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Bioactivity and GS-MS Profiling of Extracts from Static Cultures of Endophytic Fungi from Black Mangroves

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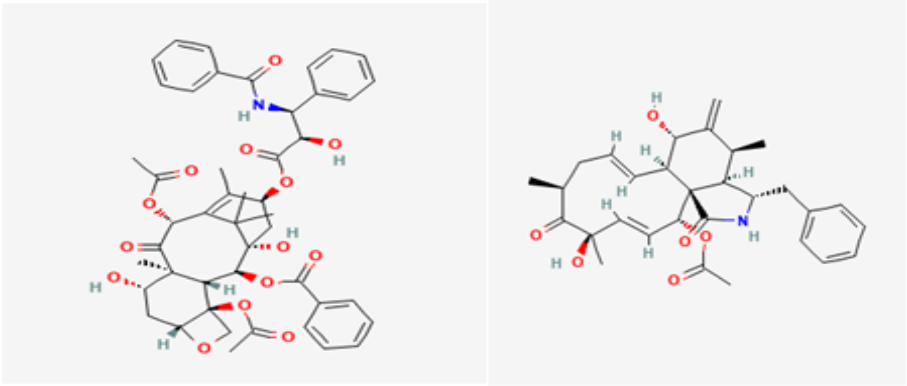
ABSTRACT

Mangrove plants and their associated micro fauna have been a rich source of bioactive secondary metabolites, though only limited antimicrobial screening of this chemo-diversity source has been reported. In this study, unidentified endophytic fungi BBMS and BBMAV were isolate from the bark of black mangroves obtained from the coastal forest of Ayensu estuary. The endophytes were cultured in static medium made of 3%$(\frac{w}{v})$ malt concentrate and 6%$(\frac{w}{v})$ glucose in distilled water. The fungi were cultured for 8 weeks and harvested. Ethyl acetate extraction of the filtrate followed by drying on a rotary evaporator afforded 1.20 g and 1.05 g of BBMS and BBMAS respectively. TLC studies and GC-MS analysis indicated that the extracts contained interesting secondary metabolites. A bioassay of the extracts was carried out on human pathogen and cocoa infesting microbes. The bioassay results indicated that the extracts were active against all the test organisms. ABTS and DDPH free radicals scavenging activities were also tested with the extracts. The results indicate that the extracts of the fungal isolates exhibited high potency of antioxidant activity.

1.0 Introduction

The interest in the search for bioactive secondary metabolites from endophytic fungi was as a result of interesting secondary metabolites obtained from exophytic and endophytic fungi. Tax*omyces andreanae*, a fungus that was found to produce taxol, was isolated by Stierle et al., [1] from T. brevifolia. This discovery offered a more feasible and useful method for producing taxol in large quantities. Taxol (1), had earlier been isolated from a pacific Yew tree, *Taxus bravefolia.*

Taxol is a renowned anticancer drug. Recently Oppong, E. K, [2] published the isolation and characterization of pure cytochalasin D (II), from endophytic fungi from a mangrove.[2]. Cytochalasin D had been reported to be antiviral. Endophytes play significant roles in how plants adapt to their environment, including stress conditions brought on by a lack of water. They provide plants with a number of advantages, including the ability to produce toxins that protect them from predators and disease [3]. Endophytic microbes isolated from different plants such as *Ginkgo biloba* L., *Taxus chinensis* (Pilg.) Rehder, and *Tectona grandis* L. f. have been found to be prevalent in nature and their association with the plants can be either obligate or facultative) [4]. Some endophytes can produce substances that alter the plant phenotype and thus increase host defenses [5].



(I) (II)

This report on Endophytes from tissues of the Black Mangrove is the first of a series of study that sought to assess the biological activity of extracts obtained from static cultures of endophytic fungi isolated from the tissues of mangrove plants growing in Winneba, Ghana. In this series, endophytic fungi isolated from Black, White and Red Mangroves will be studied.

