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ORIGINAL PAPER

Antifungal activity of 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone against *Candida albicans*: evidence for the antifungal mode of action

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Abstract The main secondary metabolite of *Senecio nutans* is 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone (4HMBA). The antifungal activity of this compound and three derivatives was assessed using *Candida albicans*. 4HMBA exhibited the highest antifungal activity among the assayed compounds. The Fractional Inhibitory Concentration (FIC = 0.133) indicated a synergistic fungicidal effect of 4HMBA (5 mg L^{-1}) and fluconazole (FLU) (0.5 mg L^{-1}) against the *C. albicans* reference strain (ATCC 10231). Microscopy showed that 4HMBA inhibits filamentation and reduces cell wall thickness. Our findings suggest that 4HMBA is an interesting compound to diminish resistance to commercial fungistatic drugs such as fluconazole.

Keywords 4-Hydroxy-3-(3-methyl-2-butenyl)acetophenone · Antifungal activity · *Candida albicans* · Filamentation · Fungal cell wall · Synergistic effect

Introduction

Candida albicans is opportunistic fungal pathogen (Fiori and Van Dijck 2012), and a common cause of invasive fungal infections in humans, producing infections that can involve any organ. There are four main antifungal drugs used in medicine against

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C. albicans: polyenes, pyrimidine analogues, azoles and echinocandins. The most used drug for candidiasis treatment is fluconazole (FLU), especially in developing countries (Flynn et al. 2009). FLU is an azole drug with fungistatic action, an aspect that favours the development of drug resistance (Sanglard et al. 2003). To overcome drug resistance in the antimicrobial therapy, a combination of drugs is suggested to be used. Drug combinations are described as synergistic, antagonistic or indifferent (i.e., no interaction) (Odds 2003). An advantage of using combinations is the synergistic effect, in which antifungal activity is greater than the individual contribution of each agent (Endo et al. 2010). In this sense, the conversion of FLU from fungistatic to fungicidal via combinations with other drugs is desirable to increase efficacy and reduce resistance development (Fiori and Van Dijck 2012).

Higher plants are interesting sources of antimicrobial agents (Soberón et al. 2007). *Senecio nutans* Sch. Bip (Asteraceae) is an aromatic plant widely used in Argentinean northwestern traditional medicine to treat respiratory disorders, cicatrizant and fungal infections (Barboza et al. 2009). The main secondary metabolite of its aerial parts is a p-hydroxyacetophenone derivative identified as 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone (4HMBA) (Loyola et al. 1985). There is only one preliminary report on 4HMBA antifungal activity (Tomás-Barberán et al. 1990), but this report did not evaluate the activity against *C. albicans* strains, instead it used *Cladosporium herbarum, Phytophthora capsici, Neurospora crassa, Penicillium spp.* and *Aspergillus spp.,* and a culture medium that is not currently recommended by CLSI. Also no study on the mode of action was performed. This article describes the antifungal activity of 4HMBA and three semi-synthetic derivatives against *C. albicans*, alone and combined with FLU. Caspofungin (CAS) was also included in combinatory assays. Antifungal activity assays included mutant strains to assess the involvement of calcineurin and a zinc finger transcriptional factor in the antifungal action. Iron-chelating activity was also investigated to evaluate a possible iron homeostasis perturbation. Time-lapse microscopic observations of germtube formation and hyphae development, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to study the morphology of *C. albicans* after 4HMBA treatment.

Materials and methods

Chemicals

Methylene blue was from Cicarelli Labs (Santa Fe, Argentina). Analytical and HPLC grade solvents were from Sintorgan Labs (Buenos Aires, Argentina). Bathophenanthroline disulfonic acid (BPS) and FLU were from Sigma-Aldrich (MO, USA). Fe_2SO_4 was from Merck (Darmstadt, Germany). Yeast extract-peptone dextrose (YEPD), Sabouraud dextrose (SD) medium, yeast extract, proteose-peptone No. 3 and agar were from Britania Labs (Buenos Aires, Argentina). CAS (Cancidas^R, Caspofungin acetate) was from Merck Sharp & Dohme (NJ, USA). RPMI 1640 medium was from Microvet Labs (Buenos Aires, Argentina). CHROMagar® Candida was from CHROMagar Microbiology, France.

Strains and media

A total of six *C. albicans* strains were employed. One reference strain was from *American Type Culture Collection* (ATCC 10231) (Tobudic et al. 2010). Four strains were from Department of Molecular Genetics and Microbiology at Duke University Medical Center (NC, USA), referred to as Day 364 (cnb1/cnb1—mutant strain lacking calcineurin B regulatory subunit—FLU hypersensitive), MCC 85 (cnb1/cnb1 + CNB1—mutant strain with a constitutive calcineurin B. Day 364 complementary strain), OCC 1.1 (crz1/crz1—mutant strain lacking Crz1 zinc finger transcription factor controlled by calcineurin—FLU hypersensitive) and OCC 7 (crz1/crz1 + CRZ1—mutant strain with a constitutive Crz1—FLU sensitive. OCC1.1 complementary strain) (Uppuluri et al. 2008). The *C. albicans* 12-99 clinical isolate strain highly resistant to FLU (overexpressing *ERG11*, *CDR1*, *CDR2* and *MDR1*) (White et al. 2002) was also employed. Strains were routinely refreshed from permanent stocks (CLSI 2008). The purity and viability of cultures were regularly evaluated by plating yeast colonies on CHROMagar® Candida. CAS was dissolved in ultra pure type 1-water, while FLU, 4HMBA and semi-synthetic derivatives were dissolved in DMSO.

