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Isolation And Characterisation of Active Fungal Endophytes from *Rhododendron Arboreum* with Superficial Disease-Causing Dermatophytes

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1.0 INTRODUCTION:

Medicinal plants can act as a reservoir of various bioactive molecules. The endophytes that reside in the various parts of the plants can act to be both healthy as well as asymptomatic. Since we know microorganisms are ubiquitous in nature and so are these endophytes (Hassan *et al*, 2017). These endophytes can be both bacterial as well as fungal. No plant species can be deprived of the presence of these endophytes. Endophyte was first brought to light by De Bary (Bacon *et al,* 2000). So, endophytes can be described as any microorganism that is present within a plant and is capable of causing any type of asymptomatic infection (De Azevedo *et al*, 2017).

If we talk about endophytic fungi, they live in the form of mycelium showing association with the plant, and that too at least for some amount of time. Usually, this endophytic fungus spends the whole or even a partial part of its life colonizing the tissues of the plants that are healthy. Several medicinal plants had already been used to isolate and identify and later on, characterize the various bioactive compounds that can be found in these plants. However, the paradigm had shifted to the discovery of different endophytic fungi that are present inside the plants and can produce similar compounds as that already available (Andra *et al*, 2016). The presence of bioactive compounds in these medicinal plants is gathering attention and thus it has led to extensive studies and research in this context.

Rhododendron arboreum is considered to be one of the most impressive species of the genus *Rhododendron.* It exhibits several characteristics it as having variable stature, being hard, having a vibrant colour, and also showing different leaf characteristics. Originally this plant was discovered in the northern central part of India and it is found in the Himalayan region ranging from Kashmir

to Bhutan and also covering the parts of the hills in Assam and Manipur. It usually grows at an elevation of 4500 to 10,500 ft and its flowering season ranges from March-April to June-September. The leaves of the plant are oblong-lanceolate and are generally crowded towards the ends of the branches. When the leaves are young, petioles are covered with lots of scales that are white in colour. The leaves are glossy green and deep impressions of veins can be seen from above (Ibrahim *et al,* 2020).

The leaves of *Rhododendron arboreum* usually report to have glucoside, ericolin, quercetin,epifriedelinol and hyperoside. The chemical analysis of these leaves reported the presence of hyperoside, ursolic acid, and epifriedelinol which are terpenoid compounds. These leaves have been also reported to contain flavone glycoside and dimethyl ester of terephthalic acid and certain other flavonoids.The majority of endophytic fungi can be isolated from various medicinal plants so they can produce either novel bioactive compounds or even anti-microbial. Therefore, in the current study, we selected two of the most important medicinal plants, i.e.,*Rhododendron arboreum* and *Aegle marmelos* for study and thus isolated a good number of endophytic fungi which were distributed unevenly in the different parts of the plants, specifically leaves and stems of both the plants.All the fungal endophytes that colonize inside the plant tissues tend to get nutrition as well as protection.

Currently, many fungal infections are regarded as among the most challenging and incurable illnesses in people. Regarding this, numerous investigations have also highlighted the high rates of morbidity and mortality brought on by these fungal infections [19]. Despite this, the general public and health professionals typically ignore them. One of the human body's largest organs is thought to be the skin. Furthermore, it is regarded as the first line of defence or barrier against both the environment and harmful microbes. Dermatophytosis, which affects 20–25% of the healthy population, is thought to be the most prevalent skin illness in humans. David Gruby made the initial discovery of dermatophytes in 1841. Even though dermatophytes exist in the human host, there is limited research on the subject. This is due, in large part, to the fact that skin infections are typically superficial and are therefore treated more as cosmetic issues than as serious medical conditions. All of these dermatophytes are therefore treatable with the majority of antifungals that are prescribed regularly. Typically, filamentous fungi are the source of this kind of infection. Thus, hyphae—which invade the skin, hair, and nails and can result in either acute or chronic infection—define dermatophytes. Five genera—Trichophyton, Epidermophyton, Microsporum, Nannizia, and Arthroderma—can be used to group these dermatophytes. These dermatophytes can produce an infection known as an annular lesion is regarded as the most prevalent one.

