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Evaluation of the antifungal photodynamic activity of *Thymophylla pentachaeta* extracts against *Candida albicans* and its virulence factors

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Abstract

Background: *Candida albicans* is one of the most common causative of opportunistic infections. Treatment of candidiasis is challenging considering the few antifungal drugs available and the increase in resistance. Antimicrobial photodynamic therapy (aPDT) is a recently developed therapeutic option that combines a non-toxic photosensitizer (PS) and light to kill the microbial pathogens. Targeting virulence, defined as the ability of a pathogen to cause overt disease, represents another attractive target for the development of novel antifungal agents. *Thymophylla pentachaeta* (DC.) Small var. *belenidium* (DC.) is an endemic plant from Argentina in which the presence of thiophenes, biologically active compounds whose antifungal activity is enhanced by irradiation with UVA, have been already described.

Purpose: The purpose of this study was to evaluate the photodynamic antifungal activity of hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) extracts from *T. pentachaeta* var. *belenidium* and their inhibitory effects on *C. albicans* virulence factors as well as biofilm formation and eradication.

Study Design/Methods: Antifungal photodynamic activity of hexane, dichloromethane, ethyl acetate and methanol extracts from different parts of the plant were assessed

with the microbroth dilution, bioautography and the time-kill assays, under light and darkness conditions. The capacities of the most active extracts of inhibiting *Candida* virulence factors (adherence to epithelial cells, germ tube and pseudomycelium formation and hydrolytic enzyme secretion) were assessed. In addition, the activity against biofilm formation and eradication has been investigated by reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) that quantifies living cells in these structures.

Results: Hex and DCM extracts from *T. pentachaeta* roots exhibited high photodynamic antifungal activity against *C. albicans* (MFCs= 7.8 µg/ml) under UVA light irradiation. Chemical analysis of active extracts (Hex and DCM from roots) revealed the presence of photoactive thiophenes. Both extracts generate reactive oxygen species through type I and II mechanisms. These extracts, at sub-inhibitory concentrations, under light conditions decreased the adherence of *C. albicans* to Buccal Epithelial Cells (BEC), inhibited germ tube formation and reduced esterase production. Finally, they demonstrated activity against preformed biofilms submitted to irradiation (MFCs= 3.91 µg/ml and 15.63 µg/ml for Hex and DCM extracts, respectively).

Conclusion: Taking together, results demonstrated the strong photodynamic effects of *T. pentachaeta* root extracts under UVA irradiation, making them valuable alternatives to the already established antifungal drugs against *C. albicans.*

Keywords

Thymophylla pentachaeta; *Candida albicans*; photoantifungal activity; thiophene; virulence factors; biofilms

Abbreviations

α-T: 2,2':5',2''-terthienyl

1,3-DPBF: 1,3-Diphenylisobenzofuran

ACN: Acetonitrile

AdhC: Adherence Control

AMB: Amphotericin B

aPDT: Antimicrobial Photodynamic Therapy

BBT: 5-(3-buten-1-ynyl)-2,2'-bithiophene

BBTOAc: 5-(4-acetoxy-1-butynyl)-2,2'-bithiophene

BBTOH: 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene

BEC: Buccal Epithelial Cells

BSA: Bovine Serum Albumin

CFU: Colony-Forming Units

DAD: Diode Array Detector

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

EC: Esterase Control

EtOAc: Ethyl Acetate

FCZ: Fluconazole

GC: Growth Control

GT: Germ Tube

GTC: Germ Tube Control

Hex: Hexane

HPLC: High Performance Liquid Chromatography

IC: Inhibitory Concentration

ICH: International Council for Harmonization

ITZ: Itraconazole

LOD: Limit of Detection

LOQ: Limit of Quantification

MeOH: Methanol

MFC: Minimal Fungicide Concentration

MIC: Minimal Inhibitory Concentration

MOPS: 3-(N-morpholino) propanesulfonic acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT: Nitro Blue Tetrazolium

PB: PS Blank

PBS: Phosphate-Buffered Saline

PS: Photosensitizer

Pz: Precipitation or halo zone

RPMI: Roswell Park Memorial Institute

ROS: Reactive Oxygen Species

RSD: Relative Standard Deviation

Rt: Retention time

SC: Sterility Control

SDA: Sabouraud-Dextrose Agar

SDB: Sabouraud-Dextrose Broth

TBF: Terbinafine

TLC: Thin Layer Chromatography

UVA: Ultraviolet A

YCB: Yeast Carbon Base

Introduction

Fungal infections produced by *Candida* spp. range from superficial skin lesions to oral thrush, vulvovaginal candidiasis and systemic infections (De Oliveira et al., 2019). Among them, invasive candidiasis that comprises bloodstream and deep organ infections, is a major cause of morbidity and mortality in the hospital setting, particularly among cancer patients and patients in intensive care units (McCarty and Pappas, 2016). Although *Candida albicans* is the predominant sp. linked to invasive candidemia, the incidence of infections due to non-*albicans Candida* spp., including *C. glabrata*, *C. krusei*, *C. parapsilosis* and recently *C. auris*, has risen sharply in recent years (De Oliveira et al., 2019).

Treatment of *Candida* infections is challenging considering that few antifungal drugs are available and that an increase in resistance has been reported (De Oliveira et al., 2019). Therefore, there is an urgent need to develop new therapies and alternative strategies to treat *Candida* infections. Natural compounds provide promising alternatives to the conventionally applied antifungal drugs.

Several virulence factors contribute to pathogenesis of *Candida*, including the capacity of adherence on epithelial surfaces and the production of enzymes that play a fundamental role in the invasion of cell membrane enabling penetration of the tissues (Mane et al., 2012). A key virulence factor of *C. albicans* is its ability to undergo morphological transformation between two forms: yeast and mycelium because it plays an important function in tissue invasion, resistance to phagocytosis and biofilm formation (Postigo et al., 2014). Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material that have the potential to initiate or prolong infections and to confer high levels of resistance to conventional antifungal therapy (Pierce and Lopez-Ribot, 2013).

Targeting virulence has become an attractive target for the development of novel antifungal agents because it expands the number of potential targets, preserves the

host microbiome and exerts a weaker selective pressure for the development of antibiotic resistance (Pierce and Lopez-Ribot, 2013).

In addition, antimicrobial photodynamic therapy (aPDT) is a recently developed therapeutic option, based on utilizing non-toxic photosensitizer (PS) and light, to induce the production of reactive oxygen species (ROS) that can kill the microbial pathogens (Dai et al., 2012). An advantage of aPDT is the difficulty that fungi have in developing resistance to it since this strategy mediated killing is a non-specific and multi-target process (Kato et al., 2013). Many naturally occurring acetylenic thiophenes are important UVA absorbing compounds known for their photodynamic effect on different organisms (Towers and Hudson, 1987; Downum et al., 1989; Romagnoli et al., 1994; 1998; Postigo et al., 2017). They have been isolated from some genera of the Asteraceae family (Tagetae tribe) such as *Thymophylla* (syn. *Dyssodia*), *Porophyllum* and *Tagetes* and their presence or absence has been used to suggest taxonomic relationships (Downum et al., 1985). In previous works, we reported the photosensitive antifungal activities of thiophene-containing extracts from *Porophyllum obscurum* (Spreng.) DC. and *Tagetes minuta* L. employing standardized methodologies based on Clinical and Laboratory Standards Institute (CLSI) consensus documents (CLSI 2017, Giacone et al., 2019; Postigo et al., 2017). *Thymophylla pentachaeta* (DC.) Small *var. belenidium* (DC.) Strother, vernacular name "perlilla", grows in USA and Argentina, on stony soils and on the edge of roads (Novara and Petenatti, 2000; Turner, 2009) in which the presence of thiophenes have been already described (Downum et al., 1985). In the present work, we studied the photodynamic antifungal activity of *T. pentachaeta* extracts and their inhibitory effects on *C. albicans* virulence factors as well as biofilm formation and eradication.

Material and methods

Chemicals and reagents

All solvents, inorganic salts, Tween 80 and dimethylsulphoxide (DMSO) were purchased from Ciccarelli (San Lorenzo, Argentina). Materials for the culture media were purchased from Britania (Buenos Aires, Argentina). Roswell Park Memorial Institute (RPMI-1640), 3-(N-morpholino) propanesulfonic acid (MOPS), amphotericin B (AMB), fluconazole (FCZ), itraconazole (ITZ), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), nitro blue tetrazolium (NBT), 1,3-diphenylisobenzofuran (1,3-DPBF), sterile fetal bovine serum and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-aminophenazone, hydroxybenzoate and horseradish peroxidase were included in the enzymatic Glycemia Kit (Wiener Lab, Rosario, Argentina). Aluminum-blacked silica gel 60 F254 TLC layers were purchased from Merck (Darmstadt, Germany).

