



LL37-rIb-AMP4 Hybrid Peptide as a Therapy for Systemic Infections of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, Vancomycin-Resistant *Enterococcus* (VRE), and Methicillin-Resistant *Staphylococcus aureus* (MRSA) Cells in a Mouse Model

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ABSTRACT

Background: Antimicrobial peptides (AMPs) are beneficial compounds that could be used as a new and effective method to suppress microbes. Both Ib-AMP4 and LL37 are antimicrobial peptides with a wide range of antimicrobial activities. This research aimed to evaluate the antibacterial potential of LL37-rIb-AMP4 hybrid protein as an antimicrobial agent against pathogenic bacteria. Therefore, its antibacterial effects against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, vancomycin-resistant *Enterococcus* (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) were investigated in vivo and in vitro.

Materials & Methods: In this study, antimicrobial peptides rIb-AMP4, LL37, and LL37-rIb-AMP4 were expressed, purified, and refolded, and their synergistic and antibacterial effects in combination with each other (LL37+rIb-AMP4) and as fusion proteins (LL37-rIb-AMP4) were tested against *A. baumannii*, *P. aeruginosa*, VRE, and MRSA cells in vitro (MIC, time kill, and SEM) and against *P. aeruginosa* and VRE cells in vivo.

Findings: LL37-rIb-AMP4 Protein with molecular weight= 28 KD was correctly produced and purified. Despite the lack of synergistic effects between LL37 and rIb-AMP4 peptides in vitro, the stability test results showed higher stability for LL37-rIb-AMP4 hybrid protein. The findings of in vivo tests confirmed that all infected mice were improved with LL37-rIb-AMP4 and no signs of bacteria were observed in their blood and spleen samples. Also, these results confirmed the stability and higher activity of LL37-rIb-AMP4 than the single form of these proteins.

Conclusion: Considering the antimicrobial potential of the produced proteins, it seems that the recombinant LL37-rIb-AMP4 protein could be considered and used as a stable and active antimicrobial drug in future studies.

Keywords: Antimicrobial peptides, Hybrid protein, LL37 peptide, rIb-AMP4 peptide

CITATION LINKS

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Introduction

Today, given the increasing prevalence of antibiotic-resistant pathogens, antimicrobial peptides (AMPs) could be considered as good candidate alternatives to antibiotics due to the wide range of activities, less toxicity, and reduced resistance of microbial agents [1].

AMPs are relatively small molecules, usually less than 100 amino acids in length, which have a sensitive task in the innate immunity of various organisms ranging from bacteria to humans, such as Ib-AMP4. In the present research, the synergistic effects of recombinant proteins on pathogenic bacteria were investigated [2].

LL-37 has human origin with extensive antimicrobial activities and It is from the human cathelicidin family and leading to bacterial death through destroying bacterial cell membranes. LL-37 is an amphipathic alpha-helical peptide manufactured by extracellular cleavage of the C-terminal end of hCAP18 protein. LL-37 is consisted of 37 amino acids, has a net positive charge of +6 at a physiological pH and a hydrophobic N-terminal domain. These properties bind it effectively to negatively charged phospholipid bacterial membranes [3]. Another AMP is Ib-AMP4, which is of plant origin and has cationic with properties amphipathic properties that gives them the ability to react with cell membranes and has antibacterial activity against Gram-positive and Gram-negative bacteria. This peptide disrupts bacterial metabolism through various mechanisms, leading to bacterial cell death, such as changes in cell surface, plasma membrane degradation, and membrane channel degradation [4].

In the present research, the synergistic effects of recombinant proteins rlb-AMP4 and LL-37 on pathogenic bacteria were investigated *in vivo* and *in vitro*.

The reversed sequence of Ib-AMP4 was used to bind the C-terminal part of the peptides

so that their N-terminal part was free, and they could perform their functions better^[5]. Synergistic effects allow the use of smaller amounts of peptides and increase the range of pathogens that may be disrupted by these peptides. Given the significance of this issue, the present research aimed to evaluate the synergistic effects of LL37-rlb-AMP4 hybrid protein and the simultaneous use of both LL37 and rlb-AMP4 peptides together (LL37+rlb-AMP4) *in vitro* and *in vivo* conditions.

