UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE ANÁLISES CLÍNICAS E BIOMEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS E FISIOPATOLOGIA

FLÁVIA KELLY TOBALDINI VALÉRIO

Evaluation of antifungal activity of natural products against *Candida* species planktonic cells and biofilm

Maringá
2017

FLÁVIA KELLY TOBALDINI VALÉRIO

Evaluation of antifungal activity of natural products against *Candida* species planktonic cells and biofilm

Tese apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito para obtenção do título de Doutor em Biociências e Fisiopatologia
Área de concentração: Biociências e Fisiopatologia Aplicadas à Farmácia

Orientador: Prof.ª Dr.ª Terezinha Inez Estivalet Svidzinski

Maringá
2017

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

V164e

Valério, Flavia Kelly Tobaldini.
Evaluation of antifungal activity of natural products against *Candida* species planktonic cells and biofilm / Flavia Kelly Tobaldini Valério. -- Maringá, 2017.
93 f. : il., color., figs., tabs.

Orientadora: Prof. Dr. Terezinha Inez Estivalet Svidzinski.

Tese (Doutorado em Biociências e Fisiopatologia) - Universidade Estadual de Maringá. Centro de Ciencias da Saúde. Programa de Pós-Graduação em Biociências e Fisiopatologia, 2017.

1.Micologia. 2.Micologia Médica. 3. Antifungicos - Produtos Naturais. 4.Leveduras. 5.Candidíase.6.Propolis. 7. *Stevia reubaudiana*. I.Svidzinski, Terezinha Inez Estivalet, orient. II. Universidade Estadual de Maringá. Centro de Ciencias da Saúde. Programa de Pós-Graduação em Biociências e Fisiopatologia.

616.969 CDD 21.ed.

Cicilia Conceição de Maria
CRB9/1066
CC-003916

FOLHA DE APROVAÇÃO

FLÁVIA KELLY TOBALDINI VALÉRIO

Evaluation of antifungal activity of natural products against *Candida* species planktonic cells and biofilm

Tese apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Biociências e Fisiopatologia pela Comissão Julgadora composta pelos membros:

COMISSÃO JULGADORA

Prof.ª Dr.ª Terezinha Inez Estivalet Svidzinski
Universidade Estadual de Maringá (Presidente)

Prof.ª Dr.ª Paula Aline Zanetti Campanerut-Sá
Universidade Estadual de Maringá

Prof.ª Dr.ª Érika Seki Kioshima Cotica
Universidade Estadual de Maringá

Prof.ª Dr.ª Vânia Aparecida Vicente
Universidade Federal do Paraná

Prof. Dr. João Carlos Palazzo de Mello
Universidade Estadual de Maringá

Aprovada em: 27 de julho de 2017
Local de Defesa: sala 112-B – Bloco T-20, *campus* da Universidade Estadual de Maringá

AGRADECIMENTOS

Deus a ti sou muito grata por este presente maravilhoso que é a vida! Agradeço também pelas pessoas que o Senhor colocou em meu caminho. Algumas delas me inspiram, me ajudam, me desafiam e me encorajam a ser cada dia melhor.
Eu Te agradeço, Senhor, por todas as coisas boas e más que me aconteceram. Cada uma delas, ao seu modo, me fizeram chegar onde eu cheguei, e me fizeram ser quem eu sou. Foi a minha jornada de tropeços, vitórias e derrotas, que me fez enxergar o verdadeiro significado e beleza da vida.

Aos meus pais, minhas irmãs e meus cunhados, amo muito vocês!

Ao Eduardo, meu companheiro, pela compreensão e incentivo.

À minha segunda família, Carlos, Marilza, Mariana e Isadora, e todos que completam essa família o meu muiiito obrigada.

Aos colegas da Micologia-UEM quero agradecer o apoio, companheirismo e amizade ao longo destes anos.

Aos colegas e amigos do grupo de Biofilms da UMINHO agradeço muito, por toda receptividade ao longo da minha estadia, pela amizade e atenção.

Aos meus amigos mais próximos, Pat, Janine, Isis, Paula, Lu, Diana, Pris, o meu muito obrigada! Obrigada pela amizade, pelo apoio e por todos os momentos de alegria. Vocês são pessoas especiais em minha vida.

Quero agradecer à **Professora Terezinha,** minha orientadora, por ter confiado nas minhas capacidades e ter prestado todo o apoio e orientação necessários à execução deste trabalho. Obrigada também pela motivação e pela disponibilidade sempre demonstrada.

À minha segunda orientadora, Sonia Silva, obrigada pela oportunidade, obrigada pela amizade, obrigada por tudo que fizeste. Receba minha profunda gratidão!

As professoras Patricia, Erika e Melyssa, obrigada pelo apoio, pela motivação e pela disponibilidade sempre demonstrada.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) que, viabilizou o suporte financeiro.

A todos aqueles que de alguma forma contribuíram para a realização deste trabalho.

Avaliação da atividade antifúngica de produtos naturais contra células planctônicas e biofilme de *Candida* spp.

RESUMO

As infecções por *Candida* têm aumentado nos últimos anos, sendo uma importante causa de morbidade e mortalidade em pacientes hospitalizados. O gênero *Candida* apresenta susceptibilidade reduzida aos medicamentos antifúngicos comumente utilizados. Portanto, há necessidade de novas estratégias para combater infecções fúngicas e os produtos naturais estão encorajando o desenvolvimento de tratamentos alternativos. O objetivo deste estudo foi avaliar o efeito do extrato de própolis, *Hypericum androsaemum* e *Stevia reubaudiana* contra células planctônicas e biofilmes de espécies de *Candida*. Os valores de MIC, as curvas de morte, a lesão da membrana celular e a inibição da forma de filamentação foram determinados em células planctônicas de *Candida*. O efeito deste extrato sobre os biofilmes de *Candida* foi avaliado através da quantificação do número de células cultiváveis. Para extratos de própolis, os valores de CIM, variando de 220 a 880 μg/mL, demonstraram maior eficiência em *C. albicans* e *C. parapsilosis* do que nas células de *C. tropicalis*. Além disso, a própolis foi capaz de prevenir a formação de biofilmes das espécies de *Candida* e erradicar seus biofilmes maduros, juntamente com uma redução significativa na filamentação de *C. tropicalis* e *C. albicans*. Para o extracto de *H. androsaemum*, os efeitos anti-*Candida* variaram entre diferentes cepas da mesma espécie, as espécies *C. glabrata* e *C. tropicalis* apresentaram maior sensibilidade com efeito diretamente relacionado com as concentrações de extrato testadas. Também foi observado um potencial significativo de formação de biofilmes, nomeadamente para *C. glabrata* e *C. tropicalis* (redução de biofilme > 90%). Para o extracto de *Stevia reubaudiana* os valores de MIC obtidos para cepas de *Candida*, variando de 3,12 a 25 mg/mL, as cepas de *C. albicans* apresentaram o maior valor de MIC. Além disso, a capacidade de formar biofilmes de *C. tropicalis* e *C. glabrata* foram atenuados na presença de extrato de *Stevia*, com uma redução de 3 logs para *C. glabrata* ATCC 2001. Este estudo mostrou que esses extratos são potentes agentes antifúngicos com efeito sobre células e biofilmes de *Candida* spp. É importante ressaltar que esses efeitos não foram apenas observados contra *C. albicans*, mas em outras espécies NCAC. Este é um dado muito promissor, considerando que as espécies NCAC mostraram ser altamente resistentes aos agentes antifúngicos convencionais. Tais propriedades desses extratos como inibidor dos fatores de virulência de *Candida* representam caminhos alternativos e inovadores de terapia

para agentes patogênicos que são resistentes a agentes antimicrobianos clássicos.
Palavras-Chave: Candidíase. *Candida* spp. Propolis. *Stevia reubaudiana*. *Hypericum androsaemum*.

Evaluation of antifungal activity of natural products against *Candida* species planktonic cells and biofilm

ABSTRACT

Candida infections have been increasing in recent years, being even an important cause of morbidity and mortality in hospitalized patients. The genus *Candida* has been demonstrating a reduced susceptibility to the commonly used antifungal drugs. Therefore, there is a need for new strategies to combat fungal infections and natural products are encouraging the development of alternative treatments. The aim of this study was evaluate the effect of propolis, *Hypericum androsaemum* and *Stevia reubaudiana* extract against *Candida* species planktonic cells and its counterpart's biofilms. The MIC values, time-kill curves, cell membrane lesion and filamentation form inhibition were determined in *Candida* planktonic cells. The effect of these extract on *Candida* biofilms was assessed through quantification of number of cultivable cells. For propolis extract the MIC values, ranging from 220 to 880 μg/ml, demonstrated higher efficiency on *C. albican*s and *C. parapsilosis* than on *C. tropicalis* cells. In addition, propolis was able to prevent *Candida* species biofilm's formation and eradicate their mature biofilms, coupled with a significant reduction on *C. tropicalis* and *C. albicans* filamentation. For *Hypericum androsaemum* extract the anti-*Candida* effects varied among different strains of the same species, *C. glabrata* and *C. tropicalis* being the most sensible species with an effect directly related with the extract concentrations tested. A significant anti-biofilm formation potential was also observed, namely for *C. glabrata* and *C. tropicalis* (biofilm reduction >90%). For *Stevia reubaudiana* extract MIC values obtained for *Candida* strains, ranging from 3.12 to ≥ 25 mg/mL, with *Candida albicans* strains presented the highest MIC value. Moreover, *Candida tropicalis* and *Candida glabrata* ability to form biofilms was attenuate in the presence of *Stevia* extract, with a reduction of 3 orders of magnitude in the case of *C. glabrata* ATCC 2001 strain. This study showed that these extracts are potent antifungal agents with effect on *Candida* planktonic cells and biofilms. It is important to highlight, that these effects were not only observed against *C. albicans* but on other NCAC species. This is a very promising data, considering that NCAC species has shown to be highly resistant to the conventional antifungal agents. Such properties of this extracts as inhibitor of *Candida* virulence factors represent an alternative and innovative pathways of therapy for pathogens that are resistant to classical antimicrobial agents.

Keywords: Candidiasis. *Candida* spp. Propolis. *Stevia reubaudiana*. *Hypericum androsaemum*.

Tese elaborada e formatada conforme as normas das publicações científicas: *Future Microbiology* (artigo 1) Disponível em: https://www.futuremedicine.com/journal/fmb; Food Research International (artigo 2) Disponível em: www.elsevier.com/locate/foodres; e *Food Chemistry* (artigo 3) Disponível em: https://www.journals.elsevier.com/food-chemistry

SUMMARY

CHAPTER I

1. CANDIDIASIS

Fungal infections are common and affect the majority of the population at some stages in life. Yeasts of the genus *Candida* are the most frequent agents and more important due to the high frequency of colonization and infection in humans. The incidence of *Candida* infections has been increasing in recent years due to the widespread use of broad- spectrum antifungal agents and the growing numbers of immunocompromised individuals [1–3]

Candida species are opportunistic human pathogen, and are common colonizers the epithelial and mucosal surfaces, as oral cavity, gastrointestinal and genitourinary tract. *Candida albicans* colonizes 30–70% of healthy individuals [4]. However, under certain circunstances, *Candida* can show a wide spectrum of clinical presentations. This scenario covers a wide range of diseases from more superficial and milder clinical manifestations such as, oropharyngeal candidiasis or vulvovaginal candidiasis to serious infections including bloodstream infections and disseminated candidiasis, whereas the description of invasive candidiasis encompasses severe diseases such as candidemia, endocarditis, disseminated infections, central nervous system infections, endophthalmitis and osteomyelitis [5,6].

Any factor that weakens either the host or the competing microbial flora enables the fungus to overgrow and cause disease. A key variable that seems to transform a host colonized with *Candida* into a sick host is an alteration in one or more aspects of the host defense apparatus, including the normal bacterial flora, anatomical barriers, and immune system [7].

Mucosal candidiasis is extremely common and vastly more common than invasive, systemic candidiasis, although the latter receives far more attention due to the accompanying mortality. Nevertheless, mucosal candidiasis is responsible for considerable morbidity in hospitalized patients, often immunocompromised hosts but also in ambulatory, otherwise entirely healthy subjects [8]. It is a significant infectious disease problem and are often difficult to eradicate because of the high frequency of acquired resistance to conventional antifungal agents [9].

Oral candidiasis is a clinical fungal infection that is the most common opportunistic infection affecting the human oral cavity. The occurrence of oral candidiasis has been related

to several predisposing factors related to the host, including age and female gender, the use of dental prostheses, reduced salivary function, smoking habits, immunosuppressive diseases, and metabolic disorders, such as diabetes mellitus [10–13]. *C albicans*, the most prevalent yeast within the oral cavity, is a fundamental etiologic factor linked to the occurrence of oral candidiasis [14]. Nevertheless, the epidemiology of *Candida* infections has changed with the emergence of non-*albicans Candida* species, which have been increasingly described both in compromised and non-compromised hosts [14–17].

Vulvovaginal candidiasis is a common fungal infection experienced by up to 75% of women in their lifetime [18–20]. It is more likely to occur in an oestrogenised environment and therefore often affects pregnant women [20]. Recurrent vulvovaginal candidiasis affecting between 5–8% of women of reproductive age [19,20] but can greatly affect the quality of life of women, causing symptoms such as itching and soreness of the vulva, dyspareunia, dysuria and the classic 'cottage cheese-like' discharge. It is most commonly caused by *C. albicans*, but other species such as *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis* although less frequent are often cause recurrent vulvovaginal candidiasis [21].

Candidemia and invasive candidiasis, which is reported to be between the fourth and seventh most frequent causes of bloodstream infections in USA and Europe, and it is associated with high crude mortality rate, prolonged hospital stays, and high healthcare costs [22]. Five species of *Candida* (*C. albicans*, *C. glabrata, C. parapsilosis, C. tropicalis and Candida krusei*) account for more than 90% of all diagnosed cases, but their relative frequency varies depending on the population involved, geographical region, previous anti-fungal exposure and patient age [22].

For disseminated candidal infections, the most common clinical risk factors involve disruption of normal ecological and anatomical barriers which separate external compartments colonized by *Candida* from internal, normally sterile blood and tissue [23,24]. Such risk factors include the presence of central venous catheters; receipt of broad-spectrum antibacterials which facilitate fungal over-growth; gastrointestinal surgery which disrupts gastrointestinal anatomical barriers; and parenteral nutrition. Hence, the primary opportunity for *Candida* to systemically invade a mammalian host involves the elimination of bacterial competitors, allowing fungal overgrowth, and breakdown of anatomical barriers at surfaces normally colonized by *Candida*, which results in fungal penetration to deeper tissues. It is

also possible that subtle immune defects that reduce phagocyte antifungal activity may enable *Candida* to invade and proliferate in the host [25].

2. EPIDEMIOLOGY OF *Candida* SPECIES

Current data show that there are more than 150 species of *Candida*. However only 15 of these species are isolated from patients as infectious agents. These are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. lipolytica*, *C. famata*, *C. inconspicua*, *C. rugosa*, and *C. norvegensis*. The epidemiology of *Candida* infections is not constant, and the incidence rates, species distribution changes according to the site of infection and the geographic region. In the last 20–30 years, it has been determined that in 95% of infections, the pathogens involved are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* [26–28].

Among the *Candida* species, *C. albicans* has been designated as the most widespread involved opportunistic pathogenic yeast [29]. However, other non-*C. albicans Candida* (NCAC) species, such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, have been already and increasingly isolated mainly due to the indiscriminate prescription of antifungal agents [30–32]. This emergence is often associated to the advent of new medical procedures to treat cancer, the increase in invasive medical procedures, the incidence of HIV, and the widespread use of broad spectrum antibiotics [33–35].

The isolation rates of species other than *C. albicans* vary according to the features (age, underlying diseases, hospitalization ward, etc) of patient population. To illustrate, *C. parapsilosis* causes 30% of the candidemia cases among newborns whereas the rate is 10%–15% among adults. Since *C. parapsilosis* colonizes the skin, it is a common pathogen in catheter-related infections and may cause outbreaks [29,36,37]. *C. glabrata* is a more common infectious agent among older and neoplastic patients, previously exposed to azole and echinocandin. The infection with this species is associated with a high mortality rate [38]. *C. tropicalis*, is more commonly seen among leukemia and neutropenic patients and is often associated with nosocomial urinary tract infections [39–41]. *C. krusei*, on the other hand, is more common among hematopoietic stem cell recipients or neutropenic leukemia patients receiving fluconazole prophylaxis and this specie has been recognized as a potentially multidrug-resistant fungal pathogen [28,42,43].

3. PATHOGENESIS OF *Candida* SPECIES

Until a few decades ago, it was believed that *Candida* microorganisms passively

participated in the establishment of an opportunistic fungal infection, caused only by an organic weakness or an immunocompromised host. Today, there is consensus that these yeasts actively participate in the pathogenesis of the disease process, using mechanisms of aggression called virulence factors [44]. Thus, the pathogenicity of *Candida* species is mediated by a number of virulence factors that include morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes. Additionally, fitness attributes include rapid adaptation to fluctuation in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries [3,5,45].

3.1 DIMORPHISM

Candida species can grow in three cellular morphologies, depending on environmental conditions, nutrients, and temperature: yeast, pseudohyphae and hyphae. Many *Candida* spp. are capable of forming yeast and pseudohyphae, but only *C. albicans* and the genetically closely related *C. tropicalis* and *C. dubliniensis* can form hyphae as well, whereby hyphae differ from pseudohyphae because hyphae form long tube-like filaments, with parallel sides and no obvious constrictions between cells. In *C. albicans*, hyphal formation is known to promote several virulence mechanisms [46]. However, both yeast and filamentous forms of *C. albicans* can be observed in infected tissues during infection [47,48].

In general, yeast to hyphal morphogenesis is a key mechanism for *C. albicans* to evade killing by host phagocytic cells [49–51]. After ingestion of the *C. albicans* yeast form by macrophages, some *C. albicans* are killed; however, most of them survive and form hyphae in response to the phagosome environment. Phagocytosis thus induces a switch in morphology from yeast to hyphae, which elongates and eventually punctures the macrophage membrane. *C. albicans* in its hyphal form produces a number of factors that result in lysis and killing of macrophages, thereby allowing *C. albicans* to escape [52].

3.2. ADHESINS, INVASION AND BIOFILMS

Candida species are able to produce and secrete several hydrolytic enzymes, including proteases, phospholipases and haemolysins. The activity of these enzymes has been associated with candidal adhesion, cell damage and invasion of host tissue. Fungi such as *C. albicans* produce secreted aspartyl proteinases (SAPs), which play a role in supplying

nutrients for the *Candida* cells through protein degradation, facilitating penetration and invasion into host tissues in addition to evading immune responses [56,57].

The adherence of the organism to host and/or medical-device surfaces, often leading to the formation of biofilms [53]. Thus, adhesion is an extremely important step in the infection process, and the extent of adhesion is dependent on microbial, host and abiotic surface properties, such as cell-surface hydrophobicity and cell-wall composition [54,55]. An important factor that has correlated with the adhesion ability of *Candida* species is the presence of specific cell-wall proteins, often referred to as adhesins.

A further important virulence factor of *Candida* is its capacity to form biofilms on abiotic or biotic surfaces. Catheters, dentures (abiotic) and mucosal cell surfaces (biotic) are the most common substrates [58]. Biofilms form in a sequential process including adherence of yeast cells to the substrate, proliferation of these yeast cells, accumulation of extracellular matrix material and, finally, dispersion of yeast cells from the biofilm complex [59].

Preformed *Candida* biofilms have a mixture of morphological forms and consist of a dense network of yeasts, hyphae, and pseudohyphae in a matrix of polysaccharides, carbohydrate, protein, and unknown components. The formation and structure of *Candida* biofilms is influenced by the nature of the contact surface, environmental factors, *Candida* morphogenesis, and the *Candida* species involved [53].

Biofilms are difficult to eradicate with conventional therapy and thus represent a permanent focus of infection. Removal of the infected device is almost always necessary because of the drug resistance of *Candida* biofilms [60,61]. *Candida* in biofilms form was found to be 1000-fold less susceptible to diverse antifungals than planktonic ones [38,62] and are highly resistant to host immune factors. Factors such as upregulation of efflux pumps, upregulation of oxidative stress, changes in membrane sterol composition, and increased cell density might contribute to the resistance of biofilm to antifungal drug treatment [58]. Suggested mechanisms of biofilm resistance include restricted penetration of drugs through the matrix, slow growth of organisms in biofilms accompanied by changes in cell surface composition affecting their susceptibility to drugs, and unique biofilm-associated patterns of gene expression [63,64].

