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Synthesis, Characterization and the Photodynamic Activity against Some Gram Negative and Positive Bacteria of Novel Subphthalocyanine Derivative

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ABSTRACT

A specific tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)Subphthalocyaninehydroxyde (SubPc-1) derivative was synthesized and the structure of SubPc-1 was confirmed by UV-Vis, Fluorescence, FTIR, ^{1}H NMR, ^{13}C NMR and MALDI-TOF-MS spectroscopy. Its thermal stability and photostability tests were checked by using TGA and solar simulator, respectively. In addition, the singlet oxygen produced capacity of amphiphilic SubPc-1 was determined for PDT studies. Besides, the photodynamic activity of this novel product against some of gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and gram positive bacteria (*Staphylococus aureus, Enterococcus faecalis*) has been investigated by using a xenon lamp which emits light in the ultraviolet-visible region and is simulated for solar light as the illumination source. It was determined that the peripheral hydrophilic groups provided great opportunities in inactivation and solubility studies. This new amphiphilic macromolecule was especially found as an effective photosensitizer against gram positive bacteria in PDT, in particular.

Key Words: *Subphthalocyanine, photosensitizer, biological activity, bacteria, photodynamic inactivation.*

1. INTRODUCTION

Subphthalocyanines (SubPcs) are the lowest homologues of phthalocyanines, well-known two-dimensional 18 π-electron aromatic systems with unusual electrical and optical properties. They are composed of three diiminoisoindole rings N-fused around a boron core. Their 14 π-electron aromatic core along with their nonplanar cone-shaped structure make them attractive compounds due to their chemical and physical properties [1].

The first boron complex of the subphthalocyanine was introduce by Meller and Ossko in 1972 [1,2]. Research on subphthalocyanines remained fairly untouched until 1990 when the tri-*tert*-butyl substituted macrocycle was described by Kobayashi et al [3]. The interest in SubPc macrocycles suddenly grew a great deal because of their use as starting materials in the synthesis of unsymmetrical phthalocyanine and materials that have particularly high non-linear optical sensitivity in second-harmonic (SHG) and third–harmonic generation (THG). Only the boron derivatives of these compounds are known and their structures are non-planar.

SubPcs can be converted to phthalocyanines by reacting with 1,3-isoindolinediimine. Such a ring-expansion reaction of SubPcs is a very attractive way for the preparation of pure unsymetrical substituted phthalocyanines [4,5].

SubPcs are non-planar aromatic macrocycles that differ from their higher homologues, phthalocyanines, in that they comprise three isoindole units around a boron atom. Whereas SubPcs have shown very interesting photophysical properties, their ability to promote intramolecular charge or energy transfer processes in electroactive dyads, still remains unexplored [6].

Their purple colour is the basis of attractive dyes for electrolithography or printing. The properties of their exited states make them good photosensitizers with potential applications in photodynamic therapy (PDT) [1,7]. This therapy is an innovative treatment for several types of cancer and based on the administration of a photosensitizer, which is selectively incorporated in tumor cells. The subsequent exposure to visible light in the presence of oxygen specifically inactivates neoplastic cells [8-21].

It is widely believed that during PDT, the photosensitizer is excited to its triplet state. After that, it transfers the energy to the triplet state oxygen O_2 ($^3\Sigma_g$) in order to form the excited-state singlet oxygen, O_2 ($^1\Delta_g$), through the so called Type II mechanism. The O_2 ($^1\Delta_g$) species are believed to be the chief agent in PDT line of work. However, Type I mechanism involves electron transfer from the excited photosensitizer to the triplet state oxygen or to the biological substrate. Both Type I and Type II mechanisms may be involved during PDT period [5,10,11, 19-23].

