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Photodynamic inactivation of oropharyngeal *Candida* strains

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ABSTRACT

Oropharyngeal candidiasis (OPC) is an infection frequent in immunocompromised patients. Photodynamic therapy is an alternative to conventional treatments, based on the utilization of compounds that inhibit or kill microorganisms only under the effect of light, process known as Photodynamic Inactivation (PDI). In the present study, PDI of Candida spp. by the natural product α-terthienyl (α-T) was investigated following the guidelines of CLSI M27-A3, under UV-A light irradiation.

The optimal values of two variables, exposure irradiation time (ET) and distance to the irradiation source (DIS) were established by employing Design Expert Software (DES). For this purpose, a panel of *Candida* strains isolated from OPC (*C. albicans, C. tropicalis, C. parapsilosis and C. krusei*) was employed and optimal values were 5 min (ET) and between 6.06 and 6.43 cm (DIS) with a desirability factor of 0.989. α-T plus UV-A light in the optimal conditions caused a complete reduction in viable cells in 5 min which was demonstrated by viable cells reduction assays and confocal microscopy after vital staining (propidium iodide/fluorescein diacetate). The germ tube formation of *C. albicans* was inhibited by α-T at sub-inhibitory concentrations. Results showed that α-T plus UV-A light could constitute an alternative for OPC treatments at the optimal conditions determined here.

Introduction

Oropharyngeal candidiasis (OPC) is an infection frequent in immunocompromised patients undergoing cancer, HIV, poor nutrition, metabolic disorders and using removable denture, among others (Bulacio et al., 2012) that causes inflammation of the oral mucosa, pain and dysgeusia and constitutes a risk factor for *Candida* dissemination (Mang et al., 2010). OPC is primarily caused by *Candida albicans* but there is an emergence of other *Candida* spp. different from *C. albicans* as infecting or co-infecting organisms (Odds et al., 1989) which produce infections with more severe symptoms than those due to *C. albicans* alone. The antifungal drugs in current use are not completely effective (Kathivaran et al., 2012) against OPC and resistance and drug-drug interactions have gained importance in the last years (Borelli et al., 2008).

Photodynamic therapy (PDT) is a promising alternative to conventional antifungal treatments since it is based on the utilization of a kind of compounds that inhibit or kill microorganisms only under the effect of light, process known as Photodynamic Inactivation (PDI) (Prates et al., 2009; Dai et al., 2009). 2,2′,5′,2″-Terthiophene (α-T) (synonyms: alpha-terthienyl or 2,5-di(2-thienyl)thiophene) (Fig. 1), a natural product first isolated from *Tagetes erecta* and then from *Eclipta, Bidens, Porophyllum* and *Tessaria* genera (Asteraceae) (Chan et al., 1979; Towers et al., 1979), is the first member of a new class of phototoxic compounds which has demonstrated antifungal (Camm et al., 1975; Towers et al., 1976, 1979; Romagnoli et al., 1994, 1998; Wang et al., 2007), antibacterial (Arnason et al., 1981), antileishmanial (Takahashi et al., 2011) and nematicidal (Bakker et al., 1979) activities, under irradiation with ultraviolet (UV) light.

Regarding antifungal activity, α-T showed to be photoactive against *C. albicans* (Towers et al., 1976, 1979; Hudson et al., 1993) and *Candida utilis* (Kagan et al., 1980) and also demonstrated activity against *Trichophyton mentagrophytes* and other species of dermatophytes (*Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton violaceum*, *Epidermophyton floccosum*, *Microsporum canis, Microsporum gypseum* and *Microsporum cookie* including its teleomorph, *Nannizzia cajetani*) under UV-A irradiation (Romagnoli et al., 1994, 1998). It also produced important ultra-structural changes within the hyphae after irradiation, such as damage to the nuclear membrane, mitochondria and endoplasmic reticulum (Romagnoli et al., 1994; Mares et al., 1990).

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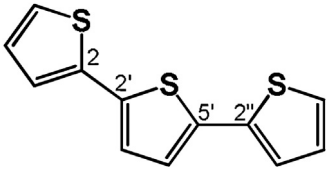

Fig. 1. Structure of 2,2′,5′,2″-terthiophene (α-terthienyl).

In spite of the several reports on the light-induced antifungal properties, the optimal conditions for obtaining the best photosensitizing antifungal properties of α-T have not been investigated yet. In addition to this lack of studies, the previous reports about the antifungal activities of α-T were performed with different non-standardized methodologies (Mares et al., 1993; Romagnoli et al., 1998; Mares et al., 2002, 2004).

In the present study, the PDI of *Candida* spp. by α-T was investigated for the first time following the guidelines of CLSI M27-A3 methodology (CLSI, 2008) in parallel in the dark and under UV-A light irradiation and the optimal values of each of two variables, exposure irradiation time (ET) and distance to the irradiation source (DIS) were established by employing Design Expert Software (DES) (Hooda et al., 2012). Optimization was performed using a panel of resistant and susceptible strains of *Candida* genus (*C. albicans, C. tropicalis, C. parapsilosis* and *C. krusei*) isolated from OPC in patients with head and neck cancer undergoing radiotherapy.

