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Antibacterial potential of endophytic fungi from *Neocarya macrophylla* against ESBL-producing Gram-negative bacteria

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Abstract

The increasing prevalence of antibiotic resistance makes the search for novel antibiotics an urgent priority. This study focused on isolating, identifying, and screening endophytic fungi associated with Neocarya macrophylla for their antibacterial potential. Stem and leaf samples from healthy N. macrophylla plants were randomly collected from Jega, Kebbi state, Nigeria; surface-sterilized and then cultured to isolate fungal endophytes. The isolated fungi were identified via molecular techniques. The antimicrobial activity of the extracts obtained from the isolated endophytic fungi was evaluated using the spot on the lawn technique against extensively beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae. Seven fungal species were identified from the plant samples. Aspergillus species were most prevalent (71%) followed by Fusarium oxysporum and Alternaria alternata (14 % each). Antibacterial assays against E. coli and K. pneumoniae revealed that A. niger isolate NMST_01 exhibited the highest antibacterial activity with inhibition zones of 10.7 ± 0.6 mm and 9 ± 1 mm against E. coli and K. pneumoniae, respectively. A. fumigatus strain NMST_02 and A. niger isolate NMST_03 also demonstrated moderate antibacterial activity. In contrast, A. pseudonomiae, Alternaria alternata, and A. nidulans exhibited no antibacterial activity. This study represents one of the first descriptions of the culturable endophytic fungi associated with N. macrophylla in Nigeria. The endophytes associated with N. macrophylla were predominantly Aspergillus sp. and they exhibited remarkable antibacterial activity against the tested organisms. Continued research on these endophytic fungi could lead to the discovery of valuable natural products with great pharmaceutical applications.

Keywords: Antibacterial, Alternaria, Aspergillus, Endophytic fungi, Fusarium, Neocarya macrophylla.

INTRODUCTION

The rapid spread of multi-drug resistant (MDR) Gram-negative bacteria is posing a grievous threat to the global public health. A 2021 study revealed that global annual mortality due to antibiotic resistance exceeds one million, surpassing the previous estimate of 700,000 from the 2016 O'Neil report (Murray *et al.*, 2022). Projections suggest that without concerted efforts to stem this tide, it could surpass 10 million by 2050 (Murray *et al.*, 2022). Additionally, MDR Gram-negative bacteria infections contribute significantly to increase healthcare costs and length of hospitalization (Olowo-Okere, Ibrahim, Sani, *et al.*, 2018).

Amidst the worsening antibiotic resistance crisis, there has been a substantial decline in research and development of new antibiotics. Data has shown that the rate of dissemination of antibiotic resistance globally far outpaced all efforts to mitigate the crisis. (Butler & Paterson, 2020). The divestment of many pharmaceutical companies from research and development of new antibiotics has further complicated the problem.

The rediscovery of known compounds that characterised the traditional Waksman's platform has necessitated exploration of previously uncultured microorganisms that may yield compounds with novel mechanisms of action. Endophytic fungi have emerged as a goldmine of varieties of natural products with great potential against MDR Gram-negative bacteria (Gupta *et al.*, 2023; Manganyi & Ateba, 2020).

Endophytes are hosts of microbes asymptomatically colonizing internal tissues of higher plants. They are an essential component of plant microbiomes (Harshitha *et al.*, 2023). Endophytic fungi form symbiotic relationships with plants, offering conferring selective advantages such as growth promotion and protection from invasion of pests and pathogenic organisms (Grabka *et al.*, 2022; Harshitha *et al.*, 2023). These microorganisms are known for their potential to produce an array of bioactive secondary metabolites with pharmaceutical, agricultural, and industrial importance (Abba *et al.*, 2014; Akinduyite & Ariole, 2018; Okezie *et al.*, 2022).

