Antioxidant activity of the endophytic fungi isolated from the Medicinal plants

A thesis submitted in partial fulfilment of the requirement
For the award of the degree of

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IN
BIOTECHNOLOGY

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CERTIFICATE

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

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DECLARATION

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

Anmol

Place: Patiala

Date: 20-AUGUST-2018

Anmol

Place: Patiala

Date: 20-AUGUST-2018

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LIST OF ABBREVATIONS

%	Percent
°C	Degree centigrade
TLC	Thin layer chromatography
ml	Millilitre
µg	Microgram
mg	Milligram
µl	Microlitre
m	Meter
nm	Nanometer
g	Gram
EC50	Effective concentration
RPM	Revolutions per minute
TPC	Total phenol content
TFC	Total flavanoid content
DPPH	2,2-Diphenyl-1-picrylhydrazyl
UV	Ultraviolent
v/v	Volume by volume
w/v	Weight by volume
PDA	Potato dextrose agar
PDB	Potato dextrose broth
GC-MS	Gas chromatograph-mass spectrometer
Rf	Retention factor
RT	Retention time
M.W	Molecular weight

ABSTRACT

The field of free radicals has been gaining attention, it has been known that free radicals are an important component of our body's developing system and also holds significance in signal transduction pathways. The source of free radicals is either body's normal essential metabolism or exogenous sources such as heavy metals, industrial pollutants, tobacco smoke etc. Diseases such as cancer, cardio-vascular and some neurological disorders are called as salient "Free radical diseases".

The main aim of this work was to study antioxidant potential of crude extracts obtained from endophytic fungi which were isolated from two medicinal plants *Eucalyptus terreticornis* and *Tinospora cordifolia*. DPPH assay, total phenolic content, total flavanoid content was performed to check for antioxidant potential of the crude fungal extracts. Based upon results, best activity giving fungi was selected and phytochemical analysis, fractionation of extract through TLC was performed and the activity of the extracted compound was also checked. GC-MS analysis of the fraction giving maximum activity was carried out to know the type of compounds present in the extract.

In preliminary screening, fungal isolates obtained were screened for antioxidant activity out of which maximum activity was shown by fungal extract EB i.e 73.6% at 1000µg/ml, total phenolic content and total flavanoid content of the four isolates (GS1, GS3, GR3 and EB) showed that EB has the highest phenolic content i.e 222 mg of QE/g of dried extract and highest flavanoid content of 124 mg of GAE/g of dried extract.

EC_{50} of EB i.e 50% of scavenging activity was observed at a concentration of 350µg/ml and 80% scavenging activity was observed at 1500µg/ml. Qualitative methods such as phytochemical analysis and thin layer chromatography indicated the presence of various compounds. Extraction of TLC fractions yielded four fractions, all the four fractions further gave antioxidant activity out of which third fraction gave the maximum activity 65.3% at 100µg/ml.

GC-MS analysis of the third fraction indicated the presence of bioactive compounds 1,2Benzenedicraboxylic acid,diisooctyl ester, 3-tert-butyl-5-chloro-2-hydroxybenzophenone, 4-amino-1,3-diphenyl-quinolin-2-(1H)-one, Octadecane, Phenylseleno-5-methyldihydro-2(3H)-furanone, Benzaldehyde glycerol acetal and α-tocopherol which may contribute to the extract's antioxidant activity.

Endophytic fungi can thus be a novel source of antioxidant compounds and can play a very important role in food, pharmaceutical and other industries.

CHAPTER-1 INTRODUCTION

Our body has various optimised mechanisms in order to maintain normal cellular functions, these metabolic functions further result in the production free radicals. Free radicals are a group of atoms or atom which have unpaired or odd number of electrons in there valence shell (Fig1). Free radicals which are produced inside the body (reactive oxygen species, reactive nitrogen species, and reactive sulphur species) either due to normal physiological processes or Exposure to exogenous components like UV light, other environmental pollutants which can increase free radical stress on our body (Fig 2). Once formed they start a chain reaction like dominoes.

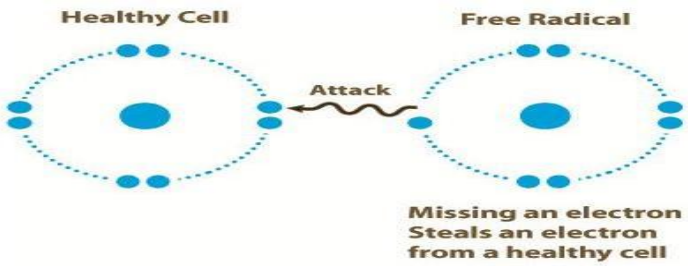

Fig 1: Free radicals attack on healthy cell.

There is a very little yet a major difference between free radicals and oxidants. Some of the examples are shown in Table 1, as already discussed free radicals are highly reactive species and there formation occurs during several courses of reactions and they are formed in small concentrations. Since, they are usually highly reactive they cannot be isolated in pure form. On the other hand, oxidants are substances which have strong affinity for electrons they do not burn but they support combustion and oxidants are generally formed of electronegative elements and in comparison to free radicals they can be isolated in pure form. Hence, it is safe to say all free radicals are oxidants but not all oxidants are free radicals.

Table 1: Free radicals and Oxidants

	Free radicals	Oxidants
1	Hydroxyl ($OH^{\bullet}$)	Ozone (O_3)
2	Superoxide ($O_2^{\bullet -}$)	Hypochlorous acid (HOCl)
3	Peroxyl ($ROO^{\bullet}$)	Nitrous acid (HNO_2)
4	Nitrogen dioxide ($NO_2^{\bullet}$)	Hydrogen peroxide (H_2O_2)
5	Nitric oxide ($NO^{\bullet}$)	Dinitrogen trioxide (N_2O_3)

Molecular and cellular damage by these reactive species is believed to be one of the major causes in ageing, cancer, neural disorders and various other diseases. If free radicals overwhelm the bodies capability to control them a condition called "oxidative stress" is induced. It is ironic how oxygen is an indispensible element of our life, and yet under certain conditions it has deleterious effects on the human body (Lobo *et al* 2010).

The source of free radicals is either body's normal essential metabolism or exogenous sources such as heavy metals, industrial pollutants, tobacco smoke etc (Fig 2). Free radicals production happens in our body in a continuation due to both non-enzymatic and enzymatic processes. Enzymatic reactions which act as a source of free radicals are prostaglandin synthesis, cytochrome P-450 system, and certain respiratory chain reactions. And other causes that is non-enzymatic reactions is seen when oxygen reacts with organic compounds due to ionizing radiation.

Diseases such as cancer, cardio-vascular and some neurological disorders are called as salient "Free radical diseases". These are elucidated further ahead:

1. Initiation of cancer is either due to oncogene activation or chromosomal defects. It is possible that endogenous free radicals may partake in such defects further causing tumour formation. Another example can be formation of hydroxyled DNA bases which is an important event in chemical carcinogenesis (Huy *et al*, 2008). This adduct formation disrupts normal cell functioning and can also alter gene transcription. A highly significant correlation exists between death rates due to leukaemia, ovarian, rectum cancers and consumptions of fats among elderly which may be an example of greater lipid per-oxidation (Droge.W 2002).
2. Coming to cardio-vascular diseases (CVD) which is a multifactorial etiology and is associated with number of risk factors such as hypertension, high cholesterol levels, improper diet and physical inactivity (Huy *et al*, 2008). However, there has been number of debates if oxidative stress is a primary or secondary cause of CVD.
3. In neurological disorders oxidative stress plays an important role in the loss of neurons and also causing dementia (such as Alzheimer). Production of ß-amyloid, a toxic peptide which is found in Alzheimer patient's brain is major symptoms caused by oxidative stress resulting in neurodegenerative process.

Many other incidents have also been seen in which excess free radicals in humans had adverse affects such as nephropathy, ocular diseases, and retardation in fetus development due to increased concentration of reactive oxygen species or reactive nitrogen species

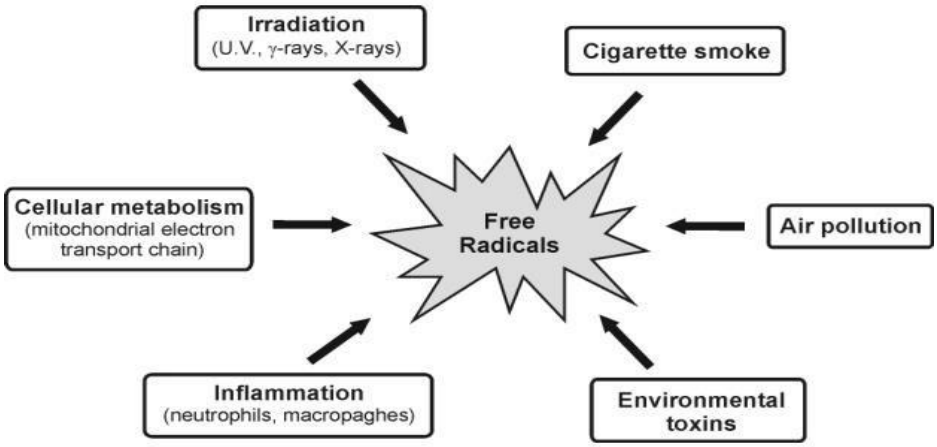

Fig 2: Factors resulting in free radicals production (João P. Silva et al, 2010)

Although, free radicals can have harmful affects yet at the same time they also act beneficial for human system but only if present at low concentration or below critical levels. Certain reactive oxygen species (ROS) and reactive nitrogen species (RNS) are essential for maturation of cellular structure and also take part in immune defence system. Phagocytes such as neutrophill, macrophages and monocytes release free radicals to fight against invading pathogens (Fig 2). Many cellular signalling systems are also triggered by ROS or RNS especially in non-phagocytic cells such as cardiac myocytes, vascular smooth muscle cells and fibroblasts etc.

Antioxidants

To balance out the oxidative stress caused by free radicals antioxidants comes into play. Antioxidants are substances which voluntarily donate electrons to free radicals to neutralize there action and in return antioxidants can readily get oxidised. Antioxidants are produced inside the human system and can also be supplemented exogenously.

There are two classes of antioxidants:

1. Enzymatic antioxidants
2. Non-enzymatic antioxidants

Enzymatic antioxidants

- Superoxide dismutase (SOD)- It is a class of enzymatic antioxidants which catalyze dissociation of superoxide anion into hydrogen peroxide and oxygen. Many isoforms of SOD's are present in plants and animals. These isoforms of SOD are majorly classified on the basis of the type of the metal cofactor associated with them. In plants Mn-SOD is present in peroxisomes as well as chloroplasts and CuZn-SOD is present in cytosol, chloroplast and apoplast. Where as in humans, chordates and other mammals various forms of SOD are distributed throughout their system such as SOD1 (with reactive centres copper and zinc) in cytoplasm, SOD2 (reactive centre is manganese) in mitochondria and SOD3 (reactive centres is copper and zinc) is present in extracellular region.
- Catalase – It is one of the prominent enzymes present in human system which results in decomposition of hydrogen peroxide into water and hydrogen. It is largely present in peroxisomes.
- Glutathione – it is a class of enzymes including glutathione reductase, glutathione S transferase, glutathione peroxidises. These enzymes have selenium as cofactors and they help in detoxification metabolism of hydrogen peroxide and organic peroxides.

