

**ANTIMICROBIAL AND ANTI ADHESIVE PROPERTIES OF
PRODIGIOSIN EXTRACTED FROM *SERRATIA MARCESCENS*
AGAINST BACTERIA ISOLATED FROM PATIENTS WITH
LEUKEMIA.**

Dr. Taif H. Hussan* and Sawsan H. Authaman

Department of Biology, College of Science, Al-Mustansiriya University, Baghdad-Iraq.

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***Correspondence for
Author**

Dr. Taif H. Hussan

Department of Biology,
College of Science, Al-
Mustansiriya University,
Baghdad-Iraq.

ABSTRACT

In this study, Prodigiosin extracted from *Serratia marcescens*. The optimum condition of pigment production including growing at different temperature for different incubation periods of times were studied. Aerobic condition for 72 hours at 30 C° was fitting to Prodigiosin production. Additionally, Prodigiosin was extracted by Chloroform and methanol and purified by acid precipitation and using Thin layer Chromatography method. Prodigiosin activity were studied, antimicrobial, anti adhesive and antibiofilm activities were evaluated against some cancer infection causative bacteria including Gram positive and Gram negative types , isolated from blood samples of Iraqi patients suffering from leukemia. Prodigiosin showed inhibitory

effect against leukemia causative bacteria at concentration (0.2) mg/ml against all these bacteria and showed inhibitory effect on adherence and biofilm formation of these bacteria, *E. coli* more sensitive to Prodigiosin, when gave the highest inhibitory percentage (46.5) % followed by *Pantoea sp.* (37.3) %, while *A. baumannii* was more resist (inhibitory percentage 2.7%).

KEYWORDS: Prodigiosin, Serratia, Antimicrobial, Anti adhesive, Leukemia.

INTRODUCTION

Serrtia are members of enterobacteriaceae.^[1] gram negative bacteria, motile, non spore former, bacilli shape with rounded end which their length ranged from 0.9-2Mm and their diameter from 0.5-0.8Mm. catalase positive but oxidase negative.^[2] *S. marcescens* is indole-negative,

lysine decarboxylase and ornithine decarboxylase-positive. Acid is typically produced only from sucrose and D-sorbitol and not from arabinose, L-rhamnose, D-xylose, cellobiose or D-arabitol.^[3] prodigiosin red pigment that bound to the cell membrane of bacterial cell.^[4,5] it's not diffusible in the medium, not dissolve in water but dissolve in alcohol compound and some of organic solvent, ex:- (chloroform), (bromoform), (benzene), (acetone), (Di methyl sulfoxide DMSO).^[6]

Bacteria are remarkable decision makers that are capable of responding to a multitude of environmental challenges through changes in gene expression.^[7] Many Gram-negative bacteria form microbial communities denoted as biofilms, which confer tolerance to environmental stress and bactericides.^[8] Gram-negative bacteria were responsible for only 27% of nosocomial bloodstream infections in the United States.^[9] In a person with leukemia, the bone marrow produces abnormal white blood cells that are called leukemia cells and leukemic blast cells. The abnormal cells can't produce normal white blood cells. Leukemia cells divide to produce copies of themselves. The copies divide again and again, producing more and more leukemia cells. Unlike normal blood cells, leukemia cells don't die when they become old or damaged. Because they don't die, leukemia cells can build up and crowd out normal blood cells. The low level of normal blood cells can make it harder for the body to get oxygen to the tissues, control bleeding, or fight infections. Also, leukemia cells can spread to other organs, such as the lymph nodes, spleen, and brain. An estimated 48,610 new cases of leukemia are expected in 2013. Leukemia is a cancer of the bone marrow and blood and is classified into four main groups according to cell type and rate of growth: acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myeloid (AML), and chronic myeloid (CML). Almost 90% of leukemia cases are diagnosed in adults 20 years of age and older, among whom the most common types are CLL(38%) and AML (30%). Among children and teens, ALL is most common, accounting for 75% of leukemia cases, From 2005 to 2009, overall leukemia incidence rates increased slightly by 0.4% per year.^[10]

AIMS OF THE STUDY

The aims of this study was to determine the antibacterial and anti adhesive properties of Prodigiosin produced by *S. marcescens* against some bacteria causing leukemia infections in Iraqi patients.

