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Isolation, Identification and Molecular Characterization of Endophytic Fungi from Belpharis Maderaspatensis

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Abstract: *Endophytes are an increasingly important area of research in many fields because of their chemical diversity and their ability to produce many novel secondary metabolites that can be utilized for fuel, medicine and agriculture. It is their chemical diversity that sparks profound interest in these organisms. The endophytic fungi are symbiotic fungi that grows between the intercellular spaces in the plant tissue without any visible change in the morphology of the leaf. They are non-pathogenic and help in the survival of the plant during stress conditions and helps in the nutrition uptake. The fresh leaves and stem of the plant was placed in PDA. The agar showed 5 different species of fungi, the fungi without visible spore was chosen as the target fungi. The fungal DNA was isolated and amplified in PCR using ITS Primer. It was identified by Gene sequencing and BLAST; the obtained sequence was then uploaded to the International Gene Bank and the accession number (MN577295.1) was obtained. Upon GC-MS analysis of ethyl acetate extract of Colletotrichumgloeosporioides revealed the presence of 39 bio active compounds. The Antimicrobial sensitivity test showed good zone of inhibition against Klebsiella pneumonia, E.coli and the zones were on par with third generation antibiotic Amikacin. Hence the endophyte Colletotrichumgloeosporioide spossess undeniable uses in both agricultural and pharmaceutical industries..*

Keywords: *Blepharismaderaspatensis*, *Colletotrichumgloeosporioides*, BLAST, GC-MS, Antimicrobial sensitivity

I. INTRODUCTION

De Bary (1866) coined the word "endophyte" which was originally given to any organism found within a plant. The meaning of the phrase has changed over time, and it is currently often used to refer to "fungi and bacteria that, for all intents and purposes, Invade the tissues of living plants as part of their life cycle generate silent and undetectable infections totally within the body in which plant tissues are affected, but there are no signs or symptoms of disease. Endophytic fungi are very unique in the branch of fungi because they are almost present present in every plant inside the inner cellular spaces. The word endophyte and endophytic fungi are used to describe the internal mycota of the living plants. Endophytes are harmless and are helpful in increasing the growth of the plant and its ability to tolerate the biotic factors and abiotic factors such as drought, insects, disease causing parasite pathogens and herbivores. In relation with plant the endophyte receives carbon for energy from the plant host. Endophytes like fungi, bacteria and virus have found to occur in the tropical and rainforest where the diversity of the plants are high and lead to new discovery of anti-microbial anti-cancer and new miscellaneous bioactive compound (Verma, V. C., Kharwar, R. N., &Strobel, G. A. 2009)

Endophytic fungi are very diverse and their DNA vary with their host plant and hence paves way for the production of unique, novel and exclusive bioactive compounds and secondary metabolites (Kusari, S., Hertweck, C., & Spiteller, M. 2012)All plants synthesis chemical compounds which give them an evolutionary improvement, such as protecting against herbivores or, in the example of salicylic acid, as a hormone in plant defences. The anti-inflammatory and antinociceptive tests suggest that *Blepharismaderaspatensis* is effective against inflammatory and nociceptive pains

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with a more pronounced effect (Sowemimo, A., et all 2013). The membrane stabilising action of different extracts of*Blepharismaderaspatensis* (L.) Heyne ex Roth. and *Blepharismolluginifolia* Pers.whole plants were performed on the human erythrocyte membrane. Among the test samples tested for membrane stabilization, *Blepharismaderaspatensis* ethyl acetate extract showed better membrane stability with 323.3±15.7 IC_{50} (µg/ml), followed by with same *Blepharismaderaspatensis* alcoholic extract with 377.6±21.3 IC_{50} (µg/ml) in comparison with the standard Ibuprofen which has 479.0±17.4 IC_{50} (µg/ml)(H.S. Neelambika et al., 2014). The pharmacognostic study of the *Blepharismaderaspatensis* has been carried out for the first time. This could be useful in the preparation of the herbal section of proposed pharmacopoeia. Using chemical fertilizers is not environmentally friendly and cause the plant to mutate and produce ill effects to the people who consume them so avoiding them is not only environmentally friendly but also result in sustainable agricultural practise, bio fertilizers are eco-friendly and using microbiome to increase the fertility of the soli and increase the nutrient uptake by the plant, endophytic fungi play a huge role in it.(Bhardwaj, D., Ansari, M., Sahoo, R., &Tuteja, N. 2014).

II. Materials and Methods

2.1 Sample Collection

The plant *Blepharismaderaspatensis* was collected from Atri Health products in Chennai, India. The leaves and stem were sterilized and kept in Potato Dextrose Agar and incubated for 10 days in an incubator at 35°C. Based on the visible morphological difference in the 10-day old fungal culture, each of the different fungi was again sub-cultured in a freshly prepared PDA plate. The fungi without visible spores were selected and the DNA was isolated.

