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Determination of Bioactive Compounds and Antimicrobial Capabilities of Purified *Nymphaea lotus* Linn. (Nymphaeaceae) Extract to Multidrug Resistant Enteric Bacteria

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Authors' contributions

This work was carried out in collaboration between both authors. Author OJA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OJA and STA managed the analyses of the study. Author STA managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The increase in multidrug resistance bacteria is a major issue of concern to researchers and healthcare experts. There is a continuous effort by the researchers to innovate new methods of prevention and treatment of infections caused by multidrug resistance bacteria. The occurrence of bioactive compounds and antimicrobial capabilities of *Nymphaea lotus* Linn. (Nymphaeaceae) extract against multidrug resistant enteric bacteria were studied by assessing *in-vitro* antibacterial properties of the extract using agar well diffusion technique. Fourier Transform Infrared Spectrophotometer (FTIR) spectra revealed the presence of thirty (30) functional groups in purified ethanol extract of *N. lotus* root. Also, Gas Chromatography-Mass Spectrophotometry revealed the

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presence of different bioactive compounds (5-Nonanol, 2-Pentanone, 4-hydroxy-4-methyl-, Azulene, Tetradecanoic acid, 2-Chloroethyl vinyl sulfide, Decanoic acid, ethyl ester, Phytol, 1-Hexadecyne, 2-octenoic acid, Methyl-n-hexadecyl ketone, Hexadecanoic acid, methyl ester, and n-Hexadecanoic acid) in purified ethanol extract of *N. lotus* root which can be used in production of antimicrobial drugs. Five fractions of purified ethanol extract of *N. lotus* root (fraction 3-7) had significant zone of inhibition. Fraction four had the highest zone of inhibition 23.67 ± 0.88 mm while fraction seven had the least zone of inhibition 2.67 ± 1.33 mm.

Keywords: Enteric bacteria; phytochemical; bioactive compound; antimicrobial; Nymphaea lotus.

1. INTRODUCTION

Pathogenic enteric bacteria has been a major cause of morbidity and mortality [1,2] due to increase in multidrug resistance in treatment of infections caused by such bacteria. Approximately over 1.8 million people die from diarrheal diseases annually, many of which have been linked to diseases acquired from the consumption of contaminated waters and seafood [3].

Gastrointestinal infections are the most common diseases caused by enteric bacteria. Examples are salmonellosis (*Salmonella* sp.), cholera (*Vibrio cholerae*), dysentery (*Shigella* sp.) and other infections caused by *Campylobacter jejuni*, *Yersinia* sp. and *Escherichia coli* O157:H7 and many other strains. *E. coli* O157:H7 can cause infections successfully due to its low infectious dose (ID), with not more than ten cells [4]. Antimicrobial resistance (AMR) is one of the major public health challenges of the 21st century that threatens the effective prevention and treatment of infections caused by bacteria, parasites, viruses and fungi [5]. The problem of AMR is especially alarming due to antibiotic resistance in bacteria. For many decades, to a large extent, bacteria causing common or chronic infections have developed resistance to emerging antibiotic. This has led to the need for proactive steps to avert a developing global crisis in health care. The World Health Organization (WHO) has long back recognised the need for an improved and coordinated global effort to contain AMR. In 2001, the WHO Global Strategy for Containment of Antimicrobial Resistance has provided a framework of interventions to slow down the emergence and to reduce the spread of antimicrobial-resistant microorganisms [5].

In 2012, WHO releases the evolving threat of antimicrobial resistance – Options for Action [6], which proposed combination of interventions that include strengthening health care systems and surveillance; improving use of antimicrobials in hospitals and in community; infection prevention and control; encouraging the development of appropriate new drugs and vaccines; and political commitment [5]. Multidrug resistant enteric bacteria have been found responsible for common or severe gastrointestinal infections. Needless to say, such infections remain a major concern to public health.

It is worth mentioning that worldwide, many people favor herbal medicine over synthetic medicine. The ancient record well documented the curing nature of herbal remedies used in: Indian medicine, Chinese, African, Egyptian, Greek, Roman and Arab System of medicine which dates back to about 5000 years [7]. About 500 plants with medicinal use are mentioned in ancient texts and around 800 plants have been used in indigenous systems of medicine. Indian subcontinent is a vast repository of medicinal plants that are used in traditional medical treatments [7], which also forms a rich source of knowledge.

