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In vitro antibacterial activity study of endophytic fungi extracts from the medicinal plants *Catharanthus roseus* and *Centella asiatica*

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Abstract

Endophytes are the microorganisms present in living tissues of various plants, establishing mutual relationship without apparently any symptoms of diseases. Endophytes have received attention of the scientific community due to their capacity to produce novel bioactive compounds. In the present study, bio prospecting of fungal endophytes from different medicinal plant was studied and screened for their antimicrobial potential. Several fungi were isolated from different parts of the plant, isolated from the leaves showed significant antimicrobial potential. The crude extract of this fungal isolate with Hexane, Ethyl acetate and Methanol were screened for their antimicrobial potential. The extract by ethyl acetate showed significant antimicrobial activity against E. coli, S. typhimurium, B. cereus, B. subtilis, K. pneumoniae and S. aureus. The antimicrobial activity was highest against E. coli, followed by S. typhimurium and B. cereus . The present study helped to justify the traditional use of ***Catharanthus roseus and Centella asiatica*** *against human pathogenic bacteria. Further, it is confirmed that the antimicrobial activity is attributable to the presence of endophytic fungi. It also justifies that the studies on isolation and identification of these bioactive compounds can be a crucial approach to search of novel natural products.*

Keyword:- Catharanthus roseus and Centella asiatica; Endophytic fungi; antibacterial activity

Introduction

There is a steady demand and need for new antimicrobial agents as infectious diseases are still a worldwide problem and the development of resistance by the pathogens is a growing concern [1, 2]. The problem extends beyond the clinical application of antimicrobial drugs and many microorganisms of agricultural concern are also known to have acquired resistance to commonly used antimicrobial chemicals [3], which indicates an increasing want for new bioactive compounds. Historically, a majority of the compounds have been isolated from the natural environment, particularly plants, and have been used in the treatment of many diseases and illnesses. Many of the drugs available commercially are derived from these natural products and have become potential drug sources [4]. While plants have been a major source of new lead compounds for drug discovery, attention has more recently turned to endophytes as these microorganisms are seen as having great potential as sources for new bioactive compounds [5]. Thus, there is a growing need for new, environmentally-friendly antimicrobial agents that may be used safely in agriculture [6].

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Endophytic fungi, which colonize plants internally without apparent adverse effects, occur ubiquitously in plants [7] and do not have pathogenic effects on its hosts [5]. They produce a number of compounds which can inhibit pathogens. They are relatively not much explored and offer potential sources of novel natural products for exploitation in medicine, agriculture and the pharmaceutical industry [8].

The present study is aimed at the isolation and identification of endophytic fungi from *Catharanthus roseus* and *Centella asiatica,* screening for their antimicrobial activity.

Materials and methods

Sample collection

The sample was collected before 1hr the work should have done within 2 hrs of sample collection. Most frequent method used to detect and quantify Endophytic fungi involves isolation from surface sterilization of host tissue (bark, leaf).Surface sterilization of plant tissue usually accomplished by the treatment with strong disinfectant for some times followed by sterile rinse. A standard method utilizes dips in both ethy1 alcohols NaOCL (bleach). Ethy1 alcohol acts as a surfactant and NaOCL are the actual sterilizing agent.

Surface Sterilization

Different parts of fresh healthy *Catharanthus roseus* and *Centella asiatica* plants were cut into small pieces (5 mm × 2 mm) using sterile blade and washed with sterile distilled water. The samples were surface sterilized by dipping into 2% sodium hypochlorite for 60 seconds and 70% ethanol for 5 seconds, rinsed with sterile water and allowed to surface dry under sterile conditions [9]. The surface sterilized samples were placed on Potato Dextrose Agar (PDA) plates amended with 50 mg/L tetracycline to suppress the bacterial growth and incubated at 28ºC to 30ºC for 2 to 3 days. The hyphal tip of endophytic fungi growing out from the plant tissue was transferred to fresh PDA plates amended with 50 mg/L tetracycline. After incubation at 30ºC for 7 to 14 days, purity of the culture was determined by colony morphology.Colonization rate (CR) was expressed as percentage of total number of isolates obtained from different tissue segments divided by total number of isolates obtained from overall tissue segments incubated. Isolation rate IR) was calculated as number of isolates obtained from segments divided by total number of segments [10].

Then the identification of endophytes was done through microscopic examination. For that the isolated endophytes were firstly pure cultured into the PDA medium. Then the morphological examination was performed by scrutinizing the culture and characteristics' of the spore attachment by staining the fungus with lacto phenol cotton blue.

