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Mechanistic studies of *Candida albicans* photodynamic inactivation with *Porophyllum obscurum* hexanic extract and its isolated thiophenic compounds

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ABSTRACT

Porophyllum obscurum (Spreng) DC (Asteraceae) hexanic extract (*PoHex*) from aerial parts has demonstrated antifungal activity under UVA irradiation against *Candida* spp. isolates from patients with oropharyngeal candidiasis and four thiophenes were isolated as responsible of the activity. In the present work, we studied the photomechanisms whereby *PoHex* and their thiophenes produce photoinactivation of *C. albicans*.

Reactive Oxygen Species generation by *PoHex* and thiophenes was evaluated: the production of superoxide anion, employing the NBT reduction assay; hydrogen peroxide, through the formation of a red quinoneimine; and singlet oxygen by using the 1,3-DPBF bleaching method. The action of ROS in fungal cells was investigated by evaluating binding of photosensitizer, leakage, apoptosis and stress sensibility that were performed by following M27-A3 guidelines, in parallel under "light" and "darkness" conditions.

Results showed that the photosensitive antifungal activity of *PoHex* required oxygen and both type I (production of superoxide anion and hydrogen peroxide) and type II (production of singlet oxygen) reactions were involved. In addition, we found that ROS generated by *PoHex* did not cause release of cytoplasmic components due to membrane damage nor apoptosis of *C. albicans*. Treatment with *PoHex* and UVA increased cells sensitivities to osmotic stressors; did not reduce resistance to additional oxidative stress and possibly affected the structure of the cell wall. In addition, 2,2′:5′2″terthiophene, the most active PS present in *PoHex* and the only one that generate single oxygen, at Minimal Fungicide Concentration, did not cause leakage nor apoptosis and did not increase sensitivities to osmotic and oxidative stressors.

Results demonstrated that Photodynamic Inactivation employing *PoHex* under UVA does represent an alternative for topical antifungal therapy for oropharyngeal candidiasis.

1. Introduction

Candida albicans is an opportunistic fungus that causes both superficial and systemic infections, especially in immunocompromised individuals or with serious underlying diseases [1]. It is also an important cause of morbidity and mortality in hospitalized patients, being Oropharyngeal candidiasis (OPC) the most common fungal infection among patients with acquired immune deficiency syndrome [2]. OPC is a mucosal infection that causes inflammation, pain and dysgeusia and constitutes a risk factor for *Candida* dissemination [3] Treatment of candidiasis relies on a limited drug arsenal, composed of three major classes of antifungal drugs: polyenes, azoles and echinocandins, compromised by problems of selectivity, toxicity, and the development of resistance [4,5].

Photodynamic Inactivation (PDI) is a promising method to eradicate pathogenic fungi. The technic involves an accumulation of a nontoxic

Abbreviations: 1,3-DPBF, 1,3-diphenylisobenzofuran; AMB, amphotericin B; CFU, colony-forming unit; CTLC, centrifugal thin layer chromatography; DES, design expert software; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; FCZ, fluconazole; Hex, hexane; ITZ, itraconazole; MFC, Minimal Fungicide Concentration; MIC, Minimal Inhibitory Concentration; MOPS, 3-(N-morpholino) propanesulfonic acid; NBT, nitro blue tetrazolium; OPC, oropharyngeal candidiasis; PBS, phosphate-buffered saline; PDI, Photodynamic Inactivation; *PoHex*, *Porophyllum obscurum* hexanic extract; PS, photosensitizer; RB, Rose Bengal; ROS, reactive oxygen specie; SD, standard deviation; SDS, sodium dodecyl sulfate; TLC, Thin Layer Chromatography; UVA, ultra-violet A; YPD, yeast extract-peptone-dextrose

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photosensitizer (PS) in the microbiological tissue, that in photoexcited estates, at correct wavelength, can react whit molecular oxygen producing cell death [5,6]. The combination of PS, light and oxygen can produce reactive oxygen species (ROS) that are highly reactive with biological components such as amino acids, constituents of DNA, proteins, and lipids damaging essential cell components or irreversibly altering metabolic activities [8]. ROS can be created through two photochemical mechanisms, either a type I or a type II. In type I reactions, electrons transfer directly from the excited PS and generate oxygen radicals such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). In a type II reactions, the excited PS transfer energy to triplet oxygen and the singlet oxygen ($O_2(^1\Delta_g)$) production becomes imminent. Then, ROS initiate further oxidative reactions in a closed environment, like the microbial cell wall, lipid membranes, enzymes, or nucleic acids [9,10].

Compared to the traditional treatments, PDI has the advantage of dual selectivity; the PS can be delivered to a target cell or tissue, and in addition, the illumination can be spatially directed to the lesion. Other advantages are: broad spectrum of action, efficient inactivation of antibiotic-resistant strains, low mutagenic potential and the lack of selection of photo-resistant microbial cells [11].

Many studies have reported the use of photoactive compounds extracted from plants for inactivation of microorganisms [12]. In a recently published work, we evaluated *Porophyllum obscurum* (Spreng) DC (Asteraceae) as a source of new PS with potential use in PDI [13]. The phytochemical investigation of the hexanic extract (*PoHex*) from aerial parts (the most active), established the presence of four photoactive thiophenes: 2,2′:5′2″terthiophene **1**, 5-(3-buten-1-ynyl)-2,2′-bithiophene **2**, 5-(4-acetoxy-1-butenyl)-2,2′- bithiophene **3** and 5-(4-hydroxy-1-butenyl)-2,2′- bithiophene **4** (Fig. 1). *PoHex* has also demonstrated activity against 25 clinical strains of *Candida* spp. isolates as etiological agents of OPC. In the present work, bearing in mind that thiophenes are photoactive natural products [13], we decided to study the photomechanisms whereby *PoHex* and its isolated constituents produce photoinactivation of yeasts such as *C. albicans*. That being said, we investigated the oxygen dependence and ROS generation of the extract and its isolated components. In addition, in order to investigate their photoinactivation impact in yeast, we evaluated if *PoHex* and thiophene **1** can induce apoptosis, cause damage in fungal membrane or alter their responses to oxidative and osmotic stress.

2. Experimental

2.1. Preparation of PoHex and isolation of PS

PoHex employed in the current work has been prepared in our previous one [13]. Briefly, aerial parts (comprising leaves and stems) of *P. obcurum* were collected in Las Chacras, province of San Luis (Argentina) at 33,246359 lat. S; 66,272505 long. W, on October 2012; and was deposited at the Herbarium of the Universidad Nacional de San Luis (L.A. Del Vitto & E.M. Petenatti # 9436). Plant material (100 g) was extracted by maceration with hexane (Hex), with continuous shaking. After filtration (Whatman no. 1 paper), solvent was evaporated to dryness under reduced pressure in a rotary evaporator (Büchi R-205, Essen, Germany) to give semisolid residue. Thiophenes were obtained by preparative centrifugal thin layer chromatography (CTLC) carried out on a Chromatotron Model 7924 T (Harrison Research, Palo Alto, CA, USA) and preparative Thin Layer Chromatography (TLC) yielding **1** (39.3 mg), **2** (18.1 mg), **3** (8.7 mg) and **4** (10.8 mg) (Fig. 1).

