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Effect of Energy Radiant Laser on Photoantimicrobial to Degradation *Staphylococcus epidermidis* Biofilm Cells Mediated Sensitizer of Nano Silver-Chlorophyll Jatropha Leaf

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Abstract

Chlorophyll compounds have been widely developed in photoinactivation research as organic photosensitizer agents, especially those extracted from green plants. Besides being natural and containing antimicrobial substances, the characteristic electronic properties of chlorophyll atomic have a long lifetime at the triplet level, so it is highly probable to produce Reactive Oxygen Singlet (ROS) while light activates. This phenomenon can potentially be applied in the mechanism of Photodynamic Therapy (PDT) or Photodynamic Inactivation (PDI) in various types of pathogenic bacteria that trigger infectious diseases. Laser light that activates photosensitizer molecules produces singlet oxygen which is reactive and toxic to microbial pathogens. In this study, will be found the efficacy of Jatropha leaf extracts combinate nano silver to inactivate biofilm cells of Staphylococcus epidermidis after being inducted by a red laser. Analysis of the research data quantitatively and qualitatively described the reduction in the number of biofilm cells and damage to the morphology of the biofilm cells with various energy radiant lasers applied through a scanning electron microscope (SEM) profile. The results showed that the photosensitizer agent, which combined Jatropha extracts with nano silver, obtained an inhibitory effect of 60% for the non-oxygenated group and 80% for the oxygenated group.

Introduction

Inactivation photodynamics is a part of photodynamic therapy devoted to killing pathogenic microbes. Microbial death induced by the activity of ROS compounds formed during irradiation occurs through a non-specific inhibition of bacterial cell metabolism [1-2]. The term non-specific in this case refers to the part of the bacterial cell that is the target of destruction or deactivation of the metabolic system of the bacterial cell, which is not specific, as is the case with some of the performance of antibiotic agents [1]. In some types of antibiotic agents, the targeted inhibition target depends on the spectrum of agent performance; for example, beta-lactams such as penicillin or vancomycin attack and damage the bacterial cell wall, chloramphenicol and tetracyclines inhibit cell protein synthesis, trimethoprim can inhibit essential enzymes in folate metabolism or nitrofurantoin which affects nucleic acid metabolism [3-4].

In addition to inhibiting specific parts of the cell, the use of antibiotic agents also has limitations in stopping microbial growth, such as the occurrence of microbial resistance due to genetic changes in bacteria for some time before the performance of antibiotics can kill bacterial cells, including when bacterial cells undergo phenotypic changes by forming biofilms so that the antibiotics agent are not able to penetrate the exopolymer matrix (EPS) [3-4]. The weakness of applying this antibiotic agent opens research opportunities to find the right strategy to cause pathogenic bacterial cells to die soon. PDI is an alternative treatment developed with an efficiency that is relatively fast and easy to implement.

The principle of PDI mechanisms differs from the performance of antibiotics, namely the presence of ROS compounds produced when photons of light interact with the photosensitizer molecule. A series of known light-induced processes include photophysics, photochemistry, and photobiology. Photophysics includes the absorption and excitation stage (electronic transition). Photochemistry includes chemical reaction processes and generating of radical compounds or Reactive Oxygen Species (ROS), especially singlet oxygen. Photobiology is the event of the inactivation metabolism and destruction of wall cell bacteria resulting in further lysis and death [2-5]. The toxicity and reactivity of ROS compounds can damage cell membranes, inhibit cell division systems, and damage cell DNA chains [4-5]. Damaged cell membranes allow photosensitizers to be transferred into the cell, thereby damaging cell organs such as lysosomes, mitochondria, and the nucleus [4-6].

One of the light sources often used in PDI is a laser with monochromatic characteristics, very narrow bandwidth, and coherence. Molecules more efficiently respond to monochromatic light with a single bandwidth characteristic, so light absorption is more optimal. The energy irradiation of the light photons influenced light's output power and intensity. Therefore, a laser with a specific wavelength, results in a flux of irradiation energy or energy dose (term practice in PDT applications). After the photosensitizer molecule is activated, orbital electrons move to the singlet energy level and then the triplet energy level before returning to the ground energy level. This electronic transition cycle will occur during irradiation [12-13]. The following illustrates the target cell that is attacked by the ROS compound until the death of cell necrosis occurs in Figure 1.

