








# Comparing the Antibacterial Effects of 660 nm Laser Therapy on *Staphylococcus aureus* Using Phycocyanin and Methylene Blue: An In-Vitro Study

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## Abstract

**Background:** *Staphylococcus aureus* is a leading cause of oral and nasopharyngeal infections, with antibiotic-resistant strains posing significant health challenges. Photodynamic therapy (PDT) is a recommended alternative antimicrobial treatment.

**Objectives:** This study aimed to evaluate and compare the antibacterial effects of 660 nm laser irradiation in combination with phycocyanin and methylene blue (MB) on *S. aureus* colonies.

**Methods:** In this in vitro study, the effects of 660 nm laser irradiation (100 mW for 100 s) were analyzed on *S. aureus* colonies using varying concentrations of phycocyanin (0.00001 to 0.005 mg/mL) and MB (0.00001 to 0.02 mg/mL). Data were analyzed using the two-way random block design test.

**Results:** The results demonstrated that laser irradiation combined with MB at concentrations of 0.005 and 0.002 mg/mL significantly reduced the number of *S. aureus* colonies compared to other treatments (P-value < 0.01). Methylene blue without PDT was effective in destroying all colonies at concentrations  $\geq 0.01$  mg/mL. Laser irradiation combined with MB at concentrations of 0.001, 0.0005, and 0.0002 mg/mL, or with phycocyanin at concentrations of 0.02, 0.01, and 0.005 mg/mL, reduced the number of colonies. However, laser irradiation alone, laser combined with phycocyanin at concentrations  $\leq 0.002$  mg/mL, or laser combined with MB at concentrations  $\leq 0.0001$  mg/mL, did not significantly reduce colony numbers (P-value > 0.05).

**Conclusions:** This study demonstrated that MB combined with laser irradiation was more effective in eliminating *S. aureus*. The findings suggest that adding photosensitizing agents enhances the antibacterial effects of laser irradiation.

**Keywords:** *Staphylococcus aureus*, Photodynamic Therapy, Photosensitive Substance, Phycocyanin, Methylene Blue

## 1. Background

*Staphylococcus aureus* is a highly hazardous bacterium. Although it is part of the human body's normal microflora, it has the potential to cause various infections, ranging from minor skin conditions to severe diseases such as osteomyelitis (1). The increasing prevalence of antibiotic-resistant bacteria, including community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), presents a significant global health threat (2-4).

The overuse and misuse of antibiotics have created a vicious cycle, whereby bacterial strains develop resistance, necessitating the use of stronger or additional antibiotics, which in turn accelerates the evolution of resistance (3-5). This defective cycle of antibiotic resistance has led to the emergence of increasingly virulent and difficult-to-treat strains of *S. aureus*, further complicating public health efforts. The critical nature of these infections has resulted in a rise in the use of anti-staphylococcal antibiotics, which exacerbates the resistance problem.

Addressing antibiotic-resistant *S. aureus* remains a significant health challenge. Recognizing this urgency, the World Health Organization (WHO) has designated *S. aureus* as a top priority for new drug development. This underscores the pressing need for innovative treatments, such as photodynamic therapy (PDT), to combat this growing threat (1, 3-5).

Photodynamic therapy is one of the recommended antimicrobial treatments (5-10). While PDT has primarily been utilized as a cancer treatment due to its effective antitumor properties, its potential as an antimicrobial therapy has received comparatively less attention (5, 6, 9, 10). In PDT, photosensitizer molecules play a crucial role in inducing targeted cell damage through photo-oxidative stress. These molecules absorb visible light of a specific wavelength and transfer the energy to molecular oxygen, generating reactive oxygen species (ROS). The ROS then induce photo-oxidative stress on organic molecules such as lipids and proteins in the bacterial envelope, ultimately leading to non-specific bacterial death with no possibility of resistance development (5, 6, 10). As the effectiveness of PDT is heavily dependent on the chemical structure of the photosensitizer molecules, empirical testing of both established and new formulations is essential. This study seeks to explore the effects of PDT on *Staphylococcus aureus* using two photosensitizer molecules: Phycocyanin and methylene blue. Phycocyanin is a blue pigment commonly employed in the food industry to enhance product aesthetics. Methylene blue (MB), initially developed as a textile dye, is also used as a surgical stain. Both components possess effective antioxidant properties and demonstrate potential as photosensitizers in PDT applications (11-13). However, no prior research has adequately compared the efficacy of these two mediators.

