Iraqi Journal of Science
ISSN: 0067-2904

The anti- Leishmaniasis activity of Purified Bacteriocin Staphylococcin and Pyocin Isolated from *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Mais E. Ahmed[1], Issra S. Mousa[1], Mohammad M.F Al-Halbosiy[2], Entsar J. Saheb*[1]
[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
[2]Biotechnology Research Center, AL-Nahrain University, Baghdad, Iraq.

Abstract

Tropical illnesses caused by parasites proceed to cause socioeconomic devastation that reverberate worldwide protozoan parasites, like *Leishmania*. This parasite has an enormous public health problem in many countries. There is a growing requisite for new control methods for many of these illnesses due to the increasing drug resistance showed by the parasites and problems with drug poisonousness. In this study, fifty-five patients (burns and wounds) were collected from patients from Al-Yarmouk Hospital and Teaching Baghdad Hospital during the period from November, 2015 to January, 2016. Cultural and morphological characteristic examination, biochemical tests were conducted and confirmed the diagnosis by antibiotics sensitivity test and Vitek-2 system. The results identified thirty-three *Staphylococcus aureus* and thirty *Pseudomonas aeruginosa* from skin burn and wounds. Vitek 2 system gave confirmation of positive results for both strains with a probability 98-99%. The *S. aureus* isolate (S3) and *P. aeruginosa* (P 5) was chosen among bacterial isolates as a good producer for crude both bacteriocins according to their widest inhibition zone by well diffusion assay WDA. Two steps method extraction were used for bacteriocin purification, first via ammonium sulphate at 70% and next step by Sephadex G-50 gel filtration chromatography. The two new drugs staphylococcin and pyocin at different concentrations was used for the treatment of *L. tropica* and *L. donvani*. All concentration of Staphylococcin showed no inhibitory activity on promastigotes of *L. tropica* and *L. donvani*. While the concentration of 32.5 μgmL^{-1} of pyocin showed the maximum cytotoxic effect on promastigotes of *L. tropica* and *L. donovani*, where the inhibition rate (IR%) were 87.1% and 87.9% respectively. As part of the research objectives is the discovery of new treatments against leishmaniasis also benefit from improved models.

Keywords: Pyocin, Staphylococcin, Anti- Leishmaniasis.

تاثير البكتريوسين الستافيلوكوكسين والبيوسين المنقى من بكتيريا المكورات العنقودية و الزائفة الزنجارية ضد داء الليشمانيات

ميس عماد احمد[1]، اسراء سالم موسى[1]، محمد محمود فرحان الحلبوسي[2]، انتصار جبار صاحب*[1]
[1]قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.
[2]قسم التقنيات الاحيائية، جامعة النهرين، بغداد، العراق.

*Email: ejsaheb@ualr.edu

الخلاصة

لا تزال الأمراض الاستوائية التي تسببها الطفيليات تسبب ضررا اجتماعيا واقتصاديا في جميع أنحاء العالم، مثل الليشمانيا. ولا يزال هذا الطفيلي يشكل مشكلة صحية عمومية كبيرة في العديد من البلدان. وهناك حاجة متزايدة إلى تدابير جديدة لمكافحة العديد من هذه الأمراض بسبب تزايد مقاومة الأدوية التي تظهرها الطفيليات والمشاكل المتعلقة بسمية الادوية المستخدمة. تم جمع خمسة وخمسين عينة سريرية (الحروق والجروح) من مرضى مستشفى اليرموك ومستشفى بغداد التعليمي خلال الفترة من تشرين الثاني 2015 وحتى كانون الثاني 2016، وتم إجراء فحوصات مظهرية واختبارات كيميائية ، وأكد التشخيص من قبل المضادات الحيوية باستخدام اختبار الحساسية ونظام فيتيك-2. حددت النتائج ثلاثة وثلاثين من *Staphylococcus aureus* وثلاثين *Pseudomonas aeruginosa* من حرق الجلد والجروح. أعطى نظام فيتيك 2 تأكيد النتائج الإيجابية لكلا سلالات مع احتمال 98-99٪. تم اختيار العزلة (S3) *S. aureus* و *P. aeruginosa* (P 5) بين العزلات البكتيرية كمنتج جيد للبكتريا الخام على حد سواء وفقا لأوسع منطقة تثبيط لها من خلال فحص النشر الجيد WDA. تم إجراء تنقية البكتريوسين من خلال خطوتين بواسطة كبريتات الأمونيوم في 70٪ الخطوة الثانية من قبل سيفادكس G-50 الترشيح هلام اللوني. تم استخدام عقاري المكورات العنقودية الجديدة والبيوسين بتركيز مختلفة لعلاج الليشمانيا الجلدية والليشمانيا الحشوية. تركيز المكورات العنقودية لم يظهر أي نشاط مثبط على الطور المسوط من الليشمانيا الجلدية والليشمانيا الحشوية. في حين أن تركيز 32.5 ميكروغرام - 1 من البيوسين أظهر التأثير السام للخلايا على الطور المسوط من الليشمانيا الجلدية والليشمانيا الحشوية ، حيث كان معدل تثبيط (إر٪) 87.1٪ و 87.9٪ على التوالي. إن عملية اكتشاف وتطوير مضادات الليشمانيا الجلدية والليشمانيا الحشوية الجديدة سوف توفر نماذج مطورة ضد الاصابة بهذه الطفيليات.

