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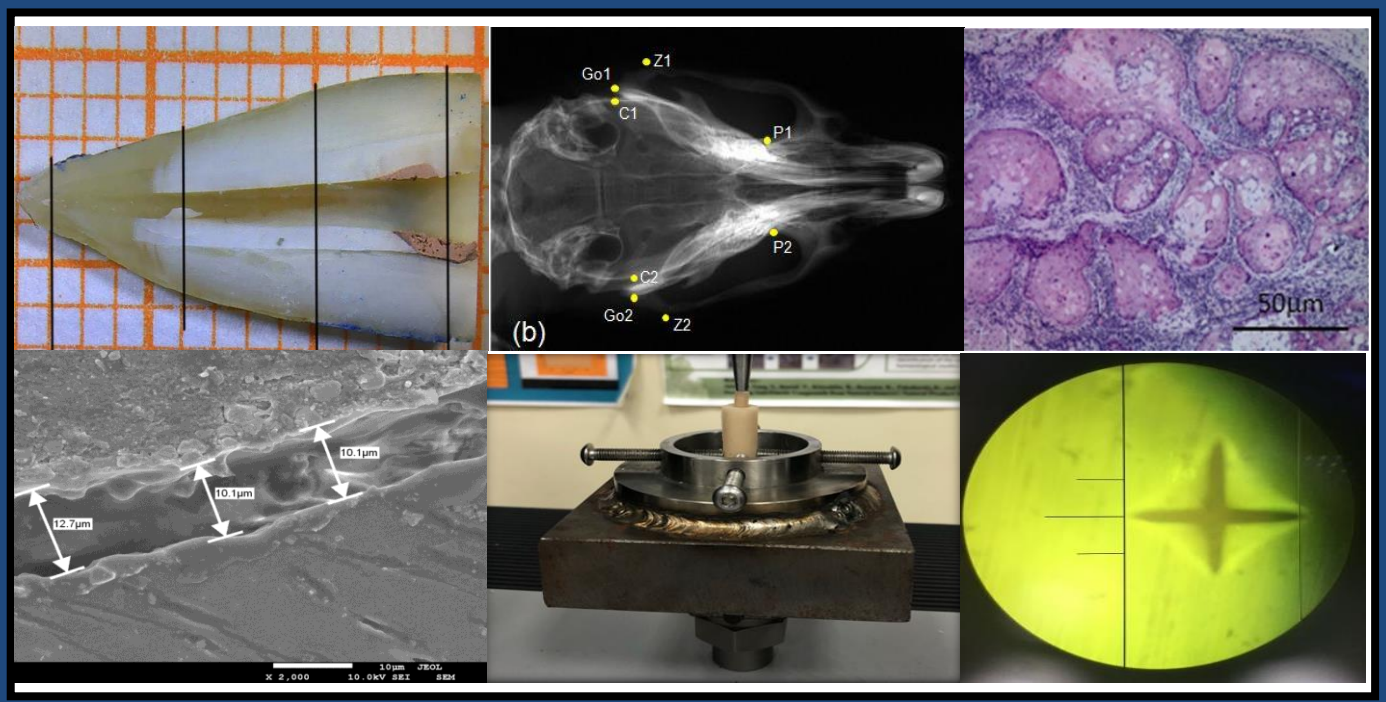

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Effectiveness of Photodynamic Inactivation with Exogenous Photosensitizer Curcuma longa Extract Activated by Laser Diode 403 nm on Staphylococcus Aureus

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Abstract

Photodynamic Inactivation (PDI) is a modality of antimicrobial therapy. Oxygen peroxidation in cell membranes has caused antimicrobial effects by inhibiting cell metabolism. The effectiveness of PDI depends on light sources, photosensitizer agents (Ps) and oxygen. This study is to investigate the antimicrobial effect of PDI using Curcuma longa (CL) extract as a photosensitizer (Ps) agent activated by 403 nm laser diode on Staphylococcus aureus (S. aureus).

