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Original Article

Controversy Between *In Vitro* Biological Activities of a Novel Designed Antimicrobial Peptide and Its *In Silico* Predicted Activities

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HIGHLIGHTS

- A novel 10 residues anti-microbial peptide was designed using bioinformatics tools.
- *In vitro* analysis showed that this novel AMP did not have efficient antimicrobial activities.
- Stringencies of bioinformatics criteria thresholds setting may result in better design.

ABSTRACT

Due to their unique mechanisms of action, antimicrobial peptides (AMPs) are promising candidates to combat different infectious diseases. They usually non-specifically interact with the bacterial cell membrane, create pores in their membrane and increase its permeability which causes the death of pathogens. In the design and development of AMPs, *in silico* strategies have been developed to enhance the function and activity of natural peptides. In this study, *in silico* approaches were used to develop a novel AMP with several extra bioactivities. Then, the designed AMP were analyzed through computational methods by *in vitro* experiments. Bioinformatics research revealed a 10-amino-acid peptide (LVSARIRCPK) having antibacterial, anti-biofilm, antiviral, antifungal, and anti-inflammatory effects. However, only the antiviral capabilities of the peptide were validated in the experimental analysis of antibacterial, antifungal, and antiviral activities. This data suggests that; while bioinformatics approaches have greatly advanced in recent years, more optimization work has to be done in order to attain high accuracy and minimize mistakes.

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Introduction

Bacteria seem to become progressively resistant to common antibiotics, resulting in the treatment failure of many antibiotics, even those that have just been

launched (Alsaggar *et al.*, 2022). Therefore, antimicrobial resistance (AMR) is a serious worldwide health concern in the 21st century, which it is estimated to kill tens of millions of people each year by 2050 (Cornaglia, 2009; Shankar, 2016; Ting *et al.*, 2020; Alsaggar *et al.*, 2022). Inadequate patient adherence to treatment standards, overmedication, and misuse of antimicrobial agents in healthcare services, as well as antibiotic abuse in the agricultural industry are all factors contributing to the global rise in antibiotic resistance (Papo and Shai, 2005; Patrulea *et al.*, 2019; Magana *et al.*, 2020; Oliveira *et al.*, 2020). Addressing AMR's fundamental effects and mechanisms at the microbe, person, and population levels, antibacterial management in the healthcare and agricultural domains, and the invention and development of novel types of antimicrobial treatment are all necessary components in combating AMR (Ting *et al.*, 2020).

Antimicrobial peptides (AMPs) are widely found in the nature and have been detected in a large number of species as a portion of the innate immune response to pathogenic microorganisms (such as bacteria, fungi, viruses, etc.). They are typically called host defense peptides (HDPs) (Cipcigan *et al.*, 2018; Koo and Seo, 2019; Patrulea *et al.*, 2020; Ting *et al.*, 2020; Alsaggar *et al.*, 2022). Innate AMPs are structurally and functionally diverse gene-encoded compounds with a vast spectrum of actions against various diseases in many species. AMPs, despite their wide range of physicochemical and structural characteristics, sources and mechanisms of function, exhibit several similar traits. Surprisingly, they are mostly small molecules with less than 100 amino acid residues, a positive net charge that ranges between +2 to +11, and a high concentration of hydrophobic amino acids (naturally 50%) (Huang *et al.*, 2010; Fry, 2018; Chegini *et al.*, 2017; Mookherjee *et al.*, 2020; Moretta *et al.*, 2021). AMPs have the potential to combat microorganisms that are resistant to common antibiotic agents. By interacting with and damaging bacterial cell membranes, these compounds provide widespread antimicrobial activity against harmful pathogens. Furthermore, they can block the synthesis of key macromolecules in vital biosynthetic pathways, such as DNA and proteins (Bahar and Ren, 2013; Kumar *et al.*, 2018).

Despite the abundance of antimicrobial peptides in nature, finding and extracting them from natural sources is expensive and time-consuming. Furthermore, they have drawbacks that make their use as antimicrobial medicines challenging. Only a few examples reported physical/chemical instability, decreased action in high salinity, enzymes digestibility in the human cytoplasm, limited activities compared to conventional antibiotics, and finally, risk of toxicity to eukaryotic organisms (Liu *et al.*, 2018; Cardoso *et al.*, 2020; Neff *et al.*, 2020). As a consequence, the design and identification of antimicrobial peptides, mimicking the behavior and function of natural AMPs while not having their shortcomings, by computers and artificial intelligence have recently attracted much attention. A number of databases have been created that contain applicable and useful information about natural and synthetic peptides. There are also varieties of servers available that can predict the different biological properties of peptides. The computational design can help scientists to save time and money, although it is unclear how accurate these servers and databases are at forecasting (Fathi *et al.*, 2022). In the present study, we designed a new antimicrobial peptide with free online servers available and then, the designed AMP was experimentally tested regarding the antibacterial, antifungal, and antiviral properties in the laboratory. We investigated the accuracy of these servers and current algorithms by comparing the findings of bioinformatics and computer analysis with the results of experimental tests.

