Curcumin as Efflux Pump Inhibitor Agent for Enhancement Treatment Against Multidrug Resistant *Pseudomonas aeruginosa* Isolates

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**Abstract**

*Pseudomonas aeruginosa* is considered as a developing opportunistic nosocomial pathogen and is well-known for its multidrug resistance that can be efficiently treated by a combination of antibiotics and efflux pump inhibitors (EPI). Therefore, the purpose of this study was to investigate the effect of curcumin as an EPI for the enhancement of the effectiveness of antibiotics against multidrug resistant (MDR) isolates of *P. aeruginosa*. Susceptibility patterns of suspected bacteria were determined using the disc diffusion method and resistant bacteria were identified using chromogenic agar and 16S rDNA. The effects of curcumin on the enhancement of antibiotics’s activity was evaluated using the broth microdilution method. The susceptibility patterns for 50 (67.6%) suspected *P. aeruginosa* isolates showed that 36 (72%) of these isolates were resistant to one of the used antibiotics, whereas only 21 (42%) were MDR. The highest percentage of resistance was observed to ceftazidime (66%) followed by ciprofloxacin and levofloxacin (40%). Only 35 isolates were specified by chromogenic agar and 16S rDNAs as *P. aeruginosa*. The minimal inhibitory concentration (MIC) of 35 isolates for ciprofloxacin resistant was between 4 and 128 µg/ml while for ceftazidime was between 64 and 512 µg/ml. After the addition of 50 µg/ml curcumin with ciprofloxacin, there was a significant increase in the sensitivity (p ≤ 0.01) of 13 MDR *P. aeruginosa* isolates whereas no differences in the sensitivity to ceftazidime were recorded before and after addition of curcumin. In conclusion, the results of this study show that curcumin can decrease the MIC value of ciprofloxacin in MDR isolates of *P. aeruginosa* and can be used as a native compound to enhance the treatment of resistant isolates with ciprofloxacin.

**Keywords:** *P. aeruginosa*, MDR, ciprofloxacin, curcumin.
1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium regarded to cause opportunistic infections which are frequently health-care associated. P. aeruginosacan cause continual and lethal infections amongst immune-compromised individuals and outcomes in more than 11% of nosocomial infections[1]. A serious therapeutic challenge is presented by P. aeruginosaduring treatment of each of the nosocomial community-acquired infections, and the determination of excellent antibiotics to initiate remedy is an integral step to optimize the clinical results [2]. However, therapy of P. aeruginosainfections has become a great challenge due to the potential of this bacterium to withstand many of the currently available antibiotics. Carbapenem-resistant P. aeruginosahas been recently listed by the World Health Organization (WHO) as one of three bacterial species for which there is a crucial requirement for developing new antibiotics [3]. Moreover, excessive use of antibiotics during treatment accelerates the development of multidrug resistant (MDR) P. aeruginosa strains, leading to the ineffectiveness of the empirical antibiotic remedy towards this microorganism [4].

Overexpression of multidrug efflux pumps is one of the essential mechanisms of drug resistance in bacteria. The resistance-nodulation-cell division (RND) family is one of the most clinically relevant multidrug efflux systems in Gram negative bacteria [5]. P. aeruginosaperserves various RND-type multidrug efflux systems that share common substrates. They are also accountable for special phenotypes inherent to their expression. Substrates of these pumps include aromatic hydrocarbons, detergents, antibiotics, biocides, dyes, homoserine lactones, and organic solvents [6,7].

The efflux pumps’ inhibition seems to be a promising way to restore antibacterial potency. In recent years, a large number of EPIs have been discovered and tested, including synthetic molecules, antibiotics and natural products [8]. Pseudomonas aeruginosa is considered as a developing opportunistic nosocomial pathogen and is well-known for its multidrug resistance that can be efficiently treated by a combination of antibiotics with EPIs. A number of potent EPIs including quinoline derivatives, carbonyl cyanide m-chlorophenylhydrazone (CCCP), phenylalanylarginyl ß-naphthylamide (PAßN), and 1-(1-Naphthylmethyl)-piperazine (NMP) have been reported to enhance...
antibiotic impacts against antibiotic-resistant Gram-negative bacteria, but the toxic nature of these materialshampers their proceed into potential clinical applications [9]. A steroidal alkaloid compound, known as conessine, could act as an EPI to retain the antibiotic activityto P. aeruginosa by inhibiting efflux pump systems [10]. Anumber of phytochemical products and plant extracts have demonstrated their potential as synergists or potentiators of other antibacterial agents.

