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# Antimicrobial Photodynamic Inactivation of *Staphylococcus aureus* Virulence Factors: *In Vitro* Study

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**Abstract**
A therapeutic alternative for the treatment of infectious diseases has arisen from antimicrobial photodynamic inactivation (PDI). The effect of photodynamic therapy (PDT) on *Staphylococcus aureus* strains was evaluated in this study. The *Staphylococcus aureus* suspension ($10^8$ CFU/mL) was incubated with the dyes Methylene blue (MB), toluidine blue O (TBO) and Rose bengal (RB) for in vitro photodynamic inactivation studies at a concentration of 20 μM using Period of incubation (30 min) and the fluence of light (10 J/cm2 of 635-nm light). The photodynamic inactivation impact on virulence factors i.e catalase, βhemolysis, lipases, thermonuclease, and coagulase of *Staphylococcus aureus* has been studied. The antimicrobial photodynamic inactivation showed that the reduction of total viable cellsof

*Staphylococcus aureus*. The study showed that 20μM of MB and TBO both were found good photodynamic action, achieving a reduction of viability of bacterial culture. Photodynamic antimicrobial inactivation suppressed the expression of virulence factors, possibly inactivating virulent strains of *Staphylococcus aureus*, relative to commonly used antibiotics, meaning that this therapy would become a powerful and efficient alternative to antibiotics to control infections of pathogenic staphylococcus.

**Keywords:**photodynamic inactivation, *Staphylococcusaureus*, dyes andVirulence factors

## Introduction

Antimicrobial photodynamic inactivation (aPDI) is the advanced strategy for the prevention of life-threatening illnesses with multidrug resistance. The growing frequency of drug resistance among pathogenic bacteria is currently facing microbiologists and health care practitioners (Nakonechny and Nisnevitch, 2011). If no action is taken, the number of fatalities arising from pathogenic bacteria per year will rise to 10 million by 2050 (Ventola, 2015). Therefore, scientists are now focused on discovering novel biocidal agents or approaches to deal successfully with increasing drug resistance. The question of rapidly increasing resistance, however, is still present and unresolved. Scientists are seeking to remove this issue and are concentrating on finding an alternative method to multidrug-resistant pathogenic bacteria (Wainwright *et al.*, 2016; Zhu, *et al.*, 2020). Antimicrobial photodynamic inactivation or photodynamic antimicrobial treatment is an alternative method to fighting resistant microorganisms, like pathogens, fungus, parasites and viruses (Awad*et al.*, 2016; Hamblin, 2016).

*Staphylococcus aureus* causes an infection of the skin, and the prevalence of infections of *Staphylococcus aureus* associated with health care and the environment has risen in recent years (Lowy, 1998). In particular, community-associated infections are caused by increased antibiotic resistance among strains of *Staphylococcus aureus* as well as the capacity to cause disease in a relatively younger and healthier population of community-associated strains (David and Daum, 2010).

*Staphylococcus aureus* is a pathogenic and opportunistic bacterium responsible for gastrointestinal, urinary, and skin infections. In extreme cases, infection with *Staphylococcus aureus* may result in chronic illnesses. In latest days, cases of infection obtained by the group of peoples with *Staphylococcus aureus* have been growing, leading to transmission mechanisms of *Staphylococcus aureus* outside the hospital environment. *Staphylococcus aureus* is an opportunistic bacterial pathogen that causes a variety of infections including, skin infections to serious, invasive diseases (Alekshun, 2006).

*Staphylococcus aureus* expresses a number of factors of virulence that help to create infection by encouraging tissue adhesion, invasion of tissues, and preventing the immune response of the host. *Staphylococcus aureus,* a pathogen that is difficult to treat, is capable of developing resistance to several antibiotic types. The development and dissemination of *Staphylococcus aureus* methicillin-resistant strains, referred to as methicillin-resistant *Staphylococcus aureus* (MRSA), has resulted in high morbidity, high mortality and increased cost of treatment. The development of new classic antibiotics is not supposed to solve the issue of resistance drugs for too long. (Chambers and DeLeo, 2009), the treatment of MRSA infections will require non-traditional antimicrobial approaches. Ideally, modern antimicrobial techniques should be non-invasive to hosts and non-toxic to host, but effective and rapidly-acting, minimising chances of resistance (Kossakowska*et al.,* 2013; Calin and Parasca, 2009; Almeida *et al.*, 2014; Alves *et al.*, 2014; Almeida *et al.*, 2014;)

