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Novel Cecropin-Like Peptides from *Rhynchophorus ferrugineus* Gut: A Potential Solution against Multidrug-Resistant *Pseudomonas aeruginosa*

Iheukwumere, I. H.[1] and Iheukwumere, C. M.[2]

[1]Department of Microbiology, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Anambra State.
[2]Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra State.

*Corresponding author email: ik.iheukwumere@coou.edu.ng / ikpower2007@yahoo.com

Abstract

The rise of multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) poses a significant threat to global health. Limited effective antibiotics and increasing resistance rates create an urgent need for novel therapeutics. There is a dearth of research on cecropin-like peptides from *Rhynchophorus ferrugineus* gut, which could potentially exhibit potent antimicrobial activity against MDR-PA, offering a promising alternative to conventional antibiotics. This study aims to identify and characterize these peptides, evaluating their efficacy against MDR-PA. This study aimed to identify novel cecropin-like peptides from *Rhynchophorus ferrugineus* gut as potential alternatives to combat multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) infections. Three *P. aeruginosa* strains (LG03, F065, F076) were characterized using cultural, morphological, and molecular techniques. Antibiotic susceptibility testing revealed 43.59% resistance to conventional antibiotics, with 70.59% exhibiting multi-drug resistance. Cecropin-like peptides were extracted and evaluated for inhibitory activity against MDR-PA isolates. Eluate 5 showed the highest inhibitory activity (17.00-21.50 mm inhibition zones). Statistical analysis revealed significant differences in resistance patterns ($p < 0.05$) and inhibitory activity among eluates ($p < 0.05$). The study suggests cecropin-like peptides from *R. ferrugineus* gut may be a potential alternative or adjunct to conventional antibiotics against MDR-PA infections, warranting further investigation.

Keywords: Multidrug-resistant, Cecrosins, Antibiotics, Eluate, Infections

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Introduction

The escalating crisis of antimicrobial resistance (AMR) poses one of the most formidable threats to global public health in the 21st century. The World Health Organization has declared AMR a top global health priority, with multidrug-resistant (MDR) bacteria rendering many conventional antibiotics ineffective (Murray *et al*., 2022). Among the most critical pathogens, *Pseudomonas aeruginosa* stands out as a particularly daunting challenge. This Gram-negative opportunistic pathogen is a leading cause of nosocomial infections, including ventilator-associated pneumonia, severe burn wound infections, and sepsis, particularly in immunocompromised individuals (Pang *et al*., 2019). The intrinsic resistance of *P. aeruginosa* to numerous antibiotic classes, coupled with its remarkable capacity to acquire further resistance determinants through horizontal gene transfer and form protective biofilms, has led to the emergence of strains that are resistant to nearly all available therapies, including carbapenems and colistin (Reynolds and Kollef, 2021). This alarming trend underscores the urgent and unmet need for novel antimicrobial agents with distinct mechanisms of action that can circumvent existing resistance pathways.

In the pursuit of new antimicrobials, nature has proven to be an invaluable reservoir of bioactive compounds. Specifically, antimicrobial peptides (AMPs) have garnered significant attention as promising alternatives to traditional antibiotics. These evolutionarily conserved components of innate immunity exhibit potent, broad-spectrum activity against bacteria, fungi, and viruses, often through mechanisms involving direct disruption of microbial membranes, which makes the development of resistance considerably more challenging than with conventional antibiotics (Mahlapuu *et al*., 2020). Insects, which lack an adaptive immune system, rely

heavily on a sophisticated arsenal of AMPs produced in response to infection, making them a particularly rich source for discovery (Wu *et al*., 2018). Among these, cecropins—a family of linear, amphipathic α-helical peptides first isolated from the giant silk moth *Hyalophora cecropia*—are renowned for their potent activity against Gram-negative bacteria (Steiner *et al*., 1981).

The red palm weevil, *Rhynchophorus ferrugineus*, is a destructive pest of palm trees worldwide. While its ecological impact is well-documented, its gut microbiome and the associated immune effectors, particularly AMPs, represent an underexplored frontier. The insect gut is a dynamic interface constantly challenged by a high load of ingested microbes, necessitating a robust local immune response. It is hypothesized that the gut of *R. ferrugineus* harbors a diverse repertoire of potent AMPs, including novel cecropin-like peptides, that have evolved to effectively manage microbial threats, including highly resistant strains. This unique selective pressure makes the weevil's gut a promising source for novel antimicrobials capable of combating recalcitrant pathogens like MDR *P. aeruginosa*.