CL extract was prepared by maceration of turmeric rhizome using 96% ethanol with concentration 0.15% (Ps1) and 0.3% (Ps2), respectively. The samples were divided into three groups; control (C1), treatments with Ps1 (C2) and Ps2 (C2'), treatment with laser irradiations (T1), PDI treatment with Ps1 (T2) and Ps2 (T3). The bacterial growth has been monitored by ELISA reader and measured by Colony Counter. The percentage of bacterial reduction was analyzed by one-way ANOVA test.

PDI treatment with CL extract is more effective to reduce S. aureus compared without exogenous CL extract. The highest reduction was given at high level irradiation with an energy density of 15.83 J/cm2 where treatment with Ps1 and Ps2 gave 79.18% and 85.48% reduction, respectively.

Exogenous photosensitizer addition in PDI can increased bacterial reduction to 85.48% with 0.3% CL extracts at high level irradiation. CL extracts as exogenous photosensitizer activated by laser diode provides an increase the effectiveness of PDI on S. aureus.

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Introduction

Staphylococcus aureus (S. aureus) is a gram-positive bacterium, which normally lives on the skin, nose, throat, and other organs in human[1]. The growth of S. aureus in humans can cause various skin diseases such as acne vulgaris, dermatitis, and cellulites. S. aureus is one of the bacteria that has high antibiotic resistance, so that it could counteract antibiotics, even though it could withstand the immune system. Several previous reported related to S. aureus bacteria in various countries tend to increase every year[2-3]. Therefore, alternative treatments are carried out to eliminate S. aureus bacteria selectively and effectively by using photodynamic inactivation[4]. Photodynamic Inactivation (PDI) is a therapy modality that uses photosensitizer (Ps) agent, light source and oxygen to produce Reactive Oxygen Species (ROS)[5-6]. Several studies exemplified a successful PDI process to reduce bacteria such as PDI to reduce gram-negative bacteria[4], PDI using chlorophyll on A. actinomycetemcomitans[7], PDI using silver nanoparticles to reduce C. albicans biofilm[8], and non-surgical treatment with PDI[9].

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Photosensitizer agent is a light absorbent agent. Nowadays, there are many kinds of introducing photosensitizer on PDI regime. Endogen photosensitizer (Ps) is a natural photosensitizer which being produced itself by the bacteria called as endogenous porphyrins. Exogenous Ps such as chlorophyll, methylene blue, and curcuma longa extract, is an additional Ps from an outside system to accelerate the absorbance light energy level. Several studies showed the additional Ps could increase the bacteria reduction[10]. The successful PDI treatment also determines by the suitability of the light wave spectrum due to related with light absorbance mechanism. Organic Ps are kind of Ps which extracted from plants. The advantage of organic Ps is well known to be nontoxic material like curcuma longa extract. Curcuma longa (CL) has several benefits such as anti-inflammatory to overcome pain and antibacterial agent to kill bacteria. The effect of CL concentrations and laser energy density in the PDI process need to be investigated further[10].
Based on the principle of a Photodynamic reaction[5], the three main components in PDI are light source (LS), photosensitizer (Ps), and oxygen to produce Reactive Oxygen Sipecies (ROS). ROS is reactive products which would inactivate certain objects[6]. Several studies have shown that PDI therapy that the use of LS and Ps can inactivate bacteria[10]. Photodynamic therapy in the area of the skin has been reported to reduce several bacteria, namely S. aureus, S. epidermis, and P. acnes[11].

Besides being able to inactivate the bacteria, PDI also require the addition of Ps. Some effective Ps for this therapy are porphyrin, chlorophyll, malachite green, methylene blue, and curcumin[12]. The choice between LS and Ps must be equated first to the wavelength so that a Photodynamic reaction occurs[13]. PDI has been reported that by using LS in the form of blue laser wavelength of 405 nm and Ps in the form of chlorophyll can reduce S. mutant bacteria by 78% [14]. Previous study with low dose of 0.1% doxycycline activated by LS 409 nm can inactivate A. actinomycetemcomitans bacteria 88.79% nothing differences with only LS treatment 88.50%[15].

