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Olivier Ndogo Eteme¹

ndogoeteme@gmail.com

Université de Yaoundé I

Mbarga Opono

Université de Yaoundé I

Esther Del

Université de Yaoundé I

Patrick Hervé

Université de Yaoundé I

Ernestine Nkwengoua Zondegoumba²

Université de Yaoundé I

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Synthesis, Proteolytic Stability, and Rapid Bactericidal Activity of Seven Synthetic Antimicrobial Peptides Against Selected Gram-Negative Bacteria

Olivier Ndogo Eteme^{1,2*}, Mbarga Opono Hermine Salome³, Esther Del Florence Moni Ndedi³, Patrick Hervé Betote Diboué^{3,4,5}, Ernestine Nkwengoua Zondegoumba²

¹ Department of Chemistry, University of Pennsylvania, Philadelphia, PA, USA

² Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon

³ Department of Microbiology, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon

⁴ Department of Galenical Pharmacy and Pharmaceutical Law, Faculty of Medicine and Biomedical Sciences, University of Yaoundé I, Yaoundé, Cameroon

⁵ Centre for Research on Medicinal Plants and Traditional Medicine, Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé, Cameroon

* Corresponding author: Olivier Ndogo Eteme; email: ndogoeteme@gmail.com

Abstract

Purpose: Antimicrobial resistance has intensified interest in peptide-based antibacterial agents, but therapeutic development requires balancing antibacterial potency with biological stability. This study evaluated seven synthetic antimicrobial peptides (P1-P7) for antibacterial activity, proteolytic stability, membrane-associated effects, and killing kinetics.

Methods: P1-P7 were prepared by Fmoc solid-phase peptide synthesis, purified by HPLC, and characterized by LC-MS. Stability was evaluated against pepsin, trypsin, chymotrypsin, and EndoGluC. Antibacterial activity was assessed by broth microdilution against selected Gram-negative bacteria. Extracellular DNA/RNA release and time-kill experiments with *Escherichia coli* M/20-03 were used to assess cellular integrity and bactericidal kinetics.

Results: The peptides showed approximately 97% or higher chromatographic purity and distinct proteolytic-stability profiles. P6 remained detectable for more than 4 h with all four proteases. P7 showed the most consistent antibacterial activity, with MICs of 8.14-130.21 µg/mL (3.56-56.87 µM). Extracellular nucleic-acid release was consistent with compromised cellular integrity. Complete loss of recoverable *E. coli* M/20-03 occurred within 10 min for P1, 20 min for P7, 40 min for P2, and 105 min for P3 under the conditions tested.

Conclusion: P7 combines the strongest overall antibacterial profile with rapid killing, whereas P6 provides the greatest proteolytic stability. These complementary properties identify both peptides as candidates for sequence optimization aimed at improving potency, selectivity, and biological stability.

Keywords: antimicrobial peptides; solid-phase peptide synthesis; proteolytic stability; antibacterial activity; bactericidal kinetics; membrane integrity

I- Introduction

Antimicrobial resistance (AMR) is a major global health challenge driven by antimicrobial use and selection across human, animal, and environmental settings (Coque et al. 2023; Salam et al. 2023; Tang et al. 2023). Antimicrobial peptides (AMPs) are attractive candidates because many combine rapid bactericidal activity with membrane-associated mechanisms that differ from those of numerous conventional antibiotics (Zhang et al. 2021; Rima et al. 2021; Bucataru and Ciobanasu 2024). Their potential nevertheless remains constrained by mammalian-cell toxicity, limited bioavailability, and susceptibility to proteolytic degradation (Wang et al. 2021; Min et al. 2024; Dad et al. 2025). Sequence composition, charge, hydrophobicity, and structural organization can substantially influence both antibacterial potency and protease susceptibility (Jiang et al. 2008; Chen and Jiang 2023). Recent work in peptide therapeutics has emphasized designs that preserve activity while improving biological stability and safety, including non-canonical residues and other structural modifications (Enninfu et al. 2026). Experimental evaluation should integrate peptide synthesis and analytical characterization with antibacterial susceptibility, proteolytic stability, and mechanism-oriented assays rather than relying on predicted structure alone. In this study, seven synthetic peptides (P1-P7) were prepared by Fmoc solid-phase peptide synthesis and evaluated

against selected Gram-negative bacteria. The aims were to compare MIC/MBC profiles, determine sequence-dependent differences in susceptibility to four proteases, assess whether peptide exposure is associated with loss of cellular integrity through extracellular nucleic-acid release, and characterize bactericidal kinetics against *E. coli* M/20-03.

II- Materials and Methods

Peptide synthesis and analytical characterization

Peptides were synthesized using the solid-phase peptide synthesis (SPPS) method with Fmoc-Wang resin (substitution degree 0.52 mmol/g, Sigma-Aldrich, USA) as the solid support. The resin was activated by two washes with dichloromethane (DCM, Sigma-Aldrich, USA) for 30 minutes, followed by two washes with dimethylformamide (DMF, Sigma-Aldrich, USA) for 1 hour, and then two additional washes with DCM for 30 minutes. The Fmoc protecting group was removed using a 4-methylpiperazine solution. After 20 minutes of agitation, the solution was discarded, and the resin was washed with DCM and DMF as previously described. The first amino acid was coupled by mixing hydroxybenzotriazole (HOBt) with the corresponding amount of amino acid in DMF. The mixture was dissolved using a sonicator for 2 hours and then transferred into the column containing the resin. Diisopropylcarbodiimide (DIC) was subsequently added, and the reaction mixture was stirred for 2 hours to allow N→C coupling. Coupling efficiency was monitored using the Kaiser test (the appearance of a blue precipitate indicating incomplete coupling). Subsequent amino acids were added sequentially, repeating the deprotection, washing, coupling, and Kaiser test steps (Yoo and Pagel 2007; Connah et al. 2019).

