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Isolation of microbial endophytes from some ethnomedicinal plants of Jammu and Kashmir

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ABSTRACT

Endophytes are the plant-associated microorganisms that live within the living tissues of their host plants without causing any harm to them. Almost all groups of microorganisms have been found in endophytic association with plants may it be fungi, bacteria or actinomycetes. They stimulate the production of secondary metabolites with a diverse range of biological activities. They have been known to produce enormous variety of strange and wonderful secondary metabolites, some of which have profound biological activities that can be exploited for human health and welfare. Some of the endophytic microorganisms can produce the same secondary metabolites as that of the plant thus making them a promising source of novel compounds. During the present investigation, endophytes were isolated for the first time from the symptomless leaves, stem, fruits and roots of the four selected ethnomedicinal plants. The ethnomedicinal angiosperms include Digitalis lanata (wooly foxglove), Digitalis purpurea (purple foxglove), Plantago ovata (psyllium/isabgol), Dioscorea bulbifera (air potato). A total of one hundred and thirty two isolates of microbial endophytes were isolated. Dioscorea bulbifera belongs to the dioscoreaceae family and the rest belong to the plantaginaceae family. The major constituents of these plants belong to the steroidal and iridoid family of secondary metabolites, having enormous applications in the medicinal/pharmaceutical arenas.

Keywords : Endophytes, Secondary metabolites, Ethnomedicinal, Steroid, Iridoid.

INTRODUCTION

Medicinal plants are gaining global attention owing to the fact that the herbal drugs are cost effective, easily available and with negligible side effects. The beneficial effects of the medicinal plants in health care can be well judged from the WHO estimate that around 80% of the world population uses them in some form or the other. It is important to note that homeopathy and modern medicine have their roots in medicinal plants. The compounds derived from medicinal plants form the ingredients of analgesics, antibiotics, heart drugs, laxatives, anti-cancer agents, ulcer treatments, contraceptives, diuretics etc.

Compounds from plants are referred as plant secondary metabolites, phytochemicals, anti-nutritional factors, plant xenobiotics etc. Some of the major plant secondary metabolites or phytochemicals that occur in plants include protease inhibitors, lectins, alkaloids, non-protein amino acids, cyanogenic glycosides, saponins, steroids and tannins. Secondary metabolites have complex and unique structure and their production is often enhanced by both biotic and abiotic stresses [1]. Plant based natural constituents can be derived from any part of the plant like bark,

leaves, flowers, fruits, roots, seeds etc. [2]. The most important bioactive compounds from medicinal plants include terpenes, alkaloids, phenolic compounds, steroidal compounds and flavonoids [3].

Endophytes are defined as microorganisms which inhabit inside of healthy plant tissues and are now considered as ubiquitous symbionts of plants from their surprisingly common detection from many plant species [4]. Common endophytes include a variety of bacteria, fungi and actinomycetes, and they can be isolated from wild [5] or cultivated crops [6] of either the monocots [7] or dicots [8]. Among the microbial group the most frequently isolated endophytes are the fungi. Endophytic fungi are considered as an outstanding source of bioactive natural products because there are so many of them occupying millions of unique biological niches growing in different types of environment. Plants infected with fungal endophytes are often healthier than endophyte free ones [9].

Digitalis is a genus comprising of about 20 species of herbaceous biennials, perennials and shrubs in the foxglove family Plantaginaceae. The use of *Digitalis* extract containing cardiac glycosides for the treatment of heart conditions was first described by William Withering in 1785, which is considered the beginning of modern therapeutics (Silverman). Treatments with drugs based on *Digitalis* extracts are some of the best known pharmaceutical products used to strengthen cardiac diffusion and to regulate heart rhythm [10]. Cardiac glycosides from *Digitalis* are a class of natural products that are used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias [11]. The most familiar glycosides from *Digitalis* are digoxin and digitoxin, which are derived from *Digitalis lanata* and *Digitalis purpurea* respectively. Their mechanism of action in the heart are well known and involve inhibition of the plasma membrane Na^+/K^+-ATPase [12], leading to increased intracellular Na^+ and Ca^{2+} and decreased intracellular K^+ [13]. The increased intracellular Ca^{2+} promotes muscle contraction and cardiac contractile force. *Digitalis* has been reported to produce cardiotonic drugs and is widely used in the treatment of various heart conditions namely atrial fibrillation, atrial flutter and sometimes heart failure that cannot be controlled by other medications.

