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Study Antibacterial Activity of Pyocyanin Extracted from *Pseudomonas Aeruginosa*

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Abstract: The study aimed to detect Pseudomonas aeruginosa pyocyanin genes and evaluate the antibacterial activity of pyocyanin produced by P. aeruginosa isolated from burn infections. A total of 224 specimens were collected from burn patients. Culture traits, biochemical testing, and Vitek-2 system confirmation were used to make the identification. P. aeruginosa was identified in 109 (48.6%) of bacterial isolates, 30 (27.5 %) produced pyocyanin, 45 (41.3 %) produced other pigments and 34 (31.2 %) not produced any pigments. Genetically, study included used PCR technique to investigation predominance of pyocyanin pigment production genes, exhibited higher. Chloroform was used to extract pyocyanin from P. aeruginosa, and FTIR was used to identify it. The extract of pyocyanin showed broad-spectrum antibacterial activity, with the greatest activity against Gram negative. It had little effect on Staphylococcus aureus and P. fluorescens and but shown significant antibacterial efficacy against Proteus mirabilis followed by Acinetobacter baumanni, Escherichia coli and Klebsiella pneumonia.

Key points: Pyocyanin extraction, Pyocyanin activity, phz S, phz M gene, and Pseudomonas aeruginosa.

Introduction

The extreme high number of spread of antimicrobial resistance (AMR) is a one of the most serious challenges that threaten global health (Rodrigues *et al.,*2019). Consequently, there is an ongoing need to develop a novel class of more affordable and safer natural compounds with high selectivity and specificity against microbial infections (Paillié-Jiménez *et al*.,2020).

Nowadays, microorganisms have gained attention for being biological agents that can combat many health-related problems owing to producing arsenals of bioactive compounds as secondary metabolites such as toxins, alkaloids, antibiotics, and pigments (Satapute *et al.,*2019). Moreover, natural and eco-friendly bio-pigments have drawn a lot of interests due to their high safety profile, increasing consumer acceptance, and their ability to reduce health related problems (Shouman *et al.,*2023). Among all natural sources, bacterial pigments are an appealing target and more desirable than other plant or animal sources. This is attributed to their rapid development, sustainable availability, and simplicity of controlling microbial cell factories for high production yields. Phenazines are a crucial class of these pigments, which are produced by some microorganisms; the most common is *Pseudomonas aeruginosa* which produces the phenazine pigment Pyocyanin (Gonçalves and Vasconcelos, 2021 & Zhou *et al*.,2022)

Pyocyanin gives *P. aeruginosa* cultures and fluids like lung sputum from patients with cystic fibrosis its distinctive blue or blue-green hue (Muhaidi *et al*.,2018 and Das *et al*.,2022). 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, the genes phzM and phzS

are the most important as they encode the two-step conversion of Phenazine 1 caroylic acid (PCA) to pyocyanin, phenazine - specific methyltransferase enzyme (PhzM) converts PCA to 5-methylphenazine-1-carboxylic acid betaine and the flavin-dependent monooxygenase enzyme (PhzS) catalyzes the hydroxylative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin (Seiffein and Ali, 2021& Marey et al.,2024). The final step is the decarboxylation of MPCBA by PhzS enzyme , which yields pyocyanin, the phzABCDEFG operon's products in P. aeruginosa manufacture phenazines from chorismic acid, Therefore, *phzM* and *phzS* are the two crucial genes responsible for pyocyanin biosynthesis, Figure (1) (Marey *et al*.,2024)

