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Research Article

Antimicrobial Properties and Characterization of Secondary Metabolites Obtained from Curvularia lunata, an Endophyte of *Azadirachta indica*

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Abstract

Background: Endophyte phytochemicals have piqued interest in drug development programs due to their enormous potential for assisting in the discovery of novel biologically active compounds. **Aim and objectives:** The study was aimed to isolate, evaluate the antimicrobial properties and characterize the secondary metabolites of *Curvularia lunata* endophyte isolated from *A. indica.* **Method:** *C. lunata* isolated from *A. indica* leaves using standard extraction protocols were screened for their potential antimicrobial activities using agar diffusion well method. HPLC-DAD, GC-FID, GC-MS, and FTIR techniques were used to characterize secondary metabolites. **Results:** At 1 mg/mL, growth inhibition of bacteria by the extracts of *C. lunata* were observed with *Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis,* and *Candida albicans* with zones of inhibition diameters of 6, 5, 4, and 4 mm respectively. The vacuum liquid chromatography sub-fraction of the extract showed the best antimicrobial activity. 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 flavonones and catechins. The results of GC-MS analysis also showed the presence of hexadecanoic acid, and 1,6-Anhydro-beta-D-glucopyranose. FTIR results established the presence of functional groups like carboxylic acids, which may be associated with some of the detected compounds. **Conclusion:** *C. lunata* (OM337582) extract of *A. indica* have potent antimicrobial activity which could be related to a high content of flavonoids and alkaloids.

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

INTRODUCTION

Azadirachta indica, sometimes known as neem, is a mahogany tree in the Meliaceae family. It is indigenous to the Indian subcontinent and the majority of African countries, including Nigeria. It grows best in tropical and semi-tropical climates. Neem oil is extracted from its fruits and seeds[1]. Plant compounds have been proven to have a vital role in illness prevention and therapy by increasing antioxidant activity, inhibiting bacterial growth, and modulating genetic pathways[2]. The therapeutics role of several plants in diseases management is still being enthusiastically researched due to their fewer side effect and affordable properties[3]. Many pharmacologically active medications are obtained from natural resources, including medicinal plants, as is widely acknowledged[4]. In many countries, different types of preparations based on plants or their elements are quite popular in the treatment of various disorders. *A. indica* has a complex of different compounds, including as nimbin, nimbidin, nimbolide, and limonoids, which play a role in disease treatment by modulating numerous genetic pathways and other activities. Quercetin and ß-sitosterol were the first polyphenolic flavonoids isolated from fresh neem leaves and had antifungal and antibacterial properties[5]. Biological and pharmacological actions such as antibacterial, antifungal, and anti-inflammatory have been described[6]. Anti-inflammatory, antiarthritic, antipyretic, hypoglycemia, anti-gastric ulcer, antifungal, antibacterial, and anticancer actions have previously been confirmed by researchers[7,8,9,10]. The goal the study was to determine the antibacterial properties and chemical makeup of endophytic fungus extracts derived from *A. indica* leaves, as well as perhaps discover the secondary metabolites responsible for antimicrobial action.

MATERIALS AND METHODS

MATERIALS

Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research), Centrifuge (EPPENDORF, GERMANY), Vortex Mixer, Block Heater (WEALTEC CORP, TAIWAN), Microwave Oven (SCANFROST, CHINA), Pipettes, Digital Scale, Microcentrifuge Tubes, Gel Tank, Gel comb, Scientific Power Pack (CLEAVER SCIENTIFIC, TAIWAN), Gel Documentation System (VILBER, GERMANY).

Plant Collection and authentication

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

Test organisms

The potential antimicrobial property of *Curvularia lunata* 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 of the fungus from plant tissues

The isolation procedures previously reported[11], were followed with mild modifications. The leaves 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[12]. 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.

Identification of isolated endophytic fungi

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[13]. 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. Morphological identification was done following the standard taxonomic parameters, including colony diameter, texture, color, conidia, hyphae dimensions, and morphology[14].

