EFFECTIVENESS OF BACTERIAL BIOFILMS PHOTODYNAMIC INACTIVATION MEDIATED BY CURCUMIN EXTRACT, NANODOXYCYCLINE AND LASER DIODE

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Abstract

Biofilms have higher levels of antibiotic resistance compared to bacteria, so the alternatives are needed as therapy for diseases caused by biofilm infections. Photodynamic Therapy (PDT) has the advantage of being a safe alternative that involves molecular-level photochemical reactions. The use of different types of exogenous photosensitizers (PS) was done to compare their effectiveness. Turmeric extract containing curcumin has good effectiveness in PDT, whereas nanodoxycycline as an antibiotic has a fairly broad absorption spectrum and is effective as PS. The purpose of this study is to compare the effectiveness of photodynamic therapy on infections by *Aggregatibacter actinomycetemcomitans* causing periodontitis using exogenous organic and non-organic photosensitisers (PS). The *A. actinomycetemcomitans* biofilm had been grown on 96-well microplate for 72 hours incubation time. The samples were divided into three groups, treated with Laser diode, Laser + Turmeric Extract 0.5%, and Laser + Nanodoxycycline 0.1%. Treatment was done with a variety of exposure times: 30, 60, 90, 120, and 150 seconds. The data were analyzed using ANOVA test. The results of data analysis showed that diode laser irradiation treatment with endogenous porphyrin, diode laser with Curcumin and diode laser with nanodoxycycline produced significantly different biofilm reductions. Treatment with diode laser irradiation at various energy densities (4.15, 8.28, 12.44, 16.59, and 20.73 J/cm^2) showed no significant difference in reducing bacterial biofilm. The treatment with diode and curcumin, and the treatment with diode laser irradiation and nanodoxycyclin showed a significant difference. Diode laser irradiation of 20.73 J/cm^2 with irradiation time of 150 seconds resulted in the greatest reduction of biofilm 14.94%, diode laser irradiation + Curcumin 47.82%, and diode laser irradiation + nanodoxycyclin 53.76%. Therefore, PDT using a diode laser combined with exogenous PS extract of curcumin and nanodoxycycline is more effective to reduce bacterial biofilms.

Keywords: photodynamic inactivation, a. actinomycetemcomitans, biofilm, curcumin, extract, nanodoxycycline, laser diode.

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ЭФФЕКТИВНОСТЬ ФОТОДИНАМИЧЕСКОЙ ИНАКТИВАЦИИ БАКТЕРИАЛЬНЫХ БИОПЛЕНОК С ИСПОЛЬЗОВАНИЕМ ЭКСТРАКТА КУРКУМИНА, НАНОДОКСИЦИКЛИНА И ЛАЗЕРНОГО ДИОДА

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Резюме

Биопленки обладают более высоким уровнем устойчивости к антибиотикам по сравнению с бактериями, поэтому необходима разработка новых подходов к лечению инфекционных заболевания, вызванных бактериальными биопленками. Одним из возможных методов лечения таких заболеваний является фотодинамическая терапия (ФДТ). В качестве фотосенсибилизаторов применяли куркумин и антибиотик нанодоксициклин. Провели сравнительное изучение эффективности фотодинамической терапии инфекций, в патогенезе которых участвовали *Aggregatibacter actinomycetemcomitans*, вызывающие пародонтит, с использованием двух указанных фотосенсибилизаторов. Биопленку *A. actinomycetemcomitans* выращивали на 96-луночном микропланшете в течение 72 ч инкубации. Образцы были разделены на три группы. В первой группе проводили обработку биопленок диодным лазером, во второй – 0,5%-ым экстрактом куркумы и диодным лазером, в третьей – 0,01%-ым раствором нанодоксициклина и и диодным лазером. Время воздействия составляло 30, 60, 90, 120 и 150 сек. Полученные данные были проанализированы с использованием теста ANOVA. Результаты анализа данных показали, что эффективность воздействия на биопленки значительно отличалась в группах с облучением диодным лазером, облучением диодным лазером с куркумином и облучением диодным лазером с нанодоксициклином. Режимы облучения диодным лазером при различных плотностях энергии 4,15; 8,28; 12,44; 16,59; и 20,73 Дж/см^2 не показали существенного воздействия на бактериальную

биопленку. Облучение диодным лазером (20,73 Дж/см^2, время облучения 150 сек) привело к наибольшему уменьшению биопленки на 14,94%, облучение диодным лазером с куркумином – на 47,82%, облучение диодным лазером с нанодоксициклином – на 53,76%. Таким образом, ФДТ с использованием диодного лазера в сочетании с экзогенными фотосенсибилизаторами куркумином и нанодоксициклином показали свою эффективность в отношении бактериальных биопленок.

Ключевые слова: фотодинамическая инактивация, A. Actinomycetemcomitans, биопленка, куркумин, экстракт, нанодоксициклин, лазерный диод.

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Introduction

Indonesia has oral and dental health problems at 25.9% of its national population [1]. One of the common oral diseases that can infect almost 50% of the world's population is periodontitis [2, 3]. The number of sufferers of this disease has reportedly increased consistently over the last decade [2]. Periodontitis can be caused by bacterial activity as a parasite that exceeds the average amount in the mouth. One of them is *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) [4, 5]. These bacteria include gram-negative bacteria that can form biofilms [6, 7, 8]. Biofilms from *A. actinomycetemcomitans* can stick to the tooth surface and form an extracellular matrix, so the level of resistance to antibiotics is high [8, 9, 10]. Therefore, effective and safe alternative treatments for periodontitis are needed, especially the disease caused by *A. actinomycetemcomitans*.

One alternative treatment for infection by bacteria developed in health is Photodynamic Therapy (PDT). This therapy utilizes Reactive Oxygen Species (ROS), which are produced through photochemical processes between light sources and chemical molecules called photosensitisers (PS) [5, 11]. The photochemical process will occur if the wavelength of the absorption of PS is matched with the light source used in PDT. PDT is claimed to be a safe therapy because it only damages the parasitic part of the target object. Therefore, as one of the main components in PDT, PS should not be toxic to healthy cells [11].

There are two types of PS used in PDT, namely endogenous PS and exogenous PS. Endogenous PS is usually an enzyme produced naturally by bacteria, such as the porphyrin in the *A. actinomycetemcomitans* [12]. Porphyrins have a maximum absorption at wavelengths of 400–450 nm [13]. Several studies suggest adding exogenous PS to maximize the effect of PDT [8, 14]. Various kinds of exogenous PS are developed for PDT, both from an organic and non-organic materials. PS from organic matter is usually obtained through extraction or isolation of a particular substance, for example, chlorophyll and curcumin [14, 15, 16]. On the other hand, the use of non-organic materials, such as antibiotics, catalysts, or dyes, was also developed as PS [17, 18, 19].

This study aims to compare the effects of PDT that occur when two types of exogenous PS are used in organic and non-organic compound. *Curcuma longa* or turmeric extracts contains curcumin. The use of curcumin as PS in PDT has a significant effect on decreasing the number of bacterial colonies [20, 21]. Several studies report the benefits of using curcumin as antimicrobial, anticarcinogenic, and anticancer agent [22]. As an ideal PS, the amount of curcumin used must be minimal but effective so that the effects arising are only due to the photodynamic process [23].

