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Original Research Article

Effect of Different Purified *Pseudomonas* exopigments on Vero-Cell Line and Some Pathogenic Bacterial Isolates

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Abstract

Genus *Pseudomonas* includes different species, each has the ability to produce different types of characteristic exopigments with their various manners of activities. This study includes production of *Pseudomonas* pigments like Pyocyanin and fluorescin in liquid culture media and used to examine their antibacterial activity. Also their cytotoxic effects on normal cell (Vero cell line) were used *in vitro*. The results revealed that pyocyanin pigment has growth stimulatory effect on vero-cell line caused increase in the growth of the cells number, especially at a concentration 30-40%, while fluorescin has cytotoxic inhibitory effect on vero-cell line causing inhibition in growth of the cells number, the inhibition ranged from 55-75%, especially at a concentration 30-40%. *Pseudomonas* exopigments are effectual antigrowth agents against different bacterial isolates, as well as human cells lines.

Key Words: *Pseudomonas,* Pyocyanin, Fluorescin, Vero cells, HPLC.

الخلاصة

يضم جنس الزوائف عددا من الانواع المختلفة التي لها القابلية على انتاج انواع متعددة ومختلفة من الصبغات المميزة والتي لها تأثيرات مختلفة. تضمن البحث انتاج واستخلاص عدد من هذه الصبغات، كالصبغة الخضراء المزرقة (البايوسيانين) والبايوفردين الصفراء. حيث تم فحص قابلية هذه الصبغات على تثبيط النمو البكتيري لعدد من انواع البكتريا المرضية، فضلا عن الكشف عن تأثيرها على نمو خطوطخلايا الكلى الطبيعية والمستخلصة من كلى القرد الافريقي. حيث بينت النتائج ان صبغة البايوسيانين لها تأثير محفز لنمو خلايا الكلى الطبيعية وخاصة عند تركيز 30–40%، بينما كان لصبغة البايوفردين تأثير مثبط للنمو خاصة عند تركيز 55–75%. أن الصبغات الخارجية لبكتريا الزوائف هي عوامل فعالة في تثبيط النمو للخلايا المختلفة المدروسة.

Introduction

Recently, too much usages of antibiotics cause the emergence of greatly resistant strains of bacteria, this is a difficult for critical care physicians because there are now several microbes that can only be effectively treated with a restricted group of antimicrobial members. Multi-drug resistant bacterial infections are related to increase morbidity and mortality, length of hospital admissions and fees of care. To lessen the selection burden for resistance, it was very significant to search for new antibiotics [1].

Pseudomonas aeruginosa is a major source of bacteraemia in victims of burns, catheterized patients with UTIs, and nosocomial pneumonia in patients with endotracheal tube in ICU. Also, it is the main cause of morbidity and mortality in patients with cystic fibrosis with long-term colonization of the lungs with this bacteria, due to abnormal airway epithelial layer [2].

These infections are difficult to cure, due to the natural resistance of this organism to antibiotics, *P. aeruginosa* produces many proteins, several of them have significant biological effects against different pathogens, that cause these different diseases [3].

Pseudomonas aeruginosa is an optimistic pathogen of humans. It is a member of the bacterial group Pseudo-monadaceae, that is widely spreading in the environment. It is widely disseminated in the nature and is commonly present in hospital environment. It causes diseases in immune-compromised humans [4].

Pseudomonas yields a number of extracellular pigments, as the phenazines is the most major case. The important and significant characteristic feature of *P. aeruginosa* is the production of the soluble type pyocyanin pigment: a phenazine compound, which is a water-soluble blue green pigment.

Pyocyanin had been implicated as an eminent reversible dye with its redox potential like that of menaquinone. It has different and special effects on the prokaryotic cells as a pharmacological agent; these biological activities are related to the resemblance in its chemical structure to flavoproteins, isoalloxazine, mononucleotide, flavin and flavin adenine dinucleotide compounds. Also, it is used for phytopathogens control [5]. Additionally, downstream processing and bioprocessing of pyocyanin pigment for aqua-culture uses have been informed [6].

