CHAPTER- III

MATERIALS AND METHODS

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3.1 Plants selection and samples collection

The plants were selected after thorough literature survey keeping in view their medicinal/pharmaceutical applications and by interacting with local tribal population. Further, plants were also considered based on their medicinal properties and usage by the tribal communities of Assam to treat day to day ailments, especially to cure stomach related disorders. The plants were identified and authenticated by referring herbarium of Department of Botany, Gauhati University, Assam, India and a voucher specimen of each plant species was deposited in the Department with an accession number. Healthy leaves of the selected plant species were collected from different sites. From each site five individual healthy plants were selected randomly and leaves were collected in sterile polyethene bags. The samples were immediately brought to the laboratory and processed for isolation of endophytic fungi.

3.2 Processing of plant samples

Healthy leaves of the collected plant samples were separated and washed under running tap water so as to remove epiphyllous debris followed by washing thoroughly in double distilled water. This was followed by washing them with mild 10% teepol bio-detergent. The leaves were then rinsed with sterile double distilled water for 2-3 times to remove surface debris, if any.

3.3 Development of sterilization protocol and test for efficacy

The surface of the sample leaves was subjected to a treatment with different surface sterilizing agents to remove epiphytic microorganisms. A standard surface

sterilization protocol was developed for each plant species following available literature as well as through trial and error method. For *Houttuynia cordata*, leaves were surface sterilized following Jena and Tayung (2013) with slight modifications. Surface sterilization was performed by sequentially dipping the leaf samples in 70% ethanol (EtOH) (3min), followed by 0.5% sodium hypochlorite (NaOCl) (2 min) and then rinsed thoroughly with sterile distilled water (1 min) and allowed to surface dry under sterile conditions. Similarly, for *Eryngium foetidum*, leaves were surface sterilized following Sushma *et al*. (2018) with slight modifications. Leaf samples were sequentially dipped in 70% ethanol (2 min) followed by 0.5% sodium hypochlorite (1 min), rinsed twice thoroughly with sterile distilled water (1-2 min) and finally allowed to surface dry under sterile conditions. However, for *Zanthoxylum oxyphyllum*, the leaves were surface sterilized by sequentially dipping them in 70% ethanol (2 min), followed by 0.5% sodium hypochlorite (1 min) and then rinsed twice with sterile distilled water (1 min each time). Leaves were then allowed to surface dry under sterile conditions. To ensure effective surface sterilization, one of the surface-sterilized leaves from each plant species was rubbed over Potato Dextrose Agar (PDA) plate and incubated to check the growth of any contaminant fungi. All of these processes were carried out under sterile conditions in the Laminar Air Flow Chamber. Thus, the efficiency of surface sterilization procedure was tested as per the method described by Schulz *et al*. (1993). Absence of any contaminant or fungal growth proved the efficacy of the protocol used.

3.4 Isolation of endophytic fungi

Surface sterilized leaves were punched into circular fragments of about 0.5 mm diameter with the help of a sterile puncture. These fragments were then placed in three

different mycological media namely Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Water Agar (WA) for all the three sample plant species supplemented with streptomycin sulphate (50µg/ml) and incubated at 25±2° C for 2-3 weeks. For samples that showed less or no growth of endophytic fungi in these three media, a modified media amended with the Plant Extract and Agar (PEA) was used to trigger the growth of endophytic fungi. The plated fragments were observed once a day for the growth of endophytic fungi. The hyphal tips of fungi growing out of the surfaced sterilized leaf tissues were immediately transferred to PDA slants and stored at 4°C for further study. Absence of growth of fungi on the medium after 7 days of incubation it indicated that sterilization procedure was effective in removing the surface fungi.

