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

Sulaiman D. Sulaiman, Ghusoon A. Abdulhasan*
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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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 andefflux 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 was determined using the disc diffusion method andresistant bacteria were identified using chromogenic agar and *16S rDNA*. The effectsof curcuminon the enhancement of antibiotics's activity was evaluated usingthe broth microdilution method.The susceptibility patterns for 50 (67.6%) suspected*P. aeruginosa*isolates showed that 36 (72%) of these isolateswere resistant to one of the used antibiotics,whereasonly 21 (42%) were MDR. The highest percentage of resistance was observedtoceftazidime (66%) followed by ciprofloxacin and levofloxacin (40%)**.** Only 35 isolates were specified by chromogenic agar and *16S rDNA*as *P. aeruginosa.*The minimal inhibitory concentration (MIC) of 35 isolates for ciprofloxacin resistant was between 4 and128 µg/ml while for ceftazidime was between 64and 512 µg/ml. After the addition of 50 µg/ml curcumin with ciprofloxacin, there wasa significant increase in the sensitivity ($p \leq 0.01$) of 13 MDR *P.aeroginosa* isolates whereas no differences in the sensitivity to ceftazidime were recorded before and after addition ofcurcumin. 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.aeroginosa*,MDR, ciprofloxacin, curcumin.

الكركمين كعامل مثبط مضخة التدفق لتحسين العلاج ضد عزلات *Pseudomonas aeruginosa* المقاومة للادوية المتعددة

سليمان داود سليمان ، غصون علي عبد الحسن*
قسم علوم الحياة، كليةالعلوم، جامعة بغداد، بغداد، العراق

*Email: ghusoona@scbaghdad.edu.iq

الخلاصة

تعتبر بكتريا *Pseudomonas aeruginosa* من مسببات الأمراض الانتهازية وهي معروفة بمقاومتها للأدوية المتعددة والتي يمكن معالجتها بكفاءة من خلال مزيج من المضادات الحيوية مع مثبط مضخة التدفق (EPI). لذلك ، كان الغرض من هذه الدراسة هو التحقيق في تأثير الكركمين كمثبط لمضخة التدفق لتعزيز فعالية المضادات الحيوية ضد عزلات بكتريا *P. aeruginosa* المقاومة للأدوية المتعددة. تم تحديد نمط الحساسية للبكتيريا باستخدام طريقة نشر القرص ثم شخصت البكتيريا المقاومة باستخدام أجار chromogenic و *16S rDNA*. وقد تم التحقيق في تأثير الكركمين على تعزيز نشاط المضادات الحيوية بأستخد طريقة microdilution مرق. تم عزل 50 عزلة (67.6٪) من البكتريا المشتبه بها كعزلات *P. aeruginosa* من خلال طريقة نشر القرص و36 (72٪) عزلة كانت مقاومة لأحد المضادات الحيوية المستخدمة بينما21 (42٪) عزلة فقط كانتمقاومة للأدوية المتعددة. وأظهرت أعلى نسبة من المقاومة لمضاد السيفتازيديم (66٪) تليها سيبروفلوكساسين والليفوفلوكساسين (40٪). بأستخدام أجار chromogenic و *16S rDNA* شخصت35 عزلة على انها *P. aeruginosa*. اظهرت نتائجMICان 35 عزلة مقاومة للسيبروفلوكساسين يتراوح بين 4−128 ميكروغرام/مل بينما كان للسيفتازيديم ما بين 64−512 ميكروغرام/مل. بعد إضافة 50 ميكروغرام/مل من الكركمين مع سيبروفلوكساسين ، لوحظ زيادة كبيرة في حساسية ($p\leq 0.01$) 13 عزلة من بكتريا *P.aeroginosa* المقاومة للأدوية المتعددةبينما لا توجد اختلافات في حساسية العزلات لمضادالسيفتازيديم قبل وبعد إضافة الكركمين. في الختام ، أظهرت نتائج هذه الدراسة أن الكركمين يمكن أن يخفض قيمة MIC للسيبروفلوكساسين في عزلات *P. aeruginosa* المقاومة للأدوية المتعددة ويمكن استخدامه كمركب أصلي لتعزيز علاج العزلات المقاومة مع سيبروفلوكساسين