2.0 Materials and method

2.1 Sample collection and treatment

The bark of a black mangrove was collected from the mangrove forest along the Ayensu estuary in Sankor and Muni lagoon in Akosua village, Winneba. The samples were collected into plastic containers in their source water and transported to the culture room. The samples collected were rinsed with distilled water and surface-washed with absolute alcohol to remove extraneous matter [6]. The samples were surface sterilized by immersing them in 99% ethanol solution for 10 minutes.

2.2 Culturing procedure

The sterilized mangrove plant materials were cut into 1 cm long portions and inoculated directly into culture media ($3\%\left(\frac{w}{v}\right)$malt extract and $6\%\left(\frac{w}{v}\right)$glucose) in the 250mL conical flask using sterile scissors and labelled as BBMS and BBMAV indicating they were taking from Sankro and Akosua village respectively. The sub-culture was incubated at a temperature of $29^{\circ}C$ for 4 weeks and monitored periodically. After four weeks, fragments of the mycelia from the sub-culture were cut using sterile forceps and spatula and inoculated into sterilized 2dm3 of flat-bottomed flask containing$1dm^3$ of $3\%\left(\frac{w}{v}\right)$) malt extract and $6\%\left(\frac{w}{v}\right)$) glucose media. Prior to using the fungal broth cultures for solvent extraction of bioactive substances, they were cultured at $29^{\circ}C$ for eight weeks under periodic monitoring.

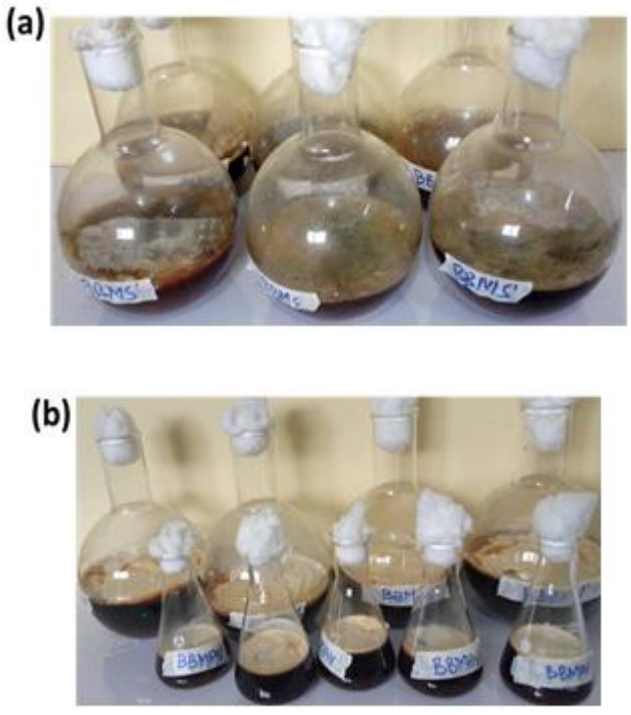


Figure 1. (a) eight weeks old culture of BBMS (b) eight weeks old culture of BBMAV

2.3. Solvent Extraction and TLC Studies

The eight weeks old fungal cultures were harvested and filtered with muslin cloth. Neat ethyl acetate was used to extract the secondary metabolites of the fungal isolate. The solvent was dried on a rotary evaporator to obtain 1.20g 1.05g of BBMS and BBMAV respectively.

TLC study of BBMS and BBMAV was conducted using Chloroform: methanol: acetic acid (45:3:3) and Petroleum ether: ethyl acetate: acetic acid (25:23:3) solvent systems respectively indicated that the extracts contain a mixture of compounds.

2.4 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The extracts were examined using a Shimadzu GCMS QP2020 gas chromatography-mass spectrometer. The GC operation parameters used were Column oven Temp: 50^0 C, Injection Temp: 250^0 C, Split ratio: 50.0, Pressure: 84.0 KPa, Column flow: 1.62 mL/min and Linear velocity: 49.2 cm/sec. Column used was Rtx-5ms (30×0, 25µm×0.25mm) and Injection volume of 1.0µL. These were coupled with MS operation Parameters of Ion source temp: 250^0C, Interface temp: 270^0C and mass scan range: 80 -550 m/z. NIST 14, NIST14s and Wiley 8' libraries were used for the identification of peaks while quantifications were done using area normalization for total ion counts (TIC), and 5 reference ions were selected to improve sensitivity.

