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In vitro antibacterial photodynamic action on nosocomial *S. aureus* using porphyrins as photosensitizers

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A B S T R A C T

The antibacterial photodynamic therapy (aPDT) exerted by porphyrins has been ascribed for the production of singlet oxygen species. This research paper evaluates the singlet oxygen production capacity and photodynamic efficacy of two neutral porphyrins, *meso*-5,10,15,20-tetrakis(4-hydroxylphenyl)porphyrin (m-THPP), *meso*-5,10,15,20-tetrakis(3-pyridyl)porphyrin (m-T3-PyP) and one cationic *meso*-5,10,15,20-tetrakis(N-methylpyridinium-3-yl)porphyrin tetra-iodide (m-T-3Py^{+}P4I^{-}) on nosocomial *Staphylococcus aureus* (ATCC #25923) strain. Absorption, emission and excitation spectra were utilised to estimate the singlet oxygen quantum yields, ($\varphi\Delta$), of the porphyrins. The singlet oxygen yields of the porphyrins were in 0.005 and 0.15 range and are considered adequate for the photoinactivation of bacteria. The antibacterial photodynamic therapy experiment revealed that the *Staphylococcus aureus colonies* were totally eliminated at a concentration of 2.50 mg/mL under light emitting diodes blue light of 470 nm wavelength for 60 minutes. This investigation further upholds the perception of aPDT as an auspicious substitute for treating antibiotic-resistant bacterial infections.

Capsule Summary: The antibacterial photodynamic therapy (aPDT) exerted by porphyrins was evaluated and the porphyrins were found to be highly active against panel of selected microbial strains.

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INTRODUCTION

Bacterial infections are usually treated using synthetic antibacterial agents. Nevertheless, several researches on health issues have indicated that resistance of bacteria to antibiotics has increased tremendously, increasing nosocomial infections in recent years. This development constitutes a big healthcare problem in hospitals which also results in high morbidity and mortality, especially in

developing countries (Ferri *et al.,* 2017; Amos-Tautua *et al.,* 2019a). Hence there is a need to search for other alternative remedies to treat infections caused by bacteria with antibacterial photodynamic therapy (aPDT) being a prospective technique. Antibacterial photodynamic therapy is centered on the proficiency of photosensitisers, including porphyrins, to react with molecular oxygen and light of a specific wavelength to destroy bacteria cells through reactive oxygen species (ROS) (Bonnett R., 2000; Almeida, 2020). In general, singlet oxygen is recognised as the principal causative lethal agent for the photoinactivation of bacteria (Fakayode *et al.,* 2017; Amos-Tautua *et al.,* 2021). Porphyrins have been regarded as proficient photosensitising drugs for the photo destruction of bacteria (Stojiljkovic *et al.,* 2001). Likewise, meso-substituted porphyrins have found specific biomedical applications, such as the treatment of bacterial infection, in the detection and treatment of neoplastic diseases (Lazzeri and Durantin, 2003; Vallecorsa *et al.,* 20212, Schulz *et al.,* 2022).

Considering the worldwide health problems caused by antibiotic resistant bacteria, this work was done to investigate the photochemical efficiency of two neutral porphyrins namely, *meso*-5,10,15,20-tetrakis(4-hydroxylphenyl)porphyrin (m-THPP), *meso*-5,10,15,20-tetrakis(3-pyridyl)porphyrin (m-T3-PyP) and one cationic *meso*-5,10,15,20-tetrakis(N-methylpyridinium-3-yl)porphyrin tetra-iodide (m-T-3Py$^+$P4I$^-$) on *Staphylococcus aureus* (ATCC #25923) strain. *Staphylococcus aureus* was particularly chosen for this investigation because it is believed to be one of the notable pathogens, accountable for 20% of hospital acquired nosocomial infections (Murray *et al.,* 2005).

