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Isolation and Identification Of Endophytic Fungi From Indigenous Medicinal Plants

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A collection of fascinating communities known as endophytic fungus (EF) inhabit the intracellular and intercellular gaps of host tissues, providing advantages to both the host and endophytic fungi. Endophytic fungi (EFs) are one of the endophytes that have garnered the most scientific attention due to their ability to produce biostimulants for the production of essential oils as well as new chemicals, antibacterial and antifungal substances, antioxidants, and anti-carcinogenic molecules. Natural compounds that are used to treat a wide range of illnesses can be found in medicinal plants in large quantities, which makes them a valuable resource for the discovery of novel drugs. Since endophytic microbes can produce a wide range of secondary metabolites from their hosts, including those with important biological activities, the interaction between them and host plants has been intensively explored. The hunt for new bioactive substances that may be isolated from fungal endophytes is one of the primary objectives of current biotechnology advancements. After being isolated from 51 samples of ten distinct plants, 133 fungi were then examined for antibacterial activity. In the present study, ascomycetes, which include *Candida, Aspergillus, Penicillium*, among others, made up the majority of the fungi were isolated from various indigenous medicinal plants such as Neem (*Azadirachta indica*), Jatropha (*Jatropha gossypiifolia*), Tulsi (*Ocimum sanctum*), Peepal (*Ficus religiosa*), Eucalyptus (*Eucalyptus teriticornis*), Geloy (*Tinaspora cordifolia*), Chinease wisteria, Turmeric (*Curcuma longa*), Moringa (*Moringa oleifera*) and Ashoka (*Saraca asoca*). Among the isolated molds, *Rhizopus, Mucor*, and *Rhizomucor*, were also present. The most common types of molds in this area included *Aspergillus* species, *Alternaria* species, *Pencililum* species, *Fusarium* species, and *Verticillum* species. *Aspergillus* spp. is the most abundant fungi found in all the samples. The objective of this investigation was to isolate the endophytic fungi from the various medicinal plants' roots, barks, and leaves which were later evaluated for antimicrobial activity

Keywords: Endophytes; Enzymes; Novel compound; Secondary-metabolites; Symbiosis.

Certain microorganisms, such as fungi and bacteria, which reside inside plants are called endophytes. They infect healthy tissue at some point in their life cycle without doing any harm[1]. Certain biological activities of structurally distinct secondary metabolites are also produced by endophytic fungi, which live in healthy living tissue without generating any negative effects[2]. It can produce metabolites for competence with co-occurring endophytes, hosts, and pathogens in order to colonize the host and provide sustenance[3]. One million of the roughly 1.5 million fungal species

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on Earth are endophytic. They live mostly inside the stems, leaves, flowers, and seeds of plants; they can spread either vertically or horizontally[4,5] .

Endophytes coexist in the tissues of plants without harming them; in fact, they have a symbiotic connection with the plants[6]. According to Ladoh[7] and Jeffrey[8], the endophytes create bioactive substances that aid the host plant in improving its nutritional status, resistance to pests and diseases, and ability to withstand physical stress. Since they have been shown to have antibacterial and antimalarial properties as well as the ability to function as enzymes, some of the bioactive compounds produced by endophytes inhabiting different plant species could be exploited in the food, pharmaceutical, and agricultural industries. Alkaloids, terpenoids, steroids, quinones, flavanoids, phenols, tannins, anthraquinones, phenolic acids, and peptides are examples of these substances. According to reports, the endophytic fungus *Taxomyces andreanae*, which belongs to the hypomycete class, produces the anti-cancer drug taxol[9].

Endophytes disrupt the metabolism of the host plant and take their nutrients from it, while the plant controls the metabolic processes of the microbes to release compounds that could protect the endophytes. Endophytes communicate with other species by chemical signals, which may be essential for the host's survival and ability to adjust to a variety of biological and environmental obstacles[9].

