Effect of Purified prodigiosin from *Serratia Marcescens* on the Inhibition of Breast Cancer (MCF-7 and CAL-51 Cell Line)

Taif H. Hassan[1], Sawsan H. Authaman[1], Khawlah J. Khalaf[1]

[1]Department of Biology, College of Science, Mustansiriyah University, Baghdad-Iraq.

Abstract

The prodigiosin pigment was extracted from *Serratia marcescens* bacteria by using different methods such as by using ethyl acetate and acetone and purified by using column cahromatography , Detection and characterization of the pigment was done by using Thin layer chromatography and λ max, The purified Prodigiosin was dissolved in DMSO solvent and prepared in four concentration (1000, 500, 250, 125) µg/ml at exposure time 24 hrs so it was used in treating MCF7 and CAL-51 cell line, the result showed that the inhibition ratio against MCF-7 was significant ($p < 0.001$) and maximum ratio of inhibition was 83% at conc. of 1000 µg/ml , 78% at 500 µg/ml , 73% at 250 µg/ml and 72% at 125 µg/ml while inhibition ratio against CAL-51 was also significant ($p < 0.001$) and 78% inhibition ratio was seen at conc. of 1000 µg/ml , 61% at 500 µg/ml , 27% at 250 µg/ml and 14% at 125 µg/ml , effect of the solvent DMSO on tumor cells also was studied and compared to the effect of prodigiosin and result showed that there was no effect or slightly effect of the solvent DMSO on MCF-7 tumor cells.(Inhibition was 6% ,13% ,16% , 22% for conc. 125 , 250 , 500 , 1000 µg/ml respectively) also there was no effect or slightly effect of the solvent DMSO on tumor CAL-51 cells.(Inhibition was 2% , 8% , 21% , 26% for conc. 125 ,250,500, 1000 µg/ml respectively) .

Keywords: *Prodigiosin, MCF-7, CAL-51, DMSO, λ max*

Introduction

Serratia marcescens are pigmented and produce the red pigment, so the organism has been regarded in medical laboratory experiments as nonpathogenic bacteria [(1)], Prodigiosin, is a group of natural red pigments, the scientist who first gave the name Prodigiosin to the red pigment produced by *Serratia spp.* was Kraft in 1902, after his successes in extraction of pigment from this bacteria [(2)], This pigment is a promising drug therapy due to its reported characteristic of having ant metastatic [(3)], anti-proliferative and immunosuppressive activit [(4)], Prodigoisin pigment induces apoptosis in hematopoietic cancer cells with no marked toxicity in benign cells [(5)], and has cytotoxicity effects on cancer cell and induces apoptosis in HT-29 and T47D cancer cell lines [(6)], it also induces cell death and morphological changes which is a clear signal of apoptosis in gastric cancer cell line HGT-1 [(7)] , which increase the possibility of its therapeutic application as an antineoplastic drug. Prodigoisin appear its content of anticancer compounds [(8)], Prodigioisin pigment was regarded as anticancer agent [(9)], the effect of this pigment was studied on many tumor cell line, the main aspect of this pigment is that its inhibitor effect appears on tumor cells only while not effected normal cells, by its effect on cell cycle arrest and induce programmed cell death that's the cause behind its use as treatment for tumor cells [(10)].

This study aimed to investigate the cytotoxicity effect of prodigiosin pigment on two breast cancer cell line MCF-7 and CAL-51.

Materials and Method

The selected isolate of *S. marcescens*, which isolated and identified in college of science-Mustansiriyah University was inoculated into 250ml flasks containing 50ml of peptone glycerol broth, (pH=8). The culture was incubated at 30 °C for 72 hrs with shaking (160 rpm), and then centrifuged at 10000 rpm for 15 minutes. The

Corresponding author:
Prof. Dr. Sawsan H. Authaman
Department of Biology, College of Science,
Mustansiriyah University, Baghdad-Iraq.
Email: dr.susan28@yahoo.com

resulting supernatant was taken and ethyl acetate was added in equal volume of the supernatant. the resulting pellet was re suspended in 1 ml of acetone in percentage 1:1 (W/V) and mixed gently. Then it was transferred into small tube to mix with the previously supernatant and concentrated in incubator at 40 °C for 24 hrs. The extract was sterilized using membrane filter (0.22 µm) and stored at 4 °C until purification step [11].

