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Antimicrobial Properties of 9, 12-Octadecadienoic Acid Isolated from Leaf Extracts of *Acalypha Fimbriata* (Euphorbiaceae)

Ufuoma Shalom Onoabedje*, Christopher Obodike Ezugwu & Efeturi Abraham Onoabedje

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Abstract: *Linoleic acid is an essential polyunsaturated fatty acid found in natural substances with numerous interesting biological and nutritional properties. Acalypha fimbriata is one of the well spread species of the Acalypha genus that is used for centuries as medicinal herb. There is however limited scientific work on this plant. The aim of the present study is to isolate and characterized bioactive compounds from the leaves of Acalypha fimbriata. The powdered leaves (650g) were extracted sequentially with n-hexane, ethyl acetate and methanol via maceration to obtain n-hexane, ethyl acetate and methanol extract respectively. The ethyl acetate extract was pre-adsorbed on celite and then subjected to repeated column chromatography over silica gel G. The fractionation and purification process resulted to a pure sample which was coded, AFE4. The structural elucidation was done via Nuclear Magnetic Resonance (NMR) and High Resolution – Liquid Mass Spectrometry (LC-HRMS). The antimicrobial study of the isolated compound was done. Based on the NMR (^{1}H and ^{13}C- DEPT) spectroscopic and LC-HRMS data, the isolated compound was identified as 9, 12-octadecadienoic acid (Linoleic acid) [$C_{18}H_{32}O_2$; Mol. Wt = 280]. The compound showed a significant broad spectrum of antimicrobial activity against the test organisms- Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Salmonella typhi, Candida albicans and Aspergillus niger with zones of inhibition ranging from 0 – 20 mm. Statistical analysis showed there were significant differences ($p<0.05$) in antimicrobial effects among the various concentrations of the isolated compound and the standard drugs against the test organisms. The observed antimicrobial action of A. fimbriata as reported in literature could be linked to the presence of linoleic acid.*

Keywords: *Acalypha fimbriata, Euphorbiaceae, Linoleic acid, Antimicrobial, Medicinal plants*

Ufuoma Shalom Onoabedje*
Department of Pharmacognosy and Environmental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.
Email: Onoabedjeufuomashalom@gmail.com
Orcid id: 0009-0004-7861-6873

Christopher Obodike Ezugwu
Department of Pharmacognosy and Environmental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enuguu Sate, Nigeria.
Email: oechristo@yahoo.co.uk
Orcid id: 0000-0002-1331-5159

Efeturi Abraham Onoabedje
Department of Pure & Industrial Chemistry, Faculty of Physical Sciences, University of Nigeria, Nsukka, Nigeria
Email; efeturi.onoabedje@unn.edu.ng
Orcid No: 0000-0002-3316-8016

1.0 Introduction

Medicinal plants have been useful in the prevention and treatment of diverse disease conditions globally. World Health Organisation projected that 80% of the population of Asia, Africa and Latin America depend on traditional medicine for their basic health care [Okunye et al. 2023; WHO-AFRO 2010]. In Africa, majority of the populace rely on traditional medicine for their primary healthcare need. In addition, natural product extracted from medicinal plants directly or

indirectly form the basis of over 30% of modern medicine; for example , aspirin (analgesic) , vinblastine (anticancer), artemisinin and quinine (antimalaria), reserpine (antihypentensive), and ephedrine (nasal decongestant) [WHO-AFRO, 2010]. Despite the numerous values derived from the use of medicinal plants, most of these plants has not been studied in detail to ascertain the phytochemicals responsible for the expressed pharmacological effects [Cragg and newman, 2013; Fabricant and Farnsworth, 2001; Verpoorte, 1998, 2000]. *Acalypha fimbriata* is a member of the Euphorbiaceae. Plants from Acalypha genus are enthnomedicinally employed in treatment of varieties of physiological disorders such as diabetes, jaundice, hypertension, fever, liver inflammation, schistosomiasis, dysentery, respiratory problems including bronchitis, asthma, pneumonia scabies, eczema and mycoses in Africa [Seebaluck, Gurib-Fakim, Mahomoodally, 2015]. Many Acalypha species are traditionally used throughout East and West Africa, particularly in Nigeria in treating infections, ulcers, anthelminthes, asthma, sylphilis, rheumatism, asthma, cough, coryza and other diseases [Soladoye, *et al.*, 2008; Seebaluck, Gurib-Fakim, Mahomoodally, 2015]. The leaves, stems, and roots are used in disease mixtures and decoctions.

