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Studies on the Antimicrobial Properties and GC-MS Analysis of Ethyl Acetate Extract of *Annona muricata* Seeds

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ABSTRACT

The increasing resistance profile of many bacterial and yeast strains to conventional antimicrobial agents has heightened the search for bioactive compounds from higher plants that would serve as lead molecules in the discovery and development of new drugs. This study determined the antimicrobial properties and chemical constituents of the ethyl acetate extract of *Annona muricata* seeds. Crude extract obtained by maceration of pulverized seeds of *Annona muricata* in ethyl acetate was evaluated for antimicrobial activity against six clinical bacterial isolates (*Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella aerogenes)* and one fungus (*Candida albicans*) using agar-well diffusion method. GC-MS method was used to determine the chemical constituents of the extract. The extraction yielded 19.93% of an oily yellow crude mass. The extract inhibited the growth of all test bacteria with inhibitory zone diameters range of 23.5 - 17.02 mm and 25.90 - 31.50 mm for the standard antimicrobial agents. The GC-MS analysis identified 48 chemical constituents in the extract with 9,12-octadecadienal, 1,14-tetradecadienal, phthalic and fatty acids accounting for 15.26, 7.77, 2.53 and 11.20% respectively. The data from this study revealed the possible antimicrobial potentials and chemical constituents of *Annona muricata* seeds and hence the scientific basis for its traditional use in the treatment of some infections.

Keywords: Antimicrobial activity, *Annona muricata*, ethyl acetate extract, GCMS

INTRODUCTION

Herbal medicine constitutes the most important aspect of traditional medicine. Study of medicinal plants is essential for the promotion of proper use of herbal medicine and their potentials as sources of new drugs in the treatment of infections [1].

It has been estimated that 14-28% of higher plant species are used medicinally and 74.0% of pharmacologically active plant derived components had ethnomedicinal use [2]. About 80% of the world population relies chiefly on traditional medicines for their primary health care needs [3,4]. The importance of the active ingredients of plants in medicine has stimulated significant scientific interest in the biological activities of these substances [5]. Plant derived medicines are culturally acceptable because of their efficacies, affordability, availability, low toxicity and few side effects on the human body [6]. Literature reports have shown that bioactive natural compounds exhibiting antimicrobial activities have been isolated mainly from cultivatable microbial strains. Thus there is the need to exploit the untapped natural resources of plants producing biologically active metabolites; this is with a view of enrolling them to alleviate the current health care challenges, like increasing cost of chemotherapy, emergence of multidrug resistant microbial strains and unmet clinical outcome.

Cinnamon, clove, thyme and lantana extracts have been shown to inhibit the growth of multidrug resistant *Pseudomonas aeruginosa* [7]. Amongst over 250,000 plant species on the planet, only about 6.0% have been screened for phytochemicals and biological activity [8]. This research gap provides a viable avenue for search of alternative therapies from among plants kingdoms.

Annona muricata commonly called soursop is gaining worldwide popularity and acceptability as a 'miracle tree' in the field of medicine. The plant is traditionally used to treat various ailments; its leaves have been used to treat various types of cancer, asthma, diabetes bacterial infections such as pneumonia, diarrhea, urinary tract infections and skin diseases [9]. A decoction of bark, leaves, and root had been reported to possess sedative, antispasmodic, and hypotensive effects respectively [10]. Soursop has found its uses in many areas. It is consumed as a desert fruit. It is made into a fruit jelly with addition of some gelatin or used in the preparation of beverages, ice cream and syrups. The fruit makes an excellent drink or ice cream after straining. In the Philippines, young soft fruits with seeds are used as vegetables. In an evaluation of lesser known tropical fruits, *Annona muricata* has been reported as one of the most promising fruits, attributed to its aromatic qualities and suitability for processing for its distinctive therapeutic applications. It has also been found to be a promising new anti-tumour agent in numerous *in vitro* studies [11].

In spite of the numerous uses of *A. muricata*, in the treatment of various ailments, there is scanty literature reports on the antimicrobial and chemical constituents of the seeds, which is about 4.0% of the whole fruit. This study was carried out to evaluate the antimicrobial

properties and chemical constituents of ethyl acetate extract of the seeds of *A. muricata.*

MATERIALS AND METHODS

Preparation of crude extracts

The fresh fruits of *Annona muricata* used for the study were purchased from one of the urban fruit markets in Benin City, Nigeria and identified at the Department of Plant Biology and Biotechnology, University of Benin, Benin City. A voucher specimen (UBH I0235) was deposited in the departmental herbarium. The fruits were washed in water and the seeds were extracted, rinsed and sun dried for five days to a constant weight. The fleshy parts of the seeds were separated from the husks and then milled into coarse powders using a kitchen blender (Kenwood BL460, England). Approximately 800 g of the coarse powders were divided into two portions of 400 g each. Each portion was macerated using 2.0 L of ethyl acetate for 72 h with stirring at intervals. The mixture was filtered using Whatman filter paper No. 1. The filtrate was concentrated in a rotary evaporator (Stuart, UK) at a speed of 5.0 rpm to obtain an oily, yellowish crude mass extract weighed and kept at 4.0 °C in an airtight container until required for use.