Isolation of genomic DNA from Fungi

The genomic DNA was extracted from 7-day old fungal cultures grown in Potato Dextrose broth. DNA was extracted from the fungal mycelium as described by Doyle and Doyle (1987). Before isolation of DNA, Cetyltrimethylammonium bromidebuffer (100 mMTris, 1.4 M NaCl, 1.11% EDTA and 2% CTAB) was pre-heated at 60°C in a water bath. After the mycelia were ground with three volumes of cetyltrimethylammonium bromidebuffer using sterile mortar and pestle then it was incubated at 65°C for 30 min, then equal volume of phenol:chloroform (1:1) was added, the slurry was mixed gently and centrifuged at room temperature for 10 min at 10,000 rpm. The supernatant was re-extracted with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged as above. The clear supernatant was transferred into new eppendorf tube and 100% ethanol was added, and centrifuged as above. The pellet was then washed with 75% ethanol and re-suspended in DNAse free water. Purity and quantity of the isolated DNA was estimated by UV spectrophotometer with 250-260 nm. The isolated DNA was stored at -20°C for further use.

Agarose gel electrophoresis of DNA

Appropriate amount of agarose was mixed in 1X agarose gel buffer. The mixture was heated in a boiling water bath by rotating the flask occasionally until agarose was dissolved. The agarose was cooled to 65-70°C in a water bath and was poured on to the gel tray to a thickness of 3-5 mm, the comb was inserted before adding the agarose. The gel was allowed to polymerize for 30 minutes. The gel was placed in the electrophoresis tank and filled with 1X agarose electrophoresis buffer. The comb was removed carefully from the gel. One volume of DNA sample was mixed with 5 volumes of DNA sample buffer. It was then applied to the wells of the gel. The sample was run until the bromophenol blue has migrated approximately 2/3 of the way through the gel. The gel was stained after electrophoresis with ethidium bromide and illuminated under UV at 254-366nm.

Amplification and Sequencing

The universal primers available for amplify specific region of fungal rDNA (ITS1, 5.8S, and ITS2 gene regions and portion of the 18S and 28S genes – White *et al*., 1990) was used in the present study. Two oligoes for forward and reverse primers were used to carry out amplification of ITS1, 5.8S, and ITS2 gene regions and portion of the 18S and 28S genes of ten *B. bassiana* isolates. The forward primer sequence used was ITS1-5'-TCCGTAGGTGAACCTGCGG-3' and reverse primer sequence used ITS4-5'-TCCTCCG- CTTATTGATATGC-3'.

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Specific Amplification of rDNA-its Sequence

A clean work surface was used for all PCR preparations. PCR mix was prepared in a laminar air flow chamber. The ITS amplification reaction mixture (30µl) consisted of 2X master mix (Amplicon, Denmark) with 10 ng of template DNA and 10 pmol of each primer. PCR amplification was performed in Agilent SureCycler 8800® gradient PCR machine. The following cyclic conditions were performed: initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation for 45 sec at 94°C; annealing for 1 min at 59°C; extension for 1 min at 72°C followed by a final extension at 72°C for 5 min. The amplified PCR products were electrophoresed along with 1 kb Ready- to -Use DNA marker on 1.2% gel at 100 volts for 45 min at room temperature using 1X TAE buffer (Tris–acetate 40mM pH 8.0, EDTA 1mM, pH 8.0). The PCR products were stained with ethidium bromide and visualized and photographed using Gelstan gel documentation system.

rDNA-its Sequencing

The amplified ITS fragment was purified and sequenced using DNA sequencing services (Eurofins Scientific, Bangalore) employing the same primer used for PCR amplification. The sequences were edited using Bioedit software v 7.0.9 (Hall, 1999). The edited sequences were blast with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) using the algorithm Blast N. The output of BLAST searches was sorted based on maximum identity with other genus or species names in GenBank records. The sequence-based identity showed cut-off of 97% or greater was considered as significant, and best hit was defined as the sequence with highest maximum identity to the query sequence. The gene sequences of the fungal isolates were deposited in GenBank and accession numbers were obtained.

Solvent Extraction

The fungi were cultivated on PDB by inoculating selected endophyte cultures in 250 mL Erlenmeyer flask containing 100 mL of the medium. The flask was incubated at 28°C for 2 weeks with periodical shaking at 150 rpm. After the incubation period, the fermentation broth of the fungus was homogenized by adding 10% methanol to it. Metabolite was extracted by solvent extraction procedure using ethyl acetate and methanol as organic solvents. To the filtrate equal volume of solvents were added, mixed well for 10 min and kept for 5 min till the two clear immiscible layers formed. The upper layer of solvent containing the extracted compounds was separated using separating funnel. Solvent was evaporated and the resultant compound was dried in rotator vaccum evaporator to yield the crude.

GC-MS analysis:

The crude ethyl acetate extract of *Colletotrichumgloeosporioides* was sent to Applied Biosystems for Gas chromatograph interfaced (GC-MS).

Antimicrobial Study

The antimicrobial activity of*Colletotrichumgloeosporioides* ethyl acetate extract was done using agar well diffusion method. A stock solution (1mg/ml) of the compound and the dilutions of the stock solution containing 0.5, 1.0, 1.5 and 2.0mg/ml were prepared in dimethyl sulfoxide (DMSO). The Gram-negative bacteria *Klebsiellapneumoniae* and Proteus *Pseudomonas aeruginosa* and the Gram-positive bacteria *Staphylococcus aureus* and *proteusmirabilis* were used as test strains. Bacterial inoculum was prepared for each strain was adjusted to the McFarland standard 0.5 Scale. Bacterial lawn culture was made on Muller Hinton Agar (MHA) plates. Prepared various concentration of the compound were loaded in well made on a MHA plate. The amikacin (30µg) was used as standard reference drugs. The plates were incubated at 37°C for 18 - 24h and the zone of inhibition was measured in mm and compared with standard antibiotic discs.