2.2. Irradiation source and conditions

The UVA light radiation source was a set of three 100 W lamps (Alic, Buenos Aires, Argentina), emitting a 315–400 nm black-light, aligned perpendicular to the samples, placed in a mobile platform that allows varying the distance between them. Under these conditions, the entire area of the microplate was illuminated uniformly. All experiments were carried out in parallel under “light” and “darkness” conditions. In light experiments with *PoHex*, UVA irradiations were performed at a distance to 12 cm from the sample for 60 min; while in assays with thiophenes **1**-**4**, UVA irradiations were performed at a distance to 6 cm from the sample during 5 min. These conditions of irradiation (distance and time) have been previously optimized using DES package (Stat-Ease, Inc., Minneapolis, USA) version 7.0.3, following the design described by Postigo et al. [14]. In darkness experiments, assays carried out in the same conditions but microplates were wrapped with aluminum foil to avoid exposure to light. The photon flux was determined by ferrioxalate actinometer under the same irradiation conditions of the antimicrobial assays. According to the procedure outlined by Kuhn et al. [15], the radiant flux incident upon the actinometer was calculated to be 1.2 J/cm^2 for *PoHex* photosensitive assays and 3.4 J/cm^2 for thiophenes photosensitive assays.

2.3. Microorganism and culture conditions

C. albicans ATCC 10231, provided by *American Type Culture Collection* (ATCC), Rockville, MD, USA, was used in this study. It was grown on Sabouraud-dextrose agar (SDA, Oxoid, Basingstoke Hampshire, UK) slants for 48 h at 30 °C, maintained on slopes of SDA and was conserved in 20% glycerol at −20 °C. Inoculum was obtained according to reported procedures and adjusted in sterile distilled water to the concentration desired for each assay [16].

2.4. Influence of oxygen in the antifungal photosensitive activity

The experiments were performed in 96-well flat-bottomed microtiter plates by following M27-A3 guidelines [16]. PS stock solution of *PoHex* or thiophenes (100 µL) were serially two-fold diluted with RPMI-1640 culture medium buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, MO, USA). Then, 100 µL of inoculum suspension (0.5–2.5 × 10^3 CFU/mL) was added to each well, reaching final concentrations of PS between 0.24–1,000 µg/mL for *PoHex* and 0.12–250 µg/mL for thiophenes. The microplates were agitated at 150 rpm for 5 min on an orbital shaker platform Innova

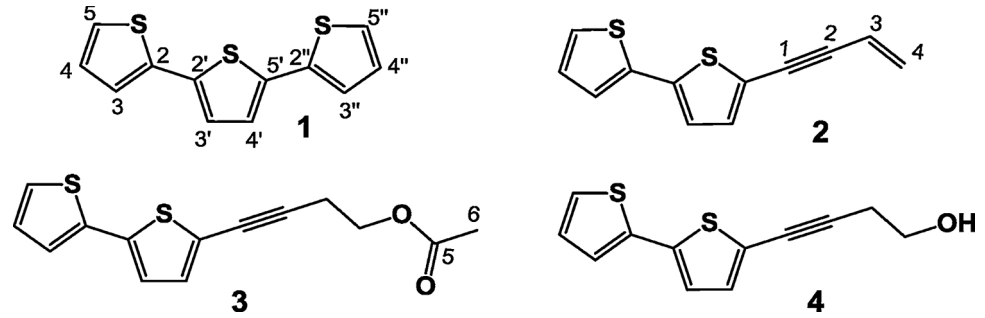

Fig. 1. Thiophenes isolated from hexanic extract of *Porophyllum obscurum* [13].

4000 (New Brunswick Scientific, NJ, USA).

To determine the oxygen demand, the antifungal photodynamic activity was determined simultaneously under four conditions: low-oxygen atmosphere/light; low-oxygen atmosphere/darkness; normal-oxygen atmosphere/light and normal oxygen atmosphere/darkness.

For low oxygen experiments, microplates were introduced into a GENbag anaer (bioMérieux, Illinois, USA) and low-oxygen atmosphere was created by H_2-CO_2-generating packs AnaeroGenCompact AN0010C (Oxoid, Basingstoke, UK). After 30 min of incubation, microplates were submitted to irradiation (light) or kept in darkness. Then, the bags were opened and aliquots (50 μL) from each well were plated onto 100-mm Petri dishes containing SDA and, after incubation at 30 °C for 48 h, the number of CFU/mL were quantified. Minimal Fungicide Concentration (MFC) was defined as the lowest concentration that causes total growth inhibition (CFU/mL = 0). Each experiment was assayed in triplicate, and data were expressed as modal value. Normal-oxygen experiments were carried out in the same conditions under normal atmosphere. The maximum amount of DMSO used for all negative controls was negligible (*i.e.* ≤ 0.2% vol:vol/DMSO:medium).

C. krusei ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains in accordance with the guidelines of the CLSI document M27-A3 [16]. Commercial antifungal drugs such as amphotericin B (AMB), fluconazole (FCZ) and itraconazole (ITZ) (Sigma-Aldrich) were also included in the assays as standard positive controls. Rose bengal (RB, Sigma Aldrich), irradiated with 200 mW blue LED (wavelength range of 435–475 nm, 95 J/cm^2) was included as photodynamic positive control [17].

2.5. Steady state analysis of ROS production

2.5.1. Determination of superoxide anion production

The formation of $O_2^{\cdot -}$ was detected with an indirect bioassay that measures the reduction of nitro blue tetrazolium (NBT) at 575 nm [18]. This procedure was performed in 96-wells microplates. PS solutions (100 μL), at a concentration of 125 μg/mL in phosphate-buffered saline (PBS), were incubated with 100 μL of NBT (0.1% w/v). The concentration of PS used in the assay was chosen in order to better visualize the effects. Determinations were performed in parallel, under light and darkness conditions. After irradiation, microplates were incubated 40 min at 37 °C. The reaction was stopped with the addition of 100 μL of HCl (0.1 M) and the content of each well was centrifuged at 10,000*g* for 30 min. The supernatant was discarded, and the sediment was dissolved with 200 μL of DMSO. Absorbance at 575 nm was measured using a VERSA Max microplate reader (Molecular Devices, USA). Each experiment was assayed in triplicate, and data were graphically represented as the mean ± SD. Control experiments were carried out under the same conditions but without adding the PS.

2.5.2. Determination of hydrogen peroxide production

The formation of H_2O_2 was quantified through the formation of a red quinoneimine, after reaction with 4-aminophenazone and hydroxybenzoate in the presence of peroxidase [19]. The colored complex produced was proportional to the H_2O_2-generated and was measured by the increase in absorption at 505 nm with regard to the basal situation (in absence of PS). This procedure was performed in 96-wells microplates. PS solutions (100 μL) at a concentration of 125 μg/mL in PBS, were incubated with 100 μL of reagent solution containing 4-aminophenazone (0.5 mM), hydroxibenzoate (12 mM) and horse-radish peroxidase (1 kU/L) (Wiener Lab, Rosario, Argentina) [20]. The concentration of PS used in the assay was chosen in order to better visualize the effects. Determinations were performed in parallel, under light and darkness conditions. Absorbance was read at 505 nm in a VERSA Max microplate reader after 30 min of incubation at 37 °C. Each experiment was assayed in triplicate, and data were graphically represented as the mean ± SD. Control experiments were carried out under the same conditions but without adding the PS.