Several studies showed that it is common for photodynamic processes to utilize antibiotics as PS even though some bacteria have high resistance, such as *A. actinomycetemcomitans* [2]. Increased antibiotics resistance in bacteria can be caused by excessive use of antibiotics, thus stimulating bacteria to produce a protective form of extracellular matrix called biofilm [2], [17]. The advantage of using antibiotics as PS in PDT due to bacterial infection is an alternative function when the use of antibiotics alone is not effective enough in dealing with bacterial infections. One antibiotic with a tetracycline group that has a broad absorption spectrum is doxycycline [17]. In low doses, the doxycycline use as PS can minimize its effects as an antibiotic without causing resistance [24]. So that doxycycline can be absorbed more optimally by biofilms, doxycycline particle size is converted to nano or nanodoxycycline.

With an effective wavelength absorption range of curcumin extract at 300–500 nm and nanodoxycycline at 200–425 nm, the laser diode is appropriate for this study [17, 25]. The match between the wavelength of light and the wavelength spectrum of PS are the keys to the success of PDT [23]. When the energy received by PS is excessive, the molecule will experience excitation, and ROS is created when the excited molecule reacts with oxygen [11]. In addition, the advantage of a laser diode as a light source is that the output beam is coherent and monochromatic so that the beam diameter is smaller and more focused compared to other conventional light sources [26, 27].

Materials and Methods

Bacterial Biofilm

This study used pure isolates of *Aggregatibacter actinomycetemcomitans* ATCC 43718 obtained from the Faculty of Dentistry, Universitas Airlangga. *A. actinomycetemcomitans* biofilms were cultured on a 96-well microplate using Tryptone Soy Broth (TSB) media. Previously, bacteria were grown in a Tryptone Soy Agar (TSA) medium suspended for 6 grams/liter of yeast extract and 8 grams/liter of glucose [28]. Biofilm cultures were incubated for 72 hours at 37°C anaerobically using a candle jar [8], [28]. Optical Density (OD) of the growing biofilm was calculated using Elisa Reader.

Curcuma longa Extractions

The extraction used the maceration method. The dried turmeric rhizome has been finely immersed in 96% ethanol (C_2H_6O) solvent in a 1 gr: 10 ml [29]. Maltodextrin was added as much as 15% of the mass of the filtrate to increase the volume and final weight of the extract results and speed up the drying process [30]. Then, the ethanol in the filtrate was evaporated by using a rotary evaporator. The final filtrate, after evaporation, was then dried using an oven at 40°C. The extract powder was stored at room temperature in a dark cupboard. *Curcuma longa* as PS was a solution with sterile distilled water.

Nanodoxycycline

Doxycycline ($C_{22}H_{24}N_2O_8 \cdot H_2O$) in the form of powder was crushed for 5 hours using a mortar. Then the sample was ground using 3D High Energy Milling (HEM) to obtain nano-sized doxycycline powder. The process lasts for 2 hours using milling balls (1:20). The grinding sample is filtered using a 7.5 µm mesh. Nanodoxycycline as PS was a solution with distilled water and filtered using PTFE 0.2 µm.

Light Source

Laser Diode characterization using Jasco CT-10 Monochromator and Thorlabs PM100D Power Meter to determine the peak wavelength and intensity of the beam output. The Laser Diode specification used has a peak wavelength (403.00 ± 0.24) nm and an output beam intensity of (138.25 ± 0.01) mW/cm^2 for the beam diameter (0.20 ± 0.01) cm^2. The value of energy density laser diode can be obtained using Equation 1.

$$\text{Energy Density}\left(\text{J cm}^{-2}\right) = \text{Intensity}\left(\text{W cm}^{-2}\right) \times \text{Time exposure}\left(\text{s}\right) \quad (1)$$

Sample Treatments

The *A. actinomycetemcomitans* bacteria sampled in this study were 100 µl biofilm culture grown on 96-well microplate for 72 hours incubation time. TSB medium in each well was removed through the rinsing process using PBS with a pH of 7.4. Then exogenous PS was added in the form of 0.5% Curcumin extract or 0.1% Nanodoxycycline. After the addition of exogenous PS, biofilm culture was incubated anaerobically using a candle jar for 30 minutes, and then the laser diode exposure was performed. The sample had been treated and incubated for 24 hours. The sample taken from incubator were washed with PBS three times. The staining/coloring process uses 200 µl of crystal violet in each well for 15 minutes. The sample was rewashed using distilled sterile water three times to remove crystal violet, and then was given 100µL 33%/well glacial acetic acid (GAA) and measured using ELISA reader S/N 17539 (Bio-rad, US) on 595nm [8]. The results of the research data (OD) were converted to log CFU/ml.

The samples were divided into three treatments, Group X was treated with laser diode, Group Y was treated with laser diode+Curcumin Extract 0.5%, and Group Z was treated with laser diode+nanodoxycycline 0.1%. Treatment of laser diode with a variation of irradiation time, which were 30, 60, 90, 120, and 150 seconds with energy densities of 4.15, 8.28, 12.44, and 16.59 J/cm^2. The data were analyzed using the ANOVA test. Each treatment has a control group, namely a negative control group, a positive control group of nanodoxycycline, and a positive group of curcumin. The percentage reduction in the treated sample was compared with the control group for each treatment.

Statistical Analysis

The percentage of biofilm reduction was obtained by reducing the log CFU/ml value of the treated sample with the control group. The study results in the form of log CFU/ml were statistically analyzed by using the Statistical Package for Social Science (SPSS) version 21. The statistical test conducted was Two Way ANOVA and the Tukey test, with $p < 0.05$, so that a significant difference between the control and treatment group has a data confidence level of 95%.

Results and Discussion

Curcuma longa Extracts

Curcumin extract solution with a concentration of 0.5% is the organic exogenous PS in this study. One of the curcumin extract's contents that has the most advantage in PDT is curcumin. To know how effective curcumin extract as an exogenous PS, it is necessary to compare the absorbance between curcumin standard and curcumin extract. Fig. 1 showed a comparison of the standard absorbance of curcumin standard and curcumin extract by using the Genesys 30 Spectrophotometer.

Curcumin standard, the chemical formula [$HOC_6H_3(OCH_3)CH{=}CHCO]_2CH_2$, used in this study, was derived from Curcuma longa powder with EC number 207–280–5. Through Fig. 1, it is known that the peak absorbance of both solutions was at 423 nm. The standard absorbance value of curcumin was higher than turmeric extract. This result was caused by the curcumin content in curcumin standard was far more than the amount of curcumin in curcumin extract.

