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Efeito *in vitro* do extrato de capim-limão em biofilmes de *Candida albicans*, na viabilidade de células sanguíneas humanas e sobre a resina acrílica

Dissertação apresentada ao Programa de Mestrado em Odontologia da Universidade CEUMA para obtenção do título de Mestre em Odontologia, com área de concentração em Odontologia Integrada.

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São Luís (MA)
2015

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DADOS CURRICULARES

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Dedicatória

A **Deus,** que me acompanha e abençoa sempre, em todos os momentos de minha vida, e não me desamparou um segundo sequer nesta jornada.

Aos meus pais, **David Moises Madeira** e **Leibe Prazeres Barros**, que sempre me ensinaram os reais valores da vida, os principais deles: Humildade e Sabedoria. Todo amor e dedicação dados por vocês me possibilitaram vivenciar mais este momento. A educação de casa é a base de tudo e sem vocês isto seria impossível.

A minha irmã, **Perla Dalei Barros Madeira**. *"Mana"* nossos pais nos ensinaram o dom da Humildade e da Sabedoria, e pelo simples fato de ter me formado Cirurgião-Dentista primeiro, sei que acabo servindo de espelho para você. Isso me faz ser um profissional muito mais dedicado, responsável e competente para poder lhe ensinar coisas boas da vida e da nossa profissão.

A minha avó **Leonise Prazeres Barros *(in memoriam)***. Minha princesa, agora minha anjinha, onde você estiver sei que estará contente assim como eu. Cheguei a mais uma vitória na vida e como você sempre falava "*importante que foi nos estudos",* prometo continuar sempre estudando para que você e nossa família possam continuar sempre se orgulhando (Amor Materno e Eterno).

Ao meu Avô **Benedito de Souza Barros**, meu grande exemplo de luta, força, perseverança, determinação, seriedade e honestidade. Ao senhor vovô, todos os agradecimentos do mundo, se hoje sou formado, devo também ao senhor pela confiança e apoio em todos os sentidos.

A minha professora **Letícia Machado Gonçalves**. Deus coloca pessoas certas na hora certa e no lugar certo. Como ele nunca me desampara, colocou na minha vida uma profissional extraordinária, exemplar, extremamente competente no que faz, dedicada e apaixonada pela pesquisa e pela ciência. O dom do estudo Deus deu a todos, é só procurar; porém o dom da pesquisa ele deu a poucos que procuram no escuro, novos caminhos que possam levar a grandes descobertas que serão de grande valia para a humanidade. Nunca permita que esse dom se guarde ou se acabe. A você todos os meus agradecimentos.

Agradecimentos

À Universidade CEUMA, representada pelo **Prof. Ms. Marcos Barros**.

À Pró-Reitoria de Pós-Graduação, Pesquisa e Extensão, representada pelo **Prof. Dr. Valério Monteiro Neto**.

Ao Programa de Mestrado Acadêmico em Odontologia representado pelo coordenador **Prof. Dr. Matheus Coelho Bandéca** e pelo coordenador adjunto **Prof. Dr. Rudys Rodolfo de Jesus Tavárez**.

À **Fundação de Amparo à Pesquisa e Desenvolvimento Tecnológico do Maranhão (FAPEMA),** pelo auxílio financeiro obtido através do auxílio PAEDT 36/2014.

A **todos os professores** do programa do Mestrado em Odontologia da Universidade CEUMA, por toda dedicação e conhecimentos a mim repassados, em especial ao **Prof. Marcos André dos Santos da Silva** por confiar e acreditar no meu potencial e no meu trabalho. Ao senhor professor todos os meus agradecimentos.

Aos professores **Marco Aurélio Benini Paschoal** e **Eduardo Martins de Sousa** por toda dedicação e auxílio no decorrer deste trabalho.

A **Letícia Torres** por sua incansável e valiosa ajuda. Sempre pontual atenta e prestativa, sua participação neste trabalho foi fundamental para encontrarmos grandes resultados.

Aos colegas de turma: **Washington Reis, João Batista, Reidson Stanley, Adrícyla Rocha, Ana Paula Dias, Iara Campos, Ana Claudia e Greyce Castelo**, pelo companheirismo nas batalhas do dia-a-dia. Vocês estarão sempre no meu coração!

Aos colegas da Coordenação, em especial, a **Erimônica Câmara Pereira** por todo auxílio, confiança e dedicação.

A todos os funcionários dos laboratórios da pós-graduação, em especial, a **Margareth Penha** por todo auxílio e dedicação.

Sumário

Resumo

Madeira PLB. **Efeito *in vitro* do extrato de capim-limão em biofilmes de *Candida albicans*, na viabilidade de células sanguíneas humanas e sobre a resina acrílica**. [Dissertação de Mestrado]. São Luís: Universidade CEUMA; 2015.

A candidose associada ao uso de próteses (CAP) é uma patologia comumente observada em usuários de próteses dentais removíveis, sendo a *Candida albicans* o seu principal agente etiológico. Considerando o desafio atual para o controle da CAP por meio de terapias convencionais, a busca por substâncias antifúngicas a partir de fontes naturais se tornou uma tendência na literatura médico-odontológica. Dessa forma, este estudo teve por objetivo investigar o uso do extrato de capim-limão como substância auxiliar no tratamento da CAP. Foi avaliado o efeito do extrato de capim-limão (ECL) em biofilmes de *C. albicans*, na viabilidade de células humanas, bem como na percepção de cor, rugosidade e resistência da resina acrílica. Inicialmente foi investigado o efeito do ECL em suspensão planctônica de *C. albicans* por meio dos testes de Concentração Inibitória Mínima (CIM), Concentração Fungicida Mínima (CFM) e tempo de morte celular. Para os testes em biofilmes, discos de resina acrílica foram confeccionados e tiveram sua rugosidade de superfície padronizada. Após a formação da película adquirida, biofilmes de *Candida albicans* foram desenvolvidos e os efeitos do ECL nas concentrações CIM, 5 X CIM e 10 X CIM foram investigados durante o período de formação e após seu desenvolvimento de 72h (*i.e.* biofilmes maduros). Os biofilmes foram analisados por meio de quantificação celular, atividade metabólica e microscopia de fluorescência. A toxidade de diferentes concentrações de ECL em células do sangue periférico humano foi verificada através do teste de MTT. Os efeitos no substrato de resina acrílica foram mensurados por meio dos testes de percepção de cor, rugosidade de superfície e resistência à flexão após 28 dias de imersão em ECL em diferentes concentrações. A exposição em água destilada foi utilizada como controle em todos os experimentos. Os

resultados foram analisados pelo teste ANOVA seguido de Tukey, considerando o nível de significância de 5%. A concentração mínima necessária para inibir o crescimento de *C. albicans* foi 0,625 mg/mL, enquanto a concentração fungicida mínima foi de 2,5 mg/mL. A presença do ECL sobre o biofilme em formação e sobre o biofilme maduro reduziu a quantidade de células significativamente comparada ao grupo controle ($p<0,05$), sendo a concentração inibitória mínima suficiente para reduzir aproximadamente 90% das células do biofilme ($p<0,0001$). A exposição do ECL em biofilmes maduros reduziu significativamente a quantificação celular e atividade metabólica em todas as concentrações testadas ($p<0,05$). A exposição das células do sangue periférico ao ECL na concentração CIM resultou em viabilidade similar ao grupo controle ($p>0,05$). Não foram observadas diferenças estatisticamente significantes na percepção de cor, rugosidade e resistência à flexão após a imersão da resina acrílica na concentração CIM ($p>0,05$). É possível concluir que a imersão em extrato de capim-limão em sua mínima concentração inibitória foi efetiva na redução de biofilmes de *C. albicans*, sem que houvesse efeitos deletérios nas propriedades da resina acrílica. A CIM apresentou-se como uma concentração segura a ser utilizada em células do sangue periférico humano.

DESCRITORES: Produtos Naturais, Capim-limão, *Candida albicans*, Biofilmes.

Abstract

Madeira PLB. ***In vitro* effects of lemongrass extract on *Candida albicans* biofilms, human cells viability and denture surface.** [Dissertation]. São Luís: CEUMA University; 2015.

Candida-associated denture stomatitis (CADS) is an infection commonly observed in denture wearers, being *C. albicans* the major etiological factor. Considering the current challenge in CADS control using conventional therapies, the search for alternative antifungal substances from natural products has become a trend in medical literature. Thus, the purpose of this study was to investigate the *in vitro* effects of lemongrass extract (LGE) as an auxiliary treatment for *Candida*-associated denture stomatitis. It was investigated the effects of LGE in *C. albicans* biofilms, viability of human cells, and in the color perception, surface roughness and flexural strength of acrylic resin. Minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and time-kill assays were performed for LGE against *C. albicans*. For biofilm analysis, disc discs were fabricated using a denture acrylic resin with surface roughness standardization. *C. albicans* biofilms were developed on saliva-coated discs and the effects of LGE at MIC, 5 X MIC or 10 X MIC were investigated during biofilm formation and after biofilm maturation. Biofilms were investigated for cell counting and metabolic activity and microscopic analysis. The cytotoxicity of different concentrations of LGE to human polymorph nuclear cells (PBMC) was analyzed by MTT assay. The effects of LGE in the acrylic resin were verified by changes measured in roughness, color and flexural strength after 28 days of immersion. Data were analyzed by ANOVA followed by Tukey test at 5% significance level. The minimal concentration of LGE required to inhibit *C. albicans* growth was 0,625 mg/mL, while MFC value was 2,5 mg/mL. The presence of LGE during biofilm development resulted in a reduction of cell counting ($p<0.05$), considering the MIC sufficient to reduce approximately 90% of biofilm cells ($p<0.0001$). Exposure of LGE after biofilm maturation also had a significant antifungal effect and metabolic activity at all concentrations tested

(*p*<0.05). When compared to PBS group, the exposure of PBMC to LGE at MIC resulted in similar viability (*p*>0.05). No differences were verified in color alteration, roughness surface or flexural strength after immersion in LGE at MIC compared to control group (*p*>0.05). It could be concluded that immersion of denture surface in LGE was effective in reducing *C. albicans* biofilms, with no deleterious effects on acrylic properties at MIC concentration. MIC also represented an effective and safe concentration for use.

KEYWORDS: Natural Products, Lemongrass, *Candida albicans*, Biofilms.

Introdução

Durante anos a política de saúde bucal no Brasil foi pautada na extração dentária como base para prevenção de dor, cárie, infecção, dentre outros problemas bucais. Esta prática curativa se reflete na população brasileira atual, em que, mesmo com o desenvolvimento na área da Odontologia e com os investimentos feitos para a melhoria na saúde bucal, ainda é grande o número de indivíduos desdentados parciais e/ou totais (1, 2). Os dados obtidos pelo SB Brasil 2010 demostraram melhorias nos indicadores de saúde, principalmente da população adulta, com redução na demanda por próteses dentais. Entretanto, o grande problema ainda se concentra na população idosa (65-74 anos), dos quais 23% ainda necessitam de próteses totais e 15% de próteses parciais (3). Dessa forma, ainda é notável a necessidade de reabilitação desta população com próteses dentais removíveis.

