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Purification and characterization of one novel cationic antimicrobial peptide from skin secretion of *Bufo kavirensis*

[*Bufo kavirensis*'in deri salgısından yeni bir katyonik antimikrobiyal peptidin saflaştırılması ve karakterizasyonu]*

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ABSTRACT

Objective: Antimicrobial peptides are the most important agents in innate immune system. These peptides have suitable activities against different pathogens. The aim of this study was to purify new antimicrobial peptides from skin secretion of *Bufo kavirensis*.

Material and Methods: Purification of peptides was carried out by reverse phase-HPLC and its sequence was determined by using MS/MS. The antimicrobial activity was tested by Radial Diffusion Assay (RDA) and Minimal Inhibitory Concentration (MIC) methods.

Results: This peptide is composed of 20 amino acids with the sequence of: ILGPVLGLVGRLAGGLIKRE. There is no similarity between this new and novel peptide which is named Maximin Bk and other antimicrobial peptides. The Maximin Bk showed considerable antimicrobial activity against gram positive and gram negative bacteria (Minimum Inhibitory Concentrations (MIC 8.1 to 20.78 µg/ml) as well as fungi (MIC, 25.7 to 35.6 mg/ml). Maximin –K showed virtually low hemolytic activity, 5% hemolysis in a concentration of 100 µg/mL.

Conclusion: The new antimicrobial peptide from skin secretion of *Bufo kavirensis* showed higher antimicrobial activity against gram negative than against gram positive bacteria and fungi. In base of high antimicrobial and low hemolytic activity, Maximin Bk is the potent peptide for treatment of various microbial diseases.

Key Words: Antimicrobial peptides, *Bufo kavirensis*, Hemolysis, MIC, RP-HPLC

Conflict of Interest: The authors have no conflict of interest.

ÖZET

Amaç: Antimikrobiyal peptidler doğuştan gelen bağışıklık sistemi için en önemli faktördür. Bu peptidlerin farklı patojenlere karşı aktiviteleri bulunmaktadır. Çalışmanın amacı *Bufo kavirensis*'in deri salgısından yeni antimikrobiyal peptidleri saflaştırmaktır.

Gereç ve Yöntemler: Peptidlerin saflaştırılmasında ters faz-HPLC yöntemi, dizi tayininde MS/MS kullanılmıştır. Antimikrobiyal aktivite Radyal Difüzyon Yöntemi ve Minimal İnhibiyon Konsantrasyonu (MIC) ile saptanmıştır.

Bulgular: Yirmi amino asitten oluşan peptidin dizisi ILGPVLGLVGRLAGGLIKRE şeklindedir. Maximin Bk olarak adlandırılan bu yeni peptid ile diğer antimikrobiyal peptidler arasında herhangi bir benzerlik bulunmamaktadır. Maximin Bk'nın gram pozitif ve gram negatif bakterilere (MIC 8.1-20.78 µg/ml) ve aynı zamanda mantarlara (MIC 25.7-35.6 mg/ml) karşı oldukça güçlü antimikrobiyal aktivitesi bulunmaktadır. Buforin K düşük hemolitik aktiviteye sahiptir (100 µg/mLderişim % 5 hemolize neden olmaktadır).

Sonuç: *Bufo kavirensis*'in deri salgısından elde edilen bu yeni peptid gram negative, gram pozitif bakterilere ve mantarlara karşı güçlü antimikrobiyal aktiviteye sahiptir. Yüksek antimikrobiyal, düşük hemolitik aktivitesine bağlı olarak, Maximin Bk çeşitli mikrobiyal hastalıkların tedavisinde kullanılabileck potansiyel bir peptiddir.

Anahtar Kelimeler: Antimikrobiyal peptidler, *Bufo kavirensis*, hemoliz, MIC, RP-HPLC

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Antimicrobial peptides (AMP) are one of members of innate immune system that have been isolated from a vast number of organisms including bacteria, insects, plants and humans [1]. The majority of these peptides have a cationic and amphipathic property. More than approximately 600 cationic peptides have been discovered in all organisms from microbe to man. These cationic peptides have a broad antimicrobial activity against different microbes such as bacteria, viruses, fungi and etc [2]. They have variety size from 12-50 amino acids [2, 3]. These peptides have the wide variations in their mass, amino acid residue composition, charge, three-dimensional structure and biological characteristics [4]. The skin of amphibian has glands that are rich resources for antimicrobial peptides [5, 6]. Antimicrobial peptides are secreted by these dermal glands and they play a defensive role against harmful microorganisms [5, 7].To date, a lot of antimicrobial peptides such as berevinins, temporins, magainin and dermaseptins have been isolated and purified from amphibians [8-14]. These peptides have several activity such as antibacterial, antifungal, antiviral, anticancer and spermicidal [9, 15-18]. These peptides are suitable alternative to the conventional antibiotic drugs, because of emergence of resistant bacteria and fungi [19]. Twenty species of amphibian exist in Iran: six salamanders in three genera in two families and fourteen frogs and toads in four genera in four families [20]. In Iran, there is several antimicrobial peptides, such as Temporin-Ra and Temporin-Rb [21], isolated from marsh frogs. The toad is widely distributed through the Palearctic and just lives Southwest Asia in Turkey and northern Iran. *Bufo kavirensis* is one of toad that lives in Kavir desert, south of Tehran, which is formed a major part of Iran. No studies have examined the skin secretions of this species [20]. The purpose of this study was identification of new antimicrobial peptides from skin secretion of *B. kavirensis.*

