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Activity of compounds isolated from *Baccharis dracunculifolia* D.C. (*Asteraceae*) against *Paracoccidioides brasiliensis*

SUSANA JOHANN*†, FLÁVIA BERALDO OLIVEIRA*†, EZEQUIAS P. SIQUEIRA*, PATRICIA S. CISALPINO†, CARLOS A. ROSA†, TÂNIA M.A. ALVES*, CARLOS L. ZANI* & BETANIA B. COTA*

*Laboratório de Química de Produtos Naturais, Centro de Pesquisas René Rachou, Belo Horizonte, Minas Gerais, and †Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Paracoccidioidomycosis is a prevalent systemic mycosis in Latin America which requires prolonged treatment with highly toxic antifungals. *Baccharis dracunculifolia* is a medicinal plant in Brazil that is a candidate in the search for new drugs. Fractions of the hexanic extracts were obtained using chromatographic procedures and assessed using an antifungal assay with *Paracoccidioides brasiliensis* (Pb18), tumor cell lines and amastigote forms of *Leishmania*, *L. amazonensis*. Four compounds were isolated, i.e., ursolic acid (**1**), methyl linolenate (**2**), caryophyllene oxide (**3**), and trans-nerolidol (**4**). Compounds **2**, **3** and **4** displayed antifungal activity against four isolates of *Paracocci dioides* with MIC values ranging from 3.9–250 µg/ml. Only caryophyllene oxide showed differences in the MIC values against Pb18 when the medium was supplemented with ergosterol, which suggested that the compound interacts with ergosterol. Ursolic acid was active in the cytotoxic assays and showed leishmanicidal activity. Scanning electron microscopy demonstrated that compounds **2**, **3** and **4** decreased the cell size and produced an irregular cell wall surface on *P. brasiliensis* cells. The present results showed the biological activities of the isolated compounds and revealed that these compounds may affect the cell surface and growth of *P. brasiliensis* isolates.

Keywords Paracoccidioidomycosis, methyl linolenate, caryophyllene oxide, transnerolidol

Introduction

Paracoccidioides brasiliensis is a fungal pathogen that causes one of the most prevalent systemic mycoses in Latin America, paracoccidioidomycosis (PCM). When analyzed as the underlying cause of death, PCM is the most important of all systemic mycoses [1]. *P. brasiliensis* consists of at least three distinct cryptic phylogenetic species, i.e., S1,

PS2 and PS3 [2]. Pb01 and Pb1578 are isolates that are representative of a new phylogenetic species that is Pb01-like. Teixeira *et al.* [3] recommended that the 'Pb01-like' cluster be formally described as the new species *P. lutzii*. The treatment of PCM is usually prolonged, many patients receive therapy for one to two years, and the infection is generally fatal in the absence of drug therapy. Currently, ketoconazole and itraconazole are the drugs of choice over sulphonamides and amphotericin B (serious forms of PCM), but patient-relapse rates are high [4]. *Baccharis* is one of the largest genera of the *Asteraceae* family, which includes more than 500 species that are primarily distributed in southern and southeastern Brazil, Uruguay, Paraguay, Argentina and Bolivia [5]. Several *Baccharis* species have

been extensively used in folk medicine for the treatment and prevention of anaemia, inflammation, diabetes, and stomach, liver and prostate diseases [6]. Previous studies of *Baccharis* plant species showed that the hexane fraction of the aerial parts is active against clinical isolates of *P. brasiliensis*. Interestingly, the hexane fraction displayed minimum inhibitory concentration (MIC) values ranging from 7.8–30 µg/ml without evidence of cytotoxicity of macrophage cells [7].

Protozoan parasites of the *Leishmania* genus are the etiological agents of leishmaniasis, which is a disease that is distributed worldwide. Leishmaniasis has a broad spectrum of clinical manifestations based on the causative species and the immunological status of the host. The treatment of choice is a pentavalent antimony (sodium stibogluconate [Pentostam] or meglumine antimoniate [Glucantime]), both of which are parenteral drugs that are moderately toxic [8]. Amphotericin B is also used to treat leishmaniasis, but it has several side effects, such as arrhythmia, nephrotoxicity and hepatotoxicity [9].

Our research group has adopted a strategic approach to identify the active natural products that have the potential to be lead compounds for the treatment of fungal diseases, cancer and leishmaniasis, all of which have had a significant impact on public health due to their high levels of morbidity and mortality. The present study is the first report the antifungal activities, the biological activities against tumor cells and the leishmanicidal effects of four compounds isolated from the aerial parts of *Baccharis dracunculifolia*.