MATERIALS AND METHODS

1- Microorganisms

Isolates of *Serratia marcescens* were collected from different clinical samples of Iraqi patients in Baghdad hospitals, then identified throughout cultural, microscopical and biochemical test according to.^[11] The isolates were used to extract Prodigiosin pigment and test the anti bacterial and anti adhesive properties of the Prodigiosin produced by *S. marcescens* including Gram positive and negative isolates. These isolates were isolated from blood samples (69 samples) of Iraqi patients suffering from leukemia, then identified throughout cultural, microscopical, biochemical tests according to.^[11] Also Ready-made strips of API-20E system was used for the bacterial identification and definitive identification was done using Vitek 2 system compact (Bio Merieux, 2010).

2- Selection the best isolate for Prodigiosin production.

S. marcescens isolates were activated by picked 4-5 colonies of each isolate from original culture and suspended into a test tube containing 5ml of brain heart infusion broth, The turbidity was adjusted to obtain approximately 0.75 at 620 nm. Using spectrophotometer. All tubes were incubated at 37c° for 72 hrs., The optical density was recorded at 499 nm and at 620 nm. The amount of pigment was calculated using the following equation:-

$$\text{Prodigiosin U/Cell} = \frac{[\text{O. D}_{499} - (1.3831 \times \text{O.D}_{620})]}{\text{O. D}_{620}} \times 1000$$

O.D₄₉₉: Represent pigment absorption

O.D₆₂₀: Represent bacterial cells absorption

1.3831: constant.

1000: Avoid dealing with numbers less than one.

3- Prodigiosin production and extraction^[12]

*The pigment was extracted by adding distill water to the cell suspension (*S. marcescens* inoculated into Brain heart infusion broth and incubated at 30c° for 72hrs). in percent 1:1 (V/V).

*The culture mixture was shaken and then centrifuged (6000 rpm for 15min).

*The sedimented cell debris was mixed with Three ml of chloroform, so the pigment in the lower layer was separated from the lower layer.

*One ml of 0.2 N Hcl was add to the lower layer and centrifuged at 10000 rpm for 15 min.

One ml of 0.4 N NaOH was added to the sediment and poured in glass petridish and incubated at 30°C for 48 hrs.

4- Purification of Prodigiosin^[13]

The pigment was purified using thin layer chromatography. The TLC plates of silica gel (20×20cm) were used, the developing solvent which contains Ethyl acetate and Chloroform and acetone (65:30:5) was standardized and poured into the chromatography tank, that was saturated with a mobile phase. The R_f value of chromatography was observed in the TLC plates. The isolated prodigiosin was estimated using the following equation:-

$$R_f = \frac{\text{Distance of sample}}{\text{Distance of mobile phase}}$$

Pigment spot was scraped and dissolved in 5ml of methanol and centrifuged (6000 rpm for 15 minutes) to get rid of silica gel residue. Then measured the optical density at wave length range 200-700nm and methanol was used as blank.

The purified pigment was stored in clean sterile tube and covered with aluminum paper at 4°C.

5- Antibacterial activity

A- Agar well-diffusion method^[14]

In this test, approximately (0.1)ml of 24 hrs broth culture of bacteria adjusted to 0.5 Macfarland standard was aseptically introduced and evenly spread using sterile swab on the surface of sterile Muller-Hinton agar plates. Four wells of about 6mm diameter were aseptically cut on agar-plate using a sterile cork borer.

Fixed volumes (100µl) of prodigiosin extract was then introduced into each well with the help of a micropipette. A control well was made with the extracting solvent.

The plates were incubated at 37°C for 24 hrs, and the diameter of any resulting zones of inhibition was measured in mm. this was repeated three times.

B- Tube method^[15]

This was done by mixing 0.5ml prodigiosin with five ml of Muller-Hinton broth. And an activated bacterial culture adjusted to 0.5 Mcfarland standard was inoculated (0.05 ml) into

the tubes, incubated at 30c° for 24 hrs. and the optical density for each tube was read at 600nm, using spectrophotometer.

