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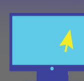
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The Analysis of Potency of Castor Leaf Extract (*Jatropha Curcas* L.) After Radiating with A Red Laser to Inhibit the Growth of Oxygenated *Staphylococcus Epidermidis* Biofilm

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Abstract. Photoinactivation known as *Antimicrobial Photodynamic Therapy* (aPDT) is part of the *Photodynamic Therapy* (PDT) system which is intended to inhibit the growth of pathogenic microbes that trigger infectious diseases in the human body. Photoinactivation developed in research has used natural photosensitizer agents extracted from plants that contain antimicrobial substances. This study aims to determine the optimal performance of castor leaf extract through irradiation with a red laser to kill *Staphylococcus epidermidis* biofilm cells whose oxygen level has been increased previously. The analytical method used is XTT staining assay with the *Optical Density* (OD) value as an indicator of the number of cells still active metabolize. The results obtained showed that the OD value for control group (P-) about (1.644±0.138), established a decrease after 10 minutes of irradiation of (0.926±0.220). This shows that the effectiveness of castor leaf extract after being activated by a high-potential red laser inhibits the growth of *S. epidermidis* biofilm cells.

INTRODUCTION

S. epidermidis is a gram-positive bacterium that is spherical in shape, usually arranged in an irregular series like grapes. These bacteria are anaerobic and can cause infectious diseases, generally causing swelling (*abscesses)* of the skin or acne [1,2]. Transmission of these microbes can be through patient contact with equipment that has been contaminated because of the ability to form biofilms on the surface of the tool. Biofilms are resistant to the activity of the antibiotic immune system which is the main cause of failure of conventional antibiotics. The use of antibiotic therapy which is long-term and undisciplined will provide opportunities for microbes to become more pathogenic [3].

The occurrence of resistance properties in biofilms encourages the application of more effective and optimal methods, especially to reduce the risk of prolonged infection. One of the methods developed is PDT system or specifically in the handling of microbes known as aPDT or photoinactivation. aPDT is an alternative antibacterial, antifungal, and antivirus treatment against drug-resistant microorganisms. aPDT utilizes the principle of light transmission and is able to penetrate into the biofilm matrix. The interaction of a nontoxic photosensitizer and a low-energy light source will generate reactive oxygen species and trigger a cascade of biological events that lead to apoptosis and the death of microorganisms. This approach will help to reduce the microbial population and biofilm [4].

In a previous study, the Radachlorine compound activated by a laser diode (λ_{660}) has the potential to reduce the growth of *P. aeruginosa* [5]. The potency of the chlorophyll compound of papaya leaf extract after incubation for 3 hours before laser irradiation, showed that the percent inactivation of *C. albicans* biofilms was significantly different. The result of irradiation with a blue laser (λ_{450}) was more effective than red laser irradiation (λ_{650}) for the two biofilm

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conditions without or with modified oxygenation [6]. The suitability of the diode laser wavelength with the photosensitizer absorption spectrum also affects the effectiveness of bacterial inactivation, where the right match will increase the photosensitization process to produce more reactive compounds that are toxic and reactive to bacterial death [7].

This research was directed to see the effectiveness of photoinactivation of *S. epidermidis* with chlorophyll photosensitizer of castor leaf extract which was activated with a red laser. The analysis used is the XTT staining assay, the inhibitory effect is calculated based on the colorimetric reading of orange color as an indicator of the amount metabolically active cells.

MATERIALS AND METHODS

The research phase begins with the extraction of chlorophyll pigment from Castor leaf using nhexane and ethyl acetate as solvents, followed by Spectrum UV-Vis characterization and phytochemical tests. The aPDT application uses a red laser source with a wavelength of 650 nm with exposure times of 2, 4, 6, 8, and 10 minutes. In vitro, the microbial culture of *S. epidermidis* was suspended in 8% glucose Nutrient Broth as nutrients for biofilm formation.

