ANTIBACTERIAL PHOTODYNAMIC THERAPEUTIC STUDIES OF METALLATED PORPHYRIN AGAINST CHRONIC WOUND COLONISING BACTERIAL ISOLATES

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ABSTRACT
Wound infections have become life threatening as a result of treatment failures caused by multi-drug resistant pathogens. The search for newer compounds potent against antibiotic resistant bacteria associated with wounds is crucial. Hence this study investigated the application of antibacterial photodynamic therapy using meso tetra-(4-phenyl) porphyrin (TPP), metallated with zinc, tin and silver (ZnTPP, SnTPP and AgTPP), meso tetra-(4-sulphonatephenyl) porphyrin (TPPS) and the corresponding metallo meso tetra-(4-sulphonatephenyl) porphyrin (MTPPS) as photosensitizers. The in-vitro toxicity and photo-toxicity properties on four chronic wound colonizing multi-drug resistant bacterial strains: Staphylococcus aureus, Klebsiella sp., Proteus sp., and Escherichia coli were assessed using agar well diffusion method. Photo-toxicity of the compounds was investigated using 100 Watt tungsten lamp. Inhibitory activity of porphyrins tested against these bacterial strains showed Staphylococcus aureus to have both lowest (11±0.0 mm) and highest (33±1.1 mm) susceptibility to SnTPPS and ZnTPPS respectively. The sequence of data also showed appreciable improvement in the antimicrobial activities of five metalloporphyrins (SnTPP, AgTPP, ZnTPPS, SnTPPS and AgTPPS) exposed to light rays than when tested against bacterial strains in dark condition. ZnTPPS exhibited the best activity with improved photo-toxic activities against all bacterial strains (Staphylococcus aureus 33±1.1 mm, Klebsiella sp. 32±0.7 mm, Proteus sp. 28±0.7 mm and Escherichia coli 30±1.4 mm) examined in this study.
Keywords: Wound, Bacteria, Antibiotics, Porphyrins, Antibacterial Photodynamic Therapy

INTRODUCTION
Wound infections are one of the most common hospital acquired infections and it continues to be a challenging problem as a significant cause of disease burden [1]. Bacterial colonization of wounds has been reported to causing life-threatening skin infections and their proliferation at wound sites causes physical damage, emotional distress and financial loss to victims [2, 3]. Human factors such as antibiotics abuse and misuse have been reported to play critical roles in bacterial resistance to drugs used in wound treatment [4, 5]. The emergence of multi-drug resistance in bacteria has global health implications and has featured prominently as the cause of morbidity and mortality in bacteria infected patients [6]. Bacteria that are drug resistant increases the progression of chronic wounds by forming drug resistant microbial biofilms which creates a condition that promotes the inability of the dermal and epidermal tissues to respond to treatment [7].
Antimicrobial resistance is a current topic of concern attracting series of advocacy worldwide in recent times. This became imperative due to the widely accepted information on the gradual loss in antibiotics effectiveness over some period of time [8, 9]. Several bacteria have been reported to have developed resistance to some antibiotics and as such demonstrating more resistance as the year progresses. More pronounced over the years include methicillin-resistant Staphylococcus aureus, (MRSA) [10, 11] and vancomycin-resistant Enterococcus spp [12]. Rapid decline in the potency of antibiotics most importantly beta-lactam antibiotics against Gram negative bacilli has been reported [13]. There is need to source for alternative therapeutics potent enough to combat lingering worries on antimicrobial resistance widely experienced in the management of wound infections.
Antibacterial photodynamic therapy (aPDT) entails using light to destroy bacteria. The efficacy of aPDT in the treatment of resistant pathogenic strains has been well documented [14-16]. In this case, a compound used as a photosensitizer, absorbs light, gains energy and gets into an exited state. On returning to the
ground state the excited photosensitizer molecules release converting molecular oxygen to its radical species, generating more radical species such as superoxide and peroxides. These radical species oxidize biomolecules causing cell damage then death of the cells [17]. Another route is the change of triplet oxygen to reactive singlet oxygen which damages cells [18]. It is also possible that the process of cell damage is independent on oxygen, rather the biomolecules are converted to radicals by absorbing the energy released by the photosensitizer instead of oxygen [19].

