**Antimicrobial Activity of *Pterocarpus santalinoides, Crescentia cujete,* and *Azadirachta indica*** **Leaf Extracts on Extended Spectrum Beta Lactamases (ESBL) producing *Escherichia coli***

**ABSTRACT**

**Aim**: The study investigates the antimicrobial activity of three selected plant leaf extracts—*Pterocarpus santalinoides, Crescentia cujete,* and *Azadirachta indica* on extended-spectrum beta-lactamase (ESBL) producing *E. coli* in clinical isolates from wound and stool specimens.

**Study area**: Clinical specimens, including stool and wound swabs, were collected from patients at Gregory University Clinics, Uturu.

**Study design and methodology**: Standard microbiological methods were employed for bacterial isolation and identification. ESBL-producing isolates were identified using screening and phenotypic confirmatory tests. Molecular analysis was conducted to detect ESBL genes in positive isolates, involving plasmid DNA isolation and PCR analysis. *Escherichia coli* was most frequently isolated from stool samples than wound samples.

**Results:** Fifty eight potential ESBL producing organisms were identified, of which 18 (39.1%) were positive by phenotypic confirmatory disc diffusion test (PCDDT) and 9 (37.5%) by double-disc synergy test (DDST) as the most *E. coli* ESBL producing organisms. The nine *E. coli* confirmed by double-disc synergy test (DDST) as potential ESBL producing isolates were subjected to ESBL genotyping using blaCTX−M, blaVEB , blaSHV and blaTEM. Only one (11.1%) had ESBL blaTEM and eight (88.9%) were negative to all the ESBL genes tested. The antimicrobial susceptibility of the plantsleaf extracts against the ESBL *E. coli* isolates showed that extracts of *Pterocarpus santalinoides* produced the highest zones of inhibition of 17.9 mm to 30.9 mm, followed by *Azadirachta indica* leaf extracts having 10.8 mm to 21 mm and the least zones of inhibition were produced by *Crescentia cujete* leaf extracts (8 mm to18.1 mm).

**Conclusion:** The findings suggest that the selected plant leaf extracts possess significant antimicrobial properties against ESBL-producing bacteria, highlighting their potentials as alternative therapeutic agents in the treatment of infections caused by multidrug-resistant organisms.

**Keywords:** antimicrobial activity,extended-spectrum beta-lactamase, plasmidDNA*,* antimicrobial susceptibility, genotyping, zones of inhibition

**1.0 INTRODUCTION**

Disease causing bacteria especially Extended Spectrum Beta Lactamase (ESBL) producing bacteria are spread worldwide and the emergence of antimicrobial resistance, in recent times, is considered one of the most critical public health issues worldwide (Chao *et al*., 2022). The appearance of antibiotic-resistant gram-negative microorganisms paved way for the occurrence of infections that can only be treated by a limited number of antimicrobial agents, and this has become a major challenge for antimicrobial therapy. Bacterial resistance to antimicrobial agents is a medical problem with public health, socioeconomic, and even political implications (Abdel-Massih *et al*., 2010).

Among bacterial pathogens, extended-spectrum beta-lactamases have been recognized as a significant contributing factor in antimicrobial resistance, particularly in hospital settings. ESBL-producing bacteria, usually found in infections like that of wounds and stools, have demonstrated resistance against a wide range of beta-lactam antibiotics, hence limiting therapeutic choices and increasing morbidity and mortality rates (Chang *et al*., 2022). While synthetic antibiotics are in the phase of their development, the appearance of resistant strains often requires the search for alternatives by investigating natural antibacterial agents.

Recently, plant-based traditional medicines from diverse cultures have aroused scientific interest due to their potential to yield novel antimicrobial compounds. Various leaf extracts from plants were assessed against multidrug-resistant bacteria (Ekeleme *et al*., 2013) and they were found to exhibit antibacterial properties (Ekeleme *et al*., 2024), however, only a few of these studies focused on their action against pathogens that produce ESBLs. *Pterocarpus santalinoides* exhibits strong antimicrobial activity against bacteria such as *S. aureus, E. coli,* and *B. subtilis* with ethanol extracts of its leaves (Mphande et al., 2022). *Crescentia cujete* leaves show extraordinary antimicrobial efficacy with 94% inhibition of microbial growth on cultures (Parvin *et al.,* 2015). *Azadirachta indica* has been found to have broader antimicrobial activity. *A. indica* is most researched based on areas like dentistry and food preservation, with the implications of combating diverse microbes (Wylie and Merrell., 2022). Determining the action of these selected plant extracts on such bacteria would hopefully bring useful results to alternative therapies and contribute toward solving the problems associated with antimicrobial resistance (AMR).

Therefore, the aim of this work was to study the antibacterial potential of three different plant leaf extracts against *E. coli* isolates of ESBL-producing organisms derived from wound and stool specimens.

**2.0 MATERIALS AND METHODS**

**2.1** **Study designs**

An experimental study design was adopted for this study which enabled a one-time assessment that determined the antimicrobial activity of three selected plant leaf extracts on extended spectrum beta lactamases (ESBL) of *E coli* isolates from wound and stool specimens.

**2.2 Area of the study**

The area of study was Gregory University Clinic, Uturu, Abia State, Nigeria. The Gregory University Clinic is a Primary healthcare institution, typically offering a broad range of medical services, including specialized care, research, and training. It is affiliated to Government General Hospital, Amachara, Abia State.

**2.3 Study Population**

The population under consideration produced 200 samples. Hundred (100) samples each were collected from stool and wound specimens of patients visiting Gregory University Clinic. The study involved the collection and analysis of specimens from these samples. The focus is on isolating the extended spectrum beta lactamases (ESBL) *E coli* isolates from wound and stool specimens. These samples were collected from various patients in the hospital.

**2.4. Determination of Sample size**

A total of two hundred (200) samples were collected from the patients visiting the clinic.

The sample size was determined with the use of the formula (n= (Z/2)2 P (1- P) /d2). By reviewing similar previous studies, the maximum sample size was obtained from a study conducted in southern Nigeria, with a prevalence/proportion of stool and wound infections was 85.0% (0. 85) (Ekeng *et al*., 2021).

As a result, n= (Z/2)2 P (1- P) /d2 with a margin of error (d = 0.05) and a 95% confidence interval. p = 0.281, d = 0.05, Z = 0.05 = Z/2 = 0.025 = 1.96.

As a result, n= (1.96)2x 0.85(1-0.85)/ (0.05)2 =195.92 = 196

Then, adjusting for a 2% non-response rate, the minimum sample size would be 199.92 = 200

**2.5 Sampling Technique**

A purposive sampling technique was adopted in the selection of the patients with wound and those who came for bacterial stool analysis. A total of two hundred (200) samples were collected using sterile swab stick for wound and stool using sterile container. The 200 samples were divided into two (2) groups. This gave hundred (100) samples for each group. Subsequently, these samples were analysed to detect the presence of Extended-Spectrum Beta-Lactamase (ESBL)-producing bacteria.

**2.6 Collection of Plant leaf and preparation of powder**

The leaves of *Pterocarpus santalinoides, Crescentia cujete* and *Azadirachta indica* were obtained from Uturu, Abia State, Nigeria and were authenticated at the Department of Biology, College of Natural and Applied Sciences of Gregory University, Uturu. The leaves were washed and air dried at room temperature and then pulverized using electric blender to obtain powder. The fine powder obtained was stored in sterile air-tight containers in a dark place to prevent oxidation and for further extraction procedure.

