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Effect of Pyocyanin on gene expression of *norA* gene in *Staphylococcus aureus* and *SMR* gene in *Serratia marcescense*

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Abstract---Background: The antibiotics active against Multidrug resistant bacteria have several limitations for their use, which include less clinical experience, During the continuing COVID-19 epidemic, limiting use has been difficult because most hospitalized COVID-19 patients receive antibiotics as a defense against subsequent bacterial infection. , Efflux pumps remove drugs and are linked to biofilm formation, making infections more difficult to treat due to a lack of therapeutic choices and necessitating new antimicrobial tactics. Pyocyanin is a secondary blue redox-active metabolite and a large family member of phenazines, tricyclic compounds. Phenazine has been utilized as a biological pesticide against several harmful microorganisms because of its antibacterial properties. Methods: Total RNA from (Staphylococcus *aureus* obtained from respiratory tract diagnosed as MDR and *Serratia marcescens* obtained from co-infection Covid 19) were isolated. The gene expression of the *norA* gene efflux pump of *S. aureus*, which was normalized to the *rpoB* HKG, and the *smr* gene efflux pump of *Serratia marcescens*, which was normalized to the *Adnalyate Kinaze enzyme*, was studied using quantitative real-time RT PCR on the extracted RNA. Result: The level of expression (*smr gene*) efflux pump for *S. marcescence* during treated with Pyocyanin decreased dramatically mean significant downregulation of gene expressions for the *S. marcescence* in compared to level of gene expression HKG (*Adnalyate Kinaze enzyme*) and the expression level of (*norA gene*) efflux pump of *S. aureus* during treated with Pyocyanin not decreased for the S. aureus in compared to level of gene

expression of control (*rpoB gene*) HKG. Conclusion: gene expression revealed inhibited *smr gene* efflux pump by Pyocyanin in *Serratia marcescence* that mean can be used against Multidrug resistance gram negative bacteria . and regard *Staphylococcus aureus* showed not effected on overexpression *nora* gene efflux pump.

Keywords---pyocyanin, efflux pump, Co- infection, COVID19.

Introduction

Antibiotic resistance is a problem for the world's health that involves the spread of microbes and genetic material among people, animals, and the environment. [1]. However, advancements in public health and medicine during the 20th century significantly lessened the burden brought on by infectious diseases. Since antibiotic-resistant infections impact both industrialized and developing nations equally, it is crucial to examine the worldwide rise of antibiotic resistance. [2] Some of the self-resistance mechanisms used by bacteria to avoid autotoxicity include antibiotic efflux, antibiotic modification, self-sacrifice, target repair, and protection. [3]. Antibiotic resistance is conferred by membrane protein efflux pumps, which extrude structurally different substances and lower their intracellular concentration. [4], The availability of drugs to inhibit the particular target is decreased by the presence or increased expression of efflux pumps. A reduced concentration of the antimicrobial agent reaches the target as a result of continual efflux. [5] They serve an important role in defending bacterial cells from harmful substances such antibiotics, disinfectants, conservatives, and detergents. [6].

The *norA* gene efflux pump, which is the most extensively researched efflux system in *Staphylococcus aureus*, is frequently used as a model to study efflux-mediated resistance in this pathogen. [7] , The SMR antimicrobial transporter family is part of the broader drug/metabolite transporter (DMT) superfamily. The *smr* gene pumps are secondary active transporters that are powered by ion motive factors, such as protons. [8]. Co- infection with Covid 19 , Numerous COVID – 19 patients are treated with antibiotics, which could increase antibiotic resistance. About half of the COVID-19 patients who died had bacterial and fungal coinfections, and some of the infections were resistant to antibiotics. Antimicrobial-resistant diseases have been linked to deaths in previous pandemics. [9] The use of broad-spectrum antibiotics has been prevalent among COVID-19 patients in hospitals, much exceeding reported secondary infection rates. [10] In addition to efforts to produce novel antibiotics, researchers are also interested in finding efflux pump inhibitors (EPI) [11]. Pyocyanin, a growth pigment produced by *P. aeruginosa*, is one of the key compounds that allows it to thrive in competitive situations. [12] Pyocyanin is a redox-active chemical that can take and provide electrons. [13], Pyocyanin can increase intracellular levels of reactive oxygen species (ROS), which can lead to direct DNA damage as well as oxidative damage to cell cycle components. [14].

