UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

RAFAELA ALVES DA SILVA

Antifungal activity of Punicalagin isolated from *Punica granatum* and synergism with Nystatin against *Candida albicans*: cellular metabolism, detection of virulence genes and proteomic analysis

Atividade antifúngic

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Orientador: **Profª. Drª. Vanessa Soares Lara**

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ABSTRACT

Antifungal activity of Punicalagin isolated from *Punica granatum* and synergism with Nystatin against *Candida albicans*: cellular metabolism, detection of virulence genes and proteomic analysis

Despite therapeutic advances, opportunistic fungal infectious diseases have increased in prevalence and have become a universal public health problem. *Candida albicans* (CA) is a commensal fungus that under certain environmental conditions can act as an opportunistic pathogen and colonize mucous membranes and tissues causing local and systemic infections. The emergence of drug resistant strains, as well as the increase of immunosuppressed patients, has limited therapeutic options. Therefore the aim of this study was to evaluate 1) The antifungal activity of Punicalagin (P), alone or in combination with Nystatin (N), against two CA strains. 2) The cytotoxicity effect of **P** and **N**, as well as the combination of both, on human primary cells. In the first step, yeasts of CA ATCC 90028 and SC5314 were exposed to **P** and **N** for 24 hours. The Minimal Inhibitory Concentration (MIC) of **P** (50 µg/mL) and **N** (3.9 µg/mL) were determined by the broth microdilution assay. The Checkerboard Assay was performed to verify the synergism between the combinations (8: PN, P8 N/4, P/8 N/2, P/8 N, P/4 N/2, P/4 N, P/2 N/4 and P/2 N/2), which were selected from the 56 combinations initially tested. The fungal metabolism was assessed by the metabolic reduction assay XTT (24h) and Minimum Microbicidal Concentration (MMC) was determined by Colony-forming unit (CFU/mL, 24h). The evaluation of virulence factors gene expression was amplified and quantified by PCR real time (Polymerase Chain Reaction-RT-qPCR, only for P/8 N/4 in 24h). The analysis of proteins related to essential biological processes of the fungus was performed by LC-MS/MS (24h). MIC and MMC of **P** were significantly reduced in the presence of **N**, indicating synergism between both. Once the antifungal potential of **P** was verified, we proposed to evaluate whether this drug acts through the same mechanism of action of **N**, altering the permeability of the cell membrane of the fungus (binding ergosterol). The evaluation of gene expression has demonstrated upregulation of some genes that may be related to a defense mechanism associated with stress or cell death. The proteomics analysis revealed alterations in the expression of several proteins in the fungi exposed to P/8 N/4 in relation to negative control, correlated with important biological processes, such as, energy metabolism,

stress response, drug metabolism, among others. In the second step, cytotoxicity assays involving **P** and **N**, as well as the combination of both, were undertaken on human palate epithelial cells (HPEC) and human gingival fibroblasts cells (HGF) by Alamarblue dye. Similarly, **P** cytotoxicity was reduced when used in combination with **N**. Based on these *in vitro* results, the synergistic antifungal activity produced between **P** and **N** suggested that the combination of drugs, at the concentrations tested, may be a topical therapeutic or preventive alternative to be used in cases of superficial candidiasis, such as denture stomatitis.

Keywords: *Candida albicans,* Oral candidiasis, *Punica granatum,* Antifungals agents.

RESUMO

Atividade antifúngica de Punicalagina isolada de *Punica granatum* e sinergismo com Nistatina sobre *Candida albicans*: metabolismo celular, detecção de genes de virulência e análise proteômica

Apesar dos avanços terapêuticos, doenças infecciosas por fungos oportunistas têm aumentado em prevalência e tornaram-se um problema universal de saúde pública. *Candida albicans* (CA) é um fungo comensal que, sob certas condições ambientais, pode atuar como um patógeno oportunista e colonizar mucosas e tecidos causando infecções locais e sistêmicas. O surgimento de cepas resistentes às drogas convencionalmente utilizadas, assim como aumento de pacientes imunodeprimidos, tem limitado as opções terapêuticas. Portanto o objetivo deste estudo foi avaliar: 1) a atividade antifúngica de Punicalagina (P), com ou sem a associação de Nistatina (N), contra duas cepas de CA. 2) O efeito citotóxico de **P** e **N**, assim como suas combinações, em culturas primárias humanas de células epiteliais de palato (CEPH) e de fibroblastos gengivais (FGH). Na primeira etapa, leveduras de CA ATCC 90028 e SC5314 foram expostas a **P** e **N**, por 24 horas. As concentrações inibitórias mínimas (CIMs) de **P** (50 µg/mL) e **N** (3,9 µg/mL) foram determinadas pelo método de diluição em caldo. O Ensaio Checkerboard foi realizado para verificar o sinergismo entre as combinações (8: PN, P8 N/4, P/8 N/2, P/8 N, P/4 N/2, P/4 N, P/2 N/4 e P/2 N/2), as quais foram selecionadas a partir de 56 combinações inicialmente testadas. O metabolismo fúngico foi avaliado pelo método do XTT (24h) e a Concentração Mínima Microbicida (CMM) foi realizada através de Unidades formadoras de colônias (UFC/mL, 24h). A avaliação da expressão gênica de fatores de virulência foi amplificada e quantificada por PCR em tempo real (Reação em Cadeia da Polimerase-RT-qPCR, apenas para P/8 N/4 em 24h). A análise proteômica para identificação de proteínas alteradas foi realizada por LC-MS/MS (24h). A MIC e MMC de **P** foram significativamente reduzidas na presença de **N**, indicando sinergismo entre ambas. Confirmado o potencial antifúngico, o Ensaio de mecanismo de ação (teste do ergosterol) foi realizado e não foi confirmada a ação de **P** através da ligação ao ergosterol. A avaliação da expressão gênica demonstrou regulação positiva de alguns genes que pode estar relacionado a um mecanismo de defesa associado ao estress ou a morte celular. A análise proteômica revelou proteínas diferencialmente expressas

