

## ORIGINAL ARTICLE

# Effect of subinhibitory concentrations of selected antibiotics and propolis on pyocyanin and biofilm production among *Pseudomonas aeruginosa* isolates in Alexandria, Egypt

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## ABSTRACT

**Key words:**

**Pyocyanin, Biofilm forming capacity, *phzS*, *phzM*, SubMIC antibiotics**

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**Background:** *Pseudomonas aeruginosa* is a highly virulent microorganism that is implicated in various types of infections. It is armed with an arena of virulence factors that is mostly controlled by quorum sensing. The pigment pyocyanin and biofilm formation are of the important defense mechanisms used by the organism to establish infection. **Objective:** detection and quantification of biofilm mass and pyocyanin along with effect of subinhibitory concentrations of antibiotics on their magnitude. **Methodology:** In the present study, fifty *Pseudomonas aeruginosa* isolates were obtained from clinical laboratories from all over Alexandria governorate, Egypt. The isolates were tested for certain quorum sensing dependent virulence factors and the effect of subinhibitory concentrations of certain antibiotics, in addition propolis extract, was assayed. **Results:** the sub MIC of selected antibiotics and propolis inhibited pyocyanin production. On the other hand, they had variable effects on the formed biofilm mass. **Conclusion:** the effect of sub inhibitory concentrations either on biofilm formation or pyocyanin production is very important to be tested and followed to predict bacterial behavior and assist in tailoring therapeutic regimen.

## INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen influencing patients who have been immunocompromised. It is known to be one of the primary drivers of dismalthness and mortality in patients who are experiencing cystic fibrosis (CF) and accounts for 80% of infections in the world. *P. aeruginosa* strains can be life-threatening, and are becoming the world's public health threat because of various mechanisms to adapt, survive and resist multiple classes of antibiotics <sup>1</sup>.

The main variables in the forestalling of wound mending are infections. Non healing wounds are an optimal place for bacterial growth, in addition to, colonization that can easily reach the underlying tissues in case of tissue injury. The infection rate in acute injuries was reported to be 12 % and in chronic injuries 38% <sup>2</sup> due to production of various virulence factors as exotoxins like exotoxin A, rhamnolipids, elastase and biofilm forming capacity, that have an important role in preventing the healing of injuries and inflammatory processes accompanying it. <sup>3</sup>

*P. aeruginosa* invades viable adjacent tissue by permeating subcutaneous lymphatics and multiplying <sup>4</sup>. Ear infections particularly swimmers' ear or otitis media, that might be severe, are accompanied by purulent discharge and perforation of the drum even with antibiotic prescription.

Pyocyanin is a blue pigment composed of two subunits of N-methyl-1-hydroxyphenazine. Seven genes, namely *phzCDEFGMS*, are used by *P. aeruginosa* to produce pyocyanin <sup>5</sup>. The genes *phzM* and *phzS* are the most important as they encode the two-step conversion of Phenazine 1 caroylic acid (PCA) to pyocyanin, enzyme *PhzM* converts PCA to 5-methylphenazine-1-carboxylic acid betaine and the enzyme *PhzS* catalyzes the hydroxylyative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin <sup>5</sup>.

The pyocyanin amount varies greatly by pH environmental condition, presence of certain anions or availability of preferred carbon source <sup>6</sup>.

High level of pyocyanin were detected in infections caused by *Pseudomonas aeruginosa* with the highest accompanying lung infections and the lowest urine infections. Such levels eventually lead to cellular death due to increased oxidative stress <sup>7</sup>. It was also suggested that it has a role in biofilm formation and apoptosis and its effects was studied on lung, urinary tract, wound and ear tissues <sup>7</sup>. In addition to direct effects on cells, pyocyanin was found to induce antibiotic tolerance in *Pseudomonas aeruginosa* strains, such behavior precedes the acquisition of antibiotic resistance <sup>8</sup>. On the other hand, it was also noted that pyocyanin is implicated in biofilm formation among other phenazines in addition to its effect on colony morphology <sup>9</sup>.