Materials and Methods

Bacterial strains

Gram Negative and Positive bacteria selected for this study (*Serratia marcescens* from co infection Covid19 patients , and (*Staphylococcus aureus* obtained from respiratory tract diagnosed as MDR) The objective of this study was to see how pyocyanin effect on efflux pumps (*norA* in *S. aureus*) and (*smr* in *S. marcescens*) that contribute to drug resistance.

Antibiotic Susceptibility Testing

Antibiotic susceptibility test to all bacterial isolates in current study done by using (VITEK-2) system, drug sensitivity test results measured using AST-N222 cards and AST-592 cards .traditional broth microdilution procedures were used to determine the minimum inhibitory concentration (MIC). .

Molecular study

Total RNA extraction

Extraction of total RNA from treatment and control groups of (*S. aureus* , *S. marcescens*) were done by using (TRIzol® reagent kit) according to instruction of manufactured company as steps below:

- Bacteria were first pelleted, then lysed in 1 ml of AccuZolTM by repeated pipetting cycles of the cell lysate.
- For every 1ml of AccuZolTM, two handred l of chloroform were added, and the mixture was rapidly shaken for 15 seconds.
- Five minutes were spent incubating the mixture on ice.
- At 4C, the mixture was centrifuged for 15 minutes at 12000 rpm.
- An equal volume of isopropyl alcohol was added when the aqueous phase was transferred to a new 1.5 ml tube.
- The tubes were combined by flipping them four to five times, and they were then incubated at -20C for 10 minutes.
- After centrifuging the sample at 12000 rpm for 10 minutes at 4C, the supernatant was carefully removed.
- One milliliter of 80% ethanol was added and well combined using a vortex.
- The supernatant was carefully removed after a centrifuge at 12000 rpm for 5 minutes at 4C. The pellet had been dried.
- Before use, the RNA was dissolved in RNase-free water by running the solution through the tip of a pipette a few times.

cDNA synthesis

Synthesis and amplification of cDNA were performed by using EasyScript one step gDNA Removal and DNA synthesis supermix as following :- RNA, primer and water were mixed and incubated at 65°C for 5 minutes, on ice for two minutes then other components (Table 1) were added.

Table 1
Reverse transcriptase master mix with their volumes for cDNA synthesis

Component	Volume
RNA	10 pg- 500ng
Random primer (0.1µg/µl)	1 µl
2xES reaction mix	10 µl
RT/RI enzyme	1 µl
gDNA remover	1 µl
RNase-free water	To 20 µl

Following the completion of the cDNA first strand synthesis reaction, the components in the tube should be well mixed and collected using pulse centrifugation for 30 seconds. the reaction mixture was incubated at 25 °C for 10 minutes with Random Primers; however, if Oligo (dT) or Gene-Specific Primer was used, this step of incubation should be skipped. for the cDNA synthesis process, the reaction mixture was incubated for 50 minutes at 42°C. The process was finally stopped by heating it to 85°C for 5 minutes and then freezing it on ice. The newly generated first-strand cDNA can be used right away or stored at -20°C for long periods of time.

Reverse transcriptase PCR (RT-PCR) technique

RT-qPCR is performed in a reaction solution of 20µL volume using BrightGreen qPCR MasterMix by mixing the following materials:

- 0.6 µL of forward (F) primer.
- 0.6 µL of reverse (R) primer.
- 10µL of BrightGreen 2X qPCR mastermix.
- ≤ 500 ng of cDNA
- Nuclease - free H2O to 20 µl.