Porphyrin is one of the many compounds that are being investigated in antibacterial photodynamic therapy. This compound is a macrocyclic molecule made up of four pyrrole rings joined together by methine bridges. It has an extensive conjugated pi bond system consisting of 22 pi electrons and 18 are delocalized, hence, absorbs light within the ultra-violet and visible regions of the electromagnetic spectrum [20]. It has been found that neutral or anionic photosensitizer’s molecules more readily bind and destroy Gram positive bacteria but Gram negative bacteria are resistant [21]. In a report by Tavares et al. [22], a single treatment of aPDT using 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)-porphyrin triiodide (Tri-Py^-Me-PF) was reported to be very effective against Vibrio fischeri and Escherichia coli. After ten generations of partially photosensitized cells, none of the bacteria recovered their viability nor developed resistance to the aPDT.

It has been reported that apart from the etiologic causes of chronic wound, it is important in addressing wound care [7]. Moreover, the increasing loss of activity of antimicrobial agents in the treatment of microbial infection is worrisome and it is imperative in the search for newer compounds potent against antibiotic resistant bacteria associated with wounds. Hence, this study investigates the antibacterial photodynamic therapy (aPDT) activities of metallated derivatives of neutral (TPP) and anionic (TPPS) porphyrin porphyrins as an alternative treatment on some selected wound isolates.

**MATERIALS AND METHODS**

**Preparation of porphyrin and metalloporphyrins**

Free base TPP, TPPS and their corresponding metalloporphyrins (M) which included Zinc (Zn), Tin (Sn) and Silver (Ag) (Figure 1) were synthesized and characterized using UV-Vis spectroscopy, IR spectroscopy, ^1^H-NMR and ^13^C-NMR spectroscopy.

![Figure 1: Structures of the porphyrin molecules (TPP, TPPS, MTPP, MTPPS)](image)

**Synthesis of Meso-tetra(4-phenyl) porphyrin, (TPP)**

TPP was prepared according to Linsdey et al. [23] and Nascimento et al. [24] with little modification. Benzaldehyde (7.32 mL, 0.0721 mol), freshly distilled pyrrole (5.0 mL, 0.0721 mol), propanio acid (14.0 mL) and nitrobenzene, (6.0 mL) were refluxed for 2 h.
Propanoic acid and nitrobenzene served as catalyst and oxidant respectively. At the expiration of time, the resulting black slur was homogenized with methanol and allowed to stand for 24 h to allow the methanol and other volatile compounds to evaporate. TPP was purified by column chromatography using a solvent system of dichloromethane/n-hexane ratio 4:1 and deep wine coloured eluent (desired product) was collected into a flask and the solvent was evaporated on a rotary evaporator. TPP was recrystallized using a dichloromethane methanol mixture (ratio) and the purple crystals was collected by filtration and air dried.

**Synthesis of Free Base Meso –Tetra (4-sulphonatophenyl)porphyrin, (TPPS)**

Following a procedure describe by Srivastava and Tsutsu, [25], TPP, (2.0 g, 3.261 mmol) was dissolved in concentrated tetraoxosulphate (VI) acid (30 mL) in a 50 mL round-bottomed flask equipped with a drying tube under reflux at 70 °C for 120 h with constant stirring. At the expiration of the time, the reaction vessel was cooled in ice for 20 minutes with continuous stirring before adding 150 mL of deionized water dropwisely. NaOH solution (0.5 M, 20 mL) was added slowly to the mixture with gentle stirring to neutralize unreacted acid and convert TPPS to its sodium salt. This was indicated with a colour change from green to purple. The solution was left in the oven for 20 h at 80 °C to remove water molecules present. In other to remove Na₂SO₄ formed, the dried product was dissolved in methanol and the insoluble sulphonate was filtered out followed by evaporation of the methanol in vacuo to obtain the reddish brown–solid TPPS.