nos fungos expostos a P/8 N/4 em relação ao controle negativo (sem tratamento), as quais estão relacionadas ao processo de metabolismo energético, resposta ao estress e metabolismo de drogas, dentre outros. Na segunda etapa, CEPH e FGH foram expostas a **P** e **N** e suas combinações por 24h para realização dos ensaios de viabilidade por Alamarblue. Combinada à **N**, a Punicalagina apresentou-se menos citotóxica do que de forma isolada. Com base nesses resultados *in vitro*, o sinergismo antifúngico produzido entre **P** e **N** sugere que a combinação das drogas pode ser uma alternativa terapêutica tópica para ser utilizada nos casos de candidose localizada, como por exemplo, a estomatite protética.

Palavras-chave: *Candida albicans,* Candidíase oral, *Punica granatum,* Agentes antifúngicos.

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1 Introduction

1 INTRODUCTION

Infectious diseases caused by opportunistic fungi have grown abruptly, becoming a public health problem. The high rates of morbidity and mortality associated with these diseases, the increase in the number of immunocompromised patients and the emergence of conventional antifungal resistant strains along with the adverse effects of current therapies, show the importance of the discovery of new drugs (BROWN et al., 2012; PACHAVA et al., 2013; ORGANIZATION, 2014).

Candida albicans (CA) is a commensal fungus and the most common opportunistic pathogen in humans. This fungus colonizes mucosal and abiotic surfaces and can cause local and systemic infections, from candidiasis to candidemia (MAVOR; THEWES; HUBE, 2005; SALERNO et al., 2011; LEE et al., 2014). Oral candidiasis is the most common fungal infection in humans, especially in immunocompromised patients, as well as in the elderly and users of removable total dentures, in this case known as denture stomatitis (DS), clinically manifested as spots or erythematous areas in the palate region (CHANDRA et al., 2001a; SHERMAN et al., 2002; SALERNO et al., 2011; WILLIAMS et al., 2013).

Currently, conventional treatments for DS include topical application of Nystatin (**N**), Amphotericin B, Chlorhexidine, Miconazole and Clotrimazole, are recommended as first-line treatment for uncomplicated cases of oral candidiasis (LYU et al., 2016). **N** is a polyene antifungal that acts by binding ergosterol and disrupts the major lipidic component of the fungal cell membrane resulting in the formation of porin channels. These pores disrupt the integrity of the fungal plasma membrane allowing the efflux of cations, such as K+, and leads to the leakage of cytosolic components which results in cell death, thus are fungicidal drugs (NIIMI; FIRTH; CANNON, 2010; SPAMPINATO; LEONARDI, 2013). Although **N** has excellent therapeutic effectiveness, some disadvantages related to the use of nystatin include: bad taste, drug interation, gastrointestinal side effects such as nausea, vomit and epigastric pain, especially in childrens or elder people. In addition, a relatively high cost of medication and the administration of doses four times per day contribute to low drug adherence (MARTINSON et al., 2009; BAKHSHI et al., 2012; KOVAC; MITIC; KOVAC, 2012; MANSOURIAN et al., 2014; MUKHERJEE et al., 2017).

Although oral Nystatin is the major antifungal agent used to treat oral Candidiasis in HIV-infected patient in resource-limited settings, studies have been conducted to identify lower-cost alternatives to replace the current treatment. Mukherjee et al. conducted a preclinical study with topical gentian violet which has anti-*Candida* potential and has been recommended by the World Health Organization (ORGANIZATION, 2014). This topical treatment showed no statistical differences with Nystatin, besides presenting a substantially lower acquisition cost than Nystatin (MUKHERJEE et al., 2017) .

Resistance of *Candida* strains to polyenes such as Nystatin and Amphotericin B is rare, having been found a nystatin resistance of 11.3% for non-*Candida albicans* species (MOHAMADI; MOTAGHI, 2014) . A study showed a lower susceptibility to Nystatin and Fluconazole in 9 clinical isolates when compared with the reference strain of *C. albicans* ATCC 90028 (SARDI et al., 2016) .

