in the extract. Appearance of reddish brown precipitate in the interface indicated the presence of terpenoids.

Detection of extracellular enzyme production (Sunitha et al., 2013)

Extracellular enzyme production by endophytic fungi was assessed using qualitative and quantitative techniques. These techniques help to screen large number of fungi in a relatively short time. Qualitative screening was carried out by using specific

indicative media. After incubation at room temperature, the zone of enzyme activity surrounding the fungal colony was measured.

Amylase enzyme

Amylase production was assessed by growing endophytic fungi on glucose yeast extract peptone agar media (Glucose, 1 g; Yeast extract, 0.1 g; Peptone, 0.5 g; Agar, 15 g, distilled water, 1 L) with 0.2% soluble starch. The pH of the medium was adjusted to 6.0. This indicative media was autoclaved and cooled at room temperature. Selected endophytic fungal cultures were inoculated into the media and the plates were incubated. After incubation, plates were flooded with 1% iodine in 2% potassium iodide. The zone of clearance around the blue background indicated the production of amylase.

Lipase enzyme

The lipase production was assessed by growing endophytic fungi on Peptone agar media (Peptone 10 g; Sodium chloride 5 g; Calcium chloride dehydrate 0.1 g; Agar, 15 g; distilled water 1 L; pH 6.0) with 1% Tween 20. Tween 20 was sterilized and added separately to the media before pouring in petri dish. The visible precipitate around the colony indicated the presence of lipase. The precipitate was formed due to the formation of calcium salts of lauric acid liberated by lipase.

Cellulase enzyme

The cellulase production was detected by growing endophytic fungi on glucose yeast extract peptone agar media with 0.5% carboxy-methyl cellulose. After incubation, the plates were flooded with 0.2% aqueous congo red solution and destained with 1 M NaCl for 15 min. The clear zone around the colony showed the presence of cellulase.

Protease enzyme

Glucose yeast peptone agar media with gelatin at pH 6.0, was used to detect the production of protease by endophytic fungi. 8 % of Gelatin was sterilized separately and added to GYP medium at the rate of 5 ml per 100 ml of media. The clear zone around the colonies showed the presence of protease which was enhanced when the plates were flooded with saturated ammonium sulphate.

Laccase enzyme

Glucose yeast peptone agar media was amended with 0.05 g of 1 napthol/liter. pH of the media was adjusted to 6. Media was autoclaved and inoculated with endophytic cultures to detect the production of laccase. The laccase production was indicated by change from colourless media to blue colour due to the oxidation of 1-napthol.

Quantitative analysis of extracellular enzymes (Patil et al., 2015)

Amylase activity

All endophytes were allowed to grow on Potato dextrose broth along with 1% starch. After incubation, filtrate was obtained. To 1ml of filtrate, 0.5ml of 1% starch was added and incubated for 30 minutes at 37°C. After incubation, the reaction was stopped by adding few drops of Dinitrosalicylic acid as indicator and diluted with 5ml of distilled water. Reddish brown colour was observed and read at 540nm using spectrophotometer. Quantification was done by comparing with different concentration of maltose solution.

Proteolytic activity

To 1ml of filtrate, 1ml of 1% casein was added and incubated at 45˚C for 1 hour. The reaction was stopped by adding 3ml of 0.5M trichloroacetic acid and the solution was centrifuged at 5000rpm for 30minutes. Absorption of filtrate was measured at 275nm. Quantification was done by comparing with different concentration of tyrosine solution.

Cellulolytic activity

For quantitative estimation, 1ml of filtrate, 1ml of citrate acetate buffer 0.5 M (pH 5) and 2.5 ml of 1% carboxymethylcellulose (CMC) was added and incubated for 1 hour at 37˚C. The reaction was stopped by adding 1ml of DNS reagent and kept in boiling water bath for 5 minutes. Absorbance was read at 540nm against blank using spectrophotometer. Simultaneously, standard curve of glucose was developed under identical condition.

Lipase

The lipase activity was assessed using olive oil as substrate. Before adding the sample, 5ml of olive (5ml of olive oil emulsion in 20 ml of 0.1M phosphate buffer) should be pre incubated at 37 ºC for 10 min. After incubation, 1ml of test sample was added and

incubated at 40˚C for 30 minutes with shaking at 120rpm. The reaction was stopped by adding 15ml of acetone-ethanol (1:1). Reaction mixture releases free fatty acids and titrated against 0.05N NaOH along with few drops of phenolphthalein indicator. One unit of lipase activity was defined as the amount of enzyme which produces 1µmol of fatty acids per minute under assay conditions.

Detection of organic acid

Qualitative analysis of organic acid (Zarina and Qureshi, 2013)

Selected endophytic fungi were subjected to screening of organic acid production by determining acid unitage value. Plate based assay with modified Czapek's dox agar media was used. Along with Czapek's Dox Agar media, 5ml of Triton X-100 and 0.2g of bromocresol green (pH indicator) was added. A loopful of spore was inoculated into the media and incubated at 30˚C for 4-7 days. Formations of yellow zone around the colony indicated the organic acid production. Acid unitage values were calculated by dividing the diameter of yellow zone by diameter of the colony.

Antimicrobial activity (Garcia et al., 2012)

Microorganisms used for antimicrobial activity

Totally four important multi drug resistant pathogenic bacteria were used for *in vitro* antibacterial screening of endophytic fungi. The gram negative bacteria namely *Klebsiella pneumonia, Escherichia coli* and gram positive bacteria namely *Enterococcus* sps. and *Staphylococcus aureus* were used for antibacterial assay and fungi namely *Candida albicans* and *A.flavus* were used for *in vitro* antifungal assay. The pathogens were obtained from PSG Institute of Medical Science and Research, Coimbatore.

Preparation of culture filtrate

In the broth systems, culture filtrates were extracted with equal volume of solvents (Methanol and ethyl acetate) and concentrated to dryness. Dried extracts were evaluated for its antimicrobial properties by re dissolving in specified solvents (20mg/ml). Finally, 20,40,60,80 and 100µl aliquots of this solution were used for antimicrobial assay by agar well diffusion method.

Agar well diffusion method

For *in vitro* antimicrobial studies, 1ml of diluted inoculum of test organisms were spread on Nutrient agar (Bacteria) and Potato dextrose agar media (Fungi). Wells were punched into agar media and filled with various concentration of endophytic fungal extract. DMSO was used as negative control.

For Antibacterial activity, Chloramphenicol was used as positive control and the plates were incubated at 30˚C for 24 hours. For antifungal activity, Flucannozole was used as positive control and plates were incubated at 27±2˚C for 48 hours. Experiments were performed in triplicates and after incubation, the diameter of zone of inhibition (mm) around each of the well was measured.

Antioxidant activity (Srinivasan et al., 2010)

Antioxidant activity of ethyl acetate extracts of endophytic fungi was determined using DPPH free radical scavenging assay

Quantitative estimation of Antioxidant compounds

Total phenolic content

Total phenolic content of endophytic fungal extract were estimated using Folin-ciocalteau method. 2ml of extracts were oxidized with 0.5N Folin-ciocalteau reagent for 5 minutes. Then the reaction was neutralized with a pinch of sodium carbonate and incubated for 2hours at room temperature under dark condition. Appearance of blue colour after incubation was measured using spectrophotometer at 760nm. Quantification was done by comparing with gallic acid standard and expressed as Gallic acid equivalent.

Total flavonoid content

Extracts of selected endophytic fungi were subjected to estimation of total flavonoid content using Aluminium chloride colorimetric method. 100µl of extract was mixed with 2ml of distilled water and 0.15ml of 5% sodium nitrate solution and incubated for 6 minutes. After incubation, 0.15ml of 10% $AlCl_3$ was added and allowed to stand for another 6minutes. Then 2ml of 4% sodium hydroxide solution was added. Final volume was made upto 5ml using distilled water and incubated for 15 minutes at room temperature. Absorbance was read at 510nm using UV-Vis spectrophotometer. Rutin was used as standard for quantification of flavonoid and results were expressed as rutin equivalents.

Estimation of DPPH free radical scavenging activity

For DPPH assay, aliquots of different concentration of extracts were added to 2ml of freshly prepared DPPH methanolic solution (0.1mM). The mixture was incubated at room temperature for 10minutes and absorbance was read at 517nm using spectrophotometer. Ascorbic acid was used as standard. Experiment were performed in triplicates and radical scavenging activity was calculated as follows,

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

Total antioxidant activity

Total antioxidant activity of endophytic fungi was assessed using phospho-molybdenum method. Different concentration of samples/standard (20-100µg/ml) was mixed with 1ml of ammonium molybdate (4Mm) followed by 1ml of disodium hydrogen phosphate (28 Mm). After the addition of 2ml of sulfuric acid (0.6M) the mixture was incubated at 37˚C for 30 minutes. Ascorbic acid was used as standard. Standard curve obtained from ascorbic acid was used to calculate the antioxidant concentration in the fungal extract.

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Absorbance of control- Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Estimation of phytohormones (Nath et al., 2014)

Quantitative analysis of IAA

Endophytic fungal samples were inoculated in PDB with 1mg of tryptopan and without tryptopan and incubated at 25±2˚C for 10 days. After incubation, the culture filtrate was separated and centrifuged at 12000rpm for 10minutes. For estimation of IAA, 1ml of supernatant was mixed with 2ml of salknowski reagent and incubated under dark condition for 30 minutes. Appearance of pink colour indicated the presence of IAA and absorbance was measured at 535nm using UV-Vis spectrophotometer. Quantification was carried out by comparing with standard IAA curve (20-100µg).

Quantitative analysis of Gibberellic acid

To the 10ml of filtrate, 0.5ml of 1M Zinc acetate solution was added and shaken for 3-5 minutes. 0.5ml of 1M Potassium ferrocyanide was added and mixture was centrifuged for 15 minutes. After centrifugation, 2.5ml of supernatant was taken and added with 8ml of absolute ethanol and 90ml of HCl (30%). The mixture was incubated at 20˚C in water bath for 1 hour and absorbance was read at 254nm. Diluted HCl was taken as control.

Statistical Analysis

All experiments were carried out in triplicates and results are expressed as mean ± SD (n=3). To analyse the differences among mean values of different assays one-way analysis of variance (ANOVA) was conducted. The data were analyzed using SPSS (2.0) software.

RESULTS

Optimization of media for biomass and metabolite production by endophytes

Standardization of basal medium

The effect of different media on growth and bioactive metabolite production in *Aspergillus* sp., *A. terreus*, *P.javanicum*, *C.globosum* and *T.radicus* was presented in Fig. 22-26. Among the tested media, maximum biomass (6.27±0.19 g/l) was observed in *P.javanicum* using PDB and minimum biomass (1.75±0.40 g/l) in *Aspergillus* sp., using SDB. Similarly, maximum metabolite was produced by *P.javanicum* (1.08±0.01g/l) using PDB followed by MEB. Minimum production was observed in *P.javanicum* (0.40±0.01g/l) using SDB media.

Comparative study of growth Vis-a-vis secondary metabolite production indicated a statistically significant higher biomass and bioactive metabolite production in Potato dextrose broth by four isolates. *A. terreus* alone exhibited high biomass and metabolite production in CDB.

Fig.22: Influence of different media on growth and metabolite production by *Aspergillus* sp.

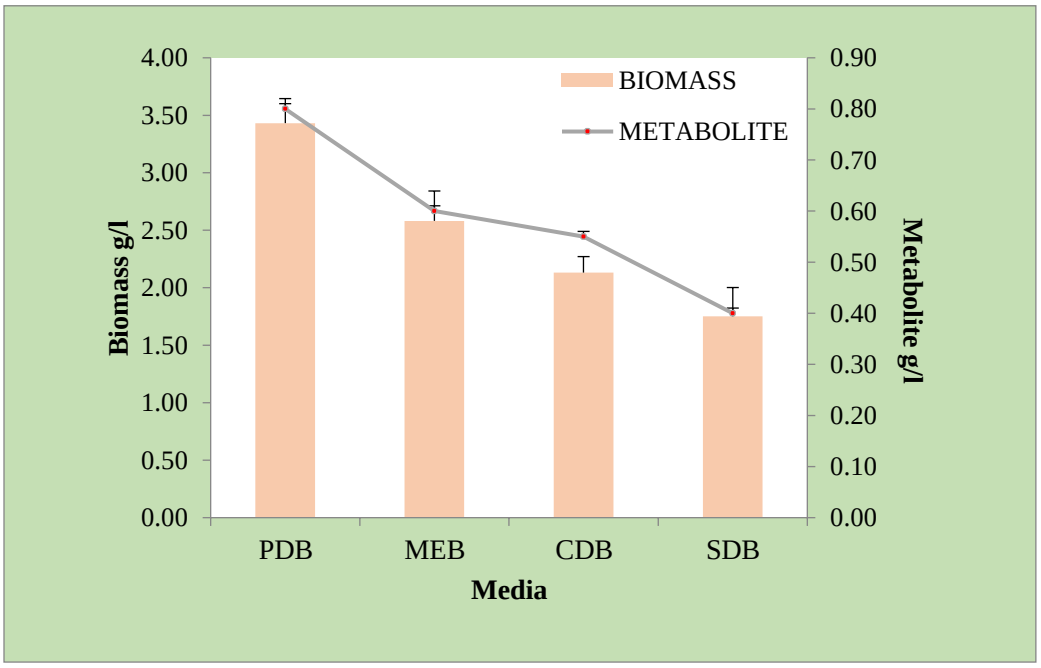


Fig.23: Influence of different media on growth and metabolite production by *Aspergillus terreus*

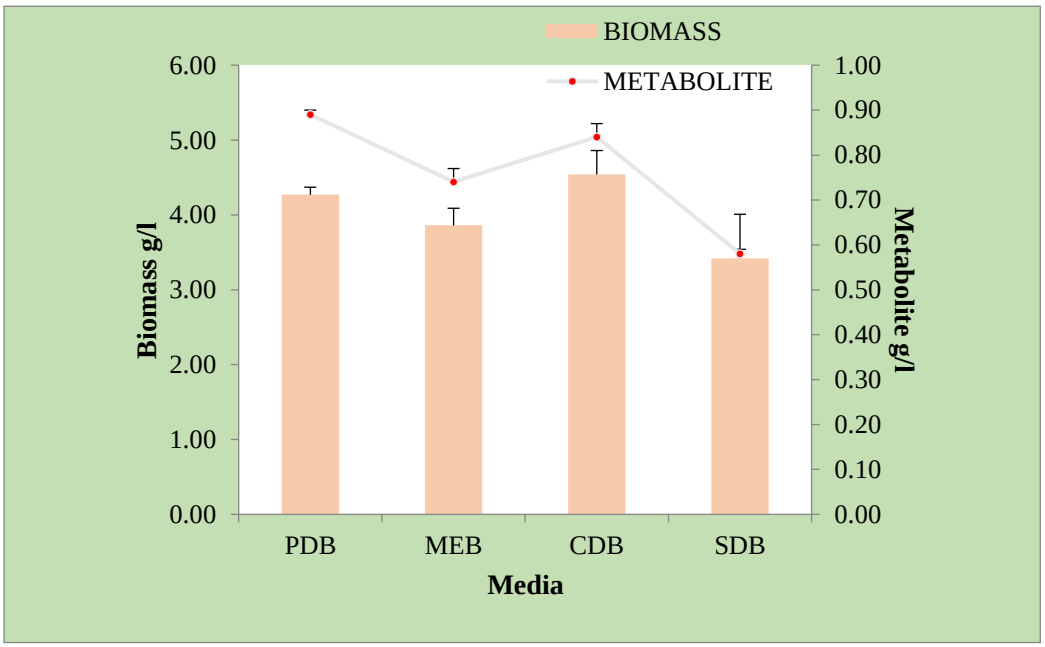


Fig.24: Influence of different media on growth and metabolite production by *P.javanicum*

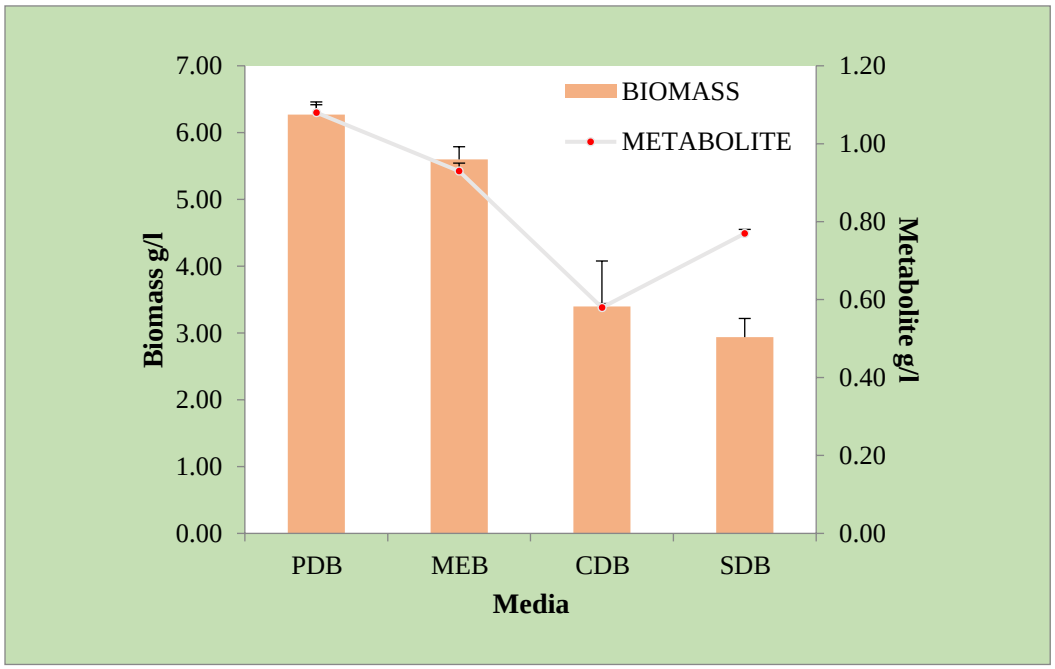


Fig.25: Influence of different media on growth and metabolite production by *C.globosum*

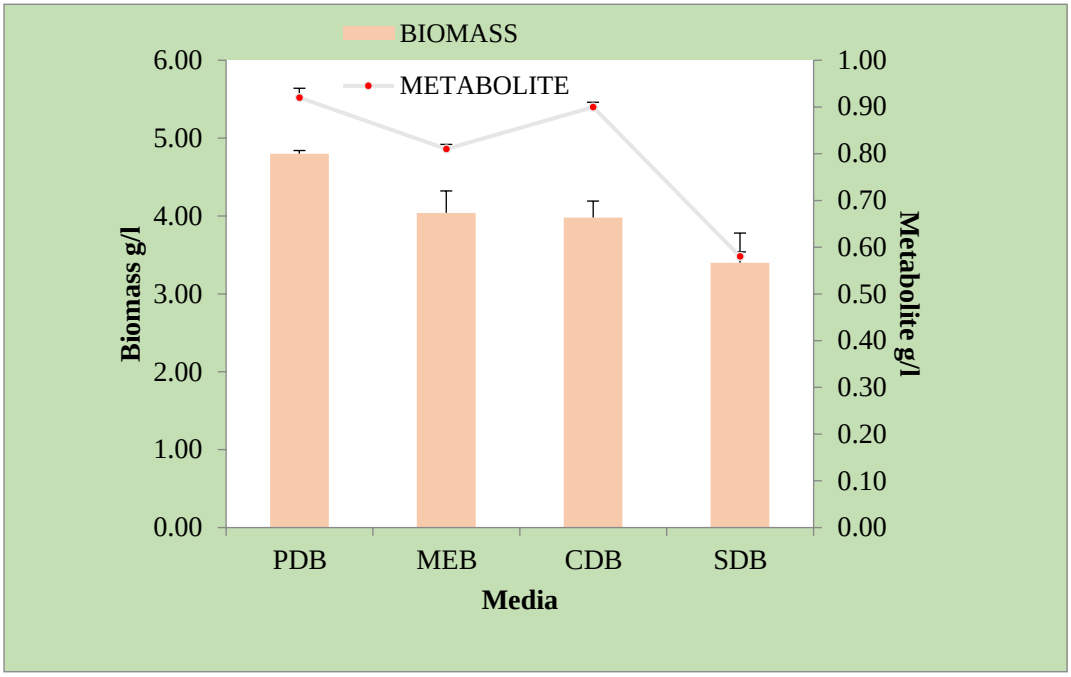
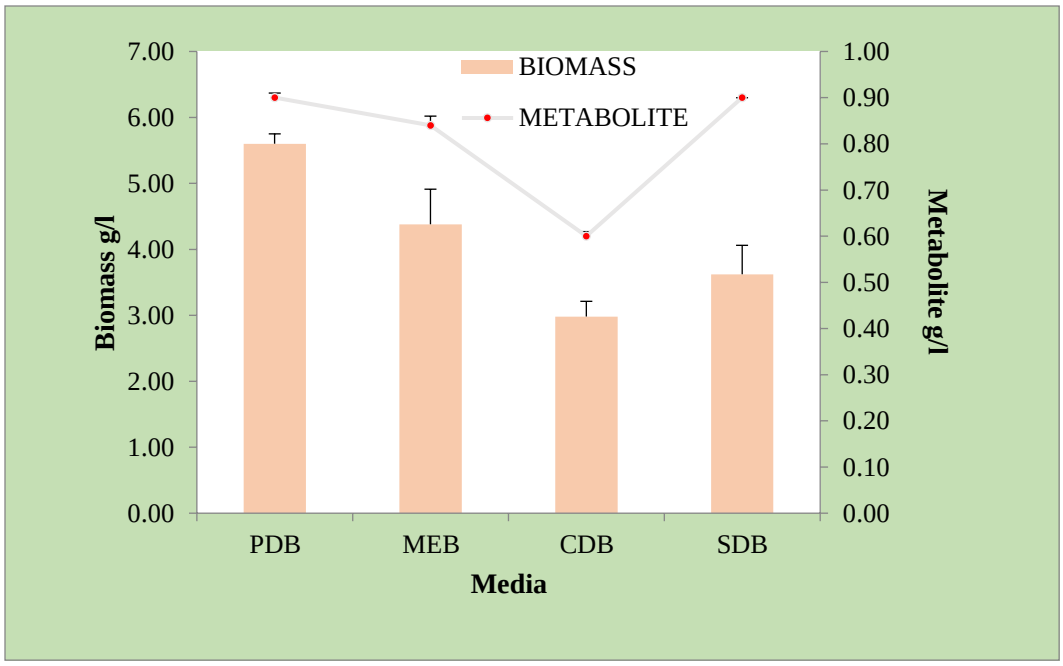


Fig.26: Influence of different media on growth and metabolite production by *T.radicus*



Effect of pH on biomass and secondary metabolite production by endophytic fungi

In *Aspergillus* sp. the maximum fresh weight (12.83±0.14g/l), dry weight (2.30±0.08g/l) and secondary metabolite production (123.94±0.07µg/l) was noted at pH 7 in PDB media. The minimum biomass and secondary metabolite production was recorded at pH 3 (Table-22).