Non-enzymatic antioxidants

- Ascorbic acid – also called vitamin C in both plants and animals, Humans do not have the capability to synthesize this endogenously. Therefore, it is to be supplemented by diet. Ascorbic acid being a reducing agent, can neutralise ROS.
- Melatonin- also known as N-acetyl-5 methoxytryptamine is a powerful antioxidant which has the capability to cross the blood brain barrier. It is also called suicidal or terminal antioxidant because upon oxidising with free radicals it does not have any capability to undergo into its reduced state since it forms stable end products.
- Tocopherols and tocotrienols- also called vitamin E specifically α-tocopherols have great usage in protecting the membrane against free radicals attack since it is a lipid soluble antioxidant, it neutralises free radicals produced in membrane due to lipid peroxidation.

A wide variety of plants have been studied for new source of natural antioxidants, especially phenolic and flavanoid compounds derived from plants were proven to be effective free

radical scavengers. Endophytic fungi are microorganisms concealed within healthy host plant were a less familiar group of microorganism and they are less studied till now. They are a dependable source of novel bioactive compounds which can be used and exploited in various fields for agricultural, medicinal and industrial purposes. Depending upon today's scenario of increasing cases of disease resistance and introduction of emerging new diseases we need a more profound focus of what should be the various targets to encounter the disease's mechanism in order to terminate their action. Endophytic fungi have a capability to produce novel products and can also produce the same products as the plants in which it is residing.

There are many reports which showed that endophytic fungi have varied bioactive potential such as anti-cancer, antioxidants, antimicrobial, and anti-inflammatory due to the production of various chemical species such as alkaloids, terpenoids, steroids, flavanoids, phenolics and many more.

Further endophytic fungi induce various advantages in plants such as enhanced secretion of plant growth stimulants, increased disease resistance and profound ability to withstand adverse environmental stress. One of the most famous examples of compounds produced by endophytic fungi is "Taxol" isolated from *Taxus brevifolia.* Taxol production by fungal fermentation is a cheaper method (Page *et al* al , 1999). There are more than 15 genera of fungi having the capability to produce paclitaxel as well as it's analogues.

Plants used for the isolation of endophytic fungi was Giloy (*Tinospora cordifolia)* and Gum tree (*Eucalyptus terreticornis)* and they have notable medicinal applications and potent antioxidant properties. The main objective of the project is antioxidant screening of crude fungal extracts obtained from the isolated endophytic fungi and fractionation of crude fungal extracts along with the antioxidant assay of the eluted fractions.

Objective

- Isolation and screening of endophytic fungi from medicinal plant having antioxidant activity.
- Separation and antioxidant assay of fractions.
- Identification of bioactive molecules.

CHAPTER-2 REVIEW OF LITERATURE

Free radicals and antioxidants in disease and health

Free radicals have dual role they can be both harmful as well as beneficial. Free radicals are known to be having a major affect in cellular signalling and immune response. Hence, it's safe to say that free radicals at low concentration are vital for human system health. They are formed by both intracellular process (during normal cell metabolism) and exogenous sources (smoking, pollution, radiation) (Lien Ai Pham-Huy *et al*, 2008). Endogenous factors like ageing, inflammation, ischemia, immune cell activation and excessive stress can also result in free radical formation. Once the concentration of free radical increases to an extent creating an imbalance with the level of antioxidant a condition of "oxidative stress" is induced in our body. This condition results in deleterious effects such as oxidation of lipid, proteins, nucleic acid, therefore, render permanent damage to cell and its components. Due to the multiple implications of diseases caused by free radicals imbalance, we turn to antioxidant therapy for help since it provides a promising avenue in the near future, though much debate is still in action regarding antioxidant supplementation and disease prevention.

Oxidative damage caused by free radicals

Once there is an imbalance introduced among free radicals and antioxidants resulting in the increase in levels of free radicals it induces a state called oxidative stress. The state of oxidative stress can result in serious oxidative damage (Lobo et al, 2010) by damaging various important bio- molecules such as nucleic acid, proteins.

Lobo et al (2010) discussed DNA susceptible to oxidative damage is a major cause of ageing and cancer and also concluded that mitochondrial DNA is more prone to oxidative damage.

Further they also explained that certain amino acids such as methonine, cystein, arginine and histidine are more prone to oxidative damage. Oxidative stress on proteins effect the functional property of main components such as enzymes, receptors which can hamper membrane transport, cellular functions and cell membrane and signal transduction pathway.

Physiological roles of free radicals

Imbalance between free radicals and body's antioxidant levels is usually counteracted by enzymatic antioxidants (glutathione peroxidase, superoxide dismutase, catalase) and non-enzymatic antioxidants (glutathione, thioredoxin, conenzyme Q). Free radicals play a dual role in our body (João P. Silva et al, 2010) as these species are strictly regulated to be maintained at lower and measurable concentration. João P. Silva reported that each cell is characterised by the concentration of electron in the cellular constituents which is also known as the redox state of the cells.

When there is a sudden increase in free radicals concentration the redox state of cell shifts towards oxidising state. Free radicals are involved in various signalling pathway and free radical production during a immune response results in body's first line of defence. Certain reactive species such as Reactive oxygen species (ROS) is produced by isoform of NAD(P)H oxidase during phagocytic response by the macrophages. On the other hand ROS also seem to be involved in the cell adhesion mechanism which results in various important processes such as embryogenesis, cell differentiation, cell growth, and also wound healing.

Endophytes as bioactive resource

De bary introduced the term endophyte in year 1866 which means any organism that resides inside the plants tissue. There is an abundance of microorganisms in our ecological niche. Various regions such as marine environment, thermal vents, and deserts are densely populated with microorganisms of various characteristics. After decades of research it has been made sure that plants act as major reservoir for untold organisms (Strobel, 2003). These microorganisms can be bacteria or fungi, resulting in production of various novel bioactive compounds which may or may not be beneficial to plant. Exploitation of this unique potential of endophytes has proven to be a boon and revolutionary in medicine and agricultural industry. It is inevitable that all the plants species showing production of any bioactive compound is a host to at least one endophyte. Many Endophytes produces pharmacologically important compounds such as Javanicin from *Chloridium spp.,* Munumbicins from *Allamanda spp.* show antibacterial activity against *Pseudomonas spp* (Nithya and Muthumary, 2011), phenolic comopounds , dihydroxynapthol from *Xylaria spp* showing activity against *Streptococcus pyrogens* and *Herpes virus*, cajanol showing prominent anticancer activity One of the major anticancer drug also known as the blockbuster drug called Paclitaxal (Taxol) it was obtained from the inner bark of a tree called *Taxus brevifolia*

Once introduced in the market it was sure that soon there will be a shortage of this drug due to it increase demand, therefore,research was conducted to screen out microorganism producing this drug (Kayser et al, 2007).

Certain endophytes also show anti-viral activity but this area of research is still in its infancy because of absence of optimised antiviral screening protocols or system in the drug discovery programmes. However, there still has been positive development in this field by the isolation of human cytomegalovirus protease inhibitors (Cytonic acid A and B) from *Cytonaema spp* residing in *Quercus spp*. (Kayser et al, 2007).

Endophyte host plant relationship

An endophytic fungus infects the host plant and does not cause any visible symptoms (Petrini 1991). Extensive amount of studies are being in action now a days to understand the complexity of endophyte interaction with their host plant.

Hye Seung yoo et al (2017) did an analysis to understand this relationship, their study were the first to opt for a hypothetical approach to evaluate the influence of host plant enzyme production on endophytic fungi and their frequency of colonization. The study showed increase in enzyme production by host plant hampers the growth of endophytic fungi to an extent but does not destroy it completely. It also concluded an interesting fact that although host plant defence mechanism restricts endophytic fungi growth yet it cannot influence the antifungal capability of endophytic fungi despite its poor growth.

Zabalgogeazcoa.I (2008) explained in a diverse manner how fungal endophytes and their secondary metabolites production are beneficial to host plant. Since, fungal endophytes are known to produce many secondary metabolites (such as alkaloids, terpenoids, phenolic compounds, flavanoids etc) which also enhances host plant characteristics by providing resistance to nematodes, viruses and other bacteria and fungi and it further increase survival chance of its host plant.

Regina S. Redman et al (2001) explained that although lifestyle chosen by fungal endophytes depends somewhat upon physiological make up of plant yet it is still unclear if it is genetically predetermined for fungal endophytes to act as a symbiont of pathogen in its life cycle spent inside the host plant.

Antioxidants related with endophytic fungi

Majority of the plants in an ecological niche are inhabitant of at least one endophytic fungi and a great amount of work has been conducted to know about the antimicrobial, anticancer and even antiviral capability of endophytic fungi but a little is known about the antioxidant potential (Yadav et al, 2014).

Most of the medicinal plants existing in nature are home to diverse number of fungal endophytes. Antioxidants have been proven to be a promising treatment for various neurodegenerative diseases (Parkisons and Alzheimer's) as well as other terminal diseases such as cancer and heart related problems.

Manila yadav et al (2014) studied antioxidant potential of endophytic fungi isolated from *Eugenia jambolana* commonly known as Jamun various assays were performed in order to conclude presence of phenolic and flavanoid compounds. Further total phenolic and flavnoid content was also checked in order to confirm antioxidant potential.

Jin long cui et al (2015) reported a study about antioxidant potential of fungal endophytes isolated from an endangered alpine plant *Rhodalia spp*. It was the first report comprising antioxidant potential about *Rhodalia* plants. From the HPLC analysis it was suggested that the two fungal strains showing maximum antioxidant activity were producing compounds salidroside and p-tyrosol respectively. These compounds were also the two main active compounds produced by *Rhodalia* plant.

Medicinal plants as a potential source of endophytes and other beneficial bioactive compounds

Since ancient times medicinal plants have been the most prominent source of new medicines. With the increase in disease resistance microorganisms and introduction of new type of diseases there is a desperate need of bioactive compounds which can serve as the new lead in pharmacological industry.

Main examples of drugs obtained from the medicinal plant are paclitaxal an anti-cancer drug isolated from a tree called *Taxus brevifolia*. Artemisinin which is an anti-malarial drug isolated from *Artemessia annua*, quinine which is another anti-malarial drug extracted from bark of *Cinchona officinalis* are among the few drugs which are obtained from medicinal plants and till date are the most frequently and popularly used in medicine.

Since it is widely observed that fungal endophytes have a potential to produce same compounds as their host plants there has been an increase interest in isolation of fungal endophytes from medicinal plants to meet the increasing demand in the field of medicine. It is relatively easy to perform an upscale fermentation on fungi and reuse it for longer period as well as to a certain extent preserves its characteristics and also It also protects the plant from getting extinct.

Tinospora cordifolia also known as Giloy or Gudduchi (Table 2) are a medicinal plant which is well known for its antioxidants and antimicrobial activity. Upadhyay et al (2002) studied antioxidant potential of *Tinospora cordifolia* and concluded that there is a major abundance of phenolic compounds in the plant which is responsible for its significant antioxidant activity.

Another plant which is of great interest is *Eucalyptus tereticornis* also called Gum tree or *Eucalyptus* (Table 2) is well known for anti-bacterial and anti-oxidant activity. Essential oils produced by eucalyptus tend to have many therapeutic benefits. Kaur et al (2011) analysed essential oil extracted from *Eucalyptus tereticornis* for its antifungal and antioxidant property. Through GC-MS analysis it was concluded that terpenes were present in abundance in essential oil and the main components were α-pinene and 1,8-cineole .

