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Examination of Antimicrobial Resistance Genes among Multidrug Resistant *Klebsiella pneumoniae* and *Escherichia coli* Isolated from Local Herbal Drugs in Anambra, Nigeria

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Abstract

Background: Herbal medicines are derived from the plants or plant extracts containing therapeutic substances. There has been an overwhelming increase in the production and distribution of herbal medications, and the trend has instigated concern about the quality of these products, leading to various reports on the contamination of these herbal medicines by pathogenic microorganisms.

Objective: Hence, the aim of this research is to determine the antibiogram and antibiotic resistance genes of *Klebsiella pneumoniae* and *Escherichia coli* isolated from local herbal drugs in Anambra metropolis.

Methodology: Different samples of herbal remedies used for this study were randomly obtained from different markets in Anambra metropolis in order to identify *Escherichia coli* and *Klebsiella pneumoniae*. Identification of the bacterial isolates was accomplished by the observation of colonial characteristics, Gram reaction and biochemical tests results. The molecular characterization and antibiotic characterization of *Klebsiella pneumoniae* and *Escherichia coli* was determined using standard molecular techniques: DNA extraction and PCR identification techniques.

Results: The isolates showed general antimicrobial resistance to most of the antibiotics, with higher percentage of the *Klebsiella pneumonia* isolates exhibiting higher antimicrobial resistance than the *Escherichia coli* isolates. The SHV and RPO E genes were found in *Escherichia coli*, while the antibiotic resistant genes: SHV, RPO S AND RPO E were found in *Klebsiella pneumoniae*. However, the NDM, KPC, TEM, CTX M, AAC and OXA genes were not detected in any of these organisms.

Conclusion: This study confirms that herbal medicines are reservoirs for resistant enteric bacteria which possess genes for antibiotic resistance with significant public health implications.

Keywords: *Klebsiella pneumoniae, Escherichia coli*, Antibiotic resistance, Local herbal drugs

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Introduction

There is an overwhelming increase in the production and distribution of herbal medications, and the trend has instigated concern about the quality of the products being sold in Anambra State, Nigeria. The demand for these herbal products is increasing, and their prevalence use has been reported (Amaeze *et al*., 2018; Fakeye *et al*., 2019). Although the demand for herbal mixtures is high, research has pointed out the harmful effects (Akande-Sholabi *et al*., 2020; Dadzie *et al*., 2020; Oreagba *et al*., 2011). Enterobacterales are inhabitants of the intestinal flora and are among the most common human pathogens, causing infections such as cystitis, pyelonephritis, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections (Yeliz *et al*., 2021). Enterobacterales (CRE) over the past two decades has attracted worldwide attention, for its indication that most currently available broad-spectrum antibiotics may no longer be a therapeutic option for some patients. It is therefore mandatory to maintain the clinical efficacy of carbapenems (imipenem, ertapenem, meropenem, doripenem), which have become antimicrobial drugs of last resort (Shahid *et al*., 2022). Among these enterobacteriaceae, *K. pneumoniae* and *Escherichia coli* are the most prevalent.

Recent studies have examined the microbiological and phytochemical properties of herbal medications sold in Nigeria. These products often contain beneficial phytochemicals like alkaloids, flavonoids, and saponins (Okeke *et al*., 2021; Enemor *et al*., 2015). However, many samples were found to be contaminated with potentially pathogenic bacteria, including *E. coli*, *Staphylococcus* sp., and *Salmonella* sp. (Archibong *et al*., 2017; Ejimofor *et al*., 2023). Unregistered herbal preparations generally showed higher levels of microbial contamination compared to registered ones (Archibong *et al*., 2017). Despite contamination concerns, some herbal extracts demonstrated antimicrobial activity against multidrug-resistant bacteria, with inhibition zones ranging from 3-18 mm (Ndukwu and Akani, 2023).

