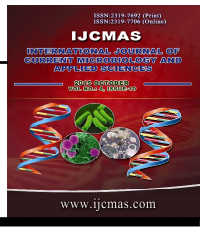
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 889-900

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 889-900**
http://www.ijcmas.com



Original Research Article

Characterization of Anuran Skin Peptides: An Alternative to the Classical Therapeutic Agents Used for MDR Pathogens

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Keywords

Rana ridibunda, Antimicrobial peptides, Brevinin-2-RA6, LC-MS/MS

A B S T R A C T

Amphibians cutaneous glands, spread over the skin, contain different bioactive molecules (peptides, proteins, steroids, alkaloids and biogenic amines) that possess effective therapeutic activities against different microbial pathogens. The amphibian peptides array is species specific. The peptidyl skin secretions were purified using Sephadex G50 gel filtration column, the protein-containing fractions were collected and assayed for antimicrobial activity against Gram positive MRSA and Gram negative ESBL bacteria (*Klebsiella pneumonia* and *Escherichia coli*), as well as *Candida albicans*. Fractions that showed substantial antimicrobial activity were subjected to polyacrylamide gel electrophoresis. The bands were eluted and sequenced by LC-MS/MS. One distinct polypeptide with amino acid sequence homologous to brevinin-2-RA6 antimicrobial peptide precursor with a molecular weight of 8 kDa was identified. The present study is a novel and the first peptidomic study for antimicrobial peptides identification of the skin secretions of *Rana ridibunda*. The present finding highlight on anuran amphibians as a potential source of new antimicrobial peptides. However, more detailed studies on the antimicrobial and anticancer activity of Brevinin- homologue Asmaa (Brevinin-HAs) *in vivo* is underway to explore its various activities.

Introduction

Genus *Rana* was one of the most diverse and widely distributed groups of anuran amphibians having more than 250 reported species around the world. The anuran skin presents morphofunctional and behavioral protective adaptations against a number of adverse factors in the terrestrial environment, in which the cutaneous glands play an essential role in the defense against infection by microorganisms on the body surface. These glands produce a secretion composed of a complex mixture of

substances with diverse array of antimicrobial peptides (AMPs) ranging from 10-50 amino acid in length against bacteria, fungi, protozoa, virus and cancer (Calderon *et al.,* 2009, 2010, 2011) and diverse pharmacological and therapeutic potential as: antidiabetic (Marenah *et al.,* 2004, 2005, 2006), antiprotozoal (Mangoni *et al.,* 2005), antiviral (Zhao *et al.,* 2005), antineoplastic (Nasu *et al.,* 2005), analgesic (Doyle *et al.,* 2003), contraceptive (Clara *et al.,* 2004), wound healing (Zhang *et al.,* 2005), behavioral change (Sila *et al.,* 2006), and endocrine activity (Gullner *et al.,* 1983). AMPs can represent a suitable alternative to the conventional antibiotics which proved ineffective in combating multidrug resistant (MDR) bacteria and fungi (Guani-Guerra *et al.,* 2010). AMPs have been classified into several families on the basis of amino acid sequence similarities that include: gaegurins (24–37 residues), brevinin-1(17–24 residues) and brevinin-2 (30–34 residues), ranalexin (20 residues), ranatuerin-1 (25 residues) and ranatuerin-2 (33 residues), esculentin-1 (46 residues) and esculentin-2 (37 residues), palustrin (31 residues), japonicin-1 (14 residues) and japonicin-2 (21 residues), nigrocin-2 (21 residues), rugosins (33–37 residues) and temporin (10-14 residues) (Duda *et al.,* 2002; Matutte *et al.,* 2000; Conlon *et al.,* 2004). AMPs isolated from Ranid frogs have very high sequential similarity and are grouped under Brevinin family (Conlon *et al.,* 2004). The numbers of AMPs in the skin secretions vary between species. While some species exhibited more than two dozens of peptides in a single frog (Hancock and Rozek, 2002). Matutte *et al.* (2000) demonstrated the presence of a single antimicrobial peptide (brevinin-1SY in the adult wood frog *Rana sylvatica*). Limited therapeutic options against MDR pathogens demand the urgent bioprospection of new bioactive molecules from the biological diversity as a source for more efficient (low toxicity and major potency) mechanisms of microorganism elimination (Calderon *et al.,* 2009; Vaara, 2009). The goal of the present research is to purify and characterize the peptides isolated from the skin gland secretions of the marsh frog *Rana ridibunda* inhabiting the fresh water of Jezzine, South Lebanon as a potential antimicrobial agent against MDR pathogens.

