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***Original Research Article***

# Antibacterial Activity of the Essential Oil of *Ocimum gratissimum* L. against Multidrug-resistant Enterotoxigenic *Escherichia coli*

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ABSTRACT

Enterotoxigenic Escherichia coli (ETEC) is a major cause of bacterial diarrhoeal diseases, particularly in infants. Increased antibiotic resistance in ETEC intestinal infections intensifies diarrheal morbidity, leading to poor treatment outcomes and acute intestinal pathology. This study aimed to characterise ETEC and evaluate Ocimum gratissimum L. essential oil (OgEO) as an anti-diarrheic agent. One hundred sixty-one fecal samples were collected from participants with confirmed diarrhea and persistent stooling. Escherichia coli from fecal samples was biotyped using the API test kit. Obtained colonies were further examined for greenish metallic sheen on Eosin Methylene Blue. The identified E. coli were screened for haemolytic activity, antibiotic susceptibility, enterotoxins, and colonisation factor production. E. coli isolates exhibiting α-haemolysis, β-haemolysis, multidrug resistance, and enterotoxigenic characteristics were characterized using 16S rRNA gene sequencing. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of OgEO were assayed against the selected multidrug-resistant and enterotoxigenic E. coli strains. Escherichia coli (16.1%) from suspected enteric bacterial pathogens were recorded. Among the E. coli isolates, 30.8% exhibited complete α-hemolysis, β-hemolysis (30.8%), and γ-hemolysis (38.4%). All the isolates showed resistance to 40% of the antibiotics, including ampiclox, imipenem, amoxicillin-clavulanate, cefuroxime, and cefixime. The isolates produced heat-stable (33%), heat-labile (25%) toxins, and colonisation factors (33%). OgEO exhibited significant antibacterial activity (MBC= 250 µg/mL; MIC=62.5 µg/mL) against the ETEC strains ($p<0.05$). OgEO demonstrated antibacterial activity against multidrug-resistant E. coli, showing promise as a candidate for developing anti-diarrhoeal agents.

**Keywords**: Antibacterial activity, Antibiotic resistance, Escherichia coli, Haemolytic activity, Ocimum gratissimum essential oil.

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## Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of bacterial diarrhoea infections and traveler's diarrhoea, increasingly leading to intestinal pathology and diarrhoeal illnesses, primarily in children from developing nations.[1] ETEC is a virulent strain of *Escherichia coli* that synthesizes potent heat-stable (ST) and heat-labile (LT) enterotoxins associated with traveler's and infantile diarrhoea.[2] Transmission of ETEC is by consumption of contaminated food and water, which enhances high rates of transmission even at a very low infective dose.[3] Primarily in endemic nations, infants are at risk of ETEC infections, causing over 99 million diarrhoeal infections and responsible for over 55,000 deaths in 2015.[4] Produced colonisation factors (CFs) from ETEC mediate intestinal cell wall degeneration, leading to severe intestinal morbidity.[5] Improper antibiotic usage and poor prescription practices have increased resistant strains that adversely affect clinical outcomes.[6]

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ETEC infection symptoms range from mild self-limiting sickness to cholera-like conditions, with watery diarrhoea lasting 3-5 days being the most prevalent symptom.[7]

There is growing interest in exploiting plant metabolites for medicinal purposes due to antibiotic resistance.[10] *Ocimum gratissimum* L. (*Lamiaceae*) is widely distributed in tropical regions. The herb is often used in traditional medicine for intestinal infections, particularly diarrhoea.[11] Oil extract from *Ocimum gratissimum* L. is a promising antibacterial candidate with active ingredients (such as citral, thymol, triterpenes, flavonoids, alkaloids, saponins, linaol, eugenol, methyl cinnamate and camphor) which exert bacteriostatic in addition to bactericidal activities and has been widely reported to be active against pathogenic intestinal *Escherichia coli.*[12] The potency of *O. gratissimum* in curing many illnesses are due to its antioxidant and antimicrobial properties, together with its ability to improve the antioxidant systems. [11] Previous studies have posited that *O. gratissimum* has many ethno-medicinal properties, such as anti-inflammatory, [12] anti-diarrhoeal,[13] antibacterial,[14] anti-oxidative properties.[15] Continuous dissemination of ETEC strains in several communities in southwestern Nigeria has resulted in huge morbidity and occasional death.[8] Poor detection of ETEC infection largely contributes to re-infection, continuous transmission, and increased antibiotic resistance, intensifying the disease burden.[9] To prevent dissemination, the provision of evidence-based data on ETEC strain serotype, antimicrobial resistance pattern, clonal type, and virulence determinants is of great importance. From African nations and Nigeria in particular, there are reports of prevalent ETEC strains having clonal relatedness with other global strains among children and travellers.

