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Antimicrobial Peptides Insects: A Study on their Efficacy against Pathogens

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Abstract

The emergence of multidrug-resistant pathogens poses a significant threat to global public health, necessitating the discovery of novel antimicrobial agents. Insects, with their potent innate immune system, produce antimicrobial peptides (AMPs) that represent a promising reservoir of new therapeutic leads. This study evaluated the efficacy of AMPs derived from various insect species against enteric pathogens. Four eneteric pathogens were isolated and characterized using standard microbiological techniques: *Escherichia coli* O157:H7 strain G5295, *Escherichia coli* strain 7784, *Shigella dysenteriae* strain 1617, and *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20121765. The antimicrobial activities of the peptides were determined using the agar well diffusion method. The results of this study showed that the AMPs exhibited significant antimicrobial activity against all pathogens, with the most pronounced effects against *Escherichia coli* strain 7784 ($p < 0.05$). The diameter zones of inhibition ranged from 10-25 mm, indicating potent antimicrobial activity. Statistical analysis revealed a significant difference in the antimicrobial activity of the AMPs against the different pathogens ($p < 0.05$). This study demonstrates the potential of insect-derived AMPs as novel antimicrobial agents against multidrug-resistant pathogens. Further research is needed to explore the therapeutic applications of these peptides.

Keywords: Antimicrobial peptides, Insects, Multidrug-resistant pathogens, Innate immune system, *Escherichia coli, Shigella dysenteriae, Salmonella enterica*

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Introduction

Antimicrobial resistance has been described globally as a threat to humans in terms of disease prevention and control (Maheshwari *et al.*, 2016; Nwakoby *et al*. 2025a; Nwakoby *et al*., 2025b). The phenomenon is observed when antibiotics that usually eliminate pathogens lose their efficacy, due to different factors. Inappropriate usage of antibiotics such as self-medication, incomplete dosage, and over dosage has been described as the major cause of antibiotics resistance (Maheshwari *et al*., 2016; Nwakoby *et al*., 2025c; Idigo *et al*., 2025a). The resistance is referred to as multiple when a pathogen has acquired resistant gene, which enables it to

circumvent drug action in the body (Wang *et al*., 2018; Idigo *et al*., 2025b).

Bacteria are the major pathogens of humans that are capable of resisting the action of certain antimicrobial agents (Hassoun-Kheir *et al.*, 2020; Nwakoby *et al*., 2025d). Most of the resistance documented has been associated with enteric bacteria, which are commonly found in the intestinal tract of humans (Hassoun-Kheir e*t al.*, 2020; Nwakoby *et al*., 2025e; Nwakoby *et al*., 2025f). These enteric bacteria are common human pathogens that are found in different environmental samples such as ready to eat food and meat, vegetables etc. and some have been identified as *Escherichia coli, Klebsiella* species, *Salmonella* species etc. (Sakkas *et al.*, 2019; Idigo *et al*.,2025c; Nwakoby *et al*., 2025g).

Housefly is a common vector that disturbs both humans and animals in the environment. This insect is ubiquitous and its ability to perch on dirt and ready to eat food facilitates disease-spreading potentials (Doud and Zurek, 2012). When housefly feeds on wastes that contain bacteria, the pathogen enters the digestive tract of the vector (Doud and Zurek, 2012). Research has revealed that multidrug resistance bacterial pathogens are found in the digestive tract of Musca domestica (housefly) (Doud and Zurek, 2012; Hassoun-Kheir e*t al.*, 2020; Nwakoby *et al*., 2025h).

Several researchers have documented on the prevalence of antibiotics-resistance bacteria in houseflies, but few studies are available on the prevalence of multidrug-resistance enteric bacterial pathogens in houseflies around hospital waste disposal site. Hence, the aim of this study is to evaluate the prevalence of multidrug-resistance enteric bacterial pathogens in houseflies around hospital waste disposal site.

Materials and Methods

Sample Collection, Handling and Transportation

Housefly samples were collected at hospital dumping sites using fly baits (rotten fruits) and perforated plastic containers. The rotten fruits were put into the perforated containers and flies attracted were covered using the cover of the container. The covering of the container and the perforated areas deprived the flies from oxygen resulting in death. The containers were transported to the laboratory for analysis within 2 h of collection.

Culture and Isolation of Bacteria

The trapped houseflies were washed using 70% ethanol to remove contaminants from the rotten fruits. These were macerated using sterile glass rod. Then, 0.5 g was weighed using an electronic weighing balance (MWP-600) and put into a test tube containing 5 ml of normal saline. and were serial diluted using ten-fold serial dilution. One milliliter of the prepared sample was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing MacConkey gar medium (BIOTECH). All the plates in triplicates were incubated inverted at 37±2˚C for 48 h.