Introduction

Antimicrobial peptides are produced by many microorganisms as a method of their protection. Antimicrobial peptides are typically relatively short (12 to 100 amino acids), positive charged , amphiphilic, and isolated from single-celled microorganisms, insects and other invertebrates, plants, amphibians, birds, fish, and mammals, including humans [1]. To date, hundreds of such peptides have been recognized form gram positive and negative bacteria indicating their importance in the innate immune system .The expression of these antimicrobial peptides can be constitutive or can be inducible by infectious and inflammatory stimulate [2].

Leishmania is a protozoan parasite that causes leishmaniasis. This illness represents an international health problem that is predominant in Europe, Africa, Asia and the Americas. Up to 20 million persons are affected. Leishmaniasis is a vector-borne metazoonosis illness, caused by obligate intra macrophage protozoa of the genus *Leishmania* [3]. There are three chief types of leishmaniases: visceral Leishmaniasis (VL) or (kala-azar), cutaneous Leishmaniasis (CL), and mucocutaneous Leishmaniasis. If left untreated, leishmaniasis is a life-threatening infection. Among parasitic diseases, mortality from leishmaniasis is second only to malaria, with an estimated 20,000 to 40,000 deaths each year [4].

The conventional treatment of leishmaniasis is pentavalent antimonials, considered a gold standard in the treatment, are identified to be very toxic to individuals, and Amphotericin B when there is no response to treatment with antimonials while efficiency of pentamidine the third drug of choice, is less well known treatment are expensive. Additional weakness is the ineffectiveness of these drugs against many species of *Leishmania*. Thus, alternative approaches in the treatment of leishmaniasis are urgently wanted [5]. In recent years, one class of compounds that have been investigated as antileishmanials is the antimicrobial peptides (AMPs) [6]. In the former decade, attention has gained excessive momentum due to its potential as both therapeutic antibiotics and as a natural food preservative [7]. Many antimicrobial peptides have now been identified in invertebrates, and they are documented as playing an important role in protection from pathogenic organisms. The role of antimicrobial peptides and the regulation of their expression, including the signaling cascades involved, are well understood for *Drosophila* [8].

Prior study showed a novel anti-infective treatments host defense peptides bacterocin from two sorts' bacteria *S. aureus* (MRSA) and *P. aeruginosa*. Evolutionarily conserved defense molecules are an intriguing alternative. Bacterocins are essential compounds of the defense mechanisms of organisms extending from bacteria to animals [9].

Material and Methods

Isolation and identification of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A total of 30 clinical specimens of *S. aureus* and *P. aeruginosa* were collected from the Pathology Hospital in Iraq. Wound swab on MacConkey agar plates and on Mannitol Salt Agar plates were transferred immediately to the microbiology laboratory for further isolation of bacterial pathogens. The plates were incubated at 37^0C for 24 hrs. After incubation, the isolated colonies were identified on the base of morphological, cultural and biochemical features [10].

Antibiotic Resistance Test

The isolates pattern was by Kirby-Bauer disc [11]. Diffusion technique studied susceptibility of the isolates were done and interpreted depending on (NCCLS) National Committee for Clinical Laboratory Standards recommendations. The antibiotic concentration per disk was as following Table-1.

Table1-Antibiotic Discs Used in the Study

Antibiotics	**Concentration *P.* (*aeruginosa* µg/disc)**	**Antibiotics *S. aureus* (µg/disc)**	**Concentration**
Azithromycin	30	Imipenem	10
Cefixime	5	Linezolid	5
Erythromycin	5	Oxacillin	5
Gentamycin	5	Vancomycin	30

VITEK 2 System

A selected number of resistant bacterial isolates were selected to confirm their diagnosis as *S. aureus* and *P. aeruginosa* using the vitek 2 system.

Parasite cultivation

Promastigotes of *L. donovani* and *L. tropica* were cultured at 26◦C in RPMI1640 media. Parasites were inoculated at a density of 1×10^5 cells/ml and grown for 3 days to get exponentially growing log phase cells, which were used for most experiments.

