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(RESEARCH ARTICLE)



The isolation, identification and antimicrobial activities of endophytic fungi from *Azadirachta indica*

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Abstract

Endophytic microorganisms are the storehouse of novel secondary metabolites that can serve as an excellent source of drugs for different ailments. In this study, we isolated endophytic fungi associated with *Azadirachta indica* collected from Agulu, Awka, Anambra State, South Eastern Nigeria, identified some of their bioactive secondary metabolites and evaluated their antimicrobial properties. The endophytic fungi were isolated following standard method and the pure culture of the isolates identified morphologically and molecularly. The fungi were subjected to solid-state fermentation on rice medium and the metabolites were extracted using ethyl acetate. The fungal crude extracts were screened for antimicrobial activity. The extracts were further subjected to high-performance liquid chromatography-diode-array detection (HPLC-DAD) analysis for the identification of the bioactive compounds. A total of four endophytic fungi identified as *Aureobasidium* sp. (AIL1), *Sodaria* sp. (AIL3), *Aspergillus* sp. (AIS1) and *Penicillium* sp. (AIS2) were isolated from the *Azadirachta indica*. The crude extracts at 1 mg/mL, showed varying degrees of antimicrobial activity against the test fungi and bacteria inhibition zone diameter (IZD) ranged from 2 to 11 mm. The minimum inhibitory concentrations (MICs) of the fungal extracts ranged from 0.0625 to 0.25 mg/mL. The HPLC-DAD analysis revealed the presence Glucobrassicin, Ferulic acid, 4-methoxybenzaldehyde, 12-Hydroxy-16-scalarin, 12-O-deacetyl-12-epi-scalarin, Ixoside, Citreodrimene F, and Cytosporin D, some of which has been reported to have shown antimicrobial activity. *Azadirachta indica* possess sundry groups of fungal endophytes that contain secondary metabolites with interesting antimicrobial properties that could be explored in drug discovery.

Keywords: *Azadirachta indica*; Endophytic fungi; Molecular identification; HPLC-DAD analysis; Antimicrobial activity

1. Introduction

Infectious and parasitic diseases account for approximately half of the deaths worldwide [1]. Plants have served as sources of medicinal bioactive compounds against numerous forms of ailments for centuries. All plants are inhabited internally by diverse microbial communities comprising bacteria, archaea, fungi, and protista [2, 3]. Amazingly, in recent

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years, microorganisms associated with plants rather than the plants themselves have proved to offer material and products with high therapeutic potential [4].

The term “endophyte” refers to all microorganisms that colonize internal plant tissues for all or part of their lifetime [3]. Endophytes are an under-investigated group of microorganisms that represent a plentiful and renewable source of bioactive and chemically new compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial realms [5]. The association between fungal endophytes and their host plant is due to the result of unique adaptations which enable the endophytes to harmonize their growth with that of their host [6]. They are a taxonomically and ecologically heterogeneous group of organisms; mainly belonging to Ascomycota, coelomycetes, and hyphomycetes [7, 8]. Recently, many known, as well as new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant, etc., have been identified [9,10,11,12,13,14,15].

Azadirachta indica is a fast-growing evergreen tree common in Afro-Indian regions as well as America [16]. Extracts of the fresh leaves of this plant are reported in folk medicine for their antimicrobial, antimalarial, anthelmintic, antiviral, antiulcer actions [17,18].

An endophyte *Geotrichum* sp., isolated from the leaves of the *A. indica* has been reported to produce two new chlorinated epimeric 1, 3-oxazinane derivatives that have significant activity against the nematodes *Bursaphelenchus xylophilus* and *Panagrellus redivevus* [19]. ‘Lavanicin’ an antibacterial naphthaquinone was also isolated and characterized from the endophytic *Chloridium* sp. obtained from root tissues of the *A. indica* A Juss. This highly functionalized naphthaquinone exhibited strong antibacterial activity against *Pseudomonas* sp. which are pathogenic to both humans and plants [19]. This study aims at isolating some endophytic fungi from the leaves and stem of *A. indica*, as well as secondary metabolites of these fungi and then investigating the biological potentials of the isolated metabolites. To the best of our knowledge, this is the first report on the isolation of the fungi –*Sordaria* sp., and *Aureobasidium* sp. from *A. indica*.

