Original Article

Synergic phototoxic effect of visible light or Gallium-Arsenide laser in the presence of different photo-sensitizers on *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

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ABSTRACT

Background: According to the development of resistant strains of pathogenic bacteria following treatment with antimicrobial chemotherapeutic agents, alternative approaches such as lethal photosensitization are being used. The aim of this study was to evaluate the effect of visible light and laser beam radiation in conjugation with three different photosensitizers on the survival of two main periodontopathogenic bacteria including *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in different exposure periods.

Materials and Methods: In this *in vitro* prospective study, strains of *P. gingivalis* and *F. nucleatum* were exposed to visible light at wavelengths of 440 nm and diode laser light, Gallium-Arsenide, at wavelength of 830 nm in the presence of a photosensitizer (erythrosine, curcuma, or hydrogen peroxide). They were exposed 1-5 min to each light. Each experiment was repeated 3 times for each strain of bacteria. Data were analyzed by two-ways ANOVA and least significant difference *post-hoc* tests. $P < 0.05$ was considered as significant. After 4 days the colonies were counted.

Results: Viability of *P. gingivalis* was reduced 10% and 20% subsequent to exposure to visible light and diode laser, respectively. The values were 65% and 75% for *F. nucleatum* in a period of 5-min, respectively. Exposure to visible light or laser beam in conjugation with the photosensitizers suspension caused significant reduction in the number of *P. gingivalis* in duration of 5-min, suggesting a synergic phototoxic effect. However, the survival rate of *F. nucleatum* following the exposure to laser with hydrogen peroxide, erythrosine and rhizome of Curcuma longa (curcumin) after 5-min was 10%, 20% and 90% respectively.

Conclusion: Within the limitations of this study, the synergic phototoxic effect of visible light in combination with each of the photosensitizers on *P. gingivalis* and *F. nucleatum*. However, the synergic phototoxic effect of laser exposure and hydrogen peroxide and curcumin as photosensitizers on *F. nucleatum* was not shown.

Key Words: Laser therapy, periodontitis, pathogenic bacteria, phototherapy

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INTRODUCTION

Surgery and application of antibacterial agents are the most-used methods of removal of periodontal pathogenic bacteria. However, the main problem with these approaches is that there is a considerable difficulty in maintaining therapeutic levels of these agents for a sufficient period of time in the oral

cavity, which reduces their therapeutic efficacy. Furthermore, antibacterial therapy is associated with the development of resistant strains of pathogenic bacteria, which would exacerbate the condition.[1] However, several alternative approaches such as photodynamic therapy (PDT) have been assessed and used for deactivation and eradication of pathogenic bacteria in the oral cavity.[2-4]

Photodynamic therapy is a new method for treatment of local or even systemic infections (e.g., blood infections).[5-8] Photosensitization has numerous advantages that can cover the disadvantages of the currently used chemical or mechanical methods. Removing pathogenic bacteria in a short time (within few minutes), no risk of surrounding host tissue injury, no risk of bacterial resistance and very low risk of induction of mutations, preserving normal microbial flora in the oral cavity, low cost and echo-friendly nature of this method are some of the considerable advantages of photosensitization.[9,10]

The mechanism of PDT is a selective destruction of target cells (microorganisms) in the presence of a photoactive agent and after exposure to light. These photoactive agents (photosensitizers) are not toxic in nature and specifically accumulate in the target cells. Only after exposure to visible light it becomes toxic and destructs the cells. Photosensitizers are commonly aromatic molecules that have a long-lived triplet excited state. Several photosensitizers have been introduced and examined during recent years.[9,11-14] For instance, chlorin e6-pentalysine is a potent antimicrobial agent that has a highly lethal effect on *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum* and *Actinomyces viscosus*.[7] Another important factor in photosensitization technique is the light source. Gas discharged lamps, halogen, xenon and ultraviolet lamps are some of these sources that produce light with different wavelengths.[9,13,15,16] Laser is another source that produces light with a constant range and has become more common these days. However, it is associated with several disadvantages: It is expensive and needs an expert team for the procedure, and always there is a possibility for surrounding tissue damage.[17-19] Because the visible light has none of these disadvantages, and it can be radiated to the whole area, it is thought as a good replacement for the laser beam.

