

In Vitro Evaluation of the Antibacterial Effect of Photodynamic Therapy with Methylene Blue

Maria Helena Chaves de Vasconcelos Catão¹, Ana Luzia Araújo Batista¹

¹Department of Dentistry, State University of Paraíba, Campina Grande, PB, Brazil.

Author to whom correspondence should be addressed: Maria Helena Chaves de Vasconcelos Catão, Rua Alfredo Farias Pimentel, n 61, Sandra Cavalcanti, Campina Grande, PB, Brazil. 50.410-675. Phone: +55 83 991345863. E-mail: mhelenact@zipmail.com.br.

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Abstract

Objective: To evaluate, *in vitro*, the effect of photodynamic therapy (PDT) compared to laser therapy and the use of a photosensitizer alone. **Material and Methods:** The following therapies were used: PDT, laser therapy and photosensitizer alone. For PDT, methylene blue (MB) at different concentrations and red laser InGaAlP 660nm were used. For the use of low-power laser (LPL) alone, red laser InGaAlP 660 nm and infrared laser AsGaAl, 830 nm, both in continuous emission were used. Standard ATCC strains of *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) species were used. The antibacterial effect of PDT was quantified by the diameter of the inhibition halos. **Results:** PDT (LPL 660 nm, 320 J/cm²) with MB at concentration of 50 µg/mL showed antibacterial efficacy only when tested against *S. aureus* and *E. coli* strains, as well as with the isolated use of MB at the same concentration. Using LPL alone, whether red or infrared, with different dosimetry, no antibacterial effect was observed. In none of the therapeutic modalities used, *P. aeruginosa* inactivation was observed. **Conclusion:** Antibacterial effects of PDT (LPL 660 nm + MB 50 µg/mL) were observed for *S. aureus* and *E. coli*, as well as with the isolated use of MB (50 µg/mL). For *P. aeruginosa*, no antibacterial effect with any of the protocols recommended in the study was observed.

Keywords: Microbiology; Bacteria; Low-Level Light Therapy; Photochemotherapy.

Introduction

The oral cavity is colonized by approximately one thousand species of microorganisms and most are organized as biofilms [1]. The dental biofilm is formed through an orderly and dynamic process where there is need for fixation and proliferation of bacteria on tooth surfaces, which may lead to the growth of the adhered species and appearance of additional species [2].

The accumulation of the bacterial biofilm complex results in diseases induced by the most prevalent bacteria: caries and periodontal disease. Current treatment of subjects with plaque related diseases involves mechanical removal of the biofilm and the use of antiseptics and antibiotics [3,4]. However, due to the overuse of antibiotics, there has been a rapid increase in the number of antibiotic-resistant bacteria. Bacteria replicate rapidly, and mutations can easily occur with the use of a single antibiotic [5].

One of the great advances in the twentieth century was the development of laser devices and their applicability in Health Sciences. Laser (Light Amplification by Stimulated Emission of Radiation), for being a differentiated light, has been used in several researches, and can be of two types: low-power laser (LPL) and high-power laser (HPL). Laser can be used individually (laser therapy) and also as a component for PDT, by means of specific wavelengths for each photosensitizer – PS [6]. PDT consists of the association of a PS agent, usually exogenous, and a light source with adequate wavelength with the objective of causing microbial death due to the formation of reactive oxygen species, causing cell damage and death [1,3,7].

In Dentistry, the most widely used PSs are methylene blue (MB) and toluidine blue (TB) [8,9], which are capable of interacting with the cell membrane, inactivating gram-positive and gram-negative bacteria, acting mainly by damaging the cytoplasmic membrane and DNA, and may reach multiple cellular targets [7], which hinders the appearance of resistant microorganisms [10].

In view of the diversity of microorganisms present in the oral cavity and the need to balance the endogenous flora to maintain the health of the individual as a whole, this study evaluated the *in vitro* antibacterial effect of PDT with MB at in different concentrations on three bacterial strains *P. aeruginosa*, *E. coli* and *S. aureus* compared to the use of LPL and MB alone.