Plantago is a genus of about 200 species of small, inconspicuous plants commonly called plantains and belongs to the family Plantaginaceae. *Plantago* species have been used since prehistoric times as herbal remedy. The herb is astringent, anti-toxic, antimicrobial and anti-inflammatory. *Plantago ovata* also known as Psyllium, Ispaghula and Isapgol is one of the important species of this genus and has a long history of use throughout the world. Some of the uses of psyllium in traditional medicine are as laxative, emollient, demulcent, and diuretic. Its seed contains mucilage, fatty oil, large quantities of albuminous matter, the pharmacologically inactive glucoside, namely aucubin (C_{13} H_{19} O_8 H_2O) and a plantiose sugar [14]. It is a diuretic, alleviates kidney and bladder complaints, gonorrhea, arthritis and hemorrhoids [15].

Dioscorea is a genus of over 600 species of flowering plants in the family Dioscoreaceae. They are commonly known as yams and have been traditionally used to lower glycemic index. *Dioscorea bulbifera*, the 'air potato', has been used in the Chinese system of medicine to treat diseases of the lungs, kidneys and spleen, and many types of diarrhoea. Diosgenin and related steroidal saponins commonly found in *Dioscorea* plants have shown antitumor, antifungal, and anti-inflammatory activities [16,17]. Mbiantcha *et al*. (2010) studied the analgesic and anti-inflammatory properties of extracts from the bulbils of *Dioscorea bulbifera* in mice and rats [18]. Earlier chemical investigations of *D. bulbifera* yielded flavonoids, clerodane diterpenoids and a few steroidal saponins [19-22].

In the present study, efforts have been made to isolate endophytes inhabiting the four selected ethnomedicinal plants i.e. *Digitalis purpurea*, *Digitalis lanata*, *Plantago ovata* and *Dioscorea bulbifera*. The isolated endophytic assemblage was then arranged according to the type of endophyte (fungi, bacteria, actinomycete) and accessioned accordingly depending upon the type of the plant from which they have been isolated.

MATERIALS AND METHODS

Selection of the Plant material

The medicinal plants used in the present study were selected after proper literature survey using internet, books and journals of the departmental and central library of the University of Jammu. Keeping in view the medicinal/pharmaceutical applications, following medicinal plants were selected for this study - *Digitalis purpurea*, *Digitalis lanata*, *Plantago ovata* and *Dioscorea bulbifera.*

Collection of the plant material

The medicinal plants selected for the present investigation were collected from their natural habitats. *Digitalis purpurea* and *Digitalis lanata* were collected from Gulmarg (altitude 2730 meters, district Baramulla, J & K State), *Plantago ovata* was collected from Jammu University, Germplasm Collection (district Jammu) and *Dioscorea bulbifera* from district Rajouri of J&K state. Disease free parts of the plants were cut with the help of a sterile scalpel and placed in zip-lock plastic bags to store the material at 4^0C until isolation.

Isolation of endophytes

(a) Surface sterilization of the plant matertial

The method most frequently utilised to detect and quantify endophytes involves isolation from surface-sterilized host plant tissues. Endophytic isolation was carried out under aseptic conditions. Different symptomless parts of the selected ethnomedicinal plants such as stem cuttings, leaves, fruits and roots were used for the isolation of endophytes [23].

The collected plant material used for the isolation was first surface sterilized following the method of Santos *et.al*. (2003) with few modifications [24]. Plant material was first cleaned by washing several times under running tap water and then cut into small segments. Surface sterilization was performed by sequentially rinsing the plant material with 70% ethanol (C_2H_5OH) for 30 seconds, then with 0.01% mercuric chloride ($HgCl_2$) for 5 minutes followed by 0.5% sodium hypochlorite (NaOCl) for 2-3 minutes and finally with sterile distilled water for 2-3 times. Plant material was then dried in between the folds of sterile filter papers.

(b) Isolation of the endophytes

After proper drying, the surface sterilized plant material i.e. leaves were cut into smaller pieces and each piece was placed on potato dextrose agar (PDA) medium supplemented with chloramphenicol (100 mg/ml). Similarly, stem, fruits and roots were cut vertically into small segments to expose the inner surface and then inoculated on the PDA plates. Six explants were put on each PDA plate. All the plates were incubated at 28ºC to promote the growth of endophytes (Fig.1) and were regularly monitored for any microbial growth. On observing the microbial growth, sub culturing was done. Each endophytic culture was checked for purity and transferred to freshly prepared PDA plate. Appropriate controls were also set up in which no plant tissues were inoculated.

(c) Maintenance of endophytes

The purified endophytic isolates were transferred separately to PDA slants and accessioned accordingly depending upon the plant and plant parts from which they have been isolated. Finally all the purified endophytes were maintained at 4ºC till further used.

