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Curcumin and restoration of ciprofloxacin susceptibility to clinical isolates of *Pseudomonas aeruginosa* with mutated genes involved in ciprofloxacin resistance

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic nosocomial infection implicated in bacteremia in patients with compromised host defenses. Resistance to ciprofloxacin and imipenem, which are considered as suitable therapeutic options, is increasing in *P. aeruginosa*. Curcumin is a diferuloylmethane with antimicrobial properties. The present study was conducted to find the molecular effects of curcumin on clinical isolates with mutated genes involved in ciprofloxacin resistance. Fifty-two clinical isolates of *P. aeruginosa* were obtained from several hospitals and laboratories in Guilan province, northern Iran. Susceptibility to five antibiotics was evaluated by disc diffusion and broth dilution (MIC) methods. Furthermore, PCR-sequencing was carried out to evaluate mutations in topoisomerase subunits, five negative regulators of efflux pumps and *oprD* gene in these isolates. The effects of curcumin on the expression of *mexB* and *mexY* were evaluated using Q-RT-PCR. Of 52 *P. aeruginosa* isolated strains, 32-44% resistance to amikacin, ciprofloxacin, imipenem and gentamicin was observed. All isolates had mutation in *gyrA*. Some isolates had mutation in other topoisomerase subunits, some negative regulator genes and *oprD* gene. Curcumin (400µg/ml) along with ciprofloxacin (subMIC) increased ciprofloxacin susceptibility in four isolates. In these isolates, the expression of *MexB* and *MexY* efflux pump genes were downregulated. It seems that among *P. aeruginosa* isolates with various mutations in important genes in antibiotic resistant pathways, curcumin can intelligently sensitize isolates to these drugs.

1. Introduction

Curcumin is the lipophilic polyphenol (Adamczak et al., 2020), the yellow pigment, and the major bioactive substance and major component (Praditya et al., 2019) of rhizomes of turmeric (*Curcuma longa L.*) (Pakizehkar et al., 2020b). Turmeric has been used as an important spice in foods in tropical countries in the south and southwest of Asia such as Iran, Malaysia, India, China and Thailand. In some of the above countries, this spice has been used in traditional medicine to treat infection, dermatologic diseases, stress, and depression (Kocaadam and Şanlier, 2017). The antiviral activity of curcumin against viruses such as HIV, HSV, HBV, HCV and HPV has been confirmed. The antibacterial property of curcumin against

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Staphylococcus, *Streptococcus*, *Bacillus*, *Listeria* and *Pseudomonas* species has been studied (Praditya et al., 2019). Curcumin also has antifungal (Praditya et al., 2019), anti-oxidant, anti-cancer and anti-inflammatory activities. Low solubility in water, low absorption and rapid degradation are restrictions of this herbal compound for therapeutic purposes(Pakizehkar et al., 2020a). Lipid nanoparticles such as micelles, liposomes and polymersomes are biodegradable options to deliver compounds such as turmeric to cells. One of the advantages of polymersomes over micelles is the ability to encapsulate hydrophilic biomaterials as well as hydrophobic ones. In addition, polymersomes are more stable and storable compared to liposomes (Pakizehkar et al., 2020b).

Pseudomonas aeruginosa , as an important cause of nosocomial infections (Kim et al., 2016), is the most common pathogen isolated from patients with nosocomial pneumonia and secondary infections after severe injury and burns (Li et al., 2012). *P. aeruginosa* has intrinsic and acquired resistance to different antibiotics due to mutations in the target enzymes (Nouri et al., 2016) and the extrusion of different antibiotics by efflux pump systems (Alguel et al., 2010). Due to intrinsic and acquired antibiotic resistance in *P. aeruginosa*, treatment of this organism is difficult (Henrichfreise et al., 2007). Fluoroquinolones such as ciprofloxacin and carbapenems such as imipenem are suitable therapeutic options for the treatment of serious infections caused by *P. aeruginosa.* Mutations in gyrA, *gyrB*, *parC* and *parE* reduce the affinity of DNA gyrase or topoisomerase for ciprofloxacin and induce resistance. Furthermore, upregulation of efflux pumps increases the expulsion of ciprofloxacin from *P. aeruginosa* cells and induces resistance. Overexpression of efflux pumps occurs through mutations in negative regulatory genes (Rehman et al., 2019). *MexR*, *NalC* and *NalD* are repressors for MexAB-OprM operon, and *mexZ* acts as a repressor of MexXY operon (Solé et al., 2015). Overexpression of mexCD-oprJ operon was observed in ciprofloxacin resistant isolates with mutation in *NfxB* gene. Mutations inhibit the dimerisation and binding of NfxB to promoter of mexCD-oprJ operon, increasing the production of the MexCD-OprJ efflux pumps (Rehman et al., 2019). Imipenem and meropenem, as two carbapenems, are the most important drugs used to treat resistant isolates of *P. aeruginosa* (Shirani et al., 2016). Carbapenems flow through a membrane porin, OprD, to the *P. aeruginosa* cytoplasm (Wolter et al., 2004). However, mutations or loss of *OprD* gene (Ocampo-Sosa et al., 2012) is the most common mechanism of resistance to imipenem in *P. aeruginosa* isolates (Queenan and Bush, 2007).

