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Efficacy of pyocyanin produced by *Pseudomonas aeruginosa* as a topical treatment of infected skin of rabbits

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ABSTRACT

Pseudomonas aeruginosa produced the maximum amount of pyocyanin pigment (64.90 µg/ml) after 5 days of incubation in liquid peptone water medium. *In vitro* antimicrobial activity of the purified pyocyanin was demonstrated by agar well diffusion test. Gram positive bacteria were more susceptible to the antibiotic action of pyocyanin than Gram negative 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* was only affected at twenty folds of the original concentration. *P. aeruginosa* and *Shigella flexneri* were resistant to pyocyanin at all concentrations. Gram positive bacteria were more susceptible to *P. aeruginosa* pyocyanin (MICs 0.06 mg/ml) than Gram negative bacteria. Fungi that were non-susceptible to pyocyanin at original concentration (1 : 0.5 v/v), were affected when the concentration of pyocyanin increased to twenty fold of the original pigment extract. Thus, the inhibition zones of fungal growth reached 19 mm. The minimum inhibitory concentrations of pyocyanin against the affected bacteria and fungi ranged from 0.06 to 1.2 mg/ml. In an animal model, pyocyanin was applied topically to treat wounded skin of rabbits which infected with *S. aureus, K. pneumoniae* or *C. albicans*. Maximum healing and hair growing of the pyocyanin treated area of rabbit skin was observed after 14 -20 days in all cases compared to the untreated infected control.

KEYWORDS

Pseudomonas aeruginosa;
Pyocyanin;
*S*kin infection;
Rabbits;
Topical treatment.

INTRODUCTION

Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species[13,28]. 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[2,27]. Several recent investigations have dealt with the problem of antibiotic resistance in *P. aeruginosa*[12,14]. Multidrug resistant *P. aeruginosa* strain caused an outbreak in a neurosurgery ward[31]. Furthermore, *P. aeruginosa* and

Acinetobacter baumannil infections were recorded in the healthcare setting[26]. *Staphylococcus aureus* and *P. aeruginosa* strains showed different resistance pattern to various antibacterials. Strains of *Klebsiella pneumoniae, Acinetobacter baumannii, Citrobacter freundii, Enterococcus faecalis, Proteus mirabilis, P. vulgaris, Streptococcus agalactiae* and *Vibrio cholerae*, were resistant to 4-9 antibiotics[30].

An alarming increase in bacterial strains resistant to existing antimicrobial agents demands a renewed effort to seek agents effective against pathogenic bacteria resistant to current antimicrobials. In this concern, phenazines have been of great interest to pharmaceutical and clinical research groups for the last 50 years[23]. Antibiotic-resistant microbes, such as methicillin-resistant *Staphylococcus aureus* (MRSA), seriously threaten human health. The first case of MRSA infection was reported in the United Kingdom in 1961[37]. At present, MRSA infections have become pandemic in many medical institutions (hospital-acquired MRSA) and the community (community acquired MRSA) worldwide[11,22]. MRSA infections cause many health problems. It has become a primary cause of skin and soft tissue infections among persons without extensive exposure to professional healthcare[34]. Moreover, the overuse of vancomycin in treating MRSA infections and the existence of vancomycin-resistant enterococci has led to the emergence of vancomycin-intermediate *S.aureus*[3,4]. In order to prevent additional MRSA infections, more effective antimicrobial drugs are needed.

Fixed oil of *Nigella sativa* seeds enhance healing of staphylococcal-infected skin of mice by reducing total and absolute differential WBC counts, local infection and inflammation, bacterial expansion and tissue impairment[5]. A new antimicrobial peptide, ctriporin, was cloned and characterized from the venom of the scorpion *Chaerilus tricostatus*, an animal which has not yet been explored for toxic peptide resources. The MICs of ctriporin against *Staphylococcus aureus, Bacillus thuringiensis, Bacillus subtilis, Micrococcus luteus*, and *Candida albicans* are 5 to 20 ìg/ml. Meanwhile, its MIC against clinical antibiotic-resistant bacterial strains is 10 ìg/ml. Furthermore, the potential for ctriporin to be used as a topical antibiotic for treating staphylococcal skin infections was investigated. External use of the peptide ctriporin dramatically decreased the bacterial counts and cured skin infections in mice. In addition, ctriporin demonstrates antimicrobial efficacy via the bactericidal mechanism of rapid cell lysis[16]. Crude extracts of *Suaeda aegyptiaca* were applied on mice skin that previously infected with the fungus *Trichophyton rubrum*. Animals infected with *T. rubrum* were treated with combination of crude extracts of (100) mg/ml. After nine days of the treatments with crude extracts of *S. aegyptiaca*, the incision was completely covered with newly formed epithelium and the hair seen in comparison with mycodin ointment indicating the efficiency of this extract as antifungal agents[1].