While the less virulent species is *P. fluorescens*, that can cause opportunistic infections in humans in acute form, and it has been stated in clinical lab. samples from the lungs, stomach, and mouth. The most public site of its infection is the bloodstream. Most described cases are bacteremic cases, attributed to transfusion of the products of contaminated blood [7].

The secretion of the fluorescent pyoverdine pigment, (that is officially known as fluorescein), is what conveys *P. fluorescens* with its fluorescence properties under the Ultra-Violet light. Fluorescin is a high-affinity iron-chelating compound (siderophore), that is vital for bacterial growth, gaining of iron from the environment, and survival. Fluorescin is considered as the chief siderophore member of *P. fluorescens* [8].

This research aims to study the inhibitory effect of pyocyanin and fluorescin exopigments against different bacterial isolates, with their effects on human normal cells.

Materials and Methods

Clinical isolates:

Different clinical isolates were obtained from Microbiology lab/college of medicine, University of Babylon, Iraq. These isolates included *P. aeruginosa, P. fluorescens, Klebsiella pneumoniae, E. coli, Staphylococcus aureus, S. epidermidis,* and *S. haemolyticus*.

Purification of pigments (pyocynin and fluorescins) by HPLC:

High performance liquid chromatography (HPLC) was performed at Ultra-Violet visible detector set at 340 nm and the used column is (250×4.6 mm) and C18.5 µm particle size was used. The mobile phase was 100% acetonenitrile. The flow rate was 1.2 $ml.min^{-1}$. The temperature of column was conserved at 30° C. The peaks obtained were compared with the standard

Effect of pyocyanin and fluoresceins on bacterial growth

After purification of pigments, the effect of these exopigments on the bacterial growth was tested by using enzyme-linked immunosorbent assay (ELISA) reader spectrophotometer. Bacterial growths and suspensions were prepared for all tested bacterial isolates, 96 micro-well plate was filled with the prepared bacterial suspensions and the prepared extracted pigments in tenth dilutions to get concentrations of 10, 20, 30, 40 and 50% and incubated for 18-24 hours. Later on, values of the optical density (OD) from the ELISA reader were taken in a mode of absorption which covered the growth of bacteria in each sample. Three values of OD for each sample were recorded and

their means were calculated with obtaining the standard deviation.

Human Cell lines:

The Vero cell is a model for kidney epithelial cell. The cell line was kindly obtained and cultured at cancer research lab/college of medicine, University of Babylon, Iraq.

The viable cells number was determined by using crystal violet assay before and after using the pigments. The pyocyanin and fluoresceins pigments were cultured on 96 well plate with Vero cell line separately with different volume of pigments. Then the 96 well plate was incubated at 37C° for 24 hours. The plated cells were divided into five groups: group 1 as a control (not treated); group 2 (add 10 µl of pigment);group 3 (add 20 µl of pigment); group 4 (add 30 µl of pigment); group 5 (add 40µl of pigment). The final cells volume with toxin were 200µl. The plated cells were incubated at 37°C for 24 hours before counting [9].

Crystal Violet Assay:

The cultured cells were washed with 100µl of Phosphate buffer saline (PBS) and 200µL of the crystal violet staining solution was added to the cells in the 96 well plate. These cells were fixed and stained for 20 minutes at room temperature. Next plate was generously rinsed and allowed to dry overnight. The next day the dried and stained cells were measured at an absorbance of 570nm by micro plate reader [10].

Results

In order to show the antibacterial effects of the extracted *Pseudomonas* extracellular pigments (Pyocynine and Fluorescin) against different Gram positive and negative bacterial isolates; the results at figures1 and 2 showed the inhibitory effects of these two pigments. The two pigments showed bactericidal effects, as the bacterial counts were reduced significantly, as it measured by optical density.