The resistance mechanism is probably due to loss of function of ERG6 or ERG3 gene through mutation, and involved in ergosterol biosynthesis, leading to the low content of ergosterol membrane detected in some resistant fungi (FICHTENBAUM et al., 2000; KANAFANI; PERFECT, 2008; NIIMI et al., 2010; SPAMPINATO; LEONARDI, 2013; MANSOURIAN et al., 2014). However, azole-resistant *C. albicans* is often found in HIV-infected patients with oropharyngeal candidiasis, resulting in cases of refractory candidiasis or therapeutic failure (SKIEST et al., 2007; SPAMPINATO; LEONARDI, 2013). Another point is that there are few potential targets of action to be explored in fungi that are not shared with human cells, both eukaryotes (ROCHA, 2002; CROSARIOL, 2010; DENNING; HOPE, 2010). For these reason leads us to find new strategies for *Candida*-related infections. Antifungals usually present some toxicity, since there are few targets of action in fungi that are not shared with human cells, both eukaryotes

Medicinal plants, used in traditional medicine, have been considered a valuable source of antimicrobial agents, which conjugated to antifungal formulations traditionally used in the clinic, intensify its antimicrobial activity against resistant strains (NASCIMENTO et al., 2000; ALAVARCE et al., 2015; DA SILVA et al., 2017). This interaction between drugs is known as synergism (ENDO et al., 2010; MERTAS et al., 2015). Therapies that use the combination of drugs can increase the action spectrum, improve the antifungal activity and reduce the associated side effects (SUN et al., 2017).

There are several mechanisms proposed for antifungal synergy through the drugs combination: (1) One type of interaction is inhibition of different stages of the same biochemical pathway. (2) Changes in the cell wall or cell membrane permeability promoted by an antifungal agent resulting in increased penetration of other antifungal agent. (3) Inhibition of carrier proteins. (4) Inhibition of different targets in fungal cells simultaneously, as targets of the cell wall and cell membrane (JOHNSON et al., 2004).

Punica granatum (*P. granatum*) is a fruit belongs to the *Lythraceae* family, popularly called "pomegranate" and a potential medicinal plant. Since ancient times, *P. granatum* has been used for the treatment of various diseases. Recently, the plant has attracted increasing interest of researchers in analyzing its composition and biological properties. As referred their antimicrobial properties, inhibits the growth of methicillin resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes, Yersinia enterocolitica, Escherichia coli, Human influenza* H3N2 and *C. albicans* (AJAIKUMAR et al., 2005; SEERAM et al., 2005; ADAMS et al., 2006; LANSKY; NEWMAN, 2007; ALTHUNIBAT et al., 2010; ENDO et al., 2010; GLAZER et al., 2012; MANSOURIAN et al., 2014). Almeida et al. (2018) used the *P. granatum* hydroethanolic extract associated with a denture adhesive that resulted in the significant interference of the development of *C. albicans* biofilms formed on thermopolymerizable acrylic resin specimens. Another study showed the use of a gel containing the extract of *P. granatum* as an antifungal agent and demonstrated promising effects for the clinical treatment of denture stomatitis (CÉSAR DE SOUZA VASCONCELOS et al., 2003).

The ellagitannins are the main polyphenols present in the *P. granatum*. Compounds such as granatins A and B, punicalagin and punicalin were isolated from the pericarp and are the main compounds responsible for the antimicrobial activity. Chemical analysis performed in one study showed that pomegranate phenolic compounds showed high levels of hydrolyzable tannins such as punicalin, punicalagin, pedunculagin and punigluconin (CATÃO et al., 2006; DUDONNÉ et al., 2009).

A therapeutic option to be used in combination with **N** is Punicalagin (P), an ellagitannin isolated from *P. granatum*, and one of the main components responsible for its antifungal activity. *In vitro* studies has previously been demonstrated the synergistic antifungal effect between **P** and commercially available Fluconazole using *C. albicans*, suggesting that the use of **P** in combination with other antifungals, such as **N** may be an interesting therapeutic strategy for the treatment of oral candidiasis (CATÃO et al., 2006; ENDO et al., 2010; ANIBAL et al., 2013).

Currently, elucidating the molecular mechanisms related to the ability of the *Candida* species to cause infections may be important for the development of new drugs that have as target virulence factors of the pathogen and less undesirable effects to the patients.

Many factors have been involved in the increase of CA pathogenicity, including production of phospholipases, adhesins (ALS Family), hyphae formation, expression of drug resistance genes and production of secreted aspartyl proteinases (SAP) (RÜCHEL et al., 1992; HUBE, 1996; CHAFFIN et al., 1998; WU et al., 2000). The use of Real-time reverse transcriptase–polymerase chain reaction (RT–qPCR) for the amplification of specific mRNA allows the study of the gene expression of these virulence factors.

Studies have shown that the development of mutant strains, whose genes encode proteins responsible for energy metabolism, stress response and biosynthesis of macromolecules that are essential for the fungus, have virulence and consequently attenuated host damage. Thus, investigating which proteins are exclusively or differentially expressed after antifungal treatments contribute to the understanding of several mechanisms involved in *Candida* species pathogenicity (TSANG; BANDARA; FONG, 2012; AOKI et al., 2013).

Here we aim to determine *in vitro* the antifungal activity of Punicalagin and Nystatin, alone and in combination, against two strains of CA associated with oral infections. The discovery of new antimicrobial components is of great relevance, particularly for Dentistry, since infections of the oral cavity of bacterial and fungal origin are relatively common problems, resulting in chronic inflammatory diseases such as *Candida*-associated denture stomatitis.

2 Discussion

2 DISCUSSION

Due to the increase of resistant strains to the conventional antifungal agents, the search for new therapeutic alternatives has been the focus of several studies (ENDO et al., 2010; MERTAS et al., 2015; SUN et al., 2017).

The antifungal drug combinations is a potential strategy to control the evolution of drug resistance and can be an effective solution for *Candida* infections, improving the action spectrum, increasing therapeutic efficacy and reducing toxicity and side effects (SUN et al., 2017).

