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PRÓPOLIS: PRODUTO NATURAL COM ATIVIDADE ANTIBIOFILME SOBRE O GÊNERO *Candida*

CAROLINA RABELO FALCÃO BEZERRA

São Luís - MA
2020

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PRÓPOLIS: PRODUTO NATURAL COM ATIVIDADE ANTIBIOFILME SOBRE O GÊNERO *Candida*.

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Saúde do Adulto da Universidade Federal do Maranhão para obtenção do Título de Mestre em Saúde do Adulto.

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Orientador: Profª Drª Geusa Felipa de Barros Bezerra.
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Dedico este trabalho ao Pedro, que deu novo sentido à minha vida e me fez descobrir minha melhor versão: ser mãe.

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"Quando os ventos das mudanças sopram, umas pessoas levantam barreiras, outras constroem moinhos de vento."

Érico Veríssimo

RESUMO

A própolis verde, tem sido uma opção terapêutica devido a sua diversidade fitoquímica e antifúngica com ação sobre diferentes espécies de *Candida*, demonstrando efeitos fungistático e fungicida satisfatórios, tanto em experiência *in vitro* quanto *in vivo.* Uma forte relação entre presença de aparatologia ortodôntica fixa e aumento do risco ao favorecimento de estagnação de biofilme tem demonstrado que os fungos possuem fatores de virulência associado ao desenvolvimento de doença humana e aumento da resistência a agentes antimicrobianos. Este estudo avaliou a influência do extrato etanólico de própolis verde na adesão e biofilme de *Candida albicans* ATCC 443-805-2, *Candida tropicalis* ATCC 1036-09-2 e *Candida parapsilosis* ATCC 726-42-6 em material odontológico. O extrato etanólico de própolis verde (EEPV) foi preparado a partir de 200 g da própolis verde diluído em 500 mL de álcool etílico PA, armazenado em frasco âmbar e conservado a temperatura ambiente, com agitação por 2h/dia durante 8 dias. Posteriormente foi rotoevaporado e liofilizado e a análise fitoquímica foi realizada por cromatografia líquida de alta eficiência. A aderência das espécies de *Candida* foi realizada em fragmentos de aço inoxidável e resina acrílica e quantificada em câmara de Neubauer contando o número de células leveduriformes aderidas aos fragmentos. A formação de biofilme foi determinada pela contagem do número de unidades formadoras de colônias (UFC). A intensidade da adesão e formação do biofilme foi classificada em negativa, fraca, moderada, forte e muito forte. Quinze compostos foram identificados no extrato de própolis verde, tendo como majoritários 3-hidroxibiochanina A, Galato do trímero [epi]catequina e Ácido Carmínico. As espécies estudadas foram capazes de aderir e formar biofilme na superfície de aço inoxidável e resina acrílica e a intensidade de adesão das células leveduriformes foi fraca em todos os tempos de incubação, com exceção de *C. parapsilosis* e *C. tropicalis* que em 12 h apresentou intensidade moderada. Quanto à formação de biofilme (24 e 48 h) observou-se no metal que a *C. albicans* teve intensidade moderada em 24 e 48 h; *C. parapsilosis* em 24 e 48 h teve intensidade muito forte; *C. tropicalis* em 24 h teve intensidade forte e em 48 h muito forte. Enquanto que na resina, todas as espécies nos tempos 24 e 48 h tiveram intensidade forte, exceção da *C. tropicalis* que em 48 h teve intensidade muito forte. O extrato de própolis verde apresentou atividade antifúngica e foi capaz de inibir tanto a adesão quanto a formação de biofilme a partir de 2,5 µg/mL. Este estudo respalda a hipótese de que a própolis verde possui atividade antifúngica e interfere nos fatores de virulência de *C. albicans*, *C. parapsilosis* e *C. tropicalis* e pode ser um aliado na prevenção das infecções orais pelo gênero *Candida* em indivíduos que usam próteses e aparelhos ortodônticos.

Palavras-chave: Própolis Verde; *Candida albicans; Candida tropicalis; Candida parapsilosis;* aderência; biofilme. Material dentário.

ABSTRACT

Green propolis has been a therapeutic option due to its phytochemical and antifungal diversity acting on different species of Candida, showing satisfactory fungistatic and fungicidal effects, both in vitro and in vivo. A strong relationship between the presence of fixed orthodontic appliances and an increased risk of favoring biofilm stagnation has shown that fungi have virulence factors associated with the development of human disease and increased resistance to antimicrobial agents. This study evaluated the influence of the ethanol extract of green propolis on the adhesion and biofilm of Candida albicans ATCC 443-805-2, Candida tropicalis ATCC 1036-09-2 and Candida parapsilosis ATCC 726-42-6 in dental material. The ethanol extract of green propolis (EEPV) was prepared from 200 g of green propolis diluted in 500 mL of ethyl alcohol PA, stored in an amber flask and kept at room temperature, with stirring for 2 hours / day for 8 days. It was subsequently rotoevaporated and lyophilized and the phytochemical analysis was performed by high performance liquid chromatography. The adhesion of Candida species was performed on stainless steel and acrylic resin fragments and quantified in a Neubauer chamber counting the number of yeast cells adhered to the fragments. Biofilm formation was determined by counting the number of colony forming units (CFU). The intensity of adhesion and biofilm formation was classified as negative, weak, moderate, strong and very strong. Fifteen compounds were identified in the green propolis extract, with 3-hydroxybiochanin A, trimer gallate [epi] catechin and carminic acid as major compounds. The studied species were able to adhere and form biofilm on the surface of stainless steel and acrylic resin and the intensity of adhesion of the yeast cells was weak at all incubation times, except for C. parapsilosis and C. tropicalis, which in 12 h moderate intensity. As for the formation of biofilm (24 and 48 h), it was observed in the metal that C. albicans had moderate intensity in 24 and 48 h; C. parapsilosis at 24 and 48 h had very strong intensity; C. tropicalis in 24 h had a strong intensity and in 48 h very strong. While in resin, all species at times 24 and 48 h had a strong intensity, except for C. tropicalis, which at 48 h had a very strong intensity. The green propolis extract showed antifungal activity and was able to inhibit both adhesion and biofilm formation from 2.5 µg / mL. This study supports the hypothesis that green propolis has antifungal activity and interferes with the virulence factors of C. albicans, C. parapsilosis and C. tropicalis and can be an ally in the prevention of oral infections by the genus Candida in individuals who use prostheses and orthodontic appliances.

Keywords: Green Propolis; Candida albicans; Candida tropicalis; Candida parapsilosis; adherence; biofilm. Dental material.

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LISTA DE SIGLAS E ABREVIATURAS

ATCC	American Type Culture Collection
EEPV	Extrato Etanólico de Própolis Verde
HIV	Vírus da Imunodeficiência Humana
HPLC	Cromatografia líquida de alta eficiência
HPLC-DAD MS	Cromatografia líquida de alta eficiência acoplado a espectrômetro de massa
QS	Quorum Sensing
UFC	Unidades Formadoras de Colônias

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1 INTRODUÇÃO

O uso de produtos naturais na Odontologia constitui alternativa viável e eficaz na prevenção e combate de diversas patologias da cavidade oral. Inúmeros produtos com ação terapêutica têm sido relatados na literatura (FREIRES *et al.,* 2010), dentre eles, a própolis. O emprego da própolis com finalidade medicinal remonta a antiguidade, tendo iniciado junto com antigas civilizações como a egípcia, grega e romana e perdura até os dias atuais com ampla utilização em múltiplas finalidades terapêuticas médicas e veterinárias (BANDEIRA-REIDEL, 2014). As variadas propriedades farmacológicas da própolis despertaram o interesse da Odontologia em utilizá-la, principalmente como antimicrobiano, sendo sua utilização no tratamento da cárie dentária como método terapêutico comprovado (DE-CARLI *et al.*, 2011). Na Odontologia, a própolis tem sido recomendada como terapia natural para manutenção da higiene oral, como um antisséptico para tratamento intracanal e tratamento de mucosite oral (MAUREIRA *et al.*, 2017).

Estudos têm mostrado uma forte relação entre presença de aparelhos ortodônticos fixos com o aumento do risco de formação de biofilme (REGALADO, 2009). As próteses dentárias são fabricadas com materiais que favorecem o acúmulo de biofilme, tornando-se reservatórios de microorganismos, que estão associados ao desenvolvimento de doenças sistêmicas, como endocardite bacteriana, pneumonia aspirativa, infecção intestinal e doença pulmonar obstrutiva crônica (BADARÓ *et al*., 2019).

A própolis verde brasileira é encontrada apenas nos estados de São Paulo e Minas Gerais, produzida por abelhas *Apis melífera* através dos brotos de *Baccharis dracunculifolia* DC (AUGUSTO-OBARA *et al.*, 2019). Própolis é uma substância resinosa que as abelhas, especialmente *Apis mellifera*, coletam de ramos e flores. Tem uma complexa composição química e é conhecida por ser rica em polifenóis (principalmente flavonoides), ceras, resinas, bálsamos, aminoácidos e outros óleos (TOBALDINI-VALERIO, *et al.*, 2016). Própolis está relacionada com a flora de cada região visitada pelas abelhas e com o período de coleta da resina (LUSTOSA *et al.,* 2008). Propriedades terapêuticas, como antimicrobiana, antioxidante, anticancerígena, antiviral, imunomoduladora, cicatrização de feridas e efeito antisséptico tem sido descrito em relação a própolis (RIGHI *et al*., 2011).

A ação da própolis sobre leveduras de diferentes espécies de *Candida* têm mostrado efeitos fungistático e fungicida satisfatórios, tanto em experiência *in vitro* quanto *in vivo*. A

Candida albicans apresenta maior patogenicidade e é principalmente encontrada nas lesões de candidose da mucosa oral, apesar de que outras espécies como *C. tropicalis, C. krusei, C. parapsilosis e C. guilliermondii*, aumentam durante a evolução da doença. Esta patologia é frequentemente encontrada em idosos, principalmente em portadores de prótese, crianças na primeira infância, pacientes que fizeram uso prolongado de antibióticos, diabéticos e imunosuprimidos, especialmente os acometidos pelo HIV/AIDS (PINA *et al*., 2017; OLIVEIRA-JÚNIOR *et al.,* 2018).

As doenças causadas por fungos podem ocorrer em pessoas saudáveis, mas os pacientes imunocomprometidos são o principal grupo de risco para infeções fúngicas. Os casos de resistência fúngica e a dificuldade de tratamento tornam as infecções fúngicas um problema de saúde pública (PARENTE-ROCHA *et al.*, 2017).

As infecções por *Candida* representam 80% de todas as infecções fúngicas no ambiente hospitalar, incluindo circulação sanguínea, infecções no trato urinário e no local cirúrgico. Fungemias são agora um grande desafio para hospitais terciários em todo o mundo devido à sua alta prevalência e taxas de mortalidade. A incidência de candidemia em hospitais públicos terciários no Brasil é aproximadamente 2,5 casos por 1000 internações hospitalares (COLOMBO *et al*., 2013). As infecções sanguíneas por *Candida* estão aumentando devido a um atraso na escolha do antifúngico inicial. A resistência de *Candida* aos agentes antimicóticos pode resultar na demora de um tratamento adequado e contribuir para alta mortalidade de aproximadamente 40% (CHAPMAN *et al.*, 2017).

As altas taxas de morbidade e mortalidade causadas por infecções fúngicas estão associadas com o atual arsenal antifúngico limitado e a alta toxicidade dos compostos. Além disso, identificar novos alvos de drogas é um desafio porque há muitas semelhanças entre células fúngicas e humanas. Os alvos antifúngicos mais comuns incluem síntese de RNA fúngica e componentes da parede celular e da membrana, embora novos alvos antifúngicos estejam sendo investigados. No entanto, os fungos desenvolvem mecanismos de resistência, como a superexpressão de proteínas, bomba de efluxo e formação de biofilmes, enfatizando a importância de entender esses mecanismos (SCORZONI *et al.,* 2017).

O biofilme é definido como uma comunidade formada por estruturas de microorganismos altamente associados ou ligados um ao outro formando uma matriz extracelular de proteção contra a resposta do hospedeiro. Para os fungos a matriz formada impede a atividade terapêutica dos medicamentos nas superfícies abióticas dificultando uma boa resposta terapêutica para o hospedeiro. Nos últimos anos, estudos têm demonstrado o papel do biofilme de fungos como um dos fatores de virulência associado ao desenvolvimento

de doença humana e o aumento da resistência a agentes antimicrobianos (SINGH *et al*., 2011; RAMAGE *et al*., 2016).

A cavidade oral pode ser considerada como reservatório para uma variedade de espécies oportunistas de microorganismos que causam infecções em indivíduos de baixa imunidade. Os microorganismos oportunistas mais representados incluem *Staphylococcus aureus, Pseudomonas aeruginosa* e *Candida albicans*. Infelizmente, devido ao uso excessivo e indevido de antibióticos, a maioria desses microorganismos tornam-se resistentes aos medicamentos, levando a dificuldades na cura de doenças infecciosas relacionadas e reduzindo as opções terapêuticas (ASSAF *et al.*, 2016).

As infecções fúngicas oportunistas constituem uma séria ameaça para saúde e bem-estar humano. Durante muitos anos atribuiu-se a *Candida albicans* ser a levedura mais patogênica e oportunista. No entanto outras não-*C.albicans* tais como as espécies *C. glabrata, C. tropicalis* e *C. parapsilosis* estão cada vez mais sendo isoladas, principalmente devido à prescrição indiscriminada de agentes antifúngicos. A patogênese da candidíase é comum a todas as espécies de *Candida* e é facilitada por vários fatores de virulência, dentre os quais, destaca-se a capacidade de adaptação para uma variedade de habitats diferentes, com a consequente formação de biofilmes em superfícies diversas. A persistência do gênero *Candida* na aderência e biofilme em superfície abiótica se deve ao fato da tolerância destas leveduras a elevadas doses de anti-fúngicos, o que complica a terapia das infecções nosocomiais (JABEUR *et al.*, 2016).

As espécies de *Candida* normalmente existem como comensais, mas podem se tornar agentes patogênicos oportunistas com a capacidade de causar infecções superficiais e sistêmicas. A prevalência de infecções oportunistas aumentou dramaticamente ao longo das últimas décadas, e isso é particularmente evidente em indivíduos imunocomprometidos. Embora a maioria dos casos de candidíase seja atribuído a *C. albicans,* nas últimas décadas métodos de diagnóstico aprimorados e níveis mais altos de resistência a certos antifúngicos levaram ao aparecimento das espécies não-*albicans*, particularmente *C. glabrata, C. parapsilosis e C. tropicalis.* Além disso, a patogenicidade das espécies de *Candida* é facilitada por uma série de fatores de virulência, incluindo dimorfismo, secreção de enzimas hidrolíticas (como proteases, lipases e hemolisinas) e a capacidade de aderir e formar biofilmes em dispositivos médicos e / ou a mucosa do epitélio do hospedeiro (ARAÚJO; HENRIQUES; SILVA, 2017).

2 JUSTIFICATIVA

Pouco se sabe das preferências fúngicas por substratos específicos e a sua interação com o ambiente abiótico. Enzimas e outras substancias produzidas pelos fungos são de grande relevância na formação de aderência e biofilme em superfícies abióticas.

O presente trabalho se justifica pela necessidade de estudos que possam conhecer fatores de virulências das espécies do gênero *Candida*, em especial *C. albicans C, parapsilosis e C. tropicalis* no fenômeno de aderência e ou/ biofilme em superfícies abióticas, especificamente acrílico e aço inoxidável, materiais presentes em reabilitações orais (prótese dentária e aparelhos ortodônticos). Utilizou-se a própolis verde como nova opção terapêutica na interferência da formação de aderência e/ou biofilme em superfícies abióticas *in vitro*.

3 REFERENCIAL TEÓRICO

3.1 Própolis

A própolis é um material resinoso produzido por abelhas utilizando resinas vegetais, exsudatos e pólen coletado de plantas (AUGUSTO-OBARA *et al*., 2019) Figura 2.