Materials and Methods

Design of the novel peptide with desired antimicrobial and cell penetrating activities

A four-step workflow was employed to design new peptide. The initial step was to use the Uniprot database (https://beta.uniprot.org/) (The UniProt 2021) to find a protein sequence. Regenerating islet-derived protein 3-alpha (REG3A) (UniProtKB-Q06141 (REG3A HUMAN) was chosen as the protein model, because that is a bactericidal C-type lectin. Then, the REG3A sequence was analyzed in the second stage utilizing four free web servers:

a) $CAMP_{R3}$, an online antimicrobial peptide predictor web server, (http://www.camp3.bicnirrh.res.in/index.php) (Waghu and Idicula-Thomas, 2020).
b) dPABBs, an online antibiofilm peptide predictor web server, (https://ab-openlab.csir.res.in/abp/antibiofilm/index.php) (Sharma *et al.*, 2016).
c) Met-iAVP, an online antiviral peptide predictor web server, (http://codes.bio/meta-iavp/) (Schaduangrat *et al.*, 2019),
d) CellPPD, an online webserver to predict peptides with cell penetrating activity (https://webs.iiitd.edu.in/raghava/cellppd/index.html) (Gautam *et al.*, 2015).

The peptide with all of these properties antimicrobial, antibiofilm, antiviral, and cell penetrating was chosen and characterized using a variety of online servers grounded on its anticipated physicochemical

characteristics, including positive net charge, molecular weight, theoretical pI, instability index, hydrophobicity, GRAVY (grand average hydropathy), hydrophobic moment, and aliphatic index. Except for hydrophobic moment and hydrophobicity, which were examined by HeliQuest (http://heliquest.ipmc.cnrs.fr) (Gautier *et al.*, 2008), these parameters were evaluated using the ExPASy tool, ProtParam. The iAMPpred (http://cabgrid.res.in:8080/amppred/index.html) (Meher *et al.*, 2017) and AIPpred (http://thegleelab.org/AIPpred/index.html) (Manavalan *et al.*, 2018) tools were used to indicate antifungal and anti-inflammatory possibilities. A few biological features were also anticipated. The antigenic prediction tool (http://imed.med.ucm.es/Tools/antigenic.pl) (Molero-Abraham *et al.*, 2015) was used to compute the allergic potential, while HemoPI (https://webs.iiitd.edu.in/raghava/hemopi/index.php) (Chaudhary *et al.*, 2016) and ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) (Gupta *et al.*, 2013) were used to assess the hemolytic and toxic prospects, respectively. Antimicrobial peptides' helicity has been a key property for successful membrane penetration and subsequently, pore formation. As a result, in the fourth phase of the workflow, the three-dimensional structure of the peptide was simulated using the publicly accessible PEP-FOLD web server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEPFOLD3/) (Lamiable *et al.*, 2016). HeliQuest was used to estimate wheel projection (http://heliquest.ipmc.cnrs.fr) (Gautier *et al.*, 2008). Ultimately, PHD-prabi was used to forecast the peptide's secondary structure (https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_phd.html) (Deléage and Roux, 1987).

There are four distinct algorithms on the $CAMP_{R3}$ webserver to predict antimicrobial regions within protein sequences: support vector machines (SVM), random forests (RF), artificial neural network (ANN), and discriminant analysis (DA). All four prediction algorithms were applied to design a new antimicrobial peptide, while the selected prediction methods for antibiofilm and cell penetrating peptide were SVM- and SVM+modif-based methods, respectively. The chosen threshold for all properties was 0.5.

Phylogenetic analysis

The ADP3 database (one of the most comprehensive antimicrobial peptide databases) (https://aps.unmc.edu/) (Wang *et al.*, 2015) was used to align the designed peptide with others on this server to see how similar it was to the existing sequences of antimicrobial peptides. Then, 10 sequences with the highest similarity to the new peptide were extracted. They were re-aligned by the Clustal Omega online server from the EMBL-EBI online site (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira *et al.*, 2022), and finally, a phylogenetic tree was drawn.

Peptide synthesis

The designed peptide was synthesized by GenScript Inc (USA). Reversed-phase high liquid chromatography (HPLC) was utilized to purity and verify the peptide. The molecular weight of the peptide was also confirmed using LC/Mass (6410 QQQ, Agilent, USA). The peptide was lyophilized and kept at -80 °C. Then, the peptide solution was prepared in ultrapure water and kept at -20°C in 10 $mg.mL^{-1}$ stock solutions.

Microorganism strains

Escherichia coli (PTCC 1276), *Klebsiella Oxytoca* (PTCC 1402), *Streptococcus Pyogenes* (PTCC 1522), *Staphylococcus Aureus* (PTCC 1337), *Candida Albicans* (PTCC 5022), *Human papillomavirus* (HPV), *Hepatitis B* (HBV) and *Severe acute respiratory syndrome (SARS) -associated coronavirus 2 (SARS-CoV2)* (patient sample) were studied at Janat Medical and Clinical Diagnosis Laboratory (Shahed Shahr– Shahriar, Iran).