Studies have revealed enormous biosynthetic capacity and metabolic diversity of endophytic fungi (Harshitha *et al.*, 2023). The significance of endophytic fungi in producing novel bioactive compounds, such as anticancer, antifungal, and antimicrobial agents, further underscores their importance (Gupta *et al.*, 2023; Manganyi & Ateba, 2020). More importantly, endophytic fungi have yielded varieties of secondary metabolites with unprecedented chemical and biological characteristics, mostly non-overlapping with those produced by other microorganisms (Jha *et al.*, 2023; Sharma *et al.*, 2023). Taxol (paclitaxel), one of the most effective and successful anticancer drugs, was extracted from a fungus, *Taxomyces andreanae* (Vélëz *et al.*, 2022). Several important compounds with potent antimicrobial activities have also been extracted from various endophytic fungi. This includes Clavatol from *Torreya mairei*, Sordaricin from *Fusarium sp.*, Jesterone from *Pestalotiopsis jesteri*, and Javanicin from *Chloridium sp.* (Gouda *et al.*, 2016).

Despite increasing research interest in endophytic fungi from various plant species, there is limited data on those associated with *Neocarya macrophylla*, a medicinally important plant native to West Africa. *N. macrophylla*, a shrub or tree from the Chrysobalanaceae family, commonly known as gingerbread plum, has traditionally been used to treat various ailments. Bioactivity profiling has shown it possesses antivenom, analgesic, anti-inflammatory, and antimicrobial activities (Olowo-Okere *et al.* 2018; Jega *et al.*, 2021). While *N. macrophylla* is known for its medicinal properties, its fungal endophytes remain largely uncharacterized, limiting our understanding of their ecological roles and potential pharmaceutical value.

This study thus aimed to explore the diversity and potential of *N. macrophylla* endophytes in the production of bioactive metabolites for use against antibiotic-resistant Gram-negative bacteria. This research will enhance our understanding of fungal biodiversity and fungal-host plant interactions while also paving the way for discovering and utilizing these fungi and their natural products in pharmaceutical applications.

METHODS

Collection and identification of plant material

Leaf and stem samples of ten (10) mature and healthy *N. macrophylla* were randomly collected from Jega Local Government Area in Kebbi State. The collected plant materials were identified at the Herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Usmanu Danfodiyo University, Sokoto. After confirming the plant's identity and authenticity, a voucher number was obtained, and the voucher specimen was deposited and preserved for future research and validation. The plant tissues were placed in sterile plastic bags, stored at 4°C, and transported to the laboratory and processed within 24 hours.

Culturing and isolation of endophytic fungi

The isolation of endophytic fungi from the collected plant material was done using a previously described method (Hussein *et al.*, 2024). Plant tissues underwent a thorough surface sterilization protocol to prevent contamination from epiphytic microorganisms. The leaves and stems were first washed under running water to remove dirt and debris. Each sample was then subjected to sequential immersion in 70% ethanol for 2 minutes, followed by a 1-minute treatment in 1% sodium hypochlorite. To remove residual sterilizing agents, the tissues were rinsed thrice in sterile distilled water. Following surface sterilization, each sample was cut into 0.5 cm^2 segments under aseptic conditions.

The sterilized tissue segments were placed on potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) supplemented with 150 mg/L of chloramphenicol to inhibit bacteria growth. Plates were incubated at 28°C ± 2 °C for 7-14 days, during which fungal colonies that emerged from the plant tissues were regularly monitored and sub-cultured onto fresh PDA plates to obtain pure fungal isolates.

Aliquots of the final rinse water were plated onto PDA and SDA plates as negative controls to confirm successful surface sterilization and rule out external contamination. No fungal growth was observed in the control plates, validating the effectiveness of the sterilization procedure.

Identification of the isolated endophytic fungi

Fungal isolates were initially characterized based on their macroscopic characteristics. Genomic DNA was extracted from approximately 100 mg of fungal mycelia using the ZymoBIOMICS™ DNA/RNA miniprep extraction kit, following the manufacturer's instructions. The internal transcribed spacer (ITS) region of ribosomal DNA, a common marker for fungal identification, was amplified using ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS1 (TCC GTA GGT GAA CCT GCG G) primers in 25 µL PCR reactions, following a previously published protocol (Sarsaiya *et al.*, 2020). Briefly, the 25 µL reaction mixture comprised 2.0 µL of primer, 12.5 µL of 2×Master Mix, 8.5 µL of RNase-free distilled water, and 2.0 µL of template DNA. The reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, United States) under the following pre-optimized conditions: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 40 seconds, followed by a final extension at 72 °C for 5 minutes. PCR products were analysed on a 1% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer, using a 1kb plus DNA ladder. Electrophoresis was performed at 120 V for 30 min, and the gel was stained with 0.1 µg/mL ethidium bromide. Images were captured using the Bio-Rad Gel Doc™ XR+ system. The amplified PCR products were then purified, sequenced on an ABI 3500XL genetic analyser (Thermo Fisher Scientific, United States). The reads were assembled and corrected using Geneious Prime® (Version 2024.0.7), and the sequences were compared to the NCBI GenBank database using BLASTN with default settings (Johnson *et al.*, 2008).

