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ANTIMICROBIAL PROPERTIES OF ENDOPHYTIC FUNGI ISOLATED FROM MEDICINAL PLANT *CAMELLIA SINESIS*

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ABSTRACT

The purpose of this work was to evaluate the antimicrobial potential of endophytic fungi isolated from leaves of *Camellia sinesis* plant. Many endophytes were isolated by using potato dextrose agar medium. In that one potent strain was taken for further study. The fungal culture was extracted with ethyl acetate and used as a crude extract for checking antimicrobial activities. The crude fungal extract showed different inhibitory activity against all pathogens. It showed significant activity against *Pseudomonas* sp. The fungal strain was identified as *Penicillium* sp. through 18S ribosome RNA sequence analysis. The *Penicillium* sp. showed 100% gene sequence similarities with *Penicillium funiculosumstrainTS08 and Penicillium sp.*12-2018S. The sequence of identified strain was submitted in NCBI and obtained accession number HQ214674 with name *Penicillium* sp. pr NN-2010. The bioactive compounds responsible for antibacterial activity were identified by GC-MS and NMR. The major compound present in crude extract was identified as 3-(3-azidopropyl)-1H-indene (15.39%) and 3-Cyano-1,2-dimethylindole. In Nuclear magnetic resonance analysis most of the peaks range were between the aromatic groups. These results indicate that the bioactive metabolites isolated from fungal *Penicillium* sp.could be promising source as antibacterial agents.

KEYWORDS: ***Camellia sinesis*, endophytic fungi, antimicrobial activity, GC-MS, NMR**

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INTRODUCTION

Antimicrobial resistance became one of the major problems worldwide. Therefore there is an urge to investigate new bioactive compounds[1]. The current trend is about opting the natural products. Natural products are of microbial, plant and animal origin. The current research focuses on the bioactive compounds obtained from endophytes. Endophytes are endosymbionts which colonize in the healthy tissues of the plants without causing any potential harm to it. In endophytes peculiarly fungi have proved to be an important source for bioactive compounds which have wide range of applications in medical field[2-3]. Many antimicrobial compounds isolated from endophytes, belonged to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids[4].

In the present study, the plant *Camellia sinesis* was chosen. *Camellia sinesis* is the most widely consumed beverage in the world, and its polyphenolic compounds have been found to possess widespread biologic functions and health benefits [5-6]. The phytochemical screening of tea revealed the presence of alkaloids, saponins, tannins, catechin and polyphenols[7-9]. The current study is investigation about isolation and identification of the fungal endophytes from the medicinal plant *Camellia sinesis* and also to evaluate the antimicrobial activity of the fungi.

MATERIALS AND METHODS

Source of endophytic fungi

The plant material *Camellia sinesis* was collected from Ooty, Tamilnadu, India. The plant material were taxonomically identified and authenticated by Dr. GVS. Murthy, Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. The voucher Specimen was deposited there with register number BSI/SRC/5/23. The leaves samples from plant were randomly cut off with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to laboratory within 12hr and stored at 4^0C until isolation procedures were completed.

Isolation of endophytic fungi

The isolation of endophytic fungi from *Camellia sinesis* was carried out by using standard method[8]. The fungal isolates were identified based on their morphological and reproductive characters using standard identification manuals[10].

Fermentation and extraction

Two or three pieces of the grown culture cut from the plate were inoculated into 1000ml Erlenmeyer flask containing 300ml potato dextrose broth for 15 days at 25°C, 120rpm. The fungal culture was filtered to remove mycelium. The filtrate was extracted with ethyl acetate (1:1 ratio) three times. The organic phase was evaporated to dryness and used for determination for antimicrobial activity. The crude extract was dissolved in DMSO to obtain different concentrations.

Bacterial and fungal strains

Bacterial strains used in this study were *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa,E.coli, Salmonella paratyphi, Proteus* sp.*, Shigella sonnei, Serratia* marcescens and *Bacillus subtilis* and fungal strains used are *Aspergillus niger* and *Aspergillus flavus.* All the strains were confirmed by morphological, cultural and biochemical characteristics and maintained in slants for further use.

