Antibacterial Potential of Endophytes Isolated from Catharanthus Roseus

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***Abstract* - Many researchers have been working on cancer treatments and therapies since years, in that natural products have always been a source of many high value compounds like alkaloids. Catharanthus roseus is a flowering plant belonging to Apocynaceae family. It mainly consists of vinca alkaloids which are used for treatment of breast cancer, lymphoma, neuroblastoma and testicular cancers. But the cost and yield factors have been major concerns for plant-based drugs. It has been reviewed that for the production of 1 gm of vinca alkaloid we need 500 kg of leaves. It is estimated that global requirement of these alkaloids is 3 kg per annum and 1 kg costs around 3 million dollars. Endophytes are microorganisms that live in symbiotic association with plant cells and produce similar phytocompounds. Identification of such endophytes from its natural sources like Catharanthus roseus would help in paving pathway for alternate sources of vinca alkaloids in a cost-effective manner which would help in meeting the world requirements. The present study aims to isolate fungal endophytes from Catharanthus roseus and study their antibacterial activity against pathogenic microorganisms Myroidesgitamensis and Xenorhabduskhoisanae isolated from wound swabs. A total of 48 pure fungal cultures were isolated using Hyphal tip method. Out of the 48 fungal cultures tested, 7 extracts namely VRT1A, VRT1B, VRT2A, VRT6A, VRL2B, VRL3B and VRT5A have given positive results for antibacterial activity by disc diffusion assay. Further purification and analytical studies would help in identifying novel antibacterial drugs from these Endophytes.**

***Index Terms* - Alkaloids, Endophytes, Catharanthus roseus , Hyphal tip method, Disc diffusion assay.**

I.INTRODUCTION

Medicinal plants comprise approximately 8000 species and account for about 50% of all the higher flowering plant species of India. Over the past decade, herbal medicines have been accepted universally and they have an impact on both world health and international trade. Over one and a half million practitioners in the Indian System of Medicine use plants in preventive, promotive and curative applications which include diabetes, cancer and other diseases[1,3,10].

Catharanthes roseus is a medicinal herb found in many tropical and subtropical regions around the world. It produces many important metabolites which are of high pharmaceutical value. Among them vinca alkaloids are most efficient chemotherapeutic agents which are being used to treat different kinds of cancer. Isolation of these alkaloids from plants is not sufficient to meet world requirements due to very low yields.

Several techniques like callus culture, hairy root culture, shoot cultures, metabolic engineering have been used to increase the yield of these high value compounds. [2,4,9] Despite these continuous efforts desired levels of production of these metabolites has not been achieved. In this regard, endophytes are new ray of hope for increase the production levels of these compounds.

Endophytic fungi live in symbiotic association with the plants, and they produce the same natural products/ phyto compounds as produced by their host. Compounds derived from endophytic fungi have wide applications in medicine, agriculture and food industry. Thus, increasing the production of pharmaceutically high value products using these endophytic fungi is advantageous. Previous studies have shown that *C.roseus* forms host for a wide range of endophytic fungal species such as *Alternaria* and *Fusarium.* Previous studies reported the production of high value compounds like taxol, camptothecin, podophyllotoxin, from endophytic fungi isolated from plants *Taxus*, *Camptotheca acuminate* and *Sinopodophyllum* respectively. [5,6,8] The present study aims to isolate endophytes from *Catharanthus roseus* and study their antibacterial activity against few pathogenic micro-organisms isolated from wound swabs.

II MATERIALS AND METHODS

A *Identification and collection of plant material*

The plant *Catharanthes roseus* has been identified and its twigs and leaves have been collected from the Osmania university campus premises. Its surface is washed gently under running tap water. Now only the healthy leaves and twigs are selected for further fungi isolation.

B *Sterilization of Leaves*

The leaves are sterilized with 70% alcohol for 1 minute followed by sterilization with sodium hypochlorite for 30 seconds and then with 70% alcohol for 30 sec. Now, the leaves are washed with distilled water for some time and then rinsed with sterile water. After the surface sterilization, the leaf sample is immediately inoculated into freshly prepared PDA plate with antibiotic streptomycin sulphate at a concentration of 200mg/Lit.

