Evaluation of Ciclopirox as a Virulence-modifying Agent Against Multidrug Resistant *Pseudomonas aeruginosa* Clinical Isolates from Egypt

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Targeting the pathogen viability using drugs is associated with development of drug resistance due to selective pressure. Hence, there is an increased interest in developing agents that target bacterial virulence. In this study, the inhibitory effect of ciclopirox, an antifungal agent with iron chelation potential, on the microbial virulence factors was evaluated in 26 clinical MDR *Pseudomonas aeruginosa* isolates collected from Alexandria Main University Hospital, a tertiary hospital in Egypt. Treatment with 9 µg/ml ciclopirox inhibited the hemolytic activity in 70% isolates, reduced pyocyanin production, decreased protease secretion in 46% isolates, lowered twitching and swarming motility, and decreased biofilm formation by 1.5- to 4.5-fold. The quantitative real-time PCR analysis revealed that treatment with ciclopirox downregulated the expression levels of alkaline protease (*aprA*) and pyocyanin (*phzA1*). Ciclopirox is used to treat hematological malignancies and the systemic administration of ciclopirox is reported to have adequate oral absorption with a satisfactory drug safety profile. It is important to calculate the appropriate clinical dose and therapeutic index to reposition ciclopirox from a topical antifungal agent to a promising virulence-modifying agent against *P. aeruginosa*, a problematic Gram-negative pathogen.

Keywords: Ciclopirox, virulence factors, *Pseudomonas aeruginosa*, biofilm, gene expression

Introduction

In the current battle between humankind and pathogens, it seems that bugs are still having the upperhand. The continuous rise in reported resistance to different antimicrobial agents and the shortage in the development of new antibiotics over the past decades are two facts weighing the results of this battle to the favor of the microorganisms [1]. Since the development of a novel antimicrobial agent may cost millions of dollars and may take a considerable number of years to happen, an alternative approach is becoming nowadays more

and more appealing. This approach intends to repurpose older drugs to combat infections caused by certain problematic pathogens [2] or to hinder the bacterial virulence instead of bacterial viability [1].

One of the old drugs that has come back to the clinical stage is ciclopirox. Ciclopirox is an off-patent antifungal agent that has been developed about forty years ago and which possesses several advantages such as a wide safety margin, a profound efficacy and the ability to affect novel targets including resistant Gram-negative pathogens [2, 3]. In Gram-negative bacteria, ciclopirox was reported to affect galactose metabolism, as well as lipopolysaccharide (LPS) biosynthesis; two pathways extremely significant for bacterial growth and virulence [2].

Out of different members of Gram-negative bacteria,

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P. aeruginosa has been regarded as a cardinal opportunistic pathogen [4] responsible for about 10–15% of nosocomial infections all over the world [5]. *P. aeruginosa* can result in ventilator associated pneumonia, urinary tract infections, multi-organ system failure, burn wounds infection and sepsis in the ICU [6]. The high morbidity and mortality rates associated with *P. aeruginosa* infections are due to the organism's ability to accommodate to environmental changes, to develop resistance to various antimicrobial agents and to express multiple virulence factors [5].

P. aeruginosa is characterized by numerous cell-associated virulence factors, such as flagella, pili, biofilm and LPS. Aside from enabling motility, the flagellum indirectly participates in membrane permeabilization [4], while the type IV pili facilitate the bacterial adherence and are in charge of the twitching motility needed for the initial attachment and biofilm development [7]. Besides escaping the host immune system, biofilms possess an antibiotic resistant nature that facilitates the bacterial endurance in chronic infections [4]. The flagellum and type IV pili, together, mediate the swarming motility of *P. aeruginosa* on semisolid surfaces [8]. The LPS of *P. aeruginosa* is involved in the activation of the host immune responses [4]. This organism expresses, as well, various extracellular virulence factors including proteases, hemolysins, cytotoxin, pyocyanin, siderophores, exotoxin A and exoenzymes [5]. Proteases produced by *P. aeruginosa* play a part in tissue destruction associated with eye and lung infections [4]. A blue redox-active exoproduct, pyocyanin, is responsible for inducing neutrophil apoptosis and impairing neutrophil-mediated host defenses *in vivo* [7]. The siderophores are used to scavenge iron required for the organism's respiration and biofilm formation [9]. In addition, *P. aeruginosa* synthesizes a secretory apparatus (Type III) which enables it to inject the toxins from the cytoplasm into the target cell [4], thus, promoting the apoptosis of eukaryotic cells [10].