MATERIAL AND METHODS

Chemicals

4-hydroxybenzaldehyde, pyrrole, dimethyl sulfoxide deuterated (DMSO-d_6), chloroform deuterated ($CDCl_3$), pyridine-3-carboxaldehyde, 1,3-diphenylisobenzofuran (DPBF), ethyl acetate (EtOAC), n-hexane, propionic acid, ethanol, acetone, dimethyl sulfoxide (DMSO), methanol (MeOH), dichloromethane (DCM), petroleum ether, methylene blue (MB), chloroform ($CHCl_3$), N,N-dimethylformamide (DMF), diethyl ether, methyl iodide (CH_3I), were procured from Sigma Aldrich (Munich, Germany). Column chromatography was conducted using silica gel 60-200 mesh. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 plates and visualized using ultra-violet (UV) lamp at 355 nm wavelength Tryptone Soya Broth (TSB), and Tryptone Soya Agar (TSA), were bought from Sigma Aldrich (Munich, Germany). Gram (+) *Staphylococcus aureus* (ATCC # 25923*)* strain was gotten from Davies Diagnostics, Johannesburg, South Africa. The antibacterial photodynamic therapy was performed at the Pharmaceutical Microbiology Unit, Department of Pharmacy, University of the Witwatersrand, Johannesburg, South Africa.

Light source

A 470-nm wavelength blue light emitting diodes (LED) Array lamp (LIU470A; Thorlabs, Newton, New Jersey, USA) consisting of 20 individual bright blue LEDs with an intensity of 4.0 mW/cm^2 (as measured from 100 mm away along the emission axis) was used for the aPDT experiments. The LED lamp with an output of 253 mW was powered by a 24 V power supply (LIU-PS; Thorlabs, Newton, New Jersey, USA).

Porphyrin photosensitizers

Synthesis of m-THPP

m-THPP was prepared according to Amos-Tautua *et al* (2019b) as presented in Figure 1. Basically, a solution of 4-hydroxybenzaldehyde (1), (2.258 g, 18.490 mmol) in propionic acid (75 mL) was refluxed for 15 min. Freshly distilled pyrrole (2), (1.25 mL, 18.632 mmol) was added quickly and the resulting mixture was refluxed for another 2 hours. Propionic acid (50 mL was removed under vacuum, and the remaining solution was cooled to room temperature and neutralized with a saturated solution of 5% $NaHCO_3$ (3×75 mL). The crude m-THPP (3) was precipitated and washed with chloroform (3×75 mL) and finally re-dissolved in 100 mL of ethanol. Crude m-THPP (1 g) was dissolved in 10 mL of MeOH, adsorbed on silica gel, and chromatographed on a silica gel (60-200 mesh) column eluting with degassed EtOAC-Hexane mixture (2:1 v/v). Pure m-THPP was obtained as a dark violet solid (Figure 3A). Yield: 3.24% (120 mg); *Rf* = 0.63 (ethyl acetate and hexane, 2:1); m.pt. >300^{o}C; IR (cm^{-1}): 3273, 2924, 2852, 1734, 1586, 1349, 1225, 750; UV-vis (C_2H_5OH) λ max (nm): Soret (419), Q-bands (520, 556, 595 and 652); Fluorescence emission λ max (nm):657 and 723; ^{1}H NMR (500 MHz, DMSO-d6) (δ) in ppm: 2.898 (s, 2H, inner pyrrole N-H); 7.975 - 7.992 (d, 8H, J=8.0 Hz, ortho-Ar-H); 7.186 - 7.202 (d, 8H, J=8.0 Hz, meta-Ar-H); 8.851 (s, 8Hβ, pyrrole); 9.959 (s, 4H, Ar-OH). ^{13}C NMR (500 MHz, DMSO-d6) (δ) in ppm: 59.69, 113.89, 119.94, 130.83 – 135.43 and 157.34; ES-MS [m/z]: 679.2306 [M+H]+, (678.7744 calculated for $C_{44}H_{30}N_4O_4$).

Synthesis of m-T3-PyP

The synthesis of m-T3-PyP was prepared according to Amos-Tautua *et al* (2019b) with slight modification (Figure 2). Pyridine-3-carboxaldehyde (1), (2.30 mL, 2.574 g, 24.031 mmol) and 1.60 mL (24.031 mmol) of freshly distilled pyrrole (2), were added to 80 mL of refluxing propionic acid in a 100 mL round-bottomed flask at 145°C for 1 h. Propionic acid was reduced by heating (down to 10 mL), and the mixture was cooled to room temperature and treated with 5% $NaHCO_3$ solution to neutralize the acid. The crude T3-PyP (3), was washed with acetone (50 mL) and filtered. 0.3 g was dissolved in 5 mL of $CHCl_3$, adsorbed on silica gel and chromatographed on a silica gel (60 - 200 mesh) column and eluted with degassed $CHCl_3$: acetone (3:7 v/v) mixture. Pure

