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The Therapeutic Effect of Prodigiosin Pigment Produced From Serratia Marsenies Bacteria on Injure Wounds In Rats

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ABSTRACT
Serratia marcescens isolates were collected from urinary tract infections, and diagnosed phenotypically and biochemical tests. The diagnosis of the isolates was confirmed using Vitik2 device. To confirm the strain producing the pigment, the bacterial strains were examined using the PCR technique. The pigment was extracted using the organic solvent methanol, then confirmed using the TLC technique. Then the dry pigment was collected also absorbance was measure in spectrophotometer.
optimum conditions for pigment production were tested, which gave the highest production on B. H. I. media with a mixture of fatty acids and sucrose added, at a temperature of 28 C for a moving incubation while (150 / min) for 72 hours. At a pH of 7.
Tests of the pigment inhibitory effectiveness against pathogenic bacteria in the laboratory, and the pigment therapeutic effectiveness against wounds created in the body of the living organism, showed positive results compared to the antibiotics used in the experiments.

Keywords: PCR, Progesterone pigment, Antimicrobial, Serratia marcescens

INTRODUCTION

Prodigiosin pigment is one of the important secondary metabolites of Serratia marcescens bacteria. It is a red pigment that is insoluble in water, while it dissolves in organic solvents such as chloroform, methanol, acetone and ethyl acetate (Haddix and Shanks, 2018).
The chemical formula is methyl-3-amyl-6methoxyprodigiosin2. It is a linear three-ring pyrrole compound, consisting of the union of two compounds together: monopyrrole, which is a volatile compound, with dipyrone, which is a stable compound. This union produces the prodgiosinpigment with the chemical formula (C20H25N3O), which is a weak base due to the presence of nitrogen atoms in its structure. Its acid dissociation constant value is 7.2. This pigment is characterized by its biological and inhibit active against Gram positive and Gram negative pathogenic microorganisms. (Vu Trong Luong1 et.al 2021).
Several laboratory experiments have reported that this pigment not only that a considerable restrained impact on pathogenic microorganisms, like Pseudomonas aeruginosa, and Staph.aureus , but can inhibit fungi of the genus Epidermomyces , Trichoderma, and Microsporum. As for immunosuppressive activity, as well as the proliferative capacity of spleen cells in rats. (Haddix and Shanks, 2018).

Objectives of Research:
1- Extraction and purification of prodigiosin pigment through secondary metabolism of Serratia marcescens bacteria isolated from urinary tract infections.
2- Testing the inhibitory effect of the pigment in vitro by comparing the inhibitory effect of the pigment with antibiotics.

3- Testing the therapeutic effect of the pigment against wounds created in the body of the living organism in vivo and contaminated with pathogenic bacteria.

MATERIALS AND METHODS

Collection of bacterial isolates

Collection of bacterial isolates from clinical sources of patients with urinary tract infection and wound infection, hospitalized and arriving at the Medical City / Baghdad, after confirming and diagnosing them using rapid phenotyping and their ability to produce pigment, as well as the characterization of the Vitek device and PCR technology.(David J ,et.al ,2022) .

The identified isolates were frozen in glycerol for use in laboratory experiments. Testing the optimum conditions for pigment production:

Media

Nutrient agar media was prepared, and after autoclaving, allowed is cool to 45°C, then poured into sterile dishes, and used for colony morphology and stain production.

Brain Heart infusion Broth media

This media was prepared for broth Brain Heart infusion media, and after autoclaving at 121°C and 15 lb/in2 for 15 minutes, was allowed to cool to 45°C, then poured into sterile dishes, and used for colony morphology and stain production.

Brain Heart infusion Broth media + sucrose

This media was prepared is Broth Brain Heart infusion media and after autoclaving at 121°C and 15 lb /in2 for 15 minutes, 2% sucrose by volume of media was added, allowed to cool to 45°C, then poured into sterile dishes, and used to detect colony morphology and stain production.

Incubation period

Prepared 50 ml of the optimal media for the production of pro pigment was prepared, as the media was inoculated with Serratia marcescens bacteria growing on B.H.i. sucrose media at a temperature of 28 °C for 72 hours, with an inoculum ratio of 5% of the volume of the media. Production was estimated at different incubation periods including (24 , 48 , 72 , 96) hours, as the optimal incubate period was determined. After assay in absorbance while a wavelength of 530nm, in spectrophotometer, and ensuring the purity of the pigment.

Temperature

Prepared 50 ml of the optimal media for the production of pro pigment was prepared, as the media was inoculated with the bacteria. Serratia marcescens growing on the B.H.i sucrose media in temperature 28 °C for 72 hours, with an inoculum ratio of 5% of the volume of the media, then the media was incubated at different temperatures including 25, 28, 30, 37, 40, 45 °C for 72 hours, as the optimal temperature for production is determine, after assay on absorbance at a wavelength of 530nm, in the Spectrophotometer, and ensuring the purity of the pigment.

