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Antifungal photosensitive activity of *Porophyllum obscurum* (Spreng.) DC.: Correlation of the chemical composition of the hexane extract with the bioactivity

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ABSTRACT

We report *Porophyllum obscurum* as a source of new photosensitizers with potential use in Photodynamic Therapy as an alternative for oropharyngeal candidiasis treatment. The antifungal photosensitive activity of different extracts from *P. obscurum* was evaluated by using microdilution and bioautographic assays. The Minimum Fungicidal Concentration for hexanic extract under UV-A irradiation was 0.98 μg/mL, but it was inactive in experiments without irradiation. The bioassay-guided fractionation of this extract led to the isolation of four thiophenes responsible for the photosensitive activity: 2,2′:5′2″terthiophene, 5-(3-buten-1-ynyl)-2,2′-bithiophene, 5-(4-acetoxy-1-butenyl)-2,2′- bithiophene and 5-(4-hydroxy-1-butenyl)-2,2′- bithiophene, with Minimum Fungicidal Concentrations ranging 0.24-7.81 μg/mL under UV-A irradiation. The activity of the hexanic extract was evaluated against 25 clinical strains of *Candida* spp. isolates as etiological agents of oropharyngeal candidiasis. No differences in susceptibility were observed in strains resistant and susceptible to conventional antifungal drugs. Qualitative and quantitative chemical analyses of seven samples of *P. obscurum* collected in four different phenological stages were carried out showing that full flowering stage possesses the highest thiophenes content. These data also allowed us to establish a correlation between the thiophene composition of the different extracts and their antifungal photosensitive activity, according to a second order polynomial model with the equation: $y = 11.2603\text{-}0.6831{*}x + 0.0108{*}x^2$. The thiophenes isolated were the responsible of antifungal photosensitive activity and can be used for the future standardization of the extract. Results showed that *P. obscurum* hexanic extract could be potentially developed as an Herbal Medicinal Product to be applied as a photosensitizer in Photodynamic Therapy.

Abbreviations: ACN, acetonitrile; AHP, American Herbal Pharmacopoeia; AMB, Amphotericin B; ANOVA, analysis of Variance; ATCC, American Type Culture Collection; CCC, CEREMIC culture collection; CEREMIC, Centro de Referencia de Micología; CFU, colony-forming units; CLSI, Clinical and Laboratory Standard Institute; CTLC, Centrifugal Thin Layer Chromatography; DAD, diode array detector; DCM, dichloromethane; EA, ethyl acetate; EMA, European Medicines Agency; FCZ, fluconazole; ITZ, itraconazole; Hex, hexane; HIV, Human Immunodeficiency Virus; HPLC, High Performance Liquid Chromatography; HRMS, High Resolution Mass Spectrometry; ICH, International Conference on Harmonization; Met, methanol; MFC, minimal fungicide concentration; MIC, minimal inhibitory concentration; MOPS, 3-(N-morpholino)propanesulfonic acid; MTT, methylthiazolyltetrazolium chloride; NMR, nuclear magnetic resonance; OPC, oropharyngeal candidiasis; PDT, photodynamic therapy; *PoDCM*, *Porophyllum obscurum* dichloromethane extract; *PoEA*, *Porophyllum obscurum* ethyl acetate extract; *PoHex*, *Porophyllum obscurum* hexane extract; *PoMet*, *Porophyllum obscurum* methanol extract; PS, photosensitizer; R, resistant; ROS, reactive oxygen species; RSD, Relative Standard Deviation; S, susceptible; SDA, Sabouraud Dextrose Agar; SDD, Susceptible Dose Dependent; TLC, thin layer chromatography; UV, ultraViolet

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1. Introduction

Oropharyngeal candidiasis (OPC) is the most frequent fungal opportunistic infection in immunocompromised patients, such as those with neutropenia, intensive care patients, organ transplant recipients, Human Immunodeficiency Virus (HIV) infected, patients with uncontrolled diabetes, poor nutrition or metabolic disorders and users of removable dentures [1–3]. Head and neck radiotherapy and chemotherapy were associated with a significantly increased risk for oral fungal infection [3]. OPC may present as painful infection that affects the quality of life since causes inflammation of the oral mucosa, pain and dysgeusia, and may extend to the esophagus or result in systemic infection [4]. Prompt diagnosis and management of this infection in patients are important to prevent these complications. OPC is primarily caused by *Candida albicans* and there is an emergence of other species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [2].

Treatment of OPC is challenging due to the side effects that can develop during therapy, and to the fact that a prolonged exposure to antifungals develops drug resistance, which has become increasingly common [5,6]. Furthermore, the treatment of these infections is limited to a number reduced of available antifungal drugs commercial. Due to all of this and the high prevalence of OPC, there is an intense interest in the development of new strategies to prevent and treat this disease [7].

Photodynamic therapy (PDT) has emerged as an alternative to conventional treatments because it overcomes the above mentioned drawbacks. It is a developing light-based therapy for various applications in oncology (prostate cancer, for instance), dermatology, ophthalmology, and, more recently, for antimicrobial therapy [8,9]. PDT consists of the combination of a photosensitizer (PS) with low-intensity harmless light and oxygen, that generate reactive oxygen species (ROS) that trigger a cascade of biological events that lead to death of microorganisms [10]. The powerful and broad-spectrum antimicrobial effect as well as the fact that the irradiation can be spatially directed to the lesion [11], targeting PS straight to its destination, has highlighted this therapy as a promising alternative for localized infections [8].

A great challenge in the field of PDT is the development of natural PS that can be safely used in the treatment of fungal pathogens [12]. PSs are present in certain plants as a defense mechanism developed to protect themselves from the action of microbes and other predators. This action is triggered when these secondary metabolites are excited to higher energy levels by absorbing solar or artificial radiation at a particular wavelength range [13,14]. In recent years, there has been an increase in the number of publications reporting on natural products as a source of PS for antimicrobial PDT [15]. Natural PSs include anthocyanins and betalains [16], chlorophyll derivatives [17], polyacethylenes, thiophenes, quinines [18] and anthraquinones [19], among others. Thiophenes have shown to be responsible for the antifungal activities under UV-A irradiation of extracts from some plants of the family Asteraceae [20,21].

The genus *Porophyllum* (Asteraceae) comprises 25 species spread from the southwestern United States, Mexico, Central America, Antilles, and South America with Argentina as the Southernmost country. Six species inhabit the Argentinean central-western region [22,23] and are annual or perennial plants with secretory cavities in oil-bearing leaves and bracts which emanate a strong foul odor. Infusions of some *Porophyllum* species are reputed in folk medicine for their activity against cramps and venereal diseases and for their diaphoretic and antispasmodic properties [24–27]. Previous studies showed that this genus possesses antibacterial, anti-inflammatory and insecticide properties [28,29]. Regarding its antifungal activity, Downum et al. [30] described the UV-A light mediated antimicrobial activity of *Porophyllum* spp. (*P. gracile* Benth., *P. ruderale* (Jacq.) Cass. var., *P. macrocephalum* (DC.) Cronq. and *P. scoparium* A. Gray) from arid and semiarid regions of United Stated and Mexico, and Meckes-Lozoya and Gaspar [13] demonstrated the antifungal activity of *P. macrocephalum* against *C. albicans*, after irradiation at 350 nm.