Worldwide, dermatophytosis is thought to be caused by both non-dermatophytic and dermatophytic types of fungi. However, it is prone to demonstrate and govern these divergences due to many factors such as climate, location-specific characteristics, and others [14]. Humans are now more vulnerable to a class of pathogenic fungi as well as to all fungi that are thought to be a source of contamination or act as contaminants, as evidenced by the abrupt rise in chronic diseases such as diabetes mellitus that has been linked to an increase in life expectancy among the general population and other underlying factors [15]. The different types of dermatophytic infections involve- [17]

(a) Tinea capitis- Dermatophytosis of hair and scalp
(b) Tinea corporis- Lesion affecting the body
(c) Tinea manuum- infection of one or both hands
(d) Tinea unguium- infection in the nail or onychomycosis
(e) Tinea pedis- infection in the foot

(f) Tinea cruris- dermatophytosis of groin

The present study was thus conducted to isolate and identify different types of endophytic fungi in neem leaves along with their antagonistic and synergistic activity against pathogenic dermatophytes.

2.0 METHODOLOGY:

2.1 Collection of Plant Samples:

i] (*Rhododendron arboreum)*: Fifteen (15) symptomless leaf samples were collected from Dharmshala (different areas), Himachal Pradesh in the month of September 2022. All these samples were collected in clean sterilised paper bags and to keep them fresh, the bark of the leaves was inserted in cut pieces of sponges which were properly dipped in water also small punctures were made in the clean paper bags so that proper aeration takes place. All these samples were then brought to the Student Research Laboratory of Sharda School of Allied Health Sciences, Sharda University, Proper Sterilisation (Initial step):

Proper surface sterilization of the leaves as well as stems was done by washing them with running tap water for 10 mins. Excessive dirt and debris weretried to remove with the running water. These leaves and stems were then allowed to air dry for some time.

2.2 Preparation of Media & Autoclaving:

To isolate these endophytic fungi from the plant sample, Potato Dextrose Agar (PDA) media was prepared. Prepared media along with the petriplates were then autoclaved for 45 minutes at 121℃.

2.3 Isolation of endophytic fungi from *Rhododendron arboreum*:

The frequently used method to isolate and later on identify the fungal endophytic fungi is proper surface sterilization. The surface sterilization is properly done by using distilled waterto minimize the microbial load from the sample surface and 70% ethanol. The leaves are properly dipped first in distilled water and kept for almost 5 minutes. Then the leaves are allowed to air dry for some time. Then the leaves are dipped in 70% ethanol for around 3-5 minutes. Again, they are allowed to air dry. Following this, the leaves are once again dipped in distilled water for almost 5 mins and they are allowed to air dry properly. The surface sterilization was done with sodium hypochlorite (NaOCl) and 75% ethanol and by using autoclaved double-distilled water for rinsing. All these steps are carried forward in a Laminar air flow chamber. Then with the help of a sterilized blade, the leaves are cut into small pieces. The efficiency of the surface sterilization is done for each segment of the tissue following the imprint method (Lacava *et al* 2022).

The cut leaves segments are then inoculated onto the medium of Potato Dextrose Agar (PDA) in a Petri dish. These plates were incubated for 4-5 days at 25℃ in an incubator having proper humidity control (Machado *et al*, 2020). These plates were then sealed properly with parafilm to avoid desiccation of the medium and any kind of contamination. After this, the isolation of endophytic fungi was done by transfer of hyphal tips to fresh potato dextrose agar plates to obtain pure cultures for their identification.

2.4 Preservation of endophytic fungi:

These purified fungal isolates were then aseptically transferred separately to the PDA slants with proper labelling at 4℃.

2.5 Identification of endophytic fungi:

All the isolated strains were then properly identified. Identification of these strains was done with the help of lactophenol cotton blue staining and then they were observed at 40x under microscope.