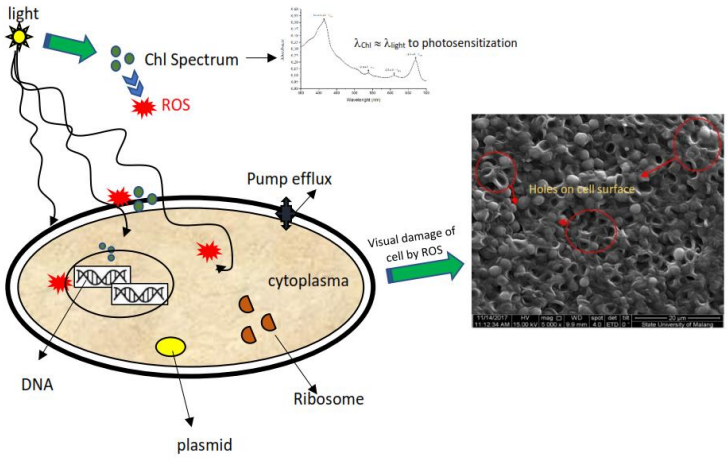


Figure 1. Destroying strategy of ROS compound in bacteria cell

According to figure 1, the light photon beam absorbed by the chlorophyll molecule (Chl) suitable to its optimum absorption wavelength band produces any ROS compounds, which will further damage the microbial cell wall and allow ROS to diffuse into cell organelles and attack the cell's DNA and other cell organelles. This stage will induce disruption of the metabolic activity of microbial cells. Through cell observation, one form of damage to cell morphology can be seen from the SEM visualization in Fig. It can be seen that there are holes on the surface of the cells, which are expected to be damaged cells.

Equally important, the component that must be considered in PDI is the presence of photosensitizer agents that affect ROS products during radiation. The main properties that a photosensitizer must have chemical purity, high quantum yield, toxicity in the dark, and ease of dissolving when removed [12-16].

Research in recent years has shown the potential development of chlorophyll as a photosensitizer from plants such as *papaya* leaf [5-6] and *alfalfa* leaf [15] succeeded in killing more than 80% of pathogenic microbes. However, previous studies have opposed using *papaya* leaf chlorophyll extract applied to *Candida albicans* biofilms in 2017-2019 [5-6,9].

This study aims to obtain a profile of the photoinactivation of *Staphylococcus epidermidis* biofilm base on radiation energy variation. The quantitative data refers to the value of OD as microbial viability and should be verified by SEM visualization damage. The data analysis used was to determine the percentage of microbial cell death through the hypothesis that the higher the percentage, the more optimal the treatment given.

Theory and Calculation

The photoinactivation system involves the absorption of photons by the photosensitizer that causes the sensitizing agent to undergo one or more transitions and usually appears in a triplet excited state, which is a photophysical process in photodynamic. Photoinactivation requires three important components a light source, a photosensitizing agent, and target oxygen. These three components are integrated through the processes involved: photophysical, photochemical, and photobiological [4-6].

Photochemical interactions between molecules and light are chemical reactions after molecules absorb light photon energy and go through a photophysical process. Photophysical processes include absorption, energy transition, electron transfer, and energy transfer [4].

Photochemical interactions that occur in the principle of photoinactivation, pass through two forms of processes, namely radiative processes which produce light emission as the end product of the process, and non-radiative processes which do not produce light emissions but form complex chemical reactions with molecules around the tissue, especially oxygen molecules. The released fluorescent emission can occur when the singlet transition molecule returns to the ground state, while the phosphorescent emission can occur when the triplet transition molecule ($^3S_0$) in the basic energy returns to the ground state [4,6].

In the photoinactivation mechanism, the mechanism that is expected to occur is that molecules that are excited to the singlet level will move to the triplet level by intersystem crossing. The oxygen molecules in nature are at the triplet level so there is an opportunity for chemical reactions between the triplet molecules to react with triplet oxygen to generate ROS compounds. Molecules that have a high level of absorptivity at a certain wavelength will occupy the higher singlet energy levels S_2, S_3, and another higher level. The state in the singlet state is unstable and the molecule has excess energy, so the molecule tends to return to the basic energy level through several possible mechanisms [4-6,16].