T3-PyP was obtained as a purple solid (Figure 3B): Yield: 0.7% (105 mg); *Rf* = 0.65 ($CHCl_3$: acetone, 3:7 v/v); m.pt. >300°C; IR (cm^{-1}): 3325, 2921, 2849, 1710, 1630, 1407, 795, 618; UV-vis (C_2H_5OH) λ max (nm): Soret (418), Q-bands (487, 521, 557 and 613); Fluorescence emission λ max (nm):650, 716 and 784; ^1H NMR ($CDCl_3$-d_4). Chemical shifts (δ) in ppm: -2.833 (s, 2H, inner pyrrole N-H); 7.751 - 7.776 (dd, 4H, J=5.5 Hz, meta-Ar-H); 9.451 (s, 4H, ortho-Ar-H); 8.513 - 8.526 (d, 4H, J=6.5 Hz, ortho-Ar-H); 9.055 - 9.068 (dd, 4H, J=1.5 Hz, para-Ar-H); 8.852 (s, 8Hβ, pyrrole). ^{13}C NMR (500 MHz, $CDCl_3$-d_4) Chemical shifts (δ) in ppm: 153.65, 149.38, 116.77, 122.06, 137.65 and 140.87; ES-MS [m/z]: 619.2324 $[M+H]^+$ (618.7280 calculated for $C_{40}H_{26}N_8$).

