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AVALIAÇÃO DE UM CONDICIONADOR TECIDUAL CONTENDO *TERPINEN-4-OL* E *CINAMALDEÍDO* ANTI-*Candida albicans*

Laura de Fátima Souto Maior

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Declaro para os devidos fins, que Laura de Fátima Souto Maior, defendeu o Trabalho de Dissertação do Programa de Pós-Graduação em Odontologia/UFPB - Nível Mestrado – Área de Concentração em Ciências Odontológicas, sob o Título: "Avaliação de um Condicionar Tecidual contendo Terpinen-4-ol e Cinamaldeído Anti Cândida *albicans*", no dia 27/01/2016. A candidata foi aprovada, devendo entregar toda a documentação exigida pelo Programa, e o resultado deverá ser homologado pelo Colegiado do Curso posteriormente.

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Universidade Federal da Paraíba – Centro de Ciências da Saúde
Programa de Pós-graduação em Odontologia – Campus I – Cidade Universitária
João Pessoa – PB CEP – 58051-900 Fone: (83) 3216-7797
www.ufpb.br/pos/odontologia

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Banca Examinadora

Prof. Dr. Paulo Rogério Ferreti Bonan
Orientador - UFPB

Prof. Dr. : Lúcio Roberto Cançado Castellano
Examinador - UFPB

Prof. Dr. Ronaldo Rodrigues Sarmento
Examinador externo

DEDICATÓRIA

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RESUMO

Introdução: estomatite protética é uma lesão multifatorial que ocorre no palato sob uma prótese removível. Tem como principal fator associado à infecção por *Candida albicans*. A incorporação de agentes antifúngicos a condicionadores de tecido para reembasamento de próteses tem sido uma alternativa para a redução do desenvolvimento de biofilmes aderentes em suas superfícies. *Terpinen-4-ol* e *Cinanamaldeído* são fitoconstituintes de óleos essenciais e apresentam inúmeras propriedades biológicas ativas, com destaque para efeitos antifúngicos e com potencial uso em condicionadores protéticos. **Objetivo:** comparar *in vitro*, a eficácia antifúngica dos fitoconstituintes em condicionador de tecido Softone e o efeito dessa modificação sobre a dureza Shore A. **Materiais e Métodos:** determinou-se a Concentração Inibitória Mínima (CIM) e a Concentração Fungicida Mínima (CFM) dos fitoconstituintes pela técnica de microdiluição e plaqueamento dos subcultivos. A mesma técnica foi utilizada para verificar se os fitoconstituintes interagem com a parede ou membrana da célula fúngica, na presença do sorbitol e ergosterol, respectivamente, determinando, assim, o mecanismo de ação. O método de difusão em ágar foi empregado para avaliação antifúngica dos fitoconstituintes isolados ou incorporados ao condicionador em diferentes concentrações (0.0156; 0.125; 0.25; 0.5; 1; 5;10; 20, 30 e 40%) e períodos (24, 48h, 4 e 7 dias). Em biofilme, a viabilidade celular foi determinada espectrofotometricamente pelo ensaio de redução do sal de tetrazólio (TTC), após 24h e 48h, nas mesmas concentrações do ensaio acima. Testes de dureza, em durômetro Shore A, foram realizados sobre os corpos modificados pelos fitoconstituintes (5, 10, 20, 30 e 40%) após imersão em saliva artificial a 37°C nos períodos de (baseline, 24, 48h, 4 e 7dias). O teste Kruskal-Wallis seguido de Wilcoxon (α=0,05) foi empregado no ensaio de disco difusão e biofilme. Para avaliação da dureza utilizou-se Análise de Variância (ANOVA) e pós teste de Tukey (α=0,05) **Resultados:** Os valores da CIM e da CFM foram de 1.125 e 2.250µg/mL para o *Terpinen-4-ol* e de 156 e 156µg/mL para o *Cinamaldeído*, respectivamente. Ensaios sobre o modo de ação sugerem que *Cinamaldeído* atue causando danos à membrana e a parede da célula fúngica. *Terpinen-4-ol* parece não ter atividade sobre as estruturas da célula fúngica aqui estudadas. Em ágar, os fitoconstituintes não mostraram atividade antifúngica expressiva até que a

concentração no condicionador fosse aumentada além de 5%. *Cinamaldeído* e *Terpinen-4-ol* (20-40%) quando misturados ao condicionador, inibiram completamente o crescimento fúngico. O ensaio de redução com TTC demonstrou que a incorporação de *Cinamaldeído* (10-40%) inibiu efetivamente a formação de biofilme nos dois períodos avaliados, e que, o condicionador modificado com *Terpinen-4-ol*, apresentou células viáveis até mesmo na concentração máxima (40%). A adição dos fitoconstituintes a Softone resultou no aumento da dureza do material durante os períodos de avaliação, no entanto, essa variação é considerada aceitável e não iterferiria com a utilização clínica de condicionador modificado em até sete dias. **Conclusão:** A incorporação de *Terpinen-4-ol* e *Cinamaldeído* ao material resiliente pode ser considerada como uma modalidade terapêutica promissora para a estomatite protética, com a vantagem de menor participação ativa dos pacientes.

Palavras-chave: Estomatite sob Prótese*, Candida albicans*, Reembasadores de Dentadura, *Terpinen-4-ol, Cinamaldeído,* Dureza.

ABSTRACT

Introduction: Denture Stomatitis it is a injury multifactorial what occurs at the palate under a prosthesis removable. Has as main factor associated the infection for *Candida albicans.* The incorporation in agents antifungals in tissue conditioners for relining the prosthetics has been a alternative for the reduction of development in biofilms adherents on their surfaces. *Terpinen-4-ol* and *Cinnamaldehyde* are phytoconstituents of essential oils with have several properties, especially antifungal effects and potential use in conditioners. **Objective:** to compare in vitro antifungal efficacy of phytoconstituents in conditioner Softone and the effect of such modification in Shore A hardness. **Methodology:** The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of phitoconstituents were determined by microdilution and plating of subcultures. The same technique was used to verify if the phitocontituents interact with the fungal cell wall or membrane, in the presence of sorbitol and ergosterol, respectively, thus determining the mecanism of action. The method of diffusion in agar was used for antifungal evaluation of isolated phitoconstituents or incorporated into the conditioner at different concentrations (0.0156; 0.125; 0.25; 0.5; 1; 5;10; 20, 30 e 40%) and periods (24, 48h, 4 e 7 dias). In biofilm the viability cell was determined spectrophotometrically in reduction of salt in tetrazolium (TTC) after 24 and 48h At the same concentrations in the above assay. Hardness tests in durometer Shore A were carried out on the specimens modified by phitoconstituents (5, 10, 20, 30 and 40%) after immersion in artificial saliva at 37°C in periods (baseline, 24, 48h, 4 and 7 days).. The Kruskal-Wallis and Wilcoxon (α=0.05) was used in the disk diffusion assay. To evaluate the hardness was used Analysis of Variance (ANOVA) and Tukey post test (α=0.05). **Results:** The values of MIC and MFC were 1.125µg / ml and 2.250µg / ml to *Terpinen-4-ol* and 156µg/mL and 156µg/mL for *Cinnamaldehyde*, respectively. Essays on the mode of action suggests that *Cinnamaldehyde* acts causing damage to the membrane and the wall of the fungal cell. *Terpinen-4-ol* seems to have no activity on fungal cell structure this study. In agar the phytoconstituents have not shown significant antifungal activity until the conditioner concentration was increased above 5%. *Cinnamaldehyde* and *Terpinen-4-ol* (20-40%) when mixed with the conditioner, completely inhibited fungal growth. The reduction assay ttc Demonstrated that incorporation of

Cinnamaldehyde (10-40%) effectively inhibited biofilm formation in both periods and, modified conditioner *Terpinen-4-ol* showed viable cells even at the maximum concentration (40%). The addition of the phytconstituents in Softone resulted in Increased hardness of the materials during the evaluation period, however, this variation is considered acceptable and non interfering with the clinical use of modified conditioner in seven days. **Conclusion:** The incorporation of *Terpinen-4-ol* and *Cinnamaldehyde* in tissue conditioner can be considered a promising therapeutic modality for denture stomatitis, with the advantage of less active Involvement of the patients.

Key-words: Denture Stomatitis, *Candida albicans*, Denture Liners, *Terpinen-4-ol*, C*innamaldehyde*, Hardness.

SUMÁRIO

1. INTRODUÇÃO

A estomatite protética (EP) consiste em uma inflamação crônica na mucosa de suporte de próteses dentárias removíveis, caracterizada clinicamente por áreas eritematosas de diferentes graus (NEWTON, 1962; WILSON,1998). A doença representa a lesão bucal mais frequentemente encontrada nos usuários, apresentando uma prevalência de até 65% (WILLIAMS and LEWYS, 2011). A combinação entre fatores sistêmicos como imunidade comprometida, deficiências nutricionais, desordens endócrinas, antibioticoterapia e, fatores locais como trauma, uso contínuo da prótese, alergias, higiene insuficiente e alterações no fluxo e no ph salivar, afetam o equilíbrio comensal predispondo a EP. (WILSON,1998; WEBB *et al*.,1998).

