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# Biochemical Characterization and Molecular Identification of *Escherichia coli* Isolate from Abattoir Wastes and Its Susceptibility to Ethanolic Root Extract of *Azadirachta indica* (neem)

**Johnson Oshiobugie Momoh [a*] and Oluremi Nurudeen Olaleye [b]**

*[a] Department of Chemical Sciences (Biochemistry Unit), College of Basic Science, Lagos State University of Science and Technology (LASUSTECH), Ikorodu, Lagos State, Nigeria.*
*[b] Department of Biological Sciences (Microbiology Unit), College of Basic Science, Lagos State University of Science and Technology (LASUSTECH), Ikorodu, Lagos State, Nigeria.*

***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

***Article Information***

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## ABSTRACT

**Aim:** The study evaluates the molecular identification of *Escherichia coli* isolate from abattoir wastes and its susceptibility to ethanolic root extract of *Azadirachta indica* (neem).
**Methodology:** The isolation, cultural characterization and identification of *Escherichia 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 *Azadirachta indica* (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

**Corresponding author: E-mail: mjohnson_2008@yahoo.com and momohjohnson2008@gmail.com;*

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, sparfloxacin and resistant to tarivid with weak response. The study shows that ethanolic root extract of *A. indica* exhibited strong response antimicrobial activity against *Escherichia coli* with zone of inhibition of 19.48±0.23 and 21.36±0.54 at concentration of 250 and 500 mg/dl respectively. At 100 mg/ml, the extract showed little or no response with zone of inhibition of 9.33±0.33. Azithromycin solution at 12.50 and 25.00 mg/ml showed strong response and potent response against *E. coli* with zone of 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. The ethanolic root extract of *A. indica* and azithromycin solution have MIC and MBC values of 31.25, 15.63, 62.50 and 31.25 mg/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 *Azadirachta indica* (neem) contains phytochemicals, possesses antibacterial activity and may prevent pathogenic diseases caused by *Escherichia coli.*

*Keywords: Antimicrobial activity; Escherichia coli; GC-MS analysis; root of Azadirachta indica.*

## 1. INTRODUCTION

"Natural plant products have shown an important role in 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].

*"Azadirachta indica* (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 *Azadirachta indica* (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 *Azadirachta indica* which include 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 Enterobacteriaceae and was first isolated and characterized in 1885 by the German scientist and pediatrician Theodore Escherich" [11]. "*Escherichia coli* is a Gram negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded animals, The bacterium is a prokaryotic living organism without a nuclear membrane, it replicate rapidly and in habits the intestinal track of animals and humans" [11]. "Most *Escherichia coli* are harmless; some are pathogenic, causing both severe 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 contacts such 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-to severe 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 only diarrheagenic *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 *Azadirachta indica* against

*Escherichia coli* isolate from abattoir centre in Ikorodu, Lagos, Nigeria.

## 2. METHODOLOGY

### 2.1 Collection and Identification of *Azadirachta indica* Root

The *Azadirachta indica* root was obtained from Lagos State University of Science and Technology Ikorodu in Lagos State, Nigeria. The plant was identified using PictureThis plant identifier.

### 2.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of *Azadirachta indica* root

"GC-MS analysis of the *Azadirachta indica* 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].

#### 2.2.1 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.

### 2.3 Preparation of Ethanolic Root Extract of *Azadirachta indica*

The root of *Azadirachta indica* 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 *Azadirachta indica* 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$^{o}$C. The concentrated root extract of *Azadirachta indica* was dried to complete dryness in an aerated oven at 40$^{o}$C for 48 hours. The root extract of *Azadirachta indica* was later stored in a refrigerator at 4$^{o}$C.

### 2.4 Calculation of Percentage Yield

The percentage extractive yield of the ethanolic root extract of *Azadirachta indica* was calculated using the formula below.

% extraction yield (w/w) = (weight of extraction recovered / initial weight of powder of *A. indica*) × 100.

#### 2.4.1 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 *Azadirachta indica* were based on the intensity of the colour change.

### 2.5 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$^{o}$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$^{o}$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.

#### 2.5.1 Biochemical tests

"A lope full was taken from growing colony, inoculated into 10 ml nutrient broth then incubated at 37$^{o}$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].

### 2.5.2 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 30 µg 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.

### 2.5.3 Procedure for DNA isolation

Eight ml of absolute ethanol was added to each bottle of wash buffer 1 concentrate and 32 ml 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 10 minute. 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.

