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Isolation and Characterisation of Active Fungal Endophytes from *Aegle marmelos* and their relation to communicable skin infections

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A B S T R A C T

The rising threat of communicable fungal skin infections, particularly dermatophytosis, has intensified the demand for innovative and effective therapeutic strategies. Silver nanoparticles (AgNPs) have emerged as potent antimicrobial agents with broad-spectrum activity, including against pathogenic fungi. However, conventional methods of synthesising AgNPs often engage noxious chemicals, causing major environmental and health risks. In response, green synthesis using biological organisms, particularly endophytic fungi, has gained momentum as a sustainable and eco-friendly alternative. Endophytic fungi, which reside asymptomatically within plant tissues, possess remarkable metabolic capabilities, including the ability to reduce silver ions into stable nanoparticles. This study explores the potential of fungal endophytes and their application in the treatment of fungal skin infections. By harnessing the natural biosynthetic machinery of these fungi, it is possible to develop biocompatible nanoparticles with enhanced antifungal efficacy. The research aims to contribute to the development of safer, greener, and more effective nanotechnological approaches for managing dermatological fungal infections.

Keywords: Endophytes, dermatophytes, communicable diseases, plant tissues

Introduction

In recent years, considerable interest has arisen in developing eco-friendly and sustainable methods for synthesising metal nanoparticles. Silver nanoparticles, among the many metal nanoparticles, have been the subject of extensive research owing to their unique physicochemical characteristics, which include antimicrobial, anti-inflammatory, and wound-healing properties. This makes them a promising candidate for a range of biomedical applications. Traditional chemical and physical approaches to silver nanoparticle synthesis often require substantial energy and financial resources, and they often rely on toxic substances. These practices carry the risk of negative effects on health and the environment.[1] Fungal endophytes have been identified as a great source for producing silver nanoparticles and are known to exist within plant tissues without appearing to cause any harm. These fungi can convert silver ions into metallic silver, which produces nanoparticles with distinct sizes and shapes. Furthermore, because endophytic fungi are easily cultivated, widely accessible, and don't require complicated extraction or purification procedures, using them to synthesise nanoparticles has several benefits.

Fungal endophytes, residing within plant tissues without causing visible harm, are a ubiquitous and often overlooked facet of plant biology. Far from being passive inhabitants, these microscopic fungi engage in complex interactions with their hosts, influencing a wide array of plant processes, from growth and development to defence against pathogens and herbivores. Despite their inconspicuous nature, the pervasive influence of fungal endophytes demands a thorough understanding of their diversity, ecology, and evolutionary history.[2] This review delves into the multifaceted world of fungal endophytes, exploring their roles as hidden drivers of plant health, resilience, and ecological interactions. This vast and often overlooked group of fungi plays a crucial role in plant ecology and evolution, shaping plant-microbe interactions, and community dynamics, and even influencing the success of invasive species. The intimate association between endophytes and their hosts has sparked a burgeoning field of research, unveiling the multifaceted nature of these symbiotic relationships. One of the primary areas of interest lies in understanding the mechanisms by which endophytes colonise their hosts. While traditional methods for detecting endophytic fungi rely on invasive techniques such as direct isolation or molecular detection, recent advancements have explored non-invasive approaches. The impact of endophytes on plant physiology and defence has garnered significant attention. Remarkably, the presence of specific endophytes enhanced the growth of adapted neighbours while suppressing the growth of evolutionarily naive neighbours, highlighting the intricate role of endophytes in shaping plant community dynamics. Beyond their ecological implications, endophytic fungi have emerged as a promising source of bioactive compounds with potential applications in agriculture and medicine. This finding underscores the potential of endophytes as biocontrol agents and sources of novel antimicrobial compounds.[3]

Aegle marmelos, also known as "Bael", belongs to Rutaceae. It has the potential to play a vital role in primary medical care. It has been in use for over 5000 years by various ethnic populations that live in the Indian subcontinent. The Indian traditional medicine system has the potential to cure many ailments and also has various bioactive compounds present in it. Several components are used for their various medicinal properties, like anaemia, wound healing, swollen joints, etc. They are reported to have chemical compositions like alkaloids, coumarins, terpenoids, etc. This plant can also cure various fungal infections.[4]

