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Comparison of the effect of ethanol and potassium iodide in antibacterial photodynamic therapy on gram negative pathogens

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ABSTRACT

Objectives: Antibiotics is the most common treatment for bacterial infections. However, bacteria can change their genetic material, develop antibiotic resistance and cannot be treated. This brings the need for new treatment methods. Antibacterial Photodynamic Therapy is becoming a promising approach to treat bacterial infections. It is based on the use of photosensitizer to be activated by light with an appropriate wavelength and it will result in reactive oxygen species which are responsible for the destruction of pathogens. In this study, Antibacterial Photodynamic Therapy was examined for the treatment of *Pseudomonas aeruginosa* with the help of ethanol and potassium iodide.

Methods: The effect of Chlorin e6-based photoinactivation was studied on Multidrug resistant *P. aeruginosa* upon irradiation with 655-nm diode laser. Then ethanol and potassium iodide was added to the mechanism separately to increase the efficacy of photoinactivation. After each application, serial dilution method was used for the determination of viable cells.

Results: Outcomes showed that only Antibacterial Photodynamic Therapy causes a mortality rate of 75%. Addition of ethanol causes a mortality rate of 93% and addition of potassium iodide causes a mortality rate of 99.9% with less amount of Chlorin e6 and light dose.

Conclusions: Chlorin-e6 based photoinactivation did not provide high mortality rate on *P. aeruginosa.* The use of ethanol and potassium iodide increased the effect of photoinactivation. The highest mortality rate was obtained with potassium iodide. It was understood that potassium iodide was a better concomitant agent to increase the bactericidal effect of Antibacterial Photodynamic Therapy on *P. aeruginosa.*

Keywords: Antibacterial photodynamic therapy, chlorin e6, ethanol, potassium iodide, *pseudomonas aeruginosa*

Bacteria are single cell organisms that can infect and contaminate any part of the body. Bacteria are generally classified as gram positive and gram negative. While gram positive bacteria have a thick cell wall, gram negative bacteria have a thin cell wall. However, gram-negative bacteria are usually more harmful because their cell wall is tighter and their outer membrane is hidden by a capsule. If any treatment is not applied, these bacterial strains can even cause death in progressive stages [1]. The most com-

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mon treatment of infections is the use of antibiotics. But, excessive use and misuse of antibiotics result in multidrug resistance which makes antibiotics become nonfunctional against multidrug resistant bacteria via horizontal gene transfer, mutations, etc. [2].

The resistance of pathogenic microorganisms to antibiotics has led to the search for alternative treatment methods for local infections [3]. A potential alternative is antibacterial photodynamic therapy (aPDT) that is defined as photoinactivation of microorganisms via reactive oxygen species that are produced by photosensitizers (PS) activated with appropriate wavelength of light. The best known advantage of aPDT is that both antibiotic-susceptible and resistant bacterial strains do not resist to photoinactivation and there is no possibility to induce resistance to aPDT after repetitive applications [4].

Strong light absorption capacity and high quantum yield of reactive oxygen species are the desired characteristics of an ideal PSs used in aPDT. Other desired properties are to show low toxicity and target selectivity. The wavelength of the light to be used must be selected according to the absorption spectrum of the PS. If the wavelength of the light reaches its peak value in the absorption spectrum of PS, the effect of the reaction will be high. In order to obtain the ideal therapeutic effect, the energy dose of light and the concentration of PS should be properly optimized during the applications [5]. aPDT leads to progressive lipid peroxidation by free radical attack on bacteria. This leads to cell death by the formation of lipid radicals, the elimination of cellular lipids resulting in oxidative damage to the cells. [4-7].

Chlorin e6 (Ce6) is a second-generation PS which is low toxic, easily synthesized, selectively accumulated in target tissue, and has high photosensitizing efficacy [8-10]. Ce6 absorbs 630-680 nm of wavelengths strongly [10]. For this reason, red part of the visible spectrum is an ideal choice to activate Ce6 [11]. Besides, the wavelengths around 600-nm is in therapeutic window in which the wavelengths can be safely used for the treatment of various diseases without giving serious harm to the biological tissues. Because of the limited absorption capacity of the biological tissue towards these wavelengths, they can travel deeper inside the biological tissues until its target without losing its energy. These features of the wavelengths in the therapeutic window makes them favorable in the therapeutic light applications [12, 13]. Ce6 is more effective at destroying gram-positive bacteria, sometimes it needs some additional strategies to overcome problems related to gram-negative species [3]. The use ethanol (EtOH) as an adjuvant can be a good strategy to enhance the effect of PS. It is known that EtOH only at low concentrations (0.075% - 0.01%) slows the growth rate of bacteria in stationary phase [14]. In other studies, unlike water, the use of solvents with less polarity such as EtOH enhances the effect of aPDT on biofilms [15]. In the direction of this information, the use of EtOH as an adjuvant was thought to provide an advantage to increase the effect of PS.

