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ANTIMICROBIAL ACTIVITY OF METALLO TETRA (4-CARBOXYPHENYL) PHTHALOCYANINE USEFUL IN PHOTODYNAMIC THERAPY

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Abstract

The antimicrobial effect of synthetic photosensitizer zinc tetra(4-carboxyphenyl)phthalocyanine was carried out on the human pathogenic gram negative and gram positive bacteria *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 27853, *Klebsiella pneumoniae* ATCC 1705, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 43300, and on three yeast *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. Growth inhibition assay was conducted using a microdilution protocol including an appropriate control without photoactivation of the photosensitizer under blue light (450 – 495 nm). The results showed percentage inhibition as high as 100% to all yeasts, E. coli and *K. pneumoniae* and values ranging from 12.25 to 72.22% against others microorganisms. In addition, very low MIC values (~ 10 µg/ ml) to *C. krusei* and *K. pneumoniae* were found when compared against treatments using the antibiotics Clotrimazole and Gentamicin respectively. Finally, the analysis of MIC values showed statistically significant differences between treatments with or without photoactivation. These results support the potential use of tetra (4-carboxyphenyl) phthalocyanine and photodynamic therapy for the treatment of infectious diseases as a future alternative approach.

Keywords: Tetra(4-carboxyphenyl)phthalocyanine, pathogen inhibition, MIC, Singlet oxygen, ROS.

Introduction

In recent years Photodynamic therapy (PDT) has emerged as a promising technique to treat drug-resistance microorganisms, confirming that this is a effective in vitro method against bacteria, fungi, viruses and protozoa [1,2]. The first report of the World Health Organization (WHO) on antibiotic resistance, focuses on the three main microorganisms (*Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*), which are associated with the occurrence of nosocomial infections [3]. One alternative for the treatment of infections caused by these bacteria could be the Antimicrobial Photodynamic Therapy (APDT) because its lethal effect; which is based on the activation of a photosensitizer (PS) by visible light, allowing the formation of reactive species oxygen (ROS) as singlet oxygen, producing a phototoxic effect on the cell. Besides, ROS does not require to enter into the cell for inhibiting or killing, avoiding the development of resistance mechanisms [4]. Recently, the routinely use of commercial antibiotics to treat hospital infections has increased the number of resistant microorganisms. For this reason, it is necessary to design alternative methods that allow the development of new strategies to combat them properly. PDT has several favorable highlights for the treatment of infections that are caused by pathogens such as: broad spectrum of action, few secondary effects, and efficient inactivation of microorganism resistant to antibiotic and the prevention of microorganism regeneration after treatment [5,6]. Therefore, intensive studies have been focused to define the scope and field of application of this technique [7]. This technique is unconventional therapy to inhibit growth of microorganisms that have developed resistance to different types of antibiotics. PDT is based on visible light excitation of a photosensitizer (PS) in the presence of basal triplet state $^3O_2$ ($\Sigma_1 g^1-$) [8–10].

Organic and metal-organic molecules can be used as photosensitizer if they fulfill three characteristics: (a) high quantum yield of $^1O_2$ production, (b) high molar absorptivity in the visible range of the electromagnetic spectrum and (c) low toxicity. It is well known that the most photosensitizers used for PDT are porphyrins, chlorins and bacteriochlorins. However, phthalocyanines, purpurines and texapirinas are considered promising alternatives [11]. As other photosensitizers, phthalocyanines are able to produce singlet oxygen and this quality is the reason why PcTc have been extensively investigated, mainly in the fields of photobiology and photomedicine. Some of these studies include the use of phthalocyanines in wastewater treatment, and as a decontaminating agent in the treatment of cancer and bacterial photoinactivation [12–15]. Among the various kinds of photosensitizers that have been studied, phthalocyanines, are considered as molecules of particular interest due to the strong absorption they have at the visible region (700nm) of light radiation, allowing deep penetration, high efficiency in the generation of singlet oxygen, high photostability and easy chemical modification. Similarly, in order to potentiate the ability to act as photosensitizers and enhance bactericidal action, have been led to replacement of the macrocyclic ring peripheral or have been synthesized in its cationic form [16]. The aim of this study was to evaluate the *in vitro* antimicrobial effect of photosensitizer Zn tetracarboxiphthalocyanine against Gram positive and Gram-negative bacterial strains and on three *Candida* species using photodynamic therapy.

