RESEARCH ARTICLE

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Anticancer and Immunomodulatory Activities of Prodigiosin Extracted and Purified from *Serratia marcescens*

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Abstract

Background: Prodigiosin is a naturally occurring compound produced by various bacteria, including *Serratia marcescens*. It is known for its diverse biological properties. The present study was conducted to extract and purify prodigiosin from *Serratia marcescens* and investigate its anticancer and immunomodulatory activities. **Methods:** S. presence was isolated from soil samples and characterized. Different solvents were used to extract prodigiosin from *Serratia marcescens*. The cytotoxic activity of prodigiosin was tested against human rhabdomyosarcoma (RD), rat embryo fibroblasts (REF), and human breast cancer MDA-MB-231 (MDA) epithelial cell lines. Albino mice were divided into six groups: Negative control (normal saline); positive control (injected with 100 µ l of Serratia marcesence); groups A-D were injected with 100 µ l of prodigiosin (1, 3, 6, and 9 µ g/mouse, respectively). After 14 days of treatment, whole blood samples were collected for immunomodulatory analysis. **Results:** The study found that the highest yield of prodigiosin (65-230 mg/l) was obtained with methanol as the extraction solvent. Prodigiosin had a cytotoxic effect on cancer cells, particularly against MDA epithelial cells. However, it did not have a cytotoxic effect on normal cells. Immunological analysis revealed significant differences ($p \leq 0.01$) in absolute neutrophil counts between the positive control and prodigiosin-treated groups, with the highest value in group C and the lowest in group A. Immunological analysis showed significant differences in neutrophil counts, IL-4, and IL-10 levels between prodigiosin-treated groups and the control group. **Conclusion:** *Serratia marcescens* prodigiosin showed cytotoxic effects on cancer cells and boosted IL-10 and IL-4 serum levels, acting as an immunomodulator.

Keywords: Prodigiosin- *Serratia marcescens*- immunomodulation- anticancer

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Introduction

Serratia marcescens, a member of the Enterobacteriaceae family, is a nosocomial Gram-negative pathogen. The pathogen was previously considered a harmless, non-pathogenic, saprophytic water organism, and its recognized red colonies made it popular as a biological marker [1]. Secondary metabolites of bacterial origin include enzymes, pigments, antibiotics, and other substances that may benefit humans in several ways [2, 3].

Prodigiosin is a secondary metabolite with a wide range of functions. *Serratia marcescens*, *Pseudomonas magneslorubra, Vibrio psychroerythrous*, and other bacteria are known to make prodigiosin [4]. Prodigiosin is a cell wall-associated antibiotic that belongs to the prodiginine family of polypyrrole bioactive compounds [5]. Many terrestrial (soil) and marine bacterial strains, including *Serratia* sp0ecies, primarily *S. marcescens*, produce prodigiosin as a secondary metabolite [6]. Researchers have become more interested in prodiginine compounds over the last thirty years due to their immunosuppressive, anticancer, and antibacterial properties [7].

Prodigiosin has been closely studied because of its anticancer and immunosuppressive activities on a range of drug-resistant cancer cell lines, including MDR1, BCRP, MRP2 [8], and K562 human chronic myelogenous leukemia cells [9]. Prodigiosin, on the other hand, does not affect normal cells and has antibacterial and antifungal properties. More than 60 distinct cancer cell lines have been used to assess the anticancer activity of prodigiosin. The capacity of prodigiosin to inhibit cancer was thought to stem from its ability to induce apoptosis in cancerous cells [10]. Little granules close to the cell nucleus, the nucleus, the cytoplasm, and the mitochondrial membrane have all been reported to contain prodigiosin [11, 12]. Prodigiosin induces cell apoptosis by four distinct mechanisms: signal transduction interference, pH changes in the cell, DNA damage, and cell cycle alteration. When prodigiosin and Cu2+ are present, prodigiosin intercalates into the DNA sequence (prefers the AT sequence) in the tiny groove position, promoting oxidation that damages DNA and causes cell death [13].

The present study aimed to extract and purify prodigiosin from *Serratia marcescens*, and investigate its anticancer and immunomodulatory activities.