Nestas reabilitações, as próteses dentais removíveis geralmente são confeccionadas com resina acrílica à base de poli (metil metacrilato), por suas boas qualidades estéticas, biocompatibilidade e custo-benefício (4). Após a instalação na cavidade bucal, as próteses dentais são expostas à saliva, sendo passíveis de formação de uma película de proteínas salivares, a qual favorece a adesão de microrganismos (5). A adesão destes microrganismos na superfície das próteses, caso não seja interrompido por meio de higienização adequada ou por tratamento com antifúngicos, resultará no desenvolvimento de comunidades microbianas conhecidas como biofilmes (6-8).

O acúmulo de biofilmes na superfície da prótese, além de ser antiestético e contribuir para halitose, funciona como reservatório de microrganismos com potencial de causar infecções locais e/ou sistêmicas (9, 10). Dentre as infecções locais, a candidose associada ao uso de próteses é a mais comumente encontrada, podendo afetar até 70% dos usuários (7). Esta infecção está associada à presença de fungos do gênero *Candida* spp., tendo como principal espécie envolvida em sua etiologia a *C. albicans* (6, 11). Do ponto de vista sistêmico, os microrganismos encontrados no biofilme das

próteses podem ser deglutidos ou aspirados, e levar ao desenvolvimento de infecções sistêmicas, tais como a pneumonia aspirativa (9, 12). Diante deste contexto, destaca-se a importância do adequado controle dos biofilmes formados na superfície das próteses, não somente para saúde bucal, mas também para saúde geral de seus usuários.

O método mais popular para controle do biofilme sobre a prótese é a escovação com dentifrício, sendo de fácil acesso e baixo custo (12-14). No entanto, a resina acrílica apresenta poros em sua superfície e, em algumas situações, as cerdas da escova não conseguem acessar e limpar adequadamente estes poros (15). Somado a isto, sabe-se que muitos usuários de próteses são pacientes idosos, os quais podem apresentar acuidade visual diminuída ou mesmo perda da habilidade motora (13). Nestes casos, a escovação adequada desta superfície também pode ser comprometida (16).

A imersão de próteses em soluções desinfetantes ou limpadores químicos tem sido sugerido como um importante método auxiliar para a eliminação dos biofilmes (17-20). Dentre as soluções desinfetantes, o hipoclorito de sódio é amplamente empregado, sendo capaz de realizar uma limpeza eficiente (17). Apesar de seus resultados favoráveis, apresenta desvantagens, tais como sabor e odor desagradáveis, além de alteração da resistência e cor da resina acrílica quando usada em regime prolongado (21, 22). Adicionalmente, pode promover corrosão de ligas metálicas, contraindicando seu uso para higienização de próteses parciais removíveis (23).

Os limpadores químicos são substâncias disponíveis comercialmente, cuja ação de limpeza ocorre inicialmente pelo efeito mecânico exercido pela efervescência produzida quando o produto é dissolvido na água, resultando em uma solução alcalina de peróxido de hidrogênio (24). Vários estudos já investigaram o efeito de diferentes limpadores químicos em biofilmes formados na superfície de próteses (15, 18, 25, 26), no entanto, observa-se que este método é falho na remoção de biofilmes de *C. albicans* (25, 26).

Em situações que a candidose associada ao uso de prótese torna-se uma infecção persistente ou mesmo recorrente, tem-se preconizado a prescrição de antifúngicos, dentre os quais se destaca o grupo dos polienos e dos azóis (*i.e.* nistatina e fluconazol, respectivamente) (27). O uso

indiscriminado destes agentes antifúngicos, principalmente depois da “Era da AIDS” , parece ter sido o fator preponderante para o aumento na resistência das cepas de *C. albicans* (28, 29). Além disso, a complexa estrutura dos biofilmes formados na superfície das próteses parece dificultar a difusão e, consequentemente, limitar ação dos agentes antifúngicos, tornando este tipo de infecção recorrente na cavidade bucal (10, 30, 31).

Percebe-se que as limitações oferecidas pelo controle químico/mecânico dos biofilmes, bem como a resistência antifúngica, têm implicações importantes no insucesso do tratamento da candidose associada ao uso de próteses. Dessa forma, a busca por substâncias antifúngicas a partir de fontes alternativas se tornou uma tendência na literatura médico-odontológica (32). Considerando que a maioria dos agentes antifúngicos existentes no mercado é de origem sintética, o interesse por insumos naturais voltou a receber a atenção dos cientistas na busca de princípios ativos para a formulação de produtos inovadores, eficazes e de baixa toxicidade no controle das infecções fúngicas (33-36). Diminuir custos no tratamento das infecções e favorecer a população com novas opções de terapia consiste em um grande desafio para a ciência.

Dentre os insumos naturais, as plantas utilizadas na medicina popular representam uma opção promissora para a prevenção e tratamento de infecções fúngicas (33, 34, 36). Dentre estas plantas, o *Cymbopogon citratus*, popularmente conhecido como “capim-limão” ou “capim-santo” tem atraído a atenção de pesquisadores pelo seu efeito antibacteriano (37, 38) e antifúngico (39-44). O capim-limão é uma planta nativa do Sudoeste asiático, sendo vastamente encontrada no território brasileiro e comumente citada em levantamentos de plantas medicinais e estudos etnobotânicos. A medicina popular costuma preparar o chá ou abafo a partir de suas folhas, sendo utilizado por sua ação calmante, digestiva, espasmolítica e antimicrobiana (34).

Por seus efeitos antimicrobianos, pode-se inferir que o capim-limão seja um possível método auxiliar no controle ou prevenção de biofilmes de *C. albicans* na superfície de próteses dentais removíveis (43). Porém, no processo de seleção e indicação de uma substância para o tratamento da candidose, alguns aspectos devem ser considerados. Entre eles, o efeito da substância

nos biofilmes formados, nas células do hospedeiro e no substrato da prótese, ou seja, na resina acrílica.

Cabe ressaltar que apesar da ação antifúngica do capim-limão ser reconhecida na literatura, estes estudos avaliaram o comportamento das leveduras apenas em estado planctônico (39-44). Neste sentido, sabe-se que *C. albicans* encontra-se na superfície da prótese predominantemente organizada em biofilmes (7, 11), e que o estado planctônico difere substancialmente dos biofilmes no que diz respeito à capacidade de resistir a antifúngicos (31). Dessa forma, a literatura ainda é escassa a respeito do efeito do capim-limão em biofilmes de *C. albicans*.

Outro aspecto a ser ponderado é a manutenção da viabilidade das células humanas após o contato com o capim-limão (45), garantindo a segurança de uso em estudos clínicos futuros. Considerando que o processo inflamatório observado na candidose associada ao uso de próteses é caracterizado pela presença de linfócitos e monócitos (46), avaliar o potencial citotóxico de diferentes concentrações da substância nestas células é de suma importância.

Dentre as principais desvantagens do uso de substâncias químicas auxiliares para controle do biofilme da prótese estão as alterações que estas podem induzir na resina acrílica (17, 21, 23, 47, 48). A descoloração da base acrílica e redução na resistência à flexão são os principais efeitos observados após o uso destas substâncias em longo prazo, estando diretamente relacionado com quesitos de estética e longevidade da prótese (48). Além disso, possíveis alterações na rugosidade de superfície podem facilitar a deposição de microrganismos bem como dificultar a completa remoção dos biofilmes (47).

Considerando o contexto exposto, este estudo teve por objetivo investigar o uso do extrato de capim-limão como substância auxiliar no tratamento da candidose associada ao uso de prótese. Desta forma, foi avaliado o efeito do capim-limão em biofilmes de *C. albicans*, na viabilidade de células humanas, bem como possíveis alterações na cor, rugosidade e resistência da resina acrílica.

Capítulo 1

Title: *In vitro* effects of lemongrass extract on *Candida albicans* biofilms, human cells viability and denture surface*

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* Este artigo foi submetido ao período *Plos One*

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Abstract

The purpose of this study was to investigate whether immersion of denture surface in lemongrass extract (LGE) have effects on *C. albicans* biofilms, human cells viability, and in denture surface. Minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and time-kill assays were performed for LGE against *C. albicans*. For biofilm analysis, discs were fabricated using a denture acrylic resin with surface roughness standardization. *C. albicans* biofilms were developed on saliva-coated discs and the effects of LGE at MIC, 5XMIC or 10XMIC were investigated during biofilm formation and after biofilm maturation. Biofilms were investigated for cell counting, metabolic activity and microscopic analysis. The cytotoxicity of different concentrations of LGE to peripheral blood mononuclear cells (PBMC) was analyzed by MTT. The effects of LGE in acrylic resin were verified by changes measured in roughness, color and flexural strength after 28 days of immersion. Data were analyzed by ANOVA followed by Tukey test at 5% significance level. The minimal concentration of LGE required to inhibit *C. albicans* growth was 0,625 mg/mL, while MFC was 2,5 mg/mL. The presence of LGE during biofilm development resulted in a reduction of cell counting ($p<0.05$), considering the MIC sufficient to reduce approximately 90% of cells ($p<0.0001$). Exposure of LGE after biofilm maturation also had a significant antifungal effect at all concentrations ($p<0.05$). When compared to control group, the exposure of PBMC to LGE at MIC resulted in similar viability ($p>0.05$). No differences were verified in color perception, roughness or flexural strength after immersion in LGE at MIC compared to control ($p>0.05$). It could be concluded that immersion of denture surface in LGE was effective in reducing *C. albicans* biofilms, with no deleterious effects on acrylic properties at MIC concentration. MIC also represented an effective and safe concentration for use.

Introduction

Candida albicans is the main pathogen responsible for the development of *Candida*-associated denture stomatitis (CADS), an infection commonly observed in denture wearers [1]. If not interrupted by appropriate hygiene or antifungal treatments, the yeast cells adhere to the acrylic denture surface, being predominantly organized as biofilms [2,3].

Mechanical brushing is a simple and widely-used hygiene method to control biofilms in dentures [4]. However, visual limitations and poor hand function presented by some denture wearers [5] in combination with the challenges imposed by denture design can limit biofilm removal [6]. Although chemical immersion solutions have been recommended as a complementary method for mechanical hygiene [7], it is observed that this treatment also fails in removing *C. albicans* biofilms [8,9]. In cases where CADS is a persistent infection, antifungal agent prescription becomes a daily routine (e.g. fluconazole and nystatin) [10]. *C. albicans* resistance to drug compounds is the one of the major problems in maintaining the survival and propagation of biofilm, which may ultimately lead to treatment failure [11,12].