Extraction of Crude Bacterocin

The isolates of *S. aureus* and *P. aeruginosa* were cultured on Tryptic Soay broth (TSB) (2)% inoculated with 6×10^8 cell/ml and incubated at 37 °C for 24 hours under aerobic conditions [12]. Cells were harvested by centrifugation at 6000 rpm for 15 minutes, the cell-free supernatant was referred to as crude MRSAcin extract which heated to 80° C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15 minutes [13] followed by filtration of the supernatant through a 0.2 µm pore-size nylon syringe filter. The supernatant was stored at -20 °C.

Partial Purification of Bacterocin

The supernatant was using after filters gradually. The supernatant was precipitated with ammonium sulfate [14] and added to constant stirring at 4°C till the level of 75% concentration of the protein to attain the optimum precipitation. The precipitate was recovered by centrifugation at 15,000 rpm for 50 min at 4°C and solubilized in 200 mL of 50mM sodium phosphate buffer pH 7.0. The resulting pellet and designated as crude preparation [15].

Estimation of protein by Lowry's method

The Lowry's method was analyzed for protein using the samples [16].

Gel Filtration chromatography (Sephadex G-75)

Sephadex G-50 of Gel was prepared according to Pharmacia catalogue [17]. It was allowed to washing several times with 500ml of D.W. at room temperature for remove impurities about 1hr. Gel was put in 500 ml of solution1 for 30 min and decanted with vacuum pump to remove all the solution

and washed with D.W. The gel was put in 500 ml of solution no. 2 for 30 min and decanted with vacuum pump, washed with D.W. as above till the pH of the gel became neutral. The gel was poured on the column (2×60) cm and left at room temperature then 5ml for each fraction was collected, absorbance of each fraction was read at 280 nm and plot was drawn between fraction number and its absorbance. It was collected separately and the performed peaks were concentrated by dialyzed and sucrose against 0.02 M phosphate buffer, pH 7 overnight.

In vitro Antileishmanial activity

The antileishmanial was evaluated for Staphylococcin from *S. aureus* and Pyocin from *P. aeruginosa* against promastigote forms of *L. tropica* and *L. donovani* according to [18, 19]. The cell viability MTT assay was used. Briefly, 100 µLwell^{-1} *Leishmania* promastigotes (10^6 cell mL^{-1}) were cultured in 96-well tissue culture plate. Prepare different concentrations of Staphylococcin and pyocin test solution (1.953, 3.906, 7.812, 15.625, 31.25, and 62.5 µgmL^{-}) and (1.015, 2.031, 4.062, 8.125, 16.25 and 32.5µgmL^{-1}) respectively and 100 µL was added of various concentrations to each well and incubated at 26◦C for 24h. After incubation, 10µL of MTT solution (5 mg mL^{-1}) was added to each well and incubation was continued for a further 4 hours. Finally, 50 µL of solubilization solution of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. Promastigotes were cultured in complete medium without treated with Staphylococcin and pyocin solution as a positive control, and in a complete medium only as a blank. The experiment was performed in triplicate. The ELISA reader was used to measure absorbance for each well at 620 nm. The mean absorbance for each group of replicates was calculated. The live cells, percentage of viability and inhibition ratio were calculated according to the formula

$$\text{Inhibition}\ (\%) = (\text{AC} - AS\ / \text{AC}) \times 100$$

Where Ac and As are the optical density for medium and Staphylococcinp or pyocin samples, respectively.

Results and Discussion

Antibiogram profile results for *S. aureus* isolated from clinical samples showed that 90% isolates as Oxacillin resistance. The isolates showed multi-resistant to Oxacillin, Imipenem, Pencillin G and Linezolid while sensitive to vancomycin (Fig 1), while 80% of *P. aeruginosa* resistance Azithromycin and multi-resistant to erythromycin, gentamycin and sensitive to cefixime Figure-1.

