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In vitro Photodynamic Antimicrobial Activity of Protoporphyrin IX in the Presence of Hydrogen Peroxide against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration between all authors. Author FA did the antimicrobial experiments, survival assay and wrote the manuscript. Author CR performed the bacterial growth. Authors FA and KU performed the fluorescence measurements. Authors FA and CR managed the literature searches. Authors NM and PRA helped in interpretation of results. Author SG designed the study, coordinated the group study (Head of Group) and corrected manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study reports on *In vitro* investigation of photodynamic antimicrobial activity of protoporphyrin IX (PPIX) in the presence and absence of Hydrogen peroxide (H_2O_2) against *S. aureus* and *P. aeruginosa*.
Place and Duration of Study: Department of Medical Physics, Anna University, Chennai between December 2013 and February 2014.
Methodology: A light-emitting diode (LED) was used as a light source to irradiate PPIX. The antibacterial effect was analyzed by standard plate counting method. Steady-state fluorescence spectroscopy technique was used to monitor the damage at protein level.
Results: We found that the antibacterial effect is dependent on PPIX concentration as well as H_2O_2 concentration and light dose. PPIX-H_2O_2 combination showed higher bacterial

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reduction of 6.5 log_{10} and 2.7 log_{10} for *S. aureus* and *P. aeruginosa* respectively, when the light dose increased to 70 J/cm^2. Fluorescence spectroscopic characterization showed a considerable change in the intensity of emission of tryptophan present in the microorganisms between pre- and post- APDT.
Conclusion: PPIX-H_2O_2 is a promising combination for APDT against Gram positive and Gram negative bacteria. The LED seems to be a very good option for PDT because of its low cost and miniature in size.

Keywords: Photodynamic therapy; light-emitting diode; hydrogen peroxide; protoporphyrin ix; Staphylococcus aureus; Pseudomonas aeruginosa; antimicrobial photodynamic therapy.

1. INTRODUCTION

The worldwide increase in antibiotic resistance among different classes of Gram-positive and Gram-negative bacteria has led to a search for alternative antimicrobial therapies. *Staphylococcus aureus* is considered as one of the most common human pathogens, which causes a wide range of diseases such as wound infections, syptic arthritis, osteomyelitis and endocarditis [1,2]. This bacterial pathogen has increasing ability to resist many antibiotics such as tetracycline, erythromycin, penicillin, methicillin and vancomycin [3]. *Pseudomonas aeruginosa* is an opportunistic pathogen and it causes infections with a high mortality rate due to its resistance to many antimicrobials [4]. This bacterial pathogen excretes many extracellular virulent factors that contribute to degradation of the immune system and destroy the tissue integrity of the host [5]. In this context, extensive research for alternative antimicrobial treatment was carried out against the multidrug resistant pathogens such as *S. aureus* and *P. aeruginosa*. Numerous published reports have shown that pathogenic bacteria that are resistant to antibiotic treatment can be inactivated successfully with Photodynamic Therapy (PDT), which is referred as Antimicrobial Photodynamic Therapy (APDT) [6,7].

APDT involves non-toxic components, which include light activated dye known as photosensitizer (PS) and harmless visible light of an adequate wavelength in the presence of molecular oxygen [8]. Excitation of the PS produces reactive oxygen species (ROS) that affect the integrity and function of bacterial cell walls, nucleic acids or enzymes, which result in cell death [9-12]. Although PDT has been considered for treatment of certain cancers [13], it is also used for the treatment of some oral diseases such as oral candidiasis [14] and root canal infections [15]. Other applications of PDT at a less developed stage include treatments for arthritis [16], psoriasis [17], Barretts's esophagus [18], atherosclerosis [19], and restenosis [20]. Furthermore, PDT is increasingly being used in dermatology for a wide range of neoplastic, inflammatory, and infectious cutaneous conditions [21].

In many studies, it has been shown that Gram positive bacteria are susceptible to APDT when compared to their Gram negative counterparts [22]. In Gram positive bacteria, the cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid, which allows the photosensitizer (PS) to cross easily [23]. However, the Gram negative bacteria are surrounded by outer membrane and inner cytoplasmic membrane separated by peptidoglycan-containing periplasm. The Gram negative bacteria can be destroyed only by increasing the permeability of the outer membrane using different chemicals or biological agents as suggested by Bertoloni et al. [24]. In this, it has been

shown that APDT using PPIX is not effective against Gram negative bacteria such as *P. aeruginosa* [25].

In PDT, it is required to use a stable, wavelength-unique, homogeneous, and large-area irradiation light source. Currently, various laser and non-laser light sources have been considered for PDT [26]. Although, laser light sources are not only very expensive, but also a specially designed optical beam delivery system is needed to broaden the beam for the irradiation of wide area. On the other hand, conventional light sources can be easily coupled with appropriate optical filters to irradiate the target area uniformly [27]. However, these conventional lamps may lead to serious thermal effect, which should be avoided during PDT. With the recent advances in optoelectronic devices, Light Emitting Diodes (LEDs) have been considered as an alternative light source for PDT. This is because, LEDs offers many advantages compared to other conventional light sources for PDT such as, less hazardous, less expensive, thermally nondestructive, and readily available [28].