Molecular identification

Molecular identification of the endophytic fungus isolated from *A. indica* was performed using the standard protocol. Genomic DNA was extracted using Quick-DNATM Fungal/Bacterial Miniprep Kit; Zymo Research), according to recommended protocol with slight modification. About 50-100mg (wet weight) pure fungi cells that had been resuspended in up to 200µl of water was added to a ZR Bashing Bead Lysis Tube (0.1mm & 0.5mm). 750 µl Bashing BeadTM Buffer was added to the tube and vortexed at maximum speed for ≥ 15minutes. ZR Bashing BeadTM Lysis Tube (0.1 & 0.5mm) was centrifuged in a microcentrifuge at 10,000 x g for 1 minute. Up to 400µl of the supernatant was added to a Zymo-SpinTM III-F Filter in a Collection Tube and centrifuged at 8000 x g for 1 minute. 1,200µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube from step 4 after which 800µl of the mixture was transferred to a Zymo-SpinTM IIC Column in a Collection Tube and Centrifuged at 10,000 x g for 1 minute. The flow through was discarded from the Collection Tube and the step repeated. 200µl DNA Pre-Wash Buffer was added to the Zymo-SpinTM IIC Column in a new Collection Tube and Centrifuged at 10,000 x g for 1 minute. 500µl g-DNA Wash Buffer was added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-SpinTM IIC Column was then transferred to a clean 1.5ml microcentrifuge tube and 60 µl DNA Elution Buffer was added directly to the column matrix. Centrifugation was at 10,000 x g for 30 seconds to elute the DNA.

Agarose Gel Electrophoresis

Two (2) % agarose gel was prepared by dissolving 1.2g of Agarose in 60ml of 1X TAE Buffer. The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50ºC. 3µl of Ethidium Bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray, with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and 1X TAE Buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed. 5 µl of DNA was mixed with 2 µl of loading dye and loaded into the holes. The tank was connected to the power pack and set to run at 100volts for 20 minutes. The band were viewed using the gel documentation system.

Polymerase Chain Reaction Protocol

A 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 (ITS1 5'- TCCGTAGGTGAACCTGCGG -3' and ITS4 5'- TCCTCCGCTTATTGATATGC -3'); 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 oa thermalcycler. Amplification conditions for the PCR was as follows for fungi: Initial denaturation for 30secs at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 20secs, primer annealing at 54C for 45secs and strand extension at 72^{o}C for 1 min. Final extension at 72^{o}C for 5 min on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with Ethidium bromide[15,16].

DNA Sequencing

DNA sequencing of the fungal and ITS region was carried out according to the method described by Stephen Davis[17], and Sharma KK[18].

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[19]. Blocks of actively developing pure fungal isolates were transferred to rice media under aseptic conditions, blocked with cotton wool and foil, and kept on the fermentation shelf for 21 days after chilling. After fermentation, ethyl acetate was used to extract the fungal secondary metabolites, and flasks were left undisturbed for 48 hours with intermittent agitation. The extract filtrates were concentrated using a rotary evaporator set to 7 rpm and 50°C. For the biological assay and other analyses, the filtrates were utilized.

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

The HPLC study on the fungal extract was done using a Dionex 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[20].

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. Chemical tests and compositions were determined by the ratio between the area and mass of internal standard and the area of the identified fungal secondary metabolites. The concentration of the different fungal secondary metabolites observed were expressed in mg/L[21].

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)[22].

Fourier transformed infrared

The analysis was performed using a Buck Scientific M530 USA FTIR. This apparatus was used in conjunction with a deuterated triglycine sulfate detector and a potassium bromide beam splitter. The spectra were obtained and manipulated using the Gram A1 software. Approximately 1.0 g of materials were combined with 0.5 ml of nujol before being placed on the salt pellet. FTIR spectra were acquired at frequency ranges of 4,000–600 cm-1 and co-added at 32 scans and 4 cm-1 resolution during the measurement. The transmitter dislplayed the values of FTIR spectra[23,24].