In addition, the fungicidal capacity of α-T, at the optimal time and distance conditions, was investigated through viable cell reduction, confocal microscopy and inhibition of germ tube formation.

Materials and methods

Chemicals

Solvents were analytical grade or were purified by standard procedures prior to use. The absorption spectra were taken with a spectrometer Beckman DU-640 (Fullerton, EEUU). α-T (purity 99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The stock solution was prepared in DMSO (Cicarelli, San Lorenzo, Argentina) and stored at 4 °C in the dark.

Microorganisms and culture conditions

C. albicans ATCC 10231 provided by *American Type Culture Collection* (ATCC), Rockville, MD, USA, was employed for determining the antifungal PDI (APDI), fungicidal studies and germ tube inhibition. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were employed as quality controls in antifungal susceptibility testing as recommend by CLSI. Twenty clinical *Candida* strains, provided by Centro de Referencia en Micología – CEREMIC (CCC, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), Rosario, Argentina), were also employed for APDI. They were isolated from denture-related oral mucosal lesions and classified as susceptible, susceptible dose-dependent or resistant to one of the drugs Amphotericin B (AMB), Fluconazole (FCZ) or Itraconazole (ICZ), established with the CLSI M27-A3 antifungal microdilution test (CLSI, 2008). Their voucher specimens are detailed in Table 2. Each isolate was maintained by sub-culturing the strain once every seven days on Sabouraud-Dextrose Agar (SDA, Difco Laboratories, Sparks, MD, USA) at 35 °C in aerobiosis. Inocula of cell suspensions were obtained according to reported procedures and adjusted to 2.5×10^3 colony-forming units *per* ml (CFU)/ml in sterile 0.145 M saline solution (CLSI, 2008).

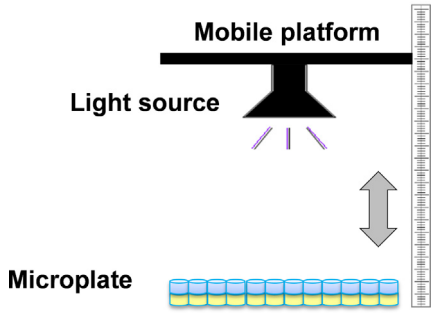

Fig. 2. Device used to vary the distance from the UV-A lamp to the microplate.

Irradiation source

Experiments were performed in a device in which the 100 W fluorescent lamps (Alic, Buenos Aires, Argentina) were placed in a mobile platform that allowed vary the distance to the test microplates (Fig. 2). Fluence value was calculated for the optimal distance obtained (Zhu and Finlay, 2008).

APDI test

The APDI was determined in parallel in two 96-wells flat-bottomed microtitre plates with the CLSI M27-A3 methodology (CLSI, 2008). One microplate was subjected to irradiation before incubation and the other one was maintained in the dark (Fig. 3).

100 μl of stock solution of α-T was serially two-fold diluted with RPMI-1640 buffered to pH 7.0 with MOPS (Sigma–Aldrich, St. Louis, MO, USA) into the wells of one line of the microplate (by triplicate), obtaining a series of Test Wells (TW). Then, 100 μl of inoculum suspension was added to each well, reaching to final concentrations of α-T between 250 and 0.03 μg/ml. In addition, Growth Control Wells (GCW) containing compound-free medium and inoculum, Blank Test Wells (BTW) including compound, culture medium and sterile water instead of inoculum and Sterility Control Wells (SCW), containing the compound in the culture medium and sterile water,

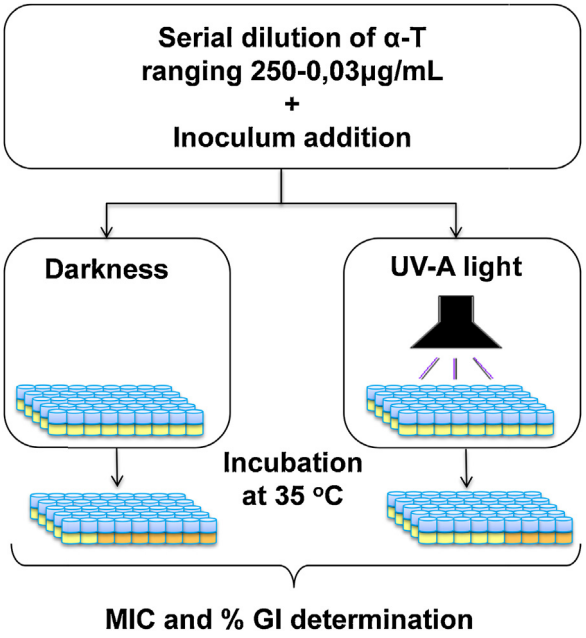

Fig. 3. APDI test. Evaluation of MIC (Minimal Inhibitory Concentration) and % Growth Inhibition (% GI) under UV-A irradiation (right). In parallel, the same assay performed in the dark (left).

were included in the plate. One of the microplates (to which the lid was removed) was submitted to irradiation under aseptic conditions in a laminar air-flow chamber. The non-irradiated plate was wrapped with aluminum foil to avoid exposure to light. Both plates were then incubated in a moist, dark chamber at 35 °C and the growth of survival cells was assessed at 24 h by measuring the optical density of each well in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. The growth inhibition percentage (% GI) in each well was calculated as follows: $\%\ GI = 100 - [(OD_{TW} - OD_{BTW})/(OD_{GCW} - OD_{SCW})] \times 100$. Results were expressed as arithmetic averages. MIC (Minimal Inhibitory Concentration) was defined as the lowest concentration that causes total inhibition of growth and was expressed as modal value.