PH

Prepared 50 ml of the optimum media for producing pro pigment was prepared, then the media was inoculated with Serratia marcescens bacteria growing on B.H.i. sucrose media at 28°C for 72 hours, with an inoculum ratio of 5% of the volume of the media. Different concentrations of pH were adopted, including 3, 4, 5, 6, 7, 8, 9, 10. The media was incubate at 28°C to 72 hours, after optimum pH for pigment production was determined.

Type of incubation

After establishing the optimal conditions for the production of pro pigment, which included the type of the culture media, the incubation period, the temperature and the pH value, two types of static incubation were used - static and moving Shaker, using the moving incubator Shaker. The flask containing 50 ml of B.H.i. sucrose media was fixed in the moving incubator at different speeds 0, 50, 100, 150, 200, 250 shakes / minute, after which the optimal type of incubation for the production of the pigment was determined.

Extraction of pigment

The pigment was extracted using a centrifuge at 10,000 rpm for 15 minutes for the B.H.I broth culture media on which Serratia marcescens bacteria were grown.

The supernatant was ignored and the precipitate was taken and mixed with methanol at a concentration of 95%, then the precipitate was centrifuged again, after which the supernatant was placed in test tubes after ignoring the precipitate using a separation tube, and a drop of H2SO4 was added to the first tube to turn the solution pink or

red, while a drop of ammonia was added to the second tube to turn the solution yellow, and this is a color detection of the pigment.
The extract was placed in watch glasses and left in the incubator at a temperature of 45 C to obtain the dry pigment powder.

Purification of dye using TLC technique

Add acetic acid - ethyl ether - Hexane according to the proportions respectively 70 - 29 - 1%, in a 900 ml glass beaker, in which 100 ml of the above mixture is placed, the pigment is loaded on the TLC plate by placing drops of the pigment at a distance of 2 cm from the bottom, then the plate is placed in the mixture and covered by placing a cover on the beaker, waiting for the pigment to spread upwards, then measuring the RF. (Hurst, M. R. H. et al ,2018) .

Measurement of absorbance pigment

Absorb of pigment were measure on spectrophotometer whit wavelength 350 to 800nm to determine the absorbance of the pigment.
The bacterial cell culture showed absorbance at 620nm. For pigment absorbances, the broth were centrifuged using methanol and supernatant taken for measure of absorbances at 534nm. The following formula is used for estimation of prodigiosin.
Prodigiosin (unit per cells) = [OD534- (1. 381× OD620)] ×100
OD620
Where, OD534= Absorbance of pigment
OD620= Absorbance of bacteria cells culture
1. 381= Constant
Purification of prodigiosin pigment can be carried out using thin layer chromatographic technique (TLC).The separation of pigment using TLC plate which is coated with silica gel. (Guo, Z, et al, 2020).

Efficacy of prodgiosenpigment in inhibiting the growth of pathogenic bacteria

The sensitivity of pathogenic bacterial isolates to 3 different concentrations of prodgiosenpigment was tested. The method of spreading in agar by drilling was followed according to Bhagwat, Ashlesha, and Unnati Padalia.(2020) , as the method included transferring 3-1 pure colony test tubes 5 ml of n.s. solution, to form a bacterial suspension and compare the growth with the standard McFarland tube after comparing the turbidity of the growth formed with the turbidity of the standard McFarland turbidity constant solution, which gives a cell count of quasi 1.5 x (10)^8 CFU / ml, then transferring 0.1 ml of the bacterial suspension and spreading it homogeneously on the solid Muller Hinton agar media. The plates were left after spreading for 15-20 minutes to dry their surfaces, then making 4 holes with equal distances in the solid Muller Hinton agar media with a diameter of 5 mm using a cork drill, and 0.2 One milliliter of prodgiosenpigment solution according to the three concentrations specified in the study and a hole in which the antibiotic Amikacin was placed as a comparison point, then plates was incubate at 37 °C for 24 hours , after which the inhibition zones around the holes were measured in millimeters, using a regular ruler, and the inhibition zones were compared for each concentration for each of the pathogenic bacterial isolates.

Determining the effectiveness of prodgiosenpigment in wound healing:

The experiment were conduct in the animal house, affiliated to the University of Mosul / College of Veterinary Medicine, where 20 rats were used in this study, approximately 50 to 60 days old, with weights (200 - 350 grams), as they were placed in cages, and they were taken care of cleaning and sterilizing with disinfectants , and all animals were putting to the same situation of light and temperature , as the temperature was 2 ± 25 C̊ and the intensity of light / darkness 12/12 hours, and the animals were 35% wheat, 33% yellow corn, 20% soybeans, 10% protein, and 1 % dried milk with the addition of 1% of each of vitamins and minerals Food and water were provided to them add libitum throughout the 14-day experiment.

