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ANTIMICROBIAL PROPERTIES OF EXTRACTS OF ENDOPHYTIC FUNGI ISOLATED FROM THE LEAVES OF SPONDIAS MOMBIN IN NIGERIA

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ABSTRACT

Drug resistant infections have become a very disturbing and challenging aspect of clinical practice necessitating the need to explore new frontiers of drug development. In the present study, four endophytic fungi (MR1, MR2, LB1 and LB2) were isolated from healthy leaves of the medicinal plant *Spondias mombin* using standard procedures. These fungi were fermented on local rice for twenty one days thereafter extracts of MR1, MR2, LB1 and LB2 were obtained using ethyl acetate and concentrated using a rotary evaporator at a reduced temperature. The fungal crude extracts were subjected to antimicrobial activity screening as well as phytochemical analysis in order to identify the secondary metabolites produced by each fungus. The results showed that MR1, LB1 and LB2 were the only active fungal extracts. At 1 mg/mL, MR1 extract was observed to be the most active inhibiting all the bacteria test organisms. The active fungal extracts exhibited good antibacterial activity against the bacterial isolates, the activity was observed to be broad-spectrum in action, inhibiting the growth of both the gram positive and gram negative test isolates at tested concentrations. The inhibition zones of the potent fungal extracts as well as their MICs

ranged between 2 to 6 mm and 0.5 – 1 mg mL^{-1} respectively. The secondary metabolites detected in the fungal extracts included Alkaloid, flavonoids and terpenoids previously established to be active against different bacteria isolates. The activities demonstrated by these fungal extracts may be attributed to the presence of the bioactive metabolites present in them. As a result of the remarkable anti-bacterial activity demonstrated by MR1 in comparison to other fungal extracts in this work, MR1 was further identified to be *Fusarium equiseti* combining macroscopic, microscopic as well as standard molecular techniques. Thus, this work clearly indicates that *F. equiseti* is a potential source of bioactive drug like molecules for development of new antibiotics.

KEYWORDS: Resistance, Endophyte, *Fusarium equiseti*, *Spondias mombin*, Antimicrobial, Secondary metabolites.

INTRODUCTION

Challenges and increasing resistance of microorganisms to antibiotics is accumulating globally, thus the urgency for novel bioactive compounds.[1] There has been a decrease in the discovery and development of newer antimicrobial drugs and this has been associated to the increase in resistance profiles of microorganism being faced globally. Due to discovery of the intrinsic chemo diversity identifying these compounds has led to the adoption of newer drug discovery techniques involving the adjustments of fungi culture conditions.[1]

In recent years, endophytic fungi have been reported to produce a plethora of bioactive metabolites[2] with interesting biological activities such as anti-inflammatory,[3] Antibacterial,[4,5] anticancer,[5,6] and antioxidant,[7] anti-diabetic, and insecticidal[6] activities. These have further provided additional evidence as well proved this group of microorganism to be important and reliable source of drug like molecules.

Fig. 1: Spondias mombin.

Spondias mombin is found almost in every part of the world and is commonly called "hog plum or yellow mombin" and belongs to the family *Anacardiaceae*. It is a flowering plant which is up to 15 to 20 m (60 ft) high and 1.5 m (4.9 ft) in girth, moderately buttressed and grows in rainforest and coastal areas.[8] *Spondias mombin* flowers between March to May, fruits appear between July to September which are edible fleshy, yellow fresh fruits with sour taste, has a single spiny fibrous kernel and wrinkled when dry.

This plant has been identified to exhibit anti-inflammatory activity,[9] antibacterial and molluscicidal activities,[10] and its leaf and bark decoctions have been observed to be a remedy for diarrhea, dysentery, gonorrhea.[8] Phytochemical analysis on extracts from *Spondias mombin* contains alkaloids, Saponins, and Terpenoids.

MATERIALS AND METHODS

Endophytic fungi isolation, Purification and Identification

Sampling site

Samples of plant under study were collected from a garden located at the Faculty of Pharmaceutical Sciences (Agulu Campus), Nnamdi Azikiwe University, Awka, Nigeria.