Objectives: This study also aimed to investigate the inhibitory effects of these peptides on infectious microorganisms.

Materials and Methods

Materials, chemicals and bacterial strains:

Enzymes used include: polymerase, DNA polymerase, Taq polymerase, restriction, ligase, and RNase enzymes, were prepared from Thermo Scientific, Fermentas. The PCR product purification kit was prepared from Roche (Germany), IPTG was purchased (Thermo Scientific, Waltham, USA) and the protein purification kit was obtained from Qiagen (USA). The antibacterial activities of the hybrid peptide synthesized (LL37-rlb-AMP4) in this study were assessed against vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Also, *Escherichia coli* DH5 α (Stratagene, La Jolla, Calif) was employed as a recombinant protein-cloning host, *E. coli* BL21 (DE3) (National Institute for Genetic Engineering and Biotechnology, Tehran, Iran) was applied as a recombinant protein expression host, and pET32a (Novagene) was used as a recombinant protein expression vector.

Gene construction: The entire gene sequence of the reversed sequence of Ib-AMP4 corresponding sequence (UniProt: P85148), comprising *Xho*I 3', histidin tag,

and 5' *BamHI*, was designed and codon-optimized for *E. coli* expression. The entire gene sequence of LL37, comprising *XhoI* 3', histidin tag, and 5' *BamHI*, was designed and codon-optimized for *E. coli* expression.

The complete LL37-rlb-AMP4 gene sequence, comprising 5' *BamHI*, LL37 sequence (EMBL: M16645), reversed Ib-AMP4 sequence corresponding sequence (UniProt: P85148), and *XhoI* 3', was designed and then codon-optimized for *E. coli* expression. This construct was synthesized by Biomatik Company (Cambridge, ON, Canada) and then included into the plasmid pET32a pET32a (Novagen, Madison, USA) as the expression vector.

Preparation of competent cells, transformation and ligation: *E. coli DH5 α* competent cell as a recombinant protein-cloning host and *E. coli BL21 (DE3)* competent cell as a recombinant protein expression host were prepared by calcium chloride method [6]. To prepare competent cell, 0.5 of the culture medium containing bacteria was poured into 50 ml of sterile LB medium and incubated at 37 °C and 220 rpm. When the OD of the LB culture medium reached 0.5, It was placed in ice for 10 min. Then it was centrifuged at 4100

Table 1) Amounts of material for ligation.

(PCR product)	8 μ l
pET32a	2 μ l
T4 DNA Ligase	1 μ l
Buffer	2 μ l
PEG	1 μ l
DDW	6 μ l
Total	20 μ l

rpm for 10 min and 4 °C. 30 ml of CaCl₂-MgCl₂ solution was poured on the sediment and centrifuged, 2ml of CaCl₂ solution was poured on the sediment and mixed. At this stage, susceptible cells are ready for use and kept at -70°C [5].

Then, synthesized plasmids were transferred into *E. coli DH5 α* for maintenance base on the standard protocol [7]. For ligation reaction, synthesized plasmids and pET32a expression vector were digested with *BamHI* and *XhoI* fast restriction enzymes base on the manufacturer's guidelines. Then the product was analyzed by electrophoresis [8]. Then, *T4 DNA ligase* was used for ligation of the digested synthesized plasmids into the pET32a expression vector. for this purpose, table 1 show amounts of material for ligation. values were poured into a microtube and incubated at 16 °C overnight [9].

Expression, purification and refolding optimization: Synthesized plasmids were transferred to *E. coli BL21 (DE3)* competent cells according to the Standard thermal shock protocol [10]. Finally, in three conditions, 50 μ l and 100 μ l, and bacterial deposits were cultured in nutrient agar (NA) containing ampicillin (100 mg/mL) and incubated at 37 °C for 24h. Then, transformed colonies were cultured on NA and nutrient broth (NB) containing ampicillin (100 mg/mL) and incubated at 37 °C for 24h. After the screening tests, one of the recombinant colonies grown on plates containing ampicillin (100 mg/mL) was cultured in 2 mL of NB + ampicillin (100 mg/mL) medium.