A phenolic compound, called curcumin, is derived from the rhizomes of the plant Curcuma longa. This compound has been studied and proved to exertConsiderable antimicrobial, anti-cancer, anti-inflammatory, and antioxidant properties [11]. Curcuminenhanced the impacts of antibiotics and, therefore, altered their susceptibility patterns which can be attributed to efflux pumps inhibition [12][13]. For this reason, the aim of this study is to investigate the effects of curcumin as an EPI that enhances the activity of antibiotics against the multidrug resistance of P. aeruginosa isolates.

2. Material and methods

1- Identification of bacterial isolates

Between September and December 2018, 74 isolates were collected from clinical specimens by laboratories at Al- Wasiti hospital and the Teaching Hospital of Baghdad Medical City. The isolates were cultured on MacConkey agar (Himedia, India) and then sub cultured on Cetrimide agar (Himedia, India). According to the results of disc diffusion test, the suspected MDR colonies were further identified using chromogenic agar (Himedia, India) and 16S rDNA gene.

2- Disk Diffusion

According to the Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2016), the disk diffusion method was determined on Muller-Hinton Agar medium using Gentamycin (10 µg/disc), Ceftazidine (30 µg/disc), Ciprofloxacin (10 µg/disc), Aztreonam (30 µg/disc), Amikacin (30 µg/disc), Levofloxacin (5 µg/disc), Piperacillin (100 µg/disc), and Imipenem (10 µg/disc). The plates were aerobically incubated overnight at 37 °C.

3- Identification of isolates using 16S rDNA

3.1- DNA extraction

The DNA of suspected MDR isolateswasextracted using Genomic DNA Mini Kit (Geneaid, Thailand) according to the instructions of manufacturer. The extracted DNA was stored at -20°C until used.

3.2- Amplification of 16S rDNA

Conventional PCR was performed to amplify the pass gene of 16S rDNA [14]. The reaction consisted of 5 µL PCR premix (i-taq) (Intron, Korea), 1 µL of forward primer (GGGGGATCTTCCGACCTCA), 1 µL of reverse primer (TCCTTAGAGTGGCCACCCC), and 100 ng/ µL of DNA template, while the volume was completed to 25 µL with D.W. The thermal programme was optimized and performed in a master cycler (Eppendorf, Germany) as in the follow subsequent steps: 2 min at 94 °C, 25 cycles of 25 sec at 94 °C, 25 sec at 56.4 °C as well as 40 sec at 72 °C, and a final elongation step at 72 °C for 5 min. Following gel electrophoresis, the isolates that showed 956 bp bands were considered as P. aeruginosa.

4- Curcumin extraction

Curcumin was extracted using Soxhlet and the rhizomes of turmeric were dried in an oven (105 °C, 3 h). Using a mortar, the dried rhizomes were triturated and a uniform powder with a particle size of 0.18 mm was obtained by screening through an 80 mesh size sieve. Ground turmeric powder (15 g) was measured, embedded in a thimble, and placed in the Soxhletwhich was gradually filled with acetone as the extraction solvent. The extraction experiment (60 °C, 8 h) was performed upon completion of the extraction. The acetone was separated from the extract using a rotary evaporator under vacuum at 35 °C. The residue (oleoresin) was dried and measured for further use.

5- High-Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) technique (Dionex, USA) with a UV detector (280 nm) was used for measuring curcumin concentration, according to published methods with some modifications [15]. The column was C18 with dimensions of 250x4.6 mm; the mobile phase composed of acetonitrile and water at ratio of 50/50; the flow rate was 1 ml/min at room temperature. The extracted powder for HPLC analysis, the standard curcumin with various concentrations of 1, 2.5, 8, 10 ppm, was intended and dissolved in grade 50% acetonitrile and then injected into the HPLC device. The standard calibration curve for curcumin was then developed. To determine the curcumin content of the unknown samples, a specific concentration of each sample (5 ppm) was made, passed through a 0.45 µm filter and injected into the
system. By using the peak area at specific retention times and the standard curve, the quantification of the extracted curcumin was performed. The amount of extracted curcumin (% w/w) from turmeric was measured as follows: The extracted solutions were also analyzed for presence of curcumin by UV-vis spectrophotometer (Dionex, USA).