N. lotus is an important component of the Egyptian vascular aquatic plants which is widely distributed. It is commonly encountered in the irrigation and drainage canals in the Nile delta. It has been receiving much attention from the ecological, medicinal and environmental scientists due to its ability to absorb and accumulate heavy metals from polluted water. Nevertheless, worldwide water lilies, apart from their ecological interest, appear to have also historical importance [8]. The syrup of the roots was used as an anti-inflammatory, and in fever, and the seeds were used for hemorrhoids [9]. Nutritionally, the tuberous rhizomes and seeds of the plant could be eaten, the first either boiled or roasted, and the latter in bakery [10]. In the current study, we investigated the bioactive compounds and antimicrobial capabilities of purified *N. lotus L.* extract to multidrug resistant enteric bacteria and the results obtained are presented in the present communication.

2. MATERIALS AND METHODS

2.1 Materials Used in the Study

N. lotus (Water lilies), conical flask, , human volunteers, paper tape, muslin clothes, ethanol, aluminum foil, Chloroform, syringe, micropipette, ice pack, nitrogen gas, ethanol, distilled water and n-hexane.

2.2 Source of Microorganism

Enteric bacteria used in this study were stock cultures from our project. They were isolates from Ogbese river water and stored at microbiology department microorganism bank of Federal university of Technology, Akure (Nigeria).

2.3 Collection of *Nymphaea lotus*

Water lilies (*Nymphaea lotus*) were collected from stagnant water at Okitipupa local government area of Ondo State, Nigeria. The plant was identified and authenticated by experts at the Crop, Soil and Pest Department, Federal University of Technology, Akure, Nigeria (Family: Nymphaeaceae. Genus: Nymphaea. Species: *N. lotus*). The roots were separated from the whole plant and collected in containers**.** Later, they were washed with running clean tap water and dried at room temperature. The dried roots were milled separately to a fine powder, and stored in an airtight container till used.

2.4 Preparation of Extracts from *Nymphaea lotus*

The plant was extracted with ethanol using the method described by [11]. The dried *N. lotus* roots (200 g) were shifted into a sterile plastic container. The dried *N. lotus* roots were homogenized in 1litre of ethanol solvent and then filtered using Whatman No. 1 filter paper. The solvent was evaporated at low pressure using rotary evaporator. The extract thus obtained was used in different experiments [12].

2.5 Storage of Stock Concentration of *Nymphaea lotus* Extracts

The 100% stock concentration of *N. lotus* extract was obtained and stored at 4°C in a well corked universal bottle. It was reconstituted with DMSO to a required concentration at each use.

2.6 Partial Purification of *Nymphaea lotus* Root Extract

Partial purification was carried out using column chromatography as described by [13]. Twenty grams of extract of *N. lotus* root was subjected to column chromatography on silica gel (100 – 200 mesh – Merck) packed and eluted with mixture of n-Hexane, chloroform, ethyl acetate, ethanol, methanol and water of increasing polarity to obtain fractions respectively.

2.7 Determination of the Retention Factor (Rf) of *Nymphaea lotus* Root Extract

The retention factor of extract of *N. lotus* root was determined by developing Thin Layer Chromatography; 30g silica gel G (with $CaSO_4$ as binder) was placed in a beaker and shook vigorously with 60-65ml of distilled water for about 1min, transferred to the applicator and spread uniformly on the plate 20x20 cm. The thickness of the layer was 0.25 mm. Plates were allowed to dry for 5-10min in dust free conditions. Gel was activated prior to use for 5min at 110^{o}C in a hot oven. Gel was divided into a number of lanes by drawing lines with a lead. Different known volume (5, 10µl) of the sample extracts were spotted in various lanes carefully with a ball pen or micro syringe on the line 2.5 cm away from each other. The plate was developed in a solvent system in a chromatographic tank for about 50 min and Rf value was calculated.

