

Effects of Curcumin on Biofilm Production and Associated Gene in Multidrug-Resistant *Acinetobacter baumannii* Isolated from Hospitalized Patients

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Article type: ABSTRACT

Original Article

Multi-drug-resistant (MDR) *Acinetobacter baumannii* has become a major global healthcare concern due to its opportunistic infections and high antibiotic resistance. This investigation is intended to investigate curcumin's potential anti-bacterial and antibiofilm impacts on MDR *A. baumannii* and to present a promising strategy for fighting against infections caused by this pathogen. This cross-sectional investigation comprised 34 MDR *A. baumannii* clinical isolates. The Kirby-Bauer disc diffusion method evaluated the sensitivity of isolates to multifaceted anti-bacterial agents. The microdilution broth method quantified curcumin's minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The efficacy of curcumin in inhibiting MDR *A. baumannii* biofilm was assessed via 96-well microtiter plates. The expression of the biofilm-associated protein (*bap*) gene was evaluated by employing quantitative real-time PCR (qRT-PCR). Within the 34 MDR *A. baumannii* isolates, the highest resistance was noted for trimethoprim/sulfamethoxazole and ciprofloxacin, with all 34 isolates (100%) indicating resistance. The lowest resistance was noted for ampicillin/sulbactam, with 22 isolates (64.7%) exhibiting resistance. The MICs of curcumin ranged from 0.625 to 2.5 mg/ml, while the MBCs varied between 1.25 to 5 mg/ml. Curcumin reduced biofilm formation by 25% to 91%, depending on the concentration. In contrast to the untreated control, the average relative activity of the *bap* gene in MDR *A. baumannii* isolates declined by 62.07%. The findings indicate that curcumin demonstrates antimicrobial and anti-biofilm activities against MDR *A. baumannii*. The downregulation noted in the *bap* gene further supports the curcumin's anti-biofilm impact.

Received:

2024.12.01

Revised:

2024.12.15

Accepted:

2024.12.23

Keywords: *Acinetobacter baumannii*, Multidrug-resistance, Curcumin, Biofilm

Cite this article: Javadi K, *et al.* Effects of Curcumin on Biofilm Production and Associated Gene in Multidrug-Resistant *Acinetobacter baumannii* Isolated from Hospitalized Patients. *International Journal of Molecular and Cellular Medicine*. 2025; 14(1):567-575. DOI: 10.22088/IJMCM.BUMS.14.1.567

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Publisher: Babol University of Medical Sciences

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Introduction

Acinetobacter baumannii (*A. baumannii*) is a gram-negative, aerobic, rod-shaped, non-motile bacterium (1). Multi-drug resistant (MDR) *A. baumannii* has emerged as a pathogen to cause highly opportunistic infections in healthcare facilities, worldwide (2). *A. baumannii* is the causative agent of many nosocomial infections characterized by notable morbidity and death rates. These infections include pneumonia, surgical site infections, bloodstream infections, heart valve infections, urinary tract infections, and secondary meningitis (3). *A. baumannii* is classified as a "critical" pathogen on the WHO priority pathogen list, highlighting its notable importance as a nosocomial infection (4). This pathogen tends to acquire diverse antibiotic resistance factors and can form biofilms. These two qualities remarkably contribute to therapy failure for infections caused by this bacterium (5).

Acinetobacter demonstrates resilience under adverse conditions such as desiccation, antimicrobial treatments, and nutritional scarcity. Its capacity to form intricate biofilms enables it to persist on biotic and abiotic surfaces for extended durations (6). Biofilms are intricate and interconnected communities of microorganisms that adhere to surfaces employing self-generated polymer matrices, consisting mainly of polysaccharides, secreted proteins, and extracellular DNA (7). Some bacteria use the capability to form biofilm as a resistance mechanism to survive in the presence of antibiotics. In this condition, the bacteria resist antibiotics up to 1,000 times more than those in a planktonic form (8). The biofilm formation process is intricate and subjected to regulation by many variables. For instance, in the case of *A. baumannii*, biofilm-associated protein (Bap) is essential for its initial attachment, facilitating its maturation and preserving its structural integrity in a mature form (9).

Curcuma longa, a plant of medicinal significance, belongs botanically to the Zingiberaceae family (10). This plant, colloquially known as 'turmeric,' is a prevalent spice and coloring agent renowned for its therapeutic properties (11). Curcuminoids, which are turmeric's active components, mainly comprise curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin (12). The most important of them, curcumin, gives turmeric its biological properties (13). Recent research has indicated that curcumin has multifaceted biological actions, including potent suppression of nuclear factor-kappa B and antioxidant and anti-bacterial, anticancer, and anti-inflammatory qualities (14, 15).

