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Pyocyanin: A Powerful Inhibitor of Bacterial Growth and Biofilm Formation

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Article Info

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Abstract

Pyocyanin, a pigment naturally produced by most *Pseudomonas aeruginosa* strains and noted to have a role in the well being of the organism and its pathogenicity, was studied for its role as an inhibitor of bacterial growth and biofilm formation of many clinical bacterial isolates. The organisms included in the study were 33 isolates of 11 different bacterial species. The results showed that most of the Gram-positive isolates from the 3 species included in the study: *Staphylococcus aureus, Staphylococcus saprophyticus* and *Enterococcus fecal is* were inhibited at low concentrations of pyocyanin. The results varied for the Gram-negative organisms tested. Whereas the growth of the *Escherichia coli, Citrobacter koseri, Enterobacter cloacae* and *A. baumannii* isolates was clearly inhibited even by low concentrations of pyocyanin, the growth of *Klebsiella pneumonia, Proteus mirabilis* and *Morganella morganii* was not affected even by higher concentrations of pyocyanin. The methanol extract of the pigment was highly effective in preventing the biofilm forming ability of all *S. saprophyticus*, *A. baumannii*, *E. cloacae* and *K. pneumoniae* isolates, 3 of the 4 *P. mirabilis* isolates and 2 of the 5 *E. coli* isolates. All the other isolates tested, also showed a moderate antibiofilm activity This study revealed that pyocyanin has a powerful inhibitory effect on the bacterial growth and/or biofilm forming ability of the numerous clinically significant isolates tested, a characteristic that can prove valuable in developing new drugs for the treatment and prevention of different bacterial infections, once its safety for regular use has been assessed.

Keywords: Pyocyanin; Antibacterial activity; Inhibition of biofilm formation; Bacterial pigment; *Pseudomonas aeruginosa*.

Introduction

*Pseudomonas aeruginosa*is a proteobacterial Gram-negative bacillus placed in the family *Pseudomonadaceae* based on the analysis of conserved macromolecules like the 16S ribosomal RNA [1]. Its metabolism relies mainly on aerobic respiration. It is motile and almost all of its strains are motile by the single polar flagellum that they possess [2,3]. *P. aeruginosa* can colonize many natural and artificial areas including soil, water, etc [4,5]. This is due to its ability to live not only in atmospheric conditions, but also in hypoxic atmospheres. Special features, including the different colors and pigments it produces, make *P. aeruginosa* easily recognizable. These pigments include pyocyanin (blue-green), pyoverdin (yellow, green and fluorescent), pyomelanin (light brown) and pyorubrin (red-brown) [6,7]. Pyocyanin, or the 'blue pus' (from pyocyneus), is produced by around 90-95 % of all *P.aeruginosa* isolates (Fordos, 1863; Gessard, 1984; Ran et al., 2003) [8,9,10]. Pyocyanin production is favored in media where iron is not present in high amounts, but it plays a major role in iron metabolism. Its mechanism of action relies mainly on its ability to reduce and release the iron from transferrin; this is important since *P.aeruginosa* relies crucially on iron for growth (Cox, 1986) [11].

Pyocyanin is a natural derivative of the nitrogen-containing heterocyclic compound called phenazine. It is a redox active secondary metabolite and soluble in chloroform. At the genetic level, 7 genes, namely phz C, D, E, F, G, M and S, were identified to be responsible for the biosynthesis of pyocyanin in *P. aeruginosa*. These loci were found in all *Pseudomonas* spp [12,13,14]. The phz M and phz S genes were found to be the main genes responsible for converting phenazine-1-carboxilic acid to pyocyanin. However, little information about the enzymes encoded by the phz M and phz S genes is known.