Considering the current challenge in CADS control using conventional therapies, the search for alternative antifungal substances has become a trend in medical literature [13]. In the process of developing new pharmacologically active compounds, plants used in the folk medicine represent a rational approach in quest for drugs [14-17]. Among these plants, *Cymbopogon citratus*, an herb world-widely known as lemongrass, has been reported to exert potent inhibitory effect against *Candida* species [18-22]. Thus, the application of lemongrass as an effective and easily-available antifungal agent could be considered as an alternative approach to reduce *C. albicans* biofilms in denture surface.

It is important to point out that in most studies the susceptibility tests to lemongrass were performed in planktonic cells [18-25], while most *C. albicans* cells in the oral cavity are associated with biofilms. It is known that biofilms differ substantially from planktonic cells, particularly in terms of their higher antifungal resistance [11]. In addition, investigations about the safety of lemongrass to human cells and the possible effects in mechanical or physical properties of acrylic surface [26-28] are of outmost importance in the decision to consider this

herb as a possible auxiliary treatment for CADS. Thus, the purpose of this study was to investigate whether immersion of denture surface in lemongrass extract (LGE) have effects on *C. albicans* biofilms, human cells viability, and in denture surface.

Materials and methods

Study design

The antifungal activity of lemongrass extract (LGE) against *Candida albicans* planktonic cells was verified by minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and time-kill assays. For biofilm analysis, discs were fabricated using a denture acrylic resin. *C. albicans* biofilms were developed on saliva-coated discs in specific culture medium with LGE at MIC, 5 X MIC or 10 X MIC (experimental groups) for 72 hours in order to investigate the effects of LGE during biofilm formation. In addition, the effects of LGE on 72h-mature biofilms were also investigated by biofilms immersion in distilled water (control group) and LGE at MIC, 5 X MIC or 10 X MIC (experimental groups) during 8 hours. Analyses were constituted by cell counting, metabolic activity and microscopic assays. Furthermore, the cytotoxicity effect of different concentrations of LGE over peripheral blood mononuclear cells (PBMC) was analyzed by MTT assay as well. Substrata measurements were constituted by changes in color, surface roughness and flexural strength, which were analyzed after 28 days of immersion on LGE. All tests were performed in triplicate, on three different days (N = 9). Data were analyzed by ANOVA followed by Tukey's test at 5% of significance level.

Vegetal material

Collection and botanical identification

The vegetal material of *Cymbopogon citratus* (*i.e.*, lemongrass) was grown in the experimental field of the Federal University of Maranhão, São Luís, Maranhão, Brazil. The sampling was collected from November 2013 to August 2014. Exsiccate was prepared and sent to the Ático Seabra Herbarium of the Federal University of Maranhão to botanical identification.

Preparation of the extract

The vegetal material was dried separately in a greenhouse with air circulation at 37^{o}C for 48 hours followed by trituration in a cutting mill. The remaining water was evaporated by freeze-drying. The dried and crushed material of lemongrass (200 g) was extracted through maceration with ethanol at 70% for 24 hours at room temperature. That process was repeated four times. The lemongrass extract (LGE) obtained was filtered and then concentrated under reduced pressure in a rotatory evaporator. The dried residues were straightly diluted in saline sterile solution to a final concentration of 200 mg/mL, sterilized through the process of filtration in membrane of 0.22 µg/mL, and kept in sterile amber bottles at 4^{o}C for test purposes.

Candida albicans growth conditions

Candida albicans (ATCC 18804) was aerobically cultured from original broth by incubation in Sabouraud Dextrose Agar (SDA; Difco, Detroit, MI, USA) for 24 hours at 35°C. A loop of yeast cells was inoculated into Yeast Nitrogen Base culture medium (YNB; Difco, Detroit, MI, USA) supplemented with 100 mM glucose and incubated aerobically under agitation at 35°C. During the exponential growth phase (*i.e.*, after 18–20 hours of incubation), the *C. albicans* cells were washed twice with phosphate-buffered saline (PBS, pH 7.2). Inoculum was prepared in YNB medium and standardized optically at means of ~10^{7} cells/mL (OD = 0.25 at 520 nm).

Susceptibility tests

MIC and MFC tests were determined using M27-A3 standards [29], and time-kill curves were defined as described by a previous published paper [30]. For all assays fluconazole (reference powder; Sigma Aldrich Co., St. Louis, MO, USA) was used as positive control, and negative and solvent (ethanol) was also used as controls.

Minimal inhibitory concentration

MIC assay was performed by the micro dilution test using a 96-well culture plate. The stock solutions of LGE was diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range

of 100–0.19 mg/mL were obtained. The *C. albicans* standard suspension previously prepared (~10^7 cells/mL) was adjusted in RPMI 1640 medium to contain ~10^3 cells/mL and, then, was added to all wells. The plate was incubated at 37^{o}C for 48 hours with MIC defined as the lowest concentration of LGE that was able to inhibit visible growth.

Minimal fungicidal concentration

The non-visible growth wells verified in MIC test were cultured on SDA plates, and incubated at 37°C during 48 hours. MFC was considered as the lowest concentration of LGE that killed fungal cells (≥ 99% of fungal death).

Time-kill assay

In order to determine the LGE time-kill curve, *C. albicans* inoculum was prepared and exposed to MIC value. After every 2 hours, during a time-period of 24 hours, one aliquot was plated on SDA plate and incubated at 37°C during 48 hours. The cells were counted to determine the cells growth curve.

Antifungal activity in biofilms

Discs fabrication

Round-shaped discs (10-mm diameter, 2-mm thickness) were fabricated using a water-bath poly (methylmethacrylate) (PMMA) acrylic resin (VipiCril Plus; Vipicril, São Paulo, Brazil) according to manufacturers' instructions. The discs were prepared using a stainless matrix with standardize dimensions. Processed discs were immersed in distilled water for 48 hours at 37°C to allow the release of residual monomer [31]. To simulate the inner side of a denture, the disc surfaces were ground in an horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) by using progressively smoother aluminum oxide papers (320, 400, and 600 grit). The surface roughness of all discs was analyzed by a rugosimeter (Mitutoyo Corp., Tokyo, Japan) with accuracy close to the nearest 0.01 µm and calibrated at a cutoff value of 0.8 mm, 2.4 mm percussion of measure, and at 0.5 mm/s. The mean of three measurements for each disc was calculated, and the surface roughness was standardized at 0.31 ± 0.05 µm. After these measurements, the discs were ultrasonically cleansed in purified

water for 20 minutes, and disinfect with 1.0% sodium hypochlorite for 3 minutes to remove any contaminants or artifacts.

Biofilm development

To mimic the oral cavity, the discs were coated with human salivary pellicle prior to biofilm development. Saliva was collected from a healthy volunteer who provided written formal consent according to a protocol approved by the Ethics Committee in Research of Ceuma University (#833.094). Saliva was collected in an ice-chilled polypropylene tube during masticatory stimulation with a flexible film and clarified by centrifugation (10,000 x *g* for 5 minutes at 4°C) with the supernatant filter-sterilized and used immediately.

Under aseptic conditions, each disc was placed in a 24-well culture plate and an aliquot of saliva was added to each well. The plate was incubated aerobically for 1 hour at 37°C to form the salivary pellicle. Saliva-coated discs were transferred to a 24-well culture plate, and *C. albicans* standard suspension previously prepared (~10^7 cells/mL) was added to each well and aerobically incubated at 37°C for 1.5 hour (adhesion phase). The discs were carefully washed with PBS and transferred to a new 24-well culture plate containing YNB 100 mM glucose culture medium for 24 hours. At the end of this period, the discs were washed with PBS and a new fresh medium was added and aerobically incubated for 72 hours at 37°C.

The effect of LGE in *C. albicans* biofilms were investigated in two distinct moments: during the biofilm formation and after its maturation. To the first step, LGE was added daily to the fresh culture medium at MIC, 5 X MIC or 10 X MIC during 3 consecutive days (72 hours) to allow biofilm development. The second step aimed to verify the effects of LGE in 72 h-mature biofilms and submitted to the cited above groups for 8 hours. The analysis was constituted by biofilm cell counting, metabolic activity and fluorescence microscopy.

Cell counting

For cell counting, biofilm-discs were washed twice with PBS and sonicated (7 W, for 30 s) to allow the biofilm structure disruption. Then, the sonicated solutions were serially diluted in PBS, and plated in triplicate onto

SDA. Plates were incubated aerobically for 24 hours at 37°C with the yeast cells counted and transformed on cells/mL unit by the support of a stereomicroscope.

Metabolic activity

The metabolic activity was determined with a modified XTT assay protocol [32]. Accordingly, biofilm-discs were placed in a 24-well culture plate with XTT solution (PBS supplemented with 200 mM glucose, 1 mg/mL XTT, and 0.4 mM menadione) with the plates protected from light and incubated for 3 hours at 37°C. Colorimetric changes in the supernatant were analyzed with the support of a spectophotometer adjusted at 492 nm.

Microscopic analysis

The biofilm structure was evaluated by fluorescence microscopy (Axio Imager Z2; Carl Zeiss, Oberkochen, Germany). The biofilm-discs were washed twice with PBS and stained by SYTO-9 and propidium iodide with the Live/Dead *BacLight* viability kit (Invitrogen-Molecular Probes, Eugene, OR, USA) with an incubation time of 20 minutes at 37°C and protected from light. At least five randomly representative optical fields were examined for each disc using the 63.4x immersion lens.

Human cells viability

Peripheral blood mononuclear cells (PBMC) were collected from healthy human volunteers (non-smoker donors who had not taken any drug for at least 15 days prior to sampling, aged 18–35 years old) who provided written formal consent according to a protocol approved by the Ethics Committee in Research of Ceuma University (#833.094). Cells were isolated by the standard method of density-gradient centrifugation over Histopaque®-1119. PBMC were washed and suspended in supplemented DMEN culture medium plus fetal bovine serum 10%,100 µg/ml streptomycin, and 100 U/ml penicillin. PBMCs were plated in 96-well plates (2×10^5 cells/well in 100 µL) and added 100 µL of PBS, or LGE at MIC, 1/2MIC, 5xMIC or 10xMIC. These sets were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. At the end of the culture, an aliquot of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 2 mg/mL in PBS) was added to each well and incubated for another 4 hour at 37°C protected

from light. Then, all the solution consists of complete medium and MTT was discarded. The formazan crystals were dissolved in DMSO (100uL) and the absorbance was determined at 540 nm using a spectrophotometer.