Embora apresente etiologia multifatorial, o estabelecimento de um biofilme patogênico na base da prótese tem sido o fator de maior importância a ser considerado (BULAD *et al*., 2004; CHANDRA *et al*., 2001; REDDING *et al*., 2009). Este biofilme é constituído por uma heterogeneicidade de microrganismos, sendo a *Candida albicans* o mais prevalente (PEREIRA *et al*., 2013). A associação entre o fungo patógeno, a mucosa de suporte e a superfície de acrílico da prótese, determina a chamada estomatite protética associada à *Candida spp* (WEBB *et al*., 1998; RAMAGE *et al*., 2001; BULAD *et al*., 2004). *C. albicans* é um fungo polimórfico, membro da microbiota oral em indivíduos saudáveis, quando apresentado sob a forma de levedura e, considerado patógeno oportunista, sob a forma de hifas, crescendo excessivamente, invadindo tecidos da mucosa, especialmente quando a resistência do hospedeiro é superada pela virulência do microrganismo (PEREIRA *et al*., 2013). Os fatores de virulência mais importantes relacionados a este fungo são a capacidade de adesão, organização em biofilmes e produção de enzimas hidrolíticas (RAMAGE *et al*., 2001)

Estudos apontam que o tratamento da EP associada à *Candida* deva ser primariamente direcionado à prótese, pois a presença de colônias de leveduras aderidas ao acrílico causa reinfecção da mucosa (JEPSON *et al*.,1993; CHANDRA *et al*., 2001; BULAD *et al*., 2004; REDDING *et al*., 2009; PEREIRA *et al*., 2013). A conduta clínica convencional é bastante variada, podendo incluir terapia antifúngica tópica, medicação antifúngica sistêmica, procedimentos de limpeza e desinfecção das próteses, reparo e substituição (WEBB *et al*.,1998;

WILSON,1998; NEPPELENBROEK *et al*., 2008). Materiais reembasadores protéticos, como os condicionadores de tecido, tem sido frequentemente utilizados como terapia adjunvante para EP, melhorando a adaptação e reduzindo as tensões suportadas pelos tecidos (REDDING *et al*., 2009; RADNAI *et al*., 2010).

Os condicionadores de tecido são materiais temporários, caracteristicamente macios, constituídos de um pó, polímero poli (etilmetacrilato/metilmetacrilato) e um líquido (éster aromático e etanol), resultando em um gel (PARKER and BRADEN, 2001), empregados com o propósito de amortecerem os impactos mastigatórios, reestabelecendo a saúde da mucosa (HANS *et al*., 2002). No entanto, esses materiais apresentam limitações de ordem físico-biológica que comprometem sua utilização. Alterações a nível dimensional, descoloração, pigmentação, aumento da dureza são alguns dos problemas apresentados quando do seu uso, tornando suas superfícies rugosas, posteriormente facilitando a aderência e a colonização microbiana (BROW, 1988; JEPSON *et al*., 1993; LONEY *et al*., 2000). Na tentativa de reduzir ou evitar a proliferação de microrganismos na superfície das próteses, autores vem investigando a incorporação de agentes com ação antimicrobiana a materiais macios, como os condicionadores de tecido (CATALÁN *et al*., 2008; RADNAI *et al*., 2010; FALAH-TAFT I *et al*., 2010; SHARMA and HEGDE, 2014; BUENO *et al*., 2015).

Neste contexto, eficácia de diferentes concentrações de Nistatina e Fluconazol incorporados ao condicionador Acrosoft® contra *C. albicans* foi avaliada. Em todas as concentrações testadas, a Nistatina conseguiu inibir completamente a ligação e a colonização do fungo ao condicionador tecidual, mas no caso do Fluconazol, somente a concentração de 10% mostrou uma inibição completa da colonização fúngica (FALAH-TAFTI *et al*., 2010). A atividade antifúngica *in vitro* do óleo de *Melaleuca alternifolia* e Fluconazol incorporados a um condicionador de tecido foi avaliada ao longo de sete dias. Ambos constituintes mostraram atividade antifúngica comparável nas primeiras 24h. Foi observado também, que ao sétimo dia o Fluconazol havia perdido substancialmente seu efeito antifúngico, fato evidenciado pelo recrescimento de *C. albicans*. O óleo da Melaleuca se manteve ativo, mostrando valores constantes (SHARMA and HEGDE, 2014).

Apesar do comprovado efeito antimicrobiano dos materiais modificados como método de liberação de fármacos prevenindo o acúmulo do biofilme, tem-se observado que a incorporação desses agentes pode afetar a matriz polimérica, resultando em alterações nas propriedades de resistência à tração, dureza e rugosidade superficial (URBAN *et al*., 2006). Estudos revelam aumento da resiliência de materiais macios quando misturados a agentes antifúngicos ao longo do tempo (JADHAV *et al*., 2013). Manter preservadas as propriedades desses materiais e ao mesmo tempo conservar o potencial antimicrobiano no combate aos microrganismos associados à EP seria o idealmente o necessário.

A resistência de *C. albicans* aos antifúngicos sintéticos tem sido reportada, o que impõe a necessidade pela busca de novas drogas (HITCHCOCK, 1993; CHANDRA *et al*., 2001). Metabólitos de óleos essenciais se tornaram grandes alvos por apresentarem comprovada atividade antifúngica, podendo ser utilizados como meios de prevenção e controle de infecções bucais (KHAN *et al*., 2012; VASCONCELOS *et al*., 2014; LEITE *et al*., 2014). *Terpinen-4-ol* e *Cinamaldeído* correspondem a um dos principais fitoconstituintes dos óleos essenciais da *Melaleuca alternifolia* e *Cinnamomum* spp, respectivamente, sendo considerados compostos majoritários, possuindo amplo espectro de atividades biológicas com destaque para efeitos antifúngicos. A *Melaleuca alternifolia* pertencente à família Myrticeae é uma planta nativa do nordeste australiano, comumente conhecida como “árvore do chá”, fluorescendo em áreas tropicais e subtropicais (HAMMER *et al.,* 2004; NINOMIYA *et al*., 2012). O gênero *Cinnamomum* (Lauraceae), originário de algumas regiões da Índia e da China é produtor do óleo essencial da canela, obtido da casca de *Cinnamomum zeylanicum* ou de *Cinnamomum cássia* (LIMA *et al*., 2005; TAGUCHI *et al*., 2012).

Terpinen-4-ol tem grande capacidade de penetração em mucosa, podendo ser eficaz para uso oral contra hifas de *Candida* (NINOMIYA *et al*., 2012). Apresenta também ação imunomoduladora, sendo indicado como um possível agente terapêutico para doenças inflamatórias (NOGUEIRA *et al*., 2014). *Cinamaldeído* foi considerado candidato promissor como agente antifúngico no tratamento da candidíase contra isolados de várias espécies de *Candida* resistentes ao Fluconazol (SHREAZ *et al.,* 2011). Investigando os efeitos de *Cinamaldeído* no combate ao crescimento de hifas de *C. albicans,* este fitoconstituinte foi capaz de inibir o fungo (TAGUCHI *et al*., 2012).

Tendo em vista que os microrganismos conseguem se infiltrar no material que constitui a própria prótese e ali perdurar por muito tempo, e, considerando, nesta perspectiva, as irregularidades do condicionador de tecido como facilitador do acúmulo, numa realidade em que o uso de prótese e a falta do manejo correto são coexistentes, a relevância deste estudo se sobrepõe, principalmente diante de uma escassez de relatos na literatura sobre o efeito da interação de materiais reembasadores que contêm fitoconstituintes frente à *C. albicans,* e sobre possíveis alternativas à resolução de tal problemática. É nesse enfoque que o presente estudo pretendeu avaliar de forma comparativa a atividade anti *C. albicans* de dois fitoconstituintes de óleos essenciais, o *Terpinen-4-ol* e o *Cinamaldeído*, incorporados ao condicionador de tecido Softone e, o efeito dessa modificação sobre a dureza Shore A do material.

2. CAPÍTULO

Antifungal activity and Shore A hardness of a tissue conditioner incorporated with Terpinen-4-ol and Cinnamaldehyde

Laura de Fátima Souto Maior, DDS[a], Panmella Pereira Maciel, DDS[a], Victor Yuri Nicolau Ferreira, DDS[a], Cíntia de Lima Gouveia Dantas, DDS, MSc[a], Jeferson Muniz de Lima, DDS, MSc[a], Lúcio Roberto Cançado Castellano, MSc, PhD[b], André Ulisses Dantas Batista DDS,MSc, PhD[c], Paulo Rogério Ferreti Bonan, DDS,MSc, PhD[d]

School of Dentistry, Federal University of Paraiba

Castelo Branco, Joao Pessoa, Paraiba, Brazil

[a] Graduate student, Dentistry Post-graduation Program, Federal University of Paraiba. E-mail: laurasoutomaior.lsm@gmail.com; panmellmaciel@hotmail.com; victor_dinrt@hotmail.com; cintialgouveia@hotmail.com; jefferson.idalino@gmail.com

[b]Professor, Dental Graduation Program and Health Technical School, Federal University of Paraiba. E-mail: luciocastellano@gmail.com

[c]Professor, Dental Graduation Program and Department of Restorative Dentistry, Federal University of Paraiba. E-mail: andreulisses@yahoo.com.br

[d]Professor, Dental Graduation Program and Department of Clinic and Social Dentistry, Federal University of Paraiba. E-mail: pbonan@yahoo.com

Corresponding author:

Laura de Fátima Souto Maior

Federal University of Paraiba

Campus I / Cidade Universitaria - Joao Pessoa /PB – Brazil

E-mail: laurasoutomaior.lsm@gmail.com

ABSTRACT

Purpose: This study investigated the anti-*Candida* activity and the Shore A hardness of a tissue conditioner (Softone^{TM}) modified by incorporation of terpinen-4-ol and cinnamaldehyde.