The present study was conducted to investigate the reasons for ciprofloxacin and imipenem resistance in isolates of *P. aeruginosa* in the north of Iran. The effect of curcumin encapsulated in nanoparticles was also evaluated in some resistant clinical isolates. In addition, the expression of *mexB* and *mexY* genes involved in efflux pump was assessed in curcumin-treated cells compared to untreated ones.

2. Materials and Methods

2.1. Antimicrobial agents and susceptibility testing

In this study, 300 clinical samples were obtained from several hospitals and laboratories in north of Iran (Guilan province). Fifty-two clinical isolates of *P. aeruginosa* were identified. Antibiotic resistance profiles of *P. aeruginosa* isolates were examined by disk diffusion method (Kirby–Bauer) according to CLSI guidelines 2020 for five antibiotics gentamicin (10 µg), imipenem (10 µg), amikacin (30 µg), ciprofloxacin (5µg), Ceftriaxone (30 µg), (HiMedia, India). The minimum inhibitory concentration (MIC) of ciprofloxacin and imipenem in *P. aeruginosa* isolates was determined by broth-dilution method according to the CLSI guidelines 2020.

2.2. Mutation evaluation in genes involved in ciprofloxacin and imipenem resistance

DNA extraction from some ciprofloxacin/imipenem resistant isolates of *P. aeruginosa* was carried out as previously described (Takrami et al., 2017) . Amplification of the the genes was performed using gold master mix (*Pfu*) kit (Golden Double Helix Co, Italy) and in Analaytik jena instrument (Germany) according to manufacture program. DNA sequencing was performed by Macrogen

Inc. (Korea). The oligonucleotide primers used for amplification and DNA sequencing for *gyrA*, *nalC*, *parC* and *mexR* are described in previous study (Takrami et al., 2017) , and for *gyrB*, *nalD*, *parE*, *mexZ, nfxB* and *oprD* are listed in Table 1. To determine mutation, reference genome of *P. aeruginosa* PAO1 (NC_002516.2) as the sequenced product was analyzed using CLC main workbench v3.5 and BLASTN software.

2.3. Encapsulation of Curcumin in polymersomes

Oleoyl chloride, polyethylene glycol (PEG) (400 KD) and chloroform (trichloromethane) were provided from Sigma-Aldrich (St. Louis, Missouri). Triethyl amine was obtained from EMD Millipore (Billerica, Massachusetts). Diblock polymeric polymersomes were synthesized by esterification of oleoyl chloride (0.01 mol) and PEG_{400} (0.01 mol) in the presence of triethyl amine (0.012 mol) and chloroform as solvent at 24°C for 4h. Triethylamine hydrochloride was removed by filtering and chloroform was evaporated at 40°C for 4h. Curcumin was extracted from curcuma longa by HPLC. Then, curcumin was encapsulated in diblock polymeric polymersomes (PEG_{400}-OA nanoparticles) at 1:10 ratio (curcumin encapsulated in polymersomes (CPNs)).

2.4. P. aeruginosa treatment with CPNs and ciprofloxacin

The inoculums were adjusted to contain approximately 1.5×10^8 CFU/mL (0.5 McFarland turbidity) of microorganisms. Then, 100 µL of the proper inoculums were added to each tube and a final concentration of 10^6 CFU/mL was obtained. Then, 1 ml of this bacterial solution was added to each dilution (1 mL) containing Mueller–Hinton Broth medium, curcumin encapsulated in polymersomes (CPNs) (0, 10, 25, 75, 100, 250, 400, 500, 750 and 1000 µg/mL) and ciprofloxacin (sub-MIC), and was incubated at 37°C for 24h. In addition, the controls were treated with ciprofloxacin (subMIC) alone. Then, 10 µl of each treatment was cultured in Mueller Hinton agar (MHA) at 37°C for 24h and colony formation was investigated in CPNs- and ciprofloxacin-treated isolates compared to ciprofloxacin-treated isolates.

