Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 [Special Issue V] 2014: 225-230

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Antimicrobial and Cytotoxic Peptides from the Skin Secretion of the Frog *Rana ridibanda*

Kahin Shahanipour[1], Zarrin Dokht Emami[2], Forogh Taleb[1], Leila Vahabi[1]
[1]Department of Biochemistry, Islamic Azad University, Falavarjan Branch, Falavarjan, Iran
[2]Department of Microbiology, Islamic Azad University, Falavarjan Branch, Falavarjan, Iran

ABSTRACT
Background: The increasing antimicrobial resistance has created a critical need to search for new antibiotics. Skins of amphibians produce various kinds of antibiotics that are effective against pathogenic microbes and are often secreted in response to environmental conditions. There have also been reports of the cytotoxicity of these secretions. Therefore, the purpose of this study was to study the antimicrobial and cytotoxic effects of skin secretions of the Rana ridibanda frog so that, if these effects would be confirmed, these secretions could be introduced as a source of antibacterial and antineoplasmic compounds and can be used as medicines for curing infectious and neoplastic diseases. Methodology: Rana ridibanda frogs were collected from the Anzali Lagoon in Gilan province and their skin secretions were separated, and then the active compounds were fractionally obtained after C-18 Sep-pak extraction and HPLC. Antimicrobial effects of skin extracts on microbial strains of Escherichia coli, Staphylococcus aureus, Pseudomonad aeruginosa, and Bacillus cereus were determined using disc diffusion agar and microdilution methods. Moreover, the cytotoxic effects of these secretions were studied on the breast cancer MDA-MB-231 cell line, the prostate cancer PC3 cell line, the uterus cancer, Hela cell line, and on the colon cancer HT29 cell line. Findings: Our study showed that the antimicrobial effects of skin extracts on Staphylococcus aureus and Bacillus cereus strains were considerable and were more than the corresponding effects on Escherichia coli, but there were no antimicrobial effects on Pseudomonas aeruginosa. Moreover, cytotoxic effects of frog skin extracts on the PC3 cell line were more than the corresponding effects on the other cell lines studied in the research. Results: Skin extracts of the Rana ridibanda frog have the capability of a new antibiotic drug in fighting bacteria especially Staphylococcus aureus and Bacillus cereus. In addition, further studies with the purpose of determining the effects of higher concentrations of these extracts on the PC3 cell line can result in the introduction of these skin secretions as an effective and natural cytotoxic drug.
Keywords:Rana ridibanda, Skin secretions, Antimicrobial effects, Cytotoxic effects

Received 10.05.2014 Revised 29.07.2014 Accepted 15.08. 2014

INTRODUCTION

Skin glands of amphibians are a rich source of antimicrobial compounds [1]. These compounds, are very similar to the neuropeptides and hormones in mammals, and are considered as a main component of the amphibian defensive system [2& 3]. The moist body of the frog is a suitable place for fungi and bacteria to grow, but compounds secreted from its skin largely prevent the growth of such microorganisms [4]. There are the two types of mucus- producing and granular glands in the skin of the frog and of the toad. Secretions of the mucus-producing skin glands provide a moist cover needed for breathing. The granular glands, which are also called serous or toxic glands, exist everywhere in the body but mostly concentrated around the head and the neck. They are activated by stress or injury, and the toxicity of their secretions varies in different species [5]. In ancient Chinese medicine, it was believed that amphibians had many pharmaceutical characteristics and properties and frog secretions were used as heartbeat regulators, antiarrhythmic, antidiabetic, immune system adjuster, sleep-inducer, and they also showed antimicrobial, antineoplastic, analgesic, palliative, birth control, and therapeutic effects against many diseases. Secretions of the granular glands of amphibians are effective against bacterial and fungal infections. Magainins were the first peptide family isolated from the skin of the African frog *Xenopuslaevis* that had a wide spectrum of antibacterial effects against gram- negative and gram- positive bacteria, fungal species such as *Cryptococcus neoformans*, *Candida albicans*, and *Saccharomyces cerevisiae* (6). Since then, extensive research has taken place on frog skin secretions worldwide [7, 8, 9, & 10]. Rollins-Smith

