The Cytotoxicity Effect of Pyocyanin on Human Hepatocellular Carcinoma Cell Line (HepG2)

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Abstract

Ninety eight specimens were collected from patients referring different hospitals in Baghdad in period from August to November in 2012. Specimens including (swabs from (Sputum, burn, wound, urine, ear, and eye).Sixty six isolates were identified as *Pseudomonas aeruginosa*. The isolates were identified according to morphological, cultural, biochemical characteristics and API 20E test. 90% of *P. aeruginosa* isolates produced pyocyanin pigment on King A medium in different amounts, whereas other isolates were produced other types of pigments such as (pyoverdine-yellow, pyorubin-red, and pyomelanin-black) on King B medium and also in different amounts.Quantitative assay of pyocyanin production was conducted. The results were shown that the isolate of sputum from cystic fibrosis (CF) was greatest in production of pyocyanin. Pyocyanin purified and thier cytotoxicity effect on HepG2 cell line were studied ***in vitro***. The results were shown that pyocyanin has cytotoxic effect on HepG2 cell line caused inhibition in growth of the cell line, the inhibition rate ranged from 7% to 84%.

Key word:cytotoxity, pyocyanin, HepG2cell line

التاثير السمي للبايوسيانين على خطوط خلايا الكبد السرطانيه

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الخلاصه

جمعت ثمانيه وتسعون عينه من مرضى في مستشفيات بغداد المختلفه خلال الفتره من شهر اغسطس الى نوفمبر في 2012 كانت تشمل عينات من (القشع ,الحروق,الادرار, العين والاذن).سته وستون عزله شخصت على انها بكتريا الزائفة الزنجارية,حسب الطرز المظهريه وشكل الزرع والفحوص البيوكيميائيه وفحص الابي 20 ,90% من العزلات كانت منتجه لصبغه البايوسيانين على وسط كنج آ بكميات مختلفه, بينما العزلات الاخرى انتجت صبغات اخرى (بايوفردين الصفراء, بايوروبين الحمراء, بايوميلانين السوداء) على وسط كنج ب ,تم حساب كميه انتاج صبغه البايوسيانين, اظهرت النتائج ان عزله من القشع لمريض التليف الكيسي كانت الاعلى انتاجا للصبغه, تم تنقيه صبغه البايوسيانين.واختبرت سميه بايوسيانين المنقى علىى خطوط خلايا الكبد السرطانيه اظهرت النتائج ان للبايوسيانين تاثير مثبط على نمو الخلايا السرطانيه,كان مدى نسبه التثبيط تتراوح من7%الى84 %

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Introduction

Pseudomonas aeruginosa main opportunistic human pathogen that has been known for many years [1]. Gram negative bacteria, their cells are straight or curved shapes but not helical with a diameter (0.5-1.0) × (1.5- 5.0) µm Motile by one or several polar flagella rarely non motile, Lateral flagella of short wavelength may also be formed, Aerobic bacteria, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, in some cases nitrate can be used as an alternative electron acceptor, allowing growth to occur anaerobically Its optimum temperature for growth is 37ºC, and it is able to grow at temperatures as high as 42ºC,Indeed, the ability to grow at 42ºC that distinguishes it from many other *Pseudomonas* spp. No growth occurs at 5ºC [2, 3]. *P. aeruginosa* was identified based on the colony morphology, gram staining, oxidase reaction, the production of pigments, nitrate reduction, use of citrate and malonate as carbon sources, and its ability to grow at 41°C±1; Most strains fail to grow under acid conditions (pH 4.5) [4]. *P.aeruginosa* also demonstrates the most consistent resistance to antimicrobial agents of all the medically important bacteria [5]. It has a natural resistance mechanism to many antibiotics because of a resistance transfer plasmid that code for proteins that destroy antibiotic substances [6]. It can become resistant through mutations in the chromosomal genes which regulate the resistance genes [7]. *P. aeruginosa* produces many types of soluble pigments such as pyocyanin and pyoverdin are the most common, other pigments produced are pyorubin (red), pyomelanin (brown) and pyoverdin (yellow/green) [2].

Pyocyanin The blue, redox-active phenazine pyocyanin is secreted by *P. aeruginosa* [8]. Belonging to the group of phenazine compounds, low-molecular weight, heterocyclic compounds with redox active properties, produced by *P.* aeroginosa, it is permeable to biological membranes [9], secondary metabolites [10], pyocyanin can exist in either oxidized or reduced form [11].

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* [12], also disruption of calcium homeostasis [13]; pyocyanin lead to increases the release of the neutrophil chemokine IL-8 from lung epithelial cells and up regulates the expression of the neutrophil receptor intracellular adhesion molecule (ICAM)-1 both in vitro and in vivo; these proinflammatory effects were blocked by treatment with antioxidants [14,15]. Pyocyanin inhibits proliferation of human dermal fibroblasts and induced premature senescence, even at low concentrations [16]. The aim of research is to study the effect of pyocyanin on Hepatocellular carcinoma cell line (HepG2).