A major health concern in Africa is the prevalent increase of multidrug-resistant ETEC strains, primarily in children.[18] Evaluation of *Ocimum gratissimum* L. Essential oil as anti-diarrheagenic ETEC agents is expected to improve therapeutic outcome and quality of life of the

patients with diarrhea, and serves as preventive therapeutics with less adverse side effects compared to the current synthetic drugs.[11]This study investigated the prevalence of clonally diverse enterotoxigenic *E. coli* (ETEC) and evaluated the antibacterial activities of *Ocimum gratissimum* essential oil on these ETEC strains.

## Materials and Method

*Ethical Approval*
Study approval was granted by the Ethics Committee at Covenant University (CU/HREC/OOS/508/24).

*Sample Collection and Selection of Subjects*
Faecal samples (n=161) were obtained from the patients with suspected gastrointestinal infections attending the Federal Medical Centre, Abeokuta, and Ota General Hospital, Ogun State. Patients with immunocompromised conditions (such as HIV and cancer), viral infections, patients with antibiotic prescriptions within 21 days, and pregnant women were excluded.

*Isolation of the Bacterial Species from the Samples*
Following overnight enrichment of the fecal samples in glucose broth, a loopful was sub-cultured on Eosin Methylene Blue agar (Oxoid, Basingstoke, UK) and MacConkey agar (Oxoid, Basingstoke, UK). Following 24-hour incubation at 37°C, the colonies were purified and further examined for cellular morphology and macroscopic appearance. Haemolysin production of the bacterial isolates was detected.[19]

*Biotyping*
The Analytical Profile Index for Enterobacteriaceae (API 20E) characterized each bacterial organism obtained.[20] A homogeneous suspension was made by thorough emulsification of a pure bacterial colony from 20 20-hour culture in 0.85% NaCl (5 mL). To each ampoule of API, broth culture suspension (0.5 mL) was added and incubated for 18-24 hours at 37°C. The Identification of bacteria from the Analytical Profile Index list is extrapolated from the nine-digit value identification code for the bacterial isolate.

*Antibiotic Susceptibility Test*
The Kirby-Bauer disc diffusion method was used to determine the susceptibility of identified *E. coli* isolates from the faecal samples to antibiotics.[21] Sterile swab sticks were used to apply adjusted broth of each isolate, similar to the standard 0.5 McFarland on Muller-Hinton agar (Oxoid, Basingstoke, UK). Placement of antibiotic discs were on agar plates inoculated with isolates followed by incubation for twenty-four hours at 37°C. Measurement and interpretation of resulting inhibition zones followed the Clinical and Laboratory Standards Institute guidelines. The antibiotics used include Cefexime 5µg (ZEM), Ceftriaxone sulbactam 45µg (CRO), Ampiclox 10µg (ACX), Cefuroxime 30µg (CXM), Cefotaxime 25µg (CTX), Imipenem/Cilastatin 10/10µg (IMP), Nitrofurantoin 300µg (NF), Levofloxacin 5µg (LBC), Ofloxacin 5µg (OFX), Gentamycin 10µg (GN), Amoxicillin Clavulanate 30µg (AUG) and Nalidixic acid 30µg (NA).

*LT, ST, and CF Serotyping*
The production of LT and ST was assayed with Ganglioside-GM1-ELISA and inhibition GM1-ELISA, respectively, using the commercial ELISA test kit previously described by [16]. Colonisation factor antigens (CFAs) associated with human pathogenic strains, including CFA/I, were assayed by anti-CFA/I/MAbs binding using the commercial ELISA kit (AFG Scientific, USA).

*Phylogenetic Analysis*
Genomic DNA of the identified ETEC strains was extracted, and 16S rRNA encoding the evolutionary strain was amplified and sequenced. The sequenced nucleotides that were obtained were blasted on NCBI at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Retrieved global strains from NCBI were manually aligned in MEGA software version 6 and analysed for genetic diversity from a built phylogenetic tree following Fitch parsimony methods. For every node, percentages of Fitch bootstrap were computed using 1000 heuristic search replicates and the bootstrap option in PAUP 3.1.1.