Plant material and preparation of extracts

T. pentachaeta was collected at Belgrano and Ayacucho Departaments, Sierra de las Quijadas, San Luis province, Argentina (32º34´S, 67º 10´W) by Dr. L.A. Del Vitto and Dr. E. Petenatti and a *voucher specimen* was deposited in the Herbarium of the National University of San Luis (Del Vitto and Petenatti #8999, UNSL). The plant material (100 g) was separated into roots (7.6 g), aerial parts (79.4 g) and flowers (13.0 g); air dried in shade and grinded to a fine powder using a Pulverisette 15 Cutting Mill (Fritsch Gmbh, Idar-Oberstein, Germany). Each material was successively extracted by maceration with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) 24 h (3 × 500 ml) with continuous shaking. After filtration (Whatman no. 1 paper), combined extracts of each solvent were evaporated to dryness under reduced pressure in a rotary evaporator (Büchi R-205, Essen, Germany) to give semisolid residues after complete elimination of solvent. For assay, the extracts were dissolved in DMSO at 50 mg/ml and used as stock solutions.

Chemical characterization of extracts by HPLC

The HPLC-DAD system consisted of a Hewlett–Packard 1050 instrument (Hewlett–Packard, Palo Alto, CA, USA), coupled to a G1306AX DAD. The chromatographic separation was performed with a Luna C18 column (250 x 4.6 mm i.d., 5 µm particle size) provided by Phenomenex (Torrance, CA, USA). The mobile phase used for the separation was an 80:20 mixture of HPLC-grade acetonitrile (ACN) and Milli-Q water, with 0.1% formic acid, delivered at a flow rate of 1 ml/min.

Thiophenes 2,2':5',2''-terthienyl (α-T); 5-(3-buten-1-ynyl)-2,2'-bithiophene (BBT); 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene (BBTOH); and 5-(4-acetoxy-1-butynyl)-2,2'-bithiophene (BBTOAc) were obtained in our laboratory from *Porophyllum obscurum* as previously described (Postigo et al., 2017) and were employed as standards. The purities of standards were ≥95% as determined by HPLC–DAD. ACN solutions of extracts were prepared at an appropriate concentration, filtered through a 30 mm, 0.45 µm Target Nylon Membrane Syringe Filter (Scientific Instrument Services, Ringoes, NJ, USA) prior to injection into the HPLC system. These solutions were stored at 4°C in light-resistant containers and left to attain room temperature before use.

Calibration curves of standards were constructed by using five solutions in the concentration range of 0.01-0.1 µg/ml (in triplicate) of each compound, prepared from stock solutions. The peak areas at 365 nm of each solution from standard compounds were plotted against the concentration. The Relative Standard Deviation (RSD) of standard peak areas for solutions of the same concentration was less than 2%. The linear regression equation was adjusted by calculating the slope, intercepts and R^2 coefficient using a least square equation and it was also used to estimate the concentration of the four components in the analyzed samples. The analytical methods were validated for linearity, limit of detection and quantification (LOD and LOQ) and intra- and inter-day variability, following the International Council for Harmonization (ICH) guidelines of Technical Requirements for Pharmaceuticals for Human Use (ICH,

1996). The percentage (%) of recovery was used to evaluate the accuracy of the method. The results of this validation are shown in Supplementary material. The presence of thiophenes in the extracts was determined by comparing the retention times (Rt) with those of the standard compounds.

Light conditions

All experiments were carried out in parallel under "light" and "darkness" conditions. UVA irradiations were performed with a homemade UVA light array composed by a set of three lamps (Alic, Buenos Aires, Argentina), emitting at 315-400 nm (100 W) aligned perpendicular to the microplates that illuminate uniformly their entire area. In light experiments, microplates were placed at a distance to 12 cm from the sample and irradiated for 60 min. In darkness experiments, assays were 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 as in the assays (Kuhn et al., 2004) and was calculated as 1.2 J/cm^2.

Microorganisms and culture conditions

C. albicans CCC 193-2013 was obtained from the Reference Center of Mycology (CEREMIC), Faculty of Biochemical and Pharmaceutical Sciences, Rosario, Argentina. It was maintained in SDA and conserved in 20% glycerol at −20°C. Inoculum was obtained according to reported procedures and adjusted to 0.5-2.5 × 10^3 CFU/ml according to the CLSI M27 Ed4 guidelines (CLSI, 2017).

Evaluation of the photodynamic antifungal activity

The experiments were performed in 96-well flat-bottomed microtiter plates. The culture medium employed was RPMI-1640 buffered to pH 7.0 with MOPS supplemented with 2% (v/v) glucose (CLSI, 2017). Briefly, stock solutions were two-fold serial diluted in RPMI-1640 (final volume 100 µL) and placed in a 96-well plate. Subsequently, 100 µl of inoculum suspension were added to each well, rendering final concentrations from 0.98 to 1,000 µg/ml, for each extract. The maximum amount of DMSO used was negligible (i.e.≤0.2% vol:vol/DMSO:medium). The following controls were included: (i) Standard positive controls with commercial antifungal drugs AMB, FCZ and ITZ, evaluated at final concentrations 0.03 - 16 µg/ml for AMB and ITZ; and 0.12 - 64 µg/ml for FCZ, according to CLSI guidelines (CLSI, 2017); (ii) Growth control with free culture medium and inoculum; (iii) Extract sterility control, with extract, culture medium and sterile water instead of inoculum and (iv) Medium sterility control, with culture medium and water instead of inoculum. The microplates were

homogenized at 150 x g for 5 min on an orbital shaker platform Innova 4000 (New Brunswick Scientific, NJ, USA). After 30 min of incubation, microplates were submitted to irradiation (light) or kept in darkness. Minimal Fungicide Concentration (MFC) was determined by plating 50 µl onto Petri dishes containing SDA, incubated at 30-32°C for 48 h. After incubation, the number of CFU/ml were quantified and MFC was defined as the lowest concentration that causes total growth inhibition (CFU/ml = 0). Each sample was assayed in triplicate and data were expressed as modal value. Extracts with MFC < 1,000 µg/ml only in light experiment were considered photoactives or PS. For fungistatic azoles, FCZ and ITZ, Inhibitory Concentration 50 (IC_{50}), defined as the minimum inhibitory concentration of the antifungal agents that inhibits growth of the fungus by 50%, has been informed.

Time-kill assay

Time-kill studies were carried out by a method previously described by Butassi et al. (2019). Briefly, 100 µl of the fungal suspension (cell density = 2.5×10^3 CFU/ml) was added to 100 µl of RPMI-1640 culture medium and submitted to different treatments: with extract and with irradiation (L+E+); with extract and without irradiation (L-E+); without extract and with irradiation (L+E-) and without extract and without irradiation (L-E-). In (L+E+) and (L-E+) assays, the final concentration or extracts were equal to MFC. The fungicide drug AMB was included as standard positive control. Each 30 min, 10 µl from different wells were aseptically removed, serially diluted in saline solution and inoculated on SDA plates and incubated 48 h at 35°C. After incubation, the CFU/ml of each plate was determined and mean colony count data ± SD were plotted as a function of time. The whole experiment was performed in triplicate.

Bioautographic assay

The extracts (20 µg) were dissolved in a volatile solvent and applied on thin layer chromatography (TLC) plates using a Sample Applicator ATS 4 (Camag, Muttenz, Switzerland) and developed with Hex:EtOAc (80:20) in a CAMAG glass chamber. Then, plates were photographed in Camag Cabinet UV lamp at 254 and 365 nm. Next, chromatograms were placed into a sterile Petri dish with lid. Culture medium, composed of 2% glucose, 1% peptone, 0.6% agar and 0.02% phenol red, was mixed with *C. albicans* inoculum (final concentration of $1\text{-}5 \times 10^4$ CFU/ml) and was distributed over developed TLC plates (0.1 ml/cm^2). After solidification of the medium, one of the TLC plates was irradiated with UVA (60 min from a distance of 12 cm) under aseptic conditions in a laminar air flow chamber. Then, the plates were incubated for 24 h in a moistened chamber at 30°C. Subsequently, each plate was sprayed with an aqueous solution of MTT (1 mg/ml) and incubated for another 2 h at 30°C. Yellow inhibition

zones appeared against a dark brown background (Giacone et al., 2019; Postigo et al., 2017).

Determination of superoxide anion production

The formation of $O_2^{\bullet-}$ was detected with an indirect bioassay that measures the reduction of NBT at 575 nm (Postigo et al., 2019). This procedure was performed in 96-well microplates. Extract solutions (100 µl), at a concentration of 1000 µg/ml in phosphate-buffered saline (PBS), were incubated with 100 µl of NBT (0.1% w/v). The concentration of extract 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 35-37°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 x 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, Sunnyvale, CA, 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 extract.