Another possibility to potentiate the effect of aPDT is the addition of inorganic salt during applications. One of these inorganic salts is potassium iodide (KI). It has been shown that the mechanism of action of KI involves electron transfer reactions to produce iodine and molecular radicals [16]. KI could also potentiate aPDT to produce more reactive species [17]. This reaction seems to have singlet oxygens to produce peroxy iodide, which is subsequently converted into molecular iodine and hydrogen peroxide. A mixture of extracellular free iodine, and reactive iodine radicals causes bacterial killing depending on the degree of PS binding to the microbial cells [18]. In brief, KI can potentiate the effect of photoinactivation of microorganisms. If the PS can bind to the microbial cells and be activated by light absorption in the presence of KI, this mechanism results in short lived reactive iodine species and then molecular iodine produced as a result of singlet oxygen-mediated oxidation of iodide causes bacterial killing [19].

The aim of this study was to investigate and compare the effect of EtOH and KI on the photoinactivation of *Pseudomonas aeruginosa* in the presence of Ce6 irradiated by 655-nm diode laser.

METHODS

In this study, photoinactivation efficacy of Ce6 and 655-nm diode laser was examined on multidrug resistant *P. aeruginosa* strain. Then EtOH and KI was used to increase the bactericidal effect of aPDT. Here is a list of groups which were formed and analyzed during these experiments.

1. Control group: The group which not received Ce6, KI, EtOH and laser treatment
2. Ce6 group: The group incubated only with Ce6
3. Laser group: The group treated only with laser light
4. KI group: The group incubated only with KI
5. Ce6 + KI group: The group incubated with KI and Ce6
6. EtOH group: The group incubated only with EtOH
7. Ce6 + EtOH group: The group incubated with Ce6 solution containing EtOH
8. aPDT group: The group incubated with Ce6 and irradiated by laser light
9. aPDT + KI group: The group incubated with Ce6 and KI and irradiated by laser light
10. aPDT + EtOH: The group incubated with Ce6 containing EtOH and then irradiated by laser light.

Bacteria

Multidrug resistant clinical isolate of *P. aeruginosa* was used as gram negative pathogen. Stock bacteria was incubated in 5 ml Tryptic Soy Broth allowing them to grow overnight at 37°C. Then they were centrifuged, the supernatant was discarded. Bacterial pellet was dissolved in phosphate buffered saline (PBS) to obtain a concentration of 108 CFU/ml and this bacterial solution was used in experiments.

Chemicals

Ce6 (Santa Cruz Biotechnology, Dallas, TX, USA) was used as PS. Fresh solutions in PBS were prepared at specific concentrations before each experiment and kept at dark to prevent photobleaching. 1, 2.5, 250 and 500 µM concentrations were examined throughout this study. These concentrations of Ce6 were determined according to our preliminary experiments. EtOH was used as an adjuvant to increase the effect of Ce6. The Ce6 solution was prepared together with absolute EtOH to obtain a solution containing 20% EtOH. The concentration of EtOH was defined as 20%, because it was proved previously that this concentration was effective to enhance the photodynamic action on pathogens [11]. KI pellets were dissolved in sterile dH2O to obtain 100 mM KI solution and it was used as a potentiator in aPDT. Similarly, 100 mM concentration of KI was also proved as a potentiator for the effective photoinactivation of different bacterial species [20].

Laser Device

In this study, a diode laser (PS4 III.LED, Changchun New Industries Optoelectronics Co. Ltd., Changchun, China) which emits 655-nm of wavelength was used. This wavelength is in the range of the highest absorption band of the Ce6. The output power of this laser device is adjustable and was set to 200 mW for photoinactivation. Depending on our preliminary experiments, 200 mW was found quite safe when it was applied alone. For this reason, it was chosen as an output power and the application times were adjusted manually to obtain different values of energy densities (10, 25, 50, 100, 150 and 200 J/cm^2) which was examined during the laser applications.

