Investigation of Antimicrobial Activity of Pyocyanin Produced by *Pseudomonas aeruginosa* Strains Isolated from Different Clinical Specimens

Farklı Klinik Malzemelerden İzole Edilen *Pseudomonas aeruginosa* Suşlarıyla Üretilen Piyosiyaninin Antimikrobiyal Etkisinin Araştırılması

Research Article

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ABSTRACT

Pyocyanin is the characteristic blue-green phenazine pigment produced by *Pseudomonas aeruginosa* strains. The aim of our study is to determine the highest amount of pyocyanin production in *P. aeruginosa* strains and to investigate the antimicrobial effect of pyocyanin pigment on *Escherichia coli*, *Bacillus sp.*, *Pseudomonas aeruginosa*, *Candida sp.* and *Aspergillus niger*. For this purpose, pyocyanin was extracted from *P. aeruginosa* culture supernatants and purified, and then it was measured by spectrophotometrically. *P. aeruginosa* A10 strain isolated from urine specimen was determined as the highest amount of pyocyanin producer and *P. aeruginosa* A1 strain isolated from abdomen was the lowest. The 'Agar Well Diffusion' method was carried out to determine the antimicrobial effect of pyocyanin. Accordingly, pyocyanin was effective as an antimicrobial agent on *Bacillus sp.*, *Escherichia coli* and *Candida sp.*, but there was no antimicrobial effect on *P. aeruginosa* and *A. niger*. After that 'Tube Dilution Method' was used on strains, which were determined as sensitive to pyocyanin with agar well diffusion method. An increase in the intensity of *Bacillus sp.* and *Candida sp.* strains' growth with decreasing the concentration of pyocyanin was observed.

Key Words
Pseudomonas aeruginosa, Pyocyanin, Antimicrobial Activity.

ÖZET

Pseudomonas aeruginosa suşları tarafından üretilen piyosiyanin karakteristik mavi-yeşil bir fenazin pigmentidir. Çalışmanın amacı, *P. aeruginosa* suşlarında en yüksek piyosiyanin üretiminin belirlenmesi ve piyosiyaninin *Escherichia coli*, *Bacillus sp.*, *Pseudomonas aeruginosa*, *Candida sp.* and *Aspergillus niger* üzerindeki antimiktobiyal etkisinin araştırılmasıdır. Bu doğrultuda, piyosiyanin *P. aeruginosa* kültür süpernatanından özütlendi ve saflaştırıldı, ardından spektrofotometrik olarak ölçüldü. En yüksek piyosiyanin üretiminin idrar örneğinden izole edilen *P. aeruginosa* A10 suşunda ve en düşük piyosiyanin üretiminin batından izole edilen *P. aeruginosa* A1 suşunda olduğu belirlendi. Piyosiyaninin antimikrobiyal etkisinin belirlenmesinde 'Agar Kuyu Difüzyon' yöntemi uygulandı. Böylece, piyosiyanin *Bacillus sp.*, *Escherichia coli* ve *Candida sp.*, üzerinde etkili bir antimikrobiyal ajanken, *P. aeruginosa* ve *A. niger* üzerinde antimikrobiyal etkisi görülmedi. Ardından, agar kuyu yöntemi ile piyosiyanine duyarlı olduğu belirlenen suşlara 'Tüp Seyreltme Yöntemi' kullanıldı. Azalan piyosiyanin derişimleri ile *Bacillus sp.* and *Candida sp.* suşlarının üremesinde artış gözlendi.

Anahtar Kelimeler
Pseudomonas aeruginosa, Piyosiyanin, Antimikrobiyal Aktivite.

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INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic human pathogen that may cause various infections. The virulence factors of *P. aeruginosa* such as two exotoxins and a variety of cytotoxic substances play an important role leading to different infections. Pyocyanin is one of the virulence factors of *P. aeruginosa*, which has particularly important role in lung infections. Pyocyanin is not only a virulence factor but also is significant by terms of biotechnology. Pyocyanin producing *P. aeruginosa* strains have a wide range of industrial applications such as food, mining, pharmaceuticals, textiles, leather and other industries [1,2].

Pyocyanin has various pharmacological and biological effects in prokaryotic cells [3]. Due to the ability of generating reactive oxygen species, pyocyanin get considerable attention. There are also different applications with pyocyanin as biosensors [4].