The peak wavelength of diode laser used in this study was 403 nm, then the absorbance value of curcumin extract was 0.475. The transmittance value of the curcumin

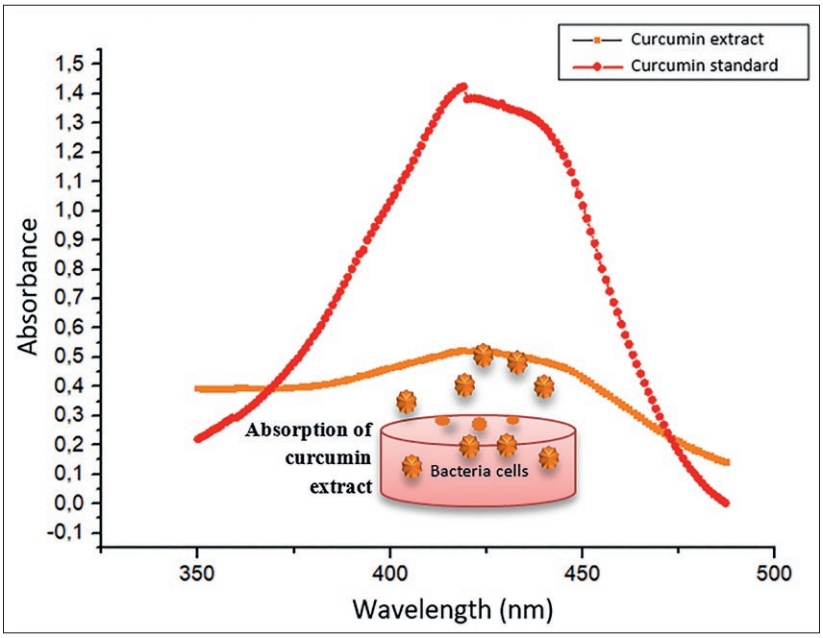

Fig. 1. The absorbance of curcumin extract and curcumin standard
Рис.1. Спектры поглощения экстракта куркумина и стандарта куркумина

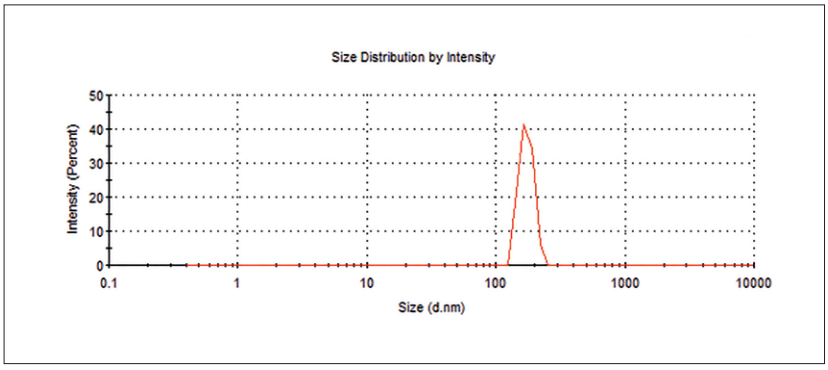

Fig. 2. Nanodoxycycline particle size distribution
Рис.2. Распределение частиц нанодоксициклина по размеру

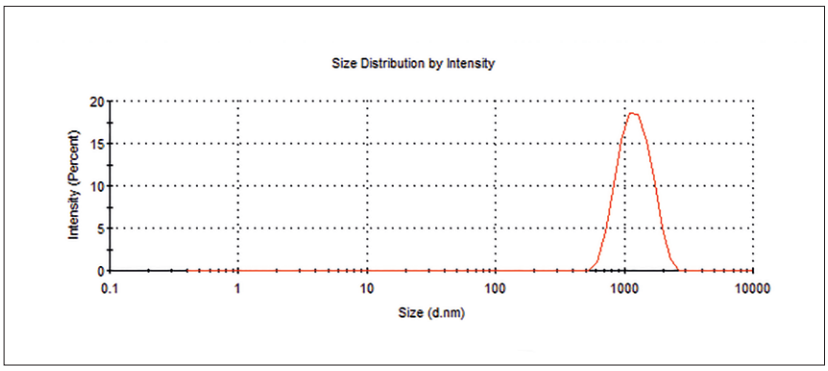

Fig. 3. Doxycycline particle size distribution
Рис.3. Распределение частиц доксициклина по размеру

extract molecule and its absorption percentage can be determined using Lambert-Beer law as follows.

$$A_1 = -\log T_1$$

$$0.475 = -\log T_1$$

$$T_1 = 0.3349$$

So, the absorption of curcumin extract is (1–0.33349) × 100% = 66.51%.

Nanodoxycycline

The 0.1% nanodoxycycline solution was the non-organic exogenous PS used in this study. The goal of reducing doxycycline particles to the nanoscale is that the molecules have a larger surface area to be more easily absorbed by the *A. actinomycetemcomitans*. The value of particle size distribution is at 141.80–220.20 nm, while the doxycycline particle distribution is at 1253.00 nm when tested using Particle Size Analyzer (PSA). The particle size distribu-

Table 1
Analysis of the Nanodoxycycline FTIR Test
Таблица 1
Результаты анализа нанодоксициклина FTIR-тестом

Peak Wavenumber (cm–1)		Functional Group wavenumber values (cm^{-1}) Значения волновых чисел функциональной группы, $см^{-1}$	Functional Group Функциональная группа
Nano doxycycline Нанодоксициклин	Doxycycline [20] Доксициклин		
3525.88	3454	3650-3400	Primary –OH group Основная -OH группа
3336.85	3300	3500-3100	–NH group –NH группа
2931.80	2964	3000-2850	C–H stretching C–H растяжение
1658.78	1672	1680-1630	C–O group C–O группа
1610.56	1618	1680-1600	C–C stretches C–C растяжение
1544.98 and 1454.33	1600 and 1400	1600 and 1475	aromatic C=C bonds ароматические C=C связи
1244.09	1245	1300-1000	C–O bond C–O связь

tion chart of the particle size analyzer test results was shown in Fig. 2 and 3.

The Fourier Transform Infrared (FTIR) test was analyzed with the results shown in Table 1 to prove that the nanodoxycycline functional groups did not experience a significant change compared with the literature. There was a shift in wavenumber but still within the same range of functional groups.

Through PSA and FTIR tests, it was known that doxycyline and nanodoxycycline had differences in terms of functional group and particle size distribution. In the absorbance test using the Genesys 30 Spectrophotometer, the difference was shown by both the absorbance wavelength and the absorbance value. Doxycycline has the highest absorbance peak, which is 2.702, at 375 nm, while the absorbance peak of nanodoxycycline occurs at a wavelength of 377 nm, which is 3.000. The graph of the absorbance value test results is shown in Fig. 4.

When molecular doxycycline is exposed to laser diode with a peak wavelength of 403 nm, the absorbance value of doxycycline is 0.808. With Lambert-Beer law, we can calculate the transmittance value and the percentage of absorption of a molecular of doxycycline.

$$A_2 = -\log T_2$$

$$0,808 = -\log T_2$$

$$T_2 = 0,1556$$

So, the% absorption of nanodoxycycline is

$$(1-0,1556)\times 100\% = 84,44\%$$

Laser Diode Characterization

The laser diode used in this study has a Gaussian output beam. The relatively small diameter of the laser beam, which was (0.20 ± 0.01) cm^2, allows for a high energy density with minimum exposure time. It is necessary to characterize the laser diode beam temperature using a Digital Constant Multimeter 89 to avoid excessive heating. The result data shows that the beam temperature of the laser output was 32.04 ± 0.02 °C, so it can be ascertained that a decrease in the level of biofilm OD is caused by PDT, not due to the thermal reactions. The characterization of the diode laser was shown in Table 2.

