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Antimicrobial efficacy of pyocyanin produced by *Pseudomonas aeruginosa* against multi-drug resistant microorganisms

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ABSTRACT

The antibiotic sensitivity test revealed that some bacteria were multiple-resistant to 3-8 antibiotics. The most effective drugs were amoxycillin, ciprofloxacin and nitrofurantoin. Yeasts exhibited different levels of sensitivity to the tested antibiotics. The addition of different stress factors enhanced the synthesis of pyocyanin by *Pseudomonas aeruginosa*. Maximum incriment of the pigment production was attained by adding ganoderma extract (159.7 μg ml^{-1} after 6 hrs) representing 254 %. A high antibacterial activity of pyocyanin against the tested Gram positive bacteria. *Streptococcus pneumoniae* was the most affected and it showed largest zones at different concentrations of pyocyanin (1:1 and 1: 0.2 v/v). Gram negative bacteria showed lower sensitivity to pyocyanin than Gram-positive bacteria. Thus, *E. coli* and *Salmonella typhi* were the only affected isolates by different concentrations of pyocyanin. Whereas, *Klebsiella pneumoniae, P. aeruginosa* and *Shigella flexneri* were resistant to all pyocyanin extracts. *Candida tropicalis* was susceptible to pyocyanin extracted by chloroform at 1:0.2 (v/v) than *Saccharomyces servisiae* which was more susceptible to pyocyanin at 1:0.5 (v/v). The antifungal effect of pyocyanin was recorded as inhibition zones ranged from 14 to 19 mm.

KEYWORDS

Pseudomonas aeruginosa;
Pyocyanin;
Antibiotics;
Antimicrobial.

INTRODUCTION

Bacterial resistance is spreading throughout the world primarily due to excessive use of antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and resistant strains of *Pseudomonas* are examples of multi-resistant bacteria that are becoming an alarming problem within the healthcare system[1]. *Enterococci* can cause bacteremia, wound infection and urinary tract infection, but serious infections of VRE usually only occurs in patients with significantly compromised host defenses[2]. *Candida* and *Pseudomonas* are other opportunistic pathogens that usually only lead to serious infections in immunocompromised individuals. Therapies for *Pseudomonas* even drug-susceptible strains have considerable defenses against antibiotics, and for *Candida* have been difficult because of the limited number of an-

tifungal agents[3].

The ability of opportunistic human pathogens to acquire resistance to a broad range of antibiotics has made effective therapy more difficult. Several recent investigations have dealt with the problem of antibiotic resistance in *P. aeruginosa*[4,5]. *Staphylococcus aureus* and *P. aeruginosa* strains showed different resistance pattern to various antibacterials. The yeast *Saccharomyces cerevisiae* is widely used in baking, brewing, wine making, and biotechnology and previously regarded as safe. Recent evidence indicates the involvement of *S. cerevisiae* in a range of superficial and systemic diseases. A number of isolates are capable of phenotypic switching and show partial or complete resistance to commonly used antifungal agents, including fluconazole[6].

Pyocyanin is a water-soluble blue-green phenazine pigment produced in large quantities by active cultures of *Pseudomonas aeruginosa*. Pyocyanin (*N*-methyl-1-hydroxyphenazine) has antibiotic activity against a wide variety of microorganisms[7-9]. Many effects of pyocyanin (PYO) and phenazine-1- carboxylic acid (PCA) on a diversity of eukaryotic hosts as well as bacteria are thought to results from oxidative stress response[10]. Pyocyanin was the major antifungal agent of *P. aeruginosa*; 1-hydroxy-phenazine also possessed activity. Pyocyanin MICs for *Candida albicans* and *Aspergillus fumigatus* were > 64 μg ml^{-1}[11]. The antifungal activity of pyocyanin produced by *P. aeruginosa* were studied. Pyocyanin was produced in King's B medium in pH 7 ±0.2 after 96 hours at 37°C and extracted by chloroform. The minimum inhibitory concentration of this compound against *C. albicans* was 40.69 μg ml^{-1}[12].

The objective of the present study was to evaluate the antibiotic resistance of some bacteria and fungi isolated from clinical specimens. The improvement of pyocyanin production by *P. aeruginosa* using some additives to the growth culture was studied. The *in vitro* antibiotic action of pyocyanin against different multidrug resistant bacteria and fungi was investigated.

