ISOLATION, ANTIMICROBIAL PROPERTIES, AND CHARACTERIZATION OF ENDOPHYTIC SECONDARY METABOLITES OBTAINED FROM *AZADIRACHTA INDICA*

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ABSTRACT

Phytochemicals of endophytes have generated significant interest in drug discovery programs due to their immense potential towards contributing to the discovery of new biologically active molecules. Reports have shown that endophytes of *Azadirachta indica* are been used in the treatment of many human disease conditions. The study was aimed to evaluate the antimicrobial properties and chemical characterization of endophytic extracts isolated from *A. indica*. Endophytic extract was isolated from *A. indica* leaves using standard extraction protocols. The extracts were screened for their potential antimicrobial activities using the agar diffusion well method. Phytochemical analyses for the identification of secondary metabolites were done using HPLC-DAD, GC-FID, GC-MS, and FTIR methods. The endophytic fungi (MR3) screened for antimicrobial activity, showed quantifiable activity. At 1.0 mg/mL, inhibition of bacterial by the endophytic fungal extract was observed in *Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis,* and *Candida albicans* with zones of inhibition diameters of 6, 5, 4, and 4 mm respectively. HPLC-DAD analysis revealed the presence of 2-carboxymethyl-3-*n*-hexylmaleic acid anhydride and aspernigin A. GC-FID analysis showed remarkably high concentrations of flavanones and catechins. The results of GC-MS analysis also showed the presence of trans-13-octadecenoic acid (282.5g/mol), hexadecanoic acid (114.14g/mol), and 1,6-Anhydro-beta-D-glucopyranose (162.14g/mol). FTIR results established the existence of functional groups like carboxylic acids, which may be associated with some of the detected compounds. Endophytic fungal extracts of *A. indica* have a potent antimicrobial activity which is related to a high content of flavonoids and alkaloids.

Keywords: *Antimicrobial, Azadirachta indica, HPLC-DAD, Chromatography, Curative.*

INTRODUCTION

Azadirachta indica, commonly known as neem or neem tree, is a tree in the mahogany family Meliaceae. It is native to the Indian subcontinent and most of the countries in Africa, including Nigeria. It is typically grown in tropical and semi-tropical regions. Its fruits and seeds are the sources of neem oil (Barstow, and Deepu,

2018). Plant products have shown an important role in diseases prevention and treatment through the enhancement of antioxidant activity, inhibition of bacterial growth, and modulation of genetic pathways (Alzohairy M., 2016). The therapeutics role of several plants in diseases management is still being enthusiastically researched due to their fewer side effect and affordable properties (Alzohairy M., 2016). It is a largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants (Singh and Sastry, 1997). Different types of preparation based on plants or their constituents are very popular in many countries in the management of various diseases condition. *A. indica* has a complex of various constituents including nimbin, nimbidin, nimbolide, and limonoids, and such types of ingredients play role in diseases management through modulation of various genetic pathways and other activities. Quercetin and ß-sitosterol were first polyphenolic flavonoids purified from fresh leaves of neem and were known to have antifungal and antibacterial activities (Govindachari *et al.,* 1998). Numerous biological and pharmacological activities have been reported including antibacterial, antifungal, and anti-inflammatory (Kher and Chaurasia, 1997). Earlier investigators have confirmed their role as anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, anti-gastric ulcer, antifungal, antibacterial, and antitumor activities (Bandyopadhyay *et al.*, 2004; Sultana *et al.*, 2007; Ebong *et al.*, 2008; Paul *et al.,* 2011). This study was meant to evaluate the antimicrobial properties and chemical composition of the extracts of endophytic fungi isolated from *A. indica* leaves, and possibly identify the secondary metabolites responsible for the antimicrobial and antimalarial activities.

MATERIALS AND METHODS

Plant Collection and authentication

New leaves of *A. indica* were collected from its natural habitat at the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus, Aniocha Local Government Area of Anambra State, Nigeria. The plant was identified by a senior technologist, Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. A voucher specimen PCG474/A/054 was deposited at the Herbarium of the Department of Pharmacognosy and Traditional Medicine, of the same institution.

Test organisms

The potential antimicrobial properties of the endophytic fungi of the plant under study were evaluated using four standard human pathogenic bacterial species including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa,* and two fungi strains, including *Candida albicans* and *Aspergillus niger*.

Isolation procedure

The isolation procedures reported by Arnold *et al,* (2000) were followed with mild modifications. Then, they were

rinsed to remove unwanted debris with sterile distilled water and blotted dry on sterile blotting paper in a lamina flow cabinet. A sterile scalpel was used to cut the leaf blade and mid-rib approximately 1cm in length. About 5-6 segments were placed aseptically on MEA - malt extract agar media which was prepared with 250 mg chloramphenicol per liter of the media to suppress bacterial growth (Pragathi *et al.*, 2013). The cut end of the plant leaf blade and midrib was made to be in contact with the media. The plates were sealed with masking tapes and then incubated at 25-28^{0}C for seven days and monitored every day. Most of the fungal growth was initiated within 5 days of inoculation. The hyphae growing from the plant materials were sub-cultured repeatedly until a pure culture is obtained.

Endophyte fermentation and extraction of metabolites

The endophyte isolated was subjected to solid-state fermentation in a 1 L Erlenmeyer flask containing sterilized rice medium which was prepared by autoclaving a mixture of 100 g of unpolished rice and 200 mL of distilled water (Terasawat and Phoolphundh, 2021). After cooling, blocks of actively growing pure fungal isolates were transferred onto the rice media under aseptic conditions, plugged with cotton wool and foil, and left on the fermentation shelf for 21 days. After fermentation, the fungal secondary metabolites were extracted with ethyl acetate, and flasks were left undisturbed for about 48 hours with intermittent agitation. A rotary evaporator was used to concentrate the extract filtrates, at a speed of 7 rpm and 50^{o}C. The filtrates were used for the biological assay.

