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Evaluation of different protocols using antimicrobial photodynamic therapy with curcumin and blue led light control of *Escherichia coli in vitro*

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ABSTRACT. *Escherichia coli* (*E. coli*) is a gram-negative bacterium extremely relevant in the context of public health, being involved in the development of several diseases worldwide. The main treatment available for infections caused by *E. coli* is the use of antibiotics. However, several strains of this bacterium have shown resistance to available antibiotics, necessitating the development of alternative treatment methodologies. In this scenario, Antimicrobial Photodynamic Therapy (aPDT) emerges as an excellent viable alternative to antibiotic use. This study aimed to standardize protocols based on aPDT utilizing curcumin as photosensitizer activated by blue LED light for the control of *E. coli in vitro*. Curcumin solutions of 60, 80, and 100 μg mL^{-1} were made and photoactivated by blue LED light with fluencies of 6.6, 13.2, and 19.8 J cm^{-2}. *E. coli* (ATCC® 25922 ™) was cultured for 24 hours at 37 °C. Bacterial suspensions of 1.5 x 10^6 CFU mL^{-1} were exposed to aPDT treatments. Control groups were established for each light dose and curcumin concentration. Protocols using 80 and 100 μg mL^{-1} curcumin solution with19.8 J cm^{-2} irradiation effectively inhibited bacterial growth, suggesting significant antibacterial activity (p-value < 0.05), and are recommended for this purpose.

Keywords: microbiology; photochemotherapy; optics and photonics.

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Introduction

Escherichia coli is a Gram-negative and aerobic facultative bacillus belonging to Enterobacteriaceae family, commonly found in the microbiota of mammals, and residing commensally in the small intestine (Jang et al., 2017). Although *E. coli* is typically part of the human microbiota, some strains have pathogenic potential and can cause several diseases such as urinary infections, diarrhea, gastrointestinal infections, and extraintestinal infections such as mastitis, meningitis, and sepsis (Balière, Rincé, Delannoy, Fach, & Gourmelon, 2016; Jang et al., 2017). Among pathogenic *E. coli* strains, enteropathogenic (EPEC), uropathogenic (UPEC) and Shiga toxin (STEC) producers are the main strains responsible for disease in humans (Balière et al., 2016). Diarrhea caused by *E. coli* results in approximately 700-800 million cases annually, leading to the death of 300,000 to 500,000 people worldwide each year (Kim, Cho, & Rhee, 2017). Urinary tract infections are also primarily caused by *E. coli* and affect over 150 million people annually in the United States (Flores-Mireles, Walker, Caparon, & Hultgren, 2015).

The conventional treatment for infections caused by *E. coli* consists involves the use of antibiotics. However, due to the misuse of antibiotics, many strains of *E. coli* have developed resistance to available antibiotics by selective genetic pressure and horizontal gene transfer among them (Alekish, Ismail, Albiss, & Nawasrah, 2018). These changes enhance the bacteria's ability to evade and resist these medications, increasing the need for alternative treatments to combat such infections (Martinez, 2014). In this context, many studies have displayed the bactericidal effect of antimicrobial photodynamic therapy (aPDT) and its

advantages as low toxicity to host cells, low cost, easy of application, and no reported antimicrobial resistance (Zhang, Zhang, Tang, Xu, & Meng, 2018; Santos et al., 2019a, 2019b; Muniz et al., 2021).

The aPDT technique consists of the photoactivation of a photosensitizer (PS) compound which can generate Reactive Oxygen Species (ROS). ROS production can occur through two mechanisms: Type I and type II. Type 1 reactions occur when the excited PS interacts with cellular molecules, producing ROS such as superoxide anion. Type II mechanisms occur when the excited PS interacts with molecular oxygen (Allison & Moghissi, 2013; Mansoori et al., 2019). These species cause a lethal cytotoxic effect through reactions with the cell membrane, biomolecules of the cytoplasm, and nucleic acids present in bacteria (Giannelli et al., 2017).