**2.7 Bacterial Isolation and Characterisation**

The clinical samples collected were inoculated into sterile petri dish plates using appropriate media. The plates were incubated at 37°C for 18-24 hours. Bacterial isolates were sub-cultured to get the pure cultures which were characterized and identified based on their motility, microscopic and colonial morphologies, gram staining reaction, biochemical tests including catalase test, methyl red test - voges proskauer (MR-VP) test, nitrate reduction test, starch hydrolysis, gelatin liquefaction test, indole test, oxidase test, urease test, triple sugar iron agar (TSI) and sugar fermentation tests etc as described in medical laboratory manual for tropical countries with reference to the Bergey's manual of systemic bacteriology (Krieg and Holt, 1994; Cheesebrough, 2005).

**2.8 Antibiotic Susceptibility Test**

Antibiotic susceptibility was determined by the Kirby Bauer disc diffusion method as described by National Committee for Clinical and Laboratory Standard Institute (Shahanara et al.,2013). Bacteria were grown on nutrient broth at 37°C for 18-24 hrs and the suspension was visually adjusted with normal saline to equal that of 0.5 MacFarland turbidity standards. The inocula were swabbed across the entire surface of Mueller-Hinton agar plate using sterile swab sticks. Inoculated plates were left to stand for at least 3 minutes and no longer than 15 minutes before the disks were applied. Commercial antibiotics disks used were: Ceftazidime (30ug), Cefotaxime(30ug), Cefuroxime(30ug), Aztreonam (30ug),Cefotetan (30ug), Ceftriaxone (30ug), amoxicillin/clavulanic acid (30ug), Gentamicin (10ug),Kanamycin (10ug), Ciprofloxacin (5ug), Ofloxacin (5ug)imipenem (10ug), Nitrofurantoin (300ug), Sulfamethoxazole/trimethroprim (30ug), Piperacillin (30ug), (TOKU-E, USA). The plates were incubated for 15 minutes at 37°C for 18 to 24 h. The inhibition zone (in diameters) around the disks were measured and interpreted according to the NCCLS guidelines (Shahanara et al*.,*2013). Isolates were considered as multidrug resistance (MDR), when it showed resistance to ≥3 antimicrobial agents (Gayathri et al*.,*2011).

**2.9 Detection of Extended Spectrum Beta Lactamase**

Three methods were used: CLSI screening method, CLSI phenotypic confirmatory method and double disk diffusion synergistic method were used.

**2.9.1 Screening Test For ESBL**

Ceftazidime, ceftriaxone, aztreonam, cefuroxime, cefotetan and cefotaxime disks were placed on a Mueller-Hinton agar plates that have been inoculated with the test organisms. The plates were incubated aerobically overnight (18-24 hours) at 37°C. Isolates showing an inhibition zone size of ≤22 mm with ceftazidime (30 μg), ≤25 mm with cefriaxone (30 μg) and ≤27 mm with cefotaxime (30 μg) were identified as potential ESBL producers and was selected for phenotypic confirmation of ESBL production (Giriyapur *et al.,*2011).

**2.9.2 Phenotypic Confirmatory Test for ESBL**

Isolates that were suspected to be ESBL producer by screening method based on their susceptibility to the cephalosporins were confirmed for enzyme production by phenotypic confirmatory disk diffusion method. Ceftazidime disk without clavulanic acid and ceftazidime with clavulanic acid combination disk was placed on the same plates. The plates with disks were incubated aerobically overnight (18-24 hours at 37°C). The isolates showing an increase in zone size of 5mm or more around ceftazidime with clavulanic acid as compared to ceftazidime alone was confirmed to be ESBL producer. No enhancement of zone indicates ESBL non-producer isolates (Giriyapur *et al.,* 2011).

**2.9.3 The Double Disc Synergy Test (DDST)**

The double disc synergy test (DDST) method described by CLSI (2013) was employed. Standardized inoculum of the test organisms were inoculated on Mueller-Hinton agar (MHA) (BIOTECH, England) using sterile swab sticks. Amoxicillin/clavulanic acid disc (20/10μg)(TOKU-E, USA) was placed at the center of the inoculated MHA. Ceftazidime (30ug), (TOKU-E, USA) and Cefotaxime (30ug), (TOKU-E, USA) were placed 15mm centre to centre from the Amoxycillin/clavulanic acid discs. The plates was incubated at 37°C for 24 hours. After incubation, enhancement of zone of inhibition of either or both the Ceftazidime and Cefotaxime discs towards the Amoxycillin/Clavulanic acid discs is indicative of ESBL production.

**2.10 Molecular analysis of ESBL positive *E. Coli*.**

The molecular analysis was involved isolation of plasmid DNA, detection of plasmid DNA molecular weight and PCR analysis of ESBL genes.

Plasmid DNA from the ESBL positive isolates of *E. coli* species were obtained using alkaline lysis method (Birnboim, 1983). Cells was grown overnight at 37oC in nutrient broth in a 1.5 ml micro tube. The growth was harvested by centrifuging at 3,000 rpm for 10 mins. The pelleted cells were then washed with sterile water at 5,000 rpm for 10 min. It was then suspended in 200µl of suspension buffer and mixed by vortexing. Lysing solution of two times the volume of sample was added into the mixture and solution were mixed by inserting gently (x5) for 5 min on ice. It was followed by the addition of potassium acetate (x 2/3 vol.) to the lysate and the content placed on ice. The solution was centrifuged at 10,000 rpm for 10 min and the clear supernatant was transferred into a new tube. Then 2x Vol. of ice cold absolute ethanol was added and placed on ice for 2 hr. The mixture was then centrifuged at 10,000 rpm for 10mins and the supernatant decanted. Following this, the pellets were washed twice in 70% ethanol. The pellets were allowed to air-dry for 2 hours. The extracted plasmid DNA were reconstituted in 30µl of Tris EDTA buffer and stored at 4 oC prior to gel electrophoresis.

For the electrophoresis, 0.8% agarose were prepared by weighing 0.8 g of agarose powder with a weighing balance. The powder were mixed with 100ml of electrophoresis buffer and then heated in a microwave oven until completely melted. Ethidium bromide was added to the gel at a final concentration of 0.5µg/ml to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60oC, it was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed, 20µl of the plasmid DNA samples was then loaded into the wells after mixing with 2µl of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The plastic tray with the gel was inserted horizontally into the electrophoresis chamber and covered with buffer. The current 70V was applied for 1hr 30 min. The distance DNA migrated in the gel was judged by visually monitoring migration of the tracking dyes. The DNA were visualized by placing the gel in an ultraviolet transilluminator in a photo documentation system (Clinix Japan, Model 1570). The size of the visible bands obtained was calculated by matching that of the isolates with the standard bands produced by HIND III marker.

The ESBL genes namely: SHV, TEM and CTX-M were detected by conventional PCR. During the PCR analysis, the concentration of extracted DNA were assessed by spectrophotometer and the reaction was done in a total volume of 25 microliters by using Master Mix Red, Taq DNA polymerase with MgCl2, amplicon (Bell *et al.,*2007). The oligonucleotide primers were used for the amplification.

List 1 : The primers, its sequences and sizes in bp are as follows

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers** | **Forward** | **Reverse** | **Band size** |
| **VEB** | GAT GGT GTT TGG TCG CAT ATC GCA AC | CAT CGC TGT TGG GGT TGC CCA ATT TT | 391 |
| **CTX-M** | ATG TGC AGY ACC AGT AAR GTK ATGGC | GGT RAA RTA RGT SACC AGA AYC AGC GG | 590 |
| **SHV** | TGT ATT ATCTC(C/T) CTG TTA GCC(A/G) CCCTG | GCT CTG CTT TGT TAT TCG GGC CAA GC | 739 |
| **TEM** | TCG CCG CAT ACA CTA TTC TCA AGA ATGAC | CAG CAA TAA ACC AGC CAG CCG GAA G | 422 |

**DNA sequencing by Sanger sequencing method and Blast analysis (CLC BIO and NCBI blast online)**

DNA sequencing was performed by Sanger (dideoxy) Sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems (Russell, 2002; Metzenberg, 2003). This result was obtained as nucleotides. Sequence analysis from resultant nucleotides base-pairs was performed by BLAST analysis by using CLO Bio software and by direct blasting on <http://blast.ncbi.nlm.nih.gov>.

