



Differential Antimicrobial Activity of Endophytes in the External vs. Internal Bark of Neem (*Azadirachta indica*): Isolation, Characterization, and Bioactive Metabolite Analysis

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Antagonistic effect, Fungal metabolites, Plant -microbe interaction

Abstract

Introduction: Endophytic fungi are beneficial microbes living harmlessly within plants and producing useful bioactive compounds. *Azadirachta indica* (Neem) has strong antimicrobial properties, but the endophytes inside its inner and outer bark are not well explored. This study compares their antimicrobial activity to identify their potential benefits.

Methods: Healthy neem bark was collected, surface sterilized, and separated into outer and inner sections. Endophytic fungi were isolated using the tissue segment method and cultured on Potato Dextrose Agar. After microscopic morphological identification, fermentation was carried out in nutrient broth, and secondary metabolites were extracted using ethyl acetate. Antimicrobial activity of these extracts was evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* using the agar disc diffusion method, with Fosfomycin as the positive control.

Results: The fungal endophytes, *C. cladosporioides* (outer and inner bark) and *P. marneffeii* (inner bark) were identified. The inner bark isolate *P. marneffeii* showed the highest antimicrobial activity, particularly against *P. aeruginosa* and *K. pneumoniae*. In comparison, the *C. cladosporioides* isolate from the outer bark exhibited moderate inhibition, especially against *S. aureus* and *P. aeruginosa*.

Conclusion: Internal bark endophytes of neem showed stronger antimicrobial activity than outer bark isolates, suggesting that deeper tissues harbour more potent and stable endophytic associations. This indicates neem endophytes may serve as promising sustainable sources of novel antimicrobial compounds, aligning with Ayurvedic bioprospecting concepts. However, since this study involved isolates from only one tree, further research on larger samples from varied ecological regions is needed to confirm broader applicability.



1. Introduction

Endophytes are hidden microbes living inside plants without causing harm. They form a mutually beneficial relationship with the host. Even though endophytes may have existed with plants for a very long time, their scientific recognition and study began only recently. [1] The term endophyte was first introduced by de Bary (1866) to differentiate them from epiphytes, which inhabit the plant surface. [2]

Endophytes are classified into different categories, such as endophytic *Clavicipitaceae*, fungal endophytes of dicots, systemic fungal endophytes, endophytes of lichens, those present in bryophytes and ferns, fungi colonizing tree bark, xylem, roots, galls, and cysts, and prokaryotic endophytes, such as bacteria and actinomycetes. [3] They obtain protection and nutrients from the host, while in return, they enhance nutrient uptake and confer resistance against various biotic and abiotic stresses.

These are not only support plant growth but also protect plants from different stresses. They help plants survive extreme conditions like hot springs, produce toxic alkaloids in grasses that stop herbivores, and protect dicot plants from pests. [4] Their interaction with pathogens depends on the host and may suppress pathogen growth, change nutrient balance, or activate host defence mechanisms. [5-6] Many endophytes produce antibiotics or antifungal compounds which reduce disease severity. [7] Some also give resistance against nematodes and insects by releasing toxic metabolites. [8] Endophytes are still a less explored part of biodiversity. They have been found in leaves, stems, bark and roots of many woody plants in temperate and tropical regions. [9-11] Many endophytes also protect plants from insect damage and act as potential sources of new antimicrobial compounds. [12]

Azadirachta indica (Neem) is an evergreen tree widely cultivated in India and commonly known as Indian lilac or Margosa. It holds major importance in Ayurveda for its strong antimicrobial properties. [13] Its broad therapeutic potential was also recognized globally, as highlighted in the 1992 U.S. National Academy of Sciences report *Neem: A Tree to Solve Global Problems*. [14] Every part of the neem tree holds medicinal value, giving it great economic importance. In Ayurveda, neem is described as *Sita* (cold in potency), *Laghu* (light to digest), *Grahi* (absorbent), and *Katu-paka* (pungent in

post-digestive effect). It stimulates the digestive fire, reduces *Vata*, and although considered *Ahridya* (unpalatable), it is highly beneficial in relieving *Srama* (fatigue), *Trit* (excessive thirst), *Kasa* (cough), *Jvara* (fever), *Aruci* (anorexia), and *Krimi* (intestinal worms). It is also indicated in the management of *Vrana* (wounds), *Pitta* and *Kapha* disorders, *Chardi* (vomiting), *Kushta* (skin diseases), *Hrillasa* (nausea), and *Meha* (diabetes). [15] Pharmacological studies over the past two decades have validated its antimicrobial, antipyretic, anti-inflammatory, and dermatological benefits, including its effectiveness against eczema, burns, ulcers, and herpes. [16-17]

