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Research Report

Aggregatibacter actinomycetemcomitans sensitivity towards chlorophyll of *Moringa* leaf after activated by diode laser

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ABSTRACT

Background: *Regular brushing teeth with scaling and root planning (SRP) cannot effectively decrease the periopatogen bacterial colonies. Even with the addition of antibiotics to support SRP, such as tetracycline given with low doses and for a long time may cause bacteria to become resistant and the effectiveness to eliminate colonies of bacteria being reduced. Photodynamic is a treatment modality that does not cause resistance and potentially to eliminate the growth of bacterial colonies. Moringa oleifera is a plant that can be easily found in Indonesia, by extracting chlorophyll of Moringa oleifera leaves, it can be used as a photosensitizer agent to increase the absorption of light on photodynamic method.* ***Purpose:*** *This study aimed to determine the potential photodynamic inactivation therapy to inactivate (eliminate) periopatogen bacterium.* ***Method:*** *This study used Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans). Laser diode 660nm as a light source with 8mm optical fiber to guide the beam, also used 20% extract chlorophyll of Moringa oleifera leaf as photosensitizer. Four diode lasers energy density exposures (2,5J.cm^{-2}, 5J.cm^{-2}, 7,5J.cm^{-2}, and 10J.cm^{-2}) are used from both at the in vitro photodynamic inactivation test.* ***Result:*** *The highest percentage of deaths occurred in the group treated with addition of photosensitizer and exposed by 660 nm diode laser with 10J.cm^{-2} energy density, which is 83.01%, compared to the results obtained in the group without addition of the photosensitizer.* ***Conclusion:*** *Chlorophyll of Moringa oleifera leaf after activated by diode laser effectively eliminates A. actinomycetemcomitans.*

Keywords: *Photodynamic inactivation; laser diode; Aggregatibacter actinomycetemcomitans; chlorophyll; Moringa oleifera*

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INTRODUCTION

The antimicrobial effects of photodynamic method first described by Oscar Raab in 1900 when observing the lethal effect of acridine red combine with light on infusoria (malaria-causing protozoa).[1] Photodynamic therapy which used to reduce the growth of bacteria, known as photoinactivation or photodynamic antimicrobial chemotherapy (PACT), and nowadays known as antimicrobial photodynamic therapy (aPDT).[2,3] Photodynamic therapy requires three main elements, including light-sensitive substance (photosensitizer), a harmless light source, and oxygen availability.[3] Bacteria had their light-sensitive substance that called endogenous porphyrin, studies have demonstrated that bacteria containing porphyrins are sensitive to visible light, in the blue as well as the red spectral region. Study shows *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* have protoporphyrin IX with 405nm soret band spectrum and peak Q band at 510 nm, 545 nm, 580 nm, 630 nm, 670 nm, and 700 nm.[4,5] Antimicrobial effect is a phototoxic response due to singlet oxygen which caused oxidative damage to cell DNA and changes in molecular mass of some cell membrane proteins and plasma membrane.[2,7,6] Oxidative damage in bacterial cells generally occurs in the DNA cells and other cell organelles, but damage to the cell organelles can differ, depended on types of bacteria and photosensitizer used.[8] The addition of exogenous photosensitizer has their role

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in improving the absorption of a light photon, where the photosensitizer is localized to the plasma membrane till enter the cell organelles such as mitochondria, lysosomes and endoplasmic reticulum causes photosensitization occurred in the area photosensitizer absorbed.[9,10]

