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Short Communication

Antimicrobial Assay and GC-MS Profile of the Extract of the Endophytic Fungus from *Annona muricata* (Annonaceae) Leaf

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ABSTRACT

Emerging cases of antimicrobial resistance in the treatment of microbial infections have really posed as a threat thus leading to the search of new drugs from natural sources. Endophytic fungi which live within plant's cells are microorganisms from which many novel metabolites with antimicrobial activity can be obtained. The aim of the study was to examine the antimicrobial activity and the phytoconstituents of the extract produced by the endophytic fungus from *Annona muricata*. From *A. muricata* leaves, the fungal endophyte was extracted and fermented. The endophytic extract produced was tested against *Pseudomonas aeruginosa*, *Streptococcus mutants*, *Salmonella typhi*, *Staphylococcus* aureus, *Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*, and *Candida albicans*. Following standard protocols, the extract was screened for phytochemicals and subjected to GC-MS analysis. With the exception of *P. aeruginosa* and *A. niger*, the results demonstrated that the extract has antimicrobial property with minimum inhibitory concentration (MIC) values in the range of 0.8 mg/ml to 1.0 mg/ml. Phytochemicals tested including phenolics, flavonoids, alkaloids, tannins, steroids, saponins and terpenes were present. Twenty (20) main compounds, mostly fatty acids, were detected by GC-MS and they may be the source of the extract's antimicrobial property. The endophytic fungus extract possesses significant bioactive compounds with a wide spectrum of antimicrobial activity and can be further explored.

***Keywords*:** *Annona muricata*, Antimicrobials, Endophytic fungi, Gas Chromatography-Mass Spectroscopy, Phytochemicals.

Introduction

Numerous health issues are still incurable despite the development of several efficient medications. In recent times, there has been reoccurrences of various infectious diseases as well as new emergence of infectious diseases. This has really posed as a threat to the health sector as many lives are being lost globally due to epidemic of these diseases. Some these diseases include Coronavirus, Lassa Fever, Ebola virus infection, Cholera, Influenza, Yellow Fever and Diphtheria.[1,2]

Medical practice over the years has had tremendous breakthroughs in different aspects of clinical medicine due to the availability of antibiotics. However, this progress in the medical practice has been threatened over time largely due to antimicrobial resistance (AMR).[3] Therefore, there is an apparent need for novel antimicrobial agents as the existing antimicrobial agents are either unaffordable or resistant to the microorganisms, causing the drugs to be quite ineffective against the microorganisms. Hence, this justifies the need to serach for novel antimicrobial agents, especially from organic sources like plants and endophytes.

Endophytes inhabit the intracellular and intercellular spaces of various plant components (fruits, leaves, stem, flowers and phloem) and they are also capable of producing metabolites with therapeutic effects as seen in various researches particularly carried out on endophytic fungi.[4,5] Protocatechuic acid, cladosporin, among other compounds with potential antimicrobial activity, were found in endophytic fungi extracted from *Ocimum gratissimum*, and *Carica papaya* leaves while a dihydrofuran-2-one derivative was identified based on nuclear magnetic resonance spectroscopic investigation from *Fusarium verticillioides* obtained from *Syzygium jambos*'s stem bark.[6,7]

Annona muricata Linn. (commonly called Soursop), is of the Annonaceae family which is a natural and ethnobotanical helpful plant all over the world. It is an evergreen tree that can reach a height of 5 to 6 meters and has huge, dark green leaves and large fruits which are also green in colour with a diameter varying between 15-20 cm.[8] The plant is indigenous to South and North America and can also be seen in different countries such as Malaysia, Nigeria, Australia and Africa.[8,9] Investigations of *Annona muricata* have highlighted its potential to offer natural therapeutic benefits for various health conditions including antibacterial, anti-inflammatory, antidiarrheal, antineoplastic, antioxidant activities, among others.[10,11,12]

This study therefore, aimed to assess the antimicrobial properties and the phytochemical constituents present in the extract produced by an endophytic fungus isolated from *A. muricata* leaves.