K. pneumoniae and *Escherichia coli* are gram-negative bacteria capable of colonizing, invading, and causing infections in different anatomical sites of the human body. They are opportunistic pathogens that can cause Gastroenteitis, pneumonia, urinary tract infections, and bloodstream infections (Martin and Bachman, 2018).

In Nigeria, the antimicrobial resistance, rate of *K. pneumoniae* was highly comparable to that of *Escherichia coli*, which ranks it second among gram-negative bacteria infections (Nwajiobi *et al.*, 2026). The presence of both beneficial phytochemicals and potentially harmful microorganisms in these herbal products highlights the need for improved quality control and further research into their safety and efficacy (Ogbale, 2020). Multidrug resistance can be described as the acquired non-susceptibility to one or more antimicrobial agents (Idris and Nadvir, 2023). This is a major menace in the treatment of infections, as increasing number of organisms are becoming resistant to many antimicrobial agents (Nwozor *et al*., 2026). Therefore ascertaining the prevalence of multidrug resistance among organisms with major antibiotic resistance prevalence by assessing the presence of resistance genes will help provide information on designing more effective control measures. The purpose of the current study is to ascertain the microbiological assessment and the presence of antibiotic resistance genes present in *K. pneumoniae*, and *E. coli* isolated from the local herbal drugs sold by roadside herbal merchants.

Methodology

Study Area

The study was conducted at the laboratory of Applied Microbiology and Brewing of Nnamdi Azikiwe University in Awka, Anambra State. The State is situated in the South-East geopolitical zone of Nigeria and ranks as the 8th most populous state in the country, with over 6.3 million people. Awka is located between latitude 6.2220° N and longitude 7.0821° E.

Sample collection and Isolation of Microorganisms

A total of 72 samples of different local herbal drugs used for this study were randomly obtained from different locations in Anambra metropolis. The samples were labeled accordingly and transported to the laboratory for microbiological analysis. Each sample was thoroughly mixed by vigorous shaking, after which 1 ml was aseptically transferred into a test tube containing 9 ml of sterile distilled water. A 10-fold serial dilution was then performed for each herbal preparation following the protocol described by Okafor and Ugwuegbulem (2022). Pour plate technique was used for bacterial enumeration. The inoculated plates were incubated at 37 °C for 24 to 48 hours. The discrete colonies on the agar plates were selected and counted. Colony counts obtained on the media were expressed as colony forming units per millilitre (cfu/ml).

Characterization and Identification of bacteria

Identification of the bacterial isolates was accomplished by the observation of colonial characteristics, Gram reaction and biochemical tests (Chessbrough, 2006). The isolation of the microorganisms was achieved using media such as: Nutrient Agar for total bacteria enumeration, MacConkey agar and Eosin Methylene Blue (EMB) agar for *E. coli* and *Klebsiella pneumoniae.* The organisms were incubated at 37°C for 24 hours.The characterization of the isolates was performed, by employing Gram staining reaction, Catalase test, Citrate test, Sugar fermentation test, Coagulase test, Motility test, Oxidase test, Urease test, Indole test, Methyl Red and Voges proskauer test as described by Bergey's Manual of Determinative Bacteriology, 9th edition (1994).

Biofilm Assay

Sterile Congo Red dye solution was aseptically added (prepared as a 0.8% w/v concentrated stock solution and filter-sterilized). The dye solution was not autoclaved, as heat would degrade it. The solution was poured into sterile petri dishes and streak with (18-24 hour) bacterial cultures to be tested. The plates were incubated aerobically at 35-37°C for 24-48 hours. Black, shiny, crystalline colonies indicated strong biofilm production while Pinkish-red colonies indicated a negative result and no biofilm production.

