ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 7, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

STRUCTURAL ELUCIDATION OF BIOACTIVE SECONDARY METABOLITES FROM ENDOPHYTIC FUNGUS

SHIV KR VERMA, MOTI LAL, MIRA DEBNATH (DAS)*

School of Biochemical Engineering IIT, Banaras Hindu University, Varanasi, Uttar Pradesh, India.
Email: m.debnath.bce@itbhu.ac.in

Received: 04 April 2017, Revised and Accepted: 04 May 2017

ABSTRACT

Objective: In this research for bioactive secondary metabolites from endophytic fungal isolate CPR5, which was later identified and characterized as *Aspergillus niger* sp.

Methods: Eight bioactive compounds (1-8) were isolated and purified using a series of chromatographic techniques and identified by applying ^{13}C and ^{1}H nuclear magnetic resonance and mass (ESI-MS spectrometry).

Results: All the compounds were tested for antimicrobial activity against a wide range of human and plant pathogenic bacteria as well as fungi. These microorganisms were, *Xanthomonas oryzae, Escherichia coli, Streptococcus pneumonia, Bacillus subtilis, Staphylococcus hyicus, Bacillus sphaericus, Staphylococcus aureus,* and *Pseudomonas aeruginosa, Candida albicans, Sclerotium rolfsii, Sclerotinia, scleratiourum,* and *Phoma exigua* minimum inhibitory concentration value have also been determine for these microorganism.

Conclusion: In this study, a total 10 endophytic fungi were isolated from the *Calotropis procera*, show a great antibacterial activity against 24 human pathogenic bacteria.

Keywords: Endophytic fungi, Antimicrobial metabolites, *Calotropis procera,* Preliminary analysis of bioactive compounds and characterization.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i7.18909

INTRODUCTION

Endophytes are the group of microorganisms that reside inside plant tissues without causing any negative sign on the host [1]. This is a topographical term and includes bacteria, fungi, *Actinomycete,* and *Algae*, which spend their whole life or a period of the life cycle in the symplast or apoplast region of healthy plant tissues without producing any disease or clinical symptoms. Studies have revealed that fungal endophytes are almost everywhere in many plant species that live on the earth, and the endophytic colonization differs from plant to plant [2]. These fungi mostly belong to the phyla *Ascomycetes, Basidiomycetes,* and *Zygomycota* [3]. Endophytes in general, are very important and viable components of microbial biodiversity. They have also been reported in mangrove community [4]. Ecological success and protection of plants are greatly affected by symbiotic microorganisms which once isolated and characterized, may also have potential for use in industry, agriculture, and medicine [4-6]. Endophytic fungi have been widely investigated as source of variety of new bioactive compounds [7]. Plant-associated microbial diversity often shows a symbiotic relationship. The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances to nullify the effects of soil toxic compounds, fungal and bacterial infection and from attack of herbivores or by enhancing plant responses [8]. There may be possibility that the medicinal characteristics of plants may result from the potential of its endophytes to generate biologically active secondary metabolites. In recent years, the biology of endophytic fungi in aerial plant tissues has become an important area for study; however, the chemistry of bioactive metabolites isolated from these organisms is main interesting area to be explored along with other biological aspects [9]. There is numerous evidence showing that bioactive substances produced by microbial endophytes may not only involve in the host-endophyte relationship, but may also have diverse applicability in medicine, agriculture, and industry. Recent reported literatures has explored that endophytic fungi have been identified as unusual productive sources of bioactive metabolites [10,11], which might be helpful to treat some of the recently observed diseases in a human being. Due to the increasing demand of medicine and destruction of medicinal plants, a huge work carried out in the field of endophytes for producing bioactive compounds that can be used in the treatment of diseases [12] Endophytic fungi have been widely investigated as source of variety of bioactive compounds. Bioactive compounds show interesting and attractive properties such as antibacterial, antifungal, anticancer, antiprotozoal, antioxidant, antiviral, antimalarial, antitubercular, immunosuppressive, antidiabetic, and antiviral. These bioactive compounds could be mainly classified as alkaloids, steroids, terpenoids, quinones, phenylpropanoids, isocoumarins, lignans, phenols, lactones, etc. These bioactive compounds used for the health care purpose for human beings [13]. Even though more than 30,000 diseases are clinically described today, less than one-third of these can be treated symptomatically, and even a fewer can be cured. The increasing occurrence of multiresistant pathogenic strains has limited the effect of traditional antimicrobial treatment. Hence, there is an urgent need for new therapeutic agents with infectious disease control [14]. The main aim of the study was to screen and isolate endophytic fungus from the latex bearing medicinal plant (*Calotropis procera*), extraction of bioactive secondary metabolite from it using ethyl acetate solvent. During our continual search for bioactive secondary metabolites from microorganisms, extracts (supernatant and cells) of the endophytic fungal strain *Aspergillus niger* sp. CPR5 showed strong antibacterial and antifungal activities. Minimum inhibitory concentration (MIC) was also determined. In addition, eight compounds have been isolated, and their structure was elucidated using nuclear magnetic resonance (NMR) and mass spectroscopy.