Antifungals usually present some toxicity, since there are few targets of action in fungi that are not shared with human cells, both eukaryotes (ROCHA, 2002; CROSARIOL, 2010). Medicinal plants may be considered as new sources of active compounds for producing antimicrobial agents, which added to the antifungal formulations traditionally used in the clinic increase their antimicrobial activity against resistant strains (ENDO et al., 2010; MERTAS et al., 2015).

Thus, the main purpose of this study was to evaluate the effect against *C. albicans* of Punicalagin (P), the major ellagitannins present in *P. granatum*, and one of the main responsible for the antifungal activity, in combination with the conventional antifungal agent, Nystatin (N). Besides, we evaluated cytotoxic effect of drugs on human cells.

The first paper of this thesis initially tested the Minimal Microbicidal Concentrations (MIC) of the drugs Punicalagin (P) and Nystatin (N) alone that were established in 50 and 3.9 µg/mL, respectively. Subsequently the Checkerboard assay, which is widely used to compare *in vitro* the efficacy of the combination of two or more antimicrobials, was performed. When used in combination, **P** and **N** demonstrated good antifungal results with reduction of their previously established MICs, with a significant increase of the antifungal activity in comparison to the drugs alone; besides identifying the synergism in 4 combinations (P/8 N/4, P/8 N/2, P/4 N/2 and P/2 N/4). In addition, fungal metabolism was dramatically reduced in some combinations (2%) compared to drugs alone, as well as the inhibition of fungal growth was very high (100%) as opposed to the results of drugs alone that were ineffective as fungicides.

Endo et al. (2010) also identified a potently synergistic action of **P** in combination

with fluconazole with the reduction of fluconazole MIC, in addition to detecting changes in fungus morphology, such as thickening of the cell wall and presence of vacuoles. Other studies proved the antifungal action of *P. granatum* and correlated with tannins, among them, Punicalagin, one of the main compounds responsible for this activity (AJAIKUMAR et al., 2005; SEERAM et al., 2005; ADAMS et al., 2006; ; LANSKY; NEWMAN, 2007 ALTHUNIBAT et al., 2010; ANIBAL et al., 2013). In the present study, **P** demonstrated high cytotoxicity in human palate epithelial cells, however, in combination with **N** showed values close to those obtained with untreated cells (negative control), an essential prerequisite for topical therapy, since it was possible to obtain lower concentrations of the drugs, with lower or no cytotoxic potential for human cells. In contrast, cytotoxicity to fungal cells was increased.

The synergistic antifungal effect produced between **P** and **N** suggests that the combination of the two compounds may be a good topical alternative for the treatment of superficial oral candidiasis, such as denture stomatitis (DS). Combined treatment of natural products and conventional drugs is one of the effective treatments against *Candida* species. This therapeutic strategy has been shown to improve drug efficacy, decrease toxicity, side effects and antimicrobial resistance problems (CUI et al., 2015; OLFA et al., 2015; MERTAS et al., 2015).

Although we cannot accurately prove by which **P** pathway acts, studies have shown that it causes morphological changes usually related to fungal cell wall and in the intracellular microbial content, which might affect metabolism and fungal growth rate (ENDO et al., 2010) . In this way we suggest that the **P** mechanism of action may be related to the fungal cell wall, similar to what occurs with the echinocandins (DUNYACH et al., 2011; SPAMPINATO; LEONARDI, 2013). In order to confirm this premise, assays such as the sorbitol test and others that evaluate the morphological characteristics, such as scanning electron microscopy are required.

In addition, the combination with drugs such as Nystatin might facilitate the entry of **P** through cell membrane and thereby potentiate its antifungal activity (ENDO et al., 2010).

Based on the results of the 1st paper, the focus of the 2nd paper was to study more deeply fungal mechanisms altered by the action of drugs alone and in combination. For the 2nd paper, we decided to study only one combination classified as synergy by the checkerboard method (P/8 N/4 and drugs alone) in order to investigate the molecular mechanisms altered, but in a drug combination that provided

at least 50 % of viable cells. The other combinations could give us a better result on the mechanisms involved in the antifungal action, however, did not provide enough material for adequate analysis. We also followed the 24-hour exposure to drugs according to the 1st paper.

The second paper demonstrated that the virulence factors of CA, ATCC and SC strains, against **P** and **N** treatment undergo complex modulations in gene expression. Regarding the virulence factors of CA, in the present study, no significant difference in the gene expression of ALS 1-5 was identified in both CA strains after treatment with **P** and **N** compared to the control. In contrast, the treatment with drug combination (P/8 N/4) in sub-inhibitory concentration, caused a significant increase in the gene expression of ALS-1, ALS-4 and ALS-5 in the ATCC strain, and ALS-4 in the SC strain compared to the control. Similar results were found in SAPs gene expression, exhibiting increased level in SAP-2, SAP-3 and SAP-9 in ATCC strain, and SAP-2 in SC strain, after the treatment with P/8 N/4 compared to the negative control (C-).