Therefore, this study aims to identify and characterize novel cecropin-like peptides from the gut of *Rhynchophorus ferrugineus* and evaluate their efficacy against multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*. We hypothesize that these peptides will exhibit potent antibacterial activity, potentially through a mechanism of action distinct from conventional antibiotics, offering a novel therapeutic strategy to address the critical gap in the treatment of MDR *P. aeruginosa* infections.

Materials and Methods

Sample Collection of water samples: Sample collection, handling and transportation: The samples used for this study were drawn from the fish pond. A total of 100 fish pond water samples were collected from five different locations in Uli community. The fish pond water samples were collected with sterile containers. The containers were thoroughly washed with detergent, rinsed with water, and then rinsed with 70% ethanol and final rinsed three times with distilled water (Iheukwumere *et al.*, 2018). The containers were placed inverted in order to drain the water inside them. The container was inverted and lowered 5 cm below the fish pond water sample, then placed vertically for the water sample to refill the sample container. This sample was covered immediately and kept in a cooler containing ice block, and this transported to the laboratory for immediate analysis (Iheukwumere *et al*., 2020).

Isolation of organisms: One milliliter (1.0 ml) water sample was aseptically transferred into a sterile test tube (Pyrex) containing 9.0 ml of the diluent (sterile normal saline) and from this; ten-fold serial dilutions were made up to 10^{-3} (Iheukwumere *et al*., 2022a).. One milliliter of the diluted sample (10-3) was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing Cetrimide agar medium (CA/BIOTECH) using pour plate method. All the plates in triplicates were incubated inverted at 37±2˚C for 24-48 h. (Iheukwumere *et al*., 2022b).

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), biochemical reactions (Cheesbrough, 2010) and molecular characterization (Iheukwumere *et al*., 2018). 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).

Gram staining technique: A thin smear was made in a cleaned grease free microscopic slide (75mm×25mm), air dried heat fixed. 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).

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 Ejike *et al.* (2017).

Biochemical characteristics of the isolates: The biochemical activity of the isolates was done using the methods described by Cheesbrough (2010) and Uba *et al.* (2020)

Indole test: The test was carried out as described by Cheesbrough (2010). Indole is a nitrogen containing compound formed when the amino acid tryptophan is hydrolyzed 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 (Amadi *et al*., 2017).

Sugar fermentation test: The test was carried out as described by Cheesbrough (2010). 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 (Nwike *et al.*, 2017). 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 (Okpalla *et al.*, 2015).

Hydrogen sulphide production: The test was carried out as described by Cheesbrough (2010). 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 (Iheukwumere *et al.*, 2017). The presence of darkened coloration was positive for Hydrogen sulphide production

Urease test: The test was carried out as described by Cheesbrough (2010). 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). 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 (Okpalla *et al*., 2015). 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). 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 (Obianom *et al.*, 2024a).

Catalase test: The test was carried out as described by Cheesbrough (2010) and (Obianom *et al.*, 2024b). 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) and (Uzoh *et al.*, 2015). 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*., 2018).

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*., 2018).

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 (Iheukwumere *et al*., 2018).

Susceptibility Patterns of the Bacterial Isolates against 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 (Okeke *et al.*, 2017).

***In vitro* activity of conventional antibiotics against the isolates using disc diffusion method:** The susceptibility of the isolates to the conventional antibiotics was done using disc diffusion method on Mueller Hinton agar. A sterile swab was used to inoculate the suspension of the isolate on the prepared and dried Mueller Hinton agar plate equally. It was then left to stay for 5 minutes. A sterile forceps was used to place the commercially prepared antibacterial discs on the inoculated plates. Within 30 minutes after applying the disc, the plates were incubated at 37˚C for 24 h. Meter rule was used underside of the plates to determine the diameter zones of inhibition in millimeter as described in the study published by Iheukwumere *et al.* (2018).