In the first stage, the carboxylic acid is activated by N,N'-diisopropylcarbodiimide (DIC) to generate an O-acylisourea intermediate, which is reactive yet unstable. Subsequently, hydroxybenzotriazole (HOBt) intervenes to produce a more stable activated ester, thereby minimizing side reactions such as racemization. In the second stage, the nucleophilic amine attacks this intermediate to form the amide bond (peptide bond), while releasing byproducts. This system is widely utilized in solid-phase peptide synthesis (SPPS) to optimize the yields and purity of the resulting peptides.

Once the complete peptide sequence was assembled, the peptide was cleaved from the resin by adding a cleavage mixture of Trifluoroacetic acid (TFA, Sigma-Aldrich, Brazil), water, and Triisopropylsilane (TIS, Sigma-Aldrich, Brazil) in a 96:2:2 ratio (v/v/v), at a volume of 100 mL per gram of peptide, for 4 hours at room temperature. The reaction was stopped by the addition of cold diethyl ether. After adding a water/acetonitrile mixture, the samples were centrifuged, the supernatant was discarded, and the peptide was lyophilized to obtain a dry powder^{26,27}.

The lyophilized samples were dissolved in double-distilled water and purified by high-performance liquid chromatography (HPLC) using a Shimadzu Prominence system equipped with a Shimadzu C18 column. The mobile phase consisted of water/acetonitrile at a flow rate of 1 mL/min for 30 minutes. Fractions containing purified peptide were collected, pooled, and lyophilized. The peptides were then quantified and characterized by LC-MS (Shimadzu/Bruker chromatograph and mass spectrometer) under conditions like those used for HPLC, but at a flow rate of 0.5 mL/min and with a solvent gradient ranging from 5% to 95% acetonitrile over 30 minutes^{26,27}.

Antibacterial susceptibility testing

After thawing and homogenization of the preserved bacterial strains, subculturing was performed in Middlebrook (MBK) 7H9 broth supplemented with 2% glycerol, 7% Tween 80, 10% OADC, and PANTA. The cultures were incubated for 7 days. The bacterial inoculum was then standardized to the turbidity of a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL, and the optical density was measured at 625 nm; values between 0.08 and 0.10 were accepted for inoculum preparation.^{28,29}

Antibacterial activity of the antimicrobial peptides (AMPs) was evaluated by a broth microdilution assay performed in Middlebrook 7H9 medium, with Alamar Blue used as the growth indicator. Briefly, 100 μ L of Middlebrook 7H9 broth supplemented with 10% OADC and PANTA was dispensed into each well of a 96-well microplate. A 100 μ L aliquot of antimicrobial peptide stock solution (100 mg/mL in sterile distilled water) was added to wells 1A-1D, followed by twofold serial dilution through the eleventh row. After homogenization, 100 μ L of bacterial suspension was added to the first three test columns. All assays were performed three times in duplicate. Wells in the twelfth row containing Middlebrook 7H9 and inoculum served as growth controls, whereas wells containing Middlebrook 7H9 and peptide without inoculum served as negative controls. Plates were covered, sealed with parafilm, and incubated at 37°C for 7 days. Thereafter, 50 μ L of 0.02% Alamar Blue solution was added to two test columns and to the control column, while the third test column was reserved for determination of the minimum bactericidal concentration (MBC). Plates were incubated for an additional 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration at which no visible color change indicative of microbial growth was observed.^{30,31} Methodological principles were interpreted with reference to AMP susceptibility-testing recommendations (Wu et al. 2017; Mercer et al. 2020).

The minimum bactericidal concentration (MBC) was determined by transferring 50 μ L from wells containing peptide concentrations greater than or equal to the MIC into a new microplate containing 150 μ L of Middlebrook 7H9 broth. The assay was performed in duplicate. Plates were incubated at 37°C for 7 days, after which 50 μ L of Alamar Blue was added to each well and incubation was continued for 24 h. The MBC was defined as the lowest peptide concentration at which no color change indicative of microbial growth was observed.^{30,31}

Methodological consideration. All MIC and MBC values reported in this study were generated under the Middlebrook 7H9/OADC/PANTA assay conditions described above, including the 7-day incubation period. Accordingly, these values should be interpreted as study-specific experimental endpoints under the conditions used here. Direct numerical comparison with MIC/MBC values obtained using different media, supplementation, inoculum handling, or incubation conditions should be made cautiously.

Proteolytic stability assay

Each peptide was evaluated separately against pepsin, trypsin, chymotrypsin, and EndoGluC. Peptides were examined at 128, 256, and 512 μM , as represented in the degradation profiles, and residual peptide was monitored at serial time points for up to 20 h by chromatographic analysis. For each protease, the concentration of intact peptide remaining at each sampling point was plotted against time. Stability was interpreted comparatively from persistence of the intact-peptide signal; persistence beyond 4 h was considered evidence of comparatively prolonged stability under the conditions used. The exact protease activity, buffer composition, pH, and quench conditions are not recoverable from the manuscript source record and should be cross-checked against the original laboratory notebook before final submission.

Membrane-integrity assay

Membrane integrity was assessed in *Escherichia coli* M/20-03 after exposure to each peptide at its experimentally determined minimum bactericidal concentration (MBC). Untreated bacteria served as the negative control, while ciprofloxacin and colistin were included as antimicrobial comparators. After treatment, cells were separated from the extracellular medium and DNA and RNA released into the supernatant were quantified and expressed as $\mu\text{g/mL}$. Increased extracellular nucleic-acid concentrations relative to the untreated control were interpreted as evidence consistent with loss of cellular integrity. Because the source manuscript does not preserve the instrument model, calibration procedure, exposure duration, or centrifugation settings, these operational details should be verified from the original experimental record before submission.

Time-kill assay

Time-kill experiments were performed with *E. coli* M/20-03 using P1, P2, P3, and P7 at the bactericidal concentrations used in the study, with ciprofloxacin and colistin as comparators. Viable bacterial recovery was followed over a 240-min observation period. Aliquots were collected at serial time points, cultured for viable-count determination, and the results were expressed as $\log_{10}$ recoverable cells per 100 μL . Complete killing was defined operationally as the absence of recoverable cells at the corresponding sampling point. The source record used for manuscript preparation does not contain the exact starting inoculum, plating medium, dilution scheme, or analytical limit of detection; these parameters should therefore be verified from the original laboratory record before final submission.