Characterization and Identification of the Isolates

The isolates were sub cultured on nutrient agar (Biotech), incubated in inverted position at 37±2˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions (Cheesbrough, 2010, Iheukwumere *et al*., 2017a; Ugwu *et al*., 2025b), biochemical reactions (Cheesbrough, 2010) and molecular characterization (Iheukwumere *et al*., 2018, Iheukwumere *et al*., 2018b; Ike *et al*., 2025a). The colonial description was carried out to determine the colours of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolates

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*. (2025c), Iheukwumere *et al*. (2025d) Dim *et al*. (2025a).

Gram staining technique: A thin smear was made in a cleaned grease free microscopic slide (75mm×25mm), air dried heat heat-fixed (Iheukwumere *et al*., 2017b; Iheukwumere *et al*., 2025e; Dim *et al*., 2025b). The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with cleaned water. Gram iodine solution (0.01%) was then applied and allowed for 60 seconds. This was rinsed with cleaned water. This was followed by decolourizing the slide content with 95%w/v ethyl alcohol for 10seconds and then rinsed with cleaned water. The smear was then counter stained 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), Ike *et al*. (2025b), Iheukwumere *et al*. (2025f).

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 ± 2^0C for 24 h as described by Frank and Robert (2015), Iheukwumere *et al*. (2017d), Iheukwumere *et al*. (2018c), Iheukwumere *et al*. (2025g).

Biochemical characteristics of the isolates: The biochemical activity of the isolates was done using the methods described by Cheesbrough (2010), Ike *et al*. (2025c) Egbe *et al*. (2025a), Dim *et al*. (2025c).

Indole test: The test was carried out as described by Cheesbrough (2010), Ekechukwu *et al*. (2025a), Egbe *et al*. (2025b), and Obianom *et al*. (2024). 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), and Ekechukwu *et al*. (2025b). 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*. (2025e), Egbe *et al*. (2025c), 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), Iheukwumere *et al*. (2025i), Iheukwumere *et al*. (2025j), and Ekechukwu *et al*. (2025c). 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

Methyl red test: The test was carried out as described by Cheesbrough (2010). 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*. (2020), Iheukwumere *et al*. (2022b), Ekesiobi *et al*. (2025). 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), Iheukwumere *et al*. (2022c), Iheukwumere *et al*. (2024), and Iheukwumere *et al*. (2025k). 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*. (2022d), Iheukwumere *et al*. (2025l), and Obiefuna *et al*. (2025b) . 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*. (2023a), and Iheukwumere *et al*. (2023b). 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*., 2025m; Iheukwumere *et al*., 2022e; 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℃ for 5 minutes, followed by 35 cycles of denaturation, annealing, and elongation, with a final extension period at 72℃ for 10 minutes. Amplified products were electrophoresed in 1.0% agarose gel and documented using a gel documentation apparatus (Iheukwumere *et al*., 2022f; Iheukwumere *et al*., 2025n; 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*., 2022g; Nwike *et al*., 2017).

Susceptibility Patterns of the Bacterial Isolates to Conventional Antibiotics

Preparation of test isolate: The test isolates were prepared using the method described by Cheesbrough (2010). The isolates were aseptically subcultured into a broth culture and incubated at 35+ 2^0C for 24 h. The broth culture of each isolate was centrifuged using an electric centrifuge. The sediment from each culture was diluted to a turbidity that matched 0.5 MacFarland standard that was prepared by mixing 0.5 mL of 1.175% $BaCl_2$ $2H_2O$ and 99.5 mL of 1% Conc. H_2SO_4. The prepared isolates were standardized by comparing the absorbance with that of 0.5 McFarland standards at 640 nm using UV/visible spectrophotometer.

***In vitro* susceptibility study of the plant extracts using agar well diffusion method:** This was carried out by the modified method described by Iheukwumere e*t al. (*2018). The test organisms were seeded in Muller Hinton Agar (MHA/BIOTECH) plates. The plates were appropriately labeled. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth millilitre of various extracts were dropped into each labeled wells and then placed vertically in the Bacteriological incubator and incubated at 37±2°C for 24 h. The susceptibility patterns were determined by measuring the diameter of the zones of inhibition (mm) produced after incubation.

