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RESEARCH ARTICLE

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Design of new truncated derivatives based on direct and reverse mirror repeats of first six residues of Caerin 4 antimicrobial peptide and evaluation of their activity and cytotoxicity

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Abstract

Caerin 4 is a family of AMPs isolated from the frog called *Litoria caerulea*. In silico drug designing methods and using machine learning algorithms for AMPs design can reduce their usage restrictions such as production costs and the time required for investigation of their activity and toxicity. In this study, two short peptides were designed based on direct and reverse mirror repeats of GLWQKI conserved sequence from Caerin 4 family that called dCar12 and rCar12. Also, Caerin 4.1 was synthesized without primary GLWQKI sequence and named $Car_{7\text{-}23}$. Following the synthesis of peptides, their antimicrobial properties, cytotoxicity, secondary structure, and mode of action were further evaluated. Results indicated that rCar12 had a good antibacterial activity (at an MIC of 3.9–62.5 μg/ml), while $Car_{7\text{-}23}$ did not have any antimicrobial properties. Cytotoxicity of rCar12 at MICs range was <5%, which is much less than Caerin 4.1. In conclusion, rCar12 with reverse mirror repeat has different functional properties compared with dCar12. These results corroborate the fact that in two peptides with identical residues and length, the position and arrangement of amino acids are very important concerning peptide function. Moreover, GLWQKI sequence is highly crucial for the antimicrobial activity of Caerin 4 antimicrobial peptide family.

KEYWORDS

antimicrobial peptide, caerin, in silico drug design, *Litoria caerulea*, machine learning algorithm

1 | INTRODUCTION

According to world Health Organization comments, emergence of antimicrobial resistance is becoming a major threat for public health (Prestinaci, Pezzotti, & Pantosti, 2015). Considering the worldwide increasing prevalence of resistance to traditional antibiotics, it has become all the more necessary to find new antibiotics (Madanchi et al., 2018). Nowadays, understanding the biological and biomedical importance of antimicrobial peptides (AMPs) might be regarded as an advance toward new and resistance-free therapies for infectious diseases (Gaspar, Veiga, & Castanho, 2013). AMPs are a group of molecules produced by innate immune system of all living organisms (Gupta, Bhatia, Sharma, & Saxena, 2018; Mahlapuu, Håkansson, Ringstad, & Björn, 2016), showing a broad spectrum of activities against Gram-negative and Gram-positive bacteria, including antibiotic-resistant bacterial strains and certain fungi, viruses, and parasites (Wang, Zeng, Yang, & Qiao, 2016). AMPs have other biological functions such as apoptosis

induction, wound healing, immune modulatory features, and even anticancer properties. These molecules can be classified into four groups based on their structures: α-helical peptides, β-sheet peptides, extended peptides, and loop peptides (Tavares et al., 2013). The limitations associated with the use of natural AMPs can be attributed to their toxicity, immunogenicity for human, low stability in human plasma, and high costs of synthesis (Maccari, Nifosi, & Di Luca, 2013; Peters, Shirtliff, & Jabra-Rizk, 2010), hence designing of new AMPs or modifying natural peptides to enhance their properties are necessary.

In silico methods and computer-based design for AMPs can reduce the limitations of natural AMPs and improve their antimicrobial properties and selectivity (Maccari et al., 2013). For a computer-based design, it is further important to understand the physicochemical properties of peptides such as peptide amino acid composition, the position of amino acids, net charge, hydrophobicity, amphipathicity, peptide length, molecular weight (Mw), hydropathicity (GRAVY index), isoelectric pH (pI), Boman index, and secondary and 3D structure (Madanchi et al., 2018), all of which, except for structure, depend on the amino acid content of the peptide. The 3D structure of peptides, in addition to amino acid content, depends on their location and arrangement in the peptide. GRAVY is an abbreviation for grand average of hydropathicity index. Proteins with a positive GRAVY index are more hydrophobic, while those with a negative GRAVY value are more hydrophilic and tend to be more water soluble (Kyte & Doolittle, 1982; Madanchi et al., 2018). Boman index (its unit is kcal/mol) is a parameter differentiating the action mechanism of hormones such as peptide (protein-protein binding) and the antimicrobial activity of peptides (protein–membrane binding; Boman, 2003; Osorio, Rondón-Villarrea, & Torres, 2015).

Caerin 4 is a family of AMPs isolated from the Australian green tree frog called *Litoria caerulea* (Waugh, Stone, Bowie, Wallace, & Tyler, 1993). From Caerin 4 family, three peptides called Caerin 4.1, Caerin 4.2, and Caerin 4.3 are introduced in APD3 (Antimicrobial Peptide Database 3) data bank (Waugh et al., 1993). These peptides have antibacterial (especially against Gram-negative bacteria), anti-viral, anti-fungal, anti-parasitic, and anticancer activities (Waugh et al., 1993). In this study, two peptides with 12 amino acids were designed based on direct (called dCar12) and reverse mirror repeats (called rCar12) of GLWQKI sequence from Caerin 4. To further assess GLWQKI role in the activity of Caerin 4, these six amino acids were removed from Caerin 4.1 and named Car_{7-23}. After the synthesis of these peptides, the antimicrobial properties, time of killing, cytotoxicity, and secondary structure of these peptides were evaluated. Eventually, LPS- and DNA-binding activities and field emission scanning electron microscopy (FE-SEM) were performed in order to study the action mode of these peptides.

2 | MATERIALS AND METHODS

2.1 | Peptide design and their synthesis

At first, Caerin 4 family sequences were obtained from APD3 data bank (http://aps.unmc.edu/AP/; Wang, Li, & Wang, 2015). Six conserved residues were selected from primary amino acids of the N-terminal of Caerin 4 members. Subsequently, to understand the effect of the orientation and arrangement of amino acid residues in activity of peptides, two new peptides were designed based on direct and reverse mirror repeats of these six amino acid sequence. Also, in a new design to study the role of the first six amino acids in the activity of Caerin 4, GLWQKI sequence was removed from Caerin 4.1. AMP possibility of these peptides was predicted by machine learning algorithms support vector machine (SVM), random forest, artificial neural network, and discriminant analysis from the Collection of Antimicrobial Peptides site (http://www.camp.bicnirrh.res.in/ 2019; Waghu, Barai, Gurung, & Idicula-Thomas, 2016). The threshold of each algorithm is between 0.5 and 1. Peptides are AMP if the threshold number is >0.5. To achieve the physicochemical properties of peptides, Antimicrobial Peptide Calculator and Predictor software from APD3 and ProtParam from Expasy (https://www.expasy.org/ 2019) were used. Helical wheel projection was carried out to predict the position of amino acids in peptides (http://lbqp.unb.br/NetWheels/). Presence of antigenic sequence in the designed peptides was predicted by Predicted Antigen Peptide software (http://imed.med.ucm.es/Tools/antigenic.pl). Afterward, all peptides were commercially synthesized by Mimotopes Pty Ltd by a solid-phase method using/N9 fluorenylmethoxy carbonyl chemistry and were supplied in 95% purity (by RP-HPLC) with satisfactory mass spectra.

