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Original Research Article

Design and synthesis of short antimicrobial peptide derivatives based on human cathelicidin

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ABSTRACT

Cathelicidin derived from human leukocytes is a 171amino acid protein that demonstrated to have antimicrobial activity. Synthesis of Antimicrobial Peptides (AMPs) are known to be expensive. In this study**,** after extraction of human cathelicidin sequence from NCBI, physicochemical characteristics such as length, amino acids composition, hydrophobicity and net charge were determined. Several truncated peptides were selected and probability of antimicrobial activity was predicted by Antimicrobial Peptide Prediction software. Purity and molecular weight of the peptides were confirmed by HPLC and mass spectrometry. Eventually, Minimum Inhibitory Concentration (MIC) of these peptides against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were examined by micro-dilution method. Two peptides were synthesized as a result of bioinformatics method. One of the two prepared peptides with 13 amino acids length showed good activity against the bacteria. MIC results of this peptide were 15.6 µg/ml (against *E. coli*), 31.2 µg/ml (against *P. aeruginosa*) and 31.2 µg/ml (against *S. aureus*). Another peptide, Cth14, showed 125 µg/ml against *E. coli*. IC_{50} of Cth13 and Cth14 were 959 µg/ml and 335 µg/ml, respectively.

***Keywords*:** AMPs, cathelicidin, peptide library, *in silico* design, MIC

INTRODUCTION

Considering the worldwide increasing spread of antibiotic resistant bacteria to classical antibiotics, finding of the new antimicrobial agents is necessary [1]. Importance of Antimicrobial Peptides (AMPs) would be regarded as new therapies for infectious diseases [2]. AMPs are a group of natural molecules that produced by the immune system of organisms [3]. Over the past two decades a large number of AMPs have been identified in plants, animals and microorganisms [4]. AMPs show a broad spectrum of activities against gram-negative and gram-positive bacteria, including antibiotic-resistant bacterial strains and some fungi, viruses, and parasites [5]. AMPs also have other biological functions such as apoptosis inducers, wound healing, immune modulatory and even anticancer properties. AMPs can be classified into four groups based on their structures: α-helical peptides, β-sheet peptides, extended peptides, and loop peptides [6]. A further obstacle to the development of AMPs as therapeutics lies in the cost associated with manufacturing large quantities at competitive costs. In addition, AMPs are susceptibility to proteolytic degradation [7]. However, some progress has been made in the recombinant expression of fusion proteins comprising multiple copies of AMPs [5,8]. Despite their diversity in AMPs structure, most antimicrobial peptides share common features that include a net positive charge and an amphipathic character, which segregates hydrophilic and hydrophobic residues to opposite faces of the molecule [9]. Thus, antimicrobial peptides probably also share common mechanisms of bactericidal action [9,10]. Although, many studies showed that bacterial membrane damage is a lethal event for bacteria, other studies demonstrated a mechanism which the peptide binds to several targets in the cytoplasmic region of the bacteria [11]. Cathelicidins are small, cationic antimicrobial peptides found in humans and other species like rabbit [12]. Cathelicidins have been isolated from many of animals such as mammals, birds, reptiles, amphibians, and some fishes [13,14]. Cathelicidins are produced as pre-pro-peptides and stored in granules of the cells. After activation of the cell, they are secreted, and the N-terminal pro-domain which includes the cathelin domain is cleaved off to form the mature that is biologically active peptide [12,13]. Studies showed that Cap18-c (amino acids 137-162) is a LPS binding domain in the C-terminal of human cathelicidins [12]. Therefore,

human cathelicidin is known as a LPS binding peptide [12]. One of the limitations of using antimicrobial peptides is their costly synthesis. One strategy to overcome this problem is truncation of the natural antimicrobial peptides by maintaining their antimicrobial activity. In this study, after truncation of LPS binding domain in the C-terminal of human cathelicidins (amino acids 137-162), probability of antimicrobial activity of these truncated peptides was predicted by Antimicrobial Peptide Prediction software. Also, physicochemical properties of these sequences were evaluated by bioinformatics software. Then, two of them were synthesized. In addition to, purity and molecular weight of the peptides by HPLC and Mass spectrometry were confirmed. Eventually, Minimum Inhibitory Concentration (MIC) of these peptides against *E.coli, S. aureus* and *P. aeruginosa* were examined by micro-dilution method. To determine the cytotoxicity by MTT assay was using Hu02 cells.