Furthermore, direct application of antifungal drugs is not yet acceptable in clinic. Side effects due to toxicity of the drugs and emergence of drug resistant strains have put limitations on the effective use of these drugs. This problem has led to the search for alternative antibiofilm agents to be used in the treatment of *Candida* infections [56].

4. THERAPEUTICS OPTIONS

Only few classes of antifungal agents are currently available to treat mucosal or systemic infections with *Candida*. The limited arsenal of antifungal drugs includes mainly polyenes, azoles and echinocandins [65–67].

Azoles (e.g., fluconazole, voriconazole and posaconazole) possess a fungistatic effect, blocking ergosterol synthesis, targeting the enzyme lanosterol 14α-demethylase (related to the ERG11 gene) and leading to an accumulation of toxic sterol pathway intermediates. Polyenes (e.g., amphotericin B and nystatin) are fungicidal, intercalating into membranes containing ergosterol, creating pores that destroy the proton gradient, which result in the outflow of the cytoplasm and other cell contents. Echinocandins (e.g., caspofungin, micafungin and anidulafungin) are also a fungicidal, targeting the synthesis of 1,3-β-glucan, a component of the *Candida* species cell wall [68,69].

The antifungal agents are the drug of choice depending on the infecting species and the clinical setting. The azole fluconazole is currently considered the first-line of drugs that is effective against most *Candida* species [70,71]. However, certain *Candida* species, such as *C. glabrata*, *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were found to have different degrees of susceptibility and were reported to have fluconazole resistance [34].

C. albicans isolates are the most susceptible to all of the antifungal agents. *C. parapsilosis* tends to have higher MICs for all of the echinocandin agents. *C. krusei* isolates have the highest fluconazole and flucytosine MICs of any of the species. Among the non-*albicans Candida* species, *C. tropicalis* and *C. parapsilosis* are both generally susceptible to azoles; however, *C. tropicalis* is less susceptible to fluconazole than is *C. albicans*. *C. glabrata* is intrinsically more resistant to antifungal agents, particularly to fluconazole. *C. krusei* is intrinsically resistant to fluconazole, and infections caused by this species are strongly associated with prior fluconazole prophylaxis and neutropenia. *Candida lusitaniae*, which accounts for 1–2% of all candidemias, is susceptible to azoles but has a higher intrinsic resistance to amphotericin B [72].

The current therapy with antifungals has serious drawbacks, in particular due to toxic effects to human cells and adverse effects [73,74]. As the drugs used to treat candidiasis are not always specific and properly prescribed (targeting the causative agent of infection), there has been a significant increase in resistance of *Candida* spp. to traditional antifungal drugs. The increasing microbial resistance rates may also be a result of long-term drug exposure or selection of strains with intrinsic resistance mechanisms [75–79]. Therefore, the development of novel strategies to minimize the toxic effects of current antifungals and improve their

effectiveness, has been strongly encouraged. The substantial attention has focused on natural products with antifungal properties, which has stimulated the search for therapeutic alternatives [66,80]. Therapies commonly called alternative, complementary and homemade have been used for centuries, and studies have intensively investigated plant species with medicinal properties to assess the feasibility, sustainability and affordability of the use of natural drugs [81,82].

5. ALTERNATIVE ANTIFUNGAL THERAPY

Opportunistic infections caused by *Candida* spp. have still been considered a recurrent health issue with high burden worldwide [83,84]. Novel antifungals are in high demand due to the challenges associated with resistant, persistent and systemic fungal infections. Thus, novel therapeutic approaches are much needed to treat *Candida* infections, including the use of naturally-occurring agents.

Natural products have continued to be a rich source of new drugs with clinically significant biological targets. Over the past 34 years, 49% of FDA-approved chemotherapeutic drugs were either natural products or directly derived therefrom [85]. There is a great interest of the pharmaceutical industry in the discovery of new molecules of natural origin or even their combination with existing drugs, in order to improve efficacy, potency, safety, tolerability, and decrease production costs, side effects and selection of resistant strains [86]. A number of studies in the literature have established the value of combined antifungal therapy against resistant strains, in particular standard drugs with naturally-occurring agents [87,88].

Natural products are promising therapeutic alternatives because they tend to display much smaller and lower intensity adverse reactions compared to allopathic drugs [89]. Natural compounds as sources for anti-*Candida* therapeutics from botanical sources have gained attention in the past decade (2004-2015) mainly because they display structural diversity and uniqueness in functional modes of action, which renders them as attractive candidates to counteract the emergence of *Candida* drug resistances [90,91].

5.1 PROPOLIS

Propolis (bee glue), is the resinous material that can be seen in different colors, is mostly collected by honey bees (*Apis mellifera* L.) from bud and exudates of the plants, mixed with bee enzymes, pollen and wax. Bees carry propolis to the bee hive where they use this dark adhesive substance to seal the walls of their hive to fortify the skeletons and

structures of combs, and also to mummify successful intruders' cadavers which bees have killed inside but cannot convey out of their hive to prevent their decomposition [92,93]. Propolis enables bees to protect their colony against hive invaders by minimizing the hive entrance size. Additionally, bees can preserve their society against several diseases, such as molds and bacterial infections, through the antimicrobial and antifungal properties of propolis [92].

The chemical groups of compounds identified in the propolis sample include flavonoids, aliphatic acids and esters, aromatic acids and esters, chalcones, terpenes, lignans, stilbenes, prenylated stilbenes, prenylated benzophenones, benzofuran, and sugarsm [94–96]. Propolis composition is completely variable, creating a problem for medical use and standardization [94,97]. Bankova (2005b) [98] has reported that the distinct chemistry of propolis from different origins could lead to different biological activities; however, different samples may display the same type of activity. A universal standardization of propolis would be difficult and complex, so that a detailed investigation of propolis composition and its botanical origin is imperative, because the composition of the plant source determines propolis composition [93]. As research progressed, more than 300 components have been identified in propolis [97].

Despite differences in propolis composition, studies at different times and in different regions have demonstrated that propolis has appreciable antibacterial [99,100], antifungal, and antiviral actions [101–103], as well as anti-inflammatory [104], anti-tumoral [105], antiulcerogenic [106,107], antidepressant, anxiolytic, and antioxidant properties [108–110], ascribed to propolis' flavonoid content.

Researches have demonstrated the antifungal activity of propolis against fungi such as *Candida* spp. [111–114]. Propolis also showed antifungal activity against dermatophytes [113,115], *C. neoformans* [116] and onychomycosis [115] and exhibited a synergistic effect with macrophages against *Paracoccidioides brasiliensis* [117]. Further, propolis has advantages such as a low cost and lack of toxicity [115].

5. 2 *Hypericum androsaemum*

Hypericum species (Hypericaceae family; nearly about 450 species) are plants with bioactive properties, being *Hypericum perforatum* L. the most widely known and with attributed anti-depressive, anti-inflammatory, antitumor, antimicrobial, antioxidant and antiviral benefits [118–120]. However, based on recent findings, other *Hypericum* species have also revealed very interesting functional properties.

The *Hypericum androsaemum* L. (Guttiferae) is a medicinal plant growing in West Europe, in damp or shady places namely, in the northern region of Portugal, where it is widely used as a medicinal herb. Infusion of *H. androsaemum* leaves has been used in traditional medicine as a cholagogue, hepatoprotector, diuretic and in kidney failure [121,122]. Hypericins, the photosensitizing naphthodianthrones characteristic of many of the species of the genus *Hypericum*, are known to be absent from *H. androsaemum* [123].

Phenolic compounds, such as flavonoids – mainly quercetin based – and two caffeoylquinic acids, [124] and phenolic acids, are known to be present in this species [125,126] and may contribute to its pharmacological effects. Xanthones can be present, but usually they occur in minor amounts in material grown in nature [124,126]. Recently, the antimicrobial, antitumor potential and the antioxidant activity of *Hypericum androsaemum* infusion was reported [118,119,127–130].

5.3 *Stevia rebaudiana*

Stevia rebaudiana Bertoni, a sweet plant native to South America [131], belonging to the Compositae (Asteraceae) family with significant economic value due to steviol glycoside sweeteners in its leaves. It is one of the 154 members of the *Stevia* genus and one of the only two species that produce sweet glycosides [132]. This plant is also cultivated in China and Southeast Asia [133]. The commercial exploitation of *S. rebaudiana* has become stronger since the 1970's, after the development in Japan of processes for the extraction and refinement of its leaf sweetener [134]. The sweet taste of *Stevia* leaves depends on the high content of stevioside and rebaudioside A which are about 250–300 times as sweet as sucrose [135] and with the advantages of not being caloric and avoiding high levels of blood sugar [136]. The high content of natural sweeteners, makes *Stevia rebaudiana* of a significant economic value in food industry in many applications as a “zero calorie sweetener”.

In addition, *S. rebaudiana* contains other metabolites with bioactive potential, such as flavonoids, alkaloids, water-soluble chlorophylls, xanthophylls, hydroxycynnamoyl derivatives (caffeoyl and chlorogenic acid derivatives), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements [137].

Likewise, *S. rebaudiana* sweetener extractives are suggested to exert beneficial effects on human health, including anti-hypertensive, anti-hyperglycemic and anti-human rotavirus activities [138–140]. It also has been shown to possess antimicrobial, antiinflammatory and antioxidant activities [141–143]. It has been reported that antibacterial activity of the extracts is due to the flavonoids, aromatic acids, terpenoids, and its esters contents [144].

JUSTIFICATIVE

Candida infections account for a high burden of morbidity and mortality. Prolonged usage of antifungal agents to treat infections caused by *Candida* species has led to the emergence of resistance to conventional drugs. Considering this, new therapeutic approaches are urgently needed to improve the outcome of the patients, as the currently available treatment options have not reduced the mortality and morbidity associated with *Candida* infections over the recent years. Moreover, it has crucial to explore alternative strategies to overcome the limitations of current therapies, especially against resilient biofilm-associated fungal infections and others virulence factors.

Possible solutions to improve the efficacy of the treatment would be the use of combined antifungal drugs and the exploration of alternatives compounds as antifungal agents (extracts of plants and isolated essential oils). One of the most promissory sources for the research of new agents is actually found in plants, which have compounds with antimicrobial properties that are only now beginning to be studied. The investigation of these active principles may be a potential area that must be explored.

OBJECTIVES

GENERAL

Investigate the effect of propolis, *Hypericum androsaemum* and *Stevia rebaudiana* extracts on clinical isolates of *Candida* spp. planktonic cells. In addition to evaluate of morphologic alteration and inhibition of virulence factors caused by these extracts on clinical isolates of *Candida* spp.

SPECIFICS

Article 1:

- Characterization of propolis extract;
- To evaluate the antifungal activity of propolis extract, against 14 *C. albicans* (12 isolates from blood and two from urine), 14 *C. parapsilosis* (13 from blood and one from urine) and 14 *C. tropicalis* (four from blood and ten from urine) and the respective reference strains;
- Determinate the time kill curves for propolis extract against three *Candida* reference strains;
- To evaluate the effect of propolis extract on filamentation form transition against four *C. albicans*, four *C. parapsilosis* and four *C. tropicalis* clinical isolates, and their respective references strains;
- To evaluate the influence of propolis extract on biofilm formation and preformed biofilms of *Candida* spp. clinical isolates, and their respective references strains;
- To evaluate the cytotoxicity of propolis extract on fibroblasts 3T3;

Article 2:

- To characterize of *H. androsaemum* extract;
- To evaluate of the antioxidant activity of *H. androsaemum* extract;
- To evaluate of the cytotoxic activity of *H. androsaemum* extract;
- To evaluate of the anti-inflammatory activity of *H. androsaemum* extract;
- To evaluate the antifungal activity of *H. androsaemum* extract, against 8 *Candida* strains belonging to the species *C. albicans*, *C. glabrata, C. parapsilosis* and *C. tropicalis* clinical isolates and the respective reference strains;
- To determinate the time kill curves for *H. androsaemum* extract against four *Candida* reference strains;
- To determinate the cell membrane lesion caused by *H. androsaemum* extract on four

Candida reference strains;

- To evaluate the influence of *H. androsaemum* extract on biofilm formation of *Candida* spp. clinical isolates, and their respective references strains;
- To characterize of *S. rebaudiana* extract;
- To evaluate the antifungal activity of *S. rebaudiana* extract, against 8 *Candida* strains belonging to the species *C. albicans*, *C. glabrata, C. parapsilosis* and *C. tropicalis* clinical isolates and the respective reference strains;
- To determinate the time kill curves for *S. rebaudiana* extract against four *Candida* reference strains;
- To evaluate the influence of *S. rebaudiana* extract on biofilm formation of *Candida* spp. clinical isolates, and their respective references strains;

References

1. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* 36(2), 288–305 (2012).
2. Rodrigues ME, Silva S, Azeredo J, Henriques M. Novel strategies to fight *Candida* species infection. *Crit. Rev. Microbiol*. 7828, 1–13 (2014).
3. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. Adherence and biofilm formation of non-*Candida albicans Candida* species. *Trends Microbiol*. 19(5), 241–7 (2011).
4. Gow NAR, van de Veerdonk FL, Brown AJP, *et al. Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat. Rev. Microbiol*. 10(2), 112–22 (2012).
5. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida a M, Mendes Giannini MJS. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol*. 62(Pt 1), 10–24 (2013).
6. McCarty TP, Pappas PG. Invasive Candidiasis. *Infect. Dis. Clin. North Am*. 30(1), 103–124 (2016).
7. Calderone RA, Clancy CJ. *Candida* and candidiasis. ASM Press, Washington.
8. Vazquez JA, Sobel JD. Mucosal candidiasis. *Infect Dis Clin North Am.* 16(4), 793–820 (2002).
9. Akins RA. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Med. Mycol*. 43(4), 285–318 (2005).
10. Sanit PV, Pavarina AC, Giampaolo ET, *et al. Candida* spp. prevalence in well controlled type 2 diabetic patients with denture stomatitis. *Oral Surgery, Oral Med. Oral Pathol. Oral Radiol. Endodontology*. 111(6), 726–733 (2011).
11. Silva MM, Mima EGDO, Colombo AL, *et al.* Comparison of denture microwave disinfection and conventional antifungal therapy in the treatment of denture stomatitis: A randomized clinical study. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* 114(4), 469–479 (2012).
12. Gendreau L, Loewy ZG. Epidemiology and Etiology of Denture Stomatitis. *J. Prosthodont.* 20(4), 251–260 (2011).
13. Mima EG, Vergani CE, Machado a L, *et al.* Comparison of Photodynamic Therapy versus conventional antifungal therapy for the treatment of denture stomatitis: a randomized clinical trial. *Clin. Microbiol. Infect*. 18(10), E380–E388 (2012).
14. Sanitá PV, Zago CE, Mima EG de O, *et al. In vitro* evaluation of the enzymatic activity profile of non-*albicans Candida* species isolated from patients with oral candidiasis with or without diabetes. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol*. 118(1), 84–91 (2014).
15. Motta-Silva AC, Aleva NA, Chavasco JK, Armond MC, França JP, Pereira LJ. Erythematous oral candidiasis in patients with controlled type II diabetes mellitus and complete dentures. *Mycopathologia*. 169(3), 215–223 (2010).
16. Manfredi M, McCullough MJ, Al-Karaawi ZM, Hurel SJ, Porter SR. The isolation, identification and molecular analysis of *Candida* spp. isolated from the oral cavities of patients with diabetes mellitus. *Oral Microbiol. Immunol.* 17(3), 181–185 (2002).
17. Belazi M, Velegraki A, Fleva A, *et al.* Candidal overgrowth in diabetic patients: Potential predisposing factors. *Mycoses*. 48(3), 192–196 (2005).
18. Sobel JD. Recurrent vulvovaginal candidiasis. *Am. J. Obstet. Gynecol*. 214(1), 15–21 (2016).

19. Foxman B, Marsh J V, Gillespie B, Sobel JD. Frequency and response to vaginal symptoms among white and African American women: results of a random digit dialing survey. *J. Womens. Health*. 7(9), 1167–74 (1998).
20. Sobel JD. Vulvovaginal candidosis. *Lancet*. 369(9577), 1961–1971 (2007).
21. Matheson A, Mazza D. Recurrent vulvovaginal candidiasis: A review of guideline recommendations. *Aust. New Zeal. J. Obstet. Gynaecol.* 57(2), 139–145 (2017).
22. Antinori S, Milazzo L, Sollima S, Galli M, Corbellino M. Candidemia and invasive candidiasis in adults: A narrative review. *Eur. J. Intern. Med*. (2016).
23. PG P, Kauffman CA, Andes D. Clinical practice guidelines for the management of Candidiasis: 2009 update by the infectious diseases society of America. *Clin Infect Dis*. 48(5), 503–535 (2009).
24. Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* 45(4), 321–346 (2007).
25. Brad Spellberg, Marr KA, Filler SG. *Candida*: What Should Clinicians and Scientists Be Talking About? In: *Candida and Candidiasis*. Calderone RA, Clancy CJ (Eds.). . ASM Press, Washington, DC, 1–7 (2012).
26. Lewis RE. Supplement: Trends in invasive *Candida* infections and their treatment: focus on echinocandins. *Curr. Med. Res. Opin.* 25(7), 1729–1762 (2009).
27. Diekema D, Arbefeville S, Boyken L, Kroeger J, Pfaller M. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn. Microbiol. Infect. Dis.* 73(1), 45–8 (2012).
28. Pappas PG. Invasive Candidiasis. *Infect. Dis. Clin. North Am.* 20(3), 485–506 (2006).
29. Guinea J, Martin G, Mannino D, *et al.* Global trends in the distribution of *Candida* species causing candidemia. (2014).
30. Estivill D, Arias A, Torres-Lana A, Carrillo-Muñoz AJ, Arévalo MP. Biofilm formation by five species of *Candida* on three clinical materials. *J. Microbiol. Methods*. 86(2), 238–242 (2011).
31. Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect. Immun.* 70, 878–888 (2002).
32. Gácser A, Schäfer W, Nosanchuk JS, Salomon S, Nosanchuk JD. Virulence of *Candida parapsilosis, Candida orthopsilosis,* and *Candida metapsilosis* in reconstituted human tissue models. *Fungal Genet. Biol.* 44(12), 1336–1341 (2007).
33. Arendrup MC. *Candida* and Candidaemia: Susceptibility and Epidemiology. *Dan. Med. J.* 60(11), 1–32 (2013).
34. Sanguinetti M, Posteraro B, Lass-Flörl C. Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses*. 58, 2–13 (2015).
35. Krcmery V, Barnes a. J. Non-*albicans Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. *J. Hosp. Infect*. 50(4), 243–260 (2002).
36. Bonassoli LA, Bertoli M, Svidzinski TIE. High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J. Hosp. Infect.* 59(2), 159–162 (2005).
37. Falagas ME, Roussos N, Vardakas KZ. Relative frequency of *Candida albicans* and the various non-*albicans Candida* spp among candidemia isolates from inpatients in various parts of the world: A systematic review. *Int. J. Infect. Dis*. 14(11), e954–e966 (2010).
38. Hajjeh RA, Sofair AN, Harrison LH, *et al.* Incidence of bloodstream infections due to *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* 42(4), 1519–27 (2004).
39. Rho J, Shin JH, Song JW, *et al.* Molecular Investigation of Two Consecutive

Nosocomial Clusters of *Candida tropicalis* Candiduria Using Pulsed - Field Gel Electrophoresis. *J. Microbiol.* 42(2), 80–86 (2004).

