ISSN: 0067-2904

Cytotoxic Effects of Prodigiosin Pigment from *Serratia marcescens* on Tumor Cell Lines

Nabaa Muwafaq Othman*, Reem Waleed Yonis
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 9/ 11/2024 Accepted: 11/8/2025 Published: 30/ 7/2026

Abstract

Prodigiosin is a secondary metabolite with red pigmentation produced by *Serratia marcescens*. Prodigiosin has been described for several biological activities, including antitumor. The study aimed to isolate *S. marcescens* that produces the prodigiosin pigment, purify it, and evaluate its activity against three human cancer cell lines, MM.1S, TP-53, and HL-60, with the HdFn normal cell line as a control. 145 urinary tract infection (UTI) samples and 35 soil samples were collected, and only 16 isolates were identified as *S. marcescens* using the VITEK system. The isolates were tested under primary and secondary screening to select the best prodigiosin-producing isolate. Isolate S2 had the highest productivity of the pigment (0.1704g/l). The pigment was purified by column chromatography, identified, and characterized using FTIR and UV-VIS spectroscopy. The pigment corresponded to prodigiosin, with a maximum absorption at 535 nm and a structural formula $C_{20}H_{25}N_3O$. High cytotoxic effects of prodigiosin were observed against TP-53 and HL-60 cell lines. The MM.1s were more pigment resistant. The cytotoxic activity showed IC_{50} of 94.59 and 57.96 against HL-60 and TP-53 cell lines, respectively, and did not affect the normal cell line at all. Prodigiosin pigment exhibits cytotoxic effects on tumor cell lines and is safe for normal cell lines.

Keywords: prodigiosin, *Serratia marcescens*, cytotoxicity, tumor cell lines.

التأثير السمي لصبغة البرودجيوسين من بكتيريا السيرشيا مارسيسينس على الخطوط الخلوية السرطانية

نبا موفق عثمان* , ريم وليد يونس
التقنيات الاحيائية , كلية العلوم , جامعة بغداد , بغداد , العراق

الخلاصة

البرودجيوسين هو مركب ابيض ثانوي، ذو صبغة حمراء، تنتجه بكتيريا *Serratia marcescens*. تم وصف البرودجيوسين لعدة أنشطة بيولوجية، بما في ذلك النشاط المضاد للأورام. هدفت الدراسة إلى عزل بكتيريا *Serratia marcescens* التي تنتج صبغة البرودجيوسين، وتنقية الصبغة وتقييم نشاطها ضد ثلاثة خطوط خلايا سرطانية بشرية MM.1s وTP−53 وHL−60، وخط خلايا طبيعية HdFn كعينة للتحكم. تم جمع 145 عينة من عدوى المسالك البولية (UTI) و35 عينة من التربة، وتم تحديد 16 عزلة فقط على أنها *Serratia marcescens* وفقًا لنظام VITEK. تم اختبار العزلات تحت الفحص الأولي والثانوي لاختيار

*Email: naba.osman2306@sc.uobaghdad.edu.iq

أفضل عزلة منتجة للبرودجيوسين. العزلة S2 كان لديها أعلى إنتاجية للصبغة (0.1704 جرام/لتر). تم تنقية الصبغة بواسطة التنقية بالعمود column chromatography، وتحديدها، وتوصيفها بواسطة FTIR وUV–VIS. كانت الصبغة المنتجة مطابقة للبرودجيوسين مع اعلى امتصاص عند 535 نانومتر وتركيب كيميائي $C_{20}H_{25}N_3O$. تمت ملاحظة تأثير سمي عالي للبرودجيوسين على خطوط خلايا TP–53 وHL–60. كانت خلايا MM.1s أكثر مقاومة للصبغة, كانت قيمة IC_{50} 94.59 و57.96 ضد خطوط الخلايا السرطانية HL–60 وTP–53 على التوالي، ولم تؤثر على الخلايا الطبيعية بشكل واضح.