At present, numerous fungal infections are considered to be some of the most difficult and untreatable diseases in humans. On this issue, many studies have pointed out the elevated morbidity and mortality rates caused by these fungal infections. The skin is believed to be one of the largest organs in the human body. Moreover, it is considered the first line of defence or barrier against both environmental factors and harmful microbes. It is believed that dermatophytosis, which impacts 20–25% of healthy individuals, is the most common skin disease among humans. As evidenced by the rapid rise in chronic diseases like diabetes mellitus linked to increased life expectancy and other underlying factors, humans are now more susceptible to a class of pathogenic fungi and all fungi considered sources of contamination or contaminants.[5]

In this research paper, we will highlight how various fungal endophytes can be beneficial in combating communicable fungal skin infections. Thus, they can pose to help in treating them with the help of naturally isolated endophytic fungi.

Methodology

Collection of plant samples

(Aegle marmelos): Ten (10) symptomless leaf samples were collected from CSIR-IIM, Jammu & Kashmir & local areas of Jammu in January 2023. All of these samples were then collected in clean and sterilised bags, and to keep the leaves fresh, the bark of the leaves was inserted into wet sponges. Also, these bags were punched with small holes for proper aeration. Later on, these samples were brought to the place of experiment.

The surface of the leaves and stems was properly sterilised. They were washed with running water for almost 10 minutes to eradicate dirt and debris. They were then allowed to air dry for another 15 minutes.[6]

Media Preparation and Autoclaving

Potato Dextrose Agar [PDA] was prepared to isolate and grow fungal endophytes from the plant. The prepared media and Petri plates were autoclaved for 45 minutes at 121.

Isolation of fungal endophyte from *Aegle marmelos*

The surface sterilisation of leaves was done with purified water to lower the microbial load from the sample. It was also washed with 70% ethanol. The plant sample (leaves) was first dipped properly in distilled water and kept like that for 5 minutes. Later, the leaves were allowed to air dry. These leaves were then dipped in 70% ethanol for approximately 3-5 minutes, followed by air drying. The leaves were again dipped in distilled water for 5 minutes and were then allowed to air dry. The surface of the leaves

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was sterilised with sodium hypochlorite (NaOCl) and 75% ethanol. These steps were carried out in a laminar air flow chamber. With the help of a sterilised blade, leaves were cut into small pieces.

These cut leaves are then inoculated into the media (PDA). The plates were incubated at 25 degrees Celsius for 4-5 days. The plates were then sealed with parafilm to avoid desiccation and contamination. Later, hyphal tips were transferred to fresh media to obtain pure cultures for further identification.[7]

Preservation of isolated endophytes

The purified fungal isolates were aseptically transferred separately to potato dextrose slants at 4 degrees Celsius.

Identification of fungal endophytes

All the isolated strains were identified with the help of lactophenol cotton blue staining and were observed under 40x resolution.

Colonising Frequency of Plant Endophytes

The colonising frequency of each fungal endophyte was calculated –

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

The total number of plant segments observed

Collection of dermatophyte samples

A total of 15 skin and nail isolates were procured from Doon Hospital, Dehradun. These samples were then properly preserved[8].

Media Preparation (Dermatophytes)

The dermatophyte isolates that were collected and preserved were subsequently cultivated on Potato Dextrose Agar (PDA) and Sabouraud's Dextrose Agar (SDA). Petri plates and media were autoclaved at 121°C for 45 minutes before growing them on media. Following the autoclave process, the plates were filled with both types of media, and the collected isolates were streaked and spread onto both media. After that, the plates were kept at 27°C for approximately 72 hours.

