Biochemical Characterization andMolecular identification of *Escherichi coli* Isolatefrom abattoir wastes and its susceptibility to ethanolic root extract of *Azadirachtaindica*(neem)

ABSTRACT

Aim: The study evaluates the molecular identification of *Escherichi coli* isolate from abattoir wastes and its susceptibility to ethanolic root extract of *Azadirachtaindica* (neem).

Methodology: The isolation, cultural characterization and identification of *Escherichi coli* isolate using biochemical methods and molecular identification (polymerase chain reaction) were carried out using standard methods.Gas Chromatography-Mass Spectrometry (GC-MS) and phytochemical analysis of the ethanolic root extract of *Azadirachtaindica* (neem) were determined using standard methods. Antibacterial activities were determined by disc diffusion and agar well diffusion methods. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) were determined using standard procedures.

Results: The result of the phytochemical screening shows that the plant contains phenolic compounds, flavonoids, carbohydrate, alkaloids, saponins, tannin and cardiac glycosides. forty compounds were identified in the root of *A.indica*using GC-MS analysis with 9-Octadecenoic acid, (E)-being the most abundant with peak area of 28.13% and retention time of 22.044.The organism was confirmed to be *Escherichia coli* using polymerase chain reaction with specific primer pairs and later resolved in an agarose gel electrophoresis for 16S rRNA gene with band size of 253 base pair. The organism was susceptible to gentamycin,amoxicillin, pefloxacin, sparfloxacinand resistant to tarividwith weak response. The study shows that ethanolic root extract of *A.indica* exhibited strong response antimicrobial activity against *Escherichia coli* with zone of inhibition of19.48±0.23 and21.36±0.54 at concentration of 250 and 500 mg/dl respectively. At 100 mg/ml, the extract showed littleornoresponse with zoneof inhibition of 9.33±0.33.Azithromycin solution at 12.50 and 25.00 mg/ml showed strong responseand potent response against *E. coli* with zoneof inhibition of 23.37±0.48 and 37.34±0.65 respectively. At 5.00 mg/ml, azithromycin antibiotic exhibited weak response against *E. coli* with zone of inhibition of 15.37 ± 0.33. Theethanolic root extract of *A.indica*andazithromycin solution have MIC and MBC values of 31.25, 15.63, 62.50 and 31.25mg/ml respectively against*E. coli.*The ethanolic root extract of *A.* indica was bactericidal with MBC/MIC ratio of 2.00. The plant has

antimicrobial activity against *Escherichia coli* and may prevent pathogenic diseases caused by the organism.

Conclusions: The root of*Azadirachtaindica* (neem)containsphytochemicals, possessesantibacterial activity and may prevent pathogenic diseases caused by *Escherichia coli.*

Keywords: Antimicrobial activity,*Escherichia coli.* GC-MS analysis, root of*Azadirachtaindica,*

INTRODUCTION

Natural plant products have shown an important rolein diseases prevention and treatment through inhibition of bacterial growth, improvement of antioxidant activities in the body and modulation of genetic pathways. Studies have shown that pharmacologically active drugs are derived from natural products like medicinal plants [1, 2].

Azadirachtaindica(neem) tree belongs to the family Meliaceae which is found in abundance in tropical and semitropical regions of the world in countries like Nigeria, India, Nepal, Bangladesh and Pakistan. It is a fast-growing tree with 20–23m tall and the trunk is straight and has a diameter around 4-5 ft. The leaves are compound, imparipinnate, with each comprising of 5–15 leaflets. Neems fruits are green drupes which turn golden yellow on ripening. Taxonomic classification of *Azadirachtaindica*(neem) include: Order; Rutales, Suborder; Rutinae, Family;Meliaceae, Subfamily; Melioideae, Tribe; Melieae, Genus;*Azadirachta,* and Species *indica*[3].Numerous studies have shown different biological and pharmacological activities of *Azadirachtaindica*whichinclude antimalarial, anti-inflammatory, antiarthritic, antioxidant and free radical scavenging activities, antipyretic, hypoglycemic, antibacterial, antigastric ulcer, antifungal and antitumour activities [4-10].

Escherichia coli (*E. coli*) belongs to the Family Enterobacteriaceaeand was first isolated and characterized in 1885by the German scientist and pediatricianTheodore Escherich [11].*Escherichia coli* is a Gramnegative,rod-shaped bacterium that is commonlyfound in the lower intestine of warm-bloodedanimals, The bacterium is a prokaryotic living organism withouta nuclear membrane, it replicate rapidly andinhabits the intestinal trackof animals and humans [11]. Most *Escherichia coli*are harmless;some are pathogenic, causing bothsevere intestinal and extra intestinal diseases in human [12]. The organism is associated with high rates of mortality and morbidity in animals [13]. Several pathogenic *E. coli* are characterized by different genes associated with their virulence. Many enteric infections caused by *E. coli* are transmitted by inter-human contactssuch as those caused by Enteroaggregative*E. coli* (EAggEC), Entero-invasive *E. coli* (EIEC), or Enteropathogenic*E. coli* (EPEC) [14, 15]. Shiga toxin-producing *E. coli* (STEC) or Enterotoxigenic*E. coli* (ETEC) are primarily transmitted to humans through the consumption of contaminated food and water [16, 17]. Shiga toxin-producing *E. coli* cause a wide range of human diseases, including mild-tosevere diarrhoea to haemorrhagic colitis (HC) and the life-threatening haemolytic uremic syndrome (HUS) [17] and are characterized by the production of potent cytotoxins. STEC are food-borne pathogens [17-19] and are the onlydiarrheagenic*E. coli* pathogroup with an ascertained zoonotic origin, with ruminants being regarded as the main animal reservoir [20, 21].The study evaluates the Gas chromatography–mass spectrometry (GC-MS),phytochemical analysis and antibacterial activity of ethanolic root

extract of *Azadirachtaindica* against *Escherichia coli*isolate from abattoircentre in Ikorodu, Lagos, Nigeria.

METHODOLOGY

Collection and Identification of *Azadirachtaindica*root

The *Azadirachtaindica*rootwas obtained from Lagos State University of Science and Technology Ikorodu in Lagos State, Nigeria. Theplant was identified using PictureThis plant identifier.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of *Azadirachtaindica*root

GC-MS analysis of the *Azadirachtaindica*root was carried out on an Agilent technology 7890 GC system equipped with a mass spectrometric detector (MSD) as described by Momoh et al. [22].

Detection of components

Analysis of mass spectrum GC-MS was conducted by the database of the National Institute Standard and Technique (NIST) which contained more than 62,000 patterns. The spectrum of the unidentified compound was compared with the spectrum of the identified compounds stored in the National Institute Standard and Technique library. The names, molecular weight, structure of the compounds in the test material were ascertained.

Preparation of ethanolic root extract of *Azadirachtaindica*

The root of *Azadirachtaindica*was washed thoroughly with distilled water to remove dirt's, it was air dried under shade in the Biochemistry Laboratory, pulverized into coarse power using industrial machine. Extraction was carried out by dispersing 120g of the grounded *Azadirachtaindica*plant material into one litre of 80% ethanol at room temperature. Shaking and maceration was done for 72 hours. The solution was filtered by passing it through cotton wool followed by Whatman filter paper and the resultant filtrate was concentrated using rotary evaporator at a temperature not exceeding 40°C. The concentrated root extract of *Azadirachtaindica*was dried to complete dryness in an aerated oven at 40°C for 48 hours. The root extract of *Azadirachtaindica*was later stored in a refrigerator at 4°C.

Calculation of Percentage Yield

The percentage extractive yield of theethanolic root extract of *Azadirachtaindica*was calculated using the formula below.

$$\% \text{ extraction yield (w/w)} = \frac{\text{weight of extraction recovered}}{\text{initial weight of powder of } A.\ indica} \times 100.$$

Phytochemical analysis

The presence of alkaloids, flavonoids, saponins, tannins, cardiac glycosides and carbohydrate were determined by qualitative methods [23-25]. The simple qualitative analyses of the ethanolic root extract of *Azadirachtaindica*were based on the intensity of the colour change.

