Isolation and Screening of Endophytic Fungi from *Achillea millefolium* L - A Medicinal Plant of Western Himalayas

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**Abstract:** The aim of the present study was to isolation of endophytic fungi of selected medicinal plant from the western Himalayas. A total of 09 strains of Endophytic fungi have been isolated from the different parts of the medicinal plant. Among nine, crude ethyle extract of three endophytic fungi were screened for antimicrobial activity against gram positive and gram negative microorganisms and the inhibition zone, were compared with positive control streptomycin 10 µg disc and negative control dimethoxysulphoxide (DMSO). Phytochemical analysis of ethyle acetate extract revealed the presence of alkaloids, flavonoids, saponins, phenols, terpenoids, steroids, cardisglycosides, tannins in Achelila melfollium indicated that the plant can be a potential source of novel natural antimicrobial agents.

**Keywords:** Western Himalayas, *Achillea millefolium* L., endophytic fungi, antibacterial activity, phytochemicals.

1. INTRODUCTION

Endophytes are microorganism which are present within healthy plant tissues without causing any harm to their host (Tan and Zou, 2001). They play an essential role to provide protection to the host from infectious agents and also synthesise bioactive natural products, which defend the plants against pathogens (Ezra, 2004). Some of the endophytic microorganisms have been found to produce the same secondary metabolites as that of endophytic fungi have a great potential to provide metabolites such as alkaloids, terpenoids, cardiac glycosides, steroids, flavonoids, phenols, tannins and peptides (Tan and Zou, 2001; Strobel G, 2004). Endophytic fungi are precursors of biologically active novel compounds like anticancer drugs, antibiotics and immunosuppressive agents (Strobel and Daisy, 2003). The most important role of endophytic fungi is to carry nutrient recycling pathway by degradation of dead or dying host plants (Strobel, G.A, 2002). Endophytic fungi that live inside the tissues of living plants are unexplored group of microorganisms that have enormous potential for new pharmaceutical substances (Hawks worth, 2001).

*Achillea millefolium* (L) or yarrow is a flowering plant in the family Asteraceae. The herb is purported to be an antiphlogistic (Benedek et al., 2007; Burk et al., 2010), gastrointestinal disorders (Noureddini and Rasta, 2008), anti-inflammatory (Popovic et al., 2008). It contains isovaleric acid, salicylic acid, sterols, flavonoids, bitters, tannins and coumarines. The plant also has a long history as a powerful healing herb used topically for wound, cuts and abrasions (Alma R. Hutchens, 1973). In the present study we focus on the isolation and identification of endophytic fungi from medicinal plant of western Himalayas and screening them for antibacterial activity and to identify the phytochemical compounds in the extract of endophytic fungi.

2. MATERIAL AND METHOD

2.1 Collection of plant materials

The plant material of *Achillea millefolium* L. (Specimen Vochur No.2356(KASH)), were collected from Pahalgam, Jammu and Kashmir District
. Anantnag. Plant showing no visual disease symptom were collected during July-August. The plant materials were brought to the laboratory in sterile bags and stored 4°C until processed. The authenticated specimens of the collection were deposited in the herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir, India.

2.1.1 Isolation of endophytic fungi

Plant material was washed with running tap water and surface sterilized with 90% ethanol for 1 minute and 1% sodium hypochlorite for 1 minute. The plant material was subsequently washed thrice with sterilized distilled water and blotted with sterilized filter paper. All the work was performed in the laminar air hood. The material were longitudinally cut in to 0.5 to 1 cm section and directly placed on a sterilized Petri dish containing potato dextrose agar, supplemented with chloramphenicol (100 mg/ml) and penicillin G (100 units/ml) to eliminate any bacterial growth (Fig.1). The plates were sealed with parafilm and incubated at 28°C for 2 weeks until fungal growth started. The hyphal tips of fungi which grew out from sample segments were isolated and sub cultured onto Potato dextrose agar (Fig.2). (Raper and Thom, 1949; Raper1,965; Ellis, 1971; Ellis, 1976; Booth, 1977; Chirstensen, 1978; Pit, 1979; Pit, 1985; Moub, 1993).

2.1.2 Maintenance of endophytes

Fungal isolates were preserved in NCIM Resource Centre Pune with accession number KU235487-KU235491.

2.1.3 Cultivation of selective fungi for the production of metabolites

The each selective fungi isolated were inoculated into 500 ml Erlenmeyer flask containing 250 ml potato dextrose broth and incubated at 28°C for two weeks under stationary condition with intermittent shaking. After incubation period, the broth culture was filtered through sterile cheesecloth to separate the mycelia and filtrate (Strobel G, 1996).