Material and methods: Agar diffusion, microdilution and mechanism of action methods were performed to determine to evaluate the antifungal activity of phytoconstituents. Then, phytoconstituents in varying concentrations were incorporated into the tissue conditioner. The anti-*Candida* effect of the modified conditioner was evaluated through agar punch well and biofilm formation methods. Shore A hardness of the experimental liners was evaluated after baseline, 24h, 48h, 4 days and 7 days immersion on artificial saliva.

Results: The phytoconstituents incorporated into Softone showed completely inhibited fungal growth in concentrations of 20-40% and did not present significant antifungal activity until their concentrations where higher than 5%. There were differences between non-modified Softone and M5; M10; C10 and T10% ($p<.05$). The groups containing 10-40% of cinnamaldehyde incorporated into Softone were able to completely inhibit the biofilm. Concentrations below 40% of Terpinen-4-ol showed unsatisfactory biofilm inhibition. The T40% and C40% groups presented the lowest Shore A hardness values. Hardness values from groups T40% at 7 days ($p=.476$), C40% at 4 days ($p=.058$) and T20% ($p=.058$), C20% ($p=.205$), T30% ($p=.154$) and C30% ($p=.874$) after 48 hours, did not differ from the Control group.

Conclusions: Cinnamaldehyde incorporated into Softone inhibited *Candida* biofilm formation at concentrations of 10-40%, being more effective than terpinen-4-ol modification despite of halo inhibition observed by both products.

Clinical Relevance: All modifications showed a very similar pattern of hardness being useful for clinical practice.

Keywords: *Candida*; Denture Stomatitis; cinnamaldehyde; terpinen-4-ol; tissue conditioner.

CLINICAL IMPLICATIONS

The incorporation of Cinnamaldehyde into tissue conditioners may be a promising alternative for the treatment of Denture Stomatitis due to biofilm inhibition and anti-*Candida* effects.

INTRODUCTION

Denture Stomatitis (DS) is the inflammatory process most frequently observed in removable denture users[1]. It presents a multifactorial etiology, combining systemic and local factors such as chronic diseases, immunosuppression, xerostomia, poor hygiene, prosthesis trauma, and continued use of a removable dental prosthesis[2]. The formation of a biofilm on the denture base is the major factor in the origin, establishment, and maintenance of the disease[3–5]. In such biofilms, *Candida albicans* stands out as being the most common species[5]. *C. albicans* is a commensal fungus of the oral cavity, and its pathogenicity is attributed to both immunodeficiency and local chronic irritation[5,6].

Studies show that treatment of *Candida*-associated DS should be primarily directed to the prosthesis, and there are several treatment options available[3–5]. Prosthodontic denture materials such as tissue conditioners have often been used as an alternative therapy for DS, improving adaptation and reducing the stresses supported by the tissues[4,7,8]. These materials are temporary soft materials, composed of a powder, polymer poly (ethyl methacrylate/methyl methacrylate), and a liquid (aromatic ester and ethanol), that when mixed, results in a gel[9]. Dimensional changes, discoloration, pigmentation, and increased hardness are some of the problems presented by these materials during clinical use, making their surfaces rougher, which subsequently facilitates microbial colonization[10,11].

The incorporation of antimicrobial agents into tissue conditioners have been investigated[7,12–15]. The effectiveness of different concentrations of nystatin and fluconazole mixed with conditioner Acrosoft against *C. albicans* was evaluated, and nystatin could completely inhibit fungal binding and colonization of the tissue conditioner, but fluconazole, only in a concentration of 10%, showed complete inhibition of fungal colonization[13]. The *in vitro* antifungal activity of the phytotherapeutic oil of *Melaleuca alternifolia* and fluconazole incorporated to Viscogel conditioner was also evaluated, showing promising results of this herbal agent on microbial colonization [14].

C. albicans resistance to conventional drugs induces the search for new antimicrobial agents. The metabolites of plant essential oils have become significant targets[16,17]. Terpinen-4-ol and cinnamaldehyde are respectively the major phytoconstituents of *Melaleuca alternifolia*, and *Cinnamomum spp*, essential oils, and are considered to have broad spectrums of biological effects, like antifungals agents, for example[18–20]. The plant *Melaleuca alternifolia*, belonging to the Myrtaceae family is native to northeastern Australia and is commonly known as the "tea tree," fluorescing in tropical and subtropical areas[18,21]. The genus *Cinnamomum* (Lauraceae), is a native of parts of India and Ceylon, and it is a producer of cinnamon essential oil obtained from the bark of either *Cinnamomum zeylanicum* or *Cinnamomum cassia*[20,22].

Considering the limitations of tissue conditioners and their importance in the treatment of DS, the objective of this study was to evaluate the *in vitro* inhibition of *Candida albicans* growth and the Shore A hardness of a conventional tissue conditioner (Softone; Bosworth Co.) modified by the incorporation of two essential phytoconstituents, terpinen-4-ol and cinnamaldehyde at various concentrations and times of evaluation. The null hypotheses were that the incorporation of terpinen-4-ol and cinnamaldehyde into the tissue conditioner

would have no effect on the colonization of *Candida albicans* on the tissue conditioner and would affect the Shore A hardness of the experimental material.

MATERIALS AND METHODS

Two types of phytoconstituents and a tissue conditioner (Softone) described in Table 1 were used.

Microorganisms

The standard reference strain of *Candida albicans* used in this study was obtained from the American Type Culture Collection (ATCC 11006). Suspensions of the reference strain were prepared in Sabouraud Dextrose Broth (SDB, Culture Medium; KASVI) to a concentration of approximately 10^6 CFU/mL (530 nm, abs 0.08–0.1, 0.5 of McFarland scale)[23].

Screening to evaluate the antifungal activity of Terpinen-4-ol and Cinnamaldehyde

The agar diffusion method was used for antifungal evaluation of the phytoconstituents [24]. For this, 20mL of Sabouraud Dextrose Agar (SDA, Culture Medium; HIMEDIA Put Laboratories Ltd), was melted and cooled to 45-50°C and dispensed in sterile Petri dishes. Upon solidification of the agar, 1mL of fungal suspension at a concentration of 10^6 CFU/mL was inoculated. The phytoconstituents were first diluted in sterile distilled water and 5% of Tween 80 (Sigma-Aldrich), to a pattern concentration of 15% [25]. The density of each of the substances was taken into consideration. Sterile 6 mm diameter filter paper discs were soaked in 50µL of 15% phytoconstituents and placed on the plate containing medium and inoculum. The assay was performed in triplicate (n=3). As positive control, 0.1% Miconazole (Dilecta) and as negative control, sterile distilled water and 5% of Tween 80 were used.

After 48h at 37°C, the diameters of the inhibition zones of *C. albicans* growth were measured at four distinct points for each disk, in millimeters, using a digital caliper (0.01mm/0.0005") (Stainless hardened; ZAAS-10004). The arithmetic means of the measurements obtained for each disc was performed for the calculation of the diametric image.

Determination of Minimum Inhibitory Concentration (MIC) by broth microdilution technique

The MIC of the phytoconstituents was determined using the microdilution technique according to the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI)[23]. Briefly, an initial volume of SDB was added to 96-well U-bottom microdilution plates. Then phytoconstituents at a 16% concentration were placed into the first well of the plate and serially diluted. *C. albicans* inoculum was added to each well at a final concentration of 2.5 x 10^3 CFU/mL. The plates were incubated at 37ºC for 24 h. Miconazole at 0.1% was used as positive control and a solution of distilled water and Tween 80 at 5% as negative control. Also, microbial viability and sterility controls were checked. Aliquots of 50µL of TTC (2,3,5-triphenyl tetrazolium chloride dye; Sigma-Aldrich) were added to all wells to verify microbial viability. The microplates were incubated for another 24h[26]. The MIC corresponded to the lowest concentration of phytoconstituent that inhibited the visible growth of the studied strain. The assay was performed in triplicate (n=3).

Determination of Minimum Fungicide Concentration (MFC)

According to the MIC results, aliquots from the wells corresponding to the MIC and the two immediately higher concentrations were subcultured on Sabouraud Dextrose Agar

(SDA, Culture Medium; HIMEDIA Put Laboratories Ltd) plates and incubated at 37°C for 24h[27]. The Minimum Fungicidal Concentrations (MFC) was considered as the lowest concentration that prevented the visible growth of subculture on the solid medium.

