ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 3, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

ANTAGONISTIC AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM NEEDLE OF *CUPRESSUS TORULOSA* D.DON

RINKY BISHT, DEEKSHA SHARMA, PAVAN KUMAR AGRAWAL*

Department of Biotechnology, G.B. Pant Engineering College, Ghurdauri, Pauri, Garhwal, Uttarakhand, India.
Email: p_k_agarwal@rediffmail.com

Received: 17 February 2016, Revised and Accepted: 27 February 2016

ABSTRACT

Objective: This study was undertaken to investigate antagonistic and antibacterial activities of the endophytic fungi isolated from living symptomless needle of *Cupressus torulosa* D.Don from Pauri, Garhwal region of Uttarakhand. The emergence of antibiotic-resistant microorganisms calls for inventive research and development strategies. Inhibition of these pathogenic microorganisms may be a promising therapeutic approach. The screening of antimicrobial compounds from endophytes may be a promising way to meet the increasing threat of drug-resistant strains of human and plant pathogens.

Methods: A total of five different fungal endophytes were isolated from the needle of *C. torulosa* D.Don using potato dextrose agar medium. These fungal isolates morphotypically characterized. These isolates were further tested for antagonistic activity by the dual culture technique. Among five endophytic fungi, only two fungal endophytes were cultured to examine their antimicrobial properties and phytochemical analysis. Antimicrobial activity was evaluated for crude hexane extracts against human pathogen *Staphylococcus aureus*, *Bacillus subtilis,* and *Salmonella typhimurium* using an agar diffusion assay.

Results: A total of five fungal endophytes characterized as such as *Cladosporium* sp., *Aspergillus* sp., *Fuzaium* sp., *Curvularia* sp., *and Diaporthe* sp. In which, only two endophytic fungal isolates such as PCTS23 and WCTS21 characterized morphotypically as *Cladosporium* sp. and *Curvularia* sp., respectively, were able to show strong antagonism activity against fungal pathogen. The fungal isolate PCTS3 was more active against *Macrophomina phaesolina* with antagonistic index 88.88 while WCTS21 was more active against *F. solani* with antagonistic index 80. The fungal crude extract of WCTS21 produced the highest zone of inhibition 12 mm for *S. aureus,* whereas crude extract of PCTS23 from hexane crude extract has shown the highest zone of inhibition of 10 mm against *S. aureus.* A preliminary qualitative phytochemical analysis of fungal crude extracts also revealed the presence of bioactive metabolites such as flavonoids, alkaloids, phenols, saponins, steroids, tannins, and terpenoids in endophytic fungi.

Conclusions: This study concludes that endophytic fungi isolated from *C. torulosa* D.Don could be a potential source for bioactive metabolites and may be used in pharmaceutical industry. The generated data has provided the basis for its application in the pharmaceutical industry in the form of traditional and folk medicine.

Keywords: Endophytic fungi, *Cupresus torulosa*, Antibacterial activity, Phytochemical analysis, Bioactive metabolites, Antagonistic activity.

INTRODUCTION

There is an ever-growing need for new and useful compounds to provide assistance and relief in all aspects of the human condition. Both human pathogens and fungal phytopathogens are prone to develop "drug" resistances. The effectiveness of the older types of antibiotics can decrease substantially. In addition, because of safety and environmental problems, many synthetic agricultural agents have been and still are targeted for removal from the market. The removal of such agents creates a need to find alternative ways to control farm pests and pathogens. Fungal endophytes belong to ascomycetes and deuteromycetes (mitosporic fungi), and very few members of basidiomycetes family generally occur as endophytes [1]. The presence of endophytic fungi in plant tissues was discovered more than 75 years ago from *Lolium* grass [2].

Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice to avoid replication in the study of natural products to assist in solving not only plant diseases but also human and animal health problems. Endophytes are chemical synthesizer inside plants, in other words, they play a role as a selection system for microbes to produce pharmacologically active substances with low toxicity toward mammalians [3] and presented a list of all approved agents from 1981 to 2006, from which a maximum number of natural drugs are produced by endophytes [4].

There are many pieces of evidence that bioactive compounds produced by endophytes could be alternative approaches for discovery of novel drugs. Stierle *et al*. were reported endophytic fungus as a sustainable alternative source of taxol [5]. Natural products are adapted to a specific function in nature. Thus, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotypes. Endophytic fungi inhabit a biotype that is not well studied [6].

Endophytic microorganisms are a significant reservoir of novel bioactive secondary metabolites including antimicrobial, antiinsect, anticancer, antidiabetic, and immunosuppressant compounds with their great potential applications in agriculture, medicine, and food industry [7,8]. These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones [9]. World health problems caused by drug-resistant bacteria and fungi are increasing.

Approximately 4000 secondary metabolites of fungal origin have been described to possess biological activities [10]. The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganisms [11]. Most of the natural products from endophytic fungi are antimicrobial, anticancer agents, biological control agents, immunosuppressive agents, and other bioactive compounds by their different functional roles.