Table-22: Effect of pH on biomass and secondary metabolite production by *Aspergillus* sp.

pH	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
3	#5.90±0.29d	0.48±0.03b	44.96±0.08d
5	10.23±0.27b	2.83±0.15a	50.03±0.09b
7	12.83±0.14a	2.30±0.08a	123.94±0.07a
9	7.13±0.26c	2.37±0.14a	46.08±0.39c
$F_{(3,11)}$	73.730***	35.646***	2.105***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

In *A.terreus* maximum fresh weight (12.08±0.04g/l), dry weight (1.73±0.03g/l) and secondary metabolite production (102.23±0.01µg/l) was recorded at pH 7. The minimum biomass and secondary metabolite production was recorded at pH 3 (Table-23).

Table-23: Effect of pH biomass and secondary metabolite on production by *A.terreus* sp.

pH	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite(µg/l)
3	#7.14±0.02d	2.74±0.02a	57.09±0.07d
5	9.84±0.04c	2.15±0.02b	77.19±0.01b
7	12.08±0.04a	1.73±0.03c	102.23±0.01a
9	10.54±0.02b	1.20±0.00d	62.85±0.01c
$F_{(3,11)}$	23.510***	58.018***	1.572***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

In *T.radicus*, maximum fresh weight (15.74±0.01g/l), dry weight (1.75±0.01g/l) and secondary metabolite production (94.20±0.01µg/l) was recorded at pH 7. The minimum biomass and secondary metabolite production was recorded at pH 9 (Table-24).

Table-24: Effect of pH on biomass and secondary metabolite production by *T.radicus*

pH	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite(µg/l)
3	#9.18±0.02c	0.83±0.02c	54.19±0.01c
5	13.28±0.01b	1.17±0.01b	65.85±0.01b
7	15.74±0.01a	1.75±0.01a	94.20±0.01a
9	8.67±0.01d	0.67±0.02d	42.88±0.00d
$F_{(3,11)}$	35.54***	35.115***	1.766***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *C.globosum* maximum fresh weight (13.17±0.01g/l), dry weight (1.61±0.01g/l) and secondary metabolite production (124.48±0.01µg/l) was recorded at pH 7. The minimum biomass and secondary metabolite production was recorded at pH 9 (Table-25).

Table-25: Effect of pH on biomass and secondary metabolite production by *C.globosum*

pH	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite(µg/l)
3	#7.18±0.01d	0.64±0.00c	47.87±0.04d
5	9.74±0.00b	0.92±0.02b	110.36±0.02b
7	13.17±0.01a	1.61±0.01a	124.48±0.01a
9	8.14±0.01c	0.76±0.02d	51.64±0.02c
$F_{(3,11)}$	20.89***	20.915***	1.143***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

In *P.javanicum* maximum fresh weight (10.21±0.01g/l), dry weight (0.95±0.00g/l) and secondary metabolite production (113±17µg/l) was recorded at pH 7. The minimum biomass and secondary metabolite production was recorded at pH 3 (Table-26).

The cultures, pH and their interactions showed significant ($p<0.05$) fresh weight, dry weight and secondary metabolite production. Effects of pH on all true endophytes were maximum at pH 7. Among the five true endophytes the maximum effect of pH was recorded in *T.radicus* at pH 7 and minimum pH effect was recorded in *P.javanicum* at pH 3 (Plate-12).

Table-26: Effect of pH on biomass and secondary metabolite production by *P.javanicum*.

pH	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
3	[#]5.22±0.02d	0.43±0.01d	67.41±0.01d
5	8.38±0.02b	0.74±0.01b	97.94±0.02b
7	10.21±0.01a	0.95±0.00a	113.17±0.01a
9	6.87±0.02c	0.57±0.01c	82.61±0.02c
$F_{(3,11)}$	61.09***	18.968***	5.480***

[#]Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

Effect of temperature on biomass and secondary metabolite production

Optimum temperature for enhancing the biomass and secondary metabolite production by different true endophytic fungus was determined by varying the incubation temperature to 10-35˚C. In *Aspergillus* sp., fresh weight (11.49±0.00 g/l), dry weight (1.39±0.02 g/l) and secondary metabolite production (72.91±0.01µg/l) was maximum at 25˚C. The minimum biomass and secondary metabolite production was recorded at 10˚C (Table-27).

Table-27: Effect of temperature on biomass and crude metabolite production by *Aspergillus* sp.

Temperature	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
10	[#]2.69±0.01f	0.39±0.01e	13.10±0.05f
15	3.49±0.02e	0.69±0.01d	34.82±0.01e
20	7.80±0.02c	0.95±0.00b	57.32±0.01c
25	11.49±0.00a	1.39±0.02a	72.91±0.01a
30	9.23±0.00b	1.03±0.02b	61.24±0.00b
35	7.23±0.02d	0.83±0.01c	42.72±0.02d
$F_{(5,17)}$	11.59***	17.242***	4.119***

[#]Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

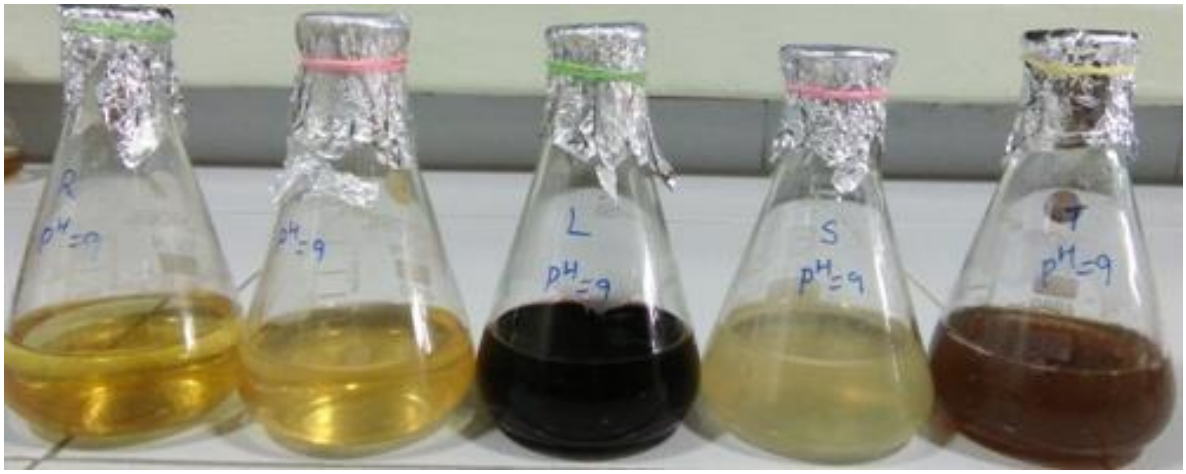
*** Significant at P< 0.001 level.

In *A.terreus*, maximum fresh weight (10.52±0.01 g/l), dry weight (1.27±0.02 g/l) and secondary metabolite production (72.22±0.01µg/l) was maximum at 25˚C followed by 8.17±0.02g/l of fresh weight, 0.92±0.01g/l of dry weight and 61.49±0.01µg/l of secondary metabolite production at 30˚C. The least effect of temperature on *A.terreus* was noted at 10˚C (Table-28).

Plate-12: Effect of pH on Biomass and metabolite production by different endophytic fungi

P.javanicum *T.radicus* *A.terreus* *Aspergillus* sp. *C.globosum*

P.javanicum *Aspergillus* sp. *A.terreus* *C.globosum*



P.javanicum *A.terreus* *T.radicus* *Aspergillus* sp. *C.globosum*

Table-28 : Effect of temperature on biomass and crude metabolite production by *A.terreus*

Temperature	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
10	#3.16±0.01f	0.45±0.02e	15.83±0.01f
15	5.62±0.02e	0.59±0.00d	20.24±0.03e
20	7.43±0.01d	0.89±0.01b	45.20±0.01d
25	10.52±0.01a	1.27±0.02a	72.22±0.01a
30	8.17±0.02b	0.92±0.01b	61.49±0.01b
35	7.30±0.01c	0.73±0.01c	45.93±0.02c
F (5,17)	14.38***	15.99***	3.399***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *T.radicus* fresh weight (12.87±0.04 g/l), dry weight (12.72±0.02 g/l) and secondary metabolite production (72.87±0.02µg/l) was maximum at 25˚C. The minimum biomass and secondary metabolite production was recorded at 10˚C (Table-29).

In *C.globosum* fresh weight (12.51±0.02 g/l), dry weight (2.69±0.03 g/l) and secondary metabolite production (74.93±0.02µg/l) was maximum at 25˚C. The minimum biomass and secondary metabolite production was recorded at 10˚C (Table-30).

In *P.javanicum* fresh weight (15.64±0.02 g/l), dry weight (3.73±0.02 g/l) and secondary metabolite production (102.68±0.01µg/l) was maximum at 25˚C. The minimum biomass and secondary metabolite production was recorded at 10˚C (Table-31). The optimum temperature for biomass and secondary metabolite production was found to be 25˚C. Among the true endophytes maximum biomass and metabolite production was observed in *T.radicus*, followed by *C.globosum*. The increase in temperature above 35ºC, did not affect the biomass and metabolite production by the cultures. The statistical analysis revealed that cultures, temperature and their interactions were significant ($p<0.05$).

Table- 29: Effect of temperature on biomass and crude metabolite production by *T.radicus*

Temperature	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
10	#2.83±0.02f	0.27±0.02f	17.22±0.01f
15	4.17±0.02e	0.55±0.02e	20.40±0.02e
20	8.62±0.01c	0.94±0.02c	38.24±0.01d
25	12.87±0.04a	12.72±0.02a	72.87±0.02a
30	10.29±0.01b	1.11±0.02b	64.72±0.03b
35	7.78±0.02d	0.84±0.01d	45.12±0.03c
$F_{(5,17)}$	11.61***	32.02***	5.28***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

Table- 30: Effect of temperature on biomass and crude metabolite production by *C.globosum*

Temperature	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
10	#2.73±0.02f	0.35±0.02f	28.24±0.03f
15	5.17±0.01e	0.74±0.01e	35.61±0.02e
20	7.38±0.02d	1.16±0.02c	42.70±0.02c
25	12.51±0.02a	2.69±0.03a	74.93±0.02a
30	10.61±0.02b	1.84±0.03b	66.22±0.02b
35	8.17±0.02c	0.94±0.01d	37.93±0.02d
$F_{(5,17)}$	16.77***	79.23***	3.57***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

Table- 31: Effect of temperature on biomass and crude metabolite production by *P.javanicum*

Temperature	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
10	#4.62±0.02f	0.48±0.01f	17.92±0.05f
15	7.18±0.02e	0.72±0.02e	38.92±0.02e
20	10.82±0.01b	1.19±0.02b	69.19±0.03d
25	15.64±0.02a	3.73±0.02a	102.68±0.01a
30	8.49±0.02c	1.03±0.02c	98.17±0.01b
35	7.93±0.02d	0.93±0.02d	75.24±0.03c
$F_{(5,17)}$	16.10***	14.27***	4.631***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

Effect of Carbon source on biomass and crude metabolite production

Best carbon source for enhancing the growth and metabolite production by different endophytic fungi was determined by varying the carbon source in the medium. Dextrose, sucrose, maltose and fructose was used as carbon sources.

In *Aspergillus* sp. the maximum fresh weight (11.63±0.03 g/l), dry weight (1.34±0.05 g/l) and secondary metabolite production (52.55±0.05µg/l) was noted on dextrose supplementation. The least fresh weight (10.52±0.02 g/l), dry weight (0.89±0.05 g/l) and secondary metabolite production (48.89±0.03µg/l) was recorded on Fructose supplementation (Table-32).

Table. 32- : Effect of Carbon source on biomass and metabolite production by *Aspergillus* sp.

Carbon source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite(µg/l)
Dextrose	11.63±0.03a	1.34±0.05b	52.55±0.05a
Sucrose	10.65±0.03c	1.16±0.03c	44.22±0.04d
Maltose	11.34±0.03b	1.72±0.03a	50.69±0.04b
Fructose	10.52±0.02d	0.89±0.05d	48.89±0.03c
$F_{(3,11)}$	38.46***	68.99***	8.266***

[#]Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *A.terreus*, the maximum fresh weight (11.61±0.02 g/l), dry weight (1.33±0.04 g/l) was noted on dextrose supplementation but maximum metabolite production (78.22±0.06µg/l) was recorded on maltose supplementation. The least fresh weight (10.38±0.04 g/l), dry weight (0.95±0.03 g/l) and secondary metabolite production (33.95±0.14µg/l) was recorded on fructose supplementation (Table-33).

In *T.radicus*, the maximum fresh weight (14.18±0.04 g/l), dry weight (2.19±0.06 g/l) and secondary metabolite production (110.81±0.05µg/l) was noted on sucrose supplementation. The least fresh weight (9.28±0.04g/l), dry weight (0.69±0.04g/l) and secondary metabolite production (54.63±0.04µg/l) was recorded on fructose supplementation (Table-34).

Table-33: Effect of Carbon source on biomass and metabolite production by *A. terreus*

Carbon source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Dextrose	11.61±0.02a	1.33±0.04b	67.23±0.04b
Sucrose	10.59±0.02c	1.08±0.01c	64.58±0.04c
Maltose	11.38±0.04b	1.48±0.02a	78.22±0.06a
Fructose	10.38±0.04d	0.95±0.03d	33.95±0.14d
$F_{(3,11)}$	37.49***	69.62***	5.490***

[#]Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

Table-34: Effect of Carbon source on biomass and metabolite production by *T.radicus*

Carbon source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Dextrose	12.68±0.02b	1.83±0.04b	76.74±0.05b
Sucrose	14.18±0.04a	2.19±0.06a	110.81±0.05a
Maltose	10.16±0.02c	0.95±0.02c	65.22±0.03c
Fructose	9.28±0.04d	0.69±0.04d	54.63±0.04d
$F_{(3,11)}$	60.50***	30.18***	3.320***

[#]Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *C.globosum*, the maximum fresh weight (13.81±0.04 g/l), dry weight (2.59±0.04 g/l) and secondary metabolite production (112.68±0.05µg/l) was noted on dextrose supplementation. The least fresh weight (8.62±0.02g/l), dry weight (1.09±0.06g/l) and secondary metabolite production (76.30±0.02µg/l) was recorded on fructose supplementation (Table-35).

Table-35: Effect of Carbon source on biomass and metabolite production by *C.globosum*

Carbon source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Dextrose	13.81±0.04a	2.59±0.04a	112.68±0.05a
Sucrose	13.71±0.03b	2.43±0.04b	105.76±0.07b
Maltose	12.16±0.02c	1.86±0.04c	97.46±0.24c
Fructose	8.62±0.02d	1.09±0.06d	76.30±0.02d
$F_{(3,11)}$	68.99***	22.81***	1.48***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

In *P.javanicum*, the maximum fresh weight (12.40±0.03 g/l), dry weight (2.58±0.03 g/l) and secondary metabolite production (118.67±0.02µg/l) was noted on sucrose supplementation. The least fresh weight (9.15±0.03g/l), dry weight (1.28±0.03g/l) and secondary metabolite production (54.25±0.04µg/l) was recorded on fructose supplementation (Table-36).

Table-36: Effect of Carbon source on biomass and metabolite production by *P.javanicum*

Carbon source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Dextrose	12.17±0.01b	2.41±0.02b	110.41±0.02b
Sucrose	12.40±0.03a	2.58±0.03a	118.67±0.02a
Maltose	11.73±0.06c	1.85±0.02c	76.42±0.02c
Fructose	9.15±0.03d	1.28±0.03d	54.25±0.04d
$F_{(3,11)}$	16.06***	55.05***	1.070***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

The results revealed that the endophytic fungi showed significant ($p<0.05$) biomass and metabolite production on carbon source supplementation. The supplementation of carbon source positively affected the biomass and metabolite production. The highest fresh weight, dry weight and metabolite production was observed in *T.radicus* on Dextrose supplementation. Least biomass and metabolite production was noted in *C.globosum* on fructose supplementation (Plate-13).

Plate-13: Effect of carbon source on Biomass and metabolite production by different endophytic fungi

T.radicus *Aspergillus* sp. *C.globosum* *A.terreus* *P.javanicum*

A.terreus *C.globosum* *T.radicus* *P.javanicum* *Aspergillus* sp.

A.terreus *Aspergillus* sp. *P.javanicum* *C.globosum* *T.radicus*

T.radicus *Aspergillus* sp. *P.javanicum* *C.globosum* *A.terreus*

Effect of Nitrogen source on Biomass and metabolite production

In the present study, the selected endophytic fungi was grown in Potato Dextrose Broth (PDB) with organic nitrogen source yeast extract and inorganic sources of nitrogen such as sodium nitrate and ammonium chloride for the biomass and metabolite production in 2% (w/v) concentration. In *Aspergillus* sp., the maximum fresh weight (10.56±0.03 g/l), dry weight (0.92±0.02 g/l) and secondary metabolite production (72.23±0.06µg/l) was noted on sodium nitrate supplementation. The least fresh weight (7.84±0.03 g/l), dry weight (0.41±0.01 g/l) and secondary metabolite production (52.20±0.01µg/l) was recorded on Ammonium chloride supplementation (Table-37 and Plate-14).

Table-37 : Effect of nitrogen source on biomass and metabolite production by *Aspergillus* sp.

Nitrogen source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Yeast extract	#9.41±0.03b	0.65±0.04	66.86±0.03
Ammonium chloride	7.84±0.03	0.41±0.01	52.20±0.01
Sodium nitrate	10.56±0.03a	0.92±0.02	72.23±0.06
$F_{(2,8)}$	2.013***	97.508***	5.900***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *A.terreus*, the maximum fresh weight (9.71±0.01 g/l), dry weight (1.24±0.03 g/l) and secondary metabolite production (66.29±0.03µg/l) was noted on sodium nitrate supplementation. The least fresh weight (7.62±0.02 g/l), dry weight (0.74±0.05 g/l) and secondary metabolite production (34.23±0.03µg/l) was recorded on ammonium chloride supplementation (Table-38).