Material and Methods

Synthesis of the Zinc Phthalocyanine

All reagents used in this work were analytical grade. We have synthesized Zn phthalocyanines (PcTcZn) using the Achar method: 0.0480 mol of Zn sulfate (II) were mixed with 0.176 moles of trimellitic anhydride, 1.0 ml of urea, 0.085 moles of ammonium chloride in 10 mL of nitrobenzene, using ammonium tetramolybdate as catalyst of the reaction. The mixture was heated to reflux for 4 h at 185 °C, and after that, the dye was purified and recrystallized with 1.0 mol/L NaOH and 1.0 mol/L HCl up to obtain the PcTcZn[17].

Material Characterization

The physicochemical properties of the PcTcZn were studied using both measurements of UV-Vis Spectrophotometry and Fourier Transform Infrared Spectroscopy (FT-IR). The absorption spectrum was obtained using a Lambda 4 Perkin Elmer spectrophotometer. FT-IR spectra (KBr) of the compounds were recorded on a Bruker Tensor 27 spectrometer in the spectrum region between 4000 - 500 cm^{-1}.

Biological test

The antimicrobial activity of PcTcZn was evaluated on the strains: Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300*, Klebsiella pneumoniae* ATCC 1705*, Staphylococcus aureus silvestre* ATCC 25923*, Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus fecalis* ATCC

27853, *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. Minimal Inhibitory Concentration (MIC) was calculated following the recommendations of the CLSI-2012 for microdilution (IC50) by using the Graphpad Prism 5.0 statistical package. A serial dilution set of each PS was prepared using DMSO as solvent (4.90, 9.80, 19.70, 37.40, 74.70µg/mL). The final volume was 100µL and the final concentration of DMSO in the assay did not exceed 1%. Each treatment consisted of suitable culture medium (nutrient broth for bacteria strain and Sabouraud dextrose broth for yeast strait), containing PS concentration and microbial strains. Each treatment was carried out by triplicate and exposed to visible light irradiation for photochemical activations. Afterwards, the cultures were incubating at 37°C and the growth was monitoring at OD_{600} until reaching the stationary phase. Irradiation, negative and positive controls were used; the negative control contained all except PS and submitted to irradiation and finally the positive control contained all except PS, which was substituted by Gentamicin (20µg/mL), or Clotrimazole (0.1%) as reference antibiotic.

Statistical Analysis

The inhibition percentage was calculated from log phase to each microorganism using the following equation.

$$\%Inh = \frac{OD_{NC} - OD_{T}}{OD_{NC}} * 100$$

OD_{NC}= Absorbance of negative control
OD_{T}= Absorbance of treatment

In order to describe the effect of the factors on the response variable, such as the type of microorganism treatments (with and without irradiation) and TcPc-Zn concentrations; a multivariate ANOVA were performed using the SPSS software package.

Results

UV-Vis spectrum of sensitizers

Figure 1 shows the UV-Vis absorption spectrum to PcTcZn dissolved H_2SO_4. The absortion spectrum shows typical Sorest band around 350nm, that corresponds to π→π* transition, along, two bands located at λPcTcZn = 689nm and λPcTcZn = 770nm which can be assigned to α-Q-Bands and β-Q-Bands respectively, that were generated by metallation of the macrocycle.

Due to the fact that the spectra only show two Q-bands, the results represent a phthalocyanines-metal complex [18].

