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Antimicrobial, Antioxidant Activity and Phytochemical Analysis Of An Endophytic Fungi Penicillium Oxalicum Isolated from a Gymnosperm Tree Cupressus Torulosa D.Don. Of Garhwal Region

[1]Rinky Bisht, [2] Arun Bhatt and [3]Pavan Kumar Agarwal
[1], [2],[3]Department of Bio-Technology, G.B. Pant Engineering College Ghurdauri, Pauri, Uttarakhand, India
[1]rinky22rimky@gmail.com, [2]arun.bhatt@reddifml.com [3]p_k_agarwal@rediffmail.com

Abstract- ***Penicillium oxalicum*** **was isolated as an endophyte from leaves of medicinal plant, Cupressus torulosa. Chloroform extract of the P. oxalicum was evaluated for antimicrobial (antibacterial and antifungal) and antioxidant activity. Chloroform extract was effective against Escherichia coli, Staphylococcus aureus and Salmonella typhi. Fungal crude extracts showed inhibitory activity against pathogenic microorganisms, with an average zone of inhibition varied between 9 mm to 10 mm, and the largest zone was of 10 mm against E. coli and B.subtilis. The antifungal activity of fungal crude extract was checked against the pathogens Fusarium solani and Fusarium oxysporim. *P. oxalicum* extract show maximum inhibition of 83.33% against F.oxysporium. Minimum inhibitory concentration (MIC) of chloroform extract evaluated by tube broth dilution method was recorded as 12.5mg/mL against S.typhi and 6.25mg/mL for B. subtilis and S.aureus. Phytochemical analysis revealed the presence phenolic compounds and anthraquinones. *P. oxalicum* was also examined for in vitro antioxidant activity by DPPH radical scavenging assay. The chloroform extract of the fungal endophytes showed potent antioxidant activity with IC50 value of 17.47µg/mL compared to the IC50 value of standard ascorbic acid, 9.0mg/mL.**

***Index Terms*— Endophytic fungi, Penicillium oxalicm, Antimicrobial activity, Minimum inhibitory concentration (MIC), Antioxidant activity**

I. INTRODUCTION

There is a desire for new and helpful compounds to produce assist and relief in all aspects of the human condition. each human pathogens and fun¬gal phytopathogens are liable to develop ‘‘drug’’ resis¬tances. The effectiveness of the older kinds of antibiotics will decrease considerably. Additionally, due to safety and environmental issues, many artificial agricul¬tural agents are and still area unit targeted for removal from the market. The removal of such agents creates a requirement to find other ways to control farm pests and pathogens. There is AN imperative need to work towards the invention of safer antifungal agents that are expected to be renewable, non-petrochemical, naturally eco-friendly, and simply procurable [1], [2]. Natural products are custom-made to a particular perform in nature. Thus, the explore for novel secondary metabolites should consider organisms that inhabit novel bio¬types. Endophytic fungi inhabit a genotype that's not well studied [3]. The presence of endophytic fungi in plant tissues was discovered more than 75 years past once Rajagopal (2012) reported such fungi from Lolium grass [4]. The up to date advance of analysis on endophytic fungi began once Bernstein and Dodgson (1977) reported the presence of endophytes in needles of pseudotsuga menziesii [5]. Endophytic fungi from medicinal plants can be a good supply of functional secondary metabolite [6]. Endophytic fungi embrace affluent sources of bioactive compounds and recently various novel bioactive substances are isolated from these microorganisms [7]-[9]. The study on endophytic fungi from medicative plants has received much attention in recent years as they're believed to be an excellent supply of biologically active compounds. *Cupressus torulosa* is belonging to Cupressaceae. it's a documented healthful plant that has been used as drug, stimulant, anti-inflammatory drug and antiseptic, for respiratory disease and wound healing in people medicines. The oil compositions of *C. torulosa* have additionally been studied earlier, that report monoterpenoids (pinenes, sabinene terpinen-4-ol and myrcene) because the

