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Original Article

The effect of Zinc Oxide nanoparticles on *Pseudomonas aeruginosa* biofilm formation and virulence genes expression

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Abstract

Introduction: Due to increased resistance to antimicrobial agents, infectious diseases remain a public health problem worldwide. The current study was designed to examine the effect of Zinc Oxide nanoparticles (ZnO–np) against the biofilm formation ability of *P. aeruginosa* clinical isolates and to study its effect on the expression level of the genes involved in biofilm formation and virulence factors production.

Methodology: The MIC of ZnO–np against *P. aeruginosa* was determined by the broth micro dilution method. The effect of ZnO–np on the biofilm-forming isolates of *P. aeruginosa* was monitored by the microtiter plate method. *P. aeruginosa* isolates were tested for the expression of different biofilm and virulence genes using real-time rt-PCR.

Results: ZnO–np significantly down-regulated the expression level of all biofilm and virulence genes of *P. aeruginosa* clinical isolates except the *toxA* gene.

Conclusions: This study demonstrates the promising use of ZnO–np as an anti-biofilm and anti-virulence compound.

Key words: Biofilm; expression; *P. aeruginosa;* rt-PCR; ZnO–np.

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Introduction

P. aeruginosa is an important cause of community and hospital-acquired infections, especially in immune-compromised patients. The formation of biofilm by *P. aeruginosa* is the key to its chronic colonization in human tissues. Due to the many clinical implications, biofilms formed by *P. aeruginosa* are the most frequently studied biofilm models. Bacteria within biofilm show marked resistance to antibiotics, reduction in growth rates and secretion of different surface molecules and virulence factors, which can enhance their pathogenicity by several hundred folds [1]. Biofilm formation by *P. aeruginosa* depends on the cell-to-cell communication, quorum-sensing (QS) systems. *P. aeruginosa* recruits at least four different QS networks interrelated to each other, namely *las*, *rhl*, *iqs*, and *pqs*. These systems possess transcriptional regulators *LasR, RhlR, IqsR, and PqsR*, respectively, which trigger the expression of selected genes linked to virulence [2]. Increased resistance to antimicrobial agents is a major public health problem worldwide [3]. One of the most promising strategies for overcoming microbial resistance is the use of nanoparticles [4]. The exact mechanisms of action of nanoparticles are not yet known, it may be dependent on factors such as composition, surface changes, properties and concentration of nanoparticles [3]. One of the famous nanoparticles is ZnO–np which is one of metal oxide nanoparticles. Zinc Oxide is a polar inorganic compound. It appears as a white powder, nearly insoluble in water with many applications, such as antimicrobial, wound healing, UV filtering properties, high catalytic and photochemical activity, due to its unique combination of interesting properties such as selective toxicity toward bacteria, with minimal effects on human and animal cells, stability in a hydrogen plasma atmosphere and low price [5].

ZnO disrupts membrane integrity via the production of reactive oxygen species that destroy bacteria [6]. In addition, the production of hydrogen peroxide and Zn2+ has shown a key role in the antibacterial activity of nanoparticles [7]. However pathogenic microorganisms are able to protect themselves against inhibitory compounds by the formation of biofilms [8]. Recently, the antibacterial and antibiofilm activities of ZnO nanoparticles have been investigated. Therefore, the current study was designed to examine the effect of ZnO-np of smaller size against the biofilm formation ability of *P. aeruginosa* clinical isolates and to study its effect on the expression level of the genes involved in biofilm formation and virulence factors production of *P. aeruginosa* clinical isolates.

Methodology

Written informed consents were obtained from all patients or his caregiver with the help of doctors at the surgery department, Minia University Hospital before collecting the samples. The study was approved by the Ethical Committee of Minia University, Faculty of Medicine (code number: 32 A).

Bacterial strains

The study was carried out on non-repeated clinical isolates of *P. aeruginosa* were obtained from patients with burn or surgical infected wounds at the surgery department, Minia University Hospital during the period between April 2018 and January 2020. All samples were cultured on Cetrimide agar media then the isolated organisms were identified by standard microbiological techniques (colony morphology, Gram staining and biochemical reactions). In vitro biofilm forming abilities of the studied isolates was examined in 96 well micro-titer plates containing Luria Bertani broth medium following the same steps as the previous published papers. We have selected 100 *P. aeruginosa* isolates that were biofilm producers from all other isolates to perform this study. *P. aeruginosa* PAO1 as standard biofilm-producing strain and P1A isolate as non-biofilm producing strain were included as positive and negative controls respectively.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

