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Research Article

Biological screening of endophytic fungi extracted from different parts of medicinal plants

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Abstract
Endophytic fungi reside in the internal plant tissues and are potential sources of the natural products that may be used in the discovery of novel antibacterial, antifungal and antitumor agents. The recent work was conducted to isolate and identify endophytic fungi from the three medicinal plants *Papaver somniferum* (Opium poppy), *Cassia fistula* (Amaltaas) and *Catharanthus roseus* (Madagascar periwinkle) and to conduct antimicrobial activities of the crude extract obtained from these endophytic fungal strains. A total of four endophytic fungal strains were isolated of which two of them were isolated from the roots of *P. somniferum*, one from leaves of *C. fistula* and one from the mid rib of *C. roseus* using Sabouraud Dextrose Agar and labelled as PS3, PS4, CF1 and CR1 respectively. Three of the bioactive fungal strains were identified as *Aspergillus spp.* and one of genus *Curvularia* based on the cultural and morphological characteristics using bright field microscopy. After identification, these endophytic fungal strains were grown using Czapek yeast extract broth medium for the production of crude metabolites. The crude isolates were tested against 4 human infectious bacterial species including two gram negative bacteria i.e. *Salmonella typhi* and *Pseudomonas aeruginosa*, and two gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus.* Different concentration of crude extract were used i.e. 24 mg/ml, 12 mg/ml and 6 mg/ml, among them the concentration 24mg/ml showed best antibacterial activity. The crude extract was also tested against two pathogenic fungal strains i.e. *Aspergillus niger* and *Alternaria* solani. From the results, it is concluded that the crude extract from these endophytic fungal strains may be potential source in the discovery of novel antibacterial and antifungal drugs.

Keywords: Antimicrobial activities; Crude Metabolites; Endophytes; Endophytic fungi; Fungi; Medicinal plants

Introduction

The term endophyte is derived from two words "Endo" meaning "Inside" and "Phyte" meaning "Plant''. It was first used by German scientist Heinrich Anton De Bary in 1809 to describe any microorganism that live inside the plant's tissues or cells without causing any harm to the host plant is called endophyte **[1]**. Scientists have been concluded that endophyte can be major source for the discovery of new natural products that can be utilizes in the production of new drugs. About 300,000 higher plant species contain one or more endophytes **[2]**. Endophytes have wide range of genetic diversity and biological novelty and are utilized in the pharmaceutical industries (e.g. taxol, an antitumor drug) and agriculture **[3]**. Those fungi that live and proliferate inside the host plant tissues or cells for whole or part of its life cycle without triggering any damage to the host plant are known as endophytic fungi **[4]**.

Endophytic fungal strains were isolated from various plants that consist of fodders

(Alfalfa, Sorghum and Clover), fruits (Citrus, Banana, and Pineapple), crops (Sugarcane, Marigold, Coffee), Cereal grains (Maize, Rice, and Wheat), vegetables (Soybean, Radish, Tomatoes, Carrot, Lettuce, Sweet potatoes), and some trees (Pine and Yew) [5]. Medicinal plants are the potential source of endophytic fungi with crucial natural products of pharmaceutical significance [6]. *Papaver somniferum* (opium poppy) is counted one of the most vital medicinal plant, belonging to family Papaveraceae, which is a source of about 80 alkaloids of various classes that has pharmaceutical importance [7]. The first alkaloid Morphine was isolated from opium poppy which is used as sleep inducing drug [8]. Other alkaloids such as codeine and thebaine are also isolated from *P. somniferum* that are used as muscular relaxant and antimicrobial agents [9]. *Catharanthus roseus* (Madagascar periwinkle) is a well-known ornamental and medicinal plant throughout the world belonging to plant Family of *Apocynaceae* [10]. Many of the metabolites extracted from *C. roseus* are beneficial for human use, it produces about 130 terpenoid indole alkaloids (TIA), of which vinblastine and vincristine are pharmaceutically essential anti-neoplastic compounds [11]. *Cassia fistula* (Amaltaas) is cultivated as ornamental plant and also known by the name of "Disease Killer" because of its medicinal activities, belonging to the Family *Caesalpiniaceae* [12]. The extract of C. fistula flower exhibit an anticancer, antioxidant, antimicrobial, and antidiabetic properties [13].