MATERIALS AND METHODS

Peptides design and bioinformatics studeis

Initially, after extraction of human cathelicidin protein sequence from NCBI (https://www.ncbi.nlm.nih.gov/), LPS binding domain in the C-terminal of human cathelicidins (amino acids 137-162) was selected and truncated by an *in silico* truncation library. Next, the probability of antimicrobial activity of these truncated peptides was predicted by machine learning algorithms such as Support Vector Machine (SVM), Random Forest (RF), Artificial Neural Network (ANN), and Discriminant Analysis (DA). To achieve these algorithms, the Collection of Antimicrobial Peptides ($CAMP_{R3}$) site (http://www.camp.bicnirrh.res.in/ 2019) was used. The threshold of each algorithm is between 0.5 and 1. Peptides are AMP if this threshold number is more than 0.5. In the following, five of the highest scores sequences from the algorithm were selected and their physicochemical properties such as pI, length, amino acids composition, hydrophobicity and net charge were evaluated by Antimicrobial Peptide Calculator and Predictor from APD3 and ProtParam from Expasy (https://www.expasy.org/ 2018). Presence of antigenic sequence in the designed peptides was predicted by Predicted Antigen Peptide software (http://imed.med.ucm.es/Tools/antigenic.pl). Eventually, two new peptides (Cth13 and Cth14) with the best specifications and Cap18-c as a positive control were selected for synthesis. The PEP-Fold3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/2018)

servers were used to predict the tertiary structure of peptides.

Synthesis of peptides

Two new peptide derivatives (Cth13 and Cth14) and Cap18-c as natural peptide control were synthesized by the solid-phase synthesis method according to Fluorene-9-Methoxycarbonyl (Fmoc)-polypeptide active ester chemistry by Mimotopes Pty Ltd (Clayton, Victoria, Australia) [15]. Synthesized peptides were purified by C18 Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) to 95 % purity. Molecular weights of synthetic peptides were determined by mass spectrometry analyses on a Sciex API100 LC/MS mass spectrometer (Perkin Elm Co., Norwalk, CT, USA) in the positive ion mode.

Bacterial strains, cell line, Chemicals and Compounds

To perform antibacterial tests from *S. aureus* (ATCC 25923), *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used. All bacteria strains were stored in Nutrient broth (NB) supplemented with glycerol (25 %) and maintained at −70 ℃. Human skin fibroblast cell line (Hu02 cell line) was used for toxicity test. This cell is cryopreserved at liquid nitrogen at -196 ℃ (90 % FBS+10 % DMSO at about 1×10^6 cells/vial). NB, Mueller Hinton Broth (*MHB),* Mueller Hinton Agar *(*MHA), ethanol, Phosphate-Buffered Saline (PBS), penicillin and streptomycin were purchased from Merck Millipore Company (Merck, Darmstadt, Germany). Fetal Bovine serum (FBS) and RPMI medium were obtained from Gibco Company (Gibco, England). Cell culture antibiotics (penicillin, streptomycin and cyclosporin), trypsin, trepan blue dye, NaOH, HCl, MTT dye (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Dimethyl Sulfoxide (DMSO) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Antimicrobial assay

The antimicrobial activity of the peptides was investigated by using a serial dilution titration method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines, to determine MIC of the peptides against three types of bacterial strains [16]. Briefly, bacteria were grown overnight at 37 ℃ in *MHB* and were diluted in the same medium. 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 give a final inoculum of 5×10^5 colony-forming units (CFU)/mL. The plates were incubated at 37 ℃ for 24 h and 48 h, and the MIC was

determined [17]. Then, 100 µL of the initial inoculum of 5×10^5 CFU/mL was platted on Mueller-Hinton agar as the positive control, and 100 µL of the 24 h inhibitory concentration test samples was platted on MHA to determine the Minimal Bactericidal Concentrations (MBC) [18].