Tests to evaluate the mechanism of action

Sorbitol and ergosterol assays were performed to indicate the antifungal mode of action of phytoconstituents against specific targets, whether it involved a direct interaction with the C. albicans cell wall structure (sorbitol assay) or the ionic permeability of the membrane of this microorganism (ergosterol assay).[28]

Sorbitol Test

The same microdilution technique was used to determine the MIC of the phytoconstituents in the presence of Sorbitol (D-sorbitol Anhydrous; INALAB), an osmotic protector. For this assay, the inoculum of *C. albicans* was prepared with sorbitol at a final concentration of 0.8 M [17,29]. As a positive control, 5µl/mL Caspofungin (Sigma-Aldrich) was used, due to its known enzyme activity which disturbs the integrity of the yeast cell wall[28]. Viability, Sterility, and Negative Controls were also conducted. The microplates were incubated at 37°C for 24h with 50µL of TTC dye added to each well, and the inhibition of growth was evaluated after incubation for 24h at 37°C.The assay was performed in triplicate (n=3).

Ergosterol Test

To verify the phytoconstituents interaction with exogenous ergosterol (Sigma-Aldrich), the microdilution technique was used as earlier described. Initially, ergosterol was prepared and diluted with 96% Ethanol (Sigma Aldrich), and Tween 80. From this mixture, ergosterol was diluted at concentrations of 200 and 400 µg/mL in SDB. Two inoculum cultures were obtained according to the concentrations determined at 37°C for 48h. The assay was performed in triplicate for each concentration. For Positive Control Nystatin (Neo Chemical) was used and for negative control 96% ethanol and Tween 80, which were

used for the preparation of ergosterol solutions. Growth and Sterility Controls were also performed. As with the previous assay, TTC was added to all wells, and final reading of the results was carried out at 48h[17,29].

Agar Diffusion test of the experimental liners

Cinnamaldehyde concentrations of 0.0156; 0.125; 0.25; 0.5; 1; 5; 10; 20, 30 and 40% were tested (C0.0156%; C0.125%; C0.25%; C0.5%; C1%; C5%; C10%; C20%; C30% and C40% groups, respectively) and Terpinen-4-ol (T0.125%; T0.25%; T0.5%; T1%; T5%; T10%; T20%; T30% and T40% groups). The T0.0156% group was not tested, because the MIC for terpinen-4-ol, was higher, as observed in the previous screening.). The groups M1%; M3%; M5% and M10% (Miconazole at concentrations of 1, 3, 5 and 10%, respectively) were used as positive control and the non-modified Softone tissue conditioner as negative control.

The Agar diffusion test was performed using a modification of the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI)[24]. Briefly, after solidification of the agar, 0.5 mL of the fungal suspension 10^6 UFC/mL was inoculated. After drilling the SDA with a steel die (6mm diameter), Softone tissue conditioner was mixed and inserted into the wells on agar culture plates.[13] It was established a proportion of 1.5 v/v (powder/liquid). For each 1mL of the tested solutions, 0.75mL was composed of the conditioner liquid, and 0.25mL of varying concentrations of phytoconstituents, homogenized in a sterile glass beaker for 30s. The powder was added and mixed according to the manufacturer's instructions. The modified material was transported and dispensed into the wells. The assay was performed in triplicate for each concentration tested (n=3). The plates were incubated at 37°C and after 24h, 48h, 4 and 7 days fungal growth inhibition halo diameters were measured, in millimeters, using a digital caliper as previously described.

Cell Adhesion testing and Biofilm Formation

Preparation of test specimens

In this test, all groups of the previous test were used, in addition to Nystatin as a positive control. Six discs for each concentration of the test materials and controls were evaluated. Stainless steel rounded matrixes of 10mm diameter and 1.5mm thick previously sterilized were used for specimens preparation. Powder and liquid were dispensed and mixed as previously described. After the manipulation, the resulting mixture was placed in the centers of the matrices and pressed between two sterile glass coverslips and maintained until gelation was completed (4-5min) (n=6).

Cell Adhesion and Biofilm Formation

Specimens were placed in 24-well culture plates containing 1mL of the previously standardized fungal suspension (10^6 UFC/mL). The specimens fully submerged were incubated in an orbital incubator under a constant rate of 100 rpm at 37°C for 2h, to promote the adhesion of microorganisms to the surface of the specimen. After the set time for cell adhesion, the specimens were removed and gently washed in another 24-well plate containing 1mL of PBS buffer solution (phosphate buffer solution), to remove non-adhered cells. After washing, the specimens were immersed in 1mL of SDB culture medium and incubated for 24h and 48h at 37°C for biofilm formation[15].

Colorimetric evaluation and quantification of cells

The colorimetric tetrazolium salt reduction method was used for quantification of the fungal biofilm formed on the specimens. For this, TTC solution at a concentration of 1mg/mL, glycosylated PBS solution at 200mM and menadione solution (0.007g of powder in 100mL of acetone at 0.4mM) suitably mixed were used, which allowed the analysis of the metabolic activity of the viable cells in the formed biofilm [15,30].

After the 24h and 48 h incubation, specimens were removed from the culture medium and transferred to a 24-well plate containing 1mL of TTC stock solution, prepared as described. The plates were then placed in an incubator at 100rpm, at 37°C for 3h to complete dye diffusion in the biofilm. After, an aliquot of 800µL of the content corresponding to each sample was transferred into microtubes and centrifuged at 100 rpm for 2 min in a centrifuge for cell sedimentation. After it, 100µL of the resulting supernatant was collected and dispensed in a 96-well culture plate for absorbance reading by spectrophotometer (Glomax-Mult; Promega Ltd). The absorbance value of the TTC solution was also measured, and used as a reference value (blank), corresponding to the minimum value to be subtracted from the absorbance of the other supernatant readings [15].

Shore A hardness test

Preparation of test specimens

To evaluate the effect of the addition of phytoconstituents in the Shore A hardness of experimental liners, tests were performed at baseline, 24h, 48h, 4 days and 7 days immersion in artificial saliva (Dilecta, Brazil) at 37°C. Specimens of modified conditioner species (70mm x 50mm x 6mm) were made through a prefabricated PVC matrix held down for two bolted glass plates[31]. The handling of the material occurred as shown in the above assays. Five concentrations of terpinen-4-ol and cinnamaldehyde were used (5, 10, 20, 30 and 40%); two for Miconazole (5 and 10%) and Nystatin 100,000 IU/mL (n=12).

Shore A Hardness measurements

Considering the specifications of ISO (the International Organization for Standardization) 10139-2[32] 12 hardness measurements were performed on each specimen, with at least 6mm distance between them, and 12mm from the edge[33] using a portable digital durometer (Shore A; Tecno TEC-35539A). Hardness values were determined for each group by the

average of the measurements obtained, in Shore A unit on a scale of 0 to 100, for each period.

Statistical analysis

Data were analyzed by IBM SDSS Statistics 20 (IBM Corporation). Differences between the *C. albicans* inhibition zones in the diffusion test and *C. albicans* biofilm formation from control and experimental groups were determined by the nonparametric Kruskal-Wallis test followed by Wilcoxon test. Shore A hardness alterations were evaluated by two-way analysis of variance (ANOVA) and Tukey post-test for multiple comparisons. The significance level was 5% (α=.05).

RESULTS

Screening to evaluate the antifungal activity of Terpinen-4-ol and Cinnamaldehyde

The studied phytoconstituents presented inhibition halos on the inoculum of *C. albicans*. The 15% terpinen-4-ol resulted in inhibition halos that ranged from 6.32mm to 11.11mm. The 15% cinnamaldehyde resulted in immensurable inhibition halos, with overlapping halos boundaries, indicating a high effect against *C. albicans* and high diffusion in the culture medium. The negative control (distilled water and Tween 80) did not present inhibition halos, as expected. The positive control (Miconazole 0.1%) resulted in inhibition halos ranging from 23.11 mm to 33.03 mm.

Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs)

The MIC and MFC values of phytoconstituents against *C. albicans* ranged from 156 to 2.250 mg/mL, as shown in Table 2. The MFC/MIC ratio indicated that the phytoconstituents used had a fungicidal activity against *C. albicans* (MFC/MIC < 4)[34]. The methodology was validated by the absence of fungal growth in the sterility control and the

presence of viable strains in the growth control. It was observed that the Positive Control (0.1% Miconazole) affected the growth of the fungal strain. The vehicle (distilled water and Tween 80) used for the preparation of the phytoconstituents emulsion did not affect microbial growth.

Mechanisms of Phytoconstituents Action

Two possible mechanisms of action of phytoconstituents in the *C. albicans* strain (disturbance of cell wall biosynthesis or cell membrane permeability) were studied. The results showed that cinnamaldehyde might act on the cell wall of *C. albicans*, such as caspofungin, used as a positive control, since the susceptibility of the strains to these materials was decreased in the presence of sorbitol, an osmotic protector (Table 3). Higher concentrations of cinnamaldehyde (> MIC) were required to disturb the protected cell wall when compared to medium with no sorbitol[28]. It was also observed an increase in MIC for the cinnamaldehyde with addition of exogenous ergosterol (Table 3), which suggests that this phytoconstituent binds to the exogenous ergosterol, and this could potentially lead to the formation of pores in the membrane and the perturbation of ionic permeability[28], as observed for Nystatin, used as a positive control for this mechanism of action. Terpinen-4-ol showed no increase in the MIC when supplemented with exogenous sorbitol or ergosterol, which may suggest that its antifungal mechanism may be dependent on other factors, then the disturbance of cell wall biosynthesis or cell membrane permeability.