Isolation of Secondary Metabolites

Isolation of secondary metabolites from liquid media was carried out by the described method[11]. The culture media and the myceliawere separated from each other by filtration. The mycelia were soaked in methanol and the methanolic extract was collected after 7 to 10 days of soaking. Organic solvents, hexane and ethyl acetate were used to extract the filter. The filtrate was extracted three times with equal volume of Hexane, Ethyl acetate. Each solvent was subjected to liquid – liquid extraction for 3 to 4 times. Solid residues were obtained by evaporating organic extracts under reduced pressure.

Test Microorganisms

Antibacterial activity of metabolites isolated from endophytic fungi was screened against pathogenic and non pathogenic bacteria using agar well diffusion method. Six bacteria, gram positive *B. subtilis* (NCIM No. 2063), *S.aureus* (NCIM No. 2079) , *B. Cereus* (NCIM No. 2155) and gram

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negative *E. coli* (NCIM No. 2345), *K. Pneumonia* (NCIM No. 2706) and *S.typhimurium* (NCIM No.2501) were grown on nutrient agar media and used for antimicrobial activity. 0.5 McFarland standard suspension was used for this assay.

Study of Antimicrobial Activity by cup Diffusion Method

The study of antimicrobial activity of Endophytic fungi was checked Against six pathogenic bacteria *B. subtilis* (NCIM No. 2063), *S.aureus* (NCIM No. 2079) , *B. Cereus* (NCIM No. 2155) and gram negative *E. coli* (NCIM No. 2345), *K. Pneumonia* (NCIM No. 2706) and *S.typhimurium* (NCIM No.2501) All these works were done aseptically under laminar airflow. After the spreading of pathogenic organisms, coated plates were punched with borer of 6mm diameter wells .The fermented broth of each Endophytic fungal samples were applied into bores by micropipette **.** It was taken under consideration that the sample should never be overflowed the bore or well and come out into the medium. Then the plates were left for 20 minutes in undistributed condition and then incubated at 37^0C in incubator for some days to show the zone of inhibition .Each Endophytic sample was checked sample was checked separately for antimicrobial activity against each pathogenic organism .After the occurrence of inhibition zone shown by Endophytic fungal extract the diameter of the inhibition zone was measured and for that inner radius of the zone was taken. Three times the diameter was taken for each zone and mean value was finally taken. The antimicrobial activity was measured in millimeter (mm) as clear zone of inhibition [12].

Results and Discussion

For this study two different medicinal plants were selected . These are collected from different areas of Panskura. The name of plant and their scientific name used during study are given in table 1.1. We used different tissue parts (leaf and bark) of these plants. Here we studied two different medicinal plants for two different locations that's why here we used serial no 1 and 2 for two different location for motioning their tissue origin . We used "p" for leaf and "b" for bark in their code name. At first the tissue parts of these plants were surface sterilized by following the method of using home hold bleach NaOC1 (as mentioned in materials and method section). Then those surface sterilized tissue parts were pressed on the Potato Dextrose Agar (PDA) plate and allowed for growth of fungal colony of different color on the PDA plate . We also found that every tissue parts of these medicinal plants not showed fungal colony. After isolation of endophytic fungi from surface sterilized medicinal plant tissues each isolated fungus were inoculated into separate Cazepeck Dock medium in sterile condition for huge growth at 25^0C incubator for 5-7 days . We also simultaneously prepared one NA for the each isolated Endophytic fungi . After the huge fungal cells (in beads form) were found in the broth the culture was filtrated out through filter papers for getting the Endophytic fungal extract . Then the filtrate was taken into an eppendroff and subjected to centrifuge at 5000 rpm for 5 minutes to remove the fungal cells from the filtrate . The fungal cells were then pelleted down. Then the supernatant of the culture was taken into another eppendroff and used for the further study of antimicrobial activity against the tested pathogen. For studying the antimicrobial activity we followed the cup diffusion method .Where we used six pathogenic bacteria named – *B. subtilis* (NCIM No. 2063), *S.aureus* (NCIM No. 2079) , *B. Cereus* (NCIM No. 2155) and gram negative *E. coli* (NCIM No. 2345), *K. Pneumonia* (NCIM No. 2706) and *S.typhimurium* (NCIM No.2501).During the study of antimicrobial activity each time we look those plant tissues extracts as a control . What we had done here that we had done here that we took different tissue parts (leaf and bark) separately and grind

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it and dissolved then in to sterile distilled water and finally vortexes and filtrate then and we used those filtrated solution as a same (for control) during antimicrobial activity test .Then we found some Endophytic fungi showed antimicrobial activity against the tested pathogen . Then we measured the diameter of those inhibition zone in mm. (as listed in table 1.2 and fig1.1).

