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Comparative study between standard pyocyanin and extracted pyocyanin and synergistic with antibiotics against multidrug bacteria isolated from different clinical samples

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Abstract--Background: Multidrug Resistance forms in Gram positive and Gram-negative bacteria are difficult to treat, and medications to be taken with caution may be unsuccessful. The emergence of treat MDR Gram-negative and Gram-positive Infections bacterial is still a major issue. Aims: The goal of this study is reliable develop new agents or new treatment methods that are capable of overcoming drug resistance in these organisms. Methods: 536(85.3%) samples from out and inpatients who admitted to Al Diwaniyah Teaching Hospital in Diwaniyah city were collected from various site of infection, 92 (46%) *Pseudomonas aeruginosa* were investigated by a qualified consulting doctor. After cultivation in special media (MacConkey agar and Nutrient agar) to detect, *Pseudomonas aeruginosa*, the samples were identified using the Vitec 2 compact system, Pyocyanin was extracted by standard methods from *Pseudomonas aeruginosa* Principal antimicrobial compounds present in pyocyanin were identified by HPLC. Other pathogenic bacteria obtained for exposure to Pyocyanin like *Escherichia coli*, *K. pneumoniae*, *S. aureus* from laboratories. This diagnosis was confirmed by Vitek 2 system. *Serratia marcescens* and *Proteus mirabilis* obtained from sputum patients with co- infection Covid 19. as well as MIC susceptibility testing to all isolates measured using AST-N222 cards and AST-592 cards. Finally, measured Synergistic effect of Pyocyanin with some antibiotics. Conclusion: Pyocyanin is a natural compound showed a broad

spectrum antimicrobial effect on multidrug resistant bacteria investigated by well diffusion and microdilution toward Gram negative and less activity for Gram positive bacteria. The antimicrobial effect of both Pyocyanin and Antibiotic (Meropenem, Minocycline and Gentamicin) used in current study can be enhanced by their combined application, investigated that Pyocyanin (Standard and extracted) exhibited broad spectrum antimicrobial activity on the (*Proteus mirabilis* and *Serratia marcescens*) that isolated from patients co-infection with Covid 19.

Keywords---standard pyocyanin, extracted pyocyanin, antibiotics, multidrug resistant isolates

Introduction

Pyocyanin is a nitrogen-containing (C₁₃H₁₀N₂O) extracellular phenazine derivative pigment generated by *Pseudomonas aeruginosa* as a secondary metabolite (secondary phase: stationary phase) [1]. Pyocyanin is made up of two N-methyl-1-hydroxyphenazine subunits.[2] The most crucial and deadly toxin secreted by *P. aeruginosa* is pyrocyenin. As a result, PYO is an superb and one-of-a-kind biomarker candidate for the indirect detection of *P. aeruginosa*. [3]. These pigments are required for bacterial pathogenicity and biological control, but not for bacterial growth and proliferation.[4] Pyocyanin allows *P. aeruginosa* to outcompete other invading organisms by enhancing oxidative metabolism and generating reactive oxygen species (ROS), resulting in potent antibacterial actions[5] Rather than *Pseudomonas spp.*, it acts as an antibacterial agent, selectively inhibiting gram-positive and gram-negative bacteria.[6] pyocyanin that makes it more resistant to medicines and capable of surviving in harsh environments pyocyanin that makes it more resistant to medicines and capable of surviving in harsh environments[7]. When PYO is present as a competitive bacterium in cystic fibrosis patients, it plays a key function. Can impede cellular respiration, ciliary function, and the V-ATPase, alter the redox cycling of glutathione, deactivate catalase, and result in neutrophil death. Other organisms are harmed by the creation of ROS by pyocyanin. It is believed that several of these processes contribute to *P. aeruginosa*'s pathogenicity.[8]