C Sterilization of twigs

The twigs are cut into 1 inch length in size and surface sterilized in 70% ethanol for 90 seconds followed by sodium hypochlorite for 60 seconds, followed by 70% ethanol for 30 seconds. Now the twigs are washed with distilled water for some time and then rinsed in sterile water.After the surface sterilization, the twig sample is immediately inoculated into freshly prepared PDA plate with antibiotic streptomycin sulphate at a concentration of 200mg/Lit.

D Incubation

The inoculated PDA plates are sealed with parafilm completely and then incubated under normal fluorescent light at 27˚ C and are to be checked daily for the appearance of mycelia growth.[5]

E *Sub-culturing*

As soon as the mycelia starts growing in the plate it is transferred into the freshly prepared PDA plate without antibiotic using sterile needle and incubate at 27 ˚C for the mycelium growth. Repeat the sub culturing step until we separate individual mycelium that grows in the parent plate

F Storage

The pure fungal cultures are isolated and stored in sterile water taken in autoclaved 2ml screw cap vials.

G Growth of pure cultures in PDB media

The above obtained pure cultures are grown in 100 ml of PDB media in 250 ml conical flasks at 120 rpm and at room temperature in orbital shaker for a period of 07 days. After sufficient growth is observed, the fungi are filtered using muslin cloth and filtrate is subjected to solvent extraction with equal volumes of ethyl acetate. The collected organic extract is concentrated using rotary vacuum evaporator and further used for the antibacterial studies.[11]

H *Isolation of pathogenic micro-organisms from wound swabs*

Cotton wound swabs were collected from Apollo Hospitals, Hyderabad. Since they are common medical waste no ethical committee permissions are required. By serial dilution technique, the microbial Inoculum sample was prepared. By inoculating the sample on nutrient agar plates consisting of antifungal agent by spread plate technique. The bacterial colonies formed after an incubation period of 24hrs. Each individual colony is considered as a pure culture. These pure cultures are grown in 50 ml of LB broth for a period of 24 hours followed by absorbance measurement at 600nm which confirms the occurrence of bacterial growth. Once maximum growth is observed, they are again inoculated back on Nutrient agar plates by streak plate technique to confirm the purity of the obtained cultures. In streak plate method, based on the morphology, we can identify the pure culture formation[12-14].

I *Bacterial identification using 16s RNA sequencing*

The obtained bacterial cultures were sent for 16s RNA sequencing for bacterial identification. The sequencing was done by Triyat Genomics Lab, Pune, India. Based on the sequencing report, BLAST analysis was performed and species identification was done [15]

J Antibacterial activity of fungal extracts

The obtained pure bacterial cultures were grown on Nutrient agar plates for 24 hours. After 24 hours of incubation, filter paper discs were dipped in fungal organic extracts and then placed on bacterial culture plates and left for incubation at room temperature. Formation of clear zones around the filter paper discs indicates the inhibition of bacterial growth by fungal extracts[7]. As reference molecules, we have also incubated the bacterial culture plates with standard molecules like Quercetin, Gallic acid (10mg/10ml solutions) which were also known to have antibacterial activity as per literature. These standards were purchased from Merck. The formed zone of inhibitions radius was measured on a scale and noted down.The experiment was done in triplicates and have taken the average value

III. RESULTS AND DISCUSSION

A Endophytes isolated from Catharanthus roseus leaves and twigs.

A total of 48 pure fungal cultures were isolated by Hyphal tip method. They are named with the codes as mentioned in table 1 and table 2 for leaf samples and twig samples respectively.