A hypothesis for the increase in virulence factors gene would be a response of fungi to antifungals administered in sub-inhibitory concentrations. Some studies have also demonstrated increased gene expression of ALS and SAP in strains treated with fluconazole subdoses (WU et al., 2000; BARELLE et al., 2007). Barelle et al. (2007) showed that the gene induction was involved to a defense mechanism of CA stress-related. The authors also demonstrated that the up-regulation of different SAPs is under different controls, such as the yeast-hyphae transition, nevertheless these effects appear to be transient *in vivo*. Wu et al. (2000) identified an increase in the expression of SAPs and did not correlate this increase to cell death or non-specific release of SAP, since it did not detect a reduction in the number of CFUs and no significant release of enolase, an enzyme constitutive of the glycolytic pathway. Thus, they correlated that exposure to sub-inhibitory doses of fluconazole may result in increased extracellular production of SAP by strains capable of overexpressing genes related to a multidrug resistance efflux pump (MDR1), associated to an increase in CA virulence *in vivo* (WU et al., 2000). However, in our results, we can correlate the up-regulation of some genes with the cell death process, because in CFU studies P/8 N/4 demonstrated a reduction in fungal growth compared to the negative control (data not shown). Additionally, although enolase had its expression decreased relative to the control, identified by proteomic analysis, this could be connected to the drug exposure time, which was 24 hours, differently of the short exposures reported in those studies.

A similar result was observed in strains resistant to some fungicides such as fluconazole and itraconazole (COPPING et al., 2005; COSTA et al., 2010).

In agreement with these results, the therapy with another fungicide, caspofungin, promotes an increase of SAP-5 gene expression and does not modulate the expression of the others SAPs and PLB1 (RIPEAU et al., 2002).

Dimethylamino dodecyl methacrylate (DMADDM) has antimicrobial activity and has been incorporated in several dental materials. This strategy has been shown to be effective against fungi, because interfere in adhesion, which may be occasioned by the decreased expression of some virulence factors such as ALS-3 and HWP1 (ZHANG et al., 2016). In the present study, ALS-3 and HWP expression was reduced in relation to the control in the two strains treated with P/8 N/4. The intervention in fungus adhesion processes may be crucial in oral candidiasis treatment, preventing the fungus attachment to the mucosal surfaces and devices such as total removable dentures (ANTLEY; HAZEN, 1988; ELLEPOLA; SAMARANAYAKE, 1998).

The virulence of microorganisms also depends on their hemolytic abilities. Regarding to fungi species, phospholipases are correlated with this ability and PLB1, PLB2, PLC and PLD are the most described in the literature (LEONOV et al., 2017). The present study did not detect statistical significance of gene expression, except of the PLB1 gene expression which was increased in the ATCC strain after P/8 N/4 treatment. However, the treatment with fluconazole inhibited the PLC expression (WILLIAMS et al., 2013) and caspofungin treatment did not affected PLB1 gene expression (RIPEAU et al., 2002), as well as DMADDM did not modified PLD gene expression (ZHANG et al., 2016).

These results demonstrate the complexity of fungus behavior towards antifungals agents. The mechanism of action, time of treatment, and drug concentration are determinant in the success of the fungal infections control. To understand the mechanisms involved in fungal resistance, the use of antifungals at sub-inhibitory doses may be interesting for *in vitro* studies; however, it is difficult to extrapolate to what occurs *in vivo*. Thus, these results encourage the development of clinical trials to evaluate the efficacy of this combination (**P** and **N**) and other combinations by changing the concentrations of each drug.

In agreement with the results of the gene expression, through the proteomic analysis it was possible to detect a large amount of altered proteins in relation to the

control without treatment, proteins associated to biological processes important for fungus viability related to energy metabolism, translation, metabolic processes and stress response. The biological processes affected through the reduction of these proteins may be associated with damaged and fragile cells formation, reduced metabolic activity and growth.

The increase of heat shocks proteins is generally related to the greater tolerance of fungi to stress, allowing the cells to survive in unfavorable conditions (BENTLEY et al., 1992; BURNIE et al., 2006). In our study, all heat shocks proteins had their expression decreased after treatment with P/8 N/4, for the two strains evaluated.

Glycolytic enzymes are relevant during the CA pathogenesis, behaving as main inducers of host immune response and are the main allergens during candidiasis (STROCKBINE et al., 1984; SHEN et al., 1991; ISHIGURO et al., 1992; SWOBODA et al., 1993; GIL-NAVARRO et al., 1997).

Enolase (ENO1) is one of the most abundant glycolytic enzymes in the *C. albicans* cytosol. It binds to human plasminogen and this interaction promotes an increase in the fibrinolytic capacity of the fungus, facilitating invasion and dissemination. Both Enolase and Heat shock protein 70 (HSP70p) are proteins that have been reported as important antigens in various infectious diseases (BIANCO et al., 1986). In our study, these two proteins had decreased expression in relation to the control after treatment with P/8 N/4 and **N**, in ATCC and SC strains. In addition, three glycolytic enzymes were identified: phosphoglycerate kinase (PGK), glyceraldehyde phosphate dehydrogenase (GAPDH), which demonstrated laminin and fibronectin binding properties, and alcohol dehydrogenase (ADH) (PENDRAK; KLOTZ, 1995; CHAFFIN et al., 1998; GOZALBO et al., 1998).

In our results, the PGK expression was decreased in the treated strains in relation to the negative control, thus suggesting interference in one of the resistance mechanisms of the fungus.

Glyceride-3-phosphate dehydrogenases (GAPDH) are a family of proteins that have several activities in different locations within the cell, besides to have a well characterized role in the glycolysis process (SIROVER, 1999). A study showed that in yeast of *S. cerevisiae* secretion of GAPDH protein within the cell wall is enhanced by some stress conditions, such as starvation (GIL et al., 2001). In our results there was an increase in GAPDH expression in the P/8 N/4 or **N**-treated strains. This suggests that the expression is could be associated to the stress response provided by the use

of antifungal drugs.