2.8 Determination of the Chemical Properties and Functional Groups of *Nymphaea lotus* root Extract

The chemical properties and functional groups of extract of *N. lotus* root was determined using fourier transform infrared spectroscopy analysis (FTIR) as described by [14]. A FT-IR spectrometer (Infrared spectrometer Varian 660 MidIR Dual MCT/DTGS Bundle with ATR) was used to confirm the chemical structure of all samples. Before analysis, the samples were dried in an auto- desiccator for 24 hours. Samples were directly applied to a diamante crystal of ATR and resulting spectra of them were corrected for background air absorbance. Potassium bromide (KBr) disks were prepared from powdered samples mixed with dry KBr in the ratio of 1:100. The spectra were recorded in a transmittance mode from 4000 to 500/400 cm^{-1} at a resolution of 4 cm. Infrared spectrum was Fourier transformed and recorded in the

absorption mode. The refractogram obtained from FT-IR spectroscopy between wave number and absorption is tabulated below. IR solution software is employed for getting the spectrum. The region of IR radiation helps to identify the functional groups of the active components present in extract based on the peaks values of the FTIR spectrum [14]. When the extract was passed into the FTIR, the functional groups of the components were separated based on its peaks ratio.

2.9 Determination of Bioactive Compounds of *Nymphaea lotus* Roots Extract

The chemical structures of extract of *N. lotus* root bioactive compounds were determined using Gas Chromatography - mass spectrometry (GC – MS) as described by [15]. Analysis was done using a Infrared spectrometer Varian 660 MidIR Dual MCT/DTGS Bundle with ATR The column temperature was initially maintained at 200°C for 2 min, increased to 300°C at 4°C/min, and maintained for 20 min at 300°C. The carrier gas was Nitrogen at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300°C with a split ratio of 50:1. A sample volume of 1µL in chloroform was injected using a split mode, with the split ratio of 50:1. The mass spectrometer was set to scan in the range of m/z 1-1000 with electron impact (EI) mode of ionization, runtime were 40 minutes. Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC – MS compounds present in the samples were identified. Samples of ethanol extract of *N. lotus* root and replicates were continuously injected as one batch in random order to discriminate technical from biological variations. Additionally, the prepared pooled samples were used as quality controls (QCs), which were injected at regular intervals throughout the analytical run to provide a set of data from which the repeatability can be assessed.

2.10 Sensitivity Pattern of Bacterial Isolates to Partially Purified Extracts of *Nymphaea lotus* Root

Each of the fractions obtained from partial purification of *N. lotus* root extract was screened for antimicrobial activity on selected isolated enteric bacteria by performing disc diffusion method as described by [11]. *N. lotus* root extract fractions were being reconstituted using 30% v/v Dimethyl sulfoxide and sterilized (by filtration) using sterile injection filters of 0.22 µm pore size. *N. lotus* root extract fractions to be screened were reconstituted to concentration of 50 mg/ml respectively. A disc shaped sterile paper was soaked overnight inside the various plants extracts fractions respectively. The discs were placed on the agar wells in each of the test bacterial seeded agar aseptically. The negative control for the experiment was 30% aqueous DMSO while ciprofloxacin (0.63 mg/ml) was used as the positive control. All the plates were incubated at 37°C for 24 hours after which the zones of inhibitions were measured and compared with CLSI chart [16].

3. RESULTS

After the partial purification of *N. lotus* Root extract, *N. lotus* root extract had 7 fractions. The Thin layer Chromatogram of ethanol extract of *N. lotus* root is represented in Plate 1. Retention factor of the ethanol extract of *N. lotus* Root is 0.87.

3.1 Determination of the Chemical Properties and Functional Groups of *Nymphaea lotus* root Extract

The peaks and the functional groups of fourier transform infrared spectroscopy analysis of *N. lotus* root extract after partial purification is represented in Table 1 and Fig. 1. Purified extract of *N. lotus* root had thirty functional groups (Fluoro compound, Aliphatic primary amine, Halo compound, anhydride, Vinyl ether, alcohol, Cabocylic acid, Cyclic alkene, Unsaturated ketone, aldehyde, alkyne, Carbon dioxide, Aliphatic primary amine, aldehyde, alkane, Benzene derivative, Secondary alcohol, sulphide, alkene, Akyl aryl ether, Sulfonyl chloride, Aromatic compound, δ-lactone, Imine/oxime, alkane, alcohol, Thiocyanate, Amine salt, esters, alkene, Nitro compound, Sulphate, Sulphone, Akyl aryl ether, Aromatic ester, Fluoro compound, Amine salt, Sulphonyl chloride, Tertiary alchohol, α,β-unsaturated ketone, alkyne, allene, Sulfonic acid, α,β-unsaturated ester, Alphatic primary amine, amine, Sulfonyl chloride, Vinyl ether, Aromatic amine, Alphatic ketone, isothiocyanate, thiocynate, conjugated alkene and anhydride).

