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A comparative study on cytotoxicity and apoptotic activity of pyocyanin produced by wild type and mutant strains of *Pseudomonas aeruginosa*

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ABSTRACT

Nutrient agar medium with various concentrations of cefotaxime was used for isolation spontaneous mutants from wild type strain of P.aeruginosa PHA-1. Eighty-two mutants were successfully isolated with the viable count 52×10^{7}, these mutants were confirmed as spontaneous not physiological adaption mutants by reculture on the same medium. Then, wild type PHA-1 and mutants were examined for production pyocyanin; a blue greenish pigment was clearly noticed on King A medium. Remarkably the mutant strain named S300-8 was distinguished in productivity in comparison with wild type strain PHA-1; the amount of pigment was 56.0667mg/l and 74.53mg/l respectively. In addition, pyocyanin produced by mutant strain S300-8 revealed a potent efficacy against growth of cancer cell line RD; the low concentration of this pigment caused 65% of dead cells after 72hrs of incubation whereas the cytotoxicity was improved by increasing the concentration of pigment with period of exposure time. Moreover, pyocyanin from mutant strain S300-8 induced apoptosis more than wild type PHA-1; the percentage of DNA fragmentation in RD cell was 80% and 73.3% respectively.

Keywords: *P.aeruginosa*, Pyocyanin, Antiproliferation, Apoptosis, Spontaneous Mutants.

INTRODUCTION

The human pathogen *Pseudomonas aeruginosa* produces pyocyanin, ablue-pigmented phenazine derivative, which is known to play a role in virulence [1, 2]. Pyocyanin is produced from chorismic acid via the phenazine pathway, nine proteins encoded by a gene cluster. Phenazine-1- carboxylic acid, the initial phenazine formed, is converted to pyocyanin in two steps that are catalyzed by enzymes PhzM and PhzS. It has been shown that PhzM is only active in the physical presence of PhS, suggesting that a protein-protein interaction is involved in pyocyanin formation [3].Phenazines are associated with antitumor activities [4, 5]. Cells that are actively respiring, such as tumor cells, appear to be more susceptible to reactive oxygene species (ROS) generation caused by pyocyanin. Additionally, pyocyanin known to interfere with topoisomerase I and II activities in eukaryotic cells have been identified [6]. The development of anticancer pyocyanin is very important in biotherapy, thus this work was aimed to isolating pyocyanin-producing mutants from wild type strain of *P. aeruginosa* PHA-1and comparing their toxicity on cancer cell line RD.

MATERIALS AND METHODS

Bacteria and Growth Conditions

The wild type strain of *Pseudomonas aeruginosa* PAH-1 was kindly obtained from Microbiology Laboratory in Department of Biology, College of Science, University of Baghdad, Iraq. This strain was regularly grown on 0.03% cetrimide agar medium at 37°C for 24hrs[7,8].

Isolation of Spontaneous Mutants from Wild Type Strain PHA-1

Serial dilutions from overnight Nutrient broth culture of wild type strain PAH-1 was prepared, 0.1ml of each dilution was transferred and spreaded on Nutrient agar gradient plates [9] containing various concentrations of cefotaxime (100, 200, 400, 600, 800, and 900 µg/l)[10].The plates were incubated at 37°C for 18hrs. After incubation period, the number of growing colonies was counted. In addition, another test was done to investigate that these colonies are spontaneous and not physiological adapted mutants by re-culturing on Nutrient agar antibiotic medium; the colonies which failed to grow were considered as adapted isolates.

Detection of Pyocyanin-Producing *P.aeruginosa* Strains on solid medium

King A medium was prepared according to the method described by Atlas and Snyder (2006)[7].The exponential growth of wild type stain PHA-1 and mutants were separately cultured on medium, then the plates incubated at 37ºC for 48hrs.

Production of Pyocyanin

The bacterial strains, PAH-1 and S300-8, were cultured on glycerol-alanine minimal medium [11] and incubated at 37 °C for 24hrs. After incubation period, the color of medium was changed from white to blue. The production of pigment can be induced by exposure the cultures to the light source for 24hrs at 25°C, this process changed the color of medium to the dark blue [12].