### 2.5.4 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), 25 pmol 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.

### 2.5.5 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].

### 2.5.6 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 × $10^8$ cfu/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.

### 2.5.7 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 *Azadirachta indica*. 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 *Azadirachta indica* was added into each 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 *Azadirachta indica* 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 inhibitory responses were classified as potent response, ++++, zone diameter >30 mm; strong response, +++, zone diameter between 21-30 mm; moderate response, ++, zone diameter between 16-20 mm; weak response, +, zone diameter between 10-15 mm; and little or no response, zone diameter <10 mm [34, 36].

### 2.5.8 Minimum inhibitory concentration (MIC) of the ethanolic root extract of *Azadirachta indica*

Minimum inhibition concentration is the lowest concentration of the root extract of *Azadirachta indica* 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 *Azadirachta indica* was determined using standard method [34]. The MIC of the ethanolic root extracts of the 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 *Azadirachta indica* (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 cell suspension of *E.coli* was added to each of the separate tubes. The tubes were incubated aerobically at 37°C for 18 hours. Negative control tube was made by pouring 1ml of normal saline instead of the ethanolic root extracts of *Azadirachta indica*. The lowest concentration of the dilution without bacterial growth was considered as the minimum inhibition concentration.

### 2.5.9 Minimum Bactericidal Concentration (MBC) of the ethanolic root extract of *Azadirachta indica*

The MBC of the ethanolic root extract of *Azadirachta indica* 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 the ethanolic root extract of *Azadirachta indica* at which no colonies of the *Escherichia coli* was seen was 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.

## 2.6 Statistical Analysis

All analyses were carried out in triplicate determination and results were expressed as mean ± SD. Students -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.

## 3. RESULTS

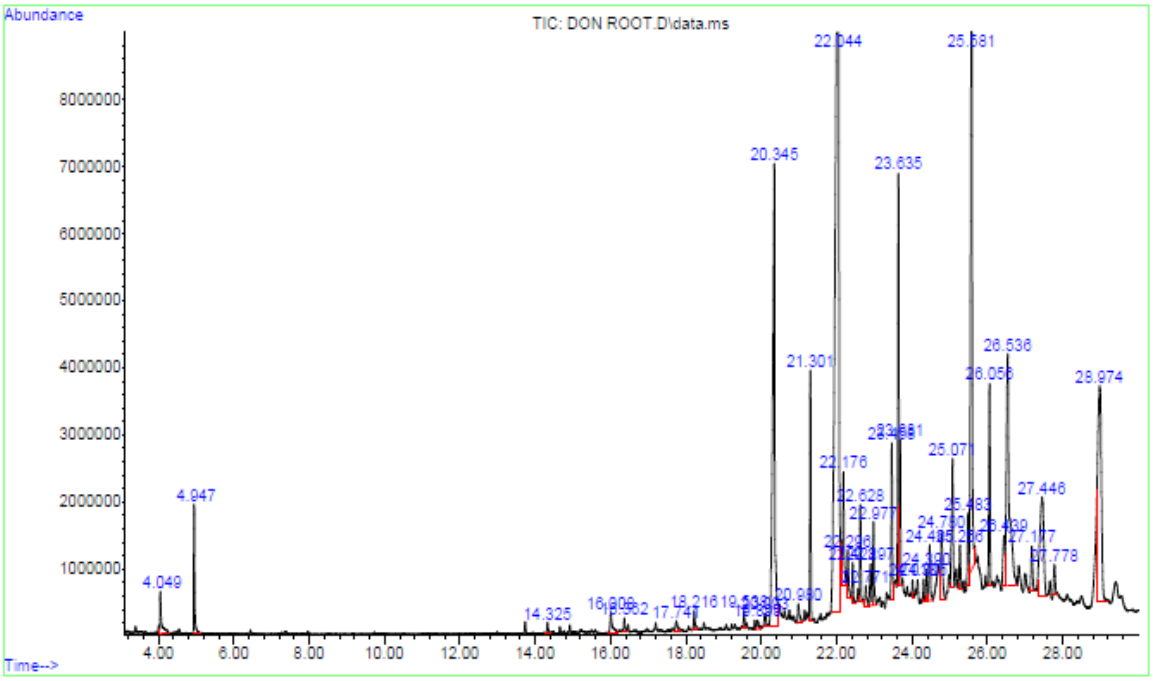
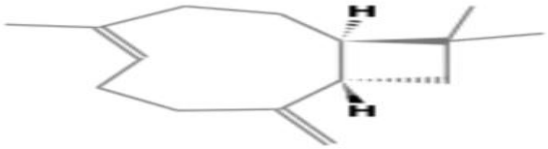



