Effect of prodigiosin isolated from Serratia marcescens on wound infections induced by clinical isolates of bacteria in a murine model.

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Abstract:

Prodigiosin is a red bio-dye that is produced by *Serratia marcescens* and another bacterium. It is made up of a linear tripyrrole chemical structure and is classified as an alkaloid. Researchers paid attention to prodigiosin pigment due to its ability to exhibit a diverse range of biological actions, including antibacterial, antifungal, antimalarial, and anticancer properties.

Objective: This study aims to assess the effectiveness of prodigiosin in treating superficial infections caused by antibiotic resistant bacteria.

Methods: The red pigment found in bacterial biomass was identified as prodigiosin by studying its structure with UV-visible spectrophotometry and high-performance liquid chromatography (HPLC). The antibacterial activity against pathogenic bacteria was assessed in vitro using agar well diffusion method. A mouse experimental wound model was used to initiate infection by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The topical treatment was administered 4 hours after the injury and continued until the wound was fully healed.

Results: The pigment exhibited a maximum absorbance peak at 535 nm. All strains that were tested showed sensitivity and produced significant areas of inhibition zones against *P. aeruginosa* (16.50±1.36mm) and *S. aureus* (20.83±2.40mm). Topical applications of prodigiosin (0.1% prodigiosin in Vaseline) have been proven to be beneficial. The scar healing was completed, with no remaining lesions and no signs of redness. The highest rate of decrease was shown at 95 mg/ml for *S. aureus (*C0.003±0.003b*) followed* by *P. aeruginosa (*D0.02±0.008b*).*

Conclusion: Prodigiosin, showed great potential as a natural substitute for synthetic drugs due to its potent antimicrobial properties against superficial skin infections caused by bacteria.

Key words: Prodigiosin, bacterial pigment, antimicrobial, superficial infection

تأثير البرودجيوسين المعزول من Serratia *marcescens* على التهابات الجروح المستحثة بواسطة العزلات البكتيريا السريرية في نموذج الفئران.

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خلاصة

المقدمة :بروديجيوسين هي صبغة حيوية حمراء تفرز من بكتريا *Serratia marcescens* وبكتريا أخرى. تتكون من التركيب الكيميائي ثلاثي البيرول الخطي وتصنف على أنها مادة قلوية. أهتم الباحثون بصبغة البرودجيوسين نظرًا لقدرتها على أظهار مجموعة متنوعة من التأثيرات البيولوجية بما في ذلك الخصائص المضادة للبكتريا والفطريات، الملاريا، والسرطان.

الهدف: هدفت هذه الدراسة إلى تقييم مدى فعالية البرودجيوسين في علاج الأخماج السطحية الملوثة بالبكتريا المرضية المقاومة للمضادات الحيوية.

طرائق العمل: تم التحري عن الصبغة الحمراء الموجودة في الكتلة الحيوية لبكتريا *S. marcescens* من خلال دراسة تركيبها باستخدام القياس الطيفي المرئي للأشعة فوق البنفسجية وتحليل كروماتوغرافيا سائل عالي الأداء (HPLC). قيمت فعالية البروديجيوسين المنتجة من *Serratia marcescens* ضد بكتريا الزائفة الزنجارية والمكورات العنقودية الذهبية باستخدام طريقة الانتشار في وسط أكار مولر-هينتون وأستخدم نموذج الجرح في الفئران لأحداث الاصابة.

اعطى العلاج الموضعي بعد 4 ساعات من الإصابة وأستمر حتى تم شفاء الجرح بالكامل. **النتائج:** أظهرت الصبغة ذروة الامتصاص القصوى عند 535 نانومتر. كانت جميع السلالات التي تم اختبارها حساسية للصبغة وأنتجت مناطق تثبيط كبيرة ضد الزائفة الزنجارية (16.50 ± 1.36) والمكورات العنقودية الذهبية (20.83 ± 2.40). كما أظهرت المعاملات لمرهم الفازلين مع البرودجيوسين بتركيز 0.1% فعالية سريعة في التئام الجروح. اكتملت عملية الندبة، دون وجود أي جرح أو علامات احمرار. أعلى معدل شفاء ظهر عند 95 ملجم/مل بالنسبة للمكورات العنقودية الذهبية (0.003±0.003)تليها الزائفة الزنجارية (0.02±0.008).