2.5.3. Determination of singlet oxygen production

The singlet oxygen, production was determined using the 1,3-diphenylisobenzofuran (1,3-DPBF) bleaching method [21] with modifications. 1,3-DPBF is an established singlet oxygen quencher and the efficiency of PS at generating $O_2(^1\Delta_g)$, through its absorption decrease, monitored spectroscopically [22]. Stock solutions of *PoHex* (1.95 μg/mL), **1, 2, 3** and **4** (2.5 μg/mL, each) and 1,3-DPBF (50 mM) were prepared in DMSO. The concentrations of PS used in the assay were chosen in order to better visualize the effects. For the analysis, two equal reaction mixtures containing 100 μL of 1,3-DPBF and 100 μL of PS solutions were placed in two 96-well microtiter plates. Determinations were performed in parallel, under light and darkness conditions. Samples were taken every 60 s and the absorbance of 1,3-DPBF was monitored at 410 nm for a period of 30 min in a VERSA Max microplate reader. The experiment was performed in octuplicate and data obtained was process as linear least-squares fit of the semi logarithmic plot of ln (A_0/A) vs time, and the values of Stern-Volmer constants (k_{obs}) were obtained

2.6. Binding of photosensitizer to C. albicans

To determine the capacity to PS to bind to *C albicans* cells, the antifungal photodynamic activity was determined simultaneously under four conditions: with washing/light; with washing/darkness; without washing/light and without washing/darkness.

Four suspensions of *C. albicans* (5×10^3 CFU/mL) were incubated with solutions of *PoHex* and **1** at MFC (0.98 and 0.24 μg/mL respectively), in darkness 1 h at 28 °C (dark incubation). Then, in order to remove extracellular unbound PS, in two of them the pellets were centrifuged, washed twice with sterile distilled water and adjusted again to a final concentration of 5×10^3 CFU/mL. The other two remained unwashed. Then, aliquots of resulting suspensions (100 μL) were added to a 96 wells microplate with 100 μL of RPMI. Two suspensions (one washed and one unwashed) were irradiated with UVA light; and the others were kept in darkness. Control experiments were carried out under the same conditions, but without adding the samples (*PoHex* and **1**).

Aliquots from all experiments (50 μL) were sowed on 100-mm Petri dishes containing SDA and, after incubation at 30 °C for 48 h, the number of CFU/mL were quantified. Each experiment was assayed in triplicate, and data were graphically represented as the mean ± SD.

2.7. DNA fragmentation

Cells of *C. albicans* cultured by shaking in RPMI at 30 °C were harvested, washed twice with PBS and adjusted to 2.5×10^7 CFU/mL. Then, cells were treated with *PoHex* or **1** at different concentrations (MFC/4, MFC/2, MFC and MFCx2) and irradiated with UVA. Previously, MFC of PS against an inoculum of 2.5×10^7 CFU/mL was determined being 7.81 μg/mL, both for *PoHex* and **1**. Controls included: light control (in absence of PS/light), PS control (with PS at the highest concentration/darkness) and negative control (in absence of PS/darkness). A positive control was carried out with H_2O_2 (20 mM) [23]. Treated *C. albicans* cells were transferred to Falcom and pelleted, resuspended in 500 μL lyses buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3%, β-mercaptoethanol 1%). Subsequently, *C. albicans* total chromosomal DNA was extracted according to reported protocol [24]. Then, 10 μl of DNA solution with 10 μl of bromophenol blue was loaded onto a 1.0% (w/v) agarose gel (Bioexpress, Kaysbille, UT, USA) impregnated with GelGreen^{TM} (Biotium, California, EE. UU.) and subjected to electrophoresis. The gel was subsequently visualized using a UV transilluminator (VersaDoc, Bio-Rad, Hercules, CA, USA).

2.8. Cellular leakage assay

Cells of *C. albicans* cultured by shaking in Sabouraud-dextrose broth

Table 1
MFCs (μg/mL) values obtained for *Candida albicans* ATCC 10231 against *PoHex* and **1**-**4**, under low- and normal-oxygen atmosphere both under light and darkness conditions.

Condition	Atmosphere	low-oxygen	normal-oxygen	low-oxygen	normal-oxygen
	Light exposure	light	light	darkness	darkness
MFC	*PoHex*	250	0.98	< 1000	< 1000
(μg/mL)	**1**	7.81	0.24	< 250	< 250
	2	62.50	7.81	< 250	< 250
	3	62.50	7.81	< 250	< 250
	4	250	3.90	< 250	< 250
	RB	> 250	62.5	< 250	< 250
Antifungal	AMB	–	–	–	0.50
drugs	FCZ	–	–	–	2
MIC (μg/mL)*	ITZ	–	–	–	0.25

AMB: amphotericin B, FCZ: fluconazole; ITZ: itraconazole; RB: Rose Bengal For antifungal drugs, the Minimal Inhibitory Concentrations (MIC) were informed.

at 30 °C to early stationary phase (18 h growth) were harvested and washed twice with MOPS buffer. Then, re-suspended in cold clean MOPS and adjusted to a concentration of 2.5×10^7 CFU/mL with MOPS buffer, pH 6.0. The procedure was performed 24-wells microplates. Volumes of cells (500 μL) and PS (500 μL) were added to each well, reaching final concentrations equal to MFC, MFCx2 and MFCx4. Determinations were performed in parallel, under light and darkness conditions. Then, the contents of wells were transferred to centrifuge tubes, pelleted and the supernatants were collected for absorbance analysis at 260 nm in a Beckman DU-640 spectrophotometer (Fullerton, EEUU). Percentage of release were determined considering 100% the absorbance produced by cells treated with 1.2 M $HClO_4$ at 100 °C, 30 min [25]. Results were represented as the means of values from at three independent assays. Controls included: 0% cellular leakage (cells of *C. albicans* without irradiation in absence of PS) and light control (cells of *C. albicans* with irradiation in absence of PS).

2.9. Red blood cell lysis assay

The hemolysis was evaluated through the amount of hemoglobin released from the suspension of red blood cells relative to the value for the completely hemolyzed sample with deionized water. Human blood was provided by the blood bank of the Hospital Provincial del Centenario, Rosario This research has obtained ethical approval from the ethical review board at the College Biochemical and Pharmaceutical Sciences, National University of Rosario. (Res. Nº 091/2019). A 4%-suspension of freshly defibrinized blood was prepared by adding 2 mL of blood to 50 mL sterile NaCl physiological solution (saline). Stock solutions of PS solubilized in DMSO (< 2%) were further diluted with physiological solution to yield final test concentrations ranging MFC/8 – MFCx16 in microplate of 96 wells U-bottom. Red blood suspension (100 μL) was added to each well, gently mixed, irradiated with UVA and then microplates were incubated at 37 °C for 24 h. Determinations were performed in parallel, under light and darkness conditions. The hemolysis was qualitatively visualized as the presence or absence of a red button in the well. Then, 100 μL of the supernatants were carefully transferred to a flat bottom microplate and the absorbance was measured at 540 nm [26]. Percentage of hemolysis was determined related to 100% control value of hemolysis, achieved treating erythrocytes with deionized water. Controls in absence of PS with irradiation and in darkness were included.