## METHODOLOGY

### Identification of the isolates

Fifty *Pseudomonas aeruginosa* isolates were obtained as swabs samples from clinical laboratory all over Alexandria governorate, Egypt. The identity of the isolates was confirmed by standard bacteriological method. The organisms were further tested for the production of pyocyanin, rhamnolipids, protease production using skimmed milk agar<sup>10</sup> and divided according to origin into ear infection, wounds, urinary tract, lung infections.

### Antibiotics resistance pattern of the *Pseudomonas aeruginosa* isolates

*Pseudomonas aeruginosa* isolates were subjected to Kirby Bauer test using the following antibiotic discs, amikacin, ampicillin, ampicillin /sulbactam, amoxicillin /clavulanic acid, cefadroxil, cefoperazone. cefoperazone/sulbactam, cefotaxime, cefepime, cefoxitin, ceftazidime, cefuroxime sodium, ceftriaxone, colistin, polymyxin, doxycycline, ertapenem, gentamicin, imipenem, linezolid, meropenem, minocycline, moxifloxacin, ofloxacin, ciprofloxacin, levofloxacin, rifampicin, Tazobactam. tazobactam/piperacillin, teicoplanin, tigecycline, tobramycin, trimethoprim -sulphamethoxazole, vancomycin.

Plates were incubated at 37 C, measured zones diameters were interpreted into sensitive, intermediate and resistant<sup>11</sup>.

### Determination of biofilm formation by all isolates

Assay of biofilm forming capacity of all isolates was done using modified microtiter plate technique as described<sup>12</sup>. In brief, 200  $\mu$ l of the overnight bacterial culture of the isolates, diluted to a count of  $10^6$  CFU/ ml, were distributed in triplicate among the wells of the flat bottomed microtiter plates, incubated overnight, tested for growth, then, plates were emptied, left to dry in air. The biofilm mass was fixed using absolute ethyl alcohol, then 1% crystal violet (150  $\mu$ l) was added for 5 minutes. Plates were then emptied washed, left to dry in air and 160  $\mu$ l 33 % glacial acetic acid were added to each well. The readings were taken using ELISA reader at 630 nm and interpretation was according to the following criteria:  $OD \leq OD_c$  no biofilm mass

$OD_c < OD \leq 2 \times OD_c$ , weak biofilm formation  
 $2 \times OD_c < OD \leq 4 \times OD_c$ , moderate biofilm formation

$4 \times OD_c < OD$  strong biofilm formation<sup>12</sup>.  
 (Where; OD: optical density and  $OD_c$ : optical density of control).

### Detection and quantification of pyocyanin

#### • Phenotypically:

*Pseudomonas aeruginosa* isolates were streaked on pseudomonas agar with 10% glycerol and incubated at room temperature for 96 hours<sup>13</sup>. The pyocyanin was extracted using chloroform as follows, the agar with the growth from culture incubated for 96 hrs were cut into small pieces and extracted with chloroform, the blue colored form was transferred to aqueous phase by using 0.01 N HCl, the red color formed was then re-alkalinized using 0.01 N NaOH drops till no change in blue color was noticed. The approximate concentrations were determined by measuring the absorbance of the acidic solutions directly after the extraction, to minimize degradation and multiplying the OD at 520 nm by 17.072.

#### • Genotype: Detection of pyocyanin biosynthetic genes:

A Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) was performed for the DNA isolation, according to the instructions of the manufacturer. The DNA samples were kept until use at -20°C. The prevalence of *phzM* and *phzS* genes was assessed by simplex PCR. The amplification procedure was performed as previously mentioned<sup>14</sup>. The sequences of the primers used in this study are shown in table 1. The primers were procured from Sigma Oligos, India. A total volume of 25  $\mu$ l was used for PCR and included 0.5  $\mu$ l DNA extract, 10 picomoles of each primer and 12.5  $\mu$ l 2X MyTaq HS Red Mix. The DNA amplification was performed in a DNA thermal cycler (Tpersonal Thermocycler biometra, Applied biosystem, USA). For PCR products separation, 1% agarose gel in TBE buffer was performed. Gel electrophoresis was used for 1 hour at a voltage of 100 V, gel was finally stained for ten minutes in 2  $\mu$ g/ml ethidium bromide and visualization was under UV transilluminator (BIORAD, Italy).