MTT assay

To investigate the toxicity of Cth13 and Cth14 peptides and Cap18-c, Hu02 cell line was cultured at 1×10^5 cell/well in 96-well plates for 24 h under optimal conditions (37 ℃, 5 % CO2 in humidified incubator). Then, the growth media (10 % FBS) was removed and the cells were washed two times with PBS. New maintenance RPMI (Gibco, Carlsbad, CA, USA) medium (10 % FBS) containing 0.5, 5, 50, 500, and 1000 µg/mL of each peptide were added and the cells were incubated for 24, 48, and 72 h [19]. Quintet wells were analyzed for each concentration and column elution buffer was used as a control. A 10 µL solution of freshly prepared 5 mg/mL MTT in PBS was added to each well and incubated for an additional 4 h. Next, the media was removed and isopropanol was added at 100µL/well. Plates were then shaken gently to facilitate formazan crystal solubilization. The absorbance was measured at 545 nm using a microplate reader (STAT FAX 2100, USA) [20].

Percentage of cell viability was measured as follows:

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

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

Statistical analysis

All results are reported as mean±SD. Statistical analysis for the comparison of MIC and toxicity values of Cth13 and Cth14 was carry out by a t-test by SPSS Statistics 22.0 software (SPSS Inc. Chicago, IL, USA).

RESULTS

Design and synthesis of peptides

At first, human cathelicidins peptide sequence was obtained from the NCBI database (Figure 1). In the following, of the twenty truncated sequences based on LPS binding domain in the C-terminal of human cathelicidins (amino acids 137-162) (Figure 1), ten truncated sequences with 6 to 15 residues were selected (Table 1) and probability of antimicrobial effects of these truncated peptides was predicted by machine learning algorithms (Table 1). According to prediction scores and physicochemical properties, Cth14n and Cth12n were selected and in order to increase the probability of having antimicrobial properties, some of mutations were created in sequences of their

peptides and their antimicrobial properties were again predicted (Table 1 and Figure 1). Also, physicochemical properties of these new sequences and Cap18-c such as hydrophobicity, net charge, pI, GRAVY index and Boman index were predicted The last two peptides were called Cth13 and Cth14 (Table 2). Results of Predicted Antigen Peptide software predicted that there are no antigenic determinants in newly designed peptides while Cap18-c has one antigenic determinant in its sequence at position14 to 21 (Figure 2). The prediction results of the 3D structure in peptides showed that α-helix and random coil are major structure in the AureinN2 peptide (Figure 3). All peptides were commercially synthesized by Mimotopes Pty Ltd (Clayton, Victoria, Australia) by a solid-phase method using N-9 fluorenylmethoxy carbonyl (Fmoc) chemistry and were supplied in 95 % purity (by RP-HPLC) with satisfactory mass spectra.

Table1. Ten of selected sequences for prediction of probability of antimicrobial activity and two modified sequences

Peptides name	Peptides sequence	Score of algorithms			
		SVM	RF	ANN	DSA
Cap18-c	DFFRKSKEKIGKEFKRIVQRIKDFL	0.769	0.632	AMP	0.952
Cth15n	DFFRKSKEKIGKEFK	0.569	0.451	AMP	0.172
Cth14n	DFFRKSKEKIGKEF	0.405	0.379	AMP	0.062
Cth13n	DFFRKSKEKIGKE	0.556	0.399	AMP	0.058
Cth12n	DFFRKSKEKIGK	0.853	0.413	AMP	0.192
Cth11n	DFFRKSKEKIG	0.487	0.3385	AMP	0.028
Cth10n	DFFRKSKEKI	0.508	0.328	AMP	0.041
Cth9n	DFFRKSKEK	0.541	0.385	AMP	0.022
Cth8n	DFFRKSKE	0.074	0.3495	AMP	0.003
Cth7n	DFFRKSK	0.123	0.4195	AMP	0.115
Cth6n	DFFRKS	0.001	0.366	AMP	0.016
Cth14	WFFRKSKIKIGKKF	1.00	0.946	AMP	0.981
Cth13	WFFRKSKIKIGKI	0.999	0.956	AMP	0.978

Table2. Predicted physicochemical properties of peptides

Peptides name	Physicochemical properties				
	Hydrophobicity (%)	Net charge	pI	GRAVY index	Boman index