Materials and Methods

Sample collection

Adult specimens *Rana ridibunda* of both sexes were collected from fresh water habitat in Jezzine, South Lebanon during different seasons of the year since skin gland secretions are expected to vary during hibernation breeding and normal activities. Skin gland secretions were collected according to the method described by Li *et al.* (2007) with some modifications. The frogs were stimulated to release the skin secretions by subjecting them to chloroform vapor for 2–5 minutes. The secretions were collected by washing the frogs with 0.1 M NaCl solution containing 0.01 M EDTA. One hundred ml of the collected washing solution were centrifuged under cooling for 5 minutes at 13000 rpm and the supernatant was collected and lyophilized. The lyophilized powder was resuspended in 10 ml of 0.1 M NaCl solution containing 0.01 M EDTA. The solution was sterilized using 0.2 µm millipore filter. The filtrate was kept refrigerated at 4°C for further use.

Microorganisms

Three different MDR bacterial strains were used throughout the present work: Gram positive methicilin-resistant *Staphylococcus aureus* (MRSA), Gram negative ESBL (*Klebsiella pneumoniae* and *Escherichia coli*

(kindly provided by Ain W Zain hospital and American University Hospital of Beirut). The bacterial isolates were identified phenotypically as described by Beregy's manual of determinative bacteriology, were maintained on nutrient agar slants and stored at 4°C with regular monthly transfer, 25% glycerol was added to the cultures for long preservation. *Candida albicans* was kindly provided by Elias Hrawi Governmental Hospital, identification was further confirmed using the Simplified Identification Method (SIM) key (Deak, 1986). It was maintained on Sabouraud-Dextrose agar slants and stored at 4°C with regular monthly transfer.

Inoculum preparation and standardization

From a freshly prepared culture of each isolate (16 to 24 hrs old grown on nutrient agar), 4 to 5 colonies were emulsified in 5 ml of 0.9 % saline solution to achieve a turbid suspension matching 0.5 McFarland standardized tube corresponding to 1.5 x 10^8 CFU/ml (Mahon *et al.*, 1998) and 3x10^4-3x10^5 CFU/ml (NCCLS, 1998) as bacterial and fungal inocula respectively.

Estimation of protein content

Absorbance was measured spectrophotometrically at 280nm (A280) to calculate protein concentration in reference to standard curve.

Peptide purification

Gel Filtration chromatography on Sephadex G-50

Preparation of Sephadex G-50

Ten grams of sephadex G-50 were allowed to swell in 400 ml of 0.1M phosphate buffer pH 6 for 1 hr, and the mixture was filtered. The gel was washed with 1 liter 0.2 M HCl followed by elution of the phosphate buffer and stored for 3–4 days at 4◦C to ensure complete swelling. The excess eluent was removed by decantation and the swollen gel degassed under reduced pressure.

Column packing and equilibration

The gel suspension adjusted to form thick slurry was poured carefully into the column either down side of the chromatographic column (20 Cm long X 2.7 Cm diameter) or down a glass rod to avoid bubble formation. The column outlet was opened to allow the gel to settle into the column. The column was equilibrated with 2.5 M phosphate buffer pH 6 at flow rate of 2 ml/5 min. once a column has been prepared, a layer of eluent should always be maintained above the column surface.

Sample preparation and application

The lyophilized powder was resuspended in10 ml of 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA. The solution was then sterilized by passing through a 0.2 µm syringe filter. The eluent above the gel surface was allowed to drain away, and then the solution was carefully layered onto the top of the bed. The column outlet was then opened and the sample was allowed to drain into the bed surface; the column wall was washed into the bed with small amount of eluent. The proteins were eluted with 0.5 M potassium phosphate buffer, pH 6. The flow rate was 2 ml/ 5 min. Samples of effluent fractions were subjected to protein estimation at 280 nm. The proteins were collected and stored at -30°C or below. Elution was performed with the same buffer, and 2 ml fractions were collected. The absorbance of the eluted fractions was monitored at 280 nm.

Concentration of peptide

The pooled protein fractions were placed in cellophane/ dialysis bag and covered with cold sucrose. Water and low molecular weight substances were dialyzed against sucrose. The concentrated extract was dialyzed against 0.1 M phosphate buffer pH 6 at 4°C.