Materials and methods

Materials

TFA (trifluoroacetic acid) and acetonitrile were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Methanol, formaldehyde, acetic acid, sodium chloride, sodium hydroxide, ethanol, TCA, acetone, glycerol and chloridric acid were purchased from Merck Chemical Co. Analytical and semi-preparative columns were purchased from Macherey Nagel GmbH Co. (St. Neumann Neander,Düren, Germany). Ultrafiltration membranes with cut off 1 and 10 kDa were procured from Millipore (Bedford, MA, USA). All other chemicals were used of analytical grade.

Extraction

Adult *B. kavirensis* of both sexes were captured from different locations of desert in Yazd, Iran. The collection of skin secretions was according to our reported method [21]. The animals were washed with distilled water. The dorsal part of the frog was stimulated several times with a 4-6 volt electrical current. The extracts were collected and centrifuged at 10 000 rpm for 15 minutes and the supernatant was lyophilized for subsequent analyses.

Peptide purification

To purification and isolation of low molecular weight compounds, lyophilized extract was dissolved in distilled water and filtered through an ultrafiltration membrane with cut off 10 kDa (Bedford, MA, USA). The filtrated solution was concentrated using of ultra membrane (1 kDa) and lyophilized. Then, the lyophilized extract was solubilized into the smallest volume of distilled water. This material was submitted to fractionation under reverse phase-HPLC with C-18 column (10 × 250 mm) at a flow rate of 2 ml/min, by using a gradient from 5 to 65% (v/v) solution B (0.098% TFA in acetonitrile) during 80 minutes. The elution was monitored with UV detector. According to the absorbance in wavelength 220 nm, each peak was manually collected and lyophilized in a freeze-dryer. Each fraction was assayed to find the major fraction containing antimicrobial peptides. For further purification, the peak of interest (presenting antimicrobial activity) was purified using the method described above except that the elution was conducted using an increasing gradient of solution (0.5% per minute)

Evaluation purity of peptide

To evaluate purity, 40 µg of each peak was checked on Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE Tricine-SDS-PAGE). A stacking gel (6.5% (w/v and also separating gel two-part of 10% and 15% (w/v) were employed. Afterward, the gel was stained with silver according to silver staining [22].

Antimicrobial tests

The antimicrobial effects of collected peptides were investigated with a Radial Diffusion Assay (RDA), as described [23]. An aliquot of bacteria with titer of 4×10^6 CFU was mixed with 10 ml of medium containing both 0.03% TSB and 1% agarose. Holes were created in the medium using a punch, the peptide sample was loaded into the wells and the plates were incubated for 3 h at 37 °C. After three-hour incubation, the secondary medium enriched with 6% TSB and 1% agarose was poured into the plate and were incubated at 37 ºC for 18 hours. Then, the plate was stained for 24 hours using a solution containing the following: 37% formaldehyde, 15 ml; methanol, 27 ml; water, 63 ml; and Coomassie brilliant blue R-250, 2 mg. The plates were destained for approximately 10 min with an aqueous solution of 10% acetic acid and 2% dimethylsulfoxide.

For antifungal activity, One ml of fungal suspension was inoculated in 20 ml of Potato dextrose agar and was poured into the germ culture plates. The holes were then created by the punch in the medium and filled by the plant extract. The plates were incubated for 7 days at 30 to 35°C. Results were recorded during this period [24, 25].

Peptide sequencing by MS/MS

Peptide sequencing was carried out using mass spectrometry in positive ionization mode on a MALDI-TOF/TOF instrument. Purified and lyophilized peptide was reconstituted with 10 µL of 0.1% trifluoroacetic acid (v:v). 1 µL aliquot of each peptide solution was applied directly to a ground steel MALDI target plate immediately by an equal volume of a freshly-prepared 5 mg/mL solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% trifluoroacetic acid (v:v). Bruker flex Analysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra. De novo sequencing was performed by hand, allowing for a maximum mass error of 0.5 Da for any given fragmentation ion. Deduced b- and y-ion series were overlaid onto their fragmentation spectra using the Bruker flex Analysis software (version 3.3).

Peptide sequence analysis

Peptide sequences analysis was done with blast from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the neighbor-joining method. This peptide was aligned with different protein using the CLUSTAL (2.0) multiple sequence alignment. The alignment was then adjusted manually. Then, the phylogenetic tree was drawn and estimated the reproducibility of the tree topology.

