CHAPTER 3 MATERIALS AND METHODS

3.1 Sampled sites and plants

Plant material was collected from botanical garden of institute of agriculture science, Banaras Hindu University (BHU), Varanasi (25.5-N 82.9-E, elevation 279 ft. /85 m (height from sea level), India, in March 2013. Identification of plant was ascertained on the basis of external morphology characteristic features. A complete mature and healthy plant was rooted out from soil surface. The samples were collected in sterile polythene bags and brought to the laboratory in an icebox. Samples were preserved at 4 ^{0}C in a refrigerator to avoid any plant tissue and fungal cell damage and processed for isolation of endophytic fungi immediately.

3.2 Isolation of entophytic fungi

Prior to surface sterilization, roots were individually removed from rest of the plant and thoroughly washed in running tap water for more than 10-12 times to remove soil particles from root surfaces, then shaken in a 500ml flask containing 200 ml of sterile distilled water with the addition of 4-5 drops of detergent, Tween 80 (SRL Chemical Co., St. Louis, Mo, USA). Thereafter, the processed samples were dipped in 75% ethanol for 1.5 min, then in 4% sodium hypo chloride (SRL Chemical) for 2 min. The tissues were then washed two times with sterile double distilled water and allowed to surface dry in sterile conditions (sulze et al., 1993; Gu, 2005). Complete sterilization was confirmed by inoculating a small volume of last washing water into petri dishes containing peptone 5 (g/l), malt extract 5 (g/l), sodium chloride 1.0 (g/l) and agar 2.5% and pH of the medium was adjusted to 5.5. After surface sterilization, roots were cleaved aseptically into small pieces of 4-5 mm^2 area section and transferred to sterile Petri dishes containing sterile Wickerham medium (Malt extract (3g/l),

Yeast extract (3 g/l), peptone (5 g/l), dextrose (10 g/l), and agar (2.5 gm/l). pH of medium was adjusted to 5.5. The inoculation medium was supplemented with streptomycin sulphate (150 µg/ml) and Chloramphenicol (100 µg/ml) to check bacterial growth. The Petri dishes were incubated at 28 °C, and regularly observed till the outcome of endophytic fungal hyphae. After 4-5 days it was observed that hyphae tips begin to originate and radiating from segments (Fig. 1), which were transferred to Petri dishes containing PDA medium devoid of antibiotics. Dishes were regularly monitored for formation of spore by stereo and light microscopy. After incubation at room temperature the fungal strains under investigation was found to grow exclusively out of the plant tissue. The identification of the endophytic fungus was based on their characteristic morphology of colony, color and pattern of spore production. Each isolate was then grown and examined to make certain that it is originated from single hyphae. All fungi present were isolated, sub cultured and preserved on PDA medium for further identification.

