Chapter 2

2. Materials and Methods

2.1 Isolation of Endophytic fungi

Healthy leaves and twigs were collected from *Eucalyptus globulus* plants grown in the Poornaprajna Institute of Scientific Research (PPISR) (13° 17’N and 77 ° 40’E) campus. The fresh samples were washed under running tap water and sterilized in series with 70% ethanol for 1 min, 4.0 % sodium hypochlorite (NaOCl) (v/v) for 2 min and further cleaned by passing through two sets of sterile distilled water and then placed on Potato dextrose agar (PDA) plates supplemented with 200 mg/L streptomycin to inhibit the growth of bacteria and incubated at room temperature. Replica plating was done by taking some imprints of surface sterilized plant samples on PDA (Nairn and Chanway 2002, Johnston-Monje and Raizada 2011).

The leaves and twigs of *Eucalyptus citriodora* plants were collected from locations near-Poornaprajna Institute of Scientific Research (13° 17’N and 77 ° 40’E) and processed within one hour of their collection. Isolation of endophytic fungi was carried out using the protocol described by Strobel *et al.* with slight modifications (Strobel *et al.* 2004). The healthy plant tissues were washed under running tap water and samples (leaves and twigs) were cut into size of an inch. *Eucalyptus citriodora* samples were surface sterilized sequentially with 70 % alcohol for 90 seconds, 1% sodium hypochlorite for 90 seconds, rinsed twice with sterile water and processed as before. All the plates were incubated at room temperature for fungal growth.

Similarly, endophytic fungi were isolated from fresh leaves and twigs of *Simarouba glauca* collected from the PPISR campus. In brief, leaves and twigs of *Simarouba*
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glaucawere washed with tap water and surface sterilized using 70% ethanol for 1 min and 1% sodium hypochlorite for 2 min, rinsed in sterile water for two times and processed as explained above.

2.1.1 Efficacy of surface sterilization

Surface sterilization was optimized for each plants species to obtain only endophytic fungi, the plant tissues of all the three selected plants were surface sterilized with different ratios of ethanol and sodium hypochlorite for varied time and final method is explained above.

2.1.2 Storage of stock cultures

The isolated fungal strains were preserved by three different methods.

A. The actively growing endophytic fungal isolate in PDA was taken and 4-5 fungal discs were punched using a sterile cork borer. All the discs were stored in sterile Milli-Q water and stored in 4°C.

B. Each fungal isolate was placed in sterile cryo vials containing 10% sterile glycerol and the vials were stored in liquid nitrogen (Espinel-Ingroff et al. 2004, Homolka 2013, Baker and Jeffries 2006).

C. PDA slants were made in culture tubes and each fungal isolate were placed on agar slants and allowed to grow for 3-4 days. Then the tubes were stored in 4°C. The viability of preserved culture was retrieved by sub culturing them into new media every month (Abd-Elsalam et al. 2010, Diogo et al. 2005, McGinnis et al. 1974).

2.2 Pathogens used in the study
Clinically important pathogenic microorganisms were used for in-vitro antimicrobial screening of endophytic fungi and their extracts. The Gram positive strain *Staphylococcus aureus* (MTCC 4381), Gram negative strains *Pseudomonas aeroginosa* (MTCC 4676), *Mycobacterium smegmatis* (MTCC 943) and *Salmonella typhimurium* (MTCC 3232) was used. The fungi *Candida albicans* (MTCC 183) was used as fungal pathogen. All pathogens were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Nutrient agar (NA) was used as growth media for subculturing and maintaining the bacterial pathogens. *Candida albicans* (MTCC 183) was grown on Yeast extract Peptone Dextrose Agar (YEPD).

### 2.3 Screening for anti-microbial activity by dual-culture method

The preliminary antimicrobial assay was carried out for all the isolated fungal strains by Dual culture method. Antimicrobial activity of isolated endophytic fungi was tested based on the protocol of Rehman et al. and Wang et al. with slight modifications (Rehman et al. 2011, Wang and Liang 2014). The petri dishes containing respective media for the growth of bacteria and fungi were prepared and 100 μL of test organism was spread over the surface of the agar media using sterile cotton swab. Actively growing fungal culture discs from PDA plates were cut using a sterile cork borer and placed on the surface of the respective agar media seeded with test bacteria or fungi. These plates were sealed with parafilm and kept in at 4°C for 12 hours for complete diffusion of antimicrobial compounds if any, thereafter the plates were incubated at room temperature for 12 hours for organisms such as *Pseudomonas*.
aeroginosa, Mycobacterium smegmatis, Salmonella typhimurium and Candida albicans. The anti-microbial activity was observed and recorded.