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III. Results and Discussion

3.1 Isolation of Endophytic Fungi

The fresh leaves and stem of *Blepharismaderaspatensis* were washed with double distilled water and then it is washed with 70% ethanol.They were placed in freshly prepared PDA as in figure 1.1 and kept for incubation at 35ºC for 5 days.

Figure 1.1

Figure 1.2

After 3 days of overnight incubation, we observed five different species of fungi based on their morphological characters which was in figure 1.2a – 1.2e. They are sub-cultured individually and inoculated into a new PDA plate and incubated at 35ºC for 10 days. The fungi without visible spore was chosen as the target and figure 1.2.a and figure 1.2.b are predicted as *Aspergillusniger*.

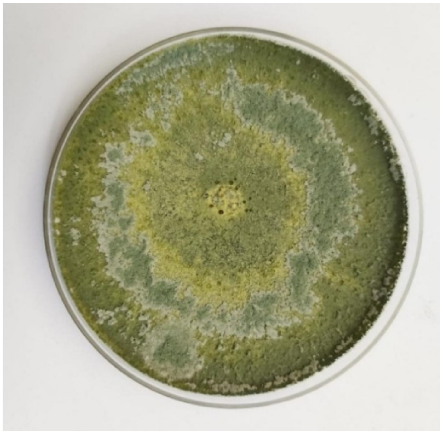

Figure 1.2.a

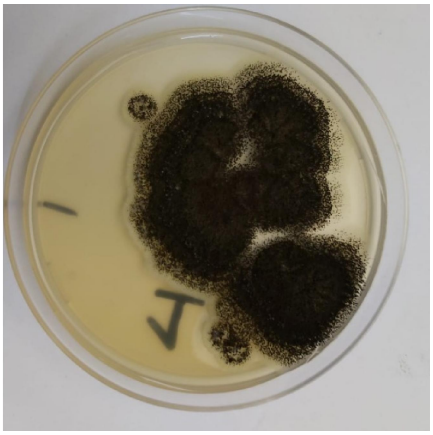

Figure 1.2.b

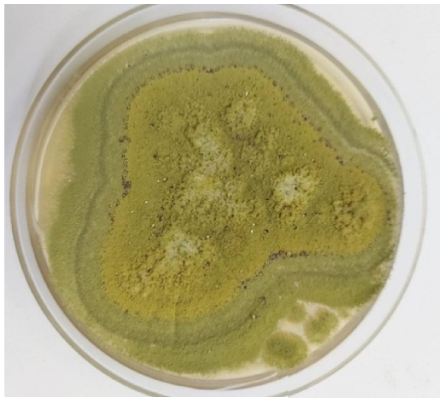

Figure 1.2.c

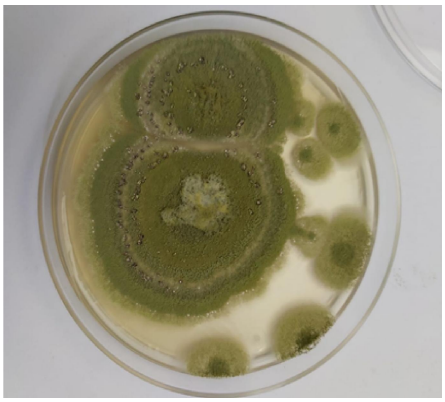

Figure 1.2.d

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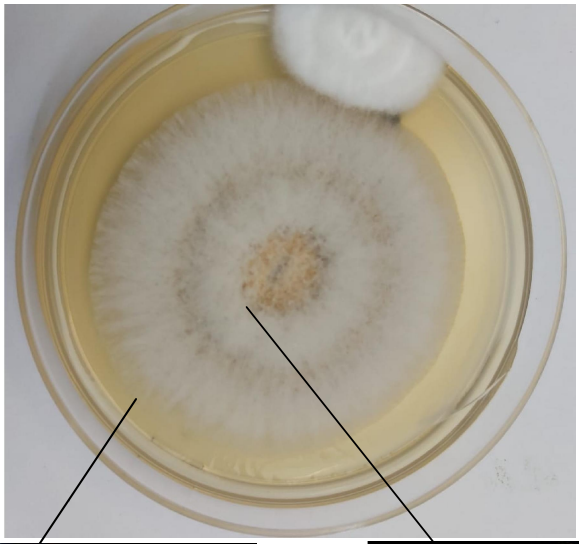
Figure 1.2.e

After selecting the fungal culture in the figure 1.2.e, we viewed the fungi under a microscope with a magnification of 40X and used Lacto phenol cotton blue as the staining dye. Fungus is filamentous around the edges of the culture and in the centre, the fungus was more distinctive and we suspect it to be *Fusariumoxysporum.*