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 (Postigo et al., 2019). The colored complex produced was proportional to the H_2O_2 generated and was measured by the increase in absorption at 505 nm spectrophotometrically with regard to the basal situation (in absence of extracts). This procedure was performed in 96-well microplates. Extracts solutions (100 µl) at a concentration of 250 µg/ml in PBS, were incubated with 100 µl of reagent solution containing 4-aminophenazone (0.5 mM), hydroxybenzoate (12 mM) and horseradish peroxidase (1 kU/L) (Meiattini et al., 1978). The concentration of extracts 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 measured 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 extracts.

Determination of singlet oxygen production

The singlet oxygen production was determined using the 1,3-DPBF bleaching method (Postigo et al., 2019). 1,3-DPBF is an established singlet oxygen quencher and the efficiency of extracts at generating $O_2(^1\Delta g)$ can be monitored spectrophotometrically through its absorption decrease. Stock solutions of extracts (1000 µg/ml) and 1,3-DPBF (50 mM) were prepared in DMSO. The concentrations of extracts used in the assay were chosen in order to better visualize the effects. For the analysis, reaction mixtures containing 100 µl of 1,3-DPBF and 100 µl of extracts solutions were placed in two 96-well microtiter plates and illuminated under UVA light. The rate at which 1,3-DPBF was consumed was followed spectrophotometrically by observing the decrease of an absorption band at 410 nm, every 60 s for a period of 40 min, in a VERSA Max microplate reader. Control with 100 µl of 1,3-DPBF and 100 µl of pure DMSO, instead of extract solution was included. Darkness experiments were carried out under the same conditions without irradiation. The experiments were performed in octuplicate and the mean values ± SD of absorbance were graphically represented as a function of time.

Adherence to buccal epithelial cells (BEC) inhibition assay

The adherence to BEC was assessed using a methodology previously described with modifications (Butassi et al., 2019). Medium and conditions for evaluation of antifungal activity (see above the *'Evaluation of the photodynamic antifungal activity'* sub-section), except that, after treatment with sub-lethal concentrations of extracts and light irradiation, aliquots of each well were incubated with BEC in order to evaluate adherence. BEC were collected from healthy human subjects by gently rubbing the inside of the cheeks with sterile swabs which were then agitated in 12 ml of PBS. Then, they were washed twice in PBS to remove unattached microorganisms and re-suspended in the same buffer to a final concentration of 5×10^5 cells/ml counted in a Neubauer chamber. *C. albicans* was grown for 24 h at 30-32°C on Sabouraud-dextrose broth (SDB). Cells in the budding yeast phase were washed twice in PBS and re-suspended in the same buffer to a final concentration of 2.5×10^7 cells/ml counted in a Neubauer chamber. Then, in order to calculate sub-lethal concentrations, MFC values have been obtained employing this inoculum size. Solutions of extracts (100 µl), at MFC/2 and MFC/4, were put in a 96-well microtiter plate (GreinerBio-One GmbH, Frickenhausen, Germany); and 100 µl of *Candida* suspensions were added. The microplates were homogenized at 150 g for 5 min on an orbital shaker platform and then, were submitted to irradiation or kept in darkness.

For the assay, aliquots (100 µl) from the contents of the wells were taken, transferred to sterile tubes containing BEC suspension (100 µl), mixed and incubated on an orbital shaker at 35-37°C for 1 h. BEC were collected on hydrophilic polyvinylidene fluoride 0.47 µm pore size filters (Merck Millipore, Billerica, MA, USA) and washed with 60 ml of PBS to remove unattached fungi. Thereafter, the filter was removed carefully with

forceps and placed firmly on a glass slide with the BEC against the glass surface and then gently removed, leaving the BEC adhered to the glass slide. The preparations were air-dried, fixed, stained using the Gram-Nicolle technique and observed by light microscopy (Eclipse E100, Nikon Corp., Tokyo, Japan) at 1000x magnification. The number of yeasts adhered to 100 BEC was counted. Adherence controls (AdhC) with yeasts without any treatment under both light and darkness conditions was included. FCZ (at its IC_{50} = 0.03 µg/ml) was used as standard positive drug. Each test was performed in triplicate and results were expressed as mean ± SD and plotted.

Germ-tube inhibition assay

Germ tube (GT) formation was induced using the same methodology and conditions described above for the evaluation of antifungal activity (see *Evaluation of the photodynamic antifungal activity*), except that, after treatment with sub-lethal concentrations of extracts and light irradiation, aliquots were diluted in a medium containing sterile fetal bovine serum as an inducer of GT formation. (Postigo et al., 2014). Serial dilutions of extracts ranging from MFC/64 to MFC/2 were made in a 96-well microtiter plate where 100 µl of a *C. albicans* suspension was added. The microplates were homogenized at 150 x g for 5 min on an orbital shaker platform and then, were submitted to irradiation or kept in darkness. For the assay, aliquots (100 µl) from the contents of the wells were taken and transferred to sterile tubes containing fetal bovine serum (100 µl), incubated at 35-37°C for 2 h and aliquots were taken to count the cell number microscopically. Controls included: GT control (GTC), employing yeast without any treatment under light and darkness conditions. FCZ (at its IC_{50} = 0.03 µg/ml) was used as standard positive drug. The total cell number and cells with GT were counted by light microscopy using the Neubauer chamber at 400x magnification. The percentage of cells with GT (%GT) was calculated as: %GT = GT x 100 / TC, being GT, the number of cells forming GT and TC, the total cell number. For quantification, cells were considered germinated if they had a GT at least as long as the diameter of the yeast. Protuberances showing a constriction at the point of connection to the mother cell, typical for pseudohyphae, were excluded (Butassi et al., 2019). Each test was performed in triplicate and results were expressed as mean ± SD and plotted.

Morphogenesis of Candida albicans on solid media

The effect of extracts on the pseudomycelium formation of *C. albicans* was evaluated using Spider medium assay according to Silva-Rocha et al. (2015) with modifications. Briefly, solutions of extracts (100 µl), rendering a final concentration equal to MFC/2 and MFC/4, were placed in a 96-well microtiter plate and 100 µl of inoculum were added. The microplates were homogenized at 150 x g for 5 min on an orbital shaker platform and then, were submitted to irradiation or kept in darkness. Then, aliquots

(10 µl) were aseptically removed and spotted on the surface of Spider medium (nutrient agar 13.5 g, mannitol 10 g, KH_2PO_4 2 g, agar 14.5 g, distilled water 1000 ml). The plates were incubated at 30°C for seven days for subsequent observation of morphology of colonies, which were inspected with optical microscope and imaged using a digital camera (Olympus DP73, Shinjuku, Tokyo, Japan). FCZ (at its IC_{50} = 0.03 µg/ml) was used as standard positive drug. The assay was performed in triplicate.

Lytic enzyme inhibition assay

Solutions of extracts (100 µl), rendering a final concentration equal to MFC/2 and MFC/4, were placed in a 96-well microtiter plate and 100 µl of inoculum were added. The microplates were homogenized at 150 x g for 5 min on an orbital shaker platform and then, were submitted to irradiation or kept in darkness. Then, aliquots (10 µl) from the contents of the wells were placed onto the appropriate culture medium (see below), left to dry at room temperature and the plates were incubated at 35-37°C for four days. Controls included: enzyme producer control, employing yeast without any treatment and light control, employing yeast treated only with light. The assay was conducted in triplicate. The level of enzymatic activity, termed the Pz interval, was established as the ratio between the colony diameter and the colony diameter+halo zone and was classified as: absence of enzymatic activity (Pz = 1.0); positive activity (1.0>Pz≥0.64); or strongly positive activity (Pz<0.64). A significant increase in Pz index, for treated cells compared to control ones, was indicative of lytic-enzyme inhibition. FCZ (at its IC_{50} = 0.03 µg/ml) was used as standard positive drug.

Egg yolk agar medium was employed in phospholipase secretion assays. The culture medium, composed of 1% peptone, 3% glucose, 1 M NaCl, 5 mM $CaCl_2$ and 1.5 % agar, was autoclaved. When the medium was cooled (50°C), 8% v/v sterile egg yolk emulsion was added and poured into Petri dishes (10 ml in 90-mm Petri dishes) (Giacone et al., 2019). Phospholipase activity produces a dense precipitation zone around the enzyme-expressing colony.

YCB-BSA (Yeast Carbon Base-Bovine Serum Albumin) agar medium was used as an inducer of aspartyl proteinases secretion. The culture medium, composed of 0.1 % KH_2PO_4, 0.5 % $MgSO_4$, 1 % glucose and 1.5 % agar, was adjusted to pH 5.0 and autoclaved. When the medium was cooled (50°C), 0.2 % BSA (as the sole nitrogen source) was added and poured into Petri dishes (10 ml in 90-mm Petri dishes) (Giacone et al., 2019). Cleavage of BSA by aspartyl proteinase results in a clearance zone around the colony that expresses enzymes.

