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Antimicrobial and Antidiabetic Activity of an *Penicillium Oxalicum* Isolated from *Cupressus Torulosa*

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Abstract—*The aim of the present work was to study preliminary antimicrobial and antidiabitic activities of the endophytic fungi Penicillium oxalicum by in vitro method. Methanolic and chloroform extract of Penicillium oxalicum tested for antimicrobial activity against Bacillus subtilis, Escherichia coli, and Staphylococcus aureus by agar well diffusion assay. Chloroform extract of Penicillium oxalicum was the most active to Gram-negative bacteria. The extracts also exhibited antidiabetic activity by inhibition of α-amylase activity. Crude methanolic and chloroform extracts of Penicillium oxalicum showed promising inhibition activity on α-amylase with an IC 50 value of 46.73 and 59.20µg/mL respectively. Whereas, the control acarbose had 26.76 µg/mL of IC 50 value for α-amylase at similar experimental condition.*

1. INTRODUCTION

Endophytic fungi is one of possible natural resources for new medicinal compound sources.Endophytic microbes are microorganism together with Actinomycetes, or fungi spend half or whole of its life within intra-oranimate thing tissue of its healthy host while not giving any symptoms[1]. Metabolites isolated from the fungal endophytes i.e alkaloids, terpenoids, quinines, isocoumarin derivatives, flavanoids, phenols, peptides and phenolic acids area unit smart sources of novel antibiotics, immunosuppressant and metastatic tumor compounds having various structural teams and showing antibacterial drug, antifungal, anticancer[2], antiviral, antioxidant, insecticide, antidiabetic and immunological disorder activities[3]. Endophytic fungi are thus a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity[4]. Novel antibiotics, antimycotics, immunosuppressants, anticancer, antidiabetic compounds are few natural products obtained from endophytic fungi[5].

Endophytic fungi from medicinal plants could be a great source of functional secondary metabolite[6]. Distinctly from plants, endophytes are often cultured quickly and also the biomass will be accumulated by giant scale fermentation. Production of bioactive compounds can be exaggerated by biotechnology of endophytic fungi so as to satisfy demands whereas keeping multifariousness and property system.

In view of the on top of concerns, *Cupressus torulosa L.* was selected for this study. It is a documented medicinal plant that has been used as drug, stimulant, anti-inflammatory and antiseptic, for common cold and wound healing in folk medicines. The essential oil compositions of *C. torulosa L.* have also been studied earlier, which report monoterpenoids (pinenes, sabinene terpinen-4-ol and myrcene) as the major constituents of the oil compositions[6].The volatile oil obtained from the cones of C. torulosa L. have been reportable to own medicament activity each in carrageenen- and polyvinyl pyrrolidine-induced rat pawedema models and are reported to own antimicrobial activity by a two-fold serial dilution methodology. The essential oil of the leaves is employed to treat rheumatism and pertussis and as an astringent, we report the isolation of endophytic fungi from genus Cupressus torulosa and the antimicrobial, antidiabetic activity of the crude alcohol and chloroform extract.

The leaves were collected of *C. torulosa D.Don* from hilly areas of Uttarakhand state specifically Pauri, Garhwal region belonging to the range of mountains region. The plant material was delivered to the laboratory in sterile bags and processed inside many hours when sampling. Fresh plant materials were used for isolation work to cut back the possibility of contamination.

2. 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 of Endophytic fungi

Tissues of the leaves of *Cupressus torulosa* D.Don were cut into 5 mm long segments then surface sterilized by method of Arnold[7] with minor modification. Segments were surface sterilized by successive immersion for 1 min in 75 % Ethanol, treated for1 min in 0.1 % mercuric chloride, followed by several washing for in sterile distilled water. The time of the dilution and immersion in ethanol and merchuric 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 medium. The dishes were sealed with parafilm and incubated at 27ºC ± 2ºC for 2-4 days in incubator.

2.3. Identification of endophytic fungi

Fungal growth and sporulation was 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, colour and morphology of fruiting bodies and spores. Fungal isolates were stained with Lactophenol cotton blue and examined under light microscope.

