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ORIGINAL RESEARCH ARTICLE

Evaluation of Antibacterial Activity of Selected Medicinal Plant on Extended Spectrum β-lactamase Producing *Salmonella enterica* serovar Typhimurium

Christiana Inuaesiet Edemekong[1,2]*, Anulika Goodness Chukwujekwu[3], Maria Ezinne Okorie[4], Evangeline Udenweze[3], Ijeoma Frederica Obodoechi[1], Ifeoma Precious Ogbonna[5,6] and NseAbasi Peter Livinus[1]

[1]Department of Public Health, Faculty of Health Care Services, Federal University of Allied Health Science, Trans-Ekulu, Enugu, Nigeria
[2]Department of Biotechnology, Faculty of Science, Ebonyi State University, Abakaliki, Nigeria
[3]Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra, Nigeria
[4]Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Federal University of Allied Health Science, Trans-Ekulu, Enugu, Nigeria
[5]Department of Microbiology and Parasitology, David Umahi Federal University of Health Science, Uburu, Ebonyi State, Nigeria
[6]International Institute for Infectious Disease, Biosafety and Biosecurity, Uburu, Ebonyi State, Nigeria

ABSTRACT
The growing prevalence of ESBL-producing *Salmonella enterica* serovar Typhimurium in typhoid fever patients, with resistance to most antibiotics, has heightened the need for plant-based remedies. This study was designed to evaluate the antibacterial activity of selected medicinal plants on ESBL-producing *Salmonella enterica* serovar Typhimurium from a clinical sample in Nigeria. A total of 200 blood samples were collected from patients suspected of typhoid fever and analyzed using standard microbiological techniques for isolation and characterization on the VITEK 2 system. *Salmonella enterica* serovar Typhimurium was screened for ESBL genes by Polymerase Chain Reaction specific primers. The antibacterial activity of ethanol extract from siam weed, bush cane, neem, Brazilian tea leaves, garlic, and ginger cloves was performed using the Agar well diffusion Technique. Of the 200 samples, *S.* Typhi accounted for 159(79.5 %). The amplification of the ESBL gene revealed a high proportion of CTX-M (*n*=159/100 %) followed by SHV (*n*=81/50.9 % and TEM (*n*=52/32.7 %). The results of the antibacterial activity of Brazilian tea, siam weed, neem, and ginger indicate that the extracts were effective at 100mg/ml, 50mg/ml, and 25mg/ml concentrations ranging from 100-46. 0%. Garlic had no antibacterial effect at 25mg/ml concentration, while All ESBL-producing *Salmonella enterica* serovar Typhimurium demonstrated resistance to bush cane extract across various concentrations. Since this study marks the first report of the antibacterial activity of these medicinal plant extracts on strain harboring such genotype, their prudent and judicious utilization is required in ethnobotanical/human medicine in treating ESBL-associated infectious diseases. There should be guidelines and regulations on the appropriate use of antibiotics to avert drug resistance and the spread of β-lactamase and other resistant determinants to susceptible strains.

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INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a major pathogenic serotype or serovars associated with human disease acquired from various animal and their food products (Thung *et al.,* 2018; Worku *et al.,* 2022). The common risk predictor of *S.* Typhi infection is marked by poor hygiene and low socioeconomic status (Akinyemi *et al.*, 2022; Awol *et al.*, 2021; Jajere, 2019). *S.* Typhi causes typhoid fever, which manifests as Salmonellosis accompanied by abdominal pain, cough, fever, malaise, headache, and nausea (Popa and Papa, 2021; Basnyat *et al.*, 2021). The fatality rate of disease accounts for 10-30% of cases; when properly managed, the fatality rate may decrease and account for 1-4 % (Akinyemi *et al.*, 2022; Popa and Papa, 2021). In the 1970s, the management and treatment of *S.* Typhi were achievable with effective first-line therapeutic agents such as β-lactams (ampicillin), trimethoprim-sulfamethoxazole and chloramphenicol (Akinyemi *et al.*, 2022).

However, the rapid emergence and dissemination of multi-drug resistant (MDR) isolates have truncated the efficacy of these therapeutic agents (Saeed *et al.,* 2020*;* Pereira and Shah, 2020).

Correspondence: Christiana Inuaesiet Edemekong. Department of Public Health, Faculty of Health Care Services, Federal University of Allied Health Science, Trans-Ekulu, P.M.B. 01473, Enugu, Nigeria. ✉ christianainuaesietedemekong@gmail.com
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Over the years, in the absence of no available treatment option, β-lactams have been extensively used to treat typhoid fever. The increase and extensive use of these drugs has resulted in an outbreak of β-lactams -resistant *S.* Typhi (Mather *et al.,* 2013; Saeed *et al.,* 2020*;* Pereira and Shah, 2020; Gberikon *et al.,* 2020; Ibrahim *et al.,* 2022).