Antimicrobial activity

For antimicrobial evaluation, agar well diffusion method was performed by standard method[10]. Mueller Hinton agar plates were inoculated with overnight culture of each bacterial suspension, by evenly spreading out with sterile cotton swabs. But for antifungal activity

Sabouraud dextrose agar plates were inoculated with overnight culture of each fungus. The Agar wells were prepared by scooping out the media with a sterile cork borer (7mm in diameter). The wells were then filled with 20µl, 40µl, 60µl, of the fungal crude extract that was already dissolved in DMSO. The plates were then incubated at 37°C for 24 h and the zone of inhibition was recorded and compared with the control (i.e. a well filled with DMSO solution only).

Molecular identification of endophytic fungus

Molecular identification of isolate was conducted by analysing the endophytic fungus 18S ribosome RNA sequence using polymerase chain reaction (PCR) cloning technology. Genomic DNA was extracted from fungal mycelia. A pair of primer Fungal forward: 5'-TCC GTA GGT GAA CCT GCG G-3' and Fungal reverse: 5'-TCC TCC GCT TAT TGA TAT GC-3 were used to amplify the highly specific for endophytic fungi targeting the gene encoding for 18SrRNA. Amplification was carried out in a thermal cycler (Eppendorf Mastercycler 5330). The molecular weight markers Generuler 100bp and 1 kb DNA ladder (Fermentas, Lithuania) were used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR products were stained with ethidium bromide and visualized by an image analyser (ChemiImager 5500, Alpha Innotech, CA, USA). The analysis and comparison of the sequence were performed with nucleotide Basic Local Alignment Search Tool (BLAST) of GenBank. Alignment and similarity comparison were initially conducted by the Clustal W method and the phylogenetic tree was constructed using Clustal W with the neighbour joining method. A boost strap analysis of 1000 replicates was carried out using MEGA 5.05 software.

GC-MS analysis and NMR analysis

The GC-MS analysis was performed at South India Textile Research Association, Coimbatore, Tamilnadu, India. The interpretation on mass spectrum GC-MS performed based on the database of Natioanl institute standard and technology having more than 62000 patterns. The spectrum of the unknown compound was compared with the spectrum of the known compound in the NIST library. The compound name molecular formula molecular weight were ascertained and tabulated and Nuclear magnetic resonance analysis was performed in Pondicherry University, India.

RESULTS

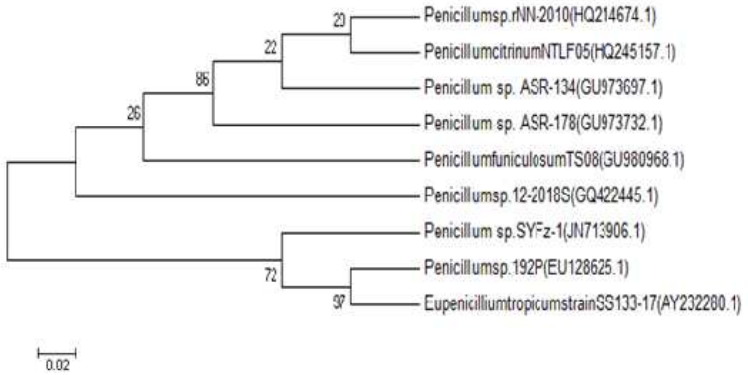
In the present study, the endophytic fungi were isolated by using two different mycological media namely Potato Dextrose Agar (PDA), and Malt Extract Agar (MEA). Maximum endophytes were isolated in PDA. The fungal culture was extracted with ethyl acetate and the crude extract was used for checking antimicrobial activity. The crude fungal extract showed inhibitory activity against all bacterial tested pathogens except *Proteus* sp. and no activity was showed against fungal pathogen. The highest zone of inhibition observed against *Pseudomonas aeruginosa*., *Serratia* marcescens *and Staphylococcus aureus.* The extract showed weak activity against *Bacillus subtilis, Klebsiella pneumoniae, Salmonella paratyphi., and Shigella sonnei.* The extract has very least activity for *E.coli* (Table 1)

Table 1
Antibiogram pattern of ethylacetate extract of endophytic fungus Penicillium sp.

