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Identification of the cell targets important for propolis-induced cell death in *Candida albicans*

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ABSTRACT

Candida albicans is the most common fungal pathogen of humans, forming both commensal and opportunistic pathogenic interactions, causing a variety of skin and soft tissue infections in healthy people. In immunocompromised patients *C. albicans* can result in invasive, systemic infections that are associated with a high incidence of mortality. Propolis is a complex mixture of several resinous substances which are collected from plants by bees. Here, we demonstrated the fungicidal activity of propolis against all three morphogenetic types of *C. albicans* and that propolis-induced cell death was mediated via metacaspase and Ras signaling. To identify genes that were involved in propolis tolerance, we screened ~800 *C. albicans* homozygous deletion mutants for decreased tolerance to propolis. Fifty-one mutant strains were identified as being hypersensitive to propolis including seventeen genes involved in cell adhesion, biofilm formation, filamentous growth, phenotypic switching and pathogenesis (*HST7*, *GIN4*, *VPS34*, *HOG1*, *ISW2*, *SUV3*, *MDS3*, *HDA2*, *KAR3*, *YHB1*, *NUP85*, *CDC10*, *MNN9*, *ACE2*, *FKH2*, and *SNF5*). We validated these results by showing that propolis inhibited the transition from yeast-like to hyphal growth. Propolis was shown to contain compounds that conferred fluorescent properties to *C. albicans* cells. Moreover, we have shown that a topical pharmaceutical preparation, based upon propolis, was able to control *C. albicans* infections in a mouse model for vulvovaginal candidiasis. Our results strongly indicate that propolis could be used as a strategy for controlling candidiasis.

1. Introduction

Candida albicans is a dimorphic fungus that normally resides as a commenal organism within the gastrointestinal tract of humans and other warm blooded animals. *C. albicans* is the most common fungal pathogen of humans and is responsible for causing a variety of skin and soft tissue infections in healthy people and more virulent systemic diseases in immunocompromised patients. The fact that *C. albicans* can survive in healthy hosts and also cause a spectrum of diseases in immunocompromised patients is a matter of significant biological interest, as well as clinical and economic importance (Noble and Johnson, 2007). *Candida* spp. are present in approximately 50% of the population and around 80% of women have suffered from *Candida* infections, with around 5% of these infections recurring (Odds et al., 2004; Ramsdale, 2008). In immunocompromised individuals, *C. albicans* can produce mild superficial infections of the mouth and vagina. In severely immunocompromised patients, this fungus can produce a disseminated, systemic infection that is associated with a high incidence of mortality (Odds et al., 2004). *C. albicans* is the fourth most common hospital infection and the rate of resistance to traditional antifungal therapies is high (Yano and Fidel, 2011; Shapiro et al., 2011; Krcmery, 2000; Lamagni et al., 2001; Ramsdale, 2008).

The striking feature of the morphology of *C. albicans* is its ability to proliferate and switch rapidly from budding yeast-like, to pseudohyphal or hyphal growth (Sudbery, 2011; Sudbery et al., 2004; Odds et al., 1998; Whiteway and Bachewich, 2007). The morphological plasticity of *C. albicans* is a virulence determinant and plays a fundamental role in the infection process. During

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mucosal infections, hyphae invade epithelial and endothelial cells causing damage, probably through the release of hydrolytic enzymes (Zhu and Filler, 2010). *C. albicans* is perfectly adapted to growth in its human host and forms hyphae under a variety of environmental conditions that reflect the diversity of microenvironments that *C. albicans* encounters within the host (Sudbery, 2011). Hyphae are formed in response to the presence of serum, neutral pH, 5% CO_2 (the partial pressure of CO_2 in the blood) and N-acetyl-D-glucosamine (GlcNAc) (Mardon et al., 1969; Sudbery, 2011).

The diploid genome of *C. albicans* is approximately 30 Mb and contains 8 pairs of homologous chromosomes (Jones et al., 2004; van het Hoog et al., 2007). Signal transduction pathways and transcription factors involved in the induction of hyphal growth have been recently reviewed in detail by Shapiro et al. (2011). The expression of hyphal growth-specific genes is negatively regulated by a complex consisting of the Tup1 co-repressor and NRG1 or the filamentous growth regulator type Rox1p (Rfg1). Cells lacking any of these repressors constitutively grow as long pseudohyphae, as the expression of hyphal-specific genes is not inhibited (Kadosh and Johnson, 2005; Murad et al., 2001; Sudbery, 2011). The up-regulation of the expression of hyphal-specific genes is accomplished by several transcription factors, including Efg1, Chp1, Cph2, Tec1, Flo8, Czf1, Rim101 and Ndt80 (Shapiro et al., 2011; Sudbery, 2011; Stoldt et al., 1997; Lane et al., 2001; Cao et al., 2006; Davis et al., 2000; Sellam et al., 2010). Efg1 is necessary for the formation of hyphae in response to serum, neutral pH, CO_2 and GlcNAc in liquid media and on solid spider media. Cph1 and its upstream activation pathway are needed only for forming hyphae on solid spider media, but not in liquid media. Consequently, it was suggested that Efg1 was the main regulator of the formation of hyphae under most conditions. Cph1 and Efg1 are activated by upstream signaling pathways such as the MAPK and cyclic AMP pathways (cAMP), respectively (Shapiro et al., 2011; Biswas et al., 2007). The Ras1 GTPase regulates both the cAMP and MAPK pathways (Shapiro et al., 2011; Sudbery, 2011; Stoldt et al., 1997; Leberer et al., 2001).

Propolis is a complex mixture of several resinous substances, which are collected from plants by bees. Propolis has been used in folk medicine for centuries. Its chemical composition complex, since more than 300 compounds including polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids, and inorganic compounds, have been identified in different propolis samples (Lustosa et al., 2008; Pereira et al., 2002; Franco et al., 2000). Propolis has attracted attention because of the variety of biological and therapeutic properties displayed. Propolis has antibacterial (Koru et al., 2007; Park et al., 2002), antiviral (Vynograd et al., 2000), antiprotozoal (Menna-Barreto et al., 2009), anti-inflammatory (Ramos and Miranda, 2007), antioxidant (Simões et al., 2004; Russo et al., 2002), wound healing (Berretta et al., 2012), immunomodulatory (Sforcin, 2007), and anti-tumor properties (Kimoto et al., 1998). Several authors have also shown that propolis can be used to treat *Candida* infections (Aguero et al., 2010; Dalken-Dota et al., 2010; Ota et al., 2001; Salomão et al., 2008; Siqueira et al., 2009). Recently, we utilized *Saccharomyces cerevisiae* as a model organism plus genetics, cell biology, and transcriptomic to determine how propolis affects eukaryotic cells. We found that propolis was able to induce the apoptosis cell death response, while increased exposure to propolis caused a necrosis response. More specifically, we found that the most prominent pathways were related to oxidative stress, the mitochondrial electron transport chain, vacuolar acidification, the regulation of macroautophagy associated with protein targeting to the vacuole, cellular response to starvation, and the negative regulation of transcription from RNA polymerase II promoter (de Castro et al., 2011).

In the present study, we evaluated the antifungal activity of propolis on *C. albicans*. We investigated specifically if propolis could activate signaling and metabolic pathways related to cell death. In addition, we determined the influence of propolis on the growth of a collection of *C. albicans* deletion mutants aiming to discover tolerance mechanisms that were impacted by propolis. Propolis demonstrated fungicidal action against all three *C. albicans* morphogenetic types and propolis-induced cell death was shown to be mediated via metacaspase and Ras signaling. Interestingly, we have identified several mutants that correspond to genes involved in either morphological transition or in the maintenance of a specific morphotype. We validated these results by showing that propolis can inhibit the transition to hyphal growth. Moreover, we have shown that a medicine based upon propolis was able to control a *C. albicans* infection in a mouse model of vulvovaginal candidiasis. Our results strongly indicate that propolis represents a promising strategy for controlling candidiasis.

2. Materials and methods

2.1. Strains, media and culture methods

The *C. albicans* strains used were: SC5314 (Gillum et al., 1984), CAI4 (*ura3::imm434/ura3::imm434 iro1/iro1::imm434*) (Fonzi and Irwin, 1993), BWP17 (*ura3*::imm434/*ura3*::imm434 *iro1/iro1*::imm434 *his1*::hisG/*his1*::hisG *arg4/arg4*) (Wilson et al., 1999), DAY286 (ura3::λimm434 his1::hisG pARG4::URA3::arg4::hisG, ura3::λimm434 his1::hisG arg4::hisG) (Davis et al., 2002), 3153A, and 529L (wild type). Solid and liquid complete YPD media (2% w/v glucose, 1% w/v yeast extract and 2% w/v peptone), which only differed in the addition of agar (2% w/v), were used for strain cultivation. Since propolis was dissolved in ethanol (60% alcoholic extract), control treatment was vehicle control (5.45% ethanol).

