

# Molecular characterization and bioactivity evaluation of two novel bombinin peptides from the skin secretion of Oriental fire-bellied toad, *Bombina orientalis*

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**Abstract** Following the exploration of biochemicals in amphibian defensive skin secretion, great attention has been focused on the novel bioactive peptides with unique molecular structures and complicated features and functions. In this study, the skin secretion of Oriental fire-bellied toad, *Bombina orientalis*, was acquired to search peptides with therapeutic potential. Using “shotgun” cloning technique, a full-length peptide precursor co-encoding two novel bombinin peptides was cloned from the skin secretion-derived cDNA library of *B. orientalis*. The deduced peptides were identified as one bombinin-like peptide (BLP) (GIGSAIL-SAGKSIKGLAKGLAEHF-NH<sub>2</sub>) and one bombinin H-type peptide (BH) (IIGPVLGLVGKALGGLL-NH<sub>2</sub>). The primary structures of both peptides were confirmed through reverse-phase HPLC fractionation and mass spectrometry. Secondary structural prediction revealed Bombinin-BO1 and Bombinin H-BO1 adopted  $\alpha$ -helical structural features. In addition, the two peptides exhibited broad-spectrum antimicrobial effect against Gram-positive and Gram-negative bacteria and yeast. Meanwhile, the anticancer activity assay indicated both peptides exerted significant anticancer

effects against human hepatoma cell lines tested (Hep G2/SK-HEP-1/Huh7). The peptides reported here for the first time may represent novel lead compounds for the design/development of new therapeutics for human infection and neoplastic disease.

**Keywords** Bombinin · “Shotgun” cloning · Mass spectrometry · Antimicrobial peptide · Anticancer peptide

## Introduction

Amphibians are well known to represent valuable biological resources and the source of potential therapeutic tools through folk medicines and traditional knowledge (Novkovic et al. 2012; Pukala et al. 2006). The skin secretions of the dorsal glands of amphibians provide a rich chemical pool of host-defense compounds, which are part of the animal’s own defense system against microorganisms and predators (Conlon and Sonnevend 2010). The secretions contain a great variety of peptides with diverse bioactivities including antimicrobial, anticancer, antiviral, analgesic, immunomodulatory and some of them are neuropeptide analogs (Clarke 1997; Stevens et al. 2007; van Zoggel et al. 2012). The functional diversity of amphibian skin peptides has made it a unique source of leads and structural templates from which new therapeutic agents may be developed. Moreover, such peptides could also be used to investigate the taxonomic relationships between different amphibian species in phylogenetic studies.

In this paper, two novel bombinin homologues have been identified from the skin secretion of Oriental fire-bellied toad, *B. orientalis*. The genus *Bombina*, belonging to the toad family Bombinatoridae, is a group of small toads (most species no longer than 4.1 cm or 1.6 in) distributed in the

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moderate climate areas in Europe and Asia (Simmaco et al. 2009). This toad genus is an excellent source of bioactive peptides with antimicrobial and pharmacological activities. So far, more than 200 peptides from this toad taxon have been reported in the scientific literature and their structures have been deposited in genomic and proteomic data banks such as the Universal Protein Resource Consortium (UniProt) (Lai et al. 2002; Miller 1990; Simmaco et al. 1991, 2009). One of the best known *Bombina* skin peptides is bombesin, which is a homolog of mammalian gastrin-releasing peptide, and also a tumor marker for neuroendocrine carcinomas (Gonzalez et al. 2008; Chejfec et al. 1985). Besides, several other bioactive peptide families have been identified from the *Bombina* species, including bradykinins, maximakinins, bombinins, prokineticins, thyrotropin-releasing hormone (TRH), and trypsin/thrombin inhibitors (Chen and Shaw 2003; Chen et al. 2005; Mangoni et al. 2000).

Bombinin is a group of antimicrobial peptides (AMPs) only detected in the defensive skin secretion of *Bombina* frogs (Gibson et al. 1991). The bombinin peptide family contains two subfamilies. One subfamily contains bombinin and bombinin-like peptide (BLP), which have a similar structure with original bombinin (Simmaco et al. 1991; Gibson et al. 1991). Member of the other subfamily was discovered from the same sequence of the bombinin precursor. As they are more hydrophobic and have more hemolytic activity compared with bombinins, they were named as bombinin H-type peptides (BHs) (Mignogna et al. 1993). Although bombinins (or BLPs) and BHs possess different amino acid compositions, they share several common structural features, such as C-terminal amidation, positively charged, and amphipathic helices in membrane mimetic environments (Gibson et al. 1991; Mangoni et al. 2000). BLPs are considered as promising antimicrobial agents owing to their obvious antibacterial effect without appreciable hemolytic capacity. They have been reported to exert broad-spectrum antimicrobial activities against Gram-positive and Gram-negative bacteria, as well as fungus. Nevertheless, the BHs generally showed lower antimicrobial effect but higher hemolytic activity (Coccia et al. 2011; Gibson et al. 1991; Miele et al. 2000).

Here, we have isolated and characterized two novel bombinin peptides, named Bombinin-BO1 and Bombinin H-BO1, respectively, from the skin secretion of Oriental fire-bellied toad, *B. orientalis*. The primary structures of both peptides were determined by combinations of “Shotgun” cloning and mass spectrometry (MS) techniques. Molecular cloning of the cDNA encoding precursor of these bombinins was achieved from skin cDNA libraries of *B. orientalis*. The amino acid sequences deduced from the nucleotide sequences of cloned precursor cDNAs corresponded exactly with those obtained by mass spectrometric techniques. Bioassays on both bombinin peptides have shown they have antimicrobial effects against *S. aureus*, *E.*

*coli*, and *C. albicans*. Moreover, both peptides also exhibited potent anticancer effects on human hepatoma (Hep G2/SK-HEP-1/Huh7) cell lines.

## Materials and methods

### Acquisition of *B. orientalis* skin secretion

Oriental fire-bellied toads, *B. orientalis* ( $n = 10$ , both sexes, 4–6 cm snout-to-vent lengths), were obtained from a commercial source in China. All toads were maintained in our purpose-designed amphibian terrarium under a 12/12-h light/dark cycle at 20–25 °C and were fed with *Tenebrio molitor* three times per week. Toad skin secretion was obtained from the dorsal skin using gentle massage as previously described (Hou et al. 2015). The secretions were washed from the skin using distilled–deionized water and dissolved in lysis buffer for RNA extraction, or in 0.05% trifluoroacetic acid (TFA) for peptide identification.

### “Shotgun” cloning of *B. orientalis* skin secretion-derived cDNA library

Lyophilised skin secretions (10 mg) were dissolved in 1 ml of cell/tissue lysis buffer supplied by Takara, JP. Utilizing TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Tokyo, Japan) as described by the manufacturer, the total RNA was isolated and subsequently transcribed into cDNA library using reverse transcriptase. Then a 3'RACE-PCR procedure was employed to amplify the full-length bombinin peptide precursor nuclear acid sequence that was coupled to the 3' end of cDNA library using a SMARTer RACE kit (Clontech, Mountain View, CA, USA). Briefly, the 3'RACE reactions employed a NUP primer (supplied with the kit) and a sense primer (S1: 5'-GATGAWKTTTAAGTACATA RTTGCRGT-3') (W = A/T; K = T/G; R = A/G) that was designed to a highly conserved domain of the 5'-untranslated region of previously described bombinin peptide precursor cDNAs from *Bombina* species (Bai et al. 2014). The thermal cycling was completed using the following parameters: initial denaturation step: 94 °C for 1 min; 35 cycles, in each cycle: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 3 min. The RACE products were purified and cloned by means of a pGEM-T Easy Vector System (Promega, Madison, WI, USA) and then sequenced by Sangon Biotech (Shanghai, China). The nucleotide sequences were edited using Chromas and Vector-NTI software programs (Invitrogen, Carlsbad, CA, USA). The amino acid sequences alignments were performed using AlignX software (Invitrogen, Carlsbad, CA, USA).