Agar diffusion test for the experimental liners

The results for the agar diffusion test are shown in Table 4. It was observed that the inhibitory effect on *C. albicans* growth was dose-dependent, as the wider halos were formed in groups with increased concentrations. The phytoconstituents did not present significant antifungal activity until their concentrations where higher than 5%. There was a

statistically significant difference between non-modified Softone (control) and M5; M10; C10 and T10% ($p<0.05$), for all time-periods evaluated (24, 48h, 4 and 7 days. The group T10% had the same results than M5% after 48h, 4 days and 7 days.

Cinnamaldehyde and terpinen-4-ol in concentrations of 20-40% when added to the conditioner completely inhibited fungal growth, and therefore were not evaluated statistically. Terpinen-4-ol in concentrations of 5 and 10% showed complete inhibition of fungal growth in the period of 24h, but in the periods of 48h, 4 days and 7 days there was fungal growth with the formation of inhibition halos. This finding indicates a fungiostatic effect of Terpinen-4-ol at concentrations of 5 and 10%. The C10% group showed statistical similarity to the group M10% for all evaluated times and presented inhibition halos larger than the M10% after 48h, 4 days and 7 days. For Miconazole, the degree of inhibition increased as the concentration increased, but with a reduction in the fungicidal effect.

Cellular Quantification

The results of the reduction assay (colorimetric method tetrazolium) with supplemented in 24h and 48h is demonstrated in Table 5. The phytoconstituents incorporated into Softone had no significant biofilm inhibitory effect at concentrations below 5%, as well as miconazole. The groups containing concentrations of 10-40% of Cinnamaldehyde incorporated into Softone were statistically similar to each other and to the groups M5%, M10% and Nystatin at times of 24h and 48h. In addition, they were able to completely inhibit the biofilm and had the lowest absorbance values (approximately zero). The T40% group showed statistical difference for the C10% groups ($p = .006$); C20-40% ($p = .004$); M5% ($p = .045$) and Nystatin ($p = .013$) in the 24h period, but presented a statistical similarity for the C10% groups; C30%; C40%; M10% and Nystatin ($p > .05$) in the 48h period. Concentrations of less than 40% Terpinen-4-ol incorporated into the tissue conditioner showed no satisfactory inhibition of biofilm.

Shore A hardness test

Shore A hardness values of all groups and time intervals are presented in Table 4. Two-way ANOVA showed that both factors (materials and time interval) were significant in Shore A hardness values changes of the evaluated groups ($p < .05$). Groups M5%, C5%, and C10% presented the highest Shore A values after 7 days (21.66; 21.75 and 21.75, respectively) and had no statistical difference between them ($p > .05$) (Figure 4).

Control, T40% and C40% groups presented the lowest Shore A hardness values after all time interval tested (24h, 48h, 4days and 7days), with exception for the control group at 48h. Hardness values from groups T40% at 7 days (p=.476), C40% at 4 days (p=.058) and T20% (p=.058), C20% (p=.205), T30% (p=.154) and C30% (p=.874) after 48 hours, did not differ significantly from the Control group.

DISCUSSION

Many synthetic drugs are used in the treatment of fungal infections and fungal resistance to these drugs has been reported in the literature. The difficulty in treating Candida infections is due to their exchange between yeast and hyphae forms which facilitates the development of a biofilm, antifungal resistance factor[35]. Thus, there is an urgent need for new antifungal agents in the fight against *Candida* infections[35,36]. Studies on active constituents of natural medicines promise to be the solution[36]. Many plants show significant anti-*Candida* activities, making them promising candidates for therapy[35]. Two phytoconstituents, cinnamaldehyde[16,19,20,22] and terpinen-4-ol1[18,21], were selected for the present research, because of their biological effects, demonstrated in previous studies.

The agar diffusion test, used for microbiological screening of antimicrobial substances[37] evaluates the antimicrobial activity by the measurement of inhibition halos around the specimens[38]. In the present study, 15% Cinnamaldehyde resulted in broad and immensurable inhibition halos, with overlapping halos boundaries, indicating potential

fungicidal activity and adequate diffusion in the solid medium, since it presented zones of inhibition larger than 14 mm in diameter, considered as high anti-*Candida* activity[37].

The results corroborate Shreaz *et al*. (2011)[19], in which, cinnamaldehyde in a solid medium completely inhibited the growth of *Candida* species, indicating potential fungicidal activity. Also, cinnamaldehyde was considered a strong fungal inhibitor (MIC less than 500µg/mL), according to Aligiannis et al. (2001)[27] As for 15%, terpinen-4-ol there was a reduced response, with halos were smaller halos, ranging from 6.32mm to 11.11mm. However, when the MIC of Terpinen-4-ol was evaluated, a relatively low concentration was observed, with Terpinen-4-ol being considered a moderate inhibitor since the MIC value ranged between 600 and 1500 µg/mL, according to the classification of Aligiannis et al. (2001)[27]. This can be explained by the difficult diffusion of the terpenoid on the culture medium, preventing physical contact with the microorganism, as well as by its volatility characteristics, which may have interfered with the results[14]. Miconazole presented halos ranging from 23.111mm to 33.033mm, with an inhibitory response predominantly observed with the presence of turbid halos, indicating the fungistatic nature of the azole[1]. The pure Softone conditioner presented small halos of inhibition in 24h that increased in 48h, seen as well in the results obtained by Bueno *et al*., (2015)[15].

Cinnamaldehyde had lower MIC and MFC values, representing greater antifungal activity than Terpinen-4-ol. This finding corroborates the studies of Ninomiya et al. (2012) [21] and Shreaz et al. (2011) [19] in resistant strains. Ninomiya *et al*. (2012)[21] found MIC values for Terpinen-4-ol of 1250µg/mL and 5000µg/mL in resistant strains of *Candida*. Shreaz *et al.* (2011)[19] studied the effect of Cinnamaldehyde against resistant *Candida* isolates, obtaining MICs from between 100-500µg/mL.

Tests with sorbitol and ergosterol provide information about the mode of action of the studied phytoconstituents[17,29]. In this study, the results, due to the increase in MIC values

showed that cinnamaldehyde antifungal activity seems to be related to the biosynthetic pathways of the cell wall as well as to the ionic permeability of the membrane for the strain tested, according to the positive controls used. Cinnamaldehyde for acting on the cell wall of *C. albicans*, an essential structure for the survival of fungal strains becomes an important target for clinical application since the cell wall is absent in mammalian cells[39]. Khan et al. (2012)[16], also points out that Cinnamaldehyde has a strong antifungal action related to inhibition of ATPase in the plasma membrane and intracellular pH reduction. According to the results, terpinen-4-ol appears to have no action on the biosynthetic pathways of the cell wall nor the ion permeability of membrane for the studied strain. In the study by Hammer et al. (2003)[18], demonstrated that components of Melaleuca oil increase the permeability of yeast cells and the fluidity of the plasma membrane, but this mechanism of action was not related to terpinen-4-ol. Moreover, in the study by Hammer et al. (2003)[18] was also discussed that the components of this oil have several antifungal mechanisms not yet understood.

The experimental liners (Softone) display maximum effect between 24h and 72h[14]. According to the manufacturer, Softone was formulated to remain functioning for four to seven days. It was selected for the study because it presents no intrinsic antifungal activity [15]. The product´s instructional material does not report any compound with antimicrobial action in its composition; even so, we observed the presence of inhibition halos on agar, probably caused by the plasticizers leaching. Concentrations tested were selected because of MIC and MFC values obtained in this study, values of the previous studies[7,12,20,40], and the standard therapeutic dosage of the commercially used synthetic antifungal.

The incorporation of antifungal agents into to tissue conditioners is considered to be a reliable and promising treatment option for treatment of Denture Stomatitis [41]. Many studies have evaluated the incorporation of a variety of medications as Nystatin[12,13,15,42],

Miconazole[7,15,42], Fluconazole[13,40], Chlorhexidine[15,31,42] among others, in addition to products of natural origin, including essential oils as Melaleuca alternifolia oil[12,25,40,43] and Origanum oil[44], which contain terpinen-4-ol in its composition. However, the incorporation of phytoconstituents derived from essential oils, such as Terpinen-4-ol and Cinnamaldehyde, into tissue conditioners have not been evaluated.

To simulate a possible *in vitro* treatment for Denture Stomatitis, antifungal agents were incorporated into tissue conditioners, that may act as drug delivery systems during their clinical use [13,40,45]. Promising results were observed with cinnamaldehyde-modified Softone in concentrations of 5 up to 40%, demonstrating a potent anti-Candida effect (inhibition zones greater than 14mm)[27], there by exhibiting adequate inhibition by contact, necessary for topical treatments. For Miconazole, the degree of inhibition increased with the increase in concentration, but with a reduction in fungicidal effect, results also observed by Radnai*et al*. (2010)[7], when incorporating Miconazole into Visco-gel.

Metabolic activity of viable cells in the biofilm was assessed by colorimetric assay and quantification of the mass of these cells by spectrophotometry. The higher absorbance values demonstrate presence of viable cells, therefore the greater cellular activity implies higher staining and consequently higher values of absorbance[15,30]. The results of the reduction test demonstrated that the antimicrobial effect of the incorporation of cinnamaldehyde depends on the incorporated concentration, corroborating with the study of Taguchi et al. (2013) [22] when using cinnamaldehyde in *Candida albicans* cultures. Incorporation of cinnamaldehyde into soft liner in concentrations of 10%-40% completely inhibited the viability of the tested strain.