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Materials and Methods

Isolation and identification of producer isolates

Soil samples were obtained from several locations in the Diyala orchards in Baghdad, Iraq These samples were serially diluted and inoculated in nutrient broth. The culture was incubated for 24 hours at 37°C. Then, the culture was purified to obtain pure bacterial isolates. A Gram stain was used to detect morphological aspects of colonies in bacterial isolates, including form, size, boundaries, and pigmentation [14]. Biochemical characteristics were employed with the Vitek-2 method to confirm the isolation of S. presence.

Extraction of prodigiosin

Ethanol, methanol, and acetone were used as solvents to extract crude prodigiosin. The culture of S. presence was obtained from the Petri plates and washed with distilled water. The washed pellet was dissolved in 4 mL of each solvent, and the absorbance was measured at 530 nm. After that, prodigiosin was purified by silica gel column chromatography, and the elution was done using deionized water. The fractions were analyzed at the same wavelength, and the active fractions were combined for further assays. The prodigiosin concentration was determined using the previously developed colorimetric technique by [15]. where the absorbance of the supernatant was determined at 530 nm and the prodigiosin concentration was calculated using the following formula:

$$Prodigiosin\frac{g}{L} = \frac{O.D.530x323.4}{7.07x104}\ X\ Dilutedfactor$$

Where 323.4 refers to the molecular weight of prodigiosin (E 530 = 7.07x10^4 M^{-1}cm^{-1} O.D.), 530 refers to the optical density at 530 nm (molar extension coefficient of prodigiosin at 530 nm, E 530 = 7.07x10^4 M^{-1}cm^{-1}), the dilution factor refers to the final volume divided by the volume of the sample.

Determination of the cytotoxic effect of purified prodigiosin
a) Viable cell counting before treatment with prodigiosin

Trypan blue exclusion was used to count the viable cells in the human rhabdomyosarcoma (RD), rat embryo fibroblasts (REF), and human breast cancer MDA-MB-231 (MDA) epithelial cell lines. Under a microscope, it is easy to discern between dead and viable cells because dead cells quickly take up trypan blue compared to viable cells. The procedure was conducted according to [16]. Phosphate buffered saline (PBS; 1.6 mL), trypan blue (0.2 mL), and cell suspension (0.2 mL) were combined. An aliquot of 20 mL of sample was placed on the edge of the slide, covered with a cover slip, and ran into the counting chamber. After 1.2 minutes, counting was completed using a light microscope at 40x objective. Then, the number and percentage of viable and non-viable cells were determined.

b) Viable cell counting before treatment with prodigiosin

The plate of cell cultures in the microtitration was exposed to prodigiosin concentrations throughout the log phase of development, and the effect was assessed following regeneration. The cytotoxicity of prodigiosin was evaluated according to the method described by [17]. After trypsinization for seeding, the cell suspension was seeded in a microtitration plate at a density of 50,000 cells/mL of RPMI-1640 as growth media. At 37°C, the plates were incubated for 24 hours. Serial concentrations (15.62, 31.25, 62.5, 125, and 250 µg/mL) of prodigiosin were made. Only 20 µL of each concentration of cells was pipetted into each well after the cells had been incubated for 24 hours (three duplicates for every concentration being evaluated). Each well of the control group received 20 µL of maintenance media. The exposure times were 24, 48, and 72 hours. The plates were covered in self-adhesive film before being placed back in the incubator at 37 °C.

The media was removed after the exposure period, and the cells were then gently washed with 0.1 ml of sterile PBS. An aliquot of 0.1 mL of maintenance media was added to each well and then incubated at 37°C for 24 hours. Upon completion of the recovery period, 10µl of the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution was applied to each well on the microtitration plate. Then the plate was incubated for 4 hours at 37°C. Using a micro-ELISA (enzyme-linked immunosorbent assay) reader, the optical density of each well was measured at a wavelength of 492 nm.