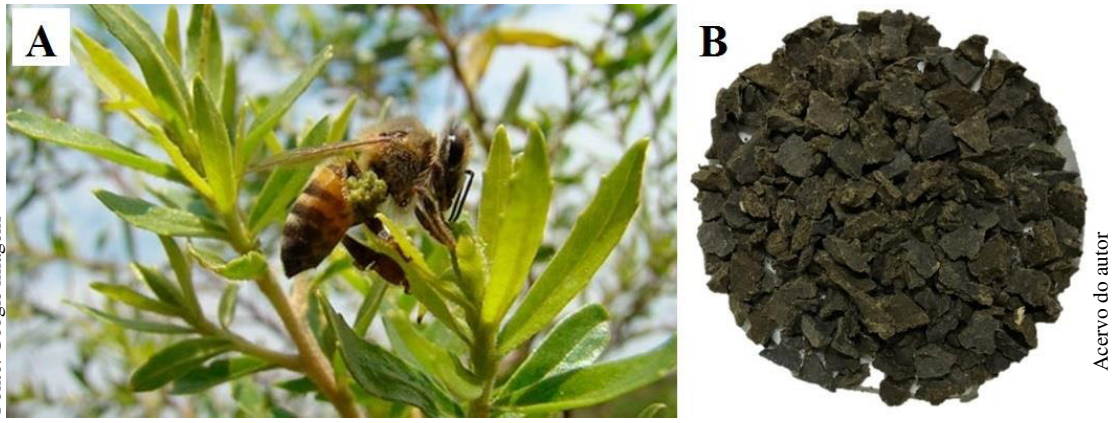

Figura 1: (A) *Apis melífera* polinizando o *Baccharis dracunculifolia* (Alecrim-do-campo) apiário greenme.com.br. (B) Própolis verde *in* natura do apiário Rosita Betim- MG.

A composição química da própolis varia de acordo com sua origem botânica e geográfica, sendo esta variabilidade a provável causa das diferenças observadas na atividade biológica da própolis com origens diferentes (HERRERA *et al*., 2010). Sua composição química inclui 50% de resina, 30% de cera, 10% de óleos essenciais, 5% de pólen e 5% de outras substâncias, incluindo minerais e compostos orgânicos como ácidos fenólicos ou seus ésteres, flavonoides, terpenos, aldeídos aromáticos e álcool, ácidos graxos, etilenos e B-esteróides (DJAIS *et al*., 2019).

Apesar de usada na medicina tradicional há séculos, somente nas últimas décadas suas propriedades e benefícios foram avaliados cientificamente (HERRERA *et al*., 2010). A própolis tem sido muito usada na medicina popular por seus efeitos antimicrobianos, antioxidantes, anti-inflamatórios, antitumorais e antimodulatórios (GOMAA; GAWEESH, 2013; FALCÃO *et al*., 2014; PIPPI *et al*., 2015). Nos últimos anos, o uso de própolis se popularizou devido não apenas às suas propriedades benéficas, mas também ao surgimento de alimentos e cosméticos suplementados que contêm própolis em sua composição, além dos extratos disponíveis no mercado (HERRERA *et al*, 2010).

Entre todos os tipos de própolis brasileiras, a própolis produzida pela espécie *Apis mellifera*, conhecida como própolis verde e coletada de exsudatos da planta *Baccharis dracunculifolia* , é uma das mais estudadas no mundo devido às suas propriedades farmacológicas, tais como ações antibacterianas, antifúngicas e antioxidantes. Estudos têm demonstrado que compostos fitoquímicos como os encontrados na própolis verde podem interagir e modificar a estrutura das biomembranas das células-alvo, levando as proteínas da membrana a perder suas funções (PAZIN *et al.*, 2019).

Atualmente, há um número crescente de estudos em diferentes especialidades médicas utilizando substâncias naturais, como chás ou extratos de várias plantas. Dentre os extratos naturais utilizados na odontologia, a própolis destaca-se por suas propriedades anti-inflamatórias, analgésicas e antimicrobianas (MAEKAWA *et al*., 2013). Esmeraldo et al (2013) utilizou própolis verde em estudo experimental para tratamento de pulpotomia de dentes decíduos de ratos como material alternativo para tratar infecção da polpa dentária. Tratamento de estomatite aftosa com própolis verde foi estudado por Rodríguez-Archilla, Raissouni (2017).

Pina et al (2017) demonstrou a própolis um produto não citotóxico e não tem potencial mutagênico tanto oral quanto tópico. A ação da própolis sobre leveduras de diferentes espécies de *Candida* têm mostrado efeitos fungistático e fungicida, tanto em experiência *in vitro* quanto *in vivo* (DE OLIVEIRA-JÚNIOR *et al.*, 2013).

3.2 Gênero *Candida*

Candida é um dos patógenos mais comuns que infectam seres humanos, causando várias infecções fúngicas mucosas e sistêmicas, como candidíase oral, vaginite e candidemia. Ela está relacionada a uma variedade de condições clínicas, variando de estomatite a doenças sistêmicas que ameaçam a vida em pacientes imunocomprometidos. As infecções por *Candida* são frequentemente resistentes e, se não tratadas adequadamente, costumam ser recorrentes. (GOMAA; GAWEESH, 2013).

A candidíase é definida como a infecção fúngica bucal mais prevalente, sendo geralmente diagnosticada clinicamente por meio da descamação do epitélio bucal, além de seu aspecto eritematoso com presença de placas brancas destacáveis sobre a mucosa, cansando sensação de ardência e prurido (ALMEIDA *et al*., 2012). Esta patologia é frequentemente

encontrada em idosos, principalmente em portadores de prótese, crianças na primeira infância, pacientes que fizeram uso prolongado de antibióticos, diabéticos e imunosuprimidos, especialmente os acometidos pelo HIV/AIDS (OLIVEIRA-JUNIOR *et al*., 2017).

Candidíase sub-protética pode causar queimação oral e faríngea, aumento da sensibilidade interferindo na alimentação, distúrbio do paladar e do olfato, disfagia e aumento do volume da mucosa oral interferindo na estética e na estabilidade do uso de próteses removíveis, levando a alterar a qualidade de vida dos indivíduos infectados. Além disso, a presença de candidíase oral está associada a lesões potencialmente malignas e ao câncer bucal, o que torna seu tratamento adequado essencial (OLIVEIRA-JUNIOR *et al*.,2017).

Com relação à virulência de *Candida,* a capacidade de adesão ao acrílico é uma condição prévia para a colonização e o desenvolvimento de biofilmes em superfícies de dentaduras. Portanto, a inibição da adesão poderia ser eficaz para tratar ou prevenir a ocorrência de estomatite protética. Alguns estudos sugerem a modificação das resinas acrílicas para diminuir o fator de adesão de *Candida spp*., realizando modificações químicas da carga superficial das resinas como recurso para retardar ou prevenir o efeito da adesão das leveduras (BERGAMO *et al*., 2018).

Mutação fisiológica (infância e envelhecimento), imunossupressão, diminuição das células de defesa como neutrófilos e linfócitos (associado ao HIV, tratamento de quimioterapia), a administração prolongada de medicamentos esteroidais e indivíduos que utilizam próteses dentárias ou aparelhos ortodônticos são grupos de risco para candidíase bucal (BARBOSA; FARIA, 2014).

O aumento no número de infecções causadas por *Candida spp.* é atribuída à seleção de microrganismos resistentes a agentes antifúngicos, seja pelas limitadas opções terapêuticas, como também pelo uso inadequado de drogas antifúngicas. A resistência prolonga e aumenta os custos das hospitalizações e se torna uma ameaça aos pacientes imunossuprimidos, aumentando as taxas de morbidade e mortalidade associadas a essas infecções (PIPPI *et al*., 2015).

Diversos mecanismos contribuem para o fenômeno de resistência de cepas de *Candida* aos antifúngicos sintéticos azólicos, como fluconazol, miconazol e intraconazol, entre eles se destacam a super expressão ou mutação do gene *ERG11*, que codifica a enzima alvo dos azóis, a lanosterol 14-á-desmetilase; a superexpressão de genes *CDR1, CDR2 e MDR1* que codificam bombas de efluxo; alterações do gene *ERG*-3 que codifica a enzima 5-6 esterol dessaturase, importante na síntese do ergosterol, bem como alterações na composição lipídica da membrana plasmática fúngica, o que dificulta o influxo do fármaco na célula. Ressalta-se

que estes mecanismos podem ocorrer simultaneamente, contribuindo para ampliar o fenômeno de resistência (ALMEIDA *et al*., 2012).

Embora a maioria dos casos de candidíase tenha sido atribuída a *Candida albicans*, espécies não-*Candida albicans* (NCAC) como *Candida parapsilosis, Candida tropicalis* e *Candida glabrata* foram identificadas como patógenos comuns nas infecções fúngicas (SILVA *et al*., 2009).

Entre as espécies de importância médica, *C. albicans, C. krusei, C. glabrata, C. tropicalis* já se mostraram sensíveis à própolis produzida por diferentes abelhas em todo o mundo, incluindo Brasil, Argentina, Estados Unidos, Croácia, Turquia e Irã. A promissora atividade antimicrobiana da própolis também foi observada em fungos resistentes às drogas convencionais (PETER *et al*., 2019).

Acervo do autor

Figura 2: ***C. Albicans***

3.2.1 Candida albicans

A levedura *Candida albicans* um microrganismo comensal que ocorre na cavidade oral de 50% a 70% dos indivíduos saudáveis, podendo crescer sob condições anaeróbicas ou aeróbicas (DJAIS, 2019).

A maioria das infecções por *C. albicans* está associada à formação de biofilmes em superfícies bióticas (tecidos humanos) ou abióticas (dispositivos médicos), sendo que a adesão é o passo inicial para a colonização e o estabelecimento da infecção. *C. albicans* é citada como a espécie de maior patogenicidade, predominantemente encontrada em lesões de candidíase na mucosa bucal, porém, observa-se o aumento no número de espécies não-*albicans* no curso da infecção, como *C. tropicalis, C. krusei, C. parapsilosis e C. guilliermondii* (ALMEIDA *et al*., 2012; BERGAMO *et al.*, 2018).

C. albicans distingue-se de outras espécies de fungos pela sua capacidade de formar células de levedura e hifas sob diferentes condições ambientais, sendo estas hifas um importante componente estrutural dos seus biofilmes, visto que contribuem para a estabilidade arquitetural geral do biofilme, atuando como um suporte para as células de levedura e outras hifas. Assim, a capacidade de formar hifas e a capacidade dessas hifas de aderir umas às outras e às células de levedura são críticas para o desenvolvimento e manutenção normais do biofilme (DJAIS *et al*., 2019).

Mesmo com o emprego de diversos medicamentos, existem evidências de cepas resistentes de *C. albicans* frente a derivados azólicos, especialmente em pacientes HIV positivos com diagnóstico de candidose bucal. Diante do crescimento do número de patógenos resistentes aos antimicrobianos atualmente utilizados, verifica-se a necessidade de que sejam introduzidos novos agentes antimicrobianos no arsenal terapêutico (ALMEIDA *et al*., 2012).

3.2.2. Candida parapsilosis

As células de *C. parapsilosis* exibem formas ovais, redondas ou cilíndricas. Quando cultivadas em ágar Sabouraud dextrose, as colônias de *C. parapsilosis* são brancas, cremosas, brilhantes e lisas ou enrugadas. Ao contrário de *C. albicans e C. tropicalis*, que podem existir em múltiplas formas morfogenéticas, *C. parapsilosis* não forma hifas verdadeiras e existe tanto na fase de levedura quanto na forma pseudo-hifa (TROFA; GÁCSER; NOSANCHUK, 2008).

Nas últimas décadas, a prevalência combinada de espécies de *Candida* não-*albicans* superou as infecções por *C. albicans* em várias regiões geográficas do mundo, ressaltando a necessidade de compreender sua patobiologia para desenvolver um tratamento eficaz e

prevenir futuros surtos. *C. parapsilosis* é considerada a segunda ou terceira espécie de *Candida* mais frequentemente isolada dos pacientes. Além de ser altamente prevalente, sua biologia difere marcadamente da de *C. albicans*, destacando-se diferenças na virulência, mecanismos de resistência a drogas regulatórias e antifúngicas e os grupos de pacientes em risco. Tais características específicas da espécie também podem influenciar seu reconhecimento e eliminação pelo hospedeiro e a eficácia de drogas antifúngicas (TÓTH *et al*., 2019).

C. parapsilosis é tipicamente um comensal da pele humana, sendo notória por sua capacidade de crescer em nutrição parenteral total e de formar biofilmes em cateteres e outros dispositivos implantados, em propagação hospitalar por transporte manual e por persistência no ambiente hospitalar. As mãos dos profissionais de saúde são os principais vetores na aquisição exógena de *C. parapsilosis*, representando uma grande ameaça para os pacientes que interagem com os profissionais de saúde colonizados, particularmente quando ocorrem violações nos protocolos padrão de lavagem das mãos contribuindo para surtos nosocomisais. *C. parapsilosis* é uma preocupação especial em neonatos gravemente enfermos, causando mais de um quarto de todas as infecções fúngicas invasivas em bebês com baixo peso. Além disso, é o organismo fúngico predominante isolado em muitas unidades de terapia intensiva neonatal (UTIN), onde está frequentemente associado à mortalidade neonatal (TROFA; GÁCSER; NOSANCHUK, 2008; SILVA *et al*.,2009). Embora testes de suscetibilidade *in vitro* apresentem alta sensibilidade nesta espécie, resistência clínica tem sido observada (PIPPI *et al*., 2015).

3.2.3 Candida tropicalis

Candida tropicalis é considerada a segunda espécie de Candida mais virulenta, precedida apenas por *C. albicans*, sendo reconhecida como um produtor de biofilme muito forte, superando a *C. albicans* na maioria dos estudos. Além disso, produz uma ampla gama de outros fatores de virulência, incluindo: adesão a células epiteliais e endoteliais bucais; a secreção de enzimas líticas, como proteinases, fosfolipases e hemolisinas, transição broto-hifas (também chamada morfogênese) e o fenômeno denominado troca fenotípica (ZUZA-ALVES *et al.*, 2017; ARASTEHFAR et al., 2019).

Candida tropicalis surgiu como o segundo ou terceiro agente mais comum de candidemia, principalmente em pacientes oncológicos. Além disso, tem sido relatado o aumento da incidência de *C. tropicalis* como agente causador de infecções nosocomiais do trato urinário (SILVA *et al*., 2009).

A grande maioria dos casos de candidíase são tratados com fluconazol por causa do alto custo das equinocandinas. No entanto, um número crescente de estudos de candidemia mostrou um aumento significativo nos isolados de *C. tropicalis* resistentes aos azóis e anfotericina B. O isolamento de *C. tropicalis* resistente ao azol limitará ainda mais as opções de tratamento disponíveis e comprometerá a vida dos pacientes, especialmente nos países em desenvolvimento. Além disso, pacientes infectados com C. tropicalis experimentam maior hospitalização e maior mortalidade em comparação com os infectados por *C. albicans* (ARASTEHFAR et al., 2019).

Estudos epidemiológicos destacam *C. tropicalis* como a mais resistente aos antifúngicos em comparação com *C. albicans*, particularmente na cavidade oral. Os crescentes níveis de resistência às terapias antifúngicas tradicionais e os altos níveis de mortalidade criaram uma necessidade urgente de desenvolver novas estratégias para combater essas infecções (FERNANDES et al., 2020).

3.3 Aderência e Biofilme

A formação do biofilme inclui diferentes etapas: adesão da levedura a um substrato, proliferação celular, formação de hifas, produção e acumulação de matriz extracelular, e por fim dispersão destas células. O comportamento dos micro-organismos quando em biofilmes é regulado pelo fenômeno chamado de Quorum sensing (QS), no qual, os micro-organismos liberam sinais químicos e expressam genes de virulência dependente da densidade celular (SANTOS, 2018).