2.4 Secondary metabolites extraction

Secondary metabolites extraction was carried out by using the method explained by Tayung et al. 2011. Endophytic fungal isolates that shown anti-microbial activity by dual culture method, were used for extraction of bioactive compounds. Each fungal isolate was inoculated into 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered by using sterile muslin cloth to separate the mycelia and filtrate. The filtrate was added with equal volume of ethyl acetate, mixed well for 10 minutes and kept for 5 minutes until the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelia were ground properly in a pestle and mortar using ethyl acetate as solvent and filtered using muslin cloth. Both mycelia and culture filtrate extracts were pooled together and dried using rotary vacuum evaporator at 40°C. This procedure was adopted to obtain both intracellular and extracellular metabolites (Petrini 1992). The extract residue was stored at 4°C until used for antimicrobial assay.
2.5 In-vitro studies on antimicrobial activity of fungal extracts

2.5.1 Secondary antimicrobial assay by well and disc diffusion method

The selected bacterial and fungal isolates were grown in 30 ml culture tubes containing 10 ml of respective broth and incubated at 35 ± 2°C for 12-24 hours for bacteria and 30 ± 2°C for 12-24 hours for Candida albicans. All the test microorganisms were set to 0.5 McFarland’s standard (0.05 ml of 1.175% Barium chloride (BaCl₂·2H₂O) in 9.95 ml of 1% Sulphuric acid (H₂SO₄) by addition or dilution with respective broth. 0.5 McFarland’s standard corresponds to approximately 1.5 × 10⁸ CFU/ml. 100 µl of 0.5 McFarland’s standard culture was spread on nutrient media in 10 cm petri plates using a sterile cotton bud.

Antimicrobial activity of secondary metabolite was tested by disc and well diffusion method explained elsewhere (Tong et al. 2011, Maria et al. 2005). The sterile growth media plate specific for test organisms was prepared and inoculated with the test organism. 50 µl of 10 mg/ml ethyl acetate crude extract was added on to a sterile 6 mm paper disc (Hi-Media) using a micropipette and allowed to dry. Discs containing compounds were placed on the microbial inoculated plates. In the same plate, 6 mm diameter wells were made using a sterile cork borer and 50 μL of 10 mg/ml sample was added to each well. The experiment was carried out in triplicates. Ciprofloxacin (10 µl of 10 mg/ml) was used as positive control for bacteria and Flucanozole (10 µl of 10 mg/ml) was used as fungal positive control. Similarly, 20 µl of DMSO as well as ethyl acetate were used as negative controls. The plates were incubated at 35 ± 2°C for 12-24 hours for bacteria of Pseudomonas aeruginosa, Mycobacterium smegmatis and Salmonella typhimurium, 30 ±2 °C for 12-24 hours for Candida albicans. The antimicrobial activity exhibited by zone of inhibition around the disc and well was measured in mm.
2.5.2 Minimum Inhibitory Concentration (MIC)

MIC was evaluated using microplate-liquid dilution method against *Mycobacterium smegmatis*, *Psuedomonas aeruginosa* and *Candida albicans* (Buatong et al. 2011). Ten milligram of test compounds dissolved in 100 µl of 10 % DMSO. The 50 µl solution was serially diluted in 96 well micro plate (1000, 500, 250, 125, 62.5, 31.25 and 15.6 µg/ml). The selected bacterial and fungal isolates were grown in 30 ml culture tubes containing 10 ml of respective broths and incubated at 35 ± 2°C for 12-24 hours for bacteria and 30 ±2 °C for 12-24 hours for *Candida albicans*. The bacterial cultures counts 3.48 x 10⁷ (*Psuedomonas aeruginosa*), 5.6 x 10⁷ (*Mycobacterium smegmatis*) and 4.2x10⁷ (*Candida albicans*) was added to diluted compounds separately. Ten micro litre of the reaction mixture was spread on a agar plate and incubated for the microbial growth. The minimum concentration of compound inhibiting the microbial growth shows no growth on the plates are considered as MIC. The Ciprofloxacin and Flucanozole were used as positive control and DMSO served as negative control.

