UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA E PARASITOLOGIA APLICADAS

# PRÓPOLIS VERMELHA BRASILEIRA COMO ALTERNATIVA ANTI-*Helicobacter pylori*

MARIANA BRENTINI SANTIAGO

UBERLÂNDIA – MG
2024

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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Tese apresentada ao Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de Doutora.

**Discente:** Ma. Mariana Brentini Santiago
**Orientador:** Prof. Dr. Carlos Henrique Gomes Martins (ICBIM/UFU)

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Mariana Brentini Santiago

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S235 Santiago, Mariana Brentini, 1993-
2024 PRÓPOLIS VERMELHA BRASILEIRA COMO ALTERNATIVA ANTI-Helicobacter pylori [recurso eletrônico] / Mariana Brentini Santiago. - 2024.

Orientador: Carlos Henrique Gomes Martins.
Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Imunologia e Parasitologia Aplicadas.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.te.2024.192
Inclui bibliografia.
Inclui ilustrações.

1. Imunologia. I. Martins, Carlos Henrique Gomes,1968-, (Orient.). II. Universidade Federal de Uberlândia. Pós-graduação em Imunologia e Parasitologia Aplicadas. III. Título.

CDU: 612.017

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

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| Programa de Pós-Graduação em | Imunologia e Parasitologia Aplicadas | | | | |
| Defesa de: | Tese de Doutorado nº 160 | | | | |
| Data: | 28/02/2024 | Hora de início: | 14:00 | Hora de encerramento: | 17 h 29 min |
| Matrícula do Discente: | 11923IPA008 | | | | |
| Nome do Discente: | Mariana Brentini Santiago | | | | |
| Título do Trabalho: | Própolis vermelha brasileira como alternativa anti-Helicobacter pylori | | | | |
| Área de concentração: | Imunologia e Parasitologia Aplicadas | | | | |
| Linha de pesquisa: | Biologia das interações entre patógenos e seus hospedeiros | | | | |
| Projeto de Pesquisa de vinculação: | Ensaios Biológicos Antimicrobianos in vitro e in vivo de Produtos Naturais e Sintéticos | | | | |

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Iniciando os trabalhos o(a) presidente da mesa, Prof. Carlos Henrique Gomes Martins, apresentou a Comissão Examinadora e a candidato, agradeceu a presença do público, e concedeu a Discente a palavra para a exposição do seu trabalho. A duração da apresentação, o tempo de arguição e resposta foram conforme as normas do Programa.

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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**Referência:** Processo nº 23117.011990/2024-85 SEI nº 5189899

Dedicatória

*Dedico esta conquista ao meu avô*
*Sebastião Pereira Brentini*
*Tenho certeza que estaria orgulhoso de mim*
*Saudades eternas*

## AGRADECIMENTOS

Gostaria de expressar meus sinceros agradecimentos a todas as pessoas e instituições que desempenharam papéis fundamentais na realização desta tese de doutorado:

Em primeiro lugar, agradeço aos meus pais, Ana Maria Pereira Brentini Amparado e Cid Santiago Amparado, por sempre fornecerem suporte e incentivo para investir nos estudos, acreditando na ciência e na sua importância para a sociedade.

À minha família, em especial aos meus irmãos, Milena Brentini Santiago e Cássio Brentini Santiago, por serem uma base de apoio constante, independente da situação.

Um agradecimento especial à minha sobrinha, Ana Júlia Blanco Brentini, pelo carinho e amor demonstrados ao longo desse período.

À minha namorada, Daniela Silva Gonçalves, por ser um porto seguro durante momentos desafiadores enfrentados ao longo dessa etapa da minha vida.

Ao meu orientador, Prof. Dr. Carlos Henrique Gomes Martins, que sempre testou meus limites e me motivou a ir cada vez mais longe. Agradeço pela paciência, ensinamentos e conselhos que contribuíram não apenas para o meu crescimento profissional, mas também pessoal. Obrigado por acreditar em mim e no meu potencial.

A todos os colegas e professores que colaboraram com estes estudos, em especial aos colegas de pós-graduação Samuel Cota Teixeira e Rafael Borges Rosa, e aos professores Murilo Vieira da Silva, João Paulo Silva Servato, Sérgio Ricardo Ambrósio, Rodrigo Cassio Sola Veneziani, Jairo Kenupp Bastos e Robinson Sabino-Silva.

Aos alunos do Laboratório de Ensaios Antimicrobianos da Universidade Federal de Uberlândia (LEA-UFU), que foram meus companheiros de trabalho nos últimos quatro anos, auxiliando no meu desenvolvimento profissional.

A todos os professores do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas da Universidade Federal de Uberlândia (PPIPA-UFU), obrigado por compartilharem seus conhecimentos.

Aos amigos, em especial a Eduardo Ribeiro Gomide, por estarem sempre ao meu lado, prontos para escutar, apoiar e aconselhar.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pelo auxílio financeiro no projeto temático "Realização de estudos químicos, analíticos, biológicos, farmacológicos e tecnológicos para preenchimento das lacunas no desenvolvimento do setor brasileiro de própolis", processo 2017/04138-8.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES), pela concessão da minha bolsa de estudos durante o curso.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico, pela concessão de bolsa de estudos de Desenvolvimento Tecnológico e Industrial (DTI-A).

**RESUMO**

A bactéria *Helicobacter pylori* é um bacilo Gram-negativo microaerófilo. Devido a sua capacidade de colonizar as mucosas do estômago, é comumente associada a diversas doenças gastrointestinais graves, como gastrite crônica, câncer, entre outras. O tratamento padrão utilizado causa fortes efeitos colaterais, resultando em altas taxas de falha. Além disso, o surgimento de cepas resistentes aos antibióticos usados torna necessário o desenvolvimento de novas opções terapêuticas capazes de eliminar a bactéria. Neste sentido, os produtos naturais representam fontes promissoras de compostos únicos que exibem propriedades biológicas capazes de abordar diversas questões de saúde, o que despertou um interesse crescente em seu estudo visando o desenvolvimento de terapias alternativas seguras. A rica biodiversidade brasileira tem sido alvo de investigações para descobrir novas fontes de potenciais ativos. A própolis vermelha, encontrada no Brasil, contêm em sua composição compostos únicos, aumentando o interesse em avaliar suas propriedades biológicas. O objetivo do presente estudo foi avaliar a atividade anti-*H. pylori* do extrato de própolis vermelha brasileira por meio de ensaios *in vivo* e *in vitro*. Nos ensaios realizados, a própolis vermelha apresentou resultados promissores contra a *H. pylori* em modelos experimentais *in vitro* e *in vivo*, sendo capaz de atuar contra a bactéria em seu modo planctônico e agregada em biofilme. Com base nos dados obtidos, conclui-se que a própolis vermelha brasileira apresenta promissora atividade anti-*H. pylori*, sendo de interesse científico e podendo contribuir para o desenvolvimento de novos fármacos e/ou terapias alternativas capazes de combater a infecção pela bactéria. Isso encoraja futuras pesquisas que aprofundem ainda mais o conhecimento científico dessas valiosas fontes naturais.

**Palavras-chave:** Produtos naturais, própolis vermelha brasileira, *Helicobacter pylori*, antibacteriano, antibiofilme.

