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Araştırma makalesi

Comparison of Antimicrobial Activity in the Skin Secretion of Same Anurans from Turkey

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Abstract

Membrane-active cationic antimicrobial peptides (CAMPs) are a new class of antibiotics produced by amphibian skin. In this study, Frog (*Bombina bombina, Rana dalmatina, Rana macrocnemis, Bufo bufo, Bufo viridis, Bufo verrocosmisscus, Pelodytes caucasicus, Pelophylax ridibunda, Pelophylax caralitana*) skin secretion was obtained from the dorsal skin using gentle transdermal electrical stimulation. Antimicrobial effects of prepared extracts on the tested microorganisms were determined by using different solvents. In vitro antimicrobial activity studies were carried out by Agar-Disc Diffusion Method. Antibacterial and antiyeast potential of different extracts were obtained from the skin secretions were assessed in terms of zone of inhibition of bacterial growth. According to our findings, all the extracts of skin secretion were obtained from different anurans, exhibit antimicrobial activity.
Keywords: *CAMP, Frog, antimicrobial.*

Özet

Membranla aktif katyonik antimikrobiyal peptidler (CAMP'ler), amfibi deri tarafından üretilen yeni bir antibiyotik sınıfıdır. Bu çalışmada yumuşak transdermal elektrik stimülasyonu ile dorsal deriden Kurbağa (*Bombina bombina, Rana dalmatina, Rana macrocnemis, Bufo bufo, Bufo viridis, Bufo verrocosmisscus, Pelodytes caucasicus, Pelophylax ridibunda, Pelophylax caralitana*) cilt sekresyonu elde edildi. Hazırlanan özütlerin test edilen mikroorganizmalar üzerindeki antimikrobiyal etkileri farklı çözücüler kullanılarak belirlenmiştir. In vitro antimikrobiyal aktivite çalışmaları Agar-Disc Difüzyon Yöntemi ile gerçekleştirildi. Farklı ekstraktların antibakteriyel ve antiyeast potansiyeli elde edildi, cilt sekresyonları bakteri üremesini engelleme bölgesi açısından değerlendirildi. Bulgularımıza göre, cilt sekresyonunun tüm özleri farklı anuranlardan elde edildi, antimikrobiyal aktivite sergiledi.
Anahtar Kelimeler: *KAMP, Kurbağa, Antimikrobiyal.*

1. Introduction

Skin secretions from many species of Anura contain a wide range of compounds with biological activity (Conlon,2011). The source of these compounds are the dermal granular glands and biochemically, the constituent molecules are representative of many classes

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including biogenic amines, peptides, proteins, alkaloids and heterocyclics (Zhou et all, 2006) These compounds are of great importance for the amphibians to regulate their physiological balance, to resist infection by microorganism, and to escape from being preyed upon by natural predators (Xiang Mo et all,2014). Recently, these secretions were also reported as a rich source of multiple antimicrobial peptides effective against multidrug resistant strains, providing instructive lessons for the development of new and more efficient nanotechnological-based therapies for infectious diseases treatment (Calderon et all, 2011). Membrane-active cationic antimicrobial peptides (CAMPs) are a new class of antibiotics produced by almost all forms of life, however, amphibian skin is one of the richest sources. Although prior studies have shown that these peptides possess potent antimicrobial activity against multidrug-resistant pathogens in a controlled environment, little is known of their effects within a living organism (Uccelletti et all,2010) . Recently, the total antimicrobial activity of skin secretion was found to be modulated by the natural flora and frogs kept in a sterile environment did not produce AMPs (Mangoni et al,2001).Therefore, there would not be a surprise that presence of skin secretions with new antimicrobial activity from different ecological conditions. Studies on this subject is limited to a few types of frog in Turkey.

The aim of this study is to test the antimicrobial activity of different anura skin secretions against Gram (+), Gram (-) bacteria and *Candida albicans* cultures.

