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RESEARCH ARTICLE

Molecular Phylogenetics Reveals the Diversity of Antagonistic Fungal Endophytes Inhabiting Medicinal Plants in Nigeria

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Abstract Antimicrobial resistance is a major health concern around the world. There is a need for novel antimicrobials from less-explored biological niches. In this study, fungal endophytes inhabiting Nigerian ethnomedicinal plants were isolated. The antimicrobial activity of these endophytes was investigated using agar plugs and cell-free broth assays. Endophytic fungal isolates were identified by sequencing the internal transcribed spacer (ITS) region, and molecular phylogenetics was done using molecular evolutionary genetics analysis version 11. Fifty-one fungal endophytes were recovered from medicinal plants used. Their fungus colonization frequencies vary depending on the plant parts, with the *Crinum glaucum* bulb having the highest colonization frequency (%CF = 96%). Several fungal genera were discovered using ITS sequencing and BLAST identity, including *Aspergillus* spp., *Penicillium* spp., *Trichoderma* spp., and *Rhizopus* spp. Using preliminary antimicrobial tests, 17 of the isolates demonstrated antimicrobial activity against at least one of the eight tested pathogens. The phylogenetic analysis of these isolates revealed that 11 of them are divergent strains, emerging as a monophyletic clade. These endophytes are reported for the first time in the Nigerian medicinal plants investigated. In conclusion, endophytic fungi associated with ethnomedicinal plants in Nigeria could be a source of new endophytes, which could lead to the development of novel antimicrobials.

Keywords Endophytic fungi · Medicinal plants · ITS sequencing · Phylogenetic analysis · Antimicrobial activity

Abbreviations

MEGA	Molecular evolutionary genetic analysis
MUSCLE	Multiple sequence alignment by log expectation
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
CF	Colonization frequency
ITS	Internal transcribed spacer
PDA	Potato dextrose agar
NA	Nutrient agar
MHA	Mueller Hinton agar
IZ	Inhibition zone
IZD	Inhibition zone diameter
BLAST	Basic local alignment search tool
LPCB	Lactophenol cotton blue
rDNA	Ribosomal deoxyribonucleic acid
BlastN	Nucleotide basic local alignment search tool

Significance Statement: Antimicrobial resistance is on the increase and a threat to global health. Endophytes from Nigerian ethnomedicinal plants are less explored for antimicrobial potential. Hence, several fungal endophytes were screened for their biosynthetic potentials.

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Introduction

Antimicrobial resistance to commercially available antimicrobial drugs is a serious issue being faced by health services and has grown to be a global concern. This hypothesis has been supported by a variety of factors, including the widespread and occasionally ineffective use of antibiotics, unsanitary living circumstances, frequent travel, an increase in the number of immunocompromised patients, and a delay in infection detection [1]. As a result, a comprehensive search for new, effective, and less toxic antimicrobial drugs is required, which can be aided by the exploration of new niches and habitats. We can employ and aim for purified bioactive chemicals from plants and microbes to tackle the problem of multidrug resistance.

Endophytic fungi's synthesis of bioactive secondary metabolites has become a recurring study topic in recent decades, as these microorganisms represent a less investigated biological niche for their broad biotechnological potential [2]. Despite this focus, research on tropical endophytes, particularly those isolated from medicinal plants found in these habitats, is limited. Furthermore, the pharmaceutical industry's state of the art has remained stagnant for the past 30 years, allowing pathogenic infections to advance one step further, resulting in the development of resistance to existing treatments [3]. Endophytes appear to be present in all plants in natural environments and invade the inter- and intracellular areas of plant tissues without causing obvious harm [4]. The three most prevalent types of endophytes are fungi, bacteria, and actinobacteria. Endophytes are microbes that have a close association with their hosts and can synthesize a variety of chemicals that stimulate vegetative growth, competitiveness, and host protection from herbivores and pathogens [4]. Endophytic fungi are a valuable source of novel bioactive compounds for pharmacological, industrial, agricultural, and biotechnological uses because they represent a diverse range of microbial adaptations that have evolved in unique and unusual habitats [5].

Morphology, ultrastructure, physiology, tissue biochemistry, ecology, and chemotaxonomic features are examples of traditional biological information used to classify fungi into major groupings [6]. Since these processes take time, it will take centuries or millennia to describe all the fungal species on the earth before they go extinct, according to the regression relationship between the number of recorded fungi and years [7]. The knowledge of molecular phylogeny has shown a surprising amount of fungal diversity. Molecular phylogeny has helped to identify several new species. The use of an internal transcribed spacer (ITS) and amplicon sequencing has raised the number of fungal operational taxonomic units considerably. Many visually similar taxa may represent separate lineages, according to phylogenetic analyses, and many well-known species are species complexes [8]. Using DNA sequence data to establish phylogenetic relationships among fungal lineages can aid in the detection of cryptic species (two or more different species classed as a single species) that share morphological or physiological characteristics. A DNA barcode is a brief, uniform, and universal gene marker that can be used to quickly identify the species of fungi [7].

Endophytic fungi are renowned for producing a variety of beneficial secondary metabolites. Bioactive substances are structurally classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones. Plants used in traditional medicine have been a crucial source for the hunting of endophytic fungi capable of producing new bioactive compounds. It is believed that the metabolites produced by fungi endophytic populations are the source of therapeutic properties exhibited by these plants [9]. Despite this, the endophytic composition of a variety of medicinal plants, especially those from Nigeria, has not been investigated. *Piper guineense* Schumach. (leaf), *Euphorbia laterifolia* Schum. & Thonn. (stem), *Allium ascolonicum* L. (root, leaves, and bulb), *Crimum glaucum* A. Chev. (bulb), *Xylobia aethiopica* A. Rich (fruit), and *Pacrilima nitida* Stapf (bark and seed) were all examined for their association with fungal endophytes. These are well-known medicinal plants in Nigeria, and they have been shown to possess antibacterial, anti-inflammatory, anticonvulsant, antitussive, and wound-healing properties [10–12]. These medicinal plants were chosen in the current study in search of endophytic fungi with the ability to produce bioactive compounds with antimicrobial activity.