For every set of isolate, a read was BLASTED and the resultant top hits for every BLAST result showing species name was used to name the specific organism.

**2.11 Antimicrobial Activity of the Plant Extracts**

The antimicrobial activity of the plants extract (Cold water, Hot Water and methanol) were determined by agar well-diffusion method according to Ahmed et al*.*, (1998). 0.5 McFarland standard (approx. 108cfu/mL) were prepared using the test organisms and 0.1ml of the bacteria species were mixed in Mueller Hinton Agar medium and poured in pre-sterilized Petri plates. A cork borer (6mm diameter) were used to punch wells in solidified medium and were filled with extracts of 50µl of 500mg/ml final concentration of extracts. Selected solvent (i.e. cold water and hot water) were used as negative control. The efficacies of the extracts against the bacteria were compared with the broad spectrum antibiotic Chloramphenicol (positive control). The plates were incubated at 37oC for 24hrs in an incubator and the diameters of the zones of inhibition were measured in millimeter. Each sample was assayed in triplicate and the mean values were observed. The antimicrobial activity were interpreted from the size of the diameter of zone of inhibition measured to the nearest millimeter (mm) as observed from the clear zones surrounding the wells.

**ETHICAL CONSIDERATION AND CONSENT**

Ethical clearance was obtained from Gregory University Uturu hospital Imo State. Informed consent was also obtained from patients. A detailed explanation as regards the nature of the study was given to the patient, parents of subjects eligible for the study, and approval was obtained before collection of their specimen. They were also assured of absolute confidentiality.

**3.0 RESULTS AND DISCUSSION**

In this study, the results of the investigation into the antimicrobial activity of three selected plant leaf extracts against *Escherichia coli* as an extended-spectrum beta-lactamases (ESBLs) from wound and stool specimens were presented. The outcomes of our research shed light on the potential of these natural compounds as a source of antimicrobial agents in the fight against *Escherichia coli* ESBL-producing pathogens.

The *Escherichia coli* was isolated from patients’ stool and wound samples following morphological, biochemical and cultural characteristics of the microorganisms are represented in Table 1.

**Table 1: Morphology and biochemical characterization of** *Escherichia coli* **from clinical samples**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Microscopy | | | | Biochemical Reactions | | | | | | | | | | Carbohydrate  utilization | | | | | |  |
| **COLONY FEATURES** | Cell Arrangement | spore | Motility | Capsule | Catalase | Oxidase | Coagulase | Indole | Nitrate | MethylRed | V.P | Urease | H2S | citrate | Glucose | Sucrose | Lactose | maltose | mannitol | xylose | Identity |
| Small pink shiny smooth colonies on MacConkey Agar (MA). | Gram negative short rods, singles and some in groups | - | + | - | + | - | - | + | - | + | - | - | - | - | + | ± | + | + | + | + | *Escherichia coli* |

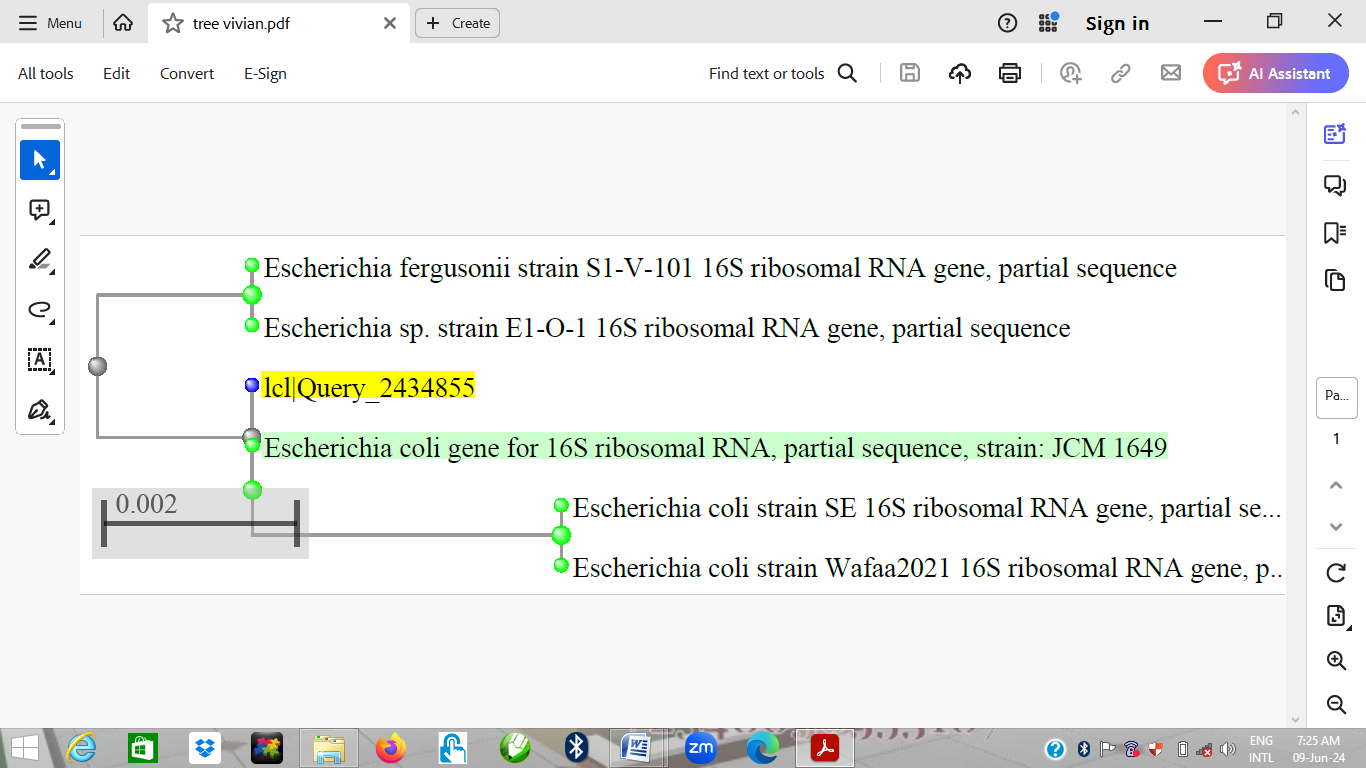
**Key:** + = Positive, - = Negative, V.P = Voges-Proskauer, NA = Nutrient Agar, MA = MacConkey Agar

**The nucleotides sequence of the** *Escherichia coli* **ESBL isolate is** presented in Table 2 based on 16S rRNA.

The blasting of the sequence results was done using the online blast software at <http://blast.ncbi:nlm.nih.gov/blast.cgi>. The result for every set of isolate was taken from the top hit of the blast showing species name and the strain number. Following this, the bacteria identified was *Escherichia coli (strain* JCM 1649)*.*

|  |  |  |  |
| --- | --- | --- | --- |
| **Table 2: Nucleotides sequence of the ESBL isolates** | | | |
| Sample code | Isolation code | Gene sequence **(**nucleotides obtained**)** | Organism/  strain code |
| 1231 | S10 | ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTAGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAG | *Escherichia coli strain* JCM 1649 |

The result of the relatedness of the isolated strains compared by neighbor-joining tree based on the 16S rRNA gene sequences of the strains of the isolate and other related taxa is presented in Figure 1



92

95

100

98

97

Fig. 1: Neighbour-joining tree based on 16S rRNA gene sequences of *Escherichia coli* strain JCM 1649 and other related taxa. Bootstrap values >50%, based on 1000 subsets, are given at branch points. Bar. 0.002 substitutions per nucleotide position, values are percentage relatedness.

