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Production of prodigiosin from *Serratia marcescens* and its Cytotoxicity activity

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ABSTRACT

The Cytotoxicity activity of prodigiosin from *Serratia marcescens* (MTCC 97*) were tested under *in vitro* conditions against cancer cell line. The anticancer activity of prodigiosin against HeLa cell was also showed satisfying results. The test was carried at different concentration 0, 10, 20, 30, 40µg/ml and the percentage of cell viability was recorded at 24, 48, 72 and 96 hours. The percentage of cell death was 54% after 96 hours. The IC_{50} value was 35µg/ml for 48 hours. This paper communicates cytotoxicity activity of prodigiosin. However further research are required to study the mechanism of cancer cell line inhibition.

Key words: Prodigiosin, *Serratia marcescens*, cytotoxicity activity, cancer cell line

INTRODUCTION

Microbial products recently been widely used for therapeutic treatment. Such products are called secondary metabolites or bioactive compounds which include pigments, enzymes, steroids and antibiotics. Prodigiosin and Prodigiosin-like pigments are example for bioactive compound produced by many organisms like Gram-negative and Gram-positive bacteria, *Actinomycetes* spp, *Serratia marcescens*, *Vibrio psychrorythrus*, *Pseudomonas magnesiorubra*, and *Alteromonas rubra* are some of the organisms produce Prodigiosin. From *Actinomycetes* spp, *Actinomadura pelletieri*, *Actinomadura madurae*, *Streptoverticillium rubrireticuli*, and *Saccharopolyspora* are novel species. *Serratia marcescens* is ubiquitous in the environment. Cultures can frequently be taken from the mammal body fluids such as blood, urine, sputum, and cerebrospinal fluid, also from sewage water, soil, foodstuffs, animal skins and milk tins etc. *Serratia marcescens* (formerly *Bacillus prodigiosus, -is, -um*), is an aerobic, facultative anaerobic, motile, spore-forming, Gram-negative enteric saprophytic rod-shaped bacterium.(Mody, et. al, 1990).Prodigiosins are red pigmented family naturally occurring (tripyrrylmethane structure) linear tripyrrole ring, Undecylprodigiosin, Cycloprodigiosin, Metacycloprodigiosin, dipyrrolyldipyrromethane depends on various organism. Those are emerging broad spectrum of Compounds having distinct biological activities like antibacterial, antifungal, antiprotozoal (Croft, et. al., 2002), cytotoxic (Nakashima,et al.,2005) antitumour (Castro,1967, Perez-Tomás et al., 2003),antimalarial, antidiabetes, antioxidants, nonsteroidal anti-inflammatory drugs, dyeing of silks and Wools . It also secretes DNAase, lipase, gelatinase, exoenzymes, chitinases, and many extracellular proteases. Most strains secrete catalase, nitralase, lysine, decarboxylase and acetoin. It does not secrete oxidase (Hubert, 1969). The literature reveals that the study on the Cytotoxicity activity of prodigiosin is very scantly, In the present study an attempt was made on prodigiosin production from *Serratia marcescens* and its cytotoxicity activity.

MATERIALS AND METHODS

Serratia marcescens

Serratia marcescens was isolated soil sample and maintained in Germ plasm, Department of Microbiology, Bharathidasan University, Tiruchirappalli-24, India.

Production of prodigiosin

The production of prodigiosin was carried out using different media such as trypticase soya broth, nutrient broth, nutrient glycerol broth medium, peanut broth medium, tryptone yeast extract HIVeg broth and yeast extract malt extract broth medium was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure, Inoculate the culture and incubate for 24 hours at 28°C to identify the growth and pigment production.

Effect of pH and temperature on prodigiosin Production

Trypticase Soya broth medium was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure, Inoculate the culture in different pH ranges from 4 to 10, and incubate for 24 hours at 28°C to identify the growth and pigment production. However, inoculate and incubate the organism in various temperature ranges from 4, 10, 28, 37 and 50°C. Observe the results in Colorimetric analysis at 540nm.