الاستنتاج: بينت الدراسة أن البروديجيوسين بديل طبيعي بسبب خصائصه المضادة للبكتريا المسببة لأخماج الجلد السطحية.

الكلمات المفتاحية: البروديجيوسين، الصبغة البكتيرية، مضادات البكتريا، العدوى السطحية.

Introduction

Over the past few years, there has been a significant increase in antibiotic resistance, as reported by the World Health Organization WHO (1). Bacterial resistance is the capacity of a bacterium to resist or tolerate the impacts of antibiotics and refers to the capacity of a bacterium to resist the growth-inhibiting or lethal effects of an antimicrobial agent at doses that can be achieved in a clinical setting(2). At the same time, there has been a decrease in the rate at which new antibiotics are being developed. This issue is of significant scientific importance in both hospital and community settings (1). There has been a global attention in the demand for microbial pigments, this is because they are more biodegradable and environmentally friendly and have lower toxicity and allergic reactions compared to synthetic pigments (3,4). Prodigiosin (PG) belongs to a category of bioactive pigmented compounds that are produced through microbial fermentation. Prodigiosin, a crimson pigment, is mostly synthesized by strains of *Serratia marcescens* and other bacteria. It exhibits numerous potential therapeutic effects (5–7). It is a compound with a tripyrrole structure and chemical formula C20H25N30, which has a broad range of biological properties (8). Prodigiosin is widely recognized as a very promising therapeutic agent because of its

abilities to combat bacteria, viruses, malaria and inhibit tumor growth (9). It has antibacterial activities against a wide spectrum of Gram-positive and Gram-negative bacteria that grow in similar environments (10,11). Despite the availability of several systemic antibiotics, topically applied drugs are still commonly employed. Compared to systemic therapy, topical application offers several potential benefits. These include the ability to deliver high and sustained concentrations of medication directly to the infected area, requiring a lower quantity of antibiotic, improving patient compliance, reducing systemic side effects, and potentially decreasing the likelihood of antimicrobial resistance(12).Wound healing is a natural biological process that is influenced by numerous circumstances, ultimately determining its rate and quality. The overall state of the affected organism, the cause, location, and occurrence of infection, as well as hereditary factors that may or may not make one more susceptible to scarring disorders(13).

Collection of specimens

This study obtained a total of 134 clinical specimens collected from various sources, such as wounds, burns, urine, sputum, brain abscess, aspiration fluid, and urethral swabs. The specimens were collected from the laboratories at the Teaching Hospitals of Baghdad Medical City and Nasiriyah City, as well as the Biotechnology Department of the College of Science at the University of Baghdad. The specimens were gathered from November 2022 to April 2023.

Isolation and Identification of bacteria

All the clinical specimens were cultured on nutrient agar and MacConkey agar(14,15). *S. aureus* was identified by culturing on mannitol salt agar and coagulase tube method was used. The VITEK 2 automated microbial identification system, developed by Biomerieux in Marcy-Étoile, France, has been employed for the purpose of identifying isolated bacteria. The GN ID card was employed to identify *S. marcescens* and *P. aeruginosa*, whereas the AST-P580 ID card was used to identify *S. aureus.*

Prodigiosin extraction and purification:

The biomass was carefully removed from the Petri dish surface (16), and the pigment was extracted using acetone and ethyl acetate in an equal volume. The cell pellet was vortexed, centrifuged at 10,000 rpm for 15 minutes, and then repeatedly centrifuged to produce a white pellet (17). The UV-VIS spectrophotometer, which measures wavelengths between 300 and 700 nm, was used to analyze the liquid component (18). A powdered pigment was formed by allowing the supernatant to evaporate at room temperature for 24 hrs. after which it was collected in a sterile petri dish (19).