There are four groups of design research, namely negative control group (P-) just biofilm without anything treatment, positive control group (P+) as an antimicrobial treatment, the laser group (L) as a group with radiating treatment, and laser with chlorophyll group (PL) as the main treatment namely aPDT group. For the control groups made five replicas associate with exposure time variation, and for the groups treatment of Laser (L_1, L_2, L_3, L_4, and L_5) or Laser with chlorophyll (PL_1, PL_2, PL_3, PL_4, and PL_5) each of made with three replicas. The light sources using the red laser with specification (634 nm; 0.321 mW; and 0.192 cm^2). Prior to the chlorophyll incubation, oxygen gas flowed at a rate of 2 liters/minute for 2 minutes for increasing the oxygen level in biofilms. The *S. epidermidis* biofilm formation procedure and XTT (*2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide*) test were referred to in the previous study procedure [8].

The color gradation of the XTT assay test results is an assessment of the number of surviving microbial cells through the OD degree indicator (λ_{490}). The comparison between the OD of the control group and the OD of the treatment group resulted in the percentage of inhibition as the effect of the aPDT treatment, through equation (1):

$$\%\text{inactivation} = \left(\frac{(\text{OD}_{\text{negative control}} - \text{OD}_{\text{treatment}})}{\text{OD}_{\text{negative control}}}\right) \times 100\% \quad (1)$$

Figure 1 shows the stages of the research with consist of 3 main stages, namely the extraction of chlorophyll in castor leaf followed by characterization, the stage of biofilm formation on 96-well microplates, the stage of toxicity to determine the MIC (*minimum inhibitory concentration*) value, the stage of the suitability between λ_{chl} optimum with λ_{laser}, photoinactivation stage, staining stage and analysis. The TLC test and phytochemical screening are supporting characteristics as purification of chlorophyll compounds in the extract as well as proving the presence of antimicrobial active substances. These two data are not presented in this article. A toxicity test was conducted to obtain the minimum inhibitory concentration applied in the treatment.

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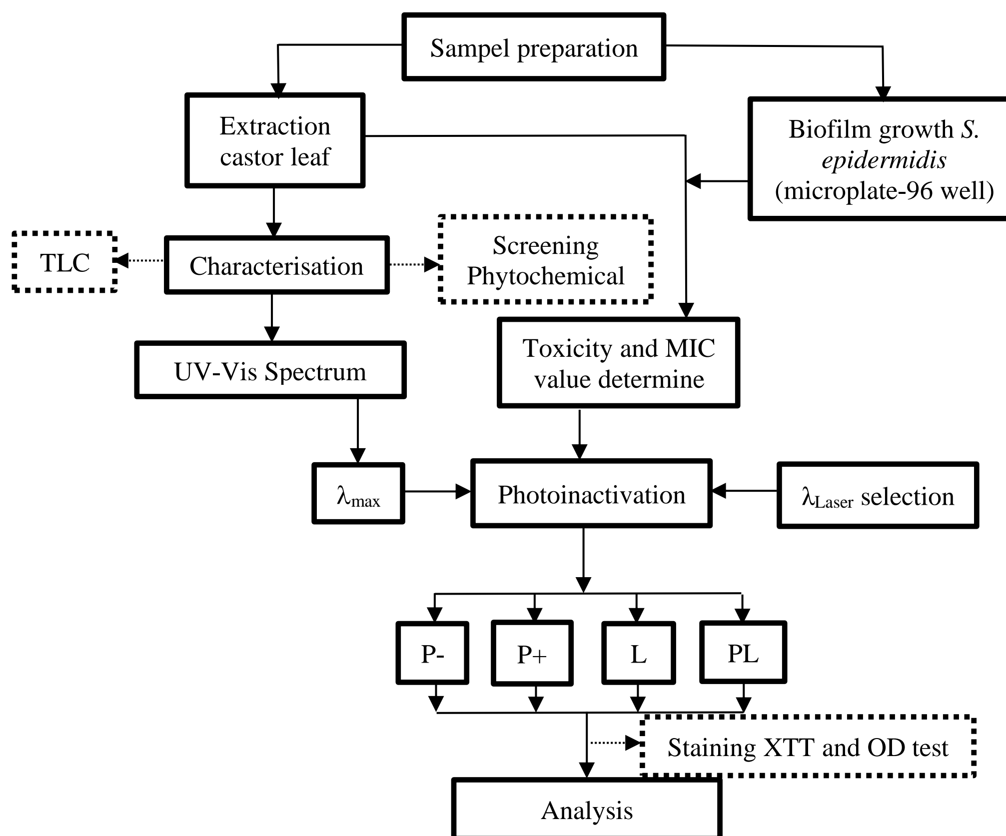

