

Plumbagin Disrupts Biofilm Integrity and Resistance Gene Expression in Carbapenem-Resistant *Acinetobacter baumannii*

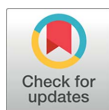
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Carbapenem-resistant *Acinetobacter baumannii* (CRAB) has appeared as a leading cause of hospital-acquired infections, resulting in high mortality rates and limited treatment options. The development of novel antibacterial agents has lagged behind the rapid spread of antibiotic-resistant bacteria; thus, alternative therapeutic strategies are urgently needed. In this study, we investigated plumbagin, a natural compound derived from *Plumbago zeylanica* L., for its potential antibacterial and antibiofilm activities against CRAB. MIC and MBC determinations showed that plumbagin significantly inhibited growth and exerted bactericidal activity at low concentrations. Biofilm inhibition concentration and biofilm eradication concentration assays revealed that plumbagin both prevented biofilm formation and eradicated mature biofilms. Consistent with these findings, XTT reduction assays showed a marked decrease in metabolic activity after plumbagin treatment, and confocal laser scanning microscopy with COMSTAT analysis confirmed reduced biofilm biomass and decreased viability of biofilm-embedded cells. Further, quantitative polymerase chain reaction confirmed the downregulation of the carbapenem-resistance gene *bla*_{OXA-23} and biofilm-related genes, including *bfpR*, *csuA/B*, *ompA*, and *bap*. Collectively, these results reveal plumbagin as a therapeutic candidate against CRAB.

Keywords: Plumbagin, Carbapenem-resistant *Acinetobacter baumannii*, Antibacterial, Antibiofilm, Carbapenem-resistant gene, Biofilm-associated genes



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Introduction

Acinetobacter baumannii is a clinically important opportunistic pathogen, particularly in patients who are hospitalized and those receiving care in intensive care units, and is notorious for its persistence in healthcare environments [1, 2]. To manage these infections, carbapenems are broad-spectrum agents administered to treat severe Gram-negative bacterial infections, including *A. baumannii*, and are considered last-resort options [3].

However, the rapid emergence and spread of carbapenem-resistant *A. baumannii* (CRAB) threaten the clinical efficacy of carbapenems [4]. Between 2009 and 2018, CRAB became a major issue in the United States of America (USA), and resistance rates in Mediterranean countries were as high as 90% [5]. A global survey across 80 countries further indicated prevalence rates of 76.2% in Asia and 69.4% in the USA [6]. In 2019 alone, CRAB infections were associated with an estimated 57,700 deaths globally [7]. Hence, the World Health Organization (WHO) designated CRAB in 2024 as a pathogen of highest priority [8].

The expression of the class D β -lactamase gene *bla*_{OXA-23} primarily mediated carbapenem resistance in CRAB [9, 10]. In addition, biofilm formation also contributes substantially to antimicrobial resistance [11]. The biofilm matrix, composed of polysaccharides, nucleic acids, and extracellular matrix, protects bacterial cells from antibiotics and various environmental stresses, thereby further enhancing resistance [12]. During the initial attachment stage, the chaperone–usher pili system mediates surface adhesion, while in the maturation stage, the biofilm-associated protein (Bap)

promotes structural maturation and attachment to host cells [13]. These processes are mainly regulated by key genes such as *csuA/B*, *bfmR*, *ompA*, and *bap* [14].

With the growing prevalence of antibiotic-resistant pathogens and the clinical challenges posed by biofilm-associated infections, plant-derived compounds possessing antimicrobial properties have received increasing attention as potential therapeutic options [15, 16]. Among these compounds, plumbagin, a naturally occurring molecule isolated from *Plumbago zeylanica* L., has been widely investigated due to its diverse biological activities, including anticancer, anti-inflammatory, and antioxidant effects [17–19]. In addition, previous studies have demonstrated that plumbagin exhibits pronounced antibacterial, antifungal, and biofilm-inhibitory activities against multiple pathogenic microorganisms, such as *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans* [20–22].

Despite these reported activities, the efficacy of plumbagin against CRAB remains insufficiently characterized, even though CRAB is well known for its multidrug resistance and strong biofilm-forming capacity. Addressing this gap, the current study assesses the antibacterial and antibiofilm potential of plumbagin toward CRAB, particularly focusing on biofilm disruption and resistance-associated gene regulation.