The reaction was evaluated on living and metabolically active cells, not dead cells, because the tetrazolium ring was cleaved in active mitochondria. The principle is based on the metabolism of mitochondrial dehydrogenases of MTT from a pale-yellow substrate into dark blue insoluble products. The following equation was used to compute the percentage inhibition:

$$Inhibition\ \% = 1 - \frac{Optical\ Density\ of\ Test\ Wells}{Optical\ Sensity\ of\ Control\ Wells}\ X\ 100\%$$

Experimental groupings and treatments

The albino mice were divided into six groups of five, and their weights were measured before treatment. For 14 days, all mice were injected intramuscularly (i.m.). The negative control group was injected with 100 µl of normal saline, while the positive control group was injected with 100 µl of *Serratia marcesence* (1.5×8^{10}cfu/mL). Group A was injected with 100 µL of prodigiosin (1 µg/mouse); Group B was injected with 100 µl of prodigiosin (3 µg/mouse); Group C was injected with 100 µL of prodigiosin (6 µ g/mouse); and Group D was injected with 100 µl of prodigiosin (9 µg/mouse). After 14 days of treatment, whole blood samples were collected in two Eppendorf tubes (EDTA for WBC and ANC and plan tubes to get serum). The serum was kept at -20°C until utilized for immunological assays.

Determination of the immunomodulatory activity of purified prodigiosin Blood cell count

Blood samples were obtained from the albino mice. White blood cell (WBC) count, absolute neutrophil count, and neutrophil percentage were determined according to [18]. White cells can be counted manually or using automatic counters in specially built chambers (Neubauer). The cover slip was placed on top of the grid

area in the chamber, and the blood suspension was diluted with a dilution solution (1:20 for WBC count). Using a micropipette, the hemocytometer (Neubauer chamber) was filled. The counting chamber was then placed on the microscope's stage, and the cells were allowed to settle for 2-3 minutes before counting the number of WBCs in four large corner squares (i.e. in 0.4 cu/mm of diluted blood). The number of cells was determined using the formula:

White Blood Cells Count=Total Cells X 50

Absolute neutrophil count

In cases of morphologic abnormalities, WBC differentiation was done manually. The differential count was determined using the Giemsa stain. A drop of blood was thinly distributed over a glass slide and air-dried. The neutrophil count was computed by multiplying the total WBC count by the percentage of each. As described by [18], the following formula was used to determine ANC:

ANC= (Total WBC X % (PMN_S+ Bands))/100

Where: PMNs: Poly morphonuclear cell

Measurement of cytokine levels in immunized albino mice

ELISA was used to determine the levels of *IL-4* and *IL-10* in male albino mice. Elab Science provided all the ELISA kits. The processes were carried out according to the manufacturer's guidelines. All reagents, standards, and samples were prepared, and before the experiment, the sample was centrifuged again after thawing. The wells were filled with the standard or sample (100 µL) at 37°C, and incubated for 90 minutes. The liquid was drained, and each well was quickly filled with 100 µl biotinylated detection Ab working solution. The preparation was incubated at 37°C for 60 minutes. Then, the solution was aspirated 3 times, and the plate was washed. After that, 100 microliters of HRP conjugate working solution were added at 37°C, and incubated for 30 minutes. It was then aspirated 5 times and the plate was washed. A substrate reagent of 90 µl and a 50 µL stop solution were added and incubated for 15 minutes at 37°C. The plate was immediately read at 450 nm.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS; version 20), and the values are expressed as means ± standard errors of the mean (SEM). Differences between groups were evaluated by analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

Results

Producers of prodigiosin and extract of prodigiosin

Figure 1A and B shows that twelve of the twenty soil samples could produce 65-230 mg/l prodigiosin. Using multiple biochemical tests, the red-colored bacterial strain isolated from Baghdad City, Iraq soil was identified as belonging to the *Serratia* genus. The intracellular bright red pigmentation, fishery-urinary culture specific odor, Gram-negative, rod shape of cells, negative oxidase test, non-lactose fermentation on MacConkey plates, negative indole test, and positive for motility, catalase, and Voges Proskauer tests were the main features. The best producer was *Serratia marcescens* 3. Different solvents were used to extract prodigiosin from *Serratia marcescens* 3 and it was found that methanol was the best solvent for the extraction process, as depicted in Figure 1b. The prodigiosin was then purified by silica column chromatography.