An ideal light source should provide high output power at the wavelengths required for photosensitizer activation. Laser, LED, and coherent metal halide lamps are most commonly used for photoinactivation. The use of unfiltered light sources with emission in the ultraviolet range should be minimized because their absorption spectrum can have a mutagenic effect. Similarly, emissions in the infrared range can cause uncontrolled heating of cultured tissues or cells. The intensity of a suitable light source for the photoinactivation of fungi is in the range of 10 to 100 mW/cm^2 [4-5,14].

$$Energy\ Radiant\ (E) = Power\ density\ (PD) \times Time\ (T) \qquad (1)$$

$$Power\ density\ (PD) = \frac{Output\ power\ of\ light\ (P)}{irradiated\ area\ (A)} \qquad (2)$$

Jatropha curcas is one of the traditional plants in South Sulawesi, which is widely used as a wound healer. *Jatropha curcas* belongs to the *Euphorbiaceae* family, the genus of *Jatropha* with the species *Jatropha curcas* Linn. Phytochemical screening Jatropha contains strong saponins, flavonoids, tannins, and steroids in various solvents [19]. This study suspects that Jatropha contains sufficient levels of chlorophyll with an optimal light absorption spectrum, so it has the potential as a photosensitizer agent.

Important equations for analyzing Jatropha chlorophyll levels are presented below [20]:

$$Chlorophyll\ total\ (\frac{mg}{L}) = 22.2\ (A_{645}) + 8.02\ (A_{663}) \qquad (3)$$

To measure the effectiveness of the photoinactivation treatment with a specific chlorophyll photosensitizing agent based on the microbial cell density level (optical density value from

the ELISA reader), the popular term "*percentage of inhibition*" is calculated using the following formula [21]:

$$\%inhibition = \frac{(A_{control} - A_{treatment})}{A_{control}} \times 100\% \quad (4)$$

Experimental Method

The research phase began with the extraction of chlorophyll pigment from Jatropha leaf using n-hexane and ethyl acetate solvents. There are two stages of separation filtrate of Jatropha well done to take their pure pigment chlorophyll, such as fractionation with column chromatography and detection of compound bands with thin layer chromatography (technical improvement from reference [5,20]). The nano silver (Np) solution is used as an additional agent to optimize the effectiveness of photoinactivation and as a photosensitizer too. Followed by UV-Vis Spectrum characterization and Photoluminescent test.

Application of the photoinactivation mechanism use a 650 nm red laser source (laser specifications used) with exposure times of 2, 4, 6, 8, and 10 minutes. In vitro, the microbial culture of *Staphylococcus epidermidis* suspended nutrient broth content of glucose 8% as a nutrient to the forming biofilms in the bottom of microplate well-98, with an incubation period of three days before harvest.