*Escherichia coli* was frequently isolated from stool samples than in the wound samples (Table 3). The Chi-square test (χ2 cal. = 14.30; χ2 tab. = 7.82, df = 3) indicates a statistically significant difference between the samples (p < 0.05).

**TABLE 3: Frequency of *Escherichia coli*****from the various clinical samples collected from Gregory University Hospital**.

|  |  |  |  |
| --- | --- | --- | --- |
| Clinical samples | No of samples collected | *Escherichia coli* | Χ2  Cal. |
|
| Stool | 100 | 32 | 14.30 |
| Wound swab | 100 | 26 | 21.96 |

The screening for extended spectrum beta lactamase (ESBL) using ceftazidime, ceftriaxone, aztreonam, cefuroxime and cefotaxime discs are shown in Tables 4. *Escherichia coli* that showed an inhibition zone size of ≤22 mm with ceftazidime (30 μg), ≤25 mm with cefriaxone (30 μg) and ≤27 mm with cefotaxime (30 μg) were identified as potential ESBL producers (positive) and were phenotypically confirmed for ESBL production.

The *Escherichia coli* isolated from stool and wound samples as potential ESBL producers had 34.4% and 65.4% for ceftriaxone respectively and were less positive for cefuroxime. The number of *Escherichia coli* isolates from both the stool sample and the wound swab, the calculated Chi-square values–χ2 = 0.98 (stool sample) and χ2 = 4.28 (wound swap)–were less than the tabulated Chi-square value (χ2 = 9.49, df = 4), indicating that the differences in the number of *Escherichia coli* isolates from the various screened clinical samples detected by different antibiotics were not statistically significant (p > 0.05).

Table 4: Number of *Escherichia coli* isolates from various clinical samples screened for potential ESBL producers

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Antibiotics used | | | | | | | | | | | |
| Clinical samples | No. of isolates screened | **Ceftazidime** | | **Ceftriaxone** | | **Aztreonam** | | **Cefotaxime** | | **Cefuroxime** | | Χ2-cal.  + (-) |
| Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| Stool | 32 | 10 (31.3) | 22 (68.8) | 11(34.4) | 21(65.5) | 9 (28.1) | 23(71.9) | 10 (31.3) | 22(68.8) | 7 (21.9) | 25 (78.1) | 0.98(0.41) |
| Wound swab | 26 | 11 (42.3) | 15(57.7) | 17 (65.4) | 9(34.6) | 12 (46.2) | 14(53.8) | 16 (61.5) | 10(38.5) | 8 (30.7) | 18(69.2) | 4.28(4.15) |

ESBL = Extended spectrum β-lactamases

The *Escherichia coli* isolates that were identified as potential ESBL producers were confirmed for enzyme production by phenotypic confirmatory disk diffusion method. Ceftazidime (30 μg) disk without clavulanic acid and ceftazidime with clavulanic acid (30 μg/10 μg) combination disk were placed on the same plates. The isolates that showed an increase in zone size of 5mm or more (≥5 mm) in the presence ceftazidime with clavulanic acid than ceftazidime alone were interpreted as ESBL producer. No enhancement of zone indicated ESBL non-producer isolates.

The double disc synergy test (DDST) method was also employed. Standardized inoculum of the test organisms were inoculated on Mueller-Hinton agar (MHA) (BIOTECH, England) using sterile swab sticks. Amoxicillin/clavulanic acid disc (20/10μg) was placed at the centre of the inoculated Mueller-Hinton agar (MHA). Ceftazidime (30ug) and Cefotaxime (30ug) were placed 15 mm away from the Amoxycillin/clavulanic acid discs. Extension of zone of inhibition of either or both the Ceftazidime and Cefotaxime discs towards the Amoxycillin/Clavulanic acid discs was interpreted as ESBL producer.

The 58 potential ESBL producing organisms were identified, 18 (39.1%) were positive by phenotypic confirmatory disc diffusion test (PCDDT) and 9 (37.5%) by double-disc synergy test (DDST) as the most *E. coli* ESBL producing organisms (Table 5).

|  |  |  |  |
| --- | --- | --- | --- |
| **Table 5: Number of ESBL producers among the Potential ESBL producing isolates (%)** | | | |
| **Organisms** | Potential ESBL producers | PCDDT (ESBL) (%) | DDST (ESBL) (%) |
| *Escherichia coli* | 58 | 18 (39.1) | 9 (37.5) |

KEY: ESBL = Extended spectrum β-lactamases, PCDDT= Phenotypic confirmatory disc diffusion test, DDST= double disc synergy test

### The nine *E. coli* confirmed by double-disc synergy test (DDST) as potential ESBL producing isolates were subjected to ESBL genotyping using blaCTX−M, blaVEB , blaSHV and blaTEM. Only one (11.1%) had ESBL blaTEM and eight (88.9%) were negative to all the ESBL genes tested (Table 6)

### Table 6: ESBL genotyping using blaCTX−M, blaVEB , blaSHV and blaTEM genes

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S/N** | **LAB I.D** | **VEB** | **CTX-M** | **SHV** | **TEM** |
| 1. | *Escherichia coli* | - | - | - | + |

The antimicrobial susceptibility of the plantsleaf extracts against the ESBL *E. coli* isolates is shown in Table 7. The extracts of *Pterocarpus santalinoides* produced the highest zones of inhibition of 17.9 mm to 30.9 mm, followed by *Azadirachta indica* leaf extracts having 10.8 mm to 21 mm and the least zones of inhibition was produced by *Crescentia cujete* leaf extracts (8 mm – 18.1 mm).

|  |  |  |  |
| --- | --- | --- | --- |
| Table 7: Antimicrobial susceptibility of the plants leaf extracts against the ESBL *E. coli* harboring **blaTEM** gene | | | |
|  | Zone of inhibition diameter (mm) | | |
| Plants leaf extracts | Methanolic (50µl) | Aqueous extract (hot water) (50µl) | Aqueous extract (cold water)  (50 µl) |
| *Azadirachta indica* | 21.0±0.9 | 11.0±0.7 | 10.8±0.3 |
| *Pterocarpus santalinoides* | 30.9±0.1 | 24.5±0.2 | 17.9±0.1 |
| *Crescentia cujete* | 18.1±0.2 | 10.0±0.2 | 8.0±0.1 |

Interpretative standard: Clinical and Laboratory Standard Institute (CLSI) (2006).

This study investigated the antibacterial activity of three selected plant leaf extracts on extended spectrum beta lactamases *E. coli* from wound and stool specimens. The outcomes of our research shed light on the potential of these natural resources as a source of antimicrobial agents in the fight against ESBL-producing pathogens.

*Escherichia coli* was the most frequently isolated organism from stool samples. The finding supports the report of Alikhani et al (2013), who noted that pathogenic strain of Escherichia coli are a common cause of acute infectious diarrhoea. However, pathogenic strains can cause gastrointestinal diseases as reported by Mueller & Tainter (2023).. Wanke & Sears (2007) showed that pathogenic strains of *E. coli*, such as *E. coli* O157:H7, can cause severe diarrhoea, urinary tract infections (UTIs), and other complications.