There are numerous studies on the antibacterial effects of photodynamic method; for instance, researches on several periopathogenes, such as *Aggregatibacter actinomycetemcomitans* (A.a), *Fusobacterium nucleatum* (F.n), *Porphyromonas gingivalis* (P.g), *Prevotella intermedia* (P.i), and *Streptococcus sanguis* (S.s).[11] Based on those previous researches, the highest bacterial (P.i, P.g, S.s, A.a and F.n) death are triggered by an exposure of a diode laser (665nm 100mW diode laser for 60 seconds at 21.2J.cm^{-2} energy density) by administration of methylene blue (MB) as a photosensitizer.[11] Another photodynamic research using diode laser 660 nm 30 mW combined with administration of malachite green (MG) as a photosensitizer can produce a bactericidal effect on A.a, which bacterial deaths up to 97.2% by three minute exposure time (5.4J. cm^{-2} energy density) and up to 99.9% by five minute exposure time (9J.cm^{-2} energy density).[12] Another in vitro photodynamic research using 405 nm laser diode by 75 second exposure time (25J.cm^{-2} energy density) combined with administration of chlorophyll as a photosensitizer with 1,5 cm distance can cause the *S. mutant* bacteria death, up to 74%.[13]

This study was perform to obtain an appropriate energy density of 660nm diode laser in photodynamic inactivation in vitro test with exposure time less than 1 minute using *A. actinomycetemcomitans* bacteria. This research also aimed to analyze the optimization of bactericide by adding chlorophyll extract derived from *Moringa oleifera* leaves as an exogenous photosensitizer to increase the ability to absorb light.

A. actinomycetemcomitans bacteria were selected as the test targets since they are flora in the oral cavity that potentially induce periodontal disease, especially localized aggressive periodontitis.[14] *A. actinomycetemcomitans* bacteria classified into*Haemophilus spp, A. actinomycetemcomitans, Cardiobacterium hominis, Eikenellacorrodens,* and *Kingella kingae* (HACEK) group of pathogens which potentially triggering endocarditis infections.[15,16]

MATERIALS DAN METHOD

A strain of bacteria used as samples in this research was *A. actinomycetemcomitans* (43718 ATCC, USA). Materials and laboratory equipment were used including *chlorophyll* extract derived from *Moringa oleifera* leaves, tryptic soy agar (TSA) Oxoid CM0131 (Thermo Fisher Scientific inc. UK) and tryptic soy broth (TSB) - CASO broth (Merck Millipore, Germany) as a medium to growing bacteria, 660 nm diode laser instruments, digital thermometer (TH3 Sanwa Electric Instrument Co. Japan), millimeter paper block, monochromator (CT-10 JASCO Inc. USA) and power meter (PM100D Thorlabs Inc. USA).

This study examined the effects of photosensitizer and diode laser spectrum with four (4) different energy densities. There were a group with administration of photosensitizer, and another group without administration of photosensitizer for further comparison.

The bacterial strain, were grown in sterile TSB media then incubated in anaerobic jars containing candles, sealed for 24 hours at a temperature of 37^{o} C. As explained above, there were two groups, namely a treatment group without the administration of photosensitizer and a treatment group with the administration of photosensitizer. The sample group with the photosensitizer was a bacterial culture treated with the administration of chlorophyll extract (*Moringa oleifera*) with a concentration of 20%.

A.actinomycetemcomitans bacteria were cultured in TSB medium then incubated in anaerobic jars (used candles to burn down oxygen) sealed at a temperature of 37^{0} C for 24 hours. After the bacterial colonies grew, dilution was performed. 0.1 ml of each dilution tube were taken then put on TSA media to be incubated in anaerobic jars at 37^{0} C for 24 hours. Observation was carried out using total plate counting (TPC) method to obtain eligible samples (most close to 300 colonies).[17]

Laser irradiation was performed at 1 cm distance from the surface of the sample in a PCR tube. 660 nm diode laser can produce 15.3 mW, 25.7 mW, 35 mW, and 45.5 mW power outputs (measured after guided by 8mm multimode optical fiber from 1cm distance). Four energy densities (2.5J. cm^{-2}, 5J. cm^{-2}, 7.5J. cm^{-2}, and 10J. cm^{-2}) were used in the both treatment groups as shown in Figure 1.

This research used quadruplication technique for each treatment. Calculation was performed using TPC method. To obtain percentage of viability of bacterial colonies, the following formula was used.[13]

$$\frac{\sum \text{colonies of treatment} - \text{colonies of control}}{\text{colonies of control}} \times 100\%$$

The exposure area characterization was conducted to ensure the exposure area of the diode laser which guided by 8mm multimode optical fiber. The results showed that the exposure area of the 660 nm diode laser was 0.162 ± 0.01 cm^2.