Fig. 1. Inflorescences of *Porophyllum obscurum.*

Porophyllum obscurum (Spreng.) DC. (Fig. 1) known for its vernacular names “kilkina” “pus-pus”, “quirquiña”, “ruda blanca”, “yerba de la gama”, “yerba del ciervo” and “yerba del venado” grows in dry, sandy, stony or saline soils up to 2500 m in southern Brazil, Paraguay, Uruguay and Argentina, where it flourishes and fructifies from November to May each year [31]. However, there have been no studies about the light-mediated antifungal activity of *P. obscurum*.

The aim of this paper was to investigate *P. obscurum* as a source of new PS with potential use in PDT as an alternative for OPC treatment. The antifungal photosensitive activity of different extracts from *P. obscurum* was evaluated, followed by the bioassay-guided fractionation that allowed the isolation of four thiophenes that display the photosensitive activity. These assays were also conducted against 25 clinical strains of *Candida* spp. isolates as etiological agents of OPC. Then, a qualitative and quantitative chemical analysis of seven samples of *P. obscurum* was carried out, employing the four photosensitive isolated thiophenes as standards [32]. These data allowed a correlation between the compositions of the different extracts and their antifungal photosensitive activity. Finally, the optimal harvesting time could be determined and active markers for extract standardization could be identified.

2. Material and methods

2.1. General experimental procedures

The solvents were of analytical grade or purified by standard procedures prior to use. Silica gel GF_{254} (0.040–0.063 mm) were purchased at Merck (Darmstadt, Germany). UV–vis absorption spectra were taken with a Beckman DU-640 spectrometer (Fullerton, USA). Fluorescence spectroscopy was performed with a spectrometer Fluoromax-4 Horiba (Kyoto, Japan). ^{1}H and ^{13}C NMR spectra were performed on Bruker

Avance II (300 MHz) spectrometer (Bremen, Germany), using $CDCl_3$ as solvent. ^1H and ^{13}C NMR chemical shifts were referenced to $CDCl_3$ residual signals, in the case of $CDCl_3$ at δ_H 7.26 and the central peak at δ_{13C} 77.0. Signals were designated as follows: *s*, singlet; *d*, doublet; *dd*, doublets of doublets. The IR spectra were recorded with a Shimadzu 21 Prestige spectrophotometer of thin films held between KBr cells. EIMS were obtained in a MS Turbo Mass Perkin Elmer (Waltman, USA) with an ionization energy of 70 eV. MS and MS–MS were carried out as followed: source type: ESI, ion polarity: positive and negative, scan begin: 200 *m/z*, scan end: 2000 *m/z*, set nebulizer: 0.4 bar, set dry heater: 180 °C, set dry gas: 4.0 L/min, set capillary: 4500 V, set end plate offset: −500 V, set collision Cell RF: 1000–1500 Vpp. Collision energy 35 eV. Accuracy was verified by infusing a 10 mM solution of Na-formiate (Sigma- Aldrich) dissolved in MeOH:H_2O (50:50). Because of the improved performance reached using direct infusion to ESI, we report only MS and MS/MS data obtained in the negative mode. For the analyses of mass spectra, the Data Analysis 4.0 SP1 software (Bruker Daltonik GmgH, Germany) was used.

2.2. Plant material

Aerial parts (comprising leaves and stems) of *P. obcurum* from a same population, were collected in Las Chacras, province of San Luis (Argentina) at 33,246359 lat. S; 66,272505 long. W, at four different phenological stages throughout seven periods: October and December 2012; February, April, October and December 2013 and April 2014. All specimens were authenticated by one of the authors, Prof. Dr. Elisa Petenatti, and each batch was deposited at the Herbarium of the Universidad Nacional de San Luis (L.A. Del Vitto & E.M. Petenatti # 9436).

2.3. Extraction and isolation of PS

Aerial parts of *P. obscurum* (100 g) of each collection were successively extracted by maceration with hexane (Hex), dichloromethane (DCM), ethyl acetate (EA) and methanol (Met) 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 which constituted the *PoHex*, *PoDCM*, *PoEA* and *PoMet*, respectively. Chlorophylls were eliminated by treatment with freshly activated charcoal.

PoHex from the sample collected on October 2012 were selected for further chromatography fractionation.

Preparative centrifugal thin layer chromatography (CTLC) was carried out on a Chromatotron Model 7924T (Harrison Research, Palo Alto, CA, USA). The circular plates were coated with silica gel GF_{254}; the layer thickness was 2 mm (prepared from slurry formed of 65 g of silica gel and 130 mL of water). The mobile phase consisted of mixtures of solvents of increased polarity from Hex (100%) to EA (100%) that was delivered by the pump at a flow-rate of 1 mL/min. UV detection was carried out at 254 nm and fractions of 1–2 mL were collected [33].

Preparative Thin Layer Chromatography (TLC) was performed in 20 × 20 cm glass plates covered with 2 mm of Silica gel GF_{254} and developed using 80:20 or 90:10 Hex:EA mixtures as the mobile phases. After being developed, the TLC plates were revealed under UV-light (254 and 365 nm). Compounds were separately removed by scraping off the silica and washed with methanol.

The development of the preparative TLC from the active fractions afforded four thiophenic compounds: 2,2′:5′2″terthiophene **1** (39.3 mg), 5-(3-buten-1-ynyl)-2,2′-bithiophene **2** (18.1 mg), 5-(4-acetoxy-1-butenyl)-2,2′- bithiophene **3** (8.7 mg) and 5-(4-hydroxy-1-butenyl)-2,2′- bithiophene **4** (10.8 mg) [34].

2,2′:5′2″Terthiophene **1**: Yellow powder. IR (KBr) ν_{max} (cm^{-1}): 1645 (C_4H_3S). ^1H RMN ($CDCl_3$, 300 MHz) *δ*: 7.03 (2H; dd; *J* = 3.8, 5.2 Hz; H-4 and H-4″), 7.09 (2H; s, H-3′ and H4′), 7.19 (2H; d; *J* = 3.6 Hz; H-3 and H-3″), 7.23 (2H; d; *J* = 5.2 Hz; H-5 and H-5″). ^{13}C RMN ($CDCl_3$, 75 MHz) *δ*: 123.7 (C3 and C-3″), 124.3 (C-5 and C-5″), 124.5 (C-3′ and C-4′), 127.9 (C-4 and C-4″), 136.2 (C-2′ and C-5′), 137.1 (C-2 and C-2″). EIMS *m/z* 248 (M^+,100), 203 (20). HRESIMS (positive ion mode) *m/z* 248.9865 $[M^+H]^+$ (calcd. for $C_{12}H_8S_3$, 248.9861).