1. Introduction

Serratia spp. are Gram-negative bacteria belonging to the Enterobacteriaceae family. They are distinct from other genera because they produce three unique enzymes: DNAase, lipase, and gelatinase. These bacteria can be found in water, soil, plants, humans, and animals [1]. *Serratia spp.* are responsible for about 1.4% of nosocomial septicemia cases, including Respiratory Infections. It can cause pneumonia, particularly in hospitalized patients, urinary tract infections (UTIs) often associated with catheter use in hospitals, wound and eye infections, it can infect wounds or surgical sites and cause conjunctivitis or keratitis and also bloodstream infections: In severe cases, *S. marcescens* can lead to bacteremia, which may progress to sepsis, particularly in immunocompromised patients. [2]. A notable feature of *Serratia spp*. is the production of the red pigment prodigiosin, an intracellular natural compound also produced by *Pseudomonas spp., Streptomyces spp*., and *Vibrio spp*. [3] . Prodigiosin is a secondary metabolite, a tripyrrole red pigment family member characterized by a 4-methoxy, 2-2 bipyrrole ring system [4]-[5]. Prodigiosin functions as a proapoptotic agent against many tumor cell lines. They also play an important role in numerous cellular targets, including multidrug-resistant cells with low or no reported side effects [6]. Prodigiosin has several promising biological activities, including immunosuppressive, antifungal, antiviral, antimicrobial, antimalarial, and antiproliferative properties[7]. It is particularly noteworthy for its ability to induce apoptosis in carcinogenic cells, selectively targeting malignant cells while having minimal impact on normal cells[8]. Research has demonstrated significant anti-cancer effects of prodigiosin, making it a potential therapeutic drug. Due to their promising therapeutic implications, the detailed mechanisms of its apoptotic action and its evaluation as an anticancer agent continue to be areas of active investigation [9]. This study aims to isolate *S. marcescens,* which produces prodigiosin pigment, purify it, and evaluate its cytotoxic activity against three human tumor cell lines. The HL-60, TP53, and MM1.S cell lines were likely chosen for this study due to their distinct characteristics and relevance to cancer research. HL-60 cells are a leukemia cell line that lacks p53 function, making them a suitable model for studying cancer in the absence of a functional tumor suppressor [10]. The TP53 cell line is genetically engineered to lack the P53 gene [11]. MM1.S is a multiple myeloma cell line with the P53 function[12]. By comparing these cell lines, it can investigate the effects of prodigiosin on tumor cell lines in the presence and absence of *p53* gene.

2. Experimental part

Collection, isolation, and identification of Specimens

One hundred forty-five samples were obtained from patients with urinary tract infections from various hospitals in Baghdad City, and thirty-five soil samples were collected between October 15, 2023, and January 15, 2024, as per the ethical approval CSEC/0624/0047, dated June 28, 2024. These samples were cultured on MacConkey agar as a selective medium for *Enterobacteriaceae* Gram-negative bacteria [13], and on nutrient agar. *S. marcescens* was obtained from urine samples by culturing samples on MacConkey agar plates from UTI infection patients [14]. The urine samples were cultured on an over-dried sterile agar plate

using a sterile loop and incubated at 37 °C and 30 °C in the absence of light for 48 hours for the pigment production [15]. Soil samples were cultured on nutrient agar plates and incubated at 30 °C for 48h. To identify red colony isolates of *S. marcescens*, a single colony was transferred to a new medium and incubated further. Pure isolates were analyzed phenotypically using colony morphology and microscopic inspection. [16]. Gram stain was carried out to define the cell form, size, configurations, and Gram staining of bacteria using a 100X objective lens of a compound light microscope with oil immersion [16]. Some biochemical tests, including oxidase, catalase, urease, methyl red, motility test, and carbohydrate fermentation like lactose, sucrose, and glucose as well as the VITEK2 system, were performed to identify *S. marcescens*[17,18]. 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 a plate assay with nutrient agar with some modifications. By culturing all the isolates and incubating them for 48 hours at 30 °C. The medium contents were (starch 10g, peptone 5g, $CaCl_2.2H_2O$ 8.82 g, $FeSO_4.4H_2O$ 0.33 g, $MgSO_4.7H_2O$ 0.61 g, $MnSO_4.4H_2O$ 2g, agar-agar 15g)[19], which were dissolved in 1L of distilled water, pH was adjusted to 7, autoclaved, and then poured into dishes. Red colonies were chosen for the next step.