One important part of PDI is the photosensitizer. Various studies have discussed the new Ps for PDT, which was curcumin, one of the active compounds in CL. This compound has many functions such as antimicrobial, anti-cancer, anti-inflammatory, etc[12]. Curcumin from the turmeric (C. longa) extract has been used for an inflammation and the result was effective[16]. Teow et al. (2016) explained that curcumin was effective as an anti-bacterial agent of S. aureus[17]. It has been reported by Pandit et al. (2015) that curcumin nanoparticle can effectively break down E. coli, S. aureus and P. aeruginosa in vitro and has been used for the treatment of the other diseases[18].

This study aims for further investigation of the PDI mechanism on S. aureus activated by diode laser. In the other studies showed that laser irradiation also has antimicrobial effects[19]. Therefore, this study will discuss photo inactivation therapy using LS in the form of a blue laser for the inactivation of S. aureus, with the exposure times used to refer to previous studies, namely 30s, 60s, 90s, 120s, 150s and 180s [20]. The addition of Ps in the form of CL extract was combined at concentrations of 0.15% and 0.3%[21]. This study aimed to determine the optimum energy density and effect on each treatment that can inactivate S. aureus.

Materials and methods

Extraction of Curcuma longa

Rhizomes of Curcuma longa was obtained from Singosari, Malang, East Java, Indonesia in September 2018. Specimens were identified by the Indonesian Institute of Sciences, Plant Conservation Center of Purwodadi Botanical Garden with registration number 1562/IPH.06/ HM/X/2018. The rhizome was peeled, washed and cut into pieces, then dried at room temperature 36^{o}C for 4 days. The dried rhizome was pureed to powder and stored in a closed place. Extract CL was obtained from maceration of plant material (100 g) using ethanol 96% (C_2H_6O) (plants: solvent, 1: 10, w/v), for 3 x 24 hours[22]. Extract CL was added with maltodextrin DE-10, then evaporated using a rotary evaporator. The results of CL extract were used for two concentrations of 0.15% and 0.30%.

Determination of Curcuminoid levels in CL extract

Preparation of standard curcuminoid from curcumin <95 (Sigma Aldrich) which was dissolved with ethanol p.a which functions as a solution with 5 main concentrations (65, 80, 95, 110, and 130 ppm). The 1 ml liquid extract was

dissolved in ethanol p.a, then the sample was applied using a 5µl aluminum 60F254 TLC plate (20 x 10 cm; Merck, Germany). The optimized mobile phase used chloroform eluent: methanol: glacial acetic acid (94: 5: 1). The scanned plate used CAMAG TLC Scanner with wavelength 350 nm, then counted specificity covers purity. Measurement of levels was determined by a densitometer, which performed a regression calculation between standards and samples.

S. aureus Culture

The strain used in this study was S. aureus ATCC 25923 obtained from the Surabaya Center for Health Laboratory. Bacterial colonies were grown for 24 hours on Tryptone Soy Agar (TSA) media and incubated at 37 °C. The bacteria were cultured using Tryptone Soy Broth (TSB) media. The bacteria are diluted to six times dilution by adding physiological water to each dilution. To determine the optical density (OD) value of bacteria from 0.2 to 0.5 using a wavelength of 595 nm an ELISA reader was used[23].

Light Source for In Vitro Treatment

Laser diode was used with wavelength λ = 403 ± 22.34 nm, output power 26.83 ± 0.01 mW and laser beam spot 0.25 ± 0.05 cm^2 at 2 cm of irradiation distance. Variation in exposure time were 30s, 60s 90s 120s, and 150s.