Materials and Methods

Plant materials

A. muricata fresh leaves were procured from Ajuona in Nsukka, Enugu State (6.8787ºN, 7.4092ºE), Nigeria in January, 2022. The identification and authentication of the leaves were carried out by Mr Felix Nwafor of the Pharmacognosy and Environmental Medicine Department at the University of Nigeria Nsukka, following which the plant specimen was deposited in the herbarium with voucher number PCG/UNN/0415. Subsequently, isolation and purification procedures were employed to obtain the endophytic fungus sample.

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Isolation and purification of the endophytic fungus
Endophytic fungus was aseptically extracted from the leaf following the methods previously described [13] with few modifications. The freshly collected leaf samples were surface sterilized. The dust and debris were removed from the healthy samples, surface sterilized, rinsed and cut into small pieces before aseptically placing them on solidified potato dextrose agar (PDA) contained in a petri dish with antibiotic to inhibit the growth of bacteria. Chloramphenicol (0.01%) was used. The agar plates were covered, sealed, and incubated at 27°C for 5-7 days. Daily checks were conducted on the samples to monitor fungal growth. After the incubation period had elapsed, various strains of fungi were observed in the plates and one strain was isolated by sub-culturing on fresh PDA medium. Sub-culturing was performed twice to obtain a pure fungal strain. The pure isolate was then examined microscopically to identify its morphological features.

Fermentation and extraction of fungal extract
The pure isolate was cultivated in a sterilized solid rice medium in an Erlenmeyer flask which was maintained at 27 ± 2°C. Fungal growth in the fermentation medium was monitored for 21 days. Thereafter, ethyl acetate (about 500 ml) was used for the extraction of the medium and the mixture was filtered through a muslin followed by a filter paper. The resultant filtrate was vacuum-vaporized using a rotary evaporator at 45°C to obtain the endophytic fungal extract.[14]

Antimicrobial Test
Agar dilution method [15] was used to assess the antimicrobial assay. It was conducted using 0.5 McFarland turbid equivalents against pure strains of *Pseudomonas aeruginosa, Streptococcus mutants*, *Bacillus subtilis, Escherichia coli*, S*taphylococcus aureus, Salmonella typhi*, *Aspergillus niger* and *Candida albicans* which were obtained from the Pharmaceutical Microbiology and Biotechnology Laboratory, University of Nigeria, Nsukka.

Determination of Minimum Inhibitory Concentrations (MICs)
Various concentrations (0.1 mg/ to 1.0 mg/ml) of the extract were prepared in the vehicle (50% dimethyl sulfoxide, DMSO) and added to the sterile glucose enriched molten agar in a sterile Petri dish. Ciprofloxacin (15 μg/ml) and fluconazole (30 μg/ml) were used as controls. The molten agar plates containing different concentrations of the agents were allowed to gel. The plates were labelled and then each plate was divided into eight equal segments with a permanent marker. Each microorganism was inoculated by streaking on a labeled segment of the agar plate under strict aseptic conditions. The plates were placed in inverted position in a bacteriological incubator at 37°C for 24 hours. The MIC of each agent was noted.

Test for the presence of Phytochemicals
The testing of the phytochemicals present in the extract was done by standard procedures as described. [16]

2.6 Gas Chromatography-Mass Spectrometry (GC-MS) analysis
GC-MS analysis was performed using a Shimadzu Japan QP – 2010 plus GC-MS. It was outfitted with a split injector, a 20 m x 0.22 mm fused-silica capillary column with a thickness of 1.00 µg, and an ion-trap mass spectrometer detector interface. Helium gas was employed at a columnar velocity flow rate of 1.8 ml/min. An interface temperature of 250°C and an ion source temperature of 200°C were employed. The analysis was done for 24.0 minutes in total. Peak identification was done by comparing with NIST mass spectra library.[17]