isolatedand purified several peptides from skin secretions of the *Rana tarahumarae* and *Rana muscosa*, and proved their antimicrobial effects. Conlon et al (2012) showed that alyteserin was a cationic and alpha helical peptide of frog skin secretions with great antibacterial activity against some gram-negative bacteria such as drug resistant strains of *Acinetobacter baumannii*, *Stenotrophomonasmaltophilia*. Asgharian et al. (2011) studied the antimicrobial, hemolytic, and cytotoxic activities of skin secretions of the *Ranaridibanda* collected from Minoodasht in Golestan province and concluded that they had considerable antimicrobial effects against both the resistant and susceptible strains of *Staphylococcus aureus* to methicillin. In their study, a greater antibacterial effect was also demonstrated against both the resistant and susceptible strains of *Escherichia coli* and *Enterococcus faecalis* to vancomycin, but no antimicrobial effects against *Pseudomonas aeruginosa* and *Candida albicans* was observed, and the hemolytic and toxic effects of skin extracts were insignificant. Since many bacteria and pathogens have become resistant to antibiotics, thediscoveryof new antibiotics seems to be a necessity. According to this, the search for new antibiotics is greatly demanded all over the world. During recent years, many reports havebeenfocusedonisolatingthefrogskinsecretions with antimicrobial properties. *Rana ridibanda* is a swamp frog with dark spots of various sizes on the backwhich is widely distributed in northern Iran, especially in Gilan province, but, up to now, no comprehensive research has been conducted on its antimicrobial and cytotoxic effects. As several types of peptides with totally different effects have been isolated from various species of frogs in different places in the world, we decided to study skin secretions of *Rana ridibanda* found in northern Iran (especially in Gilan province). In this research, frog skin secretions were isolated and tested against some selected bacteria. Moreover, according to the reports on the antineoplastic effects of frog skin secretions, we studied the cytotoxic effects of skin secretions of *Rana ridibanda* on the cervix cancer cell line Hela, the prostate cancer PC3 cell line, the colon cancer HT29 cell line, and the breast cancer MDA-MB-231 cell line.

MATERIALS AND METHODS

Methodology

This was an empirical study conducted in the research laboratory of the Islamic Azad University of Falavarjan in 2012. Samples of Rana ridibanda were collected from the swamp at Bandar-e Anzali in Gilan province in the last month of the summer of 2012. One hundred and fifteen frogs were collected in several trips made to the swamp area. The frogs were first washed in tap water andafterwardwithdistilledwater and then the sampling was performed under sterile conditions. Instead of employing painful electric stimulations, the frogs were stimulated by injecting 10 nmol/gr norepinephrin (Sigma) according to their body weight. The frogs were then put in 5 ml of a buffer solution containing 50 mM sodium chloride and 25 mM sodium acetate pH = 7 for 10-15 minutes (11). The frogs were then treatedwith one percent hydrochloric acid (12).

Isolation of peptide fractions

The acidified buffer solution containing the peptides was passed through the Sep-18C-pak cartridge. To do this, the sample was acidified by TCA (trichloroacetic acid) and HCL and was passed through the Sep - 18 C-pak together with six milliliters of water and methanol mixed at a 1:1 ratio. The external solution was discarded, the cartridge was washed three times with water, TCA, and HCL mixed at the ratio of 1:1:1, and the resulting solution was dried for two to three hours at 40 °C in a fereserayer α4 machine and was further dried in a vacuum oven. The obtained powder was weighed and stored to be used.

Culturing the bacteria and the antibacterial efficiency of the extract

In order to study the antibacterial properties of the frog extract, five different dilutions were prepared and were tested on four pathogens of *Escerichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* using the disc diffusion method. First, lawn cultures of the mentioned bacteria with the dilution equivalent to 0.5 McFarland (1.5×10^8 cells per mm) on Mueller- Hinton agar (MHA) were prepared (three plates for each bacterial species).

50 µl of each dilution was added to blank discs with a diameter of 6 mm. The discs impregnated with various dilutions of the extract were put in culture media at regular intervals so that each plate had discs containing five different dilutions of the extract. One chloramphenicol disc for gram-positive bacteria and one gentamicin disc for gram-negative bacteria were put as the positive control, and a disc containing sterile broth was taken as the negative control. The plates were kept at 37 °C. After the 24 hours, the diameters of the inhibition zones were measured and recorded.

