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Molecular Identification and Analysis of Chemical Constituents from Endophytic Mycoflora of *Annona senegalensis* Pers using Gas Chromatography-Mass Spectrometry and Fourier Transform Infrared Spectroscopy

Ikechukwu A. Onah[1*], Dinebari P. Berebon[1], Thaddeus H. Gugu[1], Patrick N. Onyi[1], Franklin C. Kenechukwu[2], Emmanuel S. Onah[3], Emmanuel C. Ibezim[2], Anthony A. Attama[2]

[1]*Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Nigeria*

[2]*Department of Pharmaceutics, University of Nigeria, Nsukka, Nigeria*

[3]*Department of Ophthalmology, Enugu State University Teaching Hospital Parklane, Enugu, Nigeria*

Corresponding Author: *Ikechukwu A. Onah Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Nigeria, E-mail: tonyonah4u@gmail.com*

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ABSTRACT

Standardized means of identification of endophytic organisms could become a necessary tool for the discovery and optimization of their vast bioactive metabolites. This study aimed to isolate, identified endophytic fungi from the Root (RT) and Leaves Blades (LB) of Annona senegalensis Pers using molecular techniques and analyzed the crude metabolites using Gas Chromatography-Mass Spectrometry (GC-MS) and Fourier Transform Infrared Spectroscopy (FTIR). Endophytic fungi unknown (RT1), Botryosphaeria laricina (RT2), Didymella macrostoma (RT3) and Oxydothis ragae (LB1) associated with Annona senegalensis Pers were successfully isolated and identified. Based on the 18S rRNA gene sequences, four endophytic fungi, RT1, RT2, RT3 and LB1 were assigned GenBank accession numbers MW672177, MW672178, MW672179 and MW672180 inclusive under submission code SUB7061858. The result showed four endophytic fungi; unknown (RT1), Botryosphaeria laricina (RT2), Didymella macrostoma (RT3) and Oxydothis ragae (LB1). The analysis of the crude metabolites reveals quite several chemical compounds most of which have been documented to have pharmacological activities, including N-hexadecanoic acid (8.34%), linoleic acid ethyl ester (40.5%), 1–H–2–benzopyran–1–one, 6–bromo–5–flouro–3–4–dihydro (4.20%), ethyl oleate (13.8%), methyl stearate (14.45%), 10-octadeconoic acid (16.65%), 3–Allyl–6–methoxy phenol (3.17%), caryophyllene (5.77%), eugenol (3.17%), tridecaenoic acid (18.57%), oleic acid (7.34%) among others. In conclusion, this study has shown that endophytic fungi isolated from Annona senegalensis could be rapidly identified using molecular techniques and their metabolites analyzed using GC-MS and FTIR.

Keywords: Endophytic fungi, *Annona senegalensis*, Metabolites, Tissues

INTRODUCTION

Over time, endophytic organisms get integrated into the tissues of their host plants and spend part or the whole of their lives without noticeable harm to the host plant. These organisms (bacteria, actinomycetes and fungi), during their long period of co-evolution with host plants, adapted themselves to the niches and formed a completely compatible symbiont via gene regulation. In this micro-symbiotic-environment, host plants provide endophytes with photosynthetic products and minerals for their normal growth while `endophytes promote the growth and chemical

defense of host plants by directly providing valuable metabolites or transferring the corresponding genes to the host genome [1-3].

In past years, endophytic studies were not eliciting so much interest among the scientific community probably due to the focus on plant extract exploration. However, recent identification and exploration of the bioactive compounds from endophytic organisms have gained attention due to their high presence in various interior tissues of almost all higher plants and have led to the development of novel molecules that could enhance agricultural production as well as improve health challenges. These organisms produce important compounds of pharmaceutical interest such as anticancer, antibiotics, anti-inflammatory agents, antioxidants and surprisingly, hydrocarbons that can be used as fuel [4].

