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 their 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: cytotoxicity, pyocyanin, HepG2 cell line

التاثير السمي للبايوسيانين على خضوع خلايا الكبد السرطانيه

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الخلاصة
جمعنتم اثنين وثمانية عينات من مرضى في مستشفيات بغداد المختلفة خلال الـ 48 شهر من شهر أغسطس إلى نوفمبر في 2012 كانت تحتوي عينات من (القشع، الحروق، الإدراز، العين والاذن). نستنتج عزلة مثبتة على انسان بكتيريا الزائفة الزنجارية، حسب الطريقة المظهرية وروح التصوير البيليوميكنائي وفحص الابي 20% من العزلات كانت منتجة لصبغة البايوسيانين على وسط كنج أ بكميات مختلفة، بينما العزلات الأخرى انتجت صبغات أخرى (بايوفردين الصفراء، بايوروبين الحمراء، بايوالميلين السوداء) على وسط كنج ب بكميات كمية. نستنتج عزلة البايوسيانين. اظهرت النتائج أن عزلة من القشع لمرض التليف الكبيسي كانت الأعلى ناتجا للصبغ، تم تتقيع صبغة البايوسيانين، واخترت سمية بايوسيانين وفتح عظمة خطوة خضوع خلايا الكبد السرطانية، نستنتج أن للبايوسيانين تأثير مثبط على نمو الخلايا السرطانية، كان مدى نسبة التثبيط تتراوح من 7% إلى 84%. 

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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 37°C, and it is able to grow at temperatures as high as 42°C, Indeed, the ability to grow at 42°C that distinguishes it from many other *Pseudomonas* spp. No growth occurs at 5°C [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. aeruginosa*, 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 37°C 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 at 37 °C for 24 hr, 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 24 hrs at 25°C, to change the color of medium to dark blue [21].

Extraction and purification of pyocyanin pigment

Chloroform at ratio:2:1 (v/v) was added to the production culture and left for 2 hrs; the color was changed to the blue. Then, the blue layer was collected and washed with acidified water (0.1 M HCL), in order to convert the blue pigment to (red). The acidified layer then neutralized by adding 1 M 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 0.05 M HCl, this step also repeated several times to have pyocyanin with high purity. At last, the water layer was adjusted to pH 7.5 by 0.1 M 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. C18, the mobile phase contained 0.01 M 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 0.9 ml/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 cm² tissue culture flask with 2 ml trypsin solution incubated for 2 min at 37°C in an incubator supplemented with (5%) CO₂ until the cells detached from the flask surface single cell suspension made by gently taping of the 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 trypsin 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 200 μl/well to the 96 well plate using automatic micropipette containing (10⁴ cell/well), Plate incubated overnight at 37°C in an incubator supplemented with (5%) CO₂ 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 CO₂ incubator supplemented with (5%) CO₂ 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, 100 μl/well of eluent solution were added to each well, Optical density for the plate measured by using ELISA reader at 492 nm wavelength 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 Discussion

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

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>Number of isolates</th>
<th>No. of pyocyanin producers</th>
<th>Pyocyanin producers %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burn</td>
<td>6</td>
<td>5</td>
<td>8.3%</td>
</tr>
<tr>
<td>Urine</td>
<td>12</td>
<td>10</td>
<td>16.66%</td>
</tr>
<tr>
<td>Wound</td>
<td>9</td>
<td>9</td>
<td>15%</td>
</tr>
<tr>
<td>Ear</td>
<td>9</td>
<td>9</td>
<td>15%</td>
</tr>
<tr>
<td>Eye</td>
<td>18</td>
<td>16</td>
<td>26.66%</td>
</tr>
<tr>
<td>Sputum</td>
<td>12</td>
<td>11</td>
<td>18.33%</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>60</td>
<td>100%</td>
</tr>
</tbody>
</table>

The best pyocyanin production isolate was isolated from Patient lung with cystic 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 cytotoxicity on HepG2 cell figure 2 ELIZA result showed inhibition rate of the cell line, Pyocyanin cytotoxicity on HepG2 results illustrated in table-2.

Figure-2 Inhibition rate of pyocyanin on HepG2 cell line
Pyocyanin showed high cytotoxicity in the concentration 450 µg/ml since the inhibition rate was (84.02 ± 0.34). Its cytotoxicity 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 it to 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$_2$O$_2$) 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 toxicity of the media and destroy the adjacent cells [32].

**Reference:**

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**Table 2 - Inhibition rate of Pyocyanin on HepG2 cell line**

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

* (P<0.05)