40. Nucci M, Colombo AL. Candidemia due to *Candida tropicalis*: clinical, epidemiologic, and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagn. Microbiol. Infect. Dis.* 58(1), 77–82 (2007).
41. Weinberger M, Leibovici L, Perez S, *et al.* Characteristics of candidaemia with *Candida albicans* compared with non-*albicans Candida* species and predictors of mortality. *J. Hosp. Infect.* 61(2), 146–154 (2005).
42. Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Buitrago MJ, Monzon A, Rodriguez-Tudela JL. Head-to-head comparison of the activities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeasts and filamentous fungi. *Antimicrob. Agents Chemother.* 50(3), 917–21 (2006).
43. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin. Microbiol. Rev.* 20(1), 133–163 (2007).
44. Tamura NK, Fernanda M, Negri N, Bonassoli LA, Inez T, Svidzinski E. Fatores de virulência de *Candida* spp isoladas de cateteres venosos e mãos de servidores hospitalares Virulence factors for Candida spp recovered from intravascular catheters and hospital workers' hands. *Med. Trop. Rev. da Soc. Bras. Med. Trop.* 40(401), 91–93 (2007).
45. Mayer FL, Wilson D, Hube B, *et al. Candida albicans* pathogenicity mechanisms. *Virulence*. 4(July), 119–128 (2013).
46. Thompson DS, Carlisle PL, Kadosh D. Coevolution of morphology and virulence in *Candida* species. *Eukaryot. Cell*. 10(9), 1173–82 (2011).
47. Corner BE, Magee PT. *Candida* pathogenesis: Unravelling the threads of infection. *Curr. Biol.* 7(11), R691–R694 (1997).
48. Eckert SE, Sheth CC, Mühlschlegel FA. Regulation of morphogenesis in *Candida* species. In: *Candida. Comparative and functional genomics*. , 263–291 (2007).
49. Tsui C, Kong EF, Jabra-Rizk MA. Pathogenesis of *Candida albicans* biofilm. *Pathog. Dis.* 74(4), ftw018 (2016).
50. De Bernardis F, Arancia S, Sandini S, Graziani S, Norelli S. Studies of Immune Responses in *Candida* vaginitis. *Pathogens*. 4(4), 697–707 (2015).
51. Netea MG, Joosten LAB, van der Meer JWM, Kullberg B-J, van de Veerdonk FL. Immune defence against *Candida* fungal infections. *Nat. Rev. Immunol.* 15(10), 630–642 (2015).
52. Krysan DJ, Sutterwala FS, Wellington M. Catching fire: *Candida albicans*, macrophages, and pyroptosis. *PLoS Pathog.* [Internet]. 10(6), e1004139 (2014).
53. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm Formation by the Fungal Pathogen *Candida albicans*: Development, Architecture, and Drug Resistance. *J. Bacteriol.* 183(18), 5385–5394 (2001).
54. Henriques M, Gasparetto K, Azeredo J, Oliveira R. Experimental methodology to quantify *Candida albicans* cell surface hydrophobicity. *Biotechnol. Lett.* 24(13), 1111–1115 (2002).
55. Chaffin WL. *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* 72(3), 495–544 (2008).
56. Gauwerky K, Borelli C, Korting HC. Targeting virulence: A new paradigm for antifungals. *Drug Discov. Today*. 14(3–4), 214–222 (2009).
57. Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martínez JP. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol. Mol. Biol. Rev.* 62(1), 130–80 (1998).
58. Fanning S, Mitchell AP, Daniels K, Lu K, Srikantha T. Fungal Biofilms. *PLoS Pathog.*

8(4), e1002585 (2012).
59. Finkel JS, Mitchell AP. Genetic control of *Candida albicans* biofilm development. *Nat. Rev. Microbiol.* 9(2), 109–118 (2011).
60. Kojic EM, Darouiche RO. *Candida* Infections of Medical Devices. Clin. Microbiol. Rev. 17(2), 255–267 (2004).
61. Pappas PG, Mycoses Study Group. Candidemia in the intensive care unit: miles to go before we sleep. *Crit. Care Med.* 39(4), 884–5 (2011).
62. Nett JE, Andes D. Fungal biofilms: *in vivo* models for discovery of anti-biofilm drugs. *Microbiol. Spectr.* 3(3), E30 (2015).
63. Douglas LJJ. *Candida* biofilms and their role in infection. *Trends Microbiol*. 11(1), 30–36 (2003).
64. Kumamoto CA. *Candida* biofilms. *Curr. Opin. Microbiol.* 5(6), 608–11 (2002).
65. Dimopoulos G, Antonopoulou A, Armaganidis A, Vincent J-L. How to select an antifungal agent in critically ill patients. *J. Crit. Care*. 28(5), 717–727 (2013).
66. Kathiravan MK, Salake AB, Chothe AS, *et al.* The biology and chemistry of antifungal agents: A review. *Bioorg. Med. Chem.* 20(19), 5678–5698 (2012).
67. Denning DW, Hope WW. Therapy for fungal diseases: opportunities and priorities. *Trends Microbiol.* 18(5), 195–204 (2010).
68. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* 11(2), 382–402 (1998).
69. Pappas PG, Kauffman CA, Andes DR, *et al.* Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 62(4), civ933 (2015).
70. Lalla R V, Patton LL, Dongari-Bagtzoglou A. Oral candidiasis: pathogenesis, clinical presentation, diagnosis and treatment strategies. *J. Calif. Dent. Assoc.* 41(4), 263–8 (2013).
71. Serpa R, Franca EJG, Furlaneto-Maia L, Andrade CGTJ, Diniz A, Furlaneto MC. *In vitro* antifungal activity of the flavonoid baicalein against *Candida* species. *J. Med. Microbiol.* 61(Pt_12), 1704–1708 (2012).
72. Cruciani M, Serpelloni G. Management of *Candida* infections in the adult intensive care unit. *Expert Opin. Pharmacother.* 9(2), 175–191 (2008).
73. Gabler IG, Barbosa AC, Velela RR, Lyon S, Rosa CA. Incidence and anatomic localization of oral candidiasis in patients with AIDS hospitalized in a public hospital in Belo Horizonte, MG, Brazil. *J. Appl. Oral Sci.* 16(4), 247–50 (2008).
74. Epstein JB, Dawson JR, Buivids IA, Wong B, Le ND. The effect of a disinfectant/coolant irrigant on microbes isolated from dental unit water lines. *Spec Care Dent.* 22(4), 137–41 (2008).
75. Ying Y, Zhao Y, Hu X, *et al.* In vitro fluconazole susceptibility of 1,903 clinical isolates of *Candida albicans* and the identification of ERG11 mutations. *Microb. Drug Resist.* 19(4), 266–73 (2013).
76. Fernández-Ruiz M, Puig-Asensio M, Guinea J, *et al. Candida tropicalis* bloodstream infection: Incidence, risk factors and outcome in a population-based surveillance. *J. Infect.* 71(3), 385–94 (2015).
77. Liao X, Qiu H, Li R, *et al.* Risk factors for fluconazole-resistant invasive candidiasis in intensive care unit patients: An analysis from the China Survey of Candidiasis study. *J. Crit. Care*. 30(4), 862.e1-5 (2015).
78. Seifi Z, Zarei Mahmoudabadi A, Zarrin M. Extracellular enzymes and susceptibility to fluconazole in Candida strains isolated from patients with vaginitis and healthy individuals. *Jundishapur J. Microbiol.* 8(3), e20162 (2015).
79. Freitas EM de, Monteiro LC, Fernandes MB da S, Martelli Junior H, Bonan PRF,

Nobre SAM. Antifungal susceptibility in vitro determined by the Etest® for *Candida* obtained from the oral cavity of irradiated and elderly individuals. *Braz. Dent. J.* 26(2), 99–104 (2015).

80. Paiva JA, Pereira JM. New antifungal antibiotics. *Curr. Opin. Infect. Dis.* 26(2), 168–174 (2013).
81. Iwu M. International Conference on Ethnomedicine and Drug Discovery. *J. Altern. Complement. Med.* 6(1), 3–5 (2000).
82. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA. The formulation of an effective topical antibacterial product containing Ocimum gratissimum leaf essential oil. *Int. J. Pharm.* 224(1–2), 177–83 (2001).
83. Rodriguez-Tudela JL, Alastruey-Izquierdo A, Gago S, *et al.* Burden of serious fungal infections in Spain. *Clin. Microbiol. Infect.* 21(2), 183–189 (2015).
84. Denning DW, Gugnani HC. Burden of serious fungal infections in Trinidad and Tobago. *Mycoses*. 58 Suppl 5, 80–4 (2015).
85. Newman DJ, Cragg GM. Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79(3), 629–661 (2016).
86. Svetaz LA, Postigo A, Butassi E, Zacchino SA, Sortino MA. Antifungal drugs combinations: a patent review 2000-2015. *Expert Opin. Ther. Pat.* 26(4), 439–453 (2016).
87. Pippi B, Lana a. JD, Moraes RC, *et al. In vitro* evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. *J. Appl. Microbiol.* (2015).
88. Han B, Chen J, Yu Y, Cao Y, Jiang Y. Antifungal activity of *Rubus chingii* extract combined with fluconazole against fluconazole-resistant *Candida albicans*. *Microbiol. Immunol.* 60(2), 82–92 (2016).
89. Vicente MF, Basilio A, Cabello A, Peláez F. Microbial natural products as a source of antifungals. *Clin. Microbiol. Infect.* 9(1), 15–32 (2003).
90. Kamba AS, Hassan LG. Phytochemical screening and antimicrobial activities of *Euphorbia balsamifera* leaves, stems and root against some pathogenic microorganisms. *African J. Pharm. Pharmacol.* 4(9), 645–652 (2010).
91. Addy BS, Owodo HT, Nana R, Gyapong K, Umeji CO, Mintah N. Phytochemical Screening and Antimicrobial Study on the Leaves of *Morinda lucida* (Rubiaceae). *J. Nat. Sci.* 3(14), 131–135 (2013).
92. Bunney MH. Contact Dermatitis In Beekeepees Due To Propolis (Bee Glue). *Br. J. Dermatol.* 80(1), 17–23 (1968).
93. Bankova V. Chemical diversity of propolis and the problem of standardization. *J. Ethnopharmacol.* 100(1–2), 114–117 (2005).
94. Marcucci MC. Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie*. 26(2), 83–99 (1995).
95. Bankova VS, de Castro SL, Marcucci MC. Propolis: recent advances in chemistry and plant origin. *Apidologie*. 31(1), 3–15 (2000).
96. Abu-Mellal A, Koolaji N, Duke RK, Tran VH, Duke CC. Prenylated cinnamate and stilbenes from Kangaroo Island propolis and their antioxidant activity. *Phytochemistry*. 77, 251–259 (2012).
97. De Castro SL. Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. *Ann Rev Biomed Sci*. 3, 49–83 (2001).
98. Bankova V. Recent trends and important developments in propolis research. *Evidence-based Complement. Altern. Med.* 2(1), 29–32 (2005).
99. Nedji N, Loucif-Ayad W. Antimicrobial activity of Algerian propolis in foodborne pathogens and its quantitative chemical composition. *Asian Pacific J. Trop. Dis.* 4(6),

433–437 (2014).
100. Jug M, Končić MZ, Kosalec I. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemotype propolis by extraction procures. *LWT - Food Sci. Technol.* 57(2), 530–537 (2014).
101. Agüero MB, Svetaz L, Baroni V, *et al.* Urban propolis from San Juan province (Argentina): Ethnopharmacological uses and antifungal activity against *Candida* and dermatophytes. *Ind. Crops Prod.* 57, 166–173 (2014).
102. Bankova V, Galabov AS, Antonova D, Vilhelmova N, Di Perri B. Chemical composition of Propolis Extract ACF® and activity against *Herpes Simplex virus*. *Phytomedicine*. 21(11), 1432–1438 (2014).
103. Kai H, Obuchi M, Yoshida H, *et al. In vitro* and *in vivo* anti-influenza virus activities of flavonoids and related compounds as components of Brazilian propolis (AF-08). *J. Funct. Foods*. 8, 214–223 (2014).
104. Wang K, Zhang J, Ping S, *et al.* Anti-inflammatory effects of ethanol extracts of Chinese propolis and buds from poplar (Populus×canadensis). *J. Ethnopharmacol.* 155(1), 300–311 (2014).
105. Novak EM, Silva MS e C, Marcucci MC, *et al.* Antitumoural activity of Brazilian red propolis fraction enriched with xanthochymol and formononetin: An *in vitro* and *in vivo* study. *J. Funct. Foods*. 11, 91–102 (2014).
106. de Barros MP, Sousa JPB, Bastos JK, de Andrade SF. Effect of Brazilian green propolis on experimental gastric ulcers in rats. *J. Ethnopharmacol.* 110(3), 567–571 (2007).
107. Barros MP de, Lemos M, Maistro EL, *et al.* Evaluation of antiulcer activity of the main phenolic acids found in Brazilian Green Propolis. *J. Ethnopharmacol.* 120(3), 372–377 (2008).
108. Reis JSS, Oliveira GB, Monteiro MC, *et al.* Antidepressant- and anxiolytic-like activities of an oil extract of propolis in rats. *Phytomedicine*. 21(11), 1466–1472 (2014).
109. Potkonjak NI, Veselinović DS, Novaković MM, Gorjanović SŽ, Pezo LL, Sužnjević DŽ. Antioxidant activity of propolis extracts from Serbia: A polarographic approach. *Food Chem. Toxicol.* 50(10), 3614–3618 (2012).
110. Castro C, Mura F, Valenzuela G, *et al.* Identification of phenolic compounds by HPLC-ESI-MS/MS and antioxidant activity from Chilean propolis. *Food Res. Int.* 64, 873–879 (2014).
111. Ota C, Unterkircher C, Fantinato V, Shimizu MT. Antifungal activity of propolis on different species of *Candida*. *Mycoses*. 44(9–10), 375–8 (2001).
112. Dalben-Dota KF, Faria MGI, Bruschi ML, Pelloso SM, Lopes-Consolaro ME, Svidzinski TIE. Antifungal activity of propolis extract against yeasts isolated from vaginal exudates. *J. Altern. Complement. Med.* 16(3), 285–290 (2010).
113. Monzote L, Cuesta-Rubio O, Campo Fernandez M, *et al. In vitro* antimicrobial assessment of Cuban propolis extracts. *Mem. Inst. Oswaldo Cruz*. 107(8), 978–84 (2012).
114. Bonvehí JS, Gutiérrez AL. The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain). *World J. Microbiol. Biotechnol.* 28(4), 1351–8 (2012).
115. Oliveira ACP, Shinobu CS, Longhini R, Franco SL, Svidzinski TIE. Antifungal activity of propolis extract against yeasts isolated from onychomycosis lesions. *J. Altern. Complement. Med.* 101(5), 493–497 (2006).
116. Fernandes FF, Dias ALT, Ramos CL, Ikegaki M, de Siqueira AM, Franco MC. The "*in vitro*" antifungal activity evaluation of propolis G12 ethanol extract on *Cryptococcus*

neoformans. *Rev. Inst. Med. Trop. Sao Paulo*. 49(2), 93–5 (2007).
117. Murad JM, Calvi SA, Soares AMVC, Bankova V, Sforcin JM. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. *J. Ethnopharmacol.* 79(3), 331–4 (2002).
118. Nogueira T, Medeiros MA, Marcelo-Curto MJ, García-Pérez BE, Luna-Herrera J, Costa MC. Profile of antimicrobial potential of fifteen *Hypericum* species from Portugal. *Ind. Crops Prod.* 47, 126–131 (2013).
119. Rainha N, Lima E, Baptista J. Comparison of the endemic Azorean *Hypericum foliosum* with other *Hypericum* species: antioxidant activity and phenolic profile. *Nat. Prod. Res.* 25(February 2015), 123–135 (2011).
120. Ramalhete N, Machado A, Serrano R, Gomes ET, Mota-Filipe H, Silva O. Comparative study on the *in vivo* antidepressant activities of the Portuguese *Hypericum foliosum*, *Hypericum androsaemum* and *Hypericum perforatum* medicinal plants. *Ind. Crops Prod.* 82, 29–36 (2016).
121. Costa AF. Farmacognosia. Volume II. Fundação Calouste Gulbenkian, Lisboa, Portugal.
122. Seabra RM, Vasconcelos MH. Análise de amostras de hipericão existentes no mercado. *ReV. Port. Farmacogn.* 17 (1992).
123. Mathis C, Ourisson G. Étude chimio-taxonomique du genre *Hypericum* I. Répartition de l'hypéricine. *Phytochemistry*. 2(2), 157–171 (1963).
124. Dias ACP, Seabra RM, Andrade PB, Fernandes-Ferreira M. The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from In vivo and In vitro biomass of *Hypericum* species. *J. Liq. Chromatogr. Relat. Technol.* 22(2), 215–227 (1999).
125. Valentão P, Dias A, Ferreira M, *et al.* Variability in phenolic composition of *Hypericum androsaemum*. *Nat. Prod. Res*. 17(2), 135–140 (2003).
126. Schmidt W, El-Mawla AMAA, Wolfender JL, Hostettmann K, Beerhues L. Xanthones in cell cultures of *Hypericum androsaemum*. *Planta Med.* 66(4), 380–381 (2000).
127. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos M de L. Antioxidant Activity of *Hypericum androsaemum* Infusion: Scavenging Activity against Superoxide Radical, Hydroxyl Radical and Hypochlorous Acid. *Biol. Pharm. Bull.* 25(10), 1320–1323 (2002).
128. Hernandez MF, Falé PLV, Araújo MEM, Serralheiro MLM. Acetylcholinesterase inhibition and antioxidant activity of the water extracts of several *Hypericum* species. *Food Chem.* 120(4), 1076–1082 (2010).
129. Ramos AA, Marques F, Fernandes-Ferreira M, Pereira-Wilson C. Water extracts of tree *Hypericum* spp. protect DNA from oxidative and alkylating damage and enhance DNA repair in colon cells. *Food Chem. Toxicol.* 51, 80–86 (2013).
130. Xavier CPR, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C. *Hypericum androsaemum* water extract inhibits proliferation in human colorectal cancer cells through effects on MAP kinases and PI3K/Akt pathway. *Food Funct.* 3, 844 (2012).
131. Carocho M, Morales P, Ferreira ICFR. Natural food additives: Quo vadis? *Trends Food Sci. Technol.* 45(2), 284–295 (2015).
132. Madan S, Ahmad S, Sing GN, *et al. Stevia rebaudiana* Bertoni : A review. *Indian J. Nat. Prod. Resour.* 1(3), 267–286 (2010).
133. Koyama E, Kitazawa K, Ohori Y, *et al. In vitro* metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem. Toxicol.* 41(3), 359–374 (2003).
134. Dacome AS, da Silva CC, da Costa CEM, Fontana JD, Adelmann J, da Costa SC. Sweet diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert.)

Bertoni: Isolation and quantitative distribution by chromatographic, spectroscopic, and electrophoretic methods. *Process Biochem.* 40(11), 3587–3594 (2005).

135. Gardana C, Scaglianti M, Simonetti P. Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. A*. 1217(9), 1463–1470 (2010).
136. Periche A, Castelló ML, Heredia A, Escriche I. Influence of drying method on steviol glycosides and antioxidants in *Stevia rebaudiana* leaves. *Food Chem.* 172, 1–6 (2015).
137. Komissarenko N, Derkach A, Kovalyov I, Bublik N. Diterpene glycosides and phenylpropanoids of *Stevia rebaudiana* Bertoni (Asteraceae). *Rast. Res.* 1(2), 53–64 (1994).
138. Jeppesen PB, Gregersen S, Alstrup KK, Hermansen K. Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects *in vivo*: studies in the diabetic Goto-Kakizaki (GK) rats. *Phytomedicine*. 9(1), 9–14 (2002).
139. Chan P, Tomlinson B, Chen YJ, Liu JC, Hsieh MH, Cheng JT. A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *Br. J. Clin. Pharmacol.* 50(3), 215–220 (2000).
140. Lee KG, Shibamoto T. Inhibition of malonaldehyde formation from blood plasma oxidation by aroma extracts and aroma components isolated from clove and eucalyptus. *Food Chem. Toxicol.* 39(12), 1199–204 (2001).
141. Tadhani MBB, Patel VHH, Subhash R. *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. *J. Food Compos. Anal.* 20(3–4), 323–329 (2007).
142. Muanda FN, Soulimani R, Diop B, Dicko A. Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. *LWT - Food Sci. Technol.* 44(9), 1865–1872 (2011).
143. Ghanta S, Banerjee A, Poddar A, Chattopadhyay S. Oxidative DNA Damage Preventive Activity and Antioxidant Potential of *Stevia rebaudiana* (Bertoni) Bertoni, a Natural Sweetener. *J. Agric. Food Chem.* 55(26), 10962–10967 (2007).
144. Choi YM, Noh DO, Cho SY, Suh HJ, Kim KM, Kim JM. Antioxidant and antimicrobial activities of propolis from several regions of Korea. *LWT - Food Sci. Technol.* 39(7), 756–761 (2006).