2.2 | Bacterial strains, cell line, reagents, and media

In order to perform antimicrobial assay, *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were employed. Mueller Hinton Broth (MHB), Mueller Hinton agar (MHA), ethanol, phosphate-buffered saline (PBS), penicillin, and streptomycin were purchased from Merck Millipore Company (Merck). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) medium 1640 medium were purchased from Gibco Company (Gibco). Cell culture antibiotics (penicillin, streptomycin, and cyclosporin), Trypsin, Trypan blue dye, NaOH, HCl, MTT dye [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Triton X-100, 2, 2, 2-trifluoroethanol (TFE), glutaraldehyde, and dimethyl sulfoxide were

procured from Sigma (Sigma-Aldrich). Human skin fibroblast cell line (Hu02 cell line, National Cell Bank of Iran, Pasture Institute of Iran) was employed for cytotoxicity test.

2.3 | Characterization of the secondary structure of peptides by Circular Dichoroism (CD)

Circular Dichoroism spectroscopy evaluations were performed to identify the secondary structure of peptides. The mean residue molar ellipticities of peptides were determined by CD spectroscopy, using a Jasco J-810 spectropolarimeter (Jasco, Japan) at 25°C with a 200 nm/min scanning speed with five scan. A 0.2–0.5 mg/ml of peptide solutions in 70% TFE was loaded on to a 1 mm quartz cell, and its spectra were scanned from 190 to 250 nm (Jiang, Vasil, Vasil, & Hodges, 2014). Eventually, the data obtained from CD were analyzed by the CAPITO web server (Wiedemann, Bellstedt, & Görlach, 2013).

2.4 | Antimicrobial assay

So as to determine the peptides' minimal inhibitory concentration (MIC), serial dilution titration method was employed. Further measured was the minimal bactericidal concentration (MBC) against the different bacterial strains according to Clinical and Laboratory Standards Institute guideline (Szabo et al., 2010). Bacteria were grown overnight at 37°C in MHB and diluted in the same medium. Twofold serial dilutions of the peptides were added to the microtiter plates in a volume of 100 µl, followed by the addition of 100 µl of bacteria to generate a final inoculum of 5×10^5 colony-forming units (CFU)/ml. The plates were incubated at 37°C for 24 hr, and MICs were determined. Next, 100 µl of the 24 hr inhibitory concentration test sample (MIC well) and its subsequent concentrations were plated on MHA and incubated at 37°C overnight to obtain the MBC (Manzini et al., 2014). All tests were conducted in triplicate, and their mean ± *SD* results were obtained.

2.5 | Evaluation of the toxicity of peptides on human skin fibroblast cells

The toxicity of peptides against Hu02 cell line was determined by MTT assay, as previously described (Kumar, Nagarajan, & Uchil, 2018), with certain modifications. Cells cultured in RPMI medium (supplemented with 10% FBS) were seeded in 96-well plates at a density of 10^5 cells/well and incubated with various concentrations of peptides. Following the incubation period, the cells were incubated with 10 µl of 5 mg/ml MTT in PBS for 4 hr at 37°C. Next, the media was removed and Isopropanol was added at 100 µl/well to dissolve the formazan crystals; the absorbance of the resulting solution was then measured using a microplate reader (STAT FAX 2100, BioTek) at 545 nm. The percentage of cell viability was calculated as follows:

$$\text{Tocixity} = \left(1 - \frac{\text{mean OD of sample}}{\text{mean OD of control}}\right) \times 100\%$$

$$\text{Viability}\,\% = 100 - \text{Toxicity}\,\%$$

2.6 | Hemolytic assay

The potential of the hemolytic effects of the peptides on human red blood cells (RBC) was determined by hemolytic assay. Fresh blood samples were primarily taken from a volunteer with blood type O. A 20% (vol/vol) suspension of human erythrocytes was prepared in PBS; the suspension was then diluted 1:20 in PBS, and 100 µl was added in triplicate to 100 µl of a 2-fold serial dilution series of the peptide in a 96-well plate. Triton X-100 (1%) and sterile 0.9% NaCl solution were used as a positive control for 100% lysis of RBCs and negative control, respectively. After incubation at 37°C for 1 hr, the plate was centrifuged for 10 min at 1,372 *g*. Next, 150 µl of the supernatant was transferred to a new 96-well plate to measure the absorbance at 414 nm using a microplate reader (STAT FAX 2100, BioTek). Percentage of hemolysis was calculated as follows (Wu et al., 2014).

$$\text{Hemolysis}\,\% = \left(\frac{\text{mean OD of sample} - \text{mean OD of negative control}}{\text{Mean OD of positive control} - \text{mean OD of negative control}}\right) \times 100\%$$

2.7 | Killing kinetics assay

Killing kinetic assay was used to determine the bactericidal activity of peptides at any point in time. Therefore, logarithmically growing *E. coli* (ATCC 25922) was suspended in fresh MHB, adjusted to 5×10^5 CFU/ml, and added to the medium containing the peptides at concentrations equivalent to 1× MIC. Following incubation for 0, 15, 30, 60, 120, 150, and 180 min at 37°C, samples were serially diluted and plated in triplicate onto MHA plates. CFU was counted following the 24 hr incubation of plates at 37°C. In this study, streptomycin and penicillin were used as antibiotic controls (Madanchi et al., 2019).

2.8 | Study of bacteria morphological changes after treatment with dCar12 and rCar12 peptides

The morphological changes of bacteria after treatment with the peptides were evaluated by FE-SEM. *S. aureus* (ATCC

TABLE 1 The probability of the antimicrobial activity of peptides and some of their physicochemical properties

Name: Sequence	Score of algorithms				Hyd[a] (%)	Nt[b]	pI[c]	BI[d]	GRAVY[e]
	SVM	RF	ANN	DA					
GLWQKI	0.195	0.297	AMP	0.632	50	+1	8.75	−0.33	−0.067
$Car_{7\text{-}23}$ KSAAGDLASGIVEGIKS	0.428	0.261	NAMP	0.282	41	0	6.07	0.57	0.235
dCar12 GLWQKIGLWQKI	0.985	0.9295	AMP	0.967	50	+2	10	−0.33	−0.067
rCar12 GLWQKIIKQWLG	0.981	0.9295	AMP	0.967	50	+2	10	−0.33	−0.067

Abbreviations: ANN, artificial neural network; DA, discriminant analysis; RF, random forest; SVM: Support Vector Machine.