Fig. 5 showed the relationship between the wavelength of the Laser Diode and its output power with the Gaussian approach. It is known that the peak wavelength of the Laser Diode was 403.00 ± 0.24 nm, with a power of 27.65 ± 0.01 mW. The output beam intensity was 138.25 ± 0.01 mW/cm^2 for diameter of the beam of 0.20 ± 0.01 $cm^{2.}$ Photochemical reactions in PDT occurred when exposure times > 1 s and power density were in the mW [31]. Therefore, five variations of the exposure time of biofilms were carried out, as summarized in Table 2.

The curcumin and doxycycline absorption spectrum were depicted at 380–780 nm based on the previous studies [11, 15, 17, 23]. When the doxycycline was in the nanoscale size, the absorption spectrum was

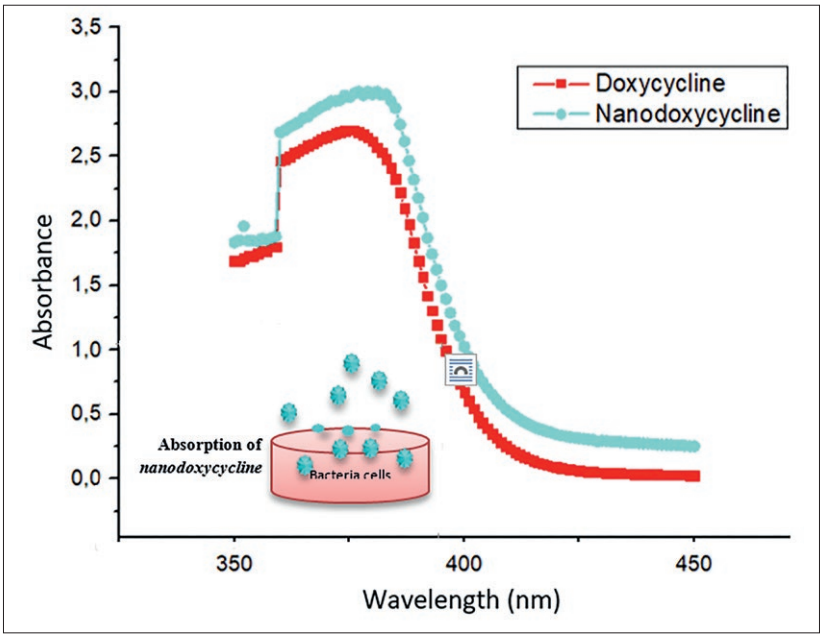

Fig. 4. Absorbance spectrum of doxycycline and nanodoxycycline
Рис.4. Спектры поглощения доксициклина и нанодоксициклина

Table 2
The characterization of laser
Таблица 2
Характеристика лазера

Wavelength (nm) Длина волны, нм	Beam Intensity (mW/cm²) Интенсивность излучения, мВт/см²	Spot Area (cm²) Размер пятна, см²	Time Exposure (s) Время экспозиции, с	Energy Density (J/cm²) Плотность энергии, Дж/см²
			30.000 ± 0.005	4,15
			60.000 ± 0.005	8,29
403,000 ± 0,007	138,25 ± 0,01	0,2±0,01	90.000 ± 0.005	12,44
			120.000 ± 0.005	16,59
			150.000 ± 0.005	20,73

shifted from 375.00 ± 0.05 nm to 377.00 ± 0.05 nm. The level of reactive oxygen formation was affected by this shifting; hence the biofilm reduction escalates too. The wavelength of the laser used in this study was 403.00 ± 0.05 nm. The percentage of photons absorbed by the curcumin and doxycycline was 67% and 84%, respectively.

The wavelength spectrum laser diode corresponds to the absorption spectrum of the exogenous PS used, namely curcumin and nanodoxycycline extracts.

Treatment Results

The results of data analysis showed that diode laser irradiation treatment with endogenous porphyrin, diode laser with curcumin, and diode laser with doxycyclin produced significantly different biofilm reductions ($p < 0.05$). Treatment with diode laser irradiation at various energy densities of 4.15, 8.28, 12.44, 16.59, and 20.73 J/cm² showed no significant difference ($p > 0.05$) in reducing bacterial biofilm. The treatment with diode laser and curcumin, diode laser with nanodoxycyclin, showed a significant difference ($p < 0.05$). Diode laser irradiation of 20.73 J/cm² with an irradiation time of 150 seconds resulted in the greatest reduction of biofilm of 14.94%, diode laser irradiation+Curcumin – 47.82%, and diode laser irradiation+nanodoxycyclin – 53.76%. The results of *A. actinomycetemcomitans* biofilm reduction is shown in Fig. 6.

The photoinactivation mechanism occurs when a biological molecule is exposed to light. A process called photophysical reaction occurs when the energy of photons is absorbed by photosensitizer molecules. The match of the wavelength spectrum between the laser diode and the exogenous PS results in a photophysical process [32]. The diode laser produces energy absorbed by PS molecules so that PS molecules get additional energy to be excited

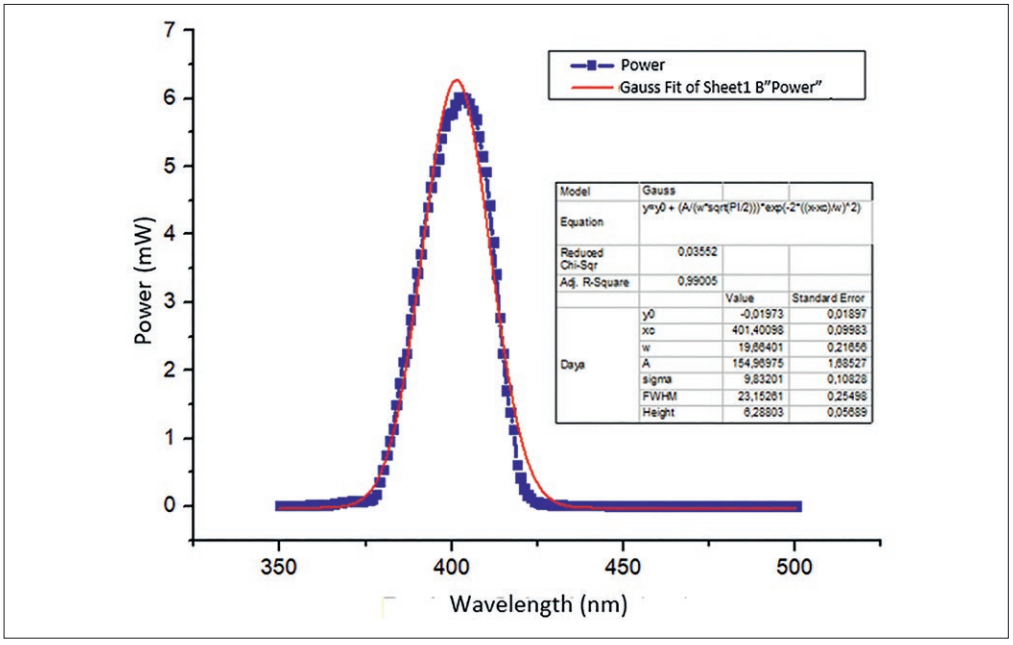

Fig. 5. Blue laser diode characterization
Рис.5. Характеристика диодного лазера

to a higher energy level. This excitation state is unstable so that the PS molecule will return to its ground state, one of which is through photochemical reactions with other molecules in the form of energy transfer or electron transfer [11]. The product of the photochemical reaction is the oxygen radicals (ROS). ROS is reactive and can damage the biofilm cell membrane, thereby disrupting the metabolic activity of cells [33].

