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Bioactive compounds and antibacterial activity of endophytic fungi isolated from Black Mangrove (*Avicennia africana*) leaves

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Abstract

The antibacterial potential of fungal endophytes from black mangrove against pathogenic marine bacteria was evaluated and the bioactive compounds identified and quantified. Black mangrove (*Avicennia africana*) healthy leaves were obtained from a Mangrove Forest in Eagle Island, Port Harcourt, Nigeria. The fungal endophytes were cultured on acidified Potato dextrose agar plates for 5 days at 28°C. The isolated fungal endophytes were identified based on microscopic and colonial morphologies. Different concentrations (0, 20, 40, 60, 80 and 100 mg/ml) of ethyl acetate extract of the fungal isolates were screened against pathogenic marine bacteria (*Salmonella spp*., *Staphylococcus aureus* and *Shigella spp*.) via agar well diffusion assay. The most active isolate was identified using molecular method. Gas chromatography – Mass spectrometry was employed in the identification and quantification of its bioactive secondary products. Fungi of the genera *Aspergillus, Penicillium, Fusarium, Collectotrichum, Phomopsis, Epicoccum* and *Rhizopus* were isolated. Two bioactive compounds were identified in ethyl acetate extract of *Fusarium sp*. which was molecularly identified as *Fusarium phyllophilum* KU350622.1. Dibutyl phthalate ($C_{16}H_{22}O_4$) is the major compound at 55.926% peak area. The results showed that fungal endophytes from black mangrove exhibited antibacterial action against pathogenic marine bacteria. The bioactive secondary products identified have vast potentials for use in agriculture and industries.

Keywords: *Avicennia africana*, fungal endophytes, antibacterial activity, bioactive compounds.

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Introduction

Mangroves are extremely useful ecosystem with different essential economic and ecological functions (Bandaranayake, 2002). Black Mangrove (*Avicennia africana*), locally known in Nigeria as Ogbun or Ofun (Odugbemi and Akinsulire, 2008), is a grey – barked small tree or shrub. The leaves have glands where salt is excreted. Yellow centred white flowers appear during the dry season at the axillary stalk (Steentoft, 1988). This ecological unit is known for intermittent tidal flooding which causes ecological factors like nutrient availability and salinity to be greatly inconsistent with definite environmental uniqueness (Holguin et al., 2006). The mangrove ecosystem, therefore, is a discrete environment inhabiting various groups of microbes (Thatol et al., 2013). Among such microbes of unique significance are marine fungi which inhabit diverse marine ecosystems and are capable of producing a number of new bioactive compounds with extensive biological activities (Amira et al., 2009).

A major group of fungi found in the marine habitat is mangrove – endophytic fungi which are found in most species of plants (Hyde, 2008; Rodriguez et al., 2009). They colonize inner tissues of plants with no obvious harmful effects (Darshan and Shishupala, 2014). They flourish under severe environment that cause

them to develop unique metabolic pathways and generate distinctive chemicals that make it possible for them to bear such tense environmental setting. A number of these chemicals are long-established to be of immense potential as a source of new agents for diverse applications in industries (Eldeen and Effendy, 2013). There is need for regular exploration for new antimicrobial compounds as a result of constant antibiotic resistance by pathogenic microbes (Pucci and Bush, 2013). Through isolation of endophytic fungi, novel species that are regarded as an exceptional source of bioactive compounds are being revealed.

Antibacterial potential of indigenous red mangrove- leaf fungal endophytes and their bioactive compounds have been documented (Ariole and Akinduyite, 2016). In the present study, endophytic fungi from the leaf of indigenous *Avicennia africana* (Black mangrove) were evaluated for their antibacterial potential. The bioactive compounds of the most active endophyte were identified and quantified.

Materials and Methods

Source of pathogenic marine bacteria: Pathogenic marine bacteria (*Shigella spp*., *Salmonella spp.* and *Staphylococcus aureus*) were got from Environmental Microbiology Laboratory culture collection, University of Port Harcourt, Port Harcourt, Nigeria.