Materials and Methods

Bacterial isolate

Ninety eight specimens were obtained from different source (ear, burn, wound, urine, sputum, and eye), were collected from patients referring different hospitals in Baghdad, The collected specimens were inoculated on MacConkey agar. To select pale colony formed, then sub-cultured on Cetramide agar to obtain pure colonies, this isolates diagnosed by morphological and biochemical tests, and re-confirmed by the API 20E profile index [17,18].

Pyocyanin detection

All *P. aeruginosa* isolates were cultured on King A **and** King B 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 [19].

Select the best isolates in pyocyanin production

Pyocyanin produced isolates were cultured in pseudomonas broth to increased pyocyanin production and incubated at 37C for 24hrs, then cooling centrifuge at 10000 r for 10 the supernatant was used and get rid the deposit. Pyocyanin extracted by adding 3 ml of chloroform and then reextracted 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 the solution was measured at 520 nm. Concentration expressed as micrograms of pyocyanin produced per milliliter of culture supernatant were determined by multiplying the optical density at 520 nm by 17.072 [20].

Production of Pyocyanin

pyocyanin produce isolates were cultured on glycerol-alanine minimal medium agar and incubated at37 °C for 24hr, the color of medium was changed from white to blue. The production of pigment can be induced by exposing the cultures to the light source for 24hrs at 25°C, to change the color of medium to dark blue [21].

Extraction and purification of pyocyanin pigment

Chloroform at ratio2:1 (v/v) was added to the production culture and left for 2hrs; the color was changed to the blue. Then, the blue layer was collected and washed with acidified water (0.1M HCL), in order to convert the blue pigment to (red).The acidified layer then neutralized by adding 1M Trisbase (pH 11.0). The re-extraction of pyocyanin from neutralized form by adding chloroform, this step was repeated for several times to extract a large amount of pigment. Thereafter, the acidified water layer was discarded and pigment 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 [22] for further purification highly Preparative Fast liquid chromatography (HFLC) was used to separate pyocyanin. It was done by using a column with ***3***µm particle size 50×4.6 mm I. D, C-18, the mobile phase contained 0.01M ammonium phosphate buffer A and acetonitrile B was eluted by linear gradient from 0-100% in 10 min, it was detected by UV set at 254 nm with flow rate 0.9ml/min and temperature 30°C, the purity was determined by comparing with standard pyocyanin [23].

Cell line Culture

HepG2 Cell Lines is a human cancer cell line was used in this study, it was propagated and maintenance on minimal essential medium (MEM) according to (US Biological, USA), Single cell suspension of HepG2 was prepared by treating 75 cm^3 tissue culture flask with 2 ml trypsin solution incubated for 2 min at 37°C in an incubator supplemented with (5%) CO_2 until the cells detached from the flask surface single cell suspension made by gently taping of the f,8 ml of growth medium supplemented with 10% fetal calf serum added to the flask to stop trypsin effect, collect the cells in centrifuge tube then centrifuge 1500 rpm for 10 min, discard the supernatant, test The viability of the cells by using trypan blue dye which stains the dead cells, in same time count the cells for proper cell dilution to seed the 96 flat well plate, 20 ml Of MEM made, mixed well, followed by transferring 200µl/well to the 96 well plate using automatic micropipette containing (10^4 cell/well), Plate incubated overnight at 37°C in an incubator supplemented with (5%) CO_2 until 60-70% confluence of the internal surface area of the well for HepG2 cell line [24].

Cytotoxic Assay of Pyocyanine on Hepatocellular carcinoma human (HepG2) Cell Line

The cytotoxic activity of pyocyanin on HepG2 determind according to inhibition rate (%IR) on the cell line.100 µl/well of pyocyanin was added, each concentration was added to the cells in triplicate form (450, 225, 112.5, 56.25, 28.15, 14.06, 7.03, 3.51, 1.75, 0.878, 0.439, 0.219) µg/ml with MEM medium, only cells incubated with culture media represented the negative control the 96-well cell culture plate transferred into an CO_2incubator supplemented with (5%) CO_2 temperature at 37°C for 72 hrs, After the incubation period medium discarded and 50 µl/well of neutral red dye freshly prepared were added to each well and incubated for 2 hrs, viable cells will uptake the dye, but the dead will not, Excess dye discarded then the plate washed by PBS ,100µl/well of eluent solution were added to each well, Optical density for the plate measured by using ELISA reader at 492nm wave length 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 [24].

Results and Discussions

All bacterial isolates were cultured selectively using cetrimide medium and according to microscopic characteristics, cultural, biochemical tests and API 20E test 66 (67.34) isolates from 98 were successfully identified as *P. aeruginosa*. Approximately 60 (90%) of the isolates were able to produce greenish blue pigment pyocyanin figure 1 when they allow grow on king A medium table-1.

