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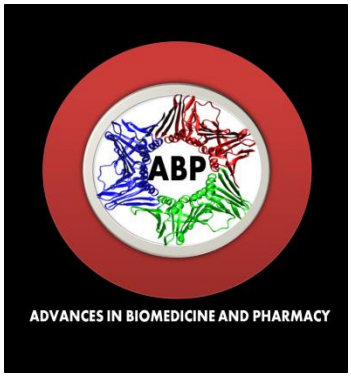

Original Article

Antimicrobial potential of fungal endophytes from selected high value medicinal plants of the Kashmir valley – India

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Abstract

The purpose of this work was to evaluate the antimicrobial potential of endophytic fungi isolated from different high value medicinal plants of Kashmir valley. Evaluation of some endophytes has been carried out for their possible antimicrobial activity from various parts of medicinal plants belonging to Kashmir valley (India). A total of twenty eight fungal endophytes were isolated from the different parts of selected medicinal plants. Dichloromethane (DCM) extracts of all the morphologically different endophytes were prepared and subsequently checked for antimicrobial activities. Eight isolates showed good activity against gram positive bacteria with two isolates showing promising activity with MIC in the range of 0.5 – 1µg/ml. All the isolated endophytic extracts were completely devoid of antifungal activity. The seven active endophytic fungal cultures were identified by ITS4 and ITS5 gene sequencing.

Keywords: Endophyte, antimicrobial, MIC, endophytes, medicinal plants

Introduction

Recently endophytes are viewed as outstanding source of secondary metabolites and bioactive antimicrobial naturalproducts. Endophytic fungi are of biotechnological interest due to their potential use as genetic vectors, metabolites [1] and biological control agents [2].In the last few decades, scientists have realized that plants are as a reservoir of unexplored numbers of potential microorganisms known as endophytes. These endophytic microbes can produce secondary metabolites such as enzymes growth hormones, antimicrobial, anti-fungal or as anticancer substances [3.4]. These secondary metabolites were proved potential drugs for treatment of newly developing disease in humans, plants and animals [5]. Due to host-endophyte co-evolution, some plants that produce bioactive natural products have associated endophytes that produce the same natural products [6]. From the microbial sources the isolation of bioactive compounds is easier and more economical for large-scale production than plant sources. The production of metabolites by microorganisms is known and explored, because the major of antibiotics are produced by fungi and bacteria [7].Antimicrobial activities have been demonstrated in a variety of metabolites biosynthesized by plant endophytes [8]. There is an increasing effort to characterize and identify the endophytic fungi isolated from the medicinal plants. Azevedo*et al*., (2000) reported that some medicinal properties of plants may be related to endophytic fungi hosted by these plants [9].

Endophytes have been intensively studied in several unexplored environments around the world. A little work in this line has been done from this state so for (which is endowed with a rich biodiversity of plant species and it has a long tradition of herbal medicine and the need for the isolation of endophytic microbes from these is the need of an hour. This led us for the isolation and exploitation of the

bioactive potential of the endophytic microbes from Kashmir valley.

In the present study an attempt has been made to isolate the endophytic fungi from the selected high value medicinal plants of Kashmir valley and assessed their antimicrobial potentials at *in vitro* level. These endophytic fungi may be the source of the potential antimicrobial drugs.

Materials and methods

Collection and identification of plant material

High value medicinal plants were collected from two localities, Sonamarg, Kashmir valley, J & K, India and from the field of CSIR-Indian Institute of Integrative Medicine (IIIM), Sanantnagar, J & K, India.Four types of plants in 5 replicates were collected randomly from each locality by individual and group visits (*Rheum emodi* wall. ex Meissn and *Hypercum perforatum* L. from Sonamarg and *Diocoria deltodia* Wall. and *Artemisia annua* L. from the fields of IIIM Sanantnager).

Isolation and purification of different endophytic strains

From each plant type, endophytic fungi were isolated as per Arnold *et al*., (2000) with some modifications [10]. Plant materials (leaf, stem and root) were thoroughly washed with running tap water followed by immersion in 70% EtOH for 1 min and in NaOCl (2.5% - 5.25%) for 3 min, drained and immersed in 70% EtOH again for 30 sec. Finally, the samples were rinsed with sterile distilled water and cut under sterile conditions into small pieces (2–3 cm). The internal tissues were cut into smaller pieces of 0.5 to 1 cm and plated on different media such as water agar, potato dextrose agar and rose bengal agar (Difco) supplemented with streptomycin sulphate (250 µg/ml, Sigma) at 28°C for 2 -3 weeks. The fungi coming out of the plant tissues were taken and grown on PDA plates free of antibiotics as a pure culture. The pure cultures were also preserved in glycerol and submitted at IIIM microbial repository. The morphological identification of the endophytic strains were done by preparing microscopic slides stained with lactophenol cotton blue as per the key of Vainio*et al*., (1998) and were examined under light microscope (Olympus, USA) [11].