Purification of Prodigiosin by column chromatography

After extraction of prodigiosin by ethyl acetate and acetone from ***S. marcescens*** (SKT14) isolate the pigment purified by using column chromatography. The crude extract was dissolved in ethyl acetate and subjected to silica gel (20gm , Merck) column chromatography (2.5×17 cm). The colored fractions were eluted with ethyl acetate, and the individual fractions were evaporated to dryness. Because of the light sensitivity of the pigments, all further purification steps were carried out in the dark. The fraction 7ml were collected and assayed for absorbance at 535nm .The purified pigment was stored at -20 °C. The Prodigiosin peak fraction was pooled and dried at 40 °C to assay pigment concentration [12].

Pigment detection by using thin layer chromatography (TLC)

The TLC plates of silica gel (20×20cm) were used, the developing solvent which contains ethyl acetate. Chloroform and acetone (65:30:5) was standardized and poured into the chromatography tank, that was saturated with a mobile phase. The sample was spotted about 1 cm from the margin of silica sheet then the strip was kept in oven for 5 minutes for drying. then allowed to run up to 3/4th of the gel about 16 cm. After that, the strip was observed under U.V. light for spot observation. The Rf value of chromatography was observed in the TLC plates. The isolated prodigiosin was estimated using the following equation:

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

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

The purified pigment was stored in clean sterile glass tube and covered with aluminum paper away from direct light exposure at 4 °C [13].

Determination of λ_{max} of prodigiosin at different optical density

The pigment was analyzed for maximum UV–vis absorbance at pH values of 2 and 9 between 200 and 700 nm, using ethyl acetate as blank. The absorbance of pigment at different wavelength and λ max was calculated. [14].

Cell line preparation

Two type of cell line were using included MCF7 cell line, this line was used from 170 passage, Primary tumor (invasive breast ductal carcinoma that originate from pleural effusion [11]. Estrogen receptors present [12], and taken from Iraqi center for medical genetics and cancer research. This line was outgrown in RPMI-1640 supplemented with 10% Fetal calf serum, When single monolayer was formed these cells was treated with Trypsin/Versin solution to divide it into another secondary cell culture. other cell line was CAL- 51 cell line , this line was used from 210 passage, is a new mammary adenocarcinoma cell line derived from the malignant pleural effusion of a patient women with metastatic breast cancer. and taken from Iraqi center for medical genetics and cancer research.

Cancer cell line

Development and growth of cancer cells

The effect of prodigiosin pigment on cancer cells was done by using two cell lines. In special culture medium in tissue culture falcon 25 cm^3 at temp. 37 °C, when the confluent monolayer was get Subculture was done by disposal from old culture medium and washing the cells by 2-3 ml of Trypsin/versene solution (Sterilizer and warm) So that it covers the surface of the cells when falcon was put in horizontal mode with shaking gently for 10-15 min. and disposal from it and replace it by another 2 ml from the same solution and falcon was incubated at 37°C for 3-5 min. Then new culture medium was added in quantity about 10-15 ml with homogenization of the cells by mixing with the new medium then cells suspension was distributed in two falcons of the tissue culture falcons in 25 cm size and 5-7 ml in each falcon[15].

Seeding (Cell culture)

Micro titer plates with 96 well were used for cancer cells culture which was obtained after formation of cancer cells monolayer and remove them from falcon surfaces and dismantled by T/V solution , after that completely nutrient culture medium supported by serum (20ml) and incubated at 37°C was added and cells was well mixed with medium ,then 0.2 ml was transferred from cells suspension by pipette from each well , every well contain not less than $1x10^5$ cell, plate surface was covered with sterilized transparent adhesive paper, then micro titer plate was moved gently stirred and incubated at 37°C over night to allow cells attachment and its growth [16].