Pseudomonas aeruginosa Gram-negative bacteria are one of the most common causes of healthcare-associated illnesses. The optimum therapeutic technique is debatable, severe patients. In the case of multidrug-resistant (MDR) and extensively drug-resistant (XDR) germs, as well as the most severe patients. [9] & [10]. *Pseudomonas aeruginosa* is difficult due to the bacteria's inherent capacity to acquire antibiotic resistance, form impenetrable biofilms, and release a huge variety of virulence factors.[11] One of the most prevalent coinfecting bacteria detected in COVID-19 patients is *P. aeruginosa*, which makes the illness worse. [12] During coinfection, *P. aeruginosa* overexpressed alginate and blocked the Type VI secretion system (T6SS), which improved biofilm development and reduced pathogenicity. This might encourage COVID-19 patients to become resistant to antibiotics and develop in vivo colonization. During coinfection, *P. aeruginosa* overexpressed alginate and blocked the Type VI secretion system

(T6SS), which improved biofilm development and reduced pathogenicity. This might encourage COVID-19 patients to become resistant to antibiotics and develop in vivo colonization. [13]

Materials and Methods

Sample collection and identification

Five hundred and thirty-six samples were collected from various site of infection (Otitis media, Burns, Surgical infections, Sputum, Urinary tract infections, Wound infections) and immediately transported to the lab after being marked with the patient's information. Samples are occasionally kept in transit media according to when they were collected till they arrived at the lab. non-sporulating, mobile, gram-negative rod with aerobic development, as determined by Gram stain morphology and straight or slightly curved appearance. The characteristic appeared blue green colonies of *P. aeruginosa* were isolated and re-streaked for purification and isolation on nutrient agar. followed by biochemical testing (Catalase test, Oxidase test, Citrate test, Indole test, Growth at 4 C and 42 C) Moreover, and as a final confirmation step all the 92 samples were subjected to VITEK-2 compact automated system to confirm the finding. In this study other pathogenic bacteria obtained for exposure to Pyocyanin like *Escherichia coli*, *K. pneumoniae*, *S. aureus* from laboratories her diagnosis as MDR was confirmed by Vitek 2 system, and *Serratia marcescens* and *Proteus mirabilis* obtained from sputum patients with co- infection Covid 19.

Antibiotic Susceptibility Test

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.

Pyocyanin Production and Extraction

After incubation for 2-3 days at 37°C in an incubator, pigment was extracted from each *P. aeruginosa* isolate using nutrient broth according Jameel *et al.*, (2017) by some modification for incubation condition, place it in the fridge for 4-5 days to enhance the pigment's appearance.

Diagnosis of the pyocyanin stain of *Pseudomonas aeruginosa* using an HPLC

The Laboratory of Drugs and Medicinal Plants/College of Pharmacy/University of Babylon employed (HPLC) of German origin and made by (Sykamn) to determine the amount of pyocyanin, with the carrier phase consisting of (acetonitrile:distilled water) (80:20). Using a (C18-ODS) separation column (25 cm * 4.6 mm) with a UV-Vis detector at 450 nm, standard pyocyanin was produced at various concentrations. The extract was then purified using a 0.25 m membrane filter, and the filtrate was centrifuged. 20 microgram syringe volume, 1 ml per minute flow rate.

Antimicrobial susceptibility assay of pyocyanin

Antimicrobial activity of Pyocyanin was evaluated using the agar well diffusion and MIC method.

Agar well diffusion method

Inoculating indicator bacteria on Muller Hinton agar plate, wells were prepared in the plate with a 4mm diameter sterile corn borer, and the wells for each culture of indicator bacteria were filled with 50 μ l of purified extracts Pyocyanin with concentrations (25,50,100) mg/ml, and then the plates were incubated at 37°C for 24 hours under dark conditions. A zone of clearing around the pure pigment was used to detect the inhibition rate. Following the incubation period, each plate was checked by measuring the diameter of the full inhibitory zone to the nearest whole millimeter with a ruler, and recording the value.