Afterwards 300 μ L of the overnight bacterial culture was added to 50 mL of 100 mg/mL ampicillin-containing NB and subjected to incubation at 37 °C while stirring at 220 rpm. The OD of the cells was occasionally measured at 600 nm, and when the opacity reached 0.6, 1 mM IPTG was inserted to stimulate protein expression. After four hrs of stimulation, then was centrifuged at

5000 rpm for 20 min, and all transformed stimulated bacterial colonies were collected and maintained at -20 °C. Then 12% SDS-PAGE was used to confirm the protein [11]. Recombinant proteins were purified by 8M urea and Ni-NTA kit according to the standard protocol [12]. The quality and quantity of the purified proteins were evaluated using 12% SDS-PAGE, and following relation respectively:

The amount of protein (mg/mL) = $(OD_{280} \times 1.55) - (OD_{260} \times 0.76)$

For refolding optimization, the dialysis procedure was performed in 20 mM PBS (phosphate-buffered saline) at pH=7 and 4 °C for 24 hrs. Finally, the quality of the refolded proteins was evaluated using 12% SDS-PAGE [13], and their quantity was investigated using the following relation: $(OD_{280} \times 1.55) - (OD_{260} \times 0.76)$ = The amount of protein (mg/mL)

For concentration of the refolded proteins, an amicon filter (Pore size = 10 kDa) (Merck Millipore, Darmstadt, Germany) was used to concentrate dialyzed proteins [14].

***In vitro* tests**

Determination of antibacterial minimum inhibitory concentration (MIC): Bacterial inoculum was prepared following the CLSI (Laboratory and Clinical Standards Institute) standard protocol M07-A10 [14]. MIC was measured based on the CLSI M07-A10 (2022) protocol. Briefly, 50 µL (initiate concentration) of each refolded protein was inseminated in one of the wells of a 96-well plate, and then two-fold serial dilutions were made in the next wells to assess the antibacterial activity of the produced proteins at different concentrations. The prepared bacterial inoculum (1×10^6 CFU/mL) was inserted into the wells and incubated at 37 °C for 24 hrs. After this period, 20 µL of 0.02% resazurin dye (w/v) was added into the wells and subjected to incubation for 2 hrs. The concentration of the proteins

in the last blue well was considered as MIC number [15].

Synergy study: A checkerboard dilution test was used to investigate the synergistic effects of rlB-AMP4 and LL37 against all the studied microorganisms. The synergistic effects of the combination of these two proteins were measured through determining the fractional inhibitory concentrations (FIC) *in vitro* as well as the FIC index (FICI) using the this formulas:

FIC of each peptide = (MIC of peptide in the combination)/(its MIC value)

FICI = FICA + FICB [16].

The degree of synergy was interpreted as follows: FICI less than 0.5 was considered as synergistic effect and between 0.5 and 0.75 as partial synergistic effect, while FICI between 0.76 and 1.0 was considered as additive effect, between 1.0 and 4.0 as no difference, and more than 4.0 as antagonistic effect.

Time-kill curves: Bacterial cell suspension was prepared at a concentration of 0.5 McFarland. Also, rlB-AMP4, LL37, and LL37-rlB-AMP were used at $2 \times$ MIC concentrations alone or in combination. After adding antimicrobial agents to bacterial suspensions, each falcon was cultured on BHI (brain-heart infusion) agar at 0, 20, 40, 60, 80, 100, and 120 min post-addition and then subjected to incubation at 37 °C for 24 hrs for colony counting. After the incubation time, the removal pattern of live bacterial cells and synergistic effects were determined. A decrease of ≥ 1 log₁₀ compared to the initial inoculum concentration indicated antibacterial activity. In the synergy assay, a decrease of ≥ 2 log₁₀ and $1 \leq \log_{10} \leq 2$ indicated synergistic and additive effects, respectively [17].