Extraction of the fungal endophytes

The endophytic fungal strains were grown on potato dextrose broth at 180 rpm, 28 °C for 10-15 days in an incubator shaker (New Brunswick, USA). The grown culture of each endophyte was homogenized with 10% methanol under ultra sonicator. The fermented broth was then extracted with Dichloromethane three times after which Na2SO4 (40µg/ml, Merck) was added to further remove the aqueous layer within the mixture. The mixture was then transferred to a round bottom flask and dried using a rotary evaporator and weighed to constitute the crude broth extract.

Screening of the endophytic fungal extracts for antimicrobial activity

For the screening the antimicrobial activity the following media were used such as Muller Hinton Agar, Muller Hinton Broth, Sabroud's Dextrose broth and Sabroud's Dextrose agar (Becton-Dickinson, Cockeysville, MD, USA; DIFCO laboratories) and the extracts were tested against following bacteria: Gram positive *Staphylococcus aureus* ATCC 29213, *Methicillin resistant Staphylococcus aureus* ATCC 15187, *Vancomycin resistant enterococcus faecalis* (VRE), Gram negative *Escherichia coli* ATCC 25292 , *Pseudomonas aeruginosa* ATCC 27853. Fungal strains *Candida albicans* ATCC 90028, *C. albicans* V-0-191 and *Aspergillusniger* ATCC 16404 and *A. fumigates*. All the cultures were procured from the American Type Cultures Collection (Manassas, VA, USA). Bacterial cultures were maintained on Tryptone soya agar, fungal cultures were maintained on Sabroud's Dextrose agar, and stored at -70 °C containing 50% glycerol. Antibiotic were obtained from sigma –Aldrich, Ciprofloxacin and Amphotericin- B was used as a standard antibacterial and antifungal agent for this study. The concentrations of the stock solutions were 1mg/ml.

Minimum inhibitory concentration (MIC) assay

MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute (Wayne 2006). Bacterial and fungal suspensions were prepared by suspending 18 h grown bacterial culture and 24h fungal culture in sterile normal saline. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards (equivalent to 1.5 x 108 CFU/ml) and 1.0 McFarland standards (equivalent to 1.5×108 colony forming units (CFU)/ml) for fungal stains at wavelength 625nm. The extract stock solutions were prepared in 100% dimethyl sulfoxide (DMSO; Merck, Mumbai India) and 2-fold serial dilutions were prepared in Mueller Hinton Broth for bacteria and Sabroud's Dextrose broth for fungal in 100 µl volume in 96-well U bottom microlitre plates (Tarson, Mumbai, India) . The above mentioned bacterial and fungal suspension were further diluted in the MHB and SDB and 100 µl volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of 5×105 CFU/mL in the well and the final concentration of extracts ranged from 0.5 to 256 µg/ml. Ciprofloxacin and Amphotericin B was used as standard Antibacterial and antifungal agent for this study at a concentration ranged from 0.03-16 µg/ml. The bacterial plates were incubated at 37°C for 18 h and fungal plates at 28°C for 42 h and were visually read for the absence or presence of turbidity. The minimum concentration of the

compound concentration showing no turbidity was recorded as MIC.

Identification of the potent fungal endophytes

Identification of the fungal endophytes was based on their internal transcribed spacer ribosomal DNA (ITSrDNA) sequences. A pair of primers ITS4 (sequence: 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS5 (5′-TCC TCC GCT TAT TGA TAT GC-3′) was used for ITS-rDNA amplification, using the polymerase chain reaction (PCR).The consensus sequences were subjected to BLAST searches of the NCBI Gen Bank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification and to find out the sequence homology with closely related organisms. All the endophytic fungal ITS-rDNA sequences were deposited in Gen Bank and the accession number was assigned to each sequence.

Results

Isolation of the fungal endophytes

From all the four selected medicinal plants total 28 fungal endophytes have been isolated. Based on the morphology of the endophytes 14 different endophytes have been selected for antimicrobial activities. The selected endophytes are IIIM1, IIIM2, IIIM3, IIIM4, IIIM5, IIIM6, IIIM7, IIIM8, IIIM9, IIIM10, IIIM11, IIIM12, IIIM13, and IIIM14.

Molecular identification and characterization of endophytic fungi

The bioactive strains were identified up to the genus level using ITS1-5.8S-ITS2 approach. Among the identified fungi, *Pichiakudriavzevii* (query cover 100%, max identity 99%), *Fusariumoxysporum* (query cover 97%, max identity 99%), *Mucorcircinelloide* (query cover 99%, max identity 99%), *Mucorcircinelloide* (query cover 99%, max identity 87%), *Trametesversicolor* (query cover 94%, max identity 95%), *Polyporales sp.* (query coverage 100%, max identity 99%), *Bjerkanderaadusta* (query coverage 98%, max identity 99%), *Fusariumtricinctum* (query coverage 100%, max identity 100%).The sequences was submitted to NCBI and accession numbers assigned to them by NCBI are KC741444, KC831587, KC831588, KC831589, KC831590, KC831591, and KC831592.