### Peptidomic analysis of novel peptides in skin secretion

A further 5 mg of lyophilized skin secretion was dissolved in 1 ml of TFA and centrifuged at  $3000\times g$  for 10 min for clarification of microparticle. Before separation, the column was equilibrated in TFA/water (0.05/99.95, v/v) at a flow rate of 1 ml/min for 20 min, then 200  $\mu$ l of the sample supernatant was directly injected onto the HPLC and eluted using a gradient formed from 0.05/99.95 (V/V) TFA/water to 0.05/29.95/70.0 (V/V/V) TFA/water/acetonitrile on a Jupiter C-5 analytical column (5  $\mu$ , 250  $\times$  10 mm, 300  $\text{\AA}$  pore, Phenomenex, Torrance, CA, USA) in 240 min at a flow rate of 1 ml/min by a pumping system with the detection wavelength of 214 nm. Fractions (1 ml) were collected every 60 s and the molecular masses of peptides in each fraction were analyzed by electrospray ion trap mass spectrometer (Waters Corporation, Milford, MA, USA). Subsequently, the peptides with the molecular weight close to novel precursor cDNA-encoded peptides were chosen for further structural characterization using Solarix FT-ICR-MS (Bruker Daltonics, Billerica, MA, USA).

### Solid-phase peptide synthesis

Solid-phase synthesis is a process by which chemical reactions can be performed on a solid support to synthesize a wide range of compounds. Here, this process was carried out to synthesize the two bombinin peptides using an automated solid-peptide synthesizer. First, all of the amino acids (10 mg) were weighed and mixed with HBTU activator and added to the amino acid tubes. The synthesizer worked by coupling carboxyl-activated amino-protected amino acids at the N-terminal end of the resin-bound peptide chain. After the peptide synthesis was completed, the resin was washed with DCM several times, disposed with cleavage reagent (95/2.5/2.5 (V/V/V) TFA/water/Tips) for 6 h to get the peptide remove from the resin. The peptides were purified by HPLC (Agilent Technologies, Santa Clara, CA, USA) and the structures were authenticated by a LC/MS system (Waters Corporation, Milford, MA, USA).

### Prediction of physicochemical properties of peptides and their secondary structures

Physicochemical properties and secondary structures are critical factors in controlling the bioactivity of peptides. So it is important to use reliable *in silico* methods to rapidly estimate the peptides' physicochemical properties and secondary structures before analysis. The online bioinformatic tool, Heliquest (<http://heliquest.ipmc.cnrs.fr/>) was used to predict the peptides physicochemical properties, such as hydrophobicity, hydrophobic moments and net charges (*Z*). The secondary structures of peptides were estimated through

software modeling by I-TASSER online server (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>).

### Minimum inhibitory concentrations and minimum bactericidal concentrations assays

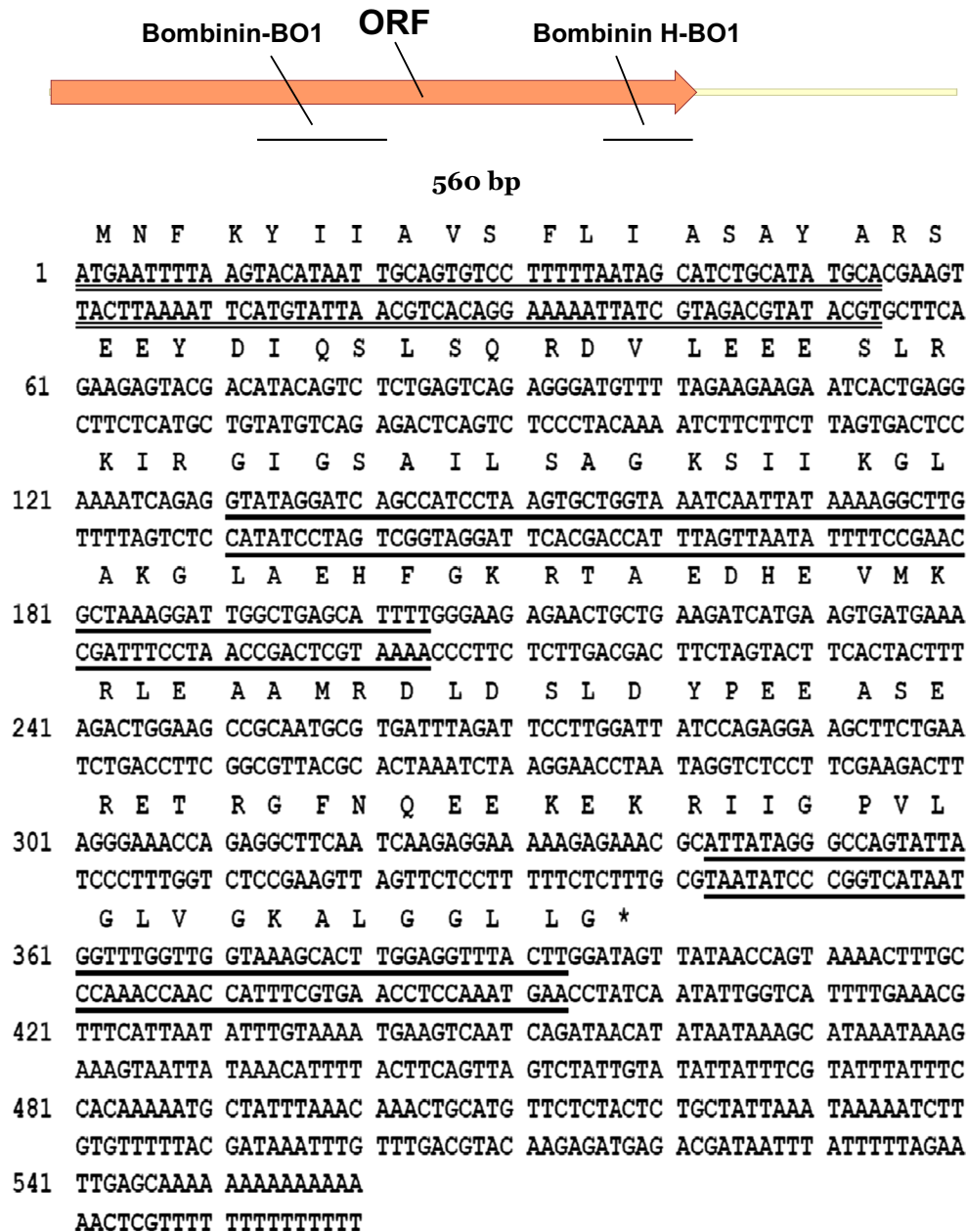
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays are basic methods used in the laboratory to determine the efficiency of new antibiotics to inhibit microbial growth. The antimicrobial activities of the novel peptides were tested against a series of Gram-positive, *S. aureus* (CPCC100520), Gram-negative, *E. coli* (CPCC100521), and yeast, *C. albicans* (CPCC400416), strains. These three microorganisms were incubated in Mueller–Hinton broth (MHB) at 37 °C for 16–20 h and the culture was diluted with sterile broth to a desired OD (550 nm). These dilutions of  $1.0 \times 10^6$  cfu/ml for the bacteria and  $0.5 \times 10^6$  cfu/ml for the yeast served as the inocula for the 96-well plates and viable cell counts on inoculums were performed to validate. Bombinin-BO1 and Bombinin H-BO1 with concentrations ranging from 1 to 256 mg/L were added to the 96-well plate and incubated for 24 h at 37 °C. After that, the MIC value was determined as the lowest concentration of the peptide that caused the absorbance value (550 nm) similar to the negative control. Then 10  $\mu$ l of the inhibited culture medium was inoculated onto Mueller–Hinton agar (MHA) plates and incubated at 37 °C overnight. The minimum concentration of peptide which resulted in no colonies growth on the plates was defined as MBC.