The antibacterial effect of pyocyanin was first reported in 1940, when its effect on the inhibition growth of *Escherichia coli* was tested, the pigment was named colicin back then [15]. The purified form of pyocyanin exhibited a concentration dependent bactericidal effect [16]. The mechanism behind the antibacterial effect of pyocyanin was investigated and it was concluded that pyocyanin prevents the cells from performing their active metabolic transport process by interacting with the cell membrane respiratory chain [17]. When *E.coli* was treated with pyocyanin, the oxygen supply to these cells was depleted, in addition to H_2O_2 production, and diversion of the electron flow, causing toxicity to *E. coli* cells [18]. Pyocyanin was also reported to have an anti-staphylococcal effect, for pyocyanin extracted from *P. aeruginosa* isolated from the sputum of Cystic fibrosis (CF) patients inhibited the growth of *Staphylococcus aureus* [19]. Pyocyanin also showed a bactericidal effect against *Salmonella* Paratyphi and *E. coli* [20]. It is now accepted that pyocyanin accounts for 90-95 % of the ability of *P. aeruginosa* to inhibit bacterial growth. In fact, pyocyanin isolated from *P. aeruginosa* 4B strain, was shown to exhibit an antibacterial effect against food spoilage bacteria like *Listeria monocytogenes* and *Bacillus cereus* [21]. In addition, when pyocyanin from *P. aeruginosa* DSO-129 was produced in a low cost micro-bioreactor with high processing, it was shown to have an inhibitory effect against several microorganisms including *S. aureus, Staphylococcus epidermidis, Bacillus subtilis, Micrococcus luteus* and *saccharomyces cerevisiae* [22].

The inhibition of fungi like *C. albicans* by pyocyanin was also clinically proven in patients with lung infections, as infection by *C. albicans* recurred after pyocyanin suppression [23]. Furthermore, *Aspergillus fumigatus* and *Candida albicans*, isolated from the sputum of Cystic fibrosis patients, were also inhibited by pyocyanin isolated from *P. aeruginosa* [24].

This study aimed at determining whether pyocyanin had the ability to inhibit the growth and/or the formation of biofilms by different Lebanese clinical isolates.

Materials and Methods

Bacterial isolates

The bacterial isolates used in the study were clinical isolates courteously provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center- Rizk Hospital (LAUMC- RH) and 4 isolates from a community health center referred to in the study as public isolates (PI). In total, the isolates tested were the following: 5 isolates of *Escherichia coli*, 5 isolates of *Staphylococcus* aureus, 4 isolates of *Proteus mirabilis,* 4 isolates of *Klebsiella pneumoniae*, 4 isolates of *Enterobacter cloacae,* 3 isolates of *Citrobacter koseri, 2* isolates of *Staphylococcus saprophyticus,* 2 isolates of *Enterococcus fecalis,* 2 isolates of *Acine* to *bacter* baumannii, and 2 isolates of *Morganella morganii*. The identity of the isolates was confirmed standard tests [25]. The identity of the Gram-negative bacilli was confirmed using the API 20E test strips (Biomerieux-France).

Aqueous extraction and purification of pyocyanin and testing for antibacterial effect

Aqueous extraction and purification of pyocyanin

pyocyanin was extracted using the method previously described by Wilson et al. (1987), [26] but as amended by Abou Raji El Feghali and Nawas (2018) [27]. Major amendments were related to media used to grow the organism, incubation and holding time and the addition of a step to the acid/base extraction, to optimize the yield of pyocyanin.

Antibacterial activity of pyocyanin

The antibacterial effect of the aqueous extracts was performed by using the well agar diffusion method using Mueller-Hinton agar (MHA) as recommended [28]. Using a sterile swab, the MHA plates were seeded with the organism taken from a saline tube containing the fresh test organism and adjusted to have a turbidity equivalent to that of a 0.5 McFarland standard. Using a cork borer, an 8.5 mm well was placed in the middle of the plate. The well was filled with increasing volumes of pyocyanin extract, from 200µl to 700µl [29]. The plates were then incubated at 35°C for 24 h, after which the of zones of inhibition of growth around the wells (whenever present) were measured using a caliper. The reported results were the averages of the diameters measured for each of the zones of inhibition of growth that surrounded the wells for each concentration of the tested extract for each test organism.

Methanol extraction and testing for anti-biofilm activity

Preparation of the methanol extract

In the extraction and purification procedure used in the study, [27] and in the final steps after the dissolution of pyocyanin crystals with chloroform, 0.05M HCL was added to the mixture and the red aqueous phase extracted and neutralized drop by drop by 0.05M NaOH until the blue color was recovered. Afterwards, methanol was added to the mixture making it 80% of the total volume.

Effect of the methanol extract on biofilm formation

Preparation of the bacterial isolates

From fresh agar plates, each of the test organisms was used to inoculate a 10 ml trypticase soy broth (TSB) tube with 1% glucose. The inoculated TSB tubes were left in the incubator at 37°C for 24 h, after which, the culture tubes were diluted 100 times with fresh media.