Additionally, this activity was heightened by nearly increase the concentrations to 40-50% for all tested isolates, so the activity of pyocyanin as antibiotic is dependent on the concentration, where the bacterial viability was decreases by increasing the pigments concentration.

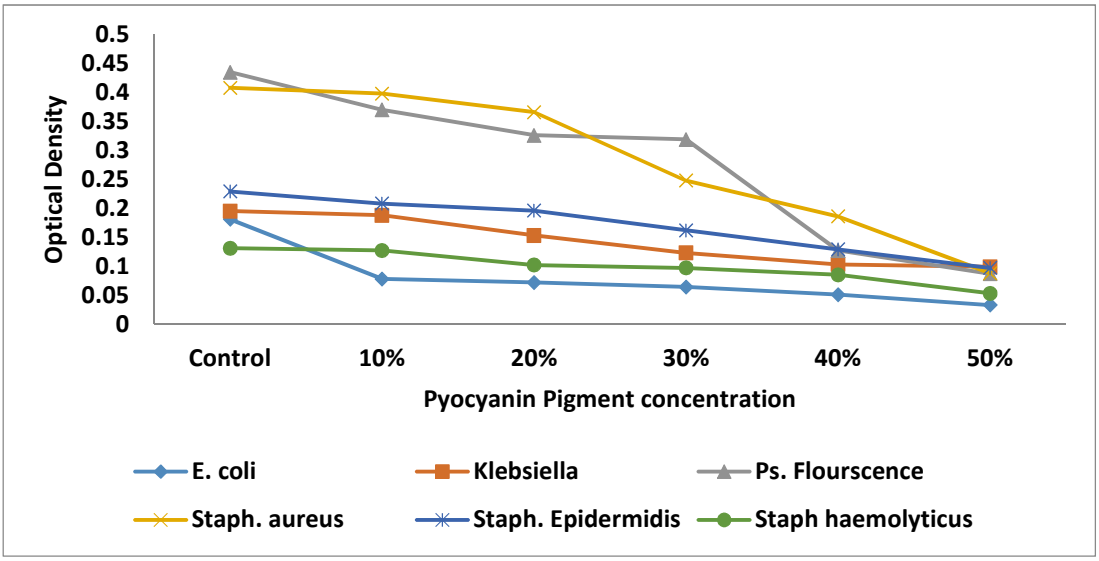

Figure 1: Effect of Different concentration of Pyocyanin on the Bacterial Count of Different pathogenic Bacterial Isolates.

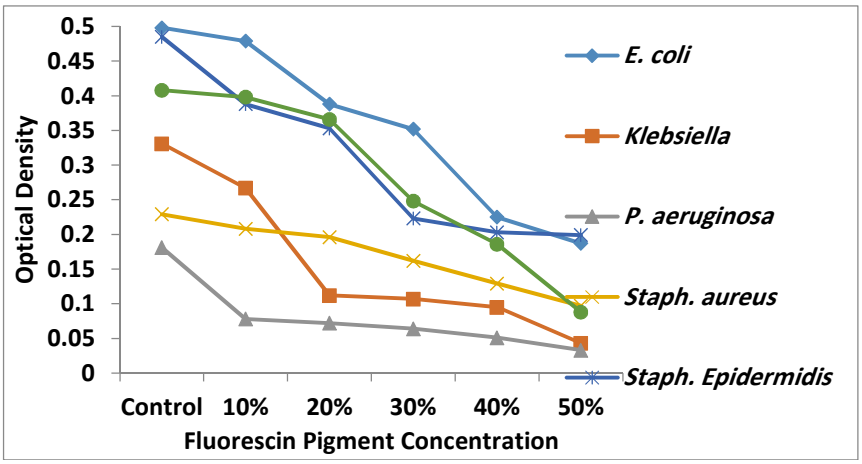

Figure 2: Effect of Different concentration of Fluorescin on the Bacterial Count of Different pathogenic Bacterial Isolates.

