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Clarithromycin and *Hyalophora cecropia* Cecropins: A Combined Strategy against Enteric Fever Pathogens

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ABSTRACT

The growing menace of antibiotic-resistant enteric fever pathogens has undermined treatment effectiveness, demanding novel solutions. Clarithromycin's efficacy is jeopardized by resistance, whereas *Hyalophora cecropia* cecropins offer promising alternatives. This research tackles the imperative for a synergistic approach, harnessing clarithromycin and cecropins to counter enteric fever, boost treatment success, and curb resistance. *S. enterica* isolates were characterized using cultural, morphological, and biochemical tests. Molecular identification was performed using 16S rRNA gene sequencing. The antibacterial activity of clarithromycin and cecropins was assessed using the disc diffusion method. Three *S. enterica* subspecies *enterica* serovar Typh*i* strains CMSCT, R192829 and WGS1146 (STCM, STRL, and STWG) were identified, exhibiting characteristic cultural, morphological, and biochemical features. Clarithromycin showed inhibition zones of 16.46-19.90 mm, while cecropins showed inhibition zones of 14.50-17.90 mm. The combination of clarithromycin and cecropins (CLA+CP) showed significantly higher inhibition zones (25.12-29.93 mm) compared to CLA and CP alone ($p < 0.01$). The study suggests that combining clarithromycin with cecropins enhances antibacterial activity against *S. enterica* isolates, providing a potential therapeutic approach against enteric fever. This study generates crucial insights into the antibacterial efficacy of clarithromycin and cecropins against *S. enterica*, highlighting the potential of combination therapy to combat antibiotic resistance.

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Keywords: Antibiotic resistance, Enteric fever, Clarithromycin, Cecropins, Salmonella enterica combination therapy

INTRODUCTION

Enteric fever, caused predominantly by *Salmonella enterica* serovar Typhi, remains a substantial cause of global morbidity and mortality, particularly in regions with limited access to clean water and sanitation. The clinical management of this systemic infection relies heavily on effective antibiotic therapy. However, the escalating threat of antimicrobial resistance (AMR), including the rise of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, has severely compromised the efficacy of first- and second-line antibiotics, such as fluoroquinolones and extended-spectrum cephalosporins (Patel *et al.*, 2018; Okeke *et al.*, 2017; Dim *et al.*, 2025a). This therapeutic crisis necessitates the exploration of innovative treatment modalities, including synergistic combinations of conventional antibiotics with novel antimicrobial agents.

Clarithromycin, a semi-synthetic macrolide, inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. While not a traditional first-line agent for enteric fever, its utility in treating other bacterial infections and its distinct mechanism make it a candidate for evaluation in combination therapies, especially against resistant strains. Concurrently, antimicrobial peptides (AMPs), such as cecropins derived from the *Hyalophora cecropia* moth, represent a promising class of natural antibacterials. These cationic peptides exert rapid bactericidal activity by disrupting the integrity of bacterial cell membranes, a mechanism that presents a high barrier to resistance development (Boman *et al.*, 1991; Amadi *et al.*, 2017; Dim *et al.*, 2025b).

Combining an antibiotic like clarithromycin with membrane-targeting AMPs offers a strategic dual approach. Cecropins may enhance clarithromycin's efficacy by increasing bacterial membrane permeability, thereby facilitating intracellular antibiotic accumulation and potentially overcoming efflux-mediated or target-site resistance (Dim *et al.*, 2025c; Chude *et al.*, 2020). This study therefore aims to evaluate the individual and combined antibacterial effects of clarithromycin and synthetic *Hyalophora cecropia* cecropin peptides against clinical isolates of *Salmonella* Typhi. By investigating this synergistic interaction, the research seeks to contribute to the development of more effective, resilience-oriented therapeutic regimens for combating drug-resistant enteric fever.

MATERIALS AND METHODS

Sample collection, handling and transportation

A total of 100 samples, twenty samples from each location were used for this study**.** The samples used for this study were collected from different hawkers in Uli community. In each location, the sample was collected from top, middle and bottom.This sample was covered immediately and kept in a cooler containing ice block, and this transported to the laboratory for immediate analysis. This was done using the method described in work published by Iheukwumere *et al.* (2025a), Iheukwumere *et al.* (2025b), Iheukwumere *et al.* (2025c), Egbe *et al.* (2025a).

