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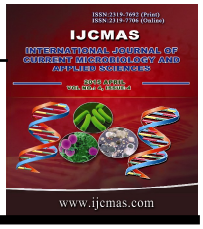

Original Research Article

Antimicrobial Effect of *Rana ridibunda* Skin Gland Peptides against Multidrug Resistant Pathogens

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K e y w o r d s

Amphibian, Antimicrobial peptides, *Rana ridibunda*, Skin glands, Multidrug resistance

A B S T R A C T

Amphibian skin glands have gained a lot of attention for secreting a wide variety of bioactive chemical compounds, with notable significance in pharmacology and therapeutics. The continuous emergence of bacterial and fungal strains with varying degrees of resistance to common antimicrobial agents justifies the urgent need to develop new antimicrobials that can meet this challenge. In the present study, the protein secretions from the skin glands of the frog *Rana ridibunda* were collected and tested against four different microorganisms, namely, *methicilin-resistant Staphylococcus aureus*, ESBL *Klebsiella pneumonia*, ESBL *Esherichia coli* and *Candida albicans*. Inhibition zone diameters of 17, 15, 15 and 31 mm with minimum inhibitory concentration of 1.18, 4.73, 4.73 and 0.29 mg/ml were observed for *methicilin-resistant Staphylococcus aureus*, ESBL *Klebsiella pneumonia*, ESBL *Esherichia coli* and *Candida albicans*, respectively. Electron microscopic study of microbial cells treated with the peptidyl secretions demonstrated direct bactericidal and fungicidal capabilities. This promising data suggested that the antimicrobial skin gland peptides of the Lebanese frog *Rana ridibunda* are active against all the tested microbial strains and should provide basis for the development of new therapeutic agents against multidrug resistant pathogens.

Introduction

Given the continuous emergence of virulent multidrug resistant (MDR) strains of microorganisms, the scientific world has turned attention into identifying new sources for antimicrobial compounds. These include bacteria, fungi, plants and animals (Zasloff, 2002; Sang and Blecha, 2008; Wang *et al.*, 2009). It is well established in previous studies that amphibian skin represents an excellent source for antimicrobial peptides used by the animals as an innate pharmacological defensive mechanism to ensure their survival in a habitat full of a variety of pathogens. Many of these peptides belong to novel families implicating that more amphibian antimicrobial peptides are

waiting to be discovered (Zheng *et al.*, 2010). These peptides are cationic, hydrophobic, 12 to 46 amino acid residues in size, and adopt an amphipathic α-helical conformation upon binding to cell membranes. Although its precise mechanism of action is still unclear, it has been proposed that the peptides bind to charged residues on target cells and cause disruption of the cell membrane, thus mimicking the action of detergents by transforming it into peptide-coated vesicles (Matsuzaki, 1999; Shai, 1999; Yang *et al.*, 2000; Zasloff, 2002). Amphibian families including Pipidae, Hylidae, Hyperoliidae, Pseudidae and Ranidae represent a rich source for antimicrobial peptides (Lu *et al.*, 2008; Zheng *et al.*, 2010; Pei *et al.*, 2013).

Rana ridibunda, also known as marsh frog, belongs to the family Ranidae and is the most abundant frog in Lebanon where it can be found in many different watery habitats. It is a middle sized frog, reaching a body length of 70 to 95 mm, strongly aquatic and stays in close proximity to water throughout its life (A Rocha Lebanon).

In this study, we collected, concentrated and tested natural skin peptides of the Lebanese frog *Rana ridibunda* against one Gram positive bacteria (MRSA), two Gram negative bacteria (ESBL *E. coli* and ESBL *Klebsiella pneumoniae*) and one fungus (*Candida albicans*).

Materials and Methods

Chemicals and reagents

All reagents were obtained from Fluka, Germany. The culture media was purchased from Oxoid Ltd, United Kingdom. Antibiotics were obtained from acknowledged chemical suppliers (Medilic, Beirut).

Animals

Adult frogs of *Rana ridibunda* of both sexes (n=35, snout-vent length range 60–80 mm) were collected from fresh water habitat in Jezzine, South Lebanon during different seasons of the year.

Skin glands secretions collection and processing

Skin glands secretions were collected according to the method described by Li *et al.* (2007) with some modifications. 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 0.1 M NaCl solution containing 0.01 M EDTA. Hundred ml of collected washing solution were centrifuged at 4°C for 5 minutes at 13000 rpm and the supernatant was collected and lyophilized. The lyophilized powder was resuspended in 10 ml of 0.1 M NaCl solution containing 0.01 M EDTA. The solution was then sterilized by passing it through a 0.2 µm millipore filter. The filtrate was kept refrigerated at 4°C for further use. The protein content was estimated using total protein colorimetric method.