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major constituents of the oil compositions [10].The volatile oil obtained from the cones of C. torulosa are reportable to have medicine activity every in carrageenen and polyvinyl pyrrolidine-induced rat pawedema models and are reported to have antimicrobial activity by a two-fold serial dilution methodology. Endophytic fungi are a poorly investigated cluster of microorganisms that represent superabundant and dependable supply of bioactive and with chemicals novel compounds with potential for exploitation in an exceedingly large choice of pharmaceutical and industrial areas [11]. Endophytic fungi isolated from leaves of *Cupress troulosa D.don* can be a possible supply for bioactive metabolites and will be utilized in Pharmaceutical industry [12]. The compounds isolated and characterised from endophytes have potential to be used in trendy drugs, agriculture and industry. Free radicals are usually generated as bioproducts of biological reactions or from exogenous factors. The involvement of free radicals within the pathological process of a large variety of diseases is well documented. A potent scavenger of free radicals may serve as a possible preventive intervention for the diseases [13]. Antioxidants may protect the body against ROS toxicity either by averting the formation of ROS by bringing disruption in ROS attack, by converting them to less reactive molecules or by scavenging the reactive metabolites [14], [15]. The natural antioxidants were characterized from the fungal compounds [16]. Therefore the uses of antioxidants, both natural and synthetic are gaining broad significance in prevention of diseases. The current study was designed to investigate the antimicrobial and antioxidant potential of endophytic fungus *P.oxalicum* isolated from a gymnosperm tree, *C.torulosa D.Don* having medicinal value.

II. MATERIAL AND METHODS

2.1 Collection of plant material

The leaves were collected of *Cupresus 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

2.2 Isolation, identification and extraction of the endophytic fungus

For endophytic fungus isolation, healthy *C.torulosa* leaves were washed, cut into about $5mm^2$ segments and surface sterilized by sequentially dipping into 5% sodium hypochlorite (5min) and 75% ethano_l(3min), and rinsed with sterile water, then allowed surface-dry under sterile condition[11]. The leaves were then deposited on a Petri dish containing PDA(potato-dextrose-agar) medium with 200 mg/mL streptomycin to inhibit the bacterial growth, and then incubated at 27ºC ± 2ºC and checked every day until the mycelium or colony originated from the injury surface. Then individual fungal colonies being transferred on to other plates with PDA. The strain studied was identified as *Penicillium oxalicum* based on its morphology and molecular characterstics. Then this endophytic fungus was inoculated into PDB (potato- dextrose-broth) in Erlenmeyer flasks and incubated for 7 days at 28° C, 180rpm/min. Crude fermentation broths were filtered and blended for extraction with chloroform. The solvent was concentrated in a rotator evaporator at 60°C and the chloroform extract yield was 2.5%. In order to as certain whether any of the extracts of the fungus was the constituents of the PDB extracts, the chloroform extract of the sterile medium treated equally but without inoculation of the fungus was obtained simultaneously.

2.3 Screening of antibacterial activity of fungal metabolites

Antibacterial activity of secondary metabolites extracted from endophytic fungi was screened against gram positive human pathogenic bacteria such as *S. aureus , E.coli and B. subtilis* using agar well diffusion method. Bacterial pathogens were spread on Muller Hinton agar (MHA) plates. Then wells were made and three concentration of extraction were inoculated in separate wells 200 µL, 150 µL, 100 µL. Antibacterial activity was detected after an incubation of 24 to 48 h at 37°C. The presence of 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.

2.4 Screening of antifungal activity of fungal metabolites

PDA medium with 25% concentration of the solvent extracts of the test fungi were prepared. About 15 mL of the medium was poured into each petriplate and allowed to solidify. 5mm disc of 7-day-old culture of the pathogenic fungi were placed at the center of the petriplates and incubated at 27±2 °C for seven days. After incubation the colony diameter was measured in millimeter. PDA medium without the solvent extract of fungi served as control. The fungi toxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the formula

% inhibition = dc – dt/dc x 100

Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelia growth in treatment [17].