[a]Hydrophobicity ratio.

[b]Net charge.

[c]Isoelectric pH.

[d]Boman index (kcal/mol).

[e]Grand average hydropathy value of the peptide.

25923) and *E. coli* (ATCC 25922) were grown in MHB and incubated at 37°C for 24 hr. Subsequently, a suspension of bacteria (10^6 CFU/ml) was prepared in PBS (pH 7.4) and incubated with 0.5× MIC of dCar12 and rCar12 for 2, 4 and 6 hr. The bacteria were precipitated by centrifugation at 1,792 *g* for 2 min and washed three times with PBS (pH 7.4) and fixed 1 hr (at 25°C, dark chamber) with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). The samples were harvested at 1,792 *g* for 2 min and washed twice with PBS (pH 7.4). The samples were placed on the FE-SEM slides (1 cm^2) and subsequently dehydrated with ethanol gradient (at 10%, 30%, 60%, 70%, 90%, and 100%). Finally, the dehydrated bacteria were coated with gold nanoparticles using an automatic sputter coater. The samples were then observed using a FE-SEM instrument (JSM-7610F, JEOL Co., Japan and MIRA3, TESCAN Co.; Madanchi et al., 2019).

2.9 | Study of LPS neutralization by LAL method

The ability of the peptides to neutralize LPS was specified via Pierce LAL (*Limulus* amebocyte lysate) Chromogenic Endotoxin Quantitation Kit (Thermo Scientific™). This was done according to the manufacturer's instruction using *E. coli* lipopolysaccharide (011: B4) and chromogenic substrate Ac-Ile-Glu-Ala-Arg-*p*-nitroanilide. The LAL test is an extremely sensitive indicator of the presence of free, non-neutralized LPS, activating the *Limulus* coagulation cascade and leading to lysate gelling. The reaction of LAL with LPS is enzymatic, converting suitable synthetic substrates to produce a color reaction. At first, serial dilution of peptides (0.78–100 μg/ml) were pre-incubated in a microtiter plate with 1 EU/ml (0.1 ng/ml) of LPS for 30 min at 37°C in 50 μl of endotoxin-free water (EFW). Fifty microlitre EFW was further used as a negative control. Next, 50 μl of LAL was added to each well, and the plate with the lid was covered and gently placed on a plate shaker for 10 s and incubated at 37°C for 10 min. After that, 100 μl of chromogenic substrate was added to each well. Following incubation for 6 min, the reaction was stopped through adding 50 μl of stop reagent (25% acetic acid), and the absorbance was quantitated at 405 nm using a microplate reader (STAT FAX 2100). Each measurement was performed in triplicate (Ghosh et al., 2014), and polymyxin was used as a control.

2.10 | DNA binding assay

To investigate the binding ability of peptide to DNA, an electrophoretic mobility shift assay (EMSA) was performed. Plasmid pUC18 (200 ng) was mixed with different amounts of dCar12 and rCar12 peptides (0, 0.25, 0.5, and 1 μg/ml) in 15 ml of 10 mM Tris, 1 mM EDTA buffer, pH 8.0; the mixtures were incubated at room temperature for 5 min and then electrophoresed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide (Nam et al., 2014).

2.11 | Statistical analysis

In our study, all MIC data are represented as mean ± *SD*. In order to obtain statistical analysis between the MIC values of the designed peptides and Aur1.2 as natural control, a *t* test by SPSS Statistics 23.0 software for Windows (SPSS Inc.) was used. The *p* values of <.05 were considered as statistically significant.

3 | RESULTS

3.1 | Results of bioinformatics studies and peptide designing

GLWQKI conserved sequence of Caerin 4 peptides was selected. Next, two new peptides GLWQKIGLWQKI and

GLWQKIIKQWLG were designed, named dCar12 and rCar12, respectively. Then, by removing GLWQKI from N-terminal of Caerin 4.1, KSAAGDLASGIVEGIKS, called $Car_{7\text{-}23}$, was obtained. Moreover, Caerin 4.1 with GLWQKIKSAAGDLASGIVEGIKS sequence was selected as a natural positive control. AMP possibility of these peptides by machine learning algorithms showed that GLWQKI and $Car_{7\text{-}23}$ had no antimicrobial effects. Table 1 shows the probability of peptides' AMP activity and their other physicochemical properties. The hydrophobic and hydrophilic faces of dCar12 and rCar12 peptides were drawn up by helical wheel projection (Figure 1).The results of Predicted Antigen Peptide software indicated that there were no antigenic determinants in new designed peptides.

3.2 | Structural studies of peptides by CD

The data obtained from CD were analyzed by the CAPITO web server. The data from CD UV spectra of the peptides indicated a typical α-helix spectrum for rCar12 with maximum peak at 193 and minimum bands at 208 and 222 nm. The α-helix content in dCar12 and rCar12 was 45% and 76%, respectively (Table 2). The recorded spectra corresponding to dCar12 and rCar12 are illustrated in Figure 2.

3.3 | MIC and MBC of peptides

Table 3 shows the measurement results pertaining to the MICs and MBCs of peptides against the selected bacteria. Results showed that $Car_{7\text{-}23}$ had no antibacterial effects against selected bacteria, revealing that rCar12 has the highest number of antimicrobial properties. This peptide showed a good activity against *E. coli*, *B. subtilis*, and *P. aeruginosa* at a MIC of 4–8 μg/ml. Statistical analysis indicated that the antibacterial activity of rCar12 on *E. coli* was significantly higher than native Caerin 4.1 ($p < .001$) and penicillin ($p < .001$). These results further showed that the antimicrobial effects of rCar12 in *S. aureus* were equal to streptomycin ($p > .05$). Therefore, against all the selected bacteria, rCar12 is stronger than Caerin 4.1 and dCar12. Statistical analysis further revealed that it was only against *S. aureus* that the antibacterial activity of dCar12 was significantly better than native Caerin 4.1 ($p < .05$). Also, GLWQKI did not have any antimicrobial activities.