Experimental design

Prepare concentrations of the pigment extracted and purified from Serratia marcescensbacteria at 3 concentrations, by weighing 2.4g of dry pigment and dissolving on 10 ml from methanol "(for a concentration of 100%), then take 1 mill a first concentration after that addition it to 10 ml of methanol to become the consistency (50%), then take 1 ml of the second concentration and insert it 10 ml of methanol to a concentration of (25%)".
To study the effectiveness of prodgiosenpigment in treating artificial wounds in rats' skin, the animals were section into five groups:
G1: 4 animals treated with pigment at a concentration of 100%
G2: 4 animals treated with pigment at a concentration of 50%
G3: 4 animals treated with pigment at a concentration of 25%

G4: 3 animals treated with the antibiotic CEFALEXIN 100mg (positive control)
G5: 3 animals not treated with any substance (negative control).
The experiment was conducted according to what was stated inAbdhul, K, K Sakthinarayanan, and M Murugan (2018) .with modification, as the hair was removed from the back area of the animal's body, and wounds were made 7 cm long and 5 mm deep in both areas using a sterile medical scalpel. The animals were left until the next day, then an infection with streptocouccousepidermes bacteria was created, using sterile cotton swabs, and left until the next day to allow the bacteria to grow on the wounds. The treatment in the above groups was by spraying the pigment on the affected area according to each group, once a day, with recording the observations and signs appearing on the affected area daily, during the period of the experiment. Then the tissue sections of the infection were studied so result of compared to control.

RESULTS AND DISCUSSION

Collection and Identification of Bacteria Isolates

The bacteria isolated from the urinary tract were identified as belonging to the genus Serratia marcescens by different biochemical tests. Other characteristics include the shape and type of colonies growing on different growth media, negative Gram reaction, production of prodigiosin stain, in addition to their fermentation of lactose on MacConkey agar media

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Fig 1: colonies of Serratia marcescens

Diagnosis Bacteria Vitek 2 system

Bacterial isolates isolated from urinary tract infections and from the skin were diagnosed according to the type, using the Vitek 2 system, using the card for Gram-negative bacteria (diagnosis appendix). Diagnosis with the Vitek 2 system is a complementary step to confirm the results of biochemical tests due to its accurate diagnosis of the type. Isolates were selected from the Serratia marcescens bacteria, which were diagnosed by traditional biochemical methods, and the diagnosis was confirmed by this system. The results of this test showed that all bacterial isolates tested confirmed their affiliation to Serratia marcescens bacteria and Staphylococcusepidermis. Lapenda, J C et al (2015) confirmed that the diagnosis of bacterial isolates using the Vitek device confirms the results of the initial diagnosis with a high percentage of up to 98%.

Molecular Identification

DNA uproote on a B. H. I. broth culture modification of 16 S r RNA gene were performed using the universal manuals for bacteria 27 f (5′-TGCCTGGAAAGCGGCGATGG-3′) and 1492 r(
5′-CGCCAGCTCGTCGTTGTGGT-3′) the result was compared to 16 S r RNA gene sequences from the Gen Bank

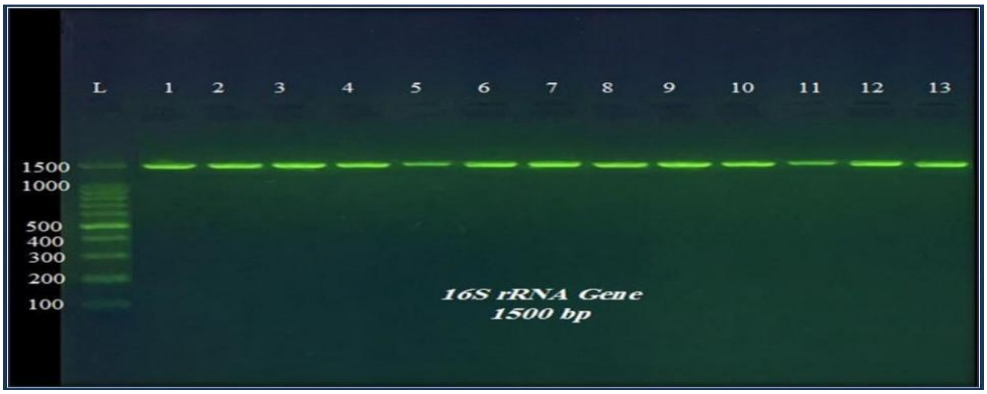

Fig 2: PCR for gene Serratia marcescens

Optimal Conditions

Media

A results showed that the highest pigment production was in B.H.I sucrose media, as the absorbance value in this media reached 2.4. These results were consistent with what was mentioned by Pore et al (2016) in his study on testing the optimal conditions for the production of Prodigosenpigment from Serratia marcescens bacterial isolates, while the absorbance value of B.H.I broth media was less than the absorbance of B.H.I sucrose media, as it reached 1.74. As for the absorbance of Nutrient broth media, it was the lowest possible, as it reached 1.31. After ensuring the purity of the pigment, B.H.I sucrose media was relied upon as the optimal media for the production of Prodigiosenpigment, as shown in Figure (3).