3.5 Morphological identification of endophytic fungal isolates

The pure fungal isolates were stained with Lactophenol cotton blue and identified based on their colonial morphology and microscopic reproductive characters observed during their growth on PDA using the standard identification manuals (Gilman, 1971; Barnett and Hunter, 1996; Damm *et al*., 2012). The fungal cultures that failed to sporulate were categorized as Mycelia Sterilia. Endophytic fungi under Mycelia Sterilia with distinct morphological features were designated as Morphotype.

3.6 Data Analysis

The relative frequency of colonization (CF%) was calculated as the number leaf segments colonized by a specific fungus divided by total number of segments plated multiplied by 100 and the dominant endophytes were calculated as percentage colony

frequency divided by sum of percentage of colony frequency of all endophytes multiplied by 100 (Hata and Futai, 1995; Tayung and Jha, 2006).

The relative colonization frequency (CF %) of endophytic species was calculated using the formula:

$$\mathbf{CF\ \% = N_{col} / N_t \times 100}$$

where,

N_{col} stands for the number of segments colonized by each endophytic fungal species

N_t stands for the total number of segments plated.

Dominant endophytes (DE) were calculated as:

DE = (CF% of individual endophyte / Sum of CF% of all endophytes) × 100

3.7 Screening for antimicrobial activity against test pathogens

The antimicrobial potency of the crude secondary metabolites of the isolated endophytic fungi was screened against a panel of clinically significant human test pathogenic microorganisms.

3.7.1 Test organisms used

The test pathogens used included one Gram positive bacterium: *Staphylococcus aureus* (MTCC 737); two gram negative bacteria: *Pseudomonas aeruginosa* (MTCC424), *Escherichia coli* (MTCC 443) and a fungus, *Candida albicans* (MTCC 227), all of which were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.7.2 Fungal Cultivation and metabolites extraction

Pure endophytic fungal isolates that were actively growing were cultivated by placing culture agar blocks (about 3 mm in diameter) on Potato Dextrose Broth (PDB) in 250ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated in a BOD shaking incubator for 2-3 weeks at 25±2 °C with a periodic shaking at 120 rpm. The culture was then filtered through sterile Whatmann No.1 filter paper to remove the mycelial mats. Each of the liquid broth thus collected was extracted with an equal volume of ethyl acetate (EtOAc) in a separating funnel by vigorous shaking for 10-15 min. In the process, the medium and cell debris got separated and the solvent thus obtained was collected. The solvent was then evaporated in a rotary evaporator and the crude ethyl acetate extract was obtained. The extracts were further concentrated and dried with magnesium sulphate ($MgSO_4$). The concentrated crude extracts were finally dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C for the determination of antimicrobial activity.

3.7.3 Determination of antimicrobial activity

Antimicrobial activity of the crude metabolites was determined by agar cup diffusion method against four selected clinically significant human test pathogens, namely, *Staphylococcus aureus* (MTCC 737), *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 443) and *Candida albicans* (MTCC 227). The bacterial and fungal test pathogens were activated by cultivating them on freshly prepared nutrient agar (NA) and Sabouraud's dextrose agar (SDA) media, respectively. Sterile Nutrient agar (NA) and Sabouraud's dextrose agar plate (SDA) plates were prepared and inoculated with 0.2 ml of overnight grown bacterial and fungal cultures containing 1.0×10^6 cells

(approximately). A lawn culture was made over each plate by evenly spreading out the inoculums with the help of a sterile cotton swab. Agar cups were then prepared in the plates using a sterile cork borer (7 mm in diameter). The cups were then filled in with 100 µL of the crude culture filtrate or metabolites obtained from the endophytic fungi and incubated at 37°C ± 1°C for 24 hrs for bacterial and at 28°C ± 1°C for 48 hrs for fungal pathogens. Antimicrobial activity was determined by the appearance of clear zones of inhibition against the target test organism around the agar cups. DMSO was used as the negative control for the assayand Streptomycin sulphate (10µg) and Fluconazole (25µg) were used as positive controls.

