ISSN: 0067-2904
GIF: 0.851

Study the Effect of Pyocyanin Extracted from *Pseudomonas aeruginosa* on DNA Fragmentation of Human Lymphocytes Cells

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Abstract
Eight isolates of *P. aeruginosa* were obtained out of 90 water samples. The isolated colonies were identified based on their morphology and biochemical characteristics, were confirmed as *P. aeruginosa* by the API 20E test system.
The percentages of *P. aeruginosa* recovery in this study were 8.8.% All isolates were able to produce greenish blue pigment (pyocyanin). Pyocyanin at all concentrations was significantly increased the percentage of fragmented DNA of peripheral blood lymphocyte cells compared to control , results showed that DNA fragmentation percentage was higher in concentration 50 µg/ml (70%,74.3%) at 24 hr,48hr respectively. In summary, results of recent study demonstrate that the pyocyanin, induces apoptosis of human peripheral blood lymphocytes .

Keywords: Pyocyanin, *P. aeruginosa*, DNA fragmentation, apoptosis.

دراسة تأثير الصبغة الخضراء المزرقة البايوسيانين المستخلصة من الزائفات الزنجارية على تجزئة الدنا لخلايا الانسان اللمفاوية

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الخلاصة
تم الحصول على ثمانية عزلات من بكتريا *P. aeruginosa* من 90 نموذج ماء الشرب. تم تشخيص العزلات اعتمادا على الصفات المظهرية والكيموحيوية ثم اكد بنظام ابي .نسبة عزل البكتريا في هذه الدراسة كانت 8.8% وجميعها منتجة للصبغة الخضراء المزرقة (البايوسيانين) . جميع تراكيز البايوسيانين حفزت تجزئة دنا الخلايا اللمفاوية مقارنة بالسيطرة وكانت نسبة التجزئة اكبر بتركيز 50 مايكروغرام بالمليليتر(70%و74.3%) عند فترة حضن 24، 48 ساعة . اظهرت نتائج الدراسة الحالية ان البايوسيانين يحفز عملية القتل المبرمج لخلايا اللمفاوية في الدم.

Introduction

Pseudomonas aeruginosa main opportunistic human pathogen that has been known for many years [1]. *P. aeruginosa* produces many types of soluble pigments such as pyocyanin (PCN) and pyoverdin are the most common, other pigments produced are pyorubin (red), pyomelanin (brown) and pyoverdin (yellow/green) [2].pyocyanin consider the first phenazine compound was discovered in nature from *P. aeroginosa* at (1890).Its characterized by thermostable ,pepsin stable and soluble in organic solvents as acetone , Phenol and its rapid soluble in chloroform for this reason used in extraction and purification of PCN [3].

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Pyocyanin causes a wide spectrum of cellular damage such as the inhibition of cell respiration, ciliary function, epidermal cell growth, and might contribute to persistence of *P. aeruginosa* [3], also disruption of calcium homeostasis [4] Pyocyanin and many of Phenazines compounds have been investigated as potential anti-cancer agents [5].

The human pathogen *P. aeruginosa* produces pyocyanin, ablue-pigmented phenazine derivative, which is known to play a role in virulence [6] Phenazines are associated with antitumor activities [7]. 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 [8].

Apoptosis was expressed by measuring the percentage of fragmented DNA. Therefore, the ability of pyocyanin to induce apoptosis of human peripheral blood lymphocyte cells at various concentrations of pyocyanin and two time of exposure was studied in this recent study.

Materials and methods:

Collection of samples:

A total of 90 samples of drinking water collected from water suppliers consumed in Baghdad at different regions included: Al-Sader city, Orfaly, Kadhimiya, Askaan, Karadah, and Zafaraniah. Prior to collection, water was allowed to run at a uniform rate for 2–3 min., in a sterile bottles containing sodium thiosulphate to a final concentration of 0.01% (w/v) to neutralize any free or combined residual chlorine. [9] .