ZnO nanoparticles (< 20 nm diameter) were purchased from Sigma–Aldrich. In order to examine the antibacterial activity of the ZnO-np, ZnO-np were suspended in sterile normal saline and constantly stirring until a uniform colloidal stock suspension was formed at a concentration of 1024µg/ml. The stock suspensions were stored at 4 °C. Before each experiment, stock suspensions were stirred for a ~2 h. The MIC of ZnO -np against *P. aeruginosa* was determined by using the broth micro dilution method in 96-well microtitre plates according to CLSI guidelines. Serial dilutions were prepared in 10 wells with concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1µg/ml and two wells were positive (including culture media and microbial suspension) and negative (including culture media) controls. Then, 10 microliters of bacterial suspension (OD620 = 0.01) were added to wells containing different concentrations of nanoparticles and the plates were then incubated overnight at 37 °C. MIC is the lowest concentration of the nanoparticles that inhibit visible bacterial growth. The concentration of ZnO-np that inhibited 50% and 90% of the isolates were measured as MIC50 and MIC90. The minimum bactericidal concentration was established by the lack of growth after re-inoculation from ZnO-np- treated media to agar medium without nanoparticles. All experiments were carried out three times [9].

Effect of ZnO-np on biofilm formation

The Effect of ZnO-np on the biofilm-forming isolates of *P. aeruginosa* was monitored by the microtiter plate method according to Samet *et al.* instructions [10]. Briefly, 190 µl of bacterial suspension (OD620 = 0.01) in Luria Bertani broth was inoculated in 96 microtiter plates. Sub-MIC concentrations of ZnO-np were added to each well excluding the positive and negative control wells. Plates were incubated at 37 °C for 24 hr. After incubation, the content of each well was gently removed. The wells were washed with phosphate-buffered saline solution to remove free-floating bacteria. Biofilms formed by bacteria were air and heat-fixed for one hour and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by washing with water and plates were kept for drying. Ethanol 95% was added to the wells and after 15 minutes the optical densities (OD) of stained adherent bacteria were determined with ELISA reader (model CS, Biotec) at 590 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate, the data were then averaged.

Effect of ZnO-np on preformed biofilm

Individual wells of microtitre plates were filled with 190 µl of bacterial suspension (OD620 = 0.01). The microtitre plates were incubated for 24 hours at 37ºC. After incubation, 10 µl of ZnO-np dilutions were added to each well. The effect of ZnO nanoparticles on the preformed biofilms was tested after 2, 4 or 6 hours incubation at 37ºC. The content of the microtitre plates was gently removed at the end of the estimated time period and then examined as described above, OD of stained adherent bacteria in wells were read at 590 nm.

Effect of ZnO-np on relative genes expression

P. aeruginosa isolates were tested for the expression of different biofilm and virulence genes using real-time reverse transcriptase-polymerase chain reaction (rt-PCR) according to the following steps.

RNA extraction

Pure bacteria were inoculated in two tubes containing 2 ml LB broth. One of the tubes contained

no nanoparticles and the other had ZnO-np. The ZnO-np concentration used herein was determined based on MIC and MBC results so that was less than MBC concentration. Tubes were incubated at 37°C, shaking 200 rpm for 6 hours (19). Bacterial RNA was extracted by the Direct-zol RNA extraction kit (Zymo research CORP, Australia) according to the manufacturer's instructions. Absorbance was assessed by a spectrophotometer (Genova, USA), and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the extracted RNA. The result within the 1.8 to 2 range was considered as acceptable purity. The quality of the extracted RNA was evaluated via electrophoresis on 1.2% agarose gel at 100 V for 60 min.

