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RESEARCH ARTICLE

ANTIMICROBIAL PEPTIDE FROM *EUPHLYCTIS HEXADACTYLUS* AND ITS EFFICACY AGAINST PLANT PATHOGENS

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ABSTRACT

The Indian green frog (*Euphlyctis hexadactylus*) is a common species existing in aquatic environment. The skin secretions of these frogs have potential antimicrobial peptides. It inhibited growth of pathogenic gram-positive and gram-negative bacteria and opportunistic fungal pathogens and also treated plant fungal diseases. The minimum inhibitory concentration (MIC) of the peptide, against the human pathogenic bacteria and fungus ranging between 128 to 512 µg / ml and 32 to 64 µg / ml respectively. The result of growth inhibition against plant fungal pathogen, maximum zone was obtained from *Fusarium oxysporum* (36 ± 0.5 mm) and minimum zone of inhibition from *Rhizoctonia solani* (24 ± 01 mm). The results clearly indicate the crude skin secretions of green frog (*Euphlyctis hexadactylus*) contain strong antifungal activity and conclusively demonstrated that these frog secretions are having immense potential as agricultural antimicrobial agents. However, the nature and sequences of these peptides are yet to be elucidated.

INTRODUCTION

The emergence of strains of microorganisms that are resistant to commonly used antibiotics, in recent years, has stimulated a search for new naturally occurring bactericidal and fungicidal agents that may have clinical utility. After discovery in 1987 of the magainins in skin secretions of the African clawed frog *Xenopus laevis*, attention has been increasingly focused upon amphibian skin peptides as potential therapeutic agents. Skin secretions generally contain multiple antimicrobial peptides with distinct spectra of activity, and it has been speculated that these peptides protect animal from invasion by a wide array of different microorganisms (Mor *et al.,*1994). The genus comprises an extremely diverse and widely distributed group of frogs with and estimated 250 species worldwide. Analysis of skin secretions and skin extracts of different species of *Ranid* frogs have led to the characterization of numerous peptides with antimicrobial activity that may be classified into different families on the basis of structural similarity (Halverson *et al.,*2000).

Skin secretion of amphibian contains a large number of biologically active compounds which are thought to play several roles, either in the regulation of the physiological functions of the skin or in defence mechanisms against predators or microorganisms (Mangoni *et al.,* 2000).

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Amphibian skin is a morphologically, bio-chemically and physiologically complex organ which fulfils a wide range of functions necessary for amphibian survival, including respiration, water regulation, antipredator, antimicrobial defence, excretion, temperature control, reproduction, etc (Clarke *et al.,*1997). Skin gland secretions are a primary source of amphibian chemicals (Daly *et al.,*1987).

The mechanism of bacterial cell lysis by peptides generally involves non specific interaction with the membrane phospholipids rather than binding to specific receptors on the cell membrane (Yeaman and Yount, 2003). On the positive side, because of their relatively non-specific mechanism of action, the development of resistance to the peptides occurs at rates that are orders of magnitude lower than those observed for conventional antibiotics (Zasloff, 2002). The major obstacles to their development as useful anti-infectives are their toxicities, particularly if they are to be administered systemically, and their short half-lives in the circulation. Thus, future therapeutic applications are more likely to involve topical rather than systemic administration.

The Green Pond Frog (*Euphlyctis hexadactylus*) is a common species of aquatic frog found in peninsular India and Sri Lanka. Size of males: 90 mm, females 130 mm; *E. hexadactylus* has a flattish snout with indistinct canthus rostalis, tympanum is distinct, equal or slightly less than a diameter of eye. This study describes the isolation and characterization of skin secretion from Indian green frog and assessing antimicrobial activity

against human pathogenic bacteria and fungi and plant fungal pathogens.

MATERIALS AND METHODS

Collection of frogs and taxonomic identification:
Adult green frogs of both sexes were collected by netting in several ponds in and around Kanchipuram, Tamilnadu, India and safely transferred to lab for further collection of secretion samples, after which they were released in original ponds. They ranged in weight from 70-140gm. The green frogs collected were taxonomically identified by Zoological Survey of India, Southern Regional Station, Chennai.

Extraction of skin secretion:
The animals [n=8, 5 male, 3 female] were anesthetized in crushed ice and subjected to mild electrical stimulation (10 V DC) at multiple sites on the dorsal region (Ali *et al.,*2003). The skin secretions were collected in a chilled beaker containing glacial acetic acid (5 ml) by washing the skin surface with water. The total volume of the secretions and washings was 250 ml. The resultant secretions were freeze dried in a freeze dryer (SubZero). Approximately 50 mg, dry weight, of skin secretion was obtained. The animal did not exhibit any deleterious effects and were returned to their pond (Kim *et al.,*2000).