## 2. Objectives

This study aimed to compare the antibacterial effects of 660 nm laser therapy with phycocyanin and methylene blue on *S. aureus* colonies.

## 3. Methods

This study was an experimental in-vitro investigation. Figure 1 illustrates a schematic representation of the study methodology.

### 3.1. Sample Size

The articles by Gholibegloo (14) and Afrasiabi (13) reported colony reduction rates ranging from 18% to 91%. To detect changes in colony numbers from 1% to 11%,

with an average reduction of 11%, a power of 81%, and an alpha error of 1% (to minimize the likelihood of a Type I error, ensuring that any statistically significant results are robust and less likely to occur by random chance), three samples were required for each subgroup.

The present study analyzed a total of seven main groups, four of which contained nine subgroups each. The remaining three groups had only one subgroup each. For each subgroup, the examinations were repeated three times, resulting in a total of 117 samples.

### 3.2. *Staphylococcus aureus* Microorganisms' Culture

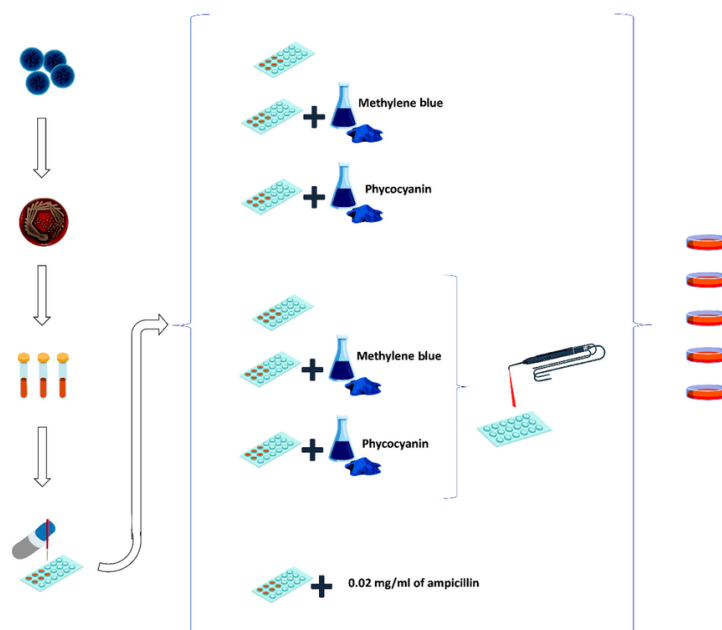
Lyophilized powder of *S. aureus* strain (ATCC433000, Pasteur Institute, Tehran, Iran) was reconstituted in nutrient broth (Catalog No. 105433, Merck, Germany) and incubated at 37 degrees Celsius (°C) for 24 hours. Following this, the culture was transferred to a blood agar medium (Catalog No. 110886, Merck, Germany) and incubated at 37°C for another 24 hours. Once the purity of the culture was confirmed, a suspension was prepared at a dilution of 0.5 McFarland ( $1.5 \times 10^8$  colony-forming units/milliliter) (15).

### 3.3. Eligibility Criteria

Only *S. aureus* strains obtained from a certified source (ATCC433000 strain from the Pasteur Institute, Tehran, Iran) were used in this study. Samples with an optical density matching the 0.5 McFarland standard ( $1.5 \times 10^8$  CFU/mL) were included to ensure consistent and comparable bacterial loads across all experimental groups. Samples exhibiting signs of contamination or impurity during the culturing process were excluded. Additionally, bacterial cultures that failed to reach the required 0.5 McFarland standard concentration or demonstrated irregular growth or inconsistency in colony-forming unit (CFU) counts during the pre-experimental phase were excluded from the experiments.