Material and Methods

Antimicrobial Activity Determination

The research activities were carried out at the Laboratory of Antimicrobial Activity Research (LPAA) of the Department of Pharmacy, State University of Paraíba, Brazil. An experimental, analytical and quantitative *in vitro* study was carried out to evaluate the antibacterial effect of PDT on different bacterial species. American Type Culture Collection (ATCC) strains: *S. aureus* (ATCC 25923) facultative anaerobic gram-positive bacterium, can be isolated from the oral cavity, being the main cause of surgical infection and multiresistance; *E. coli* (ATCC 25922) (facultative anaerobic gram-negative rod very common in the hospital environment and the etiological agent of septicemia) and *P. aeruginosa* (ATCC 27853) strict aerobic gram-negative rod present in the oral cavity (Cefar Diagnostica Ltda., São Paulo, SP, Brazil) were used.

Each strain was inoculated with the aid of a sterile platinum loop into a test whole containing 5 mL BHI (Brain heart infusion, Difco, Detroit, USA). Tubes were incubated at 37°C for 24 hours and after this period, turbidity was observed in the culture medium indicating microbial viability. After reactivation, strains were cultured to obtain isolated colonies on Blood Agar and Mueller-Hinton Agar (Difco Laboratories Inc., Detroit, MI, USA) plates in order to observe the purity of strains. Plates were incubated at 37°C for 24 hours.

To obtain the bacterial inoculum, 3 to 5 similar colonies were selected and transferred to 2.0 mL of 0.85% sterile saline (NaCl) to produce a slight turbidity of density visually equivalent to tube 0.5 of the McFarland scale, with final concentration of 10-6 CFU/mL. This bacterial inoculum was cultured approximately 15 to 20 minutes after its preparation [11].

The antimicrobial activity was verified by the disk diffusion method [11]. Tests were performed in duplicate and the results expressed in mm by the arithmetic mean of the diameter of growth inhibition halos formed around the disks. The presence of growth inhibition halos ≥ 8 mm in diameter was considered active.

Division and Description of Groups

The study was divided into three experimental groups according to the procedure to be performed:

- Group A: PDT with MB - In this group, twelve plates were used for each microorganism, two for each PS concentration (duplicate procedure), totaling thirty-six plaques. In the Petri dish where the sterile disk containing PS was located, low-power InGaAlP, red laser model Thera Lase (DMC Equipamentos Ltda, São Carlos, SP, Brasil), with wavelength of 660 nm, output of 100 mW, continuous emission, was applied at a dose of 9 J/cm², for 90 seconds, at 1 cm distance. The PS concentrations studied in this experiment were: 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.562 µg/mL by dilution in distilled water of factory concentration. After these procedures, plates were placed in the oven at 37°C for 24 hours.
- Group B: Laser therapy (LPL alone) - control
 - Red laser (InGaAlP – 660 nm): Low-power red laser InGaAlP, model Thera Lase (DMC Equipamentos Ltda., São Carlos, SP, Brasil) wavelength of 660 nm, output of 100 mW, continuous emission for 90 seconds, at 1 cm distance, was directly applied to plates previously cultured with bacterial suspension. For this, different doses (40, 80, 160 and 320 J/cm²) were tested for each bacterial strain, with duplicate procedure, totaling 24 plaques.
 - Infrared laser (AsGaAl – 830 nm): Low-power AsGaAl infrared laser, model Thera Lase (DMC Equipamentos Ltda., São Carlos, SP, Brasil), wavelength of 830 nm, output of 100 mW, continuous emission for 90 seconds, at 1 cm distance, was directly applied to plates previously cultured with bacterial suspension. For this, different doses (40, 80, 160 and 320 J/cm²) were tested for each bacterial strain, with duplicate procedure, totaling 24 plaques.
- Group C: MB alone (Control): Disks were impregnated with 20 µL of MB (Sigma-Aldrich / Merck KgaA, Saint Louis, MO, USA) solution at various concentrations with the aid of a pipette and distributed with sterile loops onto the surface of the culture medium suitable for each microorganism previously cultured with the bacterial suspension. Plates were incubated at 37°C for 24 h. Methylene blue remained for 5 minutes. In this group, twelve plates were used for each microorganism, two for each PS concentration (duplicate procedure), totaling thirty-six plates.