Standardization of Inoculum for Antimicrobial Susceptibility Testing

The procedure for inoculum preparation was meticulously carried out in alignment with the standards set by the Clinical and Laboratory Standards Institute (CLSI), as referenced by Ishaya *et al*. (2025). Once the bacterial isolates had been correctly identified, pure colonies were obtained from cultures incubated for approximately 18 hours. Using a sterile inoculating loop, between two and three well-isolated colonies were aseptically transferred into 5 ml of sterile normal saline. The suspension was then gently mixed and visually compared against a 0.5 McFarland turbidity standard. When necessary, either additional bacterial inoculum or sterile saline was added incrementally to adjust the turbidity, thereby ensuring uniformity and accuracy in cell density prior to susceptibility testing.

Antibiotic Susceptibility Testing

This in vitro procedure began with the sub-culturing of the standardized inoculum onto the surface of freshly prepared Mueller-Hinton agar plates. Characterization of the microbial resistance pattern of the isolates involved the use of the Kirby-Bauer disk diffusion method, in which 15 commercial flter disks (CECON São Paulo, Brazil), each with a diferent antibiotic or combination of antibiotics were used as follows: Clavulanic Acid + Amoxicillin (AMC, 30 µg); Amikacin (AMI, 30 µg); Nalidixic Acid (NAL, 30 µg); Imipenem (IMP, 10 µg); Aztreonam (ATM, 30 µg); Ceftazidime (CAZ, 30 µg); Cefoxitine (CFO, 30 µg); Cefotaxime (CTX, 30 µg); Ceftriaxone (CRO, 30 µg); Ciprofoxacin (CIP, 5 µg); Cefepime (CPM, 30 µg); Ertapenem (ETP, 10 µg); Sulfamethoxazole + Trimethoprim (SUT, 25 µg); Gentamicin (GM, 10 µg); Meropenem (MER, 10 µg). The disks were placed at an appropriate distance from each other on Mueller-Hinton agar plates, previously spread, with the help of a cotton swab, with a bacterial suspension of 0.5 McFarland turbidity standard, followed by incubation for 24 h at 37◦C. Susceptibility and resistance to each antibiotic was determined following the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2020).

DNA Extraction

Materials:

Quick-DNA™ Miniprep Plus Kit (Zymo Research), Centrifuge (EPPENDORF, GERMANY), Vortex Mixer, Block Heater (WEALTEC CORP, TAIWAN), Microwave Oven (SCANFROST, CHINA), Pipettes, Digital Scale, Microcentrifuge Tubes, Gel Tank, Gel comb, Scientific Power Pack (CLEAVER SCIENTIFIC, TAIWAN), Gel Documentation System (VILBER, GERMANY). Genomic DNA was extracted using Quick-DNA™ Miniprep Plus Kit (Zymo Research), according to recommended protocol.

Protocol

200 µl of each sample was added to a microcentrifuge tube. 200 µl of BioFluid & Cell Buffer and 20 µl of Proteinase K was added to it and mixed thoroughly using a vortex for 10-15 seconds and then incubated the tube at 55ºC for 10 minutes on a heating block. 1 volume Genomic Binding Buffer (i.e. 420µl) was added to the digested sample and mixed thoroughly with a vortex mixer for 10-15 seconds. The mixture was then transferred to a Zymo-Spin™ IIC-XL Column in a Collection Tube and centrifuged at 12,000 x g for 1 minute. The collection tube was discarded with the flow through. 400µl DNA Pre-Wash Buffer was added to the spin column in a new Collection Tube and centrifuged at 12,000 x g for 1 minute. The collection tube was emptied and 700 µl g-DNA Wash Buffer was added to the spin column and centrifuged at ≥ 12,000 x g for 1 minute. The spin column was then transferred to a clean microcentrifuge tube. 50 µl of DNA Elution Buffer was added directly on the matrix and incubated for 5 minutes at room temperature, then centrifuged at maximum speed for 1 minute to elute the DNA. The eluted DNA was stored ≤ -20ºC for future use (Ribeiro *et al*., 2016)

PCR Protocol for the Target Genes

An exact quantity of 12.5µl of One Taq Quick-Load 2X was Master Mixed with Standard Buffer (New England Biolabs Inc.); 0.5µl each of forward and reverse primers was added. 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to thermalcycler.