**Fig. 1. GC-MS Chromatogram of *Azadirachta indica* root**

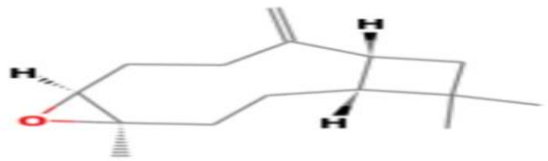
**Structure of 3-Hexen-2-one**


**Structure of 2-Pentanone, 4-hydroxy-4-methyl-**

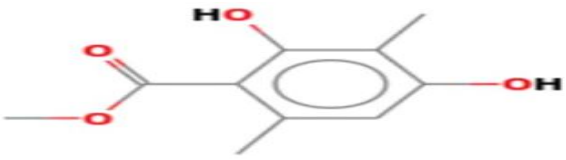


**Structure of Caryophyllene**

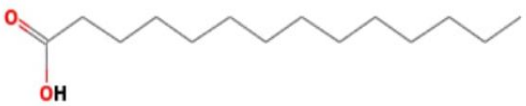

**Structure of Dodecanoic acid**



**Structure of Caryophyllene oxide**



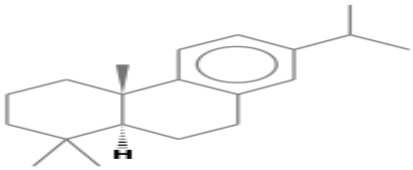

**Structure of Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester**



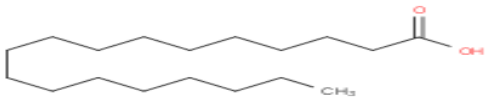
**Structure of Tetradecanoic acid**

**Structure of Hexadecanoic acid, methyl ester**

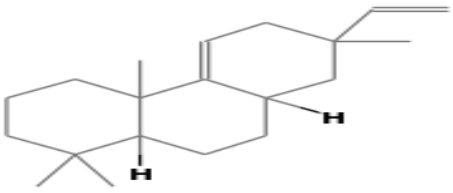
**Structure of n-Hexadecanoic acid**



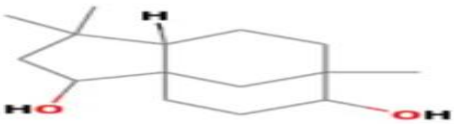
**Structure of Phenanthrene,1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-**



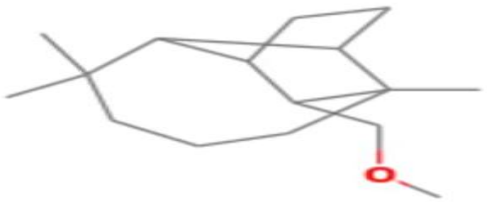
**Structure of Octadecanoic acid**



**Structure of Isopimara-9(11),15-diene**



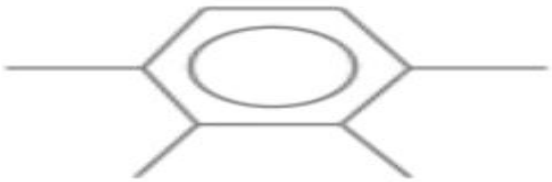
**Structure of (3S,3aS,6R,7R,9aS)-1,1,7-Trimethyldecahydro-3a,7-methanocyclopenta[8]annulene-3,6-diol**



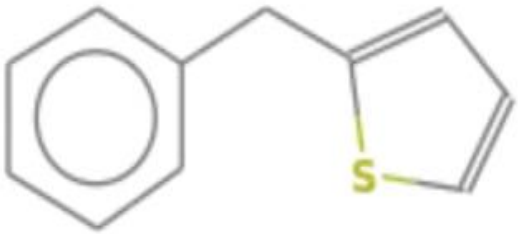
**Structure of (-)-Isolongifolol, methyl ether**

**Structure of 1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-**

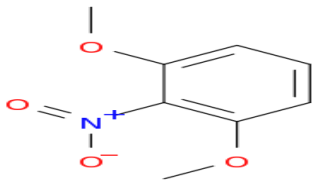
**Structure of 9-Octadecenoic acid, (E)-**