Gap and Need of the Study

Despite remarkable progress in modern medicine, major global health concerns such as cancer, diabetes, cardiovascular diseases, and antimicrobial resistance continue to pose significant challenges. [19,20] Medicinal plants remain a cornerstone of drug discovery, contributing nearly 40% of modern pharmaceuticals due to their diverse bioactive compounds and relatively fewer side effects. [21] *A. indica* (Neem) is one such important medicinal plant. However, previous research on Neem shows notable limitations. Most studies have concentrated primarily on the leaves, using crude extracts without isolating or characterizing individual endophytic fungi. [22,23] A few studies have investigated endophytic fungi in leaves, with minimal emphasis on exploring those residing in the bark. [24] Furthermore, some studies used whole-plant extracts to evaluate antimicrobial activity without identifying metabolites from bark-associated endophytes. [25] Additionally, some reports offered only generalized insights into Neem's antimicrobial potential. [26] Although Neem bark harbours diverse endophytic microbes, its layer-specific communities remain poorly explored, with most studies treating the bark as a single uniform tissue and overlooking variations between the layers in microbial diversity and metabolite production.

Need of the Study

There is a need to comparatively evaluate endophytes from the external and internal bark of neem to determine how spatial compartmentalization affects microbial diversity and metabolite production. Exploring these distinct bark layers may reveal unique bioactive



compounds and enhance targeted therapeutic utilization of Neem's medicinal potential.

Aim & Objectives

The study aims to evaluate and compare the antimicrobial potential of endophytic fungi isolated from the internal and external bark regions of the selected Neem plant. The objectives are to isolate and identify endophytic fungi from the inner and outer bark of the plant; to assess the antimicrobial activity of these isolates against selected bacterial strains; and to compare the antimicrobial efficacy between internal and external bark endophytes.

Materials and Methods

Sampling A healthy mature neem plant from a clean environment was selected for sampling. The bark surface was cleaned with 70% ethanol, and sterile tools were used to collect small pieces from both external and internal bark layers. Sterile gloves were used to avoid contamination. Samples were labeled, placed in separate sterile bags, transported to the laboratory, and processed within 24 hours. The study was conducted in the institutional microbiology laboratory over a three-month period. (August–October). [Figure 1(a)–1(b)]

[Figure 1(a)- 1(b): Collection of bark - (A) Outer bark, (B) Inner bark.]

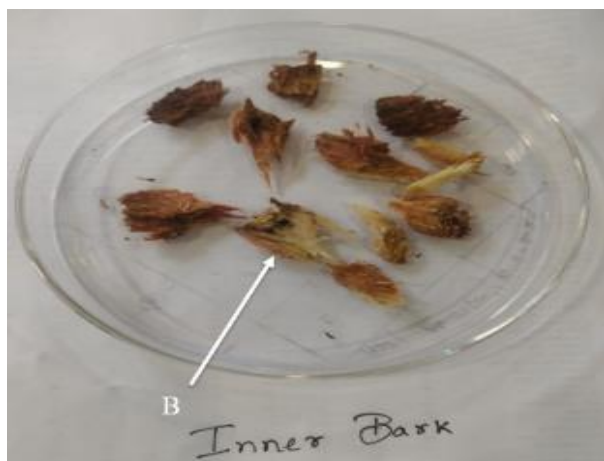


Isolation and Purification of Endophytic Fungi

Endophytic fungi were isolated from neem bark tissues using the tissue segment isolation method. The collected samples were first rinsed with sterile distilled water to remove surface debris, followed by surface sterilization under aseptic conditions. The sterilization protocol included immersion in 70% ethanol for 3 minutes, treatment with 2–4% sodium hypochlorite solution for 5 minutes, and three final rinses with sterile distilled water to eliminate residual chemicals. [Figure 2(a)–2(b)]

[Figure 2(a)- 2(b): Purification and isolation of bark - (A) Outer bark, (B) Inner bark.]