Controlling the production of bacterial biofilms is believed to be one of the most essential countermeasures against bacterial infections. As a result, attempts were made to find impactful inhibitors that can disrupt biofilm formation (16). However, whether there is a quantitative correlation within biofilm formation and antimicrobial resistance is still unclear. This investigation intended to evaluate the impacts of curcumin on the anti-bacterial activity and biofilm formation capacity of clinical isolates of MDR *A. baumannii*, as well as the expression levels of the gene associated with biofilm formation.

Materials and methods

Bacterial isolation

The study design was approved by the Ethics Committee of Shahed University (Approval ID: IR.SHAHED.REC.1402.075), and followed the statements of the Declaration of Helsinki. This cross-sectional investigation comprised 34 MDR *A. baumannii* clinical isolates collected from Hospital laboratories

affiliated with Babol University of Medical Sciences, Babol, Iran, within October 2023 and April 2024. These isolates were identified employing standard biochemical tests and confirmed by amplifying the *16S rRNA* gene (17).

Antimicrobial sensitivity evaluation

The Kirby-Bauer disc diffusion method evaluated the sensitivity of isolates to multifaceted anti-bacterial agents (18). The anti-bacterial agents evaluated comprised ampicillin/sulbactam, meropenem, gentamicin, amikacin, cefepime, trimethoprim/sulfamethoxazole, and ciprofloxacin. The test was performed on Mueller Hinton agar (MHA) employing disks from Padtan Teb Co., Iran. The findings were interpreted per the suggested protocols by the Clinical and Laboratory Standards Institute (CLSI 2024)(19). *Escherichia coli* ATCC25922 was employed as a quality control strain in the context of antimicrobial sensitivity evaluation.

Preparation of curcumin

Sigma Company (St. Louis, MO, USA) provided the curcumin component. Stock solutions were made by dissolving 100 mg of curcumin in 1 ml of dimethyl sulfoxide (DMSO), and they were kept at -20 °C until needed. The stock solutions were diluted with sterile Milli-Q water to form suspensions, which were then utilized for further evaluation.

Determination of the MIC and MBC of curcumin

MIC determination was performed following the guidelines of the CLSI 2024 utilizing the broth microdilution method, as previously explained (19). In summary, several colonies of MDR *A. baumannii* with an optical density (OD) of 0.4 at 600 nm (10^6 CFU/ml) were incorporated into a Mueller Hinton broth (MHB). Serial twofold dilutions of curcumin were prepared in triplicate, and the dilutions ranged from the highest concentration of 10 mg/ml to the lowest concentration of 0.625 mg/ml (10, 5, 2.5, 1.25, 0.625 mg/ml). This mixture was incorporated into each well on a microtitre plate and incubated for 24 hours at 37°C. MIC was quantified as the lowest concentration at which there was complete suppression of observable growth of the bacterial pathogens.

To ascertain the MBC of curcumin against MDR *A. baumannii*, 10 µL of the MHB from the wells where no bacterial growth was seen was transferred onto an MHA plate. The plates were placed in an incubator at 37 °C for 24 hours. The MBC was quantified as the minimum concentration at which bacterial growth was absent.

Biofilm formation inhibition assay

The inhibitory efficacy of curcumin against MDR *A. baumannii* biofilm was examined via 96-well microtiter plates, following the previously published protocol (20). Overnight cultures of MDR *A. baumannii* clinical isolates were diluted with LB broth and adjusted to a cell density of 0.5 Macfarland (1.5×10^8 CFU/ml). Curcumin was prepared in LB broth at 1/8 MIC, 1/4 MIC, 1/2 MIC, and 1/1 MIC. Next, LB medium with and without curcumin was inoculated with 100 µl of diluted culture, well mixed, and 200 µl was placed in each well with duplicates.

Following a 24-hour incubation at 37 °C, the planktonic cells were carefully removed from the wells, which were subsequently rinsed three times with sterile deionized water (DIW). Adherent cells were fixed using 100 µL of 99% methanol for 20 minutes, then stained with 100 µL of 1% crystal violet for 20 minutes.

