SUELEN BALERO DE PAULA

Candida SPP. ISOLADAS DA MUCOSA BUCAL DE PACIENTES PORTADORES DE HIV:

IDENTIFICAÇÃO, EXPRESSÃO DE FATORES DE VIRULÊNCIA E SENSIBILIDADE ÀS SUBSTÂNCIAS ANTIFÚNGICAS

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Orientadora: Profª Drª Sueli Fumie Yamada Ogatta.

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BANCA EXAMINADORA

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Profª Drª Sueli Fumie Yamada Ogatta
UEL – Londrina - PR

_____

Dr. Fernando César Bizerra
UNIFESP – São Paulo - SP

_____

Prof. Dr. Ricardo Sérgio Couto de Almeida
UEL – Londrina - PR

Londrina, 27 de fevereiro de 2012.

“A felicidade não se resume na ausência de problemas, mas sim na sua capacidade de lidar com eles.”

Albert Einstein

Aos meus pais, Jessé e Inês por terem sido presentes em todos os momentos, pelo auxílio e apoio. À vocês, minha gratidão e amor eterno.

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DE PAULA, Suelen Balero. ***Candida* spp. isoladas na mucosa bucal de pacientes portadores de HIV:** identificação, expressão de fatores de virulência e sensibilidade a substâncias antifúgicas. 2012. 136 f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2012.

RESUMO

A introdução da terapia antirretroviral associada aos inibidores de protease (IP) reduziu a ocorrência de infecções oportunistas em indivíduos portadores do HIV (*Human immunodeficiency virus*). Contudo, a candidíase orofaríngea continua sendo a infecção fúngica oportunista mais frequente nesses indivíduos. A sensibilidade variada e a emergência de isolados resistentes aos antifúngicos entre essas espécies reforçam a necessidade de um diagnóstico microbiológico específico para essas infecções. Além disso, a resistência apresentada pelo biofilme à maioria dos fármacos justifica o estudo de novas substâncias com potencial antifúngico. Dessa forma, o objetivo deste trabalho foi identificar, e caracterizar leveduras isoladas da cavidade bucal de pacientes HIV positivos quanto a: sensibilidade a nistatina e fluconazol; expressão dos fatores de virulência; efeito do eugenol sobre células planctônicas e sésseis de *Candida dubliniensis* e *Candida tropicalis*. Amostras de enxágue bucal de 244 pacientes foram utilizadas nesse trabalho. Do total de pacientes, 50,41% estavam colonizados por *Candida* spp., sendo que 11 pacientes apresentaram co-colonização por duas espécies. *Candida albicans* (100) foi a espécie mais prevalente, correspondendo a 74,1% dos isolados. Outras espécies foram isoladas de 35 pacientes, sendo 27 *C. glabrata*, 2 *C. krusei*, 3 *C. tropicalis* e 3 *C. dubliniensis*. A maioria dos isolados (59,26%) foi considerada sensível ao fluconazol, 22,96% e 18,52% dos isolados foram considerados sensíveis dose-dependente e resistentes ao fluconazol, respectivamente. A maioria dos isolados (86,70%) apresentou CIM entre 2 e 4 µg/mL e 14,15% apresentaram entre 8 e 128 µg/mL para a nistatina. A média relativa de HSC (hidrofobicidade da superfície celular) analisada pelo método bifásico, utilizando xileno como hidrocarboneto, foi de 63,47 ± 21,48 e a maioria dos isolados (54,81%) foi classificada como altamente hidrofóbicos. Todos os isolados foram capazes de formar biofilme na superfície de poliestireno, entretanto foi observada maior atividade metabólica em biofilmes formados por isolados de *C. tropicalis*. Cinco isolados não apresentaram atividade proteolítica em presença de albumina de soro bovino (BSA), entretanto 48,89%, 36,29% e 11,11% apresentaram alta, intermediária e baixa atividade de protease, respectivamente. Somente 36,29% dos isolados apresentaram atividade de fosfolipases extracelulares em presença de gema de ovo como substrato. A hemólise completa de sangue de carneiro foi observada em apenas 17,04% dos isolados. Foi possível verificar, por PCR em tempo real, uma redução significativa na expressão das *SAP2* de *C. albicans* isoladas de pacientes em tratamento com os IP quando comparados com aqueles sem esse tratamento. O eugenol apresentou atividade sobre células planctônicas, sobre a formação de biofilme e biofilme maduro, sendo que o tratamento com o eugenol reduziu drasticamente a quantidade de células sobre as superfícies. O tratamento com o eugenol foi capaz de reduzir a CSH, a adesão em células Hep-2 e em poliestireno. Além disso, o tratamento com o eugenol reprimiu a expressão dos genes *ALS*. Os resultados corroboram a importância da identificação e análise do perfil de sensibilidade aos antifúngicos para o planejamento terapêutico adequado para cada paciente.

Palavras-chave: *Candida* spp. Fatores de virulência. Pacientes HIV positivos. Sensibilidade aos antifúngicos. Eugenol.

DE PAULA, Suelen Balero. **Candida spp. isolated in the oral mucosa of patients with HIV:** identification, expression of virulence factors and sensitivity to antifúgicas substances. 2012. 136 f. Dissertation (Master´s degree in Microbiology) – Universidade Estadual de Londrina, Londrina, 2012.

ABSTRACT

The introduction of antiretroviral therapy associated with protease inhibitors (PI) reduced the occurrence of opportunistic infections in individuals with HIV (Human Immunodeficiency virus). However, oropharyngeal candidasis remains the most common fungic opportunistic infection in these individuals. The variable sensitivity and the emergence of resistant fungal isolates of these species reinforce the need for a specific microbiological diagnosis of the infections. Furthermore, the drug resistance presented by the most biofilms justifies a study of new substances with antifungal activity. Thus, the objective of this study was to identify and characterize yeasts isolated from the oral cavity of HIV positive patients in terms of: sensitivity to nystatin and fluconazole; expression of virulence factors; effect of eugenol on planktonic and sessile cells of *Candida dubliniensis* and *Candida tropicalis.* Oral rinse samples of 244 patients were used in this work. From all, 50.41% were colonized by *Candida* spp., and 11 patients had co-colonization by two species. *Candida albicans*(100) was the most prevalent, accounting for 74.1% of the isolates. Other species were isolated from 35 patients, 27 *C. glabrata*, 2 *C. krusei*, 3 *C. tropicalis* and 3 *C. dubliniensis.* Most isolates (59,26%) were considered sensitive to fluconazole, 22.96% and 18.52% of the isolates were considered susceptible dose-dependent and resistant to fluconazole, respectively. Most isolates (86,70%) showed MIC between 2 and 4 μg/mL and 14.15% were between 8 and 128 μg/mL for nystatin. The average for CSH (cell surface hidrophobicity) assayed by biphasic using xylene as a hydrocarbon was 63.47 ± 21.48 and most of the isolates (54,81%) were classified as highly hydrophobic. All isolates were able to form biofilm on polystyrene surface, however there was a higher metabolic activity in biofilms formed by isolates of *C. tropicalis*. Five isolates had not shown any proteolytic activity in the presence of BSA (bovine serum albumin), however 48.89%, 36.29% and 11.11% had high, intermediate and low protease activity, respectively. Only 36.29% of the isolates showed extracellular phospholipase activity on egg yolk plates. The complete hemolysis of sheep blood was observed in only 17.04% of the isolates. It was verified by Real Time PCR, a significant reduction in the expression of SAP2 of *C. albicans* isolated from patients treated with PIs compared with those without such treatment. The eugenol presented activity against planktonic cells, on biofilm formation and mature biofilm, and such treatment has drastically reduced the amount of cells on surfaces. Treatment with eugenol was able to reduce the CSH, adherence to HEp-2 and polystyrene. In addition, treatment with eugenol repressed the expression of *ALS* gene. The results corroborate the importance of identification and analysis of the sensitivity profile to antifungal agents for planning appropriate therapy for each patient.

Keywords: *Candida* spp. Eugenol. HIV-positive patients. Sensitivity to antifungal drugs. Virulence factors.

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1 REVISÃO DA LITERATURA

1.1 O GÊNERO *CANDIDA* E PACIENTES HIV POSITIVOS

Os fungos são organismos heterotróficos e estão presentes nos mais diversos ambientes. Podem ser encontrados em ambientes aquáticos, no solo participando da ciclagem de nutrientes, em associação com plantas e em superfícies mucosas de diversas espécies de animais (Sidrim; Rocha, 2004). Algumas espécies de *Candida* fazem parte da microbiota de superfícies mucosas do trato gastrointestinal e genito-urinário de animais, inclusive do homem (Soll, 2002; Kam & Xu, 2002).

O gênero *Candida* spp. compreende mais de 200 espécies de leveduras pertencentes à família *Candidaceae*, ordem *Saccharomycetales*, classe *Hemiascomycetes*, filo *Ascomycota* e reino Fungi. Estes microrganismos apresentam-se em formas arredondadas ou ovais, tamanho variando de 2 μm a 4 μm de diâmetro e crescem como colônias de aspecto cremoso em meio solidificado (Odds, 1998).

Como microrganismos oportunistas, as espécies de *Candida* podem ser responsáveis por uma variedade de quadros clínicos, desde infecções superficiais de mucosa, como candidíase vulvovaginal e orofaríngea até infecções sistêmicas (Van Burik & Magee, 2001; Kam & Xu, 2002; Soll, 2002; Eggiman *et al*, 2003; Repentigny, 2004). A colonização por *Candida* spp. da mucosa bucal de pacientes HIV positivos pode atingir aproximadamente 75% dos indivíduos. Esse valor é significativamente maior quando comparado com pacientes HIV negativos, em que 50 a 60% dos indivíduos podem ser colonizados por *Candida* spp. (Fidel, 2006; Luque *et al*, 2009).

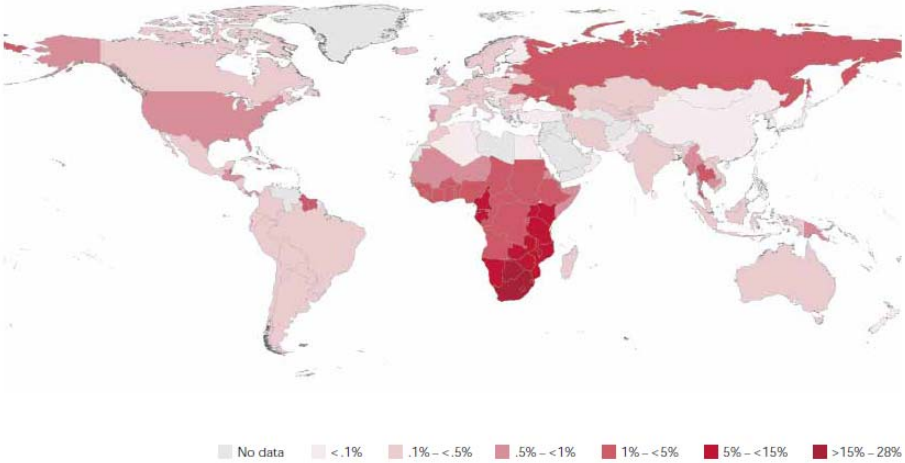
A transição de microrganismo comensal para patogênico pode ocorrer devido a inúmeras causas relacionadas às condições locais e sistêmicas do paciente (Williams & Lewis, 2011). Alguns fatores predisponentes podem aumentar a frequência de infecções por *Candida* spp., tais como: desordens hematológicas, como diferentes tipos de leucemias, tumores e transplantes; procedimentos médicos invasivos como cirurgias, implantes e uso de cateteres; tratamentos como radioterapia, quimioterapia, uso de antimicrobianos, esteróides e substâncias imunossupressoras (Guarro *et al*, 1999); tratamento de diálise (Abbott; Agodoa, 2001; Pasqualotto *et al*, 2005); distúrbios metabólicos ou hormonais (Ferrer, 2000); doenças

imunossupressoras; infecções primárias, especialmente pelo vírus da imunodeficiência humana.

Os primeiros casos reconhecidos de AIDS (*Acquired Immune Deficiency Syndrome*: Síndrome da Imunodeficiência Adquirida) ocorreram nos EUA no início de 1980. Um número de homossexuais em Nova York e na Califórnia começaram a desenvolver infecções oportunistas e raras que eram resistentes aos tratamentos (Dubois *et al,* 1981; Masur *et al,* 1981). Em setembro de 1982 essa síndrome foi nomeada como Síndrome da Imunodeficiência Adquirida pelo CDC (*Centers of Disease Control and Prevention)* (Weekly, 1982; Curran *et al*, 1985).

De acordo com dados fornecidos pela UNAIDS (*Joint United Nations Programme on HIV/AIDS*), em 2009, foi estimado um número de 33,3 milhões de pessoas portadoras do HIV (*Human Immunodeficiency Virus*: Vírus da Imunodeficiência Humana), sendo a AIDS responsável pela morte de 1,8 milhões de indivíduos, sendo responsável aproximadamente por 14% de óbitos ocorridos em adultos de 15 a 59 anos (Lopez *et al,* 2006; Anish *et al,* 2011). Do total dos indivíduos infectados, 2,5 milhões são crianças, 15,9 milhões são homens, 14,9 milhões são mulheres e a maioria (mais de 30 milhões) vive em países de baixa e média renda. Maior prevalência de infectados no mundo aparece na África Subsaariana, sendo que a África do Sul continua a ser mais severamente afetada abrangendo 11,3 milhões de pessoas com HIV em 2009 [Figura 1, (UNAIDS, 2011)].

Figura 1 - Distribuição global da infecção pelo HIV. Em 2009, cerca de 33,3 milhões de pessoas estavam infectadas pelo vírus.



Fonte: UNAIDS, 2011.

Embora o número anual de novas infecções no mundo tenha diminuído de 3,6 milhões em 1996 para 2,6 milhões em 2009, o número total de indivíduos portadores do HIV continua aumentando à medida que há maior sobrevida dos mesmos. O declínio global de novos diagnósticos de AIDS e mortes de pessoas com AIDS são em parte devido ao sucesso da terapia antirretroviral altamente ativa (*Highly Active Antiretroviral Treatment* - HAART) introduzida em 1996.

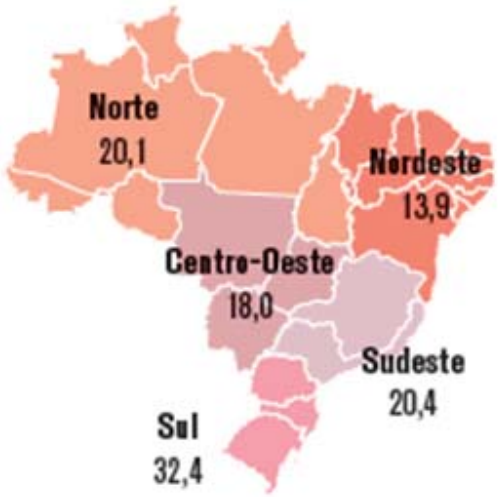
A terapia antirretroviral padrão (ART) consiste no uso de pelo menos três fármacos com o intuito de inibir a replicação viral e interromper a progressão da AIDS. A redução significativa na mortalidade de pacientes HIV positivos em uso da terapia antirretroviral parece estar associada a uma maior contagem de linfócitos T $CD4^+$ nesses indivíduos (Kitahata *et al,* 2009; Stene *et al,* 2009; Thompson *et al,* 2010; Edmonds *et al,* 2011) e diminuição da replicação viral (Ray *et al,* 2010). Além disso, alguns autores descrevem que essa terapia seja responsável pela diminuição da carga viral em secreções desses indivíduos (Vernazza *et al,* 2000; Graham *et al,* 2007; Cohen *et al,* 2010; Cohen *et al,* 2011).

De acordo com o Ministério da Saúde, de 1980 até junho de 2010, o Brasil registrou 608.230 casos de AIDS. Esse número não inclui os indivíduos que foram infectados pelo vírus e ainda não desenvolveram os sintomas da doença, onde estima-se que 630 mil sejam portadores do HIV. Somente em 2010, foram notificados 34.218 casos da doença e a taxa de incidência de AIDS no Brasil foi de 17,9 casos por 100 mil habitantes. O maior número de casos ocorre na região Sudeste e a menor porcentagem de casos acumulados encontra-se na região Norte (Figura 2).

Atualmente, ainda há mais casos da doença entre os homens do que entre as mulheres, mas essa diferença vem diminuindo ao longo dos anos. A faixa etária em que a AIDS é mais incidente, em ambos os sexos, é a de 20 a 59 anos de idade. A forma de transmissão prevalece a sexual. Nas mulheres, 94,9% dos casos registrados em 2009 decorreram de relações heterossexuais com pessoas infectadas pelo HIV. Entre os homens, 42,9% dos casos se deram por relações heterossexuais, 19,7% por relações homossexuais e 7,8% por bissexuais. O restante ocorreu por transmissão sanguínea e vertical. A diminuição de casos de AIDS em menores de cinco anos é expressiva, comparando-se os anos de 1999 e 2009. O resultado confirma a eficácia da política de redução da transmissão vertical do HIV

(Ministério da Saúde do Brasil, 2011).

Figura 2 - Taxa de incidência (por 100.000 hab) em 2009. Observando-se a epidemia por região em um período de 10 anos, 2000 a 2010, a taxa de incidência caiu no Sudeste de 24,5 para 17,6 casos por 100 mil habitantes. Nas outras regiões, cresceu: 27,1 para 28,8 no Sul; 7,0 para 20,6 no Norte; 13,9 para 15,7 no Centro-Oeste; e 7,1 para 12,6 no Nordeste. Vale lembrar que o maior número de casos acumulados está concentrado na região Sudeste (56%).



Fonte: Ministério da Saude.

Por serem os principais alvos do HIV, a progressão da AIDS envolve o declínio acentuado do número de linfócitos T $CD4^+$. Durante este estágio da doença os pacientes desenvolvem inúmeras complicações dermatológicas e orais (Tappuni & Flemming, 2001; Sierra *et al*, 2005, Goel *et al,* 2011; Ortiz *et al,* 2011; Egusa *et al,* 2011; Fidel, 2011).

As infecções oportunistas são as principais causas de morbidade e morte entre os pacientes infectados pelo HIV. Entretanto, nem todos os pacientes apresentando baixa contagem de linfócitos T $CD4^+$ desenvolvem infecções oportunistas (Rosentul *et al,* 2011). A candidíase orofaríngea (COF), a tuberculose pulmonar e criptococose são as infecções fúngicas oportunistas mais comuns em pacientes HIV-positivos (Fidel *et al,* 2011; Patel *et al,* 2011), sendo a COF, causada principalmente por *Candida albicans* (Mane *et al,* 2011). No Brasil estima-se que *Candida* spp. seja a segunda causa de morte, respondendo por cerca de 30% dos óbitos ocorridos em pacientes HIV positivos decorrentes de infecção fúngica (Prado *et al*, 2009).

Cerca de 95% de pacientes infectados pelo HIV desenvolvem pelo menos

um quadro de COF durante o curso da doença (Coleman *et al*, 1998). Além disso, esta infecção bucal pode ser considerada um marcador da imunodeficiência progressiva da AIDS (de Repentigny *et al,* 2004; Leão *et al,* 2009), já que em muitos casos pode ser a primeira manifestação dessa síndrome (Selwyn *et al,* 1992, Saramanayake *et al*, 2000). Assim a presença de COF nesses indivíduos pode estar associada à baixa contagem de células T $CD4^+$ [menores que 200 células/mm^3, (Selwyn *et al,* 1992; Nielsen *et al,* 1994; Schuman *et al,* 1998; Greenspan *et al,* 2000)] e com um aumento da carga viral (Matee *et al,* 2000; Patton, 2000; Abgrall *et al,* 2001; Campo *et al,* 2002; de Repentigny *et al,* 2004; Hamza *et al,* 2006; Butt *et al,* 2007), além da idade do paciente (Mc Carthy, 1992).

A COF, na maioria dos casos, apresenta-se assintomática, contudo alguns pacientes podem apresentar ardência, sensação dolorosa de boca seca, alteração do paladar, dor ao deglutir e/ou halitose (Fichtenbaum, 2003). Uma das complicações mais frequentes da COF é a candidíase esofágica que pode causar dores intensas e anorexia, afetando a qualidade de vida e o estado geral de saúde, assim complicando o manejo do paciente.

Dentre as diversas espécies de *Candida* isoladas da cavidade bucal, algumas já foram reconhecidas como causadoras de processos patológicos. *C. albicans* é a espécie mais prevalente em isolados clínicos, inclusive entre pacientes HIV positivos, entretanto um aumento na frequência de outras espécies de *Candida* tem sido observado nos últimos anos (Migliorati *et al*, 2004; Sánchez-Vargas *et al*, 2005; Ruhnke, 2006; Luque *et al*, 2009).

O fluconazol tem sido utilizado no tratamento primário da candidíase oral de pacientes HIV positivos, porém, os episódios recorrentes de COF conduzem ao tratamento antifúngico repetido. Como consequência desse quadro, pode ocorrer a seleção de leveduras resistentes, que contribuem para a mudança na epidemiologia das espécies de *Candida* na cavidade bucal desses indivíduos (Johnson *et al*, 1995; Melo *et al,* 2004; Migliorati *et al*, 2004; Sánchez-Vargas *et al*, 2005; Luque *et al*, 2009; Mann *et al*, 2009).

Essa mudança epidemiológica tem sido associada à alta taxa de mortalidade dos pacientes, sensibilidade variada e emergência de isolados resistentes ao fluconazol, o fármaco de escolha para o tratamento de candidíases (Collin *et al,* 1999; Melo *et al,* 2004; Migliorati *et al,* 2004; Yang *et al,* 2004; Hamza *et al,* 2008; Back-Brito *et al,* 2009; Thein *et al,* 2009; Luque *et al,* 2009; Junqueira *et al,* 2012).

