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Original Article

Evaluation of Antibacterial and Cytotoxic Effects of K4 Synthetic Peptide

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HIGHLIGHTS

- K4 peptide had strong antibacterial effect on some bacteria such as B. melitensis.
- This peptide may have a role in immunity with nitric oxide production.
- K4 antimicrobial peptide did not show any cytotoxic effect on mammalian cell.

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ABSTRACT

Introduction
With increasing antimicrobial resistance to common antibiotics, the development of alternative therapeutic strategies is necessary. Also, an antibacterial peptide with numerous behavior and different properties such as net charge, hydrophobicity, length, etc. could act against pathogenic microorganisms. In recent years, novel peptides with activity against a wide range of bacteria have been introduced.

Methods
In this study, seventeen pathogenic bacteria were chosen to study the antibacterial effect of K4 peptide using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays. The therapeutic index (TI) of this peptide was experimentally calculated by the ratio of HC50 to MIC as a parameter to represent the specificity of AMP. In silico analysis was performed to predict the physico-chemical properties, structures, and behavior of this peptide. In vitro cytotoxic effect of peptide was evaluated on the Hela cell line using MTT assay, and the amount of macrophage nitric oxide production was measured by Griess method on the J774 cell line.

Results
Peptide concentrations of 25-400 µg/ml were seen as the MIC value results for different bacteria. MBC assay showed such a result with a concentration of more than 25-400 µg/ml. The result of the hemolysis assay was 24 percent at a 1 mg/ml concentration. The amount of nitric oxide production of macrophage cell line J774 was 25.9873 µM at a 6.3 µg/ml peptide concentration.

Conclusions
K4 peptide had a strong antibacterial effect on some the bacteria such as B. melitensis. This peptide may have a role in immunity with nitric oxide production. Additionally, it enhances bacterial killing mechanism of macrophages, making this peptide a potential agent against pathogens.

Keywords: Antimicrobial Peptide; Cationic Peptide; Minimal Inhibitory Concentration; Minimal Bactericidal Concentration; Nitric Oxide

Introduction

Antimicrobial peptides have an important function in host defense against pathogens, and also their role in the regulation of the adaptive immune system, wound repair, and endogenous antibiotics are clearly demonstrated (1). These peptides could eliminate pathogenic bacteria and other microorganisms with their features. Generally, this mechanism works by the interaction between bacterial membrane and hydrophobic area of peptide, which results in bacterial membrane lysis. The initial absorption

of peptides on cell membrane with negative charge could disturb the vital process of cells via translocation into the cytoplasm. They involve cellular damage by interfering in the process of lipids synthesis on the 1 cell wall (2, 3). Several studies have shown that cationic antimicrobial peptides have strong cytotoxicity against microbial agents (4, 5). For example, Duval et al., studied cationic K4 peptide and found this peptide effective in killing the bacteria while it was not toxic to mammalian cells (6). Antimicrobial peptides with 3 to 30 amino acids usually act against bacteria. In addition, they have a powerful cationic property and resistance to temperature (7, 8). The size of some peptides and their side effects such as cytotoxicity and hemolysis are really important. To resolve this problem, small analogue and hybrid peptides were designed. Most antimicrobial peptides have an important function in killing bacteria by recruiting immune system cells and creating inflammation. In the end, they have a repair function as well (3, 9). Also, antimicrobial peptides were designed against different kinds of pathogens such as bacterial, viral, parasitic, and fungal infections. Consequently, antimicrobial synthetic peptides have been efficiently developed against several varieties of pathogens. Furthermore, antimicrobial peptides are preferred to common antibiotics because of their quick start and little resistance of bacteria (4). With rising microbial resistance to common antimicrobial agents, new therapeutic options have been considered in recent decades (10, 11). Due to the importance of intestinal pathogenic bacteria in the incidence of infection resistance, the investigation of peptide's antibacterial activity in the treatment of these infections seems vital. K. pneumonia with Carbapenemase enzyme, in particular , hydrolyzes β-lactam drugs could show drug resistance. In Dueval study, de novo cationic peptide called K4 (KKKKPLFGLFFGLF) was designed based on the natural features of Antimicrobial peptides (AMPs) as antibacterial peptides. Some of these properties include being net charge, and length, hydrophobicity percentage and enantiomers. This peptide was achieved from the antimicrobial peptides database (APD: http://aps.unmc.edu/AP/main.html) based on its suitable features such as charge, cationic property, small size, and hydrophobicity percentage. In addition to these features, the above website provided statistical information on peptide residue, structure, and function (12, 13). In the peptide sequences, there are hydrophobic residues every two or three residues. There are 4 Lys (positive net charge +4) and 4 phe and 3 leu (50% hydrophobicity) with 14 residues in K4 peptide. K4 seems to be a potent antibacterial peptide against both gram-negative and gram-positive bacteria. In 2009, Duval et al., reported that K4 peptide did not have any cytotoxic effect on Chinese Hamster Ovary cells line (CHO-K1) at the bacteriolytic concentration (6).

