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Research paper

Polarization-Controlled Photodynamic Activity of Methylene Blue Against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Photodynamic inactivation (PDI) is a promising antimicrobial strategy that utilizes a non-toxic photosensitizer (PS), molecular oxygen, and light of a specific wavelength to generate cytotoxic reactive oxygen species (ROS). Methylene blue (MB), a phenothiazinium salt with an absorption maximum around 664 nm, is a well-characterized PS that functions via both Type I (radical-forming) and Type II (singlet oxygen-forming) photochemical mechanisms. This study investigates the PDI efficacy of MB-mediated against representative Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, with a specific focus on the influence of light polarization. MB-mediated PDI was most effective under linearly polarized light in our experimental system compared with circularly polarized or non-polarized light at equivalent fluence rates. The study also confirmed a significant difference in susceptibility between the two bacterial species. *S. aureus* demonstrated markedly higher susceptibility to MB-mediated PDI than *E. coli*. This is attributed to the structural disparity in their cell envelopes: the thick, porous peptidoglycan layer of *S. aureus* facilitates greater uptake and binding of cationic MB. In contrast, the lipopolysaccharide-rich outer membrane of *E. coli* acts as a formidable permeability barrier, limiting MB access to critical cellular targets. These findings suggest that manipulating light polarization can enhance the bactericidal effects of PDI, paving the way for advances in antimicrobial therapy.

1. Introduction

The escalating crisis of antimicrobial resistance (AMR) constitutes a formidable threat to global public health, urgently necessitating the

development of alternative therapeutic modalities. Photodynamic inactivation (PDI) represents a compelling non-antibiotic strategy that employs a photosensitizer (PS), light of a specific wavelength, and molecular oxygen to generate



cytotoxic reactive oxygen species (ROS), primarily singlet oxygen (O_2) and free radicals [1, 2].

Methylene blue (MB), a cationic phenothiazinium salt, is a well-characterized PS with a strong absorption maximum in the red-light spectrum ($\lambda_{\max} \approx 664$ nm), a region that affords deeper tissue penetration [3].

MB is known to mediate bacterial death via both Type I (electron/hydrogen transfer) and Type II (energy transfer to O_2) photochemical pathways [4-6]. A key determinant of its efficacy is the structure of the bacterial cell envelope; the thick, porous peptidoglycan layer of Gram-positive bacteria, such as *Staphylococcus aureus*, facilitates the uptake of cationic MB, whereas the lipopolysaccharide-rich outer membrane of Gram-negative bacteria, like *Escherichia coli*, presents a significant permeability barrier [7-9].

The efficacy of a photochemical reaction is contingent upon photon absorption, which is maximal when the electric field vector of the incident light aligns with the transition dipole moment of the absorbing molecule. Several studies have suggested that light polarization influences photodynamic outcomes. For instance, Kamali et al. investigated the effect of light polarization on the efficiency of photodynamic therapy (PDT) for basal cell carcinoma using 5-aminolevulinic acid (ALA). They demonstrated that polarized light, particularly linearly polarized light, significantly increased the efficacy of ALA-PDT compared with non-polarized light, leading to a greater reduction in cell viability [10]. Similarly, Santos et al. found that PDT using Dimethyl-Methylene Blue Zinc Chloride Double Salt (DMMB) with a polarized light source significantly reduced the number of *S. aureus* bacteria in vitro, suggesting its potential for effective infection control [11]. However, these findings involve either a pro-drug (ALA) or an aggregating PS, and their applicability to a non-aggregating, water-soluble cationic

photosensitizer like MB remains an open and critical question. Furthermore, other reports have primarily focused on the diagnostic applications of polarized light in tissue optics rather than its role in enhancing therapeutic efficacy [12]. Using polarized light offers several advantages over non-polarized light in PDI, including increased photodynamic efficiency, lower photosensitizer concentration, reduced sensitivity to drug concentration changes, and reduced photobleaching [10]. This study aims to elucidate the influence of light polarization on the bactericidal efficacy of MB-mediated PDI against representative Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. By directly comparing the bactericidal outcomes following illumination with linearly polarized, circularly polarized, and non-polarized light at equivalent fluences, we aim to determine whether manipulating light polarization is a viable strategy for enhancing PDI using a classical photosensitizer like MB. Bacterial viability was assessed using colony-forming unit (CFU) assays to quantify cell death.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains, *Staphylococcus aureus* ATCC 33591 and *Escherichia coli* ATCC 25922, were obtained from the microbial bank of the Islamic Azad University, Central Tehran Branch. The strains were inoculated on Mueller-Hinton Agar and EMB Agar. The cultures were incubated at a constant temperature of 37°C for 24 hours and subsequently used to evaluate bacterial photodynamic inactivation.