Peptide stability test: To assess the stability of the obtained recombinant peptides, their antimicrobial activity against the studied microorganisms was evaluated by MIC test

at different time intervals after dialysis [18].

In vivo tests

Animal model: The present research was approved by the Internal Animal Ethics Committee of the Molecular and Medical Research Center, Arak University of Medical Sciences,. Syrian adult male mice with a weight of approximately 20-25 g were used in this study. They were kept in separate cages under controlled environmental and fed with a standard rodent diet.

Culture of bacteria: This method was performed on VRE as an important Gram-positive bacterium and *P. aeruginosa* as an important Gram-negative bacterium. Overnight cultures of VRE and *P. aeruginosa* on fresh BHI culture medium were prepared. The grown bacterial colonies were then subjected to centrifugation at 10000-xg for 10 min and rinsed and re-suspended in sterile PBS.

Intraperitoneal (IP) induction of systemic infection: To induce systemic bacterial infection, the intraperitoneal (IP) route was employed to inject VRE and *P. aeruginosa* bacteria. Syrian mice were infected with a volume of 1 cc of pathogenic bacteria at a concentration of 10×2^{10} CFU/mL. In this study, protein treatment was started immediately after infection of Syrian mice. Injections were done four times with 12-hour intervals for 48 hours [19].

In the present study, 20 Syrian mice were randomly and equally distributed in four groups as follows: Group 1) mice infected with VRE and *P. aeruginosa* without receiving any treatment, Group 2) mice receiving antibiotics, Group 3) mice receiving recombinant LL-37-rIb-AMP4 at two different concentrations, and Group 4) mice receiving recombinant LL-37 + rIb-AMP4 at two different concentrations (Table 2).

Supplementary assays

Negative Control group: After the death of infected mice in this group, their

blood specimens were analyzed to detect pathogenic bacteria. To do so, 2 mL of blood was taken from the heart of each mouse, cultured on sterile BHI culture medium, incubated at 37 °C for 18 hrs, and then cultured on blood agar culture medium. Specific culture media were used to confirm vancomycin resistance and the presence of VRE and *P. aeruginosa*, including SIM (sulfur, indole, motility), TSI (triple sugar iron), MHA (Mueller-Hinton agar), indole, and citrate. **Protein-treated mice groups:** In this study, 72 hrs after the start of protein treatment, one mouse from each protein-treated group was sacrificed as a sample. Afterwards approximately 5 mL of blood was taken from the heart of each mouse and cultured on sterile BHI culture medium and incubated for 18 hrs. Then 10 µL of the medium was cultivated on blood agar for confirmatory assays.

Spleen removal and culture: The spleen of one mouse from each group was removed after death and rinsed with phosphate-buffered saline. The spleen was perfused under sterile conditions in the presence of a laminar hood. Finally, approximately 100 µL of the spleen content was cultivated on blood agar.

Findings

Expression, purification, and refolding of LL37-rIb-AMP4, rIb-AMP4, and LL37 in *E. coli* BL21 (DE3) pLysS: Purified proteins were analyzed by SDS-PAGE. Due to the presence of 6histag sequences at the end of the amino acid chain of LL37, LL37-rIb-AMP4, and rIb-AMP4 recombinant proteins, they were purified using Ni-NTA kit. The molecular weight of the recombinant proteins was as follows: LL37=22 KD, LL37-rIb-AMP4=28 KD, and rIb-AMP4=25 KD (Fig. 1).The concentration of the proteins after the dialysis procedure was as follows: LL37 =0.1 mg/mL, LL37-rIb-AMP4=0.2 mg/mL,

Table 2) Different groups of mice studied in this research

Group numbers	Description	Subgroup	Different concentrations of recombinant protein (µg/mL)
Group 1	Negative control group (syrian mice infected with VRE and <i>P.aeruginosa</i> , no treatment).	-	-
Group 2	Posittive control group that were treated with antibiotic (syrian mice were treated with Imipenem and Linezolid).	-	-
Group 3	Treated with recombinant LL37-rIb-AMP4, with two different concentrations:	Group 3.A	100
		Group 3.B	50
Group 4	Treated with recombinant LL37+rIb-AMP4, with two different concentrations:	Group 4.A	100
		Group 4.B	50

and rIb-AMP4= 0.15 mg/mL. Considering that these values were not enough for subsequent tests, Amicon centrifugal filter was utilized to solve this issue. After the concentration step, the concentration of the refolded proteins was about 400 µg/mL.