Microorganisms

Three different MDR bacterial strains were used throughout the present work: *methicillin-resistant Staphylococcus aureus* (MRSA) (Gram positive), ESBL *Klebsiella pneumoniae* (Gram negative) and ESBL *Escherichia coli* (Gram negative) (kindly provided by Ain W Zain hospital and American University of Beirut Hospital). The strains were identified phenotypically as described in Bergey's manual of determinative bacteriology. The bacterial isolates were maintained on nutrient agar

slants and stored at 4°C with regular transfer at monthly intervals. 25% glycerol was added to the cultures for long preservation. *Candida albicans* was kindly provided by Elias Hrawi Governmental Hospital and was further identified using the Simplified Identification Method (SIM) key (Deak, 1986). It was maintained on Sabouraud-Dextrose agar slants and stored at 4°C with regular transfer at monthly intervals.

Inoculum preparation and standardization

From a freshly prepared culture of each isolate (16 to 24 hrs old grown on nutrient agar), 4 to 5 colonies were emulsified in 5 ml of 0.9 % saline solution to achieve a turbid suspension matching 0.5 McFarland standardized tube corresponding to 1.5 x 10^8 CFU/ml (Mahon *et al.,* 1998).

Evaluation of the antimicrobial effect of *Rana ridibunda* skin gland peptides

Antibacterial and antifungal activities of commonly used antimicrobial agents were assessed using a disc-diffusion method (Murray *et al.,* 2005). Petri dish plates were prepared with 20 ml of sterile Müller Hinton agar. The test culture were swabbed on the top of the solidified media and allowed to dry. The loaded antibiotic and antifungal discs namely: Ceftriaxone (CRO - 30 µg), Vancomycin (VA - 30 µg), Cefoxitin (FOX - 30 µg), Oxacillin (OX - 1 µg), Azotreonam (ATM - 30 µg), Cefotaxime (CTX - 30 µg), Ofloxacin (OFX - 5 µg), Gentamicin (CN - 10 µg), Tetracyclin (TE - 30 µg) and Nystatin (NS - 100 µg), were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated for 24 hrs at 37°C. Zones of inhibition were recorded in millimeters and the experiments were repeated twice.

The plate-well diffusion assay described by Kudi *et al.* (1999) was used to determine the inhibitory effect of frog skin secretions on the tested microorganisms. A sterile cork-borer (8 mm diameter) was used to make wells in the set agar. 100 µl of the microbial suspension (McFarland 0.5 standardized suspension) was swabbed over the surface of a Müller Hinton agar plate. 100 µl of the prepared frog skin secretion extract was added to each well and the plates were incubated overnight at 37°C. The zones of inhibition were then measured.

Determination of minimal inhibitory concentration (MIC) of frog skin gland secretions against the selected microorganisms

The MIC was measured by the standard microdilution method in a sterile 96-well microtiter plates (Ellof, 1998). The wells were filled with 100 µl of sterile water, and 100 µl of frog skin secretions were added to the wells by serial two fold dilution. Each well was inoculated with 100 µl of 0.5 McFarland standard bacterial suspensions diluted in Müller Hinton broth so that each well got 1.5 x 10^8 CFU/ml. The 96-well microtiter plates were covered and incubated at 37°C for 24 hrs. The MIC was defined as the lowest concentration of the frog skin secretions that exhibited total inhibition of visible microbial growth.

Determination of minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of frog skin gland secretions against the selected microorganisms

MBC and MFC were determined by inoculating the dilutions that showed no visible growth from the MIC into agar plates and SDA plates, respectively, and incubating the plates at 37°C for 24 hrs. The

lowest concentration that was lethal to the microorganism was considered as MBC in case of tested bacteria and MFC in case of *Candida albicans*.

Time-kill curve assay of frog skin gland secretions against the selected microorganisms

A 16 hrs culture was harvested, the suspension was adjusted using the McFarland standard and was further diluted in 0.9 % saline to achieve approximately 1.5 x 10^8 CFU/ml. Frog skin secretions for the selected bacteria were added to aliquots of 1 ml Müller Hinton broth followed by the addition of 1 ml of the inoculums to attain bactericidal concentration. Further samples were taken from each tube to monitor growth by measuring the absorbance at 600 nm at different time intervals (Yin *et al.*, 2002). Time-kill studies were carried out against *Candida albicans* based on the data obtained from MIC and MFC. Cells were incubated in 1 ml of Sabouraud-Dextrose broth at 35°C for 24 hrs. Further samples were taken at different time intervals for viable counts which were carried out by serial dilution of samples by 10 fold in sterile distilled water and plating on SDA. The results were estimated according to log values (Hammer *et al.*, 2002).