Amplification conditions for the PCR was as follows: Initial denaturation for 5mins at 94ºC, followed by 32 cycles of denaturation at 94ºC for 30 secs, primer annealing was achieved at different temperatures for the different target genes: (55ºC, 54 ºC, 53 ºC, 60 ºC) for 60 secs and strand extension at 72ºC for 120 secs. Final extension at 72ºC for 5 min for 1 cycle on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 1.5% agarose gel and DNA bands were visualized with Ethidium bromide (Salam and Verma, 2019).

Table 1: Primers used for the PCR Amplification

Target Genes	Product size (bp)	Primer Name	Sequence (5’→ 3’)	Annealing Temperature
NDM	660	NDM –F NDM-R	5’-GGTGCATGCCCGGTGAAATC-3’ 5’- ATGCTGGCCTTGGGGAACG-3’	55
KPC	150	KPC-F KPC-R	5’- GCTCAGGCGCAACTGTAAG -3’ 5’- AGCACAGCGGCAGCAAGAAAG-3’	55
SHV	401	SHV F SHV R	5’- CGCCTGTGTATTATCTCCCT-3’ 5’- CGAGTAGTCCACCAGATCCT-3’	54
TEM	1060	TEM F TEM R	5’- TCAACATTTCCGTGTCG -3’ 5’- CTGACAGTTACCAATGCTTA -3’	55
CTX M	720	CTX M-F CTX M-R	5’- CGCTTTGCGATGTGCAG-3’ 5’- ACCGCGATATCGTTGGT-3’	55
AAC	520	AAC-F AAC-R	5’CAGGAATTTATCGAAAATGGTAGAAAAG-3’ 5’-CACAATCGACTAAAGAGTACCAATC-3’	55
RPO E	680	RPO E-F RPO E-R	5’- CGATAGCGGGATACTGGATAAG-3’ 5’- CTGCGATTGCGTTCCTAAAG -3’	53
RPO S	338	RPO S-F RPO S-R	5’-TCGCAGAGCAACTGGATAAG-3’ 5’- CAGGCCTTCAACCTGAATCT -3’	60
OXA	720	OXA-F OXA-R	5’- TCTTTCGAGTACGGCATTAGC-3’ 5’- CCAATGATGCCCTCACTTTCC-3’	55

Agarose Gel Electrophoresis

1.5% agarose gel was prepared by dissolving 1.5g of Agarose in 100ml of 1X TBE Buffer.

The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50 ºC. 3μl of Ethidium Bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray, with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and 1X TBE Buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed. 5 μl of amplicon was loaded into the wells. The tank was connected to the power pack and set to run at 120volts for 20 minutes after which it was viewed on a gel documentation system (Ding *et al*., 2020)

Results

A total of 40 enteric organisms were obtained from local herbal drinks sold by different vendors in Anambra state metropolis. The main organisms isolated and identified for the research were *Escherichia coli* and *Klebsiella pneumoniae*. The result on table 2 highlighted the biochemical identification of the isolated enteric bacteria, using gram reaction, colonial morphology and biochemical tests.

Table 2: Biochemical Properties of the Bacterial Isolates

Biochemical Characterization	***Eschericiha coli***	***Klebsiella pneumonia***
Colonial Morphology	Cocci	Rod
Gram staining	-ve	-ve
Catalase	+ve	+ve
Citrate	-ve	-ve
Urease	-ve	-ve
Methyl Red	+ve	-ve
Voges Proskauer	-ve	-ve
Biofilm	-ve	+ve
Motitlity	-ve	-ve
Glucose	+ve	+ve
Fructose	-ve	-ve
Lactose	+ve	+ve
Mannitol	-ve	+ve
Maltose	-ve	+ve

Among the ten antibiotics used against the isolates, a higher percentage of *Klebsiella pneumonia* exhibited antimicrobial resistance to the antibiotics than the *Escherichia coli isolates*, however both organisms showed general low resistance to AM, LEV and CPX.