Cytotoxicity of purified prodigiosin

The cell line of the human breast cancer MDA-MB-231 (MDA) epithelium treated with purified prodigiosin revealed an inhibitory effect as a result of treatment with all concentrations of prodigiosin at the three exposure times. The cytotoxic effect was significant ($p < 0.05$) at

Table 1. Percentages of Inhibition on MDA-MB-231 (MDA) Epithelial, the Cell Line of Human Breast Cancer by Prodigiosin during Three Periods of Exposure

Conc.	I.R.± SD			
(µg/ml)	24 hrs	48 hrs	72 hrs	L.S.D
15.62	45.42 ± 2.42	91.56 ±4.70	36.80± 1.40	8.28*
31.25	43.13 ± 2.16	90.38± 4.52	33.69± 1.46	7.93*
62.5	55.65 ± 2.80	86.52± 3.87	36.22± 1.29	7.96*
125	33.15 ± 1.75	63.39± 2.60	25.98± 1.23	6.80*
250	28.56 ± 1.25	46.59± 2.43	23.12± 1.18	6.29*
L.S.D	6.13*	7.98*	5.89*	

I.R., inhibition rate; SD, standard deviation; L.S.D., least significant difference, hrs, hours; P, probability;*: $p < 0.05$

Table 2. Percentages of Inhibition on Rhabdomyosarcoma (RD) Human Cell Line by Prodigiosin during Three Periods of Exposure

Conc.	I.R.± SD			
(µg/ml)	24 hrs	48 hrs	72 hrs	L.S.D
15.62	51.38 ± 2.56	68.24 ±3.41	74.72± 3.62	6.52*
31.25	59.23 ± 2.61	76.19± 4.18	83.96± 3.65	6.93*
62.5	60.65 ± 2.85	63.95± 2.96	75.17± 4.07	5.86*
125	45.03 ± 2.31	42.85± 2.36	56.98± 2.23	5.48*
250	41.28 ± 2.14	44.27± 2.32	50.12± 2.87	6.59*
L.S.D	7.11*	8.26*	7.40*	

I.R., inhibition rate; SD, standard deviation; L.S.D, least significant difference; hrs, hours; P, probability; *: $p < 0.05$

Table 3. Percentages of Inhibition on Rat Embryo Fibroblasts (REF) Cell Line by Prodigiosin during Three Periods of Exposure

Conc.	I.R.± SD		
(µg/ml)	24 hrs	48 hrs	72 hrs
15.62	1.9 ± 0.06	3.5 ±0.13	3.9± 0.16
31.25	4.1 ± 0.21	5.7± 0.24	6.3± 0.23
62.5	6.8 ± 0.31	6.1± 0.27	7.9± 0.37
125	11.6 ± 0.52	9.6± 0.64	10.2± 0.57
250	13.5 ± 0.71	14.3±0.69	14± 0.63
L.S.D	2.67*	3.02*	2.17*

I.R., inhibition rate; SD, standard deviation; L.S.D., least significant difference, hrs, hours; P, probability; *: $p < 0.05$

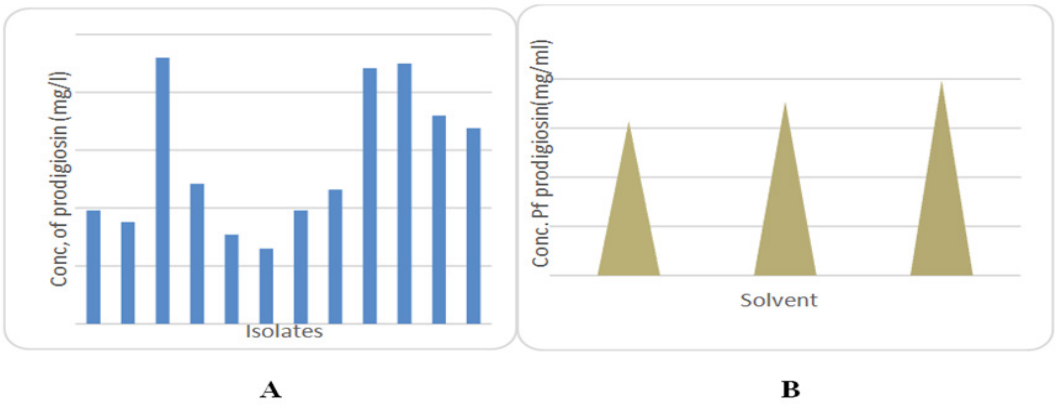

Figure 1. (A), Screening prodigiosin production by Serratia marcescens isolates; (B), Extraction of prodigiosin by different solvents