2. Materials and Methods

2.1. Specimen biodata and secretion harvesting

Adult specimens of *frogs* of both sexes *(Bombina bombina, Rana dalmatina, Rana macrocnemis, Bufo bufo, Bufo viridis, Bufo verrocosmisscus, Pelodytes caucasicus, Pelophylax ridibunda, Pelophylax caralitana*) obtain from different regions in Turkey (Table 1).

2.2. Preparation of skin secretion

Before experimentation, the frogs were washed first with tap water and then with distilled water. Skin secretion was obtained from the dorsal skin using gentle transdermal electrical stimulation as previously described by Zhou et al, 2006. Secretions were washed from the skin using deionized water and collected solutions were left in 80°C water bath for 30 min and centrifuged at 5500 rev/min for 30 min. After centrifugation, the precipitate was used in the experiments. Before using in the experiments, the precipitate was diluted with distilled water, 0.1 N HCl, 0.1 N NH4OH and 1 M phosphate buffers (pH: 4 and pH: 7) (Dülger et al., 2004 and Afsar et al, 2011).

2.3. Test microorganisms and growth conditions

In this study, a total of 11 test microorganisms were used (*Escherichia coli ATCC 25922, Staphylococcus aureus MRRL B767, Pseudomonas aeruginosa, Listeria monocytogenes ATCC 7644, Micrococcus luteus ATCC 9341, Bacillus subtilis* NRS-744*, Klebsiella pneumoniae, Salmonella typhimurium NRRL B 4420, Proteus vulgaris, Bacillus cereus* ATCC-11778*, Candida albicans*). Test microorganisms were obtained from the culture collection of Afyon Kocatepe University, Faculty of Science, Biology Department, Afyonkarahisar-Turkey. Cultures of these bacteria were grown in Nutrient Broth (NB) at 37°C for 24 h (Oskay and Sarı, 2007).

Table 1
Adult specimens of frogs obtain from different regions in Turkey

No	Species	Region	Coordinate	Altitude	N					Average
					1	2	3	4	5	
1	***Hyla orientalis***	İğne ada(Kırklareli)	41 11 13 40 59 19	39	36,88	37,6	-	-	-	37,24
2	***Bombina bombina***	Ak lake (Adapazarı)	40 52 36 30 26 02	39	37,3	37,6	36,34	-	-	37,08
3	***Bufo bufo***	Kabaca köyü (Zonguldak)	41 17 53	482	117,04	-	-	-	-	117,04
4	***Pelodytes caucasicus***	Uzun lake (Trabzon)	40 37 10 40 17 27	1164	52,28	47	42,3	43,26	44,6	45,888
5	***Rana dalmatina***	Uzun lake l (Trabzon)	40 35 27 40 20 45	1272	32	-	-	-		32
6	***Bufo verrucosissimus***	Uzun lake (Trabzon)	40 35 27 40 20 45	1272	94,6	69,34	69,29	68,68	66	73,582
7	***Rana macrocnemis***	Uzun lake (Trabzon)	40 34 38 40 23 47	1701	73,1	-	-	-	-	73,1
8	***Bufotes variabilis***	Sahara (Artvin)	41 13 47 42 27 05	1876	71,88	-	-	-	-	71,88
9	***Pelophylax caralitanus***	Eğirdir lake (Isparta)	38 12 44 30 45 05	923	132	89,82	60,6	84,48	90,5	91,48

2.4. Determination of antimicrobial activity

In vitro antimicrobial activity studies were carried out by Agar-Disc Diffusion Method according to Clinical and Laboratory Standards Institute (CLSI) Nutrient Agar (NA) was preferred as the most suitable medium for antimicrobial activity studies. 20 µl extract was implemented into a sterile 6 mm diameter disc. The turbidity of bacterial suspension was adjusted according to Mcfarland Standard Tube (0.5) with physiologic serum and suspension of the tested microorganism was spreed on the solid media plates. Filter paper discs placed on the inoculated plates. These plates, were incubated at 37 °C for 24 h for bacteria and, at 30 °C for 24 h, for yeasts (Collins etal., 1989; NCCLS 1993). The diameters of the inhibition zones were measured in millimetres. All tests were performed in duplicate.