**Synthesis of Metalloporphyrin (M = Zn, Sn, and Ag)**

The synthesis of all the metallated porphyrin were prepared according to Alders et al. [26] method. 24.22 mmol of Zn(OAc)₂, Ag(OAc)₂ or SnCl₂ and TPP (0.25 g, 0.404 mmol) was dissolved in DMF (10 mL), refluxed for 12 hours at 120 °C with continuous stirring. During the formation of the complex, the porphyrin molecules loses two of its central hydrogen atoms and becomes di-anion, electrostatic forces ensuring binding of the metal to these sites often termed as metallation [27]. Changes in the UV-Vis spectra was used to confirm the insertion of the metal into the cavity of porphyrin ring. In a separating funnel, the metallated TPP, distilled water (20 mL) and chloroform (10 mL) was added and MTPPP was extracted into chloroform. The chloroform was evaporated leaving behind the purple coloured ZnTPP, AgTPP and SnTPP, respectively. For the MTPPS series the metal salts (24.22 mmol) were dissolved in 10 mL of DMF and TPPS (0.404 mmol) was dissolved in 10 mL of methanol. The two solutions were mixed together and refluxed for 12 h at 120 °C and the metalaion reaction was monitored using UV-Vis spectroscopy. The methanol was removed by evaporation and precipitating out the MTPPPS which is insoluble in DMF. It was filtered out and washed in acetone.

**Antibacterial Photodynamic Studies**

**Source of Test Bacterial Isolates**

The bacterial strains which included Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Klebsiella sp.*, *Proteus sp.*, and *Escherichia coli*) which were initially isolated from chronic wounds were obtained from the Department of Medical Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.

**Antibiotic susceptibility testing of test organisms**

The antibiotic susceptibility of the bacterial strains was determined by the Kirby-Bauer disc diffusion method and zones of inhibition measured in millimeter (mm) were interpreted accordingly [28]. The antibiotic discs used for the Gram positive and Gram negative bacterial strains include Amikacin (30 µg), Ampicillin (10 µg), Amoxicillin/clavulanic acid (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Cefuroxime (30 µg), Chloramphenicol (10 µg), Ciprofloxacin (5 µg), Cotrimoxazole (25 µg), Erythromycin (5 µg), Gentamicin (10 µg), and Tetracycline (30 µg) (Biomark Laboratories, India). Pure colonies from the overnight culture of each of the four test strains were dislodged in sterile normal saline and vortex mixed. The cell suspensions were adjusted to 0.5 McFarland standard at 540 nm wavelength. Each strains were seeded using sterile swab sticks on Mueller-Hinton agar (MHA) plates and thereafter impregnated with the antibiotic discs. Plates were observed for zone of inhibition (mm) after 18-24 h.
Antibacterial activities of Synthesized Porphyrin derivatives

Toxicity and Photo-toxicity properties of the laboratory synthesized Porphyrin derivatives were evaluated against the four test organisms by agar well diffusion method of Gyulkhandanyan et al. [29] with modifications. Pure colonies from the overnight culture of each of the four test isolates were dislodged in sterile normal saline and vortex mixed. The cell suspensions were adjusted to 0.5 McFarland standard at 540 nm wavelength. Briefly, 30 mg of the porphyrin derivatives were dissolved in 1 mL of their respective solvents (methanol and sterile distilled water) to form a working concentration. Thereafter, 18 mL Mueller-Hinton agar (MHA) plates were evenly seeded across the set MHA plates with the standardized inoculum using sterile swab sticks. Agar wells (7 mm diameter) were drilled on the seeded Muller-Hinton agar plates using a sterile cork borer and 0.3 mL of the porphyrin derivatives working solution were dispensed in the wells for each of the test bacterial isolates. The experimental procedures were carried out in duplicates.

First sets which were observed for phototoxic activities were incubated in a controlled environment containing a light source of 100 Watt tungsten lamp placed at a distance of about 35 cm from the seeded plates as reported by Fayyaz et al. [30]. A glass tray (15 cm high and 30 cm wide) filled with about 5 L of water was placed between the lamp and the plates to absorb heat. Toxicity activities were assessed in the second sets as they were incubated at 37 °C for 18 – 24 h in a dark condition. Streptomycin sulphate (84 mg/mL) was introduced in a well as positive control, while sterile distilled water was used as negative control. Incubated plates were checked for zones of inhibition.