**Table 1: Primers used and the annealing temperatures for the amplified genes**

| Primer       | Primers sequence (5'-3') | Band Size | Annealing Temperature | Reference |
|--------------|--------------------------|-----------|-----------------------|-----------|
| <i>phzM</i>  | ATGGAGAGCGGGATCGACAG     | 875       | 54                    | 14        |
| <i>phzMR</i> | ATGCGGGTTTCCATCGGCAG     |           |                       |           |
| <i>phzSF</i> | TCGCCATGACCGATACGCTC     | 1722      | 63                    |           |
| <i>phzSR</i> | ACAACCTGAGCCAGCCTCC      |           |                       |           |

### Determination of MIC of different antibiotics against isolates under test:

The two-fold serial dilution of the following antibiotics was performed from the corresponding stock solutions (400 mg/100 ml): levofloxacin, sulphamethoxazole, ciprofloxacin, colistin sulphate, ampicillin, spectinomycin, tobramycin in addition to the use of the Ethanolic extract of Egyptian propolis (20 gm/100ml of 95% ethanol).

The experiments were performed as previously described<sup>15</sup>. Briefly, flat bottomed microtiter plates were used, each of the wells received 100 µl of the serial dilutions of the antibiotics under test and 100 µl of sterile double strength nutrient broth inoculated with 10<sup>6</sup> CFU/ml of each of the tested organisms and mixed gently. The test was done in triplicate. The plates were covered and incubated at 35-37 °C for 14 hrs. The MIC was taken as the lowest antimicrobial agent concentration showing no growth turbidity.

On what concerns propolis extract, twenty grams of propolis powder was put in 100 ml of 95% ethanol to obtain 20% W/V propolis suspension. Extraction was done at room temperature in dark for a week with shaking on intervals. Following extraction, the mixture was filtered as ethanolic extract of propolis [EEP]. Antimicrobial activity of EEP was detected by agar diffusion technique against *Pseudomonas aeruginosa* isolates using serial different (10, 5, 2.5, 1.25 and 0.625%) where 1 ml of the corresponding dilution was mixed gently with 19 ml of molten nutrient agar, left to solidify then incubated at 37 C for 24 hrs<sup>16</sup>.

### Antibiofilm activity of the antibiotic under tests:

The same arrangement in the experiment above was used, but following incubation, the content of the wells was aspirated and wells washed three times with 200 µl of sterile saline, then 200 µl of absolute methanol were applied for 15 minutes for fixation of any formed biofilm mass. Plates were then emptied and left to air dry, then 150 µl of 1% crystal violet were added in each well for 5 min. Next, plates were emptied, washed and air dried. Subsequently, 160 µl of 33% glacial acetic

acid were added to each well prior to reading using Elisa reader at 630 nm<sup>13</sup>.

### Effect of the use of subinhibitory concentrations of antibiotics on pyocyanin production

Selected pyocyanin producing and none pyocyanin producing strains were grown for 24 hours after which the cultures were diluted to an optical density (OD = 600 nm) of 0.01 and allowed to grow until OD 600 nm of 0.1–0.2 was reached. A small volume (2 mL) of each of the cultures was taken into separate tubes and antibiotics under test at concentrations below the MIC (1/2 and ¼ MIC) were added to each tube, propolis extract also was tested. The bacteria with and without antibiotics were allowed to grow for 24 hours, at 37°C. The level of pyocyanin production was measured as mentioned above and data were recorded<sup>1</sup>.