The temperature characterization of the diode laser exposure beam guided by 8mm multimode optical fiber with 1cm distance using digital thermometer for one minute show the maximum temperature reached by each use of power output as shown in Table 1.

Photosensitizer used in this research was chlorophyll derived from *Moringa oleifera* leaf extract with a concentration of 20%. To ensure the ability of chlorophyll as a photosensitizer, light absorbance characterization was carried out using a UV-Vis spectrophotometer (Genesys 10S Thermo Fisher Scientific inc. USA) Light spectrum

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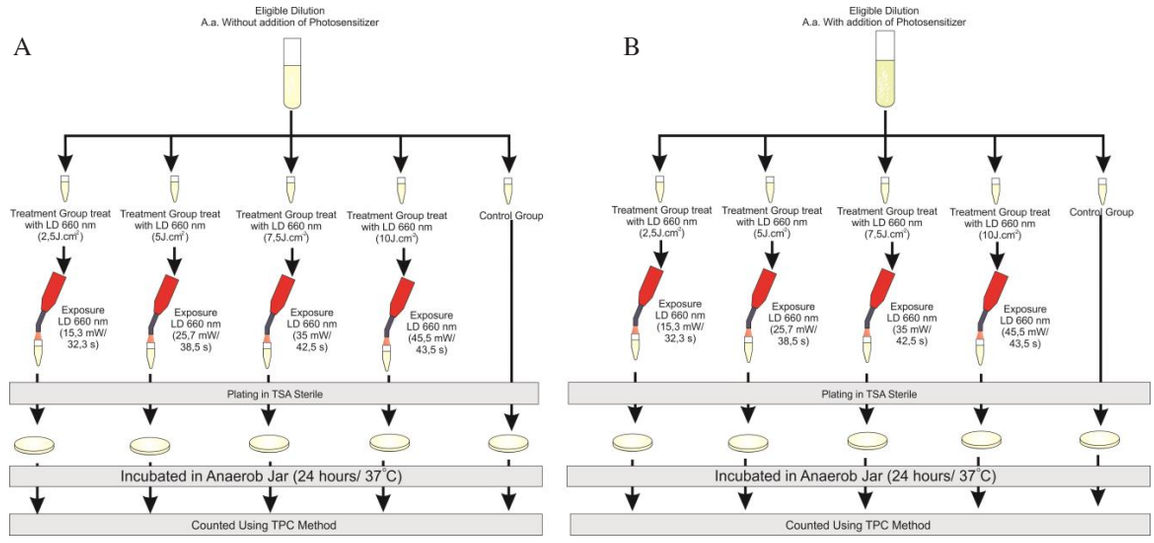

Figure 1. The treatment group A) without the addition of photosensitizer; B) with addition of photosensitizer.

Table 1. Temperature characterization of 660 nm diode laser

Output Power (mW)	Max. Temperature (°C)
15.3	30
25.7	32
35	34
45.5	35

absorbance characterization result of chlorophyll derived from *Moringa oleifera leaf* extract at the concentration of 20% are shown in Figure 2.

By using light spectrum absorbance characterization data and notice the diode laser wavelength, the percentage of energy absorbance (quantum yield) can be calculated using the following formula.[13]

$$\% \text{ absorbance} = (1 - 10^{-abs}) \times 100\%$$

The 660 nm diode laser beam had 81,84% energy absorbance. Exposure time can be calculated to obtain the energy density which can be absorbed by using power output data of the diode laser and the absorbance percentage of the photosensitizer as shown in Table 2.

One-way ANOVA statistical test was conducted to analyze the effects of the photosensitizer on the two treatment groups to ensure that the photosensitizer given was not toxic when not exposed with the laser diodes.