Alcohol dehydrogenase (ADH1p), on cell surface and cytoplasm, catalyzes the reduction of acetaldehyde to ethanol generating NAD+ and participates in multiple biological processes such as biofilm formation, fermentation and interaction with host (WANG et al., 2012). Previous studies have found that overexpression of ADH1p is related to fluconazole resistance in *C. albicans* (ZHU; LU, 2005) and that fluconazole induces ADH1 gene expression (WANG et al., 2012). In our results, we obtained an increased expression of ADH1p after **P** treatment in ATCC strain, and **N** treatment in SC strain. However, in the drug combination (P/8 N/4), the expression was decreased compared to the control. Another study similarly identified ADH1p overexpressed after separately fluconazole and tetrandrine treatment; nevertheless, when combined, there was a decrease in the expression, suggesting that ADH1p expression is involved in the synergism mechanism against *C. albicans* (ZHANG et al., 2013).

Malate dehydrogenase (MDH1p) is a considerable enzyme for the fungi bioenergetic metabolism. MDH1p participates in the glyoxalate cycle, which allows fungal cells to use fatty acids as a substrate for gluconeogenesis (TYLICKI et al., 2008). The glyoxalate cycle is required for fungal virulence. *C. albicans* exhibits a metabolic program by which the glyoxalate and gluconeogenesis cycle are activated during the early stages of infection. Our results showed a reduction of malate dehydrogenase expression in both treated strains relative to the negative control. Thus, not only the decrease of MDH1p but also of other proteins involved with the fermentative or oxidative metabolism can be effective as antifungals (TYLICKI et al., 2008).

Another interesting result was the identification of Acetyl-CoA-acetyltransferase, encoded by ERG10 gene, only in the negative controls, in both ATCC and SC strains. This data allows to assume that there was interference in the ergosterol production, as such a protein is necessary in the first step of this biosynthesis through its condensation in acetoacetyl-CoA (BUURMAN et al., 2004).

In healthy individuals, phagocytic cells, such as macrophages (EVRON, 1980), monocytes and neutrophils (SCHUIT, 1979; MARÓDI et al., 1991), act on *Candida* infections, producing various growth inhibitors and cytotoxic compounds, including microbicidal enzymes and reactive oxygen and nitrogen species (PETERSON; CALDERONE, 1978; VAZQUEZ-TORRES; BALISH, 1997). A potentially effective artifice against CA is nitric oxide (NO) (ULLMANN et al., 2004).

One way of protecting microorganisms from the toxicity of nitric oxide is through enzymes that convert NO into less toxic molecules. Flavohemoglobin is an NO-dioxygenase encoded by the YHB1 gene. In *C. albicans,* it is induced by NO and converts it to nitrate (GARDNER et al., 1998; ULLMANN et al., 2004). Amphotericin B promote oxidative damage to fungal cells and several oxidative stress response genes are overexpressed in response to the drug (LIU et al., 2005), such as YHB1, which in our results was also overexpressed compared to control after treatment with P/8 N/4 and **N** in both strains evaluated.

Hydrolytic enzymes, such as aspartyl proteinases and phospholipases, produced and secreted by *C. albicans*, as well as proteins encoded by ALS (agglutinin-like sequence) genes were not detected in the proteomic analysis, although they were expressed in the PCR assay. We hypothesize that it may have occurred because the composition, culture medium pH and fungal growth time were not adequate for the synthesis and secretion of these proteins at detectable levels by the mass spectrometer (MACDONALD; ODDS, 1980; IBRAHIM et al., 1995; CHAFFIN et al., 1998; D'EÇA JÚNIOR et al., 2011; ELLS et al., 2014).

Differences in protein expression among the both strains evaluated were not analyzed at this first moment, because initially it was not the aim of our study. Indeed, these differences are expected, since one of the strains is clinical (SC 5314), and therefore exhibit resistance and virulence factors different from the standard strain (ATCC 90028). This evaluation will be carried out later.

In this context, the results of the present study and other studies in the search for new therapeutic alternatives through the drug combination encourage the development of clinical trials to evaluate the efficacy of this combination for the treatment of oral candidosis.

3 Conclusions

3 CONCLUSIONS

1) Synergistic antifungal activity produced between **P** and **N** suggested that the combination of drugs, at the concentrations tested, may be a viable alternative to be applied as preventive treatment or topical therapy for superficial candidiasis, such as denture stomatitis.

2) P/8 N/4 appears to exert an antifungal effect on *C. albicans* by direct interaction with important proteins related to structural organization of fungus morphology and in essential metabolic energy processes. This might be related to the capacity of the drugs used in combination to cause harm to fungi structure and reduction of filamentation, resulting in defective and non-viable cells.

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Appendix

APPENDIX A – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We hereby declare that we are aware of the article "***In vitro* Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis"**, which will be included in Ph.D. thesis of the student Rafaela Alves da Silva. This article was exclusively used in this thesis and may not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, May 17th, 2018

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APPENDIX B – DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THE THESIS

We hereby declare that we are aware of the article **"Antifungal activity of Punicalagin and Nystatin used in combination against *Candida albicans*: detection of virulence genes and proteomic analysis**", which will be included in Ph.D. thesis of the student Rafaela Alves da Silva. This article was exclusively used in this thesis and may not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, May 17th, 2018

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Annexes

ANNEXES

ANNEX A – Ethics committee approval

FACULDADE DE ODONTOLOGIA DE BAURU-USP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: "INFLUÊNCIA DA INCORPORAÇÃO DOS FITOTERÁPICOS E. GIGANTEUM E P. GRANATUM SOBRE MATERIAIS RESILIENTES UTILIZADOS NA CONFECÇÃO DE PRÓTESES REMOVÍVEIS BUCAIS: TOPOGRAFIA, CARACTERIZAÇÃO QUÍMICA E AÇÃO CONTRA CANDIDA ALBICANS".