6-Determination of Minimal Inhibitory Concentration (MIC)

The modified broth microdilution method [16] was used to determine the MIC in microtiter plates. Antimicrobial stock solutions were prepared from ceftazidime (1g) and ciprofloxacin (500mg). Then two-fold serial dilutions were prepared with nutrient broth (HiMedia/ India) and the bacterial inoculums were added at a final concentration of 1×10⁶ cell/ml. Bacteria and media control solutions were also prepared. The microtiterplates were incubated overnight at 37°C. This procedure was repeated with curcumin dissolved in dimethyl sulfoxide (DMSO) using different concentrations (5, 10, 15, 20, 25, 30, 50 µg/ml) to be mixed with the inoculums. The MIC was determined by recording the lowest concentration of antibiotics that prevents the growth of the microorganism.

7-Statistical analysis

The results are presented as mean ± standard deviation (SD). Non parametric analysis Kruskal Wallis Test was used for comparison between antibiotic resistance and to determine the differences between MIC values for the studied antibiotics. A pvalue of ≤ 0.05 was considered statistically significant. The statistical calculations were carried out using the IBM SPSS Statistics 23.0.

3. Results and discussion

The cultural characteristics of 74 isolates on Cetrimide media showed that 50 (67.6%) of the isolates had characteristics common for P. aeruginosa. Cetrimide agar medium was used for the selective isolation of P. aeruginosa; this medium was used for determining the ability of the bacterial isolates to grow in the presence of 0.03% cetrimide that acts as a quaternary ammonium cationic detergent (acetyl tri-methyl ammonium bromide) inhibiting the growth of other microorganisms by releasing N and P from microorganisms [17].

Furthermore, all bacterial isolates that gave distinctive greenish blue color of P. aeruginosa and grape-like odor on Cetrimide agar were tested to identify susceptibility patterns using the disc diffusion method.

The susceptibility patterns for 50 (67.6%) suspected P. aeruginosa isolates to four different antibiotics were: 36 isolates (72%) were resistant to one of the antibiotics, one isolate (2%) was resistant to all antibiotics, and 21 isolates (42%) were MDR, i.e. resistant to three or four antibiotics. The results demonstrated significant levels of resistance (p ≤ 0.01) as shown in Figure-1. The isolates exhibited highest percentage of resistance to ceftazidime (66%) followed by ciprofloxacin (40%), aztreonam (26%), gentamicin and piperacillin (16%), amikacin (12%) and imipenem (10%).

The highest resistance rate was found in ceftazidime 66%, in consistence with the rates of 69.2% and 54% reported by local studies carried out by Othman et al. [18] and Jean-Baptiste Rona et al. [19], respectively. However, the results obtained by Al-Zaidi [20] showed 100% resistance to this antibiotic. Also, the highest resistance rate of ciprofloxacin and levofloxacin in this study corresponded to the local results published by Abdulameer and Abdulhassan [21]. In some local studies, the resistance rate was slightly higher, as in the studies of Al-Zaidi [20] which showed a resistance rate of 44.4%.

The lowest percentage of resistance which was recorded here to imipenem (10%) in the studied P. aeruginosa isolates was also noticed in another study [22] while another local study showed higher resistance that reached to 35% [23].
Figure 1-The percentage of resistance, intermediate resistance and sensitivity of 50 *P. aeruginosa* isolates, for different antibiotics that include Ceftazidine (CAZ), ciprofloxacin (CIP), levofloxacín (LEV), Amikacin (AK), Gentamicin (GN), piperacillin (PRL), Aztreonam (ATM) and imipenem (IMP).

*P. aeruginosa* has the ability to develop resistance to antibacterials either through mutational processes that change the expression and/or function of chromosomally encoded mechanisms or through the acquisition of resistance genes on mobile genetic elements such as plasmids. Developing drug resistance by both strategies can severely limit the therapeutic options for remedy of serious infections [7].

The results showed that 35 (97.2%) from 36 resistant isolates exerted positive results, as shown with chromogenic agar after 24h. Chromogenic agar is a differential medium designed specifically for the isolation of *P. aeruginosa* (PS-ID). The medium is considered the first chromogenic medium to use a chromogenic substrate for peptidase activity. The substrate, β-alanyl pentylresorufamine, is hydrolyzed by a β-alanylaminopeptidase produced by *P. aeruginosa*, resulting in the formation of greenish pigment colonies [24].

Using PCR over thirty-six isolates, it was found that the bands of 35 (97.2%) isolates were positive for 16S rDNA gene and were identified as *P. aeruginosa* as shown in Figure 2, while 1 (2.8%) were negative. The results showed that 16S rDNA is a more specific method for the detection of *P. aeruginosa* than other methods, which is consistent with the results of Alttaai et al. [25].