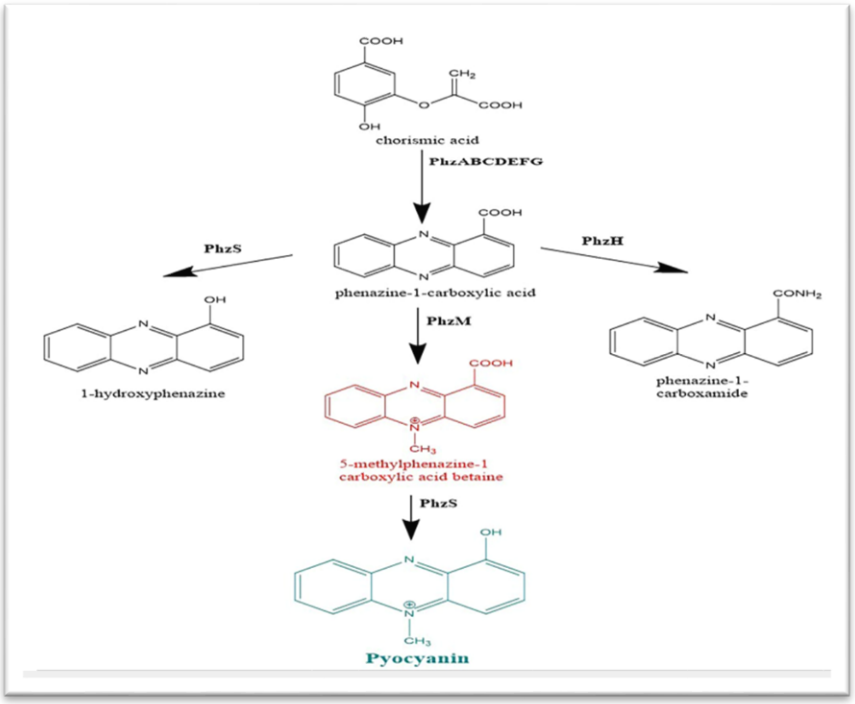

Figure 1: Biosynthetic pathway leading to pyocyanin in *P. aeruginosa* (Marey *et al*.,2024)

Burn are considered devastating form of trauma in patients with serious thermal injury, (El Hamzaoui *et al*.,2020), so many previous studies identical with this study, Forson *et al*.,(2017) they reached out of a total of 50 specimens collected from the burn patient, *P. aeruginosa* is a particularly concerning pathogen in burn patients because it is resistant to many antibiotics and can cause severe infections (Díaz-López *et al*., 2020 & Mohsin *et al*., 2020).

The bacteria *P. aeruginosa* produces the blue-green phenazine chemical known as pyocyanin, it has been shown to have potent antibacterial effects against various bacteria., it can disrupt the integrity of bacterial membranes, leading to cell lysis and death, (El-Shouny *et al*.,2011).

Materials and Methods

Between April 2022 and August 2023, 224 burn infection swabs were collected from patients at AL-Sader Medical City. Phenotypic analysis identified 109 *P. aeruginosa* isolates using the VITEK2 system.

A pure bacterial colony was grown on citrmid agar and HiFluoroTM Pseudomonas Agar Base, and the plates were then incubated at 37°C for 24 hours to identify the bacteria's capacity to create colors.

The Genomic DNA Mini Kit was used for DNA extraction by FAVOURGENE kit. Additionally, the DNA content was 74.65 mg/ml, and the purity of the solution was within the range of 1.7 ± 0.2 (Stephenson, 2003).

Table 1: Oligonucleotide primers of pigment genes

Gene	Primer	PCR products	Reference
phzM	F:GGATGGCCTTGGTCAATTCG´ R:GATCTTCCAGGGCGATACCC	233 bp	(Ali,2021)
phzS	F:CTGGTCGCCTATCCGATCTC R:GCTCTTCTCGGTCTTCGGTC	208 bp	(Ali,2021)

Pyocyanin purification, *P. aeruginosa* was cultivated in 1 L BHI broth at 37°C for three days while being shaken in order to extract pyocyanin. Following centrifugation (8000 rpm, 20 min), pyocyanin was extracted in stages using 0.1 N HCl and chloroform, and then the pH was adjusted with 0.4 M NaOH. After obtaining needle-like crystals, they were filtered, cleaned, vacuum-dried, and weighed. (Nowroozi, *et al*.,2012).

Fourier Transform Infrared Spectroscopy (FT-IR) Confirmation of Pyocyanin Extract. The pyocyanin extract underwent FT-IR analysis in compliance with Aziz et al. (2012). After placing the sample between two Thallium bromide plates and placing it within a Shimadzu infrared spectroscope, the resulting spectra were contrasted with typical pyocyanin levels.

