ABSTRACT

Amphibians cutaneous glands spread over the skin which contain different bioactive substances. So, the aim of this study is to study the effects of frog skin secretions on parasites and pathogenic bacterial isolates. Five adult frogs (Rana ridibunda) from AL-Jadria streams in Baghdad used in this study. The frogs were stimulated by single intraperitoneal injection of norepinephrine-HCl (40ng/gm body weight). Frogs skin glands secreted bioactive substances then washed .The washing solution was centrifuged, lyophilized and re-suspended and sterilized. The result of the study revealed that the concentration (500mg/ml) inhibited bacterial growth so as Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Fungi so as Candida albicans, while the concentration (250mg/ml) inhibited the growth of Salmonella typhi. All concentrations of crude extract were cytotoxic for both of Leishmania tropica and Leishmania donovani cell culture by the evidence of significant regression and correlation (P <0.071). The treatment with crud extract (500mg/ml) was active to stimulate tissue cells proliferation and eradicate pathogens inside the wound, thereby may lead to enhance healing of cutaneous wounds.

Keywords: antimicrobial, frog secretion, wounds healing

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INTRODUCTION
Skin secretions from many species of anuran (Frogs and toads) contain a wide range of compounds, that have existed interest because of their potential for drug development with promised future for this aim. Among these substances are host-defense peptides with broad spectrum antibacterial and antifungal activities and the ability to permeabilize mammalian cells (2). Rana ridibunda frog was one of the most diverse and widely distributed groups of anuran amphibious in Iraq. Rana having more than 250 reported species around the world. The anuran skin presents morph functional and behavioral protective adaptations against a number of adverse factors in the terrestrial environment, in which the cutaneous glands play an essential role in the defense against infection by microorganism on the body surface, Asoodeh, et al., (2). The present study focused on the effects of frog skin secretions on parasites and pathogenic bacterial isolates.

MATERIALS AND METHODS
Sample collection
Adult frogs (Rana ridibunda) of both sexes were collected from fresh water habitat in streams of Al-Jadria in Baghdad. Skin gland secretions were collected according to the method described by El Haj Moussa, et al., (9) with some modifications. Five frogs with different weights were subjected to the experiment (19, 30, 33, 49 and 50 gm). Each frog stimulated by intra-peritoneal injection of nor-epinephrine-HCL,(40ng/g) body weight. Then the frog left in 150 ml of 0.1 M NaCl containing 0.01M EDTA for 15 minute as a washing solution, El Haj Moussa et al., (10). The collected washing solution was centrifuged under cooling 4 °C for 5 minutes at 13000 rpm and the supernatant was collected and lyophilized. Two grams of the lyophilized powder was re-suspended in 4ml of phosphate buffer saline and sterilized by 0.2 millipore filters. The filtrate kept refrigerated at 4°C for future use.

Antimicrobial assay
The assay in present study involved the effects of crude extract on bacterial cultures of Pseudomonas aeruginosa, Staphylococcus aureus, E. coli and Bacillus subtilis, Klebsilla pneumonia, Salmonella typhi and Candida albicans El Haj Moussa, et al., (10). The assay applied with concentration 500; 250; 125 and 98.3 mg/ml. A sterile cork-borer (8mm diameter) was used for wells formation in set of nutrient agar plates and sabouraud dextrose agar for candida growth. Then plats were streaked separately by each pathogenic bacterium. Thereby we put 100 µl of each tested concentration in separated well. All plates were incubated overnights at 37°C. The zones of inhibition in millimeters were recorded and the experiments were repeated twice, AL-Ghaferi, et al., (1).

Anti-leishmanial assay
The anti-Leishmanial efficacy of the concentration (500,250, 125 and 50 mg/ml of crude extract against promastigote of L. tropica and L. donovani was evaluated. The colorimetric cell viability MTT assay was used as described by Freshney, (11) and Mahmoudvand.et al., (15). Leishmania promastigotes were cultured in 4 wells replicates of tissue culture plate that contain 100 µl well (10^6 parasite/ ml). Then 100 µl of various concentration of extract test solution added to each well separately and incubated at 26°C for 24h. After incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated at 26 °C for 4 h. finally 50 µl of DMSO was added to each well and incubated for another 10 min. promastigotes were cultured in complete medium without treatment as a control. The absorbance was measured for each well at 620 nm using ELISA reader. The live promastigotes, percentage viability and inhibition ratio were calculated as follows: GI%= {((O.D of control wells-O.D of test well) / O.D of control wells)*100

