Bioactivity of pyocyanin of *Pseudomonas aeruginosa* clinical isolates against a variety of human pathogenic bacteria and fungi species

Zahraa Jaafar Jameel¹, Anaam Fuad Hussain², Muthana Abdulkhader Al-Mahdawi¹, Nuha F. Abed Alkerim³, Eman Sabah abd Alrahman¹

**Abstract**

**Objectives:** Pyocyanin is blue pigment redox active, secondary metabolites produced by *P. aeruginosa*. The present study investigated the bioactivity of pyocyanin against certain types of bacteria and fungi causing human infections.

**Methods:** A total of 23 clinical isolates of *P. aeruginosa* were collected from patients admitted to the General Baqubah Hospital during the period from November 2016 through May 2017. All isolates were cultured on Pseudomonas agar and confirmed by biochemical tests as *P. aeruginosa*. Pyocyanin extraction was done by chloroform method and concentration was determined by multiplying the optical density at 520 nm by 17.072 expressed as µg/ml. Biological activity of pyocyanin was determined by well diffusion procedure.

**Results:** According to the source of *P. aeruginosa*, the most tested isolates were from ear infection (30%) followed by wounds (22%), burns (17%), urine (13%) and both stool and diabetic leg ulcer (9%). Antimicrobial resistant of *P. aeruginosa* isolates were the following: 19 (82.6 %) to piperacillin, followed by 10 (43.5%) to aztreonem, 8 (34.8%) to meropenem, 6 (26.1%) to amikacin, 5 (21.7%) to ciprofloxacin, 2 (8.7%) to cefotaxime. The urine isolates produced the largest amount of pyocyanin (15.894 µg/ml). Pyoacyanin have shown antimicrobial activity against the following bacteria: *Shigella spp.*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Also, pyoacyanin can inhibit the following fungi and yeast: *Aspergillus niger*, *Penicillium*

---

© Under License of Creative Commons Attribution 3.0 License | This article is available at: www.iajaa.org / www.medbrary.com
Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of human and can often lead to skin infections, otitis externa, septicemia, necrotizing pneumonia, burns and surgical wounds infections [1, 2].

P. aeruginosa is an opportunistic pathogen and it is a frequent cause of nosocomial infections, many of which are responsible for high mortality among hospitalized patients in intensive care units (ICU) and immunocompromised patients even when treated with appropriate antibiotics [1-2].

Pseudomonas aeruginosa produces a variety of cell-associated factors and toxin, some of which are responsible for its invasion and the severity of diseases. These include lipopolysaccharide (LPS), phospholipase, proteases, flagella, pili, exotoxins and pyocyanin [3-4].

Pyocyanin is a blue redox active, secondary metabolite, phenazine derivative that it exhibits a paradoxical pro-oxidant property [4]. A zwitterions that can enter biological membranes, pyocyanin can directly accept electrons from reducing agents such as NADPH and reduced glutathione (Figure 1) and endure redox cycling using oxygen as an electron acceptor resulting in the generation of the reactive oxygen species (ROS), particularly hydrogen peroxide, with resulting induction of oxidative stress and disturbance of intracellular redox homeostasis [5]. Therefore, the important mechanisms of pyocyanin to prokaryotic and eukaryotic cells are related to inhibition cellular respiration [6].

Pseudomonas spp. are also capable of producing organic volatiles, whose in vitro antifungal nature has been demonstrated against Phytophthora-

Figure 1: Chemical structure of reduced and oxidized forms of pyocyanin and mechanizm [5].

Keywords

Pseudomonas Aeruginosa, Pyocyanin, Antimicrobial Activity, Iraq.
vignae in cowpea [7]. These compounds inhibited sclerotic and ascosporic germination, and mycelial growth of Sclerotinia sclerotiorum, in vitro and in soil tests [8].

This study investigated the bioactivity of pyocyanin produced by fresh P. aeruginosa clinical isolates from patients against a variety of bacteria and fungi causing human infections.