Bacterial isolation

Faecal samples were collected from abattoirs centre in Ikorodu, Lagos, Nigeria. MacConkey agar were inoculated by the collected faecal using swabs stick and incubated at 37°C for 24 hours. The organism ferments lactose to produce pink colonies on the MacConkey agar. The representative colonies from the MacConkey agar were transferred to Eosin Methylene Blue agar and incubated at 37°C for 24 hours to observe the cultural characteristics [26, 27]. The colonies were also transferred to sorbitol MacConkey agar to check if they can ferment sorbitol. The various colonies observed in the plates were distinguished on the basis of their cultural characteristics.

Biochemical tests

A lope full was taken from growing colony, inoculated into 10 ml nutrient broth then incubated at 37°C for 24 hours. Biochemical test was carried out to identify the presence of *E. coli*, these test include: microscopy of gram stained, methyl red test, indol production,carbohydrate fermentation,citrate utilization, β-galactosidase activity, voges-proskaur test, oxidase, urease activity, ornithine decarboxylase,catalase production, lysine, ornithine decarboxylase and motility were carried out using standard methods described by [28-32].

Genomic DNA extraction

The spin column based DNA purification kit was formulated for the rapid isolation of genomic DNA for bacteria cells. The kit yields up to 30µg of DNA from the bacteria cells, and the extracted DNA was identified to be *E. coli*. The kit used was Jena Bioscience DNA extraction kit.

Procedure for DNA isolation

Eight ml of absolute ethanol was added to each bottle of wash buffer 1 concentrate and 32ml of absolute ethanol was added to each bottle of wash buffer 2 concentrate.100µl of *Escherichia coli* was added into a microcentrifuge tube,500µl of the lysis buffer was also added then vortex and

incubated at 56ºC for 10minute. The specimen was centrifuged at 10,000 rpm for a minute, 200 µl of absolute ethanol was added to the tube after spinning and was then transferred into the spin column for centrifugation at 10,000 rpm for 30 seconds. The flow-through was discarded and the collection tube was blotted on a tissue paper, 500 µl of wash buffer 1 was added to the spin column and centrifuged at 10,000 rpm for 30 seconds then the flow-through was discarded and the collection tube was blotted on a tissue paper. 500 µl of wash buffer 2 was added to the spin column and centrifuged at 10,000 rpm for a minute and the flow-through was discarded and the collection tube was blotted on a tissue paper. The spin column was centrifuged again at 12,000-14,000 rpm for 3minutes and all traces of ethanol was removed then the spin column was placed into another microcentrifuge tube.50 µl Elution Buffer or nuclease-free water was added to the centre of the column. The eluate was incubated at room temperature for 2 minutes and was centrifuged at 10,000 rpm for a minute; the DNA was eluted and stored at - 20°C.

Polymerase Chain Reaction (PCR)amplification

Polymerase chain reaction was carried out to identify the bacteria using the primer pair. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pmol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), proofreading enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.Thermal cycling was conducted in an EppendorfVapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute as indicated in the primer (F-CAGTACAGGTAGACTTCTG and R-TGGGAAGCGAAAATCCTC), 1 minute 30 seconds at 72°C for the determination of *E.coli*16S RNA. This was followed by a final extension step of 10 minutes at 72°C. The annealing temperature used was 55°C.The amplification product was

separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining under Gel documentation system. 100bp DNA ladder was used as DNA molecular weight standard.

Inoculum preparation

A loopful of isolated colonies of the organism was inoculated into 4 ml of peptone water, incubated at 37°C for 4 hours. The actively growing bacterial suspensions were then adjusted with peptone water to obtain turbidity visually comparable to that of 0.5 McFarland standards using standard procedure [33, 34]. The 0.5 McFarland standard was prepared by mixing 0.5ml of 1.75% (w/v) barium chloride dehydrate ($BaCl_2$. $2H_2O$) with 99.5 ml of 1% (v/v) H_2SO_4. This turbidity was equivalent to approximately 1 x 10^8 colony forming units per ml (CFU/ml) [33, 34].

Antibiotic Susceptibility Test (Kirby-Bauer Disk Diffusion Method)

The antibiotic susceptibility test was carried out on the *E. coli* isolate sample using Kirby-Bauer disc diffusion technique as described by Aderele et al. [33] and Momoh et al.[34]. Pure culture of the organism was inoculated into sterile nutrient broth to a density of 9 × 10^8cfu/ml and incubated for 24 hours at 37 ^oC. The suspension was used to streak for confluent growth on the surface of Muller-Hinton Agar plates with sterile swab for the disc diffusion test (Colony forming units, cfu of bacteria used and Zone sizes are measured and interpreted using Clinical and Laboratory Standards Institute (CLSI) standards) [35] after 24 hours incubation, the susceptibility profile pattern was obtained. Results were interpreted according to Clinical and Laboratory Standards Institute guidelines [35] and method described by Momoh et al.[34]. Antibiotics disc used were tarivid (10µg/disk), pefloxacin (30µg/disk), gentamycin (10µg/disk), sparfloxacin (10µg/disk), and amoxacilin (30µg/disk). For each combination of the antibiotics and the *E.coli*strain, the experiment was performed in triplicate.

Determination of diameter of zone of inhibition using agar well diffusion method

Agar well-diffusion method was employed to determine the antimicrobial activity the ethanolic root extract of *Azadirachtaindica*. Eighteen hours of broth culture of the *E.coli*strain was suspended into the sterile nutrient broth. It was standardized by gradually adding 9% normal saline to compare its turbidity to McFarland standard of 0.5 which is approximately 1 x 10^8

colony forming units per ml. Petri-dishes were prepared by loading about 25 ml of an autoclaved nutrient agar on sterile plates and left to solidify. 100 µL of a standardized culture adjusted to 0.5 McFarland of the organism was added into the different agar plates, then, the surface of each plate was drilled using a sterile cork borer (6 mm) and 3 wells were punched out on each plate.100 µL of the ethanolic root extract of *Azadirachtaindica*was addedintoeach of the wells and allowed to diffuse at room temperature for 2 hours. The plates were incubated at 37ºC for 18-24 hours for bacterial pathogen. The diameters of the inhibition zone (mm) were measured. The susceptibility of the*Escherichia coli*to the ethanolic root extract of *Azadirachtaindica* was assayed using standard method described by Aderele et al.[33] and Momoh et al. [34]. The experiment was repeated thrice, for each replicate, the readings were taken in three different fixed directions and the average values were recorded [Aderele et al. [33] and Momoh et al. [34]. The inhibitoryresponses were classified aspotent response, ++++, zonediameter >30 mm; strong response, +++, zone diameter between 21-30 mm; moderate response, ++, zone diameter between 16-20mm; weak response, +, zone diameter between 10-15 mm; and littleornoresponse,zonediameter<10mm [34, 36].

Minimum inhibitory concentration (MIC) of the ethanolic root extract of *Azadirachtaindica*

Minimum inhibition concentration is the lowest concentration of the root extract of *Azadirachtaindica* that inhibited the growth of the test organism(*E.coli*) as indicated by the absence of visible turbidity in the tested tubes compared with the control tube. The MIC of the ethanolic root extract of *Azadirachtaindica*was determined using standard method [34]. The MIC of the ethanolic root extracts ofthe plant was assayed using serial dilution method. One ml of Mueller-Hinton broth was poured into sets of different test tubes and autoclaved. Subsequently, 1 ml of 100% ethanolic root extract of *Azadirachtaindica* (2g/ml) were poured to the first separate test tubes to make a concentration of 50%, and two-fold serial dilutions were made by transferring 1 ml from one tube to another to get the following series: 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39% etc. Then, an overnight broth culture of the organism was adjusted to McFarland turbidity standard and 100 µl of the cellsuspension of *E.coli* was added to each of the separate tubes. The tubes were incubated aerobically at 37°C for 18hours. Negative control tube was made by pouring 1ml of normal saline instead of the ethanolic root extracts of *Azadirachtaindica*. The lowest concentration of the dilution without bacterial growth was considered as the minimum inhibition concentration.