2.6 Antioxidant activity of fungal ethyl acetate crude extracts

Fungal ethyl acetate crude extracts, which had antimicrobial activity were selected for antioxidant activity. The antioxidant activities were evaluated by radical scavenging activity against DPPH and ABTS.
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2.6.1 DPPH radical scavenging activity (Song et al. 2005)

Aliquots of different concentrations of fungal ethyl acetate crude extracts was added to 150µl of 0.1% freshly prepared DPPH in methanol, incubated for 20 min at room temperature and absorption measured at 517 nm using Perkin Elmer spectrophotometer. Ascorbic acid was used as standard. All tests were performed in triplicates. Antioxidant activity was evaluated as percent DPPH scavenging using the formula:

\[
\text{Percent Activity} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

All the data were analysed in triplicates. The IC\textsubscript{50} values were calculated from linear regression equation and coefficients of determination (r\textsuperscript{2}) were calculated for the relationship between pairs of variables.

2.6.2 ABTS radical scavenging activity (Huang et al. 2007)

ABTS radical was prepared by mixing equal volumes of 7 mM ABTS solution and 2.45 mM potassium per sulphate solution and allowed to stand for 12 h at room temperature in dark. Working solution was prepared freshly before each experiment by mixing ABTS radical solution with distilled water for an initial absorbance of about 0.7 at 745 nm.

Aliquots of different concentration of fungal ethyl acetate crude extract was added to 3 ml of ABTS radical solution and mixed thoroughly. After allowing standing for 5 min at room temperature, the absorbance was recorded at 745 nm using Perkin Elmer spectrophotometer. Ascorbic acid was used as standard. All tests were performed in triplicates. Antioxidant activity was evaluated as percent ABTS scavenging using the formula:
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\[
Percent\ Activity = \frac{Control - Test}{Control} \times 100
\]

All the data were average of triplicates. The IC\textsubscript{50} values were calculated from linear regression equation and coefficients of determination (r\textsuperscript{2}) were calculated for the relationship between pairs of variables.

2.7 Cell Lines and Culture Conditions

The in vitro cytotoxicity assay was carried out on a range of mammalian cancer cell lines including MCF-7, HT-1080 and C6 (from National Centre for Cell Science, Pune, India). All the cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml Penicillin, 100 μg/mL Streptomycin and 2.5 μg/mL Amphotericin-B solution, 200 mM L-Glutamine (All from HiMedia Labs, Mumbai, India); the cell lines were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO\textsubscript{2}. Following 24-48 hrs of incubation period, the adherent cells were detached using Trypsin-EDTA solution 1X/0.25 % (HI Media Labs, Mumbai, India). Cell count was carried out using the Luna automated cell counter (Logos Biosystems, India) based on trypan blue dye exclusion method. Cytotoxicity of the PEGT001 and PEGT002 (ethyl acetate crude extract and column purified fraction) on the cancer cells line was determined using MTT (-3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

2.7.1 In-vitro Cell Viability Assay (MTT Assay)

Two hundred microliter of each cell line was seeded in 96-well microplates (Corning®, USA) at a density of 25,000 cells/well and incubated for 24hrs, after
which the cells were exposed to an increasing concentration of ethyl acetate crude extract and column purified PEGT001 and PEGT002 fractions for 24 hrs. All cells were seeded in duplicates and incubated in a CO$_2$ incubator (atmospheric with 5% CO$_2$ and 37°C temperature). Treated cells were thereafter incubated with MTT (HiMedia Labs, Mumbai, India) for 3 hrs. The culture medium was aspirated and 100 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, India) was added to each well. The untreated cells were used as controls. Cell viability was determined by measuring the absorbance on a microplate reader (SPECTROstar Nano, BMG LABTECH, Germany) at 570nm. Viability was calculated as a percentage of viable cells at different test concentrations relative to the control (untreated) cells (% cell viability = (A$_{570}$ of treated cells / A$_{570}$ of control cells) ×100%). DMSO served as vehicle control (0.1% of final concentration). The concentration of fungal extracts that resulted in 50% inhibition of cell growth was calculated as the half-maximal inhibitory concentration (IC$_{50}$) by constructing a dose-response curve.