The objective of the present study was to produce a phenazine antibiotic pigment (pyocyanin) by *P. aeruginosa* isolated from clinical specimens. The *in vitro* antibiotic action of pyocyanin pigment against different multi-drug resistant bacteria and yeast was investigated. The *in vivo* healing efficacy of pyocyanin on infected skin of rabbits was also studied.

MATERIALS AND METHODS

Microorganisms

The microorganisms used for this study were isolated from clinical specimens at the Diagnostic Microbiology Laboratory of Medical Sciences College, Hodeidah University, Yemen (provided by Prof. Dr. W.A. El-Shouny, Faculty of Sciences, Tanta University, Egypt). Some bacteria were obtained from the culture collection of the Microbiology Department, Faculty of Pharmacy, Tanta University, Egypt.

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 4ÚC with monthly transfers. Whereas, fungi were maintained on Sabouroud's agar slants.

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Culture media used

The conestituents of solid media; nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar were mentioned by Cheesbrough (2000). The liquid peptone water medium is composed of (g/l); peptone 10, sodium chloride 5 and distilled water 1 litre. The medium was adjusted at pH 7.0- 7.4.

Effect of different media on pyocyanin production

Nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar were used for the growth of *P. aeruginosa* and testing the biosynthesis of pyocyanin. *P. aeruginosa* was inoculated into peptone water liquid medium and grown for 5 days at 37°C. Bacteria were then removed by centrifugation (20,000 *g* x 30 min) and the supernatant was filtered of through 0.45-µm filters. The dark blue supernatant was subjected to chloroform extraction. The obtained pigments were stored at 4°C in the dark.

Purification and quantification of pyocyanin

Pyocyanin was extracted from culture supernatant of *P. aeruginosa* by serial chloroform extractions followed by sequential extractions with acid and neutral water[9]. Briefly, 3 ml of chloroform was added to 5 ml of culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 N HCl, which converted pyocyanin to the acidic (red) form. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were calculated using an extinction coefficient at 520 nm of 17.072[15]. *Pseudomonas* proteins were removed during purification by chloroform extraction of the pyocyanin. After the completion of five separation sequences, the pH of the isolated acidified water layer could be adjusted to pH 7.5 with a minimum volume of 0.1 M NaOH. Needle-like crystals formed in the chilled solution over the following 2 hours. These were trapped on a 0.45 µm (pore size) filter, and washed with water. Finally, the pyocyanin was crystallized, dried under vacuum and weighed. It was resuspended in water and stored at 4°C until used.

In vitro antimicrobial activity test

The antimicrobial activity of pyocyanin was assayed by agar wells diffusion. Agar plates were prepared using Müller Hinton agar. The plates were seeded with 0.1 ml of test culture corresponding to 10^6 cfu/ml. Wells of 6 mm in diameter were made using a sterile cork borer in solidified agar. The test pyocyanin preparations (50 µl) were added to the wells. The extracting agents were tested as controls[36]. Plates were left for one hour at 4ÚC and then incubated for 24 h at 37ÚC (except for *Aspergillus niger* which was incubated at 28ÚC for 72 h). Inhibition zones were measured in mm and three replicates were averaged[25].

in vivo antimicrobial assay

Experimental animals

Rabbits weighing 1250 to 1500 g were used. The animals were housed one per cage under standard conditions of light and temperature with access to food and water. The experiments were carried out in accordance with institutional guidelines for animal care[18].

Preparation of inoculum.

Standard *S. aureus, K. pneumoniae* and *C. albicans* were cultured in nutreint broth medium at 37°C. A portion of culture logarithmic-phase broth (1-ml) was quantified and concentrated to 20 µl using phosphate-buffered saline (PBS) as described by fan *et al*[16].

Pyocyanin preparation.

The purified pyocyanin was prepared as an ointment in 0.5% hydroxypropylcellulose to a final concentration of 5 mg/ml[16].

Rabbit skin abrasion and infection model.