An ideal photosensitizer should be able to generate free radicals, associating with bacterial cells, and exhibiting high phototoxicity while maintaining low toxicity in the dark (Tim, 2015). Curcumin, a compound extracted from *Curcuma longa sp*., shows interesting properties for the use as a photosensitizer in aPDT, as reported in several studies (Ellerkamp et al., 2016; Almeida et al., 2017; Theodoro et al., 2017; Muniz et al., 2021). Curcumin's optimal light absorption range is between 300-550 nm, with a peak around 430 nm. When associated with blue LED (Light Emitting Diode) light which emits in the range of approximately 405-470 nm, curcumin proves to be an effective photosensitizer in aPDT technique (Araújo, Fontana, Bagnato, & Gerbi, 2014).

Given the importance of aPDT as an antimicrobial alternative to the use of antibiotics in the treatment of *E. coli*, the study aimed to standardize protocols of aPDT using different concentrations of curcumin and different doses of blue LED light for the inactivation of *E. coli in vitro*.

Material and methods

Determination of bacterial load

Escherichia coli (ATCC® 25922 ™) was obtained from the Microbiology Laboratory of the *Universidade Federal da Bahia*. The samples were in a -20 ºC freezer. At the time of culturing, the samples were thawed at room temperature, plated on MacConkey agar (Kasvi, Italy), and taken to the incubator (Prolab, São Paulo, Brazil) for 24 hours at 35 ± 2 ºC.

The bacterial load was determined by spectrophotometry. The inoculum preparation was made in a previously sterilized laminar flow (Prolab, São Paulo, Brazil) by removing 3 to 5 colonies from the culture plates, and diluting in 1 mL of sterile saline solution. After this, the solution was transferred to a quartz cuvette for reading in a spectrophotometer (SP-2000 UV, Spectrum, Shanghai, China).

The objective was to obtain 1.5 x 10^8 CFU mL^{-1} according to McFarland nephelometric scale. At this moment, some parameters were observed: 0.135 absorption at 660 nm (0.5 on McFarland scale, equivalent to 1.5 x 10^8 CFU mL^{-1}), Subsequently, serial dilutions were then made utilizing Saline Solution (pH 7.4) to obtain 1.5 x 10^6 CFU mL^{-1}. This final concentration was used for the experimental procedures.

Preparation of curcumin solutions

Curcumin was used as a photosensitizer. A solution containing distilled water (82% by volume), absolute alcohol (10% by volume) (Proquímicos, Bangu, Rio de Janeiro State, Brazil) and Tween 20 (8% by volume) (Isofar, Duque de Caxias, Rio de Janeiro State, Brazil) was prepared to solubilize the curcumin. Three different solutions were prepared with concentrations of 60, 80, and 100 µg mL^{-1}, respectively.

The solutions were prepared in a dark environment to ensure that light did not interfere with the stability of the photosensitizer. All solutions were filtered with syringe filters with 20 µL pores (TPP, Trasadingen, Switzerland) to ensure sterility.

Led light source

The LED device (RGB Biotable, São Paulo, São Paulo State, Brazil) provided a blue LED light emission of approximately 400-500 nm, with a peak at 430 nm ± 20 and energy intensity of 22 mW cm^{-2}. The irradiation times used in the experiment were 5 minutes (300 s), 10 minutes (600 s), or 15 minutes (900 s). The bacterial cells received a total fluency of 6.6, 13.2, or 19.8 J cm^{-2} respectively, according to the tested protocol. To determine such energy fluencies, the light irradiation time was calculated according to the following equation:

$$\text{Fluency } \left(\frac{\text{J}}{\text{cm}^2}\right) = \frac{\text{Power density } \left(\frac{\text{mW}}{\text{cm}^2}\right) \text{ x Irradiation time (seconds)}}{1000}$$

In Vitro experimental design and photodynamic treatment:

Nine aPDT treatment groups were tested and different protocols were evaluated (aPDT1-60, aPDT2-60, aPDT3-60, aPDT1-80, aPDT2-80, aPDT3-80, aPDT1-100, aPDT2-100 and aPDT3-100) (n = 6). Three light control groups (P-L1, P-L2, and P-L3), three curcumin control groups (P+L-60, P+L-80, and P+L-100), and one bacterial control group were also analyzed (P-L-) (n = 6). In total, 16 groups with a total sample size of 96 were tested. All procedures were performed in 24-well plates (TPP, Trasadingen, Switzerland).