Material and Methods

Chemicals and Media used in this Study

Absolute ethanol, 70% ethanol, 4% sodium hypochlorite, lactophenol cotton blue (LPCB) stain, sterile distilled water, glycerol, and immersion oil. The media used for the study include Potato dextrose agar (PDA), Mueller Hinton agar (MHA), Sabourand dextrose agar (SDA), Potato dextrose broth (PDB) and Nutrient agar (NA). All media were from Hi-media, India.

Plant Collection and Identification

Healthy parts of medicinal plants (showing no visual disease) were selected and collected from the local herbal market at Ilosha Mushin, Lagos in Nigeria. They were as follows: fruits of *X. aethiopica*, the stem of *E. laterifolia*, leaves, bulbs, and roots of *A. ascolonicum*, bulb of *C. glaucum*, bark, and seeds of *P. nitida*. These plants may harbor new fungal endophytes that are more likely to create novel antibiotics, according to our hypothesis. The plants

were identified and authenticated at the University of Lagos Herbarium, Nigeria with the voucher numbers LUH 9071, LUH 9069, LUH 9072, LUH 9070, LUH 9074, and LUH 9073 for *A. ascolonicum* L., *P. nitida* Stapf., *C. glaucum* A. Chev., *X. aethiopica* A. Rich, *P. guineense* Schumach., and *E. laterifolia* Schum & Thonn, respectively. All samples were immediately brought to the laboratory and processed for isolation of endophytic fungi within 48 h.

Surface Sterilization and Isolation of Endophytic Fungi

The surface sterilization process was done following standard procedures with little modification [13]. In a nutshell, the samples were washed under running water, cut into small 1 cm^2 pieces with a sterile scalpel, and surface sterilized by immersion in 70% ethanol for 3 min, 4% sodium hypochlorite (NaOCl) for 2 min, and again 70% ethanol for 1 min before rinsing three times in sterile water. In a sterile blot paper, the plant material was now dried. Six segments of each plant were inserted in Petri dishes before being inoculated into PDA media enhanced with 500 mg/L chloramphenicol. As a negative control, a known volume of sterile water (1 ml) used for rinsing was plated out. To validate the negative control, an impression of the surface-sterilized plant tissue is created on PDA. Plant tissues that had not been sterilized were used as a positive control. The plates were parafilm-sealed, labeled, and incubated for two weeks at 27 °C.

Purification and Preservation of Endophytic Fungi

Each morphologically different fungi colony was transferred into a new antibiotic-free PDA petri dish [14]. This was done repeatedly to obtain pure fungal isolate. Purified endophytic isolates were then transferred to PDA slants and kept at 4 °C until needed. Endophytic fungi were cryopreserved in 30 percent glycerol broth at − 40 °C for long-term preservation of isolates [15].

Morphological and Microscopical Identification

The identification of the isolated fungi was based on the description of colonies and morphological structures on PDA media. Both the front and back views were recorded. As reported in the description of medical fungi, cultural features were explored by examining spore production, types of conidia, and conidiospores [16]. A 10-day-old fungal culture was cultivated on potato dextrose agar at 28 °C, and the fungus spores were mounted on slides with 2 drops of lactophenol cotton blue (LPCB) dye using the tease mount method [17]. The slide was covered with a slide slip and viewed under the microscope at magnifications of ×40 and ×100. An Olympus CX23 microscope was used to analyze the microscopical features.

Isolation of Genomic DNA, PCR Amplification, and ITS Sequencing

Following conventional protocols, fungal genomic DNA was obtained using a fungal DNA isolation kit (GeneOmbio technologies, Pune, India). In a typical PCR experiment, the ITS4 and ITS5 genes were amplified using Universal ITS rDNA typing primers ITS4 (sequence 5′ to 3′ TCCTCC GCTTATTGATATGC) and ITS5 (sequence 5′ to 3′ GGA AGTAAAAGTCGTAACAAGG) [18]. After amplification, the products (amplicon) were purified with a PCR product purification kit and sequenced directly with an ABI PRISM Big Dye Terminator V3.1 kit (Applied Biosystems, USA) at Inqaba West Africa, Nigeria (https://inqababiotec.co.za/).

Molecular Identification

Bioinformatic analysis software was used to examine the sequences. The NCBI database server (http://www.ncbi.nlm.nih.gov/BLAST) was used to perform the BLAST analysis. The DNA sequences were compared to those in the NCBI GenBank database. The query gene sequences were deposited in the NCBI GenBank database and assigned accession codes. The accession numbers of the selected isolates are presented (Table 1) in the results section.

Phylogenetic Analysis of the Fungi Isolates

The most similar sequences to the ITS sequence of our isolates on the NCBI were selected in a FASTA file and were aligned in MEGA 11 software [19]. Multiple sequence alignment was conducted using log expectation (MUSCLE) and the phylogenetic tree was drawn using the Neighbour-joining method with 1000 replications for the bootstrap test and 0.1 scales using MEGA 11 software. The phylogenetic tree was visualized and annotated using the Interactive Tree of Life (iTOL) version 6.0 [20].

Screening of Antimicrobial Activity

Tested Microorganisms

Three pathogenic fungi strain infectious to humans were used in the antimicrobial screening tests including (*Candida tropicalis,* LUH-5852; *Cryptococcus neoformans,* LUH-5510 and *Aspergillus clavatus,* LUH-5589). These strains were provided by the Culture Collection of the Mycology Department, Lagos University Teaching Hospital (LUTH) in Lagos, Nigeria. Additionally, five bacterial strains were obtained from the National Institute for Medical Research

Table 1 Molecular identification of the selected fungal endophytic isolates harbouring antimicrobial activity