Pesquisador: Rafaela Alves da Silva Alavarce

Área Temática:

Versão: 2

CAAE: 44951715.6.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 1.114.009

Data da Relatoria: 17/06/2015

Apresentação do Projeto:

Este trabalho será desenvolvido com uma aluna de pós graduação na área de patologia e visa a avaliar se materiais utilizados para o revestimento temporário de próteses removíveis bucais, previamente incorporados especificamente com os fitoterápicos Equisetum giganteum e Punica granatum, apresentamatividade antimicrobiana e antiaderente; atividade anti-inflamatória sobre células epiteliais de palato humano (CEPH); citotoxicidade sobre células humanas e alterações de superfície. Para tanto, envolverá aparticipação de um único participante que deverá autorizar mediante o TCLE complementar, a doação de fragmento do palato duro já obtido e congelado em trabalho prévio aprovado pelo CEP.

Objetivo da Pesquisa:

O objetivo apresentado é de avaliar se materiais utilizados para o revestimento temporário de próteses removíveis bucais, previamente incorporados com os fitoterápicos Equisetum giganteum e Punica granatum, apresentam atividade antimicrobiana e antiaderente; atividade anti-inflamatória sobre células epiteliais de palato humano (CEPH); citotoxicidade sobre células humanas e alterações de superfície.

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Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.114.009

Avaliação dos Riscos e Benefícios:

Foram apresentados adequadamente:

Riscos: Os riscos envolvidos serão provenientes da cirurgia que será realizada nos pacientes durante a pesquisa "Avaliação do recobrimento radicular pela técnica de enxerto conjuntivo subepitelial associado ao condicionamento radicular com ácido cítrico ou terapia fotodinâmica - estudo clínico randomizado", já aprovada por este CEP e que irá ceder o material residual armazenado para a pesquisa em questão, e são aqueles comuns a qualquer tratamento odontológico (pequeno sangramento durante a limpeza, sensibilidade, pequeno desconforto), que ocorreriam, mesmo que não estivesse participando da pesquisa. A presente pesquisa, como irá trabalhar com material biológico armazenado não apresenta riscos. O número do protocolo de aprovação por este CEP foi solicitado e incluído no item justificativas da dispensa do TCLE do Plataforma Brasil.

Benefícios: A descoberta de novos componentes antimicrobianos é de grande relevância, particularmente para a Odontologia, já que infecções da cavidade bucal, de origem bacteriana e fúngica, são problemas relativamente comuns, resultando em doenças inflamatórias crônicas como, por exemplo, a estomatite protética associada a Candida. Levando-se em conta o aumento percentual da população idosa e que a EP acomete frequentemente idosos usuários de prótese total superior, torna-se fundamental a realização de novos estudos sobre alternativas terapêuticas para a EP, que sejam simultaneamente antimicrobiana, antiaderente, anti-inflamatória e não tóxica para os tecidos bucais, visando a melhora da qualidade de vida desta população idosa.

Comentários e Considerações sobre a Pesquisa:

Este estudo tem como objetivo avaliar in vitro se um reembasador resiliente e um condicionador tecidual, modificados por meio da incorporação prévia com os fitoterápicos E. giganteum e P. granatum, apresentam atividades antimicrobiana, antiaderente e anti-inflamatória, sem alteração de suas propriedades mecânicas (topografia) e composição química. Neste contexto, estas plantas medicinais poderiam desempenhar um papel importante no tratamento da EP.

Considerações sobre os Termos de apresentação obrigatória:

- O apoio financeiro foi descrito adequadamente e está consistente com apresentação do termo de outorga.
- A utilização desse material em pesquisas vinculadas à linha de pesquisa acima citada, será somente após a aprovação de um Comitê de Ética em Pesquisa em Seres Humanos e mediante a

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

assinatura de um documento, por parte do responsável pela guarda do material, cedendo essas células. Para utilização do material biológico cedido em pesquisas futuras, conforme item 5 da Resolução CNS 441/2011. Dessa forma, o termo de cessão de responsabilidade da Profa. Dra. Carla A. Damante foi adequadamente apresentado, bem como a justificativa de que apenas pacientes que apresentaram a cessão para futuros trabalhos da mesma linhas na assinatura do TCLE do momento da coleta cirúrgica seriam elegíveis.

- Foi informado adequadamente que o tecido todo será descartado após este trabalho, não havendo sobras.