Visual light has strong disinfectants, a reality that, unlike the antibacterial properties of ultraviolet light, is not well established. Photodynamic inactivation (PDI) arises in this sense as a photochemotherapy strategy with possible applications of antimicrobial therapy (Almeida *et al.*, 2015; Costa *et al.,* 2012; Alves *et al.,* 2013). This technique has also been shown to be effective against fungi, viruses, parasites and gram-positive and gram-negative bacteria, (Almeida *et al.*, 2011, 2015; Costa *et al.,* 2012). The effects of photodynamic inactivation on virulence factors are of utmost importance, since they can be present when the organism is present during the infection phase, but they can also be present when the bacteria is not yet present, such as in cases of poisoning, causing serious damage to the host.

The increased resistance of antibiotics to pathogenic bacteria such as *Staphylococcus aureus* has resulted to the looking for new antimicrobial methods, and a promising alternative appears The effects of photodynamic inactivation on virulence factors are of paramount importance because they may be present when the organism is present at the course of disease, but they may also be available when the bacteria are not yet present, such as in cases of poisoning, causing considerable harm to the host. to be photodynamic therapy (PDT).

Latest studies on the combination of aPDI and antibiotics reveal that the examples are caused by the susceptibility of microbial to regularly used antibiotics (Fila *et al.,* 2016).

The ability of the light-activated photosensitizer to generate reactive oxygen species at wavelengths ideal for light irradiation relies upon the photodynamic inactivation of bacteria. The various biological structures, including nucleic acids, lipids and proteins, can be oxidized by reactive oxygen species. Since the mode of action to kill microbes is non-specific and affects several sites, it is believed that it is impossible to increase resistance (Wainwright, 2005), indicating a significantly important improvement over frequently used antibiotic therapy when resistance is developed among the patients. The feature of photodynamic therapy is the ability of reactive oxygen species to activate viral factors, particularly secretory proteins (Hamblin and Hasan, 2004).
The potential of a light triggered antimicrobial agent/photosensitiser to create suitable wavelength reactive oxygen species for light irradiation depends on photodynamic inactivation of bacteria. Multiple biological structures, including proteins, nucleic acids and lipids, can oxidise reactive oxygen species. As the mode of action of microbial killing is non-specific and several areas are affected, resistance is thought to be difficult to establish (Wainwright, 2005), creating a major shift over traditional antibiotic treatment where resistance is a widespread problem. The ability of reactive oxygen species to inactivate virulence factors, particularly secreted proteins, is a very favourable feature of PDT (Hamblin and Hasan, 2004).

## Materials and Methods

### Preparation of bacterial suspensions

The bacterial culture of *Staphylococcus aureus* was obtained from the Department of

Microbiology, Alkut center hospitals,Iraq and cultured on Nutrient Agar media in the present research . The 3 mL bacterial suspension was applied to the test tubes and diluted with sterile saline (0.9 percent NaCl). The bacteria's suspensions had spectrophotometrically modified the optical density to approximately $1.0x10^8$ colony forming units (CFU) mL1(equivalent to 1.0 McFarland scale) (CLSI, 2013; Dahikar., 2018)

### Photosensitizers

Methylene blue (3,7-bis(dimethylamino)-phenothiazinium chloride; (MB), toluidine blue O (TBO), brilliant crystal blue (BCB), and Rose Bengal (RB). Every photosensitizer was processed for no more than 24 hours in the dark as an aqueous stock solution (1mM) at 4 °C.

### *In vitro* photodynamic inactivation

Bacteria at $1.0 \times 10^8$colonyforming units were incubated with the dyes Methylene blue (MB), toluidine blue O (TBO), and Rose bengal (RB), at 20 µM concentration using period of incubation (30 min), and the fluence of light (10 J/cm$^2$ of 635-nm light) for in vitro

photodynamic inactivation studies. The samples were protected from light and were incubated for 30 min in the dark, at 25–30 °C with Methylene blue (MB), toluidine blue O (TBO), and Rose bengal (RB), (20 µM), as used for the PDI experiment. Control treatments were taken as 1) Light alone, 2) dye alone, 3) dye + light, and 4) NO dye and No light. After irradiation, 0.1 mL samples were serially diluted with phosphate buffer solution. Aliquots (0.1 mL) were dispersed over the selective culture medium: Mannitol Salt Agar (Hi Media, Mumbai); after 36 hours of incubation at $37^0$C, the number of colony forming units per millilitre (CFUs/mL) on each plate was then measured and also plated on nutrient agar after incubation at the same temperature for 24-48 hours. (Sabbahi*et al.,* 2008). Each experiment has been performed three times.