The isolation of novel secondary metabolites from the endophytes is a progressive field in research [12]. Many reports demonstrated the importance of endophytic fungi in the production of antiviral agents, such as cytonic acids A and B and novel human cytomegalovirus protease inhibitors, which had been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. reported antihepatitis C virus *activity of* dihydroiso-coumarin (R)-(-)- mellein, *Trametes hirsuta*, *Aspergillus fumigates*, *Phialocephala fortinii,* and *Fusarium oxysporum* [13-15].

In this study, the endophytic fungi were isolated from living symptomless needle of *C. torulosa* D.Don isolated from Pauri, Garhwal region of Uttarakhand, and the fungal crude extracts were screened for their antibacterial activity against human pathogens. Therefore, in this study, attempts have been made to study the taxonomic position of the fungal endophytes as well as biological evaluation of its active metabolites.

METHODS

Collection of plant material

The needles were collected of *C. torulosa* D.Don from hilly areas of Uttarakhand state, namely, Pauri, Garhwal region belonging to the Himalayan region. The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used for isolation work to reduce the chance of contamination.

Isolation of endophytic fungi

The sampling procedure was designed with the intention of isolating as many endophytic fungal species as possible from the different tissues samples. Tissues of the leaves of *C. torulosa* D.Don were cut into 5.0 mm long segments then surface sterilized by the method of Raviraja [16] with minor modification. Segments were surface sterilized by consecutive immersion for 1 minute in 75% ethanol, treated for 1 minute in 0.1% mercuric chloride followed by several washing for in sterile distilled water. The time of the dilution and immersion in ethanol and mercuric chloride varies with tissues and host (at least three washing require). Under sterile conditions, tissue segments were allowed to surface-dry before plating. Five segments were then evenly placed in each 90 mm Petri dish containing potato dextrose agar (PDA) medium. The dishes were sealed with parafilm and incubated at 27±2°C for 2-4 days in incubator.

Identification of endophytic fungi

Fungal growth and sporulation were facilitated by placing the isolates onto PDA culture medium. The plates were continuously monitored for spore formation. Isolates were identified on the basis of cultural characteristics, color, and morphology of fruiting bodies and spores. Fungal isolates were stained with lactophenol cotton blue and examined under a light microscope (Olympus, USA).

Antagonistic activity of endophytic fungi

Test pathogens*, F. solani, M. phaseolina* were obtained from the Department of Biotechnology Pauri, Garhwal, Uttarakhand. Dual culture technique was adopted for antagonistic test against these pathogens on PDA plates. 5-day-old mycelia disks of 5 mm diameter of the test pathogens were placed on one corner of Petri plates containing PDA medium. Selected endophytes were inoculated on the other corner of PDA plates. Plates were incubated at 28°C for 5 days, and antagonistic index was accessed according to the following formula:

$$\text{Antagonistic index} = \frac{RM - rm}{RM} \times 100$$

RM: Radius of the pathogen in the control plate,

rm: Radius of pathogen in the dual culture plate.

Extraction of the bioactive metabolite

Extraction is done to obtain the bioactive compounds. The pure culture of the endophytic fungi is used as inoculums for the seed preparations. The pH of the media was maintained at 6.5, and bioactive metabolites extraction was carried out as described by with minor modification [17,18]. Endophytic fungal isolates were further inoculated into 250 ml erlenmeyer flasks containing 100 ml potato dextrose broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered to separate the mycelia and the filtrate. To the filtrate, an equal amount of hexane was added mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers are formed. The upper layer of hexane containing the extracted compounds was separated using the separating funnel. Cultured cells are homogenized in cell disrupter for extraction of the extracellular and intracellular compounds, thereby using hexane. The cell mass is then separated out from the supernatant by the process of extraction using muslin cloth. Solvent was evaporated and the resultant compound was dried in rotator vacuum evaporator to yield the crude metabolite. The crude extract was then dissolved in dimethyl sulfoxide (DMSO) (1 mg/ml) and kept at 4°C.

Screening of bioactive properties of fungal metabolites

Antibacterial activity of secondary metabolites extracted from endophytic fungi was screened against Gram-positive bacteria such as *Staphylococcus aureus, Bacillus subtilis*, and *S. typhymurium* of human health using agar well diffusion method. Bacterial pathogens were spread on Mueller-Hinton agar plates. Then, wells were made and three concentration of extraction were inoculated in separate wells 200, 150, and 100 µl. Antibacterial activities were detected after an incubation of 24-48 hrs at 37°C. The presence of a zone of clearance on plates was used as an indicator of bioactive nature of the strain. As positive control, streptomycin was used and DMSO was used as negative control.

