ORIGINAL ARTICLES

EFFECTIVENESS OF KATUK LEAF CHLOROPHYLL (SAUROPUS ANDROGYNUS (L) MERR) WITH BLUE AND RED LASER ACTIVATION TO REDUCE AGGREGATIBACTER ACTINOMYCETEMCOMITANS AND ENTEROCOCCUS FAECALIS BIOFILM

Permatasari P.A.D.[1], Astuti S.D.[1], Yaqubi A.K.[1], Paisei E.A.W.[1], Pujiyanto[1], Nasrul Anuar[2]
[1]Airlangga University, Surabaya, Indonesia
[2]Universiti Malaya, Kuala Lumpur, Malaysia

Abstract

In this study, the efficacy of using Sauropus androgynus (L) Merr, a katuk leaf chlorophyll photosensitizer, to reduce *Aggregatibacter actinomycetemcomitans* and *Enterococcus faecalis* biofilm was investigated. A red and blue diode laser is used as the light source. The sample was split into four groups: a negative control group, a positive control group, a blue laser treatment group (B), and a red laser treatment group (R), both with and without the addition of katuk leaf chlorophyll 1.6 mg/ml, and with varying densities of laser energy exposure of 2.5 J/cm^2, 5 J/cm^2, 7.5 J/cm^2, and 10 J/cm^2. Laser exposure and chlorophyll photosensitizer were tested using ELISA and ANOVA. At an energy density of 10 J/cm^2, the optimal bacterial mortality rate was obtained in each treatment group. Namely, in the *Aggregatibacter actinomycetemcomitans* biofilm, the negative group, the number of deaths was 73.30% using a blue diode laser and 63.25% using a red diode laser. In the positive group, the number of deaths was 86.12% using a blue diode laser and 83.29% using a red diode laser. In the *Enterococcus faecalis* biofilm, in the negative group, the number of deaths was 67.78% using the blue diode laser and 75.33% using the red diode laser, and in the positive group, the number of deaths was 71.71% using the blue diode laser and 86.41 using a red diode laser. Exposure to blue and red diode lasers activates chlorophyll in katuk leaves, killing bacteria and reducing biofilms.

Keywords: photoinactivation, blue and red diode laser, katuk leaf chlorophyll *(Sauropus androgynus (L) Merr)*, *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*.

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Contacts: Astuti S.D., e-mail: suryanidyah@fst.unair.ac.id

АНТИБАКТЕРИАЛЬНАЯ ЭФФЕКТИВНОСТЬ ХЛОРОФИЛЛА ЛИСТЬЕВ КАТУКА (SAUROPUS ANDROGYNUS (L) MERR) С АКТИВАЦИЕЙ СИНИМ И КРАСНЫМ ЛАЗЕРОМ В ОТНОШЕНИИ БИОПЛЕНКИ AGGREGATIBACTER ACTINOMYCETEMCOMITANS И ENTEROCOCCUS FAECALIS

P.A.D. Permatasari[1], S. D. Astuti[1], A. K. Yaqubi[1], E.A.W. Paisei[1], Pujiyanto[1], Nasrul Anuar[2]
[1]Airlangga University, Surabaya, Indonesia
[2]Universiti Malaya, Kuala Lumpur, Malaysia

Резюме

Изучена фотодинамическая активность фотосенсибилизатора хлорофилла листьев катука в отношении биопленки *Aggregatibacter actinomycetemcomitans* и *Enterococcus faecalis*. В качестве источника света был использован красный и синий диодный лазер. В исследование были четыре группы: группа отрицательного контроля, группа положительного контроля, группа обработки синим лазером (B) и группа обработки красным лазером (R), как с добавлением, так и без добавления хлорофилла листьев катука

в концентрации 1,6 мг/мл, а также при различной плотности энергии лазерного излучения: 2,5 Дж/см^2, 5 Дж/см^2, 7,5 Дж/см^2 и 10 Дж/см^2. Эффективность воздействия оценивали с помощью ELISA и ANOVA. Наибольшая эффективность была зарегистрирована во всех режимах воздействия (красный/синий лазер, без/с хлорофиллом) при плотности энергии 10 Дж/см^2. В биопленке *Aggregatibacter actinomycetemcomitans* в контрольных группах (только облучение) эффективность составила 73,30% при использовании синего диодного лазера и 63,25% при использовании красного диодного лазера, а в опытных группах эффективность составила 86,12% при использовании синего диодного лазера и 83,29% при использовании красного диодного лазера. В биопленке *Enterococcus faecalis* в контрольных группах эффективность составила 67,78% при использовании синего диодного лазера и 75,33% при использовании красного диодного лазера, а в опытных группах эффективность составила 71,71% при использовании синего диодного лазера и 86,41% с использованием красного диодного лазера. Таким образом, сделан вывод, что воздействие синего и красного диодных лазеров активирует хлорофилл в листьях катука, обладая бактерицидным действием бактерии и уменьшая биопленки.