One liter of water sample was collected in each bottle; the samples were carried out to the laboratory in special aseptic cool box. All the samples were analyzed within 24 hr.

Isolation

The samples were analyzed to investigate the presence of *P. aeruginosa*. Membrane filtration technique was applied. One hundred milliliter were filtrated using cellulose membrane filter of 0.45 µm pores (Millipore) and filters were transported on to selective culture media and incubated for 72 hr at 30 C°. pseudomonas agar base supplemented(Himedia,india) with 200 mg/1 cetrimide and 15 mg/1 nalidixic acid was used for detecting of *P. aeruginosa* .[10].

Identification

The suspected colonies were subcultured on Tryptic Soy Agar for 24 hr at 30 C°. All the colonies were tested for colony morphology. The l bacterial isolates were examined morphologically by Gram stain and subjected to biochemical testes [11,12] for further confirmation, API 20E were used in diagnosis as in (Biomerieux) France.

Pyocyanin detection

All *P. aeruginosa* isolates were cultured on King A medium and incubated at 37°C for 48hrs; then incubated at room temperature for one week; the isolates that shown greenish blue pigment refers to positive result of pyocyanin production were selected [13].

Pyocyanin production

Pyocyanin produced isolates were cultured in pseudomonas broth to increase pyocyanin production and incubated at 37°C for 24hrs, then cooling centrifuge at 10000 rpm for 10 min ,the supernatant was used and get rid the deposit. Pyocyanin extracted by adding 3 ml of chloroform (1:2 ratio) and then re extracted by adding 1ml of 0.2M HCL to give pink to deep red color solution in the upper layer , the top layer (0.2 M HCl) was removed and the solution was measured at 520 nm. The concentrations expressed as micrograms of pyocyanin produced per milliliter of culture supernatant and determined by multiplying the optical density at 520 nm by 17.072 [14] , then different concentrations of pyocyanin were prepared(5,15,25,50 µg/ml).

Isolation of human peripheral blood lymphocyte cells

Isolation of human peripheral blood lymphocyte cells were isolated from human blood by procedure as previously described by Shubber and Allak [15], with few modifications as in the following steps:

Blood specimens were collected from healthy volunteers, who had not taken any drugs for at least two weeks before the experiments, into heparinized tubes (20 U/ml).

A volume of 500 µl of heparinized blood was cultured in complete RPMI-1640 medium (Sigma), mitogen (Phytohemagglutinin (PHA) from the Iraqi center for Cancer and Molecular Genetics Research) and four concentrations of pyocyanin (50, 25,10,5 µg/ml) were used in sterile silicon coated tubes. Cultures were incubated for two periods 24 and 48 hr.

DNA fragmentation analysis

Apoptosis of peripheral blood lymphocyte cells was evaluated through DNA fragmentation, DNA fragments resulted from isolated lymphocyte cells was extracted according to the method described by Fernandez-botran [16] as clarified in the following steps:

1- After the end of incubation periods (24 and 48 hr), the tubes were centrifuged at 1000 rpm for 10 min - (this tubes were labeled with litter A).
2-The supernatant was removed to another tube and were labeled with letter (B).
3- One ml of TritonX-100 Ttris EDTA (TTE) solution was added to pellet in the tubes (A) and shaked well.
4- The tubes (A) were centrifuged at 14000 rpm for 10 min (in 4°C).
5- The supernatant of tubes (A) were removed to another tubes and were labeled with letter (C).
6- One ml of TritonX-100 Ttris EDTA (TTE) solution were added to the pellet in the tubes (A).
7- One ml of Trichloro acetic acid TCA (25%) were added to the tubes (A,B,C) and shaked well.
8- The tubes were incubated overnight at (4°C).
9- At the end of incubation period the tubes were centrifuged at 14000 rpm for 10 min in order to precipitation of DNA.
10- The supernatant was discarding.
11-Three hundred and twenty µL of Trichloro acetic acid TCA (5%) were added to the tubes and were put in water bath at 90°C for 15 min.
12- After the end of water bath 640 µl of Diphenylamine reagent were added and were shacked the tubes gently and incubated them over night at 37°C the color will change to blue.
13- Reading of O.D at 600 nm with spectrophotometer.
14- The DNA fragmentation percentage measured as formula:

$$F\% = \frac{B + C}{A + B + C} \times 100$$

F%: DNA fragmentation percentage
A: O.D of A tubes
B: O.D of B tubes
C: O.D of C tubes