**Spectrophotometric analysis**

To do spectrophotometric analysis, a UV / Vis spectrophotometer was used (Shimadzu, Japan). The spectrophotometric analysis of the sample were prepared by diluting the stock solution of methylene blue (MB), toluidine blue O (TBO) and Rose Bengal (RB) rose at concentration (20 µM). An acceptable fresh bacterial suspension was applied to the entire PS solution shortly before the experiments. In the visible region, where the monomer and dye dimer absorption bands are separate, the dye absorption spectrum was obtained in the 400-700 nm range.

**Enumeration of Viable Cells**

10-fold serial dilutions were prepared from each treated and control sample in sterile PBSS

(10-1 to 10-6). 100µL Aliquots is pour-plated, in duplicate, in the medium Nutrient Agar (Hi Media, Mumbai). The plates have been incubated for 48 h at 37 ° C and the number of colonies has been counted.

**Virulence Factors studies**

**To study the virulence**

Samples were checked for β-hemolysin activity, lipase and lecithinase activity, catalase activity and thermonuclease activity to evaluate the effectiveness of photodynamic inactivation therapy on the virulence factors of *Staphylococcus aureus* (Baptista *et al.*, 2015).

**β-hemolysin activity**

The β-hemolysin activity was determined by streaking Blood Agar Plates (Columbia 5% Sheep Blood Agar Plate, Hi Media, India) and observing the development of beta or gamma zone of haemolysis.

**Lipase and lecithinase activities**

For determination of Lipase and lecithinase activities, the Baird-Parker Agar Medium was used in which *Staphylococcus aureus* colonies appear in grey-blackshiny, with an opaque precipitation zone and a clear zone surrounding it for Lipase and lecithinase activities respectively.

**Catalase activity**

For determination of catalase activity, the method defined by Cappuccino *et al.,* (1986) was used. With a drop of 3 percent hydrogen peroxide solution, a layer of agar culture bacteria has been mixed on a microscope slide for approximately 28 hours and the formation of gas bubbles reveals the formation of catalase from the culture within seconds.

**Coagulase Test (Slide Test Method)**

The test was carried out following the procedure defined in Bartelt, 2000. On a clean microscope slide, a drop of saline solution (Naci 0.85 percent) was placed with limited dispersion of emulsifying fluid from one or two test colonies. The tip of the seed wire was heated at room temperature in undiluted plasma and the traces were applied to the bacterial suspension on a slide, turned on the seeding thread, and the bacterial suspension management process was repeated. Coagulase was observed when, after the random agglutination test, staphylococci accumulated within 20 seconds of applying undiluted plasma to saline on a glass tray.

**Thermocycles activity**

Nuclease, developed by *S. aureus* is thermostable, whereas it is thermolabile produced by other species. Broth cultures are measured in wells cut into the agar plate, incubated for 2 hours, followed by boiling for 15 minutes. Enzyme thermocycles developed by *S. aureus*. After the plate is incubated for 2-4 hours, aureus depolymerizes DNA in the region adjacent to the well. The metachromatic property of toluidine blue is due to the development of a pink diffusion zone or halo around the well.

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## Results and Discussion

### Photodynamic Inactivation of *Staphylococcus aureus*

Oskar R and Hermann VT discovered antimicrobial photodynamic inactivation therapy when they discovered *Paramecium* spp. Protozoa stained with acridine cytidine separated when exposed to light. Since then, PDT has improved the treatment of leukemia, mental illness and anemia. In recent years, however, there has been in order to restore the antimicrobial effect, interest of PDT, supported by a rapid increase in the antimicrobial activity of pathogenic pathogens, and PDT may be possible proposed as a treatment for a variety of local pathogens known as inactivation. antimicrobial photodynamics. (Hamblin and Hasan, 2004; Jori, 2006; Denis *et al.*, 2011). A great alternative to topical treatments is antimicrobial photodynamic inactivation treatment. It is very successful in avoiding infections induced by different vaccinations such as old ones, rendering it far less protective than antibiotics, and in the photodynamic inactivation treatment, no proof of the resistance process has been recorded (Dai *et al.*, 2010).