Antibacterial activity assay

Antibacterial activity was assessed using micro dilution method and time-kill test (Jorgensen and Ferraro, 2009; Balouiri *et al.*, 2016), with slight modification. In brief, for the micro dilution procedure, an initial 100 µL of Muller-Hinton broth medium containing $CaCl_2$ and $MgCl_2$ (target concentration of calcium of 20 $\mu g.mL^{-1}$ and magnesium of 10 $\mu g.mL^{-1}$) was added to each well of a 96-well microplate. The peptide was initially provided at a concentration of 1 $mg.mL^{-1}$. In separate wells in triple sets, serial dilutions of the peptide and polymyxin as positive control (0.48-500 $\mu g.mL^{-1}$) were prepared. Each well received 10 µL of a 1:20 dilution of 0.5 McFarland suspension (1×10^8 $CFU.mL^{-1}$), containing 5×10^6 $CFU.mL^{-1}$. The bacteria used in this study included *Escherichia coli* (PTCC 1276), *Klebsiella Oxytoca* (PTCC 1402), *Streptococcus Pyogenes* (PTCC 1522), and *Staphylococcus Aureus* (PTCC 1337). The plates were incubated for 24 hours at 35 ± 2 °C in ambient air and then, the growth of bacteria was assessed.

On the other test, for the time-kill test, 100 µL of the stock bacteria (-70 °C) were transferred to 2 mL of the LB Broth Miller medium and cultured for 24 hours. Then, 20 µL of the bacteria were re-cultured in Mueller Hinton Agar (MHA) medium. After 24 hours of incubation, the growing colonies were counted and used as negative control. Next, two 100 µL samples of the peptide (with a final concentration of the peptide of 1

mg.mL^{-1}) were mixed in separated micro tubes with 100 µL of the activated bacteria (5 × 10^5 CFU.mL^{-1}) and incubated in a shaking incubator for two time periods of 5 and 10 minutes, respectively. Then, 20 µL from each sample was cultured overnight in MHA medium. Finally, the number of colonies was count.

Antifungal activity assay

The Antifungal activity of the peptide was assessed using time-kill test (Balouiri, Sadiki et al. 2016) in a similar way to the evaluation of the antibacterial activity at Janat Medical and Clinical Diagnosis Laboratory (Shahed Shahr-Shahriar, Iran). *C. albicans* were cultured overnight on Sabouraud-dextrose-agar (SDA) at 28°C. Then, the cells were washed three times and diluted in PBS 0.1 M, pH 7.4. Next, three distinct samples of 100 μL of a suspension containing 1 × 10^6 fungal colony-forming units (CFU).mL^{-1} were incubated at 28°C for 5, 10 and 15 min with the peptide at different concentrations (0.1 mg.mL^{-1} to 1 mg.mL^{-1}). The antifungal activity was measured by culture serial dilutions of mixtures plated onto SDA plates for 48 h at 28°C, and then, the number of visible colonies (CFU) were counted. The control sample was the cells incubated in the same buffer under identical conditions but without peptides (100% survival of *Candida*). Nystatin was used as positive control.

Antiviral activity assay

The antiviral activity of the peptide was assessed by the real time reverse transcription polymerase chain reaction (real time RT-PCR) using the High PapillomaStrip kit (Oasis Diagnostics, USA), HBV Real-TM Qual (Sacace Biotechnologies, Italy) and COVID-19 One-Step RT-PCR (Pishtazteb.co, Iran) by Rotor Gene 6000 Real Time PCR Machine (Corbett Life Science, Australia). The High PapillomaStrip kit uses reverse hybridization to detect and identify 19 medium-high-risk genital HPVs (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, and 82) in DNA samples taken from cervico-uterine smear biopsies. The HBV Real-TM Qual, on the other hand, uses dual-color detection to detect the Hepatitis B virus qualitatively in human plasma while also detecting an HBV-specific Internal Control (IC). COVID-19 One-Step RT-PCR is based on a dual-target gene approach that targets both conserved RdRp region genomic sequences and nucleocapsid protein N.

Human papilloma virus –HPV (a non-enveloped DNA virus), Hepatitis-B virus -HBV (a double-stranded circular DNA virus) and severe acute respiratory syndrome coronavirus 2 (SARS CoV-2, an RNA virus) were studied at Janat Medical and Clinical Diagnosis Laboratory (Shahed Shahr – Shahriar, Iran). SARS CoV-2 had collected from hospitalized patients (Baqiyatallah Hospital, Tehran, Iran).

