UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO
DOUTORADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA - MATERIAIS DENTÁRIOS

Incorporação de óleo de copaíba (C*opaifera multijuga*) em resina adesiva experimental

Carolina Rocha Augusto

Porto Alegre, fevereiro de 2019

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO
DOUTORADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO CLÍNICA ODONTOLÓGICA - MATERIAIS DENTÁRIOS

Linha de pesquisa: Biomateriais e técnicas terapêuticas em odontologia

Incorporação de óleo de copaíba (C*opaifera multijuga*) em resina adesiva experimental

Carolina Rocha Augusto

Tese apresentada como requisito obrigatório para obtenção de título de **Doutora em Odontologia** na área de concentração em Clínica Odontológica – Materiais Dentários.

Orientador: Prof. Dr. Fabrício Mezzomo Collares

Porto Alegre, fevereiro de 2019

CIP - Catalogação na Publicação

Augusto, Carolina
Incorporação de óleo de copaíba (Copaifera multijuga) em resina adesiva experimental / Carolina Augusto. -- 2019.
80 f.
Orientador: Fabrício Mezzomo Collares.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Programa de Pós-Graduação em Odontologia, Porto Alegre, BR-RS, 2019.

1. Fitoterapia. 2. Agentes antibacterianos. 3. Adesivos dentinários. 4. Fenômenos mecânicos. 5. Propriedades físicas e químicas. I. Mezzomo Collares, Fabrício, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

“Para cultivar a sabedoria, é preciso força interior. Sem crescimento interno, é difícil conquistar a autoconfiança e a coragem necessárias. Sem elas, nossa vida se complica. O impossível torna-se possível com a força de vontade.”

Dalai Lama

DEDICATÓRIA

Dedico este trabalho aos meus amados pais **Ademar Carlos Augusto e Teresa Cristina Rocha Augusto**, pelo amor incondicional, incentivo, coragem e força. Vocês são meu exemplo de bondade, honestidade e determinação. Agradeço o carinho e apoio que recebi em todos os momentos, vocês são a base sólida e firme da minha vida. Essa vitória é tão minha quanto de vocês.

Sobretudo a **Deus**, pela sabedoria e força, que nunca me abandonou e esteve sempre ao meu lado.

AGRADECIMENTOS

A meus pais **Ademar e Cristina** que se dedicaram incansavelmente à minha felicidade e realização de todos os meus sonhos. Amo vocês.

Ao meu irmão **Antonio Carlos** pelo ombro amigo, ouvido a distância, puxões de orelha, apoio e amor sempre oferecidos.

A minha **Vovó Lena** que sempre comemora muito cada passo que dou. Agradeço por todas as orações e conversas com Deus destinadas a mim.

Aos meus melhores amigos, o trio que está comigo 24 horas por dia, **Joyce Meira**, **Rafael Saulo** e **Gisely Naura,** pelas conversas, risadas, conselhos e suporte. Aguentaram todas as minhas lágrimas, não saíram do meu lado um minuto sequer nos dias difíceis. Vocês puxaram minha orelha algumas vezes e riram comigo sempre, são o real significado de amizade.

A minha amiga **Isadora Garcia** não há palavras que conseguirão dimensionar minha gratidão. Este trabalho é nosso, sem seu apoio, parceria, cumplicidade e estímulo não teria conseguido. Além realizar tantos ensaios por mim enquanto estava em Manaus, me ajudou em tudo quando chegava a Porto Alegre. As vezes tão doce e outros momentos tão braba, contribuiu com sua inteligência, experiência e dedicação nos artigos que compões essa tese. Agradeço a amizade que construímos e mantemos mesmo à distância.

Aos meus amigos, que são minha família em Porto Alegre, **Paula Dapper** e **Pedro Mallmann**. Como essa jornada foi mais fácil por causa de vocês. Eu tive um lar, suporte e principalmente amor de família todas as vezes que vocês me acolheram. Agradeço o apoio, ombro amigo, paciência e estímulo dados nesse tempo.

Ao meu orientador, **Prof. Dr. Fabrício Mezzomo Collares** pela confiança, oportunidade, conselhos e ensinamentos. Agradeço por me incentivar sempre, não ter desistido nos momentos que estive tão ausente e por todo o suporte e estímulo na época do concurso. Tive o melhor mentor, com cobranças necessárias e orientação sempre, sendo fundamental para alcançar meus objetivos.

Aos professores **Dra. Susana Maria Werner Samuel**, **Dra. Carmem Beatriz Borges Fortes** e **Dr. Vicente Castelo Branco Leitune,** pelos exemplos, acolhimento e estímulo.

A amiga **Stéfani Becker Rodrigues** por todos os momentos de apoio e companheirismo, dentro e fora do laboratório.

Aos amigos **Thiago Mendes**, **Sybilla Dias**, **Juliana Barreto**, **Lidiane Gomes, Sarah Brasil, Lívia Coutinho**, **Felipe Muniz**, **Gabriela Meira, Rebeca Chaves, Roberto Martinho, Guilherme Cândido e Hércules Dias** pela amizade, paciência e torcida.

A toda equipe do Laboratório de Materiais Dentários (LAMAD) com a qual convivi em períodos intercalados nos últimos anos, aos que não participam mais e aos presentes, **Gabriela Balbinot, Marla Cuppini, Mariéle Mildner, Bruna Genari, Fábio Bohns, Felipe Degrazia, Fernando Portella, Nélio Dorneles, Rodrigo Tubelo, Marília Paulus** e a técnica **Rosimeri** pela ajuda que cada um forneceu a sua maneira.

A amiga **Letícia Moreira**, por todos os momentos de apoio e companheirismo, dentro e fora da UFRGS.

Aos amigos **Kamila Guedes, Cíntia Carvalhal, Wladimir Barbosa, Ghisa Benchimol** e **Rachid Zacarias** pelo incentivo e suporte em minhas ausências em sala de aula e clínica. Agradeço a torcida e força sempre dadas.

Ao professor **Dr. Emerson Silva Lima**, coordenador do Programa de Pós-Graduação em Ciências Farmacêuticas, que me acolheu de forma tão generosa desde o primeiro dia que o procurei, contribuindo de forma significativa para a realização deste trabalho. Obrigada pela oportunidade, ideias, orientações e paciência, pois tudo isso começou com o fornecimento dos óleos de copaíba. Obrigada por acreditar em mim, por todas as discussões e ensinamentos.

Ao professor **Dr. Valdir Florêncio da Veiga Junior**, maior pesquisador de copaíba do mundo, uma inspiração. Mesmo sem nos conhecermos pessoalmente, foi generoso e receptivo desde a primeira mensagem, estando sempre à disposição para compartilhar seu conhecimento e contribuir em todo o trabalho.

A **Milena Campelo Freitas de Lima**, doutoranda do Programa de Pós-Graduação em Química – UFAM, pela realização da cromatografia e todo conhecimento compartilhado sobre os óleos e temas abordados no primeiro artigo.

A toda equipe do Laboratório de Inovação e Desenvolvimento em Tecnologia Farmacêutica-UFAM, especialmente ao **Prof. Dr. Jesus Rafael Rodríguez Amado e Prof. Drª. Tatiane Pereira de Souza** e aos amigos **Rodrigo** e **Newton**.

A **Msc. Ivanildes dos Santos Bastos** e **Dr. Patrícia Puccinelli Orlandi**, da Plataforma de Bioensaios Biotecnológicos, Instituto Leônidas e Maria Deane – FIOCRUZ Amazônia, pela realização dos ensaios antimicrobianos e conhecimentos compartilhados.

A **Universidade do Estado do Amazonas**, que além de ter permitido minha formação, hoje me proporciona uma carreira que só me traz alegrias e felicidades. Agradeço em especial a direção da **Escola Superior de Ciências da Saúde** e **Coordenação do Curso de Odontologia** por apoiarem a realização deste doutorado.

A **Universidade Nilton Lins**, em especial a coordenação do curso de odontologia pelo incentivo e apoio nesta caminhada.

Ao **Programa de Pós-Graduação em Odontologia**, na pessoa do coordenador **Prof. Dr. Cassiano Kuchenbecker Rosing**. Agradeço a oportunidade de participar desse programa de excelência, do qual tenho muito orgulho de ter sido aluna.

A **Universidade Federal do Rio Grande do Sul** e a **Faculdade de Odontologia** por terem dado a oportunidade de realizar todos os trabalhos e por toda a experiência proporcionada, desde o mestrado.

RESUMO

O objetivo deste estudo foi formular uma resina adesiva experimental utilizando óleo de copaíba com concentração inibitória mínima (CIM) e caracterização química determinadas e avaliar suas propriedades físicas, mecânicas, biológicas e químicas. Doze óleos comerciais foram avaliados quanto a CIM e as amostras com menor valor foram caracterizadas por cromatografia gasosa. O óleo de *Copaifera multijuga* foi utilizado na formulação de resinas adesivas. Na resina base foi incorporado o óleo nas concentrações de 5%, 10% e 15%, em massa, além do grupo controle sem adição do óleo. As resinas foram submetidas aos ensaios de grau de conversão (GC), ângulo de contato (θ), energia livre de superfície (ELS), amolecimento em solvente (KHN inicial, KHN final e ΔKHN%), atividade antimicrobiana frente a *Streptococcus mutans*, resistência coesiva e resistência de união a microtração. Não houve alteração do GC após a incorporação do óleo ($p=0,073$), bem como o óleo não influenciou θ com água ($p=0,859$) e resistência de união imediata ($p=0,182$). Os grupos experimentais apresentaram diminuição de KHN1 ($p<0,001$), resistência coesiva ($p=0,009$) e apresentaram maior ΔKHN% ($p<0,001$). Todos os grupos apresentaram amolecimento em solvente ($p<0,001$). O θ com α-bromonaftaleno diminuiu ($p<0,001$) e a ELS aumentou ($p=0,002$) após incorporar 10 e 15% de óleo. O grupo com 15% de óleo apresentou redução na formação de biofilme ($p<0,001$) e viabilidade de bactérias planctônicas ($p = 0,004$). Portanto, a incorporação de 15% de óleo de copaíba proporcionou atividade antibacteriana na resina adesiva experimental sem comprometer GC e resistência de união imediata, indicando que a utilização de produtos naturais na formulação de sistemas adesivos resinosos é promissor.

Palavras-chave: Fitoterapia; Agentes antibacterianos; Adesivos dentinários; Fenômenos mecânicos; Propriedades físicas e químicas.

ABSTRACT

The aim of this study was to formulate an experimental adhesive resin using copaiba oil with minimum inhibitory concentration (MIC) and determined chemical characterization and to evaluate its physical, mechanical, biological and chemical properties. Twelve commercial oils were evaluated for MIC and the samples with lower value were characterized by gas chromatography. *Copaifera multijuga* oil was added in adhesive resins at 5, 10 and 15 wt.%, besides the control group without oil addition. Degree of conversion (DC), softening in solvent (KHN initial, KHN final and ΔKHN%), ultimate tensile strength (UTS), contact angle (θ), surface free energy (SFE), antibacterial activity and microtensile bond strength (μ-TBS) were evaluated. No changes in DC were observed after incorporating copaiba oil ($p = 0.073$). The addition of oil did not influence the θ with water ($p = 0.859$) and μ-TBS ($p = 0.182$). The experimental groups decreased KHN1 ($p < 0.001$), UTS ($p = 0.009$), and showed highest ΔKHN% ($p < 0.001$). All groups softened in solvent ($p < 0.001$). The θ with α-bromonaphthalene decreased ($p < 0.001$) and the SFE increased ($p = 0.002$) after incorporating 10 and 15 wt.% of copaiba oil. The group with 15 wt.% of copaiba oil presented a reduction in biofilm formation ($p < 0.001$) and planktonic bacteria viability ($p = 0.004$). Therefore, The incorporation of 15 wt.% of copaiba oil provided antibacterial activity when added to an experimental adhesive resin without compromising DC and immediately μ-TBS, which may be promising for the use of natural products in the formulation of dental adhesive systems.

Keywords: Phytotherapy; Antibacterial agents; Dentin-bonding agents; Mechanical phenomena; Physical and chemical properties.

SUMÁRIO

1. ANTECEDENTES E JUSTIFICATIVA

A longevidade dos tratamentos restauradores está relacionada a diversos fatores, contudo, a cárie secundária permanece a razão mais comum para a necessidade de substituição das restaurações (ELTAHLAH et al., 2018). O desempenho dos materiais odontológicos aplicados sobre um substrato orgânico depende da manutenção do equilíbrio dos tecidos dentais, sujeitos à ação da microbiota presente na cavidade oral (ASKAR et al., 2017). A presença de biofilme bacteriano organizado é uma das causas do início do processo de cárie, levando à destruição de tecido sadio e consequente falha no tratamento odontológico, quer seja preventivo ou restaurador (GONZÁLEZ-CABEZAS, 2010). A cárie secundária é definida como uma lesão progressiva e ativa nas margens de restaurações que (NEDELJKOVIC et al., 2015) desenvolve-se rapidamente no esmalte, próximo ao ângulo cavosupercial, quando as condições locais variam para um ambiente ácido (JOKSTAD, 2016). Desta forma, o processo de desmineralização-remineralização, igualmente à cárie primária, é influenciada por diversos fatores como hábitos de higiene dentária, dieta do indivíduo, presença de flúor, susceptibilidade do hospedeiro, composição e fluxo salivar (CHENICHERI et al., 2016). No processo, o mecanismo de redução do pH do meio ocorre mediante produção de ácidos pelas bactérias cariogênicas específicas, que formam o biofilme localizado na superfície restaurada ou no esmalte adjacente à margem da restauração (STRUŻYCKA, 2014).

A composição do biofilme cariogênico nas cáries primária e secundária são semelhantes, consistindo principalmente de *Streptococcus mutans* (*S. mutans*) e *Lactobacilli* (MO et al., 1985), localizadas predominantemente nas superfícies proximais e cervicais, regiões de fácil depósito de biofilme e difícil higienização

(MJOR, 1985). *S. mutans* são bactérias gram-positivas, acidogênicas, com capacidade de adesão a superfície dentária. Estas cepas possuem rápido metabolismo de monossacarídeos e sobrevivem em condições ácidas (STRUŻYCKA, 2014), sendo as mais avaliadas e utilizadas nos estudos *in vitro*, *in situ* e *in vivo*. Apesar da causalidade multifatorial da doença cárie (HURLBUTT; YOUNG, 2014), o aperfeiçoamento de técnicas e materiais odontológicos, com a formulação de polímeros anti-cariogênicos tem sido objeto de estudos atuais relacionados ao tema (HUANG et al, 2016; COCCO et al, 2015). Idealmente, os materiais odontológicos deveriam possuir uma característica antimicrobiana, seja para colaborar na prevenção da cárie primária ou impedir a recidiva na superfície já restaurada (NEDELJKOVIC et al., 2015; SAMPATH; HEGDE; HEGDE, 2011; PENMETSA et al, 2014).