2.10. Stress sensitivity test

The experiments were performed in 96-well microplates by following M27-A3 guidelines [16]. *PoHex* and **1** stock solutions (100 μL) were serially two-fold diluted with RPMI-1640 culture medium buffered to pH 7.0 with MOPS in order to reach final sub-lethal concentrations (between 0.12 − 0.49 for *PoHex* and 0.03 − 0.12 for **1**). Then, 100 μL of inoculum suspension (final concentration = 5×10^3 CFU/mL) was added to each well, the microplates were agitated at 150 rpm for 5 min on an orbital shaker platform Innova 4000 and irradiated. Controls included light control (in absence of PS/light), PS control (with PS / darkness) and growth control (in absence of PS/darkness).

Afterwards, the content of each well was serially 10-fold diluted with sterile distilled water yielding 5×10^3, 5×10^2 and 5×10 CFU/mL (in order to visualize individual colonies), and aliquots of 5 μL were spotted on Petri dishes containing solid media supplemented with stress-inducing compounds, incubated 48 h at 30 °C and photographed. Media employed were composed by yeast extract-peptone-dextrose (YPD) containing: NaCl (1.5 M) [27,28]; KCl (1.5 M) [28]; H_2O_2 (2 and 5 mM) [27], menadione (0.02 mM) [27]; sodium dodecyl sulfate (SDS) (0.05%) [27,28] and caffeine (7 mM) [27,29].

2.11. Statistical methods

Kruskal Wallis and Dunn's test of multiple comparisons were used to test in assays significant difference between light and darkness conditions. The *t*-test was used to assess the degree of statistical difference between the number of CFU/mL among experiments with and without washing.

3. Results

3.1. Influence of oxygen in the antifungal photosensitive activity

To investigate the influence of oxygen in the antifungal photosensitive of *PoHex* and the compounds **1** to **4**, the viability of *C. albicans* was determined with and without irradiation with UVA light (light and darkness conditions, respectively) and with normal and low content of oxygen (Table 1). No antifungal activity was observed in darkness, under both oxygen conditions (MFC > 1000 μg/mL for *PoHex* and > 250 μg/mL for thiophenes **1**-**4**). Meanwhile, an important antifungal photoactivity was observed in experiments with irradiation. *PoHex* and thiophenes exhibited the highest activities in experiments performed under normal-oxygen atmosphere/light. The activity of *PoHex* decreased more than 200 times with low-oxygen conditions in comparison with normal-oxygen experiments, increasing its MFCs value from 0.98 μg/mL to 250 μg/mL. The same effect was observed for thiophenes **1**-**4** being MFCs values in the range 0.24–7.81 μg/mL with normal-oxygen atmosphere and increasing to 7.81–250 μg/mL in low-oxygen atmosphere. Control experiments showed that the viability of *C. albicans* was not affected either by the incubation for 60 min into the anaerobiosis bag (result not shown) or by UVA irradiation [13].

3.2. Superoxide anion production (type I mechanism)

The production of $O_2^{\bullet -}$ by *PoHex* and **1**-**4** was measured by the NBT assay previously explained. Control experiments in darkness and without PS gave negligible values of absorbance suggesting that, under these conditions, there were not decomposition of NBT. *PoHex* and **2**-**4**, under UVA light irradiation, considerably increased NBT decomposition in comparison with experiment in darkness (Fig. 2). No significant differences in absorbance, in comparison with control, have been observed for **1**. These results suggested that *PoHex* stimulated $O_2^{\bullet -}$ formation, and that **2**-**4** were the responsible of this effect. Thiophene **4** stood out as the highest $O_2^{\bullet -}$ producer.

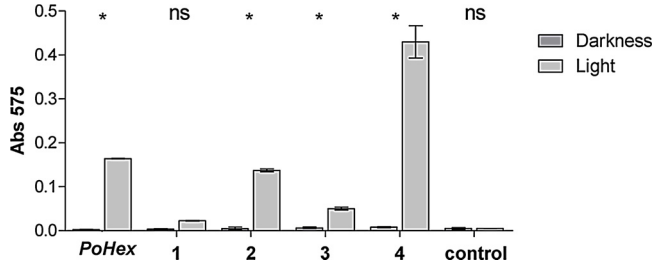

Fig. 2. Superoxide production ($O_2^{\bullet -}$) of *PoHex* and thiophenes **1**-**4** (125 μg/mL), evaluated in darkness and under irradiation (light). * $p < 0.05$; ns: no significant.

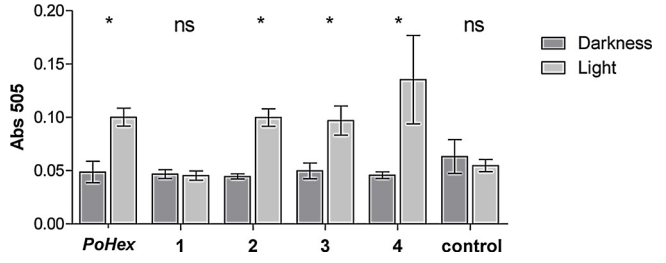

Fig. 3. Hydrogen peroxide (H_2O_2) production of *PoHex* and thiophenes **1**-**4** (125 μg/mL), evaluated in darkness and under irradiation (light). * $p < 0.05$; ns: not significant.

3.3. Hydrogen peroxide production (type I mechanism)

The production of H_2O_2 by *PoHex* and thiophenes was measured by the assay previously explained. Control experiments in darkness and without PS gave negligible values of absorbance suggesting that, under these conditions, there were not H_2O_2 production (Fig. 3). However, significant differences have been observed, for *PoHex* and **2**-**4**, between light and darkness experiments. These results suggested that *PoHex* stimulated H_2O_2 formation only when they were irradiated and that **2**-**4** were the responsible of this effect. No significant differences between light and darkness have been observed for **1**. Control experiments gave negligible values of absorbance.