Hemolytic activity assay

Hemolytic activity was measured by the method established by (Kim *et al.*, 2014). Three milliliters of fresh red blood cells (RBCs) were washed with phosphate buffered saline (pH 7.4) until the supernatant color turned transparent. Phosphate buffered saline, pH 7.4, was used to suspend the washed RBCs at a final volume of 20 mL. Then, 10 µL of peptide samples were serially diluted in phosphate-buffered saline. Next, the peptide solutions (ranging from 0.031 to 1 mg.mL^{-1}) were mixed with 190 µL of cell suspension in micro-centrifuge tubes. The tubes were gently mixed before being incubated at 37 °C for 30 minutes and then centrifuged at 4000 ×g for 5 minutes. Eventually, 100 µL of supernatant was diluted to 1mL in phosphate buffered saline, and absorbance at 567 nm was recorded to track hemoglobin release. RBC membrane injury was evidenced by the release of hemoglobin. In phosphate buffered saline and 0.2% Triton X-100, the negative control with no hemolysis and the positive control with 100% hemolysis were identified, respectively. The subsequent equation was used to compute the percentage of hemolysis:

$$\text{Hemolysis (\%)} = \frac{(As - A0)}{(A100 - A0)} \times 100$$

where A_s denotes the sample absorbance, A_{100} means the absorbance of totally-lysed RBCs in presence of 0.2 % Triton X-100, and A_0 represents the absorbance in the absence of hemolysis.

Results and Discussion

Design of novel antimicrobial peptide

In this study, REG3A protein was considered as template for AMP design, a bactericidal C-type lectin that promotes bacterial death by connecting to surface-exposed carbohydrate moieties of peptidoglycan and tunes keratinocyte proliferation and differentiation following skin damage through stimulation of the EXTL3-PI3K-AKT signaling pathway. It has been proposed to perform a favorable effect in wound healing regulation (Cash Heather *et al.*, 2006). This protein was selected to design a novel antimicrobial peptide. Since the short bioactive peptides are more preferred, consequently, just peptides with 10 amino acid residues were selected in the present study.

One peptide sequence, LPSARIRCPK, was extracted to be active against viruses, biofilms, bacteria, and fungi, as well as having cell-penetrating characteristics, following an extensive evaluation. However, to reduce

the hemolytic potency, proline in the second position of the sequence was substituted with Valine; so the final sequence of the putative peptide was L**V**SARIRCPK. It was predicted to be an antimicrobial peptide based on RF, ANN, and DA algorithms. However, when the antimicrobial activity was analyzed by the SVM algorithm, it was not well approved as an antimicrobial peptide. In other words, antibacterial potential was 52% and 50% based on RF and DA algorithms, respectively (Table 1).

Table 1. The results of AMP prediction with different methods for LVSARIRCPK sequence

The type of algorithm	AMP Probability
Support vector machine (SVM) classifier	0.063 (NAMP)
Random forest classifier	0.522 (AMP)
Artificial neural network (ANN) classifier	AMP
Discriminant analysis classifier	0.504 (AMP)

The results obtained from the analyses of anti-biofilm, antifungal, antiviral, anti-inflammatory, and cell penetrating activities are listed in Table 2. The peptide's anti-biofilm activity, according to dPABBs sever, is most likely about 87%, while the antifungal, antiviral, and anti-inflammatory potentials were 66%, 98%, and 51%, respectively.

Whilst various AMPs have been found to be of natural origin, their use has been limited due to drawbacks, including low selectivity, inadequate stability, and the challenge of isolation. The therapeutic activities of AMPs have been effectively improved by generating small artificial peptides (Ramezanzadeh *et al*., 2021). There are several databases and servers used to design and predict novel peptides. These web servers typically employ physicochemical parameters, net positive charge, hydrophobicity, the peptide's three-dimensional structure and some other common properties of peptides to forecast their capacities and potential functions (Le *et al*., 2015).

Given the important role of these servers and online tools in peptide design in recent years, many researchers have used these online servers to design new antimicrobial peptides based on the *de novo* method. For instance, Souza and colleagues designed two new peptides using free online servers and examined their function against bacteria and fungi. The results showed that although they did not have strong antimicrobial properties, the designed peptides caused damage to the membrane and bacteria cell wall and increased the generation of reactive oxygen species (ROS) in bacteria. In addition, the peptides reduced the biofilm formation, but were not strong enough to completely prevent this event. One of their designed peptides was not resistant to proteolysis, but the other was resistant to pepsin while sensitive to pancreatin. Finally, both designed peptides did not have hemolytic properties (Souza *et al.*, 2020). Klubthawee *et al.* also used physicochemical and structural characteristics together with rational engineering to create a hybrid peptide with antibacterial activities for a wide spectrum of bacteria while having less hemolytic power than parental peptides (Klubthawee *et al*., 2020).

Most of natural AMPs have amphipathic and cationic structure. Strong antibacterial action requires these certain structural characteristics. According to extensive research, at least 30% of the amino acids in the peptide structure must be cationic in order to provide the highest therapeutic index. However, if this positive charge in the peptide structure is greater than 34%, it may increase the peptide's hemolytic activity and makes it challenging to employ as a therapeutic candidate. Our newly designed peptide has a net positive charge of 3, as stated in Table 3.