Statistical analysis

MIC and MBC experiments were performed three times in duplicate, as described above. The present dataset was analyzed descriptively because the principal endpoints were MIC/MBC values, peptide persistence during proteolysis, extracellular nucleic-acid concentrations, and time to loss of recoverable bacterial cells. No inferential hypothesis-testing framework was applied to the results presented in this manuscript; consequently, no P-value threshold or multiple-comparison correction was used. Quantitative results are reported in the units generated by the corresponding assays, and interpretation emphasizes within-study comparisons under identical experimental conditions.

III- Results

Peptide synthesis and analytical characterization

Following synthesis, analytical HPLC was used to assess peptide purity. Peptides that did not meet the target purity were further purified by preparative HPLC. Analytical HPLC showed a dominant peak for each peptide, and LC-MS supported the expected molecular masses through the observed charge states. The supplementary chromatograms indicate approximately 97% or higher purity for the synthesized peptides. Table 1 summarizes the sequences, molecular weights, net charges, physical appearance, and predicted three-dimensional structures of P1-P7.

Proteolytic stability

The proteolytic-stability profiles differed substantially among the peptides (Figure 1). P6 showed the greatest stability, remaining detectable for more than 4 h after exposure to pepsin, trypsin, chymotrypsin, and EndoGluC. P2 and P7 remained detectable for more than 4 h with pepsin, chymotrypsin, and EndoGluC but were more susceptible to trypsin. P1 and P5 showed prolonged stability primarily with pepsin, whereas P3 and P4 were degraded more rapidly, within less than 1 h under the tested conditions. These data demonstrate sequence-dependent differences in proteolytic susceptibility. Because no direct secondary-structure measurements were performed in this study, the observed differences should not be assigned to a specific conformation without additional structural evidence.

Overall, the digestion experiments show that proteolytic stability is sequence dependent. The results identify P6 as the most stable peptide in the present panel and P2/P7 as partially protease-resistant candidates. Further optimization may consider strategies such as cyclization, non-natural amino acids, or other structural constraints, but these approaches were not evaluated in the present study.

The present results therefore provide an experimental basis for prioritizing peptides for subsequent optimization rather than establishing a complete structure-stability relationship.

Antibacterial activity

MIC and MBC profiles

The inhibition data in Supplementary Table 1 show that all seven peptides displayed antibacterial activity against the tested bacteria, with both bactericidal and bacteriostatic profiles. MIC values ranged from 8.14 $\mu\text{g/mL}$ to 2083.33 $\mu\text{g/mL}$ across the peptide-bacterium combinations, demonstrating substantial sequence- and organism-dependent variability.

P7 showed the most consistent antibacterial activity in the series, with MIC values ranging from 8.14 to 130.21 $\mu\text{g/mL}$ (3.56-56.87 μM). Its lowest MICs were observed against *Salmonella Typhimurium* and *E. coli* M/20-03 (8.14 $\mu\text{g/mL}$; 3.56 μM), whereas the highest was observed against *E. coli* M/30-03 (130.21 $\mu\text{g/mL}$; 56.87 μM). These data support prioritizing P7 for further optimization; however, mechanistic explanations based on charge or amphipathicity require dedicated structure-activity experiments.

The reference antibiotics showed organism-dependent activity. Ciprofloxacin MICs ranged from 0.49 to 125 $\mu\text{g/mL}$ in the supplementary dataset, whereas colistin MICs ranged from 0.24 to 125 $\mu\text{g/mL}$. Direct comparisons between peptide and antibiotic potency should therefore be made for each organism and concentration rather than generalized across the complete panel.

Membrane integrity and killing kinetics

Membrane-integrity assessment

Membrane integrity was evaluated in *E. coli* isolate M/20-03, the organism showing broad susceptibility across the peptide panel. At the corresponding MBCs, extracellular DNA concentrations ranged from 497.7 to 994.1 $\mu\text{g/mL}$ and extracellular RNA concentrations ranged from 360.6 to 612.1 $\mu\text{g/mL}$. Cells exposed to ciprofloxacin yielded 906.4 $\mu\text{g/mL}$ DNA and 612.1 $\mu\text{g/mL}$ RNA, whereas cells exposed to colistin yielded 474.6 $\mu\text{g/mL}$ DNA and 398.4 $\mu\text{g/mL}$ RNA. The detection of extracellular nucleic acids after peptide treatment is consistent with loss of membrane integrity, although these measurements alone do not establish a specific pore-forming mechanism.

Thus, the nucleic-acid release assay supports membrane damage as a component of peptide activity. Additional membrane-specific assays would be required to define the precise biophysical mechanism.

Time-kill kinetics

Time-kill experiments were performed against *E. coli* isolate M/20-03 at the tested bactericidal concentrations. Complete loss of recoverable cells occurred within 10 min for P1, 20 min for P7, 40 min for P2, and 105 min for P3 under the experimental conditions. In the same dataset, colistin required approximately 145-165 min, whereas ciprofloxacin did not achieve complete elimination within the 240-min observation period. These results demonstrate rapid killing by the selected peptides under the conditions used; they do not by themselves establish superiority *in vivo* or across other bacterial species.

IV- Discussion

This study combined peptide synthesis and analytical characterization with proteolytic-stability testing, antibacterial susceptibility measurements, a membrane-integrity assay, and time-kill analysis. The resulting dataset shows that antibacterial potency and proteolytic stability are not maximized by the same sequence, highlighting the need to optimize these properties jointly.