Table 2: Classification of *Eucalyptus* and Giloy

CLASSIFICATION	*EUCALYPTUS*	GILOY
Class	Dicotyledons	Magnoliopsida
Order	Myrtales	Ranunculaceae
Family	Myrtaceae	Menispermaceae
Genus	*Eucalyptus*	*Tinospora*
Species	*tereticornis*	*cordifloica*

As already mentioned in order to prevent extinction of medicinal plants due to their increasing demand there has been keen interest in endophytes present inside the medicinal plants. Although a lot of study has been done on fungal endophytes present inside Giloy plant yet very little is known about bioactive potential of endophytes residing inside *Eucalyptus*.

CHAPTER-3 MATERIAL AND METHODS

Material, instruments and chemicals

Materials used in this project were petri plates, flasks, test tubes, TLC plates, TLC stands, TLC applicator, TLC chromatographic chamber, 96 –well titre plates, auto pipettes (0.5 to 10µl) , BOD incubator (27°C), Laminar air hood, spectrophotometer, autoclave, MultiSkan (ELISA plate reader), rotary-evaporator, rota flasks, centrifuge, mini-centrifuge, vortex.

Chemicals used – Sodium hypochlorite (NaOCl), Milli-Q water, Ethanol (70%), ethyl acetate, methanol, DPPH, ascorbic acid, hexane, toluene, chloroform.

Media- Potato dextrose agar (PDA), Potato dextrose broth (PDB).

Source of Endophytic fungi

Two plants were selected for the isolation of endophytic fungi. Sample collection for *Eucalyptus* and Giloy was done at TIET. Sample excised were leaves, inner bark, and stem. They were transferred into sterile glass jars and were brought back to TIFAC-CORE laboratory.

Surface sterilization of collected samples

Samples collected were brought to TIFAC-CORE in glass jars for further processing. Excised plant parts stored in glass jars were covered with a mesh and were left under running water for a period of 10 minutes for the removal of debris. After this detergent was added and jars were shaken properly for proper cleaning of samples and were kept under running tap water for another 10 minutes for removal of traces of detergent. Cleaned samples were sterilized further in 70% ethanol. Sodium hypochlorite (NaOCl) was added and left for 5 minutes for disinfection purpose and another washing was performed with 70% ethanol. Finally three washings were given using deionised water in the laminar air flow.

Isolation of endophytic fungi from the plant sample

For isolation of endophytic fungi following steps were performed in laminar air flow. Plant pieces which were previously surface sterilized were dried on autoclaved tissue paper and cut upto 1 cm in length with sterile blade (Kumaresan et al, 2015). Samples were then inoculated on freshly made PDA plates supplemented with an antibiotic i.e Chloramphenicol (35µg/ml). After inoculation the plates were incubated for 7 to 14 days at 25°C ± 2°C and were checked regularly after every 2 to 3 days for the growth of endophytic fungi. Once the fungi were isolated they were given a proper code and preserved at 4°C.

Sub-culturing of isolated endophytic fungi

Colony morphology of isolated fungi was checked and was sub-cultured continuously for purification. For sub-culturing of isolated fungi, 1cm² of discs of mycelia grown on PDA plate were excised and inoculated on freshly made PDA plates carefully in order to prevent dispersal of spores. Plates were then incubated for next 7 days at 25°C± 2°C.

Preservation of master cultures was done by inoculation of fungal cultures on PDA slant and stored at 4°C.

Fermentation of isolated endophytic fungi

250 ml of PDB was made in 500ml of Erlenmeyer's flasks and was autoclaved. 1cm² discs were cut from the isolated fungal culture and inoculated in the PDB. Around two to three discs were added to the medium. It was kept for incubation at 25°C±2°Cunder static condition for 21 days. The purpose of incubation at static condition is to allow adequate production of secondary metabolites.

Extraction of crude extract from fungal broth

After 15 to 21 days of incubation, filtration was performed by using sterile muslin cloth and funnel. Filtrate was collected in sterile Erlenmeyer's flask and equal amount of ethyl acetate was added. Solvent extraction procedure was performed by taking filtrate and ethyl acetate in separating funnel and shaking it continuously for 15 to 20 minutes. Later, it was allowed to stand still until organic and inorganic phases were completely separated. Organic phase was collected in sterile flasks and Sodium sulphate was added if required for removal of water. Organic phases were further subjected to Rotary-evaporator. Evaporation was done at 32°C and at 35 to 50 RPM. The condensation temperature was 4°C. Dried Crude extracts were

collected in small glass vials and were stored for further testing of their biological activities. This extraction procedure was performed for all the filtered cultures.

Antioxidant screening through DPPH radical scavenging assay

This assay is performed in 96-well titre plate. DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a commercially available nitrogen containing radical. It is widely used to measure the intensity of compounds, which are radical scavengers. DPPH gives violet colour when in methanol. Once encountered by an antioxidant it gets stabilised by electron donation resulting in the change of colour from violet to yellow due to production of reduced product i.e diphenylpicrylhydrazine (Fig 3).

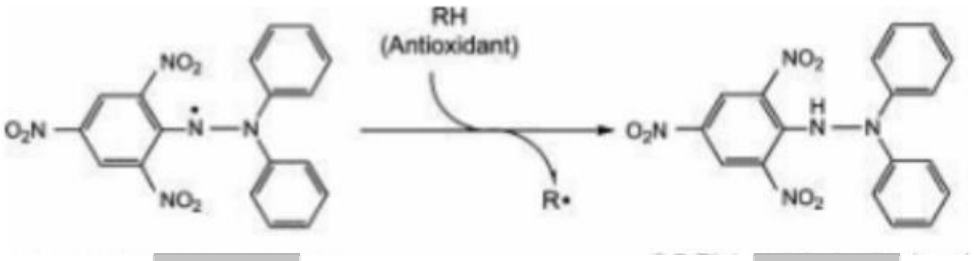

Fig 3: DPPH getting stabilised by accepting an electron from antioxidant and getting converted into its reduced form (J.lewis, 2012).

Fungal extracts were dissolved in methanol to make concentrations ranging from 100µg/ml to 1000µg/ml and further mixed with methanolic solution of DPPH (6mg/ml). Methanol was used as blank. Controls used in this assay were:

1. Ascorbic acid control (standard antioxidant control) at 6mg/ml
2. Ascorbic acid control (water is dissolved in place of ascorbic acid in DPPH methanolic solution)
3. DPPH methanolic solution without the extracts

Once the experiment was conducted in the 96 well plate it was kept in dark for incubation i.e 45 minutes. On completion of incubation, reduction of DPPH radicals was determined by measuring absorbance in microplate reader at wavelength 517 nm.

$$\text{Percentage scavenging activity} = \frac{(\text{Control's Absorbance} - \text{Extract's Absorbance})}{\text{Control's Absorbance}} \times 100$$

A template was designed at the start of the assay of 96 well plates for performance of the experiment at different concentrations:

M: Methanol i.e (200µl)

MD: Methanol (50 µl) +DPPH (150 µl)

AAS: Ascorbic acid (20 µl) + DPPH (150 µl) + Methanol (30 µl)

AAC: Distilled water (20 µl) + DPPH (150 µl) + Methanol (30 µl)

Table 3: Volume of each fungal extract in the well

Sample Concentration (µg/ml)	Total volume (µl)	Fungal extracts(µl)	Methanol(µl)	DPPH (µl)
100	200	4	46	150
250	200	10	40	150
500	200	20	30	150
1000	200	40	10	150

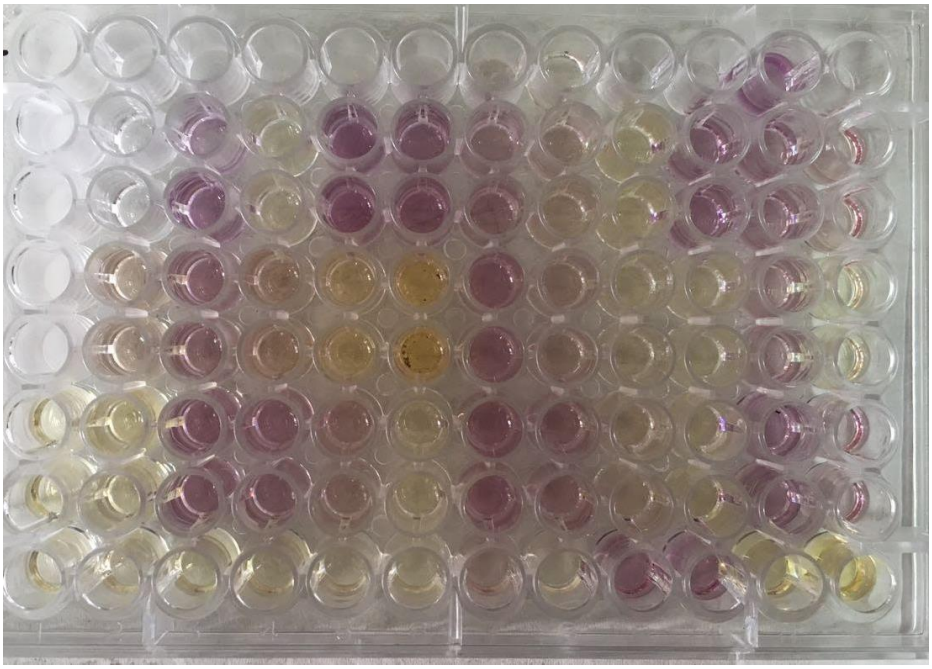

Fig 4: DPPH assay of crude fungal extract.

Estimation of Total Phenol Content (TPC) of fungal extracts

Total phenolic content measurement was done by using FolinCiocalteu reagent based assay. Gallic acid was used as standard (Yadav et al 2014). Added 1ml of extract into 10ml of deionised water, followed by addition of 1ml of Folin-Ciocalteu freshly prepared reagent.

5 minutes later, 20% of sodium carbonate (2.0ml) was added to the mixture. The mixture was incubated at room temperature in darkness for 30 minutes. Absorbance was then measured at 765 nm using UV-VIS spectrophotometer.

Same protocol was followed for Gallic acid calibration standard (50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml, 300µg/ml, 350µg/ml, and 400µg/ml). Total phenolic content value was obtained from regression equation y=0.003x—0.116 and R^2 value is 0.992.

$$\text{Total phenol content} = \frac{\text{Concentration of gallic acid equivalence x volume of fungal extract}}{\text{Weight of fungal extract}}$$

Estimation of Total Flavanoid Content (TFC) of fungal extract

Total flavanoid content was measured using the method by Azieana et al 2017. Quercetin was used as standard. Diluted 1ml of fungal extract with 4ml of distilled water and to it 5% of Sodium nitrate ($NaNO_2$) was added. After 5 to 6 minutes 10% aluminium chloride ($AlCl_3$) (w/w) was added. The mixture was further incubated for 6 minutes and 1.0 M of sodium hydroxide (NaOH) was added. Absorbance was then measured at 510 nm.

Same protocol was followed for Quercetin calibration standard (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml, 600µg/ml, 700µg/ml, 800µg/ml, and 900µg/ml). Total flavanoid content was obtained from regression equation y=0.001x+0.027, R^2 value is 0.995.

$$\text{Total flavanoid content} = \frac{\text{Concentration of Quercetin equivalence x volume of fungal extract}}{\text{Weight of fungal extract}}$$

Phytochemical analysis of fungal extracts:

Preliminary phytochemical screening of crude fungal extracts was conducted for the presence of metabolites such as alkaloids, flavanoids, carbohydrates, glycosides, terpenoids, tannins, phenolic, fats and oils.