Quantification of antimicrobial activity

To quantify of antimicrobial activity, Minimal Inhibitory Concentration (MIC) was determined by a particular method that is used for cationic peptides. Stock serial dilutions of 0.05 to 1 mg/ml (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) of the peptides were prepared and 20µl of peptide stocks were added to a solution containing 10^6 CFU/ml of bacteria which was poured into a plate. The micro plate was incubated at 37 °C for 18 hours. After this time, the absorbance of each well was read at 630 nm using an enzyme-linked immune sorbent assay (ELISA) reader (ELX800TM) and results were compared to the control samples. To determine the fungi-associated MIC, 180 micro liters of Sabouraud Dextrose Agar culture medium, 10 µl of fungal suspension (10^6 CFU/ml), and 10 µl of serial concentration of the plant extract were poured in micro plates and was incubated at 37 °C for 24 hours. The MIC was similarly defined as minimum concentration at which no growth was observed [18, 21, 22]. *Escherichia coli* PTCC2433, *Klebsiella pneumonia* PTCC4231, *Psodomonas aeroginosa PTCC2834, Agrobacterium tumefaciens* PTCC1245, *Bacillus subtilis* PTCC4533, *L. Mesenteroides* PTCC1463, *Staphylococcus aureus* PTCC1442, *Bacillus cereus PTCC1435, Asperjilus Niger*, *Asperjilus fumigates*, *Penicillium lilacinum* and *candidate albicans* were used for MIC determination. Experiments were done in triplicate.

Hemolysis assay

The hemolytic activity of purified peptides was determined using fresh human erythrocytes. Antimicrobial peptide was incubated with human red blood cells and hemolysis assay was carried out according to the method of Asoodeh, Zare-Zardini and Chamani [18, 23]. Hemolysis was also evaluated using hemoglobin release in blood agar plates, indicated by a clear zone around the wells as described above for RDA. Three ml aliquot of red blood cells were suspended in phosphate buffered saline (PBS) and mixed with warmed agar medium at 42 °C immediately poured into a bacterial culture plate. After the medium solidified, wells were created in the medium using a punch, and each well was loaded with 5 µl of a different dilution of the peptide. In one well, 5 µl of Triton X-100 (0.1%) and sterile PBS were used as a positive and negative control respectively. The hemolytic effect appeared as a clear zone around the wells.

Results

Purification of peptides from skin secretions

The skin secretions of *B. kavirensis* were fractionated into 16 fractions by RP-HPLC as indicated in Figure 1. Antimicrobial activities were found to be concentrated on fractions B-K1 to 4. As illustrated in Figure 1a, more than 16 peaks were eluted by C18 RP-HPLC. The eluted fractions indicated by an asterisk in Figure 1a to contain antimicrobial activity (Figure 1, inset and Figure 3). Therefore these peaks were subjected to further purification by using the same RP-HPLC conditions except for the use of a gradient of 0.5% eluent B per minute (Figure 1b, c, d and e). These four peaks were further analyzed by mass spectrometry and subjected to amino acid sequencing.

In vitro antimicrobial activity

The antimicrobial activity of B-K1-4 was examined by the inhibition zone assay. The results are shown in Figure 2. They showed potent antimicrobial activities against the tested microorganisms including gram-positive and gram-negative bacteria and fungi. According to the data of this figure, B-K4 resulted to be more active than B-K1, 2 and 3. All of peptides showed higher antimicrobial activity against gram-negative, gram-positive bacteria and fungi. The antimicrobial activities of these peaks are much more than 30 µg of neomycin used as a control. In addition, their antimicrobial abilities against antibiotics resistant strains (*S. aureus*) are appropriate.