3.2 Determination of the Chemical Structures and Bioactive Compounds of *Nymphaea lotus* Root

The gas chromatography-mass spectrophotometry of partially purified extract of *N. lotus* root

revealed the presence of compounds with antimicrobial activity Table 2 and Fig. 2. Partially purified extract of *N. lotus* root had eighteen peaks and eighteen bioactive compounds (5-Nonanol, 2-Pentanone, 4-hydroxy-4-methyl-, Azulene, Tetradecanoic acid, 2-Chloroethyl vinyl sulfide, Decanoic acid, ethyl ester, Phytol, 1-Hexadecyne, 2-octenoic acid, Methyl-n-hexadecyl ketone, Hexadecanoic acid, methyl ester, and n-Hexadecanoic acid).

3.3 Sensitivity Pattern of Bacterial Isolates to Partially Purified Extracts of *Nymphaea lotus* Root

The sensitivity patterns of the fractions of *N. lotus* root extract Fig. 3. Seven fractions of partially purified extract of *N. lotus* Root were tested for antimicrobial susceptibility in which five fractions of purified extract of *N. lotus* Root (fraction 3-7) had significant zone of inhibition while fraction 1-3 had no significant zone of inhibition. Fraction four had the highest zone of inhibition 23.67 ± 0.88 mm while fraction seven had the least zone of inhibition 2.67 ± 1.33 mm.

Data were obtained in triplicate and expressed as mean ± Standard Error of Mean and were statistically analysed using One Way Analysis of Variance (ANOVA) .The new Duncan Multiple Range test was used to separate and compare means of different groups. A *P*-value of < 0.05 was considered statistically significant.

Plate 1. Chromatogram of extract of *Nymphaea lotus* root

Table 1. FTIR Spectral peak values and functional groups obtained from partially purified extract of *Nymphaea lotus* Root

Run #	Peak (cm^{-1})	Functional group	Interpretation
1	3940.82	O-H Stretching vibration (Non bonded)	Alcohol
2	3880.76	O-H Stretching vibration (Non bonded)	Unidentified
3	3767.11	O-H Stretching vibration (Non bonded)	Unidentified
4	3572.40	N-H Stretching vibration	Unidentified
5	3317.58	N-H stretching	Secondary amine
6	3248.13	N-H bending vibration	Unidentified
7	3056.31	C-H Stretching	Alkene
8	2924.11	C-H Stretching	Unidentified
9	2853.81	O–H stretch, H–bonded	Unidentified
10	2661.77	O–H stretching	Carboxylic acid
11	2569.59	O-H stretching	Unidentified
12	2422.18	C-O Stretching, O-H	Unidentified
13	2350.93	O=C=O Stretching vibration	Carbondioxide
14	2175.00	S=C≡N stretching	Thiocyanate
15	2057.02	N=C=S stretching	Isothiocyanate
16	1979.10	C-O Stretching	Unidentified
17	1936.33	C=C=C Stretching	Allene
18	1851.68	Aromatic C-H bending	Aromatic compound
19	1713.31	C=O, C-Hstretching	Unidentified
20	1657.24	C=N stretching	Imine/oxime
21	1442.70	C-H bending, O–H stretching	Unidentified
22	1382.17	C-H bending	Alkane
23	1327.03	C-F, N-O , O-H, C–N stretching	Aromatic amine
24	1265.50	C-F stretching	Fluoro compound
25	1196.52	C-O Stretching	Ester
26	1088.41	C-O stretching	Unidentified
27	1050.72	C–O S=O Stretching	Unidentified
28	879.54	=C–H bend	Unidentified
29	802.59	para directing benzene ring	Unidentified
30	709.84	C=C	Alkene

4. DISCUSSION

Partial purification of extract of *N. lotus* root reveals ethanol extract of *N. lotus* root had 7 fractions. Thin layer chromatogram of ethanol extract of *N. lotus* root shows distance moved by solvent for the extract was 15.1 cm, while the distance moved by ethanol extract of *N. lotus* root was 13.1cm. The retention factor for ethanol extract of *N. lotus* root was calculated at 0.87.

Purified ethanol extract of *N. lotus* root had thirty functional groups shown in Table 1. This is also in accordance with the findings of [17] in which they reported the presence of functional groups in aqueous extract of *N. lotus* leaves.