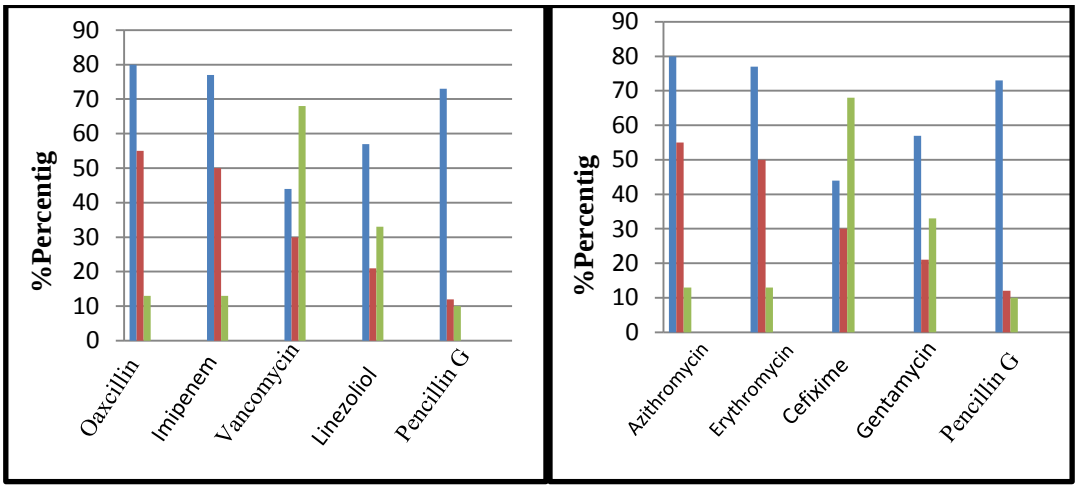

Figure 1-Antibiotic susceptibility test of *S. aureus*

Vitek 2 system

It gave confirmation of positive results for *S. aureus* and *P.aeruginosa* as a selected organism with a probability 98-99%.

Production of bacteriocin

Crude bacteriocin was heated to denaturant any proteases and heat-sensitive proteins. Ammoinum sulphat extraction completely showed recovery of Staphylococcin and Pyocin activity, extraction by ammonium sulphate 70% show inhibition zone diameter reached (17,15) mm respectively by using as indicator strain of *E.coli* Figure-2.

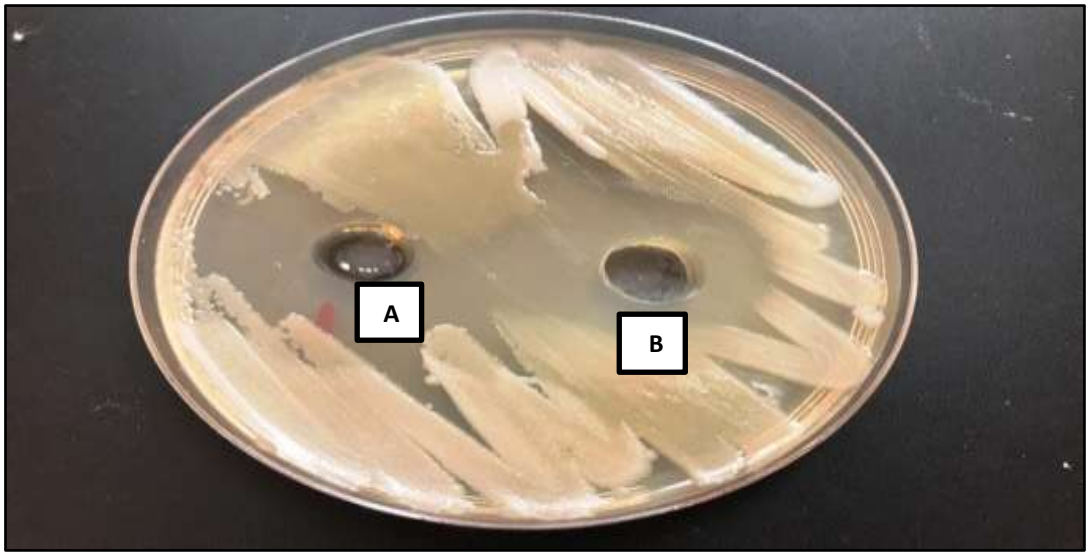

Figure 2-Antibactrial activity of the precipitation protein of A- **Staphylococcin B-pyocin** by 70%ammonium sulphat

The crude ammonium sulfate precipitate was further subjected to conventional gel filtration chromatography on Sepahdex-G75 column eluted with 50 mM sodium phosphate buffer, pH 7.0. This separation profile resulted in two major and well separated peaks designated as peak I bacterocin (Fig. 2). After chromatography, antibacterial activity of both these peaks was checked by agar well diffusion method using *E.coli* as indicator culture show inhibition zone diameter reached (21,19) mm at concentration (62.5, 32.5) µg/ml respectively Figures-(3, 4).