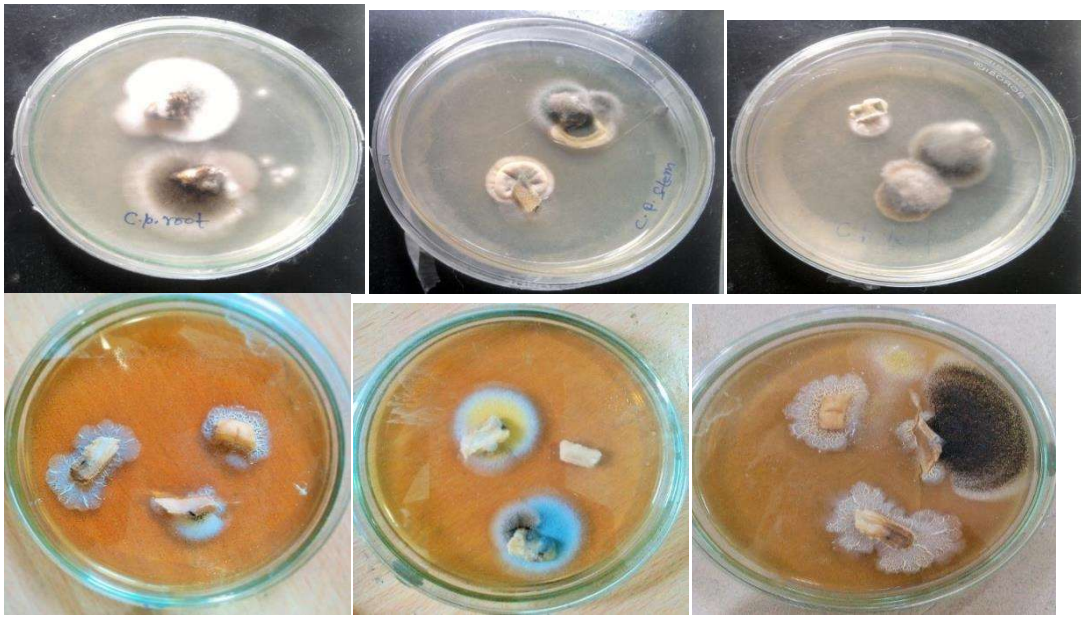


Fig. 3 Appearance of endophytic fungi from *Calotropis procera* roots Stem and leaf segments after 2-3 Days of incubation of plant materials

3.2.1 Colonization frequency (CF %)

The frequency of colonization was calculated taking the ratio of the number of morphologically distinct colony isolated (Ni) and the total number of sample fragments inoculated (N_f) Colonization Frequency (CF) was calculated as described by Hata K, Futai K, 1995.

FC% = Ni/Nf X100.

3.2.2 Steps to avoid contamination

Following steps were kept in mind during experimental procedure

- The plant material was selected carefully and during the selection process it was kept in mind that plant should not be diseased.
- Fresh plant material was chosen for isolation of fungal species
- Long term storage of plant material was avoided because long term storage sometimes results in contamination.
- The cultured plates were examined regularly and if any plates suspected for contamination, complete batch was discarded.
- Plates were regularly observed for outcome of fungal mycelium and as the growth appears, growth pattern was observed.

3.2.3 Preservation of Isolated Cultures

Pure culture was preserved on PDA slant in test tubes at 5 ^{0}C. Each tube was labeled with full batch number, code number of host plant full name of fungi and date of storage. Many replicates were prepared for each isolates and suitable media was used for each isolate.

3.3 Identification of Fungi

Preliminary identification of fungal species was done on the basis of morphological characteristics. All fungal cultures were grown on suitable culture medium (PDA medium) at specific culture conditions to make their identification easy and appropriate. During incubation period all the plates were regularly examined for production of spores. Some of the fungal species produced spore while other grown like cotton appearance. A total of 14 isolates have been identified and each isolate was cultured at small scale in liquid broth (PD broth) for production of bioactive metabolite.