Although the Acalypha genus has been generally reported to possess antimicrobial potentials, there is a dearth of information available on isolated phytochemicals with antimicrobial effects from *Acalypha fimbriata.* We have previously reported the phytochemical, pharmacognostic, antimicrobial and mosquito repellent activities of the leaf extract *of Acalypha fimbriata* (Euphorbiaceae) [Onoabedje, *et al*., 2019]. In continuation of our study on this plant we are therefore, presenting the antimicrobial activity of 9, 12-octadecadienoic acid (linoleic acid) isolated from the leaf extracts of *Acalypha fimbriata.*

2.0 Materials and Method

2..1 Collection of Plant and Authentication

Fresh leaves of *Acalypha fimbriata* were collected in the month of August 2023 from cultivated farmland in Eha-Alumona, Nsukka, Enugu State, Nigeria. They were identified and authenticated by Mr A.O. Ozioko of the International Centre for Ethnomedicine and Drug Development, InterCEDD. The plant materials were washed thoroughly under running tap water to remove soil and other extraneous materials and air-dried for two weeks. The air-dried material was ground into fine powder using an electric blender, weighed and stored in an air-tight container.

2.2 Preparation of Extract

The powdered plant material (650g) was extracted sequentially via maceration with n-hexane (2.5 L), ethyl acetate (2.5 L) and methanol (2.5 L) for 48 hours at room temperature. The content was filtered using Whattman No. 1 filter paper on a Buchner funnel. The collective filtrate was evaporated to dryness using a rotary vacuum evaporator at a controlled temperature of 40^{o}C to afford the crude extracts, and coded **AFH, AFE** and **AFM** respectively. The extracts were transferred into sterile sample containers and preserved in a refrigerator at 4^{o} C until required for the proposed experiment.

2.3 Fractionation and Isolation

A chromatographic column (50cm × 5cm) was packed with 110 g of silica gel [0.2 - 0.5 mm] using the wet packing method. About 17 g of ethyl acetate extract which was pre-adsorbed onto Celite prior to purification earlier was transferred to the column with gentle tapping on the side to ensure a uniform layer. The column was eluted via a very gentle gradient elution with the mobile phase of n-hexane – ethyl acetate starting from [95% hexane: 5% ethyl acetate] to [0% hexane: 100% ethyl acetate]. Ninety fractions (20ml each) were collected, allowed to evaporate to approximately half their initial volumes and monitored by thin layer chromatography using ethyl acetate: methanol (9:1) as solvent system. Upon concentration fraction 25 was obtained as a

light yellow oil with a distinct odour and was coded AFE_4.

2.4 Antimicrobial Screening of the Isolated Compound (AFE_4.)

Six clinical isolates (*Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, candida albican and Aspergillus niger*) were obtained from the microbial bank of the Department of Microbiology, University of Nigeria Teaching Hospital, Enugu. The organisms were standardised using a colony suspension method and matching the strain's suspension with 0.5 McFarland standard to give a resultant concentration of 1.5 × 108 cfu/ml. Antimicrobial susceptibility testing was determined using the agar well diffusion technique. From the stock solution of 25mg/ml, serial dilutions were made to obtain 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.562mg/ml. Each labelled medium plate was inoculated uniformly with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5 mm diameter was used to make wells on the medium. 0.1 ml of the isolated compound was dropped into the appropriately labelled well (Onoabedje *et al*, 2019). Ciprofloxacin (10 µg/ml) was used as a reference standard for the antibacterial test while fluconazole (5 µg/ml) was used as standard for the anti-fungal test. The plates were incubated at 37 °C for 24 h for the anti-bacteria test and 27 °C for 48 h for the anti-fungi test. The inhibition zone diameters (IZDs) produced by each concentration of the isolated compound were measured and recorded in millimetres (mm) (CLSI, 2008).

2.5 Minimum Inhibitory Concentration (MIC) Evaluation

The MIC of the compound was performed at five concentrations (25, 12.5, 6.25, 3.125 and 1.562mg/ml) employing doubling dilutions of sample in molten Mueller- Hinton agar or Sabouraud dextrose agar up to the fourth dilution. The resultant agar (1 ml) was dispensed in test tube and equal amounts of the isolated compounds (1ml) were added to the first test tube and serial dilutions done with the last 1ml being discarded. To complete the test each organism was separately suspended in 5 ml of nutrient broth or molten Sabouraud dextrose agar and incubated overnight, after which 0.1ml was added to all test tubes and incubated at 37ºC for 18 hours or 36 hours. After incubation, a loopful from each tube was sub cultured on Mueller-Hinton agar or Sabouraud dextrose agar and incubated for growth at 37 °C for 24 hours or 27ºC for 48 hours [Kasim *et al*, 2012]. The plates that showed no growth were observed. The MIC was defined as the lowest concentration of an antimicrobial agent that inhibited the visible growth of a microorganism after overnight incubation.