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[25].

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

Following microscopic and molecular identification protocols, the endophytic fungus isolated from *A. indica* was identified as *Curvularia lunata.* The fungus DNA sequence data was deposited in the NCBI database (GeneBank) with accession number OM337582.

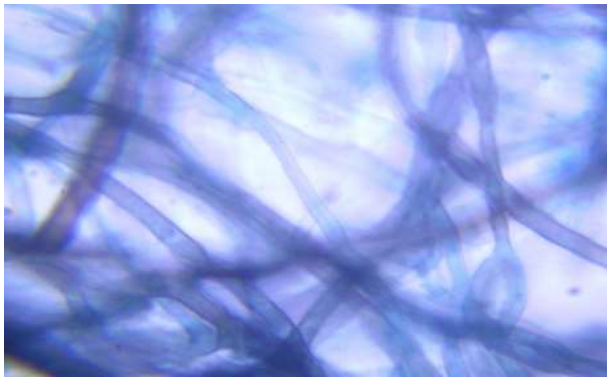


Figure 1: Microscopic characteristics *Curvularia lunata* isolated from the leaves *A. indica*

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

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

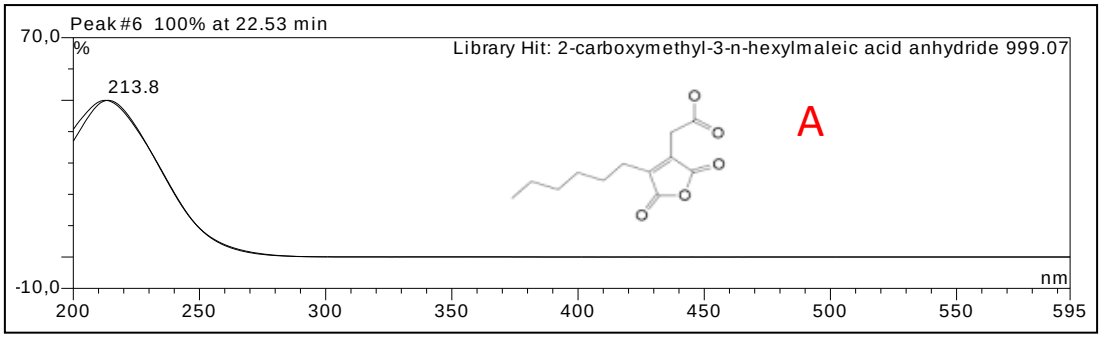
Plant source	Plant parts	Fungi codes	Identification	Class
Indica	Leaf	MR3	*Curvularia lunata*	Dothideomycetes[26].

