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Purification and Characterization of Prodigiosin Produced from *Serratia marcescens* and its Inhibitory Effect on Leukemia Cell Line

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Abstract: *Serratia marcescens* naturally produces prodigiosin, a red pigment with numerous therapeutic uses. Out of 145 UTI samples and 35 soil samples were collected, and only 16 isolates were diagnosed as *Serratia marcescens* according to the VITEK system. The isolates were tested under primary screening to select the prodigiosin-producing isolates. After that, the isolates examined secondary screening to choose the most pigmented one, Isolate S2 had the highest pigment productivity (0.1704g/l) . the optimized media consisting of sucrose as carbon source, peptone as nitrogen source, pH 7, temperature 28 °C, and 72 h incubation time resulted in the highest production of 0.367 g/L of prodigiosin. The pigment was purified by column chromatography, identified, and characterized by FTIR, UV-VIS and GC mass spectrophotometry. The pigment corresponded to prodigiosin with maximum absorption at 535 nm and structural formula $C_{20}H_{25}N_3O$.The cytotoxic effect test was done using the MTT assay. A high cytotoxic effect was observed on the HL-60 cancer cell line while no inhibitory effect was on the normal cell line.

Keywords: prodigiosin, *Serratia marcescens*, column chromatography, leukemia.

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Introduction

Serratia marcescens is an opportunistic pathogen that generally infects people with weaker immune systems. *Serratia marcescens* is a member of the genus *Serratia*, a part of the Enterobacteriaceae family (1). Eight of the 14 species of *Serratia* that are now known to exist in the genus are linked to infections in humans. *S. marcescens* is the most prevalent clinical isolate and the most significant human pathogen among the eight species of *Serratia* that have been linked to clinical infections. The other three species are *S. liquefaciens*, *S. odorifera*, and *S. marcescens* (2). These bacteria can be found in water, soil, plants, humans, and animals It causes various diseases(1), including urinary tract Infections(UTIs). It can cause pneumonia in hospitalized patients, respiratory infections, wound and eye infections, and is often linked to catheter use. It can infect wounds or surgical sites, leading to conjunctivitis or keratitis, as well as bloodstream infections(3) (4). *S. marcescens* can cause bacteremia and sepsis, especially in immunocompromised individuals especially in immunocompromised individuals or those with indwelling medical devices**(**5). *S. marcescens* produces virulence factors such as

lipases, proteases, and hemolysins, allowing it to infiltrate tissues and avoid the immune system(4). It also forms biofilms, making infections more difficult to treat and contributing to its resistance to antibiotics. The bacterium *Serratia marcescens* and numerous other species create prodigiosin, a red pigment(6). It is a secondary metabolite with a distinct tripyrrole structure(7), and its formation is regulated by environmental factors like as temperature, pH, and nutrient availability(8). *Serratia marcescens*' production of prodigiosin is of great interest due to its many bioactive qualities, particularly in medical studies. Prodigiosin has a variety of biological actions, including antibacterial, antifungal, and anticancer properties(9). Prodigiosin, which is extracted from *S. marcescens,* has been found in cancer research to trigger apoptosis (programmed cell death), making it a promising target for anticancer therapy(10). It also has immunosuppressive qualities, which are being investigated for possible therapeutic applications in immune-related illnesses(11). Research is ongoing to optimize the circumstances for prodigiosin production and purify the molecule to harness its medicinal potential in sectors like oncology and infectious disease treatment(12). The goal of this study is to extract prodigiosin pigment from *Serratia marcescens* bacteria under optimum conditions and test its inhibitory effect on leukemia cell line.

Material And Methods

Sample collection and bacterial isolation

One hundred and forty-five clinical samples were isolated from urinary tract infection patients from different hospitals in Baghdad city and thirty-five soil samples were collected during the period from 15 October 2023 to 15 January 2024 in Baghdad city. (13). These isolates were cultured on MacConkey agar as selective media for Enterobacter gram-negative bacteria(14) and nutrient agar , in order to identify *Serratia marcescens* some biochemical tests and VITEK2 system were performed. The identified isolates were prepared for screening experiments to select the pigment-producing isolates and therefore evaluated for prodigiosin production.