Regarding the cytotoxic effect of pyocyanin and fluorescin exopigments on vero cell line, results revealed that the pyocyanin pigment has growth stimulatory effect on vero-cell line, causing an increase in the cell line growth, especially at a concentration 30-40%, while fluorescin has a cytotoxic inhibitory effect on the vero cell line, that causes inhibition and decrement in growth of the cell line, as the inhibition rate varying from 55-75%, especially at a concentration 30-40% (Figures 3 and 4).

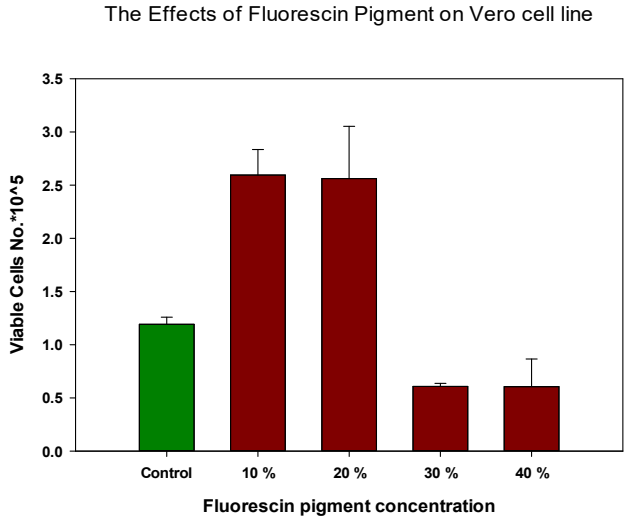

Figure 3: Cytotoxic Effect of Different Concentration of Fluorescin on the Vero-Cell line.

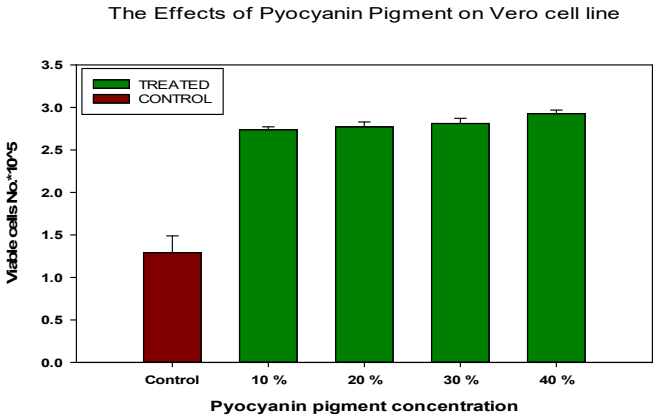

Figure 4: Cytotoxic Effect of Different Concentration of Pyocyanin on the Vero-Cell line.

Discussion

The high antimicrobial activity of pyocyanin in this study against all microbial pathogens under the test, acting as a wide spectrum antibiotic, was confirmed by several authors [11, 12].

In the same trend, El-Shouny et al. [12] revealed that growth of G+ve bacteria were entirely inhibited by the pyocyanin; Gram-negative bacteria, involving *Salmonella typhi* and *Proteus mirabilis*, were intermediately affected, while *K. pneumonia*e was completely resistant to pyocyanin. Approximate results done by several authors, also confirmed result of this study [4,13].

These pigments are important virulence factors in pseudomonas, they function as cell-to-cell signaling molecules and have an inhibitory activity against other bacteria. Pyocyanin which produced by *P. aeruginosa*, is considered as antibiotic that can act as competitive agents in microbial societies [14].

The repressing activity of pyocyanin was due to its unique redox potential. It was also proposed that, during respiration, pyocyanin becomes diminished and univalent diminish oxygen to the toxic superoxide radical. The resistance of various bacteria to pyocyanin would therefore be depending on availability of oxygen, catalase possessed by the organism and levels of superoxide dismutase [12].