Suspensions of *Staphylococcus aureus* bacterial cells were exposed to 30 min of PDI therapy and aliquots were administered prior to (0 min) and after 30 min of treatment. PDI has successfully inactivated *Staphylococcus aureus*. Reductions greater than 5 log CFU mL-1 for *Staphylococcus aureus* were observed after 30 minutes of treatment under the tested conditions. In general, however, the photoinactivation trend was distinct. between the strains of Staphylococcus aureus, as seen by the reduction rates of log10.

**Figure 1a: Reduction of cell viability of the *Staphylococcus aureus* (CFU/mL) after treatment of light and Methylene Blue (MB)**

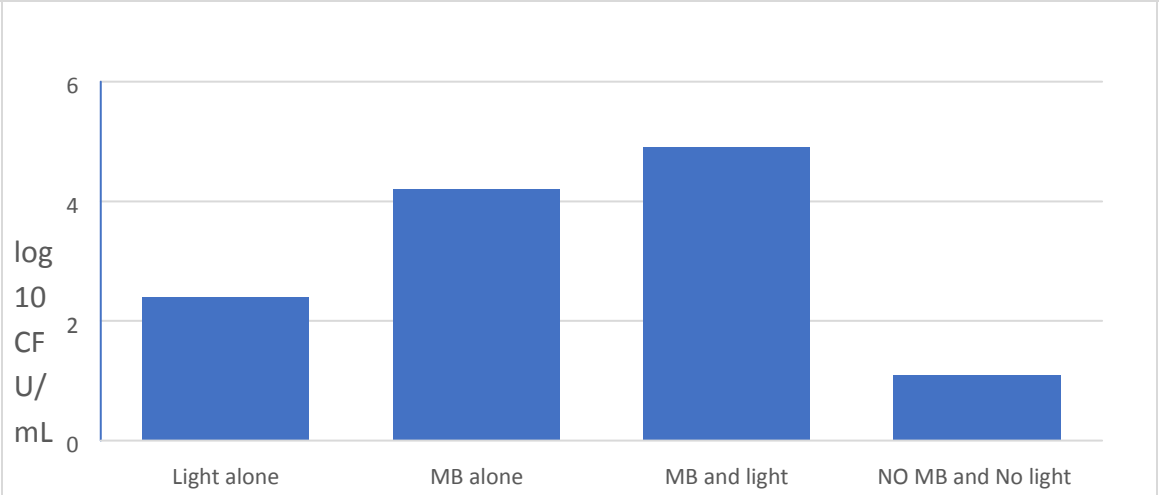


**Figure 1b: Reduction of cell viability of the *Staphylococcus aureus* (CFU/mL) after treatment of light and Toluidine blue O (TBO)**