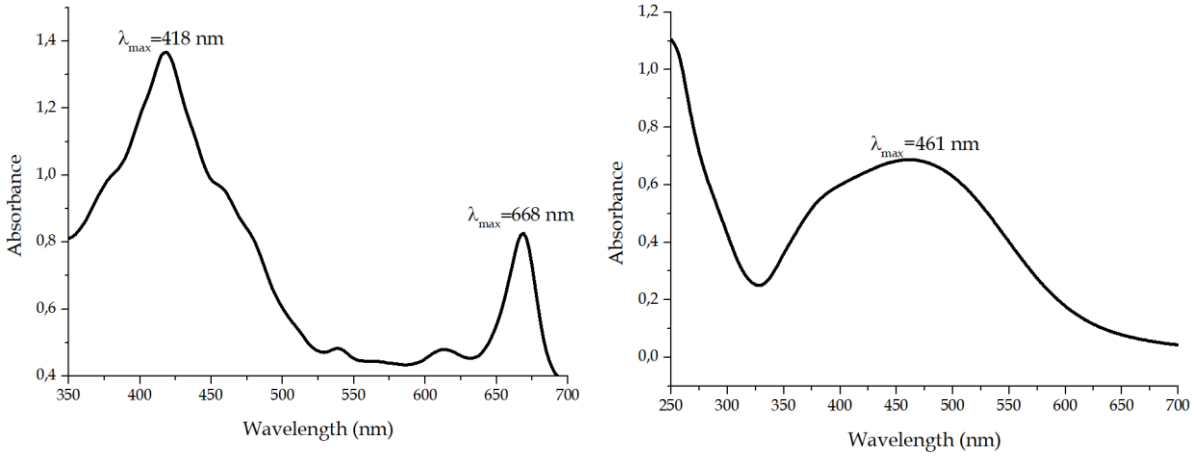
There are four groups of design research, namely negative control group (P-) is biofilm sample without anything treatment, positive control group (P+) as an antimicrobial treatment alone, the laser group (L) as a group with radiating treatment, and laser additing photosensitizer group (PL) as the primary treatment namely PDI group. The control group should be made in five replicas that associated with exposure time variation. The treatment group consist of Laser alone (sample code: L1, L2, L3, L4, and L5) and laser additing photosensitizer (sample code: PL1, PL2, PL3, PL4, and PL5). All groups should made be three replicas. The light sources using the red laser with specifications (650 nm; 321 mW; and 0.502 cm^2). Before the chlorophyll-nano silver (kinds of photosensitizer used) was added, oxygen gas flowed at a rate of 2 L/min for 2 minutes to increase the oxygen level in biofilms. The *Staphylococcus epidermidis* biofilm formation in microplate-98 well and XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay test was referred to in the previous study procedure [5].

Result and Discussion

Characteristics of chlorophyll of Jatropha Leaf Extract and Nano silver as a photosensitizer agent

Absorbance spectrum and photoluminescent spectrum are characteristics of an extract related to the optical phenomenon. These two properties give an idea of the wavelength of the molecule when it absorbs light (excited) and when it emits its energy in the form of fluorescent light before returning to basic energy levels. Chlorophyll has two absorption peaks which are referred to as the *Soret band* and *Q-band*. The *Soret band* represents the purple to the blue region of the spectrum, while the *Q-band* represents the red region of the spectrum. The terms *Soret band* and *Q-band* indicate the electron excitation of the chlorophyll molecule qualitatively at various absorption rates at certain wavelengths. The *Soret band* as an indicator of absorbed

energy is shown with a strong oscillatory curve, while the *Q-band* as an indicator of absorbed energy is less with a weak oscillation curve [15-16].



(a) (b)

Figure 2. The Profile UV-Vis Spectrum of (a) Chlorophyll of Jatropha leaf extract; (b) Nano silver liquid

Figure 2 shows Jatropha leaf extract UV-Vis spectrum profile with optimal absorption peaks occurring at 418 nm and 668 nm of wavelength. This value is slightly different from previous studies for chlorophyll of other green plants. For example, *papaya* leaf are excited at wavelength of 414 nm and 668 nm [5,6], and chlorophyll from other plants studied by Milencovic and Christina obtained optimum absorption peaks at wavelength of 411 nm & 662 nm [17] and wavelength of 412 nm and 662 nm [18]. Based on Figure 2 shows one of the absorption peaks of Jatropha leaf extract, namely in the red spectrum. The UV-Vis spectral characteristics of nano silver show that the maximum absorption peak is at a wavelength of 461 nm. This characteristic is different from Jatropha leaf extract. Still, nano silver is believed to carry chlorophyll to diffuse into the biofilm layer. It will reach the target microbial cells bound in the biofilm, so that biofilm cell growth is inhibited more optimally.

The extract UV-Vis spectrum profile shown in Figure 2 describes each wavelength corresponding to the absorbance level line. The corresponding absorbance value is offered at a certain wavelength point; it can be assumed as (1) the number of photons that some molecules of chlorophyll extract can absorb; or (2) the large number of extract chlorophyll molecules that will absorb photons of light at a certain wavelength. It is assumed is in line with *Lambert Beer's law*, the concentration of the test solution strongly influences the absorbance value of a solution. The higher of concentration of solution so, the greater the designation of the absorbance value.