Energy density is obtained by the following equation:

$$\text{density} = \frac{\text{output power (mW)}}{\text{Laser beam spot (cm}^2\text{)}} \text{ x time exposure (s)}$$

Sample Treatments

Curcuma longa extract was prepared by a maceration process of turmeric rhizome using 96% ethanol with concentrations of 0.15 % (Ps_1) and 0.3% (Ps_2). The samples were divided into three groups; Group C_1 as a control group without treatment, Group C_2 is a control group with Ps_1 and $C_{2'}$ is a control group with Ps_2, Group T_1 is PDI treatment with laser, Group T_2 is PDI treatment with Ps_1, and Group T_3 is PDI treatment with Ps_1. The bacterial growth was carried by ELISA, while the rate of bacterial reduction was measured by Colony Counter (CFU/ml). All the treatments were conducted in a dark room or inside Laminar Air Flow (LAF). The percentage of bacterial reduction was calculated by following equation.

$$\text{CFU/ml} = \frac{(\sum \text{colony x} \frac{1}{\text{f dilution}})}{\text{V.plate}}$$

$$\% \text{ Bacterial Reduction} = \left|\frac{\text{CFU/ml}_{\text{control}} - \text{CFU/ml}_{\text{treatment}}}{\text{CFU/ml}_{\text{control}}}\right| \text{x100\%}$$

Statistical Analysis

The obtained results were analyzed using one-way ANOVA to significantly determine the differences between all treatments exceeding 95% (p-value <0.05%).

Results

Light source characterization

The blue laser used for this study has a spot area (0.25 ± 0.05) cm^2 at a distance of 3.5 cm from the light source. The peak wavelength is known through measurements using the Jasco monochromator CT-10 at (403 ± 0.24) nm and power (26.38 ± 0.01) mW. The temperature characterization results using a stable digital multimeter at (32.04 ± 0.02) ^oC. This temperature is in the range of the S. aureus growth temperature. Meeting energy depends on the intensity(I) in formula I = P / A, this blue laser exposure has an intensity range between 10^{-3} - 1 W/cm^2 and the duration of exposure pulses > 1 s can be assumed that the interaction that occurs during the irradiation process is a photochemical reaction[6]. The parameter of sample treatment show in Table 1.

Sample Treatments	Extract CL		Laser Diode	
	Volume (µL)	Concentration (%)	Time (s)	Energy Density (J / cm ²)
Laser Irradiation	50	0.15	30	3,17
	50	0.15	60	6,33
	50	0.15	90	9,50
	50	0.15	120	12,67
	50	0.15	150	15,83
	50	0.3	30	3,17
	50	0.3	60	6,33
	50	0.3	90	9,50
	50	0.3	120	12,67
	50	0.3	150	15,83

Table 1. The parameter of sample treatment.

CL extract composition

CL extract has many active compounds, one of the active compounds used in PDI is Curcuminoid[2-4]. Specificity of peak purity and peak identity data obtained as shown in Table 2 shows that a Curcuma longa extract has a purity, value of > 0.9500, so it is stated that peak of curcuminoid in a pure Curcuma longa extract is not contaminated.

Track	Rf	Assigned Substance	Max. Signal	Display	r (s, m)	r (m, e)	Purity
1	0,82	SC	480 AU @ 419 nm		0,999622	0,995323	ok
2	0,80	SC	513 AU @ 418 nm		0,999450	0,998293	ok
3	0,77	SC	557 AU @ 417 nm		0,999487	0,998446	ok
4	0,76	SC	598 AU @ 417 nm		0,999493	0,998865	ok
5	0,76	CL	408 AU @ 419 nm		0,999595	0,997198	ok
6	0,79	CL	483 AU @ 419 nm		0,999104	0,997969	ok
7	0,80	CL	453 AU @ 419 nm		0,999559	0,996498	ok
8	0,81	CL	480 AU @ 418 nm		0,999335	0,997667	ok

Table 2. Peak purity of Standard Curcuminoids (SC) from Sigma Aldrich and Curcuminoids in the CL extract sample.
*SC = standard curcuminoid, CL = turmeric extract