Light control groups were tested for each dose of used blue LED light: 6.6, 13.2, or 19.8 J cm^{-2} (P-L1, P-L2, and P-L3, respectively). Three curcumin control groups were tested for each concentration of solution used: 60, 80, or 100 µg mL^{-1} (P+L-60, P+L-80, and P+L-100, respectively). The bacterial control group had only *E. coli* bacterial solution in sterile saline (P-L-). The nine aPDT protocols involved combinations for each concentration of curcumin solution (60, 80, or 100 µg mL^{-1}) at fluencies of 6.6, 13.2, or 19.8 J cm^{-2} in the bacterial treatment, as displayed in Table 1.

Table 1. Antimicrobial photodynamic therapy protocols for the control of *Escherichia coli in vitro*.

Dose of light	Curcumin solution concentration		
	60 µg mL^{-1}	80 µg mL^{-1}	100 µg mL^{-1}
6.6 J cm^{-2}	aPDT1-60	aPDT1-80	aPDT1-100
13.2 J cm^{-2}	aPDT2-60	aPDT2-80	aPDT2-100
19.8 J cm^{-2}	aPDT3-60	aPDT3-80	aPDT3-100

In the bacterial control group, *E. coli* was placed in contact with 1 mL of sterile saline and plated on MacConkey Agar medium through the pour plate technique. In the light groups, the bacteria were placed in 1 mL of sterile saline, exposed to the blue LED light device RGB Biotable (MMOptics, São Carlos, São Paulo State, Brazil), and also plated on MacConkey Agar medium through the pour plate technique. In all groups that we used curcumin (Control curcumin and PDT group), the bacteria were inoculated in 1mL of curcumin solution (60, 80, or 100 µg mL^{-1}) and left in contact with the photosensitizer for 5 minutes (pre-irradiation).

The aPDT protocols consisted of placing the bacteria in contact with the curcumin solutions for a pre-irradiation time of 5 minutes, and then the bacteria were exposed to a blue LED light at different fluencies according to the tested protocol. After treatment, the samples were plated on MacConkey Agar medium using pour plate technique. All plates were cultured at 37 °C for 24 hours. The counting of the colony-forming units was carried out using the ImageJ program (version 1.50b, National Institute Health, USA).

Statistical analysis

The Gaussian distribution of the data was assessed using the Shapiro-Wilk test. With our assessment it was verified that the data did not present a Gaussian distribution. Therefore, the non-parametrical Kruskal-Wallis test and Dunn's post-test were applied by using the GraphPad Prism software (version 7.0, GraphPad Program Inc., San Diego, CA, USA) for analysis. Statistical differences were considered significant at p-value < 0.05 *; p-value < 0.01 **; p-value < 0.001 ***; p-value < 0.0001 ****. To compare the nine PDT protocols tested, a 3D surface plot was created for further visualization using the software R Project for Statistical Computing version 3.4.4 (Lenth, 2009).

Results

When analyzing our aPDT protocols, differences were when these treatments were compared with all control groups (bacteria control group, light group, and non-photoactivated curcumin). Differences were also observed in comparisons between the groups treated with aPDT, as displayed in Figure 1. It is worth pointing out that only the main points assessed will be highlighted to determine the protocols that achieved the greatest reductions regarding in of *E. coli* load.

aPDT protocols decreased *Escherichia coli* bacterial load

In Figure 1a, it is observed that aPDT2-60 protocol showed differences when compared to groups that received only curcumin (P+L-60) (p-value < 0.0001), only light (P-L1) (p-value < 0.01) and control only bacteria (P-L-) (p-value < 0.05). Other aPDT protocols using curcumin solution concentrations of 60 µg mL^{-1} also had differences with these groups but displayed lower antibacterial activity.

In Figure 1b, it is shown that the aPDT3-80 protocol showed differences when compared to groups that received only curcumin (P+L-80) (p-value < 0.0001), only light (P-L1) (p-value < 0.01) and control only bacteria (P-L-) (p-value < 0.05). Other aPDT protocols using curcumin solution with a concentration of 80 μg mL^{-1} also had differences with these groups but exhibited lower efficiency in controlling bacterial load.

In Figure 1c, it is demonstrated that aPDT3-100 protocol exhibited differences when compared to groups that received only curcumin (P+L-80) (p-value < 0.001), only light (P-L1) (p-value < 0.001) and only bacteria (P-L-) (p-value < 0.05), showing a significant reduction of the microbial load. Other aPDT protocols using curcumin solution with a concentration of 100 μg mL^{-1} also displayed differences regarding these groups but presented smaller reductions in bacterial load.

