



# Eradication of *Acinetobacter baumannii* Planktonic and Biofilm Cells Through Erythrosine-Mediated Photodynamic Inactivation Augmented by Acetic Acid and Chitosan

Zahra Fekrirad<sup>1</sup> · Esmail Darabpour<sup>2</sup> · Nasim Kashef<sup>1</sup>

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## Abstract

Photodynamic inactivation (PDI) is an attractive treatment modality for multidrug-resistant bacterial infections. The effectiveness of photosensitization by anionic photosensitizers such as erythrosine B can be further enhanced by the addition of biological or chemical molecules. This study aimed to investigate the enhancement effect of acetic acid and chitosan on erythrosine-mediated PDI of *Acinetobacter baumannii* in planktonic and biofilm forms. The planktonic cell growth of three *A. baumannii* strains was subjected to PDI by using erythrosine B (50 µM) in 0.01% acetic acid and green laser light (530 nm) at fluence of 40 J/cm<sup>2</sup>. The phototoxic effect of erythrosine B (100 µM) in combination with chitosan (12.5 mg/ml) (in a solution of acetic acid) at fluence of 80 J/cm<sup>2</sup> on biofilms was also evaluated. Finally, the cytotoxicity and phototoxicity of the mentioned mixture were assessed on human fibroblasts. Planktonic cells of all three studied *A. baumannii* strains were almost eradicated by erythrosine B-mediated PDI in the presence of acetic acid. Also, PDI combined with chitosan resulted in a marked decrease in the number of viable biofilm cells (> 3 log<sub>10</sub> CFU). At the same experimental conditions, only 15% of the fibroblasts were photoinactivated. The results showed that PDI by using erythrosine B in acetic acid is very effective against *A. baumannii* planktonic cells and could eliminate them significantly. Also, chitosan enhanced the anti-biofilm efficacy of erythrosine B-mediated PDI against *A. baumannii*, suggesting that combination therapy may be useful in targeting biofilms.

## Introduction

*Acinetobacter baumannii*, the most important member of *Acinetobacter* species, is recognized as causing a broad range of severe hospital-acquired infections. The infections including skin and soft tissue infections, catheter-associated urinary tract infections, bloodstream infections, and ventilator-associated pneumonia [1]. Due to exceedingly increased resistance to disinfectants and major antimicrobials, *A. baumannii* evolved as a global threat in the health-care setting. Several strains are highly resistant to most clinically available antibiotics such as carbapenems and aminoglycosides. Current antimicrobials for treatments of carbapenem-resistant or pan drug-resistant *A. baumannii* are far from perfect

therapeutic options [2]. The ability of *A. baumannii* to form robust biofilms on most abiotic surfaces such as health-care-associated equipment, on occlusive dressings and in the wound, has also created challenges in the health treatment [3]. So, there is an urgent need for alternative drugs and/or therapies that are capable of inactivating *A. baumannii*.

Photodynamic inactivation (PDI) offers a promising approach to combat antibiotic resistance. PDI causes a rapid killing of microbial cells (in seconds) and has not produced resistance in bacteria so far. Besides, it could efficiently kill biofilm-grown cells both in vitro and in vivo [4]. PDI utilizes the combination of a non-toxic dye known as photosensitizer (PS) and visible light to produce cytotoxic reactive oxygen species (ROS) that can damage cellular components such as DNA, membrane lipids and proteins, ultimately leading to cell death. To achieve an efficient PDI, photosensitizer must bind to or penetrate the bacterial cell [5].

Erythrosine B (EB), an FDA-approved food dye, is known as a photosensitizer which belongs to the class of anionic xanthene dyes. The efficacy of erythrosine has been confirmed in the lethal photosensitization of pathogenic microorganisms [6].