1.Introduction

*Pseudomonas aeruginosa*is a Gram-negative bacterium regarded to cause opportunistic infections which are frequently health-care associated. *P. aeruginosa*can cause continual and lethal infections amongst immune-compromised individuals and outcomes in more than 11% of nosocomial infections[1]. A serious therapeutic challengeis presented by *P. aeruginosa* duringtreatment of eachof the nosocomialand 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. aeruginosa*infections hasbecome a great challenge due to the potential of this bacterium to withstand many of the currently availableantibiotics. Carbapenem-resistant *P. aeruginosa*has been recently listed by the World Health Organization (WHO) as one of three bacterial species forwhich there is a crucial requirement for developing new antibiotics [3]. Moreover, excessive use of antibiotics during treatment acceleratesthe 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 relevantmultidrug efflux systems in Gram negative bacteria [5].*P.aeruginosa*expressesvarious RND-type multidrug efflux systems that share commonsubstrates.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 beefficiently treated by a combination of antibiotics with EPIs. A number of potent EPIs including quinoline derivatives, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), phenylalanylarginyl β-naphthylamide (PAβN), 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,alteredtheir susceptibility patterns which can be attributed to efflux pumps inhibition [12][13]. For this reason, the aim of this studyis to investigate the effects of curcumin as an EPI that enhances the activity of antibiotics against the multidrug resistanceof *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 werecultured on MacConkey agar (Himedia, India) andthen sub cultured on Cetrimide agar (Himedia, India).According to the results of disc diffusion test, thesuspected 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 mediumusing Gentamycin (10µg/disc), Ceftazidime (30 µg/disc), Ciprofloxacin (10µg/disc),Aztreonam(30 µg/disc), Amikacin (30 µg/disc), Levofloxacin (5µg/disc), Piperacillin (100µg/disc),andImipenem (10µg/disc). The plates wereaerobically 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 -20ºC until used.

3.2-Amplification of *16S rDNA*

Conventional PCR was performed to amplify the pass gene of *16S rDNA*[14]. The reaction consisted of5 µl PCR premix (i-taq) (Intron, Korea), 1 µlof forward primer (GGGGGATCTTCGGACCTCA), 1 µl of reverse primer (TCCTTAGAGTGCCCACCCG), and100 ng/ µl of DNA template, while thevolume 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: 2min at 94 ºC, 25 cycles of 25sec at 94 ºC, 25 sec at 56.4 ºC as well as 40 sec at 72 ºC,and afinal elongation step at 72 ºC for 5 min.Followinggel electrophoresis, theisolates that showed 956 bpbands were considered as *P. aeruginosa*.

4-Curcumin extraction

Curcumin was extracted using Soxhlet andthe 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 screeningthrough an 80 mesh size sieve. Ground turmeric powder (15g) was measured, embedded in a thimble, and placedin the Soxhletwhich was gradually filled with acetone as the extraction solvent. The extraction experiment (60 °C, 8 h) was performedupon completion of the extraction. The acetone was separatedfrom 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 UVdetector (280 nm) was used for measuringcurcumin concentration, according to published methods with some modifications[15]. The column was C18 with dimensions of 250×4.6 mm; the mobile phase composed of acetonitrile and water at ratio of 50/50; the flow rate was 1.0 ml/min at room temperature. The extracted powderfor HPLC analysis, the standard curcumin with various concentrations of 1, 2.5, 8, 10 ppm, was intented and dissolved in grade 50% acetonitrile and then injected into the HPLC device. The standardcalibration 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 areaat 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 1×10^6 cell/ml . Bacteria and media control solutions were also prepared. The microtiterplates were incubated overnight at 37ºC. This procedure was repeated with curcumindissolved 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 recordingthe 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 calculationswere 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 bacterialisolates 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 isolatesthat 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*isolatestofour different antibioticfamiliesshowed that 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 demonstratedsignificant levels of resistance ($p\leq 0.01$) as shown in Figure-1. The isolates exhibited highest percentage of resistance to ceftazidime (66%) followed by ciprofloxacin and levofloxacin (40%), aztreonam (26%), gentamicin and piperacillin (16%), amikacin (12%) and imepenim (10%).