Collection of black mangrove leaves: Healthy black mangrove (*Avicennia africana*) leaves were collected at random from a mangrove forest located at Eagle Island in Port Harcourt, Rivers State, Nigeria with a knife sterilized with ethanol. Loss of moisture was prevented by placing them in sterile Ziploc bag. The bag was placed in ice box and sent to the laboratory within one hour for analysis.

Isolation of endophytic fungi: The method described by Suryanarayanan et al. (2003) with some modifications was employed for the endophytic fungi isolation. The leaves were carefully washed with running tap water, cut with sterile precautions into small fragments (0.5-1 cm) and surface sterilized with 1% sodium hypochlorite for 1 minute and then 75% ethanol for 30 seconds. Sodium hypochlorite and alcohol traces were removed by rinsing with sterile distilled water. The leaf fragments were dried on sterile blotting paper. The cut surfaces of the leaf tissue were placed aseptically on acidified potato dextrose agar plates. The plates were incubated at 28°C for 7 days. Tips of fungi, growing out of the leaf tissue, were selected and cultivated in pure culture on potato dextrose agar. They were identified on the basis of their cultural and microscopic characteristics (Barnett and Hunter, 1998). The pure cultures of endophytic fungal strains were maintained in potato dextrose agar slants at 4°C.

Bioactive compounds extraction: Mycelial plug (1 cm diameter) of 7 day - old culture of each fungal isolate was inoculated into 1 L Erlenmeyer flask containing 300 ml sterile potato dextrose broth. The flasks were incubated at 28°C for 21 days under static state. The liquid culture of each flask was filtered using sterile cheesecloth. Then, ethyl acetate (50 ml) was added to each filtrate and centrifuged for 10 min at 1500 rpm. The upper layer was removed and the extraction process was repeated 3 times. The pooled extract was subjected to evaporation at 45°C via rotary evaporator. The residue was mixed with Dimethyl sulphoxide (DMSO) and kept at -16°C for further use.

Agar well diffusion assay: The cell concentration of 24 h nutrient broth culture of each pathogen was adjusted to 0.5 McFarland turbidity standards and 0.1ml of each one was inoculated separately on Mueller-Hinton agar plates. Wells were made in the inoculated plates via a sterilized 6 mm diameter cork borer. Then, 0.1ml of different concentrations (20, 40, 60, 80 and 100 mg/ml) of ethyl acetate extract of each isolate were loaded in each well. Wells containing 0.1ml chloramphenicol solution (100 mg/ml) and 0.1ml sterile distilled water (0 mg/ml extract) served as positive control and negative control respectively. The plates were incubated for 24- 48 hr at 37°C. The clearance zones around the wells were measured in millimetre and used as indicator of antibacterial activity.

Molecular identification of endophytic fungi: DNA extraction was performed using Norgen's Yeast/Fungi Genomic DNA Isolation Kit. Genomic

DNA was proficiently extracted from the cells according to the method employed by Zhang et al. (2010). Spin column chromatography was used for purification. The purified genomic DNA was completely digestible with restriction enzymes. DNA quantification was carried out using DNA standard and the absorbance measured at 450 nm. Polymerase chain reaction (PCR) master mix from Norgenbiotek Canada was employed for PCR analysis which was performed according to the company's instructions. The PCR product separation was on 1.5% agarose gel. DNA ladder (100 bp) was employed as DNA molecular weight marker. Electrophoresis was carried out at 80V for 1½ h. The gel was stained with ethidium bromide and viewed using UV light. The sequence was observed by the use of Chromaslite for base calling. Then, BioEdit was employed for sequence editing. A Basic Local Alignment Search Tool (BLAST) was performed using National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Related sequences were aligned with Cluster W after downloading. The phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al., 2013).