Test for alkaloids

Wagner's reagent was made by addition of 1.25g of Iodine and 2g of potassium iodide (KI) in distilled water (5ml) and volume was made up to 100 ml. Few drops of Wagner's reagent was added in 1 ml of extract. Formation of reddish precipitate in test tube is an indication of presence of alkaloids.

Test for flavanoids

1 ml of fungal extract was taken and dissolved in diluted sodium hydroxide (NaOH) and 1ml hydrochloric acid. Disappearance of yellow colour and solution turns colourless which shows the presence flavanoids.

Test of terpenoids

2 ml of fungal extract taken was added in 2ml of chloroform. Concentrated hydrochloric acid (HCl) was taken and added to make a layer. Reddish brown colour appearance at the interface indicated the presence of terpenoids.

Test for carbohydrates

Molisch reagent was used for this test which is prepared by addition of 1g of α-napthol in 95% alcohol (60 ml). Few drops of this reagent was added in 1 ml of fungal extract and few drops of concentrated hydrochloric acid (HCl). Purple ring appearance at interface of HCl indicated the presence of carbohydrates.

Test of glycosides

2ml of fungal extract was taken in which glacial acetic acid and one to two drops of 5% ferric chloride ($FeCl_3$) was added and 500µl of concentrated sulphuric acid. Brown ring appearance at the interface showed the presence of glycosides.

Test for tannins and phenolics

5% of ferric chloride (**$FeCl_3$**) was added to 1ml of fungal extract.Appearance of blue colour is an indication of presence of hydrolyzable tannins and if there is dark green colour, it indicates the presence of condensed tannins.

Test for fixed oils and fats

This test is known as the saponification test. 1ml fungal extract was taken and alcoholic potassium hydroxide (KOH) was added following which few drops of phenolphthalein were added. Kept in water bath for heating (1 hour) and formation of soap indicated the presence of fixed oils and fats.

Radical scavenging concentration

An experiment was conducted in order to determine radical scavenging concentration i.e EC_{50} of the fungal extract. EC_{50} basically is defined as the value at which our selected extract shows 50% scavenging activity (Saha et al 2008).

Fungal extract concentrations ranging from 250µg/ml, 300µg/ml, 350µg/ml, 400µg/ml, 450µg/ml, 500µg/ml, 1500µg/ml, and 2000µg/ml were taken to find out EC_{50} value and compared with ascorbic acid which was used as standard antioxidant

Fig 5: Template design of 96 well for radical scavenging activity.

1	2	3	4	5	6	7	8	9	10	11	12
M	MD	AAS	AAC	250	300	350	400	450	500	1500	2000
M	MD	AAS	AAC	250	300	350	400	450	500	1500	2000

Qualitative analysis of fungal extract using thin layer chromatography (TLC)

Qualitative determination of fungal extract of *Eucalyptus* was done by thin layer chromatography. It is a highly elite and a versatile technique for separation of compounds based upon their affinity with the two phases (stationary and mobile phase). Most frequently used TLC stationary phases are silica gel and alumina. Here, we used Silica gel G_{254} for separation and bands were visualised under UV light.

The composition of mobile phase was optimised several times to obtain efficient separation of fungal extract (Table 4). Mobile phase was optimised by mixing solvent of varying polarity.

Table 4: Solvent optimization of selected fungal extract EB

Mobile phase for *Eucalyptus tereticornis*	Percentage	Volume
Pure ethyl acetate	100%	4ml
n-Hexane : ethyl acetate	90%	3 ml+1 ml
n-Hexane: ethyl acetate	90%	1 ml + 3 6ml
n-Hexane: ethyl acetate	30%	1.2ml+2.8ml
Pure n-Hexane	100%	4ml

Another TLC experiment was done for qualitative analysis of flavanoids in fungal extract and the solvent method obtained from Mandal et al 2013 i.e Toulene:chloroform:methanol (4:4:1 ; v/v/v) and the bands were visualised under UV light.

Extraction of compounds was also done by running the selected sample on TLC plate with solvent system of hexane: ethyl acetate (3:1), respective compound fractions were scratched off the plate and dissolved in equal volume of ethyl acetate or methanol and vortexed the mixture for 30 minutes to 1 hour. Following which centrifugation was done for 10 to 15 minutes at 10,000 RPM and supernatant was collected and pellet was discarded, supernatant was stored in open glass vials and kept at 37℃ for evaporation of solvent to obtain purified fractions.

Compounds extracted were further tested for their antioxidant activity by a DPPH assay to check for the fraction is showing the maximum antioxidant activity.

Gas chromatograph mass spectrometry (GC-MS)

Gas chromatograph mass spectrometry is a versatile equipment for analysis of volatile organic compounds. This instrument works on the principle that mixture will be separated once it is heated and mass spectrometer detects the compounds generated at molecular level. Instrument used for analysis was Perkin-Elmer Gas chromatograph and equipped with mass selective detector. Oven temperature was 100℃ and carrier gas was Helium with flow rate of 1ml/min. Injection volume was 1.0µl, length of the column was 25m with diameter of 200µm and ionization energy was 70eV. Compound identification was done by comparing mass spectral data with NIST library.

CHAPTER-4 RESULTS AND DISCUSSION

It is well maintained fact that plants have great therapeutic and medicinal value, and are reservoir of many efficient bioactive metabolites. However, research shows every plant is home to at least one endophytic fungi, which have the ability to produce secondary metabolites and hence are important source for developing new kinds of drugs (Agata et al, 2015).

Isolation of endophytic fungi from plant

In this study, pre-isolated fungal isolates of *Tinospora cordifolia* (Giloy) were taken (GS3,GR3,GS1). Isolation of fungal endophytes was done from *Eucalyptus tereticornis* (Gum tree) (Fig.6) which yielded 5 isolates from bark (EB, EB2, EB3, EB4, EB5) and no isolates were obtained from stem. The fungal endophytes were studied for their antioxidant property and tests were conducted in order to understand the antioxidant potential of the secondary metabolites.

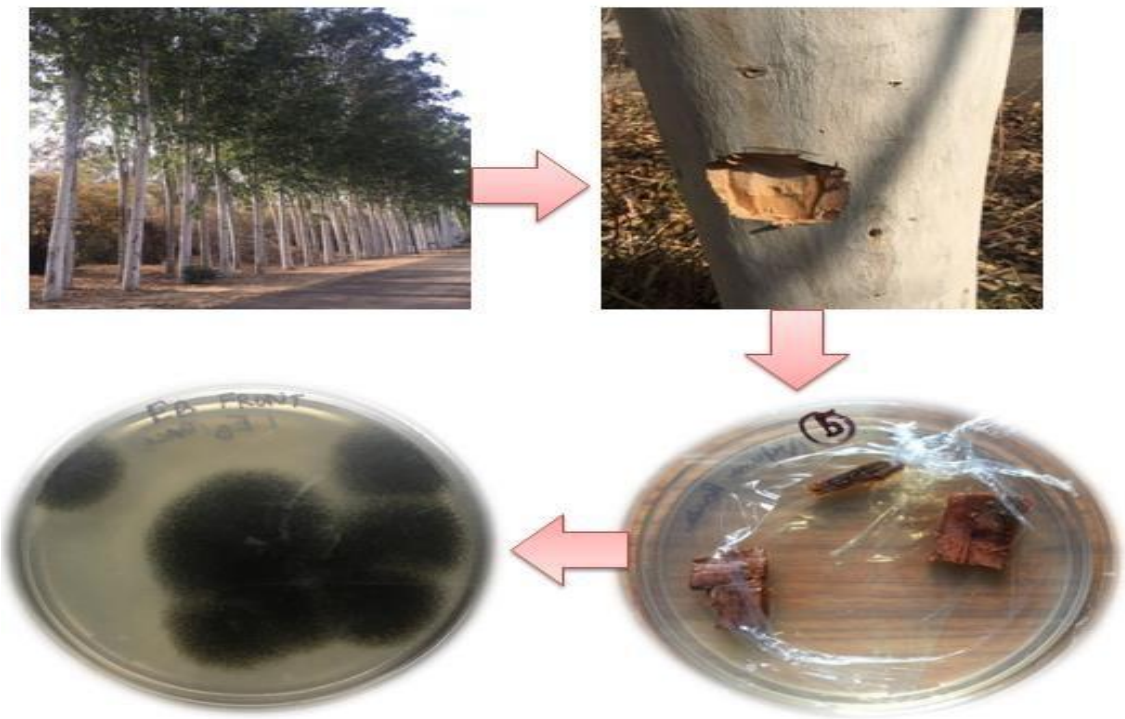

Fig 6: A) Isolation of fungal endophytes from *Eucalyptus terreticornis* **B)** inner bark pieces **C)** Bark pieces was inoculated on PDA plate **D)** Fungus isolated from bark sample.

Extraction of the crude extract from the broth

Isolated fungal endophytes were sub-cultured to get pure fungal cultures and two to three 1cm fungal discs were cut and inoculated in Potato dextrose broth (PDB), which was kept for 21 days incubation at 25 ℃± 2℃under stationary conditions. After the incubation period, the broth was filtered from mycelia following which ethyl acetate extraction of the broth was performed. The organic layer of ethyl acetate containing metabolites was evaporated to get dry residue in rotary evaporator (Fig 7).