Extraction of Pyocyanin

This pigment was extracted according to the methods described by Al-Azawi(2006)[13] with some modifications: In briefly, chloroform at ratio2:1 (v/v) was added to the production culture and left for 2hrs; the color of chloroform was changed to the blue. Then, the blue chloroform was collected and washed with acidified water, 0.1M HCL, in order to convert the blue pigment to acidic form (red). This acidified layer was then neutralized by adding 1M Tris-base, pH 11.0. The re-extraction of pyocyanin from neutralized form by adding chloroform, this step was repeated several times to extract a large amount of pigment. Thereafter, the acidified water layer was discarded and the pigment was removed from chloroform by adding 0.05M HCL, this step also repeated several times to have pyocyanin with high purity. At last, the water layer was adjusted to pH7.5 by 0.1M NaOH; a needle like crystals were formed in the chiled solution after 2hrs, these crystals were trapped on a 4.5µm filter, washed by water, dried, and weighted.

Cell Culture

A plevic rhabdomyosarcoma is a human cancer cell line (RD) with passages 245-247 was used throughout this study, it was propagated and maintenance on minimal essential medium (MEM) (US Biological, USA). As well as a normal cell line, rat embryo fibroblast (REF), was used as control. The propagation and maintenance of REF carried on RPMI-1640 medium [14].

Measurement of Cytotoxicity of Pyocyanin

The cytotoxic activity of pyocyanin produced by wild type strain PHA-1 and mutant S300-8 was examined according to the inhibition rate (%IR) [15]. Briefly, 2×10^4 exponentially growing cells were seeded in 96- well microcultureplates with various pigment's concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250, 500 µg/l) in a volume of 100µl. After incubation period (24, 48, 72hrs) at 37ºC, a 20µl of MTT [3-(4, 5-dimethlthiazol-2-yl-2-2, 5-diphenyltetrazolium bromide] was added to each well, then the plates were incubated for further 3hrs at 37ºC. The absorbance of each concentration was carried out in triplicate including untreated cell control. The inhibitory rate of cell proliferation was calculated according to equation: %IR= A-B/A×100; where A represents the absorbance of control, while B represents the absorbance of treatment [16, 17].

Determination of IC_{50}
The results of inhibitory rate (%IR) were plotted on X-axis while the concentrations of pigment were plotted on Y-axis. From the linear scale it could be measured IC_{50} of pigment [18].

Quantitation of DNA Fragmentation
RD cells at density of 5×10^5 cell/ml treated with concentration of pigment that obtained from IC_{50} and incubated 48hrs. Then suspended in 0.8ml of 10mM phosphate buffer saline (PBS), pH7.4, and 0.7ml of ice cold lysis buffer. The lysate cells transferred to microfuge tubes and centrifuged at 13000g at 4ºC for 15min to separate fragmented DNA from high molecular weight DNA. Thereafter, the fragmented DNA was suspended in 1.5ml TE buffer, added 10% TCA, and incubated at 25ºC for 10min. The centrifugation was done again at 500g for 15min and the supernatant was calculated. Then, the precipitate was resuspended in 10% TCA and boiled at 100ºC for 15min. Dimethylamine reagent (1ml) was added to the supernatant and incubated at 30ºC for 18hrs. The absorbance was measured at 600nm. The percentage of DNA fragmentation was measured according to formula: OD_{600} of the supernatant/ [OD_{600} of supernatant+OD_{600} of pellet] ×100[19].

RESULTS

Eighty-two mutants were isolated from wild type strain PHA-1; the viable count was 52×10^7 for dilution 10^{-6}. In addition, the results confirmed that these mutants are spontaneous not physiological adaption mutants through their ability to growing on Nutrient agar antibiotic medium.

After that, the ability of PHA-1 and mutants for production pyocyanin was investigated. A greenish blue pigment (pyocyanin) was appeared on King A medium. Then, the amount of this pigment was determinate; the results indicated that wild type strain PHA-1 was able to produce 56.0667mg/l, whereas the mutants exhibited different amount of pyocyanin and only one mutant designated S300-8 was distinguished with high efficiency of pigment's production; it was 74.53mg/l therfore it was selected for further study.