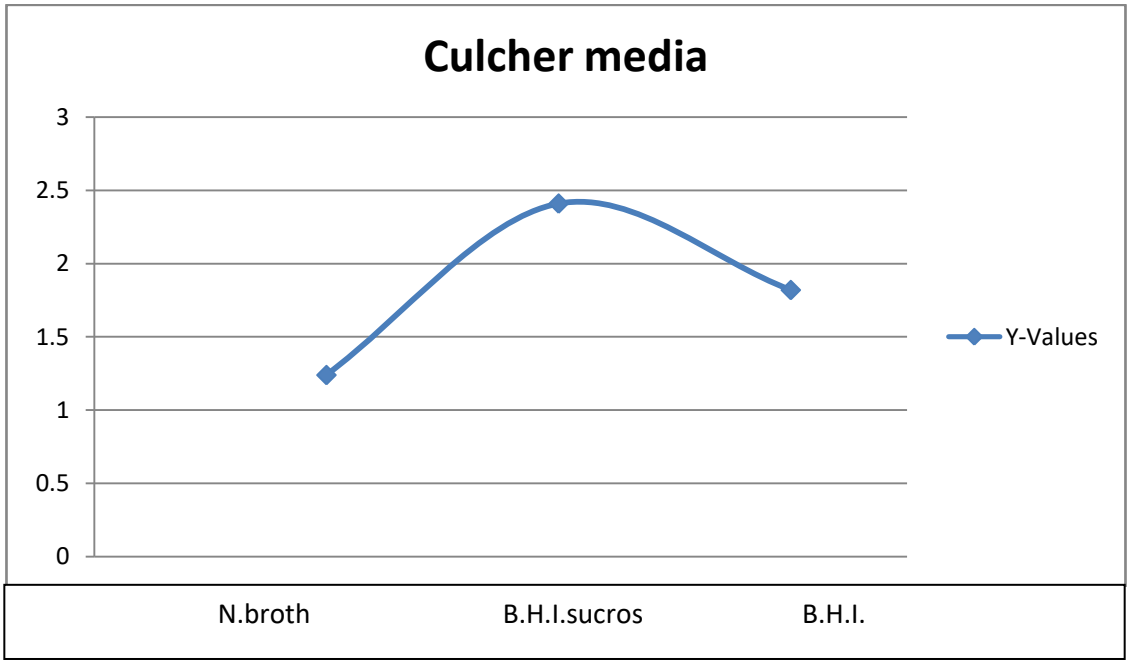

Fig 3: optimal media

Incubation period

The results in this research agreed with the results reached by Rokade et al (2017). In a study of the production of prodigosinpigment from Serratia marcescens bacteria and the conditions affecting production, confirmed that the highest percentage of pigment production appeared after 72 hours of incubation in the B.H.I sucrose medium, where it was observed that the color of the total medium changed to red, and after 96 hours of incubation, the pigment production decreased and the absorbance rate of the growth media.

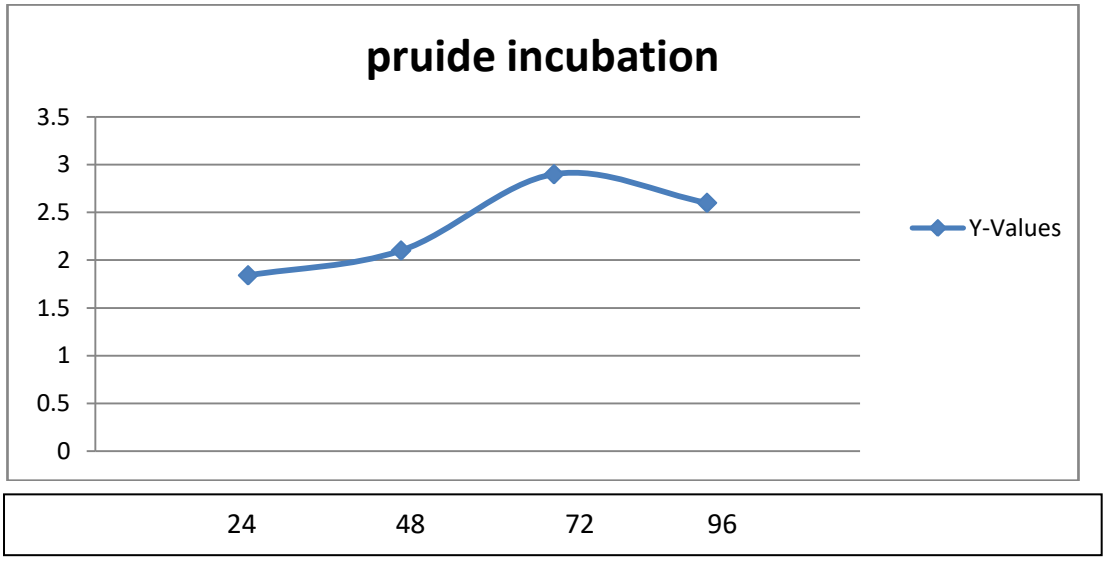

Fig 4:optimal Incubation period

Temperature

After establishing the optimal culture media and the optimal incubation period, the results shown in Figure (5) showed that the temperature of 28°C is the optimal temperature for pigment production.

These results were consistent with what was reported Vijayalakshmi et al(2016) in his study on the effect of temperature on the production of prodigiosenpigment, as the highest production was achieved at a temperature of 28°C.