The present study was designed to evaluate a new role for ciclopirox; a virulence-modifying agent, and to evaluate its efficacy in inhibiting the virulence of selected *P. aeruginosa* clinical isolates collected from Alexandria Main University Hospital (AMUH), a tertiary teaching hospital in Alexandria, Egypt.

Materials and Methods

Bacterial strains and culture conditions

Twenty-six *P. aeruginosa* strains isolated from pus, urine, bronchial lavage, pleural fluid and sputum were obtained from AMUH. These were numbered from PA1-PA26. All bacteria were stored as frozen stocks in 40% glycerol at -80°C. A fresh culture was obtained by subculturing isolates on LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1% and Bacto agar 1.5%) for 24 h at 37°C prior to each experiment.

Identification of bacteria

The bacteria were cultured on a selective medium; cetrimide agar base (Himedia, India), then were incubated at 37°C for 24 h. The obtained colonies were subcultured and identified by standard microbiological methods [11].

Antibiotic sensitivity test

Antibiotic sensitivity was carried out by the disc diffusion method according to the Clinical Laboratory Standard Institute (CLSI) [12]. Different antibiotic discs were used; cefepime, ceftazidime, ceftriaxone, ciprofloxacin, colistin, gentamycin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, piperacillin and tobramycin (BD BBL, Sensi-Disc). Sensitivity test was analyzed for the identified strains by using Müller Hinton agar (Himedia) and incubated at 37°C for 24 h.

Reagents and antimicrobial agent

All reagents and chemicals used were of a pure pharmaceutical grade. Ciclopirox olamine was purchased as Batrafen solution (10 mg/ml) (Global Napi Pharmaceuticals, Egypt) and was diluted in sterile water to the required concentration immediately prior to each experiment.

Determination of Minimum Inhibitory Concentration (MIC) of ciclopirox

Ciclopirox MICs were determined using microbroth dilution protocols performed in accordance with CLSI [13]. A volume of 100 µl of each bacterial culture (grown in double strength nutrient broth (Oxoid, USA) and adjusted to 0.5×10^6 CFU/ml using the 0.5 McFarland standard) was added to 96-well polystyrene microtiter plate. Aliquots of 100 µl of serial dilutions of ciclopirox

(2048 µg/ml) prepared in sterile water (the tested concentrations ranged from 1024 to 2 µg/ml) were added to each well and incubated at 37°C for 24 h. MIC was measured in triplicate and determined as reported before [14].

Phenotypic detection of different virulence factors

Crystal violet biofilm assay. Quantitative determination of biofilm formation before and after treatment with ciclopirox was tested by crystal violet microtiter plate assay [15, 16]. Briefly, a 96-well polystyrene plates were inoculated with a 100 µl culture of each of the isolates in double strength tryptic soy broth (Oxoid) to reach a final concentration of 0.5×10^6 CFU/ml. A volume of 100 µl of 18 µg/ml ciclopirox was then added to each well [2]. Control wells were also prepared without ciclopirox addition. The plates were incubated aerobically for 48 h without shaking at 37°C. Next, wells were gently decanted and rinsed three times with sterile PBS (pH 7.2). After air drying, the wells were fixed with 100 µl of 99% methanol per well for 20 min. Biofilms were detected by staining the wells with 200 µl of 0.1% crystal violet (Sigma) for 15 min at room temperature and then washed thoroughly with distilled water to remove any residual dye. Then, 200 µl of glacial acetic acid (33%) were added and left for 30 min to elute crystal violet from the biofilms and the absorbance of the solubilized dye was measured at 520 nm using a microtiter plate reader. Each data point was averaged from three replicate wells and the following classification was used for the determination of biofilm formation: no biofilm production ($ODs = OD_{nc}$), weak biofilm production ($OD_{nc} < ODs \leq 2OD_{nc}$), moderate biofilm production ($2OD_{nc} < ODs \leq 4OD_{nc}$) and strong biofilm production ($4OD_{nc} < ODs$) [15].

Hemolytic activity on blood agar. *P. aeruginosa* isolates, before and after treatment with 9 µg/ml ciclopirox, were streaked onto blood agar medium (Nutrient agar supplemented with 5% sheep blood, Oxoid). The formation of a hemolytic zone was indicative of hemolytic activity of tested isolates. Average hemolytic zones around the developed colonies of the control and treated isolates were then recorded after 24 h incubation at 37°C.