Analytical HPLC and LC-MS supported successful preparation of P1-P7, with the supplementary chromatograms indicating approximately 97% or higher purity. The peptides carried net positive charges of +3 to +5, consistent with their content of lysine and acidic residues. These physicochemical features provide a useful basis for future sequence-activity analysis, but the present study did not directly quantify amphipathicity, helicity, or membrane binding.

Proteolytic digestion differentiated the candidates clearly. P6 remained detectable for more than 4 h with all four proteases, whereas P2 and P7 showed selective resistance and P3/P4 were degraded more rapidly. The data therefore support the description of differential proteolytic stability rather than uniform protease resistance across the series. Future optimization could test whether structural constraints improve stability without reducing antibacterial activity. This trade-off between antibacterial activity and protease stability is consistent with recent AMP optimization studies that evaluate sequence modification alongside protease stability and cytotoxicity (Enniful et al. 2026).

All seven peptides inhibited the tested Gram-negative bacteria, but potency varied widely. P7 was the most consistently active peptide, whereas several other peptides required substantially higher concentrations against *P. aeruginosa* or *E. coli* M/30-03. The MIC/MBC profiles therefore support P7 as the leading antibacterial candidate from this panel, while P6 is more notable for stability than for antibacterial potency.

For P7, MIC values ranged from 8.14 $\mu\text{g/mL}$ (3.56 μM) against *S. Typhimurium* and *E. coli* M/20-03 to 130.21 $\mu\text{g/mL}$ (56.87 μM) against *E. coli* M/30-03. Comparisons with ciprofloxacin and colistin were organism dependent. An important limitation is that the susceptibility experiments were performed using the Middlebrook 7H9/OADC/PANTA protocol and 7-day incubation described in the Methods. Therefore, the absolute MIC and MBC values should be interpreted within this experimental system and should not be assumed to be directly interchangeable with values generated under other susceptibility-testing conditions. Within the present study, however, all peptides and comparator antibiotics were evaluated using the

same assay framework, allowing internal comparison of their relative activities. A major methodological limitation is that susceptibility testing was performed in Middlebrook 7H9/OADC/PANTA rather than a standard cation-adjusted Mueller-Hinton broth system used for routine Gram-negative antimicrobial susceptibility testing. The MIC and MBC values should therefore be interpreted as experimental, study-specific endpoints and not against clinical breakpoints. Confirmation in a standardized Mueller-Hinton-based assay would materially strengthen cross-study comparability and translational interpretation.

Extracellular DNA and RNA release after peptide exposure was consistent with compromised cellular integrity. The time-kill data further showed rapid bactericidal action for selected peptides against *E. coli* M/20-03. Together, these experiments support a membrane-associated antibacterial effect, but more direct approaches such as membrane-potential measurements, dye-uptake assays, microscopy, or lipid-vesicle studies would be needed to establish the detailed mechanism.

Conclusion

This study describes seven synthetic antimicrobial peptides with distinct antibacterial and proteolytic-stability profiles. Under the Middlebrook 7H9-based assay conditions used in this study, all peptides inhibited the Gram-negative bacteria tested, although potency varied substantially across sequences and organisms. P7 showed the most consistent antibacterial activity, with MIC values of 8.14-130.21 $\mu\text{g/mL}$ (3.56-56.87 μM), whereas P6 showed the greatest resistance to proteolytic degradation, remaining detectable for more than 4 h in the presence of pepsin, trypsin, chymotrypsin, and EndoGluC. The extracellular release of DNA and RNA after peptide exposure was consistent with compromised bacterial membrane integrity, and time-kill experiments demonstrated rapid bactericidal effects against *E. coli* M/20-03 under the conditions tested. These results support further sequence optimization aimed at combining the antibacterial profile of P7 with improved proteolytic stability, together with confirmatory susceptibility testing under additional standardized assay conditions and direct studies of membrane interaction and mammalian-cell safety.

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Data Availability

The data supporting the findings of this study are contained within the article and its Supplementary Information. Additional raw data may be made available by the corresponding author upon reasonable request, subject to availability of the original laboratory records.

Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Olivier Ndogo Eteme contributed to study conception and design, peptide-related methodology, data interpretation, visualization, and preparation of the original manuscript draft. Mbarga Opono Hermine Salome, Esther Del Florence Moni Ndedi, Patrick Hervé Betote Diboué, and Ernestine Nkwengoua Zondegoumba contributed to experimental work, data acquisition and/or interpretation, and critical review of the manuscript. All authors reviewed and approved the final manuscript.

Ethics Approval

Not applicable. This study did not involve human participants, human data, human biological material, or vertebrate animals.

Consent to Participate

Not applicable.

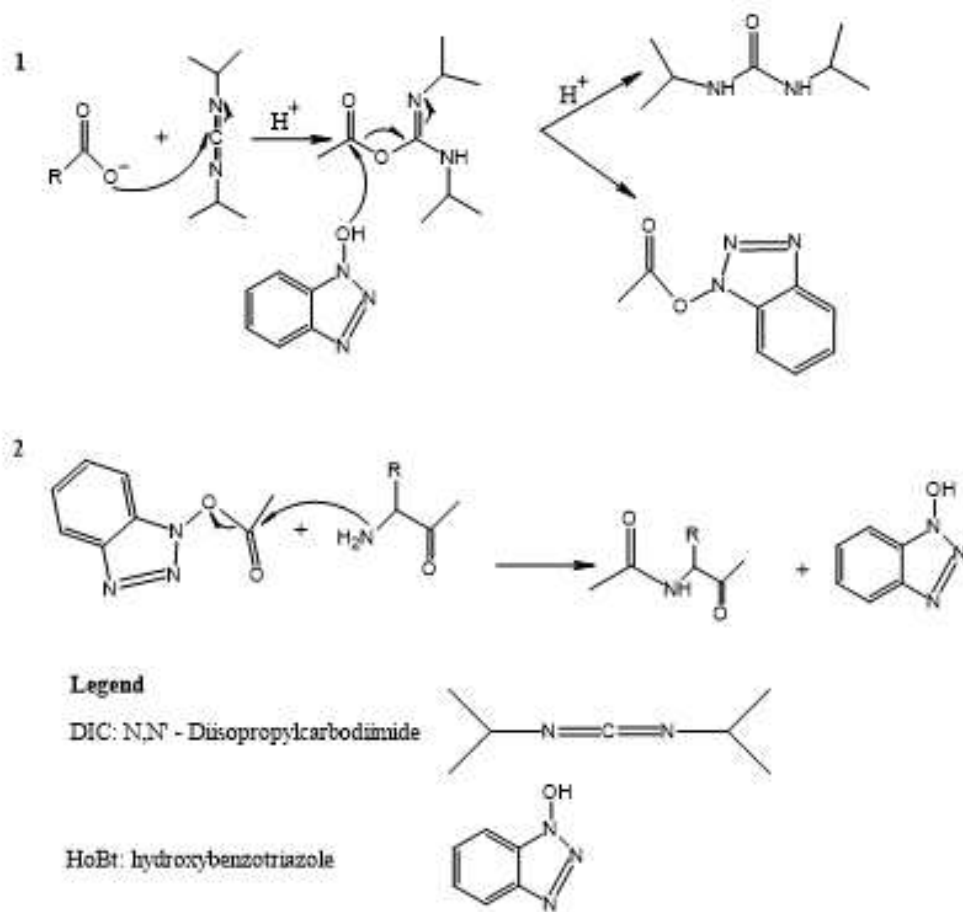
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Tables and Figures