Isolate	Plant	Plant parts	E-value	Query cover (%)	% Identity	Most similar hits	Accession no
PNS1	*Pacrilima nitida*	Stem	0.0	96	99.65	*Aspergillus aflatoxiformans*	NR_103595.1
AAL2X	*Allium ascolonicum*	Leaves	0.0	87	92.86	*Aspergillus austwickii*	NR_171607.1
CGB1	*Crimum glaucum*	Bulb	0.0	97	99.65	*Trichoderma yunnanense*	NR_007179.1
AAB2	*Allium ascolonicum*	Bulb	0.0	92	99.45	*Trichoderma yunnanense*	MN069566.1
AAB3	*Allium ascolonicum*	Bulb	0.0	91	99.07	*Aspergillus luchuensis*	NR_077143.1
ELS2	*Euphorbia laterifolia*	Stem	0.0	96	99.81	*Penicillium citrinum*	NR_121224.1
CGB8	*Crinum glaucum*	Bulb	0.0	89	80.78	*Aspergillus hancockii*	NR_171607.1
AAR2	*Allium ascolonicum*	Root	0.0	94	97.87	*Trichoderma pubescens*	NR_134419.1
AAR1	*Allium ascolonicum*	Root	0.0	86	97.22	*Rhizopus arrhizus*	NR_103595.1
AAR4	*Allium ascolonicum*	Root	0.0	92	100	*Aspergillus foetidus*	NR_077143.1
ELS1	*Euphorbia laterifolia*	Stem	0.0	90	100	*Aspergillus welwitschiae*	NR_077143.1
AAL2	*Allium ascolonicum*	Leaves	0.0	92	99.64	*Aspergillus awamori*	NR_077143.1
PNS9	*Pacrilima nitida*	Stem	0.0	96	99.81	*Penicillium citrinum*	NR_121224.1
PNS7	*Pacrilima nitida*	Stem	0.0	96	99.47	*Aspergillus austwickii*	NR_171607.1
CGB2	*Crimum glaucum*	Bulb	0.0	94	100	*Aspergillus aflatoxiformans*	NR_171606.1
ELS6	*Euphorbia laterifolia*	Stem	0.0	97	94.32	*Aspergillus foetidus*	NR_163668.1
PNS12	*Pacrilima nitida*	Stem	0.0	83	92.68	*Aspergillus aflatoxiformans*	NR_171606.1

E-value expect value, *Accession no* accession number of the most similar hit, *% identity* percentage identity

(NIMR) Yaba, Lagos including Gram-positive bacteria (*Staphylococcus aureus*, ATCC 13311; and *Bacillus subtilis*, ATCC 12022), and Gram-negative (*Escherichia coli*, ATCC 25922, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, ATCC 22595). The strains were maintained on nutrient agar plates at 4 °C and sub-cultured every month on Mueller Hinton agar (MHA) and sabouraud dextrose agar (SDA) for bacteria and fungi pathogens, respectively.

Preliminary Antimicrobial Assay

The endophytic fungi isolates were subjected to an antimicrobial assay by using the endophytic fungal agar plug inoculation technique which permits a rapid and qualitative selection of the bioactive-producing isolates. Antibacterial activity of isolated endophytic fungi was tested based on the standard protocols with slight modification [21] as follows: Petri dishes containing Mueller Hinton agar (MHA) and sabouraud dextrose agar (SDA) media for the growth of bacteria and fungi, respectively, were prepared and each test organism was spread on the surface of agar using a sterile cotton swab. Six-millimeter diameter of actively growing fungal culture disc from each PDA plate (ten days old) was cut using a sterile cork-borer and placed on the surface of respective agar media (MHA) seeded with the test bacteria, while SDA media was used for fungi. The plates were sealed with parafilm and kept in the refrigerator at 4 °C for 6 h for the complete diffusion of the endophytes' metabolites from the disc to the media in the plates. The Petri dishes were incubated at 37 °C for 24 h for bacterial growth, and at 30 °C for 48 h for fungal growth. The negative control was a plain 6 mm PDA plug. After incubation, the diameter of the zone of inhibition (IZD) was measured in millimeters by using a scale.

Fermentation in Liquid Medium

Two hundred milliliters of sterile potato dextrose broth were aseptically distributed in 500 mL sterile Erlenmeyer flasks. Three agar plugs (6 mm in size) of each isolated endophytic fungal strain were bored using a sterile cork-borer and were used to aseptically inoculate into all the flasks. The flasks were kept under stationary conditions at 27 °C for 10 days to enable growth [22]. They were examined periodically for any contamination. After 10 days, biomasses were separated, culture media were centrifuged at 5000 rpm for 45 min and the supernatant was passed through a bacteriological filter (Millipore filter, pore size 0.22 µm) in sterile conditions. The culture supernatants (cell-free broth) were collected and subjected to antimicrobial screening.

Antimicrobial Activity of Endophytic Fungal Cell-Free PDA Broth

Antimicrobial activity of culture supernatants of endophytes was tested by agar well diffusion method using Mueller Hinton agar medium for the bacteria and sabouraud dextrose agar for fungi [23]. All the overnight

bacterial cultures were adjusted to 0.5 McFarland Standard. Tested pathogens were inoculated into Mueller Hinton agar plates for bacteria and sabouraud dextrose agar for fungi using a sterile cotton swab. About a 6 mm size well was made using a sterile cork borer and an amount of 200 µL of fungal endophytic culture supernatant was added to each well. All the plates were observed for a zone of inhibition after incubation at 37 °C for 24 h for bacteria and 28 °C for 48 h for fungi. The positive control used for antibacterial strains was chloramphenicol (0.1%, 50 µL) and clotrimazole (0.1%, 50 µL) was used as a positive control for fungi pathogens. The potato dextrose broth (PDB, 200 µL) was used as a negative control in both cases [21]. Antimicrobial activities were assessed by the presence or absence of the inhibition zone and the degrees of sensitivity of the isolates to the cell-free broth were determined by measuring the diameter of the zone of inhibition in millimeters. A positive result showed that the cell-free broth displayed extra-cellular bioactive molecules.

Statistical Analysis

Colonization frequency (%) of an endophyte species was equal to the number of segments colonized by an endophyte divided by the total number of segments observed × 100 [24].colonization Frequency $= \frac{\text{Number of segments colonized by an endophyte}}{\text{Total number of segments observed}} \times 100$. Data representing inhibition zone diameters used during antimicrobial studies are presented as mean ± standard deviation (SD). Figures were drawn and analyzed in OriginPRO 2017 (https://www.originlab.com/).

Results and Discussion

Isolation of Endophytic Fungi from Medicinal Plants

Fifty-one fungal endophytes belonging to 28 species and 4 genera were isolated from the fruits, bark, stem, bulb, roots, leaves, and seeds of the selected medicinal plants (Fig. 1). The fungi colonization frequency was highest in the case of *C. glaucum* bulb with (96%) followed by *A. ascolonicum* root and bulb with (93.3%) while *X. aethiopica* fruit showed the least colonization frequency (17.5%) (Fig. 1). Our result does not agree with the report by a research group which showed that the leaves of medicinal plants have a higher colonization frequency for endophytic fungi than other plant parts [25]. This could be attributed to the type of medicinal plant whether shrub, tree, rhizomes, or bulbs. More so, the climatic condition of the originated country could greatly affect the endophytic fungi population and distribution among different plant parts.