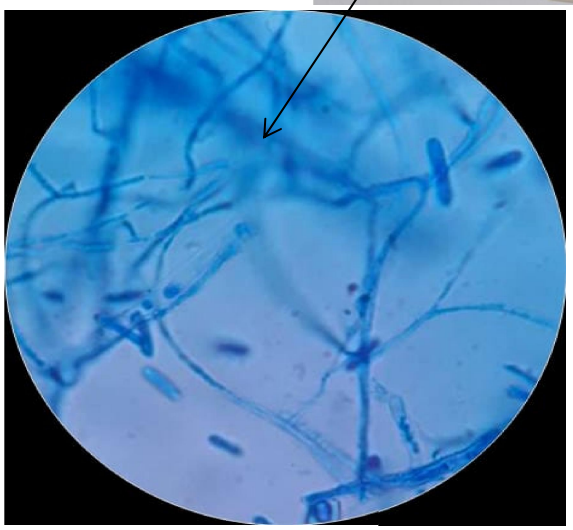


Figure 1.3

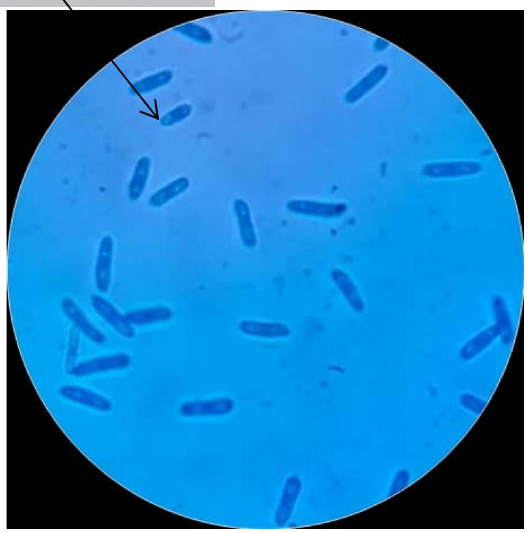


Figure 1.4

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Isolation of DNA& PCR Amplification

The genomic DNA was extracted from 5 to 7 days old fungal cultures grown in Potato Dextrose broth. DNA was extracted from the fungal mycelium by CTAB method. The extracted DNA was then run in Agarose Gel Electrophoresis and its size was found using DNA Marker 2kb, the mass of our DNA sample was 50ng and its size is 250bp.

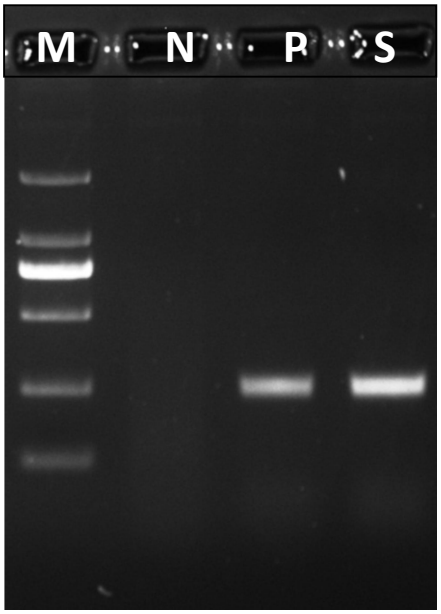

Figure 1.5 PCR

M- DNA Marker 2kb
N- Negative
P- Positive
S – Sample for ITS 1 and 2 primer
ITS1-TCCGTAGGTGAACCTGCGG
ITS2 -GCTGCGTTCTTCATCGATGC

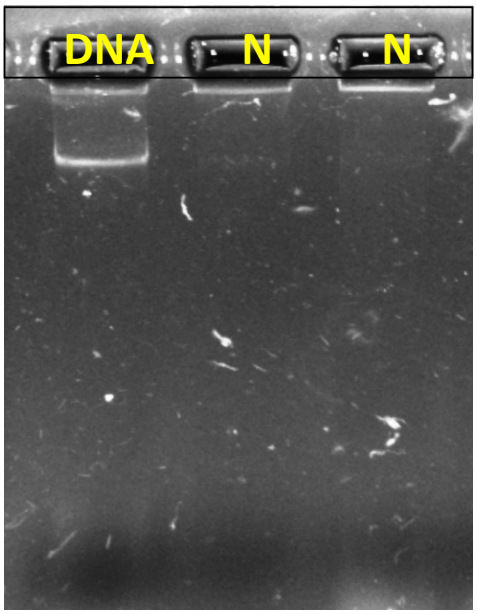

Figure 1.6 Fungal DNA with 1% agarose

N- Negative
N - Negative

DNA Sequencing:

The amplified ITS fragment was purified and sequenced using DNA sequencing services (Eurofins Scientific, Bangalore) employing the same primer used for PCR amplification. The sequences were edited using Bio edit software v 7.0.9 (Hall, 1999) which was shown in figure 1.7. The edited sequences are performed in BLAST with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) using the algorithm Blast N. The output of BLAST searches was sorted based on maximum identity with other genus or species names in GenBank records which was shown in figure 1.8. The sequence-based identity showed cut-off of 99% for *Colletotrichumgloeosporioides*was considered as significant, and best hit was defined as the sequence with highest maximum identity to the query sequence. The gene sequences of the fungal isolates were deposited in GenBank and accession numbersSUB6433168 Colletotrichum -MN577295was obtained.