A control tube (blank) was made from the broth culture without the pigment. The inhibitory effect calculated using the following equation:

$$\text{Inhibition \%} = \frac{\text{Control absorbance} - \text{test absorbance}}{\text{Control absorbance}} \times 100$$

6- Antibiofilm activity

The inhibitory effect of the Prodigiosin isolated from *S. marcescens* against biofilm formation of Gram positive and negative bacteria isolates were quantified by Co- incubation experiments using tube method described by.^[15] Each of leukemia causative bacterial suspensions in brain heart infusion broth with 2% sucrose were incubated together with Prodigiosin extract (1:1 V/V). Control tubes contained brain heart infusion broth with 2% sucrose and bacterial suspension, which adjusted with Mcfarland tube No. 0.5.

All tubes were incubated at 30 C° for 24 hrs. the tubes were decanted and washed with phosphate buffer saline (pH=7) and dried, Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with distilled water, then dried and observed for biofilm.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Experiments were performed in triplicate.

7- Anti adhesive activity

The anti adhesive activity of Prodigiosin were quantified by Co-incubation experiments according to the Procedure describe by.^[16] Each of leukemia causative bacterial suspension brain heart infusion broth with 2% sucrose (100MI) were added to 96-well flat- bottomed plastic tissue culture plates together with (100MI) of Prodigiosin. Control wells contained 180 MI of brain heart infusion broth with 2% sucrose and 20MI of bacterial suspension.

The plates were incubated at 37 C° for 24 hrs. Un attached bacterial cells were removed by washing the wells three times with phosphate buffer saline (pH=7).

After drying at room temperature for 15 min, 200ml of crystal violet (1%) was added to the wells for 20 min.

The stained attached bacterial cells were rinsed three times with PBS (pH=7), allowed to dry at room temperature for 15 min, and extracted with 200 ml of 95% ethanol, and the absorbance of each well was measured at 630 nm using ELISA Reader.

The inhibition of adhesion percentage of Prodigiosin for each pathogenic bacteria were calculated as following equation:-

$$\% \text{Inhibition of adhesion} = \frac{O.D \text{ of control} - O.D \text{ of test}}{O.D \text{ of control}} \times 100$$

RESULT AND DISCUSSION

1-*Serratia marcescens* isolates

A total of eleven *serratia marcescens* isolates were collected from patients suffering from a variety of infections during a period between September 2014 to November 2014. The bacterial isolates were preliminary were identify by culturing on blood agar and MacConkey agar plates.

On blood agar, colonies of *S. marcescens* were large, round, red colonies, smooth translucent area surrounding the colonies as a result of B- hemolysin production, which agree with.^[17]

On MacConkey agar, colonies were pale colored resulting from non lactose fermenting in the medium, and that's agree with.^[18]

The isolates were identify depending on their Gram staining and Microscopic characteristic , *S. marcescens* were found to be Gram negative bacilli, which concede with.^[19] On the other hand the biochemical characteristics of these isolates were similar as shown in table (1). All *Serratia* isolates were catalase positive, oxidase negative, and motile. and negative for indol test and urease production.

The identification of *S. marcescens* were confirmed by API-20E system and Vitek 2 system.

Table (1) Biochemical tests of *S. marcescens*

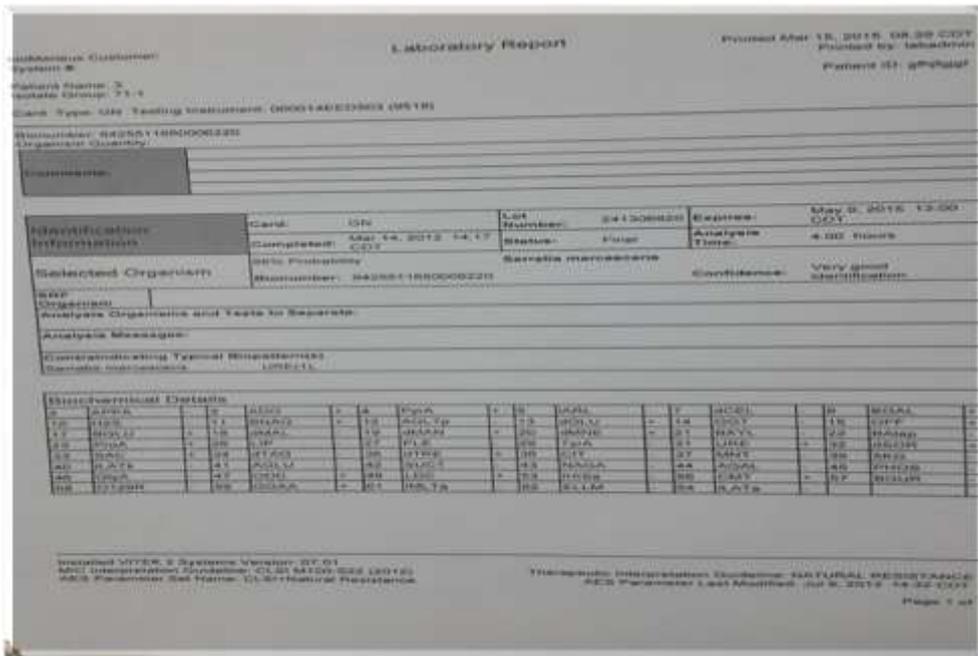
Test	Result
Catalase	+
Oxidase	-
DNase	+
Motility	+
Urease	-
Indol	-