2.5. Statistical analysis

The statistical analyses were utilized by χ^2 test and in the software SPSS version 16.0. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Identification of P. aeruginosa

Fifty-two isolates of *P. aeruginosa* were obtained from urine, burn, respiratory secretions, and prosthesis identified by biochemical tests (Table 2).

3.2. Antimicrobial susceptibility profiling

A total of 52 clinical isolates of *P. aeruginosa* were tested for their susceptibility to five antimicrobial agents. The results of the disc diffusion test are shown in Figure 1. The resistance to Ceftriaxone was reported as highest resistance (80.8%). Resistance to imipenem and gentamicin was determined in >40% of isolates. In addition, the resistance rate of isolates was 38.46% for ciprofloxacin and 32.7% for amikacin.

3.2. MIC

Evaluation of MICs of ciprofloxacin and imipenem is shown in Table 3 and 4. Imipenem-resistant isolates of *P. aeruginosa* had a MIC of 8 to 512 µg/ml. In these isolates, MIC of ciprofloxacin was 16 to 1024 µg/ml.

3.3. Mutations in genes involved in ciprofloxacin resistance

In 18 ciprofloxacin resistant isolates we found two or more mutations in topoisomerase subunits and some negative regulators of efflux pumps (Table 4). All isolates had mutation in *gyrA* gene (T83I and/or D87Y). Ten isolates had muation in *nalD* gene. Two or more isolates had mutations in *gyrB*, *parC*, *parE*, *mexZ*, *mexR*, *nalC*, *nfxB* genes.

3.4. Mutations in oprD gene

To gain insight into genetic cause of imipenem resistance in *P. aeruginosa* isolates, we amplified and sequenced the *oprD* gene. In this study, we identified 18 previously reported missense mutations, five new missense mutations (G211R, E230K, S278V, I362F, T370P), three nonsense mutations caused by a substitution, and two deletions in ten clinical strains.

3.5. Downregulation of MexB and MexY in isolates treated with CPNs and ciprofloxacin

Diblock polymeric polymersomes (Pakizehkar et al., 2020b) were used to transfer further amount of curcumin to bacterial cells. The concentration of CPNs (Figure 2), which could kill half of bacterial cells, was selected to evaluate gene expression. Our analysis using Q-RT-PCR indicated downregulation of *MexB* (Figure 3) and *MexY* (Figure 4) in four isolates treated with CPNs (400 µg/mL) and ciprofloxacin (subMIC) compared to isolates treated with ciprofloxacin (subMIC) alone (Figure5).

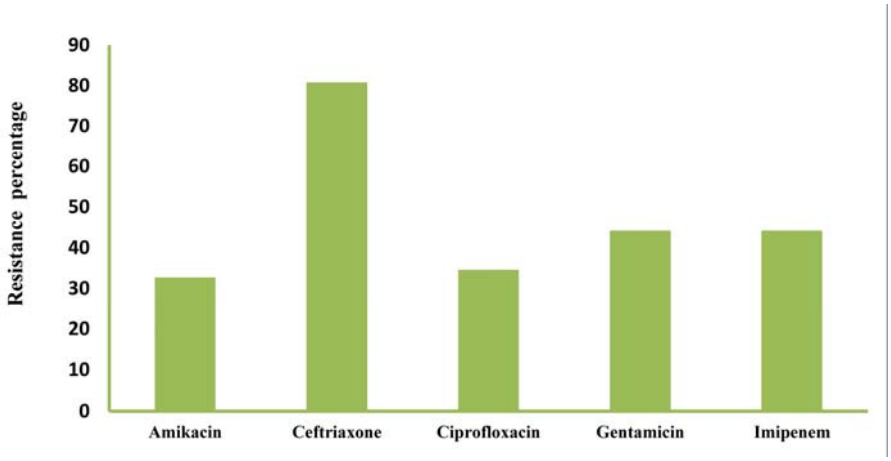

Figure 1. Antimicrobial susceptibility test. The results of disc diffusion test were evaluated for five antimicrobial agents.

Figure 2. Curcumin encapsulation in polymersome nanoparticles. After Curcumin encapsulation in these nanoparticles, Curcumin could solve in water and Inorganic solvents.