Synthesis of cationic m-T3-Py$^+$P4I$^-$

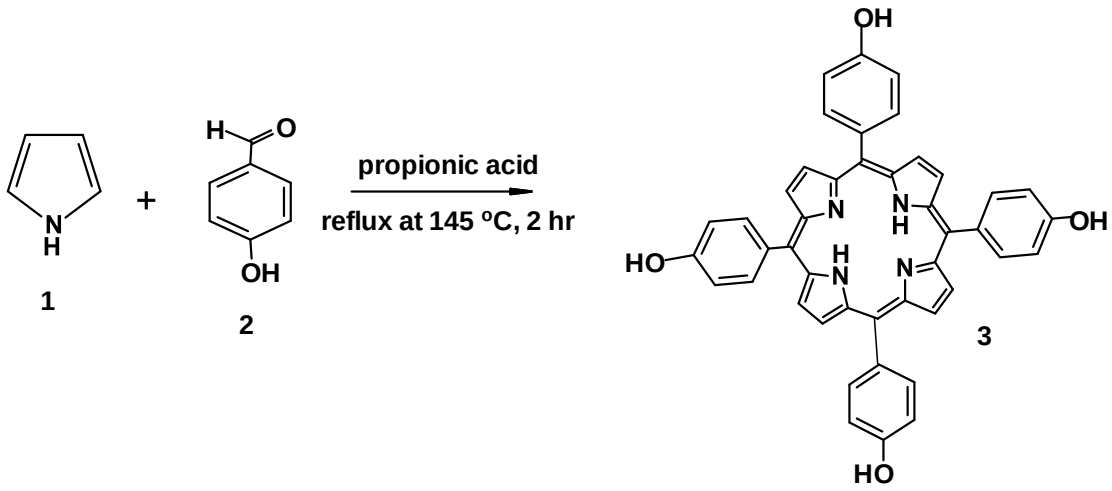

Fig. 1: Synthesis of m-THPP

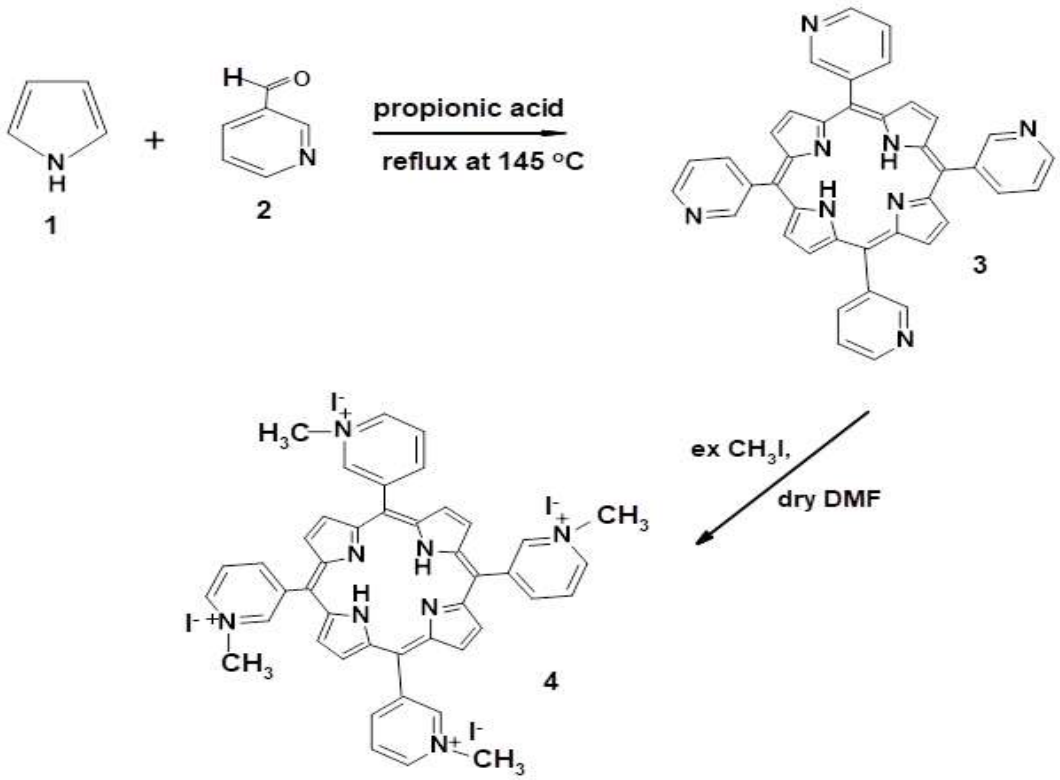

Fig. 2: Synthesis of m-T3-PyP and cationic m-T3-Py$^+$P4I$^-$

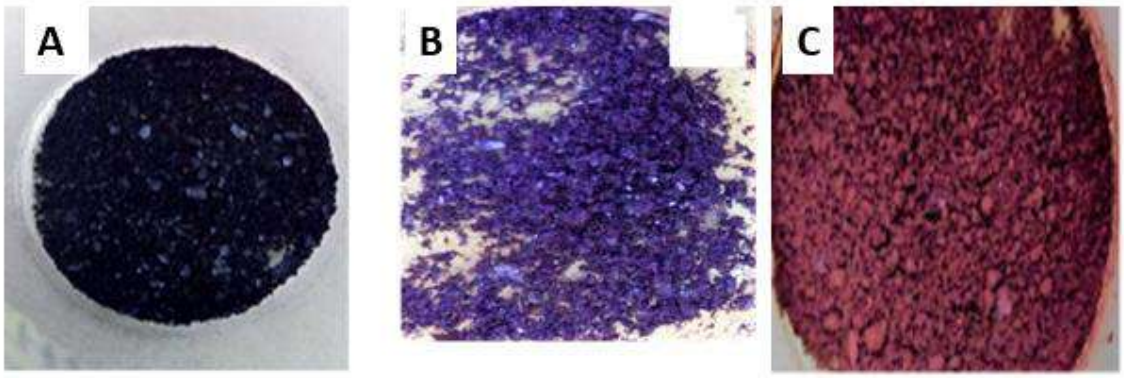

Fig. 3: Digital images of m-THPP (A); m-T3-PyP (B) *and cationic* m-T3-Py^{+}P4I^{-} (C)