Transmission electron microscopy

On the basis of MIC, MBC, MFC values and Time-Kill curve data, methicillin-resistant *Staphylococcus aureus*, ESBL *Klebsiella pneumoniae*, ESBL *Escherichia coli* and *Candida albicans* were treated with different concentrations of the frog skin secretions (1.18, 4.73, 4.73 and 0.29 mg/ml respectively). Freshly taken samples were fixed using a universal electron microscope fixative. Series of dehydration steps were followed using ethyl alcohol and propylene oxide. The samples were then embedded in labeled beam capsules and polymerized. Thin sections of cells exposed to extracts were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of uranyl acetate for 30 min and lead acetate for 2 min (McDowell and Trump, 1976). The procedure was applied to antimicrobial peptide-exposed cells. Electron micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Results and Discussion

The wide spread concern about conventional antibiotics facing resistance by microorganisms has stimulated researchers into looking for alternative therapeutics that cause less drug resistance (Ma *et al.*, 2010). The antimicrobial peptides from ranid frogs have been recognized in recent years as promising candidates for the development of therapeutically useful anti-infective agents (Xu *et al.*, 2006). In the present study, crude antimicrobial peptides were collected from the skin of the Lebanese frog *Rana ridibunda*. It showed antimicrobial activity against Gram-positive, Gram-negative bacteria and yeast.

Evaluation of antimicrobial effect of *Rana ridibunda* skin gland secretions

Screening experiments were evaluated to compare the antimicrobial effect of frog skin secretions and some common antimicrobial agents against the selected MDR pathogens. Frog skin secretions showed antimicrobial activity against the growth of the MDR microorganisms under test with some variations. Data presented in figure 1 and table 1 showed average inhibition zone diameters of 17, 15, 15 and 31 mm for

MRSA, ESBL *Klebsiella pneumoniae*, ESBL *E. coli* and *Candida albicans*, respectively. Data revealed more powerful antifungal effect of the peptide extract under test than antibacterial effect.

Determination of MIC of frog skin gland secretions against the tested microorganisms

The potency of the crude antimicrobial peptides was assessed by measuring MIC using broth dilution method. Antimicrobial peptides were tested against Gram positive MDR strain, MRSA, two Gram negative strains: ESBL *Klebsiella pneumoniae* and ESBL *Escherichia coli* and the yeast *Candida albicans*. MRSA growth was strongly inhibited with MIC value of 1.18 mg/ml. On the other hand, the tested antimicrobial peptides showed equal efficacy towards ESBL *Klebsiella pneumoniae* and ESBL *Escherichia coli* with MIC value of 4.73 mg/ml. However, the potency of the antimicrobial peptides was the highest against *Candida albicans* with MIC value of 0.29 mg/ml (table 2). Even though *Rana ridibunda* produced large quantities of peptides, their efficacy against the tested microbes was less than that of other peptides produced by many anurans as reported in several studies. For instance, antimicrobial peptides from most frogs generally have MICs in the range of 12-128 µg/ml against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans* (Lai *et al.*, 2002; Lu *et al.*, 2008; Zheng *et al.*, 2010; Park *et al.*, 2011; Liu *et al.*, 2012; Pei *et al.*, 2013). This can be explained by the fact that the antimicrobial peptides used in this study were crude, suggesting that many non-active peptides were also collected with the tested sample. Non-active peptides might be remnants of degraded proteins, antimicrobial peptide precursors, antimicrobial peptides that are active against microbes other than MRSA, ESBL *Klebsiella pneumoniae*, ESBL *Escherichia coli* and *Candida albicans*, or structural components of skin mucus not related to antimicrobial defense mechanism (Sheafor *et al.*, 2008).

Determination of MBC of frog skin gland secretions against the selected bacteria

On the basis of the previous data, MBC values were investigated for the frog skin secretion against the selected bacteria. Data presented in figures 2–4 and table 2 showed that the MBC for MRSA, ESBL *Klebsiella pneumoniae* and ESBL *E. coli* was close to the MIC values (1.18, 4.73 and 4.73 mg/ml respectively). Thus, the peptides had a bactericidal effect against the tested microorganisms with MIC index equal to 1.

Determination of MFC of frog skin gland secretions against *Candida albicans*

The minimal fungicidal concentration (MFC), which is the capacity of the frog skin secretions to kill *Candida albicans*, was investigated. It was shown that the peptidyl secretions had a fungicidal effect against *Candida albicans* with MFC value of 0.29 mg/ml (table 2). This is further demonstrated in figure 5 where the area with no growth of *Candida albicans* represents the lowest concentration of the secretions that was able to kill the cells of *Candida albicans*.

Time-kill curve assay of frog skin gland secretions against the microorganisms under test

Time-kill curves were evaluated to assess the bactericidal and fungicidal activity of frog skin secretions. Bacterial and fungal growth was assessed by measuring absorbance at 600 nm which is then plotted against the exposure time to the secretions. During time-kill curve experiments, the

maximum change in optical density was compared to a negative control (without the secretions). The time-kill test was done at concentration equivalent to 1xMBC for MRSA, ESBL *Klebsiella pneumoniae* and ESBL *E. coli* and 1xMFC for *Candida albicans*. Untreated MRSA, ESBL *Klebsiella pneumoniae*, ESBL *E. coli* or *Candida albicans* were used as negative controls and showed an increase in absorbance with time. The antimicrobial peptides used in the present study were particularly effective with respect to the time needed to exert lethal effect on the microbial growth. At MBC and MFC, the peptides were successful in killing viable cells within 10 minutes, 5 minutes, 10 minutes and 12 hours against MRSA, ESBL *Klebsiella pneumoniae*, ESBL *Escherichia coli* and *Candida albicans*, respectively (Figure 6).