Quantitative characterization of the pyocyanin, in order to quantify the isolated pyocyanin pigment, Dange *et al.* (2019) proposed the formula Pyocyanin

µg/ml=O.D. at520nm×17.072.

where the extinction coefficient is 17.072.

Antimicrobial susceptibility assay of pyocyanin (agar well diffusion), antibacterial activity was assessed on Mueller-Hinton agar by inoculating indicator bacteria and adding 50 µl of 100 mg/ml Pyocyanin extract into 4 mm wells. After 24 hours incubation at 37°C in the dark, inhibition zones were measured in millimeters. (owuama,2017).

Result and discussion

During the study period from April 2022 to August 2023, two hundred and twenty-four (224) specimens was collected and then culture on MacConkey, citrmide, Hifluoro pseudomonas, Nutrient agar, and blood agar and incubated (18_24) hours at 37 C. The results show that the result that 201 (90%) give bacterial growth and 23 (10%) give no growth. Finally, the Vitek 2 system was used for precise and accurate identification of the isolates at generic and species level, this test was applied on 109 of *Pseudomonas*.

Phenotype pigment Production of *P. aeruginosa*

The ability of *P. aeruginosa* isolates to produce pigment was tested using specific media citrmed agar and Hifluoro pseudomonas agar, the results shown that there were (75) isolates with a percentage (68.8%) were pigment producer from total (109) isolates, (34) isolates with a percentage (31.2 %) not produce pigment. From (75) isolates that pigment producer, 30 (27.5 %) isolates produce only pyocyanin, 45 (41.3 %) produced other pigments Figure (2)

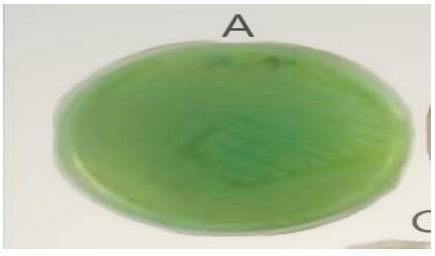

Figure 2: fluorescent pigment on cetrimide agar

The quaternary ammonium salt cetrimide, which functions as a cationic detergent and is poisonous to the majority of bacterial cells in the medium, increases *P. aeruginosa*'s synthesis of pyocyanin and pyoverdine in cetrimide agar (Sabu *et al*.,2022).

Tubes of nutrient broth and Hifluoro pseudomonas agar base exhibited a fluorescent pigment that can be seen under ultraviolet light after being infected with P. aeruginosa and cultured for 24 hours at 37°C, as seen in figure (3).

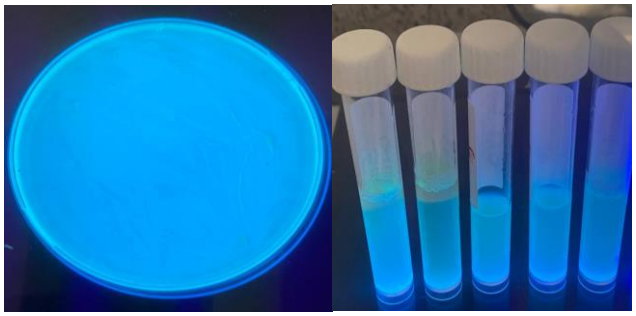

Figure 3: Shown pigment under UV.

Finding the Genes That Code for Pigment Through the PCR Method

Patients with cystic fibrosis have significant levels of pyocyanin in their sputum, and 90 to 95% of P. aeruginosa isolates generate it. (Jayaseelan *et al*.,2014).

This method has been implemented to identify the genes that produce color, and the DNA of all 30 isolates that Pyocyanin produce. In this study was detected on phenazine modifying genes *phz* M and *phz* S, based on the PCR study results for the *phzM* gene and *phz* S gene of *Pseudomonas aeruginosa,* it was observed that all 30 isolates showing pigment production gave positive results 100 % for the both of genes. figure (4 and 5 respectively).