Determination of minimum inhibitory concentration (MIC)

MIC was determined after the antibacterial activity of the fungal crude extracts by the standard method of Wariso and Ebong [19] with minor modification. Mueller-Hinton broth was made and sterilized using autoclave. 1.0 ml of the prepared broth was dispensed into the test tubes labeled from 1 to 5 using sterile syringe and needle. A stock solution containing 25 mg/ml of the extract was prepared. Then, 1 ml of the solution was dispensed into the tube 1. Subsequently, from tube 1 solution was serially transferred till tube 5 and 1 ml of the solution was discarded from it. Tube 6 was used as a control for sterility of the medium and tube 7 for viability of the organisms. An overnight culture of each of the test isolates was prepared in sterile nutrient broth. 1 ml inoculum was transferred into each tube from tube 1 to tube 7 with exception of 6, to which another sterile broth was added. The final concentration of the extract in each of the test tubes numbered after dilution 25, 12.5, 6.25, 3.125, and 1.563 mg/ml were incubated at 37°C for 24 hrs and examined for growth. The test tube in which growth failed to occur was the MIC of the culture.

Phytochemical screening of the fungal metabolites produced by the endophytic fungi

Phytochemical analysis of extracts of the fungus was performed with the standard methods with modification [20]. Chemical prospecting hexane extract of endophytic fungi was performed to observe the presence bioactive compounds already found as having antimicrobial activity, which can be present in the secondary metabolism of the endophytic fungi. These are checked for the presence of the following secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, steroids, phenols, and tannins were evaluated.

Preliminary qualitative screening of fungal metabolites produced by endophytic fungi

Alkaloids

The fungal crude extract was dissolved in 2 N HCl solutions. The mixture was treated with a few drops of Meyer's reagent (3.0 ml of

potassium iodide solution mixed 2.0 ml of mercuric chloride solution). The creamish precipitate indicates the presence of alkaloids.

Flavonoids

In the test tube containing 1.0 ml of the fungal crude extract was added with a few drops of 20% NaOH solution. Change to yellow which on addition of acid changed to colorless solution depicted the presence of flavonoids.

Phenols

The fungal extract was dissolved in 5 ml of distilled water. To this, few drops of 5% ferric chloride solution were added. A dark green indicated the presence of phenolic compounds.

Saponins

The presence of saponins was determined by the frothing test. The fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 minutes. Formations of a fairly stable emulsion indicated the presence of saponins.

Tannins

The fungal crude extract was treated with alcoholic $FeCl_3$ reagent, a bluish black, which disappears on the addition of a little dilute H_2SO_4 was followed by the formation of yellowish brown precipitate indicated the presence of tannins.

Terpenoids

About 1.0 ml of fungal crude extract was mixed in 2.0 ml of chloroform. 3.0 ml of concentrated H_2SO_4 was then added to form a layer. A reddish brown precipitate coloration at the interface formed indicated the presence of terpenoids.

RESULTS

Sample collection, isolation, and identification of endophytic fungi

A systematic study about the endophytic fungal biodiversity in conifer forest plants, *C. torulosa* D.Don grown in Govind Ballabh Pant Engineering College Campus, Pauri, Garhwal, Uttarakhand, was carried out to evaluate their capacity to produce the bioactive compound. A total of five endophytic fungi were isolated from leaves using different culture media. These endophytic fungi were characterized morphotypically using lactophenol cotton blue using scotch tape techniques (Fig. 1). Further confirm identification have been done by Forest Research Institute, Dehradun and endophytic fungi (Table 1).

Aspergillus niger (PCT S1)

Microscopic morphology of *A. niger* showing large, globose, dark brown conidial heads, which become radiate, tending to split into several loose columns with age. Conidiophores are smooth-walled, hyaline or turning dark toward the vesicle. Conidial heads are biseriate with the phialides born on brown, often septate metulae. Conidia are globose to subglobose, dark brown to black and rough-walled.

Cladosporium cladosporioides (PCT S3)

Colonies are rather slow growing, mostly olivaceous-brown to blackish brown but also sometimes gray, buff or brown, suede-like to floccose, often becoming powdery due to the production of abundant conidia. Vegetative hyphae, conidiophores, and conidia are equally pigmented. Conidiophores are more or less distinct from the vegetative hyphae, are erect, straight or flexuous, unbranched or branched only in the apical region, with geniculate sympodial elongation in some species. Conidia are 1 to 4 celled, smooth, verrucose, or echinulate, with a distinct dark hilum and are produced in branched acropetal chains.

Cladosporium sphaerospermum (PCT S13)

Colonies are rather slow growing, mostly olivaceous-brown to blackish brown but also sometimes gray, buff or brown, suede-like to floccose, often becoming powdery due to the production of abundant conidia. Vegetative hyphae, conidiophores, and conidia are equally pigmented. Conidiophores are more or less distinct from the vegetative hyphae, are erect, straight or flexuous, unbranched or branched only in the apical region, with geniculate sympodial elongation in some species. Conidia are 1 to 4 celled, smooth, verrucose or echinulate, with a distinct dark hilum and are produced in branched acropetal chains.