Antibiotics produced by other bacteria in microbial collections could function as an indicator to alarm *P. aeruginosa* to the aggregation or survival of other bacteria and the successive elevate pigment production could help *P. aeruginosa* competition with other bacteria [15].

Pyocyanin causes a broad range of cellular damage like the suppression of epidermal cell growth, ciliary function, cellular respiration and might be attributed to the persistence of *P. aeruginosa*, with disturbance of calcium homeostasis [16]. It shows high cytotoxicity in higher concentrations, while this toxicity decreases in lower concentrations; pyocyanin is considered zwitter ions especially in hydrophobic regions, that allow to it move freely throw the cytoplasmic membrane and enter the cell easily [17]. Pyocyanin increase intra-cellular reactive oxygen species, the pigment is changed to the reduced form in the cell by NADPH and NADH, and this reduced form of pyocyanin then get ability to transfer electrons to oxygen, with generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which are ROS (Reactive oxygen species), This is identified as intracellular redox cycling of the pyocyanin.

ROS development appears to occur within and around the mitochondria. Pyocyanin also been revealed to inhibit the activity of catalase directly, and even to diminish the expression of the gene encoding catalase, preventing the breakdown of hydrogen peroxide and thus increase the level of ROS indirectly [18]. The dead cell release their internal enzymes with result increase the toxicity of the media and destroy the adjacent cells [19].

Mohammed et al [20] showed that both pigments have inhibitory cytotoxic effects on HepG2cell line, as they showed that pigments had cytotoxic inhibitory activity on HepG2 cell line causing suppression in growth of the cell line, as the inhibition rate ranged from 7%-84%, according to the concentrations.

Opposite to all these facts, other studies [21,22] showed that pyocyanin has a stimulatory effect on human cells. As Ulmer revealed that *Pseudomonas* pigments have dual dose-dependent increment as well as decrement effects on immune responses *in vitro*, as it is measured by DNA synthesis of human T and B lymphocytes, interleukin-2 production. Where *Pseudomonas* pyocyanin stimulated DNA synthesis, as its stimulatory increment effect was found at lower concentrations of pyocyanine, while higher concentrations of the pigment causes a suppression of the responses [21,23].

Conclusion

Pseudomonas exopigments are effectual antigrowth agents against different bacterial isolates, as well as human cells lines.

References

1-Gorgania N, Ahlbrand S, Patterson A, Pourmanda N. Detection of point mutations associated with antibiotic resistance in *P. aeruginosa*. Int. J. Antimicrob. Agent,2009; 34(5): 414-418.

2-Bodey G, Bolivar R, Fainstein V, Jadeja L. Infection caused by *P. aeruginosa*. Rev. infect. Dis.,1983; 5: 279-313.

3-Gerene M, Shankar S, Krzysztof J, Yunixa O, George T and Bradley E. Phenazine-1-carboxylic acid, a secondary metabolite of *P. aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. Am. J. Physiol. Lung. Cell. Mol. Physiol.,2003; 285: 584-592.

4-Sudhakar T, Karpagam S, Premkumar J. Biosynthesis, antibacterial activity of pyocyanin pigment produced by *Pseudomonas aeruginosa* SU1. J. Chem. Pharm. Res., 2015; 7(3):921-924.

5-Sudhakar T, Karpagam S, Premkumar J. Production, Extraction Comparison of Spectral Analysis of Pyocyanin Diffusible Pigment from *Pseudomonas aeruginosa*. 2nd International Conference on Chemical, Biological and Medical Sciences (ICCBMS'2013). Phnom Penh., 2013; 25-26.

6-Banerjee A, supaker S, Banerjee R. Melanin from the nitrogen–fixing bacterium *Azotobacter chroococcum*: a spectro-scopic characterization, PloS One.2014; e84574.