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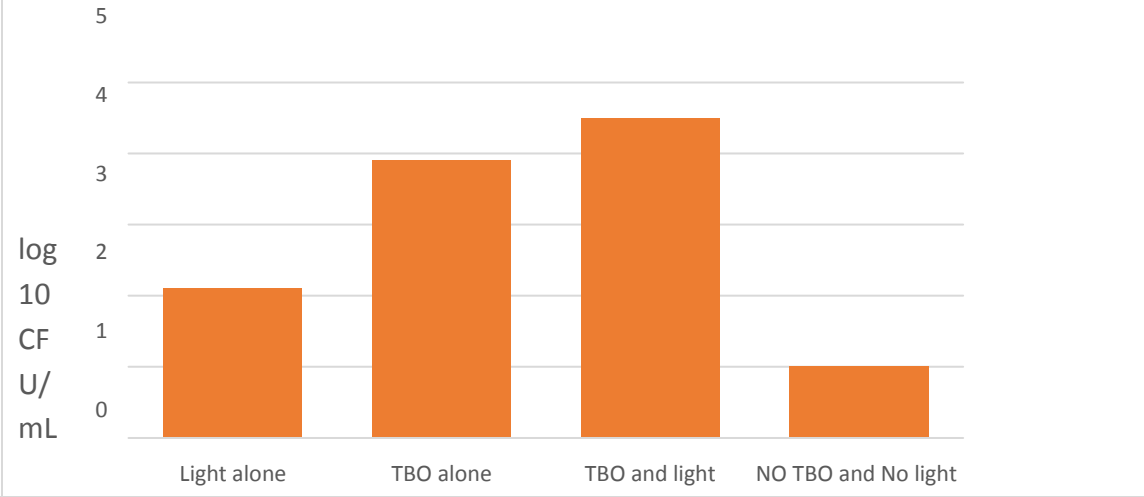
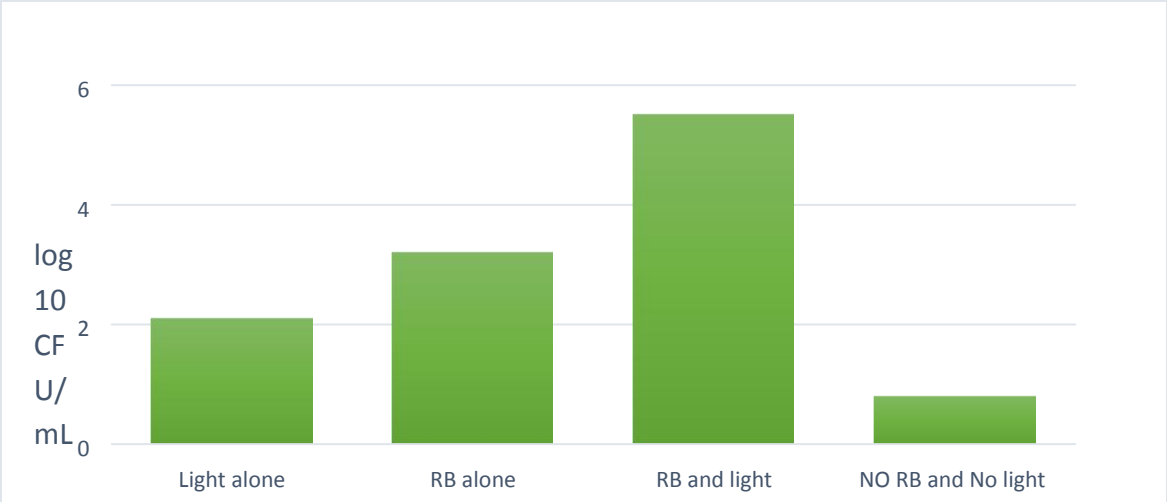



**Figure 1c: Reduction of cell viability of the *Staphylococcus aureus* (CFU/mL) after treatment of light and Rose Bengal (RB)**



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| **Table 2a: The photodynamic inactivation (MB) effect on the virulence factors of *Staphylococcus aureus*** | | | | | |
|---|---|---|---|---|---|
| Treatment | **virulence factors** | | | | |
| | β-Hemolysis | Lipase and lecithinase | Catalase | Bound Coagulase | Thermonuclease |
| Light alone | ++ | + | + | + | + |
| MB alone | ++ | ++ | ++ | + | + |
| MB + light | - | - | - | - | + |
| NO MB and No light | +++ | ++ | ++ | ++ | ++ |

| **Table 2b: The photodynamic inactivation (TBO) effect on the virulence factors of *Staphylococcus aureus*** | | | | | |
|---|---|---|---|---|---|
| Treatment | **virulence factors** | | | | |
| | β-Hemolysis | Lipase and lecithinase | Catalase | Bound Coagulase | Thermonuclease |
| Light alone | ++ | + | + | + | + |
| TBO alone | + | + | ++ | + | + |
| TBO+light | - | + | + | + | + |
| NO TBO and No light | ++ | ++ | ++ | ++ | ++ |

| **Table 2c: The photodynamic inactivation (RB) effect on the virulence factors of *Staphylococcus aureus*** | | | | | |
|---|---|---|---|---|---|
| Treatment | **virulence factors** | | | | |
| | β-Hemolysis | Lipase and lecithinase | Catalase | Bound Coagulase | Thermonuclease |
| Light alone | + | + | + | + | + |
| RB alone | + | + | ++ | + | + |
| RB + light | - | - | + | + | + |
| NO RB and No light | +++ | ++ | +++ | ++ | ++ |
| + Indicate the positive test, where – indicate negative test | | | | | |