2.2. Propolis standardized extract

Propolis Standardized Extract (EPP-AF®) (Batch 010/08) was industrially produced and kindly provided by Apis Flora (Ribeirão Preto, São Paulo, Brazil). The extract was standardized using a blend of propolis composed of raw material obtained from several sites in Brazil (Patent number PI 0405483-0, published in Revista de Propriedade Industrial n. 1778 from 01/02/2005).

The blend of raw propolis was frozen for 12 h at −20 °C and then ground to a fine powder using a blender. Propolis was then extracted from the raw material using a hydro alcoholic solution (7:3), with dynamic maceration, for 72 h at room temperature, followed by percolation (1 l/min), and finally a filtration process that first used the propolis biomass as a filter and then a 220 mesh stainless steel industrial filter. The propolis extract obtained presents 11% w/v of the dry matter and the chemical composition was evaluated by RP-HPLC (Shimadzu – C18 Shim-pack, CLC-ODS (M), 25 cm × 4.6 column, with gradient elution, with methanol and acidic water pH 2.7, plotted at 275 nm) into compounds: caffeic acid (Fluka, L. 43706045), *p*-coumaric acid (Fluka, L.3250759), *trans*-cinnamic acid (Fluka, L.21907066); the flavonoid aromadendrin-4'O-metil ether (previously isolated, identified and donated by Sousa et al. (2007) and prenylated derivative and marker of green propolis, Artepillin C (Wako, L. 016.19131), according to Berretta et al. (2012).

2.3. Medicines

To evaluate propolis efficacy *in vivo*, topical pharmaceutical preparations were developed based upon the propolis alcoholic extract (EPP-AF®), however after the complete evaporation of the alcohol and the resolubilization of propolis in propylene glycol.

Formulations G2 and G3% were prepared using poloxamer 407 as copolymer. Initially, poloxame (Lutrol® F127) was dispersed in water and maintained under hydration for 24 h at 5 °C. Meanwhile, Carbopol® was dispersed in water solution containing 0.1% potassium sorbate and 0.01% EDTA 0.01% and maintained under hydration for 24 h (Phase B). Then, under stirring, the mixture of propolis (2% and 3%, for G2 and G3%, respectively), Melaleuca (*Melaleuca alternifolia*), sweet birch (*Betula lenta*), *Mentha spicata* and rosemary (*Rosmarinus officinalis*) essential oils and propylene glycol were added gradually into the poloxamer dispersion (Phase A). Afterwards, Carbopol 940 dispersion was mixed with phase A. Under stirring, triethanolamine was used to adjust to pH 6 and also to conferred viscosity to the preparation. To prepare an emulsion (E3%), propolis (3%) was dispersed in etoxilated castor oil 40OE and castor oil (Phase A) and potassium sorbate (0.1%) and EDTA (0.01%) were dispersed in purified water (Phase B). Both phases were warmed to around 60 °C under agitation until both phases reached a homogeneous temperature. Subsequently, phase B was inverted into phase A, under agitation. When the temperature of preparation was above 40 °C, Melaleuca (*Melaleuca alternifolia*), sweet birch (*Betula lenta*), *Mentha spicata* and rosemary (*Rosmarinus officinalis*) essential oils and propylene glycol were added gradually into the emulsion (cream). To all preparations, gels (G2 and G3) and cream (E3), the respective controls were prepared without propolis and essential oils. A conventional medicine for vaginal candidiasis treatment, Clotrimazole cream (10 mg/g), was obtained from Neo Química®, Brazil, lot: LB12F1021.

2.4. Screening of the C. albicans deletion libraries

A set of approximately 800 *C. albicans* deletion mutants were screened for increased sensitivity to propolis. Fifteen deletion libraries in 96 well plates were provided by Dr. Kevin McCluskey of Fungal Genetics Stock Center (www.fgsc.net). To identify the propolis-sensitive mutants, aliquots of the cultures, from fresh 96-well plates, were spotted on YPD plates plus 5.45% ethanol as a control corresponding to the concentration of alcohol present in the 1% of alcoholic extract, or alcoholic propolis extract (Apis Flora, Ribeirão Preto, São Paulo, Brazil) at 0.5% or 1.0%. Plates were incubated at 30 °C for 5 days. Propolis sensitivity was determined by a reduction in growth.

2.5. Viability determination

C. albicans strains were incubated in YPD liquid media (150 rpm) at 30 °C for 3, 6, 9 and 16 h. After this period, 1×10^7 - cells/ml from each time point were inoculated into 50 ml of YPD liquid and exposed to different concentrations of propolis (0.125%, 0.250%, 0.5%, 1.0%) for 20 min. For the negative control, 5.45% ethanol was added to the medium. Cell viability was determined by plating appropriate concentrations of cells and counting the number of colonies compared to the negative control, which had 100% survival at all stages of the growth curve.

2.6. Evaluation of the sensitivity of C. albicans morphogenetic types to propolis

In order to evaluate the sensitivity of *C. albicans* morphogenetic types to propolis, the SC5314 strain was incubated in YPD liquid media for 16 h in a shaking incubator at 30 °C. After this period, cells were counted and 2×10^7 cells/ml inoculated into fresh liquid YPD media and return to the incubator for a further 4 h, at 30 °C (to maintain the yeast form), at 37 °C (to induce pseudohyphae), and in liquid YPD plus Fetal Bovine Serum (FBS) (20%) at 37 °C (for the formation of hyphae). Subsequently, 5.45% ethanol was added for the negative control, while for the 1% propolis treatment, 20 min and 1 h incubations were used. In order to verify the effectiveness of the transition, an aliquot of each culture was taken and observed on a Carl Zeiss microscope. After treatment, a tenfold serial dilution of the cells was plated on solid YPD media.

2.7. Dimorphic transition and Mitotracker microscopy

To analyse the effect of the propolis alcoholic extract on *C. albicans* filamentation, the SC5314 strain was grown for 16 h in liquid YPD media within a shaking incubator at 30 °C. The yeast cells were harvested by centrifugation, washed three times in filter sterilized ultra-pure water and counted using a haemocytometer. Approximately, 2×10^7 cells/ml were added to 1 ml of RPMI1640 media (to induce filamentation) containing 0.05; 0.1% or 0.2% propolis. The RPMI1640 medium without propolis was used as a negative control. The samples were then incubated for 90 min at 37 °C under shaking (100 rpm). Afterwards, the cells were collected by centrifugation (5 min at 4000 rpm) and washed with phosphate buffered saline (PBS). Before fixation, cell viability was determined by plating serial dilutions of the cells on YPD agar plates. Under those concentrations, the propolis extract was not toxic to the fungal cell (data not show). Subsequently, the cells were fixed with 3.7% l formaldehyde (J.T. Baker) in PBS for 15 min at room temperature. After fixation, the cells were washed once with PBS. To stain the mitochondria, the cells were incubated with 250 nM Mitotracker® (Invitrogen) diluted in PBS for 10 min at room temperature and washed once with PBS. To stain the nuclei, the cells were incubated with 250 ng DAPI (Invitrogen) in a PBS solution containing 42.5% glycerol for 10 min at room temperature and washed once with PBS. Finally, the stained cells were washed with PBS, mounted and viewed on a Carl Zeiss Observer Z1 fluorescence microscope (Jena, Germany) using the following filters: filter set 38 HE (wavelength excitation 470/40 nm and emission wavelength, 525/50 nm) for GFP; filter Set 49 (wavelength excitation 365 nm and emission wavelength 445/50 nm) for DAPI and filter set 63 HE (wavelength excitation 572/25 nm and emission wavelength 629/62 nm) for Mitotracker. To quantify *C. albicans* filamentous forms, at least 100 cells per sample were assessed and the filamentation percentage calculated. The experiment was repeated three times. For fluorescence analysis, mitochondria were stained in red (Mitotracker), nuclei in blue (DAPI) and the propolis alcoholic extract (autofluorescent) gave a signal under the GFP filter.

2.8. Murine model of vulvovaginal candidiasis and C. albicans biofilm formation

This protocol was performed as described by Yano and Fidel (2011) with some modifications. Briefly, female mice (BALB/c strain, at 7–8 weeks of age with a body weight of 20 to 22 g,) were housed in vented cages containing 5 animals. Three days before infection, mice were treated with β-estradiol (17 β-estradiol valerate) at 0.3 mg dissolved in 100 μl of castor oil (Sigma) and administered subcutaneously into the back of each animal, three days before infection. *The estrogen injection was* repeated weekly throughout the period of the study. *To prepare cells for the infection study*, the C. albicans 3153A strain was incubated on YPD agar plates at 30 °C for 48 h. An isolated colony was inoculated in liquid YPD medium and grown for 16 h in a shaking incubator at 30 °C. Cell cultures were spun for 5 min at 3000*g*, washed twice with PBS, counted using a haemocytometer, and diluted at a concentration of 2.5×10^6 cell/ml. Twenty μl of this inoculum was inoculated by inserting the pipette tip about 5 mm deep into the vaginal lumen.

