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The Efficacy of Photodynamic Inactivation of the Diode Laser in Inactivation of the *Candida albicans* Biofilms With Exogenous Photosensitizer of Papaya Leaf Chlorophyll

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Abstract

Introduction: Photodynamic inactivation has been developed to kill pathogenic microbes. In addition, some techniques have been introduced to minimize the biofilm resistance to antifungal properties in inhibiting cell growth. The principle of photodynamic inactivation different to antifungal drugs therapy which is resistant to biofilms. The presence of reactive oxygen species (ROS) that generating in photodynamic inactivation mechanisms can be damaging of biofilm cells and the principle of light transmission that could be penetrating in matrix layers of extracellular polymeric substance (EPS) until reaching the target cells at the base layers of biofilm. The present work aims to explore the potential of chlorophyll extract of papaya leaf as an exogenous photosensitizer to kill the *Candida albicans* biofilms after being activated by the laser. The potential of chlorophyll photosensitizer was evaluated based on the efficacy of inactivation *C. albicans* biofilm cell through a cell viability test and an organic compound test.

Methods: The treatment of photoinactivation was administered to 12 groups of *C. albicans* biofilm for four days using the 445 nm laser and the 650 nm laser. The 445 nm and 650 nm lasers activated the chlorophyll extract of the papaya leaf (0.5 mg/L) at the same energy density. The energy density variation was determined as 5, 10, 20, 30 and 40 J/cm^2 with the duration of exposure of each laser adjusted to the absorbance percentage of chlorophyll extract of the papaya leaf.

Results: The absorbance percentage of chlorophyll extracts of the papaya leaf on wavelengths of 650 nm and 445 nm respectively were 22.26% and 60.29%, respectively. The most effective treated group was a group of the laser with the addition of chlorophyll, done by the 650 nm lasers with inactivation about 32% ($P=0.001$), while the 445 nm lasers only 25% ($P=0.061$). The maximum malondialdehyde levels by treatment of the laser 650 nm were (0.046±0.004) nmol/mg.

Conclusion: The use of chlorophyll extract of the papaya leaf as a photosensitizer, resulted in the maximum spectrum of absorption at 414 nm and 668 nm, which produced a maximum reduction effect after photoinactivation up to 32% (with chlorophyll) and 25% (without chlorophyll). The utilization of chlorophyll extract of the papaya leaf would increase the antifungal effects with the activation by the diode laser in the biofilm of *C. albicans*.

Keywords: *Candida albicans* biofilms; Chlorophyll extract; Photoinactivation.

Introduction

Candida albicans is an opportunistic pathogenic fungus known as the cause of oral candidiasis infections such as cancer sores in HIV patients.[1,2] The pathogenicity of *Candida albicans* is influenced by several factors including invasion mechanism, polymorphic properties and biofilm formation.[3] A biofilm is a protective form of *C. albicans* cells from immune responses and antibiotic attacks due to the presence of an extracellular polymeric substance (EPS) matrix containing β-glucan.[4,5] Physically, the EPS matrix blocks the diffusion of antifungal compounds into the biofilm layer so that the cells embedded in the bottom of the biofilm are difficult to inhibit. Another factor that causes antifungal therapy to fail in biofilms is the limited zone of oxygen and nutrients so that the active cells accumulate in the bottom layer of the biofilm and do not

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respond to antifungal.[6]

One of the methods to inhibit the growth of the *C. albicans* cells that are not resistant to biofilms is photoinactivation. The working principle of photoinactivation is activating photosensitizer molecules through the absorption of light and the transfer of electrons to oxygen molecules to produce toxic and reactive compounds called reactive oxygen species (ROS).[7] The toxicity and reactivity of ROS compounds cause damage to cell membranes, inhibiting cell division systems and breaking DNA cell chains.[8,9] Damaged cell membranes provide photosensitizer opportunities transplanted into cells, and thereby damaging organelles such as lysosomes, mitochondria, and nuclei.[10,11] For *C. albicans* cells, cell membrane damage caused by ROS compounds causes oxidative stress. ROS compounds form covalent bonds with biomolecule components in cells, resulting in the collapse of cell biomolecular structures and causing damage and loss of cell function.[12] Phospholipids in cell membranes when reacting with ROS compounds will form lipid peroxidation resulting in a malondialdehyde compound and causing cell damage and lysis.[13] The malondialdehyde levels in *C. albicans* biofilm tissue can be used as an index of measurement of the activity of the radical compounds that attack cells.

The success of photoinactivation is determined by the type and concentration of photosensitizer as well as the output power and duration of exposure to light.[14] The maximum absorption spectrum of photosensitizer molecules is a point of reference in selecting the right wavelength of light to allow an optimum absorption rate. The amount of light photon energy absorbed by a photosensitizer molecule will determine the length of the molecular excitation stage at the triplet level and the chance of the reaction with the triplet oxygen molecule.[15] The common photosensitizer used for photoinactivation of *C. albicans* is a type of phenothiazines in the wavelength range of 600-660 nm (methylene blue and toluidine blue O), a type of porphyrin with high uptake in the blue region (hematoporphyrin), Phthalocyanines at 630-690 nm.[10] Most types of photosensitizers have high toxicity properties that require more naturally, pure chemical and non-toxic in the dark.