Data Analysis

After incubation at 37°C for 24 hours, the microbial growth inhibition area was measured. The antibacterial effect in the three groups with different bacterial species was quantified in millimeters through the diameter of inhibition halos measured with the aid of a halometer.

Data were analyzed through descriptive and inferential statistical techniques. The Mann-Whitney U for independent samples, Kruskal-Wallis, Levene's and Kolmogorov Smirnov tests were used. Data were

analyzed in the Statistical Package for Social Sciences software, version 22.0 (SPSS Inc., Chicago, Ill., USA). The margin of error of statistical tests was 5%. All the results were statistically compared in order to evaluate the antibacterial effect of PDT, LPL and the use of MB alone in the different bacterial strains.

Results

Overall, 120 samples were analyzed, 36 in photodynamic therapy, 48 in laser therapy and 36 in the isolated study.

In vitro Antibacterial Effect of PDT on Bacterial Strains

Analyzing the results of the *in vitro* antibacterial effect of PDT (LPL 660 nm; 320 J/cm² + MB at different concentrations) in standard strains used in the study, the presence of growth inhibition halo of 12 mm at 50 µg/mL MB concentration was observed only when tested against *S. aureus* and *E. coli* strains. However, at lower concentrations, both bacteria showed no inhibition halo (Table 1). No effect of this therapy was observed when tested against *P. aeruginosa* strain, due to the total absence of inhibition halo, regardless of MB concentration used (Table 1).

Table 1. Evaluation of the *in vitro* antibacterial effect of PDT (LPL 660 nm - 320 J/cm² + MB) against bacterial strains.

Microorganisms	PDT (methylene blue [µg/mL] / Diameter of Growth Inhibition Halos (mm))					
	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	3.125 µg/mL	1.5625 µg/mL
<i>Staphylococcus Aureus</i> (ATCC 25923)	12	0	0	0	0	0
<i>Escherichia Coli</i> (ATCC 25922)	12	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> (ATCC 27857)	0	0	0	0	0	0

(0) = Absence of microbial growth halos.

There is statistical evidence that for *S. aureus* and *E. coli* bacteria, the inhibition halo is significantly larger using MB concentration of 50 µg/mL than when using lower concentrations (p=0.001) (Table 2). There is no significant difference in inhibition halos according to MB concentrations for *P. aeruginosa* at 5% significance level (p=1.00).

Table 2. Analysis of the mean inhibition halo (mm) using PDT (LPL 660nm - 320J/cm² + MB) according to each bacterial strain and MB concentration.

Microorganisms	Concentration	Mean Halo	N	SD	Minimum Halo	Maximum Halo	p-value ^{1,2}
<i>Pseudomonas aeruginosa</i>	1.5625 µg/mL	0.0	2	0.00	0	0	1.000
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	
	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	0.0	2	0.00	0	0	
	Total		0.0	12	0.00	0	0
<i>Staphylococcus Aureus</i>	1.5625 µg/mL	0.0	2	0.00	0	0	0.001
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	

	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	12.00	2	0.00	12	12	
	Total	2.00	12	4.671	0	12	
<i>Escherichia Coli</i>	1.5625 µg/mL	0.0	2	0.00	0	0	0.001
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	
	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	12.00	2	0.00	12	12	
	Total	2.00	12	4.671	0	12	
Total	1.5625 µg/mL	0.0	6	0.00	0	0	
	3.125 µg/mL	0.0	6	0.00	0	0	
	6.25 µg/mL	0.0	6	0.00	0	0	-
	12.5 µg/mL	0.0	6	0.00	0	0	
	25 µg/mL	0.0	6	0.00	0	0	
	50 µg/mL	8.00	6	6.197	0	12	
	Total	1.33	36	3.825	0	12	

SD: Standard Deviation; ¹Mann-Whitney U test at 5% significance level; ²For the application of the Mann-Whitney U test, the methylene blue concentration was divided into two groups, one less than or equal to 25 µg/mL and the other equal to 50 µg/mL.