Quantitative Real-Time PCR (qPCR)

ncbi designed the primers for the PCR amplification of cDNA. Forwarde primer 5'-TCAATCCCCTTTATTTTAATATGTCA-3' and revers primer 5'-TCTTTTCCACGACAGATTGC-3' were used to amplify a 171pb region of the *norA* gene efflux pump *S.aureus*. Forwarde primer 5'-CAGCTGACGAAGAAGATAGCTATGT -3' and revers primer 5'-ACTTCATCATCCATGAAACGACCAT were used to amplify an 82pb fragment of the HKG *rpoB*. The efflux pump gene *smr* has an 184pb region. Forwarde primer 5'-GCATATTTCCTCGCCGATTA-3' and revers primer 5'-GAGGCTTTCAACATGGTGGT-3' were used to amplify *S.marcescens*. Forwarde primer 5'- GTGGACTTCGTGCTGGAGTT-3' and revers primer 5'-CTCTTCCTGATCGTCCTTGC -3' were used to amplify a 180pb fragment of the HKG *AK*.

Result

The (VITEK-2) system was used to assess the isolates' susceptibility to antibiotics. *Staphylococcus aureus* isolates obtained from respiratory tract and those isolates have been confirmed by Vitek 2 system and measured resistant precentage in VITEK AST analysis , displayed resistance to all β-lactam – antibiotics and was showed positive for the cefoxitin screen, 100% to (Tetracycline and Trimethoprim/Sulfamethoxazole) 80% for(Ciprofloxacin , Erythromycin , Clindamycin and Fusidic Acid) followed by 40% (Oxacillin and Rifampicin) also exihibits sensitive for(Gentamicin , Vancomycin ,Linezolid, Ticoplanin) The susceptibility test of the isolates and microdilution methods to determine MIC value of Pyocyanin to *S.aureus* (3.1 µg/ml)

In current study VITEK AST analysis *Serratia marcescens* obtained from respiratory tract co-infection with Covid 19 revealed high resistant rate 100% to Nitrofurantion followed 50% to Cefazolin and less resistance 10% to (Cefoxitin and Ceftriaxone)and revealed sensitivity at (Amikacin and Meropenem). and microdilution methods to determine MIC value of Pyocyanin (12.5 µg/ml) . The current study investigated the expression stability of housekeeping genes which highly conserved RNA subunit as control genes to study gene expression in Multidrug resistance bacterial (*S. aureus* , *S. marcescence*) to efflux pump regulated and determine the differences in gene expression of efflux pump gene after and before treated with Pyocyanin . Figure(1)

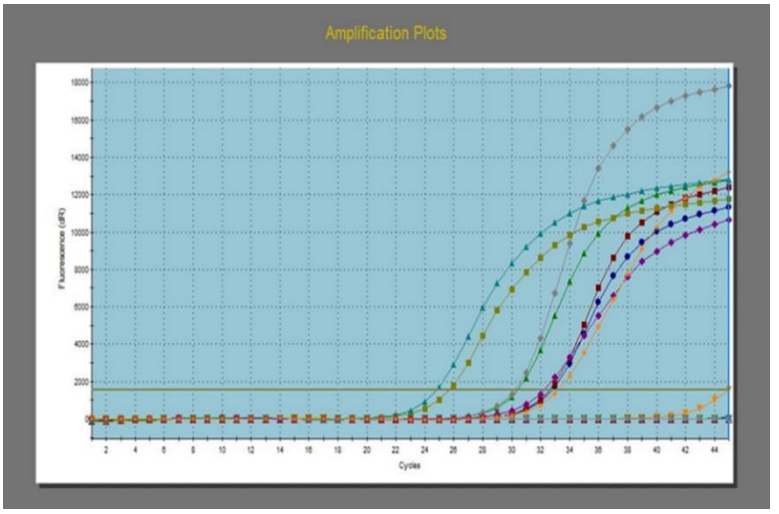

Figure 1. RT- qPCR amplification efflux pump genes to tested bacteria and control

Relative gene expression of *norA* gene efflux pump as determined by RT-PCR for *S. aureus* is found in Table These results showed that no significant change observed in over expression of *norA* genes efflux pump in *S. aureus* treated with Pyocyanin. one sample and significant change observed in other sample This might be dependent manner sample treated with pyocyanin or conditions figure 2