Optimization

The experiments were designed and analyzed by using the statistical DES package (Stat-Ease, Inc., Minneapolis, USA) version 7.0.3. In the first phase, called screening phase, all *Candida* strains of Table 2 were used and a *Total Factor Design* was built, in order to estimate whether the factors ET and DIS affected the fungal growth. They were evaluated at two levels with two replicates and the selected ranges were 1–60 min and 60–120 min for ET and 6–19 cm for DIS, being the responses measured in terms of % GI. In the second phase, called the optimization phase, a *Central Composite Design* (CCD) consisting of 11 experiments (9 experiments and 2 replicates of the central point) which were combinations of the selected factors, was applied. With CCD, the optimal values of DIS and ET to reach the maximum % GI were obtained. All experiments were performed in a *random* order to minimize the effects of uncontrolled factors that may introduce a *bias* on the measurements.

Fungicidal experiments

Minimum Fungicidal Concentration (MFC) was determined from APDI microplates as follows: 10 μl of each TW showing no-visible growth were sub-cultured on 10 cm-diameter plates containing SDA and incubated at 35 °C for 48 h. Determinations were performed by triplicate. MFC was defined as the lowest concentration showing no growth in these new plates and was expressed as modal values.

For viable cells reduction experiment, 100 μl of the fungal suspension (cell density = 2.5×10^3 CFU/ml) was added to 100 μl of RPMI-1640 culture medium containing the compound at MFC and the mixture was submitted to light irradiation at the optimal ET and DIS that were determined as described in Section "Optimization". Each minute, 10 μl of the culture mixture were removed, inoculated on SDA plates and incubated 48 h at 35 °C. After incubation, the CFU/ml of each plate was determined. The whole experiment was performed in triplicate. A viable cells reduction curve was built by plotting arithmetic averages of CFU/ml ± standard deviation (SD) *vs.* irradiation time. Simultaneously, another aliquot of 10 μl was removed at each period for confocal microscopy analysis (see Section "Confocal Microscopy").

Inhibition of germ tube formation

α-T at sub-inhibitory concentrations (0.060–0.004 μg/ml) were prepared in a microplate of 96 wells as described in Section "APDI test". Before irradiation, an aliquot of 100 μl was removed from each well (control without irradiation) and then, the microplate was irradiated at both, the optimal ET and DIS determined in Section "Optimization". Next, the whole content of each well was transferred to a sterile tube containing 100 μl of human serum as inducer of germ tube formation. In addition, a germ tube control, consisting in an irradiated compound-free medium with inoculum, was included. The contents of all tubes were vigorously stirred and then, incubated 2 h at 37 °C. After incubation, aliquots were taken to microscopically count the cells and germ tube formation. At least 200 cells were counted from each of three independent replicates. The germ tube formation was expressed as the total percentage of cells forming germ tube (% GTF).

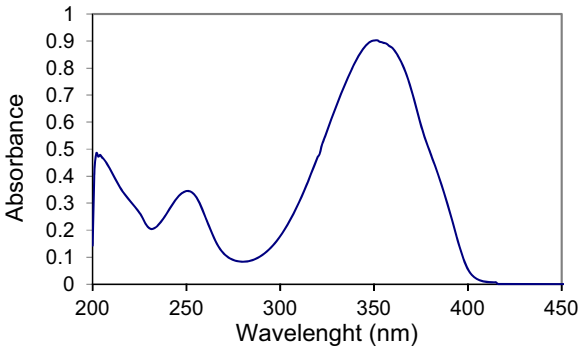

Fig. 4. UV spectrum of α-T.

Confocal microscopy

To corroborate the killing action of α-T, aliquots obtained from viable cells reduction experiments at different times (see Section "Inhibition of germ tube formation") were stained with 10 μl of aqueous propidium iodide (PI) (Molecular Probes, Eugene, USA) solution (10 μg/ml) and fluorescein diacetate (FDA) (Sigma–Aldrich, St. Louis, MO, USA) (25 μM) (Breeuwer and Abee, 2000) and then incubated 30 min at rt prior to confocal microscopy observation. For germ tube inhibition experiments, cells were stained with a 0.1% Calcofluor white (Cw; Sigma–Aldrich, St. Louis, MO, USA) aqueous solution.

All images were acquired using a Nikon C1Plus confocal microscope equipped with an Eclipse TE-2000-E2 inverted microscope and a 40× 0.95 NA Plan Apo-Chromat objective (Nikon, Melville, NY, USA). Excitation and filters were as follows: PI, 543 nm excitation, emission 605/75 nm; FDA, 488 nm excitation, emission BP 515/30 nm; Cw, 408 nm excitation, BP 450/30 nm emission.