Table-38 : Effect of nitrogen source on biomass and metabolite production by *A.terreus*

Nitrogen source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Yeast extract	#8.93±0.02b	0.94±0.03b	45.29±0.03b
Ammonium chloride	7.62±0.02c	0.74±0.05c	34.23±0.03c
Sodium nitrate	9.17±0.01a	1.24±0.03a	66.29±0.03a
$F_{(2,8)}$	1.806***	44.139***	3.021***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *T.radicus*, the maximum fresh weight (17.06±0.02 g/l), dry weight (2.89±0.05 g/l) and secondary metabolite production (112.84±0.03µg/l) was noted on yeast extract supplementation. The least fresh weight (11.20±0.02 g/l), dry weight (1.17±0.04 g/l) and secondary metabolite production (97.59±0.02µg/l) was recorded on sodium nitrate supplementation (Table-39).

In *C.globosum*, the maximum fresh weight (15.67±0.04 g/l), dry weight (1.50±0.03 g/l) and secondary metabolite production (114.61±0.04µg/l) was noted on sodium nitrate supplementation. The least fresh weight (9.12±0.03g/l), dry weight (0.77±0.01g/l) and secondary metabolite production (72.65±0.04µg/l) was recorded on ammonium chloride supplementation (Table-40).

Table-39: Effect of nitrogen source on biomass and metabolite production by *T.radicus*

Nitrogen source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Yeast extract	#17.06 ±0.02a	2.89±0.05a	112.84±0.03a
Ammonium chloride	14.73±0.03b	2.62±0.03b	105.39±0.03b
Sodium nitrate	11.20±0.02c	1.17±0.04c	97.59±0.02c
$F_{(2,8)}$	1.567***	51.79***	5.902***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

Table-40: Effect of nitrogen source on biomass and metabolite production by *C.globosum*

Nitrogen source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Yeast extract	#10.18±0.02b	0.94±0.03b	89.26±0.07b
Ammonium chloride	9.12±0.03c	0.77±0.01c	72.65±0.04c
Sodium nitrate	15.67±0.04a	1.50±0.03a	114.61±0.04a
$F_{(2,8)}$	1.296***	26.33***	1.684***

#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test. *** Significant at P< 0.001 level.

In *P.javanicum*, the maximum fresh weight (14.87±0.04 g/l), dry weight (1.35±0.05 g/l) and secondary metabolite production (104.70±0.04µg/l) was noted on yeast extract supplementation. The least fresh weight (10.92±0.04g/l), dry weight (0.72±0.03g/l) and

secondary metabolite production (78.33±0.02µg/l) was recorded on sodium nitrate supplementation (Table-41).

Table-41: Effect of nitrogen source on biomass and metabolite production by *P.javanicum*

Nitrogen source	Fresh weight (g/l)	Dry weight (g/l)	Crude metabolite (µg/l)
Yeast extract	#14.87±0.04a	1.35±0.05a	104.70±0.04a
Ammonium chloride	12.67±0.03b	0.95±0.03b	97.60±0.04b
Sodium nitrate	10.92±0.04c	0.72±0.03c	78.33±0.02c
$F_{(2,8)}$	3.279***	80.237***	1.528***

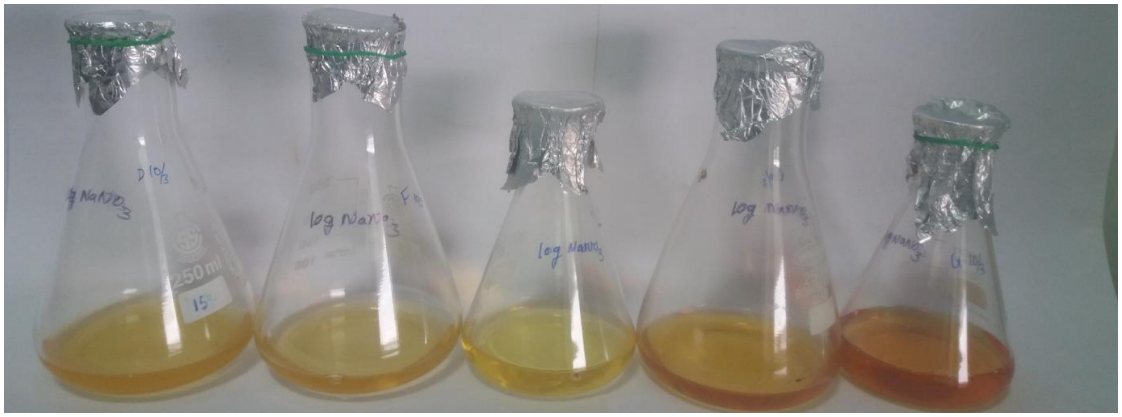
#Mean ± S.E

Means in a column followed by the same superscript (s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test.

*** Significant at P< 0.001 level.

The results revealed that the endophytic fungi showed significant (p<0.05) biomass and metabolite production on nitrogen source supplementation. The supplementation of nitrogen source positively affected the biomass and metabolite production. The highest fresh weight, dry weight and metabolite, production was observed in *T.radicus* on yeast extract supplementation. Least biomass and metabolite production was noted in *Aspergillus sp.* on ammonium chloride supplementation.

Plate- 14: Effect of Sodium nitrate on crude metabolite production in *T.radicus*



IR spectrum analysis of culture filtrate of endophytic fungi

FTIR spectroscopy of culture filtrate was carried out to find the functional groups present in extracts of endophytic fungi. The presence of different functional groups of the bioactive compounds was shown in Tables and figures. FTIR analysis of culture filtrate of *A.terreus* showed the presence of functional groups like alcohol, amine, alkyne, imine, ether and nitro compounds (Table-42 and Fig. 27).

Table-42 : Interpretation of FTIR spectrum of culture filtrate of *A.terreus*

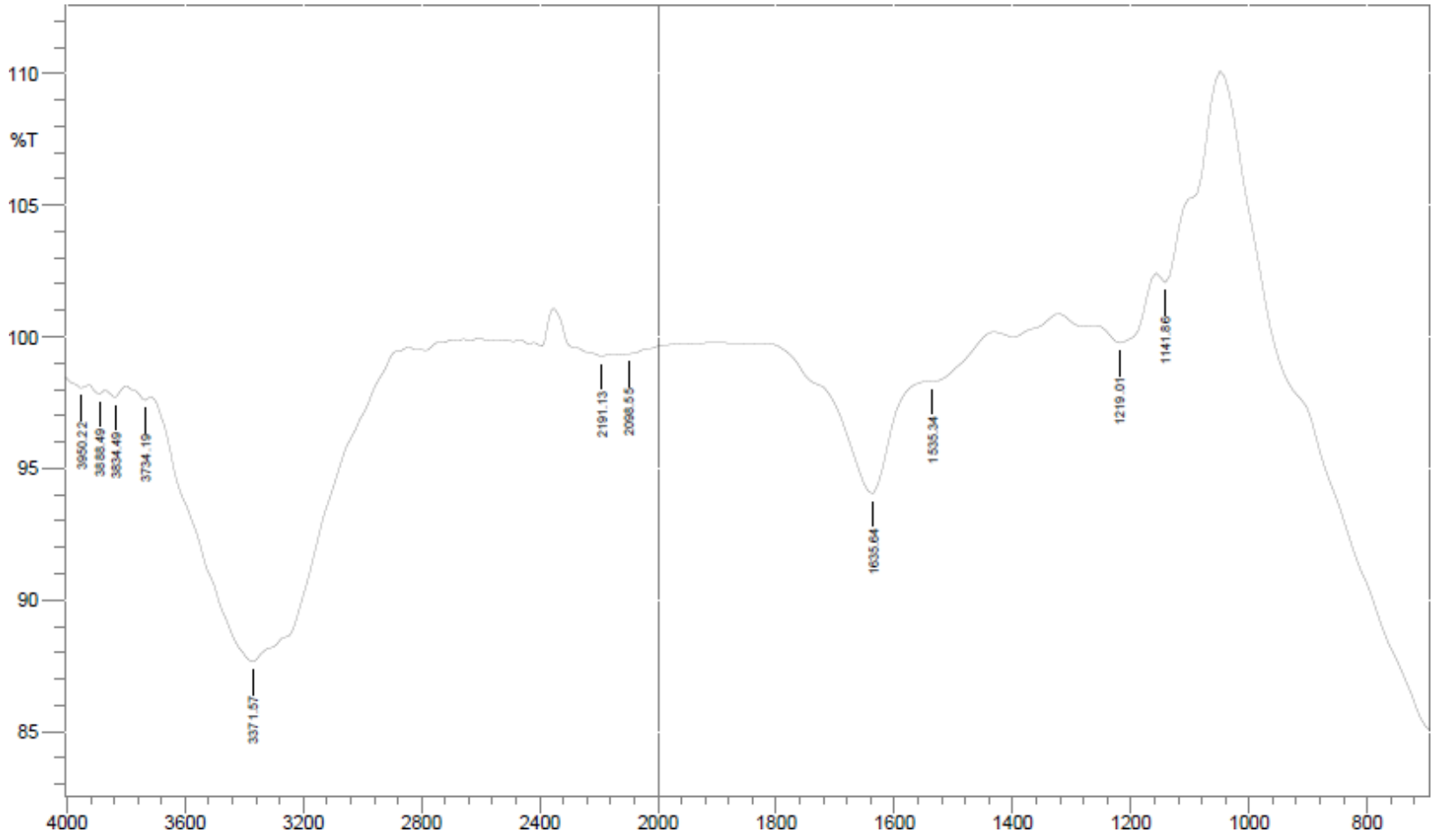
S.No	Functional group	Type of Vibration	Wave number (cm^{-1})
1.	Alcohol	OH stretching	3950, 3888, 3804,3734
2.	Amine	N-H stretching	3371
3.	Alkyne	C=C	2191
4.	Isothiocynate	N=C=S stretching	2098
5.	Imine	C=N Stretching	1653
6.	Nitro compounds	N-O stretching	1535
7.	Alkyl aryl ether	C-O stretching	1219
8.	Alcohol	C-O stretching	1141

P.javanicum also possessed some functional groups like alcohol, amine, alkyne, carbondioxide, isothiocynate, alkene and ester groups (Table-43 and Fig.28).

Table-43 : Interpretation of FTIR spectrum of culture filtrate of *P.javanicum*

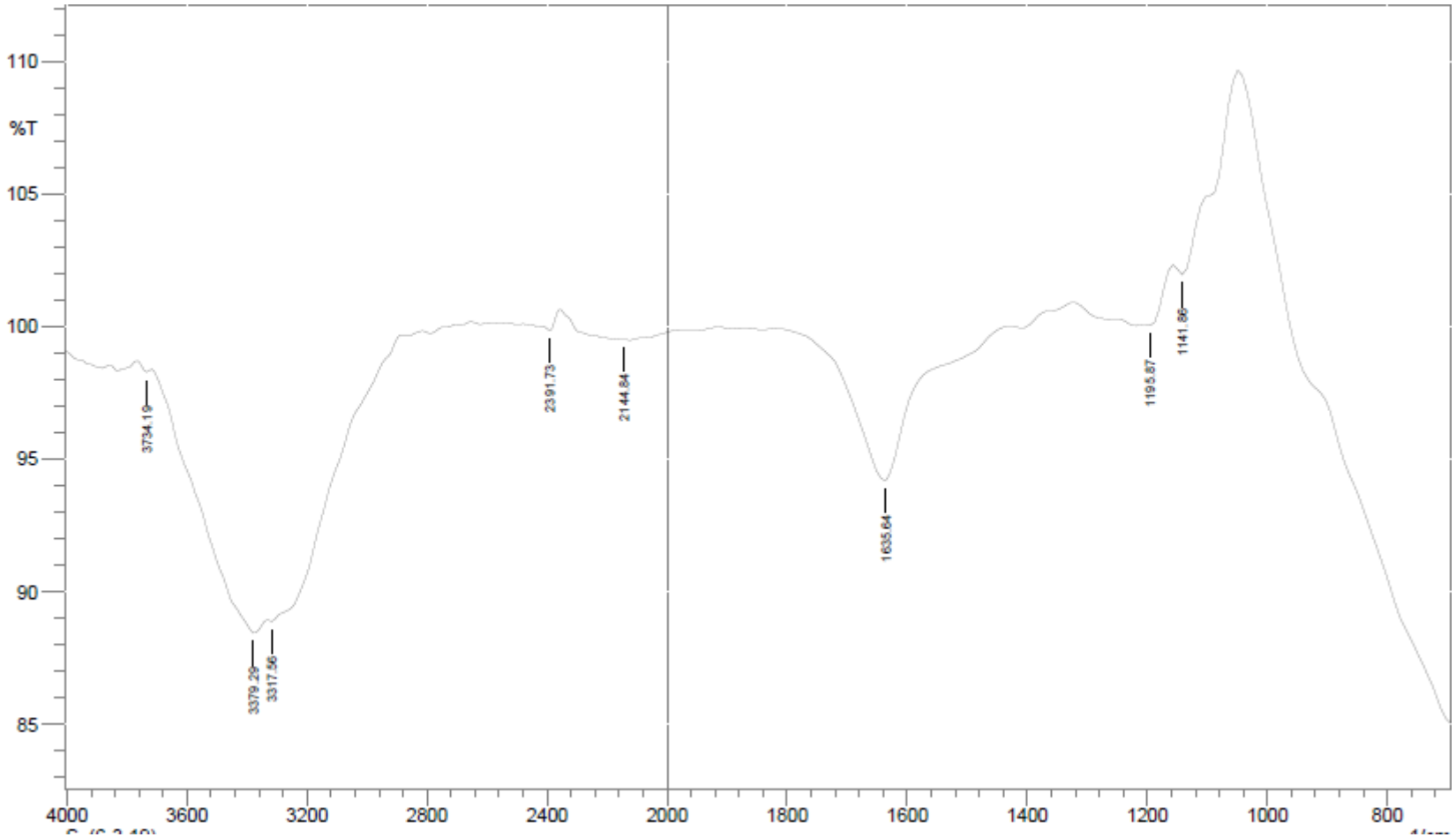
S.No	Functional group	Type of Vibration	Wave number (cm^{-1})
1.	Alcohol	OH stretching	3734
2.	Amine	N-H stretching	3379
3.	Alkyne	C-H stretching	3317
4.	Carbondioxide	O=C=O	2391
5.	Isothiocynate	N=C=S	2144
6.	Alkene	C=C	1635
7.	Ester	C-O stretching	1195
8.	Alcohol	C-O stretching	1141

Fig. 27: IR spectrum of culture filtrate of *A.terreus*



Wave number cm^{-1}

Fig.28 : IR spectrum of culture filtrate of *P.javanicum*



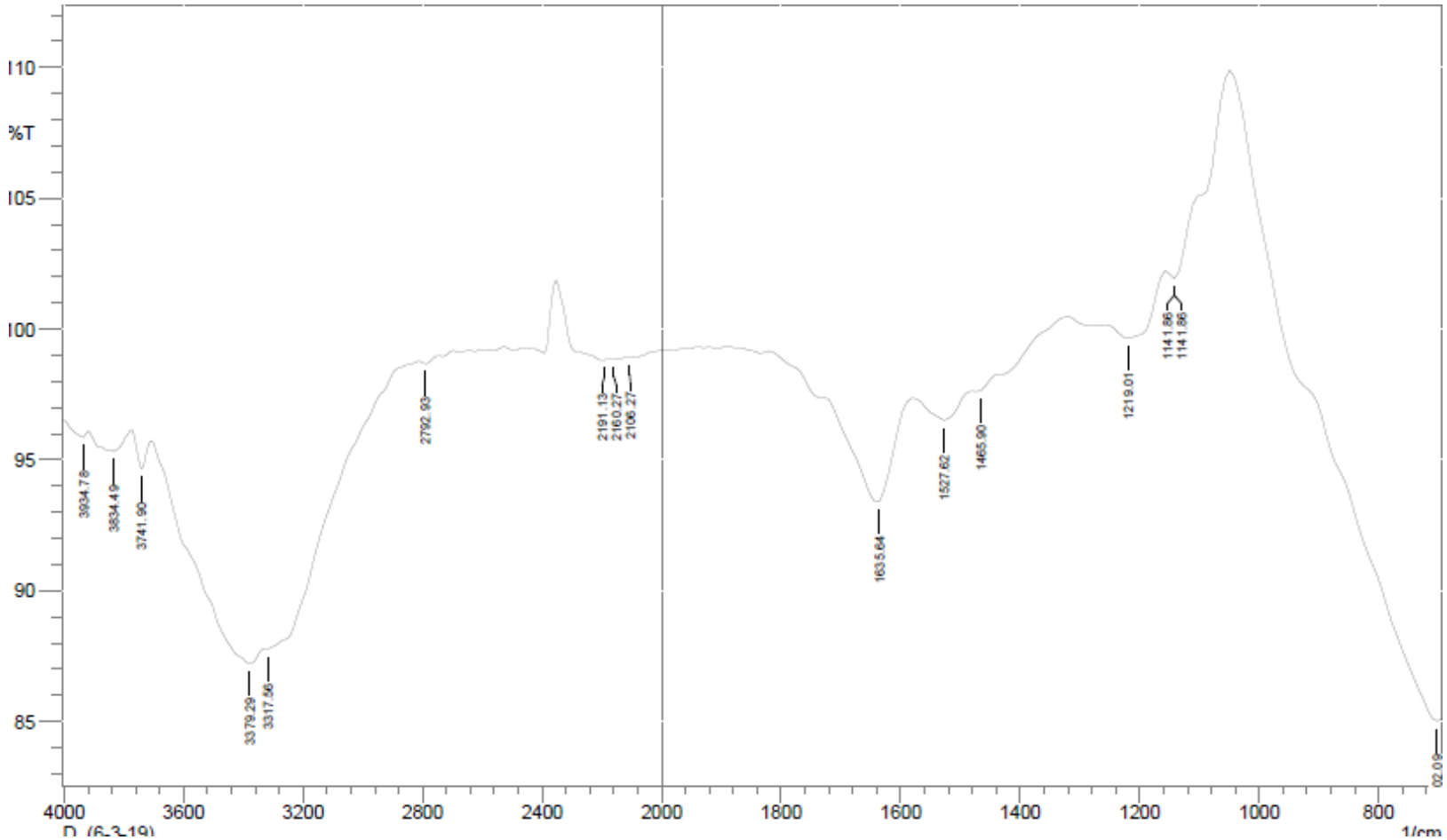
Wave number cm^{-1}

FTIR analysis of culture filtrate of *C.globosum* showed functional groups like alcohol, amine, alkyne, imine, ether, nitro compounds, aldehyde and azide groups (Table-44 and Fig. 29).