Pyocyanin is an extracellular phenazine derivative blue-green pigment which is produced as secondary metabolites by *P. aeruginosa*. Pyocyanin has also antimicrobial effects on a large number of different groups of microorganisms. Although, the inhibitory effect of pyocyanin on microorganisms are not yet fully understood, some suggestions are being developed.

The aim of this study is to produce pyocyanin pigment from different *Pseudomonas aeruginosa* strains isolated from different clinical specimens and to determine the antimicrobial activity of pyocyanin against Gram negative bacteria, Gram positive bacteria and Fungi.

MATERIALS AND METHODS

Isolation and Identification of *Pseudomonas aeruginosa*

In this study, clinically isolated ten different *Pseudomonas sp.* strains were cultured onto nutrient and EMB (Eozin-Methylen Blue) agar for primary isolation. Non-lactose fermented colonies were selected and cultured onto cetrimide agar, which is selective and differentiative media for *P. aeruginosa*, and incubated overnight at 37°C. Then selected colonies were identified by positive reaction to oxidase, IMVC, nitrate reduction and growth at 42°C.

Pyocyanin Production

Selected single colonies from cetrimide agar, were inoculated into *Pseudomonas* Broth (PB) (PB: Peptone 20 g; $MgCl_2$ 1.4 g; K_2SO_4 10 g; D.W 1000 ml) and incubated for overnight at 37°C on 150 rpm rotatory shaker [5].

Extraction and Purification of Pyocyanin

Pyocyanin was extracted from culture supernatants and measured based on the absorbance of pyocyanin in acidic solution at 520 nm [5]. The broth culture was centrifuged at 4000 rpm for 10 minutes. The culture supernatants were transferred into new test tubes and extracted with chloroform (1:2) and the aqueous phase was removed. The bottom layer was reextracted with 1 ml of 0.2 N HCl until color change was observed. Following this, the absorbance of the pigment solution was measured using spectrophotometer at 520 nm (Shimadzu-1700).

After the production and extraction of pyocyanin, pyocyanin purification was carried out [6]. Accordingly, 0.4 M borate-NaOH buffer (pH: 10) was added to the total amount of extracted pyocyanin until blue color observed. The blue colored pyocyanin was again extracted into chloroform. This step was repeated two times until obtaining a clear blue solution of pyocyanin in chloroform. The clear blue solutions of pyocyanin in chloroform were transferred to petri dish and were left overnight to evaporate chloroform. Finally, pyocyanin powders were dissolved using sterile distilled water.

Determination of Antimicrobial Effect of Pyocyanin on Bacterial and Fungal Isolates

The 'Agar Well Diffusion' method was carried out to determine the antimicrobial effect of pyocyanin on bacterial and fungal isolates. *Escherichia coli*, *Bacillus sp.*, *Pseudomonas aeruginosa* cultures were inoculated into nutrient broth and incubated overnight at 37°C. *Candida sp.* and *A. niger* cultures were inoculated into sabouraud dextrose broth and incubated overnight at 30°C. The overnight cultures of microorganisms were adjusted to 0.5 McFarland turbidity standards. Then, 100 µl of diluted bacterial suspensions were swabbed on Muller-Hinton agar plates and 100 µl of purified pyocyanin was added to the prepared

Table 1. Spectrophotometrically analysis of pyocyanin production in *Pseudomonas aeruginosa* strains.

Number of *P. aeruginosa* strains	Pyocyanin concentration (μg/ml)	Clinical Specimens
A1	0.97	Abdomen
A2	4.06	Urine
A3	6.04	Urine
A4	6.18	Ear
A5	6.76	Wound
A6	6.79	Urine
A7	7.10	Wound
A8	9.47	Urine
A9	10.11	Throat
A10	10.42	Urine

Table 2. Determination of antimicrobial activity of pyocyanin with agar well diffusion method.