Candida glabrata constitui-se na segunda espécie mais frequentemente

isolada da cavidade bucal de pacientes HIV positivos (Baradkar *et al,* 1999; Sobel *et al,* 2001; Yang *et al,* 2003; Pfaller *et al*, 2006; Hamza *et al*, 2008; Pomarico *et al,* 2009; Yang *et al,* 2010, Agwu *et al*, 2011, Patel *et al,* 2011). O isolamento de outras espécies como *C. tropicalis*, *C. parapsilosis*, *C. krusei* e *C. dubliniensis,* tem sido, também, frequentemente observado (Odds, 1988; Witzel *et al*, 2008, Back-Brito *et al*, 2009; Katiraee *et al*, 2010). Com menos frequência encontram-se *C. kefyr, C. guilliermondii, C. lusitaniae* e *C. norvegensis* (Cannon *et al*, 1995; Segal, 2005; Junqueira *et al,* 2011, Patel *et al*, 2011).

A co-colonização por duas espécies de *Candida* tem sido observada, sendo a presença de *C. albicans* e *C. glabrata* a mais comum (Sobel *et al,* 2001; Yang *et al*, 2003; Hamza *et al,* 2008; Nace *et al*, 2009). A presença de duas ou mais espécies no mesmo paciente pode predispor a candidíase recorrente, principalmente quando apresentam sensibilidade reduzida aos azóis, como *C. glabrata* e *C. krusei* (Sant´Ana *et al,* 2002).

1.2 FATORES DE VIRULÊNCIA DE *CANDIDA* SPP.

Como mencionado anteriormente, a transição de *Candida* spp. de microrganismo comensal para patógeno depende inicialmente das condições fisiológicas do hospedeiro. Entretanto, essa transição também está estreitamente relacionada à capacidade das leveduras de desenvolverem mecanismos e expressarem fatores de virulência que permitam a invasão do epitélio e a infecção desses hospedeiros (Naglik *et al,* 2003, Tamura *et al*, 2007).

Dentre os fatores associados à virulência de *Candida* spp. estão: capacidade de adesão às células epiteliais e tecidos dos hospedeiro, formação de biofilme, alteração fenotípica e secreção de enzimas extracelulares como proteases e fosfolipases (Price *et al,* 1982; Hube *et al,* 1998; Calderone; Fonzi, 2001; Haynes *et al,* 2001; Hube & Naglik, 2001; Naglik *et al,* 2003; Staib *et al,* 2001; Yang, 2003, Oksuz *et al*, 2007; Costa *et al,* 2009; Chai *et al,* 2010; Negri *et al*, 2011).

O processo de adesão às células epiteliais e proteínas do hospedeiro é um evento crítico da patogênese de *Candida* spp. Além de permitir a colonização persistente da mucosa, esse processo leva à geração de sinais para a penetração e invasão das leveduras no tecido e ao estabelecimento de um processo infeccioso no hospedeiro (Mendes-Gianini *et al,* 2005, Ramage *et al,* 2005). A adesão ao tecido hospedeiro depende da combinação de fatores

inespecíficos e específicos das leveduras, células do hospedeiro e do ambiente. Os fatores inespecíficos incluem forças eletrostáticas e interações hidrofóbicas. Por exemplo, foi mostrado que superfícies abióticas hidrofóbicas como próteses dentárias facilitam a adesão de leveduras. Por outro lado, houve uma redução significativa na capacidade de adesão de *Candida* spp. quando esses materiais foram recobertos com substâncias hidrofílicas (Yoshijima *et al,* 2009).

A hidrofobicidade da superfície celular (CSH: *cellular surface hydrophobicity*), considerada um fator inespecífico, é uma característica importante que contribui para a adesão de *Candida* spp. às células epiteliais e proteínas da matriz extracelular do hospedeiro (Hazen; Hazen, 1988; Hazen *et al,* 1991, Chaffin, 2008) e superfície abiótica (Klotz *et al*, 1985). A CSH pode ser caracterizada como uma manifestação de várias moléculas na superfície celular que estão envolvidas na virulência, e está associada à presença de determinadas proteínas na parede celular do fungo (Glee *et al,* 1995; Masuoka *et al,* 1999; Singleton *et al,* 2001). A hidrofobicidade de espécies do gênero *Candida* pode variar de acordo com condições e fase de crescimento, e da morfologia celular (Rosenberg, 2006). Células hidrofóbicas parecem exibir moléculas de fibrila de cadeias curtas, enquanto células hidrofílicas apresentam proteínas de cadeias longas altamente manosiladas (Hazen *et al*, 1990; Hazen; Hazen, 1993). A falta de uma dessas moléculas pode aparentemente não diminuir a CSH, mas pode afetar a virulência, influenciando os diferentes sítios a serem infectados (Hazen *et al*, 2001).

A CSH não pode ser considerada um fator de virulência de um microrganismo, mas pode ser um marcador da presença de outros fatores (Blanco *et al,* 2010; Raut *et al,* 2010), que em conjunto contribuem para a virulência (Cutler, 1991). Neste contexto, foi mostrado previamente por Antley e Hazen (1988) que células hidrofóbicas são mais virulentas que células hidrofílicas em infecções experimentais em camundongos. Células altamente hidrofóbicas apresentaram maior capacidade de adesão a células epiteliais, superfícies abióticas e também a moléculas da matriz extracelular [MEC, (Hazen *et al*, 1991;. Blanco *et al,* 2010)]. Além disso, as leveduras mais hidrofóbicas tendem a ligar-se ao tecido hospedeiro de forma difusa e abundante (Hazen *et al*, 1991), diferentemente de células mais hidrofílicas, que ligam-se a regiões específicas do tecido especialmente aquelas ricas em macrófagos (Cutler *et al,* 1990). Um estudo conduzido por Glee e Hazen (1995) mostrou que

células hidrofóbicas aderem-se à MEC do tecido hospedeiro através de regiões hidrofóbicas de proteínas localizadas em sua superfície celular.

Como a fase inicial da formação de biofilme é a adesão ao substrato, pode-se inferir que a CSH interfere positivamente na formação de biofilme (Polaquini *et al,* 2006). Neste sentido, outros trabalhos mostraram uma correlação positiva entre a CSH e adesão (Raut *et al,* 2010), e formação de biofilme em poliestireno (Li *et al,* 2003; Blanco *et al,* 2010).

Os mecanismos específicos do processo de adesão envolvem interações, ligante-receptor, entre *Candida* spp. e o tecido do hospedeiro (Glee; Hazen, 1995; Cotter; Cavanagh, 2000; Naglik *et al,* 2003). Em geral, as moléculas de superfície da levedura que permitem uma interação específica são chamadas de adesinas. Essas moléculas são capazes de se ligar a inúmeros tipos celulares e a diferentes proteínas da MEC, como fibronectina, colágeno tipo IV, laminina e entactina (Bouchara *et al,* 1999; López-Ribot *et al,* 1999; Sundstrom, 2002; Tronchin *et al*, 2008; Salgado *et al,* 2011). As adesinas estão relacionadas à virulência de cada espécie, já que espécies mais patogênicas como *C. albicans e C. tropicalis* possuem maior capacidade de colonizar certas superfícies do que espécies menos patogênicas como *C. krusei e C. guilhermondii* (Calderone; Braun, 1991; Repentigny *et al,* 2000; Jabra-Rizk *et al,* 2001; Zhu; Filler, 2010).

As proteínas do tipo-aglutinina (Als – *agglutinin-like sequence*) são adesinas que possuem um papel chave na adesão de *C. albicans*. Elas pertencem a um grupo de oito proteínas (Als1 a Als7, e Als9) ligadas ao GPI (glycosylphosphatidylinositol) e localizadas na camada externa da parede celular (Hoyer *et al*, 2001; 2008). O papel dessas proteínas na adesão não parece ser o mesmo para todas, já que a expressão heteróloga e a deleção dos genes *ALS* resultaram em padrões singulares de adesão a diferentes superfícies. As proteínas Als1, Als3 e Als5 medeiam a adesão a inúmeros constituintes do hospedeiro, incluindo a MEC e células epiteliais, enquanto Als6 e Als9 ligam-se a um número de substratos muito mais restrito, além de não afetarem a ligação às células epiteliais (Gaur; Klots, 1997; Fu *et al*, 2008). Expressão de Als3 está associada, além do processo de adesão, à formação do tubo germinativo, dessa forma, é um fator importante para o desenvolvimento de infecções (Coleman *et al,* 2009). Além disso a Als3 é considerada uma invasina fúngica que imita caderinas de células hospedeiras, induzindo a endocitose ligando à N-caderina nas células endoteliais (Phan *et al,* 2007) e também um receptor de ferritina, possibilitando assim

a exploração do ferro da ferritina através da ligação morfológica dependente de Als3 (Almeida *et al,* 2008), sugerindo que essa proteína única tem atributos de virulência múltiplos.

Genes *ALS* foram identificados em outras espécies de *Candida*. O domínio 5' desses genes apresenta-se conservado, entretanto o domínio 3' apresenta sequências únicas. Há diferenças significativas no padrão de expressão dos genes *ALS* até mesmo entre as espécies mais próximas como *C. albicans* e *C. dubliniensis* (Hoyer *et al,* 2001). Apesar dessas diferenças, o processamento de Als parece ser conservado entre as espécies *C. albicans, C. dubliniensis* e *C. tropicalis* e estas proteínas estão ancoradas na parede celular por ligações com β 1,6 glucanas. Entretanto, o papel dessas proteínas na biologia ou patogênese de *C. dubliniensis* e *C. tropicalis* ainda não foi estudado.

Outra molécula com um importante papel na adesão de *C. albicans* é a proteína de parede de hifa 1 (Hwp1 - *hyphal wall protein 1*). A Hwp1 também pertence ao grupo de proteínas ligadas ao GPI e é expressa exclusivamente na superfície da parede celular de formas filamentosas. Sundstrom *et al* (2002) mostraram que mutantes para ambos os alelos do gene *HWP1* aderem-se fracamente a células epiteliais e apresentaram uma redução significativa na virulência em um modelo animal de candidíase disseminada. A Hwp1 também é importante para a formação do biofilme (Nobile *et al*, 2003), e em estudos posteriores observou-se que essa adesina possui a capacidade de ligar-se a Als1 e Als3, permitindo a adesão de uma hifa de *C. albicans* a outra (Nobile *et al*, 2008).

Outro fator como a origem do isolado clínico pode afetar a capacidade de adesão de *Candida* spp.. Manfredi *et al* (2002) mostraram que isolados oriundos de pacientes diabéticos possuem maior capacidade de adesão à fibronectina quando comparados com aqueles isolados de pacientes não diabéticos. Lyon e Resende (2006) mostraram que isolados de cavidade bucal de usuários de prótese dental com sinais clínicos de candidíase apresentam maior capacidade de adesão às células em relação aos isolados de indivíduos assintomáticos.

Embora não seja um fator de virulência clássico, a formação de biofilme contribui substancialmente na patogênese das infecções microbianas. Estima-se que 65% das infecções por *Candida* spp. estão associadas a formação de biofilme (Ruhnkhe *et al,* 2006; Uppulury *et al,* 2009). O biofilme microbiano pode ser caracterizado como uma comunidade altamente organizada de células aderidas (células sésseis) à um substrato e embebidos em uma

matriz de substância polimérica extracelular (EPS) produzida pelas mesmas (Douglas, 2003). As células sésseis apresentam fenótipo alterado principalmente em relação à taxa de crescimento, a resistência aos agentes antimicrobianos, e aos mecanismos de defesa do hospedeiro (Donlan; Consterton, 2002).

Biofilmes de *Candida* spp. podem ser formados tanto em superfícies bióticas quanto sobre inúmeros dispositivos médicos: cateteres venosos, dispositivos intrauterinos, implantes ortopédicos, tubos endotraqueais, marcapassos, válvulas cardíacas, implantes dentários e lentes de contato (Donlan, 2001; Donlan, 2002; Douglas, 2003; Lanfo *et al,* 2003; Ramage *et al,* 2006; Zambrano *et al,* 2006; Storti *et al*, 2007; Buijssen *et al*, 2011). E como ocorre em biofilmes bacterianos, as células sésseis de *Candida* spp. apresentam resistência aos mecanismos de defesa do hospedeiro (Katragkow *et al*, 2010) e uma variedade de agentes antifúngicos (Hawser; Douglas, 1995; Chandra *et al,* 2001; Kuhn; Ghannoum, 2004; Melo *et al,* 2007; Bizerra *et al*, 2008; Calişkan *et al,* 2011). A resistência do biofilme aos antifúngicos será abordada posteriormente.

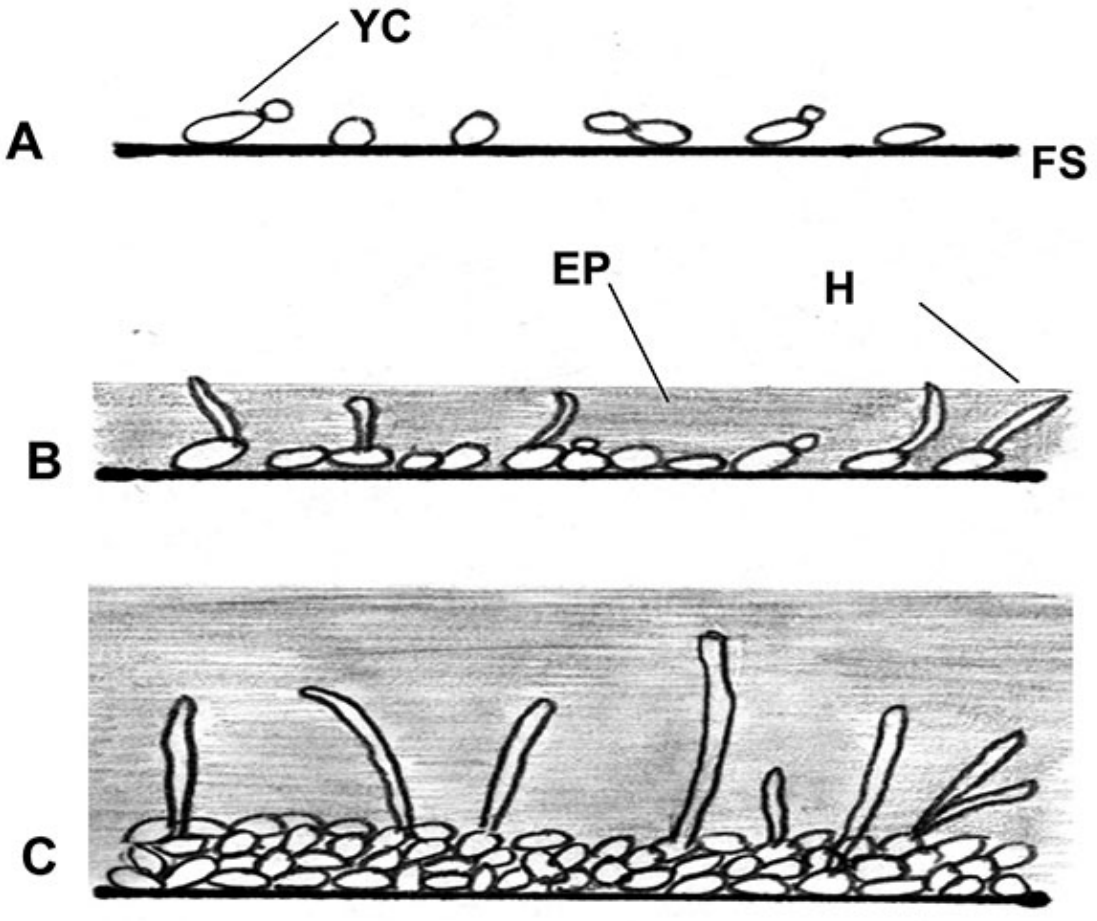
Além da variação entre espécies e entre isolados da mesma espécie, fatores ambientais também influenciam a capacidade de formação de biofilme por um microrganismo. As características físico-químicas do substrato afetam a adesão das células (Fletcher; Loeb, 1975, Chandra *et al,* 2005, Seneviratne *et al*, 2008), a presença de fluxo líquido contínuo aumenta a produção de EPS (Hawser *et al*, 1998), e mudanças no pH, temperatura, nutrientes e concentração de íons deste líquido podem levar a alterações no processo de formação do biofilme (Fletcher, 1988; Fera, 1989; Cowan, 1991).

A formação de biofilme de *C. albicans* possui fases de desenvolvimento distintas e bem definidas (Figura 3). Essas fases são caracterizadas pela adesão inicial das células leveduriformes ao substrato (fase de adesão); seguido pelo desenvolvimento de uma monocamada, formação de microcolônias e início da produção de material não-celular (fase intermediária); filamentação, proliferação, maturação e acentuada produção de material extracelular (fase de maturaçao) (Chandra *et al*, 2001; Jabra-Rizk *et al,* 2004; Heitman *et al,* 2006).

Durante a formação de biofilme de *C. tropicalis* e *C. dubliniensis* foi observado um grande aumento da atividade metabólica das células sésseis nas primeiras 24 h de adesão. A adesão de células leveduriformes ao substrato é seguida por germinação,

filamentação e formação de microcolônias, e a fase de maturação parece ocorrer entre 20 e 48 horas após a fase inicial. O biofilme maduro é caracterizado por uma grande quantidade de células leveduriformes e hifas embebidas na EPS (Ramage *et al,* 2001; Bizerra *et al,* 2008). Células sésseis de *C. tropicalis* podem apresentar resistência a anfoterecina B e fluconazol, e neste último caso parece estar relacionado com o aumento da expressão dos genes *ERG11* e *MDR1* (Bizerra *et al*, 2008).

Figura 3 - Ilustração representativa das fases de desenvolvimento do biofilme de *Candida albicans* e *Candida dubliniensis*; (A) fase de adesão 0–11 h; (B) fase intermediária 12–30 h; (C) fase de maturação 38–72 h; FS, *flat surface:* superfície plana; YC, *yeast cell:* célula leveduriforme; H, *hyphae:* hifa; EP, *exopolymeric matrix:* matriz exopolissacarídica.



Fonte: Jabra-Rizk *et al,* 2004.

A EPS é uma estrutura altamente hidratada, de aspecto gelatinoso e que envolve os microrganismos no biofilme maduro (Seneviratne *et al*, 2008), podendo concentrar

90% das fontes de carbono para esta comunidade celular (Flemming *et al*, 2000). Apesar da composição da EPS consistir principalmente de exopolissacarídeos e baixa quantidade de proteínas e ácidos nucleicos (Flemming *et al,* 2000; Donlan, 2000; Sutherland, 2001), sua composição química pode variar entre as espécies de *Candida*. A EPS de *C. albicans* é composta por 39,6% de carboidratos (32,2% correspondem a glicose), 5% de proteínas, 3,3% de hexosaminas, 0,5% de fósforo e 0,1% de ácido urônico (Mohammed *et al,* 2006). Já a EPS de *C. tropicalis* consiste principalmente de hexosamina (27,4%), seguido por outros carboidratos (5,5%, sendo 0,5% de glicose), proteínas (3,3%), ácido urônico (1,6%) e fósforo (0,2%) (Al-Fattani; Douglas, 2006). Além disso, também podem ser observadas diferenças quantitativas na EPS em que *C. albicans* e *C. tropicalis* tendem a produzir grande quantidade enquanto *C. glabrata* tende a produzir pequena quantidade dessa matriz (Al Fattani; Douglas, 2006; Seneviratne *et al*, 2008).

As Saps (*Secreted aspartyl Protease*) são as principais responsáveis pela atividade proteolítica extracelular de *C. albicans*, e sua associação com a virulência tem sido extensivamente estudada. As Saps de *C. albicans* compõem uma família de pelo menos 10 genes (*SAP* 1-10) que codificam proteínas de massa molecular entre 42 e 44 kDa (Naglik *et al,* 2003; Rehani *et al*, 2011). Análises de sequência de aminoácidos mostram que Saps 1-3 e Saps 4-6 apresentam 67% e 89% de homologia entre elas, respectivamente. Por outro lado, Sap 7 apresenta somente cerca de 20 a 27% de homologia com os demais componentes (Stehr *et al*, 2000; Hube; Naglik, 2001). Assim como a protease do HIV, essas proteínas fúngicas fazem parte da família pepsina, e caracterizam-se pela presença de dois domínios contendo o motivo Asp-Thr/Ser-Gly (ácido aspártico-treonina/serina-glicina) no qual os resíduos de ácido aspártico participam da atividade catalítica. A maioria apresenta melhor atividade em pH baixo e todas são especificamente inibidas por pepstatina A (Davies, 1999; Dunn, 2002; Naglik *et al*, 2004). As Sap 7, 9 e 10 apresentam melhor atividade em pH neutro e a Sap 7 apresenta-se pouco inibida pela pepstatina A (Aoki *et al,* 2011). Ao contrário das Saps 1 a 8 que são secretadas, as Sap 9 e Sap 10 são proteases associadas à superfície celular que atuam na integridade da parede celular e na interação com células epiteliais humanas e neutrófilos (Albrecht *et al,* 2006; Schild *et al,* 2011).

A associação entre a atividade proteolítica e virulência de *C. albicans* foi inicialmente descrita em 1965 por Staib. Neste trabalho, foi mostrado que cepas com

atividade de protease eram mais virulentas, provocando extensiva peritonite e infecção generalizada em camundongos, enquanto que as cepas não-proteolíticas não provocaram nenhuma alteração nos animais. Desde então, vários autores detectaram a atividade de protease em *Candida* spp. isoladas de infecções humanas, corroborando o papel destas enzimas na virulência dessas leveduras (Borst; Fluit, 2003; Kuriyama *et al,* 2003; Ribeiro *et al*, 2004; da Costa *et al*, 2009; Ballal; Vinitha 2009; Aoki *et al*, 2011; Dóstal *et al,* 2011).