Atribui-se a virulência do gênero *Candida* à sua versatilidade na adaptação a diferentes habitats e à sua capacidade de formar comunidades microbianas ligadas à superfície celular conhecidas como biofilme. As células de biofilme são organizadas de forma estruturada, embutidas em uma matriz de material extracelular, que geralmente é composta por carboidratos, proteínas, fósforo, glicose, hexosaminas e água. A formação de biofilme tem

importantes repercussões clínicas devido à sua maior resistência à terapia antifúngica e à proteção contra as defesas imunológicas do hospedeiro (SILVA *et al*., 2009).

Os biofilmes são considerados nichos protetores de microorganismos, destacando-se como suas principais funções fornecer aos microrganismos proteção contra formas ativas de oxigênio, desidratação, fagos, ingestão de amebas, substâncias tóxicas, incluindo desinfetantes ou antimicrobianos, bem como o sistema imunológico de um hospedeiro. Essas funções sugerem que os biofilmes aprimoram a capacidade de sobrevivência de microrganismos em um hospedeiro e aumentam sua possibilidade de sobreviver a uma terapia antimicrobiana, o que explica associações de biofilmes o desenvolvimento de infecções crônicas (ALIM; SIRCAIK; PANWAR, 2018; REBROŠOVÁ *et al*., 2019).

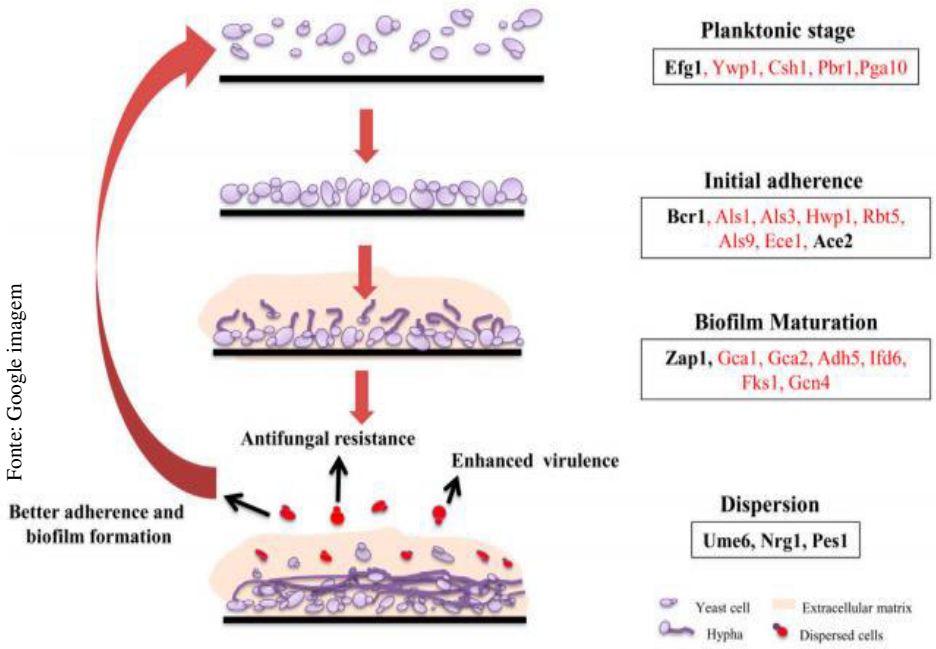

Figura 3. Fases de desenvolvimento de biofilme em *C. albicans*. O ciclo de vida do biofilme de *C. albicans* compreende a ligação de células livres de *C. albicans* à superfície, formação de hifas, produção de matriz extracelular e descolamento (dispersão) de células que podem iniciar a formação de biofilme em novos locais. Por uma questão de simplicidade, poucos genes incluindo fatores de transcrição (em negrito) envolvidos nos estágios indicados (identificados em condições in vitro e in vivo) são apresentados na caixa. As setas indicam as propriedades das células de levedura dispersas, como adesão melhorada, virulência e resistência antifúngica.
Adaptado de: Alim; Sircaik; Panwar (2018).

As próteses dentárias possuem superfícies que facilitam a acumulação de biofilmes de placas ao longo do tempo. O biofilme de *Candida spp*. na resina acrílica que compõe a prótese se desenvolve através da adesão, que ocorre de forma direta na superfície condicionada ou através de uma camada de placa de dentadura preexistente. Alguns fatores

influenciamna adesão de *Candida spp*., incluindo interações hidrofóbicas além da própria rugosidade da superfície de acrílico dos materiais protéticos (BERGAMO *et al*., 2018).

A existência de biofilmes resulta num sério problema para a saúde pública devido ao aumento da resistência dos microrganismos a agentes antimicrobianos e ao grande potencial que estes têm de causar infeções em pacientes imunossuprimidos ou portadores de próteses/implantes. Este tipo de infeção é uma epidemia silenciosa, que afeta milhares de doentes em todo o mundo, causando incapacidade e aumentando o tempo de internação, consequentemente, aumentando os custos associados ao seu tratamento, que em situações extremas pode provocar a morte (ALVES *et al*., 2016).

A utilização de produtos naturais com atividade antimicrobiana capazes de interferir no desenvolvimento do biofilme bucal, se faz uma alternativa válida e eficaz, destacando-se dentre essas substâncias à base de própolis (ALMEIDA *et al*., 2012).

4. OBJETIVOS

4.1 Geral

Avaliar *in vitro* a influência da própolis verde sobre a aderência e formação de biofilme de *Candida albicans*, *Candida tropicalis* e *Candida parapsilosis* em superfícies abióticas de material odontológico (resina acrílica e aço inoxidável).

4.2 Específicos

- Preparar o extrato etanólico de própolis verde e determinar sua composição química;
- Analisar a atividade antifúngica do extrato etanólico de própolis verde contra *C.albicans, C.parapsilosis e C.tropicalis*;
- Verificar a capacidade de aderência e formação de biofilme de *C.albicans, C.parapsilosis e C.tropicalis* em material odontológico na presença e na ausência de extrato de extrato etanólico de própolis verde;
- Determinar intensidade de aderência e biofilme das espécies *C.albicans, C.parapsilosis e C.tropicalis* em superfície abiótica de material odontológico (resina acrílica e aço inoxidável);

5 RESULTADO

5.1 Artigo

Highly Efficient Antibiofilm and Antifungal Activity of Green Propolis against *Candida* species in dentistry material

Running title: Green propolis against *Candida* biofilm in dentistry material

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Abstract

This study evaluated the influence of green propolis' extract on the adhesion and biofilm formation of *Candida* species on dentistry material. Phytochemical analysis of green propolis' extract was performed by High Performance Liquid Chromatography. Adhesion was quantified in a Neubauer chamber, counting the number of yeast cells adhered to the fragments; Biofilm formation was determined by counting the number of colony forming units (CFU). The intensity of biofilm formation adhesion was classified as negative, weak, moderate, strong and very strong. Fifteen compounds were identified in green propolis extract, mainly flavonoids. All strains were able to adhere and form biofilm on the surface of the orthodontic materials studied. In steel and resin, the adhesion intensity of the yeast cells was weak at all incubation times, except for *C. parapsilosis* and *C. tropicalis* which at 12hs showed moderate intensity. Regarding biofilm formation (24 and 48 hours), it was observed in the steel that *C. albicans* had moderate intensity at 24 and 48 hours; *C. parapsilosis* at 24 and 48 hours had very strong intensity; *C. tropicalis* at 24 hours had strong intensity and at 48 hours very strong. While in the resin, all species at 24 and 48 hours had strong intensity, except for *C. tropicalis* which at 48 hours had very strong intensity. Green propolis extract showed antifungal activity and was able to inhibit both adhesion and biofilm formation at 2.5 µg/mL. This study reinforces the idea that green propolis has antifungal activity and interferes with virulence factors of *Candida* species.
Keywords: Green Propolis; *Candida sp*, Biofilm; Dentistry material.

Background

In recent years the use of orthodontic materials has increased for aesthetic, surgical and biofunctional purposes. Polymers, ceramics, composites, resin, steels and their alloys are used in the manufacture of dental prostheses, screws and orthodontic appliances and when implanted in the oral cavity they are exposed to colonization and biofilm formation by microorganisms that live in the oral cavity. Alongside with the pH and saliva, these devices are targets of biofilm formation especially produced by *Candida* spp. (1).

A combination of factors contribute to *Candida* sp biofilm formation, salivary flow, low pH, poor oral hygiene and the type of orthodontic material contribute to biofilm colonization and formation (2). During colonization and biofilm formation, oral microbiota secrete enzymes and exopolysaccharides to colonize a surface, thus the biofilm constitutes as

a film of organic components that are absorbed from saliva forming an extracellular polymeric matrix and thus the multicellular community (bacteria or fungus) is incorporated into the extracellular matrix (ECM) (1-3).

The formation of biofilm in orthodontic materials raises concern as, when installed, increases the risk of infection, antibiotic and antifungal resistance, becoming an infectious site and obstacle for therapies. Natural products may inhibit biofilm formation, however, antibiofilm effects depends on inhibition of extracellular matrix formation, adhesin inhibition and cell attachment and inhibition of virulence factors (3).

Propolis is a resin and a natural product with medicinal properties. The production of propolis occurs from the collection of plant structures and its mixture with wax and salivary enzymes, having the modeling function of a varnish, besides protecting and sterilizing the internal and external parts of the hive, keeping the humidity and temperature (4–6).

Brazil has at least thirteen distinct types of propolis and many bioactive compounds, such as apigenin, artepilin C, vestitol, neovestitol, among others (7). There are varieties of propolis: red, green, yellow, brown, according to the flowering period. Green propolis is usually obtained from *Baccharis dracunculifolia* as a sticky exudate from leaves, flower buttons, buds, stems and fruits (8). This substance is rich in compounds such as prenylated phenylpropanoids, triterpenoids, benzoic and chlorogenic acids.

Scientific literature reports that green propolis has antifungal and antibacterial activities against *Lasiodiplodia theobromae* (9)*, Candida* spp. (10) and *Streptococcus mutans* (11), *Streptococcus acidominimus*, *Streptococcus oralis*, *Staphylococcus epidermidis*, *Veillonella parvula*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Lactobacillus acidophilus*, respectively (12). Thus, the aim of this research was to evaluate the activity of green propolis extract on the virulence factors (adhesion and biofilm) of *Candida albicans*, *C. tropicalis* and *C. parapsilosis* in dental materials (acrylic resin and steel).

RESULTS

Phytochemical screening

In the present study, the extract showed strong reaction for flavones, flavonoids and xanthones, the average intensity reaction for the presence of alkaloids, condensed tannins, hydrolysable tannins is showed on the table below (Table 1).

Table 1. Classes of secondary metabolites identified in Green Propolis Extract.

Classes of metabolites	Hydroalcoholic extract of green propolis
Fenols	+
Alcaloids	++
Condensed tannis	++
Hydrolysable tannins	++
Anthocyanins and anthocyanidins	-
Flavones, flavonols and xanthones	+++
Chacones and aurones	-
Leucoanthocyanidins	-
Catechins	-
Flavonones	++
Free steroids	
Free Pentacyclic Triterpenoids	++
Saponins	--

Subtitle: Strong (+++), medium (++), weak (+) and absent (-) reaction.

Chemical composition of green propolis hydroalcoholic extract by HPLC-DAD-MS

The profile of the compounds was analyzed by HPLC-DAD-MS (Figure 1). Fifteen compounds were identified in the green propolis extract (Table 2). The main compounds are flavonoids and phenolic acids.

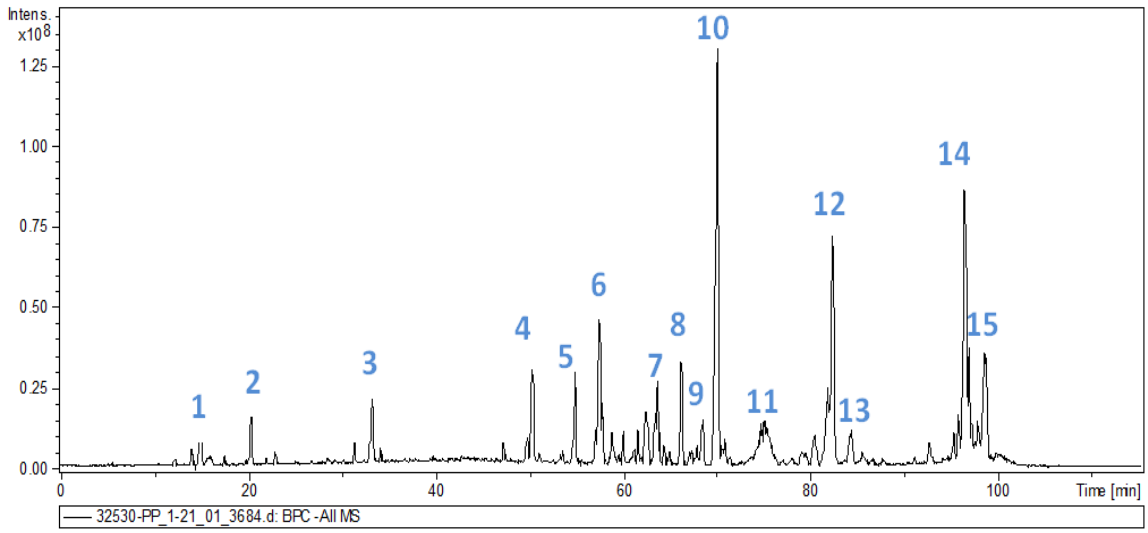

Figure 1. Chromatogram of the hydroalcoholic green propolist extract at a wavelength of 270 nm.

Isolated chemical compounds are described in Table 3, including retention time and observed mass. The spectra of each peak identified on the HPLC-DAD-MS are described in the supplementary article material. The chemical structures are described in Table 4, with their masses.

Table 2. Chemical compounds, mass, retention time (RT) from green propolis by HPLC-DAD-MS

Peek	***m/z***	**RT**	**Chemical compound**	**Chemical class**
1	515,12	15,1	Ácido 3,4- dicafeoilquínico	Ácido fenólico
2	515,08	20,5	Ácido 4,5-dicafeoilquínico	Ácido fenólico
3	301,01	33,3	Quercetina	Flavonol
4	230,99	50,4	3-(2,2-dimethylchromen-6-yl)prop-2-enoic acid	Flavonol
5	315,12	54,9	Homoferreirina	Flavanona
6	599,023	57,5	2[2-[4-(2 metilpropil)fenil]propanoiloxi]etil-4,5-diacetiloxi-9,10-dioxoanthraceno-2-carboxilato	Antraquinona
7	315,12	63,7	4',6-Dihidroxi-5,7-dimetoxiflavanone	Flavanona
8	329,17	66,3	5,7-Di-O-metilquercetina	Flavona
9	487,37	68,5	Apigenina-C-hexosil-C-deoxiexosideo	Flavonóides
10	299,06	70,1	3-hidroxibiochanina A	Isoflavonona
11	537,09	75,0	Amentoflavona	Flavonóides
12	727,34	82,3	Galato do trímero [epi]catequina	Proantocianidinas
13	613,32	84,3	Acremoxantona C	Xantona
14	491,21	96,4	Ácido Carmínico	Antraquinona
15	505,25	98,6	Peonidin-3-O(6-O-acetil)-glicosídeo	Glicosídeo

Table 3. Chemical compounds identification in green propolis by HPLC-DAD-MS

Chemical compound	**Structure**	***m/z***
1	$C_{25}H_{24}O_{12}$	515,12
2	$C_{25}H_{24}O_{12}$	515,08
3	$C_{15}H_{10}O_{7}$	301,01
4	$C_{14}H_{14}O_{3}$	230,99
5	$C_{17}H_{16}0_{6}$	315,12
6	$C_{34}H_{32}O_{10}$	599,023
7	$C_{17}H_{16}O_{6}$	315,12
8	$C_{16}H_{14}O7$	329,17
9	NI	487,37
10	$C_{16}H_{12}O_{5}$	299,06
11	$C_{30}H_{18}O_{10}$	537,09
12	NI	727,34
13	$C_{33}H_{26}O_{12}$	613,32
14	$C_{22}H_{20}O_{13}$	491,21
15	$C_{24}H_{25}0_{12}$	505,25

NI= not identified

Evaluation of antioxidant activity of green propolis extract by DPPH

The relation between the antioxidant activity (%) and the concentrations of the extract shown in the equation of the line (Y = 0.2714x + 27.966), with an R2 = 0.983 showed that the antioxidant percentage increases proportionally to the increasing concentrations of the extract, reaching 97.99% of antioxidant activity at a concentration of 275 µg / mL providing an EC50 of 81.18644 µg / mL, which is the extract of the concentration required to achieve 50% antioxidant activity (Figure 2).