3.4 Cultivation and Crude Metabolite Extraction at Small Scale

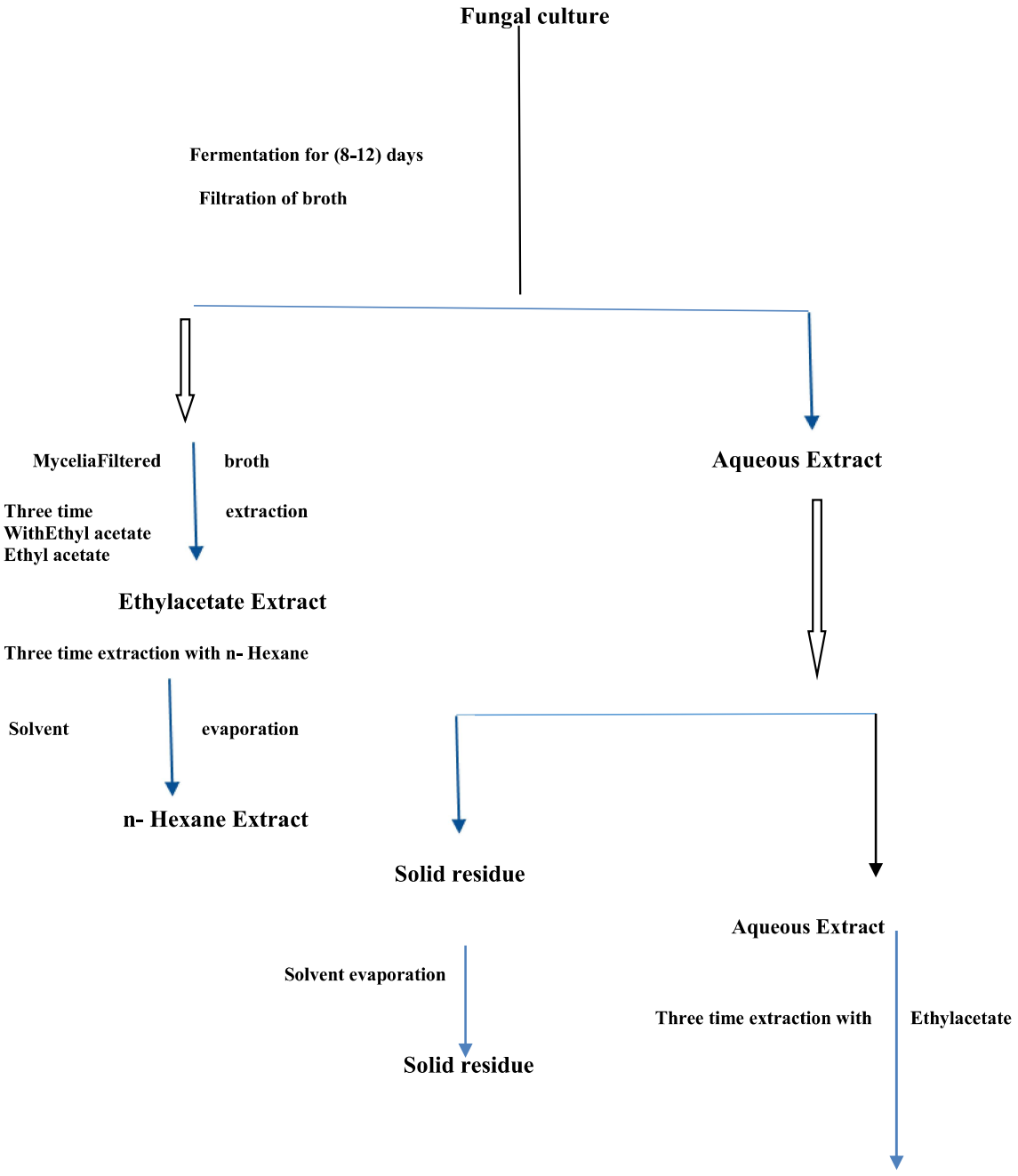
A piece of agar medium (0.3 × 0.3 cm) containing fungal hyphae was inoculated in each 500 ml Erlenmeyer flask containing150 ml sterile potato dextrose broth (PD) medium, and incubated on a rotary shaker at 150 rpm and at 28±2 ºC for 12 days. A total of 10 flasks were used in order to collect about 2 litre volume of broth. Thereafter, a total of 2 liter fermentation broth was collected for each isolate (CPR1-CPR14). The filtrate was extracted three times with an equal volume of ethyl acetate (EtOAc) and the EtOAc layer was collected. The frozen mycelia was crushed completely and extracted three times repeatedly by ultrasonic treatment and with EtOAc. Both the broth extract mycelial extract were combined and evaporated in a rotatory vacuum dryer till solid residue remained. The residue was stored at 40 ^{0}C and a small fraction of residues were again dissolved in dimethyl sulphoxide (DMSO) for subsequent analysis.

3.5 Large scale Fermentation and metabolite production

For large scale production and fermentation, same culture condition was provided as for small scale production. 5 litre Volume of broth was collected.

3.5.1 Extraction of Metabolites

Filtration process was used for removal of fungal mycelia and this was done first by using cheese cloth and then using what Mann filter paper. Thereafter filtered broth was extracted with ethyl acetate, two phases appeared: organic phase and aqueous phase were separated using separating funnel, dried in rotatory vacuum drier under 40 ^{0}C till solid residues remained for both the phases.