Materials and Methods

Bacterial Strains, Growth Conditions, and Reagents

The National Culture Collection for Pathogens (GNUH-NCCP, Republic of Korea) supplied clinical isolates (KBN12P03000, KBN12P02452, KBN12P02449, KBN12P02480, KBN12P02681, and KBN12P01964). Strains were subcultured on MacConkey agar (MAC; Difco, Becton, Dickinson and Co., USA) and inoculated into tryptic soy broth (TSB; Difco, Becton, Dickinson and Co.), followed by incubation at 37°C under static conditions. Sigma-Aldrich (USA) supplied ertapenem, imipenem, meropenem, and plumbagin. Cayman Chemical (USA) provided doripenem. Plumbagin was dissolved in dimethyl sulfoxide (DMSO), with the final solvent concentration not exceeding 1%.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays

MIC and MBC assays were performed to investigate the antibacterial effect and bactericidal effect of plumbagin, as well as to evaluate carbapenem resistance. Carbapenem susceptibility/resistance was interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Bacterial suspensions were adjusted to 0.5 McFarland (1×10^8 CFU/ml) and treated with plumbagin at concentrations of 1–16 µg/ml, followed by incubation in a 96-well plate (BD Falcon, Becton, Dickinson and Co.) at 37°C for 24 h [23]. After incubation, a Multiskan GO Microplate Reader (Thermo Fisher Scientific, USA) was used to measure absorbance at 600 nm. DMSO served as the control, and MIC was defined as the lowest concentration that inhibited $\geq 90\%$ of bacterial growth compared with the control. For MBC determination, 100 µl were taken from MIC wells showing no visible growth and plated onto Mueller–Hinton agar (MHA; Difco, Becton, Dickinson and Co.). Plates were incubated at 37°C for 24 h, and the lowest concentration at which no visible colonies were observed was defined as the MBC.

Biofilm Inhibition Concentration (BIC) Assay

The BIC assay was used to assess the biofilm-inhibitory effect of plumbagin. Bacterial suspensions were adjusted to 0.5 McFarland (1×10^8 CFU/ml), treated with plumbagin at concentrations of 1–16 µg/ml, and incubated in 96-well plates at 37°C for 24 h [24]. After incubation, the wells were washed three times with phosphate-buffered saline (PBS) and dried in a dry oven for 1 h. Biofilms were then stained with 0.5% crystal violet for 5 min and washed three times with sterilized distilled water (SDW). The bound dye was solubilized in 30% acetic acid and incubated at room temperature for 20 min. Biofilm biomass was quantified by measuring absorbance at 595 nm with a Multiskan GO Microplate Reader (Thermo Fisher Scientific). BIC values were obtained from at least six technical replicates, and data are expressed as mean $\pm$ SD.

Biofilm Eradication Concentration (BEC) Assay

A BEC assay was used to evaluate the ability of plumbagin to eradicate preformed biofilms. Bacterial suspensions were adjusted to 0.5 McFarland (1×10^8 CFU/ml), dispensed into 96-well plates, and incubated at 37°C for 24 h to enable biofilm formation. After incubation, the wells were washed three times with PBS and treated with plumbagin at concentrations of 1–16 µg/ml, and incubated again at 37°C for 24 h [25]. Biofilms were stained using the same procedure as in the BIC assay, and absorbance was measured at 595 nm with a Multiskan GO Microplate Reader (Thermo Fisher Scientific). Each BEC assay was performed at least six times independently, and the standard deviation (SD) was calculated from these technical replicates.

XTT Reduction Assay

The 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay was used to assess the metabolic activity of cells within plumbagin-treated biofilms. Biofilms, formed under the same conditions as the BEC assay, were treated with plumbagin at concentrations of 1–16 µg/ml and incubated at 37°C for 24 h [26]. A solution prepared as a 50:1 (v/v) mixture of XTT reagent and activation reagent (XTT Cell Proliferation Assay Kit, ATCC, USA) was added to each well and incubated at 37°C for 3 h. Formazan production was quantified by measuring absorbance at 475 nm, with 660 nm as the reference wavelength, using a Multiskan GO Microplate Reader (Thermo Fisher Scientific). XTT reduction assays were conducted at least six times, and the data are presented as mean $\pm$ SD.

Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to visualize cell viability in plumbagin-treated biofilms. The bacterial suspension, adjusted to 0.5 McFarland (1×10^8 CFU/ml), was dispensed into a 24-well glass-bottom imaging plate (Eppendorf AG, Germany), incubated at 37°C for 24 h, and washed three times with 0.85% saline. Plumbagin (1–16 µg/ml) was then added, and the plate was incubated for an additional 24 h under the same conditions. The cultured samples were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific) by mixing SYTO9 and propidium iodide (PI) at a 1:1 ratio, adding the mixture to each well, and incubating in the dark for 15 min. The plate was washed three times with 0.85% saline and fixed with 3.7% formaldehyde for 1 h after staining. A Zeiss LSM900 with Airyscan2 confocal laser microscope (Carl Zeiss, Germany) was used to image fluorescence signals, with emission collected at 525 nm (SYTO9) and 640 nm (PI). ZEN software version 3.13 (Carl Zeiss, Microscopy Deutschland GmbH) was used to acquire and process Z-stack images. The acquired Z-stack images were quantitatively evaluated using COMSTAT 2.0 image analysis software [27].