The discovery of chlorophyll and its derivatives such as pheophorbide, which is 20 times more effective than hematoporphyrin derivative as the first-generation sensitizer in cancer PDT,[16] became a point of reference in the development of chlorophyll from plants or bacteria, which are naturally pure as an organic photosensitizer. The chlorophyll molecule is hydrophobic and hydrophilic which the molecule structure like heme in the blood that is readily soluble in water or fat. The structure of chlorophyll molecule as same as the porphyrin that the first generation photosensitizer.[14,17] Chlorophyll pigment has a long triplet excitation stage ($\leq 10^{-8}$ seconds) so that the odds of more chemical reaction with oxygen molecules.[14,15] Chlorophyll extract has been used in photoinactivation to *Staphylococcus aureus*[13] and *Streptococcus mutant*[18] of pathogenic bacteria. In the country of Indonesia are available many green plants that have high chlorophyll content such as Papaya and Suji.[19]

The papaya leaf contains antifungal substances such as flavonoids, saponins, and tannins that play a role in damaging cell membranes and inhibiting cell division. Papaya plants are proven to have chitinase hydrolytic enzymes, which are antifungal.[27-29] Combined with the latex of papaya and fluconazole, they can inhibit the growth of *C. albicans* by degrading partial cell walls. The latex of papaya is able to reduce the polysaccharide layer in the *C. albicans* biofilm by up to 60%. Papaya leaf extract has been widely studied to inhibit the growth of bacteria and fungi but has not been applied as a photosensitizer in photoinactivation. As a sensitizer material, papaya leaf extract proved to be useful as a high light photon absorbent medium in solar cell applications.[20]

The light source in photoinactivation must have a stable output at the wavelength required for excitation, making it more efficient to use a light source with a monochrome spectrum. The commonly used light source in photoinactivation is the laser diode. Laser diodes are cheaper and easier to use and are more focused, with one characteristic strong wavelength (monochrome).

This study aims to explore and test the potency of papaya leaf chlorophyll as a photosensitizer, and its effectiveness in inactivation of the *C. albicans* biofilms. The ability of papaya leaf chlorophyll as a photosensitizer after activated at the different wavelengths was observed based on cell viability (optical density value) and the basic compound (malondialdehyde level) which accumulates in the biofilm after treatment.

Methods

Biofilm Growth

Samples of the *C. albicans* strain were obtained from a collection of microbiology laboratories of the Faculty of Dentistry at Airlangga University. Specimens were obtained using standard microbiological techniques for fungal organisms. Specimens were grown on Saburoud Dextrose Agar (Oxoid, England, UK) media and inoculated in Brain Heart Infusion Broth (Oxoid, England, UK) solution for 18 hours. Inocula for biofilm formation was diluted with sterile PBS at an optical density value of OD_{595nm}=0.5 (≈3x10^8 CFU/mL). Biofilm growth was stimulated via 2 methods, using Brain Heart Infusion Broth media for the cell viability test and using Spider media for the radical compound test.

Biofilms for the Cell Viability Test

One hundred microliters of *C. albicans* cell suspension was filled into microplate-96 wells and incubated for 90 minutes in an Incubator Shaker 37°C, 160 rpm. The plate must be washed twice with sterile PBS followed by

the addition of BHI-Broth 8% glucose (Oxoid) as much as 200 µL.[21] The culture was incubated for 4 days in an Incubator Shaker 37°C, 160 rpm. After harvest, the well was washed twice with sterile PBS followed by incubation of 100 µL papaya leaf chlorophyll extract for one hour before irradiation.

Biofilms for the Radical Compounds Test

Fifty microliters of *Candida albicans* cell suspension was dropped on a sterile cellulose filter membrane (pore 0.2 µm; Sartorius Stedim Biotech SA) grown on Spider media. After 4-day incubation, the membrane was lifted with sterile tweezers, and gently washed with sterile PBS to remove planktonic cells. Finally, the membrane was transferred into a sterile petri dish, followed with incubation of 50 µL chlorophyll extract of the papaya leaf for 1 hour before irradiation.

Photosensitizer

Papaya leaf chlorophyll extract was isolated by the maceration stage and partition, followed with fractionation by column chromatography method and by compound analysis through the thin layer chromatography method.[17] The MIC concentrations of toxicity assay results were applied to photoinactivation of the *C. albicans* biofilm of 0.5 mg/L. Before application, chlorophyll photosensitizers were characterized using UV-Vis Spectroscopy (Shimadzu UV-1800) and Luminescent Spectroscopy (PerkinElmer, LS 55) each to determine absorptivities and quantum efficiency of chlorophyll extract. The percent value of the chlorophyll absorption is used to determine the duration of exposure time of laser.