Test organisms	Zone of inhibition (mm)		
	28.85µg/20µl	57.12 µg/20µl	85.68µg/20µl
Shigella sonnei	5	7	9
Salmonella paratyphi	3	6	8
Klebsiella pneumoniae	-	-	10
Staphylococcus aureus	11	13	15
Pseudomonas aeruginosa	20	25	28
Bacillus subtilis	5	8	10
Escherichia coli	2	4	5
Proteus sp.	-	-	-
Serratia marcescens	12	13	16

In this study, fungal molecular identification of the most active isolate was done by 18S ribosome RNA sequence analysis. On the basis of blast analysis of the 18S rRNA sequence, the strain identified it belonged to the *Penicillium* sp. group. The sequence of strain submitted in NCBI and obtained accession number HQ214674 with name *Penicillium* sp. pr NN-2010. The sequence of the isolate was compared by alignment against 18S rDNA sequences, available in the GenBank Database using the BLAST program. Alignment and similarity comparison were initially conducted by the Clustal W method[12] and the phylogenetic tree was constructed using Clustal W with the neighbour joining method. A boost strap analysis of 1000 replicates was carried out using MEGA 5.05 software. The organism showed 100% gene sequence similarities with *Penicillium funiculosumstrainTS08 and Penicilliumsp.12-2018S* (Figure 1).

Figure 1
Phylogenetic tree-based on the 18S rDNA sequence homology of strain *Penicillium* sp. pr NN-2010



Neighbour joining tree based on 18S rDNA gene sequences showing relationship between the *Penicillium* sp.pr NN-2010 with other *Penicillium* species. The number at the nodes indicates the percent levels of boost strap support based on the analysis of 1000 replicates. The scale bar indicates number of changes per base position.

In GC-MS analysis the 39 compounds were identify based on peak area percentage, Retention time then the molecular formula, molecular weight (Table 2). The major constitutents are 3-(3-azidopropyl)-1H-indene, 3-Cyano-1,2-dimethylindole, Propane, 2-iodo (15.49%), Cyclo(L-Leucyl-L-prolyl), prolylleucyl anhydride, Cyclo(L-Pro-L-Leu), Pyrrolo[1,2-a]pyrazine-1,4-dione (13.88%), hexahydro-3-(2-methylpropyl)-, (3S-trans)-, 2-Methyl-5-nitro-N-propylaniline, methyl 9-oxo-8-oxabicyclo[4.3.0]nona-1(6),2-diene-2-carboxylate, 4-Isobenzofurancarboxylic acid, 1,3,6,7-tetrahydro-3-oxo- methyl ester, 5-Methyl-2-nitro-N-propylaniline (12.12%). Except the major constitutents there are 29 minor constitutents also presented (Table 2, Figure 2). In GC-MS analysis phenols also observed in the extract as minor constituent. Phenols reported for many biological activities[13]. Hence the major constituents alone or along with minor constituents may provide the antibacterial activity.