Table 1. Quantitative polymerase chain reaction primers targeting *Acinetobacter baumannii* and its virulence-associated genes.

Target gene	Primer sequence (5'-3')	Annealing temp. (°C)	Reference
<i>bfnR</i>	F: GAGCGTTTAGCGCGATTA R: TCCAAGACCACAAGATCC	53	[63]
<i>csuA/B</i>	F: ATGCGGTAAATACTCAAGCA R: TCACAGAAATATTGCCACCT	52	[63]
<i>ompA</i>	F: CTCTTGCTGGCTTAAACGTA R: GCAATTTCTGGCTTGTATTG	52	[63]
<i>bap</i>	F: AATGCACCGGTACTTGATCC R: TATTGCCTGCAGGGTCAGTT	57	[63]
<i>bla_{OXA-23}</i>	F: CCTCAGGTGTGCTGGTTATTCA R: CTCCAATCCGATCAGGGCAT	58	[63]
16S rRNA	F: CAGCTCGTGTCTGAGATGT R: CGTAAGGGCCATGATGACTT	60	[63]

RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

Relative gene expressions in response to plumbagin treatment were analyzed using qPCR. Cultures treated with plumbagin (1–16 µg/ml) were incubated at 37°C until reaching the logarithmic growth phase. Cells were pelleted by centrifugation, resuspended in proteinase K and Tris-EDTA buffer, and the NucleoSpin RNA Mini Kit (Germany) was used to extract RNA. A µDrop plate (Thermo Fisher Scientific) was utilized to measure RNA concentration and purity. ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) was used to synthesize cDNA. TOPreal qPCR 2× PreMIX (Enzynomics, Republic of Korea) with the primers listed in Table 1 was utilized for qPCR. Amplification was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) with the following cycling conditions: 95°C for 10 min (initial denaturation), followed by 40 cycles of denaturation at 95°C for 10 sec (denaturation), primer-specific annealing for 15 sec, and 72°C for 30 sec (extension). Melting curve analysis was conducted at 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Relative expression levels were normalized to 16S rRNA and calculated employing the $2^{-\Delta\Delta CT}$ method [28].

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Statistical significance was assessed using a one-way ANOVA test, with statistical significance set at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. GraphPad Prism version 10.0.0 (GraphPad Software, USA) was used for data analysis and visualization.

Results

Carbapenem-Resistant Antibacterial and Bactericidal Effect of Plumbagin against CRAB

Table 2 presents the MIC values for carbapenems and plumbagin, as well as the MBC value of plumbagin, against clinical isolates of *A. baumannii*. MIC values ranged from 128 to >256 µg/ml for doripenem, >128 µg/ml for meropenem, 256 to >256 µg/ml for imipenem, and 512–1,024 µg/ml for ertapenem. All isolates were classified based on CLSI breakpoints as carbapenem-resistant. In contrast, plumbagin demonstrated an MIC of 16 µg/ml, representing an 8-fold reduction compared with meropenem, which is a carbapenem with the lowest MIC. The MBC of plumbagin against all CRAB strains was 32 µg/ml. The strain *A. baumannii* KBN12P03000, which demonstrated the highest carbapenem MIC, was selected for subsequent experiments.

Biofilm-Inhibitory Activity of Plumbagin against CRAB

Fig. 1 illustrates the biofilm-inhibitory activity of plumbagin. Reductions in biofilm formation of 49%, 56%, and 69% were observed at 1, 2, and 4 µg/ml, respectively. Inhibition increased to 81% and 92% at sub-MIC (8 µg/ml) and MIC (16 µg/ml) levels, respectively. These results indicate a clear concentration-dependent effect. Notably, substantial inhibition (≥80%) occurred at the sub-MIC level, whereas near-complete inhibition was observed at the MIC. Collectively, these results indicate that plumbagin effectively inhibits biofilm formation in CRAB.

Table 2. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of plumbagin and carbapenems against clinically isolated *A. baumannii* strains.

Strains	Specimen	MIC (µg/ml)					MBC (µg/ml)
		Plumbagin	Doripenem	Meropenem	Imipenem	Ertapenem	Plumbagin
<i>A. baumannii</i> KBN12P03000	Blood	16	> 256	> 128	> 256	1024	32
<i>A. baumannii</i> KBN12P02452	Transtracheal aspirate	16	256	> 128	> 256	1024	32
<i>A. baumannii</i> KBN12P02449	Transtracheal aspirate	16	256	> 128	> 256	512	32
<i>A. baumannii</i> KBN12P02480	Sputum	16	128	> 128	256	512	32
<i>A. baumannii</i> KBN12P02681	Sputum	16	256	> 128	> 256	512	32
<i>A. baumannii</i> KBN12P01964	Catheter urine	16	256	> 128	256	512	32