as shown in figure (1) and table (2)



Figure (1) API-20 system used for identification of *S. marcescens* isolate.

(Table 2) Vitek2 compact system



The result in table (3) shown that most of the isolates were obtained from urine samples (six isolates) followed by abscess and blood samples (two isolate for each) and finally one isolate from sputum samples.

Table (3) Number of *S. marcescens* isolates according to the clinical sources.

Clinical sources	No. of isolates
urine	6
abscess	2
Blood	2
sputum	1
Total	11

2- Selection the best isolate for prodigiosin production.

The results in this study showed that all *S. marcescens* isolates were possessed the ability to produce prodigiosin pigment, but the *S. marcescens* isolate STS3 gave the highest pigment production (1024.5 u/cell), thus it was selected for further steps of this study.

The source of isolation, which was positive for prodigiosin production was urine. As shown in table (4).

Table(4) Production of prodigiosin from *S. marcescens* according to the source of isolates.

Isolate No.	Isolate symbol	Prodigiosin (unit/cell)	Source of isolate
1	STS4	567.98	Blood
2	STS5	613.33	Blood
3	STS9	366.8	sputum
4	STS1	191.35	urine
5	STS3	1024.55	urine
6	STS7	577.41	urine
7	STS8	1012.1	urine
8	STS10	927.7	urine
9	STS11	1009.36	urine
10	STS2	625.95	wound
11	STS6	575.10	wound

3- Influenced factors in prodigiosin production

3-1 Incubation period

Serratia marcescens isolate was incubated in three different incubation period (24, 48, 72) hours to determine the optimum incubation period for prodigiosin production. The incubation period for 72 hours was gave the optimum incubation period for pigment production (figure 2). the pigment is secondary metabolic product which synthesis in stationary phase,^[20] however as a result from the generation time of *S. marcescens*, that minimize the difference in number of bacteria and the growth phase of culture during the long period of incubation for

prodigiosin production (72 hr).^[21] mentioned that prodigiosin pigment are bounded to the cell wall and released after the death of some cells during the stationary phase.

3-2 Temperature

Different growth temperature degrees for prodigiosin production were used ranging from 26 to 36c°, as shown in figure (3), the best growth temperature for prodigiosin production ranging from 28 to 32c°, sharp decrease in pigment production was noticed at 25c°, but the production was increased gradually with increasing temperature to 28c°, and in 30c° the prodigiosin recorded the best production.

This may be as a result to the growth phase that the pigment produce in, so in low temperature (26c°) this mean longer time to reach to end of log phase or beginning the stationary phase, while the higher temperature decrease in time to reach to stationary phase, which the pigment produce^[20] and this was confirmed with the results of some researchers.^[22, 23, 24]