Ключевые слова: фотоинактивация, синий и красный диодный лазер, хлорофилл листьев катука (*Sauropus androgynus (L) Merr*), *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*.

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Контакты: Astuti S.D., e-mail: suryanidyah@fst.unair.ac.id

Introduction

In general, the bacteria *Aggregatibacter actinomycetemcomitans* and *Enterococcus faecalis* are to cause for dental and oral health issues. Due to a lack of public awareness about maintaining dental and oral hygiene, bacteria can form on the teeth and in the mouth. A gram-negative, facultative anaerobic coccobacillus that does not migrate is called *Aggregatibacter actinomycetemcomitans* (A.a.) [1]. One of the bacteria in the oral cavity that has the potential to induce periodontal disease, particularly localized aggressive periodontitis, is *Aggregatibacter actinomycetemcomitans* [2, 3]. The periodontal ligament and alveolar bone are damaged by periodontal disease caused by microorganisms [4, 5]. *Enterococcus faecalis* can form pockets in pairs, singletons, or short chains. E. faecalis is facultatively anaerobic and can cause root canal damage [6, 7].

Antibiotic overuse can lead to the development of biofilms, which have a defined structure, adhere to one another, and adhere to both living and inanimate objects [8]. As they grow, bacteria that make biofilms may be exposed to conditions that could kill them. Antibiotics, cleaning agents, and even the immune system of the host are all ineffective against the bacteria in the biofilm. Resistance to antibiotic therapy is the clinical symptom of a biofilm-forming bacterial infection [9]. The majority of bacteria in a biofilm will continue to live and proliferate, but only planktonic bacteria will be killed [10]. The photoinactivation method is effective and selective in eliminating *S. aureus* biofilm bacteria [11].

Free radicals, light, photosensitizers, PDI, and non-invasive photonics are therapeutic techniques [12]. The key to photoinactivation is photosensitization, which works by letting light in and setting off chemical reactions that make reactive oxygen species [13]. Lasers and LEDs are used to photoinactivate bacterial biofilms. Porphyrin compounds are light-sensitive photosensitizer molecules found in some bacteria. Photosensitizers are used to take in light energy, such as chlorophyll, which is used in photoinactivation therapy [14, 15]. The ability of chlorophyll to absorb light and transform it into energy is an implementation of the normal chlorophyll structure, which is primarily made up of porphyrins [16]. The comparatively lengthy (10–8 seconds) singlet chlorophyll excitation phase, which then passes through intersystem crossover to triplet excitation, is what results in the significant energy absorption of chlorophyll during photosynthesis. The closest oxygen molecule will receive the extra energy at the triplet excitation level to create reactive singlet oxygen [17].

Research using Dracaena angustifolia chlorophyll as a photosensitizing agent with a 405 nm blue laser led to an 80% reduction in S. aureus biofilm [18], 62% and 78% in S. mutant bacteria with pheophytin A and Alfalfa Medicago sativa L [19], and 22% and 60% on C. albican biofilm using 445 nm and 650 nm [20, 21].

Katuk leaves contain steroids and polyphenols, which increase prolactin levels and are anti-inflammatory and anti-diabetic. With a chlorophyll content of 1509.1 mg/kg [22], katuk leaves (*Sauropus androgynus (L) Merr*) serve as a model for a photosensitizer agent that is selective, effective, chemically stable, has a wide range of absorption wavelengths, is soluble, non-toxic, and non-toxic. In this work, blue and red diode laser light sources will be used to test the efficiency of katuk leaf chlorophyll (*Sauropus androgynus (L) Merr*) as an organic photosensitizing agent for inactivating *E. faecalis* and *A. acinomycetemcomitans* bacteria biofilms.