KBN: Korea Biobank Network

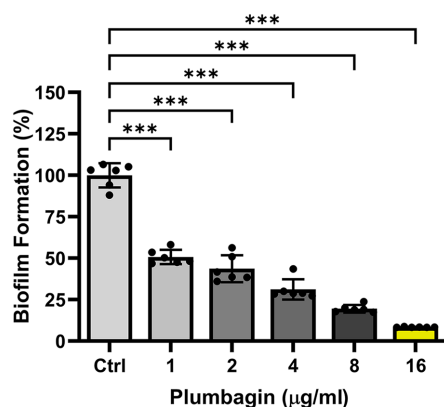


Fig. 1. Biofilm inhibition by plumbagin against the clinical isolate *Acinetobacter baumannii* KBN12P03000. Biofilm formation was quantified using 0.5% crystal violet staining, with absorbance measured at 595 nm. Data are presented as individual values (●) with error bars representing the mean $\pm$ standard deviation (SD) of six measurements. Statistical significance compared with the untreated control is indicated as *** p < 0.001.

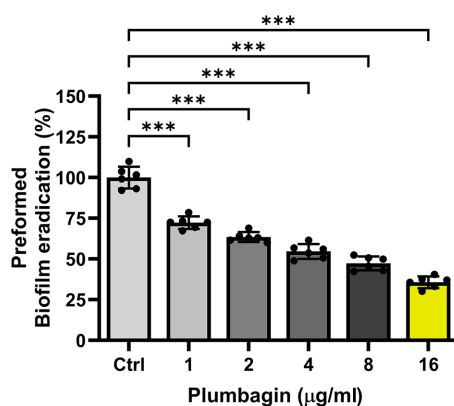


Fig. 2. Preformed biofilm eradication by plumbagin against the clinical isolate *A. baumannii* KBN12P03000. Preformed biofilms were treated with plumbagin, stained with 0.5% crystal violet, and quantified by measuring absorbance at 595 nm. The results are presented as six individual values (●), and bars correspond to the mean $\pm$ standard deviation (SD). Statistical significance compared with the untreated control is indicated as *** p < 0.001.

Biofilm Eradication Activity of Plumbagin against CRAB

Fig. 2 illustrates the effect of plumbagin on preformed biofilms. Plumbagin eradicated biofilms in a concentration-dependent manner, with rates of 28%, 37%, and 45% at 1, 2, and 4 µg/ml, respectively. Eradication rates were 53% and 64% at sub-MIC (8 µg/ml) and MIC (16 µg/ml) levels, respectively. Notably, more than half of the biofilm biomass was eradicated at these concentrations, indicating substantial activity even against established biofilms. These results reveal that plumbagin not only inhibits biofilm formation but also eradicates mature biofilms.

Impact of Plumbagin on Metabolic Activity within Biofilms

Fig. 3 illustrates the cellular metabolism of biofilms after plumbagin treatment. Plumbagin inhibited metabolic activity in a concentration-dependent manner, with rates of 8%, 22%, 37%, and 48% at 1, 2, 4, and 8 µg/ml, respectively. Inhibition rates increased to 61% at 16 µg/ml (MIC), indicating metabolic activity suppression in more than half of the biofilm-associated cells. In particular, at 16 µg/ml, inhibition reached 61%, similar to the biofilm eradication results, indicating that metabolic activity reduction measured with the XTT assay is closely associated with biofilm eradication.

CLSM Analysis of CRAB Viability after Plumbagin Treatment

Fig. 4A shows CLSM images of Live/Dead-stained CRAB cells after plumbagin treatment (green, viable; red, dead cells). In the control group, strong green fluorescence with relatively weak red fluorescence indicated that most cells remained viable. As the plumbagin concentration increased, red fluorescence progressively intensified, with a corresponding decrease in green fluorescence. This shift was more apparent in the merged images; green signals predominated in the control group, whereas red signals became increasingly dominant at higher plumbagin concentrations. At 0.5 $\times$ MIC (8 µg/ml), a substantial proportion of cells showed loss of viability, whereas at the MIC (16 µg/ml) most cells were non-viable. Overall, these results indicate that plumbagin markedly reduces CRAB cell viability in a concentration-dependent manner.