Table 4: Absolute neutrophil count for the groups under study

Groups	absolute neutrophil count Mean ± S.E cell/mm^3
(-Ve) control	26.18±2.65
(+Ve) control	360±21.85
Group A	33.13± 3.11
Group B	72.32± 9.54
Group C	210.19±6.38
Group D	148.11± 6.27

Group A, 100 µ l of prodigiosin (1 µ g/mouse); Group B, 100 µ l of prodigiosin (3 µ g/mouse); Group C, 100 µ l of prodigiosin (6 µ g/mouse); Group D, 100 µ l of prodigiosin (9 µ g/mouse); S.E., standard error

Table 5. *IL-4* and *IL-10* Titers in the Serum of Studied Groups under Study

Groups	Mean ± S.E pg/ml	
	IL-4	*IL-10*
(-Ve) control	24.43 ± 2.14	58.01 ± 1.78
(+Ve) control	112.03 ± 6.14	88.53 ± 1.49
Treat-A	52.84 ± 3.22	67.71± 1.72
Treat- B	67.30 ± 3.18	69.74 ± 1.10
Treat-C	84.07 ± 2.62	70.69±1.01
Treat-D	23.20± 1.38	72.52± 1.10

Group A, 100 µ l of prodigiosin (1 µ g/mouse); Group B, 100 µl of prodigiosin (3 µg/mouse); Group C, 100 µl of prodigiosin (6 µg/mouse); Group D, 100 µl of prodigiosin (9 µ g/mouse); S.E, standard error; IL, interleukin

all concentrations. The highest percentages of inhibition were achieved after 48 hours of incubation with values of 91.56, 90.38, and 86.52%, for the concentrations of 15.62, 31.25, and 62.5 g/ml, respectively. Meanwhile, the percentage of inhibition dropped after this concentration (Table 1). All doses of purified prodigiosin exhibited a significantly ($p < 0.05$) strong cytotoxic effect on the RD human cell line.

The inhibition percentages reached their peak at 74.72 and 83.96%, 72 hours after treatment at doses of 15.62 and 31.25 g/ml, respectively (Table 2). From the statistical analysis (Table 3), it could be observed that there was not a clear cytotoxic effect for purified prodigiosin on the normal cell line, since prodigiosin at a concentration of 250g/mL revealed a minimal level of growth inhibition without any impact on the normal cells.

Comparison of the inhibitory effect of the purified prodigiosin on RD, MDA, and REF cell lines

According to the statistical analysis, purified prodigiosin had no apparent cytotoxic effect on normal cell lines. Meanwhile, all concentrations of purified prodigiosin showed a cytotoxic effect on cancer cells, with higher effects on the MDA epithelial cell line at 48

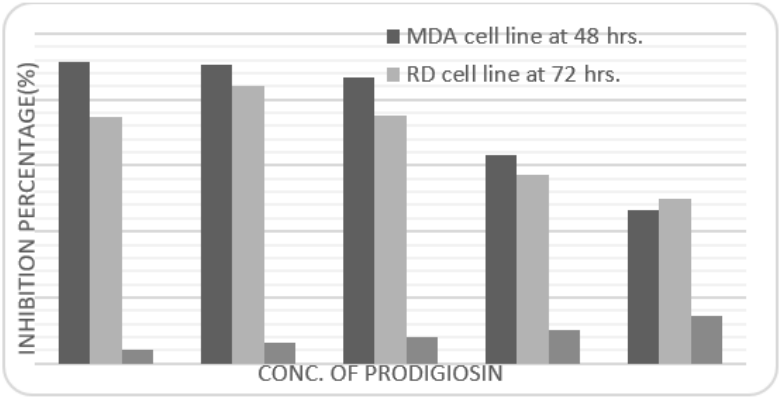

Figure 2. Comparison of the Inhibitory Effect Purified Prodigiosin on MDA-MB-231 , RD and REF Cell Lines