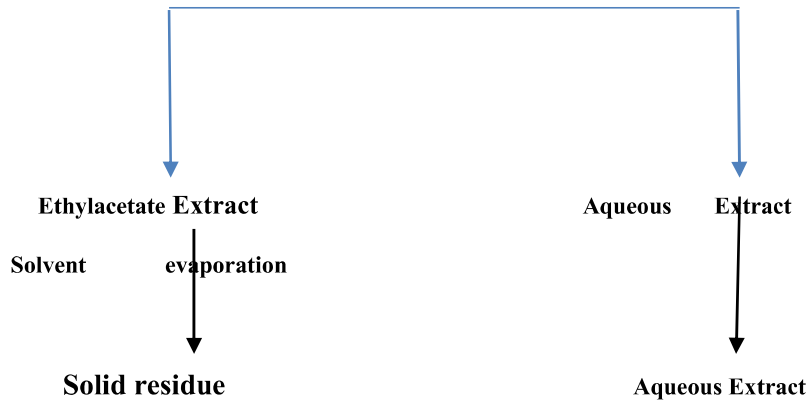


Fig. 4 Flow chart of Extraction of Metabolites: Chromatographic separation

3.6 Morphological Identification of Aspergillus species

Identification of Aspergillus isolates was done at the level of genus on Sabouraud Glucose Agar 4% (SGA4%). For maximum sensitivity and specificity of routine culture approach for identification of fungus at the level of species, four differential media including, czapek dox agar (CZ; czapek concentration 10.0 ml, K_2HPO_4 1.0 g, sucrose 30.0 g, agar 17.5 g, distilled water (DW) 1.0 litre (Maren A.Klich CBS-2002), Czapek yeast agar (CYA) (czapek concentration 10.0 ml, $K2HPO_4$ 1.0 g, powdered yeast extract 5.0 g, sucrose 30.0 g, Agar 15.0 g and DW 1.0 l, malt extract agar (MEA) (powdered malt extract 20.0 g, Peptone 10.0 g, Glucose 20.0 g, Agar 20.0 g, DW 1.0 l) and czapek yeast 20% sucrose agar (czapek concentration 10.0 ml, $K2HPO_4$ 1.0gr, powdered yeast extract 5.0 g , sucrose 200.0 g , agar 15.0 g, DW 1.0 l). Identification of fungal species was done on the basis of cultural and morphological characteristics; features like colony colour, texture and margins, as well as microscopic such as size of conidia and conidiophores and their arrangements were examined for species differentiation (Ainsworth et al., 1973) Hawksworth et a1., 1983) Slide culture method was used for microscopic study of standard strains and most of our isolates. A very

little amount of sporulated fungal culture was placed onto glass slide, drop of lacto-fuchsine was placed and 22 X 22 mm cover slip was carefully placed over the drop. During the overall process of slide preparation, entrapment of air bubble was strictly avoided. Conidial heads, stipes, color and length vesicles shape and seriation. Under compound microscope, metula (ball like structure formed by mycellia) were clearly seen, conidia size, shape and roughness are microscopic characteristics for the identification of fungal species.

3.7 Microorganisms Tested for Antimicrobial Activity

On the basis of pre-screening, it was observed that the endophytic isolate *Aspergillus niger* CPR5 showed high antibacterial and antifungal activity against *Escherichia coli, Streptococcus pneumonia, Bacillus subtilis, Staphylococcus hyicus, B. sphaericus, Staphylococcus aureus*, *pseudomonas aeruginosa,* plant pathogenic bacteria; *Xanthomonas oryzae* and human pathogenic fungus*, Candida albicans* and four plant pathogenic fungi *Sclerotium rolfsii, Sclerotinia scleratiourum, Fusarium species* and *Penicillin sp.* The inhibitory effect of the extract obtained from endophytic fungi was tested by a modified Bauer-Kirby method (Bauer et al., 1966). To test antimicrobial activity against plant pathogenic microorganisms, plant pathogenic fungi and bacteria were collected from Institute of Agriculture Science, BHU Varanasi. Each microorganism was inoculated with overnight culture of each bacterial suspension in the same way for the fungal pathogens; PDA plates were inoculated with each fungal suspension. The microbial suspensions were evenly spread out with sterile glass spreader. Sterile paper disks (6 mm diameter) was placed in the Centre of each Petri plate and loaded with 15µl of crude extract and sealed with parafilm. Bacterial plates were incubated at 36±2 ^{0}C for 24 h and fungal plates at 27 ^{0}C for 48 h, respectively (Barrios et al., 2003). The zone of inhibition was measured and expressed in diameter in

millimeter. Based on the results of preliminary screening best fungal isolates was selected as potential strains for further investigations. Three replicates of each experiment were maintained to avoid error in measurements.