5-(3-Buten-1-ynyl)-2,2′-bithiophene **2**: Yellow powder. IR (KBr) ν_{max} (cm^{-1}): 2240 (C≡C), 1645 (C_4H_3S), 1454 (CH_2 = C). ^1H RMN ($CDCl_3$, 300 MHz) *δ*: 5.56 (1H; dd; *J* = 11.22, 1.97 Hz; H-4_{ch}), 5.73 (1H; dd; *J* = 17.58, 1.97 Hz; H-4_{ch}), 6.04 (1H; dd; *J* = 17,58, 11,22 Hz; H-3_{ch}), 7.02 (2H; m, H-4 and H-4′), 7.10 (1H; d; *J* = 3,62 Hz; H-3′), 7.18 (1H; dd; *J* = 3.62, 1.16 Hz; H-5), 7.24 (1H; dd; *J* = 5.17, 1.16 Hz; H-3). ^{13}C RMN ($CDCl_3$, 75 MHz) *δ*: 83.6 (C-1_{ch}), 93.2 (C-2_{ch}), 116.7 (C-3), 122.1 (C-5′), 123.5 (C-3′), 124.1 (C-3), 124.8 (C-5), 126.9 (C-4_{ch}), 128.0 (C-4), 132.8 (C-4′), 136.9 (C-2), 138.4 (C-2′). IR (cm-1): EIMS *m/z*: 216 (M^+, 100), 171 (40). HRESIMS (positive ion mode) *m/z* 217.0142 $[M^+H]^+$ (calcd. for $C_{12}H_8S_2$, 217.0140).

5-(4-Acetoxy-1-butenyl)-2,2′- bithiophene **3**: Yellow powder. IR (KBr) ν_{max} (cm^{-1}): 2240 (C≡C), 1732 (CO−O), 1454 (CH_2 = C). ^1H RMN ($CDCl_3$, 300 MHz) *δ*: 2.10 (1H; s; H-7_{ch}), 2.78 (2H; t; *J* = 6.9 Hz; H-3_{ch}), 4.25 (2H; t; *J* = 6.9 Hz; H-4_{ch}), 7.02 (3H; m, H-3, H-4 and H-4′), 7.16 (1H; dd; *J* = 3.6, 0.95 Hz; H-3′), 7.22 (1H; dd; *J* = 5.23, 0.95 Hz; H-5). ^{13}C RMN ($CDCl_3$, 75 MHz) *δ*: 20.3 (C-3_{ch}), 20.9 (C-7_{ch}), 62.1 (C-4_{ch}), 75.2 (C-1_{ch}), 90.6 (C-2_{ch}), 122.1 (C-5), 123.3 (C-3), 124.1 (C-3′), 124.9 (C-5′), 127.9 (C-4′), 132.4 (C-4), 136.8 (C-2′), 138.1 (C-2), 170.9 (C-6_{ch}). EIMS *m/z*: 276 (M^+, 20), 216 (100). HRESIMS (positive ion mode) *m/z* 299.0178 $[M^+Na]^+$ (calcd. for $C_{14}H_{12}O_2S_2$, 299.0171).

5-(4-Hydroxy-1-butenyl)-2,2′- bithiophene **4**: Yellow powder. IR (KBr) ν_{max} (cm^{-1}): 3379 (OH), 2240 (C≡C), 1454 (CH_2 = C). ^1H RMN ($CDCl_3$, 300 MHz) *δ*: 2.73 (2H; t; *J* = 6.27 Hz; H-3_{ch}), 3.82 (2H; t; *J* = 6.27; H-4_{ch}), 7.01 (2H; m; H-3′ and H-4′), 7.05 (1H; d; *J* = 3.80 Hz; H-3), 7.15 (1H; d; *J* = 3.80 Hz; H-4), 7.22 (1H; d; *J* = 3.80 Hz; H-5). ^{13}C RMN ($CDCl_3$, 75 MHz) *δ*: 24.2 (C-3_{ch}), 61.0 (C-4_{ch}), 75.5 (C-1_{ch}), 91.6 (C-2_{ch}), 122.1 (C-5), 123.3 (C-3′), 124.1 (C-3), 124.9 (C-5′), 127.9 (C-4′), 132.4 (C-4), 136.8 (C-2′), 138.1 (C-2). IR (cm^{-1}): EIMS *m/z*: 234 (M^+, 75), 203 (100). HRESIMS (positive ion mode) *m/z* 235.0257 $[M^+H]^+$ (calcd. for $C_{14}H_{12}O_2S_2$, 235.0246).

2.4. Irradiation source

The UV-A light radiation source was a set of three 100 W lamps (Alic, Buenos Aires, Argentina), emitting a 315–400 nm black-light, placed in a mobile platform that allows varying the distance from the sample. The extracts photosensitive assays were performed at a distance to 12 cm from the sample and 60 min of UV-A irradiation, while the compound photosensitive assays were performed at a distance to 6 cm from the sample and 5 min of UV-A irradiation. The conditions of irradiation (distance and time) were optimized using DES package (Stat-Ease, Inc., Minneapolis, USA) version 7.0.3, following the previously described design by Postigo et al. [21]. 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. [35], the radiant flux incident upon the actinometer was calculated to be 1.2 J/cm^2 for extracts photosensitive assays and 3.4 J/cm^2 for compounds photosensitive assays.

2.5. Microorganisms and culture conditions

C. albicans ATCC 10231, provided by *American Type Culture Collection* (ATCC), Rockville, MD, USA, and twenty five clinical isolates of *Candida* strains isolates as etiological agents of OPC that were provided by Centro de Referencia de Micología (CEREMIC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Rosario, were used in this study. These strains were identified by the CHROMagar medium (CHROMagar Company, Paris, France) and the carbohydrate assimilation tests (ID 32C-BioMérieux, Paris, France) [36]. Isolates were maintained in Sabouraud-Dextrose Agar (SDA, Difco Laboratories, Sparks, MD, USA) and

frozen at −70 °C. Prior to each experiment, the isolates were aerobically cultured at 35 °C for 24 h. Inocula of cell suspensions were obtained according to reported procedures and adjusted in sterile distilled water to the concentration desired for each methodology [37].

2.6. Antifungal photosensitive activity

Four experimental conditions were performed to evaluate the antifungal photosensitive activity: with PS and with irradiation (P+L+); with PS and without irradiation (P+L-); without PS and with irradiation (P-L+), and without PS and without irradiation (P-L-, growth control). The experiments with both conditions irradiation and without irradiation were performed in parallel in 96-well flat-bottomed microtiter plates following the Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines [37]. 100 µL of PS stock solution (extract or compound) 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 0.24–1000 µg/mL for extracts and 0.12–250 µg/mL for pure compounds. The microplates were agitated for 5 min on an orbital shaker platform Innova 4000 (New Brunswick Scientific, NJ, USA) before irradiation. Microplates with the experiments P+L+ and P-L+ were submitted to irradiation under aseptic conditions in a laminar air-flow chamber. Instead, each microplate in dark (P+L- and P-L-) were wrapped with aluminum foil immediately after add inoculum during 60 min to extracts or 5 min to compounds to avoid exposure to light. Then, aliquots (50 µL) from all wells were plated onto 100-mm Petri dishes and, after incubation at 35 °C for 48 h, the number of CFU/mL were quantified. Assays were performed in triplicate. Minimal Fungicide Concentration (MFC) was defined as the lowest concentration that causes total growth inhibition (CFU/mL = 0) in the 100-mm dishes and was expressed as modal value. *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 [37]. 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. Strains were classified as resistant (R), susceptible- dose dependent (SDD) or susceptible (S) to commercial antifungals according to the CLSI document M27-A3 breakpoints [38].