*Extraction of Essential Oil*
Fresh leaves of *Ocimum gratissimum* were selected from the premises of the Covenant University in May 2024. The plant was identified and authenticated at the Federal University of Agriculture, Abeokuta Herbarium. The *Ocimum gratissimum* leaves were air dried for three days, then 120g of the leaves was blended with a Binatone blender (Model BLG-401) and the powder was dissolved in 1 L of distilled water. The essential oil was obtained by the hydro-distillation of the filtrate of the aqueous extract in a Clevenger-type apparatus for 6 hours with 400 mL of distilled water.[23]

*Minimum Inhibitory Concentration Determination (MIC)*
The minimum inhibitory concentration of the Ocimum gratissimum L. was determined following the standard broth micro-dilution technique.[24] The 96-well microtitre plate was used for this bioassay, and each well was labelled. Serial dilution of the extract was performed in the microtitre plate, followed by the addition of the broth containing an equal volume of identified bacteria strains (100 µL of 0.5 McFarland) and incubated for 24 hours at 37°C. Both positive and negative controls were set up. Following incubation, the addition of phenol red (20 µL) to all the wells, and the least dilution showing yellow colouration, which indicates no growth, was regarded as MIC.

*Mininmum Bactericidal Concentration (MBC)*
Sub-culturing broth dilutions at or above the MIC was performed to evaluate the MBC.[25] Following streaking onto agar, broth dilutions were incubated for 24 to 48 hours. The MBC is the lowest broth dilution of antimicrobials, which stops the growth of organisms on the agar plate. The Minimum Bactericidal Concentration (MBC) was determined after re-incubating the plates from the dilution test without visible bacterial growth at 37 ºC for 24 hours. The mode of activity of the extracts was defined as either static (if there was no growth at 24 hours but there was growth at 48 hours) or cidal (if there was neither growth at 24 hours nor 48 hours).

*Statistical Analysis*
The Statistical Package for Social Sciences (SPSS Version 24.0) was utilised to analyse the data. Frequency and percentage were used to present the data, which were analysed using descriptive statistics.

## Result and Discussion

Diarrhoeal illness is a major cause of mortality in infants worldwide, and ETEC is the cause of a notable portion of bacterial gastroenteritis.[1] Prevalence rates of 16.1% diarrhoeagenic *Escherichia coli* were observed among the participants (Fig. 1, 2). Within the same geographic area and globally, there are differences in the incidence and other epidemiologic characteristics of DEC as diarrhoeal etiologic agents. This rate is lower than the previous report in Cap-Haïtien, Haiti,19, where most strains (86.7%) were hemolytic. Most *Escherichia coli* isolates analysed demonstrate resistance to more than two antibiotic classes, resulting in multidrug-resistant diarrhoeal infections (Fig. 3). The increased prevalence of diarrhoeagenic *Escherichia coli* with multiple drug resistance has elevated intestinal morbidity, resulting in increased rates of resistant infections that could increase treatment failure and treatment costs. The effects of antibiotic resistance in diarrhoeagenic *Escherichia coli* infections present consequential restrictions to treatment and appropriate drug selection for proper patient management, as well as managing complications due to diarrhoeagenic *Escherichia coli* infections in infants. The observed varying levels of resistance to distinct antibiotic classes, including cephalosporins, aminoglycosides, fluoroquinolones, and penicillins, indicate different mechanisms these strains utilize to escape elimination by antibiotics.

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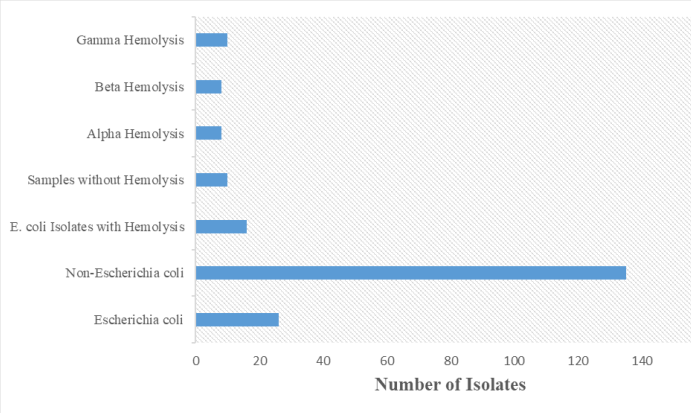