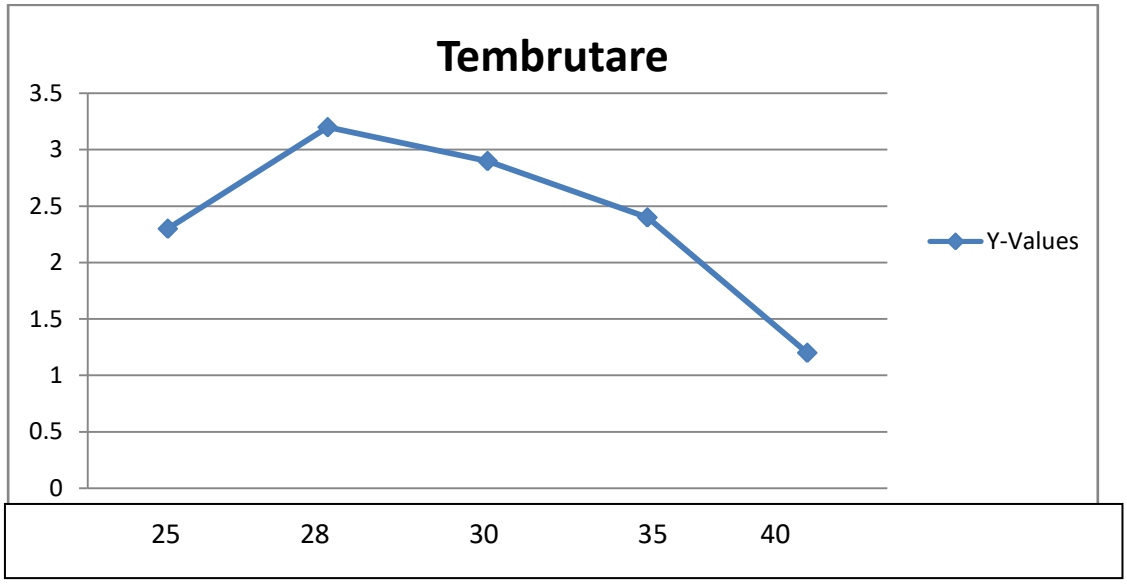

Fig 5: optimal temperature

PH value

The results shown in the figure (6) showed that the optimal pH for the production of prodgiosen pigment is 7stated that the pH value in the bacterial growth environment changes the consumption and production of energy that is important for performing all the vital functions of the bacteria, the changes that occur in the production or inhibition of protein production, or the change in the value of oxidation and reduction reactions inside the cell.

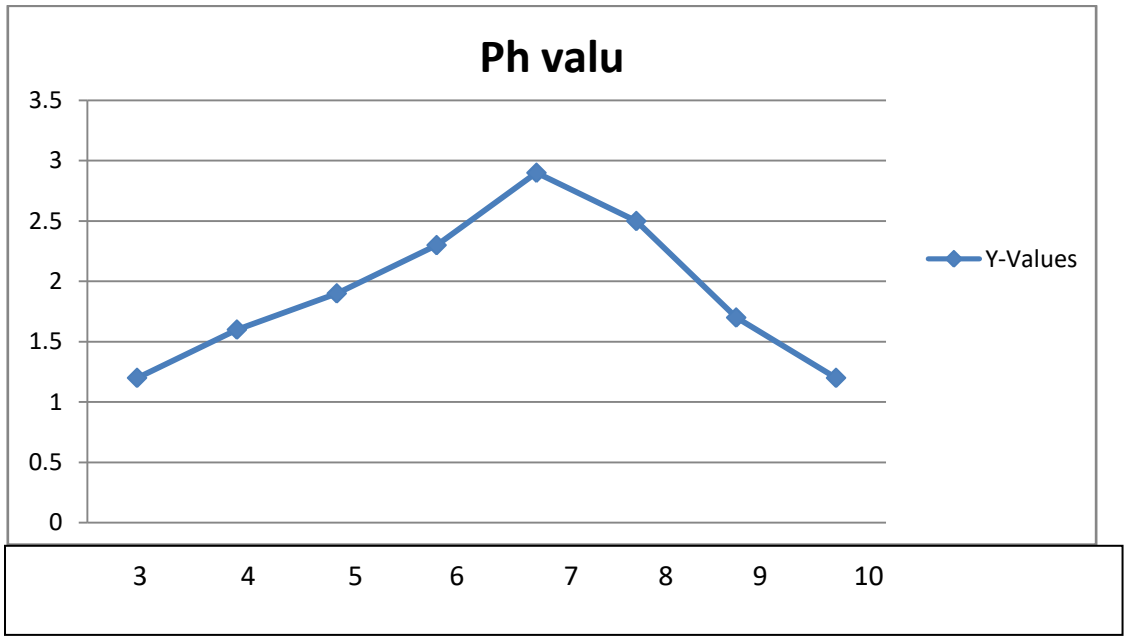

Fig 6: optimal pH

Type of incubation

The results showed that the highest production of prodgiosen pigment was at a speed of 150 vibrations/min. The highest production of pigment was 2.9 mg/L, while the production of pigment was less the lower the vibration speed of the incubator. As shown in the figure(7) These results were consistent with the results of the study of incubation and vibration speed on the production of prodgiosen pigment, as the highest production of pigment was reached at a vibration speed of 150. The importance of moving incubation lies in keeping the bacterial cells moving in the medium and homogeneous with all its components, unlike static incubation, which leads to the cells remaining stable at the bottom of the medium under the influence of gravity, which leads to the depletion of the components of the surrounding medium only. As mentioned Fantinelet al (2018). The high speed of the incubation movement leads to the formation of foam in the growth media, which hinders the diffusion of oxygen necessary for the growth of bacterial cells in the nutrient media.