MATERIALS AND METHODS

The tested Gram positive bacteria were *Staphylococcus aureus* 1, *Staphylococcus aureus* 2, *Streptococcus viridians, Streptococcus pneumoniae, Lactobacillus acidophilus* and *Dephtheroides*. Gram negative bacteria included *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri, Pseudomonas aeruginosa* and *Proteus mirabilis*. The fungi; *Candida albicans, Candida tropicalis, Saccharomyces servisiae* and *Aspergillus niger* were also used for antimicrobial testing. All stock bacterial cultures were maintained on nutrient agar slants at 4^0C with monthly transfers. Whereas, fungi were maintained on Sabouroud's agar slants.

Peptone water medium

The medium composed of (g l^{-1}); peptone 10, sodium chloride 5 and distilled water 1 litre. The medium was adjusted at pH 7.0- 7.4[13].

Antibiotics

The antibiotics; amoxycillin (30 μg), ampicillin (10 μg), norfloxacin (5 μg), cefoxitin (30 μg), cefaclor (30 μg), oxolinic acid (2 μg), nitrofurantoin (300 μg), tetracycline (30 μg) and nystatin (30 μg) were used to determine the sensitivity of the isolated microbes.

Antibiotic sensitivity test

Disc diffusion method was performed by the modified Kirby-Bauer single-disc technique described by Robert *et al*.[14] on Müller Hinton agar with the tested antibiotics.

Extraction of pyocyanin

Pyocyanin was extracted from *P. aeruginosa* isolates by serial chloroform extractions followed by sequential extractions with acid and neutral water. Pyocyanin expressed as micrograms per ml of culture supernatant. Pyocyanin calculated using an extinction coefficient at 520 nm of 17.072[15].

Effect of stress factors on pyocyanin production

Pseudomonas aeruginosa 4 was grown in peptone water medium by inoculating 50 μl of the bacterial suspension (1x10^5cfu ml^{-1}) into 5 ml broth in test tubes and incubating at 37ºC. The different stress factors; ganoderma extract (200 mg ml^{-1}), cinnamon oil, clove extract (100 mg ml^{-1}), *Sarcodiotheca furcata* algal extract (300 mg ml^{-1}) and H_2O_2 were added to the growing cultures as 50 μl after 2, 6, 18 and 24 hrs. The cultures were incubated for 5 days and then centrifuged at 3000 rpm for

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15 min. The supernatant was treated by 0.2 N HCl till the appearance of red color and the absorbance was measured spectrophotometrically at 520 nm.

Statistical analysis

One way analysis of variance (ANOVA) is used according to SPSS[16]. In the present work, each value presented in the tables is the mean of three readings ± the standard deviation (SD). The least significant difference is abbreviated as LSD and measured at $P \leq 0.05$.

RESULTS

Results in TABLE 1 show the effect of tested antibiotics on some Gram -ve bacteria. *Shigella flexneri* and *Pseudomonas aeruginosa* were highly mulit-drug resistants and were only affected by nitrofurantoin and ciprofloxacin.

TABLE 2 revealed the disk sensitivity test for some different clinical isolates of Gram positive bacteria and yeasts. The three bacteial strains tested were multiple-resistant to at least to 4 up to 8 antibiotics. Thus amoxycillin was the most effective against *Staphylococcus aureus*. Whereas, ciprofloxacin was found to be the most potent antibiotic against the tested streptococci. Concerning yeast sensitivity, *Candida albicans* was resistant to 5 drugs. *Candida tropicalis* was resistant to 3 drugs. Moreover, *Sacchromyces Servisiae* was only affected by 3 antibiotics; nystatin, nitrofurantoin and ciprofloxacin.