Effect of the extracts on biofilm formation

To test for the formation of biofilms by the isolates and possible inhibition of the process by the methanol extract of pyocyanin, a method very slightly modified from that used by Mathur et al. (2006) was used [30]. The methanol extract was

added to specified test wells of the 96 well flat-bottom tissue culture plates and the plates were left to dry in the incubator under aseptic conditions. Upon drying, 200 µl of sterile TSB were added to all the wells of the plates with 10 µl of the diluted cultures (previous section) and incubated at 35°C for 24 h. The contents of the wells were then gently discarded by repeated soft tapping, after which the wells were washed with phosphate buffered saline (PBS, pH of 7.2) several times. Then, 0.2% sodium acetate was added to fix any biofilms that may have formed, and a 0.1% solution of crystal violet was finally added to stain the biofilms, when present. Excess stain was then removed with deionized water and the plates were left to dry. The optical densities were later determined by using a microplate auto-reader at 570 nm wavelength. To have a precise result, each of the test samples (and controls) was performed in 16 wells. The reported optical densities, in the study, were the averages of the 16 readings of each sample.

Results

The aqueous extract of pyocyanin showed clear antibacterial activity against a wide range of the bacteria tested. As is obvious from Table 1, of the Gram-positive isolates, all the 5 *S. aureus* isolates were inhibited at a volume of 200 µL of the extract, while the 2 *S. saprophyticus* and *2E. fecalis* isolates were also inhibited, however, one of each was inhibited by a volume of 200 µL, while the others by a volume of 300 µL. For all, however, the antibacterial effect was shown to increase with the increase of volume of the extract added. On the other hand, the results varied for the Gram-negative isolates, for whereas the growth of *E. coli, C. koseri,* and *E. cloacae* and *A. baumannii* isolates was clearly all inhibited either by a volume of 200 µL or a volume of 300 µl, (with a clear increase in antibacterial activity with increase in volume of pyocyanin tested), yet the growth of all the *K. pneumonia, P. mirabilis*and *M. morganii* isolates was not affected by the pyocyanin extract even with the maximal volumes of pyocyanin used in this study (Table 1).

As to the ability of pyocyanin to inhibit the biofilm formation of the different isolates, Table 2 represents the optical densities read for each of the samples tested and their controls. The same results are also represented graphically for individual species in Figureures 1 to 10. The results clearly indicated that all the clinical isolates were capable of forming biofilms on the bottom of the tissue culture plates. The power of the pyocyanin methanol extract to affect the ability of the isolates to form biofilms is also obvious. The pyocyanin methanol extract was highly effective in preventing the biofilm forming ability of all *S. saprophyticus* (Figureure 2), *A. baumannii* (Figureure 6), *E. cloacae* (Figureure 9) and *K. pneumoniae* (Figureure 10) isolates, 3 of the 4 *P. mirabilis* isolates (Figureure 5), 2 of the 5 *E.coli* isolates (Figureure 4), 1 of the 2 *E. fecalis* isolates (Figureure 3) and 1 of the 5 *S. aureus* isolates (Figureure 1). All the other isolates in this study including the *M. morganii* and *C. koseri* isolates, however, showed a moderate antibiofilm activity (Figureures 7&8).

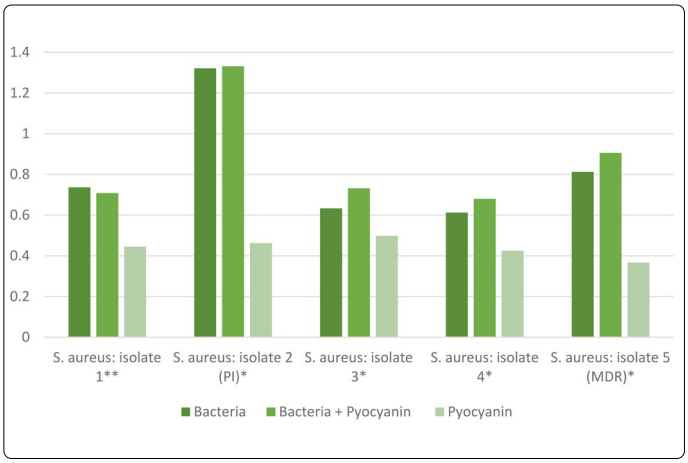
Table 1. Diameters, in millimeters, of the zones of inhibition of growth of the bacterial isolates, using different concentrations of the aqueous extract of pyocyanin. X: No zone of inhibition. PI: Public isolate, MDR: multidrug resistant, ESBL: extended spectrum beta lactamase producing