Isolation of 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone (4HMBA)

4HMBA was obtained from aerial parts of *S. nutans*, as reported previously (Lizarraga et al. 2012). Melting point (Mp), UV–Vis, MS, ^{1}H-NMR spectra were measured (Lizarraga et al. 2012). Detailed semi-synthesis of 6-acetyl-2,2-dimethyl-chromane (CHR), 4-acetyloxy-3-(3-methyl-2-butenyl)acetophenone (AcA) and 4-acetyloxy-3-(2,3-epoxy-3-methylbutyl)acetophenone (EPX) could be found as Supplementary Information. The purities and identities of 4HMBA, CHR, AcA and EPX were confirmed by typical ion fragments attained by GC–MS and ^{1}H NMR spectrum. Their chemical structures and spectroscopic data could be found as Supplementary Information.

GC–MS measurements

The GC–MS analysis of 4HMBA, CHR, AcA and EPX was carried out with a 5973 Hewlett Packard selective mass detector (quadrupole), source 70 eV, coupled to a HP 6890 GC fitted with a HP-5MS column (5 % phenylmethyl siloxane, 30 m × 0.25 mm) with helium as carrier gas (1.0 mL min^{-1}; constant flow). The oven was programmed as follows: 150 °C (0 min), 150–180 °C (3 °C min^{-1}), 180 °C (2 min), 180–234 °C (1.5 °C min^{-1}), 234 °C (2 min). Injection: 0.1 µL of a 10 % solution of the compound dissolved in methylene chloride. Injector and detector temperatures were maintained at 250 °C and 270 °C, respectively. Injection port was maintained at 250 °C, GC–MS interphase at 275 °C, ion source 230 °C, and MS Quad at 150 °C.

NMR spectra

^{1}H NMR was recorded on a Bruker AC (200 MHz) spectrometer. The sample was dissolved in $CDCl_3$ and tetramethyl silane (TMS) was used as internal standard. Chemical shifts were recorded in δ (ppm) values relative to TMS and J values are expressed in Hertz.

Disk diffusion assay

The assay was performed with *C. albicans* ATCC 10231 (FLU sensitive strain) and *C. albicans* 12-99 (FLU resistant strain). Yeast cells were obtained from a 24 h culture on SD agar (e.g., exponential growing cells). The final inoculum was adjusted to 10^3 CFU mL^{-1} in sterile distilled water and then spread uniformly onto YEPD agar plates, with and without FLU (6 mg L^{-1}) (Endo et al. 2010). 5 mm paper disks impregnated with 100 µg of pure compounds were placed onto agar plates. A paper disk impregnated with DMSO (100 µg) was included as solvent control. Diameters (in mm) of growth inhibition zones were measured after incubation at 35 °C for 24 h. Experiments were repeated at least three times.

Broth microdilution and cell viability assays

The experiments were performed in sterile multiwell microdilution plates according to CLSI reference procedure (CLSI 2008) using RPMI 1640 medium. For the ATCC 10231 and mutant strains, FLU concentrations ranged from 0.03 to 32 mg L^{-1}, while 4HMBA, CHR, AcA and EPX concentrations were from 1.25 to 160 mg L^{-1}. For the 12-99 strain, FLU concentrations ranged from 0.125 to 128 mg L^{-1} while 4HMBA, CHR, AcA and EPX concentrations were from 2.50 to 160 mg L^{-1}. CAS concentrations ranged from 0.007 to 8 mg L^{-1}. The final inoculum was obtained as previously described. Plates were incubated at 35 °C and read after 48 h for all strains except for 12-99 (24 h). Minimum inhibitory concentration (MIC) was the minimum concentration of FLU which produced 50 % decrease in optical density (MIC-2) or 100 % decrease in optical density for 4HMBA, CHR, AcA and EPX (MIC-0 or optically clear wells). Optical densities were recorded at 550 nm using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, CA, USA). To determine cell viability, 25 µL aliquots were taken from each well, serially diluted, plated on SD agar plates and incubated at 35 °C for 48 h. Experiments were repeated at least three times.

Checkerboard assays

The experiments were performed with all *C. albicans* strains by broth microdilution assays as described elsewhere (Endo et al. 2010). Viable count determinations were performed as described above. The interactions between FLU and compounds under assay were assessed through Fractional Inhibitory Concentration (FIC) (Chaturvedi et al. 2008). The interpretation of FIC was: $\leq$0.5, synergistic effect; >0.5

to <4.0, no interaction or indifference; ≥4.0, antagonistic effect (Odds 2003). The MIC-0_{FLU} and MIC-0_{CAS} were obtained for the FIC calculations, employing a criterion reported by other authors (Pfaller et al. 2004; Fiori and Van Dijck 2012). Experiments were repeated three times.