Materials and methods

Plant material and extract preparation

The aerial parts of *B. dracunculifolia* were collected at Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI) (germplasm number 138), Itajaí, Santa Catarina state, Brazil, in January 2004. The dried parts (483 g) were extracted with 80% ethanol (EtOH) over 10 days at room temperature. The hydroalcoholic extract (48 g) was solubilised in a methanol/water mixture (2:8) and partitioned successively with hexane (Hex), dichloromethane (DCM) and ethyl acetate (EtOAc). This procedure afforded 5.0 g from the Hex fraction, 1.3 g from the DCM fraction, 2.0 g from the EtOAc fraction and 5.8 g from the aqueous extracts.

Instruments

Preparative centrifugal accelerated radial thin layer chromatography (PCAR-TLC) was performed using a Chromatotron Model 7924 T (Harrison Research, Palo Alto, USA)

with rotors coated with Silica Gel 60 PF-254 (1 mm layer thickness). Thin-layer chromatographic (TLC) analyses were conducted on precoated silica gel G-60/F_{254} (0.25 mm, Merck, Darmstadt, Germany), and spots were visualised at wavelengths of 254 and 366 nm after spraying with vanillin-H_2SO_4. Column chromatography was run using silica gel 60 (70–230 mesh), and medium-pressure liquid chromatography (MPLC) was performed using an LC8 pump (Shimadzu, Kyoto, Japan). High-pressure liquid chromatography was achieved using a Shimadzu chromatographic system (Shimadzu, Kyoto, Japan) equipped with an LC6AD pump and a dual wavelength detector (SPD10A) with a Shim-pack C18 column (5 µm, 250 × 4.6 mm i.d.). We used mixtures of MeOH:H_2O as the eluent at a flow rate of 1 ml/min, and detection occurred at 210 and 254 nm. Gas chromatographic/mass spectrometric analyses were performed using a Shimadzu QP-5050A (Shimadzu, JP) instrument equipped with a PTE™-5 column (30 m length, 0.25 mm diameter and 0.25 µm internal film, Supelco, USA) using helium as the carrier gas. The following conditions were employed for all of the analyses: a helium flow rate of 27.8 ml/min; an injector temperature maintained at 250°C; an initial oven temperature of 120°C with a heating rate of 5°C/min up to 300°C, which was held for 1 min; and a 1:10 ratio split.

The mass detector was set to scan from 40–400 atomic mass units at a rate of two scans per second. Data acquisition and handling were performed using CLASS 5000 Shimadzu software. The raw data files were analysed using the Automated Mass Deconvolution and Identification System software (AMDIS) version 2.1, which was supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The elucidation of the compounds was performed using the NIST Mass Spectral Search Program, which is associated with the NIST/EPA/NIH library version 2.0. Nuclear magnetic resonance (NMR) spectra were obtained using a Brucker DRX 400 spectrometer at 400 MHz with the pulse programs provided by the manufacturer. The substances were dissolved in perdeuterated solvents containing 0.1% tetramethylsilane as the internal chemical shift standard.

Chromatographic fractionation of the extract

The Hex fraction was subjected to silica gel column chromatography (46 × 18 cm) and sequentially eluted with solvents of increasing polarity (Hex/DCM/EtOAc/MeOH). In total, 107 fractions were collected and grouped into 35 groups after TLC analysis on silica gel plates eluted with DCM/MeOH (95:5 v/v). Group 24 (754 mg) was subjected to normal MPLC separation (Sil, 25–40 mesh, Büchi column (24 × 7 cm) at 6 ml/min using increasing amounts of 5% EtOAc in Hex to generate ursolic acid (compound 1,

102 mg). An aliquot of group 15 (419 mg) was fractionated using normal PCAR-TLC elution with a step gradient of petroleum ether-EtOAc (1:0–0:1 v/v) at 3 ml/min to yield methyl linolenate (compound 2, 77 mg). Groups 11 and 12 (755 mg) were fractionated by normal MPLC (Sil, 70–250 mesh, column 20 × 4 cm at 15 ml/min) using increasing amounts of 5% EtOAc in Hex to give 13 subgroups, and caryophyllene oxide (compound 3, 70 mg) was found in subgroup 3. Subgroups 5 and 6 (152 mg) were purified by column chromatography (20–40 mesh, 20 × 7 cm, at 4.5 ml/min) using 5% EtOAc in Hex to yield trans-nerolidol (compound **4**, 25 mg) (Fig. 1).