The terpinen-4-ol modified conditioner presented viable colonies even in its concentration of 40%. In the study by Sharma and Hegde, (2014) [14], the most effective concentration of Melaleuca alternifolia oil against *C. albicans* was 30%. *Melaleuca* oil (20% v/v), when

mixed with Coe-Comfort conditioner was effective in the treatment of Denture induced Stomatitis *in vivo*[12], but *in vitro* concentrations of up to 40% of Terpinen-4-ol incorporated into Softone were not effective. This effect may be related to other compounds of the oil[40]. Miconazole was effective at concentrations of 5%, significantly reducing the fungal biofilm mass and remaining constant until evaluation at 48h. Similar results were obtained by Lamfon *et al*. (2004)[46], nystatin showed values nearly identical without relatively large time related fluctuations. Studies also prove the effectiveness demonstrated by nystatin when incorporated into a resilient material[13].

Although the microbiological tests demonstrate advantages in inhibiting the *C. albicans* growth when antimicrobial agents were incorporated on tissue conditioners, the effect of this addition can affect the Shore A hardness of the materials. During clinical use, the conditioners are already prone to dimensional instability caused by loss of plasticizer and the fluid absorption, leading to an increase of its hardness, gradual loss of cushioning effect and degradation [10,11,33]. The ability to present and adequate hardness in tissue conditioners, given its provisional nature, is a challenge. According to Craig (1997)[47] and Pavan et. al. (2007)[48] there is no clinically stablished ideal Shore A hardness value for temporary resilient materials.

Much of Shore hardness variability can be explained by the amount of plasticizer in tissue conditioner, which has the function to keep the flexibility of polymer chains preventing polymer crosslinking, which increases the rigidity of the material[33]. Plasticizers have the function of reducing the temperature of vitreous transition of the polymer making the soft material[10]. Softone presents dibutyl phthalate and butyl the benzoate with low molecular weight as a plasticizer, being easily leached in the oral cavity, leading to an increase in hardness and resulting in short-time clinical use[49], as shown in table 6. It was possible to see that groups incorporated with lower concentrations of phytoconstituents presented the

highest hardness values, comparable to nystatin groups, an standard antifungal often evaluated in similar studies as the Urban *et al*., (2015)[42]. There was avisible tendency of hardness decrease when increasing percentage of phytoconstituents were added, which can be explained by the lipophilic characteristic of this products which can function as plasticizers in the polymer matrix, compensating the reduction of plasticizer added in the mixture, keeping the softness of the material. In this study, despite the decreased resilience of the modified tissue conditioner over the seven day of evaluation, hardness Shore A values fall within the ISO specification for this category of materials[32]. Considering further research previously carried out[31] it is possible to suggest that the variations found in increased hardness would not compromise the clinical use of conditioner modified by phytoconstituents, within the period tested (7 days), which provides important data for a potential future use of an phytocosntituents modified tissue conditioner for the *in vivo* treatment of Denture Stomatitis.

The Terpinen-4-ol incorporated conditioner presented an unsatisfactory anti-Candida effect, since concentrations below 40% did not prevent biofilm formation. In addition, a statistically significant increase in Shore A hardness over time was observed, although this increase is within the specifications of the ISO standard and has occurred for all groups addressed, including the non-modified conditioner. The use of cinnamaldehyde-modified conditioner has presented an excellent anti-Candida effect in concentrations of 10 to 40% (Table 5).

The Cinnamaldehyde proved to be effective as an additive for tissue conditioning, highlight the group C10%. This group was able to inhibit the formation of biofilm and demonstrated statistical similarity to Nystatin and Miconazole, drugs used clinically in Candidiasis.

Ainda é necessário determinar os possíveis efeitos tóxicos desses fitoconstituintes nas células, para avaliar suas potenciais aplicações clínicas. Ensaios recentes em fibroblastos, apontam atividade citotóxica de Terpinen-4-ol, semelhante à Clorexidina, Maquera-Huacho et al., (2018)[50]. Ausência de toxicidade significativa de Terpinen-4-ol para *C. albicans* foi encontrada por Ramage et al., (2012) [51] fornecendo evidências *in vitro* adicionais, de que este derivado pode ser adequado para a profilaxia e tratamento da candidose orofaríngea estabelecida. García-Salinas et al., (2018)[52] demonstraram que a citotoxicidade de Cinnamaldehyde, in vitro, utilizando fibroblastos, macrófagos e linhas celulares de queratinócitos, exibiu citocompatibilidade, com menor toxicidade celular do que a Clorexidina convencional amplamente utilizada, contra infecções mediadas por bactérias.

The limitations of the present study are its *in vitro* design, which limits the extrapolation of this results to clinical situations, as well the lack of individual features such as the buccal environment, the role of immune system, patient cleaning habits, among other aspects. Further *in vitro* research to evaluate other characteristics of the Cinnamaldehyde-modified experimental conditioner are suggested, such as cytotoxicity, bond to denture-base acrylic resin and abrasion resistance, as well as clinical trials, to evaluate the clinical use of this material as alternative of treatment for Denture Stomatitis .

CONCLUSIONS

The agar diffusion antimicrobial effect of the experimental tissue conditioner resulted C. albicans growth complete inhibition for concentration of 20, 30 and 40% Cinnamaldehyde or Terpinen-4-ol. Cinnamaldehyde incorporated experimental liners at concentrations of 10-40% prevented *C. albicans* biofilm formation. Shore A hardness of control and all experimental groups increased during a 7-day period, however, this increase is within the

clinical parameters of the ISO standard, and the clinical significance of this alterations remains to be evaluated.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

Author Laura de Fátima Souto Maior, DDS[a] declares that he has no conflict of interest. Author Panmella Pereira Maciel, DDS[a] declares that he has no conflict of interest. Author Victor Yuri Nicolau Ferreira, DDS[a] declares that he has no conflict of interest. Author Cíntia de Lima Gouveia Dantas, DDS, MSc[a] declares that he has no conflict of interest. Author Jeferson Muniz de Lima, DDS, MSc[a] declares that he has no conflict of interest. Author Lúcio Roberto Cançado Castellano, MSc, PhD[b] declares that he has no conflict of interest. Author André Ulisses Dantas Batista DDS,MSc, PhD[c] declares that he has no conflict of interest. Author Paulo Rogério Ferreti Bonan, DDS,MSc, PhD[d] declares that he has no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required. Informed consent was obtained from all individual participants included in the study.

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Table 1. Description of composition, manufacturer and batch number of the resin-based denture soft lining material and the phytoconstituents used in the study.

Material	Composition	Manufacturer	Batch number
Softone	Powder: Poly (methyl methacrylate) and loading agents Liquid: Alkyl phthalate (plasticizer) and ethyl alcohol	Bosworth Company, Skokie, IL, USA	1306-255
Terpinen-4-ol (≥95%)	Liquid Density: 0.93 g/mL	Sigma- Aldrich Ltda, São Paulo Brazil	BCBF8537V
Cinnamaldehyde (≥93%)	Liquid Density: 1.05 g/mL	Sigma- Aldrich Ltda, São Paulo Brazil	MKBN5878V

Table 2. Minimum Inhibitory and Fungicidal Concentrations (MIC/MFC) of phytoconstituents against *C. albicans* inoculum. The MIC and MFC values are expressed as μg/mL.

Phytoconstituents (μg/mL)	***C. albicans***	
	MIC	**MFC**
Terpinen-4-ol	1,125	2,250
Cinnamaldehyde	156	156

Table 3. Effect of Sorbitol (osmotic protector) and different concentration of exogenous ergosterol on the MIC of phytoconstituents and positive controls (Caspofungin and Nystatin, respectively) against *C. albicans*.

Phytoconstituents	**Absence of Sorbitol and Ergosterol**	**Sorbitol 0.8M**	**Ergosterol 100µg/mL**	**Ergosterol 200µg/mL**
Terpinen-4-ol	1,125 µg/mL	1,125 µg/mL	1,125 µg/mL	281 µg/mL
Cinnamaldehyde	156 µg/mL	312 µg/mL	312 µg/mL	312 µg/mL
Caspofungin	<0.0006µg/mL	>5 µg/mL	-	-
Nystatin	<2.77 µg/mL	-	5.55 µg/mL	5.55 µg/mL

Table 4. Inhibition halos (mm) formed over some period seven days in for the concentrations of the tested groups. Control (non-modified Softone tissue conditioner); Miconazole (M); Cinnamaldehyde (C); Terpinen-4-ol (T).