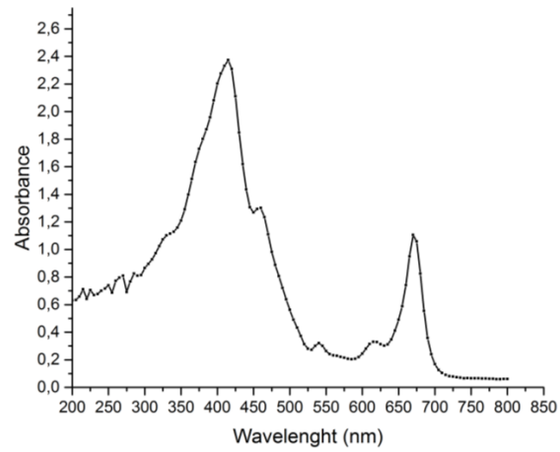

Figure 2. Light spectrum absorbance of chlorophyll derived from *Moringa oleifera* leaf extract with the concentration of 20%.

RESULTS

Certain of energy density were used in the treatment group with the photosensitizer and the treatment group without the photosensitizer. Results of TPC are shown in Table 3, while the percentages of the bacteria viability are shown in Table 4.

Table 2. Calculation of energy density

Wavelength (nm)	Output power (mW)	Exposure area (cm^2)	Exposure time (s)	Quantum yield (%)	Absorbed energy density ($J.cm^{-2}$)	Exposure energy density ($J.cm^{-2}$)
660	15.3	0.162	32.3	81.84	2.5	3.06
	25.7		38.5		5	6.11
	35		42.5		7.5	9.16
	45.5		43.5		10	12.22

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Table 3 shows the number of *A.actinomycetemcomitans* colonies in the treatment group with the photosensitizer are fewer than in the treatment group without the photosensitizer. The results of the One-Way Anova statistical test conducted to analyze the effects of the photosensitizer on the number of *A.actinomycetemcomitans* bacterial colonies in the two treatment groups, that showed there was no significant difference between the treatment group with administration of photosensitizer and the treatment group without administration of photosensitizer with a 0.069 ($p>0.05$) significance value. It can be assumed that chlorophyll photosensitizer derived from *Moringa oleifera* leaf extract with the concentration of 20% was not toxic to *A. actinomycetemcomitans* bacteria. The chlorophyll photosensitizer potentially increase the energy density absorbed to improve the process of photodynamic inactivation.

Table 4 shows the percentages of the *A. actinomycetemcomitans* bacterial viability. Negative signs indicate reduction in the growth of *A. actinomycetemcomitans* or bacterial death due to treatment or photodynamic inactivation. The highest bacterial death occurred in the treatment group with administration of photosensitizer and 660nm diode laser exposure at 10 $J.cm^{-2}$ energy density, reaching 83.01%.

DISCUSSION

The death of the *A. actinomycetemcomitans* bacteria in this research was influenced by the photophysics, photochemical, and photobiology processes due to the diode laser exposures, optimized with chlorophyll as a photosensitizer derived from *Moringa oleifera* leaf extract to increase the number of photons absorbed. The photophysics process due to exposure of the 660nm diode lasers can affect endogenous porphyrin (in the treatment group without the administration of photosensitizer) and chlorophyll moringa (in the treatment group with the administration of photosensitizer).[18] The higher photon energy absorption occurred in the group treated with the exogenous photosensitizer than in the treatment group without the exogenous photosensitizer (only bacterial endogenous porphyrin).

Chlorophyll *Moringa* which localized to the cell membrane or in the presence of bacteria endogenous porphyrin will be active when irradiated with diode laser then triggering the photochemical process which do the energy transfer to another molecules especially local oxygen that will generate oxidative agents, including radical hydroxyl, excited singlet oxygen, hydrogen peroxide, and superoxide anions, triggering photobiology process that aggressively oxidize cell membranes and organelles resulting in a damage to lipid membranes, proteins, and bacterial DNA cell leading to bacterial death.[19-22]