Extraction of cecrosins: Cecropins, a peptide antibiotic, were extracted from the gut of *Rhynchophorus ferrugineus* using a suitable solvent and thin layer chromatography (TLC). The process involved several steps. First, the guts of *Rhynchophorus ferrugineus* were dissected and homogenized in a phosphate-buffered saline (PBS) solution to release the cecropins peptide. The homogenate was then centrifuged to separate the supernatant, which contained the cecropins peptide, from the cellular debris. The supernatant was then subjected to solvent extraction using a mixture of methanol and water (1:1, v/v). The methanol-water mixture was chosen as the solvent due to its ability to effectively solubilize the cecropins peptide. The resulting extract was then applied to a TLC plate, which was developed using a solvent system consisting of n-butanol, acetic acid, and water (4:1:5, v/v/v). The TLC plate was visualized under ultraviolet (UV) light, and the band corresponding to cecropins was identified based on its retention factor (Rf) value, which was approximately 0.55. The cecropins band was then scraped off the TLC plate and eluted with a small volume of methanol. The eluted cecropins were then concentrated and purified using high-performance liquid chromatography (HPLC) (AOAC, 2019).

***In vitro* antibacterial susceptibility test:** This was ascertained using micro tube dilution method. Here, micro tube dilution plates was used. Different dilutions of the sample were prepared, 100 µL of each concentration was dropped in each well of the micro well, then 100 µL of the test isolate was added into the well. These were mixed and incubated at 37˚C for 24 h. The bacterial growth pattern was determined for the most potent minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) as described by Clinical and Laboratory Standards Institute/CLSI (2015)

Statistical Analysis: The data obtained in this study were presented in tables and figures. Their percentages were also calculated (Chukwura and Iheukwumere, 2013; Egbuna *et al.*, 2020). The sample means and standard deviations of some of the analytical data were also calculated (Uzoh *et al.*, 2015). The significance of this study was determined at 95% using one way analysis of variance (ANOVA) (Uzoh *et al*., 2017). Post-hoc analysis was conducted using Boniferroni correction test, Trend analysis was conducted using Cochran -Armitage test for dose response. Pair wise comparison was done using Fisher's Exact test as described in the study published by Iheukwumere *et al*. (2018).

Results

The study investigated the cultural, morphological, and molecular characteristics of three bacterial isolates, D1, D2, and D3, which were identified as *Pseudomonas aeruginosa* strains. The isolates exhibited similar cultural and morphological characteristics, including blue-green appearance on Cetrimide agar, smooth edges, and rod-shaped cells (Table 1). Molecular analysis confirmed the isolates as *Pseudomonas aeruginosa*, with high sequence identity (>99%) to known strains (Table 2).

The isolates were tested for susceptibility to conventional antibiotics, and the results showed varying degrees of resistance (Table 3). The overall susceptibility rate was 56.41%, with 43.59% of the isolates resistant to the tested antibiotics. The isolates exhibited multi-drug resistance, with 70.59% resistant to multiple antibiotics (Table 4). Statistical analysis revealed significant differences in resistance patterns among the isolates ($p < 0.05$).

The study also evaluated the inhibitory activity of eluates against the test isolates, and the results showed that eluate 5 had the highest inhibitory activity, with inhibition zones ranging from 17.00 to 21.50 mm (Table 5). The p-values indicated significant differences in inhibitory activity among the eluates ($p < 0.05$).

The molecular characteristics of the isolates, as presented in Table 2, showed high sequence identity to known *Pseudomonas aeruginosa* strains, indicating a high degree of similarity. The Max score, Total score, and E-value indicated significant matches, with percent identity ranging from 100%.

The antibiotic susceptibility patterns and degree of resistance exhibited by the isolates, as presented in Tables 3 and 4, highlight the need for alternative therapeutic strategies to combat antibiotic-resistant *Pseudomonas aeruginosa* infections. The study's findings suggest that the eluates may be a potential alternative or adjunct to conventional antibiotics, warranting further investigation.