Alternaria alternate (PCT S21)

A. alternata produced oblivious blackish brown colonies on PDA plates. The reverse side of the colony was dark brown and pale brown to olive brown. Conidiophore is 25-60 µm × 3-3.5 µm, and it can straight or flexuous. Individual conidiophores arise directly from substrate forming bushy heads consisting of 4-8 large catenate conidia chains. Secondary conidiophores are generally short.

Curvularia lunata (WCT S21)

Curvularia sp. is a hyphomycete (mold) fungus which is a facultative pathogen of many plant species *C. lunata* is distinguished by septate, dematiaceous hyphae producing brown, geniculate conidiophores. The poroconidia are curved slightly to distinctly, transversely septate, with an expanded third cell from the pore end of the conidium. *Curvularia* can be easily distinguished from bipolaris and *Drechslera* sp. since the conidia are non-distoseptate, that is, septate from edge to edge of the conidial wall.

Antagonistic activity of endophytic fungi isolates

The isolates were tested for antagonistic activity by the dual culture technique. A total of five endophytic fungal isolates out of 17 were showed antagonism activity (Table 2). The fungal isolate PCTS3 was more active against *M. phaesolina* with antagonistic index 88.88 while WCTS21 was more active against *F. solani* with antagonistic index 80.25. PCTS1and PCTS21 were active against both pathogens with 50-60 antagonistic index. PCTS23 have shown the lowest antagonistic index against fungal pathogens.

Production and extraction of secondary metabolites

The two isolates PCS3 and WCTS21 were found to be shown strong antagonistic activity so were selected for the production and extraction

Table 1: Morphotypic characterization fungal endophytes

Code of isolate	Source of endophytic fungi	Colony characteristics on PDA media	Probable endophytic fungus	Class
PCTS1	Leaves	Algae green	*A. niger*	Eurotiomycetes
PCTS3	Leaves	Light green fungus with a tint of green background	*C. cladosporioides*	Dothideomycetes
PCTS13	Leaves	Visible green colored fungus	*C. sphaerospermum*	Dothideomycetes
PCTS21	Leaves	Blackish gray appearance	*A. alternate*	Dothideomycetes
WCTS21	Leaves	Remarkable dark brown	*C. lunata*	Euascomycetes

PDA: Potato dextrose agar, *A. niger: Aspergillus niger, C. cladosporioides: Cladosporium cladosporioides, C. sphaerospermum: Cladosporium sphaerospermum, A. alternate: Alternaria alternate, C. lunata: Curvularia lunata*

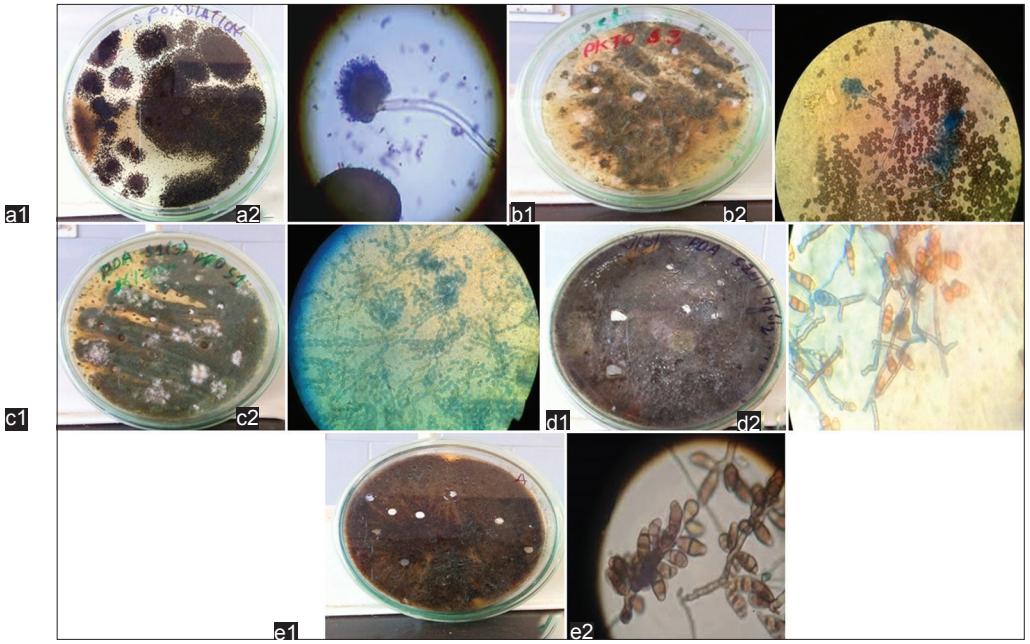

Fig. 1: (A) (1) Colony morphology on potato dextrose agar of *Aspergillus niger*; (2) shape of conidia by staining technique, (B) (1) Colony morphology on potato dextrose agar of *Cladosporium cladosporioides*; (2) shape of conidia by staining technique, (C) (1) Colony morphology on potato dextrose agar of *Cladosporium sphaerospermum*; (2) shape of conidia by staining technique, (D) (1) Colony morphology on potato dextrose agar of *Alternaria alternata*; (b) shape of conidia by staining technique, (E) (1) Colony morphology on potato dextrose agar of *Curvularia lunata*; (2) shape of conidia by staining technique

of secondary metabolites. These isolates were extracted with hexane as solvents.