2. Material and methods

2.1. Plant material

Fresh leaves and stem of *A. indica* were collected from Agulu, Awka, Anambra State, South-East Nigeria and the plant materials was authenticated by the taxonomist at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University in Agulu of Nigeria. A voucher specimen (PCG474/A/046) was deposited in the herbarium of the above Department.

2.2. Test organisms

A total of seven clinical microbial (Five bacterial and two fungi) strains: - *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Aspergillus niger* and *Candida albicans* were used to evaluate the antimicrobial activity of the isolated fungal endophyte extracts.

2.3. Isolation and purification of endophytic fungi

The collected plant materials were subjected to surface sterilization and isolation procedures following the method of Arnold et al. [20] with little modifications. Briefly, the plant materials were first washed several times under running tap water, followed by washing in distilled water. Surface sterilization was then done by sequentially rinsing the plant materials with 70% ethanol for 30s; 2% sodium hypochlorite (NaOCl) for 2–3 min; rinsing in another 70% ethanol for 2 min and finally with sterile distilled water for 2–3 times. The plant materials were then dried in between folds of sterile filter papers under aseptic conditions. After sterilization, the plant materials were further cut (aseptically) to expose the interior surface to the Malt Extract Agar. The Leaf segments were placed on 9 cm Petri plates containing malt extract agar (MEA) medium amended with chloramphenicol 500 mg/mL to suppress bacterial growth [21]. The dishes were sealed with masking tapes and incubated at 28 ±1 °C for 3-7 days. Within the days of incubation, the hyphal tips of fungi growing out from the samples were subsequently transferred onto fresh MEA plates. The procedure of transferring to fresh MEA plates carried out several times in order to obtain pure culture of fungi. The isolated endophytic fungus was subjected to solid state fermentation in 1L Erlenmeyer flask containing sterilized rice medium (prepared by autoclaving a mixture of 100 g of rice and 00 ml of distilled water). The flask was inoculated with agar blocks containing the fungus and incubated at 25-27°C for 21 days. At the completion of fermentation, the fungal secondary metabolites were extracted with ethyl acetate and then concentrated under vacuum at 40 °C.

2.4. Identification of the isolated endophytic fungi

2.4.1. Macroscopic study

Morphological study was done by plating the fungi on MEA and incubating for 7 days. The growth appearance was then noted by observing both the back and front views of the plates.

2.4.2. Microscopic study

Unknown endophytic fungi were identified by studying their cultural characteristics, spore formations and mycelium. Slides were prepared by tease mount method using Lactophenol Cotton Blue reagent and observed at $\times 40$ and $\times 100$ magnifications.

2.4.3. Molecular identification

DNA extraction and PCR

DNA was extracted from culture of endophytic fungi mycelia using a ZR Fungal/Bacterial DNA MiniPrep™ Kit from Zymo Research Corp, USA according to the manufacturer's protocols. Polymerase Chain reaction (PCR) was then conducted to amplify the internal transcribed spacer (ITS) region of the extracted DNA, including the 5.8s rDNA, using the primers ITS1 (with base sequences 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (with base sequences 5'TCCTCCGCTTATTGATATGC-3') under the following conditions: Initial denaturation at 90°C for 15 min; denaturation at 95°C for 1 min; annealing at 56°C for 1 min and extension at 72°C for 1 min. The PCR process lasted a total of 35 cycles and ended with the extension at 72°C for 10 minutes.

Sequencing

The PCR products were sequenced and the base sequences were compared to the data in Genebank. Blast search of the sequences, to identify the DNA sequence of the fungi, was performed following the link:

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

2.5. Screening for antimicrobial activity/determination of minimum inhibitory concentrations (MICs)

The antimicrobial screening and MIC determination were carried out following our previous method [15].

2.6. High performance liquid chromatography-diode array detection (HPLC-DAD) assay

HPLC analysis of the endophyte extract was carried out using a Dionex attached to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Two milligram (2mg) was reconstituted with 2 ml of HPLC grade methanol, the mixture was sonicated for 10 min and centrifuged at 3000 rpm for 5 min. Afterward 100 μ L of the dissolved samples were transferred into HPLC vials containing 500 μ L of the HPLC grade methanol for analysis. Detection was at 235, 254, 280, and 340 nm. The separation column (125 mm \times 4 mm; length \times internal diameter) was pre-filled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The absorption peaks for each of the 10 dried fungal metabolite extract were analysed by comparing with those in the HPLC-ultraviolet (UV)/visible database, which contains over 1600 registered compounds.