2.2. Photosensitizer and reagents

Methylene Blue (MB, purity $\geq 82\%$, Merck, Germany) was used as the photosensitizer. A stock solution of MB was prepared at a concentration of

1 mg/mL in sterilized phosphate-buffered saline (PBS; pH 7.2) and stored at 4°C protected from light. This stock solution was then serially diluted to obtain the required concentrations for various experimental setups.

The absorption spectrum of MB in PBS was recorded using a UV-Vis spectrophotometer over a wavelength range of 300–750 nm to confirm its characteristic absorbance maximum at ~664 nm.

2.3. Minimum inhibitory concentration (MIC) assay

To evaluate per se toxicity of MB against *S. aureus* and *E. coli*, the MIC values were determined using the broth microdilution method. Assays were performed in triplicate in 96-well plates. The tested concentration range of MB was 500, 250, 125, 62.5, 31.25 and 15.62 µg/mL, and the final bacterial concentration was 5×10^5 colony-forming units (CFU)/mL in each well. Both positive controls (bacterial suspension in broth without MB) and negative controls (broth only) were included to ensure the validity of the results. The plates were incubated at 37°C for 24 h, after which the turbidity of the wells was assessed visually, and the lowest concentration of MB that inhibited bacterial growth was considered as MIC.

2.4. Light source and optical setup

A light-emitting diode (LED) with a peak emission wavelength of 660 ± 10 nm was used as the light source, corresponding to the red absorption peak of MB. The irradiance (power density) at the sample surface was measured with a calibrated power meter and adjusted to 25 mW/cm². Three light conditions were established: Non-polarized light (NPL): The direct output from the LED.

Linearly polarized light (LPL): Generated by passing the NPL beam through a linear sheet polarizer. Circularly polarized light (CPL): Generated by passing the LPL beam through a

quarter-wave plate, with the fast axis of the wave plate oriented at 45° to the plane of linear polarization (as shown in Figure 1).

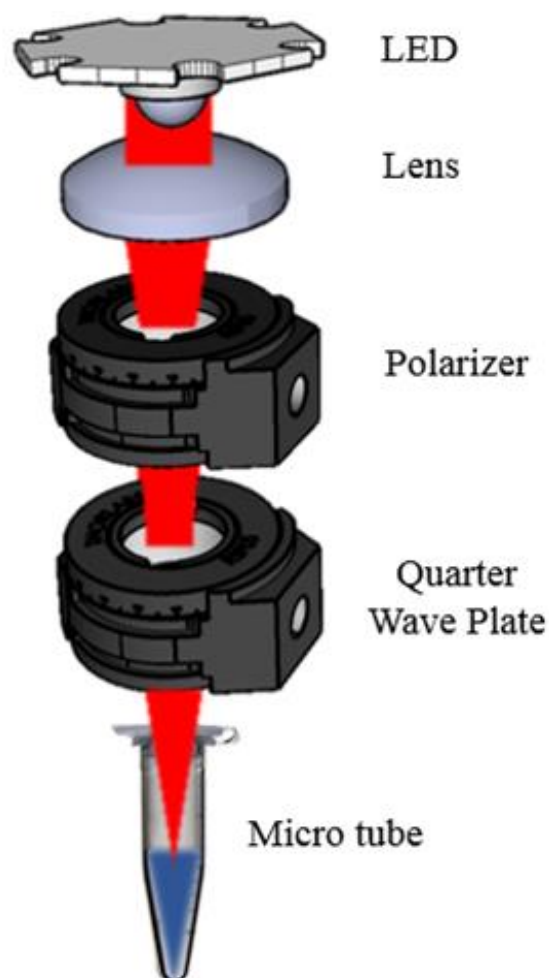


Figure 1. The schematic diagram of circularly polarized LED irradiation

2.5. Photodynamic inactivation of bacteria

Bacterial suspensions equivalent to 0.5 McFarland standard (1.5×10^8 CFU/mL) were prepared, and the accuracy of the suspension density was confirmed using a spectrophotometer. A 0.5 McFarland standard has an absorbance reading of 0.08 to 0.1 at 600 nm.