Leaf samples collection

Healthy leaves of *Spondias mombin* with no disease condition were harvested and identified by a botanist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. They were stored in polythene bags to prevent moisture loss and were transported to the laboratory within 30 min.

Isolation, Purification and Fermentation of endophytic fungi

The harvested samples were washed thoroughly under running tap water and air dried before culturing under aseptic conditions. Several endophytic fungi were isolated according to the protocol previously reported by,[5] which was modified slightly. The samples were subjected to a four-step method of surface sterilization process in other to rid of contaminants on the surface of the samples which includes washing using distilled water then subjected to surface sterilization by immersion for 2 min in 2 % v/v hypochlorite solution and 1 min in 70% v/v ethanol, and further washed three times in sterilized double distilled water for 3 min. After surface sterilization, the samples were air dried then cut into sizes and aseptically cultivated on sterile malt extract agar (MEA) plates supplemented with 50 µg/mL chloramphenicol to suppress bacterial growth. The cut end of the material was made to contact the medium. The

plates were incubated at 25 – 28ºCfor 4 to 7 days on the bench with normal day lights and dark periods. Hypha emerging out of the cultured material was sub-cultured multiple times in other to get axenic cultures. Pure endophytic fungi were fermented on rice medium in 1 L Erlenmeyer flasks for 21 days.

Extraction of secondary metabolites

At the completion of the fermentation, 500 mL of ethyl acetate were transferred into each fermentation flask, homogenized and agitated for two days. Following the stoppage of fermentation process, the homogenate were filtered and concentrated at a reduced temperature of 50ºC using a rotary evaporator. Each fungal extract was kept in the desiccator for proper drying and the weights recorded. The dried extracts were thereafter kept in the refrigerator at 4ºC prior to further analysis.

Antimicrobial activity

Test strains

The microorganisms used in this study included four standard human pathogenic bacteria species namely *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and two fungi isolates *Candida albicans* and *Aspergillus niger*. These human pathogenic microorganisms were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria and. Each of these strains was reconfirmed by standard biochemical test methods as presented by Clinical and Laboratory standards Institute (CLSI).

Agar diffusion technique

The evaluation of the antimicrobial potentials of the endophytic fungi extracts was carried out using agar diffusion assay method as described by[5] with slight modifications. Briefly in the method, sterile Mueller Hinton Agar and SDA plates were inoculated with the test culture with each of the test suspension containing 10^8CFU/mL of bacteria, 10^6 CFU/mL of yeast, and 10^4 spore/mL of fungi using surface swab technique. A sterile cork borer (8 mm in diameter) was used to make five wells on each of the MHA and SDA plates respectively. A stock concentration of 1 mg/mL of each of the extracts was prepared by weighing 4 mg of each of the endophytic fungal extract and reconstituting in 4 mL of Dimethyl sulfoxide (DMSO). Then two fold-serial dilutions of each of the fungal extract were made to get graded concentration 0.5, 0.25, 0.13, 0.06. Then, aliquots of 80 µL of each extract dilutions, was applied in each of the wells in the culture. Ciprofloxacin (5 µg/mL), and Miconazole (50

µg/mL) served as the positive controls while DMSO served as the negative control. The cultures were incubated at 37ºC for 18-24 h for the bacterial plates and 25-27ºC for 48 h for the fungal plates respectively. The antimicrobial potential for each extract were determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each extract, three replicates were conducted against each organism. Each extract was tested against all the bacterial and fungal isolates.

Phytochemical screening

In other to detect the secondary metabolites present in the MR1 fungal crude extract, tests for flavonoids, saponins, alkaloids, tannins, cardiac glycosides, terpenoids and steroids were carried out adopting standard methods as reported by.[11]

Microscopic study

In other to study and characterize the endophytic fungi, they were grown on MEA at 25 – 28ºC for 4-8 days, and the formation of mycelia was examined under a photomicrograph at the Biotechnology Laboratory, Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University Awka, Nigeria. The morphological examination of the isolated fungi was done by transferring fungal mycelia onto a glass slide containing lactophenol cotton blue stain, covered with the cover slip and was viewed under a photomicrograph at X40. However, identification of the obtained fungal isolates was confirmed by comparing the results with already established fungi morphology of several fungi presented in (Simplified fungi Identification Key, The University of Georgia).[12]

Molecular Identification of endophytic fungus by 18S rRNA gene sequencing

DNA Isolation and Polymerase chain reaction (PCR)

DNA isolation and amplification were carried out at the Molecular Research Foundation for Students and Scientist Laboratory, Nnamdi Azikiwe University, Awka using standard protocols.

