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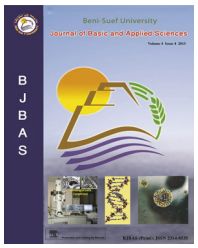

Full Length Article

In vitro antimicrobial potentials of endolichenic fungi isolated from thalli of *Parmelia* lichen against some human pathogens

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ABSTRACT

This study was undertaken to isolate and evaluate antimicrobial potentials of endolichenic fungi colonizing lichen thalli of *Parmelia* sp. A total of 19 distinct endolichenic fungi were obtained from surface sterilized fragments of *Parmelia* thalli. The dominant fungi belonged to genera *Phomopsis*, *Aspergillus*, *Penicillium* and mycelia sterilia. The colonization frequency of mycelia sterile (47.4%) was found to be the highest. The result indicated that 10.52% of the isolates showed antimicrobial activity against all the test pathogens in varying degrees, while 31.57% and 10.52% of the isolates displayed antibacterial and antifungal activity inhibiting all bacterial and fungal pathogens respectively. Among the isolates, species of *Aspergillus* and *Cytospora* and two sterile isolates showed considerable antimicrobial activity. These isolates were cultured in different media and incubation periods for optimum metabolites production. The crude metabolites of the isolates showed significant antimicrobial activity against all the test pathogens. Analyses of the crude metabolites by Thin Layer Chromatography and spectrophotometer study showed presence of bioactive components. The study suggests that endolichenic fungi colonizing lichen thalli may be a source of potential antimicrobial agents.

1. Introduction

There is an urgent need for new antimicrobial agents to combat drug resistance in bacteria and for effective treatment of systemic infection by fungi. Various antibacterial and antifungal agents have been explored, but the control of many of the bacterial and fungal diseases has not yet been achieved. Over the years, metabolites from plants and microbes are considered as important sources of drugs for therapeutic applications in several countries. Among them, 50–60% is produced by plants (alkaloids, flavonoids, terpenoids, steroids, carbohydrates, etc.)

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and 5% have a microbial origin (Demain and Sanchez, 2009). It is believed that the less explored microbial diversity might provide new and interesting metabolites with wide therapeutic applications. Therefore research is underway to study diverse and cryptic microorganisms from various habitats to obtain novel metabolites.

The symbiotic association between a fungus and an alga results into a new life form, called lichen. Lichens are widely distributed and conquered in the early developmental stages of biological communities that undergo primary and secondary successions (Logesh et al., 2012). Endolichenic fungi living in close association with the alga in the thalli of lichen are analogous to that of plant endophytes inhabiting the intercellular spaces of the hosts. They have been demonstrated to produce various antibiotics and natural bioactive compounds with multiple applications (Wu et al., 2011). However, relatively very few studies have been undertaken on endolichenic fungi, and the substances they produced have not been investigated in detail for their bioactivity and therapeutic potentials.

The objective of this present study was to evaluate the antimicrobial potentials of ethyl acetate extracts of some endolichenic fungi inhabiting the lichen thalli of *Parmelia* sp. collected from Similipal Biosphere Reserve, India, against some clinically significant human pathogens and to optimize and characterize the antimicrobial metabolites of some potent strains.

2. Materials and methods

2.1. Collection of samples and identification of lichen

The lichen thalli of *Parmelia* sp. were collected from the Joranda forest division of the Similipal Biosphere Reserve (SBR), India, located at 21° 16′ to 22° 08′ North latitude and 86 ° 4′ to 86° 37′ East longitude. The lichen thallus was light green and pale yellow in the upper cortex when dry. The lobes were round and smooth, measuring 3–8 mm, but quite often have a wrinkled appearance especially in older specimens. The lower surface is black except for a brown margin; rhizomes attached to the lower surface are black and unbranched. The thalli were mostly found associated on tree bark (Photoplate-1). Based on its morphological features the species resembles that of *Parmelia caperata*. The tree barks were ensured for the presence of the *Parmelia* sp., and healthy looking thalli were cut out from the tree bark with a sterile knife and placed into sterile plastic collection bags. The collected samples were brought to the laboratory in sterile polythene bags and processed within 24 hours of collection.

2.2. Isolation and identification of endolichenic fungi

The isolation procedure of endolichenic fungi was carried out following the method described by Li et al. (2007) with slight modification. Lichen thalli were washed thoroughly with Milli-Q water to remove dirt or debris from surfaces and then surface sterilized by immersing sequentially in 10% of 30% hydrogen peroxide (H_2O_2) solution for 4–5 min followed by rinsing with 70% alcohol for 5 s and 1% of 4% sodium hypochlorite (NaOCl) for 6 min and washed with sterile distilled water. The lichen thalli were then dried by placing over sterile filter papers and dissected aseptically to a fragment of 1 mm^2 size. The efficiency of sterilization process was verified by rubbing the sterilized fragment over Potato dextrose agar medium and incubated for growth of contaminant if any. Each fragment was then inoculated onto Potato Dextrose Agar (PDA) medium. The plates were sealed with parafilm and incubated in BOD incubator at 30 °C until growth of endolichenic fungi. The plates were observed once a day for fungal growth. After several days of incubation the colonies growing out of surface sterilized fragments were immediately isolated, sub-cultured and maintained at 4 °C in PDA slants. The isolates were identified based on their morphological and reproductive characters using the standard identification manuals (Barnett and Hunter, 1998; Gilman, 1971). The cultures that failed to sporulate were categorized as sterile mycelia.

2.3. Screening of for antimicrobial activity

Pure cultures of endolichenic fungi were cultivated on Potato Dextrose Broth (PDB) by placing agar blocks of actively growing culture (3 mm in diameter) in 250 ml Erlenmeyer flasks containing 100 ml of the medium. The flasks were incubated in BOD shaking incubator for 14 days at 29 ± 1 °C with periodic shaking at 150 rpm. The cultures were filtered through sterile cheesecloth to remove the mycelial mats. The liquid broth were collected and extracted with equal volume of ethyl acetate in a separating funnel by vigorous shaking for 15 min. The cell masses were separated, and the ethyl acetate extracts were collected for each isolate. Ethyl acetate was evaporated, and the resultant compounds were dried individually with magnesium sulfate ($MgSO_4$) and concentrated to yield the crude extracts. The crude extracts were then dissolved in 15% Dimethyl sulfoxide (DMSO) for antimicrobial bioassay. Antimicrobial activities of the crude metabolites isolated from the endolichenic fungi were determined by agar cup diffusion method against six bacterial pathogens, *Bacillus subtilis* (MTCC 736), *Staphylococcus aureus* (MTCC 737), *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 426), *Shigella flexneri* (MTCC 1457), and *Klebsiella pneumonia* (MTCC 3384), and three pathogenic fungi, *Candida albicans* (MTCC 227), *Candida krusei* (MTCC 9215), and *Trichophyton mentagrophytes* (MTCC 8476). The test pathogens were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Muller Hinton Agar (MHA) plates were inoculated with overnight culture of each bacterial suspension. Similarly for the fungal pathogens, Sabourad Dextrose Agar (SDA) plates were inoculated with each fungal suspension. The plates with the inoculated organisms were evenly spread out with sterile cotton swabs. Agar cups were prepared by scooping out the media with a sterile cork borer (7 mm diameter). The cups were then filled with 100 µl of the crude metabolites that was dissolved in DMSO to get a concentration of 1 mg/ml. The plates were then incubated at 36 ± 1 °C for 24 h and 48 h for bacterial and fungal pathogens respectively. Antimicrobial activity was determined as growth inhibition of the target organism around agar cup as appearance of clear zones.