The sterilized bark tissues were aseptically cut into small segments (approximately 0.2×0.2 cm) and inoculated onto potato dextrose agar (PDA) plates. The plates were sealed with parafilm and incubated at 27 ± 2 °C until fungal colonies appeared. To validate the sterilization process, aliquots of the final rinse water were plated onto PDA and incubated at 37 °C for 14 days. The absence of microbial growth confirmed effective surface disinfection. Emerging fungal colonies were sub-cultured by transferring hyphal tips from the colony margins onto fresh PDA plates.

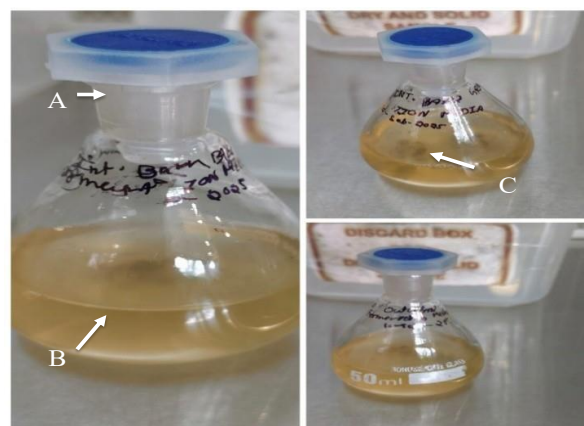
Identification of Endophytes

Fungal isolates were identified after 15 days based on their morphological and reproductive characteristics. Colony features were carefully observed, and slides were prepared from cultures using lactophenol cotton blue stain for microscopic examination.

Inoculation and Fermentation

Ten fungal inoculum discs were aseptically transferred into Erlenmeyer flasks containing fermentation medium composed of yeast extract (0.4%), beef extract (1.0%), and glucose (0.4%), with the pH adjusted to 7.0. The flasks were incubated at 28 ± 2 °C on a rotary shaker at 130 rpm for 14 days. [Figure 3]

[Figure 3: Inoculation and fermentation procedure - (A) Erlenmeyer flasks, (B) Fermentation medium, (C) Fungal inoculum discs.]



Extraction Procedure

The fermented broth was filtered under vacuum using a $0.45 \mu\text{m}$ membrane filter to separate the mycelial biomass from the culture filtrate. The filtrate was collected in Eppendorf tubes and extracted with an equal volume of ethyl acetate. The mixture was centrifuged at 10,000 rpm for 10 minutes, and the organic (ethyl acetate) layer was carefully separated. This layer was dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure to dryness in pre-weighed vials. The obtained crude metabolites were weighed and subsequently dissolved in dimethyl sulfoxide (DMSO) for antimicrobial assay.

Pathogens Used for Antimicrobial Testing

Four clinical isolates (3 gram positive and one gram negative) were selected for antimicrobial activity assays. These included *Klebsiella pneumoniae* and *Escherichia coli* isolated from urine samples of urinary tract infection (UTI) cases, *Staphylococcus aureus* obtained from bloodstream infections, and *Pseudomonas aeruginosa* isolated from pus samples. All isolates were obtained from the institutional microbiology laboratory.