Table 2. The results of anti-biofilm, Cell penetrating, anti-inflammatory, antiviral and antifungal prediction for LVSARIRCPK sequence

Sequence	CellPPD	Biofilm inhibitor	Anti-inflammatory	Antiviral	Antifungal	length
LVSARIRCPK	5.17 (CPP)	0.78	0.514	0.988	0.66	10

Table 3. The physicochemical properties of the selected peptide

Sequence	Molecular weight (Da)	Theoretical PI	Charge	Aliphatic Index	Instability index	Hydrophobicity	GRAVY	Hydrophobic Moment
LVSARIRCPK	1142.43	10.86	3	117.00	28.6(stable)	0.424	0.150	0.361

Then, according to the length of the peptide (10 residues), it consists of the appropriate cationic proportion of a therapeutic peptide (30%). Furthermore, a suitable peptide for transport through the physiological media, as well as, contact with bacterial membranes must be amphipathic. Accordingly, the hydrophobicity (H), hydrophobic moment (<H>), and the grand average hydropathy (GRAVY) were determined by the HeliQuest and ExPASy tools for the analysis and evaluation of the desired peptide's hydrophobicity and amphipathicity (Yeaman and Yount, 2003). The new peptide's hydrophobicity, hydrophobic moment, and grand average hydropathy (GRAVY) were 0.424, 0.361, and 0.150, respectively. Given that high GRAVY values represent hydrophobic peptides, and negative values imply hydrophilic peptides, the putative peptide was hydrophobic.

In addition, the bioinformatics analyses revealed that this peptide is not toxic and cannot cause allergic reactions. When the hemolytic potency of the peptide was evaluated with the HemoPI, it had a hemolytic potential of 21%, indicating that the chosen peptide is unlikely to be hemolytic (Table 4).

Table 4. The result of the calculation allergic, hemolytic and toxic potentials of the peptide

Sequence	PROB Score	antigenic prediction	Toxic potential
LVSARIRCPK	0.21	0	Non-toxin (-1.15)

PepFold results for structural and conformational information revealed that the best model was based on the nethermost sOPEP energy and the maximum Tm values as determined by this online server (Fig. 1A). The secondary structure prediction suggests adopting a conformation with a 30% extended strand and a 70% random coil. Since the α-helical configuration and amphipathicity of cationic AMPs are two significant characteristics, Helical Wheel Projections from HeliQuest were used to assess the α-helix constitution and the situation of hydrophobic and hydrophilic amino acid residues in the peptide. The helical wheel diagram is a visualization showing how α-helices in peptides and proteins behave (Pedron *et al*., 2017). The helical wheel image also confirmed the peptide's amphipathicity (Fig. 1B). The peptide exhibited theoretical molecular mass of 1142.43 Da, which was confirmed by mass spectrometry in experiment.

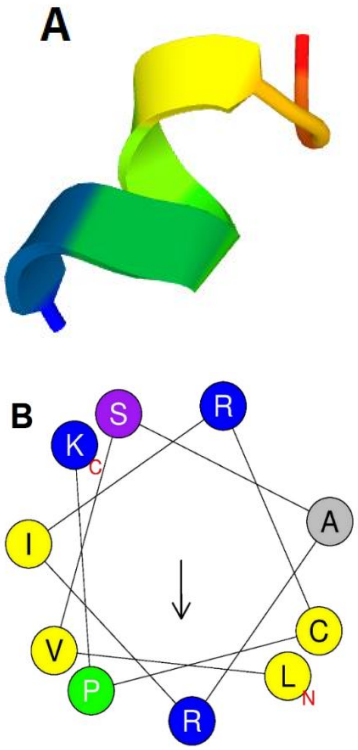

Figure 1. (A) 3D structure of the selected peptide using PEP-FOLD online tool. (B) Utilizing the HeliQuest web tool to create a helical wheel diagram of the specified peptide; at the bottom of the wheel, positively charged amino acid residues are depicted in blue, whereas hydrophobic residues are indicated in yellow. Alanine (A) is displayed in gray, whereas proline (P) is green. The arrow indicates the helical hydrophobic moment.

Phylogenetic analysis

The peptide had the most homology with Hyposin-H2 (ADP ID: AP00903) and Hyposin-H1 (ADP ID: AP00902) isolated from the skin of a frog (Thompson, Bjourson et al. 2007), with a 50 percent similarity to both peptides according the ADP3 database analysis. Both of these peptides have a wide range antibacterial activity against gram-positive and gram-negative bacteria. Another peptide with a 41.67 percent similarity was Balteatide (ADP ID: AP02411) that was a natural AMP obtained from a frog and active against the *C. albicans* and weakly against the gram-negative bacterium *E. coli* (Ge *et al*., 2014). Following that, Hyposin-H3 (ADP ID: 00904), Atr-AMP1 (ADP ID: 03130), SyCPA 116 (ADP ID: 03314), Scolopendin 2 (ADP ID: 02447), Nigroain-B1 (ADP ID: 01939), Temporin-TP2 (ADP ID: 02459), and Peptide 8361 2 were the most similar peptides to the newly designed peptide (ADP ID: 03097). Our peptide was 40% similar to Hyposin-H3 and Art-AMP, while SyCPA and Scolopendin 2 had 36.36% and 35.29% homology, respectively. Nigroain-B1 had a 35% similarity rate,

while the last two peptides had a 33.33% similarity proportion (Figure 2). Given that all of these sequences have been identified as antimicrobial peptides with respectable activities on a wide range of bacteria, from gram-positive to gram-negative, the developed peptide was expected to have the predicted characteristics and antimicrobial activity. Contrary to predictions, no appreciable antibacterial action was observed in practical experiments.