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Extraction of prodigiosin

Acid extraction

The cultural broth was washed off the agar with acetone containing 10% (vol/vol) of 3N-HCL was extracted by rotary shaking for an hour. The extraction was centrifuge at 10,000 rpm for 10 minute. The extracts were collected and the deposited cell mass was washed few times with acetone to remove all traces of acid and washed once with diethyl ether, petroleum ether, Hexane and then dried in a vacuum desiccators. The cells were at first extracted continuously with acetone to remove all traces of pigment and fat. For easy crystallization, it was necessary to remove most of the fatty contaminants. The pure dry sample of pigment was dissolved in 1 ml or less of hot absolute ethanol and was treated with a few drops of 5% (vol/vol) aqueous perchloric acid ahead of slight turbidity formation. The solution was then allowed to cool to room temperature and stored overnight at -10°C. The purple crystals of the perchlorate came out readily and another crop could be obtained from the mother liquor after further concentration and addition of some drops of 1% (vol/vol) aqueous perchloric acid. For recrystallization, the crystals were dissolved in about 0.5 ml. of hot absolute ethanol and the hot solution treated with a few drops of 1% (v/v) aqueous perchloric acid.

Methanol extraction

The culture broth of 500 ml containing pigment was mixed with an equal volume of methanol and kept the mixture in rotary shaker for 20 to 30 minute. The mixture was pour into centrifuge tube and shacked vigorously using a vortex mixer. Then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered through a Wattman number 1 (0.2 µm) filter paper. The filtrate was concentrated using a rotary evaporator and later extracted with 3.0M chloroform. The chloroform phase was collected and reconcentrated using a rotary evaporator to obtain the crude product.

Ethanol extraction

The 24 hour Culture broth containing pigment was pour into centrifuge tube. Then centrifuged at 10,000 rpm for 10 min, cell mass (pellet) was collected and then washed few times with 0.85% saline Buffered at P^H 7 with 0.01M phosphate in which the bacteria were digested by boiling with 1N NaOH for an hour in water bath, pigment was extracted from the digest with addition of equal volume of Absolute ethanol, vortex the mixture well and then once again centrifuged at 10,000 rpm for 10 min, and from ethanolic solution with petroleum ether latter the extract was dried in a water bath, dried sample was kept in deep freezer -10°C for over night to proceed Lyophilization. Red colour powdered pigmented prodigiosin obtained in lyophilized form, stored that in 4°C

Purification of prodigiosin

Column chromatography

Purification The crude product was dissolved in 20 ml of methanol and the solution was passed through a hexane-balanced silica gel column to trap the target product within the column. The loaded column was eluted with 10 M ethyl acetate to liberate the adsorbed product.

Thin layer chromatography

The extraction elute were examined by thin layer chromatography with ratio of (5:4:1), (6:3:1), methanol, ethyl acetate, chloroform, water. As faint spots showed up clearly under UV light which emits fluorescent, solvent mixture for effective separation of the impurities extracted along with the pigment by thin layer chromatography observe RF value And dried in a vacuum drier at 45°C to obtain the purified product (red powder), From this lyophilized sample identified the biological and cancer activity.

Cytotoxity activity test

Dulbeco's modified eagle medium (DMEM) were prepared, prodigiosin compound 1mg dissolved in 100µl of DMSO in different concentration 5, 15, 25, 50 µg/ml. diluted with DMEM. Then the HeLa cell line transfer to DMEM addition of 10% fetal Bovine serum, 1% penicillin and streptomycin solution (to avoid bacterial, fungal contaminant), pour it to 96 well plate containing 2ml medium, $2x10^4$ cells per ml after 24 hours addition of drug, 0.1% DMSO control, blank (without cells), incubate at 5%CO_2 for four days (24, 48, 72, 96) in different time period

Trypan Blue dye exclusion assay

Collect the medium, phosphate buffer solution (PBS) and cells totally centrifuge at 1600 rpm for 10 minute, remove supernatant, resuspended in 3ml PBS and prepare 0.4% Trypan blue 10 µl with 10 µl HeLa cells, take a drop of mixture, placed in cover slip viewed in microscope under 40x to observe the cell death by IC_{50} by MTT assay

MTT Assay

HeLa cells at 2 x 10^5 cells/well in a 96 well plate. Note: this cell number has been optimized for a colorectal tumour cell line; these wells serve as a control for the minimum absorbance, Incubate the plate overnight at 37°C in a humidified incubator, 5% CO_2. Add test compounds to the plate. Include replicates for a range of Concentrations. Include negative controls and a positive control. The final volume will be 100ìl per well. Incubate the plate overnight (or for some other appropriate time) at 37°C in a humidified incubator, 5% CO_2. Add MTT reagent (10ìl/100ìl per well of the 96 well plate). Incubate at 37°C for 3 hours. Add 1 volume (100ìl) of the stop mix solution and rock the plate at room temperature for a minimum of 1 hour. (Allows time for the formazan precipitate to dissolve) The stop mix solution must be added in a fume hood. A purple colour should be visible at this stage and should deepen over the 1 hour incubation period. After the 1 hour incubation, ensure the formazan precipitate is dissolved by pipetting each well up and down until not precipitate is visible. Read the plate on a plate reader using 570nM as test wavelength and 630nM as the reference wavelength. Record data in an excel spreadsheet.