Statistical analysis

Data obtained were statistically analyzed and expressed as mean ± standard deviation (SD) using statistical software Graph Pad Prism version 6.01.

RESULTS AND DISCUSSION

Synthesis of the Porphyrin Derivatives

Above 50 % yield of purple crystals were obtained with a melting point above 300 °C. It was found to be insoluble in water and methanol but soluble in some of the polar solvents such as CH_3OH, CHCl_3, DCM. TPPS was prepared by sulphonating TPP with conc. H_2SO_4 and a yield of about 67 %, having a melting point above 345°C. Inserting sulphonate groups on the meso-phenyl groups did not only make the compound to become negatively charged but soluble in water and methanol. From the spectroscopic results this sulphonation occurred at the para-position of the phenyl ring. The metallated TPP was insoluble in water while the metallated TPPS were soluble in water.

When the compounds were subjected to UV-visible absorption spectrum, two distinct regions were observed. An intense Soret or B-Band which occur as a result of strong transition to the second excited state (S_0 → S_2) at approximately 400 nm and four weak bands (Q-bands) occurring between 516 and 645 nm which is a weak transition to the first excited state (S_0 → S_1) (Figure 2). This is a general feature of free-base porphyrin molecule and was explained by Gouterman’s four-molecular orbital’s model theory [31]. According to this theory the two major regions arise from coupling of the two transitions between the HOMO and LUMO (\(e_g^* \sim a_{1u}; e_g \sim a_{2u}\)) and due to the closeness in energy of \(a_{1u}\) and \(a_{2u}\) orbitals, coupling of these transition dipoles made possible. The weaker absorbance around 600–650 nm (Q-bands) resulted from the transition dipoles which nearly cancelling each other while the high energy Soret band (B-band) between 400–430 nm resulted from a linear combination of the two transitions with reinforcing transition dipoles and is therefore very intense.

The UV-Vis absorption spectrum of TPPS also has the same feature of free-base porphyrin, four Q-bands at 510, 553, 582 and 628 nm and a Soret band at 413 nm. However, the electron withdrawing property of the sulphonate groups resulted in a hypsochromic shift of the Soret-bands with respect to TPP indicating an increase between the HOMO-LUMO band gaps (Figure 2a). The symmetry of the molecules changes from a \(D_{2h}\) for the unmetallated porphyrin to a \(D_{4h}\) in the metallated ones causing the HOMO to become degenerate resulting in a change in the spectra from having four Q-bands (Figure 2a) to two (Figure 2b and 2c inert) with slight shift in the B-bands [32]. These were observed in zinc and tin porphyrin complexes but the single Q-band of the silver porphyrin complexes...
Figure 2: UV-Vis spectra of (a) TPP and TPPS (b) ZnTPP, SnTPP and AgTPP (c) ZnTPPS, SnTPPS and AgTPPS
Table 1: IR data of the porphyrin molecules (cm$^{-1}$)

| Porphyrin   | $\nu_{\text{OH}}$(H$\text{H}_2\text{O}$) | $\nu_{\text{N-H}}$ | $\nu_{\text{C}=$CH(ar) | $\nu_{\text{C}=$C | $\nu_{\text{C}=$N | $\nu_{\text{S}=$O | Porphyrin | M-N |
|-------------|---------------------------------|-----------------|-----------------|-----------|------------|-----------|--------------|-----|
| TPP         | -----                           | 3312            | 3090            | 1593      | 1358       | 1380      | -----        | -----|
| TPPS        | -----                           | 3315            | 3052            | 1595      | 1350       | 1365      | 1300         | 1000 |
| ZnTPP       | -----                           | 2928            | 1593            | 1383      | 1378       | -----     | 1004         | 605 |
| SnTPP       | -----                           | 3012            | 1590            | 1413      | 1343       | -----     | 1021         | 595 |
| AgTPP       | -----                           | 3019            | 1573            | 1415      | 1348       | -----     | 1020         | 651 |
| ZnTPPS      | 3500                           | 3005            | 1573            | 1346      | 1361       | -----     | 1004         | 692 |
| SnTPPS      | 3450                           | 2996            | 1621            | 1400      | 1339       | -----     | 1040         | 640 |
| AgTPPS      | 3430                           | 3265            | 1654            | 1408      | 1342       | -----     | 1018         | 650 |