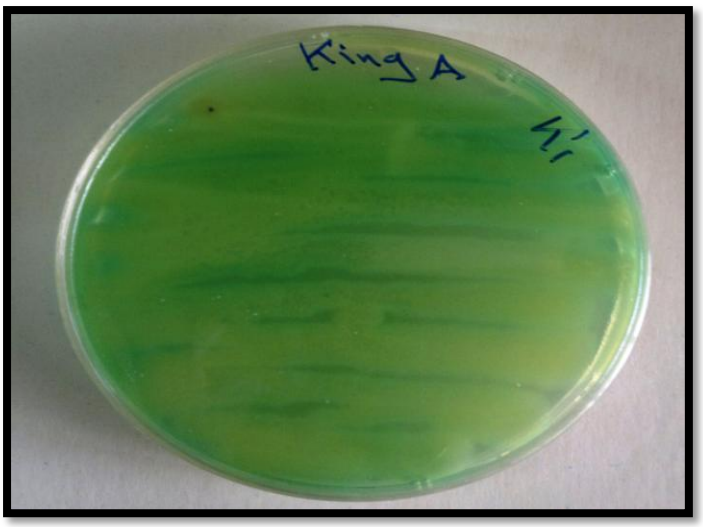

Figure 1-pyocyanin production isolate on king A medium

Table 1- The percentage of *P.aeruginosa* isolates produced pyocyanin

Source of isolate	Number of isolates	No. of pyocyanin producers	Pyocyanin producers %
Burn	6	5	8.3%
Urine	12	10	16.66%
Wound	9	9	15%
Ear	9	9	15%
Eye	18	16	26,66%
Sputum	12	11	18.33%
Total	66	60	100%

The best pyocyanin production isolate was isolated from Patient lung with cyctic fibrosis disease, this isolate used in pyocyanin production, after extraction and purification of pyocyanin, the degree of pyocyanin purity was 96% compare with stander pyocyanin. The purified pyocyanin showed cytotoxity on HepG2 cell figure 2 ELIZA result showed inhibition rate of the cell line, Pyocyanin cytotoxity on HepG2 results illustrated in table-2.

Figure-2 Inhibition rate of pyocyanin on HepG2 cell line

Table 2- Inhibition rate of Pyocyanin on HepG2cell line

No.	Concentration (µg/ml)	Inhibition rate ± SE (%)
1	450	84.02 ± 0.34
2	225	79.09 ± 0.59
3	112.5	75.67 ± 0.69
4	56.25	75.20 ± 0.72
5	28.125	74.57 ± 0.30
6	14.06	70.27 ± 0.37
7	7.03	56.13 ± 4.79
8	3.5	57.83 ± 1.98
9	1.75	37.83 ± 1.01
10	0.878	21.60 ± 1.03
11	0.439	9.57 ± 1.37
12	0.219	7.43 ± 1.95
LSD Value	---	5.083 *

* ($P<0.05$)

Pyocyanin showed high cytotoxity in the concentration 450 µg/ml since the inhibition rate was (84.02 ± 0.34). it cyototoxity decreased in lower concentrations (225, 112.5) µg/ml to be (79.09 ± 0.59, 75.67 ± 0.69)%, for the last concentrations the inhibition rate decreased, pyocyanin concentration depended effect, the concentration showed significant differences $P<0.05$.The cytotoxic effect of pyocyanin on HepG2 attributed to pyocyanin considered zwitter ions with hydrophobic regions, that allow to it move freely throw the cytoplasmic membrane and enter the cell easily [9]. Pyocyanin increase intracellular reactive oxygen species (ROS), The pigment is reduced in the cell by NADH and NADPH, and this reduced form of pyocyanin then transfers electrons to oxygen, generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which are ROS, This is known as intracellular redox cycling of pyocyanin The formation of ROS appears to occur within and around the mitochondria [25]. Pyocyanin also been shown to reduce the expression of the gene encoding catalase, and even to inhibit the activity of catalase directly, preventing the breakdown of hydrogen peroxide and thus increase the level of ROS indirectly [26] .Superoxide (O_2^-) react with nitric oxide to form highly toxic Reactive nitrogen species act together with reactive oxygen species (ROS) to damage cells, causing nitrosative stress, these two species are often collectively referred to as ROS/RNS. That damage DNA, proteins and phospholipids and modulate host immune response finally cause death to the cells [27]. Pyocyanin cause inactivation of V-ATPase which is the major enzymatic complex that is involved in non-mitochondrial ATP consumption and generation [28]. the inactivation of V-ATPase mediated mammalian injuries, such as the disruption of Calcium homeostasis (15).The mechanisms of inactivation of V_ATPase , pyocyanin redox cycle causes a dramatic increase in H2O2 [29]. Because V-ATPase regulates vesicular transport and protein sorting by maintaining a pH gradient within subcellular compartments the inhibition of V-ATPase might impair the trafficking of proteins to distinct subcellular compartments,cause cells death [30,31]. The dead cell release their internal enzyme with result increase the toxicitty of the media and destroy the adjacent cells [32].

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