Supplementary display file for International Journal of Peptide Research and Therapeutics

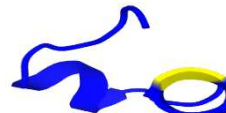


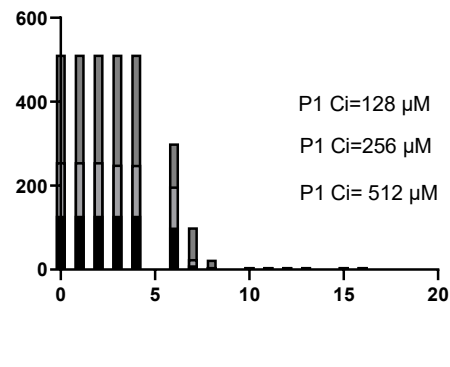
Scheme 1 : A reaction scheme illustrating the process of peptide bond formation mediated by the DIC/HOBt couple.

Table 1. Physicochemical characteristics of the synthesized peptides

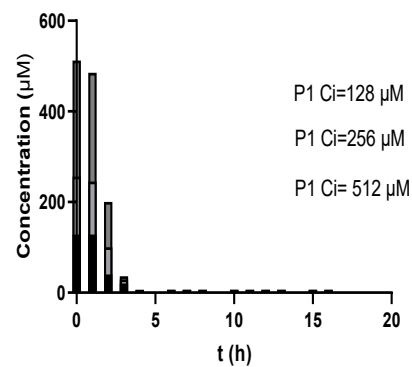
Abbreviation:

P, peptide.

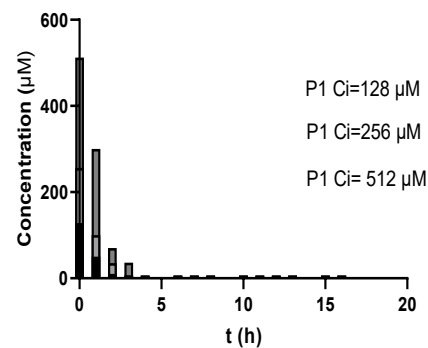
Peptide's code	Primary sequence	Molecular weight (g/mol)	Charge	Physical appearance	Three-dimensional structure
P1	KAPFGKVAKKLGKNFVALLW	2,328.84	+5	White powder	
P2	KIGGVAKKAWLKNVAKNFAFL	2,302.80	+5	White powder	
P3	DGPFNWKIGLAKVKKFIAVAN	2,315.76	+3	White powder	
P4	KAPFGKVAKKLGKNFVALWL	2,328.84	+5	White powder	
P5	DGPFNWKIGLAKVKFKIAVAN	2,315.76	+3	White powder	
P6	VAKKAAGWNVALDKIGFKNFI	2,289.72	+3	White powder	
P7	VAKKAAGWNVALDKIGFKNFI	2,289.72	+3	White powder	



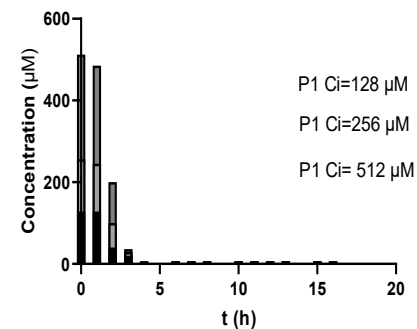
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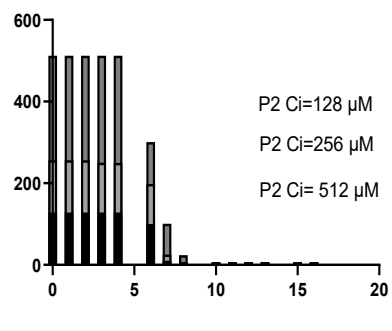


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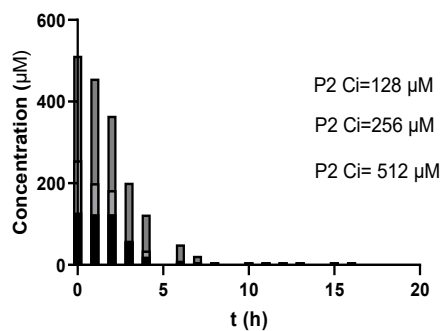