Minimum Bactericidal Concentration (MBC) of the ethanolic root extract of *Azadirachtaindica*

The MBC of the ethanolic root extract of *Azadirachtaindica* was carried out by standard method [34]. In this method, 0.1 ml aliquots of test samples taken from the non-turbid tubes of the minimum inhibition concentration assay test tubes were sub-cultured onto nutrient agar plates. The resulting plates were then incubated aerobically at 37°C for 24 hours. The lowest concentration of theethanolic root extract of *Azadirachtaindica* at which no colonies of the *Escherichia coli* was seenwas taken as the minimum bactericidal concentration. The results were compared with that of control tube using sterilized distilled water. The experiment was performed in triplicate. The MBC was taken as the concentration of the root extract that did not show any growth on a new set of agar plates. The lowest MIC value that revealed no visible growth was regarded as the minimum bactericidal concentration. The MBC/MIC values were also calculated as either bactericidal or bacteriostatic.

Statistical Analysis

All analyses were carried out in triplicate determination and results were expressed as mean ± SD. Student's -test was used for comparison. The data analysis was done using one way analysis of variance (ANOVA) Post Hoc Turkey Graph Pad prism computer software version 5.01. A P-value < 0.05 was considered significant.

RESULT

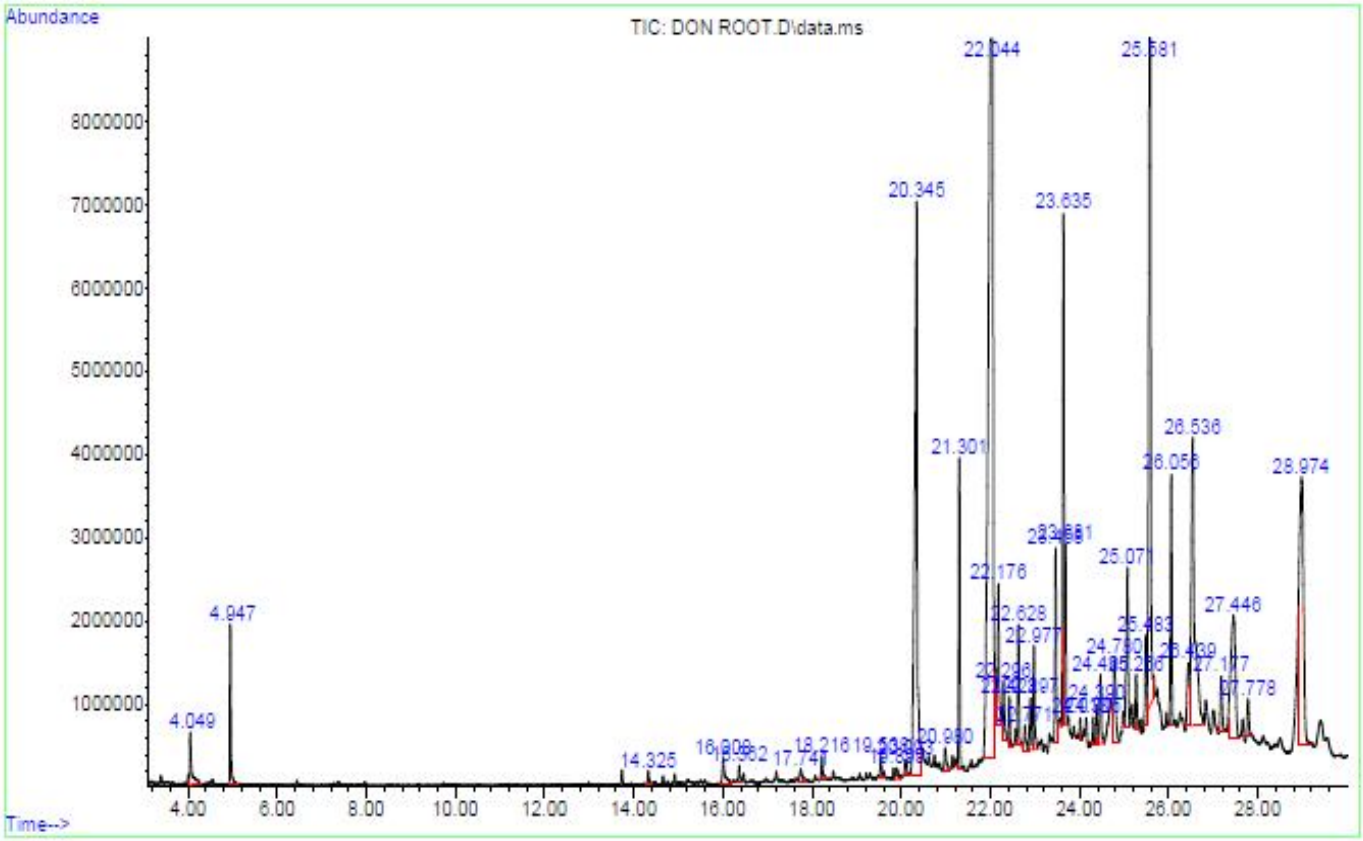
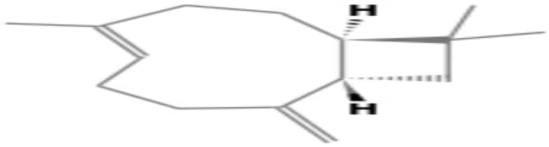
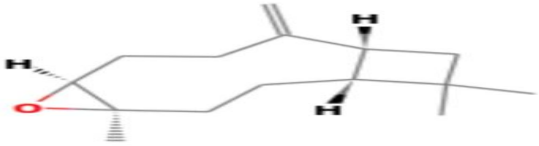
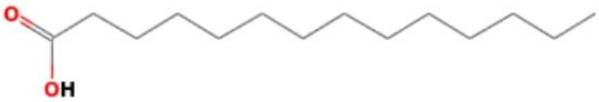
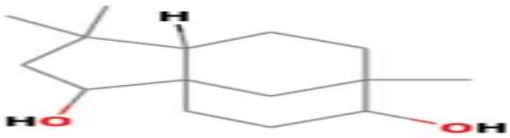
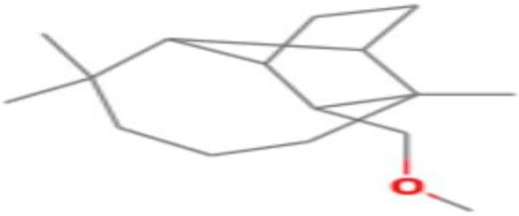
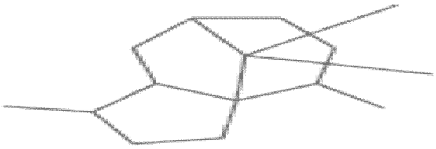