Materiais antimicrobianos somariam aos tratamentos minimamente invasivos preconizados atualmente (ERICSONA et al., 2003). Os conceitos modernos da cariologia e odontologia restauradora enfatizam a redução de riscos aliada a práticas conservadoras, como tratamentos que visam a inativação de lesões ativas, remineralização de lesões cavitadas e execução de preparos ultraconservadores (PETERS; MCLEAN, 2001). Materiais poliméricos como adesivo, selante, infiltrante e compósito resinoso são os mais utilizados neste cenário, aplicados diretamente sobre esmalte ou dentina, com adesão mecânica e química a esses tecidos (NEDELJKOVIC et al., 2015). Como agravante, as situações clínicas de fratura de restaurações podem ocorrer, com a formação de fendas marginais que se tornam retentoras de biofilme e facilitam o desenvolvimento da doença cárie (JOKSTAD, 2016). Logo, resinas antimicrobianas que consigam inibir a formação do biofilme e a

consequente desmineralização dos tecidos dentários adjacentes a restauração, seriam uma estratégia pertinente (IMAZATO, 2003; COCCO et al, 2015).

Sistemas adesivos antimicrobianos podem proporcionar um duplo efeito. Inicialmente podem desinfetar a cavidade, antes da inserção do material restaurador, para posteriormente causar um efeito bactericida e bacteriostático sobre o biofilme (NEDELJKOVIC et al., 2015). As estratégias são baseadas na incorporação de partículas de carga e no desenvolvimento de monômeros antimicrobianos (WANG; SHEN; HAAPASALO, 2014). Fluoretos (VERCRUYSSE; DE MAEYER; VERBEECK, 2001), vidro bioativo (KORKUT; TORLAK; ALTUNSOY, 2016), óxido de zinco (GARCIA et al., 2018), partículas carreadoras contendo prata (DEGRAZIA et al., 2016), clorexidina (BOUTSIOUKI et al., 2019), triclosan (RATHKE et al., 2010), compostos quaternários de amônia (COLLARES et al., 2017), quitosana (RAJABNIA et al., 2016) e antibióticos, como vancomicina e metronidazol (KUDOU et al., 2000) são algumas das possibilidades já estudadas. O sistema adesivo Clearfil SE Protect é a única opção comercial de agente adesivo antimicrobiano. Ele possui no primer um monômero funcional MDPB contendo em sua composição amônia quaternária com um grupo metacriloxil (IMAZATO et al., 2003).

A utilização de plantas, óleos e extratos naturais com finalidade terapêutica é uma prática comum e documentada desde os primórdios da civilização (AMORIM et al, 2003). A fitoterapia oferece alternativas viáveis e importantes às populações dos países em desenvolvimento, já que seu custo é diminuído. Baseada em influências culturais de populações indígenas, africanas e europeias, é uma ciência que proporciona opções acessíveis ao tratamento e prevenção de diversas doenças, com impacto cultural, social e econômico (CARVALHO et al., 2018). Nas últimas décadas o interesse pela fitoterapia aumentou expressivamente e, no Brasil, com a

implementação da Política Nacional de Plantas Medicinais e Fitoterápicos (BRASIL, 2006), houve um incentivo ao desenvolvimento de medicamentos e produtos naturais terapêuticos. Em virtude do conhecimento científico gerado por estudos a respeito das espécies botânicas e suas propriedades, materiais biotecnológicos vêm sendo desenvolvidos (GROPPO et al., 2008). Ação antibacteriana, antifúngica, antiviral, anti-inflamatória, antioxidante, anticarcinogênica, antinociceptiva, saliva estimulante e anticárie estão entre as principais atividades desempenhadas pelos fitoterápicos (FREIRES; ROSALEN, 2016). Na odontologia muitas espécies botânicas podem proporcionar alternativas de tratamentos e materiais para afecções odontológicas (FENNER et al., 2006). Estudos *in vitro* têm produzido evidências científicas que extratos e óleos naturais possuem atividade antimicrobiana frente a microrganismos patogênicos presentes na cavidade oral (CHANDRA SHEKAR et al, 2015).

As copaíbas são árvores nativas da região tropical da América Latina e África, de onde, a partir de uma incisão feita no tronco, pode-se extrair um óleo transparente, cuja coloração pode variar do amarelo ao marrom (VEIGA JUNIOR; PINTO, 2002). Esse óleo é facilmente encontrado e muito empregado na região amazônica, com grande representação social e econômica, possuindo propriedades antimicrobiana (SANTOS et al, 2008), e anti-inflamatória (VEIGA JUNIOR et al, 2007). O óleo de copaíba é constituído de sesquiterpenos, sendo os mais comuns β-cariofileno, óxido de cariofileno, α-humuleno, δ-cadineno, α-cadinol, α-cubebeno, α- e β-selineno, β-elemeno, α-copaeno; e diterpenos, como os ácidos copálico, colavênico, hardwíckico, caurenóico e cauranóico (LEANDRO et al., 2012). O gênero *Copaifera* compreende 72 espécies, sendo 16 exclusivamente encontradas no Brasil (VEIGA JUNIOR; PINTO, 2002). Em razão desta diversidade, os óleos apresentam composições químicas diferentes e variam de acordo com a espécie da árvore. Essas diferenças

também ocorrem em função da maturação da árvore, sazonalidade em uma mesma árvore, numa mesma espécie e entre espécies. Os métodos de isolamento e identificação mais frequentes são a cromatografia líquida de alta eficiência, cromatografia com fluido supercrítico com detector de infravermelho e cromatografia gasosa acoplada à espectrometria de massas (CGAR). A CGAR permite a identificação dos componentes do óleo quase na totalidade, é um método sensível, de alta precisão, sendo o mais indicado para amostras voláteis (VEIGA JUNIOR; PATITUCCI; PINTO, 1997), como o óleo de copaíba.

Estudos realizados com o óleo puro mostram sua eficácia contra *S. mutans* (BARDAJÍ et al., 2016; BARI et al., 2016; CONDE et al., 2015; DIAS et al., 2015; PIERI et al, 2012, PIERI et al, 2010; SIMÕES et al., 2016; SOUZA et al. 2011), *Streptococcus sanguinis* (BARDAJÍ et al.,2016; CONDE et al., 2015; DIAS et al., 2015; SIMÕES et al., 2016), *Porphyromonas gingivalis* (MORAES et al., 2016; DIAS et al., 2015; SOUZA et al., 2011) e *Streptococcus sobrinus* (CONDE et al., 2015; SOUZA et al. 2011). Alguns componentes isolados também apresentam resultados positivos como o β-cariofileno no *S. mutans* (PIERI et al., 2016) e ácido copálico no *S. mutans* e *S. sobrinus* (SOUZA et al., 2011). O mecanismo de ação não é completamente elucidado, porém sabe-se que a parede celular sofre efeitos diretos (BAKKALI et al., 2008). Ao avaliar as características morfológicas das células bacterianas, (SANTOS et al., 2008) nota-se uma interação com o óleo de copaíba, o que pode explicar o efeito na parede celular que se apresenta rompida e com diminuição do volume.

Sabe-se que o óleo de copaíba pode ser formulado em emulsão (BARI et al., 2016), gel de limpeza de cavidade (SIMÕES et al, 2016), cimento odontológico (VASCONCELOS et al., 2008) e cimento endodôntico experimental (GARRIDO et al.,

2010). As emulsões contendo diferentes concentrações de óleo de *C. multijuga* foram avaliadas quanto à atividade antimicrobiana (*S. mutans*, *S. oralis*, *S. salivarius* e *L. casei*) e citotoxicidade utilizando fibroblastos, com resultados positivos nestes ensaios. A atividade antimicrobiana do gel à base de óleo de *C. multijuga* apresentou resultados favoráveis frente ao *S. mitis* e *S. salivarius*. O cimento odontológico utilizando óleo de *C. multijuga* associado ao óxido de zinco e hidróxido de cálcio apresentou atividade antibacteriana frente a *S. mutans* e *S. sanguinis*, indicando ser um material promissor. O cimento endodôntico contendo óleo de *C. multijuga* foi submetido aos ensaios de tempo de presa, viscosidade, espessura de película, estabilidade dimensional, radiopacidade e solubilidade, apresentando resultados satisfatórios nestes testes (GARRIDO et al., 2010). Uma característica em comum dos materiais odontológicos citados é a ausência de estrutura polimérica, pois espera-se que a adição de um óleo em materiais com base em metacrilato atue como um plastificante.

Apesar de serem reportados diversos valores para concentração inibitória mínima (CIM) e bactericida mínima frente a diferentes microrganismos cariogênicos, o uso de um óleo padronizado é fundamental para permitir a validação do fitoterápico com uso seguro, eficaz e de qualidade. Além da identificação botânica, o estudo fitoquímico é necessário, pois muitos dados sobre a composição química e atividade farmacológica dos óleos são contraditórios (CASCON; GILBERT, 2000).

Considerando o exposto, o desenvolvimento de materiais dentários antimicrobianos com incorporação de óleo de copaíba pode ser uma alternativa aos agentes tradicionais, sendo necessário a formulação experimental de materiais poliméricos como estratégia ainda não estudada. Para tanto é necessário identificar um óleo que apresente baixa CIM, com suas características fitoquímicas

identificadas, para minimizar a concentração do óleo nos materiais a serem desenvolvidos e então avaliar sua influência sobre as propriedades mecânicas, físicas e químicas do material obtido.

2. OBJETIVO

Avaliar a concentração inibitória mínima (CIM) de óleos de copaíba de diferentes espécies e desenvolver uma resina adesiva experimental com a incorporação de óleo de copaíba com menor CIM, em diferentes concentrações, bem como caracterizar as propriedades dos materiais resultantes.

3. MANUSCRITOS

Esta tese de doutorado, cujo tema é inédito, se apresenta na forma de artigos, escritos na língua inglesa e que seguem as normas referentes aos periódicos **Phytotherapy Research**, para qual o manuscrito 1 foi submetido, e **Clinical Oral Investigations**, para qual o manuscrito 2 será submetido.

3.1 MANUSCRIPT 1

Screening for the inhibitory activity of Copaiba oil on *Streptococcus mutans*

Carolina Rocha Augusto[a], Emerson Silva Lima[b], Milena Campelo Freitas de Lima[c], Valdir Florêncio da Veiga Junior[d], Fabrício Mezzomo Collares[e]

[a]DDS, MSc, Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Laboratório de Materiais Dentários, Porto Alegre, RS, Brasil, carolina.augusto@ufrgs.br

[b]DDS, MSc, PhD, Universidade Federal do Amazonas, Laboratório de Atividades Biológicas, Faculdade de Ciências Farmacêuticas, Manaus, AM, Brasil, eslima@ufam.edu.br

[c]DDS, MSc, Universidade Federal do Amazonas, Instituto de Ciências Exatas, Departamento de Química, Manaus, AM, Brasil, mile_campelo@yahoo.com.br

[d]DDS, MSc, PhD, Military Institute of Engineering, Chemistry Section, Rio de Janeiro, RJ, Brasil, valdir.veiga@ime.eb.br

[e]DDS, MSc, PhD, Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Laboratório de Materiais Dentários, Porto Alegre, RS, Brasil, fabricio.collares@ufrgs.br

*Corresponding author:

Fabrício Mezzomo Collares

Rua Ramiro Barcelos, 2492, Rio Branco - Porto Alegre – RS – Brazil - 90035-003

Phone: +55 51 33085198

e-mail: fabricio.collares@ufrgs.br

Abstract

The aim of this study was to investigate the minimum inhibitory concentration (MIC) of commercial copaiba oils against *Streptococcus mutans* and to characterize and quantify the chemical composition of Copaiba oils with the lowest MIC. Crude copaiba oils from four copaifera species were evaluated. Essential oil was obtained by hydrodistillation and the residue was also used. MIC was performed by the microdilution technique. The oils were dissolved in serial dilutions in concentrations ranging between 200 and 1.95 µg/mL. MIC was determined based on color change, indicating cell viability. The chromatographic analyses of copaiba oils that presented the lowest MIC were performed in a gas chromatograph coupled to a mass spectrometer, and the chemical constituents of copaiba oils were identified by comparison of retention rates, mass spectra with spectrum and data available in the literature. Two oils of *C. multijuga* (samples 5 and 8) and one of *C. reticulata* (sample 4) presented the lowest values of MIC against *S. mutans* (6.25 µg/mL). The characterization of oils demonstrated that the major component was Bisabolene-β (17.98%) caryophyllene-β (51.51%) and kolavenic acid methyl ester (17.73%), respectively. Two of the evaluated species showed better antimicrobial activity, where it is possible to observe varied and distinct compositions, indicating that the combined effect among the constituents are involved at action mechanisms.

Keywords: Phytotherapy; Fabaceae; Antimicrobial agents; Dental caries; *Streptococcus mutans*.

1. Introduction

Dental caries is a multifunctional disease that depends on diet, time, individual susceptibility and microorganisms, beginning with the demineralization of enamel components and may compromise all dental tissues, leading to tooth loss (Kutsch, 2014). The presence of gram-positive bacteria, especially *Streptococcus mutans*, is an important factor for the development of caries (Struzycka, 2014), with which it is positively correlated (Kirstila, Hakkinen, Jentsch, Vilja, & Tenovuo, 1998). The bacterial metabolism results in the formation of acids, responsible for the initiation of the dissolution of the enamel (Esberg et al., 2017). The use of antimicrobial agents is a strategy to prevent this disease, such as chlorhexidine in gels, toothpastes, varnishes or mouthwash (Walsh, Oliveira-Neto, & Moore, 2015).

The use of natural oils and extracts from plants has presented promising results against bacterial activities (Chandra Shekar, Nagarajappa, Suma, & Thakur, 2015). This may be an alternative therapy, with historical and cultural representation in South America of the traditional practice of using antimicrobial agents. Copaiba oil is widely used to treat bacteria, fungal and inflammatory infections (Diefenbach, Muniz, Oballe, & Rosing, 2018; Vargas et al., 2015; Veiga Junior & Pinto, 2002), and it also presents healing properties. Its action against gram-positive microorganisms has already been shown. However, there is a dependence between copaiba oil's composition and its antimicrobial effect (Santos et al., 2008).

Copaiba oil has a complex composition, involving terpenes and sesquiterpenes (Leandro et al., 2012). β-caryophyllene is the main component responsible for the antimicrobial activity of this material (Yoo & Jwa, 2018; Pieri et al., 2016; Lucca et al., 2015). The effectiveness of herbal medicines is based on the synergy of all its

constituents. Oils from the same species may have different compositions, depending on the region where the trees were planted, the extraction period and the method of collection (Barbosa et al., 2012; Barbosa, Wiedemann, Medeiros, Sampaio, & Veiga Junior, 2013). Therefore, the antimicrobial activity varies according to the oil (Santos et al., 2008).