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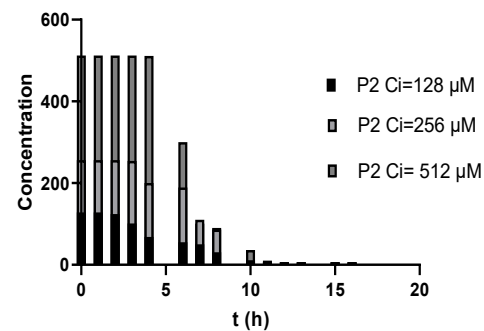
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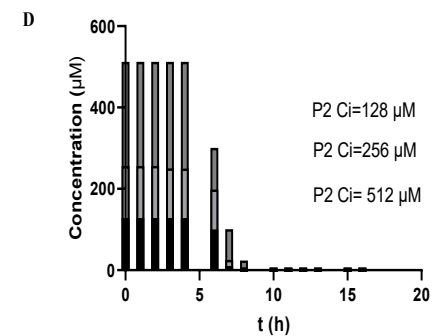
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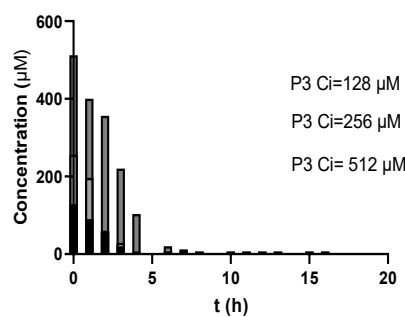


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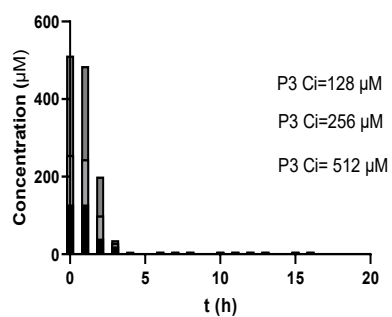


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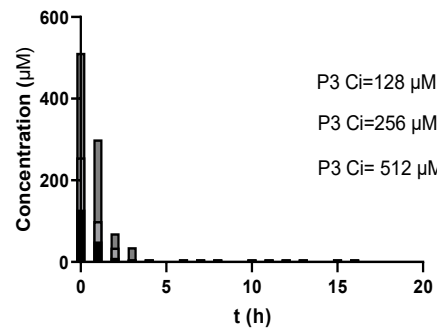
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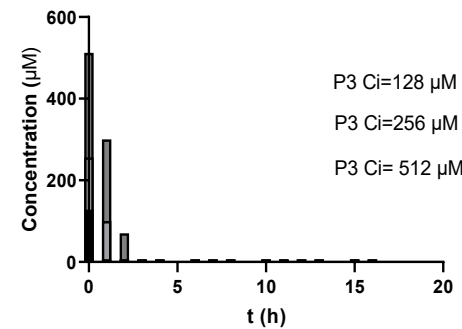
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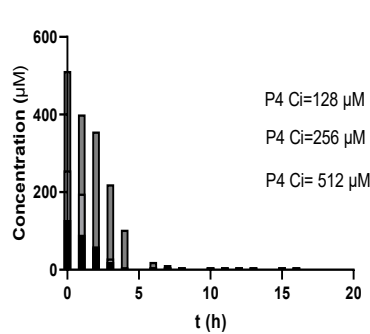
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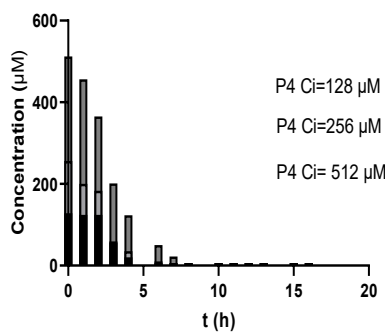
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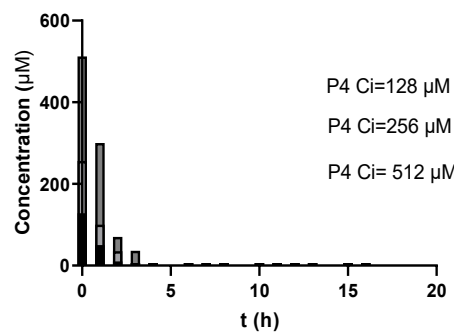
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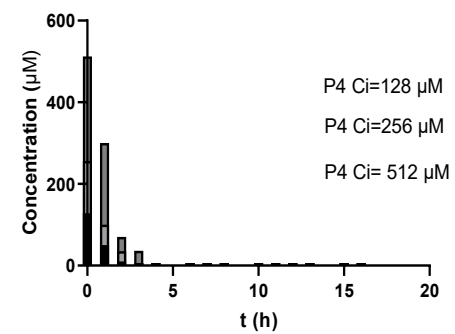
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T

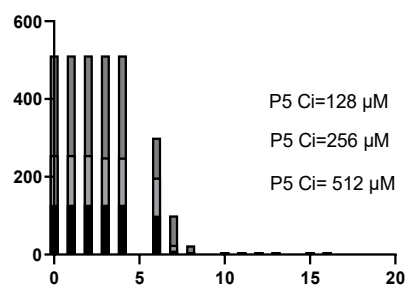


C

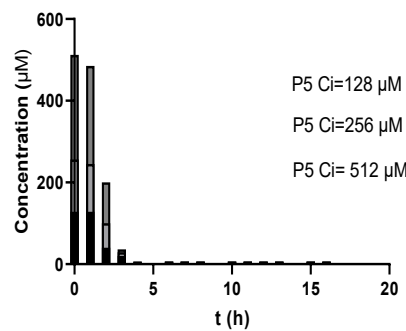


E

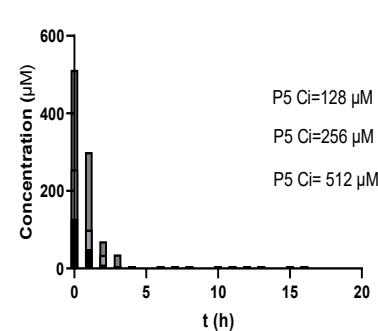
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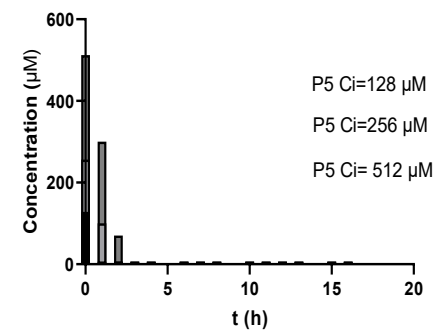
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T