The highest resistancerate was found in ceftazidime 66%,in consistence with the rates of69.2% 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]showed100% 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 wasslightly 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].

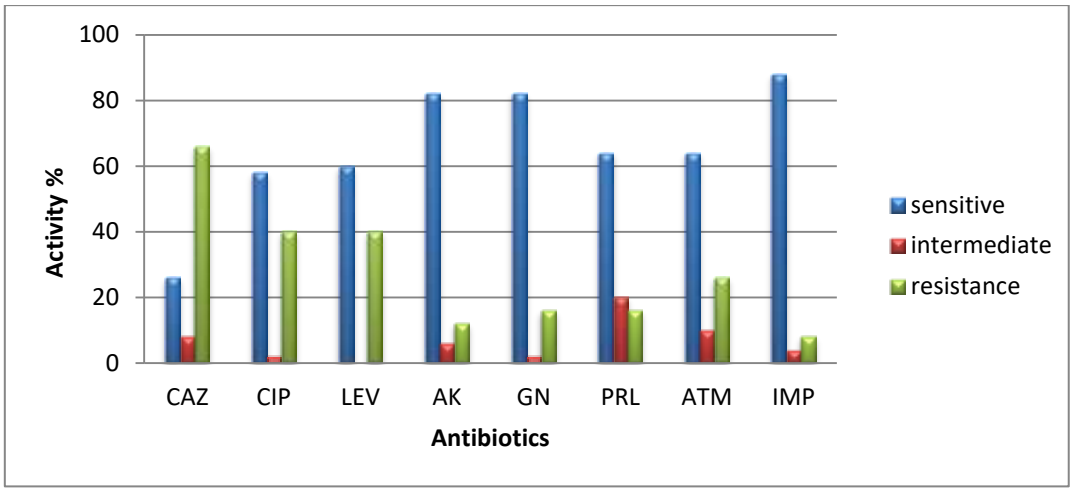


Figure 1-The percentage of resistance, intermediate resistance and sensitivity of 50 *P. aeruginosa*isolates, for different antibiotics that include Ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV),Amikacin (AK), Gentamicin (GN), piperacillin (PRL), Aztreonam (ATM) and imipenem (IMP).

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 byboth strategies can severely limit the therapeutic options for remedy of serious infections [7].

The results showed that 35(97.2%) from 36 resistantisolates exertedpositive 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 consideredas the first chromogenic medium to use a chromogenic substrate for peptidase activity. Thesubstrate, β-alanylpentylresorufamine, is hydrolyzed by a -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 iswas consistent with the results of ofAlttaai*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].