Antifungal activity

To assess the antifungal activity of the extracts, fractions and pure compounds, the clinical *P. brasiliensis* strains Pb01 (ATCC, MYA-826), Pb18 (Fungi Collection of the Faculty of Medicine of the Universidade de São Paulo, São Paulo, SP, Brazil), PbB339 (ATCC 32069) and Pb03 (clinic isolate from a patient with chronic PCM, São Paulo, Brazil, MHH Forjaz/ TIE Svidzinski) were maintained by continuous passages in YPD (yeast, peptone and dextrose) medium at 37°C. The *Paracoccidioides* isolates were used after 7 days of growth.

Determination of the minimal inhibitory concentrations (MICs)

The testing of all of the *Paracoccidioides* isolates was performed following the Clinical and Laboratory Standards Institute (CLSI) M27-A2 guidelines and modifications suggested by Johann *et al.* [7]. RPMI 1640 (Sigma, St Louis, MO, USA) and YPD media were used in these assays. The hexane fraction and the isolated compounds were tested at concentrations ranging from 1.95–250 μg/ml. Amphotericin B (Sigma, St Louis, MO, USA) and trimethoprim/sulphamethoxazole (SMT/TMP; Ducto, Daia Anapolis, Brazil) were included as positive antifungal controls. We used concentrations ranging from 0.03–25 μg/ml for amphotericin B and 1.17–600 μg/ml for SMT/TMP. Stock solutions were prepared in dimethylsulphoxide (DMSO, Amresco, Solon, USA) for amphotericin B and water for SMT/TMP, and two-fold serial dilutions were prepared as described in the CLSI document M27-A2 [10]. After the inoculation of the fungal strains, the plates were incubated at 37°C for 10 days. The tests were performed in triplicate in at least two independent experiments. The endpoints were determined visually by comparison with the drug-free growth control. The MIC is expressed in μg/ml and is defined as the lowest compound concentration for which the well was optically clear.

Minimal fungicidal concentrations

The *in vitro* minimal fungicidal concentration (MFC) of each test compound was determined by streaking 10 μl from each well that showed complete inhibition (100% inhibition or a clear well), from the last positive well (growth similar to that of the growth control well) and from the growth
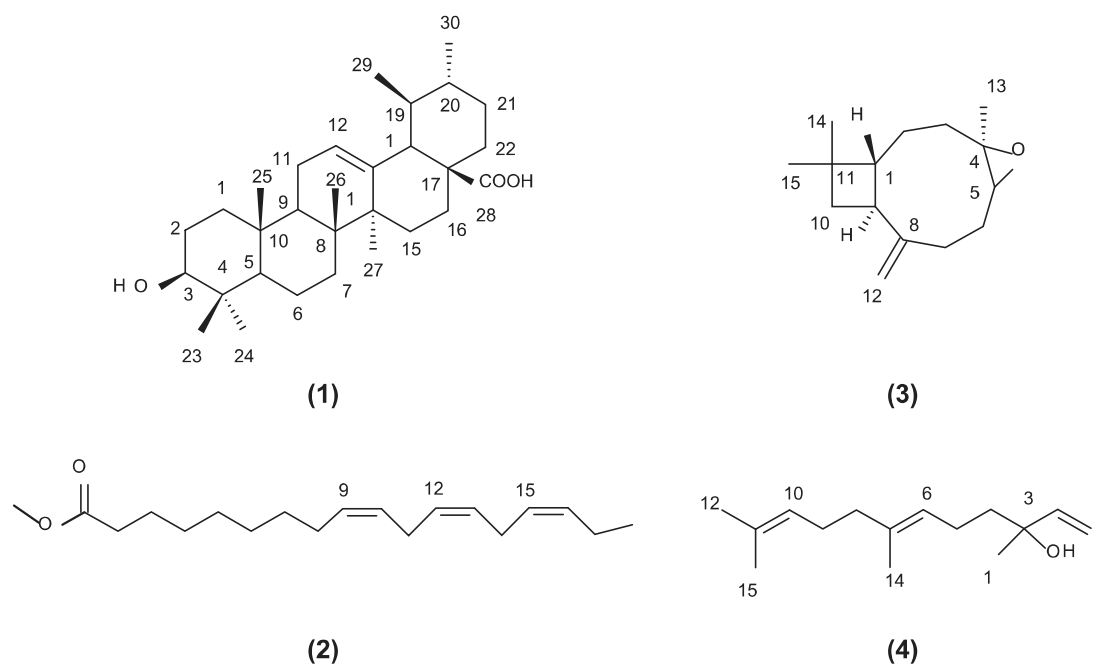
Fig. 1 Structures of the isolated compounds: ursolic acid (**1**), methyl linolenate (**2**), caryophyllene oxide (**3**) and trans-nerolidol (**4**).