We designed an *in vitro* study to compare the phototoxic effect of visible light and Gallium-Arsenide (Ga-As) laser beam exposure on two main strains of periodontal pathogenic bacteria *P. gingivalis* and *F. nucleatum*. This effect was evaluated in the absence and presence of erythrosine, the powdered rhizome of Curcuma longa (curcumin) and hydrogen peroxide as photosensitizers. These photosensitizers are less expensive and more available than other chemical agents. Thus, if these combinations are proved to be effective and can result in similar outcomes, it can be recommended for future use *in vivo* and clinical situations.

MATERIALS AND METHODS

We designed an *in vitro* study to compare the phototoxicitic effect of visible light and (Ga-As) laseron *P. gingivalis* (33,277) and *F. nucleatum* (25,586) from American type culture collection (ATCC, MAST Co., England) in the absence and presence of photosensitizer suspensions: Erythrosine, the powdered rhizome of Curcuma longa (curcumin) and hydrogen peroxide. For this purpose, the light from two sources was beamed on the two microbial colonies in the presence and absence of photosensitizer suspensions. The effect of each photosensitizer on microbial colonies was also assessed without any light.

For comparing the effect of visible light with (Ga-As) laser, we used visible light that was made by light cure device (Starlight Pro., Carasco, Italy) with a mean output of 400 mw/cm^2 and could produce visible blue light with a wavelength of 440 nm. To prevent an indirect effect of heat produced during light exposure on the survival of bacteria, we used light emitting diode light cure with a 8 mm diameter diffusing optical fiber. Laser light was made by Diode Ga-As laser device (DLT-101, BehsazGostar Co., Tehran, Iran) with a mean output of 400 mw/cm^2 which produced a light with a wavelength of 830 nm.

We used suspension of each photosensitizer in this study. For this purpose, at first, we diluted 100 ml of erythrosine solution (Farzaneh Arman Co., Tehran, Iran) with 163 ml distilled water to have a 22 μM (mol) erythrosine solution. Then, we solved 1 g of powdered rhizomes of Curcuma longa (Zagrosdaroo Co., Tehran, Iran) in 17.5 g of distilled water. We used this ratio based on the results of our pilot examinations. Finally, 0.1 ml of 30% hydrogen

peroxide solutions (Kimiafam Co., Tehran, Iran) were diluted with a 1-10 ratios for 6 times until we reached the 0.3 mM concentration.

For confirmation of an appropriate bacterial growth, we cultured the bacteria on Brain Heart Infusion Broth (BHIB). From the cultured bacteria on the blood agar (BA) environment, we made juice in 5 ml BHIB and placed the sterile tubes in the spectrophotometer and determined the concentration under 650 nm (OD_{650nm}). If the OD_{650nm} was 0.45, the number of bacteria was 10^6 colony forming unit (CFU). Furthermore, we prepared standard McFarland tubes to compare our juice tubes with them and determine the CFU/CC. For this purpose, 10 sterile tubes with exactly the same dimensions were selected and filled with different volumes of 10% sulfuric acid and 1.75% barium chloride.

For comparison of a juice tube with McFarland tubes, we used a white blank paper and drew black lines on it and compared the transparency of the tube with any McFarland tube two by two, and the most similar tube was determined. Then, we referred to the table and verified the colony count per volume (CFU/CC). The concentration in all tubes was 3×10^8 CFU/CC.

