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Isolation, identification and antibacterial screening of endophytic fungi obtained from the medicinal plant *Tinospora cordifolia* found in Ranchi District of Jharkhand

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ABSTRACT

Many medicinal plants are being used by marginal communities for treatment of various ailments. However, the potential of various endophytes surviving within traditional medicinal plants remains an important area of study for scientists and researchers. The present study elucidates the diversity of endophytes within the medicinal plants and their role in plant growth promotion and pharmacological significance. Endophytes are microorganisms, including bacteria, fungi, and actinomycetes, that inhabit intra- and intercellular plant tissues for all or part of their life cycle. Endophytic fungi are polyphyletic and ecological group of highly diverse fungi belonging mostly to ascomycetes and anamorphic fungi. It has been estimated approximately that more than one million different endophytic fungal strains inhabit about 300,000 various plant species. Endophytes are able to colonize internal plant tissues of healthy leaves, petioles, stems, twigs, fruit, flower, bark and without causing any apparent harm or pathogenicity to their host plants. Endophytes are symbiotically associated with their host plants. In many cases it is believed that microbes function as the biological defense for the plant against foreign phytopathogens. The protective mechanisms of endophytes are exerted directly by releasing metabolites to attack any antagonist or lysis of infected cells and indirectly by either promoting plant growth or by inducing host defense mechanism. The fungal endophyte produces invaluable bioactive metabolic compounds that are beneficial to humans with antibacterial, anticancer, antidiabetic, anti- inflammatory, antitumour properties etc. The major bioactive compounds include pestacin, taxol, ergoflavin, camptothecin, podophyllotoxin, isopestacin, benzopyran, phloroglucinal, tetrahydroxy-1-methylxanthone, borneol, salidroside, methyl peniphenone, lipopeptide, peniphenone etc. Despite being the aforementioned importance of metabolites produced by endophytic fungus, less than sufficient information is available on their pharmacological significance and exploration. We shall, therefore, elucidate in this research article, the fungal bioactive metabolites derived from medicinal plants and their significance.

Key Words - fungal endophytes, medicinal plants, antimicrobial, antifungal, pharmacological significance.

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INTRODUCTION

Plants popularly used as medicine traditionally by the people all over the world, according to other previous ethnobotanical literatures, are potentially of medicinal values. A wide range of medicinal plants are well studied with respect to their

phytochemical constituents and pharmacological properties, their microbiome and their physiological interactions between host and microbes are well understood. The extracts of plants remain as an important resource to fight serious diseases in the world. The data according to WHO (1993), says that 80% of the world's population depends on the traditional medicine, also a major part of the therapies involves the use of extracts of plants or active components /constituents. Medicinal plants have diverse populations of domains of microorganisms. The interdependence of the medicinal plants for the synthesis of bioactive compounds as a therapy to combat the emergence of new drug-resistant pathogens increased the search for an alternative of chemosynthetic drugs for treatment of human disorders. For example, despite the tiny nature of fungi, *Penicillium notatum* has been used as an invaluable source of novel metabolite compounds and broad-spectrum antibiotic. Since long, these antibiotics continue to dominate the market, and have awakened the scientist to search for secondary metabolites from endophytic fungus inhabiting the soil (rhizosphere) or plant endosphere.

Till date, endophytes have been recovered from all plants explored for their presence. Only a few species out of 420,000 plant species have been completely studied relative to their endophytic biology. The diversity of fungal endophytes is about 7% out of total 1.5 million fungi on earth. The fungal endophytes are therefore believed to be a precious treasure of biologically and structurally unique natural products as previously documented in several reviews. Antagonism shown by endophytic fungi against plant pathogens is a well-known phenomenon. Besides mycoparasitism, Volatile organic compound (VOCs) plays a major role in antagonism of pathogenic fungi. *Trichoderma* species significantly inhibits mycelial growth of particularly three pathogens namely *Sclerotinia sclerotiorum- TSS, Sclerotium rolfsii- CSR* and *Fusarium oxysporum- CFO.* In *Trichoderma longibrachiatum* there is the presence of several VOCs which includes hydrocarbons, ketones, alcohols, esters, aldehydes, acids, different classes of terpenes and ethers. Endophytes which are plant-associated are producing unique and important bioactive compounds, and their excellent biosynthetic process have made them to be a prospective avenues for phytochemists at present for novel discovery of metabolites to combat serious diseases prevailing at present. Endophytic fungi *Aspergillus terreus* have been isolated from healthy *Moringa oleifera* leaves and further identified for morphological characteristics and genetic traits. Extracts of the *A. terreus* contains 16 major bioactive compounds with significant pharmaceutical activities revealed by GC- MS of EACE (ethyl acetate crude extract). EACE of *A. terreus* shows effective antifungal activity against fungi which causes mucormycosis such as *Mucor racemosum, Rhizopus oryzae* and *Syncephalastrum* at 10mg/ml were found to be 20, 37 and 18 mm respectively. Therefore, the aim of this study was to isolate and identify fungal endophytes survived inside the leaves of *Tinospora cordifolia* plant. In the present study, Ranchi district was selected as the study area, due to abundance of this medicinal plant.