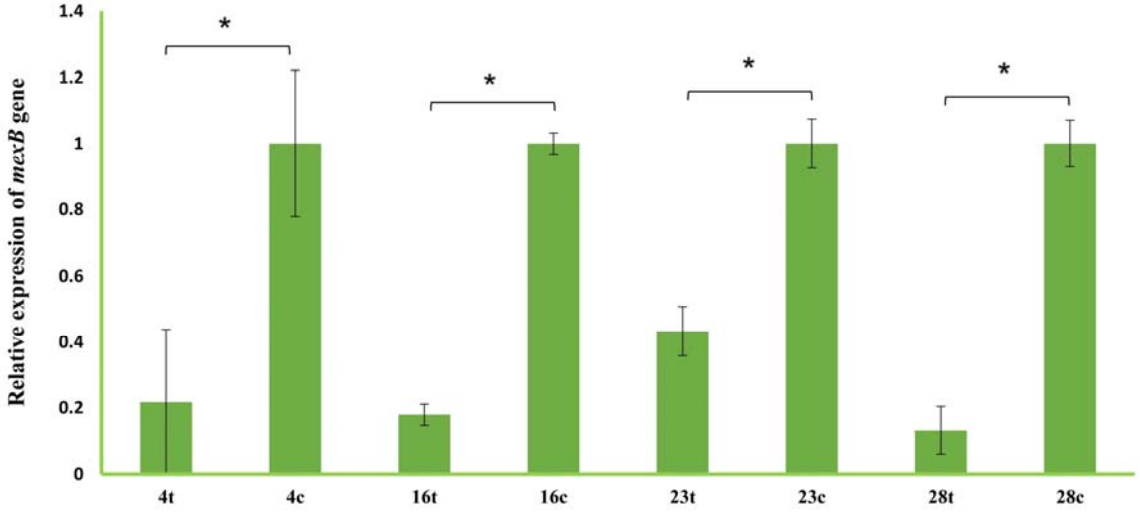

Figure 3. Relative expression of mexB gene in four Pseudomonas aeruginosa isolates (4, 16, 23 and 28) treated with CPNs (400µg/mL) and ciprofloxacin (1/2MIC) (test group=t: 4t, 16t, 23t and 28t) compared to isolates treated with ciprofloxacin (1/2MIC) (control group=c: 4c, 16c, 23c and 28c). Asterisks indicate significant differences between the two groups (*P<0.05). The results are represented as mean ± SD.

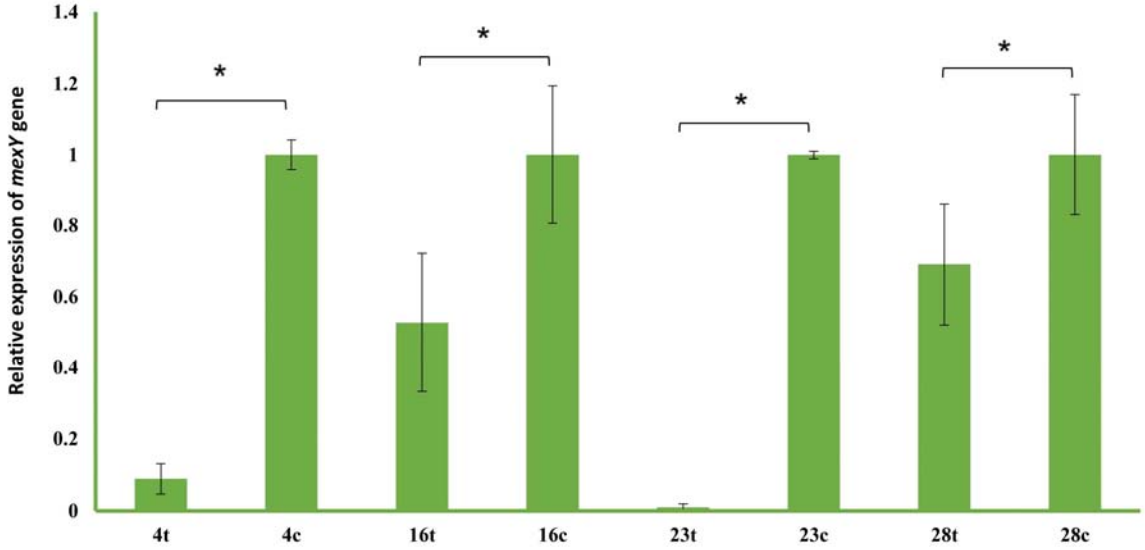

Figure 4. Relative expression of *mexY* gene in four *Pseudomonas aeruginosa* isolates (4, 16, 23 and 28) treated with CPNs (400µg/mL) and ciprofloxacin (1/2MIC) (test group=t: 4t, 16t, 23t and 28t) compared to isolates treated with ciprofloxacin (1/2MIC) (control group=c: 4c, 16c, 23c and 28c). Asterisks indicate significant differences between the two groups (*P<0.05). The results are represented as mean ± SD.