2.7.2 IC$_{50}$ Value Determination

The IC$_{50}$ for each cell line was determined by using the spectrophotometric results of different concentrations of ethyl acetate crude extract and column purified PEGT001 and PEGT002 fractions for which a linear curve fit was generated. Cell viability percentages (y-axis) were plotted against increasing concentrations of PEGT001 and PEGT002 samples. IC$_{50}$ value was estimated by using the linear equation $y = mx+c$ where 50 is substituted for y, yielding x as the IC$_{50}$ value.
2.8 Identification of fungi

2.8.1 Culture and Morphological studies: Endophytic fungi which are showing antimicrobial activity were studied for morphological characteristics (Photita et al. 2005). Each fungal isolate were grown on Potato dextrose agar (PDA, pH -5.1), Malt extract (MEA, pH -4.5) and Czapex dox agar (CZA, pH -7.5) at room temperature in dark for 7 days and studied their morphological traits such as colour of the colony, texture and shape. Microscopic images of sporulating fungi were taken.

2.8.2 Molecular identification of fungi

Genomic DNA isolation: The endophytic fungi exhibiting antimicrobial activity were used for DNA isolation. Genomic DNA was isolated as per the method explained by Cenis (Cenis, 1992). Endophytic fungal isolates, which showed antimicrobial activity, were selected for extracting genomic DNA. Each fungal isolate was grown in 50 ml conical flasks containing 20 ml of Potato Dextrose Broth and incubated at room temperature in static condition for 6-8 days. Mycelia were separated from broth by filtering through muslin cloth and the mycelial mat was grounded to powder using liquid nitrogen with the help of sterile mortar and pestle. Mycelial powder was washed with 500 µl Tris EDTA buffer and pelleted by microfuge for 10 min (Tarsons). The Tris EDTA buffer was discarded and Lysis buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 10% SDS) was added, mixed vigorously. 150 µl of 3M sodium acetate, pH 5.2 was added and incubated at -20º C for about 10 min. Tubes were then centrifuged in a microfuge for 10 min and supernatant was collected. To the supernatant equal volume of ice cold isopropanol was added and allowed to stand at room temperature for at least 5 min. The precipitated DNA was pelleted by centrifugation for 10 min. After a wash with 70 % ethanol, the pellet was air dried at
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room temperature and resuspended in 100 µl of TE buffer and stored in -20ºC until used. DNA was quantified by running on 0.8 % Agarose gel electrophoresis and also quantified by Nanodrop (Thermo scientific).

**PCR amplification of ITS regions**

The molecular identification was performed using rDNA internal transcribed spacer (ITS) regions using PCR (Road & Kong, 2000). Universal primers for fungi ITS1-TCCGTAGGTGAACCTGCGG (Forward primer) and ITS4-TCCTCCGCTTATTGATATGC (Reverse primer) was used to amplify ITS region. PCR was done in 25 µl reaction containing, 25 ng genomic DNA, 1x PCR buffer, 0.2 mM dNTP, 3.5 mM MgCl₂, 10 µM each primer, 1 unit Taq DNA polymerase.

The composition of 25 µl PCR reaction mixture as follows:

- Sterile water-12.3 µl
- 10 x PCR buffer-2.5 µl
- 10 mM dNTPs-0.5 µl
- 250 U Taq polymerase-0.2 µl
- 10 µM ITS1-0.5 µl
- 10 µM ITS4-0.5 µl
- 25 mM MgCl₂-3.5 µl
- Genomic DNA-5 µl
PCR was carried out in a thermal cycler (Technie) using following condition

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (s/min)</th>
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<tr>
<td>Initial denaturation</td>
<td>95</td>
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</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
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<td>Annealing</td>
<td>52</td>
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<tr>
<td>Extension</td>
<td>72</td>
<td>90</td>
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<tr>
<td>Final extension</td>
<td>72</td>
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35 cycles

The PCR products were purified using Qiagen columns. The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA was eluted with Tris buffer or water. QIA-quick spin columns offer 3 handling options — as an alternative to processing the spin columns in a microcentrifuge, they can now also be used on any commercial vacuum manifold with luer connectors (e.g., QIAvac 6S or QIAvac 24 Plus with QIAvac Luer Adapters) or automated on the QIAcube.