Materials and methods

Chlorophyll extraction of katuk leaves (Sauropus Androgynus (L) Merr) and antibacterial test

Using 96% ethanol, 50 grams of katuk leaves (*Sauropus androgynus (L) Merr*) were extracted. The recovered materials were then mixed with maltodextrin, which made up 20% of the filtrate's mass. The Shimidzu UV-Vis 1800 Spectrometer was used to evaluate the absorbance spectrum of the extracted materials. The disc diffusion method was then used to examine the antibacterial activity of chlorophyll on biofilm samples.

Diode laser characterization

A laser diode will be employed as the light source. Measurements of the wavelength spectrum, power stability, beam area, and temperature stability throughout treatment are all part of the light source's characterization. A temperature gun, a Thermolab PM-100 power meter, and a CT-100 wavelength meter are the instruments utilized for testing.

Bacterial culture and biofilm production

The samples were facultative anaerobe biofilms of the Gram-negative bacteria *Aggregatibacter actinomycetemcomitans* ATCC 29523 and the Gram-positive bacteria E. *faecalis* ATCC 29212. Bacterial cells were introduced to tryptic soy broth, a sterile agar medium, and 1 mL of 2% sucrose. The sample was then pipette-inserted into the microplate hole. Samples were incubated at 37 °C for 48 hours. For samples that have chlorophyll extract added, the extract will be applied before treatment, and the sample will be incubated for about two hours.

Treatment

Samples were distributed to the red laser treatment group (R) and the blue laser treatment group (B). Group B1 had the laser treatment group without the addition of chlorophyll, while group R1 was made up of each group. The katuk leaf chlorophyll (*Sauropus androgynus (L) Merr*) was also present at 1.6 mg/mL in the Groups B2 and R2 laser treatment groups. At a distance of 1 cm, a laser with energy densities ranging from 2.5 J/cm^2 to 10 J/cm^2 was utilized to treat each group. Each group has Group C_0, which stands for the negative control without the addition of chlorophyll or laser treatment, and Group C1, which stands for the treatment with chlorophyll but without laser. Laser exposure and chlorophyll photosensitizer were tested using ELISA and ANOVA to determine the effects of laser exposure and the addition of chlorophyll photosensitizer.

Results and discussion

Chlorophyll extraction of katuk leaves (Sauropus androgynus (L) Merr)

The findings of the absorbance test revealed that the chlorophyll absorbance peaks of katuk leaves were at wavelengths of 383 nm-419 nm and 500 nm-685 nm. The maximal absorbance of chlorophyll at 10% is 2.42. the following equation can be used to determine the amount of chlorophyll:

$$\text{Total chlorophyll} = [8{,}02(A663) + 20{,}20(A645)]\ \text{mg/L} \quad (1)$$

A 10% chlorophyll solution has 71.71 mg/L of total chlorophyll, according to equation 1. When employing diode laser light sources with wavelengths of 401 nm and 660 nm, 91% of the photosensitizer is absorbed. The results of the chlorophyll anti-bacterial test are shown in Table 1 for all concentration ranges, including control (0%), 2.5%, 5%, 7.5%, and 10%. The test findings revealed no clear zone on any of the disc papers, which suggests that the four concentrations are categorized as concentrations of compounds without antibacterial activity.

Diode laser characterization test results

Using a wavelength meter, a diode laser will be utilized as the light source. Its peak wavelengths are (401 ± 10) and (660 ± 7) nm, respectively (CT-100). On a blue diode laser (25.00 ± 1.71 mW) with a beam area of 0.20 cm and a red

Таблица 1

Диаметр зоны отсутствия роста в диско-диффузионном тесте

Table 1

The diameter of the clear zone in the disc diffusion test

Концентрация Concentration	Хлорофилл Chlorophyll	Диаметр зоны отсутствия роста Diameter of the clear zone			
		Бактерии Bacteria		Биопленка Biofilms	
		A.actinomycetemcomitans	*E. faecalis*	*A.actinomycetemcomitans*	*E. faecalis*
2,5%	50 µl	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm
5%	50 µl	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm
7,5%	50 µl	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm
10%	50 µl	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm	(0,00±0,05) cm