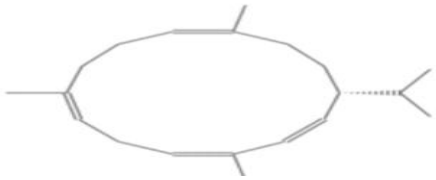
**Structure of Benzene, 1,2,3,4-tetramethyl-**



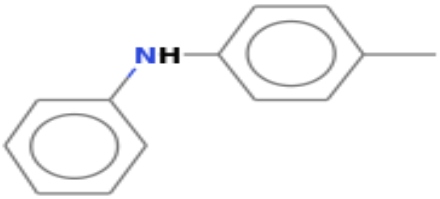
**Structure of Thiophene, 2-(phenylmethyl)-**



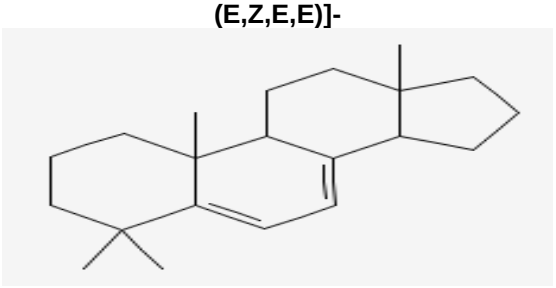
Structure of Benzene, 1,3-dimethoxy-2-nitro-



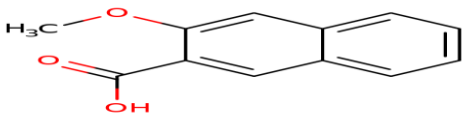
Structure of 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) -, [S-(E,Z,E,E)]-



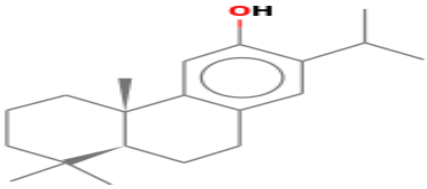
Structure of Benzenamine, 4-methyl-N-phenyl-



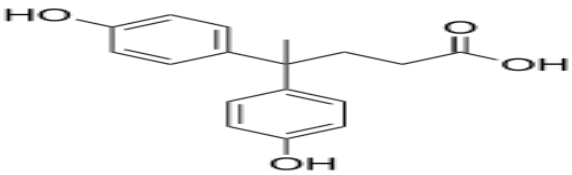
Structure of Androsta-5,7-diene, 4,4-dimethyl-



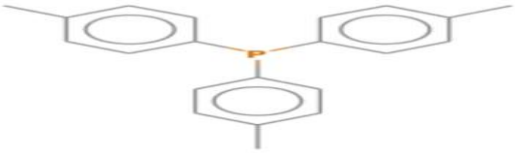
Structure of 3-Methoxy-5-naphthoic acid



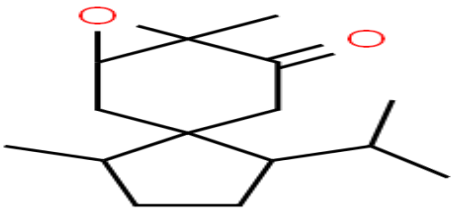
Structure of Ferruginol



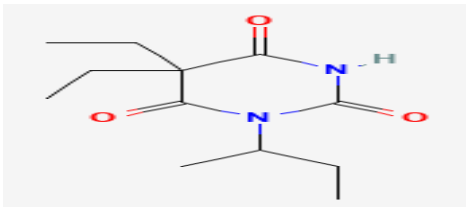
Structure of Diphenolic acid



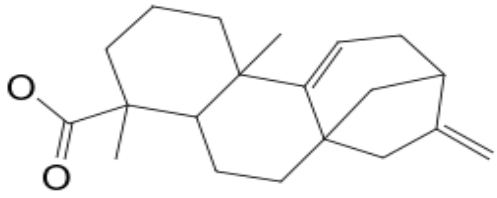
Structure of Phosphine, tris(4-methylphenyl)-



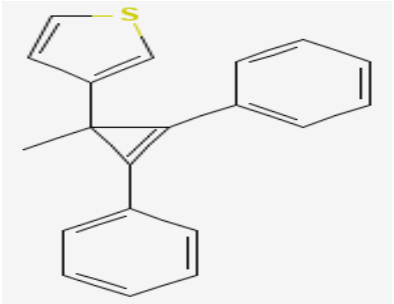
Structure of Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-