Genomic DNA was extracted using Quick-DNATM Fungal/Bacterial Miniprep Kit; (Zymo Research), according to recommended protocol with slight modification. 12.5µl of One Taq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 0.5µl each of forward and reverse primers; 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a thermal cycler. Amplification conditions for the PCR were first Initial

denaturation for 30secs at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 20secs, primer annealing at 54^{o}C for 45secs and strand extension at 72^{o}C for 1 min. Final extension at 72^{o}Cfor 5 min on an Eppendorf nexus gradient Master cycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with Ethidium bromide to confirm.

Sequencing

All the PCR products that were sequenced were first cleansed using Exo-SAP-IT (USB, Affymetrix, USA), and 1 ul of the purified product was used as a template for direct sequencing using Big Dye terminator v. 2.0 cycle sequencer, according to manufacturer's instruction. The sequences obtained were blasted in NCBI similarity check platform for proper identification.

Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out using one way analysis of variance (ANOVA) and SPSS (version 20) statistical program.

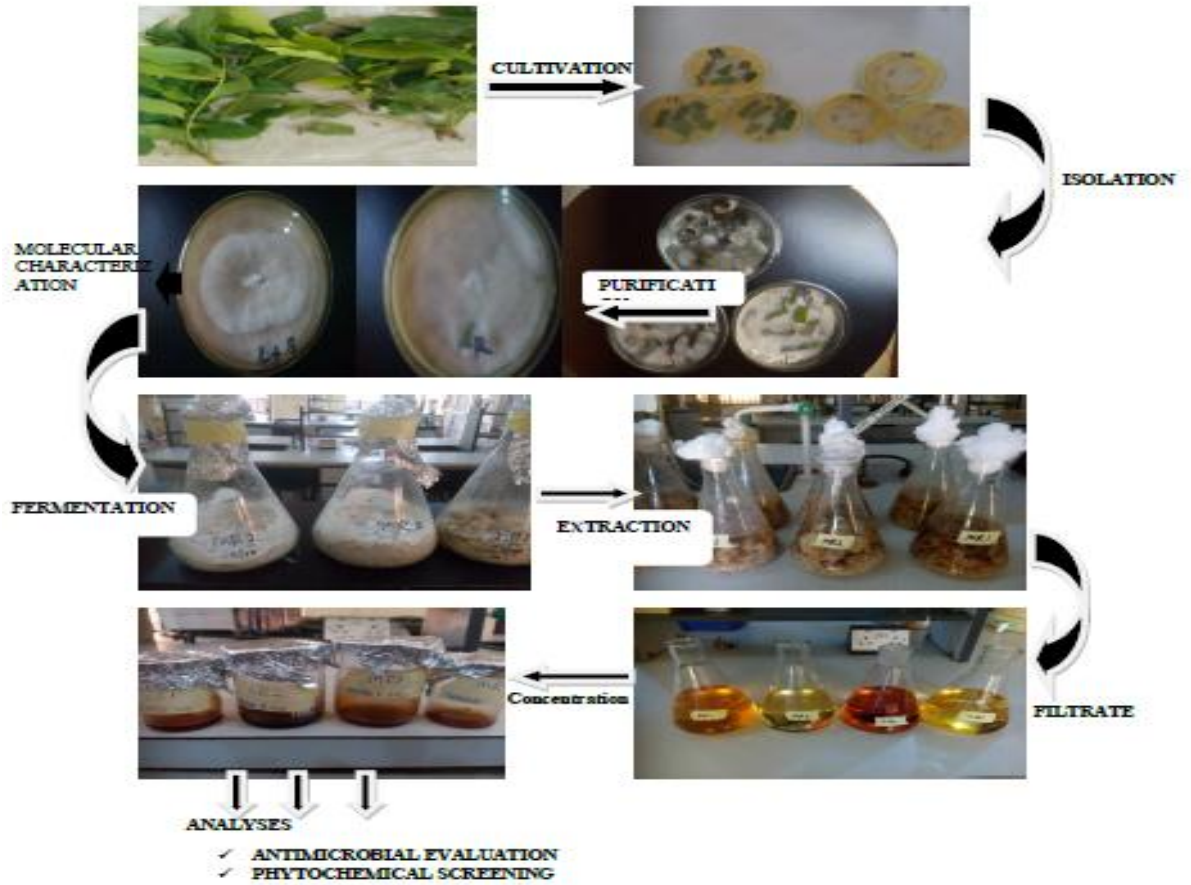


Figure 2: Graphical abstract.

RESULTS

A total of four (4) endophytic fungi were isolated from the Mid-rib and leaf blade parts of *Spondias mombin* leaf on Malt Extract Agar. Two fungi each from the mid-rib and the leaf blade respective (Table 1).

Table 1: Segments of the leaf sample that yielded endophytic fungi.

Fungal Isolate	Segment of isolation
Fusarium equiseti	Mid-rib (MR1)
Unidentified fungus	Mid-rib (MR2)
Unidentified fungus	Leaf-blade (LB1)
Unidentified fungus	Leaf-blade (LB2)