Based on equation (3), the extracted chlorophyll content of Jatropha leaf (A_{645}=0,537 and A_{663}=0,820) is:

$$Chlorophyll\ total\ \left(\frac{mg}{L}\right) = 22.2\ (A_{645}) + 8.02\ (A_{663}) = 18{,}516\ \text{mg/L}$$

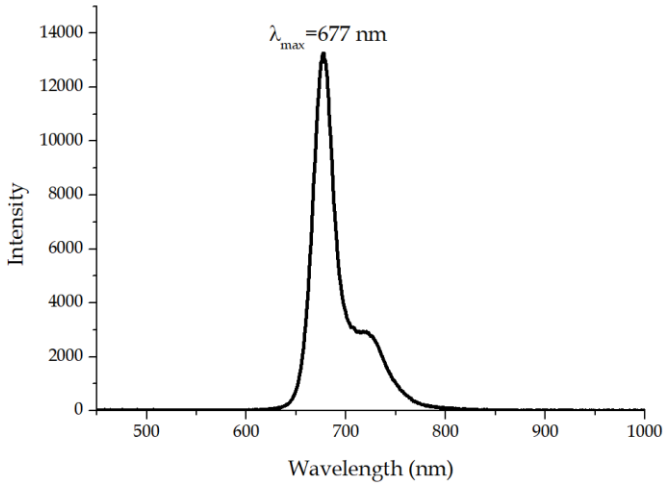


Figure 3. The Profile Photoluminescent Spectrum of Chlorophyll of Jatropha leaf extract

This profile is the same as in Figure 3, indicating that the molecule that has been excited to the singlet level returns to the basic energy level by emitting fluorescent light at 677 nm. Therefore, this data might indicate that the Jatropha leaf extract molecules will be optimally activated when absorbing red light.

The two molecular absorption spectrum profiles above are important to know to choose the wavelength of light that will induce the extract or nanosilver. This illustrates the percentage of energy absorption that will activate the extract and nanosilver molecules to induce the formation of ROS compounds.

Density Grade of *Staphylococcus epidermidis* after Photoinactivation

One of the important stages to be considered and used as supporting data in this study is the light source specification. Before carrying out the laser irradiation treatment and determining the research group design, it is necessary to calculate the radiation energy of the light source (laser) if the duration of irradiation is varied. The irradiation time was only chosen randomly following the previous research conducted by the author. All of the calculating energy radiant of laser if used the time exposure about 2 – 10 minutes, shown in table 1.

Table 1. Determination of Dose Energy in PDI mechanisms applicated (with: Output Power laser = 321 mW; and Irradiation Area A = 0,502 cm^2)

No	Time Exposure (s)	Energy Radiant (mJ/cm^2)	Symbol in Graph
1.	120	192.6	E1
2.	240	385.2	E2
3.	360	577.8	E3
4.	480	770.4	E4
5.	600	963.0	E5

Table 1 shows the results of calculating the value of laser radiation energy as the duration of exposure increases. This laser radiation energy is adjusted to the laser power against the area of the irradiation area carried out on the sample. The irradiation time varied from 2, 4, 6, 8, and 10 minutes, so changes in laser radiation energy would significantly impact the number

of microbial cell deaths. The laser radiation energy will also influence the damage potential of biofilm cell morphology which is visualized through SEM images. The calculation result of the amount of laser radiation energy used in the research is a minimum of 192.6 mJ/cm^2 and a maximum of 963.0 mJ/cm^2.

Based on the principle of photodynamic therapy with photochemical steps to generate a ROS compound to kill microbial cells, the potential of a molecule will depend on its absorption properties of light. Optimum absorption and suitability level of the wavelength of the light source used which is above 80% will be able to trigger the formation of ROS compounds with high reactivity properties [15-16].

Based on the designed treatment group, the data tabulation was arranged according to groups. All of the samples with their marked, as an explain before in methods. All of the samples with their marks, as explained before in methods. The table separated too into the sample data without and with oxygen upgrade of concentration inside of biofilm before treatment.