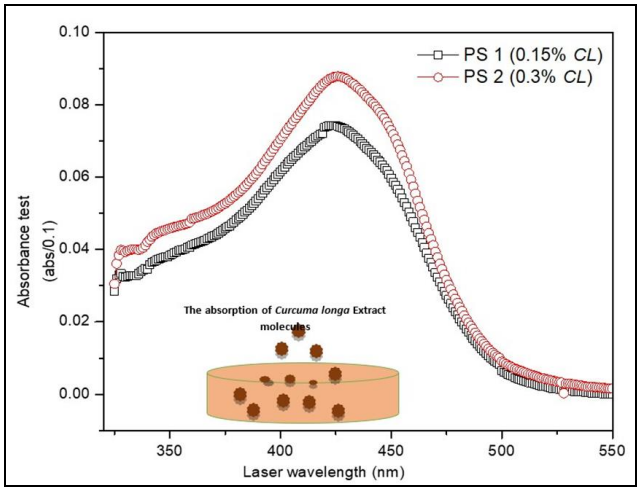

Figure 1. The characterization of the CL extract from the absorbent test.

Determination of Curcuminoid content in CL extract obtained Ps1 at 2.59% w/w and Ps2 as much as 2.96% w/w. CL extract in this study functions as photosensitizer. The antibacterial activity of CL extract was dose-dependent, so the mixing process held after CL extract ranged from 3.279 to 1.699 (log CFU/ml). In addition, antibacterial tests were also carried out in ways to see inhibitory zones.

The antibacterial test showed nothing an inhibitory zone. This showed that CL extract had antibacterial activity. The test result proved that CL extract has a wide spectrum of absorbance and a low toxicity. It could be concluded that this extract was safe to use[24-25]. CL extract has been carried out in the absorption spectrum test of CL 0.15% and 0.3% extract using Spectrophotometer Genesys 30. The absorbency test of CL 0.15% extract is in λmax (424.00 ± 0.05 - 426.00 ± 0, 05) nm with absorbance (0.742 ± 0.01). While the extract of CL 0.3% λ_{max} (426.00 ± 0.05) nm with absorbance (0.878 ± 0.01). The difference in absorbance value is shown in Figure 1 between 0.15% CL and 0.3% CL. The wavelength used as is (403.00 ± 0.05) nm with the percentage of photons absorbed by the extract of CL concentration of 0.15% by 47% and extract of CL concentration of 0.3% by 81%.

PDI treatments

There are two types of treatment factors, namely the first treatment factor consists of the laser treatment group, the CL 0.15% extract treatment group which is activated by laser, and the treatment group CL 0.3% extract which is activated by laser. The first treatment factor did not show a significant difference p = 0.00 (p-value<0.05). The second treatment factor is a variation of 30 seconds, 60 seconds, 90 seconds, 120 seconds, and 150 seconds. This second treatment factor also did not show a significant difference p = 0.00 (p-value<0.05). The most potential treatment is to increase the value of bacterial colonies in log (CFU/ml). Figure 2 shows that there are significant differences between controls and treated. It showed that the controls, both C1, C2, and C2`, have linear values in each treatment time.

The results between log (CFU/ml) and time of exposure, showed that 150 seconds was the most optimum time to decrease the value of S. aureus in log (CFU/ml). The first treatment group was 8.31 ± 0.15 log (CFU/ml), treatment 2 was 7.12 ± 0.14 log (CFU/ml), and treatment 3 was 6.17 ± 0.13 log (CFU/ml).