RESULTS AND DISCUSSION

A total of one hundred and thirty two endophytes (Table 1) were isolated from different healthy parts such as leaves, stem, fruits and roots of the four ethnomedicinal plants under investigation. Twenty one endophytes were isolated from one hundred and sixty two explants of *Digitalis purpurea*, twenty five endophytes from one hundred and sixty eight explants of *Digitalis lanata*, fourteen endophytes from one hundred and fifty six explants of *Plantago ovata* and seventy two endophytes from two hundred and seventy explants of *Dioscorea bulbifera.* Out of these one hundred and thirty two isolates, majority of the endophytes isolated were fungi followed by bacteria and a few were actinomycetes (Table-1, Fig. 2). Similar study on isolation of endophytes has been reported by other workers as well [25-29]. Caruso *et.al.* (2000) isolated 150 fungal and 71 actinomycete endophytes from the internal tissues of woody branches, shoots and leaves of different plants of *Taxus baccata* and *Taxus brevifolia* [25]; Arnold *et al.* (2000) isolated 418 endophyte morphospecies from 83 healthy leaves of *Histeria concinna* and *Ouratea lucens* in a lowland tropical forest of central panama, and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity [26]. Similarly, Jalgaonwala *et.al.* (2010) isolated 78 bacterial and 142 fungal endophytes from aerial and underground parts of various medicinal plants [27]. Teerayut *et al.* (2009) isolated 194 fungal endophytes from wild medicinal plants of Thailand [28]. Santhosh *et al.* (2011) isolated 41 endophytic fungi from 195 samples of healthy leaves and stem of a red listed endangered medicinal plant *Coscinium fenestratum* [29].

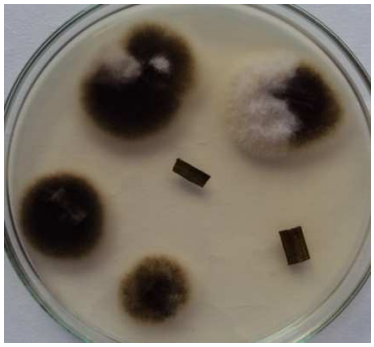
In the present study, Selection of the isolates was based on the maximum frequency of occurrence of isolates from plant parts. Some of the isolated endophytes from four selected ethnomedicinal plants are as shown below (Fig.2).

Since the discovery of the world's first billion-dollar anticancer compound - paclitaxel (Taxol) - could be biosynthesized by *Pestalotiopsis microspora* an endophytic fungus of Himalayan yew, interest in studying such endophytes for their medicinal potential has grown tremendously [30]. Natural products from endophytes have a broad spectrum of biological activity and can be grouped into several categories such as alkaloids, steroids, terpenoids, isocumarins, quinones, phenyl propanoids, lignans, aliphatic metabolites, lactones etc. [31]. Puri *et al.* (2005) isolated a novel Camptothecin producing endophytic fungus *Entrophosphora inferquens* from an important Indian medicinal plant *Nothapodytes foetida* [32]. *E. inferquens* synthesizes camptothecin having potential immunomodulatory activity. Similarly, Chen *et al.* (2007) isolated an endophytic fungus *Pencillium thomi* from the roots of *Bruguiera gymnorhiza* [33]. The separation of endophytic fungus from the root led to the isolation of a new compound 4', 5 dihydroxy -2, 3 dimethoxy 4(-hydroxy propyl)- biphenyl along with 11 known compounds. Their effect against three human cell lines was also investigated.

In the series of useful bioactive compounds from plants and their respective endophytes, the main important compounds that can be extracted from the four medicinal plants under the present investigation includes Cardiac glycosides Digoxin ($C_{41}H_{64}O_{14}$) and Digitoxin ($C_{41}H_{64}O_{13}$) from *Digitalis lanata* and *Digitalis purpurea* respectively. Similarly, steroidal saponin diosgenin ($C_{27}H_{42}O_3$) from *Dioscorea bulbifera* and a glucoside (iridoid family), namely Aucubin (C_{13} H_{19} O_8 H_2O) from *Plantago ovata*. Discovery of new chemical compounds from natural products is very important for formulating new drugs. We can say that the endophytes could be a reliable source of pharmaceutically and industrially important compounds that can be used in the treatment of various life threatening diseases along with various other industrial applications.

Table.1. Endophytes isolated from different plant parts of four ethnomedicinal plants

Plant	Endophyte	Plant part used				Total
		Stem	Leaves	Fruits	Roots	
Digitalis purpurea	Fungi	09	10	01	-	20
	Bacteria	-	01	-	-	01
	Actinomy.	-	-	-	-	-
Digitalis lanata	Fungi	10	08	05	-	23
	Bacteria	-	-	01	-	01
	Actinomy.	-	01	-	-	01
Plantago ovata	Fungi	-	13	-	-	13
	Bacteria	-	01	-	-	01
	Actinomy.	-	-	-	-	-
Dioscorea bulbifera	Fungi	02	04	-	51	57
	Bacteria	01	01	-	09	11
	Actinomy.	01	01	-	02	04
Total isolates of endophytes isolated						132