Determination of the minimum inhibitory concentration (MIC)

The MIC test was conducted on sterile 96-well microplates. Concentrations equivalent to 0.5 McFarland suspensions of -*Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, and Escherichia coli* were prepared that had been cultured on Mueller-Hinton Broth for 24 hours. Moreover, the buffer containing 50 mM sodium chloride and 25 mM sodium acetate pH =7 was used to prepare five different dilutions of

frog skin extracts. The original skin extract had a concentration of 20 µg/ml and was diluted 1:2 in five stages. The inoculated plates containing frog skin secretions were kept at 37 °C for 24 hours. The turbidities of the contents of the microplates were measured using ELISA at 650 nm, the bacterial growth at various concentrations of the skin secretions were compared to that of the control sample, and the results were recorded. The minimum concentration of the extract that did not cause any turbidity in the wells was considered as the MIC.

Study of the cytotoxic effects of frog skin secretions

The method of culturing cancer cell lines was used to study the cytotoxic activities of frog skin secretions. The breast cancer MDA- MB -231 cell line, the prostate cancer PC3 cell line, the uterus cancer Hela cell line, and the colon cancer HT29 cell line were obtained in flasks from the Iranian Pasteur Institute. Based on the NCBI cell catalogue, the codes of the cell lines used are C578, C427, C115, and C466.

The cells were cultured in a medium containing 500 milliliters of RPMI, 50 milliliters of FBS, and 10 percent antibiotic. They turned yellow after consuming the nutrients. The reason for the yellow color is that phenol red turns yellow in acidic environments. After separating pieces of explants and culturing them in fresh media, the cells were transferred to an incubator containing CO_2 (13). Following proliferation, cells were transferred to 96-well plates to study the cytotoxic effects of the extract. 12, 14, 16, 18 and 20 µl of 20 µg/ml frog skin secretions were added to the cell lines to determine the cytotoxic effects of the extract. The extents of the inhibition of the HT29, Hela, MDA-MB-231, and PC3 cell lines by the frog skin secretions were measured, while the extent of growth in the negative control samples was taken to be 100 percent. The survival percentages of cells in the presence of various concentrations of the extract were determined.

RESULTS

Results concerning the effects of frog skin secretions on the four selected pathogens determined by using the disc diffusion method

Diagram 1 shows the average diameter (of three replications) of the zones of inhibition around the discs. These results indicate that frog skin secretions had desirable antimicrobial effects against *Staphylococcus aureus* and *Bacillus cereus*, intermediate effects on the *Pseudomonas aeruginosa*, and little effect of no bacteriological value on the strain of *Escherichia coli*.

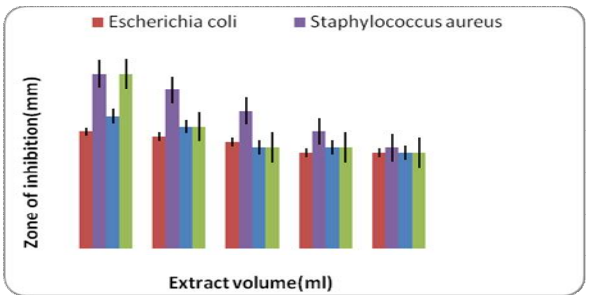

Diagram 1: the zones of inhibition obtained on selected pathogenic bacteria at different dilutions of the active materials from frog skins

Results of determining the minimum inhibitory concentration (MIC)

The MIC test was conducted on the strains of *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, and Escherichia coli*. The results are presented in Table 1. Table 1. Average absorption obtained from wells containing bacteria with six different dilutions of frog skin secretions using the microdilution method

Absorbance of wells containing bacteria in different dilutions of the extract of frog skin						Concentrationg/ml µ20 Bacteria
6/25µl	12/5µl	25µl	50µl	100µl	200µl	
350/3	0/322	20/34	0/267	2250/	1810/	*Escherichia coli*
2020/	000/2	0/181	1510/	0/183	10/16	*Bacillus cereus*
0/200	0/211	0/193	0/183	0/178	0/173	*Staphylococcus aureus*
420/2	2100/	2520/	1980/	2030/	0/195	*Pseudomonas aeruginosa*

The MIC values for *Staphylococcus aureus* and *Bacillus cereus* are significantly less than the corresponding values for the *Pseudomonas aeruginosa* and *Escherichia coli*. Comparison of the results of the MIC test with

those of the disc diffusion of skin extracts of *Rana ridibanda* shows that this extract has considerable effects on *Staphylococcus aureus* and *Bacillus cereus*.