Figure 1: Prevalence of Bacterial Isolates from Sample

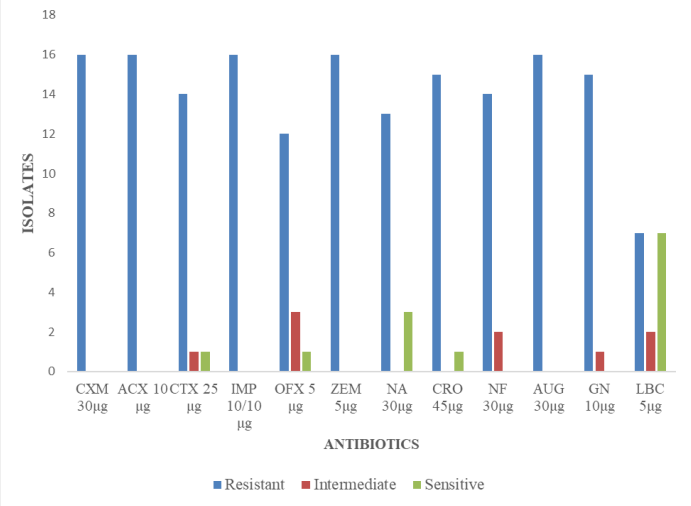


Figure 2: Patterns of Antibiotic Susceptibility in *Escherichia coli* isolates

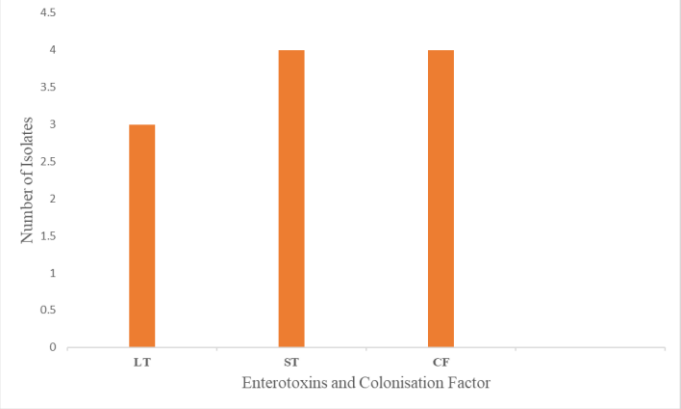


Figure 3: Enterotoxins and Colonisation Factors Producing *E. coli* Isolates

This necessitates the importance of designing a system for monitoring antibiotics for diarrhoeagenic *Escherichia coli* infections, particularly for young children, to reduce the spread of diarrhoeagenic *Escherichia coli* resistant infections.

Variations in seasonality may have accounted for the reduced ETEC isolation in this investigation. Enterotoxigenic *Escherichia coli* is a prominent bacterial cause of diarrhoea in infants. This implies that as children get older, their immune systems may improve. ST was detected in four of the tested isolates (33.3%) while the LT was detected in 25% of our tested isolates, and LT and ST were found in a single isolate. Compared to earlier studies conducted in other parts of Nigeria, the ETEC strain recovery from participants with diarrhoea in our research was significantly greater (18.4%). Previous research conducted in countries such as Ghana has verified this strong relationship between ETC and diarrhoea [22]. The low frequency of ETEC among the patients suggests that other agents (such as *Shigella* and *Salmonella* species) not investigated in this study may have caused the diarrhoea.Considering the clonal diversity of all the ETEC strains producing CF toxins from Nigeria, genetic relatedness exists with other globally identified strains. Two strains clustering with diarrhoeagenic strains from diagnosed patients indicate genetic diversity with high-level virulence (Fig. 4). A similar diversity of CF-producing ETEC pathovar was posited. [23] Steinsland reported CF toxin strains that usually clustered together with related CF production. Two sequenced ETEC strains from this study clustered with *Escherichia coli* Nissle 1917 and *Escherichia coli* strain KU from poultry. The clonal study further suggests a zoonotic transfer of *Escherichia coli* strain from poultry to the human population. Previous reports in India showed that *Escherichia coli* phylogroups A and B1 from humans were found in backyard poultry. [24, 25, 26] This clustering analysis further suggested that there is transmission of CF-producing *Escherichia coli* among the population, which could have been caused by poor hygiene or consumption of poorly cooked poultry products. Contamination from poultry fecal samples in human food samples could have caused the ETEC infection in the human population [27] A similar incidence was observed in *Escherichia coli* strains from African poultry expressing CF toxin, which was detected in food.[28] Identification of related strains from this study and other strains from children under age 2 indicates the impact of ETEC infection in childhood**.** Such ETEC infection usually leads to watery stool that are