Results

APDI of α-T

For APDI evaluation, the CLSI M27-A3 microdilution technique (CLSI, 2008), including a light irradiation step before incubation was performed, as explained in Section "Microorganisms and culture conditions", determining both the % GI at each α-T concentration and the MIC.

Experiments were divided in four groups: L+P+, with irradiation in the presence of photosensitizer; L+P−, with irradiation in the absence of photosensitizer; L−P+, without irradiation in the presence of photosensitizer and L−P−, without irradiation nor photosensitizer.

Among the wavelengths of UV spectrum, UV-A light (315–400 nm with maximum emission at 365 nm) was chosen, since α-T showed a maximum at 350 nm in the UV-spectrum (Fig. 4) (Costa et al., 2011; Dai et al., 2012).

It is known that APDI depends on several variables (Prates et al., 2011), between which ET and DIS are important factors. We determined the optimal values of these two factors for α-T to act against *C. albicans* ATCC 10231 and twenty other strains of *Candida* species isolated from patients suffering OPC.

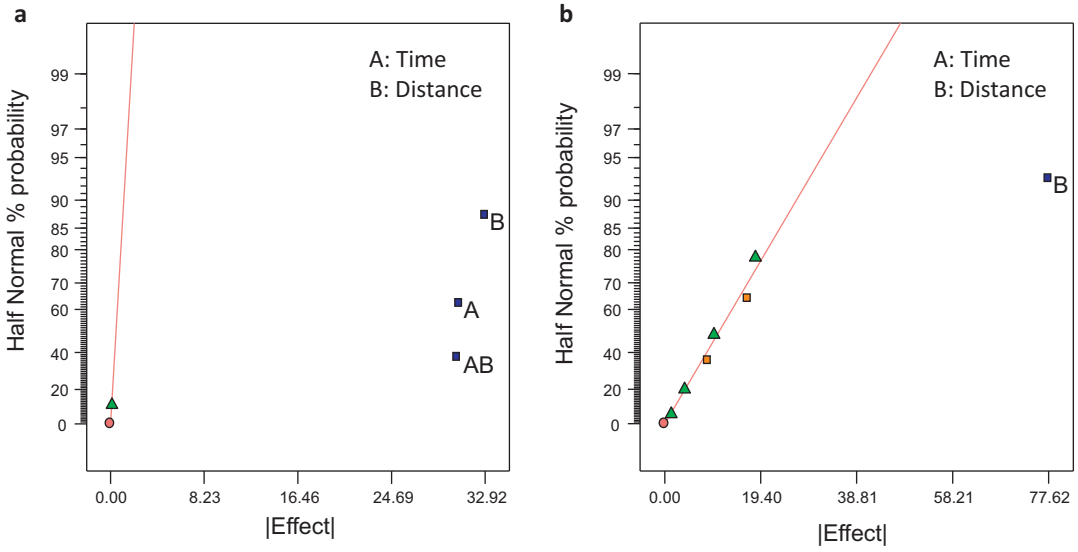

Fig. 5. Half-normal probability plots obtained in the screening phase under two conditions: (a) exposure time (ET) = 1–60 min; distance to the irradiation source (DIS) = 6–19 cm; and (b) ET = 60–120 min; DIS = 6–19 cm.

The analysis, performed using the DES, included two phases: (i) a screening phase, in which it was determined whether one or both factors have an influence in the activity; (ii) an optimization phase in which the values of each variable, that lead to the maximum % GI for all strains of *Candida* spp., were estimated.

In the screening phase, ET when ranging from 1 to 60 min (but not when the range was 60–120 min) and DIS ranging from 6 to 19 cm were found to be important factors ($p < 0.05$) for α-T APDI. This was determined after the analysis of experimental data with both the ANOVA test (data not shown) and the half-normal probability plots. These results can be clearly observed in the half-normal probability plots showed in Fig. 5.

For the optimization phase, the ranges 1–60 min for ET and 6–19 cm of DIS were used. The numerical optimization was obtained by combining the responses of all *Candida* strains into one function called Desirability function (D) represented by:

$$D = \left[\prod_{n=1}^{N} d_n\right]^N$$

D is a global function obtained by the product (Π) of the desirability functions for each individual response (d_n; $n = 1 - N$) that should be maximized choosing the best conditions of the designed variables. D ranges from 0 (value highly undesirable) to 1 (that signifies that all responses fall simultaneously within a desirable range) (Leonardi et al., 2009). The D approach to multi-response optimization is a useful technique for the analysis of experiments in which several responses have to be optimized simultaneously (Ficarra et al., 2002).

With the D values obtained in the optimization procedure, a tridimensional desirability graph was constructed (Fig. 6). From the analysis of this Figure and with the *Solutions* provided by DES (Table 1), it is clear that the optimal conditions that correspond to the maximum of desirability (0.989) are an ET of 5 min and a DIS of about 6 cm (6.00–6.43 cm) for α-T. The confluence of these values of both variables represents an excellent compromise among all the analyzed responses (Del Castillo et al., 1996).