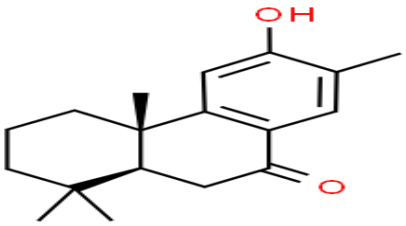
Structure of 2,4,6(1H,3H,5H)-Pyrimidinetrione, 5,5-diethyl-1-(1-methylpropyl)-



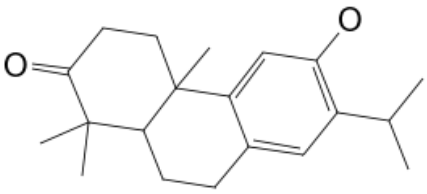
Structure of Kaura-9(11),16-dien-18-oic acid,4.alpha.)-



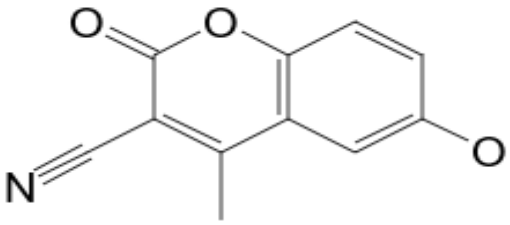
Structure of 3-Methyl-1,2-diphenyl-3-(3-thienyl)cyclopropene



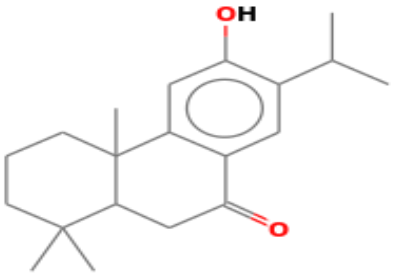

Structure of Nimbiol



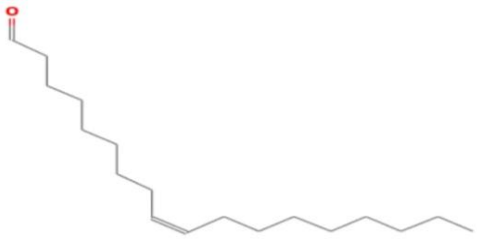

Structure of 2 (1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-



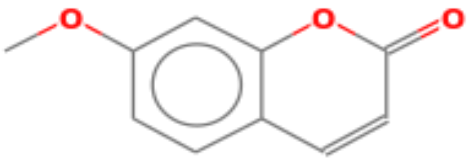

Structure of 2H-1-benzopyran-3-carbonitrile, 6-hydroxy-4-methyl-2-oxo-



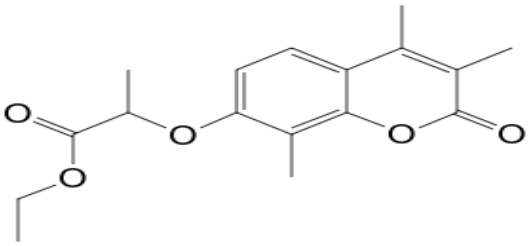

Structure of 9 (1H)-Phenanthrenone, 2,3,4,4a,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-



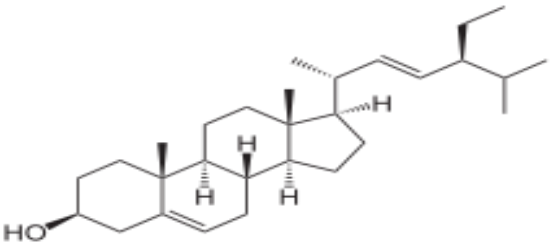

Structure of 9-Octadecenal, (Z)-



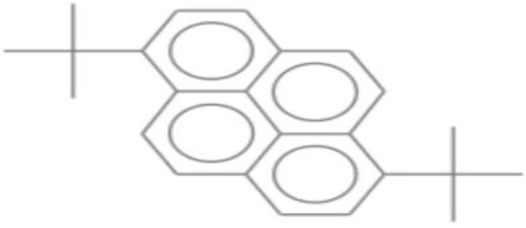

Structure of 2H-1-Benzopyran-2-one, 7-(3-methyl-



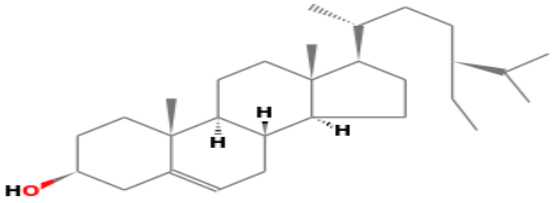

Structure of 2-(3,4,8-Trimethyl-2-oxo-2H-chromen-7-yloxy)-propionic acid ethyl ester