2.5 Bioactivity test of Extracts

2.5.1 Antimicrobial Activity Test

With a few minor modifications, the broth micro-dilution technique was carried out in accordance with the procedures outlined in document M27-A3 produced by the Clinical and Laboratory Standards Institute (CLSI) [7]. In a nutshell, 100 l of Mueller Hinton broth were poured into each of the plates' wells. 100µl each of the extract was then used to prepare well concentrations ranging from 100 – 0.1 mg/mL and wells 11 and 12 serving as positive control (Broth + organism only) and negative control (Broth with no organism only) respectively for each microorganism on the columns A- H. Voriconazole and chloramphenicol, which served as conventional medication controls against all of the test bacteria and fungi, respectively, were also subjected to the same procedures in a separate plate at concentrations of 30 ug/mL. Following the addition of 100 l of each of the 0.5 McFarland standardized test organisms on each column, the plates were incubated at 37 C for 24/48 hours, depending on whether the test organisms were bacterial or fungal strains.

Minimum Inhibitory Concentration (MIC) values were then evaluated by visual analysis by adding Tetrazolium dye after 30 minutes. Each experiment was done thrice with the MICs recorded. In order to verify if the extract were able to kill the microbial cells (bacteri-/fungi-cidal effect) the plates were also evaluated for Minimum Bactericidal (MBC) and Fungicidal Concentration (MFC). Briefly, aliquots from each well from susceptibility testing assays were transferred to plates containing Nutrient agar, which were incubated at 37ºC for 24/48 hour. Results were then evaluated by analyzing the presence or absence of growth in the Nutrient agar or SDA [8].

2.5.2 Free Radicals Scavenging Activity test (ABTS, DPPH)

A 300 µL of freshly prepared stock, 0.6 mM of 1,1-diphenylpicrylhydrazyl (DPPH) was dissolved in 100 ml of methanol as a working solution. A 150 µl of this solution was added to 50 µL of the test compound (T) with concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078 mg/mL and then incubated for 30 minutes at room temperature. The DPPH radical scavenging activity of the extracts was then determined by measurement of the

absorbance at 517nm for test compound (T) expressed as a percentage of the absorbance of a control sample (C) of DPPH without the compound. Subsequently, the antioxidant potential against ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was also determined by adding 10 mL of ABTS and 10 ml of aqueous 2.4 mM potassium persulphate to generate the ABTS free radical to form the stock solution. This stock was further diluted in 50 ml of methanol as a working solution. A 150 µL of the solution was then added to 50 µL of the prepared compound concentrations, vortexed and incubated at 30°C for 10 min. The absorbance was then recorded at 734 nm. The free radical scavenging activity for the compound and as well Ascorbic acid as a referenced positive control against DPPH and ABTS were therefore evaluated by inputting data into the relation: (22, 23).

$$\%\ Scavenging\ activity = [\frac{C-T}{C}] \times 100\%.$$

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3.0 Results and discussions

3.1 TLC Studies and GC-MS profiling of Extracts from BBMS and BBMAV

The GC-MS results of the extracts of the fungal isolates show that the extracts contained a mixture of compounds. A split ration of fifty (50) was used so as to obtain much clearer peaks. The following are tables of the GC-MS results of extracts of the fungal isolates.