In PDT it is also worth to note the role of Hydrogen peroxide (H_2O_2). H_2O_2 an oxidizing agent used widely in removing dead tissue and cleaning wounds [29]. It is reported that, H_2O_2 can be used successfully in APDT to improve the effectiveness of the PS [30,31]. Garcez et al. [32] reported the APDT effect of methylene blue (MB) in the presence of H_2O_2 against *S. aureus, Escherichia coli* and *Candida albicans*. They found that H_2O_2 is an interesting approach to improve the antimicrobial activity of MB. However, *P. aeruginosa*, a pathogen resistant to traditional chemotherapy and involved in many infections [33] was not included in their study. Furthermore, to the best of our knowledge, there is no published study on the use of PPIX combined with H_2O_2 in APDT against *S. aureus* or *P. aeruginosa.*

In this context, this paper aims to study the influence of several experimental conditions on APDT mediated by PPIX in the presence and absence of H_2O_2 against *S. aureus* and *P. aeruginosa*. A light-emitting diode was used as a light source for irradiation of the PS. Standard plate counting method was used to determine the number of surviving bacteria before and after the different APDT treatments. It was aimed also to investigate the LED as a light source in APDT. Attempts were also made to study the steady state fluorescence spectroscopic characterization of tryptophan from *S. aureus* and *P. aeruginosa* to understand the molecular changes in particular at protein level due to APDT.

2. MATERIALS AND METHODS

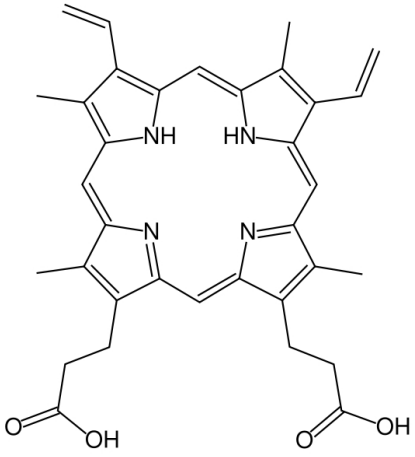
2.1 Bacterial Strains and Growth Conditions

Methicillin-sensitive *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 10145) were provided by the Centre for Advanced Studies in Botany, University of Madras. Both strains were grown in Muller-Hinton broth (MHB) (Himedia, Mumbai, India) for 16 h at 37ºC with shaking at 200rpm in LS 500 incubator/shaker (Neolab, Mumbai, India). Subsequently, the bacterial pellet was harvested by centrifugation at 10,000 rpm for 10 min and washed three times using normal saline (Na Cl 9g/L). Bacterial cells were then resuspended in normal saline to an optical density (OD) of 0.05 and 0.01 at 600 nm for *S. aureus* and *P. aeruginosa,* respectively, which corresponded to 10^6-10^7 colony-forming units (cfu)/mL.

2.2 Photosensitizer and Light Source

Protoporphyrin (PPIX) was obtained from Sigma Aldrich (St. Louis, USA). The molecular characteristics of PPIX and molecular structure are shown in Fig. 1. Stock solutions were

prepared before each experiment in sterile normal saline and kept in the dark. Before each experiment, the photosensitizer was allowed to warm up to room temperature. Hydrogen peroxide (H_2O_2) was obtained from Merck (Mumbai, India). PPIX was used at concentrations of 30, 60,100 and 200μM while H_2O_2 was used at 1, 10, 50 and 100mM. All illuminations were carried out with a portable LED (XR-C, Cree Inc., Germany) with a wavelength of 637 nm (Fig. 2). The output of the LED was measured by Field Maser GS power meter (Coherent Inc., USA) and it was found 20mW. The emission spectra of the LED were measured using spectrometer (USB 4000-VIS-NIR, Ocean Optics, USA) and are shown in Fig. 3 together with the absorption spectrum of PPIX, which was measured using UV/VIS absorption spectrophotometer (Lambda 35, Perkin Elmer, USA).