The use of β-lactam drugs as growth promoters in food-producing animals (Ibrahim *et al*., 2022; Primeau *et al.*, 2023) necessitates careful regulation to mitigate public health risks, including the rise of *Salmonella* Typhi and antibiotic resistance. This resistance is primarily due to beta-lactamase enzymes (Extended-Spectrum β-lactamases or ESBLs) produced by certain bacteria, which can hydrolyze the β-lactam ring in antibiotics, rendering them ineffective. (Joseph *et al.,* 2023). ESBLs are inhibited by *β*-lactam inhibitors (Tazobactam or Sulbactam Clavulanic acid), Cephamycine, and Carbapenems (Joseph *et al.,* 2023). There are several variants of ESBL genes: CTX-M, TEM, OXA, SHV, BES-1, PER, VEB-1, and GES (Ibrahim *et al.,* 2022; Primeau *et al.,* 2023). These genes produced by *Enterobacteriaceae* impede resistance mechanisms to antimicrobial therapy and limit the availability of antimicrobial options for the treatment of infection (Joseph *et al.,* 2023).

The distribution of clinical isolates of typhoidal *Salmonella* ESBL strain with MDR phenotype has been reported in existing literature in Nigeria (Akinyemi *et al.,* 2022; Ibrahim *et al*., 2022; Okpa *et al*., 2020), but there is a handful of information regarding the use of different herbal plant for *in vitro* antibacterial potential. Most medicinal plants such as neem, ginger, Bush cane, garlic, siam weed, and Brazilian tea plant are medicinal sources of antimicrobial agents (Circella *et al*., 2022; Robinson *et al*., 2022; Amadi *et al*., 2021; Arora *et al*., 2021; Choo *et al.*, 2020; Jagannathan *et al*., 2020; Ibrahim and Kebede, 2020; Kanth *et al*., 2016). The extracts of this medicinal plant offer a range of promising effects that can be linked to several beneficial molecular and cellular mechanisms. These mechanisms include the repair of DNA, scavenging of free radicals, and alterations in the cell cycle, all of which contribute to detoxification processes. Moreover, these extracts can promote autophagy and mitigate programmed cell death. Their anti-inflammatory properties further enhance immune surveillance, while they also exhibit significant anti-metastatic and anti-angiogenic activities. Additionally, the ability of these extracts to modulate various signaling pathways adds to their potential therapeutic value. (Amadi *et al.,* 2021; Ibrahim and Kebede, 2020; Peter *et al.,* 2022; Agbo *et al.,* 2024).

Although the use of conventional antibiotics tends to overshadow the exploration of plant-based compounds for therapeutic purposes due to the growing need for novel antimicrobial agents, there's a need to expand research to botanical and unaltered natural products as they are less likely to lead to antimicrobial resistance. This focus can enhance our strategies against infections and promote healthier alternatives.

MATERIALS AND METHODS

Sample collection and *S.* Typhi Identification

A total of two hundred (200) blood samples were collected from patients suspected of typhoid fever at the University of Calabar Teaching Hospital, Cross River State, Nigeria located at latitude 4° 57.4" N and longitude 8° 19' 10.2" E. Exactly 1 ml of the blood sample was enriched in 5ml sterile Rappaport Vassiliadis broth ™ (RVB) (Thermo Fisher Scientific™, U.S.A) and incubated for 24hrs. A loop-ful from the overnight RVB was aseptically sub-cultured onto *Salmonella-Shigella* Agar and Xylose Lysine Deoxycholate agar (Thermo Fisher Scientific™, U.S.A), respectively, and incubated at 37 ⁰C for 24hrs. The overnight cultured plates were aseptically examined for morphological characteristics of typical colonies of *Salmonella* Typhimurium on the media (Peter *et al.,* 2024). All suspected *S.* Typhi colonies were screened by Gram staining, *Microgen* ™ *Gn A+B –ID* System (Bioproducts Limited, Camberley, UK) and were further screened by polyvalent *Salmonella* antisera (Thermo Fisher Scientific™, U.S.A) for flagella "H" and somatic "O" antigen according to manufacturer's instruction. The cultured and morphologically confirmed *S.* Typhi were seeded onto CHROM agar ESBL plates (HyLabs, Rehovot, Israel). The presence of white colonies on the CHROMagar ESBL plates infers ESBL-producing *S.* Typhi and was acseptically identified using the VITEK-2 Automated System (Biomerieux, France).