In this regard, the present study was designed to evaluate the effect of antimicrobial peptides' performance on several gram-negative and positive pathogens, each of which could lead to infectious diseases (14, 15).

Methods

Peptide and Bacterial strains

The K4 peptide (KKKKPLFGLFFGLF) was chemically synthesized by Biomatik Company (CA) with ≥98% purity and mass spectrometry HPLC techniques were used to evaluate the quality of the product. The bacteria strain include: S. enteritidis, B. cereus, E. coli, S. marcescens, A. baumannii, P. vulgaris, S. aurous, V. cholera, P. aeruginosa, S. epidermidis, and N. brasiliensis were gathered from clinical diagnostic laboratories and Pasture Institute of Iran which were approved with standard microbial laboratory test.

In silico study of peptides

The primary sequence analysis and physicochemical properties of these peptides were performed online using ProtParam (http://www.expasy.org/tools/protparam.html) and other parameters including hydrophobicity, hydrophobic moments, net charge at Isoelectric pH, were determined by using Antimicrobial Peptide Database (http://aps.unmc.edu/AP/prediction/prediction_main.php). The secondary and tertiary structures of peptides were predicted online using pepstrmod server (http://osddlinux.osdd.net/raghava/pepstrmod/comb_ds.php). In silico prediction of hemolytic potency and therapeutic index (2) of peptides performed using Hemopi (http://crdd.osdd.net/raghava/hemopi/) and dserv1 server as introduced by Tossi et al., (http://split4.pmfst.hr/split/dserv1) respectively (14, 16).

MIC and MBC determination

To measure the Antibacterial activity of K4 against pathogenic bacteria, we determined the minimal inhibitory concentration (MIC) by broth microdilution method. Along with a primary inoculum of 1.5 × 108, CFU /ml is based on ways outlined by the CLSI . Different peptide Concentrations at doses of 2-400 mg/l with bacterial cultures were incubated in an incubator for 18h at 37°C. MIC was considered by the lowest peptide concentration that inhibited the growth of bacteria. Minimal bactericidal concentration (MBC) test determines the lowest concentration of antibacterial peptides to kill the organism. The experiments were done in triplicate.

Calculation of therapeutic index (MHC/MIC ratio)

The therapeutic index (TI) is often used as a parameter to represent the specificity of AMP that is experimentally calculated by the ratio of HC50 (the peptide concentration causing 50% hemolysis of red blood cells) to MIC (minimal concentration inhibiting overnight growth of

bacteria in liquid assays).

Cell line culture

Cytotoxicity of K4 peptide was surveyed on Hela (Human Cervix Tissue) cell line (Pasture Institute of Iran) at 1.6 to 200 µg/ml peptide concentration (National Cell Bank of Iran). Hela cell line was adopted and suspended in media culture. The cells were cultured in 75 cm2 cell culture flasks and after growth harvested by trypsin-EDTA. The cells were then resuspended in fresh medium, counted by Trypan Blue exclusion (99% viability) and its concentration was adjusted to105 cells/ml.

Along with, 100µl aliquot of this suspension was added to each well of 96-well cell culture plates (Corning, USA) with RPMI-1640 medium (Cambrex Bioscience) supplemented with L-glutamine (2mM, Gibco), penicillin–streptomycin (100 IU/ml penicillin, 100mg/ml streptomycin (Gibco)) and heat-inactivated fetal bovine serum (Biowest) 10% (v/v). The cells were maintained at 37°C in a humidified incubator with a 5% CO2 atmosphere.

Cytotoxicity assay

To this measure, 24 and 48h after AMP treatment, 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide (MTT) solution (USB Corporation, Cleveland, USA) was dispensed to each well as final concentration and the plates were incubated for 4h at 37°C, then MTT solution was discarded following by addition of DMSO to dissolve formazan crystals. The absorbance was read at 540nm on a 680 Microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viability was calculated as AT /AC × 100. Where AT is the absorbance of treated and AC is the control cell's absorbance.