Secondary screening (quantitative)

The isolates of pigment-producing *S. marcescens* were screened for the highest producers based on red colony morphology. *S. marcescens* was inoculated into flasks (100 mL) containing 50 mL of liquid media (starch 10g, peptone 5g, $CaCl_2.2H_2O$ 8.82 g, $FeSO_4.4H_2O$ 0.33 g, $MgSO_4.7H_2O$ 0.61 g, $MnSO_4.4H_2O$ 2g) which were dissolved in 1L of distilled water, pH was adjusted to 7 then incubated at 30 °C at 200 rpm for 48hours [19, 20]in totally dark condition since prodigiosin is sensitive to light[14]. 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 incubation time was complete, followed by centrifugation at 10,000 rpm for 15 min. The prodigiosin concentration was calculated using the molar extinction coefficient (E 535 =7.07 × 104 M^{-1} cm^{-1}) from each isolate after calculating its OD_{535} and multiplying it by 323.4 (the molecular weight of prodigiosin) [5, 9]. After that, production under optimum conditions was carried out to achieve the best output of pigment with different parameters(carbon source, nitrogen source, pH, temperature, incubation period)[21].

Prodigiosin extraction (partial purification)

The extraction of prodigiosin was performed by adding an equal volume of organic solvent [16] to the cell pellets harvested from a known volume of the *S. marcescen* culture after the end of incubation time, by centrifuging it at 8000 rpm for 15 min and agitated forcibly to solve the red pigment (prodigiosin) in the ethanol as a soluble solvent [22]. The liquid solution of ruptured cells of *S. marcescens* and ethanol was filtered through Whatman filter paper to eliminate dense dead culture bacteria cells and broth debris. [16]. The solution was then centrifuged at 5000 rpm for 10 minutes for separation. The dark red-colored supernatant was left at a temperature below 40 °C to remove excess ethanol solvent [6]. Prodigiosin's crude concentrated extract was subjected to purification.

Prodigiosin purification (column chromatography)

After the extraction of crude pigment, it was purified by column chromatography using a column of 27x1cm and silica gel (60-120) as adsorbent [9, 23]. Silica gel was heated in the oven at 80°C for 4 hours. The purification process was performed by solubilizing the pigment into 10ml of ethanol and then eluting using a chloroform: methanol: acetone solvent system (4:2:3 v/v)[9]. 50 fractions were collected (3 mL). The fractions corresponding to the purified pigment were collected, and the solvent was evaporated. Pure prodigiosin was then collected for further study.

Prodigiosin characterization

The purified red pigment was characterized by UV-VIS spectroscopy [9], with absorbance determined in the 200–700 nm range. FTIR was performed on compounds such as prodigiosin and its derivatives[3, 24].

Cell line and culture maintenance

The study was evaluated against three human cancer cell lines and human normal cell lines, Leukemia (HL-60) [9], lymphoma (TP53)[6], and myeloma (MM.1S) [25] cell lines from Nahrain University's Biotechnology Research Center. 10% heat-inactivated fetal bovine serum, 2-mM L-glutamine, 20-mM HEPES, 100 U/mL penicillin G, and 100 µg/mL streptomycin were added to Roswell Park Memorial Institute (RPMI) medium in which the cells were maintained. After being planted in tissue culture flasks, the cells were incubated for 24 to 48 hours at 37 °C with 5% CO_2 added until they reached approximately 90% monolayer confluency. Trypsin (2–3 mL, 50 mg/ml) is used in moderate trypsinization to harvest the cells for subculturing. [26].

In vitro cytotoxicity by MTT assay

The cytotoxic activity was measured using an MTT assay against HL-60, TP53, and MM.1S cells. Cells were seeded in a flat 96-well plate (100 µL per well at a concentration of 1 × 106 cells per well) and allowed to proliferate for 24 h at 37 °C in a CO_2 (5%) incubator. After incubation, the medium was replaced with a fresh medium containing increasing treatments of prodigiosin pigment (25, 50, 100, 200, and 400 µg/mL) added to each well. For a further twenty-four hours, the dish was incubated. Ten microliters of MTT solution were applied to each well after incubation. Four more hours were spent incubating the dish. To solubilize the formazan crystals, 100 µL of dimethyl sulfoxide (DMSO) was added as a solubilization solution after the medium and MTT were disposed of. A plate reader (Bio-Rad, USA) was used to measure the optical density at 570 nm after the solubilization process was finished, and the percentage of inhibition with respect to the vehicle control (untreated cells) was computed. The half-maximal inhibitory concentration (IC_{50}) of each cell line was determined after treatments were carried out in triplicate [26].