Table-44: Interpretation of FTIR spectrum of culture filtrate of *C.globosum*

S.No	Functional group	Type of Vibration	Wave number (cm^{-1})
1.	Alcohol	OH stretching	3934,3834,3741
2.	Amine	N-H stretching	3379,3317
3.	Aldehyde	C-H stretching	2792
4.	Alkyne	C=C	2191
5.	Azide	N=N=N	2160
6.	Alkyne, Isothiocynate	C=C, N=C=S stretching	2106
7.	Alkene	C=C	1635
8.	Nitro comound	N-O	1527
9.	Alkane	C-H	1465
10.	Alkyl aryl ether	C-O stretching	1219
11.	Alcohol	C-O stretching	1141

Fig.29 : IR spectrum of culture filtrate of *C.globosum*



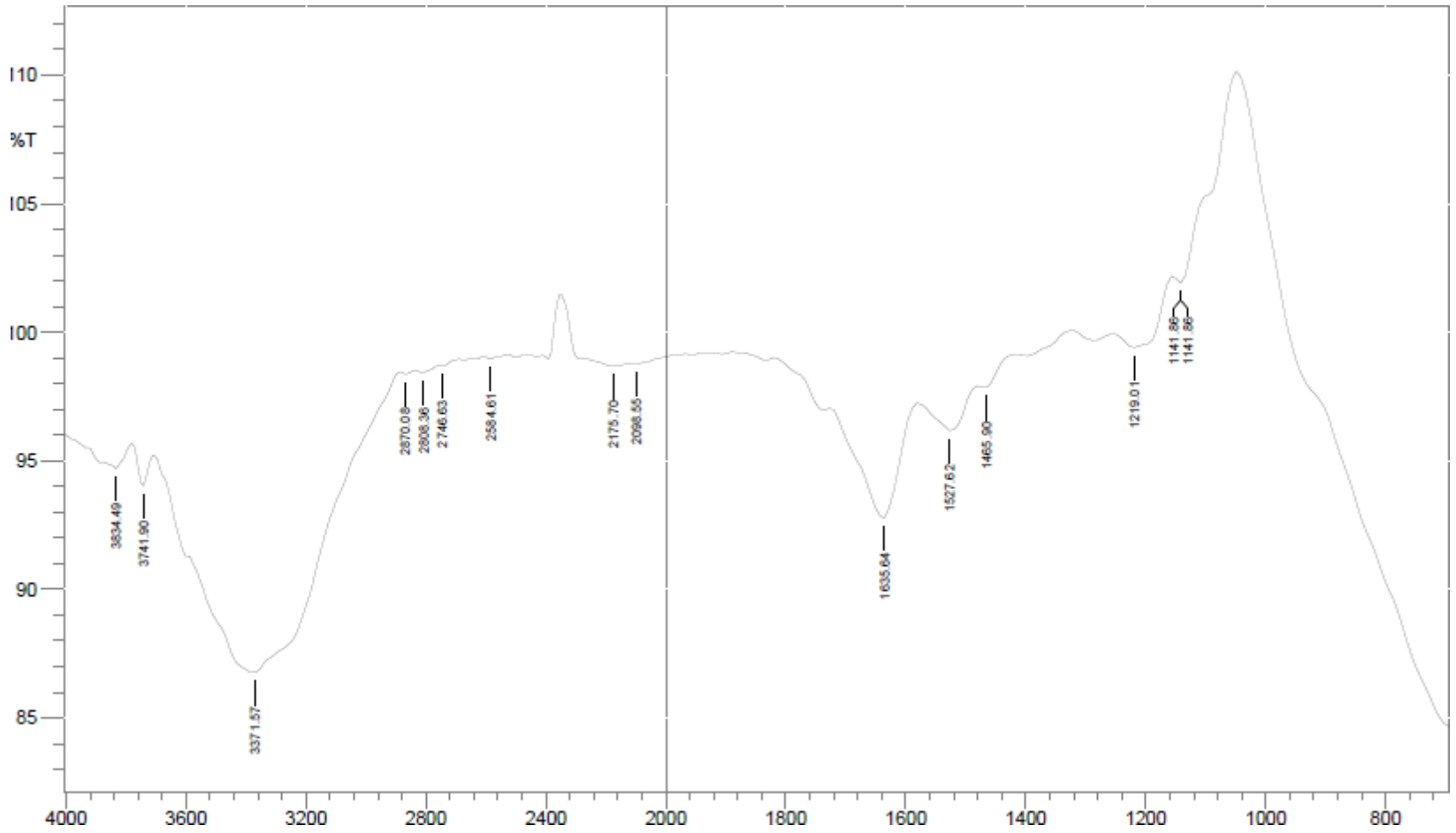
Wave number cm^{-1}

FTIR analysis of culture filtrate of *T.radicus* found to possess functional groups like alcohol, amine, alkane, thiol, thiocynate, isothiocynate, alken and nitro compounds (Table-45 and Fig. 30).

Table- 45 : Interpretation of FTIR spectrum of culture filtrate of *T.radicus*

S.No	Functional group	Type of Vibration	Wave number (cm^{-1})
1.	Alcohol	OH stretching	3834,3741
2.	Amine	N-H stretching	3371
3.	Alkane	C-H stretching	2870
4.	Aldehyde	C-H stretching	2808, 2746
5.	Thiol	S-H stretching	2584
6.	Thiocyanate	S-C=N	2175
7.	Isothiocyanate	N=C=S	2095
8.	Alkene	C=C	1635
9.	Nitro comound	N-O	1527
10.	Alkane	C-H	1465
11.	Alkyl aryl ether	C-O stretching	1219
12.	Alcohol	C-O stretching	1141

Fig. 30 : IR spectrum of culture filtrate of *T.radicus*



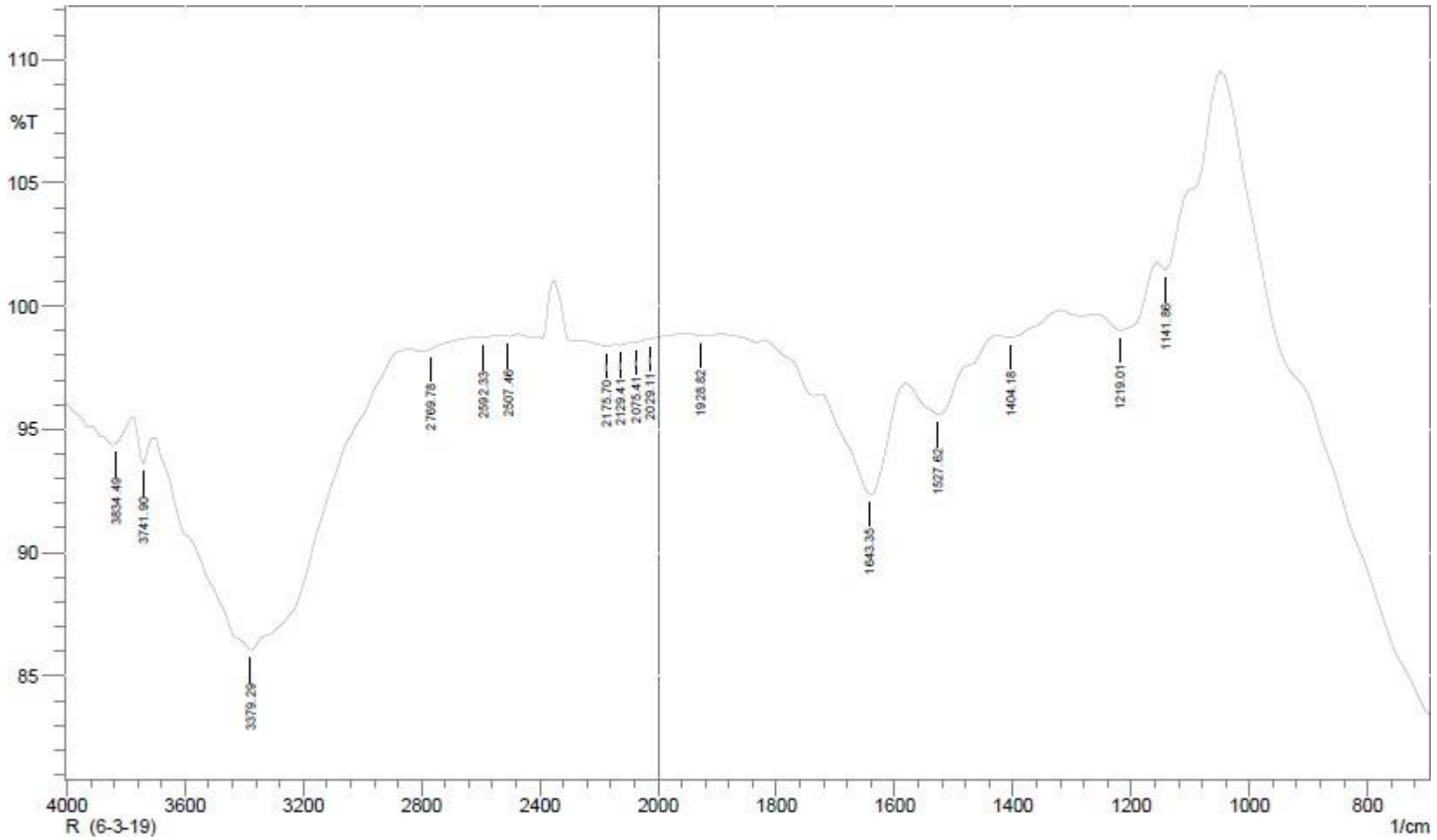
Wave number cm^{-1}

The FTIR spectrum of *Aspergillus* sp., showed the presence of alcohol, amine, aldehyde, thiol, thiocynate, Aromatic compounds and sulfonyl chloride type of functional groups (Table- 46 and Fig.31).

Table-46 : Interpretation of FTIR spectrum of culture filtrate of *Aspergillus* sp.

S.No	Functional group	Type of Vibration	Wave number (cm^{-1})
1.	Alcohol	OH stretching	3834,3741
2.	Amine	N-H stretching	3379
3.	Aldehyde	C-H stretching	2709
4.	Thiol	S-H stretching	2592
5.	Thiocyanate	S-C=N	2175
6.	Alkyne, Isothiocynate	CΞC stretching N=C=S	2129
7.	Isothiocynate	N=C=S	2075, 2029
8.	Aromatic compound	C-H	1928
9.	Imine	C=N stretching	1643
10.	Nitro compound	N-O	1527
11.	Sulfonyl chloride	S=O stretching	1404
12.	Alkyl aryl ether	C-O stretching	1219
13.	Alcohol	C-O stretching	1141

Fig.31 : IR spectrum of culture filtrate of *Aspergillus* sp.



Wave number cm^{-1}

GC-MS analysis of endophytic fungal culture filtrate

The crude extracts of culture filtrate from endophytic fungal isolates were subjected to GC-MS analysis. To reveal the retention time and mass spectra of the different components in the filtrate was compared with the mass spectra of a data library. The chromatograms of filtrate from five isolates were shown in the Fig.:32 to 36 and the identified compounds were represented in Table- 47 to 51.

GC-MS analysis of culture filtrate from *A.terreus* were found to possess 13 compounds, *C.globosum* -14 compounds, *T.radicus* - 9 compounds, *Aspergillus* sp. - 12 compounds and *P.javanicum* - 14 compounds. Phytochemicals like Benzenedicarboxylic acid and Dibutyl phthalate were commonly found in all endophytic fungal isolates.

All the compounds detected from five isolates were found to possess various properties like antimicrobial, anti-inflammatory, anti-corrosion, antioxidant, antifouling, anticancer and antimalarial activities. Some phytochemicals act as preservatives, flavoring agent, nematicide, insecticide and as pesticide. Some compounds help in the manufacturing of dyes, perfumes, soaps and nylon products etc.

Surprisingly, Cucurbitacin B major bioactive compound of *C.dipsaceus* were detected in the crude filtrate of *C.globosum*. They were present in high quantity (38.76%) in the culture filtrate and detected at retention time of 29.358.

Fig.32 :GC-MS chromatogram of crude filtrate of *A.terreus*

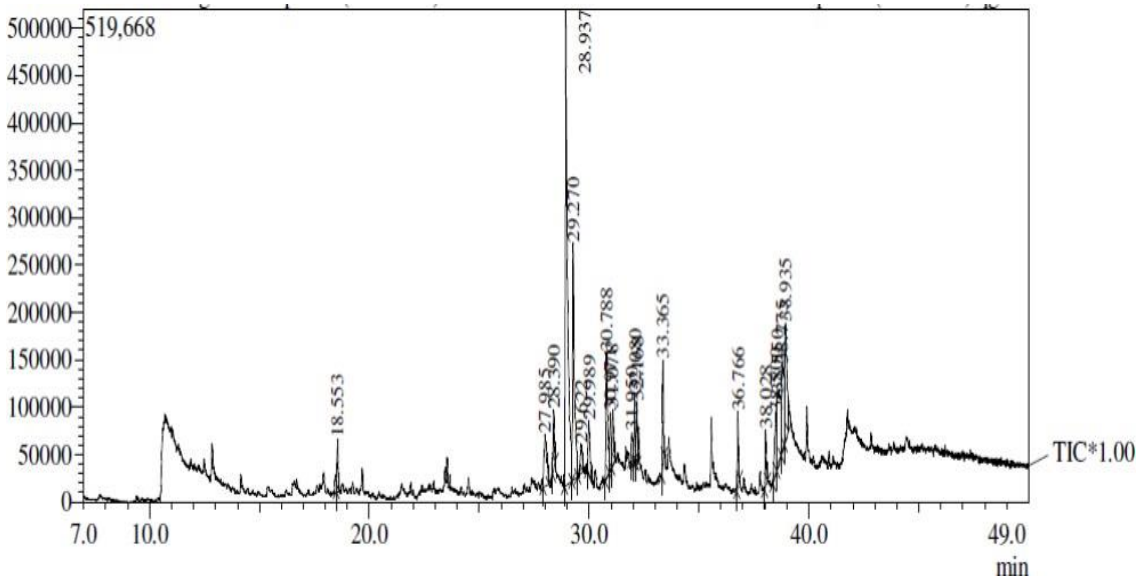


Table 47 : Compounds identified from crude extract of *A.terreus* by GC-MS

S.No.	Compound name	Retention time (min)	Uses
1.	Octadecane	18.553	Anti-corrosion agent
2.	1-Butyl 2(8-Methylnonyl)Phthalate	27.985	Unknown
3.	Heptacosanoic acid, methyl ester	28.390	Unknown
4.	Dibutyl phthalate	28.937	Plasticizer
5.	Benzenedicarboxylic acid	29.270	Flavoring agent
6.	Benzoic acid	29.622	Preservative
7.	Phthalic acid, Butyl isohexyl ester	29.989	Used in perfume and dye industries
9.	Octadecanoic acid	32.168	Antimicrobial
10.	Hexadecanoic acid	36.766	Anti-inflammatory
11.	Cyclohexanol	38.028	Act as intermediate for nylon, pesticide and in soap production
12.	Lysine	38.550	To treat cold sores
13.	Docosanol	38.935	To treat fever blisters

Fig. 33: GC-MS chromatogram of crude filtrate of *C.globosum*

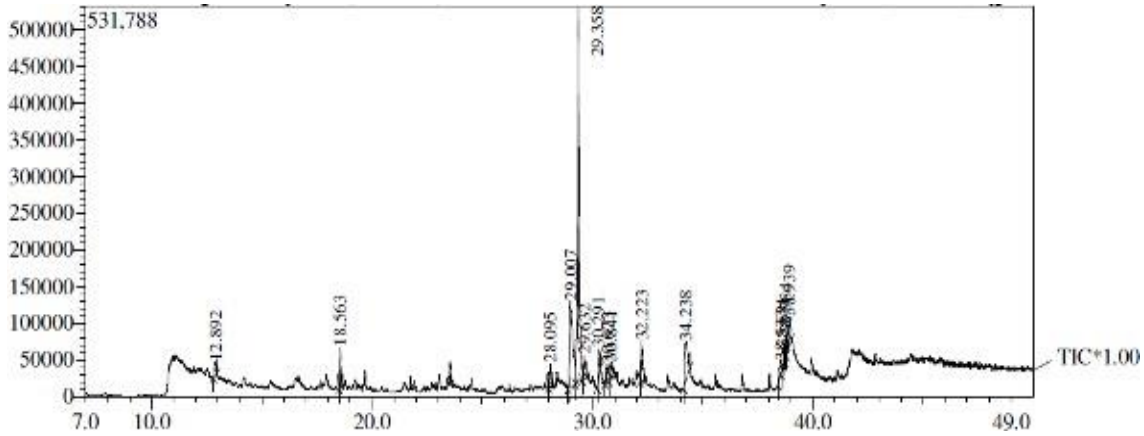


Table 48: Compounds identified from crude extract of *C.globosum* by GC-MS

S.No.	Compound name	Retention time (min)	Uses
1.	Undecane	12.892	Used to make other chemicals
2.	Octadecane	18.563	Anti-corrosion agent
3.	Isododecyl alchol	28.095	Unknown
4.	Benzenedicarboxylic acid	29.007	Flavoring agent
5.	Cucurbitacin b	29.358	Anticancer property
6.	Phosphonic acid	29.632	Fungicide
7.	Beta- Guajen	30.291	Unknown
9.	Isogeraniol	30.673	Antimicrobial activity
10.	Norvaline	32.223	Treat Alzheimer's disease
11.	Ethyl 2- hydroxycyclopentane carboxylate	38.533	Unknown
12.	Pentadecanepentol	38.631	Unknown
13.	Octaethylene glycol monododecyl ether	38.784	Used to study intestinal sodium transport mechanisms
14.	Iso-octyl phthalate	38.939	Unknown

Fig. 34: GC-MS chromatogram of crude filtrate of *T.radicus*

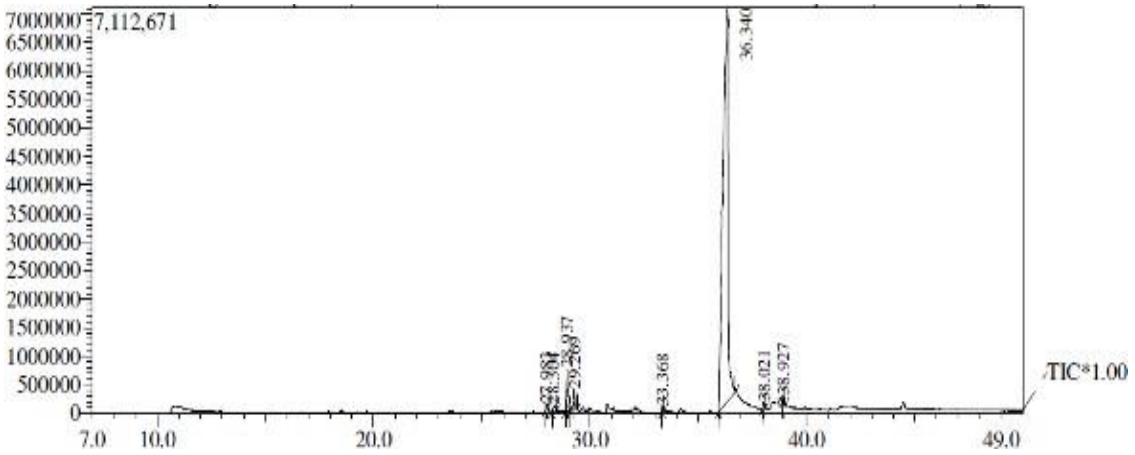


Table 49 : Compounds identified from crude extract of *T.radicus* by GC-MS

S.No.	Compound name	Retention time (min)	Uses
1.	1,2- benzenedicarboxylic acid, dibutyl ester	27.983	Insect repellent
2.	1,2- benzenedicarboxylic acid, Didodecyl ester	28.304	Plasticizer
3.	Dibutyl phthalate	28.937	Plasticizer
4.	1,2- benzenedicarboxylic acid, butyl 2- ethylhexyl ester	29.269	Unknown
5.	Hexadecanoic acid, ethyl ester	33.368	Hair and skin conditioning agent
6.	9,10- anthracenedione	36.340	Used in textile industries for manufacturing dyes.
7.	1,3,5,trisilacyclohexane	38.021	Unknown
9.	1,2- benzenedicarboxylic acid	38.927	Flavoring agent

Fig. 35: GC-MS chromatogram of crude filtrate of *Aspergillus* sp.