CHAPTER II

Article 1: Propolis: a potential natural product to fight *Candida* species infections. Tobaldini-Valerio, F. K., Bonfim-Mendonça, P. S., Rosseto, H. C., Bruschi, M. L., Henriques, M., Negri, M., ... & Svidzinski, T. I. (2016). Future microbiology, 11(8), 1035-1046. Published Online: August 9th, 2016 https://doi.org/10.2217/fmb-2015-0016

Title: Propolis a potential natural product to fight *Candida* species infections

Running Head: Propolis to fight *Candida* species

Tobaldini-Valerio, F.K.[1,3,4], Bonfim-Mendonça, P.S.[1], Rosseto, H.C.[2], Bruschi, M.L.[2], Henriques, M.[3], Negri, M.[1], Silva, S.[3*], Svidzinski, T.I.E.[1]

[1] Laboratory of Medical Mycology, Department of Clinical Analysis and Biomedicine, Universidade Estadual de Maringá, Maringá, PR, Brazil

[2] Laboratory of Research and Development of Drug Delivery Systems, Department of Pharmacy, Universidade Estadual de Maringá, Maringá, PR, Brazil

[3] CEB - Centre of Biological Engineering, LIBRO – Laboratório de Investigação em Biofilmes Rosário Oliveira, Universidade do Minho, Braga, Portugal
[4] CAPES Foundation, Ministry of Education of Brazil, Brasilia – DF 70.040-020, Brazil

Abstract

Aim: To evaluate the effect of propolis against *Candida* species planktonic cells and its counterpart's biofilms. **Materials & methods**: The MICs values, time kill curves and filamentation form inhibition were determined in *Candida* planktonic cells. The effect of propolis on *Candida* biofilms was assessed through quantification of CFUs. **Results**: MIC values, ranging from 220 to 880 μg/ml, demonstrated higher efficiency on *C. albicans* and *C. parapsilosis* than on *C. tropicalis* cells. In addition, propolis was able to prevent *Candida* species biofilm's formation and eradicate their mature biofilms, coupled with a significant reduction on *C. tropicalis* and *C. albicans* filamentation. **Conclusion**: Propolis is an inhibitor of *Candida* virulence factors and represents an innovative alternative to fight candidiasis.

Keywords: *Candida* species; propolis extract; biofilm; virulence factors

*Corresponding author: Sónia Silva
Mailing address: CEB - Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
Email address: soniasilva@deb.uminho.pt
Telephone: +351 253604408
Fax: +351 253678986

Executive summary

a. Propolis presents potential antifungal activity

b. Propolis is a stronger inhibitor of filamentous forms formation

c. Propolis is able to reduce and destroy *Candida* species biofilms

d. Propolis is a promising alternative to antifungal traditional therapy

Introduction

Candida species are human commensal microbes that commonly reside on skin, gastrointestinal tract, genitourinary system, oropharynx and upper respiratory tract without causing harm to healthy individuals [1]. However, when the host immune and defense system are debilitated or under certain favorable conditions, these species, which are opportunistic, can cause infections [2]. These infections can range from superficial, such as vulvovaginal, esophageal or oropharyngeal candidiasis, to life-threatening invasive disorders, including candidemia, which is associated with high mortality among immunocompromised populations [1].

For many years, *Candida albicans* has been reported as the predominant species responsible for the majority (60 - 80%) of infections caused by the genus *Candida* [3]. However, other non-*C. albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*, have been frequently isolated mainly due to the indiscriminate prescription of antifungal agents [4–6]. Moreover, the pathogenesis of candidiasis is common to all *Candida* species and is facilitated by a number of virulent factors, including the ability to adhere to medical devices or host cells, biofilm development and filamentous form transition [7]. From a clinical point of view, *Candida* biofilms are associated with treatment failure due to a high level of antifungal resistance [8,9]. This fact triggers serious clinical concerns, not only regarding the treatment of patient infection but also for public health [10–12].

The increasing incidence of drug-resistant pathogens, limited number of therapeutic options and the toxicity of compounds have drawn attention towards the antimicrobial activity of natural products encouraging the development of alternative treatments [13–15].

Propolis is a resinous substance that honeybees, especially *Apis mellifera*, collect from branches and flowers. It has a complex chemical composition and is known to be rich in polyphenols (mainly flavonoids), waxes, resins, balsams, amino acids and other oils, thus propolis composition varies according to the plant source [16–18]. Propolis is reported to have a wide range of therapeutic properties such as antimicrobial, antioxidant, anticancer, antiviral, immunomodulatory, wound healing, and antiseptic effect [19–28].

Thus, knowing the problems associated with *Candida* infections, the development of alternative therapies, able to attenuate microbial virulence, is of utmost importance [29]. A little knowledge is already available regarding inhibition of virulence factors of *C. albicans* by propolis [24,30,31]; nevertheless the knowledge concerning NCAC species is still scarce. Therefore, the main goal of this study was to investigate the effect of propolis on clinical

isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis* planktonic cells and their counterpart's biofilms.

Materials and Methods

- **Origin of propolis, preparation and characterization of extract**

Green Brazilian propolis was purchased from the company Mel Apinor (Wal-Luz apiary, Maringá, Paraná State, Brazil). This material was cooled at -18 °C for at least 24 h. Then *in natura* propolis was crushed in an industrial blender, packaged in plastic bags and stored in a freezer (-18 °C).

The propolis extract (PE) was prepared from the previously reduced propolis, 30% (w/w) in ethanol by turbo-extraction technique [32]. Briefly, in a glass of turbo-extractor, 30 g of propolis were mixed with 70 g of ethanol (96%, v/v) and this system was kept in the refrigerator for 24 h. After this period, the evaporated alcohol weight was completed and the mixture was subjected to turbo-extraction. Subsequently it was vacuum filtered through filter paper and stored in amber glass bottle.

For the evaluation of the quality control of the PE, the techniques used were approved by official codes and were described by many authors, namely relative density, pH, dryness residue (DR), and total phenol content (TPC) [18]. To determine the DR, an amount of 3.0 g of PE was evaporated in water bath, with slow shaking. Afterwards, the concentrated material was dried on the Ohaus-MB 200 infrared analytical balance (Pine Brook, NJ, USA), at 110°C until constant weight. The DR represents the average of, at least, three determinations. The TPC was measured by the Folin-Ciocalteau method with some modifications [33]. For that, in a 25-ml flask an aliquot of PE (2.0 μl) was mixed with 10 ml of purified water and 1 ml of phosphomolybdotungstic reagent R (Folin-Ciocalteau). Then the volume was completed with an aqueous solution of sodium carbonate 14.06% (w/v). As compensatory solution, purified water was employed. The solutions were allowed to stand, protected from light for 15 min under room temperature and then the absorbance was read in a Shimadzu double beam UV-VIS spectrophotometer (Model 1650, Tokyo, Japan) at wavelength of 760 nm. A calibration curve with different dilutions of gallic acid was used as reference. Thus, the TPC was expressed as a percentage of total phenolic substances in PE. The tests accounted for an average of six evaluations.

- ***Candida* strains**

Fourteen *C. albicans* (12 isolates from blood and two from urine), 14 *C. parapsilosis* (13 from blood and one from urine) and 14 *C. tropicalis* (four from blood and 10 from urine), were used. Three *Candida* reference strains from the American Type Culture Collection -

ATCC, namely *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 40042 were included in this work. The clinical isolates from urine and blood were selected due to the high level of resistance to commercial antifungals [34] and were obtained to archive collection of the Laboratory of Medical Mycology, Universidade Estadual de Maringá, Brazil.

In each experiment, the isolates were subcultured on Sabouraud Dextrose Agar (SDA; Merck, Munich, Germany) or on Sabouraud Dextrose Broth (SDB; Merck, Munich, Germany) overnight at 37 °C. The cellular density was adjusted using a Neubauer chamber before each assay.

- **Effect of propolis on planktonic cells**

  Antifungal susceptibility testing

The antifungal activity of PE was determined by the broth microdilution method according to CLSI standard M27-A3 [35] with some modifications for natural products [36]. For this test, the serial dilution was performed at a ratio of 2, from 1:2 to 1:1024. In this way, PE's concentrations ranged from 13.9 to 7100 µg/ml of total phenol content expressed in gallic acid. The test was carried out in RPMI 1640 (Roswell Park Memorial Institute, Gibco) with L-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-Morpholino)propanesulfonic acid (pH 7.2) as buffer (Sigma), and 2% glucose, in 96-well flat-bottomed microtitration plates (Orange Scientific, Braine-l' Alleud, Belgium). After incubation at 37°C for 72 h, minimum inhibitory concentrations (MIC) were determined by direct observation. The results of the MIC were considered relative to the TPC and were defined as the concentration of TPC that reduced 100% of the growth compared to the organisms grown in the absence of the drug. The minimum fungicidal concentration (MFC) was determined by seeding, on SDA plates, the suspensions exposed to different PE concentrations. Plates were then incubated at 37° C for 24 h. The MFC was defined as the lowest concentration of the test compound in which no recovery of microorganisms was observed. Fluconazole was used as a control (Pfizer, Brazil), and the tests were also determined according to the M27-A3 guidelines of the CLSI. The MIC of fluconazole was defined, as the lowest concentration of this antifungal that was able to inhibit 50% of growth relative to the positive control without drug. As defined by the CLSI, negative controls (medium only), positive controls (medium and yeast), and the reference strain *C. albicans* ATCC 90028 were used in each test. The cut-off levels of susceptibility to fluconazole were used according to CLSI supplement M27-S3 [37] to

identify strains as susceptible (S), dose-dependent susceptible (DDS), and resistant (R): fluconazole (S ≤ 8 μg/ml, DDS = 16–32 μg/ml, R ≥ 64 μg/ml).

Time-kill curve procedures

Time kill curves were determined for the three *Candida* reference strains, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 40042 with slight modifications to that previously described [38]. Prior to testing, fungi were subcultured on SDA and the inoculum adjusted to 1- 5×10^5 yeasts/ml, in RPMI 1640 medium, using a Neubauer chamber. Then, each *Candida* strain suspension was grown in the presence of PE at concentrations equivalent to 450 and 900 μg/ml of TPC. The RPMI 1640 medium without propolis was used as a positive control. Test suspensions were placed on a shaker and incubated at 37°C. At predetermined time points (0, 1, 2, 3, 4, 6, 8, 12, 24, 28 and 36 h), serial dilutions were performed on SDA for colony-forming units (CFU) determination. Following incubation at 37°C for 24 h, the number of CFU was determined.

Effect of propolis on filamentation form transition

To evaluate the effect of propolis against *Candida* species filamentation 4 *C. albicans,* 4 *C. parapsilosis* and 4 *C. tropicalis* clinical isolates, and their respective references strains were tested. The clinical isolates were chosen randomly. *Candida* cells were grown overnight in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium. And then, 1×10^6 yeasts/ml were incubated in RPMI 1640 medium with 10% fetal bovine serum (FBS), in the presence or absence of PE (450 μg/ml of TPC, selected in order to use a concordant concentration to all species in accordance with its MICs values), at 37°C for 4 h. Blastospore and filamentous forms were counted by observation under a phase contrast microscope, according to the criteria described by Toenjes et al. (2005) [39]. More than 100 cells were counted, in duplicate, for each strain. Additionally, images of cell morphologies were obtained, after staining the microorganisms with calcofluor white (Sigma-Aldrich, St. Louis, Missouri, EUA). The cells were visualized with BX51 Olympus epifluorescence microscope coupled with a DP72 digital camera (Olympus Portugal SA, Porto, Portugal). All images were acquired using the Olympus Cell-B software.

- **Anti-biofilm effect of propolis**

As known, biofilms are microorganism's community described as 10 a 100x more resistance than its counterpart's planktonic cells [7]. Thus, the PE concentrations used in this

part of the study were based on this concept and on our previous findings of antimicrobial susceptibility.

Influence of propolis on biofilm formation

In order to evaluate the effect of PE on *Candida* species' biofilm formation, PE was added after adhesion phase (2 h). For that, *Candida* cells were grown on SDA for 24 h at 37°C, then inoculated in SDB and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 3000 × g for 10 min, at 4 °C, and washed twice with 15 ml of phosphate-buffered saline (PBS; pH 7; 0.1 M). Cell suspensions of 1×10^5 yeasts/ml were prepared in RPMI 1640 medium, 200 µl of suspensions were inoculated into 96-well polystyrene plates, and incubated at 37 °C on a shaker at 120 rpm/min for 2 h, to allow attachment of cells to the abiotic surface. Non-adhered cells were removed by wash with sterile PBS. And then 200 µl of PE (concentrations of 500, 700 and 1400 µg/ml of TPC in RPMI 1640 medium) were added to each well. The plates were incubated at 37 °C for 24 h to allow biofilm formation. Negative controls (200 µl of only RPMI 1640 medium) were also included.

Biofilms were analyzed by CFU determination. For that, the total medium was removed and the biofilms washed once with 200 µl of PBS. Then, the biofilms were scraped from the respective wells and the suspensions vigorously vortexed for approximately 2 min to disaggregate cells from the matrix [13]. Serial dilutions were made in PBS, plated onto SDA and incubated for 24 h at 37 °C. The results were presented in terms of log of CFUs.

Influence of propolis on pre-formed biofilms

The effect of PE was evaluated on 24 h pre-formed biofilms. For that, biofilms were formed during 24 h, as described above, the medium was aspirated and the non-adherent cells were removed by washing the biofilms once with 200 µl of PBS. Then, 200 µl of PE (500, 700 and 1400 µg/ml of TPC in RPMI 1640 medium) were added to each well. The biofilms were incubated for further 24 h, at 37 °C on a shaker at 120 rpm/min. The effect of PE on *Candida* biofilms was assessed through quantification of the number of CFU as described above. The results were presented in terms of log of CFU.

Effect of propolis on biofilm structure

Candida biofilm's structure and cell morphology, after growth in the presence and absence of PE (1400 µg/ml of TPC) was characterized by scanning electron microscopy

(SEM). Biofilms were prepared as described above, but 24-well microtiter plates (orange Scientific, Braine-l'Alleud, Belgium) were used. The biofilms were dehydrated with increasing concentrations of ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and then air dried for 20 min. Samples were kept in a desiccator until analysis. Prior to observation, the bottom of the wells was removed and mounted on aluminium stubs, sputter coated with gold and imaged using an S-360 scanning electron microscope (Leo, Cambridge, USA).

- **Cytotoxicity assay**

Fibroblasts 3T3 (CCL-163) were grown in Dulbecco Modified Eagle Medium (DMEM - Gibco) containing 10% of calf bovine serum (Gibco) and 1% penicillin streptomicin (Gibco). After detachment, a suspension with 10^5 cells/ml was added to a 96 well plate and cells were allowed to grow until attaining 80% of confluence. Prior to the cytotoxicity assays, the wells were washed twice with phosphate-buffered saline (PBS). PE (concentrations from 220 to 1400 µg/ml of TPC) was added to the cells and incubated for 24h at 37 °C under 5% CO2. Cells treated with the same concentration of ethanol were used as control. Afterwards, cytotoxicity was assessed using the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, based on the reduction of MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) in DMEM without phenol red. After 4 h, the absorbance of the resulting solution was read at 490 nm. The cytotoxicity of the compound is presented as the average of three independent experiments with three replicates [40]. The percentage of cell viability (%CV) was calculated by the following equation: %CV =(Abs sample/ Abs blank) × 100, where blank is the medium with cells and MTS.

- **Statistical analysis**

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Results were compared using two-way ANOVA followed by Bonferroni multiple comparisons, using GraphPad Prism version 6 (GraphPad Software, CA, USA). The significance level was set at $p < 0.05$.

Results

- **Preparation and characterization of the propolis extract**

The green propolis sample used in this study was collected from hives located in the North of Paraná state (Brazil). The apiary is surrounded by native forest with a predominance of *Baccaris drancunculifolia* and eucalyptus reserve. Green Brazilian propolis of this region is classified as "type BRP" and, as well as their ethanolic extracts, is well chemically characterized [18,41].

The physicochemical evaluation of PE showed that pH was 5.12 ± 0.05 and relative density was 0.8722 ± 0.0009 $g.ml^{-1}$. The DR value of the PE was determined as 19.33% ± 0.01 (w/w) and the TPC value obtained was 1.42% ± 0.07 (w/w). These values are in accordance to the literature, showing the good physicochemical characteristics of it, and indicating, then, that PE can be used in the present study [18,36,41].

- **Effect of propolis on *Candida* planktonic cells**

Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations

The results of PE's MICs 50% (i.e., the concentration that was able to inhibit 50% of the isolates tested) and 90% (i.e., the concentration that was able to inhibit 90% of the isolates tested) for the different pathogenic yeasts are shown in Table 1. PE showed similar and potent inhibitory activity against all clinical isolates of *Candida* species with MICs values ranging from 220 - 880 μg/ml of TPC. In all cases the MIC value was equivalent to its correspondent MFC value.

Based in these results, PE concentrations among 450 - 1400 μg/ml of TPC were selected to be used in the following experiments.

Moreover, the percentage of fibroblasts viability, after direct contact with the PE (in these concentrations) was determined in order to allow cytotoxicity evaluation. Results shown that cytotoxicity was below 35% (15% - 35%), for all the PE's concentrations tested (data not shown).

Time-kill curves determination

The killing activity of PE, plotted from $\log_{10}$ CFU/ml versus time (36h), is represented in Figure 1. Two distinct effects were observed on the growth of the *Candida* species. At 450 μg/ml of TPC, for all species tested, slight inhibitory effect was observed until 12 h, however, after this time the resultant curves were nearly identical to those for the control. At concentration 900 μg/ml of TPC, a substantial time-dependent reduction in the number of

viable cells was observed compared with the control group. Additionally, results revealed that the PE effect was more pronounced in *C. albicans* and *C. parapsilosis* species, with a decreased of ≥99.9% (4 and 3 log) at 36 h, comparatively to control group (without PE). In fact even at 36 h the reduction observed by *C. tropicalis* did not exceed ~90% (1.5 log) of the reduction. This species had also higher MIC to PE, when compared to *C. albicans* and *C. parapsilosis*.

Propolis effect on *Candida* species filamentous forms formation

The effect of propolis on the transition of yeast to filamentous forms was evaluated (Figure 2). Four clinical isolates of *C. albicans*, *C. tropicalis* and *C. parapsilosis* species and the respective reference strains were analyzed. The results revealed that *C. parapsilosis* was unable to form filamentous forms (data not shown) and that *C. albicans* presented higher number of filamentous forms than *C. tropicalis*. It was also observed that PE (at concentration of 450 μg/ml of TPC), after 4 h of exposition, reduced approximately from 80% to 5% the formation of filamentous forms on all *C. albicans* and *C. tropicalis* strains (Figure 2).

- **Propolis anti-biofilm activity**

The second aim of this work was to evaluate the activity of propolis on *Candida* biofilms formation (Figure 3) and against *Candida* pre-formed biofilms (Figure 4). The results revealed that PE was able to reduced *Candida* biofilms, however in a species and strain dependent manner. Concerning the effect of the PE on biofilm formation the results revealed a significant reduction in the number of cultivable cells for the four clinical isolates of the each species and its respective reference strains (Figure 3A). No significant differences were observed between the three PE concentrations tested. *Candida albicans* strains presented the highest biofilm reduction (~3.5 log), followed by *C. parapsilosis* and *C. tropicalis*, with a reduction around 2.8 and 2 log, respectively, for all PE concentrations tested (Figure 3A).

The propolis effect against pre-formed *Candida* biofilms (Figure 4A) was lower comparatively to the effect on biofilm formation. In fact, at the biofilm maturation stage, no biofilm reduction was observed for 500 μg/ml of TPC for all *Candida* strains under study. Moreover, with PE concentrations ≥700 μg/ml of TPC the pre-formed biofilm reduction was similar to those observed for the biofilm formation, when compared with the control group. Concerning *C. tropicalis,* PE at 500 and 700 μg/ml of TPC was able to reduce ~1.5 and 2.4 log, respectively. This reduction was higher than the observed in the biofilm formation studies

(Figure 3A), even to for PE concentrations of the 1400 μg/ml of TPC where it was observed a reduction of ~3.5 log in the number of CFUs.