Table.1 Inhibition zone diameter (mm) of frog skin gland secretions against the tested microorganisms versus common antimicrobials

	Inhibition zone diameter (mm)			
Tested microorganism / **Antimicrobial Agent**	**MRSA**	**ESBL *Klebsiella pneumoniae***	**ESBL *E. coli***	***Candida albicans***
Frog skin secretions (3.95 mg)	17	15	15	31
Ceftriaxone (CRO – 30 µg)	11	R	9	-
Vancomycin (VA – 30 µg)	17	-	-	-
Cefoxitin (FOX – 30 µg)	15	-	-	-
Oxacillin (OX - 1 µg)	10	-	-	-
Azotreonam (ATM - 30 µg)	-	R	-	-
Cefotaxime (CTX - 30 µg)	-	R	-	-
Ofloxacin (OFX - 5 µg)	-	-	9	-
Gentamicin (CN - 10 µg)	-	-	8	-
Tetracyclin (TE - 30 µg)	-	-	7	-
Nystatin (NS – 100 µg)	-	-	-	19

Table 2: MIC and MBC/MFC of frog skin gland secretions against the tested microorganisms

Microorganism	**MIC (mg/ml)**	**MBC/MFC (mg/ml)**
MRSA	1.18	1.18
ESBL *Klebsiella pneumoniae*	4.73	4.73
ESBL *E. coli*	4.73	4.73
Candida albicans	0.29	0.29

Figure.1 Sensitivity test of the tested MDR pathogens against frog skin gland secretions. MRSA (a), ESBL *Klebsiella pneumoniae* (b), ESBL *E. coli* (c) and *Candida albicans* (d)

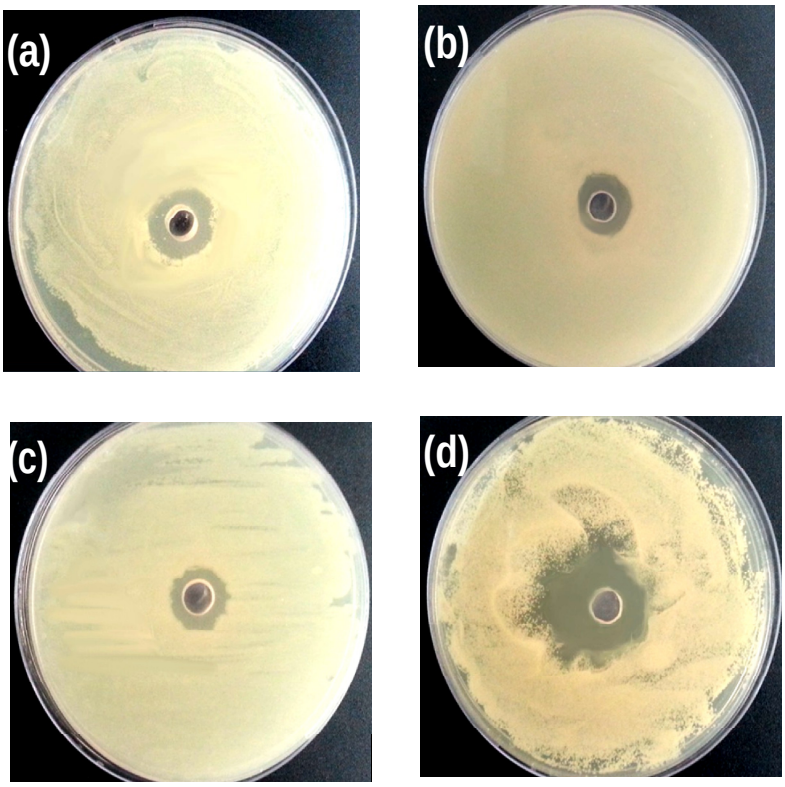


Figure.2 Determining the minimal bactericidal concentration of frog skin gland secretions against MRSA. A1→A10: serial dilutions of the peptide; A11: negative control (peptide without MRSA); A12: positive control (MRSA without peptide). Growth of MRSA was observed from 1:64 dilutions onwards (A7-A10), thus the minimum bactericidal concentration of the peptide was determined to be 1.18 mg/ml