Sequence Phylogeny

Sequences were aligned using the MAFFT v7.525 with default parameters. A phylogenetic tree was constructed with IQ-TREE v2.3.6, selecting the best-fit substitution model via ModelFinder. The maximum likelihood tree was inferred using 1000 ultrafast bootstrap replicates. The resulting tree was visualized in the interactive Tree Of Life (iTOL) (https://itol.embl.de/). The tree was rooted at the midpoint with bootstrap values displayed on internal nodes.

Extraction of bioactive metabolites

The bioactive metabolites from the isolated and identified endophytic fungi were extracted using submerged fermentation as previously reported (Eze *et al.*, 2018). In brief, a 1L Erlenmeyer flask containing potato dextrose broth was inoculated with 3 mm diameter agar plugs containing the fungi and incubated at 28°C for 14 days. At the completion of fermentation, the secondary metabolites contained in the fermentation medium were extracted with ethyl acetate and then concentrated under vacuum at 40°C using a rotary evaporator.

Antibacterial Activity Assay

The antibacterial activity of the extracts was assessed against clinical isolates of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* using the spot-on-lawn technique as described previously (Fernández-Fernández *et al.*, 2023). The test strains were sourced from our laboratory's bacterial collection, previously documented in the study by Olowo-Okere *et al.* 2018). A 0.5 McFarland standard suspension of the test bacteria was prepared from overnight cultures and evenly spread onto Mueller-Hinton agar plates. Aliquots of 5 µL of the extracts in 10% DMSO, were spotted onto the bacterial lawn. The plates were cultured in triplicates and incubated overnight at 37°C for 18 hours, after which inhibition zones were measured.

Data presentation

Descriptive statistics were employed to summarize the data. Frequency and percentage were used to present categorical variables. Results of the antibacterial study were presented as mean ± Standard deviation of sample replicates.

RESULTS

Isolation of Endophytic Fungi

Out of 32 stem and root segments studied, seven endophytic fungi were isolated. The fungal isolates displayed diverse colony morphologies, including variations in texture, pigmentation, and growth patterns. As shown in Figure 1, the fungal colonies from both leaf and stem samples exhibit different characteristics reflecting the morphological diversity of the isolates.