METHODS

Plant sample collection

Root samples of *C. procera* were collected from agricultural field of Banaras Hindu University (BHU), Varanasi (25.5-N 82.9-E, elevation

279 ft/85 m). The plant was identified on the basis of external morphology and visualization 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 ice container. Samples were preserved at 4°C and processed for isolation of desired fungus immediately.

Isolation and extraction of endophytic fungus

Before applying any chemical treatment, roots were washed in running tap water for 10-12 minutes, to remove heavy load of soil particle and microbial concentration followed by washing with double distilled water. Samples were successively surface sterilized by the method given by [15]. Sample fragments were successively surface sterilized by immersing in 70% (v/v) ethanol for 1-3 minutes followed by washing with 5% aqueous solution of sodium hypochlorite for 5 minutes and again in 70% ethanol for 30 seconds. After being rinsed in sterile water for 3 times, the roots were cut into 1-cm-length bars. The bars were longitudinally halved and incubated in Petri dishes containing potato dextrose agar (PDA) medium supplemented with chloramphenicol (50 µg/ml,) and streptomycin sulfate (250 µg/ml) to suppress bacterial growth PDA medium at 28°C until the mycelia or colonies appeared around the segments.

Identification and molecular characterization of the fungus

Morphologically fungus was studied using lactophenol cotton blue staining; isolate was identified at genus level based on mycelial morphology and for identification and characterization at molecular level, total DNA of the endophytic fungus isolate was extracted from fungal mycelia grown in PDA using PrepMAN ultra sample preparation reagent kit according to the manufacture's recommendation (nucleopore). For identification and identification internal transcribed spacer region. A pair of ITS primer IT1 (5'GTAGTCATATGCTTGTCTC 3') [Qiagen] and IT4 (5' CTTCCGTCAATTCCTTTAAG 3') [Qiagen] were used to amplify the highly specific and conserved sequence for endophytic fungi. Polymerase chain reaction (PCR) was carried out in a programmable thermal controller (Bio-rad) (Verma *et al*., 2016) 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, and 0.05 U/µl Taq polymerase) [Fermentas], 1 µl of each primer (IS1 and IS4) and double distilled water to make up the volume. The amplification was performed for 34 cycles having preset program of 1 minute at 94°C, 1 minute at 45°C, and 2 minutes at 72°C. After the final cycle has been completed, the amplification was extended for 10 minutes at 72°C. The amplified DNA fragment (approximately 400 bp) was purified and was sequenced by genetic analyzer. The sequences were compared to rDNA-ITS gene sequences in the public database using the BlastN program (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was produced using BLAST pairwise alignments.

Microorganisms tested and antimicrobial activity test

Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, and human pathogenic fungus*, Candida albicans* and three plant pathogenic fungi *Sclerotium rolfsii, Sclerotinia sclerationrum,* and *Penicillium* sp. were used to test antimicrobial activity fungal extract. Disc diffusion method [16] was followed for all the microorganisms. The microbial suspensions of particular concentration (OD=0.5) were evenly spread out with sterile glass spreader. Sterile paper disks (6 mm diameter) were placed in the center of each Petri plate and loaded with 15 µl of crude extract and sealed with parafilm. Bacterial plates were incubated at 36±2°C for 24 hrs and fungal plates at 27°C for 48 hrs, respectively. The zone of inhibition was recorded after the specified incubation period. Three replicates of each experiment were maintained to avoid error in measurements.

Cultivation and metabolite extraction

A piece of agar plug (0.3×0.3 cm^2) containing fungal hyphae was transferred in each 250 ml Erlenmeyer flask containing 100 ml sterile medium (yeas extract 3.0 g/l; malt extract 3.0 g/l; peptone 0 g/l; glucose 10 g/l), and incubated on a rotary shaker at 140 rpm and at 28±2°C for 8-10 days. A total of 50 flasks were used to collect required volume of broth. Thereafter, a total of 5 l fermentation broth was collected for each isolate (CPR1-CPR18). The filtrate was extracted 3 times with an equal volume of ethyl acetate (EtOAc) and the EtOAc layer was collected. The frozen mycelia was crushed completely and extracted 3 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. Crude extract was evaporated to make solid residue and the stock solution was prepared in the concentration of 1 mg/ml in dimethyl sulfoxide (DMSO).

Antimicrobial activity of fungal extracts

Antimicrobial activity of fungal crude extracts was examined using paper disc diffusion method. 15 µl of crude extract from stock solution (1 mg/ml) was added into sterile filter paper disc (6 mm diameter). Discs with the crude extract were placed over Petri plates inoculated with test organisms. All the plates were incubated at 37°C for 24 hrs. The zone of inhibition was measured and expressed in mm.

Spectroscopic measurements

To predict the chemical structure of nine antimicrobial compounds isolated, they were analyzed using Fourier Transform Infrared (FT-IR), NMR spectroscopy. All NMR data were collected on JEOL AL 300 FTNMR. Mass spectra were recorded (ESI-HRMS) mass spectrometer. Chemical shift was expressed in δ (ppm) and coupling constant J in Hz. ^1H and ^{13}C NMR spectra were recorded on JEOL AL 300 FTNMR spectrometer. Mass spectra were recorded on Agilent 6520 Q-TOF (ESI-HRMS) mass spectrometer. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements.