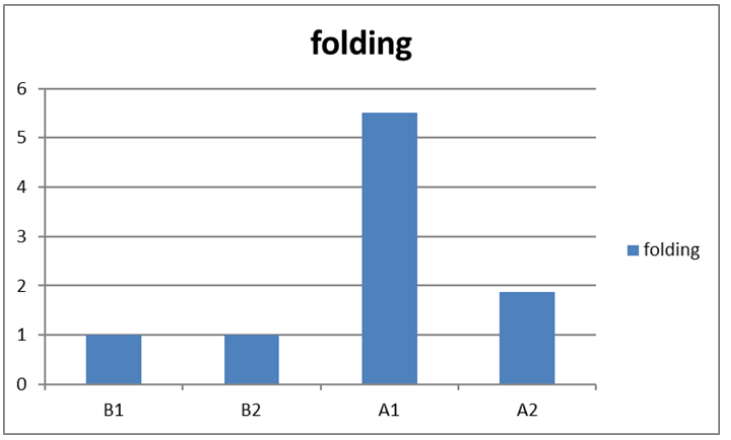

Figure 2. Mean of fold change in gene expression of *norA* genes efflux pump *S. aureus*

Table 2
fold change in gene expression of *norA* genes *S. aureus*

No.	HKG	Efflux gene	ΔCT	ΔΔCT	Folding
B1	32.83	42.7	9.87	0	1
B2	32.66	43.09	10.43	0	1
A1	30.44	37.85	7.41	-2.46	5.502167273
A2	30.24	39.76	9.52	-0.91	1.879045498

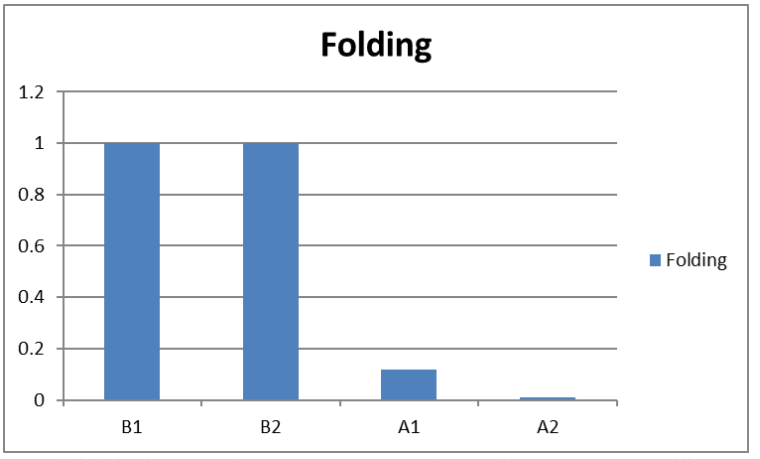

Figure 3. Mean of fold change in gene expression of *smr genes* efflux pump *S. marcescence*

The result in current stydy showed The level of expression (*smr gene*) efflux pump for *S. marcescence* during treated with Pyocyanin decreased dramatically mean significant downregulation of gene expressions for the *S. marcescence* in compared to level of gene expression HKG (Adnalyate Kinaze enzyme) .figure3

Table 3
Fold change in gene expression of *smr* genes efflux pump S. *marcescence*

No.	HKG	Efflux gene	ΔCT	ΔΔCT	Folding
B1	27.7	25.71	-1.99	0	1
B2	29.91	24.84	-5.07	0	1
A1	31.18	32.29	1.11	3.1	0.116629124
A2	31.83	33.27	1.44	6.51	0.010972226

Discussion

According this finding in Antibiotic susceptibility testing for *S. aureus* recovered as Multidrug resistance , Staphylococcus aureus acquires antimicrobial resistance in a number of ways. Some of these approaches include limiting drug uptake, changing the drug's target, enzymatic inactivation, and active efflux. The bacteria may employ one or more of these resistance mechanisms, depending on the antibiotic in question. [15] The chromosome of *S. aureus* contains more than twenty putative MDR EPs, some of which have already been discovered. *NorA* is the most well-studied of these, having been often utilized as a model for researching efflux-mediated resistance in this pathogen. [16] Bacterial infection has a significant impact on the disease's progression and prognosis, particularly in severe patients, and can result in increased requires for intensive care, antibiotic therapy, and mortality. [17] In this study *Serratia marcescens* obtained from co- infection Covid 19 patients from respirotry tract recovered as Multidrug resistance in Antibiotic susceptibility testing revealed high resistant precentage100% to Nitrofurantion , This necessitates bacterial enzymes generating metabolites to reduce. by interfering with bacterial ribosomal proteins, they impede protein synthesis. [18] . *Serratia marcescens* hospital outbreaks have been reported by Amarsy *et al.* (2020), Mendes and Casado (2022) in a COVID-19 intensive care unit.