**ABSTRACT**

The bacteria *Helicobacter pylori* is a Gram-negative, microaerophilic bacillus. Due to its ability to colonize the gastric mucosa, it is commonly associated with various serious gastrointestinal diseases such as chronic gastritis and cancer. The standard treatment often results in strong side effects, leading to high failure rates. Additionally, the emergence of antibiotic-resistant strains necessitates the development of new therapeutic options capable of eliminating the bacteria. In this context, natural products represent promising sources of unique compounds with biological properties that can address various health issues, sparking growing interest in their study for the development of safe alternative therapies. The rich biodiversity of Brazil has been a focus of investigations to discover new sources of potential bioactive compounds. Brazilian red propolis, found in the country, contains unique compounds, increasing interest in evaluating its biological properties. The aim of the present study was to assess the anti-*H. pylori* activity of Brazilian red propolis extract through *in vitro* and *in vivo* assays. In the conducted assays, Brazilian red propolis showed promising results against *H. pylori* in both *in vitro* and *in vivo* experimental models, demonstrating its effectiveness against the bacterium in planktonic form and when aggregated in biofilms. Based on the obtained data, it is concluded that Brazilian red propolis exhibits promising anti-*H. pylori* activity, representing scientific interest and contributing to the development of new drugs and/or alternative therapies capable of combating *H. pylori* infection. This encourages further research to deepen the scientific knowledge of these valuable natural sources.

**Keywords:** Natural products, Brazilian red propolis, *Helicobacter pylori*, antibacterial, antibiofilm.

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

| | |
|---|---|
| < | Menor |
| > | Maior |
| ≤ | Menor ou igual |
| ≥ | Maior ou igual |
| µg | Micrograma |
| µm | Micrómetro |
| ANOVA | One-Way Analysis Of Variance |
| ATCC | American Type Culture Collection |
| BRP | Brazilian Red Propolis |
| CFU | Colony-Forming Units |
| CHEBRP | Crude Hydroalcoholic Extract of Brazilian Red Própolis |
| CLSI | Clinical and Laboratory Standards Institute |
| CNKs | C-jun N-terminal kinases |
| COAPER | Cooperativa de Apicultores de Canavieiras |
| DMSO | Dimethylsulfoxide |
| EPS | Extracellular Polymeric Substances |
| FIC | fractional inhibitory concentration |
| FICI | Fractional Inhibitory Concentration Index |
| IL-2 | Interleucina 2 |
| kg | Quilograma |
| LEA | Laboratório de Ensaios Antimicrobianos |
| MBC | Minimum Bactericidal Concentration |
| MBEC | Minimum Eradication Concentration of Biofilm |
| mg | Miligrama |
| MIC | Minimum Inhibitory Concentration |
| MICa | Minimal Inhibitory Concentration Alone |
| MICb | Combined Minimum Inhibitory Concentration |
| $MICB_{50}$ | Minimum Inhibitory Concentration of Biofilm |
| mL | Mililitro |
| MRSA | *Staphylococcus aureus* resistente à meticilina |
| NF-κB | Nuclear Factor Kappa B |
| nm | Nanômetro |

| | |
|---|---|
| ºC | Graus Celsius |
| OD | Optical Density |
| OMS | Organização Mundial de Saúde |
| pH | Potencial Hidrogeniônico |
| S.Ds. | Standard Deviations |
| SD | Standard Deviation |
| TT | Triple Therapy |
| UFU | Universidade Federal de Uberlândia |
| VacA | Citotoxina Vacuolizante |
| VISA | *S. aureus* com resistência intermediária à vancomicina |
| VRSA | *S. aureus* resistente à vancomicina |
| WHO | World Health Organization |

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**Apresentação**

No contexto da busca por alternativas terapêuticas eficazes e seguras, produtos naturais têm despertado interesse considerável. A própolis brasileira vermelha é uma fonte promissora pela sua composição única e potencialidades terapêuticas. A presente tese de doutorado teve como objetivo investigar as propriedades antibacterianas da própolis vermelha brasileira contra a bactéria *Helicobacter pylori*.

A pesquisa busca contribuir para o conhecimento sobre o potencial terapêutico dos produtos naturais em relação a infecções bacterianas. A compreensão mais profunda das propriedades biológicas da própolis vermelha brasileira pode fornecer uma base científica sólida para a incorporação desse produto natural em abordagens terapêuticas. O impacto dessa pesquisa abrange desde a saúde pública até aplicações clínicas, oferecendo novas possibilidades para o tratamento de infecções.

Em última análise, esta tese pretende ampliar o entendimento científico sobre a propriedade antibacteriana da própolis vermelha brasileira, contribuindo para um campo dinâmico onde a natureza se revela uma valiosa fonte de soluções para desafios na área da saúde. A interdisciplinaridade entre biologia, química e medicina impulsiona a exploração de estratégias terapêuticas inovadoras, baseadas em produtos naturais, em benefício da saúde global.

Esta tese foi subdividida em quatro capítulos, conforme apresentado a seguir:

Capítulo I- **Fundamentação teórica**. Apresenta uma revisão de literatura atualizada sobre produtos naturais, evidenciando a própolis vermelha brasileira, e suas aplicações farmacológicas e antimicrobiana, e sobre a bacteria *H. pylori*.

Capítulo II – **Brazilian Red Propolis Presents Promising Anti-*H. pylori* Activity in *In Vitro* and *In Vivo* Assays with the Ability to Modulate the Immune Response**. Artigo publicado no periódico Molecules (Fator de Impacto 4.6, Qualis CAPES A2 – Ciências Biológicas III), em 2022, apresentando resultados da avaliação da propriedade antibacteriana da própolis vermelha brasileira contra a bactéria *H. pylori*, foram realizados ensaios para determinação da concentração inibitória mínima, concentração bactericida mínima e atividade sinérgica em combinação com o antibiótico tetraciclina. Adicionalmente, também foi avaliada a propriedade anti-*H. pylori in vivo*, utilizando ratos Wistar como modelo animal. Foram realizadas análises microbiológicas e histopatológicas no tecido gástrico de animais infectados e tratados com a própolis vermelha brasileira.

Capítulo III – **Unveiling The Antibacterial Potential of Brazilian Red Propolis Against the Formation and Eradication of Biofilm of *Helicobacter Pylori***. Manuscrito a ser submetido para publicação no periódico Phytomedicine (Fator de Impacto 7.9, Qualis CAPES A1 – Ciências Biológicas III), em 2024, apresentando resultados da avaliação do potencial antibiofilme da própolis vermelha brasileira, foram realizados ensaios para determinar a curva de morte e avaliar possível vazamento de nucleotídeos. Adicionalmente, foram realizados ensaios que avaliaram o potencial de inibição da formação de biofilme e erradicação do biofilme da *H. pylori* pela própolis vermelha brasileira.

Capítulo IV – **Considerações finais**. Principais conquistas obtidas com o estudo, contribuições e perspectivas futuras dos resultados da pesquisa.

Também será apresentado em ANEXO toda produção cientifica realizada em colaboração com a equipe do Laboratório de Ensaio Antimicrobianos (LEA/UFU) e outras instituições pela pós-graduanda desde seu ingresso no Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas (PPIPA/UFU).

# CAPITULO I

## Fundamentação teórica

## 1. *Helicobacter pylori*

A *Helicobacter pylori* é uma bactéria Gram-negativa, microaerófila e espiralada, com forma de S ou bastonete curvo, medindo cerca de 3 a 5 μm de comprimento por 0,5 μm de largura. Possui parede celular externa lisa e de quatro a seis flagelos unipolares embainhados, com bulbo terminal (Chmiela & Kupcinskas, 2019; Malfertheiner et al., 2023). Comumente encontrada na mucosa que reveste o estômago humano, a *H. pylori* tem sido associada a diferentes doenças gastrointestinais, principalmente úlceras gástricas e duodenais, além do câncer gástrico (Chmiela & Kupcinskas, 2019). A forma de transmissão da *H. pylori* não é totalmente conhecida; no entanto, acredita-se que ocorra geralmente durante a infância, onde as vias oral-oral e oral-fecal são consideradas possíveis formas de transmissão (Cover & Blaser, 2009; Sharndama & Mba, 2022).