Polymerase Chain Reaction Amplification of ESBL genes

The *S.* Typhi genomic DNA was extracted per the manufacturer's instructions, utilizing the advanced BioRobot EZ1 XL instrument (QIAGEN, Germany). For amplification, we employed the Master Mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany) and specific primers for polymerase chain reaction (PCR) were utilized to amplify genes encoding extended-spectrum β-lactamases (**bla$_{TEM}$**; F:ATAAAATTCTTGAAGACGAAA; R:GACAGTTACCAATGCTTAATC; **bla$_{CTX-M}$**; F:CGCTTTGCGATGTGCAG, R:ACCGCGATATCGTTGGT) according to Nwosu *et al.* (2023). The detection of amplified genes was effectively carried out using agarose gel electrophoresis with SYBR Safe (Invitrogen®, USA) alongside a DNA molecular weight marker (BenchTop pGEM® DNA Marker, Promega, Madison, WI, USA). For optimal visualization of the gels, ultraviolet illumination was employed, utilizing the BenchTop pGEM® DNA Marker (Promega, Madison, WI, USA).

Preparation of Herbal medicinal plant extract

Exactly 800 grams of Siam weed, bush cane, neem, Brazilian tea leaves, and 2500grams of garlic ginger cloves were thoroughly and properly washed separately in sterile water and air-dried at room temperature and thereafter pulverized using an automated grinder (SAISHO, china).

The pulverized herbal plant was separately mixed with 4500 ml of absolute ethanol (Guangdang Guanghua Chemical Factory Co. Ltd, China) and loaded into a soxhlet apparatus (Lincoln Mark Medical, England). The mixture was heated at 82°C for 16 hr. After the extraction, a rotary evaporator (Lincoln Mark Medical, England) was used to dry the ethanol extract of the herbal plant to obtain the solid mass for bioassay.

Preparation of Different Concentrations and Antibacterial Activity of ethanol extract of *medicinal plant*

The preparation of different concentrations for each medicinal plant was performed separately. One gram (1 g) of each medicinal plant was reconstituted with a diluent in 10 mL of 70% Dimethylsulfoxide (Guangdang Guanghua Chemical Factory Co. Ltd, China) to achieve a concentration of 100 mg/mL. Following this, a 10^{-3} serial dilution was performed, resulting in concentrations of 50 mg/mL and 25 mg/mL. The antibacterial activity of plant extracts was evaluated using the Agar well diffusion method, following the protocols established by previous researchers (Nwankwo *et al.*, 2023; Nwode *et al.*, 2024). To begin, a suspension of bacterial cells was prepared to achieve a concentration of $1x10^6$ colony-forming units per milliliter (CFU/ml), equivalent to the 0.5 McFarland turbidity standard. This suspension was then uniformly streaked onto Petri dishes containing a sterilized Mueller-Hinton agar (Thermo Fisher Scientific™, U.S.A.), and the plates were allowed to stand for 15 minutes, providing adequate time for the inoculated bacteria to pre-diffuse. Following this, wells were bored in the agar using an 11 mm sterile cork borer (Supertek®, U.S.A.), and each well was filled with 1 ml of the corresponding extract concentration. The inoculated agar plates were incubated for 24 hours at 37°C, allowing for effective interaction between the plant extracts and the bacterial cells. After incubation, the zones of inhibition were measured with a metric ruler and recorded in millimeters (mm).

RESULTS

Of the 200 blood samples, *S*. Typhi accounted for 159(79.5 %), while other bacteria isolates accounted for 41 (20.5 %), as presented in Figure 1. The amplification of the ESBL gene revealed a high proportion of CTX-M 159 (100 %) as the most predominant gene, followed by SHV 81 (50.9 %), while TEM 52 (32.7 %) was the least identified gene in *S*. Typhi (Figure 2).

Brazilian tea extract assayed at 100mg/ml, 50mg/ml, and 25mg/ml concentration displayed antibacterial activity recording 100 %, 93 %, and 50 %. Siam weed extract was 83 %, 47 %, and 10 % at 100mg/ml, 50mg/ml, and 25mg/ml concentration. Bush cane had no inhibitory effect (0.0 %) in all concentrations. Neem extract at 100mg/ml, 50mg/ml, and 25mg/ml concentration had good susceptibility values of 100 %, 85.0 %, and 67 %. Ginger extract had 100 %, 63. 0%, 46. 0% at 100mg/ml, 50mg/ml, and 25mg/ml concentration. Garlic extract recorded 100 %, 59.0%, and 0.0 % at 100mg/ml, 50mg/ml, and 25mg/ml concentration respectively (Figure 3).