FIGURE 1. Stages of experiment

RESULTS AND DISCUSSION

Spectrum Profile of Castor Leaf

As 150 g of castor leaf macerated with n-hexane and ethyl acetate solvent obtained 7 mg of thick extract was obtained, followed by the preparation of three concentration series, namely 11.2%, 5%, and 2% through suspense with a distilled water and tween 20. Calculation of the chlorophyll content obtained found in castor leaf following the Gross calculation method [9]. The TLC test results from the extract isolation process showed the presence of chlorophyll compounds in the extract after column chromatography with an eluent ratio of 1:0.6 with their number of Retention Factor (R_f) about 0.56, while the results of phytochemical screening showed that castor leaf extract contained very strong alkaloids, flavonoids, and saponins.

The chlorophyll levels of castor leaf calculation refer to the absorbance value at the wavelength 645 and 663 nm as the main criteria in the Gross methods shown from the UV-Vis spectrum in Figure 2. The absorbance levels that the wavelength respectively 0.504 and 0.769, so the chlorophyll levels obtained:

$$\text{Chlorophyll total} = (20.2 \times A_{645}) + (8.02 \times A_{663}) = 16.348\ \text{mg/L}$$

Based on the UV-Vis spectrum profile of castor leaf in Figure 2, shows two optimum absorption peaks in the Soret band and Q band as an illustration of the absorption of castor leaf extract to light. In Figure 2, the absorbance of chlorophyll is optimum at λ_{soret}=427 nm (the violet spectrum), λ_Q=669 nm (the red spectrum), two peaks are also seen in the green spectrum area (λ=539 nm) and the orange spectrum area (λ=613 nm). The spectrum obtained is a characteristic of the extract that optimally absorbs light at a certain wavelength. The terms Soret band and Q band indicate qualitatively the electron excitation of the chlorophyll molecule at various absorption levels at certain

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wavelengths. Soret band as an indicator of energy that is absorbed more is shown by a strong oscillating curve, whereas the Q band as an indicator of energy that is absorbed is less with the form of a weak oscillation curve. This shows that the maximum castor leaf extract absorbs energy in the violet region λ_{soret}=427 nm, in some other spectra it produces three other with optimum absorption wavelength points at λ_Q=669 nm. It is a similar result with the previous study [8] that chlorophyll of papaya leaf extract has the maximum absorbance at λ_{soret}=414 nm dan λ_Q=668 nm.

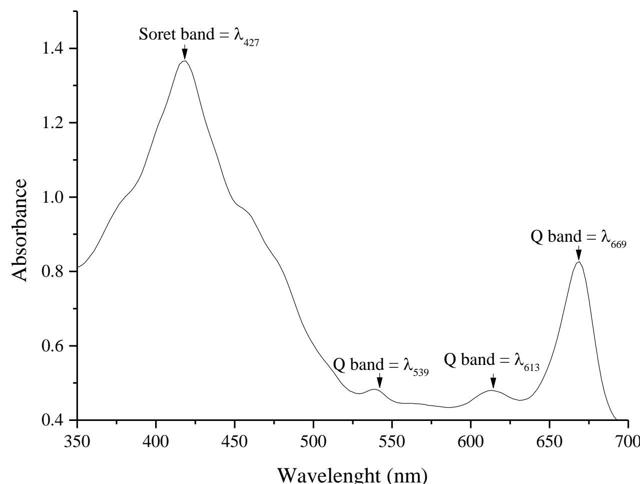

FIGURE 2. The Profile UV-Vis Spectrum of Chlorophyll of Castor leaf extract

The Cell Survive Level of *S. epidermidis* Biofilm after Photoinactivation

Data on the results of the photoinactivation *S. epidermidis* biofilm oxygenated after XTT staining indicator are the approach to the number of biofilm cells that are still alive or still actively metabolizing, written in full in Table 1.

