Natural Dyes as Photosensitizers of *Propionibacterium acnes*

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ABSTRACT
The patient's quality of life may be negatively impacted by the high prevalence of acne vulgaris among adolescents. Acne vulgaris is a skin condition in which hair follicles become clogged with dead skin cells, bacteria, and natural facial oils. It has been demonstrated that acne antibiotics raise antibacterial resistance. According to this study, natural dyes could treat *Propionibacterium acnes*-caused acne vulgaris. β-carotene, riboflavin, and curcumin mediated the activity of PDT against *Propionibacterium acnes* in vitro. Various types of light (red light λ640 nm, blue light λ423 nm, and green light λ532 nm), irradiation times of 10, 30, and 60 minutes and dye concentrations of 0.005, 0.010, and 0.015% were tested. An ELISA reader 595 nm measured the cell viability. *Propionibacterium acnes* was not inhibited by dyes alone. PDT activity was observed after being treated with the appropriate light and natural dye combination. Blue light inhibited *Propionibacterium acnes* growth more effectively than green light (41.9 vs. 14.0%). Red light, on the other hand, has no effect. With 10-30 min PDT treatment of β-carotene, riboflavin, and curcumin, bacterial survival was reduced by 41.9, 28.60, and 12.51%. Due to their cost and antibacterial effect, a concentration of 0.10% dyes was suggested for further use. Antibacterial PDT containing curcumin, riboflavin, or β-carotene may be an alternative treatment for acne vulgaris.

Keywords: *Photosensitizers, PDT, Natural dyes, Propionibacterium acnes, Acne vulgaris.*

1. INTRODUCTION

Skin disease was often considered harmless because of their low mortality rate. However, the disease can affect a person's quality of life. Acne vulgaris was a skin disease with a high prevalence in adolescents. It was ranks fourth (5.99%) by incidence of skin diseases encountered in hospital outpatients in Bangladesh [1].

Aside from physical effects such as permanent scarring and disfigurement, acne has long-term psychosocial effects on patient's quality of life. Depression, social isolation, and suicidal ideation were frequent acne co-morbidities that should not be ignored in the therapy of acne patients [2].

Acne vulgaris was activated by Propionibacterium acnes which causes inflammation of the pilosebaceous follicles [3][4]. Bacterial infections generally treated with antibiotics can cause drug resistance if given continuously and for a long time. Therefore, there is a need for alternative therapies that are more efficient. One therapy method that can be used was PDT (Photodynamic Therapy)[3].

PDT was initially used clinically for photochemotherapy due to the accumulation of photosensitizers in target cells. Furthermore, irradiation was carried out using light at specific wavelengths, encouraging the formation of reactive oxygen species (ROS) and cell death. PDT was used in skin diseases, including acne vulgaris, leishmaniasis, and skin aging [6]. Endogenous photosensitizers such as protoporphyrin IX have been used for this purpose after administration of 5-methyl aminolevulinic acid (MAL) or 5-aminolevulinic acid (ALA) precursors [7]. Chlorophyll compounds are one of the natural sensitizers used for

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antimicrobial PDT. The development of non-toxic photosensitizers derived from natural dyes has the advantage of being more economical [8].

The application of PDT using natural dyes such as riboflavin, curcumin, and β-carotene as photosensitizers to treat acne vulgaris was limited. Therefore, this study aims to examine these natural dyes' potential for treating skin diseases caused by the bacterium *Propionibacterium acne*.

2. MATERIAL AND METHODS

2.1. Light Source and Intensity

Two PDT lights (PDT Omega Light Therapy LED/red light λ640 nm, blue light λ423 nm, and green light λ532 nm) were adopted for irradiation. The power of light was measured using a lux meter (Krisbow KW06-291). The distance to the PDT LED light was 27 cm.

2.2. Photosensitizer

Riboflavin, β-carotene, and curcumin (Sigma) were prepared by dissolving dyes in DMSO and diluting them to a concentration of 1.5%. The solution was then diluted with distilled water to obtain a solution with a concentration of 0.015; 0.010; and 0.005% respectively. Dissolving is assisted by ultrasonication and a vortex mixer.

2.3. Preparation of Propionibacterium acnes

We used Mueller Hinton Agar (MHA/Himedia) for cultivation of *Propionibacterium acnes* (ATCC 11827), incubated for 18 hours at 37ºC. The bacteria were then cultured in Mueller Hinton Broth (MHB/Himedia) and incubated for 18 hours at 37ºC. *Propionibacterium acnes* used during the mid-log growth phase at a concentration of 1.5 x 10^8 CFU/ml. The optical density of the bacteria at 600 nm was measured using a UV-Vis spectrophotometer (Shimadzu 1240).

2.4. PDT Treatment

2.4.1. Effect of Light source on Bacteria Survival

Bacterial cell suspension 180 µL was distributed into a 96-well plate, then 20 µL dye (0.015%) was added. In addition, 180 µL of media and 20 µL of dye were added to the other wells. The control treatment was carried out with irradiation in the absence of natural dyes (control media, control cell, and control solvent). The plates were incubated at 37ºC and the absorbance was measured with an ELISA reader (Bio-Rad) at 595 nm. The plate was placed under a PDT light. The irradiation was carried out for 30 minutes. The temperature was maintained at room temperature. PDT activity was carried out at several types of LED, red light (λ640 nm), blue light (λ423 nm), and green light (λ532 nm). Absorbance was read with ELISA reader 595 nm. All of the experiments were evaluated in triplicate.