Results of the cytotoxic effects of skin extracts of *Rana ridibanda*

Our purpose was to find the cytotoxic effects of frog skin extracts on the breast cancer MDA-MB-231 cell line, the prostate cancer PC3 cell line, the uterus cancer Hela cell line, and the colon cancer HT29 cell line. Results of the cytotoxic study were analyzed by using the GraphPad Prism 5.0 software and the one-way ANOVA. Diagrams 2 indicate survival percentages of cancer cells in six different dilutions of skin extracts of *Rana ridibanda.*

There are significant differences between survival percentages of the prostate cancer PC3 cell line at the different dilutions of the frog skin extracts due to p-value = 0.014 < 0.05. According to Tukey's test, these differences are related to the 2, 16, and 18 dilutions, and there are no significant differences between the other dilutions considering the survival percentages. As for the other cell lines, no significant differences in survival percentages were observed.

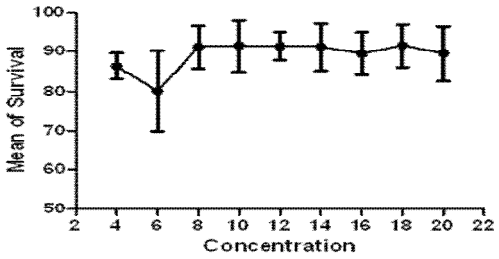

Diagram 2: The linear diagram shows average survival percentages of the HeLa cell line at various dilutions of frog skin extracts (initial concentration of 20$\mu g/ml$)

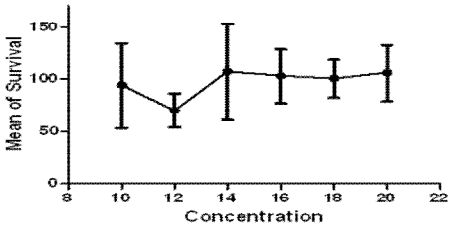

Diagram 3: The linear diagram represents the average survival percentages of the colon cancer HT29 cell line at various dilutions of frog skin extracts (initial concentration of 20 $\mu g/ml$)

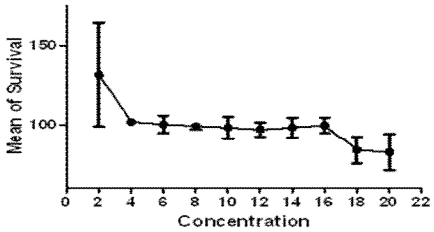

Diagram 4: The linear diagram of average survival percentages of the colon cancer PC3 cell line at various dilutions of skin extracts (initial concentration of 20 $\mu g/ml$)

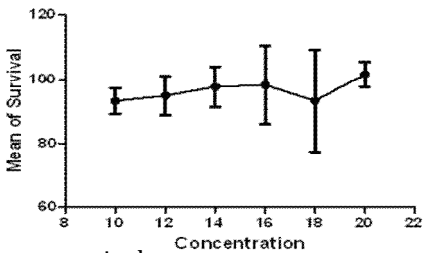

Diagram 5: The linear diagram of average survival percentages of the breast cancer MDA-MB-231 cell line at various dilutions of frog skin extracts (initial concentration of 20 $\mu g/ml$)

DISCUSSION

With the increasing resistance of microbial pathogens to antibiotics and the natural selection of resistant strains of microorganisms, the need to discover and develop compounds with new medicinal properties is growing. Many reports have been published on the therapeutic potential of biomolecules extracted from skin extracts of frogs and toads (14&15). Until now several study has reported the numerous therapeutic activities of these bio- molecules on depression, inflammation, weak memory, poor learning ability, and on neurodegenerative diseases such as Alzheimer's and Parkinson's disease. In China, India, and Vietnam, these secretions have been used for medicinal purposes for many years. Results of our research showed that skin secretions of *Ranaridibanda* have good antimicrobial effects on *Staphylococcus aureus* and *Bacillus cereus* and, intermediate effects on *Pseudomonas aeruginosa*. However, they create a small zone of inhibition for *Escherichia coli* (and, therefore, are of little value for controlling of this bacterial species). Diagram 1 shows the antimicrobial effects of different dilutions of skin secretions on various bacterial strains by disc diffusion agar method. Our results are comparable to those conducted by Goroya on antimicrobial effects of skin extracts of *Ranapipiens*, *Ranabeladeri*, and *Ranaluteiventris*. Goroya (1998) found that skin extracts of *Ranaridibanda* had greater antibacterial effects on gram-positive bacteria such as *Staphylococcus aureus* compared to gram-negative bacteria such as *Escherichia coli* and the *Candid albicans* fungus.