Blood agar medium was employed in hemolysin secretion assays. The culture medium, composed of SDA supplemented with 3% glucose, was autoclaved. When the medium was cooled (50°C), 7% sheep blood was added and poured into Petri dishes (10 ml in

90-mm Petri dishes) (Giacone et al., 2019). A translucent halo around the colony indicates a positive hemolysin activity.

Tween 80 opacity test medium was employed in esterase secretion assay. The culture medium, composed of 1 % bacto-peptone, 0.5 % NaCl, 0.01 % $CaCl_2$ and 1.5 % agar, was autoclaved. When the medium was cooled (50°C), 0.5 % v/v of Tween 80 (Sigma-Aldrich) was added and poured into Petri dishes (10 ml in 90-mm Petri dishes) (Giacone et al., 2019). The lipolytic enzymes hydrolyze the medium, releasing fatty acids, which then bind to calcium, forming a precipitation halo around the colonies expressing the enzymes.

Evaluation of the inhibition of Candida biofilms formation

Candida strain was grown for 24 h at 30-32°C on yeast extract-peptone-dextrose (YPD) broth and then, cells were washed twice in PBS and adjusted, in RPMI-1640 medium, to a final concentration of 1.0×10^6 cells/ml (Giacone et al., 2019). Solutions of extracts (100 µl), rendering final concentrations ranging from 0.06 to 1,000 µg/ml, were placed in polystyrene 96-well flat microplates and 100 µl of inoculum were added [test wells (TW)]. The microplates were homogenized at 150 g for 5 min on an orbital shaker platform, submitted to irradiation or kept in darkness and then, incubated statically for 24 h at 35-37°C. Controls included: growth control (GC), that contains extract-free culture medium and biofilm; extract blank (EB), with the extract, culture medium and without biofilm; and sterility control (SC), with culture medium and water instead of inoculum. Biofilm formation was evaluated by measuring the metabolic activity of the remaining cells based on the MTT reduction assay (Giacone et al., 2019). Absorbance of each well was determined at 540 nm in a VERSA Max microplate reader and the % of reduction in metabolic activity were calculated as follows: % of reduction in metabolic activity = $100 - [(OD_{TW} - OD_{EB})/(OD_{GC} - OD_{SC}) \times 100]$. The MFC was defined as the lowest extract concentration resulting in > 90% reduction in metabolic activity.

Evaluation of the antifungal activity against C. albicans biofilms

At first, 100 µl a standardized *C. albicans* cell suspension (see *Evaluation of the inhibition of Candida biofilms formation*), was transferred into wells of a microtiter plate and incubated statically for 24 h at 35-37°C (Giacone et al., 2019). After biofilm formation, the liquid was aspirated and each well was washed twice with PBS (200 µl) to remove non-adherent cells that remain in the wells. Solutions of extracts (200 µl) diluted in PBS giving concentrations that ranged from 0.06 to 1,000 µg/ml, were added in wells containing biofilms [test wells (TW)]. The microplates were homogenized at 150 x g for 5 min on an orbital shaker platform and then, were submitted to irradiation (light) or kept in darkness. Controls included: standard positive controls with commercial antifungal drugs AMB, FCZ and ITZ; growth control (GC), that contains

extract-free culture medium and biofilm; extract blank (EB), with the extract, culture medium and without biofilm; and sterility control (SC), with culture medium and water instead of inoculum. After incubation at 35-37°C for 2 h, viability was evaluated employing MTT assay. Absorbance of each well was determined at 540 nm in a VERSA Max microplate reader and the % of reduction in metabolic activity were calculated as follows: % of reduction in metabolic activity = 100 - $[(OD_{TW} - OD_{PB})/(OD_{GC} - OD_{SC}) \times 100]$. The MFC was defined as the lowest extract concentration resulting in > 90% reduction in metabolic activity.

Statistical analysis

Exploratory data analysis was initially performed to determine the most appropriate statistical approach for each assay. Data comparison were performed using the Kruskal-Wallis test and the Holm-Sidak method. Data were statistically analyzed using GraphPad Prism version 6.01 (San Diego, CA, USA). A *p*-value of < 0.05 was considered significant.

Results

Evaluation of the photodynamic antifungal activity

Extraction yields (%w/w) and MFC (µg/ml) of Hex, DCM, EtOAc and MeOH extracts from aerial parts, flowers and roots of *T. pentachaeta* are summarized in Table 1. None of the extracts were active against *C. albicans* in the absence of irradiation (MFC>1000 µg/ml). However, all extracts from roots; Hex and DCM extracts from aerial parts; and Hex, DCM and EtOAc extracts from flowers showed activity when UVA light was applied (Table 1). The highest activities were observed with Hex and DCM extracts obtained from roots (MFC = 7.81 µg/ml).

Chemical characterization of T. pentachaeta Hex and DCM extracts

The qualitative analysis of the Hex and DCM extracts by HPLC-DAD allowed the detection of α-T; BBT; BBTOAc; and BBTOH, previously described as having photodynamic antifungal activity (Downum et al., 1985; Postigo et al., 2017). The most polar thiophene (BBTOH) was retained at 4.24 min and was eluted before less-polar BBTOAc (Rt: 7.82 min), BBT (Rt: 9.19 min) and α-T (Rt: 11.95 min). The main constituent of the Hex extract was identified as α-T (1.28±0.05 mg/100 mg of extract) and was not detected in DCM extract (Fig. 1, Table 2). In this extract, the presence of BBT and BBTOAc was also detected at lower concentrations (0.99±0.03 and 0.27±0.02 mg/100 mg of extract, respectively). The main thiophene in the DCM extract was BBT (0.99±0.04 mg/100 mg of extract) following by BBTOAc (0.20±0.03 mg/100 mg of extract). BBT and BBTOAc were present in both Hex and DCM extracts at similar

concentrations. BBTOH was exclusively detected in DCM extract at a concentration of 0.29±0.03 mg/100 mg of extract.

Time-kill assays

The fungicidal effect of most active extracts was confirmed by time-kill curve experiments. Both, Hex and DCM extracts from roots (L+E+ conditions) reached the fungicidal endpoint (CFU/ml = 0) at 30 min (Fig. 2), but, under darkness conditions, no decrease in CFU/ml at 60 min have been observed (L-E+). There was no reduction in the number of CFU/ml in the assays without any treatment (L-E-) and those only exposed to light (L+E-), suggesting that the action of UV-A irradiation did not reduce *C. albicans* cell viability. Instead, in experiments performed with the fungicidal standard drug AMB, a complete reduction in viable cells was reached at 2 h (curve not shown).

Bioautographic assays

The TLC bioautographic assay is a useful tool to detect the photoactive compounds (Giacone et al., 2019; Postigo et al., 2017). For visualization of the major constituents, before the bioautographic assays, Fig. 3 shows TLC plates under UV 254 nm irradiation. Bioautographic assays irradiated with UVA light, after staining with MTT, revealed clear zones of inhibition (Fig. 3C) at Rf 0.64 and 0.48 in both extracts. Meanwhile, DCM extract showed another inhibition zone at Rf 0.15. Considering that several active compounds were present, the whole extracts, without purification of the active components, were used to perform the additional experiments.

Mechanism of ROS production

Superoxide anion production (type I mechanism)

The production of $O_2^{\bullet-}$ by Hex and DCM extracts from roots was measured by the NBT assay. Experiments in darkness and controls (without extract) gave negligible values of absorbance suggesting that, under these conditions, there were not decomposition of NBT. Both extracts under UVA light irradiation, considerably increased NBT decomposition compared to the experiment in darkness (p<0.05), suggesting that under these conditions, $O_2^{\bullet-}$ formation was stimulated (Fig. 4A).

Hydrogen peroxide production (type I mechanism)

The production of H_2O_2 by Hex and DCM extracts was measured with a peroxidase-catalyzed colorimetric assay. Control experiments in darkness and without extract gave negligible values of absorbance suggesting that, under these conditions, there were not H_2O_2 production (Fig. 4B). However, significant differences have been observed,

for Hex and DCM extracts, between light and darkness experiments. These results suggested that these extracts stimulated H_2O_2 formation only when they were irradiated. Control experiments under light and darkness conditions gave negligible absorbance values.

Singlet oxygen production (type II mechanism)

Singlet oxygen production (type II mechanism) by Hex and DCM extracts from roots of *T. pentachaeta*, was detected by the absorbance decrease of 1,3-DPBF at 410 nm as a function of time. A rapid decrease in the absorbance of 1,3-DBPF was observed in experiments in the presence of Hex and DCM extracts under irradiation, indicating that they require light irradiation for $O_2(^1\Delta g)$ production (Fig. 5), reaching 100% reduction of 1,3-DPBF absorption after 6 and 4 min of irradiation, respectively. Control curve indicated that irradiation of the sample effected a slower decrease in 1,3-DPBF absorption than that recorded for extracts (100% reduction of 1,3-DPBF absorption after 10 min of irradiation). Under darkness conditions the absorbance of 1,3-DPBF remains at the same level, suggesting that it was not breaking down (not shown).