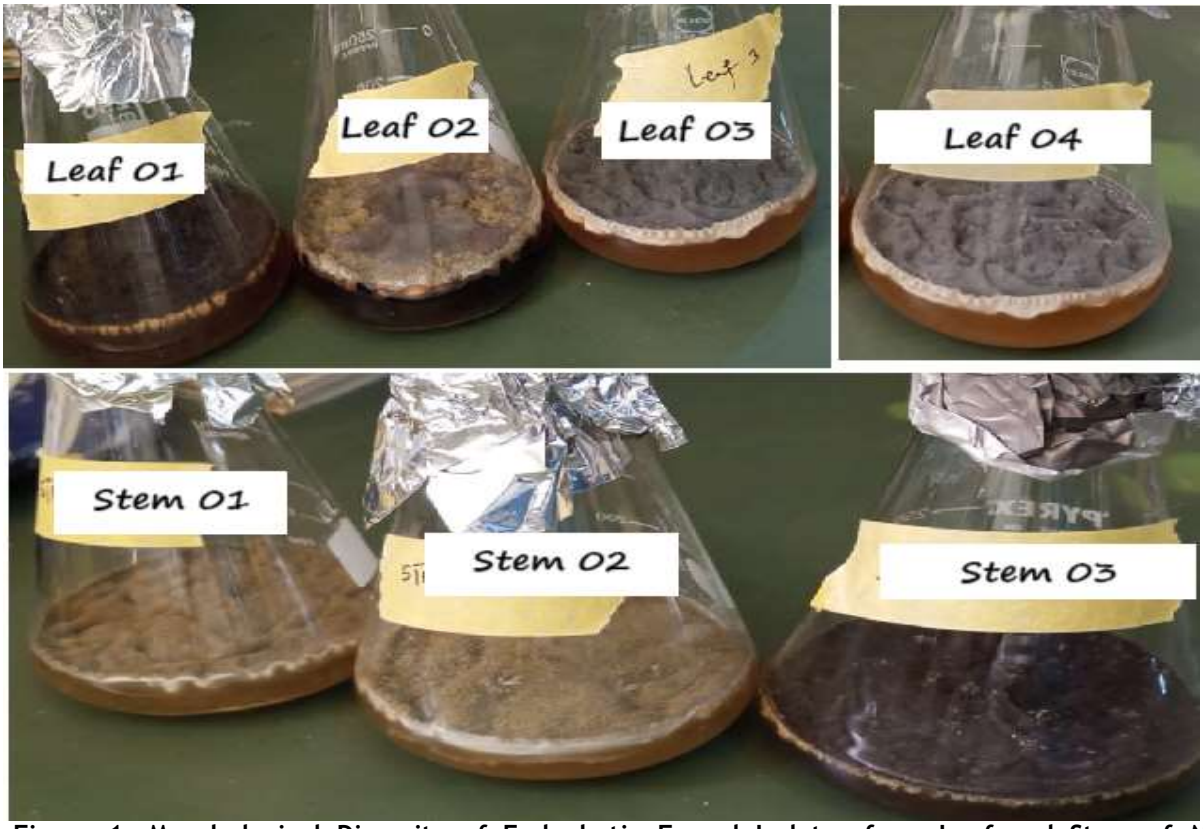

Figure 1: Morphological Diversity of Endophytic Fungal Isolates from Leaf and Stem of *N. macrophylla*

Fungal Diversity and Isolation Frequency

The electrophoretogram (Figure 2) showed successful amplification of the ITS region using ITS1 and ITS4 primers. Sequencing and subsequent BLAST analysis of the sequences of amplicons confirmed the identity of the seven (7) fungal strains with 98.92–100% similarity to known species. *Aspergillus species* were most prevalent, with 5 strains (71%), while 1 strain (14 %) each of *Fusarium oxysporum* and *Alternaria alternata* were identified. The sequences of the isolated endophytic fungi have been deposited in the GenBank under accession numbers: *Aspergillus niger* isolate NMST_01 - PQ482429, *Aspergillus fumigatus* strain NMST_02 - PQ482430, *Aspergillus niger* isolate NMST_03 - PQ482431, *Aspergillus pseudonomiae* isolate NMLF_02 - PQ482432, *Alternaria alternata* strain NMLF_03 - PQ482433, *Aspergillus nidulans* strain NMST_04 - PQ482434 and *Fusarium oxysporum* NMLF_01-PQ482424. The phylogenetic tree showing evolutionary relationship among the isolated endophytic fungi is presented in Figure 3

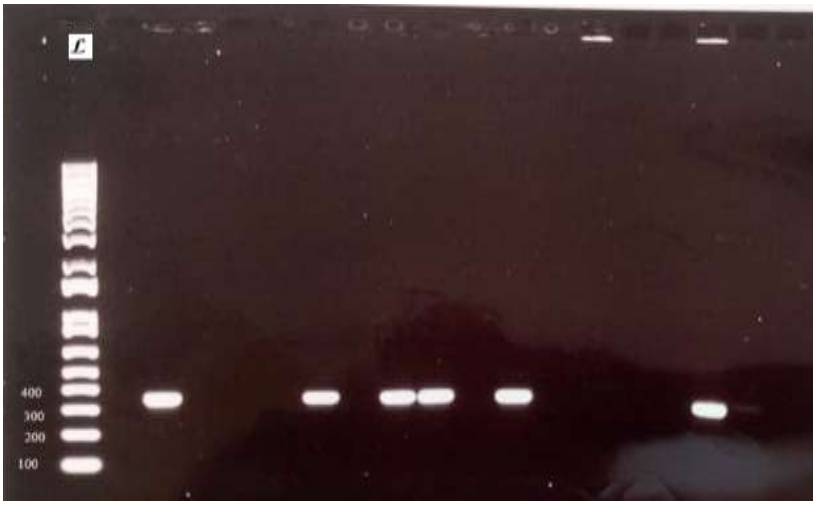

Figure 2: Electrophoretogram showing 390 bp amplicons of the ITS region.