Table 2: Yield estimate of endophytic fungi extracts of *spondias mombin*

Endophytic fungal extract	Yield (mg)
Fusarium equiseti (MR1)	4307.4
Unidentified fungus (MR2)	632.2
Unidentified fungus (LB1)	2896.8
Unidentified fungus (LB2)	2559.37

The fermentation products of the endophytic fungi isolated in this work ranged between 632.2 – 4307.4 mg. The yield estimate of MR1 was observed to be the highest.

Table 3: Results of antimicrobial evaluation of the fungal extracts against the test organisms.

Concentration (mg / mL)	Test organisms					
	Staphylococcus aureus	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***	***Aspergillus niger***
MR1(*Fusarium equiseti*)						
1	3±0	5±0	2±0	4±0	0±0	0±0
0.5	0±0	0±0	0±0	0±0	0±0	0±0
0.25	0±0	0±0	0±0	0±0	0±0	0±0
0.13	0±0	0±0	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
***LB1* (*Rhizoctoniasp*)**						
1	4±0.7	4±0	3±0.7	0±0	0±0	0±0
0.5	0±0	0±0	0±0	0±0	0±0	0±0
0.25	0±0	0±0	0±0	0±0	0±0	0±0
0.13	0±0	0±0	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
***LB2* (*Rhizoctoniasp*)**						
1	6±0.7	6±0.7	3±0	0±0	0±0	0±0
0.5	3±0.7	3±0.7	0±0	0±0	0±0	0±0
0.25	0±0	0±0	0±0	0±0	0±0	0±0
0.13	0±0	0±0	0±0	0±0	0±0	0±0

0.06	0±0	0±0	0±0	0±0	0±0	0±0
Ciprofloxacin 5 µg/mL	7	6	0	0	na	na
Miconazole 50 µg/mL	na	na	na	na	16	0

Key: na: not applicable Pos. Ctrl. positive control (Ciprofloxacin 5 µg/mL: Miconazole 50 µg/mL); Neg. Ctrl., negative control (70 % Dimetylsulfoxide);

Table 4: Phytochemical analysis of endophytic fungi crude extract of *spondias mombin* leaves.

Fungal extract	**Phytochemical Constituents/Relative abundance**								
Fusarium equiseti	Alkaloid	Saponins	Tannins	Flavonoids	Terpenoids	Steroids	Cardiac glycoside	carbohydrates	Protein
	+	-	-	+	+++	++	-	++	-

Key: (-) Absent; (+) Mildly present; (++) Moderately present; (+++) Abundantly present