Once determined the optimal conditions for APDI, the MIC of α-T, under L+P+ conditions, were determined against the panel of clinical *Candida* strains (Section "APDI test") (CLSI, 2008). ET and DIS were fixed according with the above experiments in 5 min and 6 cm, respectively. Light fluence, at these conditions, was 9.20 J/cm^2. Results of APDI of α-T under these conditions are showed in Table 2.

Table 1
Solutions provided by the Design Expert Software regarding Exposure Time (ET) and Distance to the Irradiation Source (DIS) to obtain the maximum % growth inhibition (% GI) for the twenty *Candida* tested.

Number	Solutions		Desirability
	ET	DIS	
1	5	6.00	0.989
2	5	6.27	0.987
3	5	6.20	0.987
4	5	6.43	0.986

As can be observed in Table 2, α-T was able to reduce the viability of all *Candida* strains under L+P+ conditions, showing MIC ranges between 0.12 and 0.98 μg/ml while under L−P+ conditions MIC were all higher than 250 μg/ml. To corroborate that the effect was due to α-T and not to light, fungi were submitted only to UV-A light in absence of α-T (L−P+), showing that the growth of the fungi was not inhibited under these conditions at concentrations up to 250 μg/ml. Fungal growth was neither inhibited at L+P− and L−P− conditions as expected. Antifungal drugs (FCZ, ICZ and AMB) showed similar MICs under L+P+ or L−P+ conditions (data not shown).

Fungicidal studies

MFC of α-T against *C. albicans* ATCC 10231, under L+P+ conditions, was 0.31 μg/ml and under L−P+ conditions was > 250 μg/ml. Instead, MFC of AMB was 0.98 μg/ml under both conditions. In contrast, FCZ and ICZ were not fungicide at any concentration tested.

Viable cells reduction curve was built as explained in Section "Fungicidal experiments" and is showed in Fig. 7. It can be observed that a complete reduction in viable cells (CFU/ml = 0) was reached at 5 min (fungicidal endpoint). Instead, the time-kill study performed with the standard drug AMB at its MFC, showed that it completely killed *C. albicans* ATCC 10231 after 2 h incubation time (curve not shown).

The fungicidal activity of α-T was also evaluated by staining *C. albicans* ATCC 10231 (treated with α-T and light at MFC) with PI and FDA vital dyes. PI stains nucleic acids when it crosses the damaged membrane of dead cells (Fig. 8a). In turn, FDA is an electrically

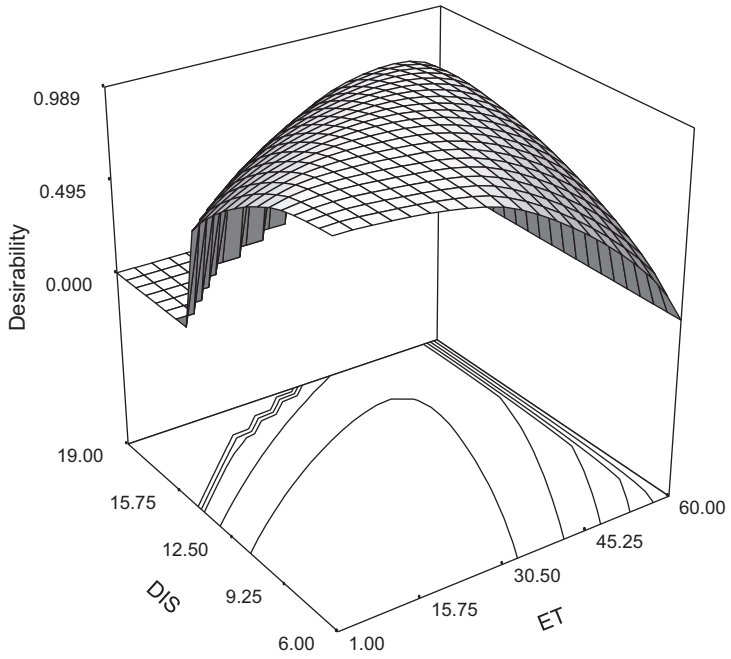

Fig. 6. Desirability function that shows the joint optimal values for Distance to the Irradiation Source (DIS) and Exposure Time (ET).

neutral molecule that can easily penetrate cell membranes where it is hydrolyzed by intracellular esterases (Fig. 8b). Living and dead cells were visually differentiated by color, orange/red fluorescence indicating uptake of PI and therefore membrane damaged dead cells, while bright green fluorescence indicates accumulation of FDA and thus viable cells (Bleve et al., 2003). This staining methodology allowed to observe that yeasts treated with α-T were all alive at the beginning of the experiment (0 min of irradiation) (Fig. 8c). The number of dead cells increased with the time of light-exposure until 5 min when they were all dead (Fig. 8d–f).

Inhibition of germ tube formation

Germ tube inhibition was analyzed in order to verify whether exposure to sub-lethal concentrations of α-T reduced *C. albicans* virulence factor. The morphology of the cells after the different

Table 2
APDI of α-T (MIC in μg/ml) against twenty *Candida* strains.