Table 1: Identified endophytic fungi from leaves and stem of *N. macrophylla*

Strains	Closest match in GenBank		
	Reference strain	Accession number	Percentage similarity (%)
NMST 01	*Aspergillus niger* isolate C16	OR229946	99.45
NMST 02	*Aspergillus fumigatus* strain TD2	OR939706	99.75
NMST 03	*Aspergillus niger* isolate SAPC2A	OL323055	99.82
NMLF 01	*Fusarium oxysporum* isolate FD3	JX036531	98.92
NMLF 02	*Aspergillus pseudonomiae* isolate DTO 267-H7	MH279417	100
NMLF 03	*Alternaria alternata* strain SOK8	KY484874	100
NMST 04	*Aspergillus nidulans* strain FGSC A4	KY074657	100

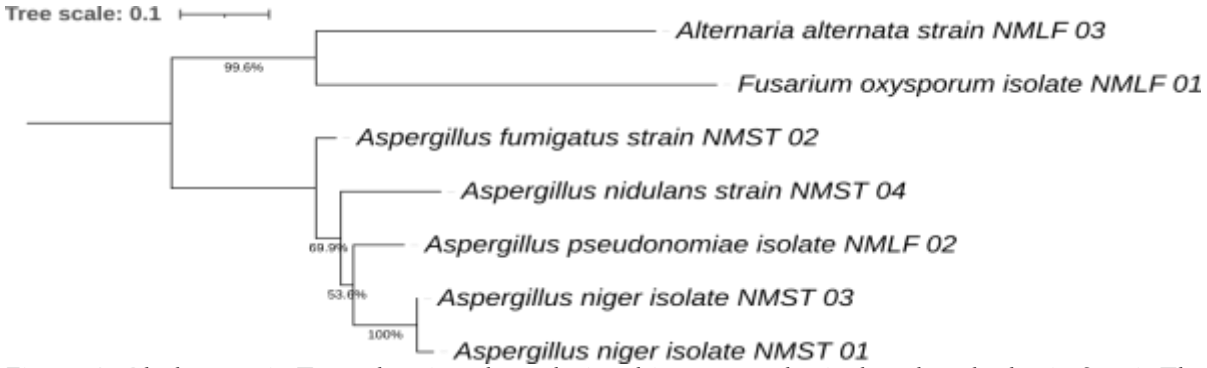

Figure 3: Phylogenetic Tree showing the relationship among the isolated endophytic fungi. The tree depicts evolutionary relationships among sequences, with branch lengths proportional to genetic distance.

Result of antibacterial assay

Among the extracts, *Aspergillus niger* isolates NMST_01 exhibited the highest antibacterial effect against *E. coli* with an inhibition zone of 10.7 ± 0.6 mm, while it showed a 9 ± 1 mm inhibition zone against *K. pneumoniae*. *Aspergillus fumigatus* strain NMST_02 demonstrated moderate activity against both bacteria, with inhibition zones of 8.7 ± 1.2 mm for *E. coli* and 9 ± 1 mm for *K. pneumoniae*. *Aspergillus niger* isolates NMST_03 also showed some effectiveness, producing inhibition zones of 9 ± 0 mm against *E. coli* and 8.7 ± 0.6 mm against *K. pneumoniae*. *Fusarium oxysporum* NMLF_01 exhibited limited antibacterial activity, with inhibition zones of 8.7 ± 0.6 mm against *E. coli* and 6.3 ± 0.6 mm against *K. pneumoniae*. In contrast, *Aspergillus pseudonomiae* isolate NMLF_02, *Alternaria alternata* strain NMLF_03, and *Aspergillus nidulans* strain NMST_04 demonstrated no antibacterial activity against either bacterial strain (Table 2; Figure 4).