3.4. Singlet oxygen production (type II mechanism)

The ability of *PoHex* and **1**-**4** to generate $O_2(^1\Delta_g)$ under UVA irradiation (type II mechanism), was assessed following the kinetics bleaching of 1,3-DPBF (Fig. 4). The values of Stern-Volmer constants (k_{obs}) were calculated and compared to each other, and the results indicated that oxygen plays a very significant role in these

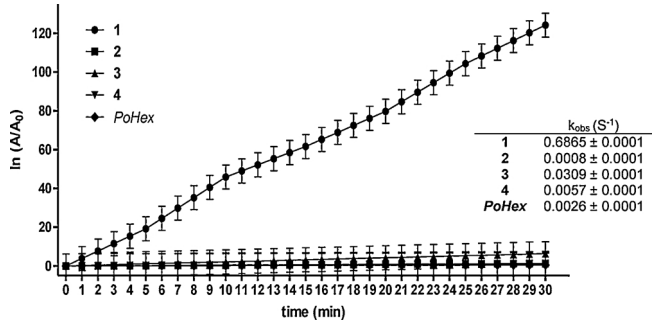

Fig. 4. Singlet oxygen [$O_2(^1\Delta_g)$] production. First-order plots for the photooxidation of 1,3-DPBF (50 mM) in DMSO, photosensitized by *PoHex* (1.95 μg/mL) and **1**, **2**, **3** and **4** (2.5 μg/mL), irradiated with UVA light. Linear least-squares fit of the semi logarithmic plot of ln (A_0/A) vs time. In the table, the values of Stern-Volmer constants (k_{obs}).

determinations. In presence of **1**, the scavenger 1,3-DPBF showed a very fast decrease in absorbance. The higher k_{obs} (0.6865), indicating a considerable singlet oxygen yield production for this thiophene. The compound **3** decrease the absorbance of 1,3-DPBF ten times slower than **1** (k_{obs} = 0.6865 and 0.0309 correspondingly). The contribution of $O_2(^1\Delta_g)$ due to **4** and *PoHex* (k_{obs} = 0.0057 and 0.0026 correspondingly), are weak in comparison of the aforementioned compounds. The value of k_{obs} for **2** is insignificant (k_{obs} = 0.0008), this denotes that **2** can act as $O_2(^1\Delta_g)$ quencher. There was no breakdown of the 1,3-DPBF in control experiments performed in darkness and without PS (data not shown), which makes it evident that under these conditions, there were not decomposition of 1,3-DPBF. Compound **1** was the only one that produced singlet oxygen and therefore, the responsible of the decrease in absorbance observed with *PoHex*. It is worth mentioning that the concentration of **1** in the assay was very low (0.14 μg/mL), considering that the concentration of **1** in the *PoHex* was 7.21 mg/100 mg [13] and the concentration of *PoHex* in the assay was 1.95 μg/mL.

3.5. Binding of photosensitizers to C. albicans

In order to test whether PS present in *PoHex* actually bound to the fungal cells, the antifungal activities were evaluated removing unbound PS by washing. A significant decrease in CFU/mL ($p < 0.05$) was observed in experiment without washing/light with both PS, *PoHex* and **1** (Fig. 5). No significant differences in CFU/mL, in comparison with untreated controls, were observed when cells were washed, both light and darkness conditions, and without washing/darkness. Furthermore, control experiments showed that the viability of *C. albicans* was not affected by the treatment (centrifugation, washes and dilutions).

3.6. DNA fragmentation

Apoptosis in *C. albicans* cells as a consequence of the oxidative modifications of biological molecules due to ROS was evaluated. DNA gel electrophoresis revealed no DNA fragmentation in negative, light and PS controls (Fig. 6). Meanwhile, H_2O_2 positive control showed DNA fragmentation pattern with a complete loss of banding and extensive migration of the small DNA fragments [23]. Cells treated with *PoHex* and **1** (at 1.95–15.62 μg/mL) and irradiation with UVA matched that of the negative control suggesting that DNA remained intact, which was indicative that apoptosis did not occur.

3.7. Cellular leakage assay

The Cellular Leakage Assay was performed to assess if the action of PS under UVA light produce fungal membrane damage [25]. This test assumes that a disruption of the membrane will cause a release of intracellular components from the fungal cells that absorb at 260 nm (nucleic acids and aromatic aminoacids). PS (at MFC, MFCx2 and MFCx4) were added to cell suspensions of *C. albicans* and irradiated. The results showed that the percentages of release produced by *PoHex* under UVA irradiation (Fig. 7A) were 10.35% at MFC (7.81 μg/mL); 13.02% at MFCx2 (15.62 μg/mL) and 13.52% at MFCx4 (31.25 μg/mL). The percentages of release produced by **1** under UVA irradiation (Fig. 7B) were 3.01% at MFC (0.24 μg/mL); 4.47% at MFCx2 (0.48 μg/mL) and 5.74% at MFCx4 (0.96 μg/mL). In both cases, values were higher under irradiation compared with experiments performed in darkness but no significant differences were observed between them ($p > 0.05$). The slight increments in leakage observed in darkness with high concentrations of *PoHex* (MFCx2 and MFCx4) were probably caused by the components of the extract that affected membrane permeability.

3.8. Red blood cell lysis assay

The hemolytic effect of *PoHex* and **1** was evaluated employing a

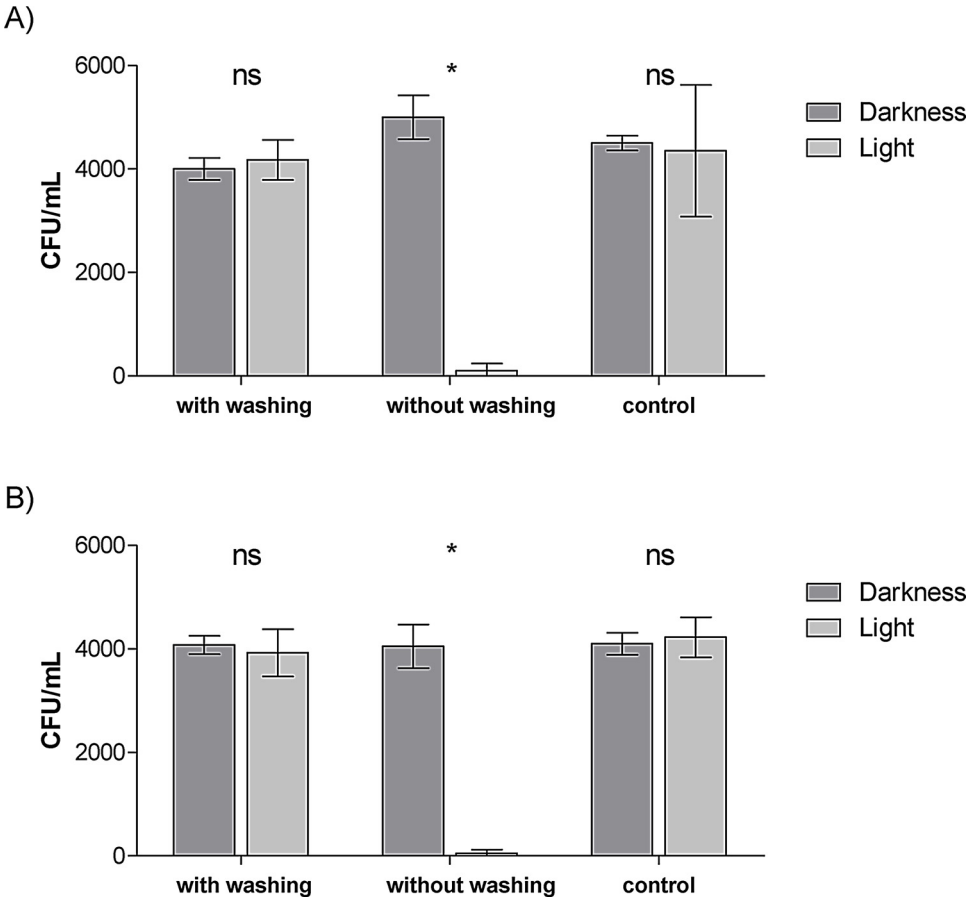

Fig. 5. Colonies Forming Units/mL (CFU/mL) obtained in experiments of antifungal activity against *Candida albicans* ATCC 10231, with and without washing, and both with irradiation and in darkness employing A) *PoHex* and B) **1**.