Table 1 Minimal inhibitory concentrations (μg /ml and μM) of isolated compounds and Hexanic fraction of aerial extracts of *Baccharis dracunculifolia* D. C. against four isolates of *Paracoccidioides brasiliensis*.

	Pb18*		Pb01	
Sample	RPMI	YPD	RPMI	YPD
Ursolic acid	>250.00 (>548.30)†	>250.00 (>548.30)	>250.00 (>548.30)	>250.00 (>548.30)
Caryophyllene oxide	250.00 (1136.40)	250.00 (1136.40)	250.00 (1136.40)	>250.00 (>1136.40)
Trans-nerolidol	125.00 (563.10)	125.00 (563.10)	125.00 (563.10)	250.00 (1126.10)
Methyl linolenato	15.60 (53.50)	15.60 (53.50)	15.60 (53.50)	31.20 (107.00)
Hexane fraction	15.60	15.60	31.20	15.60
Amphotericin B	0.06 (64.90)	0.03 (32.50)	0.12 (129.90)	0.03 (32.50)
SMT/TMP	300.00 (987.10/172.20)	300.00 (987.10/172.20)	300.00 (987.10/172.20)	300.00 (987.1/172.20)

*Pb18, Pb03 and PbB339: *Paracoccidioides brasiliensis*; Pb01: *P. lutzii*; RPMI, Roswell Park Memorial Institute medium 1640; YPD: Yeast Extract Peptone Dextrose. AmB: Amphotericin B. SMT/TMP: trimethoprim/sulfamethoxazole; †MIC results expressed in μM are showed in parentheses.

control well onto YPD plates. The plates were incubated at 37°C for 10 days. The lowest drug concentration at which no colonies were observed was taken as the MFC value [11].

Ergosterol effect

The test for ergosterol effects was performed according to Escalante *et al.* [12]. The MICs of the compounds that were active against *P. brasiliensis* (isolate Pb18) were determined as described above in the presence or absence of 500 μg/ml of ergosterol (Sigma Chemical Co., St Louis, MO, USA) in the assay media (RPMI 1640) with different lines on the same microtitre plate. Amphotericin B was used as the control drug. The MIC was determined after 10 days of incubation. This experiment was run at least three times, and the MICs in the presence and the absence of ergosterol were determined simultaneously.

Scanning electron microscopy

P. brasiliensis yeast cells were cultivated at 36°C for 8 days in YPD medium with or without (control) the isolated compounds or the Hex fraction (the subinhibitory concentration). To prepare samples for scanning electron microscopic analysis, treated *P. brasiliensis* yeast cells were fixed overnight at 4°C (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3) and harvested by centrifugation (1500 × g/10 min). The pellet was washed three times in 0.1 M sodium cacodylate buffer (1500 × g/10 min). The samples were postfixed for 1 h (1% osmium tetroxide and 0.8% potassium ferrocyanide in the same buffer) and then applied on a polylysine-coated coverslip and serially dehydrated in ethanol. The samples were dried in a critical point drier, coated with gold/palladium and viewed using a DSM 950 (Zeiss, West Germany) electron microscope. The microscopic data that are shown in the present study were obtained using microscopes and equipment at the Center for Electron Microscopy (CEMEL- ICB/Universidade Federal de Minas Gerais).