COMSTAT Analysis of CRAB Biofilm

After CLSM imaging, COMSTAT was used to quantify three-dimensional biofilm structural parameters following plumbagin treatment at

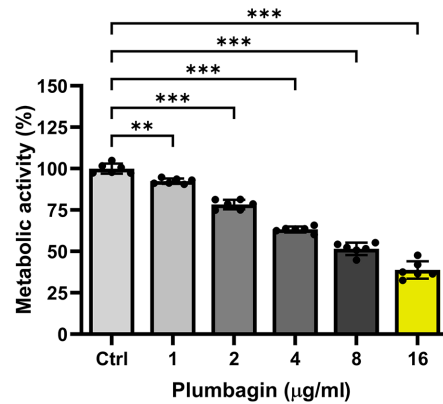


Fig. 3. Plumbagin-mediated reduction of metabolic activity in *A. baumannii* KBN12P03000. Metabolic activity after plumbagin treatment was quantitatively assessed using the XTT reduction assay. Absorbance was measured at 475 nm and 660 nm and calculated using the formula $A_{475\text{nm}}(\text{test}) - A_{475\text{nm}}(\text{blank}) - A_{660\text{nm}}(\text{test})$. Data are presented as six individual values (●) together with the mean $\pm$ standard deviation (SD). Statistical significance compared with the untreated control is indicated as ** $p < 0.01$ and *** $p < 0.001$.

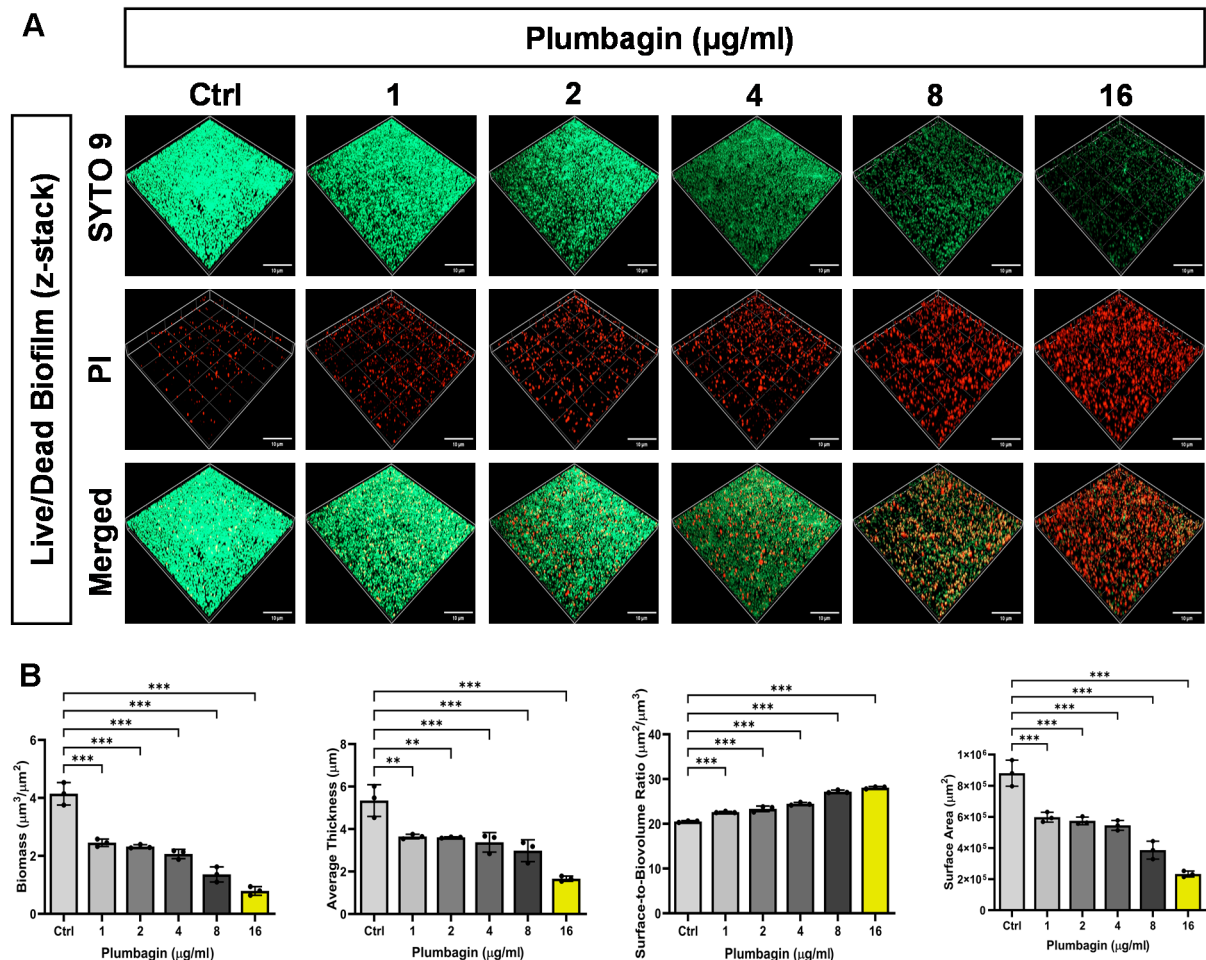


Fig. 4. (A) CLSM visualization of viability staining in *A. baumannii* KBN12P03000 following plumbagin treatment. Cells were stained with SYTO9 and propidium iodide (PI) and imaged by confocal laser scanning microscopy. Live cells are shown in green (SYTO9), while dead cells are indicated in red (PI). The merged image represents the combined fluorescence signals of SYTO9 and PI. Images were acquired at 63 $\times$ magnification using ZEN software, and the scale bar represents 10 μm . **(B) COMSTAT 2.0 analysis of CLSM Z-stacks showing quantitative biofilm structural parameters, including biomass ($\mu\text{m}^3/\mu\text{m}^2$), average thickness (μm), surface-to-biovolume ratio ($\mu\text{m}^2/\mu\text{m}^3$), and surface area (μm^2).**