Statical analysis

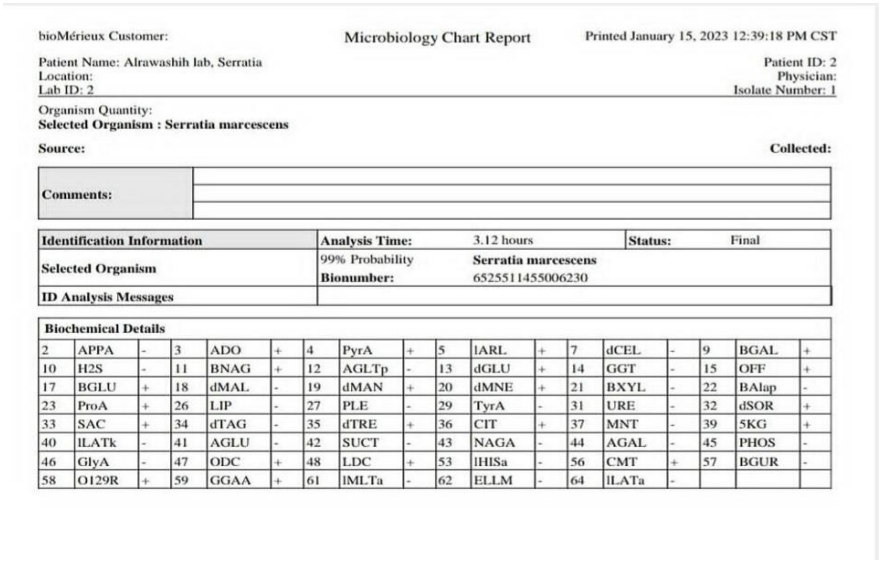
The obtained data were subjected to a two-way analysis of variance (ANOVA) to compare various groups. Results were expressed as mean and standard deviation (SD), and the curve was created using GraphPad Prism software.

3.Results and discussion

Identification of Serratia marcescens

A total of 16 *S. marcescens* isolates were obtained from 145 clinical urine samples and 35 environmental soil samples collected from various hospitals and locations in Baghdad and were named S1 to S16. The isolates showed heavy growth on MacConkey agar at both 37°C and 30°C over 48 hours. The biochemical profile revealed positive motility, catalase, and

urease tests, while tests for oxidase and methyl red were negative. Furthermore, the isolates were unable to ferment lactose and glucose. Identification and confirmation of all isolates using the VITEK 2 system (Figure 1) resulted in a high diagnosis rate of *S. marcescens*. This finding aligns with a previous study that reported the prevalence of *S. marcescens* in both clinical and environmental samples, particularly in nosocomial settings, due to its opportunistic pathogenicity and resistance to harsh conditions[1]. Recent literature highlights the growing concern regarding *S. marcescens* as an emerging multidrug-resistant (MDR) pathogen in UTIs [27]. According to *Ferguson et al.*, its ability to form biofilms, produce β-lactamases, and survive disinfectants, it makes it a frequent culprit in catheter-associated UTIs and hospital outbreaks [28]. The isolation of this organism from soil also reflects its environmental resilience and supports hypotheses about environmental reservoirs contributing to clinical infections, as suggested by *Ferguson et al.* [28].



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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 **Bionumber:**	**Serratia marcescens** 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	ILATk	-	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: VITEK2 system identification.

The 16 *S. marcescens* isolates were screened to select pigmented from non-pigmented bacteria, only 11 showed red pigment colonies at 30ºC after 48hrs, the total of 11 isolates was cultured in the broth media mentioned above, incubated at 30°C 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 was calculated, S2 isolate showed the most production of prodigiosin (0.1704 g/l) (Figure 2), after that the isolate was tested under optimum conditions, with results showing heavy production of prodigiosin with sucrose as a carbon source, peptone as a nitrogen source, pH 7, 28ºC, and 72hrs of the incubation period, as shown in Table 1.

Table 1: Summary of optimized media for prodigiosin production.