2.7. Statistical analysis

Results of the study were presented as mean \pm Standard error of mean (SEM) of sample replicate, n=5. Raw data were analyzed by one way analysis of variance (ANOVA) followed by post-hoc Turkey's test and independent students't-test. Bivariate correlation was used to assess the relationship of the biological assays. The analysis was done using statistical package for social Sciences (SPSS) version 20 for windows. Statistical significance was established when $P < 0.05$. Graphical illustration was carried out using Microsoft excel, 2007.

3. Results

A total of four endophytic fungi coded (AIL1, AIS1, AIS2 and AIL3) were isolated from *Azadirachta indica*. The isolated fungi were generally identified macroscopically and microscopically based on their morphological appearances (Fig.1 and Table 1). The endophytic fungi AIL1, AIS1 and AIS2 were identified as *Aureobasidium* sp., *Aspergillus* sp. and *Penicillium* sp. respectively (Table 1). The fungus AIL3 was further identified using molecular biology protocols (Table 2). The DNA sequences were determined using BLAST and comparing data at the NCBI GenBank database. The fungus AIL3 was identified using standard Polymerase Chain Reaction (PCR) analysis by ITS-rDNA amplification method as *Sordaria* sp., a member of Sordariaceae family respectively, with maximum identity and characteristic of 100% (Table 2).

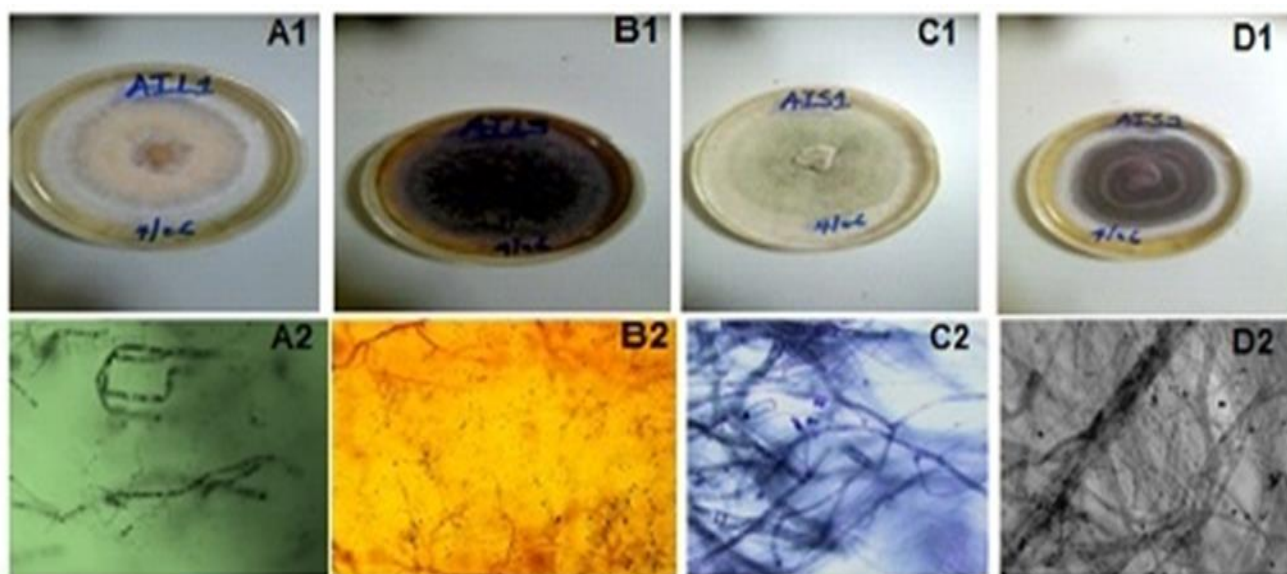


Figure 1 Macroscopic (A1, B1, C1 and D1) and microscopic (A2, B2, C2 and D2) characteristics of the isolated endophytic fungi (AIL1, AIL3, AIS1 & AIS2) isolated from the leaves and stem of the medicinal plant *Azadirachta indica* respectively

Table 1 Fungal endophytes isolated from *Azadirachta indica* the parts of the plants used, identity of the fungi, class, and references.