The aim of this study was to investigate the minimum inhibitory concentration (MIC) of commercial copaiba oils against *S. mutans* and to characterize and quantify the chemical composition of Copaiba oils with the lowest MIC.

2. Materials and methods

2.1 Obtaining copaiba oil

Copaiba oils from *Copaifera multijuga* (samples 1, 5, 6, 7, 8 and 9), *Copaifera langsdorffii* (sample 2), *Copaifera guianensis* (sample 3) and *Copaifera reticulata* (sample 4) species were obtained from tree trunks, as previously published (Santos et al., 2008). Copaiba essential oil was obtained by hydrodistillation (samples 10 and 11) (Pereira et al., 2008) and the residue was also evaluated (sample 12).

2.2 Minimum inhibitory concentration (MIC)

The *in vitro* assay was performed by the microdilution technique, performed in 96-well microtiter plates, according to (Cockerill et al., 2012). Each hole received bacterial inoculum of standard strains of *S. mutans* (NCTC 10449, INCQS 00446, Oswaldo Cruz Foundation, National Institute of Health Quality Control, Rio de Janeiro,

Brazil) incorporated within standardized brain heart infusion (Aldrich Chemical Company, St. Louis, Missouri, USA); the oils dissolved in dimethyl sulfoxide (DMSO) at 1 mg/mL in serial dilutions in concentrations ranging between 200 and 1.95 µg/mL; and 20 µL resazurin solution at 0.01% as a colorimetric indicator of oxireduction to characterize cell viability, totaling a volume of 100 µL. As positive control, imipenem and cilastatin sodium (TIENAM) was used at the same concentrations as the test samples. As negative control, only DMSO was used. The assay was prepared in triplicate for each isolate. The plates were incubated for 24 h at 37°C. MIC was defined as an oil concentration where the dye remained blue, because when there is a conversion to rose, this indicated there was bacterial growth.

2.3 Chromatographic analysis

The chromatographic analyses of copaiba oils that presented the lowest MIC were performed in a gas chromatograph coupled to the mass spectrometer (DSQ II, Thermo Scientific, Austin, Texas, USA) with a simple quadrupole and auto-injector analyzer (AI 3000, Thermo Scientific, Austin, Texas, USA). The mass spectra were obtained by electron impact, in the range between 40 and 400 µm, using 70 eV. The oven temperature programming used was initially from 90°C to 160°C with a rate of change of 2.5° C/min, and from 160°C to 290°C with a rate of change of 10°C/min up to 290°C with a final isotherm of 5 minutes. The injection module with split ratio of 1:40, flow rate of 2 $mL.min^{-1}$ and an apolar column, SE-30, coated with 100% dimethylpolysiloxane (25 m × 0.25 mm × 0.25 µm). The injector and detector temperatures were 270°C and 300°C, respectively. Copaiba oils (2 mg) was previously derivatized by an *in situ* reaction using the trimethylsilyldiazomethane reagent (TMSD)

(Aldrich Chemical Company, St. Louis, Missouri, USA), converting diterpene acids into their respective methyl esters (Migowska, Stepnowski, Paszkiewicz, Golebiowski, & Kumirska, 2010).

2.4 Identification of chemical components

The chemical constituents of copaiba oils were identified by comparison of retention rates (RR), mass spectra with spectrum and data available in the literature. For the sesquiterpene identification, homologous hydrocarbons series were used for the confirmation of RR. Similarly, previously isolated diterpene acid patterns were used for comparison of mass spectra.

3. Results

The antimicrobial screening performed by the microdilution technique is shown in Table 1. Oils of *Copaifera multijuga* (samples 5 and 8) and *Copaifera reticulata* (sample 4) presented the lowest values of MIC against *S. mutans* (6.25 µg/mL).

The characterization of oils, by the identification of chemical compounds, is presented in detail in Table 2. The chromatograph of samples 4, 5 and 8 are shown in Figures 1, 2 and 3 respectively, where the similarity between *C. multijuga* oils and difference of *C. reticulata* may be seen. Sample 4 has 45.23% sesquiterpenes, 0.83% oxygenated sesquiterpenes and 50.09% diterpenes. Sample 5 has 69.02% sesquiterpenes, 3.10% oxygenated sesquiterpenes and 23.55% diterpenes. Sample 8 has 79.11% sesquiterpenes, 5.60% oxygenated sesquiterpenes and 12.60% diterpenes.

4. Discussion and conclusion

In the present study, the antimicrobial activities of twelve samples of copaiba oil were analyzed against *S. mutans*, which is the major etiologic agent involved in dental caries (Struzycka, 2014). The antimicrobial activity of copaiba oil is the object of several studies (Diefenbach, Muniz, Oballe, & Rosing, 2018; Zimmermam-Franco et al., 2013; Souza et al., 2011; Santos et al., 2008), however as the Copaifera genus has a wide diversity of species, there is a divergence of findings. In the present study, *C. multijuga* (samples 5 and 8) and *C. reticulata* (sample 4), collected near the municipalities of Lábrea / AM, Parintins / AM and the state of Pará, respectively, presented the lowest MIC (6.25 ug/mL). In Brazil, multijuga is the most commonly found species within the genus (Veiga Junior & Pinto, 2002) so most studies used this. Besides the species, the biological properties of the oils are influenced by the planting site of the tree and the time of year collected.

Regardless of this variability, some components of the oils have greater involvement in the process of bacterial cell death than others (Leandro et al., 2012; Correia et al., 2008; Pacheco, Barata, & Duarte, 2006). *S. mutans* is a gram-positive microorganism, where it is necessary for a chemical substance to inhibit the growth and alter the cell membrane, leading to lysis, to guarantee antibacterial activity (Zacchino et al., 2017). Although several studies point out that the synergism of the chemical components is responsible for the antibacterial activity, β-caryophyllene (Yoo & Jwa, 2018; Alencar et al., 2015) and copalic acid (Leandro et al., 2012; Souza et al., 2011) are highlighted as the agents mainly responsible for the cell lysis, decreasing biofilm formation.

Copaiba oil extracted from the multijuga species has a high concentration of β-caryophyllene. As shown in Table 2, samples 5 and 8, respectively, of multijuga have 11.58% and 51.51% of this component. On the other hand, oil extracted from the reticulata species presented only 1.29%. In this sample, the sesquiterpene in greater concentration was the β-Bisabolene (18.33%). There is a great variation of composition between species, as observed in this study. Copaiba oils are composed of sesquiterpenes and diterpenes, their concentrations are differentiated between species and the chemical interaction between them is responsible for the biological properties of the oils (Silva et al., 2012; Barreto-Júnior et al., 2005).

In the present study, 39 sesquiterpenes were identified in the three samples, corroborating the data previously reported (Veiga Junior & Pinto, 2002). Sesquiterpenes, which are the volatile portion of the oils, are still classified as hydrocarbons and oxygenates. In the three oils tested, the minor components were the oxygenated sesquiterpenes, represented mainly by the caryophyllene oxide, β-sesquiphellandrene and 7-epi-α-selinene.

The production of terpenes by adult species is related to the need for protection against some aggression to the tree, such as from animals, fungi and bacteria (Dudareva et al., 2004). Changes in concentration may be observed during the year, however the differences in the percentages of these substances occurred independently of the season. The oil of the reticulata species is composed of 50.1% terpene acids and the highest concentration was of kolavenic acid (17.7%). All these active components have been studied in isolation, with controversial results (Leandro et al., 2012).

The role of each chemical constituent in the antimicrobial effect of the oils is difficult to determine precisely (Vargas et al., 2015; Silva et al., 2012). It was noted in

this study that there was a large variation among species, and even in multijuga oil, the main sesquiterpenes found varied. Although there are several studies that describe the composition and variation of copaiba oils of different species, ethnopharmacological studies have not yet reached consensus. It could be observed that of all the samples evaluated, three had better antimicrobial activity and the chromatographic analysis showed distinct compositions and concentrations, where the combined effect among them is attributed to be responsible for the results found. Therefore, when aiming to develop materials with antimicrobial potential, a scan is necessary to identify the best compound, since the mechanism of action of sesquiterpenes and terpene acids in isolation is divergent.

In conclusion, two of the evaluated species showed better antimicrobial activity, *C. multijuga* and *C. reticulata*, where it is possible to observe varied and distinct compositions, both between sesquiterpenes and terpene acids, indicating that the combined effect among the constituents are fundamental for the action.

Acknowledgements

The authors gratefully acknowledge Dr. Patrícia Puccinelli Orlandi and MSc. Ivanildes dos Santos Bastos, from Instituto Leônidas, and Maria Deane – FIOCRUZ Amazônia, [RPT11H] Biotechnological Bioassay Platform – AM, for the antimicrobial test.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Alencar, E. N., Xavier-Junior, F. H., Morais, A. R., Dantas, T. R., Dantas-Santos, N., & Verissimo, L. M., et al. (2015). Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. *Journal of Nanoscience and Nanotechnology,* 15, 880-888.

Barbosa, P. C. S., Medeiros, R. S., Sampaio, P. T. B., Vieira, G., Wiedemann, L. S. M., & Veiga Junior, V. F. (2012). Influence of abiotic factors on the chemical composition of copaiba oil (*Copaifera multijuga Hayne*): soil composition, seasonality and diameter at breast height. *Journal of the Brazilian Chemical Society*, 23(10), 1823-1833.

Barbosa, P. C. S., Wiedemann, L. S. M., Medeiros, R. S., Sampaio, P. T. B., & Veiga Junior, V. F. (2013). Phytochemical fingerprints of copaiba oils (*Copaifera multijuga Hayne*) determined by multivariate analysis. *Chemistry & Biodiversity*, (10)7, 1350-1360.

Barreto-Júnior, A. G., Biscaia-Júnior, E. C., Veiga Junior, V. F., Pinto, A. C., Carvalhaes, S. F., & Maciel, M. A. M. (2005). Ion exchange chromatography applied to the fractionation of the copaíba oil (*Copaifera multijuga*) and sacaca (*Croton cajucara*) extracts. *Quimica Nova*, 28, 719-722.

Chandra Shekar, B. R., Nagarajappa, R., Suma, S., & Thakur, R. (2015). Herbal extracts in oral health care - a review of the current scenario and its future needs. *Pharmacognosy Reviews*, 9, 87-92.

Cockerill, F. R., Wikler,M. A., Alder, J., Dudley, M. N., Eliopoulos, G. M., Ferraro, M. J., & et al. (2012). *Methods for dilution antimicrobial susceptibility. Tests for bacteria*

that grow aerobically. Wayne, PA: Clinical and Laboratory Standards Institute Approved Standard-Ninth edition (M07-A9).

Correia, A. F., Segovia, J. F., Goncalves, M. C., de Oliveira, V. L., Silveira, D., Carvalho, J. C., & et al. (2008). Amazonian plant crude extract screening for activity against multidrug-resistant bacteria. *European Review for Medical and Pharmacological Sciences*, 12, 369-380.

Diefenbach, A. L., Muniz, F., Oballe, H. J. R., & Rosing, C. K. (2018). Antimicrobial activity of copaiba oil (*Copaifera ssp.*) on oral pathogens: systematic review. *Phytotherapy Research*, 32, 586-596.

Dudareva, N., Pichersky, E., & Gershenzon, J. (2004). Biochemistry of plant volatiles. *Plant Physiology*, 135, 1893-1902.

Esberg, A., Sheng, N., Marell, L., Claesson, R., Persson, K., Boren, T., & et al. (2017). *Streptococcus mutans* adhesin biotypes that match and predict individual caries development. *EBioMedicine*, 24, 205-215.

Kirstila, V., Hakkinen, P., Jentsch, H., Vilja, P., & Tenovuo, J. (1998). Longitudinal analysis of the association of human salivary antimicrobial agents with caries increment and cariogenic micro-organisms: a two-year cohort study. *Journal of Dental Research*, 77, 73-80.

Kutsch, V. K. (2014). Dental caries: an updated medical model of risk assessment. *The Journal of Prosthetic Dentistry*, 111, 280-285.

Leandro, L. M., Vargas, F. S., Barbosa, P. C., Neves, J. K., da Silva, J. A., & Veiga Junior, V. F. (2012). Chemistry and biological activities of terpenoids from copaiba (*Copaifera spp.*) oleoresins. *Molecules*, 17, 3866-3889.

Lucca, L. G., de Matos, S. P., Borille, B. T., Dias, D. O., Teixeira, H. F., Veiga Junior, V. F., & et al. (2015). Determination of beta-caryophyllene skin permeation/retention

from crude copaiba oil (*Copaifera multijuga Hayne*) and respective oil-based nanoemulsion using a novel HS-GC/MS method. *Journal of Pharmaceutical and Biomedical Analysis*, 104, 144-148.

Migowska, N., Stepnowski, P., Paszkiewicz, M., Golebiowski, M., & Kumirska, J. (2010). Trimethylsilyldiazomethane (TMSD) as a new derivatization reagent for trace analysis of selected non-steroidal anti-inflammatory drugs (NSAIDs) by gas chromatography methods. *Analytical and Bioanalytical Chemistry*, 397, 3029-3034.

Pacheco, T. A. R. C., Barata, L. E. S., & Duarte, M. C. T. (2006). Antimicrobial activity of copaiba (*Copaifera spp.*) balsams. *Revista Brasileira de Plantas Medicinais*, 8, 123-124.

Pereira, F. J., Martins, F. T., Corrêa, R. S., Moreira, M. E. C., Costa, A. M. D. D., Santos, M. H., & et al. (2008). Isolamento, composição química e atividade anti-inflamatória do óleo essencial do pericarpo de *Copaifera langsdorffii Desf.* de acordo com hidrodestilações sucessivas. *Latin American Journal of Pharmacy*, 27, 364-374.

Pieri, F. A., Souza, M. C., Vermelho, L. L., Vermelho, M. L., Perciano, P. G., Vargas, F. S., & et al. (2016). Use of beta-caryophyllene to combat bacterial dental plaque formation in dogs. *BMC Veterinary Research*, 12(216), 1-8.

Santos, A. O., Ueda-Nakamura, T., Dias Filho, B. P., Veiga Junior, V. F., Pinto, A. C, & Nakamura, C. V. (2008). Antimicrobial activity of Brazilian copaiba oils obtained from different species of the Copaifera genus. *Memorias do Instituto Oswaldo Cruz*, 103, 277-281.

Silva, E. S., Mathias, C. S., Lima, M. C. F., Veiga Junior, V. F., Rodrigues, D. P., & Clement, C. R. (2012). Physico-chemical analysis of the oleoresin and genetic variability of copaiba in the Tapajós National Forest, Brazil. *Pesquisa Agropecuária Brasileira*, 47, 162-168.

Souza, A. B., Martins, C. H., Souza, M. G., Furtado, N. A., Heleno, V. C., de Sousa JP, & et al. (2011). Antimicrobial activity of terpenoids from *Copaifera langsdorffii Desf.* against cariogenic bacteria. *Phytotherapy Research*, 25, 215-220.