Gas chromatography-mass spectrometry (GC-MS) analysis: Agilent 7890A-5975C GC-MS system (Tao et al., 2011) was employed. Exactly 0.5 µl of the most active fungal extract was injected into the GCMS system with injector temperature of 250°C. Nitrogen was used as a carrier gas during the compounds separation which was carried out on a 60m HP-INNOWAX capillary column (0.25 mm). The flow of the carrier gas was 1ml/min with a split ratio of 10:1. The oven initial temperature was 110°C - 200°C at 10°C/min which was later elevated to 200°C - 280°C at 5°C/min and left for 9 min and ionization energy of 70 eV was employed. The mass spectra of the unidentified bioactive compounds were related with mass spectra of identified compounds in the National Institute of Standards and Technology (NIST) Database. The molecular weight, name and peak area (%) of the bioactive compounds were determined.

Results

Bacterial isolation: A total of seven (7) endophytic fungi were obtained from black mangrove (*Avicennia africana*) leaves. The fungal genera isolated were: *Penicillium, Fusarium, Collectotrichum, Rhizopus, Aspergillus, Epicoccum* and *Phomopsis* (Table 1).

Table 1: Antimicrobial activity of ethyl acetate extract of black mangrove leaf endophytic fungi against pathogenic bacteria

Isolate code	Isolate Identity	Concentration of extract (mg / ml)	Zone of inhibition (mm) ± S.D (Marine pathogenic bacteria)		
			Staphylococcus aureus	*Salmonella* sp.	*Shigella* sp.
WA1	*Penicillium* sp.	20	-	-	-
		40	-	-	-
		60	9.00±1.00	-	8.00 ±0.00
		80	12.0±0.00	-	10.00±1.00
		100	13.67±0.58	-	12.00±1.00
WB2	*Fusarium* sp.	20	9.00± 0.00	9.00± 0.00	9.00± 0.00
		40	9.67± 0.58	10.00± 0.00	9.51± 0.00
		60	13.00± 1.00	12.00± 0.00	10.00± 0.00
		80	16.00±0.00	13.00± 1.00	14.00± 1.00
		100	17.67±0.58	14.33± 0.58	15.33±0.58
WC3	*Collectotrichum* sp.	20	-	-	-
		40	-	-	-
		60	-	-	-
		80	9.00±1.00	8.33±0.58	-
		100	10.33±0.58	9.33±0.58	-
WD4	*Rhizopus* sp.	20	-	-	-
		40	-	-	-
		60	8.33±0.58	-	-
		80	10.00±1.00	-	8.33±0.58
		100	10.67±0.58	-	9.67±0.58
WE5	*Aspergillus* sp.	20	-	-	-
		40	-	-	-
		60	-	-	-
		80	9.00±1.00	-	-
		100	11.00±1.00	-	-
WF6	*Epicoccum* sp.	20	-	-	-
		40	-	-	-
		60	-	-	-
		80	9.00±1.00	-	8.33±0.58
		100	10.67±0.58	-	10.00±1.00
WG7	*Phomopsis* sp.	20	-	-	-
		40	-	-	-
		60	8.00±1.00	-	-
		80	14.00±1.00	-	8.33±0.58
		100	16.00±1.00	-	10.33±0.58
	Positive Control	Chloramphenicol (100)	25.00±1.00	21.00±1.00	23.00±1.00
	Negative Control	Distilled Water (0 mg/ml extract)	-	-	-

- = no zone of inhibition

Antibacterial activity: The result of the antibacterial assay presented in Table 1 revealed that all the endophytic fungi extracts at concentrations of 80-100 mg/ml showed antibacterial activity against at least one of the tested pathogens. However, the different concentrations (20-100 mg/ml) of ethyl acetate extracts of WB2 (*Fusarium sp*.) were active against all the tested pathogens. The zones of inhibitions obtained were between 8 and 17.67 mm. Furthermore, gram negative bacteria (*Salmonella sp*. and *Shigella sp*.) were more resistant to the fungal extracts than *Staphylococcus aureus* (a gram positive bacterium).

Molecular characteristics of the most active endophytic fungus: The fungal isolate (WB2) which was the most active fungus was identified as *Fusarium phyllophilum* KU350622.1. The phylogenetic tree is presented in Figure 1.

