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Antibacterial Activity of *Azadirachta Indica* Leaf Extracts Against Extended Spectrum Betalactamase Producing Escherichia Coli Isolated from Raw Cow Milk: A One Health Approach

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ABSTRACT

Emergence of drug resistance in *Escherichia coli* harboring extended spectrum betalactamase (ESBL) genes in both humans and animals is of major concern to global health. Antimicrobial resistance (AMR) has escalated due to the inappropriate use of antimicrobials in humans, animals, and the environment. This study aimed to evaluate the antibacterial activity of aqueous and ethanolic *Azadirachta indica* leaf extracts against ESBL producing *Escherichia coli* isolated from raw cow milk, incorporating a One Health perspective to address antibacterial resistance in human, animals and environmental health contexts. Raw milk samples (n=100) were collected from different locations within Keffi, Nasarawa state. The presence of *Escherichia coli* was investigated using cultural, morphological and biochemical technique. The presence of different ESBL resistance genes (*blaTEM, blaSHV, blaCTX-M* groups) in *E. coli* isolates were evaluated both phenotypically using double-disc synergy test (DDST) and genotypically using polymerase chain reaction (PCR) technique. *Azadirachta indica* (neem) leaves were extracted using two solvents (water and ethanol). Different concentrations of aqueous and ethanolic leaf extracts were used to detect their efficacy against ESBL producers. The results demonstrated that 30 (30%) of the isolates were contaminated with *E. coli.* The most resistant antibiotics among the isolates was amoxicillin-clavulanic acid (96.7%) and the least resistant was gentamicin (23.3%). The most frequently occurring MAR indices ranged from 0.8 (16.7%) - 0.9 (23.3%) and the least occurring MAR index is 0.2 (6.7%). ESBL production was detected in 9 out of the 30 isolated *E. coli* using Double Disc Synergy Test (DDST). 4 out of the 9 ESBL producers were confirmed to express resistance genes (*blaTEM, blaCTX-M, blaCTX-M-1* and *blaCTX-M-4).* The ethanolic leaf extracts of neem plant had antibacterial potential against ESBL *Escherichia coli* but only at very high concentration of the extracts. Therefore, it could be a potential natural alternative or a partner drug in the treatment of infections caused by ESBL *Escherichia coli* transmitted to humans or animals. The results call for multisectoral One Health strategies that combine veterinary, environmental and public health efforts to control the dissemination of antimicrobial resistance from animal sources to humans ensuring food safety and the effectiveness of the antibiotics.

Keywords: *Azadirachta indica, Escherichia coli, Extended Spectrum betalactamases, public health, antimicrobial resistance.*

1.0 INTRODUCTION

Azadirachta indica, commonly known as neem tree, is a tropical evergreen species native to the Indian subcontinent and widely recognized for its medicinal properties. Leaves, roots and stems of neem possess antibacterial and antifungal agents, making it a versatile resource in agriculture, pest control and traditional medicine (Altayb *et al.*, 2022; Wylie & Merrell, 2022). Neem's active compounds, including flavonoids, nimbin and azadirachtin, have been shown to inhibit the growth of pathogens, offering potential as alternative therapies for bacterial infections (Omar *et a*l., 2024). *Escherichia coli (E. coli)* is a gram-negative, rod-shaped, facultative, anaerobic bacterium which inhabits the intestinal tracts of human and warm-blooded animals (Islam *et al*., 2016; Hasan *et al*., 2023). E. coli has been historically used as the global indicator for the potential presence of fecal pathogens in foodstuffs particularly raw milk (Ferdous *et al*., 2021). The pathogenic strain of *E. coli* can cause several diseases both in humans and animals. This bacterium shows great genetic versatility and adaptability to continuously changing environments, and can acquire a number of antimicrobial resistance mechanisms, such as enzymes encoded by plasmid (Ribeiro *et al*., 2024).

Of particular concern is the emergence of Extended Spectrum Beta-Lactamase (ESBL)-producing *E. coli*, which show resistance to β-lactam antibiotics. ESBL producing *E. coli* has been recognized as a major multidrug resistant bacteria implicated in serious hospital and community acquired infections, especially in places where poor sanitation and inadequate hygiene practices are common (Torka, 2022; Mahmud et al., 2020). The spread of ESBL-producing *E. coli* in raw dairy products like cow milk poses a significant threat to public health due to the potential zoonotic

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transmission and horizontal gene transfer of resistant bacteria through the food chain (Gelalcha et al., 2023).
Milk is considered one of the high nutritional quality foods and whole milk is the milk as it comes from the cow and contains about 3.5% milk fat. Raw or unpasteurized milk can be contaminated by bacteria like *Escherichia coli* by improper milk processing and poor animal management in non-hygienic environment (Hasan et al., 2023). The extensive and inappropriate use of broad spectrum antibiotics in both human, veterinary medicine and agricultural settings, especially in disease prevention and promotion of growth in animal production is the major cause of selection of antibiotic resistant *E. coli*. Multi-drug resistant *E. coli* isolates characteristically exhibit non-susceptibility to at least one agent in three or more antibiotic class (Igbinosa & Chiadika, 2021).

Antimicrobial resistance has been linked to the extensive use of antimicrobial drugs in food processing, in animals and humans. *E. coli* strains are considered a serious threat to public health particularly in developing countries (Sarba *et a*l., 2023; Chung *et al*., 2016). Foodborne illnesses are a major public health concern throughout the world, with developing nations bearing majority of the burden. Multidrug resistance in E. coli is a major concern worldwide, however there was limited studies on this particularly in developing countries (Sarba *et al*., 2023).

In Nigeria, as in other developing countries, food borne diseases are frequently unreported and there is no surveillance program for food pathogens. It is also difficult to demonstrate the extent of contamination of milk which is a challenge to food safety. Foodborne infections continue to be a major public health concern in Nigeria because of the poor sanitary conditions, malnutrition, and lack of adequate medical services (Sarba *et a*l., 2023).

The isolation and identification of *E. coli* from food products are crucial steps in understanding and mitigating the risks associated with its presence. By addressing these critical aspects, this research will contribute to a better understanding of the microbial safety of raw milk sold within Keffi metropolis and support efforts to protect consumers from foodborne illnesses. This study incorporates a One Health perspective, recognizing the interconnections between human, animal, and environmental health. It seeks to develop a sustainable antibacterial strategy using neem extracts to combat antimicrobial resistance in bacteria originating from both human and animal sources.

2.0 METHODOLOGY

2.1 STUDY AREA

The research was carried out in Keffi, Nasarawa state, Nigeria. Keffi local government area (LGA) is an historic town located in Nasarawa state, North-central Nigeria between latitudes 8˚51` and 8˚53´ north of the equator and longitudes 7˚50` and 7˚51` east of the greenwich meridian. Keffi is located about 128km away from Lafia, the Nasarawa state capital and about 57km away from Abuja, the federal capital territory of Nigeria. It covers 138 square kilometers and have an average temperature of 30°C (Yakubu, 2013).