Light Sources

Illumination in photoinactivation using the laser diode (Laserland, China) at wavelengths of 445 nm and 650 nm. Laser power had measured using the JASCO CT-10 Monochromator and Thorlabs PM100D Powermeter. The result of the laser light performance test was obtained according to the specifications, for the 650 nm laser (power 0.164 W, spot area 0.432 cm^2) and for the 445 nm laser (power 0.128 W, spot area 0.418 cm^2). The laser light intensities reaching the sample surface were 0.379 W/cm^2 and 0.306 W/cm^2 for the 650 nm laser and the 445 nm laser respectively. The absorption percentage rate of papaya leaf chlorophyll extract which corresponded to the laser wavelength used was 22.36% (at 650 nm) and 60.29% (at 445 nm). The laser intensity that might be absorbed by the chlorophyll extract of papaya leaves was 0.085 W/cm^2 (for 650 nm laser) and 0.229 W/cm^2 (for 445 nm laser). Five energy density treatment that varied were 5, 10, 20, 30, and 40 J/cm^2, with the exposure time is shown in Table 1.

Chlorophyll Extract Toxicity

The toxicity of papaya leaf extract was carried out by observing the final biofilm growth of *C. albicans* after 24 hours incubation. The indicator of the biofilm cell growth of *C. albicans* is characterized by the number of cells that are still metabolizing actively through XTT staining after applying papaya leaf chlorophyll extract at various concentrations. The parameter produced by a toxicity test is the MIC value. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often they are utilized as a research tool for determining new antimicrobial in vitro activities, and data from such studies have been used to determine MIC breakpoints. The MIC value is the lowest concentration value of an extract which is thought to contain antimicrobial properties. The MIC value in photoinactivation is used because the photosensitizer agent acts as a light absorbing medium that will generate photochemical reactions and not as a significant inhibitor of the microbial cell growth.

In this research, the MIC test using Pierce methods[21] had been modified. Six series of concentration of chlorophyll extract from 0.5 mg/L to 5 mg/L had been prepared with distilled water dilution. The 21 biofilm samples contained in wells of the microplate, consisting of biofilms without extract (negative control) and biofilms treated with chlorophyll extract at various concentrations, each made triple. We have used additional 3 wells without sessile biofilm in the same microplate which was containing chlorophyll extract (5 mg/L) as an antifungal control. The time of antifungal incubation in biofilms is about one hour before staining with XTT and the optical density was determined at the wavelength 490 nm by using ELISA Reader.

Experimental Design

All treatments of the samples were carried out under dark

Table 1. The Exposure Time of Both Lasers

Treatment Code	Laser Intensity (mW/cm^2)		Laser Intensity Absorbed by Chlorophyll (mW/cm^2)		Exposure Time (s)		Energy Density (J/cm^2)
	650 nm	445 nm	650 nm	445 nm	650 nm	445 nm	
D_1	0.379	0.306	0.085	0.229	59	22	5
D_2	0.379	0.306	0.085	0.229	118	44	10
D_3	0.379	0.306	0.085	0.229	236	87	20
D_4	0.379	0.306	0.085	0.229	353	131	30
D_5	0.379	0.306	0.085	0.229	471	175	40

conditions and maintained at room temperature. The distance between the laser light and the sample was set to be 1 cm. Treatment was divided into 4 main groups of C- groups (biofilm groups without the treatment of the chlorophyll extract or lighting), C+ groups (biofilm groups with the addition of the chlorophyll extract without lighting), D_xC- groups (light-exposed biofilm without the addition of chlorophyll extract) and groups D_xC+ (biofilm exposed by laser light after the incubation of chlorophyll extract for one hour). All the groups of treatment were replicated.

Viability Measuring With XTT Assay

After the irradiation treatment, all the test biofilm groups were washed twice with sterile PBS followed by staining using 40 µL XTT 1 mg/mL (Santa Cruz), 2 µL menadione 10 mg/mL (MP Biomedical), and 158 µL sterile PBS. The microplate was incubated in the CO_2 Incubator for 2 hours followed by optical density reading with ELISA Reader at λ_{490nm}.

Determine of MDA Content

The determination of the malondialdehyde levels was performed after photoinactivation treatment against biofilm tissue of *C. albicans*. The malondialdehyde compound in biofilms was taken by using trichloroacetic acid (Merck) and thiobarbituric acid (Merck) of reagents. The concentration of malondialdehyde standard used 1,1,3,3-tetraethoxypropane (Sigma Aldrich) with concentration from 0 to 5 nmol/mL. One microliter of standard solution was added to 0.5 mL of distilled water, 1 mL trichloroacetic acid 20%, and 1 mL of thiobarbituric acid 0.67% and then the resultant solution was fed into a 10 mL reaction tube. The final mixed sample was centrifuged for 15 minutes, 3000 rpm, followed by incubation in a water bath 95°C for 45 minutes and the cooling of the solution was carried out at room temperature for 60 minutes. The last phase was reading of the absorbance of the mix solution at λ_{533nm}. The line equation of the standard curve was used as the calculation of the malondialdehyde levels of the biofilm sample. Conversion of malondialdehyde levels in nmol/mg units was obtained from the average mass of the biofilm sample test taken from the filter membrane. 25 mg of biofilm tissue was centrifuged with one mL of sterile PBS at 4°C, 10000 rpm for 15 minutes; then the resulting filtrate was used as the sample of the test solution. The same procedure as the standard malondialdehyde level test was carried out on the sample.