Prodigiosin analysis in the extracted sample

A CBM-20A apparatus (Shimadzu, Japan) with an SPD-20A UV-VIS detector and a NUCLEODUR 100-5 C8 column (4.6 × 250 mm, 5 µm particle size) from Mache Rey-Nagel were used to perform the high-performance liquid chromatography (HPLC) analysis. The total time of the analysis was 15 minutes. The injection volume used was 20 µL of a crude prodigiosin extract. The mobile phase was composed of a mixture of methanol, water, and acetonitrile (HPLC grade, Himedia, India) in a ratio of 73:20:7(20). The wavelength utilized for detection was 535 nm(6).

In vitro antibacterial susceptibility evaluation

Gram-positive (*S. aureus*) and Gam -negative (*P. aeruginosa*) bacteria that demonstrated resistance to the most antibiotics were selected. Concentrations of 25 mg/mL, 50 mg/mL, 75 mg/mL, and 95 mg/mL were

obtained (21) by dissolving prodigiosin powder in 95% methanol. The pathogenic bacteria were collected from the dishes and assessed for turbidity using the McFarland standard of 0.5 (22). After that, they were swabbed on Mueller-Hinton agar using the agar-well diffusion method (23–25). The petri dish lids were slightly lifted for a duration of 2 to 5 minutes to remove excessive surface moisture. Each well was filled with 100 µL of prodigiosin at the indicated concentrations. The experimental setup involved the use of gentamicin discs (10 µg) as the positive control, while 95% methanol was used as the negative control. The plates were placed in an incubator and maintained at a temperature of 37 °C for a duration of 24 hours. The measurement of the inhibitory zones' diameter was conducted.

In vivo therapeutic efficacy

In this study, twenty-four healthy mice weighing between 18 and 22 g were employed. Permission was given by the Ethical Community of Mustansiriyah University College of Pharmacy, in accordance with the Ethical Committee on Animal Care (file 26 on 21 April 2024). All the animals were housed in polycarbonate cages containing six animals and given food and water. The animals were divided into four groups at random, with six animals in each group. The murine model was previously dehaired and given local anesthesia before having incisions made on them. Using a scalpel, incision wounds measuring approximately 2 cm in length, width, and 0.5 cm in depth were made. Sterile cotton was used to clean the wounds before the ointment was applied. As an anesthetic, 2% xylocaine was employed at a dosage of 3 mg/kg (determined based on the animals' weights) (13). The chosen bacteria were evaluated for turbidity using the McFarland standard of 1(26). The animals were split up into four groups, with six animals in each group: The first group was shaved and disinfected with phosphate buffer saline, The second group was subjected to incision and disinfection with bacteria without any further treatment. In contrast, the third group underwent incision and disinfection with bacteria, followed by treatment with 50 mg of prodigiosin (1% prodigiosin in Vaseline) (13). Prodigiosin 95 mg (1% prodigiosin in Vaseline) was administered to the fourth group after they were incised and disinfected with bacteria. Treatment was started four hours later (27). According to development studies, at this point, there was only a 0.5 log10 difference between the initial inoculum and the bacterial counts in the wounds (27). Treatment was continued until the injury healed. Wounds cleaned with 70% ethanol should be treated once a day using a precise amount of ointment and daily measurements of the wounds were made until the course of treatment was finished. During the duration of the treatment, the dimensions of the wounds were measured, and their appearance (13).

Statistical analysis

The data was analyzed using SAS (Statistical Analysis, system - version 9.1). A two-way analysis of variance (ANOVA) was conducted to evaluate significant differences among means, considering the interaction between variables. Additionally, the least significant differences (LSD) method was used. A p-value less than 0.05 is regarded as statistically significant.