2.2 SAMPLE COLLECTION

A total of 100 raw milk samples were aseptically collected at three different locations in rural areas around Keffi, Nasarawa state. All samples were collected in sterile 50ml bottle containers and labelled suitably and then placed in an icebox and transported immediately to the research laboratory, Department of Microbiology, Nasarawa State University, Keffi (NSUK) for bacteriological analysis within 2-4hours to prevent contamination.

2.3 Isolation of *Escherichia coli*

One milliliter of each raw milk samples was aseptically transferred with a sterile pipette into nine milliliters of buffered peptone broth in test tubes and incubated at 37˚C for 24 hours as pre-enrichment step in accordance with Cheesbrough (2006). A loopful of the enriched broth was streaked on the surface of Eosin Methylene Blue agar (EMB) with a sterile inoculating loop for selective identification. Greenish metallic sheen colonies that grew on EMB agar after 24hours incubation at 37˚C were presumed to be *Escherichia spp*. The presumed colonies were stored in nutrient broth containing 30% glycerol at -80ºC in a refrigerator for future use.

2.4 Identification of *Escherichia coli*

2.4.1 Microscopic Identification by Gram Staining

Gram Staining was performed as per the procedure described by Cheesbrough (2006). Two-three colonies of test organism was picked up with a sterile inoculating loop, emulsified on a clean grease-free glass slides containing a drop of distilled water to form a thin homogenous film, the film was allowed to dry, air-dried and fixed by gently passing it through flame. Crystal violet solution was applied on each smear to stain for 60seconds and then rinsed off with distilled water. Gram's iodine was added as a mordant for 60seconds and again rinsed off with distilled water. Acetone alcohol was added as a decolouring agent and rinsed off immediately. Neutral red was added as a counter stain

and allowed to stain for 60seconds. The slides were washed with distilled water, blotted and air dried, and examined under light microscope with high power objective (100X) using a drop of immersion oil on each slide before observation. Organisms that were Gram negative, pink colored with rod shaped appearance and arranged in single or in pairs were suspected to be *E. coli.*

2.4.2 Biochemical Identification

Presumptive colonies of *E. coli* were subjected to biochemical analysis (IMViC) using the methods described by Cheesbrough (2006). The biochemical tests carried out include Carbohydrate Fermentation test, Catalase test, Methyl Red test, Voges-Proskauer (V-P) test and Indole test**.**

2.5 Antimicrobial Susceptibility Testing (AST)

The antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines using commercially available antimicrobial discs. The following antibiotics were used - Ceftriaxone (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Gentamicin (10µg), Ofloxacin (5µg), Co-Trimoxazole (Sulphamethoxazole/trimethoprim) (25µg), Amoxicillin-Clavulanic acid (30µg), Tetracycline (30µg), Levofloxacin (30µg), and Netilmicin Sulphate (30µg). Two-three discrete colonies of the test organism were inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) and the turbidity of the bacteria suspension was adjusted to turbidity equivalent to 0.5 McFarland standards for all the tests. The McFarland's standard was prepared as follows; 0.5 ml of 1.172% (w/v) BaCl2.2H2O was added into 99.5 ml of 1% (w/v) H2SO4. A sterile swab stick was soaked in the standardized bacterial suspension and the soaked swabs were pressed against the sides of the tubes to drain off some excess suspension and streaked on Mueller Hinton agar plates, the antibiotic discs were placed at the center of the inoculated plates using a sterile forceps and allowed to stand for 1 h for pre-diffusion. The plates were incubated at 37ºC for 24 h. The diameter zone of inhibition was measured in millimeters and the results were interpreted in accordance with the susceptibility breakpoint described by Clinical Laboratory Standards Institute (CLSI, 2015). The antimicrobial resistance prevalence was categorized as low ≤ 30%, moderate 30-59%, and high ≥ 60%.

2.6 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR index of the isolates was determined according to the method of Tsaku *et al.* (2019). From the result of antimicrobial susceptibility testing, MARI was calculated as:

MAR Index = No. of antibiotics to which isolate is resistant /Total No. of antibiotics tested.

2.7 Phenotypic Detection of ESBL-producing *E. coli*

Isolates that were positive for multidrug resistance or exhibits reduced susceptibility to any of the 2nd and 3rd generation cephalosporins were phenotypically confirmed for ESBL production using the double-disc synergy test (DDST). DDST was performed as a standard disc diffusion assay on Mueller-Hinton (MH) agar plates in line with CLSI criteria (CLSI, 2015). Two-three discrete colonies of the test organism was inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) in test tubes and the turbidity of the bacteria suspension was adjusted to turbidity equivalent to 0.5 McFarland standards for all the tests. The McFarland's standard was prepared as follows; 0.5 ml of 1.172% (w/v) BaCl2.2H2O was added into 99.5 ml of 1% (w/v) H_2SO4. A sterile swab stick was soaked in the standardized bacterial suspension and the excess was drained off by pressing the swab to the side of the tubes streaked on Mueller Hinton agar plates, the antibiotic discs were placed aseptically with a sterile forceps. A disc of amoxicillin/clavulanate (20µg/10µg, respectively) and a disc of cefotaxime (30µg), were placed 30 mm apart (center to center) on an inoculated agar plate. 30-µg antibiotic discs of ceftazidime and ceftriaxone was also placed on the plate, 30mm (center to center) from the amoxicillin/clavulanate disc. The plates were incubated at 37°C for 18 to 24 hours. ESBL production was suspected phenotypically when the zones of inhibition of the cephalosporins (cefotaxime 30µg and ceftazidime 30µg) increase in the presence of amoxicillin/clavulanic acid disk (20/10µg). A clear extension of the edge of the cefotaxime inhibition zone toward the disc containing clavulanate (keyhole like) was interpreted as synergy, indicating the presence of an ESBL. A≥ 5 mm increase in the inhibition zone diameter for either of the cephalosporins (cefotaxime and ceftazidime) tested in combination with amoxicillin-clavulanic acid versus its zone when tested alone confirm ESBL production phenotypically. *E. coli* ATCC 25922 was used as a standard control strain (CLSI, 2015).

2.8 Storage of Bacteria

The Bacteria was stored in the refrigerator at 4°C on nutrient agar slants and reactivated by sub-culturing on MacConkey agar and used for subsequent molecular experiments.

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2.9 Molecular Identification of ESBL producing *E. coli*

Isolates that were confirmed ESBL producers were screened to detect the presence of some ESBL resistance genes namely *blaTEM, blaSHV, and blaCTX-M*.