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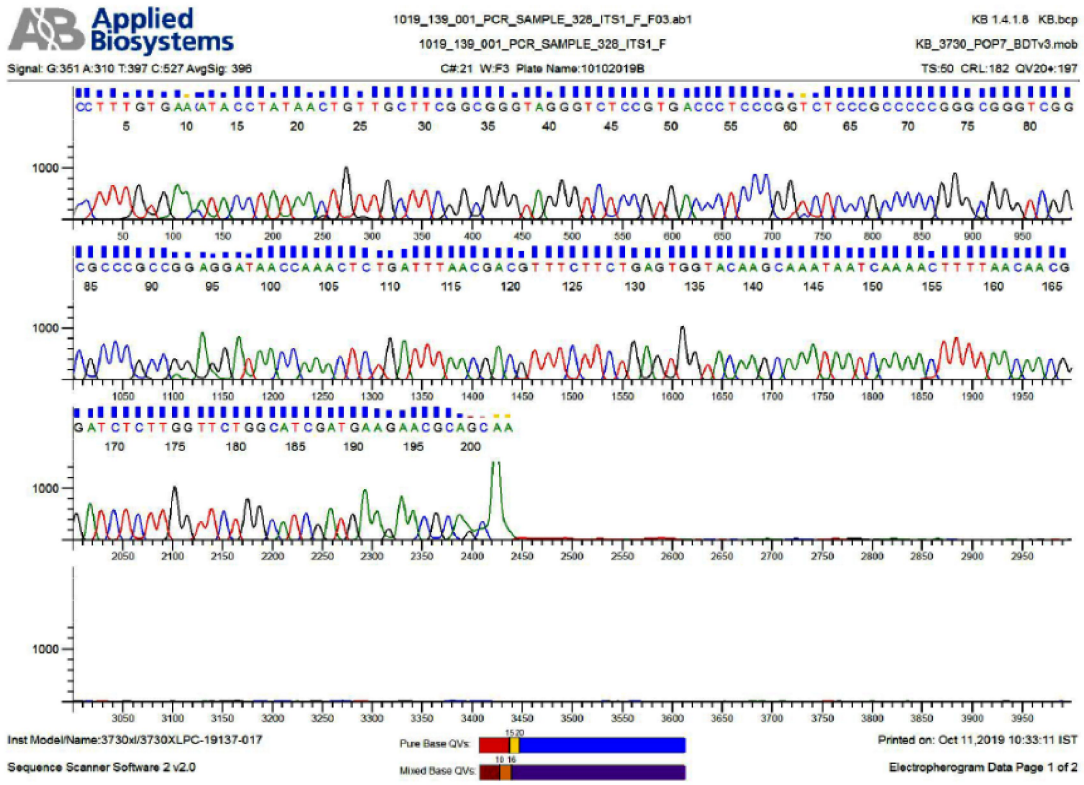

Figure 1.7 Gene sequencing Tendogram

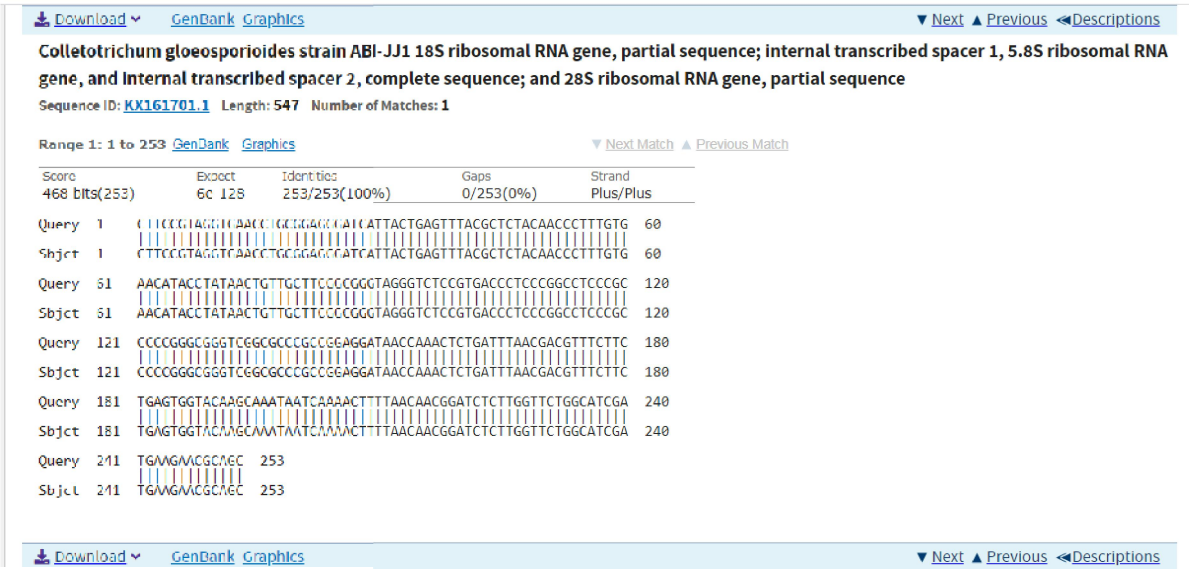

Figure 1.8 Blast results

GC-MS Analysis

GC MS analysis was done in crude ethyl acetate extract of *Colletotrichumgloeosporioides* and 39 bioactive compounds were identified. Bioactive compounds are listed in Table 1 and peaks are shown in figure 1.9.

