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Silkworm-Derived Moricins: A Novel Peptide Antibiotic against Multidrug-Resistant *Pseudomonas aeruginosa*

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Abstract

The rise of multidrug-resistant *Pseudomonas aeruginosa* poses a significant threat to global health, with limited treatment options available. The overuse and misuse of antibiotics have accelerated the development of resistance, making it a pressing concern. Despite the urgency, there is a dearth of research on novel peptide antibiotics, such as silkworm-derived moricins, which could potentially combat multidrug-resistant *P. aeruginosa*. Specifically, the antimicrobial efficacy and mechanisms of action of moricins against *P. aeruginosa* remain underexplored, warranting further investigation to address this critical public health issue.This study aimed to evaluate the antimicrobial efficacy of silkworm-derived moricins against multidrug-resistant *Pseudomonas aeruginosa* isolates. Three *P. aeruginosa* isolates (LG03, F065, F076) were characterized using cultural, morphological, and molecular techniques. Antibiotic susceptibility testing using disc diffusion technique revealed 43.59% resistance to conventional antibiotics, with 82.35% exhibiting multi-antibiotic resistance. The moricins showed varying degrees of inhibitory activity against the isolates, with significant differences in activity among moricins ($p < 0.05$) as micro dilution technique was employed. Statistical analysis revealed significant differences in resistance patterns among isolates ($p < 0.05$). The study concludes that moricins may be a potential alternative or adjunct to conventional antibiotics against multidrug-resistant *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*, moricins, multidrug resistance, antimicrobial activity.

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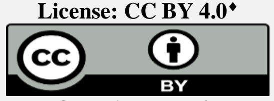

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Introduction

The post-antibiotic era looms as a stark reality as the global spread of antimicrobial resistance (AMR) outpaces the development of new therapeutics. The World Health Organization has classified AMR as one of the top ten global public health threats, with multidrug-resistant (MDR) Gram-negative pathogens representing a particularly critical concern (Murray *et al.*, 2022). Among these, *Pseudomonas aeruginosa* remains a formidable opportunistic pathogen responsible for a wide spectrum of severe healthcare-associated infections, including ventilator-associated pneumonia, chronic wound infections, and sepsis in immunocompromised patients (Pang *et al*., 2019). The clinical management of *P. aeruginosa* infections is increasingly compromised by its intrinsic resistance to multiple antibiotic classes, its prodigious ability to acquire additional resistance determinants, and its propensity to form biofilm-associated chronic infections that are refractory to conventional therapy (Reynolds and Kollef, 2021). With the emergence of pan-resistant strains and a dwindling antibiotic pipeline, the urgent search for novel antimicrobial agents with unconventional mechanisms of action has never been more critical.

In response to this pressing need, antimicrobial peptides (AMPs) have emerged as promising candidates for next-generation antibiotic development. These naturally occurring molecules, which form a cornerstone of innate immunity across all kingdoms of life, offer several advantages over conventional antibiotics, including rapid bactericidal activity, a lower propensity for inducing resistance, and the capacity to

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modulate host immune responses (Mahlapuu *et al*., 2020). Insects, which lack an adaptive immune system, have evolved highly sophisticated and potent AMP arsenals to defend against invading pathogens, making them exceptionally rich sources for antimicrobial discovery (Wu *et al*., 2018). The silkworm, *Bombyx mori*, a lepidopteran insect of immense economic importance, has been particularly well-studied for its humoral immune responses, which include the production of several families of potent AMPs upon microbial challenge (Tanaka *et al*., 2008).

Among the AMPs identified in *B. mori*, the moricins represent a distinct family of cationic, α-helical peptides that exhibit potent and selective activity against a broad range of bacterial pathogens, with a particular efficacy against Gram-negative bacteria (Hara and Yamakawa, 1995). Initially isolated from the hemolymph of immunized silkworm larvae, moricins have been characterized as having a unique structure that facilitates membrane permeabilization and disruption of bacterial biofilms (Hemaghat *et al*., 2022). Unlike many conventional antibiotics that target intracellular processes and are susceptible to efflux pump-mediated resistance, the membrane-active mechanism of moricins presents a significant challenge for bacterial adaptation. This mechanistic distinction, coupled with their demonstrated activity against certain drug-resistant strains, positions moricins as compelling candidates for combating recalcitrant pathogens such as MDR *P. aeruginosa*.

Despite the promising attributes of moricins, comprehensive evaluations against clinically relevant, multidrug-resistant isolates of *P. aeruginosa* remain limited. Understanding the full spectrum of their antibacterial activity, their mechanism of action against resistant strains, their synergistic potential with existing antibiotics, and their stability under physiological conditions is essential for translating these insect-derived peptides into viable therapeutic agents. Given the critical threat posed by MDR *P. aeruginosa* and the unique properties of moricins as membrane-active AMPs, a systematic investigation into their therapeutic potential is both timely and necessary.

