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Photoinactivation of *Candida albicans* Biofilm with Green Laser Mediated by the Papaya Leaf Extract Chlorophyll

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Abstract

This study aims to activate the effectiveness of Photodynamic Inactivation (PDI) as an antibacterial agent by using a green laser and papaya leaf chlorophyll extract to prevent Candida albicans cell death. Papaya leaf extract chlorophyll is known to have potential as a photosensitizer (PS) through its antimicrobial properties and ability to absorb optimal light photons at a wavelength range of 405–680 nm. Activation of chlorophyll molecules with appropriate light produces Reactive Oxygen Species (ROS), which are toxic to pathogenic microbes such as Candida albicans. The research method involves using PDI with a green laser light source and chlorophyll extract on Candida albicans biofilms. Four main treatment groups were applied, negative control (C-), positive controls with 10% (C1+) and 15% chlorophyll (C2+), irradiation for 60, 120, 180, 240, and 300 seconds (L1–L5), and combinations of irradiation with chlorophyll (L1F1–L5F2, where F1 for 10% chlorophyll and F2 for 15% chlorophyll), with measurements performed three times for each treatment. Living Candida albicans cells were detected using the XTT assay staining method. The results showed a significant decrease in activity in all treatment groups. Maximum activity was achieved in the L5F1 and L5F2 treatment groups with inactivation of 80% ($p<0.05$) and 83% ($p<0.05$), respectively. This study concludes that high papaya leaf extract chlorophyll concentrations combined with a green laser effectively inhibit Candida albicans biofilm.

Introduction

Infectious diseases caused by fungi, such as candidiasis, thrush, and HIV/AIDS, require serious attention due to the complex and rigid cellular characteristics of these microorganisms. The biofilms further complicate the management of these diseases, making them difficult to treat with conventional therapies such as antifungals, thereby necessitating alternative mechanisms[1]. Photodynamic inactivation is employed to treat infectious diseases through a

light-based inactivation mechanism. To achieve a more optimal treatment strategy[2][3]. Biofilm is a structured microbial community characterized by sessile cells attached to the surface and embedded in a matrix of extracellular polymeric substances produced by cells[4]. The *Candida* genera, especially *Candida albicans*, is one of the fungi species causing superficial mucosal infections and mycoses. This microorganism can produce biofilm in almost all medical devices, including the gastrointestinal tract, bronchi, genital tract, and skin. As the major cause of microbial infections in humans, biofilm is a severe problem for health services[3][5][6][7].

Photodynamic inactivation (PDI) is a process where microorganisms, such as bacteria, viruses, and fungi, are destroyed or non-activated using light. Mechanisms of photoinactivation are based on the formation of Reactive Oxygen Species (ROS), including singlet oxygen, which triggers a series of oxidation reactions in microbial cells[8]. The main molecules that become the target of ROS are proteins, cell membranes, lipids, and other cell wall components. Damage caused by oxidative stress is often irreversible and ultimately leads to cell death. The effectiveness of photoinactivation is influenced by the selection of appropriate photosensitizer structure, its concentration, and the dosage of light so that the ROS required is produced at the right time in the right site with high efficiency[4].