In vitro Antibacterial Effect of LPL Alone on Bacterial Strains

In the evaluation of the *in vitro* LPL application alone, either red laser (InGaAlP – 660 nm) or infrared laser (AsGaAl – 830 nm) with different dosimetry (40, 80, 160 and 320 J/cm²) on the growth of standard strains used in the study, it was found that in the methodological conditions used, the presence of bacterial growth inhibition halo was not observed (Table 3). It was evidenced that regardless of wavelength, red – 660 nm and infrared – 830 nm, the mean halo was 0 for all study bacteria (Table 3).

Table 3. Evaluation of the *in vitro* antibacterial effect of LPL alone (InGaAlP – 660 nm or AsGaAl – 830 nm), in different dosimetry against bacterial strains.

Microorganisms	Wave Length	Mean Halo	N	SD	Minimum Halo	Maximum Halo
<i>Pseudomonas aeruginosa</i>	(830 nm) Infrared	0.0	8	0.00	0	0
	(660 nm) Red	0.0	8	0.00	0	0
	Total	0.0	16	0.00	0	0
<i>Staphylococcus aureus</i>	(830 nm) Infrared	0.0	8	0.00	0	0
	(660 nm) Red	0.0	8	0.00	0	0
	Total	0.0	16	0.00	0	0
<i>Escherichia coli</i>	(830 nm) Infrared	0.0	8	0.00	0	0
	(660 nm) Red	0.0	8	0.00	0	0
	Total	0.0	16	0.00	0	0
Total	(830 nm) Infrared	0.0	24	0.00	0	0
	(660 nm) Red	0.0	24	0.00	0	0
	Total	0.0	48	0.00	0	0

SD: Standard Deviation.

Antibacterial Effect of MB Alone on Bacterial Strains

In the analysis of the antibacterial effect with the use of MB isolated at different concentrations on strains under study, it was evidenced that for *P. aeruginosa*, regardless of the dye concentration, the mean inhibition halo is zero. For *S. aureus* and *E. coli*, the mean inhibition halo varies depending on the dye concentration: at concentration of 1.5625 up to 25 µg/mL, the mean halo is zero, but at concentration of 50 µg/mL, the mean halo becomes 12 mm (Table 4).

Table 4. Evaluation of the *in vitro* antibacterial effect of MB alone at different concentrations against bacterial strains.

Microorganisms	Concentration	Mean Halo	N	SD	Minimum Halo	Maximum Halo	p-value*
<i>Pseudomonas aeruginosa</i>	1.5625 µg/mL	0.0	2	0.00	0	0	1.000
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	
	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	0.0	2	0.00	0	0	
	Total		0.0	12	0.00	0	
<i>Staphylococcus aureus</i>	1.5625 µg/mL	0.0	2	0.00	0	0	0.001
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	
	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	12.00	2	0.00	12	12	
	Total		2.00	12	4.671	0	
<i>Escherichia Coli</i>	1.5625 µg/mL	0.0	2	0.00	0	0	0.001
	3.125 µg/mL	0.0	2	0.00	0	0	
	6.25 µg/mL	0.0	2	0.00	0	0	
	12.5 µg/mL	0.0	2	0.00	0	0	
	25 µg/mL	0.0	2	0.00	0	0	
	50 µg/mL	12.00	2	0.00	12	12	
	Total		2.00	12	4.671	0	
Total	1.5625 µg/mL	0.0	6	0.00	0	0	
	3.125 µg/mL	0.0	6	0.00	0	0	
	6.25 µg/mL	0.0	6	0.00	0	0	
	12.5 µg/mL	0.0	6	0.00	0	0	
	25 µg/mL	0.0	6	0.00	0	0	
	50 µg/mL	8.00	6	6.197	0	12	

SD: Standard Deviation; *Mann-Whitney U test at 5% significance level.

No statistical significance was found to *P. aeruginosa* ($p=0.436$), which leads us to conclude that there is statistical evidence that the inhibition halo is the same for whatever MB concentration used. However, for *S. aureus* as for *E. coli*, it is concluded that there is statistical evidence that using MB alone at concentration of 50 µg/mL, the inhibition halo is significantly higher than using concentrations equal to or lower than 25 µg/mL (Table 5).