The antibiotic resistance profiles of *Escherichia coli* exhibit significant variability; particularly between different sample types and bacterial species Recent studies highlight substantial differences in the resistance profiles of *Escherichia coli* isolates from stool and wound samples. In stool samples, 34.4% of *E. coli* isolates were identified as potential extended-spectrum beta-lactamase (ESBL) producers for ceftriaxone. This contrasts sharply with wound samples, where 65.4% of *E. coli* isolates tested positive for ceftriaxone resistance. A study by Nisha et al. (2022) supports the observation of higher resistance rates in wound samples. The research reported that *E. coli* isolates from wound infections showed elevated resistance to multiple antibiotics, including ceftriaxone, due to increased exposure to healthcare-associated infections and antibiotic use. Conversely, stool samples, often representing community-acquired infections, may have lower antibiotic pressure, resulting in comparatively lower resistance rates. A study by Kumar et al. (2021) found similar patterns, with stool isolates exhibiting lower resistance rates to ceftriaxone than those from clinical settings like wound infections. Resistance to critical antibiotics such as ceftazidime, ceftriaxone, and aztreonam limits effective antimicrobial therapy options. This scenario often necessitates the use of alternative antibiotics, such as carbapenems. Studies by Paterson and Bonomo (2023) emphasize the clinical dilemma posed by ESBL-producing bacteria, highlighting the increased reliance on carbapenems. However, this can promote further resistance, including carbapenem-resistant Enterobacteriaceae (CRE), a growing public health concern. Research is ongoing to explore alternative treatment strategies, such as combination therapy and novel antibiotics. For instance, the study by Stone et al. (2023) investigates the efficacy of newer beta-lactam/beta-lactamase inhibitor combinations against ESBL producers, showing promise in overcoming resistance.

The Double Disk Synergy Test (DDST) employs amoxicillin/clavulanic acid, ceftazidime, and cefotaxime discs placed on Mueller-Hinton agar. The extension of the inhibition zone towards the clavulanic acid disc indicates ESBL production. In the discussed study, 37% of the isolates were identified as ESBL producers using DDST. Studies have shown varying effectiveness for DDST. A study by Jain et al. (2021) reported a detection rate of 15% using DDST, which is lower than the 37% found in the current study.

The nine *E. coli* confirmed by double-disc synergy test (DDST) as potential ESBL producing isolates were subjected to ESBL genotyping using blaCTX−M, blaVEB , blaSHV and blaTEM. Only one (11.1%) had ESBL blaTEM and eight (88.9%) were negative to all the ESBL genes tested. Recent research studies have provided additional insights into the prevalence and distribution of ESBL genes in clinical isolates, revealing similar and contrasting findings. A study by Shaikh et al. (2015) in a tertiary care hospital reported that out of 200 ESBL-producing isolates, 42.5% harbored blaTEM, 35.5% harboured blaSHV, and 22% harboured blaCTX-M genes. This distribution suggests a higher prevalence of blaTEM genes, which aligns with our findings, although our study did not test for blaCTX-M genes. In another study by Naseer and Sundsfjord (2011), 150 ESBL-producing Enterobacteriaceae isolates from different clinical samples showed that 60% of the isolates had blaCTX-M, 20% had blaTEM, and 10% had blaSHV genes. This indicates a higher prevalence of blaCTX-M genes compared to blaTEM and blaSHV, contrasting with our results where blaTEM was more common. The variation could be attributed to geographical differences, sample types, and the methodologies used. A study by Pitout and Laupland (2008) highlighted the increasing prevalence of blaCTX-M genes in ESBL-producing Enterobacteriaceae globally. They found that blaCTX-M was the most common ESBL gene, followed by blaTEM and blaSHV. The predominance of blaCTX-M in their findings differs from our study, where blaTEM and blaSHV were more prevalent. Contrastingly, a study conducted in the United States by Mendes et al. (2014) on ESBL-producing Klebsiella pneumoniae found that 70% of the isolates carried blaSHV, while only 20% had blaTEM and 10% had blaCTX-M genes.

The antimicrobial susceptibility of the plantsleaf extracts against the ESBL *E. coli* isolates showed that *Pterocarpus santalinoides* produced the highest zones of inhibition of 17.9 mm to 30.9 mm, followed by *Azadirachta indica* leaf extracts having 10.8 mm to 21 mm and the least zones of inhibition was produced by *Crescentia cujete* leaf extracts (8 mm – 18.1 mm).Recent studies by Okeke et al. (2023) corroborate these findings, demonstrating that methanolic extracts of *Pterocarpus santalinoides* show superior antimicrobial activity compared to aqueous extracts. Their research reported inhibition zones of 31 mm against *Klebsiella* species, closely aligning with the current study’s results. Conversely, Johnson et al. (2022) found that cold water extracts had limited antimicrobial activity, with inhibition zones not exceeding 20 mm against any tested isolates. This supports the observed lower efficacy of the cold water extract in the current study.A study by Singh and Agrawal (2023) indicated similar trends, with methanolic extracts of *Azadirachta indica* showing high antimicrobial activity. Research by Garcia et al. (2023) supports these results, showing that methanolic extracts of *Crescentia cujete* can produce inhibition zones of up to 28 mm against *Klebsiella* species. Across the three plant species, methanolic extracts consistently exhibited higher antimicrobial activity against ESBL isolates compared to cold water extracts. Specifically, *Pterocarpus santalinoides* methanolic extract showed the highest overall efficacy, followed by *Crescentia cujete* and *Azadirachta indica*. The superior performance of methanolic extracts suggests that organic solvents might be more effective in extracting bioactive compounds with antimicrobial properties. These findings underscore the potential of methanolic plant extracts as alternative treatments against antibiotic-resistant bacteria. The varying efficacy of different extracts highlights the complexity of plant-based antimicrobial research. Further studies are needed to identify the specific bioactive compounds responsible for the antimicrobial activity and to understand their mechanisms of action. Additionally, evaluating the safety and potential side effects of these extracts is crucial for their clinical application.

**4.0 CONCLUSION**

This study investigated the antibacterial activity of plant leaf extracts against extended spectrum beta-lactamases (ESBL) in *E. coli* isolates from wounds and stools. *Escherichia coli* had high resistance to multiple antibiotics. Methanolic extracts of *Pterocarpus santalinoides* exhibited the highest antimicrobial activity. This study highlights the potential of these plant extracts as sources of novel antimicrobial agents against ESBL *E coli* antibiotic-resistant infections.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**REFERENCES**

Abbas, R., Chakkour, M., Obaseki, E. F., Obeid, S. T., Jezzini, A., Ghssein, G., & Ezzeddine, Z. (2024). General Overview of Klebsiella pneumonia: Epidemiology and the Role of Siderophores in Its Pathogenicity. *Biology*, *13*(2), 78.

Ahmed, B. O., Omar, A. O., Asghar, A. H. and Elhassan, M. M. (2013).Increasing Prevalence of ESBL- Producing Enterobacteriaceae in Sudan Community Patients with UTIs.*EgyptianAcademic Journal of Biological Science,***5**: 17-24.

Ahmed, I., Mehmood, Z. and Mohammad, F. (1998). Screening of some Indian medicinal plants for their antimicrobial properties. *Journal ofEthnPharmacology., 62*: 183-189.

Ahuchaogu A. C. Nwaokoro and B. C. Onyekwere [2023   
gas chromatography mass spectrometry and fourier infraredspectroscopy characterization of the bioactive components ofmethanolic plant extract of Pterocarpus santalinoides. *Journal of Chemical Society of Nigeria,48*, (6,) 1113 - 1128]1113

Alikhani, M. Y., Hashemi, S. H., Aslani, M. M., & Farajnia, S. (2013). Prevalence and antibiotic resistance patterns of diarrheagenic Escherichia coli isolated from adolescents and adults in Hamedan, Western Iran. *Iranian Journal of Microbiology*, *5*(1), 42-47.