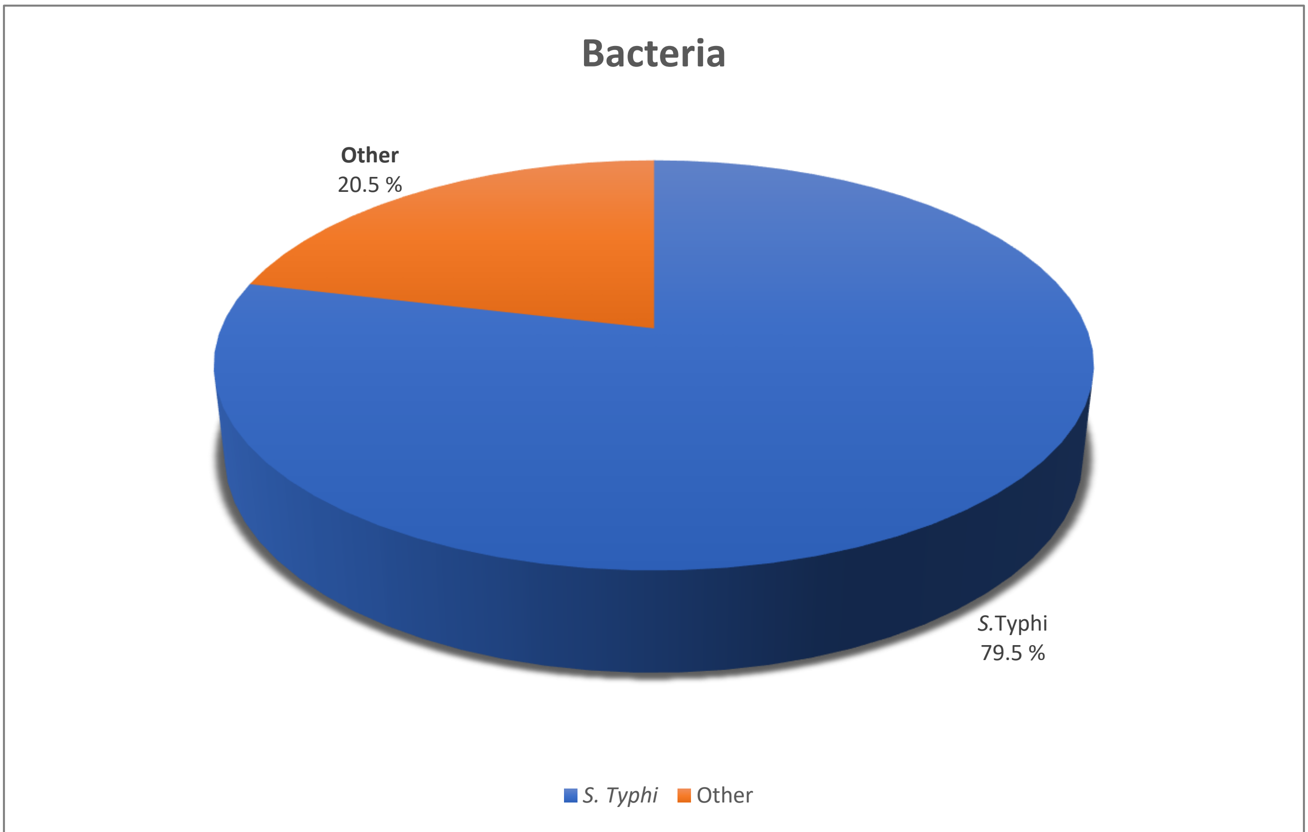

Figure 1: The percentage distribution of *S.* Typhi

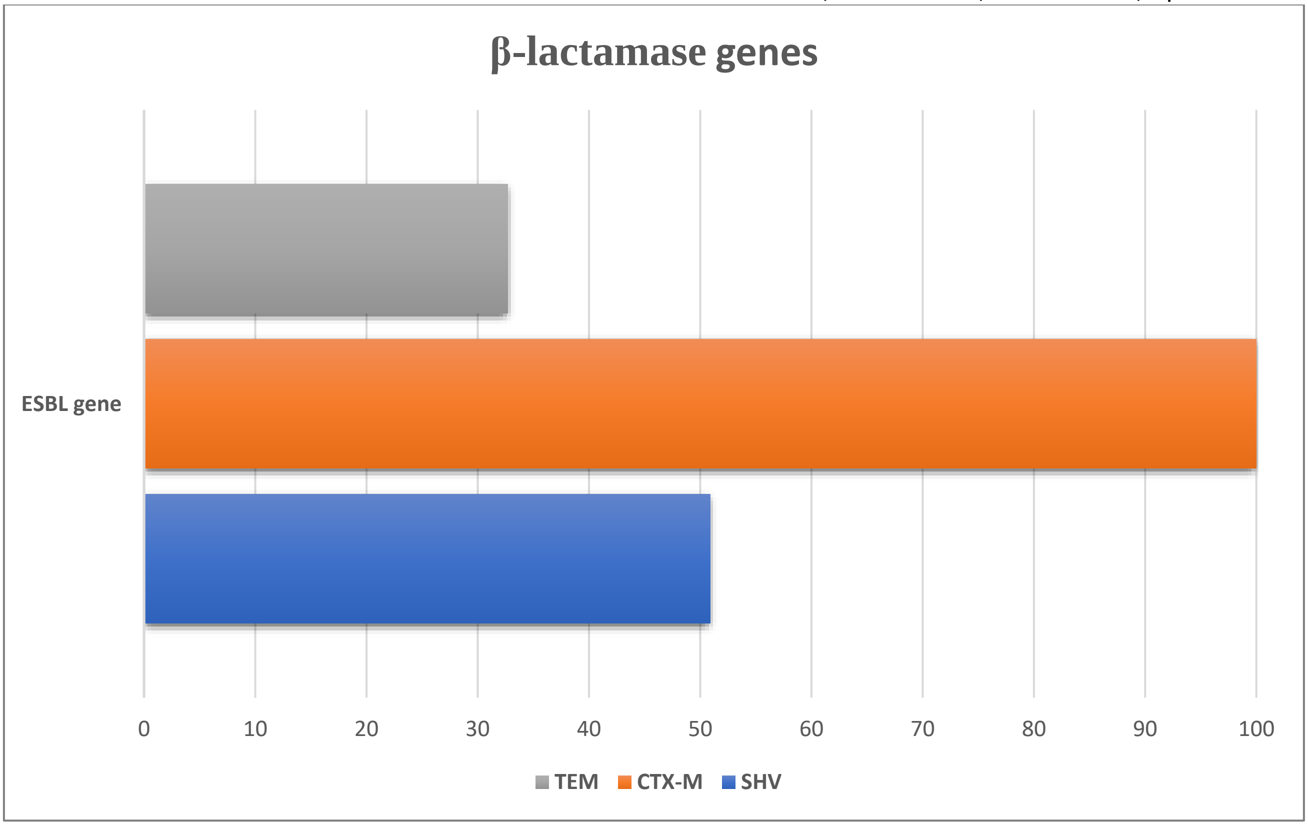

Figure 2: The frequency of amplified ESBL gene
Key: **TEM** = Temionera, **CTX-M** = Cefotaximase, **SHV** = Sulfhydryl variant