Treatment of cancer cell line by prodigiosin

Serial of half dilutions was prepared to get concentration (125, 250, 500, 1000) µg/ml dissolved in DMSO, culture medium was poured out from the well of falcons after removing of transparent adhesive paper, then 0.2 ml/well from each conc. was added about 3 well per each conc. and one column of wells regarded as negative control by adding 0.2 ml of RPMI free of serum. All plates were incubated at 37°C, Exposure time was 24 hrs only except for plate of Rat Embryo Fibroblast (REF) Exposure time was 72 hrs [17], after that all plate wells content was poured out and 0.1 ml of crystal violet solutions was added to all wells of the plates. then all plates was incubated at 37 °C for 30 minutes [18], Crystal violet was then poured out then washed by distilled water and turned over and allowed to dryness at room temp. Then O.D was recorded at 492 nm by using ELISA micro plate spectrophotometer and inhibition rate (IR) percentage was calculated as the following equation: [18]

$$\% \, Inhibition \, rate = \frac{O.D \; control - O.D \; te}{O.D \; control}$$

Results and Discussion

1. Detection of prodigiosin pigment by Thin layer chromatography (TLC)

Thin layer chromatography method regard the earliest procedure used for detection and also for purification of prodigiosin, it regard as general method for purify of secondary metabolites products [19].

During Purification of pigment from *S. marcescens* SKT14 , R_f was calculated and the result was about 0.67 as shown in (Figure 1) this result was agree with the result of some researcher which pointed out that the distance between the movement of spot sample to the distance of spot solvent movement was 0.65, while other studies pointed out the R_f was ranged from 0.9-0.95 [20].

Figure 1: Separation and detection of prodigiosin pigment (Purified) from *S. marcescens* SKT14 isolate using thin layer chromatography (TLC).

2. Determine the λ max (U.V Absorption of prodigiosin at different optical density)

The maximum absorption of prodigiosin at different wave length ranged from (200-700)nm, Figure 2 and Table 1 showed two peak of absorbance (535,539nm), maximum absorbance was at the wave length 539nm at pH values 2 and the color was red, while drop in absorbance was found at pH 9 at 400nm and color was yellow. ($p < 0.001$).

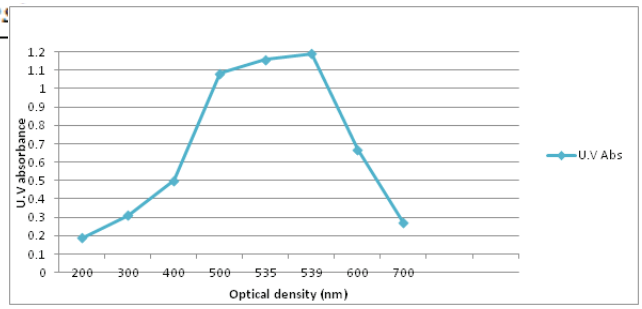

Figure 2: Determination the Lambda max U.V of prodigiosin

pigment

Table 1: lambda max U.V of prodigiosin pigment

O.D 200-700nm	U.V Abs pH=2 Mean±SD	U.V Abs pH=9 Mean±SD	P value
200	0.17±0.02 e	1.02±0.03 a	0.000
300	0.31±0.02 d	0.9±0.04 b	0.000
400	0.54±0.04 c	0.29±0.020 c	0.000
500	1.08±0.001 b	0.25±0.005 c	0.000
535	1.158±0.001 a	0.19±0.02 d	0.000
539	1.19± 0.01 a	0.14±0.01 d	0.000
600	0.67±0.03 b	0.08±0.02 e	0.000
700	0.27±0.02 d	0.02±0.005 f	0.000
P value between the groups	F=93.5 / P value 0.000		-
P value within the groups	F=71.9 / P value 0.000	F=47.09 / P value 0.000	-
*LSD	0.11	0.06	-

**LSD test was used to calculate the significant differences between tested mean, the letters (a, b, c, d and e) represented the levels of significant, highly significant start from the letter (a) and decreasing with the last one.*