Isolates	Control	200 µL	300 µL	400 µL	500 µL	600 µL	700 µL
Gram positive							
S.aureus: isolate 1	X	27.0	29.7	32.3	34.0	35.7	38.0
S.aureus: isolate 2 (PI)	X	27.7	31.0	34.3	35.7	37.0	38.3
S.aureus: isolate 3	X	28.7	30.7	32.7	34.0	36.7	38.3
S.aureus: isolate 4	X	28.0	30.3	34.5	35.3	36.7	38.0
S.aureus: isolate 5 (MDR)	X	20.3	23.3	25.7	29.7	31.3	32.7
S.saprophyticus : isolate 1	X	25.0	34.0	35.3	40.3	41.7	43.3
S.saprophyticus : isolate 2	X	X	26.7	30.0	34.3	36.7	39.7
E.fecalis : isolate 1	X	27.7	35.7	37.7	39.0	43.0	48.0
E.fecalis : isolate 2 (MDR)	X	X	22.0	25.0	29.3	32.7	35.7
Gran negative							
E.coli : isolate 1 (PI)	X	23.3	28.7	31.7	33.3	35.7	37.7
E.coli : isolate 2 (ESBL)	X	25.7	29.3	31.3	33.7	35.0	36.3
E.coli : isolate 3 (ESBL)	X	X	28.0	31.3	33.0	34.3	35.7
E.coli : isolate 4 (Non ESBL)	X	X	25.0	26.7	28.0	30.0	31.7
E.coli : isolate 5 (Non ESBL)	X	X	30.3	31.7	33.0	36.0	37.3
P. mirabilis : isolate 1	X	X	X	X	X	X	X
P. mirabilis : isolate 2	X	X	X	X	X	X	X
P.mirabilis : isolate 3	X	X	X	X	X	X	X
P.mirabilis : isolate 4 (PI)	X	X	X	X	X	X	X
M.morganii: isolate 1	X	X	X	X	X	X	X
M.morganii: isolate 2	X	X	X	X	X	X	X
C.koseri : isolate 1	X	X	21.7	25.0	26.3	28.3	31.7
C.koseri : isolate 2	X	X	20.3	25.7	27.3	28.0	29.7
C.koseri : isolate 3	X	X	21.3	22.7	25.7	27.3	30.3
E.cloacae : isolate 1	X	X	21.7	28.3	30.3	33.0	34.3
E.cloacae : isolate 2	X	X	24.7	30.3	32.0	34.3	35.7
E.cloacae : isolate 3	X	21.0	22.3	25.3	28.7	30.3	34.7
E.cloacae : isolate 4	X	21.0	25.0	27.7	30.3	32.3	35.3
K.pneumoniae : isolate 1 (PI)	X	X	X	X	X	X	X
K.pneumoniae : isolate 2	X	X	X	X	X	X	X
K.pneumoniae : isolate 3	X	X	X	X	X	X	X
K. pneumoniae : isolate 4 (ESBL)	X	X	X	X	X	X	X

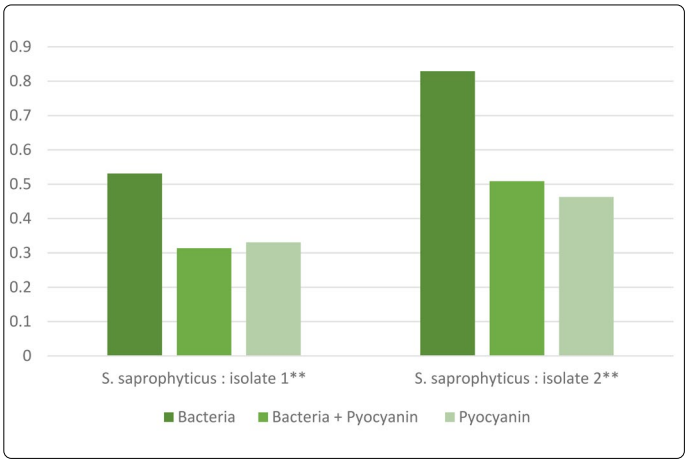
A. baumannii : isolate 1	X	20.3	24.7	27.3	29.0	31.3	33.3
A. baumannii: isolate 2	X	20.0	25.3	28.3	30.3	31.7	34.7