All over the world, the study of endophytic microorganisms is focused mainly on fungi. Endophytic fungi particularly would become an attractive source for the discovery of new leads, because of the complexity and the structural diversity of their secondary metabolites. Interestingly, any form of detailed study or exploration of endophytic fungi involving their diversity, characterization and the complexity of their chemical metabolites hinges essentially on the identification techniques.

Traditionally, endophytic fungi inside plant tissues can be identified by two basic techniques, such as direct observation and cultivation-dependent methods. While in direct observation, endophytic fungal structures within living plant tissues are directly examined under a light and electron microscope, which can show all endophytic mycobiota within the plant tissue, particularly biotrophic fungi that cannot be cultured on standard growth media, culture–dependent approach, some fungi may be missed due to failure to grow or grow slowly and are easily outcompeted by fast-growing species under artificial conditions. These limitations have, therefore, made traditional methods of endophytic fungal identification inappropriate.

Molecular techniques, such as DNA fingerprinting and sequencing methods, have been successfully employed in the detection and identification of endophytic fungi. The combination of culture-dependent and molecular techniques has been severally applied in the identification and exploration of endophytic fungal diversity and these have been adjudged the most acceptable, especially in the isolation of non-sporulation isolates that cannot be identified into any taxonomic position based on morphology and are lost in the survey of diversity using traditional techniques [5-6].

In Nigeria, *Annona senegalensis* Pers is a plant that is highly used in traditional medicine practices for treating diseases as well as in the maintenance of healthy life. It has been reported that *Annona senegalensis* possess antidiarrheal, antipyretic, anticonvulsant, anti-inflammatory as well as anti-snake venom properties. These effects have been attributed to the presence of important phytochemicals such as terpenes, anthocyanins, alkaloids, coumarins and flavonoids. Before now, several studies have tried to investigate, identify as well as isolate bioactive compounds from this plant. However, the microbial diversity of the plant has been understudied, hence the need to identify the endophytic fungi of this highly potent plant. Consequently, the objectives of this study were to isolate and identify the endophytic fungi from the leaves and root of *Annona senegalensis* Pers obtained from Mbu-Akpoti, Enugu State, Nigeria, using molecular techniques and as well, analyze the crude metabolites using GC-MS and FTIR.

MATERIALS AND METHODS

Sample collection and authentication

The various plant parts of *Annona senegalensis* used in this study (root, stem and leaves) were collected from a non-diseased, mature plant in September 2017, at Mbu-Akpoti (6º43'50.38''N, 7º41'34.58''E), in Isi-Uzo Local Government Area of Enugu State Nigeria. The freshly collected plant samples were duly identified by a taxonomist, Mr. Alfred Ozioko of the Plant Science and Biotechnology (former Botany) Department, University of Nigeria, Nsukka. Further authentication of the samples was done by a botanist Mr. Onyeukwu Chijioke John of the same department. The samples were assigned a voucher number UNH NO 9a and deposited at the herbarium collection center of the Plant Science and Biotechnology Department, University of Nigeria.

Cultivation and isolation of endophytic fungi

The freshly harvested samples (roots, stems and leaves) were washed thoroughly in running tap water followed by sterile double distilled water (ddH_2O) before processing. To eliminate epiphytic microorganisms, all the samples

were subjected to four-step surface sterilization, which include washing under running tap water-ethanol-sodium hypochlorite-distilled water by method. The cultivation and isolation of endophytic fungi were done as reported in our previous study. Sub-culturing was done at an interval of two weeks to maintain the pure cultures.