Unlike phenotypic identification, which can be influenced by variability in expression of characters or by the presence or absence of non-housekeeping genes, 16S rDNA sequencing provides specific identification of isolates with atypical phenotypic characteristics [26]. Pass primers designed for 16S rDNA-based PCR assays provide rapid, simple, and reliable identification of *P. aeruginosa*, being able to differentiate this bacterium from other phylogenetically closely related *Pseudomonas* species [14].

Figure 2-Gel electrophoresis of amplified 16S rDNA (956bp) from *P. aeruginosa* using conventional PCR. Agarose 1.5%, 80 V/cm for 1hrs., stained with safe red dye and visualized on a UV transilluminator. Lane 1-14: Amplicons of 16S rDNA. M: 100 bp DNA ladder.
The HPLC technique was used for the detection of the quality of curcumin extraction under the conditions of stationary phase: pursuit XFs 3µ C-18 250 x4.6 mm, diameter particles 5µm, Varian mobile phase: acetonitrile:water 50:50. The flow rate was 1ml/min, the column temperature was set at 35 ºC and the UV wavelength at 280nm. Chromatogram of curcumin peak is shown in Figure-3. Under these conditions, the peak of curcumin was sharp and symmetrical, in addition to the lack of interference from diluents or impurities found in the curcumin sample at this wavelength.

Figure 3- HPLC chromatograms of curcumin extracted using Soxhlet extraction at 280 nm.

The yields of the extracted curcumin obtained by using Soxhlet were considerably higher than those obtained from other methods [15]. The most common method for the determination of curcumin in turmeric samples, biological samples, or dosage forms and curcuminoids is HPLC with UV detection[27][28].

Broth microdilution method was utilized to determine the MIC of P. aeruginosa isolates to ciprofloxacin and ceftazidime. Only 35 isolates were applied for this test, for which MIC values for ciprofloxacin resistant isolates were between 4-128 µg/ml while MIC values for ceftazidime resistant isolates were between 64-512 µg/ml.

The MIC of ciprofloxacin and ceftazidime were determined after adding different concentrations of curcumin. After adding of curcumin with ciprofloxacin, therewasa significant increase in the sensitivity (p≤ 0.01) of 13 MDR P. aeruginosa isolates upon the use of 50µg/ml of curcumin as shown in Figure-4, while lower concentrations caused lower reduction in MIC. None of the MDR isolates were susceptible to curcumin alone at any concentration, indicating its efflux pump inhibition activity. There were no differences in sensitivity of the studied isolates to ceftazidime before and after the addition of different curcumin concentrations.

Figure 4- Fold change of Ciprofloxacin MIC after adding Curcumin.
These results agreed with those of Eshra and Shalaby[13] who showed significant differences between the effects of gentamicin and ciprofloxacin when mixed with curcumin on *P. aeruginosa* isolates, whereas no differences were reported among carbencillin, meropenem, and ceftazidime mixed with curcumin.

An antibiotic in combination with an EPI can be used as an effective strategy for the remedy of multidrug resistant isolates of bacteria, being able to inhibit the antibiotic extrusion and restore the antibiotic efficacy to increase bacterial cell death [29]. Because of the broad-spectrum of the properties of curcumin and its safety at high doses (12 g/day in human) [11], researchers tend to consider curcumin as a potential new complementary drug to treat different diseases. Evidence has reported that curcumin acts as an EPI against MDR in vitro, and that the reduced MIC of several antibiotics against these isolates was due to efflux-pump inhibition [30]. The antibacterial mechanism of curcumin acts through the inhibition of polymerization of the essential prokaryotic cell division protein FtsZ, thus preventing cytokinesis [31].

It was found that curcumin encapsulated in micellar/polymersomenanoparticles and ciprofloxacin had anti-*P. aeruginosa* effects, partially through downregulation of mexXand oprM genes involved in efflux pumps, as well as by trapping ciprofloxacin on bacterial cells and increasing the effects of drug in treated cells compared to cells treated with ciprofloxacin alone [32]. Furthermore, other studies found that curcuminis an EPI against other bacteria, as in a study published by Joshi et al. [33] who showed a significant inhibition of *S. aureus* NorA efflux pump with 8- and 4-fold reductions in the MIC of ciprofloxacin when combined with curcumin. Also, the curcumin-colistin combination was more effective in reducing resistance of *Acinetobacter baumannii* due to the increased ROS production and efflux pump inhibition via curcumin-aided increased membrane permeability by colistin [34].

**Conclusion**

The results of this study showed that curcumin was able to reduce the MIC value of ciprofloxacin in MDR isolates of *P. aeruginosa* and can be used as a native compound to enhance the treatment of resistant isolates with ciprofloxacin.

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