10 microtubes containing 1 mL of bacterial suspensions at a concentration of 1.5×10^5 CFU/mL (obtained by dilution of the 0.5 McFarland standard) and a non-toxic

concentration of MB, were prepared. To investigate the effect of polarized light on bacterial growth inhibition, the experiments were conducted using non-polarized (NPL), linearly polarized (LPL), and circularly polarized (CPL) for exposure durations of 5, 10, and 15 minutes. One microtube was maintained in the dark as a control.

Following light irradiation, all samples (both irradiated and unirradiated) were serially diluted with PBS to achieve a final concentration of 1.5×10^3 CFU/mL.

An aliquot (1 mL) from the appropriate dilutions was plated in triplicate onto nutrient agar plates using the pour plate method.

The plates were incubated at 37 °C for 14 to 16 hours. Bacterial survival was quantified by enumerating bacterial colonies using a colony counter.

2.6. Statistical analysis

All experiments were performed in triplicate. Data are presented as mean \pm standard deviation (SD). Statistical significance was first assessed using two-way analysis of variance (ANOVA), with polarization type and exposure time as factors. When significant effects were found, pairwise comparisons were performed using independent two-sample *t*-tests, comparing each exposure time with the corresponding 0-minute control within the same polarization group.

A *p*-value < 0.05 was considered statistically significant.

3. Results and discussion

Based on the minimum inhibitory concentration (MIC) assay, MB exhibited inhibitory activity against the growth of *Staphylococcus aureus* at a concentration of 125 μ g/mL.

However, it did not show any inhibitory activity against *Escherichia coli* at the concentrations used

in this study. The MIC results were confirmed by culturing both bacterial strains on agar plates in the presence of various concentrations of MB. For subsequent experiments, a concentration of 30 μ g/mL was selected, as this concentration was non-lethal to either bacterial species. This approach was adopted because the objective of this study was to utilize MB not as an antibiotic agent, but rather as a photosensitizer.

To investigate the effects of light polarization on the photosensitizing performance of MB, two bacterial species, *S. aureus* and *E. coli*, were exposed to three different light conditions: linearly polarized light, circularly polarized light, and non-polarized light.

The experiments were conducted at three different time points (5, 10, and 15 minutes), and a control group (without light exposure) was included for comparison.

The mean bacterial colony count \pm standard deviation (SD), expressed as (CFU/mL ($\times 10^3$)), obtained from these light-exposure experiments is depicted in [Figure 2](#).

As shown in [Figure 2](#), bacterial colony counts decreased progressively with increasing light-exposure time for all polarization types.

For *S. aureus* ([Figure 2a](#)), the reduction was most pronounced under linearly polarized light, with near-complete inhibition observed at 15 min (mean: 0 ± 0 colonies).

This corresponds to a reduction of approximately 2.0 \log_{10} units compared with the 0 min control. Circularly polarized light produced a smaller but still substantial reduction, whereas non-polarized light showed the smallest decrease at 15 min.

A similar trend was observed for *E. coli* ([Figure 2b](#)), although the overall magnitude of reduction was smaller.

Linearly polarized light again produced the greatest decline in colony counts, with significant decreases after 15 minutes.

This was followed by circularly polarized light and, lastly, non-polarized light.