Virulence factors of Candida albicans after photodynamic antimicrobial chemotherapy

Adherence to BEC cells

Adherence to BEC inhibition assay was performed in order to evaluate whether the active extracts, at sub-lethal concentrations, affected this process. Results showed that the number of yeasts adhered to 100 BEC decreased from 2324.0 ± 283.5 in AdhC (without any treatment) to 365.0 ± 84.1 and 531 ± 63.8 in cells treated with Hex extract and light at MFC/2 and MFC/4, respectively (Fig. 6). Treatment with sub-lethal concentrations of DCM extract and light also caused a remarkable decrease in yeast adherence to BEC (632.0 ± 187.7 and 874.0 ± 164.8 adhered to 100 BEC at MFC/4 and MFC/2, respectively). In the presence of the same concentrations of each extract, significant differences have been observed in the number of yeasts adhered to 100 BEC between light and darkness treatments. In the presence of the standard positive drug FCZ at its IC_{50} (0.03 µg/ml), under light and darkness conditions, the number of yeasts adhered did not decrease. No significant differences have been observed between the adhesion to BEC of yeasts treated with different concentrations of extracts in darkness with respect to adherence control (under light and darkness conditions), indicating that the action of light or extracts alone does not affect this process. Fig. 7 clearly shows that the amount of adhered fungal cells to BEC were much lower for yeasts treated with Hex extract and light in comparison with untreated cells (without extract and darkness), thus suggesting that this treatment will reduce the colonization of BEC by *C. albicans*.

Germ tube formation

The ability of *C. albicans* to form GT decreased significantly after exposure to sub-lethal concentrations of photoactive extracts and the extent of reduction depended on the concentration (Fig. 8). Hex extract, at concentrations between 0.03-0.12 μg/ml under light conditions, significantly inhibited hyphae formation (%GT 63.5 ± 4.9 to 12.5 ± 3.5, respectively) and total inhibition was obtained at concentrations ≥ 0.24 μg/ml (MFC/16). DCM extract was found to inhibit hyphal formation at 0.24-0.98 μg/ml (%GT 41.0 ± 4.2 to 4.0 ± 1.4, respectively) and was completely inhibited at 1.95 μg/ml, corresponding to MFC/4. However, the ability of *C. albicans* to form GT did not decrease after exposure to sub-lethal concentrations of both extracts in darkness compared to the GTC without any treatment. In the presence of the standard positive drug FCZ at its IC_{50} (0.03 μg/ml), under light and darkness conditions, GT formation was not inhibited.

Pseudomycelium formation

The effects of Hex and DCM extracts, under UVA irradiation, on pseudomycelium formation in *C. albicans* were also examined employing nutrient-poor Spider medium that induced pseudohyphal morphogenesis (Silva-Rocha et al., 2015). Morphology of *C. albicans* in the presence and absence of the extracts, under light and darkness conditions, showed significant pseudomycelium formation, suggesting that these extracts were not effective inhibitors (data not shown). In the presence of the standard positive drug FCZ at its IC_{50} (0.03 μg/ml), under light and darkness conditions, pseudomycelium formation was not inhibited.

Lytic enzymes production

The effect of active extracts on secretion of hydrolytic enzymes (phospolipases, proteinases, hemolysins and esterases) in *C. albicans* CCC 193-2013 cells were studied. Results demonstrated that extracts under UVA irradiation did not affect phospolipases, proteinases and hemolysins secretion (data not shown). However, esterase production decreased from Pz = 0.44 ± 0.02 in the control without any treatment to Pz = 0.76 ± 0.02 and 0.75 ± 0.02 for yeasts treated with Hex extract and light, at MFC/2 and MFC/4, respectively; and to Pz = 0.75 ± 0.03 and 0.79 ± 0.03 for yeasts treated with DCM extract and light at MFC/2 and MFC/4, respectively (Fig. 9). In the presence of the standard positive drug FCZ at its IC_{50} (0.03 μg/ml), under light and darkness conditions, did not inhibit the production of any of the enzymes evaluated.

Effects on formation and eradication of preformed biofilms

The *in vitro* effects of Hex and DCM extracts from roots of *T. pentachaeta* on biofilm formation and preformed biofilms by *C. albicans* has been investigated. Quantitative analysis using MTT assay revealed that both extracts, under UVA irradiation, significantly attenuated the metabolic activity of preformed biofilms, but no decreases were observed in experiments performed in darkness (Table 3). MFC defined as the lowest concentration of the extracts that inhibited biofilm up to 90% compared to the control, were 3.91 μg/ml and 15.63 μg/ml for Hex and DCM extracts, respectively. On the other hand, extracts did not inhibit *C. albicans* biofilm formation neither under darkness and light conditions (data not shown).

Discussion

C. albicans is the predominant pathogen among all fungal species, which is a growing problem in hospitals all over the world and leads to prolonged hospitalization (Doi et al., 2016). The treatment of *Candida* infections has become a challenge because of the emergence of drug-resistant species, along with the formation of biofilms. For these reasons, the development of alternatives to conventional antifungal agents are urgently needed. Among them, aPDT and therapies with drugs that target virulence factors and biofilms have emerged as hopeful alternatives.

(i) aPDT has recently emerged as an effective modality for the selective destruction of pathogenic microorganisms, including fungi. This therapy does not induce resistance, does not compromise the areas around the treated tissue and has a broad spectrum of action (Gonzales and Maisch, 2012). Natural PSs isolated from plants and other biological sources may be considered to be a promising approach to aPDT in antifungal therapy.

The present study proved, for the first time that the Hex and DCM extracts from roots of *T. pentachaeta* in combination with UVA light irradiation were effective in antifungal activity against both planktonic and biofilm forms of *C. albicans*. The fungicidal properties of this treatment may provide the necessary knowledge for the development of alternative antifungals considering that fungicidal agents are frequently preferred, since the recurrence rate of fungal infection is relatively low compared to fungistatic ones. In addition, time-kill studies showed that aPDT with these extracts has the capacity of killing *C. albicans* cells in 30 min, a time lower than that needed by the highly toxic antifungal drug AMB.

Chemical analysis showed that main thiophenes present in the bioactive root extracts, were α-T, BBT, BBTOH and BBTOAc whose antifungal photosensitive activity have been previously evaluated, with microdilution assay, against *C. albicans*, showing MFC values ranging 0.24‑7.81 μg/ml upon irradiation and no inhibition of growth without irradiation (Postigo et al., 2017). In addition, the antifungal activity of these compounds was also demonstrated against filamentous fungi, including

phytopathogens and dermatophytes (Mares et al., 2004; Romagnoli et al., 1998, 1994). Bioautographic assays, under UVA irradiation, revealed several inhibition zones in the extracts that were obtained at the same Rf of pure compounds, suggesting that they are the responsible of the activity observed. Similar findings have been observed in previous studies conducted of our working group in *Tagetes minuta* L. (Giacone et al., 2019) and *Porophyllum obscurum* (Spreng.) DC. (Postigo et al., 2017). The highest activities have been reported in root extracts which is the structure where these compounds usually accumulate (Giacone et al., 2019).

The mechanism of action of PS involves the absorption of a photon of light leading to its excitation to its short-lived excited singlet electronic state that can undergo an electronic transition to a triplet state. This triplet PS reacts with ambient oxygen by different photochemical pathways: type I involves an electron transfer to produce superoxide radical and then hydrogen peroxide, while type II involves energy transfer to produce excited state singlet oxygen. ROS generated can damage proteins, lipids and nucleic acids and lead to cell death (Hamblin, 2016). Analysis of ROS production indicated that Hex and DCM extracts from roots induce the formation of superoxide, peroxide and singlet oxygen in a 'mixed' photodynamic reaction of both type I and II. ROS played a significant role in the antimicrobial aPDT, reacting with polysaccharides, proteins, lipids and nucleic acids to produce molecular changes that may adversely affect fungal physiology and morphology (Wainwright et al., 2017).

Biofilms, structured microbial communities attached to a surface and encapsulated within a self-produced matrix, have the potential to initiate or prolong infections by providing a safe haven from which cells can invade local tissue and seed new infection sites. In addition, they display high levels of resistance to conventional antifungal therapy, which clearly indicates the urgent medical need for new antifungal agents and strategies (Pierce and Lopez-Ribot, 2013). aPDT employing *T. pentachaeta* Hex and DCM extracts from roots also demonstrated activity against cells in biofilms, being both preventive (i.e., inhibition of biofilm formation) as well as therapeutic (i.e., against pre-formed biofilms becoming a promising approach to combat biofilm-associated infections.