In vitro antibacterial assays

MIC and FIC determination: Table 3

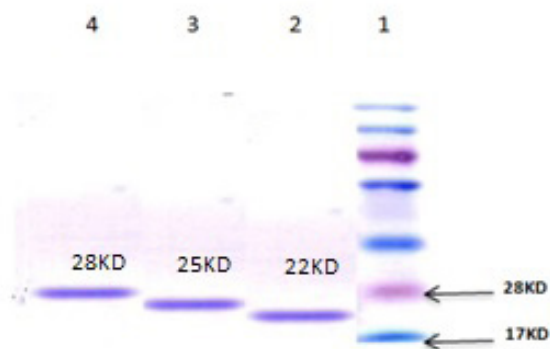


Figure 1) Purification results of recombinant proteins on 12% SDS gel. Line 1: protein ladder, line 2: purified LL-37 protein, line 3: purified rIb-AMP4 protein, and line 4: purified LL37-rIb-AMP4 protein

represents the MIC values of LL37, LL37-rIb-AMP4, LL37+rIb-AMP4, and rIb-AMP4 against Gram-negative (gr⁺) and Gram-positive (gr⁻) pathogens.

Time-kill: Time kill test was employed to evaluate the required time for the death of bacteria in the presence of the synthesized recombinant proteins. The results indicated the bactericidal activity of the synthesized recombinant LL-37+rIb-AMP4 against *A. baumannii* and VRE within 40 and 50 minutes of treatment, respectively. Regarding MRSA and *P. aeruginosa*, only the number of bacteria was reduced but did not reach zero. These results are shown in Fig. 2. The results also indicated the bactericidal activity of the synthesized recombinant LL-37-rIb-AMP4 against *A. baumannii* and VRE within 40 minutes of treatment. Regarding MRSA and *P. aeruginosa*, only the number of bacteria was reduced but did not reach zero. These results are shown in Fig. 3.

Peptide stability test: The results of stability test showed higher stability for LL37-rIb-AMP4 protein. The results of peptide stability test are shown in Table 4.

In vivo tests

Infection of mice: Intraperitoneal (IP) administration route was employed to infect mice with VRE and *P. aeruginosa*.

Positive and negative control groups: These results showed that in the negative control group, all *P. aeruginosa*-infected mice died 12 hrs after bacterial injection, and all VRE-infected mice died

Table 3) Results of MIC and FIC. A) LL37+rIb-AMP4, B) LL37-rIb-AMP4

Microorganisms	MIC ($\mu\text{g/mL}$)					
	LL-37	rIb-AMP4	LL37+rIbA-MP4	FICA	FICB	FICC
gr-						
<i>P. aeruginosa</i>	4.5	9	12.5	2.8	1.3	4.1
<i>A.baumannii</i>	1.5	12	50	33.4	4.1	37.52
gr+						
VRE	6	9	12.5	2.06	1.38	3.44
MRSA	4.5	1.5	50	11.11	33.33	44.44