Antibacterial activity

The peptide's antibacterial activity was evaluated using two different method, microdilution method and time kill test, against both gram-positive and gram-negative bacteria. The results of microdilution test to find the minimum inhibitory concentration showed that this peptide neither had effective potency to inhibit the growth of gram-positive, nor gram-negative bacteria, because at the maximum concentration of the peptide (500 $\mu g.mL^{-1}$) the bacteria have still grown.

In time-kill test, the difference between colony count in the control samples and the samples treated with the synthetic peptide (1 $mg.mL^{-1}$) demonstrated the bactericidal activity of peptides on studied pathogens. As shown in Table 5, *S. pyogenes* colony populations were 7.3×10^4 CFU/m^3 (without the presence of peptide). After 5 and 10 minutes of exposure to the peptide, the number of colonies decreased to 6.9×10^4 and 5.7×10^4 CFU/m^3, respectively. The reductions for *K. Oxytoca* were from 9.2×10^4 CFU/m^3 (without the peptide) to 8.87×10^4 CFU/m^3 and 7.54×10^4 CFU/m^3 after 5 and 10 minutes of exposure to the peptide, respectively. *E. coli* showed 10.9% and 16.75% decrease in the number of its colonies after 5 and 10 minutes of exposure to the peptide, from 6.15×10^4 to 5.48×10^4 and 5.12×10^4 CFU/m^3, respectively. Finally, the reduction percentages for *S. aureus* were 5.47% and 16.52%, from 9.08×10^4 to 8.62×10^4 and 7.58×10^4 CFU/m^3 after 5 and 10 minutes of exposure to the peptide, respectively.

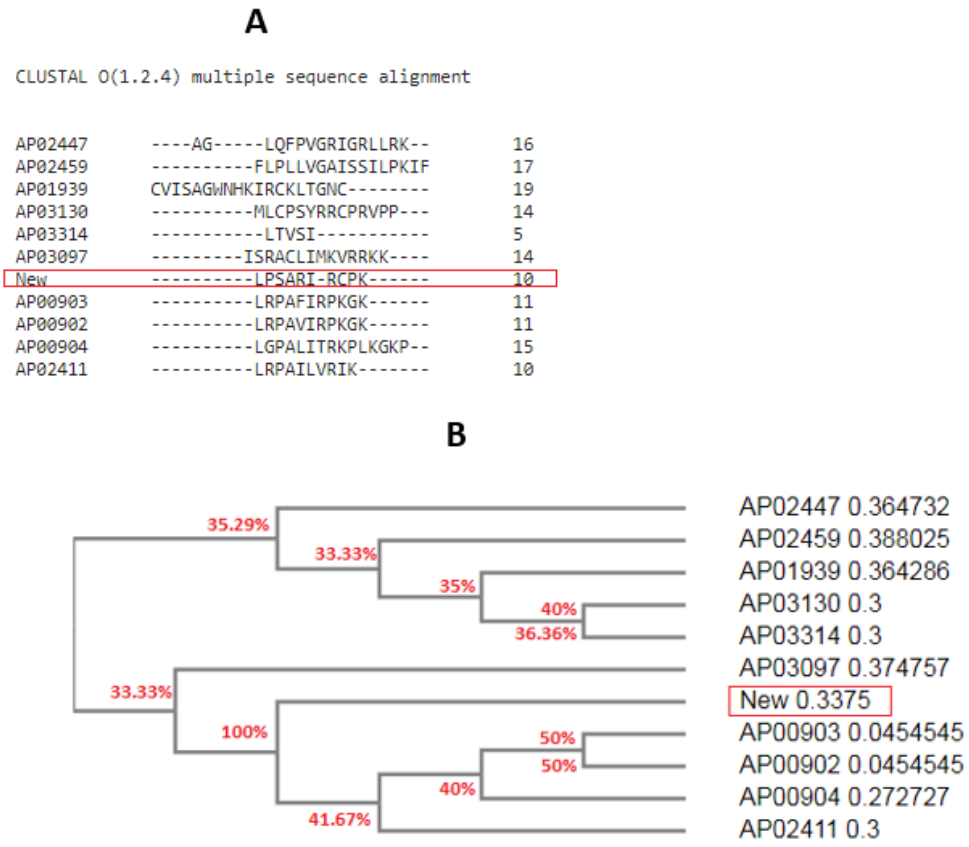

Figure 2. (A) BLAST results of 10 AMP sequences with the novel designed peptide. Peptides are indicated by ADP ID. The new peptide are shown by "new" and is marked by a red box around. (B) Phylogenetic tree. As shown in the figure, the designed peptide has the greatest similarity to Hyposin family peptides. Hyposin H1 and Hyposin H2 (ADP ID: AP00902 and AP003) have 50% homology with the new designed peptide.