2.6 Minimum Bactericidal/Fungicidal Concentration (MBC/ MFC) Determination

The value of the MBC/MFC is an extension of MIC. The agar plates showing no growth in the MIC test were used for the determination of the MBC/MFC. Equal volumes of the various concentrations of the isolated compound and Müller-Hinton or Sabouraud Dextrose Agar were mixed in micro-tubes to make up 0.5 ml of solution. 0.5 ml of McFarland standard of the organism suspension was added to each tube ((Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37 °C for 24 h and at 27 °C for 48 h. The MBC/MFC was determined by sub-culturing the test dilution on Müller-Hinton or Sabouraud Dextrose Agar and further incubated at 37 °C for 24 h (bacteria) and at 27 ºC for 48 h (fungi) (Akinyemi et al., 2005). The MBC and MFC were recorded as the lowest concentration of the isolated compound that did not permit any visible bacterial and fungal colony growth on the agar plate after the period of incubation. Ciprofloxacine (for bacteria) and fluconazole (for fungi) were used as positive controls.

2.7 Instrumental Analysis

The NMR experiments were recorded $CDCl_3$ and TMS as internal standard on a Bruker Avance DRX 400MHz and 125MHz spectrophotometer. Mass spectra were recorded on LC-HRMS performed on an

Accela 600 HPLC system with an ACE C-18 column (150 × 3 mm, 3 μm particle size) (HiChrom, Reading UK) coupled to an Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany. Thin layer chromatography was performed on pre-coated silica gel plate (0.2mm)Merck (Germany),column chromatography was carried out on silica gel G(0.2-0.5mm) MESH, Fluka,Switzerland).

2.0 Results and Discussion

The isolated compound was obtained as light yellow oil with R_f value of 0.68. The mass spectrum (Fig 3) for this compound shows a molecular ion peak [M^+ - H^+] at m/z 280, which translate to a molecular formula $C_{18}H_{32}O_2$ and was identified as 9, 12-octadecadienoic acid (linoleic acid). The ^{1}H-NMR spectrum (Fig 1 & table 1) of the compound shows deshielded resonances for olefinic protons at δ 5.35, bis-allylic protons at δ 2.73, allylic protons at δ 2.00 and a group of methylene protons at δ 2.30 which correspond with the protons attached to C-2. Other shielded resonances are at δ 0.90 for terminal methyl protons, δ 1.63 which correspond with the methylene protons attached to C-3, and δ 1.25 which correspond with the methylene protons attached to C-4 to C-7 and C-15 to C-16. The ^{13}C-NMR spectrum (fig 2 & table 2) displays two signals for the olefinic carbons δ 130.24 (C-9) and 123.57 (C-12), as well as signals for methylene carbons at δ 34.40 - 22.7 (C-2 and C-17). The terminal methyl signal (C- 18) resonates at δ 14.14, while the carbonyl signal (C-1) is observed at δ 178.74. Thus, the compound is identified as 9, 12-octadecadienoic acid. The spectral data are in agreement with that reported data in literature [Alqadeeri F.K. *et al*, 2022]. Previously we have identified via GC-MS of the ethyl acetate fraction of *Acalypha fimbriata*, derivatives of the isolated compound-linoleic acid (Onoabedje U.S *et al,* 2019).

Previous investigations of the leaf-extracts of *A. fimbriata* showed a broad spectrum of antimicrobial effects against these strains of microbes- *Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, candida albican and Aspergillus niger* (Onoabedje et al, 2019). The results of the antimicrobial studies showed that the compound exhibited remarkable