In exogenous PS utilization, a type I photochemical reaction occurs when an excited PS molecule initiates a reaction with the substrate to induce photolytic deamination in the exogenous PS fourth carbon ring system. The excited photosensitizer transfers electrons to oxygen to produce superoxide anion (O^{2-}) and forms ROS, which consists of hydroxyl radicals (*OH) and hydrogen peroxide (H_2O_2). Superoxide ionization (O^{2-}) will produce hydrogen peroxide (H_2O_2) and cause a reaction through an oxidation reaction to produce free radicals, causing bio-molecular damage. In a type II reaction, when PS is in triplet state, energy is directly transferred to the oxygen molecule to produce singlet oxygen. Excited singlet oxygen can trigger oxidative peroxidation reactions that damage biological molecules. The last reaction is a photobiological reaction when superoxide is formed in the cell (intracellular) and the peripheral area (extracellular). Hydroxyl radical reactivity causes oxidative damage. Nano-sized doxycycline can diffuse through the pores where the nutrients are transported to all parts of the biofilm region, causing wider biological damage [11].

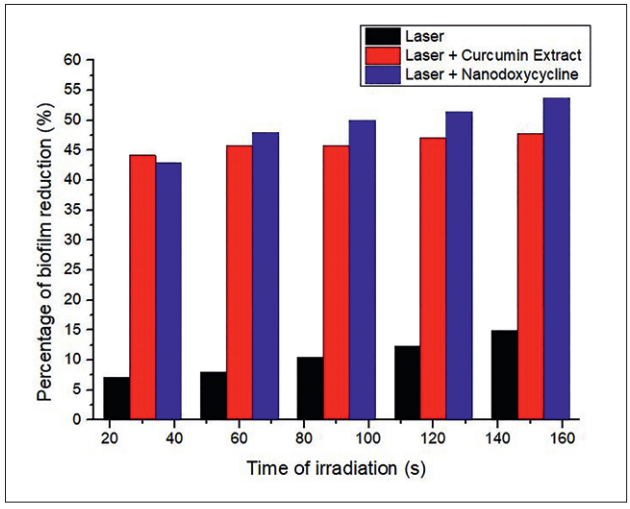

Fig. 6. Histogram Percentage of Biofilm Reduction of *A.actinomycetemcomitans*
Рис.6. Доля уменьшения биопленки *A.actinomycetemcomitans*

Oxidative damage can occur in three different locations based on the photosensitizer, namely the biofilm matrix, cell membrane, and intracellular parts. Biofilm matrix components such as lipids, proteins, and DNA are oxidized by ROS. ROS can interact with the biofilm matrix, which affects the cohesiveness and stability of EPS. It also reacts to lipids on the outside of the cell membrane and changes their morphological structure. It leads to increased photosensitizer intake and stimulation of leakage from cellular metabolites. Damage that occurs in the cell membrane causes inactivation of the membrane transport system. Damage can also occur to intracellular parts such as the nucleus and mitochondria. All of these defects change the phenotype and reduce the biofilm

[11]. The results of the research data analysis showed that the higher the energy density used, the higher the biofilm reduction. The highest decrease occurred in the use of an energy density of 20.73 J/cm^2 for each treatment.

Porphyrins as endogenous PS can reduce biofilms when PDT is used using a diode laser. The highest reduction in biofilm occurred at an energy density of 20.73 J/cm^2 of 14.94%. Porphyrin has an absorption wavelength at 400–450 nm [13], so the diode laser'wavelength used in this treatment is suitable. The relatively low biofilm reduction was due to the limited laser diode penetration of the *A. actinomycetemcomitans* biofilm layer [34]. Due to the limited light penetration, the biofilm layer at the bottom of the plate does not receive the laser diode'energy. Therefore, the ROS only reduced *A. actinomycetemcomitans* biofilm by an average of 14.94%.

The addition of exogenous PS is more effective in reducing biofilms than using only endogenous PS. As an exogenous PS, curcumin extract is relatively sufficient to reduce biofilms. One of the active components of this photosensitizer is curcumin, capable of producing ROS when exposed to light with a wavelength of more than 400 nm [35]. The average reduction in biofilm due to PDT with PS curcumin extract was 46.12%. PDT using curcumin extract was shown to be able to reduce biofilms greater than endogenous PS. However, with a greater energy density, exogenous PS nanodoxycycline was better able to reduce biofilms higher than curcumin PS.

Tetracyclines are well-established antibiotics but exhibit phototoxicity as a side effect. Anti-microbial photodynamic inactivation uses tetracyclines combined with harmless light to destroy microbial cells by reactive oxygen species. Tetracyclines (demeclocycline and doxycycline) can act as light-activating antibiotics by binding to bacterial cells and killing them only after illumination. The remaining tetracyclines can prevent bacterial regrowth after illumination has stopped. Bacteria are killed by photoactivation of tetracyclines without oxygen. Because topical tetracyclines are already used clinically, activation of blue light can increase the bactericidal effect [36].

The ability of biofilms to form extracellular matrices makes the penetration of external particles more difficult [37]. In this study, nano doxycycline particles had a wider surface area, allowing them to be more easily absorbed by *A. actinomycetemcomitans* biofilms. Although biofilms have high resistance to tetracycline antibiotics [38], through the PDT process, it has been shown that the use of antibiotics as PS increases biofilm reduction. The average reduction in biofilm *A. actinomycetemcomitans* with diode laser irradiation of 20.73 J/cm^2 and PS doxycycline was 53.76%.

In addition, the age of the biofilm affects the ability of PDT to reduce biofilms. Previous research has shown that the older the biofilm, the lower the bacterial and biofilm reduction is [39]. This behavior may be due to the limited light penetration of the biofilm. Thus, antibiotics as PS in PDT can be a therapeutic solution due to bacterial infection.