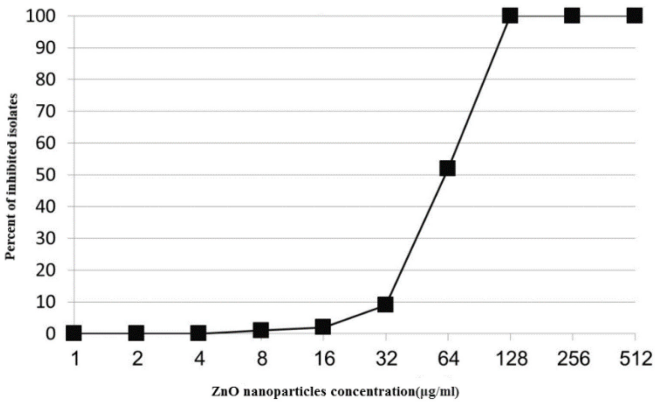
Rt-PCR

According to manufacturer instructions quantitative real-time rt-PCR was done using one step SYBR green kits (SensiFAST SYBR Lo-ROX Kit, Meridian Life science, UK) in an ABI 7500 instrument (Applied Biosystems, USA). Real-time rt-PCR reaction was prepared with a final volume of 20 µl (master mix: 10 µl, Forward primer: 0.8 µl, Reverse primer: 0.8 µl, Reverse transcriptase: 0.2 µl, RNase inhibitor, 0.4 µl, Water up to 16 µl and Template: 4 µl). Different genes and primers were listed in Table 1 [11-14]. Four negative control samples containing deionized water instead of template, one for each gene were included in the same PCR run.

We analyzed PCR results with relative quantification to *proC* (housekeeping gene) as a reference gene thus standards with known concentrations are not required. We calculated the fold changes of mRNA levels using the comparative cycle threshold (ΔΔCt). ΔCt = mean value Ct (gene of interest) - mean value Ct (reference gene), ΔΔCt=ΔCT of the test sample –Δ CT of the control sample. The negative value of this subtraction (-ΔΔCT) becomes the exponent of 2, R = 2-ΔΔCT [15].

The fold change in gene expression was normalized to the reference gene (*proC*) and relative to the control sample. Then the relative expression was confirmed by using free data analysis tools. PCR products were analyzed by gel electrophoresis, to exclude any unspecific products are present.

Figure 1. Minimal inhibitory concentration of ZnO nanoparticles.



X axis represent serial dilutions of ZnO–np that was tested in this study. Y axis represent the percent of the inhibited bacterial isolates at each concentration. The percent of the inhibited isoaltes increased with rising ZnO–np concentration.

Table 1. List of primers used for amplification.

Gene	Primer sequence	Reference
lasR	5′-AAGTGGAAAATTGGAGTGGAG-3′ 5′-GTAGTTGCCGACGATGAAG-3′	[11]
rhlI	5'-GTAGCGGGTTTGCGGATG-3' 5'-CGGCATCAGGTCTTCATCG-3'	[12]
pqsR	5'-CTGATCTGCCGGTAATTGG-3' 5'-ATCGACGAGGAACTGAAGA-3'	[12]
lecA	5′CACCATTGTGTTTCCTGGCGTTCA-3′ 5′-AGAAGGCAACGTCGACTCGTTGAT-3′	[11]
pelA	5′-AAGAACGGATGGCTGAAGG-3′ 5′-TTCCTCACCTCGGTCTCG-3′	[11]
toxA	5'…GACAACGCCCTCAGCATCAACAGC 5'…CGCTGGCCCATTCGCTCCAGC	[13]
exoS	5'…AGGCATTGCCCATGACCTTG 5'…ATACTCTGCTGACCTCGCTC	[13]
lasA	TTCTGTGATCGATTCGGCTCGGTT ACCCGGGAAGACAACTATCAGCTT	[14]
ProC	5′CAGGCCGGGCAGTTGCTGTC-3′ 5′-GGTCAGGCGCGAGGCTGTCT-3′	[11]

Statistical Analysis

All statistical analyses were performed using the SPSS program for Windows (version 20 statistical software; Texas instruments, IL, USA). A two-tailed *p*-value of < 0.05 was considered statistically significant.

Results

ZnO nanoparticles MIC and MBC

The results of the broth microdilution method showed that MIC50 and MIC90 of ZnO-np that inhibits the growth of *P. aeruginosa* clinical isolates were 64 and 128µg/ml, respectively. The range of MIC of ZnO nanoparticles for *P. aeruginosa* clinical isolates were 8-128µg/ml. Anti-bacterial activity increased with the rising concentration of ZnO nanoparticles as shown in Figure 1. The MBC of nanoparticles that kill 50 and 100% of the isolates were 128 and 256 µg/ml respectively.