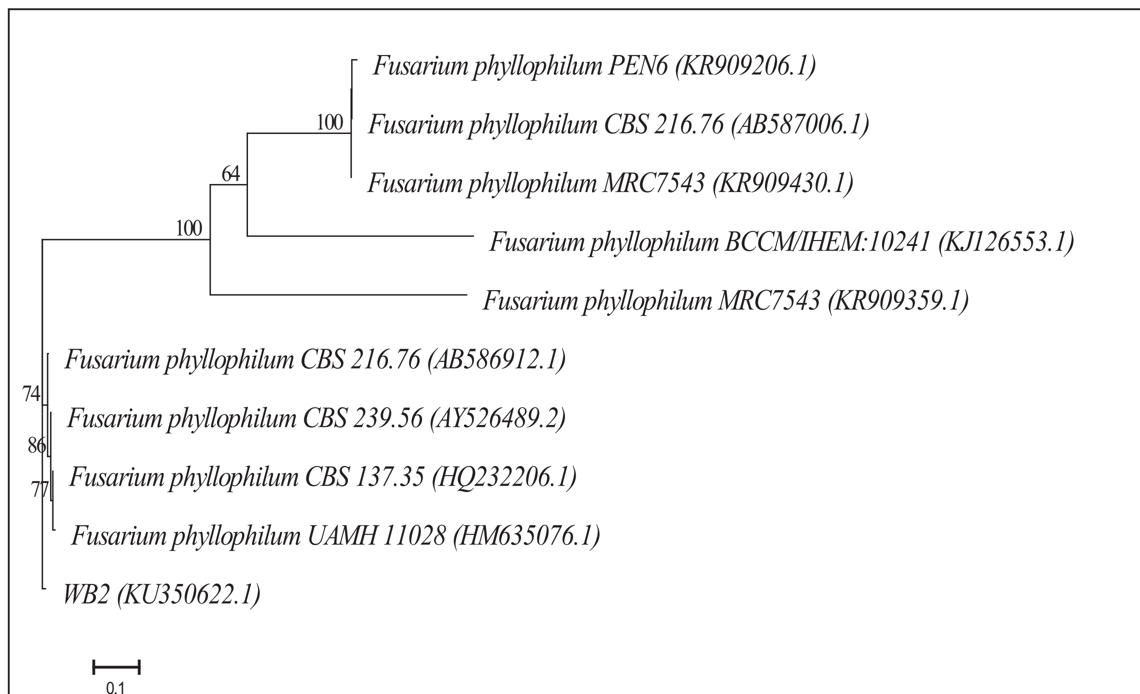

Figure 1: Phylogenetic tree of isolate WB2. Bootstrap values of >50% (based on 1000 replicates) are given in the nodes of the tree. NCBI accession numbers are given in parentheses

Bioactive compounds in the ethyl acetate extract of WB2: The bioactive compounds, their retention time, molecular weight, molecular formula, chemical structures and peak area percentage of each compound identified in the extract are presented in Table 2.

Table 2: Bioactive compounds in the ethyl acetate extract of WB2 (*Fusarium phyllophilum* KU350622.1)

S/N	Retention time (min)	Compound name	Molecular weight (g/mol)	Molecular formular	Chemical structure	Peak area (%)
1	9.210	2-Hexanoic acid,2-methyl-	128.17	$C_7H_{12}O_2$		44.074
2	14.616	Dibutyl phthalate	278.34	$C_{16}H_{22}O_4$		55.926

Gas chromatogram of bioactive compounds in the ethyl acetate extract of WB2 (*Fusarium phyllophilum* KU350622.1) is presented in Figure 2.