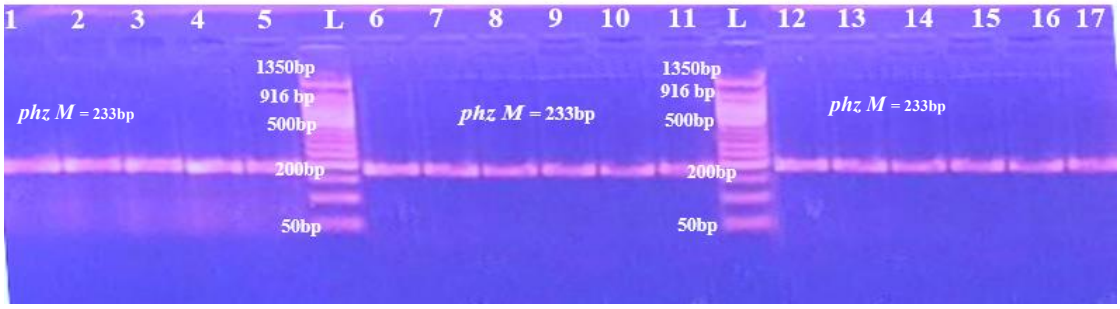

Figure 4: PCR product of *phz M gene* (233p) from *P. aeruginosa* isolates: L-molecular size marker 50bp DNA ladder, all isolates was positive of *P. aeruginosa*. The electrophoresis was performed 70 volts for 1.5 hr.

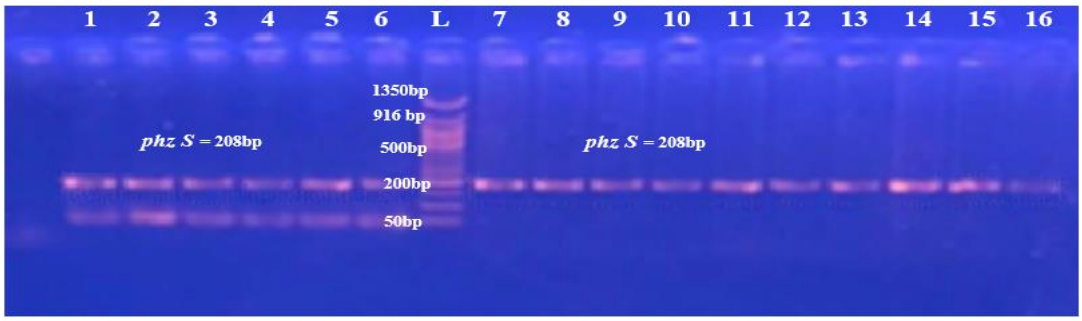

Figure 5: PCR product of *phz S gene* (208p) from *P. aeruginosa* isolates: L-molecular size marker 50bp DNA ladder. The electrophoresis was performed 70 volts for 1.5hr.

The findings of this investigation agree with those of Bogiel *et al*. (2021), research shown that the PhZ M gene and phz S gene has a 100% occurrence rate.

Many previous study were detected *phzM* gene among *P. aeruginosa* isolates, like Ali *et al*., (2021) were found that *phz M* was present (92.5%) of *P. aeruginosa* isolates, while in study of Tahmasebi *et al*.,(2022) the *phz* M were detected (58.1%) of bacterial isolates.

Seiffein and Ali (2021) were mention that The phzM gene works with phzS to produce pyocyanin and its prevalence was 90% among the isolates of study and PhzS enzyme is of great importance as phzM alone lack the capacity to methylate phenazine and thus phzS is needed to produce pyocyanin.

Extraction and chemical analysis of pyocyanin pigment

Pyocyanin pigment is a secondary metabolite that *P. aeruginosa* produces in the stationary phase, and 30 of 109 (27%) isolates of the bacteria were able to synthesize it in the current investigation. There are only one isolate chosen for pyocyanin extraction, and it had a high production rate and a high level of antibiotic resistance. Following extraction with 0.1 (N) HCl, the bluish extract of the chloroform-extracted pyocyanin became red, Figure (6).