Struzycka, I. (2014). The oral microbiome in dental caries. *Polish Journal of Microbiology*, 63, 127-135.

Vargas, F. S., Almeida, P. D. O., Aranha, E. S., Boleti, A. P. A., Newton, P., de Vasconcellos, M. C., & et al. (2015). Biological activities and cytotoxicity of diterpenes from *Copaifera spp*. oleoresins. *Molecules*, 20, 6194-6210.

Veiga Junior, V. F., & Pinto, A. C. (2002). O gênero Copaifera L. *Quimica Nova*, 25, 273-286.

Walsh, T., Oliveira-Neto, J. M., & Moore, D. (2015). Chlorhexidine treatment for the prevention of dental caries in children and adolescents. *The Cochrane Database of Systematic Reviews*, 14(4), CD008457.

Yoo, H. J., & Jwa, S. K. (2018). Inhibitory effects of beta-caryophyllene on *Streptococcus mutans* biofilm. *Archives of Oral Biology*, 88, 42-46.

Zacchino, S. A., Butassi, E., Liberto, M. D., Raimondi, M., Postigo, A., & Sortino, M. (2017). Plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, 37, 27-48.

Zimmermam-Franco, D. C., Bolutari, E. B., Polonini, H. C., do Carmo, A. M., Chaves, M., & Raposo, N. R. (2013). Antifungal activity of *Copaifera langsdorffii Desf* oleoresin against dermatophytes. *Molecules*, 18, 12561-12570.

Figures

Figure 1

Chromatogram with the main retention times of *Copaifera reticulata* (sample 4) oil

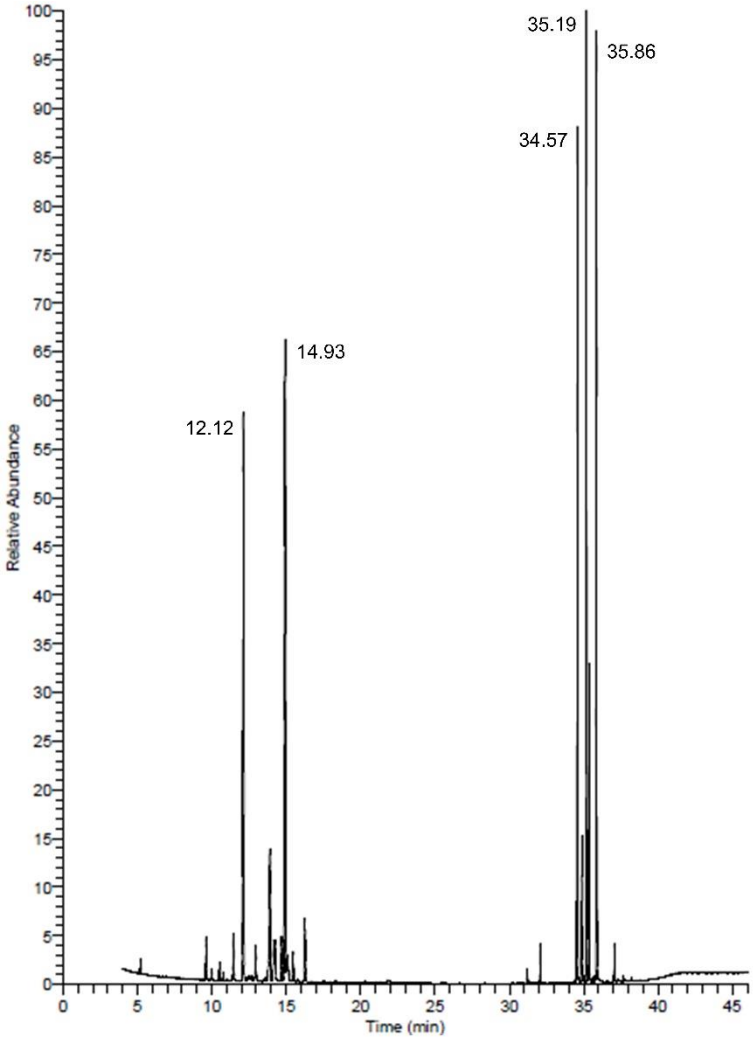


Figure 2

Chromatogram with the main retention times of *Copaifera multijuga* (sample 5) oil

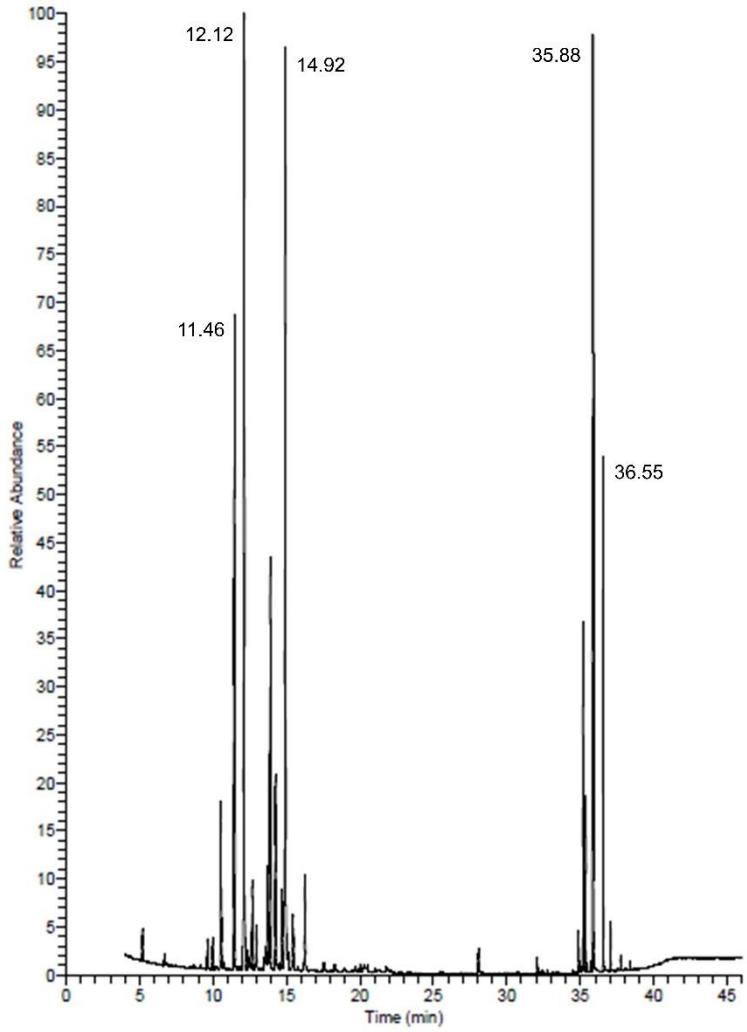
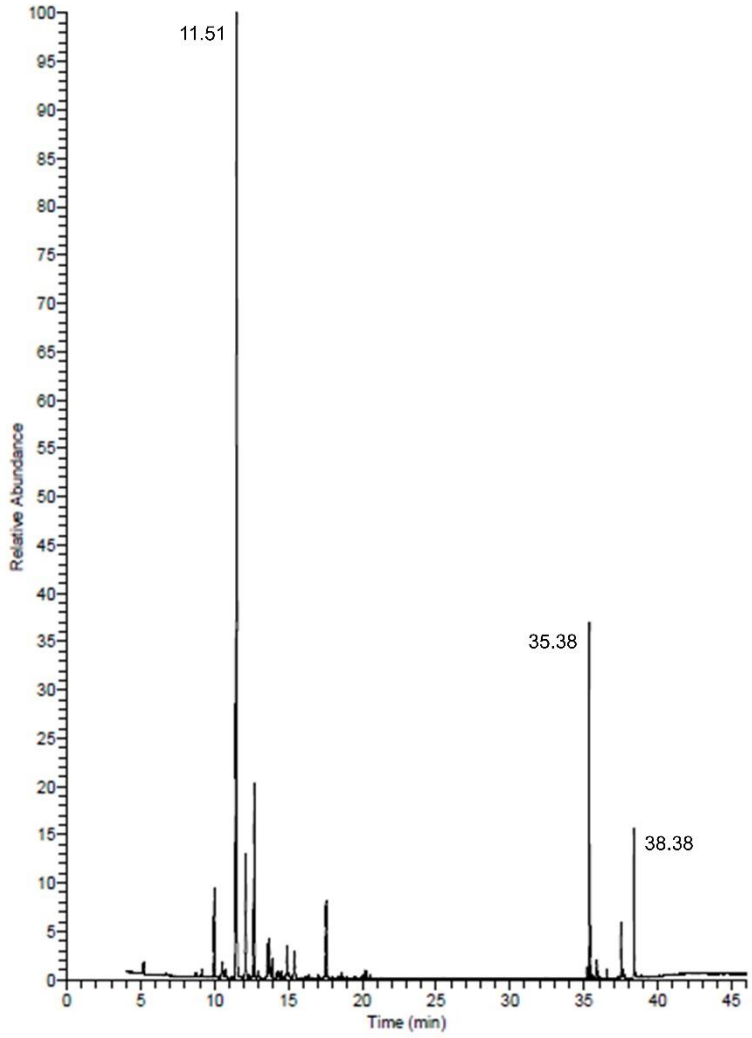


Figure 3

Chromatogram with the main retention times of *Copaifera multijuga* (sample 8) oil



Tables

Table 1

MIC of different samples of copaiba oil

Samples	MIC (µg/mL)
C. multijuga 1	25
C. langsdorffii 2	–
C. guianensis 3	12
C. reticulata 4	6.25
C. multijuga 5	6.25
C. multijuga 6	25
C. multijuga 7	100
C. multijuga 8	6.25
C. multijuga 9	25
Essential oil 10	200
Essential oil 11	–
Residue 12	200
TIENAM	1.5

Notes: (–) Exhibited no activity.

Table 2

Chemical composition of oleoresins obtained by gas chromatography

Constituents (%)	***C. reticulata* (sample 4)**	***C. multijuga* (sample 5)**	***C. multijuga* (sample 8)**
Cubebene - α	-	-	0.33
Copaene - α	0.27	0.53	3.63
Elemene - β	-	-	0.90
Cyperene	0.21	0.15	0.33
Cyclosativene	1.00	0.49	-
Caryophyllene - (*E*)	1.29	11.58	51.51

Bergamotene - α - *trans*	14.59	17.23	5.43
Farnesene - β - (*Z*)	-	0.35	0.11
Santalene – *epi* - β	0.14	0.16	-
Humulene - α	0.20	1.46	8.63
Farnesene - β - (*E*)	0.93	0.85	-
Aromadendrene - *allo*	-	-	0.29
Muurolene - γ	-	2.60	1.41
Germacrene - D	-	-	1.62
Himachalene - γ	4.35	7.68	0.90
Muurolene - α	-	0.12	0.32
Bisabolene - β	18.33	17.98	1.66
Selinene - α	1.14	3.67	-
Selinene - β	1.07	-	-
Guaiene - β - *cis*	-	-	0.44
Cadinene - δ	-	-	1.46
Farnesene - α - (*E*, *E*)	1.20	-	-
Bisabolene - α - (Z)	-	1.44	-
Caryophyllenyl alcohol	-	-	0.14
Caryophyllene oxide	-	0.24	4.51
Muurolol - α - epi	-	0.17	-
Cubenol - *epi*	-	-	0.11
Humulene epoxide II	-	-	0.29
Muurolol - α	-	-	0.43
Cadinol - α	-	0.12	0.12
Sesquiphellandrene - β	0.83	-	-
6-methyl - α - Ionone	-	0.37	-
7-epi- α -selinene	-	1.49	-
Bisabolene - γ - (*E*)	-	0.10	-
Guaiol	-	0.17	-
Junenol	-	0.13	-
Bulnesol	-	0.19	-
Bisabolol - *epi* - α	-	0.12	-
Copal acid methyl ester	-	-	7.88

Kaurenoic acid methyl ester	13.03	3.05	0.27
Caur-16-ene acid methyl ester	0.61	0.16	-
Clerod-3-en-15-oic acid methyl ester	11.71	-	-
Kolavenic acid methyl ester	17.73	-	-
Hardwíckico acid methyl ester	-	4.26	0.21
Mixture of methyl esters of labda-7,13-dien-15-oic acid and daniellic acid	-	-	0.68
Cativic acid methyl ester	1.96	-	-
Cauranoic acid methyl ester	4.42	1.68	-
Dimethyl ester of pinifolic acid	0.63	0.46	-
Dimethyl ester of ent-aghatic acid	-	0.19	-
11-acetoxy-copalic acid dimethyl ester	-	-	3.56
Mixture of methyl esters of labda-7,13-dien-15-oic acid and daniellic acid	-	13.75	-

Notes: (–) Is not present.

3.2 MANUSCRIPT 2

Copaiba oil as antimicrobial agent for experimental dental adhesive resin

Carolina Rocha Augusto[a], Isadora Martini Garcia[b], Emerson Silva Lima[c], Valdir Florêncio da Veiga Junior[d], Fabrício Mezzomo Collares[e]

[a]DDS, MSc, Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Laboratório de Materiais Dentários, Porto Alegre, RS, Brasil, carolina.augusto@ufrgs.br

[b]DDS, MSc, Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Laboratório de Materiais Dentários, Porto Alegre, RS, Brasil, isadora.garcia@ufrgs.br

[c]DDS, MSc, PhD, Universidade Federal do Amazonas, Laboratório de Atividades Biológicas, Faculdade de Ciências Farmacêuticas, Manaus, AM, Brasil, eslima@ufam.edu.br

[d]DDS, MSc, PhD, Instituto Militar de Engenharia, Seção de Química, Rio de Janeiro, RJ, Brasil, valdir.veiga@ime.eb.br

[e]DDS, MSc, PhD, Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Laboratório de Materiais Dentários, Porto Alegre, RS, Brasil, fabricio.collares@ufrgs.br

*Corresponding author:

Fabrício Mezzomo Collares

Rua Ramiro Barcelos, 2492, Rio Branco - Porto Alegre – RS – Brazil - 90035-003

Phone: +55 51 33085198

e-mail: fabricio.collares@ufrgs.br

Abstract

Objectives The purpose of this study was to formulate an experimental adhesive resin with copaiba oil (*Copaifera multijuga*) in different concentrations and evaluate its physical, mechanical, biological and chemical properties.

Materials and methods The adhesive resin was formulated with methacrylate monomers and photoinitiators. Copaiba oil was added at 5, 10 and 15 wt.%, besides the control group. Degree of conversion (DC) (n = 5), softening in solvent (KHN initial, KHN final and ΔKHN%) (n = 5), ultimate tensile strength (UTS) (n = 12), contact angle (θ) (n = 10), surface free energy (SFE) (n = 10), antibacterial activity (n = 5) and microtensile bond strength (μ-TBS) (n = 20) were evaluated.