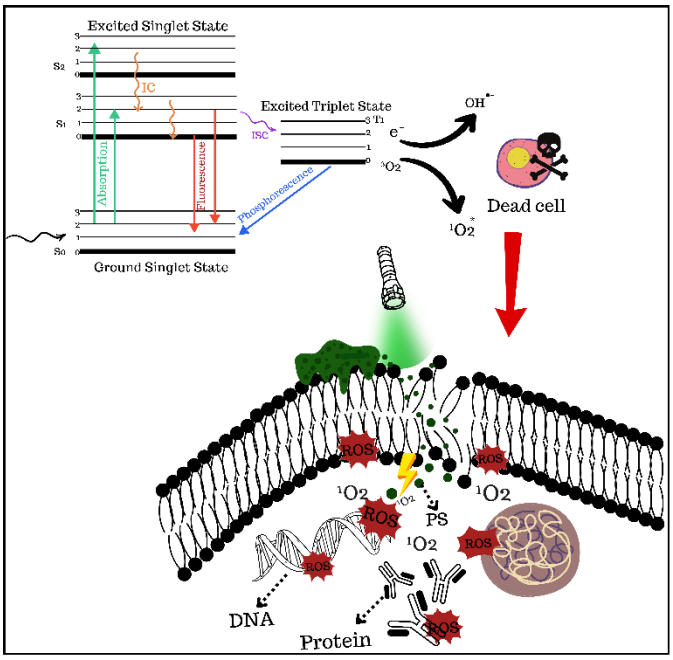

Figure 1 Mechanism of Photodynamic Inactivation (*adopted and improving from* [9])

Jablonski's diagram can be used to explain the mechanism of photoinactivation (figure 1). Photoinactivation begins with the radiation process of photosensitizer molecules with light in a specific wavelength. Photosensitizers then experience a transition from a low energy ground state (S_0) to the excited singlet state (S_n). Moreover, photosensitizer molecules can decay back to their initial state (S_0) with fluorescence emission or may experience the transition to a triplet state with Intersystem Crossing (ISC)[10]. Photosensitizer molecules in the triplet state can experience reactions type I and II. In reaction type I, the electron is transferred to organic

substrates in the cell, producing free radicals. Free radicals interact with oxygen at the molecular level and produce ROS, such as superoxide, hydroxyl radicals, and hydrogen peroxide. Reaction type II produces an electronically excited and highly reactive state of oxygen known as singlet oxygen. Singlet oxygen can destroy biomolecules in the target cells, including nucleic acids and proteins, so microorganisms can die. Contribution from Type II and II processes indicates that the damage mechanism depends on the light wavelength, oxygen, and concentration of photosensitizer[11][12][13]. ROS can damage Candida's membrane, cell wall, and lipid layer and attack cell organelles such as DNA and proteins within microbial cell organelles[7][14].

Light in PDI has a role in activating photosensitizer molecules and initiating the formation of ROS. Choosing a light source depends on the location of the target, the maximum absorption wavelength of the photosensitizer, and the light energy required. LASER (Light Amplification by Stimulated Emission of Radiation) is a light source often used in the PDI process. A diode laser is commonly used because it is lighter, portable/mobile, more stable, and cheaper. The diode laser can produce up to 8 watts of power, which is suitable for PDI medical applications that require high power. The wavelength range for diode laser that can be used in PDI is 415-690 nm[27].

Photodynamic research using photosensitizers from various chlorophyll extracts has been reported. In 2019, a study by Astuty et al. used chlorophyll extract from papaya leaves and diode lasers at 445 & 650 nm, resulting in inactivation effects of 25% and 32%, respectively[2]. In 2023, a study by Enggrianti et al. used chlorophyll extract from papaya leaves and a red laser, achieving a maximum inactivation effect of 61%[17]. Additionally, several studies have shown that the maximum absorption of chlorophyll extract from papaya leaves is in the wavelength range of 405–680 nm[2][18]. Therefore, this study focuses on applying photodynamic inactivation using chlorophyll compounds extracted from papaya leaves on Candida albicans using a diode laser at a wavelength of 530 nm. These characteristics make green lasers a promising option for applications in antimicrobial therapy, especially when dealing with pathogens resistant to conventional treatments.

The green laser has several advantages over other lasers and photodynamic therapy (PDT). One of the main advantages is its ability to produce a higher amount of reactive oxygen species (ROS) compared to lasers with longer wavelengths, such as red or infrared lasers. This makes it more effective in killing microorganisms, including Candida albicans[15]. Additionally, the green laser has a shorter wavelength, which allows it to penetrate deeper into tissue, making it more effective in treating infections that occur at a deeper level[16]. Moreover, the green laser can activate photosensitizers more efficiently, leading to a higher production of ROS and increased killing of microorganisms[15].