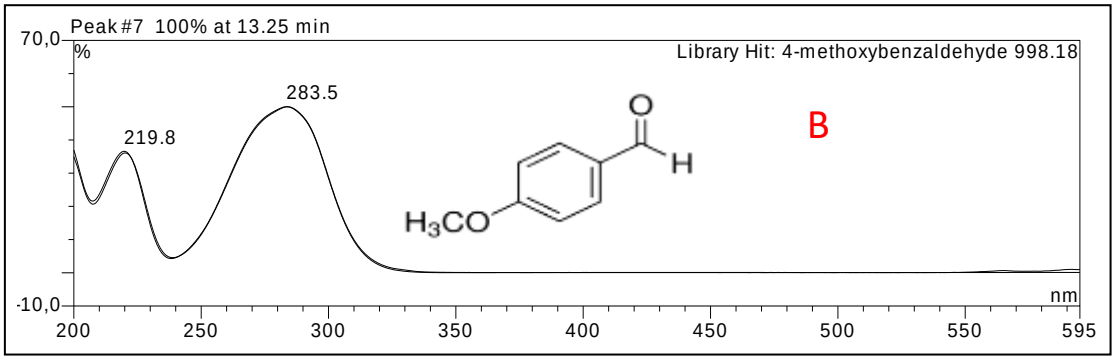
Table 2: Antimicrobial assay results

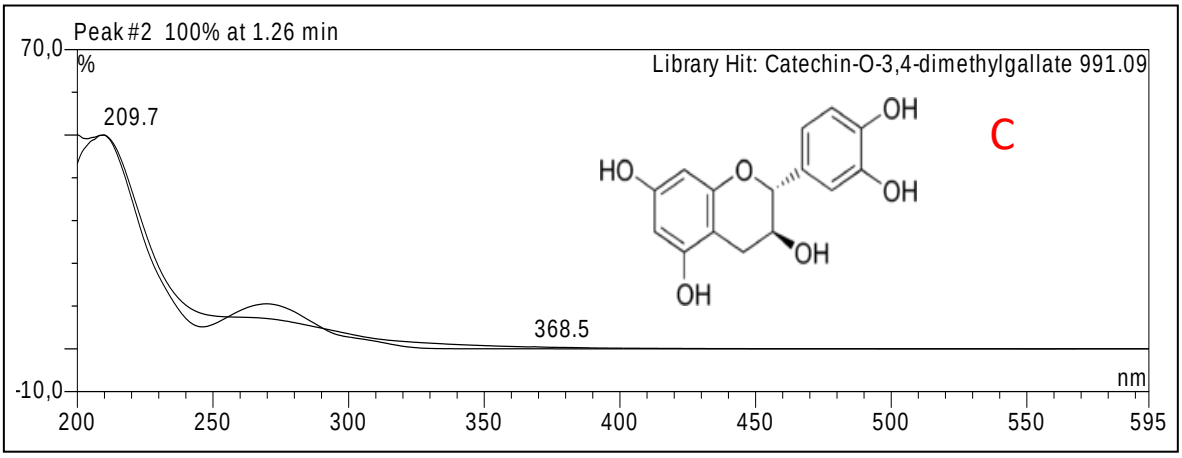
Test organism	Endophytic extract at 1 mg/mL ZOI	Positive control (Ciprofloxacin 5 µg/mL) ZOI	Negative control DMSO (100% v/v)
Staphylococcus aureus	11.5	10	0
Escherichia coli	8	7	0