The animals were divided into five groups ($n = 10$): (i) control (animals infected without treatment (gel without propolis), (ii) treated with gel containing 2% of propolis dry matter, (iii) gel

containing 3% of propolis dry matter, (iv) emulsion containing 3% of propolis dry matter and finally (v) clotrimazole (Clotrimazol vaginal cream 10 mg/g, Neo Química®, Brazil, lot: LB12F1021). The treatments were carried out for 10 days, and approximately every 12 h 60 μl of the product was applied, filling the vaginal cavity completely. In order to verify the cell morphology, an aliquot of 10 μl of the lavage was observed on a Carl Zeiss microscope.

The quantification of vaginal fungal burden was performed by inoculating a dilution of the lavage onto solid YPD media, in order to obtain about 100 colonies per plate. Counting of the colonies forming units (CFUs) was performed after incubation at 30 °C for 24 h. The animals were evaluated for 10 days post treatment and re-evaluated daily through the culture of the intravaginal lavages. The analysis of variance (ANOVA – one way) and the Bonferroni multiple comparison were performed with a 5% level of significance. Statistical analyses were performed using Prism 4 (Graph Pad).

The histopathology study was carried out following the vaginal lavage procedure and after the sacrifice. The vaginal tissue of 2 mice from each group were removed and fixed for 24 h in 3.7% formaldehyde–PBS. Samples were washed several times in 70% alcohol before dehydration in a series of alcohol solutions of increasing concentrations. Finally, the samples were diafanized in xylol and embedded in paraffin. For each sample, sequential 5-μm-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) or hematoxylin and eosin (HE) stain. Briefly, sections were deparaffinized, oxidized with 4% chromic acid, stained with methenamine silver solution, and counterstained with picric acid. For HE staining, sections were deparaffinized and stained first with hematoxylin and then with eosin. All stained slides were immediately washed, preserved with mounting medium and sealed with a coverslip. Microscopic analyses were done using an Axioplan 2 imaging microscope (Zeiss) at the stated magnifications under bright-field light.

C. albicans biofilms were formed in the wells of commercially available presterilized, polystyrene, flat-bottomed, 96-well microtiter plates as described previously by Pierce et al. (2008) and Rukayadi et al. (2011). The antimicrobial activity of propolis on *C. albicans* biofilms was monitored using the XTT [2,3 - bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay (Pierce et al., 2008). Antimicrobial activities for each isolates were expressed by the mean optical density (± standard deviation) at 490 nm wavelength (OD490).

3. Results

3.1. Propolis induces cell death in C. albicans

The tolerance of three *C. albicans* strains SC5314, 3153A and 529L, of which the last two are considered hypervirulent in the mouse model of vulvovaginal candidiasis (Yano and Fidel, 2011; Rahman et al., 2007) to propolis was assessed. All three *C. albicans* strains could not grow in the presence of 1.0% propolis (Fig. 1A). We also investigated the viability of the *C. albicans* cells at different stages of the growth curve. For this, we concentrated our attention on the SC5314 strain. Cells in the stationary phase (16 h) were more resistant to the propolis extract when compared to other stages of the growth curve (Fig. 1B). Similar results were observed for the strains 3153A and 529L (data not shown). However, the 3153A strain was more tolerant to propolis than the 529L and SC5314 strains (Fig. 1A).

3.2. Propolis inhibits all three C. albicans morphogenetic types

We evaluated the tolerance of the different morphogenetic types of *C. albicans* to propolis. The transition from yeast-like to hyphal growth was about 95%, whereas from yeast-like to pseudohyphal growth was about 60% (Fig. 2A). The three different morphogenetic types were incubated with 1% propolis (or 5.45% ethanol for the negative control) for 20 min or 1 h, under the appropriate conditions to maintain the respective morphogenetic type. A tenfold serial dilution of the cells was plated onto solid YPD. The growth of all morphogenetic types was equally inhibited by propolis at 20 min and 1 h (Fig. 2B). Similar results were observed for the strains 3153A and 529L (data not shown). Taken together, these results suggest that the alcoholic extracts of propolis, used at a concentration of 1% was able to completely inhibit the growth of all the different *C. albicans* morphogenetic types.

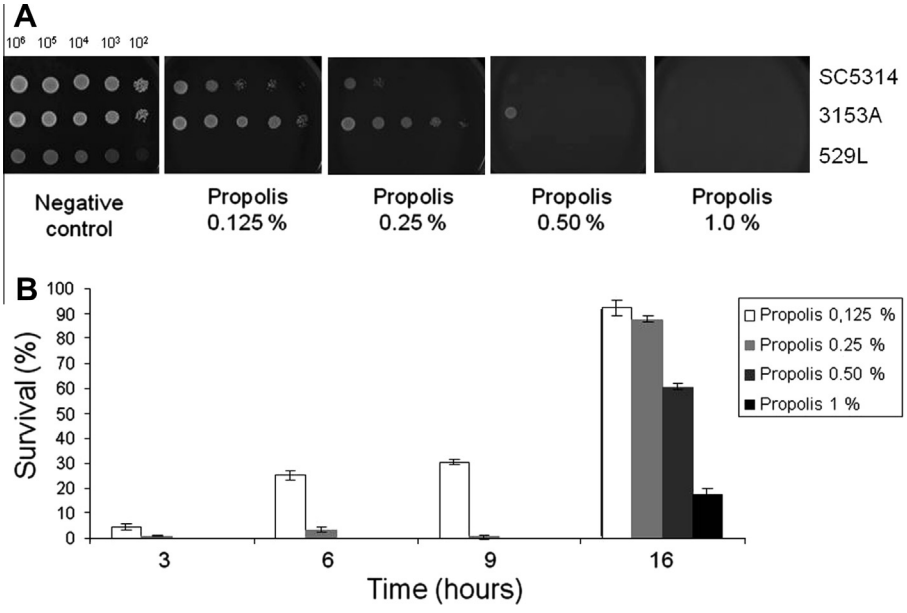

Fig. 1. Propolis induces cell death in *C. albicans*. (A) Spot dilution assays for different *C. albicans* strains in YPD medium supplemented with different concentrations of propolis. The plates were incubated at 30 °C for 72 h. (B) Viability of *C. albicans* cells in different growth phases exposed to 0.125%, 0.250%, 0.500% and 1.0% for 20 min. Cell viability was determined by plating appropriate cell concentrations and counting the number of colonies. The numbers presented are average ± standard deviation of three independent experiments.

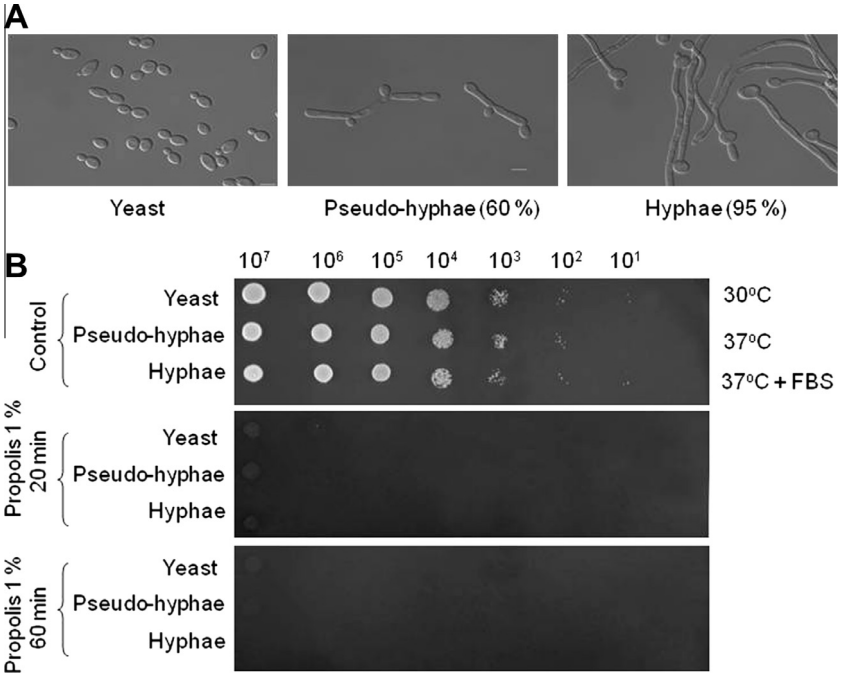

Fig. 2. *C. albicans* morphogenetic types showed equal tolerance to propolis. After induction of pseudohyphae and hyphae forms (A), cells were treated with 1% propolis for 20 min and 1 h. (B) Tenfold serial dilution were made and dripped onto plates containing YPD solid. The plates were incubated at 30 °C for 72 h. In order to verify the efficiency of the transition, a aliquot was withdrawn and checked via microscopy. Bars 5 m.