3.4 | Cytotoxicity assay

MTT assay results on Hu02 cell line revealed that the toxicity of rCar12 at its MIC range (4–32 μg/ml) was less than 5%, while native Caerin 4.1 peptide, in the same concentration, had more toxicity on Hu02 cells. At the concentration of 1,000 μg/ml, Caerin 4.1 killed 46% of the cell population, while rCar12 killed 9.38%. Statistical analysis indicated that the toxicity effect of rCar12 and dCar12 on Huo2 cells

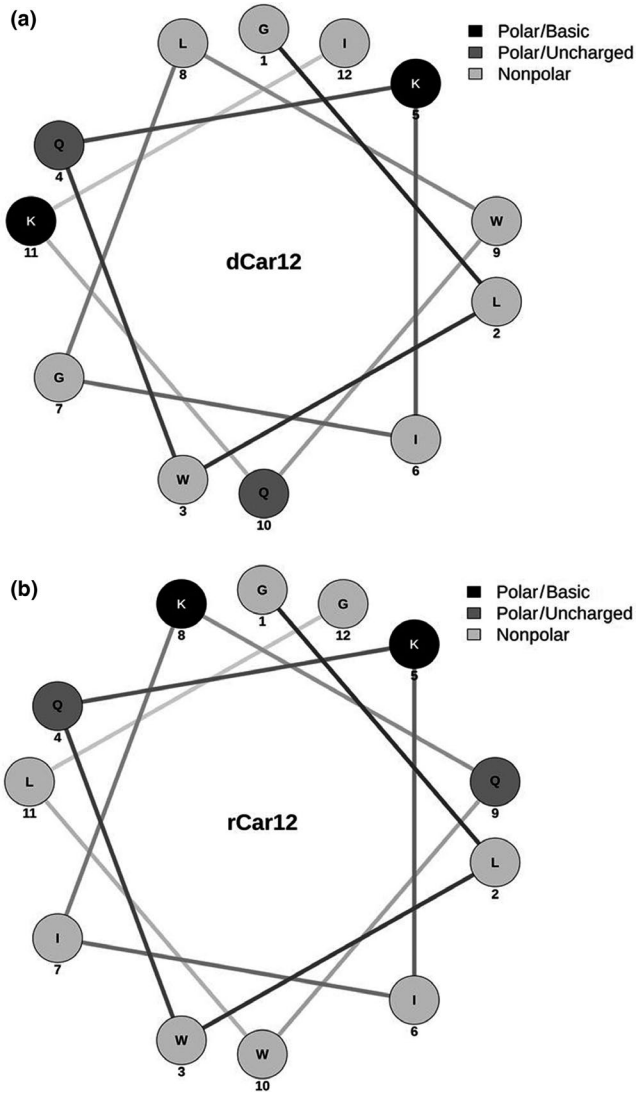

FIGURE 1 This figure shows the helical wheel projection of dCar12 (a) and rCar12 (b). In rCar12, non-polar amino acids are continuous and separated from polar amino acids. Therefore, hydrophobic and hydrophilic faces in this peptide are symmetric; in dCar12, however, hydrophobic or non-polar residues are dispersed between polar amino acids or hydrophilic face

TABLE 2 Fraction ratio of the secondary structure of dCar12 and rCar12

	Fraction of ratio (%) of secondary structure		
Substances name	Helix	β-Sheet	Random coil
dCar12	45	21	34
rCar12	76	0	24

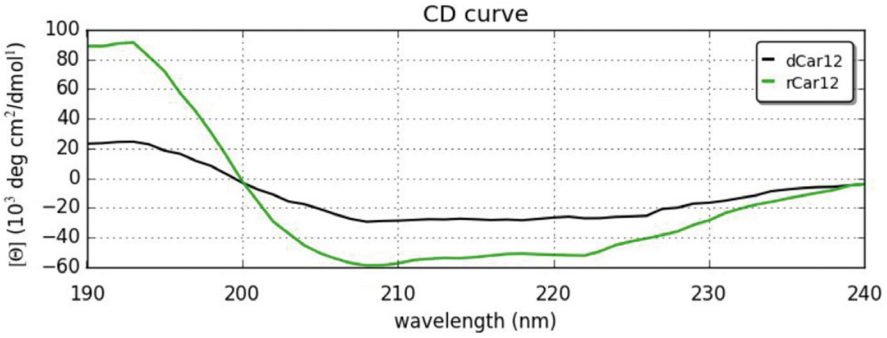

FIGURE 2 CD spectra analysis of dCar12 and rCar12 shows that both peptides in 70% TFE solution have mixed secondary structures

TABLE 3 MIC and MBC in μg/ml of peptides against the Gram-positive and Gram-negative bacteria

	Mean of (MIC/MBC) for three independent tests			
Agents	***B. subtilis***	***S. aureus***	***E. coli***	***P. aeruginosa***
Caerin 4.1	128/256	512/1,000	32/64	64/128
Car_{7-23}	>1,000	>1,000	>1,000	>1,000
dCar12	128/128	128/256	>1,000	256/512
rCar12	8/8	32/64	4/4	8/8
GLWQKI	>1,000	>1,000	>1,000	>1,000
Streptomycin	2/2	32/32	2/2	32/64
Penicillin	4/8	2/2	16/32	>1,000

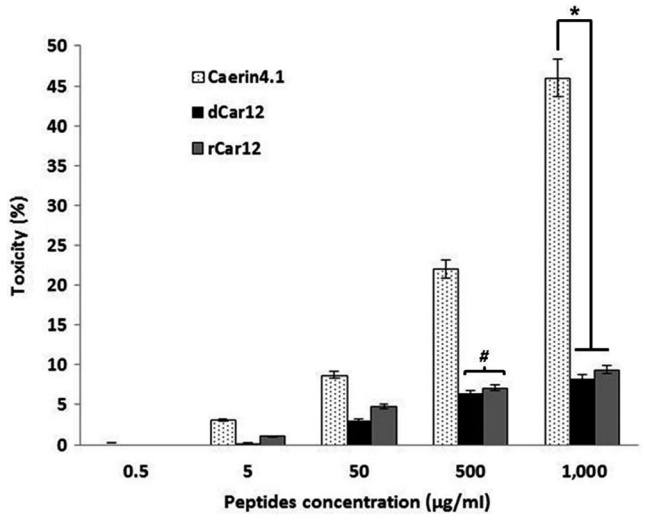

FIGURE 3 The chart displays the toxicity of dCar12, rCar12, and Caerin 4.1 peptides on Hu02 cell line. Error bars indicate standard deviation. Statistical differences were shown by stars ($^{\#}p > .05$; $^{**}p < .001$)

at all concentrations was significantly less than Caerin 4.1 ($p < .001$). Although the toxicity of rCar12 is generally very low, results showed that its toxicity was more than dCar12 at the same concentrations. According to the toxicity/concentration chart, IC_{50} of all three peptides was >1,000 μg/ml. The toxicity percentage of peptides at different concentrations is shown in Figure 3.