Hemolysis assay

Hemolytic activity of K4 peptide was appraised on human erythrocytes. In this regard, the human erythrocyte solution (20%) was preincubated for 15 min at 37°C. After that, the solution was diluted to 10% by adding different peptide concentration solutions and incubated for an additional 15 min in 37°C. The absorption of the supernatant was measured at 415nm. In this essay, Triton X100 and PBS were used as positive and negative control respectively.

%Hemolysis activity = (ODt-ODcn)/(ODcp-ODcn)×100

Macrophage cell line Nitric oxide production

The J774 cell line was obtained from the national cell bank of Iran. To measure the production of nitric oxide, the cells were cultured in 75 cm2 cell culture flasks. The cells were dissociated enzymatically with trypsin- EDTA (0.25%) and centrifuged for 5min at 1100g. Then, the cells were suspended again in a fresh medium, after that enumerated by Trypan Blue exclusion (99% viability) and the cell concentration was adapted to 105 cells/ml.

Dispensed 100 µl aliquot of this suspension to each well of 96-well cell culture plates (Corning, USA) with RPMI-1640 complete medium (Cambrex Bioscience) as above. The cells were treated with different concentrations of K4 peptide (2-200mg/l) and kept in a moisten incubator at 37°C with 5%CO2 atmosphere for 24, 48, and 96h. after incubation times, 50µL of Griess reagent include following agents such as Sulfanyl 1% saluted in phosphoric acid 5% and Naphthyl ethylene diamine dihydrochloride were added to samples and standards (different concentration of NO2Na) and incubated at temperature room for 5 min. Optical density was read by ELISA reader at 540nm. All of the reactions were performed in triplicate.

Statistical analyses

Statistical analysis was carried out using SPSS (ver.16) . Data were expressed as mean ± standard deviations. And P-value < 0.05 was considered significant.

Results

In silico study of peptides

The total number of amino acids is fourteen (n=14). Molecular weight is 1670.12 and Theoretical pI is 10.48. Along with the estimated half-life is 1.3 hours (mammalian reticulocytes, in vitro. 3 min (yeast, in vivo) 3 min (Escherichia coli, in vivo). More details were brought in Table 1. In this regard, Hydrophobicity, hydrophobic moment, and net charge of K4 peptide are 0.644 (H), 0.390 (µH), and 4 (z) respectively. Also, properties of polar and nonpolar residues of K4 peptides were brought in (Table 1).

The instability index (2) is computed to be 3.90. The result of this classifies the protein as stable. The Level of the aliphatic index is 83.57. And Level of the Grand average of hydropathicity is 0.329. Also, the Therapeutic index (2) was 2.1mg/ml (Table 1).

Helix and strand were shown as representative of secondary structure. Also, secondary and tertiary structures were drawn in (Figure 1).

PROB score is normalized. The result of peptide hemolysis prediction was 0.50 and this range is between 0 and 1, i.e., number 1 is very powerful hemolysis and number 0 has no powerful hemolysis (Table 1). Therapeutic index (2) was 2.1mg/ml and calculated for the surveyed pathogens in this study and brought in (Table 2).

The result of helical wheel diagrams shows that hydrophobic amino acids are concentrated on one side of the helix and polar or hydrophilic amino acids on the other (Figure 2).

Table 1. Calculated and predicted physicochemical characteristics of K4 peptide

PROB score	Hydrophobic residue%	Boman Index	II	TI	GRAVY	µH	H	Aliphatic index	Charge	pI	Mol wt
0.50	50%	-0.45	3.90	2.1	0.329	0.390	0.644	83.57	4	10.49	1570.30

The instability index (II), grand average of hydropathy (GRAVY), molecular weight (Mol wt), Hydrophobicity(H), Hydrophobic moment(µH).