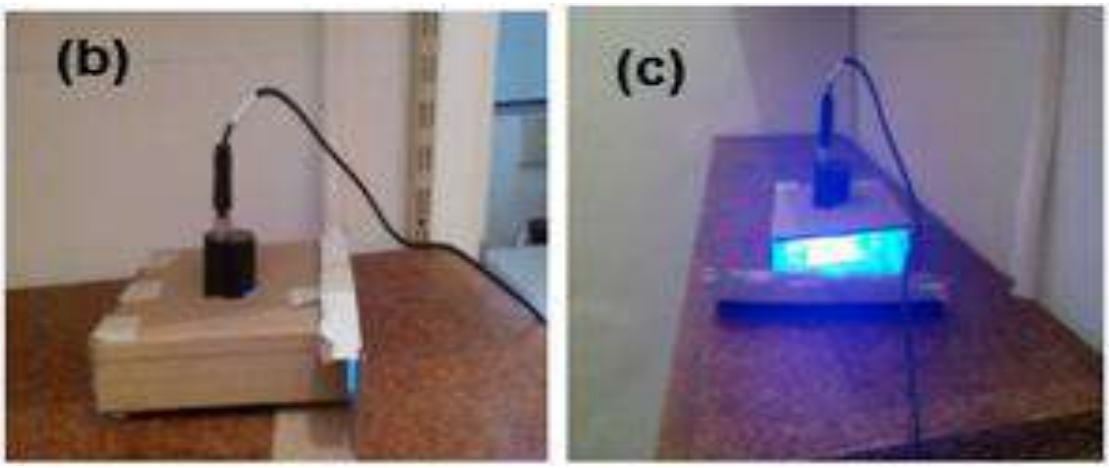

Fig. 4: A simple and cost-effective experimental set up for aPDT assay

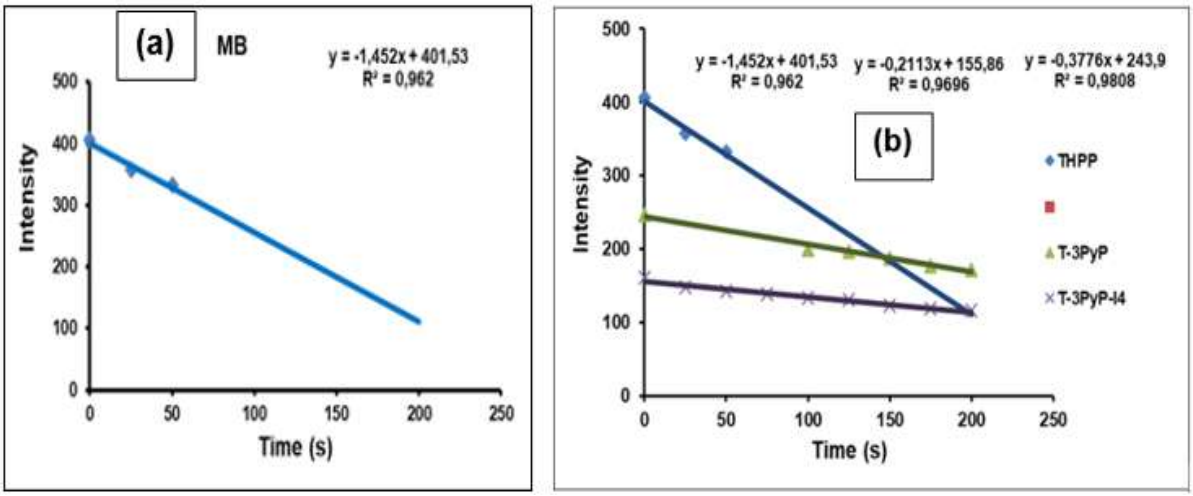

Fig. 5: Plot of fluorescence emission intensity of DPBF at 461 nm in the presence of MB (a) and different porphyrins (b) as function of irradiation time

The m-T3-PyP) was cationized according to the modified method described by Vandressen *et al.,* (2016) shown in Figure 2. Neutral T3-PyP **3,** (50.301 mg) was dissolved in dry DMF (10 mL) in a 5 mL screw cap flask and a large excess of methyl iodide (600 µL) was then added to the solution. The reaction was stirred under room temperature for 18 h in the dark and the resulting mixture was added to 20 mL of diethyl ether to precipitate the cationic m-T3-Py^{+}P4I^{-}(4) as a maroon coloured solid (Figure 3C): Yield: 83.3% (80 mg); m.pt. >400^{o}C; IR (cm^{-1}): 3445, 3396, 3035, 3015, 1635,1503, 1289 and 795; UV-vis (DMSO-d_6) λ max (nm): Soret (417), Q-bands (514, 552, 583 and 660); Fluorescence emission λ max (nm): 707 and 668; ^{1}H-NMR (500 MHz, DMSO-d_6) data (δ) in ppm: -3.125 (s, 2H, inner pyrrole N-H); 8.615-8.643 (dd, 4H,