Experimental Method

Extraction and Characterization of Photosensitizer

The maceration process was done by soaking 150 grams of papaya leaf in a mixture of methanol-petroleum Ether (3:7) solvent for 3×24 hours in a dark room. The sample solution was then filtered to obtain filtration, which evaporated to produce a thick extract in the first purification stage. The partition process was carried out by mixing the thick extract with the

solvent mixture of n-hexane: ethyl acetate (1:1). Fractionation was carried out using a chromatography column using 2 types of eluents, namely n-hexane and acetone, using the principles of SGP (*Steph Gradient Polarity*) at a ratio of 1:0 to 1:1. The mechanism of action is illustrated in Figure 2. Proof for the presence of chlorophyll pigment was tested using TLC (Thin Layer Chromatograph) followed by a UV-Vis spectrophotometric test to see the optimal absorption of light at significant wavelengths[19][20].

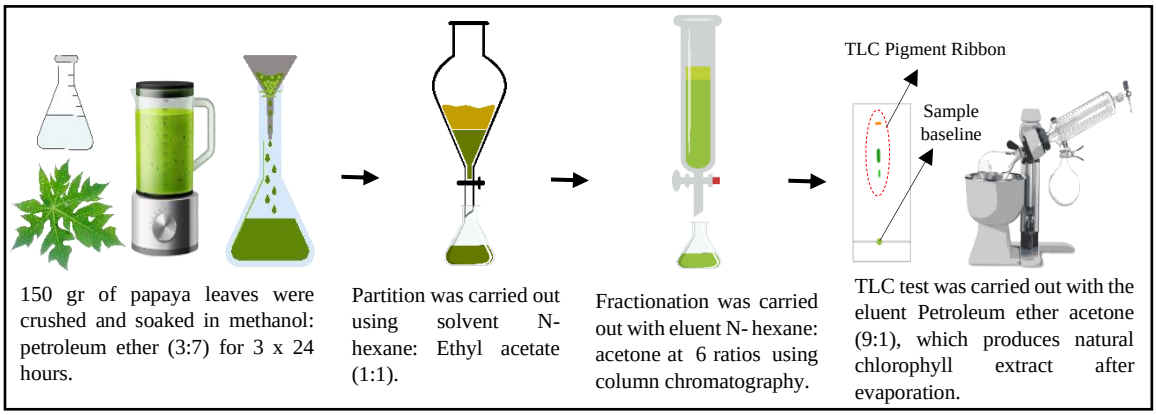

Figure 2 The Scheme of Extraction of Papaya Leaf

Antimicrobial test

The toxicity test was carried out by etching *Candida albicans* culture on the agar media. Then, the disc paper was immersed in the chlorophyll extract, antibacterial, and distilled water for 5 minutes and moved to the agar media surface using a sterile tweeze. After that, the media was incubated for 24 hours[21][22]. The mechanism underlying this process is illustrated in Figure 3.

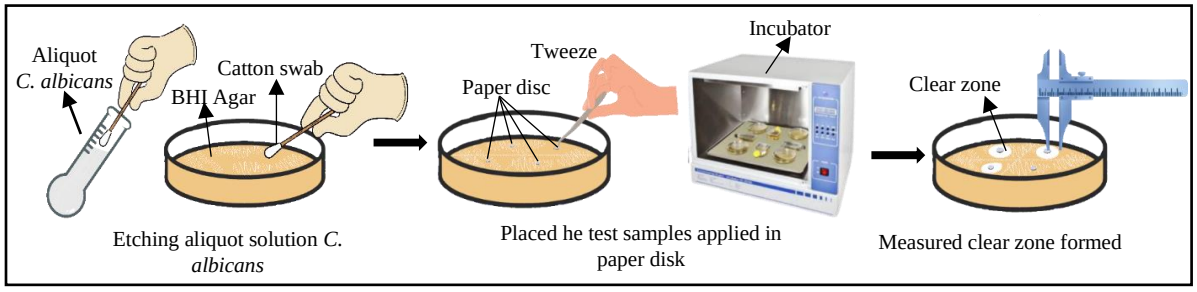

Figure 3 The scheme of clear zone testing

Laser stability test and power output determination

The laser was characterized by testing the stability of laser output power with a wavelength of 288 nm. The radiation process was carried out for 60 minutes, where laser power was observed for 10 seconds to monitor the stability of the laser. Radiation energy can be calculated using the equation[7][23]:

$$I_{laser} = \frac{P(W)}{A\ (cm^2)} \qquad (1)$$

$$D = I_{laser}(\lambda) \times t \quad (2)$$

Where $I_{absorbtion}$ is the laser intensity (W/cm^2), P is the laser output power (W), A is the area of the laser beam (cm^2), E is the radiation energy (J/cm^2), and t is the time exposure (s).