Table 2. Average optical density (O.D.) readings at a wavelength of 570 nm, of the different cultures tested in this study. PI: Public isolate, MDR: multidrug resistant, ESBL: extended spectrum beta lactamase producing.*: Moderate antibiofilm effect, **: High antibiofilm effect

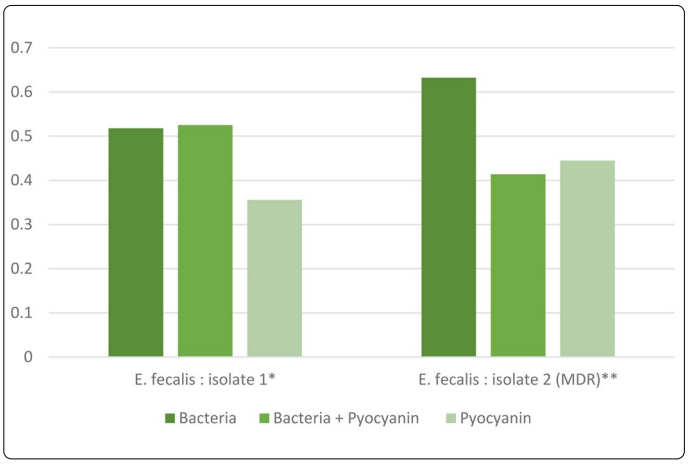
Bacterial isolates	O.D. readings of the wells containing		
	Bacteria	Bacteria + Pyocyanin	Pyocyanin
Gram positive			
S.aureus: isolate 1**	0.7363	0.7082	0.4449
S.aureus: isolate 2 (PI)*	1.3212	1.3318	0.4627
S.aureus: isolate 3*	0.6334	0.7318	0.4984
S.aureus: isolate 4*	0.6126	0.6796	0.4249
S.aureus: isolate 5 (MDR)*	0.8122	0.9056	0.3669
S.saprophyticus: isolate 1**	0.5315	0.3135	0.3307
S.saprophyticus: isolate 2**	0.829	0.5086	0.4627
E.fecalis : isolate 1*	0.518	0.525	0.3558
E.fecalis : isolate 2 (MDR)**	0.6325	0.4139	0.4449
Gran negative			
E.coli : isolate 1 (PI)*	0.4891	0.576	0.4627
E.coli : isolate 2 (ESBL)*	0.595	0.6382	0.4985
E.coli : isolate 3 (ESBL)**	0.5161	0.4512	0.4984
E.coli : isolate 4 (Non ESBL)*	0.5709	0.7197	0.4494
E.coli : isolate 5 (Non ESBL)**	0.5368	0.5281	0.4646
P.mirabilis: isolate 1**	0.5021	0.4836	0.3909
P.mirabilis: isolate 2**	0.5697	0.5568	0.4494
P.mirabilis: isolate 3*	0.4518	0.4694	0.3669
P.mirabilis: isolate 4 (PI)**	0.7437	0.6379	0.3669
M.morganii: isolate 1*	0.7719	0.7977	0.4738
M.morganii: isolate 2*	0.4046	0.4682	0.3558
C. koseri : isolate 1*	0.6013	0.8197	0.5085
C .koseri : isolate 2*	0.8871	1.2904	0.3909
C.koseri : isolate 3*	0.5816	0.6194	0.4159
E.cloacae : isolate 1**	0.8041	0.5953	0.4159
E.cloacae : isolate 2**	0.6014	0.5681	0.5038
E.cloacae : isolate 3**	0.5822	0.5223	0.5139
E.cloacae : isolate 4**	0.4395	0.3839	0.3745
K.pneumoniae : isolate 1**	0.4556	0.4018	0.4984
K.pneumoniae : isolate 2**	0.4882	0.3798	0.4446
K.pneumoniae : isolate 3**	0.5877	0.4145	0.4159
K. pneumoniae : isolate 4 (ESBL)**	0.5786	0.4459	0.3802
A. baumannii : isolate 1**	1.4349	1.0391	0.3558
A. baumannii : isolate 2**	0.8056	0.6077	0.4028