					(kcal/mol)
Cap18-c	36	+5	10.28	-0.916	3.25
Cth13	46	+5	11.39	-0.2384	1.27
Cth14	42	+6	11.33	-0.6214	1.71

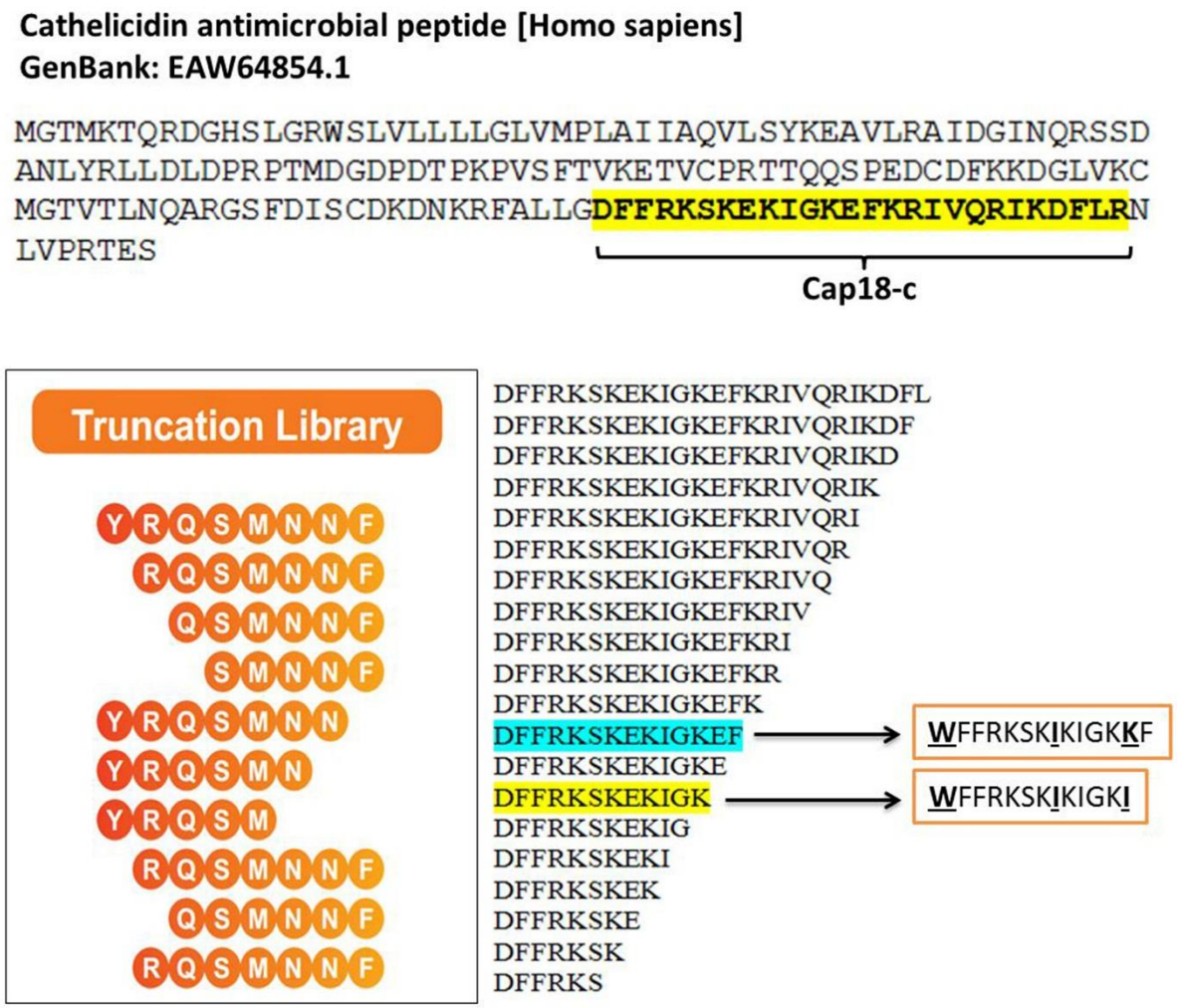

Figure 1. (A) Representation of the human cathelicidin sequence in which the Cap18-c peptide is highlighted in yellow. (B) Truncation steps from C-terminal of Cap18-c peptide.

