**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). Pure isolates were stored in nutrient broth with 20 % glycerol at –80 °C for further analysis.

**Genomic DNA Extraction**

Genomic DNA extraction was performed using a modified boiling lysis protocol, as described by Oliveira (2014). A loop of overnight bacterial culture was resuspended in 200 µL sterile distilled water and then boiled at 95 °C for 10 minutes. The mixture thus obtained was cooled instantly on ice. Cell debris was removed by centrifugation at 13,000 rpm for 5 minutes. The supernatant containing crude genomic DNA was transferred to a new tube and stored at −20 °C for future use.

**PCR Amplification of the 16S rRNA Gene**

Amplification of the 16S rRNA gene was carried out using universal bacterial primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Frank et al., 2008). The PCR reaction mixture (25 µL total volume) consisted of 12.5 µL of 2× PCR Master Mix (Thermo Scientific, USA), 1 µL each of 10 µM forward and reverse primers, 2 µL of DNA template, and 8.5 µL of nuclease-free water.

Thermal cycling conditions included an initial denaturation at 94 °C for 5 minutes; followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute; and a final extension at 72 °C for 7 minutes.

**Gel Electrophoresis and Visualization**

PCR products were analysed using 1.5% agarose gel electrophoresis stained with GelRed™ (Biotium, USA). Electrophoresis was conducted at 100 V for 45 minutes in 1× TBE buffer. DNA bands were visualized under UV light using a gel documentation system. A 100 bp DNA ladder was used as a size marker to estimate the amplicon size (1500 bp).

**Purification and Sequencing of PCR Products**

Amplicons of the expected size were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer’s protocol. Sanger sequencing of the purified products was performed bidirectionally using the same primer pair at a commercial sequencing facility (e.g. Macrogen Inc., South Korea).

**Bioinformatics and Phylogenetic Analysis**

Raw sequence chromatograms were manually inspected and edited using BioEdit v7.2.5 (Hall, 1999). Consensus sequences were compared with reference sequences using the BLASTn algorithm against the NCBI GenBank database (Altschul et al., 1990). Bacterial identities were assigned based on ≥97 % sequence similarity. Phylogenetic analysis was conducted using MEGA X (Kumar et al., 2018), employing the neighbour-joining method with 1,000 bootstrap replicates to assess branch support.

**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 (30 µg), Cefotaxime(30 µg), Cefuroxime(30 µg), Aztreonam (30 µg),Cefotetan (30 µg), Ceftriaxone (30 µg), amoxicillin/clavulanic acid (30 µg), Gentamicin (10 µg),Kanamycin (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg)imipenem (10 µg), Nitrofurantoin (300 µg), Sulfamethoxazole/trimethroprim (30 µg), Piperacillin (30 µg), (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 (20/10 μg) (TOKU-E, USA) was placed at the center of the inoculated MHA. Ceftazidime (30 µg), (TOKU-E, USA) and Cefotaxime (30 µg), (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 37 oC 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 200 µL 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 30 µl of Tris EDTA buffer and stored at 4 oC prior to gel electrophoresis.

For electrophoresis, 0.8% agarose gel was prepared in electrophoresis buffer, melted, and stained with ethidium bromide (0.5 µg/mL). The gel was cast with wells, set for 30 minutes, and loaded with 20 µL plasmid DNA mixed with 2 µL bromophenol blue. A DNA marker was included. Electrophoresis was run at 70 V for 1.5 hours. DNA migration was monitored by tracking dye. Bands were visualized under UV using a Clinix Japan transilluminator (Model 1570). DNA sizes were estimated by comparing with HIND III molecular weight 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. 108 cfu/mL) were prepared using the test organisms and 0.1 mL of the bacteria species were mixed in Mueller Hinton Agar medium and poured in pre-sterilized Petri plates. A cork borer (6 mm diameter) were used to punch wells in solidified medium and were filled with extracts of 50 µL of 500 mg/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 37 oC for 24 hrs 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* |