3.5 | Results of hemolysis assay

The results showed that the hemolytic effects ofdCar12 and rCar12 were less than 10% at the peptide concentration of 5–500 μg/ml. Accordingly, rCar12 had low toxicity at its MIC range (<6%) while Caerin 4.1 was toxic on human RBCs at its MIC range (>20%). Statistical analysis revealed that the hemolytic activity of rCar12 at its MIC range was significantly less than Caerin 4.1 at same concentration ($p < .001$). However, rCar12 had more hemolytic effects compared with dCar12 at the same concentrations. It is clear that rCar12 and dCar12 have less hemolytic activity in comparison with Caerin 4.1. Figure 4 reports the hemolysis percentages of peptides at different concentrations and the results are the mean of three independent experiments.

3.6 | Results of killing time

Killing time of *E. coli* ATCC25922 regarding dCar12, rCar12, and Caerin 4.1 was measured through monitoring CFU counts for 180 min following peptide exposure at 1× MIC. dCar12 did not show any antimicrobial effects against *E. coli*, hence the similarity of its curve to the control curve. It was further observed that rCar12 at 1× MIC showed its antibacterial activity on exponentially growing *E. coli*, within 30 min. This peptide was reduced >3log10 the initial inoculum (approximately 5×10^5 CFU/ml) following 60 min of

incubation. rCar12 peptide showed the maximum effect after 90 min. rCar12 resulted in a more than twofold decrease in the logarithmic growth of *E. coli* after 60 min of incubation, while streptomycin showed same effect within 90 min. In addition, rCar12 at 1× MIC concentration completely killed the initial inoculum within 180 min. Penicillin, on the other hand, was not able to eliminate bacteria during this period (Figure 5).

3.7 | The effect of rCar12 peptide on bacterial morphology and cell wall using FE-SEM

Since the attack on the membrane and bacterial cell wall is one of the main activity mechanisms of AMPs, FE-SEM is a useful approach to studying the effects of AMPs on the bacterial cell surfaces and morphology. In this section, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as negative control or intact cell without peptide treatments. The results showed that rCar12 peptide at 0.5× MIC concentration affected the cell surface of the bacteria. These effects were more intense and stronger against *E. coli*. Two hours after the treatment of *E. coli* cells by rCar12, cell surface roughness and shrinkage were created on the surface of *E. coli*, while *S. aureus* remained intact. After 4 hr of treatment, the amount of cell surface shrinkage increased in *E. coli* cells and a number of pores were formed on the cell surface, at which point, the surface of *S. aureus* cells was wrinkled. After 6 hr of bacterial treatment by rCar12, the pores increased on the cell surface of both bacteria, and certain *E. coli* cells were completely lysed. Also, after 6 hr of treatment, the nucleic acid was released from bacteria. Figure 6 demonstrates the FE-SEM images from bacteria prior to and after treatments by rCar12.

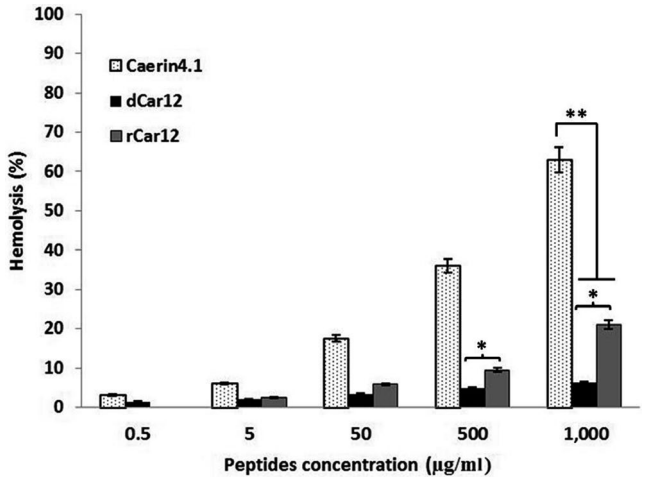

FIGURE 4 This histogram shows the hemolysis percentages of various concentrations of peptides. The results are presented as mean ± standard deviation. Error bars indicate standard deviation. Statistical differences were shown by stars (*$p < .05$; **$p < .001$)

3.8 | Neutralization of endotoxin

Quantitative LAL (Limulus Amebocyte Lysate) test was conducted so as to specify the LPS-binding activity of peptides. The experiment was carried out at the LPS concentrations of 1 EU/ml (0.5 ng/ml) and eight different peptide concentrations (from 0.78 to 100 µg/ml). The results showed that rCar12 could neutralize 51.5% of 1 EU/ml at a concentration of 100 µg/ml, while polymyxin B neutralized 71.5% of 1 EU/ml at this concentration. Furthermore, Caerin 4.1 and dCar12 neutralized 8.9% and 3.8% of 1 EU/ml at a concentration of 100 µg/ml, respectively. The results of LPS neutralization by peptides are presented in Figure 7.

3.9 | DNA-binding assay

If an AMP is attached to the DNA, its electrophoretic mobility is reduced. Therefore, EMSA test is a primary and suitable assay for the evaluation of the binding of peptide

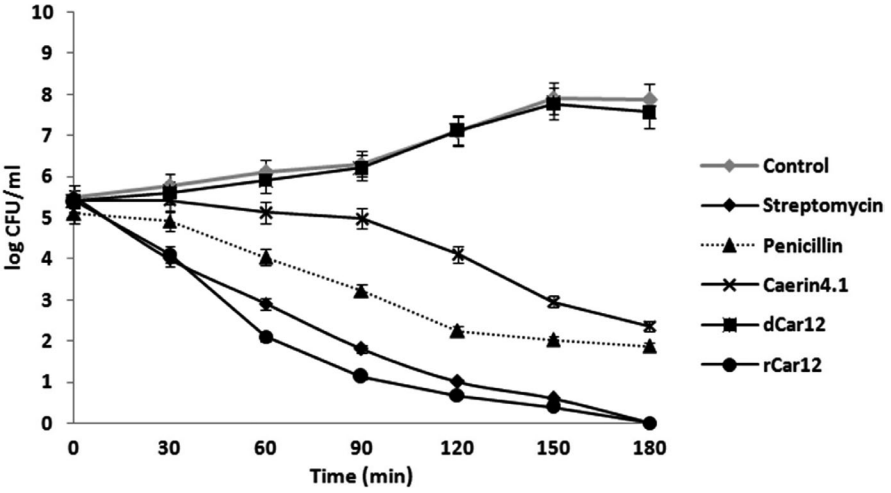

FIGURE 5 This curve shows the killing kinetic of *E. coli* ATCC25922 at 1× MIC concerning peptides and two classic antibiotics. As observed, rCar12 has the most rapid bactericidal activity against *E. coli.* Streptomycin and penicillin were used as antibiotic controls. The control group in this figure is untreated bacterial culture. Error bars indicate standard deviation