Qualitative growth test (QGT)

QGTs were performed after checkerboard assays to qualitatively determine whether *C. albicans* cells exposed to FLU and compounds under evaluation (alone or in combinations) were still alive (Xu et al. 2014). Five microlitres taken from each checkerboard assay well were punctually placed on the SD agar surface at different places and incubated for 48 h at 35 °C. Yeast growth was determined by the presence of a punctual growth zone. *C. albicans* ATCC 10231 was the strain used. Experiments were repeated at least three times.

Fungicidal activity assays

Fungicidal activity was assessed through viable count determinations after checkerboard assay performed with 10^4 CFU mL^{-1} initial inocula (Cantón et al. 2003). This inocula allowed to determine the 3 log units decrease in viable cells (e.g., >99.9 % killing) above the limit of detection (i.e., 10 CFU mL^{-1}). The minimal fungicidal concentration (MFC) was the minimal concentration of a compound under assay which caused >99.9 % growth inhibition. *C. albicans* ATCC 10231 was the strain used. Plates were incubated for 48 h at 35 °C. Experiments were repeated at least three times.

Iron chelation assay

Iron chelation was monitored through the prevention of formation of the $Fe_{II}(BPS)_3$ complex using a colorimetric assay (Maioli et al. 2010). Briefly, samples at concentrations of 25, 50, 100, 200, and 400 mg L^{-1} were dissolved in 10 mM-HEPES/NaOH buffer, pH 7.0 added with 50 µM Fe_2SO_4 and 200 µM BPS into flat-bottom 96-well plates and absorbance at 530 nm was measured after 30 min. Decrease in absorbance is inversely proportional to iron chelation activity. Chelating activity percent (CA%) was defined as the sample concentration which cause 50 % iron chelation. EDTA was used as chelating reference substance and DMSO was employed in the negative controls.

Time-lapse microscopy experiment

C. albicans ATCC 10231 was used to observe the development of germ tubes and mycelia, as described elsewhere (Barelle et al. 2003). Briefly, a glass slide was placed in a petri dish (90 mm diameter). SD agar added with 4HMBA at 10 mg L^{-1} was poured to cover the dish and the slide, and then inoculated as previously described. A coverslip was placed over the inoculated medium to generate a thin film between the glass slide and the coverslip. The marginal agar (i.e., outside the coverslip) was removed, and the coverslip edge was sealed with a lanolin, vaseline, and paraffin (1:1:1) mixture. The slide was placed over the microscope heated stage for time-lapse observations. The germ tubes and mycelia development were observed for 12 h at 35 °C.

Transmission electron microscopy (TEM)

C. albicans ATCC 10231 cells (final inoculum adjusted to 10^3 CFU mL^{-1}) were exposed to 4HMBA at 10 mg L^{-1} (sub-MIC concentration) in glass tubes containing RPMI 1640, and incubated for 48 h at 35 °C. Recovered pellets were fixed with 3 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 2 h. Postfixation was carried out in 1 % OsO_4 in 0.1 M phosphate buffer, pH 7.4 for 30 min at room temperature. Thereafter, cells were dehydrated in 30, 50, 70, 80, 85, 90 and 100 % ethanol and then embedded in SPURR (Sigma-Aldrich, MO, USA). Uranyl acetate and lead citrate (Merck, Darmstadt, Germany) were used to stain the ultrathin sections, and then observed in a Zeiss EM109 transmission electron microscope. Cell wall thickness of control and treated yeasts was quantitatively measured. Measurements of cell walls were obtained from 100 cells (n = 100) of treated and control cells.

Scanning electron microscopy (SEM)

Small drops of the fixed cells were placed on a specimen support with poly-L-lysine for 1 h at room temperature. Samples were dehydrated in graded ethanol, dried in CO_2 (critical point), coated with

gold and examined in a Jeol JSM 35 CF scanning electron microscope.

Statistic analysis

Data were analyzed by either one-way ANOVA or Student's t test. Probability levels below 0.05 ($p < 0.05$) were considered as statistically significant.

Results

Antifungal activity assays

The antifungal activities of compounds under investigation were preliminary evaluated through disk diffusion assay with and without FLU. 4HMBA showed antifungal activity against both *C. albicans* strains assayed. There were significant differences ($p < 0.05$) between the growth inhibition zones diameters for 4HMBA against *C. albicans* 10231 with and without FLU, 13.0 ± 0.2 mm and 8.0 ± 0.1 mm respectively (Fig. 1a, b), while there were no significant differences ($p > 0.05$) between the growth inhibition zones diameters for 4HMBA against *C. albicans* 12-99 with and without FLU, 7.1 ± 0.1 and 7.2 ± 0.2 mm respectively (Fig. 2c, d). These results preliminary suggested that 4HMBA antifungal activity against FLU sensitive strains could be increased by FLU presence, but not against FLU resistant strains. No growth inhibition zones were observed with CHR, AcA and EPX against the assayed strains (Fig. S2 in Supplementary Information).