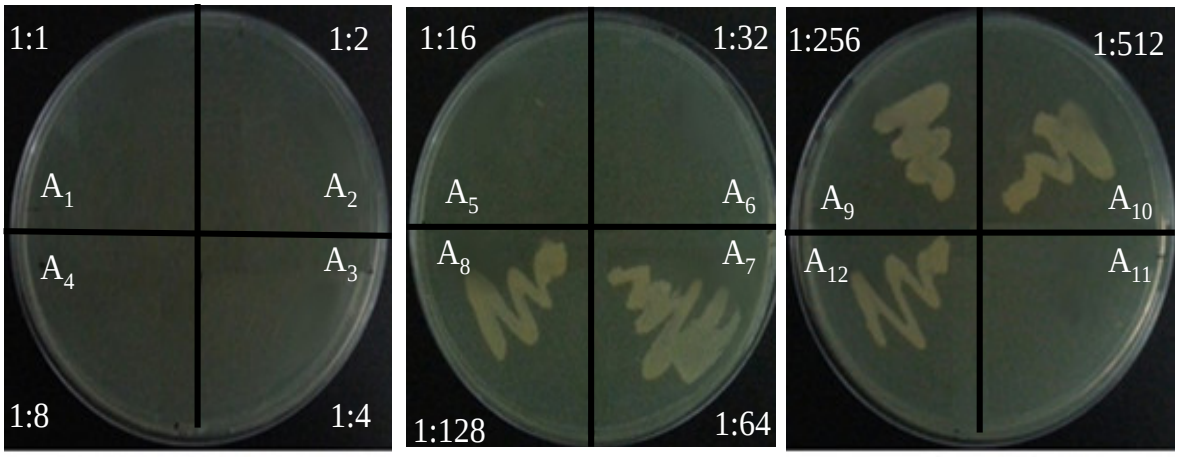


Figure.3 Determining the minimal bactericidal concentration of frog skin gland secretions against ESBL *Klebsiella pneumoniae*. B1→B10: serial dilutions of the peptide; B11: negative control (peptide without ESBL *Klebsiella pneumoniae*); B12: positive control (ESBL *Klebsiella pneumoniae* without peptide). Growth of ESBL *Klebsiella pneumoniae* was observed from 1:16 dilutions onwards (B5-B10), thus the minimum bactericidal concentration of the peptide was determined to be 4.73 mg/ml

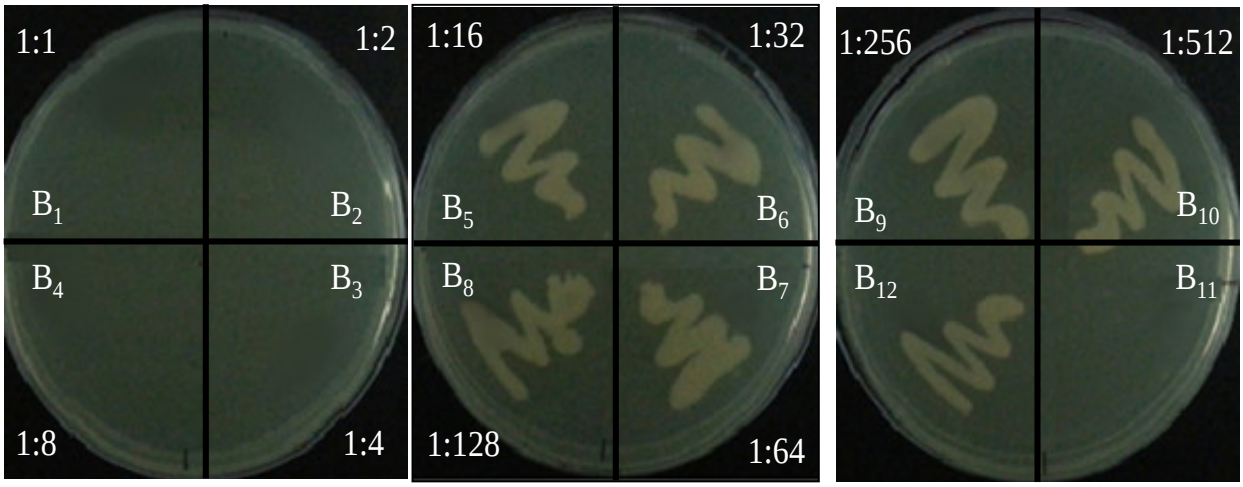


Figure.4 Determining the minimal bactericidal concentration of frog skin gland secretions against ESBL *E. coli*. C1→C10: serial dilutions of the peptide; C11: negative control (peptide without ESBL *E. coli*); C12: positive control (ESBL *E. coli* without peptide). Growth of ESBL *E. coli* was observed from 1:16 dilutions onwards (C5-C10), thus the minimum bactericidal concentration of the peptide was determined to be 4.73 mg/ml