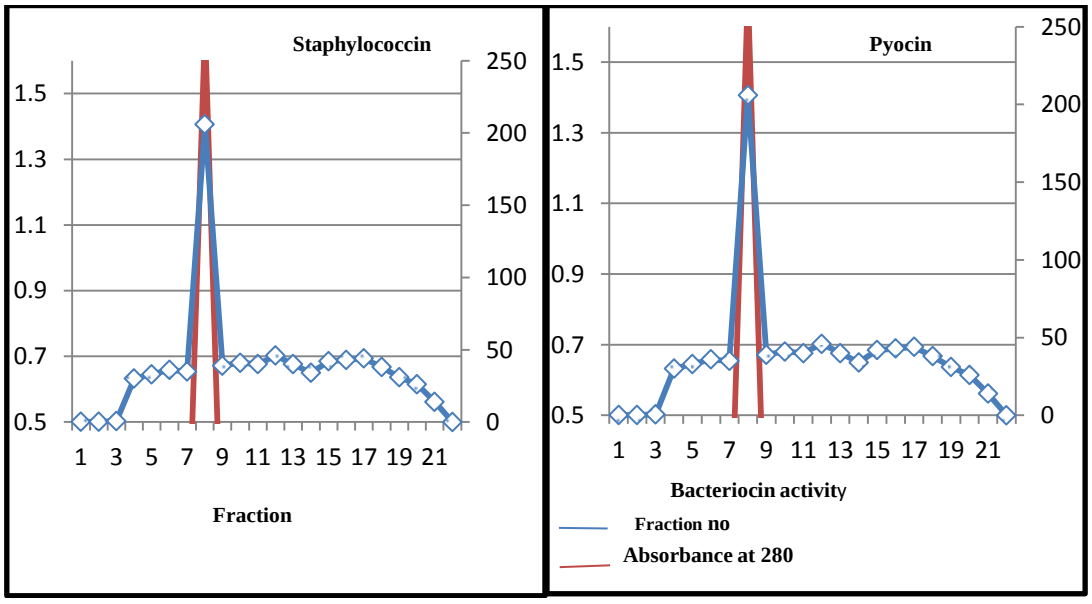

Figure 3-A and B: Purification **of** Staphylococcin and Pyocin by gel filtration chromatography, using Sephadex G-75 column with dimensions (2x60) cm that equilibrated and eluted by 20mM sodium citrate buffer (pH 7), flow rate was 40ml/hour, with 5ml for each fraction.

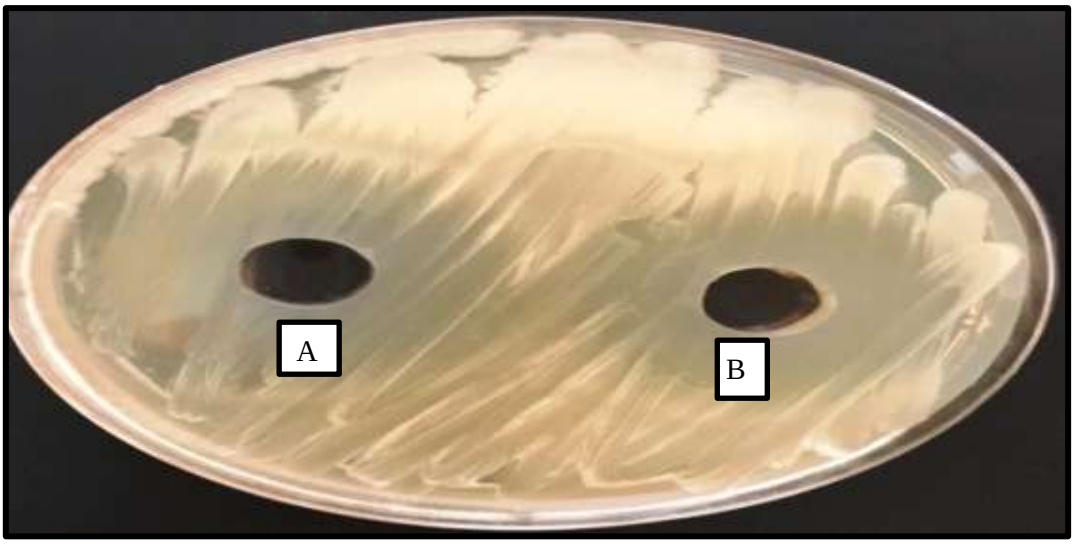

Figure 4-Antibactrial activity of the purifaction protein of bacterocin using Sephadex G-75 column A: Staphylococcin B: Pyocin

Cytotoxic effect of Staphylococcin and Pyocin on *L. tropica* by MTT assay

The cytotoxic effect of Staphylococcin and Pyocin on *L. tropica* and *L. donvani* promastigotes were evaluated at sex concentrations (1.953, 3.906, 7.812, 15.625, 31.25, and 62.5 μgmL^{-1}) and (1.015, 2.031, 4.062, 8.125, 16.25 and 32.5μgmL^{-1}) respectively for 24 h. All concentrations of Staphylococcin showed no inhibitory activity on promastigotes of *L. tropica* and *L. donvani* (Table-1). While the concentration of 32.5 μgmL^{-1} of pyocin showed the maximum cytotoxic effect on promastigotes of *L. tropica* and *L. donovani*, where the inhibition rate (IR%) were 87.1% and 87.9% respectively Table-2.