In initial experiments carried out to set up the rabbit model skin infection, three human pathogens (*S. aureus, K. pneumoniae* and *C. albicans*) were used. Before creation of the wounds, the rabbits were shaved on the dorsal surfaces. After removing hair, the skin was cleaned and disinfected with cotton swab saturated with 70% alcohol. Skin abrasion wounds were made on the dorsal surfaces of rabbits using a blade by creating 3-cm diameter scratch area both on right and left sides. The abrasion wounds only damaged the stratum corneum and the upper layer of the epidermis but not the dermis[16]. Rabbits were inoculated intracutaneously with the tested microorganisms. The intracutaneous injections containing live microbe were applied to the back of each animal previously shaved. Animals were first

inoculated with 0.5 ml of saline containing 10^7 cfu/ml of the pathogen (*S. aureus, K. pneumoniae* or *C. albicans*). At 5 min after the wounding, a pipette tip was also used to inoculate 20 µl of concentrated suspension containing 10^7 to 10^8 CFU of standard *S. aureus, K. pneumoniae* or *C. albicans* into each defined wounded area.

Treatment was conducted by application of pyocyanin ointment preparation. Approximately 100 mg (containing 5 mg of pyocyanin pigment) of the ointment preparation were topically applied to the skin area above the inoculation site in two daily doses (in the morning and the evening). The treatments were started 72 h after the challenge and lasted for 10 days. Animals were clinically monitored photographed once daily. The rabbits were followed daily for the development of deep dermal abscesses, inflammatory reaction of the inoculated area and wound size for a total of 3 weeks[18].

At the end of the experiment, the skin area corresponding to the infection site was injected with 1 ml saline solution and the saline was withdrawn again and processed for bacterial and candidal count. Suitable dilutions of the obtained saline effusion were plated on Petri plates to determine the number of living pathogens (expressed as CFU). In order to investigate the reproducibility of the infection with *S. aureus, K. pneumoniae or C. albicans,* three independent experiments were performed.

Statistical analysis

One way analysis of variance (ANOVA) is used according to SPSS[32]. 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

Production of pyocyanin on different media

Four solid media were used for the growth of five isolates of *P. aeruginosa* and detection of pyocyanin. Cultures of *P. aeruginosa* isolates elicited a blue green pigment, reminiscent of pyocyanin, during growth on the four tested media; nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar. The highest blue pigment formation was detected when *P. aeruginosa* No. 4 was grown on Müller Hinton agar. Therefore, *P. aeruginosa* No. 4 was inoculated into peptone water liquid medium and grown for 5 days at 37°C. Bacteria were then removed by centrifugation and the dark blue supernatant was subjected to chloroform extraction. The obtained pyocyanin pigment was quantified. The results in TABLE 1 showed that the pyocyanin synthesis required at least 3 days and the maximum production of the pigment (64.90 µg/ml) was attained after 5 days of incubation.

TABLE 1 : Production of pyocyanin by *Pseudomonas aeruginosa* No. 4 grown in peptone water broth for 5 days

Incubation period (days)	Yield of pyocyanin	
	$O.D._{520\ nm}$	µg/ml
1	0.00	0.00
2	0.00	0.00
3	0.289	29.47
4	0.600	62.80
5	0.690	64.90

Concentrations of pyocyanin (µg/ml) were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072.

Antimicrobial activity of pyocyanin

Agar well diffusion tests were performed on 12 isolates of Gram positive and Gram negative bacteria as well as 4 isolates of fungi to determine their susceptibility to pyocyanin. The results presented in TABLE 2 revealed a relatively high antibacterial activity of pyocyanin against the tested Gram positive bacteria. The susceptibility of bacteria to pyocyanin was slightly increased when the concentration of pyocyanin increased from the original concentration (1:0.5) to five and twenty folds. However, Gram negative bacteria that were resistant to pyocyanin at the original concentration of the tested pyocyanin, were also resistant to the pigment at the higher concentrations except *K. pneumoniae* which inhibited at twenty folds of the pyocyanin concentration recording inhibition zone of 14 mm. Fungi that were non-susceptible to pyocyanin at original concentration (1:0.5), were affected when the concentration of pyocyanin increased to twenty fold of the original pigment extract. Thus, *C. albicans* showed cloudy zone of inhibition growth (19 mm) when examined against the twenty fold concentrated pyocyanin. *A. niger* was also susceptible to pyocyanin at the twenty fold concentration recording inhibition zone of 14 mm in diameter. The minimum inhibitory concentrations of pyo-