Cytotoxic activity

The effects of the compounds on the survival and growth of the human cancer cell lines UACC-62 (melanoma), MCF-7 (breast) and TK-10 (renal) were determined using a colorimetric method that was developed at the National Cancer Institute (USA) [13]. Briefly, the cells were inoculated in 96-well plates and incubated at 37°C for 24 h in a 5% CO_2 atmosphere. The solutions of the test samples were added to the culture wells to attain the desired concentrations, and the plates were incubated for an additional 48 h. Trichloroacetic acid was added to each well to precipitate the proteins, which were stained with sulphorhodamine B. After the unbound dye was washed out, the stained protein was dissolved with 10 mM Tris, and the absorbance was measured at a wavelength of 515 nm. Using the absorbance measured in the test wells (T), the absorbance in the control wells corresponding to the initial cell inoculum (Ti) and the cells grown for 48 h without any drug (Tf), the results were calculated with the formula [(T-Ti)/(Tf-Ti)] × 100. This formula allows the quantification of both growth inhibition (values between zero and 100) and cell death (values smaller than zero). Experiments to determine the dose response curves and the EC_{50} (effective concentration to inhibit growth in 50% of the cells) were run as described above using 1:2 serial dilutions of the test compounds to reach the appropriate concentrations. The experiments were run in duplicate and repeated at least once.

Assay with amastigote-like forms of Leishmania (Leishmania) amazonensis

Promastigotes of *L. amazonensis* (strain IFLA/BR/196/PH-8) were obtained from lesions of experimentally infected hamsters. The parasites were incubated for 9 days at 26°C in Schneider's medium (pH 7.2). The promastigotes were stimulated to differentiate into amastigote-like forms by raising the incubation temperature to 32°C and lowering the pH of the medium to 6.0. After 7 days under

Table 1   (*Continued*).

Pb03		PbB339	
RPMI	YPD	RPMI	YPD
>250.00 (>548.30)	>250.00 (>548.30)	>250.00 (>548.30)	>250.00 (>548.30)
>250.00 (>1136.40)	125.00 (568.20)	250.00 (1136.40)	>250.00 (>1136.40)
125.00 (563.10)	125.00 (563.10)	15.60 (70.40)	15.60 (70.40)
62.50 (214.00)	31.20 (107.00)	3.90 (13.40)	15.60 (53.50)
31.20	15.60	3.90	15.60
0.01 (10.80)	0.03 (32.50)	0.06 (64.90)	0.03 (32.5)
300.00 (987.10/172.20)	300.00 (987.10/172.20)	75.00 (246.80/43.10)	300.00 (987.10/172.20)

these conditions, 90% of the parasites in the amastigote-like form were obtained. The parasite concentration was adjusted to 1.6×10^8 cells/ml, and 90 ml was added to each well of the 96-well plates. We also added 10 ml of the solution containing the samples and the control drug (0.2 mg/ml Amphotericin B, Fungisone, Bristol-Myers Squibb, Brazil) to each well. The plates were incubated at 32°C for 72 h, and the number of parasites was estimated using a methyl thiazolyl tetrazolium-based (MTT, Amresco, Solon, USA) colorimetric assay [14]. The results were calculated from the measured absorbance using the formula [$1-(Abs_{exp}/Abs_{contr}) \times 100$], which expresses the percentage of para-site death compared with the control without drug. All of the samples were tested in duplicate, and the experiments were repeated at least once. Experiments to determine the dose response curves and the EC_{50} (effective concentration to kill 50% of the parasites) were performed as described above using 1:2 serial dilutions of the test compounds to reach the appropriate concentrations. The experiments were performed in duplicate and repeated at least once.

Results

Antifungal, cytotoxic and leishmanicidal assays were used to guide the fractionation of the hexanic portion of the aerial parts of *B. Dracunculifolia*. We isolated the following four compounds (Fig. 1): ursolic acid (**1**), methyl lino-lenate (**2**), caryophyllene oxide (**3**) and trans-nerolidol (**4**). The structures of the isolated substances were determined using NMR spectra, GC-MS analyses and analytical TLC with standards of and comparisons to previously published data [15–18].

The four compounds were tested in antifungal assays against three strains of *P. brasiliensis* (strains Pb18, Pb03 and PbB339) and one strain of '*P. lutzii*' (strain Pb01) using RPMI 1640 and YPD media (Table 1). When the compounds were tested, the different MIC values were obtained in RPMI and YPD media. The MIC values were similar among ursolic acid, caryophyllene oxide and SMT/TMP when tested with isolates Pb18, Pb01 and Pb03. The highest MIC values for trans-nerolidol, methyl linolenate, the hexanic fraction and amphotericin B were different. In