3.3. The involvement of metacaspases and the Ras signaling in C. albicans propolis-induced cell death

Caspases are cysteine proteases that are involved in cell death in eukaryotes (Li and Yuan, 2008). We have previously shown that the *S. cerevisiae YCA1* gene (a type I metacaspase) is important for propolis-mediated cell death (de Castro et al., 2011). Interestingly, deletion of the *YCA1* functional homologue in *C. albicans*, IPF4847 (orf19.5995), results in increased resistance to oxidative stress, suggesting that in *C. albicans* this gene encodes a metacaspase responsible for programmed cell death (PCD) induced by oxidative stress (Cao et al., 2009; Aerts et al., 2009). Thus, we determined the viability of *C. albicans* strains BWP17 (wild-type) and *mca1Δ/Δ* (Aerts et al., 2009) after treatment with propolis. When the wild-type strain was exposed to 1% propolis for 10 or 20 min, there was a 70% and 94% reduction in cell viability, respectively. In the metacaspase mutant, this reduction was about 46% and 90% (Fig. 3A). These results indicate that *C. albicans MCA1* gene was important for cell death mediated by propolis.

According to Phillips et al. (2006), the apoptotic response in *C. albicans* can be triggered by exposure to H_2O_2, intracellular acidification, or a low dose of the antifungal agent amphotericin B. However, the mechanisms by which *C. albicans* undergoes apoptosis are still unclear. The Ras pathway appears to be involved in apoptotic signaling induced by acetic acid and hydrogen peroxide (Phillips et al., 2006). The artificial activation of Ras accelerates the rate at which the cells become apoptotic, but the activation of Ras alone is not sufficient to kill cells. Mutants that have Ras-cAMP-PKA signaling blocked (*ras1Δ*, *cdc35Δ*, *tpk1Δ*, and *tpk2Δ*) suppress or delay the apoptotic response, whereas mutations that stimulate signaling such as RAS^{val13} and *pde2Δ* show an accelerated rate of entry into apoptosis (Phillips et al., 2006). Thus, we decided to evaluate if these genes were involved in the cell death induced by propolis, by investigating their tolerance to propolis (Fig. 3B). The mutants *cdc35Δ* and *ras1Δ*, which block Ras signaling, showed increased viability upon incubation with propolis (Fig. 3B). In contrast, the *tpk1Δ*, *tpk2Δ*, and *pde2Δ* mutants showed greater sensitivity to propolis (Fig. 3B). Taken together, these results imply that cell death induced by propolis was mediated via metacaspase and Ras signaling.

3.4. Identification of other C. albicans genes involved in propolis-induced cell death

To identify genes that were involved in the tolerance of propolis, we screened the following *C. albicans* deletion libraries: (i) transcription factor (TF), (ii) cell wall protein (CW), (iii) kinase (K) and (iv) miscellaneous (M) homozygous mutants (available at the Fungal Genetics Stock Center). Approximately 800 different mutants were involved in this screening. These mutant libraries were grown either on plates containing 5.45% ethanol (as control) or 0.5% and 1% of alcoholic extract of propolis for 5 days at 30 °C. Fifty-one mutant strains were identified as being hypersensitive to propolis when compared with the corresponding wild-type strains (Table 1; Supplementary Table S1 and Fig. S1). The analysis of these mutants through the Candida Genome Database (http://www.candidagenome.org) allowed the identification of the possible biological processes of each gene, as well as the orthologues in *S. cerevisiae* (Supplementary Table S1). Furthermore, we have analyzed our data based upon the recently improved annotation of gene ontologies involved in biofilm formation, filamentous growth, and phenotytic switching in *C. albicans* (Inglis et al., 2013).

Protein kinases, phosphatases and transcription factors play important roles in signaling and response to changes in the external environment. The ability of *C. albicans* to rapidly detect and respond to changes in the environment is critical to its survival in the human host (Blankenship et al., 2010). We observed 11 protein kinases, 4 transcription factors and 2 tyrosine phosphatases from a total of 167 mutants as being more sensitive to propolis (Table 1 and Supplementary Table S1). The 11 protein kinases were categorized in terms of function and evolution (http://kinome.com) into the major groups of protein kinases: STE (*HST7*, *CDC15*); CaMK (*GIN4*), AGC (*RIM15*) and CMGC (*HOG1*), Atypical/PIKK/ATM (*VPS34*), Atypical/HisK (*KIS1*), Other/Dicty10 (*CTK3*) and *MSS2*,

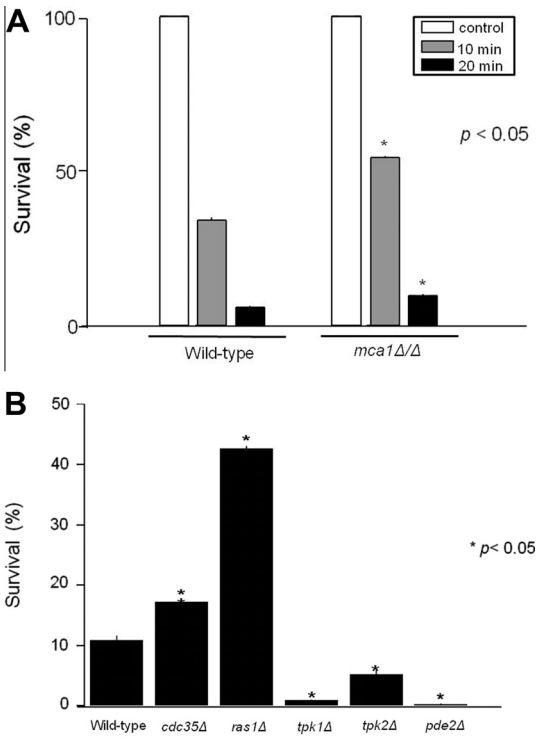

Fig. 3. The *C. albicans* metacaspase, *cdc35Δ* and *ras1Δ* mutants are more tolerant to propolis. (A) The viability of the *C. albicans* cells for wild-type and metacaspase mutant strains grown for 16 h and exposed to 1% propolis was determined by plating of appropriate concentrations of cells and then counting the number of colonies. The results are related to the mean ± standard deviation of three independent experiments. Statistical analysis was performed by the one-way ANOVA with Newman Keuls post test (*$p < 0.05$). (B) The viability of the *C. albicans* wild-type, *cdc35Δ* and *ras1Δ* mutant strains grown for 16 h and exposed to 1% propolis was determined by plating appropriate concentrations of cells and then counting the number of colonies. The results are related to the mean ± standard deviation of three independent experiments. Statistical analysis was performed by Dunnett's multiple comparisons test (*$p < 0.05$).

CKB1, that were not classified into these groups (www.cellsignal.com/reference/kinase; Table 1 and Supplementary Table S1). Interestingly, nine of these mutants (*VPS34*, *HOG1*, *SUV3*, *MDS3*, *PTP3*, *YHB1*, *CDC10*, *ACE2*, and *FKH2*) sensitive to propolis showed attenuated or an absence of virulence in different mouse models (Supplementary Table S1). Two of the four identified transcription factors, *ACE2* and *FKH2*, also have reduced virulence in the mouse model, while only the *PPT3* tyrosine phosphatase mutant has reduced virulence in an insect model (Table 1 and Supplementary Table S1).

The miscellaneous gene functions identified included chromatin remodeling (*ISW2* and *HDA2*), iron transport (*FTH1*), protein degradation (*DOA4* and *HSM3*), lipid degradation (*LIP6*), mating (*HST7*, *KAR3*), mouse virulence (*VPS34*, *HST7*, *MDS3*, *ACE2*, *YHB1*, *CDC10*), chlamydospore formation and morphology (*SUV3*, *ISW2*, *MDS3*, *CDC10*), cytokinesis (*GIN4*), septin ring formation inside the germ tube (*GIN4*, *CDC10*), caspofungin-, fluconazole-, and hydrogen peroxide-sensitivity (*HST7*, *CKB1*, *CKB2*, *VPS34*, *KIS1*, *GZF3*, *FTH1*, *SSN8*), vesicular transport (VPS34), the transport of Golgi apparatus to the vacuole (*APL3*), biofilm formation (*HIT1*, *ACE2*, *MDS3*, *CaO19.12732*), response to stress (*HOG1*), mitochondrial RNA catabolism (*SUV3*), macrophage interaction (*YHB1*), an age-dependent response to oxidative stress involved in chronological cell aging (*RIM5*) and fibroblast adherence (*VPS34*).

Actually, 16 of these mutants correspond to genes that have been annotated to the biological processes of cell adhesion, biofilm formation, filamentous growth, phenotypic switching and pathogenesis (Inglis et al., 2013), *HST7*, *GIN4*, *VPS34*, *HOG1*, *ISW2*, *SUV3*, *MDS3*, *HDA2*, *KAR3*, *YHB1*, *NUP85*, *CDC10*, *MNN9*, *ACE2*, *FKH2*, and *SNF5* (Table 1 and Supplementary Table S1). Six of these mutants correspond to genes that have orthologues predicted to play a role in the virulence of other *Candida* species or strains, but not only in the *C. albicans* SC5314 strain (Inglis et al., 2013): *VPS34*, *HOG1*, *MDS3*, *YHB1*, *CDC10*, and *ACE2* (Table 1 and Supplementary Table S1).