In addition, continued only solvent was used as negative control. 0.1 N HCl, 0.1 N NH4OH, 1 M phosphate buffers (pH: 4), 1 M phosphate buffers (pH:7) were used as positive controls. Experiments were repeated two timesand results were expressed as average values.

3. Results and Discussion

According to our findings, all the extracts of skin secretion were obtained from diffrent anurans, exhibit antimicrobial activity. Antibacterial and antiyeast potential of different extracts were obtained from the skin secretions were assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial and antiyeast activities are presented in Table 2.

The skin of amphibians is an extraordinarily rich source of antimicrobial peptides (AMPs). Several hundreds of such antimicrobial peptides have been isolated from the skin of frogs belonging to the families Bombinatoridae, Hylidae, Hyperoliidae, Leiopelmatidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae (Conlon, 2009).The main families of AMPs belong to a large group of linear amphipathic helical peptides. They are cationic, containing a variable number of positively charged residues and hydrophobic regions. These characteristics provide them with an ability to bind to negatively charged molecules and/or membrane lipids and disturb the membrane structure. This seems to be the main mechanism of induction of death of their targets (Conlon *et al.*, 2004; Smıth et al, 2005).

In general, the antimicrobial activity of frog skin peptides is tested contextually to their isolation against a small number of pathogenic, reference bacterial and fungal strains. These include the Gram-negative *E. coli* and *P. aeruginosa*, the Gram-positive *S. aureus*, and the yeast *C. albicans*. (Rinaldi, 2002). In this study, *S. typhimurium*, *K. pneumoniae E. coli, P. vulgaris* P. *aeruginosa, B. cereus, B. subtilis, S. aureus, M. luteus, L. monocytogenes* bacteria cultures and *C. albicans* yeast cultures were used. The antimicrobial activity of bombinin-like peptides or bombinins H obtain from *Bombina sp*. can be distinguished on the basis of their cytolytic properties. Bombinins were found to be active against Gram-positive (+) (*Bacillus megaterium, S. aureus*) and Gram-negative (-) (*E. coli, Yersinia pseudotuberculosis, P. aeruginosa*) bacteria as well as against *C. albicans*. (Mangoni et al, 2000; Simmaco et al, 2009).

Table 2
The results of the antibacterial and antiyeast activities (mm)

Frogs Speciment	*Salmonella typhimurium*	*Klebsiella pneumoniae*	*Escherichia coli*	*Proteus vulgaris*	Pseudomonas *aeruginosa*	*Bacillus cereus*	*Bacillus subtilis*	*Stapylococus aureus*	*Micrococcus luteus*	*Listeria monocytogenes*	*Candida albicans*
Positive control											
Sulbactam/ampicillin	9	25	-	9	20	12	13	10	17	10	-
P10/Penicillin	-	7	-	-	-	20	22	10	-	-	-
AM/Amikacin	-	-	-	-	-	21	23	21	-	-	-
Negative control											
0.1 N HCl,	-	-	10	-	-	-	7	13	11	-	-
0.1 N NH4OH	-	-	10	-	-	-	7	-	-	-	-
1 M phosphate buffers (pH: 4)	-	-	10	-	-	-	7	-	-	-	-
1 M phosphate buffers (pH:7)	-	-	8	-	-	18	7	14	20	-	-
Rana bombina											
0.1 N HCl,	13	13	12	12	10,5	11	11	11,5	12,5	9,5	12
0.1 N NH4OH	12,5	12,5	10,5	11	11	10	10	11	12	10	-
1 M phosphate buffers (pH: 4)	17	13,5	11	14,5	13	11,5	11	13	14	9,5	-