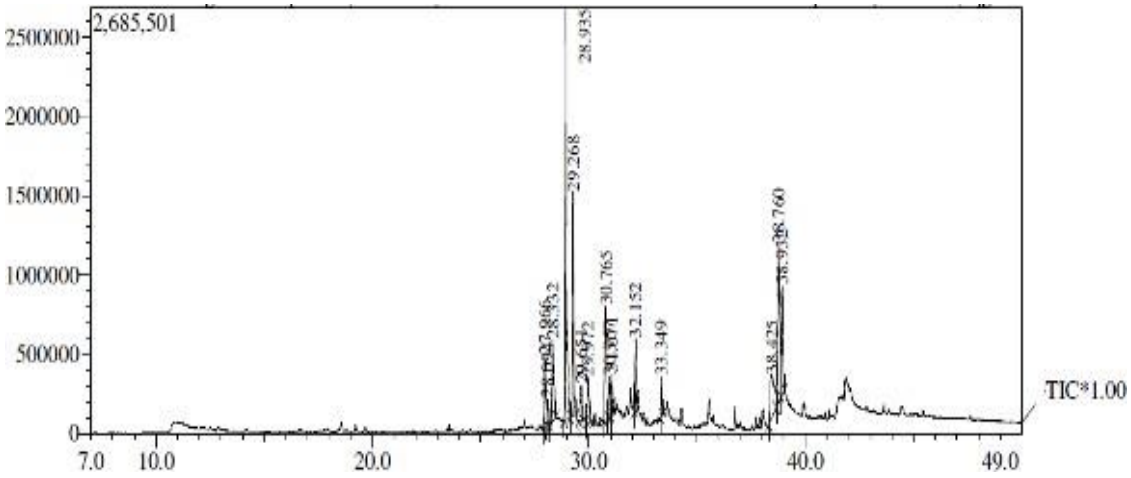


Table 50 : Compounds identified from crude extract of *Aspergillus* sp. by GC-MS

S.No.	Compound name	Retention time (min)	Uses
1.	1,2- benzenedicarboxylic acid, bis (2-methylpropyl) ester	27.966	Flavoring agent
2.	Hexadecanoic acid, methyl ester	28.332	Metabolite in plant
3.	Dibutyl phthalate	28.935	Antifungal and antimalarial agent
4.	1,2- benzenedicarboxylic acid, butyl 2- ethylhexyl ester	29.268	Unknown
5.	Ethyl13- methyl- tetradeconate	29.651	Unknown
6.	Phthalic acid, butyl isohexyl ester	29.972	Antimicrobial activity
7.	1,2- benzenedicarboxylic acid, butyl octyl ester	30.957	Unknown
9.	Diamyl phthalate	31.071	Plasticizer for nail polish
10.	Methyl stearate	32.152	As surfactants
11.	Ethyl stearate	33.349	Flavoring agent
12.	Glycerol.beta- palmitate	38.760	Improves calcium absorbtion

Fig.36 :GC-MS chromatogram of crude filtrate of *P.javanicum*

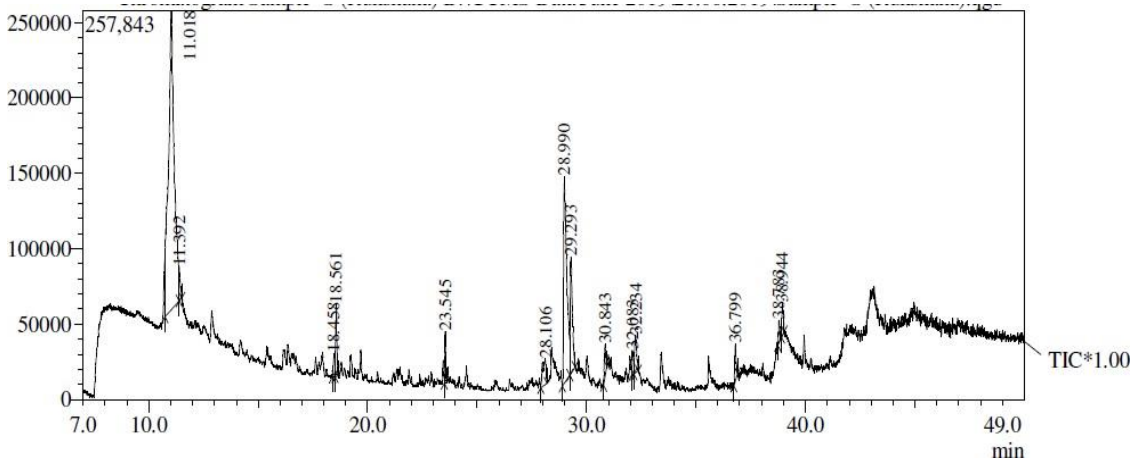


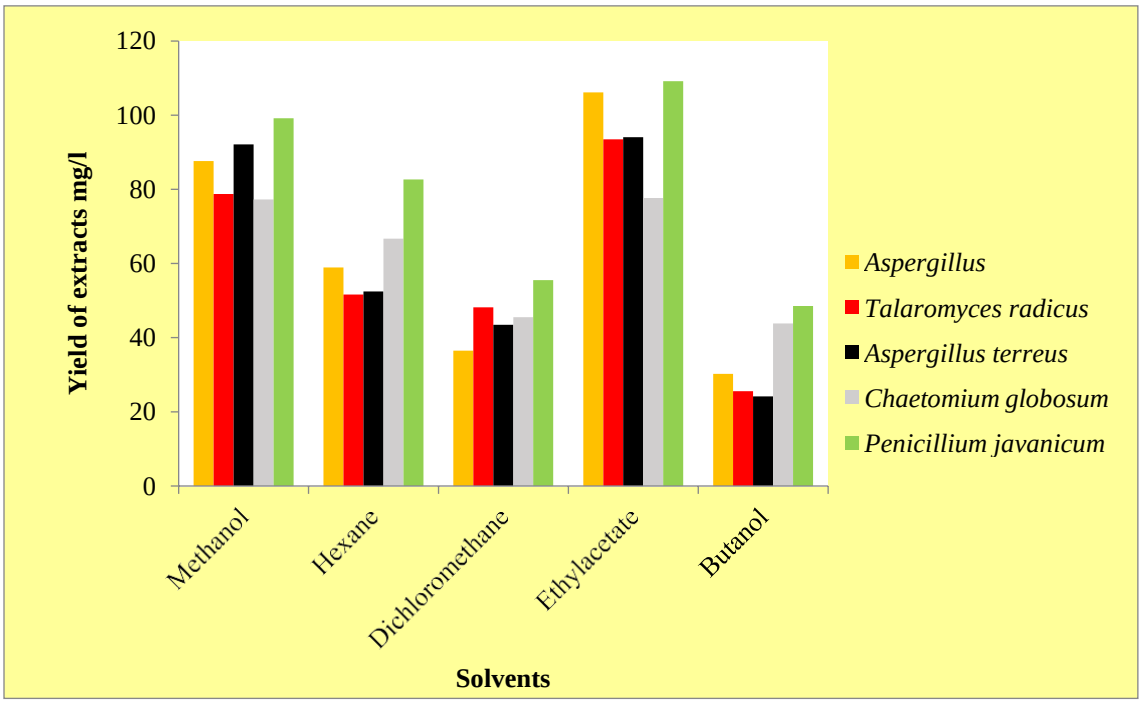
Table 51 : Compounds identified from crude extract of *P.javanicum* by GC-MS

S.No.	Compound name	Retention time (min)	Uses
1.	Naphthalene	11.018	Manufacture of dyestuffs
2.	Hexane,3,3,dimethyl-	18.458	Flavoring agent
3.	Octadecane	18.561	Anti-corrosion agent
4.	Undecane 2,7,dimethyl	23.545	Unknown
5.	Ditridecyl of phthalic acid	28.106	Toxic substance
6.	1,2- benzenedicarboxylic acid, dibutyl ester	28.990	Unknown
7.	Phthalic acid, butyl 2- pentyl ester	29.293	Antioxidant
9.	Phthalic acid, 4-cyano phenyl nonyl ester	30.843	Antifouling
10.	2,3-dimethyl-3-undecanol	32.083	Antioxidant
11.	Hexadecanoic acid, methyl ester	32.234	Antioxidant, nematicide and pesticide
12.	Hexadecanoic acid, ethyl ester	36.799	Antioxidant
13.	Fluorononane	38.783	Unknown
14.	DEHP	38.944	Plasticizer

Liquid-liquid extraction of metabolites

Crude filtrate of endophytic fungal cultures was subjected to sequential extraction using different solvents. Collected organic phase of solvents were dried and total yield of extracts were shown in the Fig.37.

Fig.37 : Yield of solvent extracts from endophytic fungi



All the isolates showed maximum extraction using ethyl acetate followed by methanol. Among isolates, *P.javanicum* gave maximum yield (109mg/l) using ethyl acetate and lower yield was obtained by *T. radicus* (22mg/l) using butanol.

Biochemical analysis

Biochemical analysis of the crude extract of different endophytic fungi was carried out to determine the presence of chemical components. The active metabolites contain chemical groups such as phenols, flavonoids, terpenoids, alkaloids, tannins, carbohydrates and saponins. The results of qualitative phytochemical analysis inferred that the crude extract of *Aspergillus* sp., *A.terreus, C.globosum* and *T.radicus* showed the presence of proteins, flavonoids, phenols, glycosides, terpenoids, alkaloids and tannins. Terpenoids, alkaloids and tannins were absent in the culture filtrate of *P.javanicum* (Table-52).

Table-52: Phytochemical screening of crude extracts of fungal endophytes (Qualitative)

Fungal endophytes	Phytochemical test							
	Proteins	**Flavonoids**	**Phenols**	**Saponins**	**Glycosides**	**Terpenoids**	**Alkaloids**	**Tannins**
Aspergillus sp.	+	+	+	-	+	+	+	+
A.terreus	+	+	+	-	+	+	+	+
C.globosum	+	+	+	+	+	+	+	+
P.javanicum	+	+	+	-	+	-	-	-
T.radicus	+	+	+	+	+	+	+	+

+: Present; -:Absent

Detection of extracellular enzymes

Culture filtrate of the endophytic fungi was subjected to extracellular enzyme analysis. All the true endophytes were able to produce the extracellular enzymes (Table-53, Plate-15) with the exception of laccase which none of the fungal isolates produced. Cellulase enzyme was actively produced by all isolates.

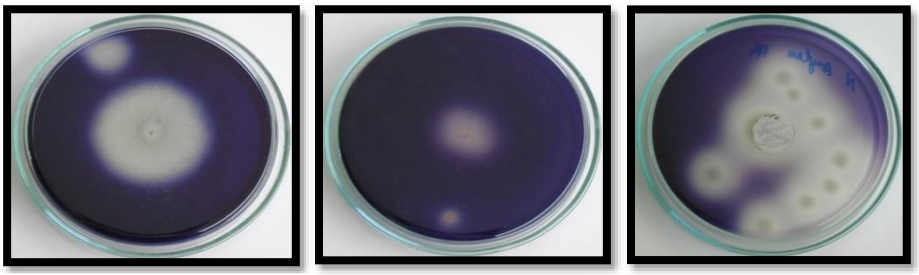
Table-53: Qualitative analysis of Extracellular enzymes from endophytic fungi

Endophytes	Enzymes				
	Amylase	**Protease**	**Laccase**	**Lipase**	**Cellulase**
C.globosum	+++	+++	-	+++	+++
A.terreus	++	+++	-	+++	+++
Aspergillus sp.	++	++	-	++	+++
T.radicus	+++	++	-	++	+++
P.javanicum	+++	+++	-	++	+++

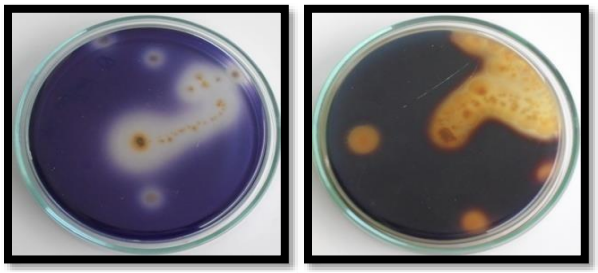
+++: high**;**++Moderate**;** +slight**;-** absent

Plate-15 :Preliminary screening of extracellular enzymes

A. Amylase



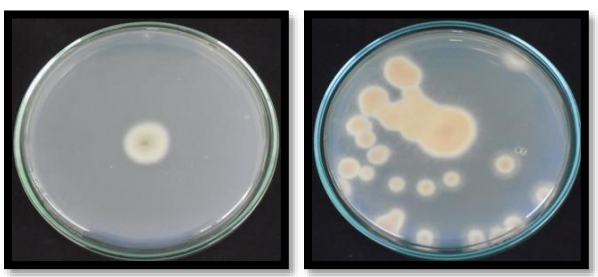
C.globosum *Aspergillus* sp. *P.javanicum*



A.terreus *T.radicus*

B. Protease

C. globosum *Aspergillus* sp. *P. javanicum*

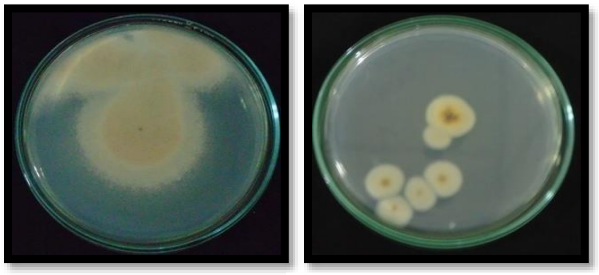


A.terreus *T.radicus*

Plate-15 :Preliminary screening of extracellular enzymes

C. Lipase

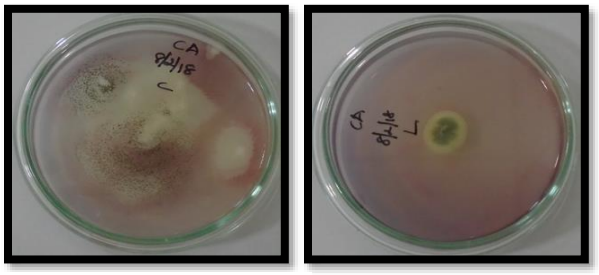
C. globosum *Aspergillus* sp. *P. javanicum*



T.radicus *A.terreus*

D. Cellulase

C.globosum *Aspergillus* sp. *P. javanicum*



T. radicus *A.terreus*

Table-54: Quantitative estimation of extracellular enzymes from endophytes

Endophytes	Enzymes (Unit of activity ml^{-1})				
	Amylase	**Protease**	**Laccase**	**Lipase**	**Cellulase**
C.globosum	#7.53±0.18	5.30±0.31	-	8.47±0.12	6.40±0.17
A.terreus	4.53±0.20	3.40±0.21	-	7.33±0.15	5.47±0.23
Aspergillus sp.,	3.77±0.09	3.87±0.12	-	5.53±0.22	4.80±0.06
T.radicus	7.47±0.15	10.40±0.21	-	4.50±0.10	6.40±0.06
P.javanicum	6.37±0.18	5.40±0.15	-	3.87±0.20	5.50±0.15

Mean±S.E

Quantitative analysis of extracellular enzymes revealed that, maximum amylase production was observed in *C.globosum* (7.53±0.18Uml^{-1}) followed by *T.radicus* (7.47±0.15Uml^{-1}). Similarly, cellulolytic activity was observed in all isolates. Prominent cellulase activity was observed in both *C.globosum* (6.40±0.17Uml^{-1}) and *T.radicus* (6.40±0.06Uml^{-1}) followed by *P.javanicum*. Among all isolates, *C.globosum* was the active producer of extracellular enzymes. None of the isolates screened demonstrated laccase activity (Table-54).

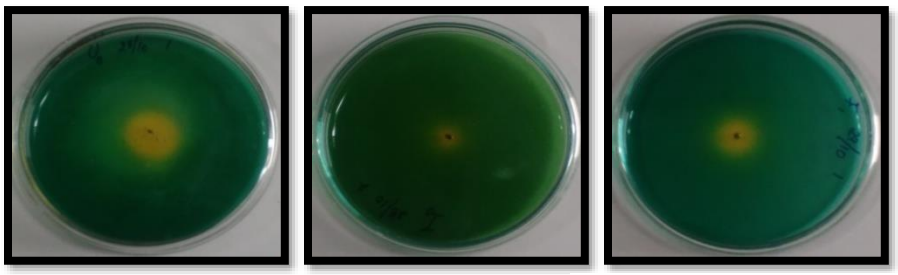
Detection of organic acids

Table-55: Quantitative estimation of organic acid production by different endophytes

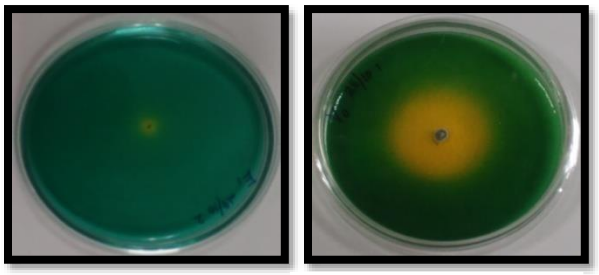
Endophytes	Zone diameter (mm) of fungal colony	Zone diameter (mm) of halo zone	Acid unitage value (AU)
C.globosum	#20.67±1.15	90.33±0.33	4.38±0.19
A.terreus	106.67±7.57	292.00±4.73	2.73±0.04
P.javanicum	11.33±1.53	82.33±1.00	7.30±0.30
Aspergillus sp.	28.33±1.53	154.33±2.85	5.40±0.05
T.radicus	11.67±1.41	62.00±1.15	5.33±0.45

#Mean ±S.E

Plate-16: Qualitative estimation of organic acid production by different endophytes



A.terreus *T.radicus* *P. javanicum*



Aspergillus sp. *Chaetomium* sp.,

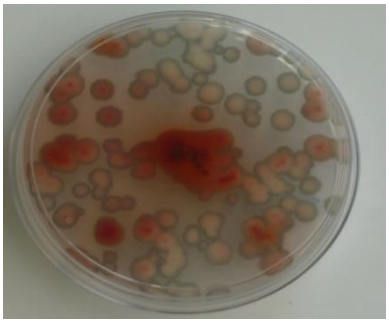
Table-55 and Plate-16 shows the production of organic acid on modified mineral salt medium. A yellow halo produced around the colony indicated the organic acid synthesizing capacity of endophytic isolates. For quantitative analysis, acid unitage values have been determined by measuring the diameter of the colony and yellow halo zone around the colony. Among isolates, *P.javanicum* possessed the maximum AU (7.30) and followed by *Aspergillus* sp. with value of 5.40 and minimum AU was obtained by *A.terreus* (2.75).

Pigment production

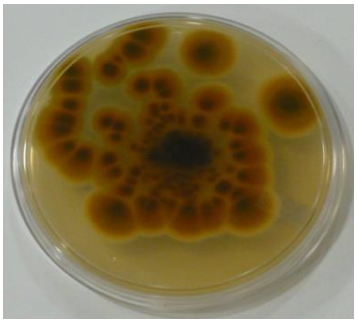
Preliminary screening of pigment producing endophytic fungi

Cultures of true endophytic fungi were screened for their pigment producing potential on PDA plates. All the five cultures possessed the capacity to produce pigments. Production of pigments on PDA plates took place by depleting the essential nutrients on plate as a result of which fungus shifts its biochemical activity towards the pathway of secondary metabolism. Apart from producing new fungal building material, this shift gives rise to other compounds (Secondary metabolite). The coloration of the fungi is due to inclusion of the pigment at various points within the structure of the fungus during growth (Plate-17).

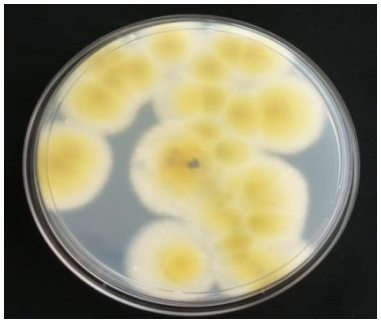
Plate-17: PDA plates showing coloured colonies of true endophytic fungi



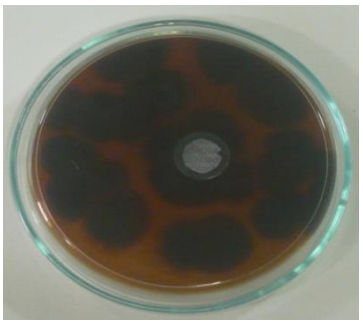
Aspergillus sp.



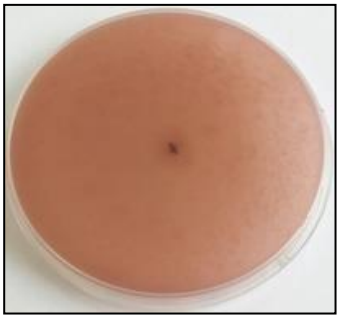
T.radicus



P.javanicum



A.terreus



C.globosum

Production of pigments under submerged state fermentation

For clear visibility of pigments, CDB was used as fermentation media for the production of pigments. Endophytic fungal cultures showed fastest ability to degrade carbon source and utilized for the mycelia growth and started producing secondary metabolites. There was considerable change in the colour of the medium of all true endophytes (Plate-18).

Antimicrobial assay

Antimicrobial activity methanolic and ethyl acetate extract of five endophytic fungi were studied by agar well diffusion method. For antibacterial assay, two gram positive bacteria like *Staphylococcus aureus*, *Enterococcus* sp. and two gram negative bacteria like and *Escherichia coli*, *Klebsella pneumonia* were used. For antifungal assay, *Candida albicans* and *Aspergillus flavus* were used. All the isolates inhibited either one or more pathogens, but compared to control (Chloramphenicol and Flucanozole) the activity is less.