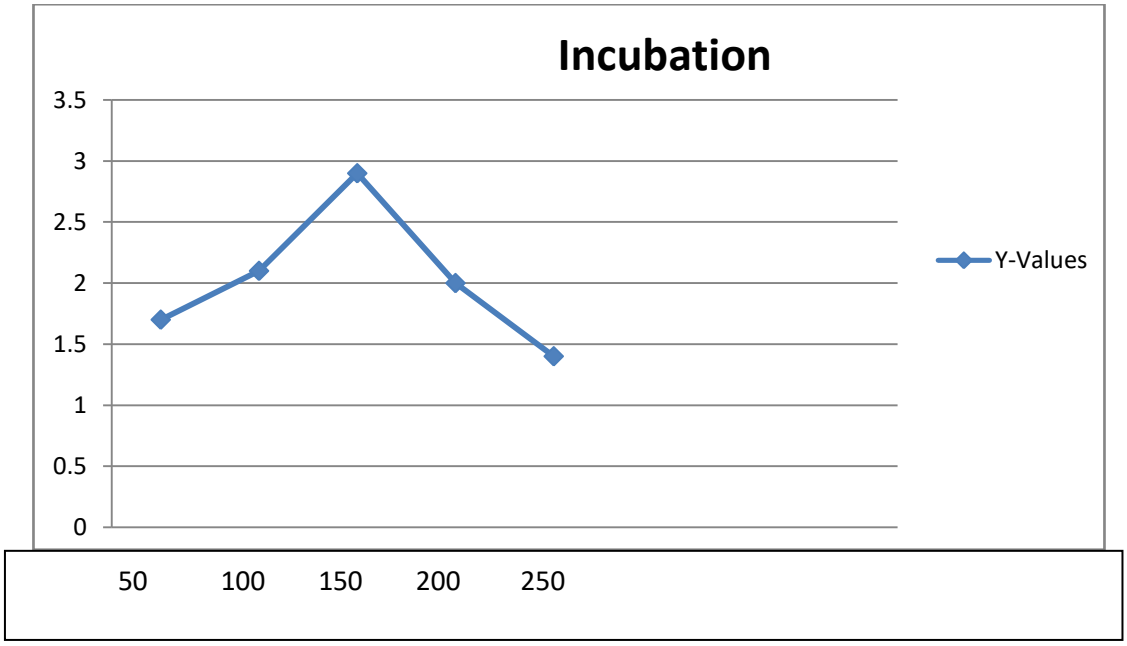

Fig 7: Type of incubation

Extraction of pigment

Presumption test was accomplished for the confirmation of prodigiosin pigment (N G Ramesh Babu et al, 2019). Using different solvents such as 95% methanol, chloroform, ethanol and ethyl acetate the centrifugation of broth was achieved. The pellet was discarded and supernatant was tested against alkaline and acidic conditions. Pink color in acid condition and yellow color in alkalizes condition confirmed a positive presumptive assay to red pigment prodigiosin. "Addition, the spectrophotometric analysis showed the peak of maximum absorbance at 533 nm (Figure8), while the TLC revealed an R.f = 0.63, corresponding to prodigiosin (Xia et al., 2016). The main absorption bands identified in the FTIR spectra of the red pigment are summarized In Table 2, and they are in accordance to the prodigiosin peaks reported in previous studies (Ramesh Babu et al, 2019).