2.9.1 Bacterial DNA Extraction

The bacterial DNA was extracted by a method as earlier described by Abimiku *et al.* (2016) with minor modification. Bacterial DNA was extracted by boiling overnight culture. Bacterial culture was inoculated into Luria-Bertani (LB) broth and incubated at 37^0C for 8 hours. Five milliliters of the LB broth culture containing the bacterial isolates in 1mL was spun at 14000 rpm for 3 minutes. The supernatant was discarded and the harvested cell pellet was resuspended in 1 mL sterile distilled water and transferred into 1.5 mL centrifuge tube and centrifuged at 14000rpm for 10 minutes. The supernatant was discarded carefully. The pellet was resuspended in 100 µL of sterile distilled water by vortexing. The tube was centrifuged again at 14000rpm for 10 min, and the supernatant was discarded carefully. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 20 minutes. The heated bacterial suspension was cooled on ice for 10mins and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20ºC for other subsequent experimentations.

2.9.2 DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. 2µl of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button (Abimiku *et al*., 2019).

2.9.3 DNA Amplification for 16S rRNA Gene of *Escherichia coli*

The 16S rRNA genes (*uidA-147bp*) of *Escherichia coli* isolates were amplified using the F: 5'-AAAACGGCAAGAAAAAGCAG-3' and R: 5'-ACGCGTGGTTACAGTCTTGCG-3' primers (Zymo research, USA) on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 25 cycles. The PCR mix included: X2 Dream Taq Master Mix (Taq polymerase, DNTPs, MgCl, Zymo research, USA), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 96ºC for 5 min; denaturation, 95ºC for 30 sec; annealing, 52ºC for 30 sec; extension, 72ºC for 30 sec for 25 cycles and final extension, 72ºC for 7 min. The product was resolved on a 1% agarose gel and visualized on a UV transilluminator.

2.9.4 DNA Amplification for *bla*TEM, *bla*SHV, *bla*CTX-M by Polymerase Chain Reaction (PCR)

The single-plex polymerase chain reaction (PCR) was performed in 45 µl total volume using the ABI 9700 Applied Biosystems thermal cycler, containing 2.5 µl MgCl2 (50 mM), 2.5 µl 10 × buffer, 0.9 µl of each primer (2.7 µM), 8 mM of dNTPs, 0.5 µl Taq DNA polymerase (Zymo research, USA) and 5 µl of DNA template. Amplification was carried out in the following program: an initial denaturation step at 95°C for 5 minutes, followed by 40 denaturation cycles at 94°C for 1 min, annealing at 55°C for bla_{CTXM-1}, 58ºC for bla_{CTX-M} and $bla_{CTX-M-4}$, 56°C for bla_{SHV}, and bla_{TEM} for 30s and extension at 68°C for 30s, and a final extension step at 68 °C for 10 minutes.

2.9.5 Detection of all the amplified genes by Agarose Gel Electrophoresis

Two percent (2%) agarose gel was used to resolve DNA fragment. This was prepared by combining 2g agarose in ten times concentration of tris-borate ethylene diamine tetraacetate (10ml 10XTB-EDTA) buffer and 90ml sterile distilled water in 250ml beaker flask and heating in a microwave for 2 minutes until the agarose is dissolved. Exactly 0.7µl of ethidium bromide was added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and casting combs were inserted. The gel was allowed to set for 30 minutes. The casting combs were carefully removed after the agarose gel had solidified completely. One times concentration (1X) TBE buffer was added to the reservoir until it covered the agarose gel. Precisely 8µl of gel tracking dye (bromophenol blue) was added to 10µl of each sample with gentle mixing. The sample was loaded onto the wells of the gel at a concentration of 10µl, the mini horizontal electrophoresis gel setup was covered and electrodes connected. Electrophoresis was carried out at 100-200mA in one hour. At the completion of electrophoresis, the gel was removed from the buffer and visualized under UV light and documented.

2.10 Preparation of Plant Materials

1. Following the method proposed by Hikaambo *et al.* (2023), the leaves of *A. indica* were removed loosely from the plant stem with the use of a knife. The leaves were washed under running tap water to eliminate dust and other foreign particles, they were washed once with

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sterilized distilled water. The leaf materials were dried under shade at room temperature for 10 days.

2.11. Preparation of Crude Powder

After about 10 days of shade drying, well dried leaves were aseptically grinded with a clean electric blender into powdered form. The product was subjected to mass sieving to obtain fine powder. Powder was kept in a glass jar with airtight container and stored until use.

2.12 Preparation of Stock Solution

2.12.1 Aqueous Extraction

Using the method developed by Ujah *et al*. (2021), the powdered leaves was extracted using maceration method, an electric weighing balance was zeroed and a filter paper was placed on the weighing balance. 200g of crude powder was weighed and soaked in 1000ml of distilled water separately and left for 48 hours in airtight plastic bottles for maceration. It was then filtered with a muslin cloth to remove the coarse residue, Whatman No 4 filter paper (125mm) was used to remove the fine residue to obtain a clear filtrate. The resultant solution was poured in a beaker and placed in a water bath at 45-55°C to evaporate excess water. Stock solution was kept in sterilized screw capped bottles in the refrigerator at 4°C for future use.

2.12.2 Ethanol Extraction

Based on the method described by Ujah *et al*. (2021), an electric weighing balance was zeroed and a filter paper was placed on the weighing balance. 200 g of the crude powder was weighed and then macerated in 200ml of 95% ethanol for 48 hours in an airtight container and covered. In ethanol extraction, it was done in closed system for proper extraction. After maceration, the solution was filtered through Whatman No 4 filter paper and the resulting solution was dried under reduced pressure in a rotatory evaporator at 45℃. Stock solution was kept in a sterilized screw capped bottles and stored at 4℃ in the refrigerator for future use.

2.13 Phytochemical Analysis

Phytochemical screening of leaf extracts was determined following standard protocols (Ujah *et al*., 2021) to identify the active compounds present in neem leaves.

2.13.1 Test for Alkaloids

Mayer's reagent was used to test for alkaloids. 2ml of leaves extract was taken in a test tube and 2-3 drops of Mayer's reagent added on it. Presence of alkaloid was indicated by the appearance of green colour precipitate in the solution.

2.13.2 Test for Saponins

Foam test: To 5 ml of each extract, 3 ml of distilled water was added and shaken vigorously for about 5 minutes, formation of frothing confirm the presence of saponins.

2.13.3 Test for Glycosides

0.5g of the extracts was dissolved in ethanol for about 10 minutes for proper extraction and filtered. To 5 ml of each filtrate, 0.3 ml of Fehlings solution A and B was added until it turn to alkaline indicating the presence of glycoside.