Excess crystal violet was washed away with DIW in three cycles, and the remaining biofilm was solubilized using 125 μ L of 33% glacial acetic acid. The OD at 570 nm was determined with a microplate reader (Bio-Rad, USA), employing *A. baumannii* ATCC19606 as the positive control for biofilm formation.

The biofilm inhibition calculation was conducted using the following formula:

$$\text{Biofilm inhibition (\%)} = [(\text{Control OD} - \text{Test OD}) / \text{Control OD}] \times 100.$$

Quantitative real-time PCR analysis of *bap* gene expression

MDR *A. baumannii* isolates were cultured in LB broth supplemented with curcumin at a sub-MIC (1/2 MIC) concentration to evaluate curcumin's effect on the *bap* gene's expression. The cultures were incubated at 37 °C for 18 hours under agitation at 80 rpm to reach the late exponential growth phase. Total RNA was isolated following the protocol provided by the bacterial RNA extraction kit (Viragene, Iran).

RNA concentration was quantified employing a NanoDrop (Thermo Scientific, USA). Afterward, a cDNA Reverse Transcription kit (Viragene, Iran) was utilized to synthesize single-strand cDNA.

Real-time PCR was performed employing a Rotor-Gene Q Real-Time PCR machine (QIAGEN GmbH, Germany). The reaction was set as 4 μ L of cDNA, 0.5 μ L of the specific primers (Table 1), 10 μ L of 2X SYBR Green qPCR master mixes (SMOBiO Technology Inc., Taiwan), and 5 μ L of RNase/DNAase free water. The gene expression level was normalized to the housekeeping gene *16S rRNA*, and the comparative expression was calculated as $2^{-\Delta\Delta CT}$ (9, 21).

Table 1. Primers used in this study.

Primer	Primer sequence	Product Size
bap	Forward: 5'- TAGACGCAATGGATAACG -3'	127 bp
	Reverse: 5'- TTAGAACCGATAACGATACC -3'	
16s rRNA	Forward: 5'- ACTCCTACGGGAGGCAGCAGT -3'	151 bp
	Reverse: 5'- TATTACCGCGGCTGCTGGC -3'	

Statistical analysis

The experiments were conducted in duplicates, and the results were presented as the mean \pm standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism software (v8.4). t-tests were utilized to determine significant differences between curcumin-treated and untreated groups, with a significance threshold set at $P < 0.05$.

Results

Bacterial identification

The bacterial identification was confirmed using standard biochemical tests, including catalase-positive and oxidase-negative reactions, an alkaline/alkaline (ALK/ALK) reaction on Triple Sugar Iron (TSI) agar, and molecular assays targeting the *16S rRNA* gene. Thirty-four isolates of MDR *A. baumannii* were collected from multifaceted clinical specimens. The specimens comprised blood (11 isolates, 32.35% of the total), urine (9 isolates, 26.47%), sputum (8 isolates, 23.52%), trachea (4 isolates, 11.76%), and wound (3 isolates, 8.82%).

Antibiotic sensitivity test

The antimicrobial sensitivity profiles of 34 MDR *A. baumannii* isolates against seven antimicrobial discs are indicated in Table 2. All isolates indicated resistance to trimethoprim/sulfamethoxazole and ciprofloxacin. Furthermore, the lowest resistance was recorded with ampicillin/sulbactam, with 22 isolates (64.7%) indicating sensitivity.

Table 2. Antimicrobial susceptibility profiles of 34 MDR *A. baumannii* isolates

Antimicrobial category	Antibiotic	Susceptible (%)	Intermediate(%)	Resistant (%)
Penicillins+ β -lactamase inhibitors	Ampicillin/sulbactam	12 (35.3)	-	22 (64.7)
carbapenems	Meropenem	2 (5.89)	-	32 (94.11)
Aminoglycosides	Gentamicin	3 (8.82)	3 (8.82)	28 (82.35)
	Amikacin	2 (5.89)	-	32 (94.11)
Extended-spectrum cephalosporins	Cefepime	3 (8.82)	-	32 (91.17)
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole	-	-	34 (100)
Fluoroquinolones	Ciprofloxacin	-	-	34 (100)

MIC and MBC of curcumin

The MIC and MBC values of curcumin were measured across 34 MDR *A. baumannii* isolates. The MIC values ranged from 0.625 to 2.5 mg/mL, with 30 isolates exhibiting 2.5 mg/mL MIC, 3 isolates at 1.25 mg/mL, and 1 isolate at 0.625 mg/mL. Similarly, the MBC values spanned from 1.25 to 5 mg/mL, with 30 isolates showing an MBC of 5 mg/mL, 3 at 2.5 mg/mL, and 1 at 1.25 mg/mL.