B *Bacterial identification by 16s RNA sequencing*

The two pure cultures coded as SS1C and SS2A that were isolated from wound swabs were identified as *Myroidesgitamensis and Xenorhabduskhoisanae* based on 16s RNA sequencing result and BLAST analysis. The genomic DNA was isolated using standard protocol provided. The isolated DNA was checked on Agarose gel electrophoresis for purity followed by PCR amplification. DNA sequencing reaction of PCR amplicon was carried out with 27F primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The blast results obtained are mentioned in Table 4 and Table 5 respectively. For the SS2A culture, maximum identity score obtained was 85.32% with Accession number NR_117921.1 and for SS1C culture, the maximum identity score obtained was 97.50 with accession number NR_125560.1.The phylogenetic evolutionary tree is represented for SS1C culture and SS2A in figure I and II respectively. For SS1C, The evolutionary history was inferred using the neighbor joining method. The optimal tree with the sum of branch length = 1.19770870 is shown. The confidence probability (multiplies by 100) that the interior branch length is greater than 0, as estimate using, the bootstrap test (500 replicates is shown next to branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in units the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st +2nd +3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 764 positions in the final dataset.For SS2A, the evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.47462866 as shown in figure. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 634 positions in the final dataset.

C *Antibacterial activity against Myroidesgitamensis* and Xenorhabduskhoisanae

All of the above mentioned culture organic extracts were subjected to disc diffusion assay. Out of all the cultures, only VRT1A, VRT1B, VRT2A, VRT6A, VRL2B, VRL3B and VRT5A have formed zone of inhibitions for both the bacteria. From the zone of inhibition values it can be observed that the fungus with code VRT6A had highest mean value of zone of inhibition for both the bacterial cultures. It was even observed that it had a higher value than the standards as well. Further studies on this fungi might help us in identifying potential biomolecules with better antibacterial properties. Table 5 explains the zone of inhibition values of the fungal extracts.

IV CONCLUSION

Lack of proper hygiene and sterility in hospitals lead to a lot of infections and diseases. Among them cotton wound swabs are one of the major routes of transmission for pathogenic micro-organisms. The multidrug resistance that these pathogenic micro-organisms develop makes it even more difficult for treating these infections. Under such circumstances the present work in which we have identified plant based endophyte culture extracts with antibacterial potential can be found effective. Further purification and analytical studies might help in identification of compounds that are present in those endophyte extracts which might lead to discovery of novel antimicrobial drugs.

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Table 1: Fungus isolated from leaf sample

Leaf Number	Fungus Code			
1	VRL1	VRL1-A		
2	VRL2	VRL2-A		
3	VRL3	VRL3-A	VRL3-B	VRL3-C
4	VRL4	VRL4-A	VRL4-B	VRL4-C
5	VRL5	VRL5-A	VRL5-B	
6	VRL6			
7	VRL7			
8	VRL8			
9	VRL9			

Table 2: fungus isolated from leaf samples

T.No	Fungus code				
1	VRT 1	VRT1-A	VRT1-B	VRT1-C	VRT1-D
2	VRT 2	VRT2-A	VRT2-B	VRT2-C	
3	VRT 3	VRT3-A			
4	VRT 4	VRT4-A	VRT4-B	VRT4-C	
5	VRT 5	VRT5-A	VRT5-B		
6	VRT 6	VRT6-A	VRT6-B		
7	VRT 7	VRT7-A	VRT7-B	VRT7-C	
8	VRT 8	VRT8-A			
9	VRT 9	VRT9-A			