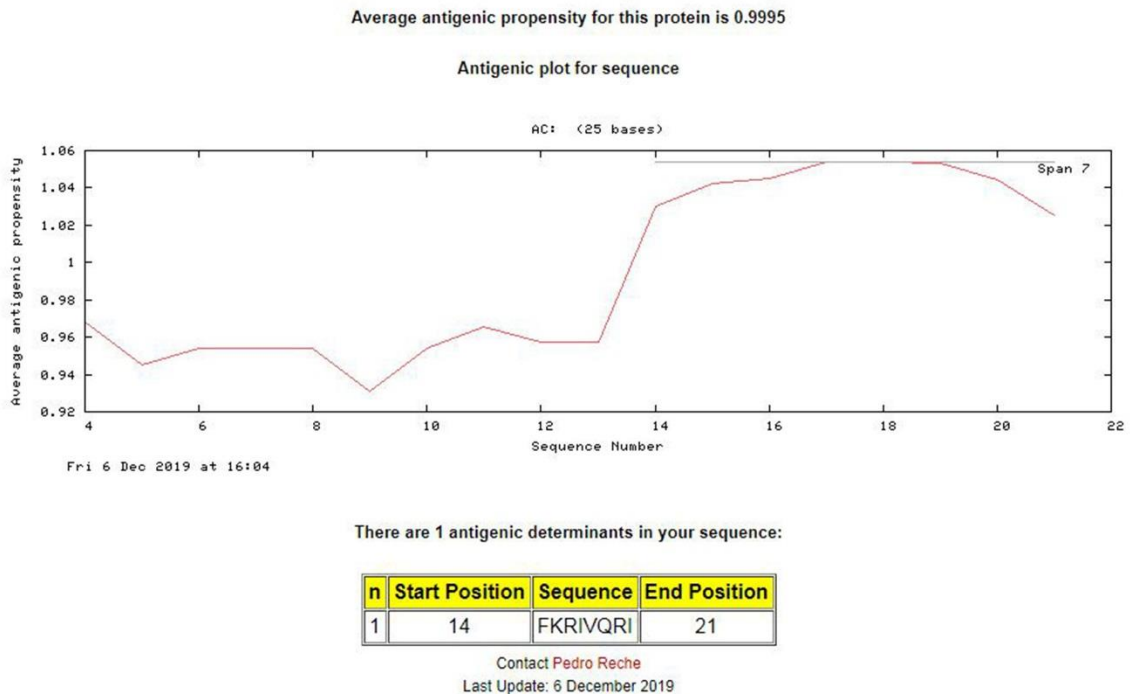

Figure 2. Results of Predicted Antigen Peptide software showed that there is 1 antigenic determinant in Cap18-c sequence at position14 to 21.

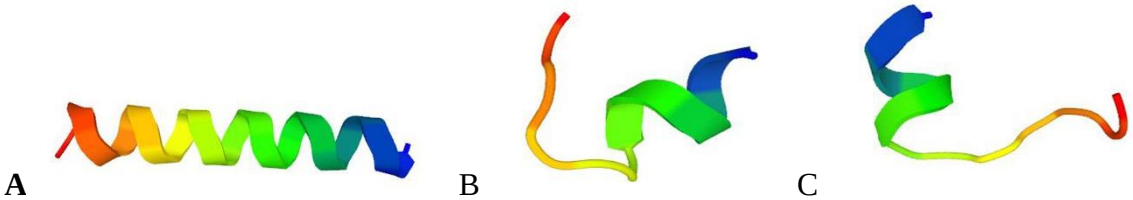

Figure 3. Prediction results of the tertiary structure of peptides by PEP-Fold3 server demonstrated that 3D structure of Cth13 (B) and Cth14 (C) is a mixture of helix and coil while Cap18-c (A) has a helix structure.