S/n	Fungi codes	Plant source	Plant parts	Identified as	Class	References
1	AIL1	<i>A. indica</i>	Leaf	<i>Aureobasidium</i> sp.	Hyphomycetes	[22,23]
2	AIL3	<i>A. indica</i>	Leaf	<i>Sordaria</i> sp.	Ascomycetes	[22]
3	AIS1	<i>A. indica</i>	Stem	<i>Aspergillus</i> sp.	Basidiomycetes	[22,24]
4	AIS2	<i>A. indica</i>	Stem	<i>Penicillium</i> sp.	Hyphomycetes	http://blast.ncbi.nlm.nih.gov/Blastin Genbank

Table 2 Molecularly identified endophytic fungus (*Sordaria* sp.)

Isolate code	Genbank number	accession	Strain reference	and % Identity	Query length	Isolate name
AIL3	KF435432.1		P44E2 [25]	100	513	<i>Sordaria</i> sp.
CGAATCTTACCCGTACGGTTGCCTCGGCGCTGGCGGTCCGGAAGGCCCTCGGGCCCCGGACCCTCGGGTCTCCCCTCGCGG GAGGCTGCCCGCCGAGTGCCGAAACCAACTCTTGATATTTTTGTCTCTCTGAGTAAACTTTTAAATAAGTCAAACTTT						

CAACACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
 TCATCGAATCTTTGAACGCACATTGCGCTCGCCAGTATTCTGGCGAGCATGCCTGTTTCGAGCGTCATTTCAACCATCAAGCT
 CTGCTTGCCTTGGGGATCCGCGGCTGCCCGGGTCCCTCAAAAACAGTGGCGGGCTCGCTAGTCACACCGAGCGTAGTAACT
 CTACATCGCTATGGTCTGCGGCGGGTTCTTsGCCGTA AAAACCCCAATTTCTAAGGTTGACCTCGGATCAGGTAGGAATAC
 CCGCTGAACTTAAGCATATCAATAA

The result of the antimicrobial activity of the fungal extracts is shown in Table 3. The fungal extracts at 1 mg/mL demonstrated varying degrees of antimicrobial activity against the test bacteria and fungi. Extract of AIL1 (*Aureobasidium* sp.) showed antibacterial activity with IZD in the range of 4 to 8 mm against *P. aeruginosa*, 2 to 7 mm against *S. typhi* and 2 to 6 mm against *B. subtilis*. It showed antifungal activity against *C. albicans*, IZD in the range of 2 to 4 mm. Extracts of AIL3 (*Sordaria* sp.) demonstrated antibacterial activity against all the bacterial strains tested with IZD ranging from 3 to 8 mm for *S. aureus*, 4 to 11 mm for *P. aeruginosa*, 2 to 6 mm for *S. typhi*, 3 to 10 mm for *K. pneumoniae* and 2 to 5 mm for *B. subtilis* but exhibited no antifungal activity against the tested fungi. Extract of AIS1 (*Aspergillus* sp.) showed antibacterial activity against Gram-negative *P. aeruginosa* (IZD, 2 to 7 mm), *S. typhi* (IZD, 3 to 6 mm) and Gram-positive *B. subtilis* (IZD, 2 to 6 mm). The fungal extract additionally showed antifungal activity against *C. albicans* (IZD, 2 to 5 mm). Extract of AIS2 (*Penicillium* sp.) demonstrated antibacterial activity against all the Gram-negative bacteria tested giving an inhibition zone diameter in the range of 2 to 8 mm for *P. aeruginosa*, 2 to 6 mm for *S. typhi* and 2 to 6 mm for *K. pneumoniae*. Antifungal activity was shown against *C. albicans* (IZD, 2 to 3 mm).

Table 3 Antimicrobial activity of the endophytic fungi (zones of inhibition in mm)

Test organisms	Endophytic fungal extract (1 mg/mL)				Positive Control	Negative control
	AIL1	AIL3	AIS1	AIS2	Ciprofloxacin (5 µg/mL)	DMSO (100% v/v)
<i>S. aureus</i>	0	8	0	0	6	0
<i>P. aeruginosa</i>	8	11	7	8	4	0
<i>S. typhi</i>	0	6	6	6	10	0
<i>K. pneumoniae</i>	7	10	0	6	24	0
<i>B. subtilis</i>	6	5	6	0	8	0
					Miconazole (50 µg/mL)	DMSO (100% v/v)
<i>C. albicans</i>	4	0	5	3	15	0
<i>A. niger</i>	0	0	0	0	8	0