Таблица 2
Параметры облучения
Table 2
Irradiation parameters

Длина волны (нм) Wavelength (nm)	Мощность лазера (мВт) Laser power (mW)	Площадь зоны облучения (см²) Laser beam area (cm²)	Время (сек) Time (s)	Плотность энергии (Дж/см²) Energy density (J/cm²)
(401± 10)	(25.00 ± 1.71)	(0.20 ± 0.01)	20	2.51
			40	5.00
			60	7.50
			80	10.00
(660 ± 7)	(39.70 ± 1,35)	(0.24 ± 0,01)	15	2.50
			31	5.12
			45	7.50
			61	10.09

diode laser (39.7 ± 1.35 mW) with a beam area of (0.24 ± 0.01) cm, the power of the diode laser was measured under stable conditions using a Thermolab PM 100 power meter. The temperature was maintained at 37 °C throughout the irradiation. Equation 2 can be used to determine the two lasers' respective energy densities [23]. Table 2 displays the duration of the laser treatment.

$$E = \frac{P}{A} x\, t \qquad (2)$$

Inactivation photodynamic test results

Fig. 1 and 2 show what happens to *A. actinomycetemcomitans* and *E. faecalis* biofilms when they are exposed to radiation.

A biofilm caused by *A. actinomycetemcomitans* was able to survive after exposure to lasers and chlorophyll photosensitizer.

Fig. 3 and 4 show the percentage reduction of *A. actinomycetemcomitans and E. faecalis* biofilms, respectively.

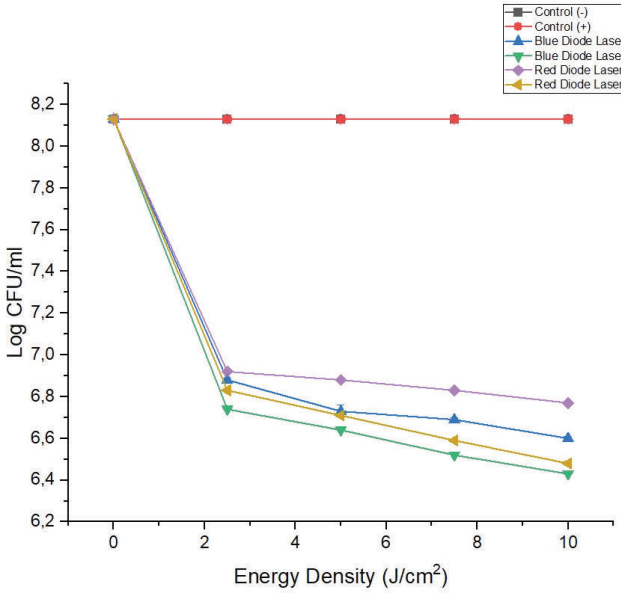

Рис. 1. Жизнеспособность биопленки *A. actinomycetemcomitans* при облучении с различной плотностью энергии с фотосенсибилизатором хлорофилл.
Fig. 1. *A. actinomycetemcomitans* biofilm viability in various laser treatments with variations in energy density and the addition of chlorophyll photosensitizer.

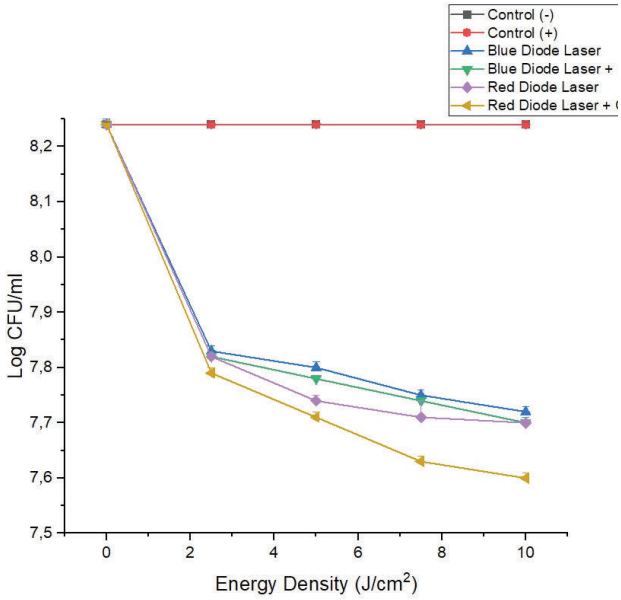

Рис. 2. Жизнеспособность биопленки *E. faecalis* при облучении с различной плотностью энергии с фотосенсибилизатором хлорофилл.
Fig. 2. *E. faecalis* biofilm viability in various laser treatments with variations in energy density and the addition of chlorophyll photosensitizer.