1 M phosphate buffers (pH:7)	12,5	14	10	12,5	11,5	9,5	10	13,5	10,5	9,5	-
Rana dalmatina											
0.1 N HCl,	9,5	14,5	11	13	11	10	9,5	10,5	12	10,5	12
0.1 N NH4OH	-	-	11	-	10	-	-	-	-	-	9
1 M phosphate buffers (pH: 4)	-	-	11	-	-	-	-	-	-	9,5	-
1 M phosphate buffers (pH:7)	7,5	14	10,5	10	9,5	9	10,5	10	-	11,5	8,5
Rana macrocnemis											
0.1 N HCl,	14	13,5	12	13,5	13,5	10,5	10,5	14,5	11	11	10,5
0.1 N NH4OH	12	13,5	13	12,5	-	14	11,5	13,5	12	7,5	-
1 M phosphate buffers (pH: 4)	9	10	9,5	-	-	11	-	9,5	-	-	-
1 M phosphate buffers (pH:7)	12,5	14,5	11	12	13,5	8	9,5	9,5	-	8,5	-
Bufo bufo											
0.1 N HCl,	11,5	16	14	10,5	-	9	9	9	-	8,5	-
0.1 N NH4OH	9	12	11	9	-	6,5	10	7,5	-	8	6,5
1 M phosphate buffers (pH: 4)	-	-	11,5	-	-	8	6,5	-	-	-	8,5
1 M phosphate buffers (pH:7)	9	-	10,5	9,5	-	8,5	8,5	8	-	9	9
Bufo viridis											
0.1 N HCl,	11	11	10,5	11	12,5	11	11	9,5	15,5	9	12,5
0.1 N NH4OH	-	-	9	-	8,5	-	-	-	-	-	-
1 M phosphate buffers (pH: 4)	-	-	10	-	-	-	-	-	-	7,5	9
1 M phosphate buffers (pH:7)	10	-	8,5	10	10	9	9	11,5	-	8	-
Bufo verrocosmisscus											

0.1 N HCl,	8,5	11,5	11	12	12	9,5	9,5	10,5	10,5	12	11
0.1 N NH4OH	-	8	13,5	-	-	-	-	-	-	-	-
1 M phosphate buffers (pH: 4)	-	-	11,5	-	-	-	-	-	-	-	-
1 M phosphate buffers (pH:7)	8,5	8	8	11	10	9,5	7,5	9,5	-	10	-
Pelodytes caucasicus											
0.1 N HCl,	9	11	10	11,5	10,5	9,5	10	10	11	10,5	10
0.1 N NH4OH	-	-	11,5	-	-	-	-	-	-	-	7
1 M phosphate buffers (pH: 4)	-	-	12	-	-	9	-	-	-	11	-
1 M phosphate buffers (pH:7)	9,5	9,5	11,5	10	8,5	8,5	10	9,5	10	9,5	6,5
Pelophylax ridibunda											
0.1 N HCl,	11	-	-	10	-	8,5	8,5	9,5	7	12	-
0.1 N NH4OH	10	9	-	10	-	12	10	11	13,5	11	-
1 M phosphate buffers (pH: 4)	12	9	8	11,5	-	15	13,5	11,5	10,5	11	6,5
1 M phosphate buffers (pH:7)	11	8	-	9,5	-	11	11	10,5	8,5	8	-
Pelophylax caralitana											
0.1 N HCl,	9,5	10	11	11,5	12	9	9	9	12,5	10	11
0.1 N NH4OH	-	9	12,5	8	-	-	10	-	8,5	-	-
1 M phosphate buffers (pH: 4)	-	6,5	12,5	-	-	-	8,5	-	-	7,5	-
1 M phosphate buffers (pH:7)	10,5	-	10,5	11	10,5	10,5	9,5	11	10,5	9	-

According to our findings, skin secretion from *Bombina bombina* was observed antimicrobial activities against both G(-) and G(+) bacteria. The highest antibacterial effect showed by 1M phosphate buffer (pH4) of *Bombina bombina* skin extract against *P.vulgaris, S. typhimurium* and *B. cereus*. Only 0.1N HCI extracts of skin secretions showed antiyeast effects Our results are in agreement with the other authors' result (Mangoni et al, 2000; Simmaco et al,2009).s.