2.4.2. Effect of Time Irradiation on Bacteria Survival

Bacterial cell suspension 180 µL was distributed into the wells of 96-well plates, then 20 µL dye (0.015%) was added. To assessed the time irradiation effect in antibacterial PDT, the light source was fixed at the best wavelength evaluated in the previous stages and the concentration was fixed at 0.015%. The PDT treatments were evaluated at various illumination times (0, 10, 30, 60 min).

2.4.3. Effect of Photosensitizers Concentration on Bacteria survival

For detecting the concentration effect in PDT antibacterial using natural dyes, the best result in previous step was chosen as a fixed condition. The PDT treatments were conducted at various concentrations of dyes (0.005, 0.010, and 0.015%).

2.5. Dark Toxicity

Dark toxicity was carried with photosensitizers in the dark (without irradiation). The cell viability was observed using ELISA reader at 595 nm.

3. RESULTS

β-carotene, riboflavin, and curcumin had low solubility in distilled water. Therefore, DMSO was used as an alternative solvent in this experiment. Unfortunately, previous studies have shown that DMSO inhibits microbes. This inhibitory effect was avoided by a 10-fold dilution of DMSO [4][5]. Therefore, to minimize the effect of DMSO inhibition on bacterial growth, DMSO was diluted with sterile distilled water to give a final concentration of <1% on each plate.

Antibacterial effects were observed using the microdilution technique [6]. Growth was indicated by diffuse turbidity monitored with a spectrophotometer. The percentage of cell viability was used as a parameter to compare the efficacy of the tested photosensitizers. The 100% viability value is given to the negative control (untreated cells). Sample viability values were determined as a percentage of this value. Suspicion of interference in the viability test was confirmed by measuring absorbance independent of medium, solvent, and dyes.

After treatment with a combination of irradiation and natural dye exposure, the viability of *Propionibacterium acnes* decreased. PDT antibacterials using β-carotene,

riboflavin, or curcumin could be a potential alternative treatment for acne vulgaris. We observed a higher effect of β-carotene to inhibit *Propionibacterium acnes* followed by riboflavin and curcumin (41.9 vs. 33.8, and 20.3%, p=0.000<0.005) using a dye concentration of 0.015% combined with irradiation of blue light (2270 lux) for 30 minutes (Figure 1).

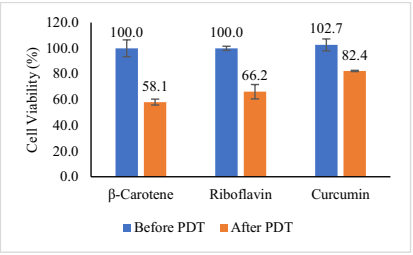

Figure 1 Bactericidal effect of β-carotene, riboflavin, and curcumin-mediated PDT against *Propionibacterium acnes*. The concentration of photosensitizers was 0.015% with blue light intensity of 2270 lux exposed for 30 min.

3.1. Effect of Light source on Bacteria Survival

The irradiation was carried out using three types of light (blue, green, and red) out of the four types found in the PDT Omega Light Therapy LED light source (red, blue, green, and yellow light). The light source has been used in several beauty clinics in Indonesia to treat different skin problems. The power outputs of blue, green, and red lights were 2270, 1388, and 759 lux, respectively.

Experiments with β-carotene confirmed that blue light irradiation caused the formation of ROS, which inhibited the growth of *Propionibacterium acnes* (Figure 2). Blue light inhibited *Propionibacterium acnes* growth more than green light (41.9 vs. 14.0%). Conversely, red light has no effect. *Propionibacterium acnes* grows well even when β-carotene exposed to the red light. Red light fails to convert β-carotene to the triplet state, which generates ROS and inhibits *Propionibacterium acnes* growth.

As well as β-carotene, curcumin and riboflavin showed PDT activity after exposure to blue light (Figure 3-4). A photosensitizer's ability to generate ROS depends on its absorption spectrum. The higher molar extinction coefficient at the specific wavelength will produce a better PDT effect [7]. In contrast to anticancer photosensitizers, antibacterial photosensitizers for topical use do not prioritize the properties of long wavelength absorption bands to promote good penetration properties. Therefore, natural dyes were potentially photosensitizers for antibacterial PDT [8]. Various mechanisms of action have been discovered in the binding of photosensitizers to bacteria, which are mainly determined by the molecular structure of the photosensitizer. The photosensitizer can bind to the bacterial cell membrane, enter the bacterial cell, or interact without direct contact (if the $^1O_2$ generated was highly efficient) [7].