Essa bactéria é considerada uma das bactérias de maior prevalência em todo o mundo, e sua relação com inúmeras mortes nas doenças gastrointestinais a torna uma ameaça global a saúde pública devido a sua resistência a alguns antibióticos (Suzuki, Kusano, Horii, Ichijima, & Ikehara, 2022). Estima-se que mais da metade da população mundial é infectada pela *H. pylori* (Hooi et al., 2017). No Brasil, estudos mostram altas taxas de infecção por *H. pylori* nas áreas urbanas e rurais, afetando até 50% das crianças nos primeiros 2 anos de vida. Em crianças com idade ≤ 10 anos, a taxa de prevalência pode atingir de 70% a 90%, e esse percentual é semelhante em adultos (Almeida Cunha et al., 2003; Braga et al., 2007; Cartagenes, Martins, Carneiro, Barile, & Corvelo, 2009; Zaterka, Eisig, Chinzon, & Rothstein, 2007).

A patogenicidade da *H. pylori* se dá por alguns fatores de virulência como sua motilidade, produção de urease, fosfolipases, lipases, mucinases, capacidade de formar biofilme, entre outros (Chmiela & Kupcinskas, 2019; Malfertheiner et al., 2023). No estômago, a bactéria move-se com auxílio de seus flagelos e de sua forma espiralada através do suco e do muco gástrico, reduzindo a viscoelasticidade da mucina. As proteases, fosfolipases, lipases e mucinases atuam sobre a camada de muco, facilitando a progressão da bactéria. A urease promove hidrólise da ureia em amônia e dióxido de carbono, o que reduz a acidez e torna o potencial hidrogeniônico (pH) do microambiente colonizado mais neutro. Esses fatores contribuem para permanência e sobrevivência da bactéria na mucosa gástrica, considerada um ambiente extremamente hostil devido à alta concentração de ácido clorídrico (Malfertheiner et al., 2023; Sharndama & Mba, 2022).

A *H. pylori* possui outros fatores de virulência codificados por genes, e são associados a uma maior agressividade da bactéria. O gene *vacA* está presente em todas as cepas de *H. pylori* sendo responsável pela produção da chamada citotoxina vacuolizante (VacA), que tem a

capacidade de formar poros e induzir a vacuolização e apoptose em células epiteliais gástricas como também inibir a proliferação e secreção de interleucina 2 (IL-2) pelas células T. Algumas cepas possuem a ilha de patogenicidade *cag* "*cytotoxin associated gene pathogenicity island – cag PAI*", é uma região cromossômica com 40kb adquirido horizontalmente, que contém cerca de 27 a 31 genes que codificam um sistema de secreção bacteriana do tipo IV, que permite a oncoproteína cagA "cytotoxin-associated gene A" codificada pelo gene *cagA*, seja liberada diretamente no citosol das células epiteliais gástricas. Após a liberação ocorre à desfosforilação de proteínas celulares, mudanças na morfologia, aumento da proliferação celular, inflamação crônica e processos de carcinogêneses (Ansari & Yamaoka, 2019; Malfertheiner et al., 2023).

Protocolos recomendam que o tratamento da infecção causada pela *H. pylori* seja realizado com a terapia tripla de primeira linha, utilizando um inibidor da bomba de prótons mais dois antibióticos (amoxicilina, claritromicina ou metronidazol). Entretanto alguns fatores podem afetar o sucesso da terapia anti-*H. pylori*, entre eles estão, a adesão ao tratamento, tabagismo, sistema imune comprometido, hipersecreção de ácido gástrico, polimorfismos associados ao citocromo CYP2C19, alta carga bacteriana e a resistência antimicrobiana considerada o principal fator para o insucesso terapêutico (Coelho et al., 2018).

*H. pylori* tem adquirido resistência a alguns antimicrobianos como claritromicina e o metronidazol. Esse aumento na resistência aos fármacos tem gerado preocupação a nível mundial, o último relato da Organização Mundial de Saúde (OMS) sobre bactérias que necessitam de novos tratamentos alternativos devido, principalmente, ao aumento da resistência bacteriana as drogas existentes, foi publicado em 27 de fevereiro de 2017. Nele a OMS divulgou uma lista de 12 bactérias resistentes a antibióticos que representam a maior ameaça para a saúde humana. Esta lista foi elaborada em uma tentativa de orientar e promover pesquisa e desenvolvimento de novos antibióticos. A lista da OMS foi dividida em três categorias (crítica, alta e média prioridade) de acordo com a urgência da necessidade de novos antibióticos. *H. pylori* resistente à claritromicina foi classificado como uma bactéria de alta prioridade, estando no mesmo grupo que *Staphylococcus aureus* resistente à meticilina (MRSA), *S. aureus* resistente à vancomicina (VRSA) e *S. aureus* com resistência intermediária à vancomicina (VISA) (Tacconelli et al., 2018).

Além da resistência a terapia, efeitos colaterais como náuseas, vômitos, diarreia e tonturas tem sido atribuídos ao uso da terapia anti-*H. pylori* (Coelho et al., 2018). Devido a estes fatores indesejáveis torna-se necessário e muito importante a pesquisa de produtos naturais com propriedades anti-*H. pylori*, que podem ser associados à terapia convencional diminuindo assim os efeitos colaterais e a resistência dessa bactéria. Neste contexto, os produtos naturais

podem ser considerados uma estratégia importante na busca por novos antimicrobianos eficazes e seguros.

## 2. Produtos naturais

As plantas medicinais são espécies vegetais que possuem componentes químicos com propriedades terapêuticas e podem ser utilizadas para prevenir, tratar ou aliviar doenças. A relação entre seres humanos e plantas medicinais remonta milênios, sendo uma das práticas mais antigas na história da medicina. Desde civilizações antigas, como a egípcia, a chinesa, a grega e a indígena, as propriedades curativas das plantas eram amplamente conhecidas e exploradas (Pirintsos et al., 2022).

Com o advento da medicina moderna, houve um afastamento gradual das práticas tradicionais em prol de abordagens farmacológicas sintéticas. No entanto, o crescente reconhecimento dos potenciais terapêuticos contidos em produtos naturais tem conduzido a uma revalorização de tais práticas, especialmente em um cenário de busca por alternativas mais seguras e sustentáveis (Heinrich, 2013).

De acordo com dados contemporâneos, a demanda por produtos naturais como complementos ou substitutos de tratamentos convencionais tem aumentado substancialmente. Estudos mostram que, entre 1981 e 2019, foram aprovados 1276 medicamentos provenientes de produtos naturais, refletindo uma mudança nas preferências dos pacientes em direção a abordagens mais naturais e menos agressivas (Newman & Cragg, 2020). Além disso, a crescente preocupação com a resistência antimicrobiana e os efeitos colaterais associados a medicamentos sintéticos tem impulsionado a investigação de produtos naturais como fontes de novos agentes terapêuticos (Li et al., 2023; Newman & Cragg, 2020).

As plantas medicinais contêm uma miríade de compostos bioativos, como alcaloides, flavonoides, terpenoides e ácidos fenólicos, que conferem a elas suas propriedades terapêuticas. Esses compostos podem interagir com sistemas biológicos do corpo humano, como enzimas e receptores, resultando em efeitos farmacológicos específicos. A eficácia das plantas medicinais está frequentemente ligada a essa complexidade química e à sinergia entre seus componentes (Pirintsos et al., 2022).

A investigação científica tem sido fundamental para desvendar os mecanismos de ação e a eficácia dos produtos naturais na saúde. Avanços tecnológicos permitiram a identificação e a caracterização de compostos bioativos presentes em plantas e organismos naturais, esclarecendo suas propriedades terapêuticas. Além disso, a biotecnologia tem possibilitado a

produção controlada de produtos naturais, visando a ampliação de seu potencial clínico (Lee et al., 2022).

A pesquisa de produtos naturais no Brasil não é apenas de importância nacional, mas também global. Compostos naturais descobertos no país têm demonstrado potencial em diversas áreas, como antimaláricos e agentes antitumorais (Oliveira et al., 2009). Além disso, a bioprospecção de recursos naturais brasileiros tem chamado a atenção da comunidade científica internacional, abrindo portas para colaborações e compartilhamento de conhecimento.

