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

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Abstract

Tropical illnesses caused by parasites proceed to cause socioeconomic devastation that reverberate worldwide protozan 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 Vitrek-2 system. The results identified thirty-three *Staphylococcus aureus* and thirty *Pseudomonas aeruginosa* from skin burn and wounds. Vitrek 2 system gave confirmation of positive results for both strains with a probability 98-99%. The *S. aureus* isolate (S3) and *P. aeruginosa* (P5) 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. donvani*, 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.

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

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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 leishmaniasis: 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 \textit{S. aureus} (MRSA) and \textit{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 \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}**

A total of 30 clinical specimens of \textit{S. aureus} and \textit{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°C 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**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration \textit{P. aeruginosa} (µg/disc)</th>
<th>Antibiotics \textit{S. aureus} (µg/disc)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>30</td>
<td>Imipenem</td>
<td>10</td>
</tr>
<tr>
<td>Cefixime</td>
<td>5</td>
<td>Linezolid</td>
<td>5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5</td>
<td>Oxacillin</td>
<td>5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>5</td>
<td>Vancomycin</td>
<td>30</td>
</tr>
</tbody>
</table>

**VITEK 2 System**

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

**Parasite cultivation**

Promastigotes of \textit{L. donovani} and \textit{L. tropica} were cultured at 26°C in RPMI1640 media. Parasites were inoculated at a density of $1 \times 10^5$ 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 \textit{S. aureus} and \textit{P. aeruginosa} were cultured on Tryptic Soay broth (TSB) (2)% inoculated with $6 \times 10^8$ 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 (10⁶ 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⁻¹) 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

\[
\text{Inhibition (\%)} = \left( \frac{\text{AC} - \text{AS}}{\text{AC}} \right) \times 100
\]

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*](image)

**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. Ammonium 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.
The crude ammonium sulfate precipitate was further subjected to conventional gel filtration chromatography on Sephadex-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 bacteriocin (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 2**-Antibacterial activity of the precipitation protein of A- **Staphylococcin B-pyocin** by 70% ammonium sulphate

**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: Antibacterial activity of the purification 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

<table>
<thead>
<tr>
<th>µg/ml Staphylococcin</th>
<th>IR (%) L. tropica</th>
<th>IR (%) L. donvani</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.953</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.906</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.812</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.625</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>62.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Concentration of R-pyocin µg/ml</th>
<th>IR (%) L. tropica</th>
<th>IR (%) L. donvani</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.015</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.031</td>
<td>51.4</td>
<td>0</td>
</tr>
<tr>
<td>4.062</td>
<td>72.8</td>
<td>56</td>
</tr>
<tr>
<td>8.125</td>
<td>83.7</td>
<td>85.3</td>
</tr>
<tr>
<td>16.25</td>
<td>81.3</td>
<td>86.5</td>
</tr>
<tr>
<td>32.5</td>
<td>87.1</td>
<td>87.9</td>
</tr>
</tbody>
</table>
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 exceeding 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**


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