Photoinactivation Treatment and XTT Assay Staining

Candida albicans cultures were grown in a microplate 96 well on BHI agar and incubated for 48 hours. Biofilm was divided into four groups: negative control group (biofilm without treatment), positive control group (biofilm with addition of 10% and 15% PS), laser treatment group (biofilm with laser treatment), and biofilm group with PDI treatment (PS addition and laser treatment). The biofilm group with laser treatment and the biofilm group with PDI treatment were given laser radiation with variations of exposure time of 1, 2, 3, 4, and 5 minutes. Using varying exposure times in laser and PDI treatments aims to identify the optimal duration for reducing biofilm. These different exposure times facilitate the evaluation of the relationship between treatment duration and effectiveness. The distance of the light source from the microplate was 2 cm[24].

XTT Assay was carried out by adding 40 µL of 1 mg/ml XTT solution, 2 µL of 10 mg/ml menadione, and 158 µL of sterile PBS in microplate in all treatment groups. The biofilm that had been stained was then incubated for 2 hours. Before being analyzed for color content, the formazan salt formed from the XTT Assay reaction against viable microbes was transferred into a new 100 µL microplate, according to the microplate treatment design template. The absorbance was then measured three times using an ELISA Reader (Thermo Scientific, USA). These measurements were subsequently subjected to ANOVA testing. The mechanism of photodynamic inactivation treatment and the XTT assay can be seen in Figure 4.

Treatment Significance Test with ANOVA

All data were processed and statistically analyzed using One-way Analysis of Variance (ANOVA) to determine the significance of differences among the observed treatments. A turkey correction was applied to the p-values to account for multiple comparisons. The significance level was set at $p < 0.05$.