Screening *Serratia marcescens* isolates for prodigiosin production

Primary screening (qualitative):

The isolates of *S. marcescens* were screened using plate assay with nutrient agar solid media with some modification. by culturing all of the isolates and incubating them for 48h at 30C.the medium contents were (starch 10g, peptone 5g, CaCl2.2H2O 8.82 g, FeSO4.4H2O 0.33 g, MgSO4.7H2O 0.61 g, MnSO4.4H2O 2 g, agar-agar 15g) dissolved in 1L distilled water PH adjust to 7, autoclaved then pour into dishes(15). Red colonies were chosen for the next step.

Secondary screening (quantitative):

The isolates of pigment-producing *Serratia marcescens* were screened for the most producing based on red colony morphology. *S. marcescens* was inoculated into flasks (100 mL) containing 50 mL of liquid media (starch 10g, peptone 5g, CaCl2.2H2O 8.82 g, FeSO4.4H2O 0.33 g, MgSO4.7H2O 0.61 g,MnSO4.4H2O 2 g) dissolved in 1L distilled water PH adjusted to 7 then incubated at30 C at 200 rpm for 48hr in totally dark condition since prodigiosin is sensitive to light(16). Prodigiosin was extracted by adding an equal volume of ethanol to the cell pellets harvested from a known volume of the *S. marcescens* culture after the end of incubation time,

by centrifugation at 10000 rpm for 15 min(17). The prodigiosin concentration calculated by the molar extinction coefficient (E 535 =7.07 × 104M -1 cm-1) from each isolate after calculating its OD535 and multiple it with 323.4 the weight of prodigiosin.

Optimization of prodigiosin production from *S. marcescens*

Prodigiosin production was studied to determine the impact of different cultural requirements using Chen and co-workers' defined media(18) for prodigiosin production. Numerous factors were investigated, such as carbon source, nitrogen source, pH value, temperature, and incubation period.

Effect of carbon source

A 10 g/L of several carbon sources, including starch, sucrose, mannitol, maltose, and glucose were added to the broth medium, the pH was set to 7. The flasks were inoculated with 3 loops full of the highest productivity isolate and the medium was shaken in a shaker incubator (200 rpm) for 48hr at 30C. then the cultures were placed in a centrifuge and the supernatant was discarded leaving the intracellular pigment to evaluate optical density at 535nm.

Effect of nitrogen source

A 5 g/L of several nitrogen sources, including peptone, yeast extract, NH4Cl, urea , and NH4SO4 were added to the broth medium, the pH was set to 7. The flasks were inoculated with 3 loops full of the highest productivity isolate and the medium was shaken in a shaker incubator (200 rpm) for 48hr at 30C. then the cultures were placed in a centrifuge and the supernatant was discarded leaving the intracellular pigment to evaluate optical density at 535nm.

Effect of PH value

In order to evaluate the pigment production, 100ml flasks containing 50ml of optimized media were inoculated with 3 loops full of the highest productivity isolate, different PH values were tasted (5,6,7,8,9) and the medium was shaken in a shaker incubator (200 rpm) for 48hr at 30C. then the cultures were placed in a centrifuge and the supernatant was discarded leaving the intracellular pigment to evaluate optical density at 535nm.

Effect of temperature

Temperatures of 28,30,32,37 were tested for pigment production, 100 ml flasks containing 50ml of optimized media were inoculated with 3 loops full of the highest productivity isolate, and the medium was shaken in a shaker incubator (200 rpm) for 48hr at 30C. then the cultures were placed in a centrifuge and the supernatant was discarded leaving the intracellular pigment to evaluate optical density at 535nm.

Effect of incubation periods

To get the most efficient pigment production, different incubation periods were carrying using 100ml flasks containing 50ml of optimized media inoculated with 3 loops full of the highest productivity isolate, the medium was shaken in a shaker incubator (200 rpm) for 24h, 48h, 72h, and 96h. then the cultures were placed in a centrifuge and the supernatant was discarded leaving the intracellular pigment to evaluate optical density at 535nm.