Table 2. Calculated experimental therapeutic indices for most of pathogens

Bacteria	S. enteritidis	B. cereus	E.coli	K. pneumoniae	P. aeruginosa	S. aureus	S. epidermidis	N. brasiliensis	Sh. Sonei	B. melitensis	E. faecalis	E. cloacae	B. abortus
TI	2	2	1	2	1	1	1	-	2	1	1	2	1

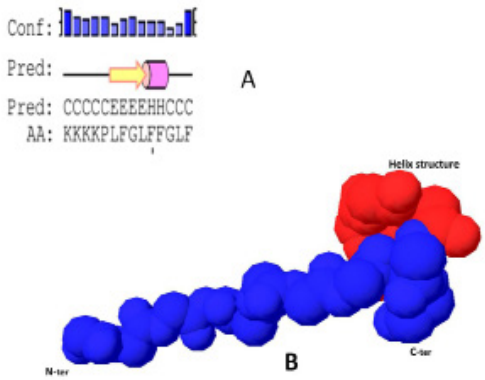

Figure 1. Secondary and tertiary structures of K4 peptide. Helix-strand as secondary and tertiary structures of this peptide was drawn.

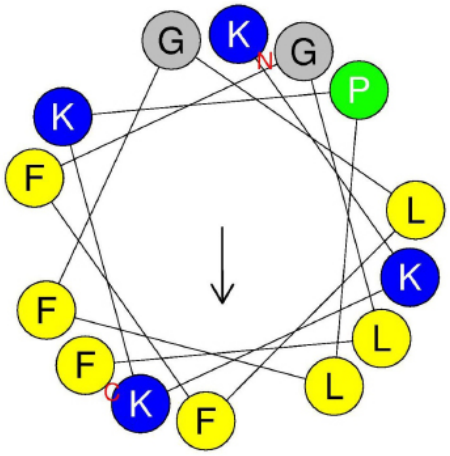

Figure 2. Helical wheel diagram of K4 peptide: The percentage of viable cells in the culture was determined after 24h (A) and 48h (B) by measuring absorption at 540nm.

Measurement of antibacterial activity

The MIC and MBC tests were conducted for seventeen pathogenic bacteria that are reported in (Table 3). The K4 peptide demonstrated an inhibitory range of 25-400 µg/ml against antibiotic resistance to pathogenic bacteria. Its bactericidal activity range was also measured. The lowest MIC and MBC value ranges were both 25 µg/ml in B. melitensis.

Cytotoxicity against human cells

The cytotoxic effects of K4 on Hela cells were evaluated with MTT assay and the dose-dependent results of assay were presented after 24 and 48 h (Figure 3A, B).

Macrophage nitric oxide production induction of K4 peptide.Murine macrophage cell line J774 was used to evaluate the K4 peptide induction of nitric oxide production on the macrophage. Here, Nitric oxide production was measured at various peptide concentrations (Figure 4).

Hemolysis effect of the peptide on erythrocytes

The hemolytic activity of K4 peptide on human erythrocyte was evaluated. The result of hemolytic activity showed that there was 24% hemolysis in 1 mg concentration of this peptide.

Discussion

Anti-microbial peptides with special features

could be useful tools in treating drug resistance to diseases (14). Therefore, we utilized K4 peptide with KKKKPLFGLFFGLF sequence to investigate its anti-microbial activity against clinical antibiotic-resistant bacteria. Results of in silico investigation revealed that K4 peptide with hydrophobicity properties equals 0.644 and 9 amino acids with a net positive charge of +4 are able to affect cell membrane bacteria with suitable efficiency. Moreover, helix-strand as a secondary structure has been shown suitable stability in this peptide. These results are compatible with Hancock's study on the role of antibacterial peptides in infections (15).

The results showed that the MIC and MBC were 25-400 and more than 400 µg/ml. Among all the bacteria, S. aureus and E. cloacae were susceptible to a dose of 50 µg/ml while P. aeruginosa, S. epidermidis, Sh. Sonei, E. faecalis, and B. abortus were inhibited at the concentration of 100 µg/ml in this study. Furthermore, the findings showed that the number of MICs in B. melitensis was the best result in this study, which will be explained. Generally, Brucellosis is considered a zoonotic disease, and this infection could proliferate through penetration into the lymphatic system and spread to other parts of the body. This disease usually could be treated by combination therapy such as tetracycline/aminoglycosides. However, due to drug resistance, this treatment could not eliminate the infection and the disease would often relapse. Drug resistance to issue in B. melitensis and other bacteria in this study could hinder treatment of the infections. To this end, the use of K4 antimicrobial peptides in this study was essential. In the present study, K4 peptide could inhibit and kill B. melitensis at the concentration of 25 µg/ml. Other bacterial infections such as the ones induced by S. aureus are resistant to methicillin and show resistance to common therapies and transfer this resistance to other strains of bacteria. In addition, P. aeruginosa and Acinetobacter baumannii could display resistance to drug treatment through efflux mechanism and β-lactamase gene which have an important role in this field. Therefore, we can partially conclude that K4 peptide as an antimicrobial peptide plays a significant role in the treatment of bacterial infections especially brucellosis.