Antibiotic Assay

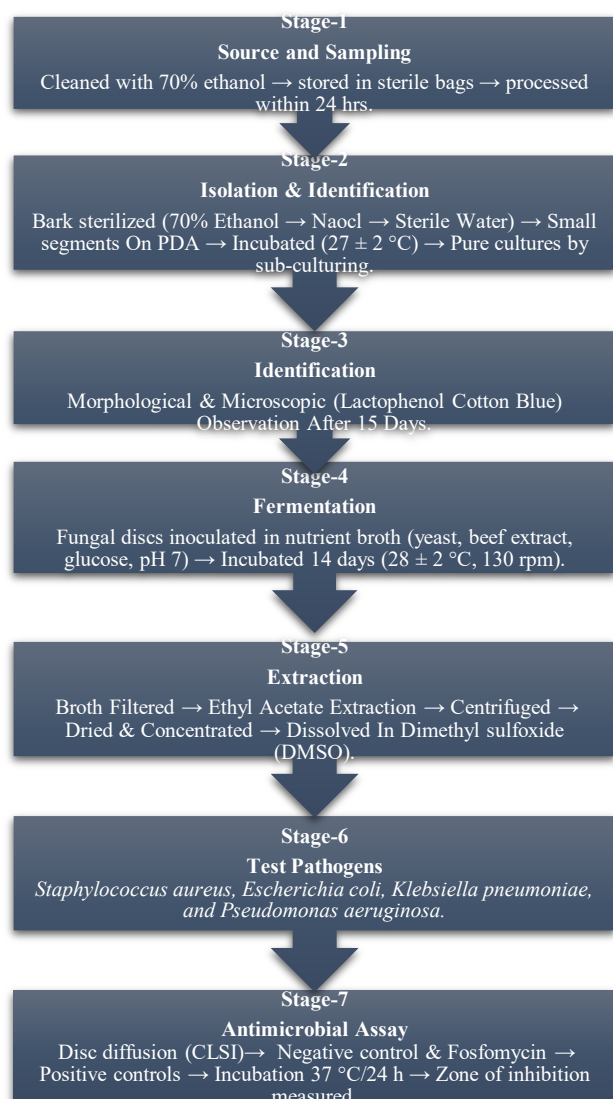
The antimicrobial activity of the fungal extracts was evaluated using the agar disc diffusion method following CLSI guidelines. [27] Test bacterial cultures were evenly spread on nutrient agar plates, and sterile discs (5 mm diameter) impregnated with the metabolite extracts were placed at three equidistant positions on each plate. A negative control disc (without extract) was included to confirm uniform bacterial growth, while a disc



containing Fosfomycin served as the positive control / standard (antibiotic).

For easy identification and handling, the three endophytic isolates were coded as follows: *C. cladosporioides* (Black outer bark)- BO, *C. cladosporioides* (Black inner bark)- BI, and *P. marneffei* (green inner bark)- GI. Plates were incubated at 37 °C for 24 hours, after which the antimicrobial activity was evaluated by measuring the diameter (in mm) of the inhibition zones surrounding each disc. Details of the methodology are described in [Figure 4]

[Figure 4: Flow Chart]

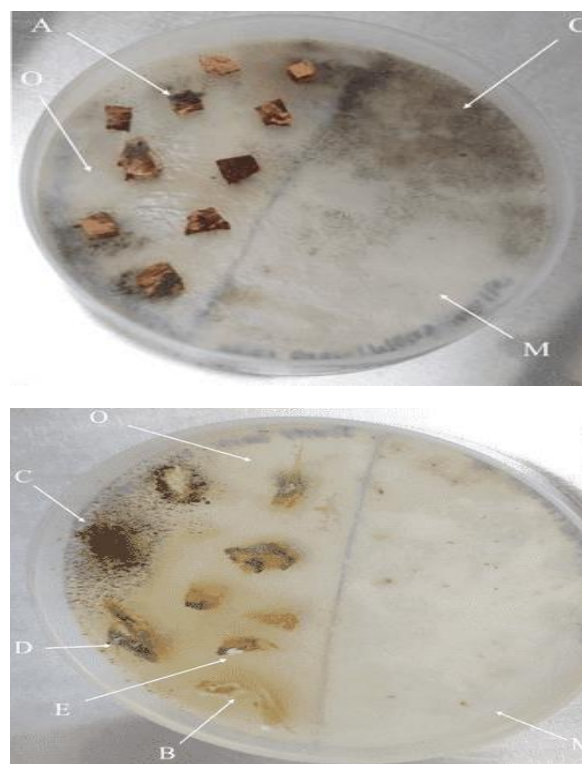


Results

Morphological identification

In this study, endophytic fungi were successfully isolated from both the external and internal bark tissues of neem (*A. indica*). From the internal bark, two distinct isolates were identified based on their morphological characteristics: *C. cladosporioides*, presenting as dark olive-green to black, slow-growing colonies, [28] and *P. marneffei*, characterized by rapidly growing, flat, filamentous colonies with a velvety to cottony texture, initially white and maturing to blue-green, grey-green, or olive-grey. [29] A third, immature white colony morphotype was also observed but could not be definitively identified due to improper growth. From the external bark, a single endophytic isolate was obtained and identified as *C. cladosporioides*, consistent with the morphology observed in the internal isolate. [Figure 5]

[Figure 5(a)- 5(b): Morphological identification: (A) Outer bark pieces, (B) Inner bark pieces, (C) Black fungal colony, (D) Green fungal colony, (E) White fungal colony.