### Statistical Analysis

Data was analyzed using IBM SPSS version 20 (Armonk, NY: IBM Corp) statistical package. Qualitative data were described and compared by applying Pearson's Chi-square test, Fisher's Exact or Monte Carlo correction. A P-value of <0.05 was regarded as statistically significant.

## RESULTS

### Identification of bacteria and detection of virulence factors

All isolates tested were classified according to their origin: 50% from cystic fibrosis, 20% wound, 20% ear infections, and 10% urine. Isolates were identified as *Pseudomonas aeruginosa* with Gram-negative staining rod with fruity odor upon growth on pseudomonas agar.

Pyocyanin production was detected in 40% of the isolates. 100, 70 and 80% of the organisms were oxidase positive, protease and rhamnolipid producers, respectively.

### Detection of antibiotic resistance pattern

The antibiotic resistance was detected by Kirby Bauer technique; most of the isolates were MDR with 100 % sensitivity to colistin, as are shown in figure 1.

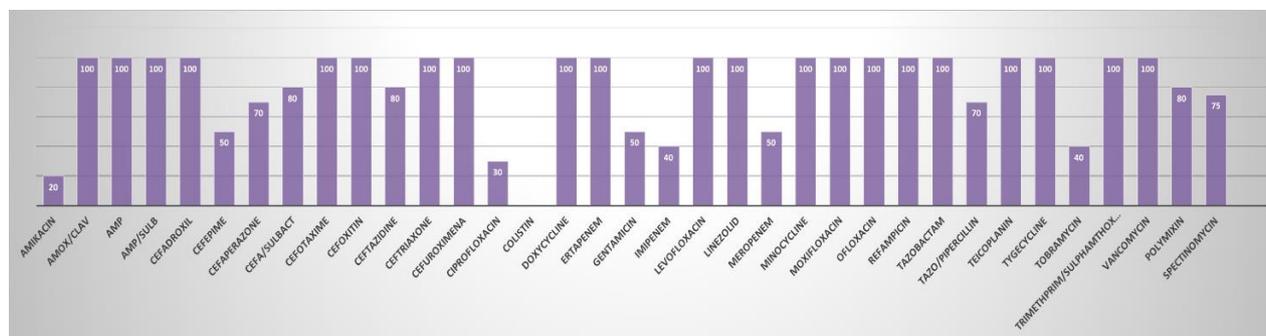


Fig. 1: Antibiotic resistance percentage among isolates under test

**Biofilm forming capacity of all isolates**

The *Pseudomonas aeruginosa* isolates were tested for their biofilm forming capacity using modified microtiter plate method. The organisms under test were all biofilm producers with 80%, 10% and 10% as strong, intermediate and weak biofilm formers, respectively.

**Detection of Pyocyanin production**

**A. Phenotypically**

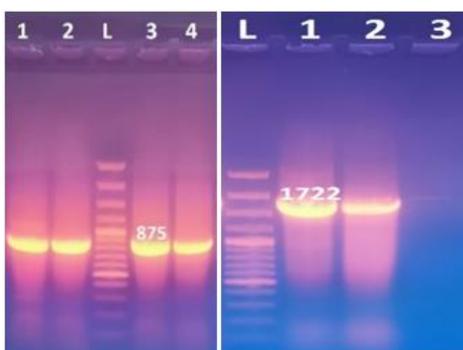
The isolates were tested for pyocyanin production through the extraction with chloroform and transfer to aqueous phase at 5 nm, only 40% of the isolates enrolled in the study were pyocyanin producers with a pyocyanin concentration that ranged between 1.2 ug/ml and 7.4 ug/ml (figure 2)



**Fig 2:** Extraction steps of pyocyanin

**B. Detection of pyocyanin biosynthetic genes**

PCR products for the phenazine -encoding gene region showed that all isolates possessed the *phzM* gene, while 90 % were positive for *phzS* gene (figure 3)



**Fig 3:** Left. Lane L, 100-bp DNA ladder; lanes 1,2,3 and 4, the 875-bp PCR product of *phzM* gene. Right: Ethidium bromide-stained agarose gel showing the band

of amplified PCR product 1722 bp *phzS* gene at lane 1 and 2. L: DNA ladder, Lane 3 is *phzS* negative

**Minimum inhibitory concentrations of all isolates against certain selected antibiotics and propolis extract:**

The following antibiotics: levofloxacin, sulphamethoxazole, ciprofloxacin, colistin sulphate, ampicillin, spectinomycin, tobramycin, in addition to the use of the Ethanolic extract of Egyptian propolis, were challenged against isolates of *Pseudomonas aeruginosa*. The MIC are shown in table 2.