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TABLE 2 : Antimicrobial activity of different concentrations of *Pseudomonas aeruginosa* No. 4 pyocyanin against some multi-drug resistant bacteria and fungi grown on Müller-Hinton agar

Tested microorganisms	Pyocyanin extraction Culture supernatant: chloroform (v/v)			MICs
	1:0.5	5 fold	20 fold	mg/ml
	Inhibition zone (mm)			
Gram positive bacteria				
Staphylococcus aureus 1	22±2	17±2	28±3	0.06
Staphylococcus aureus 2	22±3	ND	25±3	0.06
Streptococcus viridians	18±2	17±2	20±2	0.06
Streptococcus pneumoniae	12±2	18±2	26±3	0.06
Lactobacillus acidophilus	29±3	29±3	29±3	0.06
Dephtheroides	17±2	20±2	19±2	0.06
Gram negative bacteria				
Escherichia coli	29±3	29±3	29±3	0.06
Klebsiella pneumoniae	0±0	0±0	14±1	1.2
Salmonella typhi	29±3	ND	ND	0.06
Shigella flexneri	0±0	0±0	0±0	R
Pseudomonas aeruginosa	0±0	0±0	0±0	R
Proteus mirabilis	7±1	7±1	7±1	R
fungi				
Candida albicans	0±0	0±0	19±2	1.2
Candida tropicalis	18±2	9±1	14±2	0.06
Saccharomyces servisiae	19±2	10±2	22±3	0.06
Aspergillus niger	0±0	0±0	14±2	1.2
LSD	$6.82x10^{-13}$	0.055	$1.27x10^{-13}$	

Each value is the mean of three readings ± standard deviation. MICs=Minimum inhibitory concentrations, R=resistant.

cyanin against the affected bacteria and fungi ranged from 0.06 to 1.2 mg/ml.

Pyocyanin as a topical treatment for infected skin of rabbits

The pyocyanin showed potent growth-inhibitory activity against standard Gram-positive bacteria, Gram negative bacteria and *Candida albicans* at low concentrations. Moreover, the *in vitro* treatment of clinically isolated pathogens showed that pyocyanin is able to inhibit antibiotic-resistant pathogens. Therefore, it was important to explore its *in vivo* antimicrobial activity in topical treatment of animal skin infection model. In these experiments, rabbits were wounded and infected with *S. aureus, K. pneumoniae* or *C.albicans*. The infected sites were topically treated by using pyocyanin as an ointment application. Wound morphologies were observed daily for 20 days after infection.

The obtained data revealed that pyocyanin ointment displays reasonable antimicrobial activity *in vivo* against all tested pathogens. TABLE 3 showed the effect of pyocyanin as a topical treatment on rabbits s.c. infected with *S. aureus*. Skin area infected with *S. aureus* showed the highest redness and swelling after 4 days of infection, while slight pus formation was observed after 6 - 8 days. A notable increase of hair growing was recorded in skin area treated with pyocyanin ointment after 14-20 days compared to slower regenerative changes seen in the untreated control area.

In the animals that infected with *K. pneumoniae* and treated with pyocyanin ointment, wound morphologies were observed daily. At 4 days after infection, the highest redness was observed. While swelling was started at 4 days and reached its highest level after 6 days. No pus formation was observed in the infected area. An enhanced healing and hair growing of the pyocyanin treated area of rabbit skin was observed after 14 -20 days. In the untreated infected area of skin, redness still persist with swelling and slightly loss of hair for longer time than in the pyocyanin treated skin area.

TABLE 3 : Effect of pyocyanin as a topical treatment on rabbits s.c. infected with *Staphylococcus aureus*

Days	Redness	Swelling	Pus formation	Hair growing	
				Treated "L"	Control "R"
2	-	-	-	-	-
4	+++	++	-	-	-
6	++	+	+	-	-
8	+	+	+	+	+
10	-	-	-	+	+
12	-	-	-	++	++
14	-	-	-	+++	++
16	-	-	-	+++	++
18	-	-	-	++++	+++
20	-	-	-	++++	+++

L = left side, R = right side, The increase of items were represented by + to ++++++.

Thus, the time for healing by pyocyanin is less than that in the untreated control (TABLE 4).

The data presented in TABLE 5 showed that rabbits were infected with *C. albicans* and treated with pyocyanin preparation. Skin wound morphologies were observed daily. At 4 days after infection, the highest redness of skin was obtained. While swelling was started at 6 days and reached its highest level after 10 days. Pus formation was observed in the infected area after 6 -16 days, and reached its highest value at 10 days. Maximum healing and hair growing of the pyocyanin treated area of rabbit skin was observed after 14 -20 days representing two folds of hair growing obtained on the untreated control area. Generally, the time for wound healing by pyocyanin treatment was shorter than that in the untreated control.