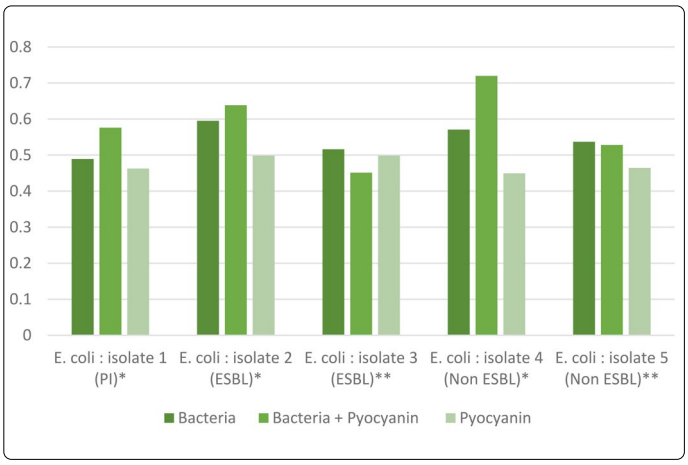
Figureure 1. Graphic representation of the data in Table 2. X axis: Samples (*S. aureus* isolates 1, 2(PI), 3, 4, 5 (MDR) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm. (PI): Public isolate, (MDR): Multi Drug Resistant



Figureure 2. Graphic representation of the data in Table 2. X axis: Samples (*S. saprophyticus* isolates 1 and 2) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.

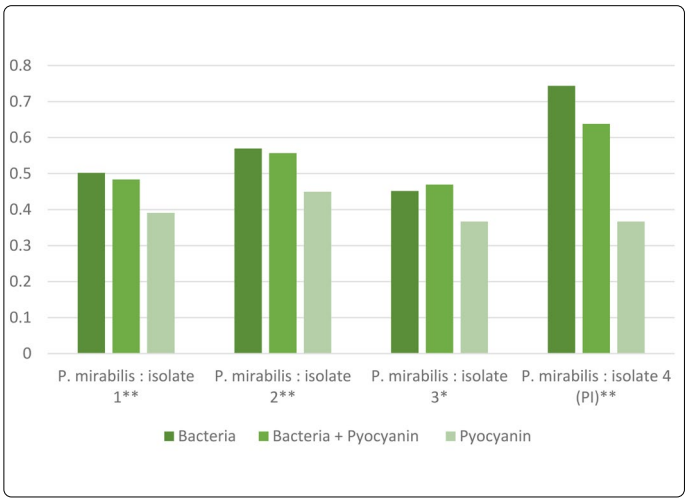


Figureure 3. Graphic representation of the data in Table 2. X axis: Samples (*E. fecalis* isolates 1 and 2 (MDR)) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm. (MDR): Multi Drug Resistant

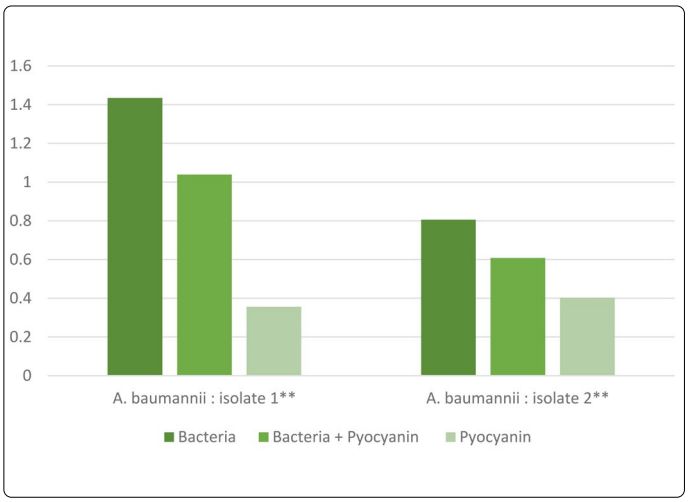


Figureure 4. Graphic representation of the data in Table 3. X axis: Samples (*E. coli* isolates 1(PI), 2(ESBL), 3(ESBL), 4, 5) tested for