The result of K4 peptide cytotoxicity test after 24h showed that there was 80% cytotoxicity at a 6.3 µg/ml concentration. In other words, cell viability at 6.3 µg/ml was 20%. In this study, the amount of NO production at 6.3 µg/ml concentration of K4 peptide treated macrophage after 48h was 25.9873 µg/ml. Hemolysis activity on human erythrocytes was also 24% in 1mg concentration of K4 peptide. In this respect, Duval et al., investigated the antibacterial activity of this cationic peptide against gram-positive bacteria such as B. megaterium and S. aureus. The results of their study showed minimal inhibitory concentration ranges of 5-10 µg/ml and 10-20 µg/ml for B. megaterium and S. aureus, respectively (6). Furthermore, Li et al., showed that MIC range of APP peptide was 2-4 µM (16). In the present study, however, K4 peptide MIC range could not show such activity against bacteria. Also, Duval et al., reported that HD50 was 6.65% at 160µg/ml concentration (6). In

Table 3. Evaluated MIC and MBC for seventeen pathogenic bacteria

Bacteria	S. enteritidis	B. cereus	E.coli	K. pneumoniae	S. marcescens	A. baumannii	P. vulgaris	V. cholera	P. aeruginosa	S. aureus	S. epidermidis	N. brasiliensis	Sh. Sonei	B. melitensis	E. faecalis	E. cloacae	B. abortus
MIC (µg)	100	200	100	200	>400	>400	>400	400	100	50	100	400	100	25	100	50	100
MBC (µg)	200	400	100	400	-	-	-	-	200	50	100	-	200	25	100	100	100

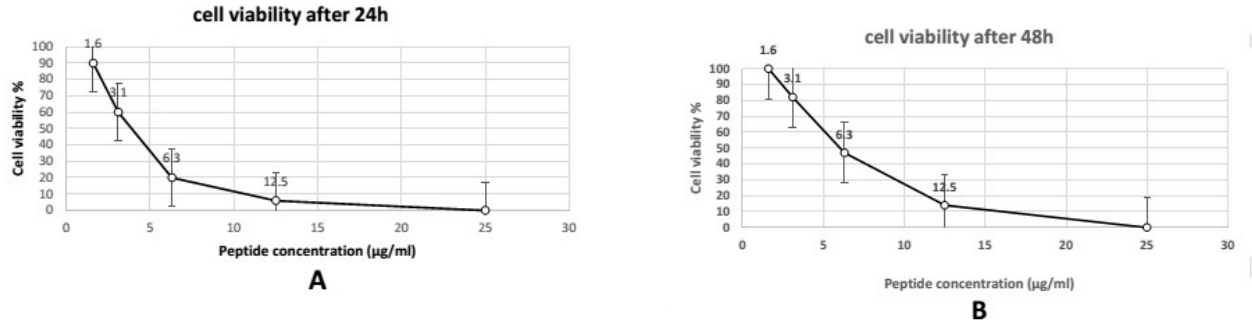

Figure 3. The cytotoxicity of K4 peptide on Hela cell line was measured by MTT assay

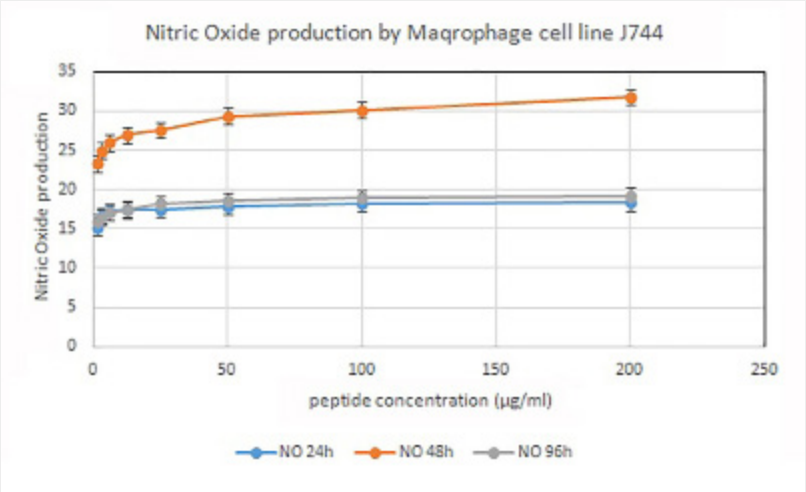

Figure 4. Nitric oxide production of macrophage J774 cell line at various peptide concentrations was measured by Griess assay after 24, 48 and 96 h