Em relação aos isolados de pacientes portadores de HIV, em geral, foi observado que essas leveduras apresentaram maior atividade proteolítica quando comparadas com os isolados de indivíduos não portadores de HIV (Ollert *et al*, 1995; De Bernardis *et al*, 1996; 1999; Migliorati *et al*, 2004; Ribeiro *et al*, 2004; Mane *et al*, 2011). Nestes pacientes, foi mostrado que os possíveis efeitos da infecção pelo HIV, como alterações no tecido do hospedeiro (Qureshi *et al,* 1995), redução no fluxo salivar (Schiodt, 1992), alterações na microbiota (Rams *et al,* 1991) e até possíveis ligações entre os vírus e as leveduras (Gruber *et al*, 1998) podem alterar o padrão de secreção de proteases e contribuir para a seleção de cepas mais virulentas (Sweet, 1997).

Estudos mostram que a inibição de Sap de *C. albicans* pela pepstatina A pode levar à redução na adesão da levedura aos queratinócitos humanos (Ollert *et al,* 1993). Segundo Monod e Borg-von Zepelin (2002), o mecanismo de ação de Sap no processo de adesão poderia ser explicado por duas hipóteses, na primeira, as proteases atuariam como ligantes presentes na superfície da levedura, sem necessariamente terem alguma função hidrolítica. Na segunda, a levedura utilizaria as enzimas para clivar proteínas da superfície da célula alterando, assim, a sua conformação, o que facilitaria a ligação entre a levedura e o tecido hospedeiro.

Além da participação no processo de adesão às células (Watts *et al*. 1998; Borg-von Zepelin *et al*, 1999; Kontoyiannis *et al*, 2001; Aoki *et al,* 2011) vários estudos mostram que as Saps podem exercer diferentes funções que incluem: digestão de moléculas para fins nutricionais (MacDonald, 1984; Kvaal *et al*, 1999); invasão e degradação de células e tecidos (Morschhäuser *et al,* 1997; Schaller *et al,* 1999; 2000; 2003; Zaugg *et al,* 2001; Felk *et al,* 2002; Villar *et al,* 2007); evasão das defesas do hospedeiro pela destruição de células e moléculas do sistema imune (Rüchel, 1986; Kaminishi *et al,* 1995; Borg-von Zeppelin *et al*, 1998; Meiller *et al*, 2009, Aoki *et al,* 2011) ou resistência a fagocitose pelos macrófagos

(Borg-Von Zepelin *et al,* 1998). Além disso, participam do "switching" fenotípico (Kvaal *et al,* 1999; Naglik *et al,* 2003).

Os genes *SAP*s são diferencialmente regulados podendo ser expressos em resposta a diferentes condições de cultivo da levedura, e tipos ou sítios de infecção. As Saps 1-3 são diferencialmente expressas por formas leveduriformes, sendo que a Sap 2 é a principal responsável pela atividade proteolítica durante o crescimento *in vitro* quando BSA (*Bovine Serum Albumin*) é o substrato presente no meio de cultura. As Saps 4-6 são expressas por hifas.

Proteínas homólogas a Saps de *C. albicans* também foram detectadas em outras espécies de *Candida,* como *C. tropicalis*, *C. dubliniensis, C. guilliermondii, C. parapsilosis* e *C. lusitaniae.* Entretanto a associação da atividade de proteases com a virulência dessas leveduras ainda não está bem definida (Zaugg *et al,* 2001; Parra-Ortega *et al,* 2009; da Costa *et al,* 2011; Negri *et al,* 2011).

Outras enzimas hidrolíticas como as fosfolipases extracelulares são particularmente importantes na virulência de *Candida* spp., pois catalisam reações de hidrólise de fosfolipídeos, o principal componente da membrana celular do hospedeiro (Ghannoum, 2000). Esta atividade resulta em desestabilização da membrana celular do hospedeiro, permitindo a invasão do fungo e consequente lise de células (Ghannoum, 2000, Oksuz *et al,* 2007; Scheid *et al,* 2010; Negri *et al,* 2011).

Além do papel ativo na invasão do tecido do hospedeiro (Pugh; Cawson, 1975; Sullivan *et al,* 1995; Samaranayake *et al,* 2005; Scheid *et al,* 2010), as fosfolipases participam da adesão de *C. albicans* às células epiteliais (Barrett-Bee *et al*, 1985; Ibrahim *et al*, 1995; Samaranayake *et al,* 2005) e interferem nos mecanismos de defesa do hospedeiro (Barrett-Bee *et al*, 1985).

A atividade de fosfolipase *in vitro* de *Candida* spp. já foi descrita por diversos autores, dentre as espécies como *C. albicans*, *C. dubliniensis*, *C. parapsilosis, C.kefyr, C. famata, C. spharicae* e *C. tropicalis*. Entre elas, *C. albicans* e *C. tropicalis* apresentaram maior atividade de fosfolipases extracelulares (Price *et al,* 1982; Borst; Fluit, 2003; Ribeiro *et al,* 2004; Fotedar; Al-Hedaithy, 2005; Samaranayake *et al,* 2005; Zeng *et al,* 2008; da Costa *et al,* 2009; Ballal; Vinitha, 2009; Scheid *et al,* 2010; D´Eça Junior *et al,* 2011; Marcos Arias *et al,* 2011).

Quatro fosfolipases foram descritas em *C. albicans* até o momento (PLA, PLB, PLC e PLD) (Niewerth; Korting 2001). Entretanto, até o momento apenas a PLB1 foi associada a virulência em modelo animal de candidíase (Oksuz *et al,* 2007), uma vez que a deleção do gene *CaPLB1* reduz drasticamente a capacidade de invasão do fungo nas células hospedeiras (Leidichi *et al,* 1998). Outros estudos que associam a atividade de fosfolipase com virulência mostram que isolados com maior atividade dessas enzimas possuem maior capacidade de adesão e invasão da mucosa bucal e provocam maior taxa de mortalidade em modelos animais de infecção (Barrett-Bee *et al*, 1985; Ibrahim *et al*, 1995).

Em outro estudo, Samaranayake *et al* (2005) analisaram a correlação entre a atividade de fosfolipase e a formação de tubo germinativo, a hidrofobicidade da superfície celular, a adesão às células epiteliais bucais e a atividade hemolítica de *C. albicans* isoladas da cavidade bucal de pacientes HIV positivos. Alta atividade de fosfolipase foi observada nesses isolados, entretanto não houve correlação com os outros parâmetros analisados. Ribeiro *et al* (2004) mostraram que *Candida* spp. isoladas, tanto da mucosa bucal quanto vaginal, de portadoras de HIV apresentaram maior atividade de fosfolipase do que isolados de mulheres não portadoras do vírus. Similarmente, Mane *et al* (2011) relataram que leveduras isoladas da cavidade bucal de indivíduos HIV positivos apresentam maior secreção de fosfolipases extracelulares em relação àquelas isoladas de pacientes não infectados.

Enquanto muitos estudos são desenvolvidos para a investigação das enzimas hidrolíticas como proteases e fosfolipases, muito pouco se sabe a respeito da atividade hemolítica exibida por diferentes espécies de *Candida*.

A capacidade de organismos patogênicos para adquirir ferro no hospedeiro mamífero tem sido mostrado como sendo de crítica importância na infecção, que estabelece. Nos seres humanos, a maior parte do ferro está localizado intracelularmente como ferritina ou como heme contendo compostos. A pequena quantidade de ferro extracelular está ligado às proteínas de ligação de ferro e de transporte transferrina e lactoferrina (Manns *et al,* 1994; Almeida *et al,* 2009). A transferrina é uma proteína sérica que se liga ao ferro com alta afinidade, restringindo esse elemento na forma livre para o crescimento microbiano. Como o ferro é essencial para uma variedade de processos metabólicos celulares, microrganismos patogênicos devem ter mecanismos para a obtenção do ferro que é sequestrado do hospedeiro. Assim, grande parte dos patógenos o adquire indiretamente de componentes que os contém

disponível, como a hemoglobina (Bullen, 1981). Muitos microrganismos possuem mecanismos que permitem destruir o grupo heme e extrair o elemento ferro do hospedeiro (Belanger *et al*, 1995). Assim, ao mediar essa atividade, essas enzimas são consideradas como um fator de virulência relevante que possibilita ao patógeno sua sobrevivência e infecção no hospedeiro humano (Otto *et al*, 1992).

Luo *et al* (2001) foram os primeiros a demonstrar os perfis variáveis de expressão de hemolisinas em diferentes espécies de *Candida* em meio suplementado com glucose e sangue de carneiro. Oito espécies de *Candida,* dentre elas: *C. albicans, C. dubliniensis, C. kefyr, C. krusei, C. zeylanoides, C. glabrata, C. tropicalis e C. lusitaniae* demonstraram alfa e beta hemólise após 48 horas de incubação em 5% de CO_2. Entretanto *C, famata, C. guilliermondii, C. rugosa* e *C. utilis* apresentaram apenas alfa hemólise. Os isolados de *C. parapsilosis* e *C. pelliculosa* não apresentaram nenhuma atividade hemolítica. Alguns autores também reportaram, recentemente, a presença de atividade hemolítica em *C. albicans*, *C. glabrata, C. krusei*, *C. parapsilosis* e *C. tropicalis* (Furlaneto-Maia *et al,* 2008; França *et al,* 2010; Favero *et al,* 2011).

França *et al* (2010) relataram a atividade hemolítica de todos os 28 isolados envolvidos no trabalho quando semeados em meio Sabouraud suplementado com 7% de sangue de carneiro, sendo que 10 foram isolados de sangue e 18 de amostras clínicas distintas (8 urina, 6 secreção traqueal e 4 lesão de unha. Destes, hemólise fraca predominou nos isolados de candidemia (4/10) , sendo que os isolados de urina (5/8) e de lesão de unha (2/4) apresentaram predominantemente hemólise forte, enquanto os isolados de secreção traqueal apresentaram hemólise muito forte (3/6), porém não houve diferença estatística entre a atividade hemolítica entre os isolados de diferentes sítos anatômicos.

Em trabalho posterior, França *et* al (2011) empregou meio Sabouraud enriquecido com 3% de eritrócitos em vez de meio contendo 7% de sangue de carneiro e revelou diferenças na atividade hemolítica entre os isolados ($P < 0,05$). Sob esta condição, todos os isolados apresentaram atividade hemolítica e 22,5% dos isolados apresentaram alta atividade hemolítica. Em geral, a maioria dos isolados de *C. parapsilosis* isolados produziram atividade fraca, ao passo que a maioria dos *C. tropicalis* apresentavam média e elevada atividade. Análise da atividade hemolítica de *C. parapsilosis* e *C. tropicalis* isoladas do mesmo sítio anatômico revelou que *C. tropicalis* isoladas de sangue apresentaram atividade

estatisticamente maior (p <0,05) em relação à *C. parapsilosis*. Não foram observadas diferenças estatísticas entre os isolados destas espécies obtidos a partir de secreção traqueal e unhas e da pele (lesões superficiais). Não foram observadas diferenças nas atividades hemolíticas entre isolados de *C. tropicalis* provenientes de diferentes sítios anatômicos, entretanto, sítios anatômicos de isolamento parece estar correlacionado com a atividade hemolítica de isolados de *C. parapsilosis*.

1.3 A TERAPIA ANTIRRETROVIRAL E AS INFECÇÕES POR *CANDIDA* SPP.

Todas as etapas do ciclo de vida do vírus são potenciais alvos para a ação de antivirais, e estes visam o retardamento da replicação do vírus no organismo. Até o momento seis classes de quimioterápicos estão liberados para uso, como monoterapia ou em combinação, em indivíduos HIV positivos pelo FDA (*U. S. Food and Drug Administration*): inibidores da transcriptase reversa nucleosídicos e não nucleosídicos, inibidores de protease viral, inibidores de entrada viral, inibidores de fusão viral e inibidores de integrase viral. Esses medicamentos integram a Terapia Antirretroviral Altamente Eficaz (HAART – *Highly Active Antiretroviral Therapy*), introduzida em 1996 com a liberação para uso das duas primeiras classes de medicamentos citadas (Cooper, Merigan, 1996, Mastrolorenzo *et al,* 2007).

O sucesso dessa terapia foi comprovado por inúmeros estudos que mostram que o uso contínuo dos antirretrovirais está associado a fatores como: uma acentuada queda na carga viral por suprimir a replicação viral; ao aumento do número de linfócitos T $CD4^+$ e melhoria das funções do sistema imunológico; diminuição da resistência ao tratamento; maior sobrevida dos pacientes e consequente redução da taxa de mortalidade (Deeks *et al*, 1997; Pallela *et al*, 1998; Cassone *et al,* 2002, Sethi *et al,* 2003; Pomarico *et al,* 2009; Stubbs *et al,* 2009; Musiime *et al,* 2011). Além disso, o uso da terapia antirretroviral no tratamento dos pacientes infectados com o HIV foi associado à diminuição na prevalência da candidíase oral e outras infecções oportunistas (Arribas *et al,* 2000; Hung; Chang, 2004; Egusa *et al,* 2008; Pomarico *et al,* 2009; Ramos-E-Silva *et al,* 2010). Em estudo multicêntrico envolvendo 6941 pacientes, os casos de candidíase caíram de 17% para 5,7% após a introdução da HAART (Babiker *et al,* 2002).

A causa da redução destas infecções em pacientes sob terapia pela HAART

ainda não está totalmente elucidada, uma vez que diferentes estudos feitos pelo acompanhamento de pacientes em tratamento antirretroviral mostraram resultados conflitantes. Segundo alguns autores essa redução ocorre devido ao aumento do número de linfócitos T $CD4^+$ e à diminuição da carga viral (Hoegl *et al,* 1998, Kaplan *et al,* 2000; Mocroft *et al,* 2003; Yang *et al,* 2006). Entretanto, outros defendem que seja improvável que a diminuição dos casos de candidíase orofaríngea ocorra pela melhora do sistema imune, uma vez que a resolução da infecção pode ocorrer antes do restabelecimento do número de células CD4+ na periferia (Cassone *et al,* 2002). Neste caso, a diminuição dos casos de candidíase orofaríngea na era HAART poderia estar associada a um potencial efeito antimicótico dos antirretrovirais sob *Candida* spp., especialmente dos inibidores de protease (IP) (Borg-von Zepelin *et al,* 1999; Gruber *et al*, 1999, Arribas *et al,* 2000, Cassone *et al,* 2002; Costa *et al,* 2006).

Segundo Cassone *et al* (1999), os IP contra HIV teriam um efeito anti-*Candida* direto pela inibição competitiva das Saps, um fator essencial para o desenvolvimento de infecção da mucosa pelas leveduras. Isto é possível devido ao fato de que Saps e a protease viral do HIV-1, pertencem à mesma classe de enzimas, como mencionado anteriorente (Davies, 1990; Mata-Essayag *et al*, 2000). De fato, inúmeros estudos têm mostrado o efeito de inibidores de proteases como amprenavir (Schaller *et al,* 2003; Braga e Silva *et al*, 2009; Tsang *et al*, 2009), saquinavir (Korting *et al*, 1999; Schaller *et al,* 2003) e ritonavir (Falkensammer *et al,* 2006) sobre a atividade de Saps. Esses estudos evidenciaram principalmente que a inibição de Saps estava associada a redução da adesão das leveduras a células epiteliais *in vitro,* após o tratamento com IPs.

Bektic *et al* (2001) estudaram o efeito dos IP saquinavir, indinavir e ritonavir na adesão de *C. albicans* a células epiteliais e na fagocitose por linfócitos polimorfonucleares. Todos IPs apresentaram atividade inibitória sobre a adesão de *C. albicans* as células HeLa S3, entretanto o ritonavir mostrou-se mais potente inibidor do que indinavir e saquinavir. Nenhum IP foi capaz de modular a fagocitose da levedura pelos linfócitos.

Braga-Silva *et al* (2009) verificaram vários efeitos do amprenavir sobre *C. albicans*, incluindo a inibição da expressão de Sap2. O crescimento celular também foi alterado, uma vez que o amprenavir na concentração de 200 μM foi capaz de reduzir o crescimento em cerca de 50%. Em concentrações superiores a 200 μM este IP provocou

alterações na superfície das células levando à perda de moléculas da superfície, além de induzir mudanças na morfologia das células, com o aparecimento de invaginações e cavitação da superfície. Além disso, o estudo mostrou que houve reduções na quantidade de esterol e glicoconjugados ricos em manose e ácido siálico e redução de cerca de 50% da formação de biofilme das células tratadas em relação ao controle.

Apesar dos inúmeros testes mostrando os efeitos dos IPs sobre *C. albicans in vitro*, poucas pesquisas mostram sua implicação *in vivo* (Cassone *et al,* 1999; Yang *et al,* 2006). Cassone *et al,* (2002) conduziram um interessante estudo comparando inúmeros fatores como incidência de candidíase orofaríngea, carga fúngica, reconstituição do sistema imune, carga viral e nível de Sap presente na saliva de pacientes HIV positivos antes e após o início do tratamento com IP em comparação a pacientes em uso de inibidores não nucleosídicos da transcriptase reversa (INNTR). Em relação à reconstituição do sistema imune e a carga viral, não houve diferenças significativas entre os dois grupos de estudos. Entretanto, os resultados mostraram um efeito anti-*Candida* e anti-Sap independente da melhora do sistema imune nos pacientes sob uso de IP uma vez que quase 100% não exibiam mais Sap em sua saliva após 30 dias de tratamento. Cerca da metade dos pacientes mantiveram este índice após dois anos, contrastando com aqueles em uso de INNTR, os quais continuaram positivos para Sap durante todo o período do estudo. Esses resultados corroboram que os IP, mas não outros antirretrovirais, possuem um efeito anti-*Candida* e anti-Sap *in vivo.*

1.4 ANTIFÚNGICOS

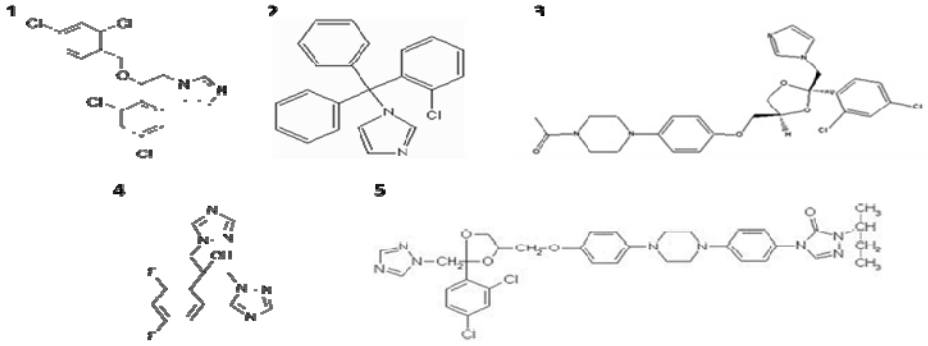
Para o tratamento da candidíase orofaríngea a terapia primária indicada é o uso de clotrimazol, nistatina ou fluconazol. Sendo que o flucanazol é recomendado para a COF moderada a grave, e a terapia tópica com clotrimazol ou nistatina para a COF leve. Para a infecção refratátia é recomendado suspensão de itraconazol, voriconazol, posaconazol ou anfoterecina B. Já para o tratamento da candidíase esofágica o fluconazol é o mais indicado, entretanto em condições em que o paciente não tolere a terapia oral podem ser empregadas anfoterecina B e equinocandinas (Pappas *et al,* 2009).

Como se pode perceber, nesses casos a utilização de antifúngicos pertencentes aos azólicos são de grande importância, entretanto, tem sido observado diversos

casos de resistência à terapia com fluconazol, principalmente em pacientes com cadidíase orofaríngea recorrente que fizeram uso anterior desse antifúngico. Nesses casos, já foi constatado que os isolados de *Candida* apresentam susceptibilidade reduzida ao fluconazol nos testes de sensibilidade *in vitro.* Além da preocupação com ocorrência de resistência durante a terapia antifúngica nos casos de COF recorrente, o tratamento profilático também representa risco para ocorrência de resistência (Muller *et al*, 2000; Pelletier *et al*, 2000; Pagani *et al*, 2002; Hamza *et al*, 2008).

Os azólicos são antifúngicos fungistáticos que (Figura 4) agem inibindo a enzima 14α-esterol demetilase, impedindo a síntese do ergosterol, que é o esterol da membrana plasmática fúngica, e consequentemente alteram a permeabilidade e fluidez da membrana fúngica (Odds, Brown e Gow, 2003; Niimi *et al,* 2010). Na forma planctônica as espécies de *Candida* geralmente são sensíveis aos azólicos, exceto *C. glabrata* e *C. krusei* que naturalmente apresentam sensibilidade reduzida e resistência ao fluconazol e voriconazol (Godoy *et al*, 2003; Tortorano *et al,* 2004; Pfaller *et al*, 2010).

Figura 4 - Estrutura química dos azólicos. **1**. Miconazol. **2**. Clotrimazol. **3**. Cetoconazol. **4**. Fluconazol. **5**. Itraconazol.

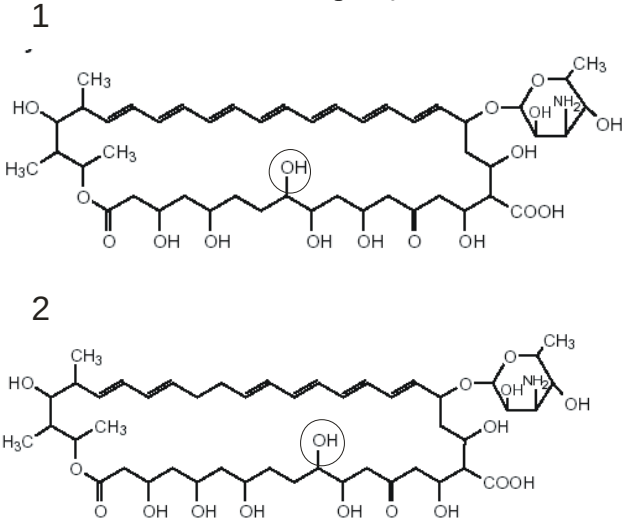


Fonte: Dias *et al,* 2009.