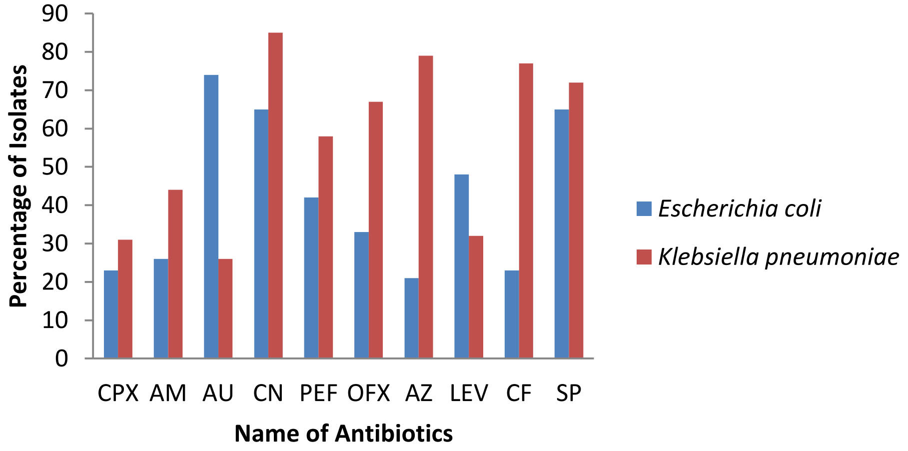

Figure 1: Percentage occurrence of Isolates to Antibiotic Resistance.

Key: CPX: Ciprofloxacin, AM: Amoxicillin, AU: Augmentin (Amoxicillin–Clavulanic acid), CN: Gentamicin, PEF: Pefloxacin, OFX: Ofloxacin, AZ: Azithromycin, LEV: Levofloxacin, CF: Ceftriaxone, SP: Sparfloxacin.

In this study, According to PCR gel electrophoresis presentation, *Escherichia coli* was positive for the antibiotic resistance gene, SHV and one stress gene: RPOE (Fig. 2), while *Klebsiella pneumoniae* was positive for the resistant gene: SHV and two antibiotic resistance stress genes: RPOE and RPOS (Fig. 3).

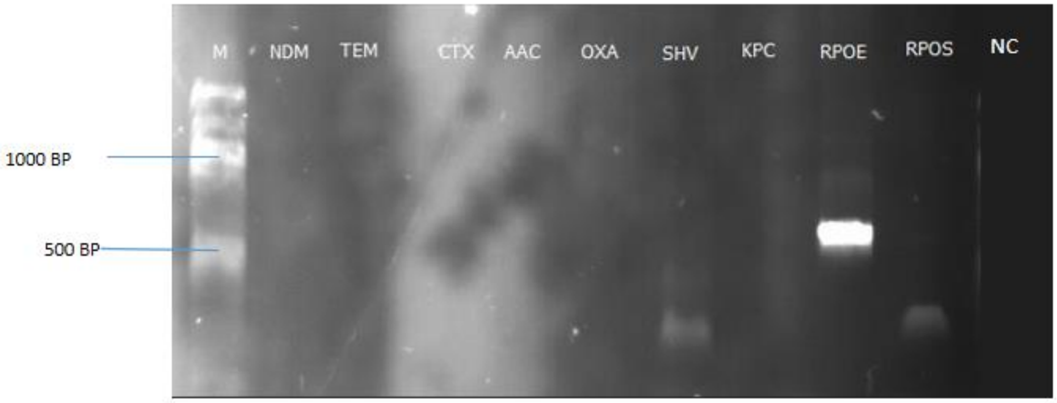

Figure 2: PCR Gel Electrophoresis for *Escherichia coli* resistance genes: SHV, RPOE, RPOS