Results

Identification of bacterial isolates

A total of 45 isolates were found out of 134 samples. There were 10 isolates of *P. aeruginosa* isolates (22.2%), 17 isolates of *S. aureus* isolates (37.7%), and18 *S. marcescens* isolates (40%) table (1)

Table (1): The numbers and percentage of bacterial isolates

Bacterial isolates	Number of isolates	Percentage of isolates (%)	Chi-square value	P-value
P. aeruginosa	10	22.2	2.53	0.28NS
S. *aureus*	17	37.7		
S. marcescens	18	40		
total	45	100		

NS=non-significant

Detecting of bacteria

S. aureus

The colonies of *S. aureus* isolates were large, spherical, convex, shiny, and golden-yellow and were positive for coagulase test. The VITEK 2 device with the AST-P580 card produced positive results.

P. aeruginosa

The morphology of ten isolates were rod-shaped. Positive outcomes were obtained with the VITEK 2 device and the AST-N222 card.

S. marcescens

The morphological identification of *S. marcescens* isolates involved the creation of smooth, spherical colonies. Out of these isolates, only two were identified as pigment producers based on the formation of red color Figure (1). *S. marcescens* was successfully identified using the compact VITEK 2 device, GN card was used.

Figure (1): S. marcescens showed red pigment on MacConkey agar at a temperature of 28 °C for 24 hours.

Prodigiosin extraction

Using a UV-VIS spectrophotometer, prodigiosin showed a reddish color in an acidic solution that is easily detectable at 530 nm (Figure 2). The outcome was in line with earlier research findings (28); however, other studies revealed that the maximum

absorption occurred at 536 nm (11) and 535 nm (18) wavelengths.

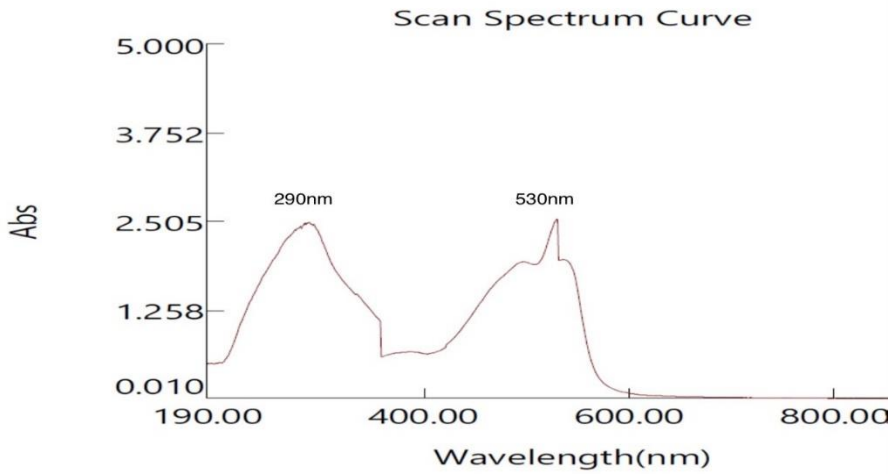


Figure (2): The absorbance profile of red prodigiosin of S. marcescens, obtained from crude extraction, measured at a wavelength of 530 nm.

Analysis using high-performance liquid chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) analysis was used to positively identify and confirm the presence of prodigiosin in the sample that was extracted.

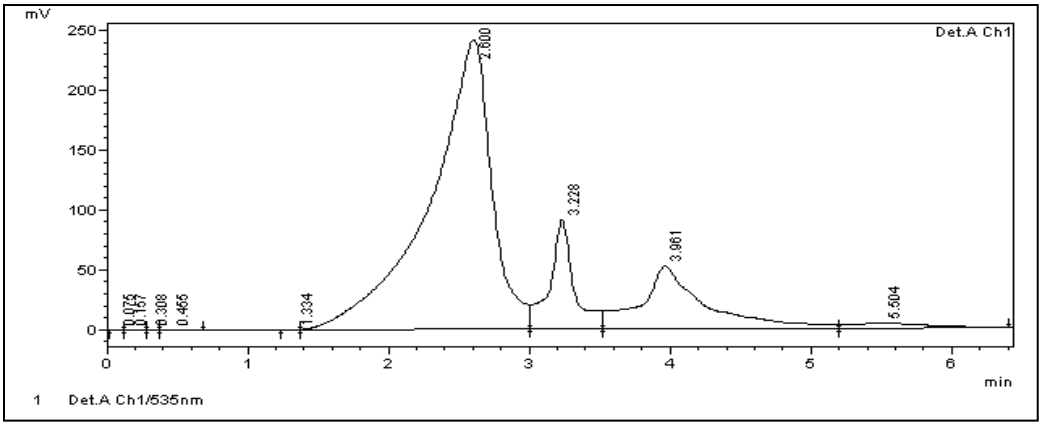


Figure (3): The identification of prodigiosin of S. marcescens in the crud extract, with a retention time of approximately 2.60 minutes, using HPLC at a wavelength of 535 nm.