7-Scott J, Boulton FE, Govan JR, Miles RS, McClelland DB and Prowse CV. A fatal transfusion reaction associated with blood contaminated with *Pseudomonas fluorescens*. Vox Sang.,1988; 54:201–204.

8-Cornelis P. Iron uptake and metabolism in pseudomonads. App. Microbiol. Biotechnol., 2010; 86:1637–1645.

9-Madlum K N, Galib R A, Ghazi, F M and Ali Z H. The Effects of Zizyphus Stem Bark and Termite Shelter Tubes on HeLa cell Growth. RJPBCS.,2015; 6(6): 620.

10-Zhenbo X,Yanrui L, Shiqi L, Dingqiang C, Bing L, Lin L et al. Crystal Violet and XTT Assays on *Staphylococcus aureus* Biofilm Quantification. Curr. Microbiol.2016.

11-El-Fouly MZ, Sharaf A M, Shahin AA, Heba M, El-Bialy A and Omara A. Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. J. Rad. Res. App. Sci., 2015; **8**: (36-48).

12-El-Shouny W A, Al-Baidani A R and Hamza W T. Antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* isolated from surgical wound-infections. Inter. J. Pharm. Med. Sci., 2011; **1**: 1-7.

13-Chandran M, Duraipandi V, Yuvaraj D, Vivek P, and Parthasarathy N. Production and Extraction of Bacterial Pigments from Novel Strains and Their Applications. RJPBCS., 2014; 5(6): 584-593.

14-Dietrich LE, Price-WhelanA, PetersenA, WhiteleyM, NewmanDK. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol. Microbiol., 2006;61: 1308-1321.

15-Liang H, L. Li Z. Dong, Surette M G and Duan K. The yebc family protein pa0964 negatively regulates the *Pseudomonas aeruginosa* quinolone signal system and pyocyanin production. J. Bacteriol., 2008; 190: 6217-6227.

16-Denning G M, Railsback MA, Rasmussen G T, Cox C D and Britigan BE. *Pseudomonas pyocyanine* alters calcium signaling in human airway epithelial cells. Amer J. Physiol., 1998;274: 893-900.

17-Lau G W, Hassett DJ, Ran H and Kong F. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med., 2004;**10**:599–606.

18-O'Malley YQ,Reszk KJ, Rasmussen GT,Abdalla MY, Denning G M and Britigan BE. The *Pseudomonas* secretory product pyocyanin inhibits catalase activity in human lung epithelial cells. American J. Physiol. - Lung Cellular and Mol. Physiol.;2003; 285(5):1077-1086.

19-FitzGerald D J,Kreitman R, Wilson W, Squires D and Pastan I. Recombinant immunotoxins for treating cancer. Inter. J. Med. Microbiol., 2004; 293: 577-582.

20-Mohammed HA, Yossef HS, Mohammad FI. The Cytotoxicity Effect of Pyocyanin

on Human Hepatocellular Carcinoma Cell Line (HepG2). 2014

21-Ulmer AJ, Pryjma J, Tarnok Z, Ernst M, Flad HD. Inhibitory and stimulatory effects of *Pseudomonas aeruginosa* pyocyanine on human T and B lymphocytes and human monocytes. Infect. Immun., 1990; **58**: 808–815.

22-ModyC H, Buser D E, Syme R M and Woods D E. *Pseudomonas aeruginosa* Exoenzyme S Induces Proliferation of Human T Lymphocytes. Infect. Imm., 1800–1805 Iraqi J. Sci., 1995**; 55(2)**: 668-674 .

23-Hall S, McDermott C, Anoopkumar-DukieS, McFarland AJ, Forbes A, Perkins AV, Chess-Williams R, Kiefel, MJ, Arora D, Grant GD. Cellular effects of pyocyanin, a secreted virulence factor of *P. aeruginosa*. Toxins, 2016; 8: 236.