Table 1 summarizes the MICs for *C. albicans* strains. Qualitative growth tests (QGTs) showed that *C. albicans* ATCC 10231 strains exposed to FLU (alone) at all assayed concentrations were viable after incubation (bottom row wells in Fig. 2a), which was in agreement with *C. albicans* FLU tolerance and FLU fungistatic action (i.e., in the presence of the MIC the growth is inhibited but the cells are not killed—Sanglard et al. 2003). Cells exposed to FLU + 4HMBA combinations showed lower diameters or lower density growth zones than those cells exposed to FLU (alone) or cells from positive experiments (Fig. 2a), which would indicate a decrease in cell viability for those cells exposed to FLU + 4HMBA. Cells treated with compounds and subsequently washed showed reduced growth, from 6.75 log units (untreated cells in control experiments) to 3.45 for cells exposed to 4HMBA (5 mg L^{-1}) + FLU (0.5 mg L^{-1}), i.e., >3 log units decrease (line with squares in Fig. 2b). Given the cells do not grow anymore within the time of the experiments, these results may indicate that 4HMBA (5 mg L^{-1}) + FLU (0.5 mg L^{-1}) combination cause the FLU shift from fungistatic to fungicidal, i.e., the loss of *C. albicans* FLU tolerance. This 3 log units decrease also allowed the use of MIC-0 criterion, as described elsewhere (Fiori and Van Dijck 2012), which yielded the FIC = 0.133 (FIC = MIC-$0_{\text{FLU in combination}}$/MIC-$0_{\text{FLU alone}}$ + MIC-$0_{\text{4HMBA in combination}}$/MIC-$0_{\text{4HMBA alone}}$), indicative of a synergistic combination between FLU and 4HMBA.

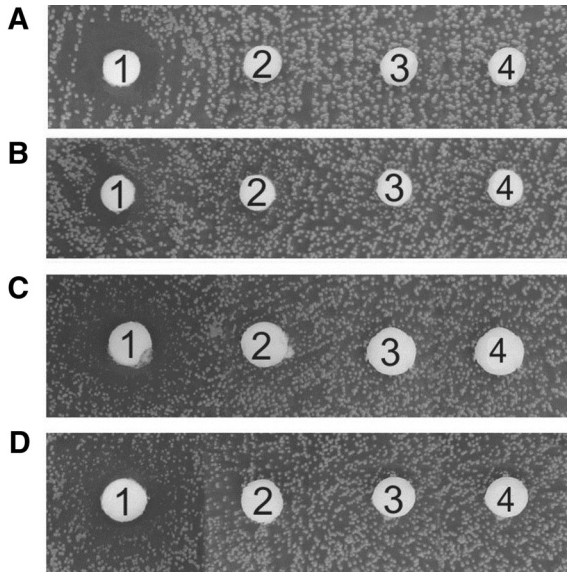

Fig. 1 Antifungal activity of 4HMBA against *C. albicans* strains by disk diffusion assay. *C. albicans* ATCC 10231 is a FLU sensitive strain, while *C. albicans* 12-99 is a FLU resistant strain. **a** *C. albicans* ATCC 10231 + FLU (6 mg L^{-1}). **b** *C. albicans* ATCC 10231 (without FLU). **c** *C. albicans* 12-99 + FLU (6 mg L^{-1}). **d** *C. albicans* 12-99 (without FLU)

QGT results showed in Fig. 2a proved that cells exposed to 4HMBA at 80 mg L^{-1} did not grow (top row wells in Fig. 2a), which suggested a fungicidal activity for 4HMBA against this strain. This observation was quantitatively confirmed through fungicidal activity assay (i.e., using 10^4 CFU mL^{-1} inocula), where no viable cells were recovered from wells exposed to 80 mg L^{-1} of 4HMBA (data not shown), thus the MFC for 4HMBA was 80 mg L^{-1}. As FLU possesses only fungistatic action, the MFC could not