2.4 Extraction of the bioactive metabolite

Extraction is done to obtain the bioactive compounds. The fungus was cultivated on Potato dextrose broth by inoculating selected endophyte cultures in Erlenmeyer flask containing Potato Dextrose Broth. The flask was incubated at 28°C for 2 weeks with periodical shaking at 150 rpm. After the incubation period, the fermentation broth of the fungus was homogenized .Metabolite was extracted by solvent extraction procedure using chloroform and methanol as organic solvents. To the filtrate equal volume of solvents were added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of solvent containing the extracted compounds was separated using separating funnel. 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 sulphoxide (DMSO) and kept at 4°C. 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 sulphoxide (DMSO) (1mg/ml) and kept at 4°C.

2.5. Screening of antimicrobial activity of fungal metabolites

Antibacterial activity of secondary metabolites extracted from endophytic fungi was screened against gram positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* of human health 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.

2.6. Determination of Minimum Inhibitory Concentration

MIC was determined after antibacterial activity of the fungal crude extracts by the standard method of Wariso with minor modification[8]. 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 innoculum 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.

2.7 Anti diabetic acivity by alpha amylase inhibition assay method

The crude mycelial extract on alpha amylase activity was determined according to the method described by Kim *et al* 2005 with some modification[9]. 100 µL of the goat pancreatic amylase (PPA) solution was added to 100 µL of test substance and incubated at room temperature (RT) for 20 minutes. The reaction was initiated by the addition of 100 µL of 1% soluble starch solution and incubated at 37°C. The reaction was arrested after 10 min of incubation by the addition of 200 µL of dinitrosalicylic acid colour reagent. The tubes were kept in a boiling water bath for 10 minutes, cooled and diluted with distilled water and absorbance was measured at 470 nm. Acarbose was used as the standard alpha-amylase inhibitor and vehicle was used as negative controls. All the assays were carried out in triplicates and average percent inhibition of enzymes by the fungal extract was calculated using the following formula.

%Inhibition=Control OD-Test OD/Control OD*100

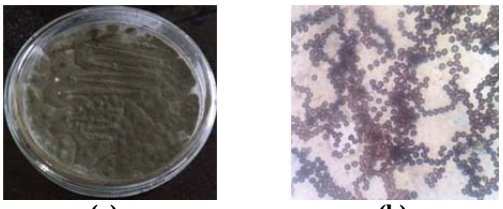
3. RESULTS AND DISCUSSION

3.1.Sample Collection, Isolation and Identification of Endophytic Fungi

A systematic study about the endophytic fungal biodiversity in a conifer forest plant, *Cupressus torulosa* D. Don. located in Govind Ballabh Pant Engineering College Campus, Pauri

Garhwal, Uttarakhand was carried out to evaluate their capacity to produce bioactive compound. *Penicillium 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 (Fig1). Further confirm identification have been done by Forest Reaserch Institute (FRI), Deharadun and endophytic fungi.

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 (Figure-1A).

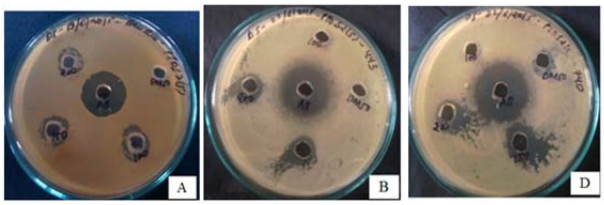


(a) (b)

Fig. 1 A: Colony Morphology on PDA of PCTS21; B: Shape of Conidia by staining techniques

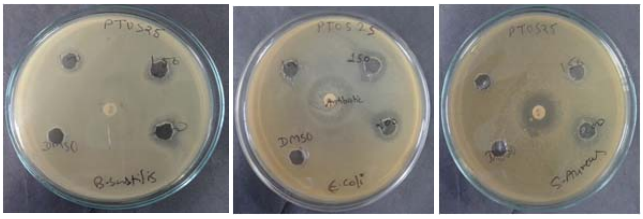
3.2. Antibacterial activity of crude extract by agar well diffusion method

The antibacterial activity at concentration of 25 mg/ml of methanol and chloroform extracts of *Penicillium 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 Table 1. The methanolic extract of *Panicillium oxalicum* produced highest zone of inhibition 17 mm, 12mm and 13.5mm respectively against *S. aureus, E.coli and B. subtilis* respectively (Fig. 2). Crude extract of *Penicillium oxalicum* from chloroform solvent have shown highest zone of inhibition of 10mm against *S.aureus* (Fig. 3).