Table 1- *In vitro* inhibitory rate (IR%) of Staphylococcin against promastigotes of *L. tropica* and *L. donvani*

µg/ml Staphylococcin	**IR (%)** ***L. tropica***	**IR (%)** ***L. donvani***
1.953	0	0
3.906	0	0
7.812	0	0
15.625	0	0
31.25	0	0
62.5	0	0

Table 2- *In vitro* inhibitory rate (IR%) of Pyocin against promastigotes of *L. tropica* and *L. donovani*

Concentration of R-pyocin µg/ml	**IR (%)** ***L.tropica***	**IR (%)** ***L.donvani***
1.015	0	0
2.031	51.4	0
4.062	72.8	56
8.125	83.7	85.3
16.25	81.3	86.5
32.5	87.1	87.9

The result revealed that by increasing the pyocin concentration, the cytotoxicity of promastigotes will increase while viability will decrease Figures-(5, 6).

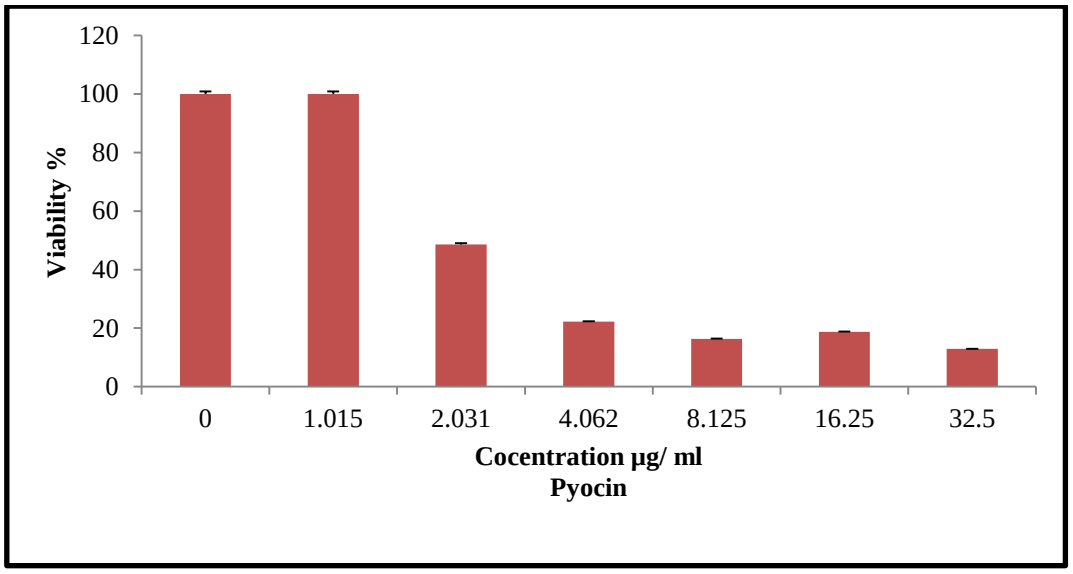

Figure 5-Cytotoxic effect of pyocin on *L. tropica* by MTT assay. Data is expressed as the Mean±S.D. obtained from triplicate experiment.

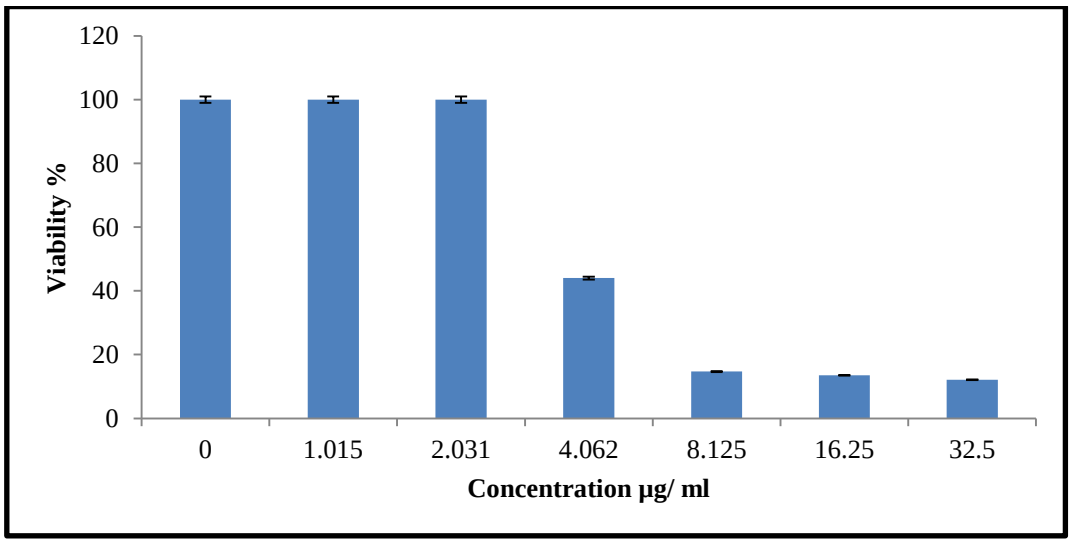

Figure 6-Cytotoxic effect of pyocin on *L. donvani* by MTT assay. Data is expressed as the Mean±S.D. obtained from triplicate experiment.