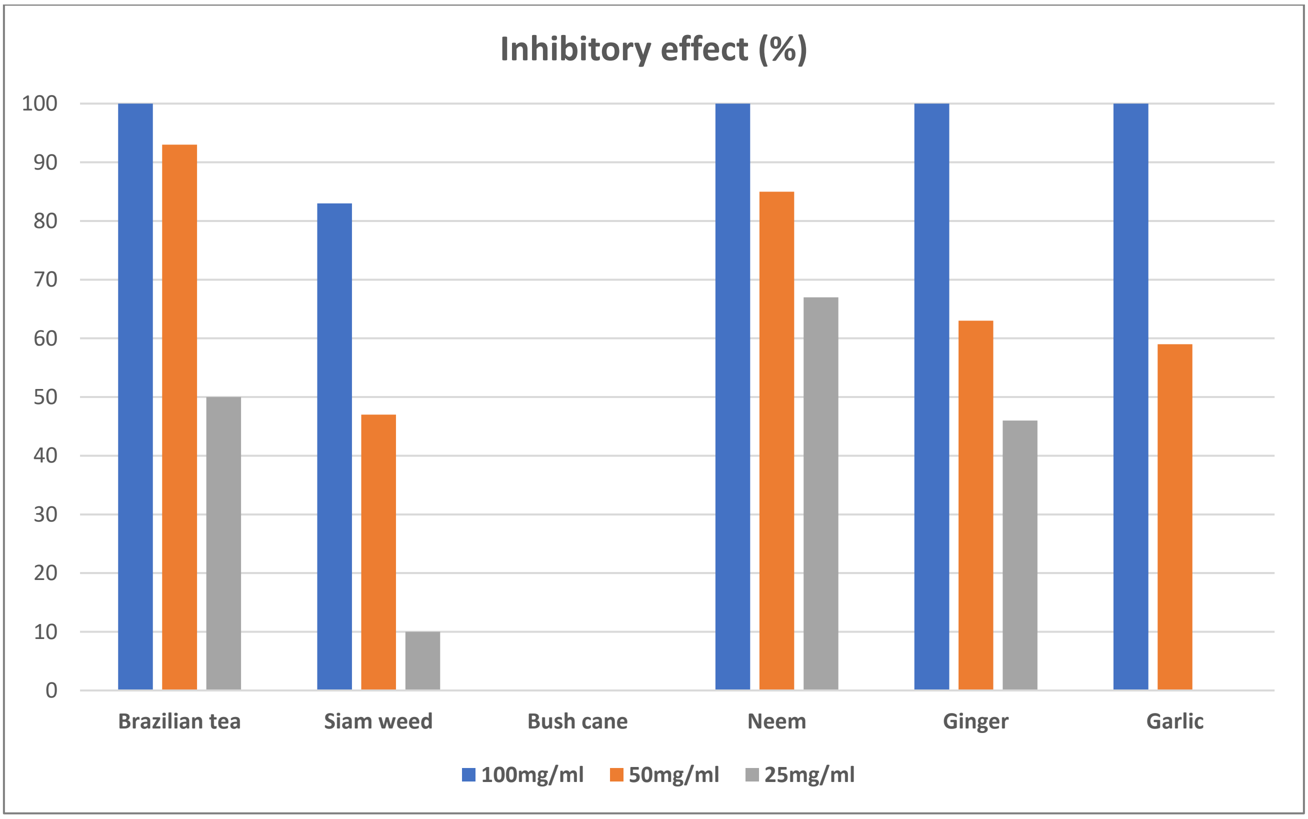

Figure 3: Antibacterial activity of selected medicinal plant

DISCUSSION

The CTX-M gene in our study emerged as the most identified ESBL genotype. This is in tandem with reports in Lagos and Benue State, Nigeria (Akinyemi *et al.,* 2022; Okpa *et al.,* 2020). The Cefotaximase (CTX-M gene) reveals the hydrolytic capacity of extended-spectrum *β*-lactamases against cefotaxime antibiotic. Bacteria that produce the CTX-M genotype are non-susceptible with cefotaxime and ceftazidime Minimum Inhibitory Concentration (MICs) resistant values (greater than 64 µg/ml) and (2-8 µg/ml) respectively (Okpa *et al.,* 2020).

The CTX-M ESBLs genotypes are rapidly disseminated and are worldwide in distribution (Rotimi *et al.,* 2008; Djeffal *et al.,* 2017; Nadimpalli *et al.,* 2019; Dor *et al.,* 2020; Egwu *et al.,* 2023). The classification of these genes is based on their amino acid sequences, dividing them into five distinct phylogenetic groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-15, and CTX-M-25 (Rotimi *et al.,* 2008; Bakeri *et al.,* 2003). Due to the enhanced capability to truncate the action of cephalosporins, most CTX-Ms variants can easily hydrolyze cefotaxime and ceftazidime, rendering these drugs ineffective (Okpa *et al.,* 2020; Rotimi *et al.,* 2008; Djeffal *et al.,* 2017; Nadimpalli *et al.,* 2019; Akinyemi *et al.,* 2015).

CTX-M-type β-lactamases, which are distinct from TEM and SHV-type extended-spectrum β-lactamases (ESBLs), were first identified in the 1980s and are closely related to the beta-lactamases produced by *Kluyvera* species (Rotimi *et al*., 2008). Understanding the mechanisms by which *Salmonella* strains acquire the gene encoding CTX-M type ESBLs from *E. coli* and *Klebsiella* species is crucial. This acquisition in community settings can lead to the dissemination of this gene among various *Salmonella* serotypes, highlighting the importance of monitoring and addressing antibiotic resistance in these bacteria (Rotimi *et al*., 2008; Nwosu *et al*., 2023). By enhancing our awareness and implementing effective strategies, we can better manage the spread of resistance genes and protect public health.