Results No changes in DC were observed after incorporating copaiba oil ($p = 0.073$). The addition of oil did not influence the θ with water ($p = 0.859$) and μ-TBS ($p = 0.182$). The experimental groups decreased KHN1 ($p < 0.001$), UTS ($p = 0.009$), and showed highest ΔKHN% ($p < 0.001$). All groups softened in solvent ($p < 0.001$). The θ with α-bromonaphthalene decreased ($p < 0.001$) and the SFE increased ($p = 0.002$) after incorporating 10 and 15 wt.% of copaiba oil. The group with 15 wt.% of copaiba oil presented a reduction in biofilm formation ($p < 0.001$) and planktonic bacteria viability ($p = 0.004$).

Conclusions The incorporation of 15 wt.% of copaiba oil provided antibacterial activity when added to an experimental adhesive resin without compromising DC and immediately μ-TBS, which may be promising for the use of natural products in the formulation of dental adhesive systems.

Keywords: Phytotherapy; Antibacterial agents; Dentin-bonding agents; Mechanical phenomena; Physical and chemical properties.

Introduction

Recurrent caries is the main reason for restoration replacement in the long term, followed by marginal defects, such as discoloration, poor anatomic form and fracture of the restoration [1]. The site where demineralization begins is adjacent to the restoration margins, where adhesion of cariogenic biofilms, allied to low quality restorations, may result in the development of the disease [2]. Modifications to adhesive resins, such as the incorporation of antibacterial agents, have been proposed to overcome this issue [3].

To avoid bacterial adhesion on resin surfaces or to reduce biofilm formation, chlorhexidine [4], zinc oxide [5], silver particles [6] and quaternary ammonium methacrylate [7] have been incorporated into polymers, however its incorporation ends up altering some properties of the materials. Alternative materials that have effective, safe and economical features, such as natural products, are emerging as viable options, considering that they are widely used in folk medicine [8, 9]. An additional consideration is that the use of herbal medicine generates a social, cultural and economic impact, particularly in the regions where they are collected [10]. Laboratory [11, 12] and clinical [13, 14] studies have demonstrated their actions in many areas and showed promising results. Thus, extracts or oils with antimicrobial action could be an alternative therapeutic addition to be tested in dental materials [15].

Copaiba oil is a product extracted from several *Copaifera* species, which is highly biocompatible [16] and has desirable characteristics, such as anti-inflammatory [17], antifungal [18] and antimicrobial action [19]. The sesquiterpenes and diterpenes present in its composition are responsible for its characteristics [9], with the concentration and type varying according to the *Copaifera* species [11, 17]. *Copaifera*

multijuga usually presents a high concentration of β-caryophyllene and copalic acid, with bactericidal and bacteriostatic action against *Streptococcus mutans* [18, 19].

Since copaiba oil (*Copaifera ssp*.) has been used against oral pathogens [20], formulating dental materials with therapeutic improvement may be a useful strategy. Copaiba oil has already been incorporated into experimental endodontic sealers [21] and cavity cleansers [22] in order to formulate antibacterial materials, using phytotherapy as an alternative to conventional materials. The purpose of this study was to formulate an experimental adhesive resin with copaiba oil (*Copaifera multijuga*) in different concentrations and evaluate its physical, mechanical, biological and chemical properties.

Materials and methods

Copaiba oil from *Copaifera multijuga* species, collected in Parintins, in the state of Amazonas, whose minimum inhibitory concentration (6.25 ug/mL) and chemical characterizations have been previously described in the literature [11], was used. All reagents used in this study were purchased from Aldrich Chemical Company (St. Louis, Missouri, USA). To perform monomer photoactivation, a light-emitting diode unit (Radii Cal, SDI, Victoria, Australia) was used with an irradiation of 1200 mW/cm^2, confirmed with a digital power meter (Ophir Optronics, North Andover, MA, USA).

Experimental adhesive resin formulation

The experimental adhesive resin was formulated mixing 66.66 wt.% bisphenol-A-glycidyl methacrylate (Bis-GMA) and 33.33 wt.% 2-hydroxyethyl methacrylate (HEMA). As photoinitiator system, camphorquinone (CQ) and ethyl 4-

dimethylaminobenzoate (EDAB) were added at 1 mol% to all groups, according to monomer moles and 0.01 wt.% butylated hydroxytoluene (BHT). Copaiba oil was added at three different concentrations – 5, 10 and 15 wt.% – to the adhesive resin, and one group remained without oil as the control group. All components were weighed using an analytical balance (AUW220D, Shimadzu, Kyoto, Japan).

Degree of conversion (DC)

Fourier-transformed infrared spectroscopy was used to evaluate the DC with a spectrometer (Vertex 70, Bruker Optics, Ettlingen, Germany) coupled to a horizontal attenuated total reflectance (ATR) device with a diamond crystal (Platinum ATR-QL, Bruker Optics, Ettlingen, Germany). The adhesive resin (3 µL) was directly dispensed onto the ATR crystal in a polyvinylsiloxane matrix with 1.0 mm thickness and 4.0 mm diameter and photoactivated for 20 s (n = 5). The software (OPUS v6.5; Bruker Optics, Ettlingen, Germany) in monitoring scan mode was used with Blackman-Harris 3-term apodization in the range 400 to 4000 cm^{-1}, a resolution of 4 cm^{-1}, and a mirror speed of 2.8 mm/s. The DC was calculated based on the intensity of the peak at 1640 cm^{-1}, related to the aliphatic carbon-carbon double bond, and the intensity of the peak at 1610 cm^{-1}, related to the aromatic carbon-carbon double bond [23].

Ultimate tensile strength (UTS)

The specimens (n = 12) were prepared in a metallic matrix with an hourglass design: 8 mm long, 2 mm wide, 1 mm thickness and with a cross-sectional area of 1 mm^2. They were covered with polyester matrix on both sides before light activation, which occurred for 20 s on each side. After polymerization, they were measured with a digital caliper, fixed in a metallic device with cyanoacrylate resin and loaded under

tension. The test was realized with a universal testing machine (EZ-SX Series, Shimadzu, Kyoto, Japan) with a load speed of 1.0 mm/min until failure. The UTS of each specimen was obtained in Newtons and divided by the constriction area of each specimen, to be expressed in megapascals (MPa).

Softening in solvent

For softening in solvent, specimens (n = 5) with 1.0 mm thickness and 4.0 mm diameter were obtained after 20 s of photoactivation on each side. They were embedded in a self-curing polymethylmethacrylate resin using a metallic cylinder-shaped matrix and wet polished (Model 3v, Arotec, Cotia, SP, Brazil) with abrasive paper (600, 1200, and 2000 grit). Final polishing was carried out with a felt disk saturated with alumina suspension (0.5 µm, Alumina, Arotec, Cotia, SP, Brazil). The embedded specimens were washed in distilled water in a sonicator. After 24 hours, three indentations (10 g/5 s) 100 µm apart were made in each specimen with a digital hardness Knoop tester (HMV 2; Shimadzu, Tokyo, Japan). The specimens were immersed in ethanol and distilled water (70:30) at 37°C for 2 hours. Before and after the immersion period, the specimens were submitted to the hardness test (KHN1 and KHN2), and the percentage of hardness reduction (ΔKHN%) was recorded.

Contact angle (θ) and surface free energy (SFE)

The specimens (n = 10) were prepared as described above in the softening in solvent section except for measures 1.0 mm thickness, 5.0 mm diameter. After 24 hours, the specimens were analyzed with an optical tensiometer Theta (Biolin Scientific, Stockholm, Sweden) using the sessile drop method, where one drop (3 µL) of distilled water (as the polar liquid) or α-bromonaphthalene (as the non-polar liquid)

was dripped over the surface of each specimen. The drop out size was 3.0 µL, drop rate 2.0 µL/s, displacement rate 20.0 µL/s, and speed dispersion of liquids 50 mm/min. The test was performed for 20 s, and the θ measured at 10 s were recorded and used to evaluate the SFE, following the Owens-Wendt-Rabel-Kaelble (OWRK) method with OneAttension software (Biolin Scientific, Stockholm, Sweden) [24].

Microtensile bond strength (µ-TBS)

The buccal surface of 80 bovine teeth (n = 20) were polished with an abrasive paper for 30 s. The dentin surface of each tooth was etched for 15 s with a 37% phosphoric acid gel (Acid Gel, Villevie, Joinville, SC, Brazil), rinsed and dried. Primer (Scotchbond multi-purpose, 3M ESPE, St. Paul, MN, USA) was applied actively for 20 s, and the solvent was evaporated for the same time. The adhesive resins were applied and photoactivated for 20 s. The restoration was performed (Z350, 3M ESPE, St. Paul, USA) in two increments of 2 mm, photoactivated for 20 s each, and stored in distilled water at 37°C for 24 hours. The teeth were sectioned in four beams (area of 0.5 mm^2 approximately) with a slow-speed saw and fixed in a metallic jig with cyanoacrylate resin. µ-TBS tests were performed at a crosshead speed of 1 mm/min using a universal testing machine (EZ-SX Series, Shimadzu, Kyoto, Japan). To calculate the bond strength in MPa, the maximum force required for restoration debonding was recorded in Newtons and divided by the adhesive area. The failure pattern was analyzed with a stereomicroscope (HMV 2, Shimadzu, Tokyo, Japan) at 10× magnification and classified as adhesive, mixed or cohesive in dentin or composite resin. The values obtained in the cohesive failures were removed from the statistical analysis.

Antibacterial activity evaluation

A direct contact inhibition evaluation was performed against *Streptococcus mutans* (NCTC 10449, INCQS 00446, Oswaldo Cruz Foundation, National Institute of Health Quality Control, Rio de Janeiro, Brazil) using specimens of experimental adhesive resins (n = 5) prepared as described in the softening in solvent section. The specimens were attached to the lid of a test plate containing 48 wells and sterilized by hydrogen peroxide plasma 58% for 48 min at 56°C. On a sterile plate with brain–heart infusion (BHI) broth with agar, 300 µL of frozen *S. mutans* in skim milk were cultured for 48 hours at 37°C, in a microaerophilic environment. The colonies on the plate were transferred to 5 mL of BHI broth with 1 wt.% of sucrose and kept at 37°C for 24 hours, in a microaerophilic environment. After this period, 100 µL of the suspension of the overnight *S. mutans* broth culture was added to each well of a sterile 48-well plate with 900 µL of BHI broth with 1 wt.% of sucrose. The specimens placed on the sterile 48-well plate were exposed to the culture broth of bacteria at 37°C for 24 hours in a microaerophilic environment. To access the initial inoculum added to each well, 100 µL was removed from the initial 5 mL of BHI broth with *S. mutans* and sucrose at 1 wt.% and added to 900 µL of a sterile saline solution. This mixture was vortexed, diluted until 10^{-6}, plated in a BHI agar and kept at 37°C for 48 hours under a microaerophilic environment. It was performed in triplicate. After 48 hours, the colonies were counted and transformed to CFU/mL, indicating an inoculum at 2.5×10^6 CFU/mL. After 24 hours of contact between the specimens' surfaces and the broth, they were removed and vortexed for 1 min in 1 mL of a saline solution and diluted until 10^{-6} dilution. Two 25 µL drops of each dilution were plated on a BHI agar and incubated for 48 hours in a microaerophilic environment. Before dilution and plating, the mixture in each

Eppendorf tube was homogenized in a vortex for 5 s. The number of colonies was visually counted, and the CFU/mL was calculated.

Antibacterial activity against planktonic bacteria was performed using the same specimens and broth cited above. After the specimens had contact with the BHI broth with sucrose at 1 wt.% and 2.5×10^6 CFU/mL of *S. mutans* for 24 hours, 100 µL was removed from each well to collect the planktonic bacteria that was in contact with the specimens' surfaces. The 100 µL from each well was diluted in 900 µL of a saline solution until 10^{-6}. The plating and counting of the number of colonies were performed as described above.

Statistical analysis

The normality of the data was evaluated using the Shapiro–Wilk test. Statistical analysis were performed with one-way ANOVA and the Tukey post hoc test for DC, UTS, KHN, ΔKHN%, θ, SFE, µ-TBS and antibacterial activity. For comparison between initial and final microhardness (KHN1 and KHN2), a paired Student's t-test was used. For all tests a significance level of 5% was considered.

Results

The DC and UTS values are presented in Table 1. The values of DC ranged from 51.09 (± 1.53)% to 54.40 (±3 .27)% with no statistical difference ($p = 0.073$). The UTS values decrease with incorporation of copaiba oil ($p = 0.009$), where the experimental groups presented less than 44.33 (± 3.35) MPa. The KHN1, KHN2 and ΔKHN% of the experimental adhesive resins are shown in Table 2. The groups with copaiba oil presented a decrease of KHN1 ($p < 0.001$). After solvent immersion, all groups

decreased in hardness ($p < 0.001$), with the control group showing less softening (ΔKHN%, $p < 0.001$). Table 3 shows the θ and SFE results. There was no statistically significant difference in θ with water ($p = 0.859$); the groups with 10 and 15 wt.% presented lower θ with α-bromonaphtalene ($p < 0.001$), which consequently increased the SFE ($p = 0.002$). The μ-TBS and antibacterial activity are shown in Table 4. Data of μ-TBS ranged from 51.24 (± 5.26) MPa to 54.95 (± 5.83) MPa and showed no statistically significant difference among groups ($p = 0.182$). The group with 15 wt.% of copaiba oil decreased biofilm formation ($p < 0.001$) and planktonic bacteria viability ($p = 0.004$). The fractures presented in the control group were 11.25% cohesive in dentin, 37.5% adhesive, 45.0% mixed and 6.25% cohesive in resin. In the group containing 5 wt.% of copaiba oil, 5.0% were cohesive fractures in dentin, 56.25% adhesive, 31.25% mixed and 7.5% cohesive resin. In the group containing 10 wt.% of oil, 10.0% were cohesive fractures in dentin, 63.75% adhesive, 23.75% mixed and 2.5% cohesive resin. In the group containing the highest concentration of copaiba oil, 8.7% of the fractures were cohesive in dentin, 60.0% adhesive, 22.5% mixed and 8.75% cohesive in resin.

Discussion

Phytotherapy is used for several health issues with reliable results. The wide variety of plant species that present antimicrobial activity [8, 15] leads to a search for an ideal species for application in the formulation of dental materials. Polymers with antimicrobial activity could be an alternative therapeutic agent to decrease recurrent caries lesions adjacent to dental restorations. The copaiba oil used, of the *multijuga* species, has had the details of its composition described in a previous study,

presenting a minimum inhibitory concentration against *S. mutans* of 6.25 μg/mL [11]. The adhesive resin containing 15 wt.% of copaiba oil formulated in this study presented antimicrobial activity without influencing the DC, θ with water and μ-TBS.