✉ Esmail Darabpour  
ismal\_dar@yahoo.com; e.darabpour@scu.ac.ir

<sup>1</sup> Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran

<sup>2</sup> Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran

However, it is known that a photosensitizer molecule that bears a net anionic charge cannot effectively exert its PDI effect against gram-negative bacteria due to their outer membrane (OM) barrier that prevents the uptake of anionic compounds [7]. Also, penetration of anionic photosensitizers into the biofilm can be restricted by negatively charged extracellular polysaccharide (EPS), which surround and protect cells.

The effectiveness of photosensitization by anionic PS can be further enhanced by the addition of biological or chemical molecules, which alter the molecular charge and structure of photosensitizer or modify the native consistence of the OM / EPS [8, 9]. Chitosan (Ch) and acetic acid (AA) may have the potential to enhance the efficacy of PDI using erythrosine. Acetic acid, an organic chemical compound, can modify the molecular charge of some photosensitizers under acidic condition that subsequently affect their activity during PDI [10]. Chitosan is a natural polycationic antimicrobial biopolymer known to be able to destabilize the biofilm architecture [11, 12].

To the best of our knowledge, there is no report in the literature on the erythrosine-mediated PDI of *A. baumannii*. The aim of this study was to investigate the effect of PDI mediated by erythrosine on planktonic (in the presence of acetic acid) and biofilm (in the presence of acetic acid and chitosan) forms of *A. baumannii*.

## Material and Methods

### Strains and Culture Conditions

*A. baumannii* ATCC BAA 747 was obtained from Persian Type Culture Collection (PTCC). Two clinical isolates of *A. baumannii* (AB1 and AB2) recovered from burn wounds (Accidents and burn center of Tehran, Iran) were also used. Both clinical isolates displayed resistance to the following antibiotics: imipenem (10 mg), ciprofloxacin (5 mg), gentamicin (10 mg), and azithromycin (15 mg). All of the synthetic antibiotic disks were produced by Padtan Teb Company (Iran). All strains were maintained in Luria–Bertani (LB) broth at 37 °C.

### Photosensitizer and Light Source

Erythrosine B (Sigma, USA) was used as photosensitizing agent. A 1 mM stock solution of EB was prepared in injectable distilled water and stored at 4 °C in the dark no longer than 2 weeks before use. Stock solution was filtrated through a 0.2 µm polycarbonate membrane and further diluted in injectable distilled water to achieve the desired concentration. The light source used in this study was a 530 nm diode laser with a maximum output power of 45 mW.

### Biofilm Formation Assay

Biofilm formation (24 h) was quantified by crystal violet assay, according to our previous studies [13]. A low cut-off (OD<sub>c</sub>) is defined as 3 × SD above the mean OD of control wells (uninoculated wells) and bacterial strains were classified into three categories: strong biofilm producer ( $4 \times \text{OD}_c < \text{OD}$ ), moderate biofilm producer ( $2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$ ), and weak biofilm producer ( $\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$ ) [14].

### Minimum Inhibitory Concentration (MIC) of Chitosan

Chitosan (low molecular weight) was purchased from Merck (Germany) and Stock solutions were prepared in injectable distilled water. The MIC values of chitosan against *A. baumannii* strains were determined by the Mueller–Hinton broth microdilution method [15].

### Photodynamic Inactivation of *A. baumannii* Planktonic Cells

For this purpose, 300 µl of bacterial suspensions ( $1\text{--}2 \times 10^8$  CFU/ml) was placed in a 96-well microplate and then incubated with EB (final concentration of 50 µM) + acetic acid (AA) (0.01%) in the dark and at room temperature for 15 min. Treated cells were exposed to diode laser with power of 45 mw for 15 min ( $40 \text{ J/cm}^2$ ). Then, 100 µl of each cell suspension, in tenfold serial dilution, was spread on nutrient agar plates. After incubation for 24 h at 37 °C, colonies were counted. All experiments were repeated three times in triplicate. Controls included bacterial suspensions incubated with 0.9% sterile saline in the dark (untreated), bacterial suspensions incubated with EB (50 µM) alone or AA alone and EB (50 µM) + AA (0.01%) in the dark [16].