Antibacterial activity of crude extract by agar well diffusion method

The antibacterial activity at concentration of 25 mg/ml of hexane extracts of endophytic fungi were tested against three human pathogens *B. subtilis*, *S. aureus,* and *Salmonella typhimurium* and had shown broad-spectrum activity which has been reported in Table 3. The crude extract of WCTS21 produced the highest zone of inhibition 12, 7, and 6 mm, respectively, against *S. aureus*, *Salmonella typhi,* and *B. subtilis,* respectively. The crude extract of PCTS3 from hexane solvent has shown the highest zone of inhibition of 10 mm against *S. aureus.* Crude extracts of fungal endophytes isolates have shown zone of inhibition from ranged 6 to 12 mm against *S. typhi*, *S. aureus,* and *B. subtilis* (Figs. 2 and 3).

MIC of fungal crude extracts

All active extracts showing potent antibacterial activity were further determined for their MIC by a tube dilution technique against *B. subtilis, S. aureus*, and *S. typhimurium* (Table 4). Fungal crude extracts have shown MIC ranged from 1.25 to 5.0 mg/ml for *S. typhimurium*, *S. aureus,* and *B. subtilis*. The crude extract of PCTS3 showed MIC of 1.25 mg/ml for *B. subtilis*, 2.5 mg/ml for *S aureus,* and 5 mg/ml *for S. typhi* which showed its efficacy as a potent antimicrobial. Whereas WCTS21 exhibited MIC of 5.0 mg/ml for *B. subtilis* and *S. aureus* and 1.25 mg/ml for *S. typhi*.

Phytochemical screening of fungal metabolites

Chemical analysis was carried out on the isolated endophytic fungal extracts to determine the presence of chemical components as a prospective source for medicinal and industrial use. Their presence is an indicator that they can be exploited as precursors in the development and advancement of synthetic drugs. The active metabolites contain chemical groups such as phenols, flavonoids, terpenoids, alkaloids, tannins, carbohydrates, and saponins. The phytochemical analyses of the crude hexane extracts of isolates have shown the presence of almost all the phytochemicals except phenols (Table 5). The fungal extract of strain WCTS21 indicated the presence of alkaloids and tannins, and fungal extract of PCTS3 indicated the presence of terpenoids, flavonoids, and tannins.

Table 2: Antagonistic evaluation of endophytic fungi against fungal pathogens

S. No.	Fungal isolates	Antagonistic index	
		F. solani	*M. phaseolina*
1.	PCTS1	59.00±0.2	51.4±0.5
2.	PCTS3	71.25±0.6	88.88±0.3
3.	PCTS21	62.50±0.15	55.55±0.2
4.	PCTS23	38.88±0.1	50.00±0.2
5.	WCTS21	80.25±0.2	69.77±0.35

F. solani: Fusarium solani, M. phaseolina: Macrophomina phaseolina

Table 3: Antibacterial activity of hexane crude extract of fungal isolates

Inhibition diameter zone (mm)			
Endophytic fungi	***S. aureus***	***S. typhi***	***B. subtilis***
WCT S21	12	7	6
PCTS3	10	11	6

B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, S. typhi: Salmonella typhi

DISCUSSION

The members of Cupressaceae plant family (*Coniferales*) are widely distributed across various geographical locations [21] and are used in ethnomedicine [22]. The asymptomatic foliar tissues of healthy plants from the Cupressaceae family are frequently colonized by various endophytic filamentous fungi [23]. The genus *Cupressus* is one of the several genera in the Cupressaceae family. Genus *Cupressus* comprises more than 20 species widely distributed throughout the world, especially in the northern hemisphere, including western North

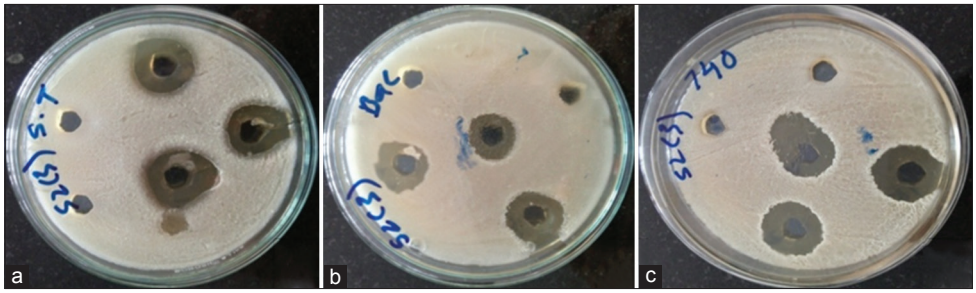

Fig. 2: Antibacterial activity of crude extract of PCTS3 against, (a) *Salmonella typhi,* (b) *Staphylococcus aureus,* (c) *Bacillus subtilis*