A biodiversidade brasileira é única e exerce influência direta no uso de produtos naturais na medicina tradicional. Com uma vasta variedade de ecossistemas, o Brasil abriga inúmeras espécies de plantas, animais e micro-organismos que têm sido empregados há séculos pelas populações locais para tratar doenças e promover a saúde (Gurib-Fakim, 2006). A tradição da fitoterapia brasileira, por exemplo, remonta às práticas indígenas e africanas, que conheciam as propriedades medicinais de plantas (Dutra, Campos, Santos, & Calixto, 2016; Ferreira, Arcanjo, & Peron, 2023).

No contexto contemporâneo, as plantas medicinais continuam a ser relevantes devido à busca por abordagens terapêuticas mais naturais e menos invasivas. A medicina tradicional e complementar tem crescido em popularidade, e muitos pacientes buscam tratamentos à base de plantas para complementar os cuidados convencionais. No entanto, a segurança, a eficácia e a padronização das preparações à base de plantas ainda são desafios a serem superados. A pesquisa científica tem um papel crucial na validação desses tratamentos e na compreensão de seus mecanismos de ação (Heinrich, 2013).

As plantas medicinais representam uma ponte entre a sabedoria ancestral e a pesquisa moderna, conectando a tradição com a inovação. Compostos bioativos derivados de plantas ou outras fontes naturais oferecem potencial terapêutico que pode ser explorado para desenvolver tratamentos seguros e eficazes. Ao unir conhecimentos tradicionais e evidências científicas, a utilização de plantas medicinais continua a desempenhar um papel fundamental na promoção da saúde e no avanço da medicina (Sofowora, Ogunbodede, & Onayade, 2013).

Neste sentido, o potencial terapêutico da própolis tem sido objeto de pesquisa devido às suas propriedades bioativas. Estudos têm demonstrado a atividade antimicrobiana da própolis contra patógenos bacterianos e fúngicos, bem como seu potencial anticancerígeno e anti-inflamatório (Wieczorek et al., 2022).

## 3. Própolis

As civilizações antigas utilizaram produtos apícolas como recursos terapêuticos valiosos na prática medicinal. A história da medicina das civilizações dos assírios, chineses, tibetanos, Incas, Egípcios e Greco Romanos é muito rica, com registro de formulações centenárias, incluindo própolis para tratar ou prevenir doenças. Na antiguidade, egípcios, gregos e romanos usavam a própolis no tratamento de feridas, lesões cutâneas, úlceras e para intervenções cirúrgicas (Ghisalberti, 1979).

No Egito, um dos principais ingredientes utilizados para embalsamar os cadáveres foi a própolis, a qual foi também usada por Aristóteles, Dioscórides, Plínio e Galneo como anti-séptico e cicatrizante de feridas. Os gregos, incluindo Aristóteles e Hipócrates, adotaram a própolis como cura interna e externa. O historiador Plínio Roman refere-se à própolis como um medicamento capaz de reduzir o inchaço e aliviar a dor (Ioirish, 1982).

O termo "própolis", "Pro" em prol da e "polis", cidade de abelhas, o que significa em defesa do pente do mel, foi descrito no século 16 na França e no século 17 a própolis foi considerada uma droga oficial (Bankova, Popov, & Marekov, 1982). Nos séculos subsequentes houve grande interesse pelas propriedades medicinais da própolis, especialmente no Leste da Europa. Em 1908, o primeiro artigo científico sobre a composição química e propriedades da própolis foi publicado (Helfenberg, 1908) e indexado ao Chemical Abstracts.

Nas últimas décadas, a própolis ganhou ampla aceitação na medicina tradicional em várias partes do mundo. Este interesse pela própolis em vários países incentivou um grande número de estudos sobre as propriedades químicas e biológicas de própolis (de Almeida-Junior et al., 2023; Wieczorek et al., 2022).

Atualmente, no mercado brasileiro e em vários outros países, é possível encontrar própolis em diferentes apresentações, tais como extratos líquidos ou secos, cápsulas, comprimidos, xaropes, cremes, entre outros. As principais atividades são como agente antimicrobiano (Przybylek & Karpinski, 2019), antioxidante (de Almeida-Junior et al., 2023), imunomodulatório (El-Seedi et al., 2021; Orsolic, 2022) anti-inflamatório (El-Seedi et al., 2021), antiviral (Zulhendri et al., 2021), entre outros, as quais foram confirmadas em ensaios *in vitro* e/ou *in vivo*.

As abelhas brasileiras são fruto do cruzamento entre as *Apis mellifera* européia e africana, o que levou a formação de uma linhagem “brasileira” africanizada altamente resistente a doenças, sem necessidade do uso de tratamentos químicos como em outros países, o que garante produtos apícolas brasileiros de excelente qualidade e livres de contaminação. No Brasil, as própolis denominadas verde, marrom e vermelha são as que possuem maior

importância econômica e se destacam nos comércio nacional e internacional (Salatino, Salatino, & Negri, 2021).

A *A. mellifera* produz a própolis vermelha brasileira através da *Dalbergia ecastophyllum*, fonte botânica da própolis vermelha brasileira e responsável pela coloração vermelha devido à presença das isoflavonas retusapurpurinas A e B em sua resina (Moise & Bobis, 2020). O interesse neste produto da *A. mellifera* deve-se aos seus numerosos efeitos biológicos, tais como: citotóxico contra várias linhagens de células tumorais, antibacteriano, antifúngico, anticariogênico, antioxidante, antiproliferativo e anti-inflamatório, dentre outros (de Freitas et al., 2017; Freires, de Alencar, & Rosalen, 2016).

A própolis vermelha brasileira teve muitos de seus compostos químicos identificados, tais como: flavonóides (liquiritigenina), chalconas (isoliquiritigenina), pterocarpanos (medicarpina), isoflavonas (formononetina), isoflavanas (vestitol, neovestitol e 7-Ometilvestitol) e benzofenonas preniladas (mistura de gutiferona E/xantoquimol e oblongifolina A) (Aldana-Mejia et al., 2021; Boeing et al., 2021; de Mendonca et al., 2015). Do ponto de vista quimiotaxonômico, esses constituintes encontrados na própolis vermelha brasleira, especialmente os isoflavonóides, são marcadores quimiotaxonômicos característicos dos membros da subfamília Papilionoideae (Fabaceae), à qual pertence o gênero *Dalbergia* (Ccana-Ccapatinta et al., 2020).

Devido ao exposto, a própolis vermelha brasileira se mostra uma fonte promissora, estimulando novos estudos que ampliem o conhecimento sobre as propriedades biológicas já reportadas na literatura, a fim de desenvolver novos fármacos ou terapias alternativas que auxiliem nos cuidados com a saúde.

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**CAPITULO II**

# Brazilian Red Propolis Presents Promising Anti-*H. Pylori* Activity In *In Vitro* And *In Vivo* Assays With The Ability To Modulate The Immune Response

Artigo publicado no periódico Molecules em 2022 (doi: 10.3390/molecules27217310).