The line equation of the standard curve is:

$$y = 0.5186x - 0.3813$$

The equation for determining malondialdehyde levels is:

$$malondialdehyde\ concentration\ \left(\frac{nmol}{mg}\right) = \frac{[(\frac{Abs + 0.3813}{0.5186})]nmol/mL}{25\ mg/mL}$$

Abs is the absorbance of the test solution.

$$\%inactivation = |\frac{OD_{control} - OD_{treatment}}{OD_{control}}| \times 100$$

Statistical Analysis

All data were tested statistically by SPSS 17.0 using One-way analysis of variance (ANOVA) and Tukey analysis at trust level of 0.05. The following equation calculated the photoinactivation effect as an indicator of the effect of the cell death of the *C. albicans* biofilm:

Results

Characteristic of Chlorophyll Extract of the Papaya Leaf

The characterization performed on papaya leaf chlorophyll extract was the absorbance spectrum and luminescent spectrum, as shown in Figure 1. Figure 2 is related to a spectrum absorbance profile of the molecules of papaya leaf chlorophyll extract; it shows the 2 optimum wavelengths λ_1=414 nm (B-band) and λ_2=668 nm (Q-band). Figure 2 is a luminescent molecule profile of papaya leaf chlorophyll extract with the wavelength of fluorescent emission at 640 nm and 720 nm after excited at 390 nm and 620 nm wavelengths. The excitation spectrum of the luminescent profile was the same as the one represented by the absorbance spectrum in UV-Vis Spectrophotometry.

The lower emission intensity compared to the intensity of the excitation indicated that some of the excited fluorophores to singlet level would experience intersystem crossing and chemical reactions with other molecules

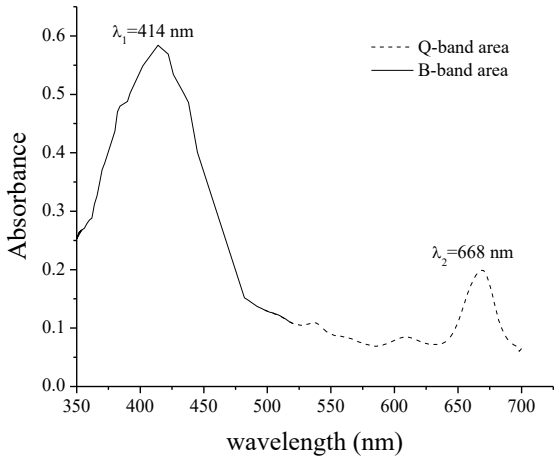

Figure 1. Spectrum Absorbance of Chlorophyll Extract of Papaya L.

such as oxygen molecules forming ROS compounds. As it is shown in Figure 2, the ratio between the maximum excitation intensity to the maximum emission intensity was a characteristic parameter of the quantum efficiency of chlorophyll extract. The quantum efficiency is an indicator of the quantum yield value of a photosensitizer molecule associated with the ability of photosensitizer to produce ROS compounds, especially singlet oxygen which is essential in photodynamic mechanisms. The values of the quantum yield of papaya leaf chlorophyll extract on the blue spectrum and the red spectrum were 0.54 and 0.86 respectively. It indicated that the quantum efficiency characteristics of the red spectrum region were more efficient than in the blue spectrum region.

The absorbance value of a laser with a wavelength of 445 nm was 60.30%, and the one with a wavelength of 650 nm was 22.29%. The suitability between absorbance characteristics of chlorophyll extract and the selected laser specifications was seen in full width at high maximum (FWHM) values of each chlorophyll extract absorption spectrum and laser power characteristics. The value of FWHM spectrum of chlorophyll extract at a wavelength of 414 nm was 88.15 nm, and at a wavelength of 668 nm was 21.04 nm. So the standard deviations of both spectrum could be written as 414.00 ± 44.08 nm and 668.00 ± 10.52 nm, respectively. The values of FWHM spectrum of both laser obtained for 445 nm and 650 nm lasers were 17.77 nm and 19.90 nm respectively. So standard deviations of spectrum for both lasers could be written as 445.00 ± 8.89 and 650.00 ± 9.95 (data was not shown). This shows that the accuracy of spectrum data of the laser was lower than the accuracy of spectrum data of chlorophyll extract. So, it could be concluded that the selection of the wavelength type of light source was based on the characteristics of the photosensitizer which was the papaya leaf chlorophyll.