Results of our research are in agreement with reported results of Rollins-Smith et al (2002), inwhich the antimicrobial effects of several peptides extracted from skin secretions of *Ranatarahumarae* was investigated. They also showed that there are certain peptides in skin secretions of the *Rana mucosa* that have antibacterial effects on *Staphylococcus aureus* (12).

Tigerinin-1 is a peptide found in skin secretions of the *Rana tigerina* frog. Sitaram et al. tested this peptide on *Staphylococcus aureus* and *Escherichia coli* at different time points and concentrations. They emphasized that the antibacterial properties of this peptide increased in accordance with its concentration (5). Therefore, the presence of Tigerinin-1 increases the antibacterial effects of skin secretions of the *Ranatigerina* on *Escherichia coli* as compared to the effects of skin secretions of the *Ranaridibanda*.

Asgharian et al. studied the antibacterial effects of skin secretions of *Ranaridibanda* found in Golestan province on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Enterococcus faecalis*. Their results indicated that antimicrobial effects on *Staphylococcus aureus* were greater than the effects on *Escherichia coli* and *Enterococcus faecalis*, but that no antimicrobial effects were observed on *Pseudomonas aureus* and *Candida albicans* (3). As *Ranaridibanda* is widely distributed worldwide, especially in Europe and Asia, and produces various products and secretions, different studies have been focused on skin secretions of *Ranaridibanda* in Golestan province and in Gilan province.In 2007, Wang conducted research on *Ranazhang* and reported peptides with five to ten amino acids in skin secretions of this frog and that these peptides showed cytotoxic effects on bacteria such as *Heliobacter pylori* and *Staphylococcus aureus*.In 1995, Nicolas showed the presence of peptides with antimicrobial properties in secretions of vertebrates and stated that these peptides had cytotoxic effects on many bacteria.

Won studied the antimicrobial activity of skin secretions of *Ranapipiens* and *Ranaesculenta* and obtained the best results (MIC = 6/5) for *Staphylococcus aureus*. He also found a higher MIC for the peptide compound Ranalexin (in skin secretions of the *Ranacatesbilina*) for strains of *Staphylococcus aureus* in comparison with *Escherichia coli* (18). These results are in agreement with those found in our study. Taking the results of various studies conducted on antibiotic properties of Rana frogs into consideration, we conclude that geographical and environmental characteristics greatly affect the peptide variety in these frogs and their properties. According to the results of our study, and the ones reported before, we can say that skin secretions of the *Ranaridibanda* contain compounds with antimicrobial effects against *Staphylococcus aureus* and *Bacillus cereus*, and that these compounds can be used as a source of new and natural antibiotics. On the other hand, our study revealed that the cytotoxic effects of these secretions on breast, as well as uterus, and colon cell lines were slight, but were considerable on the prostate cell line.Asgharian et al. studied the cytotoxic effects of skin secretions of the *Ranaridibanda* found in Golestan province on the human K562 cell line. They reported the toxicity of the extracts on these cells, as well as their slight hemolytic effects. Concerning the toxicity of skin secretions of this frog on the prostate cancer cell line observed in our study, it may be possible to find an effective drug against prostate cancer through conducting further complementary research and isolating the fractions found in these secretions.

ACKNOWLEDGEMENT

The current work was supported by a grant from Islamic Azad University of Falavarjan.