TABLE 1 : *In vitro* activity of antibiotics against some clinical isolates of Gram negative bacteria grown on Müller-Hinton agar

Tested antibiotics	Inhibition zone (mm)					
	E. coli	*K.pneumoniae*	*S.typhi*	*S.flexneri*	*P.aeruginosa*	*P.mirabilis*
Amoxycillin (30 µg)	R	R	11	R	R	10
Ampicillin (10 µg)	R	R	13	R	R	11
Cefaclor (30 µg)	R	R	R	R	R	R
Cefoxitin (30 µg)	R	R	R	R	R	R
Norfloxacin (5 µg)	15	20	28	R	R	28
Tetracycline (30 µg)	R	R	R	R	R	R
Nitrofurantoin (300 µg)	19	17	R	19	9	11
Ciprofloxacin (5 µg)	25	25	36	R	20	36
Oxolinic acid (2 µg)	15	18	20	R	R	22

R: resistant

TABLE 2 : *In vitro* activity of antibiotics against some clinical isolates of Gram positive bacteria and fungi grown on Müller-Hinton agar

Tested antibiotics	Inhibition zone (mm)					
	Gram positive bacteria			Yeasts		
	S.aureus	*Str.viridans*	*Str.pneumoniae*	*C.albicans*	*C.tropicalis*	*S.servisiae*
Amoxycillin (30 µg)	38	R	11	R	14	R
Ampicillin (10 µg)	R	R	13	8	16	R
Cefaclor (30 µg)	R	R	R	R	R	R
Cefoxitin (30 µg)	R	R	R	R	R	R
Norfloxacin (5 µg)	R	26	28	R	11	R
Tetracycline (30 µg)	R	R	R	18	15	R
(300 µg) Nitrofurantoin	R	R	R	25	23	22
Ciprofloxacin (5 µg)	R	40	36	11	21	13
Oxolinic acid (2 µg)	R	20	20	R	R	R
Nystatin (30 µg)	ND	ND	ND	20	23	23

R: resistant, ND: not detected

The optimization of pyocyanin production by using different natural substances as stressing factors during the growth of *Pseudomonas aeruginosa* was undertaken. In these experiments, the different stress factors; pyocyanin, ganoderma extract, cinnamon oil, clove extract, *Sarcodiotheca furcata* extract and H_2O_2 were added as 50 µl to the growing cultures (5 ml) of *Pseudomonas aeruginosa* No. 4 after 2, 6, 18 and 24 hrs. The cultures were incubated for 5 days and pyocyanin values were calculated. The obtained data are shown in TABLE 3. The different stress factors that increased the production of pyocyanin by *Pseudomonas aeruginosa* were ganoderma extract (159.7 µg ml^{-1} after 6 hrs), cinnamon oil (152.5 µg ml^{-1} after 6 hrs), clove extract (87.4 µg ml^{-1} after 6 hrs), *Sarcodiotheca furcata* extract (77.7 µg ml^{-1} after 24 hrs) and H_2O_2 (77.2 µg ml^{-1} after 18 hrs), respectively. The recorded percentages of improving the pyocyanin production by adding ganoderma, cinnamon oil, clove, *Sarcodiotheca furcata* extract and H_2O_2 were 254, 240, 139, 123 and 123 %, respectively. On the other hand, the low changes in the pigment biosynthesis by adding pyocyanin as a stressing agent to the growing culture of *P. aeruginosa* can be neglected.

TABLE 3 : Effect of some stressing additives on the production of pyocyanin by *Pseudomonas aeruginosa* No. 4 grown in peptone water broth for 5 days.

Induction of cultures aged (hours)	Yield of pyocyanin (µg ml^{-1})											
	Pyocyanin		Cinnamon oil		Clove extract		*S. furcata* extract		Ganoderma extract		H_2O_2	
	µg/ml	(%)	µg/ml	(%)	µg/ml	(%)	µg/ml	(%)	µg/ml	(%)	µg/ml	(%)
Control	72.4	100.0	62.8	100	62.8	100	62.8	100	62.8	100	62.8	100
2	68.9	95.2	129.3	205	85.9	137	67.3	107	76.9	122	76.9	120
6	76.7	105.9	152.5	240	87.4	139	64.9	103	159.7	254	68.2	109
18	72.4	100	140.4	220	76	121	57.3	91	146.0	232	77.2	123
24	70.4	97	148.5	236	68.7	109	77.7	123	91.8	146	73.5	117