Groups	**Time Interval**							
	24 h		**48 h**		**4 d**		**7 d**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Control	$9.52^{A,a}$	0.38	$11.24^{A,B,a,b,c,d}$	2.28	$11.97^{B,a,d,e,f,g}$	2.28	$11.03^{A,B,b,c}$	1.41
M1%	$9.24^{A,a,h}$	1.10	$9.36^{A,a,e}$	0.47	$9.57^{A,a}$	0.42	$8.86^{A,a}$	0.32
M3%	$16.08^{A,b,c}$	2.74	$15.36^{A,b,f}$	5.15	$15.56^{A,e,i,g,j}$	4.91	$14.26^{A,c,g,h,i}$	5.32
M5 %	$20.00^{A,b}$	1.70	$19.41^{A,f}$	1.23	$20.22^{A,i}$	1.53	$20.62^{A,g,j}$	1.49
M10%	$25.42^{A,g}$	0.84	$23.93^{B,g}$	0.54	$27.16^{A,l}$	1.53	$25.7^{A,g,k}$	0.34
C0.0156%	$9.53^{A,a,i}$	0.21	$10.18^{A,B,a,h,m}$	0.61	$10.55^{Ba,c,b}$	0.71	$9.67^{A,B,b,f,h}$	0.35
C0.125%	$11.31^{A,d}$	0.94	$9.65^{B,e,i,m}$	0.15	$10.48^{A,a,b}$	0.11	$10.29^{A,b,c}$	0.69
C0.25%	$11.12^{A,B,d,e}$	0.94	$9.54^{B,a,h,i}$	0.88	$11.03^{A,d,h}$	0.45	$9.12^{B,a,b,d,h}$	1.04
C0.5%	$10.47^{A,d,e,h,i}$	1.43	$10.04^{A,a,h,i}$	0.61	$10.65^{Ac,d,b}$	0.70	$10.20^{A,b,c}$	0.65
C1%	$9.97^{A,a,d,h}$	1.25	$10.14^{A,a,h,i,j,l}$	0.69	$11.44^{A,d,h}$	0.58	$9.93^{A,b,d,h}$	0.82
C5%	$19.28^{A,c,f}$	3.27	$15.39^{A,b}$	1.69	$17.06^{A,j,k}$	1.78	$15.72^{A,i}$	2.00
C10%	$22.42^{A,f,g}$	2.39	$25.41^{B,g}$	3.57	$27.70^{C,l}$	3.53	$25.89^{B,C,k,l}$	3.94
T0.125%	$10.70^{A,B,a,d}$	1.07	$10.17^{B,c,h}$	0.16	$10.58^{A,b,f}$	0.64	$10.93^{A,B,b,c}$	1.15
T0.25%	$9.80^{A,B,C,a,e}$	0.57	$9.55^{B,a,i}$	0.29	$10.23^{C,c}$	0.16	$9.59^{A,B,b}$	0.20
T0.5%	$10.03^{A,a,d}$	1.30	$10.88^{A,B,d,h}$	0.72	$12.43^{B,g,h}$	0.91	$11.00^{A,B,b,c,f}$	1.01
T1%	$10.33^{A,a,d}$	1.29	$12.49^{A,B,b,j}$	2.12	$13.69^{B,g,k}$	2.12	$11.87^{A,B,c,d}$	0.97
T5%	$>40^{A,j}$	0.00	$14,51^{B,b,l}$	1.26	$16.61^{C,j,k}$	0.95	$15.22^{B,C,i}$	2.18
T10%	$>40^{A,j}$	0.00	$17.67^{B,f}$	0.51	$21.85^{C,i}$	1.46	$22.20^{C,j,l}$	1.48

Vertically, identical superscripted small letters denote no significant differences among groups ($p < 0.05$). (Kruskal-Wallis and Wilcoxon post-test).

Horizontally, identical superscripted small letters capital letters denote no significant differences among time intervals ($p < 0.05$). (Kruskal-Wallis and Wilcoxon post-test).

Table 5. Absorbance values indicating the relative amount of viable cells (inhibition of the *C. albicans* biofim) for the groups tested after the incubation times of 24 and 48 hours. Control (non-modified Softone tissue conditioner); Nystatin 100,000 IU/mL; Miconazole (M); Cinnamaldehyde (C); Terpinen-4-ol (T).

Groups	**Time Interval**			
	24 h		**48 h**	
	Mean	**SD**	**Mean**	**SD**
Control	$0.615^{A,b}$	0.069	$0.708^{B,m,n}$	0.062
Nystatin	$0.001^{A,a}$	0.002	$0.021^{B,a,b}$	0.016
M1%	$0.471^{A,d,e}$	0.041	$0.598^{B,c,h,i,j}$	0.050
M3%	$0.525^{A,b,c}$	0.083	$0.604^{B,c,l}$	0.067
M5%	$0.003^{A,a}$	0.008	$0.013^{B,a}$	0.002
M10%	$0.011^{A,a,m}$	0.025	$0.015^{B,a,b}$	0.003
T0.125%	$0.421^{A,d,f}$	0.066	$0.656^{B,f,h,l,m}$	0.055
T0.25%	$0.509^{A,c,e,g}$	0.106	$0.578^{A,c,g,i,k}$	0.032
T0.5%	$0.394^{A,d,f}$	0.105	$0.618^{B,e,f,h,l,m}$	0.024
T1%	$0.255^{A,h,i}$	0.097	$0.614^{B,e,f,h,k,l}$	0.028
T5%	$0.427^{A,c,d,e}$	0.147	$0.644^{B,e,f,h,l}$	0.070
T10%	$0.283^{A,h}$	0.081	$0.583^{B,e,g,i,l}$	0.039
T20%	$0.179^{A,h,j}$	0.149	$0.526^{B,c,g,i}$	0.128
T30%	$0.103^{A,k}$	0.058	$0.575^{B,c,d,e,f,h}$	0.107
T40%	$0.048^{A,k,m}$	0.057	$0.069^{A,b}$	0.045
C0.0156%;	$0.285^{A,f,g,h,l}$	0.113	$0.649^{B,d,e,g,i,l,m}$	0.064
C0.125%;	$0.278^{A,h}$	0.061	$0.671^{B,d,j,k,l,m,n}$	0.072

C0.25%,	$0.192^{A,h,k}$	0.082	$0.621^{B,d,e,g,i,l}$	0.044
C0.5%	$0.205^{A,h,l}$	0.055	$0.646^{B,d,j,l}$	0.032
C1%;	$0.176^{A,i,j,k,l}$	0.052	$0.643^{B,c,g,I,k,l,m}$	0.087
C5%	$0.220^{A,h,k}$	0.116	$0.763^{B,n}$	0.078
C 10%	$0,000^{A,a}$	0.002	$0.014^{B,a,b}$	0.002
C 20%	$0,000^{A,a}$	0.001	$0.011^{B,a}$	0.002
C 30%	$0,000^{A,a}$	0.001	$0.018^{B,a,b}$	0.010
C 40%	$0,000^{A,a}$	0.001	$0.013^{B,a,b}$	0.002

Vertically, identical superscripted small letters denote no significant differences among groups ($p < 0.05$). (Kruskal-Wallis and Wilcoxon post-test).

Horizontally, identical superscripted small letters capital letters denote no significant differences among time intervals ($p < 0.05$). (Kruskal-Wallis and Wilcoxon post-test).

Table 6. Shore hardness representation as a function of the time intervals. Groups tested: Control (non-modified Softone tissue conditioner); Nystatin 100,000 IU/mL; Miconazole (M); Cinnamaldehyde (C); Terpinen-4-ol (T).

Groups	**Time Interval**									
	Baseline		**24 h**		**48 h**		**4 d**		**7 d**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Control	$4.96^{A,a}$	0.69	$9.04^{B,a}$	0.89	$14.38^{C,b,c}$	1.26	$14.29^{C,a}$	1.47	$15.54^{D,a}$	1.03
Nystatin	$10.92^{A,e,f}$	1.24	$13.25^{B,e}$	2.19	$17.75^{C,e,f}$	2.43	$18.67^{C,d}$	1.91	$17.88^{C,b}$	1.33
M 5 %	$13.46^{A,g,h}$	0.99	$15.75^{B,j,k,l}$	1.57	$19.21^{C,g}$	1.44	$20.79^{D,f}$	1.10	$21.67^{D,g}$	1.07
M 10%	$11.58^{A,f,g}$	0.76	$14.50^{B,f,g,h}$	1.51	$19.00^{C,g}$	1.19	$19.88^{C,D,e,f}$	1.61	$20.17^{D,f}$	1.81
C 5%	$14.54^{A,i}$	0.78	$16.67^{B,l}$	0.86	$17.75^{C,e,f}$	1.21	$20.75^{D,f}$	1.56	$21.75^{D,g}$	1.03
C 10%	$14.33^{A,h,i}$	0.91	$15.13^{A,h,i,j}$	1.37	$17.04^{B,d,f}$	0.96	$18.92^{C,d,e}$	1.78	$21.75^{D,g}$	1.01
C 20%	$11.04^{A,e,f}$	0.65	$13.13^{B,e}$	1.21	$13.71^{B,b}$	1.34	$16.96^{C,c}$	1.40	$18.13^{D,b,c}$	1.00
C 30%	$10.38^{A,e}$	0.74	$14.08^{B,c,e,g}$	0.41	$14.29^{B,b}$	1.74	$17.04^{C,c}$	1.42	$19.13^{D,c,e}$	1.19
C 40%	$8.08^{A,c}$	1.56	$10.92^{B,b,c,d}$	0.87	$12.08^{C,a}$	1.50	$13.29^{D,a}$	1.29	$17.29^{E,b}$	1.14
T 5%	$12.58^{A,g}$	0.92	$15.17^{B,h,i,k}$	1.68	$16.42^{C,d}$	1.78	$18.88^{D,d,e}$	0.57	$19.04^{D,c,d}$	1.18
T 10%	$11.04^{A,e,f}$	1.01	$13.92^{B,e,f,i}$	0.67	$17.00^{C,d,e}$	1.04	$16.83^{C,c}$	2.04	$17.50^{C,b}$	1.36
T 20%	$11.54^{A,f}$	1.29	$14.54^{B,f,g,i}$	1.23	$15.38^{B,c}$	1.28	$17.08^{C,c}$	1.16	$19.88^{D,d,e,f}$	1.43
T 30%	$9.25^{A,d}$	0.78	$11.92^{B,d}$	1.00	$13.63^{C,b}$	1.02	$16.79^{D,c}$	1.01	$17.38^{D,b}$	1.30
T 40%	$6.92^{A,b}$	0.51	$10.63^{B,b}$	0.91	$11.67^{C,a}$	1.91	$15.63^{D,b}$	1.00	$15.17^{D,a}$	1.19

Vertically, identical superscripted small letters denote no significant differences among groups ($p < 0.05$). (Two-way ANOVA and Tukey test).