Plate-18: Pigment production under submerged state fermentation

P.javanicum

T.radicus

C.globosum

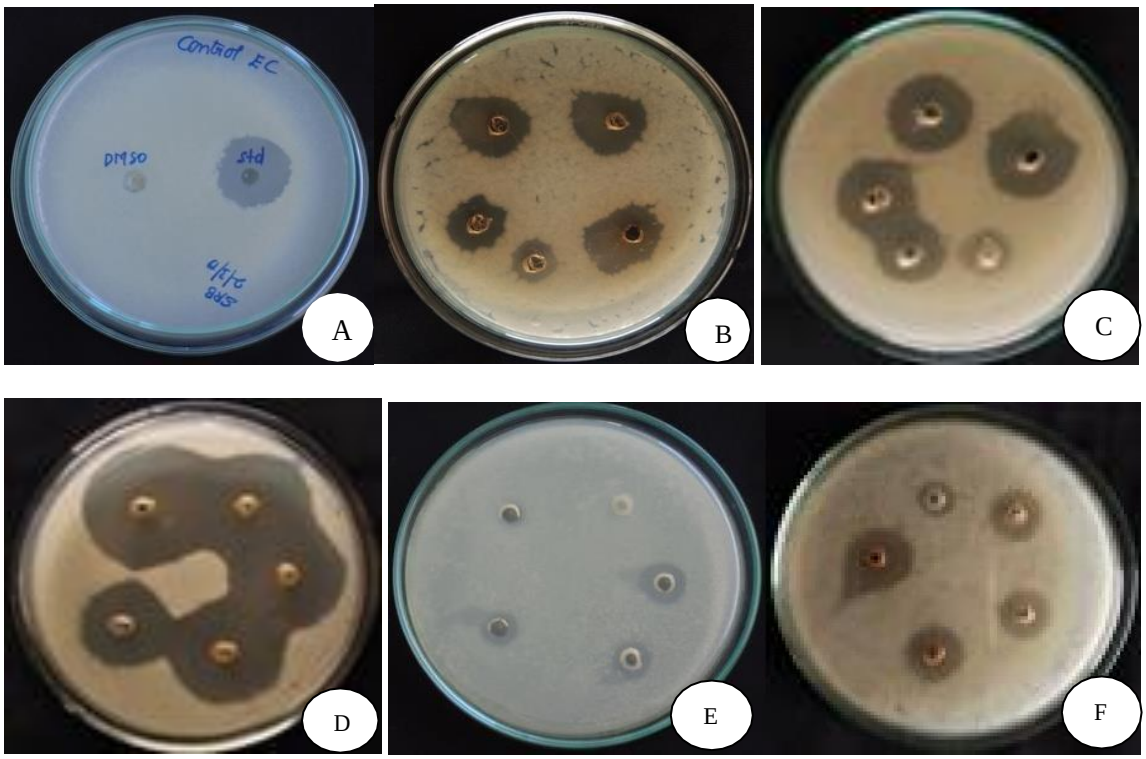
A.terreus

Aspergillus sp.

Antimicrobial activity of ethyl acetate extract of *T.radicus* showed inhibition against all pathogenic microorganisms. Zone of inhibition ranged from 31-6.3mm. But the methanolic extract was active only towards *K.pneumoniae* and *E.coli* (Table-56; Plate-19 and 20).

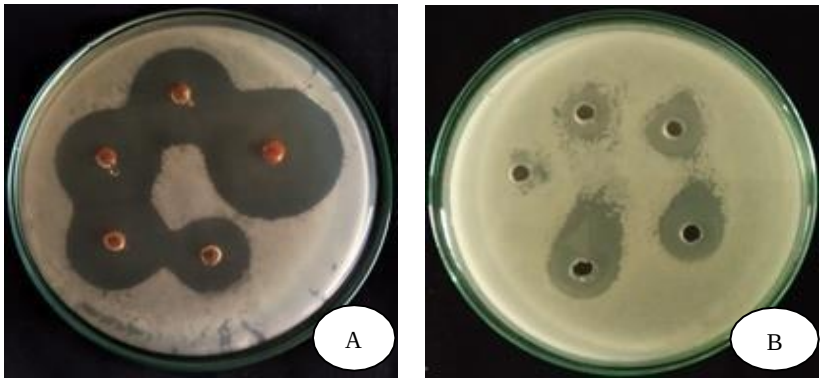
Ethyl acetate and methanolic extract of *Aspergillus* sp. showed zone of inhibition that ranged from 18mm to 4.1mm. Ethyl acetate extract of *Aspergillus* sp. showed inhibition towards *S.aureus* and *A.flavus* only at higher concentration (80-100µl). Methanolic extract also showed inhibition only at higher concentration towards *S.aureus*, *K.pneumoniae* and *C.albicans* (Table-57; Plate- 21 and 22).

Plate-19: Antimicrobial assay of solvent extracts of *T.radicus* against bacterial pathogens



A-Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *Enterococcus* sp.and *E.coli* ; E and F, Ethyl acetate extract against *Enterococcus* sp.and *E.coli*

Plate-20: Antimicrobial assay of solvent extracts of *T.radicus* against fungal pathogens



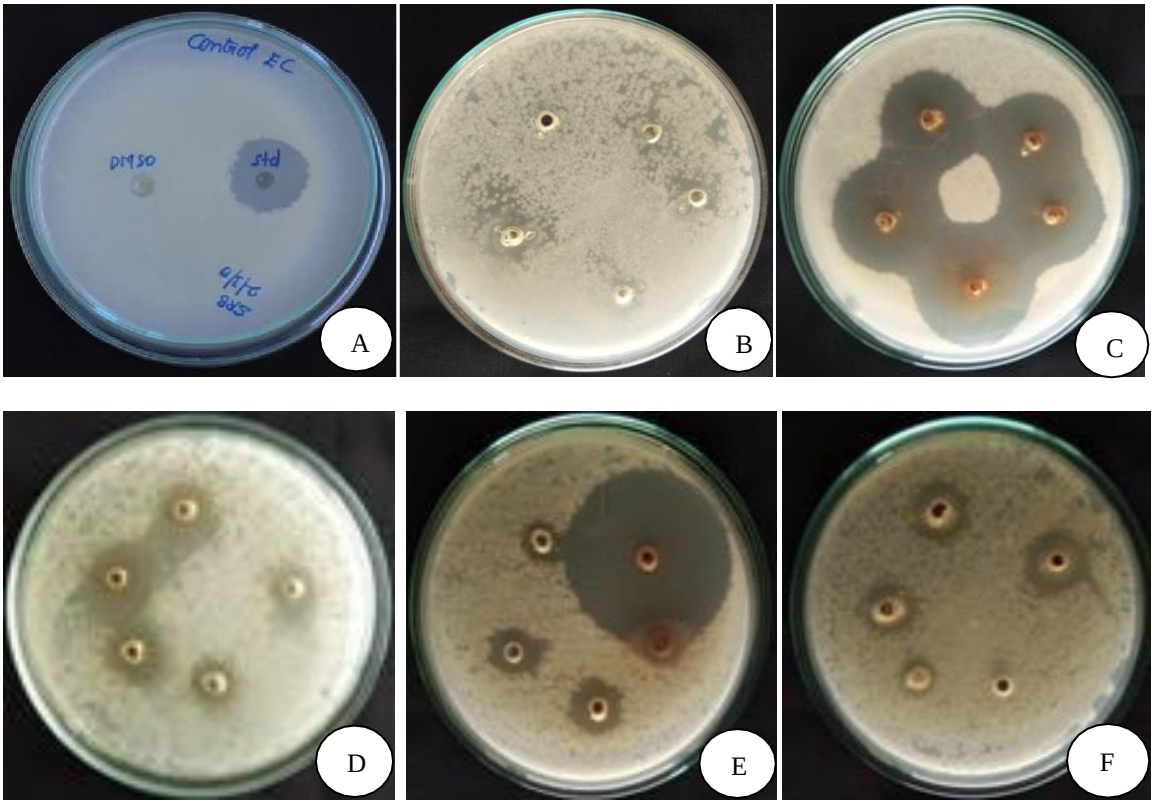
A and B-Methanolic extract against *A.flavus* and *C.albicans*

Table-56: Antimicrobial assay of solvent extracts of *T.radicus*

Extract/standard	Zone of inhibition (mm)						
	Concentration µg/ml	Bacterial pathogens				Fungal pathogens	
		S. aureus	*Enterococcus* sp.	*E. coli*	*K.pneumoniae*	*C.albicans*	*A.flavus*
Ethyl acetate extract	20	#4.67± 0.6	4.67±0.3	5.33±0.3	-	17.0±0.5	6.0±0.5
	40	7.0± 0.5	8.0±0.5	8.0±0.5	-	21.0±0.5	9.0±0.5
	60	11.0±0.5	11.6±1.2	11.0±0.5	3.67±0.3	26.0± 0.5	10.67±0.3
	80	15.0± 0.5	11.6± 0.8	15.3± 0.3	6.0± 0.5	29.0±0.5	13.0±0.5
	100	17.0±0.5	15.3±0.3	18.0±0.5	6.3±0.3	31.0± 0.5	16.0± 0.5
Methanolic extract	20	-	-	5.0± 0.5	-	-	-
	40	-	-	7.0±0.5	-	-	-
	60	-	-	7.67±0.6	-	-	-
	80	-	5.0±0.5	10.0± 0.5	-	-	-
	100	-	6.0± 0.5	11.0±0.5	-	-	-
Chloramphenicol	40	21.0 ± 2.08	17.3±0.8	23.0±0.5	11.0±0.5	-	-
Flucanozole	40	-	-	-	-	8.0±0.5	5.33± 0.3
DMSO	40	-	-	-	-	-	-

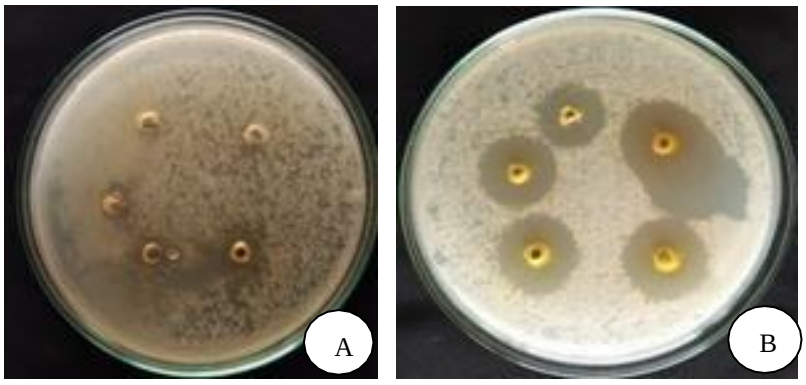
#Mean±S.E

Plate-21: Antimicrobial assay of solvent extracts of *Aspergillus* sp. against bacterial pathogens



A-Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *Enterococcus* sp.and *E.coli* ; E and F, Ethyl acetate extract against *Enterococcus* sp. and *E.coli*

Plate-22: Antimicrobial assay of solvent extracts of *Aspergillus* sp. against fungal pathogens



A and B-Methanolic extract against *A.flavus* and *C.albicans*

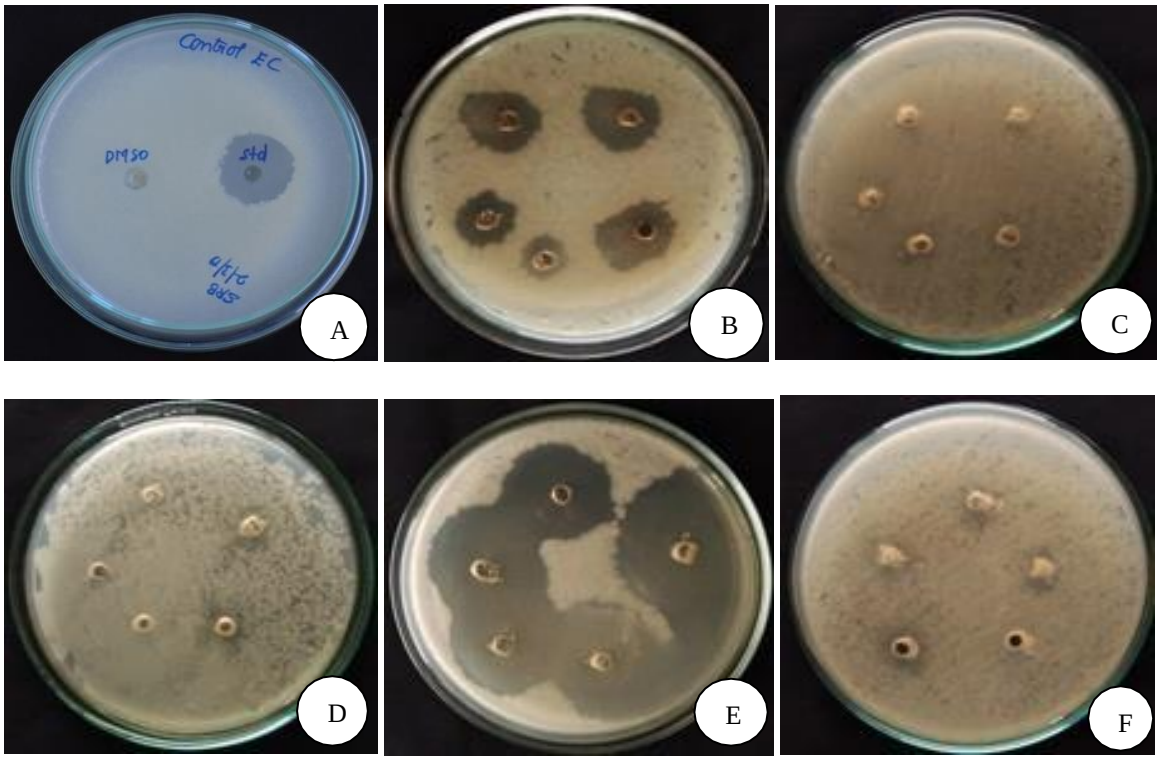
Table-57: Antimicrobial assay of solvent extracts of *Aspergillus* sp.

Extract/ standard	Zone of inhibition (mm)						
	Concentration µg/ml	Bacterial pathogens				Fungal pathogens	
		S. aureus	*Enterococcus* sp.	*E. coli*	*K.pneumoniae*	*C.albicans*	*A.flavus*
Ethyl acetate extract	20	-	#8.77±0.3	4.23±0.2	4.5±0.2	-	8.0±0.5
	40	-	10.0±0.4	6.2±0.6	4.7±0.5	-	10.0±0.5
	60	-	11.5±1.8	8.0±0.5	5.67±0.3	-	15.67±0.3
	80	-	15.6± 0.7	8.5± 0.3	6.1± 0.7	4.0±0.5	15.0±0.5
	100	4.56±0.2	18.3±0.8	10.0±0.5	6.3±0.8	5.2± 0.5	18.0± 0.5
Methanolic extract	20	-	4.6±0.2	8.0± 0.5	-	-	-
	40	-	5.1±0.5	8.6±0.5	-	-	-
	60	-	5.8±0.3	9.2±0.4	-	-	-
	80	4.9±0.2	6.2±0.5	9.6± 0.5	-	4.6±0.5	-
	100	5.1±0.6	6.9± 0.5	11.1±0.5	4.1±0.5	4.9±0.4	-
Chloramphenicol	40	20.0 ± 0.7	19.3±0.9	24.0±0.5	10.1±0.5	-	-
Flucanozole	40	-	-	-	-	6.0±0.5	6.9± 0.3
DMSO	40	-	-	-	-	-	-

#Mean±S.E

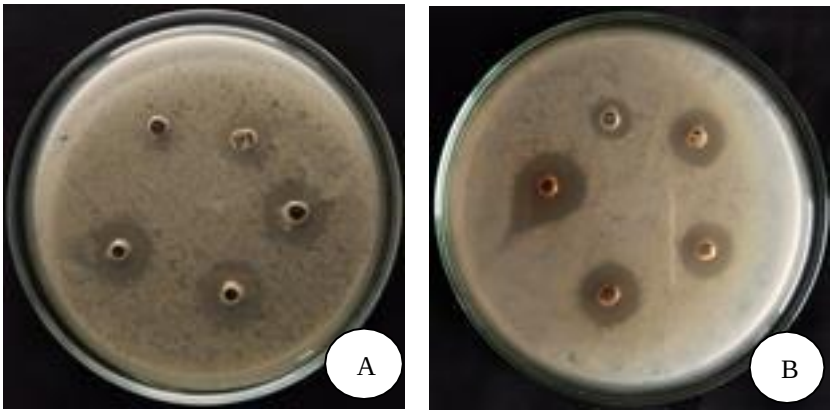
Antimicrobial activity of *A.terreus* was high towards *E.coli* (11.2mm). Methanolic extract of *A.terreus* showed inhibition towards pathogens at higher concentration (Table-58; Plate- 23 and 24).

Plate-23: Antimicrobial assay of solvent extracts of *Aspergillus terreus* against bacterial pathogens



A- Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *Enterococcus* sp. and *E.coli* ; E and F, Ethyl acetate extract against *Enterococcus* sp. and *E.coli*

Plate-24: Antimicrobial assay of solvent extracts *A.terreus* against fungal pathogens



A and B-Methanolic extract against *A.flavus* and *C.albicans*

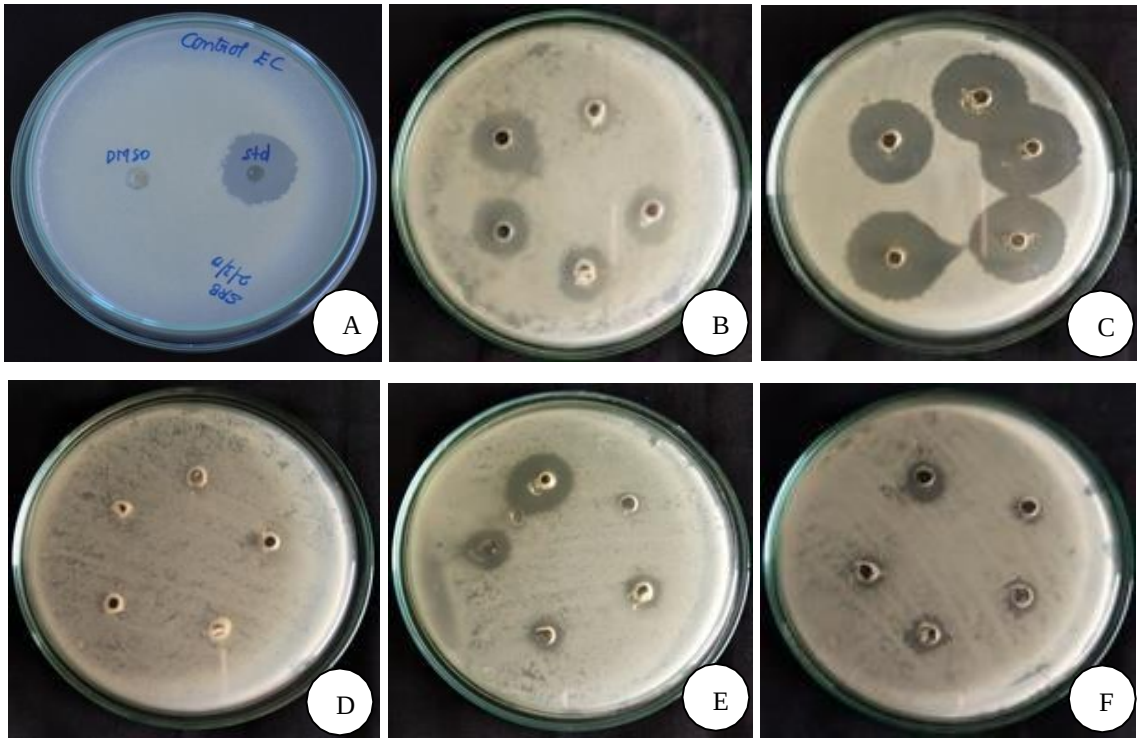
Table-58: Antimicrobial assay of solvent extracts of *A.terreus*

Extract/standard	Zone of inhibition (mm)						
	Concentration µg/ml	Bacterial pathogens				Fungal pathogens	
		S. aureus	*Enterococcus* sp.	*E. coli*	*K.pneumoniae*	*C.albicans*	*A.flavus*
Ethyl acetate extract	20	#6.9±0.2	-	7.6±0.2	-	4.1±0.5	-
	40	7.5±0.2	-	8.8±0.5	-	4.4±0.2	-
	60	7.8±0.3	-	9.3±0.5	-	4.6±0.6	-
	80	8.1±0.2	-	10.9± 0.2	4.6±0.2	5.1±0.5	-
	100	8.9±0.2	-	11.2±0.5	4.9±0.5	5.5±0.5	5.1±0.2
Methanolic extract	20	4.3±0.5	-	5.0± 0.5	3.8±0.2	7.3±0.5	-
	40	4.6±0.2	-	5.5±0.5	4.1±0.5	7.5±0.5	-
	60	5.2±0.5	-	6.2±0.4	4.4±0.6	8.3±0.2	-
	80	5.5±0.7	4.6±0.3	6.8± 0.5	4.6±0.5	8.7±0.2	4.6±0.2
	100	5.7±0.2	5.2±0.5	7.1±0.5	5.1±0.2	9.4±0.5	4.7±0.5
Chloramphenicol	40	18.0 ± 0.7	15±0.7	17±0.2	19±0.5	-	-
Flucanozole	40	-	-	-	-	8.1±0.5	7.6± 0.3
DMSO	40	-	-	-	-	-	-

#Mean±S.E

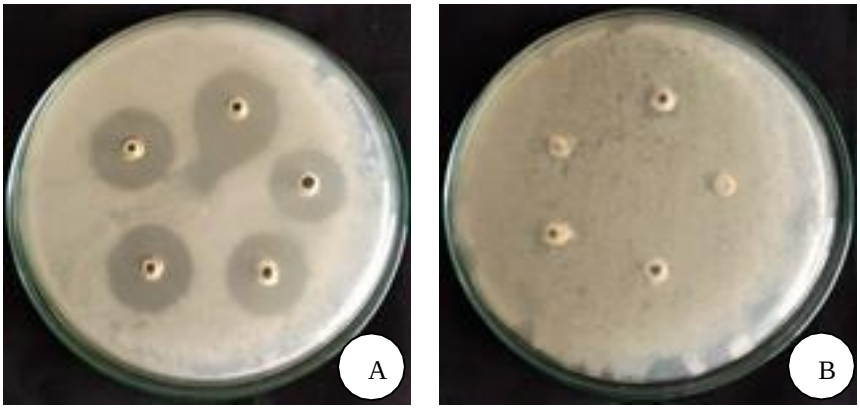
Ethyl acetate and methanolic extrct of *C.globosum* showed inhibition towards *S.aureus*. These extracts showed maximum activity at higher concentrations, against some pathogens. Methanolic extract showed less inhibition towards all pathogenic organisms (6.6- 4.1mm) (Table-59; Plate-25 and 26).