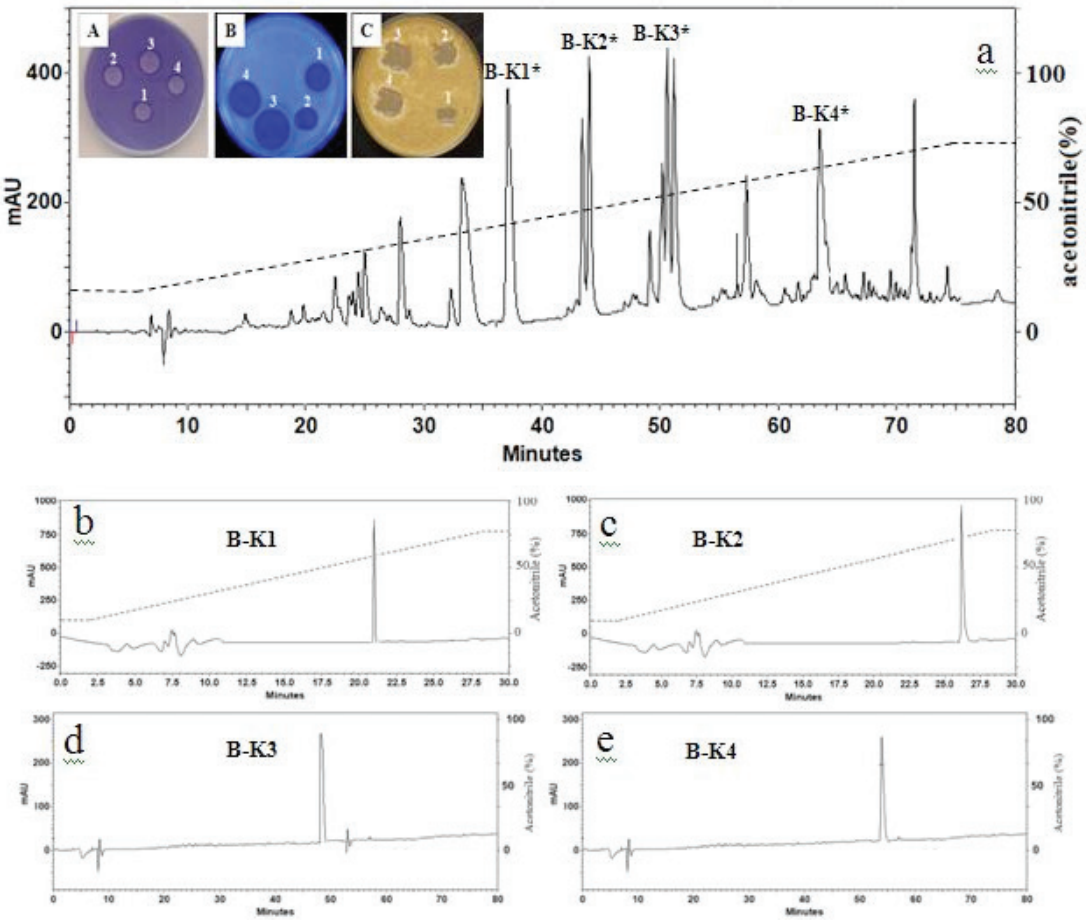

Figure 1. RP-HPLC purification of antimicrobial peptides from skin secretions of *Bufo Kavirensis*. A 400 µl aliquot of filtrated extract was loaded onto a semi-preparative C_{18} reverse-phase column. The elution was performed with a 1% acetonitrile gradient at a flow rate of 2 ml/min. The absorbance was monitored at 220 nm. 16 fractions were eluted from C_{18} reverse-phase column (**a**) * indicates active peaks with appropriate antibacterial effects. The active fractions (indicated by B-K; abbreviation of *Bufo Kavirensis*) were further purified using an analytical C_{18} column by applying a mild slope of the eluent B, at a flow rate of 1 ml/min, (**b**, **c, d and e**). The insets of figure indicates the antimicrobial activity of B-K1 (1), B-K2 (2), B-K3 (4) and B-K4 (3) against *Escherichia coli* (A), *Bacillus subtilis* (B) and *Paecilomyces variotii* (C).

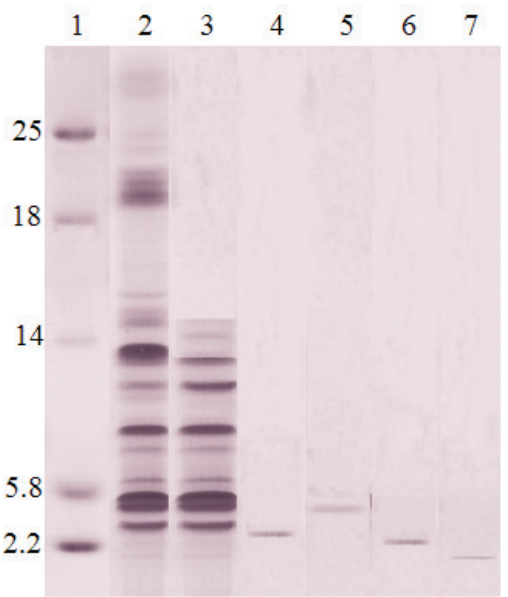

Figure 2. Tricine-SDS-PAGE of size marker (1), raw extract (2), ultrafiltrate extract (3), active fractions obtained from RP-HPLC (4, B-K1; 5, B-K2; 6, B-K3 and 7, B-K4).

Evaluation of purity of peptide

The purity of peptides B-K1-4 were assessed using analytical C_{18} RP-HPLC as well as Tricine- SDS-PAGE, as shown in Figure 3. After all uses of purification methods and according to SDS-PAGE experiment, sharp bands from each fraction were acquired on SDS-PAGE. These antimicrobial peptides had lower molecular weight than 5.8 kD. This result was well overlapping those of acquired molecular weight from peptide sequencing.

