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Study of Antimicrobial Effect of *Rana cyanophlyctis* Skin Bioactive Molecules

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Abstract

In the present study, the effect of bioactive molecules secreted by skin glands of the frog *Rana cyanophlyctis* tested against different microorganisms.Inhibition zone diameters of 15, 13, 14,14,25,13 mm were observed for *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *E.coli* respectively. Microscopic study of microbial cells treated with the frog skin secretion demonstrated direct bactericidal capabilities.This observation suggested that the antimicrobial skin gland secretion of the frog, is active against the tested microbial strains and the secretion have the potential to be used as a good source of antibacterial agents.

Key words: *Antibacterial activity, frog skin secretion.*

Introduction

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a serious threat to public health and has necessitated a search for novel types of antimicrobial agent to which the microorganisms have not been exposed (Norrby SR *et al.,* 2005).Skin of anurans (Frogs and Toads) are highly glandular and secretes mucous to protect themselves against osmotic changes and used as a defense system against various fungal, microbial and bacterial agents, and also serves the multiple roles of fluid balance, respiration and transport of essentials for survival of individuals. Amphibians have been studied and attracted special attention from a toxicological point of view. Various substances with antimicrobial activity have been isolated from skin secretions of amphibian species (Croce *et al.,* 1973;Cevikbas, 1978). The secretions from amphibian's skin have become a pivotal model for the discovery of new peptide antibiotics originating from non myeloid cells of vertebrates (Lazarus LH and Attila M. 1993). The bioactive peptides are released into the skin secretions in a holocrine fashion upon stress or injury and protect them against invasion by pathogenic microorganisms (Charpentier S *et al.,* 1998). It is believed that the simultaneous presence of antimicrobial molecules acting in synergy, provides frogs with a better shield against a wide range of harmful invaders(Lazarus LH and Attila M.1993,).For many years, people in different cultures were using therapeutic effects of amphibian skin to cure any inflammation or lesion and even scorch (Jin *et al.,* 2009 and Ostorhazi *et al.,* 2010).

This study is aimed to extract and test the secretions of frog found in Rajasthan Desert Province for antibacterial activity against six bacterial strains using the agar well diffusion method.

Materials and Methods

Experimental animals: Adult frogs *Rana cyanophlyctis* were collected from fresh water habitats (ponds) from Bikaner and Hanumangarh (Rajasthan) during monsoon season of the year.

Skin gland secretion collection and processing

The method of collection of skin secretion was adopted as described by Li *et al* (2007) with some modifications. For collection of skin gland secretion, 10 frogs were stimulated to release their skin secretions by keeping them in chloroform vapor for 2-5 minutes. The secretions were collected by washing the frogs with 0.1M NaCl solution containing 0.01 M EDTA. The solution was then sterilized by passing it through a 0.02µm Millipore filter. The filtrate was kept refrigerated for the further use.

Microorganisms used

Six different bacterial strains were used throughout the present work: *Staphylococcus aureus ,Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *E.coli.* These strains were purchased from the Micobial Type Culture collection (MTCC) and Institute of Microbial Technology, Chandigarh (India) .The bacterial isolates were maintained on nutrient agar slants and stored at 4^0C with regular transfer at monthly intervals.

Preparation of bacterial inoculums

Inoculums of employed bacterial strains were prepared in autoclaved Nutrient broth and incubated at 37^0C for 24 hours.

Evaluation of antibacterial susceptibility

Agar well diffusion method was used for the evaluation of antibacterial susceptibility. Nutrient agar medium was prepared, autoclaved at 121^0C for 15 minutes and solidified in petriplates. Each plate contained 20ml of this medium. An

inoculum (turbidity adjusted to approximately108 CFU/ml of bacterium. Each bacterial strain was uniformly spread on this medium in separate plates. Wells of 6 mm diameter were bored in solidified media (Ekpo & Etim, 2009).