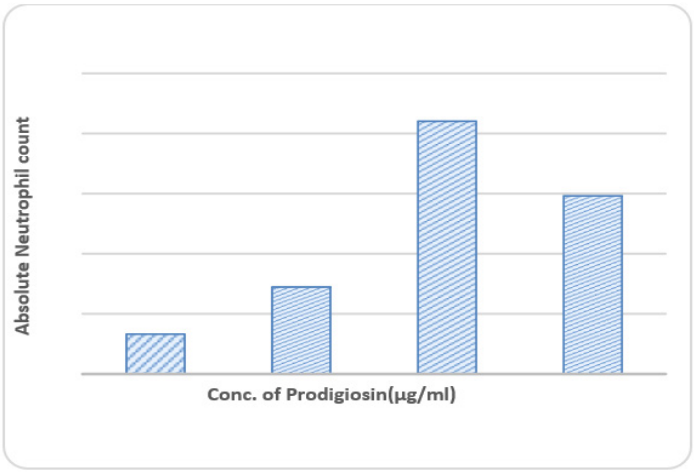

Figure 3. Effect of Prodigiosin Concentrations on Absolute Neutrophil Counts

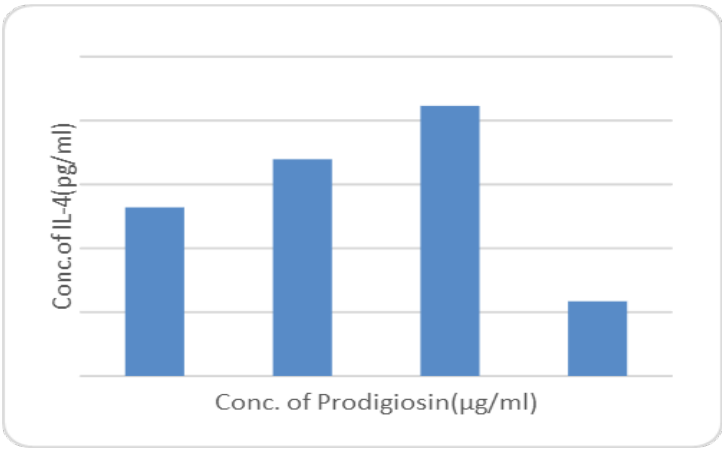

Figure 4. Effect of Prodigiosin Concentrations on *IL-4* Titers in the Serum

hours compared to the RD human cell line at 72 hours as shown in Figure (2). A secondary metabolite red pigment called prodigiosin that *Serratia marcescens* produces has intriguing apoptotic efficiency against cancer cell types while having little to no impact on healthy cells.

Immunomodulatory activity of purified prodigiosin

Absolute neutrophil counts for the study groups treated with different concentrations of prodigiosin, including 1, 3, 6, and 9 µg/mouse, and the control groups are presented in Table 4. The results indicated that the absolute neutrophil count for purified prodigiosin was 33.13, 72.32, 210.19, and 148.11 cell/mm^3, respectively, compared with the controls (Figure 3). Concerning the absolute neutrophil counts, the highest value was recorded in the positive control group injected with *Serratia* presence (360±21.85

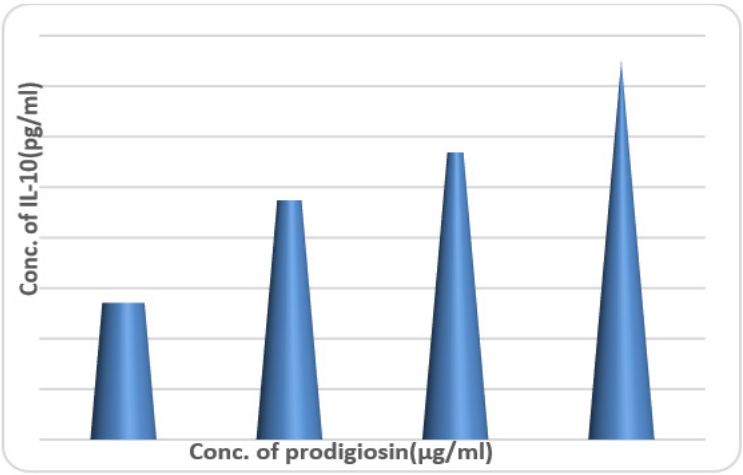

Figure 5. Effect of Prodigiosin Concentrations on *IL-10* Titers in the Serum

cell/mm^3), while in the groups that were treated with prodigiosin, the highest value (210.19 cell/mm^3) was with treatment C (6 µg of prodigiosin), and the lowest value (33.13 cell/mm3) was with treatment A (1 µg of prodigiosin), with a significant difference at $p \leq 0.01$.