Table 2: Result of antibacterial assay of isolated fungi endophytes

Extracts	Diameter zone of inhibition (mm)	
	E. coli	*K. pneumoniae*
Aspergillus niger isolate NMST_01	10.7±0.6	9±1
Aspergillus fumigatus strain NMST_02	8.7±1.2	9±1
Aspergillus niger isolate NMST_03	9±0	8.7±0.6
Fusarium oxysporum NMLF_01	8.7±0.6	6.3±0.6
Aspergillus pseudonomiae isolate NMLF_02	0	0
Alternaria alternata strain NMLF_03	0	0
Aspergillus nidulans strain NMST_04	0	0
Negative control (10 % DMSO)	0	0

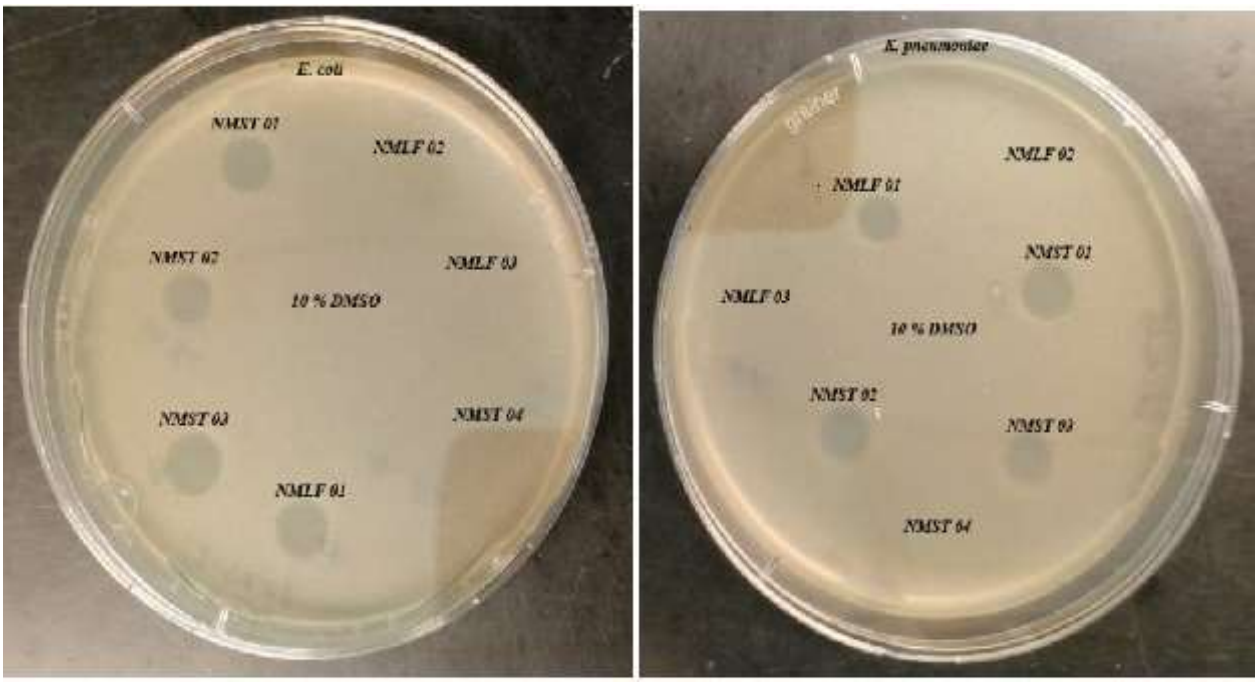

Figure 4: Antibacterial assay plate showing the activities of various extracts against the test bacteria

DISCUSSION

Medicinal plants host diverse endophytic fungi that produce beneficial secondary metabolites, enhancing their survival and adaptation. Endophytic fungi are increasingly recognized for their ability to synthesize bioactive secondary metabolites, many of which mirror or enhance the compounds produced by their host plants (Gupta *et al.*, 2023; Varghese *et al.*, 2024). This study represents Nigeria's first comprehensive description of culturable endophytic fungi associated with *N. macrophylla*.

The predominance of *Aspergillus* species among the isolated endophytes is not surprising. These fungi have been previously isolated from various medicinal plants, including *Ceriops tagal*, *Xylopia aethiopica*, and *Echinops laterifolia* (Ezeobiora *et al.*, 2023; M.r. *et al.*, 2024). *Aspergillus* species are known for their growth-promoting properties in plants, as well as their potential applications in bioremediation and the development of novel antibiotics (Leetanasaksakul *et al.*, 2024). The predominance of *Aspergillus* species in this study is consistent with their recognized roles as versatile fungal taxa capable of surviving in diverse environmental conditions. These genera are well-known for their ability to produce a broad range of secondary metabolites, including antibiotics, antifungal agents, and other bioactive compounds, which may offer protective benefits to the host plant.