2.7. Bioautographic assay

The extracts (20 µg) were dissolved in a volatile solvent and scattered on TLC plate. Then, TLC plate was developed with Hex:EA (80:20), revealed with UV-light (254 and 365 nm) and photographed with Chromato VUE® C-75, UV Viewing Cabine (UVP, San Gabriel, CA, USA). Next, chromatogram was placed into sterile Petri dishes with lids. A volume of the culture medium, composed of 2% glucose, 1% peptone, 0.6% agar and 0.02% phenol red, was mixed with an aliquot of inoculums of *C. albicans* to reach a final concentration of 1–5 × 10^4 CFU/mL and the volume was distributed over developed TLC plate (0.1 mL/cm^2).

After solidification of the medium, TLC plate was irradiated with UV-A light (60 min from a distance of 12 cm) under aseptic conditions in a laminar air flow chamber. Then, the plate was incubated for 24 h in a moistened chamber at 35 °C. Subsequently, plate was sprayed with an aqueous solution of methylthiazolyltetrazolium chloride (MTT; 1 mg/mL) (Sigma-Aldrich) and incubated for another 2 h at 35 °C. Yellow inhibition zones appeared against a dark brown background [39]. Darkness control experiment was carried out without irradiation.

2.8. HPLC analysis

2.8.1. Standards and extracts solutions

Purified active compounds **1**-**4** were employed as standards; their purities were ≥95% as determined by HPLC-DAD. Acetonitrile (ACN) solutions of the hexanic extracts from the seven periods and stock solutions of standards 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. To obtain calibration curves, aliquots of the individual stock solutions of each standard compound were diluted with ACN, yielding five solutions in the concentration range of 0.01–0.1 µg/mL. These solutions were stored at 4 °C in light-resistant containers and left to attain room temperature before use.

2.8.2. HPLC instrument and chromatographic conditions

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 × 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 ACN and Milli-Q water, with 0.1% formic acid, delivered at a flow rate of 1 mL/min. The peak areas 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.

2.9. Statistical analysis

Statistical analysis was performed with GraphPad PrismTM 4.0 (GraphPad software Inc., La Jolla, CA, USA). Data from the experiment were analyzed by two-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. p values ≤0.05 were considered significant. Non-linear regression analysis was performed using SigmaPlot10.0.

3. Results

3.1. Extraction and antifungal photosensitive activity

PoHex (3.23 g), *PoDCM* (1.19 g), *PoEA* (0.26 g) and *PoMet* (6.80 g) were obtained from 100 g of aerial parts from *P. obscurum* (from October 2012 collection). The antifungal photosensitive activity of the four extracts was determined by using the microdilution M27-A3 methodology described above. Results showed that, there were no significant differences with the number of CFU/mL obtained between the growth control (P-L-) and after exposure to only light conditions (P-L+) ($p > 0.05$), suggesting that the action of UV-A irradiation did not reduce *C. albicans* cell viability. Then, in order to identify non-photosensitive antifungal activity, the viability of *C. albicans* was also checked after incubation in the presence of each extract without irradiation. Results indicated that, in the absence of light, there were no significant differences between CFU/mL obtained with growth control (P-L-) and that one treated with the four extracts at a concentration up to 1000 µg/mL (P+L-). However, a significant reduction in *C. albicans* viability was observed for the assays subjected to antifungal photosensitive activity with *PoHex* and *PoDCM* (P+L+). *PoEA* and *PoMet* did not exhibit antifungal photosensitive activity at concentrations up to 1000 µg/mL. In the experiments with *PoHex* and *PoDCM* with irradiation (P+L+) the decrease in CFU/mL values was dependent on the PS concentration. The mean CFU/mL values obtained for the active extracts were illustrated in Fig. 2. Results indicated that concentrations of

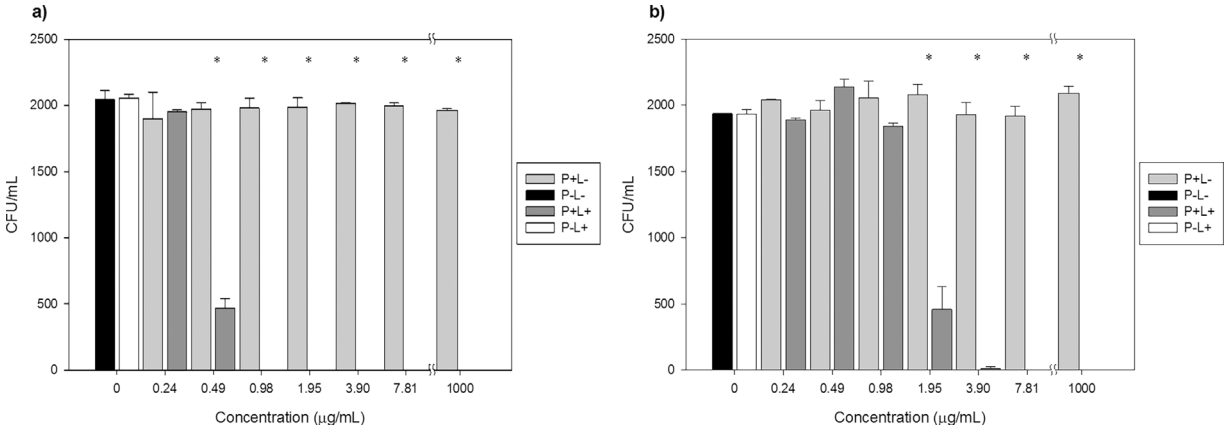

Fig. 2. Antifungal photosensitive activity. Reduction of the number of CFU/ml, of *C. albicans* ATCC 10231 (a) *PoHex* and (b) *PoDCM* with 60 min UV-A irradiation (1.2 J/cm^2) and pre-incubation in the dark. * significant difference ($p \leq 0.05$).

Table 1
Minimum Fungicide Concentration (MFC, in μg/mL) of *C. albicans* ATCC 10231 of *P. obscurum* extracts under UV-A irradiation (light) and in darkness.