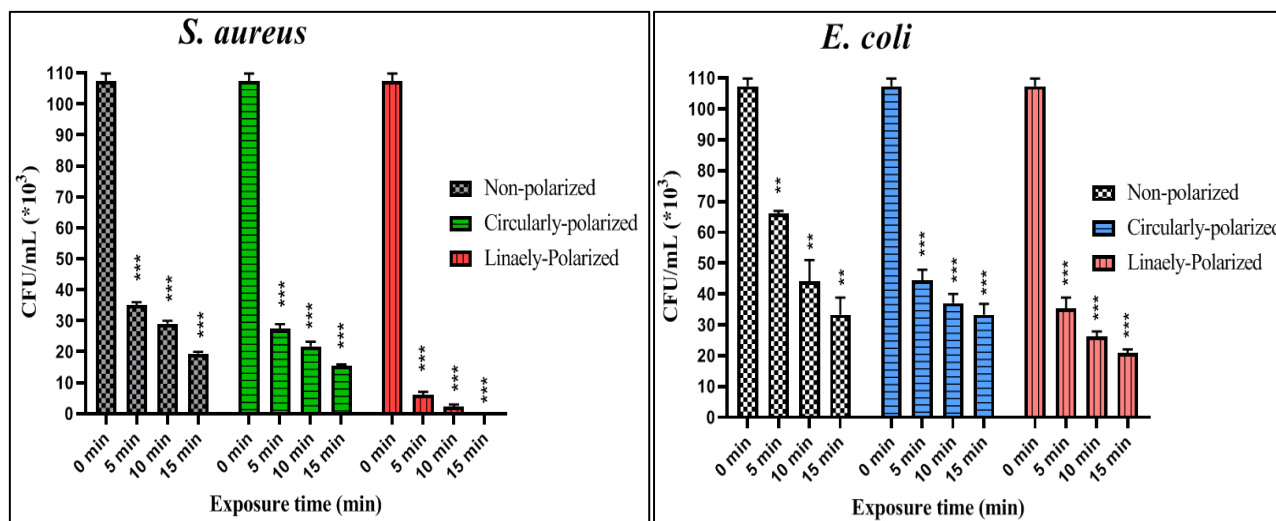


Figure 2. Comparative efficacy of PDI with different light polarizations against *S. aureus* and *E. coli*. Bar graphs illustrating the mean colony forming units (CFU) remaining after 5, 10, and 15 minutes of PDI treatment. (a) *S. aureus* and (b) *E. coli* were treated with 30 $\mu\text{g/ml}$ MB and irradiated with non-polarized, circularly polarized, or linearly polarized light. Data are presented as mean \pm standard deviation (SD) from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ represent statistically significant differences compared with the corresponding 0 min control

PDI is a process that relies on the interplay of three essential components: a non-toxic PS, visible light of an appropriate wavelength, and molecular oxygen (O_2).

In this study, MB serves as the PS. Upon irradiation, the PS absorbs light energy and transitions from its stable ground state (S_0) to a short-lived, electronically excited singlet state (S_1).

From this state, the molecule can undergo intersystem crossing to a more stable, longer-lived excited triplet state (T_1).

Once in the triplet state, the PS can initiate cytotoxic reactions through two primary mechanisms. In the Type I pathway, the excited PS directly interacts with a substrate (e.g., lipids, proteins) via electron or hydrogen transfer, producing radical ions that subsequently react with oxygen to form reactive oxygen species (ROS) such as the superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH).

In the Type II pathway, which is considered dominant for phenothiazine dyes such as MB, the triplet-state PS transfers its energy directly to

ground-state molecular oxygen ($^3\text{O}_2$), generating the highly cytotoxic singlet oxygen ($^1\text{O}_2$).

The high quantum yield of $^1\text{O}_2$ production by a single PS molecule makes PDI an exceptionally efficient antimicrobial strategy [1, 2].

The efficacy of this process begins with efficient light absorption by MB.

The absorption spectrum of MB (Figure 3) is characterized by two primary peaks in the visible region, corresponding to π - π electronic transitions (also referred to as dipole transitions).

The relative intensity of these peaks depends on the aggregation state of MB in solution [13]. Under the preparation conditions used in this study, the absorption spectrum of MB in PBS exhibited a dominant band near 664 nm, suggesting a substantial monomeric contribution. However, the possible presence of aggregated species cannot be excluded, particularly in biological media.

This alignment between the absorption peak and the light source ensures efficient photon absorption, which is the critical first step in initiating the photodynamic cascade that underpins bacterial inactivation.