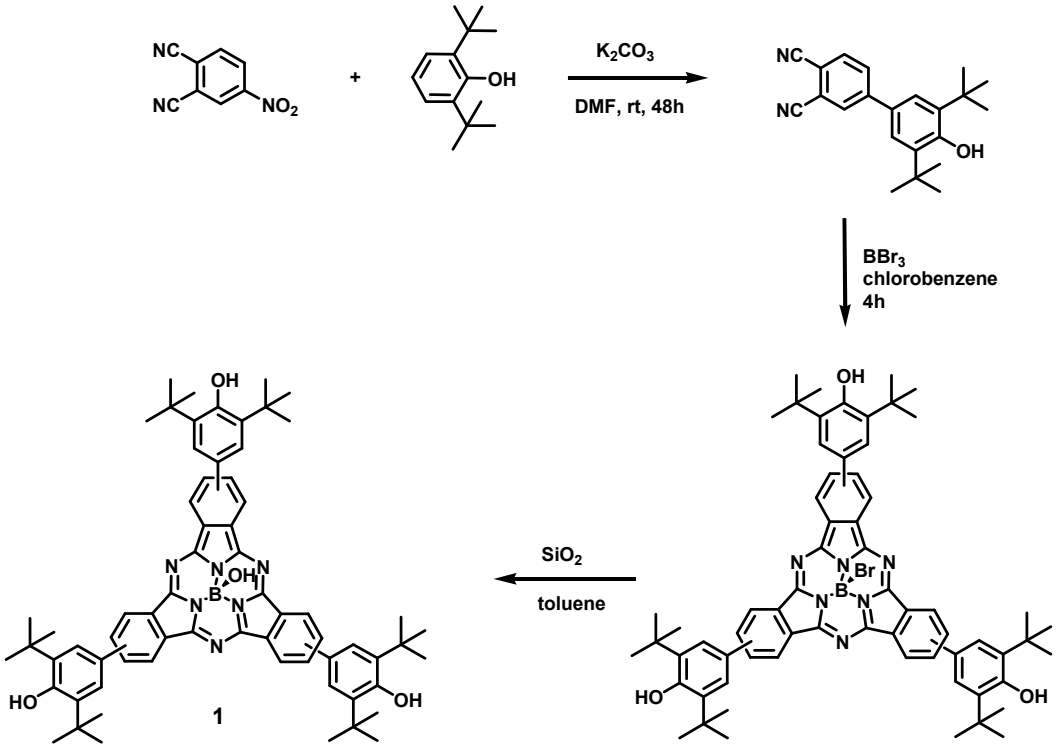
At the present time, photodynamic therapy (PDT) is receiving considerable interest for its potential as an antimicrobial therapy and photosensitizers play a critical role in this therapy. With a strong absorption at the red light region, the ability to generate singlet oxygen efficiently, and a long excitation wavelength which enables it to act deep under the skin, derivatives of phthalocyanine is an ideal parent structure as a photosensitizer. Furthermore, this treatment may be a valuable tool achieving a rapid reduction of the microbial burden perhaps even in the management of localized infections that are resistant to standard antibiotic regimens. A variety of photosensitizers from different groups including porphyrins, chlorophyll derivatives, phthalocyanines and azines have been effective in the photokilling of many Gram-positive and Gram negative bacterial pathogens in addition to parasite, fungi, and viruses. Much of the suggested antimicrobial uses of this therapy are based on results from in vitro studies [24 a,b].

Previous investigations showed that phthalocyanine derivatives can photosensitize the inactivation of various microbial pathogens[25,26]. Gram-positive cell walls typically lack the outer membrane found in Gram-negative bacteria. Gram-positive organisms are able to retain the dyestuffs because of the high amount of peptidoglycan in the cell wall. Gram positive bacteria are efficiently photoinactivated by a variety of sensitizers. Moreover, the presence of positively charged functional groups in the photosensitizer structure allows an extensive photoinduced inactivation of gram-positive and also of gram-negative bacterial cells. That is to say, the positive charge on the photosensitizer triggers to promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells. So, this association increases the efficiency of the photoinactivation processes [8,25,26,28].

Gram-negative bacteria can exhibit a remarkable resistance to negatively charged or neutral photosensitizers, unless the outer membrane translocation is artificially increased by treatment with chemical or biological agents[27,28]. On the other hand, cationic sensitizers have shown to photoinduce direct inactivation of gram-negative bacteria even in the absence of additives.

Recently, Spesia and Durantini have reported the photodynamic activity of subphthalocyanine derivatives in vitro on *Escherichia coli* cells [7]. Also, SubPcs are new molecules for photodynamic therapy and few article about this area. For this purpose, we have synthesized derivative of phthalonitrile at room temperature as a starting material [29, 30] and after that, a novel derivative of subphthalocyanine which has peripheral hydrophylic group, tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1) (Scheme 1), in this study. Firstly, its spectroscopic properties and characteristics were determined, then, testified its light stability and singlet oxygen produced capacity. A xenon lamp was used to simulate solar light in our laboratory.

Ultimately, we designed that singlet oxygen produced capacity of this new SubPc was measured before testing of PDT works. After that, the photodynamic activity of this original SubPc molecule against some gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and gram-positive (*Staphylococus aureus, Enterococcus faecalis*) bacteria. Besides, the second aim of this workout was to study and compare the photodinamic activity of this new SubPc against two differently charged microorganisms.