Sr. no.	Parameters	Optimized parameter	Conc. of prodigiosin g/l
1	Carbon source	Sucrose	0.356g/l
2	Nitrogen source	Peptone	0.286g/l
3	pH	7	0.367g/l
4	Temperature	28°C	0.354g/l
5	Incubation period	72h	0.306g/l

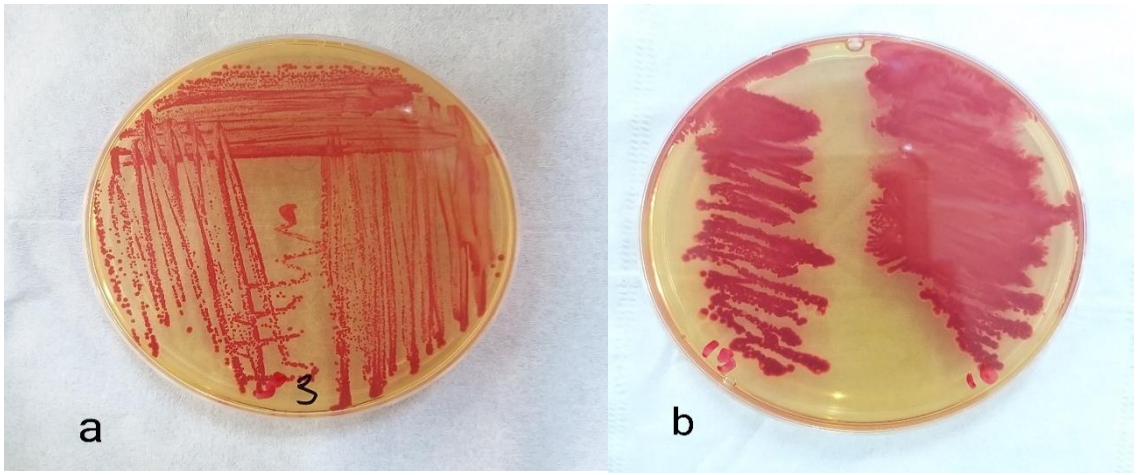

Figure 2: a) S2 isolate on McConkey agar at 30°C **b)** S2 isolate on nutrient agar.

S. marcescens S2 was cultivated under optimum conditions,500ml of broth culture, and the pigment was extracted by centrifugation at 1000rpm for 10 minutes. The crude was mixed with an equal volume of ethanol (500ml), which was reported to be the most effective solvent for dissolving the prodigiosin since the pigment is insoluble in water[29]. The solution was then filtered by filter paper and centrifuged again to get rid of broth and nutrient debris. Then the solution was poured into a petri dish and dried at 40 °C to get about 20 ml of dense prodigiosin.

Column chromatography

The purification process was performed using the column chromatography technique, resulting in 50 fractions of 3 mL each. The pigment was detected in fractions from 8 to 24 (Figure 3), which were condensed to yield approximately 0.5 g of pure pigment from three runs.

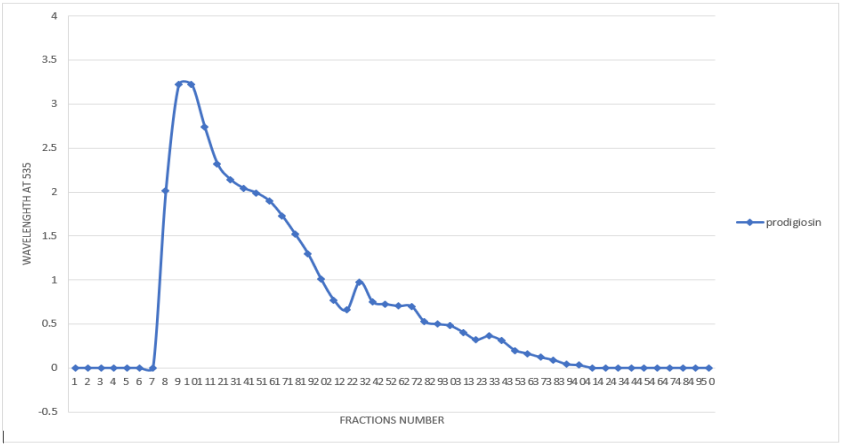

Figure 3: Spectrophotometry of all 50-column chromatography fractions at 535 nm.

Characterized by UV-VIS, the pigment showed maximum absorbance at 535nm as shown in Figure 4, corresponding to prodigiosin, and analyzed its compounds by FTIR, as in the other studies [30, 24].