Toxicity of Chlorophyll Extract to *Candida albicans* Biofilm

The experiment data of OD_{490} values from the toxicity test of papaya leaf chlorophyll extract are presented in Table 2. The data are supplemented with standard deviation (SD) values from the sample of each treatment group (chlorophyll concentration) and the percentage inhibition (inactivation effect) calculation results compared with OD_{490} values of the negative control (a group of chlorophyll concentration of 0 mg/L).

The data on the effects of inactivation were seen to increase with the increase in the concentration of papaya leaf chlorophyll extract. At chlorophyll concentrations below 2.50 mg/L, the increase in the inactivation effect increased slowly, but at chlorophyll concentrations above 2.50 mg/L a significant increase in the inactivation effect was observed. The MIC value determined from the toxicity test was 0.50 mg/L with the effect of inactivation reaching 28.905% or more than a quarter of the number of control biofilm cells (without the addition of chlorophyll extract). The chlorophyll concentration value chosen in this toxicity test did not show the concentration of chlorophyll extract which produces an inactivation effect reaching 50% or under 28%, thus providing further research opportunities.

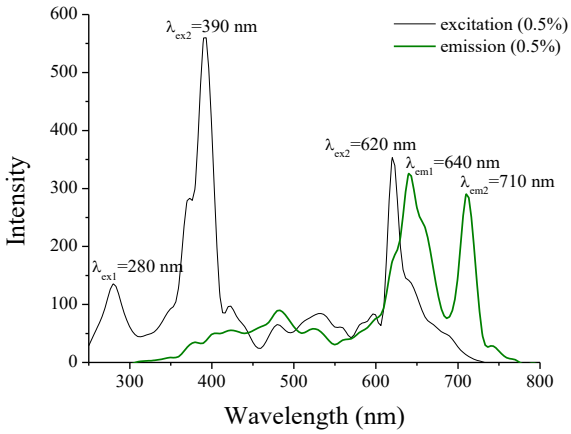

Figure 2. Fluorescence Spectrum of Chlorophyll Extract of Papaya L.

Table 2. The Data of the Toxicity Test of Chlorophyll Extract of Papaya Leaf

Chlorophyll Concentration (mg/L)	OD_{490} (Mean±SD)	Inactivation Effect (%)
0.00	1.083±0.145	0.000
0.50	0.770±0.012	28.905
1.00	0.742±0.023	31.481
1.25	0.738±0.016	31.799
2.50	0.726±0.063	32.927
3.75	0.668±0.032	38.336
5.00	0.634±0.025	41.436

Efficacy of PDI to *Candida albicans* Biofilms

Figures 3 and 4 represent a decrease in cells viability after photoinactivation treatment with a 650 nm laser (Figure 3) and a 445 nm laser (Figure 4), compared with the control groups. The green line curve was the optical density value of the chlorophyll treated groups; the red dashed line curve represented the optical density value of the laser treated groups, while the red straight line curve represented the optical density value of the laser with the chlorophyll treated groups.

The chlorophyll groups were used as the control groups against antifungal which have been made for each level of energy density. The optical density values are relatively stable from 1.086 ± 0.012 to 1.068 ± 0.018 with the inactivation effect of about 8%. The effect of photoinactivation treatment from both lasers with or without chlorophyll showed that the cell viability decrease with the increase of the energy density. The optical density value showed that the groups of 650 nm laser

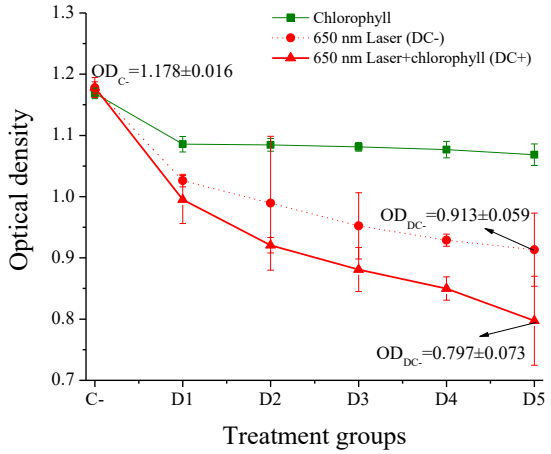

Figure 3. The Viability of *Candida albicans* biofilm with 650 nm Laser.

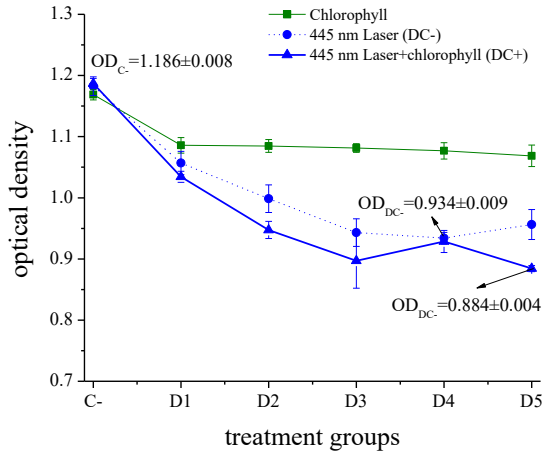

Figure 4. Viability of *Candida albicans* Biofilm With 445 nm Laser.