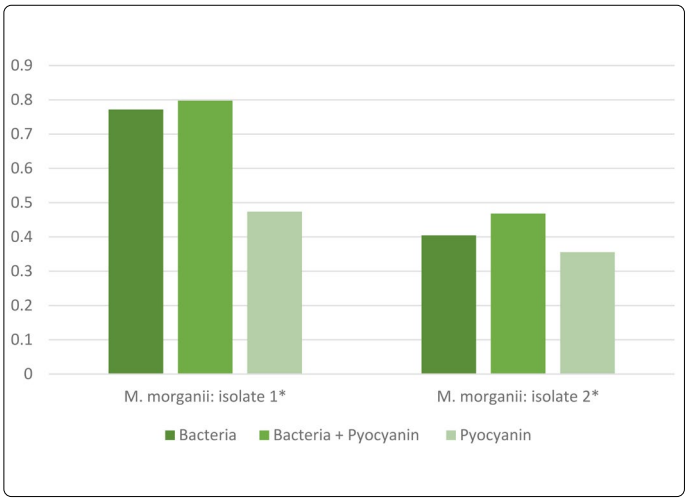
biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm. (PI): Public isolate.



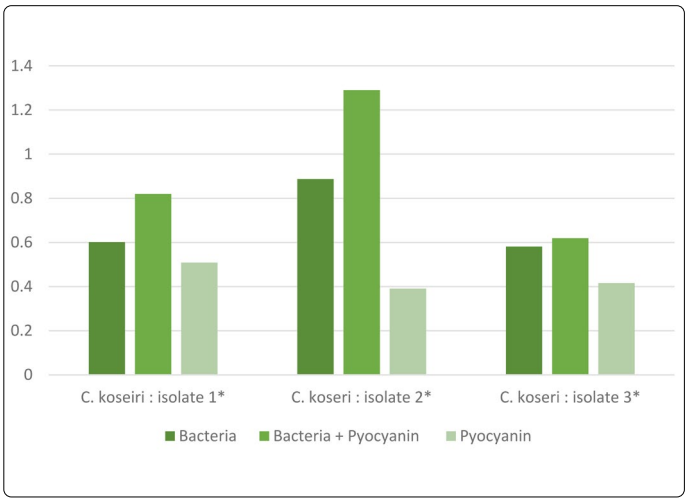
Figureure 5. Graphic representation of the data in Table 3. X axis: Samples (*P. mirabilis* isolates 1, 2, 3, 4(PI)) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm. (PI): Public isolate.



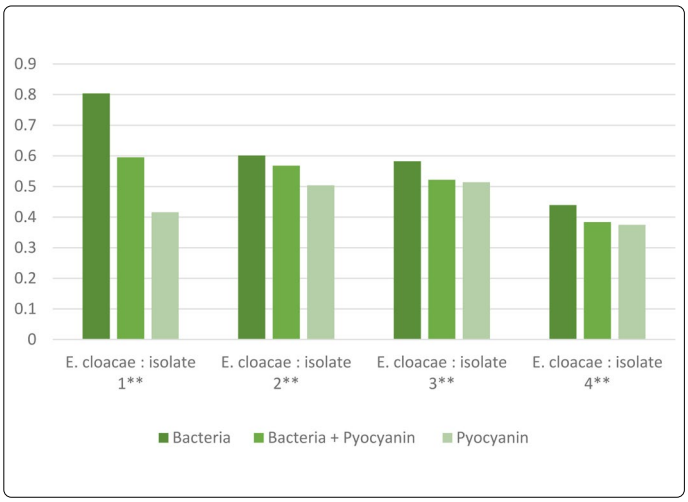
Figureure 6. Graphic representation of the data in Table 3. X axis: Samples (*A. baumannii* isolates 1 and 2) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.



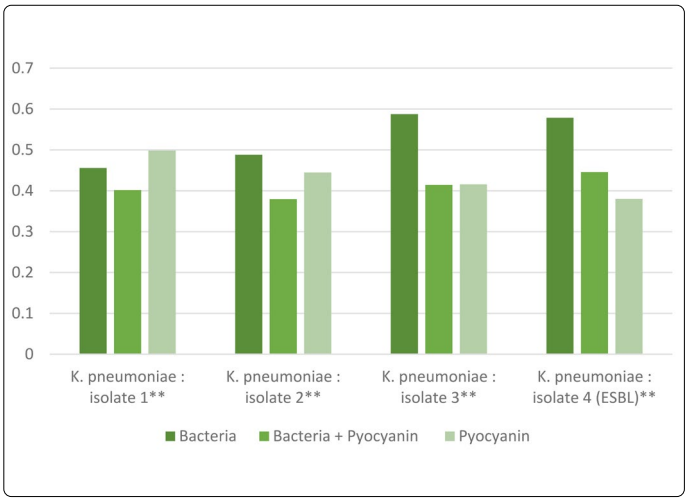
Figureure 7. Graphic representation of the data in Table 3. X axis: Samples (*M. morganii* isolates 1 and 2) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.