IR Characterization of sensitizers

Figure 2 shows the FT-IR spectra to PcTcZn that was synthesized in this work. The chemical bonding of samples were assigned for correlating signals in the spectrum to the vibration or stretching for each functional group. The absortion spectrum show a broad band at 3418 cm^{-1}, a signal that is assigned to hydroxyl groups; which is a typical signal of carboxylic acids. This broad and strong signal overlapped the signal of chemical bond stretching of N-H, along, strong signal of asymmetric stretching of C=O- located at v=1668 cm^{-1} confirm the presence of carboxylic acid group in phthalocyanines, strong signal located at v=1524 cm^{-1} can be assigned to chemical bond stretching of C=C of aromatic groups, along, signal located at v= 355 cm^{-1} is assigned to chemical bond stretching of C-O of carboxylic acid group. Finally, signals near to 1107 cm^{-1} are assigned to chemical bond bending of C-C in aromatic groups[19,20].

According to physicalchemistry analyses the next structure was proposed for TcPcZn

Biological test

The results were summarized in the tables 1-3 and show all percentages of inhibition (>80%) to all strains that were tested. Indistinctly of treatment used (unirradiated / irradiated), we observed high concentrations of inhibition. Furthermore, the yeast strains showed to be very susceptible to APDT when compared with bacteria; likewise, except *P. aeuroginosa*, the Gram-negative bacteria group increased its susceptibility to treatment with TcpCZn than Gram-positive bacteria group.

The minimum inhibitory concentrations analysis (Table 4) suggests a differential susceptibility among these microorganisms; but without statistically significant differences ($P > 0.05$). However, the results show statistically significant differences ($p < 0.05$) when the inhibition response variables to each treatment (Unirradiated / Irradiated) by microorganism (Yeast / Bacteria) were analyzed.

Discussion

The constant emergence of new resistant microorganisms to conventional antimicrobial treatments, gives relevance to develop new alternative mechanisms in order to control these kinds of germs. For this reason, alternatives as the APDT and the search for new photosensitizers

molecules has been increased during recent years [21]. This study evaluated the antimicrobial effect of molecules belonging to the zinc phthalocyanines family with the purpose of evaluating their photochemical and antimicrobial characteristics as possible alternative to control drug-resistant human pathogens.

It is noteworthy that all microorganisms tested showed susceptibility to APDT; mainly in the highest concentrations that were evaluated. The observed photoinactivation could be attributed to the cationic features of the TcPcZn and its replacement with zinc metal. Merchat et al [22] explains that the compounds capable of causing microbial photoinactivation that possess metallic substituents facilitate entry and subsequent cell damage. Effectively with this compound we observed high inhibition on Gram-positive bacteria and Gram-negative bacteria and yeast. Gram-negative bacteria could reduce the photodynamic action by physical inactivation of singlet oxygen, probably trough mechanisms related to outer membrane that can avoid the photosensitizer entry to cytoplasm. The properties of the central diamagnetic metal linked to phthalocyanines enhance two relevant parameters: the quantum yield and lifetime of the triplet, favored even at low oxygen concentrations. This mechanism was also proposed by Ben-Hur et al [23] and Rosenthal[24], stating that phthalocyanines containing paramagnetic ions (Cu, Fe, Vn) are not as efficient in producing $^1O_2$ as those linked to zinc or aluminum. In addition, as showed in Tables 1 and 3, the microbial strain *E. coli* and all strains of *Candida* had greater inhibition percentages to photodynamic therapy. These results could be attributed to photocytotoxicity generated not only by singlet oxygen, but also by the reactive oxygen species derived from photochemical pathway I. It was demonstrated several years ago that some agents that generate toxicity on *E. coli* are mostly oxygen radicals together with the action of singlet oxygen[25], probably because *E. coli* has the *nurA* gene that generates defect in the expression of the catalase enzyme[26].