Purified PCR products were electrophoresed on 1.2% agarose gel and visualized under UV transilluminator. A standard with 0.1-1 Kb size marker was used as a reference. Automated DNA sequencing was performed using an ABI 3730xl 96-capillary DNA Analyzer Machine (Eurofins, Bangalore, India) and a Blast search was done on the obtained DNA sequences to find the closest matched sequences in the GenBank database.
2.9 Optimization studies of culture conditions

2.9.1 Selection of solvent for metabolite extraction

All the fungal isolate positive antimicrobial activity were grown in 200 ml Potato Dextrose Broth (pH 5.1) in 500 ml conical flask and incubated at room temperature for 21 days under stationary condition. Ethyl acetate and Hexane were separately used as solvents for the extraction of bioactive compounds. The broth culture was filtered by using sterile muslin cloth to separate the mycelia and filtrate. To the filtrate equal volume of ethyl acetate or hexane was added, mixed well for 10 minutes and kept for 5 minutes until the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded properly in a pestle and mortar using ethyl acetate as solvent and then it was filtered using cheesecloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in rotary vacuum evaporator at 40°C. The dried extract was stored at 4°C until used for the antimicrobial assay. Both the solvent extracts were tested for its antimicrobial activity by well diffusion method against the same test organisms used in MIC method. 100 µl of 10 mg/ml ethyl acetate crude extract was added to 7 mm diameter wells, 0.5 McFarland standard organisms were used. The experiment was carried out in triplicates.

2.9.2 Standardization of media

The selection of media for increased production of bioactive compounds was done as per the procedure described by Gogoi et al (Gogoi et al. 2008). Initially PEGT002 fungal isolate was selected to grow in various media such as Potato dextrose broth (with pH ranging from 3.5, 4.5, 5.5, 6.5, 7.5), Malt extract broth (pH 4.5), New media...
(pH 6), Rice media, Czapek dox broth (pH 7.3), PDB supplemented with 0.5 mg/ml tyrosine.

### 2.9.3 Secondary metabolite extraction

Extraction of secondary metabolites was carried out as described earlier. Each endophytic fungal isolates was inoculated into 500 mL Erlenmeyer flasks containing 200 mL broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered to separate the mycelia and filtrate. To the filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded properly in a pestle and mortar using ethyl acetate as 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 at 4°C to be used for antimicrobial assay.

Each ethyl acetate crude extract of PEGT002 were tested for its antimicrobial activity by well diffusion method. Malt extract and Potato dextrose broth was selected as the best media for the growth of PEGT001, PEGT002, PECT017, PECL014, PECL011 and PEGL001, PSGL001, PSGT001 respectively.

Each fungal isolate was again grown in Malt extract broth with glucose and without glucose to find out the effect of glucose in secondary metabolite production.

The protocol of secondary metabolite extraction was slightly modified, in which equal volume of ethyl acetate was added to soak the fungi and left it overnight and stirred in
magnetic stirrer for 20 min and taken to evaporation in vacuum evaporator at 40°C. Evaporated extract was tested for its antimicrobial activity as described earlier.

2.9.4 Effect of Temperature

Two best fungal isolates were selected to observe the effect of temperature on the production of secondary metabolites. Each isolate was grown in 200 ml of Malt extract broth with 0.1% glucose in 500 ml conical flask, Incubated in dark for 21 days. One set of experiment was carried out to be incubated at 25°C for 21 days. Another set of experiment was incubated initially at 37°C for a week and transferred to 25°C for another 2 weeks.

2.9.5 Effect of Co-culturing

The protocol for co-culture was followed as that of Ho et al (Ho, To, & Hyde, 2003) Endophytic fungi were grown in 200 ml of Malt extract broth with 0.1% glucose in 500 ml conical flask was incubated at 25°C, added 1 ml of autoclaved Psuedomonas aeruginosa after 1 week, then added 1 ml of live Mycobacterium smegmatis culture after 2nd week. After 21 days metabolite extraction was done as described earlier and tested for antimicrobial activity. Each experiment was carried out in duplicate and all the antimicrobial activity was performed in triplicate.

2.10 Characterization of active molecule

2.10.1 Silica gel column chromatography

Activated silica gel (200-400 mesh) was packed onto a glass column (Diameter 20 mm × 200 mm) using ethyl acetate solvent. Ethyl acetate extract was loaded on top of
the silica gel and eluted stepwise: chloroform (100%), ethyl acetate (100%), methanol (100%) and water. About 20 fractions measuring 2 mL each were collected and tested separately for antibacterial and antioxidant activity (Zhong and Xiao 2009).