Identification of isolated endophytic fungi

Macroscopic identification

Macroscopy was carried to ascertain the different parts of the endophyte. The study was done morphologically, by plating out the fungi on malt extract agar (MEA) and incubating them for about 1 week. The hyphal growth and appearance were observed based on the back and front morphology of the fungus on the Petri dishes. Morphological identification was done following the standard taxonomic parameters, including colony diameter, texture, color, conidia, hyphae dimensions, and morphology (Mani *et al*., 2015).

Microscopic identification

The endophytic fungus was identified by studying its cultural characteristics, spore formations, and mycelium. The tease mount method was used to prepare the slides using lactophenol cotton blue reagent and detected at × 40 and × 100 lens magnifications (Wanger *et al.*, 2017). The stain contains phenol that was meant to kill the micro-organisms, lactic acid was to preserve fungal structures, and then cotton blue stains the chitin present in the cell walls of the fungi.

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

The HPLC study on the endophyte extract was done using a Dionèx attached to a photodiode array detector (UVD - 340S, Germany). A weight of 2 mg of the extract

was reconstituted with 2 ml of methanol (HPLC grade), the mixture was sonicatèd for 10 min and centrifuged at 3000 rpm for 5 min. Then 100 µL of the dissolved samples were introduced into HPLC vials containing 500 µL of methanol (HPLC grade). Detection was at 235 nm, 254 nm, 280 nm, and 340 nm, respectively. The separation column (125 mm × 4 mm; length × internal diameter) was pre-filled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopore water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The detection was at 235 nm and the absorption peaks of the fungal extracts were analyzed by comparing them with those in the HPLC-UV/Vis record (Eze *et al.,* 2019).

Quantification of phytochemicals using Gas chromatography-flame ionization detector (GC-FID)

The endophyte extract (0.1g) was weighed and transferred in a test tube and 1ml ethanol and 1mL of 50% m/v potassium hydroxide were added. The test tube was allowed to react in a watèr bath at 60°C for 60 min. After the reaction time, the reaction product contained in the test tube was transferred to a separating funnel. The tube was washed successively with 2 mL of ethanol, 1 mL of cold water, 1mL of hot water, and 3 mL of hexane, which was all transferred to the funnel. These extracts were combined and washed three times with 10mL of 1%v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulfate ($NaSO_4$) and the solvent was evaporated. The sample was solubilized in 1000 uL of pyridine of which 200 uL was transferred to a vial for analysis. The analysis of phytochemical was performed on a BUCK M910 Gas chromatography equipped with a flame ionization detector. A RESTEK 15 meter MTX-1 column (15 m x 250µm x 0.15 µm) was used. The injector temperature was 280^{o}C with a splitless injection of 2uL of sample and a linear velocity of 30 cms^{-1}, Helium 5.0 pa.s was the carrier gas with a flow ratè of 40 ml min^{-1}. The oven operated initially at 200^{0}C, it was heated to 330^{0}Cat at a rate of 3^{0}C min-1 and was kept at this temperature for 5min. the detector operated at a temperature of 320^{0}C. Phytochemical tests and compositions were determined by the ratio between the area and mass of internal standard and the area of the identified phytochemical. The concentration of the different phytochemicals observed was expressed in mg/L (Onuah *et al.,* 2019).

Gas chromatography-mass spectrometry

The GC–MS study of the biologically active compounds from the endophytic fungus extract were done with aid of Agilent Technologies GC systems (model: GC-7890A/MS-5975C, USA) furnished with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in the thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system that utilized high-energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow ratè of 1 mL/min. The initial temperature was set at 50 –150 °C with an increasing rate of 3 °C/min and a holding time of about 10 min. As a final point, the temperature was increased to about 300 °C

at 10 °C/min. 1 mL of the extract (1%) diluted with the suitable solvènts was injècted in a splitless mode. The rèlative amount of the chemical compounds presènt in each of the extracts was exprèssed as a percentage (%) based on the peak area produced in the chromatogrâm. The biologically active compounds extracted from a different endophyte of *A. indica* were identified based on Gas Chromatographic retention time on the HP-5MS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems) (Buss, 2010).

Fourier transformed infrared

Buck scientific M530 USA FTIR was used for the analysis. This instrument was coupled with a detector of deuterated triglycine sulfate and a beam splitter of potassium bromide. The software of Gram A1 was used to obtain the spectra and to manipulate them. An approximately 1.0 g of samples, 0.5 ml of nujol was added, they were mixed properly and placed on the salt pellet. During measurement, FTIR spectra were obtained at frequency regions of 4,000 – 600 cm-1 and co-added at 32 scans and 4 cm-1 resolution. FTIR spectra were displayed as transmitter values (Vander *et al.*, 2004; Ellis *et al.*, 2007).

Vacuum Liquid Chromatography (VLC)

VLC is considered as a preparative thin-layer chromatography (PTLC), as separation is carried out on TLC grade silica gel or aluminum oxide and column is dried after each fraction as in PTLC plates are dried and re-run to enhance the separation. Methanolic extract of the endophytic fungus extract was subjected to VLC procedure using DCM (100%), n-hexane (100%), ethyl acetate (100%), and methanol (100%) respectively, as solvent systems. Then four fractions were obtained which were concentrated to dryness, the resulting fractions were again subjected to VLC procedure using DCM (100%), n-hexane (100%), ethyl acetate (100%), and methanol (100%) solvent systems (Repon *et al.*, 2016).

DNA sequencing

DNA sequencing is the process of determining the nucleic acid sequence, especially the order of nucleotides in a DNA molecule. It includes any technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and drug discovery (Behjati, and Tarpey, 2013). Fasta Sequences of *Curvularia lunata* coded as A2 from molecular/taxonomic identification was conducted using Sequences ID: MT259787.1 (>OF3_ITS-1_A04_01).

Statistical analysis

Data were analyzed using SPSS computer software, Version 23. The results were expressed as mean ± SD. The results are presented as the mean ± standard error of the mean (SEM). The differences between the means of the measured parameters were compared using one-way ANOVA. The P values < 0.05 at 95% confidence were regarded as statistically significant.