Effects of LGE on acrylic resin

Acrylic resin discs disc were immersed in distilled water (control), LGE at MIC or LGE at 5 X MIC. Discs were incubated at 37^{o}C for 28 days. The immersion medium was changed daily, the disc rinsed in sterile distilled water and dried in absorbent paper. The tests were performed after 0, 7, 14, 21 and 28 days of immersion.

Color perception

At each tested time, the discs were positioned under a silicon mold with an opening aiming to contact the samples to a spectrophotometer (EasyShade Advanced 4.0; Wilcos, Germany). This mold was used in order to provide accurate repositioning and color measurement of disc surface. The color measurements were obtained using the CIE L*a*b* color system, following a previous protocol [27]. The total color alteration (ΔE) was calculated from the following equation: $\Delta E^{*} = [(\Delta L^{*})^2 + (\Delta a^{*})^2 + (\Delta b^{*})^2]^{½}$.

Surface roughness

At the same tested time, the discs were submitted to surface roughness measurement (Surftest SJ-201P rugosimeter, Mitutoyo Corp.). For each sample, three readings were performed 2.4 mm in length and cutoff value of 0.8 mm at a speed of 0.5 mm/s in the corresponding regions. The roughness of each disc was calculated by the arithmetic mean of three measurements (µm). The measure in surface roughness (ΔRa) was obtained by the difference between the roughness after immersion and baseline values.

Flexural strength

For flexural strength assay, rectangular acrylic resin discs (65 x 10 x 3 mm) were fabricated using a stainless matrix to standardize dimensions. The flexural strength (*S*) was measured using a three-point bending test in a DL 2000 universal testing machine (EMIC, São José dos Pinhais, PR, Brazil) at a

crosshead speed of 5 mm/min. The discs were subjected to flection until fracture using a previous protocol [26]. Flexural strength was calculated using the formula: $S=3PL/2bd^2$, where S is flexural strength, P is the peak load applied, L is the span length, b is the disc width and d is the disc thickness. The results were expressed in MPa.

Statistical analysis

The results were statistically analyzed by the SAS/LAB software package (SAS Software, version 9.0; SAS Institute Inc., Cary, NC, USA). Assumptions of the equality of variances and the normal distribution of errors were checked. Data were transformed as suggested by the software. Data of cell counts, metabolic activity, viability of PBMC cells and flexural strength were analyzed by one-way ANOVA followed by Tukey's HSD test, considering the immersion treatment as the study factor. Data of surface roughness and color alteration were analyzed by two-way ANOVA for repeated measures followed by Tukey's HSD test, considering the treatment and immersion periods as study factors. To all tests the significance level was set at 5%.

Results

The MIC and MFC of LGE observed for planktonic cells were 0,625 mg/mL and 2,5 mg/mL, respectively. In addition, time-kill measurements demonstrated that at MIC concentration, LGE showed its fungistatic property during the first 6 hours (Fig. 1A).

The LGE exposure during biofilm development showed a statistical difference on cell counting compared to the control group ($p < 0.05$). In addition, LGE at MIC was sufficient to reduce approximately 90% of biofilm cells ($p < 0.0001$), while LGE at 5 X MIC or 10 X MIC achieved almost a complete eradication (> 99%) of *C. albicans* biofilm (Fig. 1B). For mature *C. albicans* biofilms, the exposure of LGE for 8 hours also had a significant effect with a lower cell counting ($p = 0.001$) and metabolic activity to all studied groups in comparison to the control group ($p < 0.05$), but with no statistical differences among the experimental groups to the both studied variables ($p > 0.05$, Fig. 1C and 1D).

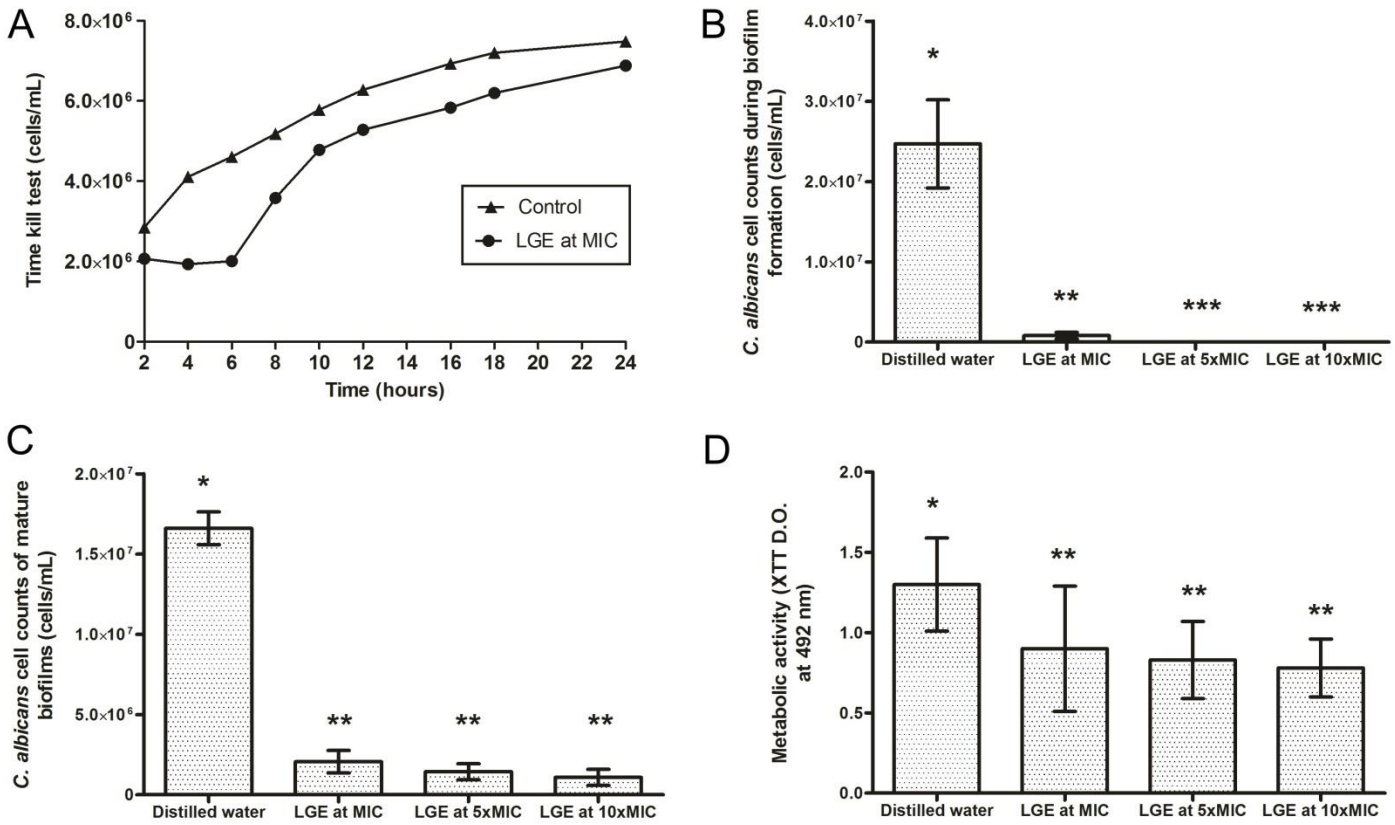

Figure 1. Effect of LGE on *C. albicans*. (A) Time-kill measurements after planktonic cells exposure to LGE at MIC; (B) Cell counts of biofilms when LGE was exposed during its development; (C) Cell counts of biofilms when mature biofilms were exposed to LGE; (D) Metabolic activity of biofilms when mature biofilms were exposed to LGE. Symbols (*, ** or ***) represents statistically significant differences between the control and experimental groups (ANOVA one way followed by Tukey test, p <0.05).

Representative microscope images of *C. albicans* biofilms were showed in Figure 2. The combined used of SYTO-9 and propidium iodide provided an effective labeling of both live and dead yeast cells under the different experimental conditions. Images showed that mature biofilms exposed to LGE tend to be less densely cellularized compared to the control group. Immersion in LGE at MIC, 5xMIC and 10xMIC resulted in a large number of dead cells (stained in red), and also a large number of black spaces, indicating the dispersion of biofilms.

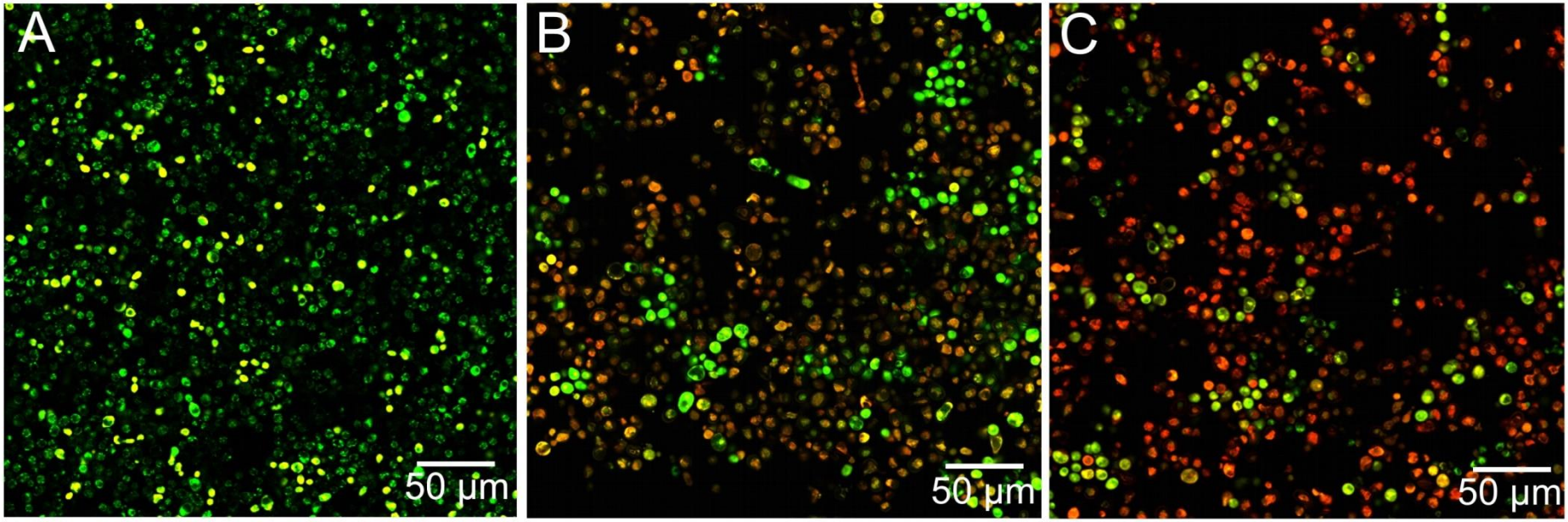

Figure 2. Microscope images of mature *C. albicans* biofilms after 8 hours of exposure to (A) distilled water, (B) LEG at MIC or (C) LEG at 5 X MIC. Live cells stain in green while dead cells stain in red.