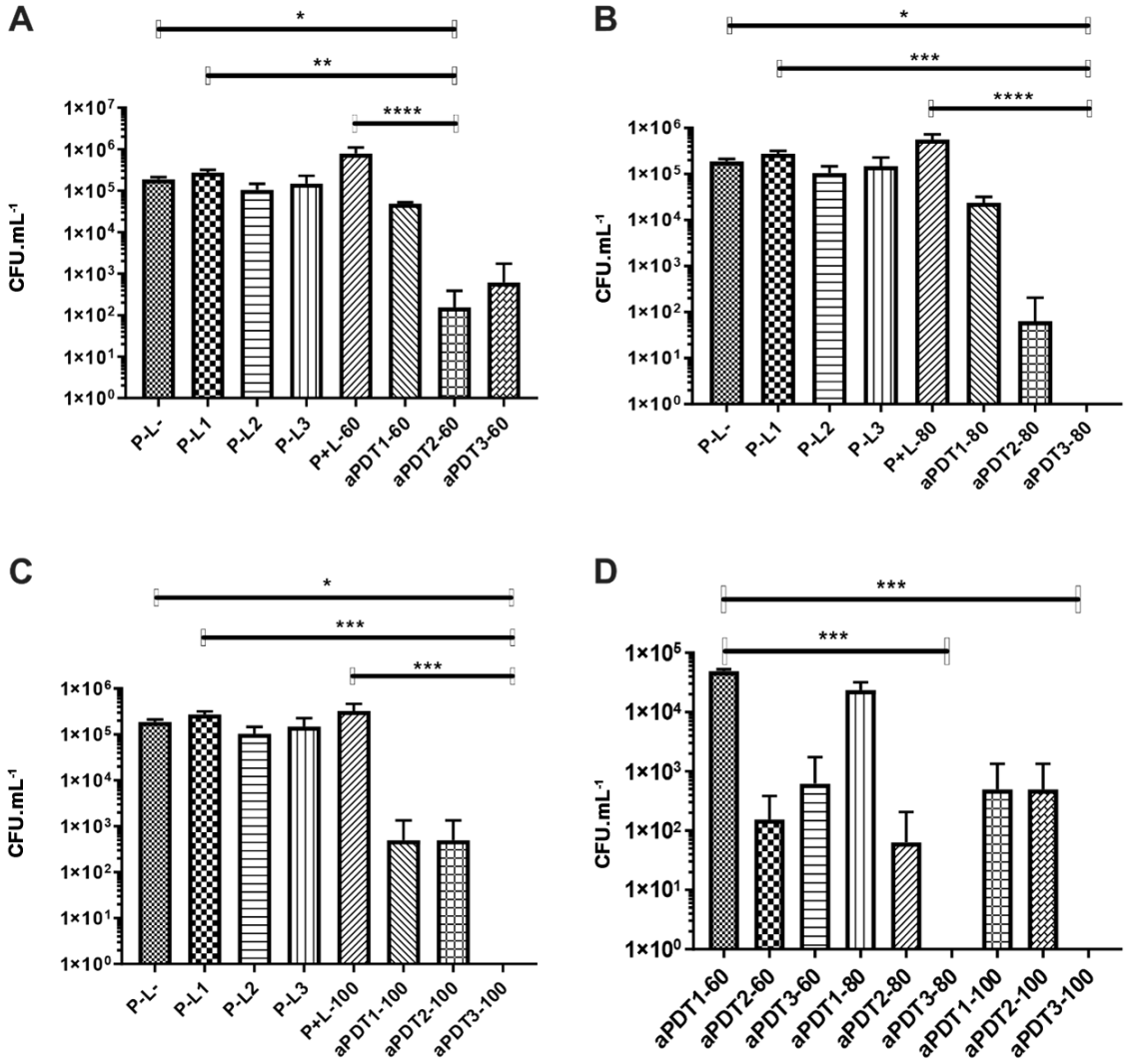

Figure 1. Antimicrobial photodynamic therapy protocols were tested at different concentrations of curcumin photoactivated by different fluencies: P-L1 and aPDT1 (6.6 J cm^{-2}); P-L2 and aPDT2 (13.2 J cm^{-2}); P-L3 and aPDT3 (19.8 J cm^{-2}). Curcumin concentrations of 60 (A), 80 (B), and 100 μg mL^{-1} (C) were tested. After performing the aPDT protocols, the results were compared to each other in order to identify the best aPDT protocol tested (D).