Scheme 1. Synthesis of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)Subphthalocyanine (SubPcB(OH)) from phthalonitrile.

2. EXPERIMENTAL

All the reagents used were chemically pure or spectroscopic grade. 4-Nitrophthalonitrile was prepared and purified according to the methods described in the literature [31]. N,N-dimethylformamid (DMF), dichloromethane, chloroform, ethylasetate, acetone, hexane, methanol and silikagel (column and preparative chromatography) were purchased from Merck. Some solvents were distilled before use. UV-Vis (Specord 600 UV-Vis spectrophotometer), PTI-Fluorescence spectrophotometer, and MALDI-TOF-MS (Applied Biosytems Voyager Instrument) spectroscopy, FTIR (Perkin-Elmer) were performed for spectra. Also, ^{1}H NMR and ^{13}C NMR spectra were carried out using a 400 MHz Varian AS NMR spectrometer. The thermogravimetric analyses were performed using a Perkin Elmer Pyris 6 TGA and DSC. Melting points were recorded with an electrothermal digital melting point apparatus. For photostability test of the product, its solutions were irratiated with xenon lamp (750 W, Philips) in a solar simulator in which has reflector surfaces and a quartz vessel was used. Light intensity was measured with Kipp&Zonen CM11 Pyranometer. Fluence rate of light source was determined to be 110 W/m^2 at a distance of 55 cm and 1033 W/m^2 at a distance of 10 cm in working systems to determined generation of singlet oxygen, photostability of SubPc and microbiological study, respectively. For microbiological study, all solutions and materials were sterilized by autoclaving.

2.1. Synthesis of 4-(3,5-di-tert-butyl-4-hydroxyphenyl) phthalonitrile

Synthesis procedure and selected data for 4-(3,5-di-tert-butyl-4-hydroxyphenyl) phthalonitrile: 1.70 g (12.30 mmol) of anhydrous K_2CO_3 was added to a solution of 2.86 g (13.80 mmol) of 2,6-di-*tert*-butylphenol and 1 g (5.75 mmol) of 4-nitrophthalonitrile in 10 mL of dry *N,N*-dimethylformamide (DMF) in 0.56 g portions at 1h intervals, under the argon atmosphere. The reaction mixture was stirred for 48 h at room temperature under argon and later, the undissolved salt was filtered and 200 mL of cold water was added, then the mixture was stirred rapidly. The resulting precipitate was filtered by vacuum and washed with water. The crude product was recrystallized twice from ethanol. Light yellow prismatic crystals. Yield: %51. m.p.: 114-116 ^{o}C, M_W:332,44 g/mol, ($C_{22}H_{24}N_2O$). IR(KBr) ν, (cm^{-1}): 3623, 3072, 2952, 2229, 1594, 1431, 1365, 1314, 1234, 1150, 1119, 889, 849, ^{1}H-NMR [400 MHz, $CDCI_3$, 298^{0}K], (ppm) δ: 7.80-7.93 (Ar, 3H), 7.37 (Ar, 2H), 5.51 (s, 1H), 1.50 (s, 18H) (Varian AS 400 Mercury), (400 MHz), ^{13}C-NMR [100 MHz, $CDCI_3$, 298^{o}K], (ppm) δ: 160.08, 153.17, 136.34, 132.15, 131.24, 130.45, 126.25, 116,12, 116.06, 115.00, 114,79, 36.82, 36.60, 32.42, 31.2 .

2.2. Synthesis of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1)

In a 50 mL two-necked round-bottomed flask, equipped with a condenser, magnetic stirrer, and rubber seal, 0.40 g (1.20 mmol) of 4-(3,5-di-*tert*-butyl-4-