activity at 25 mg/mL against the six microorganisms tested. This phytochemical displayed antimicrobial activity against the tested organisms with the zone of inhibition at various concentrations used ranging between 2 – 20 mm (table 3). The compound isolated from *Acalypha fimbriata* could be inferred to possess a dose dependent activity as increase in the concentration of the compound from 1.325mg/ml to 25mg/ml was found to result in increase in the inhibition zone diameter (table 4). Also the isolated compound showed same degree of antimicrobial effect to both Gram- positive bacteria and the yeast fungi – *Candida albican,* with a poor antibacterial profile against Gram- negative bacteria. The mold - *A. niger* showed poor susceptibility to the isolated phytochemical from *Acalypha fimbriata.* The results of the minimum bactericidal and fungicidal concentration are shown in table 5. It is worthy of note that the least MBC and MFC was shown against *Staphylococcus aureus* a Gram positive bacteria at 6.25mg/ml and *Candida albican* a yeast at 12.5mg/ml respectively. Fatty acids such as, palmitic (Hexadecanoic), oleic, linoleic acid, stearic acids etc are known to have potential antibacterial and antifungal agents [Seidel and Taylor, 2004]. Hence, the observed antimicrobial action of *A. fimbriata* as reported in literature [Kasim *et al* 2011; Onoabedje, *et al*., 2019] could be linked to the presence of this isolated compound- linoleic acid amongst other phytochemicals; thereby justifying its diverse ethnomedicinal use in the treatment of diarrhoea, skin infections, syphilis and ulcers in Nigeria [Seebaluck R *et al.*, 2015, Essiette and okoko, 2013, Odugbemi, 2008]