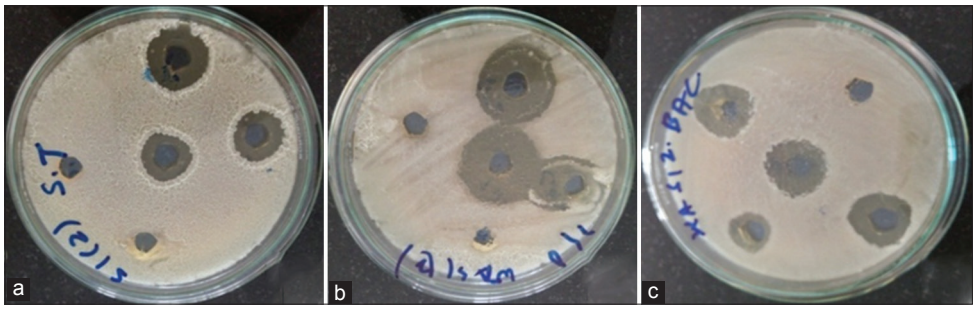

Fig. 3: Antibacterial activity of crude extract of WCTS21 against, (a) *Salmonella typhi,* (b) *Staphylococcus aureus*, (c) *Bacillus subtilis*

Table 4: MIC of the crude hexane extract of fungal isolates

Hexane crude extract (mg/ml)		
Bacterial strain	**Crude extract PCTS3**	**Crude extract WCTS21**
B. subtilis	1.25	5
S. aureus	2.5	5
S. typhi	5	1.25

MIC: Minimum inhibitory concentration, *B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, S. typhi: Salmonella typhi*

Table 5: Phytochemical screening of the crude extracts of fungal isolates

Phytochemical test	WCTS21	PCTS3
Saponins	+	+
Terpenoids	−	+
Phenols	+	−
Flavonoids	−	+
Alkaloids	+	−
Tannins	+	+

(+): Indicates the presence of fungal metabolite, (−): Indicates the absence of fungal metabolite

America, Central America, Northwest Africa, Asia, and Mexico. They are evergreen trees or large shrubs, growing to 5-40 m tall [24]. *Cupressus* genera are mainly used as diuretic, stimulant, anti-inflammatory, and antiseptic, for the common cold and wound healing in folk medicines [25]. *C. torulosa* D.Don. Bhutan cypress is a tall, large evergreen tree, widely found throughout India, Nepal, Tibet, Pakistan, and Bhutan at elevation of 1800-3300 m on limestone substrates. It is an evergreen tree that grows up to 35 m tall with whorled spreading branches with dropping branchlets.

A total of five endophytic fungi were isolated from leaves of *C. torulosa* D.Don and characterized morphotypically. Endophytic fungi are found in all divisions of fungi so have presumably evolved the association independently on many occasions. The most common endophytes are anamorphic members of the ascomycota, and some are closely related to fungi known to cause disease in plant or animal (especially insect). Biodiversity and tissue recurrence of endophytic fungi were studied in *Tripterygium wilfordii* [26]. Similarly, endophytic fungi were isolated from spikes of *Pinus roxburghii* from Pauri, Garhwal region in Himalaya [27]. Nearly 343 endophytic fungal isolates representing 60 taxa with 30 morphotypes were obtained. The endophytic assemblage comprised a number of cosmopolitan species such as *A. alternate, A. niger*, *C. cladosporioides,* and *C. lunata.*

Fungal endophytes exhibited antagonistic activity against fungal pathogen. These endophytic fungal isolates are not pathogens for their antagonism against phytopathogens. These fungi could be adapted to this host and be antagonists of their pathogens. Depending on their antagonistic capacity, they would be able to displace, reduce, suppress, or induce resistance against them [28]. Those active endophytic fungi inside the plants may play an important role in protecting the plant host against pathogenic microorganisms and have an intimate correlation with the development and physiological activity of wheat [29]. Antagonism might be due to the production of biologically active compounds in media [30].

Endophytic fungi exhibited prominent antibacterial activity. Among the species showing antibacterial properties, the fungus showing the highest inhibitory activity was identified as *Phomopsis* sp. by molecular analysis [31]. The least antibacterial activity was shown by species *Aspergillus*. Similarly, antimicrobial activities also have been reported from ethyl acetate fungal crude [27].