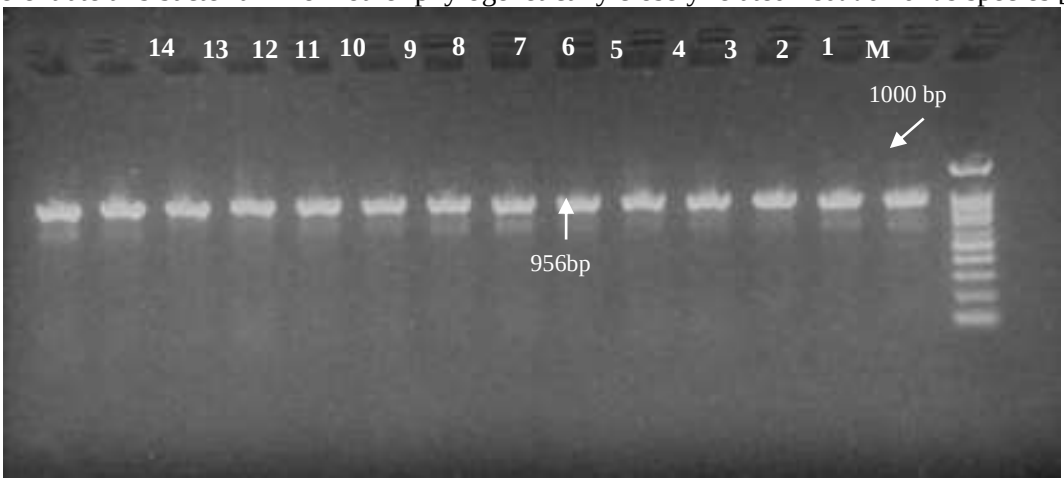
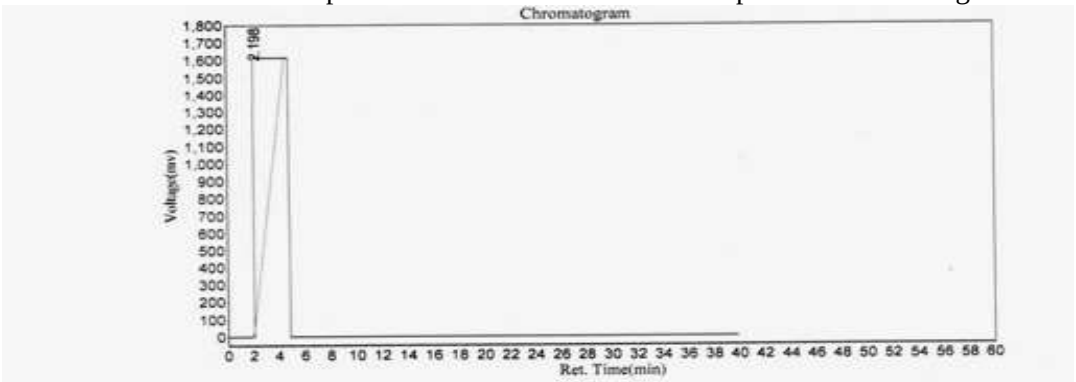


Figure 2-Gel electrophoresis of amplified *16S rDNA* (956bp) from *P. aeruginosa* using conventional PCR. Agarose 1.5%, 80 V/cm for 1hrs., stained with safe red dye and visualized on a UV transilluminator.Lane 1-14: Amplicons of *16S rDNA*. M: 100 bp DNA ladder.

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 ×4.6 mm, diameter particles 5µm, Varian mobile phase: acetonitrile:water 50:50. The flow rate was 1ml/min, the column temperature was set at35 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 ofinterference from diluents orimpurities found in the curcumin sample at this wavelength.



Results

Peak No.	Peak ID	Ret Time	Height	Area	Conc
1		2.198	1461558.500	115991208.000	100.0000
Total			1461558.500	115991208.000	100.000

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

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

Brothmicrodilution method was utilized to determine the MIC of *P.aeroginosa* isolates to ciprofloxacin and ceftazidime.Only 35 isolates were applied for this test, for whichMIC values for ciprofloxacin resistant isolates were between 4-128 µg/ml while MIC vales for ceftazidime resistant isolates were between 64-512 µg/ml.

The MIC of ciprofloxacin and ceftazidimewere determined after addingdifferent concentrations ofcurcumin. After adding of curcumin with ciprofloxacin, therewasa significantincrease in the sensitivity ($p \leq 0.01$) of 13MDR *P.aeroginosa*isolates upon the use of50µg/ml of curcumin as shown in Figure-4, while lower concentrationscaused lower reduction inMIC.None of the MDR isolates were susceptible tocurcumin alone at any concentration, indicating its efflux pump inhibitionactivity. There were nodifferences in sensitivity of the studied isolates to ceftazidime before and after the addition of different curcuminconcentrations.