Considering the PBMC culture in DMEN as the standard of 100% of viability, it could be observed that LGE reduced significantly its viability ($p < 0.05$). However, when compared to the PBS group, which represents a solution without any effect of cytotoxicity, the exposure of human cells to LGE at MIC resulted in similar viability ($p > 0.05$). Both exposures to lower or higher MIC concentrations resulted in a significant reduction in cell viability, demonstrating a discreet cytotoxicity ($p < 0.05$, Fig. 3A).

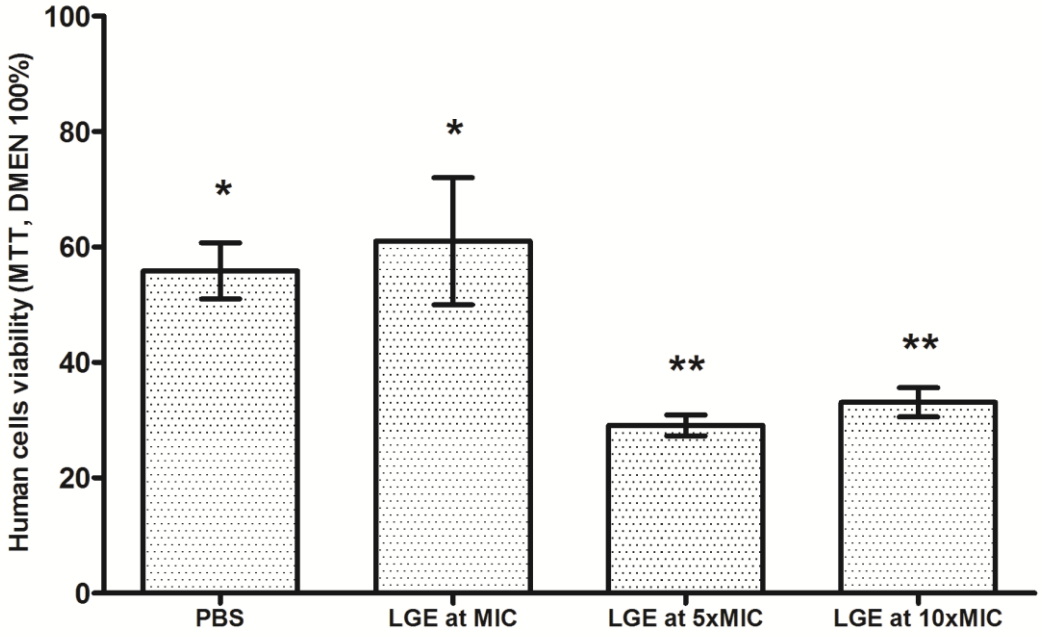

Figure 3. PBMC cells viability after exposure to different LGE concentrations. Symbols (* or **) represents statistically significant differences between the control and experimental groups (ANOVA one way followed by Tukey test, $p < 0.05$).

Regarding color stability, numerical variations were noticed in the CIEL*a*b* colorimetric system, but no significant differences between LGE at MIC and control group were found for △E values (Table 1, $p > 0.05$). However, a significant color alteration after 14 days of immersion was detected when acrylic resin was immersed in LGE at 5 X MIC. The roughness surface values to all evaluated periods of immersion in LGE demonstrated no statistically significant differences with the baseline means (Figure 4A, $p > 0.05$). Also, the mechanical property of flexural strength did not altered after LGE exposure in the different time-points tested (Figure 4B, $p > 0.05$).

Table 1. Degree of color difference (△E) of the acrylic surface after LGE exposure in different periods of time (n = 9).

	7 days	**14 days**	**21 days**	**28 days**
Distilled water	3.24 ± 1.41 (A,a)	3.33 ± 1.82 (A,a)	3.25 ± 1.52 (A,a)	3.22 ± 0.71 (A,a)
LEG at MIC	3.36 ± 0.54 (A,a)	3.05 ± 1.53 (A,a)	3.15 ± 1.86 (A,a)	3.25 ± 1.05 (A,a)
LEG at 5xMIC	3.26 ± 1.38 (A,a)	3.76 ± 0.97 (B,b)	3.88 ± 0.27 (B,b)	4.05 ± 0.61 (B,b)

Different upper case letters indicate statistical significant differences between immersion solutions. Different lower case letters indicate statistical significant differences between immersion periods (ANOVA two-way for repeated measures followed by Tukey test, $p < 0.05$).

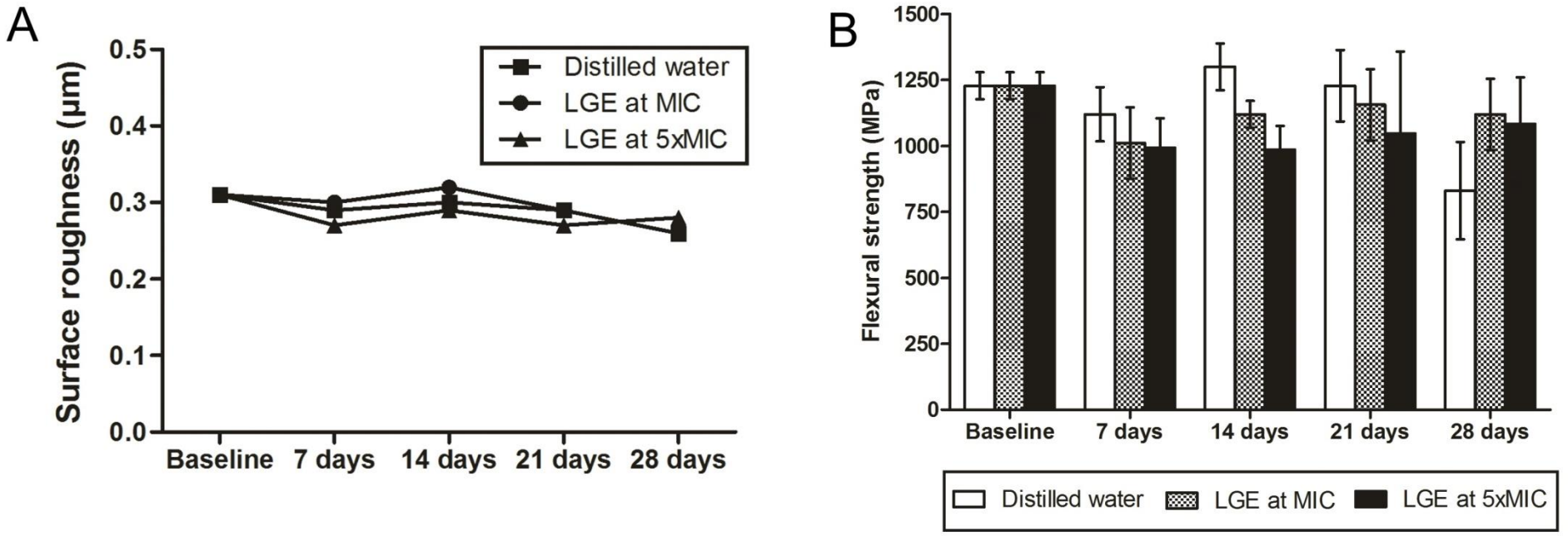

Figure 4. Differences in surface roughness (A) and flexural strength (B) after immersion in LGE at different time-points. There were no statistical differences between groups or periods of immersion (ANOVA two-way followed by Tukey test, $p < 0.05$).

Discussion

Nowadays, new compounds in all fields of fungal control have stimulated researchers regarding plant materials with antifungal properties [14-17]. *Cymbopogon citratus* (DC) Stapf (*Gramineae*) is an herb worldwide known as lemongrass. The tea made from its leaves is popularly used in folk medicine as antispasmodic, analgesic, anti-inflammatory, antipyretic, antimicrobial, diuretic and sedative [15]. In this study the extract obtained from lemongrass was examined *in vitro* as an immersion solution to reduce *C. albicans* biofilms developed on acrylic denture surface.

Susceptibility tests suggested that LGE showed a fungistatic behavior at 0,625 mg/mL against *C. albicans* planktonic cells, and also a fungicidal activity at 2,5 mg/mL concentration. Regarding MIC results, a classification for the antimicrobial activity of plant products was proposed as follows: strong inhibitors, MIC up to 0.5 mg/mL; moderate inhibitors, MIC between 0.6 and 1.5 mg/mL; and weak inhibitors, MIC above 1.6 mg/mL [33]. Regarding this classification, the LGE extract was considered as a moderate inhibitor which corroborates with the results obtained as well.

The antifungal effects of lemongrass manipulated in several formulations (*i.e.* citral, vapor phase, essential oil or hydro-alcoholic extracts) are well established, and the present data corroborates with the literature [18-22,34]. Considering that most *Candida* cells in the oral cavity are associated with biofilms and biofilms differ substantially from planktonic cells in terms of their higher antifungal resistance [11], investigate the behavior of cells organized as a biofilms when exposed to LGE is of outmost importance.

As a “start-point”, it was first observed the effects of LGE during biofilm formation. Thus, LGE at different concentrations was added to culture medium and cells quantification was performed after 72 hours of development. An increased reduction was observed in experimental groups, being 5 X MIC a sufficient concentration to eliminate biofilm cells. The fact that biofilms were grown in the constant presence of LGE could lead the extract to exert a toxic effect. Several mechanisms of action have been proposed to explain vegetal antifungal activity, including disrupting fungi cell structure causing leakage and cell death; blocking the membrane synthesis; inhibition of the spore germination, fungal proliferation and cellular respiration [35]. Therefore,

investigations about the chemical composition of lemongrass allow us to suggest that the presence of aldehydes which are known for their antibacterial and fungitoxic properties [25,35,36], could explain the achieved outcomes.

Considering time-kill results, which demonstrated that at least 6 hours of exposition to LGE are necessary to establish a fungistatic activity; and in order to simulate a denture-overnight soaking period of 8 hours, we also investigated the effects of LGE in a 72h-mature biofilm developed over an acrylic denture surface. Distilled water was employed as control group, since it is indicated for denture immersion during the sleep period. These results demonstrated that 8 hours of immersion in LGE at MIC was sufficient to reduce the metabolic activity and also the number of cells in comparison to the control group. This decrease in a short exposure time could also be explained by the presence of oxygenated monoterpenes aldehydes in vegetal composition. Citral (3,7-dimethyl-2,6-octadienal) is the major monoterpene present in lemongrass (65-85%), being a mixture of two isomeric aldehydes: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B) which possess significant antimicrobial activity [36], as discussed earlier. Beside this, lemongrass could also contain other antifungal monoterpene hydrocarbons such as camphene and limonene [24]. The fungicidal effects of LGE could be confirmed by the increased number of dead cells observed in microscope images. Future investigations should consider the exact molecular mechanisms that explain the fungal cells death in the presence of LGE.