The gas chromatography-mass spectrophotometry of partially purified ethanol extract of *N. lotus* root reveals that ethanol extract of *N. lotus* root had eighteen peaks and eighteen bioactive compounds (5-Nonanol, 2-Pentanone, 4-hydroxy-4-methyl-, Azulene, Tetradecanoic acid, 2-Chloroethyl vinyl sulfide, Decanoic acid, ethyl ester, Phytol, 1-Hexadecyne, 2-octenoic acid, Methyl-n-hexadecyl ketone, Hexadecanoic acid, methyl ester, and n-Hexadecanoic acid) which can serve in production of antimicrobial agents. This is related to the findings of [18] in which they reported the presence of bioactive compounds such as methyl and ethyl fatty acid ester compounds, hydrocarbons, oxygenated hydrocarbons and steroidal compounds in ethanolic extract from the root and rhizome and ethanolic flower extract of *N. pubescens*.

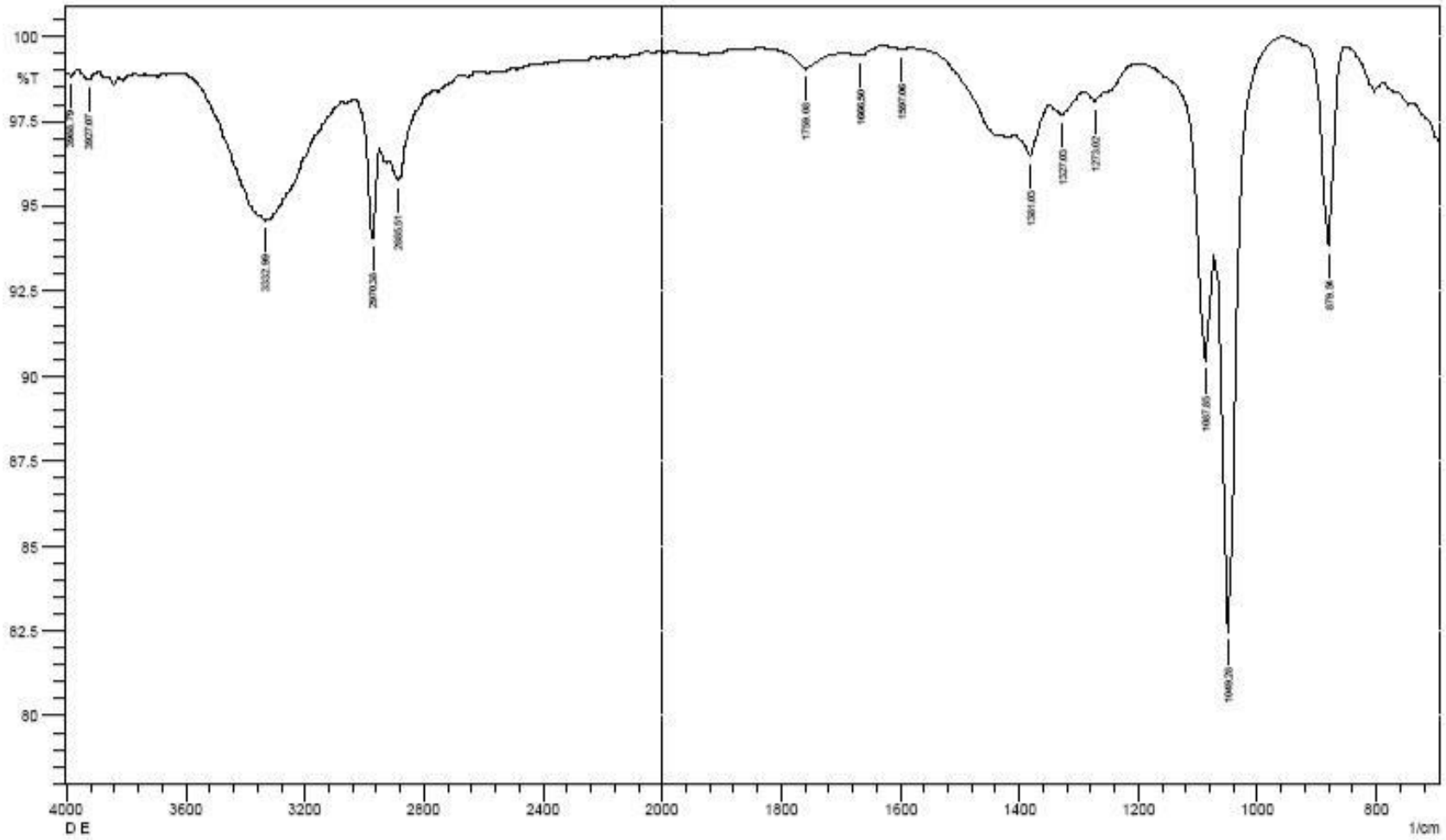
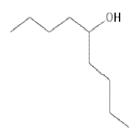
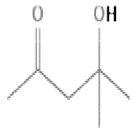
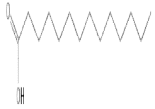
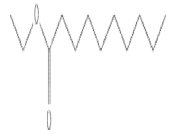
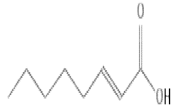
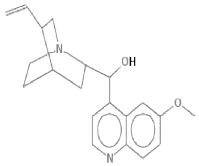
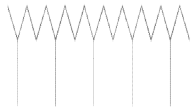
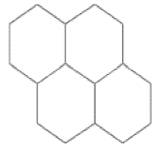