### Hemolysis assay of the two bombinin peptides

Hemolytic activities of the two bombinin peptides were assessed by incubating a 2% suspension of human erythrocytes with each of the peptides for a period of 2 h. The human blood that came from donations from the healthy volunteers was washed several times with PBS by centrifugation, then the erythrocytes were incubated with 2% Triton X-100 as the positive control, with PBS as the negative control, and with the different concentrations of peptides. After incubation, 100  $\mu$ l of each supernatant was transferred from each sample as three replicates into a 96-well plate and their absorbance was measured at  $\lambda 550$ . The percent hemolysis was calculated using the following equation: hemolysis % =  $(A - A_0)/(A_x - A_0) \times 100$ , where 'A' is absorbance of peptide treatments at  $\lambda 550$ , 'A<sub>0</sub>' is absorbance of negative control at  $\lambda 550$ , 'A<sub>x</sub>' is absorbance of positive control at  $\lambda 550$ .

### Assessment of anticancer activity

Human hepatoma cancer cells (Hep G2, SK-HEP-1 and Huh7) were seeded onto 96-well plates at a concentration



**Fig. 1** Nucleotide sequence of cloned precursor cDNA encoding the novel bombinin peptides, Bombinin-BO1 and Bombinin H-BO1 from the Oriental fire-bellied toad, *Bombina orientalis*. The putative signal

peptide domain (double-underlined), sequences encoding mature peptides (single-underlined) and the stop codon (asterisk) are shown

of  $5 \times 10^3$  cells/well and cultured overnight at 37 °C. The next day, the cells were treated with various concentrations of peptides for 6, 24 and 48 h, respectively. Following treatment, 20  $\mu$ l of 5 mg/ml MTT was added and further incubated for 4 h. Then, the culture medium was removed, and the purple formazan crystal was dissolved in 150  $\mu$ l DMSO. The resulting absorbance at 490 nm was measured using microplate reader iMark (Bio Rad, Hercules, CA, USA).

## Results

### “Shortgun” cloning of novel bombinin peptides encoded cDNA

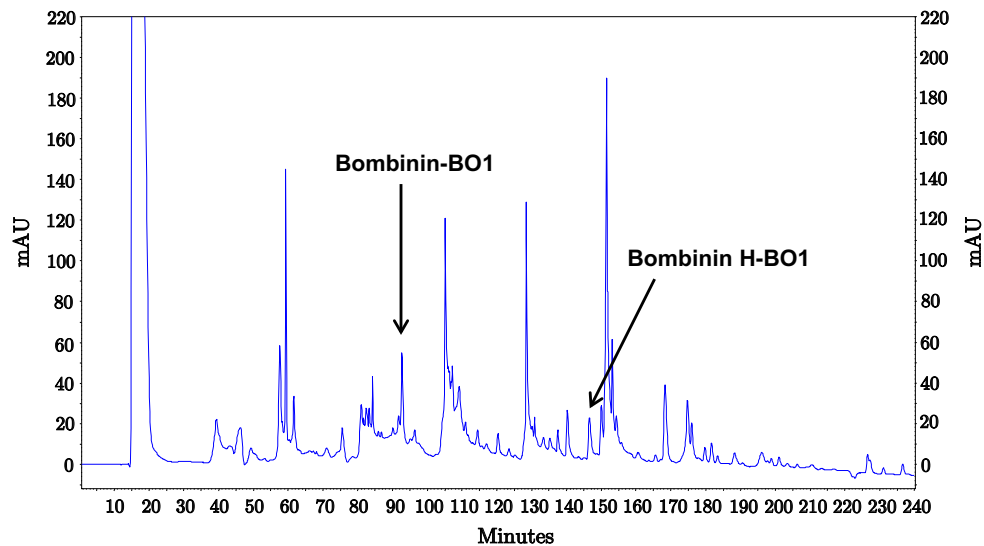
From the *Bombina orientalis* skin secretion-derived cDNA library, a full-length cDNA, co-encoded a novel BLP and a novel BH were consistently cloned and amplified. Bioinformatic analysis of both mature peptides using NCBI BLASTp

**Fig. 2** Comparison of Bombinin-BO1 (a) and Bombinin H-BO1 (b) with their homologous peptides identified from *Bombina* species in NCBI database. Different amino acid sites are shaded in gray. Gaps have been inserted into the sequence to maximize homology

A	1	10	20	
<b>Bombinin-BO1</b>	GIGSAILSAGKSI	IKGLAKGLAEHF		(LT732575.1)
<b>BLP-4</b>	GIGAILSAGKSI	IKGLANGLAEHF		(P29005.1)
<b>BLP-2</b>	GIGSAILSAGKSA	IKGLAKGLAEHF		(P29003.1)
<b>BLP-3</b>	GIGAILSAGKSA	IKGLAKGLAEHF		(P29004.1)
<b>Maximin35</b>	GIGAILSAGKSA	IKGLAKGLAEHF		(ABZ86122.1)
<b>BLP-1</b>	GIGASILSAGKSA	IKGLAKGLAEHF		(P29002.1)
<b>Maximin6</b>	GIGCAILSAGKSA	IKGLAKGLAEHF		(ABZ86157.1)
<b>Feleucin-BO1</b>	GIGCAILSAGKAA	IKGLAKGLAEHF		(CDN85505.1)
<b>Maximin36</b>	GIGCAILSVGKSA	IKGLAKGLAEHF		(ABZ86123.1)

B	1	10	
<b>Bombinin H-BO1</b>	IIGPVLGLV	GKALGGLL	(LT732575.1)
<b>Bombinin H-2</b>	IIGPVLGLV	ALGGLL	(P82286.1)
<b>Bombinin H-1</b>	IIGPVLG	VGSA LGGLL	(P82282.1)
<b>Maximin H15</b>	IIGPVLGLV	ALGGLL	(Q58T43.1)
<b>Maximin H25</b>	IIGPVLGLV	ALGGLL	(ABZ86151.1)
<b>Maximin-Hu</b>	IIGPVL	SLVGNALGGLL	(P83087.1)
<b>Maximin H3</b>	IIGPVLGLV	ALGGL-	(Q58T70.1)