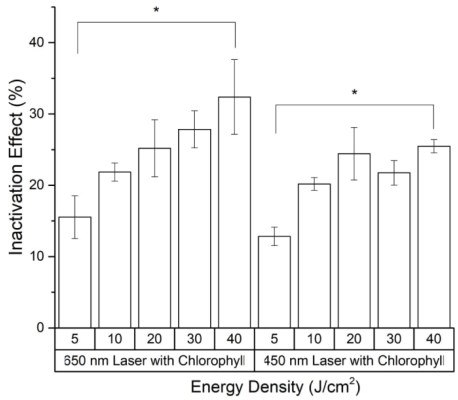

Figure 5. The Inactivation Effect for Groups of the Laser With Chlorophyll Treatment.
Note: * shows significant level in ANOVA one way test of $P<0.05$.

treatment were lower than the groups of 445 nm laser treatment. According to Figure 3, the minimum optical density value from all groups which occurred at an energy density of 40 J/cm^2 were 0.913±0.059 for the DC- groups and 0.797 ± 0.073 for the DC+ groups. Figure 4 showed that the minimum optical density value occurred at different energy densities. The optical density value for the DC- groups was 0.934 ± 0.009 (energy density 30 J/cm^2), and for the DC+ groups was 0.884 ± 0.005 (energy density 40 J/cm^2).

Based on the optical density values of both treatments of the 445 nm laser and the 650 nm laser (Figure 3 and Figure 4), the efficacy of inhibition could be determined by calculating the percentage of inactivation (formulated in methods). The data compared only groups of the laser with the addition of chlorophyll from both laser treatments (shown in Figure 5). The histogram curve for treatment with the 650 nm laser was represented by the red histogram and for treatment with 445 nm laser was represented by the blue histogram, accompanied by the value of inactivation at each density groups. The results showed that at the lowest energy density, the inactivation effects were15% and 13% for lasers with a wavelength of 650 nm and 445 nm respectively. At 40 J/cm^2 density, the differences in the inactivation effects were 32% and 25% for the lasers with a wavelength of 650 nm and 445 nm respectively. The statistical analysis showed that the photoinactivation treatments of *C. albicans* biofilms in the varying energy density of both lasers, 445 nm and 650 nm were significantly different ($P<0.05$), but the treatments between 450 and 650 showed no significant difference.

The biofilm groups of 650 nm laser treatment (Figure 3) showed that the optical densities significantly and linearly decreased with the increase in energy density. When compared with the biofilms groups of 445 nm laser treatment (Figure 4), the decrease in optical density at energy density below 25 J/cm^2 was significant and linear but after treatment at energy density 25 J/cm^2 was almost stable. The decrease in optical density profile (Figure 4) in the 445 nm laser treatment group with or without the addition of chlorophyll extract was relatively the same.

The results obtained from this study are not different from the explanation described by Calzavara-Pinton et al.[10] They explained are the relationship between the characteristics of *C. albicans* cells that have cell membranes and complex targets to the photoinactivation treatment, more effective with the use of a red laser as a light source. The attenuation principle for red light more strongly penetrate to the cell walls of *C. albicans* which were indicated easily to reach many target cells compared with blue light. In several studies on the photoinactivation of *C. albicans* photosensitizer agents of methylene blue and toluidine blue which are more effective in inhibiting microbial cell growth when combined with red light sources have been used.

In this study, the effectiveness of a red laser beam was influenced by the time of irradiation applied. The determination of irradiation time was based on absorption characteristics of chlorophyll extract between light absorption in blue regions with light absorption in different red regions of 40%. For the same energy density value, the duration of red laser exposure was 2 times longer than the duration of blue laser exposure. In this condition a greater probability of a photochemical reaction resulted in a radical oxygen species which would inactivate bacteria in the biofilm. This is shown in the results of the study.

Malondialdehyde Content Analysis

The malondialdehyde level presented in Table 2 was a result of photoinactivation of 445 nm lasers and 650 nm lasers with and without papaya leaf chlorophyll in 3 variations of energy density which were 5, 20 and 40 J/cm^2. The malondialdehyde compounds were produced from membrane lipid peroxidation mechanisms after *C. albicans* cells were exposed to light as well as the reaction to the radical or ROS compounds. The accumulation of malondialdehyde compounds in biofilms was extracted through the addition of trichloroacetic acid reagents and thiobarbituric acid. The malondialdehyde compound accumulated in biofilm tissue was an indicator of the number of cells undergoing lysis, assuming that one ROS compound produces one cell lysis or one cell death.