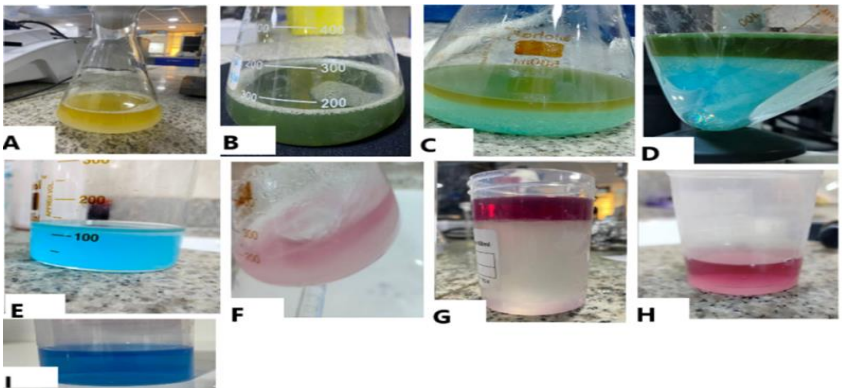

Figure 6: Bluish extract of pyocyanin converted color to red with HCl

A BHI medium inoculation, B creation of pyocyanin following three days of incubation; C addition of a chloroform layer; D and E separation of the chloroform layer; F addition of 0.1 N HCl; G separation of the aqueous layer; H addition of 0.4 N NaOH and restoration of the blue hue.

In similar study carried out by Jabbar *et al*. (2020) in Baghdad that using the king - A agar to enhanced pigment production even further, another study showed The King's A broth modified using soy as a nutrition produced the highest amount of pyocyanin, followed by the nutrient broth supplemented with sweet potatoes. (DeBritto *et al*., 2020).

Diagnosis of pyocyanin stain of *Pseudomonas aeruginosa* with FT-IR Analysis

FTIR analysis of pyocyanin revealed key functional groups: a phenazine core with O–H (3437.15 cm^{-1}), aromatic C–H (2950.73 cm^{-1}), aliphatic CH_3 (2850–2980 cm^{-1}), and C=N bond (1608.70 cm^{-1}), Figure (**7**).These peaks confirm its molecular structure.

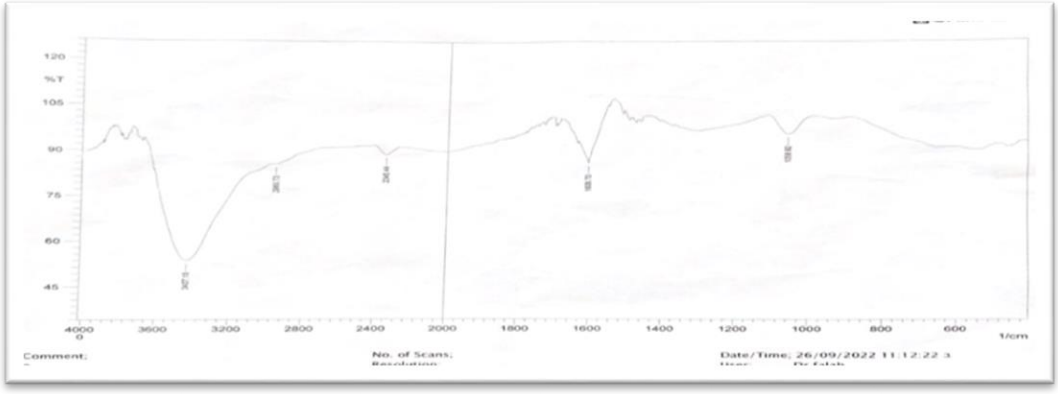

Figure 7: FTIR spectrum of pyocyanin

Noticeable pyocyanin absorbance bands in the 442 cm-1 to 2967.28 cm-1 peak region halide, alkene, aromatic, ester and nitro compounds (Ahmed, *et al*.,2016 and Sivasankara *et al.*,2021).

Antimicrobial activity of Pyocyanin

Pyocyanin (100 µg/ml) demonstrated stronger action against bacteria that are Gram-negative than those that are Gram-positive, as indicated by larger inhibition zones on Mueller-Hinton agar after 24–48 hours of incubation at 37°C. From the result appear that pyocyanin was high activity against *Proteus mirabilis* with inhibition zone (45 mm) followed by *Acinetobacter baumanni* (20 mm), *Escherichia coli* (18 mm) and *Klebsiella pneumonia* (15 mm) and the low activity appear against from gram positive bacteria *Staphylococcus aureus* with 12 mm of inhibition zone, however, no effect of pyocyanin on *Pseudomonas fluorescens* growth, figure (8).