- **Effect of propolis on biofilm structure**

SEM analysis was performed to examine the effect of the PE on *Candida* species biofilm formation (Figure 3B) and against pre-formed biofilms (Figure 4B). For that, biofilms of one clinical isolate and its respective reference strain were treated with PE at 1400 μg/ml of TPC and compared with untreated biofilms.

Examination of untreated biofilms showed the presence of different cellular morphologies in the *Candida* biofilms. *Candida albicans* and *C. parapsilosis* biofilms exhibited a blastoconidia aggregate layer with irregular clusters, while *C. tropicalis* biofilms developed a more compact and continuous structure with yeast cells more interlinked (Figures 3B and 4B - Controls). Interestingly, it was observed that *Candida* species' biofilms when treated with PE (1400 μg/ml of TPC) presented a significant reduction on the number of cells and a consistent biofilm disruption (Figures 3B and 4B - Treated). In addition, yeasts cells on biofilms treated with PE underwent morphological alterations and loss of integrity on their cell wall. Moreover, in the presence of PE, *C. albicans* biofilms presented a reduction in the number of filamentous forms.

Discussion

The incidence of candidiasis in the last two decades had a significant increment and *C. albicans* is still the most prevalent species, however, the frequency of the NCAC species has also been increasing [42–44]. This fact can be due to the lower sensibility of the yeasts to the antifungal agents most commonly used in clinical practice [45]. Moreover, the expression of the virulence factors such as morphological transition and biofilm formation has been associated to difficulties on their treatment [2,46]. The increasing incidence of drug-resistant pathogens, the limited number of therapeutic options and the toxicity of traditional compounds have drawn attention towards the antimicrobial activity of natural products encouraging the development of alternative treatments [47].

Propolis has been demonstrated important antimicrobial activity and this bioactivity has been investigated in the last years [24,48]. The antimicrobial activity of propolis is complex and has been attributed to the synergistic activity between its various potent biological ingredients, mainly phenolic and flavonoid compounds [49]. The flavonoids constitute a very important class of polyphenols, widely present in propolis [50]. The great part of propolis biological activity is attributed to polyphenols [51]. Green Brazilian propolis type BRP is rich source of phenolic substances; most of them are prenylated phenylpropanoids, and cinnamic acids, chiefly compounds bearing prenyl groups [18,36,41]. Therefore, the physicochemical analysis is fundamental for the evaluation of PE quality. The results showed the good characteristics of PE and the spectrophotometric determination of TPC was useful to characterize the amount of polyphenols. The value obtained was 1.42% ± 0.07 (w/w) of TPC, and this amount is in accordance with other researches [36,41].

Despite some work developed about the effect of propolis against virulence factors of *C. albicans* [24,30,31] scarce are the studies involving NCAC species. Thus, the main goal of this study was to investigate the effect of propolis on the three most important *Candida* species, *C. albicans, C. tropicalis* and *C. parapsilosis*. It was a goal to evaluate the effect on both planktonic cells and biofilms.

Firstly, the planktonic susceptibility of *C. albicans, C. tropicalis* and *C. parapsilosis* strains to PE was determined (Table 1). Our data demonstrated that, all *Candida* species were susceptible to PE with a MIC range of 220 to 880 μg/ml of TPC. Moreover, these work showed that PE was effective even against strains with sensitivity dose dependence to fluconazole (MIC 16 μg/ml), namely in the case of *C. tropicalis*. Therefore these results are in agreement with Dalben-Dota et al. (2010) [36] that showed *Candida* species' sensitivity to PE. These authors observed that the MIC of PE ranged from 6.14 to 3145.50 mg/ml of

flavonoids content (which are included in polyphenol content), evidencing an efficacy of this extract. A time-kill assay was performed to determine the kinetic effect of PE on *C. albicans*, *C. parapsilosis* and *C. tropicalis* growth (Figure 1). Results revealed approximately 90% of reduction on its growth for all species, with an effective reduction on *Candida* cells cultivability. In fact PE was able to reduce around 3 logs (99.9%) of *C. albicans* and *C. parapsilosis* and an approximately 1.5 log of *C. tropicalis*. These results are in accordance with the susceptibility results where, *C. tropicalis* was the species with the highest MIC value.

As previous works only showed an effective activity of PE against planktonic *Candida* species cells [36,52] this work intends to extend this knowledge, by evaluating the PE's effect on *Candida* species virulence traits, such as yeast-filamentous transition and biofilm formation ability. It is known that the formation of hyphae helps *C. albicans* to penetrate the host tissues with subsequent invasiveness that leads to the establishment of infection [53]. Thus, the ability of PE to inhibit the formation of filamentous forms was evaluated and the results revealed that PE was able to block 90% of the yeast-filamentous forms in *C. albicans* and *C. tropicalis* (Figure 2). This inhibition of yeast-filamentous' forms transition by PE, presents a very attractive option to control *Candida* infections. It was previously reported that the morphological switch from yeast to hyphae cells is important in many processes, such as biofilm formation [54]. Thus, the high capacity of PE to efficiently inhibit yeast-hyphae transition may be associated with its ability to prevent biofilm formation. Furthermore, this product has received the attention of clinicians and researchers due to its diverse pharmacological activities and low toxicity [20,55].

Biofilm formation by microorganisms is a mechanism that allows them to become persistent colonizers, to resist clearance by the host immune system and antibiotic's effect [56]. Interesting it was observed that PE was able to inhibit biofilm formation (Figures 3) and to destroy mature biofilms (Figure 4) of *C. albicans*, *C. parapsilosi*s and *C. tropicalis* strains. It is important to highlight that PE at 450 and 700 μg/ml of TPC was able to inhibit approximately 90% of biofilm formation (Figure 3A) and 1400 μg/ml of TPC was able to reduce pre-formed biofilms in 99.9% (Figure 4A). Previous studies have shown that different concentrations of propolis (0.25 - 1.25%) were able to reduce 40–45% of the *in vitro C. albicans* biofilm formation [57]. Moreover, Capoci et al. (2014) also revealed a small reduction on *C. albicans* biofilm formation (< 0.5 log) at concentration of PE lower than MIC [24]. However, the promising results obtained for *C. tropicalis* and *C. parapsilosis* were never stated before. The SEM images corroborate the biofilm disruption (Fig 3B and 4B), also

demonstrated by cultivable cells determination (Figure 3A and 4A), reinforcing the PE's capability to inhibit filamentation (Figure 2).

Conclusion

In conclusion, this study showed that PE is a potent antifungal agent with effect on *Candida* planktonic cells and biofilms. It is important to highlight, that these effects were not only observed against *C. albicans* but on other NCAC species, namely *C. tropicalis* and *C. parapsilosis*. This is a very promising data, considering that NCAC species has shown to be highly resistant to the conventional antifungal agents.

Such properties of PE as inhibitor of *Candida* virulence represent an alternative and innovative pathways of chemotherapy for pathogens that are resistant to classical antimicrobial agents.

Acknowledgments

The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (Cnpq), and Fundação Araucária for the financial support received. Flávia Tobaldini-Valerio, acknowledge the financial support of CAPES – Proc. 9469/14-1. The authors also thank FCT for the Strategic Project of the UID/BIO/04469/2013 unit, FCT and European Union funds (FEDER/COMPETE) for the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462).

References

Papers of special note have been highlighted as:

• of interest; •• of considerable interest

1. Yapar N. Epidemiology and risk factors for invasive candidiasis. *Ther. Clin. Risk Manag.* 10(1), 95–105 (2014).

2. Martins N, Ferreira ICFR, Barros L, Silva S, Henriques M. Candidiasis: Predisposing Factors, Prevention, Diagnosis and Alternative Treatment. *Mycopathologia*. 177(5-6), 223–240 (2014).

•• Excellent article for understanding the *Candida* infections.

3. Guinea J. Global trends in the distribution of *Candida* species causing candidemia. *Clin. Microbiol. Infect.* 20, 5–10 (2014).

4. Arendrup MC. *Candida* and Candidaemia: Susceptibility and Epidemiology. *Dan. Med. J.* 60(11), 1–32 (2013).

5. Sanguinetti M, Posteraro B, Lass-Flörl C. Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses*. 58, 2–13 (2015).

6. Krcmery V, Barnes a. J. Non-albicans *Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. *J. Hosp. Infect.* 50(4), 243–260 (2002).

7. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* 36(2), 288–305 (2012).

•• Excellent article for understanding the non-*C. albicans Candida* (NCAC) species infections.

8. Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol.* 11(1), 30–36 (2003).

9. Rautemaa R, Ramage G. Oral candidosis-clinical challenges of a biofilm disease. *Crit. Rev. Microbiol.* 37(4), 328–36 (2011).

10. Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int. J. Microbiol.* 2012(Article ID 528521), 14 pages (2012).

11. Uppuluri P, Chaturvedi AK, Srinivasan A, *et al.* Dispersion as an important step in the

Candida albicans biofilm developmental cycle. *PLoS Pathog.* 6(3), e1000828 (2010).

12. Uppuluri P, Pierce CG, López-Ribot JL. *Candida albicans* biofilm formation and its clinical consequences. *Future Microbiol.* 4(10), 1235–7 (2009).

13. Alves CT, Ferreira ICFR, Barros L, Silva S, Azeredo J, Henriques M. Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against *Candida species*. *Future Microbiol.* 9(2), 139–46 (2014).

14. Martins N, Barros L, Santos-Buelga C, Henriques M, Silva S, Ferreira ICFR. Evaluation of bioactive properties and phenolic compounds in different extracts prepared from *Salvia officinalis* L. *Food Chem.* 170, 378–85 (2015).

15. Sardi JCO, Almeida AMF, Mendes Giannini MJS. New antimicrobial therapies used against fungi present in subgingival sites-A brief review. *Arch. Oral Biol.* 56(10), 951–9 (2011).

16. Lustosa SR, Galindo AB, Nunes LCC, Randau KP, Rolim Neto PJ. Propolis: Atualizações sobre a química e a farmacologia. *Brazilian J. Pharmacogn.* 18(3), 447–454 (2008).

17. Pereira A dos S, Seixas FRMS, Aquino Neto FR de. Própolis: 100 anos de pesquisa e suas perspectivas futuras. *Quim. Nova*. 25(2), 321–326 (2002).

18. Franco SL, Bruschi ML, Moura LPP, Bueno JH. Avaliação farmacognóstica da própolis da região de Maringá. *Rev. Bras. Farmacogn.* 9-10(1), 1–10 (2000).

19. Righi AA, Alves TR, Negri G, Marques LM, Breyer H, Salatino A. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. *J. Sci. Food Agric.* 91(13), 2363–70 (2011).

20. Frozza CO da S, Garcia CSC, Gambato G, *et al.* Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. *Food Chem. Toxicol.* 52, 137–42 (2013).

21. Urushisaki T, Takemura T, Tazawa S, *et al.* Caffeoylquinic acids are major constituents with potent anti-influenza effects in brazilian green propolis water extract. *Evidence-based Complement. Altern. Med.* 2011(Article ID 254914), 7 pages (2011).

22. Búfalo MC, Bordon-Graciani AP, Conti BJ, de Assis Golim M, Sforcin JM. The immunomodulatory effect of propolis on receptors expression, cytokine production and

fungicidal activity of human monocytes. *J. Pharm. Pharmacol.* 66, 1497–1504 (2014).

23. Sforcin JM, Fernandes Junior A, Lopes CAM, Funari SRC, Bankova V. Seasonal effect of brazilian propolis on *Candida albicans* and *Candida tropicalis*. *J. Venom. Anim. Toxins*. 7(1), 139–144 (2001).

24. Capoci GRI, Bonfim-Mendonça PS, Arita GS, *et al.* Propolis Is an Efficient Fungicide and Inhibitor of Biofilm Production by Vaginal *Candida albicans*. *Evidence-Based Complement. Altern. Med. Artic.* 2015(Article ID 28769), 9 pages (2015).

25. Nina N, Lima B, Feresin GE, Giménez A, Capusiri ES, Schmeda-Hirschmann G. Antibacterial and Leishmanicidal Activity of Bolivian Propolis. *Lett. Appl. Microbiol.* (2016).

26. Fiordalisi SAL, Honorato LA, Loiko MR, *et al.* The effects of Brazilian propolis on etiological agents of mastitis and the viability of bovine mammary gland explants. *J. Dairy Sci.* (2015).

27. Massaro CF, Simpson JB, Powell D, Brooks P. Chemical composition and antimicrobial activity of honeybee (*Apis mellifera ligustica*) propolis from subtropical eastern Australia. *Naturwissenschaften*. 102(11-12), 68 (2015).

28. Tobaldini FK, Bonfim-Mendonca PS, Rosseto HC, *et al.* Propolis potential activity against *Candida tropicalis* adhered cells and its biofilm. *Mycoses Spec. Issue 7th Trends Med. Mycol. 9–12 Oct. 2015, Lisbon, Port.* 58(Supplement S4), 199 (2015).

29. Pfaller MA. Antifungal drug resistance: Mechanisms, epidemiology, and consequences for treatment. *Am. J. Med.* 125(1 SUPPL.), S3–S13 (2012).

30. D'Auria FD, Tecca M, Scazzocchio F, Renzini V, Strippoli V. Effect of propolis on virulence factors of *Candida albicans*. *J. Chemother.* 15(sample 1), 454–460 (2003).

31. Gomaa OM, Gaweesh AS. Variation in adhesion and germ tube formation of oral *Candida* using Egyptian propolis. *Can. J. Microbiol.* 59(3), 197–203 (2013).

32. List PH, Schmidt PC. Phytopharmaceutical Technology. Heyden & Son, Londres.

33. Teixeira ÉW, Message D, Negri G, Salatino A, Stringheta PC. Seasonal variation, chemical composition and antioxidant activity of brazilian propolis samples. *Evidence-based Complement. Altern. Med.* 7(3), 307–315 (2010).

34. da Matta DA, de Almeida LP, Machado AM, *et al.* Antifungal susceptibility of 1000

Candida bloodstream isolates to 5 antifungal drugs: results of a multicenter study conducted in São Paulo, Brazil, 1995-2003. *Diagn. Microbiol. Infect. Dis.* 57(4), 399–404 (2007).

35. Clinical and Laboratory Standards Institute. M27-A3, Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. 3rd ed. Wayne, PA.

- **Excellent standard for understanding the MIC technique.**

36. Dalben-Dota KF, Faria MGI, Bruschi ML, Pelloso SM, Lopes-Consolaro ME, Svidzinski TIE. Antifungal activity of propolis extract against yeasts isolated from vaginal exudates. *J. Altern. Complement. Med.* 16(3), 285–290 (2010).

37. Clinical and Laboratory Standards Institute. M27-S3, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Third informational supplement. (2008).

38. Klepser ME, Wolfe EJ, Jones RN, Nightingale CH. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against A *Candida albicans*. *Antimicrob. Agents Chemother.* 41(6), 1392–1395 (1997).

39. Toenjes KA, Munsee SM, Ibrahim AS, *et al.* Small-Molecule Inhibitors of the Budded-to-Hyphal-Form Transition in the Pathogenic Yeast *Candida albicans*. *Antimicrob. Agents Chemother.* 49(3), 963–972 (2005).

40. Malich G, Markovic B, Winder C. The sensitivity and specificity of the MTS tetrazolium assay for detecting the *in vitro* cytotoxicity of 20 chemicals using human cell lines. *Toxicology*. 124(3), 179–92 (1997).

41. Bruschi ML, Klein T, Lopes RS, Franco SL, Gremião MPD. Contribuição ao protocolo de controle de qualidade da própolis e de seus extratos. *Rev. ciênc. farm*. 23(2), 289–306 (2002).

42. Kauffman CA, Vazquez JA, Sobel JD, *et al.* Prospective Multicenter Surveillance Study of Funguria in Hospitalized Patients. *Clin. Infect. Dis.* 30(1), 14–18 (2000).

43. Manzano-Gayosso P, Hernández-Hernández F, Zavala-Velásquez N, *et al.* Candiduria in type 2 diabetes mellitus patients and its clinical significance. *Candida* spp. antifungal susceptibility. *Rev. médica del Inst. Mex. del Seguro Soc.* 46(6), 603–10 (2008).

44. Ruan S-Y, Hsueh P-R. Invasive candidiasis: an overview from Taiwan. *J. Formos. Med. Assoc.* 108(6), 443–51 (2009).

45. González GM, Elizondo M, Ayala J. Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-year (2004 to 2007) surveillance study. *J. Clin. Microbiol.* 46(9), 2902–5 (2008).

46. Ramage G, Martínez JP, López-Ribot JL. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res.* 6(7), 979–86 (2006).

47. Ahmad A, Khan A, Akhtar F, *et al.* Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. *Eur. J. Clin. Microbiol. Infect. Dis.* 30(1), 41–50 (2011).

48. Silva-Carvalho R, Baltazar F, Almeida-aguiar C. Propolis : A Complex Natural Product with a Plethora of Biological Activities That Can Be Explored for Drug Development. *Evidence-Based Complement. Altern. Med.* 2015(Article ID 206439), 29 pages (2015).

    • **Excellent article for understanding the potential of propolis extract.**

49. Al-Waili N, Al-Ghamdi A, Ansari MJ, Al-Attal Y, Salom K. Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in single and polymicrobial cultures. *Int. J. Med. Sci.* 9, 793–800 (2012).

50. Bankova V. Recent trends and important developments in propolis research. *Evidence-based Complement. Altern. Med.* 2(1), 29–32 (2005).

51. Burdock GA. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* 36(4), 347–363 (1998).

52. Ota C, Unterkircher C, Fantinato V, Shimizu MT. Antifungal activity of propolis on different species of *Candida*. *Mycoses*. 44(9-10), 375–8 (2001).

53. Sun L, Liao K, Wang D. Effects of magnolol and honokiol on adhesion, yeast-hyphal transition, and formation of biofilm by *Candida albicans*. *PLoS One*. 10(2), e0117695 (2015).

54. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence*.

4(2), 119–128 (2013).

55. Agarwal G, Vemanaradhya GG, Mehta DS. Evaluation of chemical composition and efficacy of Chinese propolis extract on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An *in vitro* study. *Contemp. Clin. Dent.* 3(3), 256–61 (2012).

56. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15(2), 167–93 (2002).

57. De Castro PA, Bom VLP, Brown NA, *et al.* Identification of the cell targets important for propolis-induced cell death in *Candida albicans*. *Fungal Genet. Biol.* 60, 74–86 (2013).

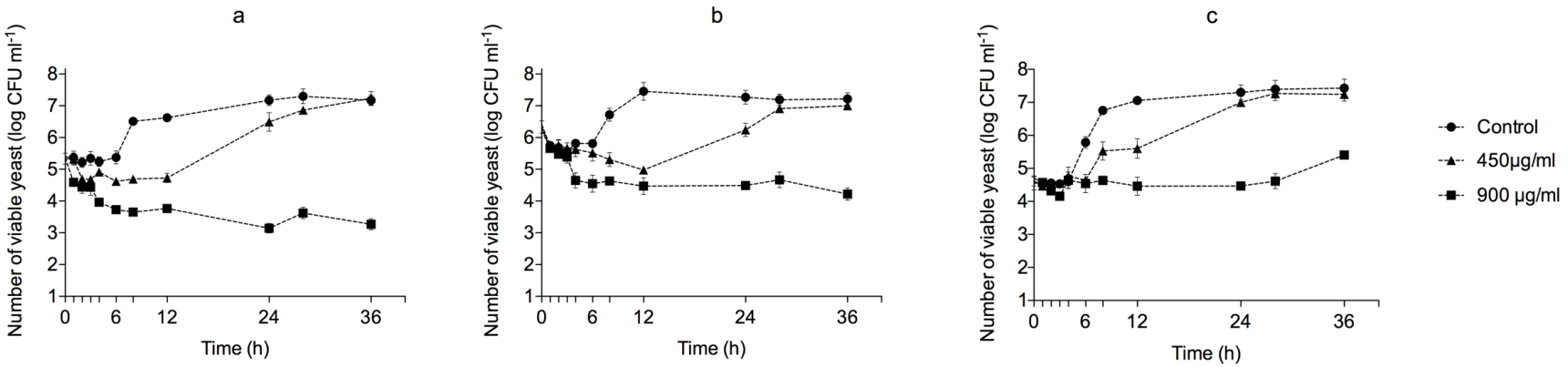


Figure 1. **Killing kinetics of propolis extract against *Candida* species**. a: *C. albicans* ATCC 90028; b: *C. parapsilosis* ATCC 22019; c: *C. tropicalis* ATCC 40042. Standardized yeast cells suspensions were exposed to 450 and 900 µg/mL of total phenol contents. At determined time intervals, samples were serially diluted and plated for colony counts. Each data point represents mean result ± standard deviation (error bars) from three experiments.