Maintenance of human cells viability after LGE exposure should be defined to ensure safety use in future clinical studies. Whereas the inflammation observed in *Candida*-associated denture stomatitis is mainly characterized by lymphocytes and monocytes [37], assess the cytotoxic potential of LGE against these cells is relevant. This study demonstrated that LGE at MIC provided a cellular viability similar to PBS (control group), although this solution is a well-known to be non-toxic for human cells due to its osmotic capacity. Although concentrations higher than MIC did not completely provided cell death, it was possible to observe a discreet cytotoxicity. Thus, according to our results, MIC seems to be a concentration that ensure satisfactory biofilm control do not resulting in significant damage to host cells. Nevertheless, cellular types present

in oral mucosa, as keratinocytes and fibroblasts, should be considered in further research.

It has been shown that immersion in certain chemical solutions can affect the color, roughness and strength of denture base resins, aspects direct related to longevity and aesthetic [26-28,38,39]. Although 28 days of LGE immersion seems to be a short period compared to the time-use of a denture, we understand that the constant exposure to the solution (changed every day) may provide a significant aging of acrylic matrix, allowing a challenging situation to the tested discs. Also, based on the similar microbiological and cytotoxic results obtained in LGE 5 X MIC and LGE 10 X MIC, the higher concentration was excluded for substratum assays.

Color stability after LGE immersion was evaluated using the CIEL*a*b* colorimetric system, a uniform three-dimensional system that has been widely used for the determination of chromatic differences by translating combinations of differences into mathematical data. In this system, color alteration (△E) is defined as the relative color change between repeated color evaluations. Considering that a color difference less than 3.7 is reported to be clinically imperceptible [40], in this study the immersion of acrylic resin in LEG at MIC did not presented a visible color alteration. This result seems to be very interesting considering that the main disadvantage of conventional chemical cleansers, as sodium hypochlorite, is their whitening property when used for prolonged periods [39-41]. Actually, the LGE at 5 X MIC was sufficient to stain the substratum surface after 14 days of immersion. The highest concentration of active compounds in this experimental group, which are green in color, may impregnate the acrylic matrix resulting in this alteration.

The surface roughness is an important factor in the adherence and entrapment of microorganisms on acrylic denture materials. So, it is crucial that solutions did not alter such property since rougher surfaces could increase biofilm formation [26]. Also, it is undesirable that a solution interfere in the substratum mechanical properties. If a treatment affects negatively the resins decreasing the strength, greater incidence of denture fractures might occur, both outside and inside the mouth [26,27]. In this study, immersion of acrylic resin in LGE did not provided significant changes in surface roughness or

flexural strength, assuring that in experimental conditions the solution was safe for use.

Conclusion

The immersion of denture surface in lemongrass extract was effective in reducing *C. albicans* biofilms with an absence of effects related to mechanical and physical substratum properties. Furthermore, the concentration of the tested extract represented an effective and safe concentration for use due to human cells outcomes. These results demonstrated the potential of using lemongrass as an alternative substance to control *C. albicans* biofilms developed on denture surface.

Acknowledgements

The authors would like to thank Foundation for Research and Scientific Development of Maranhão (FAPEMA) for the financial support (PAEDT-36-2014 and UNIVERSAL-604-14).

Conflict of interest

The author(s) declare no conflicts of interest with respect to the authorship and/or publication of this article.

References

1. Gendreau L, Loewy ZG (2011) Epidemiology and etiology of denture stomatitis. J Prosthodont 20: 251-260.
2. Goncalves LM, Del Bel Cury AA, de Vasconcellos AA, Cury JA, da Silva WJ (2014) Confocal analysis of the exopolysaccharide matrix of Candida albicans biofilms. J Investig Clin Dent.
3. Krom BP, Kidwai S, Ten Cate JM (2014) Candida and other fungal species: forgotten players of healthy oral microbiota. J Dent Res 93: 445-451.

4. Tan CM, Tsoi JK, Seneviratne CJ, Matinlinna JP (2014) Evaluation of the Candida albicans removal and mechanical properties of denture acrylics cleaned by a low-cost powered toothbrush. J Prosthodont Res.
5. Padilha DM, Hugo FN, Hilgert JB, Dal Moro RG (2007) Hand function and oral hygiene in older institutionalized Brazilians. J Am Geriatr Soc 55: 1333-1338.
6. Glass RT, Conrad RS, Bullard JW, Goodson LB, Mehta N, et al. (2010) Evaluation of microbial flora found in previously worn prostheses from the Northeast and Southwest regions of the United States. J Prosthet Dent 103: 384-389.
7. Pellizzaro D, Polyzois G, Machado AL, Giampaolo ET, Sanita PV, et al. (2012) Effectiveness of mechanical brushing with different denture cleansing agents in reducing in vitro Candida albicans biofilm viability. Braz Dent J 23: 547-554.
8. Lucena-Ferreira SC, Cavalcanti IM, Cury AA (2013) Efficacy of denture cleansers in reducing microbial counts from removable partial dentures: a short-term clinical evaluation. Braz Dent J 24: 353-356.
9. de Lucena-Ferreira SC, Ricomini-Filho AP, da Silva WJ, Cury JA, Del Bel Cury AA (2014) Influence of daily immersion in denture cleanser on multispecies biofilm. Clin Oral Investig.
10. Lalla RV, Dongari-Bagtzoglou A (2014) Antifungal medications or disinfectants for denture stomatitis. Evid Based Dent 15: 61-62.
11. Ramage G, Robertson SN, Williams C (2014) Strength in numbers: antifungal strategies against fungal biofilms. Int J Antimicrob Agents 43: 114-120.
12. Taff HT, Mitchell KF, Edward JA, Andes DR (2013) Mechanisms of Candida biofilm drug resistance. Future Microbiol 8: 1325-1337.
13. Nett JE (2014) Future directions for anti-biofilm therapeutics targeting Candida. Expert Rev Anti Infect Ther 12: 375-382.
14. Vieira DR, Amaral FM, Maciel MC, Nascimento FR, Liberio SA, et al. (2014) Plant species used in dental diseases: Ethnopharmacology aspects and antimicrobial activity evaluation. J Ethnopharmacol 155: 1441-1449.
15. Rios JL, Recio MC (2005) Medicinal plants and antimicrobial activity. J Ethnopharmacol 100: 80-84.

16. Ahmad I, Beg AZ (2001) Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J Ethnopharmacol 74: 113-123.
17. Duarte MC, Figueira GM, Sartoratto A, Rehder VL, Delarmelina C (2005) Anti-Candida activity of Brazilian medicinal plants. J Ethnopharmacol 97: 305-311.
18. Irkin R, Korukluoglu M (2009) Effectiveness of Cymbopogon citratus L. essential oil to inhibit the growth of some filamentous fungi and yeasts. J Med Food 12: 193-197.
19. Tadtong S, Watthanachaiyingcharoen R, Kamkaen N (2014) Antimicrobial constituents and synergism effect of the essential oils from Cymbopogon citratus and Alpinia galanga. Nat Prod Commun 9: 277-280.
20. Silva Cde B, Guterres SS, Weisheimer V, Schapoval EE (2008) Antifungal activity of the lemongrass oil and citral against Candida spp. Braz J Infect Dis 12: 63-66.
21. Abe S, Sato Y, Inoue S, Ishibashi H, Maruyama N, et al. (2003) [Anti-Candida albicans activity of essential oils including Lemongrass (Cymbopogon citratus) oil and its component, citral]. Nihon Ishinkin Gakkai Zasshi 44: 285-291.
22. Amornvit P, Choonharuangdej S, Srithavaj T (2014) Lemongrass-Incorporated Tissue Conditioner Against Candida albicans Culture. J Clin Diagn Res 8: ZC50-52.
23. Boukhatem MN, Ferhat MA, Kameli A, Saidi F, Kebir HT (2014) Lemon grass (Cymbopogon citratus) essential oil as a potent anti-inflammatory and antifungal drugs. Libyan J Med 9: 25431.
24. Tyagi AK, Malik A (2010) Liquid and vapour-phase antifungal activities of selected essential oils against Candida albicans: microscopic observations and chemical characterization of Cymbopogon citratus. BMC Complement Altern Med 10: 65.
25. Khan MS, Ahmad I (2012) Biofilm inhibition by Cymbopogon citratus and Syzygium aromaticum essential oils in the strains of Candida albicans. J Ethnopharmacol 140: 416-423.
26. Paranhos H, F., Peracini A, Pisani MX, Oliveira Vde C, de Souza RF, et al. (2013) Color stability, surface roughness and flexural strength of an acrylic

resin submitted to simulated overnight immersion in denture cleansers. Braz Dent J 24: 152-156.

27. de Sousa Porta SR, de Lucena-Ferreira SC, da Silva WJ, Del Bel Cury AA (2013) Evaluation of sodium hypochlorite as a denture cleanser: a clinical study. Gerodontology.
28. Goiato MC, Nobrega AS, dos Santos DM, Andreotti AM, Moreno A (2014) Effect of different solutions on color stability of acrylic resin-based dentures. Braz Oral Res 28.
29. CLSI (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard. CLSI Document M27-A3. CLSI, Pennysylvania, USA.
30. Goncalves LM, Del Bel Cury AA, Sartoratto A, Garcia Rehder VL, Silva WJ (2012) Effects of undecylenic acid released from denture liner on Candida biofilms. J Dent Res 91: 985-989.
31. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC (2006) Influence of acrylic resin polymerization methods and saliva on the adherence of four Candida species. J Prosthet Dent 96: 205-211.
32. da Silva WJ, Seneviratne J, Parahitiyawa N, Rosa EA, Samaranayake LP, et al. (2008) Improvement of XTT assay performance for studies involving Candida albicans biofilms. Braz Dent J 19: 364-369.
33. Aligiannis N, Kalpoutzakis E, Mitaku S, Chinou IB (2001) Composition and antimicrobial activity of the essential oils of two Origanum species. J Agric Food Chem 49: 4168-4170.
34. Korenblum E, Regina de Vasconcelos Goulart F, de Almeida Rodrigues I, Abreu F, Lins U, et al. (2013) Antimicrobial action and anti-corrosion effect against sulfate reducing bacteria by lemongrass (Cymbopogon citratus) essential oil and its major component, the citral. AMB Express 3: 44.
35. Wilkinson JM, Cavanagh HM (2005) Antibacterial activity of essential oils from Australian native plants. Phytother Res 19: 643-646.
36. Machado M, Pires P, Dinis AM, Santos-Rosa M, Alves V, et al. (2012) Monoterpenic aldehydes as potential anti-Leishmania agents: activity of Cymbopogon citratus and citral on L. infantum, L. tropica and L. major. Exp Parasitol 130: 223-231.