is due to the large size of the silver ion, it could not fit into the cavity thereby causing a change in conformation of the ring [33-35]. The infrared absorption spectra (Supplementary Information I) of the compound presented in showed some peaks reflecting some of the functional groups found on the porphyrin. The bands at 3314 cm$^{-1}$ and 964 cm$^{-1}$ were attributed to the presence of 2° amines (N-H) stretching and bending respectively. The bands at 3012, 1599, 1458, and 1350 cm$^{-1}$ were assigned CH (aromatic), C=C (aromatic), C=N and C-N groups respectively. Porphyrin skeletal has IR vibrational frequency around 1250 - 1001 cm$^{-1}$. All these are in agreement with literature results [24]. The IR absorption frequencies (Supplementary Information II) showed a prominent peak of ~ 3500 cm$^{-1}$ which was very broad and was assigned OH of H$_2$O which was absent in that of TPP. The bands at 3414 cm$^{-1}$ and 987 cm$^{-1}$ were attributed to the presence of 2° amines (N-H) stretching and bending respectively. The bands at 3012, 1599, 1458, and 1350 cm$^{-1}$ were assigned CH (aromatic), C=C (aromatic), C=N and C-N groups respectively. Another band of interest was the band occurring around 1145 to1114 cm$^{-1}$ assigned to S=O of SO$_3$ which again was absent in the parent porphyrin. All other IR bands were similar to those found on TPP and the TPPS. For the metallated porphyrins the band between 590 – 700 nm are indication of M-N bonds [27]. The key method of confirmation of metalation of porphyrin is the distinct change in the UV-Vis spectra. Table 1 shows the summary of the significant IR bands of the porphyrins. Anisotropy of the aromatic ring due to external magnetic field of the NMR machine affects the position of the proton NMR signals. This is because the external magnetic field ($H_0$) causes the delocalized $\pi$-electrons of the ring to move. The movement of these $\pi$-electrons generates an induced magnetic field ($H_1$). The direction of this induced field outside the ring is in the same direction as the external field ($H_0$) while the direction of the induced field inside the ring is opposite to the external field. From the $^1$H -NMR analysis of TPP (Supplementary Information III) in CDCl$_3$ at 298K it was found that the N–H protons of TPP appeared at 1.27 ppm. Any protons (N–H protons) in the center cavity of porphyrin were intensely shielded resulting in their chemical shift being farther than TMS because it opposing direction of the induced magnetic field [36]. The same diamagnetic ring current that shields the protons inside the macrocycle, deshields the protons outside of the macrocycle from the
external field. Hence, any proton outside the ring experiences have their chemical shift usually more upfield compared to that of the vinylic protons in cyclohexene (δ = 5.6 ppm). The β-pyrrole protons and meso-protons are included in this category of protons. Here the macrocycle is made up of two pyrrole units and two pyrrolene units, the pyrrole units have an aromatic sextet π-electrons while only five π-electrons are in pyrrolene units. To compensate for this shortage of π-electrons in the pyrroline, an electron is pulled from the adjacent meso-carbon.