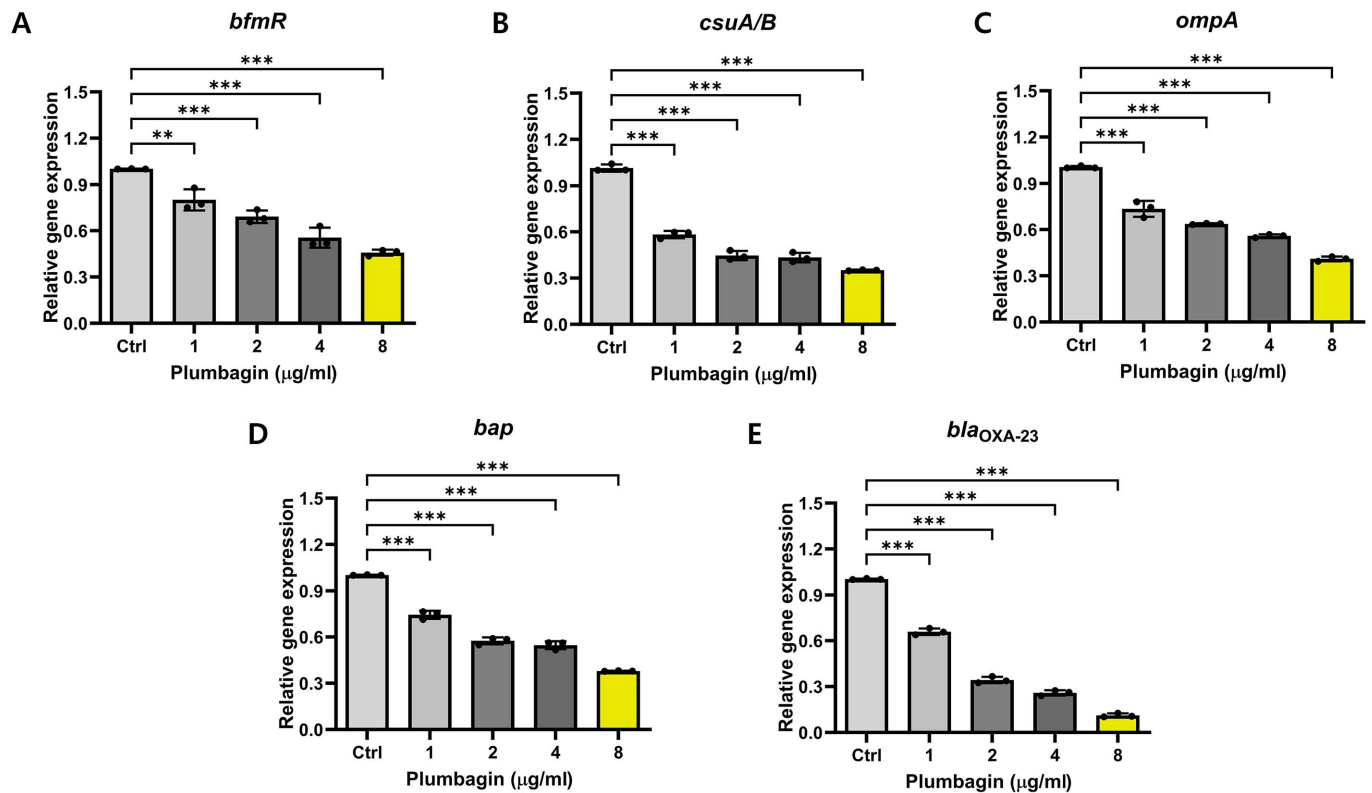


Fig. 5. Gene expression analysis in *A. baumannii* KBN12P03000 after plumbagin treatment. The expression levels of (A) biofilm master regulator gene *bfmR*, (B) attachment-related gene *csuA/B*, (C) outer membrane protein A gene *ompA*, (D) biofilm-associated protein gene *bap*, and (E) carbapenem-resistance gene *bla_{OXA-23}* were analyzed after treatment with various plumbagin concentrations. Expression levels were normalized to the 16S rRNA gene. Data are presented as individual values (●), with error bars indicating the mean $\pm$ standard deviation (SD) of three independent experiments. Statistical significance versus the untreated control is indicated as ** $p < 0.01$ and *** $p < 0.001$.

various concentrations (Fig. 4B, Compared with the control group, plumbagin significantly reduced biofilm biomass, average thickness, and surface area, at the MIC (16 µg/ml), each parameter decreased by more than 50% relative to the control. This pattern is consistent with the BEC findings and suggests that plumbagin disrupts biofilm maturation. In addition, the surface-to-biovolume ratio increased in plumbagin-treated samples, indicating a less compact, more dispersed biofilm architecture.