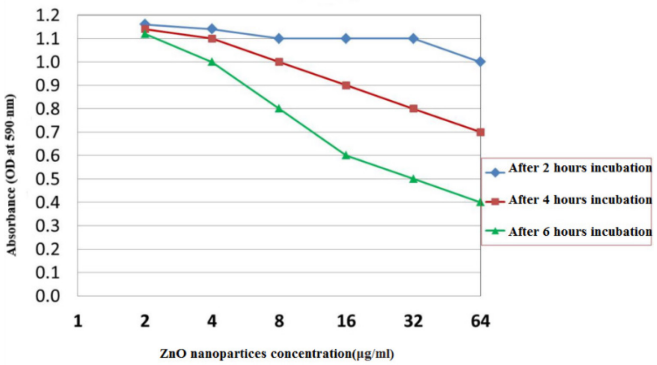
Antibiofilm effect of ZnO nanoparticles

ZnO-np showed anti-biofilm activity on all tested isolates. The anti-biofilm activity increased with the rising concentration of nanoparticles as shown in Figure 2. BIC50 and BIC90 (biofilm inhibitory concentration in 50 and 90% of the isolates respectively) were 16 and 32µg/ml respectively.

Effect of ZnO -np on the preformed biofilm

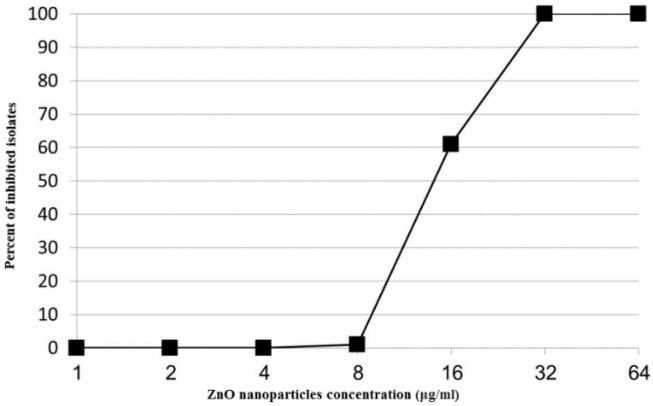
In this study, *P. aeruginosa* was employed to evaluate the effect of ZnO-np on the removal of established biofilms. The OD at 590 nm shown in Figure 3 corresponded to the amount of remaining attached biofilm biomass of *P. aeruginosa* isolates after 2, 4 and 6h treatment with ZnO-np. Treating the preformed biofilm with ZnO-np resulted in significant

Figure 3. Anti-biofilm effect of ZnO nanoparticles.



The figure represents the degree of reduction of OD values after 2, 4, 6 hs incubation of preformed bacterial biofilms with different ZnO–np concentrations at 37°C.

Figure 2. Biofilm inhibitory concentrtion of ZnO nanoparticles.



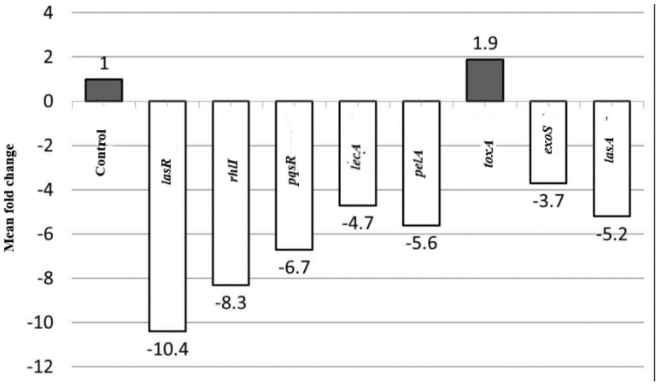
X axis represent serial dilutions of ZnO–np that was sudied in this study. Y axis represent the percent of the inhibited bacterial biofilms at each concentration. The percent of the inhibited bacterial biofilms increased with rising ZnO–np concentration.

OD reduction. The degree of reduction depends on the concentration of ZnO-np and the time of incubation between preformed biofilms and ZnO-np as shown in Figure 3. Significant reduction on the OD value (mean ± SD = 1 ± 0.03; *p* value = 0.001) was reported at 64 µg/ml concentration of ZnO-np for 2h incubation. The degree of reduction on the OD value was highly significant at 8, 16, 32 and 64µg/ml concentrations of ZnO-np incubated for 4 and 6 hours with the preformed biofilms (*p* value < 0.0001).

Effect of ZnO -np on relative genes expression

The effect of ZnO nanoparticles on the expression of different genes responsible for biofilm and virulence factors production was studied by RT-PCR. ZnO

Figure 4. Effect of ZnO nanoparticles on relative genes expression.