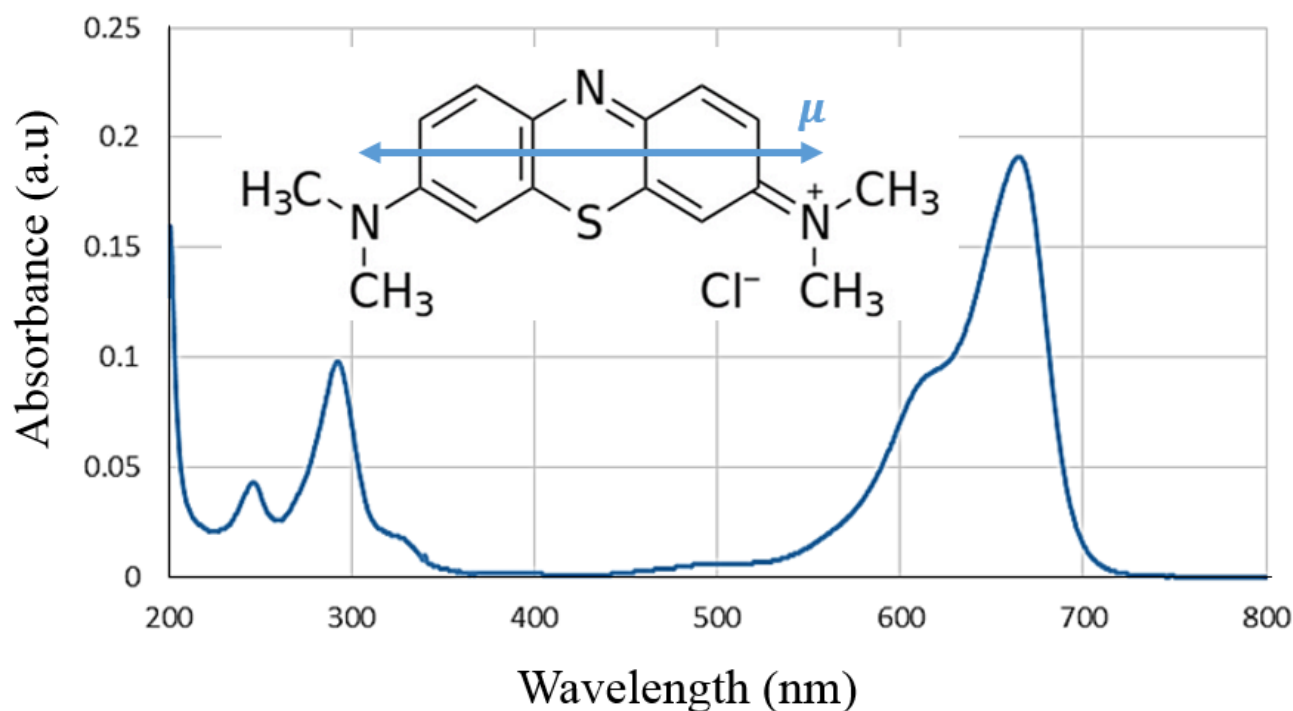


Figure 3. Optical properties of Methylene Blue. The UV-Vis absorption spectrum of aqueous Methylene Blue (MB), highlighting the primary absorption peak for the monomer at ~664 nm. The inset shows the chemical structure of MB, with an arrow indicating the orientation of its main transition dipole moment (μ)

Beyond the photochemical mechanism, the physical properties of the activating light, specifically its polarization, can significantly modulate PDI efficacy. Light is an electromagnetic wave, and its polarization describes the orientation of its oscillating electric field vector. The interaction between light and a molecule is governed by the molecule's transition dipole moment (TDM), which is a vector representing the directional charge displacement during an electronic transition. MB possesses a principal TDM (μ) aligned along its main molecular axis (as shown in Figure 3) [14]. Consequently, light absorption is maximized when the light's electric field vector is aligned with this molecular axis. **Non-Polarized Light:** This light consists of electric field vectors oriented randomly in all planes perpendicular to the direction of propagation. As a result, only a small fraction of these vectors will momentarily align with the TDM of any given MB molecule, leading to inefficient and suboptimal energy absorption.

Linearly Polarized Light: In this state, the electric field is confined to oscillate within a single plane. This confinement allows for a more selective and efficient interaction with MB molecules whose TDMs are favorably oriented relative to this plane. The absorbance (A) becomes dependent on the orientation angle (θ) between the molecule's TDM and the electric field components (E_x , E_y , E_z), as described by the equations for p-polarized (A_p) and s-polarized (A_s) light [14]:

$$A_p = a \left(\langle \cos^2 \theta \rangle E_z^2 + \frac{1}{2} (1 - \langle \cos^2 \theta \rangle) E_x^2 \right) \quad (1)$$

$$A_s = a \left(\frac{1}{2} (1 - \langle \cos^2 \theta \rangle) E_y^2 \right) \quad (2)$$

By aligning the electric field vector with the molecular absorption axis, linear polarization maximizes the probability of photon absorption. In principle, absorption by a chromophore depends on the orientation of the electric field vector relative to its transition dipole moment.