Curcumin or light when used alone were not able to decrease bacterial load

No differences were observed when comparing the bacterial control group (P-L-), the groups that received only light (P-L1, P-L2, and P-L3), and groups with only curcumin (P+L-60, P+L-80, and P+L-100) (Figure 1). This indicates that when blue LED light or curcumin were used separately under these conditions of fluency and concentration, they were not able to decrease the microbial load of *E. coli*.

In Figure 1d, all nine protocols of aPDT were compared among themselves. aPDT3-80 and aPDT3-100 protocols presented differences (p-value < 0.001) in comparison to the aPDT60-1 protocol, which had the lowest efficiency in reducing *E. coli* load.

Figure 2 shows that, with increasing concentrations of curcumin (CC) and light dose (LD), there is a decrease in microbial load based on the colony-forming units (CFU) count. It means that higher concentrations of curcumin and greater light doses, lead to a lower number of colonies.

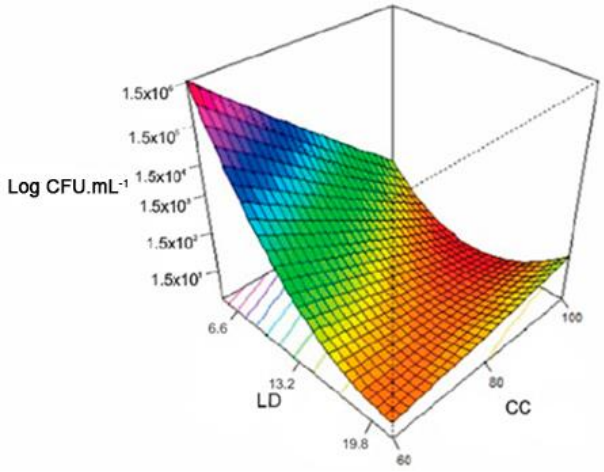

Figure 2. The nine PDT protocols were tested using a 3D surface plot for further visualization using R software. This figure shows the relationship between light dose (LD), curcumin concentration (CC) and bacterial load (Log CFU mL^{-1}). The color scale shows that the closer to the blue color scale, the smaller the reduction in bacterial load, on the other hand, the closer to the red color scale, the greater the reduction in bacterial load.

In Figure 2, the colors ranging from yellow to orange represents significant decreases in the *E. coli* load. This indicates that using a curcumin solution of 100 µg mL^{-1} with a fluency of 6.6 J cm^{-2} (5 min.) we can observe reductions, although them being minimal. With a fluency of 13.2 J cm^{-2} (10 min.) using a curcumin concentration (CC) solution of 60 µg mL^{-1}, a considerable decrease in bacterial load is also verified. The optimal point of aPDT for the control of *E. coli* in this study is represented by the red color and it is in the use of curcumin solutions of 80 and 100 µg mL^{-1}, under fluencies of 13.2 (10 min.) and 19.8 J cm^{-2} (15 min.). This shows an ideal range of different concentrations of curcumin and different doses of light for the reduction or inhibition of viable *E. coli* cells.

Discussion

This research provides valuable insights into determining an ideal aPDT protocol against *E. coli*. This is essential for the advancement of studies that seek to combat *in vivo* infections caused by pathogenic strains of *E. coli* through the application of aPDT, making possible the real applicability of this important alternative methodology for treating infectious diseases. The study found that the most effective protocols were aPDT3-80 (80 µg mL^{-1} of curcumin solution + 19.8 J cm^{-2} of blue LED light fluency) and aPDT3-100 (100 µg mL^{-1} of curcumin solution + 19.8 J cm^{-2} fluency of blue LED light). Thus, such protocols should be considered for studies targeting the treatment infections caused by *E. coli in vivo*.