Table 3: Sequences producing significant alignments for SS1C culture

Description	Max Score	Total Score	Query Cover (%)	E Value	Identity %	Accession
Myroidesgitamensis strain BSH-3 16S ribosomal RNA, partial sequence	684	684	100	0	97.50	NR_125560.1
Myroidesodoratimimus strain CCUG 39352 16S ribosomal RNA, partial sequence	684	684	100	0	97.50	NR_042354.1
Myroidesprofundi strain D25 16S ribosomal RNA, partial sequence	678	678	100	0	97.25	NR_118642.1
Myroidesprofundi strain D25 16S ribosomal RNA, partial sequence	667	667	100	0	96.75	NR_044435.1
Myroidesxuanwuensis strain TH-19 16S ribosomal RNA, partial sequence	656	656	100	0	96.26	NR_133793.1
Myroidesphaeus strain MY15 16S ribosomal RNA, partial sequence	651	651	100	0	96.01	NR_117475.1
Myroidesinjenensis M09-0166 16S ribosomal RNA, partial sequence	634	634	100	0	95.25	NR_134774.1
Myroidesmarinus strain JS-08 16S ribosomal RNA, partial sequence	634	634	100	0	95.26	NR_117408.1
Myroidesodoratus DSM 2801 strain NBRC 14945 16S ribosomal RNA, partial sequence	612	612	100	5E-175	94.25	NR_112976.1
Myroidesindicus strain UKS3 16S ribosomal RNA, partial sequence	601	601	100	1E-171	93.75	NR_145659.1

Description	Max Score	Total Score	Query Cover (%)	E Value	Identity %	Accession
Xenorhabduskhoisanae strain SF87 16S ribosomal RNA, partial sequence	337	337	79.00	4E-92	85.32	NR_117921.1
Proteus hauseri strain DSM 14437 16S ribosomal RNA, partial sequence	337	337	79.00	4E-92	85.32	NR_104767.1
Proteus mirabilis strain JCM 1669 16S ribosomal RNA, partial sequence	331	331	79.00	2E-90	85.02	NR_113344.1
Xenorhabduskoppenhoeferi strain USNJ01 16S ribosomal RNA, partial sequence	331	331	79.00	2E-90	85.02	NR_043637.1
Proteus mirabilis strain ATCC 29906 16S ribosomal RNA, partial sequence	331	331	79.00	2E-90	85.02	NR_114419.1
Proteus cibarius strain JS9 16S ribosomal RNA, partial sequence	327	327	68.00	2E-89	87.81	NR_149294.1
Xenorhabduskozodoii strain SaV 16S ribosomal RNA, partial sequence	326	326	79.00	8E-89	84.71	NR_043646.1
Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	326	326	79.00	8E-89	84.71	NR_043997.1
Proteus terrae strain N5/687 16S ribosomal RNA, partial sequence	324	324	66.00	3E-88	88.19	NR_146019.1
Methylobacterwhittenburyi strain 1521 16S ribosomal RNA, partial sequence	324	324	75.00	3E-88	85.58	NR_029242.1

Table 5: zone of inhibition values for disc diffusion assay

fungus code	zone of inhibition									
	Myroidesgitamensis (mm)					*Xenorhabduskhoisanae (*mm)				
	Plate			Avg.	S.D.	*Plate*			*Avg*	*S.D.*
	1	2	3			*1*	*2*	*3*		
VRT1A	3.5	3	3.7	3.4	0.36	3	2.2	2.9	2.7	0.43
VRT1B	2.5	3	3.5	3	0.5	3	2.9	3.2	3.0	0.15
VRT2A	0.5	0.4	0.3	0.4	0.1	0.5	0.3	0.3	0.36	0.11
VRT6A	3.5	3.9	4.2	3.86	0.35	3.9	3.5	3	3.4	0.45
VRL2B	2.5	2.1	2.3	2.3	0.2	2.5	2.4	2.5	2.4	0.05
VRL3B	2.7	2.5	2.9	2.7	0.2	3	2.5	2.7	2.7	0.25
VRT5A	3	2.9	2.5	2.8	0.26	2.5	2.1	1.9	2.1	0.30
Quercetin	3	3.5	3.4	3.3	0.26	3.9	3.5	4	3.8	0.26
Gallic acid	3.2	3.2	2.9	3.1	0.17	3.7	3.8	3.6	3.7	0.1