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Table 1 Phyto components identification using GC-MS

S. No	Rt	Name Of The Compound	Molecular Formula	Molecular Weight	Peak Area%	Biological Activity
1	4.734	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11	4.44	Treatment and prevention of Hypoxia, Anemia,
2	4.954	Benzeneacetic acid	$C_8H_8O_2$	136.14	2.46	Antimicrobial activity
3	5.425	Benzylmalonic acid	$C_{10}H_{10}O_4$	194.18	0.44	Predicted metabolite generated by Bio Transformer
4	5.600	Cyclododecane	$C_{12}H_{24}$	168.31	0.83	It is a precursor to laurolactam, a precursor to the polymer Nylon-12
5	5.659	Tetradecane	$C_{14}H_{30}$	198.38	0.44	Role as a plant metabolite and a volatile oil component.
6	6.069	Benzeneethanol, 4-hydroxy	$C_8H_{10}O_2$	138.16	2.96	Antioxidant
7	6.194	Phenol, 4-ethyl	$C_8H_{10}O$	122.16	0.71	Used as an indicator
8	6.395	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206.32	10.58	Antioxidant
9	6.791	Dodecanoic acid	$C_{12}H_{24}O_2$	200.31	0.76	Antibacterial
10	6.884	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.23	1.65	Role as a teratogenic agent, a neurotoxin and a plasticiser
11	6.912	Cyclotetradecane	$C_{14}H_{28}$	196.37	3.89	Provide nutrients
12	6.958	Hexadecane	$C_{16}H_{34}$	226.44	1.07	Component of essential oil isolated from long pepper and It has a role as a plant metabolite
13	7.066	Oxirane, hexadecyl	$C_{18}H_{36}O$	268.47	0.36	Antimicrobial
14	7.950	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	0.59	It acts as a human, algal metabolites and a role as an inhibitor
15	8.084	1-Nonadecene	$C_{19}H_{38}$	266.50	7.41	It has a role as a plant metabolite and a bacterial metabolite
16	8.118	Octadecane	$C_{18}H_{38}$	254.49	1.14	It has a role as a bacterial metabolite and a plant metabolite.
17	8.239	Oxirane, tetradecyl	$C_{16}H_{32}O$	240.42	0.64	Antimicrobial
18	8.318	Phenol, 3,5-dimethoxy-	$C_8H_{10}O_3$	154.16	1.58	It is a toxin metabolite found in

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						*Taxusbaccata*leaves
19	8.461	1,2-Benzenedicarboxylic acid,	$C_8H_6O_4$	166.13	9.86	It reduces the sperm concentration and a vulnerable effect of anti-androgen
20	8.723	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276.37	0.83	Antioxidant
21	8.794	3-Amino-2,6-dimethoxypyridine	$C_7H_{10}N_2O_2$	154.17	0.92	Antibacterial
22	8.894	5-Nitroso-2,4,6-triaminopyrimidine	$C_4H_6N_6O$	154.13	1.85	Antifungal and anti-bacterial
23	8.938	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	1.97	Ateratogenic agent, a plasticiser, a metabolite
24	9.043	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	5.71	It acts as algal metabolites
25	9.142	1-Nonadecene	$C_{19}H_{38}$	266.50	4.25	It has a role as a plant metabolite and a bacterial metabolite.
26	9.177	Eicosane	$C_{20}H_{42}$	282.54	0.92	It has a role as a plant metabolite
27	9.322	Oxirane, tetradecyl-	$C_{16}H_{32}O$	240.42	0.50	Antimicrobial
28	9.948	Benzene, propyl-	C_9H_{12}	120.19	1.97	Used to make other chemicals
29	9.985	Carbonic acid, isobutyl 2-ethylhexyl ester	$C_{13}H_{26}O_3$	230.34	2.63	Antibacterial
30	10.126	Cycloeicosane	$C_{20}H_{40}$	280.53	2.93	Helps in separation and identification of hydrocarbons
31	10.277	Trifluoroacetic acid, pentadecyl ester	$C_{17}H_{31}F_3O_2$	324.42	2.98	Used as an organic synthesis
32	10.314	Tritetracontane	$C_{43}H_{88}$	605.15	0.42	Antibacterial
33	10.539	Dodecane	$C_{12}H_{26}$	170.33	0.63	Role in plant metabolites
34	11.641	1-Heptacosanol	$C_{27}H_{56}O$	396.73	1.79	Role in marine and plant metabolites
35	11.969	1-Dodecanol	$C_{12}H_{26}O$	186.33	0.59	It has a role as a cosmetic, a pheromone, a pesticide and a plant metabolite.
36	12.786	Phthalic acid, di(oct-3-yl) ester	$C_{24}H_{38}O_4$	390.55	15.17	Acts as ananti-inflammatory, hypercholesterolemic activity.

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37	13.214	Heptacosyl acetate	$C_{29}H_{58}O_2$	438.76	1.12	Antimicrobial
38	14.915	1-Heptacosanol	$C_{27}H_{56}O$	396.73	0.59	Acts as a marine and plant metabolite.
39	16.690	Docosylpentafluoropropionate	$C_{25}H_{45}F_5O_2$	472.61	0.44	Antioxidant