MIC and MBC determination

MIC and MBC of Cth13, Cth14 and Cap18-c as positive control and two control antibiotics (penicillin and streptomycin) against *S. aureus, E. coli and P. aeruginosa* were determined (Table 3). Statistical analysis showed that bactericidal activity of Cth13 against all three mentioned bacteria was significantly higher than Cth14. Cth13 also has a significantly stronger antimicrobial effect against *S. aureus* and *P. aeruginosa* than Cap18-c. Cth14 only against *E. coli* had a considerable effect (MIC=125 µg/ml). All tests were conducted

in triplicate and their mean±SD results are shown in Tables 3.

MTT assay results

Based on the toxicity/concentration cruve, the IC_{50} for each peptide was obtained by GraphPad Prism 5.0 software. The results indicated that Cth14 has about 38-78 % toxicity at 50-1000 µg/mL while Cth13 is less toxic than Cth14 (11-50 %) at same concentration. Also, IC_{50} value of Cth13 is higher than Cap18-c and Cth14 so Cth13 is less toxic than them. IC_{50} of Cth13, Cth14 and Cap18-c were 959, 335 and 626 µg/ml respectively (Figure 4).

Table 3. MIC in µg/mL of peptides against Gram-positive and Gram-negative bacteria

Peptides name	**MIC/MBC (**µg/ml)		
	S. aureus	***E. coli***	***P. aeruginosa***
Cap18-c	>1000	31.25/ 125	500/ 500
Cth13	31.25/ 62.5	15.62/ 31.25	31.25/ 31.25
Cth14	250/ 500	125/ 125	125/ 250
Penicillin	1.95/ 3.90	15.62/ 31.25	>1000
Streptomycin	31.25/ 62.5	1.95/ 3.90	31.25/ 31.25

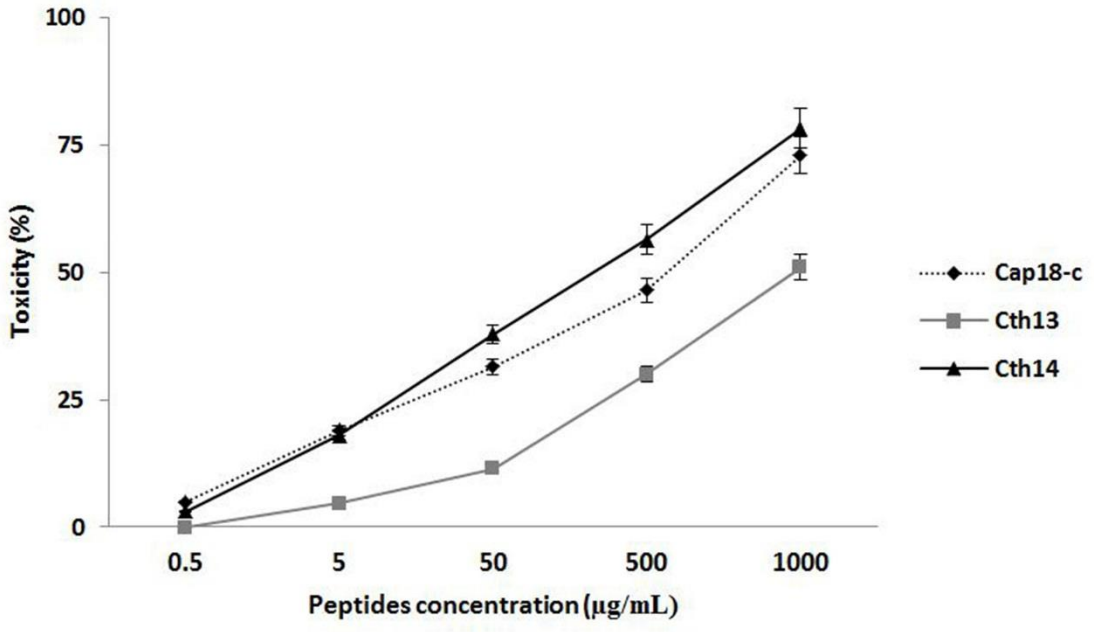

Figure4. This curve shows the toxicity of peptides. The result showed that Cth13 peptide has the least toxicity. Error bars indicate standard deviation.

