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Research Article

Isolation, characterization, and screening of secondary metabolites from endophytic fungi isolated from Nigerian *Piliostigma thonningii* for antimicrobial activities

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Abstract

Piliostigma thonningii leaves are used in Nigeria folk medicine for the treatment and management of diverse ailments. This study was carried out to investigate the antimicrobial activity of the secondary metabolites produced from endophytic fungi isolated from the leaves of *Piliostigma thonningii*. The isolation of endophytic fungi was carried out according to the standard procedures. The fungi were subjected to solid-state fermentation on rice medium and the secondary metabolites extracted using ethyl acetate. The fungal crude extracts were screened for antimicrobial activity against selected clinically important microbes using the agar well diffusion method. The fungus with the best antimicrobial activity was molecularly characterized and its secondary metabolites profiled using Gas Chromatography - Mass Spectroscopy to establish the correlation between the observed activity and its phytochemical composition. The fungal extracts showed antimicrobial activity with inhibitory zone diameter ranging from 2.00 to 9.00 mm. The secondary metabolites of *Aspergillus fumigatus* (the most bioactive endophyte) contain 50 compounds with several of them having established antimicrobial activity. The results of this study suggest that *Aspergillus fumigatus* an endophytic fungus associated with *P. thonningii* could be a promising source of novel bioactive compounds with pharmaceutical, agricultural and industrial importance.

Keywords: Antimicrobial, Endophytic fungi, GC-MS, *Piliostigma thonningii*

INTRODUCTION

There is an increasing desire for new and safer bioactive compounds in all aspects of medicine[1]. Nature notably plants, fungi, bacteria, and endophytes has consistently provided mankind with a vast array of pharmacologically active moieties capable of being explored as pharmaceutical targets for the treatment of new and existing diseases or as lead molecules for the development of synthetically derived drugs[2,3]. Endophytes inhabiting medicinal plants have been shown to be a consistent source of novel lead molecules with antimicrobial, anti-inflammatory, anticancer, and antiviral properties among others which have greatly aided drug discovery[4,5].

In Africa, *Piliostigma thonningii* Schum (family Fabaceae; common names, monkey bread and camel's foot) is a perennial tree with diverse ethnomedicinal and economic applications[6-9] like its use as an antipyretic[7,9,10] antimicrobial[11,12], anthelmintic[13], anti-inflammatory[11] agent among others. Locally in Nigeria, the plant is known as "*Abafe*" in Yoruba, "*Kalgo*" in Hausa, and "*Okpoatu*" in Igbo[9,14].

The rich plant biodiversity of Nigeria has been reported to contain diverse endophytic microbial communities which present the opportunity for the discovery of biologically important compounds of pharmaceutical and industrial importance[4,15-26]. There has been no report of the isolation or screening of secondary metabolites of any endophyte associated with *P. thonningii* hence our study seeks to isolate and investigate the secondary metabolites of an endophytic fungi isolated from leaves *P. thonningii* growing in Sokoto State, North-Western Nigeria.

METHODS

Plant collection, authentication and preparation

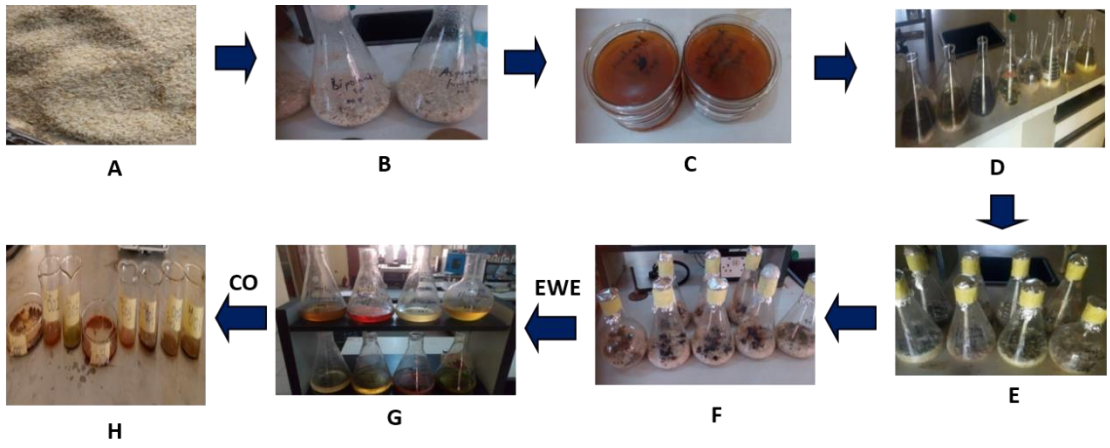
Fresh and healthy leaves of *P. thonningii* were collected, authenticated by a plant taxonomist, deposited with specimen voucher number UDUH/ANS/0137 issued.