3.8 Process optimization for enhanced metabolite production

Selected endophytic fungi showing significant antimicrobial activity were grown in different cultural conditions (different media, incubation period, pH, temperature, etc.) and their effect on metabolite production were studied. Antimicrobial activity of the potent isolates was then evaluated against selected test pathogens under these cultural conditions. They were grown in different culture media like Potato Dextrose Broth (PDB), Czapek Dox Broth (CDB), Malt Extract Broth (MEB) and Oatmeal Broth (OMB) and incubated for 21 days. The metabolites were extracted every 1 week and studied up to 3 weeks. The metabolites so obtained and determined for antimicrobial activity against the test organisms. Similarly, in another instance, keeping the culture media and incubation time constant (wherever maximum observed), the isolates were grown in different pH (5, 7 and 9) and at varied temperatures (25-40°C). The crude metabolites were extracted in all such conditions and assayed for antimicrobial activity.

3.9 Molecular identification of endophytic fungal isolates

Fungal endophytic isolates that showed prominent bioactivity were identified by ITS 18S rDNA sequencing. Resultant sequences were submitted to Genbank and were blasted against the nucleotide database using "BLASTn" Tool of the U.S. National Centre for Biotechnology Information (NCBI) for final identification of endophytes.

3.9.1 Genomic DNA isolation, amplification and sequencing

Species confirmation of the endophytic isolate that showed prominent bioactivity was determined by ITS rDNA sequence analysis. Each of the selected fungal strain was cultured on Potato Dextrose Broth (PDB) and a small amount of the fungal mycelia was suspended in 40 µL MQ water. Genomic DNA was isolated by Cetrimethyl ammonium bromide (CTAB) method (Clarke, 2009). A portion of the genomic DNA was diluted upto 50 ng/µL for use in the PCR. The nuclear ribosomal DNA and ITS region of the isolates were then amplified using the universal primers: ITS4 (50-TCCTCCGCTTATTGATATGC-30) and ITS5 (GGAAGTAAAAGTCGTAACAAGG-30). The PCR was set up using the components- 2.5 µL buffer (109), 1.5 µL $MgCl_2$ (25 mM), 2.5 µL dNTPs (2 mM), 0.2 µL Promega Taq (5 U/µL), 1.0 µL each of forward and reverse primers (5 pmol/µL), and 6.0 µL DNA from diluted extract. PCR condition was run with initial denaturation at 94ºC for 3 min. Denaturation, annealing and extension were done at 96º C for 10 s, 55 ºC for 10 s and 72ºC for 30 s, respectively in 45 cycles. Final extension was done at a temperature of 72 ºC for 10 minutes and held at 4º C temperature. After the PCR cycle, 2 µL of the product was used to check on 1 % Agarose gel. DNA sequencing of all the isolates were performed using an ABI 3730 sequencer.

3.9.2 Taxon sampling and phylogenetic analysis

The forward and reverse sequence reads thus obtained were assembled to generate the contig using CAP3, a bioinformatics tool used for the assembling of genomic DNA reads (Huang and Madan, 1999). The annotated ITS rDNA contigs were submitted to the GenBank database of NCBI and respective accession numbers were obtained for each entry. For all the query sequences (contigs) homology searches were performed when blasted against the nucleotide database using Basic Local Alignment Search Tool (BLASTn) of the U.S. National Centre for Biotechnology Information (NCBI) and the closest homologues were retrieved. Thereafter, homologous ITS sequences to the query sequences were selected randomly and retrieved from the available entries of the NCBI database. For phylogenetic analysis, multiple sequence alignments were performed for the endophytic ITS rDNA sequences of present study and downloaded sequences of their nearest neighbors using CLUSTALW software utilizing default settings, and trees were generated by the character state Maximum Parsimony (MP) method using MEGA 6.0 (Tamura *et al*., 2013). The robustness of the tree was assessed by bootstrap analysis with 1000 replications.