The total phenolic compound contents calculated by the regression equation y = 0,006x + 0,006, (R2 = 0,999), obtained by the tannic acid calibration curve (where y is the absorbance at 760 nm and x is the concentration of tannic acid in µg / mL) shows that propolis extract has total phenolic contents of 135.33 mg EAT / g (Figure 3).

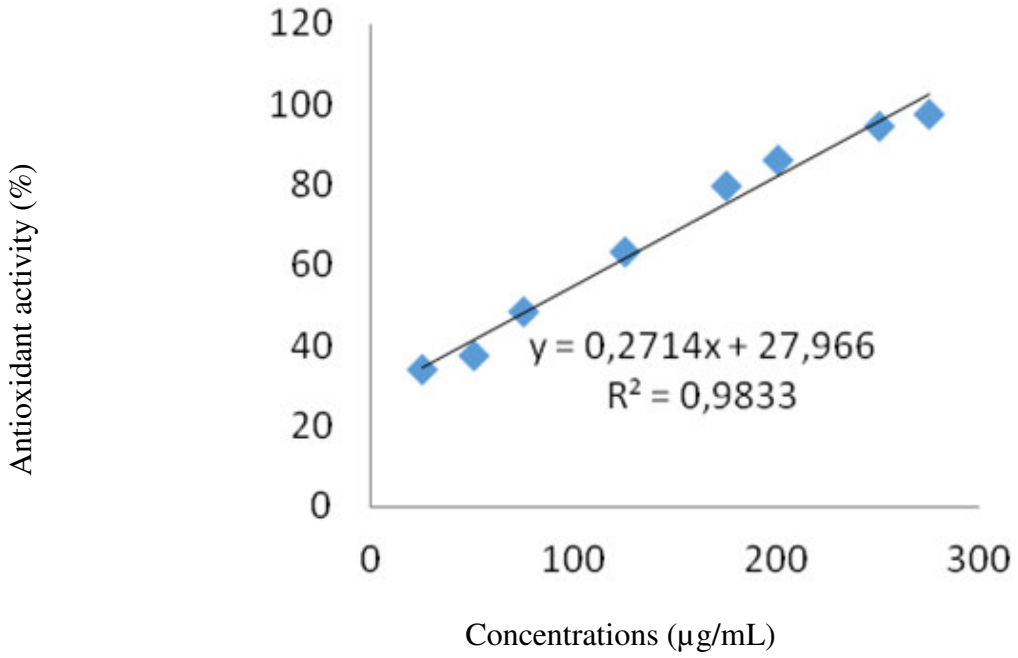

Figure 2: Curve of the percentage of antioxidant activity of the ethanol extract of propolis by the DPPH method.

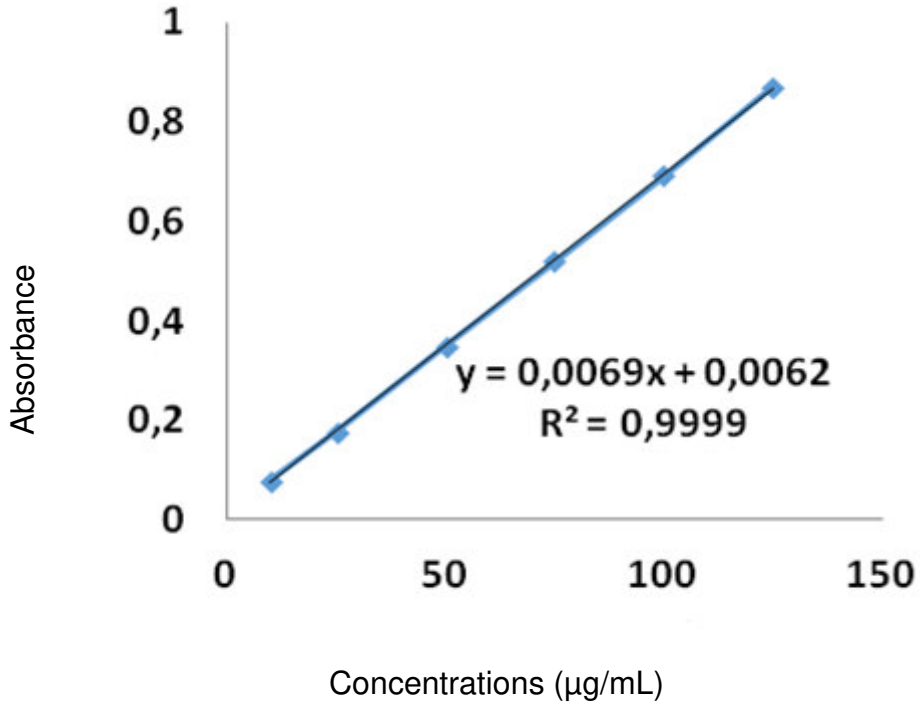

Figure 3. Standard curve of tannic acid for the quantification of phenolic compounds from green propolis extract.

Antifungal activity of green propolis extract (EPV) against *C. albicans*, *C. parapsilosis* and *C. tropicalis*

The green propolis ethanolic extract inhibited growth of the three *Candida* species evaluated (Table 5). The inhibition halo values of the green propolis ethanolic extract against three *Candida* species are shown in table 5. *C. albicans* and *C. tropicalis* were sensitive to the extracts at 2.5 to 250 µg/mL and *C. parapsilosis* was resistant at the concentrations of 0.25 and 2.5 µg/mL, while at concentrations 25 and 250 µg/mL it was sensitive.

Table 4. Antifungal activity of green propolis extract against *Candida* species by disk-diffusion.

Tested species	Means of the size of the halos (mm)/CIM (µg/mL) of green propolis extract				Control (AFB 16 µg/mL)
	0.25 µg/mL	2.5 µg/mL	25 µg/mL	250 µg/mL	16 µg/mL
C. albicans	5	15,2	17,3	20,1	25
C. tropicalis	9	13,1	14,7	16,6	25
C. parapsilosis	1	6,2	10	12,1	10

Adhesion capacity and biofilm formation of *C. albicans*, *C. tropicalis* and *C. parapsilosis* in orthodontic material (acrylic resin and steel)

All *Candida* species were able to adhere and form biofilm on the surfaces of the dental materials studied. In steel and resin, the adhesion intensity of the yeast cells was weak at all incubation times, except for *C. albicans* in 6 and 12h and for *C.parapsilosis* and *C. tropicalis* which presented moderate intensity at 12hs. Regarding biofilm formation (24 and 48 hours), it was observed in steel that *C. albicans* had moderate intensity at 24 and 48 hours; *C. parapsilosis* at 24 and 48 hours had very strong intensity; *C. tropicalis* at 24 hours had strong intensity and at 48 hours very strong. While in the resin, all species at 24 and 48 hours had strong intensity, except for *C. tropicalis* which at 48 hours had very strong intensity (Table 6).

After treatment with ethanolic extract of green propolis, adherence activity of all *Candida* species was reduced compared with the control (saline), showing the efficient activity of green propolis against virulence factors of *Candida* (Figure 4)

Table 5. Adhesion capacity and biofilm formation of *C. albicans*, *C. parapsilosis* and *C. tropicalis* on the surfaces of steel and acrylic resin of orthodontic material according to intensity.

Time (h)	*Candida* species	Materials			
		Steel		Resin	
		Number of adherent cells	Intensity	Number of adherent cells	Intensity
3	*C. albicans*	351	Weak	161	Weak
	C. parapsilopsis	175	Weak	178	Weak
	C. tropicalis	236	Weak	236	Weak
6	*C. albicans*	693	Moderate	580	Moderate
	C. parapsilopsis	208	Weak	209	Weak
	C. tropicalis	262	Weak	331	Weak
12	*C. albicans*	1566	Strong	765	Moderate
	C. parapsilopsis	459	Weak	530	Moderate
	C. tropicalis	610	Weak	520	Moderate
		Number of colonies	Intensity	Number of colonies	Intensity
24	*C. albicans*	331	Moderate	523	Strong
	C. parapsilopsis	2435	Very strong	554,3	Strong
	C. tropicalis	913,6	Strong	945,6	Strong
48	*C. albicans*	349,3	Moderate	578	Strong
	C. parapsilopsis	1012,3	Very strong	920	Strong
	C. tropicalis	1012,6	Very strong	2042,3	Very strong

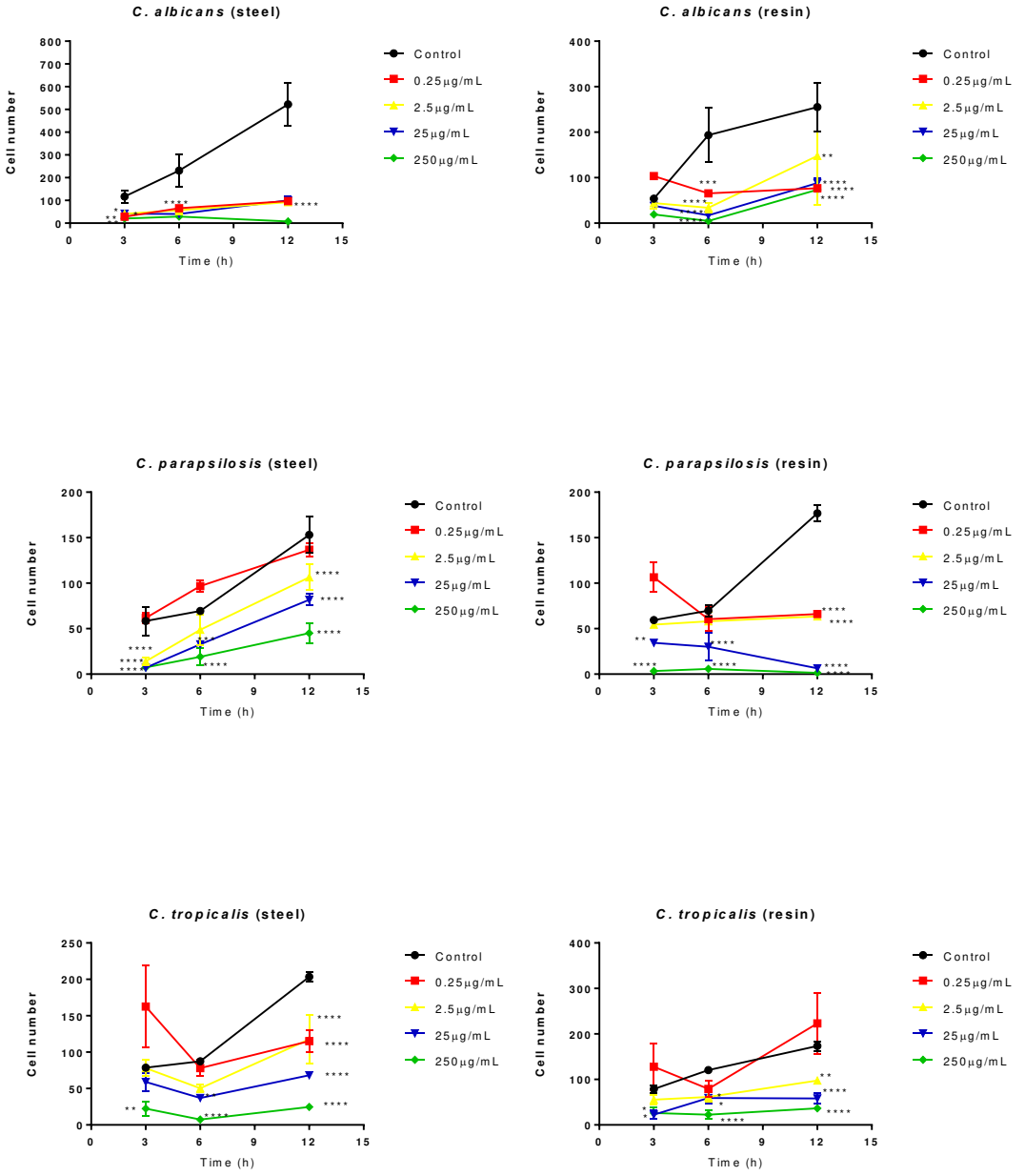

Figure 4. Influence of green propolis extract on the adhesion of *C. albicans*, *C. parapsilosis* and *C. tropicalis* to the surfaces of dental materials (acrylic resin and steel). Effect of extract against *Candida* sp. according to time and material. *p<0.05; **p<0.01;***p<0.001;****p<0.0001

Figure 5 shows the antibiofilm capacity of green propolis. All concentrations have shown the antibiofilm capacity of green propolis extract in 24 and 48 hours.

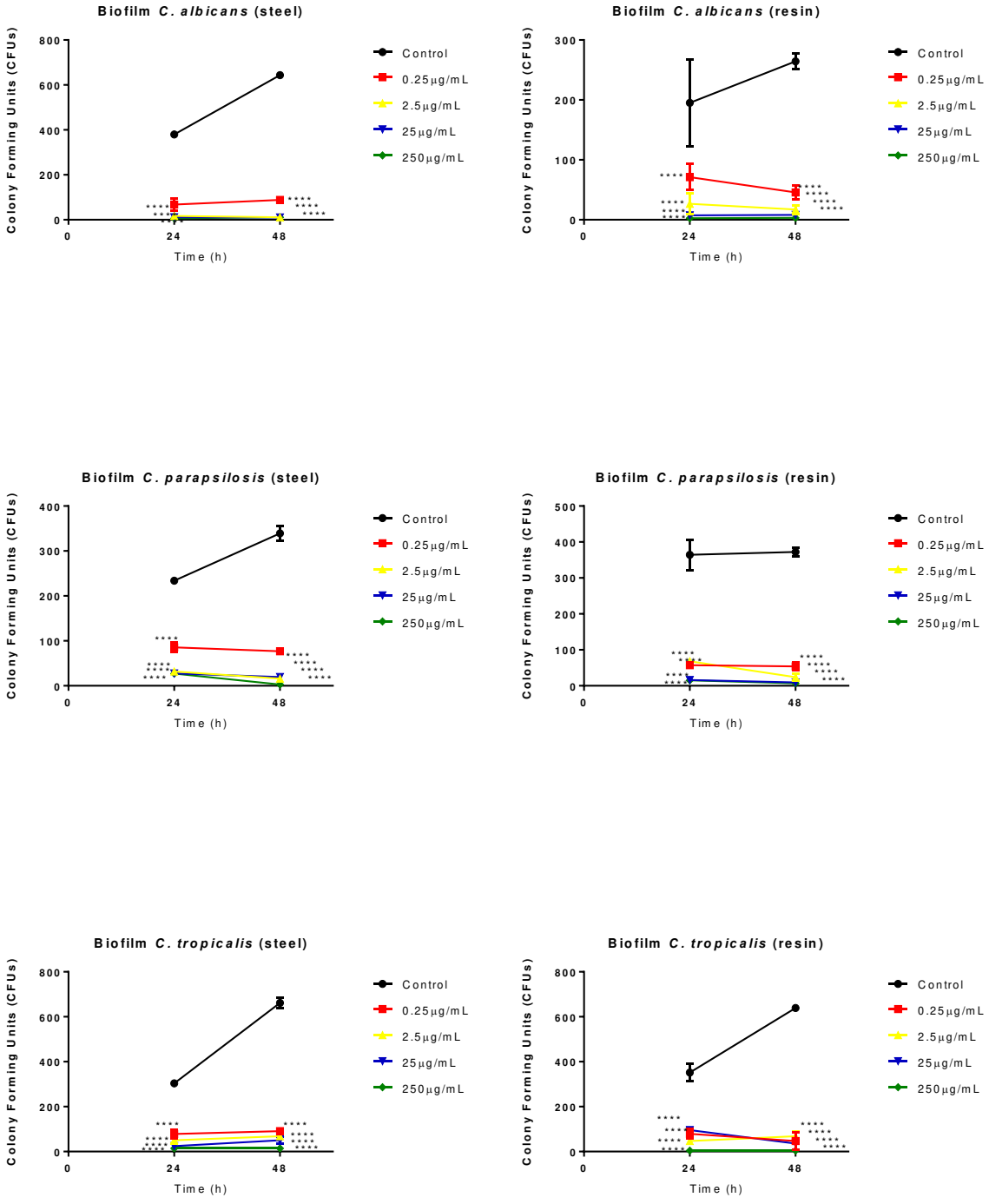

Figure 5. Influence of green propolis extract on the biofilm formation of *C. albicans*, *C. parapsilosis* and *C. tropicalis* to the surfaces of dental materials (acrylic resin and steel). Effect of extract against *Candida* sp. according to time and material. *p<0.05; **p<0.01;***p<0.001;****p<0.0001

Discussion

The presence of flavonoids, as well as phenolic, aromatic compounds and diterpene acids, in the composition of propolis are associated with several biological properties, such as antifungal (10,13).