Recomendações:

Não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Aprovado.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 17.06.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.114.009

BAURU, 18 de Junho de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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DETALHAR NOTIFICAÇÃO

– DADOS DA VERSÃO DO PROJETO DE PESQUISA

Título da Pesquisa: "INFLUÊNCIA DA INCORPORAÇÃO DOS FITOTERÁPICOS E. GIGANTEUM E P. GRANATUM SOBRE MATERIAIS RESILIENTES UTILIZADOS NA CONFECÇÃO DE PRÓTESES REMOVÍVEIS BUCAIS: TOPOGRAFIA, CARACTERIZAÇÃO QUÍMICA E AÇÃO CONTRA CANDIDA ALBICANS".
Pesquisador Responsável: Rafaela Alves da Silva
Área Temática:
Versão: 2
CAAE: 44951715.6.0000.5417
Submetido em: 10/06/2015
Instituição Proponente: Universidade de Sao Paulo
Situação da Versão do Projeto: Aprovado
Localização atual da Versão do Projeto: Pesquisador Responsável
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

Comprovante de Recepção: PB_COMPROVANTE_RECEPCAO_490

– DADOS DA NOTIFICAÇÃO

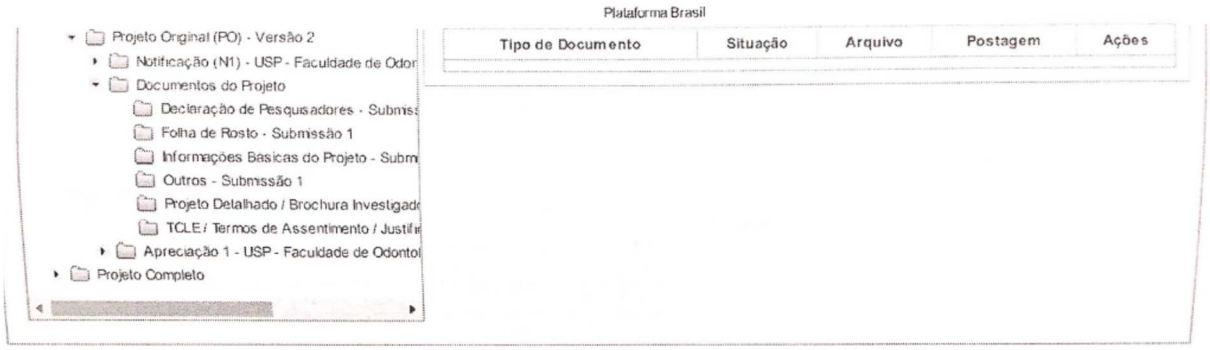
Tipo de Notificação: Envio de Relatório Final
Detalhe:
Justificativa:
Data do Envio: 26/04/2018
Situação da Notificação: Aguardando confirmação de indicação de relatoria

– DOCUMENTOS DO PROJETO DE PESQUISA

Versão Atual Aprovada (PO) - Versão 2	Tipo de Documento	Situação	Arquivo	Postagem	Ações

brasil.saude.gov.br/visao/pesquisador/gerirPesquisa/gerirPesquisaAgrupador.jsf

Maristela Petenuci Ferrari
Secretária- SRTE 43052
Setor de Apoio às Comissões
e Convênios-FOB-USP



HISTÓRICO DE TRÂMITES

Apreciação	Data/Hora	Tipo Trâmite	Versão	Perfil	Origem	Destino	Informações
N1	26/04/2018 18:15:34	Indicação de Relatoria	2	Secretária	USP - Faculdade de Odontologia de Bauru da USP	USP - Faculdade de Odontologia de Bauru da USP	
N1	26/04/2018 18:15:16	Aceitação do PP	2	Secretária	USP - Faculdade de Odontologia de Bauru da USP	USP - Faculdade de Odontologia de Bauru da USP	
N1	26/04/2018 16:23:53	Notificação enviada	2	Pesquisador	PESQUISADOR	USP - Faculdade de Odontologia de Bauru da USP	

asil.saude.gov.br/visao/administrador/4x4Novo/detalharProjetoAgrupadorApreciacao.jsf

DOCUMENTOS DO PROJETO DE PESQUISA

ersão Atual Aprovada (PO) - Versão 2
Projeto Original (PO) - Versão 2
Notificação (N1) - USP - Faculdade de Odor
Documentos do Projeto
Declaração de Pesquisadores - Submiss
Folha de Rosto - Submissão 1
Informações Básicas do Projeto - Submi
Outros - Submissão 1
Projeto Detalhado / Brochura Investigad
TCLE / Termos de Assentimento / Justific
Apreciação 1 - USP - Faculdade de Odontol
rojeto Completo

Tipo de Documento	Situação	Arquivo	Postagem	Ações
Parecer Consubstanciado do CEP	Aceito	PB_PARECER_CONSUBSTANCIADO_CEP_2669089.pdf	22/05/2018 17:30:40	

LISTA DE APRECIAÇÕES DO PROJETO

Apreciação	Pesquisador Responsável	Versão	Submissão	Modificação	Situação	Exclusiva do Centro Coord.	Ações
N1	Rafaela Alves da Silva	2	26/04/2018	22/05/2018	Aprovado	Sim	
PO	Rafaela Alves da Silva	2	10/06/2015	18/06/2015	Aprovado	Não	

ANNEX B – Manuscript submission letter confirmation from Journal of Natural Products

ScholarOne Manuscripts

Journal of Natural Products

Home

Submission Confirmation

Print

Thank you for your submission

Submitted to	Journal of Natural Products
Manuscript ID	np-2018-003913
Title	In vitro Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis
Authors	da Silva, Rafaela Lopes, Marcelo de Castro, Ricardo Ishikiriama, Bella Luna Ferrari, Tatiane Porto, Vinicius Neppelenbroek, Karin Garcia, Cindy Lara, Vanessa

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