The concentration of *IL-4* was evaluated in the serum of immunized male albino mice treated with various doses of purified prodigiosin (1, 3, 6, and 9 µg/mouse). The determination was done using sandwich ELISA according to the instructions from the manufacturer, and the results are highlighted in Table 5 and Figure (4).. The positive control group had the greatest *IL-4* level (112.03 pg/ml), followed by treatment C (6 g prodigiosin) with the highest value (84.07 pg/ml) and group D (1µg of prodigiosin) with the lowest value (23.20 pg/mL), with a significant difference at $p \leq 0.01$. The results (Table 5 and Figure 5) of the *IL-10* level in the serum of immunized male albino mice treated with different concentrations (1, 3, 6, and 9 g/mouse) of purified prodigiosin showed that the titer value of *IL-10* was 72.52 pg/ml for group D (9 µg of prodigiosin), while group A was 67.71 pg/ml, with a significant difference at $p \leq 0.01$.

Discussion

Several investigations have shown that peptone-containing cultures can enhance prodigiosin production [19]. Peptone typically contains high levels of amino acids, which could explain the rise in prodigiosin synthesis [20]. Another study by [21] revealed that methanol led to the extraction of prodigiosin at a higher level. The prodigiosin/PU-H71 combination's therapeutic potential on the MDA-MB-231 cell line was examined, and it was discovered that treating TNBC cells with both medications led to a reduction in several adherent cells with apoptotic effects. HSP90 has been recognized as an important and multimodal target in the therapy of TNBC [22]. LU-1 lung cancer, MCF-7 human breast cancer, and KB carcinoma cells in an *in vitro* study were all highly inhibited by pure prodigiosin [23]. The cancer cell membrane includes permeability properties that enable the entry of secondary metabolites inside the cancer cell, in contrast to the normal cell's efflux system, which is one of the main sources of the cytotoxicity of secondary metabolites [24].

The increasing difference in value between the groups was linked to the immune response's initial signal, which was reflected by numerous pattern recognition receptors, such as Toll-like-2. The A2 and B2 groups activate mice's immune systems. Neutrophils are phagocytic cells with high mobility. It was observed that the ability of these cells to ingest and destroy germs indicated a role in killing. The contents of their many cytoplasmic granules, which were released into the phagocytic vacuole containing the microbe, were assumed to be microbicidal]25]. Also, [26] reported that neutrophils, a type of white blood cell that engulf intruders in neutrophil extracellular traps, a web-like structure of DNA and proteins, are one of the first defense lines of the human immune response. The effect of interleukin-4 on immunological cells includes naive T cell development (into type-2 T and T follicular cells), helpers, the production of B cell antibodies, isotype changing to immunoglobulin E (IgE), basophil and eosinophil growth, mast cell activation, and macrophages [27]. The expression of *IL-10*, a potent anti-inflammatory cytokine that plays a key, and often essential, role in avoiding inflammation and autoimmune diseases, is increasing, indicating that this cytokine is important in immunological and inflammatory responses [28].

In conclusion, the red pigment of prodigiosin produced by *Serratia marcescens* revealed a cytotoxic effect against cancer cells, with a higher impact on human breast cancer MDA-MB-231 (MDA) epithelial and RD human cell lines at low concentrations in a time-dependent manner. Also, prodigiosin acts as an immunomodulator by elevating serum levels of *IL-10* (an anti- inflammatory cytokine) and *IL-4*.

Author Contribution Statement

Sahira Nsayef Muslim (SM): Conceptualization, methodology, data collection and analysis, and writing the draft manuscript; Alaa Naseer Mohammed Ali (AA) and Entkhab M. A. Alanisi (EA): Validation, reviewing the manuscript, and editing; All the authors participated in proofreading the final version of the manuscript.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Study Registration

There is no registration number or support for this project.

Declaration of conflict of interest

The authors declare that they have no competing financial or any other conflict of interests that could have appeared to influence the work reported in this paper.

Ethics committee approval

The study protocol was approved by Ministry of Health and AL-Nahrin center in Baghdad University.

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