### 3.4. Preparation of Photosensitizer Concentration

A 2 milligrams per milliliter (mg/mL) concentration of phycocyanin (Phoenix Microalgae Co., Iran) and a 0.1 mg/mL concentration of MB (Catalog No. 159270, Merck, Germany) were initially prepared. The phycocyanin concentration was subsequently reduced to 0.005 mg/mL, and the MB concentration was diluted to 0.02 mg/mL using physiological serum. Both solutions were then serially diluted with 10 mL of Dimethyl sulfoxide (DMSO) (Catalog No. 1.02952, Merck, Germany), achieving final concentrations of 0.00001 mg/mL and 0.00005 mg/mL, respectively (16, 17).



**Figure 1.** A schematic of the study methodology

### 3.5. The Examined Groups

In this study, seven main groups, divided into 39 subgroups based on different photosensitizer concentrations, were analyzed. The main groups were as follows:

- (1) *Staphylococcus aureus* in the culture medium alone
- (2) *Staphylococcus aureus* with MB at concentrations of 0.02, 0.01, 0.005, 0.002, 0.001, 0.0005, 0.0002, 0.0001, 0.00005 mg/mL, without laser irradiation
- (3) *Staphylococcus aureus* with phycocyanin at concentrations of 0.005, 0.002, 0.001, 0.0005, 0.0002, 0.0001, 0.00005, 0.00002, 0.00001 mg/mL, without laser irradiation
- (4) *Staphylococcus aureus* with laser irradiation only
- (5) *Staphylococcus aureus* with laser irradiation and phycocyanin at concentrations of 0.005, 0.002, 0.001, 0.0005, 0.0002, 0.0001, 0.00005, 0.00002, 0.00001 mg/mL
- (6) *Staphylococcus aureus* with laser irradiation and MB at concentrations of 0.02, 0.01, 0.005, 0.002, 0.001, 0.0005, 0.0002, 0.0001, 0.00005 mg/mL
- (7) *Staphylococcus aureus* with 0.02 mg/mL of ampicillin (AMP) without laser irradiation (13, 14, 18).

### 3.6. Light Source and the Photodynamic Therapy Procedure

Ten microliters ( $\lambda$ ) of *S. aureus* suspension and 10  $\lambda$  of photosensitizer substances were pipetted into each well of a microplate using a sampler. In the laser-irradiated groups, each well was exposed to a 660-nm wavelength laser (Hamerz Rad Co., Iran) with a power output of 100 milliwatts and a beam diameter of 0.8 cm. The laser was applied continuously to the samples for 100 seconds at a distance of 1 cm. The device's dosimetry was checked for accuracy (13).

The process of dispensing samples into the microplates was conducted within a laminar flow hood to ensure a sterile and dark environment. Additionally, the entire microplate was covered with thick black paper, with only the designated well openings punched out to a diameter of 0.8 cm. To prevent cross-contamination, samples were not placed in consecutive wells but in alternating wells. The minimum inhibitory concentration (MIC) was then determined for the photosensitizing substances (19).

The energy density of each irradiation can be calculated using the formula:

$$E_D = \frac{P \times t}{A}$$

**Table 1.** The Number of *Staphylococcus aureus* Colonies in 39 Examined Subgroups

Groups	Concentration	Numbers of Colonies
<i>Staphylococcus aureus</i> + culture medium	NA	1.5 × 10 <sup>8</sup>
	0.005	12 × 10 <sup>4</sup>
	0.002	84 × 10 <sup>4</sup>
	0.001	44 × 10 <sup>5</sup>
	0.0005	76 × 10 <sup>5</sup>
<i>Staphylococcus aureus</i> + MB (without irradiation)	0.0002	59 × 10 <sup>5</sup>
	0.0001	UC
	0.00005	UC
	0.00002	UC
	0.00001	UC
	0.02	10.8 × 10 <sup>4</sup>
<i>Staphylococcus aureus</i> + phycocyanin (without irradiation)	0.01	42 × 10 <sup>4</sup>
	0.005	26 × 10 <sup>5</sup>
	0.002	UC
	0.001	UC
	0.0005	UC
	0.0002	UC
<i>Staphylococcus aureus</i> + 660 nm laser	NA	0.85 × 10 <sup>8</sup>
	0.02	0.25 × 10 <sup>4</sup>
	0.01	6.7 × 10 <sup>4</sup>
	0.005	88 × 10 <sup>4</sup>
	0.002	UC
<i>Staphylococcus aureus</i> + 660 nm laser + phycocyanin	0.001	UC
	0.0005	UC
	0.0002	UC
	0.0001	UC
	0.00005	UC
	0.005	0
<i>Staphylococcus aureus</i> + 660 nm laser + MB	0.002	0
	0.001	68 × 10 <sup>4</sup>
	0.0005	168 × 10 <sup>4</sup>
	0.0002	146 × 10 <sup>5</sup>
	0.0001	12 × 10 <sup>6</sup>
	0.00005	UC
<i>Staphylococcus aureus</i> + AMP (without irradiation)	0.00002	UC
	0.00001	UC
	NA	0.15 × 10 <sup>8</sup>

