YANTIMICROBIAL EFFECT OF CRUDE FROG (*RANA RIDIBUNDA*) SKIN SECRETIONS EXTRACT ON WOUNDS HEALING IN MICE

A.J.O.AL-Khafaji M.M.F. Al-Halbosiy Y.A. Hadi S.M.AL-Chalabi B.A. Abdulhameed*
Lecturer Asst.Prof. Lecturer Asst.prof. Biologist
***Biotechnology Research Center, Al-Nahrain University**
dralij2016@gmail.com

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

مجلة العلوم الزراعية العراقية -2018 :49(4):623-631 الخفاجي وأخرون

التاثير المضادة للجراثيم والتئام الجروح للمستخلص الخام لافرازات جلد الضفدع (*Rana ridibunda*)

علي جبار عليوي الخفاجي محمد محمود فرحان الحلبوسي ياسمين علي هادي صلاح مهدي الجلبي براء عبد الهادي عبد الحميد
مدرس استاذ مساعد مدرس استاذ مساعد بايلوجي اقدم
مركز بحوث التقنيات الاحيائيه، جامعة النهرين
dralij2016@gmail.com

المستخلص

ان جلد البرمائيات تنتشر فية غدد تحتوي على مختلف المواد النشطة بايلوجيا . ان الهدف من الدراسة هو لمعرفة تاثيرات افرازات جلد الضفادع على الطفيليات والعزلات البكتيرية المرضية . استخدمت في الدراسة 5 ضفادع بالغة اخذت من جداول الجادرية – بغداد، وقد حفزت الضفادع بحقنها جرعة 40 نانو غرام/ غرام من وزن الجسم من مادة نورابينفرين –HCL في داخل تجويف البطن والتي تسبب عنها افراز جلد الضفدع مواد نشطة بايولوجيا ، بعد ذلك وضع المحلول الناتج من غسل الضفدع في جهاز الطرد المركزي ثم جفد الراشح وبعدها اذيب الباودر المتحصل عليه وعقم . لقد اظهرت الدراسة بان التركيز 500 ملغرام / مل له تاثير مثبط لنمو البكتريا *المرضية Staphylococcus aureus* ، *Bacillus subtilus* ، *Escherichia coli* ، *Pseudomonas aeruginosa* و *Candida albicans* في حين التركيز 250 ملغرام / مل ثبط نمو *Salmonella typhi*. وقد اظهرت كل تراكيز المستخلص الخام ان لها تاثير قاتل للخلايا في مزروع خلوي للطفيلين *Leishmania tropica* و *Leishmania donovani* ، وبدليل علاقة الارتباط المعنوية في التاثير (P <0.071) . ان المعاملة بتركيز 500 مليغرام/مل من المستخلص الخام له تاثير منشط لخلايا النسيج و محفز للتضاعف الخلوي، و لابادة الجراثيم المرضية داخل الجرح وبعد ذلك ربما يؤدي الى الاسراع في عملية شفاء الجروح.

كلمات مفتاحية: بايولوجي، تجويف البطن، بكتريا المرضية.

***Received:26/11/2017, Accepted:8/4/2018**

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 ^{o}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 using 0.2 millipore filters. The filtrate kept refrigerated at 4^{o}C for future use.

Antimicrobial assay

The assay in present study involved the effects of crud extract on bacterial cultures of *Pseudomonas aeruginosa* , *Staphylococcus aureus, E. coli* and *Bacillus subtilis, Klebsilla pneumonia, Salomnella 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^{o}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^{o}C for 24h. After incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated at 26 ^{o}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 intra-peritoneal injection of 100 ul/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 (mm^2) = 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).