Material and Methods

Bacterial strain isolation and identification
This study was performed from November, 2016 until May, 2017. A total of 23 P. aeruginosa isolates were collected from urine, stool, diabetes leg, ear, burns and wounds infections of patients admitted to General Baqubah Hospital. The isolates were recovered from cultures on Pseudomonas agar, MacConkey agar and blood agar. All isolates were identified as P. aeruginosa using gram-stain, blue-green pigmentation and biochemical characterization [9].

Antibiotic susceptibility testing
The antimicrobial susceptibility assay was performed on Mueller-Hinton agar using disc-diffusion method, and selection of antibiotics and growth inhibition zones were interpreted according to the Clinical Laboratory Standards Institute [11]. The antimicrobial disks: piperacillin (100µg), cefotaxime (30µg), meropenem (10µg), aztreonem (30µg), amikacin (30µg) and ciprofloxacin (5µg) were obtained from (Mast Group, UK).

Pyocyanin Production
Nutrient broth was used for the extraction of pigment from each P. aeruginosa isolate after incubation for 2-3 days at 37°C. The change in color of the pigment to bluish green indicated pigment production [9].

Pyocyanin extraction
After the color media became bluish, 5 ml of each sample was centrifuged (3000rpm/10min) and 3 ml of chloroform added to the supernatant and mixed until blue color is recovered. It was further confirmed by adding 0.2 N HCl to the blue color solution which turned to red– pink [12].

Pyocyanin quantitative assay
The absorbance of each recovered solution was measured at 520 nm. Pyocyanin concentrations were expressed as micrograms of pigment per milliliter of culture and were determined by multiplying the optical density at 520 nm by 17.072, according to the absorbance of pyocyanin at 520 nm in acid solution. [13].

Pyocyanin purification
0.4 M borate-NaOH buffer (pH= 10) was added to the pink solution until the color turned to blue and again extracted by chloroform. This step was repeated 2 to 3 times resulting pyocyanin in chloroform in a clear blue solution. Finally pyocyanin powder was collected by evaporating the chloroform and weighted then rehydration by 1ml of sterilized distilled water [14].

Biological activity of recovered pyocyanin
The assay of antibacterial and antifungal of pyocyanin was carried out in well diffusion technique [15,16]. Wells with diameter size 6mm were made using Mueller-Hinton agar petri dish. Each one isolate of the following pathogenic bacteria were tested: Escherichia coli, Klebsiella pneumoniae, Shigella, Proteus spp., Acinetobacter spp., Staphylococcus aureus and Staphylococcus epidermedis. pathogenic fungi and yeast namely Aspergillus niger, Penicillium spp., Rhizopus spp, Trichophyton mentagrophyte, Rhodotorula spp., Alternaria alternate, Trichophyton rubrum and Candida spp. The growth of each isolate was swabbed on the surface of nutrient agar and 100µl of pyocyanin solution (167mg/ml) were
added in to the well. Petri dishes were incubated at 37°C for 24-48 hours for bacteria and yeast, while kept for other fungi for up to one month. The inhibition zone in each well was measured by a ruler in mm unit to determine the biological activity of pyocyanin.

Results
A total of 23 *P. aeruginosa* isolates were investigated for production of pyocyanin. Distribution of *P. aeruginosa* isolates according to clinical source are shown in Table 1. The majority of isolates were collected from ear infections (30%), followed by wounds (22%), burns (17%), urine (13%) and each leg diabetes and stools (9%).

Susceptibility to antibiotics
The results of antimicrobial susceptibility test of 23 *P. aeruginosa* isolates have shown high resistance rate (82.6 %) to piperacillin, followed by (43.5%) to aztreonem (34.8%), to meropenem (26.1%) to amikacin, (21.7%), to ciprofloxacin and (8.7%) to cefotaxime, respectively (Table 2).

Pyocyanin extraction (quantitative assay)
A total of 6 clinical *P. aeruginosa* isolates were selected to extraction of pyocyanin according to intensity of blue pigmentation on tested agar.

Figure 2, shows the amount of pyocyanin obtained from each *P. aeruginosa* isolate according to their clinical source. The largest amount of pyocyanin (15.894 µg/ml) was recovered from one urine isolate.