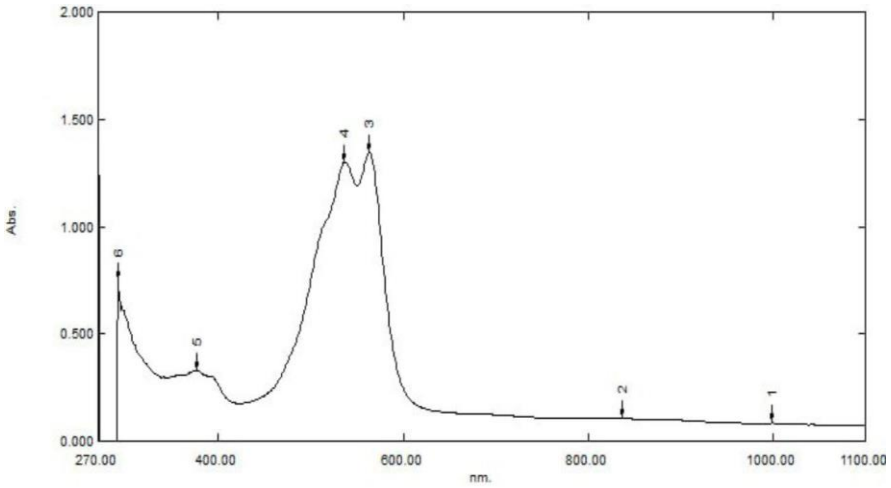

Figure 4: Spectrum of UV-Vis of prodigiosin.

Figure 5 displays the FT-IR data for the prodigiosin pigment. It is known to observe a sequence of adsorption peaks between 400 and 4000. The FT-IR spectrum of prodigiosin exhibits distinct bands at 3407, 2858, 1050, 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} is a reference to the prodigiosin carbon-carbon double bond, as described before by Sumathi *et al.,* [31].

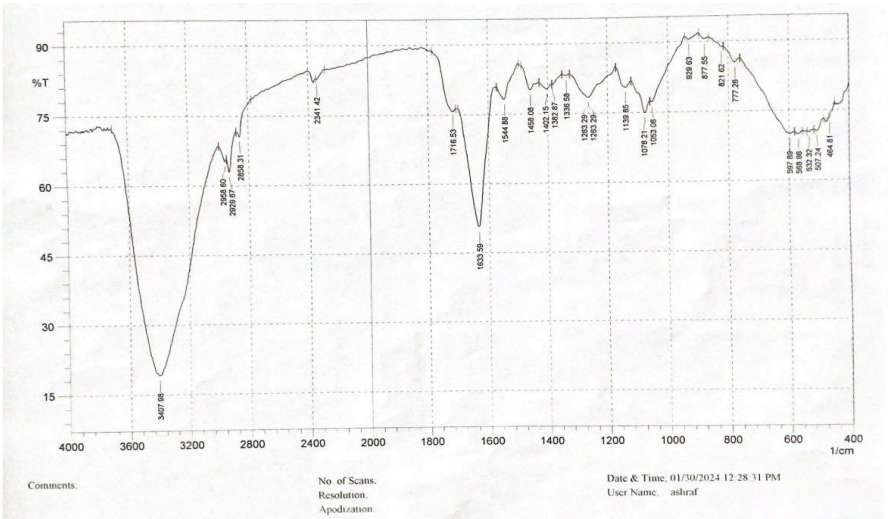

Figure 5: Prodigiosin characterized by Fourier transforms infrared (FTIR).

In vitro cytotoxicity assay of prodigiosin

The cytotoxicity of the prodigiosin pigment from the clinical isolate (S2) was tested against three tumor cell lines (HL-60, TP53, and MM.1S) with the HdFn normal cell line as a control. The MTT assay was performed as described by Koyun *et al.,* [30]. Different concentrations of prodigiosin, ranging from 25 to 400 µg/mL, for 24 hours, were used to measure the cell viability of cell lines. It has been shown that prodigiosin has a dose-dependent effect on the MM.1S cell line, with an IC_{50} value of 125.8. This effect is only observed at 400µg/mL, where the cell viability was 64.4%, as shown in Figure 6.