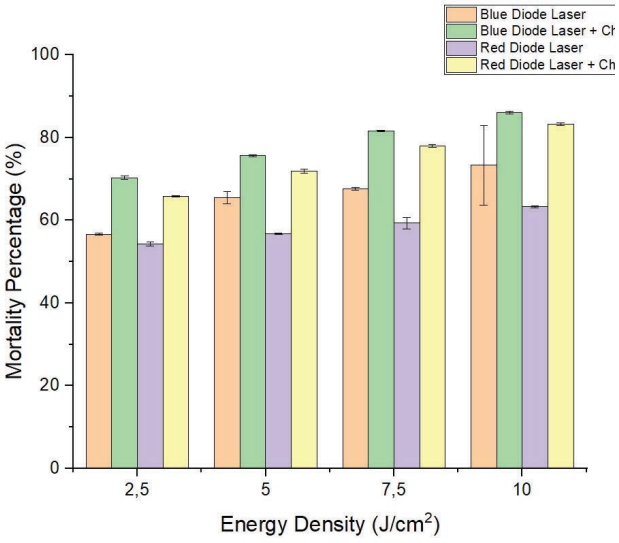

Рис. 3. Зависимость редукции биопленки *A. actinomycetemcomitans* от плотности энергии лазерного облучения.
Fig. 3. Dependence of *A. actinomycetemcomitans* biofilm reduction on laser irradiation energy density.

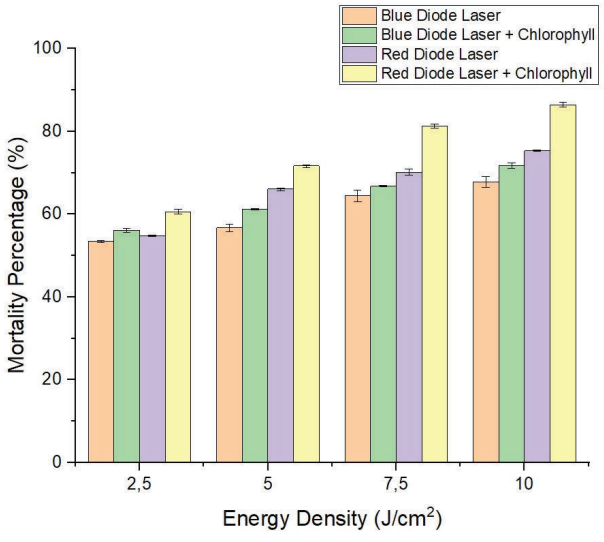

Рис. 4. Зависимость редукции биопленки *E. faecalis* в зависимости от плотности энергии лазерного облучения.
Fig. 4. Dependence of *E. faecalis* biofilm reduction on laser irradiation energy density.

The results of a factorial ANOVA test showed no significant differences between treatment groups ($p = 0$). The 10 J/cm^2 energy-dense blue diode laser treatment with 86.12% chlorophyll had the highest percentage of *A. actinomycetemcomitans* biofilm reduction, while the best decrease of *E. faecalis* biofilm was achieved with 2.5 J/cm^2.

Fluorescence test results

Testing with a fluorescent microscope to determine how many bacteria have died. Fig. 5 to 8 display the findings of the fluorescence test.

The 10 J/cm^2 energy-dense blue diode laser treatment group had the highest percentage of *A. actinomycetemcomitans* biofilm death, and the addition of chlorophyll caused 2122 cell deaths, according to the fluorescence test results. When chlorophyll was added to an energy of 10 J/cm^2, 2189 cell deaths occurred.

Chlorophyll produced by katuk leaves has no direct anti-bacterial activity. Chlorophyll becomes active as a photosensitizer exogen when exposed to the right spectrum. Triplet excitation creates radical oxygen species that render bacteria inactive due to cell membrane leakage. In this study, when blue and red diode lasers stimulated the chlorophyll in katuk leaves, radical oxygen species were made. These radical oxygen species killed bacteria and stopped *A. actinomycetemcomitan* and *E. faecalis* from making biofilms.