![Figure 2: Pyocyanin quantity (concentration (µg/ml)=O.D. 520 nm x17.072).](image)

Table 1. Distribution of *P. aeruginosa* isolates according to clinical source

<table>
<thead>
<tr>
<th>Source</th>
<th><em>P. aeruginosa</em> isolates No (%)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>Wounds</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>Burns</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Urine</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Leg Diabetes</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Stool</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Antimicrobial resistance profile of 23 *P. aeruginosa* isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>82.6</td>
</tr>
<tr>
<td>Aztreonem</td>
<td>43.5</td>
</tr>
<tr>
<td>Meropenem</td>
<td>34.8</td>
</tr>
<tr>
<td>Amikacin</td>
<td>26.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>21.7</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Biological activity of pyocyanin
The antimicrobial activity of purified pyocyanin (Needle-like crystal) at the concentration (167 mg/ml) was monitored against different microorganisms as shown in Table 3.

Gram-positive such as *Staphylococcus epidermidis, Staphylococcus aureus*; Gram-negative such as *Escherichia coli*, *proteus spp.*, *Klebsiella spp.* and *Acinetobacter spp.*; molds such as and Yeast as *Candida albicans*. Results shown in table (3) indicate that pyocyanin shows high biological activity against all mentioned organisms, except against *Klebsiella pneumonia, Acinetobacter spp.*, *Proteus* and *Escherichia coli.*
Discussion

Pyocyanin is a blue pigment redox active produced by *P. aeruginosa*. The present study investigated production, purification, characterization and bioactivity of pyocyanin pigments produced by local clinical *P. aeruginosa* isolates. The results have shown that *P. aeruginosa* isolates were most commonly associated with ear infection (30%) followed by other body sites in our region of Iraq. Many studies carried out in Iraq and other countries demonstrated wide spread of *P. aeruginosa* isolates in clinical specimens. A study of Abbas in Baqubah/Iraq, has found that *P. aeruginosa* first commonly isolated from burns cases (18.18%), followed by ear (11.6%), urine (10.8%) and wounds (6.25%) [17]. While the study of Al-Imari in Baghdad/Iraq, has reported the incidence *P. aeruginosa* isolates from certain clinical specimens as follow: urine (22.4%), ear (18.4%), sputum (16.3%), burns (14.3%) and wounds (12.3%) [18]. El Fouly and coworkers in Cairo, Egypt found that *P. aeruginosa* was the most common cause of urinary tract infection (12.5%), followed by burns (10%) and sputum (9%) [19]. The reasons of variations in the occurrence rates of *P. aeruginosa* infections may be due to underlining diseases of investigated patients, virulence factors and the local antimicrobial resistance of the organism, and other health conditions [2]. In addition, *P. aeruginosa* is able to act as opportunistic pathogens in human and patients with weakened immune systems, and especially during hospitalization in intensive care units [1-2].

The present study indicated that *P. aeruginosa* isolates have the highest resistant rate to piperacillin (82.6%) and less to other antimicrobial agents as shown in Table 2. The study of Al- Alkhozai and Alkabei in Diwanyia, Iraq, reported similar resistance rate in *P. aeruginosa* isolates to piperacillin (94%) and high resistance rate to aztreonem (88.5%) [20]. This study found a resistance rate of 43.5% to aztreonem, whereas the study in Baqubah/Iraq reported very low resistance rate of 5% to aztreonem among *P. aeruginosa* isolates [17]. The study of Ameen and coworkers in Pakistan, have found that *P. aeruginosa* resistant to aztreonem was (86.1%) [21]. It is well established that the development of resistance of *P. aeruginosa* to antibiotics is increasing locally and globally due to the overuse of antibiotics [1]. There are various multiple mechanisms contribute to developing antimicrobial resistance in *P. aeruginosa*, including active efflux, acquisition of various beta-lactamases, decrease outer membrane permeability and target modification [22-23]. Therefore, distinguishing the trends in resistance of *P. aeruginosa* becomes important for choosing the right antibiotic [24].