Prodigiosin extraction (partial purification)

The extraction of prodigiosin was performed by adding an equal volume of ethanol (19)to the cell pellets harvested from a known volume of the *S. marcescen* culture after the end of

incubation time, by centrifugation at 8000 rpm for 15 min and agitated vigorously to extract the red pigment into the ethanol as a soluble solvent (17). Next, dense culture bacteria and nutritional detritus were eliminated from the liquid solution of S. marcescens and ethanol by filtering it through Whatman filter paper. (19). The solution was the centrifuged at 5000 revolutions per minute (rpm) for 10 min for further separation and a clearer solution. The red-pigmented supernatant was left under 40C (9)in order to purify the crude concentrated extract of prodigiosin after removing the surplus alcoholic solvent.

Prodigiosin purification (column chromatography)

After extraction, the pigment was purified by column chromatography using a column of 27x1cm and silica gel (60-120) as adsorbent (12, 20)(silica gel was heated (60-120) in the oven at 80C for 4 hours). the purification process was performed by solubilizing the pigment into 10ml of ethanol and then eluting using chloroform: methanol: acetone solvent system (4:2:3 v/v), where 50 fractions were collected (3 mL)(12). The fractions corresponding to the purified pigment were collected and the solvent evaporated, pure prodigiosin was obtained.

Prodigiosin characterization

The isolated red pigment was characterized by UV-VIS, with absorbance determined in the 200–700 nm, FTIR was performed on compounds such as prodigiosin and its derivatives (6, 21) and GC mass spectrophotometry.

In vitro Cytotoxic activity

Cells were cultured using RPMI medium with various antibiotics and nutrient supplements. After 24 hours, they reached 90% confluency. The cytotoxic activity was assessed using an MTT assay against the HL-60 tumor cell line and HdFn normal cell line as control. The cells were seeded into 96-well plates and incubated for 24 hours. After adding prodigiosin pigment, the cells were solubilized in DMSO. The percentage of inhibition was computed by measuring the optical density. The IC50 value for each cell line was determined. (22).

bioMérieux Customer: Microbiology Chart Report Printed January 15, 2023 12:39:18 PM CST

Patient Name: Alrawashih lab, Serratia Patient ID: 2
Location: Physician:
Lab ID: 2 Isolate Number: 1

Organism Quantity:
Selected Organism : Serratia marcescens

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	3.12 hours	**Status:**	Final
Selected Organism	99% Probability	**Serratia marcescens**		
	Bionumber:	6525511455006230		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	lARL	+	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	+
40	lLATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	+	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

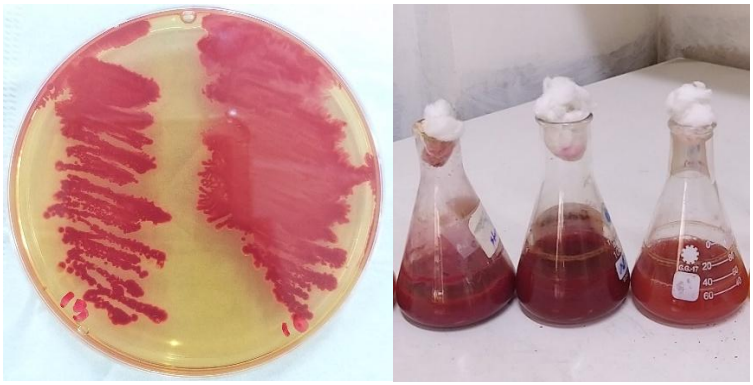
Figure (1): identification of *Serratia marcescens* using the VITEK2 system.

Results and Discussion

Isolation, cultivation, and identification of *Serratia marcescens*

A 16 bacterial isolates collected from 145 samples of urinary tract infection patients from different hospitals, the microscopic and biochemical results of 16 isolates showed heavy growth on McConkey agar at 37C and 30C for 48 h with

positive motility test, positive catalase test, negative oxidase test, positive urease test, negative methyl red test with the fermentation of lactose and sucrose but not for glucose, all of the isolates were diagnosis by VITEK2 system and confirmed to belongs to *Serratia marcescens.*



Figure(2): a)pigmented colonies of S. marcescens on solid media. b)prodigiosin production in broth media.

Screening *S. marcescens* isolates for prodigiosin production

Primary screening (qualitative)

16 *S. marcescens* isolates were screened to select pigmented from non-pigmented bacteria, only 11 showed red pigment colonies at 30C after 48hr and were chosen for the next step.