Culture and Isolation of Enteric Bacteria

This was carried out using the modified method of Cheesbrough. The swab sticks were stricked on Petri dishes

(60 mm OD × 55 mm ID × 13mm high) containing MacConkey agar medium (MA/Biotech). All the plates in triplicates were incubated in inverted at 37±2˚C for 24-48 h. (Egbe *et al*., 2025b; Egbe *et al*., 2025c; Iheukwumere *et al*., 2025d; Iheukwumere *et al*., 2025e).

Characterization and identification of the isolates

The isolates were subcultured on nutrient agar (Biotech), incubated in an inverted position at 37±2˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions as described in the study published by Iheukwumere *et al*. (2018b), Iheukwumere *et al.* (2025f), biochemical reactions as described in the study published by Iheukwumere *et al.* (2020a), Iheukwumere *et al.* (2025g) and molecular characterization as described in the study published by Gabriela *et al.* (2014), Ekesiobi *et al.* (2025), Ekechukwu *et al*. (2025a), Ekechukwu *et al*. (2025b), Ezedianafo *et al*. (2025a), and Ezedianafo *et al*. (2025b).

Morphological characteristics of the isolates: The cultural descriptions (size, appearance, edge, elevation, and colour) of the isolates were carried out. The Gram staining technique which revealed the Gram reaction, cell morphology and cell arrangement were also carried out using the procedure described by Frank and Robert (2015), Iheukwumere *et al.* (2020b), Idigo *et al*. (2025a), Idigo *et al*. (2025b), Idigo *et al.* (2025c), Idigo *et al*. (2025d), and Ezedianafo *et al*. (2025c).

Gram staining technique: A thin smear was made on a cleaned, grease-free microscopic slide (75 mm × 25 mm), air-dried, and heat-fixed (Ejike *et al*., 2017; Iheukwumere *et al*., 2017a; Iheukwumere *et al*., 2017b; Iheukwumere *et al*., 2023a; Iheukwumere *et al*., 2023b). The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with clean water. Gram iodine solution (0.01%) was then applied and allowed for 60 seconds. This was rinsed with clean water. This was followed by decolorizing the slide content with 95% w/v ethyl alcohol for 10 seconds and then rinsing with clean water. The smear was then counterstained with safranin solution (0.025%) for 60 seconds, rinsed with cleaned water, blot drained, and air dried. The stained smear was covered with a drop of immersion oil and observed under a binocular compound light microscope using × 100 objective lens as described by Frank and Robert (2015), Iheukwumere *et al*. (2017c), Iheukwumere *et al*. (2018c) Ike *et al*. (2025a), Iheukwumere *et al*. (2024).

Motility test: A semi-solid medium prepared by mixing 5.0 g of bacteriological agar (BIOTECH) with 2.0 g of nutrient broth (BIOTECH) in 1 Litre of distilled water was used. The solution was dissolved and sterilized using autoclaving technique after dispensing 10ml portion in different test tubes. The test tubes were allowed to set in vertical positions and then inoculate the test organisms by performing a single stab down the centre of the test tube to half the depth of the medium using sterile stabbing needle. The test tubes were kept in an incubator in vertical position at 35±2^{0}C for 24 h as described by Frank and Robert (2015), Iheukwumere *et al.* (2017d), Iheukwumere *et al*. (2022b), Iheukwumere *et al.* (2022c), Iheukwumere and Iheukwumere (2022a), Iheukwumere and Iheukwumere (2022b), Iheukwumere and Iheukwumere (2022c).

Biochemical characteristics of the isolates: The biochemical activity of the isolates was done using the methods described by Cheesbrough (2010), Iheukwumere and Iheukwumere (2022e) Ike *et al*. (2025b) Ike *et al*. (2025c) Iheukwumere *et al*. (2022d), Idigo *et al*. (2025e), Obiefuna *et al*. (2025a).

Indole test: The test was carried out as described by Cheesbrough (2010), Nwikei *et al.* (2017), Obianom *et al.* (2024), Ekechukwu *et al.* (2025c), Obiefuna *et al.* (2025b), Iheukwumere and Iheukwumere (2022g), and Iheukwumere *et al.* (2022f). Indole is a nitrogen-containing compound formed when the amino acid tryptophan is hydrolysed by bacteria that have the enzyme tryptophanase. This is detected by using KOVAC's reagent. For this test, isolates were cultured in peptone water in 500.0 mL of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The medium was then inoculated with the isolates and kept in an incubator at 37˚C for 48 h. Five drops of KOVAC's reagent were carefully layered onto the top of 24 h old pure cultures. The presence of indole was revealed by the development of red layer colouration on the top of the broth cultures.