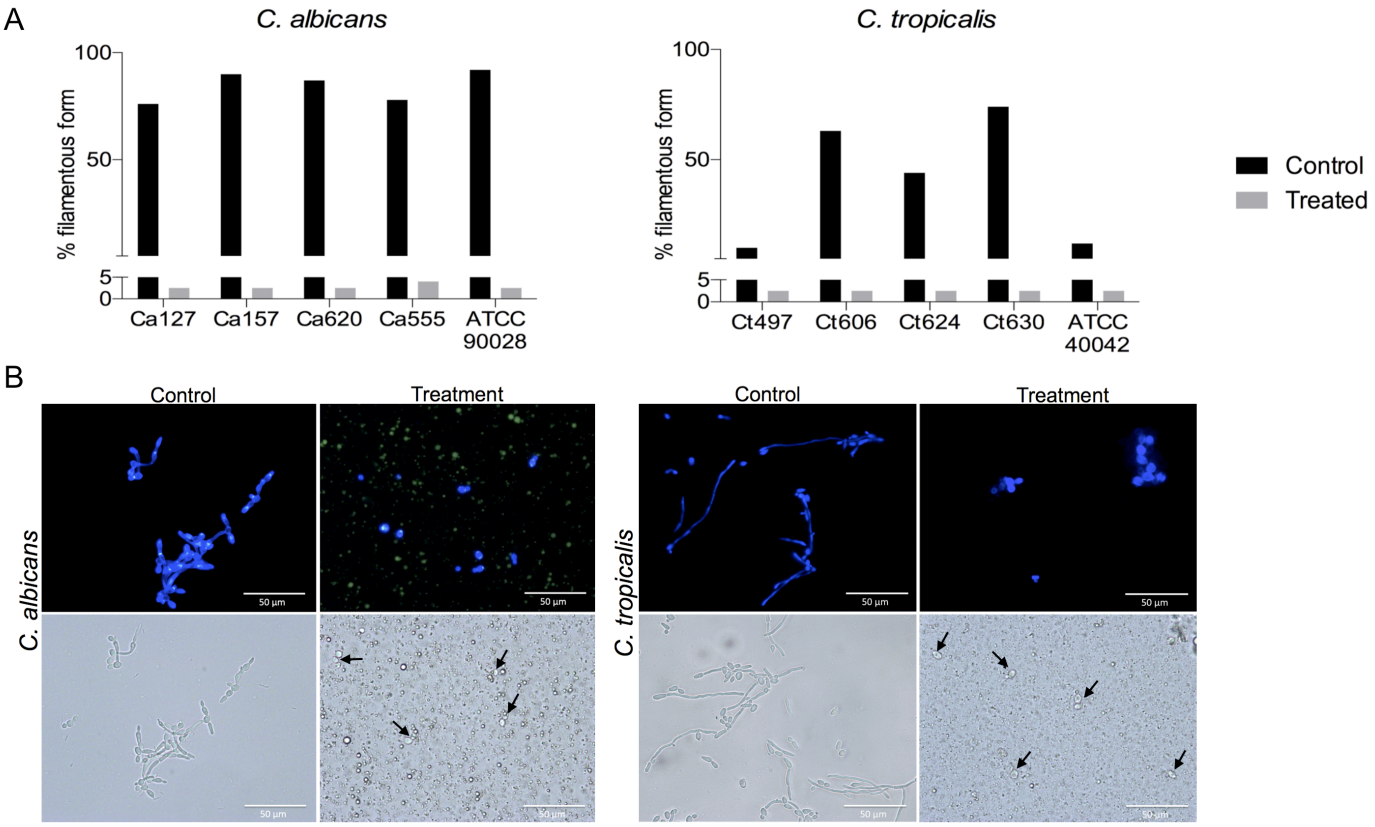


Figure 2. Inhibition of *C. albicans* and *C. tropicalis* filamentation by propolis extract in FBS RPMI 1640 medium at 37°C for 4 h. A) Percentage of the filamentous forms observed after exposing *Candida* planktonic cells to propolis (Treated). B) The images are representative of *C. albicans* and *C. tropicalis* cells obtained using a fluorescent microscope stained with calcofluor white. PE was added at a concentration of 450 µg/ml. The structures besides of the yeasts pointed out by arrows are propolis residues.

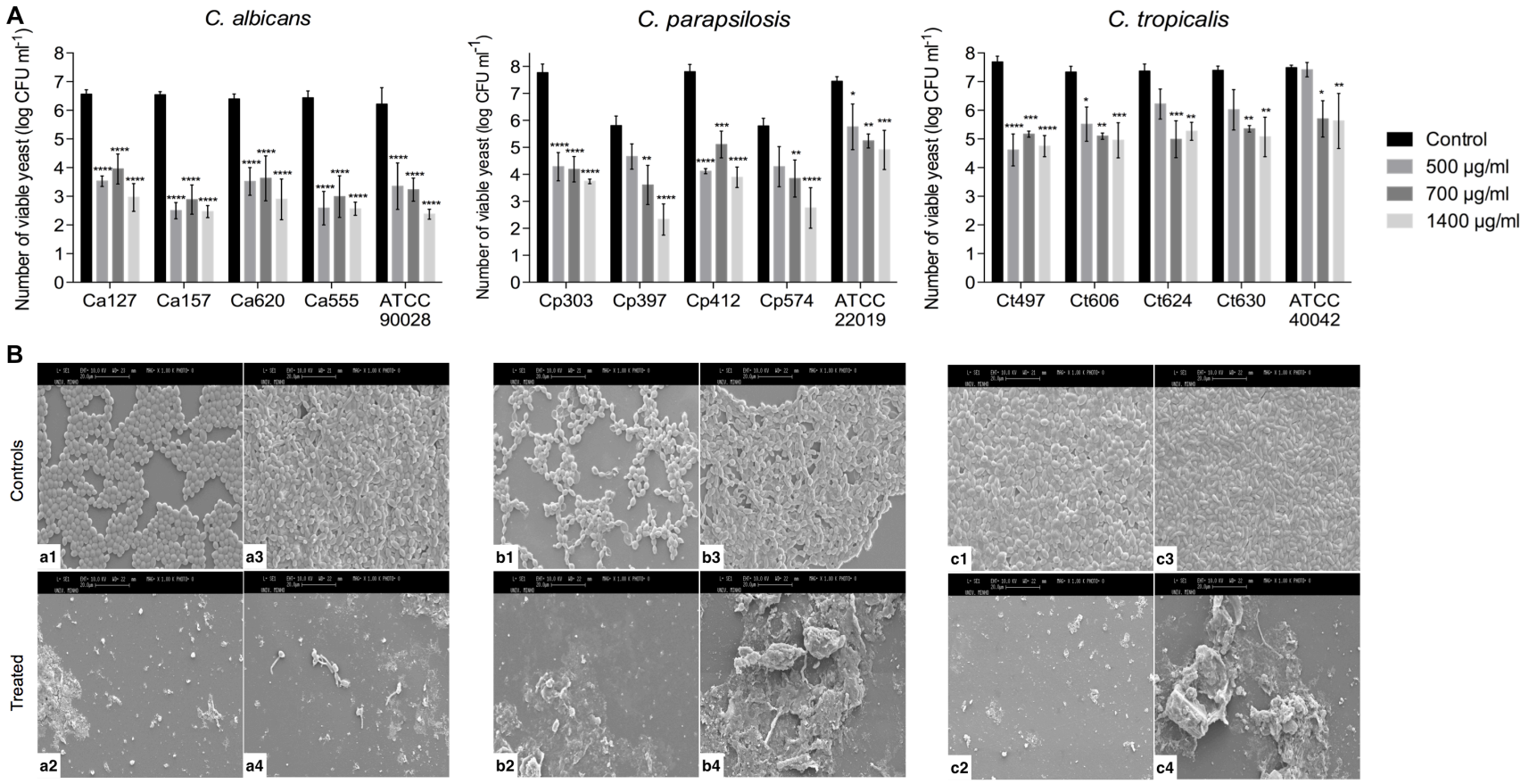


Figure 3- Logarithm of number of *Candida* cells biofilms and scanning electron microscopy images of PE effect during biofilm formation by *Candida* species. *Candida* cells, were allowed to adhere for 2 h, then propolis was added and incubated further for 24 h at 37°C. Error bars represented in graphics indicate the standard deviation. *, ** and **** correspond to $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.
In the images (a1, a2) represent isolate clinical *C. albicans* (a3, a4) ATCC *C. albicans* 90028 (b1, b2) isolate clinical *C. parapsilosis* (b3, b4) ATCC *C. parapsilosis* 22019 (c1, c2) isolate clinical *C. tropicalis* (c3, c4) ATCC *C. tropicalis* 40042.
Controls: biofilms grown in RPMI medium in the absence of propolis extract. Treated: biofilms grown in RPMI medium in the presence of 1400µg/ml of total phenol content. The bar in the images corresponds to 20 µm. Magnification x 1000.

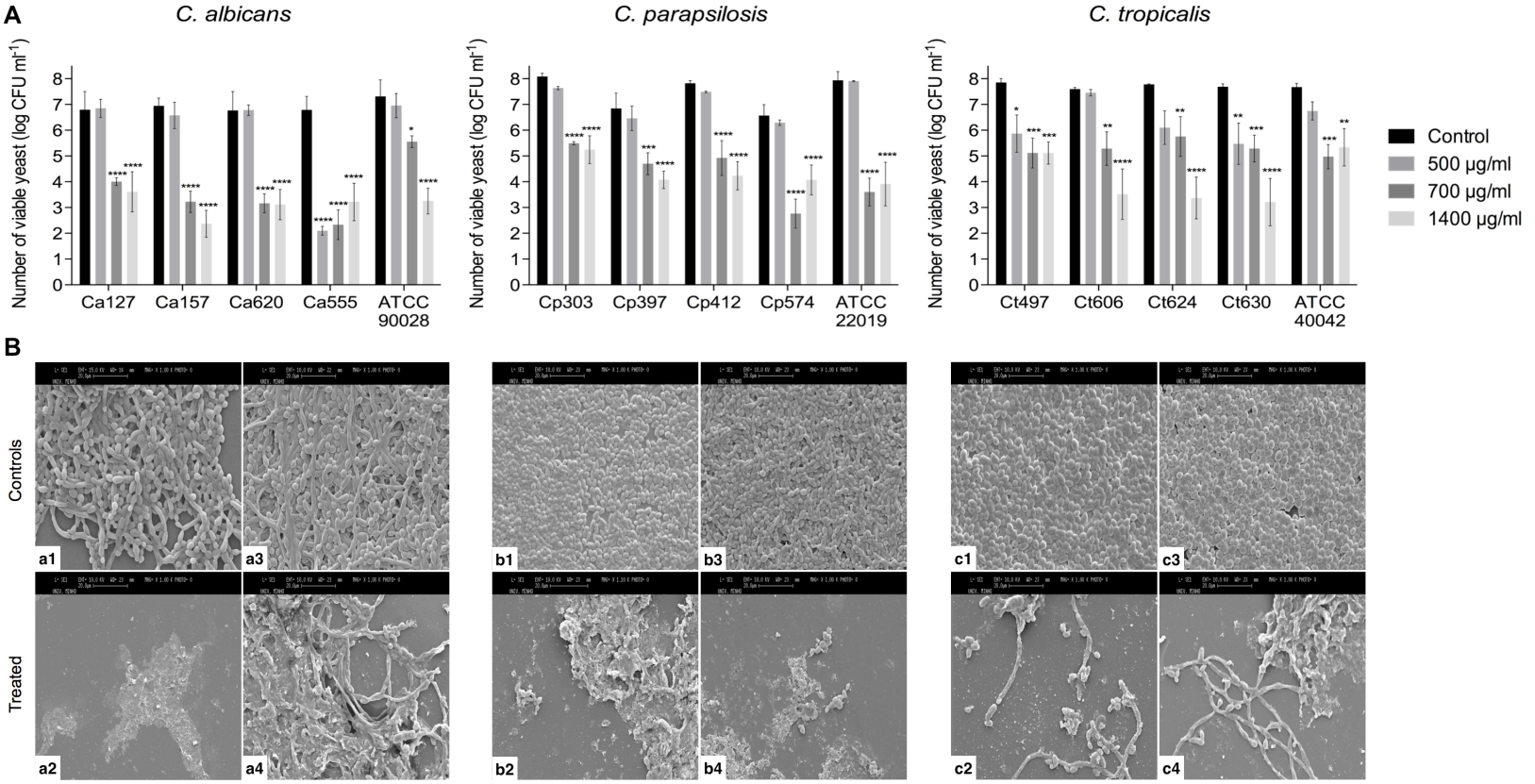


Figure 4- Logarithm of number of *Candida* cells biofilms and scanning electron microscopy images of PE effect on pre-formed *Candida* species biofilms. *Candida* cells, were allowed to form biofilm for 24 h, then propolis was added and incubated further for 24 h at 37°C. Error bars represented in graphics indicate the standard deviation. *, ** and **** correspond to $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

In the images (a1, a2) represent isolate clinical *C. albicans* (a3, a4) ATCC *C. albicans* 90028 (b1, b2) isolate clinical *C. parapsilosis* (b3, b4) ATCC *C. parapsilosis* 22019 (c1, c2) isolate clinical *C. tropicalis* (c3, c4) ATCC *C. tropicalis* 40042.

Controls: biofilms grown in RPMI medium in the absence of propolis extract.

Treated: biofilms grown in RPMI medium in the presence of 1400µg/ml of total phenol content. The bar in the images corresponds to 20 µm. Magnification x 1000.

Table 1. *In vitro Candida* species antifungal susceptibility to propolis extract (PE) and fluconazole (FLU)

	Antifungal agent	MIC (µg/mL)		
		Range	MIC 50	MIC 90
C. albicans	PE	440	440	440
	FLU	≤0.125 – 0.25 (S)	0.125	0.25
C. parapsilosis	PE	220 – 880	220	440
	FLU	0.25 – 4.0 (S)	0.5	2.0
C. tropicalis	PE	440 – 880	880	880
	FLU	0.25 – 16 (S – DDS)	0.5	8.0

MIC: minimal inhibitory concentration
MIC50 and MIC90: MIC that could inhibit 50% and 90% of the growth of the isolates, respectively.
S: susceptible; DDS: dose-dependent susceptible

Article 2: Bioactive properties and functional constituents of *Hypericum androsaemum* L.: A focus on the phenolic profile Ines Jabeur[#], Flavia Tobaldini[#], Natalia Martins, Lillian Barros, Ivone Martins, Ricardo C. Calhelha, Mariana Henriques, Sonia Silva, Lotfi Achour, Celestino Santos-Buelga, Isabel C.F.R. Ferreira (2016). Food Research International, 89, 422-431. Available online 28 August 2016. [#]Both authors contributed equally.
doi:10.1016/j.foodres.2016.08.040

Manuscript 3: *Stevia rebaudiana* Bertoni phenolic composition and anti-*Candida* planktonic cells' and biofilms' activity Flávia Tobaldini-Valério, Natália Martins, Ivone Martins, Lillian Barros, Mariana Henriques, Isabel C. F. R. Ferreira, Terezinha I E Svidzinski, Sónia Silva. Article submitted to the Journal Food Chemistry on July de 2017

Stevia rebaudiana phenolic composition and anti-*Candida* planktonic cells' and biofilms' activity

Flávia Tobaldini-Valério[1,2,3], Natália Martins[1,4], Ivone Martins[1], Lillian Barros[4], Mariana Henriques[1], Isabel C. F. R. Ferreira[4], Terezinha I E Svidzinski[2], Sónia Silva[1*]

[1]CEB, Centre of Biological Engineering, LIBRO–Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, 4710-057 Braga, Portugal.

[2]Laboratory of Medical Mycology, Department of Clinical Analysis and Biomedicine, Universidade Estadual de Maringá, Maringá, PR, Brazil.

[3] CAPES Foundation, Ministry of Education of Brazil, Brasilia – DF 70.040-020, Brazil

[4]Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1172, 5300-253 Bragança, Portugal.

*Author to whom correspondence should be addressed (e-mail: soniasilva@deb.uminho.pt, telephone +351253604401, fax +351253604429)

Abstract

Candida infections have been increasing in recent years, being even an important cause of morbidity and mortality in hospitalized patients. The genus *Candida* has been demonstrating a reduced susceptibility to the commonly used antifungal drugs. Therefore, there is a need for new strategies to combat fungal infections and natural products are encouraging the development of alternative treatments. The main objective of this work was to determine the phenolic composition of *Stevia rebaudiana* extract and to evaluate its effect against *Candida* planktonic cells and its biofilm counterparts. Minimum inhibitory concentrations (MIC) and time-kill curves were determined for *Candida* planktonic cells, while the effect on *Candida* biofilms was assessed through quantification of the number of cultivable cells.

3,5-*O*-dicaffeoylquinic and 5-*O*-caffeoylquinic acids, the most abundant phenolic compounds identified in the extract, might act as contributors for the observed biological effects. MIC values obtained for *Candida* strains, ranging from 3.12 to ≥ 25 mg/mL, with *Candida albicans* strains presented the highest MIC value. Moreover, *Candida tropicalis* and *Candida glabrata* ability to form biofilms was attenuate in the presence of *Stevia* extract, with a reduction of 3 orders of magnitude in the case of *C. glabrata* ATCC 2001 strain. Nevertheless, further studies should be carried out to determine the feasibility of using *Stevia rebaudiana* as a potential candidate for the development of new strategies to treat *Candida* infections.

Keywords: Stevia rebaudiana Bertoni; *Candida* species; Antifungal resistance; Phenolic compounds

1. Introduction

Fungal infections have been increasing in the last years, being an important cause of morbidity and mortality among hospitalized patients. Candidiasis is an opportunistic fungal infection caused by genus *Candida*, being currently responsible for up to 78% of nosocomial fungal infections (Bhatt et al., 2015; Marol & Yücesoy, 2007). Despite *Candida albicans* is the main species isolated in patients with invasive fungal infections, other non-*Candida albicans Candida* (NCAC) species have becoming increasingly common, such as *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* species (Arendrup, 2013; Deorukhkar, Saini, & Mathew, 2014). Moreover, NCAC species have demonstrated a reduced susceptibility to the antifungal drugs, mainly due to some virulence characteristics that triggers their pathogenicity, including the ability to adhere to the epithelia host, yeast-to-hypha transition, production of hydrolytic enzymes and ability to form biofilms (Lyon & de Resende, 2006; Mayer, Wilson, & Hube, 2013; Nikawa et al., 2006). Some susceptibility studies have revealed that biofilms formed by *C. albicans* may be up to 10 to 100 times more resistant to conventional antifungal drugs than planktonic cells (Baillie & Douglas, 2000; Chandra, Mukherjee, & Ghannoum, 2012; Gordon Ramage, Saville, Thomas, & López-Ribot, 2005). Therefore, there is an urgent need for upcoming strategies to combat fungal infections, namely those associated with biofilm formation.

Natural products have gained an increasing attention regarding their antimicrobial effects, including the activity of plant matrices against *Candida* species (Martins, Barros, Henriques, Silva, & Ferreira, 2015). *Stevia rebaudiana* Bertoni is a sweet plant traditionally used as a natural sweetener, with other reported biological effects, namely as antihypertensive (Chan et al., 2000; Lee et al., 2001), anti-hyperglycemic (Jeppesen, Gregersen, Alstrup, & Hermansen, 2002; Jeppesen, Gregersen, Poulsen, & Hermansen, 2000) and anti-human rotavirus (Das et al., 1992). Alkaloids, water-soluble chlorophylls, xanthophylls, hydroxycynnamoyl

derivatives, phenolic compounds, neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements are amongst to the most commonly studied bioactive molecules to which the above described effects are attributed (Komissarenko, Derkach, Kovalyov, & Bublik, 1994; Tadhani, Patel, & Subhash, 2007). Nevertheless, there are few reports determining the antifungal activity of *Stevia* extracts (Garcia, Garcia-Cela, Ramos, Sanchis, & Marín, 2011; Garcia, Ramos, Sanchis, & Marín, 2012; Muanda, Soulimani, Diop, & Dicko, 2011; Sedghi & Gholi-Toluie, 2013), and to the authors' best knowledge there are no reports available assessing the antifungal properties of ethanol:water extracts against *Candida* species.