Expression of Biofilm-Associated and Carbapenem-Resistant Genes in CRAB

Fig. 5 illustrates changes in the expression of the carbapenem-resistant gene (*bla_{OXA-23}*) and biofilm-related genes (*bfmR*, *csuA/B*, *ompA*, and *bap*) after plumbagin treatment. Expression of *bla_{OXA-23}* decreased in a concentration-dependent manner, showing approximately 4- and 9-fold downregulation at 4 and 8 µg/ml, respectively, indicating that plumbagin effectively suppresses this carbapenem-resistance gene. Furthermore, at the sub-MIC (8 µg/ml), the expression of *bfmR*, *csuA/B*, *ompA*, and *bap* was downregulated by approximately 2-, 3-, 2-, and 3-fold, respectively. These results indicate that plumbagin suppresses both carbapenem-resistance and biofilm-associated genes, thereby exerting antibacterial and antibiofilm effects against CRAB.

Discussion

Carbapenems have long been the mainstay of treatment for severe Gram-negative bacterial infections, including *A. baumannii* [29]. However, increasing clinical use has driven the rise of CRAB, thereby worsening the global public health burden [30]. In 2019, the US Centers for Disease Control and Prevention indicated that therapeutic options for CRAB infections remain severely limited, with few novel therapies in development [31]. The increase in resistant strains outpaces the discovery of new antibiotics, and research has shifted toward natural-compound-based therapeutic strategies [32, 33].

Among these, plumbagin, a natural naphthoquinone derived from *Plumbago zeylanica* L., has demonstrated antibacterial and antibiofilm activities toward pathogens, including *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [34, 35]. Furthermore, plumbagin shows selective cytotoxicity toward malignant cells while sparing normal epithelial cells, highlighting its therapeutic selectivity [36, 37]. Resistance development assays have further indicated a low propensity for resistance emergence after plumbagin exposure [38]. Despite these promising findings, its activity against CRAB remains inadequately explored. To address this gap, we assessed its antibacterial and antibiofilm activities against CRAB.

The calculated MBC/MIC ratio of 2 indicates that plumbagin exerts a bactericidal, rather than merely bacteriostatic, effect against CRAB (Table 2). Notably, the MIC observed in this study was approximately 2-fold lower than values previously reported for *E. coli* [39], suggesting a heightened sensitivity of CRAB to plumbagin under comparable conditions. While carbapenem resistance in *E. coli* often involves diverse enzymes

(e.g., NDM, KPC, OXA-48-like), resistance in CRAB is predominantly mediated by the class D β -lactamase, OXA-23 [40-46]. In this context, plumbagin treatment resulted in significant downregulation of *bla*_{OXA-23} expression. Given that OXA-23 is the primary resistance determinant in these strains, this transcriptional suppression likely compromises the bacterium's ability to hydrolyze β -lactams, thereby restoring susceptibility [45, 47]. Although direct enzyme inhibition assays are required for confirmation, these findings suggest that plumbagin's efficacy is driven by the dual action of direct bactericidal stress and the downregulation of *bla*_{OXA-23} expression.

Consistent with previous reports in *S. aureus* and *C. albicans* [21], plumbagin exhibited strong antibiofilm activity, as it suppressed biofilm development and eliminated established CRAB biofilms (Figs. 1 and 2). Remarkably, CRAB biofilms were inhibited at concentrations lower than those reported for *S. aureus* and *E. coli* [48], indicating that plumbagin is particularly effective against CRAB biofilm formation. This inhibitory effect is closely associated with *csuA/B*, *ompA*, and *bfmR* gene suppression, which are involved in initial attachment and regulatory control, together with *bap* downregulation, which contributes to intercellular aggregation and matrix accumulation during biofilm maturation [49-53]. Such transcriptional repression interferes with early adhesion, disrupts biofilm development, and weakens mature biofilm cohesion, thereby increasing their susceptibility to collapse [50]. Importantly, these inhibitory effects occurred at concentrations up to 6-fold lower than those required for other natural compounds [54], highlighting the strong ability of plumbagin to interfere with biofilm regulation and further supporting its role as an anti-biofilm candidate against CRAB (Fig. 5). In this context, previous studies have reported that plumbagin induces reactive oxygen species (ROS), disrupts cell membrane permeability, and interferes with the quorum sensing system in Gram-negative bacteria [35, 38]. While the current study did not experimentally verify these specific pathways, these known properties offer a plausible explanation for the observed results. It is possible that plumbagin indirectly modulates the *bfmRS* system, which operates as a regulatory network complementary to the quorum-sensing system. Furthermore, plumbagin-induced membrane perturbation and ROS production in *A. baumannii* likely contribute to the downstream suppression of biofilm-associated gene expression. Further studies are needed to delineate the precise signaling cascades involved.