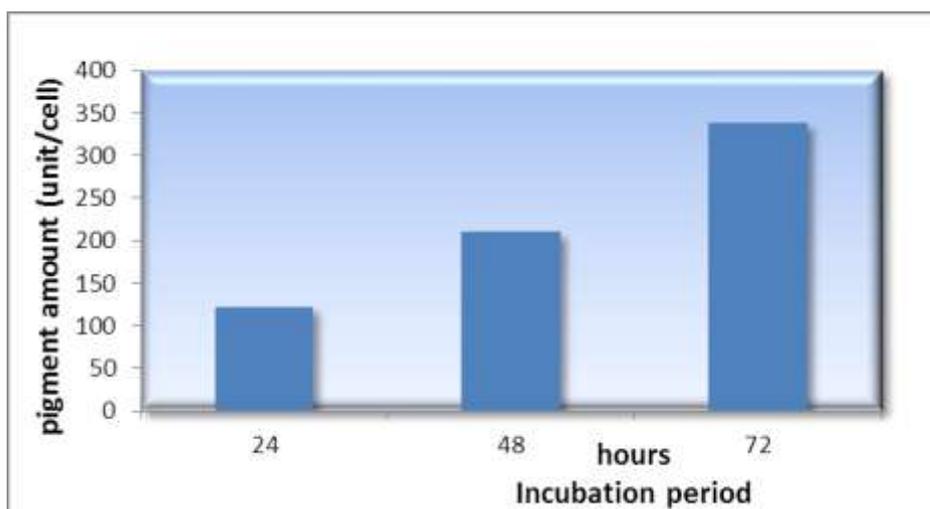


Figure (2). Production of prodigiosin pigment from STS3 isolate in 30c° in peptone glycerol broth at different incubation period.

Other studies indicated that the optimum temperature for pigment production was 28c°.^[12, 21, 25] Some change on media were described by^[12] that the nature of the medium components, play role in temperature degree for pigment production when bacteria grown in peptone glycerol broth was 30c°, while 28° was the optimum temperature for pigment production in nutrient broth. Also he indicated that the Bacteria could be produce the pigment at temperature more than 42c° when grown in oil seeds extracts medium like peanut oil seeds medium, sesame seeds, sun flower seeds.

High temperatures may lead to the suppression of enzyme activity responsible in condensing of pigment generators (prodigiosin condensing enzyme) which is sensitive to high temperatures,^[26] this explains why pathogenic isolates of *Serratia* are non pigment producer when growth in human temperatures (37c°).^[27]

3-3 pH value

Wide range of pH values of media were used to determine the best one for prodigiosin production. Highest productivity seems at pH=8 (1024.55u/cell). Figure (4).

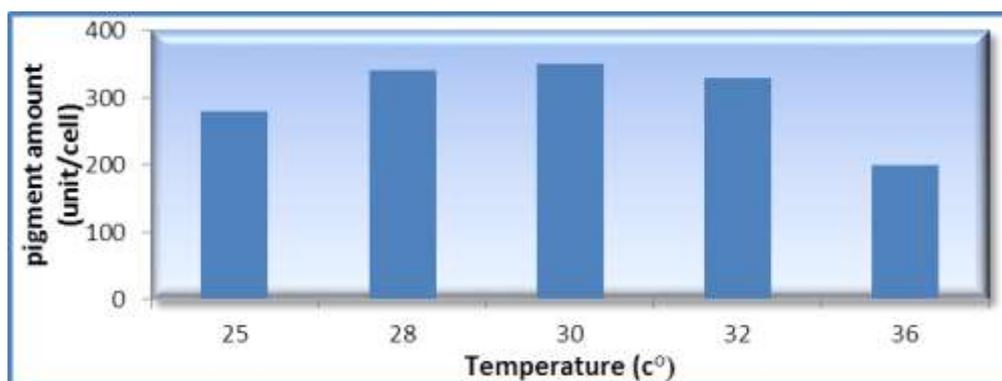


Figure (3) production of prodigiosin from STS3 grown at different temperatures.

And this result was confirmed by studies performed by ^[28] and ^[12], the reason of that is pH 8 act as inhibitor for proline Oxidase enzyme, which cause proline destruction, which regard the essential amino acid in the synthesis of pigment precursor secondary pyrrole MBC, the inhibition of this enzyme lead to increase in proline acid concentration in the medium and take advantage of these concentration in pyrrole ring synthesis for MBC, However increasing or decreasing in pH from pH 8 resulting in cutting of biological pathway which needed for the synthesis of the pigment.^[29]