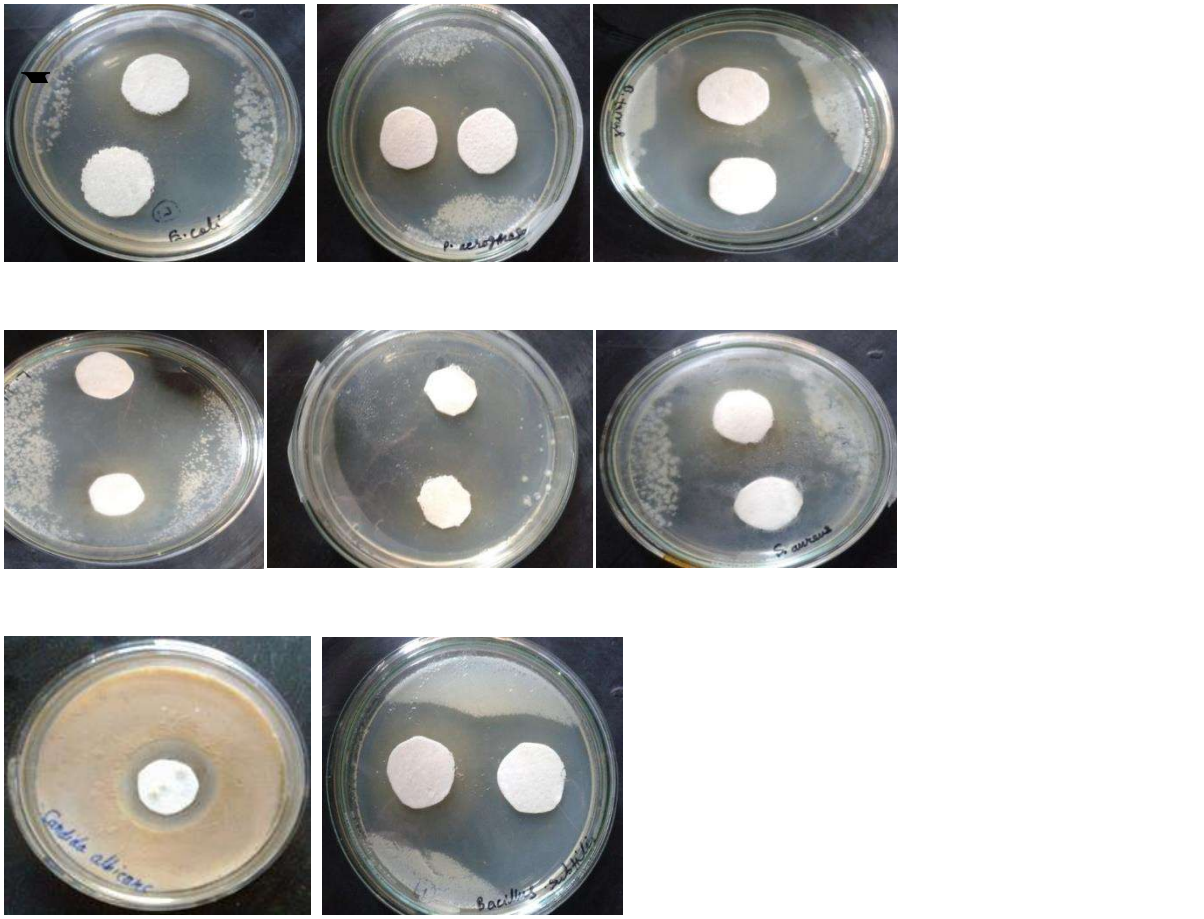


Fig. 5 Antibacterial activity test of crud metabolite (ethylacetate extract) of isolate CPR 5 against bacterial species: 1. *Pseudomonas aeruginosa;* 2. Streptococcus *pneumonia;* 3. *Bacillus subtilis,* 4. *Staphylococcus aureus*, 5.*Escherichia coli,* 6. *Staphylococcus hyicus* 7. *Candida albicans (Fungus).* 8. *B sphaericus.*

3.8 Determination of minimum inhibitory concentration

The minimal inhibitory concentrations (MICs) were determined by paper–disk assay on Luria Broth (yeast extract 5, peptone 10, NaCl 5 and agar 20 g:l, pH 7.0) and:or PDA plates seeded with 10^6 cells (and : or spores):ml suspension of tested bacteria and fungi, followed by incubation at 37 °C for bacteria (48 h) and at 28 °C for fungi (96 h), respectively. All metabolites isolated from the culture were dissolved in ethanol and applied to disks at different concentrations. The plates were incubated for 24 h at 37 °C. MIC was determined as the least concentration of the crude metabolites that inhibited the growth of the test organisms. The reference antibiotic discs were amoxicillin (10 µg/ disc) from Merk Laboratories Pvt. Ltd.