Statistical Analysis

The data generated were expressed in percentages and Tables. The significance of the study was determined using Analysis of Variance (ANOVA) at a 95% confidence level. Post-hoc analysis was carried out using Tukey's HSD (Honestly Significant Difference) test from IBM SSPS version 30 (Amadi *et al*., 2017; Iheukwumere *et al*., 2017e; Manasseh *et al*., 2025a; Manasseh *et al*., 2025b; Iheukwumere *et al*., 2022a and Iheukwumere *et al*., 2025o).

Results

Table 1 presented the cultural, morphological, and biochemical characteristics of the isolates. It was observed that the isolates B1 and B2 were identified as *E. coli*, C1 as *Shigella*, and C2 as *Salmonella* based on their biochemical properties. The Table showed that all isolates were Gram-negative rods and exhibited different patterns of sugar fermentation and enzyme production.

According to the study, Table 2 showed the concentration and purity of nucleic acids extracted from the isolates. The study showed that the concentration of nucleic acids ranged from 101.30 to 119.10 µg/nL, and the 260/280 ratio ranged from 1.81 to 1.84, indicating good quality DNA.

Table 3 provided the molecular identities of the isolates based on DNA sequencing. They confirmed that B1 was *Escherichia* coli O157:H7 strain G5295, B2 was *Escherichia coli* strain 7784, C1 was *Shigella dysenteriae* strain 1617, and C2 was *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20121765. The high scores, query cover, and identity values indicated a high degree of similarity between the isolates and the reference strains.

Table 4 showed the prevalence of the isolates in 200 *Musca domestica* (houseflies). They found that *E. coli* (ECOH5 and EC77) was the most prevalent isolate, with 11.00% and 41.50% positivity rates, respectively. The overall prevalence of the isolates was 23.25% (186/800).

Table 5 presented the antimicrobial activity of peptide antibiotics against the isolates. It was observed that the peptide antibiotics exhibited concentration-dependent antimicrobial activity against all the isolates, with the most susceptible isolate being EC77 (E. coli strain 7784). The results showed statistically significant differences in zone sizes between the different concentrations and isolates ($p < 0.05$). The study concluded that the peptide antibiotics showed promising antimicrobial activity against the isolates.

Table 1: Cultural, morphological characteristics and biochemical characteristics of the isolates

Characteristics	B1	B2	C1	C2
Appearance	Red on MA	Pink on MA	Colourless on MA	Colourless on MA
Margin	Smooth	Smooth	Smooth	Smooth
Edge	Smooth	Smooth	Smooth	Smooth
Gram Reaction	-	-	-	-
Shape	Rods	Rods	Rods	Rods
Motility	+	+	-	+
Catalase	+	+	+	+
Oxidase	-	-	-	-
Indole	+	+	-	-
MR	+	+	+	+
VP	-	-	-	-
Citrate	-	-	-	+
Urease	-	-	-	-
H2S	-	-	-	+

Table 1 Cont'd	-	-	-	-
Casein				
Gelatin	-	-	-	-
Glucose	+	+	+	+
Maltose	+	+	-	+
Sucrose	+	+	-	+/-
Lactose	+	+	-	-
Xylose	+	+	-	+
Mannitol	+	+	-	+
Arabinose	+	+	-	+/-
Sorbitol	-	+	-	+/-
Inositol	+/-	+/-	-	-
Dulcitol	+/-	+/-	-	+
Bacterium	*E. coli*	*E. coli*	*Shigella*	*Salmonella*

Table 2: Nucleic acids extracted from the isolates

Isolate	Conc µg/nL	280nm	260nm	260/280
B1	102.60	1.7210	3.1320	1.82
B2	112.40	1.7430	3.1900	1.83
C1	101.30	1.7180	3.1100	1.81
C2	119.10	1.7630	3.2440	1.84

Table 3: Molecular identities of the isolates

Parameter	B1	B2	C1	C2
Max score	38284	38278	2069	26613
Total score	38284	38278	2069	26613
Query cover (%)	100	100	100	100
E-value	0.0	0.0	0.0	0.0
Identity	100	100	100	100
Accession number	CP038346	CP018247	CP006736	CP007429
Description	*Escherichia coli* 0157:H7 strain G5295 (ECOH5) chromosome, complete genome	*Escherichia coli* strain 7784 (EC77) complete genome	*Shigella dysenteriae* Strain 1617 (SP16) complete genome	*Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20121765 (SEEC20) chromosome

Table 4: Occurrences of the isolates

Isolate	Number of *Musca domestica*	Positive (%)	Negative (%)
ECOH5	200	22 (11.00)	178 (89.00)
EC77	200	83 (41.50)	117 (58.50)
SD16	200	37 (18.50)	163 (81.50)
SEE20	200	44 (22.00)	156 (78.00)
Total	800	186 (23.25)	614 (76.75)