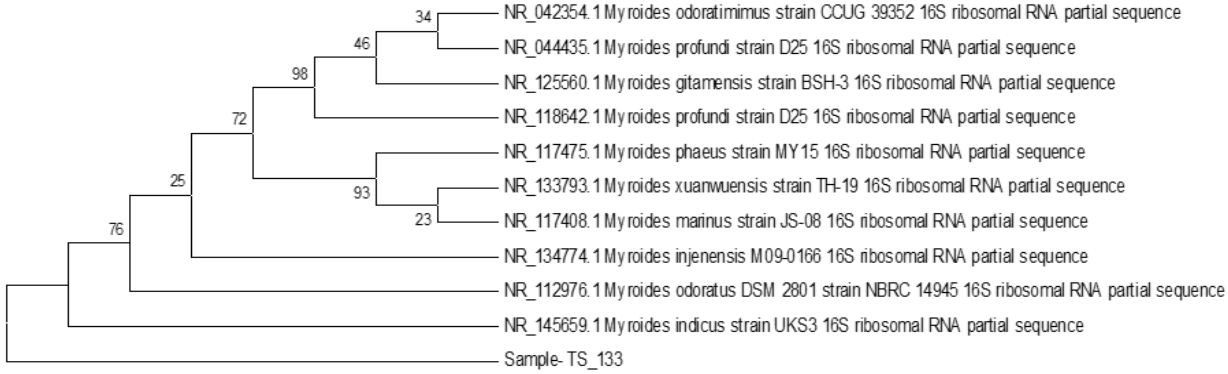

Figure 1: Phylogenetic tree for SS1C culture

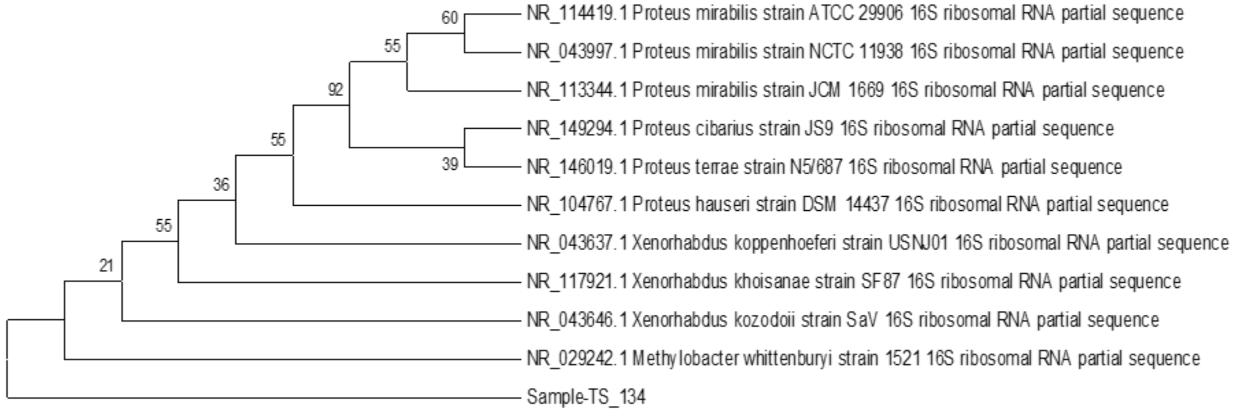

Figure 2 : Phylogentic tree for SS2A culture

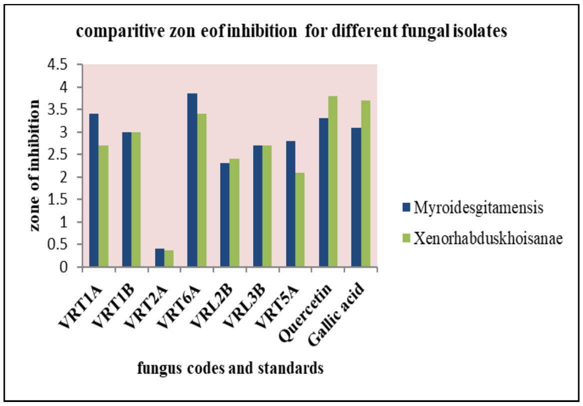

Figure 3: Zone of inhibition of different fungi isolates

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