The MICs (Minimum Inhibitory Concentrations) of green propolis extract used in the present study against *C. albicans*, *C. parapsilosis* and *C. tropicalis* were 2.5µg / mL. Siqueira *et al*. (14) reported 32-64 µg/mL of MIC for red propolis extract, showing the antifungal potential of green propolis extract against this yeast due to its sensitivity to the natural product in a concentration much lower than that found by these authors.

Sforcin *et al*. (15) reported that *C. albicans* was more sensitive to propolis from São Paulo (Brazil), located in southeastern Brazil, than *C. tropicalis*. Similar results were found in this study, where *C. albicans* was also more sensitive to propolis than C. tropicalis (the inhibition zones formed by *C. albicans* were larger than those formed by *C. tropicalis*). Propolis antifungal activity against *C. albicans* was studied by Parcker (16), and D'Auria *et al*. (17) where it was suggested that propolis extract inhibits phospholipase extracellular activity, impairing the adhesion of fungal cells to epithelial cells, which was corroborated in the present study (18).

Similar to the results found in this research, propolis extract also showed antibiofilm activity against clinical isolates and ATCC of *Fusarium* species found in patients with onychomycosis, where it was found that the biomass of the treatments decreased significantly when compared to the control, as well as the number. of viable cells (19).

Capoci *et al*. (20) observed a reduction of more than 50% of CFUs for all *Candida albicans* isolates after exposure to Propolis Extract (PES) compared to control. These results corroborate those found in this research, where there was also a reduction of CFUs of *C. albicans*, *C. tropicalis* and *C. parapsilosis* at 25 and 250 µg / mL at all abiotic materials tested.

In this study, a greater reduction in *C. albicans* biofilm formation was observed at all concentrations compared to the biofilms produced by *C. parapsilosis* and *C. tropicalis*, and the reduction of biofilm in *C. parapsilosis* was significantly higher from the concentration of *C. parapsilosis*. 25 µg/mL and *C. tropicalis* at 250 µg/mL, corroborating the work of Tobaldini-Valerio *et al*. (21) who also observed a greater biofilm reduction (~ 3.5 log) in *C. albicans*, followed by *C. parapsilosis* and *C. tropicalis*, with a reduction of approximately 2.8 and 2 log, respectively, at all Propolis Extract concentrations. tested.

The Green Propolis Ethanol Extract (EEPV) used in this study showed fungicidal, anti-adherent and antibiofilm activity on *C. albicans*, *C. parapsilosis* and *C. tropicalis* on dental materials (steel and acrylic resin) at the concentration of 2.5 µg/mL, suggesting the preventive use of this natural product in oral infections by the genus *Candida*.

Materials and Methods

Obtaining and Preparation of the Green Propolis Ethanolic Extract (EEPV).

The green propolis used in the *in vitro* assays was acquired from the Rosita Apiary (Betim-MG). Fresh propolis was stored in a dry, airless plastic bag kept under refrigeration until use. The hydroalcoholic extract of green propolis was obtained according to the methodology of Soares de Moura *et al*. (22). Approximately 200g of green propolis was diluted in 500 ml of PA ethyl alcohol, stored in an amber flask and stored at room temperature with stirring for 2h/day for 8 days. It was then filtered and rotaevaporated at 35 °C until complete solvent removal. The resulting concentrate was lyophilized and stored refrigerated until use.

Phytochemical screening.

The extract was submitted to phytochemical screening based on the methodology presented by Matos (13) to detect phenols and tannins (reaction with ferric chloride); anthocyanins, anthocyanidins, flavonoids, leucoanthocyanidins, catechins and flavanones (pH variation using hydrochloric acid and sodium hydroxide); flavonols, flavanones, flavanonols and xanthones (reaction with metallic magnesium and concentrated hydrochloric acid). The results obtained in each test were qualitatively evaluated by staining reactions and precipitate formation.

Determination of total phenolic compounds.

The determination of total phenolics of the extract occurred by the Folin-Ciocalteu method based on procedures described by Waterhouse (23), with some modifications.

Gallic acid standard curve.

For the determination of the standard curve of tannic acid, a solution of 2,000 µg.mL^{-1} was prepared which gave five different dilutions (10, 25, 50, 75, 100, 125 µg tannic acid mL-1). Thereafter, 500µL of each solution was diluted with 2.5 mL of 10% (v / v) Folin-Ciocalteu solution and 2 mL of 4% (m / v) sodium carbonate solution, then mixed in test tubes. This mixture was protected from light. After 30 minutes, the absorbance was read on a spectrophotometer at 760 nm using a quartz cuvette. The absorbance readings were plotted as a function of gallic acid concentration through the regression equation and its coefficients.

Evaluation of antioxidant activity by DPPH (2,2-Diphenyl-1-picrylhydrazy).

The antioxidant activity of the extracts was evaluated with 1,1-diphenyl-2-picrilidrazil (DPPH), according to the methodology described by Yen and Wu (24). From the extract concentrations (10, 25, 50, 75, 100, 125, 150, 175, 200 and 225 µg / mL) a reaction mixture with DPPH was prepared. Subsequently, 1.0 mL of each dilution was transferred to a test tube containing 3.0 mL of DPPH ethanolic solution (0.004%). After 30 minutes of incubation in the dark at room temperature, DPPH free radical reduction was measured by reading the absorbance using a 517 nm spectrophotometer. A blank sample was prepared using ethanol instead of the sample. Equation 1 was used to calculate the ability to sequester the free radical expressed as a percentage of radical oxidation inhibition.

Antioxidant activity (%) = [1- (Sample Absorbance / Control Absorbance] x 100.

The IC50 value (concentration of the extract needed to sequester 50% of DPPH radical) was calculated by the above equation based on the concentrations of the extracts and in their respective percentages of DPPH radical sequestration. The analyzes were performed at the Chemical Research Laboratory of the Federal University of Maranhão.

Analysis of the phytochemical composition

The analysis of the phytochemical composition of the extract was obtained by High Performance Liquid Chromatography (HPLC) coupled to mass spectrometer (HPLC-DAD MS). Chromatographic analyzes were performed at the Instrumentation Analytical Center of the Institute of Chemistry of the University of São Paulo. After solubilization, the hydroalcoholic extract of green propolis was analyzed by high performance liquid

chromatography (HPLC). Shimadzu® chromatograph (Shimadzu Corp. Kyoto, Japan) consisting of a solvent injection module with a Shimadzu LC-20AD pump and Shimadzu UV-Vis detector (SPDA-20A) was used for analysis. The column used was Supelco Ascentis C-18 (250 x 4.6 mm - 5um). HPLC was performed with an elution gradient using a mobile phase with water and 5% acetic acid and methanol (organic phase) in different proportions. The total time of the experiment was 115 minutes. The injection volume was 20 µL and chromatographic acquisition was performed at 270 nm (DAD). Data were collected and processed using LC Solution software (Shimadzu). Identification of compounds by mass spectrometry was performed in negative mode.

Dental Material and Microorganisms.

Fragments of dental material from Self-Curing Acrylic (Resin) and Orthodontic Band (Metal) types were purchased from dental shops. Three species of *Candida* were used in this study: *Candida albicans* ATCC 443-805-2, *Candida parapsilosis* ATCC 726-42-6 and *Candida tropicalis* ATCC 1036-09-2 obtained from the stock collection of the Collection of Fungi of Immunology and Mycology Laboratory - NIBA/UFMA.

Evaluation of EEPV antifungal activity.

Initially *Candida* species were cultivated on Sabouraud Agar incubated at 37ºC in a BOD greenhouse and after 24 hours were diluted in saline according to McFarland at a 05. scale. Antifungal activity was performed by the disc diffusion method on Muller-Hinton agar with 2% dextrose and 0.5 µg / mL methylene blue as recommended by the CLSI M44-A2 protocol (25). Amphotericin B was diluted in PBS 1x plus 1% DMSO to give a concentration of 16 µg / mL for positive control. To evaluate antifungal activity, 50mg of EPP was diluted in 500µl of DMSO, from this dilution it was prepared a working solution by diluting 1ml in 9ml of PBS 1x, from which concentrations of the extract of 0.25, 2.5, 25 and 250µg / mL were obtained. Inhibition halos were evaluated according to interpretation criteria of CLSI (25) and Capocci (20).

Table 6. Interpretation criteria of fungi susceptibility to green propolis extract and amphotericin B by disk-difussion (CLSI M44-A2; CAPOCCI, 2013).

Substância avaliada	Sensível (S)	Sensível dose dependente (SDD)	Re µg/mL sistente (R)
Anfotericina B	>10mm	-	≤10mm
Extrato de própolis verde	≥10mm	8-9mm	≤7mm

Adherence and biofilm formation on abiotic metal and acrylic resin surfaces.

5 cm-sized fragments of dental material (metal and acrylic resin) were made and used in this study as described by Silva *et al* (26) and Borges *et al*. (27) with modifications. The fragments were cultivated in saline with 100µl of a $1x10^4$ cel/mL suspension of *C. albicans*, *C. parapsilosis* and *C. tropicalis* and they were kept in a BOD greenhouse at times of 3, 6 and 12hs for adherence and 24 and 48hs for biofilm in triplicate. Then the fragments were washed 3x with sterile distilled water, fixed with PA alcohol and stained with violet crystal. Subsequently the fragments were added to tubes containing 3 ml 0.85% saline and vortexed for 10 minutes obtaining a fungal suspension of the cells adhered to the materials. 10µl of the adherence test suspension was added in a Neubauer chamber to count the adherent cells under light microscopy and according to the number of cells quantified the intensity of adhesion to the dental material was classified as: Negative: <50 yeast / ml; Weak: between 50 and 499 c / ml; Moderate: 500 to 999 c / ml; Strong: 1000 or more c / ml. For the biofilm test, 100µl of the suspension was added to a plate containing Muller-Hinton Agar to quantify the number of colony forming units (CFUs) and the biofilm intensity formed in the dental material was classified as: Negative: without CFU growth; Weak: growth between one and 199 CFUs, Moderate: from 200 to 499, Strong: with 500 to 1000 CFUs and Very strong: over 1000 CFUs.

Anti-adherent and antibiofilm activities of EEPV.

EEP dilutions (0.25, 2.5, 25 and 250µg / mL) were prepared as described above. To evaluate the effect of the extract, the fragments were cultivated in a tube containing 3 ml of each concentration of EEP and incubated in a BOD greenhouse at 37ºC at times of 3, 6 12hs for adhesion and 24 and 48hs for biofilm. After each period, the tubes were removed from the

greenhouse, the fragments were washed with 3X sterile distilled water, and after each wash and greenhouse drying, the fragments were fixed with PA ethyl alcohol and stained with violet crystal. Then the fragments were added in saline tube and vortexed for 10 minutes.

Statistical analysis.

The data were analyzed using the program "GraphPad Prism R" version 7. A two-way ANOVA with Tukey post-hoc was performed, where $p < 0.05$ and a confidence interval of 95% were considered.

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References

1. Mystkowska J, Niemirowicz-Laskowska K, Łysik D, Tokajuk G, Dąbrowski JR, Bucki R. The role of oral cavity biofilm on steellic biomaterial surface destruction–corrosion and friction aspects. Int J Mol Sci. 2018;19(3).
2. Ghazal ARA, Idris G, Hajeer MY, Alawer K, Cannon RD. Efficacy of removing Candida albicans from orthodontic acrylic bases: an in vitro study. BMC Oral Health. 2019 May 2;19(1):71
3. Lu L, Hu W, Tian Z, Yuan D, Yi G, Zhou Y, *et al*. Developing natural products as potential anti-biofilm agents. Chinese Med (United Kingdom) [Internet]. 2019;14(1):1–17. Available from: https://doi.org/10.1186/s13020-019-0232-2
4. Galletti J, Tobaldini-Valerio FK, Silva S, Kioshima ÉS, Trierveiler-Pereira L, Bruschi M, *et al*. Antibiofilm activity of propolis extract on Fusarium species from onychomycosis.

Future Microbiol. 2017;12(14):1311–21.

5. Devequi-Nunes D, Machado BAS, De Abreu Barreto G, Silva JR, Da Silva DF, Da Rocha JLC, et al. Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction. PLoS One. 2018;13(12):1–20.
6. Afrouzan H, Tahghighi A, Zakeri S, Es-Haghi A. Chemical composition and antimicrobial activities of Iranian Propolis. Iran Biomed J. 2018;22(1):50–65.
7. Franchin M, Freires IA, Lazarini JG, Nani BD, da Cunha MG, Colón DF, et al. The use of Brazilian propolis for discovery and development of novel anti-inflammatory drugs. Eur J Med Chem. 2018;153:49–55.
8. Bankova V, Popova M, Trusheva B. Propolis volatile compounds: Chemical diversity and biological activity: A review. Chem Cent J. 2014;8(1):1–8.
9. Martini D, Barbosa GF, Matias R, Marques Filho WC, Garcia NZT. Seasonality on the antifungal potential of green propolis collected in Campo Grande - MS, Brazil. Ciência Rural. 2017;47(3).
10. Oliveira ACP, Shinobu CS, Longhini R, Franco SL, Svidzinski TIE. Antifungal activity of propolis extract against yeasts isolated from onychomycosis lesions. Mem Inst Oswaldo Cruz. 2006;101(5):493–7.
11. Koo H, Rosalen PL, Cury JA, Ambrosano GMB, Murata RM, Yatsuda R, et al. Effect of a new variety of Apis mellifera propolis on mutans streptococci. Curr Microbiol. 2000;41(3):192–6.
12. Morawiec T, Mertas A, Wojtyczka RD, Niedzielska I, Dziedzic A, Bubiłek-Bogacz A, et al. The assessment of oral microflora exposed to 3% ethanolic extract of brazilian green propolis preparation used for hygiene maintenance following minor oral surgeries. Biomed Res Int. 2015;2015.
13. Matos FJ de A. Introdução à Fitoquímica Experimental. 3rd ed. Fortaleza: EUFC; 2009. 148 p.
14. Siqueira AB, Rodriguez LR, Santos RK. et al. Antifungal activity of propolis against Candida species isolated from cases of chronic periodontitis. Braz Oral Res 2015; 29: 29
15. Sforcin JM, Fernandes-Júnior A, Lopes CAM, Funari SRC, Bankova V. Seasonal effect of brazilian propolis on Candida albicans and Candida tropicalis. J Venom Anim Toxins. 2001;7:139-44.
16. Packer JF, Luz MMS. Método para avaliação e pesquisa da atividade antimicrobiana de produtos de origem natural. Brazilian Journal of Pharmacognosy 17(1): 102-107,

Jan./Mar. 2007

17. D'Auria FD, Tecca M, Scazzocchio F, Renzini V, Strippoli V. Effect of propolis on virulence factors of Candida albicans. J Chemother. 2003 Oct;15(5):454-60.
18. Silva-Carvalho R, Baltazar F, Almeida-Aguiar C. Propolis: A Complex Natural Product with a Plethora of Biological Activities That Can Be Explored for Drug Development. Evid Based Complement Alternat Med. 2015;2015:206439.
19. Galletti J, Tobaldini-Valerio FK, Silva S, Kioshima ES, Trierveiler-Pereira L, Bruschi M, Negri M, Estivalet Svidzinski T. Antibiofilm activity of propolis extract on Fusarium species from onychomycosis. Future Microbiol. 2017 Nov;12:1311-1321.
20. Capoci IRG, Bonfim-Mendonça PDS, Arita GS, Pereira RRDA, Consolaro MEL, Bruschi ML, et al. Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal Candida albicans. Evidence-based Complement Altern Med. 2015;2015.
21. Tobaldini-Valerio FK, Bonfim-Mendonça PS, Rosseto HC, Bruschi ML, Henriques M, Negri M, Silva S, Svidzinski TIe. Propolis: a potential natural product to fight Candida species infections. Future Microbiol. 2016 Aug;11:1035-46.
22. Soares de Moura R, Pires KM, Santos Ferreira T, Lopes AA, Nesi RT, Resende AC, Sousa PJ, da Silva AJ, Porto LC, Valenca SS. Addition of acai (Euterpe oleracea) to cigarettes has a protective effect against emphysema in mice. Food Chem Toxicol. 49(4):855-63. 2011.
23. WATERHOUSE, A. Folin-Ciocalteau Micro Method for Total Phenol in Wine. Waterhouse Lab., University of California, 8 aug. 2012. Disponível em: < http://waterhouse.ucdavis.edu/ faqs/folin-ciocalteau-micro-method-for-total-phenol-in-wine>
24. Yen G, WU J. Antioxidant and radical scavenging properties of extracts from Ganoderma tsugae. Food Chem. 1999; 65: 375-379.
25. CLSI. M44-A2: Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline—Second Edition. CLSI Doc. 2009;M44-A2(August):29(17).
26. Silva S, Negri M, Henriques M, Oliveira R, Williams D, Azeredo J. Silicone colonization by non-Candida albicans Candida species in the presence of urine. J Med Microbiol. 2010;59(7):747–54.
27. Borges KRA, Pimentel IV, Lucena LCLDS, da Silva MACN, Monteiro SG, Monteiro CA, et al. Adhesion and biofilm formation of Candida parapsilosis isolated from vaginal secretions to copper intrauterine devices. Rev Inst Med Trop Sao Paulo. 2018;60(September):1–5.