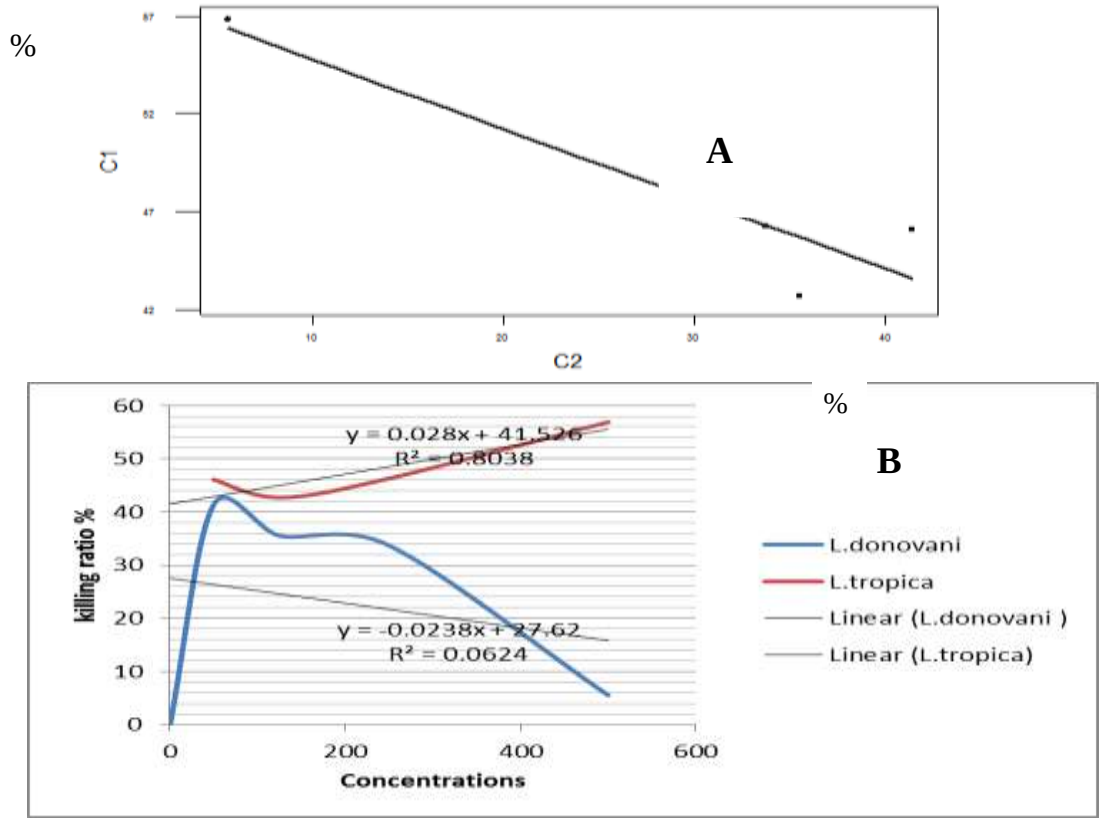

Fig.1. (A) Regression plot(Y=58.3748-0.357382X, R-Sq=0.864) for cytotoxicity effect onto *Leishmania tropica C1* and *Leishmania donovani* C2 parasites under study.(B)Shows the correlation of killing ratio and tested concentrations of crude extract onto parasites under study.

Table 1. Effects of crude extract on pathogenic Bacteria.

Conce.	*Pseudomonas aeruginosa*	*E. coli*	*Staph. aureus*	S. typhi	*Kleib.pneumonae*	*C. Albicans*	*Bacillus Subtilis*
			Inhibition Zon				
500 mg/ml	25mm	40mm	45mm	N.D	N.D		45mm
250 mg/ml	N.D	18mm	N.D	30mm	12mm	40mm	N.D
125 mg/ml	N.D	N.D	20mm	N.D	12mm	40mm	N.D
98.3 mg/ml	18mm	N.D	N.D	N.D	N.D	N.D	N.D
Control	0mm	0mm	0mm	0mm	0mm	0mm	0mm