Molecular formula: $C_{34}H_{34}N_4O_4$
Molar mass: 562.658 g/mol
Composition: C (72.58%) H (6.09 %)
N (9.96%) O (11.37 %)
Molar volume: 433.2 ± 3.0 cm^3
Density: 1.298 ± 0.06 gm/cm^3

Fig. 1. Molecular characteristics and molecular structure of PPIX

Fig. 2. The LED used in this experiment: XR-C, Cree Inc., Germany, with a wavelength of 637 nm

2.3 Effect of APDT with PPIX Alone or in the Presence of Increasing Concentrations of H_2O_2

To investigate the photodynamic effect of PPIX (30μM) alone or in the presence of increasing concentrations of H_2O_2, aliquots (100μl) of a suspension of *S. aureus* or *P. aeruginosa* was added to 100μl of PPIX-H_2O_2 in 96 wells plate (Tarsons, Kolkata, India) and incubated at 37ºC in the dark for 15 min. H_2O_2 was used at concentrations of 1, 10 and 100 mM. Samples were irradiated separately for 15 min in the dark under aseptic conditions in a laminar flow. The light dose that reached the samples surface was 18 J/cm^2. Samples used as controls include: bacteria alone without PS and light irradiation, bacteria illuminated with light and bacteria mixed with PPIX or H_2O_2 alone without light illumination and bacteria mixed with PPIX irradiated with light.

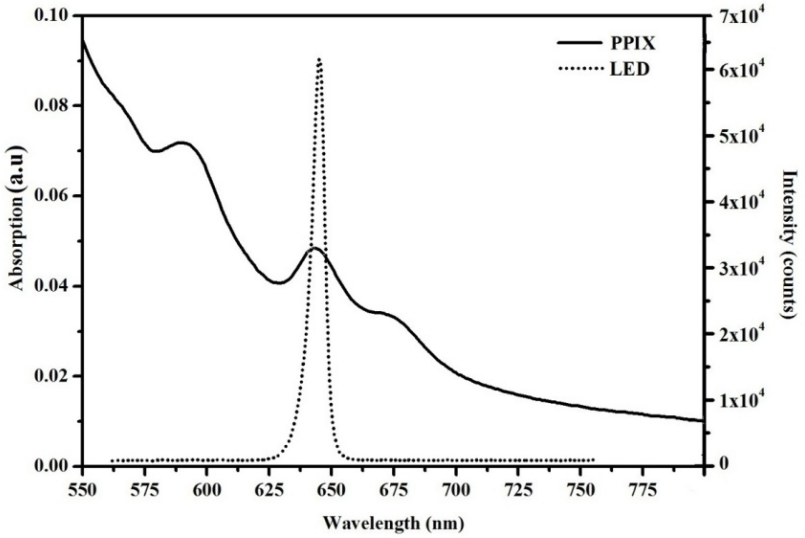


Fig. 3. Absorption spectrum of PPIX (—) and emission spectrum of LED (…..)

2.4 Effect of APDT with Increasing Concentrations of PPIX Alone or in the Presence of H_2O_2

In order to know whether increasing the PS concentration in the presence of H_2O_2 can kill more bacteria compared to when the PS is used with increasing H_2O_2 concentrations; 100μl of *S. aureus* or *P. aeruginosa* was added to 100μl of PPIX- H_2O_2 in 96 wells plate and incubated in the dark at 37ºC for 15 min. PPIX used at concentrations of 30, 60,100 and 200 μM while H_2O_2 was used at 50mM. Samples were irradiated separately for 15 min in the dark under aseptic conditions in a laminar flow. The light dose that reached the samples surface was 18 J/cm^2. Control group includes: bacteria alone without PS and light irradiation and bacteria photosensitized with PPIX alone (30, 60,100 and 200μM).

2.5 Effect of Increasing the Light Dose in APDT with PPIX Alone or in the Presence of H_2O_2

The effect of increasing the light dose for PPIX alone or in the presence of H_2O_2 was investigated. 100μl of *S. aureus* or *P. aeruginosa* was added to 100μl of PPIX-H_2O_2 in 96 wells plate and incubated in the dark at 37ºC for 15 min. The PS and H_2O_2 concentrations

used were 30μM and 50mM respectively. Each sample was irradiated separately in the dark under aseptic conditions inside a laminar flow. Each bacterium was irradiated to light from the LED for 15, 30, 45 and 60 min which equivalent to light doses of 18, 36, 54 and 70 J/cm^2, respectively. Control samples include: bacteria alone without PS and light irradiation and bacteria photosensitized with PPIX alone at light doses of 18, 36, 54 and 70 J/cm^2.

2.6 Bacterial Survival Assay

Standard plate counting method was used to determine the numbers of CFU of *S. aureus* and *P. aeruginosa*. After irradiation, 100μl aliquots were taken from each sample, 1:10 serially diluted six times in normal saline, spreaded on nutrient agar plates and incubated for 24 h at 37ºC.