Bacterial Strains	Diameter of inhibition zone (mm)
1. *Bacillus sp.*	31
2. *Escherichia coli*	12
3. *Pseudomonas aeruginosa*	R
Fungal Strains	Diameter of inhibition zone (mm)
1. *Aspergillus niger*	R
2. *Candida sp.*	15

wells in the same media, thereafter incubated at 37°C for 24 hours. Antimicrobial activity of pyocyanin was determined by measuring the inhibition zone diameters. After that 'Tube Dilution Method' was used on strains, which were determined as sensitive to pyocyanin with agar well diffusion method. So, these microorganisms were inoculated into nutrient and sabouraud dextrose broths and different concentrations of pyocyanin [1:1, 1:2, 1:5, 1:10] pigments were added in these tubes. Inoculated tubes were incubated at optimum temperature overnight. After incubation period, the growth of microorganisms was measured spectrophotometrically at 600 nm.

RESULTS

Ten different clinical *Pseudomonas* strains were identified as *P. aeruginosa* by different phenotypic methods. After identification of strains, pyocyanin production was determined in *P. aeruginosa* strains. But it was detected by spectrophotometrically that the amount of pyocyanin was different in each *P. aeruginosa* strain. So, it was indicated that, the highest amount of pyocyanin was produced by A10 strain isolated from urine specimen and the lowest amount of pyocyanin was produced by A1 strain isolated from abdomen (Table 1).

When the antimicrobial activity of pyocyanin was determined, it was seen that, pyocyanin was effective as antimicrobial agent on *Bacillus sp.*,

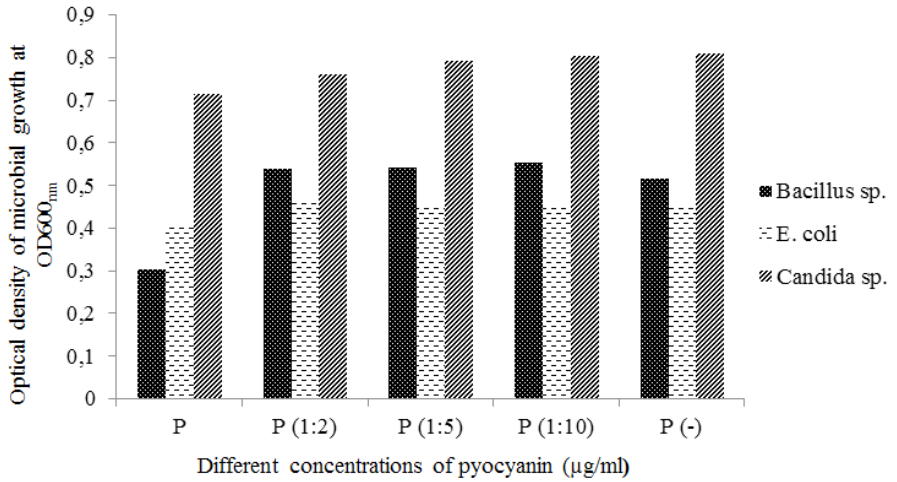

Figure 1. Optical density of bacterial growth in different concentrations of pyocyanin at 600 nm.

Escherichia coli and *Candida sp.*, but there was no effect on *Pseudomonas aeruginosa* and *Aspergillus niger* (Table 2).

In tube dilution method, with decreasing amount of pyocyanin, an increase in the intensity of *Bacillus sp.* and *Candida sp.* strains' growth was observed. So with this method, effect of pyocyanin, as an antimicrobial agent on some microorganisms, was verified (Figure 1).

All results in this study indicate that, pyocyanin was the most effective antimicrobial agent on *Bacillus sp.*

DISCUSSION

In addition to being opportunistic human pathogen, *Pseudomonas aeruginosa* is one of the most important bacteria in terms of commercial and biotechnological process. It produces pyocyanin which increases the importance of *P. aeruginosa*. Pyocyanin is also known as both virulence factor and quorum sensing signaling molecule in *P. aeruginosa* strains [7].

This study aims on pyocyanin production from different clinically isolated *P. aeruginosa* strains and investigation of the antimicrobial effect of pyocyanin. In this respect, the highest amount of pyocyanin was identified as 10.42 µg/ml in *P. aeruginosa* A10 strain (Table 1).