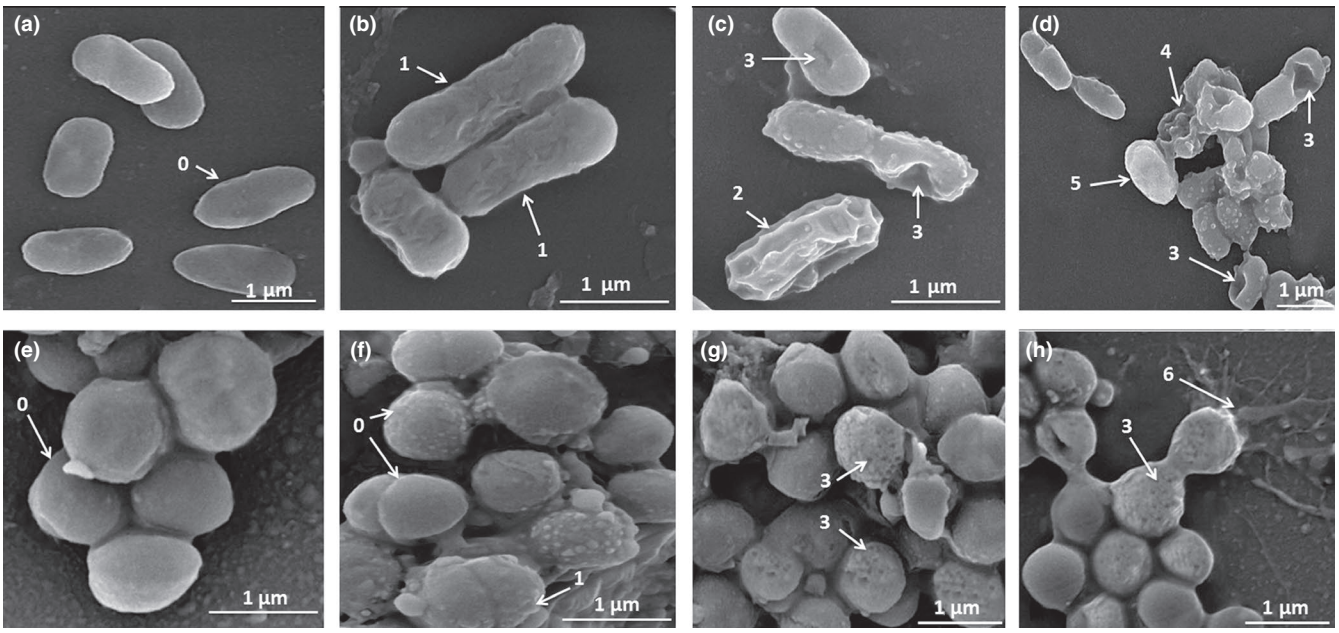

FIGURE 6 The images of FE-SEM (b–d) show *E. coli* following treatment with 0.5× MIC of rCar12 at different intervals (b, 2 hr after treatment, c, 4 hr and d, 6 hr). Figure (a) is a negative control (without peptide treatment). Images (f) to (h) demonstrate *S. aureus* treatment with 0.5× MIC of rCar12 after 2, 4, and 6 hr, respectively. Image (e) is *S. aureus* without any peptide treatment. The tags include (0) intact cells, (1) moderate cell surface shrinkage, (2) severe cell surface wrinkles, (3) cell pores and cell split, (4) full cell lysis, (5) cellular swelling with cell surface roughness, and (6) nucleic acid release

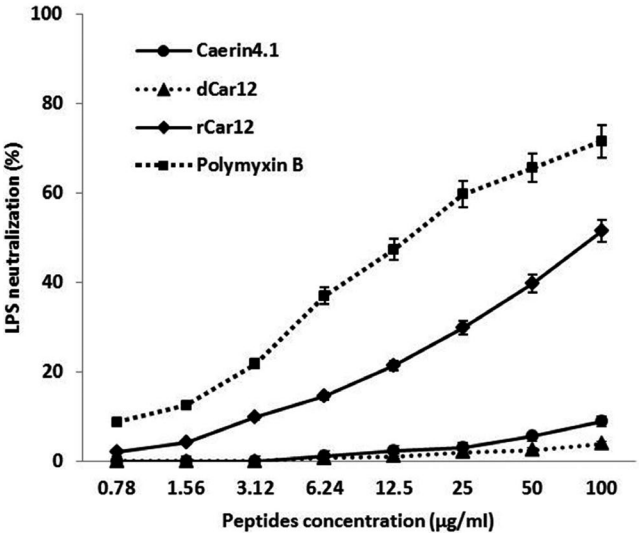

FIGURE 7 This chart shows the percentage of LPS neutralization by peptides for 1 EU/ml LPS standard solution. As is shown, rCar12 has the most LPS inhibition activity

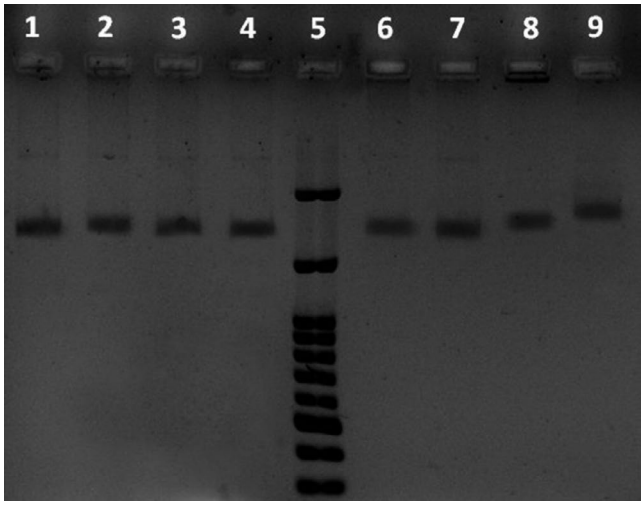

FIGURE 8 Columns 1 and 6 show the negative control. pUC18 without the peptide treatment was used as negative control. Columns 2 to 4 are related to rCar12 peptide treatments at concentrations of 0.25, 0.5, and 1 µg/ml respectively. No. 5 is the DNA size marker. Lines 7 to 9 include the DNA treated with different concentrations of dCar12 peptide (0.25–1 µg/ml, respectively). The gel photograph is displayed in the reverse mode

to DNA. The results showed that the electrophoretic mobility of DNA was not inhibited by rCar12 peptide; therefore, none of rCar12 concentrations was able to even reduce the electrophoretic mobility of pUC18. However, at the concentration of 0.5 and 1 µg/ml, dCar12 peptide was able to reduce the electrophoretic movement of DNA. Consequently, dCar12 had DNA-binding activity, while rCar12 showed a poor DNA-binding activity (Figure 8).