Minimum inhibitory concentration (MIC)

the test was done by using macrodilution method [15] Serial dilution of Pyocyanin was made to distribution on 12 test tubes at 1ml with 10 μ l bacterial inoculum after turbidity checker with control tube (only bacterial inoculum without Pyocyanin solution). PYO was concentration(50 μ g/ml) . incubated all these tubes at 37°C for 24 hrs. with dark conditions. MIC is a lowest Pyocyanin concentration that inhibited completely the bacterial growth and can detected by unaided eye. The turbidity of bacterial growth in tubes containing serial dilution of PYO compared with control tube (without PYO) and determined the concentration of tube before first one with turbidity to be the MIC.

Synergistic effect of Pyocyanin with some antibiotics

Combination of Pyocyanin with Gentamicin

To determine the synergism effect of Pyocyanin and Gentamicin by agar well diffusion, each plate containing Muller Hinton agar was inoculated with checker *S. aureus* inoculum on its surface by streaking in the disc diffusion method and by cork borer made a wells each well contained 100 μ l from both(50 μ l from each) All of these plates were incubated in the dark for 24 hours at 37°C. Following the incubation period, each plate was inspected by measuring the diameter of the whole inhibitory zone with a ruler to the nearest whole millimeter and recording the results.

Combination of Pyocyanin with Meropenem

To determine the synergism effect in this study for *Serratia marcescens* and *Proteus mirabilis* by combination between Pyocyanin with Meropenem by agar well diffusion , Same as mention above in section.

Combination of Pyocyanin with Minocycline

To determine the synergism effect for *Escherichia coli* by combination between Pyocyanin with Minocycline by agar well diffusion , Same as mention above in

section. Calculate synergistic effect: The calculation of synergistic effect according to this equation

Synergistic effect= A= ZOI for antibiotic
B= ZOI for antibiotics + Ag-NB

Result

Isolation and Identification

A total of 536 samples from out and inpatients who admitted to Al Diwaniyah Teaching Hospital in Diwaniyah city were collected from various site of infection .In This study samples from patients included Urinary tract infections 240(20%) ,Sputum 88(12%) , Otitis media 74(13%) , Surgical infections 63(15%) Wound infections 56(14%) and Burns 15(13%) . (Figure 1) explain percentage of *P. aeruginosa* isolates from Total samples .

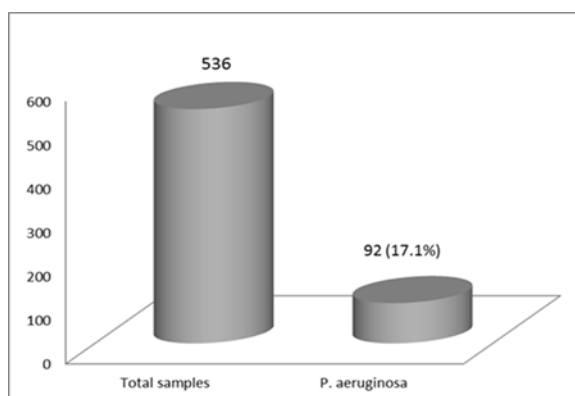


Figure 1. Total number and percentage of *P. aeruginosa* isolates in clinical sample

In this study the most tested *P. aeruginosa* isolates were from Urinary tract infections during a period of study, urine samples were collected from 240 patients with UTI, out of which 20.8% patients had an established *P. aeruginosa*.

Detection of *Pseudomonas aeruginosa*

Only 92 (17.1 percent) of 536 samples relied on bacterial isolation and identification in a laboratory. All of the isolates developed on MacConkey agar, which does not digest lactose sugar, and on Muller Hinton agar, which produces the characteristic pigment, which ranged in color from yellowish-green to bluish-green. Table 1 shows the results of biochemical testing Furthermore, all 92 samples were tested to the VITEK-2 small automated system as a last confirmation step to confirm the findings.