MATERIALS & METHODS

Collection of plant sample: Medicinal plant *Tinospora cordifolia* was collected from various places of Ranchi. Then the collected plant sample was brought to University Department of Botany, Dr. Shyama Prasad Mukherjee University, were identified and obtained. Healthy and mature plants were carefully chosen for sampling. Samples were collected randomly and brought to the laboratory in sterile bags. Leaves from these plants were gathered and stored aseptically.

Surface sterilization, isolation and culture of the endophytic fungi: Collected plant material were washed and cleaned with detergent. Surface was sterilized by treating it with 70% ethyl alcohol for 2 minutes, washed with distilled water and dipped in 10% sodium hypochlorite for 1-2 minutes. Detergent was used to wash and clean the collected plant material. It was treated with 0.1 mg/ml streptomycin for one minute after being cleaned

with distilled water once more. The effectiveness of the sterilization procedure was confirmed by the vitality test. These sterile samples were put in a petri plate, and using a sterile blade and forceps, they were sliced into tiny pieces measuring one centimeter using the tissue tear procedure. Sample cuts were put on Petri dishes with potato dextrose agar (PDA) medium, which had been pre-incubated for five days at 25°C. After that, inoculated PDA plates containing 4-5 segments of explants were sealed with parafilm and incubated for eight to ten days at 25 to 27 °C.

Regular observations were done for the development of fungal growth from the second day of incubation period. The fungi growing from the internal tissues were checked for purity and were transferred to the fresh PDA slants and then after fungal growth they were stored at 4°C for future use. After observing fungal development, individual hyphal tips of the fungus were re-inoculated on fresh PDA media and cultured for at least 12–14 days at 25–27 °C. The morphology of the fungal culture and the traits of the spores based on standard manuals and with the aid of microscopic investigations served as the foundation for fungal identification techniques. The non-sporulating strains were introduced for sporulation by culturing them on different media such as Potato Sucrose Agar media (PSA), Potato Carrot Agar media (PCA) and Water Agar media (WA). The cultures which were failed to sporulate were grouped under mycelia sterilia. This problem is common concerning with the identification of fungal endophytes.

Sterility check for the sterilized explants used for endophyte isolation: Sterility check was done by the following methods:

1. As per the method given by Pleban *et al.* (1995), the surface sterilized plant tissues were imprinted onto PDA media.
2. The last rinsing water was used to culture on PDA medium by using the method given by McInroy and Kloepper.
3. There were no microbial growth on the control petri-plate containing the PDA medium, and the surface sterilization was considered complete.

Morphological Characterization of fungal endophytes: For observing the general colonial morphology, a variety of morphological characteristics, including colony characterisation, fungal growth, colony color (front and reverse), size, form of conidiophores, and conidia, were recorded by using the criteria described by Harley and Prescott (2007) and these characteristics were used to identify the isolated fungal endophytes.

Microscopic examination: Based on the morphological characteristics such as surface texture,pigmentation and spores at the hyphal tips, the microscopic identification was done by using the standard manual of Udayaprakash *et al*., 2008. The fungal isolates were mounted on sterile slides and then stained with lactophenol cotton blue staining method was used to identify fungal endophytes. Microscope (Olympus CH20BIMF200, Japan) at a magnification of 100X was employed to capture the images of fungal endophytes. Some endophytes does not produce spores and were grouped under “sterile form” (Frohlich *et al*., 2000; Suryanarayan *et al.,* 2000). The identified fungal isolates from the respective explants were isolated and subcultured in a petri dish containing sterile PDA media. To preserve as a pure culture, the endophytic fungi was inoculated in PDA slant for further investigations.