The cytotoxicity of pyocyanin produced by both strains of *P.aeruginosa*, wild type PHA-1 and mutant S300-8, against cancer cell line RD and normal cell line REF was examined. Fig I. revealed that the low concentrations (7.8125-31.25µg/ml) of pyocyanin produced by wild type strain PHA-1 had low toxicity on RD; the inhibition rate was ranged from 28-36% after 72hrs of incubation period. Whereas increasing the concentration of this pigment result in inhibition growth of RD, so %IR of 62.5µg/ml was 54.5% and 55.6% after 48hrs and 72hrs of exposure time, and it can be noticed that the toxic efficiency of this pigment against RD was increased at the highest concentrations (250 and 500µg/ml); the inhibition rate was 60% and 64% respectively after 72hrs.

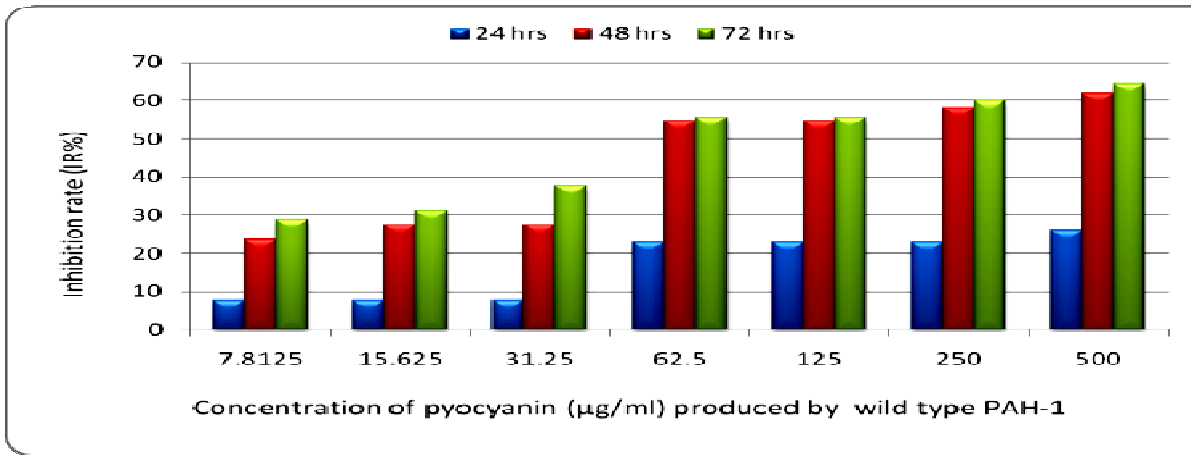

Fig. I. Cytotoxicity of Various Concentrations of Pyocyanin Produced by Wild Type Strain PHA-1 at Different Periods of Incubation on Cancer Cell Line RD

Remarkably, pyoyanin produced by mutant strain S300-8 was more efficient against growth of cancer cell line RD than that produced by PHA-1. Fig. II illustrated that this pigment was able to inhibit growth of RD and achieved

65% of dead cells when treated with low concentration, 7.8125µg/l, especially after 72hrs of incubation. Notable, an increasing of concentration of this pigment with period of exposure time caused increasing the inhibition growth of RD; the highest concentration of pigment, 500µg/ml, showed inhibition rate equal to 88% after72hrs of incubation period.