The genus *Aspergillus* comprises of hundreds of mold species that are found all over the world. It was first identified by Italian priest and biologist Pier Antonio Micheli in 1729 by observing under the microscope which reminded him shape of aspergillum (holy water sprinkler), and named the genus after it [14]. More than 60 species of aspergillus are medically and commercially essential. They also produce such secondary metabolites that are used in the preparation and improvement of drugs that are utilized to treat human infections [15]. The genus *Curvularia* belongs to a class hyphomycete found in soil that could be both pathogenic and beneficial partner for many plant species having a positive influence on plant's growth parameters [16].

In the recent work, healthy and mature plants of *P. somniferum*, *C. fistula* and *C. roseus* were collected from the gardens of Government College of Technology Kohat road and University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan. Different fragments of plant i.e. roots, stem and leaves were selected and grown on fungi relevant media. Different fungal growth were observed and then purified on the same selective media. As a result four endophytic fungi were isolated and observed under microscope for identification, of which three of them were belonging to genus *Aspergillus*, and one was belonging to *Curvularia*. The crude metabolites from these endophytic fungal strains were isolated and subjected to antibacterial and antifungal activities.

Materials and methods

Sample collection

Healthy (showing no visual disease) and mature plants were collected from the gardens of Government college of technology Kohat road and University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan. The plant samples were collected in a sterile polythene bag and then transported to Microbial Biotech laboratory at the Centre of Biotechnology and Microbiology, University of Peshawar. The collected plants were immediately processed for surface sterilization after sampling. Different parts were selected i.e. roots, stem and leaves for the isolation of endophytic fungi.

Surface sterilization

The plants were exposed to a three step surface sterilization method following the protocol of *Petrini et. al* [17] with minor changes. Initially the whole plant sample was washed with running tap water for 10

minutes to remove soil particles, adhered debris and then washed it with detergent and finally washed with distilled water. This was followed by washing with 70% ethanol for 1 minute, with 1% Mercuric chloride (HgCl2) for 2 minutes and further sterilized with distilled water for 2 minutes. This complete process was conceded in Laminar flow hood (LFH). The proficiency of surface sterilization was evaluated by making imprint of every segment of treated tissue on agar media [18].

Isolation of endophytic fungi

After the surface sterilization of plants, the stem, roots and leaves were cut into small pieces of 1-2cm long. Those small pieces were further cut transversely to put on to the plates containing Sabouraud Dextrose Agar media (Peptone: 10 gm/L, Dextrose: 40gm/L, Agar: 15 gm/L). The Sabouraud Dextrose Agar (SDA) media prepared according to the instructions of manufacturer for the isolation of desired fungal strains. Antibiotic Ciprofloxacin (200mg/L) was added to SDA media to avoid bacterial contamination. The plates were wrapped and transferred to the fungal incubator for 7-10 days at 25 ± 30 ˚C. A variety of fungal growths were observed on each petri dish after 10 days. The different types of fungal growth were sub-cultured again on SDA medium for further purification.

Preliminary antibacterial activity

The actively growing cultures of endophytic fungi (i.e. 10 days old culture) were tested against 5 pathogenic bacteria including three gram negative bacteria i.e. *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*, and two gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* following the protocol of preliminary antibacterial screening described by *Dos Santos et al.* [19]. All of the test microorganisms were collected from Microbial biotech lab University of Peshawar. A well was made with metallic cork borer in the center of each Nutrient Agar (NA) plate seeded with test microorganisms via cotton swab (Turbidity adjusted to 0.5 McFarland standard). 9 mm of the actively growing culture of endophytic fungi were placed in those wells. The plates were incubated at 37˚C for 24 hours. After completion of incubation period, the preliminary antibacterial activity was determined by measuring the diameter of zone of inhibition in millimeter using scale.

Identification of isolated endophytic fungi

The endophytic fungal strains were identified based on morphology (Shape, colony, color, topography and microscopically) following the protocol described by *Kong et al.* [20] by growing the isolates on SDA media.