Dülger et al. (2004) investigated antimicrobial activity of skin secretions from *Bufo viridis* (Laurenti, 1768). In this study, antibacterial and antiyeast activities of the *B. viridis* skin secretion 0.1N HCI extract were determined. The highest antibacterial effect showed by 0.1 N NH_4OH of Bufo viridis extract against *M.luteus*. The growth inhibition zone measured ranged from 11 to 15.5 mm for all the sensitive bacteria and 12.5mm for yeast. Bae Park et al (1996) report a novel antimicrobial peptide named buforin I and II purified from the stomach of *Bufo bufo gargarizans,* an Asian toad, which has been used as a wound-healing agent in traditional Korean medicine. Both buforin I and buforin II displayed strong antimicrobial activities against a broad spectrum of bacteria including, *B. subtilis, S.s aureus, S. mutans, S.pneumoniae, E. coli, Serratia* sp., *P. putida,* and *S. typhimurium.* Furthermore, *C. albicans, Saccharomyces cerevisiae* and *Cryptococcus neoformance* were also killed. In our results show that, Extracts of *Bufo bufo* skin secretion exhibited no antimicrobial effects against *P.s aeruginosa* and *M. luteus* However the highest antibacterial effect showed against *K. pneumoniae* (16 mm inhibition zone at 1N HCI) The most antibacterial effect showed by 0.1 N HCL. Frogs belonging to the extensive family Ranidae represent a valuable source of antimicrobial peptides (Conlon, 2009). Çevikbas (1978) reported that skin secretion of *Rana ridibunda* shows antibacterial activity at different levels. However, Afsar et al (2011) showed that, skin secretions of *Rana macrocnemis* against the yeast cultures show more antimicrobial activity than bacterial cultures. According to our findings, the highest anmicrobial activity was observed by 0.1 N HCI extract of *Rana dalmatina* and 1M phosphate buffers (pH7) extracts of *Rana macrocnemis* against *K. pneumoniae* (14.5mm). 0.1 N NH_4OH, 0.1 N HCI and 1M phosphate buffer (pH7) extracts of *Rana macrocnemis* skin secretion exhibited antimicrobial effects against *E.coli* which is resistant to tested different antibiotics.

All extracts of *Pelophylax ridibunda* skin secretion no inhibited *P. aeruginosa* growth. On the other hand, 0.1 N HCI and 1M phosphate buffer (pH7) extracts from *P. caralitana* show anti-microbial activity against *P. aeruginosa*. 1M phosphate buffer (pH 7and 4) extracts from *Pelophylax ridibunda* skin secretion also had the highest activity rate against *B. subtilis* and *M. luteus* (13.5 mm). It has been reported that sensitivity of the microorganisms to the chemotherapeutic agents changes from strain to strain (Cetin et al., 1989). According to our findings, all the extracts of skin secretion were obtained from diffrent anurans, exhibit antimicrobial activity. The present study has demonstrated that antimicrobial activity of skin secretions of differs at both the generic and ecological.

In conclusion, Amphibians, being the first group of organisms forming a connecting link between land andwater, are forced to adopt and survive in a variety of conditions laden with pathogenic microbes. Therefore, they are endowed with an excellent chemical defense systemcomposed of pharmacological and antimicrobial peptides (Boman, 1991). New peptides have been found that could inspire the design of analogues to prevent or treat infections. Peptide-based antibiotics are largely considered a potential answer to the growing problem of resistance to conventional antibiotics. In the development of AMPs as human therapeutics, peptides originating from amphibian skin and their synthetic analogues will likely play a crucial role.

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