N.D: Non determined

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

Concentration	Control O.D 620 nm	Treatment O.D 620 nm	Killing ratio	Treated parasite
500 mg/ml	0.353	0.201	56.9%	*Leishmania tropica*
250 mg/ml	0.353	0.163	46.3%	
125 mg/ml	0.353	0.151	42.7%	
50 mg/ml	0.353	0.163	46.1%	
500 mg/ml	0.163	0.154	55.2%	*Leishmania donovani*
250 mg/ml	0.163	0.108	33.7%	
125 mg/ml	0.163	0.105	35.5%	
50 mg/ml	0.163	0.0096	41.4%	

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.

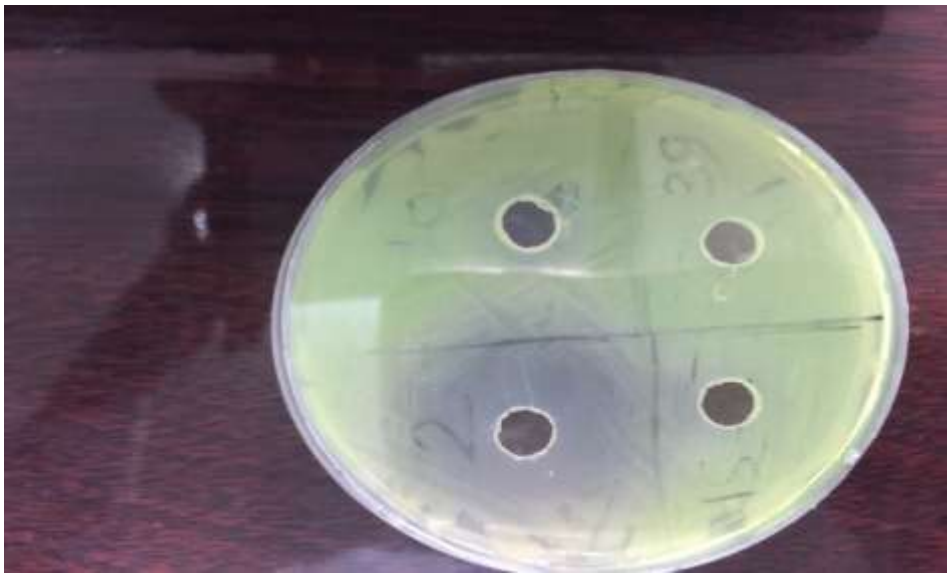

Fig.2. C-*Candida albicans* inhibition zone on sabouraud dextrose agar culture (2) that affected by testing concentration (125mg/ ml) of crude extract.