The results of this study agreed with [20] who found that only (24.41%) of tested bacteria *S. aureus* was resistance to vancomycin in USA. The current study indicates that *P. aeruginosa* is becoming resistance to commonly used antibiotic due to excessing consumption of antibiotics exerting selected present bacteria. The result confirmed the occurrence of MRD strains of *P. aeruginosa* which agree with [21] who found that the *P. aeruginosa* multi-resistance to most antibiotic such as Ceftriaxone, Cefepime and Ceftazidime at 98%, 78% and 80% respectively.

During the purification procedures, each step resulted in a considerable loss of protein concentration while specific activity increases [22]. By using this method, a good resolution of different sizes of proteins could be obtained. If some criteria follow a volume of matrix to volume of samples, low flow rate, a suitable column diameter with high length, quality of sample application, and absence of any denaturizing agents in elution buffer [23].

Pentavalent antimonials were developed in 1945, and remain the first-choice drug for both visceral and cutaneous leishmaniasis in most countries of the world. Amphotericin B and pentamidine need long courses of administration. The choice of treatment also depends on the causative *Leishmania* species. A study of 103 patients with CL in Peru displayed that among patients infected with *L. (Viannia) peruviana* (47.6%), *L. (Viannia) guyanensis* (23.3%), and *L. (Viannia) braziliensis* (22.3%), 21 of them (21.9%) did not respond to pentavalent antimonial chemotherapy. Therefore, precise diagnosis of parasite is of the paramount medical importance, because it will guide the choice of an appropriate treatment. Although spontaneous cure is the rule, the recovery rate varies depending on the species of *Leishmania*, and may require months or years to complete healing. Most of the usually used drugs are toxic and do not cure, i.e., extinction the parasite, from infected peoples. Failure to treat leishmaniasis successfully is often due to increased chemo resistance of the parasite. Because treatment is an increasing problem, the development of new treatments that can replace or complement the presently available therapeutic alternatives is necessary. Encouraging advances in chemotherapy have been made in recent years [24].

A study has revealed positive results for *S. aureus* as a selected organism with a probability 98-99% [25]. The result reported by [26] found that partial purification with ammonium sulfate precipitation bacteriocins have antimicrobial activities against food-spoiling bacteria and food-borne pathogens.

Conclusion

The results of this study showed that pyocin has dose-dependent antileishmanial activities. The antileishmanial drugs like Miltefosine can induce apoptosis in *Leishmania* through mitochondrial membrane permeability reduction, after increasing cytochrome c releasing [27] while the antileishmanial mechanism of pyocin is yet unknown. From the results of this assay, it can be concluded that pyocin produced from *P. aeruginosa* can efficiently be used as a candidate antileishmanial agent.

References

1. Wang, Z. and G. Wang. **2004**. APD: the Antimicrobial Peptide Database. *Nucleic Acids Res*. **32**: D590–D592.
2. Cunliffe, R. N. and Mahida, Y. R. **2004**. Expression and regulation of antimicrobial peptides in the gastrointestinal tract. *J. Leukoc. Biol*. **75**: 49–58.
3. Elmahallawy, E. K., Martínez, A.S., Rodriguez-Granger, J., Mallecot, Y. H., Agil, A., Mari J. M. N. and Fernández, J. G. **2014**. Diagnosis of leishmaniasis. *J. Infect. Dev. Ctries*. **8**(8): 961-972.
4. Salamm, N., Al-Shaqha, W. M. and Azzi, A. **2014**. Leishmaniasis in the Middle East: Incidence and Epidemiology. *PLoS Negl. Trop. Dis*. **8**(10): e3208.
5. Barbosaa, A. F. S., Sangiorgib, B.C.B.B., Galdinoa, S. L., Pittaa, I. R., Barral- Nettob, M., Correiac, N. A. and Pinheiroc, A. L. B. **2012**. Evaluation of Photodynamic Antimicrobial Therapy (PACT) against Promastigotes Form of the *Leishmania (Viannia) braziliensis*: In Vitro Study. *Proc. of SPIE*. **8211** (2).
6. Chadbourne, F. L., Raleigh, C., Ali, H. Z., Dennya, P.W. and Cobb, S. L. **2011**. Studies on the antileishmanial properties of the antimicrobial peptides temporin A, B and 1Sa. *J. Pept. Sci*. **17**: 751–755.
7. Perez, R. H., Zendo, T. and Sonomoto, K. **2014**. Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories*. **13**(1): S3.
8. Imler, J. L. and P. Bulet. **2005**. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. *Chem. Immunol*. Allergy. **86**: 1–21.
9. Lynn, M.A., Kindrachuk, J., Marr, A.K., Jenssen, H., Panté, N., Elliott, M.R., Napper, S., Hancock, R.E. and McMaster, W.R. **2011.** Effect of BMAP-28 Antimicrobial Peptides on Leishmania major Promastigote and Amastigote Growth: Role of Leishmanolysin in Parasite Survival. *PLoS Negl Trop Dis*. **5**(5).
10. Reid, G., Younes, J.A. and Van der Mei, H.C. **2010**. Microbiota restora- tion: natural and supplemented recovery of human micro- bial communities. *Nat. Rev. Microbiol*. **9**: 27–38.
11. Finegold, B., Baron, E.J. and Finegold, S.M. **1990**. *Diagnostic Microbiology*. 8th Ed. Mosby Company. London.