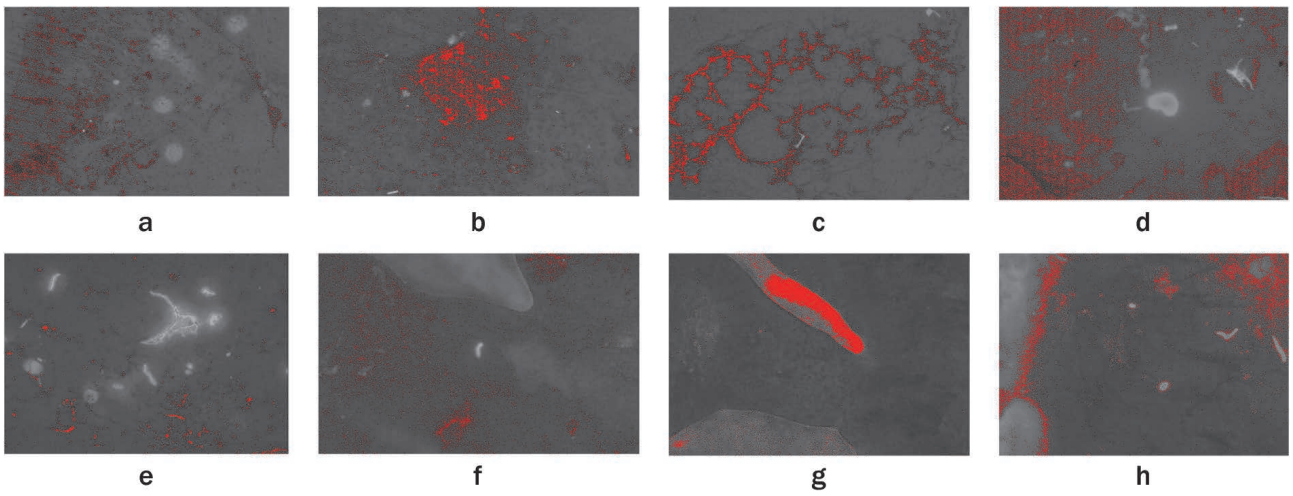

Рис. 5. Результаты воздействия на бактерии *A. actinomycetemcomitans* синим диодным лазером: a – 2,5 Дж/см^2; b – 5 Дж/см^2; c – 7,5 Дж/см^2, d – 10 Дж/см^2; и красным диодным лазером: e – 2,5 Дж/см^2; f – 5 Дж/см^2; g – 7,5 Дж/см^2; h – 10 Дж/см^2.
Fig. 5. Treatment results on *A. actinomycetemcomitans* bacteria with an energy-dense blue diode laser: a – 2.5 J/cm^2; b – 5 J/cm^2; c – 7.5 J/cm^2; d – 10 J/cm^2; and energy density red diode laser treatment: e – 2.5 J/cm^2; f – 5 J/cm^2; g – 7.5 J/cm^2; h – 10 J/cm^2.