Seventeen isolates that exhibited antimicrobial activity upon preliminary assay were identified using PCR amplification and ITS sequencing. They were identified belong to four different genera: *Aspergillus*, *Trichoderma*, *Penicillium*, and *Rhizopus*. Our findings showed that the *Aspergillus* genus was the most abundant in different parts of the medicinal plants followed by *Trichoderma* and *Penicillium* while *Rhizopus* genera are rare and were found only in the root of *A. ascolonicum*. This is not consistent with the report of Sharma et al. [25] whose study on Indian medicinal plants showed the predominance of *Colletotrichum*, *Fusarium*, *Manokwaria*, and *Oncopodium genera*. Our result is consistent with the report of Rustamova et al. [26] whose study showed *Aspergillus* spp. as the most dominant endophytic fungi isolated from the roots of the medicinal plant *Vernonia anthelmintica* in China. Their endophytic fungi isolates were specifically isolated from the root while ours were spread across different parts of the medicinal plants. There is a correlation between our result with that of Akinduyite and Ariole, whose results showed predominant fungal endophytes from medicinal plant leaves (*Avicennia Africana*) to be *Aspergillus*, *Penicillium*, *Fusarium*, *Collectotrichum*, *Phomopsis*, *Epicoccum* and *Rhizopus* genera [27].

Several plant parts showed a significant level of fungal genera diversity. The highest diversity was observed in *A. ascolonicum* (root), followed by *A. ascolonicum* (bulb), *E. laterifolia* (stem), and *P. nitida* (bark) while no antagonistic fungi colonies were isolated from *X. aethiopica* (seed), *P. nitida* (bark) and *P. guineense* (leaves). It was noticed that *Aspergillus* was the only genus isolated from *A. ascolonicum* (leaves) with antimicrobial activities (Fig. 1).

The colony morphology and microscopic features of the isolated endophytes are shown in Fig. 2. Different endophytic fungi showed different colors on PDA plates after 2 weeks of cultivation at 28 °C. Their colors ranged from black in the case of *Aspergillus and Rhizopus* genera to whitish green for *Trichoderma* and greyish for *Penicillium* genera. The substrate hyphae and microscopic characteristics showing their hyphae, microconidia, macroconidia, and conidiospores are shown in Fig. 2.

Molecular Identification of Endophytic Isolates

The sequences of isolates harboring antimicrobial activities were blasted on the NCBI site and were compared to those available in the NCBI database. The most similar hits were selected by considering their query cover and percentage identity values. Eleven isolates belonged to *Aspergillus*, three for *Trichoderma*, two for *Penicillium,* and only one *Rhizopus*. The percentage identity of the identified isolates ranged from 80.78 to 100% similarity as shown in Table 1. The GenBank accession numbers of the selected