Fermentation

Czapek Yeast Broth (CYB) medium was prepared by preparing three solutions according to the protocol described by *khattak et al.* for fermentation of isolated bioactive fungal strains [21]. Solution A (MgSO4.7H2O: 5gm/500mL, NaNO3: 20gm/500mL, FeSO4.7H2O: 0.1gm/500mL and KCL: 5gm/500mL), Solution B (K2HPO4: 10gm/500mL) and Solution C (Cu SO4.5H2O: 1gm/200mL and ZnSO4.7H2O: 2gm/200mL) were prepared in a separate flasks. Solution A (50mL), Solution B (50mL) and Solution C (1mL) were taken in 1L flask supplemented with Yeast extract (5gm) and Saccharose (15gm). After preparation of CYB medium, the isolated fungal strains were inoculated in separate flasks containing media by following the procedure determined by *Radji et al.* [22] and were incubated at 25 ± 30 ˚C and 150 rpm for 14 days in a shaker incubator.

Crude metabolites extraction from endophytic fungi

Once the incubation period was completed, 250 µl of 40%HCL were added to each flask and left for 10 minutes to acidify the medium for settling of the media constituents. The mixture was then filtered through muslin cloth to filter the mycelia. Equal volume of ethyl acetate was added to each flask and properly shaked for 20-30

minutes to mix the contents. The mixture was poured into a separating funnel and left for 10 minutes to settle down. Two separate layers were formed of which the upper layer of ethyl acetate had crude metabolites, which were separated and washed with 2 M brine solution. Once the washing with brine solution was completed, the upper layer of organic substances were collected and further purified with anhydrous sodium sulphate. The crude metabolites were concentrated using rotary evaporator at 45-50 ˚C with 150 rpm rotation. The crude extract then obtained from rotary evaporator using methanol in small valves and left for 5 days to be dried to be used further for biological activities.

Antimicrobial activities

Antibacterial activities of crude extract from isolated fungal strains

The crude metabolites isolated from four endophytic fungal strains were tested against 4 human pathogenic bacteria including two gram negative bacteria i.e. *S. typhi* and *P. aeruginosa*, and two gram positive bacteria *B. subtilis* and *S. aureus* by agar well diffusion method [23, 24]. The test microorganisms were inoculated in Nutrient broth (NB) (Turbidity adjusted to 0.5 McFarland standard) and incubated at 37 ˚C for 24 hours to make fresh culture for the activity. 100µL of test microorganisms from the broth culture was taken and spread over the NA media plates with sterile cotton swab. Wells were made in the NA media petri plates seeded with test microorganisms using metallic cork bore. Stock solution was prepared by dissolving the extract in di-methyl sulfoxide (DMSO) (24mg of crude extract/1000 µl of DMSO). Now different concentrations were poured in each well i.e. 24mg/ml, 12mg/ml, and 6mg/ml. The zone of inhibition was measured in millimeters after incubation at 37 ˚C for 24 hours. Antibiotic Ciprofloxacin was used as positive control drug and DMSO was used as negative control.

Antifungal activities of crude extract from isolated fungal strains

The antifungal activity of the crude metabolites isolated from the four bioactive endophytic fungal strains were screened against two fungal strains i.e. *Aspergillus niger* and *Alternaria solani.* For antifungal activity SDA media tubes were prepared. Stock solution was prepared by dissolving the extract in di-methyl sulfoxide (DMSO) (12mg of crude extract/1000 µl of DMSO). Now different concentrations were poured in each tube i.e. 12mg/ml, 6mg/ml, and 3mg/ml. The test tubes were left to cool for solidification to make slants. After that test organisms were inoculated in each test tube and incubated at 25 ± 30 ˚C for 3-4 days. Nystatin was used as positive control drug and DMSO was used as negative control. Once the incubation period was completed, the zones of inhibition were measured by the following formula in percentage;

$$\frac{\text{Zone of inhibition of crude extract}}{\text{Zone of inhibition of standard drug}} \times 100$$

Results

Isolation of endophytic fungi

Different growth of fungi was observed on SDA media in which we selected four bioactive strains of endophytic fungi, two from the roots of *P. somniferum*, one from leaves of *C. fistula* and one from the mid rib of *C. roseus* and labelled as PS3, PS4, CF1 and CR1 respectively. These four fungal growths were further purified on SDA media to eliminate the plants adherent compounds.