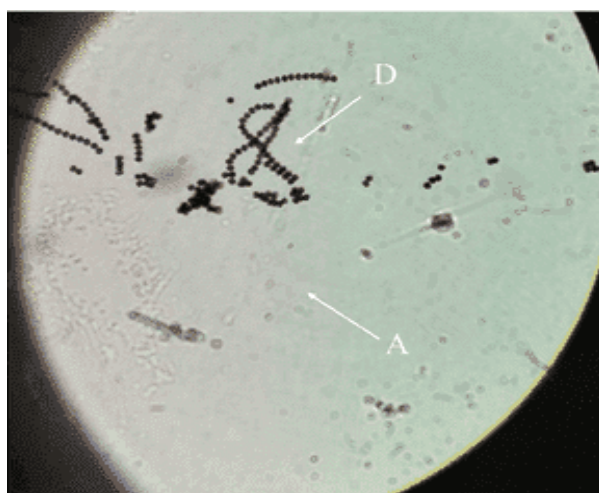
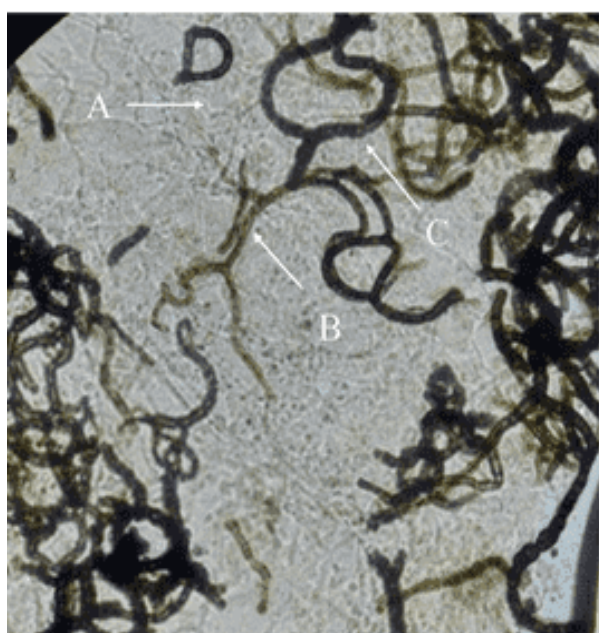


Microscopic examination of *C. cladosporioides* revealed conidia arranged in branched acropetal chains [Figure 6].

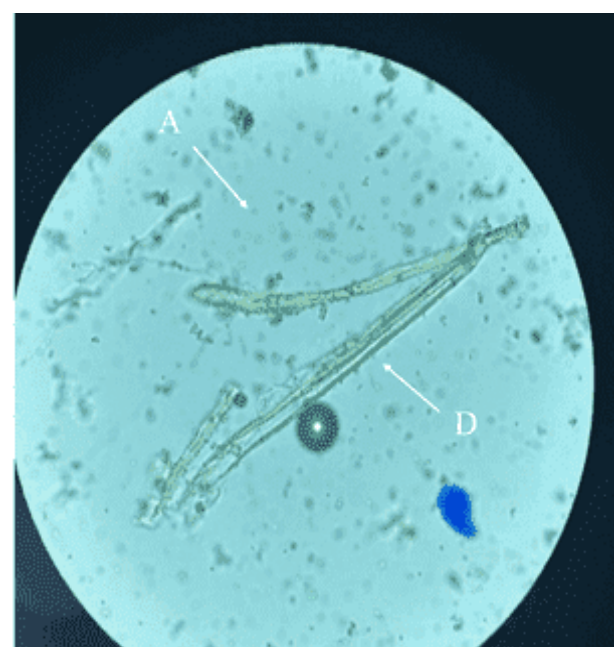
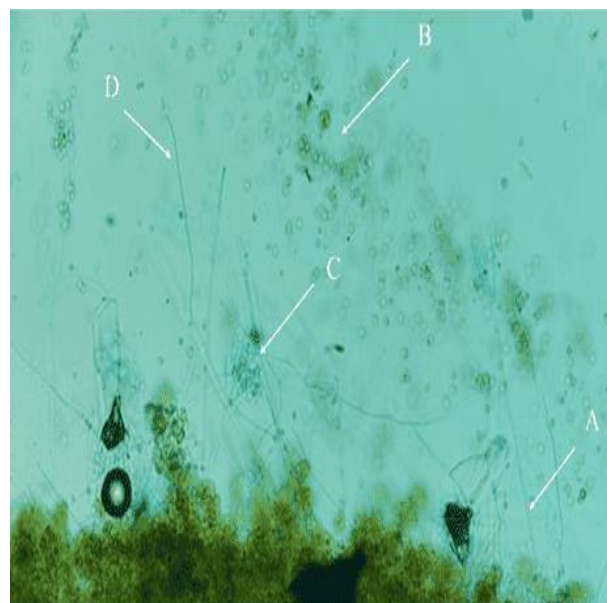