RESULTS

Endophytic fungi labeled MR3, was isolated from *A. indica.* The endophytic fungus with the best biological activity (MR3) was identified macroscopically and microscopically based on its morphological appearances and molecular identification (Figure 1 and 2). The endophytic fungus MR3 was identified as *Curvularia lunata* (Table 1)

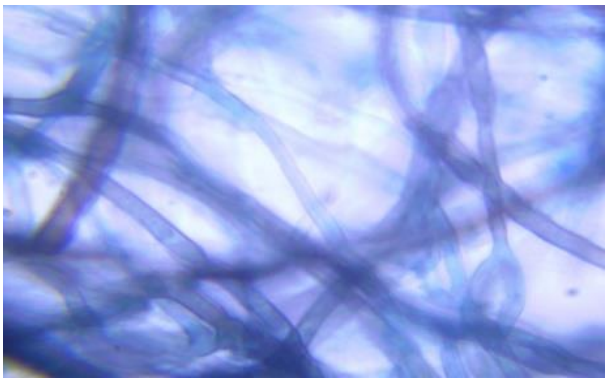


Figure 1: Microscopic characteristics *Curvularia lunata* isolated from the leaves *A. indica*

Figure 2: Macroscopic characteristics *Curvularia lunata* isolated from the leaves *A. indica*

Table 1: Fungus endophyte isolated from the lèaves of *A. indica*

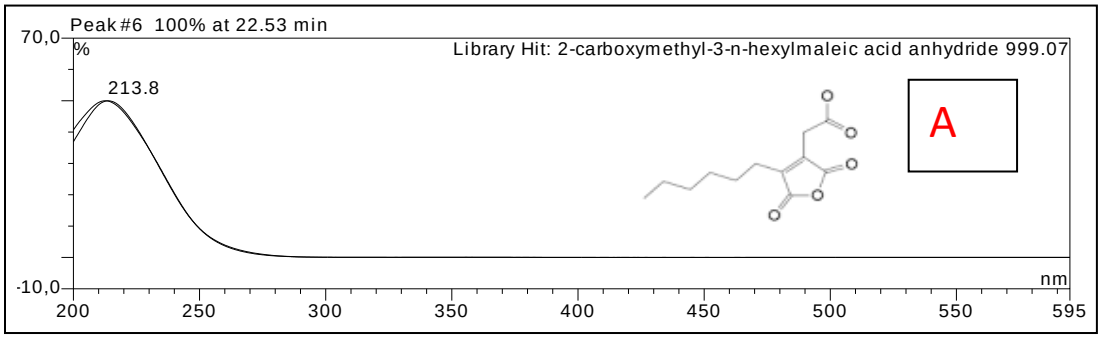
Plant source	**Plant parts**	**Fungi codes**	**Identification**	**Class**	**References**
A. indica	Leaf	MR3	*Curvularia lunata*	Dothideomycetes	(Frisvad & Samson, 2004; chemicalbook.com, 2020)

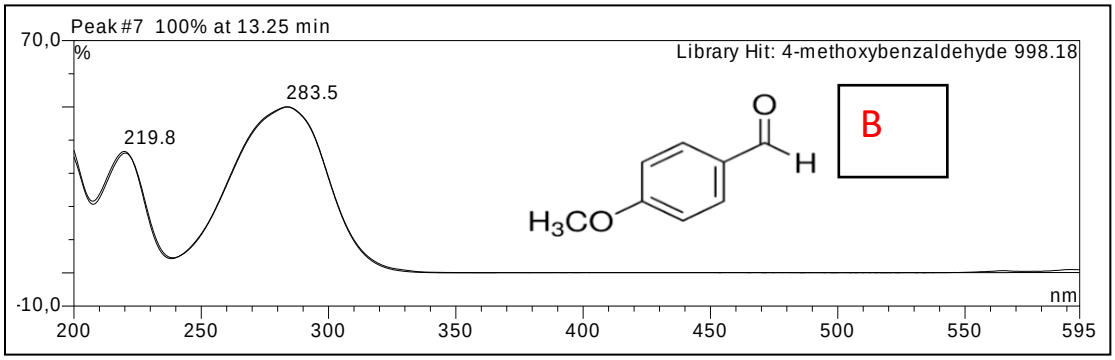
Table 2: Antimicrobial assay results

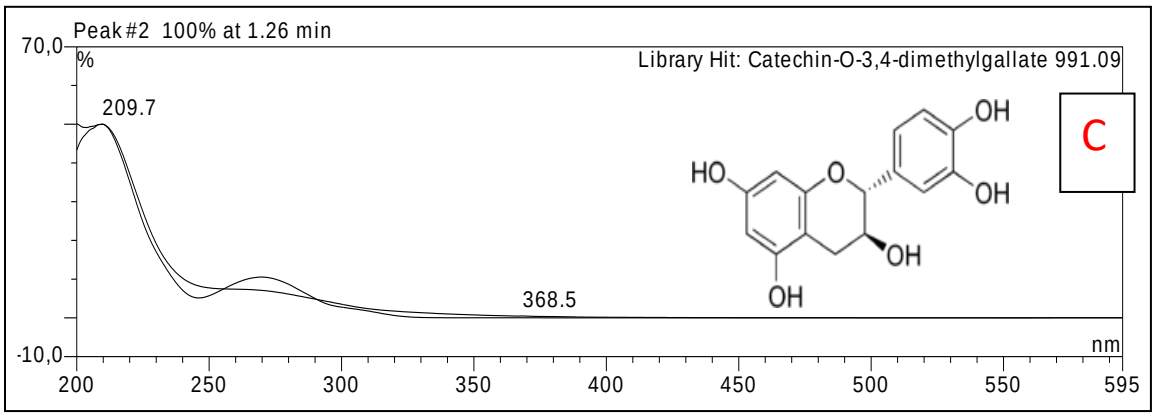
Test organism	Endophytic extract at 1 mg/mL ZOI	Positive control (Ciprofloxacin 5 µg/mL) ZOI	Negative control DMSO (100% v/v)
S.aureus	11.5	10	0
Escherichia coli	8	7	0
Pseudomonas aeruginosa	6	13	0
Bacillus subtilis	4	14	0
		Miconazole (50 µg/mL)	**DMSO (100% v/v)**
Candida albicans	4	6	Nil