### Photodynamic Inactivation of *A. baumannii* Biofilms

The overnight culture of strains was diluted (1:100) into fresh TSB (supplemented with 0.2% glucose). Then, the diluted culture was transferred into 96-well polystyrene microplates (SPL, Korea) and incubated for 24 h at 37 °C. After biofilm formation, the medium of each well was aspirated and wells were washed twice with 0.9% sterile saline to remove the planktonic cells. In the next step, 24 h-old biofilms were treated with EB (at the final concentration of 100 µM) + AA (0.01%) or EB (at the final concentration of 100 µM) + AA (0.01%) + chitosan ( $\frac{1}{2}$  MIC) for 15 min and subsequently irradiated with laser light for 30 min ( $80 \text{ J/cm}^2$ ). Controls included

biofilms incubated with 0.9% sterile saline (untreated), EB (100  $\mu$ M) + AA (0.01%), AA (0.01%) alone or EB (100  $\mu$ M) + AA (0.01%) + chitosan ( $\frac{1}{2}$  MIC) in the dark. Adherent cells in biofilms were removed from the micro-wells by scraping and by vigorous pipetting. Finally, the cells were serially diluted and plated on the nutrient agar for the determination of CFU/ml [13].

### Cytotoxicity and Phototoxicity Assay

The standard colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was performed to determine the biocompatibility of AA + EB + chitosan to human fibroblast under dark and light conditions. Human fibroblast cells (provided by the Stem Cell Technology Center, Tehran, Iran) were seeded into 96-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and incubated for 48–72 h in 5% CO<sub>2</sub>. The cells (90% confluency) were treated with AA + EB + chitosan at 37 °C for 15 min in the dark. After discarding the photosensitizer solution, cells were irradiated with green light (80 J/cm<sup>2</sup>). Fibroblast cells were also tested by AA + EB + chitosan without light irradiation (dark toxicity). Then, the cells were re-supplied with growth medium and incubated overnight at 37 °C. Following incubation, the medium was removed and cells were washed with PBS. Afterwards, MTT solution at a concentration of 0.5 mg/ml was added to medium and cells were incubated for 4 h at 37 °C. The medium was carefully removed and the crystals were dissolved by adding dimethyl sulfoxide for 15 min. The absorbance of the formazan solution was determined by an ELISA plate reader (Hiperion, Germany) at 540 nm. The cell viability was calculated based on control sample without any treatment as 100%. Each experiment was performed in triplicate [17].

### Statistical Analysis

All values were represented as mean  $\pm$  standard error. Statistical analysis was done by post hoc Tukey tests ( $P < 0.05$ ) using GraphPad Prism 6.

## Results

### Quantification of Biofilm Formation

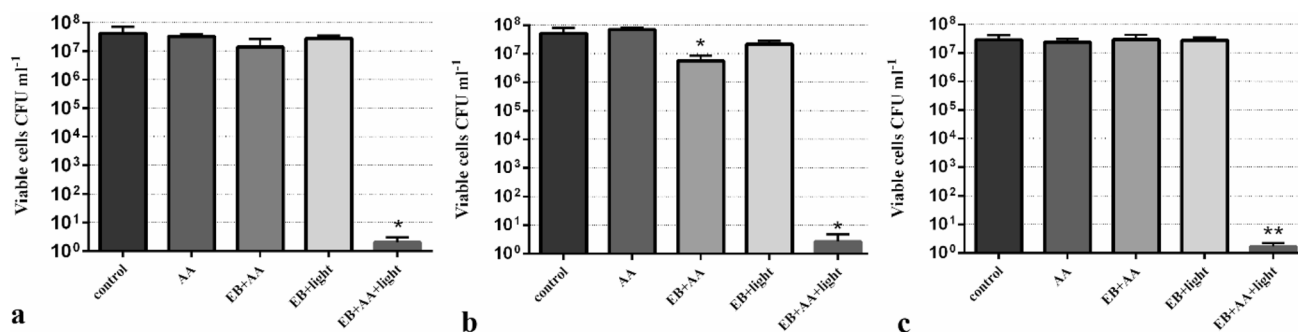
According to the crystal violet staining results, *A. baumannii* ATCC BAA 747, *A. baumannii* AB1, and *A. baumannii* AB2 were considered as weak, moderate, and strong biofilm producer strains, respectively.