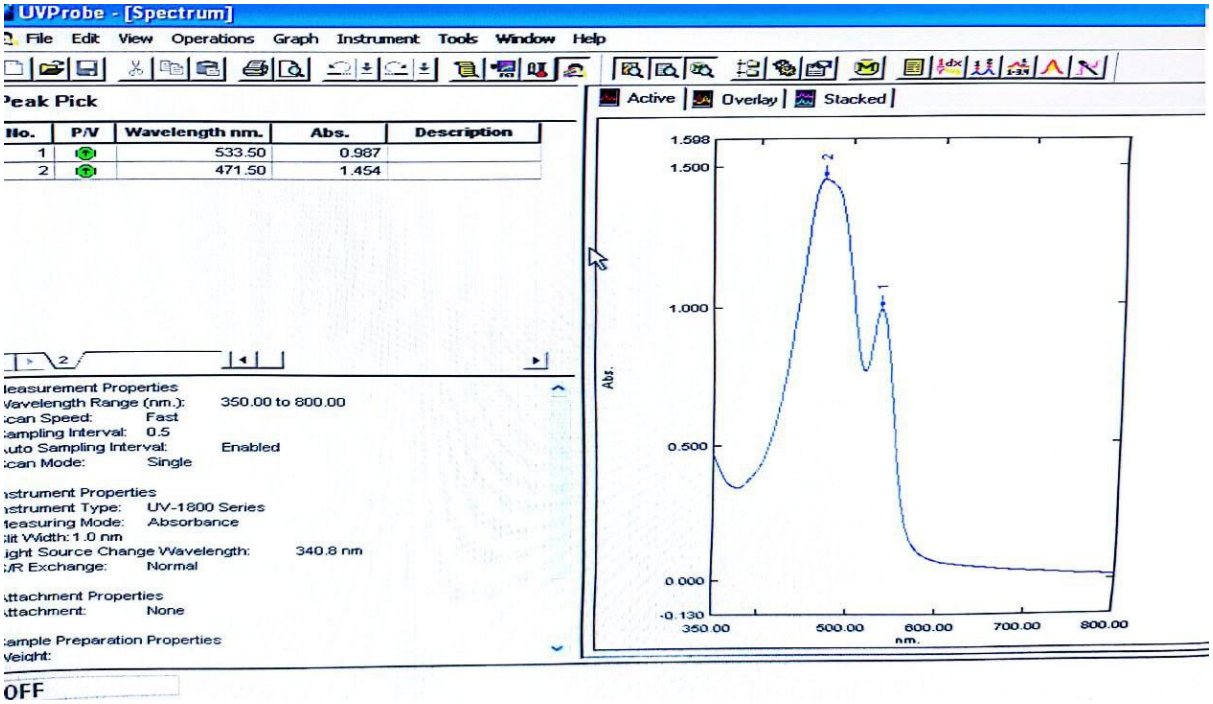

Fig 8: spectrophotometric analysis pigment

Purification of prodigiosin by TLC

Red color were anatomized by TLC using admixture of n- hexane ethyl acetate at (31; v/ v). A single red band with R.f value of 0. 63 was attained is analogous to that reported for Serratia marcescens

The color uprooted from Serratia marcescens gave an R f value of 0.63 in Thin Subcase Chromatography (TLC). The R f value of the uprooted color from Serratia marcescens KH1R was 0.64 – 0.96 Mohammed et al. reported that the R f value of prodigiosin was 0.73. (Hurst, et al, 2018). reported that the R f value of prodigiosin from Serratia marcescens insulated from rhizosphere soil samples in Salem and Namakwa sections, Tamil Nadu, India, was 0.78. This indicates that although the microbes are of the same species of S. marcescens, the R f value of colors produced by different strains within the same species shows variation.

The effectiveness pigment against pathogenic bacteria:

Effect for concentration pigment on antimicrobial activity was further studied, and the results are. The diameter inhibition zone was recorded in each case. It is evident that with increasing concentration of pigment, the zone of inhibition increased almost linearly. From the clear zone of inhibition obtained, it is apparent that pigment are bactericidal in nature and not bacteriostatic. (Hurst, et al, 2018)

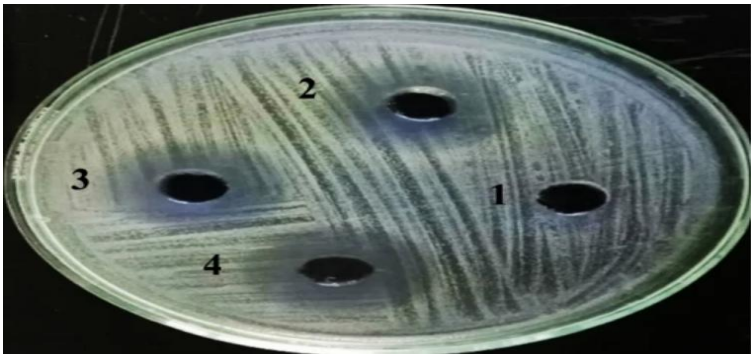

Fig 9: Inhibit zone for different constriction prodigiosin pigment
(1) Amoxicillin (2) 25 % pigment (3) 50 % pigment (4) 100 % pigment

Determining the effectiveness of pigment in wound healing

Prodigiosin pigment has antibacterial activity against many pathogenic bacterial strains that are resistant to most antibiotics, and can be used topically.

Effect is prodigiosin pigment in wound infections also their treatment, there was an urgent need for animal systems. Therefore, rats were chosen to study the effectiveness of prodigiosin pigment in inhibiting bacterial growth and the occurrence of inflammation at the site of injury and the duration of healing of those wounds compared to the negative and positive control groups under study Figure (10), as experimental wounds were created in the skin of rats and S.epidermic bacteria were chosen to contaminate the wounds, and from Table (1) the most prominent signs appearing on wound infections during the treatment period are clear.