Protease assay. Protease activity was determined using skimmed milk agar plates containing 10% w/v of non-fat

dry milk and 1% w/v of Bacto-agar [17]. Aliquots of 40 µl from overnight culture of *P. aeruginosa* isolates, before and after treatment with 9 µg/ml of ciclopirox, were added into previously cut wells in the milk agar plates and incubated at 37°C for 24 h. Protein digestion was shown as a clear zone around the wells. The average zone diameter of two independent readings was then recorded.

Qualitative and quantitative measurement of pyocyanin production

Pyocyanin pigment production was visualized by spreading *P. aeruginosa* isolates (without or with 9 µg/ml ciclopirox) on P agar plates (peptone 20 g/l, magnesium chloride 1.40 g/l, potassium sulfate 10 g/l, and Bacto agar 15 g/l (Oxoid). The final pH was adjusted to 7.2. This was followed by 24 h incubation at 37°C. Pyocyanin producing isolates manifested deep blue colonies on P agar plates [18, 19].

The quantitative pyocyanin assay of *P. aeruginosa* was adopted from Essar *et al.* [20, 21]. After growth for 24 h in LB medium (without or with 9 µg/ml ciclopirox), culture supernatants were extracted with 1–2 ml chloroform. The tubes were vortexed for a few seconds until the chloroform layer turned blue due to pyocyanin diffusion. The chloroform was acidified with a few drops of HCl, which resulted in a rapid change in color from blue to red, then analyzed spectrophotometrically [22]. All experiments were done in triplicates.

Bacterial motility

Swarming and twitching motilities of the tested *P. aeruginosa* isolates, before and after treatment with ciclopirox, were assessed as described previously [23]. Overnight cultures grown in LB were directly transferred to twitching plates (1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 1.0 g Bacto agar in 100 ml distilled water) by using sterile toothpicks. For the swarming media plates (0.8 g nutrient broth N.2, 0.5 g glucose, 0.5 g Bacto agar in 100 ml distilled water), the bacterial culture was diluted 1:100 in fresh LB medium and an aliquot of 20 µl was spotted onto the surface of the plates.

After 16 h of growth at 37°C, swarming motilities were directly observed at the air-agar interface, while twitching motility was measured at the agar-plastic interface

Table 1. Primers of the selected genes chosen for transcript analysis using quantitative RT-PCR.

Gene	Orientation	Sequence (5'-3')	Product size (bp)	Reference
<i>aprA</i>	F	GCTTCAGCCAGAACCAGAAGAT	78	24
	R	TCGACACATTGCCCTTCAAC		
<i>phzA1</i>	F	TAAACGTAATCGCGAGTTCATG	74	24
	R	TTTTATTGCGGAACGGCTATT		
<i>Rpsl</i>	F	GCAAGCGCATGGTCGACAAGA	123	25
	R	CGCTGTGCTCTGCAGGTTGTGA		

after the removal of the agar layer and staining with crystal violet [23]. Each experiment was performed using at least two independent cultures.

Molecular quantification of *aprA* and *phzA1* genes by Real-time PCR

Quantitative real-time PCR was conducted for ten isolates selected according to the results of the phenotypic detection of their virulence factors using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific Inc., USA) and used to assess the localized expression of *aprA* gene (encoding alkaline protease) and *phzA1* gene (necessary for pyocyanin biosynthesis) before and after treatment with 9 µg/ml ciclopirox. Gene specific primer pairs were synthesized in Macrogen, Korea, based on the previously published sequences [24]. The data were normalized against the house keeping gene *Rpsl* [25]. The primers of *aprA*, *phzA1* and *Rpsl* gene for the RT-PCR amplification of cDNA are listed in Table 1.

RNA isolation and reverse transcription

Total RNA was extracted from each overnight subculture of selected isolates using the TRIzol® Max™ Bacterial RNA Isolation Kit (Ambion by Life Technologies) in accordance with the manufacturer's instructions. RNA was quantified using Jenway Genova Nano, Keison products, UK.

Reverse transcription was carried out using the TOPreal™ One step RT qPCR Kit (Enzynomics). The real time PCR mixture consisted of 1 µl of TOPreal™ One step RT qPCR Enzyme Mix, 10 µl of 2X TOPreal™ One step RT qPCR Reaction Mix, 1 µl of each primer, 1 µl of total RNA, and sterile DNase-free water made up to a total reaction volume of 20 µl.