Wounds assay
Wounds healing were applied on 4 weeks age 12 albino mice by weights ranged from 25-30 gm/mouse with two categories as described by Mangoni (16). The first 3 mice acted as control group and the second 9 mice were for treatment with crude extract by using 500mg/ml concentration. The lab animals' were put in 22C with ordinary nutrition and suitable period of darkness and water in separated cages. Mice were observed for 0, 2, 4 and 6 days of treatment collectively. Each
of mice was anesthetized by intraperitoneal injection of 100 μl/mouse of mixture (1 ml of xylozin 2%-0.1 ml of ketamin 10%) and sterilized the site of injury with ethanol (70%). Mice under study were hair removed 4X4 cm from back by the electric clipper. Then making 3 cm of one longitudinal injury with depth to 2mm on dorsal site by disposable sterile surgical blade to each mouse. We put few drops of the above concentration of crude extract twice a day on the injury of the tested mice. Mice of control group were left without treatment. Mice under study were observed and we took images for both of injuries and their healing progression Mashreghi, et al., (17).

Injury area (mm²) = L*d for each mouse, and by using the formula of Wang, (20):

Healing ratio % = {(control wound area - treated wound area) / control wound area} * 100

Statistical analysis: This was performed according MINI TAB Release 11.12 32 Bit program.

RESULTS AND DISCUSSION

The results revealed an obvious effect of crude extract on all pathogens mentioned in this study, as shown in Table 1. Than the large inhibition n zone was for Candida albicans 40mm and Salmonella typhi with 30 mm of inhibition due to highest concentration (250 mg/ml). The concentration 93.8 mg/ml exhibited 18 mm of inhibition zone for Pseudomonas aeruginosa, thereby the increasing of the concentration to 500mg/ml revealed in inhibition zones ranged from (45,40 and 25mm) for the subjected pathogens under study in compare to control (Table1), (Figure 2.A-E). The parasites under study revealed within 500mg/ml of the extract to the highest killing ratio 56.9% was for Leishmania tropica versa to the lowest killing ratio was for Leshmania donovani as shows in Table 2.

The statistical analysis revealed significant regression and by the equation C1= 58.4-0.357 C2 F= 12.66 P< 0.071 C1 as constant factor represent killing ratio for Leishmania tropica versus of variable factor for Leishmania donovani values and shown in Figure-1(A&B).

Fig.1. (A) Regression plot (Y=58.3748-0.357382X, R-Sq=0.864) for cytotoxicity effect onto Leishmania tropica C1 and Leishmania donovani C2 parasites under study. (B) Shows the correlation of killing ratio and tested concentrations of crude extract onto parasites under study.
Table 1. Effects of crude extract on pathogenic Bacteria.

<table>
<thead>
<tr>
<th>Conce.</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>E. coli</em></th>
<th>Inhibition Zon</th>
<th><em>S. typhi</em></th>
<th><em>Kleib. pn eumonae</em></th>
<th><em>C. Albicans</em></th>
<th><em>Bacillus Subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mg/ml</td>
<td>25mm</td>
<td>40mm</td>
<td>45mm</td>
<td>N.D</td>
<td>N.D</td>
<td>45mm</td>
<td></td>
</tr>
<tr>
<td>250 mg/ml</td>
<td>N.D</td>
<td>18mm</td>
<td>N.D</td>
<td>30mm</td>
<td>12mm</td>
<td>40mm</td>
<td>N.D</td>
</tr>
<tr>
<td>125 mg/ml</td>
<td>N.D</td>
<td>N.D</td>
<td>20mm</td>
<td>N.D</td>
<td>12mm</td>
<td>40mm</td>
<td>N.D</td>
</tr>
<tr>
<td>98.3 mg/ml</td>
<td>18mm</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>Control</td>
<td>0mm</td>
<td>0mm</td>
<td>0mm</td>
<td>0mm</td>
<td>0mm</td>
<td>0mm</td>
<td>0mm</td>
</tr>
</tbody>
</table>