Based on the knowing about the problems associated with *Candida* infections, this work aimed to investigate the phenolic composition of *Stevia rebaudiana* ethanol:water extract and to access the antifungal potential against clinical isolates of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* planktonic cells, including their biofilms counterparts.

2. Material and methods

2.1. Plant material and preparation of the ethanol: water extracts

Leaves of *Stevia rebaudiana* (Bertoni) Bertoni were kindly provided by "Cantinho das Aromáticas", an organic and certified farm from Vila Nova de Gaia, Portugal and the botanical identification was confirmed by the botanist, Dr. Carlos Aguiar from School of Agriculture of the Polytechnic Institute of Bragança, Portugal.

The dried samples were reduced to powder and submitted to an extraction procedure, carried out at room temperature (25 °C ±1), in three independent experiments: 4 g of powder were stirred with 30 mL of ethanol: water (80:20, v/v) for 1 h, filtered and re-extracted in the same conditions. Then, ethanol was removed using a rotary evaporator (Büchi R-210, Flawil, Switzerland), and water by lyophilisation (−48 °C and 0.100 mbar, during 2–3 days, FreeZone 4.5, Labconco, Kansas City, MO, USA). Finally, stock solutions (50 mg/mL) were prepared by dissolving the obtained extract in water, from which further studies to assess the anti-*Candida* activity were performed. For phenolic compounds analysis, the extract (2.5 mg/mL) was filtered through a 0.45 μm Whatman (GE Healthcare Life Sciences) syringe filter and transferred to an amber HPLC vial for chromatographic analysis.

2.2. Standards and reagents

HPLC grade acetonitrile 99.9% and analytical grade purity ethanol were from Fisher Scientific (Lisbon, Portugal). RPMI 1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) were from Merck (Darmstadt, Germany). Phenolic compounds were purchased from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Analysis of phenolic compounds

Phenolic composition of *S. rebaudiana* extract was determined using HPLC-DAD-ESI/MS (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), under conditions previously described (Barros et al., 2012). Double online detection was carried out in a diode array detector (DAD), using 280 nm and 370 nm as preferred wavelengths and with a MS detector, performing in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. The identification of phenolic compounds was determined by comparing their retention time, UV–vis and mass spectra with those obtained with standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with the available data reported in the literature. For quantitative analysis, a calibration curve (1-100 μg/mL) for each available phenolic standard was constructed based on the area of the peak at the detection wavelength used in the HPLC-DAD analysis. To the identified phenolic compounds in which no commercial standard was available, individual quantification was performed using the calibration curves of another similar compound belonging to the same phenolic group. Analyses were carried out in triplicate and the results expressed in mg per g of dried extract.

2.4. Evaluation of the anti-Candida activity

2.4.1. Yeast strains

In this study, eight *Candida* strains were used, four of them were reference strains, namely *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750; and the other four were clinical isolates of *C. albicans* (from blood), *C. glabrata* (from urine), *C. parapsilosis* (from urine) and *C. tropicalis* (from vaginal secretion). The clinical isolates were obtained from the archive collection of the Laboratory of Medical Mycology, Universidade Estadual de Maringá, Brazil. In each experiment, *Candida* strains

were sub-cultured overnight in SDA or SDB, at 37 °C, and the cellular density was adjusted at 1×10^5 using a Neubauer chamber.

2.4.2. Effect on Candida planktonic cells

A) Minimal inhibitory concentration (MIC) determination

The antifungal activity of *S. rebaudiana* extract was determined by using the broth microdilution method according to the CLSI standard M27-A3 (Clinical and Laboratory Standards Institute., 2008), with some modifications as described for natural products (Dalben-Dota et al., 2010). For that, serial dilutions of the stevia extract stock solution (50 mg/mL) were performed at a ratio of 2, from 1:2 to 1:1024. In this way, the concentrations tested of the extract ranged from 0.05 to 25 mg/mL. The test was carried out in RPMI 1640 with L-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-morpholino)-propanesulfonic acid (pH 7.2) as buffer, supplemented with 2% glucose, in 96-well flat-bottomed microtiter plates (Orange Scientific, Braine-l' Alleud, Belgium).

After incubation at 37 °C for 24 h, MIC values were determined by direct observation, as the antifungal concentration where there was reduction of at least 50% growth by comparison with the control (cells grown without extract). *Candida* species cultivability was measured by using the drop test assay and determination of colony forming units (CFUs). Drop tests were performed dropping 10 µL of each concentration tested on SDA, while CFUs determination was performed after plating serial dilutions on SDA, and then incubated at 37 ºC during 24 h. Then, drop tests were observed and the number of colonies counted. The results were presented as total CFUs (log CFUs) and the experiments repeated in triplicate on three independent occasions.

B) Time-kill curves determination

Time kill curves were performed for the reference strains of *Candida* species selected, namely *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750, with slight modifications (Klepser, Wolfe, Jones, & Nightingale, 1997). Prior to testing, *Candida* cells were sub-cultured on SDA and the inoculum adjusted to 1 – 5×10^5 yeasts/mL, in RPMI 1640 medium, using a Neubauer chamber. Then, each *Candida* strain suspension was grown in the presence of the stevia extract at a concentration of 12.5 mg/mL (selected in accordance to the obtained MICs values). The RPMI 1640 medium without plant extract was used as a positive control. Test suspensions were placed on a shaker and incubated at 37 °C. At predetermined time points (0, 2, 4, 6, 8, 10, 12, 24 and 36 h), serial dilutions were performed on SDA for CFUs determination. Following incubation at 37 °C for 24 h, the number of CFUs was counted, and the results were presented as the log CFUs/mL. All the experiments were performed in triplicate and repeated on three independent occasions.

2.4.3. Effect on Candida biofilms determination

A) Biofilm formation

To evaluate the effect of *S. rebaudiana* extract on *Candida* biofilm formation, the extract was added at the beginning of their formation. For that, *Candida* cells were grown on SDA for 24 h at 37 °C, then inoculated in SDB and incubated for 18 h at 37 °C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 3000 *g* for 10 min, at 4 °C, and washed twice with 15 mL of phosphate-buffered saline (PBS; pH 7; 0.1 M). Volumes of 100 µL of extract (2 × final concentration) in RPMI 1640 medium were added to each well of microtiter plates. Subsequently, 100 µL of standardized yeast cell suspension (2×10^5 yeasts/mL) was added and plates were incubated at 37 °C on a shaker at 120 rpm/min for 24 h to allow biofilm formation. The extract concentrations used in this study (12.5 mg/mL and 25 mg/mL) were based on the previous findings of antimicrobial susceptibility (section 2.4.2.:

MIC and time-kill curves determination in *Candida* planktonic cells). Negative controls (200 μL of only RPMI 1640 medium) were also included.

Biofilms were analyzed by CFUs determination. For that, after 24 h the medium was removed and the biofilms were washed once with 200 μL of PBS to remove non-adhered cells. Then, the biofilms were scraped from the respective wells and the suspensions vigorously vortexed for approximately 2 min to disaggregate cells from the matrix (Martins, Ferreira, Henriques, & Silva, 2016). Serial dilutions were made in PBS, plated onto SDA and incubated for 24 h at 37 °C. The results were presented in terms of log CFUs and the experiments repeated in triplicate on three independent occasions.

2.5. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Results were compared using one-way ANOVA followed by Bonferroni multiple comparisons, using GraphPad Prism version 6 (GraphPad Software, CA, USA). The significance level was set at $p < 0.05$.

3. Results and Discussion

3.1. Phenolic compounds determination

The phenolic profile of *S. rebaudiana* extract obtained after ethanol: water extraction is shown in **Table 1**.

Sixteen compounds were detected, six of which were phenolic acid derivatives (mainly caffeoylquinic acid derivatives) and ten flavonoids (mainly quercetin and kaempferol derivatives). All these compounds were also previously identified in a study performed by Barroso et al. (2016) using *S. rebaudiana* Bertoni cultivated in the north-eastern of Portugal, aiming to access the differences in the phenolic composition when exposed to different conservation conditions. Nonetheless, in this study the plant was obtained commercially and different extraction solvents were used. However, the presence of most of the identified compounds in *S. rebaudiana* has already been described by other authors (Barroso et al., 2016; Gaweł-Bęben et al., 2015; Karaköse, Jaiswal, & Kuhnert, 2011; Karaköse, Müller, & Kuhnert, 2015; Muanda et al., 2011). Phenolic acids were present in *S. rebaudiana* ethanol: water extract with an abundance nearly to 3 times higher than flavonoids. Among the identified phenolic acids, caffeoylquinic acid derivatives represented the major compounds present in this sample, being 3,5-*O*-dicaffeoylquinic acid the most abundant, followed by 5-*O*-caffeoylquinic acid. In relation to flavonoids, quercetin derivatives were the most abundant, namely quercetin-3-*O*-rhamnoside, followed by quercetin-3-*O*-xyloside and quercetin-3-*O*-rutinoside.

3.2. Anti-Candida activity

3.2.1 Effect on planktonic cells

The high prevalence of candidiasis in recent years (Antinori, Milazzo, Sollima, Galli, & Corbellino, 2016; Lagunes & Rello, 2016) has been partly attributed to the emergence of

Candida species with acquired drug-resistance. This problem, associated with the limited number of chemotherapeutic agents and the toxicity of traditional compounds, have drawn attention towards to isolate new antifungal agents, mainly from plant extracts, with the goal of discovering new chemical structures with antimicrobial activity (Martins, Ferreira, Barros, Silva, & Henriques, 2014). In this sense, the effect of the *S. rebaudiana* extract on *Candida* planktonic cells was determined (**Figure 1**).

Figure 1 (I and **II)** shows the obtained MIC values to *S. rebaudiana* extract against eight *Candida* strains from different species and origins, with MIC values ranging from 3.12 to 25 mg/mL. **Figure 1 III** shows the logarithm of the colony forming units (CFUs) for *Candida* strains treated with *S. rebaudiana* extract, being the quantitative results in total concordance with observed in the qualitative data (**Figure 1I and II**). In fact, the most pronounced fungistatic effect of *S. rebaudiana* was observed against *C. glabrata* and *C. parapsilosis* strains, for which the two assayed strains, were inhibited in a dose-dependent manner.

A time-kill assay was performed to determine the kinetic effect of *S. rebaudiana* extract on the growth of *Candida* species (**Figure 2**). It was observed a substantial time-dependent reduction in the number of cultivable cells when compared to the control group, after exposure at 12.5 mg/mL during 24 h. The most pronounced effect was observed in the case of *C. tropicalis*, with a decrease of ≤99% cells (~1.98 logs) until 12 h, comparatively to the control group. In the case of *C. glabrata* and *C. parapsilosis* species the reduction was around 90% (1.0 log) up to 24 h, with a slight re-growth after that time, while no effects were observed on the growth pattern of *C. albicans*. These results corroborate the obtained MIC values (**Figure 1)**, where *C. albicans* presented the highest MIC value, when compared with *C. tropicalis*, *C. glabrata* and *C. parapsilosis* species.

3.2.2. Effect on Candida biofilms

Biofilms are structured communities that can cause serious medical problems, such as indwelling device-related infections. Recent data show that over 65% of all hospital infections derived from biofilms (Azevedo et al., 2014). Biofilm-associated *Candida* cells are resistant to a wide spectrum of available antifungal drugs (G. Ramage, Mowat, Jones, Williams, & Lopez-Ribot, 2009). This epidemic scenario point out the urgent need for safer and more effective antifungal therapies, that target fundamental biological processes and/or pathogenic determinants (Khan, Ahmad, Cameotra, & Botha, 2014). Therefore, one of the aims of this work was to evaluate the effect of *S. rebaudiana* extract on *Candida* biofilm formation. In the **Figure 3**, the ability of *C. tropicalis* and *C. glabrata* species to form biofilms was attenuated in the presence of *S. rebaudiana* extract with a reduction of approximately 90% in the number of cultivable cells in pre-formed biofilms at a concentration of 12.5 and 25 mg/mL extract, respectively. In fact, *C. glabrata* strains evidenced the highest reduction on biofilm formation (~1.0 to 3.2 log units) (**Figure 3B**), followed by *C. tropicalis* with a reduction around ~1.2 to 2.8 log units (**Figure 3D**) when compared with control group (p <0.01). No significant differences were observed for *C. albicans* and *C. parapsilosis* biofilms in relation to the untreated biofilms (**Figure 3A and C**). These results confirmed data obtained in MIC determination and kinetic studies, were *C. glabrata* were amongst to the most susceptible species, while *C. albicans* followed by *C. parapsilosis* were the most resistant. The differences observed may be in part attributed to the observed in the cell wall being more complex on these species than in *C. glabrata* species (Groot et al., 2008). In fact, considering the marked ability of *Candida* cells to adapt to a wide variety of different habitats with the consequent formation of surface-attached microbial communities, biofilms become 10 to 100 times more resistant than planktonic cells (Rautemaa & Ramage, 2011; Sardi et al., 2014).

4. Conclusion

The obtained results through this *in vitro* experiment indicate that *Stevia* might be a potential

candidate for the development of new strategies to manage fungal infections. Phenolic extract obtained from *Stevia* leaves seems to provide very interesting benefits on *Candida* infections management. It is important to highlight that *Stevia* acted in a non-selective manner, once positive effects were observed both against planktonic cells of NCAC species, namely *C. tropicalis* and *C. glabrata* strains. This is a very promising data, considering that NCAC species have shown to be highly resistant to conventional antifungal agents, namely in face to biofilm formation.

Considering their chemical composition in terms of bioactive compounds, *Stevia* extract may be also used for different cosmetic, food and pharmacological purposes, as an added-value ingredient for these industries. In that respect, it represents an economic alternative of great interest. Nevertheless, further studies are needed to deepen knowledge on this field as also to elucidate the effective mechanism of action of this plant extract.

Acknowledgements

The authors thank to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme Horizon PT2020 for financial support to CIMO (UID/AGR/00690/2013), LSRE (Project UID/EQU/50020/2013), S. Silva (SFRH/BPD/109053/2015) and L. Barros (SFRH/BPD/107855/2015) grants. The authors would also like to thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (Cnpq) and Fundação Araucária for financial support. FCT for Strategic Project of the UID/BIO/04469/2013 unit, FCT and European Union funds (FEDER/COMPETE) for the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). F. Tobaldini-Valério would also like to acknowledge the financial support of CAPES – Proc. 9469/14-1.

References

Antinori, S., Milazzo, L., Sollima, S., Galli, M., & Corbellino, M. (2016). Candidemia and invasive candidiasis in adults: A narrative review. *European Journal of Internal Medicine*. http://doi.org/10.1016/j.ejim.2016.06.029

Arendrup, M. C. (2013). *Candida* and Candidaemia: Susceptibility and Epidemiology. *Danish Medical Journal*, *60*(11), 1–32.

Azevedo, M. M., Ramalho, P., Silva, A. P., Teixeira-Santos, R., Pina-Vaz, C., & Rodrigues, A. G. (2014). Polyethyleneimine and polyethyleneimine-based nanoparticles: novel bacterial and yeast biofilm inhibitors. *Journal of Medical Microbiology*, *63 (pt9)*, 1167–1173.

Baillie, G. S., & Douglas, L. J. (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *The Journal of Antimicrobial Chemotherapy*, *46*(3), 397–403. http://doi.org/10.1093/JAC/46.3.397

Barros, L., Dueñas, M., Dias, M. I., Sousa, M. J., Santos-Buelga, C., & Ferreira, I. C. F. R. (2012). Phenolic profiles of *in vivo* and *in vitro* grown *Coriandrum sativum* L. *Food Chemistry*, *132*(2), 841–848. http://doi.org/10.1016/j.foodchem.2011.11.048

Barroso, M., Barros, L., Rodrigues, M. Â., Sousa, M. J., Santos-Buelga, C., & Ferreira, I. C. F. R. (2016). *Stevia rebaudiana* Bertoni cultivated in Portugal : A prospective study of its antioxidant potential in different conservation conditions. *Industrial Crops & Products*, *90*, 49–55. http://doi.org/10.1016/j.indcrop.2016.06.013

Bhatt, M., Sarangi, G., Paty, B. P., Mohapatra, D., Chayani, N., Mahapatra, A., Sahoo, D. (2015). Biofilm as a virulence marker in *Candida* species in Nosocomial blood stream infection and its correlation with antifungal resistance. *Indian Journal of Medical Microbiology*, *33 Suppl*, 112–4. http://doi.org/10.4103/0255-0857.150909

Chan, P., Tomlinson, B., Chen, Y. J., Liu, J. C., Hsieh, M. H., & Cheng, J. T. (2000). A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *British Journal of Clinical Pharmacology*, *50*(3), 215–220. http://doi.org/10.1046/j.1365-2125.2000.00260.x

Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2012). *Candida* biofilms associated with CVC and medical devices. *Mycoses*, *55*(s1), 46–57. http://doi.org/10.1111/j.1439-0507.2011.02149.x

Clinical and Laboratory Standards Institute. (2008). *M27-A3, Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard*. (Wayne, Ed.) (3rd ed). PA.

Dalben-Dota, K. F., Faria, M. G. I., Bruschi, M. L., Pelloso, S. M., Lopes-Consolaro, M. E., & Svidzinski, T. I. E. (2010). Antifungal activity of propolis extract against yeasts isolated from vaginal exudates. *Journal of Alternative and Complementary Medicine*, *16*(3), 285–290.

Das, S., Das, A., Murphy, R., Punwani, I., Nasution, M., & Kinghorn, A. (1992). Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. *Basic Sciences*, *26*, 363–366.

Deorukhkar, S. C., Saini, S., & Mathew, S. (2014). Non- *albicans Candida* infection: An emerging threat. *Interdisciplinary Perspectives on Infectious Diseases*, *2014*. http://doi.org/10.1155/2014/615958

Garcia, D., Garcia-Cela, E., Ramos, A. J., Sanchis, V., & Marín, S. (2011). Mould growth and mycotoxin production as affected by *Equisetum arvense* and *Stevia rebaudiana* extracts. *Food Control*, *22*(8), 1378–1384. http://doi.org/10.1016/j.foodcont.2011.02.016

Garcia, D., Ramos, A. J., Sanchis, V., & Marín, S. (2012). Effect of *Equisetum arvense* and *Stevia rebaudiana* extracts on growth and mycotoxin production by *Aspergillus flavus*

and *Fusarium verticillioides* in maize seeds as affected by water activity. *International Journal of Food Microbiology*, *153*(1–2), 21–27. http://doi.org/10.1016/j.ijfoodmicro.2011.10.010

Gaweł-Bęben, K., Bujak, T., Nizioł-Łukaszewska, Z., Antosiewicz, B., Jakubczyk, A., Karaś, M., & Rybczyńska, K. (2015). *Stevia rebaudiana* Bert. leaf extracts as a multifunctional source of natural antioxidants. *Molecules*, *20*(4), 5468–5486. http://doi.org/10.3390/molecules20045468

Groot, P. W. J., Kraneveld, E. A., Yin, Q. Y., Dekker, H. L., Groß, U., Crielaard, W., Weig, M. (2008). The cell wall of the human pathogen *Candida glabrata*: Differential incorporation of novel adhesin-like wall proteins. *Eukaryotic Cell*, *7*(11), 1951–1964. http://doi.org/10.1128/EC.00284-08

Jeppesen, P. B., Gregersen, S., Alstrup, K. K., & Hermansen, K. (2002). Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: studies in the diabetic Goto-Kakizaki (GK) rats. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, *9*(1), 9–14. http://doi.org/10.1078/0944-7113-00081

Jeppesen, P. B., Gregersen, S., Poulsen, C. R., & Hermansen, K. (2000). Stevioside acts directly on pancreatic β cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate—sensitivie K+-channel activity. *Metabolism*, *49*(2), 208–214. http://doi.org/10.1016/S0026-0495(00)91325-8

Karaköse, H., Jaiswal, R., & Kuhnert, N. (2011). Characterization and quantification of hydroxycinnamate derivatives in *Stevia rebaudiana* leaves by LC-MS. *Journal of Agricultural and Food Chemistry*, *59*(18), 10143–10150. http://doi.org/10.1021/jf202185m

Karaköse, H., Müller, A., & Kuhnert, N. (2015). Profiling and quantification of phenolics in *Stevia rebaudiana* leaves. *Journal of Agricultural and Food Chemistry*, *63*(41), 9188–9198. http://doi.org/10.1021/acs.jafc.5b01944

Khan, M. S., Ahmad, I., Cameotra, S. S., & Botha, F. (2014). Sub-MICs of *Carum copticum* and *Thymus vulgari*s influence virulence factors and biofilm formation in *Candida* spp. *BMC Complementary and Alternative Medicine*, *14*(1), 337.