Antimicrobial assay

The antimicrobial activities of the obtained fractions were determined using the plate-well diffusion assay described by Kudi *et al.* (1999) that was used to determine the inhibitory effect of the partially purified frog skin secretions against the tested MDR microorganisms. A sterile cork-borer (8 mm diameter) was used for wells formation in the set agar. Hundred µl of the microbial suspension (McFarland 0.5 standardized suspension) was swabbed over the surface of a Müller Hinton agar plate. Hundred µl of the purified frog skin secretion extract was added to each well and the plates were incubated overnight at 37°C. The zones of inhibition were then recorded in millimeters and the experiments were repeated twice. The fractions with antimicrobial activity were lyophilized and stored for further analysis.

Electrophoresis

Sodium dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS/PAGE) analysis was carried out for measuring the molecular weight of the partially purified peptide. SDS/PADE was performed following the method adopted by Neuhoff *et al.* (1985) with some modifications. The proteins were resolved with 18% SDS-PAGE. Following electrophoresis, the gel was fixed overnight at room temperature in 40% methanol, 7% acetic acid in deionized water. The fixing solution was discarded and brilliant blue G-colloidal concentrate solution was added (16% of concentrate, 20% methanol and 64% deionized water) and rocked for at least two hours. The gel was destained by different changes of distilled water until the gel background was clear and protein bands were clearly visible. The gel was calibrated with standard proteins with molecular weights (50, 37, 25, 20, 15 and 10 kDa). Proteins within the gel tracks were subsequently identified by LC-MS/MS.

Protein characterization using the liquid chromatoghraphy-tandem mass spectrometry (LC-MS/MS)

Following gel electrophoresis and subsequent Coomassie Brilliant Blue G staining, the protein bands of interest were eluted from the gel and subjected to tryptic digestion following the modified method adopted by Shevchenko *et al.* (1996).