Table 3 was the result of determining malondialdehyde content of biofilms sample after the photoinactivation treatment by both lasers, 445 nm and 650 nm. From the statistical analysis, it was generally seen that the irradiation treatment with chlorophyll extract from the 2 lasers, differed significantly from the control groups as well as the laser groups without chlorophyll extract. The groups with the highest malondialdehyde levels of all treatment groups were obtained at the energy density of 40 J/cm^2 (0.0467 ± 0.0035). While the laser treatment groups with the lowest malondialdehyde level were produced by 445 nm laser treatment at the energy density of 5 J/cm^2 (0.0339 ± 0.0001), the 650 nm laser treatment groups with the addition of chlorophyll were significantly different from the 445 nm laser treatment groups with or without chlorophyll ($P = 0.00$). The results of the inactivation effect at an energy density of 40 J/cm^2 for the laser groups with and without chlorophyll, compared with the control group, indicated an increase in malondialdehyde levels 18% and 29% (for the laser groups of 650 nm), and 7% and 17% (for laser groups 445 nm). The statistical analysis from Table 2 showed the groups of 650 nm laser treatment with or without chlorophyll and the groups of 445 nm laser with chlorophyll treatment significantly differed compared with the control groups ($P < 0.05$). It means that the photoinactivation treatment was more effective with the addition of the photosensitizer agents.

Table 3. The Data of malondialdehyde Levels in *Candida albicans* Biofilms After Exposure to 650 nm and 445 nm Diode Laser

Groups of Treatment	Malondialdehyde Content (nmol/mg)±SD	
	650 nm Laser	445 nm Laser
C-	0.0322±0.0001	0.0322±0.0001
C+	0.0336±0.0001[(a)]	0.0336±0.0001[(a)]
D_1C-	0.0390±0.0002[(b)]	0.0339±0.0001[(a)]
D_3C-	0.0378±0.0001[(b)]	0.0344±0.0001[(a)]
D_5C-	0.0405±0.0034[(b)]	0.0358±0.0012[(a)]
D_1C+	0.0433±0.0016[(b)]	0.0386±0.0003[(b)]
D_3C+	0.0450±0.0038[(b)]	0.0392±0.0005[(b)]
D_5C+	0.0467±0.0035[(b)]	0.0397±0.0031[(b)]

Note: index after SD on the data represented by "(a)" was no difference significant compared control (C-) with ($P > 0.05$) and "(b) was other with ($P < 0.05$)

Discussion

The development of sensitizers of generation III is directed to the effectiveness of light absorption,[30] which corresponds to the molecular nature of pigments, especially chlorophyll leaves. Chlorophyll pigments have the characteristics of 2 maximum absorbances, namely chlorophyll-*a* (430 and 662) nm during chlorophyll-*b* (453 and 642) nm so that in the photoinactivation application there are more choices to determine the wavelength of light to be used. Compared with other photosensitizers like methylene blue, chlorophyll is relatively more straightforward to produce, more stable, soluble in water, and non toxic (extracted from various green plants). Moreover, chlorophyll has properties that can minimize the release of heat energy into the environment through the formation of redox reactions.[15] The stages of the existence of molecules at an energy level are called a lifetime. In photodynamic photosensitizer, the lifetime is in an extended triplet state and is thought that it is more useful to produce a series of photochemical reactions, especially interactions with oxygen molecules that initiate the formation of ROS. The primary function of chlorophyll is as a light absorber, transferring excitation energy to the reaction center and charge separator in photosynthetic membranes.[15,31] The high absorption of chlorophyll is caused by chlorophyll excitation steps of $\leq 10^{-8}$ seconds.

The potential of chlorophyll[16] as an active sensitizer by light stimulation in tumor and cancer therapy has a structure similar to hemoglobin (chromophore in the body). The principle in photoinactivation considers that those that play a role in microbial inactivation are light sources instead of photosensitizers because photosensitizers only trigger the formation of oxygen species into highly reactive oxygen singlet.

The maximum absorption of papaya leaf chlorophyll extracts in 2 spectrums was Soret-band and Q-bands at wavelengths of 414 nm and 668 nm, which was different from the results reported by Maulana et al, with $\lambda_1 = \pm 450$ nm and $\lambda_2 = \pm 630$ nm.[20] The peak intensity λ_1 was higher

than the peak intensity λ_2 with the absorption intensity corresponding to both wavelengths of the laser about $A_{445}=0.401 \pm 0.001$ and $A_{650}=0.110 \pm 0.003$ yielding percent absorption of 60.29% and 22.26% respectively.

Photoinactivation of *Ca. albicans* biofilm has been investigated by Junqueira et al[22] and Pereira et al.[11] Junqueira et al investigated ZnPc phototoxic 0.25 mg/mL after activation by GaAlAs 660 nm 26.3 J/cm^2 lasers for 285 seconds, with the inactivation percentages of 21%, 30% and 55% for the ZnPc groups, the laser groups and the ZnPc combination laser groups, respectively. Pereira et al investigated the photoinactivation effects of species *C. albicans* biofilm with *S. aureus* and *S. mutants*. Their work explained that the bacteria has been added for 0.1 mg/mL Methylene Blue and activated by InGaAlP laser (660 nm, 100 mW, 350 J/cm^2) for 1.5 minutes with the final number of colonies is about 2.32, 3.29, and 2.81 CFU/mL with inactivation effects are 45%, 55%, and 50% for *C. albicans*, *Staphylococcus aureus* and *S. mutants*, respectively. It was indicated that *C. albicans* biofilms were more challenging to be inactivated due to the influence of complex cell walls (eukaryotic cells) containing a nuclear membrane that acted as a sensitizer barrier so that the sensitizer concentration had to be high.