Table 1.Compounds identified by GC-MS from BBMS

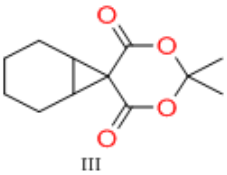
S/N	Compound name	t_R	Formular	M/Z
1	Phenylethyl Alcohol	5.4	$C_8H_{10}O$	122.1
2	2',2'-Dimethylspiro{bicyclo[4.1.0]heptane-7,5'-[1,3]dioxane}-4',6'-dione	7.3	$C_{12}H_{16}O_4$	224.1
3	Benzeneethanol, 4-hydroxy-	8.0	$C_8H_{10}O_2$	138.1
4	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.3	$C_{12}H_{16}O_5$	240.1
5	Cyclobutane, 3-hexyl-1,1,2-trimethyl-, cis-	8.5	$C_{13}H_{26}$	182.2
6	Phenol, 2,5-bis(1,1-dimethylethyl)-	8.6	$C_{14}H_{22}O$	206.2
7	4a.alpha.(2h)-Naphthol,octahydro-4.alpha.,8a.beta.-dimethyl-	8.7	$C_{12}H_{22}O$	182
8	Piperazine,1-methyl-4-(2,4,6-trimethylphenylsulfonyl)-	8.8	$C_{14}H_{22}N_2O_2S$	282.1
9	Trans-bicyclo[4.4.0]decan-1-ol-3-one	9.7	$C_{10}H_{16}O_2$	168.1
10	12-Methyl-E,E-2,13-octadecadien-1-ol	10.0	NA	NA
11	7-Methyl-oxa-cyclododeca-6,10-dien-2-one	10.3	$C_{12}H_{18}O_2$	194.1
12	Oxacyclotetradecan-2-one	10.5	$C_{13}H_{24}O_2$	212.2
13	2-Methoxy-3-hydrazinyl-pyrazine	10.7	$C_5H_8N_4O$	140.1
14	3,3-Dimethyl-5-oxocyclohexanecarbaldehyde	11.2	$C_9H_{14}O_2$	154.1
15	2(3H)-Benzofuranone, 6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-,[3S-(3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)]	13.1	$C_{15}H_{22}O_2$	234.2
16	3,5a,9,9-Tetramethyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol	13.2	$C_{15}H_{26}O_2$	238.2
17	Koiganal II	14.4	$C_{18}H_{32}O$	264.446
18	Bicyclo[2.2.1]heptane-1-carboxylic acid, 7,7-dimethyl-2-oxo-	14.4	$C_{10}H_{14}O_3$	182.1
19	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propanal	14.8	$C_{15}H_{22}O$	218.2
20	9,12-Octadecadienoic acid (z,z)-	14.9	$C_{18}H_{32}O_2$	280.2
21	Widdrol hydroxyether	16.1	$C_{15}H_{26}O_2$	238.37
22	Methyl 5,13-docosadienoate	17.3	$C_{23}H_{42}O_2$	350.3
23	(7S)-trans-bicyclo[4.3.0]-3-nonen-7-ol	17.7	$C_9H_{14}O$	138
24	Methyl 5,9-tetracosadienoate	17.9	$C_{25}H_{46}O_2$	378.3
25	(2S,3S,6S)-6-Isopropyl-3-methyl-2-(prop-1-en-2-yl)-3-vinylcyclohexanone	18.8	$C_{15}H_{24}O$	220.2

Table 2.Compounds Identified by GC-MS from BBMAV

S/N	Compound name	t_R	Formula	M/Z
1	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-	8.4	$C_{12}H_{16}O_5$	240.1
2	trans-1,10-Dimethyl-trans-9-decalol	8.8	$C_{12}H_{22}O$	182.2
3	7-Methyl-oxa-cyclododeca-6,10-dien-2-one	11.2	$C_{12}H_{18}O_2$	194.1
4	1-Oxetan-2-one, 4,4-diethyl-3-methylene-	11.7	$C_8H_{12}O_2$	140.18
5	Ethyl trans-3-methyltetrazole-5-acrylate	12.4	$C_7H_{10}N_4O_2$	182.1
6	2(3H)-Benzofuranone, 6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-, [3S-(3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)]	14.6	$C_{15}H_{22}O_2$	234.2
7	3,5a,9,9-tetramethyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol	14.7	$C_{15}H_{26}O_2$	238.2
8	tricyclo[20.8.0.0e7,16]triacontan, 1(22),7(16)-diepoxy-	16.1	NA	NA
9	1-Methyl-3,6-diazahomoadamantan-9-ol	16.2	$C_{10}H_{18}N_2O$	182.26
10	Koiganal ii	16.6	$C_{18}H_{32}O$	264.446
11	14-.beta.-h-pregna	16.6		
12	4-(5,5-dimethyl-1-oxaspiro[2.5]oct-4-yl)-3-buten-2-one	17.9	$C_{13}H_{20}O_2$	208.1
13	(Z)-9-Octadecen-4-olide	18.5	$C_{18}H_{32}O_2$	280.2
14	Methyl 5,13-docosadienoate	19.1	$C_{23}H_{42}O_2$	350.3
15	Methyl 5,9-tetracosadienoate	19.5	$C_{25}H_{46}O_2$	378.3
16	Spiro[7h-cyclohepta[b]furan-7,2'(5'h)-furan]-2,5'(3h)-dione, octahydro-8-hydroxy-6,8-dimethyl-3-methylene-, [3as-(3a.alpha.,6.	20.7	NA	NA

