

Full Length Research Paper

Antimicrobial activity in the skin secretion of brown frog, *Rana macrocnemis* (Boulenger, 1885) collected from Turkey

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In this study, antimicrobial activity of various extracts prepared from *Rana macrocnemis* skin secretion were tested against the microorganisms by disc diffusion method. *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Sarcina lutea*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Candida albicans* were used as test microorganisms. According to our results, the extracts prepared from *Rana macrocnemis* skin secretion have antimicrobial activity against the tested microorganisms.

Key words: *Rana macrocnemis*, amphibia, antimicrobial activity, skin secretion.

INTRODUCTION

The emergence in recent years of numerous strains of pathogenic microorganisms that have developed resistance to a range of formerly efficacious antibiotics constitutes a serious threat to public health. The fact that certain nosocomial pathogens are already resistant to all available antibiotics, and therefore essentially untreatable, dramatically demonstrates the need for completely new types of antimicrobial agents (Conlon et al., 2006).

The global decline in amphibian populations is increasingly becoming a cause for concern. Habitat destruction, introduction of predators and direct exposure to toxins and xenobiotics has been implicated in this decline, together with infection by pathogens particularly the chytrid fungus *Batrachochytrium dendrobatidis*. The skin is the first barrier against such pathogens and production of antimicrobial molecules in the granular glands of the skin of many, but by no means all, species of frogs constitutes an important defense mechanism. Frogs may synthesize and release multiple peptides that differ in their specificity and potency against the diverse range of pathogenic microorganisms encountered in the environment (Ohnuma et al., 2007).

Amphibians have been studied and have attracted special attention from a toxicological point of view. Various

substances with antimicrobial activity have been isolated from skin secretions of amphibian species (Cevikbas, 1978; Dülger et al., 2004; Pal et al., 2005; Conlon et al., 2006; Ohnuma et al., 2007). Several toxins in amphibian poisons have been used as experimental tools and contributed to significant progress in physiology. Some toxins (Batrachotoxins) specifically block the inactivation of the voltage regulated Na⁺ channels in nerve and muscle cells, which causes a massive inflow of Na⁺. The cells become irreversibly depolarized, which among other things produces heart arrhythmia and respiratory failure and finally cardiac insufficiency.

In humans, some amphibian toxins (Bufotenin) produce symptoms similar to those of LSD (Edstrom, 1992). In previous studies, some skin secretions showed remarkable cytotoxic activity against eukaryotic cells (Sanna et al., 1993). The aim of this study is to test the antimicrobial activity of *Rana macrocnemis* skin secretions against Gram (+), Gram (-) bacteria and yeast cultures for future possible use in providing pharmacological tools for the study of new drugs and aid in benefiting human health.

MATERIALS AND METHODS

Preparation of skin secretion

Specimens of *Rana macrocnemis* were collected from northeast

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Table 1. Inhibition zone values of extracts of *R. macrocnemis* skin secretion against some microorganisms (mm).

Test MO*	A**	B	C		D	P	NO	NA	NYS
			C1	C2					
BC	20.0	18.0	22.0	24.0	---	10.0	25.0	28.0	---
BS	14.0	16.0	20.0	20.0	---	---	13.0	32.0	---
EA	14.0	15.0	22.0	20.0	18.0	---	17.0	26.0	---
PV	---	---	---	---	---	10.0	26.0	12.0	---
SL	25.0	30.0	18.0	18.0	---	20.0	28.0	10.0	---
EC	16.0	15.0	16.0	18.0	---	---	---	26.0	---
ST	---	---	---	---	---	---	40.0	---	---
SA	20.0	23.0	26.0	28.0	---	24.0	32.0	20.0	---
CA	20.0	22.0	15.0	14.0	16.0	---	---	---	22.0

* EC: *Escherichia coli*, SA: *Staphylococcus aureus*, PV: *Proteus vulgaris*, BS: *Bacillus subtilis*, BC: *Bacillus cereus*, EA: *Enterobacter aerogenes*, SL: *Sarcina lutea*, ST: *Salmonella typhimurium*, CA: *Candida albicans*.

**A: 0.1 N HCl; B: 0.1 N NH₄OH; C: Phosphate Buffer; C1: pH=4; C2: pH=7; D: Distilled water; P: Penicillin G; NO: Novobiocin; NA: Nalidixic acid; NYS: Nystatin.

region (Borçka -Artvin) of Turkey in 2007. Collected frogs were brought to the laboratory and kept in an aquarium. Before experimentation, the frogs were washed first with tap water and then with distilled water. They were placed for 3 – 5 min in a glass jar containing a piece of cotton soaked with ether to stimulate skin secretions. The secretion accumulated on the skin was obtained by scraping the body of the animals with a spatula. The foamy secretion thus obtained was placed in a tube, 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).