Experimental Procedure

In the experiments, each group was studied in 96-well plates as 3 samples and repeated at least three times. 50 µL bacterial solutions were added to the specific wells where light was irradiated on them. Then;

- 50 µL bacterial solution was mixed with equal volume of PBS in Control and only Laser groups.
- 50 µL bacterial solution was mixed with 50 µL of Ce6 in only Ce6, Ce6+KI, aPDT and aPDT+KI groups.
- 50 µL bacterial solution was mixed with 50 µL KI in only KI, Ce6+KI and aPDT+KI groups.
- 50 µL bacterial solution was mixed with 50 µL Ce6 solution containing 20% EtOH in only EtOH, Ce6+EtOH and aPDT+EtOH groups.
- The bacteria were incubated with specific solution in each experimental group for 15 minutes.
- Bacterial solutions were treated with laser light in only Laser, aPDT, aPDT+KI and aPDT+EtOH groups.
- After each of these applications, viable bacterial cell count was determined by serial dilution method.

Statistical Analysis

Data collected from each group after colony counting was normalized by the data of control groups. Normalized data were statistically analyzed by two-tailed Student's t-test. A p value which was smaller than 0.05 was determined as statistically significant.

RESULTS

The Effect of Different Energy Doses on P. aeruginosa

10, 25, 50, 100, 150 and 200 J/cm^2 energy doses were used in Laser and aPDT groups. First of all, the effect of these energy doses was examined on bacteria without addition of any chemicals. As shown in Figure 1, the maximum bactericidal effect of only laser irradiation was obtained with 150 J/cm^2 and resulted in approximately 30% decrease in cell viability. And still none of these results were statistically significant when they were compared with control group (Fig. 1).

The Photoinactivation with Chlorin e6 on P. aeruginosa

Throughout this study different Ce6 concentration (1, 2.5, 250 and 500 µM) was examined in aPDT groups. The maximum mortality rate was obtained with 500 µM Ce6 concentration. It reduced the cell viability nearly 50% and only this concentration showed a significant bactericidal effect (Fig. 2). In aPDT groups, 250 and 500 µM Ce6 were studied together with 50, 100, 150 and 200 J/cm^2 of energy doses. 100 J/cm^2 of energy dose together with 500 µM Ce6 provided maximum photoinactivation of *P. aeruginosa.* As shown in Figure. 2, the maximum reduction in bacterial cell viability with 100 J/cm^2 of energy dose and 500 µM Ce6 was nearly 75%. This effect was quite high when compared with other parameters that was used and it resulted in a statistical significant reduction when compared with the control group. Unexpectedly, higher energy doses with the same Ce6 concentration did not provide similar or higher bactericidal effect (Fig. 2).

The effect of Ethanol in Photoinactivation with Ce6 on P. aeruginosa

Ce6 solution was mixed with absolute EtOH to obtain 250 µM Ce6 concentration in a 20% EtOH solution. The effect of this combination was examined together with 4 different laser energy doses (50, 100, 150 and 200 J/cm^2). 20% EtOH solution decreased bacterial cell viability nearly 55%. When it was mixed with 250 µM Ce6, its bactericidal effect was not high as in the group of 20% EtOH only and both of these groups resulted in significant effects. Then this solution was used in aPDT groups. Maximum reduction was obtained with an energy dose of 200 J/cm^2 that resulted in 93% cell death and it was a statistical significant antibacterial effect (Fig. 3).

The effect of Potassium Iodide in Photoinactivation with Ce6 on P. aeruginosa

In the last part of this study, 100 mM KI was used as a source reactive oxygen/iodine species to increase the effect of aPDT with Ce6. In the aPDT+KI group, firstly 500 and 250 µM Ce6 concentrations were used together with 100 and 200 J/cm^2 light energy doses re-

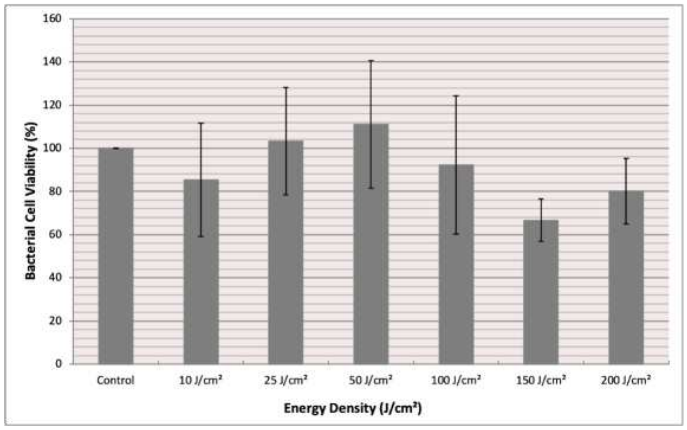

Fig. 1. Effect of light doses on the viability of bacterial cells. Cell viability for *P. aeruginosa* strains was assessed by colony counting after only laser applications and the number of viable cells was normalized with control group (Light dose: 10, 25, 50, 100, 150, and 200 J/cm2). * shows the statistical significance with respect to control group ($p \leq 0.05$).