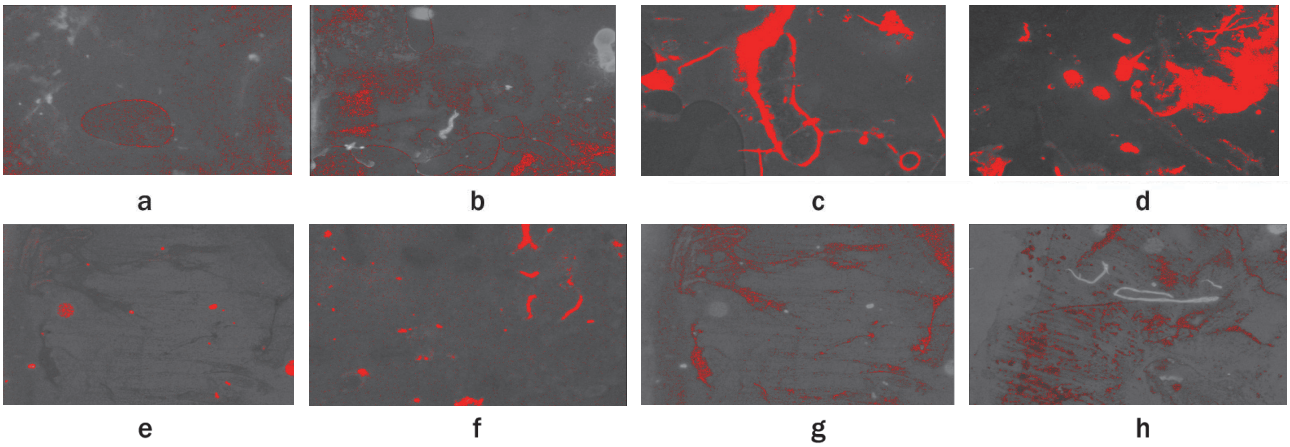

Рис. 6. Результаты воздействия на бактерии *A. actinomycetemcomitans* с добавлением хлорофилла синим диодным лазером: a – 2,5 Дж/см²; b – 5 Дж/см²; c – 7,5 Дж/см², d – 10 Дж/см²; и красным диодным лазером: e – 2,5 Дж/см²; f – 5 Дж/см²; g – 7,5 Дж/см²; h – 10 Дж/см².
Fig. 6. Treatment results on *A. actinomycetemcomitans* bacteria with the addition of chlorophyll with an energy-dense blue diode laser: a – 2.5 J/cm²; b – 5 J/cm²; c – 7.5 J/cm²; d – 10 J/cm²; and energy density red diode laser treatment: e – 2.5 J/cm²; f – 5 J/cm²; g – 7.5 J/cm²; h – 10 J/cm².

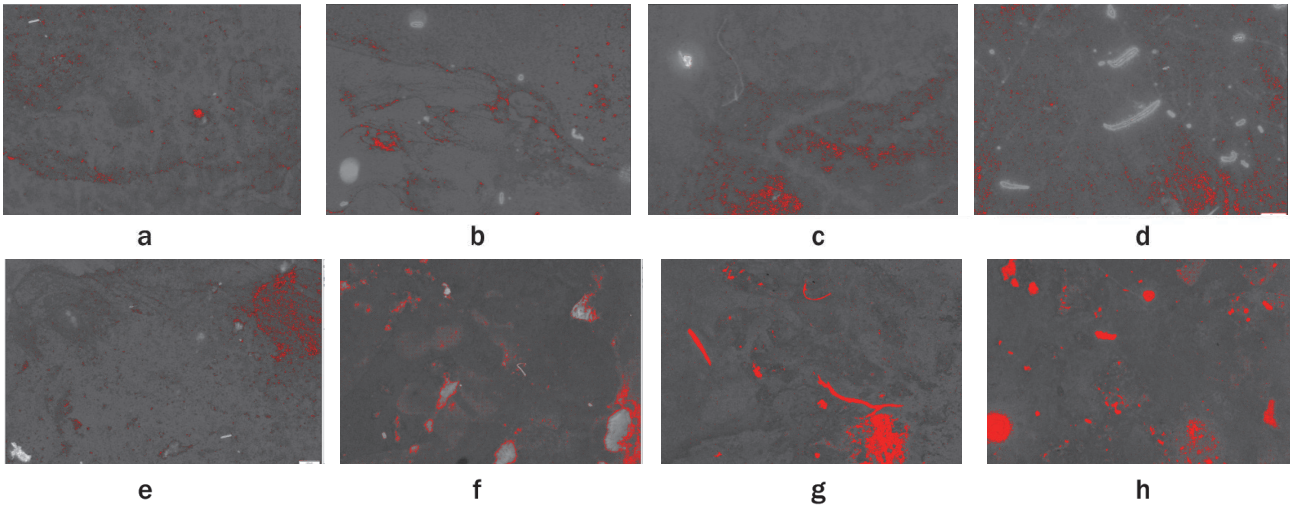

Рис. 7. Результаты воздействия на бактерии *E. faecalis* синим диодным лазером: a – 2,5 Дж/см²; b – 5 Дж/см²; c – 7,5 Дж/см², d – 10 Дж/см²; и красным диодным лазером: e – 2,5 Дж/см²; f – 5 Дж/см²; g – 7,5 Дж/см²; h – 10 Дж/см².
Fig. 7. The results of treatment on *E. faecalis* bacteria with an energy density blue diode laser: a – 2.5 J/cm²; b – 5 J/cm²; c – 7.5 J/cm²; d – 10 J/cm²; and energy density red diode laser treatment: e – 2.5 J/cm²; f – 5 J/cm²; g – 7.5 J/cm²; h – 10 J/cm².