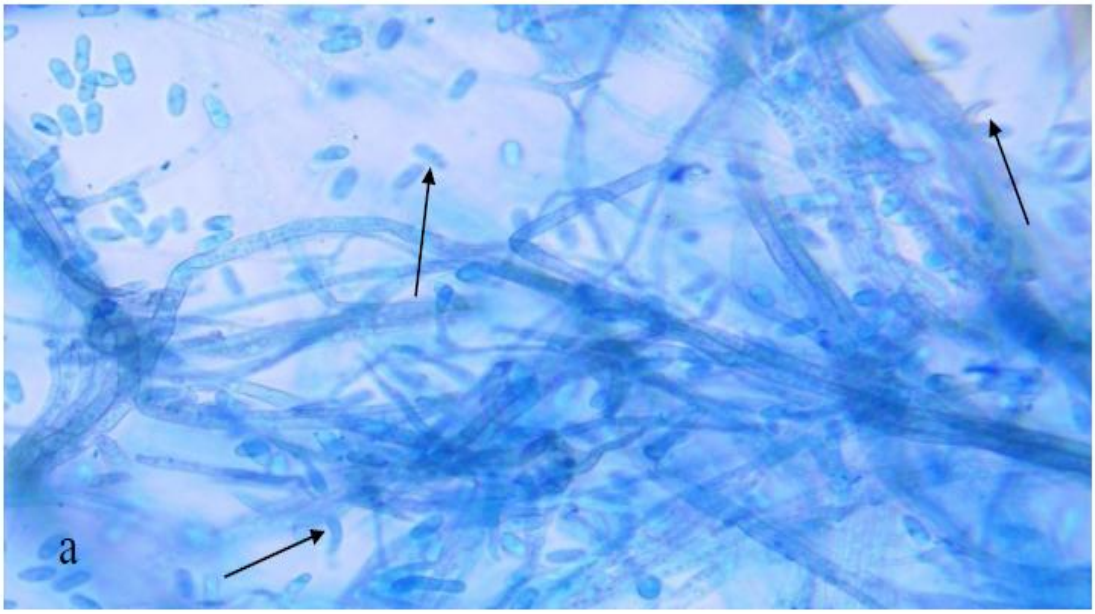
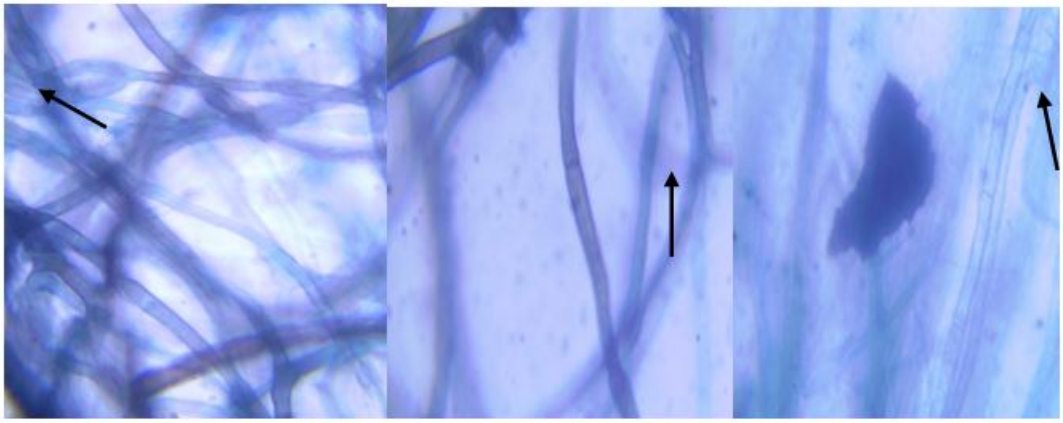


Fig. 3a: Showing the photo micrographic appearance of endophytic fungus isolate MR1 at 100X magnification. Conidia 3 or more-celled elongated to slightly curved, canoe-shaped (Probable organism: *Fusarium species).* This was compared with the photo micrographic features of *Fusarium species* presented in Simplified Fungi Identification Key, The University of Georgia.[12]



b **c** **d**

Fig. 3: (b) MR2, (c) LB1, (d) LB2, Showing the photo micrographic appearance of *Rhizoctoniaspp*at 100X magnification. Hyphae (mycelium) right angled branches (arrow); septa (Cross walls) occur near branching point.

Result of molecular identification of MR1

Blast outcome

Fusarium equiseti was returned with the highest percentage similarity in the NCBI BLAST platform. The percentage similarity score and E value were 99.60% and 0.0 respectively.