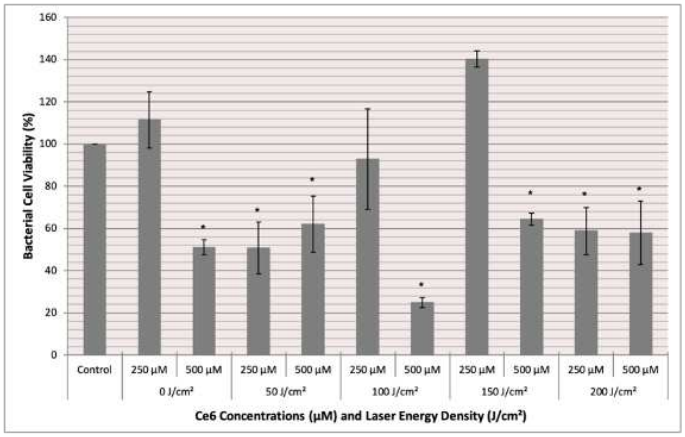

Fig. 2. Effect of Ce6 concentrations and PDT parameters on the viability of bacterial cells. Cell viability for *P. aeruginosa* strains was assessed by colony counting after Ce6 only and PDT applications and the number of viable cells in each experimental group was normalized with control group (Light dose: 50, 100, 150, and 200 J/cm2 and Ce6 concentrations: 250 and 500 µM. * shows the statistical significance with respect to control group ($p \leq 0.05$).

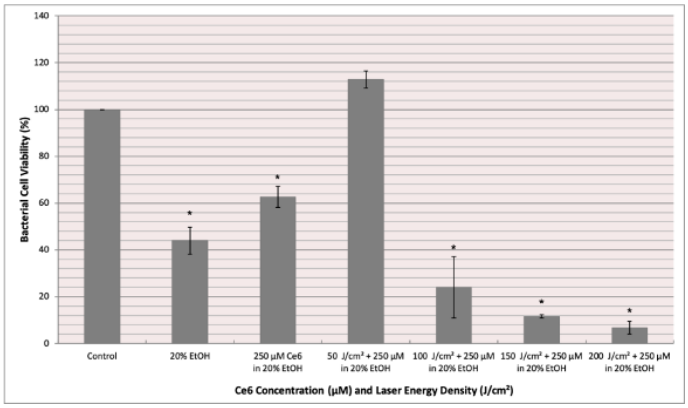

Fig. 3. Effect of 20% EtOH, 250 µM Ce6 in 20% EtOH and different PDT-EtOH parameters on the viability of bacterial cells. Cell viability for *P. aeruginosa* strains was assessed by colony counting after EtOH only, 250 µM Ce6 in 20% EtOH only and PDT-EtOH applications and the number of viable cells in each experimental group was normalized with control group (Light dose: 50, 100, 150, and 200 J/cm2 and Ce6 concentration: 250 µM). * shows the statistical significance with respect to control group ($p \leq 0.05$)

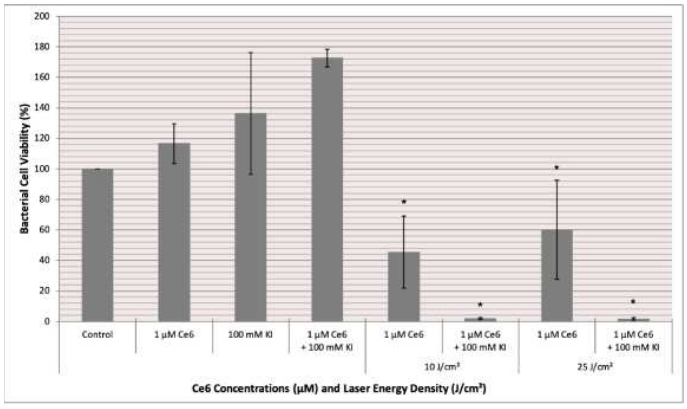

Fig. 4. 4. Effect of 100 mM KI, 1 µM Ce6 and different PDT-KI parameters on the viability of bacterial cells. Cell viability for *P. aeruginosa* strains was assessed by colony counting after 100 mM KI only, 1 µM Ce6 in PBS only and PDT-KI applications and the number of viable cells in each experimental group was normalized with control group (Light dose: 10 and 25 J/cm2, Ce6 concentration: 1 µM and KI concentration: 100 mM). * shows the statistical significance with respect to control group ($p \leq 0.05$)