Fungal DNA extraction

In extracting the fungal DNA, Quick-DNATM Fungal/Bacterial miniprep Kit (USA) was used according to the manufacturer's instructions. Here, 50 mg-100 mg (wet weight) of fungal cells that have been re-suspended in up to 200 µl of water were added to a ZR Bashing Bead™ Lysis Tube. Again, 750 µl of lysis Solution was added to the tube. The tube was secured in a bead beater fitted with a 2 ml tube holder assembly (Disruptor Genie™) and processed at maximum speed for 5 min. The ZR Bashing Bead™ Lysis tube was centrifuged in a micro centrifuged at 10,000 × g for 1 min. Further, up to 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter (orange top) in a collection tube and centrifuged (laboratory centrifuge model SM 800D Surgifield Medicals, England) at 7,000 rpm (approx. 7,000 × g) for 1 min. The base of the Zymo-Spin IV™ Spin filter was snapped off before use. Again, 1,200 µl of Fungal DNA binding buffer was added to the filtrate in the collection tube above after which 800 µl of the mixture from the collection tube was transferred to a Zymo-Spin™ IIC column in another collection tube and centrifuged at 10,000 × g for 1 min. The flow through from the collection tube was discarded and the addition of 800 µl of the mixture was repeated. A volume of 200 µl DNA pre-wash buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 × g for 1 min. In addition, 500 µl Fungal DNA wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 × g for 1 min. The Zymo-Spin™ IIC column was added to a clean 1.5 ml microcentrifuge tube and 100 µl (25 µl minimum) DNA elution buffer was added directly to the column matrix and centrifuged at 10,000 x g for 30 secs to elute the DNA. The eluted pure DNA was collected and ready for use. [7]

PCR amplification

The ribosomal RNA (rRNA) gene of the fungi was amplified according to the method of Maksun. A pair of forward primer NS1: 5'GTAGTCATATGCTTGTCTC3'and reverse primer NS4: 5'CTTCCGTCAATTCCTTTAAG3' highly specific for endophytic fungi were used to amplify, targeting the gene encoding for 18S rRNA [22]. PCR was carried out in a programmable thermal controller (MJ Mini Biorad). The PCR reaction mixture (25 µl) consisting of 10 µl template DNA, 12.5 µl PCR Master Mix (PCR Buffer, 4 mM MgCl2, 0.4 mM of each dNTP, 0.05 U/µl Taq polymerase; Fermentas), 1 µM of each primer (NS1 and NS4) and ddH2O to make up the volume. The PCR cycling condition was: denaturation at 94ºC for 1 min, primer annealing at 45ºC for 2 min and extension at 72ºC for 2 min for a total of 30 cycles and a final extension step at 72ºC for 10 min. PCR amplicons were electrophoresed in a 2% agarose gel.

Electrophoresis of PCR products

About 2 g of agarose (Laboratories Conda, Madrid, Spain) was weighed into a 250 ml flask and 100 ml of 1 × tris acetate EDTA (TAE) buffer was added giving a 2% gel. The mixture was heated in a microwave oven until it was completely melted (solution clear and transparent). The solution was allowed to cool to about 60ºC and 2 µl of ethidium bromide was added and gently swirled to avoid air bubbles. The solution was then poured into a clean-leveled casting tray with combs already inserted and allowed to solidify at room temperature for at least 30 min. The combs were carefully removed to create wells and still in the casting tray, the gel was introduced into the electrophoresis chamber filled with a sufficient amount of running buffer (1 × TAE) just slightly above the gel surface. With a micropipette adjusted to 7 µl, the PCR product was gently mixed with 1 µl of loading buffer (bromophenol blue and xylene cyanol) and gently loaded into sample wells. For the approximation of the expected band size, a 100 bp DNA ladder (NEB) was also loaded in the first wells at the same time to run simultaneously. Once the lid was closed the power leads were connected to the electrophoresis apparatus at 100 volts and allowed to run for 10 min to 30 min. The gel was visualized under a UV trans illuminator (Alliance 4.7, France) and photographs were taken with a digital camera [8].