J=7.0 Hz, meta-Ar-H); 9.995 (s, 4H, ortho-Ar-H); 9.313 - 9.327 (d, 4H, J=7.0 Hz, ortho-Ar-H); 9.555 - 9.567 (d, 4H, J=6.0 Hz, para-Ar-H); 9.259 (s, 8Hβ, pyrrole); ^{13}C NMR (500 MHz, DMSO-d_6) (δ) in ppm: 126.96, 145.98, 113.63, 140.47 and 48.99

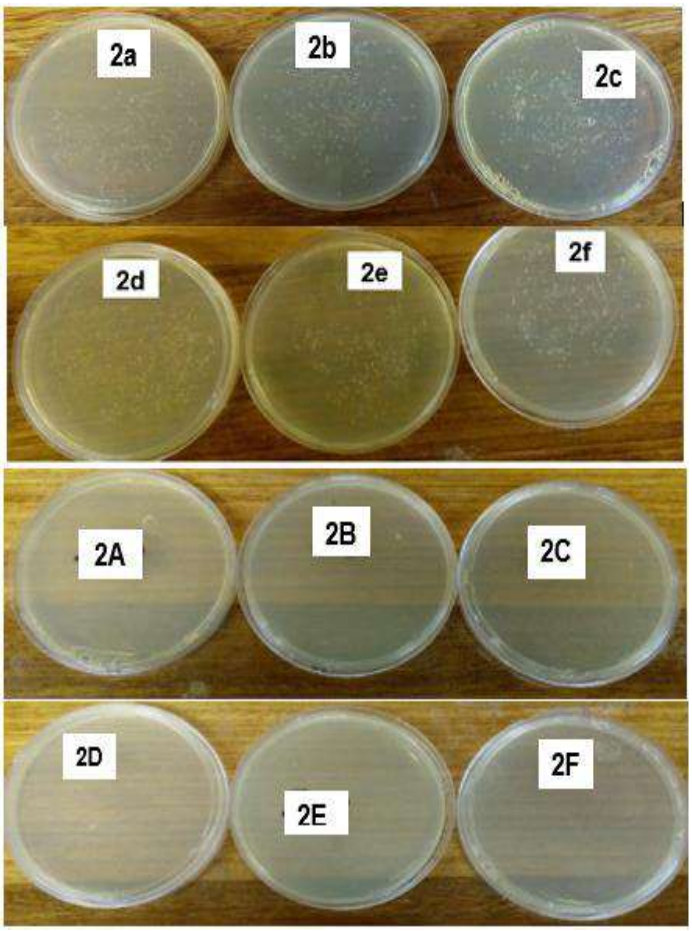

Fig. 6: Images of m-THPP elimination of *S. aureus* colonies with 470 nm blue LED light at 5.00 mg/mL concentration (2A, 2B, 2C) and at 2.50 mg/mL concentration (2E, 2F, 2G). *S. aureus* colonies in the dark control without m-THPP at 5.00 mg/mL concentration (2a, 2b, 2c) and at 2.50 mg/mL concentration (2d, 2e, 2f)

Characterization

The porphyrins, m-THPP, m-T3-PyP and m-T3-Py^{+}P4I^{-} were characterized by Fourier Transform Infrared (FTIR) spectroscopy (Spectrum two UATR spectrometer, Perkin Elmer, UK), Ultraviolet–visible spectroscopy (Perkin Elmer UV-vis Lambda 25 spectrometer, UK), photoluminescence (PL) spectroscopy (RF-6000, Shimadzu, Japan), nuclear magnetic resonance (^{1}H and ^{13}C NMR, 500 MHz Bruker). Electrospray mass spectra (ES-MS) of THPP and m-T3-PyP were recorded on a Shimadzu (Kyoto, Japan) LCMS-IT-TOF (Liquid chromatography mass spectrometry ion trap-time-of-flight) hybrid mass spectrometer.