spectively. These applications resulted in 100% mortality rate (data not shown). To find the optimum parameters that cause nearly 99.9% with nontoxic Ce6 concentration and lower energy doses, these parameters were decreased to 1 and 2.5 µM Ce6 and 10 and 25 J/cm2 energy doses and used together with 100 mM KI. As shown in Figure 4, 1 µM Ce6 was examined with 10 and 20 J/cm2 energy dose. These applications resulted in more than 98% decrease in cell viability (Fig. 4).

Then the concentration of Ce6 was increased to 2.5 µM and used with 10 and 20 J/cm^2 energy dose in the presence of 100 mM KI. The effect of 2.5 µM Ce6 with 10 J/cm^2 energy dose in the presence of 100 mM KI was again 98%. The highest mortality rate was observed with 99.97% when 25 J/cm^2 of energy dose used together with 2.5 µM Ce6 in the presence of 100 mM KI. The effect of 100 mM KI with and without these Ce6 concentrations showed no bactericidal effect (Fig. 5).

DISCUSSION

aPDT has become a promising tool to combat bacterial infections recently and Ce6 has many advantageous characteristics against pathogens which has been proved in many studies [21]. Therefore, aPDT with Ce6 was examined in this study for the treatment of gram-negative pathogens which is hard to be destroyed and need higher doses of PS and light intensities to be photoinactivated [22]. When the low concentrations of Ce6 were evaluated on *P. aeruginosa,* they did not exhibit any dark toxicity. But 500

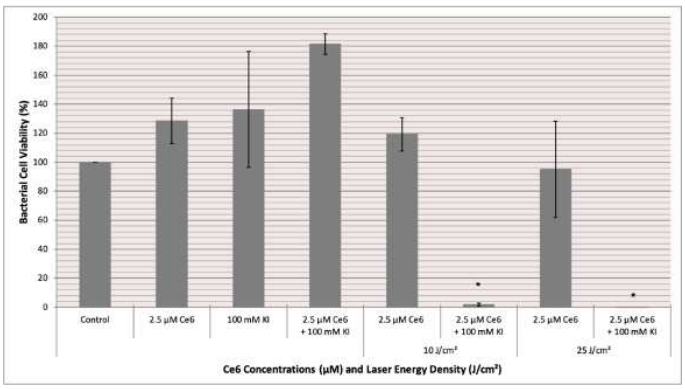

Fig. 4. Effect of 100 mM KI, 2.5 µM Ce6 and different PDT-KI parameters on the viability of bacterial cells. Cell viability for *P. aeruginosa* strains was assessed by colony counting after 100 mM KI only, 2.5 µM Ce6 in PBS only and PDT-KI applications and the number of viable cells in each experimental group was normalized with control group (Light dose: 10 and 25 J/cm2, Ce6 concentrations: 2.5 µM and KI concentration: 100 mM). * shows the statistical significance with respect to control group ($p \leq 0.05$)

μM Ce6 caused nearly 50% reduction in cell viability which can be considered as a high dark toxicity and this cannot support idea of aPDT with less side effect in neighboring areas of biological tissues [23].

On the other hand, only laser light with various intensities ranging from 10 to 200 J/cm^2 did not result in meaningful reduction in the cell viability. The maximum mortality rate of 35% was obtained at the light energy dose of 150 J/cm^2. Thus, it can be explained that only laser application was not harmful on bacterial cell viability and it is a well-known characteristics of red light on cells [24].