In Figure 1 Relative gene expression of (*norA gene*) in *Staphylococcus aureus* and (*smr* gene) in *Serratia marcescens* determined by RT-PCR for theise bacteria The overexpression of *norA* genes encoding efflux pumps to (*S. aureus*) in Figure 2 One of the resistance mechanisms predisposing microbes to high drug resistance is the expulsion of antimicrobial medicines from the intracellular environment, decreasing their concentrations to subinhibitory levels. Native efflux pump functions, particularly those encoded in core bacterial genomes and retained over extended evolutionary periods, are likely to be related to physiological requirements imposed by their niche habitats, or even fundamental biochemical demands. [21].

Figure 3 shows (*Serratia marcescens*) down-expression of the smr gene efflux pump. In the presence of Pyocyanin, the expression of these pumps can be regulated, and they have the ability to switch from resistant to sensitive phenotypes. Pyocyanin also increases the expression of redox transport and regulatory genes while decreasing the expression of Fe3+ acquisition genes and operons. On the other hand, lowering pyocyanin expression permits cells to remain physiologically unharmed. Efflux pumps can be impeded by two main processes. The inhibiting molecule binds to a specific region on the cell membrane

where the pump is located. This keeps the drugs from being washed out of the cytosol and harming the cell. A competitive inhibition approach is commonly used to interfere with a specific efflux pump. As a result, the pump remains inactive, and the medicine is kept inside for a longer period of time. The second type of obstacle is the efflux system, which would alter the efflux kinetics. [22]

Conclusion

Gene expression revealed inhibited *smr* gene efflux pump by Pyocyanin in *Serratia marcescence* that mean can be used against Multidrug resistance gram negative bacteria obtained from co-infection Covid19 patients . and regard *Staphylococcus aureus* showed not effected on overexpression *nora* gene efflux pump.

References

1. Larsson, D. G., & Flach, C. F. (2022). Antibiotic resistance in the environment. Nature Reviews Microbiology, 20(5), 257-269.
2. Chokshi, A., Sifri, Z., Cennimo, D., & Horng, H. (2019). Global contributors to antibiotic resistance. Journal of global infectious diseases, 11(1), 36.
3. Ogawara, H. (2019). Comparison of antibiotic resistance mechanisms in antibiotic-producing and pathogenic bacteria. Molecules, 24(19), 3430.
4. Brawley, D. N., Sauer, D. B., Li, J., Zheng, X., Koide, A., Jedhe, G. S., ... & Traaseth, N. J. (2022). Structural basis for inhibition of the drug efflux pump NorA from Staphylococcus aureus. Nature Chemical Biology, 1-7.
5. Singh, S., Kalia, N. P., Joshi, P., Kumar, A., Sharma, P. R., Kumar, A., ... & Khan, I. A. (2017). Boeravinone B, A novel dual inhibitor of NorA bacterial efflux pump of Staphylococcus aureus and human P-glycoprotein, reduces the biofilm formation and intracellular invasion of bacteria. Frontiers in microbiology, 8, 1868.
6. Rybenkov, V. V., Zgurskaya, H. I., Ganguly, C., Leus, I. V., Zhang, Z., & Moniruzzaman, M. (2021). The Whole Is Bigger than the Sum of Its Parts: Drug Transport in the Context of Two Membranes with Active Efflux. Chemical reviews, 121(9), 5597-5631.
7. Costa, S. S., Sobkowiak, B., Parreira, R., Edgeworth, J. D., Viveiros, M., Clark, T. G., & Couto, I. (2019). Genetic diversity of norA, coding for a main efflux pump of Staphylococcus aureus. Frontiers in genetics, 710.
8. Varela, M. F. (2019). Antimicrobial efflux pumps. Antibiotic Drug Resistance, 167-179.
9. Ukuhor, H. O. (2021). The interrelationships between antimicrobial resistance, COVID-19, past, and future pandemics. Journal of Infection and Public Health, 14(1), 53-60.
10. Clancy, C. J., Buehrle, D. J., & Nguyen, M. H. (2020). PRO: The COVID-19 pandemic will result in increased antimicrobial resistance rates. JAC-Antimicrobial Resistance, 2(3), dlaa049.
11. Tintino, S. R., Souza, V. C. D., Silva, J. M. D., Oliveira-Tintino, C. D. D. M., Pereira, P. S., Leal-Balbino, T. C., ... & Balbino, V. Q. (2020). Effect of vitamin K3 inhibiting the function of NorA efflux pump and its gene expression on Staphylococcus aureus. Membranes, 10(6), 130.