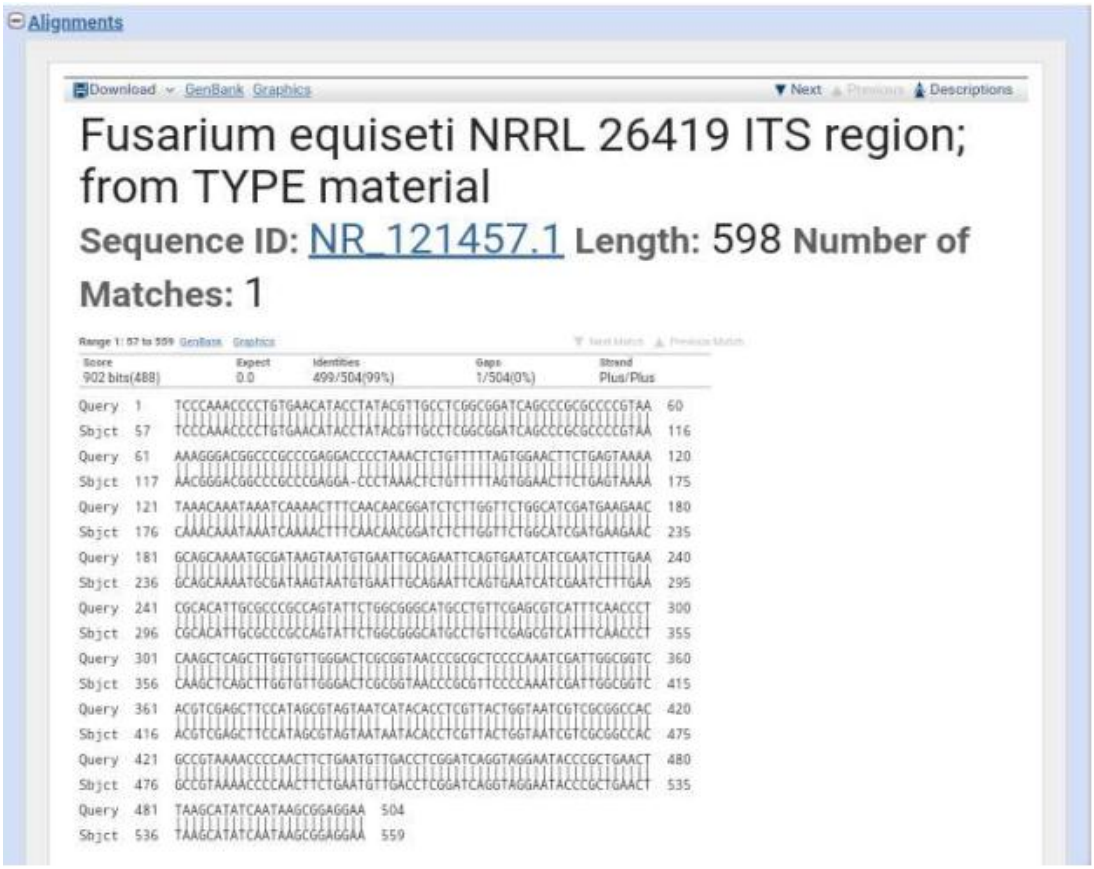


Figure 4: Sequence alignment outcome.

DISCUSSION

A total of four fungal (MR1, MR2, LB1 & LB2) endophytes were isolated from healthy leaves of *Spondias mombin* species. The number of isolates, rate of isolation and colonization obtained from the mid-rib and leaf blade parts were the same (Table 1).