Secondary screening (quantitative)

A total of 11 isolates were cultured in the broth media mentioned above, incubated at 30C for 48hr in a shaker incubator, Prodigiosin was extracted by adding ethanol to the cell pellets harvested from a known volume of the *S. marcescens* culture from each isolate. The prodigiosin concentration calculated by the molar extinction coefficient (E 535 =7.07 × 10^4M -1 cm-1).

Prodigiosin(g/l)=OD_{535}*323.4*dilution factor /7.07*10^4

Where O.D $_{535}$ Optical density at 535 nm

323.4 : Molecular weight of prodigiosin

E=7.07× 10M cm^3 (Molar extension coefficient of prodigiosin at 535)

Dilution factor = Final volume/sample volume

Table (1): Screening of *Serratia* isolates for prodigiosin production in Chen and co-workers' media at 30C.

Isolate NO.	Isolate symbol	Isolate source	Prodigiosin g/l
1	S1	UTI	0.016g/l
2	**S2**	**UTI**	**0.1704g/l**
3	S3	UTI	0.0846g/l
4	S4	UTI	Non-pigmented
5	S5	UTI	Non-pigmented
6	S6	UTI	Non-pigmented
7	S7	UTI	0.1528g/l
8	S8	UTI	0.1055g/l
9	S9	UTI	Non-pigmented
10	S10	UTI	Non-pigmented
11	S11	UTI	0.0816g/l
12	S12	UTI	0.1362g/l
13	S13	UTI	0.0168g/l
14	S14	UTI	0.014g/l
15	S15	UTI	0.1356g/l
16	S16	UTI	0.0985g/l

Isolate S2 showed a maximum prodigiosin production of 0.1704g/l and was selected for further experiments.

Optimum conditions for prodigiosin production:

Effect of carbon source

The best carbon source for prodigiosin production was determined by the existence of different carbon sources such as starch(the original media's carbon source), sucrose, maltose, mannitol, and glucose at a concentration of 10 g/l. results in the figure indicate that the highest production was when the sucrose present in the media by 0.35g/l followed by mannitol 0.33g/l and maltose 0.29g/l, and starch was 0.15g/l. Glucose showed no production of prodigiosin when present in the media indicating that the carbon source was the most important element required for organism growth.

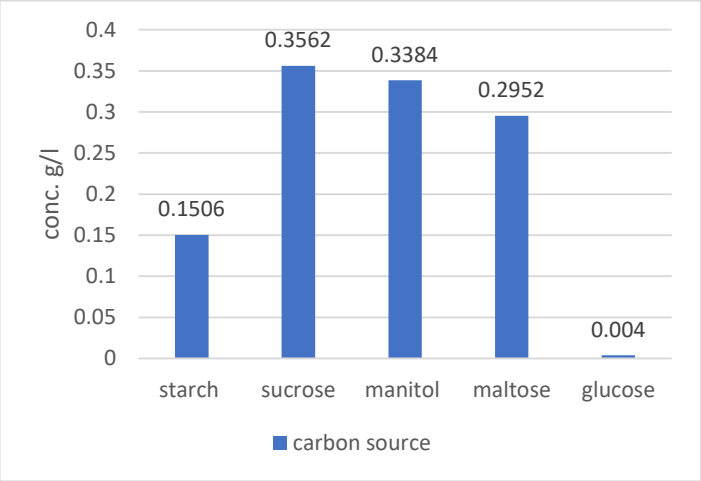


Figure (3): prodigisoin production with different carbon sources.

Effect of nitrogen source

Producing prodigiosin was also studied in the presence of different nitrogen sources like peptone (the original media's nitrogen source), yeast extract, NH4Cl, urea, and NH4SO4 at a concentration of 5g/l. results in Figure 4 showed that the peptone was the greatest nitrogen for supporting prodigiosin production by 0.28g/l followed by yeast extract 0.14g/l. urea and NH4SO4 were the worst sources of nitrogen for little or no production at all.