2.5 Determination of Minimum Inhibitory Concentration

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MIC was determined after antibacterial activity of the fungal crude extracts by the standard method of Wariso with minor modification [18]. Muller Hinton Broth was made and sterilized using autoclave. 1 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. 1mL inoculums 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, 1.563 mg/mL were incubated at 37 C for 24h and examined for growth. The test tube in which growth failed to occur was the MIC of the culture.

2.6 Phytochemical screening

Chloroform extract of *P.oxalicum* was evaluated for its phytochemical constituents [11],[19]. Alkaloids, carbohydrates, proteins and amino acids, phytosterols, phenolic compounds, flavonoids and anthraquinones were qualitatively analyzed.

2.7 In vitro Antioxidant assay

2,2 '-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay was performed as reported by Samaga et al., 2014 with slight modifications [20]. Individual fungal extract (1 mL) at different con-centrations (25, 12.5, 6.25, 3.125, 1.563µg/mL) was added to 4 mL of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in inhibition percent (I%) was calculated in following way:

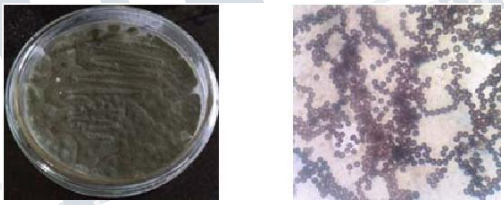
$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100;$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the fungal crude extract), and A_{sample} is the absorbance of the fungal crude extract. The values of inhibition were calculated for the various concentrations of fungus extract. Tests were carried out in triplicate.

III RESULTS AND DISCUSSION

3.1 Identification of Endophytes

The leaves materials Gymnosperm *Cupressus torulosa* D.Don were collected from Govind Ballabh Pant Engineering College Campus, Pauri, Uttarakhand, was carried out to evaluate their capacity to produce bioactive compound. The plant was taxonomically identified and authenticated by Botanical Survey of India, Dehradun, Uttrakhand. The voucher specimen was deposited there with register number 115744.. *P. oxalicum* endophytic fungi were isolated from leaves of *Cupressus torulosa* D.Don. This endophytic fungi was characterzed morphotypically using lactophenole cotton blue using scotch tape techniques (Fig.1. (a), (b). Further confirm identification have been done by using 18S rDNA from Gujrat Biotech mission. The fungal sequence was submitted in National Centre for Biotechnology Information with accession number KT355727 with the name *Penicillium oxalicum* BAB 5444. *Penicillium oxalicum* has been classified under class hyphomycetes. They have septate hyphae ending with conidiophores and produced one celled conidia. Conidiophores branch widely and produce a great number conidioconidia in branched chains. It produced olive green colour colonies (Fig. 1. (a)).



(a) (b)

Fig.1. (a): Colony Morphology on PDA of Penicillium oxalicum; (b): Shape of Conidia by staining techniques

3.2 Antibacterial activity of crude extract by agar well diffusion method

The antibacterial activity at concentration of 25 mg/mL of chloroform extracts of *P.oxalicum* was tested against three human pathogens, *B. subtilis*, *E.coli* and *S. aureus* and have shown broad spectrum activity which has been reported in the Table1. Crude extract of *P. oxalicum* from chloroform solvent have shown highest zone of inhibition of 10mm against *S.aureus* (Fig.2). The methanolic extract of *P. oxalicum* produced highest zone of inhibition 17mm, 12mm and 13.5mm respectively against *S. aureus*, *E.coli and B. subtilis* respectively [12]. The crude extract of *Alternaria alternata* was assessed for antimicrobial activity against *S. aureus* and *E. coli* are 18 and 13 respectevily [21].