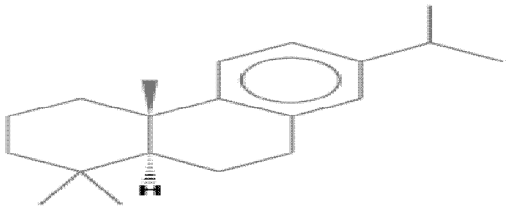
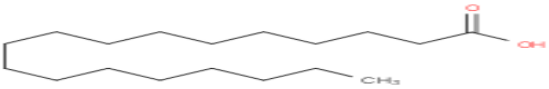
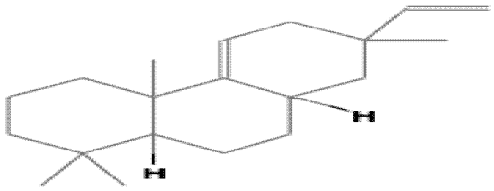
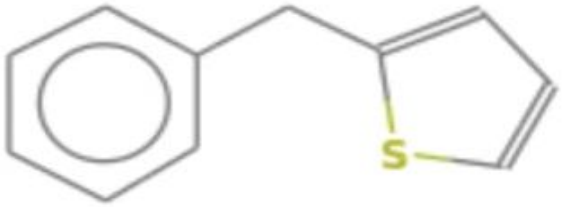
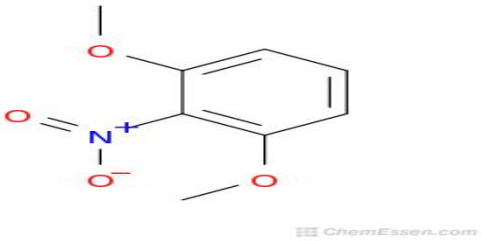
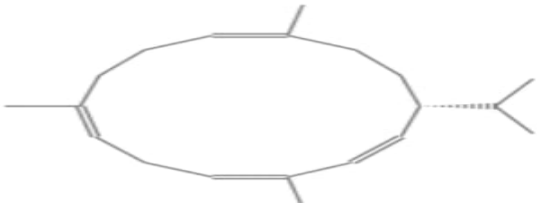
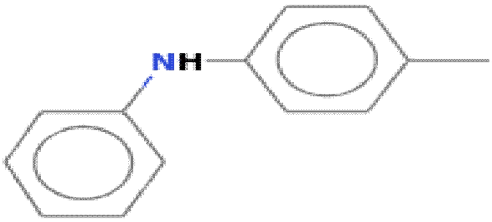
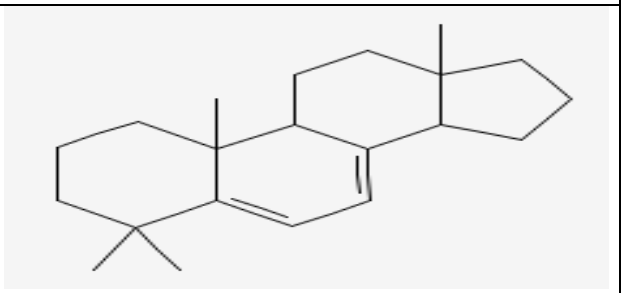
Figure 1: GC-MS Chromatogram of *Azadirachtaindica*root

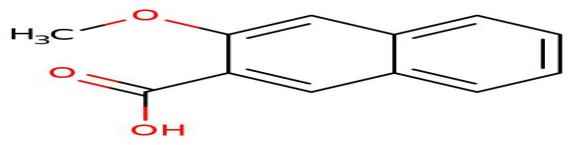
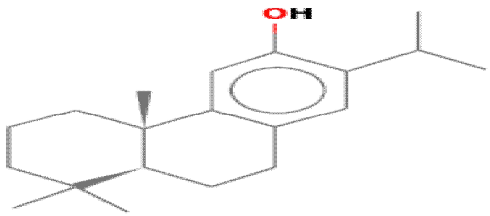
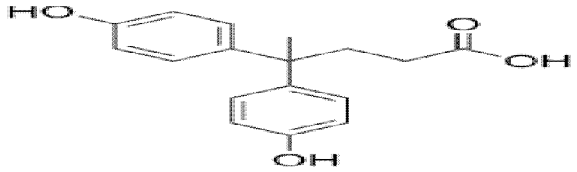
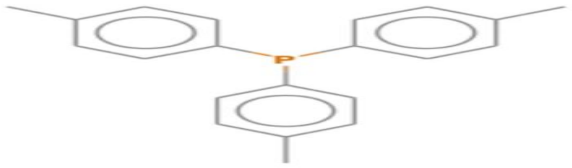
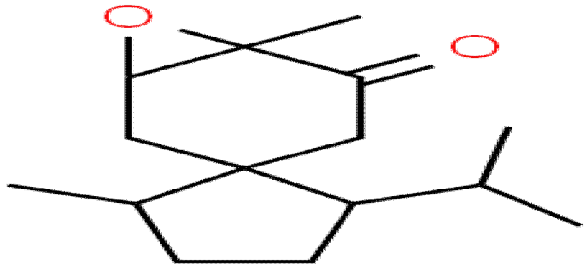
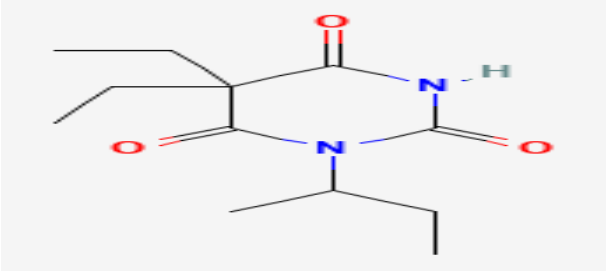
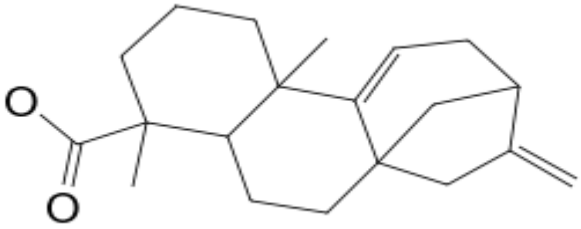
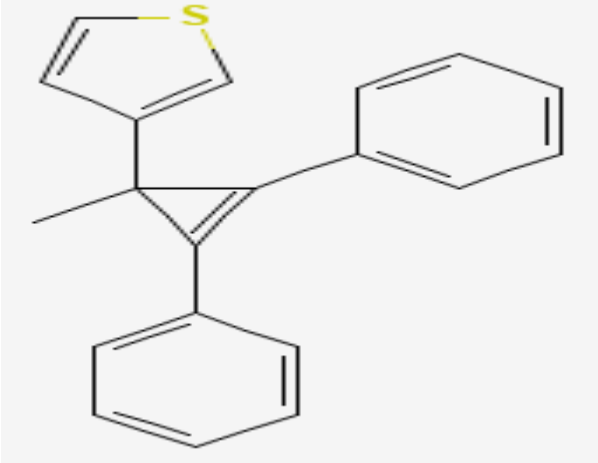
Table 1.Chemical composition of *Azadirachtaindica*root analyzed using Gas Chromatography–Mass Spectrometry