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# Brazilian Red Propolis Presents Promising Anti-*H. pylori* Activity in In Vitro and In Vivo Assays with the Ability to Modulate the Immune Response

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**Abstract:** *Helicobacter pylori* is a Gram-negative, microaerophilic, curved-rod, flagellated bacterium commonly found in the stomach mucosa and associated with different gastrointestinal diseases. With high levels of prevalence worldwide, it has developed resistance to the antibiotics used in its therapy. Brazilian red propolis has been studied due to its biological properties, and in the literature, it has shown promising antibacterial activities. The aim of this study was to evaluate anti-*H. pylori* from the crude hydroalcoholic extract of Brazilian red propolis (CHEBRP). For this, in vitro determination of the minimum inhibitory and bactericidal concentration (MIC/MBC) and synergistic activity and in vivo, microbiological, and histopathological analyses using Wistar rats were carried out using CHEBRP against *H. pylori* strains (ATCC 46523 and clinical isolate). CHEBRP presented MIC/MBC of 50 and 100 µg/mL against *H. pylori* strains (ATCC 43526 and clinical isolate, respectively) and tetracycline MIC/MBC of 0.74 µg/mL. The association of CHEBRP with tetracycline had an indifferent effect. In the stomach mucosa of rats, all treatments performed significantly decreased the number of *H. pylori*, and a concentration of 300 mg/kg was able to modulate the inflammatory response in the tissue. Therefore, CHEBRP showed promising anti-*H. pylori* in in vitro and in vivo assays.

**Keywords:** *Helicobacter pylori*; Brazilin red propolis; antibacterial; in vivo infection; synergism

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**Citation:** Santiago, M.B.; Leandro, L.F.; Rosa, R.B.; Silva, M.V.; Teixeira, S.C.; Servato, J.P.S.; Ambrósio, S.R.; Veneziani, R.C.S.; Aldana-Mejía, J.A.; Bastos, J.K.; et al. Brazilian Red Propolis Presents Promising Anti-*H. pylori* Activity in In Vitro and In Vivo Assays with the Ability to Modulate the Immune Response. *Molecules* **2022**, *27*, 7310. https://doi.org/10.3390/molecules27217310

Academic Editor: Juraj Majtan

Received: 23 September 2022
Accepted: 20 October 2022
Published: 27 October 2022

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## 1. Introduction

*Helicobacter pylori* is a Gram-negative, microaerophilic, spiral, S-shaped/curved-rod bacterium measuring about 3 to 5 µm in length by 0.5 µm in width, with a smooth outer cell wall and four to six sheathed unipolar flagella with a terminal bulb [1]. Prevalence across the world varies; however, it is known that the bacterium colonizes the gastrointestinal mucosa, the stomach antrum being its main site, of almost half the global human population [2,3]. It is believed that *H. pylori* infection occurs in childhood, where the oral-oral and oral–fecal routes are considered possible forms of transmission. When colonized by the bacterium, the child becomes a healthy carrier, and may or may not have symptoms of *H. pylori* infection during adulthood [2,4]. It has virulence and adaptation factors that are associated with the development of different gastrointestinal diseases (e.g., gastric and duodenal ulcers and gastric cancer) [3–5].

The gastric mucosa is considered an extremely hostile environment, mainly due to the high concentration of hydrochloric acid; however, *H. pylori* has mechanisms that allow it to

overcome barriers and colonize, and survive, in the site. Upon reaching the stomach, *H. pylori*, through its urease activity, neutralizes the acidic environment of the organ. Due to the presence of its flagella, the bacteria have motility and are thus is able to move until reaching the gastric epithelium, where the bacterial adhesins interact with the host's receptor cells, causing the colonization of *H. pylori*. After colonization is completed, bacteria begin to replicate and cause damage to the host tissue through the various toxins and effector proteins produced. The clinical manifestation of the infection occurs through the activation of innate immunity, triggered by the secretion of chemokines by the bacteria and activation of neutrophils [4].

Infection caused by *H. pylori* activates complex host immune responses, involving both innate and adaptive response mechanisms. Upon contact with bacteria, *H. pylori* antigens bind to receptors on gastric cells, activating nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase (CNK) pathways, followed by the release of proinflammatory cytokines [6]. Next, neutrophils and mononuclear cells infiltrate the gastric mucosa, resulting in the production of nitric oxide. Adaptive immune cells, CD4+ T lymphocytes, and CD8+ T lymphocytes are also recruited. Furthermore, the cytokine profile in patients with *H. pylori* infection, high levels of gamma interferon, tumor necrosis factor and interleukins, in addition to having proinflammatory effects during infection, suggest that the host response follows a Th1-polarized response [6].

Protocols for the treatment of *H. pylori* infection recommend that it be performed with first-line triple therapy, using a proton pump inhibitor (e.g., omeprazole) and two antibiotics (e.g., clarithromycin, tetracycline, metronidazole and amoxicillin). However, the increase in bacterial resistance to antibiotics, such as clarithromycin, has been alarming and the main factor for therapeutic failure [7]. The concern is such that in 2017 the World Health Organization (WHO) published a list containing 12 antibiotic-resistant bacteria that pose a threat to human health, and in this list the development of new antibiotics against clarithromycin-resistant *H. pylori* was classified as high priority [8]. Another problem is that the high dose during a long administration frequency and long duration of treatment is responsible for causing several side effects (e.g., nausea, vomiting, diarrhea and dizziness), accountable for poor patient compliance [7].

In this sense, natural products are sources of active substances that can be used therapeutically due to the great diversity of metabolites produced [9]. The use of these resources for medicinal purposes, whether in treatment, cure or prevention of pathologies, is one of the oldest forms of medicinal practice of humanity [10]. Brazil is the most biodiverse country in the world and many studies are carried out seeking to evaluate the biological properties of natural products, and their compounds, found in the country, in order to develop therapeutic alternatives against diseases [11].

Several natural products with anti-*H. pylori* properties are found in the scientific literature, such as *Aloe vera* [12], *Syzygium aromaticum* [13], *Canarium album* Raeusch. [14], essential oils and South African honey [15]. Due to the scientific interest in finding therapeutic alternatives in natural products to control and combat the infection of *H. pylori*, and the good results found, it is promising to look for new products that have not yet been studied that may present this property.

Propolis, popularly known as "bee glue," is a product produced by bees from buds, flowers and different plant exudates, to which bees add salivary secretions, wax and pollen for the final preparation of the substance [16]. In recent years, the scientific literature on propolis has been reporting promising pharmacological properties, where great antimicrobial potential can be highlighted [17,18]. The chemical compounds of natural products are heterogeneous and may vary according to their geographical location and the time of harvest [19,20]. Propolis is mainly composed of resin (50%), wax (30%), essential oils (10%), pollen (5%) and organic compounds (5%). Flavonoids, phenolic compounds, esters, alcohols, terpenes and beta-steroids can be mentioned as important organic compounds already identified in propolis samples [21]. To date, the scientific literature has reported

the identification of about 300 different chemical components in propolis from different sources [22].

Brazilian red propolis is a product of the *Apis mellifera* bee due to the collection of red exudates on the surface of the trunks of *Dalbergia ecastophyllum*, popularly known in Brazil as *rabo de bugio*, which is the botanical origin of red propolis [23–25]. The Brazilian red propolis presents in its chemical composition compounds that are not found in propolis samples from other sources, due to the vast Brazilian biodiversity and its botanical source. Brazilian red propolis has bioactivities reported in the literature, such as antioxidant, antibacterial, antiviral, antiparasitic, anti-inflammatory and wound healing, antitumor and antiproliferative activity, and immunomodulatory action, among others [25].

To the best of our knowledge, only one study in the literature has reported the in vitro anti-*H. pylori* activity of the Brazilian red propolis [26]. For this purpose, this study evaluated the in vitro and in vivo antibacterial activity of the crude hydroalcoholic extract of Brazilian red propolis (CHEBRP) against strains of *H. pylori*.

## 2. Results

### 2.1. CHEBRP Chemical Characterization

The main compounds identified in CHEBRP are liquiritigenin, formononetin, vestitol, neovestitol, medicarpin, 7-O-methylvestitol, a mixture of guttiferone E and xanthochymol, and oblongifolin B (Figure 1).