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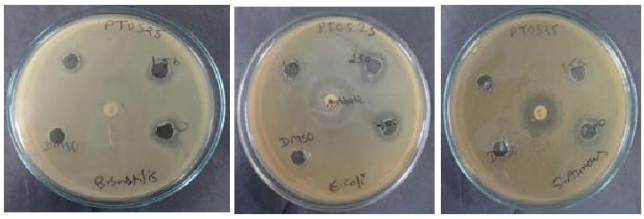


(a) (b) (c)

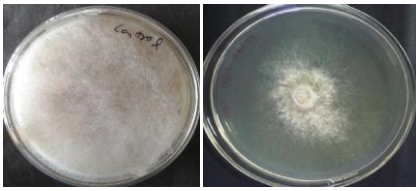
Fig.2. Antibacterial activity of Penicillim oxalicum chloroform extract against (a): Bacillus subtilis, (b): Escherichia coli, (c):Staphyloccocus aureus

Table 1. Antibacterial activity of chloroform crude extract of *P. oxalicum*

Bacterial strain	Chloroform extract of *P.oxalicum*
Inhibition diameter zone(mm)	
S. aureus	9
E.coli	10
B.subtilis	10

3.3 Antifungal Assay

Antifungal activity of chloroform crude extract of *P. oxalicum* was performed against the two fungal strain including *Fusarium solani and Fusarium oxysporum* (Fig.3 and Fig.4). The inhibition (%) of fungal extract against fungal pathogen is shown in table 2. *P.oxalicum* showed 83.33 % inhibition against *F. oxysporum* which on further chemical investigation will lead to isolation of antifungal chemicals (Table2). The ether extract of the endophytic fungus have broader antifungal activity to the Candida albicans, *Trichophyton rubrum* and *Aspergillus fumigatus*, especially the IC80 about *Trichophyton rubrum* was 4 mg/mL, equally to the control[22]. Three of the -*Streptomyces* sp. isolates strongly inhibited *Colletotrichum musae*, five were very active against *Fusarium oxysporum* and two strongly inhibited growth of both test fungi[23].



(a) (b)

Fig. 3. Antifungal activity shown by crude extracts of *P.oxalicum* against Fungal pathogen *F.solani* (a) Control (b) Test

(a) (b)

Fig. 4. Antifungal activity shown by crude extracts of *P.oxalicum* against Fungal pathogen *F. oxysporium* (a) Control (b) Test

Table 2. Antifungal activity of the chloroform crude fungal extract of *P.oxalicum*

Pathogenic Fungi	Chloroform extract of *P.oxalicum*
Percentage of Inhibition	
F.solani	61.11
F.oxysporium	83.33

3.4 Minimum Inhibitory Concentration of fungal crude extracts

Fungal crude extracts showing potent antibacterial activity were further determined for their MIC by a tube dilution technique against *B.subtilis*, *E.coli* and *S.aureus* (Table3). Fungal crude extracts have shown MIC ranged from 3.125-25mg/mL for *E.coli*, *S.aureus* and *B.subtilis*. Chloroform extract showed MIC of 6.25mg/mL for *B. subtilis* and *S.aureus* and 12.5 mg/mL for *S. typhi*. Methanolic crude extract of *P. oxalicum* showed MIC of 6.25 mg/mL for *B.subtilis*, 3.125 mg/mL for *S. aureus* and *E.coli* which showed its efficacy as a potent antimicrobial [12]. Phongpaichit *et al.* (2006) isolated 377 fungi and their fermentation broths were tested for antimicrobial activity. The results revealed that the strains of *S. aureus* (MIC 32-512 µg/mL) and *C. albicans* (MIC 64-200 µg/ mL) were inhibited by 6-10 % and 1 % of the crude ethyl acetate extracts, respectively [24].