Table 5. Analysis of the mean inhibition halo studied alone according to the methylene blue concentration for each microorganism.

Microorganisms	Concentration	Mean Halo	N	SD	Minimum Halo	Maximum Halo	p-value*
<i>Pseudomonas aeruginosa</i>	1.5625 µg/mL	0.00	2	0.000	0	0	0.436
	3.125 µg/mL	0.00	2	0.000	0	0	
	6.25 µg/mL	0.00	2	0.000	0	0	
	12.5 µg/mL	0.00	2	0.000	0	0	
	25 µg/mL	0.00	2	0.000	0	0	
	50 µg/mL	0.00	2	0.000	0	0	
	Total		0.00	12	0.000	0	
<i>Staphylococcus aureus</i>	1.5625 µg/mL	0.00	2	0.000	0	0	0.001
	3.125 µg/mL	0.00	2	0.000	0	0	
	6.25 µg/mL	0.00	2	0.000	0	0	

	12.5 µg/mL	0.00	2	0.000	0	0	
	25 µg/mL	0.00	2	0.000	0	0	
	50 µg/mL	12.00	2	0.000	12	12	
	Total	2.00	12	4.671	0	12	
<i>Escherichia coli</i>	1.5625 µg/mL	0.00	2	0.000	0	0	0.001
	3.125 µg/mL	0.00	2	0.000	0	0	
	6.25 µg/mL	0.00	2	0.000	0	0	
	12.5 µg/mL	0.00	2	0.000	0	0	
	25 µg/mL	0.00	2	0.000	0	0	
	50 µg/mL	12.00	2	0.000	12	12	
	Total	2.00	12	4.671	0	12	
Total	1.5625 µg/mL	0.00	6	0.000	0	0	
	3.125 µg/mL	0.00	6	0.000	0	0	
	6.25 µg/mL	0.00	6	0.000	0	0	
	12.5 µg/mL	0.00	6	0.000	0	0	
	25 µg/mL	0.00	6	0.000	0	0	
	50 µg/mL	8.00	6	6.197	0	12	
	Total	1.33	36	3.825	0	12	

SD: Standard Deviation; *Mann-Whitney U test at 5% significance level.

Discussion

Antimicrobial photodynamic therapy is a minimally invasive treatment that uses a PS agent associated with a specific wavelength light, promoting bacterial death [1]. The aim of the present study was to evaluate the *in vitro* antibacterial effect of the action of PDT with MB at different concentrations against bacterial strains, comparing with the use of LPL and MB alone. *In vitro* methods have advantages over *in vivo* ones such as: limited number of experimental variables and significant data are obtained within a shorter test period.

Although PDT is shown to be effective for microbiological inactivation, some variables such as PS type and concentration, microorganism species, light source and dose used may also influence [12]. *In vitro* studies with microorganisms used several light sources: laser [2,4,13-16], LED - Light Emission Diodo [3,17-19] or other sources such as fluorescent, photopolymerizing and bioluminescent lamps [20].

Another important element is the active medium used in researches: Carbon Dioxide – CO₂, Argon – Air [14], Helium-Neon-He-Ne [14,16] de YAG [14], semi-conducting diodes, such as AsGaAl [13,15], InGaAlP [1,2]. To perform the tests, InGaAlP and laser therapy alone, InGaAlP and AsGaAl were used for PDT.

Among these parameters, it is fundamental to select an effective PS that, in addition to being non-toxic, absorbs light at compatible wavelength between 620 and 660 nm [18] and evaluates the type and its concentration. In this study, phenothiazine MB was used, either alone or in combination, since it has been well accepted and widely used in dentistry studies [2,20,21]. Some studies have used other types of phenothiazine dye, such as toluidine blue – TB [1,3,13,16,17], in addition to malachite green – MG [1], safranin [14] and porphyrin [18].