Antibacterial screening of fungal isolates: The fungal isolates from *T. cordifolia* were screened for antibacterial activity against bacteria by disc diffusion method. Test bacterial strains were procured from IMTECH Chandigarh, India which include one gram positive bacteria and one gram negative bacteria viz., *Staphylococcus aureus* (MTCC 619) and *E coli* sp. (MTCC 2760) respectively. 0.1 ml of the inoculum of the test bacteria was spread into nutrient agar plates. Paper discs were immersed in the fungal extracts and then placed onto the bacterial plates in triplicate manner. The plates were incubated at 37^{o}C for 24

– 48 hrs. After the incubation, the inhibition zone was observed, measured, recorded in millimeter.

RESULT & CONCLUSION

Isolation and identification of fungal endophytes: Three fungal endophytes (Tc_1, Tc_2, and Tc_3) were isolated from *T. cordifolia* leaves, these fungal strains were identified as *Botrytis cinerea*, *Cladosporium sphaerospermum* and *Aspergillus ochraceus*. Morphological description of fungal strains from *T. cordifolia* (Fig. 1) showed that the colonies on PDA had 25–40 mm diameter, usually plane, somewhat granular; mycelium inconspicuous; conidial production moderate to heavy.

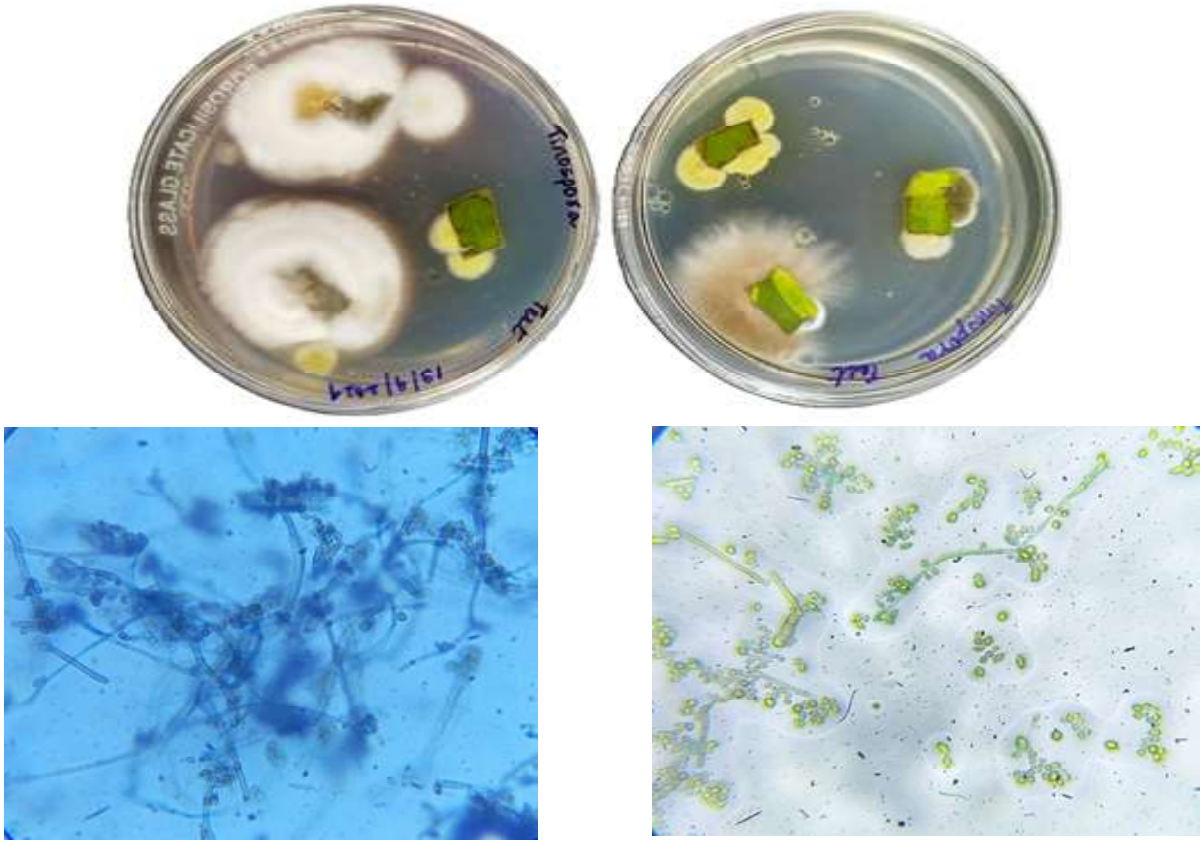

Fig. 1 Cultural morphology and microscopic picture of fungal endophyte from *Tinospora cordifolia* (Tc_1) grown on PDA and observed after 5 days of incubation.