Endophytic fungi isolation

The isolation of endophytic fungi from the plant leaves was carried out according to the procedures outlined by Eze *et al*.[15] and Abba *et al*.[16]. The plant leaves were washed thoroughly under running tap water to remove the debris adhered and finally washed with double distilled deionized water. The samples were cut into a 1 cm^2 segments before being surface sterilized by sequential washes in different concentrations of sterilizing agents (2% sodium hypochlorite solution for 2 min and 70% ethanol for 2 min), rinsed with distilled water and allowed to air dry under sterile conditions The efficiency of the surface sterilizations was confirmed by the imprint method as outlined by Schulz *et al*.[27]. The sample washed distilled water was inoculated into broth media as a control to check growth in liquid medium. The dissected tissues were inoculated on to malt extract agar (MEA) supplemented with 50 mg/L chloramphenicol Petri dishes. The Petri dishes were then monitored for 5 - 6 days of incubation at 28°C. Hyphal tips from distinct colonies emerging from leaf segments were sub-cultured onto fresh MEA plates to obtain pure colonies using the single spore technique[28,29].

Endophytic fungi cultivation, induction and extraction of secondary metabolites

A 1L Erlenmeyer flask containing autoclaved sterile solid rice medium (100 g rice and 200 mL distilled water) was inoculated with 3 mm diameter agar blocks cut out from malt extract agar plates containing pure culture of each of the fungi. The inoculated flasks were incubated at 27-30°C for 21 days to induce secondary metabolites production. At the completion of fermentation, the fungal secondary metabolites were extracted with ethyl acetate and the crude extracts concentrated using rotary evaporator at 40°C under reduced pressure. The scheme for the cultivation and extraction of the secondary metabolites from the isolated endophytic fungi is represented in Figure 1 below:



A = Local rice medium; B = Autoclaved local rice; C = Agar blocks of pure endophytic fungi culture; D = Inoculated fermentation media; E = Fermentation (Day 1); F = Fermentation (Day 21); G = Extracted secondary metabolites; H = Crude endophytic fungi extracts; EWE = Extraction with ethyl acetate; CO = Concentration and open drying.

Figure 1: Fermentation and extraction of secondary metabolites of endophytic fungi

Test microorganisms

Confirmed clinical *Staphylococcus aureus; Bacillus subtilis; Pseudomonas aeruginosa; Salmonella species; Klebsiella pneumoniae, Candida albicans*, and *Aspergillus niger* microorganisms were used for the study.

Antimicrobial assay

Antibacterial and antifungal screening of the secondary metabolites of the isolated endophytic fungi was carried out using the agar well diffusion method previously described by Akpotu *et al*.[19,20]. A concentration of 1 mg/mL was prepared for the secondary metabolites by dissolving it in 100% DMSO. A volume of 20 mL of molten Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (for the antibacterial and antifungal tests respectively) were poured into sterile Petri plates (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes (6 mm diameter) were made in the agar plates using a sterile metal cork-borer. A volume of 20 µL of the endophytic fungus secondary metabolites were put in each hole under aseptic condition, kept at room temperature for one hr to allow the agents to diffuse into the agar medium, and then incubated at 37°C for 24 h (for bacteria) and 28°C for 72 h (for fungi). Ciprofloxacin (5.0 µg/mL) and miconazole (50.0 µg/mL) were the positive controls that were used for the antibacterial and antifungal tests respectively; while 100% DMSO was used as the negative control in both tests. The inhibition zone diameters (IZDs) were measured and recorded. The procedure was conducted in triplicate, and the mean IZDs calculated. The identity of the endophytic fungus with the most promising antimicrobial activity was confirmed and subsequently profiled by Gas Chromatography – Mass Spectroscopy to identify its phytoconstituents.