Similarly, *Alternaria alternata* has been isolated from various African medicinal plants, including *Ziziphus spina-christi* (Elghaffar *et al.*, 2022). It has also been found in a range of seed products (Patriarca, 2016). Though, it exhibits significant pathogenic potential, causing diseases in a variety of plants (DeMers, 2022). Despite this, *A. alternata* can enhance plant resilience by producing bioactive compounds that deter other pathogens and herbivores, thus supporting plant health in its native ecosystems (DeMers, 2022).

The identification of *F. oxysporum* is particularly noteworthy. This endophyte has been described as a treasure trove of microbial natural products (Ahmed *et al.*, 2023). The fungus has yielded several important biologically active compounds, including gibberellic acid, which regulates plant growth and development; beauvericin, which exhibits cytotoxic and antibacterial activities; and bikaverin, known for its cytotoxic and anti-angiogenic properties against various cancer cells (Ahmed *et al.*, 2023).

The results of this study highlight the antibacterial potential of various fungal extracts against clinical isolates of *E. coli* and *K. pneumoniae*, with *Aspergillus niger* isolate NMST_01 demonstrating the most antibacterial activity. The inhibition zones of 10.7 ± 0.6 mm against *E. coli* and 9 ± 1 mm against *K. pneumoniae* suggest that this isolate may produce bioactive compounds capable of targeting these ESBL-producing pathogens.

The findings align with previous studies indicating the therapeutic potential of *A. niger* as sources of antimicrobial agents (Chigozie *et al.*, 2022; Silva *et al.*, 2022). The activity observed with *Aspergillus fumigatus* strain NMST_02 and *Aspergillus niger* isolate NMST_03 further supports the notion that metabolites of *Aspergillus* spp. from *N. macrophylla* can be effective against clinical strains of antibiotic-resistant Gram-negative bacteria.

In contrast, the lack of antibacterial activity observed in *Aspergillus pseudonomiae* isolate NMLF_02, *Alternaria alternata* strain NMLF_03, and *Aspergillus nidulans* strain NMST_04 indicates that not all fungal endophytes possess antimicrobial properties. The antimicrobial activity of *Fusarium oxysporum* NMLF_01, as observed in this study with inhibition zones of 8.7 ± 0.6 mm against *E. coli* and 6.3 ± 0.6 mm against *K. pneumoniae*, is consistent with the findings of Khattak *et al.*, 2024. Notably, *F. oxysporum* has been reported as a producer of secondary metabolites with significant growth inhibition against a broad spectrum of multidrug-resistant pathogens, including *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, through the production of bioactive compounds like ethyl iso-allocholate and 1-monolinoleoyl glycerol trimethylsilyl ether (Khattak *et al.*, 2024).

Overall, the study contributes valuable insights into the potential of fungi endophytes as a promising source of antimicrobial agents, particularly in the face of increasing resistance among pathogenic bacteria. To the best of our knowledge, this study reports the first description of endophytic fungal diversity of *N. macrophylla*. Nevertheless, the study has several limitations. The reliance on culture-dependent methods may have resulted in the underestimation of the true fungal diversity within the plant tissues. Also, the use of a limited number of microbiological growth media may further limit the number of culturable endophytic fungi. Many endophytic fungi are unculturable under standard laboratory conditions, and therefore, culture-independent techniques such as next-generation sequencing (NGS) should be employed in future studies to obtain a more comprehensive profile of the fungal community.

Additionally, this study was limited to sampling only the Jega Local Government area of Kebbi State; expanding the geographical scope of sampling to include more diverse ecosystems would likely reveal greater fungal diversity in this important medicinal plant. Seasonal variations in endophytic fungal communities should also be explored to determine how temporal factors influence fungal colonization patterns within *N. macrophylla*. Lastly, the determination of the chemical composition of the organic extract obtained from the endophytes and also the elucidation of the mode of action of the secondary metabolites should be explored in future studies.

CONCLUSION

This study represents one of the first descriptions of culturable endophytic fungi associated with *N. macrophylla* from Nigeria. The endophytes associated with *N. macrophylla* were predominantly *Aspergillus* sp. and they exhibited remarkable antibacterial activity against the tested organisms.

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Conflict of Interest: We have no conflict of interest.

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