Table 1
The characteristic and biochemical tests for *Pseudomonas aeruginosa*

Testes	Result
Gram's Stain	Negative
Growth in 42 C°	Positive
Catalase	Positive
Oxidase	Positive
Citrate	Positive
Indole	Positive

In this study different types of bacteria isolated (*Staphylococcus aureus* , *E . coli* , *K. pneumoniae*) from (urine and sputum) samples revealed MDR These isolates were obtained in advance from laboratories for the purpose of studying the effectiveness of Pyocyanin on multidrug resistant isolates , we confirmed by Vitek 2 system. also antibiotic susceptibility test results measured using AST-N222 cards and AST-592 cards. In This study Other pathogenic bacteria obtained from co- infection Covid 19 patients from respiratory tract (*Serratia marcescens* and *Proteus mirabilis*) to exposure for Pyocyanin pigment because most patients develop complications due to bacterial resistance to antibiotics the diagnosis for these bacteria confirmed by Vitek 2 system . as show in Table2

Table 2
Number of Bacterial isolates of Co- infection with Covid 19

N	Bacterial isolates	Type of samples	Site of infection
2	<i>Serratia marcescens</i>	Sputum	Respiratory tract
2	<i>Proteus mirabilis</i>	Sputum	Respiratory tract

Pyocyanin Production and Extraction

In current study Sixty three isolates of *P. aeruginosa* (68%) were able to produce greenish blue pigment (pyocyanin) is a secondary metabolite produced by *P. aeruginosa* in the stationary phase). Only one isolate selected for extraction pyocyanin which high production for this pigment.

Diagnosis of pyocyanin stain of *Pseudomonas aeruginosa* with High Performance Liquid Chromatography (HPLC)

Figure 2 illustrates how to use HPLC to identify the pyocyanin produced by the selected *Pseudomonas aeruginosa* isolation (B). as well as its comparison to the typical pyocyanin profile and the recommended separation conditions. The holding time of the standard pyocyanin (A) is (7) minutes, however the *Pseudomonas aeruginosa* Profile of Isolation's initial peak showed up at a different time, (20) minutes, proving that the pigment produced by these bacteria is pyocyanin.

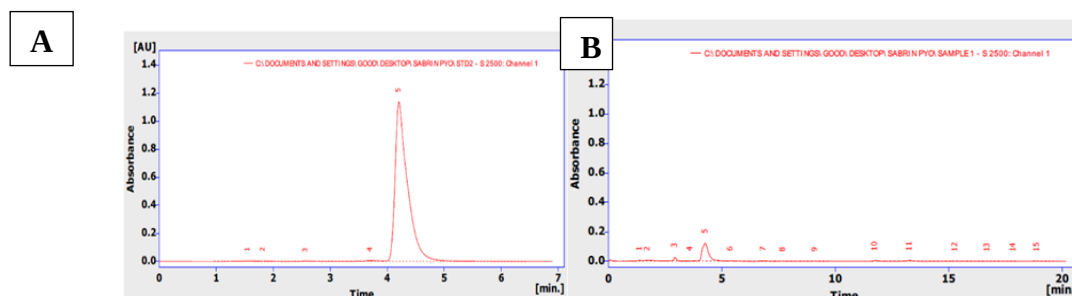
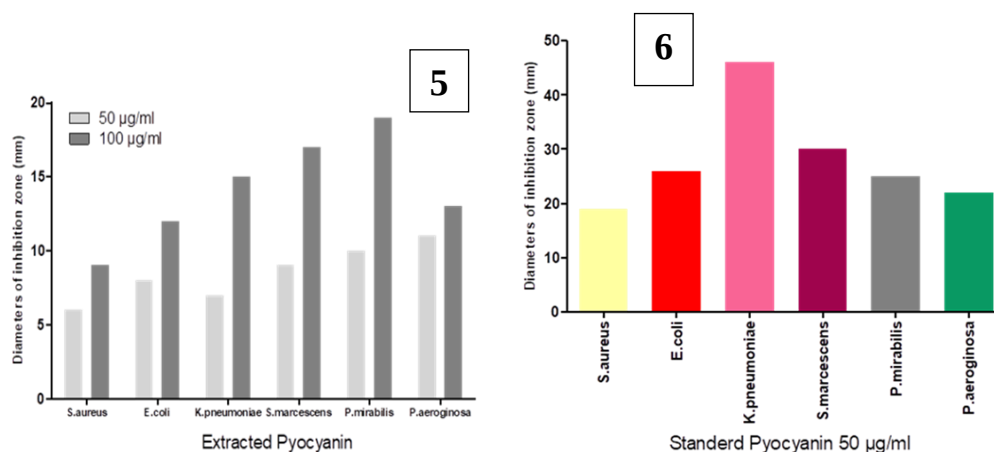