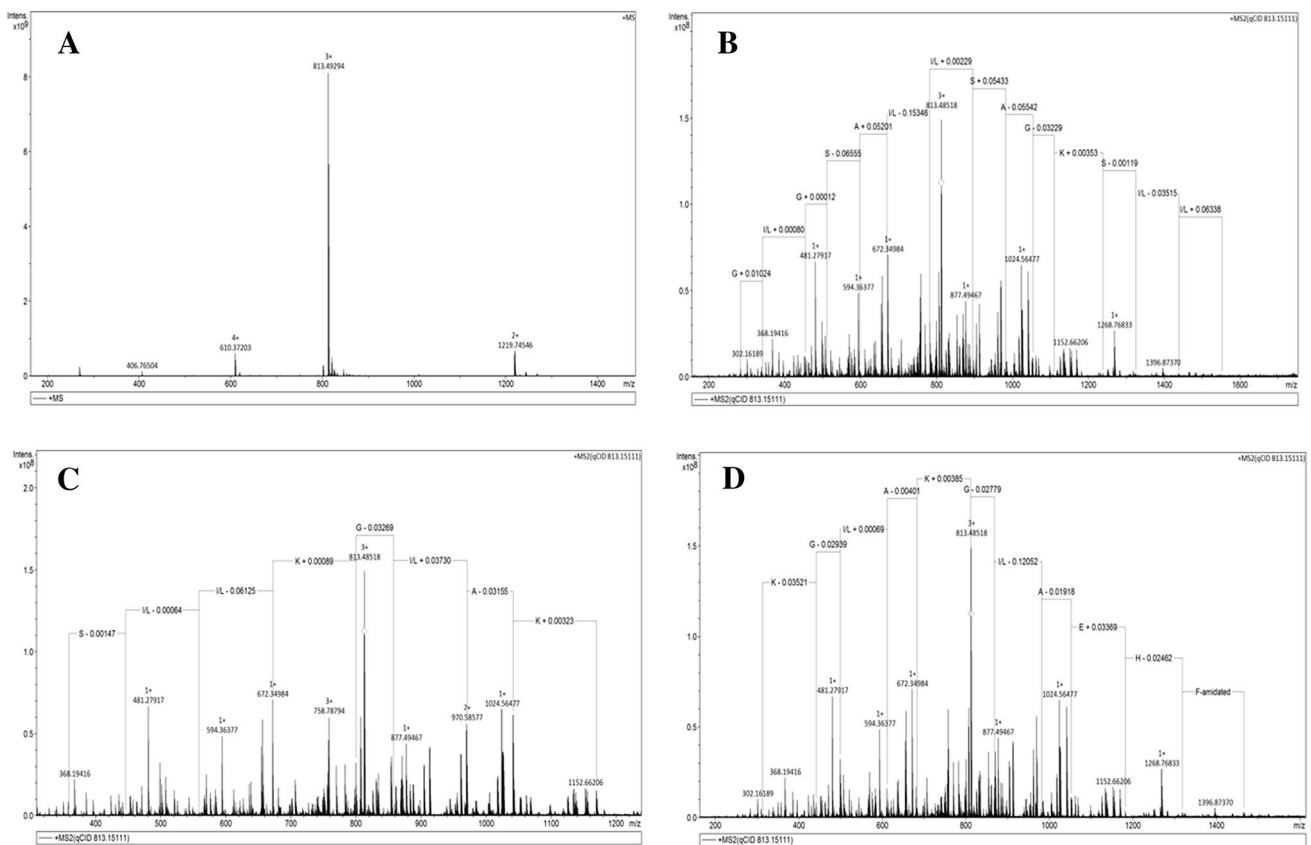


**Fig. 3** Reversed-phase HPLC chromatogram of lyophilized *B. orientalis* skin secretion. The arrows indicate the absorbance peaks corresponding to Bombinin-BO1 and Bombinin H-BO1. The X-axis

represents the retention time in 240 min. The Y-axis represents the absorbency of sample at a wavelength of 214 nm

program revealed they were new bombinins. The nucleic acids and their translated amino acid sequences of prepro-peptide open reading frame (ORF) are shown in Fig. 1. This ORF consisted of 132 amino acid residues, which contained a putative signal peptide, two acidic spacer peptides, typical

-KR- or -R- propeptide convertase cleavage sites, a single copy of a bombinin-like peptide (named as Bombinin-BO1) located in the middle of the sequence and another single copy of bombinin H-type peptide (named as Bombinin H-BO1) located at the end of the sequence. Moreover, the



**Fig. 4** a MS full scan of Bombinin-BO1 from HPLC fraction. The peptide with triply charged  $[M + H]^{3+}$  ions and doubly charged  $[M + H]^{2+}$  ions at  $m/z$  813.49 and 1219.75 was deduced as Bombinin-BO1. **b–d** Tandem mass spectrometry analysis of Bombinin-BO1

**Table 1** The primary structures and molecular mass of the novel bombinin peptides from *B. orientalis*

Peptides	Primary structure	Molecular mass (Da)
Bombinin-BO1	GIGSAILSAGKSIHK- GLAKGLAEHF-NH <sub>2</sub>	2437.89
Bombinin H-BO1	IIGPVLGLVGKALGGLL- NH <sub>2</sub>	1589.02

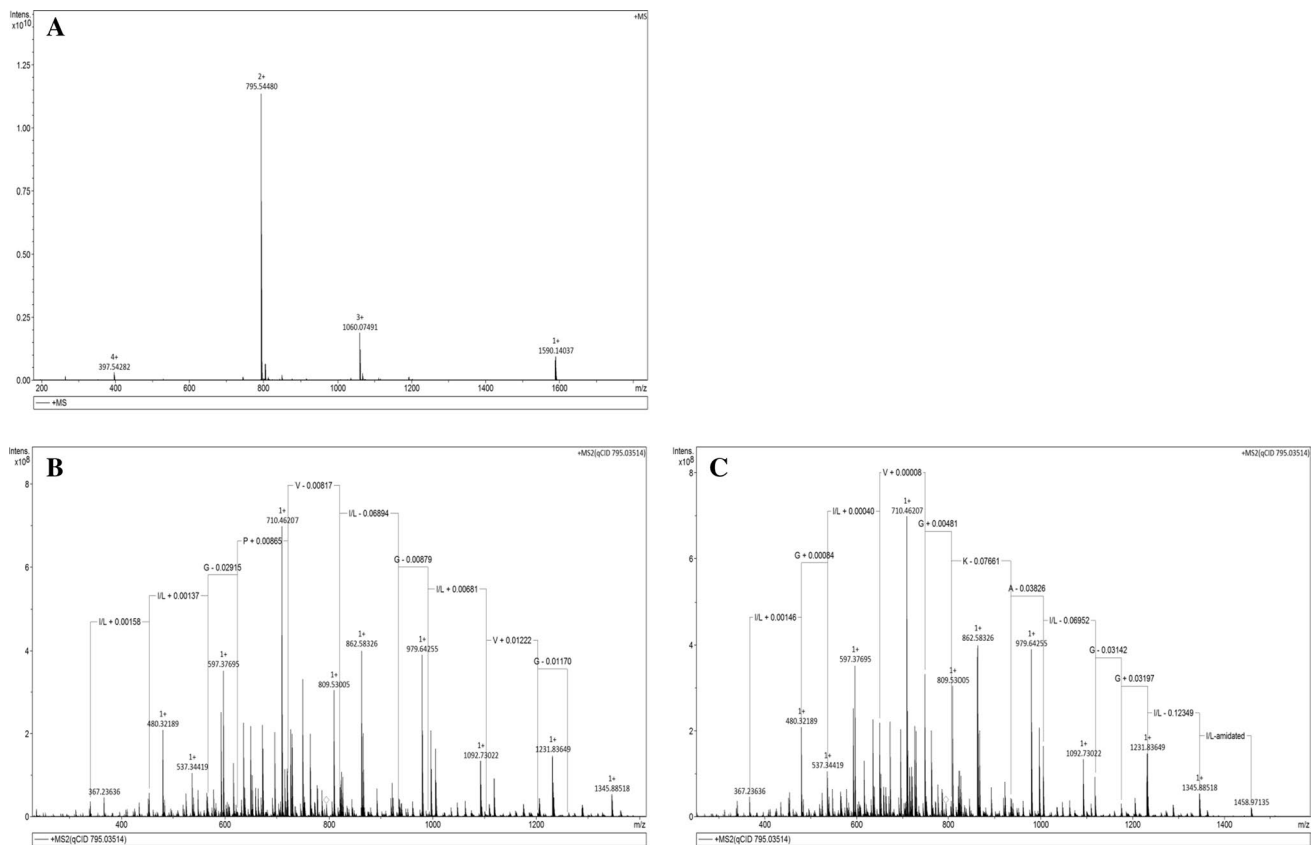
glycyl residue at C terminus of each peptide acts as an amide donor to create terminal amino acid amidation in the post-translational modification process.