Table 3. Diameter of antimicrobial activity of pyocyanin to various tested microorganisms.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Properties</th>
<th>Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shigella</em> spp.</td>
<td>Gram negative</td>
<td>25</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Gram positive</td>
<td>23</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Gram positive</td>
<td>23</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>Gram negative</td>
<td>None</td>
</tr>
<tr>
<td><em>Acinetobacter</em> spp.</td>
<td>Gram negative</td>
<td>None</td>
</tr>
<tr>
<td><em>Proteus</em> spp.</td>
<td>Gram negative</td>
<td>None</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Gram negative</td>
<td>None</td>
</tr>
<tr>
<td><em>Penicillium</em> spp.</td>
<td>Mold</td>
<td>25 (inhibition of spore formation)*</td>
</tr>
<tr>
<td><em>Aspergillus</em> nig.</td>
<td>Mold</td>
<td>25</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp.</td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Trichophytonmentagrophyte</em></td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Trichophytonrubrum</em></td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Rhizopus</em> spp.1</td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Alternaria</em> alternate</td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Rhodotorula</em> spp.</td>
<td>Mold</td>
<td>23</td>
</tr>
<tr>
<td><em>Rhizopus</em> spp.2</td>
<td>Mold</td>
<td>10</td>
</tr>
<tr>
<td><em>Candida</em> spp.1</td>
<td>Yeast</td>
<td>20</td>
</tr>
<tr>
<td><em>Candida</em> spp.2</td>
<td>Yeast</td>
<td>17</td>
</tr>
</tbody>
</table>

*: Apply for all tested fungi.

© Under License of Creative Commons Attribution 3.0 License
culture strains on King’s A medium which has potassium and magnesium salts in appropriate concentration to defeat fluorescein production (pyoverdine) [2]. Pyocyanin is blue color in alkaline and neutral pH while red color in acid pH. Any change in the pH may affect the color of pyocyanin [25]. In addition, the variation in pyocyanin production among different strains could be due to various factors such as lighting conditions. It has been reported that the concentration of pyocyanin was reduced under certain lighting conditions. This decrease was dependent on both the light intensity and wavelength and occurred with light in the ultraviolet and violet region of the spectrum [26-27].

The present study has demonstrated that pyocyanin production by all investigated clinical isolates had antimicrobial activity against Gram-positive Staphylococcus spp. more than gram negative bacteria and to some extent against yeast such Candida spp. and filamentous fungi. This result is in agreement with those results of Makarand et al. (2007) which have reported that phenazine antibiotic (chemical substance similar to pyocyanin) have antimicrobial activity against strains of Bacillus subtilis and Candida albicans [28].

The study of El fouly in Egypt, found that P. aeruginosa isolated from human urine sample produced more pyocyanin concentration than isolates from rice cultivated soil (production titer was 6.3 and 5.9 mg/ml, respectively) [19], While Ra’oof and Latif, in Iraq, has detected that the P. aeruginosa isolate from sputum produced the largest amount of pyocyanin (12.069 µg/ml) [29]. The study of Charyulu et al. (2009), has shown that the secondary metabolite from Pseudomonas which played a significant role in lyases the pathogenic bacterial including MRSA strains [30]. Also, the Sudhakar et al. (2013) showed different antimicrobial activity against E. coli, S. aureus, Proteus sp., and Klebsiella spp. [31], while the study Karpagam et al., (2013) found the pigment produced by P. aeruginosa has antifungal activity against Candida sp. and Cryptococcus neoformans [32].

In conclusion, pyocyanin produced by clinical P. aeruginosa isolates has various spectrum of antibacterial and antifungal activity against certain Gram-positive and Gram-negative bacteria, Candida spp. and filamentous in vitro.

References


The Journal is an open access peer-reviewed journal that publishes scientific papers about all aspects of antimicrobials. The journal will publish original research articles, reviews, brief reports and case reports dealing with basic and clinical antibacterial agents, antiviral, antiprotozoals, antituberculuous, antifungal and antihelminthes agents. All manuscripts must be prepared in English, and are subject to a rigorous and fair peer-review process. Accepted papers will immediately appear online. The journal aims to advance the knowledge, attitude and the research of chemotherapy in the Arabic world in cooperation with international, national scientific and public societies as well as research centers with similar aims and objectives.