Antimicrobial assay against human pathogens:
Disk diffusion method
The crude frog skin secretions were evaluated in vitro for their antimicrobial activity. The antimicrobial activities were carried out against six human pathogenic bacterial strains, *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa and Shigella dysenteriae* and two fungal strains, *Candida albicans and Aspergillus niger,* which were obtained from Department of Microbiology, University of Madras, Chennai. Sterilized antibiotic discs (6 mm) were used following the literature procedure (NCCLS, 1993; Bauer *et al.,*1966). Fresh stock solutions of the crude frog skin secretions were prepared in dimethyl sulfoxide (DMSO) according to the needed concentrations for experiments. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO as the same procedures as used in the experiments. All the bacteria were incubated at 30°C for 24 h in nutrient broth. The yeast and fungus were incubated in malt extract broth for 48 h. The discs injected with solutions (200 µg) were placed on the inoculated agar and incubated at 35°C (24 h) and at 25°C (72 h) for bacteria and yeast, respectively. On each plate an appropriate reference antibiotic disc was applied depending on the test microorganisms. In each case triplicate tests were performed and the average was taken as the final reading.

Broth dilution method
Screening for antibacterial and antifungal activities was carried out by preparing a broth micro dilution, following the procedure outlined in manual of clinical microbiology (Jones *et al.,*1984). All the bacteria were incubated and activated at 37°C for 24 h inoculation into nutrient broth, and the fungus were incubated in malt extract broth for 48 h. The compounds were dissolved in DMSO (2 mg/ml) and then diluted using caution adjusted Mueller Hinton Broth (Himedia). Two-fold serial concentrations of the extracts were employed to determine the MIC ranging from 512 µg/ml to 1 µg/ml. Cultures were grown at 37°C (12 h) and the final inoculation (inoculums) was approximately 10^6 cfu/ml. Test cultures were incubated at 37°C (24 h). The lowest concentrations of antimicrobial agents that result in complete inhibition of microorganisms were represented as (MIC) µg/ml. In each case, triplicate tests were performed and the results are expressed as means.

Against plant fungal pathogens:
Well diffusion method
The efficacy of frog skin secretions bio-active peptide was evaluated using an inhibition zone assay on potato dextrose agar plates (Simmaco *et al.,*1993). Totally five fungal plant pathogens namely, *Phomopsis sp., Botrytis sp., Fusarium oxysporum, Rhizoctonia solani* and *Colletotrichum sp.* were obtained from Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai. The frog skin secretions (200 µg) were added to the wells of potato dextrose agar plates seeded with above plant pathogens. The plates were incubated for 3 days at 25^0C. The zones of inhibition were measured after the incubation period.

Determination of peptide nature of the bioactive compounds
Even though, the fact that the antimicrobial activity of frog skin secretion is due to short peptides present in the secretion, is well established by several researchers, in the present study, attempts were made to confirm that the bioactive substances are indeed peptide in nature. These attempts include Proteinase K treatment followed by assessment of antibacterial activity, Muller- Hinton agar plates were prepared with three wells and seeded with *Staphylococcus aureus*. The frog skin section (200µg) was added in to the wells and 10µl of Proteinase K enzyme was added 2 mm apart from the wells. The plates were incubated overnight at 37^0C. The zone of inhibitions around the wells and at the site, where Proteinase K was applied, was compared (Simmaco *et al.,*1993).

RESULTS AND DISCUSSION

Adult green frogs of both sexes were collected by netting in several ponds in and around Kanchipuram and safely transferred to lab for further collection of secretion samples, after which they were released in original ponds. The green frog was identified as *Euphlyctis hexadactylus*. The results concerning in vitro antimicrobial activities of the skin secretion together with the inhibition zone (mm) and (MIC) values of compared antibiotic and antifungal agents are listed in Tables 1 and 2. The crude Indian green frog secretion tested exhibit strong or moderate antimicrobial activity. Anti bacterial activity, after 24 hours the zone of inhibition was measured, maximum zone of inhibition was obtained from *Salmonella typhi* (23.3 ± 0.5 mm) and *Bacillus cereus* (19 ± 01mm) and minimum zone of inhibition was obtained from