The figure represents the fold change decrease (-) or increase (+) in the virulence genes expression of *P. aeruginosa* clinical isolates after ZnO–np treatment.

nanoparticles significantly downregulated the expression level of all biofilm and virulence genes of *P. aeruginosa* clinical isolates except the *toxA* gene which was up-regulated as shown in Figure 4. The fold change decrease of the quorum sensing genes, *LasR, rhlI* and *pqsR* after ZnO nanoparticles treatment were 10.4, 6.3 and 8.7 fold (p value < 0.0001) respectively. ZnO nanoparticles down-regulated other genes responsible for biofilm formation; *LecA* and *PelA* genes by 4.7 and 5.6 fold (p value < 0.0004) respectively. ZnO nanoparticles also down-regulated virulence genes; *exoS* and *lasA* by 3.7 and 5.2 fold respectively (p value < 0.008). None statistically significant up-regulation of *toxA* gene after ZnO nanoparticles treatment by 1.9 fold was reported (p value = 0.37).

Discussion

In our study, we have examined the antibacterial activity of ZnO-np (< 20nm diameter) and its effect on the biofilm formation by *P. aeruginosa* isolated from hospitalized patients.

In the present study, ZnO-np were found to be effectively inhibiting the growth of *P. aeruginosa* and restrict the biofilm formation. The antibacterial and anti-biofilm effect gradually increased with raising the concentration of ZnO-np. MIC50 and MIC90 of ZnO-np for the studied isolates were 64 and 128µg/ml, respectively. BIC50 and BIC90 of ZnO-np for the studied isolates were 16 and 32µg/ml respectively. The MBC of nanoparticles was higher than the MIC indicates that ZnO-np can kill bacteria at higher concentrations. Also, treating the preformed biofilm with ZnO-np resulted in significant OD reduction. ZnO-np treatment resulted in a significant reduction in the OD value of the preformed biofilms at a concentration of 64 µg/ml for 2h incubation. Also, a significant reduction was reported at lower concentrations for an extended time of incubation. Overall, our results suggest that ZnO-np could inhibit the establishment and development of biofilm, also to remove pre-formed biofilm. Some previous studies have shown the antibacterial activity of ZnO-np. Ali et al. have studied the antibacterial effect of biosythesize ZnO-np against multidrug resistant *P. aeruginosa* isolates and the MIC values ranged from 1600–3200 ug/mL for clinical isolates obtained from different sources [16].

Hassani *et al.* have studied the antibacterial and anti-biofilm effect of ZnO-np against *P. aeruginosa* clinical isolates; they reported that MIC50 and MIC90 of their studied isolates were 150µg/ml and 175µg/ml [17]. Also, Hassani *et al.* reported that ZnO-np had an anti-biofilm effect at a concentration of 50 to 350 µg/ml. Also ZnO-np at a concentration of 100 to 350 µg/ml reduced pre-formed biofilm of *P. aeruginosa* [17]. Saadat *et al.* have studied the effect of ZnO-np with the size of 30-90 nm against *P. aeruginosa* and reported that the mean MIC of ZnO-np for the studied isolates was 300µg/ml [18]. Pati *et al.* showed that ZnO-np can disrupt bacterial cell membrane integrity, reduce cell surface hydrophobicity and down-regulate the transcription of oxidative stress-resistance genes in bacteria [19]. The toxicity of ZnO nanoparticles depends on concentration, bacterial species, and particle size [20]. In this study, we have observed that ZnO nanoparticles of smaller size (< 20 nm) has a better antigrowth and antibiofilm effect as the MIC and BIC is lower than the previous studies. This may be due to its good penetration power through cell wall of the bacteria than the larger one. We also assessed the relative expression of the genes regulating biofilm and other virulence factors production in ZnO-treated and untreated isolates using the ΔΔCt method. In Gram-negative bacteria, acylated homoserine lactones (AHLs) are common QS autoinducers. Typically, an AHL synthase produces an autoinducer that is bound by a partner transcriptional activator, regulate expression of genes that underpin group behaviors. *LasI/R* and *rhlI/R* are two principle QS systems that regulate virulence genes production in *P. aeruginosa*. *LasI* and *rhlI* synthases are responsible for the production of N-oxododecanoyl-L-homoserine lactone (C12-AHL) and N-butanoyl-L-homoserine lactone (C4-HSL) autoinducers, respectively. At a threshold concentration of autoinducers, C12-AHL binds with lasR and induces the expression of genes that control the production of elastase and proteases and also activates the *rhlI/R* system. In addition, C4-AHL binds with rhlR controlling the expression of genes encoding the production of elastase, and pyocyanin. If *lasI/R* and *rhlI/R* are interrupted, virulence factors will be inhibited. The *LasR* is a major transcriptional activator of *P. aeruginosa* QS and plays a pivotal role in the activation of many virulence genes with its ability to function as a transcriptional activator of lasI. RhlI is one of the key genes involved in *P. aeruginosa* QS signal production [21]. The Pseudomonas quinolone signal (PQS) is another QS systems that play an important role in the expression of several virulence factors as well as in inducing a protective stress response toward deteriorating environmental conditions. *PqsR* is a major transcriptional activator of PQS system, and plays a critical role in the pathogenicity of *P. aeruginosa* and is regulated by both the *las* and *rhl* system [22].