Results and discussion

Isolation and Identification of *P. aeruginosa*

Eight isolates of *P. aeruginosa* were obtained out of 90 water samples. The isolated colonies were identified based on their morphology and biochemical characteristics, and confirmed as *P. aeruginosa* by the API 20E test system.

The percentages of *P. aeruginosa* recovery in this study were 8.8 %(8 positive *P. aeruginosa* water samples), other researchers studied the present of *P. aeruginosa* in drinking water [17, 18].

The occurrence of *P. aeruginosa* in supplied water is considered as quality indicator. Drinking water has been suggested as an important source of human infections caused by the members of *Pseudomonas* . From health risk point of view, the large distribution of *Pseudomonas* spp. and as the main components of heterotrophic bacteria in supplied water and their increased resistance to clinically available antimicrobial agents has been considered as health concern particularly to immunocompromised patient and because there is the risk of transferring the resistance to other bacteria present in the human body and some pathogenic ones [19 , 20] .

Pyocyanin detection

King A medium was used for pyocyanin production, its contains salts (potassium and magnesium) in sufficient concentrations to accelerate the production of pyocyanin through enhance the genes encode of this pigment, and suppress production of fluorescein (pyoverdin)[21].

In this study, all isolates were able to produce greenish blue pigment (pyocyanin) when they allow growing on king A medium, on king A medium, *P. aeruginosa* was grown with production of pyocyanin which is considered a unique character for *P. aeruginosa* [22].

Mohammed, [23] who found that approximately 90% of their isolated *P.aeruginosa* isolates produce pyocyanin, sixty isolates from sixty-six of the total isolates produced pyocyanin.

Pyocyanin production

The pyocyanin produced from *P.aeroginosa* was variable from one isolate to another, the sample that given the higher productivity of pyocyanin was choosen .In table 1. The results observed that the

higher concentration of pigmen tfound in isolate P5 according to the optical density which determined by multiplying the optical density at 520 nm by 17.072 [14] and concentration of pyocyanin expressed as micrograms of pyocyanin produced per milliliter of culture supernatant.

Table 1-Pyocyanin concentrations determined by absorbance density at 520 nm

Isolates	O.D520 nm	Concentration µg/ml
P1	0.0992	1.69
P2	0.0632	1.08
P3	0.1442	2.462
P4	0.2733	4.666
P5	**0.3155**	**5.386**
P6	0.1444	2.465
P7	0.2772	4.732
P8	0.0833	1.422

Isolation of peripheral blood lymphocyte and DNA fragmentation assay

These cells were isolated from human blood by procedure as previously described by Shubber [15], and were used in DNA fragmentation assay.

Pyocyanin induces apoptosis of peripheral blood lymphocyte cells treated with tested pigment at various concentrations and incubated at two periods (24 and, 48).

The results showed that pyocyanin at all concentrations was significantly increased $p \leq 0.05$ the percentage of fragmented DNA of peripheral blood lymphocyte cells compared to control figure 1 and 2 results showed that DNA fragmentation percentage was higher in concentration 50 µg/ml (70%,74.3%) at 24 hr,48hr respectively.

In this study, the results showed that this pigment was induced apoptosis in a concentration-dependent fashion but in time, fashion is not significantly, because the activity of pigment did not depend on the time of exposure (24hr, 48 hr).