(ii) Virulence factors are attributes of pathogens and are generally considered not essential for their survival *in vitro,* while they are important with respect to causing disease in a host (Gauwerky et al., 2009). The advantages of targeting virulence includes decreased evolutionary pressure for the development of resistance, low cytotoxicity and rapid target inactivation (Liu et al., 2018). Hex and DCM extracts from *T. pentachaeta* roots, at sub-lethal concentrations under UVA irradiation, decreased the adherence of *C. albicans* to BEC, an intriguing characteristic of yeasts that is essential for colonization on an epithelial surface and initiation for pathogenesis. In addition, these treatments render reduced transition from unicellular yeast cells to GT but not pseudomycelium. Morphological transitions between yeast and filamentous

forms are interesting morphological features of *C. albicans* complex that play a crucial role in adherence, disease progression and biofilm formation (Butassi et al., 2019; Peters et al., 2014; Postigo et al., 2014). Hydrolytic enzymes play an important role in *Candida* growth, facilitate cell adherence, tissue penetration, modulate immune responses and therefore host invasion (Mane et al., 2012; Silva-Rocha et al., 2015). The capacity of both extracts to inhibit the production of hydrolytic enzymes was also evaluated, observing that both decrease the secretion of esterases, enzymes that degrade cell membrane ester bonds and accentuate tissue invasion.

Conclusion

Hex and DCM extracts from roots of *T. pentachaeta* under UVA irradiation demonstrated strong photodynamic effects against *C. albicans*. All these properties were attributed to the effect of thiophenes whose photodynamic antifungal activities have already been described in several plant species. This work reported for the first time that this treatment also reduces *Candida* virulence and affects the viability of the fungal biofilms becoming PDT with *T. pentachaeta* extracts a valuable alternative to the already established antifungal drugs.

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Conflict of interest

Authors declare that they do not have any conflict of interest.

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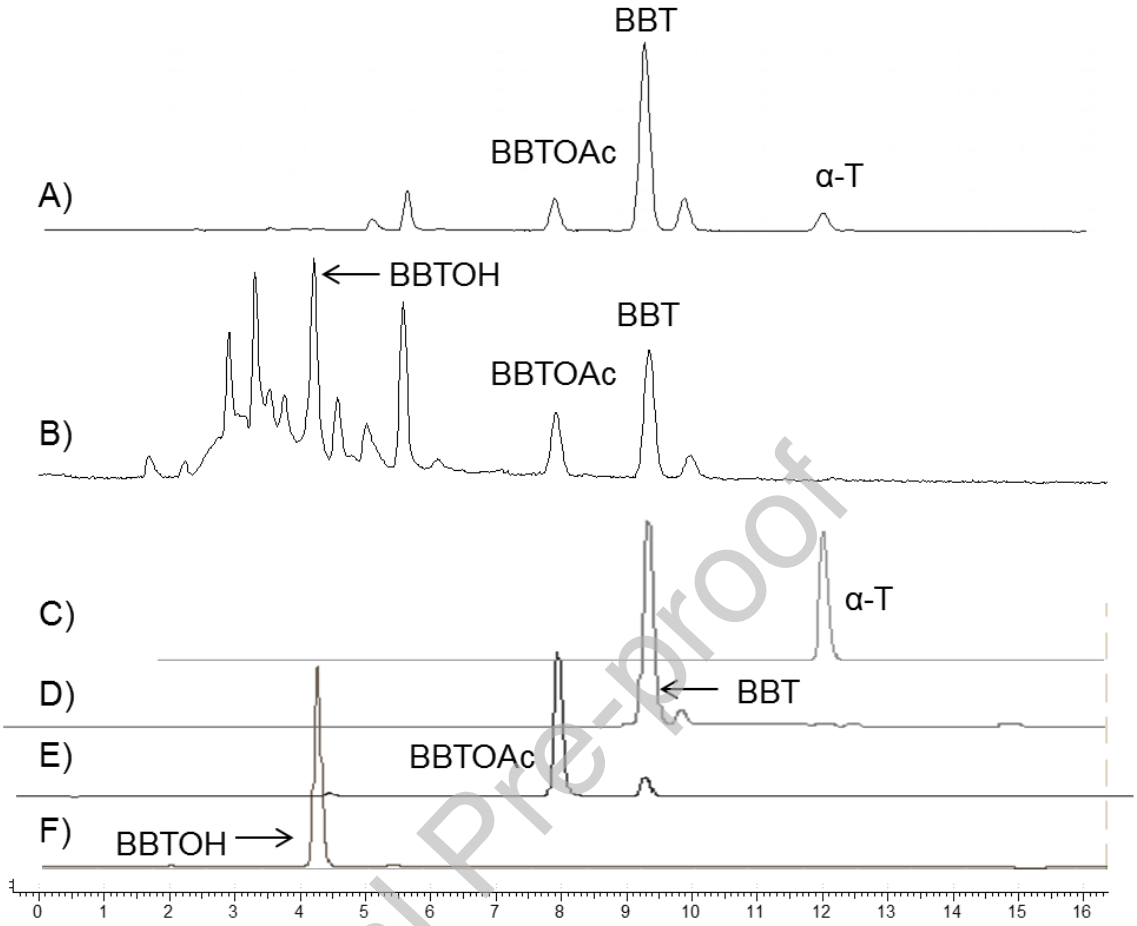

Fig. 1: HPLC–UV chromatograms of: **A**) hexane (Hex) and **B**) dichloromethane (DCM) extracts from roots of *Thymophylla pentachaeta* and standards: **C**) α-T, **D**) BBT, **E**) BBTOAc and **F**) BBTOH, at 351 nm. Retention times (Rt, min) of standards are: BBTOH (4.24 min), BBTOAc (7.82 min), BBT (9.19 min) and α-T (11.95 min).

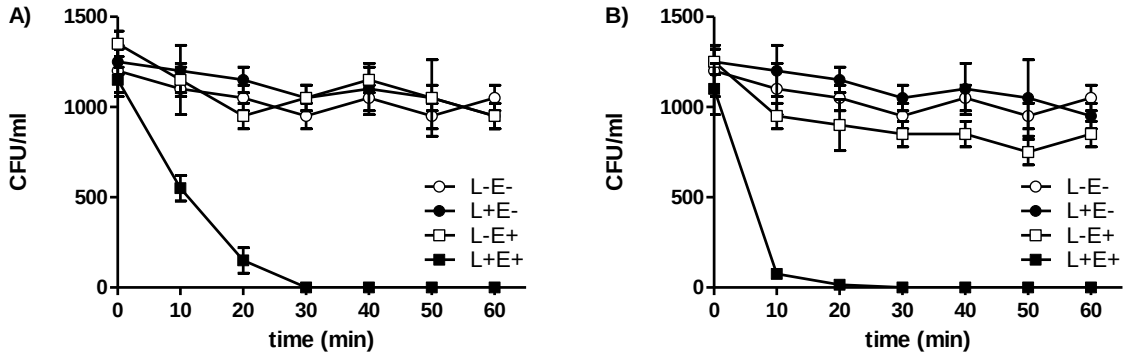

Fig. 2: Time-kill curves for **A**) hexane (Hex) and **B**) dichloromethane (DCM) extracts from roots of *Thymophylla pentachaeta* at minimal fungicide concentrations (MFC) against *Candida albicans* CCC 193-2013. **L+E+**: with extract and with irradiation; **L-E+**: with extract and without irradiation; **L+E-**: without extract and with irradiation and **L-E-:** without extract and without irradiation.

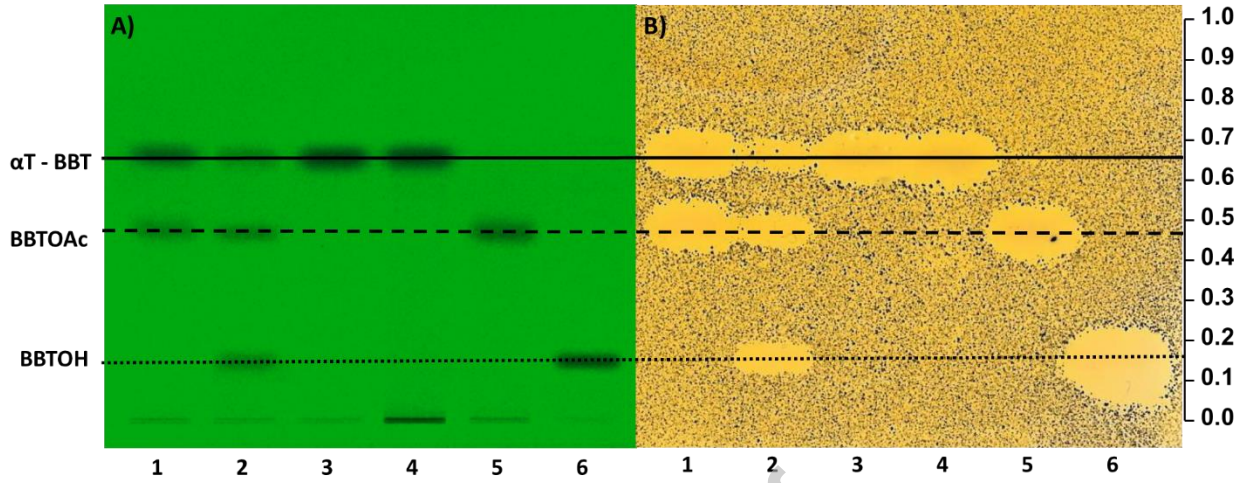

Fig. 3: Chromatographic profile of extracts from roots of *Thymophylla pentachaeta* and thiophenes **A**) under UV 254 nm and **B**) bioautography against *Candida albicans* CCC 193-2013 irradiated 60 min with UVA light (1.2 J/cm^2). Lanes **1**: hexane extract; **2**: dichloromethane extract; **3**: α-T; **4**: BBT; **5**: BBTOAc and **6**: BBTOH. Stationary phase silica gel GF_{254}; mobile phase Hex:EtOAc (80:20). Continuous line indicates α-T and BBT. Dotted line indicates BBTOAc. Dashed line indicates BBTOH.