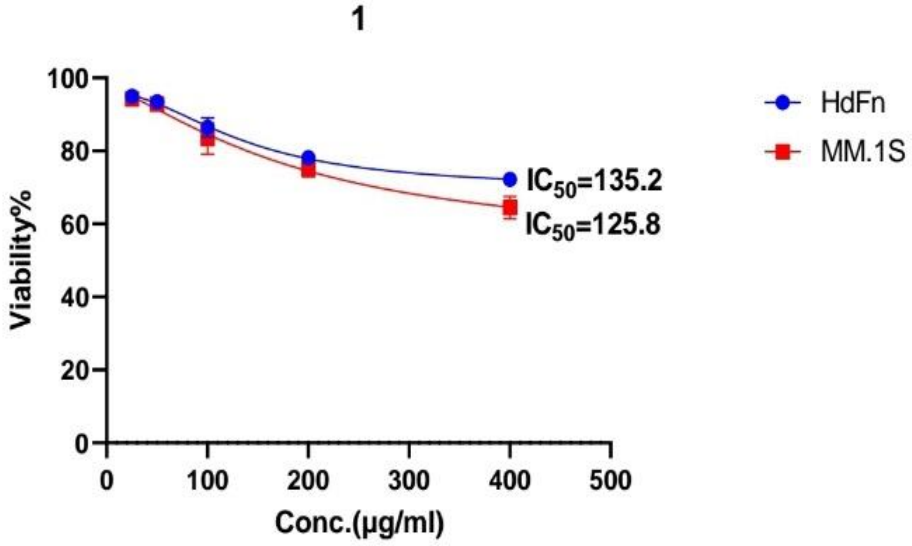

Figure 6: cytotoxic effect of prodigiosin on the MM.1S cell line.

On the other hand, treatment with prodigiosin against the HL-60 cell line showed a high effect, with an IC_{50} value of 94.5 and cell viability of 40.1, as shown in Figure 7.

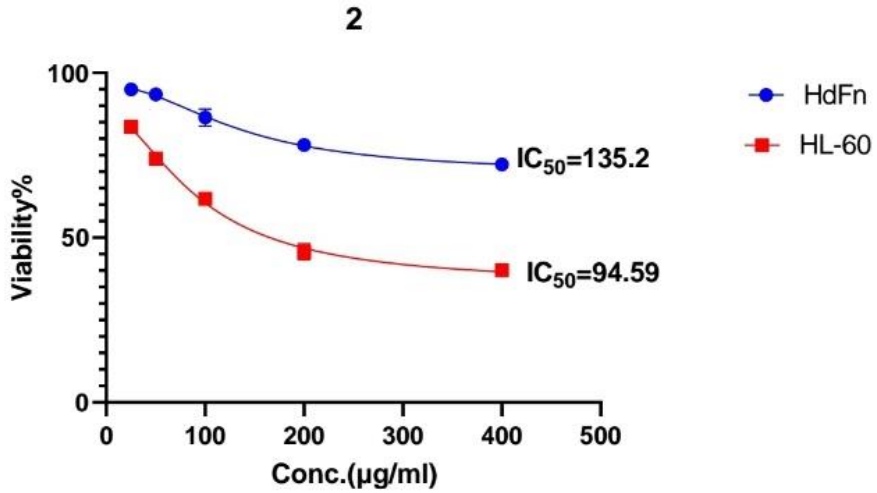

Figure 7: cytotoxic effect of prodigiosin on the HL-60 cell line.

Further, the effect of prodigiosin pigment on the Tp53 cell line showed a high impact with an IC_{50} value of 57.9 and cell viability of 48.2, as shown in Figure 8.

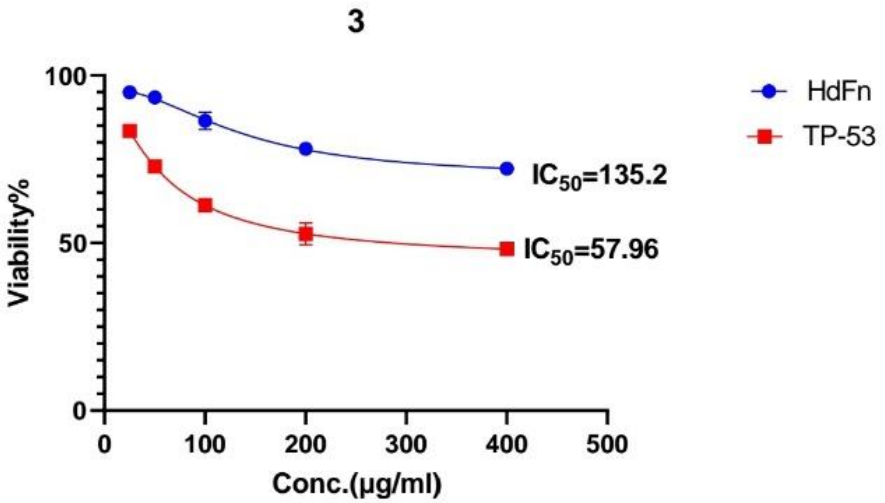

Figure 8: cytotoxic effect of prodigiosin on the Tp53 cell line.