Endophytic fungus identification

The most bioactive endophytic fungus was identified morphologically, microscopically and molecularly using DNA amplification and sequencing of the fungal ITS region[30]. Extraction of the fungal deoxyribonucleic acid (DNA) was done using Zymo fungal / bacteria DNA extraction kit (Zymo Research Corp., South Africa) according to the manufacturer's instructions. Polymerase chain reaction was carried out to amplify the ITS gene of specific DNA of the fungus using the primer pair ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4

(5′- TCCTCCGCTTATTGATATGC-3′). ExoSAP was used to purify the amplified product, and Sanger sequencing was performed with Nimagen's BrilliantDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000. The endophytic fungus was identified using Basic Local Alignment Search Tool (BLAST) sequence match procedures to compare the amplified sequence to ITS sequence data from strains in the US National Centre for Biotechnology Information (NCBI) database.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The GC-MS analysis was carried out using GC-MS-QP 2010 Plus Shimadzu system and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: Column Elite-1 fused silica capillary column (30 m × 0.25 mm 1 D × µL df, composed of 100% dimethyl polysiloxane). For GC-MS operation, an electron ionization system with an ionization energy of 70 eV was used. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1 mL minG1 and an injection volume of 2 µL. Split ratio 10:1 injector temperature (250 EC) and ion-source temperature of 280 EC were used. The oven temperature was programmed from 110 EC (Isothermal for 2 min) with an increase of 10 EC min G1 to 200 EC then 5-280 EC min G1, ending with a 9 min isothermal at 280 EC. Mass spectra were taken at 70 eV, a scan interval of 0.5 sec and fragments from 40-550 Da. Total GC running time was 60 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The mass spectra of the components were matched with the data available in the National Institute of Standards and Technology (NIST) library Ver. 2.0 2008 library where the retention time and chemical name of the components of the extract were revealed[31].

Statistical analysis

The result of the antimicrobial assay of the endophytic fungi extract was presented as mean ± standard error of mean IZDs, statistical significance was set at $p<0.05$ at 95% confidence level.

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Eight strains of endophytic fungi were isolated and identified from *P. thonningii* leaves as listed in Table I.

Table I: Endophytic fungi isolated from the leaves of *P. thonningii*

Code	Preliminary Identification
EDP 1	*Aspergillus fumigatus*
EDP 2	*Curvularia* sp.
EDP 3	*Curvularia* sp.
EDP 4	*Alternaria alternata*
EDP 5	*Bipolaris* sp.
EDP 6	*Rhizoctonia* sp.
EDP 7	*Rhizoctonia* sp.
EDP 8	*Pleospora* sp.

External factors such as weather, nutrient availability, plant species, location, soil composition and water have been reported to determine the composition of microbiomes inside plants[32].

Antimicrobial assay

The *in vitro* susceptibility of the secondary metabolites of the endophytic fungi when challenged with Gram-positive, Gram-negative bacteria and fungi is presented in Table 2. All of the crude extracts of fungi isolates inhibited at least one of the microorganisms studied.