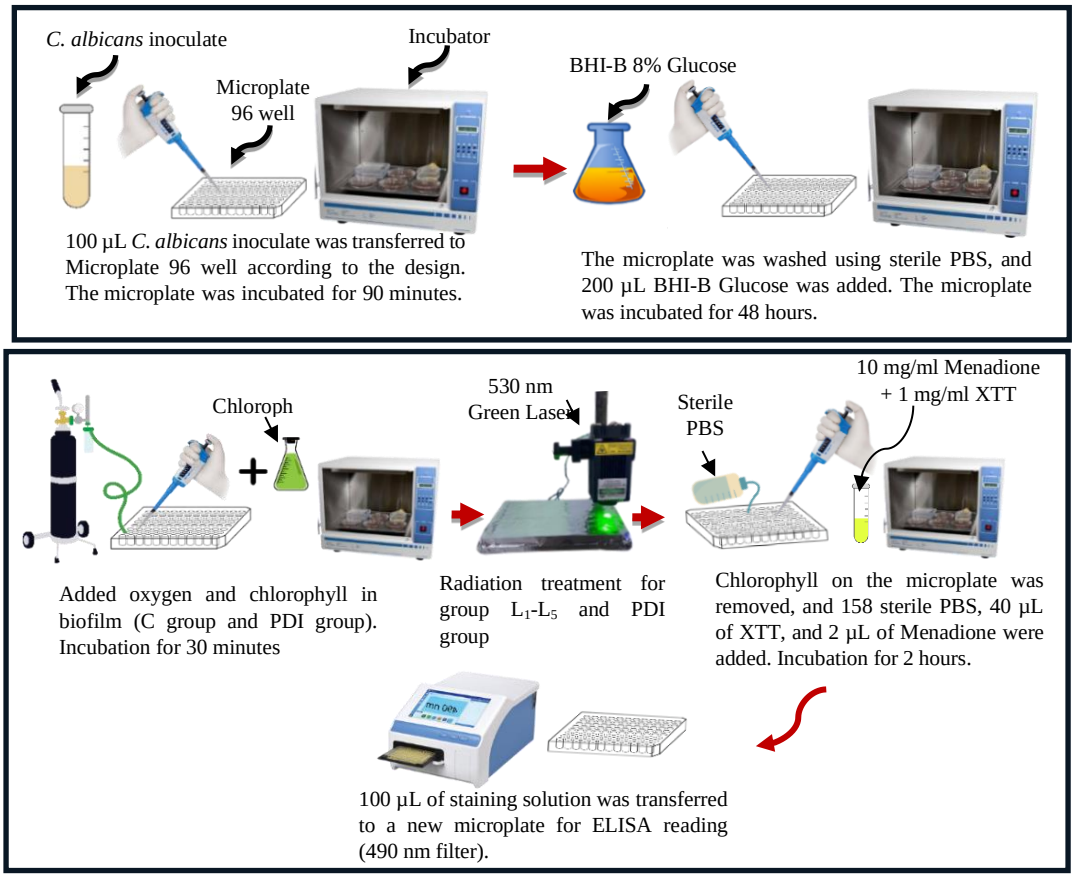

Figure 4 The scheme of photodynamic inactivation mechanisms

Results and Discussion

Optical characterization of chlorophyll papaya leaf

The results of the optical characterization test showed two prominent absorption peaks: the Soret-band and Q-band areas. The peak at a wavelength of 414 nm in the Soret-band had an absorbance value of 2.014. The peak at a wavelength of 670 m showed a Q-band area with an absorbance value of 1.133 (Figure 5). This study used a green laser as a light source with a 495-570 nm wavelength range. This is compatible with a wavelength of 536 nm with an absorbance of 0.601.

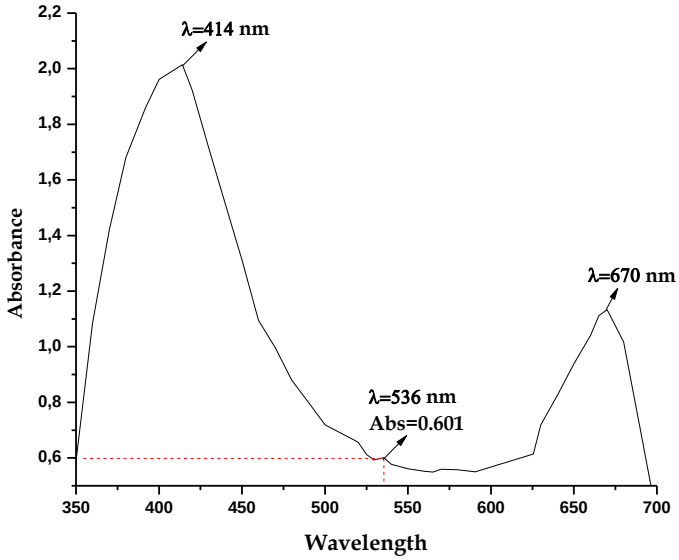

Figure 5. Absorption spectrum of papaya leaf chlorophyll extract

The Results of the Clear Zone Test

The clear zone was determined to observe the antifungal properties of papaya leaf chlorophyll extract. Antifungal activity through the detection of clear zones for *Candida albicans* culture samples was tested against several factors, particularly the antifungal properties of papaya extract at concentrations of 10% and 15%, compared to conventional antifungals and aquabidest as a negative control.