3.9 Molecular identification of endophytic fungus CPR5

Molecular identification of endophytic fungus CPR5 was conducted on the basis of 18S ribosome RNA sequence using polymerase chain reaction (PCR) cloning technology. Genomic DNA was isolated from fungal mycelia using Prep MAN Ultra Sample Preparation Reagent kit (It is used to prepare PCR ready DNA templet from a wide variety of sourses including bacteria, yest and fungi) according to the manufacture's recommendation (Nucleopore). A pair of ITS primer, IT1 (5'GTAGTCATATGCTTGTCTC3') and IT4 (5' CTTCCGTCAATTCCTTTAAG 3' Qiagen White et al., 1990) were used to amplify the highly specific and conserved sequence for endophytic fungi targeting the gene encoding for 18S rRNA (Wu et al., 2002). PCR was carried out in a programmable thermal controller (Biorad). In PCR reaction mixture of a total volume of 2.5 µl, it contained 10 µl template DNA, 14.5 µl PCR Master Mix (PCR buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP, 0.05U/µl Taq polymerase), 1 µl of each primer (IS1 and IS4) and double distilled water to make up the volume. The amplification of DNA was carried out for 34 cycles having preset program of 1

min at 94 ^{0}C, 1 min at 45 ^{0}C and 2 min at 72 ^{0}C. After the final cycle has been completed, the amplification was extended for 10 min at 72 ^{0}C. The amplified DNA fragment (approximately 400 bp) was purified and was sequenced by Genetic Analyzer. The analysis and comparison of the sequence were performed with nucleotide Basic Local Alignment Search Tool (Blastn) of GeneBank (http://www.ncbi.nlm.nih.gov) (Zhang et al., 2000).

3.10 Media optimization

All experiments in this study were carried out in triplicates shaking cultures to determine the physiological and physical conditions that would affect the antimicrobial agent production of the selected isolate.

3.10. 1 Effect of Carbon and Nitrogen Sources on Biomass and Bioactive Metabolite Production

The effect of different carbon sources on growth and bioactive metabolite production was studied by replacing glucose in the basal medium with other carbon sources like galactose, maltose, lactose, cellobiose, starch etc. Four flasks were set up for each carbon source tested. After the incubation period, to study the effect of carbon sources on biomass production, the mycelia was separated by centrifugation and dried at 70 ^{0}C until a constant weight was obtained which was expressed as mg/100 ml. The triplicate flasks were used for ethyl acetate extraction of the filtrate, in order to determine the effect of carbon and nitrogen sources on the production of bioactive metabolites. The extracts were concentrated and used for testing antimicrobial activity. The bioactivity was determined by paper disk diffusion method against *Bacillus cereus*. 15 µl of the ethyl acetate extract (1 mg/ml) was used to optimize anti microbial metabolite production. The diameters of the zones of inhibition were noted. Similarly, for optimization of nitrogen sources on the production of bioactive metabolites,

various nitrogen sources like ammonium sulphate and potassium nitrate sodium nitrate peptone and ammonium chloride were used.

3.10.2 Effect of Incubation Period on Biomass and Bioactive Metabolite Production

The isolats was inoculated into the basal medium and incubated up to 20 days in a rotary shaker at 130 rpm at 28˚C± 2. For the determination of bioactive metabolite production, 1 ml aliquots were withdrawn after every 24 hours and subjected to centrifugation. The cell free supernatant thus obtained was concentrated five-fold in the vacuum concentrator and 50 µl was used to determine antimicrobial activity. The diameters of zones of inhibition were noted

3.10.3 Effects of Initial pH on Biomass and Bioactive Metabolite Production

The initial pH levels of the basal media were adjusted from 4 to 11 and the isolates were grown for 10 days at 130 rpm and 28 ˚C. The biomass and bioactive metabolite production was estimated as described above. Similarly, the optimum temperature for growth and bioactive metabolite production was optimized by incubating the isolates at temperatures ranging from 20 ˚C to 40 ˚C. The growth was measured as dried cell mass (mg/100 ml) and bioactive metabolite production was determined on the basis of diameters of zones of inhibition.