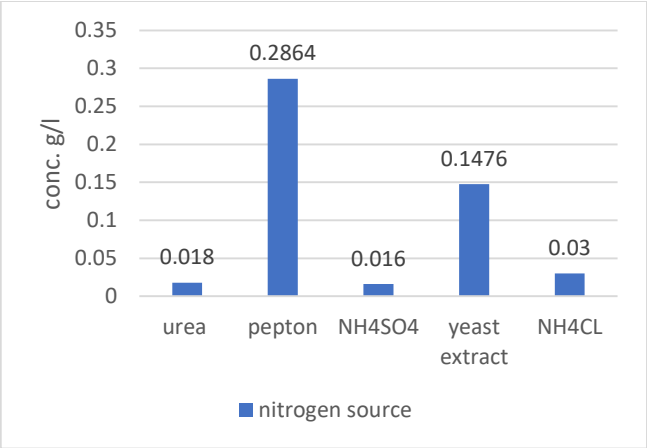


Figure (4): prodigisoin production with different nitrogen sources.

Effect of PH

To determine the best pH value for prodigiosin production, different pH values were used(5, 6, 7,8, 9) Results showed that the highest output of prodigiosin was obtained at pH 7 with 0.36g/l which agreed with results of (23). a drop in prodigiosin production happened when increasing or decreasing the pH value above or below 7.0.

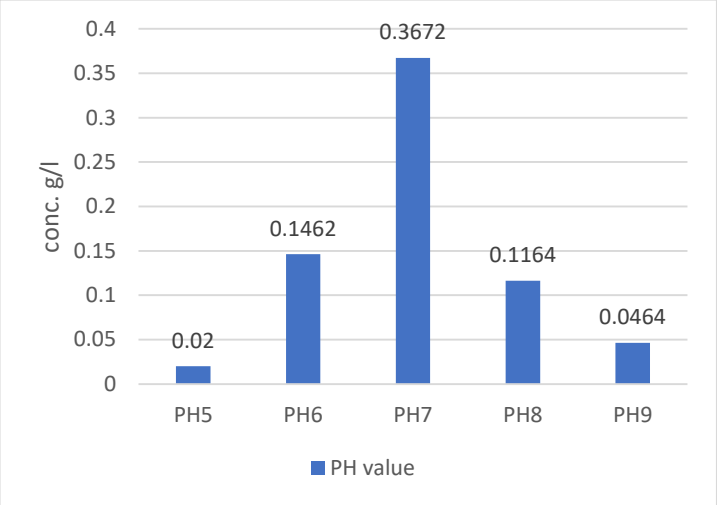


Figure (5): prodigisoin production with different PH values.

Effect of temperature

Temperature plays an important role in the growth and metabolism of any microorganism. Different temperature degrees were used to determine the best temperature degree for prodigiosin production (37,32, 30, 28). Results in figure6 showed that the highest prodigiosin production was obtained at 28C with 0.35g/l. lower yields obtained at a higher temperature of 32C with no production of red pigment at 37C.

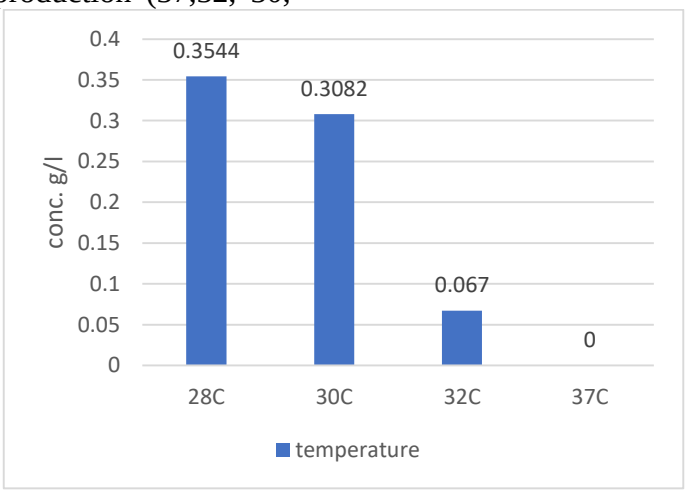


Figure (6): prodigisoin production with different temperatures.

Effect of incubation periods

To measure the optimal incubation period for prodigiosin production, the bacteria were incubated in the productive medium for several periods (one day, two days, three days, and four days). results in figure 7 showed that the highest prodigiosin production was 0.3g/l after incubation for 72 hours. From day 1 to day 3 the production increased, The production declined after 96 hours because it reported that bacteria used prodigiosin which is a protein as nutrition during stress conditions(8).