Os poliênicos (Figura 5) foram os primeiros antifúngicos descritos, ainda na década de 50. Eles se ligam fortemente ao ergosterol, causando a ruptura da membrana plasmática e extravazamento do conteúdo citoplasmático, sendo assim, fungicidas. Os poliênicos mais utilizados são a nistatina e a anfoterecina B, sendo a última utilizada pela via intravenosa (Niimi *et al,* 2010; Rogers *et al,* 2011). Contudo, a terapia com anfotericina B em suas diferentes formulações está associada com toxicidade renal (Laniado-Laborín; Cabrales-

Vargas, 2009; Wang *et al,* 2010). A introdução de formulações lipídicas (lipossomas ou complexos lipídicos) reduziu a incidência de complicações renais da anfoterecina B.

Figura 5 - Estrutura química dos derivados poliênicos. **1**. Anfotericina B. **2**. Nistatina. A diferença entre os fármacos reside na posição da hidroxila em destaque.

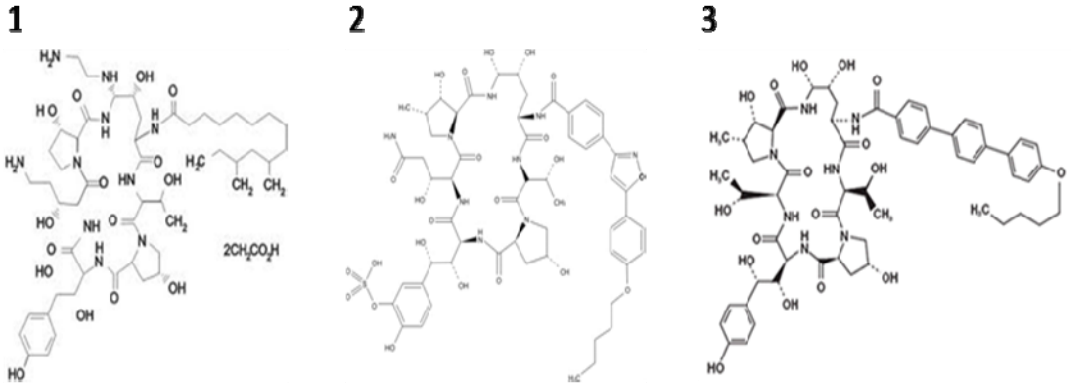


Fonte: http://www.qb.fcen.uba.ar/microinmuno/SeminarioAntibioticos2.htm.

As equinocandinas (Figura 6), mais nova classe de substâncias antifúngicas, são inibidores não competitivos da biossíntese de β-1,3-D-glucana (Douglas *et al*, 1997), componente necessário para a integridade da parede celular fúngica (Perlin, 2011; Wilke *et al,* 2011). Estes antifúngicos atuam principalmente sobre a proteína Fks1 (sub-unidade de β-1,3-D-glucana sintase), provocando a depleção de glucanas. Esta classe de antifúngicos tem sido utilizada como fármaco de escolha em substituição ao fluconazol, para o tratamento de candidíase esofágica pela sua eficiência tanto no biofilme como em células planctônicas. (Chandwani *et al,* 2009; Zilberberg, Kothari e Shorr, 2009). Casos de resistência as equinocandinas ainda são incomuns, embora espécies como *C. parapsilosis* e *C. guilliermondii* apresentam valores de concentração inibitória mínima (CIM) mais altos, e *C. glabrata* resistente tanto as equinocandinas quanto aos azólicos foi reportada recentemente (Kofla & Ruhnke, 2011; Pfaller *et al*, 2011). Este fenótipo multirresistente, que é importante ressaltar que essa seja muito rara, pode estar associado à exposição prolongada às duas classes de antifúngicos e se caracterizam por episódios recorrentes de candidemia (Sun & Singh, 2010).

Outro aspecto interessante das equinocandinas é a possibilidade de determinados fungos, entre eles espécies de *Candida*, apresentarem o efeito paradoxal. Isto é, esses fungos crescem em concentrações superiores a CIM do antifúngico. Esse efeito foi observado tanto para células planctônicas quanto sésseis de *Candida* spp. (Melo *et al*, 2007; Bizerra *et al*, 2011).

Figura 6 - Estrutura química das equinocandinas. **1**. Caspofungina. **2**. Micafungina. **3**. Anidulafungina.



Fonte: http://www.visaoacademica.ufpr.br/v5n2/ana_m.htm

A resistência dos biofilmes aos antimicrobianos torna as infecções associadas a essas comunidades extremamente difíceis de serem tratadas. Os biofilmes formados por *C. albicans* apresentam-se 30 a 2000 vezes mais resistentes que as células planctônicas aos antifúngicos fluconazol, anfotericina B, flucitosina, itraconazol e cetoconazol (Hawser; Douglas, 1995; Chandra *et al,* 2001; Kuhn; Ghannoum, 2004; Melo *et al,* 2007; Bizerra *et al*, 2008; Calişkan *et al,* 2011). Algumas hipóteses tentam explicar os possíveis mecanismos de desenvolvimento dessa resistência: 1) foi proposto que a EPS poderia limitar o acesso dos antifúngicos às células localizadas nas regiões mais profundas do biofilme devido à lenta difusão dos fármacos. Entretanto, vários autores mostraram que não há diferenças relevantes na penetração de antimicrobianos em biofilmes formados sob fluxo líquido contínuo (que formam uma matriz mais robusta) em comparação com aqueles formados sob condições estáticas (com uma matriz menos proeminente) (Baillie; Douglas, 2000; Nah; O'Toole; 2001; Al Fattani; Douglas, 2004); 2) a resistência estaria associada a utilização de bombas de efluxo. A super-expressão de genes das famílias *CDR* e *MDR* tem

sido observada em biofilmes de *Candida* spp. (Bizerra *et al,* 2008). Contudo esse mecanismo parece atuar nos momentos iniciais do biofilme, mas não durante a maturação (Mukherjee *et al*, 2003); 3) taxa de crescimento reduzida, o que poderia causar alterações na superfície celular prejudicando a ação dos antimicrobianos (Baillie; Douglas, 2000; Donlan; Costerton, 2002; Kumamoto, 2002; Douglas, 2003; D´Enfert, 2006); 4) presença de células persistentes tolerantes aos antifúngicos somente no biofilme de *Candida* spp. (LaFleur *et al*, 2006). O mecanismo da persistência em *Candida* spp. ainda não foi elucidado, entretanto em bactérias, estas células representam variantes fenotípicos e são dormentes, apresentando baixo nível de tradução (Shah *et al*, 2006).

Em vista do exposto o tratamento para a candidíase tem se tornado problemático pela toxicidade dos antifúngicos disponíveis, pelo crescimento do número de isolados resistentes e a presença de biofilme (Khan *et al,* 2012). Dessa forma, a busca por novos fármacos para o tratamento de candidíases, principalmente com atividade antibiofilme tem sido um desafio para novos pesquisadores (Ahamad *et al,* 2010).

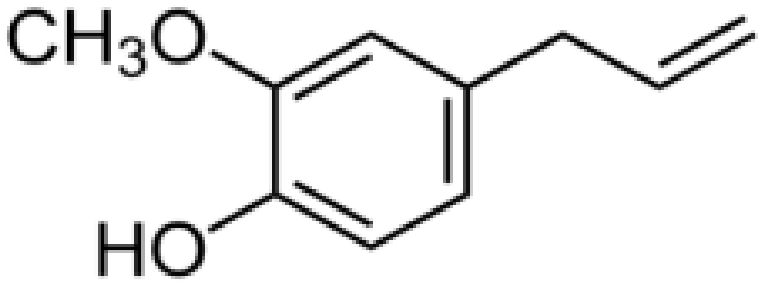
Há muito tempo as plantas têm sido usadas na medicina popular para o tratamento de infecções, devido a fácil acessibilidade e baixo custo (Rojas *et al*, 2006). Nos últimos anos diversos estudos foram conduzidos com plantas medicinais na procura de novas substâncias com atividade antimicrobiana (Ahmad e Beg, 2001; Audi *et al*, 2004; Botelho *et al*, 2007; More *et al,* 2008; Höfling *et al*, 2010).

Ocimum gratissimum L. (Lamiaceae) é uma planta herbácea medicinal e diversos compostos químicos ativos são obtidos como: eugenol, linalol, cânfora e timol (Adebolu *et al,* 2005; Lemos *et al,* 2005; Matasyoh *et al,* 2007; 2008). O eugenol (Figura 7) é amplamente utilizado, nas clínicas odontológicas, devido sua ação anestésica e analgésica (Pramod *et al,* 2010). Outras propriedades biológicas do eugenol foram descritas na literatura, dentre estas: a atividade antifúngica (Lima *et al*., 1993; Nakamura *et al*, 2004; Lemos, 2005; He *et al*, 2007; Braga *et al*, 2007; Ahmad *et al*, 2010; Marcos-Arias *et al,* 2011, Sosto *et al,* 2011) atividade antibacteriana (Janssen *et al*, 1989; Nakamura *et al*, 1999; Cimanga *et al*, 2002; Ngassoum *et al*, 2003, Devi *et al,* 2010; García-García *et al,* 2011; Horváth *et al,* 2011), atividade anti-*Leishmania* (Ueda-Nakamura *et al,* 2006), inseticida (Kéita *et al*, 2001) e antiviral (Ayisi; Nyadedzor, 2003; Chaieb *et al,* 2007). Além disso, o eugenol possui significante atividade antioxidante, anti-inflamatória, além de propriedades cardiovasculares

(Pramod *et al,* 2010) e potencial antitumorigênico (Chaieb *et al,* 2007; Hussain *et al,* 2011).

Isômeros do eugenol apresentam inibição sobre *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes* e *C. albicans* (Pauli; Kubeczka; 2010).

Figura 7 - Estrutura química do eugenol.



Fonte: Ahmad *et al,* 2010.

O óleo essencial de cravo (*Eugenia caryophyllata, Syzygium aromaticum*) apresenta atividade antifúngica contra um grande número de fungos patogênicos como *Candida* spp., *Aspergillus* spp. e dermatófitos, sendo que o eugenol corresponde a 85,3% - 88,6% do total desse óleo (Chami *et al,* 2005; Chaieb *et al,* 2007, Pinto *et al,* 2009).

A atividade *in vivo* do eugenol também foi descrita, onde o tratamento de ratos com candidíase promoveu a redução da contagem do número de colônias na cavidade vaginal (Chami *et al,* 2004) e bucal (Chami *et al,* 2005) desses animais em relação ao controle sem tratamento.

Nozaki *et al* (2010) mostraram que óleos essenciais apresentam atividade sobre isolados de *Candida* spp. e muitos deles potencializam o efeito da anfoterecina B. Neste caso, o óleo de cravo apresentou o efeito mais potente. Em outros trabalhos, 21 óleos essenciais de diversas plantas foram testados em isolados de *Candida* spp. resistentes ao fluconazol e à anfoterecina B e alguns foram mais eficientes que os fármacos já utilizados rotineiramente. Além disso, o eugenol exibiu significante sinergismo com o fluconazol e anfoterecina B (Ahmad *et al,* 2010; Zore *et al,* 2011).

O mecanismo de ação do eugenol sobre células planctônicas não foi totalmente esclarecido. Este composto interfere na arquitetura da superfície celular de *C. albicans* (Braga *et al,* 2007) além de inibir a formação do tubo germinativo (Nakamura *et al,*

2004; Pinto *et al,* 2009; Zore *et al,* 2011), processo que depende da integridade da parede celular (Ahmad *et al,* 2010). Células tratadas com eugenol apresentam redução significativa do conteúdo de ergosterol (Pinto *et al,* 2009; Ahmad *et al,* 2010a), além disso interferem na atividade de H+-ATPase resultando em acidificação intracelular e morte celular (Ahmad *et al,* 2010b; Khan *et al,* 2010).

Carrasco *et al* (2012) mostraram que um derivado do eugenol (*4-allyl-2-methoxy-5-nitrophenol*) exibiu potente atividade sobre *Candida* spp., *Cryptococcus neoformans* e dermatófitos. A atividade antifúngica desse derivado não foi revertida na presença de um suporte osmótico, como sorbitol, sugerindo que a parede celular fúngica não seja o alvo desse composto. Além disso, foi mostrado que o composto não se liga diretamente ao ergosterol o que pode revelar que a atividade antifúngica desse análogo do eugenol não esteja relacionada à alteração estrutural da membrana.

O eugenol também apresenta atividade sobre a formação e sobre o biofilme maduro de *C. albicans*, reduzindo a atividade metabólica das células sésseis em aproximadamente 80% após 48 horas de tratamento (He *et al,* 2007). Além disso, o eugenol apresenta sinergismo com o fluconazol para o tratamento do biofilme maduro (Khan *et al,* 2011).

Em conjunto, esses dados mostram o potencial do eugenol como modelo para o desenvolvimento de fármacos com eficácia tanto sobre células planctônicas quanto sésseis para o tratamento de candidíases.

2 OBJETIVO

Como descrito anteriormente, espécies do gênero *Candida* são comensais do trato gastrintestinal e genital de indivíduos saudáveis. Contudo, esses microrganismos podem ser responsáveis por uma variedade de quadros clínicos no homem, desde infecções superficiais de mucosa até infecções sistêmicas principalmente em indivíduos imunologicamente debilitados. Particularmente em indivíduos HIV positivos a COF continua sendo a infecção fúngica oportunista mais prevalente. Embora *C. albicans* seja a mais frequentemente isolada de amostras clínicas, outras espécies estão significativamente emergindo, entre elas *C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis* e *C. tropicalis*. Associado a essa mudança epidemiológica, o prognóstico das candidíases tem sido dificultado pela eficácia reduzida dos fármacos comercialmente disponíveis tanto em células planctônicas quanto na presença de biofilme, e pelo desenvolvimento de resistência microbiana.

Neste sentido, a identificação e a determinação do perfil de sensibilidade aos antifúngicos é uma prática importante para um tratamento seguro e eficaz das candidíases. O estudo de fatores de virulência pode contribuir tanto no conhecimento da patogênese da COF, quanto no desenvolvimento de estratégias para combate às infecções por *Candida* spp. em indivíduos HIV positivos. Além disso, devido aos isolados resistentes aos antifúngicos comumente encontrados, faz-se necessário a procura de novas substâncias com maior atividade sobre formas planctônicas e sésseis e menor toxicidade para células hospedeiras. Em vista disso, o objetivo principal desse trabalho foi:

Identificar e caracterizar leveduras do gênero *Candida* isoladas da cavidade bucal de pacientes portadores do HIV quanto a expressão de fatores de virulência e sensibilidade às substâncias antifúngicas.

Os objetivos específicos foram:

- Isolar leveduras da cavidade bucal de indivíduos portadores do HIV.
- Identificar as leveduras por métodos fenotípicos padrões e métodos moleculares;
- Determinar o perfil de sensibilidade ao fluconazol e nistatina pelo método da

microdiluição em caldo;

- Avaliar a atividade proteolítica, produção de fosfolipase e atividade hemolítica pelo método em placa;
- Determinar a hidrofobicidade da superfície celular;
- Avaliar a capacidade de formar biofilme sobre superfície de poliestireno;
- Avaliar a expressão de transcritos dos genes *SAP*s e correlacionar com a utilização do tratamento antirretroviral associado aos inibidores de protease;
- Avaliar o efeito do eugenol sobre a capacidade de adesão em células HEp-2, hidrofobicidade da superfície celular e formação de biofilme em poliestireno e próteses bucais de *C. dubliniensis* e *C. tropicalis*.
- Avaliar a expressão relativa de transcritos dos genes *ALS*s entre os isolados tratados ou não com eugenol.

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4 ARTIGO 1

Candida isolated from oral mucosa of HIV-infected patients, antifungal susceptibility and putative virulence factors.

Suelen Balero de Paula[1], Alexandre Tadachi Morey[1], Jussevânia Pereira Santos[1], Gilselena Kerbauy[2], Ester de Melo Sena[2], Luiz Toshio Ueda[3], Lucy Megumi Yamauchi[1] & Sueli Fumie Yamada-Ogatta[1]*

Note: In this article, the term *Candida* species is used to refer to species of *Candida* other than *Candida albicans*.

ABSTRACT

Oropharyngeal candidasis remains the most common opportunistic fungal infection in individuals HIV-infected. The variable susceptibility and the emergence of species and/or isolates resistant isolates of these species reinforce the need for a specific microbiological diagnosis of the infections. Thus, the objective of this study was to identify and characterize yeasts isolated from the oral cavity of HIV positive patients in terms of: susceptibility to nystatin and fluconazole and expression of virulence factors. Oral rinse samples of 244 HIV-patients were used in this work. Of the total of patients, 50.00% (122) were colonized by *Candida* spp., and 11 patients had co-colonization by two species. *Candida albicans* was the most prevalent, accounting for 74.1% (100) of the isolates. Other species were isolated from 35 (25.9%) patients, 27 (20.00%) *C. glabrata*, 2 (1.50%) *C. krusei*, 3 (2.20%) *C. tropicalis* and 3 (2.20%) *C. dubliniensis*. Most isolates were considered sensitive (59.26%) to fluconazole, 22.96% and 18.52% of the isolates were considered susceptible dose-dependent and resistant to fluconazole, respectively. Most isolates showed MIC between 2 and 4 µg/mL and 14.15% were between 8 and 128 µg/mL for nystatin. The average for cell surface hidrophobicity (CSH) assayed by biphasic using xylene as a hydrocarbon was 63.47 ± 21.48 and most of the isolates (54.81%) were classified as highly hydrophobic. All isolates were able to form biofilm on polystyrene surface, however *C. tropicalis* was the highest biofilm producer followed by *C. albicans*. Five isolates not showed proteolytic activity in the

[1] Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil.
[2] Departamento de Enfermagem, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[3] Ambulatório de Infectologia, Centro Integrado de Doenças Infecciosas, Instituto de Saúde do Estado do Paraná, Londrina, Paraná, Brazil.
* Corresponding author. Tel: +55-43-3371-4297; fax: +55-43-3371-4192.
E-mail address: ogatta@uel.br (S.F. Yamada-Ogatta)

presence of BSA, however 48.89%, 36.29% and 11.11% had high, intermediate and low protease activity, respectively. Only 36.29% of the isolates showed extracellular phospholipase activity in the presence of egg yolk as substrate. The complete hemolysis of sheep blood was observed in only 17.04% of the isolates. The results corroborate the importance of identification and analysis of the sensitivity profile to antifungal agents for planning appropriate therapy for each patient. Furthermore, noting that the isolates colonizing these patients express various virulence factors may colonize and cause infections in these patients already debilitated.

Introduction

Species of *Candida* are comensal microrganism of the gastrointestinal and reproductive tracts in healthy individuals. However they are one of the most frequent causative agents of opportunistic infection in immunocompromised individuals, responsible for superficial and systemic illness (Soll, 2002). Colonization of skin and mucous membrane with *Candida* spp. is one important risk factor that predispose to candidiasis (Méan *et al*, 2008).

In patients infected with human immunodeficiency virus (HIV-positive) the asymptomatic colonization of oral mucous can reach approximately 75% of individuals, and can achieve 88% when more than one sample was collected (Vargas; Joly. 2002; Delgado *et al,* 2009). This rate is significantly higher when compared with the colonization of HIV-negative patients where 50 to 60% of the individuals can be colonized by *Candida* spp. (Fidel, 2006; Luque *et al,* 2009). In fact, the oropharyngeal candidiasis (OPC) is the most common opportunistic infection in these immunocompromised patients (Fidel *et al,* 2006, Brito *et al,* 2010) and often recurs (Gianini; Shetty, 2011). It can occur at any stage during the course of HIV infection and is considered as an important marker of Acquired Immunodeficiency Syndrome (AIDS) and its progression, which is characterized by the increased viral load and decreased CD4+ lymphocyte count (de Repentigny *et al,* 2004; Mercante *et al,* 2006; Leão *et al,* 2009). In Brazil is estimated that *Candida* spp. is responsible for 30% of deaths in HIV positive patients due to fungal infections (Prado *et al,* 2009).

Although *Candida albicans* continues to be the most common etiologic agent of candidiasis, several studies have showed changes in epidemiology of these infections toward other species of *Candida* (Luque *et al*, 2009). The reasons for this

epidemiological change are unclear, but this is probably due to the increasing use of azole agents. The clinical importance of this change concern about the emergence of isolates of *Candida* less susceptible or resistant to fluconazole, an azole antifungal, currently used for the treatment of candidiasis (Horn *et al*, 2009).

In contrast to *C. albicans*, little is known about the virulence factors of *Candida* species. In this context, various traits of *C. albicans* have been considered as putative factors of virulence and infection development, such as adherence to host tissues, yeast-hyphal reversible transition (morphogenesis), phenotypic switching and biofilm formation. In addition, this microorganism secretes proteases and phospholipases that contribute to fungal pathogenicity (Price *et al,* 1982; De Bernardis *et al*, 1990; Cutler, 1991; Hube *et al,* 1998; Senet, 1998; Ghannoum, 2000; Calderone, Fonzi, 2001; Haynes *et al,* 2001; Hube; Naglik, 2001; Douglas, 2003; Naglik *et al,* 2003; Ozkan *et al,* 2005; Ramage *et al,* 2005; Oksuz *et al*, 2007; Vinitha; Ballal, 2008; Chai *et al,* 2010; Mohandas; Ballal, 2011; Negri *et al*, 2011).

In the study described here, the yeasts were isolated from the oral cavity of HIV-infected individuals with no clinical evidence of candidiasis. All isolates were identified and tested for protease and phospholipase production, hemolytic activity, cell surface hidrofobicicty and biofilm formation. Moreover the *in vitro* susceptibility pattern of the isolates to fluconazole and nystatin was evaluated. The results may contribute for the knowledge of the pathogenesis of OPC.

Material and Methods

Study population

Two-hundred forty-four HIV-infected patients attended at the Centro de Referência Dr. Bruno Piancastelli Filho in Londrina from January to April of 2010 were enrolled in this study. This is the major reference center for the management of HIV-related infections in the north of Paraná State in Brazil. All of the patients signed an informed consent form to participate in this study. The study protocol was in accordance with the Ethics Committee of the Universidade Estadual de Londrina (CONEP n^{o}. 036/10). They

were evaluated regularly by experienced infectious disease physicians. During specimen collection, none was taking antifungal agents and presenting with clinical evidence of oral candidiasis. The most recent $CD4^+$ T lymphocyte count and the antiretroviral therapy prescribed were obtained.