So, meso carbons have a tendency to be electron deficient and thus shifted further downfield than the β-protons. The β-pyrrolic protons appeared as singlet around 8.9 ppm (8H) due to the deshielding effect of the aromatic porphyrin 13C-NMR of TPP (Supplementary Information IV) had eight signals comprising five methine (a, b, c, d, and e) and three quaternary carbons (f, g and h). The methine carbons (c, d and e) are ring. The signal of the ortho phenyl proton (8H, d) appeared at 8.4 ppm due to the macrocyclic deshielding effect while those of the meta and para phenyl protons (12H, m) appeared upfield around 7.8 ppm the one on the phenyl groups (three) and other two on the pyrrole rings (β-carbons). The methine carbons signals were assigned the chemical shift 129.26 ppm (o-phenyl), 127.69
ppm \((m\text{-phenyl})\), and 126.66 ppm \((p\text{-phenyl})\), respectively. While the \(\beta\)-carbons (a and b) has the chemical shift 123.46 and 120.11 ppm, respectively. The three quaternary carbons are carbon on phenyl groups (carbon-h) which were highly deshielded appeared around 142.14 ppm. The carbon at the meso position (carbon-g) which appeared around 134.13 ppm, \(\alpha\)-carbons (carbon – f) of the porphyrin ring appeared around 131.15 ppm. The summary of the molecular characterization of TPP is presented in Table 2.

The \(^1\)H-NMR of TPPS (Supplementary Information V) had the two internal protons signal appeared at negative chemical shift (−3.0 ppm). The \(\beta\)-pyrrolic protons appeared at 8.56 ppm (8H, s) and the para protons signal of the phenyl groups disappear due to sulphonation. The remaining two signals present an integral value of 7.95 ppm (8H, d; \(o\)-phenyl) and 7.55 ppm (8H, d; \(m\)-phenyl). The electron withdrawing property of the sulphonate groups has the change in the chemical shift with respect to TPP. \(^{13}\)Carbon-NMR spectra were severely complicated by the effect of aggregation and therefore quantitative interpretation was not possible [37]. Table 3 is a summary of the molecular characteristics of TPPS.

| Table 2: Summary of the Molecular Characterization of TPP |
|---|---|
| **Parameters** | **Results** |
| **Yield:** | 0.53 g (55.4 %) |
| **UV/Vis \(\lambda_{\max} \text{nm, CHCl}_3\):** | 419, 510, 550, 590 and 645 |
| **IR (KBr pellets) \(\nu_{\max}/\text{cm}^{-1}\):** | 3314 (N-H, \(2\text{^o}\) amine), 3092 (\(\text{CH}_{\text{sp}^{2}}\)), 1599 (C=C, phenyl), 1458 (C=N), 1350 (C-N), 1001–1250 porphyrin skeletal |
| **\(^1\)H-NMR (CDCl\(_3\)) \(\delta\) (ppm):** | 8.90 (8H, s; pyrrole-H), 8.23 (8H, d; \(o\)-phenyl-H) 8.78 (8H, d; \(p\)-phenyl-H), 1.25 (2H, s; N-H) |
| **\(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) (ppm):** | 142.14 (quaternary-C, Ar); 134.53 (quaternary-C, C\(_{\text{ms}}\)); 134.15 (quaternary-C, C\(_{\text{ar}}\)); 129.26, 127.69, 126.66 (methine-C, Ar); 123.45, 120.11 (methine-C \(\beta\)-pyrrolic carbons) |

| Table 3: Summary of the Molecular Characterization of TPPS |
|---|---|
| **Parameter** | **Result** |
| **Yield:** | 0.78 g (67 %) |
| **UV/Vis \(\lambda_{\max} \text{nm, (CHCl}_3\):** | 413, 510, 553, 582 and 628 |
| **\(^1\)H-NMR (CDCl\(_3\)) \(\delta\) (ppm):** | 8.56 (8H-s; Pyrrole-H), 7.95 (8H, d; \(o\)-Phenyl-H), 7.66 (8H, d; \(m\)-Phenyl-H), 1.25 (2H, s; N-H) |
| **\(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) (ppm):** | (very poor due to aggregation) |
| **IR [(KBr pellets) \(\nu_{\max}/\text{cm}^{-1}\):** | 3448 (OH of H\(_2\)O), 3414 (N-H, \(2\text{^o}\) amine), 3012 (\(\text{CH}_{\text{sp}^{2}}\)), 1599 (C=C phenyl), 1458 and 1350 C=N and C-N groups, 1153.47 \(-1076.32 \text{ (S=O)}\). |
Antibacterial Susceptibility Profile of the Bacterial Isolates

Staphylococcus aureus was resistant to 2 of the 6 antibiotics tested while the Gram negative bacterial isolates were resistant to 6 out of the 10 antibiotics used as shown in Table 4.