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

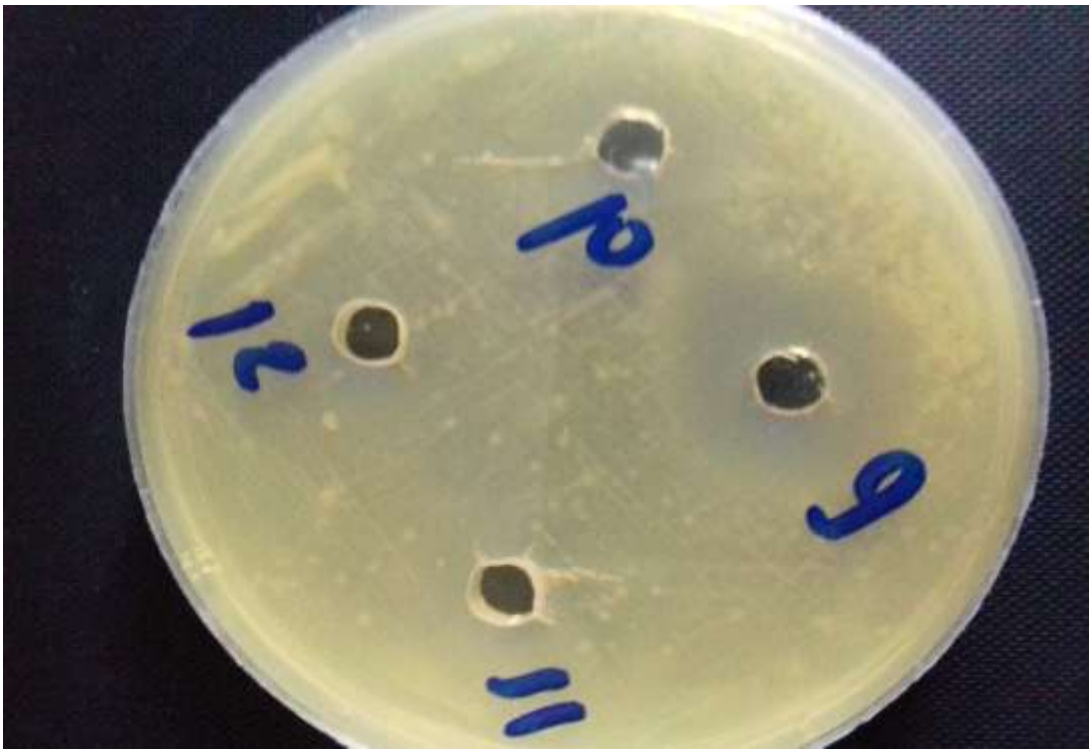

Fig.2. E-*Pseudomonas aeruginosa* inhibition zone in nutrient agar (9) that affected by testing concentration (500mg/ ml) of crude extract.

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.

Control mice n=3							
Day 0		Day 2		Day 4		Day 6	
L. mm	d. mm	L. mm	d. mm	L. mm	d. mm	L. mm	d. mm
30	2	25	2	20	1.5	10	1.0
Treated mice n=9							
Day 0		Day 2		Day 4		Day 6	
L. mm	d. mm	L. mm	d. mm	L. mm	d. mm	L. mm	d. mm
30	2	20	1.5	10	0.5	0	0
Healing ratio %							
0 %		60 %		83.33 %		100 %	

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 crud extract .The inhibition zones and cytotoxic effects were due to an interaction of crud 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) .

Acknowledgment

Of our deep pleasure we want to thank Dr. Huda S. Alagely (Bio. Tech. Res. Cen.) for her assistance to complete this manuscript.

REFERENCES

1-Al-Ghaferi, N., J. Koloziejec, N. Nowotny, L. Coquet, T. Jouenne, J. Leprince, H. Vaudry, J.D. King and J.M. Conlon, 2010.Antimicrobial peptides from skin secretions of the South-East Asian frog Hylarana erythraea (Ranidae). Peptides,31:548-554

2-Asoodeh,A., H.Naderi-Manesh, M. Mirshahi and B. Ranjbar, 2004. Purification and characterization of an antibacterial ,antifungal and non–hemolytic peptide from Rana ridibunda. J.Sci.I.Iran.,15(4):303-309.

3-Calderon,L.A.,L.P.H. Silva and ,R.G Stabeli.2010.Biodiversity,university infrastructure and bureaucracy:challenges of the bioprospective research aiming the sustainable development of the Brazillian Amazon.Revistade Estudos Universitarios ,36(3):15-41.

4-Calderon,L.A.,A.A Silva., ,P. Ciancaglini and ,R.G Stabeli.2011. nAntimicrobial peptides from Phyllommedusa frogs :From biomolecular diversity to potential nanotechnologic medical applications. Amino Acid., 40(1):29-49.

5-Calderon,L.A.,Silvap- Jardim, I.,Zuliani, J.P.,Silva, A.A.,Ciancaglini.P., ,L.H.P Silva. and,R.G Stabeli .2009.Amazonian biodiversity: a view of drug development for leishmaniasis and malaria .J. Braz .chem.Soc.,20:1011-1023.