severe with high intestinal morbidity. In childhood, primarily in infants and toddlers, prolonged ETEC infection could lead to watery stooling, which can be life-threatening if treatment is not provided.[29]

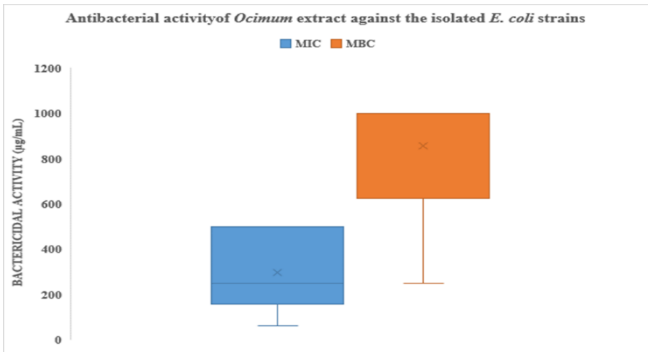


Figure 5: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Ocimum* extract against the isolated *E. coli* strains

Improper sanitation and absence of potable water have caused a surge of infant deaths from ETEC infection. Similar ETEC infection was reported in Somalia with elevated mortality due to stunted growth in childhood from diarrhoeal infections.[30] Mortality due to clonally diverse ETEC diarrhoea in infants below age five was reported in the African region, particularly Niger,[31] Guinea-Bissau,[32] and Chad,[33] then the Eastern Mediterranean Region,[35] and the Southeast Asian region.[34] OgEOs' antibacterial activity on *Escherichia coli* strains is noteworthy since it shows its potential as a natural antibacterial agent.

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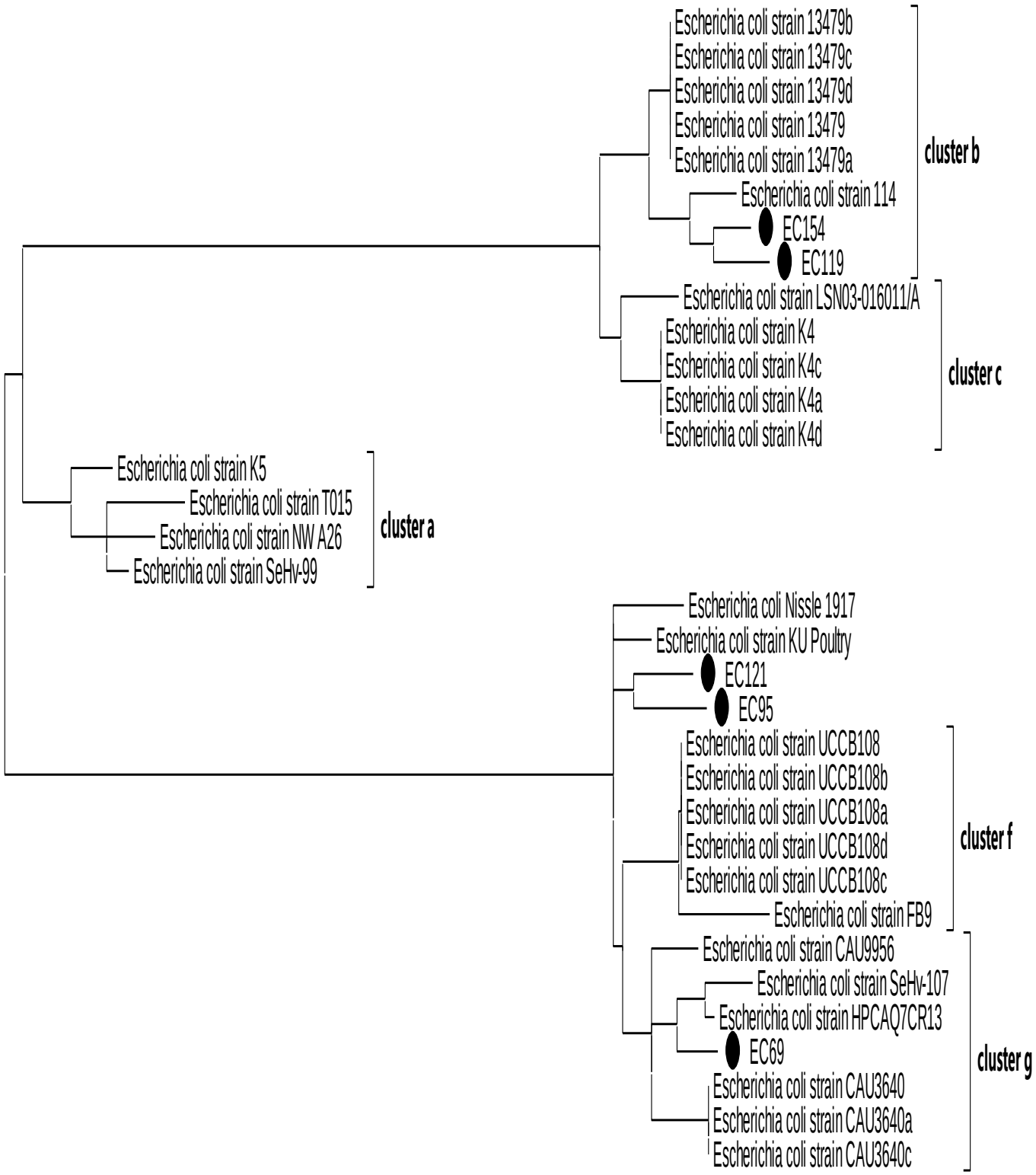