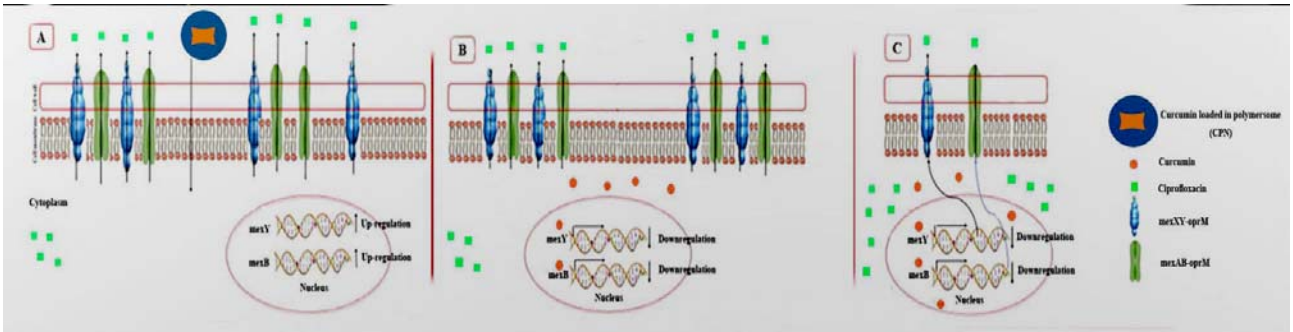

Figure5. A schematic figure of the effects of curcumin on ciprofloxacin-resistant cells. A) Before curcumin induction, there are eight efflux pumps (mexXY-/mexAB-oprM) as a result of upregulation of these pumps in bacteria with mutation in negative regulators. Polymersomes (CPNs) were still out of cells. B) After transfer of polymersomes (CPNs) to cells and curcumin to nucleus, curcumin induced downregulation of *mexB* and *mexY* genes. C) Curcumin induction decreases the number of these efflux pumps in cell membrane and results in the decrease of ciprofloxacin withdrawal.

4. Discussion

Fluoroquinolones and carbapenems are the important antibiotics to treat severe infections caused by the multidrug-resistant *P. aeruginosa*, but nowadays the increase in resistance to these antibiotics has been revealed in *Pseudomonas* infections (Rodriguez-Martinez et al., 2009). In this study, we identified the mutation profile of several genes implicated in ciprofloxacin and imipenem resistance in clinical isolates of *P. aeruginosa.* Then, we treated four ciprofloxacin resistant isolates (with identified mutation) with curcumin encapsulated in polymersome (CPNs) and ciprofloxacin. Our analysis showed that curcumin can induce susceptibility to ciprofloxacin (subMIC) with downregulation of some genes involved in efflux pump such as *mexB* and *mexY* genes.

In this study, we identified mutations in different genes involved in ciprofloxacin resistance in isolates of *P. aeruginosa* such as *gyrA, gyrB, parC, parD and mexR*. Mutations in DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*parC, parD*) are major reasons for ciprofloxacin resistance in *P. aeruginosa*. Alteration of codons 83 and 87 in *gyrA* reduces the affinity of gyrase for ciprofloxacin (Rehman et al., 2019). Bruchmann et al. showed that mutations such as *gyrA* T83I, *gyrB* E468D along with *parC* S87L and/or deletion of the efflux negative regulator-encoding gene (*mexR*, *nfxB*, or mexZ) increased MIC of ciprofloxacin (Bruchmann et al., 2013). Therefore, in our study, association of several mutations in all strains except two isolates can be the reason for increased MIC. The second reason for ciprofloxacin resistance in *P. aeruginosa* is upregulation of efflux pumps MexAB-OprM and MexXY due to mutation in their negative regulators. Mutations in *nalC* gene in our study can lead to overexpression of *mexB* gene (Campo Esquisabel et al., 2011). Two isolates with mutation p.P37PfsX117 in *mexR* (Saito et al., 2003) led to inactivation of *mexR* and in result upregulation of MexAB-OprM and MexXY. Other mutation *mexR* (p.S88RfsX116) has not hydrophobic parts (Saito et al., 2003) of protein and lack of these domains can lead to upregulation of some efflux pump genes.