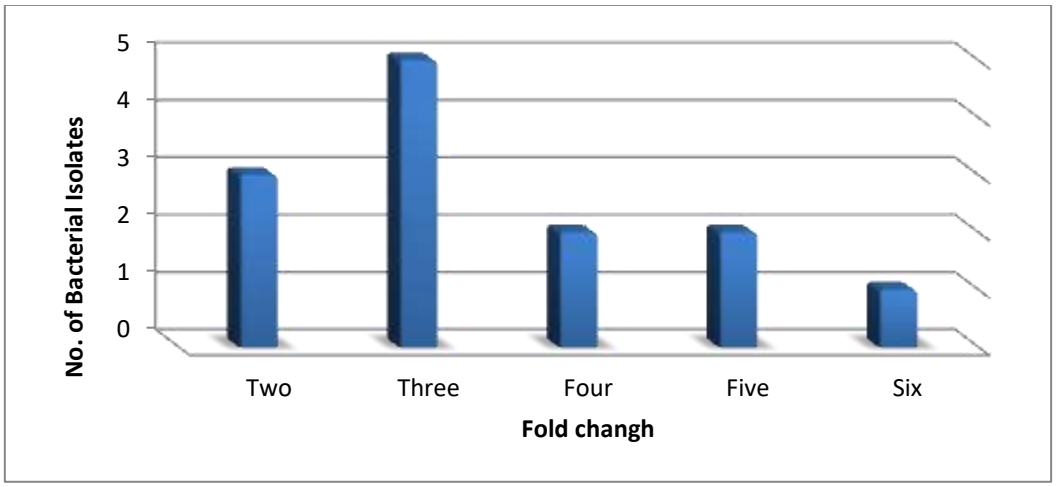


Figure 4-Fold change of Ciprofloxacin MIC after adding Curcumin.

These results agreed with those ofEshra and Shalaby[13]who showed significant differences between the effects ofgentamicin and ciprofloxacin when mixed with curcumin on *P. aeruginosa* isolates,whereas no differences were reportedamongcarbenicillin, 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 toinhibit the antibiotic extrusion and restore the antibiotic efficacy to increase bacterial cell death [29].Because of the broad-spectrum of the properties of curcuminand its safety at high doses (12 g/day in human)[11],researchers tend to consider curcumin as a potential newcomplementary drug to treat different diseases. Evidence has reported that curcuminactsas an EPIagainst MDR*in vitro*, and thatthe reducedMIC of several antibiotics against these isolates was due to efflux-pump inhibition [30].The antibacterial mechanism ofcurcuminactsthrough the inhibition of polymerization of the essential prokaryoticcell division proteinFtsZ, thus preventing cytokinesis[31].

It was found that curcumin encapsulated in micellar/polymersomenanoparticles and ciprofloxacin had anti-*P. aeruginosa*effects, partially through downregulation of *mexX*and *oprM*genes involved in efflux pumps, as well as bytrapping 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 anEPI 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 combinedwith curcumin. Also, the curcumin-colistin combination was more effective in reducing resistanceof *Acinetobacterbaumannii* 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.