Table 5. The results of antibacterial and antifungal assays

Test Microorganism	Contact time (min)	Run type	CFU/m^3	Percent remain parallel to positive control
Streptococcus Pyogenes (PTCC:1522)	0	Positive Control	7.3 ×10^4	100%
	5	Contact	6.9 ×10^4	94.52%
	10	Contact	5.7× 10^4	78.08%
Staphylococcus Aureus (PTCC:1337/29737)	0	Positive Control	9.08 ×10^4	100%
	5	Contact	8.62 ×10^4	94.53%
	10	Contact	7.58 ×10^4	83.48%
Escherichia coli (PTCC:1276)	0	Positive Control	6.15×10^4	100%
	5	Contact	5.48×10^4	89.10%
	10	Contact	5.12 ×10^4	83.25%
Klebsiella Oxytoca (PTCC:1402/8724)	0	Positive Control	9.2 ×10^4	100%
	5	Contact	8.87 ×10^4	96.07%
	10	Contact	7.54 ×10^4	81.95%
Candida Albicans (PTCC:5022/10231)	0	Positive Control	1.05 ×10^4	100%
	5	Contact	1.03 ×10^4	98.09%
	10	Contact	1.00 ×10^4	96.64%

The results demonstrated that the greatest reducing influence of the peptide was observed on *S. Pyogenes* after 10 minutes of contact (21.92%, 5.7× 10^4 CFU/m^3) (Table 6).

Table 6. The results of anti-viral assay

Cycle of threshold (ΔCt) Real time PCR method (HPV)	Negative Control	HPV (+)	HPV (+)	HPV (+)
	Treatment time	5 min	10 min	15 min
	Not curve Ct and melting	16	18	21
	Not curve Ct and melting	18-Rech	17-Rech	22-Rech
	Average (ΔCt)	17	17.5	21.5
	Percent reduction Parallel positive control (ΔCt)		15	
Cycle of threshold (Δct) Real time PCR method (HBV)	Negative Control	HBV (+)	HBV (+)	HBV (+)
	Treatment time	5 min	10 min	15 min
	Not curve Ct and melting	18	22	27
	Not curve Ct and melting	21-Rech	23-Rech	26-Rech
	Average (ΔCt)	18.5	22.5	26.7
	Percent reduction Parallel positive control (ΔCt)		17	
Cycle of threshold (Δct) Real time PCR method (COVID-19)	Negative Control	Covid-19	Covid-19	Covid-19
	Treatment time	5 min	10 min	15 min
	Not curve Ct and melting	16	28	34
	Not curve Ct and melting	18-Rech	26-Rech	32-Rech
	Average (ΔCt)	17.0	27.0	33.0
	Percent reduction Parallel positive control (ΔCt)		13	

As this is a gram-positive bacterium with extra thick peptidoglycan layer compared to gram-negative bacteria, and as this effect was not same in all gram-positive bacteria, the mechanism of action may different from direct effect on cell membrane. The peptide's average antibacterial effect was around 11%, which was very small compared to the results of previous studies and thus, could not be classified as an applicable antibacterial peptide. There are several examples that novel designed AMPs could not exert promising antimicrobial activities. Tincho *et al*. reported that some of their novel designed AMPs, which had been previously reported as HIV inhibitory AMPs, could exhibit good antimicrobial activity against gram-negative bacteria such as *P. aeruginosa* and *K. pneumonia*, but had less antimicrobial activity against gram-positive bacteria (Tincho *et al.*, 2020). In another study, it was also shown that only one of the new peptides from different ones they designed, could reduce the number of *Escherichia coli* bacterial colonies by up to 99 percent (Xu *et al.*, 2014).

Moreover, time of exposure can be an efficient factor in the antimicrobial activity. It was previously reported that more than 99 percent of the population of *K. pneumoniae* was killed after 2 hours of exposure to SET-M33, as an antibiotic peptide (van der Weide *et al.*, 2017). Therefore, our novel designed AMP would resulted in a relatively more potency, if the time of AMPs treatment had expanded. However, since the overall activities of peptide could not approve its potency, this test was not evaluated in longer exposure times. In general, the results of experimental tests supported the findings of the SVM's bioinformatics analysis while contradicting the findings of other algorithms.

Antifungal activity

In this study, *C. albicans* was used as fungal model, and exposed to the peptide for 5 and 10 minutes. Then, the rate of fungal population reduction was examined, similar to the antibacterial activity test. There was almost no antifungal effect, as the number of colonies was 1.05×10^4 CFU/m^3 in the absence of the peptide, while the population of the *C. albicans* were 1.03×10^4 and 1.00×10^4 after 5 and 10 minutes of the peptide exposure, respectively (Table 6). This finding was completely inconsistent with the corresponding server results (AIPpred). When comparing the findings of previous trials, it is clear that additional exposure time may be required to observe the peptide's antifungal effect. Chou *et al.* had designed a peptide that kill more than 90% of *C. albicans* after 1 hour (Chou *et al.*, 2021). In another study, Sonson *et al.* showed that a derivative peptide of domain-5 of high-molecular-weight kininogen killed more than 80% of the *C. albicans* cells in 15 minutes (Sonesson *et al.*, 2011).