TABLE 4 : Effect of pyocyanin as a topical treatment on rabbits s.c. infected with *Klebsiella pneumoniae*

Days	Redness	Swelling	Pus formation	Hair growing	
				Treated "L"	Control "R"
2	-	-	-	-	-
4	++	-	-	-	-
6	++	++	-	-	-
8	+	++	-	+	+
10	+	+++	-	++	+
12	+	++	-	++	++
14	-	+	-	+++	++
16	-	-	-	++++	+++
18	-	-	-	+++++	+++
20	-	-	-	++++++	++++

L = left side, R = right side, The increase of items were represented by + to ++++++.

TABLE 5 : Effect of pyocyanin as a topical treatment on rabbits s.c. infected with *Candida albicans.*

Days	Redness	Swelling	Pus formation	Hair growing	
				Treated "L"	Control "R"
2	++	-	-	-	-
4	++++	++	-	-	-
6	+++	++++	+	++	+
8	++	+++	+	++	+
10	++	+++	++	+++	++
12	+	++	++	+++	++
14	-	++	+	++++	++
16	-	+	+	++++	++
18	-	+	-	++++	++
20	-	+	-	++++	++

L = left side, R = right side, The increase of items were represented by + to ++++++.

DISCUSSION

Production of pyocyanin

Phenazine compounds have been shown to be important virulence factors in *P. aeruginosa*. In addition, they act as cell-cell signaling molecules and have inhibitory activity against other bacteria[13,28]. 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 antibiotics secreted by other microorganisms in microbial communities could serve as a signal to alert *P. aeruginosa* to the existence or aggression of other bacteria, and the subsequent increased pyocyanin production would help *P. aeruginosa* to compete with the other microbes[24].

Cultures of *P. aeruginosa* isolates elicited a blue green pigment, reminiscent of pyocyanin, during growth on the four tested media; nutrient agar, blood agar, Mac Conkey agar and Müller Hinton agar. Considerable amounts of blue pigment were produced by *P. aeruginosa* isolates when grown on Müller-Hinton agar. Pigment production began during the first 24 hrs of growth, and maximal pigment production was achieved by isolate No. 4 after 72 hrs. Daly *et al*[10]. showed that ninety-seven percent of a total of 135 clinical *P. aeruginosa* isolates elaborated detectable pigments on the modified Mac Conkey agar (MMA) within 24 hrs. Several strains of *P. aeruginosa* (83%) produced pigment on Mac Conkey agar and sheep blood agar as well, but most required 48 hrs of incubation.

in vitro airing, pH and temperature play a role in the variation of pyocyanin production[38]. Our bacterium; *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 (64.90 µg/ml) was attained after 5 days of incubation. In previous investigations, *P. aeruginosa* was grown in low- and high-phosphate succinate media. Under culture conditions of limited

phosphate, both pyocyanin production and catalase activity were enhanced[20]. Five clinical isolates (CIs) of *P. aeruginosa* were examined for the production of pyocyanin using glycerol alanine minimal medium. The level of pyocyanin produced by CI-5 was comparable to that produced by PAO1[35].

Cheluvappa *et al*[8]. found that pyocyanin concentrations from patients with (CF) colonized by *P. aeruginosa* can go up to (27.3 ìg/ml). Ra'oof and Latif[29] 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) on King's B medium and also in different amounts. Gupta *et al*[19]. found that the cultures of *P. aeruginosa* PAO1 grown in Luria broth at 37°C and 120 rpm for 16-18 hrs produced low amount of pyocyanin (8.55 µg/ml). The low pigment production could be attributed to iron deficiency in the medium.

Antimicrobial activity of pyocyanin

In vitro antimicrobial activity of the purified pyocyanin pigment against the tested microorganisms was demonstrated by agar well diffusion tests. The obtained results revealed that Gram positive bacteria were more susceptible to the antibiotic action of pyocyanin than Gram negative 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* was only affected at twenty folds of the original concentration. *P. aeruginosa* and *Shigella flexneri* were resistant to pyocyanin at all concentrations. Gram positive bacteria were more susceptible to *Pseudomonas aeruginosa* pyocyanin (MICs 0.06 mg/ml) than Gram negative bacteria. All tested fungi were sensitive to pyocyanin at MIC ranged from 0.06 to 1.2 mg/ml. This finding agreed with that obtained by Baron and Rowe[6] 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*[27]. 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*[13,28]. stated that the antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity.