The result of the Minimum Inhibitory Concentrations (MICs) of the different fungal endophyte crude extracts is shown in Table 4. Extract of AIL1 had MIC of 0.125 mg/mL for *K. pneumoniae*, 0.25 mg/mL for *C. albicans* and 0.25 mg/mL for *P. aeruginosa*. Extract of AIL3 had an MIC of 0.25 mg/mL for *S. aureus* and *B. subtilis*; 0.125 mg/mL for *S. typhi* and 0.0625 mg/mL for *P. aeruginosa* and *K. pneumoniae*. Extract of AIS1 showed MIC of 0.25 mg/mL for *B. subtilis*, *S. typhi* and *C. albicans* and 0.125 mg/mL against *P. aeruginosa*. Extract of AIS2 had MIC of 0.125 mg/mL for *B. subtilis*, *P. aeruginosa*, *S. typhi* and *K. pneumoniae* and MIC of 0.5 mg/mL for *C. albicans*. The chromatograms of the isolated fungal endophytes extracts as revealed by High Pressure Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis are shown in Figures 2 numbered 1, 2, 3 and 4 for AIL1, AIL3, AIS1 and AIS2 respectively.

Table 4 Minimum inhibitory concentrations (MICs) of the fungal extracts

Fungal extracts	Minimum inhibitory concentration (MIC) mg/mL						
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>S. typhi</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>A. niger</i>	<i>C. albicans</i>
AIL1	-	0.125	-	0.125	0.125	-	0.25
AIL3	0.25	0.25	0.125	0.0625	0.0625	-	-

AIS1	-	0.25	0.25	0.125	-	-	0.25
AIS2	-	-	0.125	0.125	0.125	-	0.50

S. aureus = *Staphylococcus aureus*, *B. subtilis* = *Bacillus subtilis*, *S. typhi* = *Salmonella typhi*, *P. aeruginosa* = *Pseudomonas aeruginosa*, *K. pneumoniae* = *Klebsiella pneumoniae*, *A. niger* = *Aspergillus niger*, *C. albicans* = *Candida albicans*

The bioactive compounds identified/detected at different peak levels are represented alphabetically. The UV spectra of the detected compounds are shown in Figure 3: these include **(A)** Glucobrassicin from **AIL1**; **(B)** Ferulic acid from **AIL3**; **(C)** 4-methoxybenzaldehyde, **(D)** 12-Hydroxy-16-scalarin, **(E)** 12-O-deacetyl-12-epi-scalarin, **(F)** Ixoside from **AIS1**; and **(G)** Citreodrimene F, **(H)** Cytosporin D from **AIS2**. Some of the detected compounds were unknown (Figures 3).

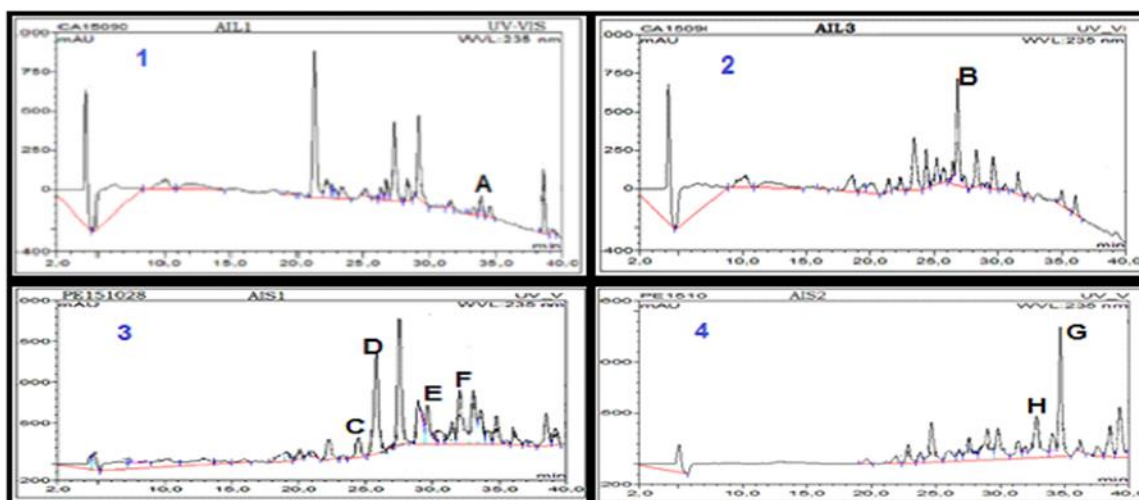


Figure 2 HPLC-DAD chromatograms (1, 2, 3 and 4) of the extracts of the endophytic fungi (AIL1, AIL3, AIS1 and AIS2) respectively isolated from *Azadirachta indica* showing the detected bioactive compounds.