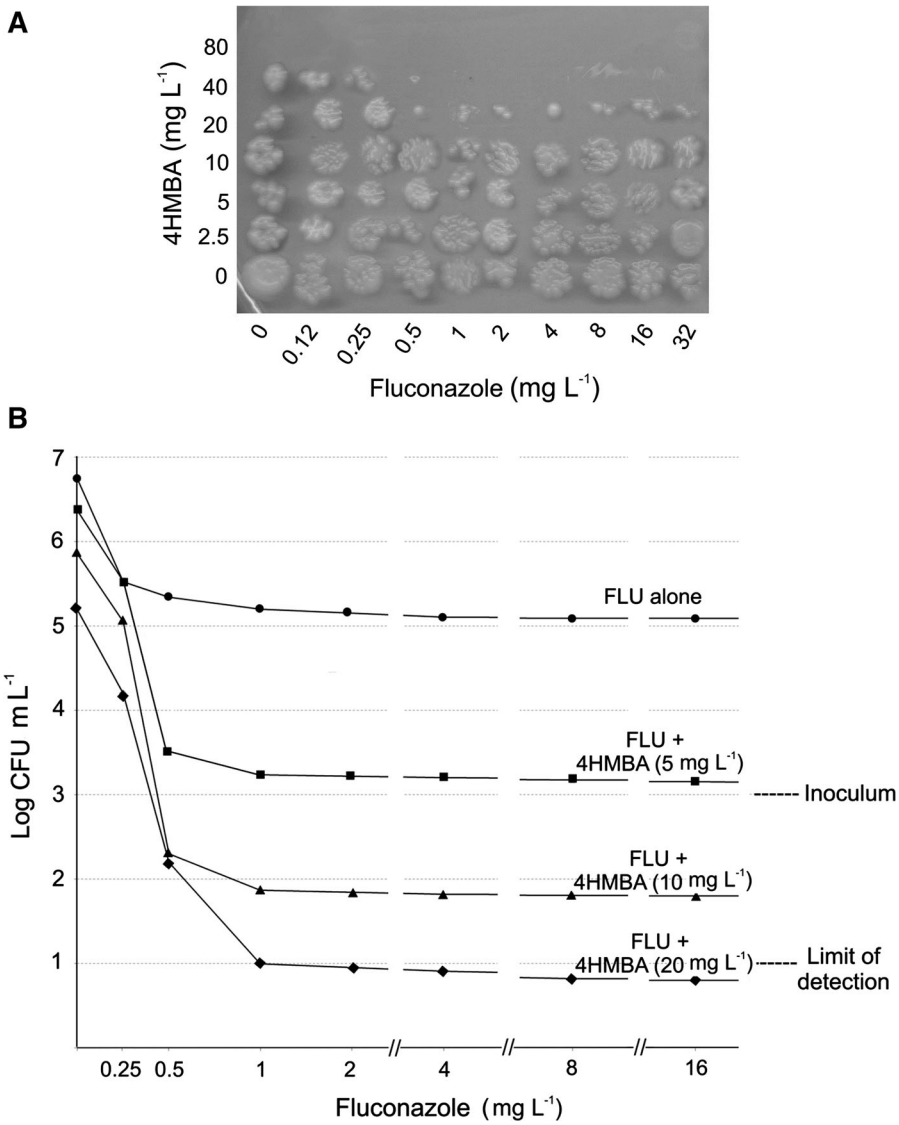

Fig. 2 **a** Qualitative growth test. **b** Viable cell count assays with 10^3 CFU/mL initial inoculum. *Circles* FLU alone; *squares* FLU + 5 mg L^{-1} 4HMBA; *triangles* FLU + 10 mg L^{-1} 4HMBA; *diamonds* FLU + 20 mg L^{-1} 4HMBA

Table 1 MIC values for *C. albicans* ATCC 10231 and 12-99

Strain	MIC-2 (mg L^{-1})		MIC-0 (mg L^{-1})		
	FLU	CAS	4HMBA	FLU	CAS
ATCC 10231	1	0.03	40	64[a]	0.25
12-99	>128	0.06	80	256[a]	0.25

MIC-2 and MIC-0: minimum concentration of compound which produced 50 % (MIC-2) or 100 % (MIC-0) decrease in optical density

[a] The $MIC\text{-}0_{FLU}$ is used for the FIC calculations given the 3 log units decrease of viable cells obtained though viability assays (criterion employed by Fiori and Van Dijck 2012)

be obtained. Fungicidal activity assay followed by viable cell count showed no cell growth for those experiments containing FLU (≥1 mg L^{-1}) + HMBA (40 mg L^{-1}) (Fig. 3), suggestive of >99.9 % viability lost of initial inoculum. These data confirm the fungicidal activity suggested for FLU + 4HMBA combination against *C. albicans* ATCC 10231.

The FLU + 4HMBA combination was also tested against *C. albicans* 12-99. The FIC = 0.625 indicated an absence of interaction between these substances against this strain. The detailed data for this experiment can be found as Supplementary Information