Table 1. The data of clear zone measure

Type of liquid	Clear zone (mm)
Aquabidest	0
Chlorophyll 10%	0.55
Chlorophyll 15%	0.575
Conventional antifungal	1.05

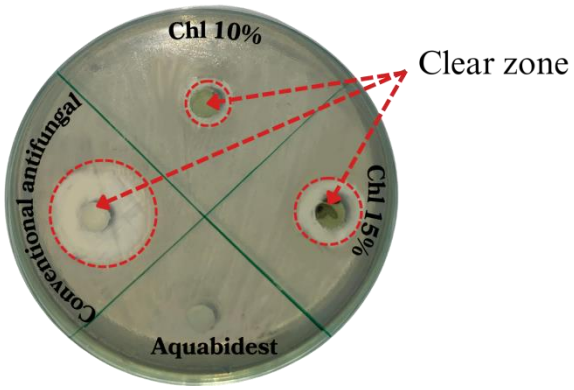

Figure 6. Toxicity test of clear zone method

The test successfully formed a clear zone around the chlorophyll for two concentrations tested, which were 10% and 15%, compared to a clear zone formed around conventional antifungal agents. The clear zone circle around the chlorophyll at a concentration of 15% was more comprehensive than the concentration of 10%, but the clear zone area around the antifungal was even more expansive. These three variations were verified by placing a paper disk containing distilled water as a negative control. The area around the distilled water did not show a significant clear area, which means that this area did not inhibit the growth of *Candida albicans* (Figure 6).

Laser Stability

The radiation energy used at each treatment time with an exposure surface area of 0.196 $cm^{2,}$ and the power used was 288 mW. These results showed that radiation energy increased two times every minute, with minimum radiation energy at $L_{1(60s)}$ of 88.163 J/cm^2 and maximum radiation energy at $L_{5(300s)}$ of 440.816 J/cm^2. This radiation energy was then implemented in each laser treatment (table 2).

Table 2. The data on radiation treatment energy

Code Treatment Laser	Area of Exposure (cm^2)	Laser Power (mW)	Laser Intensity (mW/cm^2)	Time (s)	Radiation Energy (J/cm^2)
L_1	0.196	288.0	1469.39	60	88.163
L_2				120	176.327
L_3				180	264.49
L_4				240	352.653
L_5				300	440.816

The Results of the Biofilm Cell Viability Test

A reduction in the optical density value of formazan salt was achieved through the XTT assay method, which was read in a 490 nm filter for all treatment groups. Groups C-, C_1^+, or C_2^+ were made with 5 replicas, respectively, adjusted to the number of variations in radiation energy. Changes in the optical density value of C_1^+ and C_2^+ were only influenced by variations in the measurement results. They were used to compare changes in optical density in the laser treatment group and laser treatment with a combination of 10% and 15% chlorophyll. Cell viability measurement data in optical density parameters for all control and treatment groups are presented in Figure 7.

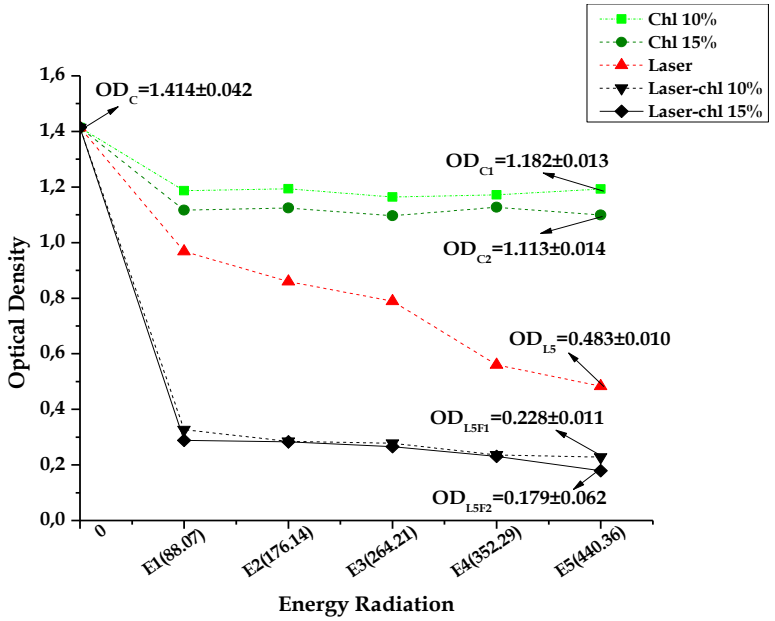

Figure 7 Optical density of laser treatment group

All treatment groups experienced a significant decrease in the increase in radiation energy provided. The maximum reduction in biofilm cell viability for all groups was in $L_{5(300s)}$ = 440,816 (J/cm^2), where for the optical density laser group, it was 0.483±0.010, laser group with the addition of 10% chlorophyll of 0.228±0.011, and laser group with the addition of 15% chlorophyll of 0.179±0.062. The graphic above also shows no significant difference in the decrease of cell viability in the laser group with the addition of 10% and 15% chlorophyll.