blood cell lysis assay that measures the release of hemoglobin as a consequence of the disruption of the erythrocyte membrane. *PoHex* and **1** solutions, at concentrations between MFC/8 to MFCx16, were incubated with erythrocytes and irradiated. No hemolytic effect has been observed in control with irradiation without PS; and in darkness with PS (even at concentration 16 times greater than MFC). *PoHex* at concentrations higher than CFM under irradiation, showed significantly higher percentages of hemolysis in comparison with controls in darkness. The same behavior has been observed with **1**, at concentrations between CFM/2-CFMx16 (0.12–3.90). In our previous work [13], we determined that the concentration of **1** in the extract employed in the assays was 7.21 mg/100 mg. Therefore, in the solutions of *PoHex* showed significantly higher percentages of hemolysis between light and darkness experiments (≥ 1.95 μg/mL), the concentration of **1** was > 0.14 μg/Ml (Table 2).

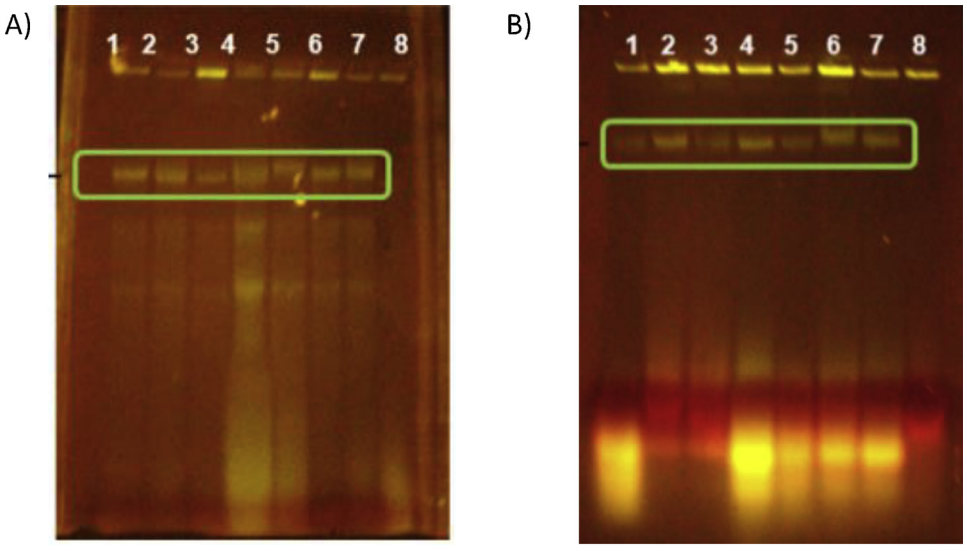

Fig. 6. DNA fragmentation of *Candida* cell cultures with: **A)** *PoHex* and **B) 1**. Street **1)** light control (in absence of PS/light); **2)** PS control (with *PoHex* or **1** at 15.63 μg/mL/darkness); **3)** PS (at 15.63 μg/mL)/light; **4)** PS (at 7.81 μg/mL)/light; **5)** PS (at 3.91 μg/mL)/light; **6)** PS (at 1.95 μg/mL)/light; **7)** negative control (in absence of PS/darkness) and **8)** positive control (H_2O_2 20 mM). Rectangle indicates not fragmented *Candida* DNA.

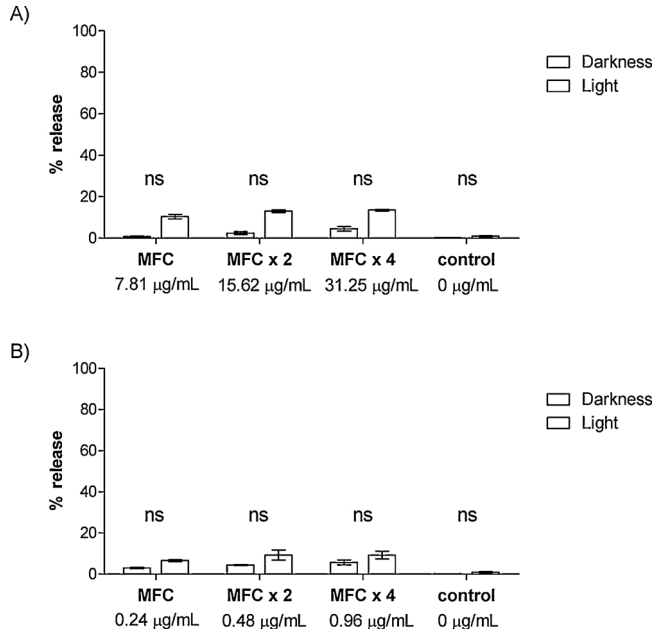

Fig. 7. Cellular leakage assay for **A)** *PoHex* (at MFC = 7.81 μg/mL; MFCx2 = 15.63 μg/mL and MFCx4 = 31.25 μg/mL) and **B) 1** (at MFC = 0.24 μg/mL; MFCx2 = 0.48 μg/mL and MFCx4 = 0.96 μg/mL), evaluated in darkness and under irradiation (light). ns: not significant.

Table 2
Hemolytic effect. Percentage of hemolysis produced for the *PoHex* and **1**, in darkness and under irradiation with UVA. *** significant; ns: not significant.

		Conc. (μg/mL)	light	darkness	
PoHex	CFMx16	15.63	9.57 ± 2.77	0.01 ± 0.02	***
	CFMx8	7.81	7.47 ± 1.36	0.09 ± 0.01	***
	CFMx4	3.90	4.85 ± 1.19	0.02 ± 0.01	***
	CFMx2	1.95	2.00 ± 0.40	0.02 ± 0.01	***
	CFM	0.98	0.54 ± 0.16	0.02 ± 0.02	ns
	CFM/2	0.49	0.02 ± 0.03	0.01 ± 0.09	ns
	CFM/4	0.24	0.06 ± 0.03	0.13 ± 0.02	ns
	CFM/8	0.12	0.02 ± 0.09	0.13 ± 0.07	ns
1	CFMx16	3.90	14.37 ± 0.27	0.09 ± 0.10	***
	CFMx8	1.95	11.31 ± 0.04	0.02 ± 0.03	***
	CFMx4	0.98	7.96 ± 0.15	0.02 ± 0.04	***
	CFMx2	0.49	4.92 ± 0.27	0.01 ± 0.03	***
	CFM	0.24	2.68 ± 0.45	0.04 ± 0.01	***
	CFM/2	0.12	0.94 ± 0.18	0.05 ± 0.01	***
	CFM/4	0.06	0.06 ± 0.00	0.06 ± 0.02	ns
	CFM/8	0.03	0.05 ± 0.04	0.01 ± 0.05	ns
control		0	0.01 ± 0.03	0.00 ± 0.01	ns