**Table 2: Minimum inhibitory concentrations values against *Pseudomonas aeruginosa* isolates**

| Antibiotic        | MIC values in ug/ml |
|-------------------|---------------------|
| Levofloxacin      | 7.8-15.62           |
| Ciprofloxacin     | 32.25-62.5          |
| Tobramycin        | 32.25-62.5          |
| Sulphamethoxazole | 1000                |
| Spectinomycin     | 125-250             |
| Colistin sulphate | 0.09                |
| ampicillin        | 1000                |
| Propolis extract  | 625-1250 ug/ml      |

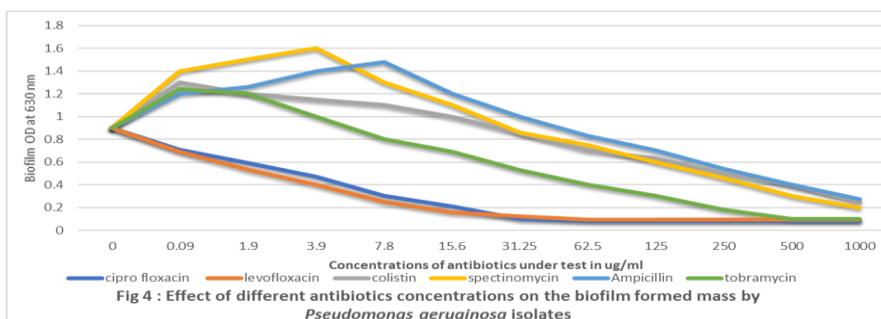
Lower value in ranges mostly was obtained in case of non-pyocyanin producers, while pyocyanin producers showed higher MIC.

**Antibiofilm activity of antibiotic under test**

The antibiofilm activity of the antibiotics under test and propolis was tested against the *Pseudomonas aeruginosa* isolates

The responses of the isolates varied towards the antibiotic used. First of all, propolis extract decreased greatly the biofilm mass formed by the isolates.

Antibiotics as levofloxacin and ciprofloxacin decreased greatly biofilm formed mass which was nearly abolished, the rest of the antibiotics induced biofilm formation at low concentrations. Such effect varied according to the antibiotics but the mass decreased gradually by increasing antibiotic concentration as shown in figure 4.



**Fig. 4:** Effect of different antibiotics concentrations on the biofilm formed mass by *Pseudomonas aeruginosa* isolates

### Effect of subinhibitory concentrations of antibiotics on pyocyanin production by *Pseudomonas aeruginosa* isolates

The *Pseudomonas aeruginosa* (pyocyanin producing and none producing) strains were cultured with ¼ and ½ MIC of all tested antibiotics in addition to propolis extract.

After 24 hours, the strains were tested for pyocyanin production. The subinhibitory concentrations of antibiotics inhibited the production of pyocyanin completely except for ampicillin, where a very low concentration of pyocyanin was produced in pyocyanin producing pseudomonas (figure 5). No change was noticed in non-pyocyanin producers.



**Fig. 5:** Left to right: 1. propolis extract effect on pyocyanin production, 2. ciprofloxacin effect, 3. tobramycin effect on pyocyanin production and 4. Ampicillin effect on pyocyanin production under UV light

### Correlation studies

Pyocyanin production was correlated with different parameters. The isolates that were negative pyocyanin producers were mostly strong biofilm formers and positive producers varied between strong, intermediate and weak ( $P < 0.001$ ), 83% of the pyocyanin negative were tobramycin sensitive and 75% of the pyocyanin

producers were resistant to tobramycin ( $p < 0.001$ ), source of infection significantly correlated with phenotypic pyocyanin production. All wound and urine isolates were pyocyanin positive, while isolates from lung infections were pyocyanin negative and ear infection isolates were equally distributed among the two groups ( $p < 0.001$ ) as demonstrated in table 3.