Supplementary Data

Chemical compounds identified by HPLC-DAD-MS

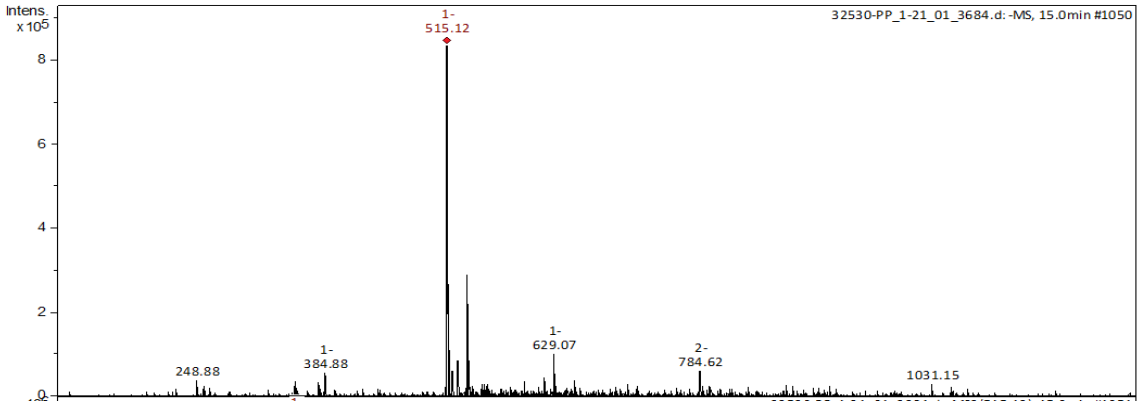


Fig S1. Mass Spectrum of $[M-H]^-$ for 1

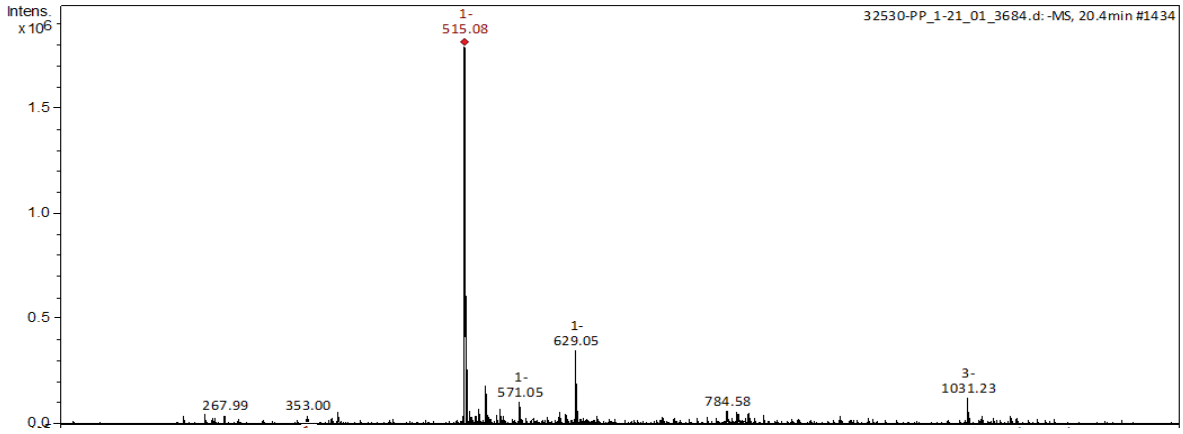
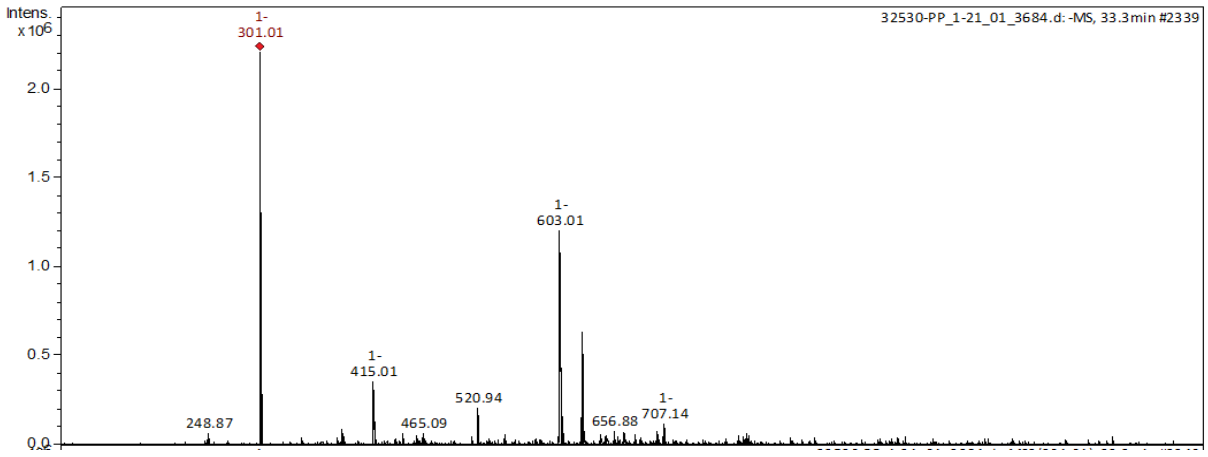


Fig S2. Mass Spectrum of $[M-H]^-$ for 2



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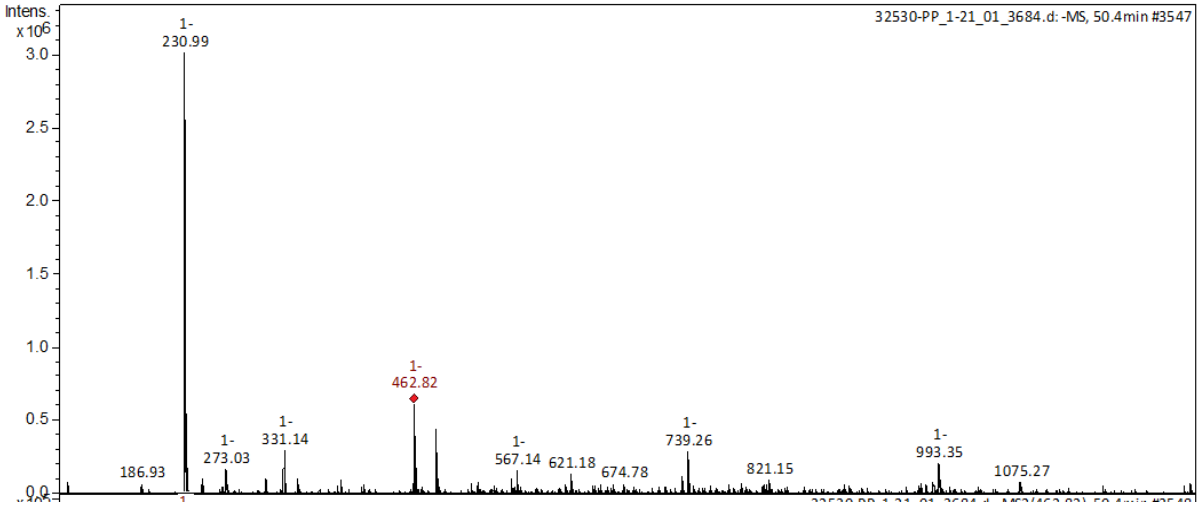

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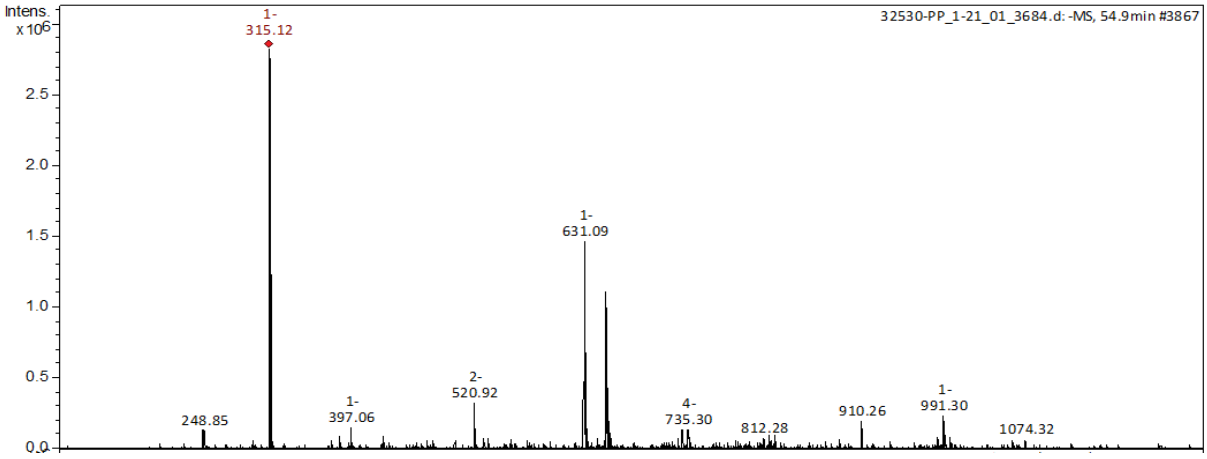