Due the frequent and misuse of antibiotics resistance of pathogenic bacteria is increasing such as Methicillin Resistant *Staphylococcus aureus* (MRSA), so it is a need of hour to searchalternative to antibiotics and develop a new antimicrobial strategy, beside this photodynamic therapy (PDT) may be an emerging and promising alternative to antibiotics. The activity of the light-activated antimicrobial agent, i.e., the photosensitizer, to generate reactive oxygen species depends on the photodynamic inactivation of the bacteria when irradiated with light of the required wavelength. The biological structures, nucleic acids, lipids including and proteins, may be oxidised by reactive oxygen species. Since microbial killing's mode of action is nonspecific and affects several locations, resistance is considered impossible (Wainwright, 2005), suggesting a major benefit as resistance is a growing issue over traditional antibiotic care. The likelihood of reactive oxygen species activating viral factors, especially secretory proteins, is a very beneficial feature of PDT (Hamblin Hasan, 2004).

In this study, the 10-log Staphylococcus aureus CFU/ml was substantially decreased by all experimental photosensitizer treatments relative to the untreated control group ($p<0.05$). Important variations were found in the groups in which Methylene Blue, Toluidine blue and Rose Bengal were added for 30 minutes (only light, only MB, MB and light, NO MB and no light) relative to that group, with only lasers (MB and light) providing a higher reduction rate of Staphylococcus aureus CFU/ml log10. Compared to MB + Light and TBO + light, the APDI RB group displayed a larger reduction in Staphylococcus aureus CFU/ml log 10. In the group where APDI was used, the use of this stain significantly reduced the log log of Staphylococcus aureus. MB+ light showed the best results in reducing Staphylococcus aureus CFU/ml log 10 (4.9 log 10). It was observed for the antimicrobial photodynamic inactivation (APDI) group that TBO + light showed the greatest decrease in CFU/ml log 10 (4.5 log 10), while RB + light showed the decrease in CFU/ml log 10 (5 log 10) (Fig. 1a, 1b and 1c). Fig. 1a, b and c show the comparison between treatment of antimicrobial photodynamic inactivation (APDI) with the three dyes. The scientific literature indicates that there may be significant microbial destruction due to the The relationship between the origin of light and the photosensitizers that absorb at this wave length, such as MB, TBO and RB (Peloi*et al.*, 2008; Lima *et al.*, 2009). The effect of photoinactivation on the virulence activity of *Staphylococcus aureus* was also explored in this study. The findings suggest that photodynamic inactivation affects all virulence factors. This is a relative advantage of frequently used antibiotics, which work only on bacterial cells and not on factors of virulence. Such findings are comparable with previous studies reported by Kömerik*et al.*, (2000) and Tubby *et al.*, (2009).

Packer et al., 2000, reported that the 633 nm wavelength photosensitizer Toluidine Blue O and red laser light could inactivate the periodontal pathogen *Porphyromonasgingivalis* proteolytic enzymes. The findings reported here, with a very substantial decrease in Staphylococcus aureus activity at methylene blue concentrations of 20μM, support these observations. The dosedependent inactivation was determined to be with a median determined methylene blue concentration (20μM) and laser light irradiation of 9.65 J/cm2, resulting in a 100% decrease in operation relative to untreated samples. Under the same

conditions, treatment of MB, TBO and RB resulted in the inactivation of Staphylococcus aureus, meaning that the inactivation of secreted β-hemolysis, lipase, lecithinase, coagulase catalase, and thermonuclease and the removal of infectious bacteria could be necessary.

**Conclusion:** The findings revealed that the photodynamic inactivation of Staphylococcus aureus bacteria was successful in decreasing the number of viable cells. The findings revealed that strong photodynamic activity was shown by both MB 20 μM and TBO, reducing the viability of bacterial cultures. Photodynamic antimicrobial inactivation (PDI), unlike widely used antibiotics, Blocks the expression of causes of virulence, essentially inactivating virulent *Staphylococcus aureus* strains, rendering this treatment a potential alternative to antibiotics to contain the staphylococcal infection.

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