Sequencing of endophytic fungi

The sequencing was done using the Big Dye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol. The reaction mixture for the cycle sequencing contained 1 µl forward primer NS1: 5'GTAGTCATATGCTTGTCTC3' and 1 µl reverse primer NS4: 5'CTTCCGTCAATTCCTTTAAG3' (Inqaba-Biotech, South Africa), 4 µl ready reaction mix (2.5X), 2 µl Big Dye Terminator Sequencing Buffer (5X), 2 µl

PCR amplicon and 10 µl deionized water making a total reaction volume of 20 µl. The reaction mix was amplified using GeneAmp PCR System 9700 commenced with a pre-denaturation at 96ºC for 1 min followed by 25 cycles of denaturation at 96°C for 10 secs, annealing at 50ºC for 5 sec and extension at 60ºC for 4 min. The nucleotide sequences were read after the PCR cycling sequencing.

Sequence data analysis

The sequencing results generated were uploaded in http://blast.ncbi.nlm.nih.gov/Blast.cgi which is an intuitive interface for analyzing DNA sequences. The assembled sequences were end-trimmed, paired in their respective forward and reverse sequences to build consensus sequences. Sequence alignment and percentage similarity searches were compared with the Genbank DNA database using the National Centre for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool (BLAST®).

Evolutionary relationships of taxa

The evolutionary relationship among the isolates was inferred using. Evolutionary relatedness was conducted in MEGA X.

Fourier Transform Infrared Spectroscopy (FTIR)

The crude extracts of the endophytic fungi were analyzed by FTIR (FTIR, Agilent Technologies) to identify the different functional groups present in the fungal extracts. The crude extract was subjected to analysis in FTIR in which the diffuse reflectance technique was followed. Two milligrams of the crude sample were mixed with Potassium bromide (KBr) to form a very fine powder. This powder was then compressed into a thin pellet which was analyzed. The KBr was also transparent in infrared light. The samples were irradiated by a broad spectrum of infrared light and the level of absorbance at a particular frequency was plotted after the Fourier transform of the data. The resulting spectrum was characteristic of the organic molecule present in the sample. The absorbance was measured at 400 nm-600 nm for the identification and quantification of organic species. Compounds contained in the extracts were identified according to established criteria.

Gas Chromatography-Mass Spectrometry (GC–MS) analysis

The protocols of Socrates were adopted for the GC–MS analysis. Precisely, ethyl acetate extracts were subjected to GC–MS analysis to identify the bioactive compounds. The sample was analyzed in a Hewlett-Packard 7890A gas chromatograph system coupled with an MSD Agilent Technology 5975C VL MSD detector. The volume of a 1 microliter sample was introduced via an all-glass injector working in the split mode, with helium as the carrier gas with a linear velocity of 32 cm/s. The HP-5MS fused silica capillary column (Length: 30 mm; Column thickness: 25 µm internal diameter -0.32 mm) was used. The temperature program was as follows: 80ºC - 240ºC at 8 degrees-/-min; 240ºC-300ºC at 12 degrees per min and a 20 min hold at 300ºC. The identification of components was accomplished using computer searches in commercial libraries.

RESULTS

Cultivation and isolation of endophytic fungi

The cultivation, emergence, isolation and purification of the endophytic fungi from the plant part cultured on the Malt Extract agar are shown in Figures 1A-1D. Figure 1A shows the cultivation of freshly collected and disinfected plant parts in sterile MEA. In Figure 1B, the emergence of fungal hyphae from the cultivated plant parts is shown. Isolation and purification of the various fungal isolates are shown in plate of Figures 1C and 1D.

Hyphal tips of actively growing fungi from these plant segments were sub-cultured till pure isolates were obtained. The root yielded three fungal isolates (RT1, RT2 and RT3), whereas midrib and leaf blade generated two fungal isolates each (LB1, LB2, MR1 and MR2).

The cultures of the different fungal isolates, RT1, RT2, RT3 and LB1 are shown in Figure 2. The hyphal growth pattern in RT2 and LB1 were circular and whitish, LB1 was slippery to the touch whereas RT3, RT2 and LB1 appear pigmented. However, RT1 was opaque from the rear while the hyphal growth was dispersed and whitish with no visible pigmentation.