3.10.4 Effect of NaCl concentration on biomass and bioactive metabolite production

The effect of salinity on bioactive metabolite produced by the isolate *S. radicinum* was carried out by incubating in various NaCl concentrations, ranging from 3-7% with 1% of carbon and nitrogen source while other parameters were kept at optimum level. The bioactive metabolite production for each sodium chloride concentration were estimated and recorded.

3.10.5 Fractionation of crude Extract

The EtOAc layer was evaporated to dryness (2.57 g) and mixed with 5g silica gel, dried at 50 ^{0}C, and then loaded on a silica gel column (30 X 8,000 mm) containing 200 g silica gel (200–300 mesh) (J. H. Zhao 2012) . The column was eluted with n-hexane and ethyl acetate gradient (100:0, 90:10,800:20, 70:30, 60:40,50:50, 40:60, 30:70,20:80,10:90,0:100,v/v). Still the column had retained the mixture of compounds. Thereafter the column was eluted with chloroform and methanol (4:1, 3:2 1:1, and v/v) finally the column was eluted to methanol and acetone (4:1, 3:2 1:1, v/v). Based on the TLC monitoring, the collected fractions (50 ml each) were combined into eight parts.

F-1, 3.5 g; F-2, 1.20 g; F-3, 0.80 g; F-4, 1.0 g; F-5, 2.5 g; F-6, .75 g, F-7, 0.550 g; F-8, 1.10 g;

Fig. 6 Different stages of column chromatography during fractionation of crude metabolite

3.10.6 Chromatographic separation

For coating nine 20 x 20 plates, about 50g TLC silica gel was dissolved in 60 ml water. The fine silica will not stay on the plate after coating if some type of binder is not included. It would literally fall or blow from the plate. The classical binder used is gypsum (G), which is $CaSO_4.½ H2O$, or Plaster of Paris. In most silica gels it is put in at a level of from 10-15% to give good binding to the glass plates. Once made, then the time available to put in the applicator and to pull across the glass plates is limited, since the binder will hydrate and the slurry will no longer be a flowing liquid. For a stronger layer, polyvinyl alcohol or polyvinyl pyrollidone can be added to a TLC grade silica gel (without Gypsum) in a level of 1-2% by weight as a polymeric binder. After making thin layer (0.25 mm) all plates were air dried followed by heat drying to makes cross-linking of binders. Slower drying of these layers insures no cracking. The next day or before use, the plates can be activated if desired, to remove more of the adsorbed water. If not using immediately, then the water still remaining on an unactuated plate will actually protect the surface more by not allowing impurities to collect on the activated silanols. The plate can be stored with this water and activated for 30 minutes at 110 °C just before use.

A mark was made at one end of each plate at a distance of 0.5 cm. 10µl of fractionated compound was loaded at mark point. Development of plates was carried out in methanol ethyl acetate and chloroform (7:1:1). All plates were inclined at 60^0 in individual beaker containing sufficient amount (100 ml) of developing solvent. Thereafter all beakers were covered with aluminum foil to avoid air contact of the system. After 15-20 min, TLC plates were removed from developing solvents and dried in oven for 30 minutes at 110 °C; orange

brown spots were appeared in each plate. When sprayed with sulphuric acid and heated mostly turned pink-orange.

Fig. 7 TLC of eight fractionated compounds.

4.10.7 Spectroscopic Measurements

In order to predict the chemical structure of nine antimicrobial compounds isolated,they were analyzed using Fourier Transform InfraRed (FT-IR), Nuclear magnetic resonance spectroscopy (NMR). All NMR data were collected on JEOL AL 300 FTNMR. Mass spectra were recorded on Agilent 6520 Q-TOF (ESI-HRMS) mass spectrometer. Chemical shift were expressed in δ (ppm) and coupling constant J in Hz.FT-IR spectra was collected.

3.10.8 Antifungal activity test of fraction

All the fractions were tested for antibacterial activity and antifungal activity F-1, F-6, F-7, F-8, F-9 were found to be active against *Phoma exigua*, *Fusarium oxysporum f. sp. lycopersici, Penicillium sp, candida albicans and candida tropicalis* were found to be sensitive for F-1, F-5, F-6, F-9 F-1, F-5, F-6, F-9 were active against *sclerotium rolfsii, sclerotium sclerotiorum.*