Moreover, some authors previously described not satisfactory inhibition of *Candida* species by Photoinactivation using phthalocyanite derivate as photosensitizer when their results were compared with positive controls [27,28]. However, in this study we reported greater inhibitory effect of TcPcZn on yeast and bacteria. The found positive results with yeast may be associated to the adhesion to the body provided by the presence of a carboxyl group. Most treatments with irradiation show higherpercentages of inhibition to bacteria and yeast that were tested by Photoinactivation with TcPcZn and statistically significant differences between treatments with and without irradiation were also found (Tables 1, 2). The action of photosensitizers are optimized when specific and appropriate lengths wave are handled [29], which increases the efficiency added to the production of singlet oxygen and other reactive oxygen species [21]. The minimum inhibitory concentrations varied between microorganisms, showing higher values (100%) for radiation treatment corresponding to low MIC values (~10µg/mL), indicating that TcPcZn molecules have a significant effect on the inhibition of these pathogens in comparison with the antibiotics used as positive controls. It is important to point out that MICs value refer to dose capable of causing adverse effects on growth of these microorganisms, becoming these findings a key for biosprospecting from synthetic compounds as alternative antimicrobial treatments, given the aforementioned problems with drug-resistant pathogens.

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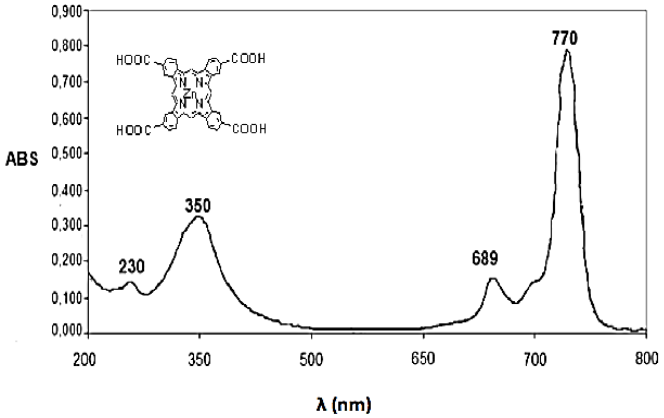

Figure 1. Absorption UV-Vis spectrum of PcTcZn dissolved in concentrated H2SO4

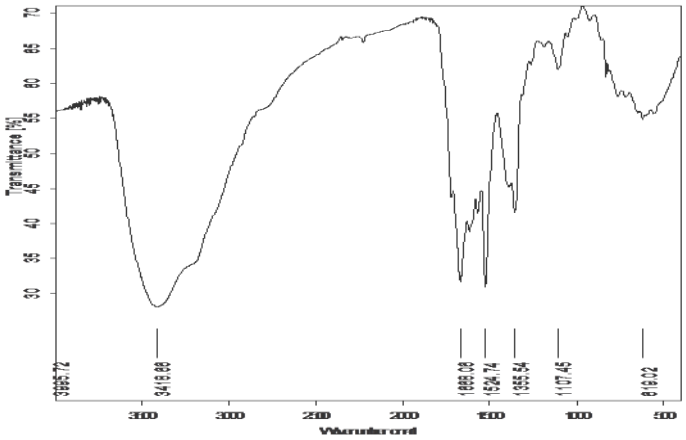

Figure 2. IR spectrum of PcTcZn

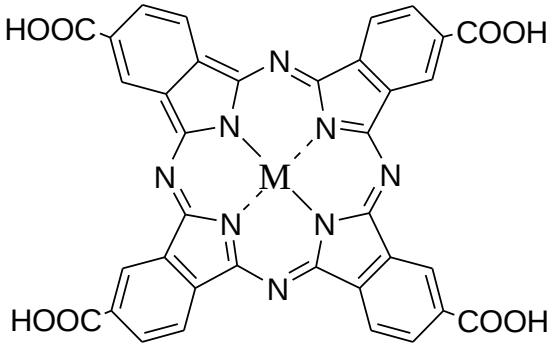

Figure 3. Chemical structure of Metallotetra(4-carboxyphenyl)phthalocyanine

Table 1. Percent inhibition obtained for five concentration of photosensitizer tested against tree Candida species. ± correspond to standard deviation