Fractionation of crude extract

Concentrated and dried crude extract (12 g), which was subjected to column chromatography over silica gel column (150 g) and 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, and 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, and v/v). Based on the thin-layer chromatography (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). The separation column (125×40 mm) was prefilled with Eurospher-10 C18 (Knauer, Germany) using a linear gradient of MeOH and 0.02% H_3PO_4 in H_2O and a flow rate of 1 ml/minute. Ultraviolet (UV) data (k max) for individual compounds were extracted from the online UV spectra provided by the instrument software. TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used for monitoring of fractions using n-hexane: EtOAc (95:5 and 90:10), CH_2Cl_2:MeOH (95:5, 90:10, 85:15, and 80:20) as well as CH_2Cl_2:MeOH:EtOAc(90:10:5 and 80:20:10). Detection was at 254 and 366 nm or by spraying the plates with anisaldehyde reagent.

RESULTS AND DISCUSSION

Morphological and molecular identification of Fungal isolate

About 18 endophytic fungal isolate have been isolated form *C. procera* plant and tested for antimicrobial activity and among the 18 isolate, only one strain, which produced the wide-spectrum antibacterial substances with the highest efficiency, was selected for morphological and molecular identification. The growth of mycelia colonies found to be opaque and almost circular when grown on PDA at 34°C for 4 days. Representative image of endophytic fungal mycelia (40X inverted microscope) was obtained in the laboratory as image taken from 1 week old liquid culture it clearly shown that dense mycelia are intermingled with each other (Fig. 1). The 18S rRNA sequencing (Fig. 2) followed by basic local alignment search tool (BLAST) and SeqMatch analysis was used for molecular conformation endophytic isolate. The 18S rRNA sequence was submitted to the DNA Data Bank of Japan and assigned an accession number (LC062385). The 18S rRNA gene sequence (Fig. 2)

was used to build a phylogenetic tree (Fig. 3), by performing automated BLASTN searches, to determine the closest type strain to the isolate under investigation. The percentage of identity was found to be 96%. Phylogenetic tree analysis indicated that 18S ribosome RNA sequence of CPR-5 strain was closely related to *A. niger* species (Fig. 2). The phylogenetic tree suggested that the local isolated strain is definitely a member of the genus *Aspergillus* and formed a common phylogenetic lineage that could be equated with a novel local species. From the results of inhibition zone it is observed that the metabolite is abroad spectrum antimicrobial agent, it inhibits growth of both Gram-positive and Gram-negative bacteria. 1 mg/ml of crude metabolite was used in the experiment. Maximum zone of inhibition was recorded. Against *Klebsiella pneumonia* (21 mm) followed by *E. coli* (20 mm). Equal zone of inhibition (18 mm) was observed for *Vibrio* sp. and *Bacillus* sp. The inhibition zones against *Pseudomonas* sp., *Salmonella typhi* 16.02, 17.02. Similarly, when antifungal activity was tested it was observe that equal zone of inhibition (18 mm) was observed for *Penicillium chrysogenum* and *S. rolfsii.* Minimum zone of inhibition (16 mm) was recorded for *Candida tropicalis.* Zone of inhibition of 18 and 17 mm was observed for *S. sclerationrum* and *Phoma exigua*. Maximum zone of inhibition was recorded for *K. pneumonia* (Table 1). These observations were obtained from crude metabolites and the results compared with fluconazole as a standard. From Table 2 it is clear that the metabolite from this isolate could be a promising source of antifungal agents. The antibacterial activity of bioactive compound produced by isolates CPR5 is comparable with chloramphenicol as standard antibiotic (Table 1).

Spectroscopic analysis of compounds

Compound 1

Dark brown solid like compound, composition (64.25%) H (10.39%) O (25.36%) molecular formula $C_{27}H_{52}O_8$, ESI-MS (m/z) 504.69698.

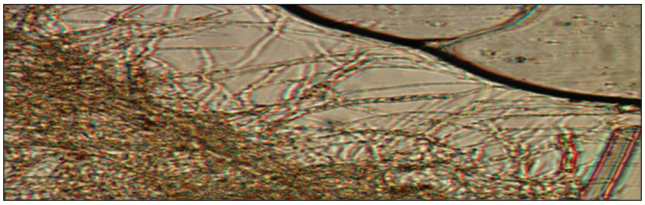

Fig. 1: Representative image of endophytic fungal mycelia (×40 inverted microscope)

^{1}H NMR (DMSO-d_6, 300 MHZ) 1.25-145 ppm (m, 20H), 3.21 (m, 2H), 3.90 (m, 1H), 0.90 (d, 6H), 1.18 (d, 3H), 1.79 (m, 1H), 4.40-1.80 (m, H-pentose), 3.58 (m, 3H), ^{13}C NMR (75 MHZ) DMSO-d_6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 85-93 ppm (C-O-C) IR (KBr) υmax (cm^{-1}) 3250, 1200 (Fig. 3).