Figureure 8. Graphic representation of the data in Table 3. X axis: Samples (*C. koseri* isolates 1, 2 and 3) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.



Figureure 9. Graphic representation of the data in Table 3. X axis: Samples (*E. cloacae* isolates 1, 2, 3 and 4) tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.



Figureure 10. Graphic representation of the data in Table 3. X axis: Samples (*K. pneumoniae* isolates 1, 2, 3 and 4(ESBL)) tested for

biofilm formation/inhibition; Y axis: optical density (O.D.) readings at a wavelength of 570 nm.

Discussion

Previous reports noted the antibacterial and antifungal activity of pyocyanin [19,23,24,31,32]. The results of this study showed that the antibacterial effect of pyocyanin on the different Lebanese bacterial strains tested, was also evident. The Gram-positive organisms including *S. aureus, S. saprophyticus* and *E. fecalis* were all inhibited even using low concentrations of the aqueous pyocyanin (200 or 300 µL). A similar result was reported by Machan and his colleagues in 1991, where pyocyanin extracted from *P. aeruginosa* from cystic fibrosis (CF) patients exhibited anti-staphylococcal activity by inhibiting the growth of *S. aureus.* These results were obtained through the cross-streak test, well plate assay and growth of mixed culture [19].

Most of the Gram-negative bacteria tested in this study, namely *E. coli, E. cloacae, C. koseri* and *A. baumannii*, were also inhibited by low concentration of aqueous pyocyanin (200 or 300 µL). The antibacterial effect of pyocyanin on certain Gram negative bacteria was also previously noted and reported, and it was shown that pyocyanin had an antibacterial effect against *E. coli* and *Acinetobacter* isolates [31,32].

It was noted in this study, however, that pyocyanin, even at the higher volumes used did not in any way affect the growth of the *K. pneumoniae, P. mirabilis* or *M. morganii* isolates tested (Table 1). Similar results were reported by previous studies. Sweedan in 2010, reported that *K. pneumoniae* and *P. vulgaris* were resistant to any antibacterial effect that pyocyanin may have, [31] while El-Shouny and his colleagues (2011) also reported that *K. pneumoniae* was fully resistant to any antibacterial activity that pyocyanin may have demonstrated [32].

It is also worth noting from Table 1 that the antibacterial effect of pyocyanin, on the different isolates that were susceptible to it, was concentration dependent and increased with increasing concentrations of the pyocyanin added. The diameters of the zones of inhibition of growth increased with increasing concentration of pyocyanin. The effect of pyocyanin, as an antibacterial agent, on the Gram-positive bacteria was evidently much stronger than that on the Gram-negative bacteria as was clear from comparing the diameters of the zones of inhibition of growth of the two groups (Table 1).

The mechanism through which pyocyanin exhibits its antibacterial activity was previously studied and it turned out that pyocyanin interacted with the respiratory chain of the cell membrane preventing the susceptible bacteria from performing their regular metabolic processes [17]. Furthermore, pyocyanin was toxic to the *E. coli* cells by depleting the oxygen supply to the cell, producing H_2O_2 and diverting the regular flow of electrons [18].

After reviewing the literature, it is believed that this study is the first to demonstrate the ability of pyocyanin methanol extracts to inhibit the biofilm formatting ability of several clinically important bacterial strains (Table 2 and Figureures 1-10). This finding is extremely important as one of the most challenging problems in antibacterial therapy remains the difficulty in targeting bacteria that are, enclosed within formed biofilms [33].

Conclusion

This study revealed that pyocyanin produced by *P. aeruginosa* has a powerful inhibitory effect on the bacterial growth and/or biofilm forming ability of the numerous clinically significant isolates tested. Such results could be supported by future studies on the rate, frequency and type of other infections in the presence of *P. aeruginosa,* like in the case of cystic fibrosis (CF) patients. Pyocyanin may turn out to be a valuable new addition to the currently existing medications as an antibacterial drug or a chemical used in the prevention of bacterial biofilm formation. Further studies are also needed to assess the safety of using pyocyanin on hosts and their microbiota.

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