Growth was monitored every 24 hours, and after 72 hours, appropriate growth was noted on the Petri plates. The plates were subsequently sealed with parafilm and covered with aluminium foil. Subsequently, the plates are kept in the refrigerator until further examination.[9]

Identification of isolated dermatophytes

The isolated dermatophytes were identified using lactophenol cotton blue staining (LPCB). Slides made of glass that were cleaned beforehand were taken. Using a sterile loop or forceps, the fungal hyphae of the dermatophyte were moved to a clean glass slide and carefully teased apart to break them (Rouzaud et al; 2018). Once they were adequately teased, a small amount of lactophenol cotton blue stain was applied, and the specimen was covered with a coverslip. The slides that had been prepared were subsequently examined under a microscope at 40x. All the isolated dermatophytes were identified based on their morphological and taxonomic characteristics[10]

Results and discussion

Collecting samples

10 samples were collected from different locations in Jammu & Kashmir[11-12].

Isolation and characterisation

Endophytes were isolated on potato dextrose agar. Proper identification and characterisation of these isolated endophytes were carried out. The identification was done based on the appearance, morphology, hyphae, and mycelial arrangement[13].

Various strains of endophytic fungi that were isolated from different locations: *Alternaria spp.* (3), *Aspergillus* (2), *Fusarium* (2) *Ulocladium* (1), *Muscodor* (1), *Rhizopus* (1)15.

Antifungal Susceptibility Test

Four antifungal medications were utilised in the antifungal susceptibility testing: itraconazole (8 µg/ml), terbinafine (16 µg/ml), griseofulvin (256 µg/ml), and fluconazole (256 µg/ml)[16-17].

Calculation of Colonization Factor

The highest colonisation frequency was observed at Location 3 (60%), followed by Location 1, Location 2, and Location 5 (30%). The lowest value was observed by Location 4 (20%)[18-19].

Table 1.Identification and characterization of endophytic fungi

Location	Number of Isolates	Species
Jammu & Kashmir (Location 1)	2	*Alternaria* (1), *Aspergillus* (1)
Jammu & Kashmir (Location 2)	3	*Alternaria* (1), *Muscodor* (1), *Fusarium* (1)
Jammu & Kashmir (Location 3)	2	*Alternaria* (1), *Aspergillus* (1)
Jammu & Kashmir (Location 4)	3	*Fusarium* (1), *Ulocladium* (1), *Rhizopus* (1)

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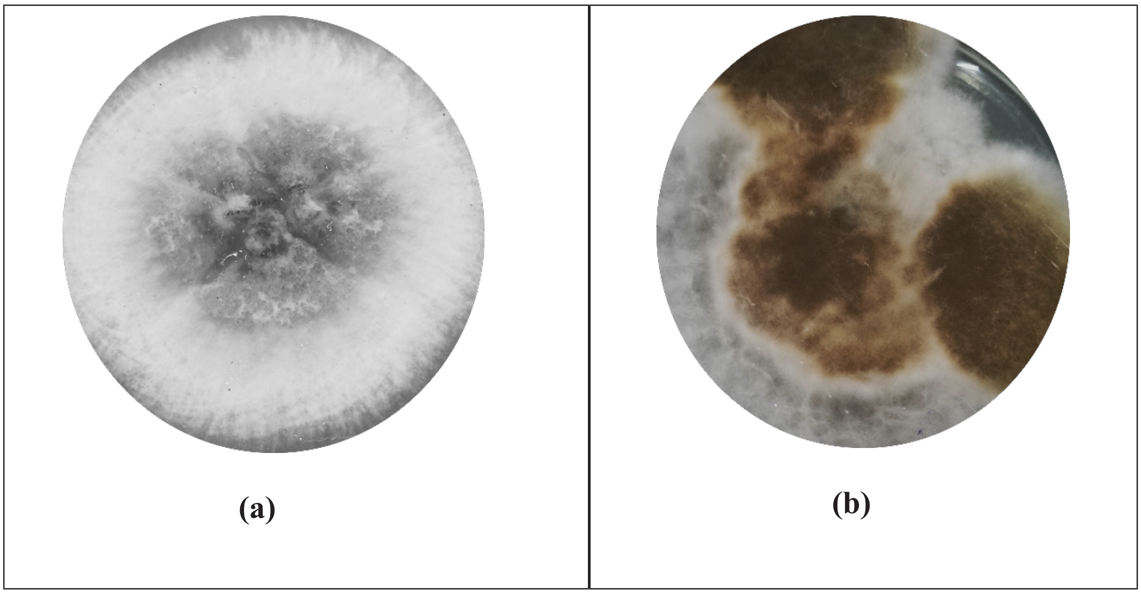