Compound 1 was observed active against:
E. coli, Streptococcus pneumonia, B. subtilis, Staphylococcus hyicus, Bacillus sphaericus, S. aureus, and *pseudomonas aeruginosa, C. albicans, S. rolfsii, Sclerotinia, sclerationrum, Fusarium species,* and *Penicillin* sp.

Compound 2

Black brown solid like, composition: C (62.65%) H (9.62%) N (5.35%) O (22.39%) molecular formula $C_{41}H_{75}N_3O_{11}$, ESI-MS (m/z) 785.539612.

^{1}H NMR (DMSO-d_6, 300 MHZ), 3.3-3.8 ppm (m, 2H-glucose), 4.43 (m,2H), 4.52 (m,1H), 7.10-7.90 (m, 4H- - NH_2), 1.96 (t, 4H), 1.33 (t, 2H), 1.29 (m, 10H), 1.25 (m, 12H), 5.62-5.7 (m, 4H), 4.85 (s, 1H), 0.96 (d, 3H), ^{13}C NMR (75 MHZ) DMSO; 172-174 ppm (-C=O), 163 ppm (amide group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C). IR (KBr) υmax (cm^{-1}) 3250, 1200, 1650, 1000, and 1062.

The results are near about similar to [17] (Fig. 4).

Compound 2 was observed active against:
Xanthomonas oryzae, E. coli, *S. pneumonia, B. subtilis, Staphylococcus hyicus, B. sphaericus, S. aureus*, and *P. aeruginosa, C. albicans, S. rolfsii, P. exigua,* and *S. sclerationrum.*

Compound 3

Dark brown solid like, composition: C (67.98%) H (10.73%) N (1.89%) O (19.40%) molecular formula $C_{42}H_{79}NO_9$, ESI-MS (m/z) 742.07796.

^{1}H NMR (DMSO-d_6, 300 MHZ), 3.3-3.8 ppm (m, 2H-glucose), 4.43 ppm (m, 2H), 4.52 ppm (m, 1H), 1.96 (t, 4H), 1.33 (t, 2H), 1.29 (m, 10H), 1.25 (m, 12H), 5.62-5.7 (m, 4H), 4.85 (s, 1H), 0.96 (d, 3H), 0.88 (m, 6H), 1.31 (m, 4H), ^{13}C NMR (75 MHZ) DMSO-d_6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax cm^{-1} 3400, 1650, 3100, 1000, and 1062. The results are near about similar to (Fig. 5) [17].

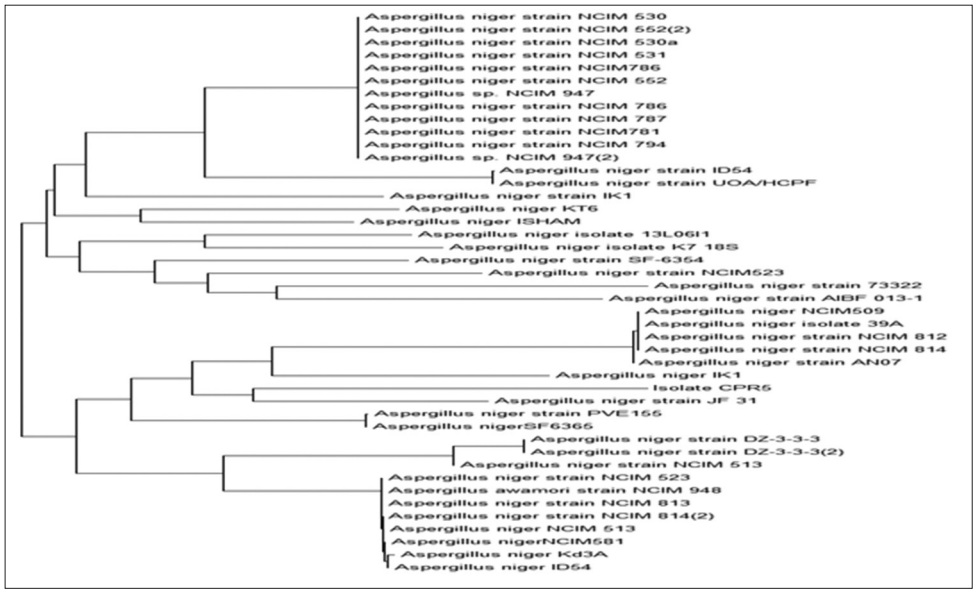

Fig. 2: Phylogenic tree based on 18S rRNA sequence showing the relationship between fungal endophytic isolate from *Calotropis procera* (isolate CPR5) and reference strains, the evolutionary history was inferred using UPGMA method

Table 1: Antifungal activity (inhibition zone) of the crude aqueous extracts of isolate CPR5