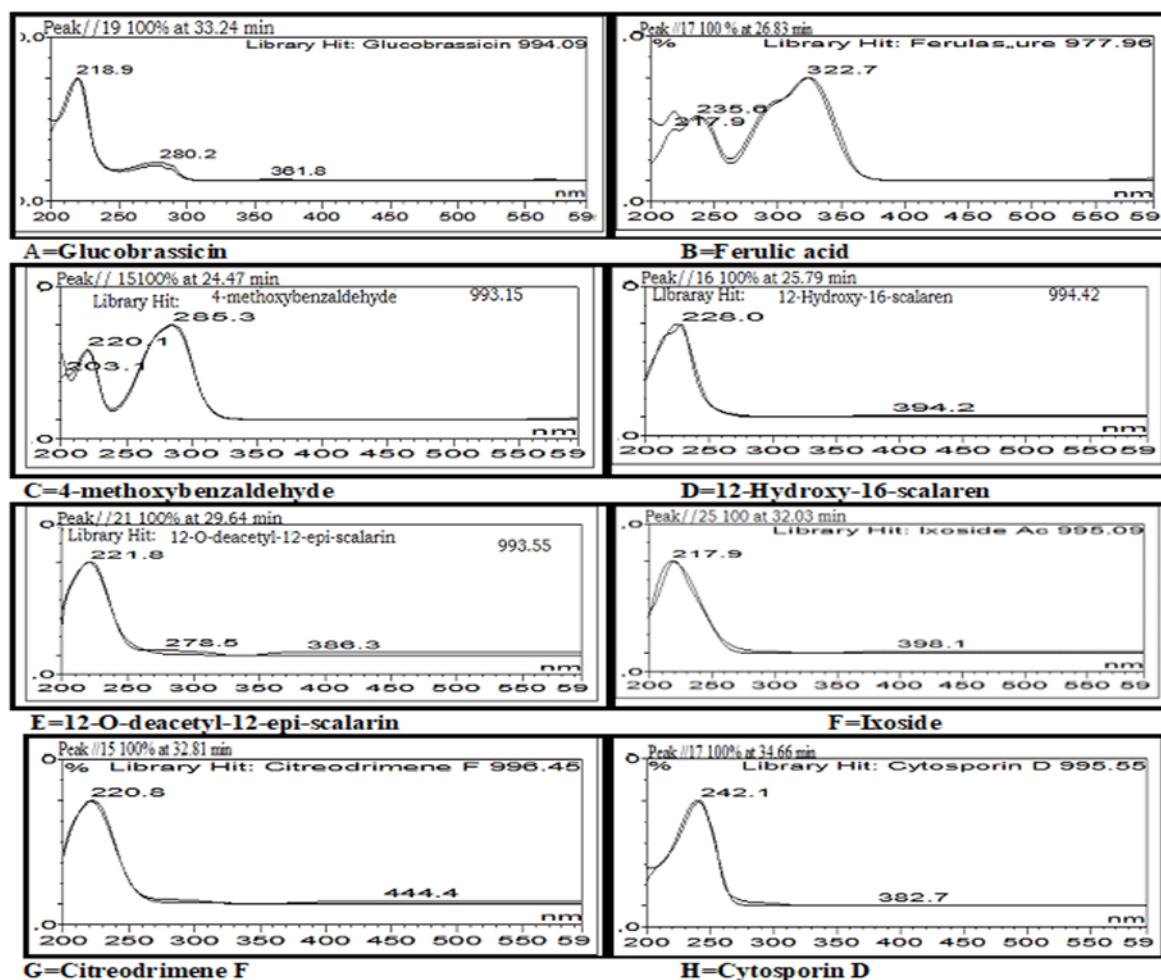


Figure 3 UV-spectra of the bioactive compounds detected (A-H)

4. Discussion

Endophytic microorganisms are ubiquitous, having been encountered in many plant species growing under natural conditions [26, 27]. Endophytic fungi, in particular, have been investigated in a variety of plants ranging from grasses [28, 29] to trees [12,15,30,31].