another study, Moghaddam et al., investigated cationic CM11 peptide cytotoxicity and showed that there was no significant cytotoxicity on Hela cell line below 6mg/l in the first 24h and 48h. Additionally, they reported HD50 at 64mg/l, which is significantly different from the results of the present study (17). According to the studies above, K4 peptide showed weak antimicrobial activity based on MIC and MBC range. In this study, K4 peptide showed a strong hemolysis activity on human red blood cells, while in other studies it has shown less such activity. Zare_Zardini et al., reported HD50 at 100 µg/ml, and only 3% of human red blood cells were lysed [20]. Several studies have shown the efficacy of cationic antimicrobial peptides in combating infections (4, 5). In this regard, Duval et al., showed that K4 peptide with cationic properties could lysis bacterial cells, and was non-toxic to mammalian cells (6). Furthermore, Lashua et al., showed that de novo engineered cationic antimicrobial peptide (WLBU2) could prevent abiotic bacterial biofilm formation (18). In another study, Marr et al., showed that three synthetic peptides (E6, L-1018, and RI-1018) could exhibit leishmanicidal activity against L. Donovani and L. major (19). Additionally, Zardini et al., showed that antimicrobial activity of Mastoparan-s against microorganisms such as gram-positive, gram-negative bacteria and fungi is really effective. They concluded that this peptide is more effective than kanamycin antibiotic. MIC range of Mastoparan-s is 15.1-28.3µg/ml for bacteria and 19.3-24.6µg/ml for fungi (5). Han et al., showed that di-PH2, di-WP2 and HHP1 antifungal peptides had strong activity against candida strains. The MIC values of these three peptides are 1–2, 2–4 and 2–4 µg/ml, respectively (20). Moreover, another assay which is important in evaluating antimicrobial peptide quality is macrophage toxicity. Thus, the identification of nitrite oxide as a critical factor in a Macrophage cell line is useful in determining bactericidal potency (21). Park et al., studied the effect of thalidomide on nitric oxide production and showed that thalidomide could inhibit nitric oxide production significantly (21). Hoyt's study of the modulatory effect of doxycycline on nitric oxide production in murine lung epithelial cells showed that doxycycline could decrease nitric oxide production from iNOS (22). Macrophage toxicity at different concentrations of K4 peptide was assessed for the first time in the present study. Our study showed that K4 peptide could induce nitric oxide production at a variety of concentrations after 48h. Most nitric oxide is produced 48h after treatment with K4 peptide. Evaluation of cationic K4 peptide with different assays demonstrated that this peptide could have some activity on a wide range of bacteria, and its adverse effect on RBC is noticeable. In the present study, the effect of this short cationic peptide on several pathogenic bacteria was investigated, and also nitric oxide production was measured for the first time. Nitric oxide production by macrophage and other myeloid cells could help eliminate bacterial infections and produce more cytokine to lysis bacteria and also has a role in stimulating innate immunity (23, 24). Taken together, inducing nitric oxide production and maintaining 47% viability cell at 25, K4 peptide seems to have an outstanding antibacterial performance.

Conclusions

The present study demonstrated that short cationic K4 peptides has inhibitory and bactericidal activities against pathogenic bacteria especially B. abortus. Therefore, it can be concluded that K4 antimicrobial peptide could have remarkable effects on important infections. In addition, this peptides may act on immune responses by preventing bacteria from adhering to cells and neutralizing bacterial

toxin and LPS, and could also reinforce innate immunity through stimu lasting nitric oxide production. In the future, we will focus on the interaction between modified K4 peptide and immunity responses.

Authors' contributions
All authors contributed equally.

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Conflict of interest
All authors declare that there is not any kind of conflict of interest.

Funding
There was no founding.

Ethics statement
Not applicable.

Data availability
Data will be provided by the corresponding author on request.

Abbreviations

AMP	Antimicrobial peptides
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide
TI	Therapeutic index

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