Identification and evolutionary relationship of endophytic fungal isolates

In the present study, endophytic fungi emerged and were properly isolated from cultured healthy tissues of *Annona senegalensis* pers on malt extract agar (MEA). Seven endophytic fungi were isolated and their metabolites were screened for antioxidant, antimicrobial and antimalarial activities. However, four isolates (RT1, RT2, RT3 and LB1) with the highest activities were selected in this study for identification. Table 1 show fungal samples isolated from different parts of *Annona senegalensis* and the identified fungal species from those samples using sequence alignment and compared with the Genbank database using the NCBI web-based site, BLAST. The root isolates (RT1, RT2 and RT3) and the leaf blade isolate (LB1) whose sequences were BLAST was identified based on the percentage similarity with the Genbank database. Their close evolutionary relationship is shown in Figure 3.

Based on the percentage similarity with the Genbank database Table 1, BLAST search and sequence alignment showed that the RT2 shared 88.94% identity with Botryosphaeria laricina (MW672178), 85.60% with Didymella macrostoma (MW672179) and 84.53% with Oxydothis ragae (MW672180). However, RT1 did not correspond to any sequence in the database. Using MEGA software, a phylogenetic tree of fungal isolates with high similarity was constructed using the Neighbor-joining method to show their close evolutionary relationship (Figure 3).

Electrophoregram of PCR product

The electrophoregram of the PCR results of the different fungal isolates from *Annona senegalensis* is shown in Figure 4. The four fungal isolates were shown in distinctive bands RT1, RT2, RT3 and LB1 implying that the DNA extraction was successful. The electrophoregram shows a band size of 1000-1100 bp for RT1, RT2 and RT3 whereas a band above 1100 bp was observed for LB1. This band separation shows a close evolutionary relationship among the isolates.

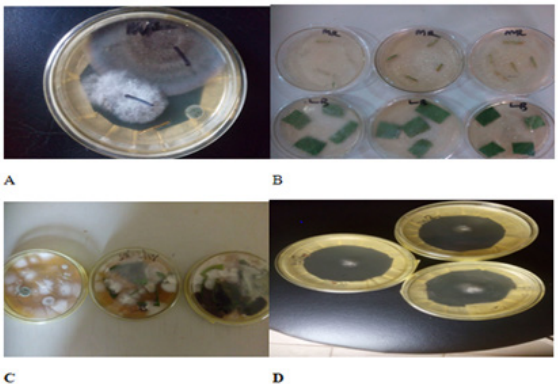

Figure 1: The cultivation, emergence, isolation, and purification of the endophytic fungi from the plant part cultured on the Malt Extract Agar (MEA). (A) Shows the cultivation of freshly collected and disinfected plant parts in sterile MEA. (B) The emergence of fungal hyphae from the cultivated plant parts is as shown. (C) Isolation of fungal isolates and (D) Purification of the various fungal isolates.

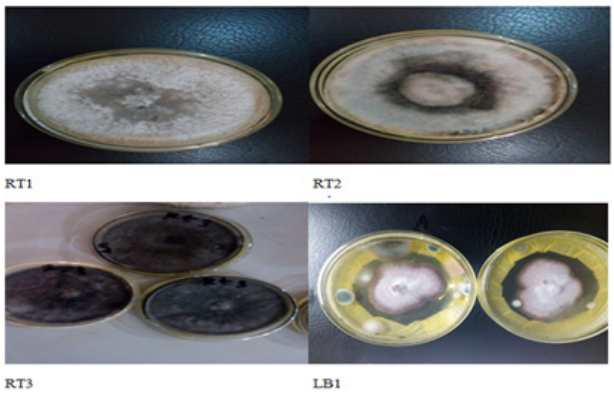

Figure 2: Views of the cultures of the different fungal isolate from plant root and leaf blade: RT1 – RT 3 (Root isolates) and LB1 (Leaf blade isolate).