hydroxyphenyl)phthalonitrile was resolved in the 10 mL chlorobenzene under the argon atmosphere. 0.075 mL (0.80mmol) of BBr_3 was added to solution. The reaction mixture was stirred under reflux for 4 hours. The purple solution was then flushed with argon. The solvent was removed under reduced pressure and the solid residue was dissolved in chloroform and precipitated by hexane repeatedly. A mixture of SubPcB(Br) and 500 mg of silica gel (230-400 mesh) in 5 ml toluene was heated at reflux for 1h. After cooling to room temperature, the solvent was removed. The crude product was purified by column chromatography on silica gel (chloroform/methanol, 10:1). Hydroxysubphthalocyanine derivative 1 was obtained as a violet solid which was further purified washing with hexane. Yield: 16 % for SubPc 1 (Scheme 1.) . ($C_{66}H_{73}N_6O_4B$), M_W: 1025.15 g/mol, m.p.>200 ^oC, UV-Vis ($CHCl_3$), λ(log ε/dm^3 $mol^{-1}cm^{-1}$): 586 nm (4.62), 536 nm(4.17), 370 nm (4.22), 290 nm (4.61), MALDI-TOF-MS (m/z): [M^+] 1024.70. IR(KBr) ν, (cm^{-1}): 3626, 2955, 1731, 1360, 1315, 1232, 714, 610, ^{1}H NMR [400 MHz, CD_3OD] (ppm) δ: 9.03 (m, 3H), 8.89 (m, 1C), 8.10 (m, 3H), 7.67 (m, 6H), 5.39 (s, 3H), 1.59 (s, 54H). ^{13}C-NMR [100 MHz, CD_3OD, 298^oK], (ppm) δ: 188.01, 175.88, 170.47, 168.33, 153.85, 152.84, 134.62, 130.25, 126,02, 116.28, 110.40, 109,69, 108,50, 82.22, 56.60, 55.20 .

2.3. The light sensitivity and thermal stability of new SubPc-1

The photostability test and photodegradation of SubPc-1 was carried out within 240 min. under the xenon lamp which emits in a wide ranged light resembled to solar light. The decomposition were monitored by following the decrease in absorption of Subphthalocyanine Q band (maximum wavelength at 586 nm) in DMSO at 30 min-intervals. Diminishing of SubPc-1 was only 10 % in 90 min.

The thermogravimetric analyses of the compound-SubPc-1 was performed using a Perkin Elmer Pyris 6 TGA in a nitrogen atmosphere at a heating rate of 20 ^oC/min. The temperature of the initial and maximum weight losses of the SubPc-1 was about 193 and 297 ^oC (midpoint), respectively

2.4. Singlet oxygen generation capacity of SubPc-1

In this study, singlet oxygen produced capacity of this novel SubPc was measured before testing of PDT works. Singlet oxygen generation capacity of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1) was investigated in DMSO and under xenon light source. The experiments were performed according to reported procedure [32]. The solutions were aerated for 5 min before beginning of the irradiation period. The fluence rate of light was determined to be 110 W/m^2. It was monitored that the change in the absorbance of the selective singlet oxygen trap 1,3-diphenyl-iso-benzofuran (50.0 μM) in the absence or presence of SubPc-1. To eliminate any potential contribution of dark reactions, we also recorded absorbance under the same conditions for 30 min in dark, followed by a 30 min period of irradiation with xenon light.

2.5. Antimicrobial activity

In vitro cytotoxic effect of the SubPc derivative was studied using gram negative and gram positive bacteria. The SubPc was solubilized in DMSO at 0.1mg SubPc-1/0.1mL DMSO, 0.2mg SubPc-1/0.1mL DMSO, concentration (stock solution), fresh aliquots from the stock solutions were diluted in PBS solution containing 1% DMSO in order to obtain the final working SubPc-1 solutions.

Our previous PDT studies, the effects of variety concentrations of DMSO against to microorganisms was observed [33]. At last, we set the DMSO concentration at 1% because gram positive bacteria are sensitive against to DMSO at higher concentration. In this manner, we are able to exclude the DMSO influence against the bacteria [33]. So, the amounts of the photosensitizer were varied 0.1 mL from stock solutions and were diluted in PBS (The total working solution is 10 mL, the concentrations of SubPc are 0.9-1.8 μM). The initial concentration of microorganism was 1.10^6 colony forming units, cfu/ml. The light intensity was determined as 1033 W/m^2. The solutions were irradiated for 240 min. A number of viable cells in this suspension that were subjected to the photosensor-light treatment or were not subjected to the photosensor- light treatment were determined by plating 10 μL aliquots of serially diluted suspensions onto blood agar plates. The blood agar plates were incubated at 35^oC for 24 h. Then, the numbers of colonies on the plates were counted.

2.5.1. Culture of microorganisms

1- *Escherichia coli* ATCC 35218

2- *Pseudomonas aeruginosa* ATCC 27853

3- *Staphylococcus aureus* ATCC 25923

4- *Enterococcus faecalis* ATCC 29212

2.5.2. Subphthalocyanine (SubPc-1) formulation

The SubPc-1 was solubilized in DMSO at 0.1mg/0.1mL, 0.2mg/0.1mL, concentration (stock solution). Fresh aliquots from the stock solutions were diluted in PBS solution containing 1% DMSO in order to obtain the final working SubPc solutions.