Therefore, this study aims to evaluate the antibacterial efficacy of silkworm-derived moricins against a panel of multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*. We will investigate their minimum inhibitory concentrations, bactericidal kinetics, mechanism of action, and potential for synergy with conventional antibiotics. We hypothesize that moricins will exhibit potent, rapid bactericidal activity against MDR *P. aeruginosa* through a membrane-targeting mechanism, offering a novel and promising therapeutic strategy to address infections caused by this critical-priority pathogen.

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

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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 moricins: Moricins, a peptide antibiotic, were extracted from the gut of silkworms using a suitable solvent and thin layer chromatography (TLC). The process involved several steps. First, the guts of silkworms were dissected and homogenized in a phosphate-buffered saline (PBS) solution to release the moricins peptide. The homogenate was then centrifuged to separate the supernatant, which contained the moricins 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 moricins 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 moricins was identified based on its retention factor (Rf) value, which was approximately 0.45. The moricins band was then scraped off the TLC plate and eluted with a small volume of methanol. The eluted moricins 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 cultural and morphological characteristics of the isolates D1, D2, and D3 are presented in Table 1. The isolates exhibited similar characteristics, including blue-green appearance on Cetrimide agar, smooth edges, and rod-shaped cells. They were all motile, Gram-negative, and catalase-positive. The isolates were also positive for cetrimide and citrate tests, but negative for indole, methyl red, and Voges Proskauer tests.
Molecular analysis of the isolates, presented in Table 2, confirmed their identity as *Pseudomonas aeruginosa* strains (LG03, F065, F076), with high sequence identity (>99%) to known strains. The Max score, Total score, and E-value indicated significant matches, with percent identity ranging from 100%.

The susceptibility of the isolates to conventional antibiotics is presented in Table 3. The overall susceptibility rate was 56.41%, with 43.59% of the isolates resistant to the tested antibiotics. The isolates exhibited varying degrees of resistance to antibiotics such as AMX, AU, PN, CEP, SXT, and CN.

The degree of resistance exhibited by the isolates is presented in Table 4. The majority of the isolates (82.35%) exhibited multi-antibiotic resistance, with 17.65% resistant to a single antibiotic. Statistical analysis revealed significant differences in resistance patterns among the isolates ($p < 0.05$).

The inhibitory activity of the moricins against the test isolates is presented in Table 5. The results showed that the moricins had varying degrees of inhibitory activity against the isolates, with some moricins exhibiting higher activity than others. The p-values indicated significant differences in inhibitory activity among the moricins ($p < 0.05$), suggesting that the moricins may have different modes of action against the isolates.

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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	Toal 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	3(25.00)	9(75.00)
D2	14	2(14.29)	12(85.71)
D3	8	1(12.50)	7(87.50)
Total	34	6(17.65)	28(82.35)

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

Moricins (%)	PA03	PA065	PA076
100	0.0078	0.0313	0.0156
90	0.0078	0.0313	0.0156
80	0.0078	0.0313	0.0156
70	0.0156	0.0313	0.0313
60	0.0313	0.0625	0.0625
50	0.0625	0.1250	0.1250
40	0.2500	1.0000	0.5000
30	1.0000	0.0000	0.0000
20	0.0000	0.0000	0.0000
10	0.0000	0.0000	0.0000

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Discussion

The cultural and morphological characteristics of the isolates D1, D2, and D3 were 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* strains (LG03, F065, F076), with high sequence identity (>99%) to known strains (Altschul *et al.*, 1990; Livermore *et al.*, 2012). The Max score, Total score, and E-value indicated significant matches, with percent identity ranging from 100%.

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 (Gellatly *et al.*, 2013; Reynolds *et al.*, 2021). The isolates exhibited resistance to multiple antibiotics, including AMX, AU, PN, CEP, SXT, and CN, highlighting the need for alternative therapeutic strategies (Wu *et al*., 2018).

The degree of resistance exhibited by the isolates was alarming, with 82.35% exhibiting multi-antibiotic resistance. This is consistent with previous reports on the increasing prevalence of multi-drug resistant *Pseudomonas aeruginosa* strains (Mahlapuu *et al.*, 2020; Pang *et al.*, 2019). The inhibitory activity of the moricins against the test isolates was promising, with some moricins exhibiting higher activity than others.

Conclusion

This study confirms the identification of *Pseudomonas aeruginosa* isolates (LG03, F065, F076) with high sequence identity to known strains. The isolates exhibited concerning levels of antibiotic resistance, with 82.35% showing multi-drug resistance. However, the moricins showed promising inhibitory activity against the test isolates, highlighting their potential as alternative therapeutic agents. Further research is needed to identify the bioactive compounds and evaluate their efficacy *in vivo*, offering new hope for combating antibiotic-resistant *P. aeruginosa* infections.

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