Alsamawi M, Joudeh AI, Eldeeb Y,Al-Dahshan A, Khan F, Ghadban W,Almaslamani M and Alkhal A (2022). Epidemiology of extended-spectrumbeta-lactamase producing Enterobacteriaceae in Qatar: A 3-yearhospital-based study. *Frontiers Antibiotics1*: 92-99

Altayar, M. A., Thokar, M. A. and Mohamma, M. A. (2012). Extended spectrum beta-lactamase- producing *Escherichia coli* in clinical isolates in benghazi, libya: phenotypic detection and antimicrobial susceptibility pattern. *Medical Journal of Islamic World Academy of Science,20*:49-56.

Amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant ß-lactamases (http://www.lahey.org/studies/webt.asp). Accessed 20 July 2007.

Arekemase, O., Kayode, O. and Ajiboye, E. (2011). Antimicrobial Activity and phytochemical analysis of Jatropha curcas plant against some selected microorganisms*. Inter J Biolo., 3(3*): 52-55.

Arpin, C., Dubois, V and Coulange, L . (2003). Extended-spectrum ß-lactamase-producing *Enterobacteriaceae* in community and private health care centers.*Antimicrobial Agents Chemotherapy, 47*: 3506-14.

Ashurst JV, Dawson A. Klebsiella Pneumonia. [Updated 2023 Jul 20]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK519004/>

Batchelor M., Threlfall E.J., Liebana E. (2005) Cephalosporin resistance among animal-associated Enterobacteria: A current perspective. *Expert Review Anti-Infective Therapy*;*3*:403–417.

Bazzaz, B. S., Naderinasab, M., Mohamadpoor, A. H., Farshahzadeh, Z., Ahmadi, S. and Yousefi, F. (2009). The prevalence of extended spectrum Beta-lactamase producing *Escherichia coli* and *Klebsiella pneumonia* among clinical isolates from a general hospital in Iran.*Acta Microbiologica et Immunologica Hungarica*, *56*: 89-99.

Bell, J., M., Chitsaz, M and Turnidge, J., D .(2007). Prevalence and significance of a negative extended-spectrum β-lactamase (ESBL) confirmation test result after a positive ESBL screening test result for isolates of *Escherichia coli* and *Klebsiella pneumoniae*: results from the SENTRY Asia-Pacific Surveillance Program. *Journal of Clinical Microbiology*.,*45***:** 1478-82.

Benton B., Breukink E., Visscher I., Debabov D., Lunde C., Janc J., Mammen M., Humphrey P.( 2007) Telavancin inhibits peptidoglycan biosynthesis through preferential targeting of transglycosylation: evidence for a multivalent interaction between telavancin and lipid II. *International Journal of Antimicrobial Agents.29*:51–52.

Boligon AA and Athayde ML. (2014) Importance of HPLC in Analysis of Plants Extracts. Austin Chromatogr.;1(3): 2.

Bonnet R. (2004) Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents Chemotherapy; 48*(1):1–14.

Bradford, P.A. (2001). Extended – spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*, *14*: 933-951.

Brisson-Noel A., Delrieu P., Samain D., Courvalin P. (1988) Inactivation of lincosaminide antibiotics in Staphylococcus: identification of lincosaminide O-nucleotidyltransferases and comparison of the corresponding resistance genes. *Journal of Biological Chemistry. 263*:15880–15887.

Bush K, Jacoby GA. (2010). Updated functional classification of β-lactamases.*Antimicrobial Agents Chemotherapy*.*54*: 969–976.

Bush, K., & Bradford, P. A. (2021).Epidemiology of β-lactamase-producing pathogens.*Clinical Microbiology Reviews*, *34*(4), e00229-20.

Canton, R and Coque, T., M .(2006). The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology*, *9*: 466-75.

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L. and Baquero, F. (2008). Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe*. Clinical Microbiology Infect*ion, *1*:44-53.

 Carattoli A. (2008) Animal reservoirs for extended spectrum β-lactamase producers. *Clinical Microbiology Infection, 14*:117–123.

Chander, A., & Shrestha, C. D. (2013).Prevalence of extended spectrum beta lactamase producing Escherichia coli and Klebsiella pneumoniae in urinary isolates.*Journal of Infectious Diseases and Antimicrobial Agents*, *30*(1), 11-16.

Chang, Y., Kim, J., & Hong, S. (2022). Antimicrobial resistance patterns of Citrobacter species isolated from clinical specimens*. Journal of Clinical Microbiology*, *60*(7), e00234-22.

Chao, M., Lai, C., & Yu, L. (2022).Epidemiology of extended-spectrum β-lactamases in Enterobacterales in Taiwan for over two decades.*Frontiers in Microbiology*, *13*.<https://doi.org/10.3389/fmicb.2022.1060050>

Cheesbrough, M. (2005). District laboratory practice in tropical countries, part 2. Cambridge University Press, Cambridge, 5:159-162.

Chen P. A., Hung C. H., Huang P. C., Chen J. R., Huang I. F., Chen W. L., et al.. (2016). Characteristics of CTX-M extended-spectrum β-lactamase-producing Escherichia coli strains isolated from multiple rivers in southern Taiwan. *Appllied Environment Microbiology,* *82*, 1889–1897.

Chen W. Y., Jang T. N., Huang C. H., Hsueh P. R. (2009). In vitro susceptibilities of aerobic and facultative anaerobic gram-negative bacilli isolated from patients with intra-abdominal infections at a medical center in Taiwan: results of the study for monitoring antimicrobial resistance trends (SMART) 2002-2006. *Journal of Microbiology Immunological Infectious.* *42*,:317–323.

Chia, J., H., Chu, C and Su, L., H. (2005) . Development of a multiplex PCR and SHV melting curve mutation detection system for detection of some SHV and CTX-M β-lactamases of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* in Taiwan.*Journal of Clinical Microbiology*.*43***:** 4486-91.

CLSI,( 2013).*Performance Standards for Antimicrobial Susceptibility Testing*. In: Twenty-third Informational Supplement. CLSI Document M100-S23, Pennsylvania.*Clinical and Laboratory Standards Institute, 12*: 34-190.

Colmenarejo C., Hernández-García M., Muñoz-Rodríguez J. R., Huertas N., Navarro F. J., Mateo A. B., et al.. (2020). Prevalence and risks factors associated with ESBL-producing faecal carriage in a single long-term-care facility in Spain: emergence of CTX-M-24- and CTX-M-27-producing Escherichia coli ST131-H30R. *Journal of Antimicrobial Chemotherapy.* *75*, 2480–2484.

Costa, D., Poeta., P and Sáenz, Y. (2006). Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal.*Journal of Antimicrobial Chemotherapy*.*58***:** 1311-2.

Coudron, P.E. (2005). Inhibitor-based methods for detection of plasmid-mediated AmpC beta-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*.*Journal of Clinical Microbiology***,** *43*:416-437.

Davies, T. A., Shang., W., Bush., K. and Flamm, R. K. (2008). Activity of Imepenem and comparator beta-lactam agent clinical isolates of streptococcus pneumonia with defined motivations in the penicillin binding domain of pbp2b and pbp2x. *Journal of Antimicrobial chemotheapyr,3*: 751-753.

Davis CP. Normal Flora. In: Baron S, (1996). Medical Microbiology.4th edition. Galveston (TX): University of Texas Medical Branch at Galveston;.Chapter 6. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK7617/>

Day M.J., Rodriguez I., van Essen-Zandbergen A., Dierikx C., Kadlec K., Schink A.K., Wu G., Chattaway M.A., DoNascimento V., Wain J., et al. (2016;) Diversity of STs, plasmids and ESBL genes among Escherichia coli from humans, animals and food in Germany, the Netherlands and the UK. *Journal of Antimicrobial Chemotherapy. 71*:1178–1182.