2.7 Measurement of Tryptophan Fluorescence

Steady-state fluorescence of tryptophan from the pre- and post- treated *S. aureus* and *P. aeruginosa* was acquired using spectrofluorometer (FluoroMax-2, ISA Jobin Yvon-Spex, Edison, NJ). The excitation source (ozone-free xenon arc lamp) connected to the excitation monochromator to obtain the light of the required wavelength. The fluorescence emission was collected using the emission monochromator connected to a photomultiplier tube (R928P, Hamamatzu, Shizuoka-Ken, Japan). Both excitation and emission monochromators gratings have a groove density of 1200 grooves/mm. The slit widths for excitation and the emission were adjusted at 5 nm during fluorescence data acquisition with an integration time of 0.1s. The generated signal is transferred to the PC through an RS232 interface. Data Max (Windows-based data acquisition program) powered by GRAMS/386® was used for processing the data. To investigate the APDT effect of PPIX (30μM) alone or in presence of H_2O_2 (100mM) on tryptophan fluorescence, 200 μl of *S. aureus* or *P. aeruginosa* suspended in normal saline (10^6 – 10^7 cell/mL) was added to 200μl of PPIX-H_2O_2 in 12 wells plate. Samples were irradiated separately with LED for 15 min. The light dose that reached the samples surface was 18 J/cm^2. Samples used as controls include bacteria alone without PS or light illumination and bacteria photosensitized with PPIX (30μM). Appropriate volume of normal saline was added to bring the total volume of each sample in the quvette to 1.50mL. Samples were excited at 280 nm and emission spectra were collected in the wavelength range from 300 nm to 540 nm.

2.8 Statistics

Each experiment was performed twice and at least in triplicate. Values are expressed as means ± standard deviation. Differences were tested for statistical significance by Student's t test. Probability values less than 5% were considered significant.

3. RESULTS AND DISCUSSION

3.1 Effect of APDT with PPIX Alone or in the Presence of Increasing Concentrations of H_2O_2

It was reported that bacterial strains showed different sensitivities against exogenous H_2O_2 due to APDT. In all bacterial strains some physiological damage has been observed, particularly to their membrane permeability and the efficiency of APDT depended on the bacterial strains [32]. The authors also found that, a high concentration of H_2O_2 has

exhibiting considerably change in membrane potential, esterase activity and intracellular pH. Funk & Krise [34] reported that, a single dose of hydrogen peroxide at lower concentration was found to produce dramatic increases in the apparent intracellular accumulation of fluorophores with different physicochemical properties in different cell types. The results were reliable with changes in lateral membrane diffusion induced by H_2O_2.

In the present study, APDT with PPIX as the function of H_2O_2 concentrations was carried out and from the results it is observed that a significant reduction in the CFU of *S. aureus* and *P. aeruginosa* (Figs. 4 and 5). The highest CFU reduction ($P<0.005$) is found to be about 2.5 $\log_{10}$ and 1.5 $\log_{10}$ for *S. aureus* and *P. aeruginosa*, respectively when PPIX irradiated in the presence of H_2O_2 (100mM). As it is shown in Figs. 4 and 5, the two groups: PDT and PDT+ H_2O_2 (1mM), only about 0.5 $\log_{10}$ and 0.2 $\log_{10}$ bacterial reductions were obtained for *S. aureus* and *P. aeruginosa* respectively. However, when H_2O_2 concentration was increased to 10mM, the antibacterial effect was also increased by 1.0 $\log_{10}$ and 0.5 $\log_{10}$ for *S. aureus* and *P. aeruginosa*, respectively. These data demonstrated that the Gram positive bacterium, *S. aureus,* seems to be more sensitive to APDT with PPIX alone or in the presence of H_2O_2 compared to the Gram negative bacterium *P. aeruginosa*. Furthermore, neither light, nor photosensitizer or H_2O_2 alone showed significant reduction in the CFU of *S. aureus* and *P. aeruginosa*. The antibacterial effect of PPIX activated with light is due to the formation of singlet oxygen and reactive oxygen species such as superoxide anions($O_2^{\bullet-}$) and hydroxyl radicals (•OH). The enhanced antibacterial effect of PPIX in the presence of H_2O_2 may be due to the changes in the membrane permeability and hence the probability of more accumulation of the PS in the cell, or it may be due to the fact that the photoreaction would cause membrane disruption which then facilitate H_2O_2 penetration into the cell [35,36].

Garcez et al. [32] reported that, there is a possibility of ROS formation due to H_2O_2. Using 60 μM of MB and H_2O_2 of 100mM with 60 J/cm^2 light dose from diode laser, the authors obtained about 1.2 $\log_{10}$, 0.9 $\log_{10}$ and 1.3 $\log_{10}$ reductions in the viability of *S. aureus*, *Escherichia coli and Candida albicans* respectively. However, in this study, 30μM of PPIX activated with 18 J/cm^2 light dose from LED in the presence of H_2O_2 (100mM) higher inactivation (2.5 $\log_{10}$) in the viability of *S. aureus* was observed.