Plate-25: Antimicrobial assay of solvent extracts of *Chaetomium globosum* against bacterial pathogens



A-Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *Enterococcus* sp. and *E.coli* ; E and F, Ethyl acetate extract against *Enterococcus* sp. and *E.coli*

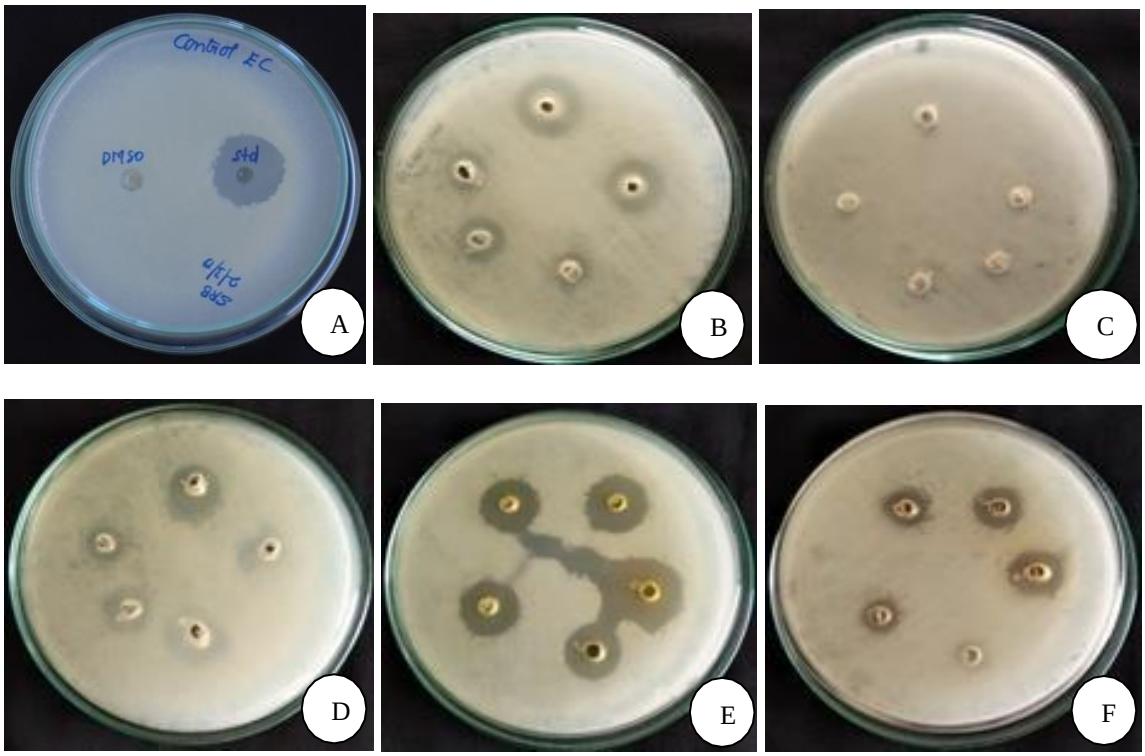
Plate-26:Antimicrobial assay of solvent extracts of *C.globosum* against fungal pathogens



A and B-Methanolic extract against *A.flavus* and *C.albicans*

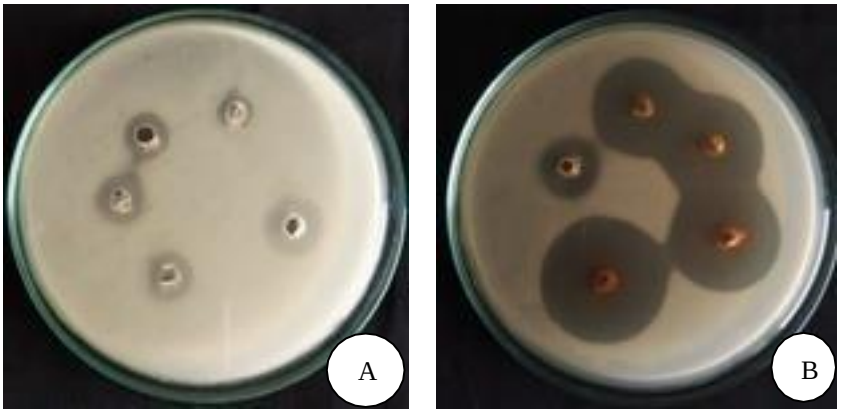
Ethyl acetate extract of *P.javanicum* showed higher inhibition against *E.coli* (8.5mm) and zone of inhibition ranged from 4.8-8.5mm and methanolic extract showed zone of inhibition that ranged from 5.3-7.5mm (Table-60; Plate-27 and 28).

Plate-27: Antimicrobial assay of solvent extracts of *Penicillium javanicum* against bacterial pathogens



A-Inhibition of pathogen by standard; B-D Methanolic extract against *S.aureus*, *Enterococcus* sp. and *E.coli* ; E and F, Ethyl acetate extract against *Enterococcus* sp. and *E.coli*

Plate-28: Antimicrobial assay of solvent extracts of *P. javanicum* against fungal pathogens



A and B-Methanolic extract against *A.flavus* and *C.albicans*

Table-59: Antimicrobial assay of solvent extracts of *Chaetomium globosum*

Extract/standard	Zone of inhibition (mm)						
	Concentration µg/ml	Bacterial pathogens				Fungal pathogens	
		S. aureus	*Enterococcus* sp.	*E. coli*	*K.pneumoniae*	*C.albicans*	*A.flavus*
Ethyl acetate extract	20	-	-	6.6±0.2	-	4.6±0.5	-
	40	-	#4.1±0.5	6.8±0.3	-	5.4±0.2	-
	60	-	4.3±0.5	6.9±0.2	-	5.6±0.6	-
	80	-	4.6±0.2	7.1±0.6	4.1±0.2	6.1±0.5	5.0±0.5
	100	-	5.1±0.5	7.3±0.5	4.3±0.5	6.5±0.5	5.1±0.2
Methanolic extract	20	-	5.2±0.3	5.2±0.2	4.4±0.2	-	4.3±0.2
	40	-	5.5±0.2	5.5±0.5	4.6±0.5	-	4.5±0.5
	60	-	5.7±0.6	5.8±0.7	4.8±0.5	-	4.8±0.5
	80	4.5±0.7	6.1±0.3	6.2±0.3	5.3±0.7	-	5.2±0.6
	100	4.7±0.2	6.6±0.7	6.5±0.5	5.8±0.5	4.1±0.5	5.5±0.7
Chloramphenicol	40	14.3 ± 0.5	15.1±0.7	17.6±0.2	11±0.5	-	-
Flucanozole	40	-	-	-	-	5.7±0.5	6.2± 0.3
DMSO	40	-	-	-	-	-	-

#Mean±S.E

Table-60: Antimicrobial assay of solvent extracts of *P. javanicum*

Extract/standard	Zone of inhibition (mm)						
	Concentration µg/ml	Bacterial pathogens				Fungal pathogens	
		S. aureus	*Enterococcus* sp.	*E. coli*	*K.pneumoniae*	*C.albicans*	*A.flavus*
Ethyl acetate extract	20	#4.2±0.5	-	7.2±0.5	-	4.4±0.5	3.8±0.6
	40	4.3±0.6	-	7.5±0.3	-	4.6±0.4	4.1±0.5
	60	4.7±0.5	-	7.8±0.5	-	4.9±0.6	4.3±0.5
	80	4.9±0.5	-	8.3±0.2	4.7±0.2	5.3±0.5	4.4±0.6
	100	5.2±0.5	-	8.5±0.5	4.8±0.5	5.8±0.5	4.8±0.7
Methanolic extract	20	-	4.2±0.3	6.2±0.2		5.2±0.5	-
	40	-	4.4±0.2	6.5±0.5		5.4±0.6	-
	60	-	4.7±0.6	6.8±0.7		5.8±0.7	-
	80	-	5.1±0.3	7.2±0.3	5.5±0.7	6.1±0.8	5.2±0.4
	100	-	5.3±0.7	7.5±0.5	5.6±0.5	6.6±0.4	5.5±0.6
Chloramphenicol	40	11.2 ± 0.5	13.1±0.7	18.1±0.2	10.0±0.5	-	-
Flucanozole	40	-	-	-	-	8.6±0.5	6.5± 0.3
DMSO	40	-	-	-	-	-	-

#Mean±S.E

Antioxidant assay of endophytic fungi

Quantitative estimation of antioxidant compounds

Total flavonoid content present in culture filtrate of endophytic fungi was assessed using Aluminium chloride colorimetric method. Among the isolates, *Aspergillus* sp. showed high (22.37mg) flavonoid content followed by *T.radicus* (18.30mg of RE/g of extract) (Table- 61).

Total phenolic content of endophytic fungi was assessed using Folin-ciocalteau reagent method. Both *P.javanicum* and *T.radicus* showed high (19 mg of GAE/ g of extract) phenolic content (Table- 61).

Table- 61: Quantitative estimation of antioxidant compound

Fungal endophytes	Antioxidant compounds (mg of standard/g of the extract)	
	Flavonoids (Rutin)	Phenols (Gallic acid)
Aspergillus sp.	#22.37±0.22	15.43±0.12
A. terreus	14.33±0.19	15.47±0.22
C. globosum	17.50±0.21	18.47±0.19
P. javanicum	14.63±0.35	19.43±0.15
T. radicus	18.30±0.23	19.63±0.20

#Mean±S.E

Total antioxidant activity assay

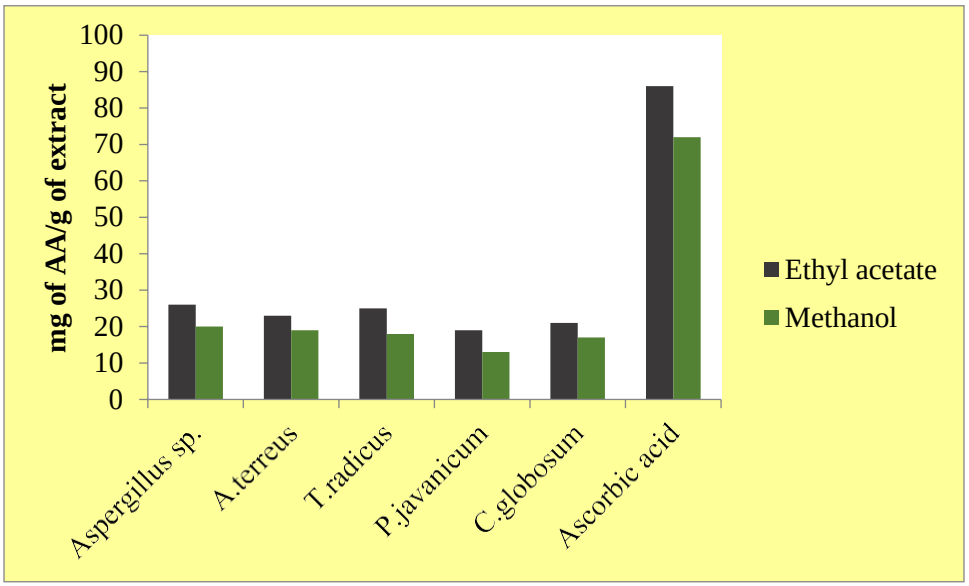
Total antioxidant activity of solvent extracts of endophytic fungi was assessed using Phosphomolybdenum method. Among the isolates, *Aspergillus* sp. showed 26mg AA/g exhibited higher radical scavenging activity compared to other isolates. Radical scavenging activity of all isolates was relatively low when compared to standard Ascorbic acid (Fig.38).

DPPH radical scavenging activity

DPPH, a stable free radical with absorption rate at 570nm was used to study the radical scavenging effects of ethyl acetate and methanolic extract of endophytic fungal isolates. The extracts used for testing were prepared at various concentration ranging from

20-100µg/ml. Along with sample, Ascorbic acid and blank methanol were taken as positive and negative control respectively. With the increase in concentration, colour of testing solution gradually changed. Extracts showed lower scavenging activity compared to control.

Fig. 38 : Total Antioxidant assay of endophytic fungi



Antioxidant activity of ethyl acetate extract of endophytic fungi showed a visible colour change from purple to yellow. Isolates like *T.radicus* and *P.javanicum* showed a high antioxidant activity (23 and 22%) (Table-62).

Methanolic extract of *A.terreus* and *T.radicus* showed a high scavenging activity (22 and 18%). Ascorbic acid taken as standard showed 97% of reducing potential (Table-63).

Estimation of phytoharmones of endophytic fungi

Quantitative analysis of IAA

Indole acetic acid present in culture filtrate of endophytic isolates were determined using Salkowski reagent. Concentration of IAA was higher in isolates when they were supplemented with typtophan. Among all isolates, *T.radicus* supplemented with tryptophan showed higher concentration of IAA (56.8 mg of IAA/ g of extract) followed by *Aspergillus* sp. (Fig.39).

Table-62: DPPH radical scavenging activity of ethyl acetate extracts of fungal endophytes

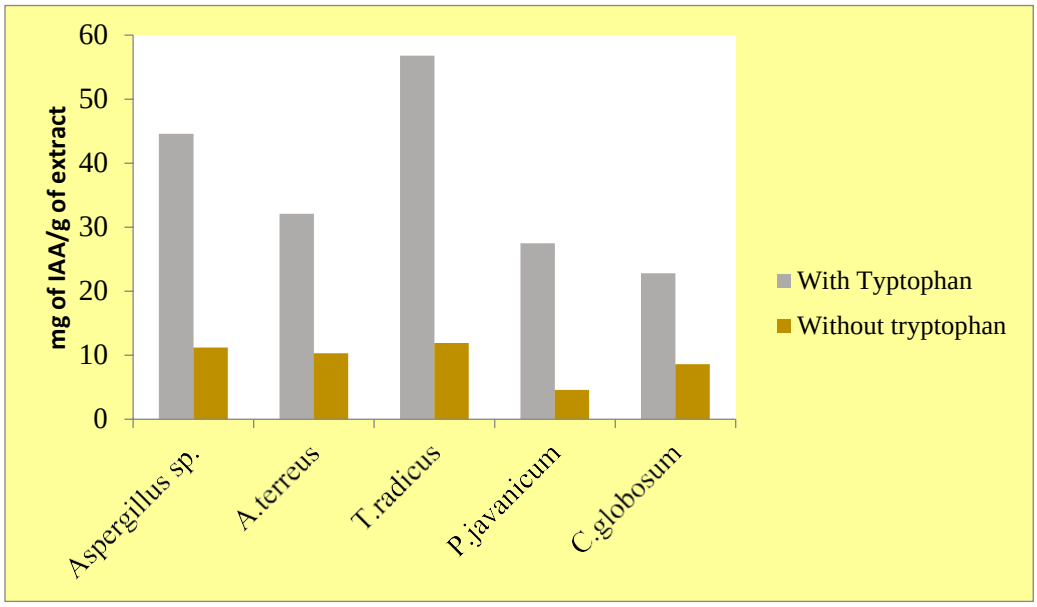
S.No	Fungal endophytes	% of inhibition at different concentration (µg/ml)				
		20	40	60	80	100
1.	*Aspergillus* sp.	#8.62±0.05	14.57±0.14	17.88±0.01	20.64±0.04	22.83±0.03
2.	*A.terreus*	7.74±0.08	9.43±0.04	11.18±0.03	16.19±0.01	18.15±0.02
3.	*C.globosum*	5.46±0.01	7.11±0.06	9.14±0.02	11.18±0.01	14.11±0.04
4.	*T.radicus*	15.59±0.11	17.33±0.05	19.05±0.03	21.08±0.01	23.12±0.01
5.	*P.javanicum*	12.12±0.01	15.32±1.01	17.10±0.03	19.31±0.02	22.06±0.01
6.	Ascorbic acid	82.44±0.06	87.13±0.02	91.65±0.03	94.17±0.01	97.66±0.08

Table-63: DPPH radical scavenging activity of Methanolic extracts of fungal endophytes

S.No	Fungal endophytes	% of inhibition at different concentration (µg/ml)				
		20	40	60	80	100
1.	*Aspergillus* sp.	#9.13±0.01	11.66±0.02	11.66±0.02	15.66±0.02	17.78±0.01
2.	*A.terreus*	10.15±0.02	13.19±0.01	13.19±0.01	18.17±0.01	22.14±0.02
3.	*C.globosum*	5.32±0.01	7.23±0.01	7.23±0.01	12.17±0.01	15.91±0.01
4.	*T.radicus*	7.26±0.01	9.13±0.02	9.13±0.02	16.22±0.01	18.23±0.02
5.	*P. javanicum*	6.18±0.01	8.22±0.02	8.22±0.02	13.17±0.01	15.16±0.02
6.	Ascorbic acid	82.44±0.06	87.13±0.02	91.65±0.03	94.17±0.01	97.66±0.08

#Mean±S.E.

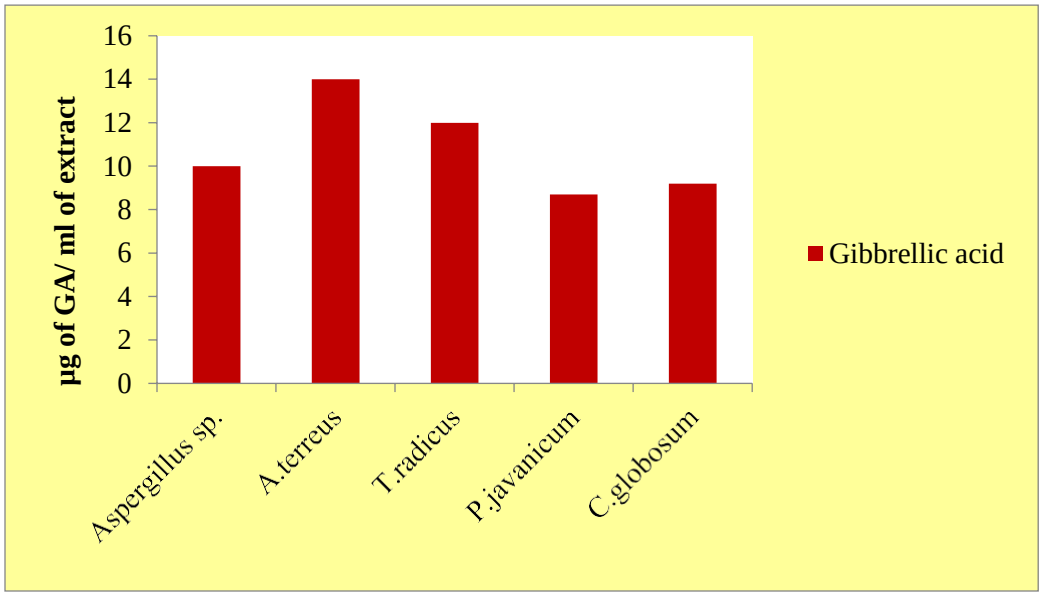
Fig.39: Quantitative analysis of IAA in culture filtrates of endophytic fungi



Quantitative analysis of Gibberellic acid

Estimation of Giberellic acid in the culture filtrate showed that concentration ranged between 8.7 to14 µg/ml. The highest activity was exhibited by *A.terreus* (14 µg/ml) followed by *T.radicus* (12 µg/ml), *Aspergillus* sp. (10 µg/ml), *C.globosum* (9.2 µg/ml) and *P.javanicum* (8.7 µg/ml) (Fig.40).