De Gheldre, Y., Avesani, V., and Berhin, C. (2003). Evaluation of Oxoid combination discs for detection of extended-spectrum ß-lactamases.*Journal of Antimicrobial Chemotherapy*, *52****:*** 591-710

Deshpande, L., M., Jones, R., N and Fritsche, T. (2006). Occurrence of plasmidic AmpC type β-lactamase-mediated resistance in *Escherichia coli*: report from the SENTRY Antimicrobial Surveillance Program .*International Journal of Antimicrobial Agents., 28*: 578-81.

Doi, Y. and Paterson, D. L. (2007).Detection of plasmid-mediated class C beta-lactamases.*International Journal of InfectiousDiseases, 11*: 191-234.

Effah, C.Y., Sun, T., Liu, S. (2020)*.* *Klebsiella pneumoniae*: an increasing threat to public health. *Annal of Clinical Microbiology Antimicrobial* 19, 1.

Egbebi, A. and Famurewa, O. (2011).Prevalence of Extended Spectrum Beta-Lactamase (ESBL) Production among KlebsiellaIsolates in Some Parts of South West Nigeria.*Journal of Microbiology and Biotechnology Research,1*(2): 64-68.

Egbule, O. S. & Odih, E. E. (2020). Prevalence of Extended-Spectrum Beta-Lactamases (ESBLs) and Metallo-BetaLactamases (MBLs) Among Healthy and Hospitalized Children in Abraka and Eku Communities, Delta State, Nigeria.*Nigerian Journal of Basic and Applied Science*, *28*(1): 07-14.

Ekeleme, U. G., Nwachukwu, N. C., Ogodo, A. C., Nnadi, C. J., Onuabuchi, I. A. and Osuocha, K.U. (2013). Phytochemical Screening and Antibacterial Activity of *Cnidoscolus aconitifolius* and Associated Changes in Liver Enzymes in Wistar Rats.*Australian Journal of Basic and Applied Sciences*, 7(12): 156-162.

Ekeleme, Uzochukwu G., Adiruo Akunna Stephanie, Chukwuocha Uchechukwu M., Nwakanma Jane C, Ikenna O. Ogini, Uche Ifeoma U. and Ikwuagwu Vivian C (2024). The Use of Indigenous Plants for Food Preservation 2024 7. African Journal of Agricultural Research https://academicjournals.org/journal/AJAR/article-in-press-

Ensor, V., M., Shahid, M and Evans, J.,T. (2006). Occurrence, prevalence and genetic environment of CTX-M β-lactamases in Enterobacteriaceae from Indian hospitals. *Journal of Antimicrobial Chemotherapy*., *58***:** 1260-3.

Estaleva C. E. L., Zimba T. F., Sekyere J. O., Govinden U., Chenia H. Y., Simonsen G. S., et al.. (2021). High prevalence of multidrug resistant ESBL- and plasmid mediated AmpC-producing clinical isolates of Escherichia coli at Maputo Central Hospital, Mozambique. *BMC Infectious Diseases.* *21*:16.

Fadare F. T., Okoh A. I. (2021). Distribution and molecular characterization of ESBL, pAmpC β-lactamases, and non-β-lactam encoding genes in Enterobacteriaceae isolated from hospital wastewater in Eastern Cape Province, South Africa. *PLoS One* 16:e0254

Fadare FT, Adefisoye MA, Okoh AI. Occurrence, identification and antibiogram signatures of selected Enterobacteriaceae from Tsomo and Tyhume rivers in the Eastern Cape Province, Republic of South Africa.PLoS One.2020; 2020.08.11.246025.pmid:33284819

Fadare, F. T., & Okoh, A. I. (2021). Distribution and molecular characterization of ESBL, pAmpC β-lactamases, and non-β-lactam encoding genes in Enterobacteriaceae isolated from hospital wastewater in Eastern Cape Province, South Africa. *PLOS ONE*, *16*(7), e0254753. <https://doi.org/10.1371/journal.pone.0254753>

 Fisher J.F., Mobashery S. vol. 8. Elsevier; 2010.Enzymology of Bacterial Resistance. Comprehensive Natural Products II; pp. 443–487.

Garcia, M. T., Hernandez, C. E., & Lopez, D. A. (2023). Antimicrobial properties of Crescentia cujete extracts against multi-drug resistant bacteria. *Journal of Ethnopharmacology*, *268*, 113-564.

Gayathri, G., Kathireshan, A. K. and Balagurunathan, R. (2011). Prevalence of extended spectrum Beta Lactamases in Uropathogenic *E. coli* and *Klebsiella* species in a Chennai Suburban Tertiary Care Hospital and its Antibiogram Pattern. *Research Journal of Microbiology***,** *6:* 796-804.

Giriyapur, R.S., Nandihal, N.W., Krishna, B.V.S., Patil, A. B. and Chandrasekhar, M. R.( 2011) .Comparison of Disc Diffusion Methods for the Detection of Extended-Spectrum Beta Lactamase-Producing Enterobacteriaceae. *Journal of Laboratory Physician*, *3*: 33-36.

Gomi R., Matsuda T., Yamamoto M., Chou P. H., Tanaka M., Ichiyama S., et al..(2018). Characteristics of carbapenemase-producing Enterobacteriaceae in wastewater revealed by genomic analysis. *Antimicrobial Agents Chemotheapyr.* 62:62.

Goodall R.R., Levi A.A. (1946) A microchromatographic method for the detection and approximate determination of the different penicillins in a mixture. *Nature*; 158:675.

Grande, R.; Vitale, I.; Niro, A.; Molinaro, G.; Prezioso, S.; Muraro, R.; Di Giovanni, P. (2021) Microbial Species Isolated from Infected Wounds and Antimicrobial Resistance Analysis: Data Emerging from a Three-Years Retrospective Study. *Antibiotic*s, 10, 1162.

Gülmez D., Çakar A., Şener B. (2010) Comparison of different antimicrobial susceptibility testing methods for Stenotrophomonas maltophilia and results of synergy testing. *Journal of Infection and Chemotherapy*. ;*16*:322–328.

Gupta P., Khare V., Kumar D. (2015) Comparative evaluation of disc diffusion and E-test with broth micro-dilution in susceptibility testing of amphotericin B, voriconazole and caspofungin against clinical Aspergillus isolates. *Journal of Clinicaland Diagnostic Research*.,*9*:2013–2016.

Heffernan, H., Woodhouse, R and Blackmore, T.(2006). Prevalence of extended-spectrum β-lactamases among *Escherichia coli* and *Klebsiella* in New Zealand: Institute of Environmental Science and Research. Report FW 06107.

Ho, P., L., Poon, W., W., N and Loke, S., L. (2007). Community emergence of CTX-M-type extended-spectrum β-lactamases among urinary *Escherichia coli* from women. *Journal ofAntimicrobial Chemother*.apy, *60***:** 140-4.

Huijbers P.M., van Hoek A.H., Graat E.A., Haenen A.P., Florijn A., Hengeveld P.D., van Duijkeren E. (2015) Methicillin-resistant Staphylococcus aureus and extended-spectrum and AmpC beta-lactamase-producing Escherichia coli in broilers and in people living and/or working on organic broiler farms. *Veterinary Microbiology. 176*:120–125.