The antimicrobial efficacy

Table (2) and figure 4,5 demonstrated that both tested bacteria; *P. aeruginosa* and *S. aureus* exhibited susceptibility to prodigiosin. The observed inhibitory zone had a high value for *S. aureus* (20.83±2.40 mm), with *P. aeruginosa* following closely behind at 16.50±1.36 mm. The negative control, consisting of methanol (95%), did not display a clearly defined zone. However,

the positive control of gentamicin demonstrated susceptibility to *P. aeruginosa* and *S. aureus.*

Table (2): Mean of zone of inhibition (mm) of prodigiosin against *P.aeruginosa* and *S. aureus*

Concentrations of prodigiosin and positive and negative control	*P. aeruginosa*	*S. aureus*
Gentamicin 10	B16.50±1.36a	A21.33±0.88a
Methanol 95%	A0.00±0.00c	A0.00±0.00d
Prodigiosin 25 (mg/ml)	A9.33±0.80b	A9.83±0.87c
Prodigiosin 50 (mg/ml)	A13.33±0.95a	A16.16±0.94b
Prodigiosin 75 (mg/ml)	A14.33±0.95a	A17.33±1.08b
Prodigiosin 95 (mg/ml)	B16.50±1.36a	A20.83±2.40a
LSD	3.21	

LSD: least significant difference
Means with a different small letter in the same column are significantly different (P<0.05)
Means with a different capital letter in the same row are significantly different (P<0.05)

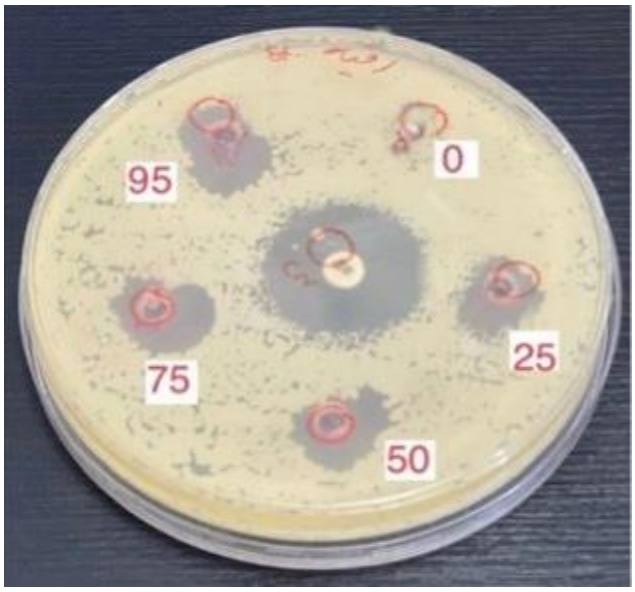


Figure (4): Effect of prodigiosin on *S. aureus* after incubation for 24 hr.