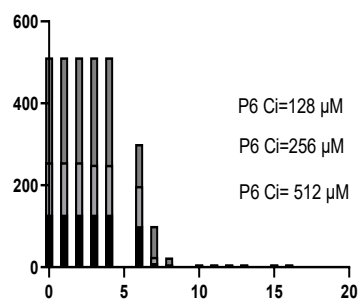


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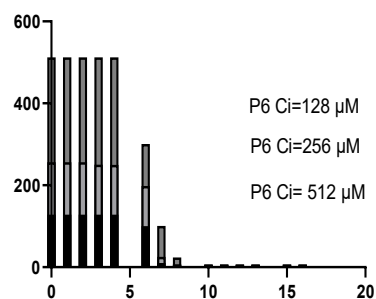


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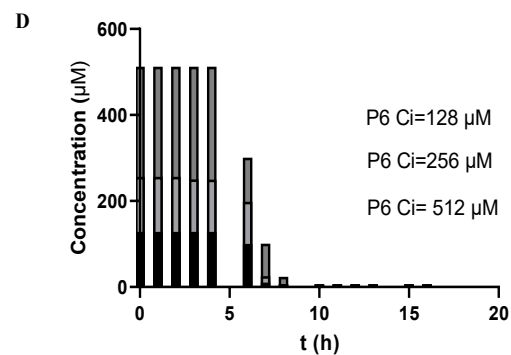
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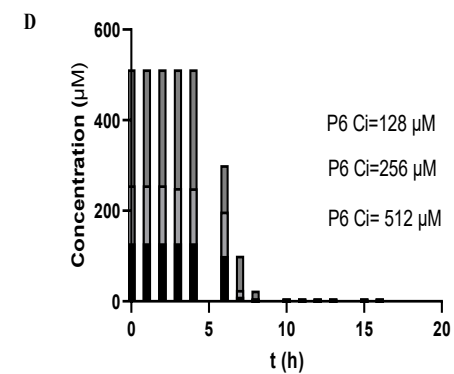
P



T



C



E

P6

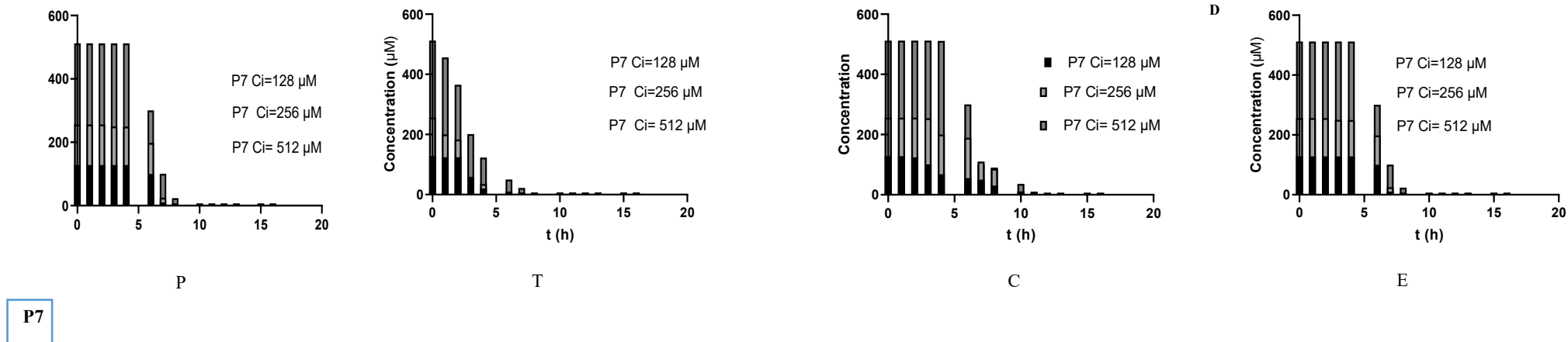


Figure 1 : Monitoring of peptide degradation kinetics by proteases.

Legend : P, pepsin; T, trypsin; C, chymotrypsin; E, EndoGluC.

Proteolytic stability was sequence dependent; P6 was the most stable, whereas P3 and P4 were degraded more rapidly.