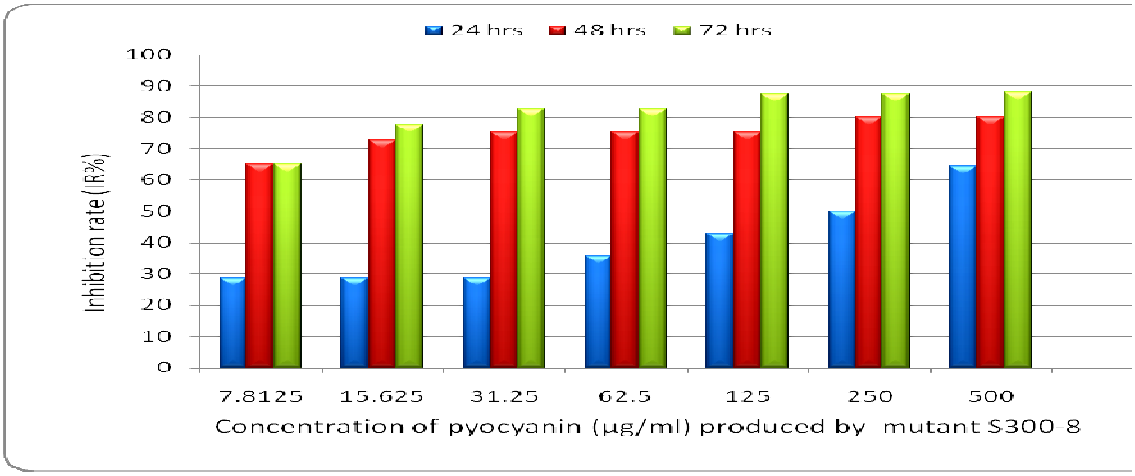

Fig. II. Cytotoxicity of Various Concentrations of Pyocyanin Produced by Mutant Strain S300-8 at Different Periods of Incubation on Cancer Cell Line RD

In contrast, this pigment either produced by PHA-1 or S300-8 had no effectiveness on the viability of REF cells at all period of incubation (Fig. III and IV).

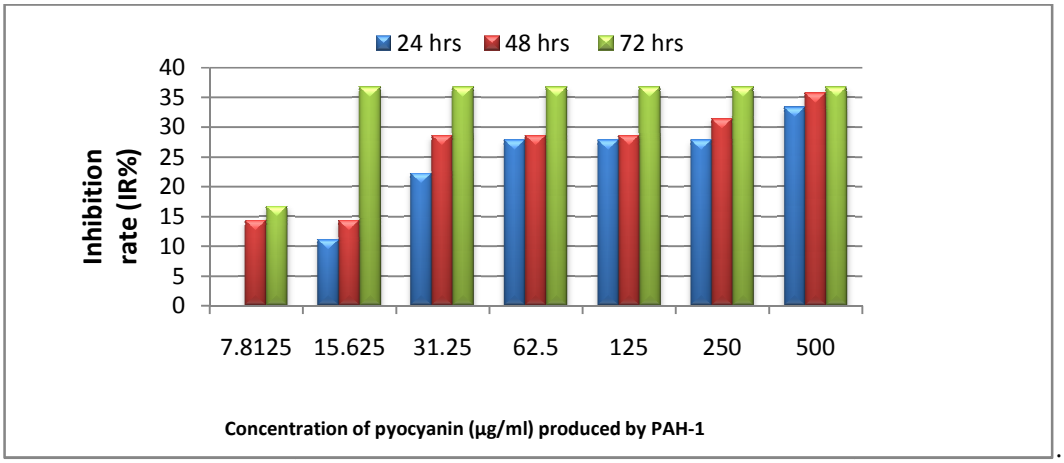

Fig. III. Cytotoxicity of Various Concentrations of Pyocyanin Produced by Wild Type PHA-1 at Different Periods of Incubation on Normal Cell Line REF

According to the measurement of IC_{50}, the concentration of pyocyanin of wild type PHA-1 and mutant strain S300-8 was 57.35 and 225µ/ml respectively, after 48hrs of incubation. Thus the concentrations above were dependent to evalute the apoptotic activity of pyocyanin. The results in Table I indicated that pyocyanin produced by mutant strain S800-3 achieved high percentage of fragmented DNA in RD cells than wild type strain PHA-1; the percentage of fragmented DNA was 80% and 73.3% respectively.