2.6 Collection of Dermatophytes samples:

A total of 20 nail, skin, and hair isolates were procured from the Department of Mycology, Sharda School of Medical Science and Research (SMSR), Sharda Hospital, Greater Noida, Uttar Pradesh, and with procured MTCC strains. These samples were properly collected and preserved.

2.7 Media Preparation (Dermatophytes):

The collected and preserved dermatophyte isolates were then grown on Potato Dextrose Agar (PDA) and Sabaraud's Dextrose Agar (SDA). Before growing them on media, Petri plates and media were autoclaved at 121°C for 45 minutes. After the autoclave, the plates were poured with both media, and the collected isolates were streaked and spread on both media. The plates were then incubated for about 72 hrs at 27 °C.

The growth was checked every 24 hrs and after 72 hrs, proper growth was observed on the Petri plates. These plates were then properly sealed with parafilm and covered with aluminium foil. These plates are then stored in the refrigerator before further investigation.

2.8 Identification of isolated dermatophytes:

The isolated dermatophytes were then identified with the help of lactophenol cotton blue staining (LPCB). Clean glass slides were taken. With the help of a sterile loop or forceps, the dermatophyte's fungal hyphae were transferred to a clean glass slide and were properly teased to break them (Rouzaud *et al,* 2018). After they were properly teased, a small drop of lactophenol cotton blue stain was added and it was then covered with a coverslip. The prepared slides were then observed under a microscope at 40x. The identification of all the isolated dermatophytes was done based on their morphological, and taxonomic features (Ardakani *et al*, 2016).

2.9 Colonizing Frequency of Plant Endophytes:

The colonizing frequency of each fungal endophyte was calculated as per Suryanarayanan *et al.* (2003).

$$CF\ (\%) = \frac{\text{Number of plant segments colonized by a single fungus}}{\text{The total number of plant segments observed}} \times 100$$

2.10 Antifungal Susceptibility Test:

Four antifungal drugs were used in the antifungal susceptibility testing: Itraconazole (8µg/ml), Terbinafine(16µg/ml), Griseofulvin (256µg/ml), and Fluconazole (256µg/ml). After being suspended in sterile tubes, the mixture was given 30 minutes to settle. Equal swaths of swabs dipped into the inocula suspensions were applied to the surface of Mueller-Hinton Agar (Alotibi *et al*, 2020).

To allow any excess surface moisture to be absorbed into the agar, lids were left unkept in a laminar flow cabinet for three minutes before applying drug-impregnated discs

For five to ten days, the plates were incubated at 25°C. The size of the zones of inhibition surrounding the discs was measured and noted during the development phase. Measurements of antifungal agent susceptibility and resistance criteria were made.

3.0 RESULT AND DISCUSSION:

3.1Collection of Samples:

12 samples were collected from different locations in Himachal Pradesh.

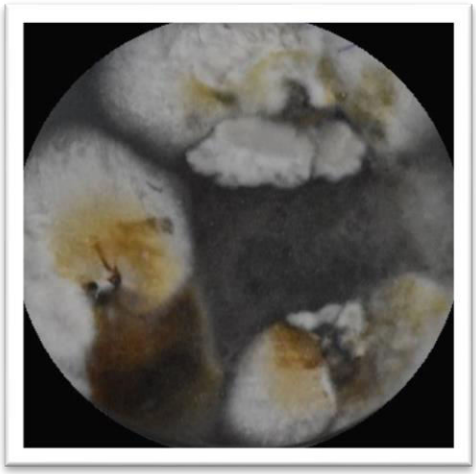
3.2 Isolation and characterization:

Endophytes were isolated on Potato Dextrose Agar. These isolated endophytic fungi were later screened for identification and characterization based on their morphology, conidial appearance, hyphae and arrangement of mycelium.