Table 2: Inhibition zone diameters (mm) obtained from the secondary metabolites of isolated endophytic fungi

Secondary Metabolites	MCOs/ IZDs (mm) at 1.00 mg/mL				
	SA	*EC*	*KP*	*AN*	*CA*
EDP 1	9.0 ± 0.7^{a}	7.0 ± 0.0^{a}	8.0 ± 0.0^{a}	4.0 ± 0.7^{a}	6.0 ± 0.0^{a}
EDP 2	-	6.0 ± 0.0^{a}	-	-	-
EDP 3	7.0 ± 1.4^{a}	6.0 ± 0.7^{a}	-	2.0 ± 0.7^{a}	3.0 ± 0.7^{b}
EDP 4	3.0 ± 0.7^{b}	-	2.0 ± 0.7^{b}	-	-
EDP 5	-	-	-	1.0 ± 0.0^{a}	2.0 ± 0.0^{b}
EDP 6	-	2.0 ± 0.0^{b}	3.0 ± 0.7^{b}	-	-
EDP 7	3.0 ± 0.0^{b}	7.0 ± 0.0^{a}	-	-	-
EDP 8	-	2.0 ± 0.7^{b}	5.0 ± 0.7^{b}	-	-
CIP (5 µg/mL)	15.0 ± 0.7^{c}	18.0 ± 0.7^{c}	15.0 ± 0.7^{c}	NT	NT
MICO (50 µg/mL)	NT	NT	NT	14.0 ± 0.0^{b}	11.0 ± 0.4^{c}
DMSO	-	-	-	-	-

Values are presented as mean ± standard error of the mean of three determinations. Values with the same and different superscripts in the same column are significantly not different and significantly different at $p < 0.05$.

Key: EDP = Endophyte; CIP = Ciprofloxacin; IZDs = Inhibition Zone Diameters; MCOs = Microorganisms; MICO = Miconazole; NT = Not Tested; (-) = No Activity; *SA = Staphylococcus aureus; EC = Escherichia coli; KP = Klebsiella pnuemoniae; AN = Aspergillus niger*; *CA = Candida albicans.*

Among the tested secondary metabolites, *A. fumigatus* exhibited the highest inhibition of the growth of all the tested pathogenic microorganisms presenting an inhibition zone with diameters measuring between 4 mm for *A. niger* and 9 mm for *S. aureus.* The other extracts displayed significantly smaller inhibition zones when compared to *A. fumigatus.* For this reason, this fungus was regarded as the most bioactive fungus.

Identification of the most bioactive endophytic fungus

The morphological and microscopic confirmation of the most bioactive endophytic fungus is presented in Tables 3 and 4, and the molecular identification techniques and confirmation from the DNA sequence data deposited in the NCBI database (GenBank) with accession number KC 119199[33] are presented in Figure 2.

Table 3: Morphological characteristics of endophytic fungus EDP 1

Characteristics	Observation
Surface colour	Green to dark green
Growth	Rapid
Margins	Entire
Reverse side	Yellow

Table 4: Microscopic characteristics of endophytic fungus EDP 1

Characteristics	Observation
Hyphae	Branched septate
Conidiophore	Present
Vesicle	Dome shaped
Conidia	Present
Phialides	Uniseriate
Fruiting body	Cleistothecia