Antimicrobial assay

The *in vitro* antimicrobial activities of isolated extracts were tested on a group of significant Gram positive and Gram-negative bacteria (Table 1) and fungal strains (Table 2)

IIIM8 and IIIM2 was the most active of the all the isolated extracts against the bacterial pathogens. However the activity of IIIM8 and IIIM2 was limited to Gram-positive bacteria only as its MIC was >256± µg/ml against *Escherichia coli* ATCC 25292 and *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative pathogens used in this study). Whereas seven extracts IIIM3, IIIM4, IIIM5, IIIM6, IIIM7, IIIM1 and IIIM8 exhibited moderate Gram-positive antibacterial activity (MIC ≈ 32-128± µg/ml). Five extract IIIM9, IIIM10, IIIM11, IIIM12 and IIIM13 on the other hand were inactive against bacterial pathogens up to the tested concentration of 256± µg/ml. All the isolated extracts were completely devoid of antifungal activity up to the highest tested concentration of 256± µg/ml.

Discussion

In the present study we evaluated the antimicrobial potential of the endophytic strains both by agar diffusion assay and MIC determination assay against a series of microbial pathogens and eight isolates showed good activity against gram positive bacteria with two isolates showing promising activity with MIC in the range of 0.5 – 1(µg/mL) in diameter .

To the best of our knowledge, for the first time wereported *invitro*antimicrobial activities of endophytic fungal isolates of high value medicinal plants of Kashmir Valley, India. The reported fungal endophytes could be used as an appropriate source to produce antibacterial. Based on the study carried out it can be concluded that the two elite endophytic fungal strains, IIIM2 and IIIM8, have the potential to act as a source of new antibacterial compounds against pathogenic microorganisms (Gram positive bacteria and drug resistant bacteria). Roots of R.emodi are reported to have antibacterial and antifungal activities [12-14]. This strongly supports our results that the endophytes from these could be appropriate source of antimicrobial secondary metabolites and it is also evident from the study that fungal endophytes are a rich source of bioactive compounds [15-19]. Likewise, our study shows that fungal endophytes isolated from these plants, especially Kashmir valley India, also will be a rich array of antimicrobial compounds and warrant more intensive study in our search for new chemical structures. These reports and our results strongly support the view that the endophytic fungi of traditional medicinal plants are promising sources of natural antimicrobial compounds [5, 20-21].

Conclusion

In an attempt made in the present study two endophytic fungal strains are expected to be a potential source for new natural bioactive molecules isolated from the high value medicinal plants of the Kashmir valley. These two endophytic fungal strains could be an alternative to the secondary metabolite production from medicinal plants. These endophytic fungal strains could be a source of large scale production of antimicrobial compounds provided using fermentation studies.

Declaration of Interest

We the authors of the manuscript declare that the work done is original and has not been published anywhere or has not been submitted anywhere for publication .All the work has been properly cited as per literature. All the authors declare that there is no conflict of interest regarding the publication of this article. There are no issues regarding the authorship, financial and institutional affiliations. We authorize the corresponding author for all the matters regarding paper publishing.

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Table 1: Antimicrobial activity of the isolated extracts against bacterial pathogens.

S.No	Tested Extracts	MIC (µg/mL)				
		S.aureus **ATCC 29213**	**MRSA ATCC 15187**	**VRE**	***E. coli*** **ATCC 25292**	***P. aeruginosa*** **ATCC 27853**
1	IIIM2	**1**	**0.5**	**1**	>256	>256
2	IIIM3	64	128	128	>256	>256
3	IIIM10	>256	>256	>256	>256	>256
4	IIIM4	64	64	64	>256	>256
5	IIIM5	32	32	64	>256	>256
6	IIIM6	32	32	32	>256	>256
7	IIIM11	>256	>256	>256	>256	>256
8	IIIM7	128	128	128	>256	>256
9	IIIM12	>256	>256	>256	>256	>256
10	IIIM13	>256	>256	>256	>256	>256
11	IIIM1	64	64	64	>256	>256
12	IIIM8	**0.5**	**0.5**	**1**	>256	>256
13	IIIM14	>256	>256	>256	>256	>256
14	IIIM9	>256	>256	>256	>256	>256
15	Cipro	0.25	16	64	0.03	0.03

Table 2: Antimicrobial activity of the isolated extracts against fungal pathogens.

S.No	Tested Extracts	MIC (µg/mL)			
		***C. albicans*ATCC 90028**	***C.albicans V-0-191***	***A. niger* 16404**	***A. fumigates***
1	IIIM2	>256	>256	>256	>256
2	IIIM3	>256	>256	>256	>256
3	IIIM10	>256	>256	>256	>256
4	IIIM4	>256	>256	>256	>256
5	IIIM5	>256	>256	>256	>256
6	IIIM6	>256	>256	>256	>256
7	IIIM11	>256	>256	>256	>256
8	IIIM7	>256	>256	>256	>256
9	IIIM12	>256	>256	>256	>256
10	IIIM13	>256	>256	>256	>256
11	IIIM1	>256	>256	>256	>256
12	IIIM8	>256	>256	>256	>256
13	IIIM14	>256	>256	>256	>256
14	IIIM9	>256	>256	>256	>256
15	Ampho- B	0.5	0.5	1	1

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