The present study carries on the exploration of natural compounds derived from endophytic fungi residing in medicinal plants. Medicinal plants represent a significant global source of innovative medications. Since ancient times, people have used herbal medicines as a kind of healing. Eighty percent of the world's population is thought to be predominantly treated by traditional medical systems. This statistic comes from the World Health Organization. These might provide future wonder drugs. Moreover, up to 80% of people in developing countries solely depend on herbal therapies for their primary healthcare, but about 25% of prescribed drugs in wealthy nations come from wild plant species. It is possible to cultivate a large number of plants for the local industrial production of herbal nutritional supplements and therapeutic items. Ensuring that the knowledge obtained is put to use is crucial, as it may improve the welfare of our people as well as yield financial gains. There is a current need to establish the necessary knowledge for the creation of traditional medicines in order to facilitate agriculture and so contribute in the elimination of poverty. In addition, there should be a strong push for the industrial manufacturing of herbal and traditional remedies in the area.

MATERIAL AND METHOD

Isolation and identification of endophytic fungi from leaves, bark, and stem of indigenous medicinal plants

Using a standardized and modified approach, endophytic fungi were isolated and identified from indigenous medicinal plants found in the near and native land of Kurukshetra, Haryana. Dust and dirt were eliminated from the samples by gently rinsing them under running water[10]. Following a thorough cleaning, stem samples were cut into long, 0.5–1 cm pieces, and leaves, under aseptic circumstances, were cut into 3–4 mm x 0.5–1 cm pieces. Depending on the kind of tissue, 1–13% sodium hypochlorite (NaOCl) was used for surface sterilization. Three sets of plant material was treated for one minute with 75% ethanol, then submerged in sodium hypochlorite and again for thirty seconds with 75% ethanol[11]. After being thoroughly cleaned with deionized sterile distilled water to eliminate any remaining sterilants, the sterilized stem and leaf explants were placed on sterile tissue paper and allowed to dry. They were then cultivated on Petri dishes filled with potato dextrose agar medium (PDA) that was enhanced with 100 g/mL streptomycin. The petri dishes were covered with parafilm, left in a dark environment, and incubated at 27±2°C for 15 days while being evaluated daily for any contamination.

The fungi that emerged from the plant explants were sub-cultured at room temperature on distinct PDA plates. The isolated fungi were identified using lacto-phenol cotton blue wet mount method under the compound microscope. The fungus known as "mycelia sterilia" were those that were unable to sporulate. The mycelia were moved into PDA agar media to measure colony characteristics. The fungal isolates were stored at 4! for preservation and further studies.

RESULTS

A total of 133 fungi (same and different strains) were isolated from 51 samples of 10 different plants and were later evaluated for antimicrobial activity (Table 1). The majority of the fungi were Ascomycetes, including *Candida, Aspergillus, Penicillium,* and *Nigrospora* etc. and some being molds- *Rhizopus, Mucor,* and *Rhizomucor* etc.

Aspergillus spp., *Alternaria* spp., *Pencililum* spp., *Fusarium* and *Verticillum* spp. were mostly found in the plants of this region. In present study neem plant exhibited the highest frequency of endophytic fungi whereas, Chinese wisteria plant had none.

The fungi isolated were cultured at 27°C for seven days. There were 2 replicates in the fully randomized experimental design. The average diameter of the colonies (measured in centimetres), the aspect of their boundaries, the colour of the mycelium, the colour of the petri dish's reverse, and the colour of the medium were all examined. The microscopic characteristics of the isolated endophytic fungi were studied under compound microscope. Using lacto-phenol cotton blue wet mount method the microscopic characteristics of isolated fungi were observed. The microscopic observations of various fungal genera are enlisted in Table 2.