DISCUSSION

In-silico methods for AMPs design are promising strategies to eliminate of the limitations of natural AMPs [19,20]. Length factor in natural peptides is one of the parameters that increases the cost of their synthesis [7]. Therefor, truncation strategy can be useful for this problem. Also, study of Liu et al showed that hemolytic and cytotoxic effects of the peptides increase with chain length [5]. We also used truncation strategy in this study. In den Hertog et al study, LL-37, a human cathelicidin peptide derivative was truncated from both N and C terminal and truncated peptides were applied against *Candida albicans* [21]. Results of this project demonstrate that LL-37 and its C-terminally truncated peptide LL-31 remain permanently associated with the perimeter of the cell. The N-terminally truncated peptide RK-31 (N terminal truncated with 31 residues) initially accumulated at the cell boundary, but transmigrated into the cytoplasm within 30 min. The C-terminally truncated peptide LL-25 transmigrated instantaneously into the cytoplasm [21]. In this research, LL-31, LL-25 and LL-31 were the most effective peptides against *C. albicans*. We were designed more truncated peptides with 13 (Cth13) and 14 (Cth14) residues that Cth13 was shown good antimicrobial activity against selected bacteria. Cth13 indicated a good antibacterial effect against Gram negative bacteria. MIC of this peptide was almost similar to that of penicillin against *E. coli* and was stronger than that of penicillin against *P. aeruginosa*. Also, Cth13 has less cytotoxicity than other peptides against the Hu02 cell line. Cathelicidins are AMPs that preferentially kill Gram-negative bacteria in vitro, purportedly by assembling into higher-order structures that perforate the membrane [22]. However, Cth13 in our study demonstrated bactericidal activity at concentration of 62.5 μg/mL against *S. aureus.* In a study, Rowe-Magnus et al. used truncated derivatives of cathelicidin in order to evaluation of antibacterial activity (against Gram negative and Gram positive bacteria) and cytotoxic effects [22]. Truncation of six C-terminal amino acids by Rowe-Magnus et al. reduced

killing of *P. aeruginosa*, without significantly affecting killing of *E. coli*. Longer C-terminal truncations caused a complete loss of bactericidal activity [22]. In our Cth13, truncations of 12 C-terminal amino acids from Cap18-c resulted that improved bactericidal activities of this peptide *against E. coli* and *P. aeruginosa*. So it can be concluded that truncation with modification in amino acid residues of peptides to improve of peptide properties can be more useful. Truncations of N-terminal and C-terminal have various effects on activity and cytotoxicity of AMPs. Jittikoon et al investigated effect of N-terminal truncation on antibacterial activity, cytotoxicity and membrane perturbation activity of Cc-CATH3 peptide [23]. In this study, Cc-CATH3 $_{(1-29)}$ as a peptide and the first four-residue truncated peptide Cc-CATH3 $_{(5-29)}$ were used in all experiments, while the eight and twelve residue truncated variants Cc-CATH3 $_{(9-29)}$ and Cc-CATH3 $_{(13-29)}$ were inactive. Cc-CATH3 $_{(1-29)}$ and Cc-CATH3 $_{(5-29)}$ indicated antibacterial activity with Minimum Inhibitory Concentrations of 2-4 and 1-2 μM, respectively. Also, for cytotoxicity, Cc-CATH3 $_{(1-29)}$ and Cc-CATH3 $_{(5-29)}$ displayed cytotoxicity with the IC_{50} values of 9.33 and 4.93 μM, respectively [23]. Therefore, truncation of more than 4 amino acids from the N-terminal of the peptide decreased its activity. In contrast, we truncated 12 amino acids from C-terminal with modification of 3 residues of cathelicidin while antimicrobial activity of Cth13 was enhanced and its toxicity was reduced.

CONCLUSION

Cathelicidin is an important AMPs that is naturally produced by the innate immune system of mammals. Today, many studies have been performed on this peptide. This study has been shown that this peptide has good potential as an antimicrobial compound. One of the limitations of using antimicrobial peptides is their costly synthesis. One strategy to overcome this problem is truncation of the natural antimicrobial peptides by maintaining their antimicrobial activity. We truncated and modified Cap18-c, a part of human cathelicidin. In this study, Cth13 a truncated peptide derivative with 13 residues has more antibacterial activity and less toxicity than parent peptide (Cap18-c). Therefore, truncation and modification in amino acid residues of peptides are good strategies to improve of antimicrobial peptide properties.

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