addition, microscopic analysis of the growth in the MIC assay using the YPD medium revealed the presence of yeast cells with typical multilateral budding. However, in the RPMI medium we observed that the fungus appeared in the mycelial form at 37°C. The hexanic fraction and methyl linolenate were the most active against the isolates of *Paracoccidioides*, with the MIC values ranging from 3.9–31.2 µg/ml and 3.9–62.5 µg/ml, respectively. Caryo-phyllene oxide and trans-nerolidol were moderately active against the fungus, in that the MIC values ranged from 125–250 µg/ml and from 15.6–250 µg/ml, respectively. In general, the PbB339 strain was the most susceptible to all of the compounds. Ursolic acid did not display antifungal activity at the concentrations tested (MIC > 250 µg/ml). Compounds **2**, **3** and **4**, however, showed MFC values that were equal to the MIC values for all of the strains of *Para-coccidioides* (Table 1). The MFC for amphotericin B was also equal to the MIC value. In addition, SMT/TMP showed fungistatic activity against the isolate Pb03.

The four compounds were tested against tumor cell lines and the amastigote forms of *L. amazonensis*. Interest-ingly, only ursolic acid was active against these targets. Ursolic acid exhibited cytotoxic (IC_{50} of 8.2 µg/ml for TK-10, 3.8 µg/ml for MCF-7 and 5.2 µg/ml for UACC-62) and leishmanicidal (EC_{50} 7.1 µg/ml) activities.

To observe the effects of the compounds on ergosterol in *P. brasiliensis* (Pb18), the MIC assay was supplemented with ergosterol. Among the four isolated compounds, dif-ferences were noted only with caryophyllene oxide in the MIC assay with the supplemental ergosterol (the MIC value increased from 125–250 µg/ml) (Table 2). A five-fold increase in the MIC value of the control drug, amphotericin B, was also found in the presence of exogenous ergosterol. In addition, cells of the Pb18 isolate of *P. brasiliensis* were treated with subinhibitory MIC values of the hexanic frac-tion and with three compounds that were active against *P. brasiliensis* to determine if the isolated compounds from the crude hexane fraction of *B. dracunculifolia* interfered with cell morphology. The Pb18 cells were examined by scan-ning electron microscopy (Fig. 2), and we observed that all of the natural products decreased the cell size and promoted irregularities on the cell surface of *P. brasiliensis*.

Table 2 Minimal inhibitory concentrations (µg/ml) of isolated substances and hexane fraction from *Baccharis dracunculifolia* against *Paracoccidioides brasiliensis*, in the presence and absence of ergoesterol in RPMI medium.

Sample	With ergoesterol	Without ergoesterol
Ursolic acid	≥ 250.00	≥ 250.00
Caryophyllene oxide	250.00	125.00
Trans-nerolidol	125.00	125.00
Methyl linolenate	15.60	15.60
Hexane fraction	15.60	15.60
Amphotericin B	2.00	0.06