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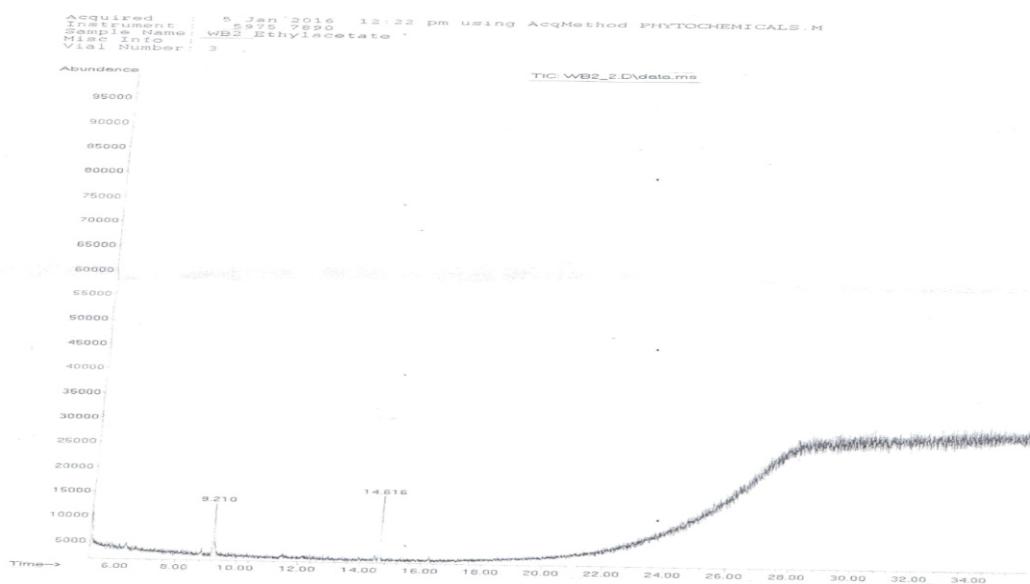
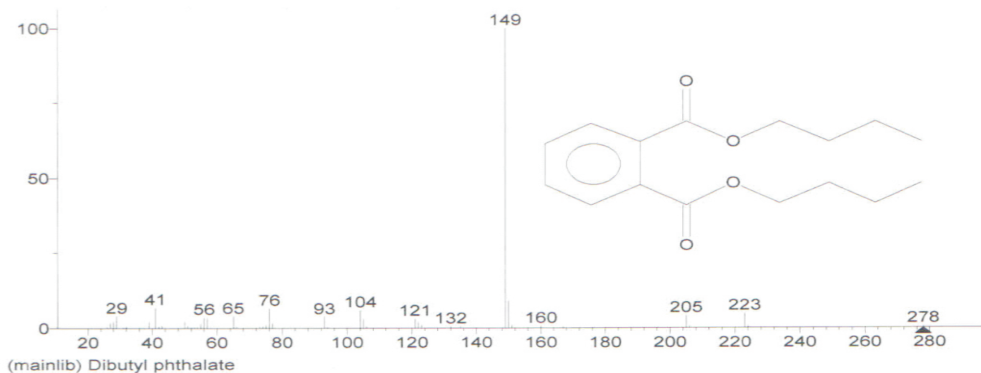
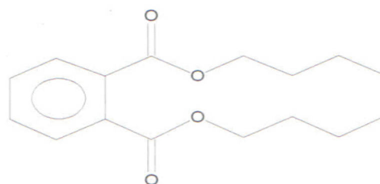


Figure 2: Gas chromatogram of bioactive compounds in the ethyl acetate extract of WB2 (*Fusarium phyllophilum* KU350622.1) from black mangrove leaves

Two compounds were detected namely; Dibutyl phthalate ($C_{16}H_{22}O_4$) at 55.926% and 2-Hexanoic acid, 2-methyl ($C_7H_{12}O_2$) at 44.074%.

The mass spectrum of the major bioactive compound, Dibutyl phthalate ($C_{16}H_{22}O_4$), is presented in Figure 3.





Name: Dibutyl phthalate
Formula: $C_{16}H_{22}O_4$
MW: 278 CAS#: 84-74-2 NIST#: 114974 ID#: 110431 DB: mainlib
Other DBs: Fine, TSCA, RTECS, EPA, HODOC, NIH, EINECS, IRDB
Contributor: NIST Mass Spectrometry Data Center, 1990.