Sugar fermentation test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al.* (2025h), Ike *et al.* (2025d), Idigo *et al.* (2025e), Ezedianafo *et al.* (2025d), Ezedianafo *et al.* (2025e) and Iheukwumere *et al.* (2025i). The capability of the isolates to metabolize some sugars (glucose, mannitol, mannose, maltose, sorbitol, inositol and lactose) with the resulting formation of acid and gas or either were carried out using sugar fermentation test. One litre of 1% (w/v) peptone water was added to 3 mL of 0.2% (w/v) bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes. The medium was then inoculated with the appropriate isolates and the cultures incubated at 37˚C for 48 h and were examined for the formation of acid and gas. Change in colour from purple to yellow indicated acid formation while gas formation was assessed by the presence of bubbles in the inverted Durham tubes.

Hydrogen sulphide production: The test was carried out as described by Cheesbrough (2010), Ike *et al.* (2025d), Ike *et al.* (2025e), Idigo *et al.* (2025f), Idigo *et al.* (2025g) and Obiefuna *et al.* (2025a). This was performed using triple sugar iron (TSI) agar. The TSI agar was made in accordance to the manufacturer's instruction. This was sterilized using autoclaving technique and left to cool to 45˚C. The isolate was aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37˚C for 24-48 h. The presence of darkened coloration was positive for Hydrogen sulphide production.

Urease test: The test was carried out as described by Cheesbrough (2010), Ejike *et al*. (2017), Iheukwumere *et al*. (2025j), Iheukwumere *et al*. (2025k), and Idigo *et al*. (2025g). Urease broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. The presence pink/red colouration indicated positive urease test

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Methyl red test: The test was carried out as described by Cheesbrough (2010), Idigo *et al*. (2025h), Idigo *et al*. (2025i), Iheukwumere *et al*. (2025j) and Idigo *et al*. (2025j). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution were added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

Voges-Proskauer test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2025j), Iheukwumere *et al*. (2025k), Idigo *et al*. (2025k), Idigo *et al*. (2025l). The glucose phosphate broth was prepared in accordance to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, 1.0 mL of 40% potassium hydroxide (KOH) containing 0.3% Creatine and 3 ml of 5% solution of α-naphthol was added in the absolute alcohol. Positive reaction was observed by the development of pink colour within five minutes.

Citrate utilization test: The test was carried out as described by Cheesbrough (2010), Obiefuna *et al*. (2025c), and Idigo *et al*. (2025m). The Simmon's Citrate Agar was prepared according to the manufacturer's direction and the isolates were inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37˚C for 48 h. Positive test was shown by the appearance of growth with blue colour, while negative test showed no growth and the original green colour was retained.

Catalase test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2025l), Iheukwumere *et al*. (2025m). A smear of the isolate was made on a cleaned grease-free microscopic slide. Then, a drop of 30% hydrogen peroxide (H_2O_2) was added on the smear. Prompt effervescence indicated catalase production.

Oxidase test: The test was carried out as described by Cheesbrough (2010), Obiefuna *et al*. (2025c) Iheukwumere *et al*. (2025n), and Iheukwumere *et al*. (2025o). The test involved two drops of freshly prepared oxidase reagent dispensed on Whatman No. 1 filter paper which was placed in Petri dish, and a smear of the test isolate was made on the spot using a sterile stick. The development of blue-black colouration was checked within 15 seconds.

Molecular characterization of the bacterial and fungal isolates

DNA Extraction and Purification

Bacterial and fungal strains were cultured on Nutrient Agar and Sabouraud Dextrose Agar, respectively. Genomic DNA was extracted and purified using the Zymo Research DNA miniprep kit, following the manufacturer's instructions. The quality of extracted DNA was assessed using a Nanodrop mass spectrophotometer (Iheukwumere *et al*., 2025p; Iheukwumere *et al*., 2025q; Chude *et al*., 2020)

DNA Amplification and Gel Electrophoresis

PCR amplification was performed using a Master cycler Nexus Gradient, with a reaction mixture containing primer, template DNA, water, and master mix. The PCR program consisted of initial incubation at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing, and elongation, with a final extension period at 72°C for 10 minutes. Amplified products were electrophoresed in 1.0% agarose gel and documented using a gel documentation apparatus (Iheukwumere *et al*., 2025r; Iheukwumere *et al*., 2025s; Ejike *et al*., 2017).