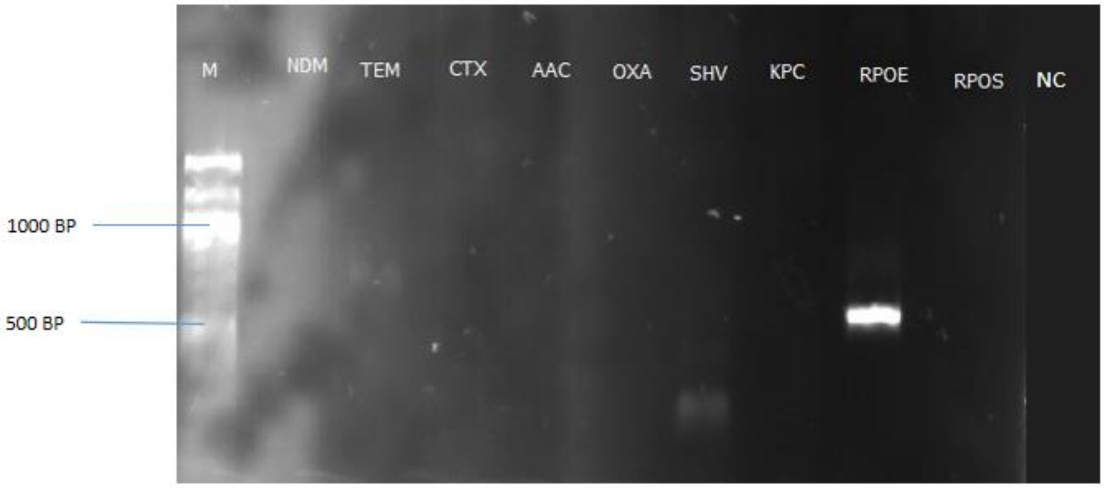

Figure 3: PCR Gel Electrophoresis for *Klebsiella pneumoniae* resistance genes: SHV, RPOE, RPOS

Table 3: Antibiotic Resistance Genes

Isolate	Sample source	NDM	KPC	SHV	TEM	CTXM	SHV	AAC	RPOE	RPOS	OXA
Klebsiella pneumoniae	Herbal drink	-	-	+	-	-	-	-	+	+	-
E. coli	Herbal drink	-	-	+	-	-	-	-	+	-	-

Discussion

The presence of the bacterial isolates: of *E. coli* and *Klebsiella pneumonia*, indicate that fecal contamination was the most probable route for the introduction of microorganisms. These findings differ from those of Nwankwo and Olime, who found no pathogenic bacteria in 60 herbal drug samples but consistently recovered Bacillus spp. Similarly, Ejimofor *et al*. (2023) reported *Bacillus subtilis*, *Staphylococcus* sp., *Salmonella* sp., and *E. coli* as contaminants of hawked herbal drugs in Onitsha, showing overlap with the present study involving *Klebsiella pneumoniae* and *E. coli* The diversity of isolates across studies could be as a result of differences in preparation methods, handling practices, and environmental sources of contamination.

The detection of *Escherichia coli* in this study is highly significant. Although some strains are harmless commensals, pathogenic variants such as enterohemorrhagic and enterotoxigenic strains are capable of causing severe gastrointestinal disease. The recovery of *Escherichia coli* has been widely documented in herbal preparations. De Sousa Lima *et al*. (2020) found *Escherichia coli* in 25.8% of herbal medicine samples, while Walusansa *et al*. (2022) reported *Escherichia coli* in 20.3% of samples sold in Kampala. Jamilu *et al*. (2020) observed *Escherichia coli* in 32.5% of herbal products, while Nwankwo and John also recorded *Escherichia coli* as the most frequent isolate at 21.9% of their samples. The repeated identification of *Escherichia coli* across diverse geographic locations strongly implicates fecal contamination as a persistent and widespread problem in the production and distribution of herbal medicines. cern.