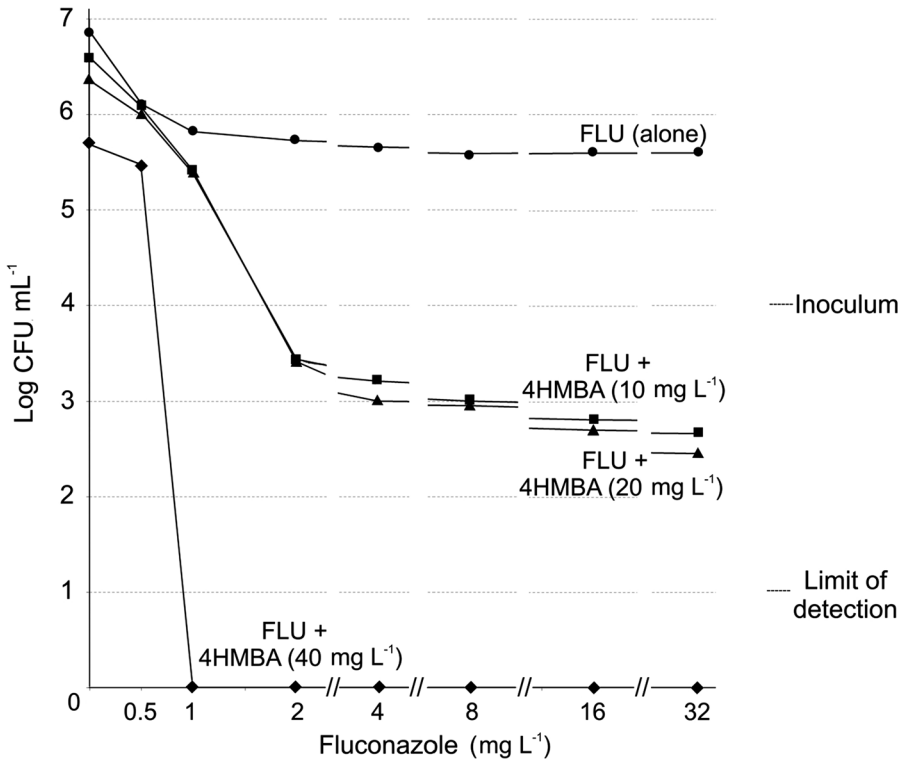

Fig. 3 Fungicidal activity assays. *Circles* FLU alone; *squares* FLU + 10 mg L^{-1} 4HMBA; *triangles* FLU + 20 mg L^{-1} 4HMBA; *diamonds* FLU + 40 mg L^{-1} 4HMBA

(Table S1). These results suggest that overexpression of *ERG11*, *CDR1*, *CDR2* and *MDR1* genes, as occurs in the 12-99 strain (White et al. 2002) affect not only FLU inhibitory activity, but also 4HMBA and 4HMBA + FLU combinatory effects.

CAS is a broad spectrum fungicidal drug which restrain cell wall synthesis by inhibiting β-glucan synthetase. CAS was included in checkerboard assays to study the effect of 4HMBA on fungal cell wall synthesis. Whether 4HMBA inhibits fungal cell wall synthesis, than an indifference CAS + 4HMBA association would be expected, given that antimicrobial compounds which act with the same mode of action have an indifference effect (i.e., non synergistic effect) on target cell (Endo et al. 2010). *C. albicans* ATCC 10231 and 12-99 were exposed to CAS + 4HMBA combinations through checkerboard assay. MIC-0 was employed as endpoint (Pfaller et al. 2004). The FIC = 0.490 for *C. albicans* ATCC 10231 was indicative of a synergistic combination, while FIC = 0.980 against *C. albicans* 12-99 was indicative of an indifference association (i.e., no interaction). The MIC values for combinatory experiments can be found as Supplementary Information (Table S1). Some FLU + drug combinations may potentiate the activity of azoles, acting in synergism with FLU, yielding a fungicidal combination (Sanglard et al. 2003). These combinations could act through inhibition of calcineurin (Reedy et al. 2010), a key enzyme in the signaling pathway leading to azole tolerance, and responsible for the FLU resistance showed by biofilms (Uppuluri et al. 2008). Under normal conditions this enzyme possesses low basal activity, which is increased when calcium signaling is activated in response of certain external signals (Sanglard et al. 2003). *C. albicans* mutant strain Day 364 (cnb1/cnb1—mutant strain lacking calcineurin B regulatory subunit—FLU hypersensitive) and its complementary strain MCC 85 (cnb1/cnb1 + CNB1—mutant strain with a constitutive calcineurin B) were employed to investigate the inhibitory effect of 4HMBA on calcineurin activity. Whether 4HMBA exerts inhibitory effect on calcineurin activity from MCC 85 strain, it would affect the FLU susceptibility of MCC 85, making this strain FLU hypersensitive, and non significant differences ($p > 0.05$) would be expected between the MICs obtained for FLU in combinatory experiments (i.e., FLU + 4HMBA) for both strains. Our results showed that there were significant differences between de MICs obtained for

FLU in combinatory experiments against both strains ($p < 0.05$), which suggests that 4HMBA would not affect calcineurin activity of MCC 85 strain. Both strains exhibited the same sensibility to 4HMBA. The detailed results could be found as Supplementary Information (Table S2). Crz1 is a zinc finger transcription factor controlled by calcineurin. In response to extracellular stress calcineurin dephosphorylates Crz1, promoting nuclear translocation and induction of genes encoding biosynthetic cell wall enzymes and homeostatic ion machinery (Onyewu et al. 2004). OCC 1.1 (crz1/crz1—mutant strain lacking Crz1. FLU hypersensitive) and its complementary strain OCC 7 (crz1/crz1 + CRZ1—mutant strain with a constitutive Crz1. FLU sensitive) were employed to investigate the inhibitory effect of 4HMBA on Crz1 activity. Whether 4HMBA exerts inhibitory effect on Crz1 activity from OCC 7 strain, it would affect the FLU susceptibility of OCC 7, making this strain FLU hypersensitive, and non significant differences ($p > 0.05$) would be expected between the MICs obtained for FLU in combinatory experiments for both strains. There were significant differences between de MICs obtained for FLU in combinatory experiments against both strains ($p < 0.05$), which suggests that 4HMBA would not affect Crz1 activity of OCC 7. Both strains exhibited the same sensibility to 4HMBA. The results could be found as Supplementary Information (Table S2). All these data lead us to conclude that the synergistic effect of FLU + 4HMBA combination against *C. albicans* would not affect calcineurin activity or the calcineurin controlled factor Crz1.