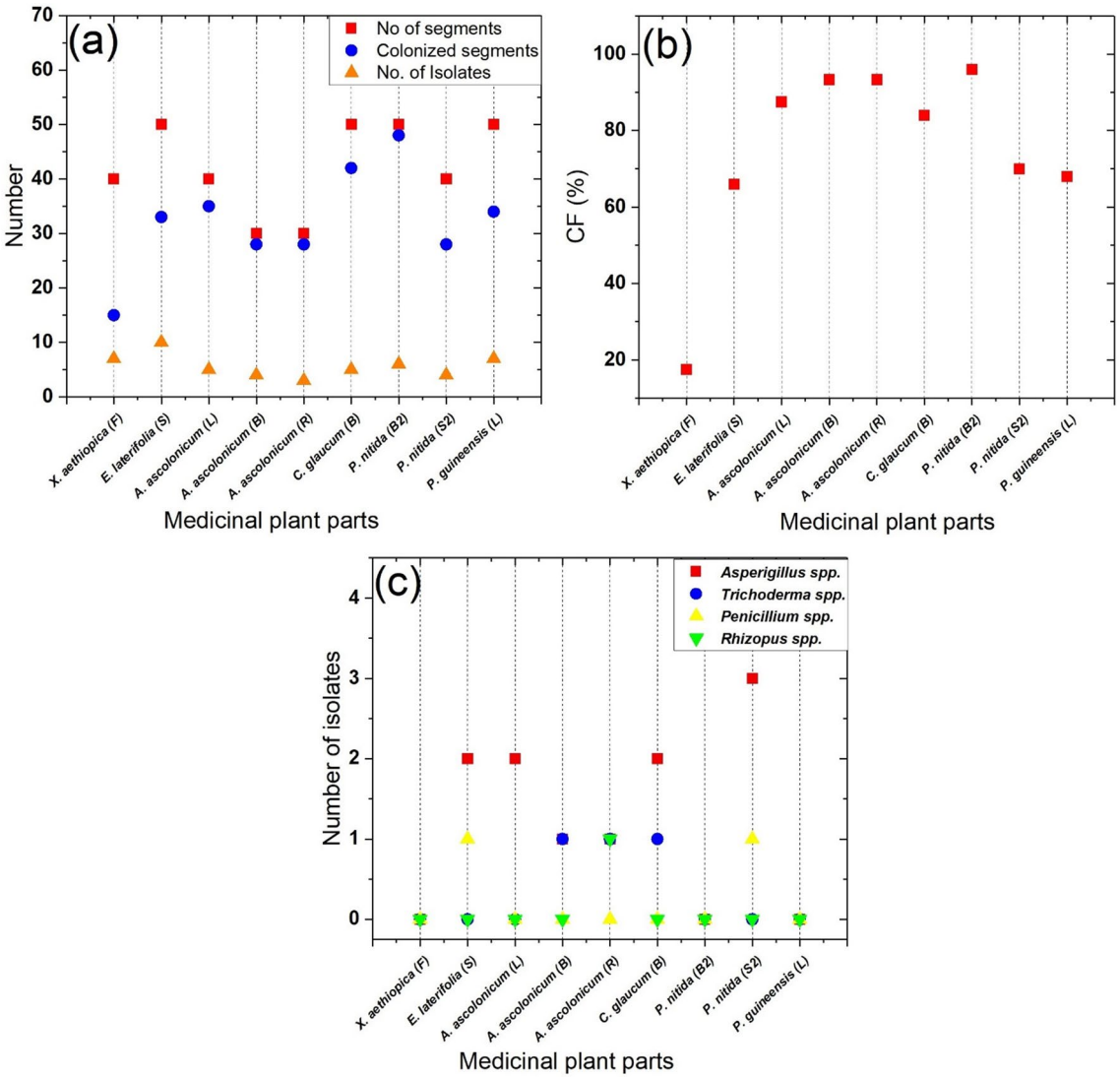


Fig. 1 The isolation of endophytic fungi among the selected medicinal plants and their colonization frequencies. (**a**) Chart showing the total number of plant segments used, the number colonized by fungal endophytes and total isolates from obtained from each medicinal plants; (**b**) Chart showing the % colonization frequency (% CF) exhibited by each of the selected plant parts. The % CF of a plant species is equal to the number of segments colonized by an endophyte divided by the total number of segments observed×100; (**c**) Endophytic fungi diversity and number of fungal isolate(s) harboring antimicrobial activity from medicinal plants parts including (F): Fruit, (S): Stem, (L): Leaves, (B): Bulb, (R): Root, (B2): Bark, (S2): Seed. The Figure was drawn and analyzed in OriginPRO 2017 (https://www.originlab.com/)

endophytic fungi harboring antimicrobial activity are listed as in Table 2.

Phylogenetic Studies on the Endophytic Fungi Isolates

The phylogenetic analysis of the selected endophytic fungi in our study showed a phylogenetic tree subdivided into 3 large clades (Fig. 3). The result showed an ancestral relationship between the endophytic fungal isolates with the closest relatives in the NCBI GenBank. *P. citrinium* (PNS9) isolated from the seed of *P. nitida* shared a close phylogenetic association with *Penicillium hetheringtonii* CBS 122392 and *Penicillium citrinum* NRRL 1841 from the GenBank with a 100% bootstrap value while *A. austwickii* (PNS7) from the seed of *P. nitida* shared close phylogenetic relationship with *A. hancockii* (CGB8) at 77% bootstrap value. *T. yunnanense* (CGB1) from the bulb of *C. glaucum* shared a phylogenetic association with *T. yunnanense* (AAB2) from the bulb of *A. ascolonicum* at a bootstrap value of 54%. This is also true with *P. citrinum* (ELS2) from the stem of *E. laterifolia* which shared an evolutionary relationship with *A. aflatoxiformans* (CGB2) from the bulb of *C. glaucum*

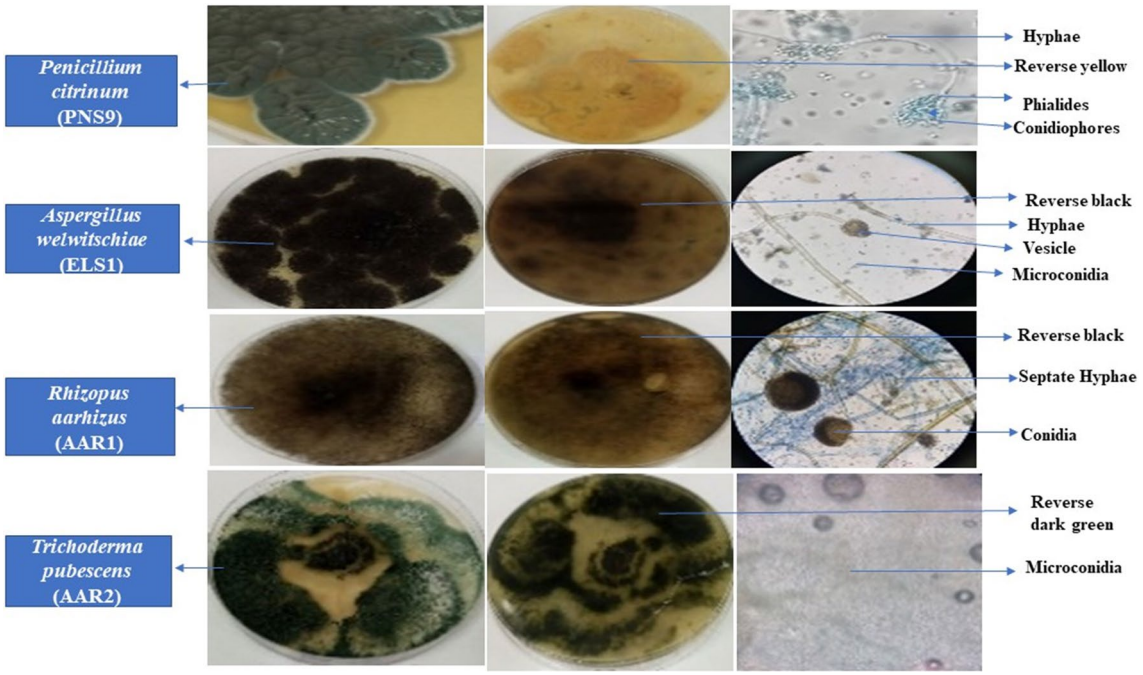


Fig. 2 Cultural and microscopic identification of *Penicillium*, *Aspergillus*, *Rhizopus*, and *Trichoderma* of Nigerian endophytic fungi from medicinal plants. They have been arranged from left to right: the front view, reverse, and microscopic view. The four represented species include *P. citrinium*, *A. welwitschiae*, *R. aarhizus*, and *T. pubescens*. The plates represent 14 days old culture on PDA incubated at 28 °C. The microscopy was observed using an Olympus CX23 model microscope (40×)

Table 2 The isolate codes and GenBank accession numbers of the selected endophytic fungi isolates harbouring antimicrobial activity