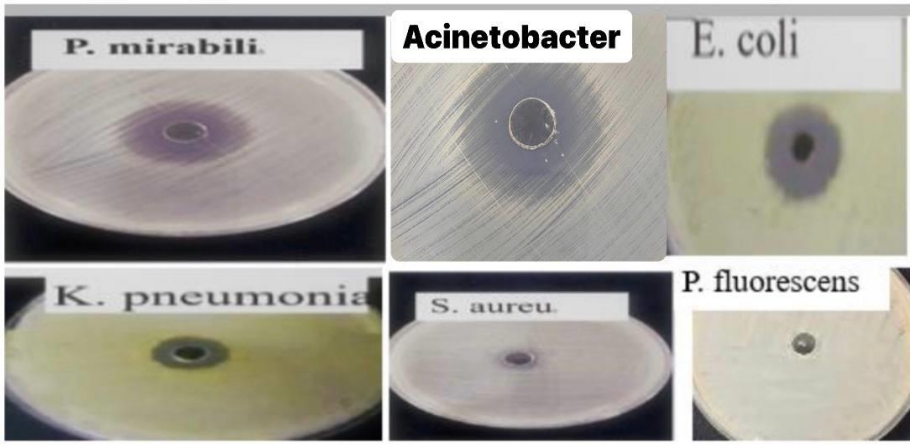

Figure 8: Antimicrobial activity of Extracted Pyocyanin on tested Bacteria at100 µg /mL

Through the passage of electrons and the buildup of ROS, particularly O_2^- and H_2O_2, following a reaction with molecular oxygen, pyocyanin causes oxidative stress in vulnerable prokaryotic and eukaryotic cells (Aonofriesei *et al*.,2004). However, the majority of microorganisms are able to produce certain metabolites that have an inhibitory effect on *P. aeruginosa*. These substances are released to prevent, but not to eliminate, possible rivals (Bonifácio *et al*.2020).

In study carrying out by Dhairh *et al*., (2022) demonstrated that pyocyanin production have antimicrobial activity toward gram negative more than gram Positive and *P. mirabilis* is more sensitive.

Study of Ghazi and Kahya, (2021). supports that results were gram positive bacteria less susceptible to Pyocyanin than gram negative bacteria this variation refers to the lipid of cell wall content of Gram positive and Gram-negative bacteria. Other study mention, on gram positive, the peptidoglycan layer and lipopolysaccharides layer could prevent the penetration of pyocyanin (Dijk et al., 2018).

Dhairh *et al*.(2022) Both Gram-positive and Gram-negative bacteria interact with pyocyanin (PYO) through their negatively charged cell walls; however, Gram-negative bacteria exhibit more ion uptake because of negatively charged LPS in their outer membrane. Increased intracellular damage results from this.

Conclusions

This study provided a timely and cost-effective method for the extraction of pyocyanin from clinical isolates of *P. aeruginosa* from patients with burn infections. It revealed that isolated pyocyanin was an effective broad-spectrum antimicrobial agent, as it showed inhibitory effects against Gram negative and Gram-positive bacteria, with more effect against most Gram-negative species. The findings of the current study indicated the efficacy of pyocyanin to inhibit colony formation. Accordingly, pyocyanin may participate in the management of clinical challenges represented by the spread of bacterial infections which traditional treatments are powerless, being a potential therapeutic alternative option.