Table 1- Identification and characterization of endophytic fungi

Location	Number of Isolates	Species
Dharmshala (Location 1)	3	*Muscodor spp.* (1), *Ulocladium spp.* (1), *Alternaria spp.*(1)
Dharmshala (Location 2)	6	*Muscodor spp.* (1), *Ulocladium spp.* (1), *Alternaria spp.*(1), *Fusarium spp.* (3)
Dharmshala (Location 3)	2	*Muscodor spp.* (1), *Fusarium spp.* (1)
Dharmshala (Location 4)	3	*Alternaria spp.* (1), *Fusarium spp.* (1), *Ulocladium spp.* (1)
Dharmshala (Location 5)	3	*Fusarium spp.* (1), *Muscodor spp.* (1), *Ulocladium spp.*(1)

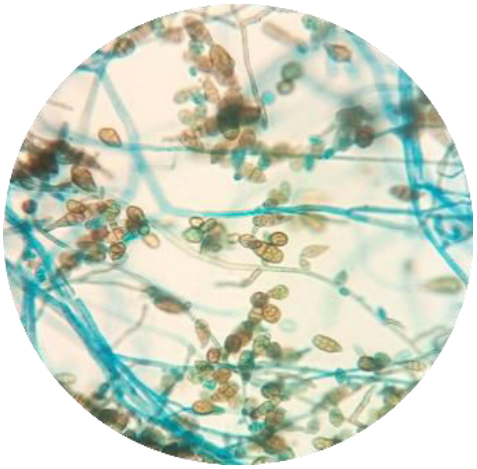
The maximum number of endophytic fungi which were isolated from all five locationswas*Fusariumspp.*(6), *Muscodor spp.* (4),and*Ulocladiumspp. (*4) followed by*Alternaria spp.* (3)being the intermediate number of endophytic fungi which were isolated from the six locations.



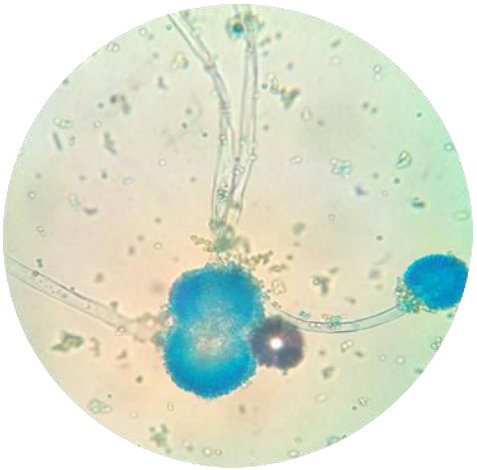
(a)

(b)

Figure 1: (a) This shows the endophytic growth of *Fusarium spp.* (b) This shows the endophytic growth of *Muscodor spp.*



(a)



(b)

Figure 2: (a) This shows the microscopic view of *Alternaria spp.*
(b) This shows the microscopic view of *Muscodor spp.*

3.3Calculation of Colonization Factor:

Table 2: Calculating the colonization factor percentage

Location	Part of Plant	CF %
Location 1	Leaves	30 %
Location 2	Leaves	60 %
Location 3	Leaves	20 %
Location 4	Leaves	30 %
Location 5	Leaves	30 %

The highest colonization frequency was observed by Location 2 (60%), followed by Location 1, Location 4, and Location 5 (30%). The lowest value was observed by Location 2 (20%).