2.13.4 Test for Terpenoids

0.5g of aqueous and ethanolic extracts was dissolved in ethanol for about 10minutes for proper extraction and filtered. To 5 ml of each filtrate, 1 ml of acetic anhydride was added followed by additional concentrated H_2S0_4. A change in colour from pink to violet indicates the presence of terpenoid.

2.13.5 Test for Flavonoids

0.5g of the extracts was dissolved in distilled water, which was filtered to 5ml of filtration, 3ml of lead ethanoate was added. Appearance of a pale yellow-brown (buff-coloured) confirm the presence of flavonoid.

2.13.6 Test for Steroids

0.5g of the extracts was dissolved in distilled water, which was then filtered. To 4ml of the filtrate, 2ml of acetic acid was added and the solution was allowed to cool in a refrigerator, concentrated H_2SO_4 was added carefully. A change in colour from violet to bluish green indicates the presence of steroidal ring.

2.13.7 Test for Phenol

0.5g of the sample was boiled with 15ml distilled water and filtered. To 2 ml of the filtrate, few drops of 10% ferric chloride solution was then added. The presence of phenolic hydroxyl group was confirmed by the formation of violet colour.

2.13.8 Test for Coumarins

Extracts solution was concentrated to yield a residue. Residue was dissolved in hot water. After cooling, the solution was divided in two test tubes. To one test tube, 10% (w/v) ammonium hydroxide was added. Other test tube was used as control. The appearance of fluorescence colour indicates the presence of coumarins.

2.14 Antibacterial activity of aqueous and ethanolic neem leaf extracts on the isolates of ESBL-producing *E. coli*

Antibacterial effects of aqueous and ethanolic neem extracts was observed using agar-well diffusion method. Two-three discrete colonies of the test organism was inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) in test tubes and the turbidity of the bacteria suspension was adjusted to turbidity equivalent to 0.5 McFarland standards for all the tests. A sterile cork borer was used to bore 6 wells that were 15mm apart on each MHA plates to be tested and the bottom of the bored wells were sealed with dissolved agar. Two-three discrete colonies of the test organism was inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) in test tubes and the turbidity of the bacteria suspension was adjusted to turbidity equivalent to 0.5 McFarland standards for all the tests. A sterile swab stick was soaked in the standardized bacterial suspension, the

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excess were drained off by pressing the swab to the side of the tubes and streaked evenly on the MHA plates. Different concentrations (100mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125mg/mL) of the aqueous and ethanolic extracts of *A. indica* leaf extracts were aseptically dispensed into each well and allowed to stand for 1hour for pre-diffusion then incubated at 37ºC for 24hours. The sensitivity of the tested ESBL *E. coli* to aqueous and ethanolic extracts were as shown by zones of inhibition after incubation. The zones of inhibition were measured using a plastic ruler in millimetres (mm) (Hikaambo *et al*., 2022).

2.15 Statistical Analysis

All data obtained were analyzed using Microsoft Excel to compute descriptive statistics, including means, averages, and percentages. For effective communication of the findings, the analyzed results were formatted and presented through structured tables and figures.

3.0 RESULTS

3.1 Isolation and Identification of *E. coli* isolated from raw cow milk

Greenish metallic sheen on EMB agar which were gram negative, rod shaped, Carbohydrate fermentation test positive, Indole positive, Methyl Red positive, Voges-Proskauer negative, and Catalase test negative were identified as *Escherichia coli.*

Table 1: Primers used in the study to amplify ESBL genes

Target genes	Primer Sequence	Amplicon size (bp)	References
blaTEM	F: 5´-TCGGGGAAATGTGCGCG-3´ R: 3´-TGCTTAATCAGTGAGGCACC-5´	872	El-Hariri *et al*., 2023
blaCTX-M	F: 5´-ACGCTGTTGTTAGGgAAGTG-3´ R: 3´-TTGAGGCTGGGTGAAGT-5´	307	El-Hariri *et al*., 2023
blaCTX-M-1	F: 5´-TTAGGAAGTGTGCCGCTGTA-3´ R: 3´- CGGTTTTATCCCCCACAAC-5´	197	El-Hariri *et al*., 2023
blaCTX-M-4	F: 5´-GACAAAGAGAGTGCAACGGATG-3 R: 3´- TCAGTGCGATCCAGACGAAA-5’	501	El-Hariri *et al*., 2023

Table 2: Cultural, Morphological and Biochemical Characteristics of *Escherichia Coli* Isolated from Raw Cow Milk

Cultural characteristics	Morphological Characteristics		Biochemical Characteristics					Inference
	Gram Stain	Shape	CFT	MR	VP	IND	CAT	
Green metallic sheen colonies on EMB agar	-	Rod	+	+	-	+	+	*Escherichia coli*

KEY: CFT = Carbohydrate Fermentation test, MR = Methyl Red, VP = Voges Proskauer, IND = Indole, CAT = Catalase, EMB = Eosin Methylene Blue, - = Negative, + = Positive

3.2 Prevalence of *Escherichia coli* isolated from raw cow milk

The overall prevalence of the isolates was 30 out of 100 raw milk samples that were collected. Market (MKT) had the highest prevalence (53.3%) followed by Total roundabout (TRB) (26.7%) and University (UNI) had the least prevalence (20.0%).

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Table 3: Prevalence of *Escherichia coli* isolated from raw milk collected within Keffi, Nasarawa State, Nigeria

Location	No. of Milk Sample Obtained	No. (%) of *E. coli* Isolated
Market	40	16 (53.3)
University	30	6 (20.0)
Total Roundabout	30	8 (26.7)
Total	100	30

3.3 Antimicrobial Resistance profile of *E. coli* isolated from raw cow milk

The isolates were highly resistant to Amoxicillin-Clavulanic acid (96.7%) followed by Tetracycline (86.7%), Cefotaxime (86.7%), Ceftazidime (66.7%), Ofloxacin (63.3%), Co-trimoxazole (63.3%) and Levofloxacin (60%) but moderately resistant to Ceftriaxone (56.7%) and Netilmicin (50%) with Gentamicin (23.3%) having the least resistance.

Table 4: Antimicrobial Resistance Profile of *Escherichia coli* isolated from raw cow milk

Antimicrobials	Disc content (µg)	No. (%) Resistance (n= 30)
Ceftriaxone	30	17 (56.7%)
Gentamicin	10	7 (23.3%)
Co-trimoxazole	25	19 (63.3%)
Levofloxacin	5	18 (60.0%)
Tetracycline	30	26 (86.7%)
Netillin (Netilmicin Sulphate)	30	15 (50.0%)
Amoxicillin-Clavulanic acid	30	29 (96.7%)
Cefotaxime	30	26 (86.7%)
Ceftazidime	30	20 (66.7%)
Ofloxacin	5	19 (63.3%)

3.4 Antimicrobial Resistance Phenotypes

The antimicrobial resistant isolates were distributed into different antimicrobial resistant phenotypes and the most common phenotypes observed were CTR-COT-TET (43.3%) and CTR-CTX-CTZ (33.3%) respectively.