DISCUSSION

The foundation of traditional medicine, medicinal plants, have been the focus of extensive pharmacological research in recent years. It is known that every plant on the planet contains at least one endophytic bacterium. One of the most diverse and unknown groups of organisms, endophytic fungus create symbiotic relationships with higher life forms and generate advantageous compounds for their hosts[12]. Nevertheless, the variety of endophytes and their capacity to generate bioactive chemicals have only been examined in a small number of plants. Numerous investigations into endophytic biodiversity, taxonomy, reproduction, host ecology, and their impact on hosts have been conducted[13]. Because they can grow in such a wide variety of odd conditions and occupy distinct biological niches, endophytes are currently regarded as an excellent source of bioactive natural compounds. There are two types of endophytic microflora: facultative and obligate. Only a portion of facultative endophytes' life cycles are spent inside plants; at other times, they may establish a relationship with the host plants' immediate rhizosphere soil by residing outside the plant. On the other hand, the obligatory strains remain within plants for the duration of their life cycles. Through vertical transmission, these microbes multiply throughout plant generations and modify plant products and metabolic processes to ensure their own survival. Plants are the bacteria' natural home since they coexist together to supply all of their needs. Endophytes, or endosymbiotic microorganisms that grow in plants, and microorganisms and their bioactive metabolites are important natural sources of potentially beneficial therapeutic compounds.

Table 1. Endophytic fungi isolated from different parts of indigenous medicinal plants

S. No.	Host Plant	Site of isolation	No. of samples	Fungi isolated
1	Neem (*Azadirachta indica*)	Leaf, Stem and Bark.	7	20
2	Jatropha (*Jatropha gossypiifolia*)	Leaf, Stem and Bark.	5	19
3	Tulsi (*Ocimum sanctum)*	Leaf, Stem and Bark.	6	10
4	Peepal (*Ficus religiosa)*	Leaf, Stem and Bark.	4	18
5	Eucalyptus (*Eucalyptus teriticornis*)	Leaf, Stem and Bark.	4	11
6	Geloy (*Tinaspora cordifolia*)	Leaf, Stem and Bark.	3	9
7	Chinese wisteria	Leaf, Stem and Bark.	3	-
8	Turmeric (*Curcuma longa)*	Leaf, Stem	5	13
9	Moringa (*Moringa oleifera*)	Leaf, Stem and Bark	6	15
10	Ashoka (*Saraca asoca*)	Leaf, Stem and Bark	8	18

Table 2. Microscopic characteristics of various endophytic fungi observed under compound microscope[18]