Plant pathogenic fungi	Zone of inhibition (mm)		Plant pathogenic bacteria	Zone of inhibition (mm)	
	Crud extract	Fluconazole (+ve control)		Crud extract	Chloramphenicol (+ve control)
Penicillium chrysogenum	3.35±0.2	10.5±0.2	*Ralstonia solanacearum*	7.15±0.2	18.4±0.2
Phoma exigua	2.21±0.2	12.4±0.2	*Xanthomonas oryzae*	6.85±0.2	13.3±0.2
Sclerotium rolfsii	4.38±0.2	10±0.2	*Xanthomonas campestris*	8.38±0.2	16.3±0.2
Sclerotinia sclerationrum	5.15±0.2	11.3±0.2	*Pseudomonas* spp.	6.0±0.2	16.3±0.2
Fusarium oxysporum	4.54±0.2	12.7±0.2	Human pathogenic fungus		
Rhizoctonia spp.	5.84±0.2	9.5±0.2	*Candida albicans*	4.54±0.2	10.5±0.2
Fusarium verticillioides	3.45±0.2	14.5±0.2			
Penicillium expansum	2.21±0.2	8.9±0.2			

Table 2: Antibacterial activity (inhibition zone) of the crude aqueous extracts of isolate CPR5 against human pathogenic bacteria

Test bacteria	Zone of inhibition (mm)	MIC	
	Crud extract (mg/ml)	Crud extract (µg/ml)	Chloramphenicol (+ve control) (µg/ml)
Escherichia coli O157:H7	22.0±0.2	130	50
Bacillus cereus	24.75±0.2	230	40
Bacillus subtilis	22.25±0.2	230	45
Staphylococcus aureus	19.0±0.2	330	50
Bacillus megaterium	16.0±0.2	200	35
Helicobacter pylori	15.12±0.2	270	70
Streptococcus pyogenes	14±0.2	310	100
M. D. R *Staphylococcus aureus*	18.0±0.2	280	80
Chlamydia pneumonia	17.15±0.2	220	70
Corynebacterium amycolatum	14.0±0.2	270	110
Mycobacterium tuberculosis	11.5±0.2	270	150
Staphylococcus epidermis	25.5±0.2	322	45
Pseudomonas aeruginosa	24.35±0.2	330	30
Listeria monocytogenes	16.5±0.2	300	75
Salmonella typhimurium	15.20±0.2	300	130
Salmonella typhi	11.19±0.2	330	150
Streptococcus pneumoniae	16.85±0.2	280	75
Staphylococcus epidermidis	15.85±0.2	300	120
Corynebacterium diphtheriae	17.0±0.2	220	80
Vibrio sp.	11.85±0.2	350	40
Clostridium difficile	14.55±0.2	300	120
Klebsiella pneumonia	28.38±0.2	220	45
Enterococcus faecalis	19.45±0.2	31	55
Enterococcus faecium	18.05±0.2	32	85

MIC: Minimum inhibitory concentration

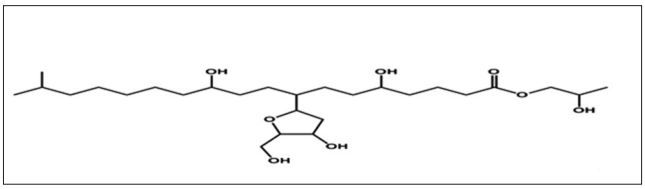

Fig. 3: 3-hydroxypropyl 5, 11-dihydroxy-8-(4-hydroxy-5-(hydroxyl methyl) tetrahydrofuran-2-yl) 17-methyleoctadecanate

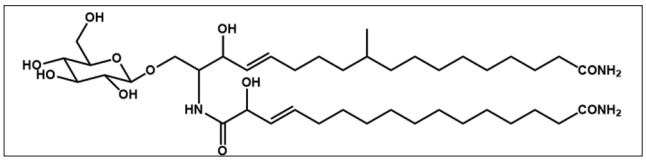

Fig. 4: (E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R, 3R, 4S, 5S, 6R)-3, 4, 5- trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) octadec-4-en-2-yl)-2- hydroxyhexadec-3-enediamide

Compound 3 was observed active against:

E. coli, *S. pneumonia, B. subtilis, B. sphaericus, S. aureus*, and *pseudomonas aeruginosa, C. albicans, S. rolfsii, P. exigua*, *Sclerotinia,* and *S. scleratiourum.*

Compound 4

Dark brown solid like, composition: C (62.97%) H (9.56%) N (3.50%) O (23.97%) molecular formula $C_{42}H_{76}N_2O_{12}$, ESI-MS (m/z) 801.05904.

^{1}H NMR (DMSO-d_6, 300 MHZ), 3.3-3.8 ppm (m, 2H-glucose), 4.43 (m, 2H), 4.52 (m, 1H), 7.10-7.90 (m, 4H- - NH_2), 1.96 (t, 4H), 1.33 (t, 2H), 1.29 (m, 10H), 1.25 (m, 12H), 5.62-5.7 (m, 4H), 4.85 (s, 1H), 0.96 (d, 3H), 2.30 (t, 2H), ^{13}C NMR (75 MHZ) DMSO-d_6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax (cm^{-1}) 1700, 1650, 1725, 3400, 1650, 3100, 1000, and 1062. The results are near about similar to (Fig. 6) [18].

E. coli, S. pneumonia, B. subtilis, S. hyicus, B. sphaericus, S. aureus, and *pseudomonas aeruginosa, C. albicans, P. exigua, S. rolfsii, Sclerotinia, scleratiourm Fusarium* sp. and *Penicillin* sp.