The conidia were smooth to verrucose or echinulate, one-to four-celled, and possessed a distinct dark hilum. *P. marneffei* was identified by its septate, hyaline hyphae (1.5–5 μm in diameter), and conidiophores that terminated in characteristic brush-like clusters, or penicillin, comprised of metulae bearing flask-shaped phialides. [Figure 7] These phialides produced round, unicellular conidia (2.5–5 μm) arranged in chains.

[Figure 6(a)- 6(b): Microscopic identification: (A) *C. cladosporioides* under microscope, (B) Mycelium, (C) Hyphae, (D) Conidia in chains.]



[Figure 7(a)- 7(b): Microscopic identification: (A) *P. marneffei* under microscope, (B) Spores, (C) Sporangium, (D) Hyphae.]



Antimicrobial Susceptibility Test (AST) Results

Antimicrobial susceptibility testing was performed after 24 hours of incubation at 37 °C against three Gram-negative bacteria- *P. aeruginosa*, *E. coli*, and *K. pneumoniae* and one Gram-positive bacterium, *S. aureus*. The observed zones of inhibition (in mm) for each extract and control are presented in [Table 1].



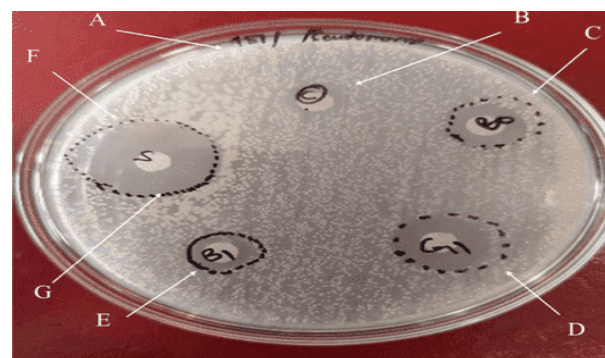
[Table 1. Zone of Inhibition Produced by Fungal Extracts Against Test Bacteria and Comparison with Positive control]

Test Organism	Positive Control	BO	BI	GI	Remarks
<i>P. aeruginosa</i>	20.0	16.0	12.0	18.0	Clear inhibition; no antagonistic effects observed
<i>E. coli</i>	24.0	15.0	11.0	16.0	Reduced activity in BI indicating antagonistic effect
<i>K. pneumoniae</i>	22.0	14.2	16.5	14.5	Mild antagonistic effects in all extracts, maximum in BI
<i>S. aureus</i>	22.0	18.0	11.0	16.0	BI showed decreased inhibition, suggesting antagonism

- PO = Positive control (Standard antibiotic: Fosfomycin)
- BO = *Cladosporium cladosporioides* (Black outer bark fungal isolate)
- BI = *Cladosporium cladosporioides* (Black inner bark fungal isolate)
- GI = *Penicillium marneffeii* (Green inner bark fungal isolate)
- Values represent mean inhibition zone diameters (mm) measured after 24 h incubation at 37 °C.
- Antagonistic effects indicate reduced inhibitory activity possibly due to internal compound interactions.