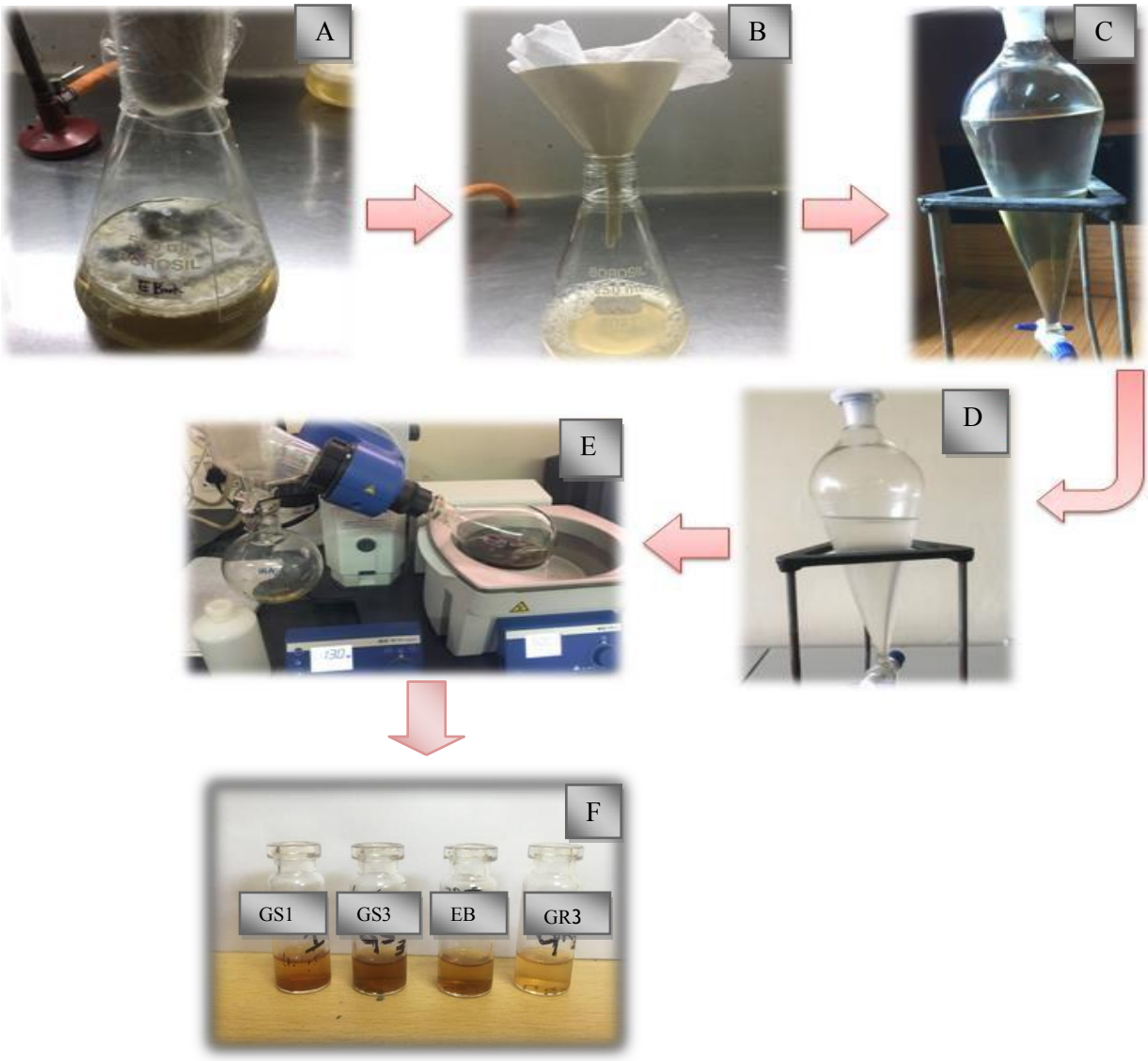

Fig 7: A) Fermented fungal broth, **B)** Filtration of the fungal broth, **C)** Solvent extraction, **D)** Organic phase collection, **E)** Rotary-evaporation of the organic phase **F)** Crude fungal extracts.

Antioxidant screening of fungal extract through DPPH radical scavenging assay

Antioxidant screening was done using DPPH radical scavenging assay in a 96- well titre microplate on fungal extracts of *Eucalyptus tereticornis* and *Tinospora cordifolia*. Among the five isolates (EB, EB2, EB3, EB4, EB5) most consistent activity was shown by EB. Colour change was also observed in the three extracts GS1, GR3, GS3 out of which EB showed the most significant antioxidant activity

Stock concentration of fungal extracts used was 5mg/ml. Control used in DPPH assay was ascorbic acid whose activity was 83.76%.

Based upon the experiment performed in triplicates EB had showed the best radical scavenging activity of 73.6% at a concentration of 1000µg/ml (Fig.8). Hence, it was selected out of the four extracts (GS1, GR3, GS3 and EB for further experimental analysis.

An endophytic fungi isolated from *Eugenia jambolana* showed an antioxidant activity of 80% (Yadav et al, 2014)

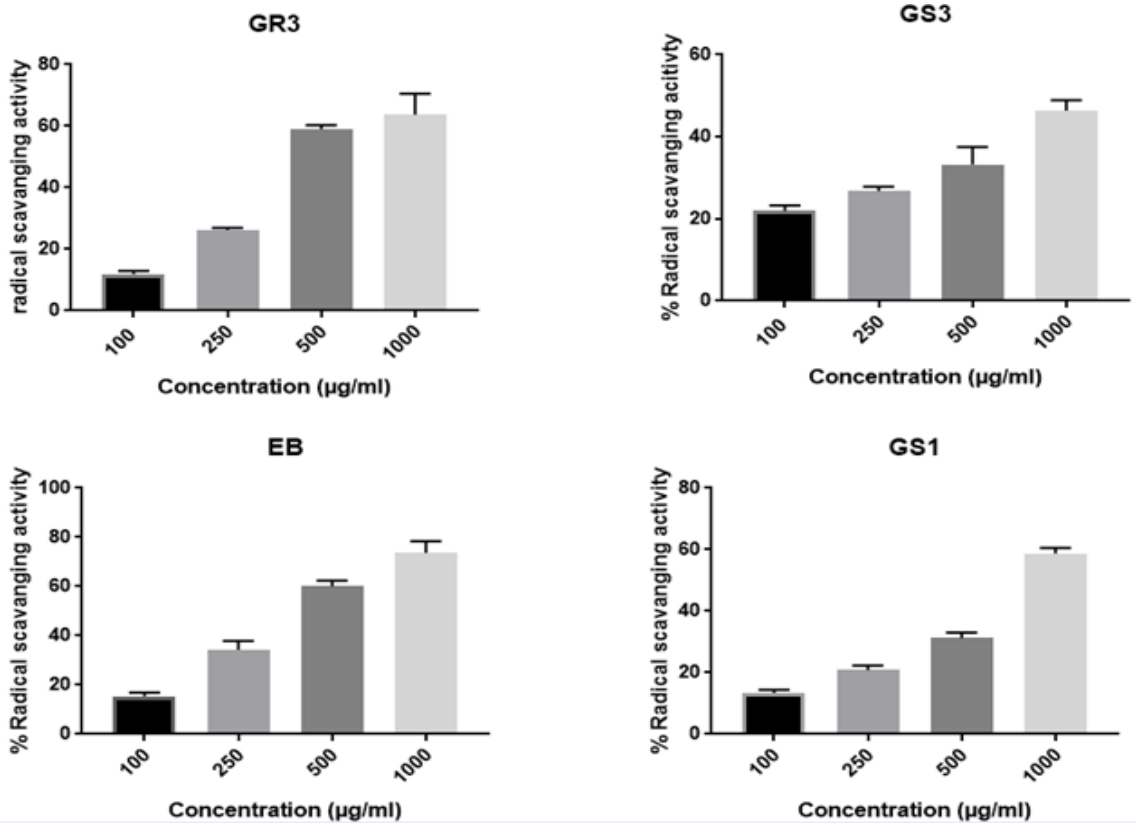

Fig 8: Effect of concentration on antioxidant acitivty of fungal extracts.

Statistical analysis was performed by using two-way ANOVA and a P-value of <0.001 was considered to be significant (Fig 9).

Table 5: Percentage scavenging activity of extracts by DPPH assay.

S.no.	Concentration (µg/ml)	GR3	GS3	EB	GS1
1	100	11.925±0.95	21.915 ±0.62	15.412±1.49	13.403±0.99
2	250	26.263±0.62	26.77±1.10	34.273±3.70	20.853±1.53
3	500	59.04±1.29	33.272±4.26	60.00±2.45	31.24±1.78
4	1000	63.746±6.77	46.385±2.54	73.646±4.71	58.787±1.70

Table 6: Two way Analysis of variance.

S.no.	Source of variations	Sum of squares	Mean sum	Degree of freedom	F (DFn, DFd)
1	Interactions	2073	230.4	9	F (9, 32) = 28.32
2	Concentration	14304	4768	3	F (3, 32) = 586.2
3	Extracts	1770	589.9	3	F (3, 32) = 72.53
4	Residual	260.3	8.134	32	

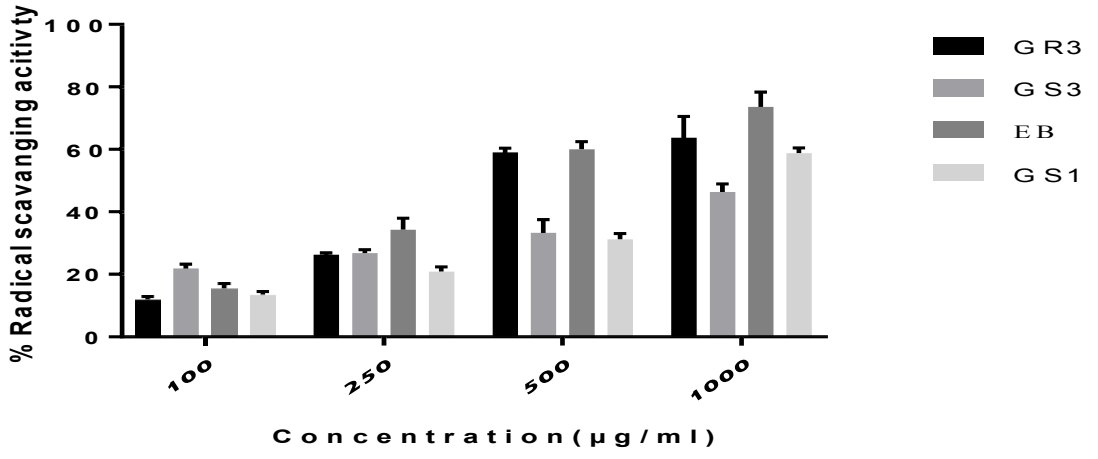

Fig 9: Antioxidant activities of fungal extracts

Total phenolic content (TPC) of fungal extracts

An experiment was conducted to find out total phenolic content of four selected fungal extracts GS1, GR3, GS3, EB to know about the concentration of phenolics present in the crude extract. Gallic acid was used as reference standard (Fig 10).

Table 7: TPC of crude fungal extract.

Crude fungal extracts	**TPC expressed as mg GAE/g dried extract**
GR3	100.4
GS1	114.4
EB	124
GS3	85

Table 8: Absorbance (765nm) of extract and standard.

Concentration (µg/ml)	Absorbance (765nm)
0	0
50	0.087
100	0.124
150	0.360
200	0.451
250	0.601
300	0.782
350	0.976
400	1.112
450	1.226
500	1.336
GR3	0.817
GS1	0.742
EB	0.523
GS3	0.531

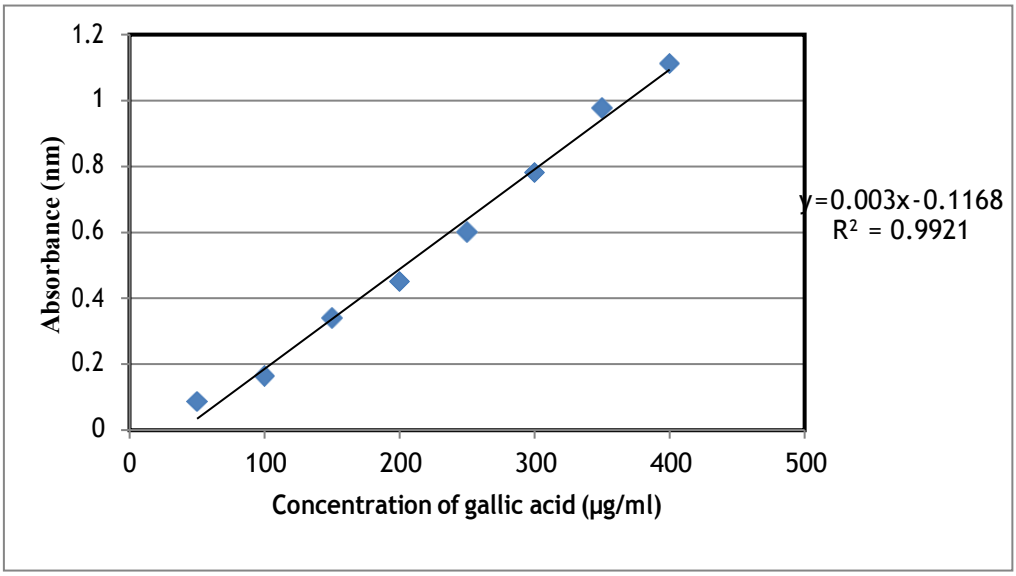

Fig 10: Standard curve of gallic acid

It was observed that highest phenolic concentration was observed in EB i.e 124 mg GAE/g of dried extract followed by lowest in GS3 i.e 85 mg GAE/g of dried extract (Table 7).