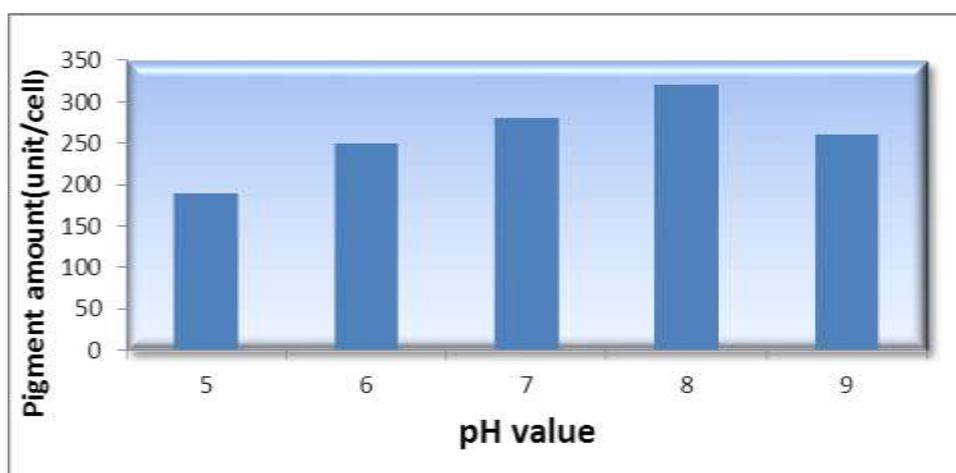


Figure (4) production of prodigiosin pigment from STS3 isolate at different pH values.

4- Extraction by chloroform in acid – base medium^[12]

This method gave very good yield and purity and was applicable for pigment production as TLC results showed. This is may be as a result the addition of chloroform as solvent and using acid solution and alkaline solution, which gave very high yield of prodigiosin, due to high polarity solvent consisted of water, chloroform.

5- Purification of prodigiosin pigment.

In this study method of ^[13] (modified method) was used for separation of pigment in which ethyl acetate used to break down cells walls and released the pigment to the medium, while methanol used to dissolve pigment after drying it, this method was repeated many times to get rid of un soluble parts then thin layer chromatography technique was carry out. Purification of pigment from *S. marcescens* STS3, R_f was calculated and the result was about 0.85 (Figure 5) this result was compatible with the result of some scientists ^[12, 30] which pointed out that the distance of spot sample movement percent to the distance of spot solvent percent was 0.65, other studies pointed out the R_f percentage ranged from 0.9-0.95 ^[32] due to using other solvents in the purification.

Pigment analysis was done using spectrophotometer in different wave length ranged from (200-700)nm, Figure ^[6] showed two peak of absorbance, maximum absorbance was at the wave length 535.55 nm. This result approached with the result of the other studies ^[30, 32] which showed that the maximum absorbance peak for pure pigment suspension was at the wave length 535 nm. Other studies showed that the maximum absorbance peak for purified prodigiosin pigment was at the wave length 539nm.^[33] This a little difference in the wave length for the absorbance of pure pigment suspension may be as the type of solvent or materials used or method of extraction and purification.