Figure 1. (a) This shows the endophytic growth of *Alternaria* (b) This shows the endophytic growth of *Aspergillus*

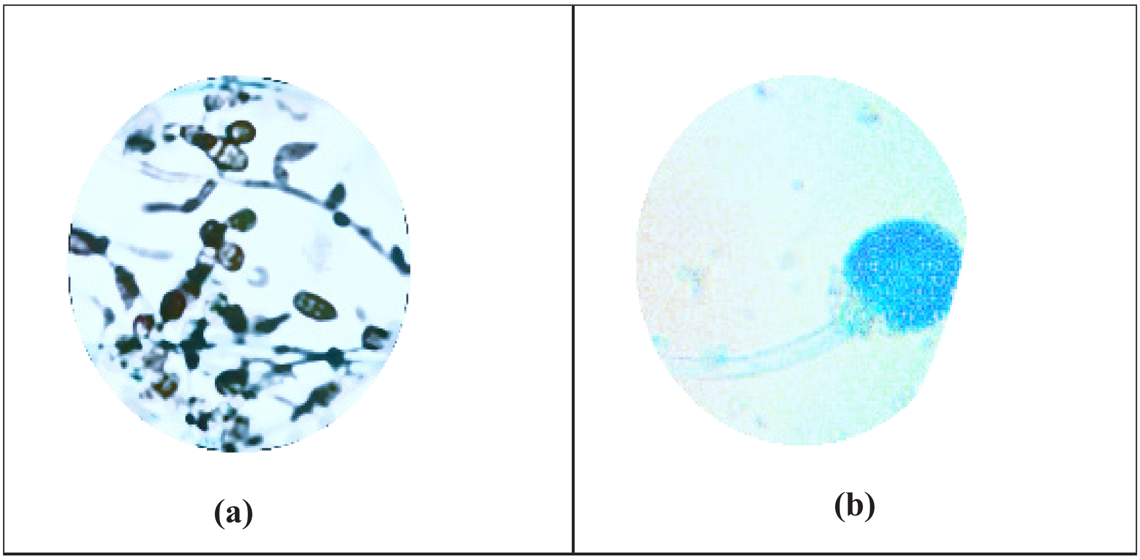

Figure 2.(a) This shows the microscopic view of endophytic growth of Alternaria spp. (b) This shows the microscopic view of endophytic growth of Aspergillus spp.

Figure 3.Plates showing antifungal susceptibility

Table 2.Identification and characterization of endophytic fungi

Location	Part of Plant	CF %
Location 1	Leaves	40 %
Location 2	Leaves	30 %
Location 3	Leaves	60 %
Location 4	Leaves	20 %
Location 5	Leaves	30 %

Activity of endophytes against dermatophytes (communicable skin infections)

A total of 9 species of dermatophytes were isolated and identified from the clinical samples and were further tested against the isolated endophytes. Fungal endophytes like *Muscodor spp*. inhibited the growth of dermatophytes[20].

Conclusion

This study's results demonstrated that various endophytic fungi occupy an ecological niche in the natural regions inhabited by the medicinal plant *Aegle marmelos*. As a result, 10 fungal strains were identified by ITS sequence analysis after being isolated from healthy *Aegle marmelos* leaves. These endophytes exhibited a range of actions that encouraged plant growth. Among these, the ability to enter tissues is provided by making various interstitial lytic enzymes, including pectinase, amylase, cellulase, and catalase. Additionally, the fungal strains showed activity against many pathogenic microorganisms, potentially enhancing plant tolerance to infections.

The various endophytes isolated from several ethnomedicinal plants can exhibit scavenging effects at different levels. It is seen that the most effective strains can be exploited as a reservoir of novel bioactive molecules. Later on, this research can help focus on various other associated factors. At this point, we have more precise knowledge about things like this group of fungi's enormous diversity, where they are found in different plant tissues and compartments, how they vary seasonally, and how the structure & composition of the population of endophytes vary and evolve along with their host plants. These endophytic fungi can help in combating various communicable skin infections, which can pose a serious problem to human society.

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