Prodigiosin revealed low cytotoxicity against normal cell line HdFn and had a moderately effect, with an IC_{50} value of 135.2 at a high dose. The findings demonstrated that the vitality of MM.1S cells viability was influenced by prodigiosin only with increased concentrations, while the vitality of Tp53 and HL-60 cells viability was significantly influenced by prodigiosin, which showed that the prodigiosin effect varies between types of cells, and the purified prodigiosin can suppress the cancer cells' growth in a selective pattern. While the non-tumor cell line of humans (HdFn cells) was unaffected by the utilization.

Table 2: Inhibition ratio of tumor cell lines when treated with purified prodigiosin.

	MM.1S		HL-60		TP-53		HdFn	
Conc. µg/mL	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
400	64.4	3.1	40.1	1.5	48.2	1.5	72.1	1.6
200	75.1	2.2	45.6	2.4	52.7	3.2	78.0	1.0
100	83.3	4.2	61.8	1.3	61.2	1.9	86.4	2.6
50	92.7	0.7	73.9	1.8	72.8	1.2	93.4	0.6
25	94.2	0.4	83.6	0.8	83.4	1.9	94.9	0.4

Results in Table 2 showing the cytotoxic activity of prodigiosin, were evaluated across different cell lines, demonstrating variable sensitivity profiles. Notably, TP-53 cells exhibited the highest sensitivity to prodigiosin, with an IC_{50} value of 57.96 µg/mL, indicating a significant reduction in cell viability with increasing concentrations. This aligns with the findings of Campas *et al*., who confirmed that prodigiosin induces apoptosis through TP53-dependent mechanisms, supporting its potential as a targeted anticancer agent [32]. In comparison, HL-60, a human promyelocytic leukemia cell line, showed reduced viability with a slightly higher IC_{50} of 94.59 µg/mL. These results corroborate the study by Lapenda *et al*., which reported high cytotoxic effects of prodigiosin on leukemia cells, suggesting its potential utility in hematologic malignancies [9]. Interestingly, the MM.1S myeloma cell line and normal human dermal fibroblasts (HdFn) demonstrated lower sensitivity to prodigiosin, with substantial cytotoxic effects observed only at concentrations of 400 µg/mL. The IC_{50} value for MM.1S cells (125.8 µg/mL) was slightly lower than that of HdFn, indicating a moderate selectivity of prodigiosin toward cancer cells over normal cells. This relatively high IC_{50} for MM.1S suggests that prodigiosin does not exert a strong cytotoxic effect on this

myeloma cell line, a finding consistent with Oancea *et al*., who observed minimal apoptosis induction in certain multiple myeloma models [33]. Recent studies have highlighted the context-dependent nature of prodigiosin's cytotoxicity. For example, Niakani *et al.,* demonstrated that the anticancer effect of prodigiosin varies depending on the expression of apoptotic regulators like Bcl-2 and caspase-3 [34]. Overall, these findings reinforce the selective cytotoxic potential of prodigiosin, particularly in TP-53 and HL-60 cell lines, while also underscoring its limited impact on MM.1S and normal fibroblasts at clinically relevant concentrations. These results warrant further investigation into the mechanistic pathways involved and the therapeutic window for effective and safe application of prodigiosin in cancer treatment.

Conclusion

The present study demonstrates that prodigiosin, a natural red pigment produced by *S. marcescens*, exhibits selective cytotoxic activity against specific cancer cell lines *in vitro*. Notably, minimal toxicity was observed against the MM.1S cell line at a concentration of 400 µg/mL, whereas a significant inhibitory and cytotoxic effect was recorded against HL-60 and TP53 cell lines across various concentrations. Importantly, the pigment showed no substantial adverse impact on the viability of the normal human dermal fibroblast (HdFn) cell line. These findings highlight the potential of prodigiosin as a promising candidate for targeted anticancer therapies and underscore the need for further research to explore its mechanisms of action and therapeutic applications.

Conflict of Interest

The authors declare that they have no conflict of interest.

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