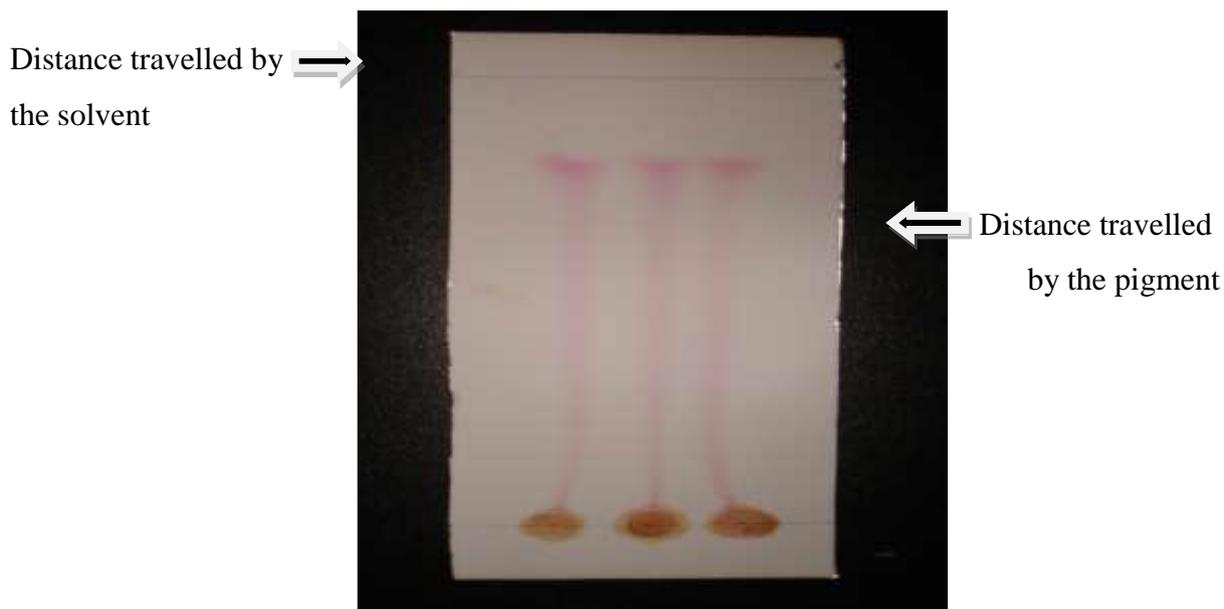


Figure (5) separation of prodigiosin pigment (Purified) from *Serratia marcescens* STS3 isolate using thin layer chromatography (TLC).

The solvent was

Ethyl acetate: Chloroform: Acetone (65:30:5 ml).

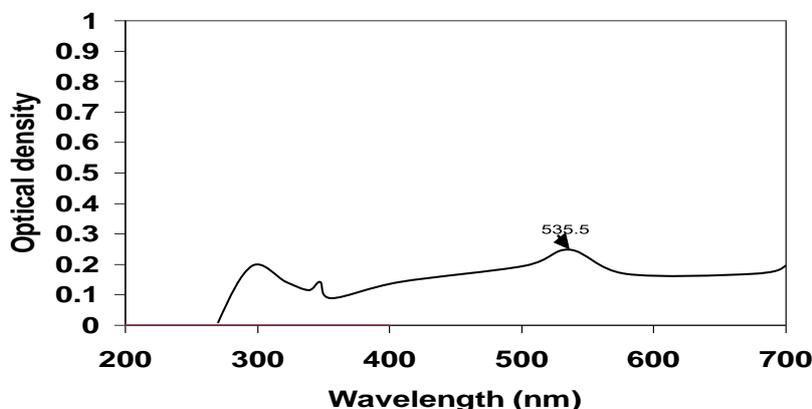


Figure (6) Absorbance curve for prodigiosin pigment purified from *S. marcescens* STS3 isolate measured with different wave length.

6- The antibacterial effect of prodigiosin(tubes method)

To determine the inhibitory effect of prodigiosin extract against bacterial isolates from patients with cancer, tubes method was used. Table (5) illustrated that the prodigiosin extract was more effective on the *S. epidermidis* than the other bacterial types, when the inhibition percentage was 80.4% against *S. epidermidis* compared with (79.3, 76.2 and 49.9) % against *A. baumannii*, *S. enterica* and *E. coli* respectively.

The results agreed with ^[34] they mentioned that Gram positive bacteria are more sensitive to the red pigment (prodigiosin) than Gram negative bacteria. Also ^[35] showed in their study that the prodigiosin extracted from *S. marcescens* have potent antibacterial activity, which confirms its use against infection with pathogenic bacteria.

Table (5) Inhibitory effect of prodigiosin on the bacterial isolates from patients with cancer using tubes methods.

Bacterial isolates	Absorbance Of growth at 600 nm		
	Without prodigiosin	With prodigiosin	Inhibition%
<i>S. epidermidis</i>	1.821	0.356	80.4
<i>A. baumannii</i>	1.837	0.380	79.3
<i>Salmonella enterica</i>	1.762	0.419	76.2
<i>E. coli</i>	2.032	1.015	49.9

7- Effect of prodigiosin on biofilm formation (Micro titration plates method).

Bacteria are remarkable decision makers that are capable of responding to a multitude of environmental challenges through changes in gene expression.^[36] Many Gram-negative bacteria form microbial communities denoted as biofilms, which confer tolerance to environmental stress and bactericides.^[37] Inhibition activity of prodigiosin pigment extract against biofilm producing bacteria was shown by reading the optical density of each isolate with and without presence of pigment to ensure of its inhibition efficiency against biofilm. The result show reduction in the ability of the bacteria isolated from different cancer Patient in the formation of biofilm as shown in table (6).