TABLE 1. The data of cell survive level of photoinactivation *S. epidermidis* biofilm cells

No	Time exposure of laser (minute)	Cell survive level			
		Negative control (P-)	Positive control (P+)	Laser only (L)	Laser + chlorophyll (PL)
1	2	2.178±0.030	1.983±0.052	1.740±0.085	1.562±0.022
2	4	2.237±0.030	1.971±0.052	1.531±0.406	1.363±0.005
3	6	1.949±0.030	2.043±0.052	1.616±0.111	1.224±0.037
4	8	2.123±0.030	1.867±0.052	1.604±0.124	0.997±0.213
5	10	2.138±0.030	1.972±0.052	1.452±0.130	0.828±0.139

After XTT assay staining on aPDT treated samples and then done absorbance test using Elisa Reader (λ_{490}). A cell viability assay is often based on assaying ongoing cellular metabolism and enzyme activity, ie measuring factors that reflect the number of living cells in a population. Tetrazolium cell viability assays rely on cellular dehydrogenases to form a colored formazan product, which is measured by absorbance. A high value (>2.000) produces a deep orange color which indicates that there are still many active cells. According to Table 1 variation, time exposure laser treatment is 2 – 10 minutes that applicated against the two groups, namely the Laser only (L) group, and the Laser+chlorophyll (PL) group. The data indicates that the longer the exposure, the more it affects the mortality rate of microbes, where the value of absorbance level (cell survive) compared with negative controls. According to Table 1, we use the words “Cell survive level” the same as kind of a word at the other data such diagram (Figure 3 and Figure 4) is written “OD_{490nm}”.

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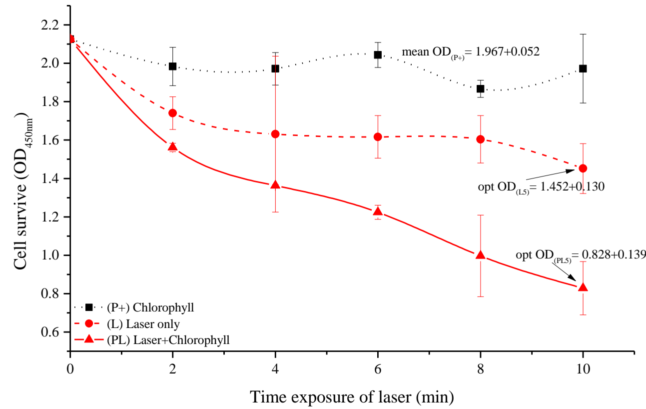

FIGURE 3. Graph of cell survive levels of *S. epidermidis* oxygenated biofilms after aPDT treatment.

Figure 3 shows that the negative control group (P-) with an average absorbance of 2.125 ± 0.030 experienced a decrease in the number of cells by 1.967 ± 0.052 in the positive control group (P +) with an inhibitory effect of 7.42%. The least values of cell survival were obtained from the results of aPDT treatment (laser+chlorophyll group), in the 2-minute irradiation treatment of 1.562±0.022 decreased to 1.224±0.037 at the 6-minute duration of exposure, and was able to achieve a cell reduction of 0.828±0.139 after 10 minutes of radiation. In the Laser only group, the number of live cells did not decrease drastically even at the duration of exposure of 4 minutes, it was seen that the number of live cells was lower than the duration of exposure at 6 and 8 minutes.

In the (P+) group, the reduction of biofilm cells looks fluctuating because the control group is not affected by light, but the number of samples is only an indicator that adjusts the amount of variation in exposure time as for the laser only (L) and laser+chlorophyll (PL) groups. In group (L) for an exposure time of 2 to 8 minutes, the values were almost constant, except for an exposure time of 8 to 10 minutes, there was a decrease in the number of cells.