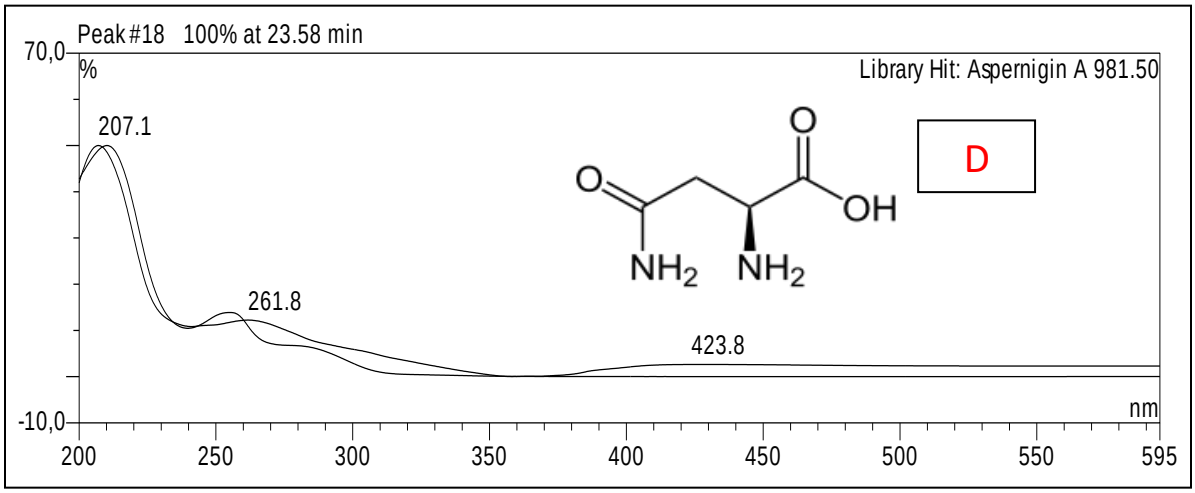
Key: *ZOI- Zone of inhibition (mm) of the antimicrobial test of Endophyte from A. indica bioactive compounds and standard antibiotics*

HPLC chromatogram, UV spectra, and structures of major compounds detected









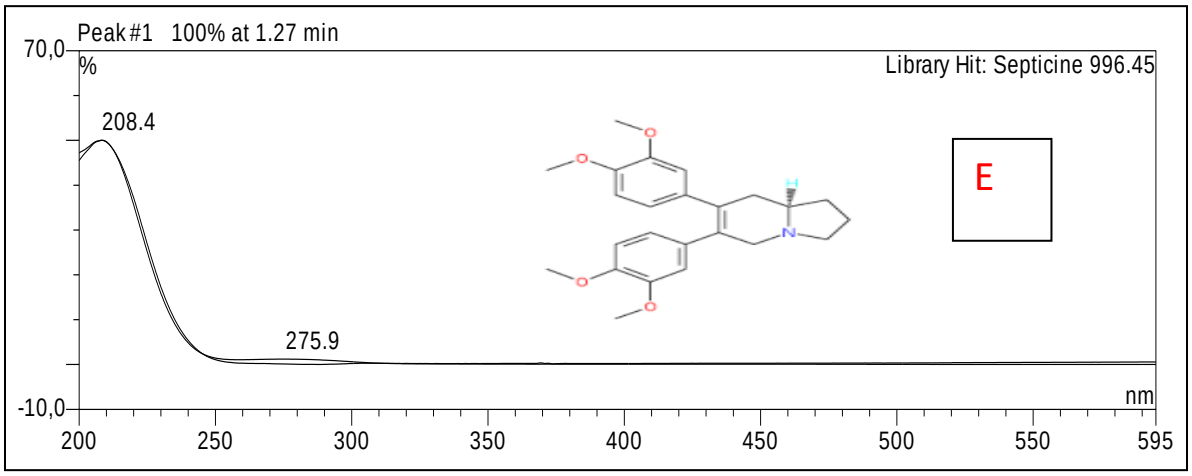
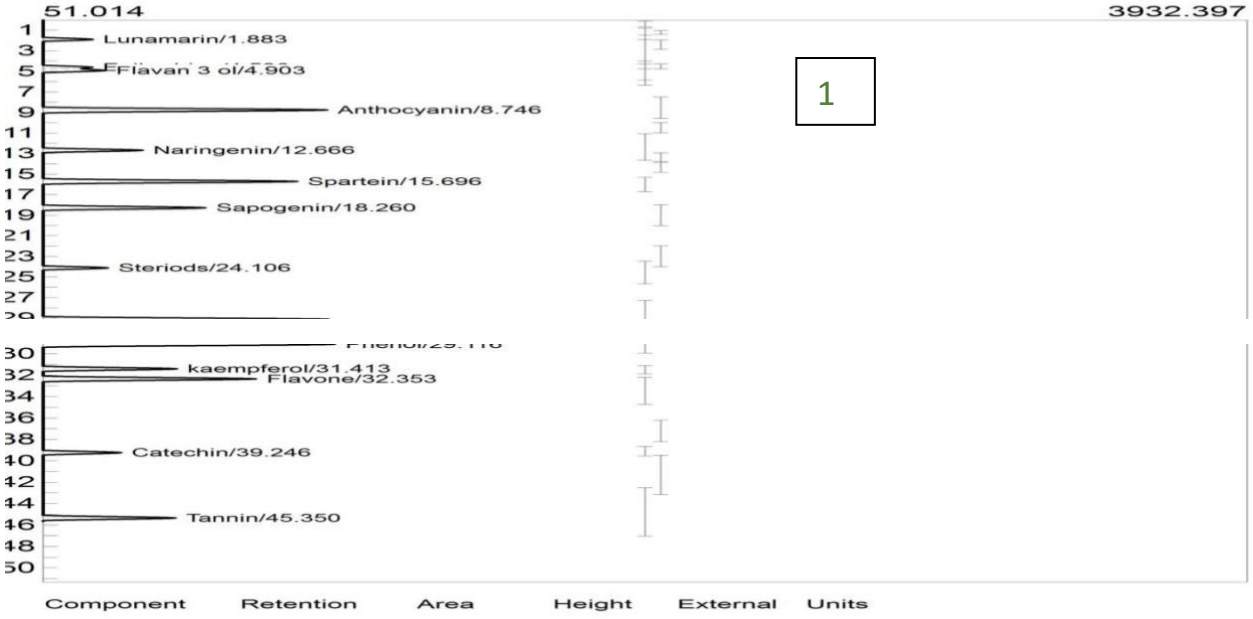