After a preliminary holding step at 50°C for 30 min, the cycling conditions used for the PCR reaction

included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. A negative (no template) control containing sterile DNase-free water instead of the total RNA template was included in each RT-PCR run. Samples were run in triplicate; virtually all individual results were within 0.5 Ct units of the averaged triplicate value.

Melting curves were then analyzed (to ensure the absence of primer dimers and other artifacts) in one cycle of 94°C, 53°C and 94°C, one minute each. Amplification curves and cycle threshold (Ct) values were determined by Stratagene MX3005P software. The Ct values for each gene amplification were converted into fold differences after normalization to the Ct of the *Rpsl* gene and according to the relative quantification method described previously [26].

Normalized expression of *aprA* and *phzA1* in the ten selected isolates was calibrated against corresponding mRNA expression of the same isolate without ciclopirox treatment. Results were presented as the relative expression of the mRNA compared to the untreated isolate.

Statistical analysis

Data of the RT-PCR data were expressed as means ± S.D. For multi-variable comparisons, one-way ANOVA was conducted, followed by Bonferroni testing using Prism 3 GRAPHPAD computer program. Differences were considered significant at p -value < 0.05. The data used in this experiment represents the mean of 3 biological replicates.

Results

Isolation and Identification

A total of 26 *P. aeruginosa* clinical isolates were collected from AMUH: 8 samples were obtained from pus, 8

Table 2. Minimum inhibitory concentrations (MICs) of ciclopirox against *P. aeruginosa* (N=26) and their joined resistance pattern.

MIC of ciclopirox (µg/ml) (No. of isolates)	Joined resistance pattern
8 (9)	TOB, GM, CAZ, CRO, CIP
16 (10)	TOB, GM, CAZ, CRO, FEP, PIP, CIP
32 (6)	TOB, GM, CAZ, CRO, FEP, PIP, CIP, OFX, CL
64 (1)	TOB, GM, CAZ, CRO, FEP, CIP, OFX, CL, NOR, MFX, LOM

TOB; Tobramycin, GM; Gentamycin, CAZ; Ceftazidime, CRO; Ceftriaxone, FEP; Cefepime, PIP; Piperacillin, CIP; Ciprofloxacin, OFX; Ofloxacin, CL; Colistin, NOR; Norfloxacin, MFX; Moxifloxacin and LOM; Lomefloxacin.

from urine, 6 from sputum, 3 from bronchial lavage fluids and one isolate from the pleural fluid. Collected isolates were subjected to microscopic examination and their identity was confirmed by using preliminary and selective biochemical tests [11].

Ciclopirox MIC and antibiotic resistance profile of clinical isolates

The MICs of ciclopirox and the joined resistance profile data of the 26 clinical isolates are presented in Table 2. Overall, there was a clear correlation between the resistance of the strains to different antibiotics and the corresponding MICs of ciclopirox. The MIC values of ciclopirox against the tested isolates ranged from 8 µg/ml to 64 µg/ml.

The clinical isolates exhibited different antibiotic resistance patterns. More than 73% of the isolates had ciclopirox MICs of ≤ 16 µg/ml. All the isolates under test were resistant to ceftazidime, ceftriaxone, tobramycin, gentamycin and streptomycin. Sixty-two percent of the isolates were resistant to ciprofloxacin while only seven isolates (27%) shared ofloxacin resistance (Table 2). However, most of the isolates tested (73%) were found to be susceptible to colistin.

Phenotypic detection of virulence factors

In the present study, 92% of the strains were biofilm formers. The majority of the isolates tested were moderate biofilm formers (20/26) (Fig. 1) and they were distributed among different specimens. Only three isolates were found to be strong biofilm formers, one isolated

Fig. 1. Distribution of isolates' biofilm strength among different sample sites using crystal violet biofilm assay.

from sputum and two from urine samples. No significant correlation was found relating the collection sample site and biofilm formation (Fig. 1). Furthermore, no detected relationship between the biofilm intensity and the resistance pattern of the organisms was observed.