Figure 2. Profile of absorbance using an HPLC device at 450nm wavelength: standard pyocyanin

In this study, the concentration of the Pyocyanin was estimated (0.99 ug/ml) in the peak area compared to stander (1.54 ug/ml).

Antimicrobial activity

In this study the common influenced bacteria to pyocyanin was *Proteus mirabilis* at 50 mg/ml (10mm) at 100mg/ml (19mm) and *Serratia marcescens* at 50 mg/ml (9mm) at 100mg/ml (17mm) followed by *Escherichia coli* at 50mg/ml (8mm) at 100mg/ml (12mm) and *Klebsiella pneumoniae* at 50mg/ml (7mm) at 100mg/ml (15mm), it may be due to its high resistance against most antibiotics. Regarding *S. aureus*, the result showed that less activity by Pyocyanin at 50mg/ml (6mm) at 100mg/ml (9mm) Figure 3



In this study, we used standard Pyocyanin pigment manufactured with high purity and under sterial conditions, especially by accredited companies, for the purpose of measuring its antibacterial activity on the same isolates. The antimicrobial activity of standard Pyocyanin pigment on Gram- positive and negative bacteria at 50mg/ml concentration, The common influenced bacteria to pyocyanin was *Klebsiella pneumoniae* (46mm) followed by *S. marcescens* (30mm), *E. coli* (26mm), *P. mirabilis* (25mm), and less activity *S. aureus* (19mm) Figure 5

Comparative Study between Standard and Extracted Pyocyanin

The results revealed that the antibacterial activity of Pyocyanin increased directly with concentration increasing to both types. Through expanding Pyocyanin concentration from 50 mg/ml to 100 mg/ml, the antimicrobial activity is improved and enhanced; therefore the pyocyanin is concentration dependent as an antibiotic activity.. Statistical analysis to antimicrobial effect of Standard Pyocyanin recover that was significant differences $p=0.0007$ among *S. aureus* isolates and control and no significant differences $p= (0.0537)$ in extracted Pyocyanin .Figure 5

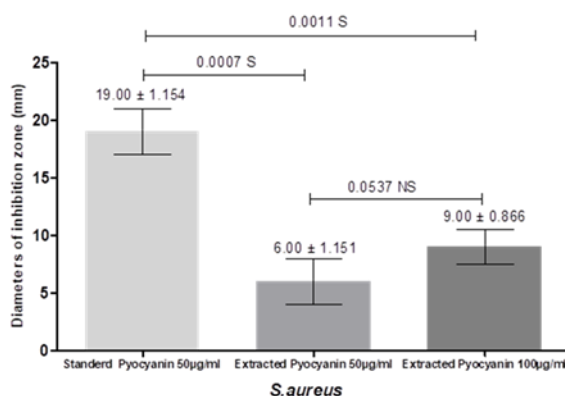
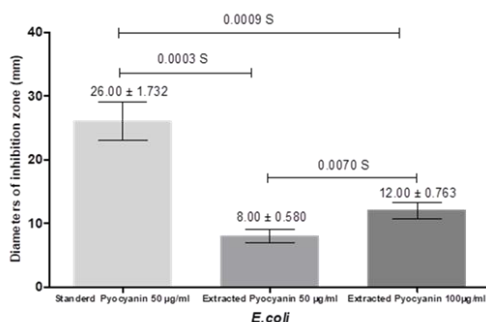