Alignment of Bombinin-BO1 and other BLPs suggested that they share very similar primary structures. The most variable residues are Ser for Ala/Gly at position 4 and -Ile-Ile- for -Ala-Leu- at position 13 and 14 (Fig. 2a). Bombinin H-BO1 also exhibited structural similarity with homologous peptides from other species of *Bombina*. The structures of greatest identity to Bombinin H-BO1 are Bombinin H-2

and Bombinin H-1 from *B. variegata*, with 94% and 88% sequence identity, respectively (Fig. 2b). The nucleotide sequence of peptide precursor has been deposited in the EMBL Nucleotide Sequence Database under the accession number, LT732575.1.

### HPLC fractionation of skin secretion and peptide characterization

Besides cDNA cloning, another way to analyze the content of skin secretions is to use the biochemical techniques of HPLC coupled with mass spectrometry along with primary structure clarification methods of MS/MS fragmentation. After a number of HPLC fractionations, differing the column chemistry and elution time intermittently, the peptide-containing fractions were separated by their different polarities and molecular masses. The putative bombinin peptides, Bombinin-BO1 and Bombinin H-BO1, were isolated from the *B. orientalis* skin secretion at the elution time of 92 and 147 min, respectively (Fig. 3). MS analyses indicated the



**Fig. 5** a MS full scan of Bombinin H-BO1 from HPLC fraction. The peptide with doubly charged  $[M + H]^{2+}$  ions and singly charged  $[M + H]^+$  ions at  $m/z$  795.54 and 1590.14 was deduced as Bombinin H-BO1. b, c Tandem mass spectrometry analysis of Bombinin H-BO1

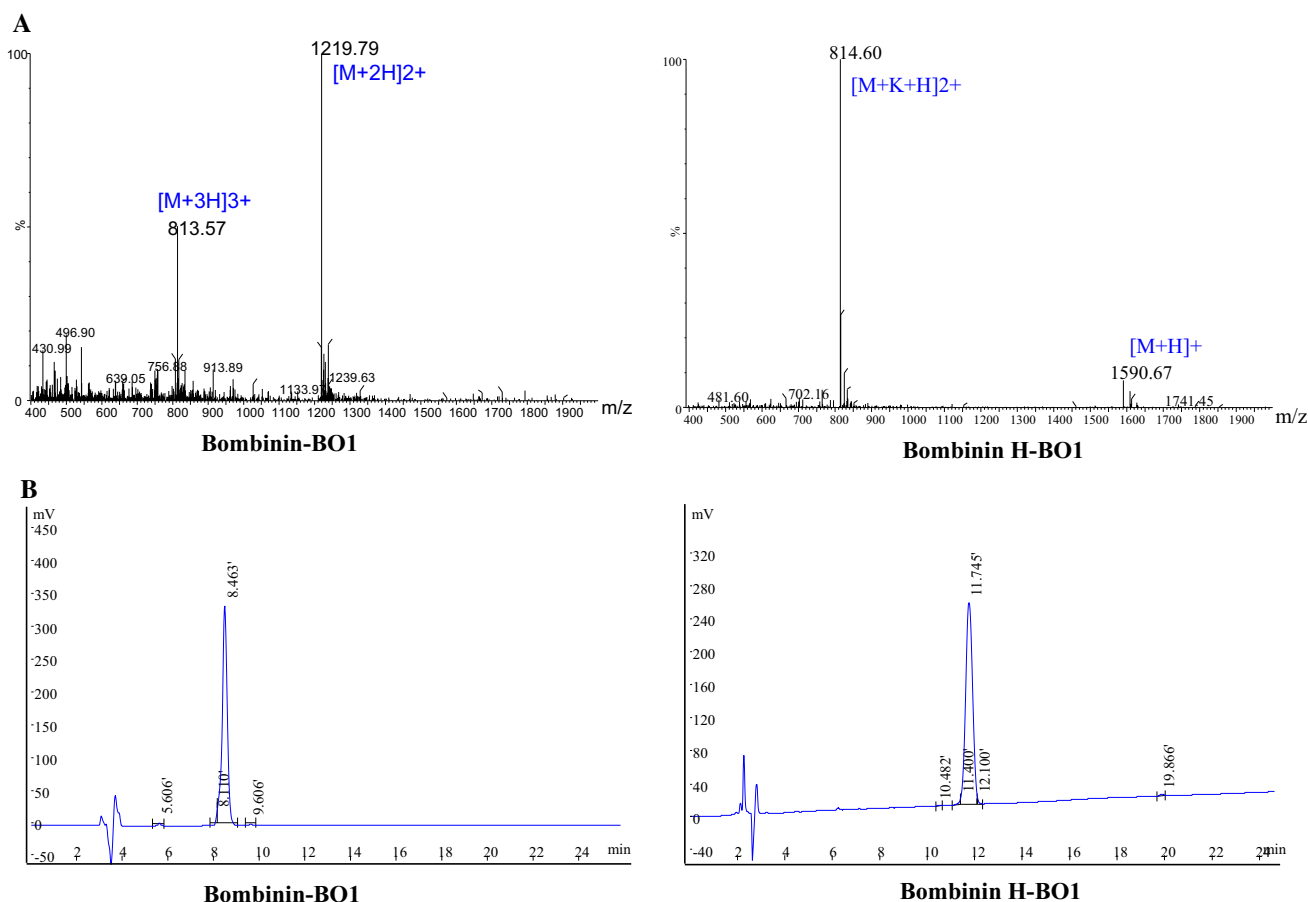
observation molecular masses of both peptides were very close to calculation molecular masses of cloned mature peptides. The sequences of each polypeptide were established by MS/MS fragmentation and were found to be: GIGSAIL-SAGKSII-KGLAK-GLAEHF-NH<sub>2</sub> (Bombinin-BO1) (Fig. 4 and Table 1) and IIGPVLGLVG-KALGGLL-NH<sub>2</sub> (Bombinin H-BO1) (Fig. 5 and Table 1).