Compound 5

Dark brown solid like, composition: C (68.61%) H (11.52%) O (19.87%) molecular formula $C_{23}H_{46}O_5$, ESI-MS (m/z402.60834 ^{1}H NMR (DMSO-d_6, 300 MHZ), 0.85 (t, 3H), 1.23-1.50 ppm (m, 2H), 2.35 (t, 2H), 3.12 (m, 2H), 1.64 (m, 1H), 1.68 (m, 2H), 3.90 (m, 1H), 3.5 (s, 3H), ^{13}C NMR (75 MHZ) DMSO-d6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax cm^{-1} 3400, 1650, and 3100. The results are near about similar to [18].

Compound 5 was observed active against:
E. coli, S. pneumonia, B. subtilis, S. hyicus, B. sphaericus, S. aureus, and *pseudomonas aeruginosa, C. albicans, S. rolfsii, Sclerotinia, scleratiourm,* and *Penicillin* sp. (Fig. 7).

Compound 6

Dark brown solid like, molecular formula; C41H78N2O10, composition: C (64.87%) H (10.36%) N (3.69%) O (21.08%), ESI-MS (m/z) 759.06542.

^{1}H NMR (DMSO-d_6, 300 MHZ), 3.71 (m, 2H), 3.99 (m, 1H), 1.25-1.29 (m, 38H), 1.31 (m, 1H), (m, 2H), 1.44 (m, 2H), 2.34 (m, 2H), 1.53 (m, 2H), 1.96 (m, 2H), 5.67-5.69 (s, 2H), 4.85 (s, 1H), 3.83 (m, 1H), 3.40-5.03 (m, H-glucose), 0.88 (m, 3H), 6.8-7.1 (s, 2H –NH_2), ^{13}C NMR (75 MHZ) DMSO-d6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax cm^{-1} - 1600, 3400, 1650, 3100, 1000, and 1062. The results are near about similar to [19].

Compound 6 was found active against:
X. oryzae, E. coli, S. pneumonia, B. subtilis, S. hyicus, B. sphaericus, S. aureus, and *P. aeruginosa, C. albicans, S. rolfsii, S. scleratiourum,* and *P. exigua* (Fig. 8).

Compound 7

Dark brown solid like, composition: C (57.34%) H (9.43%) N (2.67%) O (30.55%) molecular formula $C_{24}H_{49}NO_{10}$, ESI-MS (m/z) 523.65726 ^{1}H NMR (DMSO-d_6, 300 MHZ), 3.37 (m, 3H), 1.25-1.44 (m, 20H), 3.21 (m, 3H), 1.66 (m, 2H)., 4.89 (m, 1H), 2.27 (d, 2H), 3.58 (m, 3H), 3.40-5-03 (m, H-glucose),7.8-7.1 (s, 2H –NH_2); ^{13}CNMR (75 MHZ) DMSO; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax cm^{-1} 3200, 1600, 3400, 1650, 3100, 1000, and 1062. The results are near about similar to [17].

Compound 7 was found active against:
E. coli, S. pneumonia, B. subtilis, S. hyicus, B. sphaericus, S. aureus, P. aeruginosa and *Xanthomonas oryzae, C. albicans, S. rolfsii, S. scleratiourum,* and *Penicillin* sp., *P. exigua* (Fig. 9).

Compound 8

Dark brown solid like, composition: C (55.05%) H (9.43%) N (8.02%) O (27.50%) molecular formula $C_{24}H_{49}N_3O_9$, ESI-MS (m/z) 523.66056.

^{1}H NMR (DMSO-d_6, 300 MHZ), 3.71 (m, 2H), 1.25-145 ppm (m, 16H), 1.51 (t, 4H), 3.4 m (m, 31H), 2.61 (m, 1H), 3.0 (m, 1H), 7.16 (s, 2H), 5.11 (t, 4H), 5.75-3.40 (m, 1H glucose). ^{13}C NMR (75 MHZ) DMSO-d6; 172-174 ppm (-C=O), 163 ppm (amid group), 110 ppm (C=C), 27-38 ppm (C-C), 73 ppm (C-OH), 75 ppm (C-O-C), 134-135 ppm (C=C). IR (KBr) υmax (cm^{-1}), 3400, 1650, 3100, 1000, and 1062. The results are near about similar to [17].

Compound 8 was found active against:
X. oryzae, E. coli, S. pneumonia, B. subtilis, S. hyicus, B. sphaericus, S. aureus, and *P. aeruginosa, C. albicans, S. rolfsii, Sclerotinia, P. exigua, Scleratiourm,* and *Fusarium* sp. (Fig. 10).