Inhibition of biofilm formation by curcumin

To assess curcumin's antibiofilm efficacy against MDR *A. baumannii* isolates, the impact of sub-MIC concentrations of curcumin on biofilm formation was evaluated. The findings indicated that the application of curcumin at concentrations of 1/8 MIC, 1/4 MIC, 1/2 MIC, and 1/1 MIC resulted in a decrease in biofilm

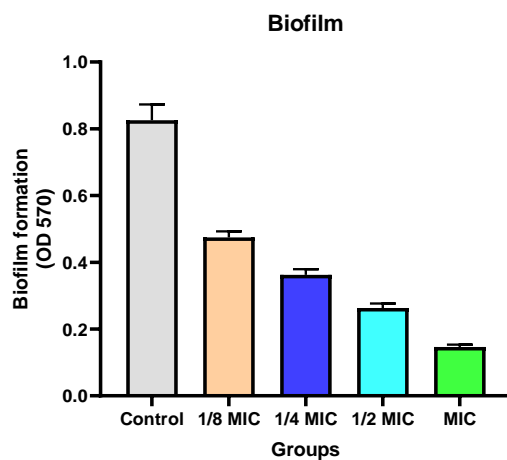


Fig. 1. Curcumin biofilm inhibition effect against MDR *A. baumannii* isolates. Error bars represent the SEM.

formation by MDR *A. baumannii* isolates ($P < 0.05$; Figure 1) as contrasted to the cells that were not treated. When utilized at a concentration of 1/8 of the MIC, curcumin reduced biofilm formation by 25% to 54%. At 1/4 MIC, the reduction was within 38% to 70%. At 1/2 MIC, the reduction was from 56% to 79%. Finally, at 1/1 MIC, the reduction was within 75% to 91% in the treated MDR *A. baumannii* isolates.

Impact of curcumin on the expression of *bap* gene

The relative expression of the *bap* gene in MDR *A. baumannii* cultures treated with curcumin was compared to that in untreated cells. The Ct values of the *bap* gene were determined, and the gene expression levels were normalized against the *16S rRNA* reference gene in the corresponding samples. As shown in Figure 2, curcumin at 1/2 MIC significantly downregulated *bap* gene expression ($P < 0.05$) across 34 MDR *A. baumannii* isolates. On average, the relative expression of the *bap* gene decreased by 62.07% compared to the untreated controls.

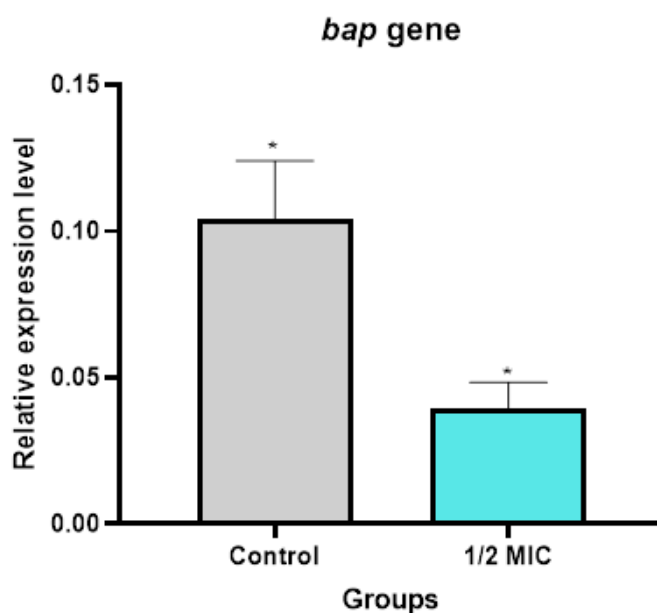


Fig. 2. Effect of curcumin on the relative expression level of the *bap* gene of MDR *A. baumannii* isolates (* $P < 0.05$). Error bars represent the SEM.

Discussion

The findings of this investigation provide compelling evidence that sub-inhibitory curcumin concentrations can inhibit MDR *A. baumannii*'s biofilm formation. The noted suppression of biofilm formation at sub-inhibitory concentrations is particularly noteworthy. Biofilms are complex, structured communities of bacteria attached to surfaces and protected by a matrix of extracellular polymeric substances. They are known to be remarkably more antibiotic-resistant than planktonic cells (22). Therefore, curcumin's capability to inhibit biofilm formation at concentrations that do not inhibit bacterium growth could have notable implications for treating biofilm-associated infections.