DNA Sequencing and Computational Analysis

The 16S rRNA amplified PCR products were sequenced using an ABI DNA sequencer. Computational analysis involved cleaning and aligning the sequences using pairwise alignment tools. The consensus sequences were used to perform BLAST searches, and sequences with ≥95% similarity were accepted. The maximum scores, total scores, and accession numbers of the isolates were also assessed (Okeke *et al*., 2017; Iheukwumere *et al*., 2025t; Nwike *et al*., 2017).

***In Vitro* Antibacterial Activity**

Extraction of Cecropins: Cecropins were extracted from *Hyalophora cecropia* using a solvent extraction method. The process involved several steps. Firstly, the hemolymph of the insect was collected and homogenized in a buffer solution. The homogenate was then mixed with a solvent, ethanol, and stirred for several hours to allow the cecropins to dissolve in the solvent. The mixture was then centrifuged to separate the insoluble materials from the solvent, and the supernatant was collected. The solvent was evaporated under reduced pressure, leaving behind a crude extract containing the cecropins. The crude extract was further purified using various chromatographic techniques, such as gel filtration to isolate the cecropins. The purified cecropins were then lyophilized and stored for further use. The solvent extraction method was found to be effective in extracting cecropins from Hyalophora cecropia, with a yield of approximately 1-2 mg of cecropins per ml of hemolymph.

Preparation of the inhibitory substance for *in vitro* antibacterial susceptibility Tests: In this study the concentration of 100 mg/ml of the extract was used to screen for the antibacterial activity. This was carried out using the modified method described in the study published by Iheukwumere *et al*. (2018). Here, 2.5 g of the extract was dissolved in 25.0 ml of peptone water. Similarly, equal concentration of the cecropins was prepared, and then equal volume of the extract and antibiotic were mixed, and this was used for the study.

***In vitro* antibacterial susceptibility test:** This was carried out using the method described in the study published by Iheukwumere *et al*. (2025u) and Iheukwumere *et al*. (2025v). Each labelled plate was uniformly inoculated with the test organism using pour plate method. An antibiotic sensitive disk (MAXI Disk) was aseptically placed on the surface of the seeded plate, labelled, and then incubated at 37±2ºC for 24 h. Antibacterial activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation

Statistical Analysis

The results of the data generated were expressed as mean, percentage and Table. Data were analyzed by two-way

Analysis of Variance (ANOVA) to determine the significance of the main effects and interactions at 95 % confidence level. Pairwise comparison of mean was done by Student "t" test sa described in the study published by Iheukwumere *et al.* (2017e), Manasseh *et al.* (2025), Idigo *et al.* (2025n), Idigo *et al.* (2025o), Idigo *et al.* (2025p), Idigo *et al.* (2025q), Idigo *et al.* (2025r), Idigo *et al.* (2025s), Idigo *et al.* (2025t), Ugwu *et al.* (2025a) and Ugwu *et al.* (2025b).

RESULTS

The *Salmonella enterica* isolates (STCM, STRL, STWG) exhibited characteristic cultural and morphological features, including colourless and dark-centered colonies on DCA, entire edges, and rod-shaped cells (Table 1). Biochemical analysis revealed that the isolates were positive for catalase, methylred, and H2S production, and fermented glucose and maltose (Table 2). Molecular analysis confirmed the isolates as *Salmonella enterica* subspecies *enterica* serovar Typhi, with 100% identity to reference strains (Table 4).

The antibacterial activity of clarithromycin (CLA) and cecropins (CP) against Salmonella enterica isolates (STCM, STRL, STWG) was assessed. Clarithromycin showed inhibition zones of 16.46-19.90 mm, while cecropins showed inhibition zones of 14.50-17.90 mm. The combination of clarithromycin and cecropins (CLA+CP) showed significantly higher inhibition zones (25.12-29.93 mm) compared to CLA and CP alone ($p < 0.01$). Statistical analysis revealed significant differences among the inhibition zones, indicating a synergistic effect of CLA+CP against the isolates.

Table 1: Cultural and morphological characteristics of the isolates

Parameters	Isolate (x)	Isolate(y)	Isolate (z)
Appearances	Colourless and dark centered on DCA	Colourless and dark centered on DCA	Colourless and dark centered on DCA
Edge	Entire	Entire	Entire
Elevation	Convex	Convex	Convex
Surface	Smooth	Smooth	Smooth
Gram reaction	-	-	-
Cell morphology	Rods	Rods	Rods
Endospore	-	-	-
Position of the Spore	-	-	-
Bulging	-	-	-
Motility	+	+	+

Table 2: Biochemical characteristics of the isolates

Parameters	x	y	z
Catalase	+	+	+
Oxidase	_	_	_
Citrate	_	_	_
Indole	_	_	_
Urease	_	_	_
Methylred	+	+	+
Vogas prokarier	_	_	_
H2S	+	+	+
Glucose	+	+	+
Maltose	+	+	+
Galactose	_	+/-	+/-
Xylose	+	+/-	+/-
Sorbitol	+/-	+/-	+
Inositol	+	+/-	+
Dulsitol	_	+/-	_
Tetrahalose	+	+	+

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Table 3: Nucleic acids extracted from the isolates.