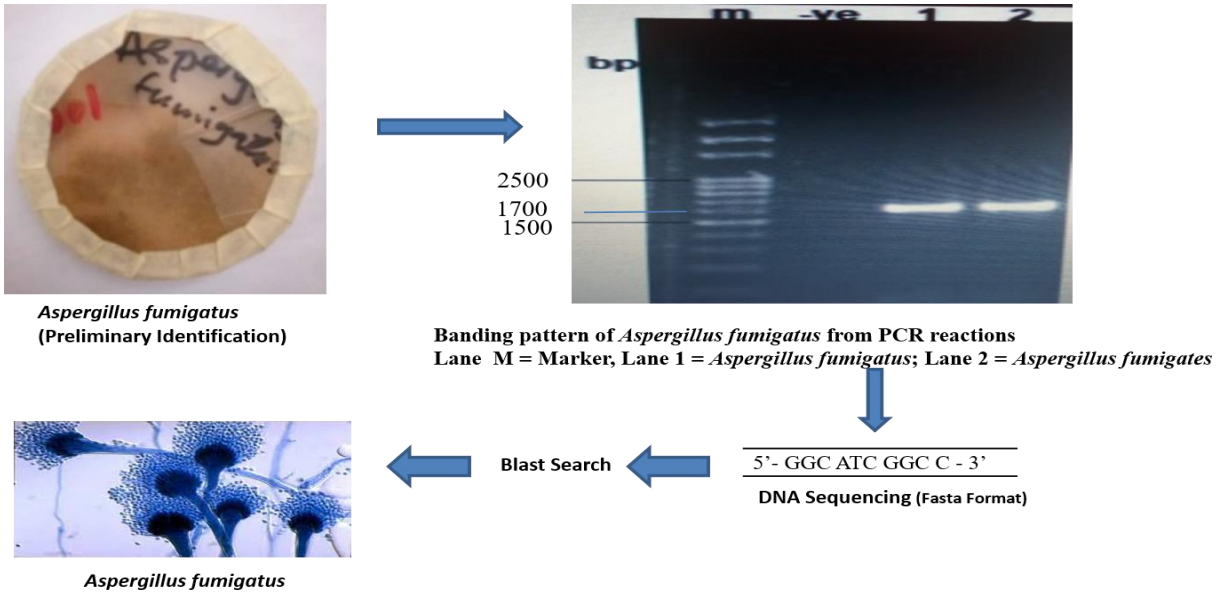


Figure 2: Molecular characterization of EDP 1

Identification of compounds by gas chromatography-mass spectrometry (GC-MS)

The GC – MS profile of the secondary metabolite of *A. fumigatus* revealed the occurrence of 50 peaks corresponding to the presence of 50 chemical compounds as presented in Figure 3 and Table 5 respectively.