Curcumin was found to have an anti-bacterial action against many bacterial pathogens. The present investigation indicated that curcumin has an anti-bacterial impact against MDR *A. baumannii* isolates with

MIC₅₀ and MBC₅₀ values of 2.5 and 5 mg/ml, respectively. In the investigation by Sasidharan *et al.*, the MIC and MBC of curcumin were evaluated for multifaceted bacterial strains. MIC and MBC for *Staphylococcus aureus*, *Bacillus subtilis*, and *E. coli* were found to be 0.25 and 0.5 mg/ml, respectively. For *Pseudomonas aeruginosa*, the MIC and MBC of curcumin were quantified to be 0.5 and 1 mg/ml, respectively. The MIC and MBC of curcumin against *Vibrio cholerae* were quantified to be 0.125 and 0.25 mg/ml, respectively (23). In another investigation by Górski *et al.*, the MIC and MBC of curcumin were evaluated for multifaceted bacterial strains. The MIC of *P. aeruginosa* ranged from 2.5 to 5 mg/mL, whereas the MBC varied from 5 mg/ml to values above 20 mg/mL (24). Compared to previous studies, the variation in MIC and MBC seen in the investigation may be attributed to many factors. These comprise the quality and purity of the curcumin utilized, the specific strains or isolates investigated, and the variations in curcumin solubility in multifaceted vehicles (such as water, DMSO, and ethanol) employed by different research groups (25, 26).

Curcumin has indicated its potential to inhibit biofilm formation across multifaceted bacterial species. The current investigation noted that a sub-inhibitory concentration (0.312 mg/ml to 1.25 mg/ml) of curcumin reduced biofilm formation in MDR *A. baumannii* isolates by 25% to 79%. In a Tanhay Mangoudehi *et al.* investigation, the biofilm formation of *Aeromonas hydrophila* was inhibited by 53–67% when treated with a curcumin concentration of 16 µg/mL. Moreover, an impressive suppression of biofilm formation, ranging from 88–93%, was attained when the bacterial cells were subjected to a higher curcumin concentration of 128 µg/mL (27). In a separate investigation, Packiavathy *et al.* reported that curcumin at a concentration of 100 µg/mL effectively reduced the biomass of biofilms in *E. coli*, *P. aeruginosa*, *Proteus mirabilis*, and *Serratia marcescens* by 52%, 89%, 52%, and 76%, respectively (28).

In *A. baumannii*, the *bap* gene plays a pivotal role in biofilm formation. The investigation demonstrated that at 1/2 MIC (1.25 mg/mL), curcumin remarkably downregulated the expression of the *bap* gene in MDR *A. baumannii* isolates. In an investigation performed by Li *et al.*, it was observed that the *srtA* and *spaP* gene expression in *Streptococcus mutans* biofilms exposed to curcumin for 5 minutes was significantly reduced by nearly one-fold compared to untreated biofilms (29). Furthermore, research by Kumbar *et al.* revealed that curcumin mitigated the formation of biofilms in *Porphyromonas gingivalis* by suppressing the expression of genes coding for adhesions, namely *fmA*, *hagA*, and *hagB* (30).

Curcumin has indicated an anti-bacterial impact against a broad spectrum of bacteria (31). Studies have indicated that curcumin could disrupt the permeability and structural integrity of bacterial cell membranes in Gram-positive and Gram-negative bacteria, resulting in bacterial cell destruction. Curcumin's lipophilic properties allow it to readily incorporate into liposome bilayers, enhancing their permeability (32). Curcumin exerts a suppressive impact on biofilm formation. It diminishes the biofilm's biomass, impedes adhesion, and disrupts its structure (33).

This research concludes that curcumin exhibits antimicrobial and anti-biofilm activity in MDR *A. baumannii*. The results indicate that curcumin impairs MDR *A. baumannii*'s biofilm formation ability. The downregulation of gene *bap*, linked to biofilm formation, supports this finding. These findings highlight curcumin's potential as an anti-biofilm agent. As a result, curcumin can potentially be a valuable tool for combating MDR *A. baumannii* infections. Further investigation is necessary to understand the processes behind curcumin's antibiofilm activity and evaluate its impactiveness *in vivo*. Future research should examine

curcumin's potential synergistic impacts with already available antibiotics, as this may lead to new therapeutic options for diseases caused by bacteria resistant to drugs.

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