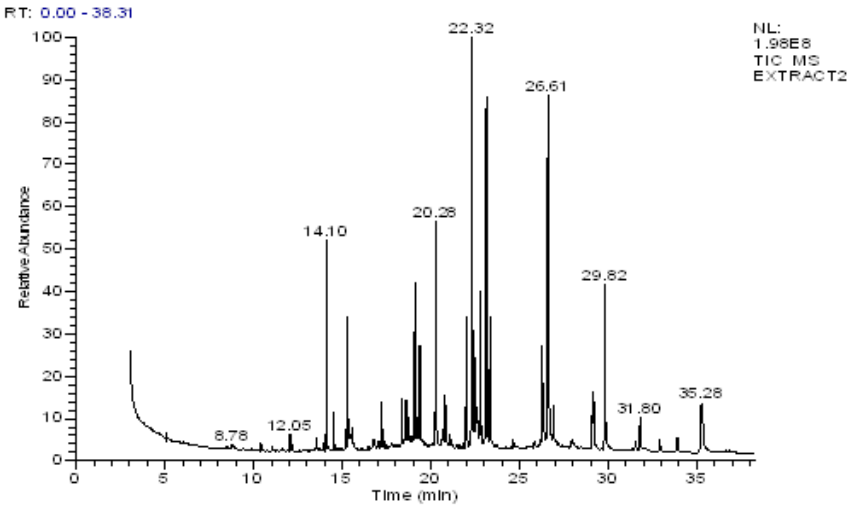


Figure 2
GC-MS chromatogram of the ethyl acetate fungal crude extract

Table 2
Compounds identified in the ethyl acetate fungal crude extract by GC-MS

Retention time	Compound name	Molecular weight	Molecular formula	Area %
26.61	3-(3-azidopropyl)-1H-indene	199	$C_{12}H_{13}N_3$	15.49
	3-Cyano-1,2-dimethylindole	170	$C_{11}H_{10}N_2$	15.49
	Propane, 2-iodo- (CAS)	170	C_3H_7I	15.49
22.32	Cyclo(L-Leucyl-L-prolyl)	210	$C_{11}H_{18}N_2O_2$	13.88
	prolylleucyl anhydride	210	$C_{11}H_{18}N_2O_2$	13.88
	Cyclo(L-Pro-L-Leu)	210	$C_{11}H_{18}N_2O_2$	13.88
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	210	$C_{11}H_{18}N_2O_2$	13.88
23.12	Methyl9-oxo-8-xabicyclo[4.3.0]nona-1(6),2-diene-2-carboxylate	194	$C_{10}H_{10}O_4$	12.12
	4-Isobenzofurancarboxylicacid,	194	$C_{10}H_{10}O_4$	12.12
	1,3,6,7-tetrahydro-3-oxo-, methyl ester (CAS)	194	$C_{10}H_{10}O_4$	12.12
	5-Methyl-2-nitro-N-propylaniline	194	$C_{10}H_{14}N_2O_2$	12.12
20.28	Cyclo(L-Pro-L-Val)	196	$C_{10}H_{16}N_2O_2$	7.76
	9-Isopropyl-1,7-dioxo-2,8 diazabicyclo[4.3.0]nonane	196	$C_{10}H_{16}N_2O_2$	7.76
	5-Chlorobenzimidazole-2-carboxylic acid	202	$C_8H_5ClN_2O_2$	7.76
29.82	Ergotamine - GC Artefact I	244	$C_{14}H_{16}N_2O_2$	5.17
	Pyrrolo[1,2-a]pyrazine-1,4-dione, 3-benzylhexahydro-, (3S,8aS)- (CAS Cyclo(-Pro-Phe)	244	$C_{14}H_{16}N_2O_2$	5.17
	3-Benzyl-1,4-diaza-2,5-dioxbicyclo[4.3.0]nonane	244	$C_{14}H_{16}N_2O_2$	5.17
	Dihydroergotamine	244	$C_{14}H_{16}N_2O_2$	5.17
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl	244	$C_{14}H_{16}N_2O_2$	5.17
14.10	4-tert-butyl-1,6-dimethyl-2-isopropylpyrimidinium-5-carboxylate	250	$C_{14}H_{22}N_2O_2$	4.39
	Phenol	206	$C_{14}H_{22}O$	4.39
	2,4-bis(1,1-dimethylethyl)- (CAS	206	$C_{14}H_{22}O$	4.39
	3,4-Dihydro-2H-1,5-(3"-t-butyl)benzodioxepine	206	$C_{13}H_{18}O_2$	4.39

In NMR analysis. it was identified that aromatic compounds were present in the extract. Aromatic compounds have various pharmacological activities. So the aromatic compounds present in the extract showed the activity of the tested pathogens (Figure 3,4). Yet the bioactive compounds in the fungal crude extract had to be isolated, purified and further invivo studies have to carry out for future study.