After verification of concentrations in each tube, 175 μl of bacterial juice and 175 μl of photosensitizer suspensions were mixed thoroughly, and poured into five 64 cell wells. Then, they were exposed 1-5 min to light by placing the fiber tips of light cure or laser device at the entry of each well, which had exactly the same diameter (8 mm). The volumes of wells were 350 ml, and thus, they were completely full. Therefore, fiber tips of light cure or laser device were at the level of suspension, and the density of transmitted energy was equal to the mean output of the device. The fluency of energy transmitted to each suspension could be calculated by multiplying the output to exposure time. For light cure and laser device, this was 34-170 J/cm^2 and 24-120 J/cm^2 at 1-5 min, respectively.

After 1-5 min exposure to light, 10 μl of the suspension was taken for determination of colony count in each group. Furthermore, as a control, at the zero time, 175 μl of bacteria juice and 175 μl of sterile BHIB environment were poured into the well and mixed completely, and 10 μl of the suspension was selected. Then, all the 10 μl suspensions were diluted 1-10 with BHIB sterile environment for 6 times and after the last dilution, 10 μl was selected

and placed at a petri containing BA environment. With a streaking technique, we spread it on the surface of the environment with a sterilized loop in several parallel lines perpendicular on the diagonal axis of the petri.

Therefore, from each well, we prepared a culture on BA environment and placed it in an anaerobic incubator for 3-5 days. To provide anaerobic situation, the incubators were connected to H_2 and N_2 cylinders. Oxygen was evacuated from incubators with pump and H_2 and N_2 were replaced. When O_2 decreased to zero, we turned off the device and kept the environment anaerobic until the end of culturing. To prevent dehydration in this 3-5 days period, full possible thickness of culture environment (5 mm) was used, and a distilled water vessel was placed in the incubator. For confirmation of bacterial purification, liquid environment was cultured on BA tubes and were placed in the anaerobic incubator. As old cultures have some amount of oxygen that might adversely affect the growth of anaerobic bacteria or even prevent the bacterial growth, we used cultures that were made in that day in a sterile condition. We evaluated bacterial growth each day for 4 days. For confirming that cultures are not contaminated and evaluating the purity of colonies, we prepared smear from each petri and stain it with Gram stain and examined it with light microscopy. Bacterial survival was determined after 4 days by counting colonies formed on the BA environments.

All these examinations were performed 3 times, and the mean of colony count was verified and used in the data analysis. Two-way ANOVA test was used to compare the bacterial survival after exposure to visible and laser light. Furthermore, least significant difference (LSD) *post-hoc* was the test for classification of means. $P < 0.05$ was considered as statistical significance. Data was analyzed by SPSS software v. 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

After 4 days, the colonies were counted. In the absence of photosensitizers, laser and visible light 5-min exposure could reduce *F. nucleatum* colony count to 75% and 65%, respectively [Figure 1a] and *P. gingivalis* to 10% and 20%, respectively [Figure 1b]. In comparison between the light sources, there was no significant difference ($P = 0.125$); however, LSD *post-hoc* comparison showed that

P. gingivalis was significantly more susceptible than *F. nucleatum* to phototoxicity (*P* = 0.001).

Regarding the toxic effect of photosensitizers on these two bacteria in the absence of light exposure, data showed that after 5-min exposure to the erythrosine, hydrogen peroxide and powdered rhizome of curcumin, *P. gingivalis* reduced to 60%. There was no significant difference between each of these photosensitizers [Figure 2a] (*P* > 0.05). The reduction in *F. nucleatum* after 5-min exposure to erythrosine, hydrogen peroxide and powdered rhizome of curcumin was 20%, 10% and 90%. The effect of curcumin on *F. nucleatum* after 5-min was significantly higher [Figure 2b]. The susceptibility of *P. gingivalis* to these photosensitizers was significantly higher than that of *F. nucleatum* (P for erythrosin = 0.05, P for hydrogen peroxide = 0.045, P for curcumin = 0.042).