3. Effect of prodigiosin on MCF7 cell line

Prodigiosin pigment in four concentrations at exposure time 24 hrs was used in treating MCF7 cell line. As shown in (table 2) inhibition ratio was significant (p value <0.001) and maximum ratio of inhibition was 83% at conc. of 1000 µg/ml, 78% at 500 µg/ml, 73% at 250 µg/ml and 72% at 125 µg/ml . also the table showed that there was no effect or slightly effect of the solvent DMSO on tumor cells.(Inhibition was 6% ,13% ,16% , 22% for conc. 125 , 250 , 500 , 1000 µg/ml respectively).

As showed by some researchers, staphyloxanthin pigment caused inhibition of MCF-7 cell line and the cell viability was 35% when 400 µg/ml of pigment was used [20], The apoptosis and inhibition of cancer cell is dose dependent and that is agree with the result of other researchers which showed that the apoptosis demonstrated a dose-dependent relationship in the early apoptotic cells [21].

Table2: Inhibition ratio of MCF7 cell line when treated at 24 hrs with purified prodigiosin .

Conc. Mg/ml	IC of prodigiosin %	IC of DMSO%	X2 test/P value
1000	83	22	0.000
500	78	16	0.000
250	73	13	0.000
125	72	6	0.000

4 Effect of prodigiosin on CAL-51 cell line

Prodigiosin pigment in four concentrations was used in treating CAL-51 cell line tumor. As shown in (table 3) inhibition ratio was significant ($p < 0.001$) and 78% inhibition ratio was seen at conc. of 1000 Mg/ml, 61% at 500 Mg/ml, 27% at 250 Mg/ml and 14% at 125 Mg/ml. also the table showed that there was no effect or slightly effect of the solvent DMSO on tumor cells.(Inhibition was 2%, 8%, 21%, 26% for conc. 125,250,500,1000 µg/ml respectively). The studies showed that some natural product that extracted from plant exhibited considerable cytotoxicity against Cal-51 triple negative breast cancer cell with even lower IC_{50}values [11].

Table 3: Inhibition ratio of Cal51 cell line when treated with purified prodigiosin .

Conc. µg /ml	IC of prodigiosin%	IC of DMSO%	X2 test/P value
1000	78	26	0.000
500	61	21	0.000
250	27	8	0.001
125	14	2	0.001

Prodigiosin pigment had significant effect against MCF-7 and CAL-51and the P values were 0.01 for 125,250,500 µg/ml while non significant for conc. 1000 µg/ml.

The effect of DMSO solvent on MCF-7 and CAL-51 showing Non significant effect for both (p value 0.64, 0.13) respectively, the p value between the two group was significant ($p <0.01$) , the p value between each conc. was also significant ($p <0.01$) for conc. 125,250,500,1000 µg /ml).

Conflict of Interest: No

Funds: Authors declare there is no fund from any institute

Ethical Clearance: The researchers already have ethical clearance from Department of Biology, College of Science, Mustansiriyah University, Baghdad-Iraq.