References

1. Driscoll, J.A., Brody, S.L. and Kollef, M.H. **2007**. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs*. Springer, **67**(3): 351-68.
2. Micek, S. T., Lloyd, A.E., Ritchie,D. J.,Reichley,R. M., Fraser,V. J. and Kollef, M. H. **2005**. *Pseudomonas aeruginosa* bloodstream infection: importance of appropriate initial antimicrobial treatment. *Antimicrob. Agents Chemother*, **49**: 1306–1311.
3. Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E.M., Houchens, C.R., Grayson, M.L., Hansen, P., Singh, N., Theuretzbacher, U., Magrini, N.; WHO Pathogens Priority List Working Group.**2018**. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis.*, **18**(3): 318-327.
4. Hirsch, E.B. and Tam, V.H. **2010**. Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Rev Pharmacoecon Outcomes Res*, **10**: 441-51.
5. Poole, K. **2004**. Resistance to beta-lactam antibiotics. *Cell Mol Life Sci,* **61**: 2200-23
6. Piddock, L.J. **2006**. Multidrug-resistance efflux pumps - not just for resistance.*Nat Rev Microbiol.*, **4**(8): 629-36.
7. Lister, D., Wolter, D. and Hanson, N. **2009**. Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clinical Microbiology Reviews*, **22**(4): 582–610.
8. Wang, Y., Venter, H. and Ma. S. **2016**. Efflux Pump Inhibitors: A Novel Approach to Combat Efflux-Mediated Drug Resistance in Bacteria. *Curr Drug Targets.*, **17**(6): 702-19.
9. Lomovskaya, O. and Bostian, K.A. **2006**. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. *Biochem Pharmacol*, **71**: 910–918.
10. Siriyong, T., Srimanote, P., Chusri, S., Yingyongnarongkul, B.Suaisom, C., Tipmanee, V. and Voravuthikunchai, C.P. **2017**. Conessine as a novel inhibitor of multidrug efflux pump systems in *Pseudomonas aeruginosa*. *BMC Complement Altern Med*, **17**: 405.

11. Moghadamtousi, S.Z., Kadir, H.A., Hassandarvish, P., Tajik, H., Abubakar, S., Zandi, K. **2014**. A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed Res Int*, **2014**: 18686
12. Negi, N., Prakash, P., Gupta, M.L. and Mohapatra, T.M. **2014**. Possible role of curcumin as an efflux pump inhibitor in multi drug resistant clinical isolates of *Pseudomonas aeruginosa*. *J ClinDiagn Res*,, **8**: DC04–DC07.
13. Eshra, K. A. and Shalaby, M. M. **2017**. Efflux Pump Inhibition effect of Curcumin and Phenylalanine Arginyl β-Naphthylamide (PAβN) against Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Burn Infections in Tanta University Hospitals.*Egyptian Journal of Medical Microbiology*, **26**(1): 113-119.
14. Spilker, T., Coenye, T. and Vandamme, P. **2004**. PCR-Based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas*species recovered from Cystic Fibrosis patients. *Journal of Clinical Microbiology*, **42**(5): 2074–2079.
15. Sahne, F., Mohammadi,M., Najafpour, G.D. and Moghadamnia. A.A. **2016**. Extraction of bioactive compound curcumin from turmeric (*Curcumalonga* L.) via different routs: a comparative study.*Pak.J. Biotechnol*, **3**(3): 173 -180.
16. Wiegand I1, Hilpert K, Hancock RE.**2008.** Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc.* **3**(2): 163-75.
17. Tang, Y. W. and Stratton, C. W. **2006**. *Advanced Techniques in Diagnostic Microbiology*. Springer Science and Business Media, USA.
18. Othman, N., Babakir-Mina, M., Chia, K. N. and Rashid, P. Y. **2014**. *Pseudomonas aeruginosa* infection in burn patients in Sulaimaniyah, Iraq: risk factors and antibiotic resistance rates. *J. Infect. Dev. Ctries*, **8**(11): 1498-1502.
19. Ronat, J.,Kakol, J., Khoury, M.N., Berthelot, M., Yun, O., Brown, V. and Murphy R.A. **2014**. Highly Drug-Resistant Pathogens Implicated in Burn- Associated Bacteremia in an Iraqi Burn Care Unit. *PLOS ONE*; **9**(8): e101017.
20. Al-Zaidi, J. R. **2016**. Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* isolated from clinical and hospital environmental samples in Nasiriyah, Iraq. *African journal of microbiology research*, **10**(23): 844-849.
21. Abdulameer, H.H,Abdulhassan, G.A. **2018**. In vitro susceptibility comparison among different fluoroquinolone generations using Pseudomonas aeruginosa. *Current Research in Microbiology and Biotechnology*, **6**(3): 1622-1625
22. Al-Charrakh A. H., Al-Awadi, S.J. and Mohammed, A.S. **2016**. Detection of Metallo-β-Lactamase Producing *Pseudomonas aeruginosa* Isolatedfrom Public and Private Hospitals in Baghdad, Iraq.*ActaMedicaIranica*, **54**(2): 107-113.
23. Hussein, Z.K., Kadhim, H.S. and Hassan J.S. **2018**. Detection of New Delhi Metallo-Beta-Lactamase-1 (blaNDM-1) in Carbapenem-Resistant *Pseudomonas aeruginosa*Isolated from Clinical Samples in Wasit Hospitals. *Iraqi JMS*, **16**(3): 239-246.
24. Lainea, L., Perrya, J. D., Leea, J.,Olivera, M., Jamesb, A.L., De La Foatac, D., Halimic, D., Orengac, S., Gallowaya, A. and Goulda, F.K. **2009**. A novel chromogenic medium for isolation of*Pseudomonas aeruginosa*from the sputa of cystic fibrosis patients. *Journal of Cystic Fibrosis*, **8**: 143-149.
25. Altaai, M.E., Aziz, I.H. and Marhoon, A.A. **2014**. Identification *Pseudomonas aeruginosa* by *16s rRNA* gene for Differentiation from Other *Pseudomonas* Species that isolated from Patients and environment. *Baghdad Science Journal*, **11**(2): 1028-1034.
26. Woo, P.C., Lau, S.K., Teng, J.L., Tse, H. and Yuen, K.Y. **2008**. Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *ClinMicrobiol Infect*.**14**(10): 908-34.
27. Koop, H., de Freitas, R., de Souza, L., Martinez, G. J. and Silveira, M. **2013**. Development and validation of a RP-HPLC–PDA method for determination of curcuminoids in microemulsions. *Chroma*, **76**(15–16): 1041-1048.
28. Syed, H.K., Liew, K.B, Loh, G.O.K. and Peh, K.K. **2015**. Stability indicating HPLC–UV method for detection of curcumin in *Curcuma longa* extract and emulsion formulation.*Food Chem.*, **170**: 321-326.