Horizontally, identical superscripted small letters capital letters denote no significant differences among time intervals ($p < 0.05$). (Two-way ANOVA and Tukey test).

Figure 1. Agar Diffusion Assay of Phytoconstituents in triplicate. (a) Cinnamaldehyde (C), presented completely clear and immeasurable halos and (b) Terpinen-4-ol (T), the halos varied between 6.32mm and 11.11mm. PC: Positive Control (Miconazole 0.1%); NC: Negative Control (distilled water and Tween 80).

Fig. 1

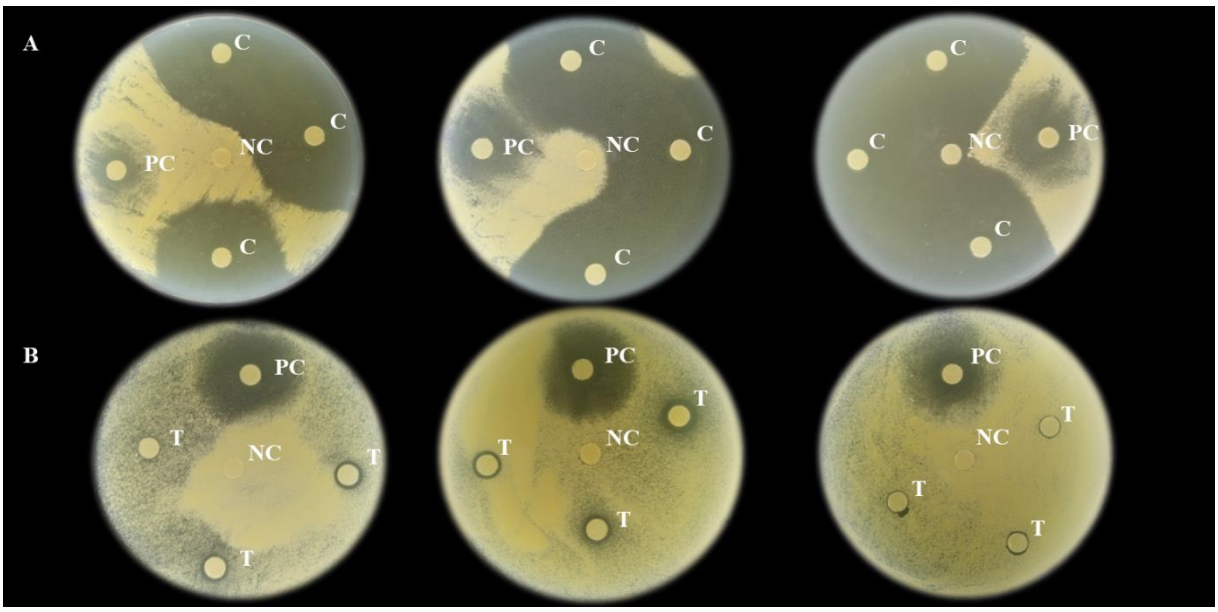
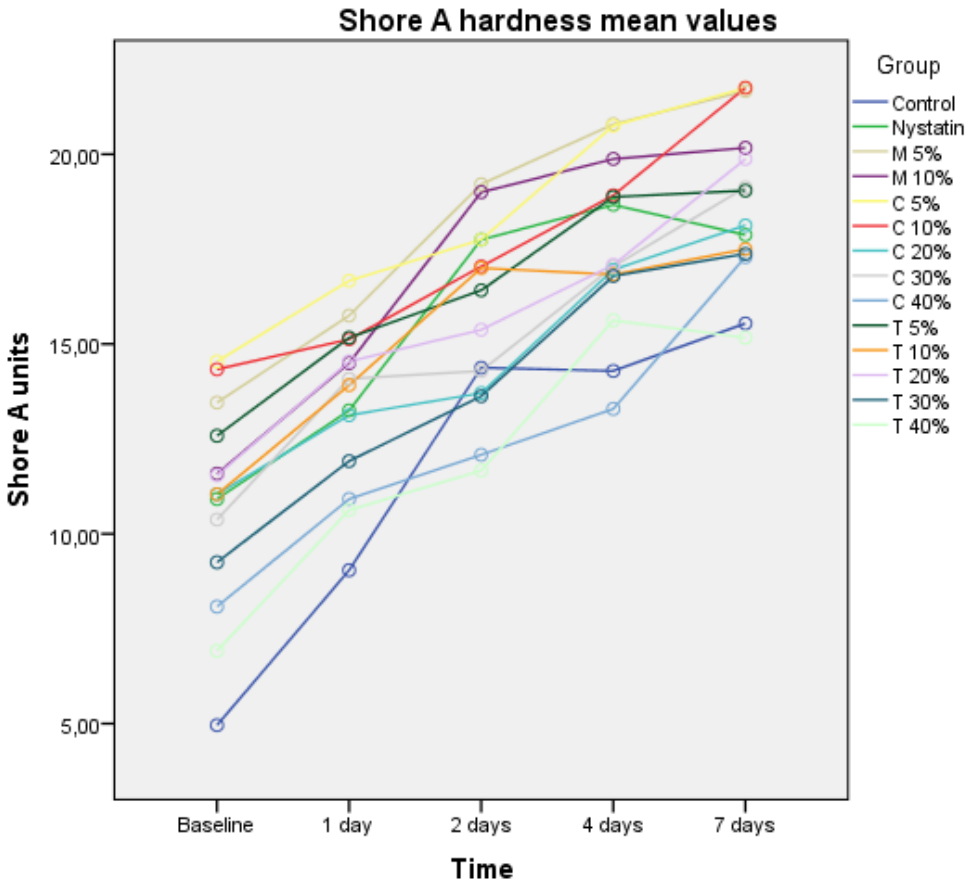


Figure 2. Representation of Shore A hardness values for all groups and over time (1 to 7 days). Control (non-modified Softone tissue conditioner); Nystatin 100,000 IU/mL; Miconazole (M); Cinnamaldehyde (C); Terpinen-4-ol (T).

Fig. 2



3. CONSIDERAÇÕES GERAIS

As falhas nas terapias convencionais para a EP leva a busca por novas técnicas de intervenção clínica. A adição de agentes com ação antimicrobianaa materiais resilientes para reembasamento em aparelhos protéticos,tem sido uma terapia alternativa no combate ao biofilme formado nas superfícies das próteses removíveis, em pacientes que são acometidos pela lesão, até a troca por um novo aparelho. Assim como destaca a literatura, e, dentro das condições avaliadas nesta investigação *in vitro,* os resultados obtidos apontam que ambos os fitoconstituintes apresentaram ação anti *C. albicans* isolados ou quando incorporados ao condicionador Softone, com destaque para o *Cinamaldeído* que se mostrou eficaz em todos os testes empregados, com expressiva atividade antifúngica, despontando como possível agente terapêutico. O efeito da adição dos fitoconstituintes sobre a dureza shore A do material revelou um aumento progressivo da dureza em relação ao tempo independente do tipo de substância incorporada, entretanto, essa variação não seria considerada expressiva o suficiente para interferir com o uso clínico, uma vez que, os valores estão totalmente dentro dos padrões determinados pela ISO. Não obstante, mais estudos *in vitro* são necessários visando à inibição de outros microrganismos presentes no biofilme, bem como, a avaliação de outros condicionadores teciduais, com o objetivo de justificar e validar o uso clínico do condicionador modificado. Testes físicos para avaliação da rugosidade também devem ser realizados, bem como, testes toxicológicos e de liberação contolada. Desta feita, e, considerando ainda o aumento de cepas resistentes aos medicamentos antifúngicos comerciais,*Terpinen-4-ol* e *Cinamaldeído* incorporados ao material resiliente podem representar relevante significância para aplicação no tratamento da EP.

4. CONCLUSÃO

A incorporação de *Terpinen-4-ol* e *Cinamaldeído* ao condicionador tecidual Softone, foi efetiva contra a cepa fúngica testada, promovendo poucas alterações na dureza do material, as quais se situaram dentro dos limites clinicamente aceitáveis. Essa técnica pode ser considerada como uma modalidade terapêutica promissora para a EP, com a vantagem de requerer menor participação ativa dos pacientes.

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