Results and Discussion

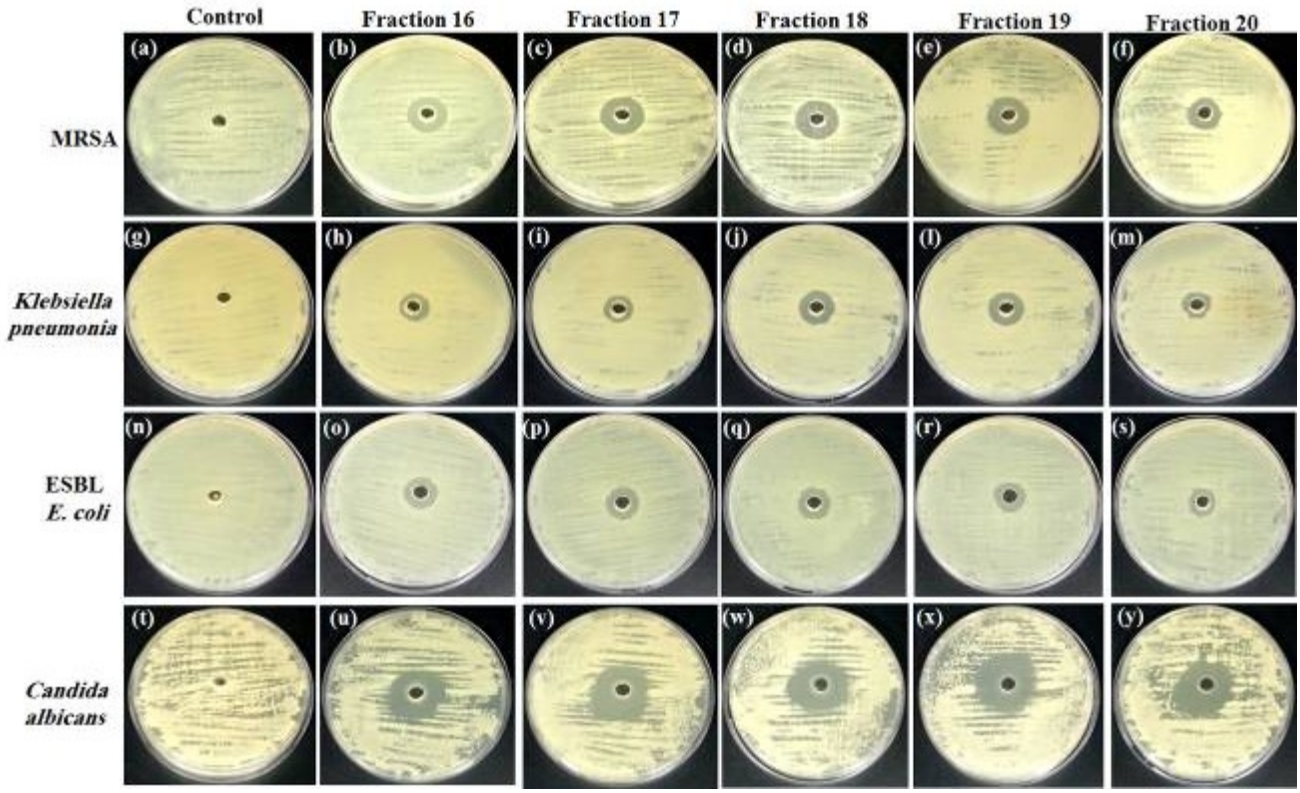
Peptide secretion purification

The ability of the crude peptidyl secretions isolated from the skin of *Rana ridibunda* to inhibit the growth of Gram positive (MRSA) and Gram negative bacteria (ESBL *Klebsiella pneumonia,* ESBL *E. coli*) and *Candida albicans* was evaluated, the peptidyl secretions were particularly effective with respect to the time needed to exert lethal effect on the microbial growth, causing the lysis of the cell membranes (El Haj Moussa *et al.,* 2015). The lyophilized sample of *Rana ridibunda* skin gland secretions was applied to Sephadex G50 gel filtration column. Each collected fraction was monitored for protein content by measuring absorbance at 280 nm. Data in figure 1 indicated that the purified peptides contained different protein components represented by different peaks.

The antimicrobial activity of the gel filtration fractions

In a trial to test the antimicrobial effect of the purified protein fractions obtained from gel filtration (50 fractions) against MRSA, ESBL *Klebsiella pneumonia*, ESBL *E. coli* and *Candida albicans* the well diffusion assay was used. Results illustrated in Plate 1 revealed that not all the collected fractions that showed a protein activity (Figure 1) displayed antimicrobial activity. Fraction 18 showed the highest antibacterial activity with average inhibition zone diameters of 21, 18 and 17 mm against MRSA, ESBL *Klebsiella pneumonia* and ESBL *E. coli* respectively, while fractions 19 and 20 exhibited the highest antifungal activity with average inhibition zone diameter of 30 mm against *Candida albicans* (Table 1). ESBL *E. coli* proved to be the most resistant bacterium followed by ESBL *Klebsiella pneumonia*. The most active fractions that showed the highest antimicrobial activity (fractions 16–20) were pooled, lyophilized and stored for further analysis by LC-MS/MS. Having broad spectrum activity, the antimicrobial peptide appears to have interesting potential for therapeutic application. Mashreghi *et al.* (2013) reported that the skin secretions of the frog *Rana ridibunda* exhibited significant healing effects on wound treatment process by preventing infections in wounds due to its antimicrobial capabilities.

Plate.1 Sensitivity test of the most Sephadex G-50 bioactive fractions (Fractions 16–20) against the MDR microorganisms under test



Characterization of antimicrobial peptides

In a trial to characterize the antimicrobial peptide(s) of *Rana ridibunda* peptidyl skin secretions, the most promising fractions that showed the highest antimicrobial activity were resolved with 18% SDS-PAGE. The resolved protein bands were visualized by Coomassie blue (Figure 2). While many protein bands were present, those with molecular weight ranging between 10 and 50 kDa (indicated by the arrows in figure 2) were considered for further analysis.

To determine the identities of these proteins, proteomic analysis by LC-MS/MS was performed. The gel slice containing bands as indicated by the arrows were cut out, digested with trypsin, and injected into LC-MS/MS apparatus. In contrast to the synthesis of multiple AMPs reported in other ranids such as the skin secretions of the *Rana palustris* containing at least 22 antimicrobial peptides belonging to eight different families (Basir *et al.,* 2000), the present study showed that of the six analyzed bands, the tryptic digest fragments of the protein band below 10 kDa on the SDS-PAGE revealed one distinct polypeptide with the amino acid sequence homologous to Brevinin-2-RA6 antimicrobial peptide precursor with a molecular weight 8 kDa, identified at 65 % confidence and was named as " Brevinin-Homolgue Asmaa" with acronym " Brevinin-HAs". The amino acid sequence of the identified peptide from the frog *Rana ridibunda* in the present study was compared with the amino acid sequence of peptides related to the Brevinin 2 family which were first identified from the skin extract of Japanese pond frog *Rana brevipoda porsa* as reported by Morikawa *et al.* (1992) (Figure 3).