### Determination of Chitosan MIC

The MIC value of chitosan against all the tested strains was 25 mg/ml.

### PDI against Planktonic Cells

Photodynamic inactivation at a concentration of 50  $\mu$ M EB (without acetic acid) was ineffective against all tested *A. baumannii* strains (in planktonic form) and only caused less than 1 log<sub>10</sub> CFU reduction in the number of viable bacteria. *A. baumannii* strains were not significantly affected by the PDI treatment even after the erythrosine concentration was raised up to 400  $\mu$ M (without acetic acid). PDI with EB in 0.01% acetic acid (AA) showed promising results against planktonic cells of *A. baumannii*. As shown in Fig. 1, AA (0.01%) alone and in combination with EB (50  $\mu$ M) in the dark caused less than 1 log<sub>10</sub> CFU reduction in the number of bacterial cells in comparison with the untreated control while EB-mediated PDI under acidic condition caused a significant reduction in viable count of the tested strains ( $> 7$  CFU/ml).



**Fig. 1** Photodynamic inactivation of **a** *A. baumannii* ATCC BAA 747, **b** *A. baumannii* AB1, **c** *A. baumannii* AB2 planktonic cells. Control: untreated group, AA: acetic acid (0.01%), EB: 50  $\mu$ M erythrosine B, light: 40 J/cm<sup>2</sup>. \*Significant at  $p < 0.05$ , \*\*significant at  $p < 0.005$

## PDI Against Biofilms

In this study, lethal PDI was defined as the treatment that resulted in a  $\geq 3 \log_{10}$  reduction in CFU. As shown in Fig. 2, PDI mediated by EB + AA did not cause lethal effect on biofilm-associated cells of *A. baumannii* ATCC BAA 747. However, the lethal effect of PDI on biofilm of this strain was observed after the addition of chitosan at sub-MIC ( $\frac{1}{2}$  MIC) to EB. AA (0.01%) alone, AA (0.01%) + EB (50  $\mu$ M), and AA (0.01%) + EB (50  $\mu$ M) + chitosan ( $\frac{1}{2}$  MIC) in the absence of light did not show significant effect on the viability of *A. baumannii* ATCC BAA 747 biofilm.

Figure 3 demonstrates that the *A. baumannii* AB1 biofilm cells were more susceptible to PDI treatment in comparison to *A. baumannii* ATCC BAA 747. Both PDI treatments mediated by EB + AA and EB + AA in combination of chitosan resulted in a lethal effect on *A. baumannii* AB1 biofilm. The AA alone, AA + EB, and AA + EB + chitosan showed dark toxicity effect on biofilm cells of AB1 clinical isolate.

The effect of PDI on biofilm of *A. baumannii* AB2 is displayed in Fig. 4. The lethal effect of PDI was achieved only by adding of chitosan to AA + EB. The AA alone, AA + EB, and AA + EB + chitosan showed dark toxicity effect on biofilm cells of AB2 clinical isolate.