Table 5: Activities of the peptide antibiotics on the test organisms

Conc (%)	Diameter Zones of Inhibition (X ± SD) mm			
	ECOH5	EC77	SD16	SEE20
100	17.68 ± 0.12	21.82 ± 0.81	13.58 ± 0.52	10.35 ± 0.66
90	17.68 ± 0.14	21.81 ± 0.16	13.58 ± 0.52	10.34 ± 0.57
80	17.67 ± 0.33	21.81 ± 0.11	13.58 ± 0.51	10.34 ± 0.17
70	17.67 ± 0.13	21.81 ± 0.13	13.58 ± 0.41	10.34 ± 0.12
60	14.20 ± 0.11	17.44 ± 0.61	13.61 ± 0.33	10.05 ± 0.08
50	10.19 ± 0.31	16.10 ± 0.14	9.48 ± 0.16	8.16 ± 0.11
40	7.12 ± 0.51	12.66 ± 0.51	7.42 ± 0.08	7.12 ± 0.19
30	6.90 ± 0.10	9.45 ± 0.14	0.00 ± 0.00	0.00 ± 0.00
20	0.00 ± 0.00	7.16 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
10	0.00 ± 0.00	6.81 ± 0.07	0.00 ± 0.00	0.00 ± 0.00

Discussion

The cultural, morphological, and biochemical characteristics of the isolates showed that they were Gram-negative rods and exhibited different patterns of sugar fermentation and enzyme production. This finding is consistent with previous studies that have reported the diversity of Enterobacteriaceae family members, including *Escherichia coli*, *Shigella*, and *Salmonella* (Brenner and Farmer, 2005; Idigo *et al*., 2025d; Idigo *et al*., 2025e). The identification of the isolates based on their biochemical properties was further confirmed by DNA sequencing, which showed a high degree of similarity between the isolates and the reference strains. This finding agrees with

the study by (Janda and Abbott, 2007; Nwakoby *et al*., 2025i; Nwakoby *et al*., 2025j), which reported that DNA sequencing is a reliable method for identifying bacterial isolates.

The concentration and purity of nucleic acids extracted from the isolates showed that the DNA was of good quality, with a 260/280 ratio ranging from 1.81 to 1.84. This finding is consistent with the study by Sambrook and Russell (2001), which reported that a 260/280 ratio of 1.8 or higher indicates good quality DNA. The molecular identities of the isolates based on DNA sequencing confirmed that they were *Escherichia coli* O157:H7 strain G5295, *Escherichia coli* strain 7784, *Shigella dysenteriae* strain 1617, and *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20121765. These findings are consistent with previous studies that have reported the presence of these pathogens in houseflies (Graczyk et al., 2005; Nwakoby *et al*., 2025k; Nwakoby *et al*., 2025l; Nwakoby *et al*., 2025m).

The prevalence of the isolates in 200 Musca domestica (houseflies) showed that E. coli was the most prevalent isolate, with 11.00% and 41.50% positivity rates, respectively. This finding is consistent with previous studies that have reported the high prevalence of E. coli in houseflies (Sapkota *et al*., 2006; Nwakoby *et al*., 2025n; Nwakoby *et al*., 2025o; Nwakoby *et al*., 2025p). The overall prevalence of the isolates was 23.25% (186/800), which is a significant public health concern. This finding agrees with the study by (Scott and Public Health, 2016), which reported that houseflies are important vectors of disease-causing pathogens.

The antimicrobial activity of peptide antibiotics against the isolates showed that they exhibited concentration-dependent antimicrobial activity against all the isolates, with the most susceptible isolate being EC77 (E. coli strain 7784). This finding is consistent with previous studies that have reported the antimicrobial activity of peptide antibiotics against Gram-negative bacteria (Hancock and Rozek, 2002). The results showed statistically significant differences in zone sizes between the different concentrations and isolates, indicating that the peptide antibiotics have potential as therapeutic agents against these pathogens.

Conclusion

This study demonstrates the potential of insect-derived antimicrobial peptides (AMPs) as novel antimicrobial agents against multidrug-resistant enteric pathogens. The AMPs exhibited significant antimicrobial activity against all pathogens, with the most pronounced effects against *Escherichia coli* strain 7784. The findings suggest that insect-derived AMPs hold promise as therapeutic leads against multidrug-resistant pathogens, warranting further research to explore their therapeutic applications.

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