The protein " Brevinin-HAs" was identified based on a peptide fragment consisting of seven amino acids ((R)GLLDTLK(N)) that exhibited a molecular mass of 758.45 Da (m/z 380.25; z = +2) as depicted in figure 4.

While the low score probably resulted from low sample abundance, the biological significance of brevivin-2-RA6 presents an interesting finding which was warrant further investigation. Asoodeh *et al.* (2004) purified a cationic antimicrobial peptide Ridibundin 1 from Iranian *Rana ridibunda*. A total of 29 peptides belonging to the brevinin-1 and -2, esculentin-1 and -2, ranatuerin-2, bradykinin and bombesin families were identified in the Caucasian Marsh frog *Rana ridibunda* from the Moscow region and the sequence of brevinin-2Ra (2990 Da molecular weight) was detected by LC-MS/MS as follows: GILDSLKNFAKDAAGILLKKASCKLSG QC-OH (Samgina *et al.,* 2008). The peptide was also identified in *Rana lessonae* and *Rana esculenta* (Samgina *et al.,* 2012). Two more antimicrobial peptides were isolated from Iranian *Rana ridibunda*, namely temporin-Rb (12 amino acids) and temporin-Ra (14 amino acids) with molecular weight of 1242.5 Da and 1585.1 Da, respectively (Asoodeh *et al.,* 2012). A single antimicrobial peptide of the brevinin-1 family (FLPVVAGLAAKVLPSIICAV TK KC) isolated from the Canadian wood frog *Rana sylvatica* inhibited the growth of *E. coli* and *S. aureus* (Matutte *et al.,* 2000). Three peptides belonging to brevinin-1 family and three belonging to ranatuerin-2 family were identified in the Tarahumara frog *Rana tarahumarae* in the US (Rollins-Smith *et al.,* 2002). Grahamin 1 (GLLSGILGAGKNIVCGLSGLC) and grahamin 2 (GLLSGILGAGKHIVCGLSG LC) related to nigrocins were isolated from skin secretions of Chinese *Rana graham*.

Table.1 Antimicrobial effect of the bioactive fractions against the MDR microorganisms under test

Tested microorganism / Fraction number	MRSA	ESBL *Klebsiella pneumonia*	ESBL *E. coli*	*Candida albicans*
	Inhibition zone diameter (mm)			
16	19.0	14.0	16.0	23.0
17	20.0	15.0	17.0	25.0
18	21.0	18.0	17.0	26.0
19	20.0	18.0	16.0	30.0
20	17.0	14.0	14.0	30.0

Figure.1 Purification profile of *Rana ridibunda* peptidyl secretions using G-50 sephadex gel filtration chromatography

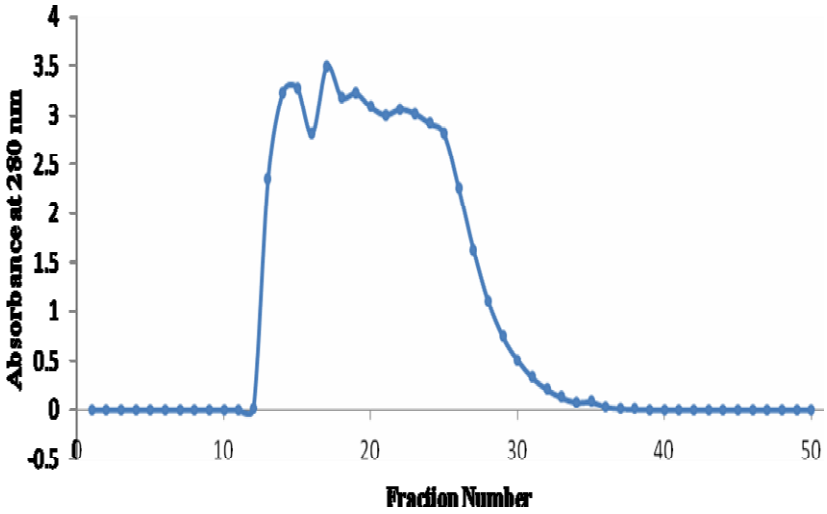


Figure.2 *Rana ridibunda* protein profile on 18% crosslink SDS-PAGE. Lane 1: size marker (kDa), Lane 2: sample (125µg), Lane 3: sample (250µg). Bands indicated by arrows were submitted for LC-MS/MS analysis