The addition of an oil to photocured resins may affect the DC and its reduction could lead to poor mechanical properties and reduced stability of the polymer [25]. The addition of copaiba oil did not influence the DC ($p = 0.073$), where all groups showed at least 51.09% of carbon-carbon double bond conversion. The complex composition of the oil, which has 28 constituents identified [11], has β-caryophyllene (51.51%) and copalic acid (7.88%) as its major components, as shown in Figure 1. Different molecular structures may have influenced this result. Among these organic components, there are molecules with no carbon double bond, therefore the incorporation of a material with lower concentrations of polymerizable groups (lower degree of functionality) reduces DC [26]. In contrast, the oil used probably did not decrease the light transmission through the material compared with the inorganic filler addition [27], in which the differences in the refractive index and opacity jeopardize the DC [28]. Copaiba oil is colorless [9] and even with 15 wt.%, the DC was above 50%, which is in accordance with commercial adhesives found in the literature [28].

Although there was no difference among the DC, the UTS ($p = 0.009$), KHN ($p < 0.001$) and ΔKHN% ($p < 0.001$) results showed a statistically significant difference, indicating that the final structure of the polymers obtained in the different groups was not the same [29]. In this study, the oil may act as a plasticizer, influencing the crosslink density and pattern of formed polymer. When this plasticization effect occurs, the unconverted monomers tend to leach, leading to a swelling of the material, decreasing the frictional forces among chains [30]. This plasticizer effect on the polymer led to less

hardness and greater softening, as result of the separation of the polymer chains by a molecule that does not form primary chemical bonds with the chain [31].

The incorporation of a hydrophobic oil into the resin could increase the θ and a high θ of an adhesive resin leads to poor wetting [24]. For the determination of SFE, two dispersive liquids were used, with different polarities [32]. Regardless of the oil concentration, the θ with water did not change ($p = 0.859$); however, the incorporation of 10 and 15 wt.% of copaiba oil decreased the θ with the α-bromonaphtalene liquid ($p < 0.001$). This apolar liquid has more dispersive forces than water [33], so a decrease in θ indicates that the incorporation of the oil produced a material with low polarity. In this scenario the SFE consequently increases and higher values of SFE indicate greater interaction between the solid and the liquid, promoting greater wettability [6]. The 10 and 15 wt.% groups had higher SFE ($p = 0.002$), so there probably was a higher dipole–dipole interaction or even hydrogen bonds, which are stronger bonds. From this result it may also be inferred that the softening occurred in the groups with oil, since materials with low polarity are prone to be soluble in organic solvent [33].

The mechanical and physical properties evaluated in this study, which presented alteration in the polymer structure and surface characteristics after the incorporation of the oil, could directly influence the bond strength, mainly to the dentin substrate [27, 33]. The bond strength was immediately evaluated by the μ-TBS test and no differences were observed among groups ($p = 0.182$). An adhesive type of fracture was the most prevalent in the groups with oil incorporation, while in the control group, the most frequent fracture was mixed, as shown in Figure 2. These results could be explained by the higher SFE presented in the test groups, where a better wetting of the adhesive resin on the dentin may have occurred; however, its lower UTS favored the type of failure presented. The increase of SFE and solvent softening is related to

the inclusion of an oil to the polymer structure; however, it did not interfere in the outcome of the bond strength.

The antibacterial activity of copaiba oil in nature against *S. mutans* is evidenced in the literature [20]; however, when integrated with a polymeric structure its effect could be reduced. In the present study, the adhesive resins containing copaiba oil were tested in direct contact with a bacterial medium, with the purpose of evaluating biofilm formation and cell viability of planktonic bacteria. The group containing 15 wt.% presented lower film formation ($p < 0.001$) and cell viability of *S. mutans* ($p = 0.004$), indicating that the components responsible for the antibacterial effect of the oil, even trapped or copolymerized in the polymer network, acted on these microorganisms. The activities of copaiba oil are possible thanks to the synergisms of its components [9]. β-caryophyllene and copalic acid are the most cited molecules in the literature as the main responsible for antibacterial activity [12, 34]. The mechanism involves destruction of the cell wall with release of the cytoplasmic content [15]. This action was reported when the bactericidal activity and reduction of biofilm formation with gram-positive microorganisms were evaluated [20]. By scanning electron microscopy, it is observed that the interaction of oil components with the cell membrane leads to destruction and alteration of volume [18]. The wetting characteristics also evidenced in the results of this study, with a decrease of the θ with α-bromonaphtalene and increase of SFE, could indicate the interaction between the oil and the components of the cell wall, leading to its rupture [15]. Furthermore, the effect against planktonic bacteria may have occurred by leaching of the oil in the group with the highest concentration.

The copaiba oil used in this study is a natural product of the Amazon region with antibacterial activity and biocompatibility [9, 16, 21, 22]. In polymeric structures in dental materials, its guaranteed antibacterial activity has never been tested in adhesive

resin. One way to overcome some issues observed in this study may be via the incorporation of oil carriers, such as nanotubules, microspheres or nanocapsules [35, 36], so as not to interfere with the mechanical-physical properties of the polymer, maintaining its antimicrobial activity, as evidenced here. Therefore, a biotechnological product using this phytotherapic may be a feasible alternative to the formulation of polymer-based materials.

Conclusion

The incorporation of 15 wt.% of copaiba oil provided antibacterial activity when added to an experimental adhesive resin without compromising DC and immediately μ-TBS, which may be promising for the use of natural products in the formulation of dental adhesive systems.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Funding: This study was not supported for source of founding.

Ethical approval: Not applicable.

Informed consent: For this type of study, formal consent is not required.

References

1. Eltahlah D, Lynch CD, Chadwick BL, Blum IR, Wilson NHF (2018) An update on the reasons for placement and replacement of direct restorations. J Dent 72:1-7. http://doi.org/10.1016/j.jdent.2018.03.001.

2. Jokstad A (2016) Secondary caries and microleakage. Dent Mater 32(1):11-25. https://doi.org/10.1016/j.dental.2015.09.006.

3. Dahl JE, Stenhagen ISR (2018) Optimizing quality and safety of dental materials. Eur J Oral Sci 126(1):102-105. https://doi-org/10.1111/eos.12422.

4. Boutsiouki C, Frankenberger R, Lücker S, Krämer N (2019) Inhibition of secondary caries in vitro by addition of chlorhexidine to adhesive components. Dent Mater [Epub ahead of print]. https://doi.org/10.1016/j.dental.2018.12.002.

5. Garcia IM, Leitune VCB, Visioli F, Samuel SMW, Collares FM (2018) Influence of zinc oxide quantum dots in the antibacterial activity and cytotoxicity of an experimental adhesive resin. J Dent 73:57-60. https://doi.org/10.1016/j.jdent.2018.04.003.

6. Degrazia FW, Leitune VC, Garcia IM, Arthur RA, Samuel SM, Collares FM (2016) Effect of silver nanoparticles on the physicochemical and antimicrobial properties of an orthodontic adhesive. J Appl Oral Sci 24(4):404-410. https://doi.org/10.1590/1678-775720160154.

7. Collares FM, Leitune VCB, Franken P, Parollo CF, Ogliari FA, Samuel SMW (2017) Influence of addition of [2-(methacryloyloxy)ethyl]trimethylammonium chloride to an experimental adhesive. Braz Oral Res 31:e31. https://doi.or/10.1590/1807-3107BOR-2017.vol31.0031.

8. Freires IA, Rosalen PL (2016) How natural product research has contributed to oral care product development? A critical view. Pharm Res 33(6):1311-1317. https://doi.org/10.1007/s11095-016-1905-5.

9. Leandro LM, Vargas FS, Barbosa PC, Neves JK, da Silva JA, Veiga Junior VF (2012) Chemistry and biological activities of terpenoids from copaiba (*Copaifera* spp.) oleoresins. Molecules 17(4):3866-3889. https://doi.org/10.3390/molecules17043866.

10. Carvalho ACB, Lana TN, Perfeito JPS, Silveira D (2018) The Brazilian market of herbal medicinal products and the impacts of the new legislation on traditional medicines. J Ethnopharmacol 212:29-35. https://doi.org/10.1016/j.jep.2017.09.040.

11. Augusto CR, Lima ES, Lima MCF, Veiga Junior VF, Collares FM (2019) Screening for the inhibitory activity of Copaiba oil on *Streptococcus mutans*. Submitted to Phytother Res.

12. Souza AB, Souza MGM, Moreira MA, et al (2011) Antimicrobial evaluation of diterpenes from *Copaifera langsdorffii* oleoresin against periodontal anaerobic bacteria. Molecules 16:9611-9619. https://doi.org/10.3390/molecules16119611.

13. Gelmini F, Beretta G, Anselmi C, et al (2013) GC-MS profiling of the phytochemical constituents of the oleoresin from *Copaifera langsdorffii* Desf. and a preliminary in vivo evaluation of its antipsoriatic effect. Int J Pharm 440(2):170-178. https://doi.org/10.1016/j.ijpharm.2012.08.021.

14. da Silva AG, Puziol PF, Leitao RN, et al (2012) Application of the essential oil from copaiba (*Copaifera langsdori* Desf.) for acne vulgaris: a double-blind, placebo-controlled clinical trial. Altern Med Rev 17(1):69-75. https://europepmc.org/abstract/med/22502624.

15. Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils – a review. Food and Chemical Toxicology 46(2):446–475. https://doi.org/10.1016/j.fct.2007.09.106.

16. Vargas FS, de Almeida PDO , Aranha ESP, et al (2015) Biological activities and cytotoxicity of diterpenes from *Copaifera* spp. Oleoresins. Molecules 20(4):6194-6210. https://doi.org/10.3390/molecules20046194.

17. Veiga Junior VF, Rosas EC, Carvalho MV, Henriques MG, Pinto AC (2007) Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne--a comparative study. J Ethnopharmacol 112(2):248-254. https://doi.org/10.1016/j.jep.2007.03.005.

18. Santos AO, Ueda-Nakamura T, Dias Filho BP, Veiga Junior VF, Pinto AC, Nakamura CV (2008) Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus. Mem Inst Oswaldo Cruz 103:277-281. http://dx.doi.org/10.1590/S0074-02762008005000015.

19. Bardají DK, da Silva JJ, Bianchi TC, et al (2016) *Copaifera reticulata* oleoresin: chemical characterization and antibacterial properties against oral pathogens. Anaerobe 40:18-27. https://doi.org/10.1016/j.anaerobe.2016.04.017.

20. Diefenbach AL, Muniz FWMG, Oballe HJR, Rösing CK (2018) Antimicrobial activity of copaiba oil (*Copaifera* ssp.) on oral pathogens: systematic review. Phytother Res 32(4):586-596. https://doi.org/10.1002/ptr.5992.

21. Garrido AD, de Cara SP, Marques MM, Sponchiado EC Jr, Garcia LF, de Sousa-Neto MD (2015) Cytotoxicity evaluation of a copaiba oil-based root canal sealer compared to three commonly used sealers in endodontics. Dent Res J 12(2):121-126. https://www-ncbi-nlm-nih/pmc/articles/PMC4387623/.

22. Bari C, Sampaio F, Conde N, et al (2016) Amazon emulsions as cavity cleansers: antibacterial activity, cytotoxicity and changes in human tooth color. Braz J Pharmacog 26(4):497–501. https://doi.org/10.1016/j.bjp.2016.03.010.

23. Collares FM, Portella FF, Leitune VC, Samuel SM (2014) Discrepancies in degree of conversion measurements by FTIR. Braz Oral Res. 28:9-15. http://dx.doi.org/10.1590/S1806-83242013000600002.

24. Degrazia FW, Leitune VCB, Samuel SMW, Collares FM (2017) Boron nitride nanotubes as novel fillers for improving the properties of dental adhesives. J Dent 62:85-90. https://doi.org/10.1016/j.jdent.2017.05.013.

25. Ferracane JL, Hilton TJ, Stansbury JW, et al (2017) Academy of Dental Materials guidance - Resin composites: Part II - technique sensitivity (handling, polymerization, dimensional changes). Dent Mater 33(11):1171-1191. https://doi.org/10.1016/j.dental.2017.08.188.

26. Collares FM, Ogliari FA, Zanchi CH, Petzhold CL, Piva E, Samuel SM (2011) Influence of 2-hydroxyethyl methacrylate concentration on polymer network of adhesive resin. J Adhes Dent 13(2):125-129. https://doi.org/10.3290/j.jad.a18781.

27. Garcia IM, Leitune VCB, Ferreira CJ, Collares FM (2018) Tantalum oxide as filler for dental adhesive resin. Dent Mater J 37(6):897-903. https://doi.org/10.4012/dmj.2017-308.

28. Gaglianone LA, Lima AF, Gonçalves LS, Cavalcanti AN, Aguiar FH, Marchi GM (2012) Mechanical properties and degree of conversion of etch-and-rinse and self-etch adhesive systems cured by a quartz tungsten halogen lamp and a light-emitting diode. J Mech Behav Biomed Mater 12:139-143. https://doi.org/10.1016/j.jmbbm.2012.01.018.

29. Schneider LF, Moraes RR, Cavalcante LM, Sinhoreti MA, Correr-Sobrinho L, Consani S (2008) Cross-link density evaluation through softening tests: effect of ethanol concentration. Dent Mater 24:199–203. https://doi.org/10.1016/j.dental.2007.03.010.

30. Malacarne J, Carvalho RM, de Goes MF et al (2006) Water sorption/solubility of dental adhesive resins. Dent Mater 22(10):973-980. https://doi.org/10.1016/j.dental.2005.11.020.

31. Ferracane JL (2006) Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater 22(3):211-22. https://doi.org/ 10.1016/j.dental.2005.05.005.

32. Combe EC, Owen BA, Hodges JS (2004) A protocol for determining the surface free energy of dental materials. Dent Mater 20(3):262-8. https://doi.org/10.1016/S0109-5641(03)00102-7.

33. Imai A, Takamizawa T, Sai K et al (2017) Influence of application method on surface free-energy and bond strength of universal adhesive systems to enamel. Eur J Oral Sci 125(5):385-395. https://doi.org/10.1111/eos.12361.

34. Pieri FA, Souza MC, Vermelho LL et al (2016) Use of β-caryophyllene to combat bacterial dental plaque formation in dogs. BMC Vet Res 12: 216. https://doi.org/10.1186/s12917-016-0842-1

35. El Asbahani A, Miladi K, Badri W et al (2015) Essential oils: from extraction to encapsulation. Int J Pharm 483(1-2):220-243. https://doi.org/10.1016/j.ijpharm.2014.12.069.

36. Alencar EN, Xavier-Junior FH, Morais AR et al (2015) Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. J Nnanosci Nanotechnol 15:880-888. https://doi.org/10.1166/jnn.2015.9187.

Figures

Figure 1**.** Scheme of major compounds of copaiba oil: β-caryophyllene (A) and copalic acid (B).