3.2 Effect of APDT with Increasing Concentration of PPIX Alone or in the Presence Of H_2O_2

As the PS represents the main component in the photosensitization process, the dependency of PS concentration as the function of light dose and H_2O_2 was also investigated. Results of lethal photosensitization of *S. aureus* and *P. aeruginosa* as the function of PPIX concentrations in the presence or absence of H_2O_2 (50mM) are shown in Figs. 6 and 7, respectively. The antimicrobial effect of PDT proportionally increases ($P<0.005$) with PPIX concentration and these results are consistent with that of previous reports [37]. It is noted that, there is a considerable reduction (2.5 $\log_{10}$) in *S. aureus* CFU using PPIX at 200μM. However, APDT in the presence of 50mM of H_2O_2 exhibited a more reduction (3.4 $\log_{10}$) even at 30μM of PPIX. Under similar concentration of PPIX (alone) and PPIX in presence of H_2O_2, it is observed that the APDT efficiency is lesser for *P. aeruginosa*. For example it is noted that 200 μM PPIX at a light dose of 18 J/cm^2, *P. aeruginosa* has exhibited only 0.5 $\log_{10}$ reduction and 1.0 $\log_{10}$ reduction with H_2O_2 (50mM). The differences in susceptibility of the Gram positive and Gram negative bacteria to the APDT in this study may be attributed to the differences in the structure of the cell wall. *P. aeruginosa* have an outer membrane, which acts as a barrier and reduce reactive oxygen species taken by the cell [38].

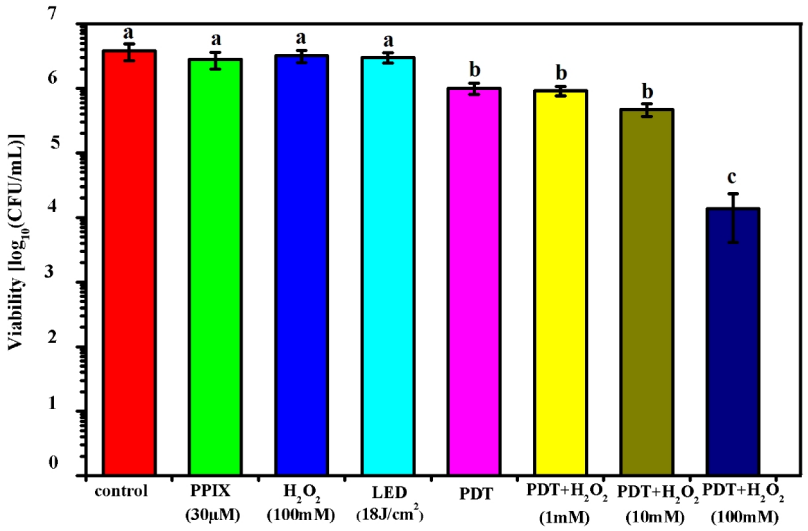


Fig. 4. The effect of increasing concentrations of H_2O_2 on lethal photosensitization of *S. aureus* using PPIX (30μM), Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments. Columns marked with different letters were significantly different (P<0.005)

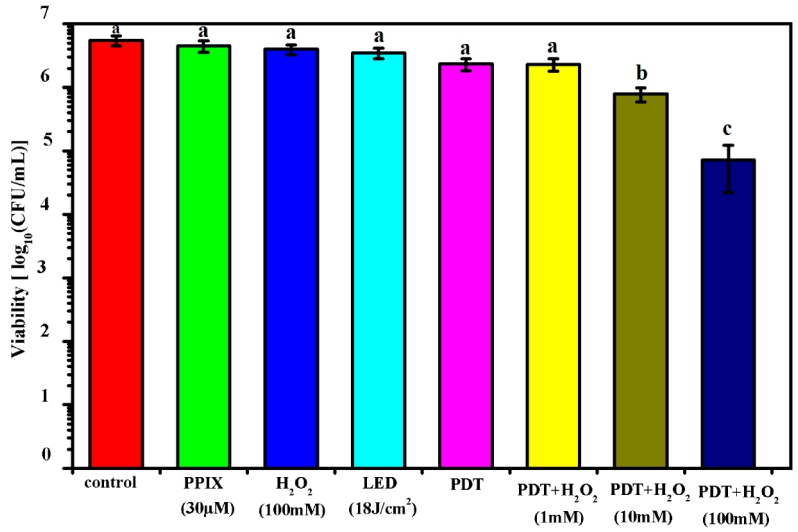


Fig. 5. The effect of increasing concentrations of H_2O_2 on lethal photosensitization of *P. aeruginosa* using PPIX (30 μM), Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments. Columns marked with different letters were significantly different (P<0.005)

It's well known that neutral PSs like PPIX are not able to photosensitize the Gram negative bacteria [39,40]. However, the results obtained in this study revealed that, the APDT effect of PPIX against *S. aureus* and *P. aeruginosa* can be enhanced in the presence of H_2O_2.