Results and Discussion

Isolation and cultivation
About seven fungi were observed on the edges of the cut leaves (*A. muricata*), each with distinct morphological characteristics and colours. However, one of the endophytes was isolated and purified. A pure isolate was obtained which was greyish-green in colour and had morphological features similar to Penicillium spp. under a microscopic view. The growth of different fungi observed from the edges of the leaves implies the existence of endophytes. An endophyte is a microorganism that inhabits the internal plant tissues and does no harm to the host plant. They are symbiotic groups of bacteria or fungi capable of invading the intercellular and intracellular regions of the host plant.[4,5]

Fermentation and extraction of isolated fungus metabolites
On the solid rice medium, the fungus grew gradually and after 21 days, had filled the entire space. During ferrmentation in the Erlenmeyer flask, metabolites were produced by the endophytic fungus and visible growth of the fungus was seen and the growth increased gradually till the entire rice medium was fully occupied by the 21st day. Some factors are known to affect the generation of metabolites during fermentation. One of these is the absence of stimuli like metal ions, host signaling, chemicals that causes stress, and metabolic precursors that could hamper the fungi during sub-culturing. [17,18]
The extraction was done with ethyl acetate which is moderately polar, hence moderately non-polar molecules would be extracted more effectively by the solvent. The primary reasons for the chemical and biological properties of ethyl acetate comes from its medium polarity, low toxicity to test organism strains as well as its high volatility. Hence, ethyl acetate is often used for the extraction of the metabolites of endophytic fungi.[19]

Antimicrobial screening result
The extract was tested against both bacterial and fungi to determine the antimicrobial activity. The MIC for *S. mutants, S. aureus, S. typhi* and *C. albicans* was 1.0 mg/ml for each while 0.9 mg/ml was for *E. coli* and 0.8 mg/ml was for *B. subtilis* as shown in Table 1. This implies that the extract possesses a broad spectrum of antimicrobial activity because it inhibited the growth of bacteria (Gram +ve and Gram –ve) as well as a fungus. Within the range of concentrations tested (1.0 mg/ml to 0.1 mg/ml), *P. aeruginosa* and *A. niger* were not sensitive to the fungal extract; this could imply that a higher concentration of the extract is needed to inhibit the growth.
The antimicrobial results suggest that the endophytic fungus produces metabolites with antimicrobial activity. Thus, the extract could be employed for the treatment of infections caused by these sensitive organisms. Previous investigators have gotten similar results. For instance, *Penicillium commune* and *Penicillium canescens* isolated from *Olea europaea* L. (olive tree) leaves were evaluated to have a very effective biostatic effect against Gram+ve and Gram-ve bacteria as compared with 30 μg/ml of chloramphenicol.[20] *Pseudofusicoccum* sp identified from Soursop leaves showed activity against *Bacillus subtilis*, *Salmonella typhi* and *Candida albicans.*[21]

Phytochemical constituents
All phytochemicals tested including phenolics, flavonoids, alkaloids, tannins, steroids, saponins, terpenes and reducing sugars were present. These phytoconsituents could account for the antimicrobial property of the fungal extract either singly or in combination. In several studies, phenolic compounds, alkaloids, flavonoids, terpenoids and saponins have been found present in extracts from *Annona muricata* with antimicrobial activity against several microorganisms.[22,23,24]