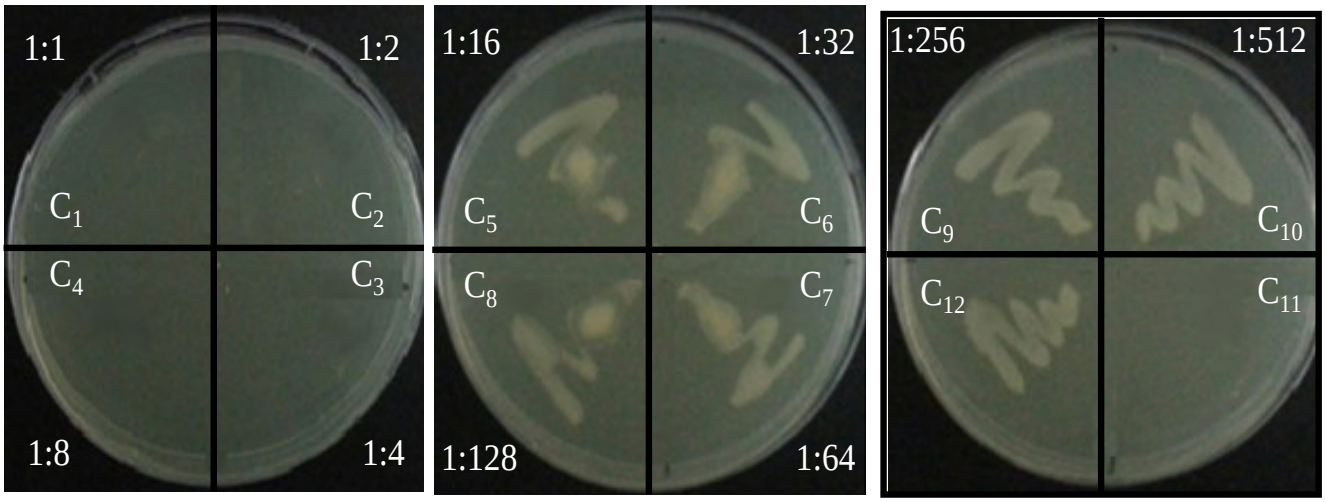


Figure.5 Determining the minimal fungicidal concentration of frog skin gland secretions against *Candida albicans*. D1→D10: serial dilutions of the peptide; D11: negative control (peptide without *Candida albicans*); D12: positive control (*Candida albicans* without peptide). Growth of *Candida albicans* was observed from 1:256 dilutions onwards (D9 and D10), thus the minimum bactericidal concentration of the peptide was determined to be 0.29 mg/ml

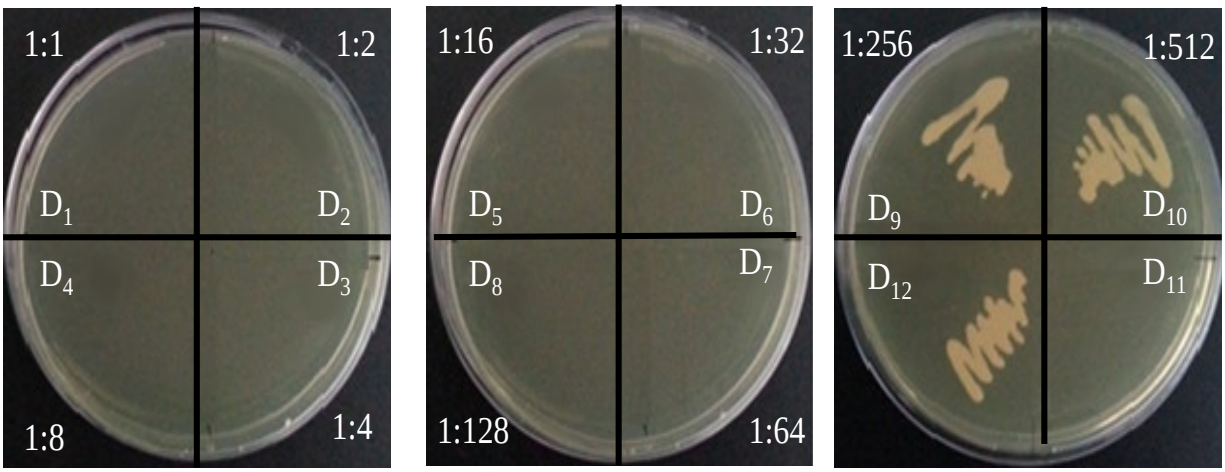


Figure.6 Time-kill curve of (a) MRSA, (b) ESBL *Klebsiella pneumoniae*, (c) ESBL *E. coli* and (d) *Candida albicans* treated with frog skin gland secretions and compared to a negative control