Pseudomonas aeruginosa	6	13	0
Bacillus subtilis	4	14	0
		Miconazole (50 µg/mL)	**DMSO (100% v/v)**
Candida albicans	4	6	0
Aspergillus niger	0	15	0

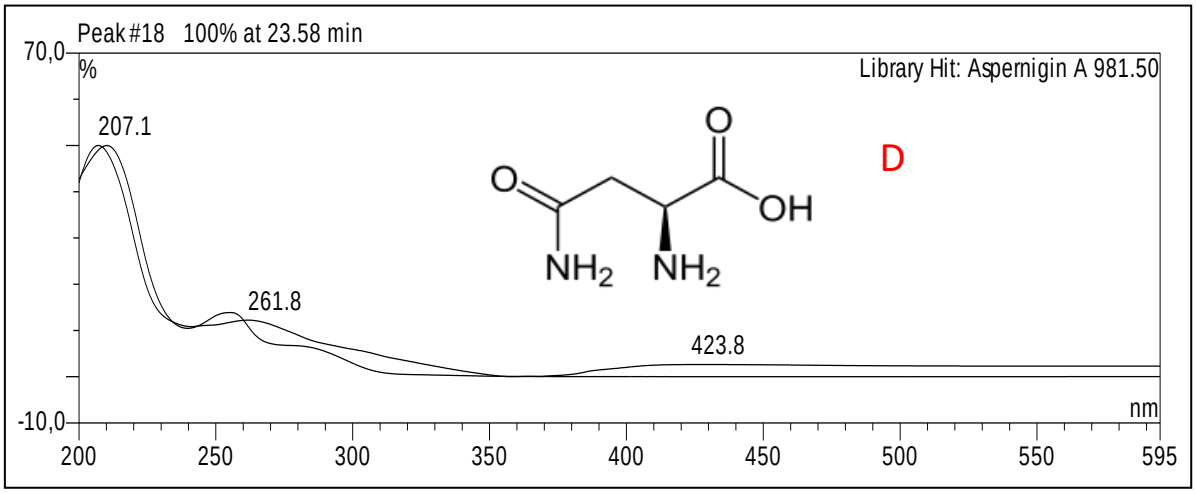
Key: *ZOI- Zone of inhibition (mm) of the antimicrobial test of Endophyte from A. indica bioactive compounds and standard antibiotics*