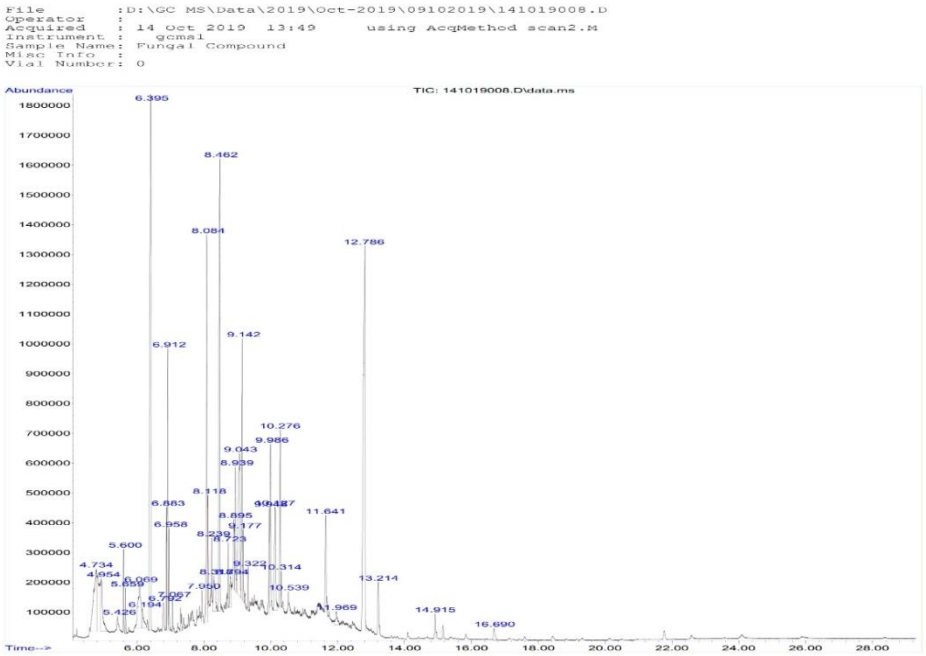

Figure 1.9: GC MS analysis of ethyl acetate extract of *Colletotrichumgloeosporioides*

Antimicrobial Sensitivity Test:

The antimicrobial activity of *Colletotrichumgloeosporioides* ethyl acetate extract was done using agar well diffusion method. The showed the zone of inhibition of 10, 14, 18 and 25 mm against *Klebsiella pneumonia* 10, 12, 14 and 18 mm against *Pseudomonas aeruginosa*, 8, 10, 12 and 15mm against *Staphylococcus aureus* 12, 15, 17 and 23 mm against *E.coli* and 9, 13, 15 and 19 against *Proteus mirabilis* at the concentration of 0.5, 1, 1.5 and 2mg respectively. In this study, the antibacterial activity exhibited by *Colletotrichumgloeosporioide*s against *Klebsiella pneumonia* and *E.coli*was almost similar that of the zone of inhibition of the Amikacin. Results was shown in figure 1.10a – 1.10e and listed in table 2.

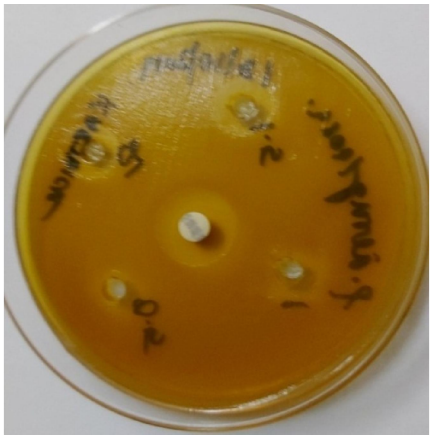

Figure 1.10a Pseudomonas aeruginosa

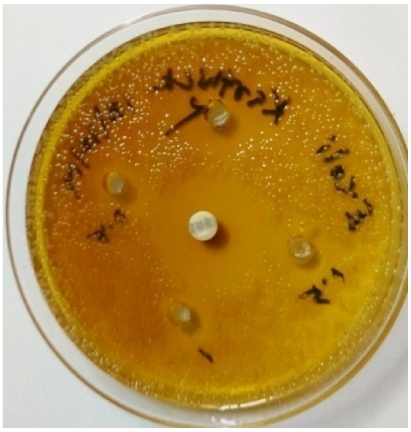

Figure 1.10bE.coli

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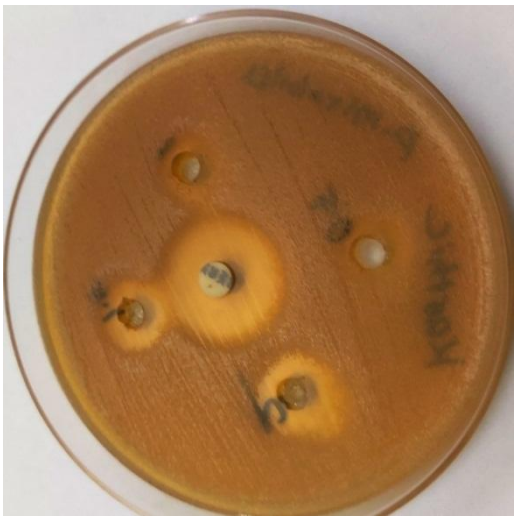