Structural characterization

The peaks B-K1, 2, 3 and 4 were analyzed by mass/mass spectrometry. The MS/MS spectrometry analysis of the peak B-K4 was demonstrated in Figure 4. The molecular weights of peaks B-K1, 2, 3 and 4 were 2675.3, 4262.9, 2279.6 and 2012.2, respectively. The observed masses (2675.3, 4262.9, 2279.6 and 2012.2) were matched with the theoretical masses (2675.13, 4262.89, 2279.62 and 2012.22). Their amino sequences are shown in Table 1. By BLAST search, B-K1, 2 and 3 showed sequence similarity to the amphibian antimicrobial Maximin

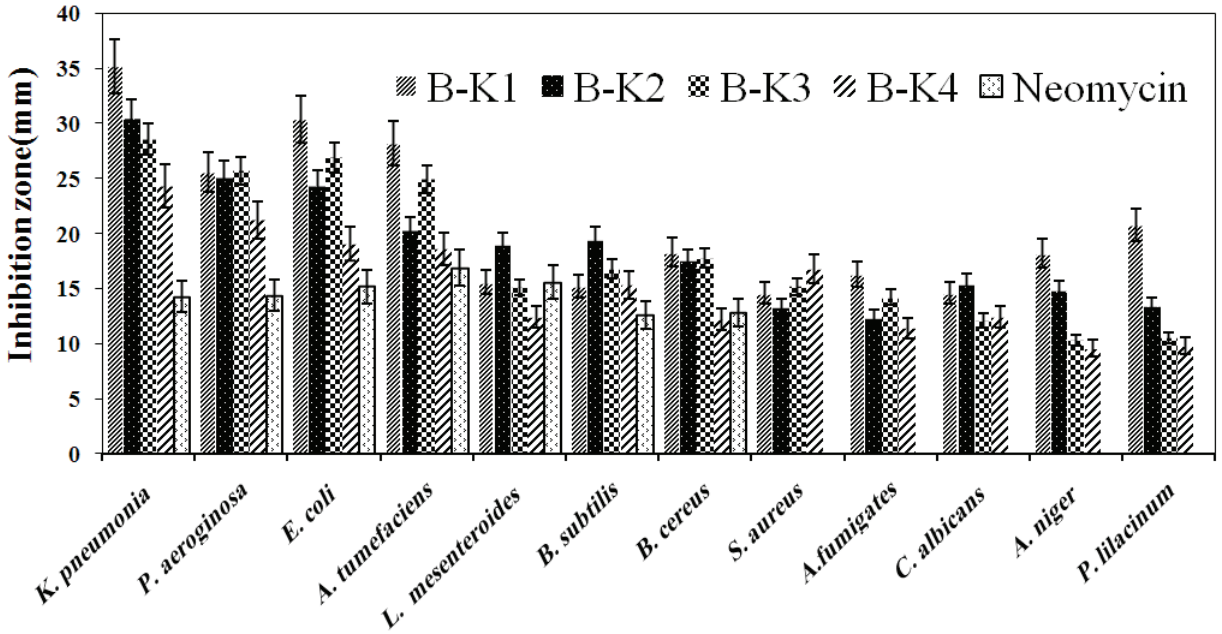

Figure 3. The antimicrobial activity of purified peptides against bacteria and fungi using the RDA method (inhibition zone). The diameter of the non-growth halo was measured and shown as diagram. S.aureus was resistant to neomycin.

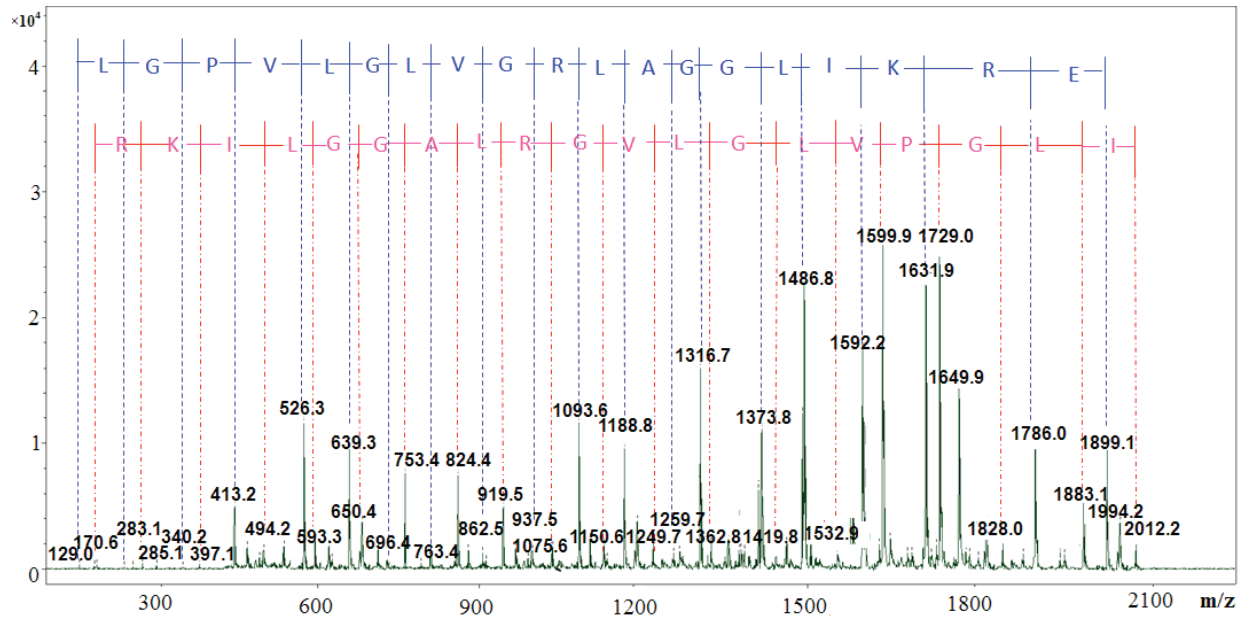

Figure 4. The MS/MS spectra of new peptide isolated from skin secretion of *Bufo Kavirensis*.