Tabulate results and calculate the percentage viability

$$\% \text{ Viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100$$

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Table. 1. Production of Prodigiosin in different media

S.no	Media	Pigment production at 28°C for 72 hours
1	Nutrient broth	+
2	Lubria's broth	-
3	Peanut broth	++
4	Trypticase soya broth	+++

Note: - = absence of Pigmentat, + = presence of Pigmentation,+ + = slightly Pigmentation,+++ = dark pigmentation.

Table 3. Cytotoxicity Activity of prodigiosin on HeLa cells

Concentration of prodigiosin (pg) µg/ml(pg)	Cytotoxicity Activity of prodigiosin on HeLa cells Time periods (hours) 0	24	48	72	96
0	100	100	100	100	100
5	100	76	73	69	61
15	100	67	61	57	54
25	100	62	58	52	49
50	100	57	53	49	46

Table 2: Effect of pH, temperature and incubation time on prodigiosin from *Serratia marcescens*

S.no	Factors	OD Value at 540 nm
1	**pH**	
	4	0.01
	5	0.04
	6	0.03
	7	0.06
	8	0.06
	9	0.03
	10	0.04
2	**Temperature(°C)**	
	4	0.01
	10	0.08
	28	0.13
	37	0.03
	50	0.05
3	**Incubation time**	**Pigmentation at 28(°C)**
	24	-
	72	++
	120	+++
	168	+++

Note: - = absence of Pigmentation,++ = slightly Pigmentation +++ = dark pigmentation

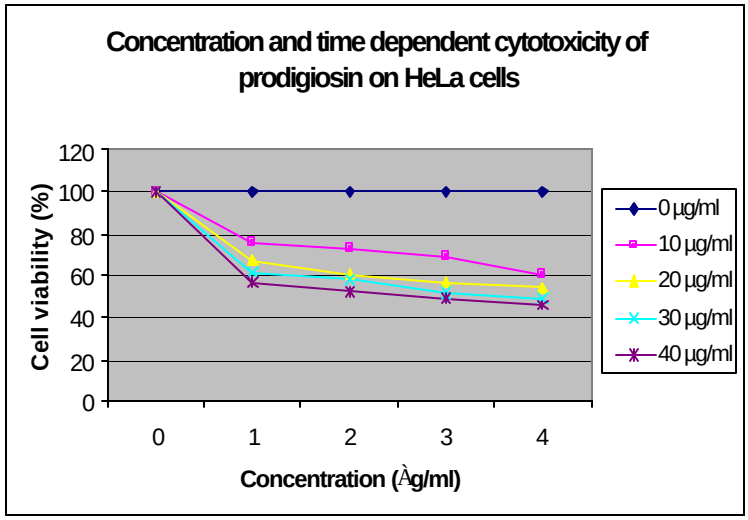

Fig .1.Concentration and time dependent cytotoxicity of prodigiosin on HeLa cells