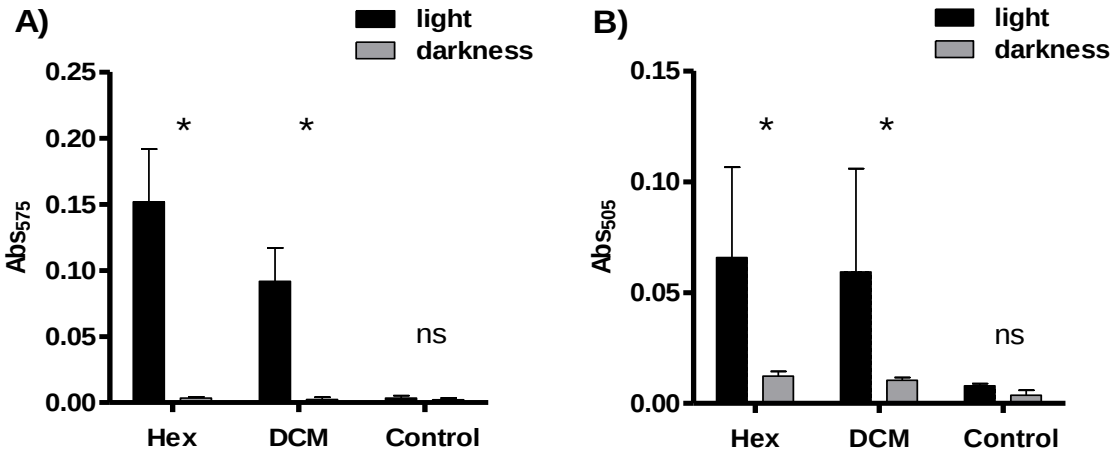

Fig. 4: **A**) Superoxide ($O_2^{\bullet-}$) and **B**) Hydrogen peroxide (H_2O_2) production of hexane (Hex) and dichloromethane (DCM) extracts from roots of *Thymophylla pentachaeta* and control without adding any extract, evaluated in darkness and under irradiation (light). * ($p < 0.05$); ns: not significant.

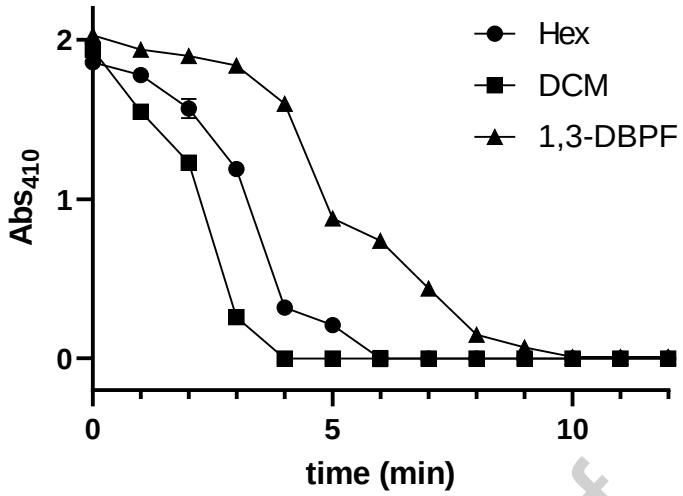

Fig. 5: Singlet oxygen production by hexane (Hex) and dichloromethane (DCM) extracts from roots of *Thymophylla pentachaeta* and control with DMSO instead of extract solution, under UVA light irradiation detected by the absorbance decrease of 1,3-diphenylisobenzofuran (1,3-DPBF) at 410 nm as a function of time.

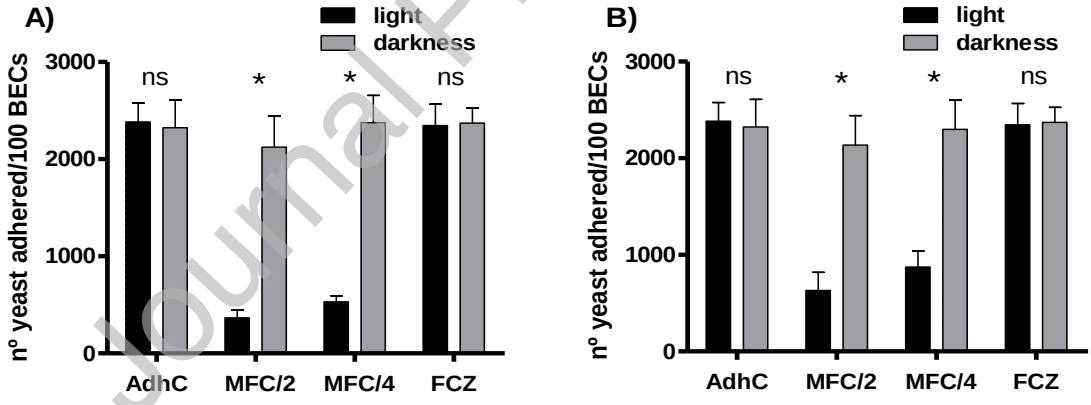

Fig. 6: Values of *Candida albicans* adherence to human buccal epithelial cells (BEC) after incubation in media containing sub-lethal concentrations of **A**) hexane (Hex) and **B**) dichloromethane (DCM) extracts and adherence control (AdhC) without adding any extract. Fluconazole (FCZ) was used as standard positive drug (at its IC_{50} = 0.03 µg/ml). Adherence to BEC is expressed as number of yeasts/BEC (N = 100). * (p <0.05), ns: not significant.

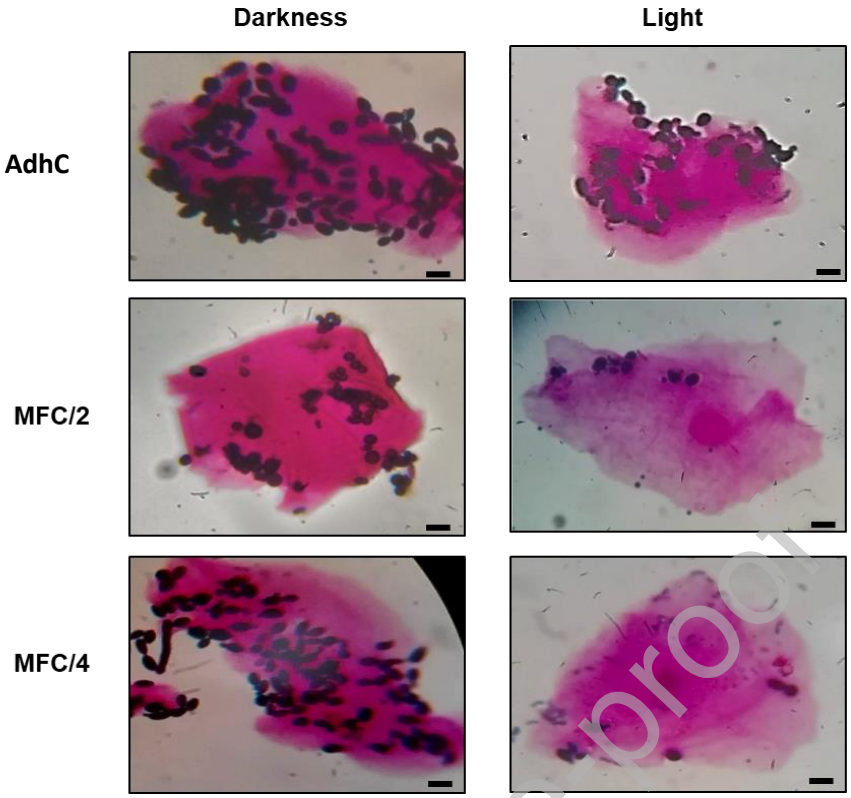

Fig. 7: Adhesion of *Candida albicans* (purple) to buccal epithelial cells (BEC, pink) treated with *Thymophylla pentachaeta* hexane extract from roots, at CFM/2 and CFM/4 and adherence control (AdhC) without the extract, under light and darkness conditions. Light microscopy at 1000x magnification. Bar: 5 µm.