3.9. Stress sensibility test

Stress sensitivity tests were carried out in order to examine if the alterations induced by antifungal photodynamic activity alter responses to oxidative and osmotic stress and cell wall integrity. Assays included the osmotic stressors, NaCl and KCl; oxidative stressors such as H_2O_2 and the superoxide generator, menadione; and cell wall stressors, SDS and caffeine. No differences in growth between light, PS and growth controls have been observed suggesting that PS alone or irradiation alone did not influence growth under any of the stress conditions tested. The treatment of cells with *PoHex* and light, impaired the growth of *C. albicans* in presence of the osmotic stressors NaCl and KCl (Fig. 8A b,c). Regarding oxidative stress, cells treated with PS and light, showed almost control levels of sensitivity to H_2O_2 (at 2 and 5 mM), and menadione (Fig. 8A d,e,f). Following treatment with *PoHex* and irradiation, cells displayed increased sensitivity to the cell wall stressor SDS; however, only a marginal effect could be observed in response to caffeine (Fig. 8A g,h). Regarding **1**, the treatment with this thiophene and light, the growth of *C. albicans* was only affected in presence of H_2O_2 at 5 mM (Fig. 8B e) but it did not increase sensibility to other oxidative stressors, such as H_2O_2 at 2 mM and menadione (Fig. 8B d,f). No growth impairment have been observed in response to osmotic (Fig. 8B b,c) and cell wall stressor (Fig. 8B g,h).

4. Discussion

Given the increasing numbers of immunocompromised patients, the number of fungal infections caused by yeasts has significantly enhanced in recent years associated with a dramatic increase of resistant microorganisms. For these reasons, it is imperative to find new and effective agents to manage such problems. PDI has proven to be a potential clinical antifungal therapy since it works equally well regardless of resistance to antifungals and which (until now) has not been shown to produce resistance. Many plants are known to produce substances that show antimicrobial activity and, in addition, that some natural compounds are being described as well-established PSs [30], such as hypericin [31], curcumins [31], anthraquinones [32], and some alkaloids [21]. In our previous studies, we investigate the antifungal photosensitive activity of extracts of *P. obscurum* as alternative for the treatment of fungal infections and found that *PoHex* and their main thiophenes could be used as effective photosensitizing agents [13]. However, these studies were preliminary, and more studies with these extracts were needed. Therefore, in the present work, in order to get inside the mechanism of photosensitization, we have studied several features related to the activity of *PoHex* and thiophenes.

The mechanisms of the antifungal activity of *PoHex* and thiophenes **1**-**4** can be classified as photodynamic [7] considering that oxygen absence had a negative effect on the antifungal photosensitive activity. When was evaluated in low-oxygen atmosphere, MFC of *PoHex* value increased almost 250-times in comparison with normal-oxygen one. The same effect was observed for individual thiophenes: **1** increased 32-times its CFM value, compounds **2** and **3**, 8-times; and the highest oxygen-dependent effect was observed for compound **4** (64-times). Then, in order to evaluate ROS production, we determined the contribution of mechanism type I or II. We could observe that thiophenes **2**-**4** were generator of $O_2^{\bullet -}$ and H_2O_2. On the other hand, in accordance with previous reports [33,34], **1** has demonstrated to be an excellent singlet oxygen producer, but it is not involved in electron transfer reactions. These results suggested that ROS played a significant role in the antimicrobial PDI of *C. albicans*, and furthermore, both type I and II reactions were involved in the photodynamic antimicrobial action of *PoHex*. Although $O_2^{\bullet -}$ and H_2O_2 may contribute to the photoactivity observed, it is regarded that singlet oxygen plays the major role in PDI [35,36], because it has high chemical reactivity, destroying microorganism more rapidly and at much lower concentrations than other biocides; and has short half-life that ensures a localized response without affecting distant cells or organs [6]. In addition, the killing action of ROS may be significantly impaired by anti-oxidant enzymes such as superoxide dismutase and catalase that protect microorganisms against $O_2^{\bullet -}$ and H_2O_2, respectively but it is unlikely that they can develop resistance to PDI by producing anti-oxidant enzymes against singlet oxygen.

To gain a better interpretation to the photoactivity of *PoHex*, the binding of PSs to *C. albicans*, apoptosis induction, damage in fungal and erythrocyte membranes and yeast response to oxidative and osmotic stress were determined. Assays have been conducted both with *PoHex* and **1** because was the most active compound in the extract; and the only one that generated singlet oxygen.

Washing of the cells prior to illumination inhibited PDI, which is indicative that PSs were removed, suggesting that molecules were not or were weakly bound to the cells. An explanation of this phenomenon is that thiophenes **1**-**4** are uncharged hydrophobic compounds,