It was found that prodigiosin extract inhibited bacterial biofilm formation on poly styrene surface and consequently caused biofilms detachment and this caused decreased in biofilm formation degree from strong (+ + +) for *E. coli* , *P. aeruginosa*, *Salmonella sp.* and *S. epidermidis* to weak (+).

Table (6) Inhibitory effect of Prodigiosin on biofilm formation by isolates from patients with cancer.

Pathogenic bacteria	Biofilm formation	
	Control	Prodigiosin extract
G+ve		
<i>S. epidermis</i>	+++	+
<i>S. aureus</i>	+++	++
<i>Streptococcus sp.</i>	+++	+
<i>Micrococcus sp.</i>	+++	++
G-ve		
<i>E. coli</i>	+++	+
<i>Salmonella sp.</i>	+++	+
<i>P. aeruginosa</i>	+++	+
<i>A. baumannii</i>	+++	++
<i>Pantoea sp.</i>	+++	+
<i>M. morgani</i>	+++	++
<i>A. hydrophilia</i>	+++	++

(+) weak (++) moderate (+++) strong.

8- Anti adhesive effect of prodigiosin

Microbial adhesion is considered the first step in the sequence of events leading to colonization, the ability to adhere is weakened by exposure to sub lethal dose of antibacterial agents. [38]

The anti adhesive effect were quantified by measuring the absorbance of stained biofilms at 570 nm with a micro titer plate reader, and the results of effect of prodigiosin extract of *S. marcescens* on biofilm formation by some of pathogenic bacteria isolated from patients with cancer were showed in table (7).

The results indicated that all isolates could adhere and have the ability to form biofilm with different thickness degrees, the absorbance value were ranged between (0.110- 0.187). the *E. coli* isolates were the best isolates could adhere with highest absorbance value (0.187). followed by *Pantoea sp.* (0.166) and *P. aeruginosa* (0.155). on the contrary *A. baumannii* isolates were the lowest isolates could adhere (0.110). And prodigiosin consequently act as anti adhesive agent and this caused decreased in absorbance values to be (0.100, 0.104, 0.117) for *E. coli*, *Pantoea sp.* and *P. aeruginosa* respectively and the inhibition percentages were (46.5, 37.3 and 24.5) % for *E. coli*, *Pantoea sp.* and *P. aeruginosa* respectively .

The mechanism of anti adhesive action of prodigiosin regards to the fact that prodigiosin may disturb membrane structure through interaction with phospholipids as well as membrane proteins.^[39]

Another explanation of antimicrobial effect of prodigiosin is the adhering property of prodigiosin to cell surface caused deterioration in the integrity of cell membrane and also break down in the nutrition cycle.^[40]

Table (7): Anti adhesive effect of prodigiosin extracted from *S. marcescens* on bacteria isolated from patients with cancer.

Type of bacterial isolates	Absorbance at 570 nm		inhibition percentage %
	Without prodigiosin	With prodigiosin	
Gram +ve			
<i>S. epidermidis</i>	0.124	0.098	20.9
<i>S. aureus</i>	0.131	0.108	17.6
<i>Streptococcus sp.</i>	0.138	0.101	26.8
<i>Micrococcus sp.</i>	0.125	0.118	5.6
Gram -ve			
<i>E. coli</i>	0.187	0.100	46.5
<i>Salmonella sp.</i>	0.135	0.107	20.7
<i>P. aeruginosa</i>	0.155	0.117	24.5
<i>A. baumannii</i>	0.110	0.107	2.7
<i>Pantoea sp.</i>	0.166	0.104	37.3
<i>Morganella morganii</i>	0.124	0.109	12.1
<i>Aeromonas hydrophilia</i>	0.141	0.129	8.5

CONCLUSION

1. Leukemia was the highest incidence of cancer in Iraqi hospitals.
2. Both Gram positive and gram negative exhibited susceptibility to the prodigiosin.
3. Prodigiosin inhibited biofilm formation and adherence factor of the bacteria isolated from leukemia patients.

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