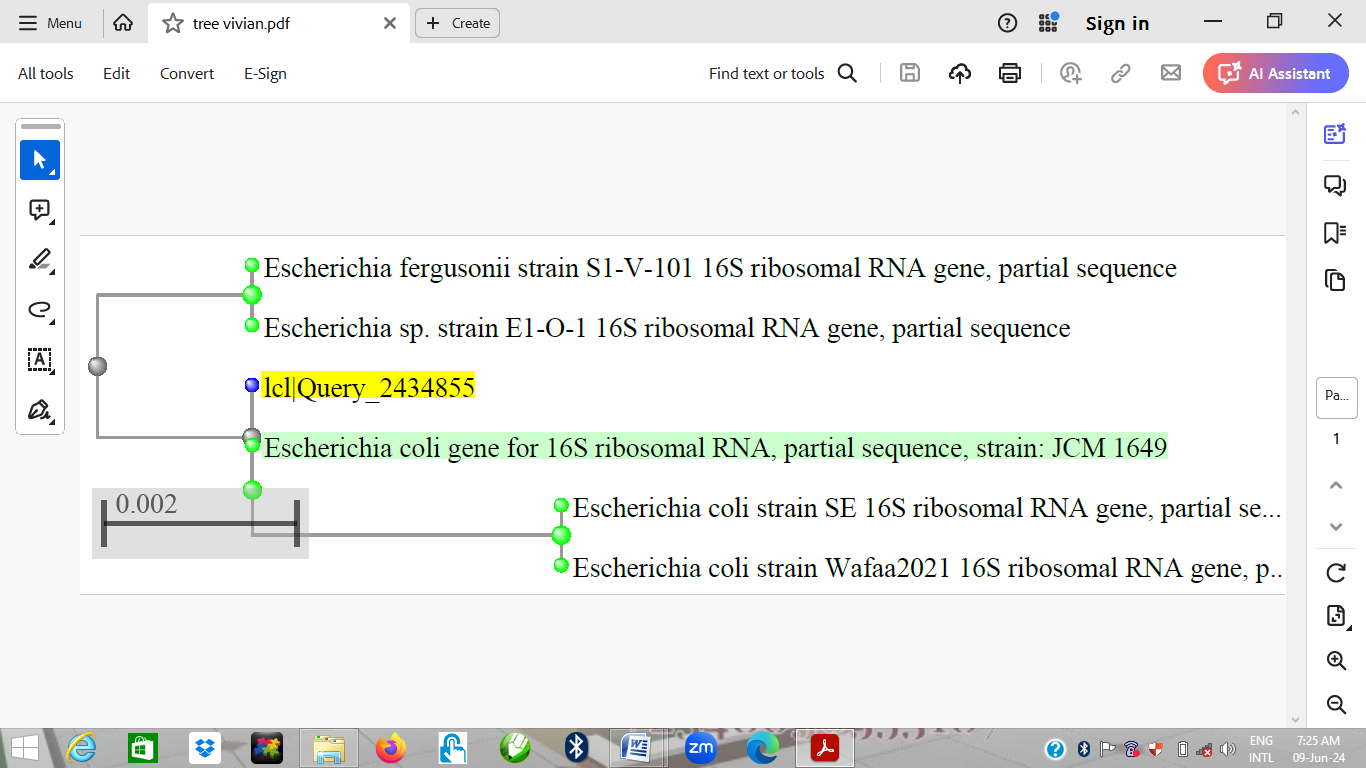
**Key:** + = Positive, - = Negative, V.P = Voges-Proskauer, NA = Nutrient Agar, MA = MacConkey Agar

**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 (20/10 μg) was placed at the centre of the inoculated Mueller-Hinton agar (MHA). Ceftazidime (30 ug) and Cefotaxime (30 ug) 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 (50 µL) | 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.

**Consent**

Informed consent was also obtained from patients.

Ethical Approval

Ethical Approval was obtained from Gregory University Uturu hospital Imo State

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.

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