12. Ali, W. Sh. **2010**. Production, purification and characterization of plantaricin from local strains of Lactobacillus plantarum. Ph.D thesis, College of Science, University of Baghdad.
13. Powell, L., Slater, S., Mirtcheva, D., Bao, Y. and Chaloupka, F. **2007**. Food store availability and neighborhood characteristics in the United States. *Preventive Medicine*. **44:** 189–195.
14. McCaughey, L.C., Josts, I., Grinter, R., White, P., Byron, O., Tucker, N.P., Matthews, J.M., Kleanthous, C., Whitchurch, C.B. and Walker D. **2016**. Discovery, characterisation and in vivo activity of pyocin SD2, a protein antibiotic from Pseudomonas aeruginosa. *Biochem J.* **1:473**(15): 2345-58.
15. Iqbal, A., Ali, S.A., Abbasi, A., Volter, W. and Rasool, S.A. **2001**. Production and some properties of Bac201: A bacteriocin like inhibitory substance from *Staphylococcus aureus* AB201. *J. Basic Microbiol*. **41**: 25-36.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. **1951**. Protein measurement with folin phenol reagent. *J. Biol. Chem*. **193**(1): 265-275.
17. Wang, J. **2010.** Potential and flux landscapes quantify the stability and robustness of budding yeast cell cycle network. *Proc Natl Acad Sci U S A.* **107**(18): 8195-200.
18. Mahmoudvand, H., Ezzatkhah, F., Sharififar, F., Sharifi, I. and Dezaki, E.S. **2015**. Antileishmanial and cytotoxic effects of essential oil and methanolic extract of *Myrtus communis* L. *Korean J. Parasitol*. **53**(1): 21–27.
19. Mosmann, T. **1983.** Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth*. **65**: 55–63.
20. Verma, N.K., Singh, G. and Dey, G.S. **2007**. Miltifosine induces apoptosis in arsenite-resistant *Leishmania donovani* promastigotes through mitochondrial dysfunction. *Exp. Parasitol*. **116:** 1-13.
21. Tiuman,T. S., Santos, A.O., Ueda-Nakamura, T., DiasFilho, B. P. and Nakamura, C. V. **2011**. Recent advances in leishmaniasis treatment. *Inter. J. l of Infec. Dis*. **15**(8): 525-532
22. Abrams, D., Barbosa, J., Albano, H., Silva, J., Gibbs, P. and Teixeira, P. **2011**. Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from "Alheira". *Food Control*. **22**: 940-946.
23. Grillon. A., Schramm, F. and Kleinberg, M. **2016.** Comparative Bactericidal Activity of Fluoroquinolones against *P.aeruginosa* and *S.maltophilia* Assessedby Minimum Inhibitory Concentrations and Time-Kill Studies. *J. pone*.0156690.
24. Cartwright, E., Paterson, G., Raven, K. and Torok E. **2013.** Use of Vitek 2 Antimicrobial Susceptibility Profile to Identify mec C in Methicillin-Resistant Staphylococcus aureus. *In J. Clin. Micro*. **51**(8).
25. Afhannaz, S. and Ajazrasool, S. **2013.** Isolation, Production and Characteriztion of Bacterocin Produced by strains form indigenous environment. *Pak. J. Bot*. **45**(1): 261-267.
26. Ogunbanwo, S.T., Sanni, A.I. and Onilude, A. A. **2003.** Characterization of bacteriocin produced by Lactobacillus plantarum F1 and Lactobacillus brevis OG1. *Afri. J. Biotech.* **2**(8): 219-227.
27. Sure, K., Bhagwat. P., Ranveer. R, Dandge, P. and Sahoo, A. **2016.** Production and Characterization of Bacteriocin Produced by Lactobacillus Viridescence (NICM 2167) Braz. *Arch. Biol. Technol*. **59**: e16150518.