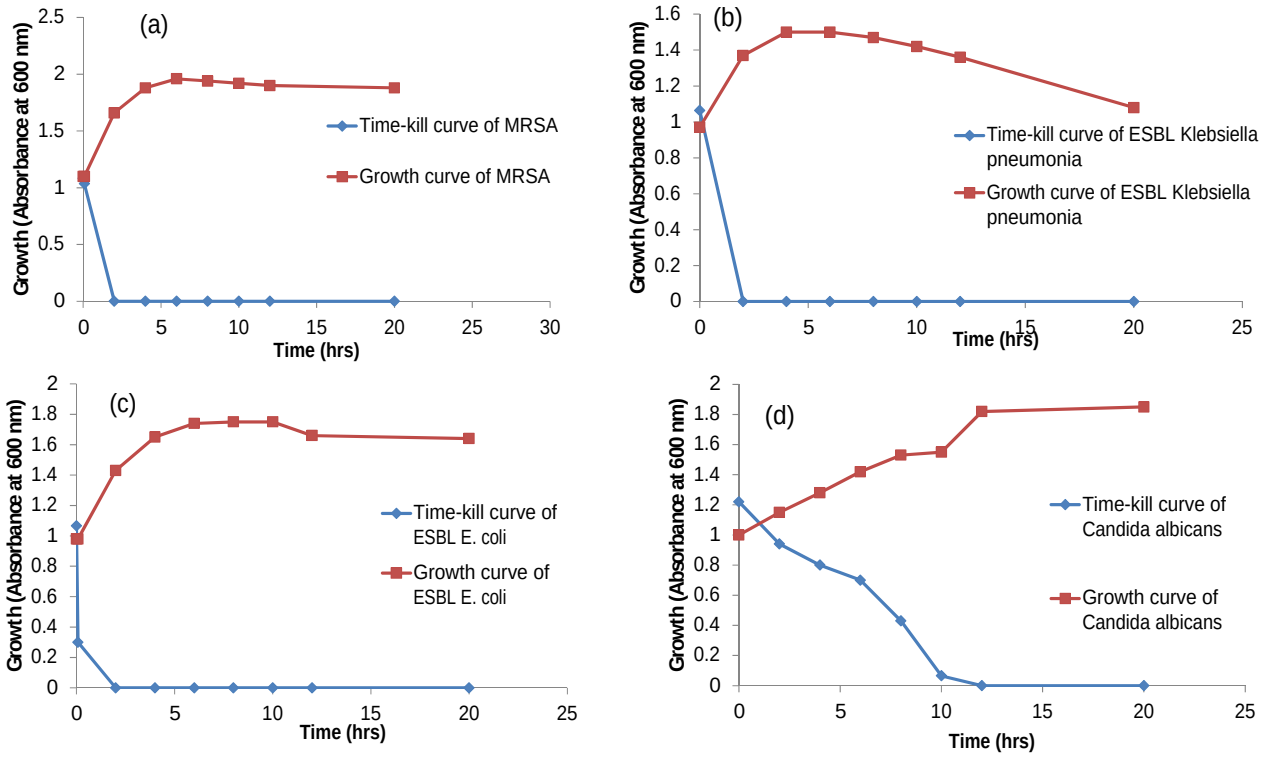
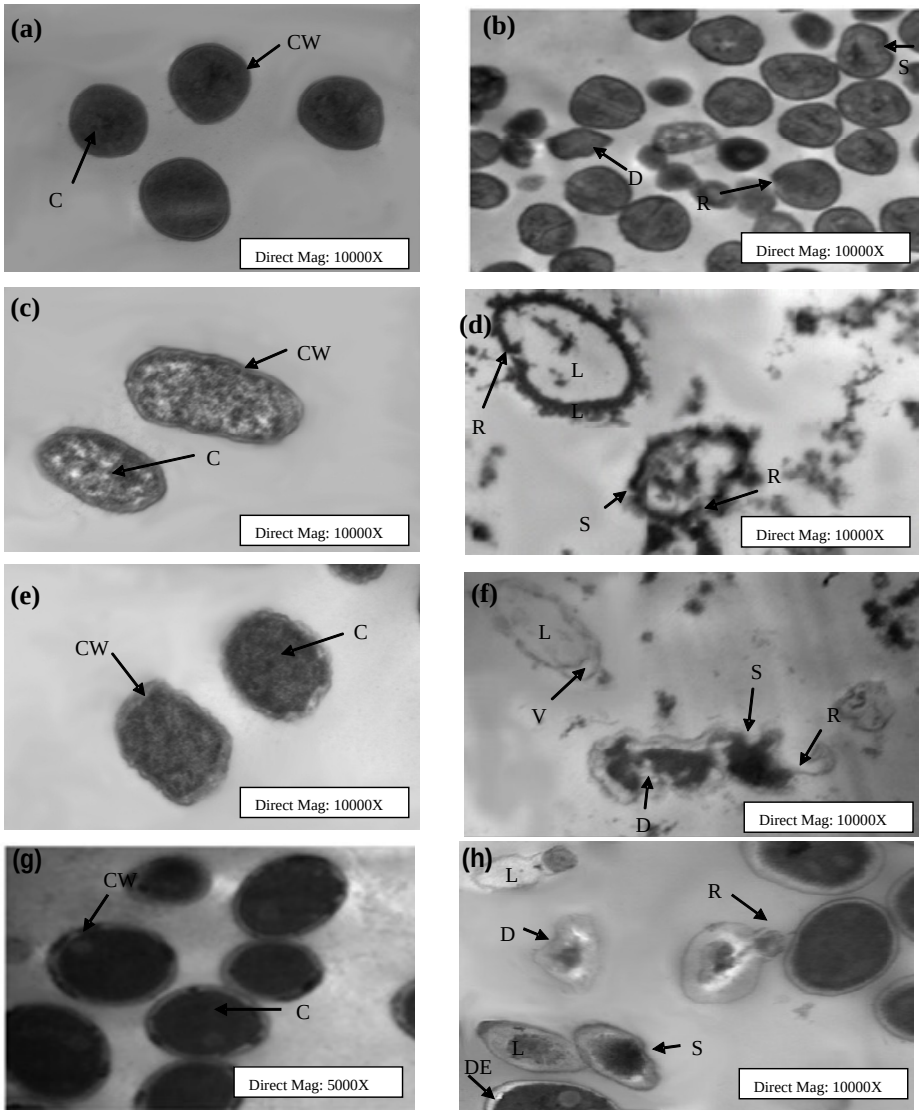