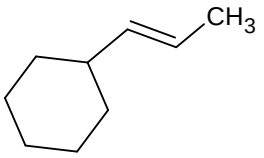
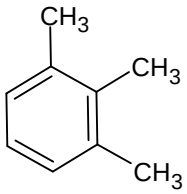
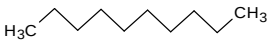
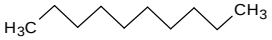
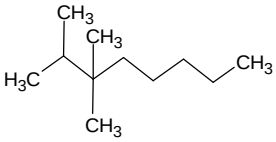
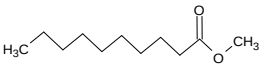
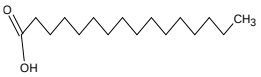
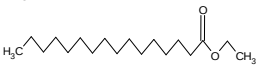
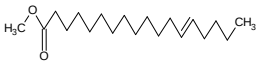
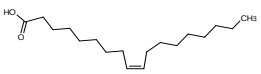
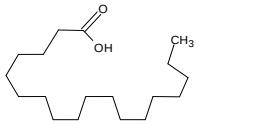
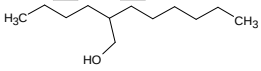
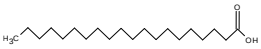
GC-MS Results
Structural representation of molecules obtained by GC-MS as well as other details of the molecules are outlined in Table 2. Twenty (20) main peaks (compounds) are seen in the chromatogram (Figure 1) based on their peak area. The first main compound from gas chromatography having a retention time of 3.272 minutes was determined to have a molecular weight of 124 mg/mol and molecular formula to be C_9H_{16} by mass spectroscopy which showed similarity with the compound cyclohexane-1-propenyl from the Mass Spectroscopy data bank. Other compounds are similarly shown in the table. Some of the compounds are likely to effect antimicrobial activity. Nine (9) compounds are saturated fatty acids which explains the oily nature of the dried extract. They include decanoic acid methyl ester, n-hexadecanoic acid, hexadecanoic acid ethyl ester, among others. Previous studies have shown that many other compounds including Penicidin, 2-methyl resorcinol, 4-O-methylphorbol 12,13-didecanoate have been identified by GC-MS analysis in endophytic fungi extracted from Soursop.[22,25]

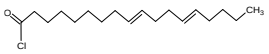
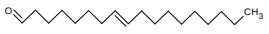
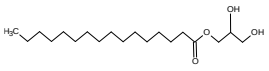
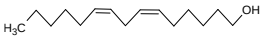
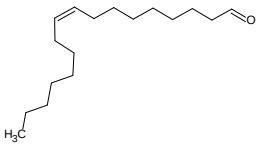
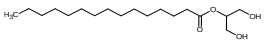
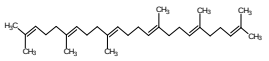
Table 1: The Minimal Inhibitory Concentration (MIC) mg/ml of the ethyl acetate extract from the endophytic fungus isolated from the leaves of *Annona muricata*

Test organisms	MIC (mg/ml)	Control		
		Ciprofloxacin (15 μg /ml)	Fluconazole (30 μg /ml)	DMSO (50%)
Pseudomonas aeruginosa	Nill	_	+	+
Escherichia coli	0.9	_	+	+
Streptococcus mutants	1.0	_	+	+
Aspergillus niger	Nill	+	_	+
Staphylococcus aureus	1.0	_	+	+
Candida albicans	1.0	+	_	+
Salmonella typhi	1.0	_	+	+
Bacillus subtilis	0.8	_	+	+

Key : (-) = no growth, sensitivity; (+) = Growth, no sensitivity.

Table 2: GC-MS analysis result of the ethyl acetate extract from the endophytic fungus isolated from the leaves of *Annona muricata*

Peak No.	R. Time (min)	Peak Area %	Name of Compound	Molecular Formula	Molecular Weight (mg/mol)	R. Index	Structure
1	3.272	1.19	Cyclohexane-1-propenyl	C_9H_{16}	124	987	
2	3.685	2.20	Benzene-1,2,3-trimethyl	C_9H_{12}	120	1020	
3	4.061	4.40	Decane	$C_{10}H_{22}$	142	1015	
4	5.337	1.08	Decane	$C_{10}H_{22}$	142	1015	
5	8.065	0.24	Octane-2,3,3-trimethyl	$C_{11}H_{24}$	156	966	
6	15.411	0.23	Decanoic acid, methyl ester	$C_{11}H_{22}O_2$	186	1282	
7	15.955	19.36	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1968	
8	16.076	1.85	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	1978	
9	17.113	0.57	11-octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	296	2085	
10	17.688	44.31	9-Octadecanoic acid (Z)	$C_{18}H_{34}O_2$	282	2175	
11	17.838	11.54	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2167	
12	18.861	0.47	2-butyl-1-octanol	$C_{12}H_{26}O$	186	1393	
13	19.508	0.77	Eicosanoic acid	$C_{20}H_{40}O_2$	312	2366	