Abbreviations: MB, methylene blue; AMP, ampicillin.

In this case, the energy density is equal to:

$$E_D = \frac{0.1 \times 100}{0.5}$$

which is equivalent to 20 J/cm<sup>2</sup> (15, 19).

### 3.7. *Staphylococcus Aureus* Colony Counting

After the intervention, all samples were cultured on blood agar medium and incubated at 37°C for 24 hours (19). Following incubation, the number of bacterial colonies per milliliter (CFU/mL) was counted to assess bacterial growth.

### 3.8. Statistical Analysis

To compare the number of colonies among the study groups, the two-way random block design test was employed after confirming the normality of the data distribution.

## 4. Results

Table 1 presents the number of *S. aureus* colonies in different subgroups.

The results demonstrated that laser irradiation combined with MB at concentrations of 0.005 and 0.002 mg/mL significantly reduced the number of *S. aureus* colonies more effectively than other subgroups (mean reduction = 100%, CI: [95%, 100%]; P-value < 0.01). This combination completely eradicated all *S. aureus* microorganisms, reducing the colony count to zero. Additionally, MB alone, without laser irradiation, was able to destroy all colonies at concentrations of ≥ 0.01 mg/mL, significantly reducing the colony count compared to other subgroups (P-value < 0.05), with a mean reduction of approximately 90% (CI: [80%, 100%]).

Furthermore, the study showed that laser irradiation combined with MB at concentrations of 0.001, 0.0005, and 0.0002 mg/mL, as well as with phycocyanin at concentrations of 0.02, 0.01, and 0.005 mg/ml, reduced the number of colonies. Phycocyanin alone decreased colony-forming units (CFUs) by approximately 30% (CI: [10%, 50%]) at higher concentrations, but its effect was less pronounced compared to MB. However, laser irradiation alone, laser irradiation combined with

phycocyanin at concentrations  $\leq 0.002$  mg/mL, and laser irradiation combined with MB at concentrations  $\leq 0.0001$  mg/mL did not significantly reduce the number of colonies (P-value  $> 0.05$ ).

## 5. Discussion

For the first time, this study compared the impact of phycocyanin and MB on the antibacterial effects of PDT. The results indicate that MB exhibits significantly greater antibacterial effects against *S. aureus* compared to phycocyanin, both with and without PDT. In a study by Virych et al. (20), a 60% reduction in *S. aureus* colonies was observed following laser irradiation with MB, with the 660 nm wavelength showing the highest efficacy among the lasers tested. Similarly, Tanev demonstrated that MB generates more ROS when exposed to laser light, further emphasizing the importance of laser wavelength. Additional research by Rineh et al. (21) found that a 668 nm laser could effectively kill *S. aureus* when used with MB. These findings support the conclusion that MB is an effective photosensitizer both alone and when combined with other agents, such as sodium citrate, silver, or zinc oxide nanoparticles, which have been shown to enhance MB's antibacterial properties. In the present study, MB completely eradicated *S. aureus* colonies when irradiated with a 660 nm laser and significantly reduced colony numbers even without laser treatment, underscoring its strong antimicrobial potential (22-24).