When pyocyanin production was examined according to clinical specimens, it was found that pyocyanin producing *P. aeruginosa* strains were frequently isolated from urine specimens. Furthermore, the highest amount of pyocyanin was produced by A10 strain which is isolated from urine, the lowest amount of pyocyanin was produced by A1 strain isolated from abdomen. It was pointed out in a similar study that the *P. aeruginosa* strains isolated from urine produced higher amounts of pyocyanin than other strains [8]. In a study with 96 clinical *P. aeruginosa* strains, all strains were able to produce different amounts of pyocyanin pigment Moreover, in that study *P. aeruginosa* strains isolated from urine had the highest pyocyanin production (20.15 g/ml), while the strains isolated from sputum showed the lowest pyocyanin level (3.80 g/ml) [9]. In a recent study, it was shown that the highest amount of pyocyanin production was determined in *P. aeruginosa* U3 strain isolated from urine specimen [2].

In particular, *Bacillus sp.* was the most sensitive bacteria against antimicrobial activity of pyocyanin pigment. Similar to our study, pyocyanin, which is extracted from *P. aeruginosa* strain, has antimicrobial activities against *Listeria monocytogenes* and *Bacillus cereus* [10]. The antimicrobial effect of pyocyanin against *E. coli* and *Candia sp.* is lower than its antimicrobial effect against *Bacillus sp.* (Table 2). Besides, due to the differences in the lipid content of cell walls in Gram-positive and Gram-negative bacteria, this can be

associated with different levels of susceptibilities of bacteria to pyocyanin [11]. On the contrary, it was determined in our study that *P. aeruginosa* and *A. niger* were resistant to pyocyanin. The inhibitory effect of pyocyanin is caused by altering the normal electron transport in respiratory chain and formation of the free oxygen radicals. It is believed that *P. aeruginosa* protect itself from toxic effects of pyocyanin with various mechanisms. In this manner, *P. aeruginosa* produces higher levels of enzymes such as superoxide dismutase (SOD), hydrogen peroxide (H_2O_2) than other aerobic microorganisms, it is more resistant to antimicrobial effect of pyocyanin. Due to the limited diffusion of pyocyanin through the outer membrane, the pyocyanin resistance in *P. aeruginosa* is associated with the blockage of the uptake of this compound [12-15].

After determining the susceptible and resistant microorganisms to pyocyanin, the efficiency of decreasing concentrations of pyocyanin were examined with tube dilution method. With the decreasing concentrations of pyocyanin, no increase in the growth of *E. coli* was observed. But decrease in the amount of pyocyanin ensured a growth increase in *Bacillus sp.* and *Candida sp.* (Figure 1). Similar to our study, it was shown in different studies that pyocyanin was more effective on Gram positive bacteria and C. *albicans* than Gram negative bacteria such as *S. typhi*, *P. mirabilis*, *E. coli*, *Acinetobacter sp.*; whereas Gram negative bacteria such as *K. pneumonia*, *P. aeruginosa*, *P. vulgaris* were resistant to antimicrobial effect of pyocyanin [16,17]. In addition to this, it was indicated that pyocyanin has a high antimicrobial activity against Gram positive bacteria (*S. aureus*; *B. subtilis*), Gram negative bacteria (*E. coli*, *Klebsiella sp.*, *S. typhi*, *Shigella sp.*, *P. vulgaris*) and *C. albicans* [2,18].

Pyocyanin, which is produced by *P. aeruginosa*, has ability to disrupt the electron transport chain of fungi and this reveals the antifungal effect [19]. Accordingly, *Kerr et al.*, demonstrated that pyocyanin has high antifungal effect on *C. albicans* and *A. fumigatus* as redox active compound [20]. Similar results detected in another study show that, pyocyanin has antifungal effect on *C. krusei*, *C. glabrata*, *C. tropicalis*, *Cryptococcus neoformans* [21]. It is also known that pyocyanin is highly effective on different *C. albicans* strains are associated with lung infections [22]. Compounds such as pyocyanin, pyrrolnitrin and pseudomonic acid have antifungal effect on various *Candida sp.* strains [23]. Pyocyanin also has considerable effect on inhibition of biofilms in *C. albicans* and *C. tropicalis* strains [24]. Pyocyanin synthesis is regulated by Quorum-sensing molecules termed as autoinducers. With the increase of cell density, the low molecular weight signal molecules are synthased, so the expression of virulence genes such as pyocyanin are regulated [25]. In the light of all these studies similar to our study, pyocyanin has more antimicrobial effect on *Bacillus sp.* and *Candida sp.* than Gram negative bacteria such as *E. coli* and *P. aeruginosa*.

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