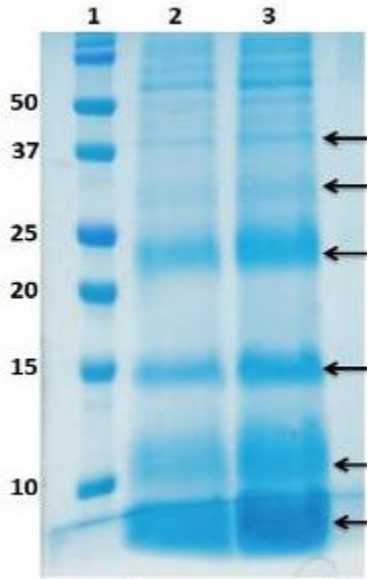
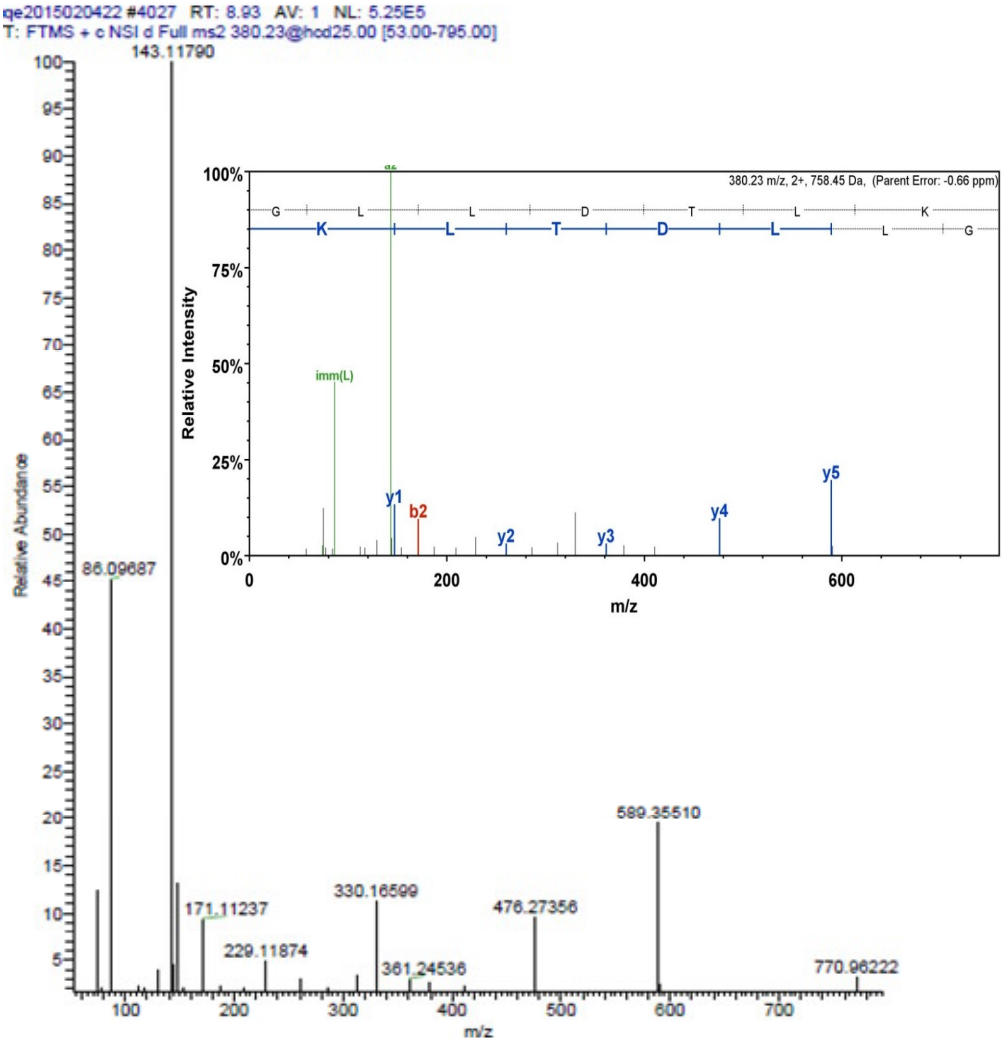