	MFC (μg/mL)	
	Light	Darkness
PoHex	0.98	> 1000
PoDCM	7.81	> 1000
PoEA	> 1000	> 1000
PoMet	> 1000	> 1000
AMB	0.50	0.50
FCZ[a]	> 64 (MIC = 2)	> 64 (MIC = 2)
ITZ[a]	> 16 (MIC = 0.25)	> 16 (MIC = 0.25)

[a] FCZ and ITZ are fungistactic drugs, so MIC (minimal inhibitory concentration) values were showed.

at least 0.49 μg/mL (for *PoHex*) and 1.95 μg/mL (for *PoDCM*) were required for a statistically significant reduction of CFU/mL in the experimental conditions compared to P-L- ($p \leq 0.05$). For these extracts, the MFCs (CFU/mL = 0) were: 0.98 μg/mL for *PoHex* and 7.81 μg/mL for *PoDCM* (Table 1). Non photosensitive antifungal commercial drugs included in the assays as positive controls (AMB, FCZ and ITZ) showed the same antifungal activity when observed under both conditions, light and darkness (FCZ and ITZ are fungistatic drugs, so Minimal Inhibitory Concentration (MIC) values were used for this purpose).

The antifungal resistance profile of the twenty five clinical *albicans* and non-*albicans Candida* strains isolates of OPC of patients with head and neck cancer undergoing radiotherapy, was determined in order to classify them according to their susceptibility towards FCZ, ITZ and AMB. Then, the photosensitive antifungal activity of *PoHex*, the most active extract, was determined for each strain. MFC values varied between 0.98 and 3.90 μg/mL. Thirteen strains (52%) showed MFC = 0.98 μg/mL and only 2 (8%) MFC = 3.90 μg/mL. No substantial differences in MFC values have been observed between resistant and sensitive strains to antifungal commercial drugs. Resistant strains showed MFC in the range 0.98–1.95 μg/mL (Table 2).

In order to investigate the number of PS present in *PoHex*, a bioautographic assay was then performed versus *C. albicans*. For visualization of the major constituents, TLC plates were observed (previous to the bioautographic assays) under UV_{254} and UV_{365} irradiation (Fig. 3a and b). In the assays, after staining by using a viability dye, three major clear inhibition zones (Rf: 0.09, 0.36 and 0.71) were detected in the plate irradiated with UV-A light (Fig. 3c), whereas no inhibition of growth *C. albicans* could be detected in the plate that was not irradiated (Fig. 3d).

Table 2
Resistance profile of *Candida* strains isolated as etiological agents of OPC. Minimal Fungicide Concentration (MFC, in μg/mL) of *P. obscurum* hexane extract under UV-A irradiation (light) and in darkness.

Specie	Voucher specimen	Antifungal profile			MFC (μg/mL)	
		FCZ	ITZ	AMB	Darkness	Light
C. albicans	CCC 182-13	S	S	S	>250	1.95
	CCC 191-13	S	S	S	>250	3.90
	CCC 192-13	S	S	S	>250	0.98
	CCC 193-13	S	S	S	>250	0.98
	CCC 196-13	R	R	S	>250	1.95
	CCC-128-15	S	S	S	>250	0.98
	CCC-129-15	S	S	S	>250	1.95
	CCC-130-15	S	S	S	>250	1.95
	CCC-131-15	S	S	S	>250	0.98
	CCC-132-15	S	S	S	>250	0.98
	CCC 188-13	S	S	S	>250	0.98
C. parapsilopsis	CCC 183-13	S	S	S	>250	0.98
	CCC 187-13	SDD	S	S	>250	1.95
	CCC 200-13	S	S	S	>250	1.95
	CCC 184-13	SDD	R	S	>250	0.98
C. tropicalis	CCC 185-13	S	S	S	>250	1.95
	CCC 186-13	R	R	S	>250	1.95
	CCC 190-13	R	R	S	>250	0.98
	CCC 194-13	S	S	S	>250	0.98
	CCC 195-13	S	S	S	>250	0.98
	CCC 197-13	R	S	S	>250	1.95
	CCC 198-13	S	S	S	>250	1.95
	CCC 199-13	S	S	S	>250	0.98
	CCC 189-13	SDD	S	S	>250	0.98
C. krusei	CCC 189-13	R[a]	S	S	>250	3.90

CCC: CEREMIC Culture Collection. R: Resistant, SDD: Susceptible Dose Dependent, S: Susceptible.

[a] *C. krusei* is recognized as inherently resistant to FCZ.

3.2. Photosensitive compounds isolation, identification and antifungal activity

In an attempt to isolate pure compounds that contribute to the observed photosensitive antifungal activity, *PoHex* was fractionated by chromatographic techniques such as preparative CTLC and TLC. The isolation of the main PSs was guided by bioautographic assays, producing four main compounds. These compounds were identified by their High Resolution Mass Spectrometry (HRMS) and NMR spectra analysis as the thiophene derivatives: 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. 4).

The antifungal photosensitive activity of thiophenes **1**-**4** was

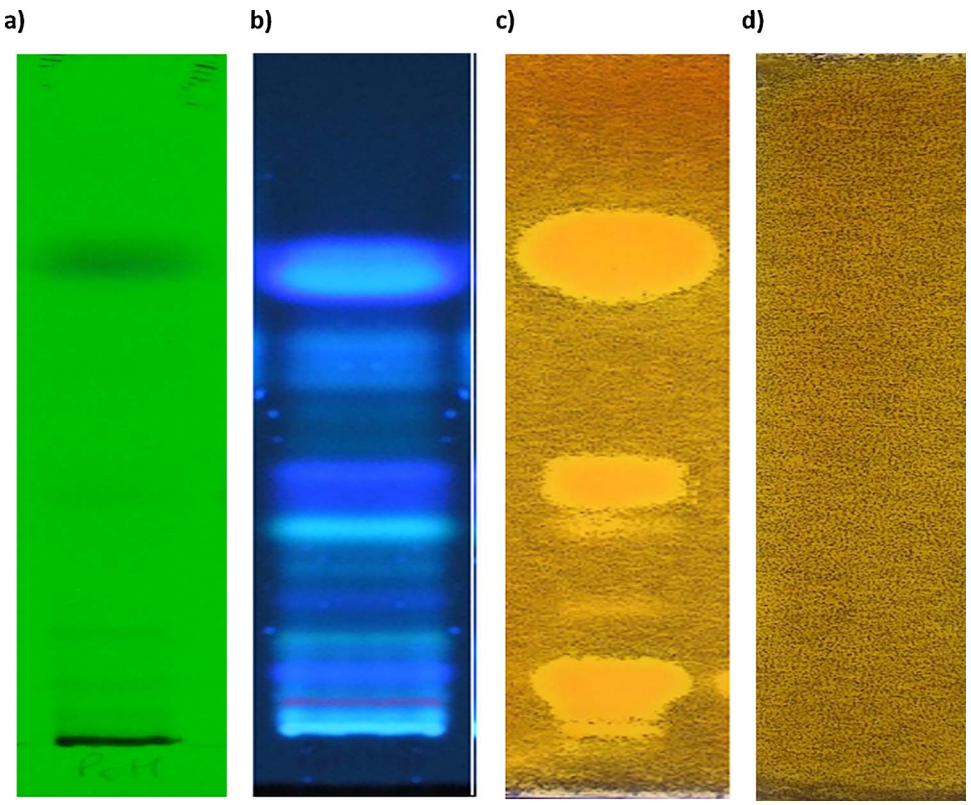

Fig. 3. Profile of *PoHex* under a) UV_{254} nm; and b) UV_{365} nm. Bioautography chromatograms against *C. albicans* c) irradiated 60 min with UV-A light (1.2 J/cm^2) and d) in darkness. Silica gel GF_{254}; mobile phase Hexane:Ethyl acetate (80:20).

evaluated against *C. albicans*, showing MFC values ranging 0.24–7.81 μg/mL upon irradiation and no inhibition of growth without irradiation (Table 3). The photosensitive antifungal activity of *PoHex* (MFC = 0.98 μg/mL, Table 1) is the result of the combination of the activities of the four thiophenes isolated.