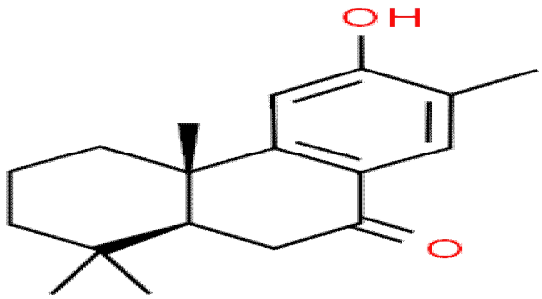
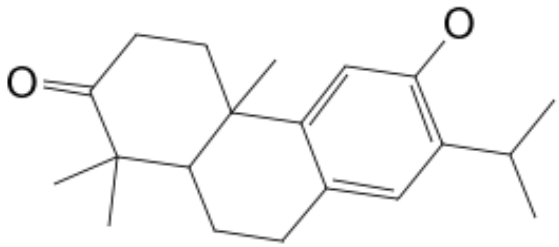
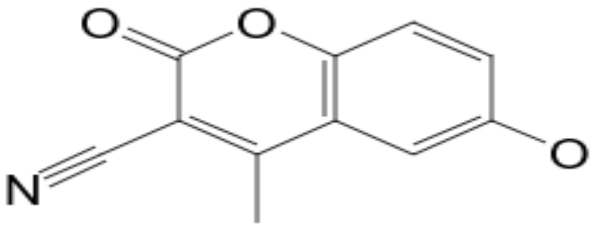
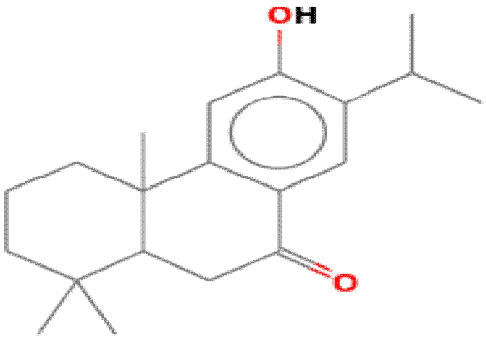
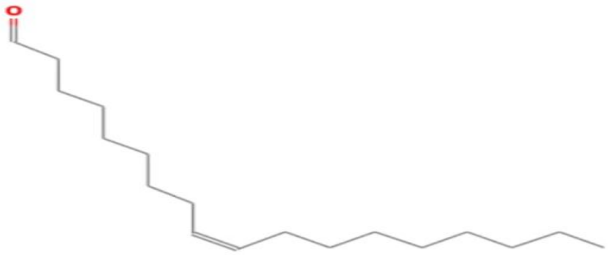
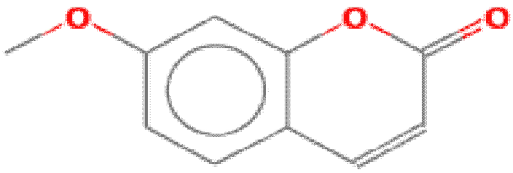
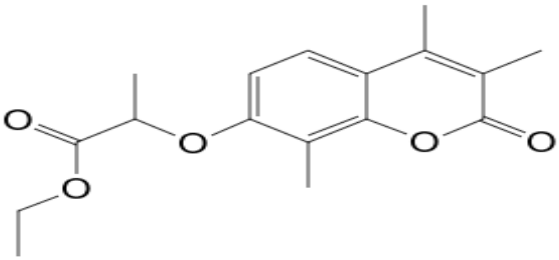
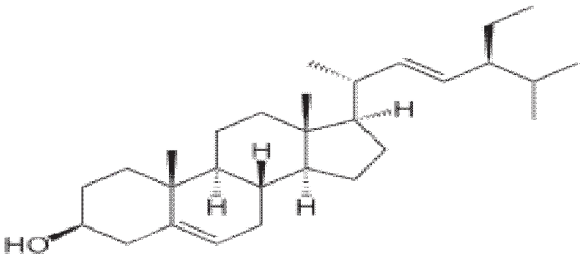
Pk#	RT	Peak Area (%)	Name of compound	Molecular Formulae	Molecular Weight (g/mol)	Ref#	CAS#	Qual.
1	4.049	0.66	3-Hexen-2-one	$C_6H_{10}O$	98.1430	3215	000763-93-9	90
2	4.947	1.30	2-Pentanone, 4-hydroxy-4-methyl-	$C_6H_{12}O_2$	116.1583	8340	000123-42-2	50
3	14.325	0.14	Caryophyllene	$C_{15}H_{24}$	204.3511	68509	000087-44-5	99
4	16.008	0.46	Dodecanoic acid	$C_{12}H_{24}O_2$	200.3178	64983	000143-07-7	99
5	16.362	0.18	Caryophyllene oxide	$C_{15}H_{24}O$	220.3505	83535	001139-30-6	92
6	17.741	0.19	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester	$C_{10}H_{12}O_4$	196.1999	61218	004707-47-5	97
7	18.216	0.28	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.3709	91415	000544-63-8	98
8	19.538	0.21	(3S,3aS,6R,7R,9aS)-1,1,7-Trimethyldecahydro-3a,7-methanocyclopenta[8]annulene-3,6-diol	$C_{15}H_{26}O_2$	238.3657	100431	002649-64-1	99
9	19.899	0.15	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	130817	000112-39-0	70
10	20.093	0.25	(-)-Isolongifolol, methyl ether	$C_{16}H_{28}O$	236.3929	98688	1000333-80-8	55
11	20.345	10.80	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241	117418	000057-10-3	99
12	20.980	0.31	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-	$C_{15}H_{26}$	206.3669	70783	025491-20-7	86
13	21.301	2.58	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	$C_{20}H_{30}$	270.4522	131010	019407-28-4	99
14	22.044	28.13	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	282.4614	142089	000112-79-8	99
15	22.176	1.44	Octadecanoic acid	$C_{18}H_{36}O_2$	284.4772	144272	000057-11-4	96
16	22.296	0.66	Benzene, 1,2,3,4-tetramethyl-	$C_{10}H_{14}$	134.2182	15205	000488-23-3	46
17	22.422	0.47	Isopimara-9(11),15-diene	$C_{20}H_{32}$	272.4681	133015	039702-28-8	86
18	22.628	1.02	Thiophene, 2-(phenylmethyl)-	$C_{11}H_{10}S$	174.262	42901	013132-15-5	25
19	22.771	0.30	Benzene, 1,3-dimethoxy-2-nitro-	$C_8H_9NO_4$	183.16136	50692	006665-97-0	35
20	22.897	0.61	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) -, [S-(E,Z,E,E)]-	$C_{20}H_{32}$	272.4681	133055	001898-13-1	90
21	22.977	0.99	Benzenamine, 4-methyl-N-phenyl-	$C_{13}H_{13}N$	183.2490	50514	000620-84-8	11

22	23.458	1.71	Androsta-5,7-diene, 4,4-dimethyl-	$C_{21}H_{32}$	284.5	144499	1000194-15-2	38
23	23.635	4.67	3-Methoxy-5-naphthoic acid	$C_{12}H_{10}O_3$	202.21	66567	007498-58-0	42
24	23.681	1.30	Ferruginol	$C_{20}H_{30}O$	286.4516	146477	000514-62-5	99
25	24.013	0.19	Diphenolic acid	$C_{17}H_{18}O_4$	286.327	146177	000126-00-1	72
26	24.305	0.20	Phosphine, tris(4-methylphenyl)-	$C_{21}H_{21}P$	304.3652	163776	001038-95-5	53
27	24.390	0.39	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	$C_{15}H_{24}O_2$	236.350	98548	061050-91-7	90
28	24.465	0.80	2,4,6(1H,3H,5H)-Pyrimidinetrione, 5,5-diethyl-1-(1-methylpropyl)-	$C_{12}H_{20}N_2O_3$	240.30	130213	1000363-29-9	49
29	24.780	1.21	Kaura-9(11),16-dien-18-oic acid,4.alpha.)-	$C_{20}H_{28}O_2$	300.44	160051	022338-67-6	51
30	24.780	1.21	3-Methyl-1,2-diphenyl-3-(3-thienyl)cyclopropene	$C_{20}H_{16}S$	288.4	148232	086738-99-0	25
31	25.266	0.55	Nimbiol	$C_{18}H_{24}O_2$	272.382	132901	000561-95-5	99
32	25.483	0.79	2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	$C_{20}H_{28}O_2$	300.4351	160072	000472-37-7	94
33	25.581	11.51	2H-1-benzopyran-3-carbonitrile, 6-hydroxy-4-methyl-2-oxo-	$C_{11}H_7NO_3$	201.18	65806	1000396-04-0	38
34	26.056	2.37	9(1H)-Phenanthrenone, 2,3,4,4a,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	$C_{20}H_{28}O_2$	300.4351	160074	000511-05-7	99
35	26.439	0.92	9-Octadecenal, (Z)-	$C_{18}H_{34}O$	266.4620	126829	002423-10-1	86
36	26.536	7.13	2H-1-Benzopyran-2-one, 7-(3-methyl-	$C_{10}H_8O_3$	176.1687	161711	006025-18-9	70
37	27.177	0.69	2-(3,4,8-Trimethyl-2-oxo-2H-chromen-7-yloxy)-propionic acid ethyl ester	304.34	$C_{17}H_{20}O_5$	163616	053604-44-7	46
38	27.446	4.17	Stigmasterol	$C_{29}H_{48}O$	412.7	244153	000083-48-7	99
39	27.778	0.45	Pyrene, 1,6-bis(1,1-dimethylethyl)	$C_{24}H_{26}$	314.4632	173585	055044-29-6	83
40	28.974	7.92	gamma.-Sitosterol	$C_{29}H_{50}O$	414.7067	245062	000083-47-6	99