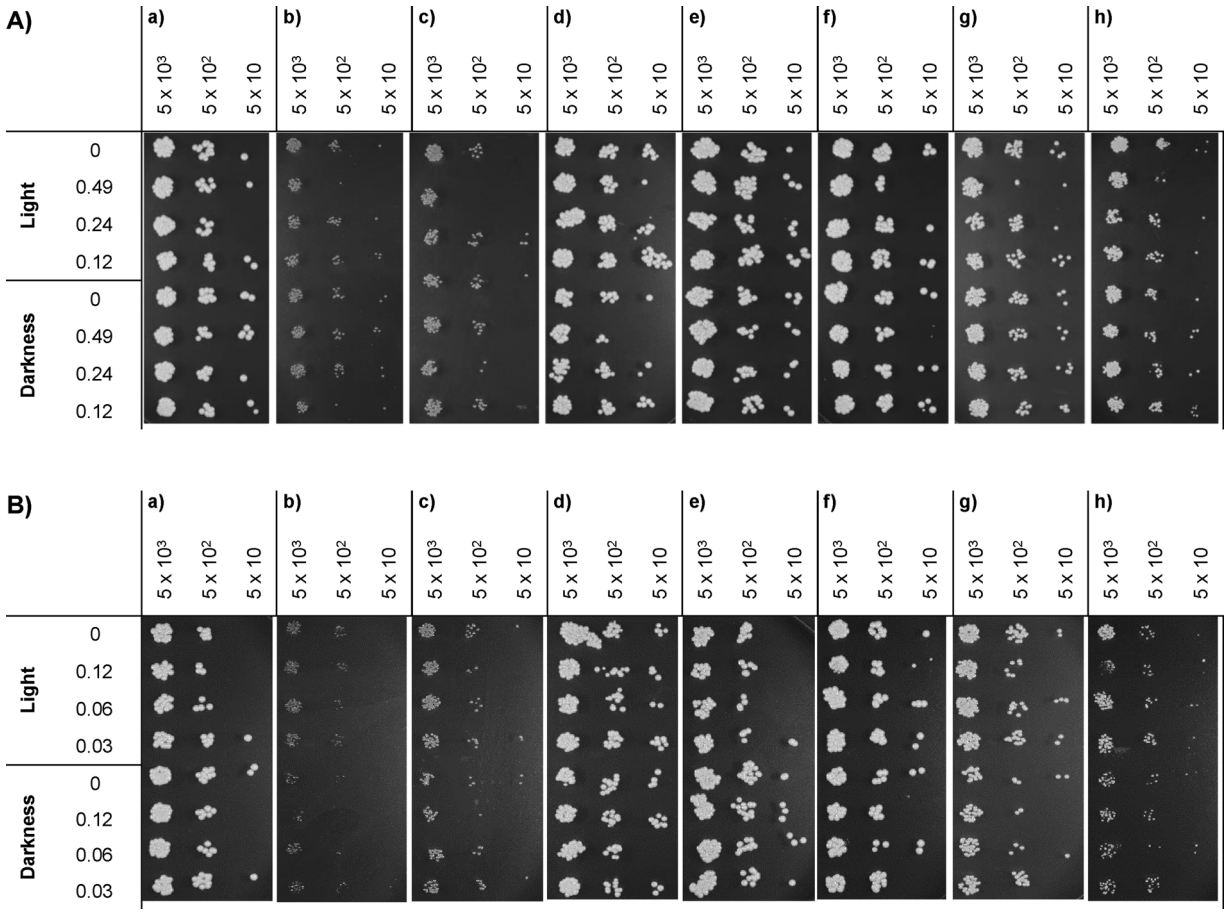

Fig. 8. Growth, after incubation for 48 h at 35 °C, of serial dilutions (5×10^3, 5×10^2 and 5×10^1 CFU/mL) of *Candida albicans* ATCC 10231, treated with sub-lethal concentrations of **A)** *PoHex* (0.12 − 0.49 μg/mL) and **B) 1** (0.03 − 0.12 μg/mL), both under irradiation and in darkness, and spotted onto solid media (YPD) supplemented with stress-inducing compounds **a)** control; **b)** NaCl 1,5 M; **c)** KCl 1,5 M; **d)** H_2O_2 5 mM; **e)** H_2O_2 2 mM; **f)** Menadione 0,02 mM; **g)** SDS 0,05% and **h)** Caffeine 7 mM.

considering that previous works stated that both hydrophilic and hydrophobic compounds exhibited much lower cellular uptake than the amphiphilic ones [37,38]. In addition, hydrophilic PSs easily solubilize in H_2O and can effectively diffuse to the cells; and positive charges would help PSs to be adsorbed on the surface of *C. albicans* [37,38]. Some authors have stated that PS does not need to be internalized for inflicting photodamage on vital structures of the cell and this behavior has the advantage that is not likely to promote resistance in microorganisms due to enhanced drug metabolism, changes in the drug binding sites, efflux pumps, etc. [6,36,39].

ROS cause an imbalance in cellular homeostasis, including damaging cytoplasmic organelles and nucleic acids, resulting in cell death by apoptosis, necrosis, or autophagy, that play pivotal role in photodynamic microbial inactivation [40]. In order to investigate which is the process that cause cell death, we evaluate if apoptosis occurred when *C. albicans* underwent photodynamic treatment with *PoHex* and **1** [23]. There are numerous assays for apoptosis being the two most common those that evaluate morphological changes (cell shrinkage, condensation of nuclear chromatin, formation of apoptotic bodies) or DNA fragmentation [41]. Results obtained employing the methodology of DNA fragmentation methodology indicated that ROS generated by *PoHex* and **1** under UVA irradiation, at conditions employed in PDI, did not induce apoptosis in *C. albicans*, suggesting that another process was the responsible of the cellular death [42,43].

One of the basic mechanisms that have been proposed to account for the lethal effect caused to microorganisms by PDI is damage caused by ROS to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport systems and enzymes [44]. A possible explanation of this phenomenon could be transformation of ergosterol into its transanular peroxide through photochemical oxygenation caused by singlet oxygen suggested by Trigos et al. [45]. We employed leakage assays which consider that any damage to the cell membrane and results showed that exposure of *C. albicans* cells to *PoHex* and UVA irradiation, in the condition employed in PDI, caused low percentages of release (lower than 15%), even at the highest concentration of PSs (MFCx4). It is worth mentioning that these values were higher than those in darkness, but the differences were not significant. The same behavior has been observed with **1** and light that causes percentages of release lower than 6% at MFCx4. It is evident that PS and UVA induced a mild damage of membrane integrity and did not cause loss of cytoplasm content in *C. albicans* cells. Then we performed the hemolysis assay by using erythrocytes as a model of biological membrane system for probing interactions between toxic compounds and the lipid bilayer [46]. The hemolytic activity of PS was assessed at different concentrations between MFC/8 to MFCx16. The results showed that the exposure to *PoHex*, for all the concentrations analyzed (under light and darkness conditions), the degree of hemolysis was lower than 10%, which allows us to suppose that *PoHex* is not toxic for erythrocytes, even in high doses. Under irradiation, **1** showed percentages of hemolysis lower than 15% at high concentrations (MFCx16) significant different from those obtained in darkness. Some authors have stated that the target action of **1** is the membrane of yeast or erythrocytes [34]. However, under the conditions employed in the present study for PDI of *C. albicans*, neither of them was been affected.

In addition, the stress assay evaluated whether PDI impaired the growth of *C. albicans* in the presence of osmotic, oxidative and cell wall

stressors. Treatment with *PoHex* and UVA increased cells sensitivities to osmotic stressors did not reduce resistance to additional oxidative stress and possibly affected the structure of the cell wall, a vital structure involved in protection, growth, and adherence. Regarding **1**, treatment under UVA only affected sensibility to oxidative stress at high concentrations of stressor (H_2O_2 at 5 mM). Kato et al [27] have observed the same alterations with methylene blue and 660 nm light and they stated that these alterations could reduce the ability of *C. albicans* to infect and lowered their resistance to immune cells.

5. Conclusions

PDI employing hexane extract of *P. obscurum* aerial parts under UVA does represent one more alternative for topical antifungal therapy, which could reduce unwanted side effects, and with the advantages of low cost, low or non-existent overdose risk. In addition, ROS have a broad spectrum of activity and can destroy numerous molecular microbial targets so the appearance of fungal resistance is improbable [6]. The activity displayed by *PoHex* was attributed to a mixture of four thiophenes that contributed to the activity by generating different ROS, through type I and II mechanisms. The most active compound in the extract, 2,2′:5′2″terthiophene generated single oxygen which is considered as the ROS that plays the major role in PDI. The mechanism that causes fungal cell death is not clear, but our results showed that ROS generated and did not induced apoptosis, suggesting that it may occur by another mechanism such as necrosis or autophagy. In addition, at the concentrations that they displayed antifungal activity, ROS did not cause a severe damage of erythrocyte membrane integrity, so the possibilities of causing hemolysis are low. These advantages encourage further investigations to confirm the potential of this natural product for application as PS in photodynamic antimicrobial chemotherapy against fungal infections.

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