The antibiotic susceptibility testing in Fig 1, revealed a concerning pattern of resistance. Most isolates demonstrated resistance to ciprofloxacin (CPX), amoxicillin (AM), augmentin (AU), gentamicin (CN), and ceftriaxone (CF). *E. coli* isolates were generally resistant, suggesting reduced efficacy of many commonly used antibiotics. These findings are comparable to those reported in earlier studies. Ahiabor *et al*. (2024) highlighted widespread resistance to β-lactams among bacterial isolates from herbal medicines. Ejimofor *et al*. (2023) found that while some herbal extracts inhibited bacterial growth, *E. coli* showed limited susceptibility, with inhibition zones between 6 and 14 mm. Nwankwo and John (2017) similarly reported that most isolates from herbal products were resistant to amoxicillin and chloramphenicol but showed susceptibility to levofloxacin (100%) and ceftriaxone (88.9%). Oshoma and Dijeh (2017) also observed resistance in *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* being the most sensitive. Previous and current studies confirm herbal medicines are reservoirs of multidrug-resistant organisms, with resistance patterns broadly consistent across different geographic studies.

Herbal products are often consumed raw or in minimally processed forms under the assumption of being safe and natural. The presence of drug-resistant pathogens not only increases the risk of direct infection but also contributes to the wider problem of antimicrobial resistance through horizontal gene transfer. This aligns with global reports that highlights herbal medicines as potential reservoirs for resistant bacteria when hygienic and quality control measures are lacking (Korir *et al*., 2017; Opuni *et al*., 2023).

Multidrug-resistant organisms are a major public health concern worldwide; of particular concern has been the emergence of resistance to carbapenem antimicrobial drugs among Enterobacterales (Awoke *et al*., 2021). Therefore, preventing transmission of carbapenemaseproducing, carbapenem-resistant Enterobacterales is a public health priority (Chidi and Ndukwu, 2023).

Carbapenemases have been slow to emerge in the Enterobacterales, but now their prevelance is increasing. They are notable for their diversity, including enzymes belonging to molecular classes such as KPC (Klebsiella pneumoniae carbapenemase), and SME, NDM. (Arpasiri *et al*., 2025).

In this study, *Escherichia coli* and *Klebsiella pneumoniae* both carried the SHV gene, this is similar to previous reports from fresh water in Thailand (Tansawai *et al*., 2019). The CTX-M and VEB were ESBLs, whereas SHV in *Klebsiella pneumoniae* and *Escherichia coli* could be narrow-spectrum β-lactamase (Castanheira *et al*., 2021).

RpoS (σS) is considered the second most important sigma factor in *E. coli*, controlling the transcription of genes associated with bacterial survival in stressful conditions and during the stationary phase. (Abram *et al*., 2021). It is important to note that while resistance to antibiotics is the most defnitive response to these toxic molecules, bacteria can also defend themselves through two other processes: tolerance and persistence...Valencia *et al*. BMC Microbiology (2024).

The occurrence of such resistant enteric bacteria in herbal drug samples raises important public health concerns. Herbal products are often consumed raw or in minimally processed forms under the assumption of being safe and natural. The presence of drug-resistant pathogens not only increases the risk of direct infection but also contributes to the wider problem of antimicrobial resistance through horizontal gene transfer. This aligns with global reports that highlights herbal medicines as potential reservoirs for resistant bacteria when hygienic and quality control measures are lacking (Korir *et al*., 2017; Opuni *et al*., 2023).

Conclusion

This study showed that the presence of Enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* point to fecal contamination as a likely source, which raises safety concerns. This is particularly because such products are often consumed without further treatment. The observed multidrug resistance patterns against commonly used antibiotics and resistance genes reveal the potential role of herbal drugs as reservoirs of resistant pathogens and the high risk of the spread of antibiotic resistant gene through horizontal gene transfer. These findings emphasize the importance of better hygiene, quality control, and regulation to protect consumers of herbal medicines in Nigeria.

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