Test microorganisms and growth conditions

In this study, a total of 9 test microorganisms were used: *Escherichia coli* ATCC 39628, *Staphylococcus aureus* ATCC 6538P, *Proteus vulgaris* ATCC 8427, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CM 99, *Enterobacter aerogenes* ATCC 13048, *Sarcina lutea* ATCC 9341NA, *Salmonella typhimurium* CCM 5445 and *Candida albicans* ATCC 10231.

Cultures of these bacteria were grown in Mueller Hinton broth (Oxoid) at 37°C for 24 h and the studied yeast was incubated in glucose yeast extract broth at 30°C for 48 h (Oskay and Sari, 2007). Test microorganisms were obtained from the culture collection of Ege University, Faculty of Science, Basic and Industrial Microbiology Department.

Determination of antimicrobial activity

In vitro antimicrobial activity studies were carried out by Agar-Disc Diffusion Method. Mueller Hinton Agar (Merck) was preferred as the most suitable medium for antimicrobial activity studies. Each extract was implemented into a sterile disc in varying concentrations starting from 20 µl. each disc was 6 mm in diameter (Solak et al., 2006).

Bacteria and yeast cultures were suspended in 4 – 5 ml Brain Heart Infusion Broth (Merck) and Malt Extract Broth (Merck). Bacteria were incubated in 37°C for 2 h. Yeast cultures were incubated in 30°C for 5 – 7 h. A visible turbidity was obtained at the end of this time. The turbidity of bacterial suspension was adjusted according to McFarland Standard Tube (0.5) with physiologic serum

and inoculation performed. Prepared bacterial suspension was mixed with a sterile applicator and excess fluid of applicator was removed by rotating the applicator to one side of the tube. We streaked the entire Mueller Hinton Agar surface in three different directions by rotating the plate 60° angles after each streaking. Yeast cultures were inoculated into Mueller Hinton Agar (10² cfu/ml). All Petri dishes after inoculation were allowed to dry for 15 – 20 min at room temperature (bacteria at 35°C and yeast at 30°C). Inhibition zone diameters were measured after 24 – 48 h (Collins et al., 1989; NCCLS 1993). In addition, continued only solvent was used as negative control disc and antibiotic penicillin G (10 IU), novobiocin (30 µg), nalidixic acid (30 µg) and nystatin (10 µg) discs were used as references. Experiments were repeated three times and results were expressed as average values.

Statistical analysis

The mean values were statistically analyzed with the MINITAB Release 13.20 program by the general one-way (unstacked) analysis of variance (ANOVA) to find out the most effective extracts and the most sensitive test organisms. Similarity (%) of microorganisms in relation to their susceptibility to the skin secretion extracts was analyzed by the multivariate cluster analysis according to the data obtained from disc diffusion assay.

RESULTS

Antimicrobial activity effects of five different extracts which were prepared by using distilled water, 0.1 N HCl, 0.1 N NH₄OH, 1 M phosphate buffers (pH:4 and pH:7), were obtained from the skin secretion of *Rana macrocnemis* against bacteria and yeast cultures and results are given in Table 1.

According to our findings, the highest antibacterial effect showed by 0.1 N NH₄OH extract against *S. lutea* with 30 mm that higher than compared antibiotics. The phosphate buffer extracts of skin secretions showed antibacterial effects likely standard antibiotics against *S. aureus*, *B. subtilis* and *E. aerogenes*. The highest antifungal activity is demonstrated by 0.1 N NH₄OH