Isolate identification

Samples of the patients were obtained by oral rinse method according to Samaranayake *et al*, (1986), with some modifications. Patients did a rinse with 10 mL of sterile distilled water for 30 seconds and spit it back in a sterile container. Each sample was centrifuged at 2700 g, the supernatant was discarded and the pellet was further suspended in 1.0 mL of 50 mmol l^{-1} sodium phosphate buffer pH 7.4 containing 0.15 mol l^{-1} NaCl (PBS). A 100-μl sample was spread on Sabouraud dextrose (SD) agar supplemented with 50 μg/ml of chloramphenicol. The cultures were incubated at 37 ^{o}C for at least 7 days under aerobic conditions and colonies were counted. Results were expressed as numbers of colony-forming units (CFU) ± standard error per milliliter. The samples were also cultured on CHROMagar Candida® medium (Probac, Brazil) for differential growth analysis. For each positive sample, colonies were examined microscopically after Gram staining, and two colonies of each sample were sub-cultured in SD agar for identification. The identity of each yeast isolate was confirmed by standard mycological methods (Kurtzman; Fell, 1998), such as germ-tube test, production of chlamydoconidia on cornmeal agar with 0.5 % Tween 80, and sugar assimilation and fermentation. Concomitantly, species identification was confirmed by a PCR-based methods (Table 1) (Donnelly *et al*, 1999; Milde *et al*, 2000; Ahmad *et al*, 2002).

Antifungal susceptibility testing

The minimum inhibitory concentration of fluconazole (Pfizer Central Research, United Kingdom) and nystatin (Sigma) for all isolates was determined by the broth microdilution assay for yeasts according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The reference strain *Candida parapsilosis* ATCC 22019 was included in each experiment as quality control. Two wells of each plate served as growth and

sterility controls. The interpretative criteria for susceptibility to fluconazole were those published in the CLSI (2008). For nystatin, the minimal inhibitory concentration (MIC) was defined as the value in which 100% growth inhibition was observed (Wingenter *et al*, 2007).

Determination of protease, phospholipase and hemolytic activities

Enzyme activity was assayed on agar plates containing 0.1% bovine serum albumin (BSA) and 4.0% egg yolk as protease and phospholipase substrate, respectively. For protease activity, the yeast isolates were previously cultured at 37 ^{o}C for 18 h in Yeast Carbon Broth (YCB) medium supplemented with 0.1% BSA pH 4.0, to induce the secretion of enzyme. To determine phospholipase activity, cell suspension was obtained from a 24-h SDB-yeast culture and the assay was carried out in SDA supplemented with 4% egg yolk, 350 μmol l^{-1} NaCl and 6.5 μmol l^{-1} $CaCl_2$, pH 4.5. For both assays, cells were counted in a hemocytometer (Neubauer Improved Chamber), and a 10-μl suspension of 10^6 yeasts was placed on the surface of the agar medium. The cultures were incubated at 37 ^{o}C for 96 h, after which the diameter of the degradation or precipitation zone around the colony was determined. Enzyme activity was determined by calculating the ratio between colony diameter and colony diameter plus degradation/precipitation zone (Dz/Pz values of 1 indicate no detectable protease or phospholipase activity, respectively). The isolates were classified according to Price *et al* (1982). Hemolytic activity of the yeasts was determined according to Luo *et al* (2001). The cells were spotted on the surface of SDA medium supplemented with 3% glucose (Sigma, USA) and 5% fresh sheep blood, pH 5.6. The plates were incubated at 37 ^{o}C in 5% CO_2 for 48 h. Hemolytic activity was indicated by a translucent halo around the inoculum site. Each isolate was tested in triplicate, and the experiments were carried out on three different occasions.

Cell surface hydrophobicity determination

The hydrophobicity of the yeasts was determined according to Anil *et al* (2002). Briefly, each *Candida* isolate was cultured at 37 °C for 24 h in SD broth. The yeasts were then harvested by centrifugation and washed twice, and the cell density was then

adjusted to 0.5 Mc Farland scale in 5 ml of PBS. A volume of 1.5 ml of this yeast suspension was added to two sterile glass tubes, and 0.5 ml of xylene (Merck, Brazil) was added to these test tubes, the other 2 ml was not added xilol and this tube was considered de control tube. Following 10 min preincubation in a water bath at 37 ^{o}C, the test tube was vigorously mixed for 1 min, and the incubation was allowed to proceed for an additional 30 min under the same conditions. The aqueous phase was carefully removed, and the absorbance was determined at 520 nm. The cell surface hydrophobicity (CSH) was expressed as the percentage decrease in optical density of the aqueous phase of the test as compared with the control [$(OD_{control}/OD_{test})/OD_{control}$]. Where the greater the change in absorbance of the aqueous phase, the more hydrophobic the yeast sample. Each assay was performed on three separate occasions with triplicate determinations each time.

Biofilm production assay

Determination of biofilm production was performed in polystyrene, flat-bottomed 96-well microtiter plates (Techno Plastic Products, Switzerland) using the procedure described in Bizerra *et al* (2008). In brief, the yeast isolates were cultured at 37 ^{o}C for 24 h in SD broth, and the cells were counted. A 20-μl SD broth suspension of 6 x 10^5 yeasts was placed into each well containing 180 μl of SD broth. The plates were incubated at 35 ^{o}C for 24 h and washed once with sterile distilled water. Approximately 100 μl of XTT-menadione were added and after 2h of incubation at 37°C was made spectrophotometric readings at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments, USA). Experiments were carried out in triplicate on three different occasions.

Statistical analysis

The results were evaluated by Student's t-test using the software GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA). The *p* values less than 0.05 were considered significant.

Results

Patients and yeast identification

A total of 244 HIV-infected patients were enrolled in this study. Of these, 122 (50.00%) were colonized by yeasts. Eleven patients were colonized by two species of *Candida* spp. The age of the HIV-positive patients ranged from 17 to 78 years (median 40.36 years old) and the majority of them were men (65.8 %). Sexual transmission was the most prevalent pathway of HIV contamination. The viral load ranged by < 50 to > 500.000, of these, 150 patients (61.47 %) showed the viral load below fifty. The median of $CD4^+$ and CD8+ lymphocyte counts were 512.88 cells/mm^3 and 1090.08 cells/mm^3, respectively. Previous use of fluconazole and nystatin during the 6 months before sampling were reported by 88 and 37 patients, respectively. A hundred fifty four patients (63.11 %) were treated with HAART combined with a viral protease inhibitor.

We found a high prevalence of *C. albicans* isolates (100 isolates; 74.08 %) in this study. *Candida* species were isolated from 35 patients (25.92 %), 27 were identified as *C. glabrata* (20.00 %),two as *C. krusei* (1.48 %), three as *C. tropicalis* (2.22 %) and three as *C. dubliniensis* (2.22%).

Fluconazole and nystatin susceptibility pattern

According to the CLSI breakpoints, the great majority of the isolates (59.26%) was considered susceptible (MIC ≤ 8 μg/ml) to fluconazole. Twenty four (17.78 %) isolates was resistant to fluconazole (MIC ≥ 64 μg/ml) of these 13 *C. albicans*, 10 *C. glabrata* and one *C. tropicalis.* Thirty-one isolates were classified as susceptible dose-dependent yeasts (8 ≤ MIC ≥ 32 μg/ml), of these 22 *C. albicans,* seven *C. glabrata* and one isolate each of *C. krusei* and *C. tropicalis*.

According Wingenter *et al* (2007), 86.7% (117) of the isolates were considered susceptible (MIC ≤ 4 μg/ml) to nystatin, and the presence of resistance to nystatin (MIC ≥ 64 μg/ml) was observed only in 3.7% (5) of the total of samples (three *C. albicans* and one isolate each of *C. glabrata* and *C. krusei*, and is important to emphasize that these

five isolates showed MIC value ≥ 128 μg/mL.

Enzyme activities and hemolytic activity

The majority of the isolates (96.29%) showed the protease activity on agar plate assay using BSA as substrate (Table 2). A large variation in protease activity was found among different isolates of each species, but no significant differences were observed between the species. However, a significant correlation ($p < 0.05$) was observed among the protease activity and the type of antiretroviral therapy, since isolates from patients teated with protease inhibitors showed lower protease activity.

Among isolates protease producers (130), 48.89% and 36.29% were classified as strong and moderately producers of extracellular proteases. Only a few isolates of *C. albicans*, *C. glabrata* and *C. tropicalis* were classified as poor producers of proteases, accounting for 11.11% of the total of isolates.

About phospholipase activity, in (86) 63.70% of *Candida* spp. was not observed the formation of a halo of precipitation around the colony, indicating that the isolates were negative for the production of phospholipase (Table 2). Only one isolate (0.74%) demonstrated a medium phospholipase activity and (48) 35.56% was considered as a low producer of phospholipase.

For the hemolytic activity, the majority (82.96%) presented partial hemolysis and formation of a green halo around the colony. Only in 23 isolates (17.04%) was observed the presence of complete hemolysis.

Cell surface hidrofobicity

The CSH was determined by measuring the difference in optical density of the aqueous phase between test and control. Thus, the greater the difference in absorbance greater cell hydrophobicity. The mean relative CSH was 63.47 ± 21.48 ranging from 4.22 ± 0.15 and 85.83 ± 0.42. Most of the isolates (54.81%) were classified as highly hydrophobic, 40.00% as moderately hydrophobic and 5.19% as weakly hydrophobic. It is noteworthy that all isolates of *C. tropicalis* were classified as highly hydrophobic.

Biofilm formation

The biofilm formation on the surface of polystyrene was monitored by the method of reduction of XTT. High metabolic activity of biofilm was observed after 24 h of incubation, indicating the capacity of biofilm formation on this surface. The mean value obtained by reading at 490 nm was 0.8157 ± 0.3723, ranging from 0.0245 ± 0.0026 to 1.700 ± 0.0037. Higher values in absorbance were observed in isolates of *C. albicans* and *C. tropicalis,* indicating higher cell density and consequently higher biofilm formation. *C. krusei* isolates showed lower values indicating the minor capacity of biofilm formation.

Discussion

In this study 50.00% of the total of patients was colonized with *Candida* species in the oral cavity of HIV-positive individuals, which is in accordance with the results of others. The isolation rate of *Candida* spp. around the world may range from 40 to 100% in these patients presenting oral candidiasis (Baradkar *et al,* 2009; Domaneshi *et al*, 2010; Mane *et al,* 2010). The species distribution of the *Candida* isolates from the oral cavity of HIV-infected patients examined in this study was similar to that reported by other authors, which *C. albicans* is the predominant isolate and among the *Candida* species, *C. glabrata* was the most frequently encountered (Baradkar *et al,* 1999; Sobel *et al,* 2001; Yang *et al,* 2003; Pfaller *et al*, 2006; Hamza *et al*, 2008; Pomarico *et al,* 2009; Yang *et al,* 2010; Agwu *et al*, 2011; Panel *et al,* 2011). The co-colonization (7.37%) by *C. albicans* and *C. glabrata* observed in this study was also reported by other authors (Sobel *et al,* 2001; Yang *et al*, 2003; Hamza *et al,* 2008; Nace *et al*, 2009).

Fluconazole has been established as a first-line antifungal for the treatment of oral candidiasis in HIV-patients. However, the continuous and indiscriminated use of this antifungal may result in colonization with species less susceptible to fluconazole and it can explain the increase of *Candida* species colonizing these patients (Sobel *et al,* 2001; Horn *et al,* 2006; Hamza *et al*, 2008; Tan *et al,* 2009). Overall, *C. albicans* isolates from HIV-infected patients have been found to be susceptible to this drug. However, isolates of *C. albicans* and non-*C. albicans* azole-resistant have been reported recently (Badiee *et al,* 2010; Kotwal *et al,*

2011; Negri *et al*, 2011). In addition, azole cross-resistance has been found among *Candida* isolates fluconazole resistant (Marcos-Arias *et al,* 2011; Yan *et al,* 2011).

All *C. dubliniensis* isolated in this study were classified as susceptible and this result is compatible with other studies. Although the inducibility of azole resistance *in vitro* has been reported, the great majority of *C. dubliniensis* isolates are susceptible to fluconazole (Martinez *et al*, 2002; Badiee *et al*, 2010; Pfaller *et al,* 2010; Yang *et al*, 2010; Nweze *et al*, 2011).

It has been showed that the azole resistance can be induced in *Candida* species. In addition some *Candida* species are less susceptibility or intrinsically resistant to fluconazole, especially *C. glabrata* and *C. krusei*, respectively (Brito *et al*, 2010). Ozhakk-Bavsan *et al* (2011) observed that 9% of the total of isolates, including five isolates of *C. krusei* and four of *C. glabrata*, were considered susceptible dose-dependent. Two isolates of *C. glabrata* and one of *C. krusei* were considered resistant to fluconazole. These results are similar to those obtained in the present study.

Several species of the genus *Candida* spp. have the ability to secrete aspartyl proteases *in vitro* (Parra-Ortega *et al,* 2009; da Costa *et al,* 2011; Negri *et al,* 2011). Studies show that Saps may perform different functions including: digesting molecules for nutrition (MacDonald, 1984; Kvaal *et al,* 1999), participation in the process of adherence to host tissue (Watts *et al,* 1998; Borg-von Zepelin *et al,* 1999; Kontoyiannis *et al,* 2001), invasion and degradation of tissues and organs (Morschhauser *et al,* 1997; Schaller *et al,* 1999; 2000; 2003; Zaugg *et al,* 2001; Felker *et al,* 2002; Villar *et al,* 2007), evasion of host defenses by damaging cells and molecules of the immune system (Ruchel, 1986; Kaminishi *et al,* 1995; Borg-von Zepelin *et al,* 1998; Meiller *et al,* 2009) or resistance to phagocytosis by macrophages (Borg-von Zepelin *et al,* 1998) and formation of hyphae and "switching" phenotype (Naglik *et al,* 2003).

In relation to *Candida* spp. isolated from patients with HIV, in general, it was observed that these yeasts showed higher proteolytic activity compared with isolates of patients HIV-negative (Ollert *et al,* 1995; De Bernardis *et al,* 1996, 1999; Migliorati *et al,* 2004, Ribeiro *et al,* 2004; Mane *et al,* 2011). In HIV-positive patients, it was shown that the possible effects of virus infection, such as changes in host tissue (Qureshi *et al,* 1995), reduced salivary flow (Schiodt, 1992), changes in the microbiota (Rams *et al,* 1991) and even

possible interactions between viruses and yeasts (Gruber *et al,* 1998) may cause changes in the pattern of secretion of protease and contribute to the selection of more virulent strains (Sweet, 1997).

Phospholipases are a heterogeneous group of proteins which are involved in many biological events including phospholipid turnover and signal transduction in eukaryotic cells. All types of phospholipases (A, B, C and D) have been reported in *C. albicans*, but only phospholipase B has hydrolase and lysophospholipase–transacylase activities detected extracellularly. The hydrolytic activity of phospholipases yields fatty acids and lysophospholipids from membrane phospholipids leading to cell lysis and host damage (Ghannoum, 2000). Indeed, there are several sources of evidence correlating phospholipases with the virulence of *C. albicans* (Ibrahim *et al*, 1995; Mukherjee *et al*, 2001).

Samaranayake *et al* (2005) analyzed the correlation between the activity of phospholipase and germ-tube formation, cell surface hydrofobicity, adherence to oral epithelial cells and hemolytic activity of *C. albicans* isolated from HIV-positive patients. High phospholipase activity was observed in these isolates, however there was no correlation with other parameters. Ribeiro *et al* (2004) showed that *Candida* spp. isolated from both the oral and the vaginal mucosa of HIV-infected patients had higher phospholipase activity than healthy women.

Hemolysins are produced by many different species of microorganisms. There is essentially no free iron in the human host, therefore, microorganisms can use these enzymes to acquire this nutrient from iron-containing compounds, such as hemoglobin. Prominent representatives of this enzyme family are streptococcal β-hemolysins. These enzymes act by forming pores in the cytoplasmic membrane of erythrocytes and a wide range of host cells, contributing to tissue damage during infection (Nizet, 2002). Several *Candida* species can produce one or more types of hemolysins on the surface of blood-enriched agar medium (Manns *et al*, 1994; Luo *et al*, 2001; Samaranayake *et al*, 2005), and *C. albicans* can use iron contained in hemoglobin for growth (Manns *et al*, 1994). The importance of this effect in the pathogenesis of *Candida* is still unclear, and further studies are needed to investigate the nature of the hemolytic factor and its role as a putative virulence factor.

Another characteristic of the *Candida* spp. strongly associated with

candidiasis is the ability to form biofilm (Douglas, 2003). The accession of these yeasts to a surface can trigger various responses including, invasion (due to formation of hyphae) or production of biofilm (Kumamoto; Vinces, 2005; Negri *et al*, 2011). Several specific factors (fungal adhesions and receptors on the host) are involved in the adhesion of the fungus to biotic or abiotic surfaces (Calderone, Braun 1991, Sundstrom 2002; Tronchin *et al,* 2008)

The formation of biofilms by *C. albicans* has been demonstrated on a number of non-biological surfaces, including medical devices (Ramage *et al*, 2006). It has also been reported that *Candida* species can form extensive biofilms *in vitro* on the surface of different types of catheters (Hawser; Douglas, 1994; Bizerra *et al*, 2008) and polystyrene (Shin *et al*, 2002; Bizerra et al., 2008). However, Jin *et al* (2003) suggested that the capacity to form biofilm does not appear to contribute to the high levels of colonization by *C. albicans* from the oral cavity of HIV-positive patients. These authors showed that there was no significant difference in the capacity to form biofilm in polystyrene plates by samples of *C. albicans* isolates from patients infected with HIV compared to non-infected patients.

The knowledge of virulence factors of fungi and their inhibitors may contribute for the development of potential antifungals, further studies are warranted to determine the virulence factors of *Candida* species, and such investigations are currently underway in our laboratory.

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Table 1 - Sequence of primers utilized for molecular identification.

Oligonucleotídeo	**Seqüência 5' → 3'**
CTSF	TCGCATCGAT GAAGAACGCAGC
CTSR	TCTTTTCCTCCGCTTAT TGATATGC
CADET	ATTGCTTGCGGCGGTAACGTCC
CPDET	TCTTTTCCTCCGCTTAT TGATATGC
CGDET	TAGGTTTTACCAAC TCGGTGTT
CTDET	ATTTTGCTAGTGGCC
DUBF	GTATTTGTCGTTCCCCTTTC
DUBR	GTGTTGTGTGCACTAACGTC
KRUS1	GGTTGACACTTCGCATA
KRUS2	CGTATGTGACCAGTGA

5 ARTIGO 2

Title: Effect of eugenol on cell surface hydrophobicity, adhesion, biofilm and *ALS* gene expression of *Candida tropicalis* and *Candida dubliniensis* isolated from oral cavity of HIV-infected patients

Eugenol inhibits the adherence properties and biofilm of *Candida dubliniensis* and *Candida tropicalis* isolated from oral cavity of HIV-infected patients

Suelen Balero de Paula[1], Thais Fernanda Bartelli[1], Vanessa Di Raimo[1], Jussevania Pereira Santos[1], Alexandre Tadachi Morey[1], Celso Vataru Nakamura[2], Lucy Megumi Yamauchi[1], Sueli Fumie Yamada-Ogatta[1,*]

ABSTRACT

Background: *Candida* spp. is comensal of the human mucosal. Adherence of these microorganisms to host cells is the first and crucial event for initial colonization or establishment of infection. The aim of this study was evaluate the effect of eugenol on the adherence of *Candida dubliniensis* and *Candida tropicalis*.
Methods: The ability of adhesion to HEp-2, cell surface hydrophobicity, biofilm formation on polystyrene and denture and the relative expression of *ALS* using Real-time-PCR between samples treated or not with eugenol was evaluated of 3 isolates of each species enrolled in this work.
Results: All isolates were capable to form biofilms on different substrates surface. Eugenol showed activity against planktonic and sessile cells of *Candida* species, including inhibitory activity formation and against mature biofilms of these species. No metabolic activity in biofilm was detected after 24 h of the treatment. By scanning electron microscopy, it can be observed that eugenol reduced drastically the amount of sessile cells of both species on the denture materials surfaces. Except for *C. dubliniensis* 219, all *Candida* species showed hydrophobic behavior as determined by the biphasic hydrocarbon/aqueous method, and the mean relative CSH was 60.82 ± 20.79 ranging from 29.48 ± 2.97 to 84.59 ± 4.32. Except for *C. tropicalis* 176, a significant difference ($p < 0.005$) in CSH of *Candida* species was observed after exposure of planktonic cells to eugenol for 1 h. Eugenol also caused a

[1] Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Microbiologia, Londrina, Paraná, Brazil.
[2] Universidade Estadual de Maringá, Centro de Ciências da Saúde, Departamento de Ciências Básicas da Saúde, Maringá, Paraná, Brazil.
* Corresponding author: Sueli Fumie Yamada-Ogatta, Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Microbiologia. Rodovia Celso Garcia Cid, PR 445, km 380. CEP 86055-900. Tel: +55-43-3371-4297; fax: +55-43-3371-4788.
e-mail: ogatta@uel.br

significant reduction on adhesion of most *Candida* species to HEp-2 cells ($p < 0.005$) and to polystyrene ($p < 0.05$). There was no significant difference in the adhesion percentage of the isolate 176 of *C. tropicalis* to both surfaces, although it had shown a reduction of 20% of adhered cells to mammalian cells after eugenol exposure. For eugenol-treated planktonic cells of most *Candida* spp. the relative quantification of *ALS* genes expression displayed significant ($p < 0.005$) downregulation compared to untreated cells.
Conclusion: The eugenol was capable to changes the CSH, adhesion capacity and *ALS* gene expression of planktonic cells of *C. dubliniensis* and *C. tropicalis*. In addition, this phenylpropanoid compound inhibited the biofilm formation and mature biofilm formed on polystyrene and denture materials of both *Candida* species.