Candida spp	Voucher specimen	APDI of α-T				Antifungal drugs					
						AMB		FCZ		ICZ	
		L+P+	L−P+	L+P−	L−P−	MIC	int	MIC	int	MIC	int
C. albicans	ATCC 10231	0.12	>250	+	+	0.25	S	1	S	4	R
C. albicans	CCC 182-13	0.49	>250	+	+	0.12	S	2	S	0.03	S
C. albicans	CCC 191-13	0.49	>250	+	+	0.12	S	2	S	0.06	S
C. albicans	CCC 192-13	0.25	>250	+	+	0.12	S	1	S	0.03	S
C. albicans	CCC 193-13	0.25	>250	+	+	0.25	S	4	S	0.004	S
C. albicans	CCC 196-13	0.49	>250	+	+	0.25	S	2	S	16	R
C. krusei	CCC 189-13	0.98	>250	+	+	0.50	S	32	[a]	0.01	S
C. parapsilosis	CCC 183-13	0.25	>250	+	+	0.50	S	1	S	0.01	S
C. parapsilosis	CCC 187-13	0.49	>250	+	+	0.50	S	16	I	0.03	S
C. parapsilosis	CCC 200-13	0.49	>250	+	+	0.50	S	8	S	0.06	S
C. parapsilosis	CCC 188-13	0.25	>250	+	+	0.50	S	2	S	0.01	S
C. tropicalis	CCC 184-13	0.49	>250	+	+	0.12	S	16	I	>16	R
C. tropicalis	CCC 185-13	0.49	>250	+	+	0.25	S	2	S	0.06	S
C. tropicalis	CCC 186-13	0.98	>250	+	+	0.12	S	>64	R	>16	R
C. tropicalis	CCC 190-13	0.98	>250	+	+	0.06	S	1	S	16	R
C. tropicalis	CCC 194-13	0.98	>250	+	+	0.12	S	2	S	>16	R
C. tropicalis	CCC 195-13	0.49	>250	+	+	0.50	S	1	S	>16	R
C. tropicalis	CCC 197-13	0.49	>250	+	+	0.50	S	1	S	>16	R
C. tropicalis	CCC 198-13	0.25	>250	+	+	0.50	S	0.5	S	0.002	S
C. tropicalis	CCC 199-13	0.25	>250	+	+	0.50	S	8	S	0.03	S
C. parapsilosis[b]	ATCC 22019					0.50		2		0.125	
C. krusei[b]	ATCC 6258					0.25		16		0.25	

+ Full growth.

[a] Isolates of *C. krusei* are assumed to be intrinsically resistant to FCZ.

[b] Quality control strain.

Interpretation of susceptibility to antifungal drugs according with M27-A3 document [31] as S: susceptible, I: susceptible dose-dependent and R: resistant. L+P+ = with irradiation in the presence of photosensitizer; L+P− = with light in absence of photosensitizer; L−P+ = without irradiation in the presence of photosensitizer and L−P− = without light or photosensitizer.

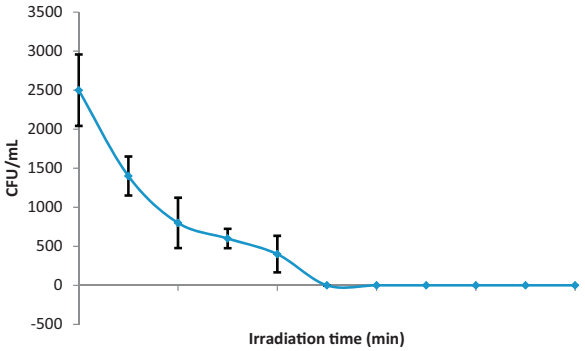

Fig. 7. Viable cells reduction curve for α-T at MFC (Minimum Fungicidal Concentration).

treatments was observed by light and confocal microscopy (Fig. 9), %GTF was calculated at different conditions and the results were summarized in Table 3. Results showed that the ability of *C. albicans* to form germ tubes significantly decreased after exposition to different sub-lethal concentrations of α-T and after 5 min of irradiation under the conditions L+P+. At the sub-inhibitory concentrations 0.060 μg/ml (MIC/2) and 0.030 μg/ml (MIC/4), the germ tube formation was completely inhibited and only budding yeasts were observed (Fig. 9a). Under the same irradiation conditions, cells treated with 0.015 μg/ml (MIC/8) and 0.007 μg/ml (MIC/16) showed marginal values (<13%) of germ tube formation and at 0.004 μg/ml (MIC/32), 100% of cells formed germ tubes (Fig. 9a). *C. albicans* did not modify %GTF neither under L−P+ treated with α-T at concentrations ≥7.81 μg/ml nor under L+P− conditions.