Figure.3 A comparison of the primary structure of the peptide "Brevinin-HAs" isolated from *Rana ridibunda* (b) with Brevinin 2 (a) first isolated from the skin extract of Japanese pond frog *Rana brevipoda porsa*. The highlighted section was the main sequence that led to the identification of Brevinin-HAs in the sample

a- Brevinin 2: **GLLDSLK**GFA ATAGKGVLQS LLSTASCKLAKTC (Morikawa *et al.*, 1992)

b- Brevinin-HAs (Identified peptide) : MFTMKKPLLL LFFLGTISLS LCEEERDADE DDGVEVTEEE VKR**GLLDTLK** NMAINAAKGA GVSVLNALSC KLSKTC

Figure.4 LC- MS/MS spectra of purified peptide isolated from skin secretions of *Rana ridibunda* and injected into LC-MS/MS apparatus. The depicted spectra led to the identification of the "GLLDTLK" peptide (inset) and eventually brevinin-2-RA6 peptide precursor



In accordance with the current study, these antimicrobial peptides contained one positive amino acid residue (Lysine) and had antimicrobial activity against *E. coli*, *S. aureus* and *C. albicans* (Xu *et al.,* 2006). Another antimicrobial peptide containing one positive amino acid residue (CKIALPYT) was isolated from the Chinese frog *Rana nigrovittata* (Ma *et al.,* 2010). Functional activities of antibacterial peptides were largely determined by their structural features as the presence of cationic amino acids which facilitates the interaction of the brevinins with the anionic phospholipids of the bacterial membranes or the negatively charged eukaryotic cell membranes. However, the number and distribution of positive charges could be the cause for selectivity for some antimicrobial peptides to bacterial membranes (Simmaco *et al.,* 1998). Ultrastructural analysis of MDR microorganisms treated with Lebanese *Rana ridibunda* peptide secretions showed that the main targets included the cell membrane and plasma membrane of the pathogens (El Haj Moussa *et al.,* 2015). Precursors of brevinin-1RTa, brevinin-1RTb, brevinin-1RTc, brevinin-2RTa, and brevinin-2RTb have been identified from the skin-derived cDNA library of *Amolops ricketti* (Wang *et al.,* 2012). Peptidomic analysis of skin secretion of the Indian Ranid frog *Clinotarsus curtipes* identified five novel antimicrobial peptides with sequence homology to brevinin family (Abraham *et al.,* 2014). A unique peptide brevinin-2R consisting of 25 amino acids (sequence revealed by spectrometric analysis as KLKNFAKGVAQSLLNK ASC KLSGQC) having antimicrobial and anticancer activities was isolated from *Rana ridibunda* and has strong homology with brevinin-2Ej and brevinin-2Ee. The antimicrobial spectrum of brevinin-2R displayed activities against *S. aureus*, *M. luteus*, *Bacillus* spKR-8104, *E. coli*, *S. typhimurium*, *P. aeruginosa*, *K. pneumonidae* and fungi, such as *C. albicans* and *C. tropicalis*. Interestingly, the hemolytic activity of the brevinin-2R peptides has been found to be approximately 10-fold less than those of the brevinin-1 family (Ghavami *et al.,* 2008; Thomas *et al.,* 2012) that was characterized by potential pharmaceutical value, highly antimicrobial activity with a low toxicity against red blood cells. Thus, Brevinin-HAs peptide identified in the present study can be considered as a good candidate for designing new therapeutic agents for use against resistant pathogens. The present finding highlights anuran amphibians from Lebanon as a potential source of new antimicrobial peptides.

In conclusion, Antimicrobial peptides from ranid frogs have been suggested as an important source of new therapeutic agents as opposed to the antibiotics that are well known for resulting in increased resistance among the microbial pathogens. In the present work, an antimicrobial peptide was isolated from the crude skin secretions of the frog *Rana ridibunda* that proved effective against various MDR microorganisms. This antimicrobial peptide was structurally classified into the family of Brevinins-2 found in *Rana* amphibians. The broad spectrum antibacterial and antifungal activities of the identified peptide renders it a promising candidate for use in the development of new therapeutic anti-infective agents. However, more detailed studies on the antimicrobial activity of Brevinin-HAs *in vivo* are required and its cytotoxic and anticancer activity should be explored

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