Similarly, the crude extract of endophytic fungus *Cochliobolus intermedius* isolated from *Sesbania grandiflora* showed antimicrobial activity [32]. A *Cladosporium* sp. endophytic fungus was isolated from *Kigelia africana,* and its extract was tested for antibacterial activity against *E. coli*, *S. aureus,* and *S. typhimurium*. Its extract has shown broad-spectrum activity against pathogens [33]. Antibacterial activity was evaluated for crude ethyl acetate extracts of endophytic fungus isolated from *Triticum durum*. All extracts showed inhibitory activity on at least one or more pathogenic microorganisms, with an average zone of inhibition varied between 7 and 25 mm, and the largest zone was of 25 and 25.3 mm against *E. coli,* respectively [34].

Phytochemical analysis of endophytic fungal extracts exhibited the presence of chemical components as a prospective source for medicinal

and industrial use. In the past few years, many valuable bioactive compounds with antibacterial, cytotoxic anticancer, insecticidal activities have been discovered from the endophytic fungi. These secondary metabolites can be further grouped as alkaloids, terpenoids, steroids, quinones, etc. Endophytic fungi can prove to be a great source of bioactive compounds and natural products. The endophytes are the rich source of a wide range of bioactive compounds that have been shown to fight against various pathogens and also cancers in humans including animals [35]. Along with this fungal metabolite analysis was carried out on endophytic fungi which showed the presence of saponins, tannins, terpenoids, etc., fungal metabolite properties of endophytic fungi shows that it has antimicrobial activity. Endophytes are sources of bioactive metabolites with tannins, saponins, flavonoids, phenols, and terpenoids being examples. The ability of an endophyte to produce some metabolites but not others has been described by Selim *et al*. [36] where different endophytes in a plant may produce different secondary metabolites hence play different functions in the plant and that the total number of metabolites in a plant extract maybe a contribution of all the endophytes that live in the plant. The available secondary metabolites could be responsible for the function of the plant extracts in defense against plant and animal pathogens. The phytochemical analysis of ethyl acetate extracts of *Colletotrichum gloeosporioides* extract showed the presence of alkaloids and steroids, whereas *F. oxysporum* extract revealed the presence of flavonoids, phenol, and phenolic compounds [37].

CONCLUSIONS

Medicines derived from endophytic fungi have made an immense contribution toward the betterment of human health and act as a source of inspiration for novel drug compounds. In the present study, antagonistic endophytic fungal strains showed promising antibacterial activity against the bacterial pathogen. Endophytic fungi are poorly investigated bunch of microorganisms that could prove to be one of the best sources of bioactive and chemically novel compounds which can be used in various pharmaceutical and industrial fields. From the above research, it can be concluded that this endophytic fungus has immense potential to be used in the area of pharmacology and as a prospective source of valuable drugs. It has been seen that some plants have been reported to produce the same natural compounds. Hence, the fungal endophytes isolate from *C. torulosa* D.Don for the production of bioactive compounds may facilitate the new product discovery process.

ACKNOWLEDGMENT

We gratefully acknowledge TEQIP-II and G. B. Pant Engineering College, Pauri, Garhwal for providing financial support.