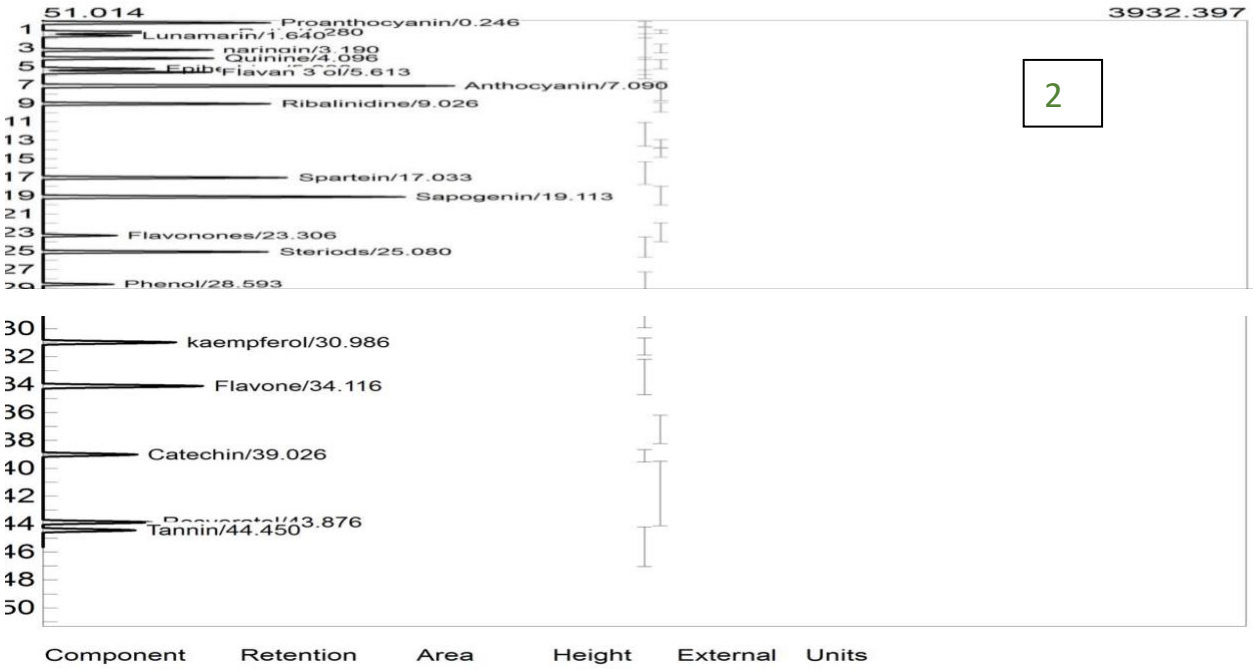
Figure 3 (A - E): HPLC chromatogram, UV spectra and structures of major compounds detected in the vacuum liquid chromatography sub-fraction of endophytic extract of *A.indica*; A = 2-carboxymethyl-3-hexylmaleic acid anhydride (RT = 22.53 min, Hit 999.07), B = 4-methoxybenzaldehyde (RT = 13.25 min, Hit 998.18), and C = Catechin-O-3,4-

dimethylgallate (RT = 1.26 min, Hit 991.09), D = Aspernigin A (RT = 1.26 min, Hit 981.50), E = Septicine (RT =1.27min, Hit 996.45).

GC-FID

The quantitative phytochemical screèning of the plants using GC-FID showed that the endophytic extract of *A. indica* is rich mainly in alkaloids and flavonoids (phenolic compounds) shown in figure 7 below.