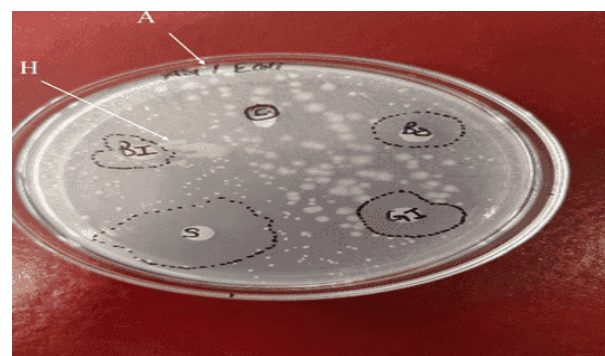
For *P. aeruginosa*, the positive control (Fosfomycin) produced a zone of inhibition measuring 20 mm. Among the test extracts, *C. cladosporioides* (BO) exhibited 16 mm, *C. cladosporioides* (BI) showed 12 mm, and *P. marneffeii* (GI) demonstrated 18 mm of inhibition. [Figure 8]

[Figure-8] Antimicrobial sensitivity test (AST) - (A) Test against *Pseudomonas aeruginosa*, (B) Negative Control disc, (C) Black outer bark disc, (D) Green inner bark disc, (E) Black inner bark disc, (F) Standard / Positive control disc (Fosfomycin), (G) Zone of inhibition (demarcated by the black outline).]



For *E. coli*, the positive control (Fosfomycin) showed a zone of 24 mm, while the fungal extracts BO, BI, and GI exhibited 15 mm, 11 mm, and 16 mm, respectively. The *C. cladosporioides* (BI) extract showed reduced inhibition, indicating a possible antagonistic effect. [Figure 9]

[Figure 9: Antimicrobial sensitivity test (AST) - (A) Test against *Escherichia coli*, (H) Antagonistic effect].

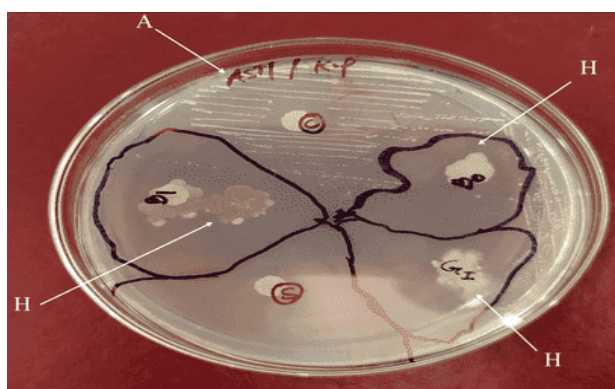


Against *K. pneumoniae*, the positive control (Fosfomycin) produced a 22 mm inhibition zone. The fungal extracts BO, BI, and GI displayed 14.2 mm, 16.5 mm, and 14.5 mm, respectively.



mm, and 14.5 mm, respectively. Mild antagonistic interactions were observed in all three extracts, with the maximum effect noted in *C. cladosporioides* (BI). [Figure 10]

[Figure 10: Antimicrobial sensitivity test (AST) - (A) Test against *Klebsiella pneumoniae*, (H) Antagonistic effect.]



For the Gram-positive *S. aureus*, the positive control (Fosfomycin) recorded a 22 mm inhibition zone, while BO, BI, and GI exhibited 18 mm, 11 mm, and 16 mm, respectively. The *C. cladosporioides* (BI) extract again demonstrated diminished activity, suggestive of an antagonistic response. [Figure 11]

[Figure-11] Antimicrobial sensitivity test (AST) - (A) Test against *Staphylococcus aureus*, (H) Antagonistic effect.



Discussion

Differential Activity of Outer and Inner Bark Isolates

Black fungal isolates obtained from both outer and inner bark samples were identified as *C. cladosporioides*. The outer bark isolate showed the strongest inhibition against *S. aureus* (18 mm) and *P. aeruginosa* (16 mm), while the inner bark isolate exhibited much lower inhibition zones (11 mm each) against the same pathogens.

At the biochemical level, outer bark isolates experience higher environmental stressors such as UV exposure, desiccation, and competition with epiphytic microbes, which stimulate the production of monoterpenes and phenolic antimicrobials that act rapidly and independently. [30] Whereas internal bark isolates operate within nutrient-rich but microaerophilic niches, favouring complex polyketide synthesis and cross-regulation among biosynthetic gene clusters. [31] Such pathways may produce antagonistic or complementary compounds, influencing the differential inhibition patterns observed between BO, BI, and GI.