Natural antimicrobial compounds generally alter cell permeability as a result causing huge loss of micromolecules and macromolecules from cell interior, this could be one basis of antimicrobial activity of antimicrobial metabolites. Another worker has reported that antimicrobial metabolite is targeted to lysosome through entry of bacterial cell wall and with

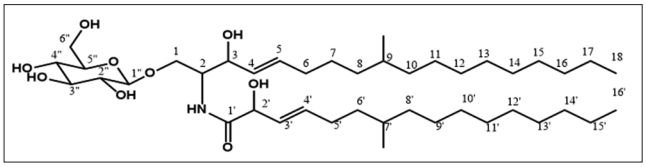

Fig. 5: (E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R, 3R, 4S, 5S, 6R)-3, 4, 5- trihydroxy-6- (hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) octadec-4-en-2-yl)-2- hydroxy-7-methylhexadec-3-enediamide

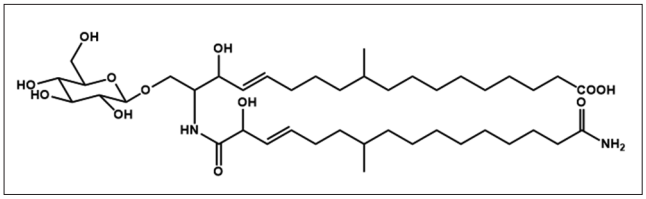

Fig. 6: (E)-17-((E)-16-amino-2-hydroxy-7-methyl-16-oxohexadec-3-enamido)-16-hydroxy-10- methyl-18- (2R, 3R, 4S, 5S, 6R)-3, 4, 5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran- 2-yl) oxy) octadec-14- enoic acid

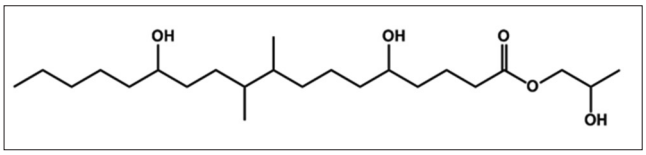

Fig. 7: Hydroxypropyl 5, 13-dihydroxy-9, 10-dimethyloctadecanoate

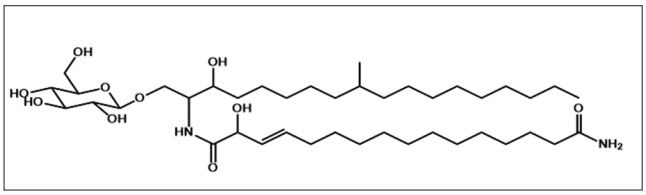

Fig. 8: (E)-2-hydroxy-N1-(3-hydroxy-9-methyl-1-(((2R, 3R, 4S, 5S, 6R)-3, 4, 5-trihydroxy- 6(hydroxymethyl) tetrahydro -2H-pyran-2-yl) oxy) octadecan-2-yl) hexadec-3-enediamide

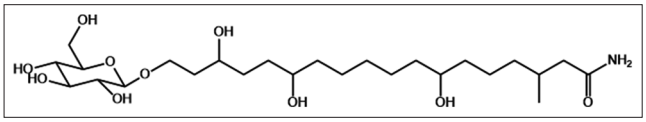

Fig. 9: 13, 16-trihydroxy-3-methyl-18-(((2R, 3R, 4S, 5S, 6R)-3, 4, 5-trihydroxy-6 (hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) octadecanamide

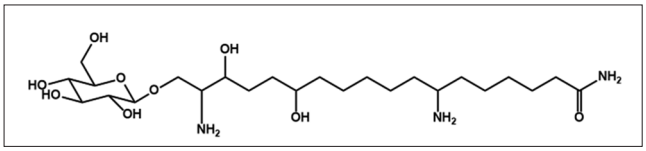

Fig. 10: 17-diamino-13, 16-dihydroxy-18-(((2R, 3R, 4S, 5S, 6R)-3, 4, 5-trihydroxy-6 (hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) octadecanamide

the bursting of lysosome, antimicrobial compounds target various component of cell cytoplasm interfering their functioning, changing osmolarity of cell resulting in cell lysis [20,21]. Another explanation might be that antimicrobial metabolite interferes with membrane function and interact with membrane protein causing deformation in structure and functionality [20]. Some antimicrobial compounds interact directly with microbial cell membrane making pores in the membrane by interacting with phospholipids of membrane. This action rapidly disrupts membrane at several position and result in leaching out of vital cell components [22] Finally, we summarize that structures' of the afforded compounds were confirmed on the bases of different spectroscopic means (NMR and MS) and comparison, and identified as 2-hydroxypropyl 5, 11-dihydroxy-8-(4-hydroxy-5-(hydroxyl methyl) tetrahydrofuran-2-yl) 17-methyleoctadecanate. Finally, compounds 2, 3, and 4 belong to group of lipid called cerebrosides. Their chemical structure shows maximum similarity with this group of an organic compound. Cerebrosides are a kind of important bioactive substances isolated mainly from sea cucumber [23]. Cerebrosides are known of their antifungal activities at low concentrations [24,25]. Compound 5 is a long chain unsaturated fatty acid which when isolated was in the form of brown oil-like liquid has also isolated some similar natural products from marine bacterial species Compound 6 was also isolated from *Aspergillus lentulus, Penicillium thymicola,* and *Penicillium corylophilum*.

CONCLUSIONS

In this study, a total 10 endophytic fungi were isolated from the *C. procera*, show a great antibacterial activity against 24 human pathogenic bacteria. All the compounds isolated from crude metabolite showed broad spectrum antibacterial activity against Gram-positive and Gram-negative bacteria and plant pathogenic bacteria. Further growing those on a large scale, modifying culture conditions and supplying some stimulants might help in getting better production of particular bioactive compound which may be a potential lead biomolecule for the synthesis of new emerging antimicrobial agents in the pharmaceutical industry.