Structure of Stigmasterol



Structure of Pyrene, 1,6-bis(1,1-dimethylethyl)




Structure of gamma.-Sitosterol

**Fig. 2. Structure of 40 different compounds obtained during GC-MS analysis of *Azadirachta indica* root**

**Table 1. Chemical composition of *Azadirachta indica* 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 |

| Pk# | RT | Peak Area (%) | Name of compound | Molecular Formulae | Molecular Weight (g/mol) | Ref# | CAS# | Qual. |
|---|---|---|---|---|---|---|---|---|
| 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 |

### 3.1 Phytochemical Analysis of Ethanolic Root Extract of *Azadirachta indica*

The Percentage extraction yield of the ethanolic root extract of *Azadirachta indica* 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).

### 3.2 Confirmation Test for *Escherichia coli*

*E. coli* was isolated from fecal samples after 24 hours incubation. The growing bacterial colonies 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 (Fig. 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.

**Table 2. The qualitative phytochemical constituents of the ethanolic root extract of *Azadirachta indica***

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

*+ indicate present*

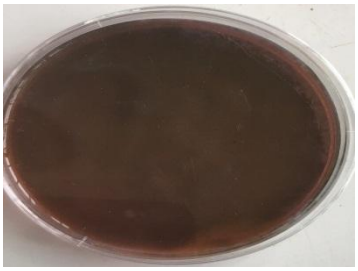


**Fig. 3. Eosin methylene blue agar showing the presence of *E. coli***

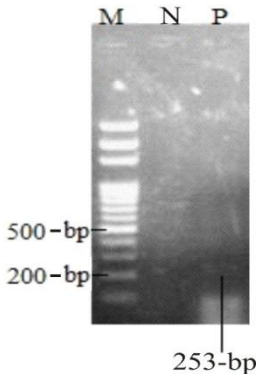



**Fig. 4. Agarose gel electrophoresis for 16S rRNA gene (253 bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel and visualized under U.V. light after staining with red stain. Lane M is 100 bp DNA ladder, lane N is negative control and lane P is the *E. coli* strain.**

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

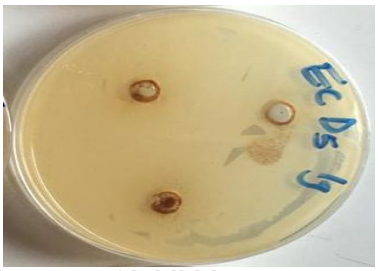


**Fig. 5b. Zone of inhibition at 100 mg/ml of the ethanolic root extract of *Azadirachta indica* against *Escherichia coli***

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

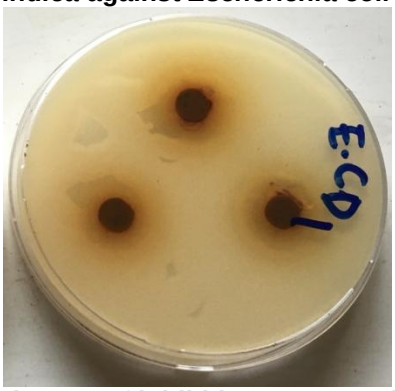


**Fig. 5d. Zone of inhibition at 250 mg/ml of the ethanolic root extract of *Azadirachta indica* against *Escherichia coli***