4. Photochemical characterization

Fig. 5 show the absorbance and fluorescende spectra of extracts and compound **1**-**4**. Table 4 show the photochemical characterization of extracts and compounds **1**-**4**. All extracts and compounds have a maximum absorption ranging 314–350°nm coinciding with the emitting range of the light radiation source used for the antimicrobial assays.

4.1. Analysis of thiophene variation in different collection batches

The content of thiophenes **1-4** and the photosensitive antifungal activity were determined in *PoHex* collected in four different phenological stages: vegetative (October), full flowering (December), flowering and fruiting (February) and full fruiting (April). Since the seven samples came from the same site but collected in different months, the variations observed in the content mostly reflected temporal variations.

Table 3
Minimum Fungicide Concentration (MFC, in μg/mL) of *C. albicans* ATCC 10231 of thiophenes isolated under UV-A irradiation (light) and in darkness.

	MFC (μg/mL)	
	Light	Darkness
1	0.24	> 250
2	7.81	> 250
3	7.81	> 250
4	3.90	> 250
AMB	0.50	0.50
FCZ[a]	> 64 (MIC = 2)	> 64 (MIC = 2)
ITZ[a]	> 16 (MIC = 0.25)	> 16 (MIC = 0.25)

[a] FCZ and ITZ are fungistactic drugs, so MIC (minimal inhibitory concentration) values were showed.

Thiophenes **1-4** were qualitatively identified and quantitatively determined using the HPLC-DAD that was first validated following International Conference on Harmonization (ICH) guidelines [41]. The most polar thiophene (compound **4**, peak 4) was retained at 4.5 min and was eluted before the non-polar thiophenes **3** (6.7 min, peak 3), **2** (10.5 min, peak 2) and **1** (11.6 min, peak 1) (Fig. 6). All seven extracts

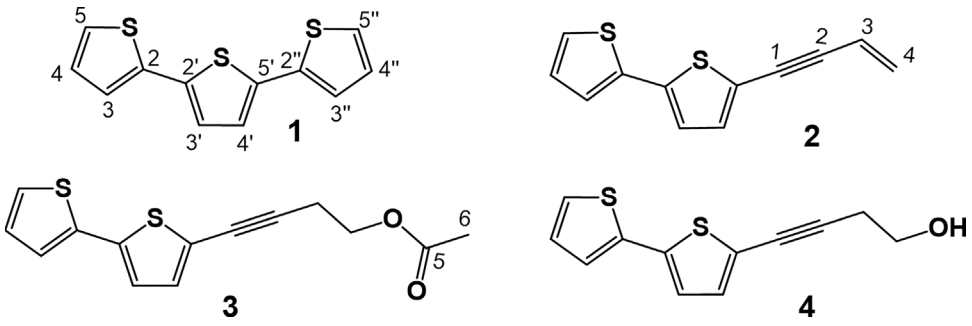

Fig. 4. Structure of the thiophenes **1**-**4** isolated from *P. obscurum* extract.

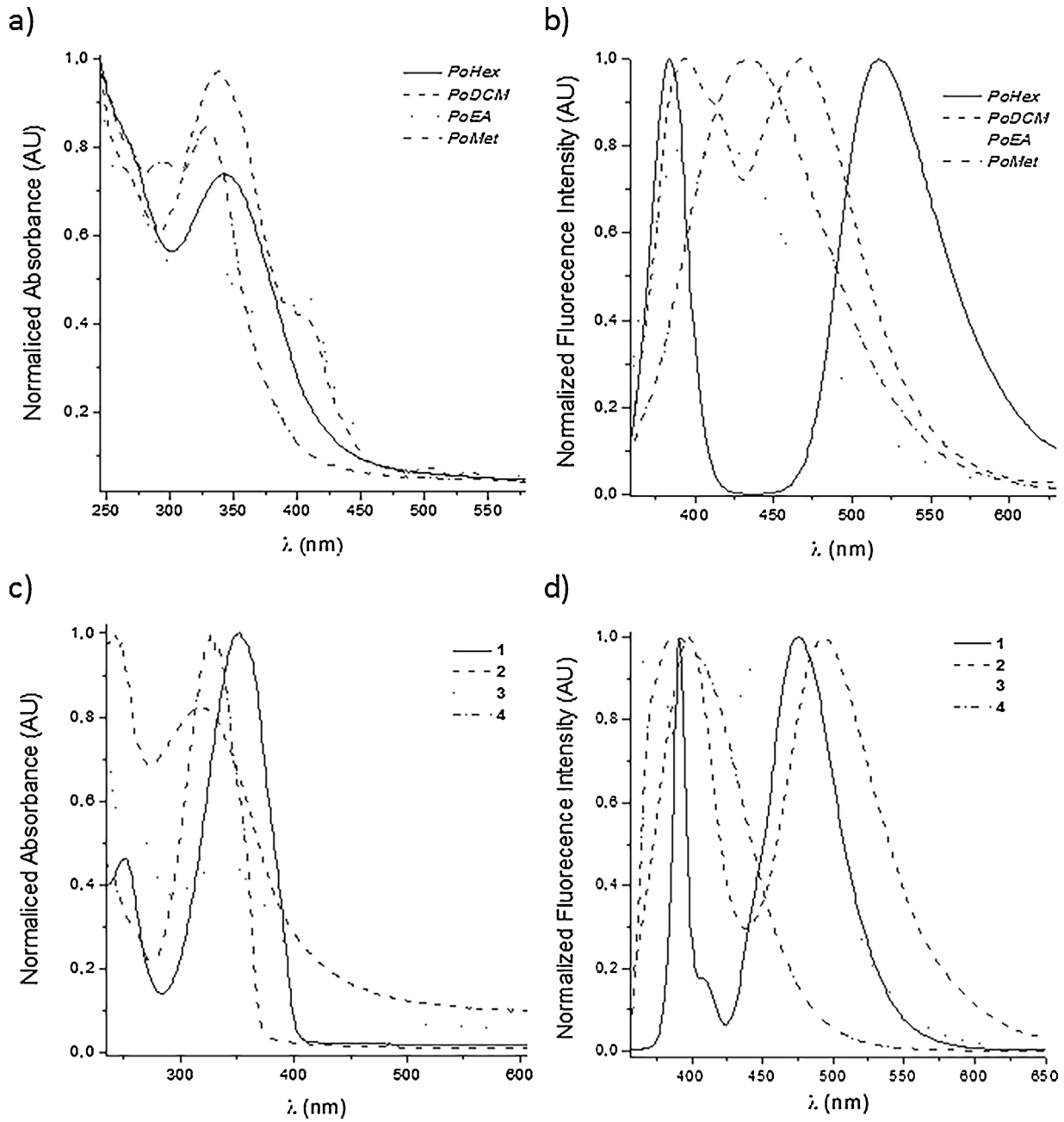

Fig. 5. a) Absorbance and b) fluorescence spectra of extracts obtained from *P. obscurum*. c) Absorbance and d) fluorescence spectra of compounds **1**-**4**.