Code	Submission number	Genus	Species	Accession no
AAB2	SUB11202196 AAB2	*Trichoderma*	*yunnanense*	ON054314
AAB3	SUB11202196 AAB3	*Aspergillus*	*Luchuensis*	ON054315
AAL2	SUB11202196 AAL2	*Aspergillus*	*awamori*	ON054316
AAL2X	SUB11202196 AAL2X	*Aspergillus*	*austwickii*	ON054317
AAR1	SUB11202196 AAR1	*Rhizopus*	*arrhizus*	ON054318
AAR2	SUB11202196 AAR2	*Trichoderma*	*pubescens*	ON054319
AAR4	SUB11202196 AAR4	*Aspergillus*	*foetidus*	ON054320
CGBI	SUB11202196 CGB1	*Trichoderma*	*yunnanense*	ON054321
CGB2	SUB11202196 CGB2	*Aspergillus*	*aflatoxiformans*	ON054322
CGB8	SUB11202196 CGB8	*Aspergillus*	*hancockii*	ON054323
ELSI	SUB11202196 ELSI	*Aspergillus*	*welwitschiae*	ON054324
ELS2	SUB11202196 ELS2	*Penicillium*	*citrinum*	ON054325
ELS6	SUB11202196 ELS6	*Aspergillus*	*foetidus*	ON054326
PNS1	SUB11202196 PNS1	*Aspergillus*	*aflatoxiformans*	ON054327
PNS7	SUB11202196 PNS7	*Aspergillus*	*austwickii*	ON054328
PNS9	SUB11202196 PNS9	*Penicillium*	*citrinum*	ON054329
PNS12	SUB11202196 PNS12	*Aspergillus*	*aflatoxiformans*	ON054330

at the bootstrap value of 54%. However, eleven endophytic fungi isolates belonging to *Rhizopus* (1), *Trichoderma* (3), *Aspergillus* (6), and *Penicillium* (1) were observed to form a monophyletic clade at the top end of the tree indicating that they have common ancestry different from other isolates and those from GenBank. Among these isolates that formed a monophyletic clade, *P. citrinum* (ELS2) and *A. aflatoxiformans* (CGB2) shared a more recent common ancestor than any other isolate. More so, *R. arrhizus* (AAR1) showed the farthest ancestral relationship with other isolates in the clade. The phylogenetic analysis of our endophytic fungal isolates showed that they are divergent. Our result is

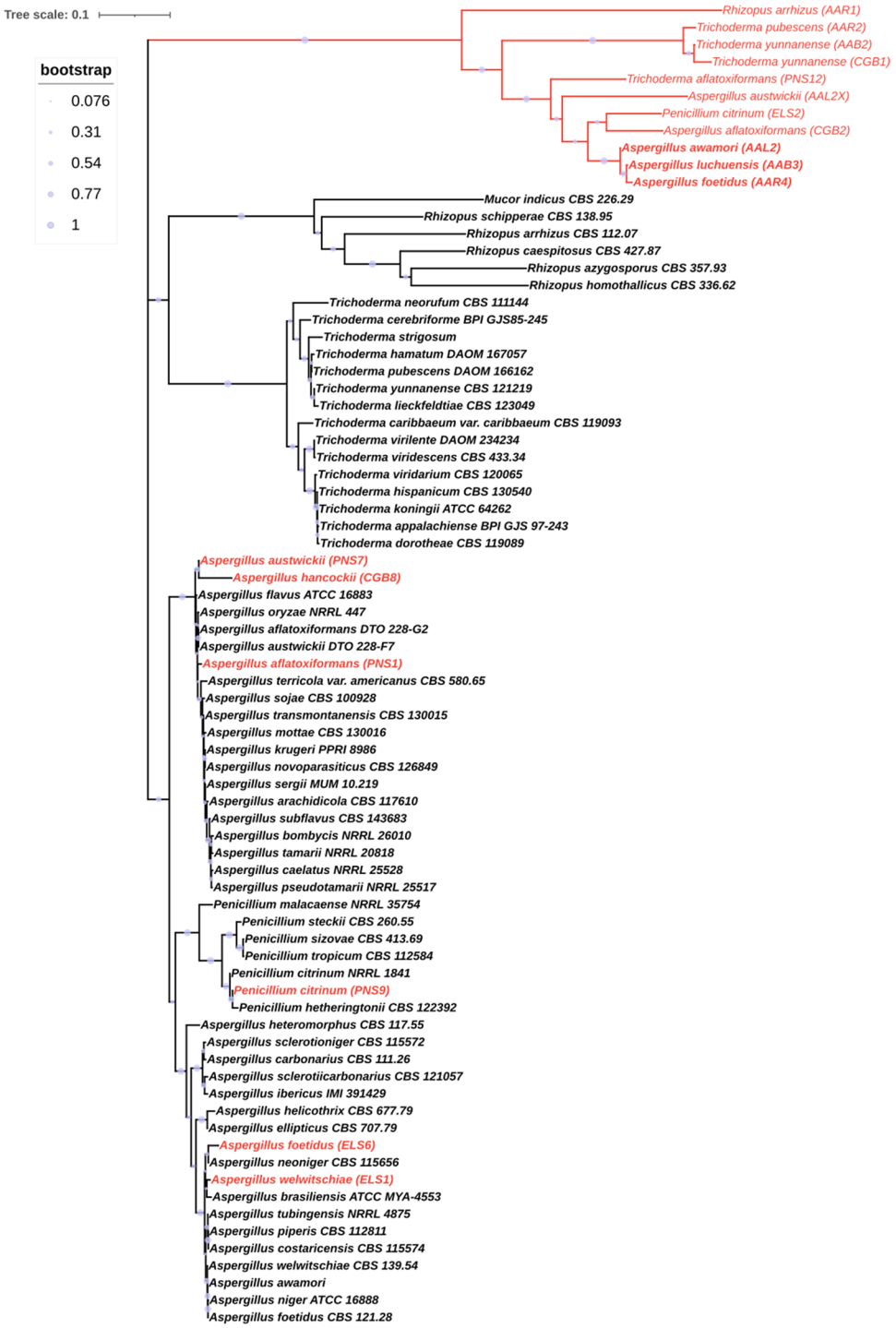


Fig. 3 Maximum likelihood tree of the isolated fungal endophytes and their closely associated taxa obtained from GenBank based on ITS gene sequence. Alignment was conducted with MUSCLE and MEGA 11.0 software was used for drawing the tree using the Neighbour-joining method while the annotation was done using iTOL version 6.0. The GenBank taxa are designated by species name with accession number while our isolates are designated by species name and isolation code names highlighted in red. Numbers at nodes are bootstrap percentages based on 1000 replications. The bar indicates 0.10 nucleotide substitutions per site

consistent with the report by Rajesh et al. [28] whose result described the phylogenetic relationship among some fungal endophytes. Those isolates that form monophyletic clade could be linked to their novelty, thus worth further studies as they could harbor novel antimicrobial compounds not produced in other isolates. They showed longer branch lengths than those in other clades which indicates that mutation occurs among them slower than when compared with other isolates with shorter branch lengths. Finally, our results confirmed the biosynthetic potential of endophytic fungi against pathogenic microbes.