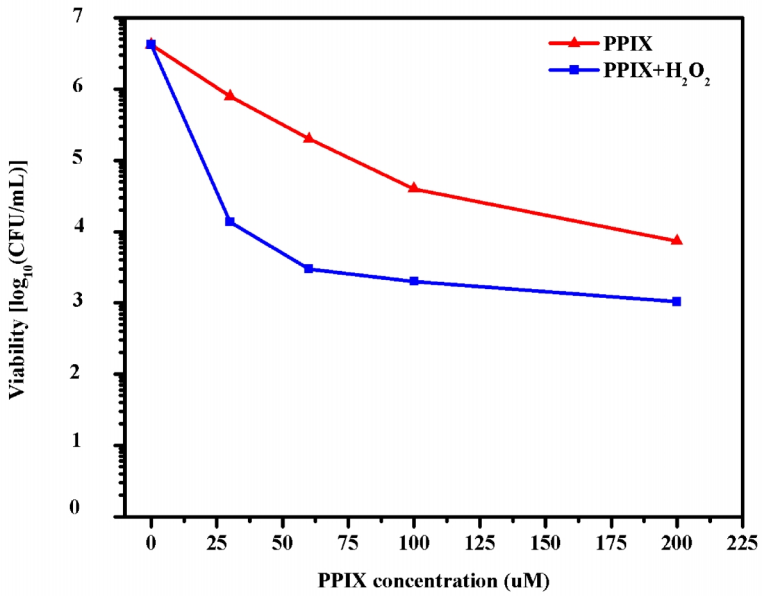


Fig. 6. Effect of increasing PPIX concentrations (30, 60, 100, 200 µM) alone or in the presence of H_2O_2 (50 mM) on the viability of *S. aureus*. Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments (P<0.005)

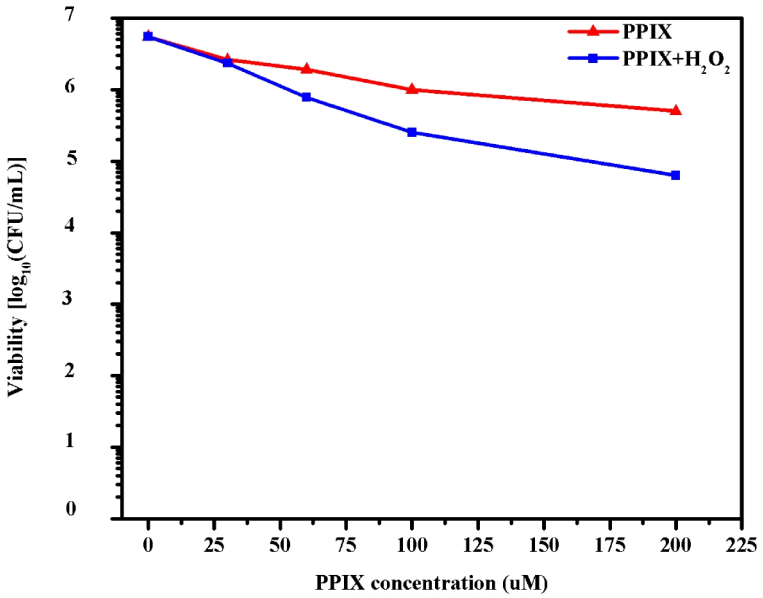


Fig. 7. Effect of increasing PPIX concentrations (30, 60, 100, 200 µM) alone or in the presence of H_2O_2 (50 mM) on the viability of *P. aeruginosa*. Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments (P<0.005)

3.3 Effect of Increasing the Light Dose in APDT with PPIX Alone or in the Presence of H_2O_2

Exposure time represents a fundamental quantity in APDT. Figs. 8 and 9 shows the dependence of APDT against *S. aureus* and *P. aeruginosa* respectively on light dose. For each bacterium, the most effective bacterial kills were seen when PPIX alone (30μM) or in the presence of H_2O_2 (50 mM) was irradiated with light dose of 70 J/cm^2. When 70 J/cm^2 light dose had been delivered to activate PPIX alone, approximately 2.0 and 1.0 $\log_{10}$ CFU reduction ($P<0.005$) was achieved for *S. aureus* and *P. aeruginosa*, respectively. However, when the same treatment was applied with adding H_2O_2 (50mM) with PPIX to the bacteria and irradiated with 70 J/cm^2 light dose, 6.5 $\log_{10}$ and 2.7 $\log_{10}$ CFU reductions was obtained for *S. aureus* and *P. aeruginosa*, respectively.

Previous study by Ganz et al. [37] reported that APDT effect was less at low concentration of PPIX. However, they observed higher reduction when PPIX illuminated for 20 min. The study of the light irradiation time demonstrates that the balance between PPIX concentration and illumination time can control the efficiency of PDT. Furthermore, for non-perfused tissues where the accumulation of PS is less, longer irradiation time should be considered to allow identical therapeutic effect.