Table 2. Data of Density Grade of Cell after PDI treatment with Chlorophyll-Nano silver

No	Group of Treatment	Density Grade of Cell (±SD)	
		Unoxygenated Biofilm	Oxygenated Biofilm
1	P- (negative control)	1.646±0.11	2.517±0.15
2	P+ (positive control)	1.281±0.09	1.904±0.15
3	L1 (2 minute)	1.312±0.17	2.252±0.02
4	L2 (4 minute)	1.151±0.07	2.195±0.16
5	L3 (6 minute)	1.097±0.11	2.051±0.21
6	L4 (8 minute)	0.963±0.03	2.032±0.21
7	L5 (10 minute)	0.834±0.20	1.891±0.19
8	PL1 (2 minute)	1.175±0.07	2.122±0.07
9	PL2 (4 minute)	0.935±0.02	2.039±0.07
10	PL3 (6 minute)	0.849±0.13	0.930±0.22
11	PL4 (8 minute)	0.636±0.21	0.676±0.11
12	PL5 (10 minute)	0.444±0.04	0.519±0.14

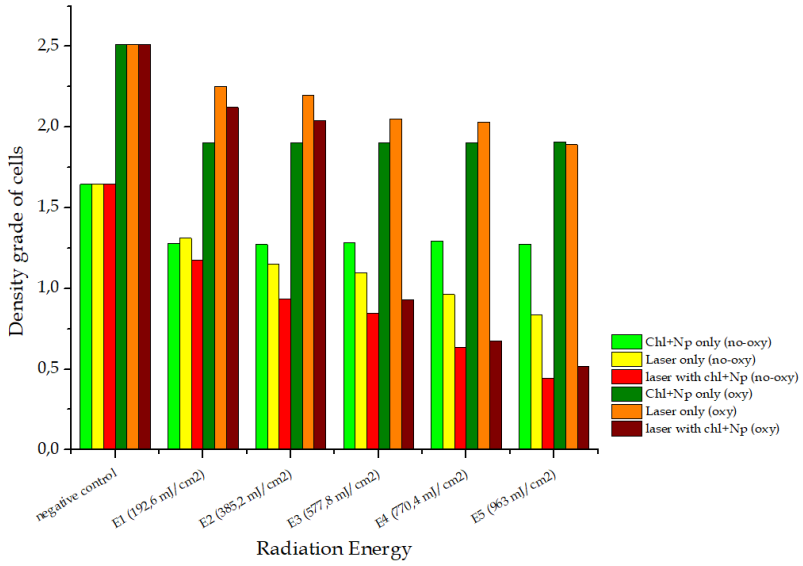
Table 2 describes the density grade of living microbial cells through the results of the XTT assay staining. This procedure only binds cells that are still actively metabolizing after the photoinactivation mechanism (inhibition of cell metabolism through light) has been carried out. It can be seen that high radiation energy contributes to a decrease in the number of microbial cells shown in the sample replicas studied. The quantitative data shown does not directly indicate the number of viable cells however the degree of orange color produced is relevant to the indicator of the formazan salt formed from the XTT assay. The darker the orange color produced, the greater the number of bound cells indicates that bound cells are a description of still alive cells. This data was compared to the negative control group (biofilm samples without any treatment) and the positive control group (biofilm samples treated alone with chlorophyll-nano silver).

The treatment between the laser without photosensitizer and the light treatment on samples that had given chlorophyll-nano silver showed significant changes. Compared with the negative control, the degree of cell density in the sample group without adding chlorophyll-

nano silver decreased twofold. In contrast with the sample group with the addition of chlorophyll-nano silver, the laser radiation energy reduced microbial cells by up to four times.

Inhibition of Biofilm *Staphylococcus epidermidis*

The bar chart profile will show the effectiveness of inhibiting microbial cell growth through photoinactivation treatment. Figure 4 shows the reduction profile of microbial cells according to the laser, photosensitizer, and combination treatment groups. Figure 4 also distinguishes the photoinactivation treatment in the two treatments, namely without or with modification of oxygenation. However, figure 5 is the main bar chart profile in this study. The profile shows the potential effect of Jatropha leaf extract and nano silver composite as a photosensitizer agent in the photoinactivation of *Staphylococcus epidermidis* biofilm cells.