Conclusion

The results of data analysis showed that diode laser irradiation treatment with endogenous porphyrin, diode laser with Curcumin, and diode laser with nanodoxycyclin produced significantly different biofilm reductions. Treatment with diode laser irradiation at various energy densities of 4.15, 8.28, 12.44, 16.59, and 20.73 J/cm^2 showed no significant difference in reducing bacterial biofilm. The treatment with diode and curcumin, and diode laser irradiation with nanodoxycyclin showed a significant difference. Diode laser irradiation of 20.73 J/cm^2 with an irradiation time of 150 seconds resulted in the most significant reduction of biofilm of 14.94%, diode laser irradiation+Curcumin – 47.82%, and diode laser irradiation+nanodoxycyclin – 53.76%. Therefore, PDT using a blue diode laser combined with exogenous PS extract of curcumin and nanodoxycycline is more effective to reduce bacterial biofilms *A. actinomycetemcomitans*.

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REFERENCES

1. Badan Penelitian and Pengembangan, *"RISET KESEHATAN DASAR"*, 2013.
2. Cionca N., Use and Misuse of Systemic Antibiotics in Periodontitis Treatment, *Oral Health Prev. Dent.*, 2017, vol. 15 (4), pp. 305–306.
3. Petersen P.E. and Ogawa H., The global burden of periodontal disease: towards integration with chronic disease prevention and control, 2012, vol. 60, pp. 15–39.
4. Akram Z. et al., Bactericidal Efficacy of Photodynamic Therapy against Periodontal Pathogens in Periodontal Disease: A Systematic Review, *Photomed. Laser Surg.*, 2016, vol. 34 (4), pp. 137–149.

ЛИТЕРАТУРА

1. Badan Penelitian and Pengembangan//"RISET KESEHATAN DASAR. – 2013.
2. Cionca N., Use and Misuse of Systemic Antibiotics in Periodontitis Treatment//Oral Health Prev. Dent. – 2017. – Vol. 15 (4). – P. 305–306.
3. Petersen P.E. and Ogawa H., The global burden of periodontal disease: towards integration with chronic disease prevention and control. – 2012. – Vol. 60. – P. 15–39.
4. Akram Z. et al., Bactericidal Efficacy of Photodynamic Therapy against Periodontal Pathogens in Periodontal Disease: A Sys-

5. Akram Z., Hyder T., Al-Hamoudi N. et al., Efficacy of photodynamic therapy versus antibiotics as an adjunct to scaling and root planing in the treatment of periodontitis: A systematic review and meta-analysis, *Photodiagnosis Photodyn. Ther.,* 2017, vol. 19, pp. 86–92.
6. Moslemi N. et al., Inactivation of *Aggregatibacter actinomycetemcomitans* by two different modalities of photodynamic therapy using Toluidine blue O or Radachlorin as photosensitizers: an in vitro study, *Lasers Med. Sci.*, 2014, vol. 30 (1), pp. 89–94.
7. Periasamy S. and Kolenbrander P.E., Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with Fusobacterium nucleatum and Veillonella *species* in saliva, *Infect. Immun*., 2009, vol. 77 (9), pp. 3542–3551.
8. de C. Goulart R. et al., Photodynamic Therapy in Planktonic and Biofilm Cultures of *Aggregatibacter actinomycetemcomitans*, *Photomed. Laser Surg*., 2010, vol. 28 (S1), pp. 53–60.
9. de C. Goulart R., Thedei G., Souza S.L.S. et al., Comparative Study of Methylene Blue and Erythrosine Dyes Employed in Photodynamic Therapy for Inactivation of Planktonic and Biofilm-Cultivated Aggregatibacter actinomycetemcomitans, *Photomed. Laser Surg.*, 2010, vol. 28 (S1), pp. 85–90.
10. Alvarenga L.H. et al., *A*ggregatibacter actinomycetemcomitans biofilm can be inactivated by methylene blue-mediated photodynamic therapy, *Photodiagnosis Photodyn. Ther.*, 2015, vol. 12 (1), pp. 131–135.
11. Astuti S.D., Ma'rifah Z.A., Fitriyah N. et al., The effectiveness of nano-doxycycline Activated by Diode Laser Exposure to Reduce S. aureus Biofilms: an in vitro Study, Proc. SPIE 10863, *Photonic Diagnosis and Treatment of Infections and Inflammatory Disease II*, 2019, 1086311.
12. Fyrestam J., Bjurshammar N., Paulsson E. et al., Influence of culture conditions on porphyrin production in Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis, *Photodiagnosis Photodyn. Ther.*, 2017, vol. 17, pp. 115–123.
13. Kempa M. et al., Physicochemical properties of potential porphyrin photosensitizers for photodynamic therapy, Spectrochim. *Acta – Part A Mol. Biomol. Spectrosc.*, 2015, vol. 146, pp. 249–254.
14. Setiawatie E.M., Astuti S.D., and Zaidan A.H., An in vitro Antimicrobial Photodynamic Therapy (aPDT) with Blue LEDs to Activate Chlorophylls of Alfalfa Medicago sativa L on Aggregatibacter actinomycetemcomitans," *J. Int. Dent. Med. Res.*, 2016, vol. 9 (2), pp. 118–125.
15. Astuti, SD., Mahmud A.F. et al., Antimicrobial photodynamic of blue LED for activation of curcumin extract (curcuma longa) on staphylococcus aureus bacteria, an in vitro study, *IOP Conf. Series: Journal of Physics: Conf. Series,* 2018, vol. 1120, 012073.
16. Cellamare B.M., Fini P., Agostiano A. et al., Identification of ROS produced by photodynamic activity of chlorophyll/cyclodextrin inclusion complexes, *Photochem. Photobiol.*, 2013, vol. 89 (2), pp. 432–441.
17. Setiawatie E.M., Lestari V.P., and Astuti S.D., Comparison of Antibacterial Efficacy of Photodynamic Therapy and Doxycycline on Aggregatibacter Actinomycetemcomitans, *Afr J Infect Dis*, 2018, vol. 12, pp. 95–103.
18. Astuti S.D., Drantantiyas N.D.G., Putra A.P. et al., Suhariningsih, Photodynamic effectiveness of laser diode combined with ozone to reduce Staphylococcus aureus biofilm with exogenous chlorophyll of Dracaena angustifolia leaves, *Biomedical Photonic*, 2019, vol.8 (2), pp. 4–13.
19. Chang G., Zhang H., Li S. et al., Effective photodynamic therapy of polymer hydrogel on tumor cells prepared using methylene blue sensitized mesoporous titania nanocrystal, *Mater. Sci. Eng. C*, 2019, vol. 99, pp. 1392–1398.
20. Sahne F., Mohammadi M., Najafpour G.D. et al., Extraction of Bioactive Compound Curcumin from Turmeric (Curcuma Longa L.) Via Different Routes: a Comparative Study," *J. Biotechnol*, 2016, vol.13 (3), pp. 173–180.