The occurrence of SHV 81 (50.9 %) and TEM 52 (32.7 %) was reiterated in other studies (Wu *et al.,* 2013; Saliu *et al.,* 2017; Onyenwe *et al.,* 2020; Ibrahim *et al.,* 2022; Egwu *et al.,* 2023). This study highlights the presence of specific strains of *S*. Typhi that produce ESBL genotypes, which may provide valuable insights into the persistent cases of Salmonellosis observed in patients, even when treated with third-generation cephalosporins. Understanding this relationship could pave the way for more effective treatment strategies in the future. The misuse and overuse of antibiotics in both human and animal populations are well-known factors contributing to the evolution of drug-resistant bacteria. This occurs through gene mutations or the horizontal transmission of resistance genes via plasmids. The emergence of resistance, particularly in bacteria such as *Salmonella* Typhi in our study that produce CTX-M, TEM, and SHV, poses a significant public health challenge. These resistant strains often lead to the failure of empirical antibiotic therapy, resulting in increased morbidity and mortality rates. Antimicrobial resistance (AMR) hinders the achievement of the Sustainable Development Goals (SDGs) and impacts food safety and food security. The rise of extended-spectrum β-lactamases (ESBLs) has resulted in higher rates of morbidity, extended hospital stays, and more costly treatment options (Husna *et al.,* 2023)

The Brazilian tea extract exerts antibacterial activity against the isolate at 100mg/ml, 50mg/ml, and 25mg/ml concentration. Based on our current understanding, this finding tends to be the first published *in vitro* assessment of this plant on *S*. Typhi. Within the Efik and Ibibio tribes in Nigeria, the Brazilian tea commonly called 'adan umoun' is one of the ethnobotanical plants that plays a significant role in traditional medicine practices as an anti-typhoid remedy.

Additionally, Brazilian tea has several ethnomedicinal benefits, including promoting weight loss, reducing inflammation, being rich in antioxidants (which help protect against inflammation, cardiovascular issues, and liver disease), lowering blood sugar and cholesterol levels, enhancing exercise performance, and increasing bone density (Lutomski *et al.,* 2020; José *et al.,* 2023). This *in vitro* study substantiates the use of this plant as an anti-typhoidal agent and also accentuates the exploration of the antimicrobial potential of this plant.

Our findings indicate that most isolates demonstrated resistance to bush cane (*Costus afer*) extract across various concentrations. However, it is noteworthy that bush cane, referred to as "Mbritem" in the Efik and Ibibio tribes, has been documented to exhibit antibacterial activities against several pathogenic bacteria, including *E. coli*, *S. aureus*, MRSA, *Klebsiella pneumoniae, Bacillus subtilis*, and *Pseudomonas aeruginosa* (Peter *et al.,* 2022; Izah *et al.,* 2019).

The phytochemical screening conducted on the rhizomes, stems, and leaves of this plant using different solvents reveals a diverse array of compounds, including glycosides, phenols, tannins, saponins, alkaloids, and triterpenes Peter *et al.,* 2022; Akpan *et al.,* 2012; Jesus *et al.,* 2016; Boison *et al.,* 2019). This indicates the potential for further exploration of these phytochemicals and their applications. The variation in our findings could be attributed to the solvent utilized during the assay, extraction process, lack of receptors that possess an affinity for the phytomolecules, regional climatic and environmental condition of the plant, plant age, etc., Despite the poor *in vitro* activity noted in this study, the ethnomedicinal potential of this tropical plant should not be overlooked, as it offers various medicinal uses, including the treatment of helminthic**,** inflammation, rheumatism, arthritis, epileptic attack**,** stomach ailments, miscarriages, cough, hepatic disorders, hemorrhoids, and diabetes (Boison *et al*., 2019).

Neem (*Azardicta indica*) also displayed good antibacterial activity, and the isolates susceptibility profile was concentration-dependent. Neem leave is well-known to possess enormous antimicrobial potential (Akhter and Sarker, 2019). The MIC and MBC of the neem leaf extract for *Samonella* species, *S. pullorum,* and *S. gallinarum* has been reported (Ali *et al.,* 2021). Several research have comprehensive review the large phytochemical abundance of neem (Wylie and Merrell, 2022; Saleem *et al.,* 2018; Gupta *et al.,* 2017).

Recent findings have highlighted the significant ethnobotanical applications of the neem plant. The leaf extract effectively helps in reducing tooth plaque and is beneficial for treating lice infestations. Neem is known for its beneficial compounds that may contribute to lowering blood sugar levels, promoting the healing of ulcers in the digestive tract, eliminating harmful bacteria, and preventing the formation of plaque in the mouth. It also exhibits immunomodulatory, anti-inflammatory, antihyperglycemic, antiulcer, and antimalarial properties (Wylie and Merrell, 2022; Saleem *et al.,* 2018; Gupta *et al.,* 2017). Incorporating neem into your wellness routine could support these aspects of health effectively.