Regarding synergic phototoxic effects of laser and visible light in the presence of photosensitizers, data showed that in the presence of erythrosine and after 5-min exposure to visible and laser light, the colony count of *P. gingivalis* reduced to 0% and 15%, respectively. After 3-min exposure to visible and laser light, the colony count of *P. gingivalis* reduced to 90% and 50%. The difference was statistically significant (*P* = 0.045). Synergic effect of visible light or laser beam with each photosentisizere on *P. gingivalis*

toxicity are shown in Figure 3a-c and for *F. nucleatum* in Figure 4a-c. In the presence of hydrogen peroxide [Figure 3b] and curcumin [Figure 3c], after 2-min exposure to visible and laser light, the colony count of *P. gingivalis* reduced to lower than 5% and 20%, respectively. The difference was not significant in this case.

In the presence of erythrosine and after 5-min exposure to visible and laser light, the colony count of *F. nucleatum* reduced to 5% and 20%, respectively [Figure 4a]. This did not show a significant difference (*P* = 0.09). However, after 3-min of exposure to visible light in the presence of erythrosine, the colony count reduced 100% that had a significant difference (*P* = 0.001). After exposure to visible light in the presence of curcumin for 4-min [Figure 4b] and

Figure 2: The effect of photosensitizers in the absence of light exposure for *Porphyromonas gingivalis* (a) and *Fusobacterium nucleatum* (b).
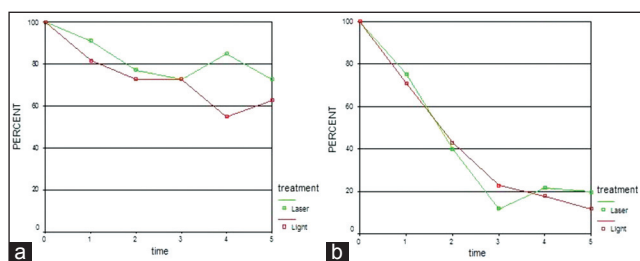
Figure 1: The effect of laser and visible light on *Fusobacterium nucleatum* (a) and *Porphyromonas gingivalis* (b) in the absence of photosensitizers.
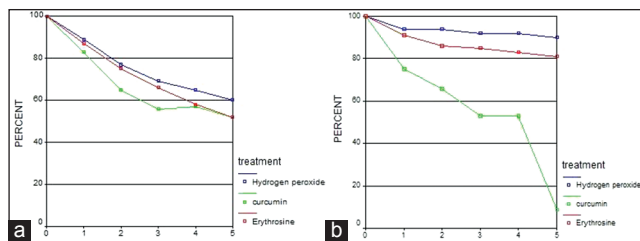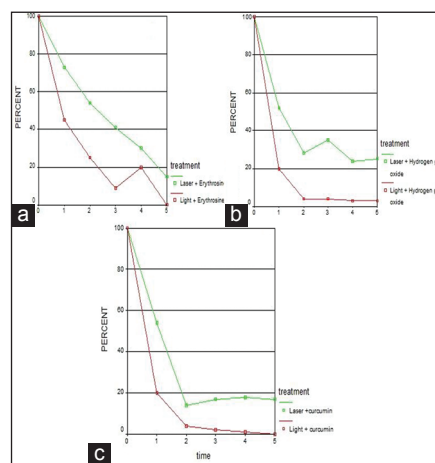
Figure 3: Synergic phototoxic effects of laser and visible light in the presence of each photosensitizers (a-c) on *Porphyromonas gingivalis*.
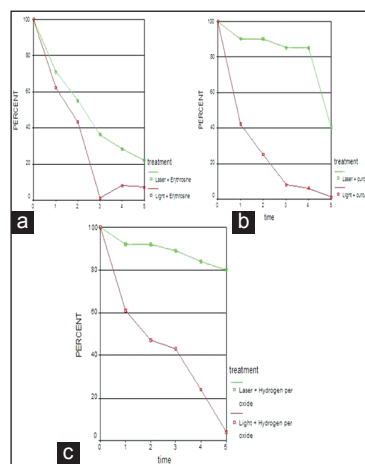
Figure 4: Synergic phototoxic effects of laser and visible light in the presence of each photosensitizers (a-c) on *Fusobacterium nucleatum*.