2.1.4 Extraction of the secondary metabolites

Extraction of secondary metabolites from the filtrate and mycelia by added equal volume of ethyl acetate and mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. Mycelium was grinded using ethyl acetate as solvent and then it was filtered. Both mycelia and filtered extracts were pooled together evaporated to dryness in hot air oven to yield the crude extract. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) and stored at 4°C to be used as stock solution for antimicrobial assay (Raviraja et al., 2006).

3. Test microorganisms

Six common human pathogens Bacillus subtilis (MTCC 44), Staphylococcus aureus (MTCC 96), Escherichia coli (MTCC739), Pseudomonas aeruginosa (MTCC 1688) and Klebsiella pneumonia (MTCC 139), were used to evaluate the antimicrobial activity of endophytic crude extracts.

3.1.1 Screening of antibacterial activity of endophytic fungi

The antibacterial activity of the three endophytic fungi crude extract were assay ed against human pathogen bacteria by Agar well diffusion method. 20 ml PDA was dispensed in each tube and autoclaved at 121°C for 15 minutes. Tubes were allowed to cool at 50°C and a fresh bacterial culture 100 µl (10^7CFU/ML) was inoculated to each tube. The medium was then poured on the Petri plates and kept for some times to solidify. Streptomycin 10 µg disc were placed on the surface of each plate as a positive control. In the same plates 6 mm diameter wells were made using a sterile cork borer and 100 µl of sample (5 mg/ml concentration) was added to the each well. DMSO was used as negative control. The plates were incubated at 37°C for overnight. The experiment was carried out in triplicate .The diameter of inhibition zone around disc and wells were measured in mm (Bauer, 1966). Results are given in table (Table 1).

4. Phytochemical Analysis of crude extract

The ethyl acetate extract of endophytic fungi was subjected to various qualitative chemical tests to determine phytochemical constituents such as alkaloid, phenols, flavonoids, saponins, steroids, cardiac glycosides and tannins (S.Gurupavithra, 2013). The results are given in table (Table 2).
5. RESULTS

![Inoculation of plant parts on potato dextrose agar plate for isolation of endophytes](image1)

Fig. 1. Inoculation of plant parts on potato dextrose agar plate for isolation of endophytes

![Some of the endophytic fungi isolated from Achillea millefolium L.](image2)

Fig. 2. Some of the endophytic fungi isolated from *Achillea millefolium* L.

<table>
<thead>
<tr>
<th>Test</th>
<th>Name of phytochemical test</th>
<th>Endophytic fungi</th>
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<tr>
<td></td>
<td></td>
<td><em>Aspergillus niger</em></td>
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<tr>
<td>Flavonoids</td>
<td>Shinoda’s &amp; Zn-HCl</td>
<td>+</td>
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<tr>
<td>Phenols</td>
<td>Ferric chloride</td>
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<tr>
<td>Tannins</td>
<td>Ferric chloride</td>
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<td>Cardioglycosides</td>
<td>Keller Kilani</td>
<td>+</td>
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<td>Alkaloids</td>
<td>Mayers &amp; Dragendorff’s</td>
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<td>Steroids</td>
<td>Liebermann-Burchard</td>
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<td>Saponins</td>
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DISCUSSIONS

Natural products play an important role in the drug discovery and development. Plants and fungi are recognized as sources of natural products. Medicinal plants harbour endophytic fungi that share characteristics with their host. The endophytes are also known to protect their host from infectious agents and provide strength to survive in adverse conditions.

_Achillea millefolium_ L. was selected for the isolation of endophytic fungi on the basis of medicinal importance and availability. For isolation of endophytic fungi were using mycological media namely PDA. Based on the ITS1 and ITS4 gene sequencing, endophytic fungi isolated from the _Achillea millefolium_ L. were identified as _Aspergillus niger_, _Aspergillus terreus_ and _Aspergillus flavus_.

Antimicrobial activity of ethyl acetate extract of three endophytes was observed against selected gram positive and gram negative microorganisms. The considerable antimicrobial activity of crude extract of endophytic fungi against human pathogens showed the broad spectrum nature of metabolites. On the basis of phytochemical analysis, crude ethyl acetate extract of endophytic fungi revealed the presence and absence of phytochemical constituents like flavonoids, phenols, tannins, alkaloids, steroids and saponins.

In the conclusion, the results of the present study reveals that the ethyle acetate extract of the _Achillea millefolium_ L. has broad range of antibacterial activity and could be good potential sources for the screening programs of bioactive natural products. The presence of various phytochemical constituents in the endophytic extract indicates that the plant can be potential source of bioactive metabolites that can be exploited for the development of effective therapeutic agents.

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REFERENCES