Figure 5. S: P-value Significant, NS: P-value non-Significant

Statistical analysis to antimicrobial effect of Standard pyocyanin recover that significant differences $p=0.0003$ among *E.coli* isolate and its control and significant differences $p= 0.0070$ in extracted pyocyanin , and antimicrobial effect of Standard pyocyanin recover that significant differences $p=0.0001$ among *K.pneumonia* isolate and its control and significant differences $p= 0.0028$ in extracted Pyocyanin . Figure 6



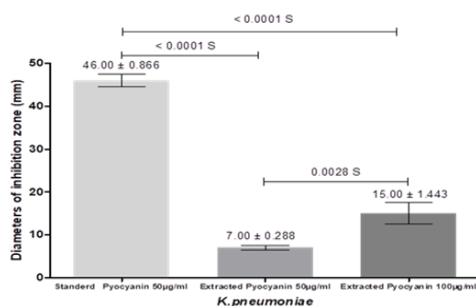


Figure 6. Zone of inhibition (mm) of Pyocyanin against *E.coli* and *K.pneumonia*

Statistical analysis to antimicrobial effect of Standard Pyocyanin recover that was no significant differences $p=0.0012$ among *P.mirabilis* isolates and control and $p=0.0022$ among *S. marcescens* isolates Standard Pyocyanin and its control and no significant differences $p=0.0055$ in extracted Pyocyanin. Figure 7

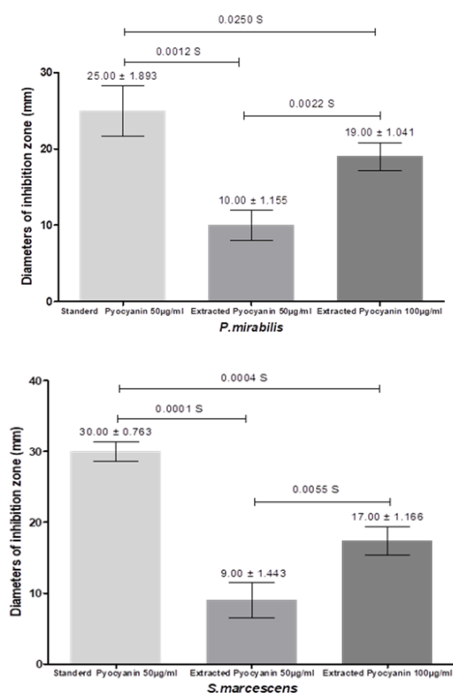


Figure 7. Zone of inhibition (mm) of Pyocyanin against *P.mirabilis* and *S. marcescens*

Effect of Combination between antibiotics and Pyocyanin Effect on Multidrug bacteria (*S. aureus*, *E. coli*, *K. pneumoniae*)

it was done by Agar well diffusion method Statistical analysis recover that Standard Pyocyanin in combination with Gentamicin more effective than alone that significant differences at $p=0.0017$ in Pyocyanin and Antibiotic alone, and significant differences at $p=0.0028$ in combination for *S.aureus*, In this study

during antibiotic susceptibility testing for *Staphylococcus aureus* exhibited more sensitive for Gentamicin(GNE) , So expremintal in combination with Pyocyanin .Figure 8

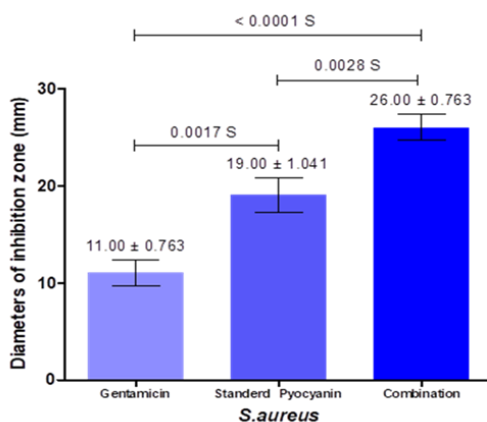
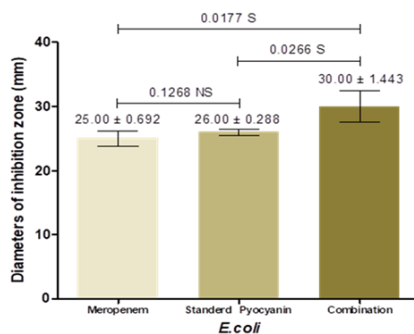