Another study by Pinto et al,[23] that studied the effects of photoinactivation on the age of biofilm *C. albicans* (6, 12, 24 and 48 hours), used exogenous photosensitizer of toluidine blue O and sources light of LED (630 nm, 0.073 W, beam area 0.38 cm^2, energy density 21.7 J/cm^2). The morphology of biofilm is change from yeast to hype happen at 12 hours growth with the value of metabolic activity after irradiation were 22% and 48% for the concentrations of Toluidine Blue O are 0.05 and 0.1 mg/mL, respectively. In other conditions investigated for 24 hours of biofilm growth, the viability reduction at each concentration of Toluidine Blue O showed the metabolite activities were 36% and 61%, respectively. This shows that the photosensitizer concentration and age of biofilm growth affect the decrease in cell viability and of the cell damage in photodynamics mechanisms.

Photoinactivation of planktonic cells from *C. albicans* by additional methylene blue (50 µM = 0.015 mg/mL) was reported by de Oliveira et al[24] for activated by diode laser 660 nm, 100 mW, 9 J with 3 minutes exposure. Oliviera et al was made a group of the laser with chlorophyll treatment to gave the inactivation effect of 68%, while the groups of the sensitizer treatment and the groups of lasers treatment are 18% and 53% respectively. Another study of de Oliveira et al[24], at higher Methylene Blue concentrations (0.1 mg/mL), after activated by a 685 nm laser, 28 J/cm^2 shows the inactivation effect was 88.6%. The energy density of 60, 120 and 180 J/cm^2 (from the Laser 660 nm, 40 mW), with the addition of methylene blue (0.15 mg/mL), resulted in the inactivation effect of 42%, 62%, and 78%, respectively.[25] In an *in vivo* study, photoinactivation of *C. albicans* at the periapical region of the tooth, with added photosensitizer calcium hydroxide, was proven to be more effective in reducing bacterial colonies by 58% compared to the treatment of calcium hydroxide without illumination, which reduced bacterial colonies only 13%.[26]

The results of the photoinactivation studies on the form of planktonic and biofilm cells of *Candida albicans* indicated that the more complex and thicker structure of *C. albicans* cell membrane could decrease the inactivation effect. The hype form tends to occur when *C. albicans* envelops its cells into the biofilm layer. Therefore, the invasion or penetration of *C. albicans* cells could pass through the mucosa more easily in the presence of filaments. It proved that biofilms were often associated with the severity of pathogenicity of *C. albicans*.

The result from measuring the malondialdehyde levels revealed that the laser groups without chlorophyll was relatively lower than the laser groups with chlorophyll. The malondialdehyde levels in the control groups were expected to arise from the lipid biofilm peroxidation process caused by the presence of toxic compounds produced by *C. albicans* cells or derived from the reaction process between thiobarbituric acid reagents and the survival of *Candida albicans* cells. The malondialdehyde levels began to increase when chlorophyll was added to the biofilm of *C. albicans*. The malondialdehyde levels were increasing with photochemical events after chlorophyll was activated by various energy density light. The result from the laser light exposure treatment indicated the highest of the malondialdehyde levels in the laser groups with the addition of chlorophyll of 0.467 nmol/mg and 0.397 nmol/mg for 650 nm and 445 nm laser, respectively.

Both tests of cell viability and malondialdehyde levels in biofilms after photoinactivation treatment with the 650 nm and 445 nm lasers showed that the most effective tendency belonged to the laser treatment groups of 650 nm. This is because the duration of the laser irradiation for the 650 nm laser was longer than the 445 nm laser for one of the energy density values. An increase in energy density affected the probability of the number of cells exposed to the rays and the number of radical compounds that were formed simultaneously. The red laser was more effective than the blue laser because of the power and absorbency intensity laser. The absorbency intensity of papaya leaf chlorophyll extract in the red region had a lower intensity than absorption in the blue region, causing lighting with a red laser to take longer to obtain the same energy density. The exposure time of the red laser resulted in a higher inactivation effect. The results of this study indicated that with the same energy density, 2 light sources with different spectra had specific inactivation characteristics in *Candida albicans* biofilms.

Conclusion

The use of chlorophyll extract of the papaya leaf as a photosensitizer resulted in the maximum spectrum of

absorption at 414 nm and 668 nm, which produced a maximum reduction effect after photoinactivation up to 32% (with chlorophyll) and 23% (without chlorophyll). The utilization of chlorophyll extract of the papaya leaf would increase the antifungal effects with the activation by the diode laser in the biofilm of *C. albicans*.

Ethical Considerations

Not applicable.

Conflict of Interests

The authors have no conflict of interest to declare.

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