The herein obtained data showed that only two yeasts; *Candida tropicalis* and *Sacchromyces servisiae* were susceptible to pyocyanin at the original concentration (1: 0.5). The antifungal effect of pyocyanin was recorded as an inhibition zones ranged from 14 to 19 mm. Fungi; *C. albicans* and *A. niger* that were non-susceptible to pyocyanin at original concentration (1: 0.5), were affected when the concentration of pyocyanin increased to twenty fold of the original pigment extract. Flaishman *et al*[17]. observed that pyocyanin, a phenazine produced by *P. aeruginosa*, suppresses wheat blotch caused by *Septoria tritici*. Anjaiah *et al*[2]. stated that production of phenazine antibiotics by *P. aeruginosa* PNA1 enabled the bacterium to suppress some phytopathogenic fungi. Stephen and Hawkey[33] indicated that pyocyanin is bactericidal for many species including *E. coli, Staphylococcus aureus* and *Mycobacterium smegmatis* which may benefit *P. aeruginosa* by elimination of competing microorganisms. 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[33]. Pyocyanin was effective on the growth of the following studied microorganisms: *Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Salmonella typhi, Klebsiella pneumoniae* and *Penicillium sp.*, while it was not effective on *P. aeruginosa* and *Fusarium* sp[29].

Pyocyanin as a topical treatment of skin infected rabbits

Pyocyanin possesses antimicrobial activity against several multidrug resistant pathogenic microbes and may be used topically in susceptible cases. Since there are no effective treatment strategies for some serious skin infections, our study focused on the effect of the external use of pyocyanin. The results suggest that pyocya-

nin may become a potential candidate for treating skin infections. *In vitro*, pyocyanin can effectively inhibit Gram-positive and Gram-negative bacteria. Moreover, pyocyanin also shows antifungal activity against *C. albicans*. A test for the efficiency of external use of pyocyanin was carried out *in vivo*. The results show that pyocyanin topical treatment can effectively protect skin from infection in a rabbit skin *S. aureus, K. pneumoniae* and *C. albicans* infection models. Morphological observation showed that pyocyanin-treated skin was back to normal.

Pseudomonads represent the major group of non-differentiating microorganisms that produce antibiotics. The antibiotic substances produced by this group of organisms are pyocyanin, pyrolnitrin and pseudomonic acid. Kaleli *et al*[21]. investigated the *in vivo* and *in vitro* anticandidal activity of *P. aeruginosa* strains against *Candida* species. Forty-four *P. aeruginosa* strains isolated from various specimens of intensive care patients were included in the study. All *P. aeruginosa* strains have pyocyanin pigment. The total inhibition rates obtained by using Sabouraud dextrose agar of *C. albicans, C. parapsilosis, C. krusei* and *C. tropicalis* were 45%, 39%, 48% and 25% respectively. In the mouse model of concomitant subcutaneous infection with *Candida* species and *P. aeruginosa* no yeast were recovered from skin cultures despite 100% detection of *P. aeruginosa*. The anticandidal activity of *P. aeruginosa*-pyocyanin may be important in the treatment of patients. Al-Ani *et al*[1]. reported that skin area of mice infected with *Trichophyton ruburm* show irregular margin with boil formation, redness and swelling (2x2) cm. This indicates the invasion of the hyphae into stratum corneum inducing a hyperkeratosis with crust. This represented by elevation of skin due to accumulation of inflammatory cells, this is in turn lead to dilation of the blood vessels causing redness of the skin.

In a conclusion, this study showed that pyocyanin, a new member of antimicrobial pigment, demonstrates a strong antimicrobial activity, including the multidrug Gram-positive and Gram-negative bacteria as well as fungi (*S. aureus, K. pneumoniae* and *C. albicans*). It is a new template for anti-infective drug design. It rapidly killed microbes and effectively cured skin infections in rabbits when applied externally. Therefore, we believe pyocyanin, because of its high antimicrobial activity and therapeutic potential in treating skin infection, shows promise in the area of clinical application as one of the alternatives to traditional antibiotics. More studies are needed to further explore the antimicrobial mechanism of pyocyanin as a promising candidate for the treatment of skin infection by topical application. *In vivo* trials are required to advocate the systemic use of the potent antimicrobials in infectious diseases.

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