Figure 1.10cProteus mirabilis

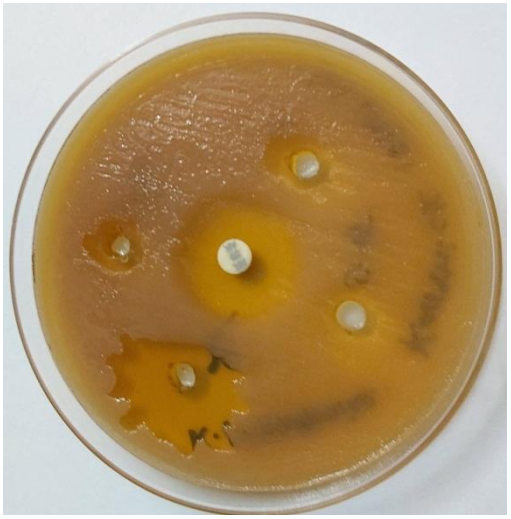

Figure 1.10dKlebsiella pneumonia

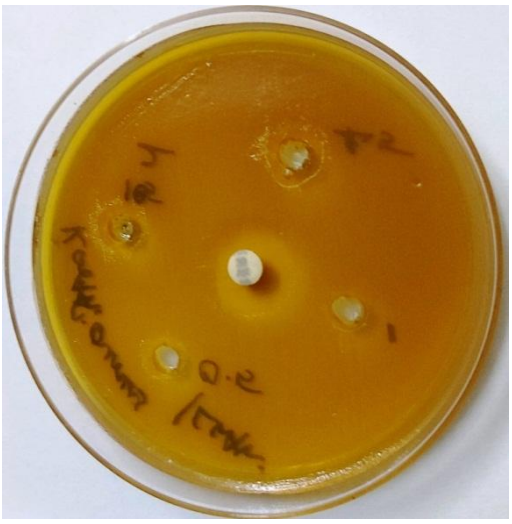

Figure 1.10e Staphylococcus aureus

Table 2: Anti-microbial sensitivity test

Organism	Zone of inhibition (in mm) of *Colletotrichumgloeosporioides* extract				
	Amikacin-30µg (Standard)	0.5 mg	1 mg	1.5 mg	2 mg
Klebsiellapneumoniae	26	10	14	18	25
Pseudomonas aeruginosa	18	10	12	14	18
Staphylococcusaureus	20	8	10	12	15
E.coli	25	12	15	17	23
Proteus mirabilis	24	9	13	15	19

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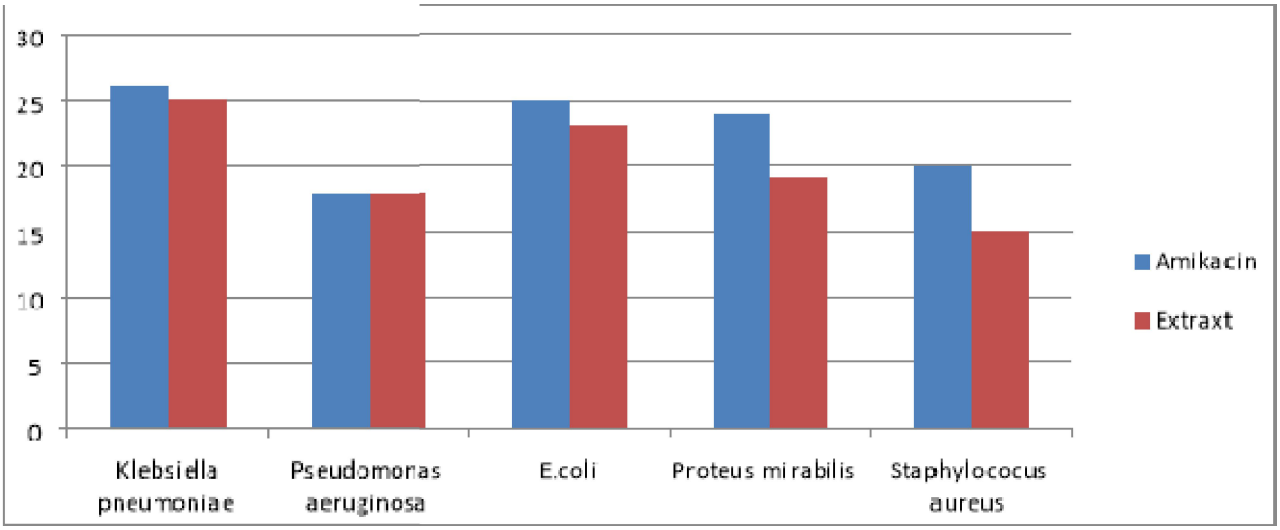

Figure 2 Antimicrobial Sensitivity test

IV. CONCLUSION

Plant sample was authenticated by Plant Anatomy Research Centre. Isolation of endophytic fungi from plant samples using PDA was performed. The DNA was extracted from fungi and plant sample using by CTAB method and the mass and size of the DNA was found out to be 50 ng and 250 bp. Molecular level identification was carried out using PCR with the help of universalInternal transcribed spacer (ITS) primers and the fungi was found out to be *Colletotrichumgloeosporioides*. After the PCR amplification, the samples were sequenced using mega software version 7 and the sequence was submitted to Gene Bank and the accession number SUB6433168 Colletotrichum -MN577295 was obtained. Secondary metabolites were extracted using ethyl acetate solvent and it was characterized using GC-MS.GC-MS analysis of secondary metabolites from the ethyl acetate extract revealed the presence of 39 bioactive compounds. Antimicrobial sensitivity test was carried out and the maximum zone on inhibition was found in *Klebsiellapneumoniae*. Further research will explore the drug potential on fungi using ADME tool and also can be utilized as a potential Biofuel source.

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