6-Conolon,J.M., ,J. Kolodziejek and,N. Nowotny 2009.Antimicrobial peptides from the skin of North American frogs.Biochem.Biophys.Acta.,1788:1556-63

7-Conolon, J.M., , M. Mechkarska ,E. Ahmed, Coquet,I.,T. Jouenne , J. Leprince,H Vaudry ,M.P. Hayes and G. Padqett-Flohr, 2011.Host defense peptides in skin secretions of the Oregon spotted frog Rana pretiosa:Implications for species resistance to chytridiomycosis .Dev. Comp.Immunol.,35:644-649.

8-Diamond,G., N. Beckloff, ,A. Weiberg and ,K.O. Kisich 2009. The roles of antimicrobial peptides in innate host defense. Curr .Pharm.Des.,15:2377-92.

9- El Haj Moussa , A. El-Dakdouki , M.H . Olama, , Z. Moussad and, E. 2015. Antimicrobial effect of Rana ridibunda skin glands peptides against multidrug resistant pathogens Int.J. Curr .Microbiol.App.Sci., 4:62-74 .

10- El Haj Moussa , Z. A.Olama Moussad, E.,Kavunja , H. and, H, El-Dakdouki .2015.Characterization of anuran skin peptides : An alternative to the classical therapeutic agents used for MDR pathogens . Int.J.Curr.Microbiol . App.Sci.,4(16) : 889-900 .

11-Freshney,R.I.,2012. Culture of Animal Cell.6th Ed. Wily-Liss,NewYork.pp.377

12-Gibson, B. W.,Tang, D. ,R. Mandrell ,M. Kelly and,E.R. Spindel 1991.Bombinin-like peptides with anti-microbial activity from skin

secretions of the Asian toad ,Bom binaorientalis. J.Biol.Chem. 266:23103-11.

13-Kim, J.B. , S. Iwamuro , F.C. Knoop and ,J.M. Conolon 2001. Antimicrobial peptides from the skin of the Japanese mountain frog, Rana ornativentris.J.Pept.Res.,58:349-56.

14 - Liu,H.,Mu ,L. Tang, J. C. Shen, ,C. Gao, M. Rong , Z. Zhang,J. Liu , X. Wu , H.Yu and ,R. Lai 2014.A potential wound healing-promoting peptide from frog skin.Int.J.Biochem.Cel.Biol.,(49):32-41

15-Mahmoudvand, H. ,F. Ezzatkhah ,F. Sharififar ,I. Sharifi and E.S Dezaki 2015:Antileishmanial and cytotoxic effects of essential oil and methanolic extract of Myrtus communis L. Korean .J. Parasitol. ,53(1).21-27

16-Mangoni, M.L. ,J.M. Saugar ,M. Dellisanti ,D. Barra ,M. Simmaco and ,L. Rivas 2005. Temporins ,small antimicrobial peptides with leishmanicidal activity.J.Biol.Chem.,280:984-990.

17Mashreghi ,M. ,M.R. Bazaz, ,N.M. Shahri ,A. Assodeh ,M. Mashreghi , M.B . Rassouli and Golmohammadzadeh ,S.2012.Topical effects of frog *Rana ridibunda* skin secretions on wound healing and reduction of wound microbial load.J.Enthopharm 145.Issue, 3:793-797.

18-Park, C.P , H.S. Kim and S.C. 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microoganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Commun., 244:253-57.

19-Rinaldi,A.C.2002.Antimicrobial peptides from amphibian skin: an expanding scenario. Curr. Opin. Chem.Biol.,6:799-804.

20-Wang,T. ,O.Gu, J. Zhao,Mei,J., M. Shao , Y. Pan ,J . Zhang ,H. Wu ,Z. Zhang and F. Liu 2015.Calcium alginate enhances wound healing by up –regulating the ratio of collagen types 1/111 in diabetic rats.Int.J. Clin.Exp.Pathol.,8(6):6636-6645.