Although the use of Ce6 was a promising drug in aPDT applications [3], our experiments showed that it was ineffective on the multidrug resistant *P. aeruginosa* strain. Even though light energy dose and drug concentration were increased in serious amounts, killing more than 99% was not obtained. The maximum reduction in cell viability nearly 75% was reached in the application of 100 J/cm^2 light dose and 500 μM Ce6 concentration (Fig. 2). According to many studies, it is shown that Gram negative bacteria opposed to aPDT due to their special wall structure [25-27]. Gram negative bacteria have a complicated many layered and hard-spun outer barrier structures [28]. This barrier excludes most of the PS, thus certain methods have to be used to confirm that PS can penetrate the bacteria and be effective on them [27]. *P. aeruginosa* strain which was used in this study cannot be destroyed efficiently solely using Ce6 in aPDT application. Therefore, this therapy needed some concomitant agents to amplify its activity.

Firstly, the effect of EtOH was examined as an adjuvant in order to increase the effect of the PS. The goal in this part of the study was to reduce the the drug concentration in a serious manner and achieve a mortality rate over 99%. 20% EtOH was chosen as a concentration to act as an adjuvant in aPDT application. Its antibacterial effect without Ce6 and light application was not significant, but still quite high. It reduced bacterial cell viability approximately 55%. Although an important decrease (around 93%) in cell viability with 20% EtOH in aPDT was obtained with the parameters of 200 J/cm2 energy dose and 250 μM Ce6, sufficient amount of antibacterial effect cannot be achieved (Fig. 3). The only advantage of using 20% EtOH as an adjuvant was to reduce the concentration of Ce6 to its half. The light dose has been increased to 200 J/cm2 when this was compared with only aPDT application. Using EtOH against P.aeruginosa raised antimicrobial effect of aPDT. This application exerts antibacterial activity including more production of singlet oxygen, destroying bacterial membrane, rapid denaturation of proteins, causing to cell lysis [29, 30]. But still the energy dose and PS concentration were quite high in this application.

Since the desired outcomes were not achieved in the applications of only aPDT and aPDT+EtOH, KI was examined as a potentiator at the last part of this study. Purpose of using KI was to increase effectiveness of PS for the production of more radical ions. For this reason, 100 mM KI concentration was chosen as an optimum parameter after literature survey [16-19]. When the effects of only KI and KI together with Ce6 were studied, the results showed that they had no lethal effects on the bacteria. Only KI treatment has increased the bacterial cell viability with a rate of 32% instead. Then, the effect of 100 and 200 J/cm2 light dose and 500 and 2500 μM ce6 concentrations respectively was evaluated together with 100 mM KI. This resulted in 100% mortality rate (data not shown). Then the concentration of Ce6 and light dose were reduced to obtain optimum energy dose and PS concentration for achieving a mortality rate of 99.9%. The highest mortality rate was 99.9%, when 25 J/cm2 light dose and 2.5 μM Ce6 were used together with 100 mM KI (Fig. 5). Results showed that nontoxic inorganic salt KI potentiates aPDT by increasing the effectiveness of Ce6 which was proved previously with different types of PSs on different strains of bacteria [17, 31, 32]. The presence of KI forms short-lived reactive iodine species and they induce the generation of more reactive species. Then these reactive iodine species damage bacterial cells [32, 33]. Use of 100 mM KI provides the opportunity to decrease the level of energy dose and PS concentration in serious levels. So, it eliminates the risk of dark toxicity and photodestruction depending upon the use of high level of PS and light dose.

CONCLUSION

Although different light doses of 655-nm of wavelength and Ce6 concentrations were used in Ce6-based photoinactivation and their levels were increased quite

high values, it did not show efficient bactericidal effect on multidrug resistant *P. aeruginosa*. Therefore, EtOH was used as an adjuvant in aPDT applications to increase the mortality rate. Serious mortality rate was obtained, but it was still not sufficient and the parameters used were still high which may cause cytotoxicity on healthy cells. Then, KI was used as a potentiator in aPDT applications. Over 99.9% mortality rate was obtained with the help of KI. When results of each three different applications were examined, the use of KI resulted in a significant reduction in light dose (4 × reduction) and drug concentration (200 × reduction) by achieving high killing capability on P. aeruginosa which is multidrug resistant gram negative strain.

Authors' Contribution

Study Conception: NTA, MÇ, EB, AK; Study Design: NTA, MÇ, EB, AK; Supervision: NTA, MÇ, EB, AK; Funding: NTA, AK; Materials: NTA, AK; Data Collection and/or Processing: NTA, MÇ, EB; Statistical Analysis and/or Data Interpretation: NTA, MÇ, EB; Literature Review: NTA, MÇ, EB; Manuscript Preparation: NTA, MÇ, EB, AK and Critical Review: NTA, MÇ, EB, AK.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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