4 | DISCUSSION

AMPs are considered as potential antibiotic candidates. These agents have a wide spectrum of antimicrobial activity and are less likely to cause microbial resistance (Nam et al.,

2014). No detailed study has been done on Caerin 4 family since its identification by Stone et al., in 1996 (Waugh et al., 1993). Moreover, no modified or synthetic peptides have been reported on the basis of Caerin 4 family. In the present research, this peptide was utilized to study the importance of the six primary amino acids of this peptide. Based on our previous statistical studies of the APD3 server G, K, L, and I amino acids are high frequent in AMPs (Madanchi et al., 2018). In this regard, GLWQKI sequence of N-terminal of Caerin 4.1 was selected. This sequence has 50% hydrophobicity, +1 charge, and high frequent amino acids in AMPs such as G, L, W, K, and I. Therefore, it can be said that GLWQKI sequence could have an effective role in the antimicrobial activity of Caerin 4 family.

The predictions of machine learning algorithms and antimicrobial assay showed that GLWQKI sequence does not have antimicrobial properties alone, while in vitro antimicrobial test indicated that when GLWQKI sequence was removed from N-terminal of Caerin 4.1, the recent peptide ($Car_{7\text{-}23}$) lost its antimicrobial properties. However, intact Caerin 4.1 (positive control) had a weak to moderate activity against the bacteria. Based on the results of antimicrobial tests regarding Caerin 4.1 and $Car_{7\text{-}23}$, and since GLWQKI exists in all three members of this family, it can be concluded that this sequence is highly crucial for the antibacterial activity of Caerin 4 family. Results of physicochemical properties prediction showed that parameters such as net charge, hydrophobicity, amphipathicity, peptide length, Mw, GRAVY index, pI, and Boman index were constant and similar in both rCar12 and dCar12. Therefore, these features only depend on the amino acid content of peptides. In the present design, different directions of GLWQKI sequence at dCar12 and rCar12 showed that these features are independent of the amino acid sequence**.**

Furthermore, the results of AMP probability prediction were not significantly different in these two peptides. As a result, it is proposed that the algorithms used in this project and other software related to the prediction of peptide functions, work based on amino acid composition and their type. These algorithms cannot accurately predict the performance of peptides with the same length and amino acid content with different in sequence. The comparison regarding the activity depends more on the secondary structure, as is reported by many researchers regarding the structure and activity of AMPs (Zelezetsky & Tossi, 2006). Our CD studies showed that helices were major structures in rCar12, while dCar12 peptide had a mixed structure. CD results indicated that dCar12 and rCar12 peptides, despite the same amino acid content and length, had different secondary structures. Therefore, it is safe to say that the secondary structure of peptides with same amino acid composition depends on amino acid arrangement in peptides. In addition to GLWQKI sequence alone lacked antimicrobial activity while it is quite similar in amino acid type to rCar12 and dCar12. Therefore, in addition to the amino acid arrangement for peptide function, the length of the peptide is also important. The experimental tests that were performed by Liu et al., showed an increase in length of peptides can enhance the antimicrobial activities and cytotoxic and hemolytic effects (Liu et al., 2007).

Generally, among all the properties studied in dCar12 and rCar12 peptides, only the secondary structure, immunogenicity, and proteolytic digestion pattern were dependent on the arrangement of amino acids. Other physicochemical properties such as net charge, hydrophobicity, GRAVY index, Boman index, and pI were independent on amino acid arrangement in peptides with the same length and residues, and only dependent on the amino acid composition. Our in vitro studies determined that dCar12 and rCar12 peptides were very different concerning antimicrobial activity, toxicity, and mode of actions, while the same regarding all physicochemical properties except for the secondary structure. The antimicrobial assay indicated that rCar12 had a good activity against the selected bacteria, whereas dCar12 showed a weak antimicrobial activity. Also, rCar12 exhibited a more rapid bactericidal effect against *E. coli* compared with Caerin 4.1, streptomycin, and penicillin at 1XMIC concentrations. In line with other studies, the increase in the hydrophobic ratio in the non-polar face of rCar12 increased the symmetry in its hydrophobic and hydrophilic faces, elevating the toxicity and hemolytic effects of these peptides (Boland & Separovic, 2006; Madanchi et al., 2018). Helical wheel projection showed that in dCar12, the hydrophilic face became more discontinuous by Q in the tenth position. Cytotoxicity assay confirmed helical wheel projection results, and it was found that dCar12 had less hemolytic and toxicity effects compared with rCar12 at the same concentrations.

The evaluation of LPS and DNA-binding activities of dCar12 and rCar12 peptides suggests that even with same amino acid compositions, net charge, and other similar properties, not all peptides can bind to LPS or DNA. Therefore, arrangement of peptide residues with same amino acids and similar length can affect the peptides' action mechanism such as LPS- and DNA-binding activities. Despite the identical amino acid content of these two peptides, the secondary structure of these two peptides is different due to the different arrangement of amino acids. It can be suggested that the different secondary structure of dCar12 and rCar12 peptides led to their different functional behavior. As the study by Nam et al., indicated that olfLBP 4N, olfLBP 5A, and olfLBP 6A peptides with same net charge (+4), have different DNA binding activity (Nam et al., 2014).

5 | CONCLUSION

The results showed that rCar12 is a good antibacterial peptide (at an MIC of 3.9–62.5 μg/ml), while $Car_{7\text{-}23}$ did not

have any antimicrobial properties. Cytotoxicity of rCar12 at MICs range was <5%, which is much less than Caerin 4.1. Also, these tests showed that GLWQKI sequence is highly necessary for the antimicrobial activity of Caerin 4 family, while it alone did not show any antimicrobial activity. As a result, rCar12 with reverse mirror repeat has different activity and properties in comparison with dCar12 with directed repeat. The results of this study indicated that certain physicochemical parameters such as net charge, hydrophobicity, amphipathicity, peptide length, Mw, GRAVY index, pI, and Boman index only depend on the amino acid content of peptides while the different arrangement of amino acids in peptides with the same length and amino acids content can alter some of other properties such as secondary structure, immunogenicity, and proteolytic digestion map of proteins. In conclusion, in peptides with the same amino acid content and length but dissimilar residues arrangement, structure and function of peptides are different.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Boland, M. P., & Separovic, F. (2006). Membrane interactions of antimicrobial peptides from Australian tree frogs. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, *1758*(9), 1178–1183. https://doi.org/10.1016/j.bbamem.2006.02.010

Boman, H. G. (2003). Antibacterial peptides: Basic facts and emerging concepts. *Journal of Internal Medicine*, *254*(3), 197–215. https://doi.org/10.1046/j.1365-2796.2003.01228.x

Gaspar, D., Veiga, A. S., & Castanho, M. A. (2013). From antimicrobial to anticancer peptides. A review. *Frontiers in Microbiology*, *4*, 294.