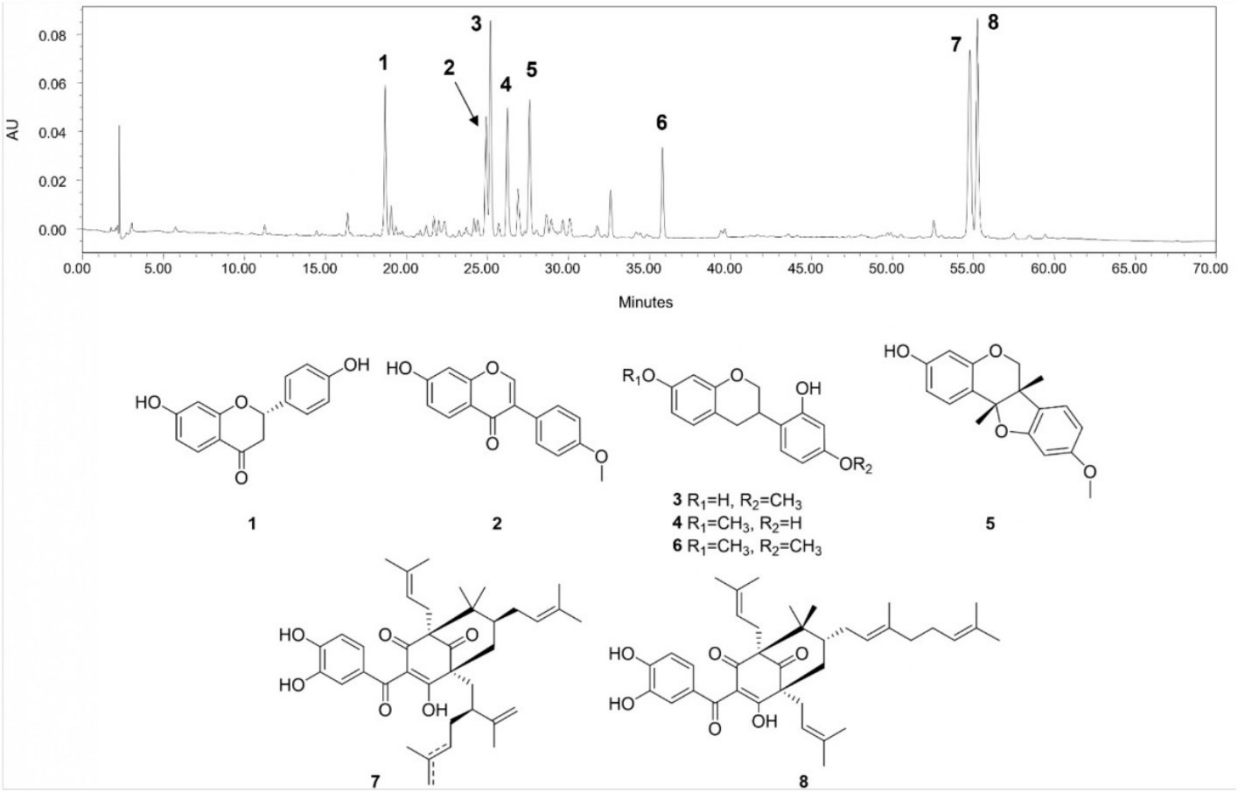



**Figure 1.** HPLC chromatographic profile (275 nm) and chemical structures of the main compounds identified in CHEBRP. Numbers correspond to liquiritigenin (**1**), formononetin (**2**), vestitol (**3**), neovestitol (**4**), medicarpin (**5**), 7-O-neovestitol (**6**), guttiferone E (**7**), and oblongifolin B (**8**).

### 2.2. In Vitro Evaluation

In vitro evaluation of anti-*H. pylori* activity of CHEBRP was performed by determining the minimal inhibitory concentration (MIC), minimal bacterial concentration (MBC), and synergistic activity.

The determination of the MIC of CHEBRP obtained results of 50.0 and 100.0 µg/mL against strains *H. pylori* (ATCC 43526) and *H. pylori* (clinical isolate), respectively. The determination of MBC obtained the same results (as found in the MIC) against the same strains. Therefore, the bactericidal effect of CHEBRP against the evaluated bacteria was determined (Table 1). Tetracycline was used as a positive control in both assays, and its MIC and MBC were determined to be 0.74 µg/mL against both strains evaluated in the present study (Table 1).

**Table 1.** Determination of Minimal Inhibitory and Bactericidal Concentrations of the crude hydroalcoholic extract of Brazilian red propolis against *Helicobacter pylori* strains (ATCC 43526 and clinical isolate).

| Strains | CHEBRP | | Tetracycline | |
|---|---|---|---|---|
| | MIC | MBC | MIC (µg/mL) | MBC |
| *Helicobacter pylori* (ATCC 43526) | 50.0 | 50.0 | 0.74 | 0.74 |
| *Helicobacter pylori* (clinical isolate) | 100.0 | 100.0 | 0.74 | 0.74 |

CHEBRP—crude hydroalcoholic extract of Brazilian red propolis. MIC—minimal inhibitory concentration. MBC—minimal bacterial concentration.

The synergistic activity of CHEBRP in combination with the antibiotic tetracycline was evaluated using the fractional inhibitory concentration index (FICI). The combination of the two substances against the strains of *H. pylori* had an indifferent effect (Table 2).

**Table 2.** Determination of the synergistic activity of the hydroalcoholic extract of Brazilian red propolis in combination with tetracycline against *Helicobacter pylori* strains (ATCC 43526 and clinical isolate).

| Strains | MICa (µg/mL) | | MICb (µg/mL) | | FIC * | | FICI ** | Outcome *** |
|---|---|---|---|---|---|---|---|---|
| | (1) | (2) | (1) | (2) | (1) | (2) | | |
| *Helicobacter pylori* (ATCC 43526) | 50.0 | 1.48 | 50.0 | 0.74 | 1.0 | 0.5 | 1.5 | Indifferent |
| *Helicobacter pylori* (clinical isolate) | 200.0 | 1.48 | 200.0 | 1.48 | 1.0 | 1.0 | 2.0 | Indifferent |

MICa—minimal inhibitory concentration alone. MICb—combined minimum inhibitory concentration. FIC—fractional inhibitory concentration. FICI—fractional inhibitory concentration index. (1)—Hydroalcoholic crude extract of Brazilian red propolis. (2)—Tetracycline. * FIC = MICb/MICa. ** FICI = FIC (1) + FIC (2). *** Synergistic interaction: FICI $\leq$ 0.5. Additive: FICI > 0.5 and <1.0. Indifferent: FICI > 1.0 and <4.0. Antagonistic interaction: FICI $\geq$ 4.0.

### 2.3. In Vivo Evaluation

For the in vivo evaluation of the anti-*H. pylori* activity of CHEBRP, the assays were performed using Wistar rats as animal models. Through microbiological and histopathological analysis, a quantitative and qualitative evaluation of the effect of defined concentrations of CHEBRP in the gastric tissue (antrum) of the rats was carried out.

The microbiological analysis performed using the technique of counting colony-forming units (CFU)/mL determined that all treatments used, except the one performed with dimethyl sulfoxide (DMSO), for both strains evaluated statistically decreased the quantity of bacteria in the antrum of rats (Figure 2).