Total flavanoid content (TFC) of fungal extracts

To know about the flavanoid content in the crude fungal extracts GS1, GS3, GR3 and EB was determined and quercetin was used as a reference standard (Fig 11).

It was seen that EB showed highest flavanoid concentration i.e 222.4 mg QE/g of dried extract and lowest concentration was seen in GS1 i.e 102.8 mg QE/g of dried extract (Table 9).

Table 9: TFC of crude fungal extracts.

Crude fungal extracts	TFC expressed as mg QE/g of dried extract
GR3	164.8
GS1	102.8
EB	222.4
GS3	116.4

Table 10: Absorbance (510nm) of extract and standard.

Concentration (µg/ml)	Absorbance (510nm)
0	0
100	0.121
200	0.305
300	0.421
400	0.513
500	0.670
600	0.730
700	0.870
800	0.982
900	1.121
1000	1.305
GR3	0.583
GS1	0.318
EB	0.439
GS3	0.284

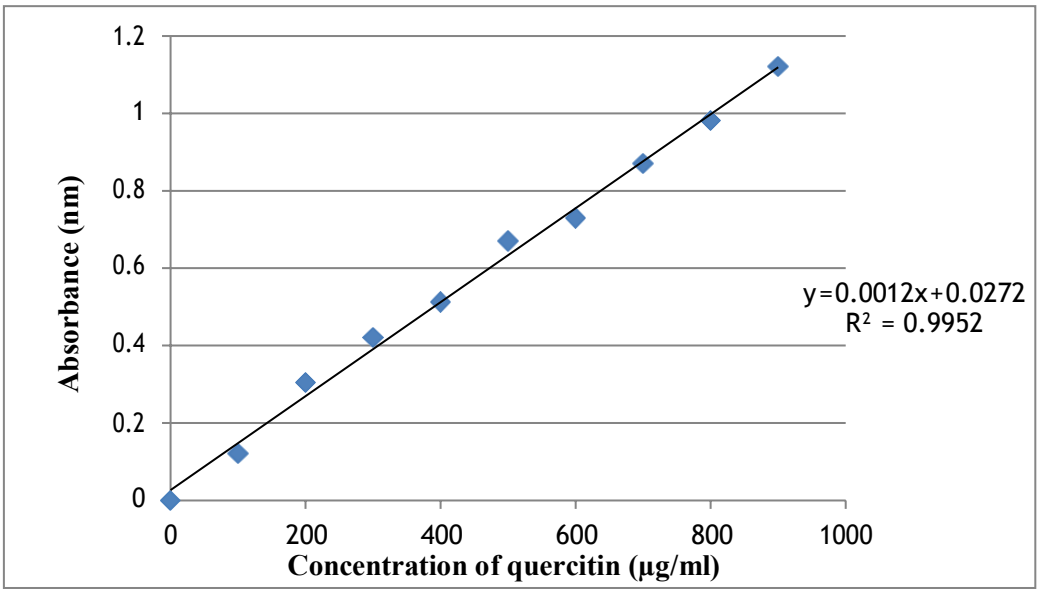

Fig 11: Quercetin standard curve

From the TPC and TFC of extracts it is observed that EB showed maximum flavanoid content i.e 222 mg QE/g dried extract as in comparison to phenolic content i.e 124 mg GAE/g of dried extract. However, EB among the four extracts EB showed highest flavanoid and phenolic content as well.

Phytochemical analysis of the fungal extract

Table 11: Phytochemical results of fungal extract EB.

Test	Fungal extract EB	Inference
Alkaloid	+ + +	Reddish precipitate
Carbohydrates	-	No inference
Flavanoids	+ +	Solution turned colourless
Fats & Fixed oils	-	No inference
Glycosides	+ + +	Brown ring appearance
Tannin and phenolics	+ +	Solution turned green
Terpenoids	+	Solution slightly turned red

(+++) Test firmly positive

(++) Positive test

(+) Weak positive test

(-) Negative test (No appearance of the expected inference)

10mg/ml of crude fungal extract EB was dissolved in methanol and used for phytochemical analysis. EB indicated presence of alkaloids, glycosides and also showed positive test for flavanoids, tannins and phenolics. However, it showed weak positive test for terpenoids and showed no presence of carbohydrates, fats and fixed oils (Table 11).

Minimum scavenging activity

In order to find out radical scavenging activity i.e EC_{50} value of the selected fungal extract (EB), DPPH assay was performed at 250µg/ml, 300µg/ml, 350µg/ml, 380µg/ml, 400µg/ml, 450µg/ml, 500µg/ml, 1500µg/ml, and 2000µg/ml (Fig 13.)

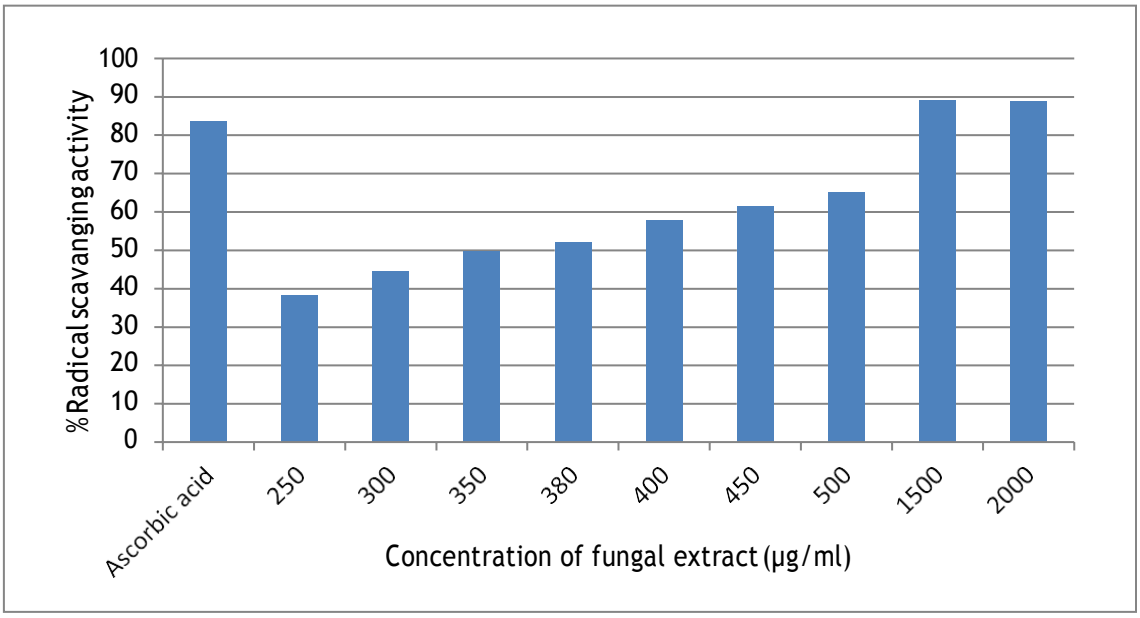

Fig 12: Radical scavenging activities of Fungal extract EB.

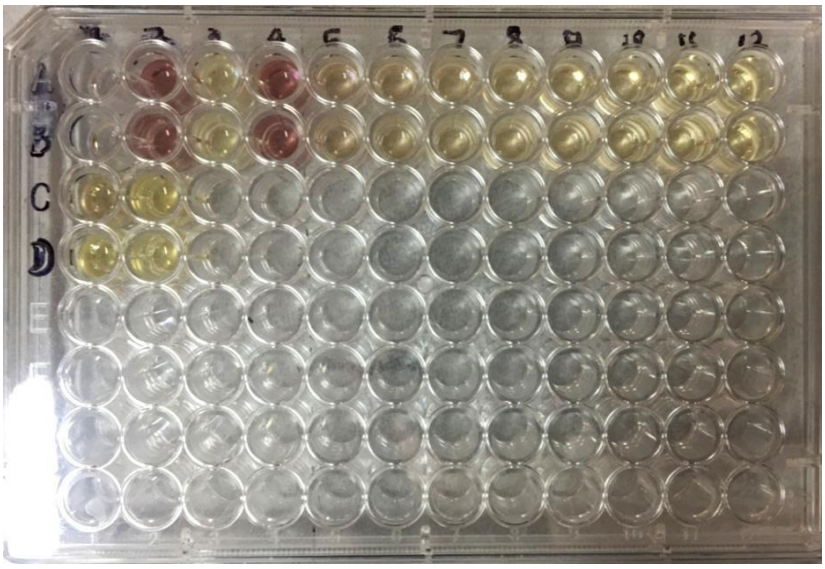

Fig 13: Radical scavenging assay performed by DPPH method

50% scavenging activity was observed at 350µg/ml and 80% activity was observed at 1500µg/ml, and 2000µg/ml. It was seen that after a concentration of 1500µg/ml no further increase in scavenging activity was observed (Fig 12).

Qualitative analysis: Thin layer chromatography (TLC) of crude fungal extract EB.

TLC analysis is a qualitative method and was followed for compound separation on silica Gel G_{254}. Solvent optimization was done using various solvents Two solvents systems, were used for EB Hexane:ethyl acetate (1:3) and another TLC was conducted for separation of flavanoids in crude methanolic extract which is Toluene:chloroform:methanol (4:4:1) (Table 12).

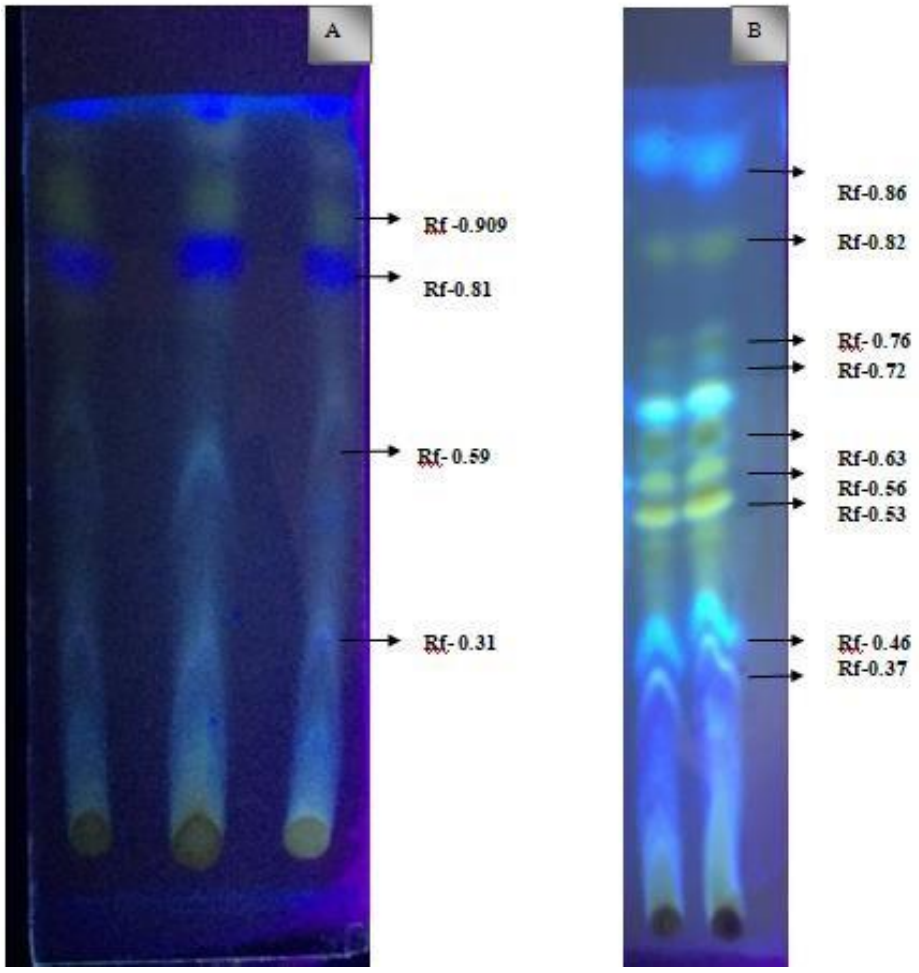

Fig 14: A) TLC analysis using solvent system Hexane:ethyl acetate (1:3) **B)**TLC analysis using solvent system Toulene:chloroform:methanol (4:4:1).