In this study, a total of four (4) endophyte fungal isolates were obtained from the leaves and stem segments of *Azadirachta indica*. Morphological (macroscopic and microscopic) characteristics (Fig. 1) resulted in the identification of three of the endophytic fungi isolates AIL1, AIS1 and AIS2 as *Aureobasidium* sp., *Aspergillus* sp., and *Penicillium* sp. respectively (Table 1). One of the endophytic fungal isolates (AIL3) was further identified by internal transcribed spacer. The BLAST search sequence similarity found against the existing non-redundant nucleotide sequence database identified the percentage of similarity between the fungus and database. The fungi AIL3 was confirmed as *Sordaria* sp., strain-P44E2 and given accession number-KF435432.1; in NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 2).

The isolated fungal endophytes belong to classes Hyphomycetes, Ascomycetes and Basidiomycetes. Out of the total fungal endophytes isolated, half (50%) of the genera are from hypomycetes. Verma et al. (2011) [32] isolated *Alternaria* sp., *Acromonium* sp., *Cladosporium* sp. and *Aspergillus* sp. from *Azadirachta indica*. The group also reported the presence of *Pestalotiopsis*, *Trichoderma*, *Curvularia* and *Penicillium* sp. Taware and Rajurkar [8] also reported the isolation of *Alternaria* sp. and *Trichoderma* sp. from some Nigerian medicinal plants. Our study is the first report on the isolation of *Sordaria* sp. and *Aureobasidium* sp. from *Azadirachta indica*. It is possible that our findings differed from the previous reports due to factors such as geographical and seasonal differences

In recent years, considerable attention has been given to fungi and this has resulted in a large number of studies involving a variety of approaches [27, 32]. Focus has also been on the exploration of secondary metabolites of fungi to obtain new bioactive molecules with potential applications in the medical, pharmaceutical, industrial, agricultural and environmental fields [12,13,31,]

HPLC analysis revealed the presence of some bioactive compounds in the extracts of the isolated fungi. Citreodrimene F and Cytosporin D were previously reported to have antimicrobial, antiplasmodial and immunomodulatory properties [33, 34, 35]. Some of the extracts of the endophytic fungal isolates contain many undetected/unknown bioactive compounds which could also be contributing to the biological activities they displayed. The undetected compounds or compounds whose spectra had no library hit/match may represent important or novel bioactive compounds with interesting pharmaceutical or industrial applications [14].

Ethyl acetate extract of *Areobasidium* sp. (AIL1) exhibited antibacterial activity against *K. pneumoniae*, *P. aeruginosa* and *B. subtilis* but lacked activity against *S. aureus* and *S. typhi*. In addition, the extract showed antifungal activity against *C. albicans* but was in-active against *A. niger*. The identity of the major bioactive compounds in the extract could not be revealed by HPLC-DAD analysis.

Extract of *Sordaria* (AIL3) was most active of all, showing a broad-spectrum antibacterial activity against the test Gram-positive and Gram-negative bacteria (*S. aureus*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae* and *B. subtilis*) though it showed no antifungal activity against the test fungi; *C. albicans* and *Aspergillus niger*. The HPLC-DAD analysis of the extract revealed that it contains the bioactive compound ferulic acid which was previously reported to inhibit the growth of both Gram-positive and negative bacteria [34]. The result of this study is consistent with their findings. Extract of *Aspergillus* sp. (AIS1) showed antimicrobial activity against *S. subtilis*, *P. aeruginosa*, *S. typhi* and *C. albicans* but no activity against *S. aureus*, *K. pneumoniae* and *A. niger*. Extract of *Penicillium* sp. (AIS2) showed antimicrobial activity against all the test Gram negative bacteria but showed no activity against the Gram-positive ones.

The antimicrobial activity of the fungal endophytes extracts against the test microorganisms (bacteria and fungi) was moderate. Thus, it could be inferred that extracts of the endophytic fungi from *A. indica* make up a reliable source of bioactive compounds, as shown by the wide range of compounds with diverse biological properties present in these extracts.

5. Conclusion

Our results showed the diversity of endophytic fungi in the leaves and stems of *A. indica* and significant of these fungi as promising source for bioactive compounds with antimicrobial potential. Additional studies are required to identify the unknown compounds detected in the extracts.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors Ujam Nonye T., Abba Chika C., Eze Peter M., Oli Angus N., Ejikeugwu Chika, Ugwu Malachy C., Okoye Festus B.C. and Esimone Charles O. declare that they have no conflict of interest.

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