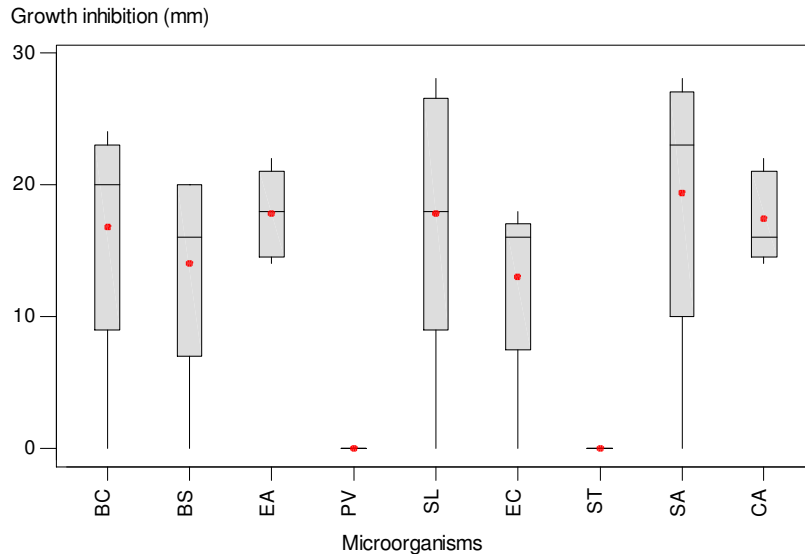


Figure 1. Mean values of microorganisms in relation to their susceptibility to the skin secretion extracts. Means are indicated by solid circles. See Table 1 for abbreviations of test microorganisms.

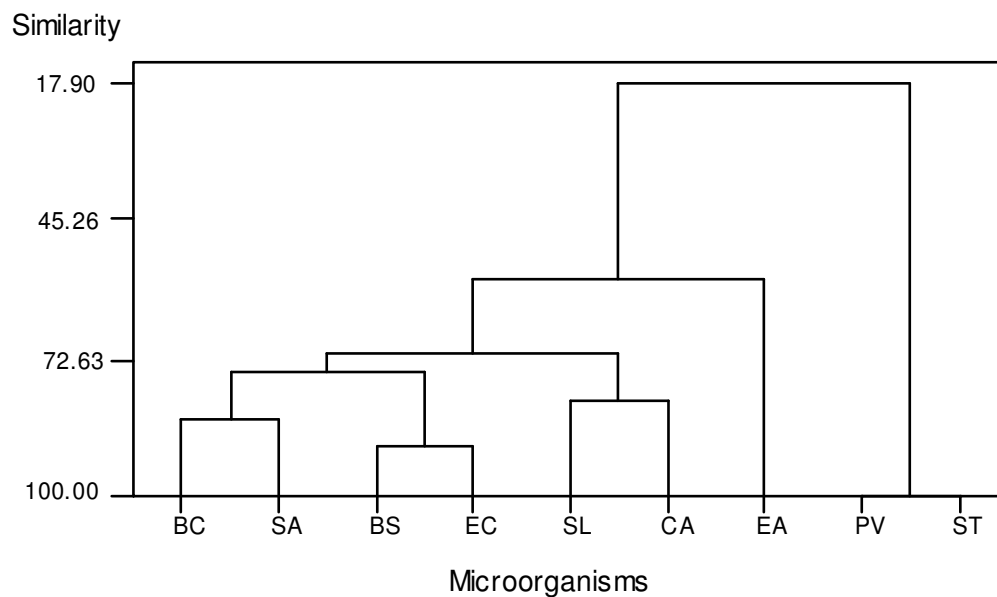


Figure 2. Similarity (%) of microorganisms in relation to their susceptibility to the skin secretion extracts. See Table 1 for abbreviations of test microorganisms.

extract against *C. albicans* (22 mm) similarly nystatin. As listed in Table 1; none of the extracts were active against *Proteus vulgaris* and *Salmonella typhimurium*. Distilled water extracts of skin secretion exhibited minor effects against *E. aerogenes* and *C. albicans* (18 and 16 mm, respectively).

Susceptibility of test microorganisms, in decreasing order was as follows: *S. aureus*, *S. lutea*, *E. aerogenes*, *C. albicans*, *B. cereus*, *B. subtilis*, *E. coli*, *P. vulgaris* and

S. typhimurium (Figure 1). Figure 2 summarizes the similarity of test microorganisms in relation to their susceptibility to the skin secretion extracts.

DISCUSSION

It has been reported that sensitivity of the microorganisms to be chemotherapeutic agents changes from

strain to strain (Smith et al., 2006). Our results are in agreement with the other authors results.

Dülger et al. (2004) investigated antimicrobial activity of skin secretions from *Bufo viridis* (Laurenti, 1768). They homogenized skin secretion with phosphate buffers (pH:4 and pH:7), 0.1 N HCl, 0.1 N NH₄OH and distilled water. These homogenates show high antimicrobial activity against yeast cultures *Rhodotorula rubra* and *Saccharomyces cerevisiae*.

Çevikbas (1978) examined antibacterial activity in the skin secretions of *Rana ridibunda*. The author reported that skin secretion of *Rana ridibunda* shows antibacterial activity at different levels. However, in our study, skin secretions of *Rana macrocnemis* against the yeast cultures show more antimicrobial activity than that of the bacterial cultures. Our findings parallel those reported in the above studies. In amphibian, antimicrobial activity of skin secretions differs at both the generic and specific levels.

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