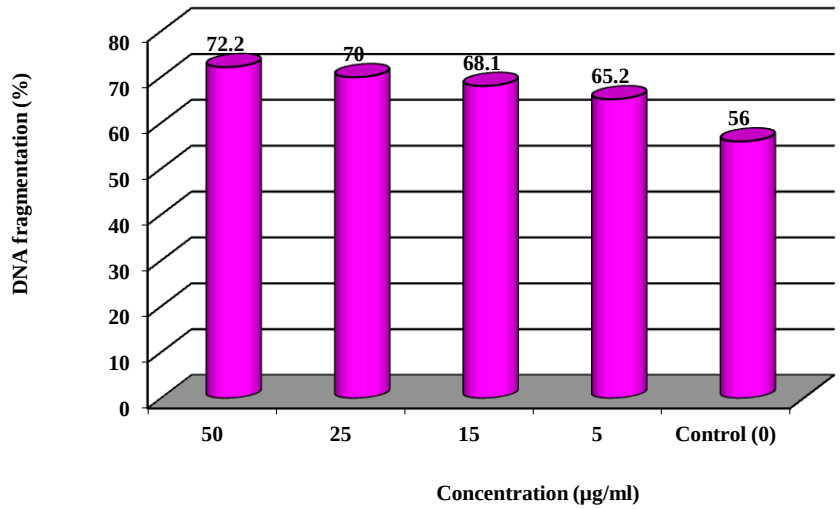

Figure.1-DNA fragmentation of peripheral blood lymphocyte cells after exposure to different concentrations of pyocyanin at incubation period 24 hr.

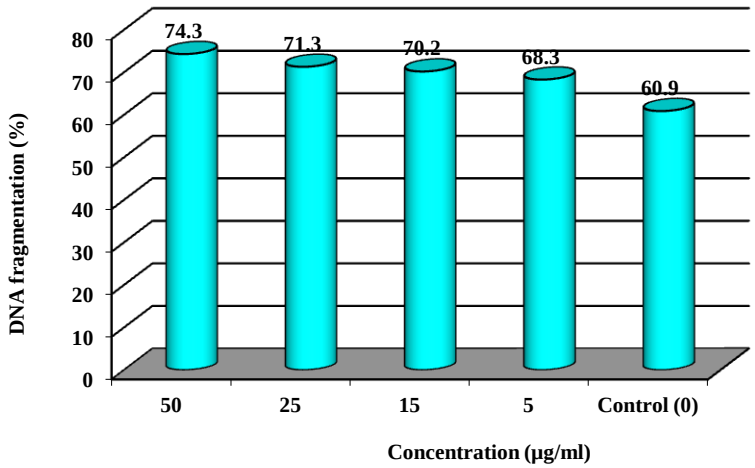

Figure 2-DNA fragmentation of peripheral blood lymphocyte cells after exposure to different concentrations of pyocyanin at incubation period 48 hr.

Pyocyanin is a blue, secondary metabolite with the ability to oxidise and reduce other molecules [24] Due to its redox-active properties, pyocyanin generates reactive oxygen species. Additional to these effects, another target of pyocyanin is caspase 3-like proteases, which can then go on to initiate apoptosis and necrosis. The cell cycle can be disturbed by the action of pyocyanin as well as hinder the proliferation of lymphocytes [3]. This is done by the generation of reactive oxygen intermediates such as hydrogen peroxide and superoxide which cause oxidative stress by damaging DNA directly or alternatively targeting other constituents of the cell cycle such as DNA recombination and repair machinery[25].

Usher *etal*., [26] also found that the predominant phenazine pigment pyocyanin, induces apoptosis , which is associated with rapid reactive oxygen intermediate (ROI) generation.

Pyocyanin exerts its toxic effects upon mammalian cells and other bacteria [27], was found to be important mediators of death of these organisms [28]. Pyocyanine has dual dose-dependent stimulatory as well as inhibitory effects on immune responses in vitro as measured by DNA synthesis of human T and B-lymphocytes [29].

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