Antimicrobial assay

Clinical isolates obtained from stock culture of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Nigeria were used for the study. These includes; *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella aerogenes* and *Candida albicans*.

Preparation of test microorganisms

Prior to use, test bacteria were sub-cultured from stock into sterile nutrient agar plates and incubated for 24 h at 37 °C while test fungus was sub-cultured in Sabouraud dextrose agar for 48 h at 35 °C. After incubation identical colonies were suspended in sterile broth for 12 h and adjusted to 0.5 McFarland standard to give an inoculum size of approximately 10^8 CFU/mL. The adjusted inocula were diluted 1:100 to give inoculum size of approximately 10^6 CFU/mL using the M07-A10 approved guideline [12].

Preparation of stock solutions

Ciprofloxacin stock solution was prepared by dissolving 5.0 mg of ciprofloxacin powder in 10% DMSO solution and making up the volume to 10.0 mL, to give a final concentration of 0.5 mg/mL while fluconazole stock solution was prepared with 20.0 mg fluconazole powder in 10% DMSO solution to give a 2.0 mg/mL final concentration solution. A working concentration of 466.5 mg/mL stock of the seed extract was prepared by dispersing 1.0 mL (933 mg) of extract in 1.0 mL of 10% DMSO solution [13].

Antimicrobial susceptibility tests

Antimicrobial susceptibility test was carried out using agar well diffusion method with some modifications [14]. Standardized inoculum of each microorganism (200 µL) was evenly spread on the surface of already set 30.0 mL sterile Mueller Hinton agar. A sterile cork borer (10 mm) was used to bore six wells in each agar plate. The agar disks were removed and wells sealed at the base with two drops of sterile molten agar. Two wells were filled with 200 µL

(0.1 mg) ciprofloxacin. Similarly, Sabouraud agar was prepared by the manufacturer for *C. albicans* where fluconazole was used as a standard with 200 uL (0.4 mg) of it filled into two separate wells. The last two wells were filled with 200 uL (186 mg) of ethyl acetate extract. Negative control (10% DMSO solution) and positive control (viability test for organisms used) were carried out for each set of experiment. The plates were all incubated in an upright position for 24 h for bacteria at 37 °C and at 35 °C for 48 h for *Candida albicans*. The inhibition zone diameters (IZD) were measured in millimeters (mm) as an index of the inhibitory action of the test agent against a given organism. All experiments were carried out in triplicates, separate set for each test microorganism.

Determination of minimum inhibitory concentration (MIC)

The agar dilution method of Afolayan and Meyer [15] was used for the determination of minimum inhibitory concentration (MIC) of the extracts, ciprofloxacin and fluconazole on susceptible isolates. Following the manufacturer's instructions, nutrient agar and Sabouraud agar were prepared separately by placing them in a hot water bath at 50 °C. The stock solution of extract was incorporated into the molten agar at different volumes to obtain a range of concentrations of between 100 - 1600 mg/mL. A twofold serial dilution of the stock solution of ciprofloxacin (0.5 mg/mL) to obtain a concentration range of 0.0125 - 0.40 µg/mL was carried out. The concentration range for fluconazole stock solution (2.0 mg/mL) after serial dilution was 0.0032 - 0.2842 µg/mL. The nutrient agar-extract and nutrient agar-ciprofloxacin as well as the Sabouraud agar-fluconazole concentrations were poured separately into sterile plates and allowed to set and then dried at 40 °C for five (5) minutes. The test organisms were separately streaked onto the surface of each of these solidified agar plates. Each set of experiments had a negative (10% DMSO solution) and a positive (viability test for used organisms) control. The plates were incubated at 37 °C for 24 h while the plate for *C. albicans* was incubated at 35 °C for 48 h. All experiments were carried out in triplicates. The MIC was defined as the lowest concentration of extract or ciprofloxacin/fluconazole that inhibited growth of test microorganisms.