The antimicrobial activity of the endophytic fungal extracts revealed the inhibitory activities of MR1, LB1 and LB2 against at least three of the four pathogenic bacteria with an average zone of inhibition that ranged between 2 and 6 mm. These activities as recorded demonstrate broad spectrum potentials by each of the potent fungal extract (Table 3). In contrast, none of the extracts had inhibitory effect against any of fungi isolates. The extract of *MR1 at* 1 mg/mL demonstrated a maximum inhibitory zone diameter of 3 mm against *Staphylococcus aureus*; 5 mm against *Bacillus subtilis;* 4 mm against *Pseudomonas aeruginosa* and 2 mm against *Escherichia coli*. While the fungal extracts LB1 and LB2 at 1 mg/mL had inhibition zones of 4 and 6 mm against (*Staph aureus*); 4 and 6 mm against (*B. subtilis*) respectively. While both extract produced and IZD of 3mm against *E. coli* (Table 3). The results showed varying Minimum inhibitory concentration by each of the fungal extract. LB2 had the lowest MIC for *Staph aureus* and *Bacillus subtilis* (0.5 mg/mL) (Table: 3c). Among the two major groups of bacteria tested, the fungal extracts were observed to be more active against the Gram positive species which included: *Staphylococcus aureus* and *Bacillus subtilis*, while *E. coli* was observed to be the most susceptible Gram negative test organism recorded in this work. Similar reports by,[13,14] also showed that the metabolites of endophytic fungi *Colleton tritium sp* and *Colleton tritium gloeosporoides* had strong antimicrobial activity against *(B. subtilis, Pseudomonas sp*) and (Multidrug-resistant *Staphylococcus aureus*) respectively. However, MR2 did not show inhibitory activity against the test organisms at tested concentrations.

Due the observed antimicrobial activity of MR1 in comparison with other fungal extracts, the extract of *MR1* was subjected to phytochemical analysis in order to detect the secondary metabolites that may be responsible for the observed antimicrobial.

The results of the phytochemical analysis revealed the presence of bioactive constituents such as alkaloids, flavonoids and terpenoids (Table 4). These bioactive secondary metabolites have been reported by several authors to have antimicrobial potentials.[15] Such as[16] and[17] who reported antibacterial activity of flavonoid rich plant extracts. The observed antimicrobial activities in this work may be due to the inhibitory actions of flavonoids against multiple cell

wall targets. Similarly, several reports exist on detected compounds produced by endophytes with potential antimicrobial activity against human pathogens. Some of these compounds includes but not limited to 4-hydroxyphenyl acetic acid, *p*-methoxycoumaric acid, indole-3-acetic acid, acropyrone and 4-hydroxyphenyl acetic acid,[5] citreo isocoumarinol, cladosporin and Acropyrone.[4] The difference in the degree of sensitivity of both Gram positive and Gram negative organisms to the extracts can be accounted for by the differences in the type of bioactive compounds present in the fungal extracts. The negative control (DMSO) had no inhibitory effect against the test microorganisms. The results of this study further confirm that endophytes are considered to de dependable reservoirs of novel bioactive compounds.[18]

MR1, on MEA was observed to appear white, with dense growth, aerial mycelium (colony) with no pigmentation (Fig.1). Photo micrographic examination of MR1 revealed the appearance of it conidia to be elongated and slightly curved, canoe-shaped (Fig. 3a). Molecular characterization of MR1 based on DNA isolation, amplification and sequencing was also carried out. The complete characterization of MR1 as against the other fungi was occasioned by the fact that this fungus produced the most active extract in this work. The macroscopic, microscopic as well as the molecular characteristics allowed for the identification of the endophytic fungus MR1 specifically to be *Fusarium equiseti*, This was supported by the result of sequencing of its 18S rRNA gene that gave a 99.60 % sequence similarity to those accessible at the BLASTN of *Fusarium equiseti*(Fig. 4).

MR1 was thus identified to be *Fusarium species*. One of several literatures on fungal diversity and their chemo diversity reveal *Fusarium sp* to be ubiquitous[19] and also a potential source of bioactive compounds, producing these bioactive compounds in form of chemical defense mechanism against predation to survive adverse situation.[20] reported the isolation of a new antifungal compound from *Fusarium sp* isolated from the plant *Selaginella pallescens*. The absence of antifungal compounds from MR1 may, however, be related to location of the plant under study. It has been severally reported that geographical location may influence the type and number of fungi which in turn affects the secondary metabolites produced.

CONCLUSION

The data presented in this study showed that extracts of endophytic fungi isolated from healthy leaves of *Spondias mombin* have compounds with antimicrobial potentials and this

study also highlights the biosynthetic capacity of *Fusarium sp* as a reliable source of bioactive compounds.

ACKNOWLEDGEMENT

"Not applicable"

CONFLICTS OF INTEREST

There are no conflicts of interest

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