The same method was used to determine the inhibitory effect of frog skin secretions on the tested microorganisms .Holes of 6 mm diameter were made into which the skin secretion (50 µl) was applied. Ampicillin was used as positive control (Ekpo & Etim, 2009).The culture plates were incubated at 37 ^{0}C for 24 hours. The antibacterial activity was measured from the formation of the clearing zone due to inhibition of bacterial growth around the treated area. Zones of inhibition were recorded in millimeters and the experiments were repeated thrice.

Results

As shown in the table 1 frog skin secretions showed antibacterial activity against the growth of six different bacterial strains. Data also presented in Figure 1 showed the average inhibition zone diameter of 15, 13,14,14,25 and 13 mm for *Staphylococcus aureus* , *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *E.coli.* respectively.

Table1. Inhibition zone diameter (mm) of the frog (*Rana cyanophlyctis*) skin secretions against the tested bacterial strains versus control antimicrobial (ampicillin)

Zone of inhibition in mm		
Bacterial Strain	**Frog Secretion**	**Control (Ampicillin)**
Staphylococcus aureus	15	12
Pseudomonas aeruginosa	13	R
Klebsiella pneumonia	14	25
Salmonella typhi	14	27
Shigella flexneri	25	25
E. coli	13	30

Data revealed more powerful antibacterial effect of the frog skin secretion against the strain *Shigella flexneri*.(Table 1&Fig.1e On the other hand *Pseudomonas aeruginosa* was found resistant to ampicillin but sensitive to frog skin secretion(Table 1).

Figure 1. Antibacterial activity of skin secretions of the frog *Rana Cyanophlyctis* against bacteria *Staphylococcus aureus*(a.) ,*Pseudomonas aeruginosa* (b.), *Klebsiella pneumonia* (c.), *Salmonella typhi* (d.), *Shigella flexneri* (e.)and *E.coli* (f.) .The formation of clearing zone around the treated area indicated antibacterial activity.

The range of inhibition zone for *Staphylococcus aureus* (15mm) was higher than those for ampicillin(12mm).The range of inhibition zone for *Shigella flexneri* was similar(25mm) for both skin secretion and ampicillin as well. However the range of inhibition zone was observed less for *Klebsiella pneumonia* (14mm), *Salmonella typhi* (14mm) and *E.coli* (13mm) in comparison to ampicillin 25mm , 27 and 30mm respectively.

Microscopic observation revealed significant changes in cell structure treated with frog skin secretion .The cells exhibited structural disorganization and become picnotic .Overall the frog skin secretion had a direct bactericidal capability.

Discussion

The findings of present study clearly showed that the skin secretion of the frog, *Rana cyanophlyctis*, has the potential to be used as a source of antibacterial agents. This study employed six different strains representing gram positive and gram negative bacteria. *Staphylococcus aureus* is a gram positive bacteria found in the human respiratory tract and on the skin .It can be virulent pathogen .Corey (2009) , reported high rate of infections caused by *S.aureus* bacterium in humans .S. *aureus* was found to be the main cause of infections morbidity and mortality in hemodialysis patients.

In present study antibacterial activity of skin secretions was found evident and even higher than that of ampicillin for some bacterial stains. As shown in the table1,skin secretion of the frog was found able to inhibit the growth of almost all tested bacterial strains.

A number of studies have also reported the antibacterial activity of various skin secretions derived from different anuran amphibians (Afsar *et al* 2011 ;Abraham *et al* 2014; Asmaa *et al 2015, Sharma KK et al 2012*).Artika *et al (2015)*repoted chemical compounds like fatty acids tri acontane, hepadecane, pregnane and myrtnol of the toad and frog skin secretions, having antibactireal activity and it was also reported that long chain unsaturated fatty acids found in frog skin secretion inhibited the activity of bacterial enoyl-acyl carrier protein reductase which is an essential component of bacterial fatty acid synthesis.

Considering the potential use of skin secretions of the frog *Rana cyanophlyctis* as source of new antimicrobial agent it is suggested to test the antibacterial activity of these secretions against multidrug resistant bacteria and therefore, further work in this area is recommended.

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