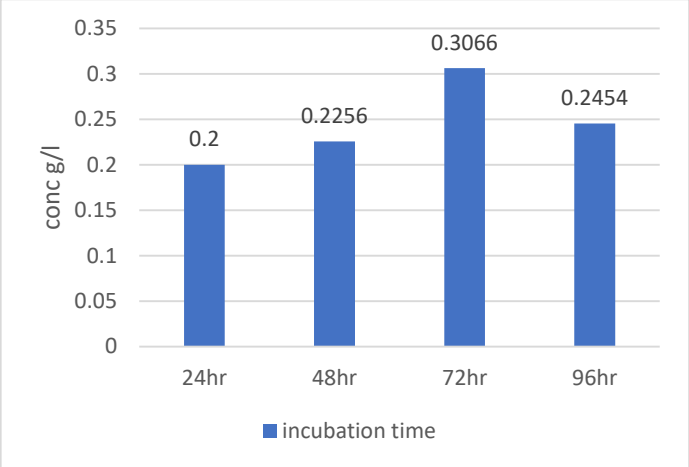


Figure (7): prodigisoin production at different incubation periods.

Purification of prodigiosin

Serratia marcescens S2 was cultivated under optimum conditions,500ml of broth culture, pigment extracted by centrifugation at 1000rpm for 10 minutes. the crud was mixed with an equal volume of ethanol 500ml that was reported to be the best solvent for prodigiosin**(19)**, the solution was filtered using filter paper and centrifugation again to get rid of agar and nutrient debris. then pour the solution in a petri dish and dry it at 40C to get about 20 ml of dense prodigiosin.

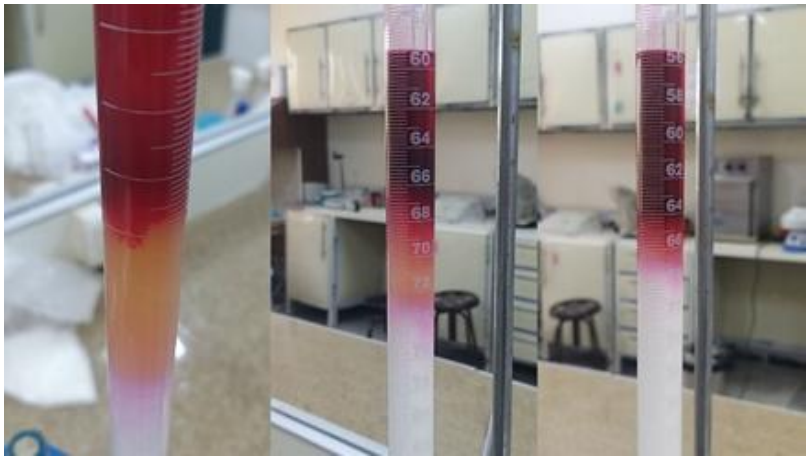


Figure (8): pigment separation by column chromatography.

Column chromatography

The purification process was carried out using column chromatography. After heating the silica gel (60-120) in an oven at 80°C for 4 hours, it was solubilized in a

mixture of chloroform, methanol, and acetone solvents (4:2:3 v/v) and allowed to sit for 24 hours. After that, pour the 10ml of pigment concentrate obtained in the previous stage, resulting in 50 fractions of 3ml, the pigment showed in fractions from 8 to 24 that were condensed to yield approximately 0.5g of pure pigment from three runs.