Our study reported that the relative expression levels of quorum sensing genes: *lasR*, *rhlI*, and *pqsR* were significantly reduced under ZnO sub-MIC treatment. The fold change decrease in the expression of *lasR*, *rhlI*, and *pqsR* genes were 10.4, 6.3 and 8.7 fold (p value < 0.0001) respectively. In accordance with our data, Saleh et al. reported that ZnO-np had a significant decrease in the relative expression of QS-genes *lasI*, *lasR, rhlI*, *rhlR*, *pqsA* and *pqsR*. Additionally, ZnO significantly decreased the pathogenesis of *P. aeruginosa* in vivo [12]. *P. aeruginosa* produces a large number of secreted and cell associated virulence factors that have been implicated in the pathogenesis of infection. Adhesion factors are crucial for the attachment of bacterial cells to the surfaces. In *P. aeruginosa* biofilms, adhesion factors such as lectins (*lecA* and *lecB*) play an important role in adhesion and biofilm formation. In this study, ZnO nanoparticles significantly down-regulated *LecA* gene expression (p value <0.0004) in biofilm-forming *P. aeruginosa* clinical isolates by 4.7 fold change.

Also, the presence of exopolysaccharides is an essential characteristic of the *P. aeruginosa* biofilm, which contributes to resistance and biofilm architecture. Pseudomonas biofilms are composed of at least three types of polysaccharides: Psl, Pel, and alginate. In the present study, the *pelA* gene was down-regulated in the presence of ZnO–np by 5.7 fold change (P-value <0.0004) in biofilm-forming *P. aeruginosa* clinical isolates.

exoS gene code for the exoenzyme S, a bifunctional enzyme, induces apoptosis of epithelial cells and macrophages and inhibits macrophages phagocytic activity. toxA gene code for exotoxin A, a major virulence factor of *Ps. aeruginosa*. This toxin binds to a specific receptor on animal cells, allowing endocytosis of the toxin. The toxin then blocks protein synthesis by ADP ribosylation of elongation factor 2, thereby triggering cell death. Other virulence gene named *lasA* code for lasA protease play an important role in *P. aeruginosa* infection.

In this study, ZnO nanoparticles significantly down-regulated *lasA and exos* genes expression in biofilm-forming *P. aeruginosa* clinical isolates by by 5.2 and 3.7 fold respectively (P-value <0.008). None statistically significant up-regulation of *toxA* gene after ZnO nanoparticles treatment by 1.9 fold was reported (p value = 0.37). Similarly, it was proved by Lee et al that ZnO-np (< 50 nm) inhibits *P. aeruginosa* biofilm formation and virulence factor production, they reported that ZnO-np at 1 mm inhibited biofilm formation by more than 95% on polystyrene surface. Also, Lee et al. showed that ZnO-np treatment resulted in significant down regulation to most of the virulence genes of *P. aeruginosa* that were studied by microarray and qRT-PCR [23].

García-Lara *et al.* previously studied the effect of ZnO-np on the virulence factors production of clinical and environmental *P. aeruginosa* strains; they reported that ZnO-np were able to inhibit most virulence factors of the majority of the strains [24].

Conclusions

This study reported that ZnO-np of smaller size (< 20 nm) has a better antigrowth and antibiofilm effect in *P. aeruginosa* clinical isolates. Also better to remove the pre-formed biofilm produced by this isolates. ZnO-np of smaller size reduced the expression of genes resposible for biofilm formation and virulence factors production in *P. aeruginosa* clinical isolates. Therefore this study confirm the promising use of ZnO-np as an anti-biofilm QS inhibitor and anti-virulence compound. More studies are needed to study the effect ZnO-np on all genes of *P. aeruginosa* as we reported that toxA gene was up-regulated. Also, the possible harmful effects of ZnO-np in vivo should be investigated.

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