Antimicrobial Studies of Endophytic Fungal Isolates

The antimicrobial activities of endophytic fungi isolates were studied by agar plug assay and agar diffusion of

cell-free broth. These isolates were tested against 5 bacterial and 3 fungi pathogens. The presence of halos shows activities against the pathogen. The results from agar plug antibacterial bioassay of endophytic fungi showed 16 of the isolates to have activity against one or more tested bacterial pathogens. *A. welwitschiae* from the stem of *E. laterifolia*, *A. awamori* from the roots of *A. ascolonicum*, and *R. arrhizus* from the roots of *A. ascolonicum* showed broad antibacterial activity with inhibitory activity across all the bacterial pathogen tested. *A. hancockii* from the bulb of *C. glaucum* showed significant activity against only *P. aeruginosa* with an inhibition zone (14 mm) (Table S1, Figs. 4, 5). Agar-plug antifungal bioassay of endophytic fungi showed that among 17 endophytic fungi identified to harbor antimicrobial activity, 11 were shown to have activity against one or more tested fungal pathogens. *P. citrinum* from the seed of *P. nitida* and *T. yunnanense* from the bulb of *C. glaucum* showed broad-spectrum antifungal activity against *C. tropicalis*, *A. clavatus*, and *C. neoformans*. *A. aflatoxiformans* isolated from the bulb of *C. glaucum* and the seed of *P. nitida* showed inhibitory activity only on *C. neoformans* with inhibition zone diameters of (13 mm and 9 mm), respectively. *A. austwickii* from leaves of *A. ascolonicum* had activity only against *A. clavatus* while *T. pubescens* from roots of *A. ascolonicum* and *A. welwitschiae* from the stem of *E. laterifolia* showed activity only against *C. tropicalis* (Fig. 4). *A. austwickii* (PNS7) demonstrated good anti-pseudomonas activity with inhibition halos of 24 ± 0.7 mm which concurs with the report by Nwakanma et al. [29], whose endophytic fungi isolated from bush mango in Nigeria were reported to show anti-pseudomonas activity with maximum inhibition value of 9 mm. It is worth noting that isolates from this study; *A. austwickii* (AAL2X), *A. welwitschiae* (ELS1), *T. pubescens* (AAR2), *A. hancockii* (CGB8), *A. foetidus* (AAR4), *P. citrinum* (ELS2), *A. aflatoxiformans* (PNS1), *A. austwickii* (PNS7), and *P. citrinum* (PNS9) demonstrated both antibacterial and antifungal activities on agar plug bioassay. This report concurs with that of An et al. [30] whose endophytic fungi *Thanatephorus cucumeris* from *Chloranthus japonicus* showed both antibacterial and antifungal activity with wide pharmaceutical applications. The potent antifungal activity exhibited by *P. citrinum* (PNS9 and ELS2) concurs with the results of Firdausi et al. where endophytic *P. citrinum* showed great antifungal activity against onion purple blotch disease caused by *Alternaria porri*.

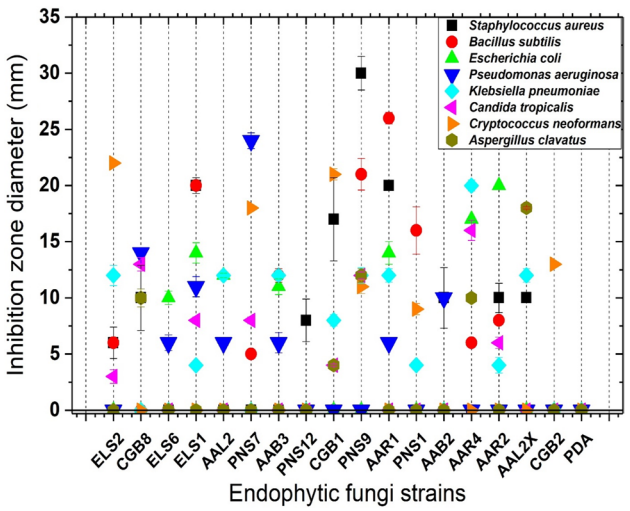
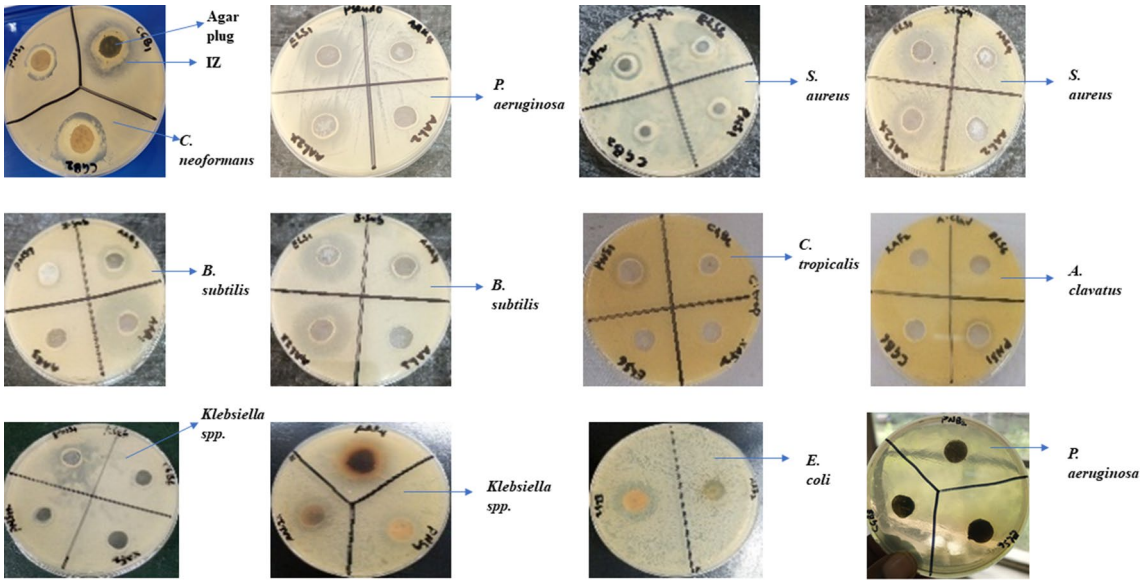