2.5.3. Photocatalytic reaction procedure

The microorganisms used in inactivation studies were *Escherichia coli* gram (-), *Pseudomonas aeruginosa* gram (-), *Staphylococcus aureus* gram (+), *Enterococcus faecalis* gram (+). The microorganism solutions were prepared with PBS solution. The amounts of the photosensitizer were varied 0.1 mL from stock solutions and were diluted in PBS (The total working solution is 10 mL). The initial concentration of microorganism was 1.10^6 colony forming units, cfu/ml. The reaction was carried out in a pyrex reactor with water-cooling jacket in order to eliminate parameter of heat. The solutions were irradiated for 240 min.

3. RESULTS AND DISCUSSION

The synthetic pathway regarding to SubPc-1 involves the cyclotrimerisation of the corresponding C-substituted phthalonitrile in the presence of boron tribromide to obtain SubPcB(Br) followed by substitution of the axial bromine atom with the -OH group. Substitution of the bromine atom of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl) subphthalocyanine with hydroxyl group in toluene in the presence of SiO_2 gave SubPcB(OH) derivative (SubPc-1) in 16% yield. The new compound of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1) was prepared starting from 4-(3,5-di-*tert*-butyl-4-hydroxyphenyl) phthalonitrile which can be seen Scheme 1. Several previous studies have illustrated the ability of the anion of 2,6-di-*tert*-butyl phenol behave as a carbon nucleophile in the preparation of biphenyl derivatives aromatic nucleophilic substitution. McKeown and co-workers found that the anion of 2,6-di-*tert*-butylphenol reacts efficiently with 4-nitrophthalonitrile as a carbon nucleophile to give 4-(3,5-di-*tert*-butyl-4-hydroxyphenyl) phthalonitrile. We synthesized this phthalonitrile derivative in moderate yield (%51) as the starting material for novel SubPc-1 .

The structure of SubPc-1 was confirmed by UV-Vis, PTI-fluorescence, FTIR, ^{1}H NMR, ^{13}C NMR and MALDI-TOF-MS spectroscopy. The absorption spectrum and the fluorescence spectrum of SubPc in DMSO are demonstrated in Figure 1.The SubPc exhibited characteristic Q band in the visible region at 586 nm and B band in the UV region at 290 nm. Absorption coefficient, logε ($dm^3mol^{-1}cm^{-1}$), value of the Q band of SubPc-1 derivative was found to be 4.61. Fluorescence emission of SubPc is observed 598 nm (λ_{exc}:586 nm).