## Cytotoxicity and Phototoxicity of the Mixture of EB + AA + Ch on Human Fibroblasts

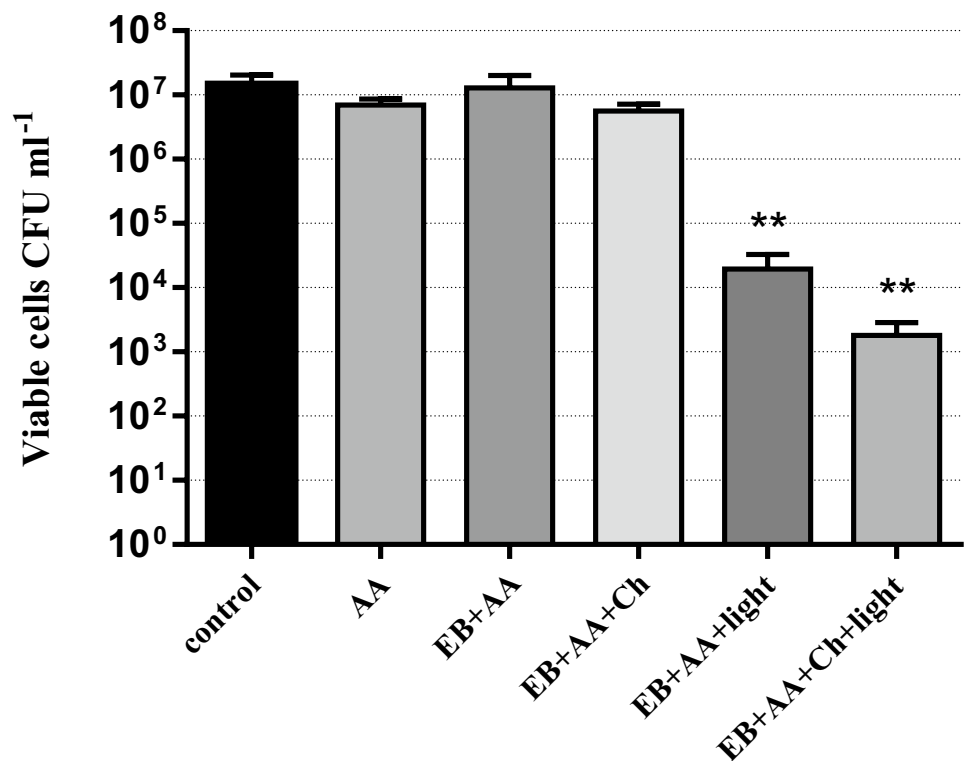
As shown in Fig. 5, exposure of fibroblasts to green light (80 J/cm<sup>2</sup>) in the presence of erythrosine B, acetic acid, and chitosan (EB + AA + Ch) did not induce significant cytotoxicity ( $p > 0.05$ ); also, incubation of fibroblasts with EB + AA + Ch without illumination did not significantly influence viability of fibroblast cells.

## Discussion

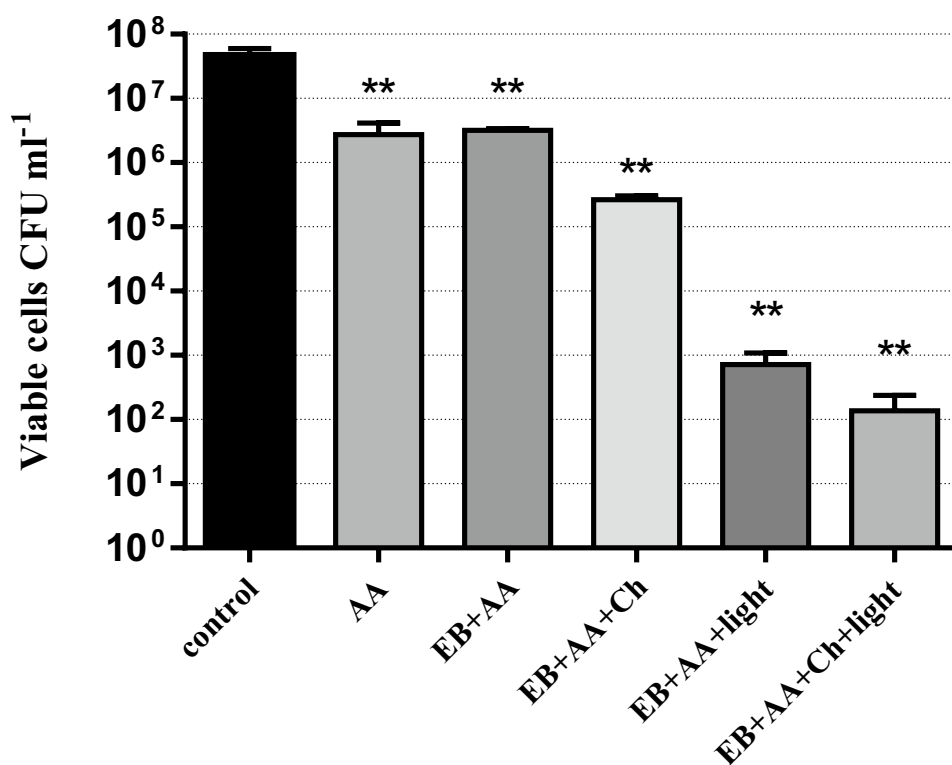
*A. baumannii* causes diverse nosocomial infections and its increased antibiotic resistance aroused the interest of biomedical researchers. The virulence factors including the ability to form biofilm and survive in harsh environmental conditions have created challenges in the management of critically ill patients in hospital intensive care units (ICUs) [18].