Table I. The Percentage of DNA Fragmentation of Pyocyanin Produced by Wild type and Mutant Strains of *P.aeruginosa*

Pyocyanin Products	% of DNA Fragmentation of Cancer Cell LineRD
Pyocyanin– Producing Wild Type PHA-1	73.3
Pyocyanin- Producing Mutant S300-8	80
Control	17.4

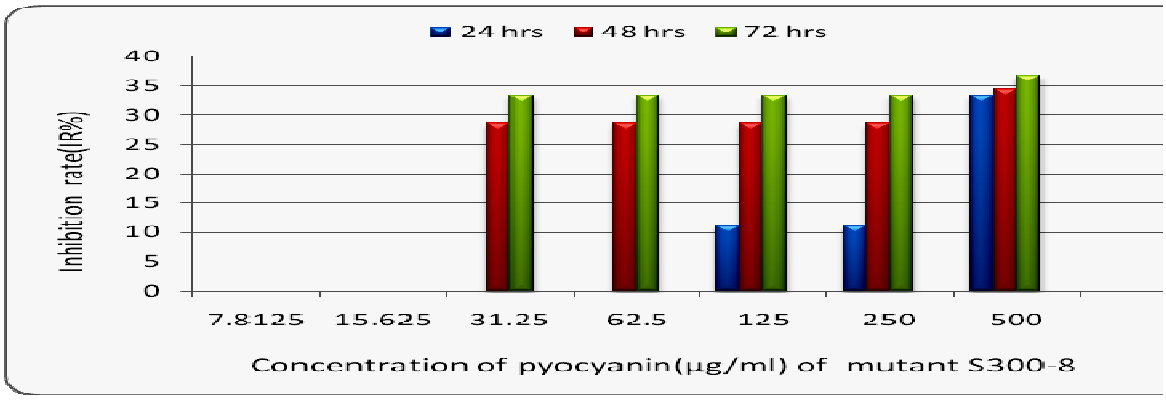

Fig. IV. Cytotoxicity of Various Concentrations of Pyocyanin Produced by Mutant Strain S300-8 at Different Periods of Incubation on Normal Cell Line REF.

DISCUSSION

Many of antibiotics can be used for isolation spontaneous mutants such as rifampicin,chloramphenicol, piperacillin, cephalexin, gentamycin, and cefotaxime [20, 21, 22]. In this work cefotaxime was selected because no effectiveness had noticed on bacterial growth, so our finding showed 82 mutants was successfully grown on nutrient agar medium containing cefotaxime with a viable count 52×10^7. To investigate that these mutants are real spontaneous not adapted mutants, a small part of selected colonies, so as wild type strain PHA-1, was separately streaked on nutrient agar medium containing various concentrations of cefotaxime; the results indicated that only mutants were successfully grown on this medium. Moreover all these mutants were cultured on King A medium and our finding confirmed their ability for production pyocyanin. Nevertheless only one mutant strain, named S300-8, was selected for further study due to the highest production in comparison with wild type strain PHA-1. Indeed, such differences in the productivity may return to the genes encoding pyocyanin which were more active than wild type strain due to the proceeding by strong promoter.

On the other hand, pyocyanin produced by mutant strain S300-8 was more efficient against proliferation of RD cells than that produced by PHA-1. This may suggest that pyocyanin produced by S300-8 may have active group in its structure that let to easily interacts with and penetrate the cytoplasm membrane of RD cells, then control the redox reactions which lead to generation of reactive oxygene species (ROS) that causes cell senescent [23, 24, 25, 26]. Moreover, our findings indicated that pyocyanin produced by mutant strain S300-8 induced DNA breaks in RD cells by cleavage poly (ADP-ribse) polymerase (PARP) which in turn activate caspases such as -9,-8, and -3 and this action indirectly induces apoptosis [27,28].

CONCLUSION

In this work the spontaneous mutants were successfully isolated from wild type strain of *P.aureoginosa* PHA-1by use several concentrations of cefotaxime. One mutant strain named S300-8 was recorded with high productivity of pyocyanin in comparison with wild type strain PHA-1. In addition pyocyanin produced by S300-8 displayed a potent toxicity against cancer cell line RD and its effect was dependent on the concentration and time of exposure, moreover this pigment showed a putative apoptotic activity by DNA fragmentation of cancer cell line RD and elimination the cells that harbor un-desirable DNA sequences.

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