Table 4
Photochemical characterization of extracts and isolated thiophenes.

PS	λ_1 (nm) (ε (M^{-1} cm^{-1}))	λ_2 (nm) (ε (M^{-1} cm^{-1}))	λ_3 (nm) (ε (M^{-1} cm^{-1}))	$\lambda_{1Emis.max.}$ (nm)[a]	$\lambda_{2Emis.max.}$ (nm)[a]	φ_F[b]
PoHex	210 (12,550)	271 (7502)	343 (23,741)	384	520	0.068
PoDCM	210 (10,587)	267 (9320)	338 (22,963)	394	468	0.084
PoEA	261 (13,780)	328 (8124)	409 (23,127)	391	424	0.043
PoMet	213 (15,036)	290 (7100)	330 (21,004)	–	433	0.056
1	210 (12,420)	252 (9220)	350 (22,650)	391	474	0.049*
2	210 (12,645)	263 (8820)	341 (22,930)	398	498	0.088
3	208 (16,700)	262 (9700)	332 (28,200)	370	458	0.082
4	207 (8270)	242 (6580)	328 (22,060)	390	–	0.050

[a] Emission Maxima.
[b] Molar fluorescence efficiency. The excitation wavelength was 360 nm and the fluorescence yield was determined for the emission between 435 and 500 nm. Solvent: Ethanol-Methanol (1:1, V/V) [40].

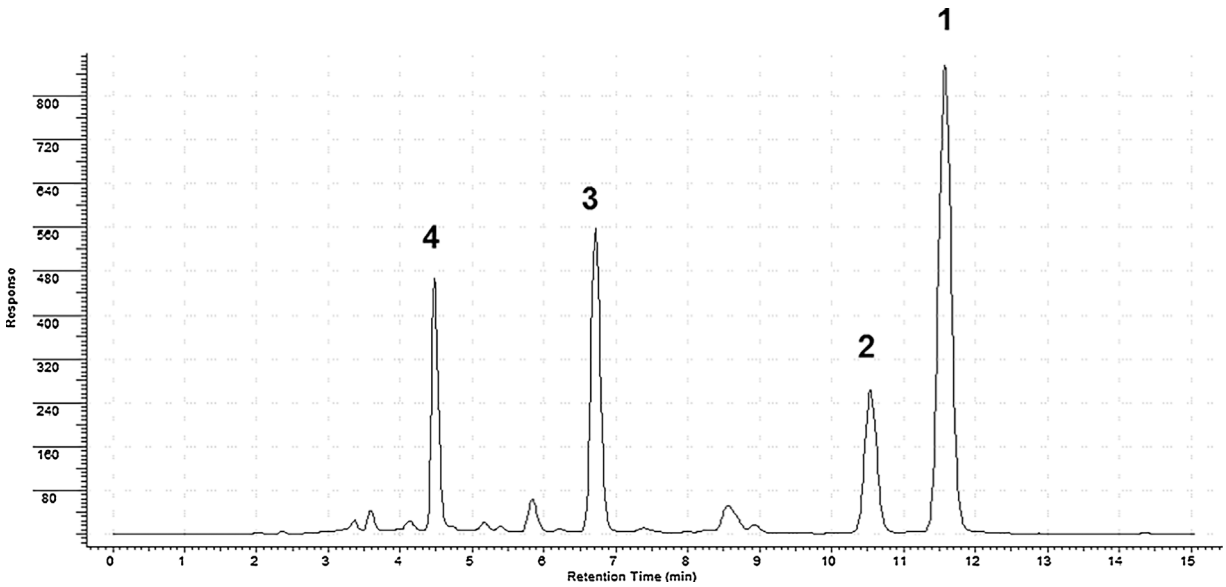

Fig. 6. Representative HPLC Chromatogram of mixed thiophenes isolated from *P. obscurum*. The compound number are the same as Fig. 5.

Table 5
Variation in thiophene composition (mg/100 mg extract) and Minimal Fungicide Concentration (μg/mL) of *P. obscurum* in batches collected in different dates.

Date of collection (month year)	Phenologic stage	Yield (g/100 g vegetal material)	Concentration (mg/100 mg extract)					CFM (μg/mL)
			1	**2**	**3**	**4**	Sum	
October 2012	vegetative	3.23	7.12	1.71	8.78	5.83	23.44	0.98
December 2012	full flowering	4.21	11.92	10.59	5.63	3.46	31.60	0.49
February 2013	flowering and fruiting	5.03	1.76	1.72	2.24	1.52	7.24	7.81
April 2013	fruiting	2.35	4.22	5.72	3.06	1.91	14.91	3.90
October 2013	vegetative	3.24	3.50	1.49	4.11	2.66	11.76	3.90
December 2013	full flowering	6.25	8.82	6.79	6.51	3.36	25.49	0.98
April 2014	fruiting	7.25	0.39	0.23	3.29	0.80	4.71	7.81

of *P. obscurum* were characterized by the presence of the four thiophenes. The total thiophene content (calculated as the sum of the four thiophenes) reached levels ranging from 4.71 to 31.60 mg/100 g of extract (Table 5) and they relative amounts varied greatly among the phenological stages. In particular, full flowering stage (December 2012 and 2013) possessed the highest amounts of total thiophene content (31.60 and 25.49 mg/100 g of extract) and fruiting stage (April 2014), the lowest (4.71 mg/100 g of extract). However, the extract of April 2013, which also corresponds to the fruiting stage, had a total thiophene content higher than April 2014. As regards the relative abundance of each thiophene, **1** was present at the highest level in extracts from December 2012 and 2013, while **3** was present at the highest level in extracts from October 2012 and 2013, February 2013 and April 2014. All extracts showed an important antifungal photosensitive activity with MFC ranging from 0.49 to 7.81 μg/mL. The highest antifungal photosensitive activity, MFC = 0.49–0.98 μg/mL, was showed in extracts with the highest total thiophene content (batches collected in October 2012, December 2012 and December 2013). On the other hand, the lowest antifungal photosensitive activity (MFC = 7.81 μg/mL) was observed in the batches collected in February 2013 and April 2014, extracts with both the lowest total thiophene content.