Table no - 1.1 Name of the medicinal plants used for the study.

Local name of the plant used for the study	Scientific name	Code name used during study
Nayantara	***Catharanthus roseus***	Cr
Thalkuni	***Centella asiatica***	Ca

Table no -1.2 Antimicrobial activity of Endophytic fungi against tested pathogens.

Sl.No	Name of Organisms	Hexane	Ethylacetate	Methanol	Cloramphenicol (+ve control)
1	*E.coli* (NCIM No.2345)	16	15	12	22
2	*S.typhimurium* (NCIM No.2501)	14	13	22	23
3	*B.cereus* (NCIM No.2155)	10	16	11	20
4	*S.aureus* (NCIM No.2079)	13	18	-	18
5	*K.pneumoniae* (NCIM No.2706)	-	12	10	18
6	*B.subtilis* (NCIM No.2063)	10	8	-	22

Table 1.3 :- Colony morphology and characterization of Endophytic fungi from *Centella asiatica* and *Catharanthus roseus.*

Name of the plant	Code name of the Endophytic fungi	Colony morphology	Microscopic appearance	
Centella asiatica	Ca	Color -Rapidly growing white colored fungi swarms over entire Plate fuzzy colony.	Conidia – (single called spores) Mycelium-septet Conidiophores-Virile long.	Fungal group-*Aspergillus sp.*

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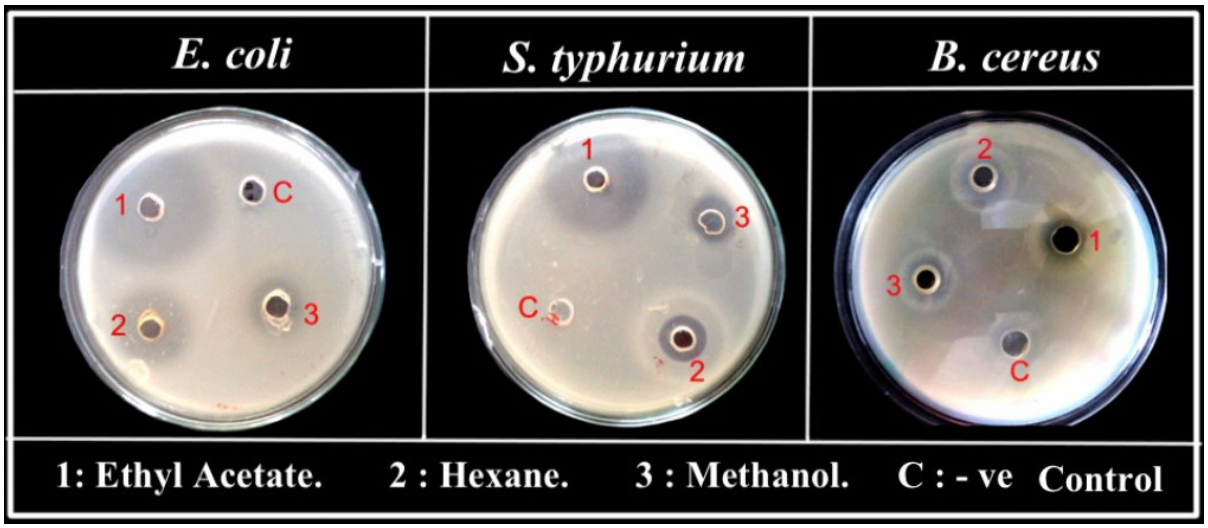
Catharanthus roseus	Cr	Color - Mature culture usually greenish.	Conidia- (single cooled spores) chains developed at the end of the stigma. Conidiophores- Branching Mycelium-septate.	Fungal group-*Penicillium sp.*

Fig-1.1 Antimicrobial activity of Endophytic fungi against tested pathogens



Conclusions

The present study reveals that the endophytic fungi *Penicillium sp and Aspergillus sp* isolated from *Catharanthus roseus* and *Centella asiatica* respectively are effective alternative sources of antimicrobial drugs. Further studies on isolation of these antimicrobial compounds and identification of bioactive compounds can be a crucial approach to search of novel natural products.

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Conflict of interest

The authors have declared no conflict of interests.

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