Based on the optical density value obtained, the percentage of inactivation can be determined to see the inhibitory ability of PDI treatment on *Candida albicans* biofilm cells.

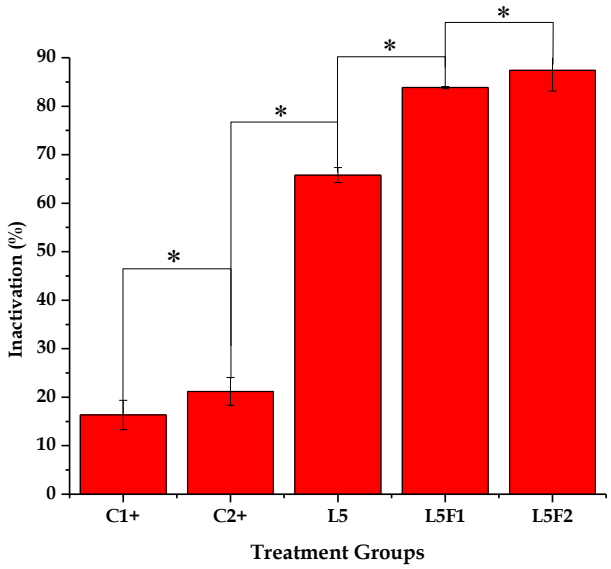

Figure 8 Diagram of the percentage level of inactivation in the PDI group
Note: *Show significant level in ANOVA one-way test of $p<0.05$

The most optimal inactivation level occurred in the longest exposure time of 300 seconds using a chlorophyll concentration of 15% and an inactivation level of 87%. On the other hand, in the same exposure time with a chlorophyll concentration of 10%, the inactivation activity produced reached 83% (figure 8).

Chlorophyll at a concentration of 15% could inhibit the growth and viability of *Candida albicans* biofilm cells. This can be seen from the clear zone testing and ELISA reading, which showed that the clear zone was more expansive around the chlorophyll and had a significant decrease in the viability of biofilm cells in the group with the addition of chlorophyll. Thus, chlorophyll can be considered a potential agent in controlling the growth of *Candida albicans*, where at a concentration of 15%, it showed a more substantial effect than at a concentration of 10%.

Discussion

Photodynamic inactivation (PDI) is one of the therapeutic approaches to overcoming pathogenic biofilm problems, especially *Candida albicans*. The effectiveness of photodynamic inactivation depends on the physicochemical properties of the photosensitizer compounds used[25]. In the literature, chlorophyll extracted from papaya leaves has been identified as a

promising photosensitizer. Research on green lasers in photoinactivation reveals several promising applications for microbial control. Their ability to effectively eliminate bacteria and viruses positions them as viable options for sterilization in the healthcare and food sectors. Moreover, their role in photodynamic therapy can enhance treatment strategies for specific infections. Incorporating green laser technology into regenerative medicine may facilitate faster healing of infected tissues[15]. However, the application of green laser in combination with chlorophyll to overcome *Candida albicans* problem continues to be studied. Thus, this study focuses on applying photodynamic inactivation using chlorophyll compounds extracted from papaya leaves on *Candida albicans* using a diode laser at a wavelength of 530 nm[26].