Table 5: Antimicrobial Resistance Phenotypes of *Escherichia coli* isolated from raw cow milk

Antimicrobial Resistance Phenotypes	Frequency (%) (n=30)
CTR-CTX-CTZ	10 (33.3)
CTR-COT-TET	13 (43.3)
CTR- GEN-COT	4 (13.3)
CTR-GEN-LEV	3 (10.0)
CTR-COT-CTX-CTZ	9 (30.0)
CTR-GEN-COT-LEV	3 (10.0)
CTZ-COT-NET-AMC	8 (26.7)
CTX-GEN-COT-TET	5 (16.7)
CTR-GEN-COT-LEV-TET	2 (6.7)
CTR-COT-NET-LEV-AMC	7 (23.3)
CTR-CTX-CTZ-GEN-OFL	1 (3.3)
CTR-COT-NET-LEV-AMC-OFL	6 (20.0)
CTX-GEN-COT-NET-LEV-AMC	3 (10.0)
CTZ-GEN-COT-TET-LEV-AMC	3 (10.0)
CTR-GEN-COT-NET-LEV-TET-OFL	2 (6.7)
CTX-GEN-COT-NET-TET-LEV-AMC-OFL	2(6.7)

KEY: CTR= ceftriaxone, CTX= Cefotaxime, AMC= Amoxicillin-clavulanic acid, NET= Netilmicin, TET= tetracycline, COT= Cotrimoxazole, LEV= Levofloxacin, CTZ= Ceftazidime, OFL= Ofloxacin, GEN= Gentamicin

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3.5 Multiple Antimicrobial Resistance (MAR) Index

The majority of the isolates exhibited MAR indices > 0.2, indicating exposure to environment with frequent antibiotic use or misuse. The most frequently occurring MAR indices ranged from 0.8 (16.7%) - 0.9 (23.3%) and the least occurring MAR index is 0.2 (6.7%).

Table 6: Multiple Antibiotic Resistance Index of *Escherichia coli* Isolated from raw cow milk

No. of Antibiotics isolate is resistant to (a)	Total No. of Antimicrobial Tested (b)	MAR Index (a/b)	Frequency (%) (n=28)
10	10	1.0	1 (3.3%)
9	10	0.9	7 (23.3%)
8	10	0.8	5 (16.7%)
7	10	0.7	4 (13.3%)
6	10	0.6	3 (10%)
5	10	0.5	3 (10%)
4	10	0.4	4 (13.3%)
3	10	0.3	1 (3.3%)
2	10	0.2	2 (6.7%)

KEY: a= Number of isolates the antibiotics to, b= Total number of antimicrobial tested, MAR= Multiple Antibiotic Resistance.

3.6 Antimicrobial Resistance Phenotypes, Multiple Antibiotic Resistance Index and ESBL production in *Escherichia coli* isolated from raw milk in Keffi Metropolis

The antimicrobial resistance phenotypes, multiple antibiotic resistance index and phenotypic screening for ESBL production in *Escherichia coli* is as shown. Only 9 (26.7%) out of the 30 resistant isolates screened showed enhanced zones of inhibition towards the amoxicillin-clavulanic acid disc when examined by Double Disc Synergy Test for ESBL production.

Table 7: Antibiotic Resistance Phenotypes, Multiple Antibiotic Resistant Index, and ESBL production in *Escherichia coli* isolated from raw cow milk

Isolates	Resistance phenotype	No. of antibiotics tested	MARI	ESBL Production
MKT1	AMC	10	0.1	-
MKT2	NET-TET-AMC-OFL-CTZ-CTX	10	0.6	+
MKT3	CTR-GEN-COT-LEV-NET-TETAMC-OFL-CTZ-CTX	10	1.0	+
MKT4	CTR-COT-LEV-NET-TET-AMC-OFL-CTZ-CTX	10	0.9	+
MKT 5	GEN-LEV-TET- AMC-CTX	10	0.5	-
MKT6	CTR-GEN-COT-NET-TET-AMC-OFL-CTX	10	0.8	-
MKT7	TET-AMC-CTZ-CTX	10	0.4	-
MKT8	CTR-COT-LEV-NET-TET-AMC-OFL-CTZ-CTX	10	0.9	+
MKT9	CTR-COT-TET-AMC-OFL-CTZ-CTX	10	0.7	-
MKT10	CTR-COT-LEV-TET-AMC-OFL-CTZ-CTX	10	0.8	-
MKT11	CTR-GEN-COT-LEV-NET-TET-AMC-CTZ-CTX	10	0.9	+
MKT12	NET-TET-AMC-CTX	10	0.4	-
MKT13	COT-TET-AMC-OFL-CTZ-CTX	10	0.6	-
MKT14	CTR-COT-LEV-TET-AMC-CTZ	10	0.6	-
MKT15	COT-LEV-NET-TET-AMC-OFL-CTZ-CTX	10	0.8	-
MKT16	CTR-NET-AMC-CTX	10	0.4	-
TRB1	GEN-COT-LEV-TET-AMC-OFL-CTZ-CTX	10	0.8	-
TRB2	CTR-COT-LEV-AMC-OFL	10	0.5	-
TRB3	CTR-COT-LEV-NET-TET-AMC-OFL-CTZ-CTX	10	0.9	+
TRB4	CTR-COT-LEV-NET-TET-AMC-OFL-CTZ-CTZ	10	0.9	-
TRB5	GEN-COT-NET-TET-AMC-OFL-CTX	10	0.7	-

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TRB6	CTZ	10	0.1	-
TRB7	CTR-GEN-COT-LEV-NET-TET-AMC-OFL-CTX	10	0.9	+
TRB8	NET-TET-AMC-CTX	10	0.4	-
UNI1	AMC-CTZ-CTX	10	0.3	-
UNI2	CTR-COT-LEV-TET-AMC-OFL-CTZ-CTX	10	0.8	+
UNI3	COT-LEV-TET-AMC-OFL-CTZ-CTX	10	0.7	-
UNI4	CTR-NET-TET-AMC-CTX	10	0.5	-
UNI5	CTR-LEV-TET-AMC-OFL-CTZ-CTX	10	0.7	-
UNI6	CTR-COT-LEV-TET-AMC-OFL-CTX	10	0.7	+

KEY: MKT= Market, UNI= University, TRB= Total Roundabout, + = presence, - = absence, CTR= ceftriaxone, CTX= Cefotaxime, AMC= Amoxicillin-clavulanic acid, NET= Netilmicin, TET= tetracycline, COT= Cotrimoxazole, LEV= Levofloxacin, CTZ= Ceftazidime, OFL= Ofloxacin, GEN= Gentamicin