tematic Review//Photomed. Laser Surg. – 2016. – Vol. 34 (4). – P. 137–149.
5. Akram Z., Hyder T. et al., Efficacy of photodynamic therapy versus antibiotics as an adjunct to scaling and root planing in the treatment of periodontitis: A systematic review and meta-analysis//Photodiagnosis Photodyn. Ther. – 2017. – Vol. 19. – P. 86–92.
6. Moslemi N. et al., Inactivation of Aggregatibacter actinomycetemcomitans by two different modalities of photodynamic therapy using Toluidine blue O or Radachlorin as photosensitizers: an in vitro study//Lasers Med. Sci. – 2014. – Vol. 30 (1). – P. 89–94.
7. Periasamy S. and Kolenbrander P.E., Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with Fusobacterium nucleatum and Veillonella species in saliva//Infect. Immun. – 2009. – Vol. 77 (9). – P. 3542–3551.
8. de C. Goulart R. et al., Photodynamic Therapy in Planktonic and Biofilm Cultures of Aggregatibacter actinomycetemcomitans//Photomed. Laser Surg. – 2010. – Vol. 28 (S1). – P. 53–60.
9. de C. Goulart R., Thedei G. et al., Comparative Study of Methylene Blue and Erythrosine Dyes Employed in Photodynamic Therapy for Inactivation of Planktonic and Biofilm-Cultivated Aggregatibacter actinomycetemcomitans//Photomed. Laser Surg. – 2010. – Vol. 28 (S1). – P. 85–90.
10. Alvarenga L.H. et al., Aggregatibacter actinomycetemcomitans biofilm can be inactivated by methylene blue-mediated photodynamic therapy//Photodiagnosis Photodyn. Ther. – 2015. – Vol. 12 (1). – P. 131–135.
11. Astuti S.D., Ma'rifah Z.A., Fitriyah N. et al., The effectiveness of nano-doxycycline Activated by Diode Laser Exposure to Reduce S//aureus Biofilms: an in vitro Study, Proc. SPIE 10863, Photonic Diagnosis and Treatment of Infections and Inflammatory Disease II, 1086311. – 2019.
12. Fyrestam J., Bjurshammar N., Paulsson E. et al., Influence of culture conditions on porphyrin production in Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis//Photodiagnosis Photodyn. Ther. – 2017. – Vol. 17. – P. 115–123.
13. Kempa M. et al., Physicochemical properties of potential porphyrin photosensitizers for photodynamic therapy, Spectrochim//Acta – Part A Mol. Biomol. Spectrosc. – 2015. – Vol. 146. – P. 249–254.
14. Setiawatie E.M., Astuti S.D., and Zaidan A.H., An in vitro Antimicrobial Photodynamic Therapy (aPDT) with Blue LEDs to Activate Chlorophylls of Alfalfa Medicago sativa L on Aggregatibacter actinomycetemcomitans//J. Int. Dent. Med. Res. – 2016. – Vol. 9 (2). – P. 118–125.
15. Astuti, SD., Mahmud A.F., Pudjiyanto et al., Antimicrobial photodynamic of blue LED for activation of curcumin extract (curcuma longa) on staphylococcus aureus bacteria, an in vitro study, IOP Conf//Series: Journal of Physics: Conf. Series. – 2018. – Vol. 1120. – 012073.
16. Cellamare B.M., Fini P., Agostiano A., Sortino S., and Cosma P., Identification of ROS produced by photodynamic activity of chlorophyll/cyclodextrin inclusion complexes//Photochem. Photobiol. – 2013. – Vol. 89 (2). – P. 432–441.
17. Setiawatie E.M., Lestari V.P., and Astuti S.D., Comparison of Antibacterial Efficacy of Photodynamic Therapy and Doxycycline on Aggregatibacter Actinomycetemcomitans//Afr J Infect Dis. – 2018. – Vol. 12. – P. 95–103.
18. Astuti S.D., Drantantiyas N.D.G., Putra A.P. et al., Suhariningsih, Photodynamic effectiveness of laser diode combined with ozone to reduce Staphylococcus aureus biofilm with exogenous chlorophyll of Dracaena angustifolia leaves//Biomedical Photonics. – 2019. – Vol. 8 (2). – P. 4–13.
19. Chang G., Zhang H., Li S. et al., Effective photodynamic therapy of polymer hydrogel on tumor cells prepared using methylene blue sensitized mesoporous titania nanocrystal//Mater. Sci. Eng. C. – 2019. – Vol. 99. – P. 1392–1398.

21. Saitawee D., Teerakapong A., Morales N.P., Jitprasertwong P., and Hormdee D., Photodynamic therapy of Curcuma longa extract stimulated with blue light against Aggregatibacter actinomycetemcomitans, *Photodiagnosis Photodyn. Ther*., 2018, vol. 22, pp. 101–105.
22. Martinez-Correa H. A. et al., Composition and antimalarial activity of extracts of Curcuma longa *L*. obtained by a combination of extraction processes using supercritical CO_2, ethanol and water as solvents, *J. Supercrit. Fluids*, 2017, vol. 119, pp. 122–129.
23. Astuti SD., Mawaddah A., Nasution AM. et al., Abdurachman, Puspita PS and Suhariningsih. Effectiveness of Photodynamic Inactivation with Exogenous Photosensitizer Curcuma longa Extract Activated by Laser Diode 403 nm on Staphylococcus aureus, *J. Int. Dent. Med. Res*, 2020, vol. 13 (1), pp. 155-–161.
24. Skidmore R., Kovach R., Walker C. et al., Effects of Subantimicrobial-Dose Doxycycline in the Treatment of Moderate Acne, *Arch Dermatol*, 2003, vol. 139, pp. 459–464.
25. Paschoal M. A., Tonon C. C., Spolidório D. M. P. et al., Photodynamic potential of curcumin and blue LED against Streptococcus mutans in a planktonic culture, *Photodiagnosis Photodyn. Ther.*, 2013, vol. 10 (3), pp. 313–319.
26. Caccianiga G., Baldoni M., Ghisalberti C. A. et al., Preliminary In Vitro Study on the Efficacy of High-Power Photodynamic Therapy (HLLT): Comparison between Pulsed Diode Lasers and Superpulsed Diode Lasers and Impact of Hydrogen Peroxide with Controlled Stabilization, *Biomed Res. Int.*, 2016.
27. Asnaashari M., Mojahedi S. M., Asadi Z., et al., A comparison of the antibacterial activity of the two methods of photodynamic therapy (using diode laser 810 nm and LED lamp 630 nm) against Enterococcus faecalis in extracted human anterior teeth, *Photodiagnosis Photodyn. Ther.*, 2016, vol. 13, pp. 233–237.
28. Kaplan J. B., Meyenhofer M. F., and Fine D. H., Biofilm Growth and Detachment of *Actinobacillus actinomycetemcomitans*, 2003, vol. 185 (4), pp. 1399–1404.
29. Paulucci V. P., Couto R. O., Teixeira C. C. et al., Optimization of the extraction of curcumin from Curcuma longa rhizomes, *Brazilian J. Pharmacogn*., 2013, vol. 23 (1), pp. 94–100.
30. Carolina M., Gregorio J., Iturriaga L. et al., Microencapsulation of betalains obtained from cactus fruit *(Opuntia ficus-indica)* by spray drying using cactus cladode mucilage and maltodextrin as encapsulating agents, 2015, vol. 187, pp. 174–181.
31. Niemz M. H., Laser-Tissue Interactions, Fundamentals and Applications, T*hird Enlarged Edition, 3rd ed. Heidelberg: Springer-Verlag*, 2007.
32. Grossweiner L. I., Grossweiner J. B., Gerald Rogers B. H. et al., The science of phototherapy: An introduction, 2005.
33. Awad M. M., Tovmasyan A., Craik J. D. et al., Important cellular targets for antimicrobial photodynamic therapy, Appl. *Microbiol. Biotechnol.*, 2016, vol. 100 (17), pp. 7679–7688.
34. Astuti S. D., Widya I. W., Arifianto D. et al., Effectiveness Photodynamic Inactivation with Wide Spectrum Range of Diode Laser to Staphylococcus aureus Bacteria with Endogenous Photosensitizer: An in vitro Study, *J. Int. Dent. Med. Res*, 2019, vol. 12 (2), pp. 481–486.
35. Sunarko S. A., Ekasari W., Astuti S. D., Antimicrobial effect of Pleomele angustifolia pheophytin A activation with diode laser to Streptococcus mutans, *Journal of Physics: Conference Series*, 2017, vol. 85 (12038).
36. Hamblin M.R and Abrahamse H, Tetracyclines: Light-Activated Antibiotic, *Future Medisinal Chemistry*, 2019, vol. 11 (18), pp. 2427–2444.
37. Stewart P. S., Mechanisms of antibiotic resistance in bacterial biofilms, 2002, vol. 113, pp. 107–113.
38. Rodrigues R. M., Gonçalves C., Souto R. et al., Antibiotic resistance profile of the subgingival microbiota following systemic or local tetracycline therapy, 2004, pp. 420–427.