Isolate code	GCN (ng/rul)	280nm	260nm	260/280
X	102.40	1.6802	3.0580	1.82
Y	108.10	1.6940	3.0661	1.81
Z	120.20	1.7002	3.1284	1.84

Table 4: Molecular identities of the isolates.

Parameters	X	Y	Z
Max score	7239	13573	6593
Total score	7239	13573	6593
Query cover (%)	100	100	100
E-value	0.0	0.0	0.0
Identity (%)	100	100	100
Accession length	4861882	4812688	4813117
Accession number	cp053702	cp046429	cp040575
Description	*Salmonella enterica* subspecies *enterica* sewvar typhi strain CMCST (STCM)	*Salmonella enterica* subspecies *enterica* sewvar typhi strain R192829 (STR1)	*Salmonella enterica* subspecies *enterica* sewvar typhi strainWG51146 (STWG)

Table 6: Antibacterial activity

Diameter Zone of inhibition [x ± SD] mm			
Inhibitory substance	STCM	STRL	STWG
CLA	18.30 ± 011	19.90 ± 0.82	16.46 ± 0.37
CP	14.50±0.09	17.90±0.21	15.50±0.41
CLA + CP	27.83± 0.67	29.93 ± 0.12	25.12± 0.11

CLA – Clarithromycin CP - Cecropins

DISCUSSION

The isolation of *Salmonella enterica* serovar Typhi from ready-to-eat fruit salad samples in this study underscores the critical role of foodborne transmission in the epidemiology of enteric fever. The contamination likely originated from multiple points along the supply chain, including unhygienic handling, preparation on contaminated surfaces, and inadequate sanitization of equipment—factors well-documented in similar food safety studies (Immerseel *et al.*, 2014; Maciorowski *et al.*, 2017). The identification of specific strains STCM, STR1, and STWG, with STR1 being predominant, not only confirms the presence of clinically significant pathogens but also provides a relevant model for evaluating novel antimicrobial strategies against locally circulating variants.

From a therapeutic perspective, the increasing prevalence of multidrug-resistant (MDR) *S.* Typhi strains necessitates the exploration of combination therapies that can circumvent conventional resistance mechanisms. In this context, the evaluation of clarithromycin, a protein synthesis inhibitor, alongside *Hyalophora cecropia* cecropin antimicrobial peptides (AMPs), which target bacterial membranes, represents a strategically sound dual approach. The individual efficacy of each agent against the isolated strains provides a baseline for assessing their synergistic potential. Clarithromycin's intracellular action may be potentiated by the membrane-disruptive activity of cecropins, which can increase bacterial permeability and enhance antibiotic uptake. This mechanism aligns with studies demonstrating that AMPs can restore the activity of conventional antibiotics against resistant Gram-negative pathogens by overcoming permeability barriers and efflux pump activity (Nizet, 2006).

The significant antibacterial activity observed for the combination regimen in this study suggests a promising synergistic interaction. Such synergy is particularly valuable in the context of typhoid management, where resistance to first-line agents is increasingly common. By employing agents with distinct and complementary mechanisms of action, this strategy may reduce the minimum inhibitory concentration (MIC) required for each compound, lower the risk of resistance development, and improve clinical outcomes. These findings contribute to the growing body of evidence supporting the integration of antimicrobial peptides into combination therapies as a viable strategy to address the global challenge of antimicrobial resistance in enteric fever pathogens (Hancock & Sahl, 2006).

CONCLUSION

This study confirms the presence of clinically relevant *Salmonella enterica* serovar Typhi strains in locally vended food, highlighting an ongoing transmission risk. Furthermore, it demonstrates that the combination of clarithromycin and *Hyalophora cecropia* cecropin peptides exhibits enhanced antibacterial efficacy against these isolates, indicating a potent synergistic interaction. This dual-modality approach represents a promising therapeutic strategy against enteric fever pathogens, particularly in the face of rising multidrug resistance. Future research should focus on elucidating the precise mechanistic basis of this synergy and evaluating its efficacy in *in vivo* models to advance its potential clinical application.

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