Antagonistic Potential of Endophytic Fungi

The ability of endophytic fungi to hinder or suppress the growth and activity of other microorganisms is a feature of their antagonistic potential. This antagonism operates through multiple overlapping mechanisms. In antibiosis, endophytes secrete bioactive metabolites such as antibiotics and toxins that inhibit pathogen proliferation, while in mycoparasitism, they directly attack and derive nutrients from pathogenic fungi. They further enhance host resistance by triggering induced systemic responses, secreting cell wall-degrading enzymes, and activating defence-related pathways that promote the formation of physical barriers and the synthesis of protective compounds. Endophytic fungi can also detoxify virulence factors produced by pathogens and compete for space and nutrients. These multifaceted mechanisms underscore the potential of endophytic fungi as sustainable biocontrol agents in integrated plant disease management. [32]

Internal bark isolates often yield richer metabolic profiles but may contain inactive analogues or secondary oxidized derivatives that dampen antibacterial efficacy. [33] This could explain the lower inhibition observed in



the inner bark, where chemical competition or metabolite instability reduces net activity.

The green colony isolated from the inner bark identified as *P. marneffeii*, which produced wider inhibition zones (16–18 mm) against all tested organisms, suggesting synergistic interactions among its metabolites. Synergism occurs when different compounds work together to enhance antibacterial activity. These results are consistent with previous studies reporting that *Penicillium* endophytes from neem and other medicinal plants produce potent antimicrobial metabolites, such as citreosein, citrinin, and terreic acid, which are effective against *P. aeruginosa* and *K. pneumoniae*. [34] Similarly, research on internal bark fungi has shown that mixtures of alkaloids and chlorinated lactones can act against *S. aureus* and *E. coli*, though their potency varies with incubation conditions and extraction methods. [35] This highlights the importance of tissue type and extraction site in determining the strength of antimicrobial activity. [36]

The tissue-specific distribution and metabolite variability of internal bark provide a stable microenvironment that supports diverse endophytes and enhanced metabolite production, while external bark faces environmental stress, limiting microbial diversity and metabolite yield. Internal bark represents a richer microbial niche, enabling sustainable drug discovery and Ayurveda-based resource management. This approach ensures formulations derive bioactive compounds from potent microhabitats while preserving parent trees and aligns with Ayurvedic sustainability principles, Good Agricultural and Collection Practices (GACP), and One Health-One Earth initiatives.

The ability of internal bark endophytes to inhibit erythromycin-resistant bacterial strains suggests that their metabolites may possess novel mechanisms of antimicrobial action. Such compounds could serve as leads for developing alternatives to existing antibiotics, addressing the global antimicrobial resistance (AMR) crisis. [37]

Strengths and Limitations

This study uniquely compared endophytic fungi from the inner and outer bark of *A. indica*, providing novel insights into tissue-specific antimicrobial potential. The

systematic isolation and screening methods demonstrated clear bioactivity differences and supported sustainable bioprospecting concepts. However, the findings are limited by the single-tree sampling and the restricted antimicrobial assay involving only three bacterial pathogens. Broader testing against fungi, resistant strains, and cancer cell lines, along with molecular and chemical characterization, is needed to strengthen the therapeutic and scientific relevance of the results.

Future Directions

Future research can focus on systematic quantification of secondary metabolites from internal and external bark endophytes using High-Performance Thin Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), and Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS). Structurally unique metabolites may further be characterized using Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier-Transform Infrared (FTIR) spectroscopy. Bioassay-guided fractionation through bioautography and microplate-based antimicrobial assays will help identify the key bioactive constituents, establishing a direct correlation between chemical identity and biological activity. Safety evaluation on mammalian cell lines, mechanism-of-action studies through molecular docking and biochemical assays, and biosynthetic gene cluster identification using integrated metagenomic and metabolomic approaches should also be explored. These advancements will support the standardization and development of endophyte-derived phytopharmaceutical formulations for preclinical and clinical applications.

Conclusion

This study demonstrates that different bark layers of neem harbours distinct endophytic communities, with the inner bark supporting greater metabolic diversity. These tissue-specific variations influence bioactive compound production, underscoring the value of micro-niche targeting for sustainable discovery of potent, eco-friendly antimicrobial agents.

Declaration of generative AI in scientific writing

We have used the AI writing tools to improve the language and readability. The content was reviewed and revised as necessary after using these AI tools.



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Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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