S. No.	Fungi isolated	Microscopic characteristics
1.	*Penicillium*	Colony colour green, *Penicillium's* vegetative structure is a multicellular mycelium. The long, septate, multinucleated, highly branching filamentous structures called hyphae make up the mycelium. At the branch end, spherical conidiospores—asexual spores produced exogenously—as well as conidiophores are present. The youngest conidium is found at the base of the series of conidia that are formed. The specialized cells in the group known as phialide produce conidia, which give the fungus a brush-like look. Ascospores are sexual spores generated internally that are located in asci organized in ascocarps.
2.	*Fusarium*	Moderate growth, peach, white, salmon pink, or violet. Various conidia are produced by *Fusarium* species. Macroconidia hyaline, curved, phragmospores, bearing a foot cell and some sort of heel; microconidia hyaline, 0-1 or septate, tiny. Additionally, chlamydospores may be present and carried on the macroconidia, terminally, or intercalary. Microconidia are generated on short, simple phialides and range in shape from oval to cylindrical or even curved and septate macroconidia, ranges 27–60 X 3–5 µm.
3.	*Aspergillus*	Colonies spread quickly, showing mycelium that is white, dark brown, black, or purple brown conidial heads. Conidiophores emerge from the substratum and are globose, radiate, smooth, and vesicle globose. In certain species, phialides are carried directly on the vesicles, but metulae are typically present and range in length from 10-15mm. Conidia are small, rough, and more or less globose, measuring 4-5 mm in diameter.
4.	*Colletotrichum*	Colonies are greyish white, with small thick felty patches and sparse aerial mycelium; conidial masses are salmon pink, and the reverse white to grey coloration occurs elsewhere. Acervuli are absent in certain cultures and the aerial mycelium has a copious grey-white color with inadequate sporulation. There are no sclerotia in any race. Septae are few. Falcate conidia with obtuse fusiform apices, measuring 15.5-26.5 X 4-5 µ. Sparse, medium-brown, clavate or circular appressoria with a complete margin, measuring 12.5-14.5 X 9.5-2.5µ.
5.	*Alternaria*	When fully grown, the fuzzy white mycelium has a pulvinate look, a central crater, and dark brown rings that alternate with light brown ones, with the white mycelium located at the periphery. A colony's diameter of 1.33 cm each day is noted. These fungus have conidiophores that are naturally pale brown in colour. The spore is 31.875 µm in length and 8.125 µm in width at its widest point. The substrate gives birth to the conidiophores. Compared to the primary conidia, the secondary conidia are shorter.
6.	*Nigrospora*	White colonies eventually turn brown or black due to a high rate of sporulation. Conidia solitary, acregenous, simple, spherical or broadly ellipsoidal, compressed dorsiventrally, black, shiny, smooth, septate, 10-16 µm diameter; conidiophores branched, flexous, colourless to brown, smooth.
7.	*Rhizopus*	Colonies are up to 10 mm high, dark greyish-brown, and produce simple rhizoids. Brownish in color, sporangiophores can grow up to 400 µm in height and 10 µm in width. They are typically generated in pairs or in groups of one to four. The round, greyish-black sporangia can have a diameter of up to 100 µm.
8.	*Verticillium*	Conidia, phialides, conidiophores, and septate hyaline hyphae are seen. Conidiophores can be simple, branching, or hyaline. Conidiophores branch at multiple levels in whorls (verticillate; like spokes in a wheel from a central axis). Phialides are carried by conidiophores. Phialides surround the conidiophore in verticils, or whorls, and are quite lengthy. Slide culture may cause disruptions to verticils.
9.	*Tricothecium*	Conidia, conidiophores, and septate hyaline hyphae are seen. Conidiophores are unbranched and lengthy. The conidia are carried by them. Conidia (8-10 x 12-18 µm) are smooth, hyaline to mildly pigmented, pear- or club-shaped, with slightly thick walls. Their conidiophore attachment site is noticeably truncated. They are arranged in an extended cluster, side by side. To generate a zigzag pattern at the tip of the conidiophore, they overlap.

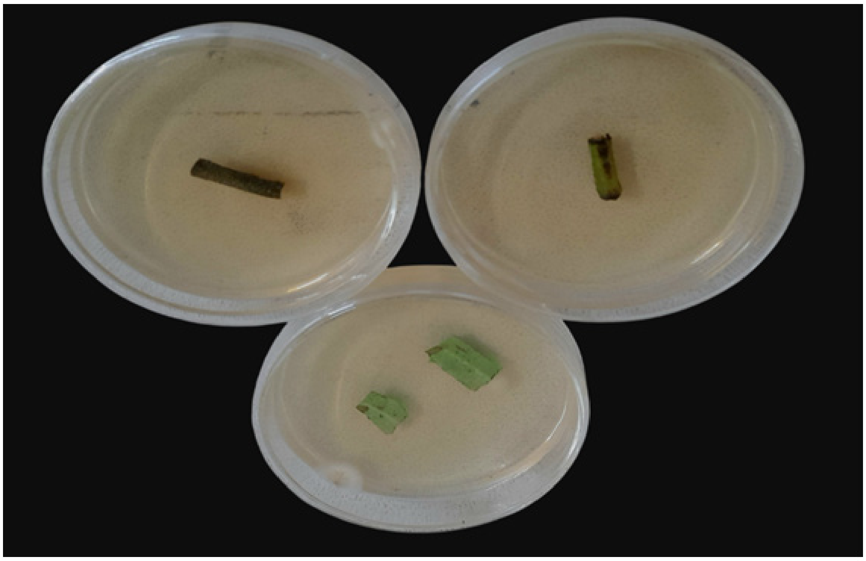

Fig. 1. Different plant parts (Bark, stem and leaves) of medicinal plants for the isolation of endophytic fungi