3.9.3 RNA secondary structure analysis

Basing upon the phylogenetic analysis, selected ITS2 sequences belonging to the identified fungal isolates of varied lifestyles (endophytic and pathogenic) were used to generate the RNA secondary structures. The minimum free energy (MFE) structures were predicted for the ITS2 query segments and their closest homologues using RNAalifold and MFOLD web servers (Zuker, 2003; Wolf *et al*., 2005; Bernhart *et al*., 2008). The structure prediction was performed under default settings with a temperature of 37 °C,

ionic conditions: 1 M NaCl, no divalent ions; maximum number of nucleotides in a bulge or loop: 30, maximum asymmetry of an interior/bulge loop: 30, percentage sub-optimality number: 5 and upper bound on number of computed foldings: 50. The structures were analyzed based on their noticeable similarities and variations. The structures chosen from different output files were the ones with the highest negative free energy if various similar structures were obtained. The consensus ITS2 secondary structures were compared among different lifestyles and differences in their folding patterns as well as in number and types of motifs were investigated.

3.10 Characterization of bioactive metabolites

The metabolites that showed significant antimicrobial activity was characterized by Fourier-Transform Infrared (FTIR) and Gas Chromatography Mass Spectrometry (GCMS) analyses at the Guwahati Biotech Park, IIT Guwahati.

3.10.1 FTIR Analysis

Infra-Red spectral analysis was performed using Thermo Nicolet iS10 FT-IR Spectromer (Thermo scientific) instrument. KBr pellet was prepared by mixing 1mg of dried ethyl acetate extract of the isolated endophytic fungi with 10mg of anhydrous potassium bromide. The spectra were recorded for thirty-two scans from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and the obtained spectra were recorded and analyzed.

3.10.2 GC-MS Analysis

The compounds were further identified using a Perkin Elmer Turbo Mass Spectrophotometer (USA) of model Claurus 680 Gas chromatography/Claurus600 Mass spectrometer (GC with Liquid Auto sampler). The column used was Perkin Elmer Elite-

5MS capillary, with column of length 60m and internal diameter 0.25mm and composed of 5% diphenyl and 95% dimethyl polysiloxane (low bleed). Carrier gas used was Helium at a flow rate of 1ml/min. 1µL of the prepared 1% of extract diluted with methanol was injected in a split less mode. Inlet temperature was maintained as 250°C. The oven temperature was programmed initially at 60°C for 5 min, then increased to 180° C at a rate of 10°C and holding time of about 5 min and then again programmed at 280°C at a rate of 20°C ending with 5 min. The MS transfer line was maintained at a temperature of 200°C and the source temperature was maintained at 180° C. The total run time was 37 minutes. GC-MS was analyzed using electron impact ionization at 70 eV. The data was further evaluated using total ion chromatogram (TIC) for compound identification and quantification. Identification of chemical compounds present in the extract was achieved by comparing their respective retention time (RT) and mass spectra fragmentation patterns with data from those stored in the library of National Institute of Standard and Technology (NIST) and also with published literatures. All identified components were summarized in terms of relative peak area percentage.

3.11 Purification and structure elucidation of metabolites

Bioactive crude extracts obtained from the potent endophytic fungal isolates were purified by analytical and preparative Thin Layer Chromatography (TLC) and Column Chromatography at the Department of Chemistry, University of Naples Federico II, Italy through a collaborative program. The extracts were purified on silica gel (Kieselgel 60, F254) using different solvent mixtures namely $CHCl_3$-*i*-PrOH, Petroleum ether- Me_2CO and *n*-hexane-EtOAc in different proportions as eluent to yield homogenous pure compounds. The spots were visualized through exposure to UV radiation or by spraying

first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 minutes. ^1H NMR spectra were recorded at 400 or 500 MHz in duterated chloroform ($CDCl_3$) on spectrometers. Identification of the compounds was carried out by comparing the NMR spectroscopic data with data already reported in literature.