Fig.2 Cultural morphology of different fungal isolates (Tc_2 & Tc_3) obtained from *Tinospora cordifolia* leaves grown on PDA medium after one week of incubation period.

The morphology of first fungal strain, of Tc_1 (Fig. 1) showed that the colonies appeared on PDA medium reached to 15–25 mm diameter, dense to floccose white mycelium, reverse uniformly pale or white centrally, colourless at the margins, branched nonseptated mycelium. The cultural morphology of the second fungal strain shown in Fig. 2 appeared greyish turquoise to dull green; reverse pale, yellowish, yellow brown. Conidiophores are borne from surface or subsurface hyphae. Fungal strain Tc_3 shows that colonies grown on PDA were 30–35 mm diameter, or covering to low and dense, dark olive to dark in old culture; after three weeks of incubation appeared nearly black. Conidia are blown out from the apices of undifferentiated conidiophores as short, irregularly branched chains of up to 12 units. They subsequently develop both lateral and longitudinal septation, forming up to six transverse septa and two to three longitudinal or oblique septa. They are typically pyriform or clavate in shape overall, and appeared tapering towards the apices to form a short beak. In culture, they are typically 30–50 and 6–15 lm, with smooth to noticeably roughened walls.

Figure 3. The agar plate pictures of the inhibition zone of fungal isolates against the growth *of S. aureus* and *E. coli*.

Table 1. Antibacterial activity of fungal isolate TC1, TC2, and TC3 against *S. aureus* and *E.coli*

Fungus code	Zone of inhibition (mm)	
	S. aureus	*E. coli*
TC1	12.30 ± 2.17	11.69 ± 0.16
TC2	15.71 ± 1.56	12.15 ± 2.61
TC3	14.80 ± 2.34	16.61 ± .67

CONCLUSION

Endophytic fungi have been isolated from the leaves of *Tinospora cordifolia*. In this study it was found that the three endophytic fungal strains *Aspergillus ochraceus*, *Botrytis cinerea* and *Cladosporium sphaerospermum* were capable of producing antibacterial compounds. Advanced research of these findings and ongoing efforts to purify, identify, and optimize fermentation is necessary. Due to the potential of these fungal endophytes to synthesize the same compound originating from their host medicinal plant, the endophytic fungi isolated from plants have acquired numerous researchers' attention in basic and applied research. All the three isolates from *Tinospora cordifolia* showed inhibition zone against both the gram positive and gram negative bacteria.

FUTURE PROSPECTS

Medicinal plants are currently on the verge of extinction, and there is growing interest in their preservation. Fungal endophytes can be crucial in this respect because of their many uses, which include giving plants tolerance to a variety of biotic and abiotic stresses, enhancing plant growth, generating a number of secondary metabolites, and having antibacterial action against harmful bacteria. The biodiversity of Jharkhand's medicinal plants and fungus may be preserved by the variety of fungal endophytes. In today's world, there is the search for effective low-risk, non-drug strategies that provides valuable alternative treatment of different health issues. There is much interest in complementary and alternates for several diseases which is growing at a significant rate. Taking into consideration, the extremely high cost and longtime duration of new drug development, and also the high drug attrition rate, it becomes an imminent task for pharmaceutical companies to explore new ways for drug research and development. Facing challenges and difficulties, there is an urgent need and opportunity to discover new antimicrobial molecules or compounds from plant sources with broad spectrum activities and immunomodulatory actions against pathogens for fighting plant pathogens and promoting growth of indigenous plant species. Significantly, through the research of endophytic fungi associated with the medicinal plants, we can preserve the traditional medicinal plants which are of utmost important to nature and as well as for the humans, as the endophytes synthesizes the same or similar bioactive compounds as that of their host. We can protect the medicinal plants from overharvesting, ecological distortions, from natural anthropogenic activities, and the destruction of the habitat by pest infestation.

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