Table 1. Primary structure, molecular weight and pI values of the peptides identified from *B. kavirensis*

Peptides	Sequence	Mass(Da)	pI	Homology peptide
B-K1	GIGTKILGGVKTALKGALKELASTYAN	2675.13	10.19	Maximin 1
B-K2	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLR KGNY	4262.9	12.41	Buforin I
B-K3	GLKDIFKAGLGSLVKGIAAHVAN	2279.6	10.25	Alyteserin-1a
B-K4	ILGPVLGLVGRLAGGLIKRE	2012.22	11.08	None found

* *B-KX (B-K:* abbreviation *of B. kavirensis and X: number of peak)*

1, Buforin I and Alyteserin-1a, respectively but B-K4 showed no sequence homology to any AMP in the database, suggesting that it is a novel AMP. It's named Maximin Bk based on a systematic nomenclature for antimicrobial peptides [24]. There are multiple basic amino acids in the sequence of this peptide as found in other antimicrobial peptides. Analysis using the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi_tool.html) showed that this new peptide had the predicted pI (isoelectric point) of 11.08.

MIC determination

Analysis of the antimicrobial activity was carried out on gram positive and gram negative bacteria as well as fungi as listed in Table 2. As shown in this table, the peptide Maximin Bk showed total inhibition of bacterial growth at very low concentrations (≤ 21 μg/ml). Maximin Bk also demonstrated fungicidal activity against four species, *Asperjilus Niger*, *Asperjilus fumigates*, *P. lilacinum* and *candidate albicans* at low concentrations (≤ 36 μg/ml). These data showed that Maximin Bk was more active against gram negative than gram positive bacteria and fungi. The sensitive strains had not capability of growth after a 6-h treatment with concentrations above the corresponding MICs. Among the tested microorganisms, the new peptide showed the strongest antimicrobial activities against *E. coli* and *K. pneumonia*. The MICs against *E. coli* and *K. pneumonia* were <9 μg/ml. This peptide exhibited antimicrobial activity against resistant hospital bacteria (*S. aureus*).

P*hylogenetic analysis*

The result of BLAST showed that Maximin Bk has a high homology to the Maximin AMPs from *Bombina maxima* (Figure 5). Phylogenetic analysis of the amino acid sequences was carried out by constructing a phylogenetic tree. The phylogenetic tree showed that Maximin Bk had the highest similarity to Maximin [25, 26]. Despite these similarities, Maximin Bk can be considered as a novel peptide that belongs to the Maximin family (Figure 5). Due to this, this new peptide was named Maximin Bk in accordance with nomenclature rules which recently suggested for peptides [24].

Hemolytic activity testing

Human red blood cells were used to examine hemolytic activity. Maximin Bk showed low hemolytic activity. At a concentration 100 μg/ml, Maximin Bk induced 5% human RBC hemolysis. It was observed that this new peptide exhibited little hemolytic effect near its MIC value (≤ 36μg/ml). Maximin Bk in range of 5-100 μg/ml showed moderate hemolytic activity hemolytic assay by RDA method (using blood agar) (Figure 6, inset). Thus, very slight hemolytic activity was obtained against human erythrocytes (<5.5% at 100 μg/ml).

Discussion

Antimicrobial peptides play key roles in innate immunity against harmful microorganisms [1]. Amphibian skins are potential storehouse to purify antimicrobial peptides. Their skins are complex organs and act as a defensive system. Upon amphibians are exposed to dangers of microorganism, their skins secrete a different antimicrobial peptides [26, 27]. Several groups had purified the number of antimicrobial peptide from skin of different toads such as *Bombina orientalis*, *Bombina maxima*, *Floodplain toadlet*, *Bufo bufo gargarizans*, *Australian toadlet* and *Yellow-bellied toad* [28-30]. B. *kavirensis* is one of toad that live in Kavir desert

Table 2. The MIC values of Maximin Bk against various microbes.

Name of organism	MIC(μg/ml)*
Gram positive	
L. mesenteroides	20.78±1
B. subtilis	19.4±0.9
B. cereus	18.5±1.2
S.aureus	16.3±1.1
Gram negative	
K. pneumonia	8.9±0.8
P. aeroginosa	10.3±0.6
E. coli	8.1±0.7
A. tumefaciens	12.1±0.8
Fungi	
A.fumigates	35.6±1.5
C. albicans	32.1±1.1
A. niger	28.6±1.5
P. lilacinum	25.7±1.2

* The concentration of an antimicrobial peptide to inhibit of bacterial or fungal growth after 24 h at 37°C.