3.5. Propolis inhibits the dimorphic transition from yeast to hyphal growth in C. albicans

In total, 12 of the 51 genes identified were involved in the morphological transitions or in the maintenance of a specific morphotype (Table 1 and Supplementary Table S1; http://www.candidagenome.org/): (i) hyphal growth and formation (*HST7*, *VPS34*, *PTP3*, *MNN9*, *SUV3*, and *MDS3*), (ii) hyperfilamentation (*ACE2*), (iii) hyphal morphology (*CDC10*), (iv) hyphal-specific gene transcription (*FKH2*), (v) hyphal downregulation (*YHB1*), (vi) pseudohyphal growth (*PTP1*) and (vi) required for pseudohyphal to hyphal transition (*GIN4*). Thus, we decided to evaluate the effect of propolis on the dimorphic transition from yeast to hyphal growth (Fig. 4). The *C. albicans* SC5314 strain was transferred to RPMI1640 media, to induce filamentation, in the presence of 0.05, with or without the addition of 0.05%, 0.1% and 0.2% propolis (with the corresponding ethanol concentration as control). These concentrations were not fungicidal. Post exposure to propolis, cell viability was checked and there was no significant induction of cell death at the used concentrations (Fig. 4A). In contrast, there was a dramatic reduction in the transition to filamentation of approximately 30% and 90%, respectively, when the cells were grown in the presence of 0.1% and 0.2% propolis (Fig. 4B).

During these tests, we have noticed that when *C. albicans* yeast, pseudohyphal or hyphal forms were exposed to alcoholic extracts of propolis for at least 10 min emitted a green fluorescence. This fluorescence was distributed along the cell and also grouped on structures that are similar to the mitochondrial shape (Fig. 5). These structures were confirmed as mitochondria by co-staining with Mitotracker (Fig. 5). These results indicate that propolis can inhibit the yeast to hyphal transitions and it has compounds that confer fluorescent properties to *C. albicans* cells, accumulating into the mitochondria.

3.6. Propolis can reduce C. albicans burden in a vulvovaginal candidiasis mouse model

To evaluate if propolis can inhibit *C. albicans* infection, we established a vulvovaginal candidiasis mouse model with the hypervirulent strain 3153A (Yano and Fidel, 2011). We prepared three different propolis formulations (G2 and G3 poloxamer based gels, and finally, E3 emulsion gelled with carbopol, containing 2%, 3%, and 3% propolis dry matter respectively) and treated cohorts of 10 infected animals by applying intravaginally 60 µl of each formulation every 12 h for 10 days. We evaluated fungal burden after 10 days of propolis applications and also after 10 days following interruption of the treatments (Fig. 6). As a positive control, animals were treated with clotrimazole cream (10 mg/g). After 10 days of propolis treatment there was a 83.5%, 87.7%, 95.4%, and 97.9% reduction in fungal burden for G2, G3, E3 and clotrimazole, respectively, when compared to the control group (Fig. 6A). After 10 days with treatment interruption, there was a 90.1%, 87.7%, 84.7% and 68.4% reduction in fungal burden for G2, G3, E3 and clotrimazole, respectively, when compared to the control group (Fig. 6B). All the treatments were different from the control but statistically similar to the clotrimazole treatment ($p < 0.05$).

Table 1
Selected *C. albicans* mutant strains that showed increased susceptibility to propolis.

Sensitivity*	Gene	*S. cerevisae* Orthologue	Function
Kinase			
–	*HST7*	*STE7*	MAP kinase
–	*CTK3*	*CTK3*	Cyclin-dependent protein kinase
–	*GIN4*	*GIN4*	Autophosphorylated kinase; role in pseudohyphal–hyphal switch and cytokinesis
+/–	*MSS2*	*MSS2*	Role in protein insertion into mitochondrial membrane from inner side
+/– – –	*CDC15*	*CDC15*	Protein serine/threonine kinase activity, ATP binding activity and role in protein phosphorylation
+/–	*RIM15*	*RIM15*	Protein kinase activity, role in age-dependent response to oxidative stress
+/–	*CKB2*	*CKB2*	Regulatory subunit of protein kinase CK2
–	*CKB1*	*CKB1*	Regulatory subunit of protein kinase CK2
+/–	*VPS34*	*VPS34*	Autophosphorylated class III phosphatidylinositol 3-kinase
+/–	*HOG1*	*HOG1*	MAP kinase of osmotic-, heavy metal-, and core stress response
+/–	*KIS1*	*GAL83*	Snf1p complex scaffold protein
Miscellaneous			
+/–	*CRD1*	*CRD1*	Protein similar to *S. cerevisiae* Crd1p, a cardiolipin synthase
+/–	*ISW2*	*ISW2*	Protein required for chlamydospore formation; similar to *S. cerevisiae* Isw2p, an ATPase involved in chromatin remodeling
–	*HDA2*	*HDA2*	Histone deacetylase activity
+/–	*SUV3*	*SUV3*	Similar to *S. cerevisiae* Suv3p, which is an RNA helicase involved in mitochondrial RNA catabolism
–	*MDS3*	*MDS3*	TOR signaling pathway component required for growth and hyphal formation
–	*MUB1*	*MUB1*	Protein monoubiquitination; have role in regulation of cell budding,
–	*HIT1*	*HIT1*	Biofilm-induced gene
–	*KAR3*	*KAR3*	Kinesin-like microtubule motor protein
–	*BUR2*	*BUR2p*	Protein with similarity to *S. cerevisiae* Bur2p, contains a cyclin domain
–	*PEX14*	*PEX14*	Protein binding, bridging activity, role in protein import into peroxisome matrix
–	*FTH1*	*FTH1*	Protein similar to *S. cerevisiae* Fth1p, a high affinity iron transporter for intravacuolar stores of iron
+/– – –	*HSM3*	*HSM3*	Ortholog(s) have role in proteasome regulatory particle assembly, mismatch repair and nucleus, cytosol, proteasome regulatory particle, base subcomplex localization
+/– – –	*UGA2*	*UGA2*	Protein not essential for viability; similar to *S. cerevisiae* Uga2p, which is succinate semialdehyde dehydrogenase
–	*LIP6*	–	Secreted lipase, member of family of lipase genes expressed differentially in response to carbon source and during infection
–	*PTP3*	*PTP3*	Protein not essential for viability; similar to *S. cerevisiae* Ptp3p, which is a protein tyrosine phosphatase
–	*PTP1*	*PTP1*	Ortholog(s) have protein tyrosine phosphatase activity
–	*APL5*	*APL5*	Phosphorylated protein, not essential for viability; similar to S. cerevisiae Apl5p, which is a subunit of the AP-3 adaptor complex involved in Golgi-to-vacuole transport
–	*YHB1*	*YHB1*	Nitric oxide dioxygenase, acts in nitric oxide scavenging/detoxification; role in virulence in mouse
+/–	*NUP85*	*NUP85*	Ortholog(s) have role in mRNA export from nucleus, ribosomal large subunit export from nucleus, nuclear envelope organization and Nup107–160 complex localization
–	*DOA4*	*DOA4*	Protein similar to *S. cerevisiae* Doa4p, a ubiquitin hydrolase
+/–	*SSN8*	*SSN8*	Protein similar to *S. cerevisiae* Ssn8p, a component of RNA polymerase II holoenzyme
+/– – –	*CaO19.4736*	*PHO8*	Ortholog(s) have nucleotide phosphatase activity, alkaline phosphatase activity
Cell wall proteins			
+/– – –	*CaO19.5412*	*ECM9*	Ortholog(s) have role in fungal-type cell wall organization
–	*CDC10*	*CDC10*	Septin, required for wild-type cell, hyphal, or chlamydospore morphology
+/– – –	*MNN9*	*MNN9*	Protein of N-linked outer-chain mannan biosynthesis
Transcriptional factor			
+/–	*ACE2*	*ACE2*	Transcription factor involved in regulation of morphogenesis
–	*MNL1*	*YER130C*	Transcription factor that activates transcription of stress response genes via SLE (STRE-like) elements
+/– – –	*FKH2*	*FKH2*	Forkhead transcription factor; morphogenesis regulator
+/– –	*GZF3*	*GZF3*	Putative transcription factor; oxidative stress-induced via Cap1p

* The sensitivity to propolis was verified by comparing the growth of mutants with their respective wild type strains (CAI4, SC5314, BWP17 and DAY286). For this, we created a legend with the signs + and –, which indicate the degree of strains susceptibility. In YPD solid medium containing 1% propolis, wild type strains grown as +. The Supplementary Table S1 shows the full dataset and the cell growth that corresponds to: +, +/–, +/– –. +/– – –.