Determination of minimum bactericidal/fungicidal concentration (MBC/MFC)

The plates with no visible growth following MIC determination were swabbed and streaked onto fresh Mueller Hinton agar and Sabouraud agar plates containing neither the extract nor the antimicrobial agents. The plates were incubated for 24 h at 37 °C for bacteria and at 35 °C for 48 h for fungi. The MBC/MFC was defined as the lowest concentration of extract and test antimicrobial agent that showed no growth for the test isolates.

GC-MS analysis

GC-MS analysis was carried out on Agilent Technologies 6890N Network GC System and Agilent Technologies 5973 Network Mass Selective Detector coupled with 7683B Series Injector. Agilent capillary column (Model No. 122-5533) with the following specifications

was used: DB-5MS (5.0% phenylmethyl siloxane as stationary phase), 0.25 x 30 mm (internal diameter) and 1.0 µm (film thickness). The carrier gas used was helium at a flow rate of 1.2 mL/min. The injection volume was 1.0 µL. The inlet temperature was maintained at 230 °C. The oven temperature was programmed initially at 50 °C for 5.0 min, then programmed to increase to 300 °C at a rate of 10 °C ending with in 25 min. Total run time was 45 min. The MS transfer line was maintained at a temperature of 300 °C. The source temperature was maintained at 230 °C and the MS Quad at 150 °C. The ionization mode used was electron ionization mode at 70 eV. Total Ion Chromatogram (TIC) was used to evaluate for compound identification and quantization. The spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NIST02 Reference Spectra Library. Data analysis and peak area measurement was carried out using Agilent Chemstation and Pherobase Software in comparison with data reported in literature.

Statistical analysis

Data were reported as mean from triplicate determinations for inhibition zone diameters ± standard error. Data analysis and peak area measurement of raw GC-MS data were carried out using Agilent Chemstation and Pherobase software.

RESULTS

The ethyl acetate seed extract of *A. muricata* was an aromatic yellowish oily extract with a yield of 19.93%. Results from the antimicrobial assay of the extract shown in Table 1 revealed that the test organisms were susceptible to the inhibitory effect of the ethyl acetate extract, ciprofloxacin and fluconazole with inhibition zone diameters of 17.02 - 23.50 mm.

Table 1: Susceptibility profile of test microorganisms to the extract and standard antimicrobial agents

Test Microorganisms	Ethyl acetate extract (mm)	Ciprofloxacin (mm)	Fluconazole (mm)
Pseudomonas aeruginosa	23.50 ± 0.64	34.50 ± 0.50	ND
Staphylococcus aureus	19.75 ± 0.39	25.90 ± 0.94	ND
Klebsiella aerogenes	ND	29.00 ± 0.90	ND
Escherichia coli	17.02 ± 0.25	26.50 ± 0.50	ND
Bacillus subtilis	19.21 ± 0.50	31.50 ± 0.50	ND
Candida albicans	20.50 ± 0.50	ND	31.50 ± 0.5

ND = Not determined

The minimum inhibitory and microbiocidal concentrations (MIC and MMC) of the ethyl acetate test extract and standard antimicrobial drugs against susceptible microorganisms are shown in Table 2. MICs and MMCs values of 25.0 mg/mL of the extract were obtained for *Pseudomonas aeruginosa* and *Bacillus subtilis* while an MIC of 400 mg/mL and MMC of 800

mg/mL of the extract were obtained for *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. *Klebsiella aerogenes* exhibited MIC and MMC values of 800 mg/mL of the extract.

The standard antimicrobial test drug, ciprofloxacin had a range of MIC and MMC values of 0.0125-0.05 µg/mL for all test bacteria while fluconazole displayed MIC and MMC values of 0.205 µg/mL for *Candida albicans*.

Table 2: Minimum inhibitory concentration/ Minimum microbiocidal concentrations of the extract and standard antimicrobial agents

Test microorganisms	Ethyl acetate extract (mg/mL)		Ciprofloxacin (µg/mL)		Fluconazole (µg/mL)	
	MIC	MMC	MIC	MMC	MIC	MMC
Pseudomonas aeruginosa	25	25	0.0125	0.0125	ND	ND
Bacillus subtilis	25	25	0.025	0.025	ND	ND
Escherichia coli	400	800	0.05	0.05	ND	ND
Staphylococcus aureus	400	800	0.025	0.025	ND	ND
Candida albicans	400	800	ND	ND	0.205	0.205
Klebsiella pyrogenes	800	800	0.0125	0.0125	ND	ND

ND = Not determined

GC-MS chromatogram obtained from the extract showed several peaks with corresponding retention times and percentage areas (Figure 1). Comparison of this chromatogram with NIST14 reference spectral library identified 48 phyto-compounds (Table 3). Predominant compounds identified with known antimicrobial properties were fatty acids (Hexadecanoic, pentadecanoic and undecanoic acids), phthalic acid, aldehyde (9,12-octadecadienal), alcohol (1,14-tetradecanediol) and unsaturated hydrocarbons (1,5-heptadiene, 2-hexene).