The neem plant is increasingly recognized for its potential as a natural drug, exhibiting a diverse array of bioceutical properties (Nagini *et al.*, 2021; Braga *et al.*, 2020; Saleem *et al.*, 2018). Its effectiveness against both Gram-negative and Gram-positive bacteria highlights its promising role in addressing bacterial infections (Al Saiqali *et al.*, 2018; Zihadi *et al.*, 2019; Ibrahim and Kebede, 2020; Bhatwalkar *et al.*, 2021). Further research into its applications may lead to significant advancements in natural healthcare solutions.

Garlic cloves extract was not exceptional. The biological activity of garlic extract has been published by existing studies (Kaur *et al.,* 2021; Bhatwalkar *et al.,* 2021; Circella *et al.,* 2022). At 25mg/ml concentration, the garlic extract was ineffective. This may result from the instability of a potent antimicrobial agent known as allicin (Choo *et al.,* 2020; Belguith *et al.,* 2010). The instability of this compound is achievable through the enzymatic reaction of allinase which transforms allicin to its precursor alliin. Although alliin is somewhat unstable, this characteristic encourages a series of non-enzymatic reactions that lead to the formation of valuable compounds such as polysulfides, vinyl dithiins, and ajoene. These compounds have shown promising antimicrobial properties, highlighting potential avenues for further research and application (Nakamoto *et al.,* 2020; Quesada *et al.,* 2020). Garlic has recently garnered attention for its potential health benefits, showing promise in combating cancer, heart disease, high blood pressure, diabetes, skin conditions, and bone disorders. These benefits are attributed to garlic's antioxidant, anti-inflammatory, and lipid-lowering effects (Ansary *et al.,* 2020**)**.

Ginger extract had a better effect on the isolates. Comparatively, similar reports have been published in animal and human *S.* Typhi strains (Gull *et al.,* 2012; Robinson *et al.,* 2022; Felicia *et al.,* 2022). The bioactive ingredient contained in ginger has been useful in the prevention and treatment of various human diseases, including asthma, diabetes, dementia, cardiovascular disease, cancer, ulcerative colitis, etc. (Kela *et al.,* 2023; Thakor *et al.,* 2023). The antimicrobial potentials of ginger have been studied extensively; therefore, it can be used to treat diseases associated with ESBL in humans.

The results of our bioassay on the medicinal plant material revealed a clear concentration-dependent effect. This supports the earlier hypothesis that the concentration of an extract is directly proportional to the degree of microbicidal activity (Peter *et al.,* 2022; Agbo *et al.,* 2024). Such findings underscore the importance of concentration in maximizing the therapeutic potential of these extracts. The significance of our *in vitro* findings, in light of the global challenges posed by antimicrobial resistance bacteria, suggests that these plants possess significant potential as a composite material with antibacterial properties. This could revolutionize the management and treatment of antibiotic-resistant *Salmonella* Typhi and related infectious diseases in human medicine.

CONCLUSION

This study demonstrates the antibacterial potential of herbal plants against *Salmonella* Typhi strains that produce TEM, CTX-M, and SHV extended-spectrum β-lactamases (ESBL). The selected medicinal plants showed effectiveness at 100 mg/ml, 50 mg/ml, and 25 mg/ml concentration. This study is the first to report the antibacterial activity of the extract on *Salmonella* Typhi with an ESBL genotype. Therefore, it is crucial to use this extract prudently and judiciously in ethnobotanical practices and human medicine for treating infections associated with ESBL. Further research should focus on the pharmacodynamics and pharmacokinetics of the medicinal plant extract. Additionally, guidelines and regulations are necessary for the appropriate use of antibiotics to prevent drug resistance and the spread of resistant strains to susceptible strains.

AVAILABILITY OF DATA AND MATERIAL

The data from this study is available upon request from the corresponding author.

AUTHORS CONTRIBUTIONS

This research was conducted through collaboration among all authors. Author CIE developed the protocol, while Authors CIE, AGC, MEO, and EU collaborated on the first draft of the manuscript. Authors IFO, IPO, CIE, and NPL were responsible for the characterization and analysis of the study. Author CIE supervised the research and provided critical revisions to the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

None

FINANCIAL SUPPORT

None

ETHICS STATEMENT

All experiments in this study were executed following ARRIVE guidelines regarding Animal and human subjects. Ethical approval with reference No:

UCTH/Vol.34/ERC/22 was obtained from the Research and Ethics Committee of the Hospital.

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