29. Kourtesi, C., Ball, A.R., Huang, Y.Y., Jachak, S.M., Vera, D.M., Khondkar, P., Gibbons, S., Hamblin, M.R. and Tegos, G.P. **2013** .Microbial efflux systems and inhibitors: approaches to drug discovery and the challenge of clinical implementation. *Open Microbiol J*, **7**: 34-52.
30. Negi, N., Prakash, P., Gupta, M. L. and Mohapatra, T. M. **2014**. Possible role of curcumin as an efflux pump inhibitor in multi-drug resistant *Pseudomonas aeruginosa*. *J ClinDiag Res*, **8**(10): DC04–DC07.
31. Rai, D., Singh, J.K., Roy, N. and Panda, D. **2008**. Curcumin inhibits FtsZassembly: an attractive mechanism for its antibacterial activity .*BiochemJ*, **410**: 147-155.
32. Rahbar Takrami, S., Ranji, N. and Sadeghizadeh, M. **2019.** Antibacterial effects of curcumin encapsulated in nanoparticles on clinical isolates of *Pseudomonas aeruginosa* through downregulation of efflux pumps.*MolBiol Rep*, **46**(2): 2395-2404.
33. Joshi, P., Singh, S., Wani, A., Sharma, S., Jain, S.K. and Singh, B. **2014**. Osthol and curcumin as inhibitors of human Pgp and multidrug efflux pumps of Staphylococcus aureus: Reversing the resistance against frontline antibacterial drugs. *Med Chem Commun*., **5**: 1540–1547.
34. Kaur, A., Sharma, P.,Capalash, N. **2018.** Curcumin alleviates persistence of *Acinetobacterbaumannii* against colistin. *Scientific Reports*, **8**: 11029.