Preliminary antibacterial activity

The isolated strains of endophytic fungi PS3, PS4, CF1 and CR1 were tested against 5 different human pathogenic bacteria including three gram negative bacteria i.e. *E. coli, S. typhi* and *P. aeruginosa*, and two gram positive bacteria i.e. *B. subtilis* and *S. aureus* for preliminary antibacterial activity. The zones of inhibition were measured in millimeter and are listed in the (Table 1). The range of zones of inhibition was 6mm to 20mm. The endophytic fungal strains PS3 and CF1 showed best activity

against test bacterial strains, while PS4 and CR1 slightly inhibited the bacterial growth.

Identification of isolated endophytic fungi

The isolated endophytic fungal strains PS3, PS4, CF1 and CR1 were studied under light microscope after performing their preliminary antibacterial testing. Based on the morphological characteristics of endophytic fungal strains, PS3 was identified as *Aspergillus spp.* 1, PS4 was identified as *Aspergillus spp*. 2, CF1 was identified as *Aspergillus spp*. 3 and CR1 was identified as genus *Curvularia spp.* Growth and results of microscopic identification are shown in the (Figs. 1, 2, 3 & 4). These endophytic fungal strains were fermented on suitable media for further studies.

Figure 1. A-Growth of PS3 on SDA media B-microscopic image of PS3

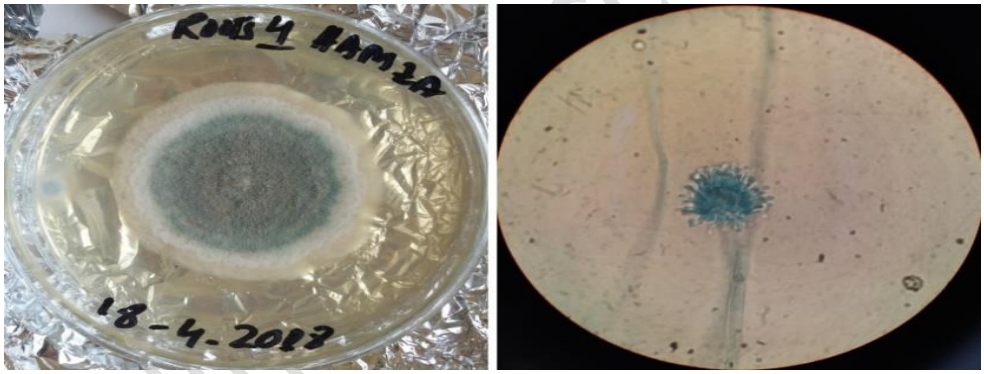

Figure 2. A-Growth of PS4 on SDA media B- microscopic image of PS4

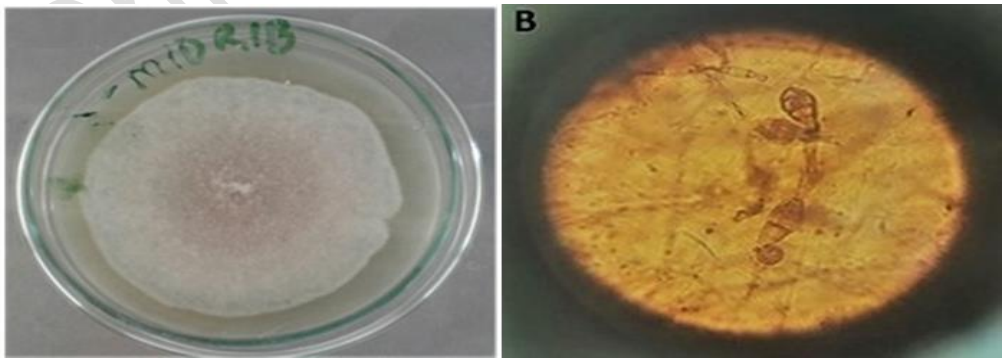

Figure 3. A-Growth of CR1 on SDA media B- microscopic image of CR1

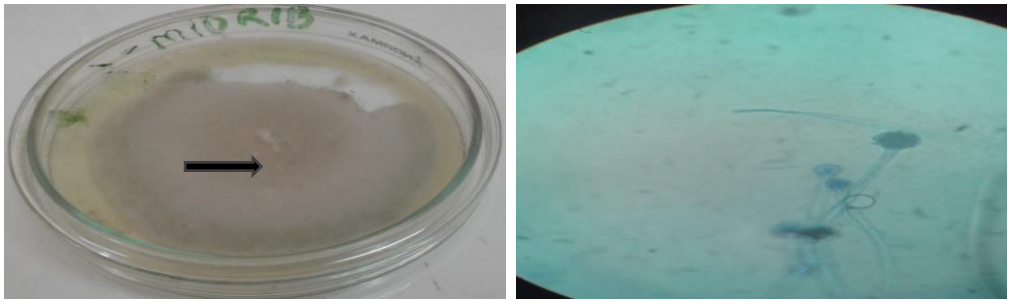

Figure 4. A-Growth of CF1 on SDA media B- microscopic image of CF1

Antimicrobial activities

Antibacterial activities of crude extract from isolated fungal strains

The crude extracts isolated from endophytic fungal strains PS3, PS4, CF1 and CR1 were screened against four human pathogenic bacteria including two gram negative bacteria i.e. *S. typhi* and *P. aeruginosa*, and two gram positive bacteria *B. subtilis* and *S. aureus* by agar well diffusion method. Different concentrations of endophytic fungal crude extract inhibit the growth of test microorganisms with different zones of inhibition and measured in millimeters. PS3 doesn't inhibited the growth of *S. aureus* while PS4, CF1 and CR1 showed the best antibacterial activity. Results of antibacterial activity are given in the (Tables 2, 3, 4 & 5).