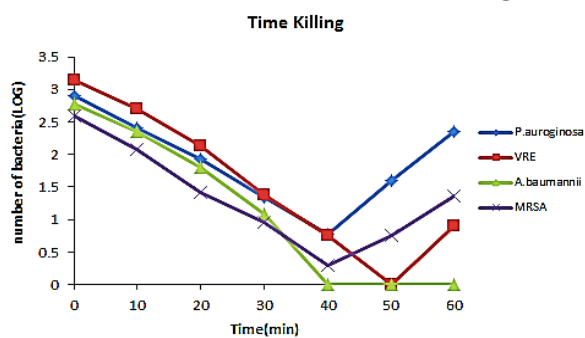
A. LL37+rIb-AMP4

Microorganisms	MIC ($\mu\text{g/mL}$)					
	LL-37	rIb-AMP4	LL37-rIb-AMP4	FICA	FICB	FICC
gr-						
<i>P. aeruginosa</i>	4.5	9	12	2.7	1.4	4.1
<i>A.baumannii</i>	1.5	12	15	10	1.25	11.25
gr+						
VRE	6	9	12	2	1.4	3.4
MRSA	4.5	1.5	25	5.6	16.7	23.3

B. LL37-rIb-AMP4

20-48 hrs after bacterial injection. After the death of infected mice in the negative control group, their blood specimens were collected and cultured on solid medium. Then bacterial growth on blood agar culture medium indicated the presence of VRE and *P. aeruginosa*.

The findings of culture and differential tests showed that the blood specimens of mice in the negative control group were contaminated with VRE and *P. aeruginosa*.

**Figure 2)** Time kill test results for LL-37+rIb-AMP4 against bacteria

These results are shown in Fig.4.

Moreover, in the positive control group, the results indicated that 100% of mice treated with linezolid (at a concentration of 2 mg/mL) survived, and no traces of bacterial pathogens were found in their spleen and blood specimens, but 40% of mice treated with imipenem (at a concentration of (15000 unit/mL) died.

Recombinant protein-treated groups: All infected mice receiving LL37-rIb-AMP4

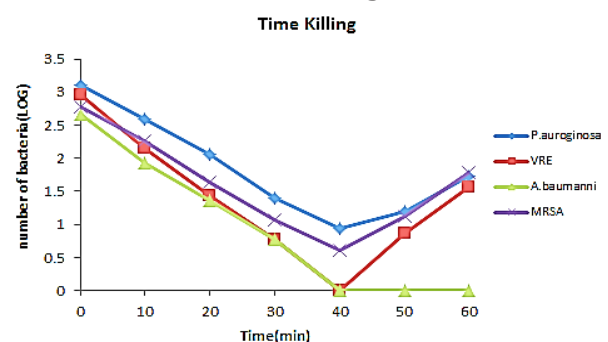
**Figure 3)** Time kill test results for LL-37-rIb-AMP4 against bacteria

Table 4) Peptide stability test results

Recombinant Peptide	Microorganisms	Number of Stability Days
LL37	<i>A.baumannii</i> <i>VRE</i> , <i>MRSA</i> , <i>P. aeruginosa</i>	3
rIb-AMP4	<i>A. baumannii</i> <i>VRE</i> , <i>MRSA</i> , <i>P. aeruginosa</i>	7
LL37-rIb-AMP4	<i>A. baumannii</i> <i>VRE</i> , <i>MRSA</i> , <i>P. aeruginosa</i>	45
LL37+rIb-AMP4	<i>A. baumannii</i> <i>VRE</i> , <i>MRSA</i> , <i>P. aeruginosa</i>	35

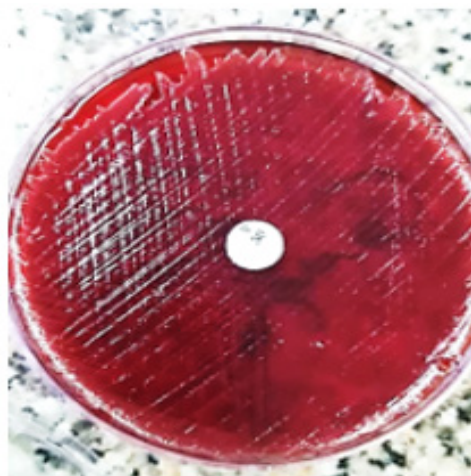


Figure 4) Results of vancomycin resistance of VRE. and LL37+rIb-AMP4 at both 50 and 100 µg/mL concentrations survived, and no signs of bacterial pathogens were found in their spleen and blood specimens.