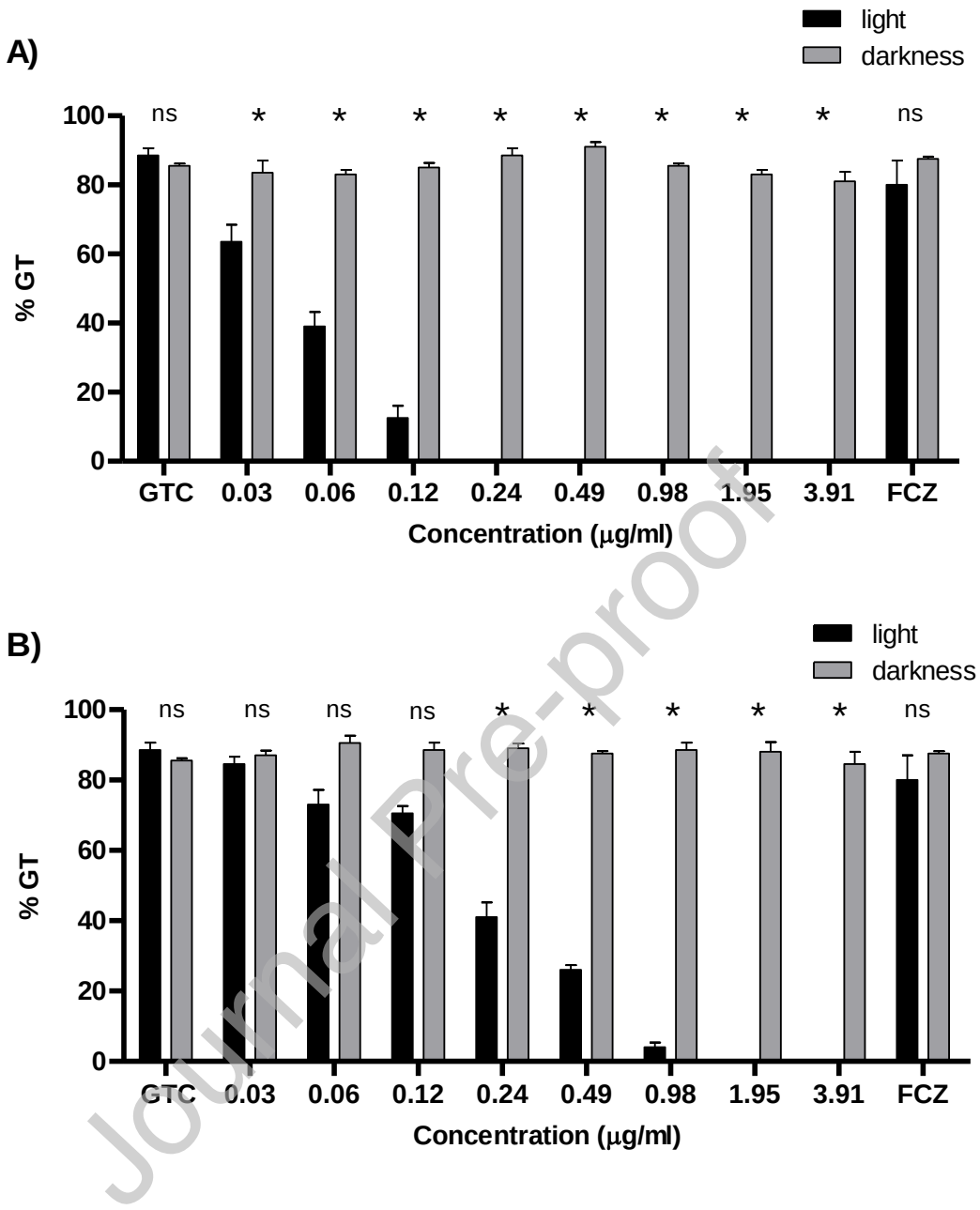

Fig. 8: Percentage of *Candida albicans* cells with germ tube (GT) exposed to sub-lethal concentrations (0.03-3.19 µg/ml) of **A**) hexane (Hex) and **B**) dichloromethane (DCM) extracts from roots of *Thymophylla pentachaeta* under light and darkness conditions. Fluconazole (FCZ) was used as standard positive drug (at its IC_{50} = 0.03 µg/ml). GTC: Germ tube control (without extract). *****(p<0.05), ns: no significant.

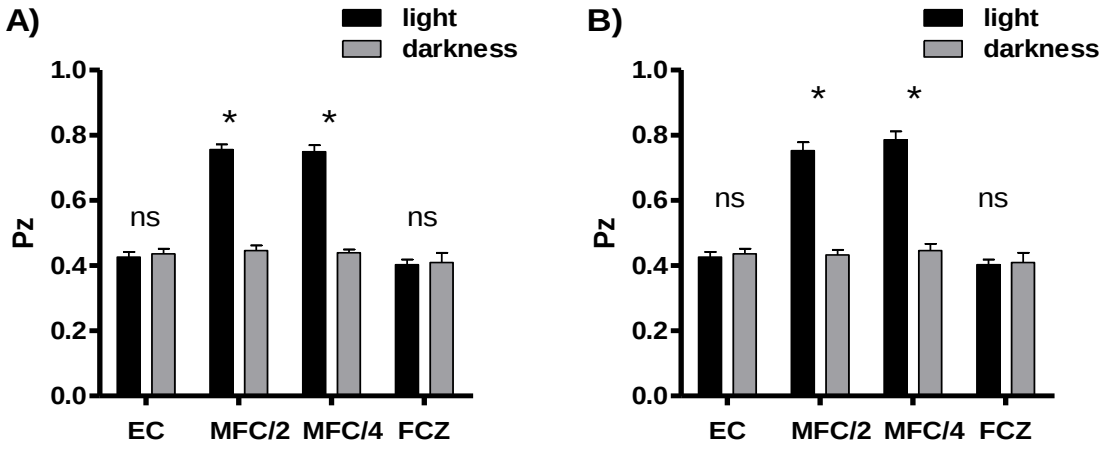

Fig. 9: Pz values of esterase secretion from *C. albicans* cells exposed to sub-lethal concentrations of **A**) hexane (Hex) and **B**) dichloromethane (DCM) extracts of *Thymophylla pentachaeta*, at CFM/2, CFM/4 and esterase control (EC) without adding any extract, under light and darkness conditions. Fluconazole (FCZ) was used as standard positive drug (at its IC_{50} = 0.03 µg/ml). *****p<0.05, ns: no significant.

Table 1: Yields (%w/w) and minimal fungicide concentrations (MFC, µg/ml) of extracts from *Thymophylla pentachaeta*, under light and darkness conditions, against *Candida albicans* CCC 193-2013.

			MFC (µg/ml)	
Part	**Extract**	**Yield**	**light**	**darkness**
Aerial parts	Hex	2.34 %	62.5	> 1000
	DCM	0.40 %	15.63	> 1000
	EtOAc	5.60 %	> 1000	> 1000
	MeOH	7.25 %	> 1000	> 1000
Flowers	Hex	8.90 %	> 250	> 1000
	DCM	7.76 %	62.5	> 1000
	EtOAc	9.53 %	125	> 1000
	MeOH	28.88 %	> 1000	> 1000
Roots	Hex	7.11 %	7.81	> 1000
	DCM	7.49 %	7.81	> 1000
	EtOAc	11.57 %	15.63	> 1000
	MeOH	15.24 %	125	> 1000
Standard positive drugs	AMB		0.5	0.5
	FCZ		0.03*	0.03*
	ITZ		0.25*	0.25*

Hex, Hexane; DCM, dichloromethane; EtOAc, ethyl acetate; MeOH, methanol. AMB, Amphotericin B; FCZ, fluconazole; ITZ, itraconazole. *For fungistatic azoles, IC_{50} has been informed.

Table 2: Thiophene composition of bioactive extracts (mg/100 mg extract)

Extract	αT	BBT	BBTOAc	BBTOH
Hex	1.28±0.05	0.99±0.03	0.27±0.02	
DCM		0.99±0.04	0.20±0.03	0.29±0.03

Table 3: Minimal fungicide concentrations (MFC, µg/ml) of active extracts from roots of *Thymophylla pentachaeta*, under light and darkness conditions, against preformed biofilms of *Candida albicans* CCC 193-2013.

		MFC (µg/ml)	
		light	darkness
Extracts	Hex	3.91	>1000
	DCM	15.63	>1000
Standard positive drugs	AMB	16	16
	FCZ	>64	>64
	ITZ	>16	>16

Hex, Hexane; DCM, dichloromethane. AMB, Amphotericin B; FCZ, fluconazole; ITZ, itraconazole.

Graphical abstract