Fig. 4 The antimicrobial activity of culturable endophytic fungi isolates by agar plug assay. The colour codes represent different pathogens used in this study. Each pathogen was spread on the surface of agar using a sterile cotton swab. Six-millimeter diameter of actively growing fungal culture disc from each PDA plate of ten days old was cut using sterile cork-borer and placed on the surface of respective agar media (MHA) seeded with the test bacteria, while SDA media was used for fungi. The Petri dishes were incubated at 37 °C for 24 h for bacterial growth, and at 30 °C for 48 h for fungal growth. Antimicrobial activities were determined by measuring the diameter of the zone of inhibition in millimeters. The potato dextrose agar (PDA) plug represents the negative control. The figure was drawn and analyzed in OriginPRO 2017 (https://www.originlab.com/)

Cell-free broth antimicrobial bioassay results of endophytic fungi showed their ability to produce certain antibacterial and antifungal compounds (Fig. 6, Table S2). *A. welwitschiae* (ELS1) and *A. austwickii* (AAL2X) showed good activities against all bacteria pathogens tested except *E. coli*. *A. awamori* (AAL2) lost its antibacterial activities against all tested bacterial pathogens. *A. hancockii* (CGB8), *A. awamori* (ELS6), *A. austwickii* (PNS7), *A. luchuensis* (AAB3), *A. foetidus* (AAR4), and *A. Aflatoxiformans* (PNS1) exhibited inhibitory activities against a single bacterial pathogen. Broad-spectrum antibacterial activity was exhibited by *A. welwitschiae* (ELS1), *T. yunnanense* (AAB2), *T. pubescens* (AAR2), *A. austwickii* (AAL2X), *A. aflatoxiformans* (PNS12) and *T. yunnanense* (CGB1). All *Penicillium* and *Rhizopus* genera tested were only sensitive to Gram-positive pathogens. Several endophytic isolates which showed antifungal activity during agar plug bioassay were observed to lose their activities including *A. austwickii* (PNS7), *T. yunnanense* (CGB1), *A. aflatoxiformans* (CGB2), *A. hancockii* (CGB8), and *T. pubescens* (AAR2). However, *A. aflatoxiformans* (PNS1) exhibited good antifungal activity across all the tested fungi pathogens with zones of inhibition of 10 ± 0.7 mm, 15 ± 0.2 mm, and 10 ± 0.9 mm ,respectively, for *C. tropicalis*, *C. neoformans* and *A. clavatus*.

Conclusions

This study tried to explore several parts of the plant including the root, bulb, bark, leaves, and stem in search of novel isolates harboring antimicrobial activity. To the best of our knowledge, this study reported here for the first time the



Kleb- *Klebsiella pneumoniae*, E- *Escherichia coli*, Pseudo- *Pseudomonas aeruginosa*, B. sub- *Bacillus subtilis*, Staph- *Staphylococcus aureus, C. neo- Cryptococcus neoformans, A. clav- Aspergillus clavatus, C. trop- Candida tropicalis*, IZ- Inhibition zone.

Fig. 5 Pictorial view of some plates showing antagonistic activities of fungal endophytes against tested pathogens. Pathogenic organisms were swabbed on MHA (for bacteria) or SDA (for fungi), then either a 6 mm agar plug or 200 µL free cell broth of 14 weeks old endophytic fungi was placed aseptically on the plate or the bored 6 mm hole. The setup allowed for the diffusion of bioactive if any at 4 °C for 6 h. Incubation was done at 28 °C and 37 °C for 48 h and 24 h, respectively, for fungi and bacteria pathogens

presence of antimicrobial harboring endophytes in Nigerian *E. laterifolia* (stem), *P. guineense* (leaves), *X. aethiopica* (fruit), *A. ascolonicum* (leaves, bulb, root), *C. glaucum* (bulb) and *P. nitida* (bark). In conclusion, Nigerian ethnomedicinal plants possess diverse genera of endophytic fungi such as *Aspergillus*, *Trichoderma*, *Penicillium,* and *Rhizopus* harboring inhibitory activities against several pathogens, confirming that endophytic fungi could be a potential source of novel antimicrobial compounds for pharmaceutical application.

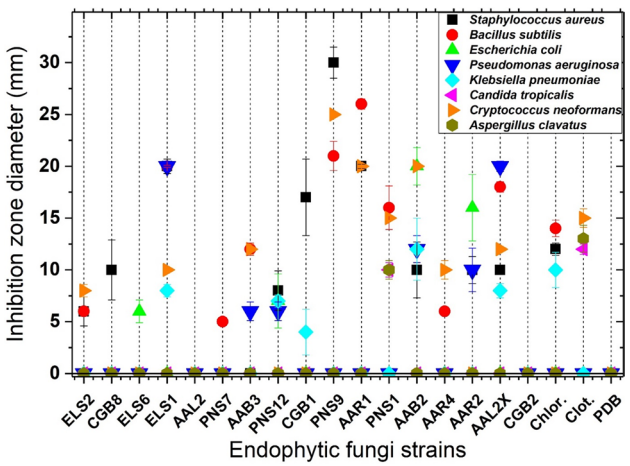


Fig. 6 The antimicrobial activity of cell-free broth from culturable endophytic isolates by agar well diffusion assay. The codes represent different pathogens used in this study. Chlor. and Clot. represent chloramphenicol and clotrimazole which served as positive controls while potato dextrose broth (PDB) represented the negative control. The figure was drawn and analyzed in OriginPRO 2017 (https://www.originlab.com/)

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Author Contributions CEE: conceptualization, methodology, validation, formal analysis, investigation, resources, data collection, writing original draft, writing review and editing, visualization. NHI: writing review and editing, project administration, funding acquisition. DHA: writing review and editing, supervision, project administration, funding acquisition, formal analysis. UEM: supervision, writing review and editing, project administration. All authors read and approved the final manuscript.

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Data Availability Sequence data are available at NCBI GenBank (https://www.ncbi.nlm.nih.gov/). Accession numbers are available in this report (Table 2).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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