The results in TABLE 4 revealed a relatively high antibacterial activity of pyocyanin against the tested Gram positive bacteria. *Streptococcus pneumoniae* was the most susceptible to pyocyanin extracted by chloroform (1: 0.2 and 1:1 v/v). Gram negative bacteria showed lower sensitivity to pyocyanin than Gram-positive bacteria. Thus, *E. coli* and *Salmonella typhi* were the only affected isolates by different chloroform extracts of pyocyanin. Whereas, *Klebsiella pneumoniae, P. aeruginosa* and *Shigella flexneri* were resistant to all extracts of pyocyanin. The data showed that only two species of fungi were susceptible to pyocyanin extracts. *Candida tropicalis* was susceptible to pyocyanin extracted by chloroform at 1: 0.2 (v/v) than *Saccharomyces servisiae* which was more susceptible to pyocyanin extracted at 1: 0.5 (v/v). The antifungal effect of pyocyanin was recorded as an inhibition zones

TABLE 4 : Antimicrobial activity of *Pseudomonas aeruginosa* No. 4 pyocyanin extracted by different volumes of chloroform against some multi-drug resistant bacteria and fungi grown on Müller-Hinton agar

Tested microorganisms	Pyocyanin extraction Culture supernatant : chloroform (v v)		
	1:0.2	1:0.5	1:1
	Inhibition zone (mm)		
Gram positive bacteria			
Staphylococcus aureus 1	15±2	22±3	0±0
Staphylococcus aureus 2	24±3	22±3	30±3
Streptococcus viridians	22±3	18±2	17±2
Streptococcus pneumoniae	32±4	12±2	39±4
Lactobacillus acidophilus	29±3	29±3	29±3
Corynebacterium sp.	21±3	17±2	13±2
Gram negative bacteria			
Escherichia coli	29±3	29±3	29±3
Klebsiella pneumoniae	0±0	0±0	0±0
Salmonella typhi	27±3	29±3	0±0
Shigella flexneri	0±0	0±0	0±0
Pseudomonas aeruginosa	0±0	0±0	0±0
Proteus mirabilis	7±1	7±1	7±1
Fungi			
Candida albicans	0±0	0±0	0±0
Candida tropicalis	17±2	18±2	0±0
Saccharomyces servisiae	14±2	19±02	0±0
Aspergillus niger	0±0	0±0	0±0
LSD	$6.48x10^{-14}$	$6.82x10^{-13}$	$1.97x10^{-15}$

Each value is the mean of three readings ± standard deviation; ND: not detected.

ranged from 14 to 19 mm.

DISCUSSION

In the last two decades, there has been an increased interest in the natural antimicrobial substances due to the spread of antibiotic resistance. Due to excessive and often unnecessary use of antibiotics in humans and animals, bacterial resistance has now been reported against every currently available antibiotic[1,2]. The results of disk sensitivity testing for the isolated microorganisms revealed that some bacteria were multiple-resistant to 3-8 antibiotics. The most effective drugs were amoxycillin, ciprofloxacin and nitrofurantoin. Livermore[3] reported that *P. aeruginosa* is inherently resistant to a wide variety of the commonly used antibiotics due to the synergy between multi-drug efflux systems or a type 1 AmpC β-lactamase and low outer membrane permeability. Diab *et al*.[4] indicated a paralleled correlation between bacterial plasmids, outer membrane proteins with antibiotic resistance. The multidrug resistance was reported for Gram positive and Gram negative bacteria. The increase in resistance to antibiotics along with the adverse side effects associated with the conventional treatments, led researchers to investigate other options in treating the multi-drug resistant infections[5,17]. Phenazine compounds, such as pyocyanin produced by *P. aeruginosa*, are antibiotics in their own right that can function as competitive agents in microbial communities. The increased pyocyanin production would help *P. aeruginosa* to compete with the other microbes[18].

P. aeruginosa No. 4 was inoculated into peptone water liquid medium and grown for 5 days at 37°C. Chloroform extraction was used to obtain pyocyanin pigment. The results showed that the pyocyanin synthesis required at least 3 days and the maximum production of the pigment (62.8 - 72.4 μg ml^{-1}) was attained after 5 days of incubation. The addition of different stress factors to the growing cultures significantly increased the production of pyocyanin by *P. aeruginosa* No. 4. The best inducers that enhanced the pigment biosynthesis were ganoderma extract (159.7 μg ml^{-1} after 6 hrs) and cinnamon oil (152.5 μg ml^{-1} after 6 hrs), respectively. Under culture conditions of limited phosphate, both pyocyanin production and catalase activity from *P. aeruginosa* were enhanced[7]. Ra'oof and Latif[19] observed that all *P. aeruginosa* isolates produced pyocyanin pigment on King's A medium in different amounts, whereas some of them produced different types of other pigments like (pyoverdine-yellow, pyorubin-red, and pyomelanin-black).