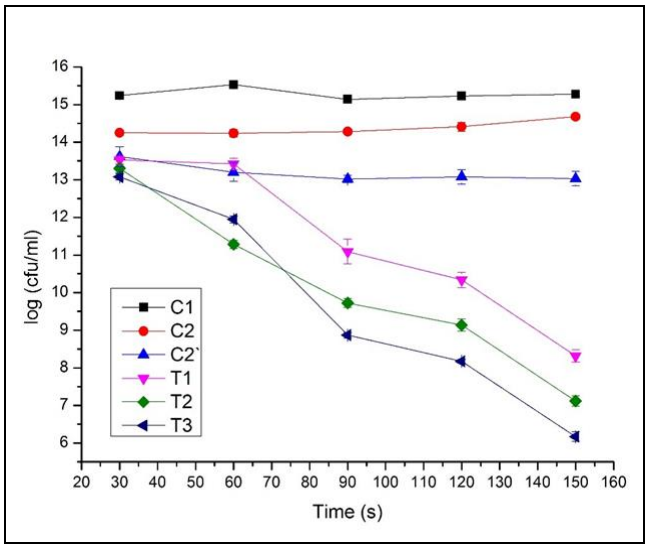

Figure 2. The S. aureus colony in log (CFU/ml) over the time of exposure.

Figure 3 showed that the bacteria reduction has decreased significantly with the longer exposure time. The most optimum

exposure time to reduce the percentage of S. aureus bacteria deaths was 150 seconds. The results explain optimum energy density of 15.83 J/cm^2 could reduce the percentage of S. aureus reduction by (79.18 ± 1.79) % when using CL extract 0.15 %, (T2). CL extract 0.3 % (T1) had the percentage of S. aureus reduction by (85.48 ± 1.79) % with the same energy density. The greater the density of energy density used, the higher the percentage of S. aureus bacteria reduction. The addition of photosensitizer in the form of CL extract could increase the percentage value of S. aureus reduction.

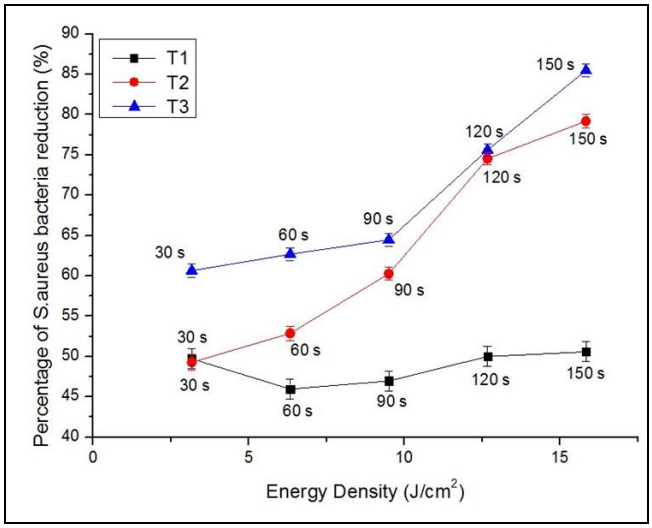

Figure 3. The bacterial reduction of S. aureus towards energy density and time of exposure.

Photodynamic effect depend on the wavelength between light sources and the length of photosensitizer absorption must be appropriate, so that photophysical and photochemical interactions occur. The uptake of photons in CL extract concentration of 0.15% was 47% and extract of CL concentrated 0.3% from 81%. The sample with 0.3% CL extract showed high S. aureus bacterial reduction of (85.48 ± 1.79) % or 8.681 log CFU/ml. Paulaucci reported that the optimization of CL extract with a concentration of 3.4% is more optimal when compared with a concentration of 1.8%. This has the provision of the value of absorptions in each concentration between 0.1 and 0.8 (abs) [22].

Discussion

From Table 2, Rf is the distance between the paths of the solvent. The greater the value of the Rf from the sample, the greater the moving distance of the compound on the thin layer chromatography plate. Assigned substance, naming curcuminoid levels in CL extract. The r (s, m) and r (m, e) are purity values of the standard curcuminoid (SC) and CL, from these values it was known that the value of the CL extract sample had a purity value > 0.999. The value of peak curcuminoid in pure CL was not contaminated with the other compounds. The basic difference was actually from the comparison between the standard curcuminoid and the turmeric extract sample. If the purity value of the sample is almost the same or has entered the standard sigma Aldrich curcuminoid track, the purity value of the curcumin content in the extract is correct.