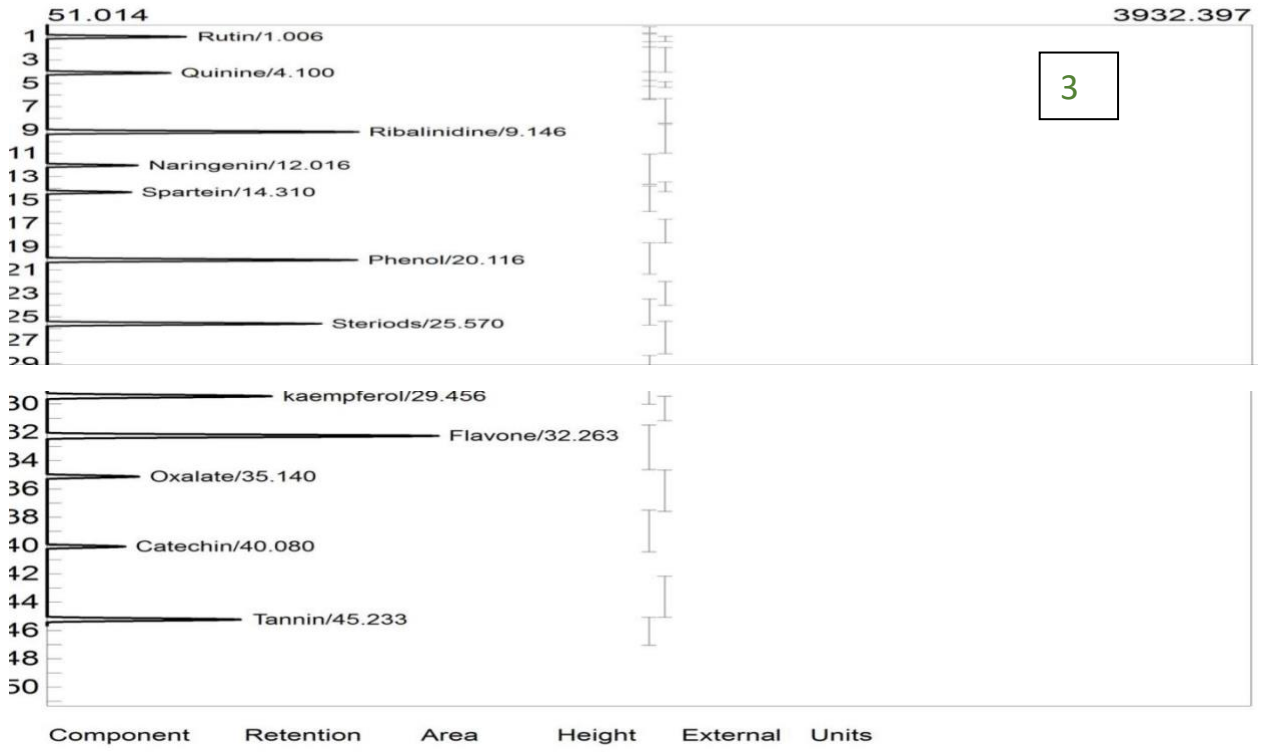
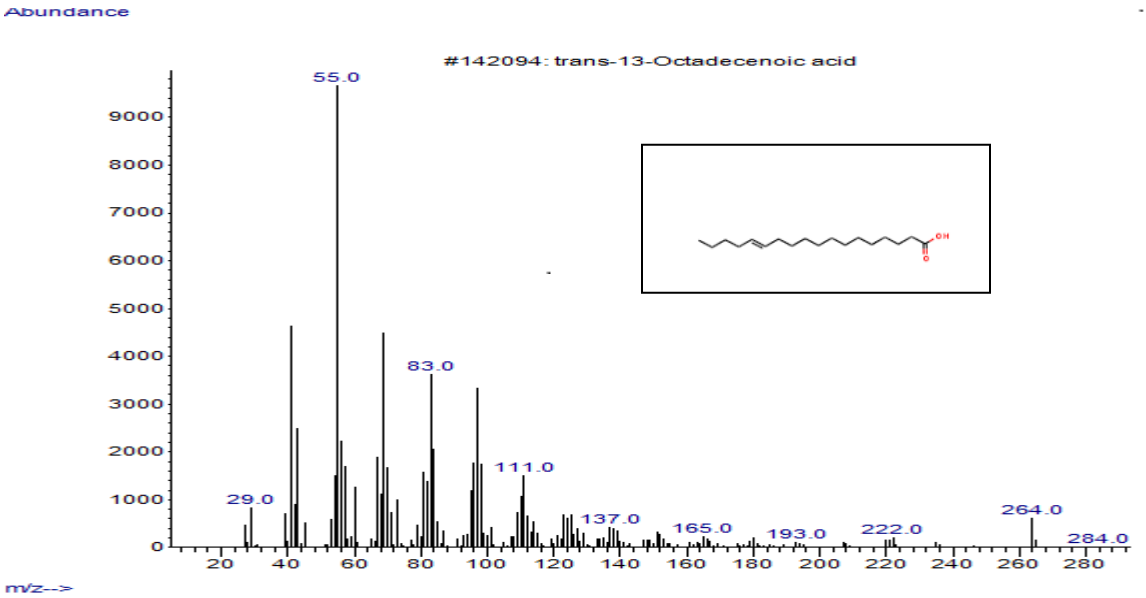
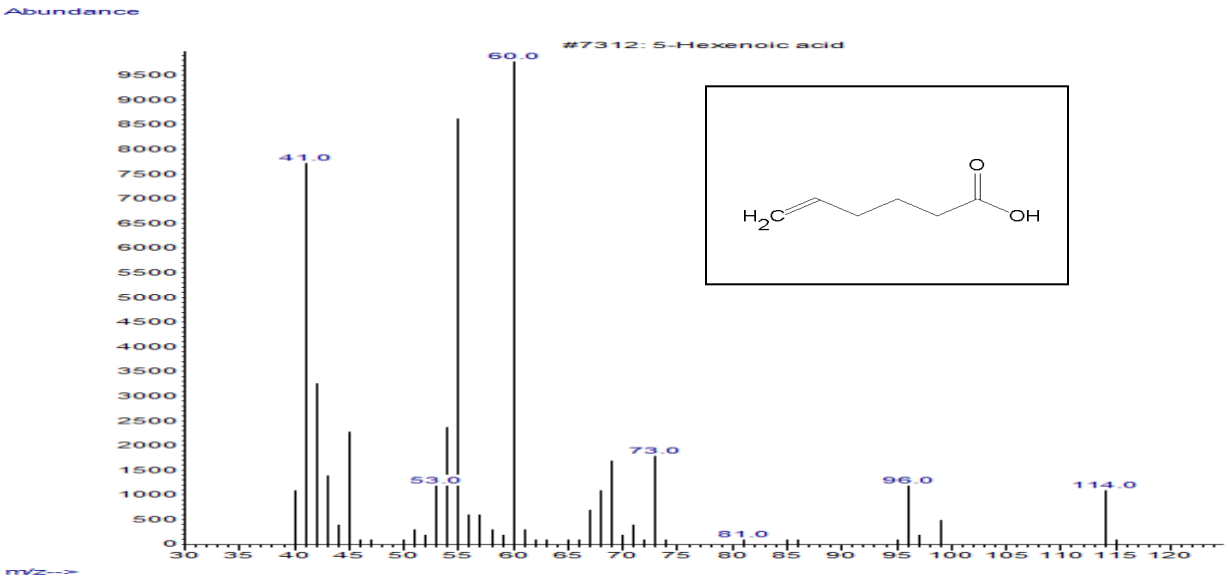


Figure 4 (1 - 3): Phytochemical constituents of the VLC sub-fractions (1-3) of the extract of *Curvularia lunata* isolated from *A. indica*

Table 3: Quantitative determination of the endophytic chemical constituents using GC-MS