C. albicans can form biofilms on denture stomatitis and medical implants and this biofilm formation can protect the fungus from host defenses and reduce their susceptibility to antifungal agents (for reviews, see Bonhomme and d'Enfert, 2013; Mayer et al., 2013; and Ganguly and Mitchell, 2011). Harriott et al. (2010) have shown that *C. albicans* 3153A can form biofilms on the vaginal mucosa *in vivo* and *ex vivo*, demonstrating typical biofilm architecture and the presence of an extracellular matrix (ECM) that co-localized with the presence of the fungus. Thus, we determined if propolis could affect the *in vitro* biofilm formation by incubating *C. albicans* biofilm in the presence of either ethanol (control) or propolis and evaluating the biofilm metabolic activity via the XTT reduction assay, which directly correlates with the metabolic activity of the biofilm. There was a 40–45% reduction in metabolic activity when the biofilm was exposed to 0.25–1.25% propolis, respectively (Fig. 6C), indicating that propolis can decrease *C. albicans* biofilm formation *in vitro*.

The previously presented results were corroborated by the histological analysis, which shows the control (without any treatment) and propolis treated groups (G3 and E3) all after 10 days of treatment (Fig. 7). The results showed that estradiol ministration maintained the pseudo-estrus condition and the presence of infection. The histological analyses of these experiments corroborated the fungal burden analysis. In the control without treatment, we observed using HE staining, tissue lesion and especially the presence of neutrophils influx (Fig. 7A, a). In addition, an absence of irritation or inflammation induced by G3 and E3 treatments, while rare immune cells were detected (Fig. 7A, c and e). The morphological aspects of the tissues displayed normal architecture and thickness in the pseudo-estrus phase, with keratin deposition

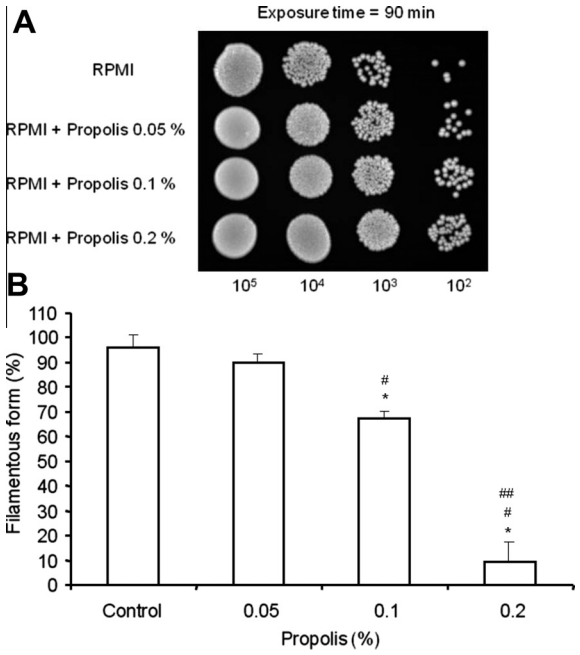

Fig. 4. Propolis can inhibit *C. albicans* the transition from yeast-to-hyphal growth. The filamentation of SC5314 strain was induced in RPMI1640 medium exposed, or not, to different propolis concentrations. Cell viability was not decreased upon propolis exposure as shown by a tenfold dilution drop-out assay (A). The influence of propolis on filamentation was evaluated by analyzing at least 100 cells per treatment on the microscope and the percentage filamentous cells was calculated (B). Statistical analyses *t* test $p < 0.01$; *different from the control; # different from 0.05%; and ## different from 0.1%.

and GMS staining indicating the absence of *C. albicans* infection (Fig. 7A, d and f). Histological slices of vaginal mucosa, 10 days after the interruption of the treatments, showed the presence of *C. albicans* in the clotrimazole treatment group, similar to that observed in the non-treated control group (Fig. 8A, b and d). In contrast, *C. albicans* was not observed in all propolis formulations (Fig. 8B).

Taken together, these results suggest that propolis can inhibit the growth *in vivo* of a *C. albicans* hypervirulent strain in a candidiasis vulvovaginal model of infection to the same extent as clotrimazole.

4. Discussion

Although *C. albicans* is commonly associated with various types of infections, such as vulvogaginal candidiasis, the development of a universal therapeutic treatment for this disease remains a major challenge, because of various problems associated with conventional treatments such as toxicity, side effects, resistance and recidivism. The use of natural products for the treatment of candidiasis has been gaining popularity both in practical use and research. Recently, Kim et al. (2012) showed a quantitative and qualitative analysis of the antifungal activity of allicin (an organic compound, derived mainly from garlic) in *C. albicans*. A wide variety of natural antifungal compounds have been discovered (Abu et al., 2011; Lee and Chee, 2010; Alireza et al., 2009; Zhang et al., 2006) and understanding the mode of action of these compounds is extremely important for the development of an effective antifungal therapy.

The health benefits and also biological activities of propolis have long been known. Propolis contains resinous substances collected by honey bees, from various plants, thus forming a complex of biologically active substances. Currently, propolis has received the attention of clinicians and researchers due to its diverse pharmacological activities and low toxicity (da Silva Frozza et al., 2012; Agarwal et al., 2012; Bankova, 2009). Here, we evaluated the antifungal activity of propolis against *C. albicans*. We observed that propolis induced cell death in *C. albicans* and also inhibited all three *C. albicans* morphogenetic types. Previous studies have shown that various stimuli, including acetic acid, hydrogen peroxide, farnesol, lactoferrin, defensins, the antifungal agent amphotericin B (Phillips et al. (2003), Lu et al., 2011; Shirtliff et al., 2009; Nikawa et al., 1993; Vylkova et al., 2007; Al-Dhaheri and Douglas, 2010; Hao et al., 2013; Zhu et al., 2011) induce *C. albicans* cells towards cell death-like apoptosis. Recently, Zhu et al. (2011)

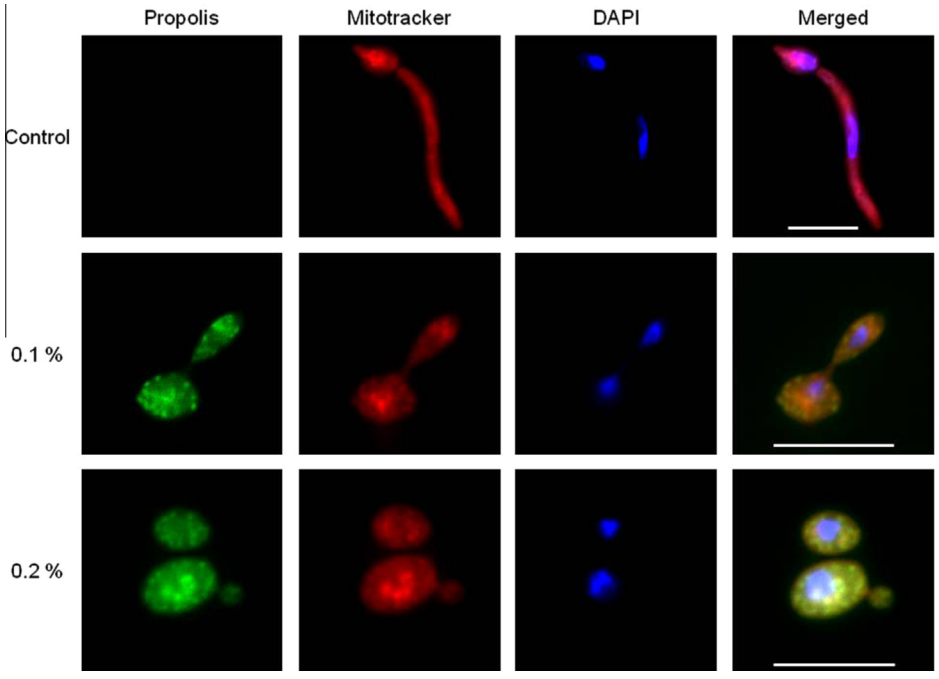

Fig. 5. *C. albicans* cells exposed to propolis auto-fluoresce. The filamentation of SC5314 strain was induced in RPMI1640 medium, as described in Fig. 4. The cells were stained with Mitotracker and DAPI, then visualized on a fluorescent microscope. Bar: 10 m.