The GC-MS results above revealed that the two fungal isolates produced different number of components in their culture medium indicating they are not the same fungus. This was evidenced in the appearance of their mycelia and the way their extracts interacted with different solvent systems during the TLC studies. Despite their differences, there were seven compounds common the two fungal isolates. These include 3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-,7-Methyl-oxa-cyclododeca-6,10-dien-2-one,2(3H)-Benzofuranone,6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-,[3S(3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)],3,5a,9,9-tetra methyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol, Koiganal ii, Methyl 5,13-docosadienoate and Methyl 5,9-tetracosadienoate.

Some of the compounds identified by the GC-MS have little information on them in literature. These compounds include 12-Methyl-E,E-2,13-octadecadien-1-ol from BBMS and tricyclo[20.8.0.0e7,16]triacontan,1(22),7(16)-diepoxy-,Spiro[7h-cyclohepta[b]furan-7,2'(5'h)-furan]-2,5'(3h)-dione, octahydro-8-hydroxy-6,8-dimethyl-3-methylene-, [3as-(3a.alpha., 6 from BBMAV. These compounds are potential novel compounds which could be explored due to high antimicrobial activity of the extracts. 2',2'-Dimethylspiro{bicyclo[4.1.0]heptane-7,5'-[1,3]dioxane}-4',6'-dione (III) was identified to be an essential component of fragrance. Other structurally interesting compounds in the crude extracts are Cyclobutane, 3-hexyl-1,1,2-trimethyl-, cis- (IV), Piperazine,1-methyl-4-(2,4,6-trimethylphenylsulfonyl)- (V),7-Methyl-oxa-cyclododeca-6,10-dien-2-one(VI), Oxacyclotetradecan-2-one (VII) and 2-Methoxy-3-hydrazinyl-pyrazine (VIII) from BBMS extract.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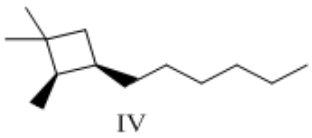
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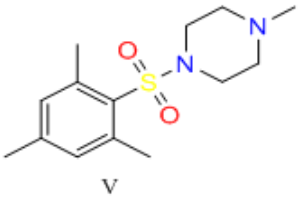
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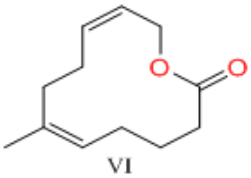
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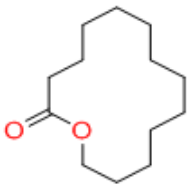
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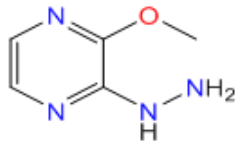
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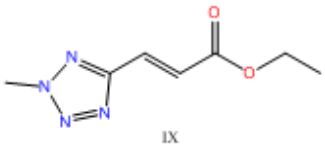
Ethyl trans-3-methyltetrazole-5-acrylate (IX), 2(3H)-Benzofuranone, 6-ethenylhexahydro-3,6-dimethyl-7-(1-methylethenyl)-,[3S (3.alpha.,3a.alpha.,6.alpha.,7.beta.,7a.beta.)] (X), 1-Methyl-3,6-diazahomoadamantan-9-ol (XI) and 4-(5,5-dimethyl-1-oxaspiro[2.5]oct-4-yl)-3-buten-2-one are also present in BBMAV.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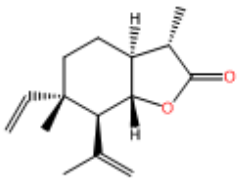
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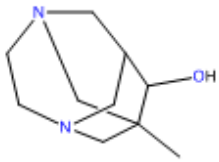
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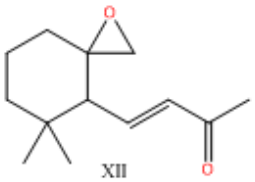
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3.2 Antimicrobial Activity of Extract