However, because MB molecules were not experimentally oriented in the present aqueous system, this concept should be interpreted at the ensemble level rather than as direct molecular alignment. This theoretical enhancement in absorbance translates directly into increased ROS generation and, consequently, greater bactericidal efficacy. Our experimental results corroborate this principle.

As depicted in Figure 2, linearly polarized light demonstrated superior inhibition of both *S. aureus* and *E. coli* growth, achieving complete eradication of *S. aureus* after 15 minutes of exposure (light dose: 22.5 J/cm²).

Circularly Polarized Light: This form of light is composed of two orthogonal linearly polarized components with a 90° phase difference, causing the electric field vector to rotate. While this ensures that a component of the electric field is always available to interact with the TDM of MB, regardless of molecular orientation, the instantaneous field strength in any single direction is lower than that of optimally aligned linearly polarized light. This results in reduced overall absorption efficiency, diminished ROS

production, and a corresponding decrease in PDI effectiveness, as confirmed by our findings (Figure 2).

The enhanced ROS generation resulting from the superior light absorption of MB under linear polarization leads to a more potent PDI effect for both bacterial species tested.

However, our results demonstrate that this enhancement is particularly pronounced in the Gram-positive *S. aureus* compared with the Gram-negative *E. coli* (Figure 4). This differential susceptibility is rooted in the fundamental structural differences between their respective cell envelopes.

The Gram-positive cell wall is composed of a thick, highly porous peptidoglycan layer. This structure presents a minimal barrier to the diffusion of cationic photosensitizers such as MB, allowing the molecules to permeate the cell envelope effectively and associate with their primary target, the cytoplasmic membrane. Consequently, the increased ROS flux generated by linearly polarized light can efficiently induce lethal oxidative damage, leading to rapid cell death.

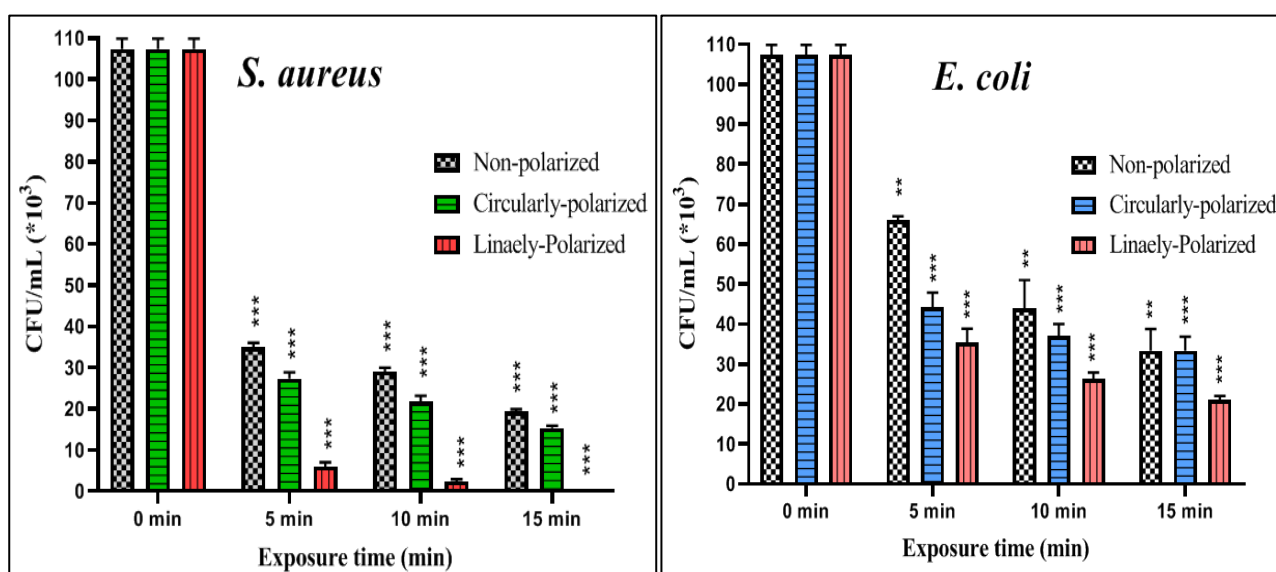


Figure 4. Comparative photographic evidence of bacterial survival of *S. aureus* and *E. coli* on agar plates following PDI. Both a) *S. aureus* and b) *E. coli* were treated with 30 µg/ml MB and irradiated with non-polarized light, circularly polarized light, and linearly polarized light, demonstrating the superior bactericidal effect of *S. aureus* by the linearly polarized condition. **p<0.01 and ***p<0.001 represent statistically significant differences compared with the corresponding 0 min control

In contrast, the Gram-negative cell envelope presents a formidable permeability barrier. Its defining feature is the asymmetric outer membrane, which is rich in lipopolysaccharides (LPS).