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









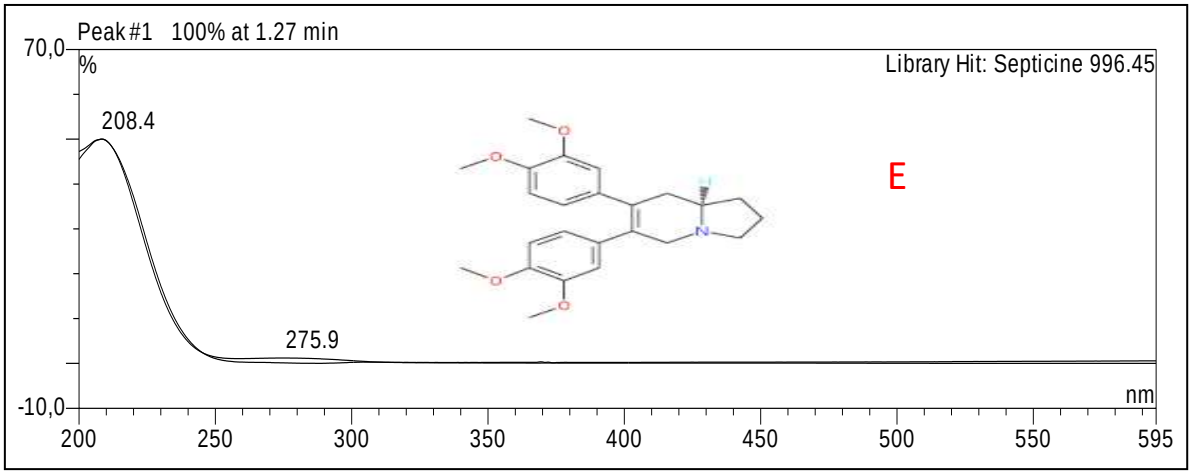
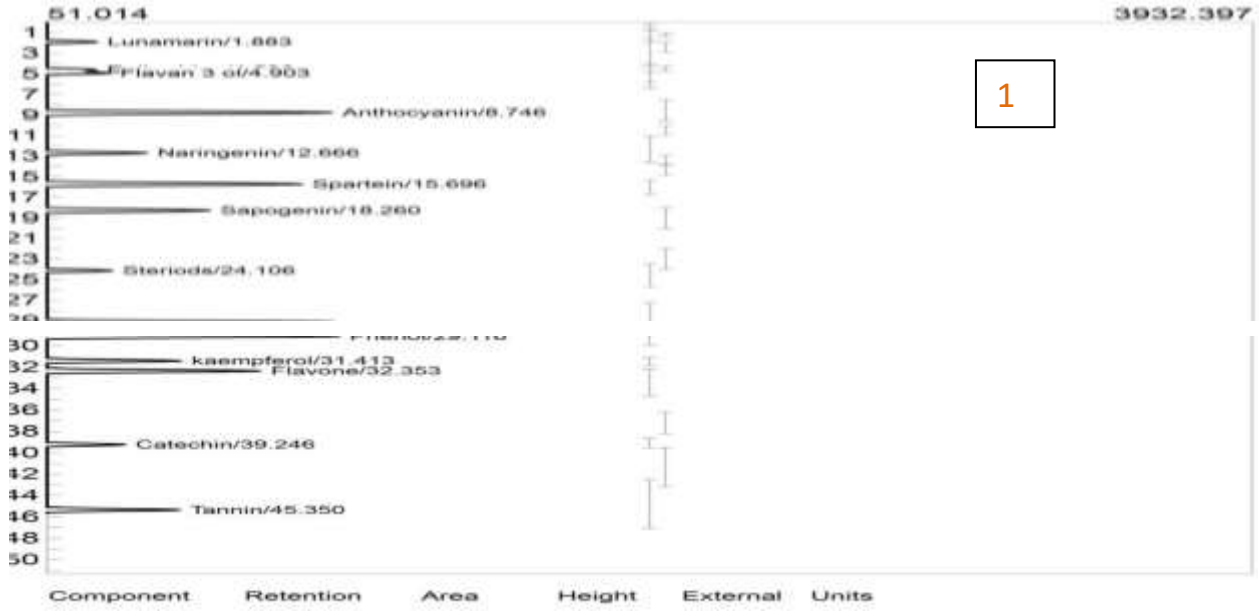