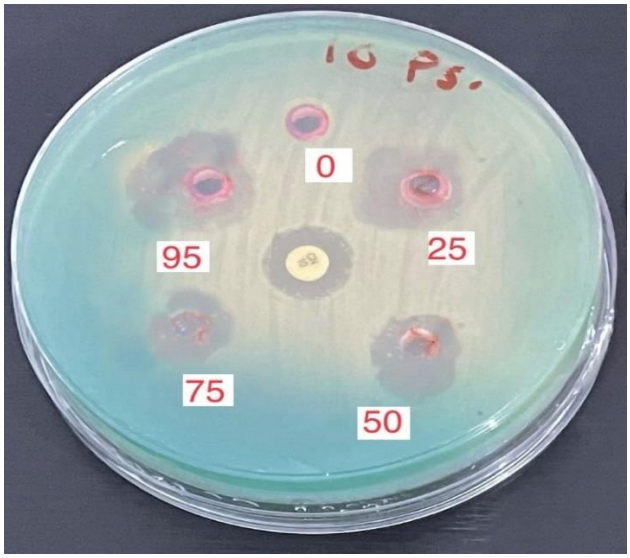


Figure (5): Effect of prodigiosin on *P. aeruginosa* after incubation for 24 hr.

The therapeutic effectiveness in vivo

The results shown in table (3) demonstrated prodigiosin's ability to heal wounds rapidly and effectively.

Table (3): The effectiveness of prodigiosin of *S. marcescens* on pathogenic bacteria in a mouse wound.

Groups	Days						
	1	2	3	4	5	6	7
Control	A3.70±0.11a	AB3.15±0.23a	B2.56±0.17a	BC1.98±0.27ab	CD1.53±0.20a	DE1.06±0.16b	E0.76±0.10a
Induced	A3.76±0.06a	AB3.60±0.14a	BC3.02±0.18a	CD2.54±0.21a	DE2.11±0.20a	EF1.88±0.22a	F1.29±0.14a
50mg/ml *p. aeruginosa*	A3.63±0.14a	B2.95±0.08b	B2.40±0.08a	C1.58±0.12bc	D1.04±0.32b	DE0.49±0.17c	E0.22±0.10b
50mg/ml *S. aureus*	A3.58±0.45a	A2.88±0.41b	B2.10±0.33b	B1.34±0.39bc	C0.91±0.57b	C0.33±0.19c	C0.13±0.09b
95mg/ml *p. aeruginosa*	A3.60±0.26a	B2.32±0.08b	C1.72±0.02b	C1.13±0.13c	D0.61±0.04c	D0.14±0.02c	D0.02±0.008b
95mg/ml *S. aureus*	A3.55±0.26a	B2.27±0.31c	BC1.56±0.56c	C0.82±0.02d	C0.33±0.002c	C0.09±0.003c	C0.003±0.003b
LSD	0.65						

Means with a different small letter in the same column are significantly different (P<0.05)

Means with a different capital letter in the same row are significantly different (P<0.05)

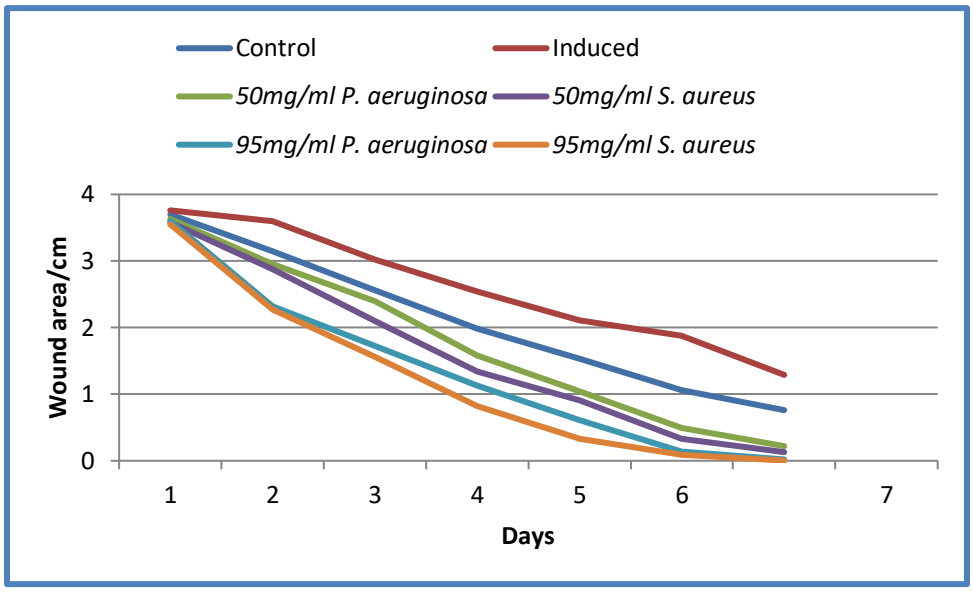
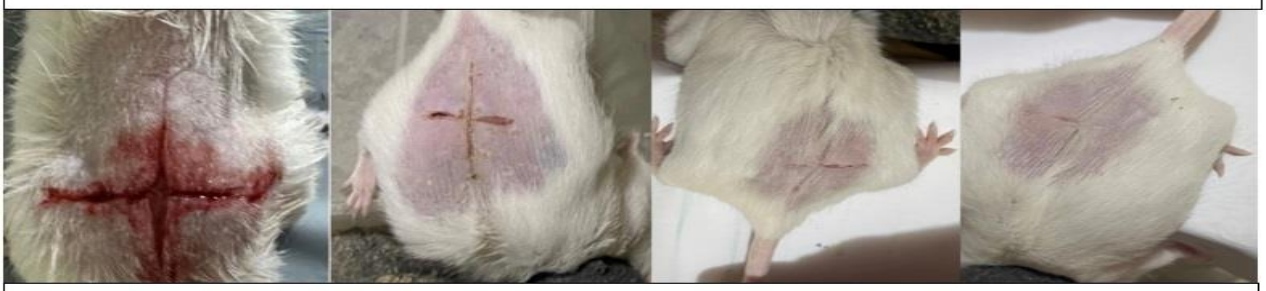