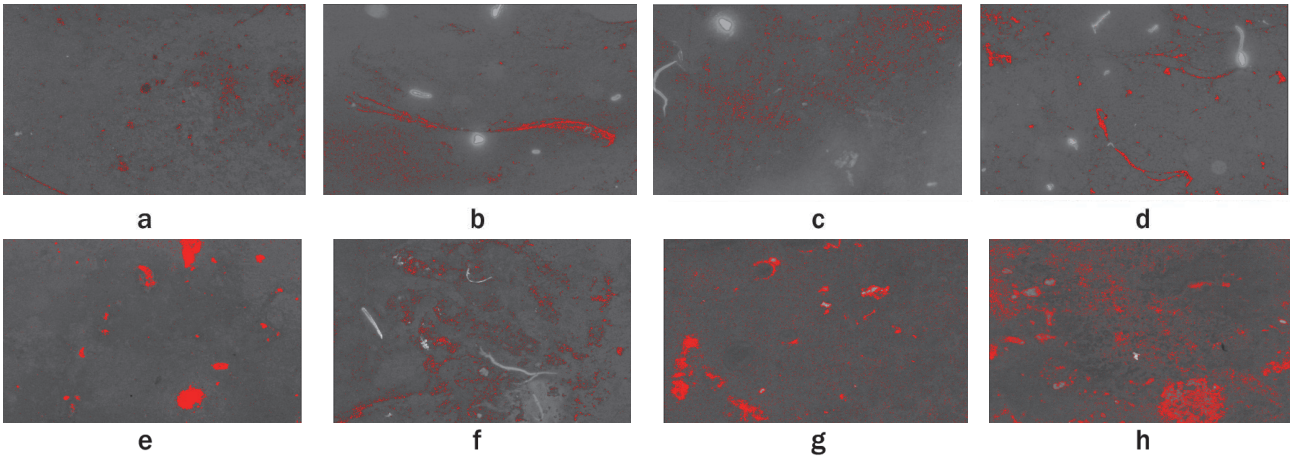

Рис. 8. Результаты воздействия на бактерии *E. faecalis* с добавлением хлорофилла синим диодным лазером: a – 2,5 Дж/см²; b – 5 Дж/см²; c – 7,5 Дж/см², d – 10 Дж/см²; и красным диодным лазером: e – 2,5 Дж/см²; f – 5 Дж/см²; g – 7,5 Дж/см²; h – 10 Дж/см².
Fig. 8. Treatment results on *E. faecalis* bacteria with the addition of chlorophyll with an energy density blue diode laser: a – 2.5 J/cm²; b – 5 J/cm²; c – 7.5 J/cm²; d – 10 J/cm²; and energy density red diode laser treatment: e – 2.5 J/cm²; f – 5 J/cm²; g – 7.5 J/cm²; h – 10 J/cm².

The *A. actinomycetemcomitans* biofilm was reduced by 86.12% in the 10 J/cm^2 energy-dense blue diode laser therapy with chlorophyll addition. The 2.5 J/cm^2 red diode laser treatment without the addition of chlorophyll had the largest reduction percentage of *A. actinomycetemcomitans* biofilm (54.34%). The energy-dense red diode laser treatment of 10 J/cm^2 with the addition of chlorophyll produced the highest percentage test results for the removal of *E. faecalis* biofilm, 86.41%. The best way to get rid of *E. faecalis* biofilm was to use a blue diode laser with a high energy density of 2.5 J/cm^2 without adding chlorophyll.

Red diode laser treatment of Gram-positive bacteria resulted in the greatest percentage of biofilm reduction. Gram-positive bacteria have fluorescence emission maxima at 622 nm and 617 nm, while gram-negative bacteria have emission peaks at 630 nm and 615 nm [24]. Gram-positive bacteria are more vulnerable to singlet oxygen, making chlorophyll more effective at killing them [25]. Gram-positive bacteria have a single layer of teichoic acid and a thick, porous peptidoglycan layer, while Gram-negative bacteria have two layers. Gram-negative bacteria are more sensitive to physical disturbances, with *E. faecalis* dying off at a higher rate than *A. actinomycetemcomitans*.

Conclusion

The 10 J/cm^2 energy-dense blue diode laser treatment with chlorophyll addition had the largest reduction percentage of *A. actinomycetemcomitans* biofilm, with a reduction percentage of 86.12%. Without the addition of chlorophyll, the red diode laser treatment reduced the *A. actinomycetemcomitans* biofilm by 54.34% at a density of 2.5 J/cm^2. While the red diode laser treatment with a 10 J/cm^2 energy density and the addition of chlorophyll had the greatest percentage reduction for *E. faecalis* biofilm (86.41%), the blue diode laser treatment with a 2.5 J/cm^2 energy density and the absence of chlorophyll had the greatest percentage reduction for *E. faecalis* biofilm (54.40%). So, when blue and red diode lasers hit katuk leaves, the chlorophyll gets turned on. This makes radical oxygen species, which kill bacteria and reduce *Aggregatibacter actinomycetemcomitans* and *E. faecalis* biofilms.

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