The obtained results revealed that Gram +ve bacteria were more susceptible to the antibiotic action of pyocyanin than Gram -ve bacteria. Thus, the Gram negative bacteria; *E. coli* and *Salmonella typhi* were the only affected isolates by the original extract of pyocyanin (1:0.5 v/v). Whereas, *Klebsiella pneumoniae, P. aeruginosa* and *Shigella flexneri* were resistant to all pyocyanin extracts. Our finding agreed with that obtained by Baron and Rowe[21] who reported that the Gram positive bacteria (*Micrococcus luteus, Staphylococcus aureus, Bacillus licheniformis, Bacillus subtilis, Paracoccus denitrificans*) were more susceptible to the antibiotic action of pyocyanin than were the Gram negative bacteria (*Escherichia coli, Proteus vulgaris, Enterobacter aerogenes* and *P. aeruginosa*). Norman *et al*.[8] reported that pyocyanin has been detected in an oil-degrading culture containing *P. aeruginosa* and is a redox-active compound capable of inhibiting the growth of pyocyanin-sensitive members of the microbial community. Price-Whelan *et al*.[9] stated that the antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity.

Our results concerning the resistance of *P. aeruginosa* No. 4 to its own produced pyocyanin pigment were coincided with those obtained by[20] who stated that if *P. aeruginosa* uses pyocyanin production to its advantage in competing with other bacteria in the same ecological habitat, it must therefore have a mechanism to insure its own protection or immunity against the bactericidal agent it produces. This immunity could be via higher concentrations of SOD and catalase or by lack of permeability. Similarly, Baron and Rowe[21] showed that all apyocyanogenic pseudomonads tested (a reddish-brown strain of *P. aeruginosa, P. denitrificans, P. fluorescens* and *P. perfectomarinus*) were totally resistant to the pyocyanin pigment, suggesting that resistance may be a characteristic of the genus. *P. aeruginosa*, the producer organism was also essentially unaffected by high concentrations of pyo-

cyanin. It is also possible that other general antibiotic resistance mechanisms play a role in pyocyanin resistance. Müller *et al.*[22] also found that pyocyanin did not affect the intracellular killing of *P. aeruginosa* in human neutrophils.

It was assumed that differential expression of catalase and SOD activities is the principal means of pyocyanin resistance[8]. The herein obtained data showed that only two species of fungi were susceptible to pyocyanin at two concentrations. *Candida tropicalis* was susceptible to pyocyanin extracted by chloroform at 1:0.2 (v/v) than *Saccharomyces servisiae* which was more susceptible to pyocyanin extracted at 1: 0.5 (v/v). The antifungal effect of pyocyanin was recorded as an inhibition zones ranged from 14 to 19 mm. Anjaiah *et al.*[23] stated that production of phenazine antibiotics, mainly phenazine-1-carboxylic acid (PCA) and minor amounts of oxychloraphine (OCP), contributed to the capacity of *P. aeruginosa* PNA1 to suppress *Fusarium* wilt of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceris* and *Pythium* damping-off of bean, caused by *Pythium splendens*. Pyocyanin is bactericidal for many species which can exist either in oxidized or reduced form, the latter being an unstable free radical which reacts rapidly with molecular oxygen. This autoxidation leads to the formation of superoxide (O_2^-) or hydrogen peroxide (H_2O_2). This killing is observed on agar plates as clear zones on lawns of sensitive bacteria[24].

It can be concluded that pyocyanin, as a new member of antimicrobial pigment, demonstrates a strong antimicrobial activity, including the multidrug Gram-positive and Gram-negative bacteria as well as yeasts. It is a new template for anti-infective drug design. Therefore, we believe pyocyanin shows promise in the area of clinical application as one of the alternatives to traditional antibiotics. More studies *in vivo* are needed to further explore the antimicrobial activity of pyocyanin as a promising candidate for the treatment of infectious diseases.

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