Fig. 1. Fourier Transform Infrared Spectrophotometer (FTIR) spectra of partially purified extract of *Nymphaea lotus* root

Table 2. Gas Chromatography-mass spectrophotometry compounds present in the partially purified extract of *Nymphaea lotus* Root

Peak #	Retention Time	Compound Detected	Mol. Formula.	Mol. Wt.	Peak Area (%)	% Composition	m/z	Structures
1	11.62	5-Nonanol	$C_9H_{20}O$	186	3.26	6.13	26, 52, 53	
2	13.85	2-Pentanone, 4-hydroxy-4-methyl-	$C_6H_{12}O_2$	116	3.26	4.21	43, 59, 116	
3	14.87	Azulene	$C_{10}H_8$	128	3.42	3.08	51, 102, 128	
4	15.83	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	8.45	8.04o	60, 73, 228	
5	16.31	2-Chloroethyl vinyl sulfide	C_4H_7ClS	122	6.56	4.99	45, 73, 122	

Peak #	Retention Time	Compound Detected	Mol. Formula.	Mol. Wt.	Peak Area (%)	% Composition	m/z	Structures
6	16.65	Decanoic acid, ethyl ester	$C_{12}H_{24}O_2$	200	7.10	5.20	88, 109, 200	
7	17.00	Phytol	$C_{20}H_{40}O$	296	4.60	8.52	71, 123, 296	
8	17.13	1-Hexadecyne	$C_{16}H_{30}$	222	9.17	6.73	55, 71, 280	
9	17.73	2-octenoic acid, Methyl-n-hexadecyl ketone, Hexadecanoic acid, methyl ester, n-Hexadecanoic acid,	$C_8H_{14}O_2$	142	8.89	4549	41, 73, 142	
10	18.82	Methyl-n-hexadecyl ketone	$C_{18}H_{36}O$	268	7.18	7.81	58, 71, 268	
11	19.04	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	4.67	6.38	74, 87, 270	

Peak #	Retention Time	Compound Detected	Mol. Formula.	Mol. Wt.	Peak Area (%)	% Composition	m/z	Structures
12	20.55	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	4.47	7.45	43, 73, 256	
13	21.45	Quinine	$C_{20}H_{24}N_2O_2$	324	8.66	5.36	56, 81, 324	
14	22.47	Tetracosane	$C_{24}H_{50}$	338	4.91	4.15	57, 71, 338	
15	23.25	Eicosane, 2,6,10,14,18-pentamethyl	$C_{25}H_{52}$	352	4.30	6.39	57, 99, 352	
16	24.73	Pyrene, hexadecahydro-	$C_{18}H_{36}O$	218	4.32	2.80	43, 58, 218	

Peak #	Retention Time	Compound Detected	Mol. Formula.	Mol. Wt.	Peak Area (%)	% Composition	m/z	Structures
17	28.14	1,14-Tetradecanediol	$C_{14}H_{30}O_2$	230	4.13	2.13	55, 82, 230	
18	32.66	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	2.66	3.89	43, 60, 242	