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References

1. Ali, G. H. (2021). Effect of subinhibitory concentrations of selected antibiotics and propolis on pyocyanin and biofilm production among Pseudomonas aeruginosa isolates in Alexandria, Egypt. Egyptian Journal of Medical Microbiology, 30(4), 129-137.
2. Aonofriesei, F.; Crâşmaru,(2004,) M. Antibacterial activity of pyocyanin produced by some Pseudomonas strains isolated from seawater. In Annals of the University of Craiova, 2004, IX (XLV); University of Craiova: Craiova, Romania, pp. 121–126
3. Aziz, L. M., Hamza, S. J. and Abdul-Rahman, I. A. (2012). Isolation and characterization of phenazine produced from mutant Pseudomonas aeruginosa. Al-Anbar Journal of Veterinary Science 5(1): 42-53.
4. Bogiel T, Prażyńska M, Kwiecińska-Piróg J, Mikucka A, Gosp-odarek-Komkowska E (2021) Carbapenem-resistant Pseudomonas aeruginosa strains-distribution of the essential enzymatic viru-
lence factors genes. Antibiotics 10:8
5. Bonifácio, T.T.C.; Arruda, R.R.A.; Oliveira, B.T.M.; Silva, J.E.G.; Vasconcelos, (2020). Exposure to pyocyanin promotes cellular changes in Candida spp. Res. J. Pharm. Biol. Chem. Sci., 11, 111–119.
6. Dange, S. S., Gulve, R. M., Deshmukh, R. B., Phatake, Y. B., & Dange, S. R. (2019). Pyocyanin: process optimization and evaluation of its antimicrobial activity. Journal of Experimental Biology and Agricultural Sciences, 7(5), 494-504.
7. Das, T., Sabir, S., Chen, R., Farrell, J., Kriel, F. H., Whiteley, G. S., ... & Kumar, N. (2022). Halogenated Dihydropyrrol-2-One Molecules Inhibit Pyocyanin Biosynthesis by Blocking the Pseudomonas Quinolone Signaling System. Molecules, 27(4), 1169.
8. DeBritto, S., Gajbar, D.T., Satapute, P., Sundaram, L., Lakshmikantha, Y.R., Jogaiah, S., Ito, S.I., (2020). Isolation and characterization of nutrient dependent pyocyanin from Pseudomonas aeruginosa and its dye and agrochemical properties. Sci. Rep. 10,
9. Dhairh, S. H., & Al-Azawi, I. H(2022). Comparative study between standard pyocyanin and extracted pyocyanin and synergistic with antibiotics against multidrug bacteria isolated from different clinical samples.
10. Dhairh, S. H., & Al-Azawi, I. H(2022). Comparative study between standard pyocyanin and extracted pyocyanin and synergistic with antibiotics against multidrug bacteria isolated from different clinical samples.
11. Díaz-López MD, Lluberas-Benítez M, González-García I, (2020). Microbiological analysis of burn wound infections in patients hospitalized in a burn center in Uruguay. Burns.;46(7):1629-1636
12. Dik, D. A., Fisher, J. F., & Mobashery, S. (2018). Cell-wall recycling of the Gram-negative bacteria and the nexus to antibiotic resistance. Chemical reviews, 118(12), 5952-5984.
13. El Hamzaoui N, Barguigua A, Larouz S et al. (2020): Epidemiology of burn wound bacterial infections at a Meknes hospital, Morocco. New Microbes and New Infections, 38:1-6.
14. El-Shouny, W. A., Al-Baidani, A. R. H., & Hamza, W. T. (2011). Antimicrobial activity of pyocyanin produced by Pseudomonas aeruginosa isolated from surgical wound-infections. Int. J. Pharm. Med. Sci, 1(1), 1-7.

15. Forson, O. A., Ayanka, E., Olu-Taiwo, M., Pappoe-Ashong, P. J., & Ayeh-Kumi, P. J. (2017). Bacterial infections in burn wound patients at a tertiary teaching hospital in Accra, Ghana. Annals of burns and fire disasters, 30(2), 116.

16. Ghazi, D., & Kahya, H. F. (2021). Purified Pyocyanin from Clinical Isolates of Pseudomonas aeruginosa Enhances Antibiotic Sensitivity Against Some Pathogenic Bacteria. Indian Journal of Forensic Medicine & Toxicology, 15(1), 999.

17. Gonçalves, T., & Vasconcelos, U. (2021). Colour me blue: the history and the biotechnological potential of pyocyanin. *Molecules*, *26*(4), 927.

18. Jabbar, A. T., Aziz, R. A., & Al Marjani, M. F. (2020). Extrection, purifactiion and characterize of pyocyanin pigment from Pseudomonas aeruginosa. Biochemical & Cellular Archives, 20(2).