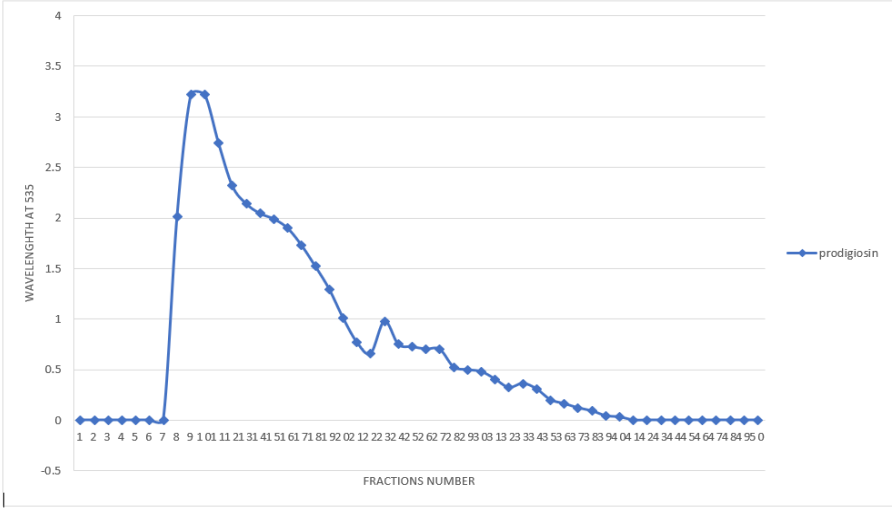


Figure (9) : column chromatography for prodigiosin purification from *Serratia marcescens* utilizing silica gel (60-120) column (27x1) cm equilibrated and eluted with chloroform, methanol, and acetone solvents (4:2:3 v/v) in rate of flow 20ml/hr. 3ml for every Fraction.

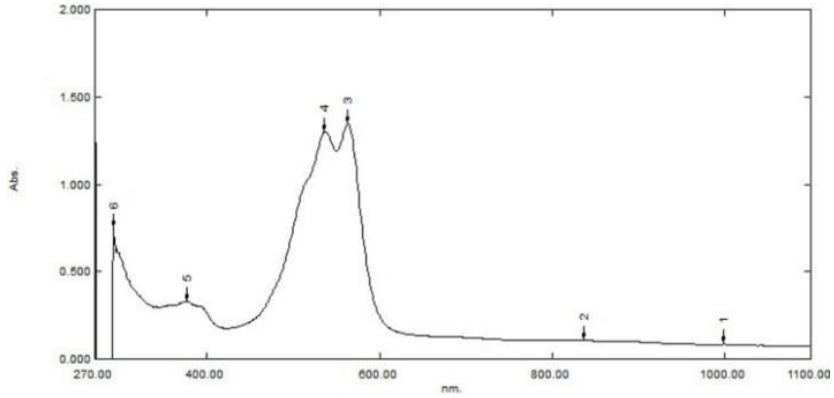


Figure (10) : UV-VIS characterization of prodigiosin.

Characterization of prodigiosin

Characterized by UV-VIS the pigment showed peak maximum absorbance at 535nm cross-ponding to prodigiosin as described before by (12). Figure 11 displayed the FT-IR data for the prodigiosin pigment. It is common to see a succession of adsorption peaks between 400-4000. FT-IR spectrum of prodigiosin shows different bands positioned at 3407, 2858, 1053, and 777 cm−1. The presence of 3407 cm−1 bands contributes to the N – H stretch. The peaks of 2858 cm−1 are typical of symmetrical stretching of aromatic methylene groups (CH). The 1050 cm−1 peak was due to the C-O stretching. The value observed at about 777 cm−1 refer to the prodigiosin carbon-carbon double bond, these results agreed with (15). Figure 12 shows GC mass spectrophotometry revealed that the red pigment had a molecular weight of 323 D m/z, indicating that the pigment isolated from S. marcescens was prodigiosin pigment.(24).

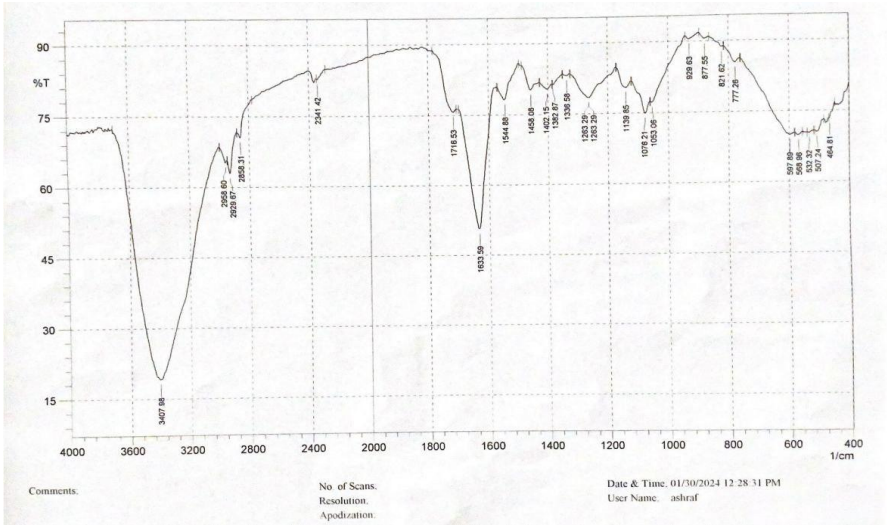


Figure (11): prodigiosin characterized by Fourier transforms infrared (FTIR).