Singlet oxygen generation of synthesized porphyrins

The singlet oxygen quantum yields of the porphyrins were determined by absorption, emission and excitation spectra performed in DMSO. The principle and method reported by Amos-Tautua *et al*., (2021) was adopted to estimate the singlet oxygen quantum yields of the synthesized porphyrins using absorption, emission and excitation spectra. Methylene blue (MB) was used as reference standard and 1,3-diphenylisobenzofuran (DPBF) as the singlet oxygen scavenger (Lutkus *et al.,* 2019). The singlet oxygen quantum φΔ was estimated using Equation 1(Adarsh *et al.,* 2010).

$$^{1}O_2^{*} = (S_1 \times Q_2 \times A_2) / (S_2 \times A_1) \quad (1)$$

Where, $^{1}O_2$* = Singlet oxygen quantum yield of sample, A_1 = Absorbance of sample at wavelength of illumination, S_1 = Slope of sample, A_2 = Absorbance of reference at wavelength of illumination, Q_2 = Quantum yield of Reference (MB), S_2 = Slope of Reference

In vitro antibacterial photodynamic therapy on *Staphylococcus aureus* (ATCC # 25923)

Sterile 96-well micro titre plates were used for the assay (0.5 mL volume). All wells were filled with 100 µL of sterile TSB. Mc Farland standard solution (10 µL) of *Staphylococcus aureus* (ATCC # 25923) was added with 180 µL of TSB media to each well. Then, 10 µL of test sample solutions at the appropriate concentrations were added to each well (See Figure 3.9.4a) and the plate was incubated for 10 min in the dark at 37°C prior to irradiation. This process was to ensure the binding of the porphyrin molecules to the cells of *S. aureus.* Thereafter, the plate was placed in a home-made cardboard box coated inside with aluminium foil and irradiated from the top by the LED-based lamp emitting blue light at 470 nm for 60 min with a fixed distance of irradiation of 15 mm from the top of the plate. The inside of the box was completely illuminated (Figure 4). After irradiation, 10 µL of solution from each well was spotted and evenly spread on TSA plates using sterile plastic spreader and incubated at 37°C overnight. The experiments were performed in triplicates. After that, the plates were examined for live bacterial growth and the surviving bacteria organisms were enumerated by colony counts.

For the dark control experiment, 10 µL of Mc Farland standard solution of *Staphylococcus aureus* (ATCC # 25923) was added to wells containing 180 µL of TSB media only without the photosensitisers. The microtiter plate was wrapped in aluminium foil and maintained at room temperature for the same 60 min illumination time. 10 µL of solution from each well was then spotted and evenly spread on TSA plates using a sterile plastic spreader and incubated at 37 °C overnight. After that, the plates were examined for live bacterial growth and the surviving bacteria organisms were enumerated as colony-forming counts. Two concentrations (5.00 mg/mL and 2.50 mg/mL) of the

photosensitisers were used for the bioassay. Both experiments were carried out in duplicate and the results reported as averages.

RESULTS AND DISCUSSION

Singlet oxygen generation of porphyrins

Table 1: Singlet oxygen yield of MB and porphyrins

Sample	Absorbance	Slope	Singlet Oxygen Yield
Methylene blue (MB)	0.169449	-17.5821	0.52
THPP	0.049314	-1.4521	0.15
T-3PyP	0.040096	-0.3776	0.05
T3-Py$^+$P4I$^-$	0.038076	-0.2113	0.03

For THPP $\frac{-1.4521 \times 0.52 \times 0.169449}{-17.5821 \times 0.049314} = 0.15$, For T-3PyP $\frac{-0.3776 \times 0.52 \times 0.169449}{-17.5821 \times 0.040096} = 0.05$ For T3-Py$^+$P4I$^-$ $\frac{-0.2113 \times 0.52 \times 0.169449}{-17.5821 \times 0.038076} = 0.03$

Table 2. *In-vitro* aPDT of as-synthesized materials on *Staphylococcus aureus* (ATCC # 25923)

Sample	No of bacterial colony (Dark)		No of bacterial colony (Light)		% SBC	% RBG
	5.00 mg/mL	2.50mg/mL	5.00 mg/mL	2.50 mg/mL		
THPP	57	71	0	0	0	100
T3-PyP	115	108	15	0	0	100
T3Py$^+$PI$^-$	127	131	0	04	3	99
*Ciprofloxacin		198		294	70	63
**Culture Control (CC)		670		547	81	0

*Ciprofloxacin = Bacterial solution + Ciprofloxacin without porphyrins
**Culture Control (CC) = only the bacteria solution alone, without porphyrins and ciprofloxacin
SBC = Surviving bacteria colony
RBG = Reduction in growth of bacteria colony

Assay controls

The followings were used as control.