Introduction

The prevalence of oral colonization by *Candida* spp. can vary among different population groups (Kumamoto and Vinces, 2005), and the presence of these fungi as commensal of human microflora is one important predisposing factor for candidosis (Pfaller; Diekema 2007). Adherence of the microorganisms to host cells and tissues is the first event required for initial colonization or establishment of infection. Moreover, the microbial surface contact can trigger various cellular behaviors, including biofilm formation (Kumamoto and Vinces, 2005), which is also strongly associated with candidosis (Rautemaa and Ramage, 2011).

Biofilms can be defined as irreversibly surface-attached communities of cells (sessile cells) embedded in a self-produced exopolymeric matrix and displaying a distinctive phenotype compared to their free-floating (planktonic cells) counterparts (Donlan and Costerton, 2002). Remarkably, sessile cells are less susceptible to a variety of antifungal agents (Hawser and Douglas, 1995; Ramage *et al.*, 2001; Bizerra *et al*., 2008; Katragkou *et al*., 2008; Melo *et al*., 2010) and to host defenses (Katragkou *et al*., 2010). Thereby, biofilms are difficult to eradicate, representing a source of re-infections. In these contexts, new antifungal agents are urgently needed, particularly those with anti-biofilm activities, for effective management of *Candida* spp. infections.

Several researchers have been showed the anti-*Candida* biofilm potential of plant derived compounds such as flavonoids (Cao *et al*., 2008) and essential oils (Alviano *et al*., 2005; He *et al*., 2007; Braga *et al.*, 2008; Rukayadi *et al*., 2010; Khan and Ahmad, 2011). Eugenol is the main active phenylpropanoid component of essential oil from many aromatic plants (De Vincenzi *et al*., 2000). The inhibitory effect of eugenol alone (Nakamura *et al*.,

2004; Chami *et al*., 2005; Pinto *et al*., 2009; Ahmad *et al*., 2010a; Marcos-Arias *et al*., 2011) and in combination with fluconazole and amphotericin B (Khan *et al*., 2012) against planktonic cells of *Candida* spp. has been previously reported. In addition, eugenol can interfere with initial phases of biofilm formation as well as with mature biofilm of *Candida albicans* (He *et al.,* 2007; Khan and Ahmad, 2011).

Although *C. albicans* continues to be the most common causative agent of candidoses in humans, other species of *Candida* have become a significant cause of such infections (Loreto *et al*., 2010; Nucci *et al*., 2010; Pfaller *et al*., 2011). *Candida tropicalis* and *Candida dubliniensis* have been regarded as high biofilm producer and sessile cells within this community have been showed to be resistant to antifungal agents (Ramage *et al*., 2001; Bizerra *et al*., 2008; Melo *et al*., 2010). In this context, here we analyzed the effect of eugenol on cell surface hydrophobicity, adhesion to human epithelial cells and polystyrene, and *ALS* expression of planktonic cells of these species. Moreover, the inhibitory activity on biofilm formed on polystyrene and denture materials was also analyzed.

Materials and Methods

Candida spp. isolates and growth conditions

Candida species used in this study included three *C. dubliniensis* (131, 219, 248) and three *C. tropicalis* (23, 150, 176) isolated from the oral cavity of HIV-infected patients. The species identification of oral isolates was carried out by standard mycological methods (Kurtzman and Fell, 1998; Sullivan *et al*., 1995). Species identification was confirmed by a PCR-based method using specific primers as described previously (Ahmad *et al*., 2002; Donnelly *et al*., 1999). *C. tropicalis* ATCC 28707 and *C. dubliniensis* ATCC MYA-646 reference strains were included as quality control. The yeasts were also maintained at -80 ^{o}C. The study protocol was in accordance with the Ethics Committee of the Universidade Estadual de Londrina (CEP/UEL n^{o}. 036/10).

Biofilm formation

Candida strains were cultured in SD broth and incubated at 37 °C for 18 h. The yeasts were harvested by centrifugation, washed twice with sterile PBS, and the cells were counted. A 20-μl SD broth suspension of 6 x 10^5 yeasts was placed into each well of flat-bottomed 96-well microtiter plates (Techno Plastic Products, Switzerland) containing 180 μl of SD broth. The plates were incubated at 37 ^{o}C 24 h. After the incubation time, the wells were washed once with sterile distilled water and the metabolic activity of the cells were quantified using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay. A 100-μl aliquot of XTT-menadione [0.1 mg/ml XTT, 1 μM menadione (Sigma Chemical Co, USA)] was added to each well, and the plates were incubated in the dark for 2 h at 37 ^{o}C. The XTT formazan product was measured at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments, USA) (Bizerra *et al*., 2008).

Antifungal susceptibility testing

The inhibitory activity of eugenol (SSWhite, Brazil) for planktonic cells of *Candida* isolates was determined by broth microdilution assays according to the Clinical and Laboratory Standards Institute (CLSI, 2008). Stock solution of eugenol was prepared in water containing 10% dimethylsulfoxide [DMSO, v/v (Sigma Chemical Co., USA)]. The DMSO final concentration in the assays did not exceed 1.0%. The substance was serially double-diluted concentrations in RPMI buffered with MOPS pH 7.0, ranging from 3,000 to 5.85 μg/ml of eugenol. Quality control *C. dubliniensis* ATCC MYA-646 and *C. tropicalis* ATCC 28707 and fluconazole (512.0 - 0.5 μg/ml, Pfizer Central Research, United Kingdom) were included in each experiment. Two wells of each plate served as growth and sterility controls. The minimum inhibitory concentrations (MIC) for fluconazole were determined at the 50% of inhibition of visual growth and for eugenol were determined at total inhibition of visual growth after 24 h incubation compared to untreated planktonic cells. To determine the minimal fungicidal concentration (MFC), a 20-μl aliquot from the wells showing no growth were aseptically transferred to SD agar plates and incubated at 37 ^{o}C for 48 h. The MFCs (Barchiesi *et al*, 2005; AAC, 49: 4989-4992) were determined as the lowest concentration of the eugenol leading fungal death. To determine antifungal susceptibilities of sessile cells,

biofilms were formed in polystyrene, as described above. After 1 h and 24 h of biofilm formation, the medium was aspirated off and each well was washed three times with sterile 0.15 M phosphate-buffered saline pH 7.2 (PBS). A 200-μl aliquot of RPMI 1640 medium containing serially double-diluted concentrations of eugenol and fluconazole was added and the plates were incubated further for 24 h at 37 ^{o}C. Controls included antifungal-free wells and biofilm-free wells. Sessile minimum inhibitory concentrations were determined at 100% inhibition ($SMIC_{100}$) and 50% inhibition ($SMIC_{50}$) compared to antifungal-free control wells using the XTT-reduction assay (Bizerra *et al*., 2008) for eugenol and fluconazole, respectively. To evaluate the time-dependent effect of eugenol, 24 h-biofilms of *Candida* species were formed in polystyrene and treated with $SMIC_{100}$ of the eugenol as described above. At determined time points (3, 6, 12 and 24 h), the metabolic activity of sessile cells was determined. All experiments were carried out in triplicate in three different occasions.

Cell surface hydrophobicity determination

The hydrophobicity of the untreated and eugenol-treated (½MIC for 1 h) planktonic cells was determined by the biphasic hydrocarbon/aqueous method according to Anil *et al*. (2001). The cell surface hydrophobicity (CSH) was expressed as the percentage decrease in optical density of the aqueous phase of the test as compared with the control $[(OD_{control}-OD_{test})/OD_{control}]$, where the greater the change in absorbance of the aqueous phase, the more hydrophobic the yeast sample. Each assay was performed on three separate occasions with triplicate determinations each time.

Adhesion of yeasts to HEp-2 cells and polystyrene

HEp-2 cells (human larynx epidermoid carcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B in a humidified 5% CO_2 atmosphere at 37 ^{o}C. For adhesion assays, HEp-2 cells were seeded in 24-well plates at 4.0 x 10^5 cells per well and incubated for 18 h. The medium was removed and each well was washed three times with sterile 0.15 M PBS. The

fresh culture medium minus the antimicrobials was added and the wells were inoculated with untreated and eugenol-treated *Candida* spp. (½MIC for 1 h) with approximately 2.0 x 10^6 yeasts, and the plates were incubated at 37 ^oC for 2 h in 5% CO_2 atmosphere. Non-adherent yeasts were removed by washing with sterile 0.15 M PBS. Adherent yeasts were harvested by treatment of the cell monolayers with 1 ml 0.5% (v/v) Triton X-100 (Sigma Chemical Co) for 10 min on ice. The viable yeasts were enumerated by dilution plating in SD agar. Experiments were carried out in duplicate on three different occasions. The percent adherence was calculated by the equation: % Adherence = (cfu_{120}/cfu_0) x 100, where cfu_{120} refers to adhered cells per ml after 2 h and cfu_0 the initial number of inoculated cells.

The adhesion on polystyrene surface was performed as described for biofilm formation with minor modifications. Briefly, untreated and eugenol-treated (½MIC for 1 h) planktonic cells were placed on each well and the plates were incubated for 2 h. The metabolic activity of adherent cells was determined using the XTT-reduction assay as described above.

Analysis of *ALS* gene expression

Real-time PCR was performed to determine the relative *ALSd1-2* and *ALSTt1-3* mRNA levels of *C. dubliniensis* and *C. tropicalis*, respectively, with *ACT1* used as a reference housekeeping gene to normalize the data. Yeast cells were cultured in SD broth at 35 ^oC for 24 h. The total RNAs were extracted using the RNAeasy kit (Qiagen Inc.) following the manufacturer's instruction and were treated with DNAse-RQ1 (Promega). The nucleotide sequences of *C. dubliniensis* and *C. tropicalis* genes deposited in the GenBank/EMBL databases were used for specific primer design with Primer Select software (DNASTAR Lasergene). The primer sequences and their features are shown in Table 1. Total RNA (1 μg) was converted to cDNA by incubation with oligo(dT) and M-MLV transcriptase reverse (Invitrogen) for 1 h at 42 ºC, according to the manufacturer's recommendation. Samples were purified by centrifugation through a Microcon YM-30 filter (Millipore). Two-step real-time RT-PCR assays were performed in a RotorGene 6000 (Corbett Life Science). 100 *n*g of cDNA and the recommended concentration of SYBR Green Master Mix (Invitrogen) were added to a 25 μl reaction mixture. The specific primers were added at a concentration of 200

*n*M in all cases. PCR conditions were as follows: 2 min at 50 ºC, 10 min at 95 ºC, followed by 45 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s. Only for *ALSd-2*, the primer annealing temperature was replaced to 58 ^{o}C. Thermal dissociation confirmed that RT-PCR generated a single amplicon.

Scanning electron microscopy (SEM)

Discs (diameter 0.8 cm) of polymethylmetacrylate (PMM, Orto Class,Clássico, Brazil) and ceramic (Noritake®, Shofu Dental Corp., USA) were aseptically placed in wells of 24-well tissue culture plates (Techno Plastic Products, Switzerland). A standard inoculum of 3.0 x 10^6 cells, from overnight cultures of the yeast strains, was prepared in 1 ml of RPMI 1640 pH 7.0 medium and used to form biofilm on these surfaces. The discs were then immersed in these cell suspensions and incubated statically at 37 ºC for 24 h. After this incubation, non-adherent organisms were removed by washing gently three times with PBS pH 7.2. One milliliter of RPMI containing eugenol ($SMIC_{100}$) was added and the plates were incubated further for 24 h. Biofilms formed on these strips were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature. Post-fixation, the cells were dehydrated with a series of ethanol washes (15, 30, 50, 70, 80, 90, 95 and 100%), critical-point dried in CO_2, coated with gold and examined with a SHIMADZU SS-550 scanning electron microscope.

Statistical analysis: The results were evaluated by Student's t-test using the software GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA). The *p* values less than 0.05 were considered significant.

RESULTS

Biofilm formation on abiotic surfaces

Biofilm formation by *Candida* species on polystyrene, PMM and ceramic was monitored using the XTT-reduction assay. All isolates were capable to form biofilms on

these different substrates surface after 24 h, as judged by the metabolic activity of sessile cells (Table 2). No significant differences on the metabolic activities were observed between the strains and isolates in each substrate analyzed. However, a significant difference ($p < 0.05$) was observed among the substrates, where the highest biofilm formation was detected on polystyrene surface, followed by PMM and ceramic. According, the mean OD_{490nm} (optical density at 490 nm ± standard deviation) value was 1.037 ± 0.089 for the polystyrene surface, whereas for PMM and ceramic were 0.776 ± 0.037 and 0.598 ± 0.051, respectively.

Antifungal activity against planktonic and sessile cells

The MICs, MFCs and SMICs of eugenol and fluconazole for the *Candida* spp. isolates and type strains are reported in Table 3. Planktonic cells of all isolates and the type strain of *C. dubliniensis* were susceptible to fluconazole. However, variations on fluconazole susceptibility were observed for *C. tropicalis*, where the type strain and isolate 176 were resistant, isolate 150 was susceptible dose-dependent, and isolate 23 was susceptible to fluconazole, considering the CLSI (2008) interpretative breakpoints. The biofilm of these *Candida* species exhibited an enhanced resistance to fluconazole. The $SMIC_{50}$ of this compound for all isolates and type strains were higher than 512 μg/ml. The MIC and MFC values of eugenol for *C. dubliniensis* and *C. tropicalis* planktonic cells ranged from 375 to 750 μg/ml and 750 to 1,500 μg/ml, respectively. Eugenol also exhibited an inhibitory effect against mature biofilms of *Candida* species that appeared to be dose-dependent (Fig. 1A). Over 80% reduction of metabolic activity of 24 h-sessile cells was observed at eugenol concentrations between 187.5 to 750 μg/ml. No metabolic activity was detected at concentrations ranging from 375 to 1,500 μg/ml, and these values were considered the $SMIC_{100}$. The inhibitory effect of eugenol against 24 h-sessile cells was also time-dependent (Fig. 1B). The metabolic activity reduction ranged from 11.1 to 31.6%, 76.6 to 85.5% and 90.6 to 93.5% after incubation in the presence of $SMIC_{100}$ eugenol for 3, 6 and 12 h, respectively. No metabolic activity was detected after 24 h of the treatment. Eugenol also interfered in biofilm formation, since treatment of 1 h-adherent cells resulted in dose-dependent reduction of their metabolic activity (data not shown). The $SMIC_{100}$ for 1h-adherent cells ranged from 375 to 750 μg/ml (Table 3).

Scanning electron microscopy of *Candida* species biofilm on denture materials

The effect of eugenol on *C. dubliniensis* (isolate 150) and *C. tropicalis* (isolate 219) biofilms formed on PMM and ceramic surfaces was monitored by SEM (Fig. 2 and Fig. 3). Mature biofilms of untreated cells of both *Candida* species consisted of a dense network of cells and minimal variations in their architecture among the species were observed. Biofilm of *C. tropicalis* has visually higher volume than those produced by *C. dubliniensis*, and it was composed of heterogeneous layer of yeast, pseudohyphae and hyphae (Fig. 3). In contrast, *C. dubliniensis* biofilm comprised a dense layer of filamentous forms and, to a lesser extent, yeast cells (Fig. 2). The treatment of biofilms with eugenol reduced drastically the amount of sessile cells of both species on the denture materials surfaces (Fig. 3). On the biofilm formed on ceramic treated with eugenol was observed a reduction of filamentation.

Effect of eugenol on cell surface hydrophobicity and adhesion to HEp-2 cells and polystyrene

To evaluate the effect of eugenol on CSH and, adhesion to mammalian cells and polystyrene features, planktonic cells of *Candida* species were exposed to subinhibitory (½ MIC) concentration of eugenol for 1 h before the assays. Except for *C. dubliniensis* 219, all *Candida* species showed hydrophobic behavior as determined by the biphasic hydrocarbon/aqueous method, and the mean relative CSH was 60.82 ± 20.79 ranging from 29.48 ± 2.97 to 84.59 ± 4.32. Except for *C. tropicalis* 176, a significant difference ($p < 0.005$) in CSH of *Candida* species was observed after exposure of planktonic cells to eugenol for 1 h (Table 4). It was observed a range of 42.3% to 75.1% reduction in the CSH of eugenol-treated cells as compared to untreated counterpart cells. Eugenol also provoked a significant reduction on adhesion of most *Candida* species to HEp-2 cells ($p < 0.005$) and to polystyrene ($p < 0.05$). There was no significant difference in the adhesion percentage of the isolate 176 of *C. tropicalis* to both surfaces, although it had shown a reduction of 20% of adhered cells to mammalian cells after eugenol exposure. For the other *Candida* isolates, it was observed a range of 46.9 to 68.9% and 27.4 to 67.8% of adhesion reduction to HEp-2 cells and

polystyrene, respectively.

Effect of eugenol on expression of *ALS* genes

The analysis of expression of all *Candida* spp. isolates revealed that *ALS* genes were overexpressed (≥ 2-fold change, p <0.005) compared with the reference gene *ACT1*. The isolates 23 of *C. tropicalis* and 248 of *C. dubliniensis* displayed the relatively high levels of *ALSt1-3* and *ALSd1-2* expression, respectively. The pattern of expression observed for *ALS* genes after eugenol treatment was similar between the *C. dubliniensis* (Fig. 4A) and *C. tropicalis* (Fig. 4B) isolates. For eugenol-treated planktonic cells of most *Candida* spp. the relative quantification of *ALS* genes expression displayed significant (p < 0.005) downregulation compared to untreated cells. There was no change in expression of *ALSt1* and *ALSt2* for isolate 176 of *C. tropicalis* after eugenol treatment.

Discussion

Eugenol has been widely used in medicine and dentistry due to its antiseptic, antimicrobial, anesthetic, analgesic, antioxidant, anti-inflammatory and cardiovascular properties (Pauli and Kubeczka, 2010; Pramod *et al*., 2010). This phenylpropanoid compound has been reported to have antimicrobial activity against planktonic cells of *C. albicans, C. dubliniensis, C. glabrata*, *C. guillermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* (Nakamura *et al*., 2004; Chami *et al*., 2005; Pinto *et al*., 2009; Ahmad *et al*., 2010a; Khan *et al*., 2011; Marcos-Arias *et al*., 2011). Moreover, this compound showed the *in vitro* synergy with fluconazole and amphotericin B against *C. albicans* (Khan and Ahmad, 2011; Khan *et al*., 2012). As reported previously (Chami *et al*., 2005; Pinto *et al*., 2009; Ahmad *et al*., 2010a; Marcos-Arias *et al*., 2011), our results showed that eugenol exhibit fungicidal activity against planktonic cells of *C. tropicalis*, including those classified as fluconazole-resistant and dose-dependent yeasts, and this effect was also observed for *C. dubliniensis*.

Previous studies reported in the literature have focused on showing the antibiofilm activity of eugenol against *C. albicans*. He *et al*. (2007) showed a dose-dependent

reduction of metabolic activity of 48 h-biofilm formed on polystyrene surface and treated with eugenol for further 48 h. In the presence of 500 μg/ml and 2,000 μg/ml of this compound, 50% ($SMIC_{50}$) and over 80% ($SMIC_{80}$) reduction were detected, respectively. Khan and Ahmad (2011) evaluated the effect of phytocompounds (eugenol, cinnamaldehyde, citral and geraniol) against 48 h-biofilm of *C. albicans*, and their results also showed a dose and time-dependent inhibitory activity of eugenol. The $SMIC_{80}$ after treatment with the compounds for 48 h ranged from 100 to 400 μg/ml. The results obtained in this study showed that eugenol displayed inhibitory activity against biofilms of *C. dubliniensis* and *C. tropicalis*, which not surprisingly exhibited enhanced resistance to fluconazole. This compound was capable to inhibit the biofilm formation as well the mature biofilms formed on polystyrene in a dose-dependent manner. SEM analysis further revealed the reduction of biofilm formed in denture materials (PMM and ceramic).

The mechanisms by which eugenol provokes the *Candida* spp. death are not completely understood. This compound caused profound changes in the morphology of planktonic cells and leakage of cytoplasmic constituents, indicating an action on the cell envelope (Nakamura *et al*., 2004; Chami *et al*., 2005). In fact, recent data showed that the fungicidal concentration of eugenol against *C. albicans* provoked a significant reduction on ergosterol content of the cell (Pinto *et al*., 2009; Ahmad *et al*., 2010a) and interfered with the H^+-ATPase activity (Ahmad *et al*., 2010b). In addition, the extensive damage of cell membrane (Pinto *et al*., 2009) may be attributed to oxidative stress mediated by reactive oxygen species (Khan *et al*., 2011).

Microbial adherence on the surface of the substrates is the initial event of biofilm formation, and the cell envelope mediates the first interaction between the microorganism and the environment. Adhesion of *Candida* spp. to different surfaces is a dynamic process, and can be mediated by hydrophobic and electrostatic forces (Klotz *et al*., 1985) and specific cell surface adhesins able to recognize the host ligands (Dranginis *et al*., 2007; Hoyer *et al*., 2008). The CSH, a nonspecific factor, is considered an important feature that contributes to adherence of *Candida* spp. on different surfaces (Klotz *et al*., 1985; Hazen *et al*., 1991; Blanco *et al*., 2010). Moreover, it has been showed that CSH of planktonic cells of *C. albicans* isolated from different origins correlates positively with biofilm formation on polystyrene capacity (Li *et al*., 2003; Blanco *et al*., 2010; Borghi *et al*., 2011). To examine the

effect of eugenol on CSH of *C. dubliniensis* and *C. tropicalis*, planktonic cells of both species were incubated previously with sub-inhibitory concentration (½ MIC) of the compound, for 1 h. The presence of ½ MIC eugenol induced a significant reduction on CSH and adhesion to polystyrene and HEp-2 cells of almost all planktonic cells of *C. tropicalis* and *C. dubliniensis*. It has been reported that *C. albicans* adhesion to polystyrene (Imbert *et al*., 2002) and epithelial cells was reduced after *in vitro* exposure to sub-inhibitory concentrations of fluconazole (Ellepola and Samaranayake, 1998), an antifungal that interferes with the ergosterol biosynthesis (Odds *et al*., 2003).