REFERENCES

1. Abdel-Wahab Y, Power G, Flatt PR, Woodhams D, Rollins-Smith L, Conlon J,2008, A peptide of the phylloseptin family from the skin of the frog Hylomantis lemur(Phyllomedusinae) with potent in vitro and in vivo insulin-releasing activity, peptides 29 , 2136–2143.
2. Ashcoroft J., Zalinger, Z, Bevier, C, Fekete, F, 2007, Antimicrobial properties of two purified skin peptides from mink frog against bacteria isolated from the natural habitat, Comparative Biochemistry and physiology, Part C, 146, 325-330.
3. Asgharian AM, Mohammadi M, 2012, Evaluating the Antibacterial, Hemolytic and Cytotoxic Activities of the Iranian RanaRidibunda Skin Extract as a New Source of Antimicrobial Compound,Tehran University Medical Journal, 69(10), 595-604.
4. Bonifacino J S, Dasso M, Harford J B, Lippincott-Schwartz J, Yamada K,2004,Short Protocols in cell biology. USA: John Wiley & Sons, 826.
5. Conlon J. Chapter 3, 2012, American Chemical Society. The Potential of Frog Skin Antimicrobial Peptides for Development into Therapeutically Valuable Anti-Infective Agents , 47–60 , 1095.
6. Conlon J., Mechkarska M, Arafat K, Attoub S, Sonnevend A, 2012, Analogues of the frog skin peptide alyteserin-2a with enhanced antimicrobial activities against Gram-negative bacteria, Journal of Peptide Science,275-270,4,18.
7. Conlon, J, Sonnerend, A, Davidson, C, Smith, D, Nielsen, P, 2004, The ascaphins: a family of antimicrobial Peptides from the skin secretions of the most primitive extent frog, Ascaphustruei, Biochemical and Biophysical Research Communications, 320, 170-175.
8. Conlon JM, Sonnevend A, Patel M, Davidson C, Nielsen PF, Pál T, et al. 2003, Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog Ranaboylii. J Pept Res,62(5):207-13.
9. Douradoa b., Leiteb J., SilvabL,Melob J ., Jr C ., Schwartza E , 2007, Antimicrobial peptide from the skin secretion of the frog Leptodactylussyphax, Toxicon, 50, 572–580.
10. Duellman W E&Trueb L. 1986. In biology of amphibians (McGraw-Hill, New York), 257.
11. Goraya J, Knoop FC, Conlon JM. 1998, Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, Ranacatesbeiana. BiochemBiophys Res Commun, 250(3):589-92.
12. GovenderT,DawoodA,EsterhuyseA,Katerere D, 2012, Antimicrobial properties of the skin secretions of frogs, S Afr J Sci. 108(5/6),1-6.
13. Gomes A.Giri B, Saha A, Mishra r, Dasgupta S, Debnath a, Gomes A,2007, Bioactive molecules from amphibian skin: Their biological activities with reference to therapeutic potentials for possible drug development, Indian j Experimental biology, 45,579-593.
14. Nicolas, P., Mor, A., 1995, Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49.277-304.
15. Rollins-Smith, L., Reinert, L., Miera, V., Conlon, J., 2002, Antimicrobial peptide defenses of the Tarahumara frog, Ranatarahumarae, Biochemical and Biophysical Research Communications, 297, 361-367.
16. Rollins-Smith, L., Woodhams, D., Reinert, L., Vredenburg, V., Briggs, C.,2006, Antimicrobial peptide defenses of the mountain yellow- legged frog, Developmenttal and Comparative Immunology, 30, 831-842.
17. Wang, X., Song, Y., Li, J., Liu, H., Xu, X., Lai, R., Zhang, K., 2007, A new family of Antimicrobial peptides from skin secretions of Ranapleuraden, Peptides, 28, 2069-2074.
18. Won HS, Kim SS, Jung SJ, Son WS, Lee B, Lee BJ.,2004, Structyre-activity relationships of antimicrobial peptides from the skin of Ranaesculenta inhabiting in Korea, Mol Cells, 17(3), 469.
19. Zairi A, Tangy F, Bouassida K, Hani K, 2009, Dermaseptins and Magainins: Antimicrobial Peptides from Frogs' Skin—New Sources for a Promising Spermicides Microbicides—A Mini Review, Journal of Biomedicine and Biotechnology, 1-8.
20. Zasloff M,1987, Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of precursor,Proc Nat AcadSci USA,84,5449.

CITATION OF THIS ARTICLE

Kahin S, Zarrin D E, Forogh T, Leila V. Antimicrobial and Cytotoxic Peptides from the Skin Secretion of the Frog *Rana ridibanda.* Bull. Env. Pharmacol. Life Sci., Vol 3 [Spl Issue V] 2014: 225-230