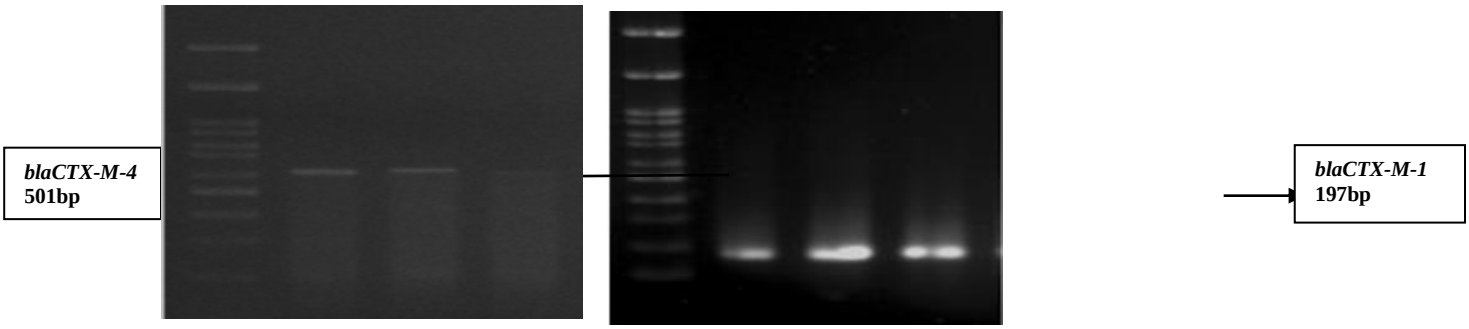
3.7 Prevalence of ESBL genes on *Escherichia coli* isolated from raw cow milk

Out of the 9 *Escherichia coli* isolates expressing ESBL production, 4 (13.3%) were confirmed to be ESBL producers. Figure 1 shows the agarose gel electrophoresis of *E. coli* isolates amplified ESBL gene. Lanes 1 and 2 represents blaCTX-M-4 (501bp) gene. Figure 2 shows the agarose gel electrophoresis of *E. coli* isolates amplified ESBL gene. Lanes 1, 2 and 3 represent the expression of blaCTX-M-1(197bp) gene. Figure 3 shows the agarose gel electrophoresis of ESBL *E. coli* isolates. Lanes 1 and 3 represent the expression of blaCTX-M (307bp) gene. Figure 4 shows the agarose gel electrophoresis of E. coli isolates amplified ESBL gene. Lane 3 represents the expression of *blaTEM* (872bp).

Table 7: Prevalence of ESBL genes on *E. coli* isolated from raw cow milk

ESBL Genes	No (%) *E. coli* (n=9)
blaTEM	4 (13.3%)
blaSHV	0 (0.0%)
blaCTX-M	4 (13.3%)
blaCTX-M-1	4 (13.3%)
blaCTX-M-4	4 (13.3%)

KEY: *bla*TEM = betalactamase gene Temoneira, *bla*SHV = betalactamase gene Sulfhydryl variable, *bla*CTX-M = betalactamase gene Cefotaxime-Munich, *bla*CTX-M-1 = betalactamase gene Cefotaxime-Munich-1, *bla*CTX-M-4 = betalactamase gene Cefotaxime Munich-4



a. **Agarose gel electrophoresis of *E. coli* isolates amplified ESBL gene. Lanes 1 and 2 represents blaCTX-M-4 (501bp) gene**
b. **b. Agarose gel electrophoresis of *E. coli* isolates amplified ESBL gene. Lanes 1, 2 and 3 represent the expression of blaCTX-M-1(197bp) gene**

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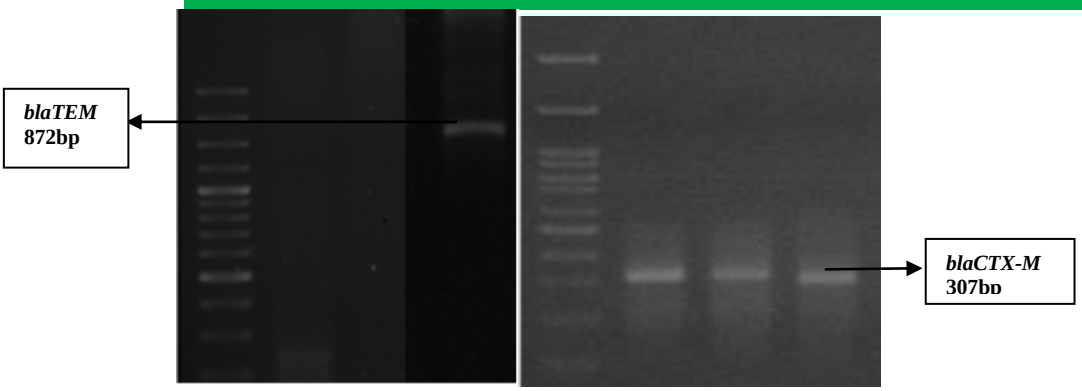

c. Agarose gel electrophoresis of ESBL *E. coli* isolates. Lanes 1 and 3 represent the expression of blaCTX-M (307bp) gene
d. Agarose gel electrophoresis of E. coli isolates amplified ESBL gene. Lane 3 represents the expression of *blaTEM* (872bp).

3.8 Phytochemical screening of *A. indica* leaves

The phytochemical screening of *A. indica* leaves is as shown in Table 8. The presence of secondary metabolites such as alkaloids, flavonoids, steroids, saponins, phenol, glycosides and absence of terpenoids and coumarins was observed from the neem leaves.

Table 8: Phytochemical Screening of *A. indica* leaves

Phytochemicals	Source-Leaves
Alkaloids	+
Saponins	+
Glycosides	+
Terpenoids	-
Flavonoids	+
Steroids	+
Phenol	+
Coumarins	-

KEY: += POSITIVE, -=NEGATIVE

3.9 Antibacterial activity of aqueous and ethanolic *Azadirachta indica* leaf extracts on ESBL *Escherichia coli* isolated from raw milk

The antibacterial activity of aqueous and ethanolic *A. indica* leaf extracts on ESBL producing *E. coli* is as shown in Table 4.9. The six concentrations in mg/ml (100, 50, 25, 12.5, 6.25, 3. 125) of aqueous and ethanolic neem leaf extracts had very low antibacterial activity against ESBL *Escherichia coli* isolates. The aqueous extracts demonstrated no antibacterial activity while the ethanolic extracts demonstrated very minimal antibacterial activity.