Table 1. Preliminary antimicrobial activity of endophytic fungi

Isolated Fungi	Zones of inhibition by pathogenic bacteria (mm)				
	E.C	P.A	S.T	B.S	S.A
PS3	8	9	15	17	20
PS4	6	5	4	9	2
CF1	12	10	12	8	11
CR1	9	7	10	6	12

E.C- *Escherichia coli*, **P.A**- *Pseudomonas aeruginosa*, **S.T**- *Salmonella typhi*, **B.S**- *Bacillus subtilis*, **S.A**- *Staphylococcus aureus*

Table 2. Antibacterial activity of PS3 crude extract

Endophytic fungal crude extract concentrations	Zones of inhibition by pathogenic bacteria (mm)			
	B.S	P.A	S.A	S.T
24mg/ml	5	10	0	6
12mg/ml	5	7	0	6
6mg/ml	2	3	0	2
DMSO	0	0	0	0
CIP	7	0	0	8

Table 3. Antibacterial activity of PS4 crude extract

Endophytic fungal crude extract concentrations	Zones of inhibition by pathogenic bacteria (mm)			
	B.S	P.A	S.A	S.T
24mg/ml	10	10	6	10
12mg/ml	5	8	0	6
6mg/ml	3	4	0	4
DMSO	0	0	0	0
CIP	6	0	12	5

Table 4. Antibacterial activity of CR1 crude extract

Endophytic fungal crude extract concentrations	Zones of inhibition by pathogenic bacteria (mm)			
	B.S	P.A	S.A	S.T
24mg/ml	9	14	13	12
12mg/ml	4	7	6	9
6mg/ml	2	3	0	4
DMSO	0	0	0	0
CIP	10	5	8	5

Table 5. Antibacterial activity of CF1 crude extract

Endophytic fungal crude extract concentrations	Zones of inhibition by pathogenic bacteria (mm)			
	B.S	P.A	S.A	S.T
24mg/ml	14	10	13	09
12mg/ml	08	10	09	05
6mg/ml	3	5	5	0
DMSO	0	0	0	0
CIP	10	5	7	3

Antifungal activities of crude extract from isolated fungal strains

The crude extracts isolated from endophytic fungal strains PS3, PS4, CF1 and CR1 were tested against two pathogenic fungal strains i.e. *Aspergillus niger* and *Alternaria solani*. Different concentrations of endophytic fungal crude extract inhibit the growth of test microorganisms with different zones of inhibition. All of the crude extract concentration showed potential activity against both of the fungal strains.