Table 4: Antibiotics Susceptibility Profile of the Bacterial Isolates using CLSI standard (Zone of inhibition in mm)

| Gram-positive Antibiotics (Concentration) | S. aureus | Gram-negative Antibiotics (Concentration) | E. coli | Klebsiella sp. | Proteus sp. |
|------------------------------------------|-----------|------------------------------------------|---------|----------------|-------------|
| Ciprofloxacin (5 μg)                     | 29 (S)    | Amikacin (30 μg)                         | 26 (S)  | 23 (S)         | 29 (S)      |
| Erythromycin (5 μg)                      | 28 (S)    | Ciprofloxacin (5 μg)                     | 25 (S)  | 23 (S)         | 16 (S)      |
| Gentamicin (10 μg)                       | 15 (S)    | Gentamicin (10 μg)                       | 17 (S)  | 17 (S)         | 15 (S)      |
| Tetracycline (30 μg)                     | 22 (S)    | Tetracycline (10 μg)                     | 16 (S)  | 17 (S)         | 15 (S)      |
| Ampicillin (10 μg)                       | R         | Cefotaxime (30 μg)                       | R       | R              | R           |
| Amoxicillin/clavulanic acid (30 μg)      | R         | Ceftazidime (30 μg)                      | R       | R              | R           |
|                                          |           | Ceftriaxone (30 μg)                      | R       | R              | R           |
|                                          |           | Cefuroxime (30 μg)                       | R       | R              | R           |
|                                          |           | Chloramphenicol (10 μg)                  | R       | R              | R           |
|                                          |           | Cotrimoxazole (25 μg)                    | R       | R              | R           |

S = Susceptible; R = Resistant

3.3 Antibacterial activities of Synthesized Porphyrin derivatives

The antibacterial evaluation of free-base TPP, TPPS and their metalloporphyrins against Staphylococcus aureus, Klebsiella sp., Proteus sp., and Escherichia coli (Figure 3 and 4) showed values of inhibition range between 11±0.0 – 33±1.1 mm. Cumulative mean values presented in Figure 5 showed no photodynamic activity in free-base TPP compounds against any of the bacterial isolates.

Figure 3: TPP and metallated derivatives (a) dark and (b) light condition
Antibacterial activities of neutral TPP, and its metalloporphyrins as shown in Table 5, indicates silver porphyrins showed moderate and improved photo-toxic activity from the initial value 12±0.6 mm to 17±1.4 mm. *Staphylococcus aureus* and *Escherichia coli* were susceptible to anionic TPPS and metallated derivatives assessed with value range of 11±0.0 – 33±1.1 mm and 13±0.3 – 33±2.1 mm as shown in Table 5. It was observed in this present study that free-base anionic TPPS and its metal-base derivatives showed excellent biocidal action on three of the test isolates (*Staphylococcus aureus*, *Klebsiella* sp. and *Escherichia coli*) with an increase zone of inhibition when exposed to light (Table 6). Potentiation of TPPS with Zinc metal improved its biocidal activities on *Proteus* sp. with an increased toxicity under light rays.

This corroborates the findings of Zoltan *et al.* [38] who observed the efficient inactivation of *E. coli* with metallated porphyrins. Also Bondi *et al.* [9] reported a significant increase in susceptibility in Gram-negative bacteria with the addition of EDTA before photo-activation. Comparison study of the metalloporphyrins showed anionic TPPS is effective across the three experimented metal as compared with TPP which showed average toxicity properties against *Staphylococcus aureus*, *Klebsiella* sp. and *Escherichia coli* when conjugated with silver. However, this does not agree with a report which showed that anionic porphyrins proved to be more effective only on Gram–positive bacteria as compared to Gram–negative bacteria [21].
Table 5: Antibacterial activity of free-base TPP and MTPP against the bacterial isolates (zone of inhibition in mm)