CL extract could produce single oxygen when illuminated by a light source with a wavelength above 400 nm. This is in accordance with this study using laser wavelength 403 ± 0.05 nm as a light source. Singlet oxygen results from photochemistry can damage biological systems, Intersystem crossing is excitation from different circumstances, and Excitation is needed in the Photodynamic process. CL extract that absorbs energy from the light source will go to a singlet state (^{1}P*), then through intersystem crossing the molecule will move to a triplet state (^{3}P *). In the Photodynamic process, molecular level chemical reactions are produced through the mechanism of type I and type II. The mechanism of type I occurs when ^{3}P * interacts with H_2O in tissues (bacterial molecules) or biological systems. When this interaction arises, there is a transfer of protons and electrons to form radical anions and cations. These radical cations that interact with oxygen produce superoxide anions (O_2^-). Hydrogen peroxide (H_2O_2) is formed when O_2^- reacts with biological molecules. In high concentrations, H_2O_2 forms radical hydroxyl if it reacts with O_2^- as well as metal or iron ions. Radical hydroxyl is very easy to diffuse through the membrane and damage cells so that microorganisms die. In type II, ^{3}P * energy directly into oxygen molecules and produces excited oxygen ($^1O_2$ *). The molecules at the triplet excitation level have a lifetime and high energy, so the energy is transferred to oxygen molecules, consequently the oxygen molecules are excited from a stable state to a very reactive level (singlet) and cause oxygen to become cytotoxic. Curcumin can turn into a radical

compound when undergoing electron transfer or being excited. In accordance with the process in type II, curcumin compounds that turn into radicals have reactive oxygen, which can damage the cell membrane of Staphylococcus aureus bacteria.

The spectrum of light sources and energy density are factors that can influence PDI results. The addition of CL as Ps and the use of energy density of blue light 24, 48, 72 J/cm^2 can reduce S. mutant[20]. In vitro studies on A. Actinomycetemcomitans mentioned that the optimal CL concentration was 0.78 µg/ml with a light energy density at 61.8 J/cm^2 for 5 minutes[24-26]. Gram-positive bacteria such as S. aureus are very helpful in the PDI process compared to gram-negative bacteria. Cell walls that are porous and do not have a solid protein layer make it easy for Ps to penetrate[8]. Mun et al (2014) showed that phosphatidylethanolamine, the main component of gram negative bacterial cell walls, was much more easily penetrated[2].
The results of previous studies indicate that the effectiveness of PDI is influenced by the wavelength compatibility of the light source used with the absorption spectrum of photosensitizing agents[27].

Low Level Laser Therapy (LLLT) therapy for the treatment of chronic periodontitis (CP) has been carried out. Treatment with LLLT 635 nm results in a reduction in periodontal inflammation in CP patients based on the reduced levels of IL-1α and IL-1β in GCF[28]. The results of non surgical periodontal therapy combined with LLLT 660 nm in patients with chronic periodontitis with Iron Deficiency Anemia (IDA) show improved scaling of periodontal pockets[29]. Sulijaya et al's results in patients with severe periodontitis using the ND-YAG laser produced a significant increase in bone density and bone grains and reduced tooth mobility[30]. The results of other studies indicate that the 810 nm diode laser does not have a significant effect on the removal of the smear layer on the root canal dentin[31].

Conclusions

Curcuma longa (CL) extracts could increase the effectiveness of antimicrobials and can be effectively used as a photosensitizer (Ps) when exposed to a blue laser. The results showed that exposure with laser wavelength adjustment around 403 nm of blue laser and energy density of 15.83 J/cm^2 at 150 seconds using a 0.3% Ps concentration could reduce the percentage of S. aureus bacteria by 85.48%. Thus, the CL extract can increase the effectiveness as a Ps when there is an addition in the form of a blue laser.

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Declaration of Interest

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