ACKNOWLEDGMENT

The authors acknowledged the facility provided from the School of Biochemical Engineering IIT-BHU Varanasi, for carrying out research work.

REFERENCES

1. Petrini O. Fungal endophytes of tree leave. In: Andrews JA, Hirano SS, editors. Microbial Ecology of Leaves. Vol. 9. New York: Springer; 1991. p. 179-97.
2. Verma SK, Kumar A, Ashish V, Das MD. Antimicrobial activity of endophytic fungal isolate in *Argemone maxicana*, a traditional Indian medicinal plant. Int J Innov Res Sci Eng Technol 2014;3(3):10151-62.
3. Luiz HR, Sonia CN, Rita Q, Moraes M, Xiaoning W, Natascha TM, *et al*. Wedge *Coniochaeta ligniaria* antifungal activity of the crypticendophytic fungus associated with autotrophic tissue cultures of the Medicin plan *Smallanthus sonchifolius* (*Asteraceae*). Symbiosis 2013;60:231-8.
4. Kumaresan V, Suryanarayanan TS. Occurrence and distribution of endophytic fungi in a Mangrove community. Mycol Res 2001;105:1388-91.
5. Teng Y, Yan C, Xing X, Chuan CD. Plant symbionts, keys to the phytosphere. Symbiosis 2013;59:1-14.
6. Silva MG, Furtado NA, Pupo MT, Fonseca MJ, Said S, Silva FA. Antibacterial activity from *Penicillium corylophilum* Dierckx. Microbiol Res 2004;159:317-22.
7. Carroll G. Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbionts. Ecology 1988;69:2-9.
8. Gao F, Dai C, Liu X. Mechanisms of fungal endophytes in plant protection against Pathogens. Afr J Microbiol Res 2010;4(13):1346-51.
9. Schulz B, Wanke U, Draeger S, Aust HJ. Endophytes from herbaceous plants and shrubs: Effectiveness of surface sterilization methods. Mycol Res 1993;97:1447-50.
10. Amal H, Abdessamad D, Proksch P. An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. J Nat Prod 2007;70(12):1939-45.
11. Shenxi C, Yang Z, Shubin N, Xingzhong L, Yongsheng C. Chen guanacastane diterpenoids from the plant endophytic fungus. J Nat Prod 2011;74(10):2052-61.
12. Tan RX, Zou WX. Endophytes: A rich source of functional metabolites. Nat Prod Rep 2001;18:448-59.
13. Lal M, Neelam T, Verma SK, Das MD. Studies of bioactive metabolites from endophytic bacterial isolate. Int J Sci Eng Res 2016;7(6):598-606.
14. Abraham G, Virgilio BG, Palma-Nicolás JP, Rivera G. Recent advances in antitubercular natural products. Eur J Med Chem 2012;49:1-23.
15. Stierle A, Strobel GA, Stierle D. Taxol and taxen production by *Taxomyces andreanae* an endophytic fungus of pacific yew. Science 1993;260:214-6.
16. Nielsen ML, Raahave JG, Justesen T. Anaerobic and aerobic skin bacteria before and after skin-disinfection with chlorhexidine: An experimental study in volunteers. J Clin Pathol 1975;28:793-7.
17. Mohamed S. Structural elucidation and bioactivity studies of secondary metabolites from endophytic *Aspergillus niger*. Indian J Appl Res 2015;5(9):3-7.
18. Mohamed S, Hamdi N, Amal ZH, Mohsen SA. Bioactive secondary metabolities from endophytic *Aspergillus fumigates*. Struct Elucidation Bioact Stud 2013;41(10):50-60.
19. Humaira N. Structural elucidation and bioactivity studies of secondary metabolites from endophytic *Aspergillus niger*. Indian J Appl Res 2015;5(9):3-7.
20. Jing T, Xiangjie M, Hao L, Jianglin Z, Ligang Z, Minghua Q, *et al.* Antimicrobial activity of sphingolipids isolated from the stems of cucumber (*Cucumis sativus* L.). Molecules 2016;15:9288-97.
21. Cateni F, Zilic J, Zacchigna M. Isolation and structure elucidation of cerebrosides from *Euphorbia platyphyllos* L. Sci Pharm 2008;76:451-69.
22. Xu J, Wang YM, Feng TY, Zhang B, Sugawara T, Xue CH. Isolation and antifatty liver activity of a novel cerebroside from the sea cucumber *Acaudina molpadiodes*. Biosc Biotechnol Biochem 2011;75:1466-71.
23. Larsen TO, Frydenvang K, Frisvad JC, Christophersen C. UV-guided isolation of alantrypinone, a novel *Penicillium alkaloid*. J Nat Prod 1998;61:1154-7.
24. Strobell G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67:491-2.
25. Verma SK, Kumar A, Lal M, Das MD. Antimicrobial activity of extract from endophytic fungus in *Calotropis procera* roots Res. Environ Life Sci 2016;9(2):212-6.