References

1. Mahlen SD. Serratia infections: from military experiments to current practice. Clin Microbiol Rev. 2011; 24(4):755-791.
2. Williamson NR, Fineran PC, Leeper FJ, Salmond GP. The biosynthesis and regulation of bacterial prodiginines. Nat. Rev. Microbiol. 2006; 4: 887–899. doi: 10.1038/nrmicro1531.
3. Zhang J, Shen Y, Liu J, Wei D. Antimetastatic effect of prodigiosin through inhibition of tumor invasion. Biochem. Pharmacol. 2005; 69: 407414. doi:10.1016 /j.bcp. 08.037.
4. Chang CCC, Chen W, Ho TFF, Wu HSS, Wei YHH. Development of natural antitumor drugs by microorganisms. J Biosci Bioeng. 2011; 111: 501–511. doi: 10.1016/j.jbiosc.2010.12.026.
5. Montaner B, Navarro S, Piqué M, Vilaseca M, Martinell M, Giralt E, Gil J, Pérez-Tomás R. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Br J Pharmacol, 2000; 131: 585-593. doi: 10.1038/sj.bjp.0703614.
6. Dalili D, Fouladdel S, Rastkari N, Samadi N, Ahmadkhaniha R, Ardavan A. Azizi E. Prodigiosin, the red pigment of *Serratia marcescens*, shows cytotoxic effects and apoptosis induction in HT-29 and T47D cancer cell lines. Nat Prod Res. 2012; 26: 2078-83.
7. Díaz-Ruiz C, Montaner B, Pérez-omás R. Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. Histol Histopathol. 2001; 16: 415-421.
8. Regourd J, Al-Sheikh AA, Thompson A. Synthesis and anti-cancer activity of C-ring-functionalized prodigiosin analogues. J. Med. Chem. 2007; 50: 1528-1536.
9. Murakami A, Dirks M, Boyd P, Hubbard B, Lingwood S, Rutka D, and Verotoxins J. Inhibit the growth of and induce apoptosis in human astrocytoma cells. J. Neurol. Oncol. 1998; 40: 137-150.
10. Deorukhkar AA, Chander R, Ghosh SB, Sainis KB. Identification of a red-pigmented bacterium producing a potent anti-tumor N-alkylated Prodigiosin as *Serratia marcescens*. Res. Microbiol. 2007; 158: 399-404.
11. Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adelaide J, Cervera N, Fekairi S, Xerri L, Jacquemier J, Birnbaum D, Bertucci F. “Gene expression profiling of breast cell lines identifies

potential new basal markers". Oncogene. 2006; 25(15): 2273-2284.

12. Pelz A, Wieland KP, Putzbach K, Hentschel P, Albert-Kand-Götz F. Structure and biosynthesis of staphyloxanthin from Staphylococcus aureus. J. Biol Chem. 2005; 280(37): 32493-32498.
13. Nakashima T, Kato Y, Yamaguchi K, Oda T. Evaluation of the anti-Trichophyton activity of a prodigiosin analogue produced by γ-proteobacterium , using stratum concerum epidermis of the Yucatan micropig . J. infect Chemother. 2005a; 11: 123-128.
14. Patil CD, Patil SV, Salunke BK, Salunkhe RB. Prodigiosin produced by Serratia marcescens NMCC46 as a mosquito larvicidal agent against Aedes aegypti and Anopheles stephensi. Parasitol Res. 2011. 109: 1179-1187. doi: 10.1007/s00436-011-2365-9.
15. Mitra A, Mishra L, Shulin LI. Technologies for deriving primary tumor cells for use in personalized cancer therapy; Trends Biotechnol. 2013; 31(6): 347-354.
16. Freshney RI. Culture of Animal Cells: A Manual of Basic Technique and .John Wiley and Sons, Ins. 2010. USA.
17. Mather JP, Roberts PE. Introduction to cell and tissue culture theory and technique. Plenum Press. 1998. New York.
18. Gao S, Yu BP, Dong WG, Luo IS, Li Y. Antiproliferative Effect of Octreotide on Gastric Cancer Cells Mediated by Inhibition of Akt/PKB and Telomerase. World J. Gastroenterol. 2003; 9(10): 2362-2365.
19. Alam Z, Murkovic M. High-performance thin-layer chromatographic method for monitoring the thermal degradation of β -carotene in sunflower oil. Journal of Planar Chromatography. 2010; 23(1): 35-39.
20. Raj DN, Dhanasekaran D, Thajuddin N, Panneerselvam A. Production of Prodigiosin from Serratia marcescens and its Cytotoxicity activity. Journal of Pharmacy Research. 2009; 2: 590-593.
21. Schmiech M, Lang SJ, Ulrich J, Werner K, Rashan LJ, Syrovets T, Simmet T. Comparative Investigation of Frankincense Nutraceuticals: Correlation of Boswellic and Lupeolic Acid Contents with Cytokine Release Inhibition and toxicity against Triple-Negative Breast Cancer Cells. Nutrients, 2019; 11(10): pii: E2341. doi: 10.3390/nu11102341.