20. Sahne F., Mohammadi M., Najafpour G. D., and Moghadamnia A. A., Extraction of Bioactive Compound Curcumin from Turmeric (Curcuma Longa L.) Via Different Routes: a Comparative Study//J. Biotechnol. – 2016. – Vol. 13 (3). – P. 173–180.
21. Saitawee D., Teerakapong A., Morales N. P. et al., Photodynamic therapy of Curcuma longa extract stimulated with blue light against Aggregatibacter actinomycetemcomitans//Photodiagnosis Photodyn. Ther. – 2018. – Vol. 22. – P.101–105.
22. Martinez-Correa H. A. et al., Composition and antimalarial activity of extracts of Curcuma longa L. obtained by a combination of extraction processes using supercritical CO2, ethanol and water as solvents//J. Supercrit. Fluids. – 2017. – Vol. 119. – P. 122–129.
23. Astuti SD., Mawaddah A., Nasution AM. et al., Effectiveness of Photodynamic Inactivation with Exogenous Photosensitizer Curcuma longa Extract Activated by Laser Diode 403 nm on Staphylococcus aureus//J. Int. Dent. Med. Res. – 2020. – Vol. 13 (1). – P. 155–161.
24. Skidmore R., Kovach R., Walker C. et al., Effects of Subantimicrobial-Dose Doxycycline in the Treatment of Moderate Acne//Arch Dermatol. – 2003. – Vol. 139. – P. 459–464.
25. Paschoal M. A., Tonon C. C., Spolidório D. M. P. et al., Photodynamic potential of curcumin and blue LED against Streptococcus mutans in a planktonic culture//Photodiagnosis Photodyn. Ther. – 2013. – Vol. 10 (3). – P. 313–319.
26. Caccianiga G., Baldoni M., Ghisalberti C. A., and Paiusco A., A Preliminary In Vitro Study on the Efficacy of High-Power Photodynamic Therapy (HLLT): Comparison between Pulsed Diode Lasers and Superpulsed Diode Lasers and Impact of Hydrogen Peroxide with Controlled Stabilization//Biomed Res. Int. – 2016.
27. Asnaashari M., Mojahedi S. M., Asadi Z., Azari-Marhabi S., and Maleki A., A comparison of the antibacterial activity of the two methods of photodynamic therapy (using diode laser 810 nm and LED lamp 630 nm) against Enterococcus faecalis in extracted human anterior teeth//Photodiagnosis Photodyn. Ther. – 2016. – Vol. 13. – P. 233–237.
28. Kaplan J. B., Meyenhofer M. F., and Fine D. H., Biofilm Growth and Detachment of Actinobacillus actinomycetemcomitans. – 2003. – Vol. 185 (4). – P. 1399–1404.
29. Paulucci V. P., Couto R. O., Teixeira C. C. C., and Freitas L. A. P., Optimization of the extraction of curcumin from Curcuma longa rhizomes//Brazilian J. Pharmacogn. – 2013. –Vol. 23 (1). – P. 94–100.
30. Carolina M., Gregorio J., Iturriaga L. et al., Microencapsulation of betalains obtained from cactus fruit (Opuntia ficus-indica) by spray drying using cactus cladode mucilage and maltodextrin as encapsulating agents. – 2015. – Vol. 187. – P. 174–181.
31. Niemz M. H., Laser-Tissue Interactions, Fundamentals and Applications//Third Enlarged Edition, 3rd ed. Heidelberg: Springer-Verlag. – 2007.
32. Grossweiner L. I., Grossweiner J. B., Gerald Rogers B. H., and Jones L. R., The science of phototherapy: An introduction. – 2005.
33. Awad M. M., Tovmasyan A., Craik J. D. et al., Important cellular targets for antimicrobial photodynamic therapy//Appl. Microbiol. Biotechnol. – 2016. – Vol. 100 (17). – P. 7679–7688.
34. Astuti S. D., Widya I. W., Arifianto D. and Apsari R. Effectiveness Photodynamic Inactivation with Wide Spectrum Range of Diode Laser to Staphylococcus aureus Bacteria with Endogenous Photosensitizer: An in vitro Study//J. Int. Dent. Med. Res. – 2019. – Vol. 12 (2). – P. 481–486.
35. Sunarko S. A., Ekasari W., Astuti S. D., Antimicrobial effect of Pleomele angustifolia pheophytin A activation with diode laser to Streptococcus mutans//Journal of Physics: Conference Series. – 2017. – Vol. 85 (12038).
36. Hamblin M.R and Abrahamse H, Tetracyclines: Light-Activated Antibiotic//Future Medisinal Chemistry. – 2019. – Vol. 11 (18). – P. 2427–2444.
37. Stewart P. S., Mechanisms of antibiotic resistance in bacterial biofilms. – 2002. – Vol. 113. – Vol. 107–113.

39. Astuti SD, Rulaningtyas R., Putra A. P. et al., The efficacy of photodynamic inactivation with laser diode on Staphylococcus aureus biofilm with various ages of biofilm, *Infectious Disease Reports*, 2020, vol. 12 (s1),8736, pp. 68–74.

38. Rodrigues R. M., Gonçalves C., Souto R. et al., Antibiotic resistance profile of the subgingival microbiota following systemic or local tetracycline therapy. – 2004. – P. 420–427.
39. Astuti S. D., Rulaningtyas R., Putra A. P. et al., The efficacy of photodynamic inactivation with laser diode on Staphylococcus aureus biofilm with various ages of biofilm//Infectious Disease Reports. – 2020. – Vol. 12 (s1):8736. – P. 68–74