Iron chelation assay

None of the assayed compounds exerted iron-chelating activity at the assayed concentrations (CA% >400 mg L^{-1}), which suggests that synergistic effect of 4HMBA + FLU combination against *C. albicans* would not be mediated by iron depletion.

Microscopy experiments

Through time-lapse microscopy experiments we found that *C. albicans* cells exposed to 4HMBA maintained the yeast-like morphological form throughout the assay. These observations were confirmed by SEM images, where a complete absence of filamentous cells was observed after treatment with 4HMBA. Noteworthy differences in biofilm density were observed between control and treated cells (Fig. 4a, b). Cells exposed to 4HMBA showed irregular budding patterns (Fig. 4c), which could be attributed to the inhibition of germtube formation. TEM images of untreated cells showed well constituted outer and inner layers divided by a low-density space (Fig. 4d), while 4HMBA exposed cells showed narrowed cell walls (Fig. 4e). There were significant differences ($p < 0.05$) between the cell wall thickness of control cells (300 ± 7 nm) and treated cells (172 ± 9 nm).

Discussion

An antimicrobial activity is labeled as "very interesting" for isolated compounds for which inhibitory concentrations are below 10 mg L^{-1} (Ríos and Recio 2005). Our results showed that 4HMBA at 5 mg L^{-1} was enough to generate a synergistic antifungal effect in combination with FLU. This FLU + 4HMBA association resulted in a fungicidal combination, which could be valuable to diminish the incidence of resistance to FLU, especially in developing countries where FLU is the most used antifungal drug against *C. albicans* (Flynn et al. 2009). It is agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are structural requirements for biocidal activity (Bachmann et al. 2002). 4HMBA has an acid hydroxyl and the lipophylicity is provided by the prenyled chain. On the other hand, the three semi-synthetic compounds (i.e., CHR, AcA and EPX) do not possess phenolic hydroxyl group and showed no significant antifungal activity alone or in combination with FLU. These data suggest that phenolic hydroxyl group might be crucial for the antifungal action of acetophenone derivatives.

Potentiation of the FLU action against *C. albicans* by iron depletion has been previously demonstrated (Prasad et al. 2006). Moreover, some hydroxylacetophenones possess iron chelating activity (Bonifait et al. 2012). These led us to evaluate this activity for the compounds under study. The absence of iron chelating activity for 4HMBA and derivatives could be attributed to the lack of structural requirements, e.g., a hydroxyl group in conjunction with a keto group, ortho-dihydroxyl groups or large number of hydroxyl groups (Khokhar and Owusu Apenten 2003).