*notes: Chl = chlorophyll; Np = nano silver; no-oxy = no oxygen; oxy = with oxygen

Figure 4. Profile of the number reducting cell *Stapylococcus epidermidis* after photoinactivation using chlorophyll of Jatropha-nanosilver

Based on Figure 4, the decrease in the number of living cells decreased with the increase in radiation energy, but with the oxygenation treatment in the sample there was a change in the number of cells in each biofilm sample replica. Although there was an increase in the number of initial cells before treatment for the oxygenation group, the reduction in cells that were still able to survive significantly decreased at the radiation energy E3 (577.8 mJ/cm^2). It is shown as a dark brown bar ending at E5 (963 mJ/cm^2).

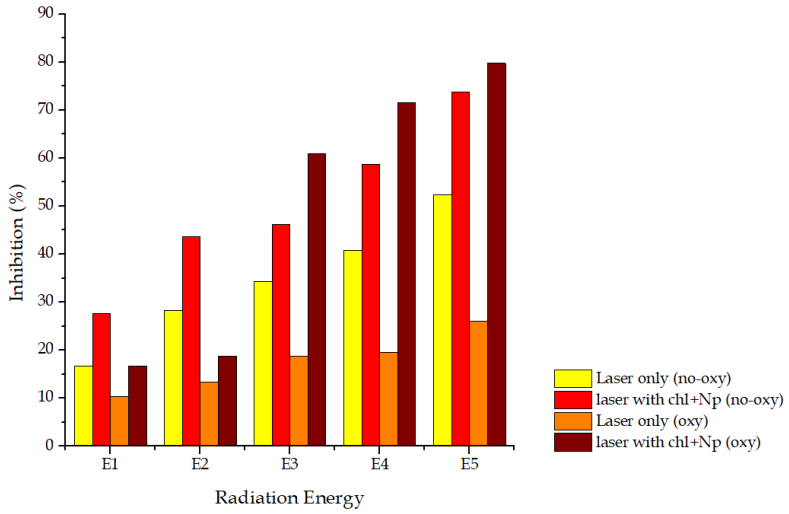


Figure 5. Effectivity of inactivation chlorophyll of Jatropha-nano silver

The cell reduction profile shown in Figure 4 corresponds to the percent value of inactivation that occurs, as shown in Figure 5. This inactivation percentage represents the reduction in cells for the treatment group against the control group cells. Figure 5 only retrieves data from the laser treatment group treated with a laser-irradiated photosensitizer agent (extract). At energy E1 to E2, the percent inactivation ranged from 28 – 43 % (for the group without oxygenation), while in the energy range E3 to E5, the percent inactivation increased to 45 – 73 % (for the group without oxygenation). Other results, for the sample group with oxygenation, the energy E3 to E5 gave a much larger percentage of inactivation, namely 60 – 80%. This is an optimal success among the results obtained by the authors from previous studies with an inhibition value of only 32% for *C. albicans* biofilms [6]. Other studies that have been reported include Ma et al. using LED (445 nm; 13.2 J/cm^2) to effectively kill *C. albicans* biofilm cells by 90.87% [2]. Kariminezhad et al. applied a laser (630 nm; 100 mW/cm^2) and successfully killed *S. epidermidis* cells [3]. Paramanantham et al. used a laser (670 nm; 97.65 J/cm^2) for 5 minutes and obtained an effectiveness of 65.68% against *E. coli* and 79.66% against *S. aureus* [22].

Profile SEM of Degradation Biofilm *Staphylococcus epidermidis*

Verification of the application of photodynamic inactivation to pathogenic microbes was carried out through SEM observations. The following shows a visualization of the damage to the biofilm morphology resulting from the photoinactivation treatment. The group of biofilm samples tested was separated between the control, using photosensitizers individually, and combining all treatments.