 Structure of 3-Hexen-2-one	 **Structure of** 2-Pentanone, 4-hydroxy-4-methyl-
 **Structure of Caryophyllene**	 **Structure of Dodecanoic acid**
 Structure of Caryophyllene oxide	 **Structure of** Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester
 Structure of Tetradecanoic acid	 Structure of (3S,3aS,6R,7R,9aS)-1,1,7-Trimethyldecahydro-3a,7-methanocyclopenta[8]annulene-3,6-diol
 Structure of Hexadecanoic acid, methyl ester	 Structure of (-)-Isolongifolol, methyl ether
 Structure of n-Hexadecanoic acid	 Structure of 1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-

 **Structure of** Phenanthrene,1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	 Structure of 9-Octadecenoic acid, (E)-
 Structure of Octadecanoic acid	 Structure of Benzene, 1,2,3,4-tetramethyl-
 Structure of Isopimara-9(11),15-diene	 Structure of Thiophene, 2-(phenylmethyl)-
 Structure of Benzene, 1,3-dimethoxy-2-nitro-	 Structure of 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) -, [S-(E,Z,E,E)]-
 Structure of Benzenamine, 4-methyl-N-phenyl-	 Structure of Androsta-5,7-diene, 4,4-dimethyl-

 Structure of 3-Methoxy-5-naphthoic acid	 Structure of Ferruginol
 Structure of Diphenolic acid	 Structure ofPhosphine, tris(4-methylphenyl)-
 Structure ofSpiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	 Structure of2,4,6(1H,3H,5H)-Pyrimidinetrione, 5,5-diethyl-1-(1-methylpropyl)-
 Structure ofKaura-9(11),16-dien-18-oic acid,4.alpha.)-	 Structure of3-Methyl-1,2-diphenyl-3-(3-thienyl)cyclopropene

 Structure ofNimbiol	 Structure of2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-
 Structure of2H-1-benzopyran-3-carbonitrile, 6-hydroxy-4-methyl-2-oxo-	 Structure of9(1H)-Phenanthrenone, 2,3,4,4a,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-
 Structure of9-Octadecenal, (Z)-	 Structure of2H-1-Benzopyran-2-one, 7-(3-methyl-
 Structure of2-(3,4,8-Trimethyl-2-oxo-2H-	 Structure ofStigmasterol

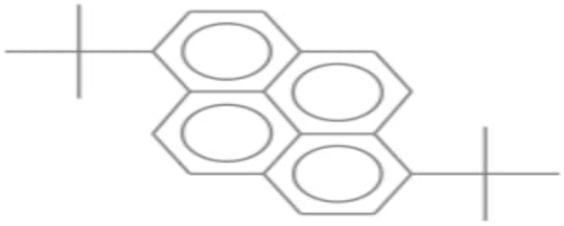
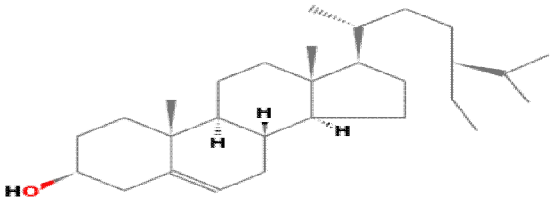
chromen-7-yloxy)-propionic acid ethyl ester	
 Structure ofPyrene, 1,6-bis(1,1-dimethylethyl)	 Structure ofgamma.-Sitosterol

Figure 2. Structure of 40 different compounds obtained during GC-MS analysis of ***Azadirachtaindica*root.**

Phytochemical analysis of ethanolic root extract of *Azadirachtaindica*

The Percentage extraction yield of the ethanolic root extract of *Azadirachtaindica*obtained in the study was 8.65 %. Phytochemical screening of the ethanolic root extract of *A.indica* shows the present of secondary metabolite like phenolic compounds, saponin, alkaloids, tannins, flavonoid, carbohydrate and cardiac glycoside (Table 2).

Table 2: The qualitative phytochemical constituents of the ethanolicroot extract of *Azadirachtaindica.*

Phytochemical constituent	Inference
Alkaloids	+ +
Tannins	+
Saponins	+
Phenolic compounds	+
Flavonoids	+
Carbohydrate	+
Cardiac glycoside	+

+ indicate present

Confirmation test for *Escherichia coli*

E. coli was isolated from fecal samples after 24 hours incubation. The growing bacterialcolonies appeared as round small, bright pink on MacConkey agar, while on Eosin Methylene Blue agar they appeared as small round colonies with green metallic sheen(Figure 3). The organism is gram negative bacterium, the result of the biochemical tests used in the identification of the organism shows that the *E. coli* gave a positive *test*to beta galactosidase, lysine decarboxylase, indol production and motility and a negative test to Citrate utilization, Urease, Hydrogen sulphide test and Voges-proskaur test. The organism was confirmed to be *Escherichia coli* using

polymerase chain reaction with specific primer pairs and later resolved in an agarose gel electrophoresis for 16S rRNA gene with band size of 253 base pair.

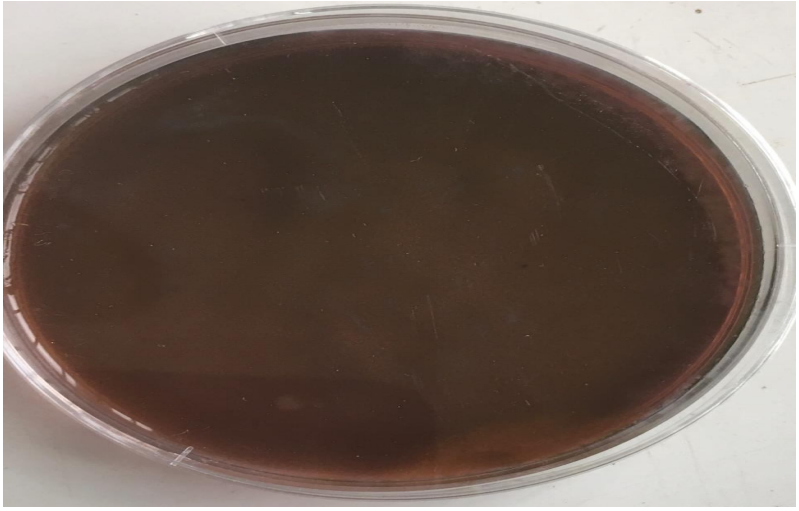


Plate 3: Eosin methylene blue agar showing the presence of *E.coli.*

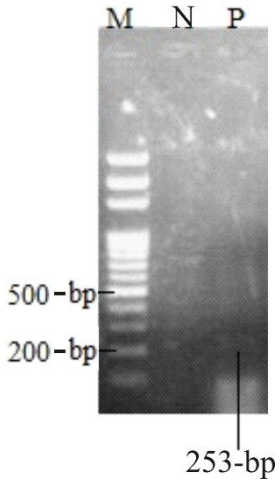


Figure 4. Agarose gel electrophoresis for 16S rRNA gene (253 bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel and visualized under U.V. light after staining with red stain. Lane M is 100 bpDNA ladder, lane N is negative control and lane P is the *E.coli* strain.