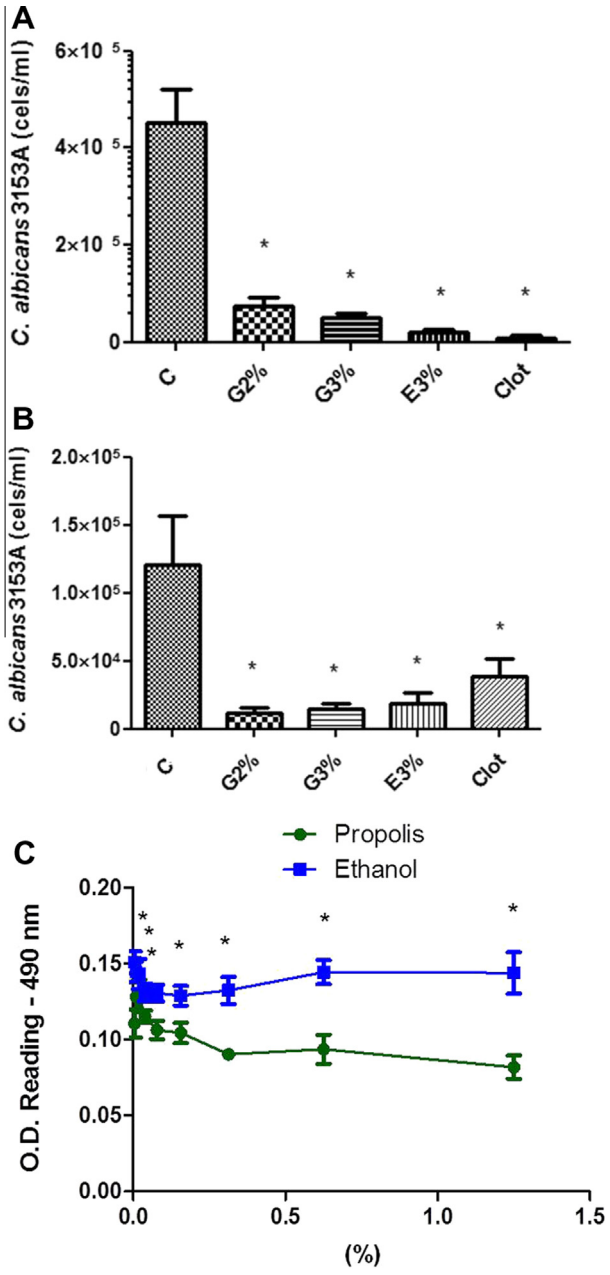

Fig. 6. Propolis can reduce *C. albicans* burden in a vulvovaginal candidiasis mouse model. (A) Fungal burden after 10 days propolis treatment and (B) after 10 days following interruption of the propolis treatment. C = control; G2%, G3%, and E3% (propolis formulations G2, G3 gels, and E3 emulsion with 2%, 3%, and 3% propolis dry matter) and Clot (clotrimazole 10 mg/g). *Indicates all the propolis treatments were different from the control but statistically similar to the clotrimazole treatment ($p < 0.05$, ANOVA *one-way* with Bonferroni post-test). (C) Inhibition of *C. albicans* 3153A strain biofilm formation by propolis. The results were the average ± standard deviation of three repetitions (*significant difference, ANOVA *one-way* – Bonferroni *post-test*, $\alpha = 0.05$).

elucidated to the mechanism behind farnesol-induced apoptosis in *C. albicans*, suggesting the potential use of this redox-cycling agent as an alternative antifungal agent. Hao et al. (2013) also showed that caspofungin (an antifungal agent in the echinocandin class) exerts activity against *C. albicans* by causing apoptosis and necrosis. However, until now, little was known about the effector molecules that are associated with the onset of PCD in *C. albicans*. Furthermore, propolis was observed to contain compounds that confer fluorescent properties to *C. albicans* cells. We proved that the fluorescent compounds from propolis, such as phenolics, were distributed throughout the cytoplasm and concentrated in the mitochondria. However, this fluorescence was not observed for *S. cerevisiae* cells treated with the same concentrations of propolis (de Castro et al., 2011). This could be due to the fact that *C. albicans* is more permeable than *S. cerevisiae* to the fluorescent compounds in propolis and/or other compounds present in *C. albicans* became fluorescent when they reacted with propolis. This propolis-induced autofluorescence did not allow us to perform caspase activity, TUNEL and propidium iodide assays, since all these techniques are dependent on fluorophores (data not shown).

Cao et al. (2009) provided the first evidence that caspase activity was involved in *C. albicans* oxidative stress-induced PCD. This caspase activity was attributed to the *C. albicans* homologue of the *S. cerevisiae* metacaspase YCA1. Our data demonstrated that cell death induced by propolis was mediated via this metacaspase, since the metacaspase mutant in *C. albicans* showed reduced sensitivity to propolis. Moreover, our results indicated that the Ras pathway was also involved in cell death induced by propolis. Ras is a member of a highly conserved family of GTP binding proteins that act as an important molecular switch in a variety of signaling pathways that control cell growth and differentiation (Leberer et al., 2001). The Ras pathway has been strongly linked to morphogenetic signals in certain fungi, and *C. albicans ras1* regulates a diverse set of phenotypes critical for both commensal and pathogenic lifestyles within the host (Piispanen et al., 2011) and it is also linked to apoptosis signaling (Cao et al., 2009). Deletion of *ras1* in *C. albicans* is able to slow down or even prevent PCD, as is the case with the *S. cerevisiae ras2* strain (D'Souza and Heitman, 2001; Feng et al.,1999; Phillips et al., 2006).

The yeast-to-hyphal morphological transition in the opportunistic pathogen *C. albicans* plays a critical role in the ability of this fungus to colonize as a commensal symbiont and cause disease as a pathogen (Zacchi et al., 2010; Mitchell, 1998; Gow et al., 2002). The immune system is able to discriminate between yeast and hyphal growth. This specific recognition of the *C. albicans* morphology, together with the detection of fungal burden and of the damage caused by the invading hyphae, might be the key for the discrimination between host colonization and infection (Jacobsen et al., 2012; Whiteway and Bachewich, 2007). Therefore, the inhibition of the yeast-to-hyphal transition or the modulation of the immune response associated with dimorphism presents a very attractive option for the control of *C. albicans* infections. We observed that *C. albicans cdc35Δ* and *ras1Δ* mutants showed a reduction in propolis-induced cell death compared with the wild-type, while *tpk1Δ*, *tpk2Δ* and *pde2Δ* mutants that showed greater sensitivity to propolis. This fact confirms the hypothesis that the disruption of Ras-cAMP-PKA signaling partially inhibits the rate of entry into apoptosis, but does not prevent it if the stimulus is strong enough, for example, by the use of propolis. The Ras-cAMP-PKA signaling pathway also plays an important role in the activation of *C. albicans* yeast to hyphal transition. Leberer et al. (2001) have shown that *C. albicans* Ras1 conveys signals to both the filament-inducing MAP kinase signaling pathway and the cAMP signaling pathway in order to promote *C. albicans* filamentous growth and virulence. Thus, Ras signaling provides an important link between polarized morphogenesis and virulence in this human pathogen. The cAMP-activated protein kinase A pathway plays a pivotal role in *C. albicans* morphogenesis and infection biology (Biswas et al., 2007). The screening of *C. albicans* deletion libraries showed several mutants that correspond to genes involved in either the morphological transitions or in the maintenance of a specific morphotype (*HST7*, *VPS34*, *PTP3*, *MNN9*, *SUV3*, *MDS3*, *ACE2*, *CDC10*, *FKH2*, *YHB1*, *PTP1*, *GIN4*) as more sensitive to propolis. This enrichment may be due to the inherent bias of the gene knockout collection, which contains a high proportion of genes required for morphogenesis. Additionally, the kinases identified are central regulators of cell