N.D: Non determined

Table 2. Cytotoxic effect of crude extract on *Leishmania tropica* and *Leishmania donovani*.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control O.D 620 nm</th>
<th>Treatment O.D 620 nm</th>
<th>Killing ratio</th>
<th>Treated parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mg/ml</td>
<td>0.353</td>
<td>0.201</td>
<td>56.9%</td>
<td><em>Leishmania tropica</em></td>
</tr>
<tr>
<td>250 mg/ml</td>
<td>0.353</td>
<td>0.163</td>
<td>46.3%</td>
<td></td>
</tr>
<tr>
<td>125 mg/ml</td>
<td>0.353</td>
<td>0.151</td>
<td>42.7%</td>
<td></td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>0.353</td>
<td>0.163</td>
<td>46.1%</td>
<td></td>
</tr>
<tr>
<td>500 mg/ml</td>
<td>0.163</td>
<td>0.154</td>
<td>55.2%</td>
<td><em>Leishmania donovani</em></td>
</tr>
<tr>
<td>250 mg/ml</td>
<td>0.163</td>
<td>0.108</td>
<td>33.7%</td>
<td></td>
</tr>
<tr>
<td>125 mg/ml</td>
<td>0.163</td>
<td>0.105</td>
<td>35.5%</td>
<td></td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>0.163</td>
<td>0.0096</td>
<td>41.4%</td>
<td></td>
</tr>
</tbody>
</table>

Fig.2.A-*Staph. aureus* inhibition zone on nutrient agar culture that affected by testing concentration (500mg/ml) of crude extract.
Fig. 2. B-*E. coli* inhibition zone on nutrient agar culture (1, 2) that affected by testing concentration (500mg/ml) of crude extract.

Fig. 2. C-*Candida albicans* inhibition zone on sabouraud dextrose agar culture (2) that affected by testing concentration (125mg/ml) of crude extract.

Fig. 2. D-*Bacillus subtilis* inhibition zone on nutrient agar culture (2) that affected by testing concentration (500mg/ml) of crude extract.
Fig. 2. *Pseudomonas aeruginosa* inhibition zone in nutrient agar (9) that affected by testing concentration (500mg/ml) of crude extract.

Mice wound healing by using crude extract revealed to a high healing ratio (100%) as shows in Table 3 at day 6 of treatment. Also by the evidence of disappearing of injury at the above period in compare with control mice as shown in Figure, 3 (C&E).

A: After 2 days of treatment and healing ratio (60%)  
B: After 4 days of treatment and healing ratio (83.33%)
C: After 6 days treatment and healing ratio (100%)

D: control, without treatment of and healing ratio (0%)

E: Control after 6 days without treatment

Fig. 3. A,B,C,D,E. Shows the effects of two treatments per a day by concentration 500mg/ml of crude extract of frog skin secretion on tested mice wound.

Table 3. Mice injury healing by using crude extract of frog skin (*Rana ridibunda*) secretions.

<table>
<thead>
<tr>
<th></th>
<th>Control mice n=3</th>
<th>Treated mice n=9</th>
<th>Healing ratio %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>L. mm</td>
<td>d. mm</td>
<td>L. mm</td>
<td>d. mm</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>0 %</td>
<td>60 %</td>
<td>83.33</td>
<td>100 %</td>
</tr>
</tbody>
</table>

As shown in study results, there were effects of crude extract by concentrations (500, 250 and 93.8 mg/ml) for both gram positive and gram negative pathogenic bacteria in agreement with Dimond, et al., (8) and Rinaldi, et al., (19). The Iraq frogs under study (*Rana ridibunda*), have skin glands which produce a secretion composed of a complex mixture of
substances with divers array of anti-microbial peptides (AMPs) ranging from (10 – 50) amino acid in length against bacteria in agreement with Calderon, et al., (3) ; Calderon, et al., (4);Conolon, et al., (6);El Haj Moussa, et al., (10);Gibson et al., (12) and Kim, et al., (13)

The recent study revealed a highly effect was on gram positive, gram negative pathogens and Leishmania tropica with some resistance in Leishmania donovani .The effects were caused by higher concentration of crude extract .The inhibition zones and cytotoxic effects were due to an interaction of crude substances with prokaryotic and eukaryotic cell membrane , thereby may lead to cytolysis in agreement with Calderon, et al.,(5) Conolon , et al.,(7); Mangoni, et al., (16) and Park , et al.,(18).

The recent study revealed to some substances that were existed in crude frogs (Rana ridibunda) skin secretions by the evidence of complete healing within 6 days (100%) in tested mice wounds. Thereby, the crude extract substances may lead to stimulate cells proliferation and the healing started from depth and along the site of wound in agreement with Liu, et al., (14) .Also this crude extract has had activity to eradicate wounds contaminated pathogenic bacteria and to reduce inflammation in agreement with El Haj Moussa , et al., (10); Liu, et al., (14) and Mashreghi , et al., (17) .

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REFERENCES