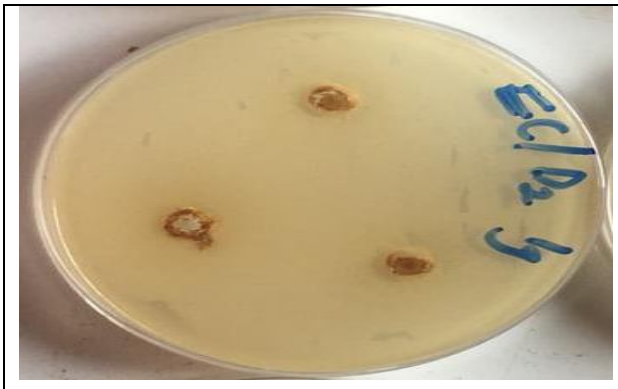


Fig 5a. Zone of inhibition at 100 mg/ml of the ethanolic root extract of *Azadirachtaindica*against *Escherichia coli*	Fig 5b. Zone of inhibition at 100 mg/ml of the ethanolic root extract of *Azadirachtaindica*against *Escherichia coli*

Fig 5c. Zone of inhibition at 250 mg/ml of the ethanolic root extract of *Azadirachtaindica*against *Escherichia coli*

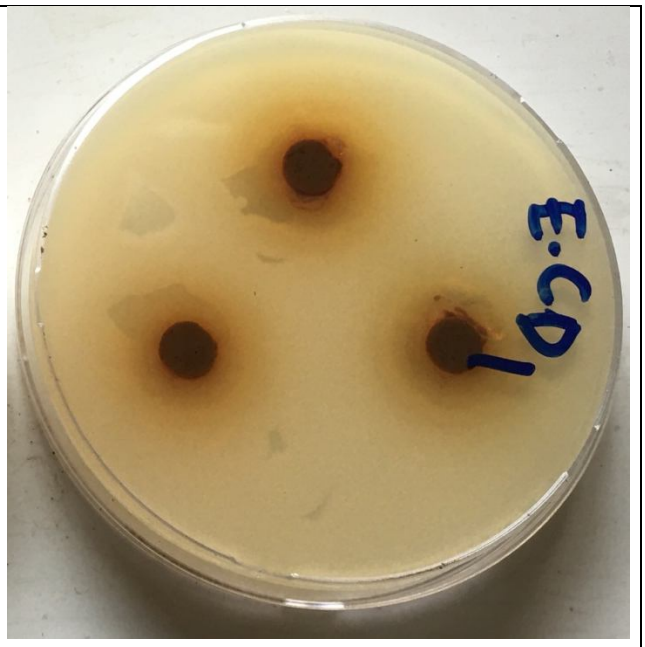


Fig 5d. Zone of inhibition at 250 mg/ml of the ethanolic root extract of *Azadirachtaindica*against *Escherichia coli*

Fig. 5e. Zone of inhibition at 12.5 mg/ml for azithromycin solution against *Escherichia coli*

Fig. 5f. Zone of inhibition at 12.5 mg/ml for azithromycin solution against *Escherichia coli*

Fig. 5g. Zone of inhibition at 25 mg/ml for azithromycin solution against *Escherichia coli*

Figure 5. Zone of inhibition of ethanolic root extract of *Azadirachtaindica*andazithromycin solution against *Escherichia coli*

Table 3.Antimicrobial susceptibility pattern of standard antibiotics agent against *Escherichia coli*

Antibiotic sensitive disc	Concentration (µg)	Diameter of zone of inhibition (mm)	Interpretation
Tarlvid (OFX)	10	14.20 ± 0.73[c]	+
Pefloxacin (PEF)	30	19.15± 1.40[b]	++
Gentamycin (CN)	10	21.13± 1.36[a]	+++
Sparfloxacin (SP)	10	18.57 ± 0.25[b]	++
Amoxacillin (AM)	30	22.40± 0.35[a]	+++

A comparison across the column was done using One way ANOVA Post Hoc Turkey test. The superscript a has the highest value followed by b and c has the lowest value. A. P<0.05 was considered statistically significant.

Table 4. Zone of inhibition of azithromycin solution and ethanolicroot extract of *Azadirachtaindica* against *Escherichia coli*

Test organism	Ethanolic root extract of *Azadirachtaindica* concentration (mg/ml)	Zone of inhibition of ethanolic root extract of *Azadirachtaindica* (mm)	Concentration of azithromycin solution used (mg/ml)	Zone of inhibition of azithromycin solution (mm)
Escherichia coli	100	9.33 ± 0.33[c]	5.00	15.37 ± 0.33[c]
Escherichia coli	250	19.48 ± 0.23[b]	12.50	23.37±0.48[b]
Escherichia coli	500	21.36±0.54[a]	25.00	37.34± 0.65[a]

A comparison across the column was done using One way ANOVA Post Hoc Turkey test. The superscript a has the highest value followed by b and c has the lowest value. A. P<0.05 was considered statistically significant.

Table 5: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for ethanolicroot extract of *Azadirachtaindica* and azithromycin solution against*Escherichia coli*

MIC and MBC values	*Escherichia coli*
MIC for ethanolic root extract of *Azadirachtaindica* (mg/ml)	31.25
MIC for azithromycin solution (mg/ml)	15.63
MBC for ethanolic root extract of *Azadirachtaindica* (mg/ml)	62.50
MBC for azithromycin solution (mg/ml)	31.25
MBC/MIC for ethanolic root extract of *Azadirachtaindica*	2.00

MBC/MIC for azithromycin solution	1.99

DISCUSSION

Medicinal plants played important role in antimicrobial activities and is used in the treatment of many diseases across the globe**.** Phytochemicals are major constitute found in medicinal plants and are responsible for their numerous bioactivities and metabolisms. For quantitative determination of compounds, gas chromatography with flame ionization detector (GC-FID) and GC-MS are usually preferred. Gas chromatography coupled with massspectrometry (GC-MS) is an establishedtechnique for reliable identification of bioactivecompounds existing in medicinal plants includinglong chain and branched chain hydrocarbons, volatile matter, alcohols, acids and esters [37-39].The different compounds identified with their retention index, peak area, molecular formulae and molecular weight are shown in Table 1. A total of 40 compounds were identified consisting of four prominent compounds and 36 minor compounds (Table 1). The four prominent compounds constitute 57.57% of the *A.indica* plantroot. The four major compounds and their percentage abundance are 9-Octadecenoic acid, (E)- (RT=22.044 and peak area=28.13%) is the most abundant compound in the root of the plant, 2H-1-benzopyran-3-carbonitrile, 6-hydroxy-4-methyl-2-oxo- (RT=25.581 and peak area=11.51%), n-Hexadecanoic acid (RT=20.345 and peak area=10.80%) and 2H-1-Benzopyran-2-one,7-(3-methyl-1H-pyrazol-1-yl)-3-phenyl- (RT=26.536 and peak area=7.13%) respectively.Study has shown that 9-Octadecenoic acid, (E)- compound has antiviral activity [40].Garbaand Garba[41] study shows that Cis-9-Octadecenoic acid isolated from *LandolphiaOwariensis* plant possess anti-diarrhoeal properties.Different study have reported that n-Hexadecanoic acid possess anti-inflammatory, antibacterial and antifungal activities [42-44].