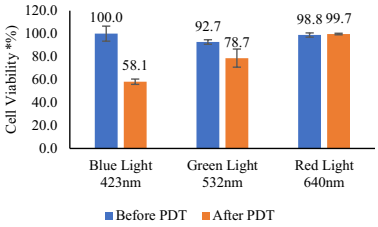

Figure 2 The Effect of various types of light exposed for 30 min on the survival of *Propionibacterium acnes*. β-carotene 0.015% was used as a photosensitizer.

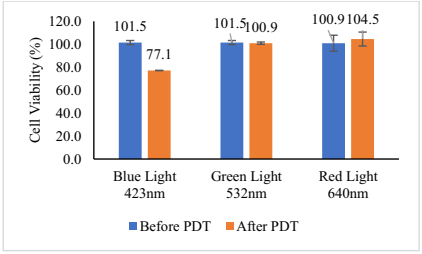

Figure 3 The Effect of various types of light exposed for 30 min on the survival of *Propionibacterium acnes*. Riboflavin 0.015% was used as a photosensitizer.

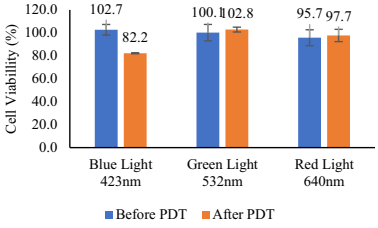

Figure 4 The Effect of various types of light exposed for 30 min on the survival of *Propionibacterium acnes*. Curcumin 0.015% was used as a photosensitizer.

3.2 Effect of Irradiation Time on Bacteria Survival

Figure 5 shows the relationship between blue light irradiation time and bacterial viability. The viability of

Propionibacterium acnes decreases with increasing irradiation time. In this experiment, we used blue light, which has a high PDT effect. Bacteria survival was reduced by 41.9, 28.60, and 12.51% with 10-30 min PDT treatment of β-carotene, riboflavin, and curcumin, respectively. Furthermore, longer illumination times (30–60 min) decreased the viability of cells using beta-carotene, curcumin, and riboflavin as photosensitizers by 16.93, 9.30, and 6.85%, respectively. Irradiation times of 10 to 30 minutes showed significant inactivation of cells compared to longer irradiation times (30 to 60 minutes). With the same exposure time, light type, and photosensitizer concentration, β-carotene could more effectively suppress *Propionibacterium acnes* growth.

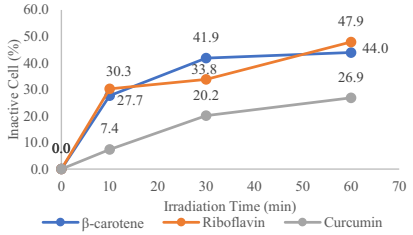

Figure 5 The Effect of Irradiation time on the survival of *Propionibacterium acnes*. The concentration of photosensitizers was 0.015% with blue light intensity of 2270 lux.

3.3 Effect of Photosensitizers Concentration on Bacteria survival

An increase in inactive cell trend was shown with increasing dye concentrations (Figure 6). We used blue light with an irradiation time of 60 minutes for this experiment. *Propionibacterium acnes* survival was reduced by 47.9, 42.7, and 39% with PDT treatment of riboflavin, β-carotene, and curcumin, respectively. Since 0.05% did not demonstrate a significant difference in activity, 0.10% dyes were recommended for further use due to their cost and antibacterial effect.

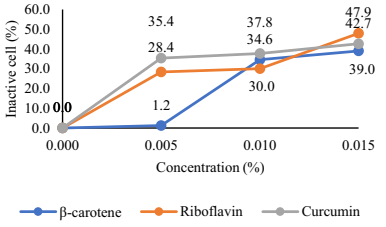

Figure 6 The effect of photosensitizers concentration on the survival of *Propionibacterium acnes*. The irradiation time was 60 min with a blue light intensity of 2270 lux.

Our findings were consistent with those of other researchers evaluating antimicrobial effects on curcumin [9]–[23]. The effect of PDT on riboflavin as an antimicrobial has been the subject of several studies [24]–[27]. However, studies focusing on the antibacterial PDT beta-carotene were limited. Among these publications, only one publication evaluated the use of riboflavin in acne vulgaris[26]. Riboflavin was able to reduce a small number of bacteria compared to using toluidine blue O as a photosensitizer.

3.4 Dark Toxicity

Curcumin, riboflavin, or beta carotene were ineffective against P acnes without exposure to irradiation (Figure 7). Dye alone at 0.015% concentration did not reduce the number of viable P acnes. Even when the solution was incubated for 30 minutes in the dark, the bacteria continued to grow.

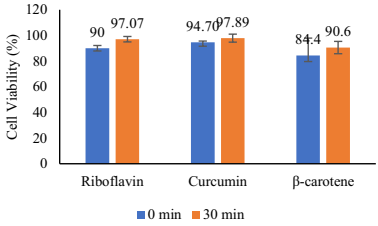

Figure 7 Percentage of cell viability of *P. acnes* grown without exposure to irradiation.

This study supports PDT as an alternative treatment for acne, especially in patients unresponsive to topical or oral antibiotic therapy. Furthermore, in vivo evaluation is required to confirm the clinical relevance of these results for better clinical application.

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