**Table 3: Relation between pyocyanin and different parameters**

|                   | Pyocyanin         |       |                   |      | p                  |
|-------------------|-------------------|-------|-------------------|------|--------------------|
|                   | Negative (n = 30) |       | Positive (n = 20) |      |                    |
|                   | No.               | %     | No.               | %    |                    |
| <b>Biofilm</b>    |                   |       |                   |      |                    |
| Strong            | 30                | 100.0 | 10                | 50.0 | MC<br>p<br><0.001* |
| Moderate          | 0                 | 0.0   | 5                 | 25.0 |                    |
| Weak              | 0                 | 0.0   | 5                 | 25.0 |                    |
| <b>Tobramycin</b> |                   |       |                   |      |                    |
| Sensitive         | 25                | 83.3  | 5                 | 25.0 | <0.001*            |
| Resistant         | 5                 | 16.7  | 15                | 75.0 |                    |
| <b>Source</b>     |                   |       |                   |      |                    |
| Wound             | 0                 | 0.0   | 10                | 50.0 | MC<br>p<br><0.001* |
| Urine             | 0                 | 0.0   | 5                 | 25.0 |                    |
| Lung infection    | 25                | 83.3  | 0                 | 0.0  |                    |
| Ear infection     | 5                 | 16.7  | 5                 | 25.0 |                    |

$\chi^2$ : Chi square test

MC: Monte Carlo

p: p value for comparing between the studied groups

\*: Statistically significant at  $p \leq 0.05$

When the presence of the gene *phzS* was correlated to biofilm, no significant correlation was detected. However, all *phzS* negative isolates were tobramycin resistant, while 67 % of those *phzS* positive were tobramycin sensitive and the rest tobramycin resistant

( $p = 0.007$ ). All isolates were *phzS* positive except for wounds infection, where prevalence was equal for *phzS* positive and negative isolates ( $p = 0.001$ ) as shown in table 4.

**Table 4: Relation between *phzS* gene and different parameters**

|                   | <i>phzS</i>      |       |                   |      | p                  |
|-------------------|------------------|-------|-------------------|------|--------------------|
|                   | Negative (n = 5) |       | Positive (n = 45) |      |                    |
|                   | No.              | %     | No.               | %    |                    |
| <b>Biofilm</b>    |                  |       |                   |      |                    |
| Strong            | 5                | 100.0 | 35                | 77.8 | MC<br>p=<br>1.000  |
| Intermediate      | 0                | 0.0   | 5                 | 11.1 |                    |
| Weak              | 0                | 0.0   | 5                 | 11.1 |                    |
| <b>Tobramycin</b> |                  |       |                   |      |                    |
| Sensitive         | 0                | 0.0   | 30                | 66.7 | FE<br>p=<br>0.007* |
| Resistant         | 5                | 100.0 | 15                | 33.3 |                    |
| <b>Source</b>     |                  |       |                   |      |                    |
| Wound             | 5                | 100.0 | 5                 | 11.1 | MC<br>p=<br>0.001* |
| Urine             | 0                | 0.0   | 5                 | 11.1 |                    |
| Lung infection    | 0                | 0.0   | 25                | 55.6 |                    |
| Ear infection     | 0                | 0.0   | 10                | 22.2 |                    |