REFERENCES

1. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, Heuvel JV, editors. Microbiology of Phyllosphere. New York: Cambridge University Press; 1986. p. 175-87.
2. Sampson K. Further observations on the systemic infection of *Lolium*. Trans Br Mycol Soc 1935;21:84-97.
3. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007;70(3):461-77.
4. Kaul S, Ahmed M, Zargar K, Sharma P, Dhar MK. Prospecting endophytic fungal assemblage of *Digitalis lanata* Ehrh. (Foxglove) as a novel source of digoxin: A cardiac glycoside. 3 Biotech 2012;3(4):335-40.
5. Stierle A, Strobel GA, Stierle DB. Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Sci 1993;260:214-6.
6. Nithya K, Muthumary J. Bioactive metabolite produced by *Phomopsis* sp., an endophytic fungus in *Allamanda cathartica* Linn. Recent Res in Sci Technol 2011;3:44-8.
7. Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D. Anticancer compounds derived from fungal endophytes: Their importance and future challenges. Nat Prod Rep 2011;28(7):1208-28.
8. Yu HS, Zhang L, Li L, Zheng CJ, Guo L, Li WC, *et al*. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol Res 2010;165(6):437-49.
9. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67(4):491-502.
10. Dreyfuss MM, Chapela IH. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In: Gullo VP, editor. The Discovery of Natural Products with Therapeutic Potential. London, United Kingdom: Butterworth-Heinemann; 1994. p. 49-80.
11. Zhang G, Sun S, Zhu T, Lin Z, Gu J, Li D, *et al*. Antiviral isoindolone derivatives from an endophytic fungus Emericella sp. associated with Aegiceras corniculatum. Phytochemistry 2011;72(11-12):1436-42.
12. Guo B, Wang Y, Sun X, Tang K. Bioactive natural products from endophytes: A review. Prikl Biokhim Mikrobiol 2008;44(2):153-8.
13. Singh SB, Ondeyka JG, Tsipouras N, Ruby C, Sardana V, Schulman M, *et al*. Hinnuliquinone, a C2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. Biochem Biophys Res Commun 2004;324(1):108-13.
14. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, Amna T, *et al*. The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 2006;122(4):494-510.
15. Gimenez C, Cabrera R, Reina M, Gonzalez-Coloma A. Fungal endophytes and their role in plant protection. Curr Org Chem 2007;11:707-20.
16. Raviraja NS. Fungal endophytes in five medicinal plant species from Kudremukh Range, Western Ghats of India. J Basic Microbiol 2005;45(3):230-5.
17. Radji M, Sumiati A, Rachmayani R, Elya B. Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity. Afr J Biotechnol 2011;1:103-7.
18. Bhardwaj A, Sharma D, Jodan N, Agrawal PK. Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus rouxburghii*. Arch Clin Microbiol 2015;6(3):1-9.
19. Wariso BA, Ebong O. Antimicrobial activity of Kalanchoe pinnaata (Ntiele. Lam) pers. W Afr J Pharmacol Drug Res 1996;12:65-8.
20. Devi NN, Shankar DP, Sutha S. Biomimetic synthesis of silver nanoparticles from an endophytic fungus and their antimicrobial efficacy. Int J Biomed Adv Res 2012;3(5):409-15.
21. Fralish JS, Franklin SB. Taxonomy and Ecology of Woody Plants in North American Forests. New York: Wiley; 2002.
22. Greenwald J, Brendler T, Jaenicke C, editors. PDR for Herbal Medicines. 3rd ed. Montvale, NJ: Thomson; 2004.
23. Moghaddam MH, Soltani J. Psychrophilic endophytic fungi with biological activity inhabit Cupressaceae plant family. Symbios 2014;63:79-86.
24. Pierre-Leandri C, Fernandez X, Lizzani-Cuvelier L, Loiseau M, Fellous R, Garnero J. Chemical composition of cypress essential oils: Volatile constituents of leaf oils from seven cultivated *Cupressus* species. J Essent Oil Res 2003;15:242-7.
25. Tumen I, Süntar I, Keles H, Küpeli Akkol E. A therapeutic approach for wound healing by using essential oils of *Cupressus* and *Juniperus* species growing in Turkey. Evid Based Complement Alternat Med 2012;2012:728281.
26. Kumar DS, Hyde KD. Biodiversity and tissue recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Divers 2004;17:69-90.
27. Bhardwaj A, Sharma D, Agrawal PK. Isolation and characterization of endophytic fungi from spikes of *Pinus rouxburghii* growing in Himalayan region. World J Pharm Res 2014;3(9):568-79.
28. Larran S, Perello A, Simon MR, Moreno V. The endophytic fungi from wheat (*Triticum aestivum* L.). World J Microbiol Biotechnol 2007;23:565-72.
29. Tian XL, Cao LX, Tan HM, Zeng QG, Jia YY, Han WQ, *et al*. Study on the communities of endophytic fungi and endophytic actinomycetes from rice and their antipathogenic activities *in vitro*. World J Microbiol Biotechnol 2004;20:303-9.
30. Castillo UF, Strobel GA, Ford EJ, Hess WM, Porter H, Jensen JB, *et al*. Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL 30562, endophytic on *Kennedia nigriscans*. Microbiology 2002;148:2675-85.
31. Desale M, Bodhankar MG. Antimicrobial activity of endophytic fungi isolated from Ocimum sanctum Linn. Am Int J Contemp Sci Res 2014;1(3):17-22.
32. Garcia A, Rhoden SA, Bernardi-Wenzel J, Orlandelli RC, Azevedo JL, Pamphile JA. Antimicrobial activity of crude extracts of endophytic fungi isolated from medicinal plant *Sapindus saponaria* L. J Appl Pharm Sci 2012;2(10):35-40.
33. Idris A, Ietidal A, Idris M. Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia* Africana. Egypt Acad J Biol Sci 2013;5(1):1-9.

34. Sadrati N, Daoud H, Zerroug A, Dahamna S, Bouharati S. Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (Triticum durum). J Plant Protect Res 2013;53(2):128-36.
35. Bhardwaj A, Agrawal PK. A review fungal endophytes: As a store house of bioactive compound. World J Pharm Pharm Sci 2014;3(9):228-37.
36. Selim SA, El-Alfy S, Al-Ruwaili M, Abdo A, Al-Jaouni S. Susceptibility of imipenem-resistant *Pseudomonas aeruginosa* to flavonoid glycosides of date palm (*Phoenix dactylifera* L.) Tamar growing in Al Madinah, Saudi Arabia. Afr J Biotechnol 2012;11(2):416-22.
37. Ramesha A, Srinivas C. Antimicrobial activity and phytochemical analysis of crude extracts of endophytic fungi isolated from *Plumeria acuminata* L. and *Plumeria obtusifolia* L. Eur J Exp Biol 2014;4(2):35-43.