Discussion

OPC is a common infection caused for different *Candida* spp in immunocompromised patients usually wearing dentures, being FCZ, ICZ or AMB the antifungal drugs of choice for its treatment (Mang et al., 2010). However, these drugs are ineffective for some immunosuppressed patients, show different degrees of toxicity and have led to antifungal resistance (Espinel-Ingroff, 2008). PDT does represent an actual alternative for OPC treatment since the topical

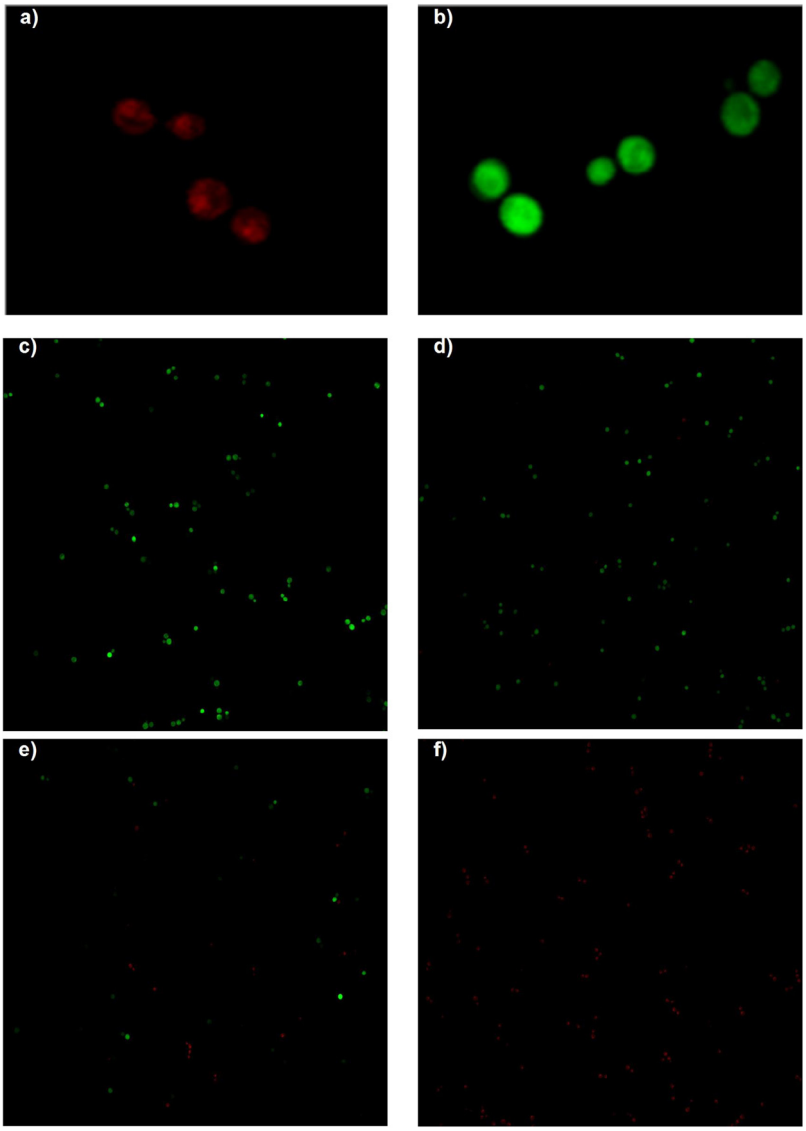

Fig. 8. Propidium iodide (PI) (red) and fluorescein diacetate (FDA) (green) staining of *C. albicans* observed with confocal microscope. Examples of staining in dead yeasts (a) and living yeasts (b) observed at 400× magnification. Cells exposed to α-terthienyl (α-T) at Minimal Fungicidal Concentration (MFC) without UV-A light exposition (0 min) (c) and at different exposure times: 3 min (d); 4 min (e) and 5 min (f) observed at 100× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

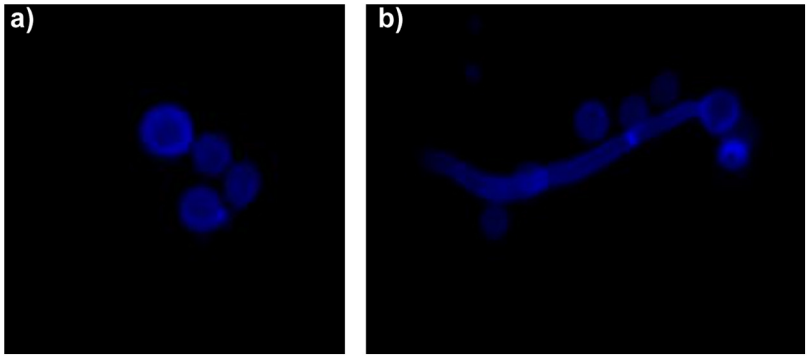

Fig. 9. Germ tube formation after α-T (at sub-inhibitory concentrations) and light treatment stained Calcofluor white (Cw). (a) Budding yeast observed 0.030 μg/ml α-T (MIC/4) and (b) germ tube formation at 0.004 μg/ml α-T (MIC/32).

Table 3
% Cells with germ tube (% GTF) formation of *C. albicans* ATCC 10231 treated with α-terthienyl (α-T) after irradiation with UV-A light (Light) during 5 min at 6 cm and without irradiation (Dark).