Table 1. In vitro screening for antimicrobial activity of crude frog skin secretions (200µg/disc)

Microorganisms	Zone of inhibition in mm		
	Crude frog skin secretions (200µg/disc)	Tetracycline[a]	Amphotericin-B[b]
Staphylococcus aureus	10.3 ± 0.5	21.6 ± 01	-
Bacillus cereus	19.1 ± 01	18.2 ± 01	-
Escherichia coli	13.6 ± 0.5	14.3 ± 0.5	-
Salmonella typhi	23.3 ± 0.5	15.7 ± 0.5	-
Psedumonas aeruginosa	12.5 ± 0.5	21.9 ± 01	-
Shigella dysenteriae	11.5 ± 01	19.8 ± 01	-
Candida albicans	26.4 ± 0.5	-	23.5 ± 0.05
Aspergillus niger	28.7 ± 0.5	-	26.0 ± 0.05

Legend: [a]The concentration of used standard drugs was 30 µg/ disc, [b]The concentration of used standard drugs was 50 µg/ disc, Average ± standard deviation (*n*=3).

Table 2. The minimum inhibitory concentration (MIC, µg/ml) of the crude frog skin secretions

Microorganisms	The minimum inhibitory concentration (MIC, µg/ml)		
	Crude frog skin secretions	Tetracycline	Amphotericin-B
Staphylococcus aureus	256	4	-
Bacillus cereus	128	8	-
Escherichia coli	512	32	-
Salmonella typhi	128	8	-
Psedumonas aeruginosa	256	8	-
Shigella dysenteriae	128	4	-
Candida albicans	64	-	64
Aspergillus niger	32	-	64

Table-3: Crude frog skin secretions against plant fungal pathogens

S.NO	Plant pathogens	Zone of inhibition(mm)
1.	*Phomopsis sp.*	30 ± 0.5
2.	*Botrytis sp.*	32 ± 0.5
2.	*Fusarium oxysporum*	36 ± 0.5
4.	*Colletotrichum sp.*	28 ± 0.5
5.	*Rhizoctonia solani*	24 ± 01

Legend: Average ± standard deviation (*n*=3)

Staphylococcus aureus (10.3 ± 0.5 mm). Antifungal activity, after 48 hours the zone of inhibitions was measured, maximum zone (28.7 ± 0.5 mm) of inhibition was obtained from *Aspergillus niger* as compared to standard drug, Amphotericin-B.

The antibacterial activity of the frog skin secretions of *Euphlyctis hexadactylus* was measured in terms of MIC. The crude frog skin secretions showed MIC of 256, 128, 512, 128, 256 and 128µg/ml respectively, against *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli, Salmonella typhi, Psedumonas aeruginosa* and *Shigella dysenteriae*. In antifungal activity, minimum inhibitory concentration of 64 and 32µg/ml were seen respectively, against human pathogenic fungus *Candida albicans and Aspergillus niger*. This finding ascertains the importance of MIC determination against disc diffusion method in case of diagnosis and management of the important pathogens. Previous studies have led to the isolation of antimicrobial peptides from the Asian frogs *R. ornativentris* (Kim *et al.,* 2001), *Rana rugosa* (Park *et al.,*1994; Suzuki *et al.,*1995), and *Rana nigromaculata* (Park *et al.,*2001) and from the European frogs *Rana esculenta* (Ali *et al.,*2003) and *R. temporaria* (Simmaco *et al.,*1998). The efficacy of crude green frog skin secretions was evaluated against plant fungal pathogen. The results are depicted in Table-3 and maximum zone of inhibition was obtained from *Fusarium oxysporum* (36 ± 0.5 mm) and minimum zone of inhibition was obtained from *Rhizoctonia solani* (24 ± 01 mm). These results clearly indicate the crude green frog (*Euphlyctis hexadactylus*) skin secretions contain strong antifungal activity. Attempts were made to confirm that the bioactive substances are indeed peptide in nature. These attempts include Proteinase K treatment followed by assessment of antibacterial activity. The zone of inhibition was not found around the Proteinase K wells, hence this result clearly indicates as frog secretions contain peptide. The present study is the first report on antimicrobial peptides from Indian green frog skin, *Euphlyctis hexadactylus* and conclusively demonstrated that these frog secretions are having immense potential as agricultural antimicrobial agents. However, the sequence of the peptide of this secretion is to be elucidated using HPLC, NMR, FTIR, and Peptide sequencing studies.

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