	% Inhibition					
	Candida albicans		***Candida krusei***		***Candida parapsilosis***	
[ ]/treatment	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**
74.7	68.99 ±3.8	100.00 ±0.8	70 ±3.1	99.50 ±1.0	55.53 ±6.0	100.00 ±0.0
37.4	36.29 ±11.0	77.52 ±5.3	46.29 ±19.0	58.00 ±6.9	15.92 ±5.5	67.37 ±3.0
19.7	42.92 ±8.7	68.00 ±8.4	46.15 ±4.8	54.95 ±5.0	29.32 ±3.6	55.26 ±7.6
9.8	91.46 ±6.8	37.81 ±10.4	52.03 ±11.6	55.32 ±6.7	62.72 ±6.5	52.63 ±6.3
4.9	63.12 ±11.8	32.71 ±18.8	48.13 ±19.0	57.52 ±6.4	57.28 ±11.5	53.42 ±5.5

Table 2. Percent inhibition obtained for five concentration of photosensitizer tested against tree Gram positive bacteria species. ± correspond to standard deviation

	% Inhibition					
	Enterococcus faecalis		***Staphylococcus aureus***		**MRSA**	
[ ]/treatment	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**
74.7	26.48 ±1.8	32.91 ±1.8	11.54 ±6.9	10.73 ±3.4	75.45 ±7.9	72.22 ±1.0
37.4	19.41 ±6.4	30.01 ±1.36	ND	ND	ND	68.59 ±3.5
19.7	2.63 ±0.9	ND	ND	ND	34.01 ±7.9	100.03 ±7.4
9.8	ND	ND	ND	ND	ND	17.37 ±1.8
4.9	ND	ND	ND	ND	14.27 ±3.1	16.10 ±3.9

ND: Percent inhibition below zero or no detected at these concentrations

Table 3. Percent inhibition obtained for five concentration of photosensitizer tested against tree Gram negative bacteria species. ± correspond to standard deviation

	% Inhibition					
	Pseudomonas aeruginosa		***Escherichia coli***		***Klebsiella pneumoniae***	
[ ]/treatment	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**	**Unirradiated**	**Irradiated**
74.7	21.23 ±0.6	12.25 ±0.0	100.00 ±2.3	100.00 ±2.3	ND	100.00 ±2.1
37.4	9.39 ±4.0	11.03 ±1.73	76.82 ±2.8	78.45 ±4.3	26.76 ±6.2	ND
19.7	ND	ND	67.56 ±3.1	39.26 ±3.8	34.29 ±3.5	69.43 ±2.1
9.8	ND	ND	45.23 ±0.9	43.37 ±4.7	17.83 ±1.3	ND
4.9	ND	ND	30.28 ±3.1	51.23 ±3.2	40.00 ±5.3	ND

ND: Percent inhibition below zero or no detected at these concentrations

Table 4. Minimum Inhibitory concentration values (MIC) for the nine tested microorganisms in units of µg/mL. Numbers in parentheses correspond to the value of R2 for each MIC calculated.

	MIC (µg/mL)	
Microorganism	**Treatment**	
	Unirradiated	**Irradiated**
C. albicans	ND	32.17 (0.90)
C. krusei	10.61 (0.45)	36.51 (0.86)
C. parapsilosis	ND	34.89 (0.66)
E. faecalis	ND	22.02 (0.94)
S. aureus	63.51 (0.93)	39.25 (0.98)
MRSA	80.93 (0.83)	16.03 (0.86)
P. aeruginosa	25.17 (0.96)	22.48 (0.97)
E. coli	76.08 (0.96)	39.85 (0.97)
K. pneumoniae	10.43 (0.97)	10.43 (0.42)

ND: MIC no detected