To further examine the effect of eugenol on *Candida* species adhesion properties, the expression of *ALS* (agglutinin-like sequence) genes was analyzed in the presence of ½ MIC eugenol. The *ALS* gene family of *C. albicans* encodes cell-surface glycoproteins implicated in adhesion on several surfaces (Liu and Filler, 2011; Wächtler *et al*., 2011). These genes are differentially expressed under a variety of conditions and between *C. albicans* strains (Dranginis *et al*., 2007; Hoyer *et al*., 2008). In addition, the participation of *ASL* genes during *C. albicans* biofilm formation has been showed by several authors (Garcia-Sánchez *et al*., 2004; Nobile *et al*., 2008; Liu and Filler, 2011). Although *ALS*-encoding genes have been identified in *C. dubliniensis* and *C. tropicalis* (Hoyer *et al*., 2001), no further studies have been analyzing the role of these proteins on their biology or pathogenesis. In this study, a significant downregulation of *ALS* gene expression was observed for almost all eugenol-treated planktonic cells. No significant changes in expression of *ALSt1* and *ALSt2* for isolate 176 of *C. tropicalis* were observed after eugenol treatment. Similarly, the presence of the compound did not result in changes in the adherence on polystyrene and HEp-2 cells of this isolate. Although these findings may suggest that ALS proteins contribute to adhesion of both *Candida* species, further studies are needed to understand this process.

In conclusion, the results obtained in this study showed that besides the fungicidal activity, eugenol was capable to changes the CSH, adhesion capacity and *ALS* gene expression of planktonic cells of *C. dubliniensis* and *C. tropicalis*. In addition, this phenylpropanoid compound inhibited the biofilm formation and mature biofilm formed on polystyrene and denture materials of both *Candida* species. These findings corroborate the effectiveness of eugenol against planktonic and sessile cells of *Candida* species other than *C. albicans* reinforcing the potential of this compound as antifungal. In this context, initial *in*

vivo studies have been showed the safety and efficacy of the topical use of eugenol for the treatment of vaginal (Chami *et al*., 2004) and oral (Chami *et al*., 2005) candidosis in rats. Further studies are warranted to confirm its efficacy in the prophylaxis and/or treatment of biofilm-associated candidosis in human.

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Table 1 - Description of primers used in real time PCR for analysis of *ALS* gene expression of *Candida dubliniensis* and *Candida tropicalis*.

Target gene[a]	Sequence of the primer (5' to 3')	Amplicon size (bp)	Accession number[b]
ALSd-1	F: ACAGTGTCACCTTTACCGATG	177	XM_002419486
	R: CGAGCACACCAGACTTATATCC		
ALSd-2	F: AGTGACGGAGACAACCAAAG	155	XM_002421088
	R: GTACTAGAAGGAGCAACAGGTG		
ALSt-1	F: GCAAAGCTTTTCATACACCACTCA	212	AF201686
	R: TCACTGTTAACATATCCCGACCAA		
ALSt-2	F: GGGTTACATATGATGCTTCGATG	269	AF211865
	R: GAGTTGCTGTGTCTCCAATTGTTG		
ALSt-3	F: GTGTGAAGGTTCTGTCCAAAGAG	274	AF211866
	R: CTGTGTCACCAATAGTACCAGT		
ACTd	F: GGTGACGACGCTCCAAGAGC	176	XM_717232
	R: TCGTCCCAGTTGGAAACAATACCG		
ACTt	F: TGTCTTGGATTCTGGTGATGG	230	XM_002549283
	R: CAATTTCTCTTTCAGCGGTGG		

[a] *ALSd* and *ALSt*: coding for agglutinin-like sequence from *C. dubliniensis* and *C. tropicalis*, respectively, *ACTd* and *ACTt*: coding for actin from *C. dubliniensis* and *C. tropicalis*, respectively. [b] The nucleotide sequences of *C. dubliniensis* and *C. tropicalis* genes deposited in the GenBank/EMBL databases used for specific primer design. [b] Anneling temperature.

Table 2 - Metabolic activities of biofilm formed by *Candida dubliniensis* and *Candida tropicalis* on different substrate surfaces. A significant difference ($p < 0.05$) was observed among the substrates, where the highest biofilm formation was detected on polystyrene surface, followed by PMM and ceramic.

Isolates	Metabolic activity (OD)[a]		
	Polystyrene	PMM[b]	Ceramic
Candida dubliniensis			
ATCC MYA-646	0.855 ± 0.029	0.711 ± 0.056	0.499 ± 0.055
131	1.045 ± 0.032	0.795 ± 0.058	0.628 ± 0.056
219	0.989 ± 0.033	0.751 ± 0.054	0.566 ± 0.057
248	1.094 ± 0.034	0.810 ± 0.055	0.637 ± 0.056
Candida tropicalis			
ATCC 28707	0.978 ± 0.029	0.745 ± 0.058	0.559 ± 0.056
23	1.136 ± 0.032	0.815 ± 0.056	0.638 ± 0.057
150	1.100 ± 0.034	0.801 ± 0.056	0.635 ± 0.057
176	1.056 ± 0.031	0.786 ± 0.057	0.624 ± 0.054

[a] Metabolic activity of sessile cells was determined by the XTT-reduction assay. The XTT formazan product was measured at 490 nm. [b] Polymethylmetacrylate.

Table 3 - Antifungal concentrations of eugenol against planktonic and sessile cells of *Candida dubliniensis* and *Candida tropicalis* strains

Isolates	Eugenol				Fluconazole	
	MIC[a]	MFC[b]	SMIC-1[c]	SMIC-24[d]	MIC[e]	SMIC[d]
Candida dubliniensis						
ATCC MYA-646	375	750	375	375	8	>512
131	750	1,500	750	1,500	4	>512
219	375	1,500	750	1,500	8	>512
248	375	1,500	750	750	4	>512
Candida tropicalis						
ATCC 28707	375	750	375	375	128	>512
23	375	1,500	750	750	8	>512
150	750	1,500	750	1,500	32	>512
176	375	1,500	750	1,500	64	>512

[a]MIC: minimum inhibitory concentration of the antifungal which resulted in total inhibition of visible planktonic cells growth; [b]MFC: minimal fungicidal concentration of the antifungal which resulted in total planktonic cells death; [c]SMIC-1: minimal inhibitory concentration of the antifungal which resulted in total reduction of metabolic activity of sessile cells, using the XTT-reduction assay, after 1 h of adhesion; [d]SMIC-24: minimal inhibitory concentration of the antifungal which resulted in total reduction of metabolic activity of sessile cells, using the XTT-reduction assay, after 24 h of biofilm formation [e]MIC was defined according to CLSI (2008) guidelines for fluconazole broth microdilution assays; The results are expressed as μg/ml.

Table 4 - Effect of eugenol on cell surface hydrophobicity, and adhesion to human ephitelial cells and polystyrene

Isolates	CSH[a]		Adhesion to HEp-2 cells[b]		Adhesion to polystyrene[c]	
	Untreated	Treated[d]	Untreated	Treated[d]	Untreated	Treated[d]
Candida dubliniensis						
131	67.97 ± 5.61*	39.22 ± 6.97	92.00 ± 5.60#	35.00 ± 5.27	0.450 ± 0.001″	0.302 ± 0.001
219	29.48 ± 2.97*	15.58 ± 3.16	45.00 ± 4.16#	14.00 ± 4.53	0.405 ± 0.002″	0.209 ± 0.001
248	69.20 ± 9.10*	16.00 ± 6.11	90.00 ± 5.21#	30.00 ±4.73	0.384 ± 0.001″	0.216 ± 0.002
Candida tropicalis						
23	72.00 ± 8.22*	21.00 ± 5.63	92.00 ± 5.12#	46.00 ± 4.33	0.397 ± 0.004″	0.288 ± 0.003
150	41.66 ± 4.72*	23.75 ± 5.21	81.00 ± 5.06#	43.00 ± 5.84	0.335 ± 0.002″	0.108 ± 0.001
176	84.59 ± 4.32	81.16 ± 3.19	45.00 ± 3.12	36.00 ± 3.21	0.395 ± 0.005	0.393 ± 0.002

[a] Percentage of cell surface hydrophobicity (CSH) determined by the difference in the optical density (OD) of the aqueous phase between test and control. The greater the change in OD of the aqueous phase, the more hydrophobic the yeast sample.
[b] The percent adherence was calculated by the equation: % Adherence = (cfu_{120}/cfu_0) x 100, where cfu_{120} refers to adhered bacterial cells per mL after 2 h and cfu_0 the initial number of inoculated cells.
[C] The metabolic activity of cells was determined by the XTT-reduction assays after 2 h of adhesion on polystyrene surface.
[d] Planktonic cells were eugenol-treated for 1 h with ½MIC before the assay.
Significant differences on CSH (*), adhesion to HEp-2 cells (#) and to polystyrene (″) properties when compared to eugenol-treated counterpart cells (* and #, $p < 0.005$; ″, $p < 0.05$).

Figure 1 - Activities of eugenol against mature biofilm of *Candida* spp. Activity of eugenol at different concentrations (5,86 - 3000,00 μg/ml) (A) and different hours of treatment with the MIC value of eugenol of each isolate (3 – 24 hours) (B). Values are expressed as the average percentage of optical density (OD) of wells containing treated biofilms compared to that of control wells (considered to be 100%) for the XTT assays.

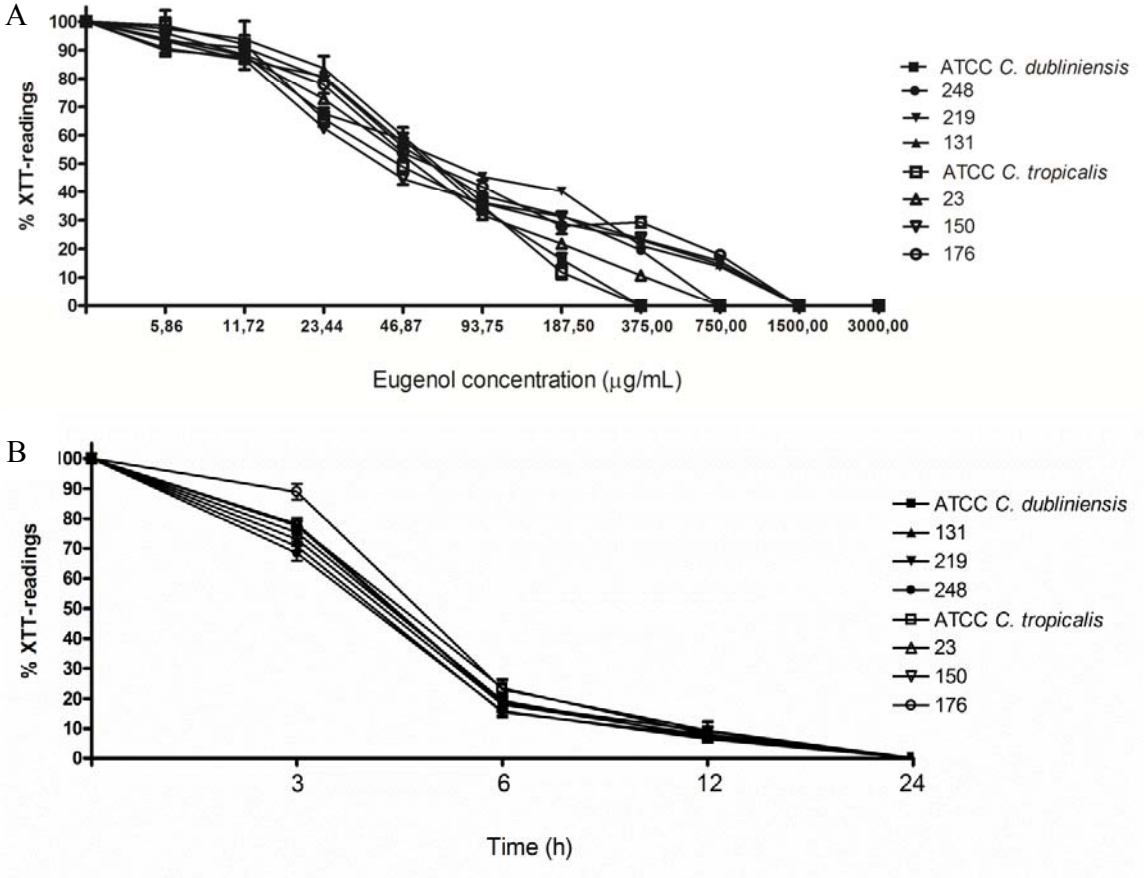

Figure 2 - Scanning electron microscopy of *C. dubliniensis* biofilm formed on the surface of PMM. Biofilm formation after 24 hours (A, C) and mature biofilm treated with $SMIC_{100}$ for 24 hours (B, D).

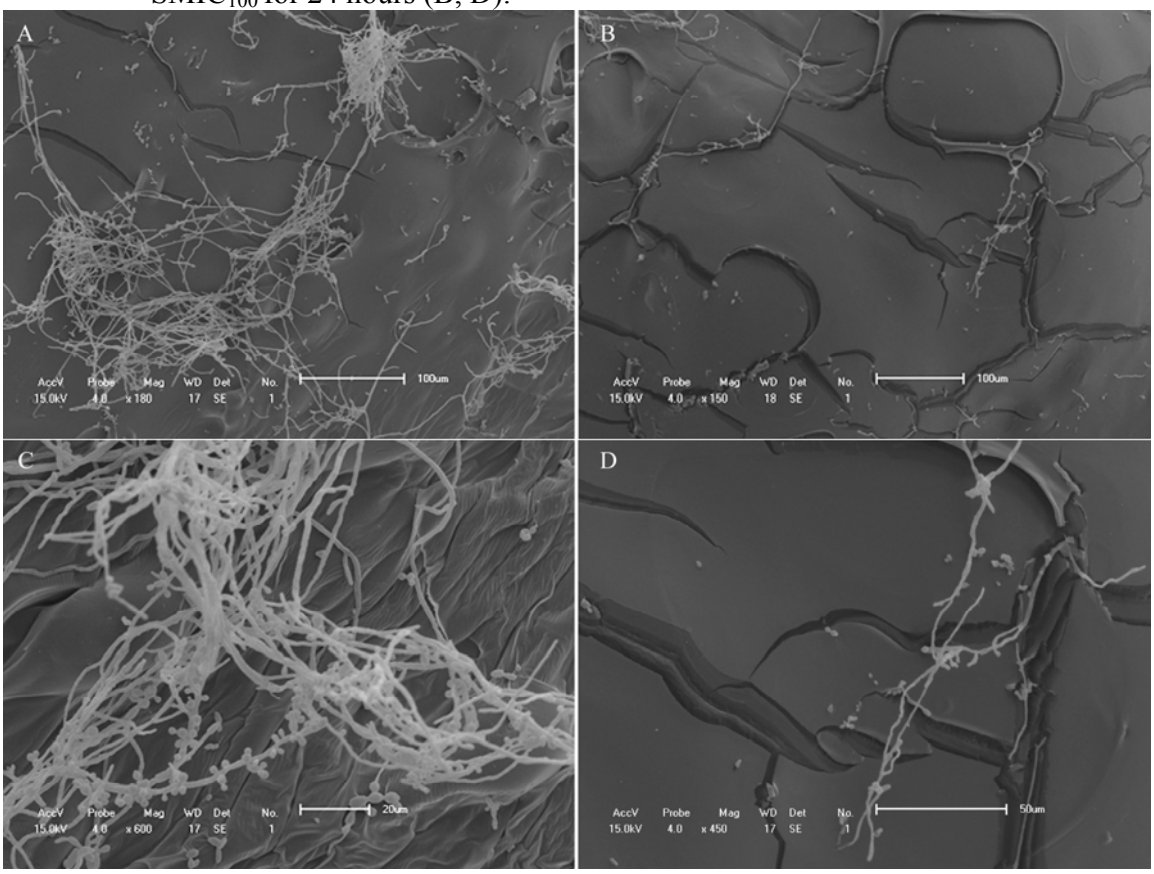

Table 4 - Effect of eugenol on cell surface hydrophobicity, and adhesion to human ephitelial cells and polystyrene

Isolates	CSH[a]		Adhesion to HEp-2 cells[b]		Adhesion to polystyrene[c]	
	Untreated	Treated[d]	Untreated	Treated[d]	Untreated	Treated[d]
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131	67.97 ± 5.61*	39.22 ± 6.97	92.00 ± 5.60#	35.00 ± 5.27	0.450 ± 0.001″	0.302 ± 0.001
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23	72.00 ± 8.22*	21.00 ± 5.63	92.00 ± 5.12#	46.00 ± 4.33	0.397 ± 0.004″	0.288 ± 0.003
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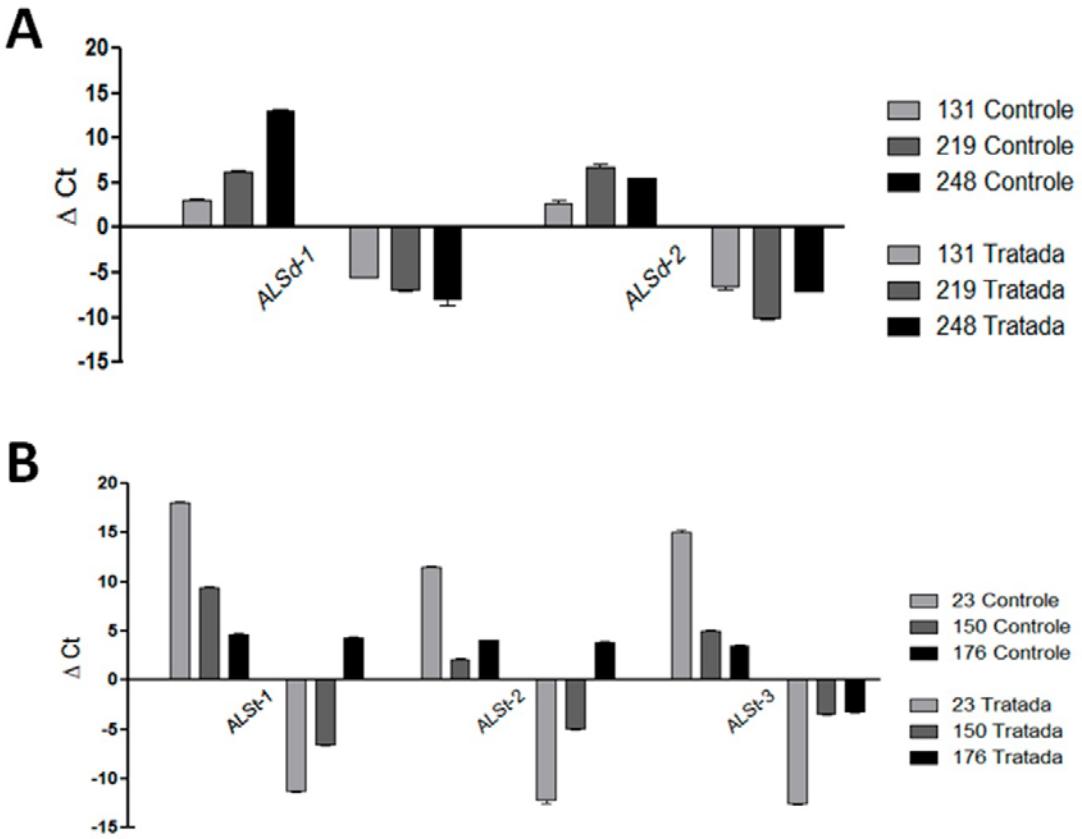
176	84.59 ± 4.32	81.16 ± 3.19	45.00 ± 3.12	36.00 ± 3.21	0.395 ± 0.005	0.393 ± 0.002

[a] Percentage of cell surface hydrophobicity (CSH) determined by the difference in the optical density (OD) of the aqueous phase between test and control. The greater the change in OD of the aqueous phase, the more hydrophobic the yeast sample.
[b] The percent adherence was calculated by the equation: % Adherence = (cfu_{120}/cfu_0) x 100, where cfu_{120} refers to adhered bacterial cells per mL after 2 h and cfu_0 the initial number of inoculated cells.
[C] The metabolic activity of cells was determined by the XTT-reduction assays after 2 h of adhesion on polystyrene surface.
[d] Planktonic cells were eugenol-treated for 1 h with ½MIC before the assay.
Significant differences on CSH (*), adhesion to HEp-2 cells (#) and to polystyrene (″) properties when compared to eugenol-treated counterpart cells (* and #, $p < 0.005$; ″, $p < 0.05$).

Figure 4 - *ALS* gene expression of *C. dubliniensis* (A) and *C. tropicalis* (B). For eugenol-treated planktonic cells of most *Candida* spp. the relative quantification of *ALS* genes expression displayed significant ($p < 0.005$) downregulation compared to untreated cells



ANEXOS

6 ANEXO A

Materiais e métodos

6.1 ISOLAMENTO E IDENTIFICAÇÃO DAS LEVEDURAS

6.1.1 Pacientes

Um total de 244 pacientes HIV positivos atendidos no Centro de Referência Dr. Bruno Piancastelli Filho localizado na cidade de Londrina no período de janeiro à abril de 2010 foram convidados a participar do estudo. Após serem informados sobre o teor da pesquisa, aqueles que concordaram, assinaram um termo de consentimento para utilização das amostras de leveduras isoladas da mucosa bucal. Posteriormente foi realizada consulta no prontuário de cada paciente para obtenção dos dados referentes à contagem de linfócitos $CD4^+$ e $CD8^+$, carga viral, terapia antirretroviral e contato prévio aos antifúngicos fluconazol e nistatina. Além disso, dados sobre a forma de contágio, idade dos pacientes, sexo e data do diagnóstico foram coletados dos prontuários.