Figure 4: Clonal diversity of prevalent diarrhoeagenic enterotoxigenic *Escherichia coli* with other global strains

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Table 1: Phenotypic identification of the *Escherichia coli* isolates from the samples using API

| LABEL | Gram | Motility | Glucose | Lactose | Mannitol | Maltose | Indole | Methyl Red | Voges proskauer | Citrate | $H_2S$ | Sucrose | Urea | Oxidase | Coagulase | Catalase | ONPG | ADH | LDC | ODC | INO | RHA | SAC | MEL | AMY | ARA | IDENTIFICATION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ECO 48** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 69** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 42** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 100** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 81** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli***** |
| **ECO 89** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli***** |
| **ECO 95** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 154** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 001** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli***** |
| **ECO 119** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 121** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |
| **ECO 118** | GNB | + | + | + | + | + | + | + | - | - | - | NA | - | - | NA | + | + | + | - | + | + | - | + | + | + | + | ***Escherichia coli*** |

KEY: + = Positive - = Negative NA = Not applicable GPB = Gram Positive Bacilli GNB = Gram Negative Bacilli GPC = Gram Positive Cocci NG = No Growth; **differential colonial appearan

In this study, the MIC values varied between 62.5 and 500 mg/mL (Fig. 5), and the MBC values deviate from those in another study, where the Minimum inhibitory concentrations (MICs) for Escherichia were between 300 and 1200 mg/mL.[35] OgEO demonstrated significant antibacterial activity against the tested isolates based on the minimum inhibitory concentration (MIC) analysis. The antibacterial activity of OgEO might be due to its inherent phytochemicals, which enhanced the antimicrobial effects on the ETEC isolates.

Study limitation: Study participants' demographic data were not available to aid evaluation of the spread of ETEC, and low levels of consent from parents for collecting samples from children further limited the number of samples collected.

## Conclusion

The increased prevalence of multidrug-resistant diarrhoeagenic *Escherichia coli* intensifies the need for definitive clinical diagnosis and antibiotic prescription. Diarrhoeagenic *Escherichia coli* strains showing hemolytic activity aggravate intestinal morbidity and tissue degradation, leading to more severe intestinal disease. The *Ocimum gratissimum* essential oil showed significant bactericidal activity against the resistant ETEC strains. This essential oil is a potential anti-ETEC agent that could be exploited to formulate anti-diarrheic agents for clinical management of diarrheic conditions.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by the authors.

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