Husna, A., Rahman, M. M., M. Badruzzaman, A. T., Sikder, M. H., Islam, M. R., Rahman, M. T., Alam, J., & Ashour, H. M. (2023). Extended-Spectrum β-Lactamases (ESBL): Challenges and Opportunities. *Biomedicines*, *11*(11).<https://doi.org/10.3390/biomedicines11112937>

Jacoby, G. A., & Munoz-Price, L. S. (2022).The new beta-lactamases.*New England Journal of Medicine*, *352*(4), 380-391.

Jain, R., Shukla, R., & Tiwari, R. (2021).Comparative analysis of phenotypic methods for detection of ESBL-producing Enterobacteriaceae.*Journal of Clinical Pathology*, *74*(7), 426-430.

Jean S. S., Hsueh P. R., Lee W. S., Chang H. T., Chou M. Y., Chen I. S., et al..(2009). Nationwide surveillance of antimicrobial resistance among Enterobacteriaceae in intensive care units in Taiwan. *Eur. Journal of Clinical Microbiology and Infectious Diseases.* *28*:215–220.

Jeong, S., H., Bae, I., K and Kwon, S., B. (2005) .Dissemination of transferable CTX-M-type extended-spectrum B-lactamase-producing *Escherichia coli* in Korea.*Journal of AppliedMicrobiol*ogy, *98***:** 921-7.

Johann, D.D. Pitout Kevin, B. Laupland. (2008) Extended Spectrum β- Lactamase- Producing Enterobacteriaceae: An emerging public-health concern.*Lancet Infectious Diseases*.*8*:159-66

Johnson, T. R., Peters, J. O., & Adewumi, A. (2022). Comparative efficacy of Pterocarpus santalinoides extracts against clinical bacterial isolates. *African Journal of Traditional, Complementary and Alternative Medicines*, *19*(1), 47-54.

Jorgensen J.H. & Ferraro M.J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical InfectiousDiseases*, *49*:1749–1755.

Kalayama, Y., Zhang, H. Z. and Chambers, H. F. (2011).PBP mutations producing very-high- level resistance in beta-lactamase.*Antimicrobial Agents Chemotherapy*, *48*: 453-459.

Madec J.Y., Haenni M., Nordmann P., Poirel L. (2017) Extended-spectrum beta-lactamase/AmpC- and carbapenemase-producing Enterobacteriaceae in animals: A threat for humans? *Clinical Microbiology and Infection, 23*:826–833.

Maha, H. D.,Naser, E. B., Mutasim, E. I. & Mohamed, E. H. (2020). Prevalence of extended-spectrum β-lactamase (ESBL) and molecular detection of bla TEM, bla SHV and bla CTX-M genotypes among Enterobacteriaceae isolates from patients in Khartoum, Sudan. *Pan African Medical Journal, 37*: 213.

Marston A. (2011) Thin-layer chromatography with biological detection in phytochemistry. *Journal of Chromatography, 1218*:2676–2683.

Martin M, Guiochon G. (2005) Effects of high pressure in liquid chromatography. *Journal of Chromatography A.*; 1090: 16-38.

Martine´z-Martine´z, L., Herna´ndez-Alle´s, S., Albertı´s, S., Toma´s, J. and Benedi, V. (1996). In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins.*Antimicrobial Agents Chemotherapy*, *40*: 342-348

Maslikowska J.A., Walker S.A., Elligsen M., Mittmann N., Palmay L., Daneman N., Simor A. (2016) Impact of infection with extended-spectrum beta-lactamase-producing Escherichia coli or Klebsiella species on outcome and hospitalization costs. *Journal of Hospital Infection. 92*:33–41

Matsuoka M., Sasaki T. (2004) Inactivation of macrolides by producers and pathogens. *Current Drug Targets Infectious Disorder, 4:*217–240.

Mendes, R. E., Jones, R. N., Woosley, L. N., Cattoir, V., Castanheira, M., & Flamm, R. K. (2014). Application of next-generation sequencing for characterization of surveillance and clinical trial isolates: Analysis of the fluoroquinolone-resistant Escherichia coli population and characterization of the fluoroquinolone-resistant Klebsiella pneumoniae population. *Journal of Clinical Microbiology*, *52*(4), 1277-1281.

Metri, B. C., Jyothi, P. and Peerapur, V. ( 2011). The prevalence of ESBL among enterobacteriaceae in tertiary care hospital of North Karnataka India. *Journal of Clinical and Diagnostic Research*, 5:470-475.

Moor, C., T., Roberts, S and Simmons, G .(2006). Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae: emergence and risk factors for infection in the community setting, Auckland, New Zealand. *Journal of Hospital Infection*. In press

Moubarek, C., Daoud, Z and Hakime, N., I.(2005). Countrywide spread of community- and hospital-acquired extended-spectrum β-lactamase (CTX-M-15)-producing Enterobacteriaceae in Lebanon. *Journal of Clinical Microbiol*ogy, *43*: 3309-13

Mphande, I., Kataba, A., Muzandu, K., & Gono-Bwalya, A. (2022). An Evaluation of the Antibacterial Activity of *Pterocarpus tinctorius* Bark Extract against Enteric Bacteria That Cause Gastroenteritis. *Evidence-based complementary and alternative medicine : eCAM*, *2022*, 7973942. https://doi.org/10.1155/2022/7973942

Mueller M, & Tainter, CR. (2023) Escherichia coli Infection. [Updated 2023 Jul 13]. In: StatPearls [Internet]. Treasure Island (FL): <https://www.ncbi.nlm.nih.gov/books/NBK564298/>

Mugnaioli, C., Luzzaro, F and De Luca, F. (2006). CTX-M-type extended-spectrum β-lactamases in Italy: molecular epidemiology of an emerging countrywide problem. *Antimicrobial Agents Chemotherapy*, *50*: 2700-6.

Munday, C., J., Xiong., J and Li, C.(2004). Dissemination of CTX-M type β-lactamases in Enterobacteriaceae isolates in the People.s Republic of China. *International Journal of AntimicrobialAgents.,23***:** 175-180.

Naseer, U., & Sundsfjord, A. (2011). The CTX-M conundrum: Dissemination of plasmids and Escherichia coli clones. *Microbial Drug Resistance*, *17*(1), 83-97.

Nisha, N., Patil, V., & Shah, R. (2022).Comparative study of antibiotic resistance in Escherichia coli from different clinical samples.*Journal of Infectious Diseases*, *226*(5), 785-793.

Rodriguez-Bano, J., Navarro, M.,D and Romero, L .(2006). Bacteremia due to extended-spectrum β-lactamase-producing *Escherichia coli* in the CTX-M era: a new challenge. *Clinical InfectiousDiseases*.,*43***:** 1407-14.

Roula Abdel-Massih, Elias Abdou, Elias Baydoun, and Ziad Daoud (2010).Antibacterial Activity of the Extracts Obtained from *Rosmarinus officinalis, Origanum majorana, and Trigonella foenum-graecum* on Highly Drug-Resistant Gram Negative Bacilli.*Journal of Botany*, *2010*: 1- 9.

Upton, A., Mohiuddin, J and Bathgate, T .(2007). High prevalence of CTX-M-15 extended-spectrum β-lactamase among contacts of patients with shigellosis due to *Shigella flexneri* carrying CTX-M-15. *Journal Antimicrobial Chemother*apy, *60***:** 906-8.

Vink J., Edgeworth J., Bailey S. L. (2020). Acquisition of MDR-GNB in hospital settings: a systematic review and meta-analysis focusing on ESBL-E. *Journal of Hospital Infection* 106, 419–428. doi: 10.1016/j.jhin.2020.09.006.

Wylie, M. R., & Merrell, D. S. (2022). The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. *Frontiers in pharmacology*, *13*, 891535. https://doi.org/10.3389/fphar.2022.891535