Ghosh, A., Datta, A., Jana, J., Kar, R. K., Chatterjee, C., Chatterjee, S., & Bhunia, A. (2014). Sequence context induced antimicrobial activity: Insight into lipopolysaccharide permeabilization. *Molecular BioSystems*, *10*(6), 1596–1612. https://doi.org/10.1039/C4MB00111G

Gupta, S., Bhatia, G., Sharma, A., & Saxena, S. (2018). Host defense peptides: An insight into the antimicrobial world. *Journal of Oral and Maxillofacial Pathology*, *22*(2), 239. https://doi.org/10.4103/jomfp.JOMFP_113_16

Jiang, Z., Vasil, A., Vasil, M., & Hodges, R. (2014). "Specificity determinants" improve therapeutic indices of two antimicrobial peptides piscidin 1 and dermaseptin S4 against the gram-negative pathogens *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Pharmaceuticals*, *7*(4), 366–391. https://doi.org/10.3390/ph7040366

Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of cell viability by the MTT assay. *Cold Spring Harbor Protocols*, *2018*(6), pdb-prot095505. https://doi.org/10.1101/pdb.prot095505

Kyte, J., & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, *157*(1), 105–132. https://doi.org/10.1016/0022-2836(82)90515-0

Liu, Z., Brady, A., Young, A., Rasimick, B., Chen, K., Zhou, C., & Kallenbach, N. R. (2007). Length effects in antimicrobial peptides of the (RW) n series. *Antimicrobial Agents and Chemotherapy*, *51*(2), 597–603. https://doi.org/10.1128/AAC.00828-06

Maccari, G., Nifosi, R., & Di Luca, M. (2013). Rational development of antimicrobial peptides for therapeutic use: Design and production of highly active compounds. In Méndez-Vilas A. (Ed.), *Microbial pathogens and strategies for combating them: Science, technology and education*, pp. 1265–1277. Badajoz, Spain: Formatex Research Center.

Madanchi, H., Akbari, S., Shabani, A. A., Sardari, S., Farmahini Farahani, Y., Ghavami, G., & Ebrahimi Kiasari, R. (2018). Alignment-based design and synthesis of new antimicrobial Aurein-derived peptides with improved activity against Gram-negative bacteria and evaluation of their toxicity on human cells. *Drug Development Research*, *80*(1), 162–170. https://doi.org/10.1002/ddr.21503

Madanchi, H., Khalaj, V., Jang, S., Shabani, A. A., Ebrahimi Kiasari, R., Seyed Mousavi, S. J., … Sardari, S. (2019). AurH1: A new heptapeptide derived from Aurein1. 2 antimicrobial peptide with specific and exclusive fungicidal activity. *Journal of Peptide Science*, *25*(7), e3175.

Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial peptides: An emerging category of therapeutic agents. *Frontiers in Cellular and Infection Microbiology*, *6*, 194. https://doi.org/10.3389/fcimb.2016.00194

Manzini, M. C., Perez, K. R., Riske, K. A., Bozelli, J. C., Santos, T. L., da Silva, M. A., … Cuccovia, I. M. (2014). Peptide: Lipid ratio and membrane surface charge determine the mechanism of action of the antimicrobial peptide BP100. Conformational and functional studies. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, *1838*(7), 1985–1999. https://doi.org/10.1016/j.bbamem.2014.04.004

Nam, B.-H., Moon, J.-Y., Park, E.-H., Kim, Y.-O., Kim, D.-G., Kong, H., … Seo, J.-K. (2014). Antimicrobial activity of peptides derived from olive flounder lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI). *Marine Drugs*, *12*(10), 5240–5257. https://doi.org/10.3390/md12105240

Osorio, D., Rondón-Villarrea, P., & Torres, R. (2015). Peptides: A package for data mining of antimicrobial peptides. *R Journal*, *7*(1), 4. https://doi.org/10.32614/RJ-2015-001

Peters, B. M., Shirtliff, M. E., & Jabra-Rizk, M. A. (2010). Antimicrobial peptides: Primeval molecules or future drugs? *PLoS Path*, *6*(10), e1001067. https://doi.org/10.1371/journal.ppat.1001067

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health*, *109*(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000030

Szabo, D., Ostorhazi, E., Binas, A., Rozgonyi, F., Kocsis, B., Cassone, M., … Otvos, L. (2010). The designer proline-rich antibacterial peptide A3-APO is effective against systemic *Escherichia coli* infections in different mouse models. *International Journal of Antimicrobial Agents*, *35*(4), 357–361. https://doi.org/10.1016/j.ijantimicag.2009.10.015

Tavares, L. S., Silva, C. D. S. F. D., Souza, V. C., Silva, V. L. D., Diniz, C. G., & Santos, M. D. O. (2013). Strategies and molecular tools to fight antimicrobial resistance: Resistome, transcriptome, and antimicrobial peptides. *Frontiers in Microbiology*, *4*, 412. https://doi.org/10.3389/fmicb.2013.00412

Waghu, F. H., Barai, R. S., Gurung, P., & Idicula-Thomas, S. (2016). CAMPR3: A database on sequences, structures and signatures of antimicrobial peptides. *Nucleic Acids Research*, *44*(D1), 1094–1097.

Wang, G., Li, X., & Wang, Z. (2015). APD3: The antimicrobial peptide database as a tool for research and education. *Nucleic Acids Research*, *44*(D1), 1087–1093.

Wang, S., Zeng, X., Yang, Q., & Qiao, S. (2016). Antimicrobial peptides as potential alternatives to antibiotics in food animal industry. *International Journal of Molecular Sciences*, *17*(5), 603. https://doi.org/10.3390/ijms17050603

Waugh, R. J., Stone, D. J. M., Bowie, J. H., Wallace, J. C., & Tyler, M. J. (1993). Peptides from Australian frogs. The structures of the caerins and caeridins from *Litoria gilleni*. *Journal of Chemical Research*, (4).

Wiedemann, C., Bellstedt, P., & Görlach, M. (2013). CAPITO—a web server-based analysis and plotting tool for circular dichroism data. *Bioinformatics*, *29*(14), 1750–1757. https://doi.org/10.1093/bioinformatics/btt278

Wu, X., Wang, Z., Li, X., Fan, Y., He, G., Wan, Y., … Zhang, H. (2014). In vitro and in vivo activities of antimicrobial peptides developed using an amino acid-based activity prediction method. *Antimicrobial Agents and Chemotherapy*, *58*(9), 5342–5349.

Zelezetsky, I., & Tossi, A. (2006). Alpha-helical antimicrobial peptides—Using a sequence template to guide structure–activity relationship studies. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, *1758*(9), 1436–1449. https://doi.org/10.1016/j.bbamem.2006.03.021

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