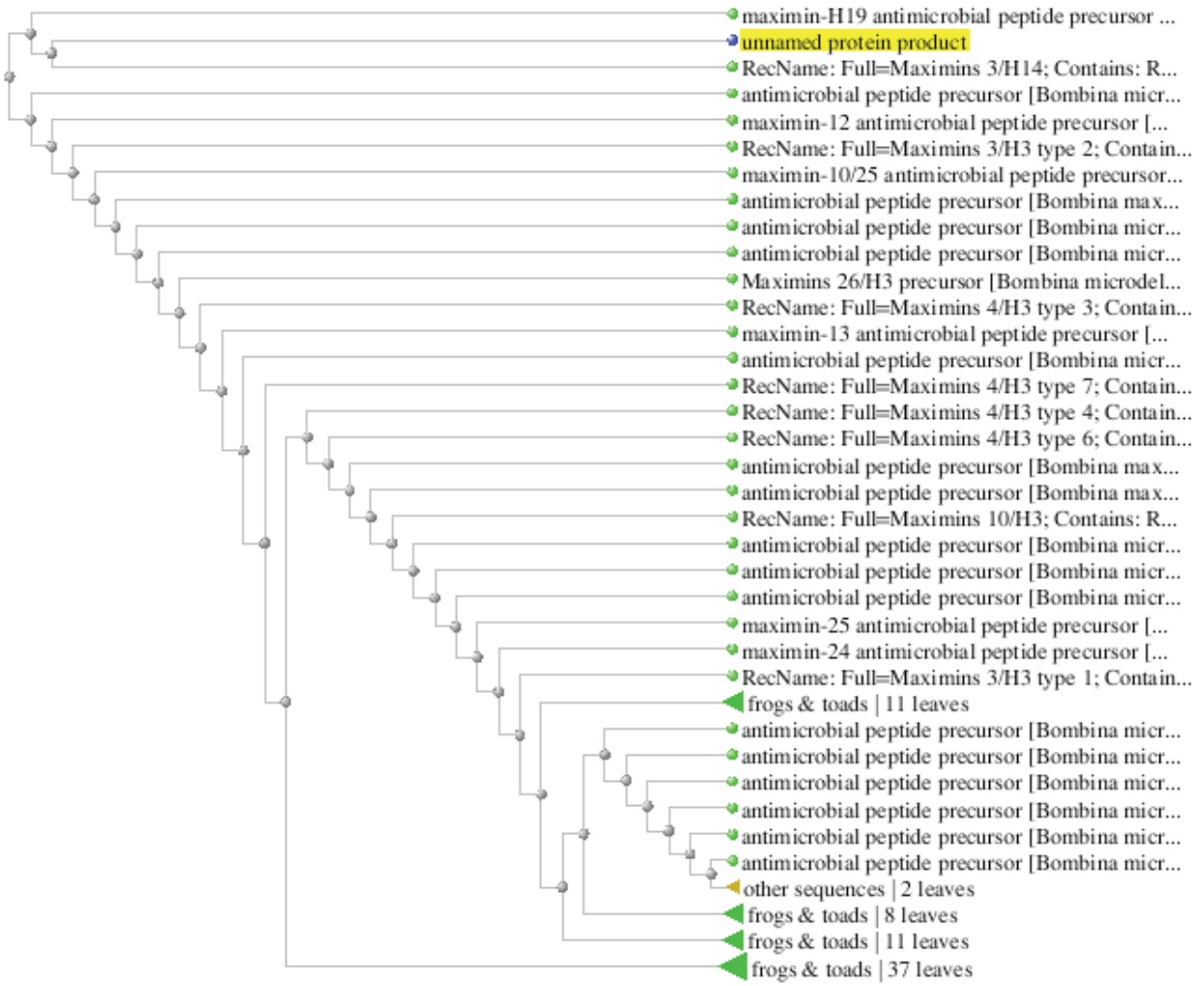

Figure 5. The phylogenetic tree of Maximin Bk. The alignment was carried out with CLUSTAL (2.0) multiple sequence alignment. Amino acid sequences were incorporated into the tree using the neighbor-joining method. The name of each sequence is typed at the end of the corresponding branch. Maximin Bk was shown as unnamed protein.