Discussion

Millions of people die every year due to infectious diseases. Among pathogenic bacteria, *P. aeruginosa* is of particular importance due to its high prevalence.

P. aeruginosa is naturally resistant to a significant number of antibiotics. Another antibiotic-resistant bacterium is VRE whose resistance to existing antibiotics is increasing day by day [20].

Scientists are trying to develop and replace new drugs to overcome antibiotic resistance, and today, extensive research on antimicrobial peptides is underway [21]. One of the effective ways to increase the effectiveness and sustainability of antibacterial peptides against microbial pathogens is the hybridization of antimicrobial peptides [22]. Thus, in the current research, the antimicrobial peptide sequences of LL37 and rIb-AMP4 were hybridized, and then *in vitro* and *in vivo* antibacterial activities of LL37-rIb-AMP4 hybrid peptide and a combination of LL37 and rIb-AMP4 (LL37+rIb-AMP4) were evaluated against drug-resistant VRE and *P. aeruginosa*.

Sadelaji et al. (2022) showed that the produced Ib-AMP4 peptide exhibited antimicrobial activity against MRSA infection [23]. Moreover, previous studies have shown that Ib-AMP4 protein exhibits inhibitory activity against Gram-negative and Gram-positive pathogens [24].

Fan et al. (2013) indicated that Ib-AMP4 exhibited enhanced antimicrobial effects and killed bacteria by destroying the plasma membrane and acting on the lipopolysaccharide (LPS) layer [25].

Turner et al. (1998) showed that the produced LL37 protein (10 µg/mL) had significant antibacterial effects on *S. epidermidis*, *P. aeruginosa*, and *Listeria monocytogenes*. Moreover, several investigations have shown that LL37 peptide exhibits inhibitory activity against Gram-negative and Gram-positive pathogens [26]. The most critical property of antimicrobial peptides is their potential to neutralize LPS due to their high positive charge and hydrophobicity,

which enables them to attach to LPS or LTA in bacterial membranes and neutralize their inflammatory activity [27]. The findings of laboratory experiments in the current research indicated that the LL37-rIb-AMP4 hybrid peptide killed bacteria faster and was more stable than LL37 + rIb-AMP4. In addition, the antimicrobial peptide LL37-rIb-AMP4 was more stable compared to the LL37 and rIb-AMP4 peptides alone and had a high advantage after *in vitro* purification and dialysis. The *in vivo* test results confirmed that LL37-rIb-AMP4 at lower concentrations (50 µg/mL) was more successful in controlling VRE and *P. aeruginosa* than LL37 + rIb-AMP4. This may be due to the higher stability of the LL37-rIb-AMP4 hybrid compared to LL37 + rIb-AMP4. In this study, the results of MIC and FIC tests *in vitro* showed that the LL37-rIb-AMP4 peptide did not have a great synergistic effect, but the *in vivo* test results showed that treatment with LL37-rIb-AMP4 reduced mortality and minimized spleen damage. Therefore, given the *in vitro* and *in vivo* antimicrobial potential of the synthesized peptides, it seems that with more research and clinical trials, new antibiotics could be produced to treat patients. Accordingly, it is possible to reduce the loss of life and property, which is the most important goal of the health system in the world.

Conclusion

The results show the higher efficiency of LL37-rIb-AMP4 hybrid protein against *P. aeruginosa*, *A. baumannii*, MRSA, and VRE cells *in vitro* and *in vivo*.

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Ethical permissions: The current research was done after obtaining the approval of the Ethics Committee of Arak University of Medical Sciences, Arak, Iran (Ethics code: 95-228-7).

Conflicts of interests: The authors declare no conflict of interest in this study.

Authors' contributions: Hamid Abtahi, Ehsanollah Ghaznavi-Rad, and Ehsan Zarei-Mehrvarz designed the research plan and coordinated the data analysis. Shabnam Sadoogh Abbasian and Ali Sadoogh Abbasian write the article.

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Consent to participate: Not applicable.

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