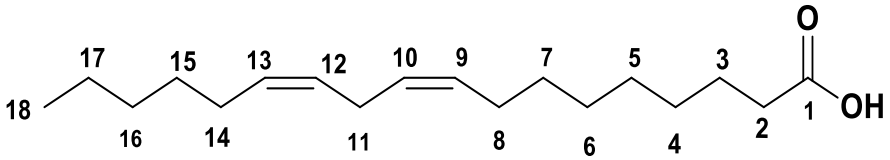

Fig. 1: Structure of the isolated Compound - 9, 12- octadecadienoic acid

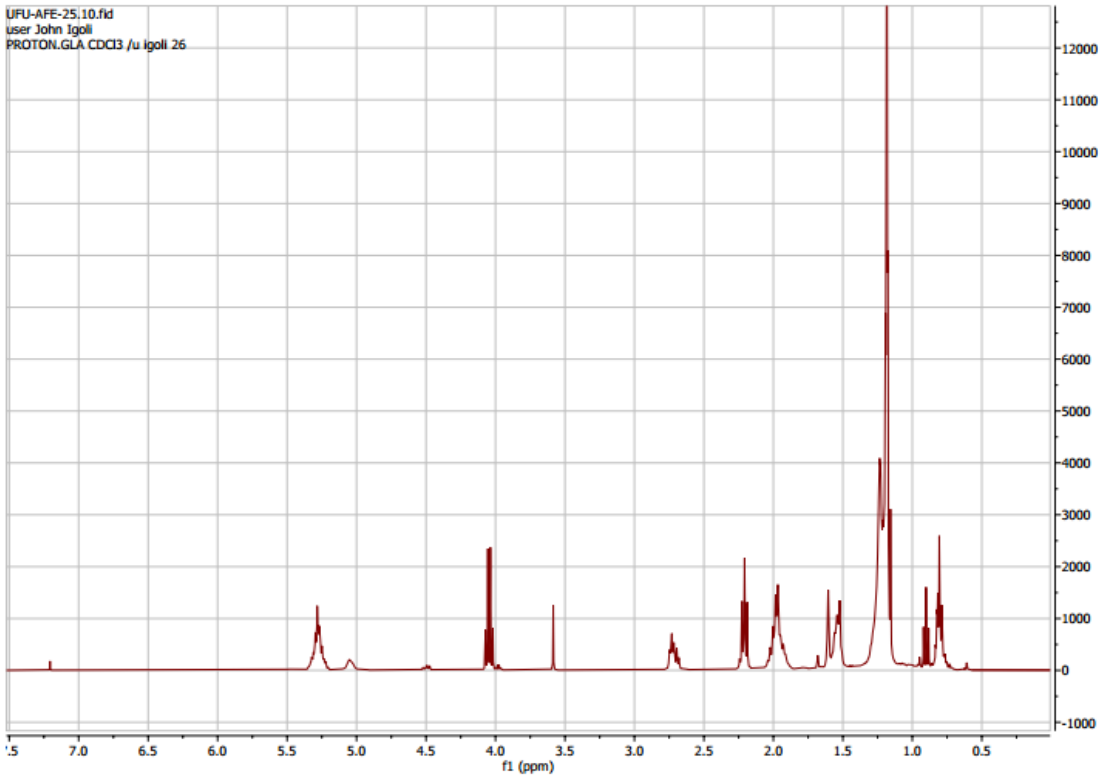

Fig. 2: ^{1}H NMR of 9, 12- octadecadienoic acid

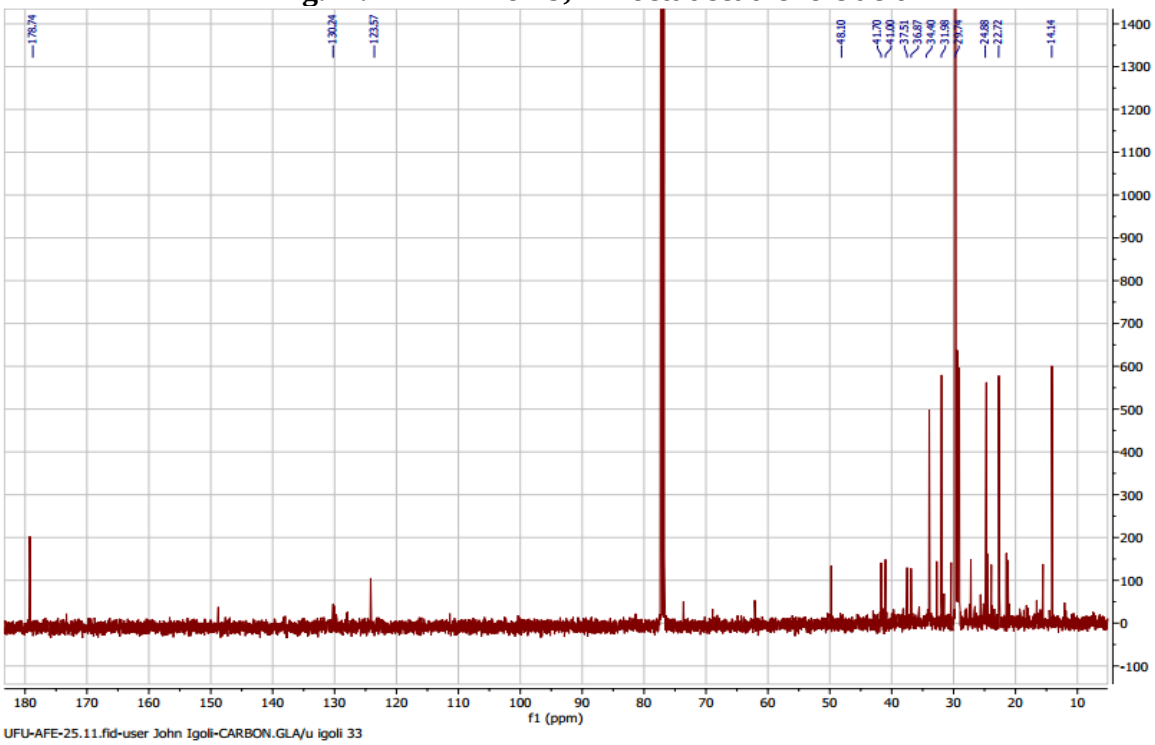

Fig. 3: ^{13}C (DEPT) Spectrum of 9, 12- octadecadienoic acid

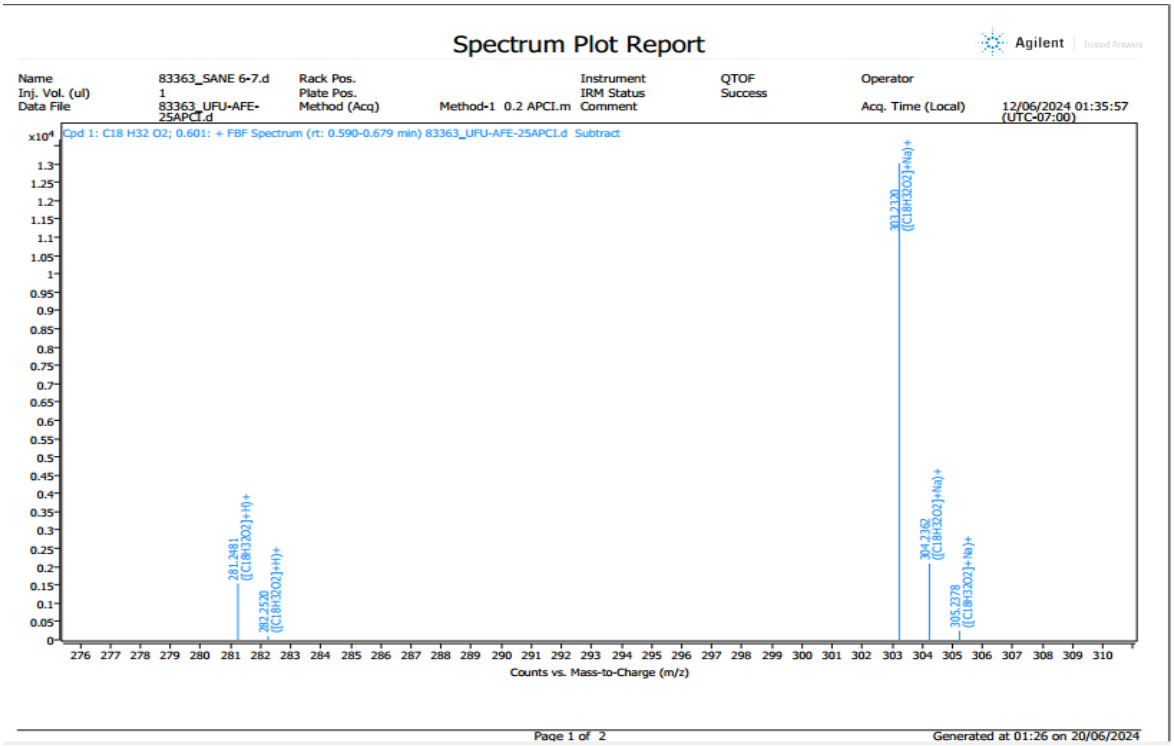

Fig. 4: LC-MS of 9, 12- octadecadienoic acid

Table 1: ^{1}H spectral data for compound 3[400 MHz, CDCl3, d (ppm)]

Protons	**^{1}H (ppm)**
Olefinic	5.35
Bis-allylic	2.73
Allylic	2.00
Methylene	2.34
Terminal methyl	0.90
Methylene proton attached to C-3	1.63
Methylene carbon attached to C4 – C7 and C15- C16	1.25

Table 2: ^{13}C NMR spectral data for compounds 3[125 MHz, CDCl3, d (ppm)]

POSITION	**^{13}C (ppm)**
COOH	178.74
C-9, C-13	130.24
C-10	-
C-12	127.9
C-2	34.40
C-16	31.98
C-14	-
C-4 to C-7 and C-15	29.74
C-11	-
C-3	24.88
C-17	22.72
C-18	14.14

Table 3: Diameter of Zone Of Inhibition (mm) of linoleic acid

Organism	Concentration (mg/ml)					Ciprofloxacine (µg/ml)				Fluconazole (µg/ml)			
	25	12.5	6.25	3.125	1.25	10	5	2.5	1.25	10	5	2.5	1.25
S. aureus	20	13	9	7	2	25	17	11	6				
S.Pyogene	16	12	9	5	-	23	13	8	6				
S. typhil	10	5	-	-	-	20	18	11	7				
E. coli	7	3	-	-	-	22	16	11	8				
C. albican	13	9	9	5	2	-				22	13	9	4
A. niger	7	3	-	-	-	-				20	14	8	5

Table 4: Minimum Inhibitory Concentration (MIC) of the isolated compound

Organism	Linoleic Acid (mg/ml)	Ciprofloxacin (µg/ml)	Fluconazole (µg/ml)
S. aureus	1.325	1.25	-
S. Pyogene	3.125	1.25	-
S. typhil	12.5	1.25	-
E. coli	12.5	1.25	-
C. albican	3.125	-	0.625
A. niger	12.5	-	0.625

Table 5: Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration (MBC/ MFC) of linoleic acid

Organism	Linoleic acid (mg/ml)
S. aureus	6.25
Strep. Pyogene	12.5
S. typhil	25
E. coli	25
C. albican	12.5

4.0 Conclusion

9, 12-octadecadienoic acid (linoleic acid) was isolated and characterized from leaf extracts of *Acalypha fimbriata* (Euphorbiaceae) for the first time. The isolated compound showed antibacterial and antifungal activity and may play a contributory role in the prevention and treatment of infections caused by these strains of microorganisms. This study therefore, could provide scientific basis for support of the use of *A. fimbriata* in the treatment of various infections in Nigeria.

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5.0 References

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Declaration

Consent for publication

Not applicable

Availability of data

Data shall be made available on demand.

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Authors' contributions
All the authors contributed to the work. COE designed the work while all other authors were involved in the computation, development of the draft manuscript and correction.