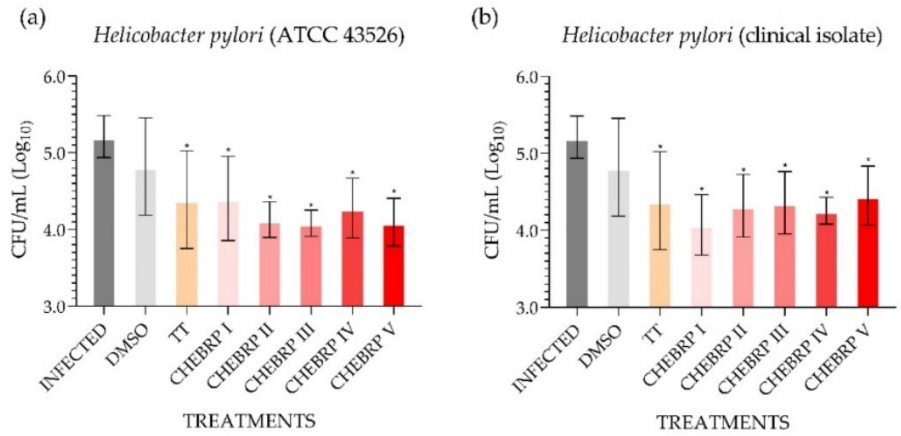



**Figure 2.** Quantitative determination of the presence of *Helicobacter pylori* in the stomach mucosa (antrum) of Wistar rats. DMSO—dimethyl sulfoxide. TT—triple therapy (amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg). CHEBRP—concentrations of crude hydroalcoholic extract of Brazilian red propolis (I: 18.25 mg/kg; II: 37.5 mg/kg; III: 75.0 mg/kg; IV: 150.0 mg/kg and V: 300.0 mg/kg). (**a**) Determination of the quantity of *Helicobacter pylori* (ATCC 43526) in the gastric antrum after treatments. (**b**) Determination of the quantity of *Helicobacter pylori* (clinical isolate) in the gastric antrum after treatments. * $p < 0.05$ compared to control (infected).

Histopathological analyses were performed to qualitatively evaluate the inflammatory infiltrate, activity, epithelial atrophy and presence of metaplasia. In the evaluation of the histological sections, it was determined that CHEPBR at a concentration of 300.0 mg/kg and triple therapy (TT: amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg) were the only treatments performed that were capable of decreasing the density of *H. pylori* (ATCC 43526 and clinical isolate) and modulating the inflammatory response (Tables 3 and 4).

Microscopic images were obtained for hematoxylin and eosin (HE) and with modified Giemsa staining (Figures 3 and 4, respectively). The same pattern of inflammation and bacterial infection was observed (both strains analyzed) for the untreated and those treated with CHEBRP at concentrations of 18.25, 37.5, 75.0 and 150.0 mg/kg. The same pattern was also observed for the groups treated with TT and CHEBRP at 300.0 mg/kg.

These last two treatments were the only ones capable, according to the updated Sydney classification [27,28], of decreasing chronic inflammation from moderate to mild, neutrophil polymorph infiltration (activity) from mild to absent, macrophages from mild to absent, tissue damage from mild to absent, apoptotic bodies from mild to absent, lamina propria edema from moderate to absent, epithelial/glandular atrophy from mild to absent, intestinal metaplasia from mild to absent, and bacterial (*H. pylori* ATCC 43526 and clinical isolate) density from moderate to mild.

**Table 3.** Qualitative analyses of gastric tissue (antrum-pyloric) of the experimental groups during *Helicobacter pylori* (ATCC 43526) infection in rats.

| Histological Criteria | Uninfected | Infected | DMSO¤ | TT¤ | CHEBRP I¤ | CHEBRP II¤ | CHEBRP III¤ | CHEBRP IV¤ | CHEBRP V¤ |
|---|---|---|---|---|---|---|---|---|---|
| Chronic inflammation | + ** | ++ | ++ | + | ++ | ++ | ++ | ++ | + |
| Neutrophil polymorph infiltration (activity) | - | + | + | - | + | + | + | + | - |
| Eosinophils | - | - | - | - | - | - | - | - | - |
| Macrophages | - | + | + | - | + | + | + | + | - |
| Lymphocytes | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Plasma cells | + | + | + | + | + | + | + | + | + |

**Table 3.** *Cont.*

| Histological Criteria | Uninfected | Infected | DMSO¤ | TT¤ | CHEBRP I¤ | CHEBRP II¤ | CHEBRP III¤ | CHEBRP IV¤ | CHEBRP V¤ |
|---|---|---|---|---|---|---|---|---|---|
| Giant foreign body cells | - | - | - | - | - | - | - | - | - |
| Fibroblast | - | - | - | - | - | - | - | - | - |
| Tissue damage | - | + | + | - | + | + | + | + | - |
| Necrotic tissue | - | - | - | - | - | - | - | - | - |
| Apoptotic bodies | - | + | + | - | + | + | + | + | - |
| Lamina propria edema | - | ++ | ++ | - | ++ | ++ | ++ | ++ | - |
| Epithelial/glandular atrophy | - | + | + | - | + | + | + | + | - |
| Intestinal metaplasia | - | + | + | - | + | + | + | + | - |
| *Helicobacter pylori* (ATCC 43526) density * | - | ++ | ++ | + | ++ | ++ | ++ | ++ | + |

DMSO—dimethyl sulfoxide. TT—triple therapy (amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg). CHEBRP—concentrations of crude hydroalcoholic extract of Brazilian red propolis (I: 18.25 mg/kg; II: 37.5 mg/kg; III: 75.0 mg/kg; IV: 150.0 mg/kg and V: 300.0 mg/kg). ¤ Infected groups. * Modified Giemsa staining. ** Normal resident cells. Scored for intensity: (-) absent, (+) mild, (++) moderate, as previously described by the updated Sydney classification [27,28].

**Table 4.** Qualitative analyses of gastric tissues (antrum-pyloric) of the experimental groups during *Helicobacter pylori* (clinical isolate) infection in rats.

| Histological Criteria | Uninfected | Infected | DMSO¤ | TT¤ | CHEBRP I¤ | CHEBRP II¤ | CHEBRP III¤ | CHEBRP IV¤ | CHEBRP V¤ |
|---|---|---|---|---|---|---|---|---|---|
| Chronic inflammation | + ** | +++ | ++ | + | ++ | ++ | ++ | ++ | + |
| Neutrophil polymorph infiltration (activity) | - | + | + | - | + | + | + | + | - |
| Eosinophils | - | - | - | - | - | - | - | - | - |
| Macrophages | - | + | + | - | + | + | + | + | - |
| Lymphocytes | + | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Plasma cells | + | + | + | + | + | + | + | + | + |
| Giant foreign body cells | - | - | - | - | - | - | - | - | - |
| Fibroblast | - | - | - | - | - | - | - | - | - |
| Tissue damage | - | ++ — ulcer | + | - | + | + | + | + | - |
| Necrotic tissue | - | - | - | - | - | - | - | - | - |
| Apoptotic bodies | - | ++ | + | - | + | + | + | + | - |
| Lamina propria edema | - | ++ | ++ | - | ++ | ++ | ++ | ++ | - |
| Epithelial/glandular atrophy | - | ++ | + | - | ++ | ++ | ++ | ++ | - |
| Intestinal metaplasia | - | + | + | - | + | + | + | + | - |
| *Helicobacter pylori* (clinical isolate) density * | - | ++ | ++ | + | ++ | ++ | ++ | ++ | + |

DMSO—dimethyl sulfoxide. TT—triple therapy (amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg). CHEBRP—concentrations of crude hydroalcoholic extract of Brazilian red propolis (I: 18.25 mg/kg; II: 37.5 mg/kg; III: 75.0 mg/kg; IV: 150.0 mg/kg and V: 300.0 mg/kg). ¤ Infected groups. * Modified Giemsa staining. ** Normal resident cells. Scored for intensity: (-) absent, (+) mild, (++) moderate, (+++) severe, as previously described by the updated Sydney classification [27,28].