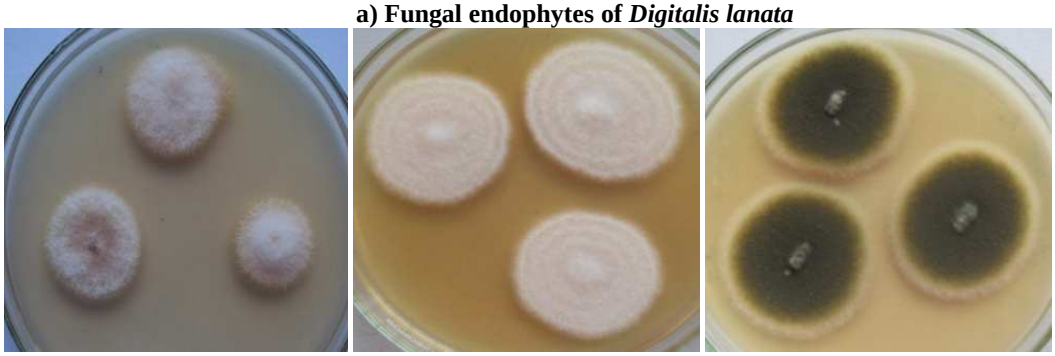


Leaf Inoculation

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

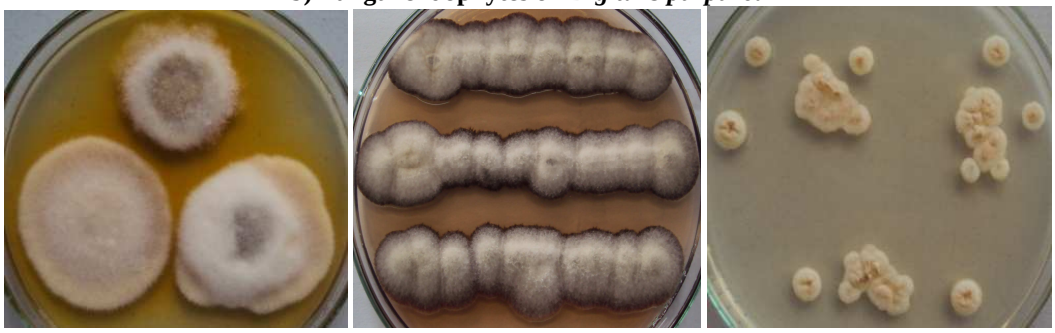
DLL-1 **DLS-9** **DLF-3**

a) Fungal endophytes of *Digitalis lanata*



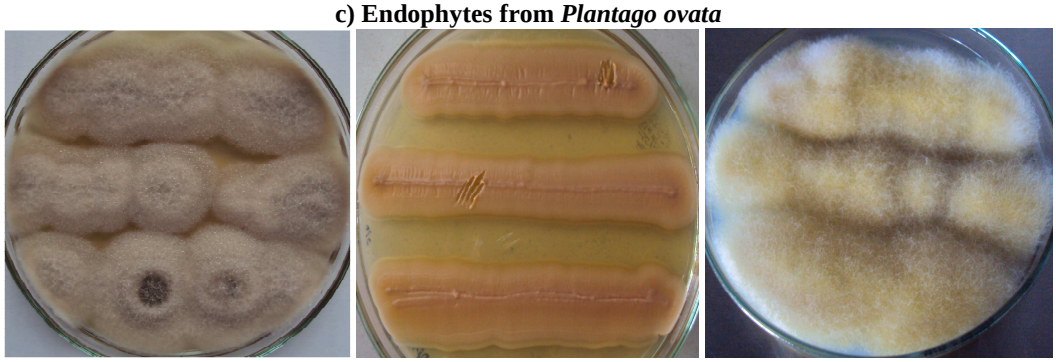
DPL-10 **DPS-7** **DPF-1**

b) Fungal endophytes of *Digitalis purpurea*



POL-3 **POL-8** **POL-13**

c) Endophytes from *Plantago ovata*



DBR-9 **DBR-14** **DBR-27**

d) Endophytes from *Dioscorea bulbifera*

Fig.2. Some of the microbial endophytes isolated from four ethnomedicinal plants.

CONCLUSION

Keeping in view the importance of the four selected ethnomedicinal plants, their pharmaceutical applications and biological activity, the endophytes isolated from these plants can be further characterized chemically for bioactive secondary metabolites. This study can be further worked out on a pilot scale for process optimization and scale up studies of potential novel bioactives from these endophytes. Endophytes as drug source will help to conserve biodiversity and drug resistance as they are an alternate source of drugs. Therefore, in future the traditional methods of drug discovery may be replaced by endophytes.

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