Phytochemical Compound	Abundance (%)	Retention time (RT) minutes	Molecular Formula	Molecular Weight (g/mole)	Biological activity reported
Trans-13-Octadecenoic acid	57.97	16.61	$C_{18}H_{34}O_2$	282.5	Antimicrobial (28)
Hexadecanoic acid	43.89	55.06	$C_6H_{10}O_2$	114.14	Cytotoxic (27)
1,6-Anhydro-beta-D-glucopyranose	22.48	50.02	$C_6H_{10}O_5$	162.14	Antibiotic (26)


Abundance
#142094: trans-13-Octadecenoic acid
55.0
83.0
111.0
29.0
137.0
165.0
193.0
222.0
264.0
284.0
m/z-->


Abundance
#7312: 5-Hexenoic acid
60.0
41.0
53.0
73.0
96.0
114.0
81.0
m/z-->

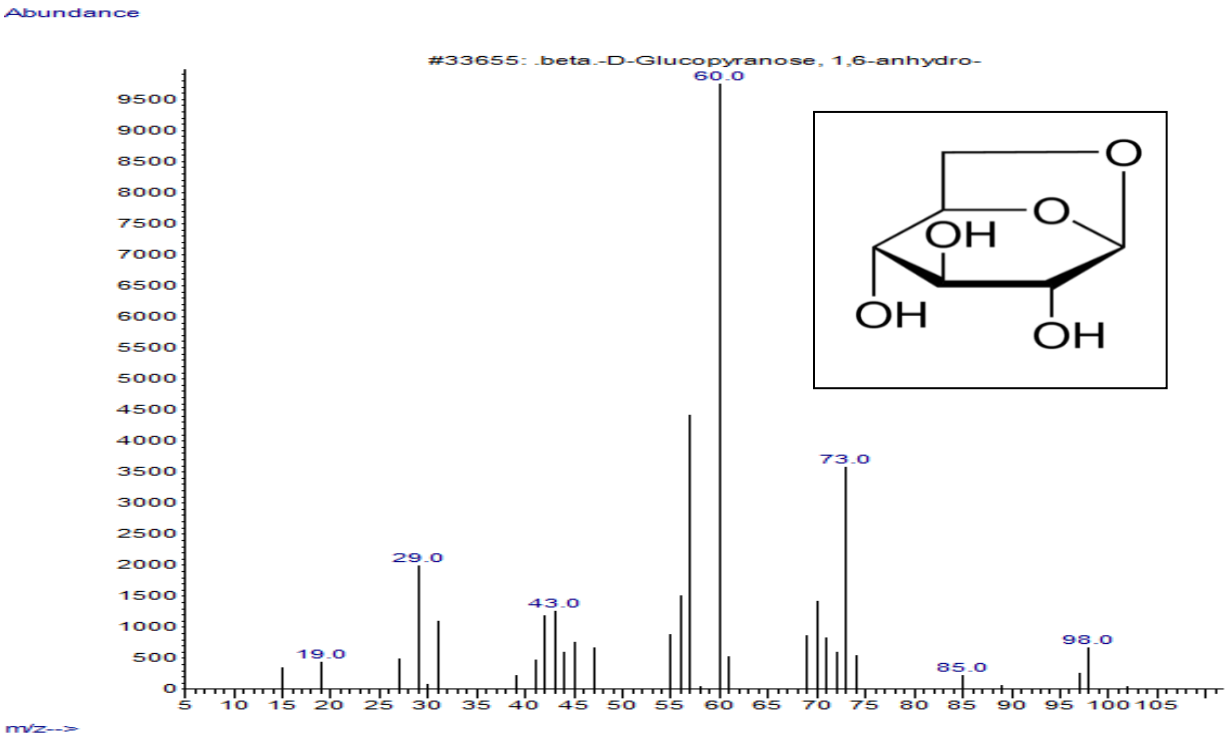


Figure 5. GC-MS analysis of the isolated extract

FT-IR Analysis

Fourier Transformed Infrared (FTIR) technique is an important tool used to identify the characteristic functional groups, which are instrumental in the determination of functional groups and organic compounds inherent in any given sample. Results of the FTIR spectra are recorded in the table below:

Table 4. Quantitative determination of the endophytic functional groups using FTIR (Sample MR3)

S/N	Frequency	Functional group	Compounds
1	715.2362	C-Br	Bromo C-Br symmetric stretch
2	827.5784	C-CI	chloro C-Br symmetric stretch
3	1219.542	R-0-R	Ether C0 symmetric stretch
4	1337.928	$H_2C=CH_2$	Ethene CH symmetric stretch
5	1441.636	$H_2C=CH_2$	Ethene CH symmetric stretch

6	1614.245	RNH_3	1^0 amine NH stretch
7	1843.481	RC00R	Cyclic ester C00 stretch
8	2009.913	RC00H	Carboxylic acid C0 stretch
9	2097.522	RC00H	Carboxylic acid C0 stretch
10	2285.879	$R_2C=0$	Carbonyl C0 stretch
11	2450.537	R-C≡N	Nitriles CN antisymmetric stretch
12	2553.440	R-C≡N	Nitriles CN antisymmetric stretch
13	2643.685	CH_2	Methylene CH symmetric stretch
14	2758.091	CH_2	Methylene CH symmetric stretch
15	2927.338	R-S-C≡N	Thiocyanate SCN antisymmetric stretch
16	3017.612	RCH0H	1^0 alcohol 0H stretch
17	3140.970	RCH0H	1^0 alcohol 0H stretch
18	3242.058	RCH0H	1^0 alcohol 0H stretch
19	3377.973	R_2CH0H	2^0 alcohol 0H stretch
20	3588.887	R_3CH0H	3^0 alcohol 0H stretch
21	3694.784	R_3CH0H	3^0 alcohol 0H stretch
22	3809.405	R_3CH0H	3^0 alcohol 0H stretch
23	3924.964	R_3CH0H	3^0 alcohol 0H stretch

From the table of results above for sample M, the absorption bands around 715.2362cm^{-1} and 827.5784cm^{-1} were assigned to C-Br and C-CI stretching vibration of the halogenous compound.