14	19.957	0.92	9,12-octadecadienoyl chloride (Z,Z)	$C_{18}H_{31}ClO$	298	2139	
15	20.348	1.99	9-octadecanal	$C_{18}H_{34}O$	266	2007	
16	20.703	1.17	Hexadecanoic acid-2,3-dihydroxypropyl ester	$C_{19}H_{38}O_4$	330	2482	
17	21.963	0.93	(Z)6-(Z)9-pentadecadien-1-ol	$C_{15}H_{28}O$	224	1771	
18	22.254	4.97	9-Hexadecanal	$C_{16}H_{30}O$	238	1808	
19	22.465	1.33	Pentadecanoic acid-2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{18}H_{36}O_4$	316	2399	
20	23.529	0.47	2,6,10,14,18,22-Tetracosahexane-2,6,10,15,19,23-hexamethyl	$C_{30}H_{50}$	410	2914	

R. Time- Retention time; R. Index- Retention Index

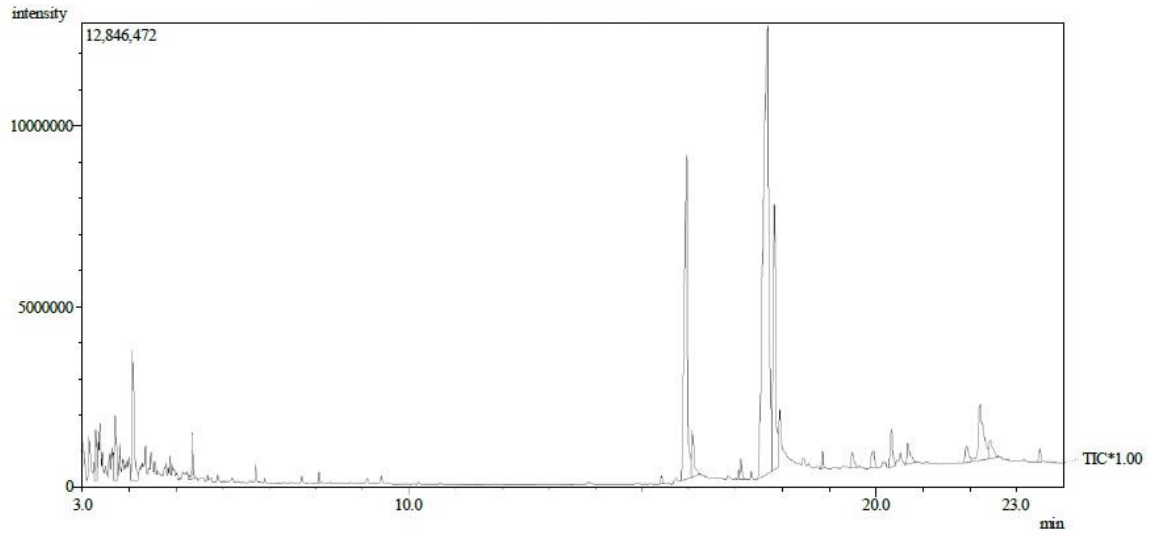


Figure 1: GC- MS Chromatogram showing the major peaks of the molecules present.

Conclusion

The endophytic fungus isolated from *A. muricata* leaf produced phytochemicals having a broad spectrum of antimicrobial activity as seen in the present study. Twenty (20) major compounds of which are mostly fatty acids may have contributed to the wide spectrum anti-microbial activity of the extract. Hence, it is imperative that further studies should exploit the area of endophytic fungi and their metabolites as an innovative and alternative source of antimicrobial agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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