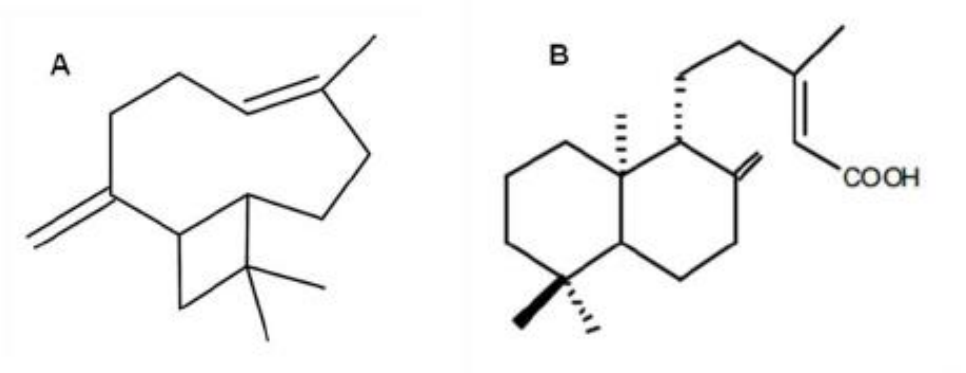
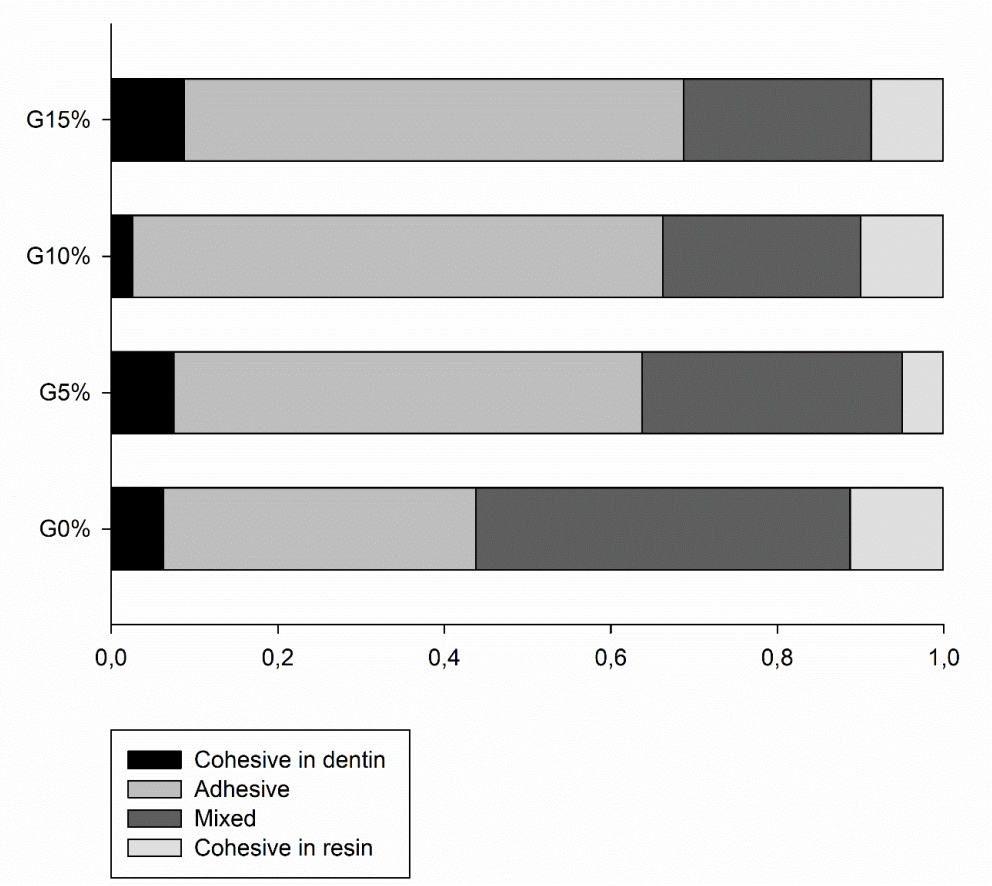


Figure 2. Failure pattern analysis of immediate μ- SBS test of experimental adhesive resins.



Tables

Table 1. Mean and standard deviation values of degree of conversion (DC) after 20 s of photoactivation and ultimate tensile strength (UTS) of the experimental adhesive resins.

Groups	DC (%)	UTS (MPa)
$G_{0\%}$	54.40 (± 3.27) ^{A}	49.73 (± 4.90) ^{A}
$G_{5\%}$	52.03 (± 0.25) ^{A}	44.01 (± 6.86) ^{B}
$G_{10\%}$	52.26 (± 0.90) ^{A}	43.23 (± 3.83) ^{B}
$G_{15\%}$	51.09 (± 1.53) ^{A}	44.33 (± 3.35) ^{B}

Different capital letters indicate statistically significant difference in the same column ($p < 0.05$).

p-value for DC = 0.073; p-value for UTS = 0.009.

Table 2. Mean and standard deviation values of initial Knoop hardness number (KHN1), final Knoop hardness number (KHN2) and Knoop hardness variation (ΔKHN%) of the experimental adhesive resins.

Groups	KHN1	KHN2	ΔKHN (%)
$G_{0\%}$	15.18 (± 0.79)^{Aa}	4.61 (± 0.83) ^{b}	69.76 (± 4.06) ^{B}
$G_{5\%}$	12.17 (± 1.01) ^{BCa}	2.56 (± 0.48) ^{b}	79.05 (± 3.07) ^{A}
$G_{10\%}$	11.05 (± 1.15) ^{CDa}	1.91 (± 0.74) ^{b}	83.11 (± 5.16) ^{A}
$G_{15\%}$	9.76 (± 0.98) ^{Da}	1.58 (± 0.34) ^{b}	83.88 (± 2.72) ^{A}

Different capital letters indicate statistically significant difference in the same column ($p < 0.05$).

Different small letters indicate statistically significant difference in the same line ($p < 0.05$).

p-value for KHN1 < 0.001; p-value for KHN2-KHN1 < 0.001; p-value for ΔKHN < 0.001.

Table 3. Mean and standard deviation values of contact angle (θ) and surface free energy (SFE) of experimental adhesive resins.

Groups	**Contact angle**		**SFE** (mN/M)
	Water	**α-bromonaphtalene**	
$G_{0\%}$	80.12 (± 3.06) ^{A}	28.27 (± 7.25) ^{A}	43.17 (± 1.91) ^{C}
$G_{5\%}$	81.41 (± 6.94) ^{A}	23.27 (± 3.56) ^{A}	43.85 (± 1.46) ^{BC}
$G_{10\%}$	79.67 (± 6.32) ^{A}	12.84 (± 3.63) ^{B}	46.41 (± 2.00) ^{A}
$G_{15\%}$	80.14 (± 5.13) ^{A}	16.43 (± 5.00) ^{B}	45.56 (± 2.15) ^{AB}

Different capital letters indicate statistically significant difference in the same column ($p < 0.05$).

p-value for θ with water = 0.859; p-value for θ with α-bromonaphtalene < 0.001; p-value for SFE = 0.002.

Table 4**.** Mean and standard deviation values of immediate microtensile bond strength (μ-TBS) and direct contact inhibition assay against biofilm formation, planktonic bacteria in CFU/mL after logarithmic transformation of experimental adhesive resins. Different capital letters indicate statistically significant difference in the same column ($p < 0.05$).

Groups	μ-TBS (MPa)	Biofilm formation	Planktonic bacteria viability
$G_{0\%}$	52.70 (± 6.88) ^{A}	6.08 (± 0.11) ^{A}	7.00 (± 0.54) ^{A}
$G_{5\%}$	54.95 (± 5.83) ^{A}	5.92 (± 0.45) ^{A}	7.10 (± 0.10) ^{A}
$G_{10\%}$	51.24 (± 5.26) ^{A}	5.70 (± 0.49) ^{A}	6.93 (± 0.39) ^{A}
$G_{15\%}$	54.66 (± 7.35) ^{A}	4.89 (± 0.16) ^{B}	6.16 (± 0.41) ^{B}
$G_{negative}$	-	-	7.02 (± 0.26) ^{A}

p-value for μ-TBS = 0.182; p-value for biofilm formation <0.001; p-value for planktonic bacteria viability = 0.004.

4. CONSIDERAÇÕES FINAIS

As técnicas e materiais adesivos estão em constante aperfeiçoamento permitindo tratamentos restauradores com altas taxas de sucesso (ELTAHLAH et al., 2018). Com o intuito de aumentar a longevidade das restaurações diretas e indiretas, os estudos concentram-se no desenvolvimento e/ou identificação de novos monômeros, partículas de carga e sistema iniciador-ativador. Uma característica considerada ideal para as resinas adesivas, sendo ainda um desafio, é possuir atividade antibacteriana (HUANG et al., 2016). É necessário identificar um material que não interfira nas propriedades mecânicas, físicas, químicas e biológicas da resina e que mesmo após a polimerização o efeito antimicrobiano continue atuando.

No presente estudo, óleo de copaíba (*Copaifera multijuga*) foi adicionado a uma resina adesiva experimental contendo os monômeros Bis-GMA e HEMA. A proposta de utilizar este fitoterápico como alternativa aos agentes tradicionais deve-se a vasta literatura existente sobre os efeitos antimicrobianos deste óleo, particularmente da espécie *multijuga* frente a microrganismos cariogênicos. O primeiro estudo específico a relatar essa atividade foi em 1999, onde BANDEIRA et al. avaliaram a atividade antimicrobiana do óleo de copaíba associado ao hidróxido de cálcio ou óxido de zinco frente a *Streptococcus mutans* e *Pseudomonas aeruginosa*. As propriedades medicinais do óleo de copaíba, especialmente no Brasil e região Amazônica, são datadas a época do descobrimento, no século XVI, pois os índios o utilizavam como cicatrizante e anti-inflamatório (VEIGA JUNIOR; PINTO, 2002). A identificação e descrição de seus componentes químicos permitiu sua aplicação comercial na indústria farmacêutica, consolidando sua atuação como fitoterápico. A composição fitoquímica dos óleos inclui mais de 60 sesquiterpenos e 30 ácidos terpênicos, cujas

concentrações variam de acordo com a espécie, sazonalidade, além de fatores biológicos externos (VEIGA JUNIOR; PINTO, 2002; CASCON; GILBERT, 2000). As propriedades do óleo não são atribuídas a um único constituinte e sim ao sinergismo entre as moléculas presentes nele (BARBOSA et al., 2013).

A primeira etapa deste estudo foi identificar um óleo que apresentasse alta ação antibacteriana frente a *S. mutans*, pois a literatura apresenta dados controversos com relação a espécie, concentração bactericida e inibitória mínima. Foram avaliadas doze amostras, de quatro espécies (*C. multijuga, C. langsdorffii, C. guianensis* e *C. reticulata*), sendo 9 óleos, 2 óleos essenciais e 1 resíduo da destilação. Com a determinação da CIM, a composição química dos óleos com menores valores foi determinada, cujo resultado foi compatível com estudos prévios de análise fitoquímica das espécies *multijuga* e *reticulata* (VEIGA JUNIOR et al., 2007). A amostra 8, por apresentar, dentre as três avaliadas a maior concentração de β-cariofileno e ácido copálico, foi escolhida para formulação das resinas adesivas.

Nas condições descritas neste trabalho, a incorporação do óleo de copaíba não resultou em alteração no grau de conversão, ângulo de contato com água e resistência de união a microtração imediata. A resina adesiva contendo 15% de óleo de copaíba apresentou diminuição na formação de biofilme e redução da viabilidade celular de *S. mutans*. Portanto a incorporação de um material de caráter untuoso e hidrófobo a uma mistura monomérica foi possível e mesmo após a polimerização apresentou atividade antibacteriana.

A incorporação do óleo influenciou as propriedades de resistência coesiva, dureza e amolecimento do polímero obtido após imersão em solvente. Apesar do grau de conversão não ter sido alterado, a estrutura polimérica obtida nos diferentes grupos não foi a mesma. O óleo atuou como um plastificante e influenciou a densidade das

ligações cruzadas. Quanto maior a concentração do óleo, menor a dureza do material, entretanto não houve diferença no amolecimento entre os grupos experimentais. Os componentes químicos do óleo, cujo principais são o β-cariofileno e ácido copálico, podem ter favorecido a separação das cadeias por não terem participado das ligações químicas no processo de polimerização. Não há na literatura dados sobre a utilização do óleo em estruturas poliméricas, apenas em cimentos odontológicos (VASCONCELOS et al. 2008; GARRIDO et al., 2010), onde propriedades como tempo de presa, viscosidade, espessura de película, estabilidade dimensional, radiopacidade e solubilidade não foram alteradas.

Uma resina adesiva ideal deve possuir, além de boas propriedades mecânicas e físicas, uma alta capacidade de molhamento do substrato, favorecendo a formação de uma interface adesiva de qualidade (DAHL; STENHAGEN, 2018). O ângulo de contato entre um líquido e uma superfície é um dos métodos para verificar a capacidade de um material molhar um substrato. Por meio deste ensaio, utilizando água e α-bromonaftaleno foi possível determinar a energia de superfície (IMAI et al., 2017). No presente estudo o ângulo de contato com α-bromonaftaleno diminuiu nos grupos contendo 10 e 15% de óleo, o que consequentemente resultou num aumento da energia de superfície. Esta caraterística de melhor molhamento dos grupos com maior concentração de óleo de copaíba repercutiu positivamente no ensaio de resistência de união a microtração. Por outro lado, esta propriedade poderia ter prejudicado os resultados da atividade antimicrobiana, entretanto, tanto na avaliação de biofilme quanto de células planctônicas o grupo com 15% de óleo foi eficaz. Quando há um aumento da energia de superfície espera-se um acúmulo maior de biofilme, apesar desta relação não ser totalmente estabelecida (CHEN et al., 2013). Todavia, os compostos químicos do óleo responsáveis pelo efeito antibacteriano

foram capazes de agir sobre o biofilme e as bactérias planctônicas, permitindo assim obter um polímero antimicrobiano. Portanto, a resina adesiva experimental obtida após incorporação de 15% de óleo de copaíba apresentou grau de conversão e resistência de união a dentina semelhante ao grupo controle, apresentando atividade antibacteriana frente *S. mutans*.

Como perspectiva, além do uso do óleo de copaíba em resina adesiva como proposto neste estudo, a forma de apresentação pode ser modificada e incorporada em outros materiais poliméricos. Sistemas de liberação controladas de fármacos, como o encapsulamento em uma membrana de polímero natural ou sintético pode permitir o aumento da disponibilidade e estabilidade do óleo (EL ASBAHANI et al., 2015). Existem diversas partículas carreadoras, como tubos, esferas e cápsulas, as quais podem ser em escala micro e nanométrica. Além disto diferentes técnicas de obtenção de nanoemulsões podem ser utilizadas (DIAS et al.; 2014; LUCCA et al., 2015; LUCCA et al., 2018). ALENCAR et al. (2015) produziram um sistema de emulsão nano estruturada baseada no óleo de copaíba (*C. langsdorffii*) que apresentou atividade antimicrobiana frente a *Staphylococcus, Pseudomonas* e *Candida*. BONAN et al., (2015) desenvolveram nanofibras contendo óleo de copaíba (*Copaifera sp.*) avaliando ângulo de contato, perfil de liberação e atividade antimicrobiana, com resultados positivos frente a *Staphylococcus aureus*. Portanto, o desenvolvimento de partículas carreadoras contendo óleo de copaíba que possam integrar a estrutura polimérica poderão ser adicionadas em diversos materiais dentários, inclusive em outros tipos de adesivos resinosos, como os autocondicionantes.