2.10.2 Thin Layer chromatography

The bioactive compound was eluted on F$_{254}$ silica coated plates using n-butanol-acetic acid-water (4:1:2) as solvent system. The bands were visualized under sunlight, UV light at 280 nm (Laminar air flow hood) and 365 nm (Transilluminator) and R$_f$ value was calculated (Cimpoiu 2006).

2.10.3 UV-Visible spectrometry

The UV-Visible spectrum of the compound isolated from PEGT001 and PEGT002 was recorded on a Perkin Elmer spectrophotometer at room temperature. Twenty five µg/mL of column purified compound of PEGT001 and PEGT002 was used to record spectrum range of 200 to 800 nm.

2.10.4 Infra-red (IR) spectrometry

The IR spectrum of compounds from PEGT001 and PEGT002 was recorded on a Perkin-Elmer FT-IR spectrometer at room temperature. About 10 mg of sample in KBr pellet was used to record the spectrum from between 4000 and 400 cm$^{-1}$ frequencies (Policegoudra et al. 2010).

2.10.5 Estimation of total phenolics

A 20 µl of sample aliquots of fungal extracts or Gallic acid (20-100 µg/ml) was mixed with water (1.58 ml) followed by addition of 20 µl of 2N Folin-Ciocalteau’s reagent. The mixture was vortexed thoroughly and was incubated at room temperature for 5
min. Then 100 µl of 20 % sodium carbonate was added and vortexed the samples thoroughly and kept at room temperature in dark for 30 min. Absorbance was recorded at 730 nm. The total phenolic value was compared with that of calibration curve of Gallic acid standard at various concentrations (5, 10, 15, 20, 25 and 30 µg/ml) (Lu et al. 2011).

2.10.6 Qualitative test for flavonoids

Ethyl acetate extract of fungi was taken in a test tube and few drops of 1N NaOH solution was added. The solution should appear an intense yellow colour now. By addition of few drops of 1 N HCl, sample should turn yellowish or colourless. This test confirms the presence of flavonoids.

2.10.7 LC-ESI-MS analysis

LC-MS analysis was carried out in Thermo Finnigan Surveyor HPLC system with PDA detector (Length: 4.6 (ID) X 150 mm, pore size: 3 µm and particle size: 120 Å) connected with Thermo LCQ Deca XP MAX equipped with Xcalibur software. Five microlitre of sample was injected with the help of a micro syringe. The mobile phase used was 100% acetonitrile and 100% methanol under gradient conditions. Flow rate used was 0.5 mL/min. PDA detection was carried out at Channel A (230 nm), Channel B (256 nm) and Channel C (380 nm). HPLC Column was equilibrated with 100% methanol and eluted for 30 minutes in gradient condition by increasing the acetonitrile concentration 0 to 100%. The column was again equilibrated with 100% methanol for next 10 minutes. Samples were analyzed both in positive and negative electronic ion spray ionization with a mass range 60 m/z to 1000 m/z (Chang and Wong 2004). Phenol explorer database was used to screen the compounds based on molecular mass (Neveu et al. 2010).
2.10.8 UHD Q-TOF analysis

The silica gel column purified compounds of PEGT002 were analysed by Agilent 6540 UHD Q-TOF system with new photonis fast bipolar detector. The column used was Eclipse plus C\textsubscript{18} (3 mm X 100 mm). Five microlitre of sample was injected with the help of a microsyringe. The mobile phase used was 0.5% formic acid in methanol for negative mode ionization and 100 % methanol for positive mode ionization. The flow rate was 0.5 ml/min. The experimental parameters used for QTOF was Nebulizer-40 psig, Drying gas-10 L N\textsubscript{2}/min at 325°C, Sheath gas-10 L N\textsubscript{2}/min at 350°C, Fragmentor-180 V, Nozzle voltage-8 V. Samples were analyzed both in positive and negative ionization with a mass range 100 m/z to 3000 m/z.