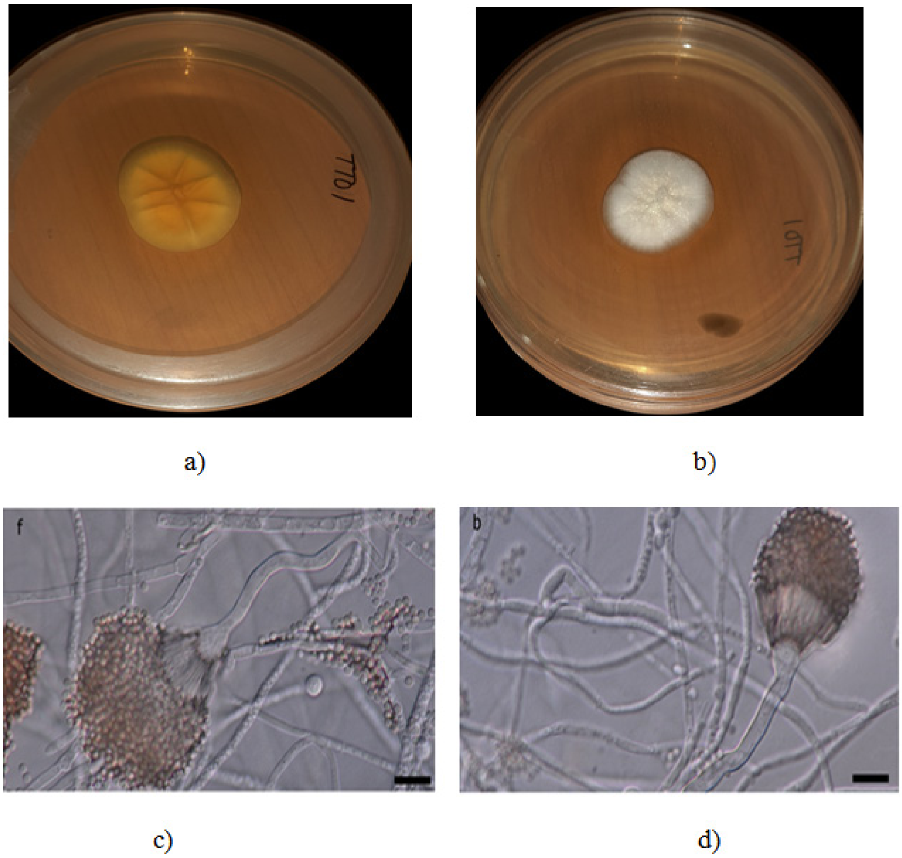

Fig. 2. (a, b) Macroscopic and (c, d) Microscopic morphological characteristics of *Aspergillus terreus*, the most abundant endophytic fungi isolated. (Microscopic images provided and Characterised from CSIR-Institute of Microbial Technology (CSIR-IMTECH), Chandigarh)