Changes in the virulence pattern of the studied *P. aeruginosa* clinical isolates upon ciclopirox treatment are shown in Table 3. All of the twenty-six tested *P. aeruginosa* isolates were found to possess hemolytic activity before treatment with ciclopirox olamine, while about 70% of the isolates lost their activity after treatment. When testing pyocyanin production, 24 isolates (92.3%) and 3 isolates (11.5%) were found to be pyocyanin producers before and after ciclopirox treatment, respectively. As for protease production, a difference of 5 mm or more in proteolytic zone was considered an acceptable decrease in protease production [27]. Twelve isolates (46%) exhibited a prominent reduction in protease secretion upon ciclopirox treatment while 5 isolates totally lost their protease production ability after treatment (Fig. 2). A total of 24 and 20 isolates were found to be positive for twitching and swarming motility, respectively. Furthermore, 6 and 10 isolates lost their twitching and swarming motility, respectively, upon ciclopirox treatment (Table 3).

Ten isolates were selected for further tests since they displayed significant changes in virulence tests after treatment with 9 µg/ml of ciclopirox. The MIC of ciclopirox against these isolates ranged from 16–64 µg/ml. The quantitative fold decrease in their biofilm forma-

Table 3. Effect of ciclopirox olamine on different virulence factors of *P. aeruginosa* clinical isolates.

Isolate code	Swarming		Twitching		Hemolysis		Pyocyanin production		Protease production**	
	U	T*	U	T	U	T	U	T	U	T
PA1	+	-	+	-	+	-	+	-	11	10
PA2	+	+	+	+	+	+	+	-	24	20
PA3	+	-	+	-	+	-	+	-	20	11
PA4	+	-	+	-	+	+	+	-	18	16
PA5	+	+	-	-	+	+	+	-	22	16
PA6	+	-	+	-	+	+	+	+	20	19
PA7	-	-	+	-	+	-	-	-	14	11
PA8	-	-	+	+	+	-	+	-	21	8
PA9	+	+	+	+	+	+	+	-	22	20
PA10	-	-	+	-	+	-	+	-	20	10
PA11	+	-	+	+	+	-	+	-	19	0
PA12	+	+	+	+	+	-	+	-	19	0
PA13	+	-	+	+	+	-	+	-	20	11
PA14	+	+	+	+	+	+	+	-	23	14
PA15	+	+	+	+	+	-	+	+	21	15
PA16	-	-	+	+	+	-	+	-	18	11
PA17	+	-	+	-	+	-	+	-	19	12
PA18	+	+	+	+	+	+	+	+	18	13
PA19	+	+	-	-	+	+	+	-	18	18
PA20	+	+	+	+	+	-	-	-	18	11
PA21	-	-	+	+	+	-	+	-	20	12
PA22	+	-	+	+	+	-	+	-	21	12
PA23	+	-	+	+	+	-	+	-	18	0
PA24	-	-	+	+	+	-	+	-	20	0
PA25	+	-	+	+	+	-	+	-	18	9
PA26	+	-	+	+	+	-	+	-	21	0

*U (Untreated sample), T (treated sample).

**Zone diameter in mm.

Fig. 2. Loss of protease activity in isolates PA23 and PA24 where (u) represents supernatant of untreated corresponding isolate and (t) represents supernatant of corresponding isolate treated with 9 µg/ml of ciclopirox olamine.

tion and pyocyanin production was demonstrated in Fig. 3. The selected strains were either moderate or strong biofilm formers. After ciclopirox overnight treatment, all of the isolates became non-biofilm formers with a decrease in biofilm formation ranging from 1.5- to 4.5-fold (Fig. 3). Among our selected strains, pyocyanin was significantly produced before treatment with ciclopirox. That was manifested as a blue green pigment around the colonies grown on P agar plates and quantitatively upon extraction with chloroform. However, after ciclopirox treatment, there was a significant reduction in the amount of the pyocyanin produced ranging from 4-fold decrease in case of isolate PA26 to more than 12-fold decrease in case of PA10 (Fig. 3).

Fig. 3. Fold change in biofilm formation and pyocyanin production after overnight treatment with 9 µg/ml ciclopirox among ten *P. aeruginosa* selected isolates. The error bars represent SDs.

Effect of ciclopirox olamine on *aprA* and *phzA1* gene expression

The effect of overnight treatment with 9 µg/ml ciclopirox on the expression of the virulence genes *aprA* (alkaline protease gene) (Fig. 4A) and *phzA1* (pyocyanin biosynthesis gene) (Fig. 4B) among ten selected isolates

of *P. aeruginosa* grown at 37°C was measured by quantitative RT-PCR (Fig. 4).