### Synthesis, identification and purification of novel peptides

The two peptides were successfully synthesized by solid-phase chemical synthesis technology. Following deprotection and cleavage from the resin, the peptides were lyophilized and then characterized by LC/MS. The detected molecular ions in accordance with molecular masses of respective predicted peptides were observed (Fig. 6a). The purities of the final products were proved to be approximately 95% (Fig. 6b).

### Physicochemical properties of Bombinin-BO1 and Bombinin H-BO1

The physicochemical properties of both peptides were predicted and are summarized in Table 2. Bombinin-BO1 and Bombinin H-BO1 have positive charges and a certain degree of hydrophobicity, suggesting both peptides may have antibacterial effects. Helical wheel plots of Bombinin-BO1 and Bombinin H-BO1 revealed that both peptides possessed amphipathic structures (Fig. 7a). In Bombinin-BO1, the hydrophobic side chains contained Phe (F), Ile (I) and Leu (L) at lower side of the peptide and the side chains of the hydrophilic Lys (K) and Ser (S) residues were located on the opposite side. Similarly, the hydrophobic face of Bombinin H-BO1 consisting of Ile (I), Leu (L) and Val (V) was oriented at one side of the peptide and the hydrophilic face containing Lys (K) were located on the other side.



**Fig. 6** a ESI mass spectra of two synthetic bombinin peptides. b HPLC purification of synthetic replicates of two novel peptides, and the purity was proved to be approximately 95%

### Secondary structural prediction of two peptides

The secondary structures of both peptides were predicted using online protein prediction tool, I-TASSER. As shown in Fig. 7b and Table 2, Bombinin-BO1 had two predicted helical domains representing 76.0% of the structure. In Bombinin H-BO1, about 67.4% of the structure was helical, with a remainder coil at both ends of the peptide.

### Measurement of antimicrobial and hemolytic activities

The antimicrobial activities of peptides were evaluated through testing their MIC and MBC values against three types of standard organisms: the Gram-negative bacteria, *E. coli*, the Gram-positive bacteria, *S. aureus*, and the yeast, *C. albicans*. The MIC and MBC obtained with the peptides are shown in Table 3 and Fig. 8. Bombinin-BO1 was found to be effective against all three tested microorganisms with MICs of 26.3  $\mu\text{M}$  against *E. coli* and *S. aureus*, and 52.5  $\mu\text{M}$  against *C. albicans*. The MBC concentrations were 26.3  $\mu\text{M}$  for *E. coli*, and 52.5  $\mu\text{M}$  for *S.*

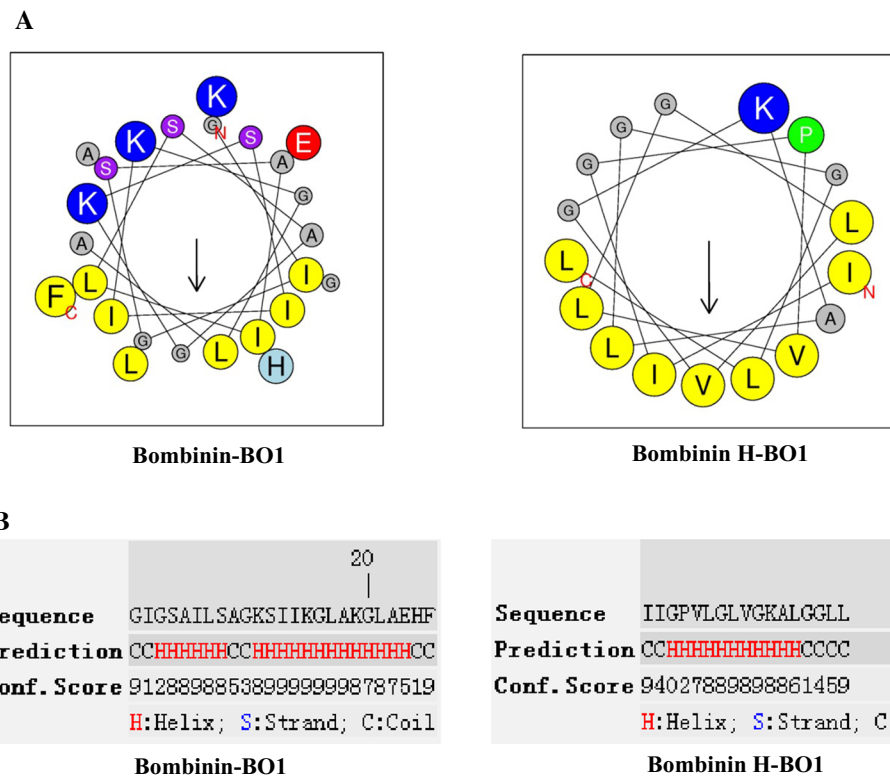
**Table 2** Secondary structures and physiochemical properties of two bombinin peptides, showing helicity, hydrophobicity, hydrophobic moment and net charges

Peptides	%Helix	Hydrophobicity (H)	Hydrophobic Moment ( $\mu\text{H}$ )	Net charge
Bombinin-BO1	76.0	0.469	0.449	2
Bombinin H-BO1	64.7	0.858	0.491	1

*aureus*, and 105.0  $\mu\text{M}$  for *C. albicans*. Besides, Bombinin-BO1 exhibited moderate hemolytic effect on human red blood cells. It caused only 2.89% hemolysis of the red cells at a concentration of 26.3  $\mu\text{M}$  and 38.05% hemolysis at 52.5  $\mu\text{M}$ . In contrast, Bombinin H-BO1 showed weak antibacterial action against *C. albicans* (161.1  $\mu\text{M}$  for both MIC and MBC), and less effect on *E. coli* and *S. aureus*, whereas this peptide caused 42.03% hemolysis of the red cells at a concentration of 40.3  $\mu\text{M}$  while reaching 100% hemolysis at 161.1  $\mu\text{M}$ .