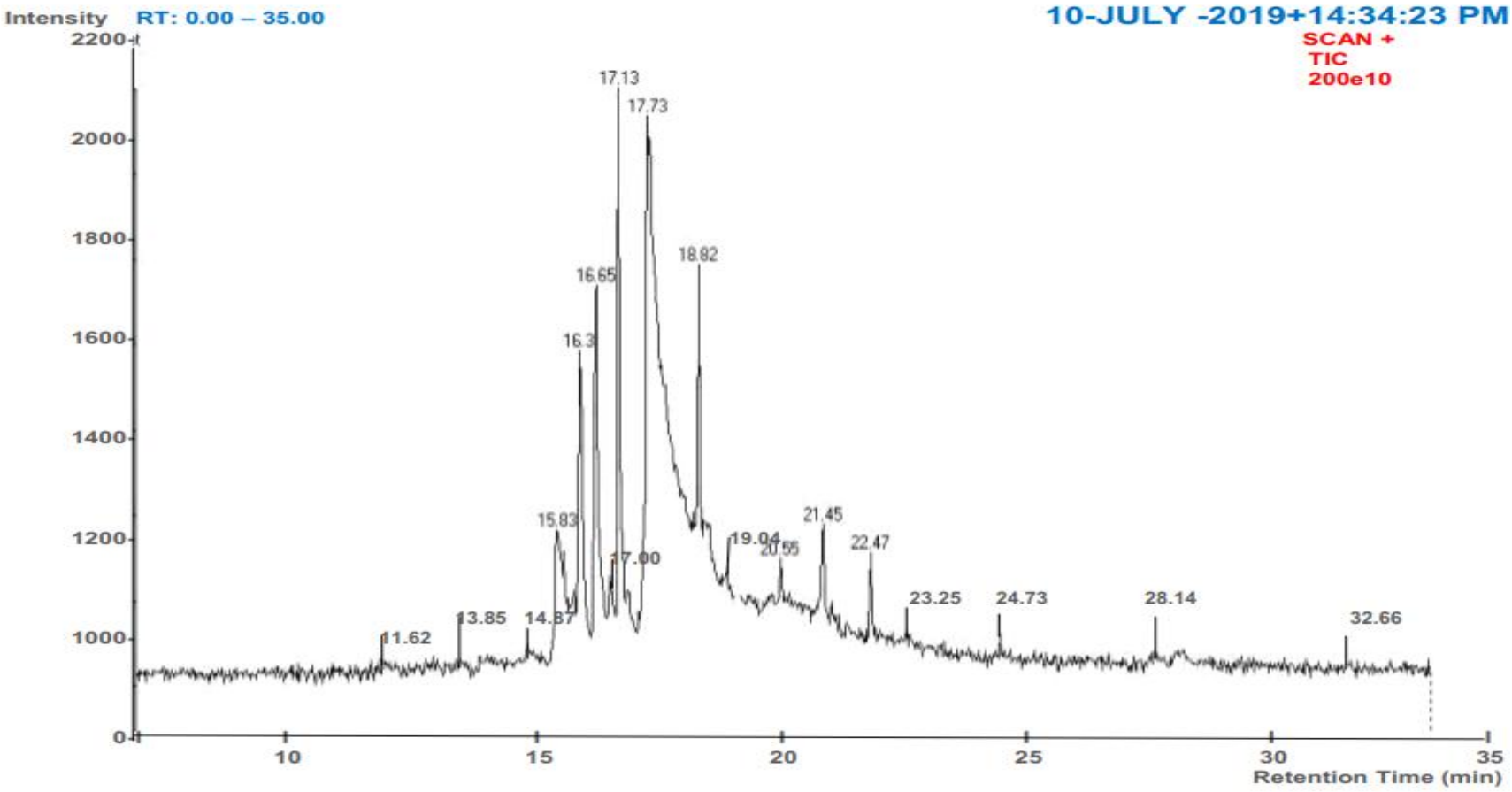


Fig. 2. Gas Chromatography-Mass Spectrophotometry Spectra of partially purified extract of *Nymphaea lotus* root