Figure 3: (A - E): HPLC chromatogram, UV spectra and structures of major compounds detected in the vacuum liquid chromatography sub-fraction of endophytic extract of *A.indica*; A = 2-carboxymethyl-3-hexylmaleic acid anhydride (RT = 22.53 min, Hit 999.07), B = 4-methoxybenzaldehyde (RT = 13.25 min, Hit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



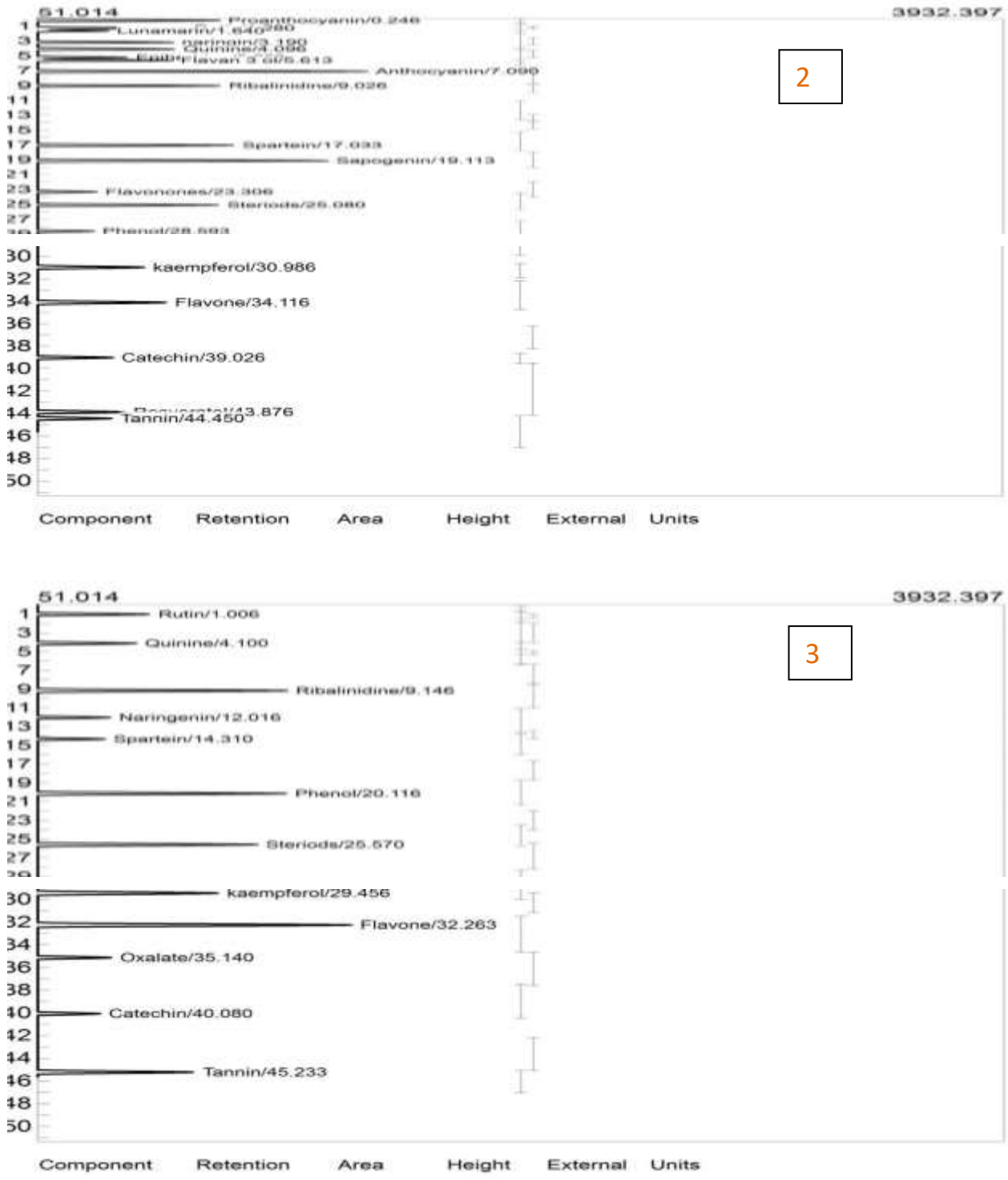


Figure 4 (1 - 3): Chemical constituents of the VLC sub-fractions of *Curvularia lunata* isolated from *A. indica*

Table 3: Quantitative determination of the endophytic secondary metabolites using GC-MS

Fungal metabolites	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)