Fig S5. Mass Spectrum of $[M-H]^-$ for 6

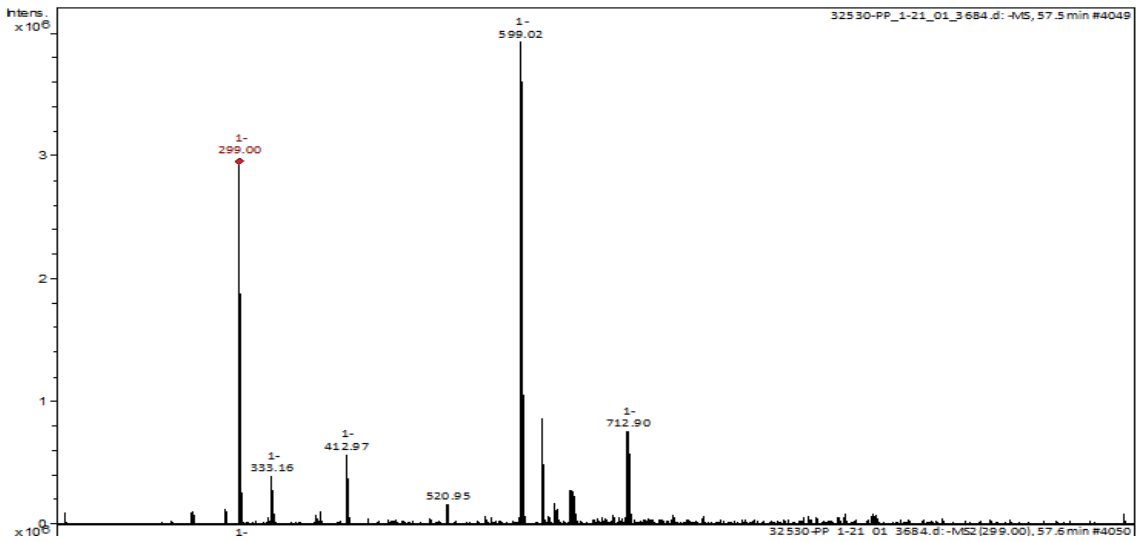

Fig S6. Mass Spectrum of $[M-H]^-$ for 6

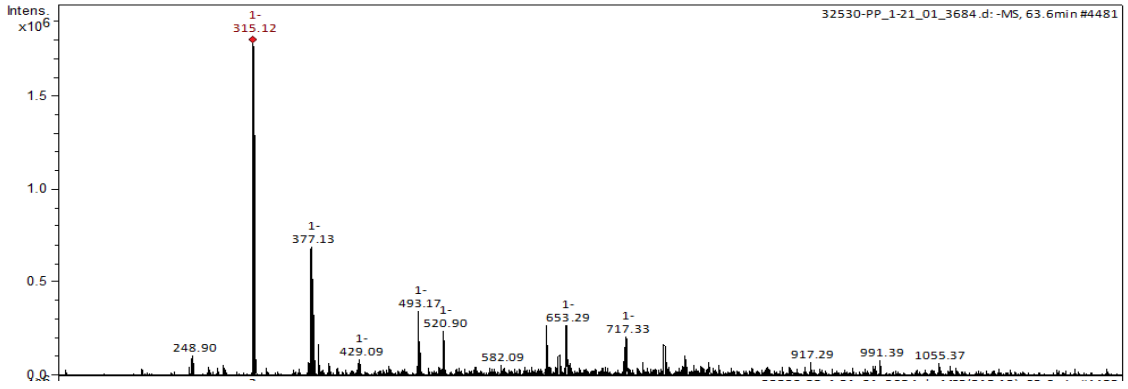

Fig S7. Mass Spectrum of $[M-H]^-$ for 7

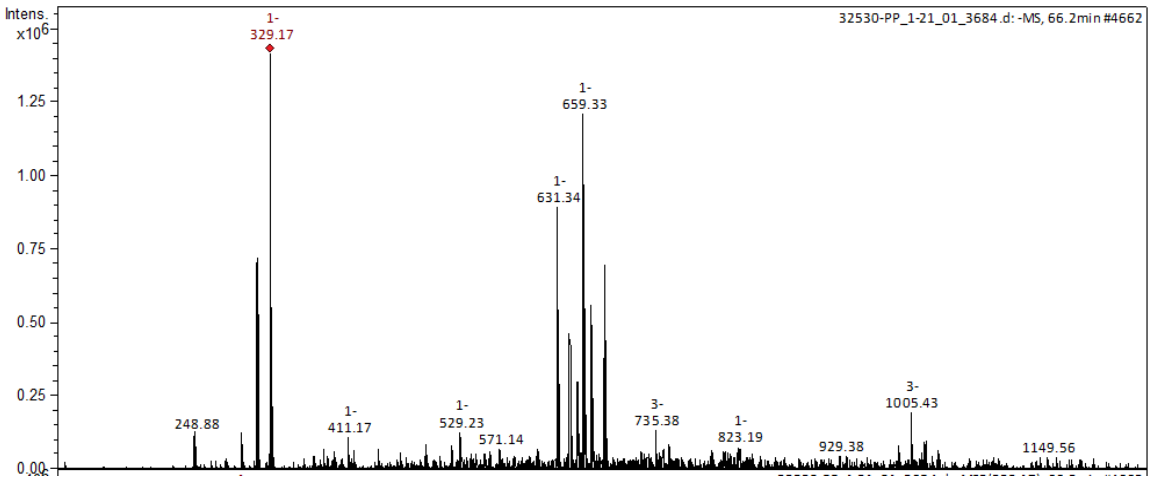

Fig S8. Mass Spectrum of $[M-H]^-$ for 7

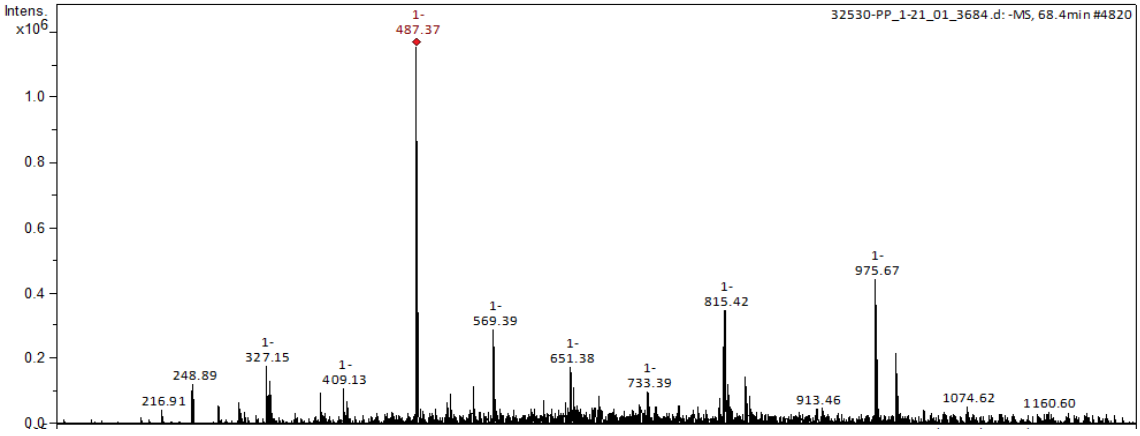

Fig S9. Mass Spectrum of $[M-H]^-$ for 8

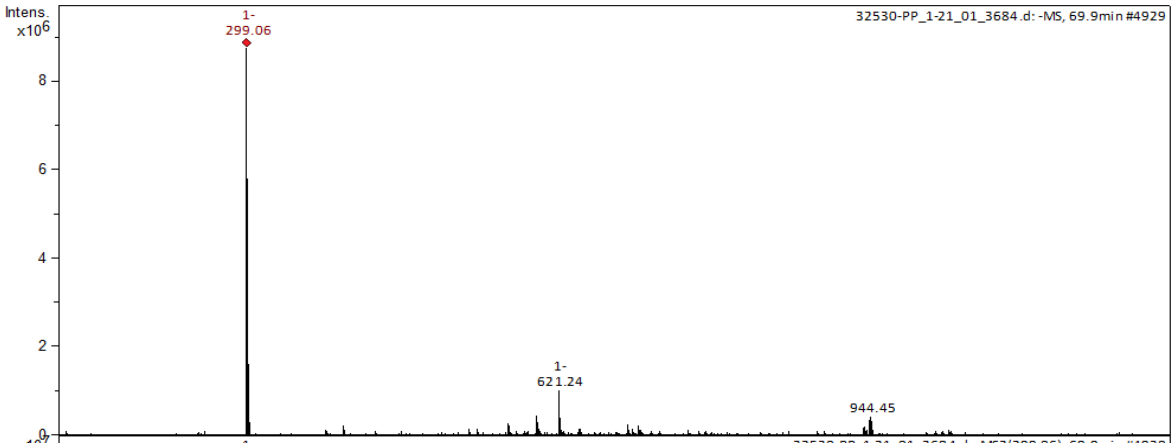

Fig S10. Mass Spectrum of $[M-H]^-$ for 10

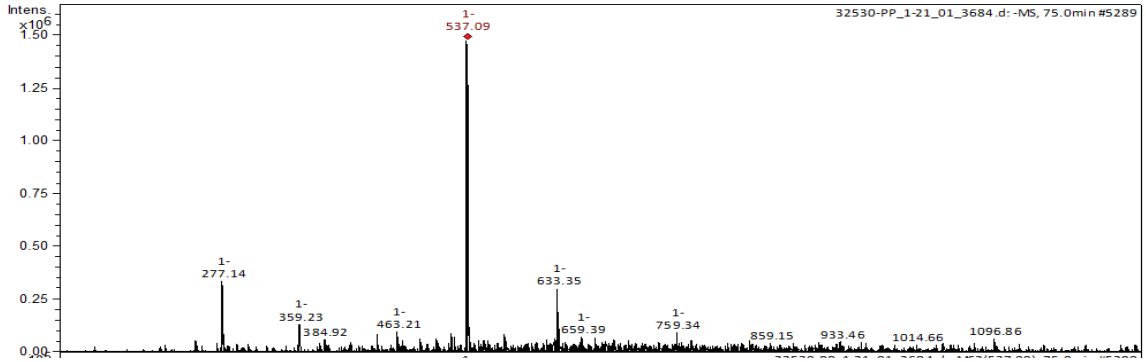

Fig S11. Mass Spectrum of $[M-H]^-$ for 11

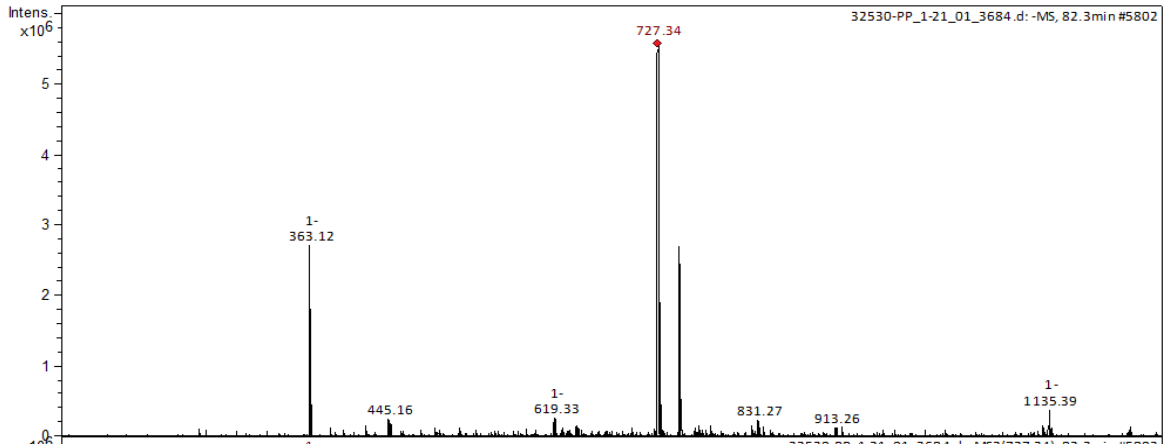

Fig S12. Mass Spectrum of $[M-H]^-$ for 12

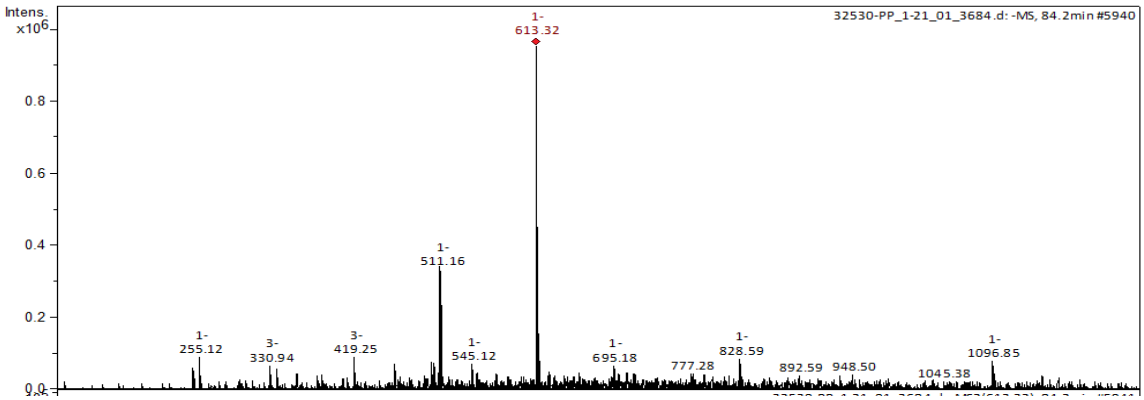

Fig S13. Mass Spectrum of $[M-H]^-$ for 13

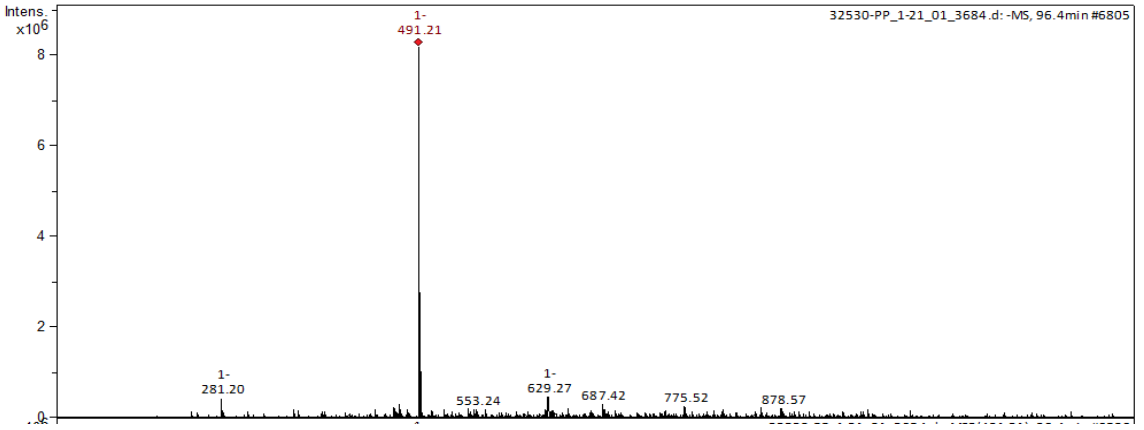

Fig S14. Mass Spectrum of $[M-H]^-$ for 14

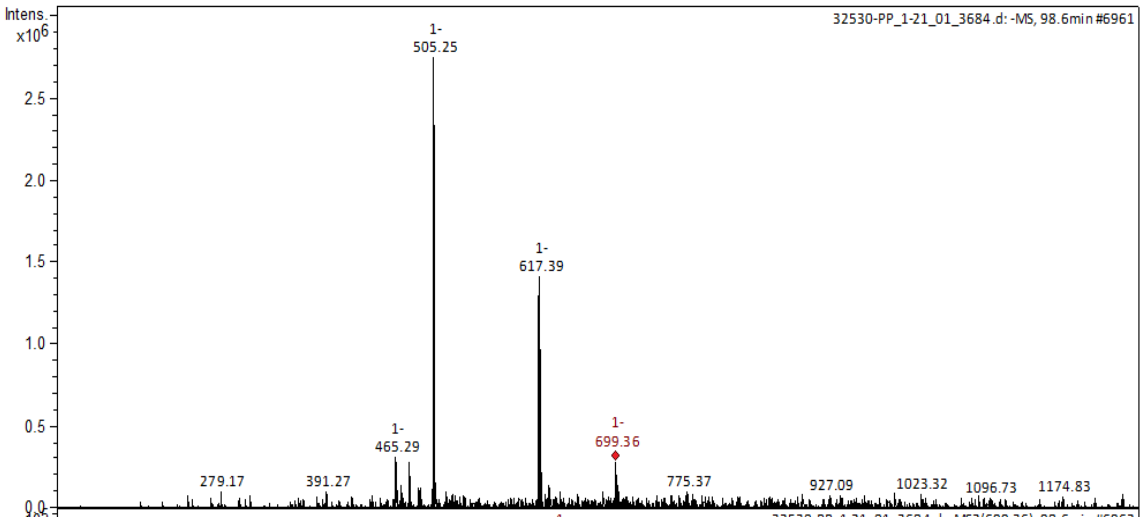

Fig S15. Mass Spectrum of $[M-H]^-$ for 15

6 CONCLUSÃO

- O Extrato Etanólico de Própolis Verde (EEPV) utilizado neste estudo apresentou atividade fungicida, antiaderente e antibiofilme contra *C. albicans*, *C. parapsilosis* e *C. tropicalis* na superfície abiótica dos materiais odontológicos (aço e resina acrílica);
- Espécies de *Candida* estudadas foram capazes de aderir e formar biofilme em superfície abiótica no material odontológico (aço e resina);
- O metal foi mais suscetível à adesão e formação de biofilme que a resina acrílica.

REFERÊNCIAS

ALIM, D.; SIRCAIK, S.; PANWAR, S. L. The Significance of Lipids to Biofilm Formation in Candida albicans: An Emerging Perspective. J. Fungi 2018, 4, 140; doi:10.3390/jof4040140.

ALMEIDA, L.F.D.; CAVALCANTI, B.Y. W.; LIRA JÚNIOR, B.R.; LIMA, E.O.; CASTRO, R.D. Efeito antifúngico de tinturas de própolis e romã sobre espécies de Candida. *Revista Cubana de Estomatología*, v. 49, n. 2, p. 99-106, 2012.

ALVES, M. J. et al. Biofilme bacteriano e infeção hospitalar. **Saúde**: Conexões e Sustentabilidade para o Entendimento Global, p. 110-122, 2016.

ARASTEHFAR, A.; DANESHNIA, F.; HAFEZ, A.; KHODAVAISY,S.; NAJAFZADEH, MOHAMMAD-JAVAD; CHARSIZADEH, A.; ZARRINFAR, H. et al. Antifungal susceptibility, genotyping, resistance mechanism, and clinical profile of Candida tropicalis blood isolates. Medical Mycology, 0, 1–8. 2019. doi:10.1093/mmy/myz124.

ARAÚJO, D.; HENRIQUES, M.; SILVA, S. Portrait of Candida species biofilm regulatory network genes. *Trends in microbiology*. v. 25, n. 1, p. 62-75, 2017.

ASSAF, A.M; AMRO, B.I.; MASHALLAH, S.; HADDADIN, R.N. Antimicrobial and anti-inflammatory potential therapy for opportunistic microorganisms. *J Infect Dev Ctries*. 10(5):494-505, 2016. 2016

AUGUSTO-OBARA, T.R.; OLIVEIRA, J.; GLORIA, E.M.; SPOTO, M.H.F.; GODOY, K.; VIEIRA, T.M.F.S. et al. Benefits of superfine grinding method on antioxidant and antifungal characteristic of Brazilian green propolis extract. *Scientia Agricola*. v. 76, n. 5, p. 398-404, 2019.

BADARÓ, M.M.; LEITE-FERNANDES, V.M.F.; MARTIN, L.T.; OLIVEIRA, V.C.; WATANABE, E.; PARANHOS, H.F.O. et al. Antibiofilm Activity of an Experimental Ricinus Communis Dentifrice on Soft Denture Liners. *Brazilian Dental Journal*, v. 30, n. 3, p. 252-258, 2019.

BANDEIRA-REIDEL, R.V. *Potencial antifúngico e antibiofilme sobre isolados patogênicos de Candida não-albicans de diferentes tipos de própolis brasileiras.* (Dissertação de Mestrado). Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Porto Alegre-RS, 2014. 107 p. http://hdl.handle.net/10183/101512

BARBOSA, M.B.; FARIA, M.G.I. Produtos naturais como nova alternativa terapêutica para o tratamento de candidíase bucal. *Revista UNINGÁ Review*. Vol.20,n.1,pp.103-107. 2014.