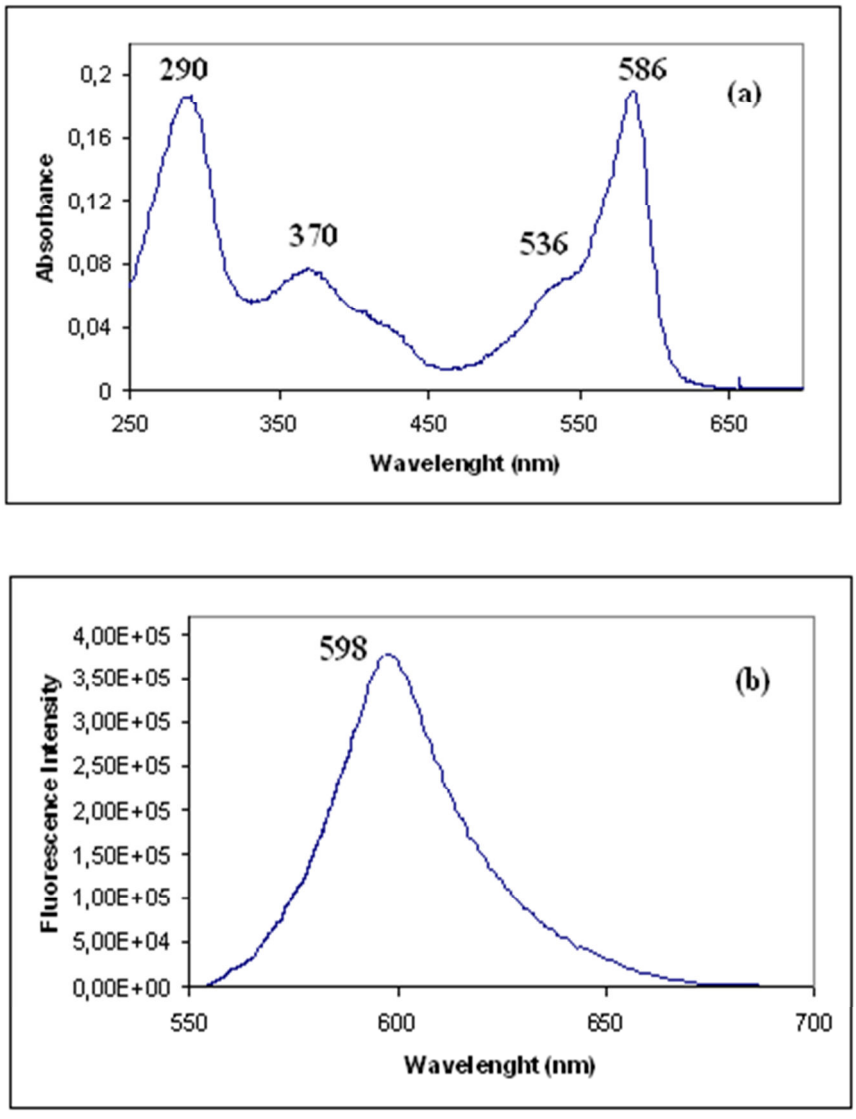


Figure 1. Absorption (a) and fluorescence (b) spectra of SubPc-1 in DMSO (10^{-6} M), λ_{exc}:586 nm, log ε ($dm^3mol^{-1}cm^{-1}$)= 4.61, λ_{emis}: 598 nm.

3.1. Testing of the light sensitivity and thermal stability of novel SubPc-1

The photostability test of SubPc-1 was carried out within 240 min under the xenon lamp which emits in a wide ranged light similar to solar light. The decomposition were monitored by following the decrease in absorption of Subphthalocyanine Q band. Figure 2. shows the absorption spectra changes observed on photolysis in DMSO. Diminishing of SubPc-1 was only 10 % in 90 min. However, it was 65 % in 240 min. In that state, the light stability of sensitizer (SubPc-1) allows for antimicrobial applications such as PDT. Yet, the mechanism of photodegradation of sensitizer (SubPc-1) might be complicated and differs in solution and in biological media.

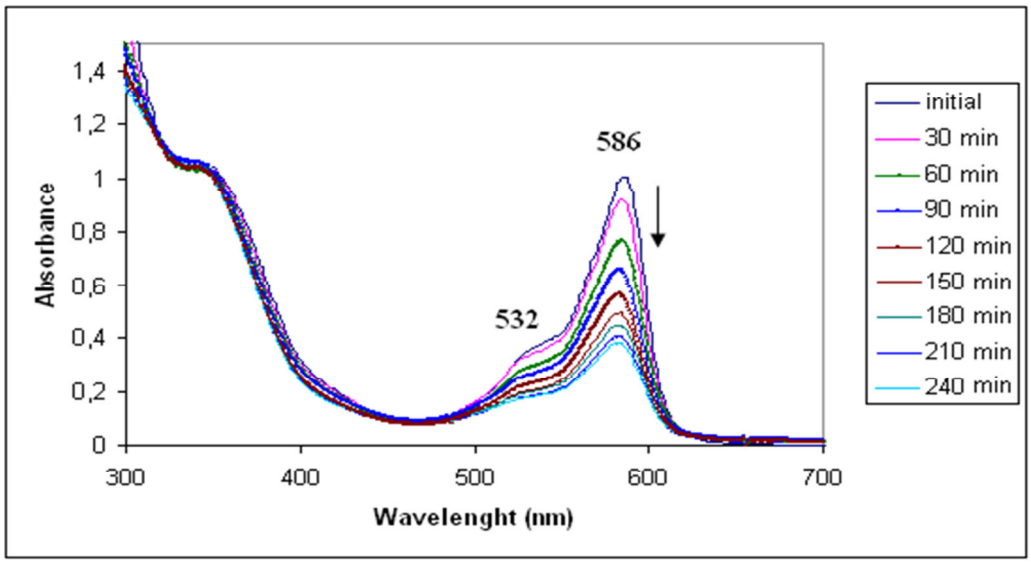


Figure 2. Absorption spectra changes of SubPc-1 irradiated with xenon lamp in DMSO (Δt=240 min)

The thermogravimetric analyses of the compound-SubPc-1 was performed using a Perkin Elmer Pyris 6 TGA in an inert atmosphere. The temperature of the initial and maximum weight losses of the SubPc-1 was about 193 and 297 ^{o}C (midpoint) respectively. That's way, it can be thought that this characteristic likely provides that the catalytic application fields of these kind of materials are in a wide range.