hydrogen peroxide for 5-min [Figure 4c], only 5% of *F. nucleatum* survived. LSD *post-hoc* comparison showed that the difference of *F. nucleatum* colony count reduction after visible light and laser beam in the presence of erythrosine was significant ($P = 0.001$). In addition, data showed the synergic phototoxic effects of visible light in conjugation with any of the photosensitizers on both bacteria; however, the synergic phototoxic effects of laser light in the presence of hydrogen peroxide and curcumin on *F. nucleatum* was not verified.

After 5-min exposure to visible light in the presence of any of the photosensitizers, all the *P. gingivalis* bacteria were killed [Figure 5a]. However, after 3-min, the colony count reduced to 10% with erythrosine, 55% with hydrogen peroxide and 90% with curcumin, which had a significant difference ($P = 0.001$). After 5-min exposure to laser light in the presence of any of the photosensitizers, 80% of the *P. gingivalis* bacteria were killed [Figure 5b]. The effect of exposure to visible light in the presence of any of the photosentisizer for *F. Nucleatum* is shown in Figure 5c. In addition, exposure to laser in the presence of hydrogen peroxide, curcumin and erythrosine could reduce the *F. nucleatum* colony count 20%, 60% and 80%, respectively. The LSD *post-hoc* comparisons showed that it had a significant difference ($P = 0.001$) [Figure 5d].

The results of two-sided variance analysis regarding the phototoxic effects of various treatment and time exposures (visible and laser light in the absence and presence of the photosensitizers) on *P. gingivalis* and *F. nucleatum* are given in Table 1 and the plots in Figures 6 and 7 show the distribution of bacteria after various time and treatments.

DISCUSSION

Periodontal diseases are caused by the invasion of pathogenic bacteria and host reactions to it that induces connective tissue damage and alveolar bone destruction. *P. gingivalis* and *F. nucleatum* are two of the main pathogenic bacteria in the periodontal tissues that cause periodontitis in adults.[3,20] The current routine treatment used for this disease is mechanical debridement in conjugation to surgery and antibiotic therapy. However, this might be only associated with a temporary reduction in the bacterial load. While most of the pathogenic microorganisms after surgery may survive, a subsequent use of antimicrobial
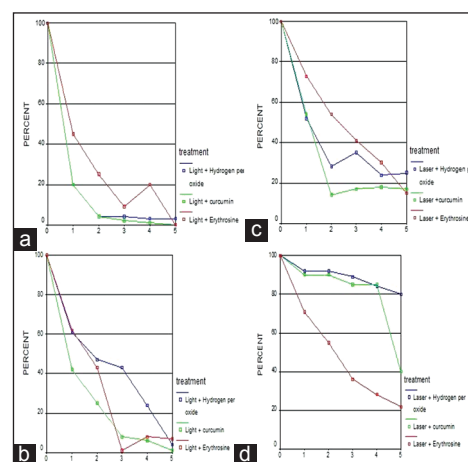
Figure 5: The synergic effect of each photosensitizers with visible light or laser on *Porphyromonas gingivals* survival rate (a and b) and *Fusobacterium nucleatum* survival rate (c and d).
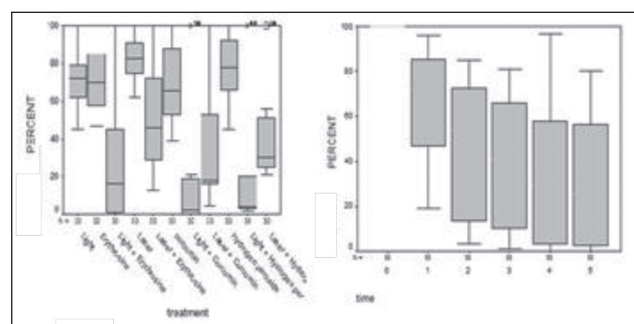
Figure 6: Distribution of *Porphyromonas gingivalis* after various time and treatments.
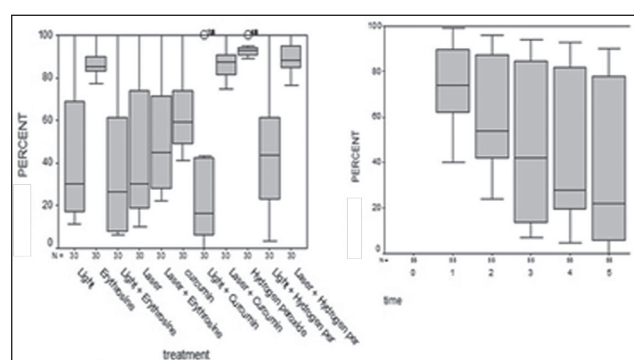
Figure 7: Distribution of *Fusobacterium nucleatum* after various time and treatments.