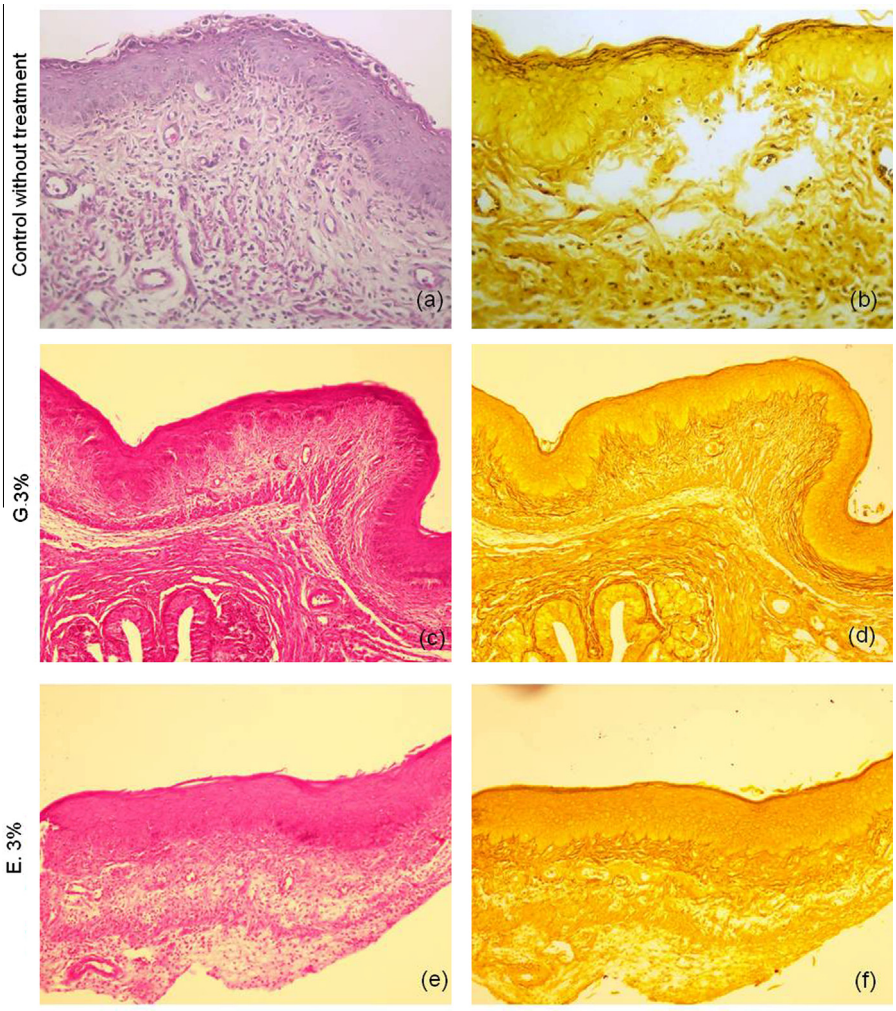

Fig. 7. Histological slices of vaginal mucosa of mice 10 days of treatment. Negative control group without any treatment (a and b), presentation of G3 (c and d) and E3 (e and f) treated groups (treatments applied every 12 h). Tissular irritation, inflammation and neutrophils influx were seen (HE staining) in the control infected group (a). *C. albicans* presence in this group was showed by GMS staining (b) demonstrating that vaginal candidiasis model was successfully implemented (×40 magnification). Pictures of G3 and E3 treatments demonstrated that propolis restored the lesions caused by *Candida* presence when compared to control group (a and b) and that these treatments do not show the presence of *Candida*, corroborating the data shown in Fig. 6.

growth (both yeast and hyphal), and may represent a more general defect in fitness. We validated these results by showing that propolis can inhibit the yeast-to-hyphal al transition.

The compounds present in the propolis that are inhibiting the yeast-to-hyphal transition are yet to be discovered. However, farnesol and eugenol have already been identified in the Brazilian red propolis (Righi et al., 2011). *E*-nerolidol has been identified in the essential oil extracted from *Baccharis dracunculifolia*, the most important plant source of the Brazilian green propolis (Parreira et al., 2010; Johann et al., 2012), which has been shown to have hyphal-inhibiting activity (Shareck and Belhumeur, 2011). In *C. albicans* dimorphism and biofilm formation is affected by farnesol (Langford et al., 2009), while Hall et al. (2011) recently showed that farnesol directly inhibits adenylyl cyclase activity both *in vitro* and *in vivo*. *E*-nerolidol and dodecanol induced a reduction in both *C. albicans* and *C. dubliniensis* filamentation (Martins et al., 2007). Eugenol induced a severe modification in the *C. albicans* morphogenesis (Braga et al., 2007). Martins et al. (2012) have injected intraperitoneally a cocktail with different concentrations of isoamyl alcohol, phenylethanol, nerolidol, and farnesol in a murine model of hematogenously disseminated candidiasis. They demonstrated this treatment increased survival and decreased organ fungal burden when compared to the control mice. Thus, it is possible that the combination of these compounds and other unknown substances present in propolis are able to inhibit the yeast-to-hyphal transition.

Finally, we have shown that both the propolis based gels (G2 and G3) and cream (E3) were partially able to control vulvovaginal candidiasis in a mouse model. Our results strongly indicate that propolis represents a promising alternative therapeutic with great potential to control vulvovaginal candidiasis. Taken into consideration the fact that propolis is a complex substance with several possible antifungal active compounds, it may be more difficult for resistance to evolve in *C. albicans*, as it would require several concomitant mutations. Moreover, it is possible to increase its therapeutic value by combining propolis with other antifungal agents, such as azoles or caspofungin and with possible different delivery systems (pharmaceutical vehicles). Another promising aspect of this combined therapy is the fact that propolis inhibits the yeast-to-hyphal transition which contributes to the overall virulence of *C. albicans* and may even constitute a target for the development of antifungal drugs. Indeed, the inhibition of *C. albicans* morphogenesis has been suggested as a good target for drug development and controlling *C. albicans* infections (Saville et al., 2006; Shareck and Belhumeur, 2011). Actually, a large number of small molecules such as farnesol, fatty acids, rapamycin, geldanamycin,

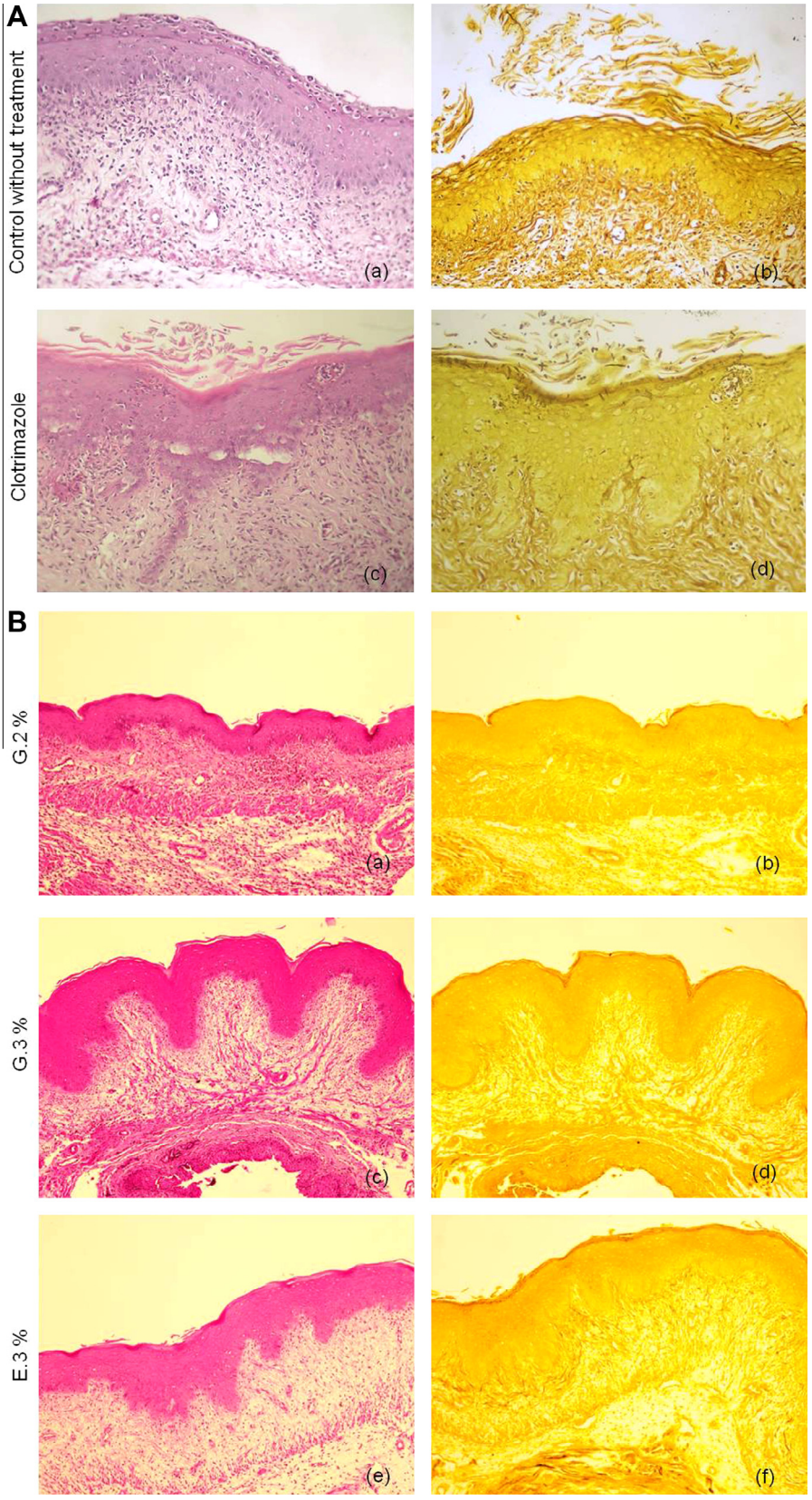

Fig. 8. Histological slices of mice vaginal mucosa 10 days after the interruption of the treatments, showing (A) infected control group (a and b) and Clotrimazole treated group (c and d). Histological analyses demonstrated that *C. albicans* infection causes vaginal lesion with neutrophils influx (a and c, HE staining). Also, the presence of *C. albicans* in both groups was shown by GMS staining (b and d). Propolis treatments (B) demonstrated restored vaginal tissue in all groups: G2 (a), G3 (c) and E3 (e), since normal tissue can be seen in all pictures with the absence of inflammation. The absence of *C. albicans* corroborated previous results seen in Fig. 7 (×40 magnification).

histone deacetylase and cell cycle inhibitors have been reported to modulate the yeast-to-hyphal transition in *C. albicans* (for a revision, see Shareck and Belhumeur, 2011). Once more, this opens the possibility to apply propolis in a combined therapy with these compounds.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.07.001.

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