BERGAMO, V.Z.; LANA, D.F.D.; PIPPI, B.; GUERREIRO, I.C.K.; FUENTEFRIA, A.M. Novas tendências de combate ao biofilme de *Candida* em próteses dentárias. *Clinical and biomedical research.* 38;2, p. 155-166, 2018. https://doi.org/10.4322/2357-9730.79880

CHAPMAN, B.; SLAVIN, M.; MARRIOTT, D.; HALLIDAY, C.; KIDD, S.; ARTHUR, I. et al. Changing epidemiology of candidaemia in Australia. *Journal of Antimicrobial Chemotherapy*. v. 72, n. 4, p. 1103-1108, 2017. doi: 10.1093/jac/dkw422.

COLOMBO, A.L.; GUIMARÃES, T.; CAMARGO, L.F.A.; RICHTMANN, R.; QUEIROZ-TELLES, F.; SALLES, M.J.C., et al. Brazilian guidelines for the management of candidiasis – a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical. *The Brazilian Journal of Infectious Diseases*. v. 17, n. 3, p. 283-312, 2013. http://dx.doi.org/10.1016/j.bjid.2013.02.001

DE OLIVEIRA, J.R.; DE CASTRO, V.C.; VILELA, P.G.F.; CAMARGO, S.E.A.; CARVALHO, C.A.T.; JORGE, A.O.C. Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry. *BMC complementary and alternative medicine*, v. 13, n. 1, p. 208, 2013.

DE-CARLI, A.D.; ZÁRATE-PEREIRA, P.; DE-CARLI, G.; ZAFALON, E.J.; ZÁRATE, C.B.R.; YASSUMOTO, L.M. Ação da própolis de apis mellifera associada ao fluoreto de sódio sobre o biofilme dental: ensaio clínico duplo cego randomizado. *Revista Odontológica do Brasil Central*. v. 19, n. 51, 2011.

DJAIS, A.A.; JEMMY.; PUTRI, N.; PUTRI, A.R.; DARWITA, R.R. BACHTIAR, B.M. Formação de biofilme de candida albicans exposta ao extrato etanólico de própolis. *International Journal of Applied Pharmaceutics.* v. 11, p. 210-213, 2019.

ESMERALDO, M. R. A.; CARVALHO, M. G. F. de; CARVALHO, R. F. L. de; COSTA, E. M. M. B. de. Inflammatory effect of green propolis on dental pulp in rats. Braz Oral Res., (São Paulo) 2013 Sep-Oct;27(5):417-22

FALCÃO, S.I.; VALE. N.; COS, P.; GOMES, P.; FREIRE, C.; MAES, L., et al. In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. *Phytotherapy Research*, v. 28, n. 3, p. 437-443, 2014.

Fernandes, L.; Oliveira, A.; Henriques, M.; Rodrigues, M.E. Honey as a Strategy to Fight Candida tropicalis in Mixed-Biofilms with Pseudomonas aeruginosa. Antibiotics, 9, 43; 2020. doi:10.3390/antibiotics9020043.

FRANCHIN, M.; FREIRES, I.A.; LAZARINI, J.G.; NANI, B.D.; CUNHA, M.G.; COLÓN, D.F., et al. The use of Brazilian propolis for discovery and development of novel anti-inflammatory drugs. *European journal of medicinal chemistry*. v. 153, p. 49-55, 2018.

FREIRES, I.A.; ALVES, L.A.; JOVITO, V.C.; ALMEIDA, L.F.D.; CASTRO, R.D., et al. Atividades antibacteriana e antiaderente in vitro de tinturas de Schinus terebinthinfolius (Aroeira) e Solidago microglossa (Arnica) frente a bactérias formadoras do biofilme dentário. *Odontologia Clínico-Científica (Online)*, v. 9, n. 2, p. 139-143, 2010.

GOMAA, O.M.; GAWEESH, A.S. Variation in adhesion and germ tube formation of oral Candida using Egyptian propolis. *Canadian journal of microbiology*, v. 59, n. 3, p. 197-203, 2013.

HERRERA, C. L.; ALVEAR, M.; BARRIENTOS, L.; MONTENEGRO, G.; SALAZAR, L.A. The antifungal effect of six commercial extracts of Chilean propolis on Candida spp. *Ciencia e investigación agraria.* v. 37, n. 1, p. 75-84, 2010.

JABEUR, I.; TOBALDINI, F.; MARTINS, N.; BARROS, L.; MARTINS, I.; CALHELHA, R.C., et al. Bioactive properties and functional constituents of Hypericum androsaemum L.: A focus on the phenolic profile. *Food Research International.* v. 89, p. 422-431, 2016.

LUSTOSA, S.R.; GALINDO, A.B.; NUNES, L.C.C.; RANDAU, K.P.; ROLIM NETO, P.J. Própolis: atualizações sobre a química e a farmacologia. *Revista Brasileira de Farmacognosia*. v. 18, n. 3, p. 447, 2008.

MAEKAWA, L.E.; VALERA, M.C.; OLIVEIRA, L.D.; CARVALHO, C.A.T.; CAMARGO, C.H.R.; JORGE, A.O.C. Effect of Zingiber officinale and propolis on microorganisms and endotoxins in root canals. *Journal of Applied Oral Science.* v. 21, n. 1, p. 25-31, 2013. http://dx.doi.org/10.1590/1678-7757201302129

MAUREIRA, Natalia et al. Suscetibilidade de Cepas de Candida Oral e Extrato Etanólico de Propileno Chileno de Olmué. **Revista internacional de odontostomatologia**, v. 11, n. 3, p. 295-303, 2017.

OLIVEIRA-JÚNIOR, J.K.; VIEIRA, L.E.M.; BARNABÉ, L.É.G.; SANTOS, M.L.; FIGUEIREDO, V.S.A.; SILVA, D.F., et al. Análise do Efeito Antifúngico da Própolis Sobre Espécies de Candida albicans e Não-Albicans. *Revista Uningá Review*, v.30, n.3, pp.45-51, 2017.

OLIVEIRA-JÚNIOR, J.K.; VIEIRA, L.E.M.; BARNABÉ, L.É.G.; SANTOS, M.L.; FIGUEIREDO, V.S.A.; SILVA, D.F., et al. Análise do Efeito Antifúngico da Própolis Sobre Espécies de Candida albicans e Não-Albicans. *Revista Uningá Review*, v. 30, n. 3, 2018.

PARENTE-ROCHA, J.A.; BAILÃO, A.M.; AMARAL, A.C.; TABORDA, C.P.; PACCEZ, J.D.; BORGES, C.L., et al. Antifungal resistance, metabolic routes as drug targets, and new antifungal agents: an overview about endemic dimorphic fungi. *Mediators of inflammation*. v. 2017, 2017.

PAZIN, W.M.; ANTOS, S.N.; QUEIROZ, S.C.N.; BAGATOLLI, L.A.; SOARES, A.E.E.; MELO, I.S.; ITO, A.S. Bioactivity and action mechanism of green propolis against Pythium aphanidermatum. *Anais da Academia Brasileira de Ciências*. v. 91, n. 2, 2019.

PETER, C.M.; WALLER, S.B.; PICOLI, T.; OSÓRIO, L.G.; ZANI, J.L.; MEIRELES, M.C.A., et al. Análises químicas e citotóxicas de três variedades de própolis brasileira (própolis verde, própolis jataí e própolis marrom) e sua atividade in vitro anti-Sporothrix brasiliensis. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia.* v. 71, n. 3, p. 819-827, 2019.

PINA, G.M.; LIA, E.N.; BERRETTA, A.A.; NASCIMENTO, A.P.; TORRES, E.C.; BUSZINSKI, A.F., et al. Efficacy of propolis on the denture stomatitis treatment in older adults: A multicentric randomized trial. *Evidence-Based Complementary and Alternative Medicine*. 2017. doi: 10.1155/2017/8971746.

PIPPI, B.; LANA, A.J.; MORAES, R.C.; GÜEZ, C.M.; MACHADO, M.; DE OLIVEIRA, L.F., et al. In vitro evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on Candida spp. J Appl Microbiol. 118(4):839-50. 2015. doi: 10.1111/jam.12746.

RAMAGE, G. Comparing apples and oranges: considerations for quantifying candidal biofilms with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium carboxanilide] and the need for standardized testing. *Journal of Medical Microbiolog***y**. v. 65, p.259–260, 2016.

REBROŠOVÁ, K.; ŠILER, M.; SAMEK, O.; RŮŽIČKA, F.; BERNATOVÁ, S.; JEŽEK, J., et al. Identification of ability to form biofilm in Candida parapsilosis and Staphylococcus epidermidis by Raman spectroscopy. *Future microbiology*. v. 14, n. 6, p. 509-517, 2019. Doi: 10.2217/fmb-2018-0297.

REGALADO, Fabiano Ferreira. *Ação da própolis de Apis Mellifera no controle do risco de cárie dentária e gengivite em pacientes submetidos a tratamento ortodôntico*. 2009. Dissertação de Mestrado. https://repositorio.ufms.br:8443/jspui/handle/123456789/340.

RIGHI, A.A.; ALVES, T.R.; NEGRI, G.; MARQUES, L.M.; BREYERD, H.; SALATINO, A. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J Sci Food Agric; 91: 2363–2370. 2011. DOI 10.1002/jsfa.4468

RODRÍGUEZ-ARCHILLA, A.; RAISSOUNI, T. Randomized clinical trial of the effectiveness of complementary therapies for recurrent aphthous stomatitis. Med Clin (Barc). 2017;149(2):55–60

SANTOS, Juliana Guimarães dos. *Influência de extratos vegetais nos fatores de virulência de Candida albicans em biofilme*: estudo in vitro. Dissertação (Mestrado em Biopatologia Bucal) - Pós-graduação em Biopatologia Bucal - Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos, 2018. 50 f.

SCORZONI, L.; DE PAULA E SILVA, A.C.; MARCOS, C.M.; ASSATO, P.A.; DE MELO, W.C.; DE OLIVEIRA, H.C., et al. Antifungal therapy: new advances in the understanding and treatment of mycosis. *Frontiers in microbiology*. v. 8, p. 36, 2017.

SILVA, S.; HENRIQUES, M.; MARTINS, A.; OLIVEIRA, R.; WILLIAMS, D.; AZEREDO, J. Biofilmes de espécies não *Candida albicans Candida*: quantificação, estrutura e composição matricial. *Medical Mycology*. 47, 681-689. 2009. https://doi.org/10.3109/13693780802549594.

SINGH, A.V.; VYAS, V.; PATIL, R.; SHARMA, V.; SCOPELLITI, P.E.; BONGIORNO, G., et al. Quantitative characterization of the influence of the nanoscale morphology of nanostructured surfaces on bacterial adhesion and biofilm formation. *PloS one*. v. 6, n. 9, p. e25029, 2011. doi: 10.1371/journal.pone.0025029.

TOBALDINI-VALERIO, F.K.; BONFIM-MENDONÇA, P.S.; ROSSETO, H.C.; BRUSCHI, M.L.; HENRIQUES, M.; NEGRI, M. et al. Propolis: a potential natural product to fight Candida species infections. *Future microbiology*. v. 11, n. 8, p. 1035-1046, 2016. doi: 10.2217/fmb-2015-0016.

TÓTH, R.; NOSEK, J.; MORA-MONTES, HM.; GABALDON, T.; BLISS, J.M.; NOSANCHUK, J.D. et al. Candida parapsilosis: from Genes to the Bedside. Clinical Microbiology Reviews. 32;2;e00111-18. 2019.

TROFA, D.; GÁCSER, Á.; NOSANCHUK, J.D. Candida parapsilosis, um patógeno fúngico emergente. *Clinical microbiology reviews*, v. 21, n. 4, p. 606-625, 2008.

ZUZA-ALVES, D.L.; SILVA-ROCHA, W.P.; CHAVES, G.M. An update on Candida tropicalis based on basic and clinical approaches. *Frontiers in microbiology*. v. 8, p. 1927, 2017.

ANEXOS

ANEXO A: **Comprovante de submissão – Plos One – A1**

PLOS ONE

Highly Efficient Antibiofilm and Antifungal Activity of Green Propolis against Candida species in dentistry material

--Manuscript Draft--

Manuscript Number:	PONE-D-20-01892
Article Type:	Research Article
Full Title:	Highly Efficient Antibiofilm and Antifungal Activity of Green Propolis against Candida species in dentistry material
Short Title:	Green propolis against Candida biofilm
Corresponding Author:	Geusa Felipa de Barros Bezerra, Ph.D Universidade Federal do Maranhão São Luís, Maranhão BRAZIL
Keywords:	Green propolis; Candida sp; Biofilm; Dentistry material
Abstract:	Background This study evaluated the influence of green propolis' extract on the adhesion and biofilm formation of Candida species on dentistry material. Methods Phytochemical analysis of green propolis' extract was performed by High Performance Liquid Chromatography. Adhesion was quantified in a Neubauer chamber, counting the number of yeast cells adhered to the fragments; Biofilm formation was determined by counting the number of colony forming units (CFU). The intensity of biofilm formation adhesion was classified as negative, weak, moderate, strong and very strong. Fifteen compounds were identified in green propolis extract, mainly flavonoids. Results All strains were able to adhere and form biofilm on the surface of the orthodontic materials studied. In steel and resin, the adhesion intensity of the yeast cells was weak at all incubation times, except for C. parapsilosis and C. tropicalis which at 12hs showed moderate intensity. Regarding biofilm formation (24 and 48 hours), it was observed in the steel that C. albicans had moderate intensity at 24 and 48 hours; C. parapsilosis at 24 and 48 hours had very strong intensity; C. tropicalis at 24 hours had strong intensity and at 48 hours very strong. While in the resin, all species at 24 and 48 hours had strong intensity, except for C. tropicalis which at 48 hours had very strong intensity. Green propolis extract showed antifungal activity and was able to inhibit both adhesion and biofilm formation at 2.5 µg/mL. Conclusions This study reinforces the idea that green propolis has antifungal activity and interferes with virulence factors of Candida species.
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ANEXO B: **Regras de Submissão**

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Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value i parentheses after each value. Read more about SI units.
Drugs	Provide the Recommended International Non-Proprietary Name (rINN
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Allergens	The systematic allergen nomenclature of the World Health Organization/Internationa Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-commit should be used for manuscripts that include the description or use of allergenic protei manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manusc publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

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Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service."

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Middle section	*The following elements can be renamed as needed and presented in any order:* • Materials and Methods • Results • Discussion • Conclusions (optional)
Ending section	*The following elements are required, in order:* • Acknowledgments • References • Supporting information captions (if applicable)
Other elements	• Figure captions are inserted immediately after the first paragraph in which the figu cited. Figure files are uploaded separately. • Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.

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Parts of a Submission

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Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of cigarette smoke exposure on innate immunit A *Caenorhabditis elegans* model Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-random controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

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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.

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Acknowledgments

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Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.
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A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.
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Source	Format
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Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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- *PLOS ONE* guidelines, for clinical trials requirements
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- EQUATOR, for specific reporting guidelines for a range of other study types

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- Include details of any corrections applied to account for multiple comparisons. If corrections were not applied, include a justification for not doing so
- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).
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Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in theInternational Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

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

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A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research*, including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the

approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention ("death as an endpoint"), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How "Humane" Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.
- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected

- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select “Research Article” as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants’ experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.
See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Please review *PLOS ONE's* requirements for reporting blot and gel results and providing the underlying raw images.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such asAntibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to initial submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, databases, or tools as the primary focus of the manuscript if they meet the following criteria:

Utility
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability

If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a database, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper. If the primary focus of a manuscript is the presentation of a new tool, such as a newly developed or modified questionnaire or scale, it should be openly available under a license no more restrictive than CC BY.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials and software.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Hymenogaster huthii. Stielow et al. 2010, sp. nov.
[urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording. Note that this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum using the prefix http://www.indexfungorum.org/Names/NamesRecord.asp?RecordID=.

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the

electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist or Standards for reporting qualitative research (SRQR) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

Give Feedback