agents such as antibiotics can improve the results.[1,21] The emergence of antibiotic resistance and the risk of adverse reactions secondary to antibiotic use have developed a need for the introduction of a replacement method for removal of pathogenic microorganisms.[21] Phototherapy is one of the useful substitutes for antibiotic therapy in the treatment of periodontitis. Several studies have assessed and

Table 1: The results of two-sided variance analysis regarding the phototoxic effects of various treatment and time exposures (visible and laser light in the absence and presence of the photosensitizers) on *P. gingivalis* and *F. nucleatum*

Bacteria	Source of variation	Sum of squares	Degree of freedom	Mean of squares	Test value	*P* value
P. gingivalis	Time	179,758	5	35,951	1474	<0.001
	Treatment	162,380	10	16,238	665	<0.001
	Time and treatment	42,319	50	846	36	<0.001
	Error	6439	264	24	—	—
	Total	390,896	329	—	-—	—
F. nucleatum	Time	142,773	5	28,554	4216	<0.001
	Treatment	166,489	10	16,648	7231	<0.001
	Time and treatment	53,671	50	1073	271	<0.001
	Error	1044	264	3.9	—	—
	Total	363,977	329	—	—	—

P. gingivalis: Porphyromonas gingivalis; F. nucleatum: Fusobacterium nucleatum

verified the toxic effects of phototherapy with various photosensitizers on the periodontal pathogenic bacteria such as *P. gingivalis*. For instance, Feuerstein *et al.* and Soukos *et al.* have confirmed the reduction of *F. nucleatum* and *P. gingivalis* after exposure to visible light (with a wavelength of 400-500 nm).[2,22] In addition to visible light, the phototoxic impacts of Neon Helium laser beams (with a wavelength of 623 nm) and Argon laser (with a wavelength 488-514 nm) on black-pigmented porphyrin-producing bacteria such as *P. gingivalis* and *Prevotella intermedia* in the absence of photosensitizers have been showed. The results of this study was in agreement with the findings of previous studies.[18,19]

Between *F. nucleatum* and *P. gingivalis*, the phototoxic effect of visible or laser light in the presence of photosensitizeres was more prominent in the second bacteria. The reason that *P. gingivalis* is more susceptible to phototoxic effects is that this bacteria contains a considerable amount of endogenous porphyrin and thus it is more sensitive to destruction by visible light in the lab environments and dental plaque.[23,24]