Figure (6) Wound-narrowing values of wound healing on mice (n=3)

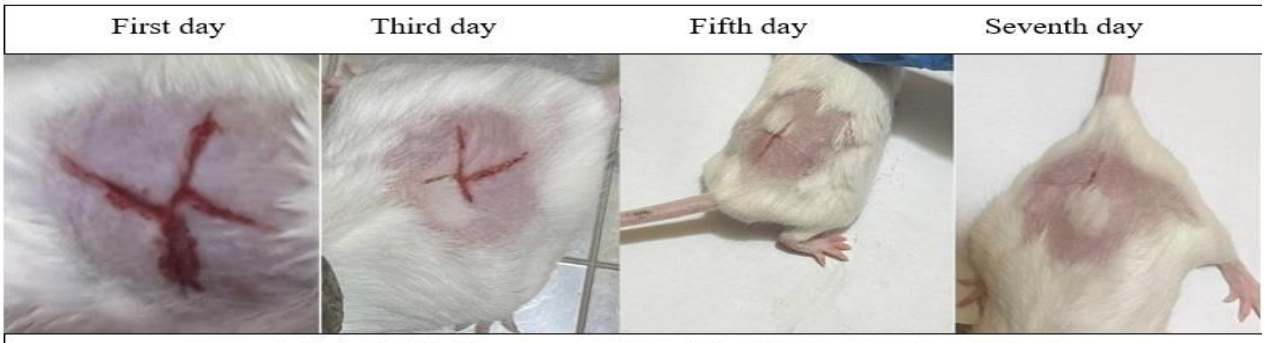
Results revealed that the differences among wound areas on the first day were not significant, while the differences among groups were significant ($P<0.05$) for all other days. In general, the highest wound area was detected in two groups (control and induced) from the 2nd day to the 7th day. Concerning the differences among days within each group, results showed a significant decrease ($P<0.05$) in all groups with different rates of decrease. The highest rate of decrease was shown at 95 mg/mL for *S. aureus,* followed by *P. aeruginosa figure (7,8).*

Group infected with *P. aeruginosa* and treated with 50 mg of prodigiosin.



Group infected with *P. aeruginosa* and treated with 95 mg of prodigiosin.

Figure (7): photographs show the rate of wound contraction and the extent of wound contraction several days after the excision and infection with *P. aeruginosa*.



Group infected with *S. aureus* and treated with 50 mg of prodigiosin.

Group infected with *S. aureus* and treated with 95 mg of prodigiosin.