E. coli is an important pathogen that causes a wide variety of clinical conditions such as intestinal infections, urinary tract infections, bloodstream infections (Johnson & Russo, 2018). The onset of diseases caused by *E. coli* antibiotic-resistant strains hinder the treatment of these infectious conditions, thus showing the need to develop alternative therapies to conventional treatment (Chong, Shimoda, & Shimono, 2018). Our study displayed that aPDT with curcumin and blue LED light effectively reduced the microbial load of *E. coli in vitro* through a dose-dependent manner. Since gram-negative bacteria present more defense mechanisms than gram-positive (Demidova & Hamblin, 2005), it is relevant for the expansion of alternative treatments rather than traditional medicinal therapies.

The efficiency of aPDT antimicrobial activity against *E. coli* may be related to the use of curcumin, a hydrophobic compound (Lee et al., 2013). As a hydrophobic compound, curcumin can penetrate gram-negative bacterial cells, which have a negative charge due to the presence of LPS (Santezi, Reina, & Dovigo, 2018). Therefore, this treatment with curcumin can perform its functions in generating reactive oxygen species after being photoactivated by a blue LED light.

Antimicrobial photodynamic therapy was efficient in reducing the bacterial load in all of the nine protocols of aPDT. Differences in these reductions were observed, indicating that some treatments were more efficient

than others. Higher *E. coli* load reduction was observed when bacterial cells were exposed to aPDT with a fluency of 19.8 J cm^{-2} of blue LED light and in the presence of 80 and 100 µg mL^{-1} curcumin solution concentration. Our results corroborate with other studies using aPDT, given that it has been reported that in the presence of blue LED light a 120 µM concentration of curcumin promoted reduction in the cellular viability of *C. albicans*, *C. glabrata*, and *S. mutans* in biofilm after 24 hours of incubation (Quishida et al., 2016). In addition, it was found that the levels of reactive oxygen species are elevated according to the increase of time and curcumin concentration, causing damage to the cellular structure of the bacteria. At the time of 510s (approximately 10 minutes) and 2 µmol L^{-1} curcumin, concentration, a reduction of 0.95 log in the count of *E. coli* was obtained (Tao et al., 2019).

Recently, aPDT has been found to be effective t in reducing the microbial load of *C. albicans* using curcumin concentrations of 20, 40, and 80 µM. In this study, it was observed that the reduction in *C. albicans* microbial load is associated with the use of curcumin, mainly at 80 µM, along with a light dose of 18 J cm^{-2}, similar to the dose of 19.8 J cm^{-2} that we used. in our study (Dovigo et al., 2013). This fact demonstrates that it is possible that the protocols established here may be applicable to other microorganisms in different anatomical sites. However, more studies are needed to assess the efficacy in different cases.

Other studies have demonstrated the antimicrobial efficiency of curcumin without photoactivation and blue LED light without the presence of photosensitizers. It has been reported that the use of 2000, 4000, and 8000 µM curcumin concentrations or blue LED light doses of 24, 48, and 72 J cm^{-2}, separately, caused a decrease in the bacterial load of *Streptococcus mutans in vitro* (Paschoal et al., 2013). This behavior was not observed in our study, since the reductions of *E. coli* loads were only significant with the use of aPDT with both curcumin and blue LED light. On the other hand, when comparing the aPDT protocols employed, we used lower concentrations of curcumin and light doses and obtained excellent results in reducing the bacterial load of *E. coli*.

Other photosensitizers have been reported to be efficient in aPDT, such as methylene blue. In our study, the aPDT3-80 and aPDT3-100 protocols were able to inhibit the bacterial load using a curcumin solution of 80 and 100 µg mL^{-1} applying blue LED light with a fluency of 19.8 J cm^{-2}. A similar result was reported using 100 µg mL^{-1} methylene blue solution and 50 J cm^{-2} red LED light fluency in reducing the bacterial load of *E. coli* (Carvalho et al., 2014). Curcumin shows to be more interesting for *in vivo* application in this situation, as it requires less light irradiation to exert its antibacterial function, thereby reducing the animals' exposure to the treatment.

Conclusion

This research highlights aPDT as an effective antimicrobial therapy against *E. coli*. In this *in vitro* model, the aPDT3-80 and aPDT3-100 protocols were the most effective, with aPDT3-80 recommended due to its lower light exposure while still significantly inhibiting bacterial growth. These findings provide valuable insights into aPDT treatment for gram-negative bacteria in planktonic cultures and could guide future biofilm and in vivo applications. Further research is needed to explore aPDT's effects on other gram-negative bacteria to establish it as a viable alternative antimicrobial therapy.

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