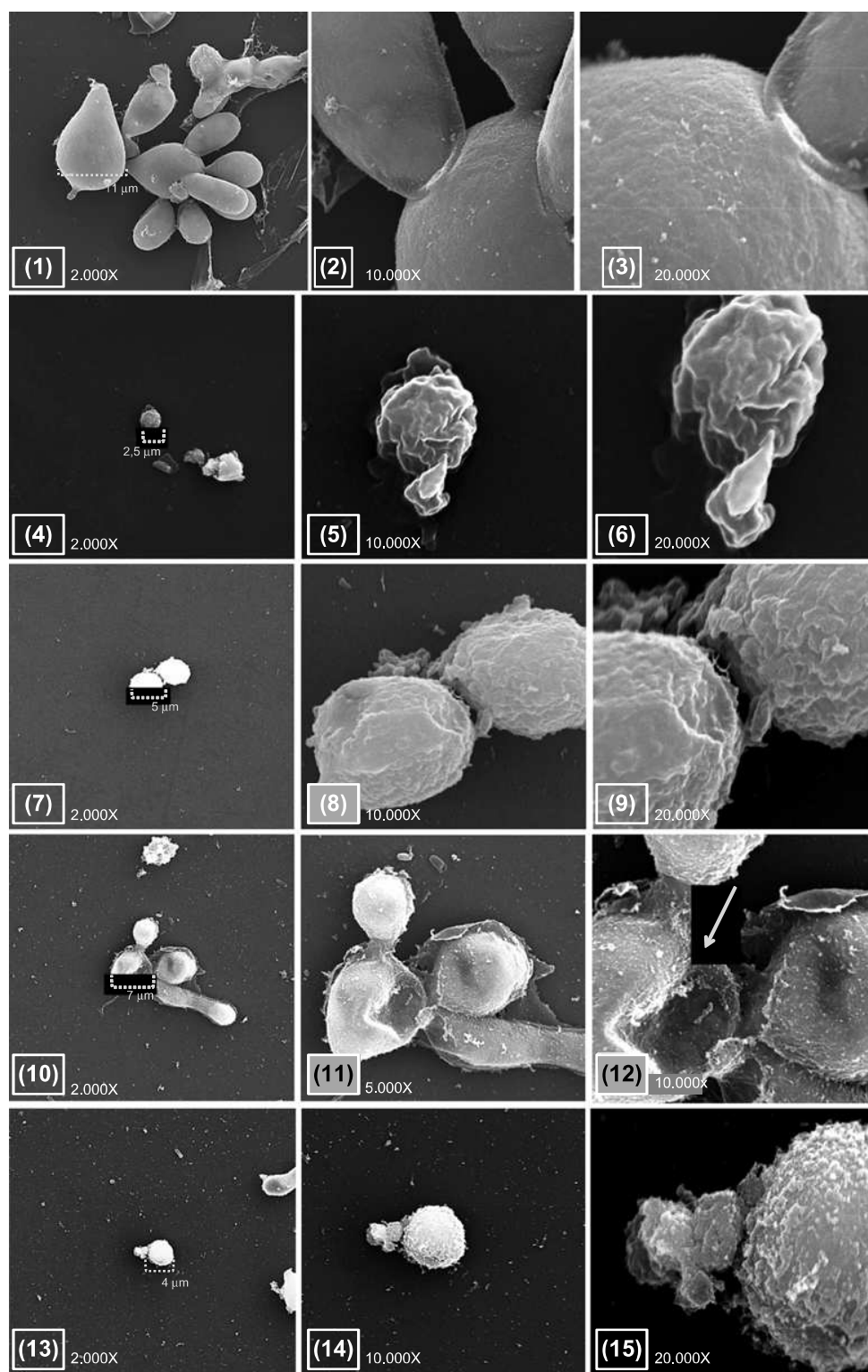**Paracoccidioides brasiliensis*: isolate Pb18.

Discussion

There are only a few studies on the antifungal activity of extracts and compounds from *B. dracunculifolia*. Duarte *et al.* [19] used samples of *B. dracunculifolia* grown in the experimental field of the Research Center for Chemistry, Biology and Agriculture (CPQBA), State University of Campinas, São Paulo, Brazil, and reported that the ethanolic extract and the essential oil of the leaves of this plant did not have antifungal activity against *C. albicans*. In addition, a study by Johann *et al.* [20] tested the hexane extracts of all of the aerial parts of *B. dracunculifolia* from Itajaí, Santa Catarina State, and observed that this extract was not active against *C. albicans*. Taken together, these two studies showed that there were no differences in the activities of the *B. dracunculifolia* extracts collected in two different sites against *C. albicans*. Interestingly, the hexane extract of *B. dracunculifolia* was active against *C. tropicalis* and *C. glabrata* (the MIC values were 250 and 1000 µg/ml, respectively) [20]. Johann *et al.* [20] observed that *Cryptococcus neoformans* was susceptible to concentrations of 30 µg/ml of this hexane extract [20]. In the present study, the *Paracoccidioides* isolates were more susceptible than the *Candida* species, but the results were similar to those of *C. neoformans* [20].

The liquid-liquid extraction with solvents of increasing polarity suggested that nonpolar metabolites of *B. dracunculifolia* are active against tumor cell lines, *L. amazonensis* and *P. brasiliensis*. In the present work, caryophyllene oxide and methyl linolenate were identified in *B. dracunculifolia* for the first time. Silva-Filho *et al.* [21] isolated ursolic acid from *B. dracunculifolia* and demonstrated that it was active against *L. donovani* (IC_{50} 3.7 µg/ml). Ursolic acid appears to be the main cytotoxic substance from the hexane fraction, which corroborated the results of previous studies that have described the apoptotic effects of ursolic acid on human non-small cell lung cancer A549 cells [22,23]. Methyl linolenate has been shown to inhibit the cell division of *Saccharomyces cerevisiae* [24], and the present study demonstrated that it was one of the most

active substances against the four isolates of *P. brasiliensis* that were tested. Indeed, the MIC values were nearly identical to the crude hexanic fraction. Interestingly, previous studies have found trans-nerolidol and/or (E)-nerolidol in *B. dracunculifolia*, *B. latifolia* and *B. salicifolia* [25,26]. In addition, previous studies have shown that (Z)-nerolidol has leishmanicidal activity against the promastigote forms of *L. amazonensis* (IC_{50} of 85 µM), *L. brasiliensis* (IC_{50} of 74 µM) and *L. chagasi* (IC_{50} of 75 µM) [27] and antiparasitic activities against *Plasmodium falciparum* (100 µg/ml, 100% growth inhibition) [28], *Babesia bovis* (IC_{50} of 4.7 µg/ml), *B. bigemina* (IC_{50} of 0.7 µg/ml), *B. ovata* (IC_{50} of 0.45 µg/ml) and *B. caballi* (IC_{50} of 5.2 µg/ml) [29].