In our study, mutation in *oprD* gene was observed in 10 imipenem resistant isolates. 18 mutations were observed in previous studies (Rodriguez-Martinez et al., 2009) (Liu et al., 2013) (Rodriguez-Martinez et al., 2009). It appears that these mutations affected imipenem uptake by resistant isolates. Furthermore, five new missense mutations were identified in imipenem-resistant isolates including G211R, E230K, S278V, I362F, and T370P. It seems that the new mutations were located in extracellular loops of OprD, decreased imipenem affinity to OprD, and as a result, decreased imipenem uptake.

Curcumin has a particle size of 500–800nm, which is impaired in cellular uptake and leads to low bioavailability (Praditya et al., 2019). Different methods to improve its stability and drug delivery were evaluated. Herein, we used curcumin loaded in diblock polymeric polymersomes, nanoparticles with spherical shape, an appropriate mean size of 259.5±1.5 nm, the acceptable polydispersity index of ~ 0.465, and the zeta potential of (-8.74±0.2)

(Pakizehkar et al., 2020b). One advantage of polymersomes was that curcumin loaded in polymersomes was easily dissolved in water and there was no need for DMSO as a cytotoxic solvent.

In the present study, four isolates of *P. aeruginosa* with different identified mutations in genes involved in resistance to ciprofloxacin and other antibiotics were treated with curcumin (loaded in polymersomes) and ciprofloxacin (subMIC). Our analysis revealed that ciprofloxacin (subMIC) induced death in isolates with different mutations in the presence of CPNs (400µg/mL) in a synergic manner, while this dose of ciprofloxacin could not induce death in the absence of CPNs. Kali *et al.* showed that curcumin had maximum synergy with ciprofloxacin in Gram-positive and with amikacin, gentamicin and cefepime in Gram-negative bacteria (Kali et al., 2016). Bahari *et al.* revealed that curcumin along with gentamicin and azithromycin can inhibit *P. aeruginosa* quorum sensing (Bahari et al., 2017). One study reported that curcumin (32mg/l) could change 8 isolates to ciprofloxacin-sensitive isolates from among 15 ciprofloxacin-resistant isolates (Kali et al., 2016). Rudrappa *et al.* found the MIC of curcumin for PAO1 strain to be 30 µg/mL (Rudrappa and Bais, 2008). Different concentrations of curcumin in different studies are unknown. In our study however, diversity of mutations in several genes involved in ciprofloxacin resistance may be affected by CPNs concentration.

Our quantitative analysis showed that the expression of *mexB* and *mexY* genes in isolates treated with CPNs and ciprofloxacin (subMIC) decreased compared to isolates treated with ciprofloxacin (subMIC) alone. In a study, Shariati *et al.* showed that *mexB* gene expression decreased after curcumin treatment in MDR isolates of *P. aeruginosa* and PAO1 (Tahmasebi Birgani et al., 2015). Arya et al. reported that vanillin capped gold nanoparticles (VAuNPs) downregulated *mexB* and *OprM* expression in clinical isolates of P. aeruginosa. One study revealed that VAuNPs reduced the MIC of meropenem tenfold and induced cell damage (Arya et al., 2019). Similar to the study of Arya et al., our analysis suggested that CPNs decreased the number of efflux pumps of mexAB-oprM and mexXY-oprM localized in bacterial membranes, partly through downregulation of *mexB* and *mexY*. Therefore, lower amount of ciprofloxacin, after uptake by bacterial cells in the presence of CPNs, was pumped out of cells due to the decreased localization of efflux pumps.

Conclusion

Overall, it seems that CPNs in ciprofloxacin resistant strains (with different mutated genes) can restore susceptibility to ciprofloxacin through 1) downregulation of some genes and 2) decreasing the localization of efflux pumps in cell membranes.

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Declarations

Authors' Contribution:

S.R.T. and N.R. contributed for the conception and design of this article. S.R.T., M.A., H.M.T., P.S.G. and N.R. contributed for analysis and interpretation. S.R.T. and N.R. were involved in statistical analysis. S.R.T. and N.R. contributed in writing and provided critical revision for this article. N. R. gave final approval for this article. All authors agree to be accountable for all aspects of the work.

Conflict of Interest: The authors declare no conflicts of interest.

Research Involving Human Participants and/or Animals: This work does not involve experiments using humans or other animals.

Informed Consent: Not applicable to this work.

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