Curvularia lunata isolate Amankwa5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MT259787.1 Length: 568 Number of Matches: 1

Range 1: 48 to 568 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
963 bits(521)	0.0	521/521(100%)	0/521(0%)	Plus/Plus

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Query  5    GCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGG  64
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Sbjct  48   GCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGG  107

Query  65   CGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGTTGCAATCAGCGT  124
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Sbjct  108  CGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGTTGCAATCAGCGT  167

Query  125  CAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATC  184
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Sbjct  168  CAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATC  227

Query  185  GATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCG  244
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Sbjct  528  GGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA  568
```

Figure 6. Fasta Sequences of *Curvularia lunata* coded as A2 from molecular/taxonomic identification

>OF3_ITS-1_A04_01: Sequences ID: MT259787.1

GACTGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACT

TGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCT

TTTTTATGCAGTTGCAATCAGCGTCAGTACAAACAATGTAAATCATTTAC

AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG

AAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTT

TGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGC

GTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGG

TCGCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTC

GCAGCGCAGCACATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCA

AGCCTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC

TTAAGCATATCAATAAGCGGAGGAA

DISCUSSION

Antimicrobial activities and elemental analysis of endophytic extracts isolated from *Azadirachta indica* leaves were, successfully conducted using the agar diffusion well method and different spectrophotometric methods. In the antimicrobial assay, the fungal extract inhibited the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans* at 1 mg/mL, with zones of inhibition diameters of 6, 4, 8, and 4 mm respectively and as much as 11.5mm against *S. aureus* (Table 2). The microscopic and macroscopic characteristics *of Curvularia lunata* isolated from the leaves *A. indica* was identified (Figure 1, 2, and Table 1). Furthermore, the elemental analysis was performed to ascertain the chemical constituents from the extract. For HPLC-DAD, the chromatogram of the endophytic extracts revealed the presence of major compounds of which most were from the phenolic group (Figure 3). These include β-sitosterol-3-O-a-D-glucopyranoside, catechin-O-3, 4-dimethygallate, septicine, 4-methoxybenzaldehyde, indol-3-carbaldehyde, aspernigin A, protocatechuate, chloramphenicol, p-hydroxyphenyl-acetic acid, 2-carboxymethyl-3-n-hexylmaleic acid anhydride, catechin-O-3,4-dimethylgallate and had very high precisions as high as 99%.Beta-sitosterol-3-O-beta-D-glucopyranoside is a steroidal glycoside that has been reported to selectively inhibit the activity of mammalian DNA polymerase lambda in vitro, which provides important enzymatic activities for base excision repair (BER). From the FTIR results, using sample MR3 for example, (Table 4), the absorption bands around 715.2362cm^{-1} and 827.5784cm^{-1} were assigned to C-Br and C-CI stretching vibration of the halogenous compound. The peak value around 1219.542cm^{-1} was assigned to the C=O stretching vibration of the ether compound. The absorbance around 1337.928cm^{-1} and 1441.636cm^{-1} was assigned to the C=C stretching vibration of the ethene compound. The medium band around 1614.245cm^{-1} was assigned to NH stretching vibration of 1^0 amine compound while the value around 1843.481cm^{-1} was assigned to COO stretching vibration of cyclic ester compound. The absorbance around 2009.913cm^{-1}, 2097.522cm^{-1}, and 2285.879cm^{-1} were assigned to COO stretching vibration of carboxylic acid and C=O stretching vibration of carbonyl compound respectively. The peak values around 2450.537cm^{-1} and 2553.440cm^{-1} were assigned to CN anti-symmetric vibration of nitrile compounds respectively. The weak bands around 2643.685cm^{-1}, 2758.091cm^{-1}, and 2927.338cm^{-1} were assigned to CH and SCN stretching vibration of methylene and thiocyanate compounds respectively. The absorption bands around 3017.612cm^{-1}, 3140.970cm^{-1}, 3242.058cm^{-1}, 3377.973cm^{-1}, 3588.887cm^{-1}, 3694.784cm^{-1}, 3809.405cm^{-1} and 3924.964cm^{-1} were assigned to OH stretching vibration of 1^0, 2^0 & 3^0 phenolic

compounds respectively (Table 4 and Figure 4).

The HPLC chromatograms, UV-spectra, and chemical structures of the detected compounds are shown in Fig 3. Also, GC-MS discovered that the endophytic extract from *A. indica* possesses many promising compounds. The chromatogram of the GC-MS analysis carried out showed the presènce of seventeen compounds. The results of GC-MS analysis showed the presènce of trans-13-Octadecenoic acid, n-Hexadecanoic acid, Methyl stearate, 9,17-Octadecadienal, Erucic acid, 9,12-Octadecadienoic acid (z,z)-methyl ester, 9-Octadecenoic acid, methyl ester, Oleic Acid, Hexadecane, Methyl tetradecanoate, Tetradecanal, 2-Heptadecanone, Hexadecanoic acid methyl ester, 9-Octadecenal (Z), 2-Methyl-Z,Z-3,13-octadecadienol, Z-2-Octadecen-1-ol at different percentage concentrations. The most abundant having bioactivities associated with the plant (Table 3, Figure 5). These constituents have been reported to confer antibacterial properties to medicinal plants (Divya and Mukesh, 2017; Rhian *et al.*, 2020). DNA sequencing was successfully done to identify different genetic information in the isolated endophyte: *Curvularia lunata* (Figure 6).

CONCLUSION

From isolated compounds, five endophytic extracts were screened for their potential antimicrobial activities, MR3 showed the highest activity. Test microorganisms' growth was inhibited by the fungal extract at 1 mg/mL. The vacuum liquid chromatography sub-fraction (MR3M) of the endophyte showed the best antimicrobial activity. HPLC-DAD revealed the presence of 2-carboxymethyl-3-*n*-hexylmaleic acid anhydride and aspernigin, A while GC-MS showed, hexadecanoic acid, and 1,6-Anhydro-beta-D-glucopyranose and FTIR confirmed the presence of carboxylic acids functional groups. Therefore, from the results obtained, the endophytic extracts of *A. indica* have potent antimicrobial activities with several peptide linkages as shown in the sequence result.

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