MBC/MFC and MIC analysis were carried out on the extracts of the fungal isolates. In order to verify if the extract was able to kill the microbial cells (bacteri-/fungicidal effect) the plates were also evaluated for MBC and MFC. The results of the antimicrobial activity of human pathogenic microbes are presented in table 3 and that of cocoa infesting fungi in table 4 below.

The MIC is the lowest concentration of a drug that inhibits bacterial growth so you will have no turbidity in your culture media. But MBC is the lowest concentration that kills bacteria [9]. Usually, the concentration which is considered as MBC is higher than the concentration for MIC. Lower MIC and MBC values indicated higher efficacy [10]. From the above table, The MIC and MBC for BBMS are the same for each test organism hence has the same MBC/MIC ratio of 1 indicating a stronger bactericidal activity against all tested organism.

BBMAV on the other hand exhibited a strong activity against the test organisms. It recorded a least MIC value of 1.56mg/mL against E. coli, Staphylococcus aureus and Candida albicans and 3.125mg/mL against Klebsillia pneumonia, Methicilin resistant Staphyloccocus aureus, Samonella typhii and Streptococcus mutans. BBMAV exhibited a stronger bactericidal activity against all tested organism except against E. coli of which it is bacteriostatic. Comparatively, BBMAV exhibited greater bioactivity against the tested organisms than BBMS despite having more compounds than BBMAV.

Black pod disease is a protozoal disease of Cocoa trees [11]. This pathogen if left untreated can destroy all yields.

Annually, the pathogen can cause a yield loss of up to 1/3 and up to 10% of total trees can be lost completely. There is much research carried out in recent times and control efforts that go into the management of Phytophthora sp. pathogens. There are several methods available in order to control black pod disease such as cultural, chemical and biological control [12].

BBMS exhibited the activity against *Phytophthora palmivora and Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 1 respectively. BBMAV also recorded MIC and MFC/MIC values of 3.125mg/mL and 2 respectively indicating a high fungicidal activity against *Phytophthora palmivora*. It however showed a comparatively lower activity against *Phytophthora megakarya* with MIC and MFC/MIC values of 12.5mg/mL and 4 respectively.

3.3 Free Radical Scavenging Activities (antioxidant activities) of Crude Extract

The antioxidant activities of the extract from mangrove fungal isolates were tested against DPPH and ABTS by measuring their scavenging potentials for the extracts. The ABTS and DPPH scavenging activities for natural products are widely used methods for the determination of the antioxidant properties of natural compounds. The ABTS and DPPH scavenging activity of the extract was determined by measuring the absorbance of the concentrations of samples ranging from 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078 mg/mL using ascorbic acid as the reference compound. The higher the percentage scavenging activities of the extracts, the greater the antioxidant potentials. The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of samples. According to Jadid *et al*. [13], extracts which possess IC_{50} values ranging from 50 to 100 mg / mL is considered to exhibit intermediate antioxidant activity.

The percentage scavenging activities and the IC_{50} of the extracts at the various concentrations are displayed in table 5 and 6 below;

The data above reveal that BBMS exhibited potent antioxidant activity of 62.62% to 68.69% at concentrations of 0.0625 to 1.00mg/mL and IC_{50} of 4.919663 mg/mL against ABTS while it recorded percent scavenging activities of 76.12% to 100% at same concentrations DPPH. This implies that the extracts of BBMS if more potent against DPPH than ABTS.

BBMAV extract exhibited potent antioxidant activity of 67.52% to 84.35 at concentrations 0.0625 to 1.00mg/mL against ABTS and 58.93% to 81.25% at same concentrations against DPPH. This indicates comparatively, BBMAV is slightly more potent against ABTS than DPPH. However, it showed potency against DPPH up to a concentration as low as 0.0078 mg/mL.