Table 12: R_f value of fungal extract EB

SOLVENT SYSTEM	No. of fractions	R_f VALUE
Pure hexane	0	-
Hexane:ethyl acetate (1:3)	4	0.909
		0.81
		0.59
		0.31
Toulene:chloroform:methanol (4:4:1)	9	0.86
		0.82
		0.76
		0.72
		0.63
		0.56
		0.53
		0.46
		0.37

Separated compounds were scratched of TLC plate which was run in solvent system Hexane:ethyl acetate (1:3) in order to extract compounds, and there antioxidant activity was checked through DPPH scavenging assay(Fig 15).

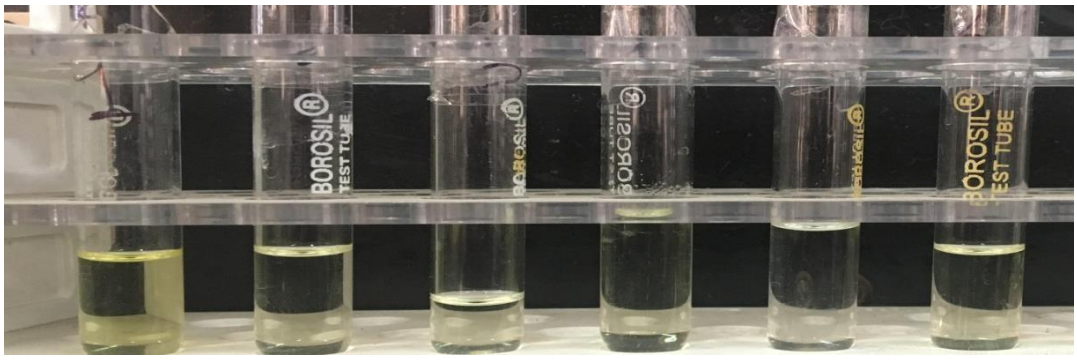

Fig 15: Compounds extracted from TLC plates dissolved in ethyl acetate.

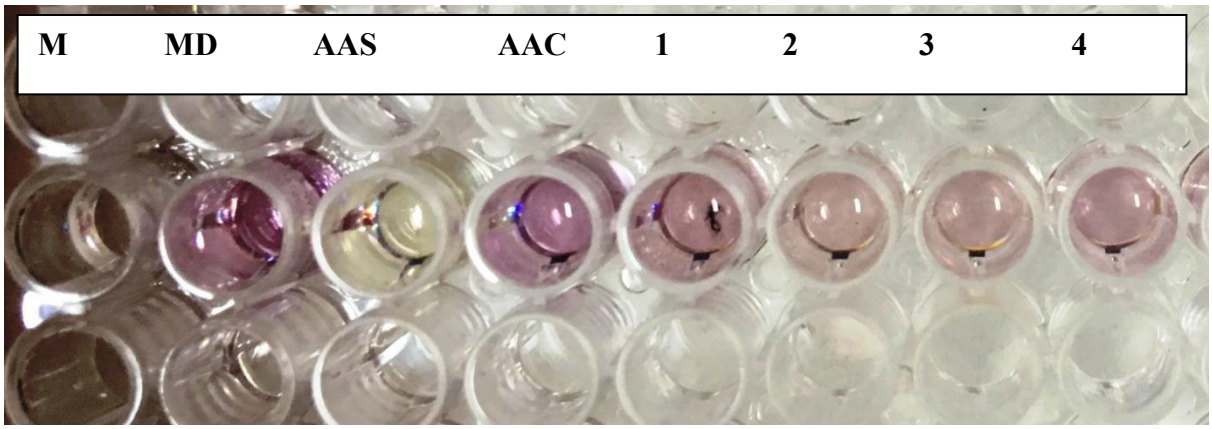

Fig 16: Antioxidant assay (DPPH assay) of extracted compound fraction

All the four fractions showed antioxidant activity, with highest activity of 65.37% shown by fraction 3 at 100µg/ml (Table 13).

Table 13: Antioxidant activity of TLC fractions at 100µg/ml

Fractions	% Scavenging acitivty
1	56.68
2	60.85
3	65.37
4	51.44

4.7 Gas chromatograph mass spectrometry (GC-MS)

Fraction showing the highest antioxidant activity i.e Fraction 3 was characterized for compounds through GC-MS. The spectral data indicated the presence of bioactive compounds (Fig 17). The compounds were analysed and reported with the help of NIST database and reported literature (Table 14).

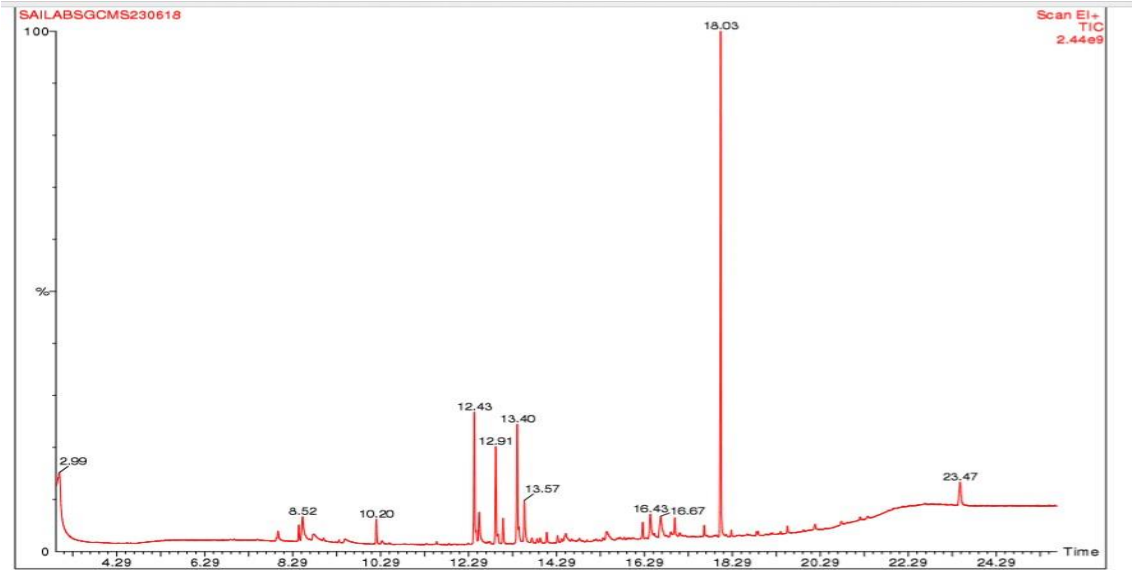

Fig 17: Total ion chromatogram of fraction 3 analysis

Table 14: List of Compounds present in fraction 3.

S.No	Compound name	Reported M.W	Obtained M.W	Biological activity reported
1	Octadecane	254.5	254	Antimicrobial activity (Rasekhi et al,2014)
2	3-tert-butyl-5-chloro-2-hydroxybenzophenone	288.77	288.4	Antioxidant activity (Scott et al, 1979)
3	Phenylseleno-5-methyldihydro-2(3H)-furanone	272.01	272	Anti-inflammatory and anti-bacterial property (Husain et al 2009), antioxidant activity (Sasaki et al 1998)
4	4-amino-1,3-diphenyl-quinolin-2-(1H)-one	312.01	312	Antioxidant activity (Chung et al, 2001),antimicrobial activity
5	1,2Benzenedicraboxylic acid,diisooctyl ester	390.5	389.09	Anticancer activity (Save et al, 2015)

6	Benzaldehyde glycerol acetal	180.2	180	Antioxidant property (Dodson et al, 2014)
7	α-tocopherol	430.7	430	Antioxidant acitivty (Yamauchi,1997)

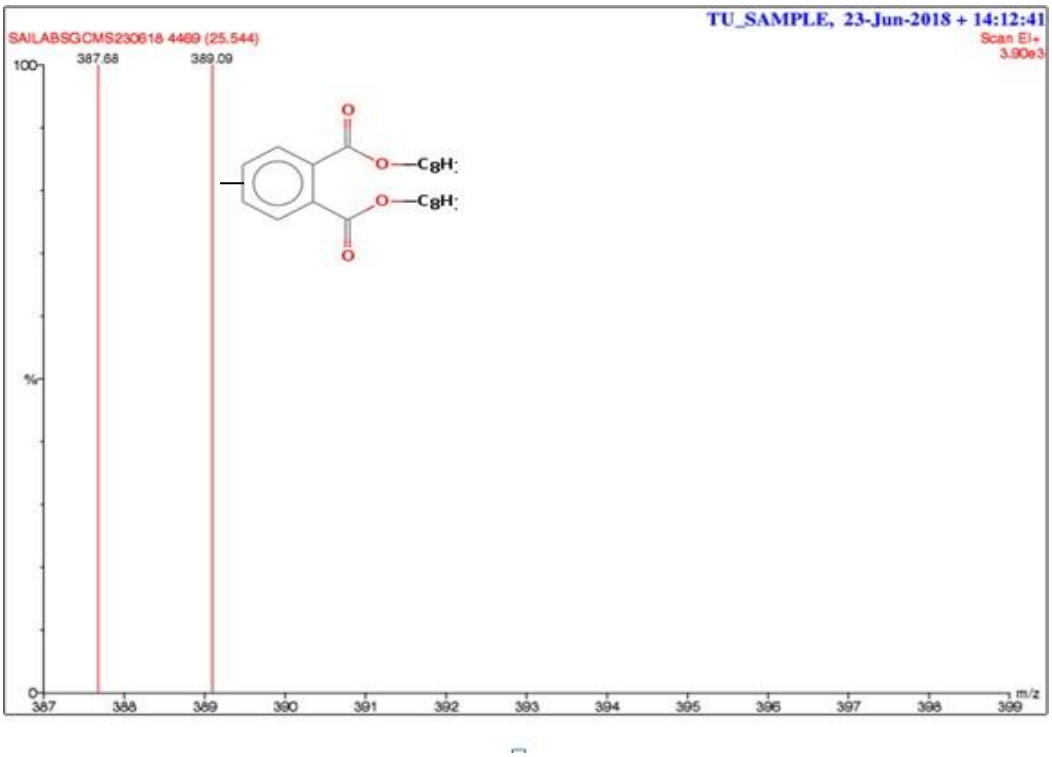

Fig 18:1,2 Benzenedicraboxylic acid,diisooctyl ester showing mass spectra peak at 389.09.