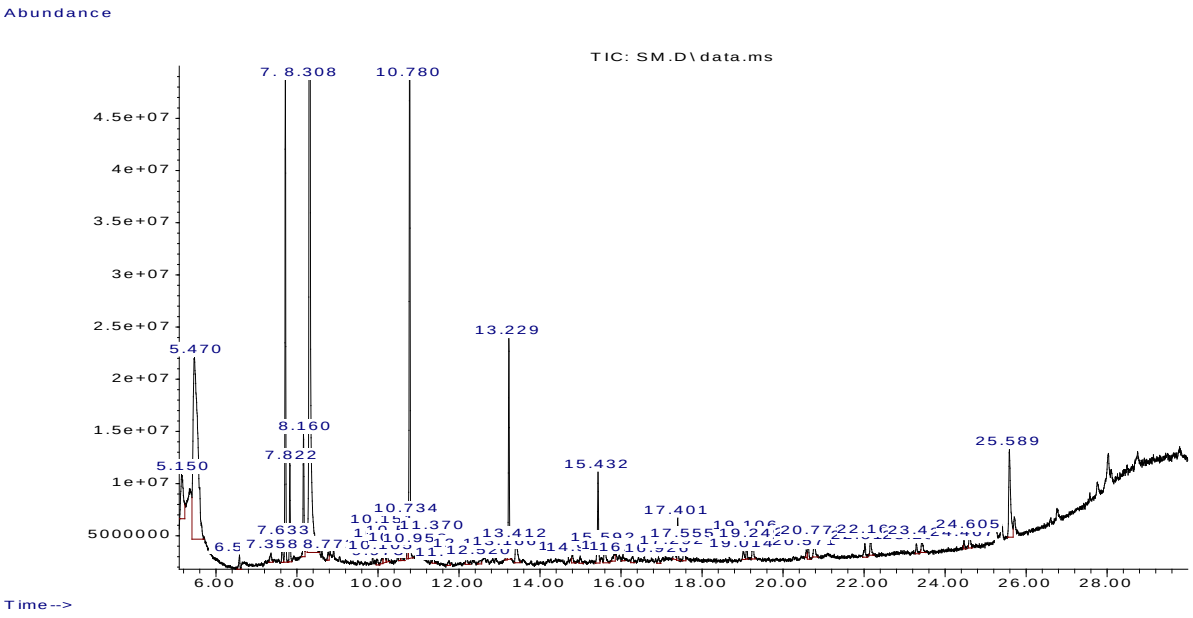


Figure 3: Gas ion chromatogram of the secondary metabolites of *A. fumigatus*

Table 5: GC-MS identified compounds of *A. fumigatus* secondary metabolites

S/N	RT (min)	% Area	Name of compound
1	5.150	2.54	Cyclotrisiloxane, hexamethyl-
2	5.470	18.14	Tetrasiloxane, decamethyl-
3	6.580	0.33	Silane, triethoxymethyl-
4	7.358	0.34	tert-Butyldimethylsilyl 2,3-dimethylbenzoate
5	7.633	0.52	4-Formyl-2,5-dimethoxy-6-methyltropone
6	7.713	8.43	Disiloxane, 1,3-diethoxy-1,1,3,3-tetramethyl-
7	7.822	1.82	2-(3-Methylphenyl)isoindole-1,3-dione
8	8.160	2.46	Pentasiloxane, dodecamethyl-
9	8.308	28.97	1-Amino-2-(dimethylamino)-4-hydroxyanthracene-9,10-dione
10	8.772	0.23	Cyclotetrasiloxane, octamethyl-
11	9.973	0.18	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene
12	10.105	0.26	13H-Dibenzo[a,i]carbazole
13	10.151	0.80	2-Hydrazino-4,6-dimethylpyrimidine
14	10.231	0.35	2,5-Dihydroxybenzaldehyde
15	10.534	0.39	Benzo[h]quinoline, 2,4-dimethyl-
16	10.597	0.31	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid
17	10.734	0.94	Fenoterol
18	10.780	11.30	Cyclopentasiloxane, decamethyl-
19	10.958	0.28	2,4-Dihydroxybenzoic acid
20	11.370	0.58	Cyclododecane
21	11.753	0.18	Acetamide, N-[4-(trimethylsilyl)phenyl]-
22	12.188	0.37	2-Ethylacridine
23	12.520	0.23	Tris(tert-butyldimethylsilyloxy)arsane
24	13.166	0.26	2H-Pyrrol-2-one, 1,5-dihydro-1-(4- methoxyphenyl)-5,5-diphenyl-
25	13.229	4.22	Cyclohexasiloxane, dodecamethyl-
26	13.412	1.07	4,6'-Dimethoxy-2'-(tert.-butyldimethylsilyl)oxychalcone
27	14.803	0.22	1,4-Bis(trimethylsilyl)benzene
28	14.992	0.20	Arsenous acid, tris(trimethylsilyl) ester
29	15.432	2.01	Cycloheptasiloxane, tetradecamethyl-
30	15.592	0.72	5-Methylsalicylic acid
31	15.850	0.34	4-Quinolinecarboxylic acid, 2-chloro-
32	16.050	0.18	Cyclotrisiloxane, hexamethyl-
33	16.291	0.24	4-tert-Butylphenol
34	16.926	0.19	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
35	17.292	0.45	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-
36	17.401	0.85	3,4-Dihydroxyphenylglycol
37	17.555	0.62	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-
38	19.014	0.28	5-Methyl-2-phenylindolizine
39	19.106	0.47	Cyclononasiloxane, octadecamethyl-
40	19.249	0.55	1,2-Bis(trimethylsilyl)benzene
41	20.571	0.18	Silicic acid, diethyl bis(trimethylsilyl) ester
42	20.622	0.39	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-
43	20.771	0.67	1H-Indole-2-carboxylic acid, 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester
44	22.018	0.45	1-Benzazirene-1-carboxylic acid
45	22.161	0.50	Formic acid, 1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5-a]pyrimidin-5-yl)-, methyl ester
46	23.289	0.28	1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-
47	23.426	0.49	Formic acid, 1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5-a]pyrimidin-5-yl)-, methyl ester
48	24.467	0.17	2'-Hydroxy-5'-methylacetophenone
49	24.605	0.51	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-
50	25.589	3.51	Bis(2-ethylhexyl) phthalate