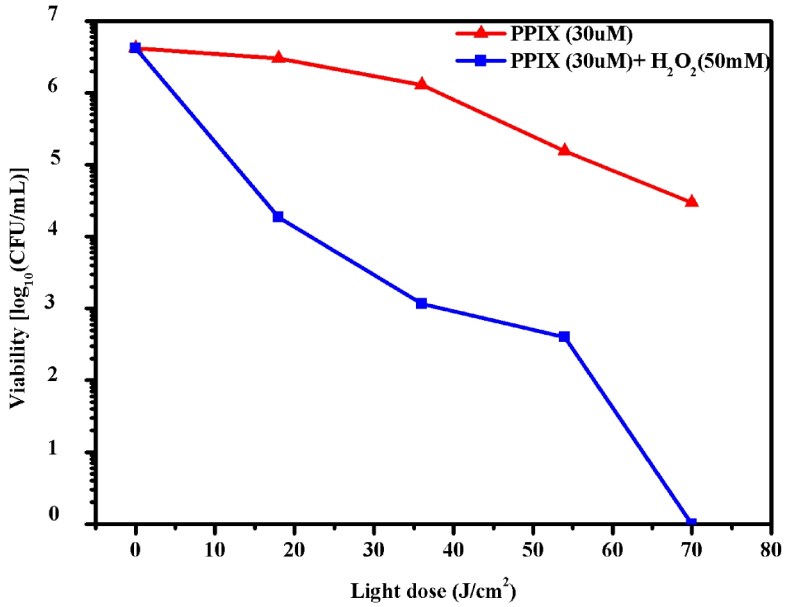


Fig. 8. Effect of increasing the light dose (18, 36, 54, 70 J/cm2) using PPIX (30μM) alone or in the presence of H_2O_2 (50mM) on the viability of *S. aureus*. Incubation time 15 min. Data are means ± standard deviation of three experiments ($P<0.005$)

3.4 Measurement of Tryptophan Fluorescence

All bacteria cells own proteins with the aromatic amino acid, tryptophan, which is the dominant cellular fluorophore in the ultraviolet spectral region [41]. Tryptophan shows a broad emission from 320 to 400 nm. However, the maximum emission wavelength is greatly

dependent on its local environment and protein structure [42]. Fluorescence spectra of tryptophan from bacteria can give further information about the mechanism of antimicrobial action.

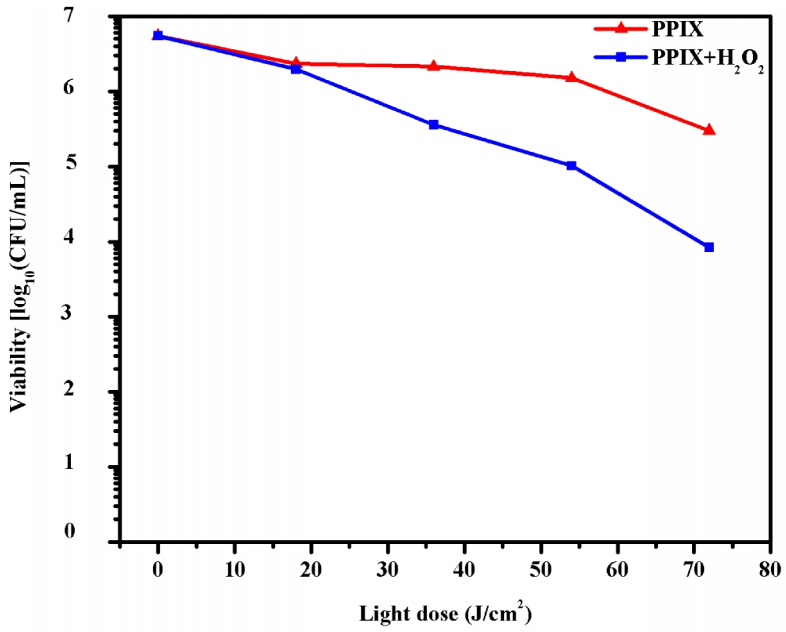


Fig. 9. Effect of increasing the light dose (18, 36, 54, 70 J/cm^2) using PPIX (30 µM) alone or in the presence of H_2O_2 (50mM) on the viability of *P. aeruginosa*. Incubation time 15 min. Data are means ± standard deviation of three experiments (P<0.005)

The APDT effects on tryptophan fluorescence from *S. aureus* and *P. aeruginosa* are presented in Figs.10 and 11 respectively. The overall fluorescence from post- treated *S. aureus* and *P. aeruginosa* exhibited a significant reduction (P<0.005) in the intensity. The emission peak at 340 nm is attributed to the key amino acid, tryptophan which is present in the bacteria [43]. From the inset of Figs. 10 and 11, it is found that there is a reduction in the peak emission at 340 nm due to APDT with respect to control group. The decreases in tryptophan fluorescence intensity may correspond to protein damage as suggested by Manpreet et al. [44]. The fluorescence spectra of *P. aeruginosa* exhibited a red shift after APDT; however this shift was not shown in the spectra of *S. aureus*. The noticed red shift may not relate to a change in tryptophan emission from *P. aeruginosa* but a contribution of the pyoverdine emission at 340 nm [45]. The intrinsic fluorescence of tryptophan for the control and APDT group for *S. aureus* and *P. aeruginosa* correlates with that of CFU results. Moreover, the results obtained in this study showed that APDT can have a significant effect on the fluorescence properties of bacteria.