(a) (b) (c)

Fig. 2. Antibacterial activity of Penicillium oxalicum methanol extract against A: Bacillus subtilis, B:Eschericia coli, C: Staphyloccocus aureus



(a) (b) (c)

Fig. 3. Antibacterial activity of Penicillim oxalicum chloroform extract against A: Bacillus subtilis, B:Eschericia coli, C: Staphyloccocus aureus

Table1: Antibacterial activity of methanolic and chloroform crude extract of fungal isolates

Inhibition diameter zone(mm)			
Endophytic Fungi	*S. aureus*	*E.coli*	*B.subtilis*
Methanolic extract of *Penisiliium oxalicum*	17.0	12.0	13.5
Chloroform extract of *Penisiliium oxalicum*	9.0	10.0	10.0

3.3. Minimum Inhibitory Concentration 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, E.coli* and *S.aureus* (Table 2). Fungal crude extracts have shown MIC ranged from 3.125-25mg/ml for *E.coli, S.aureus* and *B.subtilis*. 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. Whereas chloroform extract showed MIC of 6.25mg/mL for *B. subtilis* and *S.aureus* and 12.5 mg/mL for *S. typhi*.

Table 2: Minimum Inhibitory Concentration of the crude methanol extract of fungal isolates

	MIC		
Endophytic fungi	***S.aureus***	***E.coli***	***B.subtilis***
Methanolic extract of *Penicillium oxalicum*	3.125	3.125	6.25
Chloroform extract of *Penicillium oxalicum*	25	12.5	6.25

3.5. Alpha Amylase Inhibition Assay

Both chloroform and methanol extracts have were tested for alpha amylase inhibitors and they shown significant inhibition for alpha enzymes. Percentage inhibitions of alpha amylase by endophytic fungal isolate at various concentrations was calculated and plotted in the Fig. 1 for both metanolic and chloroform extract of *P. oxalicum*. Methanolic crude extract of *P. oxalicum* and chloroform crude extract of *P. oxalicum* have IC values of 46.73 and 59.20µg/mL respectively. The IC value of these two extracts of *P. oxalicum* was more than IC value of standard drug acarbose (26.76µg/mL) when assayed under similar condition. Similar antidiabetic activity by endophytic fungi were observed by Edward et al and Ramdanis et al [10,11], however the mode of action was α-glucosidase inhibition

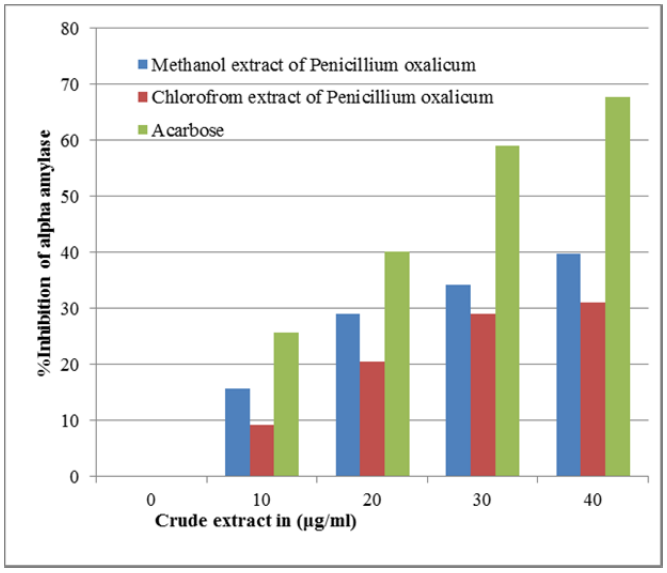


Fig. 4: Percent inhibition of alpha-amylase with different concentration of fungal extract

4. ACKNOWLEDGEMENT

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