Conversely, phycocyanin demonstrated much weaker antibacterial effects in this study. Although phycocyanin has been widely utilized in cancer treatment and has shown antimicrobial potential, few studies have assessed its effectiveness against *S. aureus*. One study by Chakroun et al. (25) reported that phycocyanin alone possesses antibacterial effects on *S. aureus*, but results have been inconsistent across studies. Several factors may explain these discrepancies. A key factor could be phycocyanin's optimal light absorption range, which lies between 580 - 630 nm. Since this study employed a 660 nm laser, phycocyanin may not have absorbed sufficient light energy to generate the ROS needed for bacterial eradication. Additionally, *S. aureus* produces acidic byproducts under certain growth conditions, and phycocyanin is sensitive to low-pH environments, where it tends to aggregate and lose its efficacy. These environmental factors, combined with variations in experimental design, such as differences in bacterial growth conditions, light exposure durations, and photosensitizer concentrations, likely contribute to the varying outcomes observed in studies investigating phycocyanin's antibacterial effects (24-26).

It is also essential to compare the mechanisms of ROS production between MB and phycocyanin, as this could explain their differing antibacterial efficacies. When activated by laser light, MB generates a substantial amount of ROS, including singlet oxygen, which is highly toxic to bacterial cells. This ROS generation is well-documented and constitutes the primary reason for MB's effectiveness in PDT. In contrast, while phycocyanin has been shown to produce ROS, the efficiency and types of ROS generated may differ. Phycocyanin primarily absorbs light in the 580-630 nm range, and its ability to generate ROS under the 660 nm laser used in this study may have been suboptimal. This disparity in ROS generation mechanisms and efficiencies likely accounts for MB's superior antibacterial performance compared to phycocyanin (11, 27, 28).

The combination of low-power lasers and photosensitizers in PDT represents a promising approach for eradicating bacteria and promoting wound healing. One of PDT's key advantages over conventional antibiotics is its ability to circumvent the development of bacterial resistance. Unlike antibiotics, which target specific bacterial pathways, PDT operates by generating ROS that cause widespread oxidative damage to bacterial cells, affecting proteins, lipids, and DNA. This non-specific mechanism significantly reduces the likelihood of bacteria, including *S. aureus*, developing resistance to PDT. Achieving resistance to ROS would require multiple simultaneous genetic changes, a challenge that is exceedingly difficult for bacteria to overcome (29-31).

Despite the promising results for MB, there are still no studies examining the simultaneous effects of PDT and phycocyanin on *S. aureus*, and the limited research on phycocyanin's antimicrobial properties presents conflicting data. For instance, Safari et al. found that phycocyanin is more effective against gram-positive bacteria, such as *Streptococcus iniae*, but less effective against gram-negative species (32). These discrepancies may arise from variations in experimental conditions or differences in bacterial physiology, highlighting the need for further investigation.

While this study provides valuable insights, it has limitations. The experiments were conducted in vitro under controlled conditions, and the results may not fully translate to clinical settings. Consequently, animal and clinical trials are recommended to evaluate the efficacy of PDT with MB or phycocyanin in real-world scenarios. To effectively implement this method in clinical practice, optimizing the dosage of photosensitizers and calibrating light exposure will be



crucial. Photodynamic therapy holds particular promise for treating localized infections, such as chronic wounds, infections surrounding medical implants, and nasal decolonization in high-risk patients. Monitoring bacterial load and tissue response during treatment will be essential to ensure therapeutic success and prevent damage to healthy tissues (33, 34).

### 5.1. Conclusions

In the present study, MB was able to destroy all *S. aureus* colonies at concentrations of 0.01 mg/mL or higher without laser irradiation. Additionally, 0.002 and 0.005 mg/mL concentrations of this photosensitizer completely eradicated all colonies when combined with laser irradiation. However, phycocyanin only insignificantly reduced the number of colonies. Therefore, MB appears to be a more effective photosensitizer for PDT compared to phycocyanin. Unfortunately, no prior studies have compared the antibacterial effects of phycocyanin and MB in PDT, making this study the first of its kind. Thus, further research on this topic is recommended.

### Footnotes

**Authors' Contribution:** Study concept and design: S. L., H. E., and M. K. R.; analysis and interpretation of data: R. Sh.; drafting of the manuscript: R. Sh. and M. M.; critical revision of the manuscript for important intellectual content: S. L. and H. E.; statistical analysis: R. Sh.

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**Data Availability:** The dataset presented in the study is available on request from the corresponding author during submission or after publication.

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