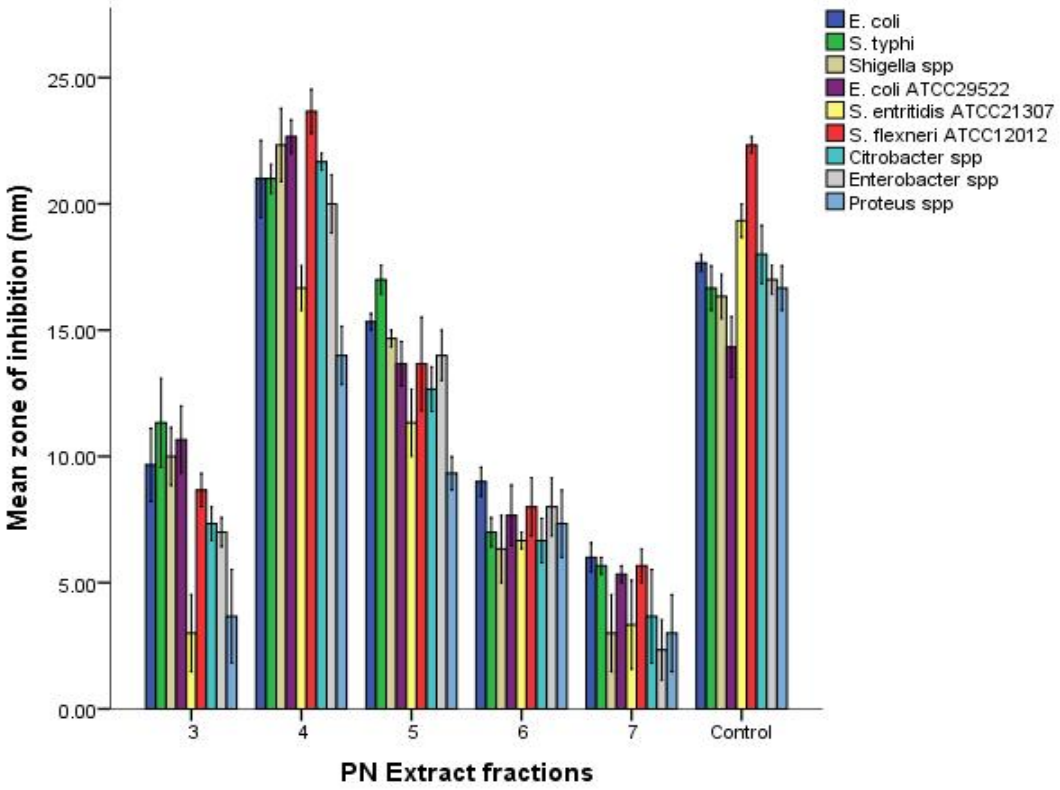


Fig. 3. Sensitivity pattern of bacterial isolates to the fractions of partially purified extract of *Nymphaea lotus* root

The sensitivity patterns of the purified fractions of ethanol extract of *N. lotus* root shows that fractions of the purified extract were able to inhibit most of the enteric bacteria isolates. Fraction five had the highest zone of inhibition 23.67 ± 0.88 mm while fraction seven had the least zone of inhibition 2.67 ± 1.33 mm. This is in accordance with [19] findings which showed that *S. aureus, S. pyogenes* and *E. coli* isolated from wounds were highly susceptible to *N. lotus* with the zone of inhibition ranging from 8 to 25 mm while *K. pneumoniae* and *P. aureginosa* were moderately susceptible to this antimicrobial substance with the zone of inhibition ranging from 8 to 15 mm.

This is also in accordance with [20] findings which confirmed that fungi derived from Nymphaea specie, *Eupenicillium levitum* FNL036 (FNL036CH) gave the strongest antifungal activity against *Cryptococcus neoformans* and *Talaromyces marneffei* with minimum inhibitory concentration of 0.5 µg/mL and affected cell morphology of the test microorganisms.

5. CONCLUSION

The findings of the present study demonstrated that *N. lotus* (ethanolic root extract) possessed several bioactive compounds. This compounds includes 5-Nonanol, 2-Pentanone, 4-hydroxy-4-methyl-, Azulene, Tetradecanoic acid, 2-Chloroethyl vinyl sulfide, Decanoic acid, ethyl ester, Phytol, 1-Hexadecyne, 2-octenoic acid, Methyl-n-hexadecyl ketone, Hexadecanoic acid, methyl ester, and n-Hexadecanoic acid which had antimicrobial effect against multidrug resistant enteric bacteria. The findings from this study suggests Ethanol extract of *N. lotus* root has bioactive compounds that can be useful in production of antimicrobials against multidrug resistance enteric bacteria.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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