|          | TPP | ZnTPP | SnTPP | AgTPP | Streptomy 
|----------|-----|-------|-------|-------|cin Sulphate |
|          | Dark | Light | Dark  | Light | Dark  | Light | Dark  | Light |
| **S. aureus.** | 0    | 0     | 16 ± 0.7 | 13 ± 0.7 | 12 ± 0.6 | 17 ± 1.4 | 35 ± 1.4 |
| **Klebsiella sp.** | 0    | 0     | 0     | 0     | 12 ± 0.6 | 16 ± 1.4 | 27 ± 2.8 |
| **Proteus sp.** | 0    | 0     | 0     | 0     | 0     | 0     | 27 ± 1.4 |
| **E. coli** | 0    | 0     | 0     | 0     | 14 ± 0.6 | 12 ± 0.6 | 15 ± 0.6 | 35 ± 2.8 |

Table 6: Antibacterial activity of free-base TPPS and MTPPS against the bacterial isolates (zone of inhibition in mm)

|          | TPPS | ZnTPPS | SnTPPS | AgTPPS | Streptomy 
|----------|------|--------|--------|--------|cin Sulphate |
|          | Dark | Light | Dark  | Light | Dark  | Light | Dark  | Light |
| **S. aureus.** | 18 ± 0.8 | 18 ± 0.8 | 31 ± 1.4 | 33 ± 1.1 | 11 ± 0.0 | 15±0.7 | 14 ± 0.7 | 15 ± 0.7 | 35 ± 1.4 |
| **Klebsiella sp.** | 20 ± 1.4 | 16 ± 0.6 | 30 ± 1.2 | 32 ± 0.7 | 0 | 17±0.7 | 15 ± 2.8 | 17 ± 0.8 | 27 ± 2.8 |
| **Proteus sp.** | 0 | 0 | 23 ± 2.1 | 28 ± 0.7 | 0 | 0 | 0 | 0 | 27 ± 1.4 |
| **E. coli** | 33 ± 2.1 | 17 ± 0.7 | 26 ± 0.7 | 30 ± 1.4 | 13 ± 0.3 | 20±0.7 | 14 ± 0.4 | 17 ± 0.7 | 35 ± 2.8 |
Consequently, the zone of antimicrobial inhibition values recorded under light conditions were higher for anionic TPPS compounds than in dark condition, showing some levels of improved photo-toxicity. Photo-activation of some of these metalloporphyrins explains the photo-killing attributes and enhancement strategy for neutral (TPP) and anionic (TPPS) porphyrins. Studies have also shown that the absorbance of these light rays activates the production of reactive oxygen species such as singlet oxygen and superoxide dismutase [39, 48]. This reactive oxygen causes cell disruption otherwise known as respiratory burst in bacteria cells [41].

*Staphylococcus aureus* (Gram-positive) was observed as the most susceptible organism in the study and corroborated the report of Hanakova et al. [14], showing their susceptibility to the photosensitisers at different concentrations. This could be attributed to the thick but porous peptidoglycan layer and the simple inner cytoplasmic bilayer membrane structure of Gram (+) cells [42]. This permits the diffusion, penetration and toxicity actions of some porphyrin compounds assessed in this study. However, *Proteus* sp. an organism that is more pronounced in wound infections was susceptible to ZnTPPS only. The swarming pattern, high cell surface hydrophobicity expressed by this Gram-negative bacterium could have possibly reduced its electrostatic interaction with compounds, thus, conferring a mode of resistance on the bacterial isolates as observed in this study [43].

**CONCLUSION**

It was observed in this study, that metallated neutral TPP and anionic TPPS compounds showed antibacterial potential against the studied isolates. The antibacterial effectiveness of these compounds were also observed especially in TPPS with an increased antibacterial activity on Gram–positive and Gram–negative bacteria isolates when exposed to light rays. Hence, this may suggest the antimicrobial potential of anionic TPPS and its metal derivatives (zinc, tin and silver) that could be used in antimicrobial photodynamic therapy (aPDT) for wound decontamination to ensuring rapid healing process.

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