Table 9: Antibacterial activity of aqueous and ethanolic neem leaf extracts on ESBL *Escherichia coli*

Organism	Diameter Zone of Inhibition (mm)											
ESBL producing *E. coli*	**Aqueous Extracts** (mg/ml)						**Ethanolic Extracts** (mg/ml)					
	100	50	25	12.5	6.25	3.125	100	50	25	12.5	6.25	3.125
	NZ	NZ	NZ	NZ	NZ	NZ	10	NZ	NZ	NZ	NZ	NZ

KEY: NO ZONE OF INHIBITION

4.0 DISCUSSION

Medicinal plants contain a wide variety of secondary metabolites, such as glycosides, alkaloids, flavonoids, and terpenoids. Many of these compounds have antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties. Herbal extracts had been

demonstrated to effectively reduce bacterial load, improve overall health, and enhance innate immunity in laboratory animals. Plant-based therapies are very cheap, readily available locally, safe, and pose fewer risks than conventional drugs. Among medicinal plants, *Azadirachta indica* (Neem) is the most referenced in both traditional Indian and Ayurvedic veterinary medicine. *Azadirachta indica* (Neem) leaves, bark, seeds, and oil contain a wide variety of biologically active substances, including azadirachtin, nimbin, quercetin, tannins, and many flavonoids. These substances achieve their antibacterial effects through mechanisms such as tearing the cell wall, stopping protein synthesis, damaging nucleic acid replication, and interfering with quorum sensing (Varma *et al*., 2025). The development of antibiotic resistance and the emergence of infectious diseases in bacteria resulting in decreased action or failure of existing antibacterial agents, has resulted in an urgent need for the discovery of new, safe, and efficient antibacterial agents. Food animals constitute huge reservoirs of resistant bacteria that can be disseminated in the environment and in animal-source foods (Ribeiro *et al*., 2024).

The presence of *Escherichia coli* observed in raw cow milk sold within Keffi metropolis is similar to the results obtained in similar studies in Nigeria by Bello *et al.*, 2025. The isolation of *Escherichia coli* from raw cow milk in the study area suggests that the milk may have been contaminated as a result of poor hygiene during milking and poor sanitary condition of the environment. The isolation of *E. coli* is an indication of fecal contamination from the environment by human or animal, and during milk collection and processing, making it unsafe for human consumption. Milk can be contaminated in the processing stages. It might get contaminated from the outer surface of udder, hands of peoples dealing with processing, storage equipment and surrounding environments. Not every *Escherichia coli* that present in milk are harmful for human health except the pathogenic *Escherichia coli* that contains bacterial toxins that can transmit diseases to human body. The microbial quality of the milk can be affected by feeding style and housing strategies. The use of water for washing the milking equipment might be another source of contamination (Hasan *et al*., 2023).

In the current study, the prevalence of *Escherichia coli* was 30% isolated from raw cow milk collected within Keffi Metropolis. This findings is in agreement with recent studies with a prevalence rate of 35.8% from raw milk and its products (Wulgo *et al*., 2022). A higher prevalence of 46% and 48.9% was reported in Maiduguri, Borno state, North-eastern Nigeria from raw milk of cows, sheep and goats and in Kwara State, Nigeria from marketed raw cow milk respectively (Bello *et al*., 2025; Ghali-Mohammed *et al*., 2022). The variations in prevalence of *E. coli* in milk can be attributed to dairy farming type, geographic location, milking equipment, milking technique, duration of milk transportation, and level of sanitary practices. The overall prevalence of 30% indicates a substantial contamination rate in Keffi's raw milk supply, posing risks for foodborne illnesses like gastroenteritis or urinary tract infections. The market exhibited the highest rate (53.3%), potentially due to higher exposure to environmental contaminants, poor refrigeration, or cross-contamination from vendors. The university's lower rate (20%) might reflect better hygiene awareness among sellers, while the roundabout's (26.7%) falls in between, possibly influenced by traffic-related pollution.

The AMR profile revealed that the *Escherichia coli* isolates showed resistance to the antibiotics tested in decreasing order: amoxicillin-clavulanic acid (96.7%), tetracycline (86.7%), cefotaxime (86.7%), ceftazidime (66.7%), ofloxacin (63.3%) and cotrimoxazole (63.3%), levofloxacin (60.0%), ceftriaxone (56.7%), netilmicin (50.0%) and gentamicin (23.3%). Our study is in contrast with studies in Kwara State, Nigeria which showed reistance to cefotaxime, ampicillin, gentamicin and chloramphenicol (Ghali-Mohammed *et al*., 2025) and another study in Sudan showed the highest resistance to gentamicin followed by Azithromycin, Oxytetracycline, Cotrimoxazole-trimethoprim and nalidixic acid (Nahar *et al*., 2023). A recent study in Southwestern Nigeria by Akindele *et al*. (2025) showed similar prevalence with high resistance to amoxicillin-clavulanic acid (100%) and levofloxacin (61.9%). Another study by Tama *et al*. (2021) reported resistance of *E. coli* to gentamicin (52.2%) and amoxicillin-clavulanic acid (61.1%). The indiscriminate or excessive use of these drugs in both human and animal healthcare settings can contribute to the development and spread of antimicrobial resistance in the different study areas. The resistance of isolates to these antibiotics may be due to antibiotic misuse, ineffective empiric antibiotic therapy, poor dosing regimen of antimicrobial agent, and prolong therapy of infection caused by this organism may also likely be the reason for the resistance to antibiotics mentioned (Nahar *et al*., 2023).

The most common phenotypic pattern resistance of *E. coli* from raw cow milk were CTR-COT-TET (43.3%) and CTZ-CTZ-CTX (33.3%) and the least phenotypic pattern resistance were CTX-GEN-COT-NET-TET-LEV-AMC-OFL (6.7%) and CTR-CTX-CTZ-GEN-NET-LEV-TET-AMC-OFL (3.3%). The antimicrobial

resistance profile of the *E. coli* isolates revealed the presence of multiple antibiotic resistance, with several isolates exhibiting Multiple Antibiotic Resistance Index (MARI) greater than 2. The most common MAR indices were 0.9 and 0.8 (21.4%). MARI greater than 0.2 indicates that an organism probably originated from an environment where antibiotics were frequently used and reflects sustained antimicrobial selective pressure in the study area. All isolates exhibited resistance to at least two agent, indicating pervasive exposure to antibiotics in the dairy ecosystem. These high MAR index isolates, all from raw cow milk points to the fact that antibiotic resistance in humans does not only come from hospitals, but also from food producing animals (Tama *et al.*, 2021; Tsaku *et al*., 2019).

Nine (9) of the 30 isolates (26.7%) were phenotypically confirmed to be positive for ESBL production. The production of ESBL in betalactam resistant isolates observed in this study was expected and is an indication that ESBL enzymes may be responsible for the inactivation of the third generation cephalosporins. The prevalence of ESBL producers in *Escherichia coli* isolates jointly resistant to ceftazidime and cefotaxime observed in this study was higher than 13.9% reported by Mohammed & Kurawa (2025) in persons with urinary tract infections in Kano metropolis but was lower than 73.7% from poultry droppings in Karu, Nasarawa State reported by Tama *et al*., (2021).