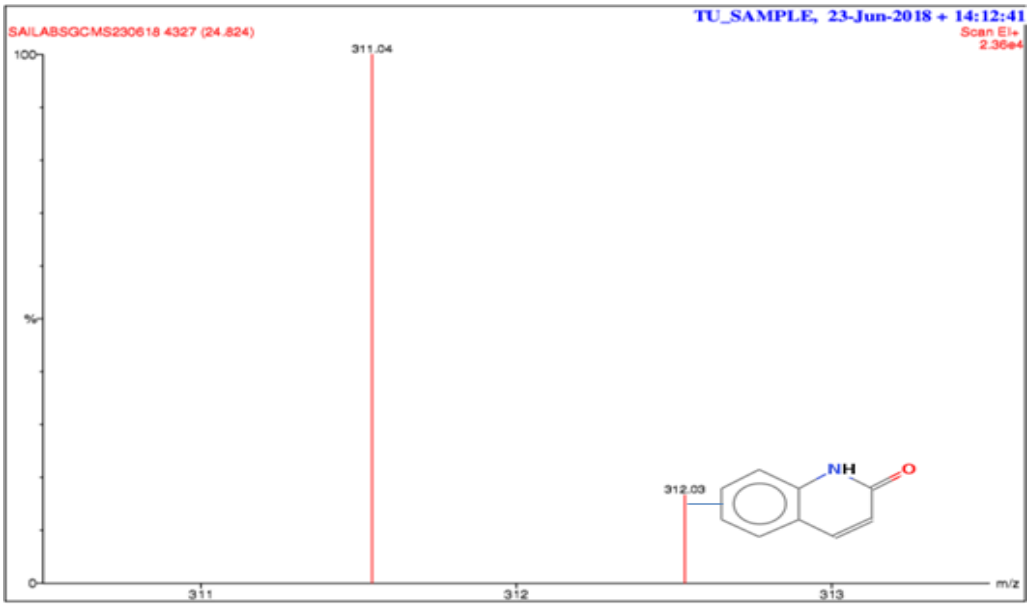

Fig 19: 4-amino-1,3-diphenyl-quinolin-2-(1H)-one showing mass spectra peak at 312.03.

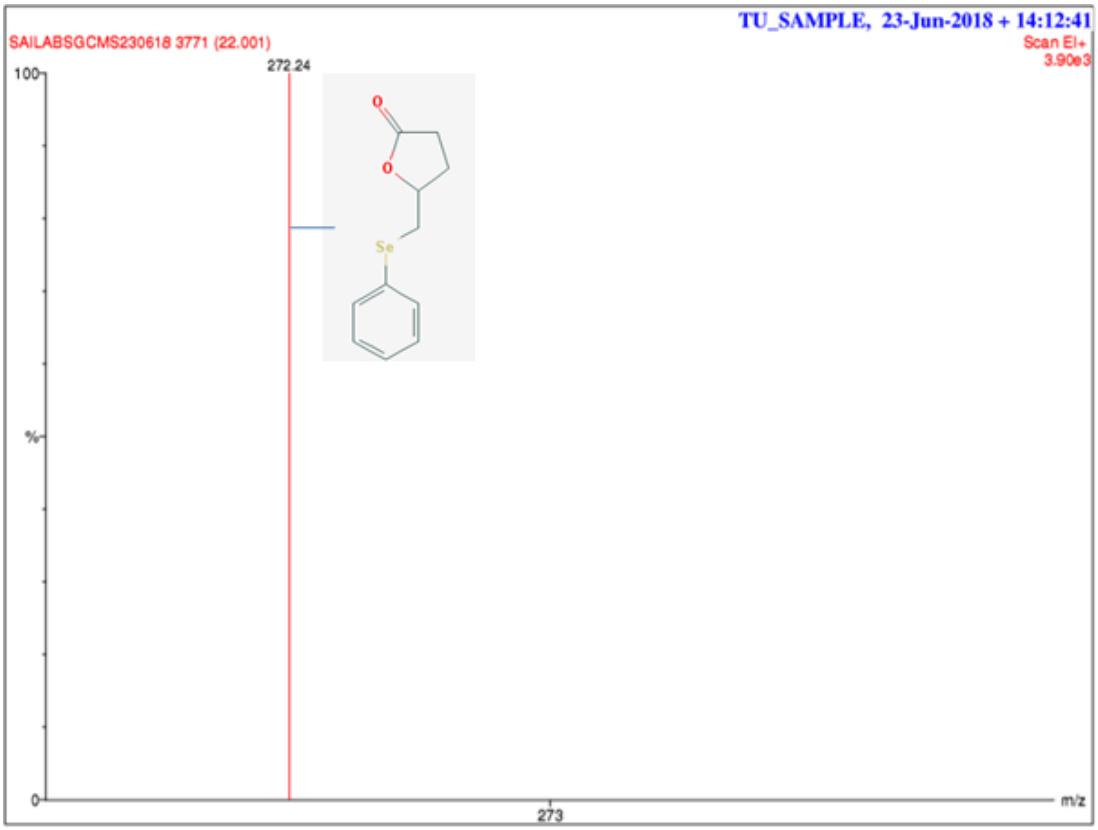

Fig 20: Phenylseleno-5-methyldihydro-2(3H)-furanone showing mass spectra peak at 272.24.

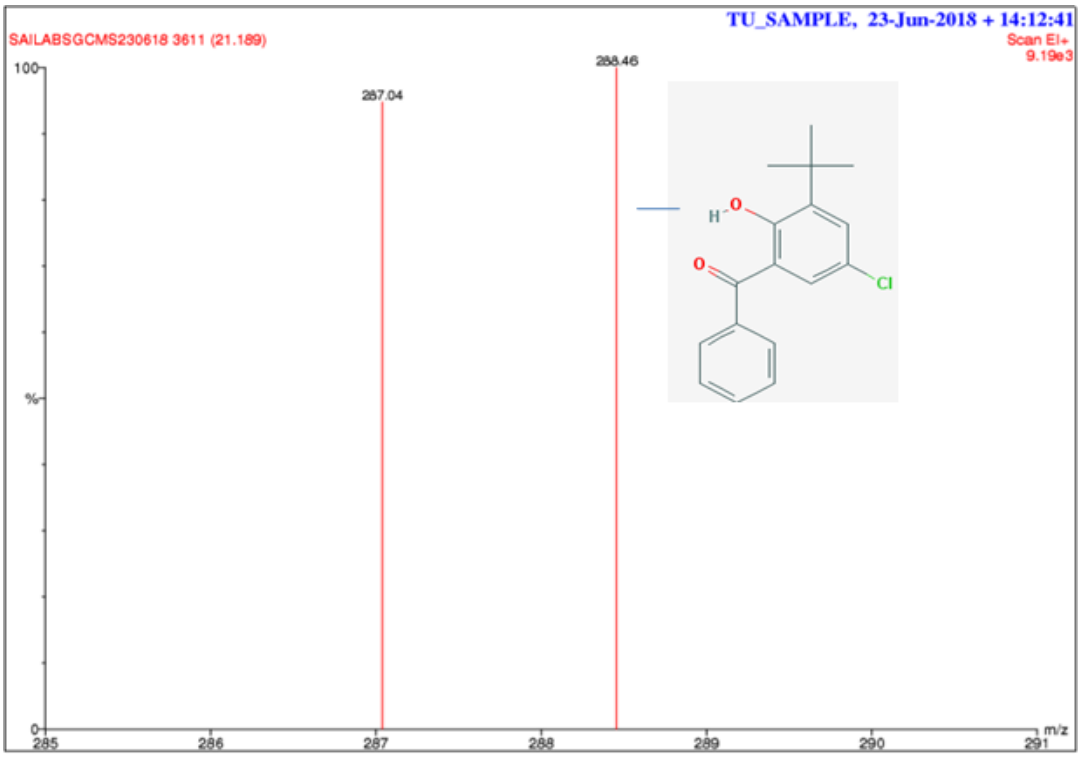

Fig 21: 3-tert-butyl-5-chloro-2-hydroxybenzophenone showing mass spectra peak at 288.46.

GC-MS analysis was conducted on fraction 3 and bioactive compounds were detected (Table 14). Highest retention peak i.e 18.03 was shown by 1,2Benzenedicraboxylic acid,diisooctyl ester(Fig18). Compounds showing various biological activities were detected like 3-tert-butyl-5-chloro-2-hydroxybenzophenone is known to show a good antioxidant activity (Scott et al, 1979), many other classes of compounds which showed prominent antimicrobial activity were also detected such as 4-amino-1,3-diphenyl-quinolin-2-(1H)-one, Phenylseleno-5-methyldihydro-2(3H)-furanone (Husain et al 2009).Analysis also showed presence of fatty acids which have been known to show antimicrobial activity (Rasekhi et al,2014).

Various compounds such as Benzaldehyde glycerol acetal, α-tocopherol, 4-amino-1,3-diphenyl-quinolin-2-(1H)-one were detected as well and are reported to show antioxidant activity along other biological activities as well.

CONCLUSION

Exploitation of this unique potential of endophytes has proven to be a boon and revolutionary in medicine and agricultural industry. It is inevitable that all the plants species showing production of any bioactive compound is a host to at least one endophyte.

There are various studies on the antioxidant activity of fungal endophytes isolated from plants. As a part of the current research towards finding novel therapeutic agents an attempt has been made in this study to screen endophytic fungi showing maximum antioxidant activity and characterization of compound responsible for its activity.

In preliminary screening, fungal isolates obtained were screened for antioxidant activity out of which maximum activity was shown by fungal extract EB i.e 73.6% at 1000µg/ml, total phenolic content and total flavanoid content of the four isolates (GS1, GS3, GR3 and EB) showed that EB has the highest phenolic content i.e 222 mg of QE/g of dried extract and highest flavanoid content of 124 mg of GAE/g of dried extract.

EC_{50} of EB i.e 50% of scavenging activity was observed at a concentration of 350µg/ml and 80% scavenging activity was observed at 1500µg/ml. Qualitative methods such as phytochemical analysis and thin layer chromatography indicated the presence of various compounds. Extraction of TLC fractions yielded four fractions, all the four fractions further gave antioxidant activity out of which third fraction gave the maximum activity 65.3% at 100µg/ml.

GC-MS analysis of the third fraction indicated the presence of bioactive compounds 1,2Benzenedicraboxylic acid,diisooctyl ester, 3-tert-butyl-5-chloro-2-hydroxybenzophenone, 4-amino-1,3-diphenyl-quinolin-2-(1H)-one, Octadecane, Phenylseleno-5-methyldihydro-2(3H)-furanone, Benzaldehyde glycerol acetal and α-tocopherol which may contribute to the extract's antioxidant activity.

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ANNEXURE-1

1. Composition of potato dextrose broth

ingredients	Gram/litre
Potato infusion	200
Dextrose	20

Final Ph 5.1 at 25℃

2. Composition of potato dextrose agar

ingredients	Gram/litre
Potato infusion	200
Dextrose	20
Agar	20

Final Ph 5.1 at 25 ℃

3. Wagner reagent-Dissolve 1.27g iodine and 2g Potassium iodide (KI) in 5ml of distilled water and make final volume upto 100ml

4. Ninhydrine solution – 2g of ninhydrin dissolved in 100ml of aceton

5. Molisch reagent- Dissolve 1g of alpha-napthol in 60ml of 95% alcohol