Figure 9. Combination antibacterial effect of Pyocyanin and Gentamicin on *S. aureus*

Statistical analysis recover that Standard Pyocyanin in combination with Minocycline(MIN) more effective than alone that no significant differences at $p=0.1268$ and significant differences at $p=0.0005$ in Standard Pyocyanin and Antibiotic alone for *E.coli* and *K. pneumoniae* respectively and significant differences at $p=0.0266$ and no significant differences at $p=0.0672$ for *E.coli* and *K. pneumoniae* respectively in combination Figure 10. In this study during antibiotic susceptibility testing for *E.coli* and *K. pneumoniae* exhibited more sensitive for Minocycline , So expremintal in combination with Standard Pyocyanin



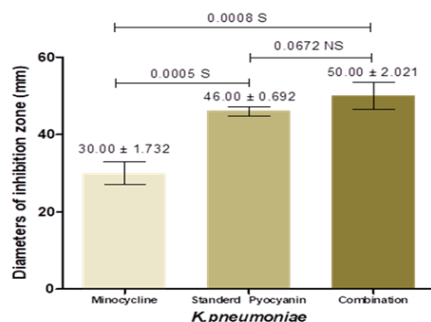
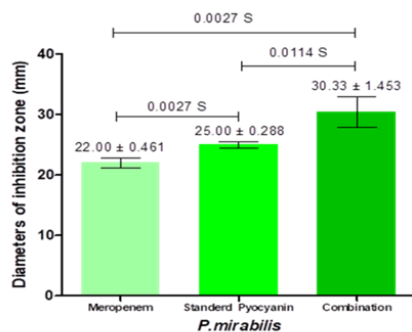


Figure 10. Combination antibacterial effect of Pyocyanin and Minocycline on *E.coli* and *K. pneumoniae*

The majority of the Minocyclines examined here (Fig. 10) showed MICs that were sensitive ≥ 1 , intermediate ≥ 8 , and resistant ≥ 1 in *E. coli* and *K. pneumoniae*, respectively.

Effect of Combination between antibiotics and Pyocyanin on isolates from co-infection Covid19

In this study during antibiotic susceptibility testing for *P. mirabilis* and *S. marcescens* which obtained from co-infection Covid19 patients, exhibited more sensitive for Meropenem, So experimental in combination with Standard Pyocyanin. Statistical analysis recover that Standard Pyocyanin in combination with Meropenem more effective than alone that significant differences at $p=0.0154$ in Combination, for *P. mirabilis* and *S. marcescens* that significant differences in Pyocyanin and Antibiotic at $p= (0.0027, 0.0047)$ respectively and significant differences at $p= (0.0114, 0.0006)$ respectively in Combination. Figure 11