**Fig. 7 a** Schiffer–Edmundson helical wheel plots of Bombinin-BO1 and Bombinin H-BO1. Both peptides showed alpha-helical amphipathic structural features. The hydrophobic face is indicated by an arrow. **b** Predicted secondary structures of two novel peptides using protein structure and function prediction tool, I-TASSER



**Table 3** The antimicrobial and hemolytic activities of Bombinin-BO1 and Bombinin H-BO1

Peptides	MIC			MBC			Hemolysis ( $\mu\text{M}$ )
	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	Human Red cells
Bombinin-BO1	64 mg/L (26.3 $\mu\text{M}$ )	64 mg/L (26.3 $\mu\text{M}$ )	128 mg/L (52.5 $\mu\text{M}$ )	64 mg/L (26.3 $\mu\text{M}$ )	128 mg/L (52.5 $\mu\text{M}$ )	256 mg/L (105.0 $\mu\text{M}$ )	128 mg/L (52.5 $\mu\text{M}$ )
Bombinin H-BO1	> 256 mg/L (> 161.1 $\mu\text{M}$ )	> 256 mg/L (> 161.1 $\mu\text{M}$ )	256 mg/L (161.1 $\mu\text{M}$ )	> 256 mg/L (> 161.1 $\mu\text{M}$ )	> 256 mg/L (> 161.1 $\mu\text{M}$ )	256 mg/L (161.1 $\mu\text{M}$ )	32 mg/L (40.3 $\mu\text{M}$ )

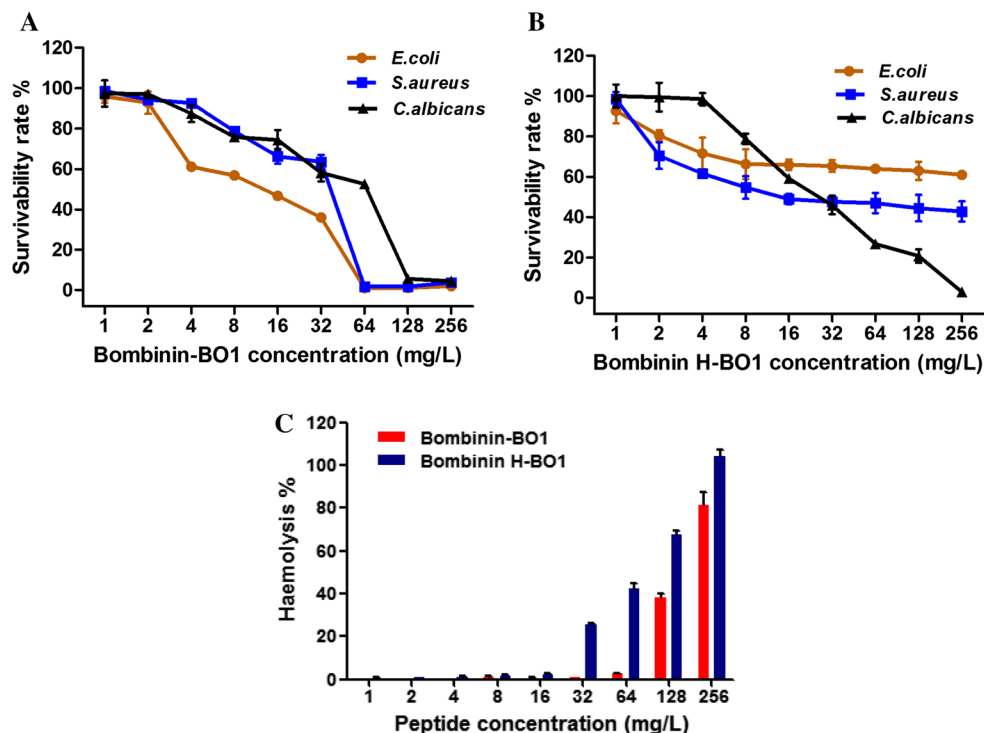
### Bombinin-BO1 and Bombinin H-BO1 inhibited the proliferation of human hepatoma cell lines

The anti-proliferative effects of two novel peptides on three human hepatoma cell lines (Hep G2, SK-HEP-1 and Huh7) were investigated by MTT assay, a colorimetric assay for measurement of cell viability. Bombinin-BO1 was found to inhibit all the three hepatoma cell lines' proliferation dose and time dependently over 48-h observation periods (Fig. 9a–c). Bombinin-BO1 had higher growth inhibitory potency on SK-HEP-1 and Hep G2 cells than on Huh7 cells, with IC<sub>50</sub> values of 0.76, 3.75, and 3.91  $\mu\text{M}$ , respectively, after 48 h treatment. Similarly, Bombinin H-BO1 was shown to inhibit three hepatoma cell lines' growth in a dose-dependent manner (Fig. 9d–f). SK-HEP-1 and Hep G2 cells were more sensitive to this peptide than Huh7 cells, and the IC<sub>50</sub> values at 48 h were 3.61, 8.08, and 8.42  $\mu\text{M}$ , respectively.

### Discussion

Amphibian skin secretions contain incredibly complex cocktails of bioactive molecules, especially bioactive peptides. So far, the most interesting aspect of potentially therapeutic peptides present in frog skin secretions is their antimicrobial and anticancer properties (Jenssen et al. 2006; Rinaldi 2002). Frogs or toads produce antimicrobial peptides as their defense against invading microorganisms. Antimicrobial peptides usually have activity against a broad spectrum of microorganisms, such as Gram-positive and Gram-negative bacteria, viruses, protozoa, yeasts and fungus (Galdiero et al. 2015). They are usually characterized by relatively short chain length (12–100 amino acid residues), positively charged (net charge of + 2 to + 9), basic in nature (lysine or arginine rich) and amphipathic (Wang and Wang 2004). Recent research demonstrated that many amphibian antimicrobial peptides possessed potent antitumor effect without

**Fig. 8** Antimicrobial activities of Bombinin-BO1 (a) and Bombinin H-BO1 (b) against *E. coli*, *S. aureus*, and *C. albicans*. Three replicates were recorded in each assay. c Hemolytic effects of two bombinin peptides on human red blood cells. Three replicates at each point were recorded. (positive control: 2% Triton X-100; negative control: equal volume of PBS)



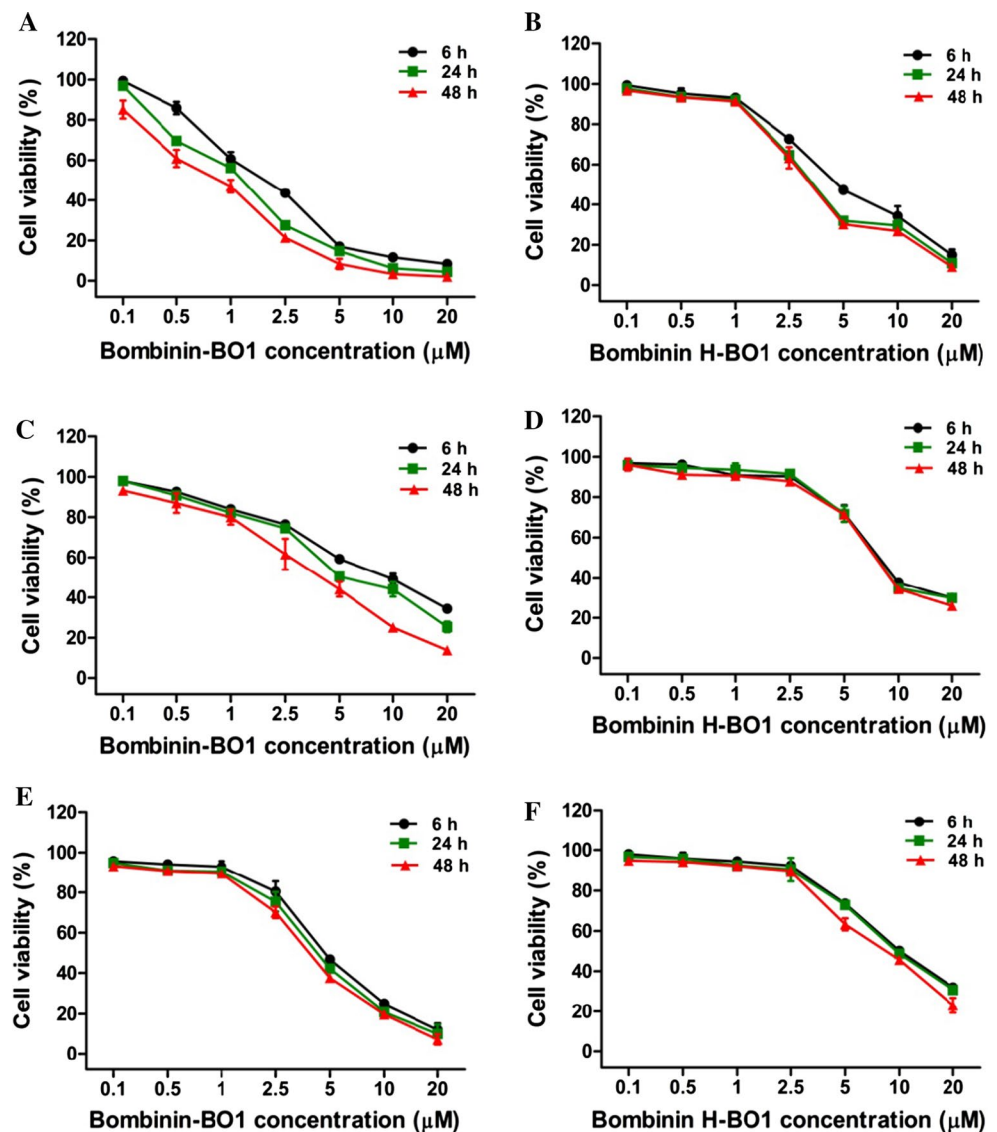
showing obvious toxicity, which has brought great attention for medicinal scientists (Oelkrug et al. 2015).