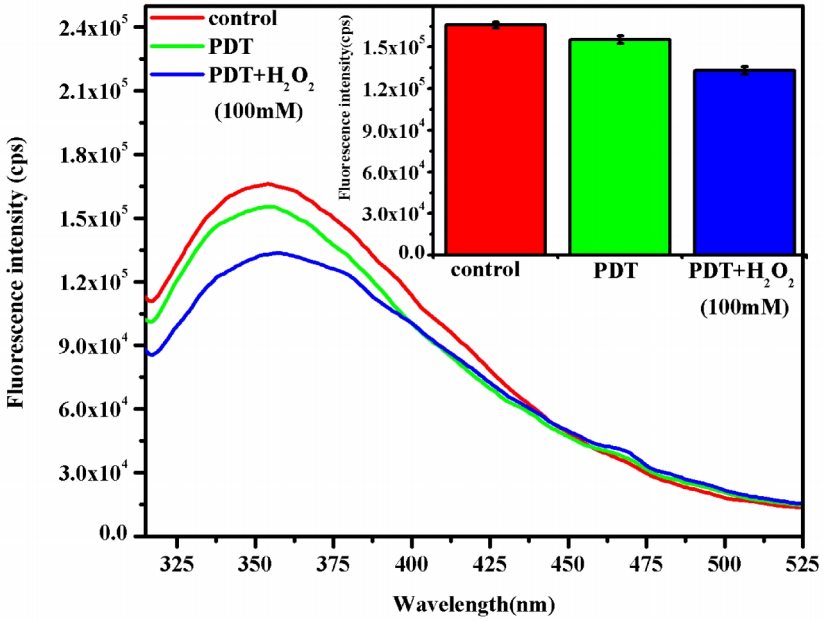


Fig. 10. The effect of APDT using PPIX (30 µM) alone or in the presence of H_2O_2 (100 mM) on tryptophan fluorescence from *S. aureus*. Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments (P<0.005)

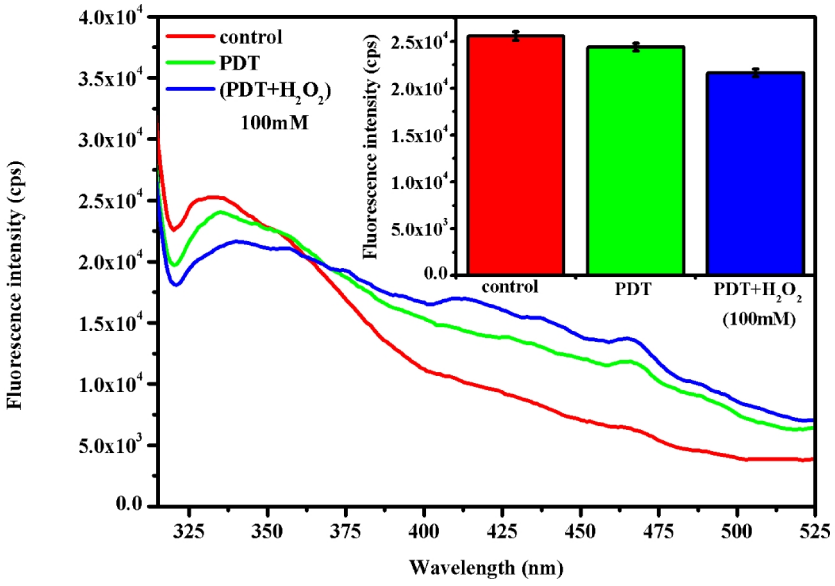


Fig. 11. The effect of APDT using PPIX (30 µM) alone or in the presence of H_2O_2 (100 mM) on tryptophan fluorescence from *P. aeruginosa*. Incubation time 15 min and the light dose 18 J/cm^2. Data are means ± standard deviation of three experiments (P<0.005)

4. CONCLUSION

The results showed in this study suggest that PPIX-H_2O_2 is a promising combination for APDT against Gram positive and Gram negative bacteria. APDT withPPIX-H_2O_2 seems significantly effective at very high H_2O_2 concentration against *S. aureus,* but its only mildly to moderately effective against *P. aeruginosa*. We achieved higher bacterial killing when PPIX in the presence of H_2O_2 illuminated with increasing light doses from the LED. The LED seems to be a very good option for PDT because of its low cost and miniature in size. Steady state fluorescence spectroscopy may be considered to characterize the molecular changes at protein level due to APDT as well as to monitor APDT efficiency. The optimized properties of PS as well as specific delivery systems will determine if APDT for bacterial infection could be considered as an alternative approach to traditional antibiotic therapy. Although, after extensive well-designed preclinical and clinical trials, this novel therapeutic approach may be considered in clinical practices for the treatment of superficial infections.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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