Results of antifungal are listed in the (Tables 6, 7, 8 & 9). The zones of inhibition was measured by the following formula in percentage;

$$\frac{\text{Zone of inhibition of crude extract}}{\text{Zone of inhibition of Nystatin}} \times 100$$

Table 6. Antifungal activity of PS3 crude extract

Endophytic fungal crude extract concentration	Zones of inhibition by pathogenic fungi (%)	
	A. niger	*A. solani*
12gm/ml	10%	10%
6gm/ml	90%	90%
3gm/ml	20%	30%
Nystatin	100%	100%

Table 7. Antifungal activity of PS4 crude extract

Endophytic fungal crude extract concentration	Zones of inhibition by pathogenic fungi (%)	
	A. niger	*A. solani*
12gm/ml	80%	80%
6gm/ml	80%	70%
3gm/ml	90%	80%
Nystatin	100%	100%

Table 8. Antifungal activity of CR1 crude extract

Endophytic fungal crude extract concentration	Zones of inhibition by pathogenic fungi (%)	
	A. niger	*A. solani*
12gm/ml	80%	90%
6gm/ml	75%	70%
3gm/ml	40%	50%
Nystatin	100%	100%

Table 9. Antifungal activity of CF1 crude extract

Endophytic fungal crude extract concentration	Zones of inhibition by pathogenic fungi (%)	
	A. niger	*A. solani*
12gm/ml	100%	80%
6gm/ml	70%	70%
3gm/ml	50%	60%
Nystatin	100%	100%

Discussion

Plants are widely used as therapeutic agents for providing natural metabolites that functions as source of all the bioactive drugs [25]. Approximately 22,000 species of microbial secondary metabolites are identified as bioactive compounds in which about 8,600 are of fungal source. Half of the identified fungal secondary metabolites require further evaluation that whether they have any bioactivities [26]. Microbes are becoming resistant against antimicrobial agents day by day therefore discovery of new antimicrobial agents is essential. Endophytic fungi can be identified and classified on the basis of morphological characteristics [27].

In the current study, different parts of three medicinal plants *P. somniferum C. fistula and C. roseus* were placed on the relevant media for isolation of endophytic fungal strains. Although, different growth of endophytic fungi observed on relevant media but four of them were selected after performing their preliminary antibacterial testing against five human pathogenic bacterial strains. The endophytic fungal isolates indicate that these medicinal plants can be one of the source of various beneficial fungal strains. All of the endophytic fungal strains were identified via light microscope of which three were from genus *Aspergillus* and one from genus *Curvularia*. The four endophytic fungal isolates were grown in CZAPEK broth for the extraction of crude metabolites. The crude metabolites extracted from these endophytic fungal isolates were further screened for their biological activities.

The results of antimicrobial activities of crude extract isolated from these endophytic fungal strains i.e. PS3, PS4, CF1 and CR1 showed that they contain antimicrobial compounds, which are active against the tested microorganisms. Of the four tested bacterial strains, *P. aeruginosa* was the most sensitive to the crude extract. This was confirmed by the observation of clear zone around the well. Crude extract from these endophytic fungal strains also showed best activity against *S. typhi* and *B. subtilis*, while crude extract from endophytic fungal strain PS3 showed less activity against *S. typhi* and *B. subtilis*. The crude extract isolated from PS4 showed best antifungal activity, while PS3 showed less activity against the pathogenic fungal strains. The crude extract of CF1 and CR1 showed 100% efficiency against the pathogenic fungal strains. This results indicated that the endophytic fungal strains PS3, PS4, CF1 and CR1 contains active antimicrobial metabolites that can be further used as antimicrobial drugs.

Conclusion

In this study, to the best of our knowledge it was the first time to isolate endophytic fungi from the medicinal plants *Papaver somniferum, C. fistula and C. roseus*. The four isolated endophytic fungal strains i.e. PS3, PS4, CF1 and CR1 are capable of generating valuable amount of crude bioactive metabolites screened in preliminary test. Furthermore, it was revealed that crude metabolites extracted from these bioactive endophytic fungal strains has shown potential antimicrobial activities against four human pathogenic bacterial strains and two pathogenic fungal strains. From these results, it was concluded that by modifying culture conditions like changing pH, changing growth media and supplying some stimulant may help for better production of bioactive compounds

on a large scale. These endophytic fungal strains could be the potential way for generating useful pharmaceutical and medical products such as antibiotics and antifungal drugs.

Authors' contributions

Conceived and designed the experiments: HU Rehman & B Ismail, Performed the experiments: HU Rehman & A Khurshid, Analysed the data: HU Rehman, Contributed materials/ analysis/ tools: A Khurshid & B Ismail, Wrote the paper: HU Rehman

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