3.4 Isolation and characterisation of dermatophytes:

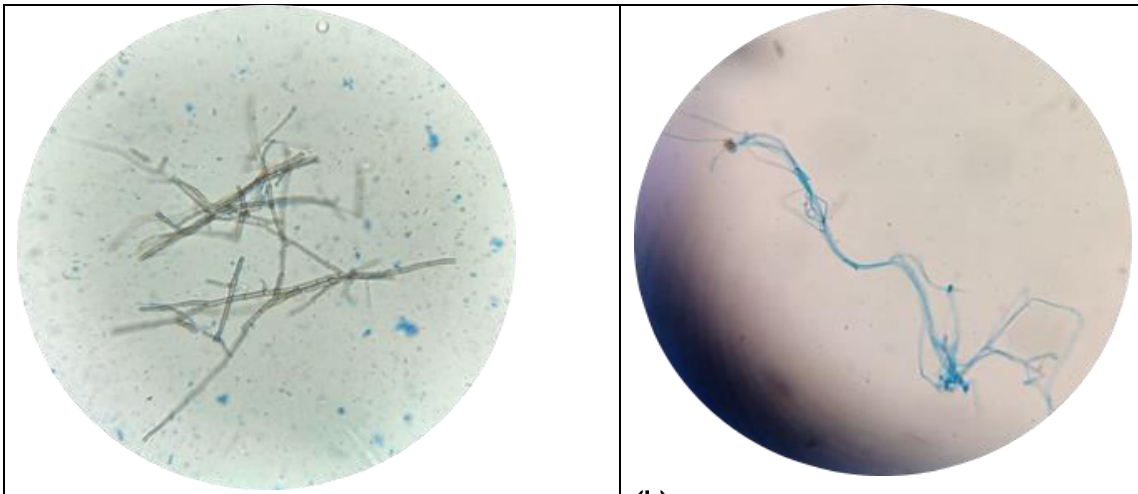
A total of 9 species of dermatophytes were isolated and identified from the clinical samples and were further tested against the isolated endophytes.

The isolated species are as follows-

1. MTCC 96- *Trichophyton rubrum* [VD1]
2. MTCC 80- *Epidermophyton floccosum* [VD2]
3. MTCC 19- *Microsporum gypseum* [VD3]
4. S7- *Trichophyton spp.* [VD4]
5. S9- *Trichophyton spp.* [VD5]
6. S6- *Trichophyton spp.* [VD6]
7. S2- *Epidermophyton spp.* [VD7]
8. S3- *Microsporum spp.* [VD8]
9. S8- *Trichophyton mentagrophytes* [VD9]

(a) (b)

Figure 3: (a) This shows the dermatophytic growth of *Trichophyton mentagrophytes spp.* (b) This shows the dermatophytic growth of *Microsporum spp.*



(a) (b)

Figure 4: (a) This shows the microscopic view of *Microsporum spp.* (b) This shows the microscopic view of *Trichophyton spp.*

3.3 Sensitivity pattern of endophytes against pathogenic dermatophytes:

Table 3: The overall sensitivity pattern of fungal endophytes against the isolated dermatophytes

Endophytes	Dermatophytes	Dermatophyte growth	Endophytic growth
Fusarium (6)	*Trichophyton spp.*	--	++++
Ulocladium (4)	*Trichophyton mentagrophytes*	--	+++++
	Microsporum spp.	++++	--
Alternaria	*Trichophyton spp.*	+	+
	Microsporum spp.	++++	--
Muscodor (4)	*Trichophyton spp*	--	+++++
	Microsporum spp.	++++	---

Fusarium spp. was effective in inhibiting the growth of *Trichophyton spp.* Also, *Ulocladium spp*. inhibited the growth *of Trichophyton mentagrophytes.Muscodor spp.* too inhibited the growth of *Trichophyton spp.*

4. CONCLUSION:

In the current scenario, endophytes are considered to be one of the less valued groups of microorganisms that can synthesize various bioactive compounds which can in return be used to combat various pathogens. They are also considered to be very useful for drug discovery. Numerous bioactive chemicals, such as those that are antibacterial (vanillin, essential oils), antifungal and antiviral (alkaloids), antioxidant (eugenol), anti-inflammatory (cineole), etc., can be produced via biotransformation techniques. In this research work, some of the isolated fungi were effective against some strains of pathogenic dermatophytes. In this regard, the effective species can be later on used as a bioactive formulation.

In the present study, the sensitivity pattern of endophytes was observed against pathogenic dermatophytes and the inhibition pattern shows valuable results which provide evidence forutilizing endophytes as totals for biological control against dermatophytic fungi. The present study showed that the formulation of volatile compounds of those fungi can be used as an anti-dermatophytic agent in the future, which can prove as a novel discovery in the pharmaceutical industry. They can thus be used in standard herbal preparation for the betterment of human society.

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