Table 1: shows the most prominent signs appearing on the wound area of the skin of rats during the treatment period

No.	group	Healing time (day)	Signs appearing during the treatment period
1	100%prodigiosin dye treatment	5	Redness of the tissue in the area of injury and healing without a trace
2	Treatment with prodigiosin dye 50%	7	Redness, swelling and healing without scarring
3	Treatment with prodigiosin 25% dye	9	Redness and swelling of the tissue with healing and remaining scar
4	Positive control of CEFALEXIN	12	Redness and necrosis of the tissue, simple healing with scarring
5	negative control	14	tissue redness and necrosis

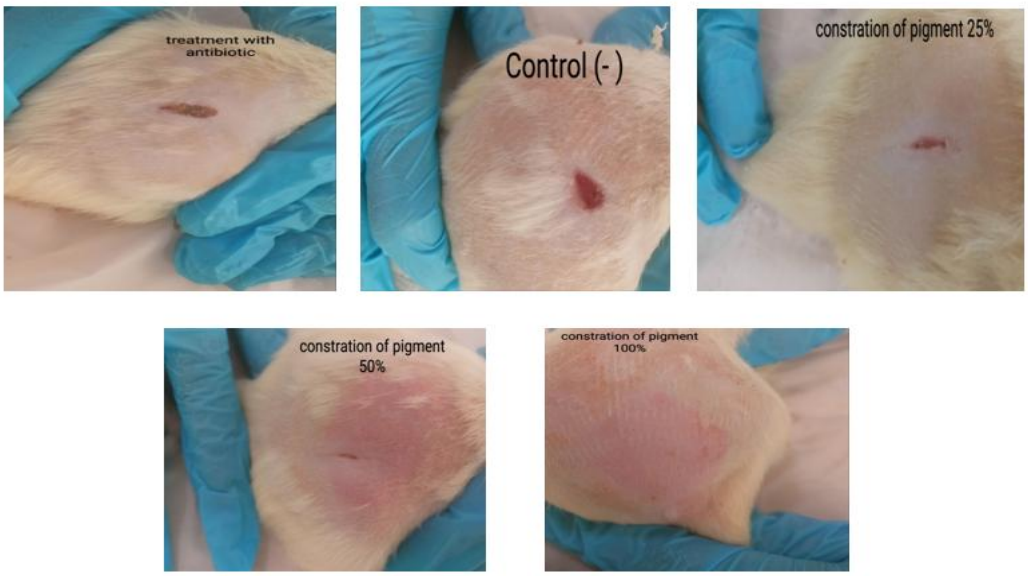

Fig 10: stage of healthy experimental wounds were created in the skin of rats.

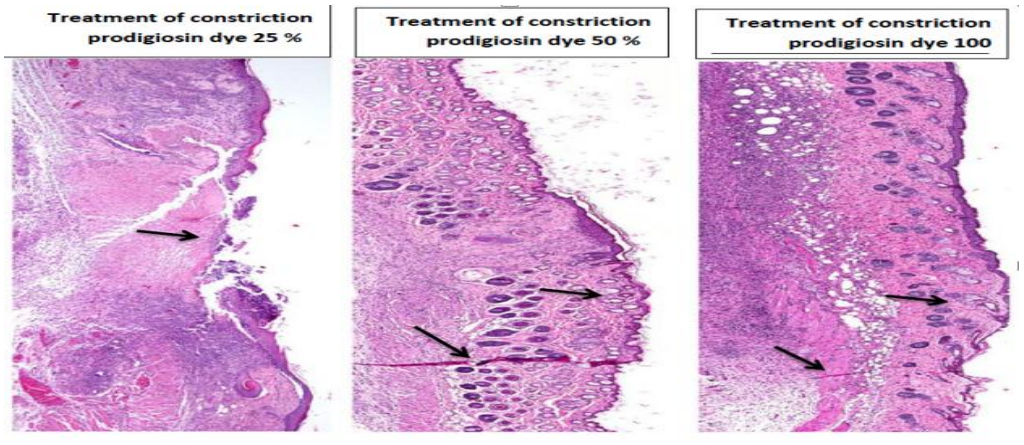

Fig 11: histology of rat skin wounds treated with prodigiosin pigment at three different concentions .

From the above table and the tissue sections of the rats' skin, and the wound injury images, it is clear that the most affected group was the first group, as the healing period and wound recovery were days due to the use of a high concentration of prodigiosin pigment of 2.4 g mg/ml at a concentration of 100%, as this group has a high inhibitory effect against S. epidermic bacteria and its toxic products that contaminate the wound area, while the wound treatment period increased in the second group because the pigment concentration was 50%, which led to a decrease in the inhibitory effect of the pigment and a decrease in its spread in the wound area. As for the third group, the wound healed with the effect remaining after the healing period, and this confirms the spread of metabolic products in the wound area before limiting their spread and inhibiting them due to the low concentration of the pigment in this group, which led to healing at a late time with the effect remaining. As for the fourth group, the effectiveness of the antibiotic CEFALEXIN was low against the virulence of S. epidermic bacteria, which resulted in additional effects on the stages of wound healing, which is the occurrence of necrosis with inflammation of the wound area, due to the bacteria's resistance to the effectiveness of the antibiotic and its low effectiveness in limiting the spread of bacteria in the area of infection, while the fifth group took the longest period to heal the wound due to the absence of any antibiotic or pigment in that group to affect and inhibit the bacteria contaminating the wound.

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