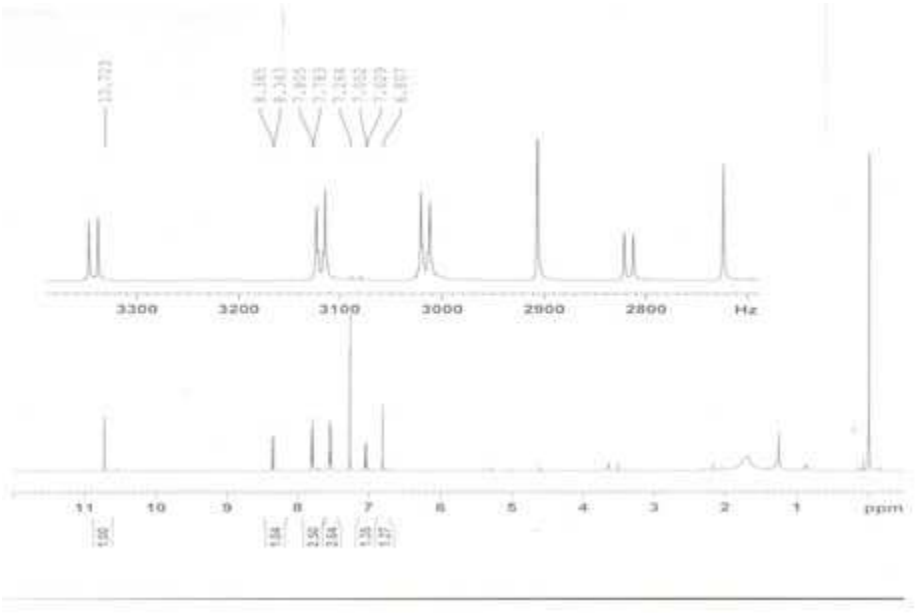


Figure 3
Proton NMR analysis of Penicillium* sp.pr NN-2010 *ethyl acetate extract

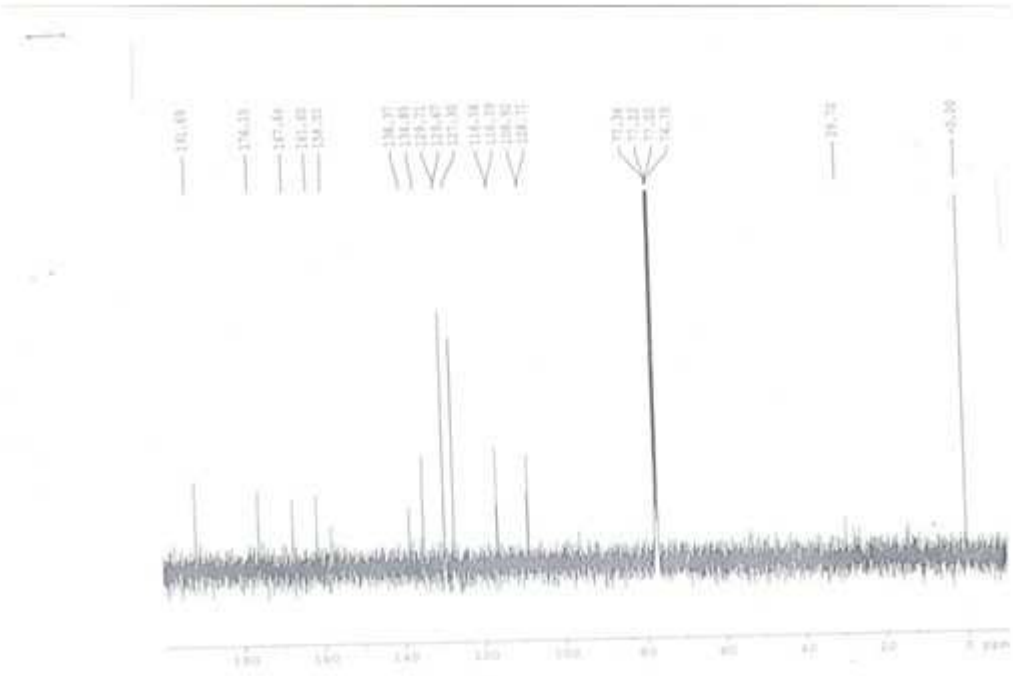


Figure 4
C-13 NMR analysis of** Penicillium* sp. pr NN-2010 ***ethyl acetate extract

CONCLUSION

The present investigation was an attempt to search for antibiogram pattern from an alternate natural source endophytic fungus. The study revealed the presence of good antibacterial activity for the crude extracts of *Penicillium* sp. against *Pseudomonas* sp. So the *Penicillium* sp.prNN-2010 could be a good source for bioactive compounds and the isolated compounds may be further check in invivo model as an antibacterial agent.

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