The levels of *aprA* and *phzA1* gene expression were normalized to the levels of *Rpsl* gene expression and then compared with the levels of expression in corresponding untreated isolates. The transcripts of target

Fig. 4. Relative gene expression of *aprA* (A) and *phzA1* (B) determined by quantitative reverse transcriptase PCR for *P. aeruginosa* cultures. Total RNAs were prepared from ten selected isolates after overnight treatment with 9 µg/ml ciclopirox and calibrated against the corresponding untreated isolate. The levels of transcripts were normalized to the level of *rpsl* expression then compared to the level of expression by untreated cultures. No effect of the treatment corresponds to relative expression of 1. Results are expressed as the means and standard deviations of three independent determinations. The error bars represent SDs. The *p*-values indicate significance where **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001.

genes were calculated using the Pfaffle method or $\Delta\Delta C_t$ method [26].

The results depicted in Fig. 4A clearly show that a statistically significant but variable downregulation in the expression of *aprA* gene occurred upon treating the samples with subinhibitory concentration of ciclopirox with fold change ranging from 0.45- to 0.03-fold. The *p*-value was < 0.05 among isolates PA25 and PA26, while isolates PA8, PA13 and PA21 showed a highly significant reduction in the expression of the target gene with a *p*-value of < 0.001 compared to no effect of the treatment which corresponds to relative expression of 1.

The effect of subinhibitory concentration of ciclopirox on the ten selected pseudomonas cultures displayed different results as respect to the relative expression of *phzA1* gene as shown in Fig. 4B. Isolates PA10 and PA26 showed an unexpected significant upregulation in the gene under test compared to the expression of the isolates without treatment with fold changes in expression equals to 3.73 and 1.93, respectively. In contrast, the significance of ciclopirox treatment of the other isolates ranged from highly downregulation values in both PA8 and PA21 to acceptable values, though significant, in case of PA25 and PA1. However, no significant effect was observed on the expression of isolate PA12.

Discussion

P. aeruginosa is one of the clinically significant opportunistic pathogens causing infections that are life-threatening, and in most cases, difficult or challenging to eradicate [28]. The problem is aggravated by the fact that most of the isolated *P. aeruginosa* strains are reported to be multidrug resistant (MDR) ones [29]. In South Asia, the Middle East and the Mediterranean, modern medicine is already under threat from these MDR *P. aeruginosa* isolates [30].

In the present study, 26 clinical isolates of *P. aeruginosa* collected from hospitalized patients admitted to AMUH were found to be resistant to at least 3 classes of antibiotics and, thus, were recognized as MDR isolates [31]. A study conducted in the ICU of a parallel tertiary hospital, Alexandria University Students' Hospital, reported a percentage of 19% of MDR *P. aeruginosa* [32]. The tested isolates in the present study remained susceptible to colistin, an antibiotic described as salvage

therapy for *P. aeruginosa* infections [33, 34]. However, because of its significant nephrotoxicity and neurotoxicity, colistin has a limited clinical use [35]. The lack of appearance of new classes of antibiotics for the treatment of Gram-negative infections for more than 40 years [36] has outlined the importance on focusing on agents that disarm bacteria, thereby decreasing or completely nullifying their pathogenicity.

Ciclopirox, an old antifungal agent, has been evaluated in this study for its ability to inhibit the virulence factors expressed by *P. aeruginosa* isolates under investigation. Ciclopirox was previously reported to inhibit the growth of some Gram-negative bacteria [37, 38], but its efficacy had not been widely tested against antibiotic resistant bacteria. In the current study, the MIC₅₀ of ciclopirox was found to be 16 $\mu\text{g/ml}$ for MDR *P. aeruginosa*. An MIC range of 10 to >30 $\mu\text{g/ml}$ was reported by Carlson-Banning *et al.* for clinical *P. aeruginosa* isolates [2]. A number of observations suggested that ciclopirox olamine might act as a chelator of iron ions [39–41], this could explain the inhibition of the cell growth observed in this work.

A concentration of 9 $\mu\text{g/ml}$ of ciclopirox was used in this study to test its effect on virulence factors without affecting the bacterial growth. Hemolytic phospholipases secreted by *Pseudomonas* species are reported to directly cause lysis of human and sheep erythrocytes [42]. A percentage of 70% of tested isolates lost their hemolytic activity after ciclopirox treatment (Table 3). However, among *Candida albicans* isolates, subinhibitory concentration of ciclopirox treatment caused a strong upregulation of secreted phospholipases compared to the untreated ones [43].