3.2. Singlet oxygen generation capacity of SubPc-1

In this paper, singlet oxygen produced capacity of new SubPc which was measured before testing of PDT works. Singlet oxygen generation capacity of tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1) was investigated in DMSO. The experiments were performed similar to reported procedure [32, 33]. For this purpose, xenon light source was, first time, used to excite SubPc-1 derivative in our work. The fluence rate of light was determined to be 110 W/m^2. The change in the absorbance of the selective singlet oxygen trap 1,3-diphenyl-iso-benzofuran (50.0 μM) in the presence of SubPc-1 was exhibited in Figure 3. Before beginning of the irradiation period, the solutions were aerated for 5 min. Under these experimental conditions, the absorbance at 416 nm of 1,3-diphenyl-iso-benzofuran in the absence SubPc changed quite slowly. We also recorded absorbance under the same conditions for 30 min in dark to eliminate any potential contribution to the absorbance resulted from dark reactions, After that, it was followed by a 30 min period of irradiation with xenon light (Figure 4). Any absorbance differences did not observe in dark.

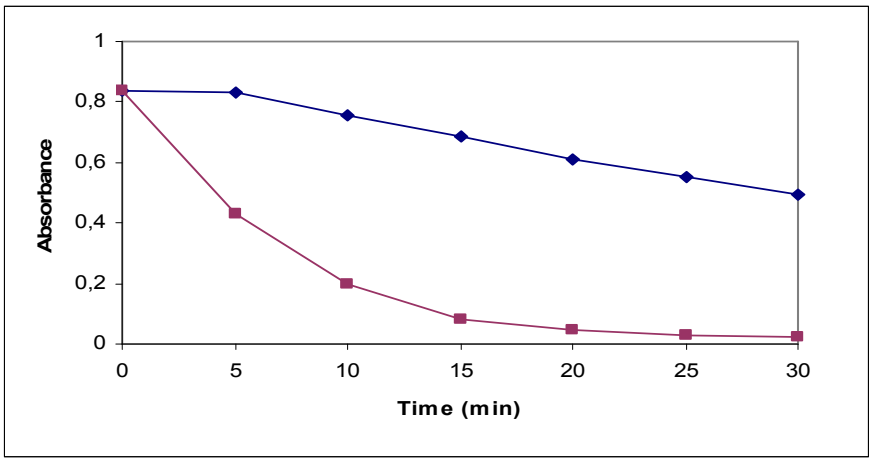


Figure 3. The change in the absorbance of 1,3-diphenyl-iso-benzofuran (50.0 µM) in DMSO under xenon light illumination in the absent and presence of SubPc-1 (diamonds:no added SubPc, squares: with SubPc, at 416 nm)

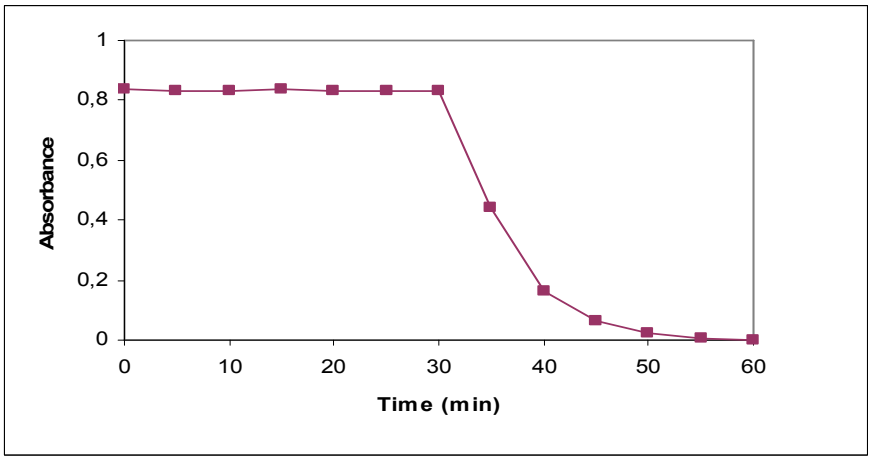


Figure 4. The change in the absorbance (at 416 nm) of 1,3-diphenyl-iso-benzofuran (50.0 µM) in DMSO in the presence of SubPc-1; illumination starts after 30 min dark treatment.