37. Sinha S, Jothiramajayam M, Ghosh M, Mukherjee A (2014) Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes. Food Chem Toxicol 68: 71-77.
38. Felipucci DN, Davi LR, Paranhos HF, Bezzon OL, Silva RF, et al. (2011) Effect of different cleansers on the surface of removable partial denture. Braz Dent J 22: 392-397.
39. Savabi O, Attar K, Nejatidanesh F, Goroohi H, Badrian H (2013) Effect of different chemical disinfectants on the flexural strength of heat-polymerized acrylic resins. Eur J Prosthodont Restor Dent 21: 105-108.
40. Moon A, Powers JM, Kiat-Amnuay S (2014). Color stability of denture teeth and acrylic base resin subjected daily to various consumer cleansers. J Esth and Rest Dentistry 26: 247-255.
41. Salloum AM (2014) Effect of 5.25 % sodium hypochlorite on color stability of acrylic and silicone based soft liners and a denture base acrylic resin. J Indian Prosthodont Soc 14: 179-186.

Conclusão

Considerando as limitações deste estudo, é possível concluir que a imersão em extrato de capim-limão em sua mínima concentração inibitória foi efetiva na redução de biofilmes de *C. albicans*, sem que houvesse efeitos deletérios nas propriedades da resina acrílica. A CIM apresentou-se como uma concentração segura a ser utilizada em células do sangue periférico humano.

Referências

1. Chaves SC. Oral health in Brazil: the challenges for dental health care models. Braz Oral Res. 2012;26 Suppl 1:71-80.
2. Silva AE, Demarco FF, Feldens CA. Oral health-related quality of life and associated factors in Southern Brazilian elderly. Gerodontology. 2013 Jul 14.
3. Brasil. Ministério da Saúde. Coordenação Nacional de Saúde Bucal. SB Brasil 2010: Pesquisa Nacional de Saúde Bucal 2010. Nota para a Imprensa de 28/12/2010. Saúde BMd, editor2010.
4. Carlsson GE, Omar R. The future of complete dentures in oral rehabilitation. A critical review. J Oral Rehabil. 2010 Feb;37(2):143-56.
5. Siqueira WL, Custodio W, McDonald EE. New insights into the composition and functions of the acquired enamel pellicle. J Dent Res. 2012 Dec;91(12):1110-8.
6. Emami E, Taraf H, de Grandmont P, Gauthier G, de Koninck L, Lamarche C, et al. The association of denture stomatitis and partial removable dental prostheses: a systematic review. Int J Prosthodont. 2012 Mar-Apr;25(2):113-9.
7. Gendreau L, Loewy ZG. Epidemiology and etiology of denture stomatitis. J Prosthodont. 2011 Jun;20(4):251-60.
8. Lalla RV, Dongari-Bagtzoglou A. Antifungal medications or disinfectants for denture stomatitis. Evid Based Dent. 2014 Jun;15(2):61-2.
9. Preshaw PM, Walls AW, Jakubovics NS, Moynihan PJ, Jepson NJ, Loewy Z. Association of removable partial denture use with oral and systemic health. J Dent. 2011 Nov;39(11):711-9.
10. Seneviratne CJ, Jin L, Samaranayake LP. Biofilm lifestyle of Candida: a mini review. Oral Dis. 2008 Oct;14(7):582-90.
11. Figueiral MH, Azul A, Pinto E, Fonseca PA, Branco FM, Scully C. Denture-related stomatitis: identification of aetiological and predisposing factors - a large cohort. J Oral Rehabil. 2007 Jun;34(6):448-55.

12. Glass RT, Conrad RS, Bullard JW, Goodson LB, Mehta N, Lech SJ, et al. Evaluation of microbial flora found in previously worn prostheses from the Northeast and Southwest regions of the United States. J Prosthet Dent. 2010 Jun;103(6):384-9.
13. Padilha DM, Hugo FN, Hilgert JB, Dal Moro RG. Hand function and oral hygiene in older institutionalized Brazilians. J Am Geriatr Soc. 2007 Sep;55(9):1333-8.
14. Tan CM, Tsoi JK, Seneviratne CJ, Matinlinna JP. Evaluation of the Candida albicans removal and mechanical properties of denture acrylics cleaned by a low-cost powered toothbrush. J Prosthodont Res. 2014 Jul 19.
15. Paranhos HF, Salles AE, Macedo LD, Silva-Lovato CH, Pagnano VO, Watanabe E. Complete denture biofilm after brushing with specific denture paste, neutral soap and artificial saliva. Braz Dent J. 2013;24(1):47-52.
16. Pellizzaro D, Polyzois G, Machado AL, Giampaolo ET, Sanita PV, Vergani CE. Effectiveness of mechanical brushing with different denture cleansing agents in reducing in vitro Candida albicans biofilm viability. Braz Dent J. 2012;23(5):547-54.
17. de Sousa Porta SR, de Lucena-Ferreira SC, da Silva WJ, Del Bel Cury AA. Evaluation of sodium hypochlorite as a denture cleanser: a clinical study. Gerodontology. 2013 Dec 12.
18. Nishi Y, Seto K, Kamashita Y, Take C, Kurono A, Nagaoka E. Examination of denture-cleaning methods based on the quantity of microorganisms adhering to a denture. Gerodontology. 2012 Jun;29(2):e259-66.
19. Vieira AP, Senna PM, Silva WJ, Del Bel Cury AA. Long-term efficacy of denture cleansers in preventing Candida spp. biofilm recolonization on liner surface. Braz Oral Res. 2010 Jul-Sep;24(3):342-8.
20. Uludamar A, Ozkan YK, Kadir T, Ceyhan I. In vivo efficacy of alkaline peroxide tablets and mouthwashes on Candida albicans in patients with denture stomatitis. J Appl Oral Sci. 2010 May-Jun;18(3):291-6.
21. Savabi O, Attar K, Nejatidanesh F, Goroohi H, Badrian H. Effect of different chemical disinfectants on the flexural strength of heat-polymerized acrylic resins. Eur J Prosthodont Restor Dent. 2013 Sep;21(3):105-8.

22. Salloum AM. Effect of 5.25 % sodium hypochlorite on color stability of acrylic and silicone based soft liners and a denture base acrylic resin. J Indian Prosthodont Soc. 2014 Jun;14(2):179-86.
23. Felipucci DN, Davi LR, Paranhos HF, Bezzon OL, Silva RF, Pagnano VO. Effect of different cleansers on the surface of removable partial denture. Braz Dent J. 2011;22(5):392-7.
24. Jagger DC, Harrison A. Denture cleansing--the best approach. Br Dent J. 1995 Jun 10;178(11):413-7.
25. de Lucena-Ferreira SC, Ricomini-Filho AP, da Silva WJ, Cury JA, Del Bel Cury AA. Influence of daily immersion in denture cleanser on multispecies biofilm. Clin Oral Investig. 2014 Mar 4.
26. Lucena-Ferreira SC, Cavalcanti IM, Cury AA. Efficacy of denture cleansers in reducing microbial counts from removable partial dentures: a short-term clinical evaluation. Braz Dent J. 2013;24(4):353-6.
27. da Silva WJ, Goncalves LM, Seneviratne J, Parahitiyawa N, Samaranayake LP, Del Bel Cury AA. Exopolysaccharide matrix of developed Candida albicans biofilms after exposure to antifungal agents. Braz Dent J. 2012;23(6):716-22.
28. Niimi M, Firth NA, Cannon RD. Antifungal drug resistance of oral fungi. Odontology. 2010 Feb;98(1):15-25.
29. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of Candida biofilm drug resistance. Future Microbiol. 2013 Oct;8(10):1325-37.
30. Goncalves LM, Del Bel Cury AA, Sartoratto A, Garcia Rehder VL, Silva WJ. Effects of undecylenic acid released from denture liner on Candida biofilms. J Dent Res. 2012 Oct;91(10):985-9.
31. Ramage G, Robertson SN, Williams C. Strength in numbers: antifungal strategies against fungal biofilms. Int J Antimicrob Agents. 2014 Feb;43(2):114-20.
32. Nett JE. Future directions for anti-biofilm therapeutics targeting Candida. Expert Rev Anti Infect Ther. 2014 Mar;12(3):375-82.
33. Vieira DR, Amaral FM, Maciel MC, Nascimento FR, Liberio SA, Rodrigues VP. Plant species used in dental diseases: Ethnopharmacology aspects and antimicrobial activity evaluation. J Ethnopharmacol. 2014 Sep 29;155(3):1441-9.

34. Rios JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005 Aug 22;100(1-2):80-4.
35. Ahmad I, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J Ethnopharmacol. 2001 Feb;74(2):113-23.
36. Duarte MC, Figueira GM, Sartoratto A, Rehder VL, Delarmelina C. Anti-Candida activity of Brazilian medicinal plants. J Ethnopharmacol. 2005 Feb 28;97(2):305-11.
37. Ushimaru PI, Barbosa LN, Fernandes AA, Di Stasi LC, Fernandes A, Jr. In vitro antibacterial activity of medicinal plant extracts against Escherichia coli strains from human clinical specimens and interactions with antimicrobial drugs. Nat Prod Res. 2012;26(16):1553-7.
38. Mayaud L, Carricajo A, Zhiri A, Aubert G. Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. Lett Appl Microbiol. 2008 Sep;47(3):167-73.
39. Irkin R, Korukluoglu M. Effectiveness of Cymbopogon citratus L. essential oil to inhibit the growth of some filamentous fungi and yeasts. J Med Food. 2009 Feb;12(1):193-7.
40. Tadtong S, Watthanachaiyingcharoen R, Kamkaen N. Antimicrobial constituents and synergism effect of the essential oils from Cymbopogon citratus and Alpinia galanga. Nat Prod Commun. 2014 Feb;9(2):277-80.
41. Silva Cde B, Guterres SS, Weisheimer V, Schapoval EE. Antifungal activity of the lemongrass oil and citral against Candida spp. Braz J Infect Dis. 2008 Feb;12(1):63-6.
42. Abe S, Sato Y, Inoue S, Ishibashi H, Maruyama N, Takizawa T, et al. [Anti-Candida albicans activity of essential oils including Lemongrass (Cymbopogon citratus) oil and its component, citral]. Nihon Ishinkin Gakkai Zasshi. 2003;44(4):285-91.
43. Amornvit P, Choonharuangdej S, Srithavaj T. Lemongrass-Incorporated Tissue Conditioner Against Candida albicans Culture. J Clin Diagn Res. 2014 Jul;8(7):ZC50-2.
44. Korenblum E, Regina de Vasconcelos Goulart F, de Almeida Rodrigues I, Abreu F, Lins U, Alves PB, et al. Antimicrobial action and anti-corrosion effect against sulfate reducing bacteria by lemongrass (Cymbopogon

citratus) essential oil and its major component, the citral. AMB Express. 2013;3(1):44.