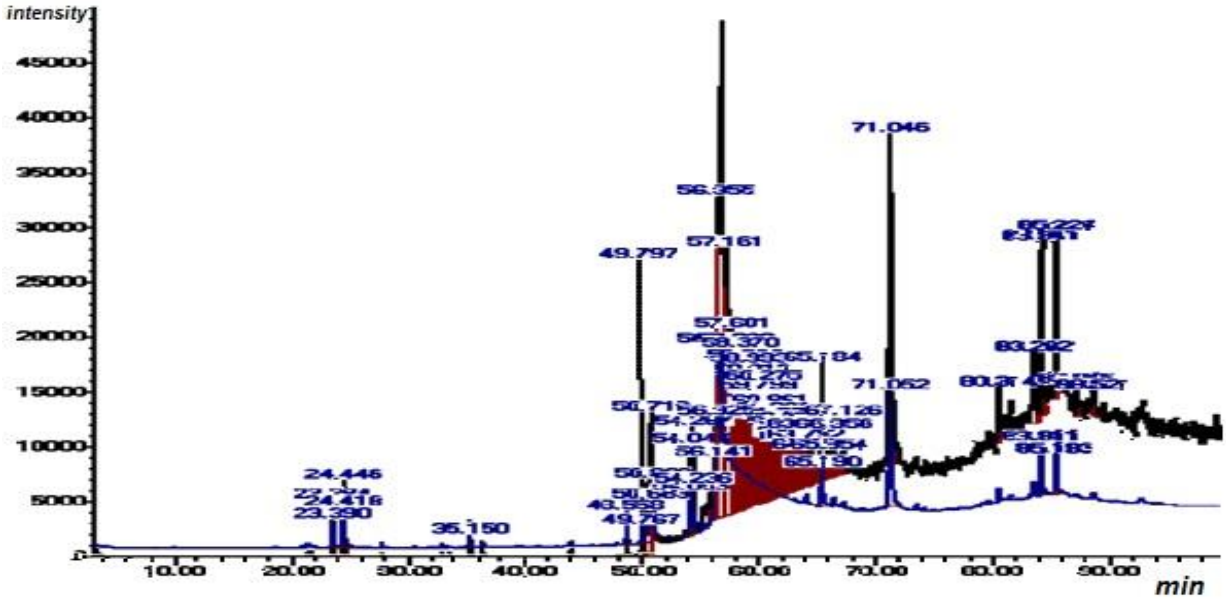

Figure 1: GC-MS chromatogram of the ethyl acetate extract of *A. muricata* seed

Table 3: Chemical components of the ethyl acetate extract of *A. muricata* seed

Peak number	Retention time	Area (%)	Chemical compound
1	23.384	1.38	Methallyl cyanide cyclopentene
2	49.797	2.53	Phthalic acid
3	50.713	3.08	n-hexadecanoic acid
4	50.860	0.56	Pentadecanoic acid
5	54.047	0.69	11-(12-cyclopenten-1-yl) undecanoic acid
6	54.267	0.59	6-hepten-3-ol
7	56.172	3.37	2,7-octadien-1-ol
8	56.355	4.02	9-oxabicyclo-6-nonane
9	56.758	15.26	9,12-octadecadienal
10	57.161	7.77	1,14-tetradecadienal
11	57.601	4.04	2-hexene
12	57.930	2.92	1,5-heptadiene
13	58.187	1.22	1-nonyne
14	58.370	1.94	Oxirane
15	58.736	2.98	Cyclohexeanebutanoic acid
16	58.553	1.13	Carvone oxide
17	58.993	2.10	Cis-methyl-2-(2-propenyl) cyclopropane
18	59.213	4.10	10-Triepoxydecane
19	59.652	0.88	4-cyclohexene-12-diol
20	59.799	1.94	Cyclopropane carboxylic acid
21	60.275	2.92	Chlorohexane methanol
22	60.532	0.95	6-nitro-1-nonyne
23	60.715	0.94	3-butenyl-oxirane
24	60.861	0.90	3-hepten-1-ol
25	61.081	1. 68	2,2-(1,4-butanediyl)bis-2-hexene
26	61.301	0.59	Cis-1-methyl-2-(2-propenyl) cyclopropane
27	61.374	0.79	11-(2-cyclopenten-1-yl) undecanoic acid
28	61.777	1.60	2-hexene
29	62.070	1.64	3-butenyl-oxirane
30	62.400	1.02	1,2-triepoxydecane
31	62.656	0.76	6-nitro-oxirane
32	62.876	0.66	5,10-dioxatricyclodecane
33	63.059	0.62	1,8-nonadiene
34	63.059	0.61	6-nitro-5-cyano-1-pentene
35	63.792	0.56	6-bromo-cyclopentane