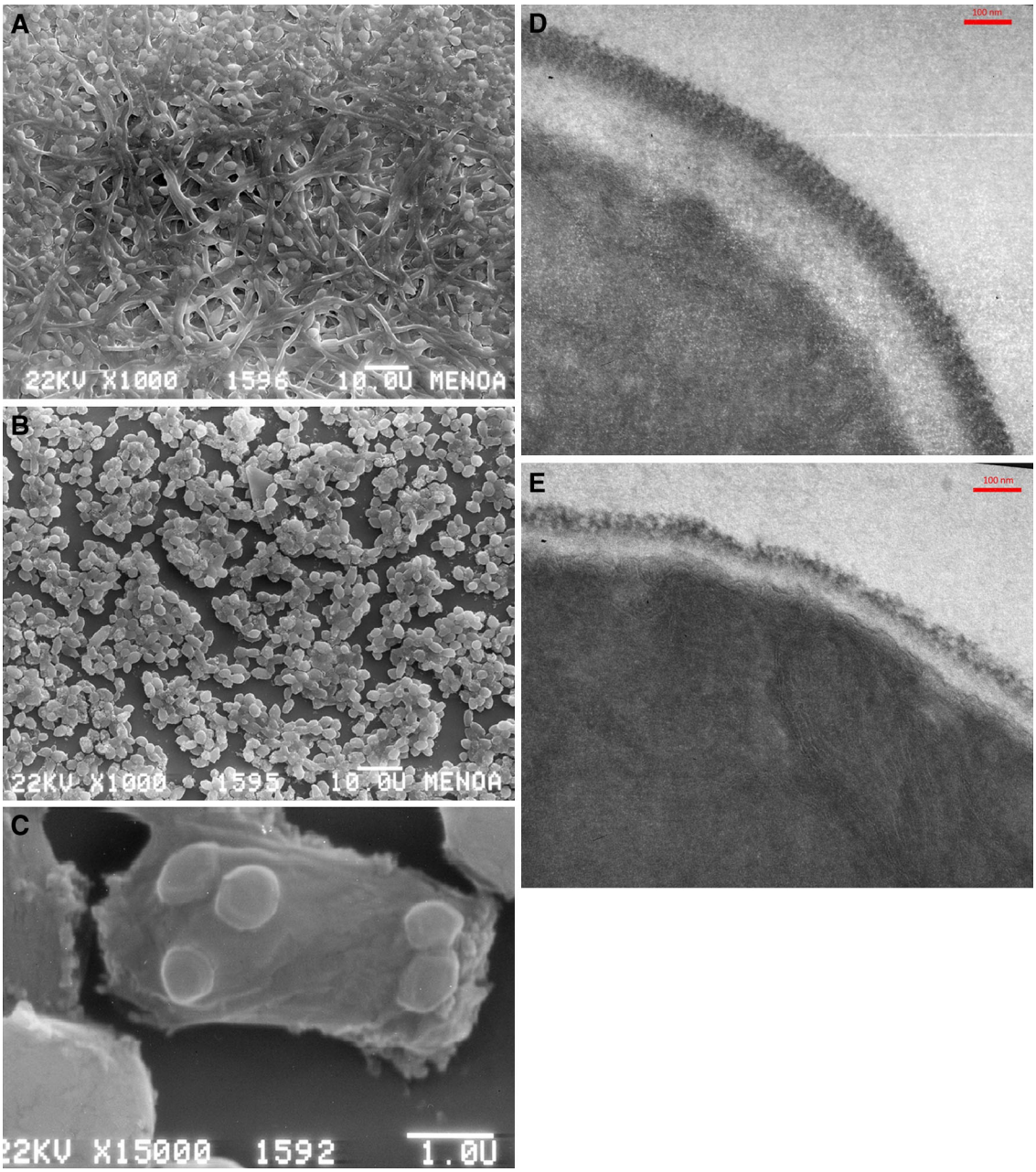

Fig. 4 **a** *C. albicans* cells in control experiments (e.g., not exposed) within a high density biofilm observed by SEM (magnification ×1000). **b** *C. albicans* cells exposed to 4HMBA observed without filamentation by SEM (magnification ×1000). **c** *C. albicans* cell exposed to 4HMBA showing irregular budding patterns by SEM (magnification ×15,000). **d** *C. albicans* cell in control experiment showing no alterations on cell wall, observed by TEM (magnification ×50,000). **e** *C. albicans* cell exposed to 4HMBA showing narrowed cell wall, observed by TEM (magnification ×50,000)

These observations lead us to conclude that the synergistic effect for FLU + 4HMBA would not be related to iron homeostasis.

Steinbach et al. (2004) have reported that some calcineurin inhibitors delay fungi filamentation, yielding a synergistic combination with CAS. We evaluated

4HMBA effect on calcineurin pathway of *C. albicans*. Our results showed that 4HMBA does not interfere with the calcineurin signaling pathway, so consequently 4HMBA may be exerting the inhibitory effect by acting on a different target.

Usually, antimicrobial compounds which act through different modes of action have a synergistic effect on target cells (Endo et al. 2010). Therefore, the synergistic effects of 4HMBA + FLU and 4HMBA + CAS against *C. albicans* ATCC 10231 could be attributed to the fact that FLU action is directed to ergosterol biosynthetic pathway, CAS inhibits cell wall β-glucan synthesis, and 4HMBA inhibits cell transition between yeast-form (non virulent) to filamentous-form (virulent). Boeck et al. (2005) have reported hydroxyl chalcones (structurally related to hydroxyl acetophenones) with inhibitory activity on fungal cell wall assembly. Our findings suggest 4HMBA could have an inhibitory effect on the *C. albicans* cell wall, but the synergistic combination showed by 4HMBA + CAS would suggest that the target of 4HMBA may be different than that of CAS.

Recent reports have shown that the co-occurrence of a hydroxyl and a lipophilic alkyl group in ortho relationship is helpful for the antifungal activity (Ma et al. 2013). Our results match these findings, because 4HMBA possess the ortho relationship between hydroxyl and prenyl groups.

Conclusions

Microscopy observations showed 4HMBA affects *C. albicans* filamentation, which may explain the synergistic effects observed for FLU + 4HMBA and CAS + 4HMBA, taking into consideration that compounds with different targets usually synergize their actions. Moreover, FLU + 4HMBA resulted in a fungicidal combination. 4HMBA phenolic hydroxyl group could play a key role in the antifungal activity, because the three semi-synthetic derivatives, which lack phenolic hydroxyl group, showed no significant antifungal activity. Our results provide valuable information regarding structural issues to consider for further chemical modifications in order to preserve antifungal activity. Further assays should clarify the cell target for 4HMBA and the action mechanism, though this study sets the first step for those researches.

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