The optimal treatment for multidrug-resistant *A. baumannii* hospital-acquired infections has not been established. So, there is a serious demand for new therapeutic approaches for efficient treatment of planktonic as well as biofilm cells. PDI targets a broad-spectrum of microorganisms, independently of their antimicrobial resistance profiles and the effect of photodynamic inactivation (PDI) on *A. baumannii* has been reported previously [19–21]. To the best of our knowledge,

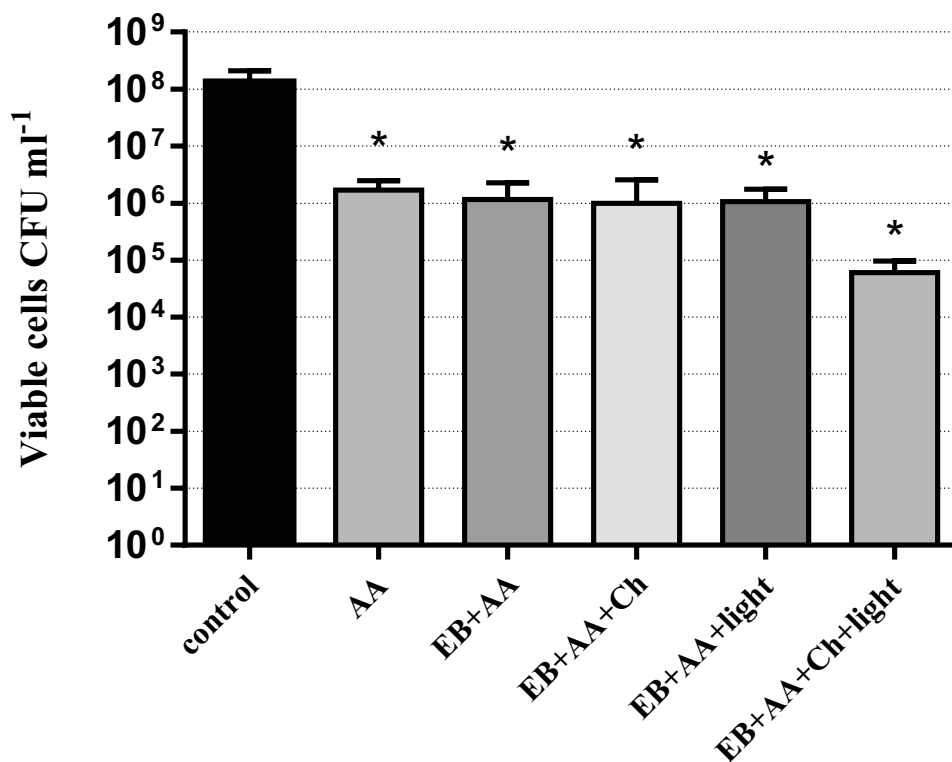
**Fig. 2** Photodynamic inactivation of *A. baumannii* ATCC BAA 747 biofilm. Control: untreated group, AA: acetic acid (0.01%), EB: 100  $\mu$ M erythrosine, Ch: chitosan ( $\frac{1}{2}$  MIC), light: 80 J/cm<sup>2</sup>. \*\*Significant at  $p < 0.005$