Beta-lactamase encoding gene *blaTEM* was identified in 4 isolates (Plate 4), while *blaSHV* was not detected in any of the isolates, and *blaCTX-M*, *blaCTX-M-1* and *blaCTXM-4* was detected in 4 of the isolates (Plate 1, 2 and 3). Equal prevalence of *blaTEM* (13.3%) and *blaCTX-M* (13.3%) reflects their global dominance in ESBLs, with *CTX-M* groups indicating recent evolutionary adaptations. The predominance of the *blaCTX-M* variant of ESBL over the others among the Enterobacteriaceae in humans and food animals, including dairy cattle. It is hypothesized that the frequent occurrence and the dominance of the *blaCTX-M* variant over the others in ESBL-*Escherichia coli* may be related to its low fitness cost to the host, successful dissemination through MGEs, and rapid response to beta-lactam antibiotics selection pressure. Absence of *blaSHV* may denote regional gene pool differences (Gelalcha *et al*., 2023).

This study detected 4 (13.3%) extended spectrum beta lactamase producing *E. coli* out of the 100 raw milk samples investigated. The prevalence of ESBL *E. coli* isolates from raw cow milk samples in our study was 13.3% in the findings of this study, which is higher than 5.92% and lower than 25.01% reported in some studies in Nasarawa state (North central, Nigeria) and Oyo state (Southwestern, Nigeria) respectively (Aliyu *et al*., 2024; Adesanwo *et al*., 2024). A similar study carried out in Bangledash on raw milk detected the prevalence of *blaTEM* and *blaCTX-M* genes in *E. coli* at 6.35% and 25.39% respectively (Ahmed *et al*., 2025). Variations in prevalence rates across studies may be due to differences in sample size, population characteristics or testing protocols. The findings of this study underscore the concerning prevalence of *E. coli* particularly strains exhibiting antibiotic resistance. The excessive use of antimicrobials in treatment and prevention of diseases leads to drug residues contaminating the environment, milk and meat of the animal. Food producing animals are found to be a reservoir and risk factor for ESBL producing *E. coli* especially in Cattle (Akindele *et al*., 2025; Mohammed and Kurawa, 2025). In communities such as Keffi where raw milk is consumed without pasteurization, these presents a direct risks to human health. Consumption of contaminated milk can lead to illness, and enzymes such as *blaTEM*, *blaSHV*, and *blaCTX-M* complicate treatment by breaking down extended spectrum cephalosporins and monobactams.

The study revealed the presence of secondary metabolites including alkaloids, flavonoids, saponins, glycosides, phenols, and steroids whereas terpenoids and coumarins were absent in the leaves. The presence of alkaloids suggests that *A. indica* extracts may have biological actions, including anti-cholinergic, anti-tumor, anti-hypertensive, cough expectorant, anesthetic, analgesic, muscle relaxant, anti-pyretic, and anti-malaria. Flavonoids and phenols are known to disrupt bacterial cell membranes, inhibit nucleic acid synthesis and interfere with essential metabolic processes while alkaloids inhibit bacterial enzyme systems. Saponins further enhance antimicrobial effects by increasing membrane permeability and causing leakage of intracellular components (Muhammed *et al*., 2026). Some flavonoids have natural antimicrobial properties and can help inhibit the growth of bacteria, fungi, and viruses, which can be beneficial for food preservation and combatting infections. The absence of terpenoids and coumarins shows that neem leaves may be deficient in these phytochemical groups. Terpenoids, which are renowned for their varied biological actions, are frequently present in plant essential (Bolaji *et al*., 2024).
The antibacterial activity of aqueous leaf extracts against ESBL *E. coli* demonstrated complete resistance at all concentrations whereas ethanolic leaf extracts demonstrated stronger efficacy (10mm) at the highest concentration (100mg/ml) used and complete resistance on the remaining concentrations (50, 25, 12.5, 6.25 and

3.125mg/ml). Several studies have indicated that the ethanol leaf extract were more inhibitory than the aqueous extract. A study carried out in Kaduna State (Northwestern), Nigeria showed that ethanol had greater potency than the aqueous extracts and was more effective in extracting antibacterial compounds from neem leaves (Muhammed *et al*., 2026). The superior performance of the ethanol extract may be attributed to its ability to dissolve a broader range of phytochemicals, including moderately polar and nonpolar compounds, which are often responsible for antimicrobial activity. The observed concentration-dependent inhibition in the ethanolic extracts suggests that bioactive compounds present in the extract such as flavonoids, tannins, terpenoids, and azadirachtin may disrupt bacterial cell walls, inhibit enzyme activity, or interfere with nucleic acid synthesis (Thamer *et al*., 2024; Hikaambo *et al*., 2022).

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tatistical analysis confirmed that the level of efficacy or inhibition of aqueous extracts to the test organisms differs significantly from ethanol extracts (p value=0.0016) but showed no signicant difference between concentrations (p value=0.45) of the aqueous and ethanol extracts used. Based on the findings of this study, *A. indica* showed a remarkable antibacterial potential against ESBL. Although the efficacy was found to decrease at lower concentrations, the ethanol extracts has shown considerable activity, justifying further exploration for its therapeutic applications. The antibacterial activity of *A. indica* demonstrates a strong dose-dependent effect, signifying its potential as a promising antibacterial agent comparable to conventional antibiotics. This antibacterial activity is due to the bioactive compounds of *A. indica*, which works by disrupting bacterial cell walls and inhibit critical enzyme systems in ESBL. It is difficult to understand what optimum dosages can be used and what other factors can influence or retard the medicinal properties of neem leaves (Khairnar *et al.*, 2025).

This study was conducted using a One Health approach highlights the interconnected risks to human, animal and environmental health in the emergence and dissemination of multidrug resistant *E. col*i in Keffi, Nasarawa state. It also emphasizes that antimicrobial resistance in dairy systems cannot be addressed in isolation, integrated interventions targeting farm hygiene, rational antibiotic use, milk pasteurization and continuous surveillance are essential to mitigate this public health threat.

4.1 CONCLUSION

The ethanolic leaf extracts of neem plant had antibacterial potential against ESBL *Escherichia coli* but only at very high concentration of the extracts. Therefore, it could be a potential natural alternative or a partner drug in the treatment of infections caused by ESBL *Escherichia coli* transmitted to humans or animals. The results call for multisectoral One Health strategies that combine veterinary, environmental and public health efforts to control the dissemination of antimicrobial resistance from animal sources to humans ensuring food safety and the effectiveness of the antibiotics.

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