 $\chi^2$ : Chi square test

FE: Fisher Exact

MC: Monte Carlo

p: p value for comparing between the studied groups

\*: Statistically significant at  $p \leq 0.05$ 

## DISCUSSION

In the present study, the *Pseudomonas aeruginosa* isolates under test were mostly MDR with different resistance patterns. Such types of *Pseudomonas* are reported to be present in burn infections and cystic fibrosis<sup>17</sup>. In our study, 100 % sensitivity to colistin was detected among the isolates, despite their varying sources. Our results are in accordance with a study in turkey where all *Pseudomonas* isolates were MDR yet Colistin sensitive<sup>18</sup>. On the contrary, some studies found low levels of resistance among *Pseudomonas aeruginosa* isolates as in a retrospective study in Brazil. In addition, emerged colistin resistance among *Pseudomonas* isolates was reported in a hospital in Tehran<sup>19,20</sup>. A study in Egypt reported elevated colistin resistance level up to 21%<sup>21</sup>. The ability of losing and acquiring the colistin resistance, has been related to a certain genetic reversion in *Pseudomonas aeruginosa*<sup>22</sup>.

The rhamnolipids were detected in 80 % of the *Pseudomonas aeruginosa* isolates in the present study. The rhamnolipids, a biosurfactant that is usually associated with *Pseudomonas*, is considered as a very important virulence factor that has a role in dispersing and removing biofilm of other species when present in the supernatant of relative *Pseudomonas* culture<sup>23</sup>, production of rhamnolipid is known to be controlled by quorum sensing genes and was detected to be enhanced under anaerobic condition and presence of glycerol in the growth medium<sup>24</sup>.

When protease enzyme production was examined in our study, it was found that 70 % of the isolates produced halo regions around the streaks indicating protease production, the proteases comprise an array of enzymes : elastase A (LasA), elastase B (LasB), alkaline protease (AP), protease IV (PIV), *Pseudomonas* small protease (PASP), large protease A (LepA), MucD, and

*Pseudomonas aeruginosa* aminopeptidase (PAAP), that enhance and promote the virulence of *Pseudomonas* against other organisms specially *Staphylococcus aureus* and might be enhanced by certain antibiotics as ciprofloxacin<sup>25</sup>.

In our study, all the isolates were tested for the presence of phenazine modifying genes *phzM* and *phzS*. All the isolates were carrying the *phzM* genes that are known to be in the form of a dimer and is highly implicated in pyocyanin production. As stated before<sup>26,27</sup>, the organism lacking one of the two genes are probably non pyocyanin producers. Some *Pseudomonas* were reported to have low frequency of those genes such strains were regarded as low virulent strains<sup>28</sup>. The *phzM* gene works with *phzS* to produce pyocyanin and its prevalence was 90% among the isolates in the current study. This enzyme is of great importance as *phzM* alone lack the capacity to methylate phenazine and thus *phzS* is needed to produce pyocyanin<sup>29</sup>.

When testing phenotypic production of pyocyanin, it was found that only 40% of the isolates produced pyocyanin with a level of production between 1.2 ug/ml and 7.4 ug/ml.

Although they were all grown at the same pH and temperature in the laboratory, but such variable results could be attributed to factors as the origin of the samples, sensitivity to pH changes, incubation period and glycerol concentration in the media. In fact, it was detected by others that the expression of the *phzM* and *phzS* gene are environment dependent.<sup>30</sup>

In our study, when the protease test was performed on skimmed milk agar it was interestingly detected that presence of special nutrient (milk in skimmed milk agar) triggered production of pyocyanin in fifteen of the originally non-producing strains. These findings are in correlation with a previous study which showed that

quorum sensing genes could interact in rich media to activate the phenazine operon promoters<sup>31</sup>.

When the MIC of the antibiotics under test were detected in the current study, it revealed that pyocyanin producers strains had an MIC of 2 fold higher than non-pyocyanin producing with some antibiotics as ciprofloxacin, levofloxacin, tobramycin and spectinomycin, while high resistance was shown against ampicillin and sulphamethoxazole, and high sensitivity was observed against colistin. That was in accordance with previous study where loss of pyocyanin production correlated to a decrease in MIC<sup>32</sup>. Propolis extract showed an MIC range of 625 -1250 ug/ml, the use of propolis against wound infections is gaining a very good reputation as propolis has a dual effect of healing wounds by its ability to regenerate tissues and its antimicrobial activity useful in fighting infections<sup>33,34</sup>.