Figure 3: Mass spectrum of the major bioactive compound (Dibutyl phthalate) in the ethyl acetate extract of *Fusarium phyllophilum* KU350622.1 from black mangrove leaves

Discussion

Avicennia africana leaves appear to harbour uniquely diverse endophytic fungal communities. It is of immense importance to recognize that surface sterilization notwithstanding, the endophytic fungal isolates still sporulated. This can be ascribed to their distinctive ability to carry on in unfavorable environmental condition. Fungi endophytes have also been isolated from mangrove plant leaves (Chaeprasert et al., 2010; Joel and Bhimba, 2011; Costa et al., 2012; Job et al., 2015). Furthermore, fungi endophytes of numerous plants have been reported (Suryanarayanan et al., 2003; Marquez et al., 2008; Sun et al., 2012). The higher resistance exhibited by Gram negative than Gram positive which Alias et al. (2010) and Ling et al. (2016) also reported, can be ascribed to the existence of outer membrane that acts as an obstacle to antimicrobial agents in cells of gram negative bacteria. Bhimba et al. (2012) reported that ethyl acetate extracts of fungi from leaves of *Avicennia marina*, *Avicennia officialis* and *Rhizophora mucronata* demonstrated antibacterial action against human pathogenic bacteria. The antibacterial activities of the black mangrove leaf- endophytic fungal extracts can be attributed to the presence of bioactive compounds. Endophytes are well-known for their great biotechnological capability as source of bioactive novel compounds for industrial, agricultural and medical applications.

Dibutyl phthalate, the major bioactive compound produced by ethyl acetate extract of WB2 (*Fusarium phyllophilum* KU350622.1), is a bioactive ester. It acts as antimalarial, antibacterial, antifungal, antitumor, anticancer, and herbicide. It is also employed as plasticiser in the manufacture of polyvinyl chloride and other plastics and as additive in the manufacture of ink. Dibutyl phthalate isolated from ethyl acetate extract of the stem bark of *Ipomoea carnea* exhibited considerable activity against the tested pathogenic strains (Khatiwora et al., 2012). Antibacterial activities of dibutyl phthalate against *Salmonella typhimurium* and *Staphylococcus aureus* have been reported (Srinivasan et al., 2009).

Mangrove endophytes have been known as rich sources of natural products with distinctive structures and attractive bioactive characteristics of secondary metabolites (Suryanarayanan et al., 2009). The metabolites aid the host plant to withstand biotic and abiotic pressure, enhance rate of growth and reproduction capacity (Arnold and Herre, 2003; Strobel and Daisy, 2003). The correlation amid host plants and their endophytes ranges from phytopathogenesis to mutuality (Bharathidasan and Panneerselvam, 2011). Extracellular hydrolases are produced by endophytic fungi to acquire nourishment from host and as a resistance device against invasion by pathogens (Sunitha et al., 2013). The chemical and therapeutic properties of the plant could be as a result of contact with its endophytic fungi (Zhao et al., 2010). Fungal endophyte (*Fusarium phyllophilum* KU350622.1.) isolated from *Avicennia africana* leaves revealed its potential to yield potent bioactive compounds for drug discovery programmes.

Conclusion

This study showed the presence of endophytic fungi in Avicennia africana leaf. The fungal endophytes were of the genera- *Penicillium, Fusarium, Collectotrichum, Rhizopus, Aspergillus, Epicoccum* and *Phomopsis*. Their antibacterial potential offers a promising source of bioactive compounds that can be exploited in the biotechnology field. Two bioactive compounds namely -Dibutyl phthalate ($C_{16}H_{22}O_4$) and 2-Hexanoic acid, 2-methyl ($C_7H_{12}O_2$) were detected in the ethyl acetate extract of the most active isolate molecularly identified as *Fusarium phyllophilum* KU350622.1. Therefore, *Fusarium phyllophilum* KU350622.1 could be a good source for Dibutyl phthalate.

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