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

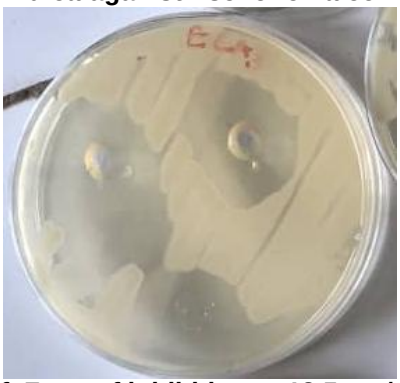


**Fig. 5f. Zone of inhibition at 12.5 mg/ml for azithromycin solution against *Escherichia coli***

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

**Fig. 5. Zone of inhibition of ethanolic root extract of *Azadirachta indica* and azithromycin 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 ethanolic root extract of *Azadirachta indica* against *Escherichia coli***

| Test organism | Ethanolic root extract of *Azadirachta indica* concentration (mg/ml) | Zone of inhibition of ethanolic root extract of *Azadirachta indica* (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 ethanolic root extract of *Azadirachta indica* and azithromycin solution against *Escherichia coli***

| MIC and MBC values | *Escherichia coli* |
|---|---|
| MIC for ethanolic root extract of *Azadirachta indica* (mg/ml) | 31.25 |
| MIC for azithromycin solution (mg/ml) | 15.63 |
| MBC for ethanolic root extract of *Azadirachta indica* (mg/ml) | 62.50 |
| MBC for azithromycin solution (mg/ml) | 31.25 |
| MBC/MIC for ethanolic root extract of *Azadirachta indica* | 2.00 |
| MBC/MIC for azithromycin solution | 1.99 |

## 4. 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 mass spectrometry (GC-MS) is an established technique for reliable identification of bioactive compounds existing in medicinal plants including long 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* plant root. 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]. Garba and Garba [41] study shows that Cis-9-Octadecenoic acid isolated from *Landolphia Owariensis* plant possess anti-diarrhoeal properties. Different study have reported that n-Hexadecanoic acid possess anti-inflammatory, antibacterial and antifungal activities [42-44].

In one of 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 *Azadirachta indica* 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-benzoyl-gedunin, 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 polyphenolic compounds possess cancer chemopreventive effects related to their antioxidant effect [51] via their ability to scavenge free radicals [52] and induce apoptosis [53]. A mechanistic study has revealed that alkaloids arrest cell division, thus inhibiting cancer 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 et al. [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* ethanolic root extract are responsible for the plant antibacterial activity 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 rRNA with 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 *Azadirachta indica*. 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 16-20 mm while 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 *Azadirachta indica* exhibited strong response antimicrobial activity against *Escherichia coli* with zone of inhibition of 19.48±0.23 and 21.36±0.54 at concentration of 250 and 500 mg/dl respectively. At concentration of 100 mg/ml, the extract showed little or no response with zone of inhibition of 9.33±0.33. Azithromycin solution at 25 mg/ml showed potent response against *Escherichia coli* with zone of 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 *Azadirachta indica.* The antibacterial activity of *Azadirachta indica* and guava extracts against 21 strains of foodborne pathogens was investigated and the result of the study suggested that *A. indica* and guava extracts possess compounds containing antibacterial properties that can potentially be useful to control foodborne pathogens and spoilage of micro-organisms [56]. The antibacterial activity of the leaf, bark, seed, and fruit extracts of *Azadirachta indica* (neem) on bacteria isolated from adult mouth revealed that the extracts showed antibacterial activity against all the test bacteria used [57]. Furthermore, the fruit and seed extracts showed antibacterial activity only at higher concentrations [57]. In a study carried out by Hikaambo et 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 extract with zone of inhibition of 8.7 ± 0.58 mm (p-value = 0.072). The antibacterial activity of ethanolic root extract of *Azadirachta indica* 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]. The ethanolic root extract of *Azadirachta indica* and azithromycin solution have MIC and MBC values of 31.25, 15.63, 62.50 and 31.25 mg/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 *Azadirachta indica* is a potential 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* and azithromycin solution. Hikaambo et al [58] study shows that the MIC of *A. indica* aqueous extract was 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 activity of ethyl acetate extract and butanol fraction presented greater activity against *Streptococcus mutans* and *Streptococcus mitis* presenting an MIC of 50 µg/ml for these strains, and the strain *Enteroccocus faecalis*, the hydroethanolic extract and aqueous 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 *Azadirachta indica* against *Escherichia coli.* Aderele et 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 *Azadirachta indica* and azithromycin solution have bactericidal effects on *Escherichia coli* and may be used in the treatment of the organism.

## 5. CONCLUSION

A total of 40 compounds were identified in *Azadirachta indica* 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 *Azadirachta indica* shows the presence of secondary 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* was 31.25 while its MBC value was 62.50. MBC/MIC ratio value (2.0) showed that *Azadirachta indica* extract had bactericidal effects.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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