The blue-green pyocyanin siderophore produced by *P. aeruginosa* cultures has been shown *in vitro* to induce apoptosis in neutrophils, as well as to inhibit the phagocytosis of apoptotic bodies by macrophages [44]. A percentage of 93% of the tested isolates produced pyocyanin. This percentage decreased to 12% upon ciclopirox treatment (Table 3) with a quantitative reduction ranging from 4-fold to more than 12-fold (Fig. 3).

The proteases secreted by *P. aeruginosa* have established roles in sepsis and ocular infections where they can degrade immunoglobulins and fibrin and disrupt epithelial tight junctions [45]. A total of 12 isolates (46%) exhibited a prominent reduction in protease secretion

and 5 isolates totally lost their protease production ability after treatment (Fig. 2). A similar finding has been reported when ciclopirox was tested against *C. albicans* where a decrease in protease secretion was reported [43]. Moreover, in a pancreatic cell model, ciclopirox was found to decrease caspases, a family of cysteine proteases, by forcing the cells to elevate the cleavage level of procaspase 3 [46].

During an infection, *P. aeruginosa* can adhere to host epithelial cells through the binding of its flagellum which is essential for the swimming and swarming motility of the organism [47]. The four pili localized at a cell pole of the organism are involved in twitching and swarming motility, as well as in the formation of biofilms [45]. Treatment with 9 µg/ml of ciclopirox resulted in decrease of twitching and swarming motility in 37.5% and 85% of the isolates, respectively (Table 3). Similar results were previously proven among *Salmonella typhimurium* strains where ciclopirox was found to decrease the bacterial motility by 91.4% compared to a control motile strain [48].

Twitching motility is an important factor in the development of biofilm, where highly organized structured communities of bacteria attached to one another and to a surface decrease the penetration of antibiotics and host defense molecules [49]. A percentage of 92% of the isolates under test were biofilm formers with different degrees of strength (Fig. 1). When ten strong to moderate biofilm formers were tested quantitatively, the fold decrease in biofilm formation ranged from 1.5 to 4.5-fold after ciclopirox treatment (Fig. 3). Similarly, a study investigating the effects of limiting iron levels in *P. aeruginosa* isolates found that biofilm development was significantly decreased by iron removal [50]. Disruption of biofilm formation is certainly a desirable goal that might prevent the repelling of antibiotics by biofilm structures of *P. aeruginosa*.

The effect of overnight treatment with ciclopirox on the expression of virulence genes *aprA* and *phzA1* among ten selected isolates of *P. aeruginosa* was measured by quantitative RT-PCR (Fig. 4). The levels of expression of the selected housekeeping gene; *rpsL*, a 30S ribosomal protein, did not change when cells were treated with subinhibitory concentrations of ciclopirox. This indicated that essential functions of the bacterial cells were not directly affected by treatment with this

drug. However, the fact that genes encoding alkaline protease production and pyocyanin biosynthesis (*aprA*) and (*phzA1*), respectively, were downregulated in the presence of ciclopirox (Fig. 4) showed that this drug has multiple direct or indirect effects on the cells. It was interesting that although most of the strains treated with ciclopirox experienced downregulation with variable significance regarding *phzA1* gene, the gene was markedly upregulated in PA10 and PA 26 (Fig. 4B). This can be explained by the fact that pyocyanin production in *P. aeruginosa* is a complex process involving two almost identical operons termed *phz1* and *phz2*, which drive the production of phenazine-1-carboxylic acid which is further converted to pyocyanin [51].

The downregulatory effect of ciclopirox on genes encoding alkaline protease and pyocyanin production in *P. aeruginosa* has not been reported yet in the literature. Thus, it needs further studies as it seems to be an encouraging pathway that might account for the antipseudomonal activity of ciclopirox *in vivo*.

Due to the current interest in ciclopirox for treatment of hematological malignancies such as multiple myeloma [52], systemic administration of this drug has been investigated in clinical trials with a resultant satisfactory drug safety profile and an adequate absorption following oral administration [53]. Calculation of the appropriate clinical dose and assessment of therapeutic index will be certainly required as a further step to permit the repositioning of ciclopirox from its role as merely a topical antifungal agent to a promising virulence-modifying agent against one of the most problematic Gram-negative pathogens, *P. aeruginosa*.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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