12. Raji El Feghali, P. A., & Nawas, T. (2018). Pyocyanin: a powerful inhibitor of bacterial growth and biofilm formation. Madridge J Case Rep Stud, 2(2), 101-107.
13. Alatraktchi, F. A. A., Svendsen, W. E., & Molin, S. (2020). Electrochemical detection of pyocyanin as a biomarker for Pseudomonas aeruginosa: A focused review. Sensors, 20(18), 5218.
14. Dong, L., Pang, J., Wang, X., Zhang, Y., Li, G., Hu, X., ... & You, X. (2020). Mechanism of pyocyanin abolishment caused by mvaT mvaU double knockout in Pseudomonas aeruginosa PAO1. Viru lence, 11(1), 57-67.
15. Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. AIMS microbiology, 4(3), 482.
16. Costa, S. S., Sobkowiak, B., Parreira, R., Edgeworth, J. D., Viveiros, M., Clark, T. G., & Couto, I. (2019). Genetic diversity of norA, coding for a main efflux pump of Staphylococcus aureus. Frontiers in genetics, 9, 710.
17. Chen, X., Liao, B., Cheng, L., Peng, X., Xu, X., Li, Y., ... & Ren, B. (2020). The microbial coinfection in COVID-19. Applied microbiology and biotechnology, 104(18), 7777-7785.
18. Gardiner, B. J., Stewardson, A. J., Abbott, I. J., & Peleg, A. Y. (2019). Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. Australian prescriber, 42(1), 14.
19. Amarsy, R., de Ponfilly, G. P., Benmansour, H. A., Jacquier, H., Cambau, E. E., & Mégarbane, B. (2020). Serratia marcescens outbreak in the intensive care unit during the COVID-19 pandemic: A paradoxical risk?. Medecine et maladies infectieuses, 50(8), 750-751.
20. Mendes, J. C., & Casado, A. (2022). Serratia marcescens outbreak in a COVID-19 intensive care unit–Are there any factors specific to COVID-19 units that facilitate bacterial cross-contamination between COVID-19 patients?. American journal of infection control, 50(2), 223-225.
21. Henderson, P. J., Maher, C., Elbourne, L. D., Eijkelkamp, B. A., Paulsen, I. T., & Hassan, K. A. (2021). Physiological functions of bacterial “multidrug” efflux pumps. Chemical Reviews, 121(9), 5417-5478.
22. Dey, N., Kamatchi, C., Vickram, A. S., Anbarasu, K., Thanigaivel, S., Palanivelu, J., ... & Ponnusamy, V. K. (2022). Role of nanomaterials in deactivating multiple drug resistance efflux pumps–A review. Environmental Research, 204, 111968.
23. Suryasa, W., Sudipa, I. N., Puspani, I. A. M., & Netra, I. (2019). Towards a Change of Emotion in Translation of Kṛṣṇa Text. *Journal of Advanced Research in Dynamical and Control Systems*, *11*(2), 1221-1231.
24. Gandamayu, I. B. M., Antari, N. W. S., & Strisanti, I. A. S. (2022). The level of community compliance in implementing health protocols to prevent the spread of COVID-19. International Journal of Health & Medical Sciences, 5(2), 177-182. https://doi.org/10.21744/ijhms.v5n2.1897