**Fig. 3** Photodynamic inactivation of *A. baumannii* AB1 biofilm. Control: untreated group, AA: acetic acid (0.01%), EB: 100  $\mu$ M erythrosine, Ch: chitosan ( $\frac{1}{2}$  MIC), light: 80 J/cm<sup>2</sup>. \*\*Significant at  $p < 0.005$



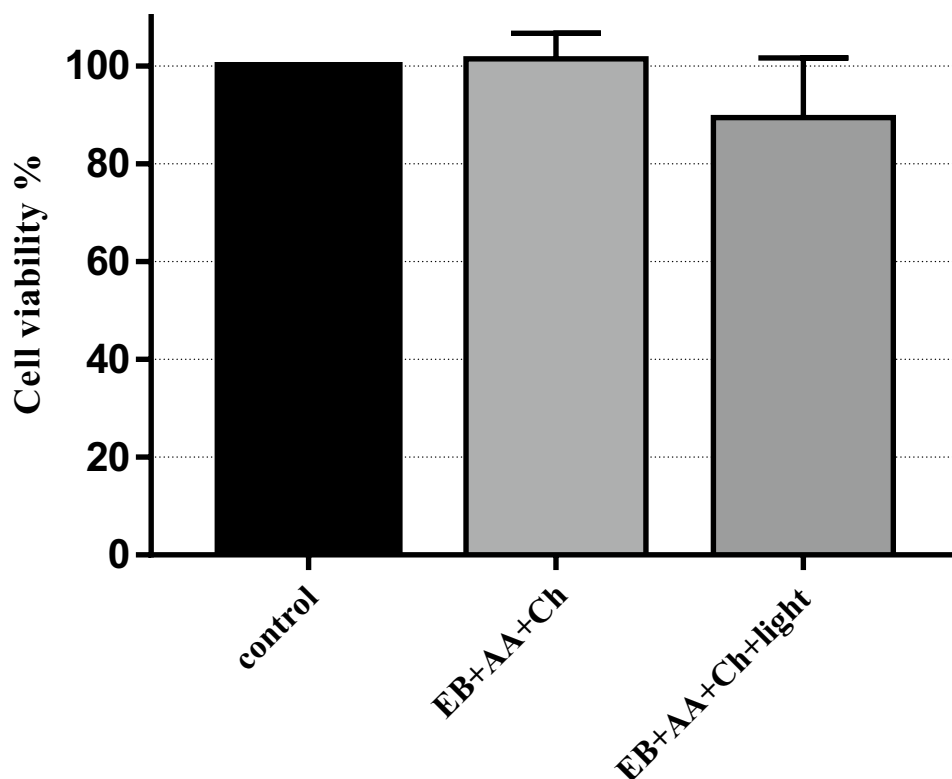
**Fig. 4** Photodynamic inactivation of *A. baumannii* AB2 biofilm. Control: untreated group, AA: acetic acid (0.01%), EB: 100  $\mu$ M erythrosine, Ch: chitosan ( $\frac{1}{2}$  MIC), light: 80 J/cm<sup>2</sup>. \*\*Significant at  $p < 0.005$



EB-mediated PDI has not been employed against *A. baumannii*. In this study, antibacterial effects of EB-mediated PDI against *A. baumannii* planktonic and biofilm cells were

evaluated. PDI using EB (range 50–400  $\mu$ M) did not cause any reduction in the numbers of CFU/ml of planktonic cells in comparison to the control group. Gram-positive bacteria