2.5. Antimicrobial Analysis Results

In this paper, the activity of this novel macromolecule against both gram positive and gram negative bacteria was determined and evaluated. Firstly, the microbial inactivation was not observed in the absence of SubPc under xenon light and in the dark conditions. In the presence SubPc-1, the microbial inactivation of gram positive bacteria *S. aureus, E. fecalis* was observed under light. However, same success was not observed against gram-negative bacteria *E.coli* and *P. aurogenosa.* The photoinhibitory activity of SubPc against *S. aureus, E. faecalis* are shown Figure 5., Figure 6., respectively.

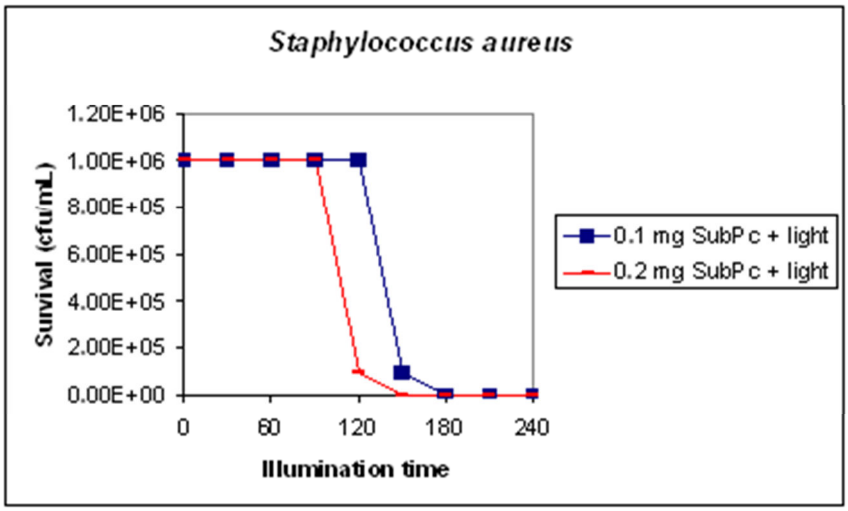


Figure 5. Effect of SubPc-1 on *S.aureus* survival. Initial concentration: $1x10^6$ cfu/ml.

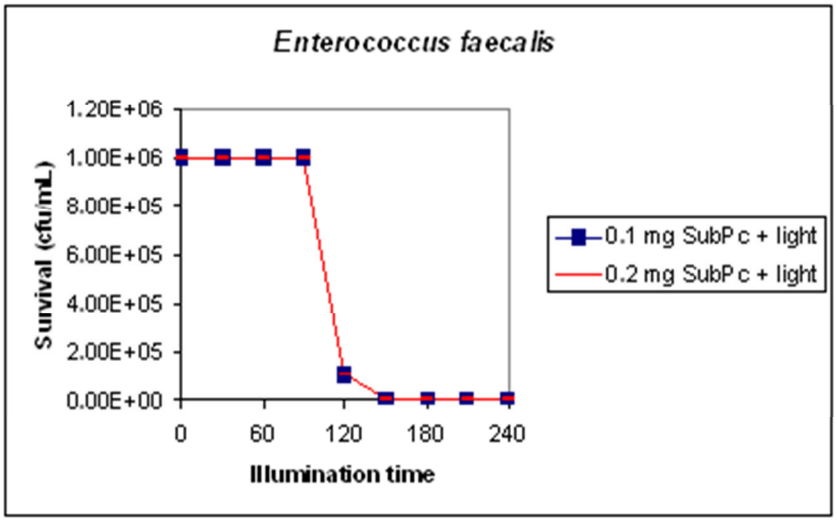


Figure 6. Effect of SubPc-1 on *E.faecalis* survival. Initial concentration: $1x10^6$cfu/ml.

In conclusion, this study shows that tris(3,5-di-*tert*-butyl-4-hydroxyphenyl)SubPcB(OH) (SubPc-1) derivative is interesting as new amphiphilic photosensitizer for photoantimicrobial therapy. This novel product is especially more selective against gram positive bacteria. As a matter of fact, the chosen microorganisms were approximately inactivated by using SubPc-1 within 120 min in PDT. As a consequence, it was observed that this new product had a potential targeting selective efficiency and its photostability and also singlet oxygen produced capacity was quite suitable for the purpose of PDT and cancer studies.

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