Figure.7 TEM of MRSA (b), ESBL *Klebsiella pneumoniae* (d), ESBL *E. coli* (f) and *Candida albicans* (h) treated with its corresponding MIC; 1.18, 4.73, 4.73 and 0.29 mg/ml peptide sample, respectively, and compared to a control (a, c, e and g, respectively). C: cytoplasm, CW: cell wall, D: damage, DE: detachment of plasma membrane from the cell wall, L: leakage of cellular content, R: rupture, S: shrinkage, V: vacuolization



Transmission electron micrograph assay

The effect of the peptidyl secretions on the MDR microorganisms under study was further investigated by observing its morphology under a Transmission Electron Microscope (TEM). Images of control (untreated) microorganisms and treated one are depicted in figure 7. Figure 7b demonstrated that upon the treatment of MRSA with 1.18 mg/ml of frog skin peptide (MIC), the cells exhibited morphological changes such as deformation and shrinkage of cell wall as well as condensation of DNA, all of which could lead to cell death.

Ultrastructural analysis of ESBL *Klebsiella pneumoniae* (Figure 7d) treated with 4.73

mg/ml of frog skin peptides (MIC) revealed morphological alteration exhibited by cell wall rupture and shrinkage of cytoplasm leading to leakage of cellular content and eventual cell death. Moreover, loss of typical structural organization and structural integrity was observed. Microscopic observation revealed significant changes in the ultrastructure of ESBL *E. coli* treated with 4.73 mg/ml (MIC) of frog skin peptides (Figure 7f). The cells exhibited structural disorganization due to irreversible cell wall damage in addition to leakage of cytoplasm, vacuolization, severe ruptures as well as presence of highly condensed areas. Peptide-induced morphological changes of *Candida albicans* (MIC = 0.29 mg/ml) were observed in figure 7h.

The majority of cells displayed leakage of cytoplasmic content, in addition to the presence of cell debris and membrane damage. Detachment of the plasma membrane from the cell wall and loss of integrity of nuclear material were also noted. Overall, the frog skin peptide had a direct bactericidal and fungicidal capability as illustrated in figure 7. The difference in the cell surface of *Candida albicans* compared to bacteria does not seem to affect the mechanism of action of the frog skin peptides. The main targets included the cell membrane and plasma membrane leading to leakage of cytoplasm and cellular damage and eventually death.

There is an increasing resistance of microbial pathogens to antibiotics as a result of misuse and subsequent natural selection of resistant strains. This situation results in a higher morbidity and mortality rates coupled with increased health-care costs. Thus, the need to develop new classes of antibiotics seems indispensable. The present study proved that the Lebanese amphibian skin glands are rich source of biologically active compounds that exert antimicrobial effect showing direct bactericidal and fungicidal capability against Gram positive, Gram negative bacteria and *Candida albicans*. In addition to the range of pharmacologically active peptides present, amphibian skin gland secretions contain a broad spectrum of antimicrobial peptides. Antimicrobial peptides are a relatively new class of potential antibiotics which are fast acting, possess broad-spectrum activity and are able to escape many of the currently known mechanisms of drug resistance. They have been shown to be active against MDR Gram-negative and Gram-positive bacteria and fungi. It is believed that it has properties that play important roles in the interaction with the membrane of target cells and/or in the mechanism that eventually causes cell lysis. However, toxicity to healthy host cells remains a major concern and has affected the clinical development of therapeutics based on antimicrobial peptides. Peptides from only a few species have been studied and screening of other species is expected to yield new antimicrobial agents. The proteomic research work on frog's skin gland peptides in Lebanon is quite limited and further work in this area is recommended.

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