Fig.40: Quantitative analysis of Gibberellic acid in culture filtrates of endophytic fungi



DISCUSSION

Natural products from plants, microbes and marine organisms act as most important source for discovery of potential drug molecules and tested for their diverse pharmaceutical applications. Among them endophytic microbes also play a major role in recent days for production of novel metabolites. Human beings greatly depend on natural remedies to overcome the disease incidences. Although, many chemicals are available, they are not only costly, they are also harmful to health, ecosystem, environment and make pathogenic organisms develop resistance against chemicals. Therefore, to overcome negative impact of synthetic chemicals, natural sources have been widely used.

Cultivation of fungal endophytes in different media signalized the ability for growth and secondary metabolite production. Recent researches proved that the culture parameters, i.e. nutrient supplements, nutrient concentrations and culture conditions of the media affects the growth and metabolite production by the endophytic fungi. The quality of medium based on its composition greatly influenced the growth and metabolite production both quantitatively and qualitatively. In the present study, maximum growth and metabolite production by four endophytic fungi *Aspergillus* sp., *T.radicus*, *C.globosum* and *P.javanicum* was observed on Potato dextrose broth (PDB), which is in corroboration of the findings of Bhattacharya and Jha (2011), Pradeep et al., (2013), Kalyani et al., (2016), Suzuki and Iwahashi (2016) and Devi et al., (2018) who suggested PDB as best medium for growth and secondary metabolite production in fungi.

Under *in vitro* condition, growth and secondary metabolite production by fungal endophytes mainly depended on various cultural conditions such as carbon source, nitrogen source, temperature and pH. The present study focused on the optimization of suitable condition for the selected isolates, for higher level of biomass and metabolite production.

The pH of the medium determines the rate of growth, metabolite production and other metabolic process of fungi. Most of the microbes showed maximum growth and metabolite production at pH ranging between 5.5 and 8.5 (Thongwai and Kunopakarn, 2007). With the increase in pH growth and metabolic process decreased rapidly. Our results corroborated with the results of previous studies using *Aspergillus nidulans*

(Geweely, 2011), *Aspergillus fumigatus* (Astuti et al., 2013), *Aspergillus terreus* (Mathan et al., 2013). Merlin et al., (2013) reported that pH 7 was suitable for growth and metabolite production by *Fusarium solani*.

Our results (pH 7) were in contradiction with the results of Bhattacharyya and Jha (2011) where the optimum pH for enhancing growth and metabolite production in *Aspergillus* sp. was reported to be 5.5. Jain and Pudir (2011) and Fathima et al., (2016) reported pH 6 proved to be the best for maximum growth and secondary metabolite production in *Aspergillus terreus* and *Chaetomium* sp.

Incubation temperature naturally influences the overall growth and development of any microbe by affecting their physiology and metabolite production. Optimal temperatures are required for vegetative growth and metabolite production. *Aspergillus* sp., *Aspergillus terreus, T.radicus, P.javanicum* and *C.globosum* showed maximum fresh weight, dry weight and metabolite production at temperature 25˚C and beyond this it gradually declined. Different studies proved that temperature is one of the major conditions affecting the growth rate and metabolite production. Several existing literature also stated that incubation temperature ranging between 20 and 30 °C was detected to be optimum for mycelial growth and metabolite production (Kok and Papert, 2002).

Our results were similar to the findings of Ritchie et al., (2009), Parmar et al., (2010), Jain and Pundir (2011), Merlin et al., (2013), Abo-Elmagd (2014) and Fathima et al., (2016) where they detected 25˚C as the optimum temperature for growth and metabolite production of *Rhizoctonia solani, Fusarium oxysporum, A.terreus, Fusarium solani, Chaetomium madrasense* and *Chaetomium globosum*

In order to design the effective medium, the role of different carbon sources like Dextrose, Sucrose, Maltose and Fructose was evaluated for their impact on growth and metabolite production. Among the various carbon sources tested on fungal endophytes, glucose was found to be the best carbon source for both biomass and metabolite production by *Aspergillus* sp., *A.terreus* and *C.globosum*. *T.radicus* and *P.javanicum* exhibited maximum growth and metabolite production when sucrose was used as carbon source. Bhattacharyya and Jha (2011) and Verma et al., (2017) reported that all carbon sources

helped *Aspergillus* sp. to enhance the growth and metabolite production. Abo-Elmagd (2014) and Zhang et al., (2017) reported dextrose as the best carbon enhancing growth and metabolite production of *Aspergillus terreus* and *C.madrasense*.

Our results contradicted with the results of Mathan et al., (2013), who reported that sucrose acted as best carbon source for enhancing the growth and metabolite production of *Aspergillus terreus*.

Peighamy-Ashnaei et al., (2007) described the importance of various nitrogen sources in maximizing the growth rate of the fungal strain and the antibiotic production. In the present study, it was observed that yeast extract showed the maximum growth and metabolite production by fungal endophytes like *T.radicus* and *P.javanicum*. Requirement of nitrogen source greatly differ from one microbe to another. Our results were similar to the findings of Ismaiel et al., (2010), Jain and Gupta (2012) and Shukla et al., (2014) reported that sodium nitrate as best nitrogen source for growth and metabolite production by *A.terreus*, *Penicillium* sp. and *Fusarium roseum*. Verma et al., (2017) achieved maximum growth and metabolite production when yeast extract was used as nitrogen source.

Preliminary chemical analysis of crude filtrate of endophytic fungi was done to determine their presence, as they lead to further exploration. The active metabolites contain chemical groups such as phenols, flavonoids, terpenoids, alkaloids, tannins, carbohydrates and saponins. Almost all the components were present in *Aspergillus* sp., *A.terreus*, *P.javanicum*, *C.globosum* and *T.radicus*. Similar results were obtained by Devi et al., (2012) and Bharadwaj et al., (2015) in *Penicillium* sp. Satari et al., (2018) reported the presence of tannins, steroids, glycosides, flavonoids, terpenoids, alkaloids, phenols, saponins and anthraquinones from the ethanolic extract of endophytes *A.niger*, *A.terreus* and *A.flavus* isolated from *Achillea millefolium*. Thakur and Sahani (2019) showed the presence of alkaloids, phenols, flavonoids, steroids and terpenoids, from the ethyl acetate, methanolic and chloroform extracts of *Aspergillus*.

In order to study the structure of compounds and to detect the functional groups of chemical constituents present in the culture filtrate, FTIR analysis was performed. In the present study IR spectrum of selected fungal endophytes showed some major peaks which proved the presence of alcohol, amine, alkyne, imine, ether, nitro compounds, aldehyde,

azide, thiol, thiocynate, isothiocynate and sulfonyl chloride. Our results corroborated with the results of Hameed et al., (2017) who proved the presence of some functional groups like amine, alcohol, imine in the culture filtrate of *Aspergillus niger*. Similar reports were observed by Kanjana et al., (2019) in endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*.

Crude filtrate of fungal endophytes possessed important bioactive compounds and metabolites involved in various metabolic processes. Components of culture filtrate were analyzed using GC-MS. Culture filtrate of fungal endophytes *Aspergillus* sp., *A.terreus*, *C.globosum, T.radicus* and *P.javanicum* were found to produce high and low molecular weight compounds with certain biological activities. In the present study, twenty one volatile metabolites were detected and some of the important compounds are Dibutyl phthalate, Diamyl phthalate, Benzenedicarboxylic acid, Octadecanoic acid. Our results were similar to the findings of Prabukumar et al., (2015); Sharma et al., (2016) and Elgorban et al., (2019) where Benzedicarboxylic acid was a major compound observed in endophytes of *Nigrospora sphaerica, Fusarium oxysporum, Pestalotiopsis neglecta* and *Alternaria* sp. some of the results that corroborated with our results are as follows,

Valderramas et al., (2008), Karmakar et al., (2011), Devi and Singh, (2013) and Murugan et al., (2017) reported octadecane, an alkane hydrocarbon, with anticancer, antioxidant and antimicrobial properties. It is also reported as active compound from endophytes, *Collectotrichum gloeosporiodes* and *Penicillium* sp.

In the present study Dibutyl phthalate was recorded as major bioactive compound from *T.radicus* and *Aspergillus* sp. previously it was reported from *Fusarium phyllophilum* (Akinduyile and Ariole, 2018). It acts as strong antimicrobial, antimalarial, antifungal, antitumor, anticancer agent.

Al-Bari et al., (2006) recorded Phthalic acid as strong antimicrobial agent. Among five isolates it is present only in culture filtrate of *P.javanicum*. Some endophytic fungi like *Phomopsis* (Senthilkumar et al., 2014), *A.egypticus* (Gulyamova et al., 2018) also reported to possess Phthalic acid.

Octadecanoic acid, an important saturated fatty acid found in *A.terreus* have been previously reported from endophytes like *Pestalotiopsis sp.* (Li et al., 2013),

Pestalotiopsis neglecta (Sharma et al., 2016), *T.purpureogenus* (Kumari et al., 2018), *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* (Kanjana et al., 2019).

Hexadecanoic acid is widely used in industrial applications. In the present study, *T.radicus* and *Aspergillus* sp. were found to possess this compound. Some of the endophytic fungal cultures like *Trichothecium* sp. (Anisha and Radhakrishnan, 2017), *A.terreus* (Rani et al., 2017), *Talaromyces purpurogenous* (Kumari et al., 2018), *Cryptococcus neoformans* and *Microsporum canis* (Abayomi and Patrick, 2018) were also reported to produce hexadecanoic acid.

Cyclohexanol is an intermediate product which helps in the production of some chemicals, plasticizer and in pesticides. This was the first report on endophytic fungi producing cyclohexanol. In addition to cyclohexanol, other compounds like Lysine, Docosanol, Undecane, Cucurbitacin B, Phosphonic acid, Isogeraniol and norvaline were reported for first time in culture filtrate of *Aspergillus* sp., *T.radicus, A.terreus, P. javanicum* and *C.globosum*.

Anthracenedione and their derivatives act as excellent anticancer agents. In the present study, *T.radicus* was found to possess this compound. But it has been previously reported in endophytes like *Halorosellinia* sp. and *Guignardia* sp. (Zhang et al., 2010). In the present study, Naphthalene an important insect repellent compound was reported from *P.javanicum*. It has been previously reported from culture filtrate of endophytes *Muscodor vitigenus* (Daisy et al., 2002), *Phomopsis* (Li et al., 2019).

Apart from parameters like pH, temperature, incubation time, carbon and nitrogen, type of solvent used for extraction was optimized for enhanced recovery of secondary metabolites (Venugopalan and Srivastava, 2015; Tian et al., 2016). The suitable solvent used for extraction of secondary metabolites from endophytes depends on the nature of compounds to be isolated (Sarker et al. 2005). In the present study, selected fungal endophytes *Aspergillus* sp., *A.terreus*, *T.radicus*, *C.globosum* and *P.javanicum* gave high extractive value when extracted with ethyl acetate solvent, which is similar to the findings of Dai and Mumper, (2010); Khoddami et al. (2013); Devi and Prabakaran (2014); Goutam et al., (2016); Rajeswari et al., (2016) and Dhayanithy et al., (2019).

Fungal enzymes are important in agriculture, industry and in human health. Sometimes, they can withstand any temperature and pH. Potential of fungal enzymes will be more when compared to plant and animal derived enzymes. They can be used in manufacturing food, beverages, confectioneries, textiles and in leather industries (Maria et al., 2005).

The true endophytes obtained from *C.dipsaceus* produced different types of extracellular enzymes like amylase, protease, lipase and cellulase. The qualitative analysis of present study agrees with those of Gopinath et al., (2005), Maria et al., (2005), Amirita et al., (2012), Bhagobathy and Joshi (2012), Sunitha et al., (2013) and Uzma et al., (2016) who reported the capacity of endophytic fungi to produce extracellular enzymes. Isolates like *Aspergillus* sp., *Penicillium* sp., *Chaetomium* sp. and *Talaromyces* sp. isolated from various plants produced Amylase, Cellulase, Protease and lipase.

Quantitative analysis of enzyme production was carried out in selected fungal endophytes. Our results were similar to the findings of Maria et al., (2005) were two strains of *Aspergillus* sp. isolated as endophytes from *Acanthus ilicifolius* and *Acrostichum aurem* exhibited cellulase, lipase and protease activity at the rate of 4.2, 6.9 and 3.8 enzyme units.

In contrast to our findings, Tuppad and Shishupala (2014) and Shubha and Srinivas, (2017) reported less quantity of Amylase, cellulase, protease and lipase from endophytes like *C.crispatum, A.fumigatus, A.sydowi, A.japonicus, A.terreus, P.citrinum* and *Talaromyces rotundus*.

Laccase is an active enzyme that damages the host cells and none of true endophytes produced this enzyme. Maria et al., (2005), Uzma et al., (2016) and Subha and Srinivas (2017) also supported the view that true endophyte doesn't produce laccase enzymes.

Some fungi naturally have the capacity to produce some useful organic acids. *Penicillium* sp. produces Qunic, Tartaric, Oxalic, Citric, Malic, Succinic and fumaric acids (Sharma and Mishra, 1981), *Aspergillus* sp. produce citric, gluconic, malic and itaconic acids (Yang et al., 2017), *Rhizopus* produce lactic and fumaric acids. Our results were similar to the findings of Sharma and Mishra (1981); Khan and Gupta (2017) and Sahoo and Gupta (2017) who reported that *Aspergillus, Alternaria, Penicillium*, Sterila, *Mucor, Trichoderma, Fusarium, Phoma* and *Paecilomyces* produced organic acid. Among them *A.niger* (0.125 Moles) and *Penicillium* sp. (0.037 Moles) were significantly higher producers of organic acids.

Fungal isolates, which are recognized as true endophytes from *C.dipsaceus* had the capability to produce pigments. *Aspergilus* sp. and *A.terreus* was to found to produce high quality of pigment which excreted intense brown colour. Our results were similar to the results of Celestino et al., (2014) who reported that *A.calidoustus* isolated from amazon soil has the capacity to produce intense brown colour pigment which had the potential to use as natural dye.

Another true endophyte *P.javanicum* was found to produce yellow pigment. Similar to our findings Mendez et al., (2011) and Afshari et al., (2015) reported that *P.purpurogenum* and *P.aculeatum* have the capacity to produce yellow pigment. *C.globosum* produced dark purple coloured pigment. Our results were corroborated with the results of Brewer et al., (1968); Pornsuriya et al., (2008); Yang et al., (2012) and Soumya et al., (2014) who reported that *C.globosum* have the capacity to produce purple coloured pigment.

In the present study, *T.radicus* produced brown coloured pigment. Similar to our results, Barbosa et al., (2018); Ogbonna, (2018); Rajeshkumar et al., (2019) reported that different species of *Talaromyces* like *T.verruculosus, T.pigmentosus, T.mycothecae, T.brasiliensis* and *T.purpurogens* has the capability to produce brown pigment. Morphological studies on *T.radicus* by Yilmaz et al., (2014) clearly stated that brown pigment production on reverse side of the colony.

Endophytes are now recognized as an excellent source for secondary metabolites with antimicrobial properties (Strobel and Daisy, 2003) which is used for medicinal purposes. The test pathogens choosen for this study has the ability to cause diseases like wound gallstone, Diarrhea, urinary retention and pneumonia. As shown in Table -1, different parts of *C.dipsaceus* are used to cure these ailments in traditional medicines. Therefore, true endophytes identified from this plant were tested against the pathogens.

In the present study, extracts of all five isolates showed strong inhibition towards pathogenic organisms. Similar to our results, Maria et al., (2005), Raviraja et al. (2006) Meenupriya and Thangaraj, (2010), Selim et al., (2011), Deepake et al., (2012), Prabavathy and Vallinachiyar, (2012), Lu et al., (2013) and Mathan et al., (2013) reported that the crude extract of endophytic fungi, *Aspergillus* sp., *Penicillium* sp., *C.globosum* and

T.radicus showed strong antagonistic effect towards pathogens like *B.subtilis*, *K.pneumoniae*, *P.aureginosa, S.aureus, A.fumigatus* and *A.niger* and *C.albicans*. This is may be due to fewer amounts of active compounds present in the extracts of endophytes (Radu and Kqueen, 2002). In contrast to this result, *C.globosum* isolated from *Catharanthus roseus* showed strong inhibitory effect towards *C.albicans* (Bashyal et al., 2005).

The level of inhibition varied among endophytes, this might be due to the amount of antimicrobial compounds or by their activities. Sometimes it may be due to difference in mode of action, level of isolate inoculation and activity of biochemical constituents produced by the isolates. All the above studies clearly stated that endophytes isolated from different host plants inhabiting different ecosystems act as excellent source for natural antimicrobial compounds.

Phenols and flavonoids act as important source of primary and secondary antioxidant compounds. They are important chemical constituents responsible for reducing lipid peroxidation (Yadav et al., 2014). The DPPH radical scavenging activity of ethyl acetate and methanolic extract of endophytic fungi and ascorbic acid showed high scavenging effect with increase in concentration of fungal extracts. Similarly, DPPH activity of endophytes *Aspergillus* sp., *C.globosum* and *Penicillium* sp. isolated from *Nerium oleander* (Hung et al., 2007), *Tabebuia argentea* (Govindappa et al., 2013), *Lobelia nicotianifolia* (Murthy et al., 2011), trees of sacred forests (Bhagobaty and Joshi, 2012), *Lannea coromendalica* (Premjanu and Jaynthy, 2014), *Eugenia jambolana* (Yadav et al., 2014), *Hugonia mystax* (Abirami and Boominathan, 2016), *Thymus vulgaris* (Ali et al., 2018), *Achillea millefolium* (Satari et al., 2018), *Adiantum capillus* (Selim et al., 2018) and *Catharanthus roseus* (Dhayanithy et al., 2019) showed positive correlation between antioxidant capacity and total phenolic content. Isolates showed 70-80% inhibition activity.

Eventhough radical scavenging activity of fungal extracts were low when compared to synthetic antioxidants like Rutin and Ascorbic acid, it can be prescribed as a safe antioxidant source as the synthetic antioxidant are reported to pose certain side effects. Therefore, it can be suggested as safe and economical antioxidant source. Similar to our result, leaf and fruit extract of *Cucumis melo* and *C.dipsaceus* were reported to possess radical scaving activity (Arora et al., 2011; Nivedhini et al., 2014).

Now-a-days many studies focus on harmless and environment friendly techniques. Large number of researches has been carried out with the goal of identifying suitable endophyte that have the ability to enhance plant growth and performance. In the present study, five true endophytic isolates were subjected to initial screening of IAA and Gibbrellic acid production because they were considered as important in agricultural point of view. Production of phytohormones by endophytes helps the plant to tolerate and overcome abiotic and biotic environmental stresses (Lubna et al., 2018).

Current findings showed that all endophytic fungi had the ability to produce IAA and Gibbrellic acid at significant level. Similar results were previously reported by Hassan (2002), in fungal species like *A.flavus*, *A.niger* and *P. corylophilum*. Waqas et al., (2012) also obtained lower level of IAA (29mg) and Gibbrellic acid (9µg) in culture filtrates of *Phoma glomerata* and *Penicillium* sp., which is similar to the present findings where *T.radicus* showed only 56mg of IAA and *Aspergillus* sp. (9.2mg). Eventhough, concentration of IAA and GA was low, it is enough to compensate the deficiency of GA and IAA and helped to restore normal growth (You et al., 2015). Therefore, endophytes may have a potential use in agriculture to alleviate environmental stress and reduce agricultural cost.

CONCLUSION

Endophytic fungi from plants were considered as important source of different bioactive metabolites that can be used for the development of various therapeutic agents. In the present study selected endophytic fungi namely *Aspergillus* sp., *A.terreus*, *Penicillium javanicum*, *Chaetomium globosum* and *Talaromyces radicus* were screened for the presence of bioactive metabolites. These endophytic fungi were grown in different broth and their metabolites were extracted using different solvents. Based on the present study, it may be concluded that, selected endophytic fungi have the capacity to produce phytochemicals, extracellular enzymes, pigments, plant growth harmones and possessed antimicrobial and antioxidant property. The study proves that these endophytic fungi associated with *C.dipsaceus* have diverse biological applications.