When each photosensitizer was used separately, phototherapy could reduce the colony count of *P. gingivalis* up to 60% in the presence of erythrosine. The role of erythrosine as a photosensitizer has been confirmed in this study. There are some investigations that focus on the antimicrobial characteristics of erythrosine, which exists in the dental plaque detectors. Several studies have found similar results. For instance, Conlon and Berrios declared that in the presence of erythrosine the colony count of the periodontal pathogenic bacteria is affected. They suggest that being a cyclic

substance in the category of Xanthenes, it can absorb the visible light and initiate the photochemical reactions.[15] Erythrosine has several advantages on the currently used photosensitizers. One of them is its attachment to the dental plaque structure that increases its acceptability.[25]

There was a significant difference between the phototoxic effects of these photosensitizers. Erythrosine alone and hydrogen peroxide alone had the lower toxic effect in comparison to powdered rhizome of curcumin. The reason might be the production of free radicals and active forms of oxygen, especially hydroxyl ion, which initiates phototoxic reactions.

Data of this study confirmed the synergic phototoxic effect of visible light in combination with each of the photosensitizers on the *P. gingivalis* and *F. nucleatum*. However, the synergic phototoxic effect of laser exposure and hydrogen peroxide and curcumin as photosensitizers on *F. nucleatum* was not proved. This can be explained by the lower energy of the laser device in comparison to the light cure which produced visible light. In addition, previous investigations revealed a lower toxicity of the laser beam in the infra-red range in comparison to visible blue light on bacteria (especially Gram-negative bacteria).[17,18,26] The reason was alleged to be the difference in the photochemical mechanism.

Completely similar to the results of our study, several investigations confirmed that low output laser in the presence of suitable photosensitizers can significantly reduce the periodontal pathogenic bacteria *in vivo* and *in vitro*.[27,28] Therefore, we can suggest conjugation of laser phototherapy to the currently used mechanical debridement for decreasing the bacteria of periodontal pockets in order to get a better result.

This study was performed *in vitro*, and therefore, several other factors must be taken into consideration while recommending the results of this study to *in vivo* situation. For instance, the temperature increments are not comparable to the oral cavity situation. *In vitro*, temperature was not increased to as high as 37°C. Some other factors such as saliva production, gingival crest fluid and photosensitizer solution compatibility to the oral cavity should be considered. Because of these factors and many others, to verify the clinical efficacy of phototherapy, future studies must be performed *in vivo* situation.

Regarding the application of phototherapy in the oral cavity for eradicating the pathogenic bacteria, some investigations even declare that the efficacy of *in vivo* phototherapy might be better than the *in vitro* situation.[4,29-32] One reason might be the impact of other stressors present in the oral cavity that makes the bacteria more susceptible to photosensitization. In addition, suspension environment of the oral cavity might be another factor that increases the efficacy. It is shown that the necessary energy for phototoxic reactions is absorbed more in the bacterial biofilm and plaque in comparison to the suspension fluid as they have a lower molecular distribution. This makes the bacterial colony more susceptible to damage by phototoxic reactions.[31,33] However, there are some parameters that lessen the efficacy of *in vivo* phototherapy such as the presence of saliva.[4] If it is proved to be of lower efficacy, increasing the concentrations of photosensitizers or the number phototherapy episodes may solve the problem, as it is now proved that this method has a minimal risk of host tissue damage such as gingival ulceration or connective tissue inflammatory response.

CONCLUSION

According to the findings of this study, visible light and with lower efficacy laser had synergic phototoxic effects with curcumin, hydrogen peroxide and erythrosine as photosensitizers on two periodontal pathogenic bacteria *P. gingivalis* and *F. nucleatum*. Thus, application of visible light with a blue spectrum and enough energy dose in the presence of these photosensitizers with a defined concentration in a period of at least 3-min can be considered as a good therapeutic modality for the treatment of periodontitis. Three-minute exposures to light can reduce the colony count of these bacteria and thus can be recommended

as a therapeutic modality or a supplementary therapeutic and prophylactic regimen along with scaling and root planning and periodontal flap surgery to increase the removal of pathogenic bacteria and enhance the therapeutic outcome.

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