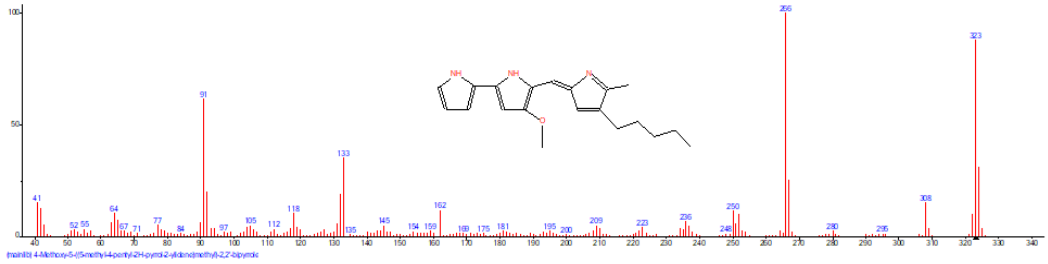
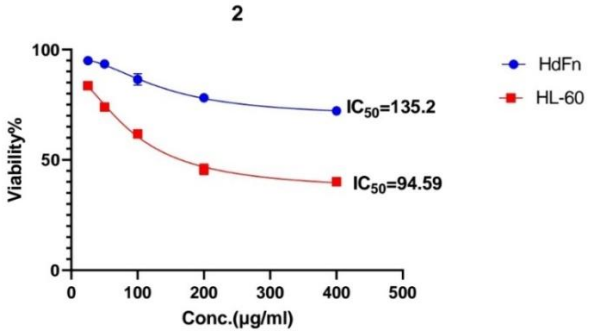


Figure (12): prodigiosin characterized by GC mass spectrophotometry.

In vitro cytotoxic activity of prodigiosin pigment

Cytotoxicity of prodigiosin pigment from the clinical isolate of *Serratia marcescens* was tested against leukemia cell line HL-60 with HdFn normal cell line as control employed MTT assay(25). Different concentrations of prodigiosin ranging from 25 to 400ug/ml for 24 hours were used to measure the cell viability of cell lines as done before by(22). Results showed a high effect, with an IC_{50} value of 94.5 and cell viability of 40.1, as shown in Figure 13 which agreed with the results of (12) and (26). The data shows a clear dose-dependent response in both HL-60 and HdFn cell lines across decreasing concentrations. HL-60 cells exhibit significantly lower mean values at higher concentrations, indicating higher sensitivity, with viability increasing as the concentration decreases (from 40.1 at 400 µg/mL to 83.6 at 25 µg/mL). In contrast, HdFn cells maintain relatively high viability throughout, ranging from 72.1 to 94.9, suggesting greater resistance. The mean values and standard deviations further highlight that HL-60 cells are more affected by the treatment, while HdFn cells display a more stable and less variable response. These results suggest that the tested compound or condition selectively affects HL-60 cells more than HdFn cells.



	HL-60		HdFn	
Conc.	Mean	SD	Mean	SD
400	40.1	1.5	72.1	1.6
200	45.6	2.4	78.0	1.0
100	61.8	1.3	86.4	2.6
50	73.9	1.8	93.4	0.6
25	83.6	0.8	94.9	0.4

Figure (13): MTT assay results of synthesized prodigiosin on HL-60 cells and HdFn cell line.

Conclusion

The yield of prodigiosin was increased at optimal conditions. The media developed by Chen and co-workers demonstrated a greater prodigiosin yield. The best carbon source was sucrose, and the best nitrogen source was peptone with PH 7 and 28C temperature. for the best output, 72h of incubation is recommended. *Serratia marcescens* clinical isolates may also produce pigment. The pigment showed a high cytotoxic effect on cancer cell line HL60 while it did not affect normal cell line at all. Because of pigment's anti-proliferative, anti-bacterial, and anti-mycotic properties, it is recognized to have a wide range of medical applications. Therefore, future research will concentrate on prodigiosin's medical uses.

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