In oneof our study, Momoh et al.[37], seven bioactive compounds were found in the root hexane extract of *A.indica*. These compounds are: Hexadecanoic acid, methyl ester (7.51%), 9-Octadecenoic acid, methyl ester (70.14%), Methyl stearate (6.78%), (E)-9-Octadecenoic acid ethyl ester (4.03%), cis-11-Eicosenoic acid, methyl ester (2.79%), Methyl 18-methylnonadecanoate (3.04%) and Docosanoic acid, methyl ester (5.72%).Some of the compounds found in these hexane extract of *A.indica* are found in the current plant used for the antimicrobial study.The GC-MS of *A. indica*has also been reported by other research studies to contain bioactive compounds [45-47].Some of the compounds obtained above in the GC-MS

analysis are also found in our result. It has been reported that the most important active constituent in *Azadirachtaindica*is azadirachtin and others are nimbolinin, nimbidin, nimbin, nimbidol, sodium nimbinate, salannin, quercetin and gedunin,The leaves contain ingredients such as nimbin, nimbolide, nimbanene, n-hexacosanol and amino acid, ascorbic acid, 6-desacetylnimbinene,nimbandiol, 7-desacetyl-7- benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, nimbiol and 17 hydroxyazadiradione[48-50].

The result of the qualitative phytochemical analysis shows that the ethanolic root extract of *A. indica* contain some secondary metabolites like phenolic compounds, flavonoids, carbohydrate, alkaloids, saponins, tannin and cardiac glycosides (Table 2).Secondary metabolites have different biological activities in human.Flavonoids and polyphenoliccompounds possess cancer chemopreventive effects related to their antioxidant effect [51] viatheir ability to scavenge free radicals [52] and induce apoptosis [53].A mechanistic study has revealed that alkaloids arrest cell division, thus inhibitingcancer proliferation [54].Adeniyi et al. [4] study shows that *A.indica*methanolic root extract contain secondary metabolite like phenolic compunds, flavonoids, alkaloids, saponins, tannin etc. Manda etal. [55]study shows that *A.indica*methanolic root extract possess carbohydrate, flavonoids, alkaloids, phenolic compound, saponins, tannin. Amino acids and protein were absent.The presences of these secondary metabolites in *A. indica*ethanolicroot extract are responsible forthe plant antibacterialactivity against *E.coli*.

Escherichia coli was isolated from abattoirs centre in Ikorodu, the organism was confirmed by morphological characterization, biochemical characterization and PCR technique using 16S rRNAwith specific primers with band size of 253 base pair when passed through 1.5% agarose gel during electrophoresis. Pathogenic *E. coli* contaminate the environment as a result of the discharge of untreated abattoir faeces and effluent. Lack of enforcement of good hygiene practices may ease the release and persistence of multiple pathogenic *E. coli* strains in the abattoir environment, making such a setting a unique favorable environment for bacteria to bacteria interaction and exchange of genetic material possibly leading to the emergence of new pathogenic strains with shuffled virulence features. The *E. coli* was tested against some selected antibiotics and ethanolic root extract of *Azadirachtaindica*. Gentamycin and amoxacillin antibiotics showed strong response with zone diameter between 21-30 mm against *Escherichia coli*, pefloxacin and sparfloxacin showed moderate response with zone diameter between 16-20mmwhile tarivid showed weak response with zone diameter less than 15 mm (Table 3).Neem and

its components play an important role in the inhibition of growth of numerous microbes such as bacteria,viruses, and pathogenic fungi. The study shows that ethanolic root extract of *Azadirachtaindica* exhibited strong response antimicrobial activity against *Escherichia coli* with zone of inhibition of19.48±0.23 and21.36±0.54 at concentration of 250 and 500 mg/dl respectively. At concentration of 100 mg/ml, the extract showed littleornoresponse with zoneof inhibition of 9.33±0.33.Azithromycin solution at 25 mg/ml showed potent response against *Escherichia coli* with zoneof inhibition of 37.34±0.65 and strong response with zone of inhibition of 23.37±0.48 at concentration of 12.50 mg/ml. At a lower concentration (5.00 mg/ml), azithromycin antibiotic exhibited weak response against *Escherichia coli* with zone of inhibition of 15.37 ± 0.33. The antibacterial activities of the *A. indica*extract and azithromycin solution increases with increase in their concentration.Different studies have shown the antimicrobial activity of *Azadirachtaindica*.The antibacterial activity of *Azadirachtaindica*and guava extractsagainst 21 strains of foodborne pathogens was investigated and the result of the study suggested that *A. indica*and guava extractspossess compounds containing antibacterial properties thatcan potentially be useful to control foodborne pathogens andspoilage of micro-organisms [56].The antibacterialactivity of the leaf, bark, seed, and fruit extracts of*Azadirachtaindica*(neem) on bacteria isolated from adultmouth revealed that the extracts showedantibacterial activity against all the test bacteria used [57].Furthermore, the fruit and seedextracts showed antibacterialactivity only at higher concentrations [57].In a study carried out by Hikaamboet al.[58], it was observed that the extracts of *A. indica* displayed antibacterial activity against *E. coli* in a dose-dependent manner and the aqueous extract with zone of inhibition of 10.67 ± 0.58 mm produced better antibacterial properties against *E. coli* than the ethanolic extractwith zone of inhibition of 8.7 ± 0.58 mm (p-value = 0.072).The antibacterial activity of ethanolic root extract of *Azadirachtaindica*against *Escherichia coli* pathogen was investigated for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MIC or MBC values are the lowest concentration of an antimicrobial agent necessary to inhibit bacterial growth or kill bacteria respectively [33]. Study has shown that MIC test is important in the laboratory to confirm the resistance of microorganisms to an antimicrobial agent and also used it to monitor the activity of new antimicrobial agents[33].Theethanolic root extract of *Azadirachtaindica*andazithromycin solution have MIC and MBC values of 31.25, 15.63, 62.50 and 31.25mg/ml respectively against for *E. coli*(Table

5).According to Gibbons [59] and Van Vuuren[60],crude extracts of natural products with an MIC lower than 1000 µg/ml are considered relevant and extracts with an MIC below 100 µg/ml are considered promising as potential antimicrobial agents. Therefore, based on this study, it can be considered that the ethanolic root extract of *Azadirachtaindica*is apotential antimicrobial agent against different microorganisms. The result of this study showed that the gram-negative bacterium (*Escherichia coli*) was susceptible to the ethanolic root extract of *A.indica*andazithromycin solution.Hikaamboet al[58] study shows that the MIC of *A. indica* aqueous extractwas found to be 10 mg/ml while the ethanolic extract has an MIC of 20 mg/ml against *E. coli.* In another study, it was observed that the antimicrobial activityof ethyl acetate extract and butanolfraction presented greater activity against *Streptococcus mutans*and *Streptococcus mitis*presenting an MIC of 50 µg/ml for these strains, and the strain *Enteroccocusfaecalis*, the hydroethanolic extract andaqueous fraction were most promising samples with an MIC of 50 µg/ml and MIC of 25 µg/ml, respectively [61]. All the above studies support the result obtained in our research concerning the antibacterial activity of ethanolic root extract of *Azadirachtaindica*against *Escherichia coli.*Adereleet al. [33]study has shown that calculated MBC/MIC ratio is bactericidal if the values of MBC/MIC ratio are less than or equal to 4 and bacteriostatic if the MBC/MIC ratio is > 4. The ethanolic root extract of *Azadirachtaindica*and azithromycin solution have bactericidal effects on *Escherichia coli*and may be used in the treatment of the organism.

CONCLUSION

A total of 40 compounds were identified in *Azadirachtaindica*using GC-MS analysis.9-Octadecenoic acid, (E)- (RT=22.044 and peak area=28.13%) was the most abundant compound found in the root of *A. indica* plant. The phytochemical screening of the ethanolic root extract of *Azadirachtaindica*shows the presence ofsecondary metabolites like cardiac glycoside, alkaloids,carbohydrates, tannins, phenolic, saponins and flavonoids. The ethanolic root extract of *A.indica* was effective on *Escherichia coli*.This is an indication that the extract has antibacterial activity.The MIC value of *A.indica*against *E.coli*was31.25 while its MBC value was62.50.MBC/MIC ratio value(2.0) showed that *Azadirachtaindica*extract had bactericidal effects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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