[α-T] (μg/ml)	% GTF	
	Light	Dark
0.06 (MIC/2)	0	100
0.03 (MIC/4)	0	100
0.015 (MIC/8)	6	100
0.007 (MIC/16)	13	100
0.004 (MIC/32)	100	100
7.81	–	100
0	100	–

administration of a photosensitizer in the oral mucosa followed by irradiation are not complex processes and have the extra advantage of avoiding systemic toxicity and interactions with systemic antifungal drugs (Wainwright and Crossley, 2004; Donnelly et al., 2007). In addition, PDT does not generate fungal resistance (Dai et al., 2009), is of low cost and possesses low overdose risk.

In the present work, the optimal ET and DIS conditions that led α-T to display the best anti-candidal activity were studied. Although the light-induced antifungal properties of α-T (Towers et al., 1976, 1979; Kagan et al., 1980; Hudson et al., 1993; Romagnoli et al., 1998), have been reported, previous evaluations of this activity were performed employing non-standardized methodologies and nothing was reported on the conditions for obtaining a maximum effect.

In the present work, the APDI evaluation was performed following the guidelines of CLSI M27-A3 methodology that established consensus' procedures to facilitate the agreement among laboratories in the measuring the antifungal susceptibility of yeasts, assuring confident and reproducible results. Besides, the optimal conditions for obtaining the best photosensitizing antifungal activity of α-T have been investigated with DES, with the aim of finding the suitable combination of ET and DIS that results in a maximum fungal % GI for α-T.

Traditionally, optimization experiments have been carried out using one-dimensional search with successive modification of variables. This procedure not only was time-consuming but sometimes led to misinterpretation of results, especially because the interaction between different factors was overlooked (Francis et al., 2003). In addition, it is well known that it is practically impossible to achieve, using this mode of search, an appropriate optimum value in a finite number of experiments (Hooda et al., 2012). In contrast, optimization employing DES has some advantages that include a low number of experiments, availability for multiple factor experiments, search for relativity between factors and finding out the most suitable conditions and forecast responses (Sayyad et al., 2007).

Results showed that the optimal DIS for α-T to kill fungi was about 6 cm, being this distance highly appropriate for UV-A irradiation of OPC in clinical practice. Regarding the optimal ET, results of the present investigation demonstrated that 5 min of UV-A light (9.20 J/cm^2) is the optimal time for α-T to display the best antifungal activities (MICs between 0.12 and 0.98 μg/l) against a panel of twenty clinical *Candida* species. It is known that ET is a crucial factor in PDT, being times longer than 30 min, clinically inapplicable (Mima et al., 2010). Therefore, α-T appears as an interesting candidate for developing a new anti-OPC drug. In addition, α-T could constitute a promising treatment to overcome the problems of antifungal resistance, since it showed effectiveness not only against sensitive but also resistant *Candida* strains to FCZ or ITZ, two drugs of choice for the treatment of OPC.

An extra advantage of α-T is its fungicidal (MCF = 0.31 μg/ml) rather than fungistatic activity against *C. albicans*. To be fungicidal is extremely important, particularly in HIV patients, because prophylactic use of fungistatic drugs has been associated with an increased frequency of innate or acquired drug resistance in clinical isolates (Monk and Goffeau, 2008). In addition, time-kill assays of α-T against C. albicans showed a total reduction in viable cells within 5 min of ET.

The killing effect of α-T at the optimal conditions was corroborated by cell viability tests using a combination of FDA and PI that are based on the esterase activity of intact living cells and detection of plasma membrane damage, respectively. Non-viable, permeabilized cells were marked using the fluorescent probe PI that is incorporated and stains the nucleic acids only in cells with damaged membranes (Corliss and White, 1981), suggesting that one of the effect of α-T is to produce cytoplasmic membrane or fungal cell-wall lesions (Pina-Vaz et al., 2005).

The inhibition of germ tube formation by α-T was also studied. C. albicans is able to switch, in response to external stimuli, between growing isotropically (yeast) or apically (hyphal and pseudohyphal). The germ tube which is the initial elongating structure formed during this transition (Kato et al., 2013) is considered a virulence mechanism and plays an important function in tissue invasion and resistance to phagocytosis (Jayatilake et al., 2006). Results showed that α-T inhibited germ tube formation in *C. albicans* at much lower concentrations than MIC (MIC/8) indicating that this cell function is very sensitive to α-T-mediated APDI (Sardi et al., 2013). So, α-T can be used to inhibit not only the growth of *C. albicans* but also its potential virulence after the appropriate combination of light and photosensitizer at optimized conditions.

Conclusion

The use of DES allowed us to find the optimal conditions for the *in vitro* antifungal activity of α-T against standardized and clinically

isolates of *Candida* species, which were the producers of infections in the oral cavity of immunocompromised patients. From the results obtained here, it is clear that α-T irradiated with UV-A light during 5 min from a distance of about 6 cm could constitute a good alternative for the treatment of OPC. Under these conditions, α-T was able to kill a panel of twenty susceptible or resistant strains of *Candida* spp at concentrations as lower as 0.31 μg/l.

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