Klepser, M. E., Wolfe, E. J., Jones, R. N., & Nightingale, C. H. (1997). Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against A *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, *41*(6), 1392–1395.

Komissarenko, N., Derkach, A., Kovalyov, I., & Bublik, N. (1994). Diterpene glycosides and phenylpropanoids of *Stevia rebaudiana* Bertoni (Asteraceae). *Rast. Res.*, *1*(2), 53–64.

Lagunes, L., & Rello, J. (2016). Invasive candidiasis: from mycobiome to infection, therapy, and prevention. *European Journal of Clinical Microbiology and Infectious Diseases*, 1–6. http://doi.org/10.1007/s10096-016-2658-0

Lee, C. N., Wong, K. L., Liu, J. C., Chen, Y. J., Cheng, J. T., & Chan, P. (2001). Inhibitory effect of stevioside on calcium influx to produce antihypertension. *Planta Medica*, *67*(9), 796–799. http://doi.org/10.1055/s-2001-18841

Lyon, J. P., & de Resende, M. A. (2006). Correlation between adhesion, enzyme production, and susceptibility to fluconazole in *Candida albicans* obtained from denture wearers. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, *102*(5), 632–638. http://doi.org/10.1016/j.tripleo.2005.12.015

Marol, S., & Yücesoy, M. (2007). Molecular epidemiology of *Candida* species isolated from clinical specimens of intensive care unit patients. *Mycoses*, *51*, 40–9. http://doi.org/10.1111/j.1439-0507.2007.01435.x

Martins, N., Barros, L., Henriques, M., Silva, S., & Ferreira, I. C. F. R. (2015). Activity of phenolic compounds from plant origin against *Candida* species. *Industrial Crops and*

Products, *74*, 648–670. http://doi.org/10.1016/j.indcrop.2015.05.067

Martins, N., Ferreira, I. C. F. R., Barros, L., Silva, S., & Henriques, M. (2014). Candidiasis: predisposing factors, prevention, diagnosis and alternative treatment. *Mycopathologia*, *177*(5–6), 223–40. http://doi.org/10.1007/s11046-014-9749-1

Martins, N., Ferreira, I. C. F. R., Henriques, M., & Silva, S. (2016). *In vitro* anti-*Candida* activity of *Glycyrrhiza glabra* L. *Industrial Crops & Products*, *83*, 81–85. http://doi.org/10.1016/j.indcrop.2015.12.029

Mayer, F. L., Wilson, D., & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence*, *4*(2), 119–28. http://doi.org/10.4161/viru.22913

Muanda, F. N., Soulimani, R., Diop, B., & Dicko, A. (2011). Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. *LWT - Food Science and Technology*, *44*(9), 1865–1872. http://doi.org/10.1016/j.lwt.2010.12.002

Nikawa, H., Egusa, H., Makihira, S., Okamoto, T., Kurihara, H., Shiba, H., … Hamada, T. (2006). An *in vitro* evaluation of the adhesion of *Candida* species to oral and lung tissue cells. *Mycoses*, *49*(1), 14–17. http://doi.org/10.1111/j.1439-0507.2005.01176.x

Ramage, G., Mowat, E., Jones, B., Williams, C., & Lopez-Ribot, J. (2009). Our current understanding of fungal biofilms. *Critical Reviews in Microbiology*, *35*, 340–355.

Ramage, G., Saville, S. P., Thomas, D. P., & López-Ribot, J. L. (2005). *Candida* biofilms: an update. *Eukaryotic Cell*, *4*(4), 633–8. http://doi.org/10.1128/EC.4.4.633-638.2005

Rautemaa, R., & Ramage, G. (2011). Oral candidosis-clinical challenges of a biofilm disease. *Critical Reviews in Microbiology*, *37*(4), 328–36. http://doi.org/10.3109/1040841X.2011.585606

Sardi, J. D. C. O., Pitangui, N. D. S., Rodríguez-Arellanes, G., Taylor, M. L., Fusco-Almeida, A. M., & Mendes-Giannini, M. J. S. (2014). Highlights in pathogenic fungal biofilms. *Revista Iberoamericana de Micología*, *31*(1), 22–29. http://doi.org/10.1016/j.riam.2013.09.014

Sedghi, M., & Gholi-Toluie, S. (2013). Influence of salicylic acid on the antimicrobial potential of *Stevia* (*Stevia Rebaudiana* Bertoni, Asteraceae) leaf extracts against soybean seed-borne pathogens. *Tropical Journal of Pharmaceutical Research*, *12*(6), 1035–1038. http://doi.org/10.4314/tjpr.v12i6.25

Tadhani, M. B. B., Patel, V. H. H., & Subhash, R. (2007). *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. *Journal of Food Composition and Analysis*, *20*(3–4), 323–329. http://doi.org/10.1016/j.jfca.2006.08.004

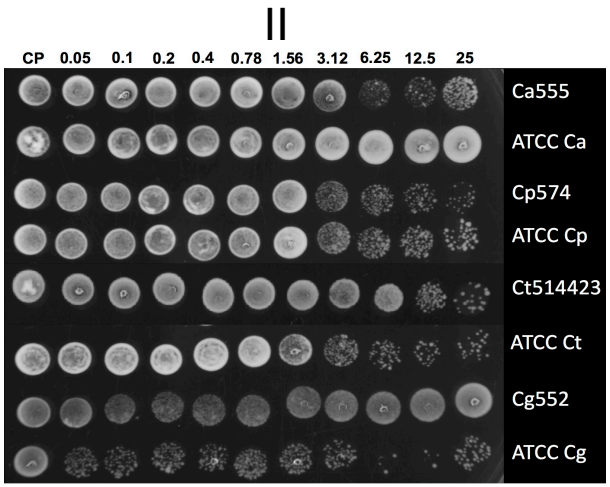
Table 1. Phenolic compounds identification and quantification in *Stevia rebaudiana* Bertoni leaves.

Compounds	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)
1	5.1	328	353	191(100),179(45),161(6),135(66)	3-*O*-Caffeoylquinic acid	5.08 ± 0.04
2	7.4	328	353	191(72),179(80),173(100),161(9),135(77)	4-*O*-Caffeoylquinic acid	3.6 ± 0.2
3	8.1	328	353	191(100),179(3),161(6),135(4)	5-*O*-Caffeoylquinic acid	43.6 ± 0.1
4	19.4	354	609	301(100)	Quercetin-3-*O*-rutinoside	6.74 ± 0.01
5	20.7	354	463	301(100)	Quercetin-3-*O*-glucoside	1.13 ± 0.01
6	21.1	328	515	353(87),335(45),191(41),179(76),173(91),161(17),135(25)	3,4-*O*-Dicaffeoylquinic acid	2.9 ± 0.3
7	21.3	358	579	301(100)	Quercetin-*O*-pentosyl-deoxyhexoside	1.11 ± 0.01
8	21.7	348	447	285(100)	Kaempferol-3-*O*-glucoside	1.96 ± 0.01
9	22.9	328	515	353(90),335(8),191(100),179(89),173(14),161(8),135(46)	3,5-*O*-Dicaffeoylquinic acid	50.0 ± 0.1
10	23.7	358	433	301(100)	Quercetin-3-*O*-xyloside	9.19 ± 0.01
11	24.8	350	447	301(100)	Quercetin-3-*O*-rhamnoside	15.3 ± 0.2
12	25.5	328	515	353(81),335(3),191(21),179(73),173(100),135(21)	4,5-*O*-Dicaffeoylquinic acid	17.3 ± 0.2
13	26.8	350	417	285(100)	Kaempferol-*O*-pentoside	0.99 ± 0.01
14	27.5	350	417	285(100)	Kaempferol-*O*-pentoside	0.23 ± 0.01

15	28.9	336	771	609(85),301(27)	Quercetin-*O*-caffeoyl-*O*-rutinoside	0.39 ± 0.01
16	29.3	346	431	285(100)	Kaempferol-*O*-deoxyhexoside	1.21 ± 0.01
					Total phenolic acid derivatives	122 ± 1
					Total flavonoids	38.2 ± 0.2
					Total phenolic compounds	161 ± 1

I

Strains	Source	MIC (mg/mL)
C. albicans		
Ca555	Blood	6.25
ATCC 90028	Reference	≥ 25
C. parapsilosis		
Cp574	Urine	3.12
ATCC 22019	Reference	3.12
C. tropicalis		
Ct514423	Vaginal secretion	12.5
ATCC 750	Reference	3.12
C. glabrata		
Cg552	Urine	≥ 25
ATCC 2001	Reference	3.12



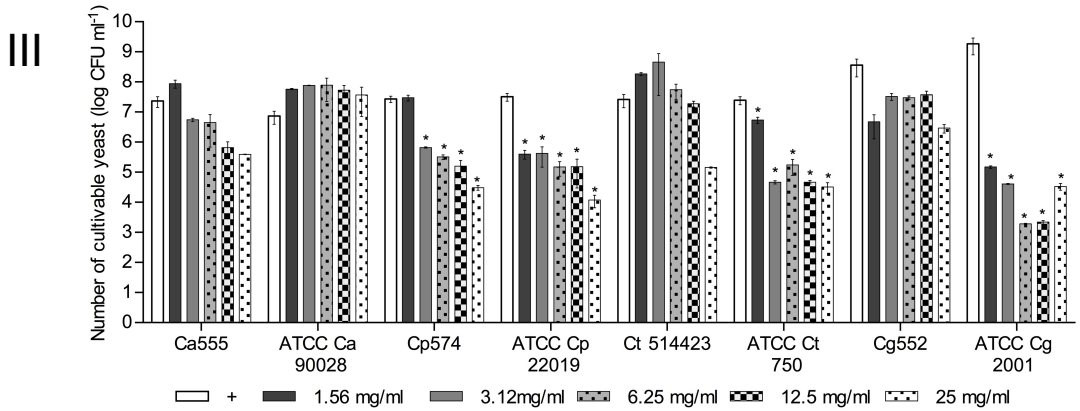


Figure 1. *In vitro* effects of the *S. rebaudiana* extract on *Candida* strains planktonic cells. I) Minimal inhibitory concentrations (MICs values), II) serial drop tests III) and Logarithm of number *Candida* species cells grown in the presence of increased concentrations of *S. rebaudiana* extract during 24 h. Error bars represent standard deviation. Statistical *p* value (represented by *) indicates concentrations that are significantly different from control. *$p < 0.05$.

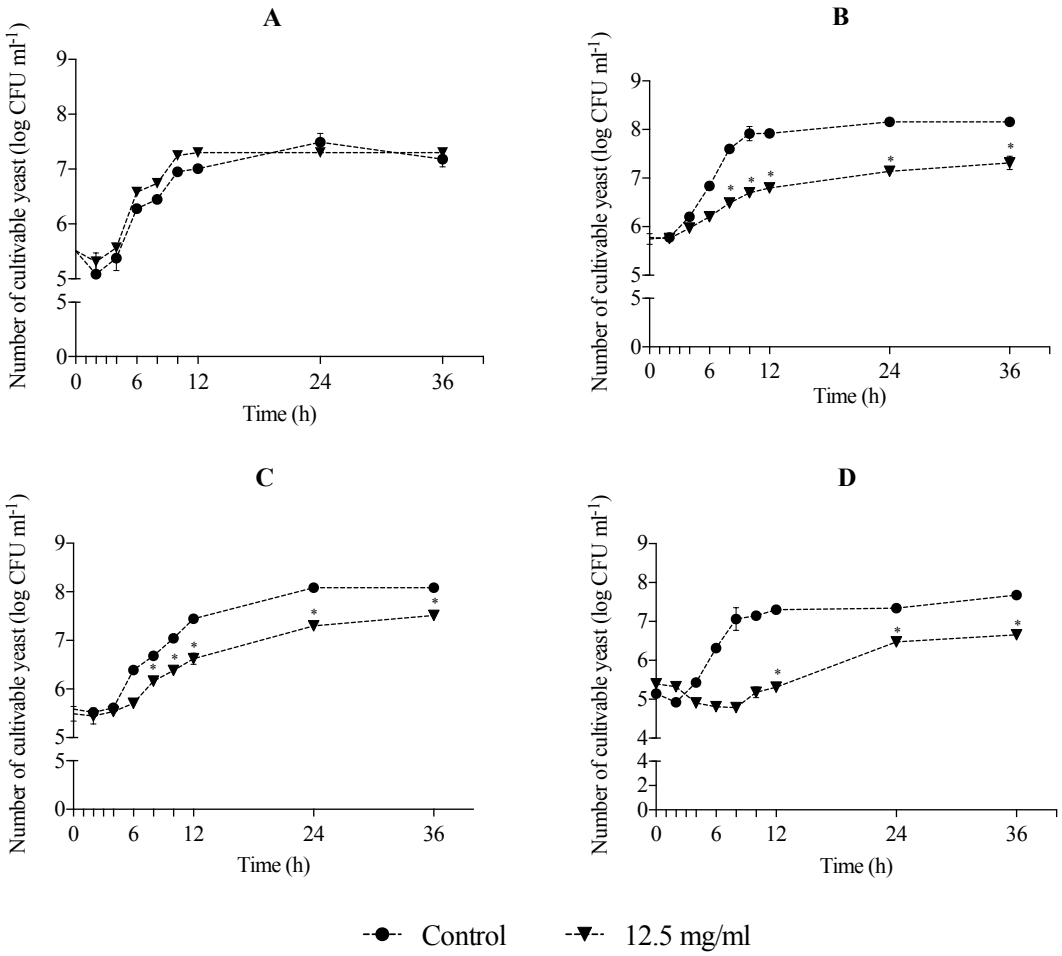


Figure 2. Killing kinetics of the *S. rebaudiana* extract against *Candida* species. A: *Candida albicans* ATCC 90028; B: *Candida glabrata* ATCC 2001; C: *Candida parapsilosis* ATCC 22019; D: *Candida tropicalis* ATCC 750. Standardized yeast cells suspensions were exposed to 12.5 mg/mL of *S. rebaudiana* extract. At determined time intervals, samples were serially diluted and plated for colony counts. Each data point represents mean result ± standard deviation (error bars) from three experiments. Statistical *p* value (represented by *) indicates concentrations that are significantly different from control. *$p < 0.05$.

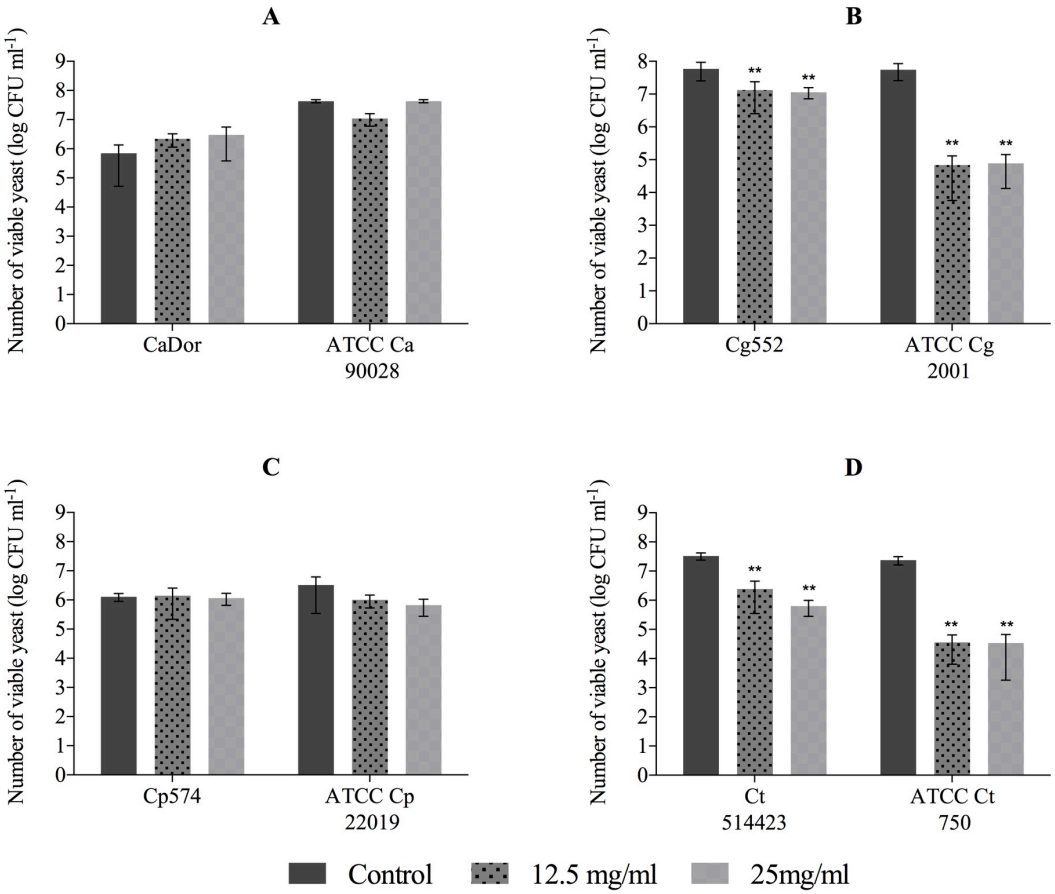


Figure 3. Effect of the *S. rebaudiana* extract on *Candida* strains (n=8) biofilms. Logarithm of number of colony forming units (CFUs) of different *Candida albicans* (n=2) (A), *Candida glabrata* (n=2) (B), *Candida parapsilois* (n=2) (C) and *Candida tropicalis* (n=2) (D) biofilms strain, treated with different concentrations of the *S. rebaudiana* extract. *Candida* cells were allowed to form biofilm for 24 h with extract at 37°C. Controls: biofilms grown in RPMI medium in the absence of extract. Error bars represented in graphics indicate the standard deviation; ** correspond to $p < 0.01$.

CHAPTER III

GENERAL CONCLUSIONS

This study showed that propolis, *H. androsaemum* and *S. rebaudiana* extracts are potent antifungal agents with effect on *Candida* planktonic cells and biofilms. It is important to highlight, that these effects were not only observed against *C. albicans* but on other NCAC species, namely *C. tropicalis*, *C. glabrata* and *C. parapsilosis*. This is a very promising data, considering that NCAC species has shown to be highly resistant to the conventional antifungal agents. Such properties of this extracts as inhibitor of *Candida* virulence factors represent an alternative and innovative pathways of therapy for pathogens that are resistant to classical antimicrobial agents.

Total phenolic content, present in the propolis extract, was shown to be responsible for the anti-*Candida* effects observed. Furthermore, for *H. androsaemum* and *S. rebaudiana* extract, 5-o-caffeoylquinic acid followed by 3-o-caffeoylquinic acid were the most abundant phenolic compound, and the observed anti-*Candida* effect may be directly related by its abundance at the concentrations tested.

Considering the chemical composition in terms of bioactive compounds, this extracts may be also used for different cosmetics, medicinal and pharmacological purposes, as also an added-value ingredient for food industries. In that respect, it represents an economic alternative of great interest.

FUTURE PERSPECTIVES

The work described in this thesis provided a useful insight into several aspects of antifungal potential of natural products, leading to interesting new questions for further research. Some of the suggestions that should be taken into consideration for future investigations are:

- To increase our understanding of antifungal properties these compounds. The investigations may help to understand the mechanism of action, specially in what concerns its role in biofilm activity, which are particularly problematic in clinical settings;
- The potential cytotoxicity of the compounds should be assessed, in order to facilitate use as antifungals in human therapy;
- To study the effect of the combination of the conventional antifungal agents and plants extracts;
- To evaluate antifungal activity against a panel of opportunistic fungi.