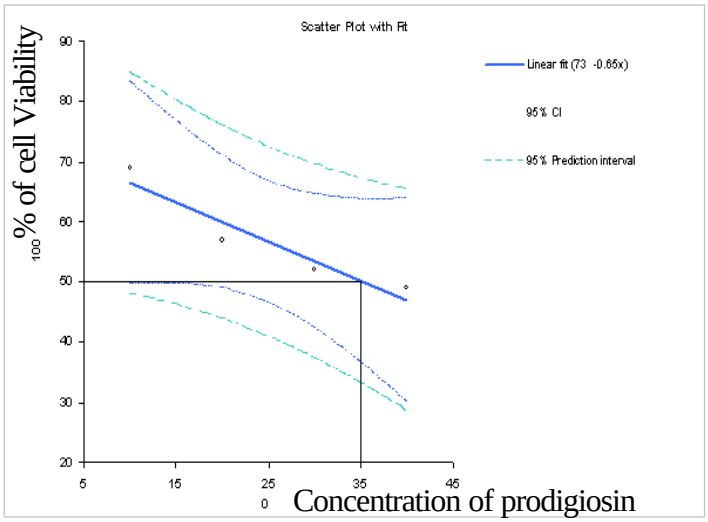

Fig. 2. IC_{50} value of prodigiosin against HeLa cells

RESULTS AND DISSCUSSION

Serratia marcescens are the major producers of prodigiosin production by (Gerber, 1975). The production of prodigiosin was carried out using different media such as trypticase soya broth, nutrient broth, nutrient glycerol broth medium, peanut broth medium, tryptone yeast extract HIVeg broth and yeast extract malt extract broth medium. Based on the prodigiosin production on first, third, fifth, seventh day the medium was selected for optimal prodigiosin production. Among the six medium, only one medium Trypticase soya broth was selected as suitable medium for prodigiosin production (Table 1). This finding is similar to the finding of (Giri *et al*. 2004).method by Hubbard and Rimington (1949). The extracted prodigiosin was purified using column chromatography. The purified prodigiosin was separated by thin layer chromatography Silica gel plate was used, the solvent system was 10M ethyl acetate and methanol at ratio (6:4) was and its Rf value is 0.9 to 0.95, prodigiosin was observed as pink color spot under direct visualization whereas the spot shows fluorescent emitting light under UV light observation. The effect of pH, temperature and incubation period of prodigiosin production was optimized using trypticase soya broth. The production of prodigiosin was evaluated on pH 4 to pH 10 among the pH 7 and pH 8 shows maximum pigment production was observed and facilitate the easy recovery of the pigment whereas in acidic pH 4, 5, 6 and alkaline pH 9 and 10 the production of prodigiosin was observed within the biomass, it is not diffused in the broth (Table 2).

The prodigiosin production was studied using different temperature ranges from 4, 10, 28, 37 and 50°C. Among the various temperatures, 28°C is suitable for the maximum prodigiosin production whereas temperatures 10°C bacterial growth with very slight pigment production was observed. Whereas the temperature 37°C and 50°C bacterial growth was observed. However, it suppresses the pigmentation and produces the white mutant colonies so it completely fails to produce prodigiosin. (Table 2), Alonzo *et al.,* (1979), Sole *et al.,* (1994) were identified optimum pH for prodigiosin production.

The prodigiosin production was evaluated in different incubation time period such as day 1 to 7 days. The significant pigment production was observed in fifth and seventh day of incubation whereas the pigment production completely reduce after seventh day of incubation (Table 2).

Serratia marcescens was grown in Trypticase soya broth at optimized condition 28°C, pH 7 ±0.2 for 5 to 7 days after the production. The pigmented broth was extracted using three different extraction method such as methanol, ethanol and acid extraction method. Acid extraction method was most appropriate than the other extraction The effect of prodigiosin on the viability of different concentration of HeLa cell line was studied. Cell lines were incubated for 24 hours with several doses of prodigiosin, ranging from 1 mg/100ml dissolved in DMSO (5, 15, 25, 50mg/ml) and cell viability was then determined by the MTT assay. A significant dose-dependent decrease in the number of viable cells was observed with an IC_{50}. When HeLa cells were exposed to prodigiosin for different time periods (24, 48, 72, 96) hours and washed in Phosphate Buffer Solution (PBS) and fresh growth medium without prodigiosin was added, the number of viable cells and death cells was identified only by adding Trypan blue suggesting that the effect of prodigiosin observe under 40x in microscope. It was less sensitive to prodigiosin exposure, although showed a constant decrease in its viability. an important decrease was observed in the viability of the HeLa cells, which showed a slight reduction in the number of viable cells. The cytotoxic effect of prodigiosin on these cell lines was found significant (Table 3; Fig1 & 2). The induction of Prodigiosin on lymphocytic leukemia is already reported by Campas et al.,(1990). Díaz et al., (2001) reported the Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. The cytotoxic sensitivity of the human small cell lung doxorubicin-resistant carcinoma (GLC4/ ADR) cell line to prodigiosin was evaluated by Llagostera, et. al.,(2005). Prodigiosin *induces* apoptosis in haematopoietic cancer cell lines was studied by Montaner, et. *al* (2000). The cytotoxicity of prodigiosin on HeLa cells will demonstrates future anticancer drug development for cervical carcinoma.

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