A.actinomycetemcomitans grows well at 20-42º C.[23] The exposure of 660 nm diode laser at 2,5$J.cm^{-2}$, 5$J.cm^{-2}$, 7,5$J.cm^{-2}$ and 10$J.cm^{-2}$ energy density produce the optimum temperature for *A.actinomycetemcomitans* growth shown at Table 1, thus concluded that bacterial killed should not occurs due to photothermal effect. This study using <100 $W.cm^{-2}$ laser diode power density and $<10^3$ s exposure time, so the light interaction that occurs is the photochemical reaction.[19]

Table 3. Bacterial colonies count at both treatment groups

Energy Density ($J.cm^{-2}$)	Colonies count in the treatment group without administration of photosensitizer ($CFU.ml^{-1}$)	Colonies count in the treatment group with administration of photosensitizer ($CFU.ml^{-1}$)
0 (Control)	$5.23.10^{10} \pm 1.1.10^9$	$4.76.10^{10} \pm 4.1.10^9$
2.5	$3.79.10^{10} \pm 2.6.10^9$	$1.19.10^{10} \pm 3.2.10^9$
5	$3.69.10^{10} \pm 2.5.10^9$	$1.14.10^{10} \pm 1.7.10^9$
7.5	$2.69.10^{10} \pm 3.6.10^9$	$1.12.10^{10} \pm 2.1.10^9$
10	$2.44.10^{10} \pm 3.6.10^9$	$8.05.10^9 \pm 9.7.10^8$

Table 4. Viability of the bacteria at both treatment groups

Energy Density ($J.cm^{-2}$)	Viability percentages of the treatment group without administration of photosensitizer (%)	Viability percentages of the treatment group with administration of photosensitizer (%)
2.5	-27.47 ± 6	-75.22 ± 5.6
5	-29.49 ± 5.3	-75.72 ± 5.4
7.5	-48.5± 7.6	-76.48 ± 3.9
10	-53.42 ± 7	-83.01 ± 2.4

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In this research, the highest percentage of killed bacteria was obtained in the group treated with the administration of photosensitizer by 10J.cm^{-2} energy density, which is -83.01 ± 2.4 %. The bacteria death at group treated without the administration of photosensitizer by 10J.cm^{-2} energy density only reaching -53.42 ± 7%. The viability of bacteria by 2,5J.cm^{-2}, 5 J.cm^{-2}, 7,5 J.cm^{-2} energy density also showed more bacteria death in the treatment group with the addition of photosensitizer compared to the group without photosensitizer. Tukey HSD comparison test between energy density and the *A.actinomycetemcomitans* bacterial viability showed there was no significant difference between 2,5J.cm^{-2} and 5 J.cm^{-2} energy density (sig.0,745), which is by using the lower energy density (2,5J.cm^{-2}) it can obtain bacterial death reaching 75% at treatment group with administration of photosensitizer.

Energy density, exposure time and wavelenght of light source with the administration of photosensitizer have a very important role in inactivation of bacteria, the result at this research showed exposure energy density of diode laser and administration of photosensitizer plays important role in inactivation that occurs at *A.actinomycetemcomitan* death.[3,10-13,20,22]

Photodynamic therapy effectively killing pathogens, including strains that have resistance to antibiotics therefore the photodynamic method is more effective than giving antibiotics.[24] Ecologically, commensal microorganisms and pathogenic bacteria can be found in mouth.[25-27] Commensal microorganisms are bacteria found on the surface of epithelial cells at human body, including oral epithelium, which in normal condition is favorable be the barrier and contributes to homeostasis as well as the host's body defense.[26,27] Pathogenic bacteria tend to give inflammatory responses through virulence factors expressed.[25] Two bacteria with two different behaviors in the mouth ecosystem which mutually pressing (antagonistic) resulting no excessive growth of pathogenic bacteria in periodontal tissues. Conversely, excessive growth of pathogenic bacteria can cause damage to teeth supporting collagen.[28]

It can be concluded that chlorophyll extract derived from *Moringa oleifera* leaves showed no direct toxic effect for *A. actinomycetemcomitans*. Chlorophyll of Moringa oleifera leaf after activated by diode laser effectively eliminates *A. actinomycetemcomitans*.

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