It is also important to note that biofilm inhibition and eradication assays can overestimate eradication because they may retain signal from dead cells and cellular debris [55]. To overcome this, we used the XTT reduction assay, which quantifies the metabolic activity of biofilm cells by measuring the reduction of tetrazolium salt to formazan [56-58]. The XTT assay results corroborated the biofilm eradication data, demonstrating that plumbagin effectively suppresses metabolic activity within established biofilms (Fig. 3). These findings suggest that reduced biofilm signals are accompanied by metabolic suppression. In support of this, CLSM images showed a gradual increase in red fluorescence, indicative of non-viable cells, as the concentration of plumbagin increased [59]. In addition, COMSTAT analysis of CLSM Z-stacks revealed an overall reduction in biofilm biomass, with the most pronounced decrease at the MIC. Collectively, these results indicate that plumbagin not only impairs cellular viability but also weakens the structural integrity of CRAB biofilms (Fig. 4A and 4B). Interestingly, previous studies have reported that plumbagin reduces extracellular DNA (eDNA) in other bacterial species [60], raising the possibility that the observed biofilm disruption may be related to decreased eDNA. However, further studies are warranted to verify this mechanism.

Overall, gene downregulation involved in initial attachment and maturation, BIC/BEC confirmed biofilm biomass inhibition and eradication, XTT revealed decreased metabolic activity, and CLSM imaging combined with COMSTAT analysis revealed an increased number of non-viable cells and a marked reduction in biofilm biomass. These outcomes are interconnected, collectively demonstrating that plumbagin concurrently induces inhibition of biofilm formation, structural weakening, and cellular viability reduction in CRAB. In addition, the reduced expression of the carbapenem-resistance gene *bla*_{OXA-23}, together with the concomitant decreases in MIC and MBC values, supports the antibacterial and bactericidal effects of plumbagin.

In summary, the evidence indicates plumbagin as a promising therapeutic candidate for CRAB. However, as this study was conducted using a single strain exhibiting the highest level of carbapenem resistance, the results may represent strain-specific effects. Moreover, the present work focused exclusively on CRAB isolates; therefore, the antibacterial effect of plumbagin on carbapenem-susceptible strains was not evaluated. Future investigations, including both resistant and susceptible isolates, are needed to comprehensively assess the overall antibacterial potential of plumbagin across the *A. baumannii* species. Moreover, as this study was limited to *in vitro* analyses, the antibacterial and antibiofilm efficacy of plumbagin within a complex host environment remains to be established. Consequently, additional validation using relevant animal infection models is necessary to confirm plumbagin's safety and therapeutic potential *in vivo*. In this regard, preclinical pharmacokinetic studies have reported low toxicity when plumbagin was administered orally to rats at 150 mg/kg [61]. Further, selective cytotoxicity without detectable toxicity to normal epithelial cells has been demonstrated [36, 37], supporting the feasibility of oral therapy development. This study did not investigate the antibiofilm activity of plumbagin on medical devices, and future investigations on catheter surfaces will be crucial to identify its potential to reduce the persistence of hospital-acquired infections [62]. Finally, to advance its development, additional investigations into its pharmacodynamics and overall safety profile are warranted.

Conclusion

This study confirmed the antibacterial and bactericidal activities of plumbagin, revealing growth inhibition of carbapenem-resistant *A. baumannii* (CRAB) at 16 μ g/ml and complete bacterial eradication at 32 μ g/ml. Beyond planktonic growth inhibition, plumbagin exhibited both biofilm-preventive and biofilm-eradicating effects. XTT reduction assay quantitatively demonstrated decreased metabolic activity in established biofilms, which was further supported by CLSM observations. COMSTAT-based analysis further quantified biofilm structural alterations, revealing an overall reduction in biofilm biomass following plumbagin treatment. These phenotypic effects were accompanied by downregulation of the carbapenem-resistance gene *bla*_{OXA-23} and several biofilm-associated genes (*bfmR*, *csuA/B*, *ompA*, and *bap*). Overall, these findings support plumbagin as a potential anti-CRAB candidate *in vitro*. However, current evidence is limited, and further investigations are warranted to establish its efficacy and safety.

Author Contributions

Min-Ji Youn: Conceptualization of the study, data curation, investigation, validation, visualization, writing—original draft, and writing—review and editing. **Yong-Bin Eom:** Design and conceptualization of the study, funding acquisition, investigation, project administration, resources, supervision, validation, writing—original draft, and writing—review and editing.

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