The morphological and microscopical characterization of fungi are the most adopted methods, they are not enough for fungal identification as most fungi possess the same morphological features hence the need to adopt molecular techniques for correct species identification[33,34]. Molecular characterization is a rapid procedure that helps in distinguishing morphologically, similar fungal species by the application of PCR technology using an internal transcribed spacer (ITS)[35]. The results from the morphological, microscopy, and DNA characterization of the bioactive endophytic fungus confirm it to be *Aspergillus fumigatus* in agreement with the report of Shalini and Amutha[33]. This is the first report for the isolation of any endophytic fungi from the genus Piliostigma.

The crude extract of *Aspergillus fumigatus* was the most effective against all the challenged microorganisms with the highest inhibition level observed with *S. aureus*, when compared to the extract of other endophytic fungi. The observed antifungal activity against *Aspergillus niger* and *Candida albicans* strains is in agreement with the report of Phongpaichit *et al.*[36] who reported a 1.00% inhibition of the crude ethyl acetate extracts against *C. albicans* (MIC 64 - 200 µg/ mL) when 377 endophytic fungi was isolated from *Garcinia* sp. with their fermentation tested for their antimicrobial activity.

The lower antibacterial activity of *Aspergillus fumigatus* extract experienced with the Gram-negative bacteria of *E. coli* and *K. pnuemoniae* might be as a result of their complex cellular walls of the bacteria in agreement with the report of Fernandes *et al.*[37].

The antimicrobial results from this study shows difference among different strains from the same genus or species, for example, *Curvularia* sp. 1 and 2 (EDP 2 and EDP 3 respectively) with *Curvularia* sp. 2 showing stronger antimicrobial activity among the all challenged microbes (Table 2). The same observation was noticed among EDP 6 and 7 (*Rhizoctonia* sp. 1 and 2 respectively*)* which exhibited different antimicrobial potency (Table 2) among the same microorganism *(Escherichia coli)*. This disparity is similar to those reported by Souwalak *et al.*[38].

The GC-MS results revealed the presence of several phytoconstituents responsible for the investigated pharmacological activity. The cyclic, unsaturated cyclotrisiloxane, hexamethyl has been reported to possess antimicrobial[39] and antibacterial[40]. Compounds containing the carbazole moiety as present in 13H-Dibenzocarbazole have been reported as an important class of agents against numerous diseases and are conceivably useful in clinical studies[41]. Scientific literature reports have established a wide range of pharmacological activities by compounds encompassing pyrimidines nucleus as seen in 2-Hydrazino-4,6-dimethylpyrimidine such as antibacterial[42] and antifungal[43] activities. The antimicrobial potency of 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde similar to the compounds 2,4-dihydroxybenzoic acid and 2,5-dihydroxybenzaldehyde detected in the secondary metabolite have been shown to exhibit marked activity against *T. chinense*, *E. coli*, *Staphylococcus aureus*, *Vibrio cholera* and *Salmonella thypimurium*[44]. Similarly, Bis (2-ethylhexyl) phthalate has been reported to showed significant antibacterial activity against *E. coli* and *S. aureus*[45,46], *Bacillus subtilis*[47] and *Pseudomonas* sp. PBO1[48].

CONCLUSION

The findings from the study confirm the secondary metabolites of *A. fumigatus* as an important source for antioxidant and antimicrobial compounds and hence might represent an alternative source for the production of therapeutic agents of pharmaceutical relevance. The potentials of this fungus are of great interest and warrants further investigation.

Conflicts of Interest

None to declare

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Authors' contributions

OFBC conceived and designed the study. ECJ co-supervised the study alongside OFBC. OC carried out the study and drafted the manuscript. CO and ICJ analyzed and interpreted the data. CO, ECJ, ICJ and OFBC critically revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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