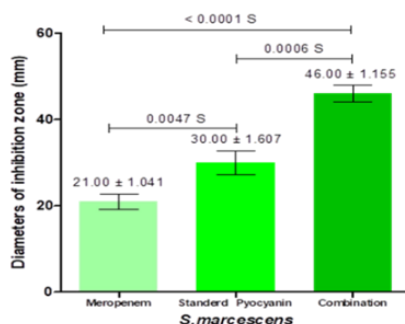


Figure 11

Discussion

According to the findings in this study Extracted Pyocyanin at (50mg/ml , 100mg/ml)concentrations had antimicrobial activity toward Multidrug resistant isolates Gram-Negative more than Gram Positive bacteria *S. aureus* . gram positive bacteria were less vulnerable to Pyocyanin than gram negative bacteria, according to the findings. This difference refers to the lipid content of Gram-positive and Gram-negative bacteria's cell walls. It is obvious that the peptidoglycan and lipopolysaccharide layers could inhibit pyocyanin penetration [16] . This explains why pyocyanin has less of an effect on *S. aureus*. In the interactions between bacterial cell walls and PYO, the surface charge and electronegativity of the cell wall are also thought to play a role. Gram-positive and Gram-negative bacteria both have negatively charged cell walls, as is well known. An outer membrane containing negatively charged LPS molecules surrounds Gram-negative cell walls. This gives most Pyocyanin constructions a larger affinity for the positive ions they produce, resulting in increased absorption and total ion buildup, which causes intracellular harm. High activity was seen against *Klebsiella pneumoniae* and *S.aureus*, whereas moderate activity was observed against *Bacillus subtilis*, *Salmonella typhi*, and *E.coli*, according to Bavithra and Santhymurthy (2019).

This study's conclusion Pyocyanin was extracted and found to have antibacterial action against multidrug-resistant bacteria isolated from co-infections. *Proteus mirabilis* was the most commonly impacted bacteria to pyocyanin, followed by *Serratia marcescens*, which could be related to its great resistance to most medicines, according to Covid 19. The emergence and spread of multidrug-resistant *P. mirabilis* isolates, including those that produce ESBLs, ampC cephalosporinases, and carbapenemases, is becoming more common. For a wild-type phenotype, *P. mirabilis* produces no chromosomally encoded b-lactamase, resulting in complete susceptibility to all b-lactams [18] . Due to their capacity to produce beta-lactamases, many strains of *Serratia marcescens* can multiply antibiotic resistance. Additionally, they have the capacity to acquire plasmid-mediated extended spectrum -B-lactamases, which confer resistance to a variety of B-lactamase antibiotics. [19] Due to limited treatment options and the risk that empiric antimicrobial therapy (AMR) could worsen antibiotic resistance, the COVID-19 pandemic has raised concerns regarding secondary infections [20]. Hospitalization of COVID-19 patients, particularly in ICUs, predisposes them to

adverse outcomes, with healthcare-associated infections (HAIs)/secondary infections being one of the most prominent. according to other research by Lehmann *et al.*, (2021) and Khurana *et al.*, (2021). *Serratia marcescens* hospital outbreaks have been reported by Amarsy *et al.* (2020), Mendes and Casado (2022) in a COVID-19 intensive care unit.[24]

In this study antibacterial activity of Pyocyanin increased directly with concentration increasing to both types. Through expanding Pyocyanin concentration from 50 mg/ml to 100 mg/ml, the antimicrobial activity is improved and enhanced; therefore the pyocyanin is concentration dependent as an antibiotic activity. Synergistic activity showed that antibacterial activity of Standard Pyocyanin in combination with antibiotics on Multidrug resistant isolates were increased and enhanced on tested bacteria inhibited growth It's possible that in Figure (9) for *S. aureus*, pyocyanin increased the activity of gentamicin's binding to the 16s rRNA at the 30s ribosomal subunit, interfering with the translation of mRNA and, as a result, causing the creation of truncated or ineffective proteins. [25]. Figure (10) When combined with Minocycline, *E. coli* and *K. pneumoniae* become more susceptible to antibiotics because Minocycline binds to a specific location in the 30S ribosomal subunit and prevents aminoacyl tRNA from attaching to the ribosomal acceptor site [26]. Figure(11) shows that there are substantial differences between Pyocyanin and antibiotics, and that Pyocyanin combined with Meropenem is more effective than Pyocyanin alone.

Conclusion

Pyocyanin is a natural compound showed a broad spectrum antimicrobial effect on multidrug resistant bacteria investigated by well diffusion and microdilution toward Gram negative and less activity for Gram positive bacteria ,The antimicrobial effect of both Pyocyanin and Antibiotic(Meropenem , Minocycline and Gentamicin) used in current study can be enhanced by their combined application, investigated that Pyocyanin (Standard and extracted) exhibited broad spectrum antimicrobial activity on the (*Proteus mirabilis* and *Serratia marcescens*) that isolated from patients co-infection with Covid 19 .

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