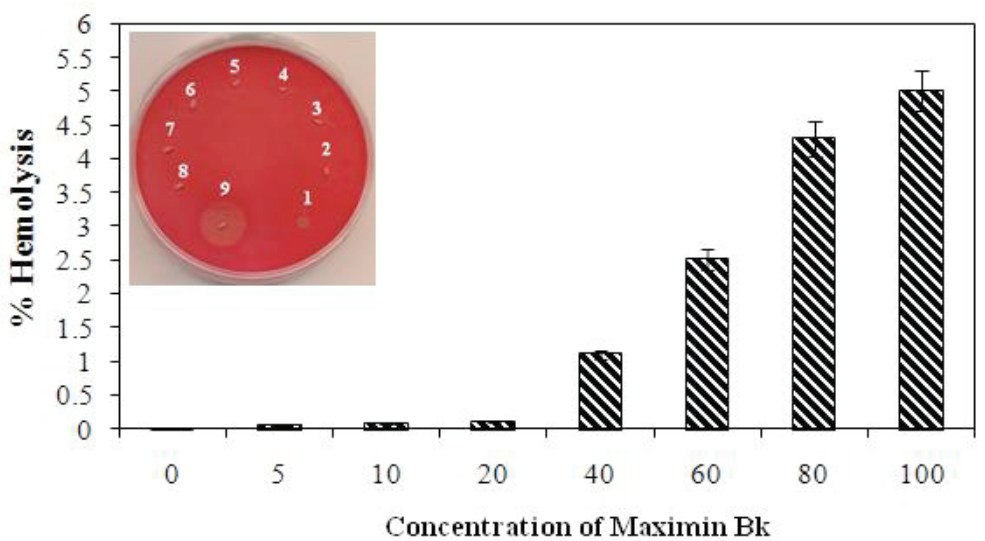

Figure 6. Hemolytic activities of the antimicrobial peptide, Maximin Bk, isolated from *Bufo Kavirensis*. The insets of figure indicates the Hemolysis assay of Maximin Bk in the range of 5-100 µg/ml based on the method of RDA (1, 100 µg/ml; 2, 80 µg/ml; 3, 60 µg/ml; 4, 40 µg/ml; 5, 20; 6, 10 µg/ml; 7, 5 µg/ml; 8, PBS and 9; Triton X-100).

Table 3. Comparison of the MIC values against bacteria between Maximin Bk and some AMPS derived from different amphibians

Peptide	Sources	MIC (μg/ml)	References
Ascaphin-1	*Ascaphus truei*	>50	[36]
Ascaphin-7	*Ascaphus truei*	25	[36]
Odorranain-HP	*Odorrana grahami.*	30	[35]
Brevinin-1CDYa	*Rana chensinensis*	12.5	[29]
Japonicin-1CDYa	*Rana chensinensis*	25	[29]
Temporin-CDYb	*Rana chensinensis*	>100	[29]
RTCI	*Rana Temporaria Chensinensis*	62.5	[37]
Brevinin -2GHa	*Hylarana guentheri*	14.9	[38]
Brevinin -2GHb	*Hylarana guentheri*	16.5	[38]
Brevinin -2GHc	*Hylarana guentheri*	35.5	[38]
Guentherin	*Hylarana guentheri*	9.8	[38]
Temporin-GH	*Hylarana guentheri*	44.3	[38]
Dybowskin-112,	*Rana dybowskii*	25	[39]
Dybowskin-26.25	*Rana dybowskii*	15	[39]
Tk	*Amolops loloensis*	15	[40]
Maximin Bk	*B. kavirensis*	<20	This study

south of Tehran, Iran [20]. In this study, one novel antimicrobial peptide was purified and sequenced from the skin secretion of *B. kavirensis*. This peptide was named Maximin Bk. According to chromatogram, *t*his new peptide is hydrophobic (Figure 1), as reported by other authors [15]. The estimated amino acid sequences of purified peptide, experimental molecular masses and iso-electric point are summarized in Table 1. Based on Table 1 and gel electrophoresis patterns (Figure 2), this peptide, like many antimicrobial peptides, has low molecular weight [31-33]. Most antimicrobial peptides are 10–50 amino acids in length and the new peptide, Maximin Bk, has 20 amino acids [15, 34]. The net charge and total hydrophobic ratio of this new peptide is +2 and 50 %, respectively. Net charge and number of amino acids in Maximin Bk is similar to those of other reported antimicrobial peptides from toads [30, 35, 36]. Computer alignments with published sequences of other antibacterial peptides revealed striking similarities between parts of this peptide and Maximin [30, 35], as shown in Figure 5. This new peptide belongs to the Maximin family. Maximins constitute a family of antimicrobial peptides isolated from skin secretions of the Chinese red belly toad Bombina maxim [28]. All maximins except Maximin 9 and Maximin S4 have broad antimicrobial activity against gram-positive and gram-negative bacteria and they have spermicidal activity also cytotoxic property against tumor cells. Maximin Bk also exerted antimicrobial activities against gram negative and gram positive bacteria and fungi (Table 2). Many hypotheses have been presented that how antimicrobial peptides kill microbes. This new peptide could target cell walls, cell membrane, cytoplasm or other sites as some other antimicrobial peptides [17]. The MIC comparison between Maximin Bk and the other peptides reported so far show that this peptide has low MIC values. As shown in Table 3, the MIC value of Maximin Bk is much lower than Ascaphins [37], Odorranain-HP [38], Brevinin-1CDYa, Brevinin-2GHa, Temporin-GH, Japonicin, Dybowskin-112 [39, 40], and so forth. Moreover, Maximin Bk has a low percentage of hemolytic property. Therefore, Maximin Bk might provide novel candidate for treatment of microbial disease. However, more detailed researches as an indication of its antimicrobial activity in vivo are required.

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