36	63.939	0.71	1,6-hexanediol
37	64.304	0.56	9,10-triepoxydecane cyclopentane
38	65.184	2.62	1,15-pentadecanediol
39	65.954	0.63	6-nitro-1,6-octadiene
40	66.356	0.69	Cis-2,4-dimethylthiane
41	67.126	0.64	3-ethylheptanoic acid
42	71.046	6.26	9,12-octadecadienal
43	80.314	0.74	Beta-d-galactopyranoside
44	83.282	1.62	Cyclopentane undecanoic acid
45	83.941	2.64	6-nitroundec-5-ene
46	85.224	2.50	Trans-cyclopentane undecanoic acid
47	86.945	0.46	Undec-10-ynoic acid
48	88.521	0.57	Alpha-d-galactopyranoside

DISCUSSION

The antimicrobial potential and chemical composition of the ethyl acetate seed extract of *Annona muricata* were evaluated. Studies on the possible antidiabetic, antioxidant, anticancer and antibacterial activities of various extracts of the leaves, fruit pulps, peels and seeds of *Annona muricata* have been extensively reported [16-20]. The extractive percentage yield of the seeds (19.93%) obtained from this study was high especially when compared with previously reported figures; aqueous (6.15%), methanol (9.46%) and ether (2.41%) yields of the seeds [21]. The yellowish colour of the seed may suggest the ability of the extractive solvent (ethyl acetate) to extract pigment as previously reported from the seeds [22]. The break point of inhibition zone diameter (IZD) of an antimicrobial active crude plant extract has been categorised into low (12-18 mm), moderate (19-22 mm) and strong (23-38 mm) or equal or greater than 10.0 mm [23,24]. The obtained data from this study are indicative of the fact that *A. muricata* seed extract could be moderately active against test microorganisms (with IZD range of 17.02 - 23.50 mm). Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) are quantitative indices used to measure the efficacy of crude extract against microorganisms [25]. In this study the MIC values of the extract varied from 25 - 800 mg/mL. This variation may be due to the inherent structural differences of microbial test strains. The pure standard antimicrobial agents were expectedly more efficacious with the observed low inhibitory and bactericidal concentrations. Obtained values of MIC and MBC against *Pseudomonas aeruginosa* and *Bacillus subtilis*, were suggestive of a moderate bactericidal activity of the ethyl acetate extract against these test microorganisms. However, the higher MBC/MFC values (800 mg/mL) exhibited by *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* may be consistent with a slow bacteriostatic action.

GC-MS is one of the preliminary steps toward identification and of understanding the nature of active phytocompounds [26,27]. The observed antimicrobial activity of the seeds extract in this study may be due to the abundance of an unsaturated aldehyde, 9,12-octadecadienal (15.26%), which was the major quantitative phytochemical identified. This compound which is a secondary degradation product of octadecanoic fatty acid has been previously reported to have antimicrobial activity [28,29]. In earlier studies, fatty acids such as hexadecanoic, pentadecanoic, undecanoic and phthalic acids have been associated with antibacterial and antifungal activities [30]. This activity might have resulted from the ability of these acids to disrupt the bacterial cell membrane causing increased fluidity, permeability changes, cytoplasmic leakages and the consequent unstable bacterial cell and lysis [31]. Antibacterial and antifungal activity of biological compounds like 1,14-tetradecanediol have also been reported [32]. A possible synergistic and/or additive combination of these compounds could explain the observed antimicrobial activity of the extract. Further studies of the extract for isolation and evaluation of these compounds may lead to new and novel bioactives with antimicrobial properties.

CONCLUSIONS

This study has provided data for the antimicrobial activity of the ethyl acetate seed extract of *A. muricata*. The extract had a moderate antimicrobial inhibitory activity against test microorganisms. The main bioactive compounds were; fatty acids, alcohols and aldehydes. A combination of their bioactivities could be the scientific bases of their ethnomedicinal usage and further evaluation may present the active principles as potential antimicrobial agents.

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