1. *Only light*: bacterial samples were irradiated with light in the absence of any porphyrins. This is to rule out any inactivation due to light and heating effects.
2. *Only porphyrins*: bacterial samples with porphyrins were kept in the dark to assess the cytocidal effect (reduction of surviving bacterial growth) the porphyrins.
3. *Culture Control* using bacterial samples alone (not treated by either light or porphyrins) kept in the dark.
4. A broad-spectrum antibiotic, Ciprofloxacin with an initial concentration 0.01 mg/mL was used as a reference standard and positive control

The percentage survival of bacterial colony (SBC) was calculated using equation (2).

$$N_1/ N_0 \times 10 \quad (2)$$

Where, N_0 represents the number of bacterial colonies in samples before the irradiation (dark) and N_1 is the number of bacterial colonies in samples after light irradiation. The percentage of reduction of bacterial growth (RBG) by the as-synthesized materials was calculated using the formula Eq. 3 (Where CC is culture control).

$$\text{Reduction } (\%) = \frac{\text{Average of CC} - \text{Average of sample}}{\text{Average of CC}} \quad (3)$$

The plots of fluorescence intensity values of DPBF at 461 nm in the presence of MB and THPP, T-3PyP and T3-Py$^+$P4I$^-$ samples as function of irradiation time are shown in Figure 5. Other results such as R^2 values, slopes and estimated singlet oxygen quantum yield is given in Table 1 and φΔ for the standard MB is 0.52 as determined in various solvent media (Lutkus *et al.,* 2019).

The estimated singlet oxygen quantum yields φΔ for m-THPP, T-3PyP and T3-Py$^+$P4I$^-$ were 0.15, 0.05 and 0.03 respectively and the values are considered adequate for photodynamic action (Redmond and Gamlin, 1999; Fakayode *et al.,* 2017).

In vitro antibacterial photodynamic therapy on *Staphylococcus aureus* (ATCC # 25923)

The aPDT experiment was performed both in the dark (without light illumination) and light irradiation at two different concentrations of 5.00 and 2.50 mg/mL of the test samples. Table 2 provides the results of the aPDT experiment.

Cationic m-T3-Py$^+$P4I$^-$, m-THPP, and m-T3-PyP at the two concentrations totally inhibited the growth of *S. aureus* colonies (RGB) about 100% in each case when irradiated with blue LED. Likewise, the percentage survival of bacterial colony (SBC) of *S. aureus* when treated with the porphyrin photosensitizes and after irradiation was zero

compared to the results obtained for the positive control (ciprofloxacin with bacteria) and culture control (only bacteria) which are 70% and 81% respectively. The results confirmed the success of the photoinactivation of *S. aureus* by the as-synthesized porphyrins. Cationic m-T3-Py^{+}P4I^{-} and m-THPP completely eradicated the bacterial colonies at both concentrations of 5.00 and 2.50 mg/mL (Figure 6), whereas m-T3-PyP only eliminated the bacterial colonies completely at the lower concentration of 2.50 mg/mL. It can be explained that the photoinactivation of *S. aureus* by these porphyrins could be as a result of production of ROS during irradiation with the blue LED light. It is well known that porphyrins exert their photodynamic action on bacteria due to the presence of reactive oxygen species especially the singlet oxygen which is the major cytotoxic agent for the destruction of bacterial cell (Hamblin, 2016; Kou *et al.* 2017). Singlet oxygen attacks several targets such as proteins, cholesterol and DNA bases within a bacterial cell (Maclean et al., 2008; Zampini et al., 2017).

CONCLUSIONS

The results presented in this work have confirmed the photoinactivation of *Staphylococcus aureus* (ATCC # 25923) by 5,10,15,20-tetrakis(4-hydroxyphenyl) porphyrin, 5,10,15,20-tetrakis(3-pyridyl)porphyrin and cationic 5,10,15,20-tetrakis(N-methylpyridinium-3-yl)porphyrin tetra-iodide. The significance of this research demonstrated that aPDT produces extensive and faster inactivation of *Staphylococcus auerus* than the traditional antibiotic ciprofloxacin. This also paper reports the development of a simple and cost-effective aPDT set-up that requires less expensive materials and apparatus.

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