Photosensitizer is a substance that can absorb light at a specific wavelength and trigger photochemical reactions in forming ROS compounds. ROS produced from the photochemical process enables the deactivation of target cell proteins and inactivation of the cell metabolism system induced by light[28]. Thousands of natural and synthetic photoactive compounds have potential as photosensitizers. Currently, several studies are trying to identify the potential of natural photosensitizers isolated from medicinal plant extracts and observe their effectiveness after being combined with light. Plant extract enriched with chlorophyll can be used as photosensitizer because chlorophyll is the main compound of the plant that has properties of absorbing light, especially in visible light[29][30].

A study in phytopharmaceuticals has revealed various medicinal properties that can be obtained from green leaf extracts. Leaf extracts have anti-inflammatory properties, are effective in treating dengue fever, provide liver protection (hepatoprotection), are anti-oxidant to fight free radicals, have a potential for anti-cancer, and show anti-microbial activities[31][32][33]. A study conducted by Jing Ma et al. using natural photosensitizer, which is curcumin, with radiation using LED at a wavelength of 455 nm showed an inhibitory effect on *Candida albicans* biofilm, reaching 90.87%[34]. A study conducted by Irene et al., which studied the effect of papaya leaf extract in various concentrations on the growth of *Candida albicans* in disc paper, showed that the maximum zone of inhibition is at a concentration of 100% of 23.61 mm, while at a concentration of 10%, the inhibition zone formed is 7.39 nm[35]. Zhang et al. also conducted a zone of inhibition test in *Candida albicans* using papaya leaf extracts with various concentrations. The results showed that the most significant antifungal activities occurred at higher concentrations of extracts. The increase in effectiveness at higher concentrations is caused by the more substantial number of active components in papaya leaf extracts at the concentration[36]. This study also shows antimicrobial activity from papaya leaf extracts due to the presence of a flavonoid compound, which was confirmed by a clear zone test.

Several studies regarding PDI have been conducted, including a survey by Astuty et al. (2019) regarding the effectiveness of PDI on *Candida albicans* biofilm using PS of papaya leaf chlorophyll and light source in the form of diode laser at a wavelength of 445 nm and 650 nm and intensity of laser beam of 0.379 W/cm^2 and 0.306 W/cm^2, respectively. This study results in a maximum reduction effect after photoinactivation of up to 32% (with chlorophyll) and 25% (without chlorophyll)[2]. Another study by I. Buchovec et al. (2022) regarding inactivation of A. baumannii microbial biofilm used PS riboflavin and chlorophyllin. The light source used was LED at a wavelength of 402 nm and 440 nm. The application of PS riboflavin and

chlorophyllin in PDT at a radiation dose of 84 J/cm^2 results in the inactivation of 6.7 $\log_{10}$ and 5.7 $\log_1$, respectively[37].

Papaya leaf extract chlorophyll shows absorption peaks at a spectral 414 nm, 536 nm, and 670 nm. This absorption peak is relevant to be applied in PDI because it is in the spectral range known as the therapeutic window. Chlorophyll has a specific affinity with microorganisms. When chlorophyll is exposed to light at a specific wavelength, chlorophyll produces Reactive Oxygen Species (ROS) that can induce fatal damage to *Candida albicans*. The previous study has verified the ability of chlorophyll in photodynamic inactivation against *Candida albicans* when it is activated using a specific wavelength. Furthermore, the flavonoid content in papaya leaves makes chlorophyll from papaya leaf extracts a potential photosensitizer in overcoming *Candida albicans* biofilm[38].

Photodynamic inactivation treatment in *Candida albicans* biofilm was achieved by comparing two concentrations of 10% and 15% for five variations of exposure time, obtaining an optimum exposure time of 300 seconds. The effectiveness of photoinactivation obtained was 83% for a concentration of 10% and 87% for a concentration of 15%.

Conclusion

The chlorophyll of papaya leaf has the potential to inhibit the growth of *Candida albicans* biofilm after the formation of reactive oxygen species (ROS) compound by photoinactivation mechanisms. The effectiveness inhibition is significant with increased exposure time to the laser and concentration of chlorophyll. The significance of this research lies in combining green laser with papaya leaf extract chlorophyll, achieving an optimal inhibition rate of Candida albicans biofilm cells at 87%. Therefore, this combination presents a promising approach for photodynamic inactivation therapy.

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