This layer acts as a highly selective shield, significantly restricting the uptake of many antimicrobial agents, including photosensitizers. Furthermore, the passage of molecules across this membrane is tightly regulated by protein channels known as porins, which can further limit the intracellular accumulation of MB. While linear polarization still yields a greater bactericidal effect than other light forms against *E. coli*, the intrinsic resistance conferred by the outer membrane mitigates the overall efficacy of the treatment [15, 16].

The findings of this study establish that light polarization is a critical, yet often overlooked, parameter for modulating PDI efficacy. By optimizing the polarization state of the incident light to match the transition dipole moment of the photosensitizer, it is possible to significantly enhance bactericidal outcomes.

This principle offers a promising strategy for potentiating existing PDI protocols against both Gram-positive pathogens and, albeit to a lesser but still significant extent, resistant Gram-negative pathogens.

Several limitations should be considered when interpreting these findings.

The aggregation state of methylene blue under our experimental conditions was not systematically assessed; although the UV–Vis spectrum showed a strong band near 664 nm consistent with a substantial monomeric contribution, some aggregation may still have occurred and could affect absorption and ROS generation. In addition, light scattering in the turbid bacterial suspensions was not quantified and may have altered light propagation and partially reduced polarization within the sample. Dissolved oxygen and temperature were also not monitored during

irradiation, so oxygen depletion or minor heating, especially at longer exposure times, cannot be fully excluded. These factors do not invalidate the observed polarization-dependent differences, but they should be considered when interpreting the underlying mechanism and addressed in future studies.

4. Conclusion

PDI is recognized as a promising alternative to conventional antibiotics due to its broad-spectrum activity and low potential for inducing resistance. This study provides compelling evidence that light polarization is a critical and highly effective parameter for modulating the efficacy of MB-mediated PDI. Our findings conclusively show that 660 nm linearly polarized light significantly enhances bactericidal activity against both the Gram-positive *S. aureus* and the Gram-negative *E. coli* compared with circularly polarized and conventional non-polarized light. In particular, linearly polarized light significantly improved the bactericidal activity of MB-based PDI, with complete eradication of *S. aureus* observed after 15 minutes (22.5 J/cm²) of exposure. This enhanced efficacy is mechanistically linked to the fundamental interaction between the light's electric field vector and the transition dipole moment of the MB molecule. Our results suggest that linearly polarized light enhances PDI efficacy under the present conditions, possibly through polarization-dependent differences in excitation efficiency that ultimately increase ROS generation. However, direct molecular alignment was not measured in this study.

The primary novelty and contribution of this work lie in its systematic application of a fundamental photophysical principle to a practical antimicrobial challenge. While the physical properties of polarized light are well understood in optics and materials science, its application as a tool to enhance antimicrobial PDI has remained a

largely unexplored frontier. Previous studies have primarily focused on optimizing PDI by modifying the chemical structure of the photosensitizer or using adjuncts to increase bacterial uptake. By demonstrating that PDI can be significantly potentiated without altering any chemical components, this research introduces a new and valuable physical dimension to the optimization of photodynamic treatments.

The implications of these findings are significant, particularly in the context of the global antimicrobial resistance (AMR) crisis. Our work suggests a pathway to make this promising therapy even more potent. Although this study provides a robust *in vitro* proof-of-concept, future research is needed to build on these findings. Key future directions should include: (i) investigating the effect of polarization on other classes of photosensitizers with different molecular symmetries; (ii) evaluating this approach in more complex and clinically relevant microbial structures, such as biofilms, which are notoriously resistant to antimicrobial agents; and (iii) translating these findings to preclinical models to assess efficacy in a more complex biological environment.

Competing interests

The authors declare no competing interests.

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