**Fig. 5** Cell viability of human fibroblasts following treatment with EB + AA + Ch in the presence and absence of light. Control: untreated group, AA: acetic acid (0.01%), EB: 100  $\mu$ M erythrosine, Ch: chitosan ( $\frac{1}{2}$  MIC), light: 80 J/cm<sup>2</sup>



are susceptible to EB-mediated PDI [22, 23], while gram-negative bacteria, such as *A. baumannii*, are less susceptible [24] or resistant to EB-mediated PDI [25, 26]. Gram-positive bacteria have a permeable cell wall that does not restrict the penetration of PSs. In contrast, the cell envelope of gram-negative bacteria consists of an outer membrane which makes a barrier (physical and functional) between the cell and its environment and thus many of the commonly used PS in PDI could not penetrate this gram-negative barrier. Furthermore, the outer membrane of gram-negative bacteria is impermeable to anionic compounds due to its negative surface charge [27].

PSs bearing an anionic charge such as EB need agents that increase their penetration into the outer membrane of gram-negative bacteria. So we examine the effect of acetic acid (0.01%) on the erythrosine-mediated PDI of *A. baumannii*. The results showed the addition of acetic acid (0.01%) to EB, potentiated the PDI against *A. baumannii* cells. The planktonic cells of *A. baumannii* were almost eradicated by illumination in the presence of EB and AA. The mechanisms behind the additive effect of acetic acid on EB-mediated PDI are unknown. It seems that the low pH (pH < 3.5) associated with the presence of acetic acid can cause the formation of erythrosine monoanionic form that would be potentially more effective than dianionic form in the PDI efficacy of gram-negative bacteria [28]. In fact, the monoanionic form of erythrosine possesses a higher binding affinity to the bacterial outer membrane than dianionic form.

The augmentation effect of acetic acid on EB-mediated PDI was not enough to cause lethal effect on *A. baumannii* biofilm cells. Microcolonies as the basic structural unit of biofilms are usually composed of 10–25% cells and 75–90% extracellular polymeric substances (EPS); EPS can serve as a mechanical barrier for the penetration of the photosensitizer and the diffusion of light [29, 30]. Thus, agents that affect the biofilm's structural organization would allow better penetration of both photosensitizer and light into the biofilm structure and result in a greater photodynamic efficacy. In this study, chitosan enhanced the anti-biofilm efficacy of erythrosine B-mediated PDI against *A. baumannii*. Enhancement of the efficacy of EB-mediated PDI using chitosan might be attributed to the following three reasons: (1) the ability of chitosan to disrupt the biofilm structure and thus helping the PDI to act on the cells that are released from the biofilm, (2) the ability of chitosan to permeabilize the outer membrane of gram-negative bacteria such as *A. baumannii*, and (3) the ability of cationic chitosan to act as a drug carrier for delivery of erythrosine to biofilm [12, 31, 32].

For clinical use, an ideal PDI should produce little effect on mammalian tissues in the area of infection. So, the cytotoxicity and phototoxicity of the mixture of chitosan + acetic acid + erythrosine were assessed on human fibroblast cells that are involved in wound healing process. The mixture of EB + AA + Ch with and without illumination did not significantly influence on cell viability and thus this mixture has the potential for clinical use in antimicrobial treatment.



## Conclusions

PDI mediated by erythrosine + acetic acid can cause a lethal effect on *A. baumannii* in planktonic form while the lethal effect of PDI on *A. baumannii* (ATCC BAA 747 and AB2) biofilm can be achieved only by adding chitosan to EB in the presence of acetic acid. Our results also provide evidence that fibroblast cells can survive under PDI conditions (used in this study) that are lethal to *A. baumannii* cells. In summary, PDI mediated by the mixture of erythrosine, acetic acid, and chitosan can be considered as a new therapeutic option for biofilm-related wound infections caused by multidrug-resistant *A. baumannii*.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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