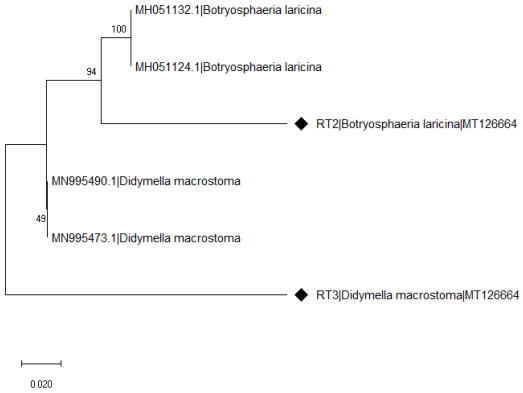

Figure 3: Evolutionary relationship of the recovered fungal isolates and their closest GenBank relatives

Table 1: Identified fungal species from different parts of *Annona senegalensis* Pers

Fungal code	Sample location	Submission code given	Genbank code generate	% Gene Similarity	Fungal identity
RT1	Root	SUB7061858 RT1	MW672177	-	Unknown
RT2	Root	SUB7061858 RT2	MW672178	88.94%	Botryosphaeria laricina
RT3	Root	SUB7061858 RT3	MW672179	85.60%	Didymella macrostoma
LB1	Leaf blade	SUB7061858 LB1	MW672180	84.53%	Oxydothis ragae

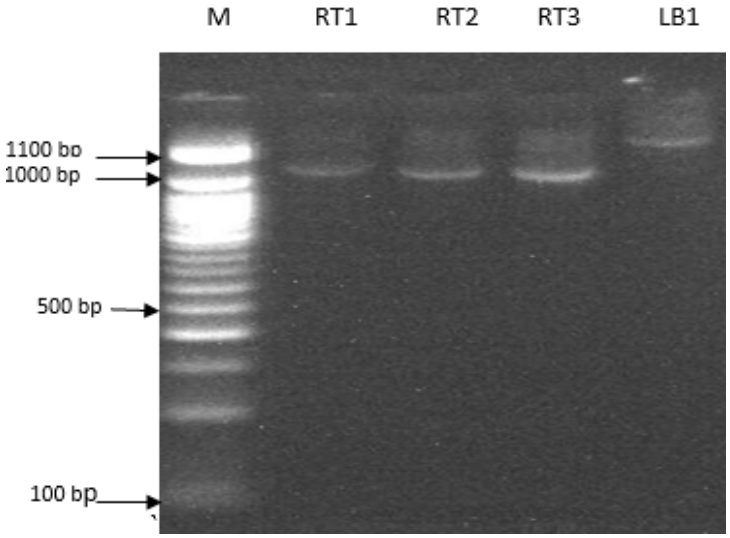

Figure 4: Amplified 18S rRNA sequence showing a band size of 1000 bp for RT1, RT2, RT3 and 1100 bp for LB1. M indicates 100 bp molecular weight markers.

GC-MS analysis of the crude endophytic fungal extract from Annona senegalensis pers

The GC-MS profile of the crude extracts from the various endophytic fungal isolates from *Annona senegalensis* Pers are shown in Figure 4. The relative abundance and the various peak areas characteristic of chemical compounds present in each fungal metabolite are essential in identification of the chemical constituents of the bioactive compounds.

DISCUSSION

Long periods of evolution of endophytic fungi with their host plants contributed to the vast morphological, metabolic and phylogenetic diversification of these microorganisms. Therefore, identification of these fungi using molecular

techniques is essential for precision in their classification as well as in improved knowledge of their bioactive compounds. Similarly, in related scientific studies various plant parts were cultured on potato dextrose agar and endophytic fungi were obtained from their samples. Deeksha equally isolated endophytic fungi from their plant samples and described the isolates based on their colony morphology [9-11].