As for spectinomycin, usually used to treat gonorrhea, but used for treatment of *Pseudomonas* infections when colistin resistant. it showed an MIC of 125 to 250 ug/ml. These results were higher than those obtained in a recent study Li,<sup>35</sup> where MIC was 32 ug/ml.

In our study, antibiofilm activity of the antibiotics under test and propolis was determined. Subinhibitory concentrations of antibiotics had variable effect on the biofilm mass, while ciprofloxacin, levofloxacin and propolis extract has an inhibitory effect on biofilm formation as shown by decrease in formed biofilm mass (OD 630). Such results on propolis were in accordance with those of De Marco,<sup>36</sup> who found that propolis had an antibiofilm activity against *Pseudomonas aeruginosa*. For ciprofloxacin and levofloxacin, it was proved that they have a capability of killing bacterial cells in biofilms in very high concentrations<sup>37</sup>. Regarding fluoroquinolones, they had an antibiofilm activity at subinhibitory concentrations against streptococcus biofilms<sup>38</sup> and showed an inhibitory effect on *Pseudomonas aeruginosa* biofilm with different results according to the drug tested.

While the antibiotics ampicillin, colistin sulphate, sulphamethoxazole, tobramycin, spectinomycin induced biofilm formation at subinhibitory concentration such result was completely reverted by increasing concentration most probably due to bacterial killing. The results are similar to those reported previously on aminoglycosides where this class of antibiotics induced biofilm formation in *Pseudomonas aeruginosa*<sup>39</sup>. In addition, colistin was reported to induce the biofilm formation in *Acinetobacter baumannii*<sup>40</sup> through enhancement of protein expression. Besides, colistin was recorded to increase quinolone signaling thus inducing biofilm<sup>41</sup>.

Concerning the effect of the subinhibitory concentration of antibiotics on pyocyanin production, all the antibiotics in the study were tested against pyocyanin producing *Pseudomonas* and non-pyocyanin

producing *Pseudomonas*. It was detected that all the antibiotics under test inhibited the production of pyocyanin completely. As for ampicillin, the amount of produced pyocyanin decreased by 70 %. The pyocyanin is known to be variably affected by subinhibitory concentration of compounds as mentioned before, in a study where sub MIC concentrations of the royal jelly induced pyocyanin production<sup>42</sup>. A recent study done in 2021 found that ciprofloxacin induced pyocyanin production, while with tobramycin its levels remained unchanged<sup>8</sup>. The pyocyanin level is strain specific, substances that are enriching are possibly responsible of increasing amount of pyocyanin as for glycerol and skimmed milk.

## CONCLUSION

The results could be attributed to an effect on quorum sensing gene expression or on the activity of the enzymes responsible for pyocyanin formation; and the degree of effect might be attributed to the activity of the antibiotic where the antibiotics affecting protein synthesis and DNA was more powerful than those affecting cell wall.

Statistical correlation revealed that pyocyanin levels were higher in isolates from wound infections and they were strong biofilm formers, it was detected previously that pyocyanin quantities are strain specific<sup>43</sup>. Such results are of great importance because *Pseudomonas* isolates loose ability to produce pyocyanin under low antibiotics concentrations but tend to hide in biofilms, hence, empiric antibiotic treatments and abrupt antibiotic cessation might induce biofilm formation as a defense mechanism, in an attempt to overcome the loss of pyocyanin weapon, specially that both behaviors are controlled by quorum sensing.

Propolis offers a great treatment for wound infections caused by pyocyanin producing strong biofilm formers *Pseudomonas aeruginosa* as it both eliminates biofilm and decreases pyocyanin production.

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### Competing interests statement

The authors declare that there are no conflicts of interest.

### Contributors' statement

Both authors equally contributed to the manuscript.

### Authors contribution:

**Both authors equally contributed in the manuscript**

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