2.11 DNA protection assay

DNA protection assay studies were performed using the protocol explained elsewhere with slight modification (Blanco-Ayala et al. 2013). TEV-PMHT plasmid DNA protective capability of antioxidant compounds from PEGT001 and PEGT002 was analyzed by using hydrogen peroxide. Isolation of plasmid DNA was done by Mini prep method. Three microlitre of plasmid was incubated with aliquots of 5 mg/ml column purified compound at room temperature for 10 min. Then 2 µl of 100 mM hydrogen peroxide and 1µl of 20 mM ferric chloride were added. The final reaction mixture was made upto10μl with milliQ water and incubated at room temperature for 30 min. The Plasmid DNA was analyzed on 1% agarose gel. Vitamin C was used as positive control. The results were observed by comparing intensity of control band versus test band.
2.12 Fenton reagent catalysed protein oxidation

Protein protection studies were followed as per the method of Davies et al (Davies and Delsignore 1987). Five milligram of BSA was dissolved in 1 ml of phosphate buffer saline (pH-7.4) and 10 µl of this BSA solution was incubated with various aliquots of column purified 2 mg/ml compound at room temperature for 10 min. Then 2 µl of 20 mM copper sulphate and 3 µl of 100 mM hydrogen peroxide were added. The final mixture was made up to 25 µl with milli Q water and incubated for 1 hr at room temperature. Vitamin C also processed similarly like test compounds and used as a positive control.

Damage to protein was quantified by 12 % SDS-PAGE. Samples were mixed with loading buffer (10% glycerol, 2% SDS, 25 mM Tris–HCl (pH 6.8), 5% mercaptoethanol, 0.1% bromophenol blue and heated at 100°C for 5 min. The protein samples were loaded on to 12 % polyacrylamide gel and electrophoresis was run at 150V until the loading stain reaches end. Gel was removed and stained with coomassie brilliant blue for 30 minutes and destained excess stain using destaining solution (Water: Methanol: Glacial acetic acid ,50:40:10). Stained protein bands were photographed and analyzed the results. Bovine serum albumin was used as control.

2.13 Metal catalysed Lipid peroxidation

Lipid protection studies were performed as per the procedure of Devasagayam et al. with modifications (Devasagayam et al. 2003). One milliliter of egg yolk was thoroughly homogenized in 24 mL of 1M Tris buffer (pH 6.8). Hundred microlitre of egg homogenate was incubated with column purified PEGT001 and PEGT002
compound with various concentrations (0.05-0.3 mg/mL) at room temperature for 10 min. Then 10 µl of 25 mM Ferric chloride was added to induce lipid peroxidation. The final volume of the mixture was made up to 300 µl by adding 1M Tris buffer (pH 6.8) and incubated at 37°C for 15 min, followed by addition of 100 µl glacial acetic acid. The reaction mixture was centrifuged at 3500 rpm for 15 min in room temperature. Two hundred microlitre of TBA was added to the supernatant and heated the mixture at 95°C for 15 min. After cooling, samples were centrifuged at 3000 rpm for 15 min in room temperature and absorbance of supernatant was measured at 532 nm. The control was all solutions without the sample. Vitamin C was used as standard. The percentage inhibition ratio was calculated from the following equation:

\[
\text{Percent Activity} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

2.14 Crystallization

A 50 µl (10 mg/ml) of column purified PEGT001, PEGT002 and Vitamin-C (dissolved in methanol) were added to petridish containing 50 µl of 0.1% DPPH (dissolved in methanol) and incubated at 25°C. Formation of crystals were monitored regularly using Polarizing microscope.

2.15 Rule of Five (Lipinski rule)

The \textit{in-vivo} adsorption, distribution, metabolism, and excretion (ADME) studies of drug-like molecules are costly and time-consuming. Rule of five proposed by Lipinski was the most reliable method to predict ADME properties. This method was based on a set of rules such as, Molecular mass≤500, number of hydrogen bond
donors≤5, number of hydrogen bond acceptors≤10, octanol-water partition coefficient≤5. This method states that violating more than one rule by a compound expected not to have drug-like properties (the rule of five for non-oral route). Based on this method, the bioactive compounds from this study were screened for drug-likeness.

Additional to rule of five, we also used OSIRIS property explorer to predict the toxicity features (mutagenicity, tumorigenicity, reproductive effect, and irritability) of the isolated compounds. The drug-like properties were calculated on the fly, when the desired molecule was drawn in OSIRIS drug discovery informatics system (organic chemistry portal 2012).