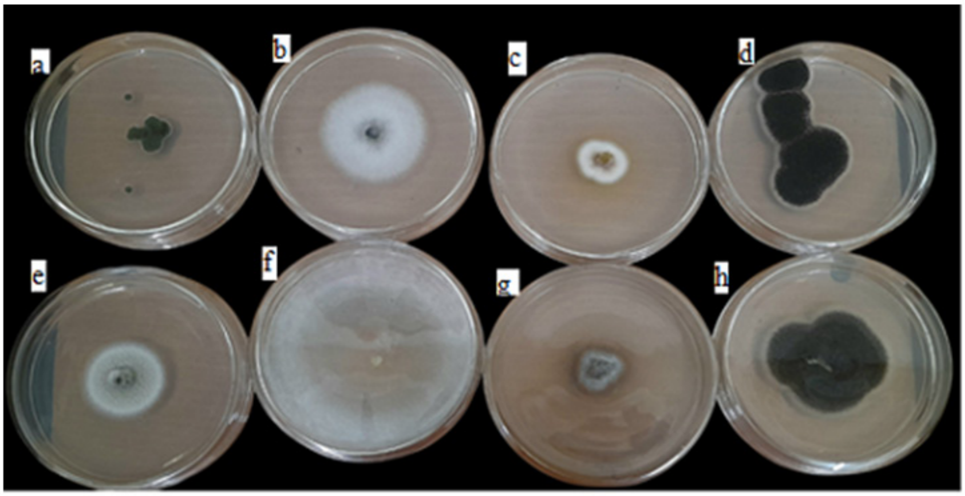

Fig. 3. Macroscopic images of endophytic fungi isolated from plants.(a-*Penicillium,* b-*Fusarium,* c-*Verticillium,* d-*Aspergillus,* e-*Colletotrichum,* f-*Nigrospora,* g-*Rhizopus, h-Alternaria*)

Endophytic fungi (EFs) are one of the endophytes that have garnered the most scientific attention due to their ability to produce biostimulants for the production of essential oils as well as new chemicals, antibacterial and antifungal substances, antioxidants, and anti-carcinogenic molecules. In addition to acting as biological control agents and strengthening plants' resistance to biotic and abiotic stress, they may increase nutrient solubilization in the plant rhizosphere, which would encourage host plant development.

In recent years, there has been a surge in scientific interest in comprehending, elucidating, and harnessing the plant/microbe interaction, as the potential benefits of endophytic fungal-plant associations have been recognized[14]. It is suggested that when a plant and an endophyte have a symbiotic relationship, the microorganisms and the plant biosynthesise the same biomolecule. Myrtucommulones, camptothecin, paclitaxel, and deoxypodophyllotoxin from *Myrtus communis, Taxus brevifolia, Camptotheca acuminata*, and *Juniperus communis* are a few examples of these. It is thought that this modification increases the microbe's chances of surviving in the plant tissues' microenvironment[15]. Endophytes occupy a distinct ecological niche where they balance commensal, parasitic, or mutualistic symbiosis with a host plant. This symbiosis is mostly controlled by chemicals, and endophytes produce distinctive metabolites. These organisms are being investigated more and more since they are essential to the creation of natural products, especially when the plant is important for pharmaceuticals. This is because the endophyte metabolites released within the plant tissue may also contribute to the plant's medicinal qualities, in addition to the plant's metabolome[16].

One of the main goals of recent developments in biotechnology is the search for novel bioactive compounds that may be extracted from fungal endophytes. Even so, only a very small portion of fungal endophytes have been identified and investigated[17, 18].

CONCLUSION

The present study demonstrated isolation of endophytic fungi from various indigenous medicinal plants. Neem plant exhibited the highest frequency of endophytic fungi and Chinese wisteria plant had none. The results indicated that the most abundant endophytic fungi found was *Aspergillus* spp. Current study also examined the colonies' average diameter, their boundaries, the mycelium's color, the reverse color of the petri dish, and the medium's color. The microscopic characteristics of isolated endophytic fungi were observed with lacto-phenol cotton blue wet mount method for characterization.

The hunt for further endophytes is currently ongoing and should continue because there are still a large number of them that have not yet been identified despite the identification of many fungal endophytes and the experimentation on their biomolecules. They may have more promise than we could have ever imagined. Furthermore, different endophytic fungi can be better manipulated through the use of cutting-edge technologies like genetic engineering, opening up previously undiscovered possibilities and providing additional insight into their undiscovered advantages.

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Conflict of interest

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Data Availability Statement

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Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials**.**

Author Contributions

Tamanna Tandon - Conceptualization, Collection of data and Writing the Original Draft; Tarun Kumar – Discussion and review; Dr. Pranay Jain* - Visualization, Supervision and Analysis of Data.

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