4.2. Correlation between thiophenes content and activity

The changes in the photosensitive antifungal activity of *PoHex*, with respect to temporal variations, could be attributed to the different total thiophene composition. In order to investigate the impact of the total thiophene content on the *in vitro* photosensitive antifungal activity of *P. obscurum* extracts a correlation analysis between them was performed. For this purpose, MFC values were used as parameters of biological activity, and the mean concentration values of the total thiophenes concentration as the parameter of chemical composition. A statistically significant positive relationship was established and a second order polynomial model was fitted $y = 11.2603\text{-}0.6831*x + 0.0108*x^2$, where y is the biological activity (MFC) and x is the chemical composition (total thiophene content) (Fig. 7). The analysis of regression show a coefficient of determination $R^2 = 0.9663$ and adequate residual

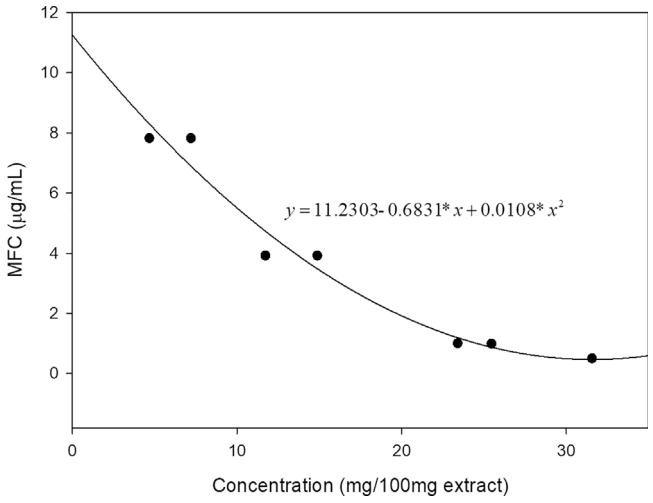

Fig. 7. Fitted regression function between MFC and the concentration of total thiophenic compounds.

Table 6
Best fit value and standard error for each parameter with nonlinear regression ($y = y_o + a * x + b * x^2$).

Parameter	Value	Std Err
y_o	11.2603	1081
a	−0.6831	0.1457
b	0.0108	0.004

distribution and behavior (Table 6). Therefore, higher total thiophene content higher antifungal photosensitive activity.

5. Discussion

Our results showed that the extracts and compounds have a maximum absorption ranging 314–350 nm, that coinciding with the range of irradiation UV-A employed for Downun et al. when demonstrate that some *Porophyllum* species (*P. gracile, P. scoparia* and *P. ruderale* var. *macrocephalum*) possessed photosensitive antifungal activity [30]. The emitting range of the light radiation source used for the antimicrobial assays coinciding with the absorption range.

The present study proved, at the first time, the photosensitive antifungal activity under UV-A of *P. obscurum* hexanic and dichloromethanic extracts against *C. albicans*. Hexanic extract showed the best photosensitive activity, also against a panel of twenty five *Candida* strains at MFC values between 0.98 and 1.95 μg/mL. These *Candida* strains panel were isolated from OPC in patients with head and neck cancer undergoing radiotherapy including non-*albicans Candida* species and multidrug resistant strains. Non-*albicans Candida* species have become increasingly problematic among the immunocompromised patients and that each species has a unique antifungal resistance profile [3]. These observations support the idea that PDT may be an alternative therapy for treating etiological agents of OPC resistant to conventional antifungals.

Four thiophene compounds (**1**-**4**) were isolated from the *PoHex*. A careful revision of literature revealed that this is the first report on the presence of thiophenic compounds in *P. obscurum* that were isolated by bioguided fractionation. Previous phytochemical analysis of this species demonstrated the presence of volatile monoterpenes [42] phenolic compounds such as quercetin, isorhamnetin, escopoletin, chlorogenic acid, caffeic acid and derivatives [43].

Thiophenes and their derivatives are natural products that play the role of chemical defenses in plants [44]. They are biologically active compounds, under UV-A radiation [45,46]. They are widely distributed in many tribes of the Asteraceae family particularly found in *Tagetes*, *Berkheya*, *Dyssodia*, *Echinops*, *Bidens* [34,47] and some other species of *Porophyllum*, such as *P. gracile* [45,47], *P. lanceolatum* [47], *P. riedelli* [48], *P. ruderale* [46,49,50] and *P. scoparium* [46,50]. The photosensitive antifungal activity of the thiophenes **1**-**4** under UV-A have been previously demonstrated against filamentous fungi, including phytopathogens and dermatophytes [51–56]. In a previous work [21], we have already investigated the antifungal UV-A-dependence of **1** against *Candida* by using an optimized design based on the guidelines of CLSI M27-A3 methodology [37]. The present study we determined the MFC of the compound **1**-**4** against *C. albicans* under just 5 min of irradiation. The MFC values ranging 0.24-7.81 μg/mL, the compound **1** exhibited the highest antifungal photosensitive activity (MFC = 0.24 μg/mL), higher than AMB (0.5 μg/mL).

Environment variations during plant growth may lead to significant differences in chemical composition and content. Several reports have emphasized the existence of marked chemical differences among extracts from different samples collected at different times [56]. As the chemical composition may potentially affect its biological activities, the photosensitive antifungal activity of the extracts, from plants collected at different times, was recorded. The composition of **1**-**4** was investigated at first time in samples of *P. obscurum* collected in the same place at different phenologic stages. The four thiophenes were presence in all seven extracts, but their relative amounts varied greatly. We observed a great variation in the total thiophene content between extracts of the same phenologic stage and different months of collection. Full flowering stage (December 2012 and 2013) possessed the highest amounts of total thiophene content, this data is in agreement with the observed in *Tagetes paula* by Downum and Towers [57]. They reported that the thiophene concentration levels increase as the plant get older, reaching a maximum during the reproductive stages. The high content of thiophenes during these critical stages may guarantee species reproduction and survival by reducing the risk of pathogen attacks [58]. Then a correlation between total thiophenes concentration and antifungal photosensitive activity of which extract has been established. We observed a statistically significant positive correlation that respond to a second order polynomial model. These data allowed us to establish that a higher total thiophene content higher antifungal photosensitive activity.

These results are useful to determine the optimal harvesting time of *P. obscurum* for its application as an antifungal herbal product for PDT. It must be taken into account that an extract has an enormous number of compounds, so that the quantification of the total of its components is impracticable. For this reason, it is necessary to select markers. Besides as discussed in the monographs of the American Herbal Pharmacopoeia (AHP), the use of single or multiple chemical markers was important to quality control to a medicinal product. The European Medicine Agency (EMA) categorizes chemical markers into analytical markers and active markers. The latter are defined as constituents or groups of constituents which are generally accepted to contribute to the activity [32,59]. According to this definition and considering that thiophenes **1**-**4** were the responsible of antifungal photosensitive activity, they can be classified as active markers of *P. obscurum* and can be used for the future standardization of the *P. obscurum* extract for develop an Herbal Medicinal Product.

The *PoHex* showed an important antifungal photosensitive activity against *Candida* spp. under irradiation UV-A *in vitro* at low concentrations even against multidrug resistant strains. All these results provide support and justification for the investigation of *P. obscurum* for a potential develop as an Herbal Medicinal Product to be applied as PS in PDT for treatment OPC.

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