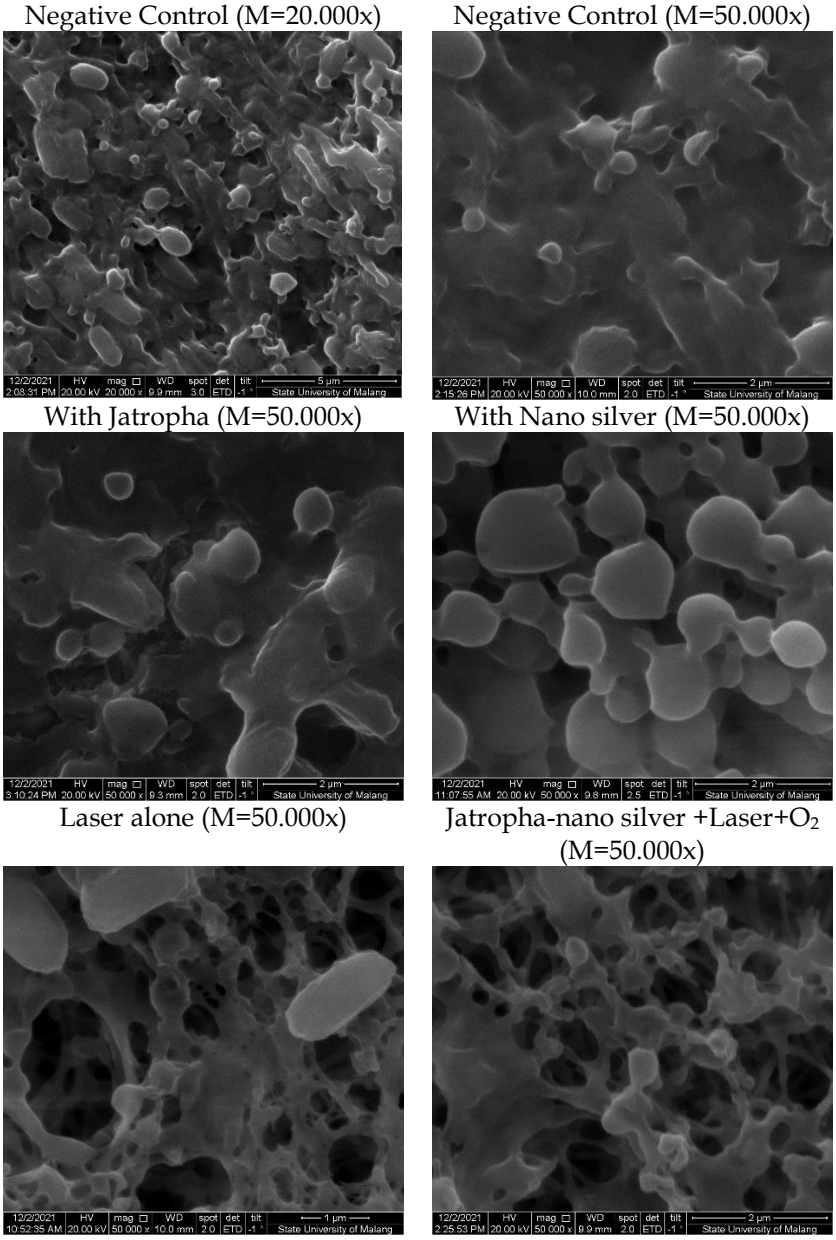


Figure 6. SEM Visualisation of *Staphylococcus epidermidis* biofilm after PDI treatment

Figure 6 shows the magnification of the control group is 20,000x and 50,000x. SEM visual results of the control biofilm contained a layer of the extra polymer matrix (EPS) that binds the biofilm cells. Some parts have a thicker coating than others. 50,000x magnification clearly shows the presence of membranes that limit the microbial colonies.

The Jatropha and nano silver groups are both treatments that apply a photosensitizer alone (without laser induction), whereas the group of nano silver is more effective than the Jatropha group. Visual SEM of the Jatropha group biofilm still showed the thickness of the EPS matrix in the biofilm. The laser group showed more significant changes. In addition to the EPS matrix, which can be broken down and decomposed by the activity of ROS compounds, some target

microbial cells are also separated from their colonies, and some show smaller microbial parts than intact cells. In the group that combined laser+photosensitizer+oxygenation, it showed optimal potential. Biofilms can be penetrated and damaged by the activity of ROS compounds so that they can release some intact microbial cells from the EPS matrix.

This visualization provides evidence that applying photodynamic inactivation treatment through the effect of light-activating photosensitizer molecules and the adequacy of oxygen around the target will result in the process of cell death of pathogenic microbes.

Conclusion

The Jatropha leaf extract has chlorophyll content at 18.516 mg/L, potentially applied as a photosensitizer after being activated with a 650 nm laser. The percent inhibition to *Staphylococcus epidermidis* biofilm cells obtained a maximum value of 80% by time exposure of 10 minutes.

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