4.0 Conclusion

The levels antimicrobial activity of crude extracts of the fungal isolates as well their antioxidant activities are interesting and needs to be explored for their potentials. Further studies would be carried out on the crude extracts to isolate the various components of the crude and carry out bioassay on the isolated compounds.

Table 3.Results of MBC and MIC of crude extract on human pathogenic microbes (mg/ml)

ORGANISMS	BBMS			BBMAV		
	MIC	MBC	MBC/ MIC	MIC	MBC	MBC/ MIC
EC	25	25	1^{bc}	1.56	12.5	8.0^{bs}
KP	50	50	1^{bc}	3.125	6.25	2.0^{bc}
MRSA	25	25	1^{bc}	3.125	6.25	2.0^{bc}
PA	50	50	1^{bc}	3.125	6.25	2.0^{bc}
ST	50	50	1^{bc}	3.125	6.25	2.0^{bc}
SA	50	50	1^{bc}	1.56	6.25	4.0^{bc}
SM	50	50	1^{bc}	3.125	6.25	2.0^{bc}
CA	50	50	1^{fc}	1.56	6.25	4.0^{fc}

EC- E. coli KP- Klebsillia pneumonia MRSA- Methicilin resistant Staphyloccocus aureus PA- Pseudomonas aeruginosa ST- Samonella typhii SA- Staphylococcus aureus SM- Streptococcus mutans CA- Candida albicans.

Table 4.Results of MFC and MIC of crude extracts on Cocoa infesting fungi (mg/ml)

Organism	Phytophthora palmivora			Phytophthora megakarya		
SAMPLES	MIC(mg/mL)	MFC(mg/mL)	MFC/MIC	MIC(mg/mL)	MFC(mg/mL)	MFC/MIC
BBMS	12.5	12.5	1^{fc}	12.5	12.5	1^{fc}
BBMAV	3.125	6.25	2^{fc}	12.5	50	4^{fc}

Table 5. Results for Antioxidant Activity of BBMS.

CONC.	ABTS activity [IC_{50}=4.919663±0.00 mg/mL]				DPPH activity [IC_{50} = 8.120576±0.00 mg/mL]		
	EXP1	EXP2	MEAN±SD		EXP1	EXP2	MEAN±SD
1.0	66.36	71.03	68.69±3.30		100.00	100.00	100.00±0.00
0.5	65.42	70.09	67.76±3.30		95.09	98.07	96.58±2.10
0.25	64.49	67.76	66.12±2.31		94.35	97.62	95.98±2.31
0.125	63.08	63.08	63.08±0.00		82.59	84.23	83.41±1.16
0.0625	62.62	62.62	62.62±0.00		74.55	77.68	76.12±2.21
0.0313	40.19	40.19	40.19±0.00		73.36	76.34	74.85±2.10
0.0156	31.78	31.78	31.78±0.00		49.55	52.68	51.12±2.21
0.0078	22.90	22.90	22.90±0.00		47.02	50.30	48.66±2.31

Table 6. Results For Antioxidant Activity Of BBMAV

CONC.	ABTS activity [IC_{50} =5.01697±0.00 mg/mL]				DPPH activity [IC_{50} = 8.542313±0.00 mg/mL]		
	EXP1	EXP2	MEAN±SD		EXP1	EXP2	MEAN±SD
1.0	86.92	81.78	84.35±3.63		79.76	82.74	81.25±2.10
0.5	85.05	80.37	82.71±3.30		72.77	75.89	74.33±2.21
0.25	76.17	71.03	73.60±3.63		69.35	72.32	70.83±2.10
0.125	72.43	67.76	70.09±3.30		63.10	66.07	64.58±2.10
0.0625	72.90	62.15	67.52±7.60		57.44	60.42	58.93±2.10
0.0313	29.44	29.44	29.44±0.00		57.14	60.42	58.78±2.31
0.0156	19.16	19.16	19.16±0.00		56.99	60.27	58.63±2.31
0.0078	18.22	18.22	18.22±0.00		52.83	54.32	53.57±1.05

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