MB and TB are efficient PSs against planktonic bacteria and have also been evaluated due to their efficacy when organized in biofilms, since microorganisms have advantages such as increased resistance to antimicrobial agents and increased protection against the host immune system [1]. Recently, developments of antimicrobial hydrogels have attracted significant attention and incorporation of photosensitizers have been suggested as a promising approach. In fact, the capabilities of hydrogel served as an excellent wound dressing and drug depot to release drugs in a sustained manner and achieve high local drug concentration have been well demonstrated [22].

S. aureus and *E. coli* are the two most common multidrug resistant pathogens [23], which justifies the recent search for new alternatives [10] and photodynamic inactivation seems to be effective against several classes of microorganisms without causing resistance [12]. In view of the large microbial complex, we chose to use three different types of bacteria with different morphologies, namely: *P. aeruginosa* (strict aerobic, non-fermenting gram-negative rod); *E. coli* (facultative anaerobic, gram-negative rod) and *S. aureus* (facultative anaerobic, gram-positive). In general, gram-positive bacteria are more susceptible than gram-negative bacteria, which is justified by the more complex structure of the latter, including the presence of two lipid layers [7].

These results showed that both PDT (660 nm + MB 50 µg/mL) and MB alone (50 µg/mL) were effective against *S. aureus* (gram-positive) and *E. coli* (gram-negative) strains, which coincides with the findings of previous research [1,13,14]. Other studies [15,16,18,23,24] have found inhibitory effects only against *S. aureus*, unlike other studies that did not obtain effects on the same strains with the use of PDT or with PS alone [3,16].

In none of the therapeutic modalities used, inactivation against *P. aeruginosa* strains was not obtained, perhaps because it was a gram-negative bacterium with its more complex structure [7]. These data corroborate other reports [17,21], which may be justified by the difficulty of obtaining inhibitory or bactericidal effects in gram-negative bacterial species, especially *P. aeruginosa*.

The use of LPL has the advantage of achieving a bactericidal effect without inducing damage to the host tissue [18]. The biological effect and/or the elimination of bacteria with isolated laser use has been studied, mainly in *in vitro* studies. In the present study, the use of LPL alone, whether red laser (InGaAlP – 660 nm) or infrared laser (AsGaAl – 830 nm), regardless of dosimetry and wavelength, was innocuous on the bacteria under study, corroborating previous study [16].

The phenomenon of bacterial viability in the action of LPL alone is still widely discussed and despite its analgesic, anti-inflammatory and biomodulatory effects, do not present antimicrobial effect [25]. The development of resistance to PDT seems to be unlikely, since in microbial cells, singlet oxygen and free radicals interact with cell structures in the most diverse metabolic pathways [1]. However, a recent study evaluating the efficacy of PDT found selection of resistant mutants [26], explained by the characteristics of the survival curve, suggesting that persistence is a factor to be considered.

The comparison between studies involving laser is very limited due to methodological differences, various types, and to different PS and protocols. Therefore, specific protocols should be developed for each type of wavelength, in addition to the development and validation of the methodology in order to guarantee a direct comparison among studies.


According to our search, this was the first work that involved the use of these three specific bacteria. However, it has limitations due to the treatment being directed to each species in isolation, whereas most infections involve several species in the same pathological site. In addition, bacteria isolated from patients may show greater resistance because they had previously undergone antibiotic treatments and the effectiveness of a treatment under ATCC bacteria does not reflect a clinical reality.


Conclusion

PDT using red laser (InGaAlP 660 nm) associated with MB at concentration of 50 µg/mL, as well as the isolated use of MB at this concentration showed antibacterial effect against *S. aureus* and *E. coli* strains. Lasertherapy alone, regardless of laser type, wavelength and dosimetry used in the study did not present

antibacterial effect against any of the bacterial strains used. Regardless of the therapeutic modality used in this study, none showed antibacterial effect against *P. aeruginosa* strains.

Authors' Contributions

MHVC  0000-0001-7681-3225 Conceptualization, Methodology, Validation, Investigation, Resources, Data Curation, Writing - Original Draft Preparation), Supervision and Project Administration.

ALAB  0000-0002-7780-060X Writing - Review and Editing and Visualization.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

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