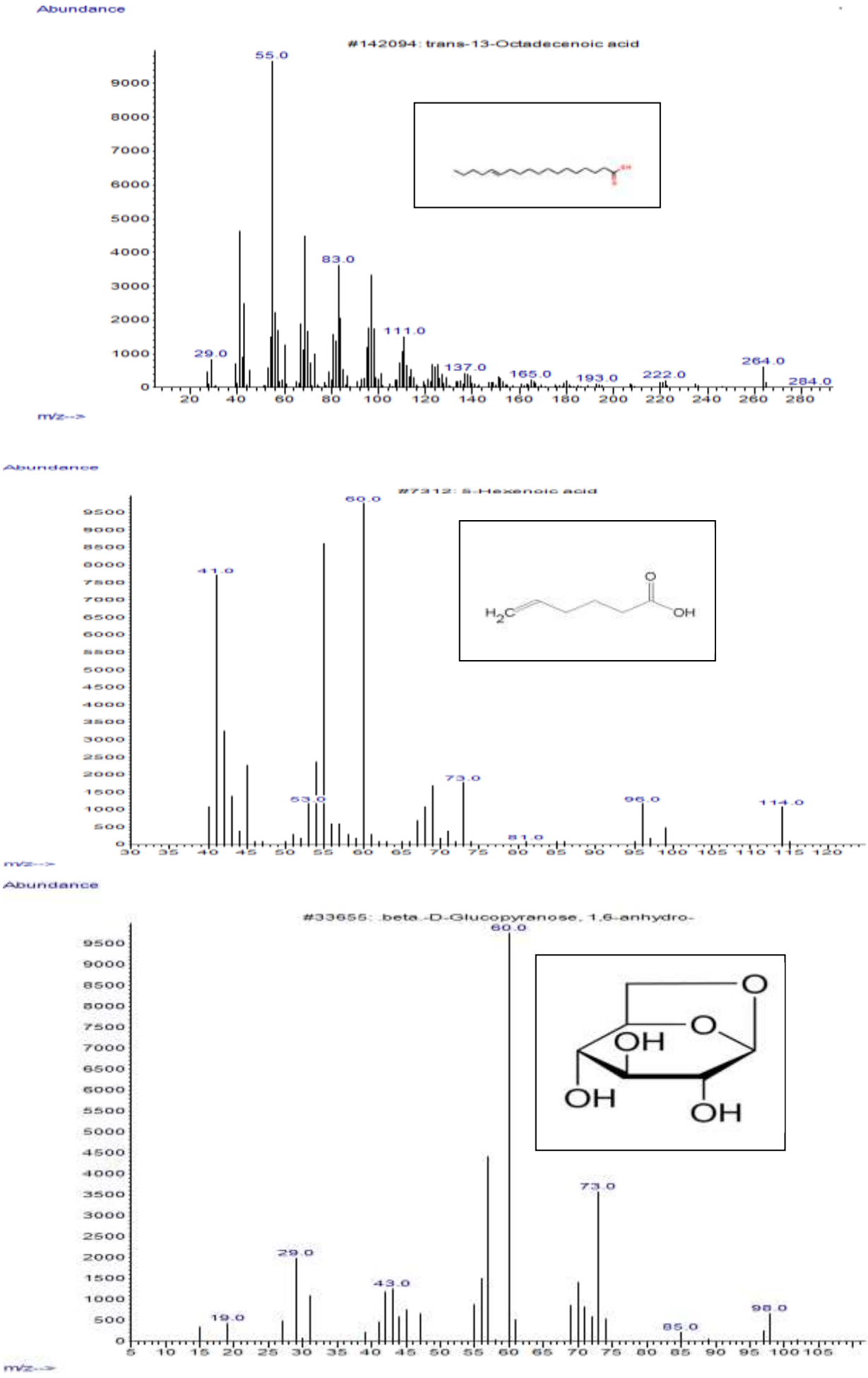


Figure 5: GC-MS analysis of the isolated extract of *Curvularia lunata*

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 functional groups using FTIR of *Curvularia lunata* extract.

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.

DISCUSSION

Antimicrobial activities and secondary metabolites of C. *lunata* extracts isolated from *A. 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 *Staphylococcus aureus* (Table 2). The microscopic and molecular characteristics *of Curvularia lunata* isolated from the leaves *A. indica* was identified (Figure 1, 2, and Table 1). Furthermore, the chemical analysis was performed to ascertain the secondary metabolites from the extract. For HPLC-DAD, the chromatogram of the *C. lunata* extract 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, 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%. Most of these compouds have been reported to have antimicrobial activity. 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 of *C.lunata*, (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 Figure 3. Also, GC-MS discovered that *C. lunata* extract from *A. indica* contains many promising compounds. The chromatogram of the GC-MS analysis carried out showed the presence of seventeen compounds. The results of GC-MS analysis showed the presence 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 *Curvularia lunata* 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 property to medicinal plants[27,28]. BLAST program available at NCBI GenBank databases (National Center for Biotechnology Information, website: www.ncbi.nlm.gov/blast) was used to align DNA strand forward and reverse sequence. The finalized sequence was blasted against the collection of non-redundant NCBI nucleotide sequence database. Accession number of *C. lunata* was obtained and seen at NCBI GeneBank[29].

CONCLUSION

C. lunata (OM337582) showed very significant antimicrobial activity. HPLC-DAD revealed the presence of 2-carboxymethyl-3-n-hexylmaleic acid anhydride and aspernigin A while GC-MS analysis showed, hexadecanoic acid, and 1,6-Anhydro-beta-D-glucopyranose. FTIR confirmed the presence of carboxylic acids functional groups. Therefore, from the results obtained, the endophytic extract of *A. indica* have potent antimicrobial activity with promising secondary metabolites.

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