Este trabalho foi aprovado pelo Comitê de Ética envolvendo seres humanos da Universidade Estadual de Londrina, com o número 036/10.

6.1.2 Coleta e Isolamento das Amostras

As amostras foram coletadas de acordo com a metodologia descrita em Samaranayake *et al* (1986), com modificações. As amostras foram coletadas, em recipiente esterilizado, de cada paciente após o bochecho por 30 segundos com 10 mL de água destilada esterilizada. Logo após a coleta, os frascos contendo o material biológico foram transportados para o laboratório para o seu processamento. O material resultante do bochecho foi centrifugado por 10 min a 4.000 rpm, e após foi ressuspendido em 1 mL de PBS (pH 7,2) esterilizado. Cerca de 10 μL da suspensão foram semeados na superfície do meio Sabouraud Dextrose Agar (SDA). As culturas foram incubadas a 37 ^oC até 7 dias em condições de aerobiose.

6.1.3 Identificação

6.1.3.1 Identificação Fenotípica

Diferentes colônias crescidas no meio Sabouraud foram submetidas à coloração de Gram, e as leveduras foram semeadas no meio HiCrome Candida® (Himedia) para observar o crescimento diferencial das espécies de *Candida*.

A identificação fenotípica de cada isolado foi realizada por métodos fenotípicos padrões descritos por Kurtzman & Fell (1998), tais como formação de tubo germinativo, produção de clamidoconídeos, e assimilação de carboidratos. A diferenciação entre *C. albicans* e *Candida dubliniensis* foi realizada pelo SDA suplementado com 6,5% de cloreto de sódio (Akgül & Çerikçioğlu, 2009).

6.1.3.2 Identificação Molecular

O DNA genômico foi extraído pelo método descrito por Ausubel *et al* (1999) com algumas modificações. Assim, os isolados clínicos foram cultivados em 3 mL de meio Sabouraud líquido a 37 ºC sob agitação durante 24 horas. As células foram centrifugadas a 13.000 rpm e lavadas com 1,5 mL de água destilada esterilizada. O sedimento foi ressuspendido em 500 µL de tampão de lise TENTS (10 mM Tris HCl pH 8, 0, 1 mM EDTA , 100 mM NaCl, 2% v/v Triton X-100, 1% SDS, e 2 pérolas de vidro de 2 mm). Em seguida foram adicionados 400 µL de fenol saturado e a suspensão foi homogeneizada vigorosamente por 3 minutos. A fase aquosa foi removida, após centrifugação a 13.000 rpm por 5 minutos, e transferida para novo tubo e desproteinizada com fenol-clorofórmio-álcool isoamílico (25:24:1,v/v). O DNA presente na fase aquosa foi precipitado com etanol absoluto por pelo menos 2 horas, lavado com etanol 70% (v/v), seco e ressuspendido com 50 µL de água deionizada estéril. O DNA foi tratado com RNAse A (1 mg/mL) a 37 ºC por 1 hora e as concentrações foram determinadas em espectrofotômetro utilizando a relação da DO obtida entre 260 e 280 nm.

A identificação molecular foi realizada utilizando a metodologia de semi-nested PCR (Ahmad, 2002) utilizando oligonucleotídeos universais (CTSF e CTSR) para o

primeiro ciclo de amplificação e o oligonucleotídeo reverso utilizado no primeiro ciclo e oligonucleotídeo específico (CADET, CGDET, CTDET e CPDET) para o segundo ciclo de amplificação. Essa metodologia permite a identificação das espécies *C. albicans*, *C. tropicalis*, *C. glabrata* e *C. parapsilosis.* Para a identificação de *C. dubliniensis* (DUBF e DUBR) *(*Donnelly *et al*., 1999), *C. krusei* (KRUS1 e KRUS2) e *C. glabrata* (GLAB1 e GLAB2) foram utilizados primers específicos *(*Milde *et al*, 2000).

As espécies identificadas foram estocadas em Sabouraud Dextrose Agar a 4 °C para uso posterior.

Tabela 1 - Sequência dos iniciadores utilizados na identificação molecular dos isolados de *Candida* spp..

Oligonucleotídeo	**Seqüência 5' → 3'**
CTS	TCGCATCGAT GAAGAACGCAGC
	TCTTTTCCTCCGCTTAT TGATATGC
CADET	ATTGCTTGCGGCGGTAACGTCC
CPDET	TCTTTTCCTCCGCTTAT TGATATGC
CGDET	TAGGTTTTACCAAC TCGGTGTT
CTDET	ATTTTGCTAGTGGCC
DUB	GTATTTGTCGTTCCCCTTTC
	GTGTTGTGTGCACTAACGTC
KRUS	GGTTGACACTTCGCATA
	CGTATGTGACCAGTGA
GLAB	GTGCAGATATGTCGCTATTACCTTTGG
	CGACTGGTTGACGATAATCAGAGGAGATGGG

6.2 Determinação do Perfil de Sensibilidade aos Antifúngicos

6.2.1 Estoque dos antifúngicos

6.2.1.1 Fluconazol (Sigma)

O pó liofilizado foi diluído com água destilada esterilizada a uma

concentração final de 5 mg/mL, sendo armazenado a -20°C por até 3 meses.

6.2.1.2 Nistatina (Sigma)

O pó liofilizado foi diluído em uma solução de dimetilsulfóxido – DMSO (Merck) a uma concentração final de 5 mg/mL, sendo armazenado a 4 °C por até 3 meses.

6.2.2 Teste de Sensibilidade aos Antifúngicos

A determinação da concentração inibitória mínima (CIM) para os antifúngicos foi realizada pela técnica de microdiluição em caldo, segundo normas preconizadas pelo Clinical Laboratory Standards Institute (CLSI, 2008). *Candida parapsilosis* ATCC 22019, gentilmente cedida pela FIOCRUZ, Rio de Janeiro, Brazil, foi incluída como controle de qualidade da reação. Controles de crescimento dos isolados também foram realizados. As placas foram incubadas a 37 °C por 48 h com leitura a cada 24 h. As concentrações testadas foram 128 µg/mL à 0,25 µg/mL, 64 µg/mL à 0,125 µg/mL para fluconazol e nistatina, respectivamente. A CIM para o fluconazol foi determinada pela menor concentração que inibiu 50% do crescimento das leveduras e estas foram classificadas de acordo com os critérios descritos no CLSI (2008). A CIM para a nistatina foi determinada pela menor concentração capaz de inibir 100% do crescimento das leveduras (Kuriyama *et al.* 2005; Hamza *et al.* 2008).

A interpretação dos valores de CIM considerados para os antifúngicos fluconazol e nistatina está descrita na tabela 2. Para o fluconazol considerou-se as normas da CLSI (2008) Norma M27-A3. A nistatina não possui valores estabelecidos pela CLSI (2008) e os critérios utilizados foram descritos em estudos anteriores por Wingenter *et al* (2007).

Tabela 2 – Interpretação dos valores de concentração inibitória mínima dos isolados de *Candida* spp.

Antifúngico	Concentração inibitória mínima (μg/mL) para:		
	S	**SSD**	**R**
Nistatina *	≤ 4,0	8,0 a 32,0	≥ 64,0
Fluconazol **	≤ 8,0	16,0 a 32,0	≥ 64,0

S – sensível; SSD – sensível dose-dependente; R – resistente. *A CIM foi determinada de acordo com padrões de Wingenter *et al.* 2007; ** A CIM foi determinada de acordo com padrões da CLSI (2008), norma M27-A3.

6.3 DETERMINAÇÃO DOS FATORES DE VIRULÊNCIA

6.3.1 Determinação da Atividade de Protease e Fosfolipase

As amostras foram testadas quanto à habilidade em crescer e produzir um halo de degradação/precipitação em meios de culturas solidificados contendo 0,1% de albumina sérica bovina (BSA) e 4,0% de gema de ovo como substratos para protease e fosfolipase, respectivamente. Para tanto, as amostras foram previamente incubadas a 37 ^{o}C por 24 h sob agitação constante (160 rpm) em caldo Sabouraud para o ensaio de protease e fosfolipase. O número de células foi determinado pela contagem direta em câmara de Neubauer e cerca de 1 x 10^6 células (10 μL) foram inoculadas na superfície de meio específico para cada atividade enzimática (meio mínimo suplementado com 0,1% de BSA para atividade de protease, e 4,0% de gema de ovo, 350 μM NaCl, 6,5 μM $CaCl_2$ para atividade de fosfolipase). As placas foram incubadas a 37 ^{o}C e após 96 h o valor de Pz foi calculado pela razão entre o diâmetro das colônias e do diâmetro da colônia mais o halo de degradação/precipitação. As amostras foram classificadas de acordo com Price *et al*. 1982, onde: atividade nula (Pz = 1,00), baixa atividade (0,64 ≤ Pz < 1,00), média atividade (0,30 ≤ Pz ≤ 0,64) e alta atividade Pz ≤ 0,30)

6.3.2 Determinação da Atividade Hemolítica

A atividade hemolítica das leveduras foi determinada de acordo com Luo *et al.* (2002). O

inóculo celular foi preparado como anteriormente e a mesma densidade celular foi semeada na superfície do meio SDA suplementado com 3% de glicose e 5% de sangue fresco de carneiro. As placas foram incubadas a 37 °C por 48 h. A atividade hemolítica foi indicada por um halo translúcido ao redor do local do inóculo.

6.3.3 Determinação da Hidrofobicidade da Superfície Celular (HSC)

A HSC das leveduras foi determinada de acordo com o procedimento descrito por Anil *et al*. 2002. Para tanto, as leveduras foram previamente cultivadas em caldo Sabouraud a 37 ^{o}C por 24 h. A densidade celular foi ajustada de acordo com a escala ½ de McFarland em 5 mL de PBS. Foram transferidos 1,5 mL da suspensão nos dois tubos testes e 0,5 mL de xileno foi adicionado a eles. Um tubo controle, contendo 2 mL da suspensão, foi mantido sem xilol. Após incubação em banho-maria a 37 ^{o}C durante 10 min, os tubos foram agitados vigorosamente e depois submetidos a uma nova incubação, nas mesmas condições de temperatura por mais 30 min. A densidade óptica da fase aquosa foi determinada a 520 nm. A HSC foi expressa como porcentagem da diminuição da densidade óptica da fase aquosa do teste quando comparada com o controle. sendo que valores abaixo de 30% são consideradas fracamente hidrofóbicas, de 30-70% são moderadamente e acima de 70% são consideradas altamente hidrofóbicas.

6.3.4 Formação de Biofilme

A determinação da formação do biofilme foi realizada segundo a metodologia descrita por Bizerra *et al.* (2008). O biofilme foi formado em meio RPMI 1640 em placas de poliestireno (96 poços). As amostras foram cultivadas por 24 h a 37 °C em meio Sabouraud e as células foram contadas. Uma suspensão (20 µL) contendo 6 x 10^5 leveduras foi adicionado ao poço contendo 180 µL de meio RPMI. As placas foram incubadas a 37 ºC por 24 horas sem agitação. A atividade metabólica do biofilme foi determinada utilizando a metodologia de redução do XTT. Uma alíquota de 100 µL de XTT-menadiona [0,1 mg / mL XTT, 1 mM menadiona] foi adicionada a cada poço e as placas foram incubadas no escuro por 2 horas e a leitura espectrofotométrica foi realizada a 490 nm em espectrofotômetro

(Universal Microplate Reader modelo ELx 800, Bio-Tek Instruments INC)

6.4 Expressão dos genes SAP e ALS

6.4.1 Extração de RNA e Síntese de cDNA

As leveduras foram previamente cultivadas em Sabouraud caldo por 24 horas sob agitação à 37 °C.

A extração de RNA das leveduras foi realizada utilizando o kit RNAeasy Mini Kit (Qiagen). O DNA complementar (cDNA) foi obtido pela reação de transcritase reversa utilizando o oligonucleotídeo-dT para a síntese da primeira fita. A reação foi realizada no volume final de 20 μL contendo tampão para RT 5X (Tris-HCl 250 mM pH 8,3; KCl 375 mM; $MgCl_2$ 15 mM), DTT 0,1 M e dNTPs (10 mM cada), seguido de incubação a 37 ^{o}C por 2 minutos e adição de 200 U da enzima transcritase reversa (M-MLV Reverse Transcriptase, purificada de *E. coli* contendo o gene *pol* do vírus MML – Moloney Murine Leukemia). Após incubação por 60 minutos a 37 ^{o}C, a reação foi bloqueada com o aumento da temperatura a 65^{o} C por 15 minutos.

6.4.2 PCR em Tempo Real

Para a análise da expressão dos genes SAPs e ALSs foi realizada a PCR em tempo real no termociclador Rotor Gene 3600, utilizando-se o sistema SYBR Green de detecção. Cerca de 15 ng de cDNA, 200 mM de cada oligonucleotídeo específico e a concentração recomendada de SYBR Green Master Mix (Invitrogen) foram misturados em um volume final de 20 μL. O gene que codifica a actina de cada espécie de *Candida* foi utilizado como controle da reação. A análise da expressão foi realizada por quantificação relativa (Pfaffl, 2006; Hayes *et al*, 2011). Os oligonucleotídeos iniciadores foram desenhados a partir de sequências nucleotídicas dos genes correspondentes de *Candida* spp. depositadas em banco de dados.

Tabela 3 - Sequência nucleotídica de oligonucleotídeos iniciadores utilizados para análise da expressão dos genes SAP e ALS de *Candida* spp.

Gene[a]	Sequência do Iniciador (5'para 3')	Tamanho do fragmento (bp)	Número de Acesso[b]
SAPd-1	F: GATGCCTTCCATGCTGAATTG	128	XM_002421028.1
	R: CTCAGAAGCAGGAACAGAGATC		
SAPd-2	F: AGGATACTGTTGGGTTTGGTG	292	XM_002422241.1
	R: TCGGTATTGGAAGTAACTGGC		
SAPa-2	F: AGGATACCGTTGGATTTGGTG	245	XM_705969.1
	R: AGCATTATCAACCCCACCG		
SAPt2	F: ATCCAAACCAATTCAGCGACAGT	286	AF115320
	R: AAACCAGCAACGGCACCATTA		
SAPt3	F: CACAAGAAATCGCCGACACAAT	255	AF115321
	R: AAAACGGCGTACGCCAGTCTC		
SAPt4	F: CATCAGATTCAACAACTGGTGAG	297	FJ558626
	R: TTTCCTGGTAAGTCACAATCAACA		
ACTa/d	F: GGTGACGACGCTCCAAGAGC	176	XM_717232
	R: TCGTCCCAGTTGGAAACAATACCG		
ACTt	F: TGTCTTGGATTCTGGTGATGG	230	XM_002549283
	R: CAATTTCTCTTTCAGCGGTGG		
ALSd-1	F: ACAGTGTCACCTTTACCGATG	177	XM_002419486
	R: CGAGCACACCAGACTTATATCC		
ALSd-2	F: AGTGACGGAGACAACCAAAG	155	XM_002421088
	R: GTACTAGAAGGAGCAACAGGTG		
ALSt-1	F: GCAAAGCTTTTCATACACCACTCA	212	AF201686
	R: TCACTGTTAACATATCCCGACCAA		
ALSt-2	F: GGGTTACATATGATGCTTCGATG	269	AF211865
	R: GAGTTGCTGTGTCTCCAATTGTTG		
ALSt-3	F: GTGTGAAGGTTCTGTCCAAAGAG	274	AF211866
	R: CTGTGTCACCAATAGTACCAGT		
ACTd	F: GGTGACGACGCTCCAAGAGC	176	XM_717232
	R: TCGTCCCAGTTGGAAACAATACCG		
ACTt	F: TGTCTTGGATTCTGGTGATGG	230	XM_002549283
	R: CAATTTCTCTTTCAGCGGTGG		

[a]*SAPd, SAPa* e *SAPt*: codifica a aspartil protease secretada de *C. dubliniensis, C. albicans* e *C. tropicalis*, respectivamente, *ACTa/d* e *ACTt*: codifica a actina de *C. dubliniensis*, *C. albicans* e *C. tropicalis*, respectivamente. [b] As sequências nucleotídicas dos genes de *C. dubliniensis* e *C. tropicalis* estão depositados no GenBank/EMBL e estas sequências foram utilizadas para a construção dos iniciadores específicos.

6.5 ATIVIDADE ANTIMICROBIANA DO EUGENOL SOBRE CÉLULAS SÉSSEIS E PLANCTÔNICAS DE AMOSTRAS DE *C. TROPICALIS* E *C. DUBLINIENSIS*

Para determinar o efeito do eugenol foram utilizadas 3 amostras de *C. tropicalis* (23, 150, 176) e 3 amostras de *C. dubliniensis* (131, 219, 248) isoladas de cavidade bucal de pacientes HIV positivos.

A inibição do crescimento das leveduras pela ação do eugenol para células planctônicas de *Candida* spp. foi determinada pelo método de microdiluição em caldo de acordo com o CLSI, 2008. A substância foi diluída em RPMI tamponado com MOPS pH 7,0 (3000 – 5,85 μg/mL). O controle de qualidade foi feito utilizando amostras padrões *C. dubliniensis* ATCC MYA-646 e *C. tropicalis* ATCC 28707 e fluconazol. A concentração inibitória mínima foi determinada pela inibição total do crescimento visual após 24 h de incubação em comparação com células planctônicas não tratadas. Para determinar a concentração mínima fungicida (CMF) uma alíquota de 20 μL dos poços não mostrando crescimento foram assepticamente transferidos para placas de Sabouraud Dextrose Agar e incubadas a 37 °C por 48 h. A CMF foi determinada como a menor concentração do eugenol capaz de levar a morte das leveduras.

Para a determinação da sensibilidade das células sésseis os biofilmes foram formados em placas de poliestireno de 96 poços de fundo chato de acordo com Bizerra *et al,* 2008. Depois de 1 e 24 horas de adesão o meio foi aspirado e cada poço foi lavado 3 vezes com PBS estéril. Uma alíquota de 200 μL de RPMI contendo as concentrações de eugenol foi adicionada e as placas foram incubadas por 24 horas a 37 °C. A concentração inibitória mínima das células sésseis (CIMS) foi determinada pela redução de 100% da atividade metabólica em relação ao controle sem a presença de eugenol utilizando o ensaio de redução do XTT como descrito anteriormente. Os experimentos foram realizados em triplicata em três ensaios diferentes.

Para a determinação da hidrofobicidade celular (CSH), teste de adesão em células epiteliais e expressão dos genes ALS as leveduras foram tratadas por 1 hora com a metade do valor da CIM de cada isolado. Em contrapartida, uma outra população não foi tratada a fim de comparar os resultados de células tratadas em não tratadas com o eugenol.

6.5.1 Teste de Adesão em Células HEp-2

A adesão das amostras em superfície biótica foi analisada em células de carcinoma de laringe (HEp-2). Para tanto, uma suspensão de 4 x 10^5 células/mL foi cultivada em placas de poliestireno de 24 poços contendo meio Dulbecco´s modified Eagle´s medium (DMEM, Invitrogen) em estufa com 5% de CO_2 à 37 ºC por 48 horas. As leveduras foram cultivadas por 24 horas a 37 ºC em caldo Sabouraud, o eugenol foi adicionado a essas culturas nas concentrações da metade da CIM a 37 °C por 1h. Após esse período as leveduras foram lavadas e a densidade celular foi determinada pela contagem direta em câmara de Neubauer. Cerca de 2,0 x 10^6 céls/mL foram adicionados à cultura de células HEp-2 e após um período de adesão de duas horas em uma atmosfera com 5% de CO_2 a 37 ºC o meio foi removido, os poços foram lavados com PBS, e em seguida foram adicionados 100 µL de Triton X-100 (0,5 %) (Sigma). As células foram removidas dos poços, e por raspagem, e desta suspensão, 20 µL foram diluídos seriadamente (razão 10) e alíquotas (100 µL) correspondentes a diferentes diluições foram plaqueadas em meio SDA. As placas foram incubadas a 37 °C por no mínimo 24 horas e após este período, foi feita a contagem de unidades formadoras de colônias (UFC). A adesão foi expressa em porcentagem e o resultado obtido pela equação: % de adesão= UFC'/UFC^0 x 100, em que UFC' representa o número de UFC que ficaram aderidas após duas horas e UFC^0 o número inicial de células inoculadas. Neste teste cada amostra (3 de *C. tropicalis* e 3 de *C. dubliniensis)* foi dividida em 2 grupos, um grupo previamente tratado com a metade da CIM do eugenol por 1 hora e um grupo não tratado.

6.5.2 Microscopia Eletrônica de Varredura

Próteses dentárias foram assepticamente colocadas em poços de placas de 24 poços. Um inóculo de 3 x 10^6 foi preparado em 1 mL de meio RPMI 1640, e usado para formar biofilme nestas superfícies. As próteses foram então imersas nessas suspensões celulares e incubadas a 37 º C por 24 h. Após a incubação, as células não aderidas foram removidas após a lavagem por 3 vezes com PBS. Um mL de RPMI contendo o valor da CIM do eugenol foi adicionado e as próteses foram incubadas por mais 24 h. Os biofilmes formados nas próteses foram fixados em tampão cacodilato 0,1 M (pH 7,2). As células foram

desidratadas em etanol, foi então realizado o ponto crítico e a metalização com ouro para a observação no microscópio eletrônico de varredura SHIMADZU SS-550.

6.6 ANÁLISE ESTATÍSTICA

Os dados foram analisados através de métodos não paramétricos, teste de Spearman e teste de Mann-Whitney, por meio do GraphPad Prism versão 5.00 para Windows (GraphPad Software Inc.; San Diego, CA, USA). Para ambos os testes considerou-se um nível de significância de 5%.

Além disso, os resultados obtidos da determinação da hidrofobicidade celular, adesão em células epiteliais e expressão dos genes foram analisados pelo programa GraphPad Prism 5.0 através do teste One Way-ANOVA com posterior análise entre os grupos pelo teste de Tukey.