In this study, two novel antimicrobial peptides have been discovered from the skin secretion of *B. orientalis* using multiple peptidomic approaches, including molecular cloning, HPLC fractionation and mass spectrometry. Searching the nucleotide sequence and deduced amino acid sequence of the two peptides in NCBI-BLAST databases indicated both novel peptides were new members of the bombinin peptide family. They were identified as a BLP (Bombinin-BO1) and a BH (Bombinin H-BO1) and both of them were co-encoded in the same peptide precursor. The existence of BLP was first reported in skin of *B. variegata* in the 1970s. Later, from the cloned bombinin cDNA precursor, the sequence of BH was discovered (Dennison et al. 2006; Gibson et al. 1991). Alignment of Bombinin-BO1 and Bombinin H-BO1 with other peptides in each group suggested they share very similar structural features, respectively (Fig. 2). Bombinin-like peptides consist of 25 or 27 residues and generally have a common Gly-Ile-Gly- motif at the N terminus and a highly conserved sequence Lys-Gly-Leu-Ala-Lys-Gly-Leu-Ala-Glu-His-Phe-(Ala-Asn) at the C terminus. The amino acid substitution sites in most mature peptides are located at position 4 (Ser/Ala/Gly), 6 (Ile/Leu) and 13–14 (Ile/Ala-Ile/Leu). Bombinin H-type peptides are usually heptadecapeptides exhibiting more conservative structure than the former. They contain two internal -Gly-Pro-Val-Leu- and -Ala-Leu-Gly-Gly-Leu- motif in the peptidic backbone. Thirteen amino acids in the total of seventeen are invariant, and the

variable four positions are limited to certain types of amino acids, for example, 2-Ile/Leu; 7-Gly/Ser; 8-Leu/Met; 11-Lys/Ser/Asn. In addition, the C-terminal amidation of both peptide groups could be essential for their structural stability.

As bombinin are groups of antimicrobial peptides, the antimicrobial activities of Bombinin-BO1 and Bombinin H-BO1 were evaluated by determining their MIC values against three standard model microbes. As expected, both peptides exhibited a broad spectrum of antimicrobial activity against Gram-negative bacteria, *E. coli*, Gram-positive bacteria, *S. aureus*, and the yeast, *C. albicans*. The efficacy of Bombinin-BO1 against Gram-negative bacterium over Gram-positive bacteria was observed, with the same MIC and MBC twofold less for the former than the latter. This was probably due to the distinct compositions of the cell walls and membranes. Bombinin-BO1 was an  $\alpha$ -helical cationic peptide, the mode of action of which was proposed through the membrane-targeted disruption process: the Gram-negative bacteria—*E. coli*—has thinner cell walls and thinner peptidoglycan layers than *S. aureus*; therefore, easier to be penetrated. In comparison, Bombinin H-BO1 only showed moderate activity against a range of microbial species. It was found to inhibit the growth of yeast with MIC of 161  $\mu$ M. Next, hemolytic assay was employed to evaluate the toxicity of both peptides to the human normal cells. Bombinin-BO1 caused hemolysis of human erythrocyte at 128 mg/L (52.5  $\mu$ M), which was twice of the concentration needed to inhibit the growth of *E. coli* and *S. aureus*. Bombinin H-BO1 showed a higher hemolytic activity at 32 mg/L

**Fig. 9** Bombinin-BO1 and Bombinin H-BO1 inhibited the proliferation of human hepatoma cell lines, SK-HEP-1 (a, b), Huh7 (c, d) and Hep G2 (e, f) in 48 h. Cells were treated with Bombinin-BO1 and Bombinin H-BO1, respectively, and incubated for 6, 24 and 48 h. Cell viability was measured using MTT assay. Data shown indicate the mean  $\pm$  SEM. The experiment was performed in triplicate



(40.3  $\mu$ M). This could be due to that Bombinin H-BO1 is more hydrophobic, but the higher hydrophobicity increased its hemolytic activity rather than the antimicrobial effect.

Most recently, a growing number of reports have demonstrated that many antimicrobial peptides exhibited both antimicrobial and anticancer activities (Lapis 2010; Wan et al. 2015). Some of these are  $\alpha$ -helical cationic peptides with the similar mechanisms against bacterial and cancer cells, which involved disrupting membrane function of the target tumor cells, resulting in an excessive flux of ions and small molecules across the cytoplasmic membrane bilayer, ultimately leading to cell lysis (Dehghan Esmatabadi et al. 2017). Compared to normal cells, cancer cell surfaces generally overexpress anionic O-glycosylated mucin and high molecular mass glycoprotein. The negatively charged membranes may contribute to the selective

cytotoxicity of antimicrobial peptides on cancer cells (Dennison et al. 2006; Gaspar et al. 2013). The possible anti-proliferation effect of both peptides was assessed on three human hepatoma cells. The results revealed Bombinin-BO1 and Bombinin H-BO1 exhibited potent anticancer effect, especially on SK-HEP-1 cells with IC<sub>50</sub> as 0.76 and 3.61  $\mu$ M, respectively. Additionally, it was believed that both peptides could selectively kill cancer cells as their hemolysis concentrations on human red cells were 52.5 and 40.3  $\mu$ M, which were far above the IC<sub>50</sub>s against hepatoma cells.

Nowadays, numerous oncology drugs have been developed to treat cancers; however, their severe side effects to healthy human cells and the increasing of multiple drug resistance have become the main cause of chemotherapy failure (Deslouches and Di 2017; Otvos 2017). The bombinins

discovered in this paper showed selective cytotoxicity for cancer cells have great potential to become a class of anticancer drug candidates.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

**Research involving human participants and/or animals** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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