Figure (8): photographs show the rate of wound contraction and the extent of wound contraction several days after the excision and infection with *S. aureus*.

Discussion

The prodigiosin pigment was extracted using organic solvents since a fraction of the pigment was attached to the bacterial cell wall, while a minor fraction was released externally. The present study utilized the technique of employing ethyl acetate and acetone, which is distinguished by its simplicity, efficiency, and capacity to acquire substantial amounts of the pigment (29). The pigment secreted into the culture medium was extracted using ethyl acetate, whereas the pigment from the cell remains was extracted using acetone. Acetone breaks the bacterial cell walls and releases the bound pigment into the medium(30). HPLC was employed because to its superior performance enhancement capabilities in comparison to alternative chromatography methods. High-performance liquid chromatography (HPLC) can detect and analyze small amounts of a chemical, making it a suitable method for identifying trace compounds. This was done by finding a peak at a wavelength of 535 nm. Prior studies supported the validity of this result (31). A peak was seen at approximately 2.6 minutes of retention duration, as depicted in (Figure 3). The observed retention duration in this investigation aligned with the retention time reported in other studies (11,18). The findings of this study clearly demonstrated that the *S. aureus* showed the largest zone of inhibition, while the *P. aeruginosa* revealed the smallest zone of inhibition. This is because the Gram-negative bacteria are different from those of Gram-positive bacteria, mostly due to the presence of an outer membrane. This barrier increases the resistance of bacteria by preventing the passage of hydrophobic compounds, such as prodigiosin(32). The lipopolysaccharide layer is believed to exhibit resistance to the infiltration of inhibitory chemicals, such as prodigiosin, into the cellular environment (33). The outer membrane of the bacterium functions as a defensive barrier that inhibits the entry of hydrophobic substances, such as prodigiosin, hence enhancing the bacteria's capacity to resist external stressors. The cellular membranes of Gram-positive bacteria can be penetrated by prodigiosin. Additionally, it exerted a negative impact on cellular growth by selectively targeting the DNA of the bacterial cell, leading to cellular suppression (34). The results supported the findings established by the researchers (35). Another research has indicated that disks with a concentration of 500 g/l of the prodigiosin have the potential to impede the growth of *S. aureus* (22±0.33mm) (10). A study demonstrated the antibacterial effects of prodigiosin on *P. aeruginosa* (6 mm) (33). The findings from this study indicated that the pigment, when utilized in vitro at a concentration of 95 mg/ml, demonstrated the most noticeable influence on pathogenic bacteria. In contrast, the effects observed were similar when concentrations of 50 mg/ml and 75 mg/ml were employed. Therefore, based on the in vitro findings, a concentration of 95 mg/ml was chosen for the in vivo experiment due to its substantial influence, while a concentration of 50 mg/ml was selected because it had an effect comparable to that of 75 mg/ml. Previous research conducted experiments on rats using crud prodigiosin and found that when topical prodigiosin was applied, complete healing of the skin occurred after 6 days(13). Another study treated skin infections in mice with a concentration of 95 mg/ml and demonstrated a potential inhibitory action against both Gram-positive and Gram-negative bacteria on the fifth day(36).The mouse model of skin wound infection serves as a representation of secondary skin infections that can arise after accidental injuries, surgical procedures, burns, or when an underlying skin illness is superimposed (27). The two groups (control and induced) had the greatest wound area, with each group demonstrating varying rates

of reduction. *S. aureus* had the most significant rate of decrease at a concentration of 95mg/ml, followed by *P. aeruginosa*. These findings demonstrated the efficacy of a well-established topical therapy in animals.

Conclusion:

HPLC demonstrated superior efficacy in detecting prodigiosin, as evidenced by the distinct peak observed at 535 nm. It has been observed that prodigiosin exhibited greater effectiveness against the Gram-positive bacterium *S. aureus* in comparison to the Gram-negative bacterium *P. aeruginosa*. prodigiosin has a rapid, powerful wound healing potential with a dose-dependent effect. It is promising as a natural antibacterial agent replacement for the synthetic drug.

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