Caryophyllene oxide, which has also been isolated from petroleum ether extracts of the bark of *Annona squamosa*, has been shown to have central and peripheral analgesic and anti-inflammatory activity in Swiss albino mice [30]. Park *et al.* [31] investigated the effects of β-caryophyllene oxide on the PI3K/AKT/mTOR/S6K1 and MAPK activation pathways in human prostate and breast cancer cells (signalling cascades play an important role in cell proliferation, survival, angiogenesis and metastasis of tumor cells) and suggested that β-caryophyllene can interfere with multiple tumorigenesis signalling cascades. Indeed, β-caryophyllene is a potential therapeutic candidate for both the prevention and the treatment of cancer. In the present experiments, caryophyllene oxide demonstrated weak activity (MIC values > 125 µg/ml) against the *Paracoccidioides* strains and no activity against *L. amazonensis* or the cancer cell lines that were tested. Interestingly, caryophyllene oxide was the only isolated substance that had its MIC value increased in the presence of exogenous ergosterol. According to Escalante *et al.* [12], if binding to ergosterol was responsible for a compound's activity, then the exogenous ergosterol would prevent the fungal ergosterol from binding to the membrane. Thus, the MIC values are enhanced for caryophyllene oxide in the presence of exogenous ergosterol. In the present study, the control drug, amphotericin B, also showed increased MIC levels in the presence of exogenous ergosterol. The interaction of amphotericin B with ergosterol has been demonstrated in several studies [12,32]. Caryophyllene oxide could be binding to the ergosterol, but additional assays are necessary to evaluate if caryophyllene oxide is binding ergosterols in the cellular membrane of *P. brasiliensis*.

Derengowski *et al.* [33] showed that the cell walls of *P. brasiliensis* (Pb18) treated with farnesol remained intact when observed by scanning electron microscopy. In the present study, however, all of the compounds that we tested induced changes to the surface of the cells of *P. brasiliensis* and decreased the cell size. The Pb18 cells that were treated with caryophyllene oxide were wrinkled compared

Fig. 2   The cell morphology of *Paracoccidioides brasiliensis* (Pb18) treated with methyl linolenate, caryophyllene oxide trans-nerolidol or the hexanic fraction of *Baccharis dracunculifolia* by scanning electron microscopy. (1–3) Pb18 without any of the isolated compounds (negative control); (4–6) Pb18 treated with caryophyllene oxide; (7–9) Pb18 treated with trans-nerolidol; (10–12) Pb18 treated with methyl linolenate; (13–15) Pb18 treated with the Hex fraction. The arrow in #9 shows the depression that occurred in the *P. brasiliensis* cells that were treated with trans-nerolidol.

with the untreated cells, which had intact cell walls. The *P. brasiliensis* cells that were treated with methyl linolenate exhibited a drastic change in cell morphology, and the *P. brasiliensis* cells treated with trans-nerolidol exhibited cell surface squashing and depression. In addition, the cells treated with trans-nerolidol showed desquamation of the cell surface. In a study of oenothein B from *Eugenia uniflora* using scanning electron microscopy, the authors observed that the *P. brasiliensis* Pb01 ('*P. lutzii*') cells that were treated with oenothein B were squashed and depressed and cellular contents had leaked out of the cells [34].

In the present study, we observed that all of the compounds that were isolated from the crude hexanic fraction of *B. dracunculifolia* demonstrated biological activities. Three of the isolated compounds (i.e., methyl linolenate, caryophyllene oxide and trans-nerolidol) exhibited antifungal activity against four clinical isolates of *P. brasiliensis*. Methyl linolenate exhibited the best antifungal activity and resulted in significant changes in the cell morphology of *P. brasiliensis*. In addition, the mode of action of caryophyllene oxide was suggested to be through binding to ergosterol.

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