Com base nos resultados apresentados neste estudo e considerando as limitações do mesmo, o óleo de copaíba apresenta-se como alternativa viável para

adição como agente antimicrobiano em materiais dentários de estrutura polimérica. O trabalho apresentado é pioneiro em polímeros e um precursor para estudos que podem utilizar outras apresentações do óleo para formulação de adesivos, infiltrantes, selantes e resinas compostas antimicrobianas.

REFERÊNCIAS

1. ALENCAR, E. N. et al. Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions. **J. Nanosci. Nanotechnol**., v. 15, n. 1, p. 880-888, Jan. 2015.

2. AMORIM, E. L. C. et al. Fitoterapia: instrumento para uma melhor qualidade de vida. **Infarm**., v. 15. n. 1/3. p. 66-69, Jan.-Mar. 2003.

3. ASKAR, H. et al. Risk of caries adjacent to different restoration materials: systematic review of in situ studies. **J. Dent**., v. 56, n. 1, p. 1-10, Jan. 2017.

4. BAKKALI, F. et al. Biological effects of essential oils – a review. **Food Chem. Toxicol**., v. 46, n. 2, p. 446–475, Feb. 2008

5. BANDEIRA, M. F. et al. Estudo preliminar da atividade antibacteriana do óleo essencial e da resina da *Copaifera multijuga* (óleo de copaíba), associados ao óxido de zinco e ao hidróxido de cálcio. **J. Bras. Clin. Estet. Odontol**. v. 3, n. 17, p. 46–51, Set. 1999.

6. BARBOSA, P. C. S. et al. Phytochemical fingerprints of copaiba oils (*Copaifera multijuga Hayne*) determined by multivariate analysis. **Chem. Biodivers.**, v. 10, n. 7, p. 1350-1360, Jul. 2013.

7. BARDAJÍ, D. K. et al. *Copaifera reticulata* oleoresin: chemical characterization and antibacterial properties against oral pathogens. **Anaerobe.**, v. 40, p. 18-27. Aug. 2016.

8. BARI, C. et al. Amazon emulsions as cavity cleansers: antibacterial activity, cytotoxicity and changes in human tooth color. **Rev. Bras. Farmacogn.**, v. 26, n. 4, p. 497-501, Aug. 2016.

9. BONAN, R. F. et al. In vitro antimicrobial activity of solution blow spun poly(lactic acid)/polyvinylpyrrolidone nanofibers loaded with Copaiba (*Copaifera sp*.) oil. **Mater. Sci. Eng. C. Mater. Biol. Appl.**, v. 48, p. 372-377, Mar. 2015.

10. BOUTSIOUKI, C. et al. Inhibition of secondary caries in vitro by addition of chlorhexidine to adhesive components. **Dent. Mater.**, Jan. 2019. doi: 10.1016/j.dental.2018.12.002 [Epub ahead of print].

11. BRASIL, 2006. Política nacional de plantas medicinais e fitoterápicos. (http://bvsms.saude.gov.br/bvs/publicacoes/politica_nacional_fitoterapicos.pdf) (acesso em 5 de janeiro de 2019).

12. CARVALHO, A. C. B. et al. The Brazilian market of herbal medicinal products and the impacts of the new legislation on traditional medicines. **J. Ethnopharmacol.**, v. 212, p. 29-35, Feb. 2018.

13. CASCON, V.; GILBERT, B. Characterization of the chemical composition of oleoresins of *Copaifera guianensis Desf. Copaifera duckei Dwyer* and *Copaifera multijuga Hayne*. **Phytochemistry**, v. 55, n. 7, p. 773-778, Dec. 2000.

14. CHANDRA SHEKAR, B. R. et al. Herbal extracts in oral health care - a review of the current scenario and its future needs. **Pharmacogn. Rev**.; v. 9, n. 18, p. 87-92, Jul.-Dec. 2015.

15. CHEN, M. et al. Novel strategies for the prevention and treatment of biofilm related infections. **Int. J. Mol. Sci.**, v. 14, n. 9, p. 18488-18501, Sep. 2013.

16. CHENICHERI, S. et al. Insight into oral biofilm: primary, secondary and residual caries and phyto-challenged solutions. **Open Dent. J.**, v. 30, n. 11, p. 312-333, Jun. 2017.

17. COCCO, A. R. et al. A systematic review about antibacterial monomers used in dental adhesive systems: current status and further prospects. **Dent. Mater.**, v. 31, n. 11, p. 1345-1362, Nov. 2015.

18. COLLARES, F. M. et al. Influence of addition of [2-(methacryloyloxy)ethyl] trimethylammonium chloride to an experimental adhesive. **Braz. Oral. Res.,** v. 31, e31, May. 2019.

19. CONDE, N. C. O. et al. In vitro antimicrobial activity of plants of the Amazon on oral biofilm micro-organisms. **Rev. Odonto Cienc.**, v. 30, n. 4, p. 179–183, Jan. 2015.

20. DAHL, J. E.; STENHAGEN, I. S. R. Optimizing quality and safety of dental materials. **Eur. J. Oral. Sci.**, v. 126, n. 1, p. 102-105, Oct. 2018.

21. DEGRAZIA, F. W. et al. Effect of silver nanoparticles on the physicochemical and antimicrobial properties of an orthodontic adhesive. **J. Appl. Oral. Sci.**, v. 24, n. 4, p. 404-410, Jul.-Ago. 2016.

22. DIAS, D. O. et al. Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. **Ind. Crops. Prod.**, v. 59, p. 154–162. Aug. 2014.

23. DIAS, F. G. G. et al. Endodontics pastes formulated with copaiba oil: action on oral microbiota and dentin bridge formation in dogs. **Ciência Rural.**, v. 45, n. 6, p. 1073–1078, Jun. 2015.

24. EL ASBAHANI, A. et al. Essential oils: from extraction to encapsulation. **Int. J. Pharm.**, v. 483, n. 1-2, p. 220-243, Apr. 2015.

25. ELTAHLAH, D. et al. An update on the reasons for placement and replacement of direct restorations. **J. Dent.**, v. 72, p. 1-7, May. 2018.

26. ERICSON, D. et al. Minimally invasive dentistry - concepts and techniques in cariology. **Oral Health Prev. Dent.**, v. 1, n. 1, p. 59-72. Feb. 2003.

27. FENNER, R. et al. Plantas utilizadas na medicina popular brasileira com potencial atividade antifúngica. **Revista Brasileira de Ciências Farmacêuticas**, v. 42, n. 3, Jul.-Set. 2006.

28. FREIRES, I. A.; ROSALEN, P. L. How natural product research has contributed to oral care product development? A critical view. **Pharm. Res.**, v. 33, p. 1311–1317, Jun. 2016.

29. GARCIA, I. M. et al. Influence of zinc oxide quantum dots in the antibacterial activity and cytotoxicity of an experimental adhesive resin. **J. Dent.**, v. 73, p. 57-60, Jun. 2018.

30. GARRIDO, A. D. et al. Laboratory evaluation of the physicochemical properties of a new root canal sealer based on *Copaifera multijuga* oil-resin. **Int. Endod. J.**, v. 43, n. 4, p. 283–291, Apr. 2010.

31. GONZÁLEZ-CABEZAS, C. The chemistry of caries: remineralization and demineralization events with direct clinical relevance. **Dent. Clin. North Am.**, v. 54, n. 3, p. 469-478, Jul. 2010.

32. GROPPO, F. C. et al. Use of phytotherapy in dentistry. **Phytother. Res.**, v. 22, n. 8, p. 993-998, Aug. 2008.

33. HUANG, K. S. et al. Recent advances in antimicrobial polymers: a mini-review. **Int. J. Mol. Sci.**, v. 17, n. 9, Sep. 2016.

34. HURLBUTT, M.; YOUNG, D. A. A best practices approach to caries management. **J. Evid. Based Dent. Pract.**, v. 14, p. 77-86, Jun. 2014.

35. IMAI, A. et al. Influence of application method on surface free-energy and bond strength of universal adhesive systems to enamel. **Eur. J. Oral Sci.**, v. 125, n. 5, p. 385-395, Oct. 2017.

36. IMAZATO, S. Antibacterial properties of resin composites and dentin bonding systems. **Dent. Mater.**, v. 19, n. 6, p. 449-457, Sep. 2003.

37. JOKSTAD, A. Secondary caries and microleakage. **Dent. Mater.**, v. 32, n. 1, p. 11-25. Jan. 2016.

38. KORKUT, E. et al. Antimicrobial and mechanical properties of dental resin composite containing bioactive glass. **J. Appl. Biomater. Funct. Mater.**, v. 14, n. 3, p. e296–e301, Jul. 2016.

39. KUDOU, Y. et al. Addition of antibacterial agents to MMA-TBB dentin bonding systems--influence on tensile bond strength and antibacterial effect. **Dent. Mater. J.**, v. 19, n. 1, p. 65-74, Mar. 2000.

40. LEANDRO, L. M. et al. Chemistry and biological activities of terpenoids from copaiba (*Copaifera spp*.) oleoresins. **Molecules**, v. 17, n. 4, p. 3866-3889, Mar. 2012.

41. LUCCA, L. G. et al. Anti-inflammatory effect from a hydrogel containing nanoemulsified copaiba oil (*Copaifera multijuga Hayne*). **AAPS PharmSciTech**, v. 19, n. 2, p. 522-530, Feb. 2018.

42. LUCCA, L. G. et al. Determination of beta-caryophyllene skin permeation/retention from crude copaiba oil (*Copaifera multijuga Hayne*) and respective oil-based nanoemulsion using a novel HS-GC/MS method. **J. Pharm. Biomed. Anal.**, v. 104, p. 144-148, Feb. 2015.

43. MJOR, I. A. Frequency of secondary caries at various anatomical locations. **Oper. Dent.**, v. 10, n. 3, p. 88–92, Summer. 1985.

44. MO, S. S. et al. The microfloral analysis of secondary caries biofilm around Class I and Class II composite and amalgam fillings. **BMC Infect. Dis.**, v. 17, n. 10, p. 241, Aug. 2010.

45. MORAES, T. S. et al. In vitro evaluation of *Copaifera oblongifolia* oleoresin against bacteria causing oral infections and assessment of its cytotoxic potential. **Curr. Pharm. Biotechnol.**, v. 17, n. 10, p. 894–904, Aug. 2016.

46. NEDELJKOVIC, I. et al. Is secondary caries with composites a material-based problem? **Dent. Mater.**, v. 31, n. 11, p. e247-77, Nov. 2015.

47. PENMETSA, R. K. et al. An in vitro evaluation of antibacterial properties of self-etching dental adhesive systems. **J. Clin. Diagn. Res.**, v. 8, n. 7, p. ZC01-ZC05, Jul. 2014.

48. PETERS, M. C.; MCLEAN, M. E. Minimally invasive operative care. I. Minimal intervention and concepts for minimally invasive cavity preparations. **J. Adhes. Dent.**, v. 3, n. 1, p. 7-16, Mar. 2001.

49. PIERI, F. A. et al. Bacteriostatic effect of copaiba oil (*Copaifera officinalis*) against *Streptococcus mutans*. **Braz. Dent. J.**, v. 23, n. 1, p. 36-38, Jan.-Fev. 2012.

50. PIERI, F. A. et al. Use of β-caryophyllene to combat bacterial dental plaque formation in dogs. **BMC Vet. Res.**, v. 12, n. 216, p. 1-8, Oct. 2016.

51. PIERI, F. et al. Clinical and microbiological effects of copaiba oil (*Copaifera officinalis*) on dental plaque forming bacteria in dogs. **Arq. Bras. Med. Vet. Zootec.**, v. 62, n. 3, p. 578–585, Jun. 2010.

52. RAJABNIA, R. et al. Anti-*Streptococcus mutans* property of a chitosan: containing resin sealant. **J. Int. Soc. Prev. Community Dent.**, v. 6, n. 1, p. 49-53, Jan.-Feb. 2016.

53. RATHKE, A. et al. Antibacterial activity of a triclosan-containing resin composite matrix against three common oral bacteria. **J. Mater. Sci. Mater. Med.**, v. 21, n. 11, p. 2971–2977, Nov. 2010.

54. SAMPATH, P. B. et al. Assessment of antibacterial properties of newer dentin bonding agents: an in vitro study. **Contemp. Clin. Dent.**, v. 2, n. 3, p. 165-169, Jul. 2011.

55. SANTOS, A. O. et al. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus. **Mem. Inst. Oswaldo Cruz.**, v. 103, n. 3, p. 277-281, May. 2008.

56. SIMÕES, C. A. et al. Antibacterial activity of Copaiba oil gel on dental biofilm. **Open Dent. J.**, v. 10, p. 188-195, May. 2016.

57. SOUZA, A. B. et al. Antimicrobial activity of terpenoids from *Copaifera langsdorffii Desf.* against cariogenic bacteria. **Phytother. Res.**, v. 25, n. 2, p. 215-220, Feb. 2011.

58. STRUŻYCKA, I. The oral microbiome in dental caries. **Pol. J. Microbiol.**, v. 63, n. 2, p. 127–135, Nov. 2014.

59. VASCONCELOS, K. R. F. et al. Avaliação in vitro da atividade antibacteriana de um cimento odontológico à base de óleo-resina de *Copaifera multijuga* Hayne. **Rev. Bras. Farmacogn.**, v. 18, p. 733-738, Dez. 2008.

60. VEIGA JUNIOR, V. F. et al. Controle de autenticidade de óleos de copaíba comerciais por cromatografia gasosa de alta resolução. **Quim. Nova**, v. 20, n. 6, p. 612-615, Nov.-Dez. 1997.

61. VEIGA JUNIOR, V. F.; PINTO, A. C. O gênero *Copaifera* L. **Quim Nova**, v. 25, n. 2, p. 273-286, Abr.-Mai. 2002.

62. VEIGA JUNIOR, V. F. et al. Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne - a comparative study. **J. Ethnopharmacol.**, v. 112, n. 2, p. 248-254, Jun. 2007.

63. VERCRUYSSE, C. W. et al. Fluoride release of polyacid-modified composite resins with and without bonding agents. **Dent. Mater.**, v. 17, n. 4, p. 354–358, Jul. 2001.

64. WANG, Z. et al. Dental materials with antibiofilm properties. **Dent. Mater.**, v. 30, n. 2, p. e1–16, Feb. 2014.