45. Sinha S, Jothiramajayam M, Ghosh M, Mukherjee A. Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes. Food Chem Toxicol. 2014 Jun;68:71-7.
46. Altarawneh S, Bencharit S, Mendoza L, Curran A, Barrow D, Barros S, et al. Clinical and histological findings of denture stomatitis as related to intraoral colonization patterns of Candida albicans, salivary flow, and dry mouth. J Prosthodont. 2013 Jan;22(1):13-22.
47. Paranhos H, F., Peracini A, Pisani MX, Oliveira Vde C, de Souza RF, Silva-Lovato CH. Color stability, surface roughness and flexural strength of an acrylic resin submitted to simulated overnight immersion in denture cleansers. Braz Dent J. 2013;24(2):152-6.
48. Goiato MC, Nobrega AS, dos Santos DM, Andreotti AM, Moreno A. Effect of different solutions on color stability of acrylic resin-based dentures. Braz Oral Res. 2014 Jan-Feb;28(1).

Anexo 1

CENTRO UNIVERSITÁRIO DO MARANHÃO - UNICEUMA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Potencial antifúngico de extratos naturais sobre biofilmes de Candida albicans desenvolvidos em superfície de poli(metil metacrilato)

Pesquisador: LETÍCIA MACHADO GONÇALVES

Área Temática:

Versão: 1

CAAE: 36679214.0.0000.5084

Instituição Proponente: Centro Universitário do Maranhão - UniCEUMA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 833.094

Data da Relatoria: 30/09/2014

Apresentação do Projeto:

A candidose associada ao uso de prótese dental, patologia prevalente em usuários de próteses totais e/ou parciais confeccionadas em poli (metil metacrilato), além de contribuir para a halitose, pode levar ao desenvolvimento de infecções sistêmicas. A Candida albicans atua como o principal agente etiológico, o que está diretamente relacionado a sua virulência e capacidade de aderência à superfície da prótese. Atualmente, o maior desafio no restabelecimento da saúde bucal destes pacientes está na resistência fúngica aos tratamentos convencionais. Neste contexto, a busca de substâncias alternativas que auxiliem na prevenção e tratamento desta infecção e que sejam acessíveis à população, como os extratos naturais, torna-se de grande importância na esfera médico-odontológica.Este trabalho tem como objetivo verificar o potencial antifúngico dos extratos naturais de Coriandrum sativum (coentro) e Cymbopogon citratus (capim-limão) sobre biofilmes de C. albicans desenvolvidos em superfície de poli (metil metacrilato). Este objetivo será alcançado avaliando-se primeiramente a susceptibilidade de células planctônicas de C. albicans através das análises de concentração inibitória mínima (CIM), concentração fungicida mínima (CFM) e tempo de morte celular (time-kill). A ação sobre os biofilmes será avaliada por meio de contagem celular, atividade metabólica e estrutura dos biofilmes. Inicialmente, a susceptibilidade de células planctônicas C. albicans (um isolado de referência e dois isolados clínicos) aos extratos será investigada através dos ensaios de CIM, CFM e time-kill. Para análise dos biofilmes, corpos-de-prova (cps) de resina acrílica a base de PMMA serão confeccionados conforme indicação dos fabricantes, e terão sua rugosidade de superfície padronizada. Após, sobre os cps contendo película de saliva humana, biofilmes de um isolado de referência (ATCC 90028) e dois isolados clínicos de C. albicans serão desenvolvidos. No grupo experimental, extrato de coentro ou capim-limão nas concentrações de CIM, 5xCIM ou 10xCIM será adicionado ao meio de cultura durante a formação dos biofilmes. O meio será trocado a cada 24 h e, após 72 h, os biofilmes desenvolvidos serão investigados quanto à contagem celular, atividade metabólica e estrutura. Todos os experimentos serão realizados em triplicata, em três momentos distintos.

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UF: MA **Município:** SAO LUIS
Telefone: (98)3214-4265 **Fax:** (98)3214-4212 **E-mail:** cep@ceuma.br

Objetivo da Pesquisa:

Este trabalho tem como objetivo verificar o potencial antifúngico dos extratos naturais de Coriandrum sativum (coentro) e Cymbopogon citratus (capim-limão) sobre biofilmes de C. albicans desenvolvidos em superfície de poli (metil metacrilato). Este objetivo será alcançado avaliando-se primeiramente a susceptibilidade de células planctônicas de C. albicans através das análises de concentração inibitória mínima (CIM), concentração fungicida mínima (CFM) e tempo de morte celular (time-kill). A ação sobre os biofilmes será avaliada por meio de contagem celular, atividade metabólica e estrutura dos biofilmes.

Avaliação dos Riscos e Benefícios:

Não existe nenhum tipo de risco previsível durante o exame clínico, preenchimento da ficha clínica e coleta da saliva ou isolados clínicos. Desse modo, sua participação neste estudo não oferece nenhum tipo de risco para a sua saúde. Além do mais, o tratamento odontológico que você irá receber é semelhante ao que você estaria recebendo se não fizesse parte desta pesquisa.

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma pesquisa relevante e com aplicabilidade na área odontológica.A pesquisa apresenta relevância científica e certamente contribuirá para esclarecer aspectos importantes a respeito do tema. A metodologia é adequada aos objetivos propostos. A equipe executora apresenta-se capacitada para desenvolver a pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos obrigatórios foram anexados devidamente preenchidos.

Recomendações:

Nenhuma

Conclusões ou Pendências e Lista de Inadequações:

Projeto aprovado

Situação do Parecer:

Aprovado

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Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

SAO LUIS, 15 de Outubro de 2014

Assinado por:
Eduardo Durans Figuerêdo
(Coordenador)

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Anexo 2

PLOS ONE Manuscript Guidelines

As of January 2015, our manuscript guidelines have been updated in line with our new submission requirements. Please review the information below to properly prepare and format your submission. For a detailed overview of what has changed, please see our PLOS Blogs post.

Format Requirements
Title
Authors and Affiliations
Abstract
Introduction
Materials and Methods
Results, Discussion, and Conclusions
Acknowledgments
References
Figure Legends

1. Format Requirements
PLOS ONE does not consider presubmission inquiries. All submissions should be prepared with the following files:

Cover Letter
You should supply an approximately one page cover letter that:

- Concisely summarizes why your paper is a valuable addition to the scientific literature
- Briefly relates your study to previously published work
- Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)
- Describes any prior interactions with PLOS regarding the submitted manuscript
- Suggests appropriate PLOS ONE Academic Editors to handle your manuscript (view a complete listing of our academic editors)
- Lists any opposed reviewers

Manuscript Organization
Manuscripts should begin with the ordered sections:
Title
Authors
Affiliations
Abstract
Introduction

Acknowledgments
References
Supporting Information Captions

Figures should be cited in ascending numeric order upon first appearance. Each figure caption should then be inserted immediately after the first paragraph in which it is cited in the article file.
Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file. Find more information about preparing figures here.
Tables should be cited in ascending numeric order upon first appearance. Each table should then be inserted immmediately after the first paragraph in which it is cited in the article file.
The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.
There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors' preference. In most cases, internal sections include:

Materials and Methods
Results
Discussion
Conclusions (optional)

Manuscript File Requirements
Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Word files must not be protected.

2. Guidelines for Standard Sections
Title
Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.
The full title must be 250 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Authors and Affiliations
All author names should be listed in the following order:
First names (or initials, if used),
Middle names (or initials, if used), and
Last names (surname, family name)
Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

This information cannot be changed after initial submission, so please ensure that it is correct.

Abstract
The abstract should:
Describe the main objective(s) of the study
Explain how the study was done, including any model organisms used, without methodological detail
Summarize the most important results and their significance
Not exceed 300 words

Abstracts should not include:
Citations
Abbreviations, if possible

Introduction
The introduction should:
Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
Define the problem addressed and why it is important
Include a brief review of the key literature
Note any relevant controversies or disagreements in the field
Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods
This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.
We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. Further information about formatting Supporting Information files, can be found here.
Methods sections of papers on research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.
Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication. A list of recommended repositories for different types of data can be found here.
Methods sections of papers using cell lines must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

Methods sections of papers adding new taxon names to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the PLOS ONE Publication Criteria for more information.

Acknowledgments

People who contributed to the work but do not fit the PLOS ONE authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named. Funding sources should not be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

References

General guidelines

- PLOS uses the reference style as outlined in the ICMJE sample references, also referred to as the “Vancouver” style.
- References must be listed at the end of the manuscript and numbered in the order that they appear in the text.
- In the text, citations should be indicated by the reference number in brackets.
- Authors may cite any and all available works in the reference list.
- Authors may not cite unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”).
- If an article is submitted to a journal and also publicly available as a pre-print, the pre-print may be cited.
- If related work has been submitted to PLOS ONE or elsewhere, authors should include a copy with the submitted article as confidential supplementary information, for review purposes only.
- Authors should not state 'unpublished work' or 'data not shown,' but instead include those data as supplementary material or deposit the data in a publicly available database.

- Journal name abbreviations should be those found in the NCBI databases.

Reference formatting
Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial. References should be formatted as follows:

Published papers.
1. Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun, B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588.

Websites or online articles
1. Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14.

Books
1. Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters

Deposited articles (preprints, e-prints, or arXiv)
1. Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014.

Figure Legends
Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here. Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations. Figures should be cited in ascending numeric order upon first appearance. Each figure caption should be inserted immediately after the first paragraph in which they are cited in the article file. Further information about figure captions can be found in the Figure Guidelines.

Supporting Information Captions
Because Supporting Information is accessed via a hyperlink attached to its captions, captions must be listed in the article file. Do not submit a separate caption file. It is acceptable to have them in the file itself in addition, but they must be in the article file for access to be possible in the published version.
The file category name and number is required, and a one-line title is highly recommended. A legend can also be included but is not required. Supporting Information captions should be formatted as follows.
Title is strongly recommended. Legend is optional.

Please see our Supporting Information guidelines for more details.

Tables

Tables should be cited in ascending numeric order upon first appearance. Each table should be inserted immediately after the first paragraph in which it is cited in the article file. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting Information. Please ensure that table formatting conforms to our Guidelines for table preparation.

3. Specific Reporting Guidelines

Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed

Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:

Why written consent could not be obtained

That the Institutional Review Board (IRB) approved use of oral consent

How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

Explicitly describe their methods of categorizing human populations

Define categories in as much detail as the study protocol allows

Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency

Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about PLOS ONE policies regarding human subject research, see the Publication Criteria and Editorial Policies.