19. Jayaseelan S, Ramaswamy D, Dharmaraj S (2014) Pyocyanin: Production, applications, challenges and new insights. World J Microbiol Biotechnol 30:1159–1168. https://doi.org/10.1007/s11274-013-1552-5

20. Marey, M. A., Abozahra, R., El-Nikhely, N. A., Kamal, M. F., Abdelhamid, S. M., & El-Kholy, M. A. (2024). Transforming microbial pigment into therapeutic revelation: extraction and characterization of pyocyanin from Pseudomonas aeruginosa and its therapeutic potential as an antibacterial and anticancer agent. Microbial Cell Factories, 23(1), 174.

21. Mohsin, M. I., Al-Shamarti, M. J., Mohsin, R. I., & Al-Sahaf, S. (2020). Role of Interleukin-36 in Response to *Pseudomonas Aeruginosa* Infection. Indian Journal of Forensic Medicine & Toxicology, 14(3).

22. Muhaidi, M. J., Aziz, L. M., & Ahmed, M. N. (2018). Geneti evaluation of phenazine synthesized by Pseudomonas aeroginosa. Iraqi Journal of Agricultural Sciences, 49(2).

23. Nowroozi, J., Sepahi, A. A., & Rashnonejad, A. (2012). Pyocyanine biosynthetic genes in clinical and environmental isolates of Pseudomonas aeruginosa and detection of pyocyanine's antimicrobial effects with or without colloidal silver nanoparticles. Cell Journal (Yakhteh), 14(1), 7.

24. Owuama, C. I. (2017). Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. African journal of microbiology research, 11(23), 977-980.

25. Pailliè-Jiménez, M. E., Stincone, P., & Brandelli, A. (2020). Natural pigments of microbial origin. *Frontiers in Sustainable Food Systems*, *4*, 590439.

26. Rodrigues, G., Silva, G. G. O., Buccini, D. F., Duque, H. M., Dias, S. C., & Franco, O. L. (2019). Bacterial proteinaceous compounds with multiple activities toward cancers and microbial infection. *Frontiers in microbiology*, *10*, 1690.

27. Sabu, N., Thomas, N. A., Thimmaiah, C., Joseph, A. P., Jobe, J., & Palose, P. S. (2022). Comparative Evaluation of Chlorhexidine and Cetrimide as Irrigants in Necrotic Primary Teeth: An In vivo Study. Journal of Pharmacy & Bioallied Sciences, 14.

28. Satapute, P., Paidi, M. K., Kurjogi, M., & Jogaiah, S. (2019). Physiological adaptation and spectral annotation of Arsenic and Cadmium heavy metal-resistant and susceptible strain Pseudomonas taiwanensis. *Environmental pollution*, *251*, 555-563.

29. Seiffein, N. L., & Ali, G. H. (2021). Effect of subinhibitory concentrations of selected antibiotics and propolis on pyocyanin and biofilm production among Pseudomonas aeruginosa isolates in Alexandria, Egypt. Egyptian Journal of Medical Microbiology, 30(4), 129-137.

30. Shouman, H., Said, H. S., Kenawy, H. I., & Hassan, R. (2023). Molecular and biological characterization of pyocyanin from clinical and environmental Pseudomonas aeruginosa. *Microbial Cell Factories*, *22*(1), 166.

31. Sivasankara Narayani, S., Saranya, P., Lokesh, P., & Ravindran, J. (2021). Identification of Bioactive Compounds, Characterization, Optimization and Cytotoxic Study of Pyocyanin against Colon Cancer Cell Line (HT-29). Journal of Chemical and Pharmaceutical Research, *13*(6), 01-18.

32. Stephenson, F.H. (2003). Calculations in molecular Biology and Biotechnology. Academic Press, Animprint of Elsevier, USA.

33. Tahmasebi, H., Dehbashi, S., Nasaj, M., & Arabestani, M. R. (2022). Molecular epidemiology and collaboration of siderophore-based iron acquisition with surface adhesion in hypervirulent Pseudomonas aeruginosa isolates from wound infections. Scientific Reports, 12(1), 1-11

34. Zhou, H., Yang, Y., Shang, W., Rao, Y., Chen, J., Peng, H., ... & Rao, X. (2022). Pyocyanin biosynthesis protects Pseudomonas aeruginosa from nonthermal plasma inactivation. Microbial Biotechnology, 15(6), 1910-1921.