QUANTIFICATION OF BIOMOLECULES

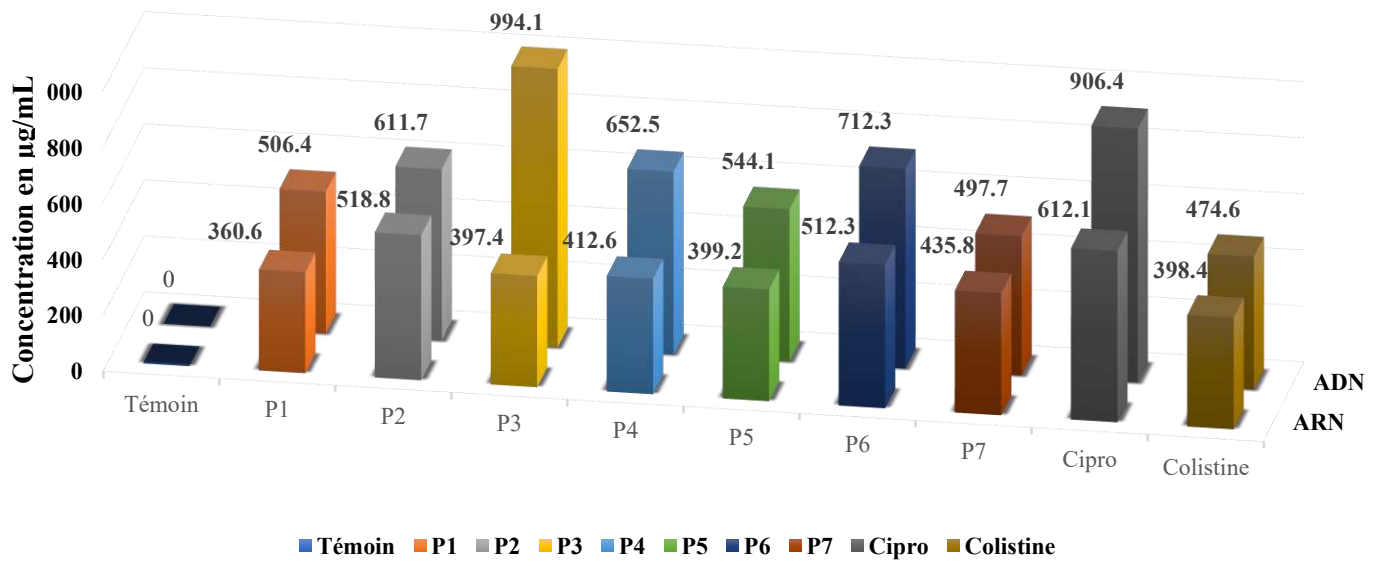
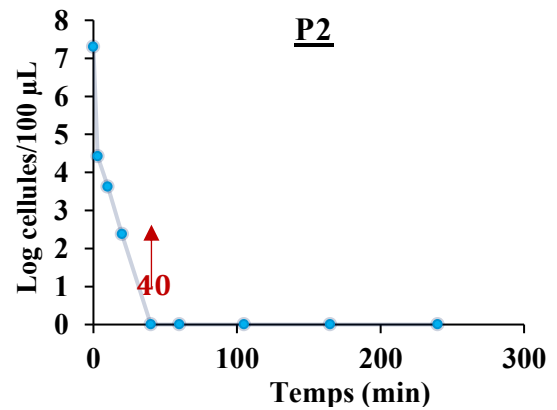
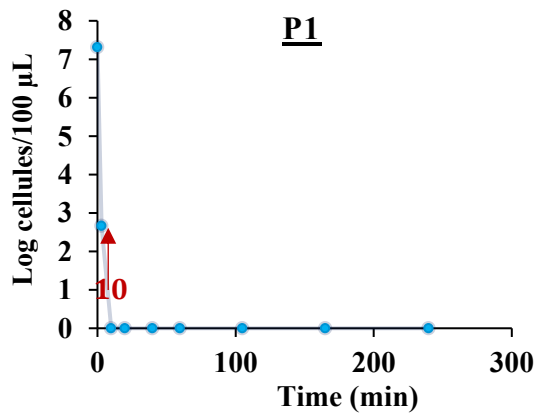


Figure 1. Proteolytic degradation kinetics of P1-P7 in the presence of pepsin (P), trypsin (T), chymotrypsin (C), and EndoGluC (E). Peptide persistence was sequence dependent, with P6 showing the greatest stability under the tested conditions.

Figure 2. Extracellular DNA and RNA measured after antimicrobial treatment of E. coli M/20-03.



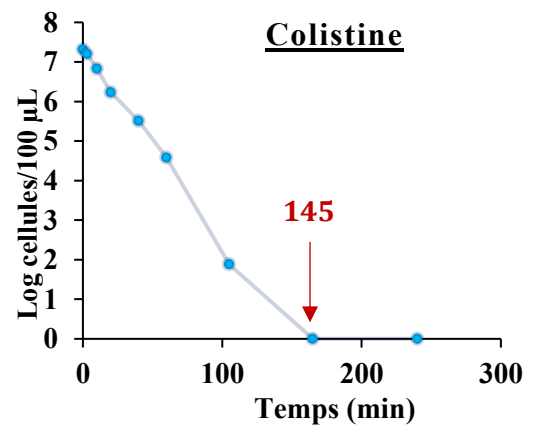
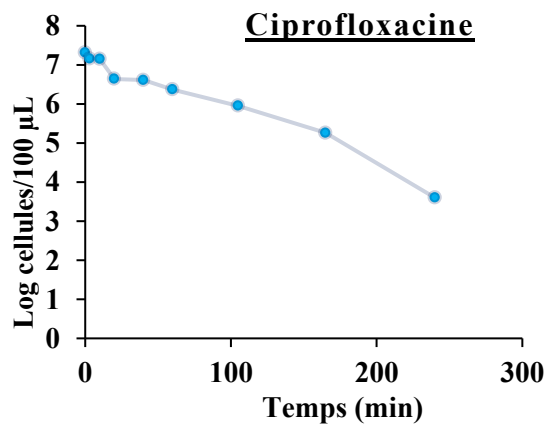
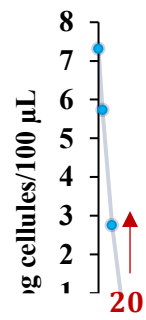
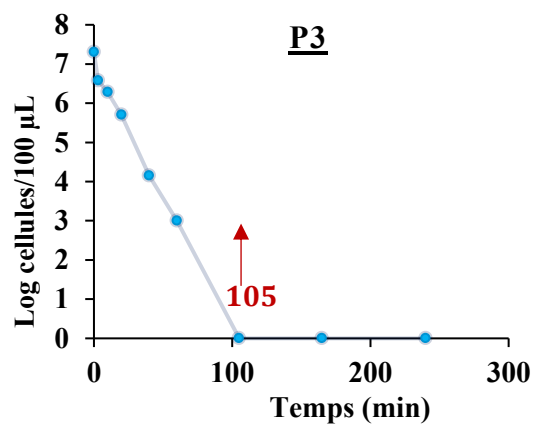


Figure 3. Time-kill kinetics of *E. coli* isolate M/20-03 after exposure to selected antimicrobial peptides, ciprofloxacin, and colistin.

Supplementary Files

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- [IJPRTSupplementaryInformationv2.docx](#)