The recovered fungal isolates exhibited a close evolutionary relationship with GenBank relatives. However, recent researches have variously described fungal identification using morphological characteristics as misleading due to hybridization, cryptic speciation and convergent evolution. In addition, morphological characterization may not provide accurate grouping within an evolutionary framework, mainly at the species level. Consequently, this research applied molecular techniques in the identification of these isolates. NS1 and NS4 primer pairs were used to amplify the specific gene of the endophytic fungi targeting the specific gene encoding for 18S rRNA. Similarly, it has been reported that fungal 18S rRNA can be amplified and sequenced using NS1 and NS2 primers, especially where the research interest is in the phylogenetic classification of fungi at a higher taxonomic level. In the present study, sequenced amplicon from the endophytic DNA extract was matched with the Genbank database using NCBI- web-based site, BLAST to identify RT1, as unknown, RT2-Botryosphaeria laricina, RT3-Didymella macrostoma and LB1 as Oxydothis ragae.

The high presence of phenolic and carboxylic functional groups in the extracts from various fungal isolates might have contributed to the antimicrobial as well as antioxidant potential of the extracts. The resulting spectra were characteristics of the organic molecules present in RT1, RT2, RT3 and LB1. The peaks indicated the presence of carboxylic acids, strong broad O–H stretch (3008.91, 2922.53, 2355.65), aldehyde, ketone; strong O=H and C=H stretch respectively (1710.26), aromatic compounds, medium strong ring C=C stretch (1517.17). The exhibition of various pharmacological activities of the extracts depends on the presence of the functional groups in the metabolites. These functional groups also determine the structure of the secondary metabolites [38-39]. In the displayed characteristic absorption spectrum of functional groups in RT2 metabolites, it has several peaks that comprise carboxylic acid O–H stretch, amines, N–H symmetric, amides N–H asymmetric (3343.62), alkane, C–H stretch (2922.72 cm^{-1}), aldehydes, C=O stretch (1707.54 cm^{-1}). Esters and alcohol C–O stretch and O=C–O–C symmetric respectively at (1039.51 cm^{-1}), alkanes, strong=C–H bend (887.67, 924.67). Given all these functional groups and their pharmaceutical prospects, it is pertinent to note that further exhaustive exploration of this medicinal plant would yield novel moieties that can be used in combating many drug-resistant organisms.

Most of the compounds did not come out as clear single peaks rather there were clustered bands implying that the extracts were not pure samples. Several other related studies, however, used crude extracts for compound identification before purification. The GC-MS spectrum further corroborated the functional groups present in these extracts [12].

The FTIR and GC-MS analysis reports of these metabolites reveal quite several chemical compounds most of which have been documented to have pharmacological activities. Some of these bioactive compounds include N-hexadecanoic acid (8.34%), linoleic acid ethyl ester (40.5%), I H–2–benzopyran-I-one, 6-bromo-5-flouro-3-4-dihydro (4.20%), ethyl oleate (13.8%), methyl stearate (14.45%), 10-octadeconoic acid (16.65%), 3–allyl–6–methoxy phenol (3.17%), caryophyllene (5.77%), eugenol (3.17%), tridecaenoic acid (18.57%), oleic acid (7.34%) among others. These quite agree with the report of Samuel et al., on the chemical compounds isolated from *Annona senegalensis*. The discovery of these chemical compounds and their likely pharmacological potential has raised hope of developing novel moieties that can address the pressing need for newer drugs to replace already existing ones with the attendant ineffectiveness, safety issue and cost.

CONCLUSION

The present study has explored the under-searched endophytic fungi from *Annona senegalensis*. Out of the seven endophytic fungi isolated from this plant, three have been molecularly identified and assigned the following accession numbers (Botryosphaeria laricina MT126664 and Didymella macrostoma MT126665) in the Genbank database. The GC-MS analysis of the crude extract of these fungal metabolites showed varied chemical structures of the component compounds present in the fungal extract some of which included; saturated and unsaturated fatty acids, phenolics, steroids, organo bromides and dyes. Further purification of the bioactive compounds and exhaustive investigation of these extracts for pharmacological activities would be undertaken in future studies.

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