Antiviral activity

The peptide's antiviral activity on HPV, HBV and SARS CoV-2 was assessed using real-time RT-PCR. In this test, the concentration of objective nucleic acid in the sample is negatively correlated to the number of cycles essential for the fluorescent signal to cross the threshold (Ct: cycle threshold). In fact, ΔCt is considered as an index for the reduction in viral load (Xu *et al.*, 2015).

The results showed that the peptide had the least effect on HPV and the most effect on the SARS CoV-2 ($\Delta Ct_{HPV} = 21$, 15min, $\Delta Ct_{SARS\ CoV\text{-}2} = 34$, 15 min) (Table 6). Considering SARS CoV-2 is an RNA virus, it may be reasonable to assume that this peptide could target and destroy viruses containing RNA genetic material. However, because only one RNA virus has been tested, more samples must be tested in order to draw more precise results, which was not attainable in this experiment. The prior researches demonstrating that natural antimicrobial peptides, particularly beta-defensin and LL-37, which act as antimicrobials in the most sensitive mucosal areas, could act as "disruptive" of viral attachment, entry, and infection. These AMPs are attractive targets to investigate as potential anti-SARS-CoV-2 medicines due to their various modes of action against many viruses, including respiratory viruses (Ghosh and Weinberg, 2021). Through a computer simulation, Mustafa *et al.* found that a small peptide called P9, generated from mouse beta-defensin 4, interacts with MERS-CoV's type I transmembrane glycoprotein S2 domain. In another recent research published by Wang *et al.*, the intestinal-defensin HD5, secreted from paneth cells in the small intestine, was capable to interact with ACE2 (Angiotensin-converting enzyme 2). These researchers discovered that HD5 was coupled to many ACE2 locations required for binding to CoV-2's S protein receptor-binding domain (S-RBD), and HD5 prevented S protein-expressing pseudovirions from penetrating ACE2-expressing intestinal absorptive cells. Therefore, it might be the intrinsic defense of enterocytes against SARS CoV-2 infection (Mustafa *et al.*, 2019). However, our peptide needs more design optimization to be considered as a promising antiviral candidate.

Hemolytic effect

The peptide's hemolytic activity was compared to that of Polymyxin B as a peptide antibiotic. The results showed that, like Polymyxin B, the peptide did not cause substantial hemolysis at the same dose. This finding

confirmed the prediction of HemoPI. Therefore, regarding human safety, the novel designed peptide can be an acceptable candidate.

Conclusion

It's critical to find new antibiotic agents since drug-resistant bacteria are one of the world's most pressing problems today. One possible source of new antibiotics is antimicrobial peptides (AMPs), a crucial element of most complex organisms' ancient, non-specific innate defense mechanisms that serve as the first line of protection from microbial infection (Kim *et al.*, 2014). The employment of computational approaches is expanding day by day due to time and cost savings. Various studies in the last decade have focused on *in silico* methodologies to design and develop antimicrobial peptides. However, there is still a long, hard road ahead of improving prediction server accuracy and sensitivity while lowering mistake rates (Chegini *et al*., 2019). In this study, a new AMP was designed and the results of bioinformatics analysis were evaluated by laboratory findings. Antibacterial, anti-biofilm, anti-fungal, antiviral and anti-inflammatory capabilities were predicted for the novel designed AMPs with 10 amino acid residues, using different servers. Then, among these sequences, the peptide with the best predicted value in all of these activities was selected. However, *in vitro* investigations revealed limited antimicrobial and antiviral activity, contrary to *in silico* results. Just the peptide's low hemolytic activity was confirmed through *in vitro* analysis, which is similar to the predicted value. As it could not approved as potent antimicrobial agent, its effect on inflammatory cytokines was not *in vitro* evaluated. In addition, it was observed that this novel AMP sequence had better antiviral activity against the RNA virus like SARS CoV-2 than DNA ones, thus, it may be concluded that its antiviral function may be rather selective and target specific. Respectively, it had not a broad-spectrum activity as bioinformatics tools suggested. In conclusion, novel peptide design or peptide combining with other antimicrobial compounds or nanoparticles, needs to improve its activity and efficacy.

Ethical Statement

This work did not contains any animal and human studies. This work was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (approval code: IR.SBMU.PHARMACY.REC.1400.265).

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Competing Interests

Authors declare no conflict of interests.

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Authors' Contribution

Maryam Tabarzad and Maryam Ghobeh designed the study and experimental methods. Fariba Fathi performed the computational and laboratory experiments and analyze the results. Maryam Tabarzad and Fariba Fathi wrote the manuscript. Arash Mahboubi supervised the antimicrobial tests. All authors read and confirmed the manuscript.

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