Table 3. Minimum Inhibitory Concentration of the crude chloroform extract of fungal isolates

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Bacterial pathogen	Chloroform extract of *P.oxalicum*
	MIC(mg/mL)
S. aureus	25
E. coli	12.5
B.subtilis	6.25

3.5 Phytochemical screening

Phytochemical analysis of chloroform extract of *P.oxalicum* showed presence of phenolic compounds and anthraquinones. These phytochemicals may be responsible for antimicrobial and antioxidant activity evaluated in present study. The preliminary screening of fungal metabolite compounds in ethyl acetate extract of endophytic fungi from *Pinus roxburghii* showed the presence of different fungal metabolite, phenolic compounds, steroids, tannins, alkaloids and flavonoids [11]. *Aspergillus niger* and *Fusarium oxysporum* yielded the tannin, flavonoids, tepenoids, phenol and saponins from ethanol extract [25].

3.6 Antioxidant activity

Scavenging effect on DPPH radicals

To determine the effects of chloroform extract of *P. oxalicum* on *in vitro* antioxidant activity, the DPPH scavenging rate was studied. The DPPH radical contains an old electron, which is accountable for the absorbance at 517 nm and also for a visible deep purple color. DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance. The antioxidant activity of sample was expressed in terms of IC50 value which was calculated from the graph after plotting inhibition percentage against extract concentration (Fig. 5). The chloroform extract of *P. oxalicum* exhibited antioxidant activity with IC50 value of 17.47mg/mL compared to the IC50 value of ascorbic acid 9.01mg/mL (Table 4). Ethyl acetate extracts of *Penicillium* sp. 2, and *Aspergillus* sp. had an anti-oxidation activity to linoleic acid, and an inhibition percentage which ranged from 78.961±3.183% to 73.977±1.102%, respectively [26]. The scavenging activity of ethanol extract of the *A. niger* and *Penicillium* sp. reached 88.61% and 86.72% respectively while at the concentration, that of *Tricho- derma* sp. was 51.66% [27].

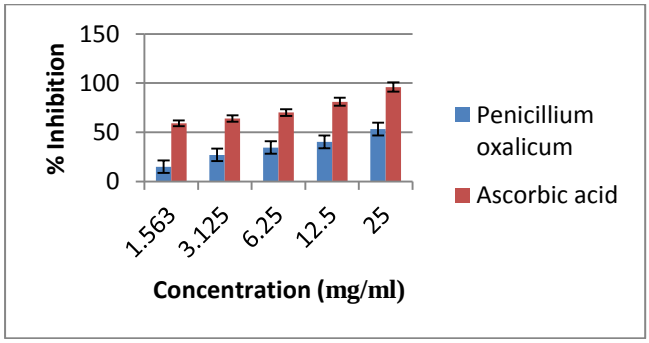

Fig. 5. Free radical scavenging effect of P. oxalicum chloroform extract against DPPH radicals

Table 4. DPPH radical scavenging activities of chloroform extract of endophytic fungus P.oxlaicum. and ascorbic acid

Test	DPPH radical scavenging activity (%)					
	1.563	3.125	6.25	12.5	25	IC50
	(mg/mL)					
Chloroform extract of *P.oxalicum*	15	27	34.47	40.21	53.2	17.4
Standard (Ascorbic acid)	59	64	70	81	96	9.01

The results showed that increase in concentration ascorbic acid increased % radical scavenging activity. The extract showed an inhibition in free radical production, almost a near equipotent effect with standard antioxidant ascorbic acid.

IV. CONCLUSION

Medicines derived from endophytic fungi have made immense contribution towards the betterment of human health and act as a source of inspiration for novel drug compounds. Fungal endophytes from medicinal plants are under attention as these plants tend to produce natural products advantageous for us. Investigations have been carried on leaves of *C. torulosa D.Don*. The present study provides evidence that isolated endophytic fungi *Penicillium oxalicum* is capable to provide high antimicrobial activity against the bacterial and fungal pathogen. In this study, antioxidant method (DPPH) showed that the chloroform endophytic extracts of *P. oxalicum* contains antioxidant activities. Therefore, natural antioxidants and presence of phenol compounds in endophyte *P. oxalicum* has the

capability to be used in pharmaceutical industry. From the above research it can be concluded that this endophytic fungi has immense potential to be used in the area of pharmacology and as a prospective source of valuable drugs.

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