For the group (PL) the number of cells that were still alive from the shortest to the longest irradiation treatment, a relatively decreasing curve was obtained. This indicates that aPDT treatment by laser radiation combined with a photosensitizer agent is fully effective for killing microbes. This statement is in line with the results obtained in previous studies [4,5,7,8,13].

Effect aPDT Laser Against to *S. epidermidis* Biofilms

The data substituted in equation (1) are those listed in Table 1. The value of cell survive (OD_{490nm}) for all treatment groups was then compared with the negative control group. The calculated data are not listed in the table but are plotted directly in the graph shown in Figure 4.

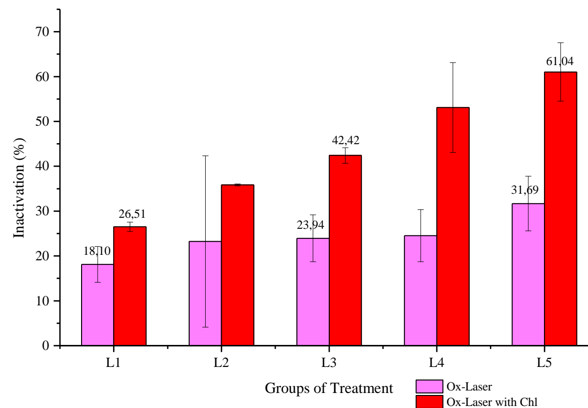

FIGURE 4. The histogram profile of percent inactivation which obtained aPDT treatment to *S. epidermidis* biofilm cells

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Based on Figure 4, the percentage of inactivation for the two treatment groups, namely the laser only group (L) with a pink histogram ranging from (18.10 – 31.69) % and the laser+chlorophyll (PL) group with a red histogram ranging from (26.51 - 61.04) %. For the laser group alone, the difference in the percentage of inactivation between the L_1 to L_3 groups was 5.84%, an increase of 7.75% when the exposure time was longer 4 minutes (than L_3 group) which occurred in the L_5 group. For the laser+chlorophyll group, the difference in the percentage of inactivation between the PL_1 to PL_3 groups was 15.91% with each inactivation effect of 26.51% to 42.42%, while the increase in the percentage of inactivation from the (PL_3) to (PL_5) group with a percent value inactivation at (PL_5) reached 61.04%, meaning an increase of 18.62%.

Castor leaf contain alkaloids that can affect or inhibit antimicrobial growth and aPDT can effectively reduce the number of bacteria present in the biofilm. When compared with previous studies, the addition of AgNP in aPDT after treatment with diode Laser 650 nm that combinated *Methylene blue* (MB) significantly strengthened the bactericidal potential of the applied therapy [10,11]. In line with other studies, aPDT was shown to be effective in increasing the death of *S epidermidis* biofilm cells when the *Toluidine Blue O* (TBO) concentration was 60 nmol/mg with an irradiation time of 20 minutes [12]. Likewise, with S aureus, the most effective treatment group on *C.albicans* biofilm was the laser group with the addition of chlorophyll of papaya leaf extract [13]. The activation treatment with a 650 nm diode laser resulted in an inhibitory effect of 32% (P=0.0001) while with a 445 nm laser just 25% (P=0.061).

Application of aPDT to planktonic *S. epidermidis* cells compared with *C. albicans* resulted in more than 3log10 cell death after activation with (red LED+MB) for S. epidermidis cells and (UV+RBF) for *C. albicans* cells. Radiation with red LED+MB in the *Staphylococcus* genus was the most prominent, resulting in 4log10 or greater cell reduction in *Candida* bacteria [4].

CONCLUSION

The potential of Castor leaf extract after isolated by chromatographic column has chlorophyll content at 16.348 mg/L and from phytochemical test results prove positive castor leaf extract contains antimicrobial substances. It is can be applied as a photosensitizer for inactivation to the growth of *S. epidermidis* biofilm cells, with the optimum inactivation occur at laser+chlorophyll groups 5th (PL_5) as 61,04% by time exposure 10 minute.

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