Table 1: Cultural and Morphological Characteristics of the Isolates

Parameter	D1	D2	D3
Appearance on Cetrimide agar	Blue-green	Colourless	Blue-green
Appearance on Nutrient agar	Blur-green	Bluish	Blue-green
Edge	Smooth	Smooth	Smooth
Surface	Smooth	Smooth	Smooth
Motility	+	+	+
Gram Reaction	-	-	-
Cell morphology	Rods	Rods	Rods
Catalase	+	+	+
Cetrimide test	+	+	+
Citrate	+	+	+
Indole	-	-	-
Methyl red	-	-	-
Voges Proskauer	-	-	-
Oxidase	+	+	+
Glucose	-	-	-
Maltose	-	-	-
Fructose	+	+/-	+/-
Galactose	-	-	-
Inositol	-	-	-
Xylitol	-	+/-	-

Table 2: Molecular characteristics of the bacterial isolates

Isolate code	Max score	Total score	Query cover (%)	E-value	Percent identity (%)	Accession Number	Description
D1	1672	1672	100	0.0	100	CP129520.1	*Pseudomonas aeruginosa* strain LG03 (PA03)
D2	1821	1821	100	0.0	100	CP115810.1	*Pseudomonas aeruginosa* strain F065 (PA065)
D3	1692	1692	100	0.0	100	CP115198.1	*Pseudomonas aeruginosa* strain F076 (PA076)

Table 3: Susceptibility of the isolates to conventional antibiotics

Isolate	Number	Susceptible Strain (%)	Resistant strain (%)	Implicated Antibiotic
D1	26	14(53.85)	12(46.35)	AMX, AU, PN, CEP, SXT, CN
D2	36	22(61.11)	14(38.89)	AMX, S, PN, SXT, CEP.
D3	16	8(50.00)	8(50.00)	AU, AMX, S, PN, SXT, CEP
Total	78	44(56.41)	34(43.59)	

Table 4: Degree of resistance exhibited by the isolates

Isolate	Number of resistant strain	Single antibiotic resistant strain (%)	Multiantibiotic resistant strain (%)
D1	12	4(33.33)	8(66.67)
D2	14	4(28.57)	10(71.43)
D3	8	2(25.00)	6(75.00)
Total	34	10(29.41)	24(70.59)

Table 5: Inhibitory activity of the eluates against the test isolates

Eluate	PA03 (mm)	PA065 (mm)	PA076 (mm)
1	0.00	0.00	0.00
2	0.00	0.00	0.00
3	9.50	8.00	8.00
4	13.50	12.00	11.00
5	21.50	17.00	19.00
6	8.50	6.50	7.00
7	0.00	0.00	0.00
8	0.00	0.00	0.00
9	0.00	0.00	0.00
10	0.00	0.00	0.00
11	0.00	0.00	0.00

Discussion

The study's findings on the cultural, morphological, and molecular characteristics of the isolates are consistent with previous reports on *Pseudomonas aeruginosa* (Holt *et al.*, 1994). The blue-green appearance on Cetrimide agar and rod-shaped cells are characteristic features of this bacterium. Molecular analysis confirmed the isolates as *Pseudomonas aeruginosa*, with high sequence identity (>99%) to known strains, indicating a high degree of similarity (Altschul *et al*., 1990).

The antibiotic susceptibility patterns of the isolates revealed varying degrees of resistance, with an overall susceptibility rate of 56.41%. This is concerning, as *Pseudomonas aeruginosa* is known to exhibit multi-drug resistance (Livermore *et al.*, 2012). The isolates exhibited resistance to multiple antibiotics, with 70.59% resistant to more than one antibiotic

The study's findings on the inhibitory activity of cecrosins against the test isolates are promising. Eluate 5 showed the highest inhibitory activity, with inhibition zones ranging from 17.00 to 21.50 mm. The emergence of antibiotic-resistant *Pseudomonas aeruginosa* strains highlights the need for alternative therapeutic strategies (Gellatly *et al.,* 2013). The study's findings suggest that the cecrosins may be a potential alternative or adjunct to conventional antibiotics, warranting further investigation. Further research is needed to identify the bioactive compounds responsible for the inhibitory activity and to evaluate their efficacy *in vivo*.

Conclusion

The study's findings highlight the importance of continued surveillance of antibiotic resistance patterns and the need for alternative therapeutic strategies to combat *Pseudomonas aeruginosa* infections. The cecrosins show promise as a potential alternative or adjunct to conventional antibiotics, and further research is warranted to explore their therapeutic potential.

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