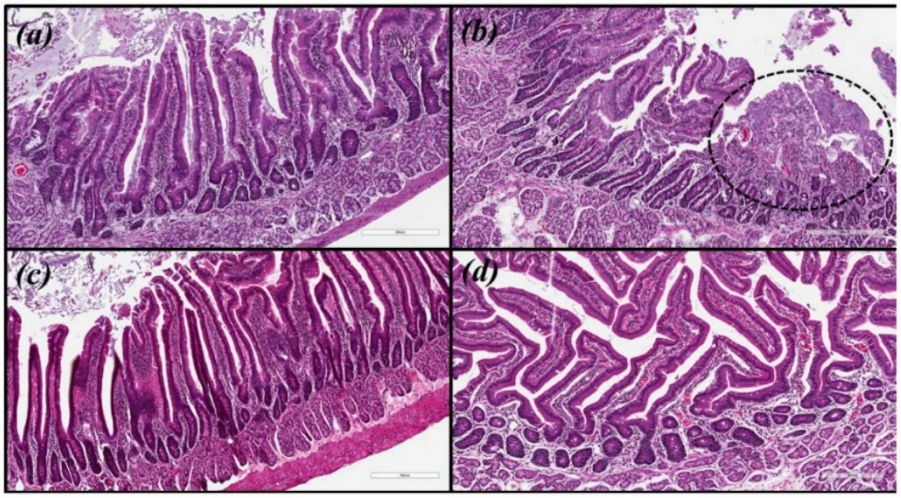



**Figure 3.** Biological tissue images (antrum-pyloric) of rats stained with hematoxylin and eosin (HE). (**a**) Uninfected group. (**b**) Infected group—the circled area demonstrates the ulceration and the intense mononuclear inflammatory infiltrate in the submucosa. (**c**) Group infected and treated with triple therapy (amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg)—note the integrity of the epithelial lining and the lower density of lymphocytic infiltrate in the submucosa. (**d**) Group infected and treated with 300 mg/kg of crude hydroalcoholic extract of Brazilian red propolis—note the integrity of the epithelial lining and the lower density of lymphocytic infiltrate in the submucosa.

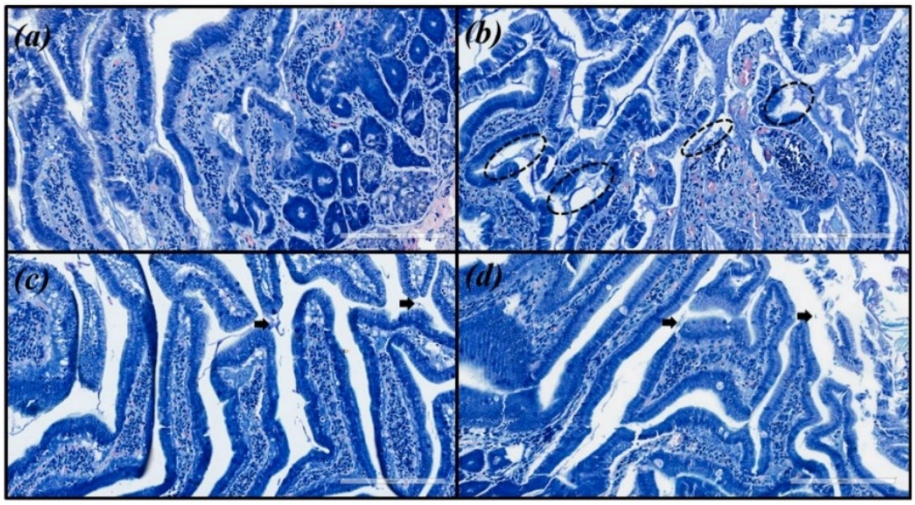



**Figure 4.** Images of the biological tissue (antrum-pyloric) of rats stained with modified Giemsa. (**a**) Uninfected group. (**b**) Infected group—black circles indicate stained *H. pylori* (blue) that are attached to the gastric epithelial cells (moderated density). (**c**) Group infected and treated with triple therapy (amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg)—the arrows point to occasionally stained *H. pylori* (mild density). (**d**) Group infected and treated with 300 mg/kg of crude hydroalcoholic extract of Brazilian red propolis—the arrow point to occasionally stained *H. pylori* (mild density).

## 3. Discussion

In the scientific literature, no other studies were found in which the anti-*H. pylori* activity of CHEBRP was determined. Therefore, the results found in this study, in which these properties of CHEBRP were evaluated by in vitro and in vivo analyses, are unprecedented.

In the present study, CHEBRP presented an MIC value of 50.0 and 100.0 µg/mL against *H. pylori* (ATCC 43526) and *H. pylori* (clinical isolate), respectively. In their study, Holetz et al. [29] considered good antibacterial activity when extracts have an MIC value lower than 100.0 µg/mL, moderate activity when MIC is from 100.0 to 500.0 µg/mL, weak activity when the concentration varies from 500.0 to 1000 µg/mL, and inactive when it is over 1000 µg/mL. Considering the same criteria used by the authors [29] and the results obtained in the present study, CHEBRP showed promising anti-*H. pylori* activity, since, against *H. pylori* (ATCC 43526) the activity was good and against *H. pylori* (clinical isolate) the activity was moderate.

Baltas et al. [30] evaluated the antimicrobial activity of 15 samples of propolis, acquired from different areas of Turkey, against the strain *H. pylori* J99 through the agar-well diffusion assay. At a concentration of 75.0 µg/mL, the zones of inhibition ranged from 31 to 47 mm. Boyanova et al. [31] evaluated the ethanolic extract of Bulgarian propolis against 94 strains of *H. pylori* (clinical isolate), also through the agar-well diffusion assay, at concentrations of 9000, 18,000 and 27,000 µg per well, and found zones of inhibition that ranged from 7 to 48, 7 to 56.5, and 9 to 60 mm, respectively. Villanueva et al. [32], also chose concentrations of 9000, 18,000 and 27,000 µg per well to evaluate the antibacterial activity of 22 Chilean propolis extracts against 10 strains of *H. pylori* through the agar-well diffusion assay. The authors determined zones of inhibition that ranged from 35 to 77, 31 to 60 and 26 to 54 mm in wells containing 27,000, 18,000 and 9000 µg/well of propolis, respectively. Mendonça et al. [26] evaluated the Brazilian red propolis extract, also through the agar-well diffusion method, and determined that 80 µL of Brazilian red propolis at a concentration of 100,000 µg/mL causes 13.0 ± 2.0 mm inhibition of *H. pylori* (ATCC 43504) The present study used the broth dilution assay to determine the MIC of CHEBRP against strains of *H. pylori*, due to the difference in methodology used, it is not possible to compare the results obtained in this study with those obtained by the authors described above. However, it is possible to verify that the propolis extract from different regions has good antibacterial activity against *H. pylori*, which, with further studies, could become a therapeutic alternative.

Widelski et al. [33] determined the MIC/MBC of propolis extract samples—four from Poland, four from Ukraine, one from Kazakhstan, and one from Greece—against *H. pylori* (ATCC 43504). The results found ranged from 15.0 to 60.0 µg/mL. In the present study, against *H. pylori* (ATCC 43526), CHEBRP presented an MIC/CBM of 50.0 µg/mL, a lower result than that found by Widelski et al. [33] from a propolis sample collected from Poland against *H. pylori* (ATCC 43504), which obtained an MIC/MBC of 60.0 µg/mL. The other nine extracts evaluated by the authors obtained MBC of 30 µg/mL against this strain. In the present study, the CBM concentration of CHEBRP was only 20 µg/mL higher against *H. pylori* (ATCC 43526). These results suggest that the data obtained in this present study are within the expected range, since they are in agreement with the observations of previous studies.

In previous studies, our research group evaluated the MIC and MBC of the hydroalcoholic crude extract from the leaves of nine *Copaifera* spp. against *H. pylori* (ATCC 43526). MIC and MBC were determined for: *C. trapezifolia* (12.5/25 µg/mL), *C. duckei* (12.5/12.5 µg/mL), *C. oblongifolia* (50.0/50.0 µg/mL), *C. langsdorffii* (25.0/100.0 µg/mL), *C. pubiflora* (100.0/100.0 µg/mL), *C. paupera* (50.0/50.0 µg/mL), *C. reticulata* (50.0/50.0 µg/mL), *C. multijuga* (25.0/25.0 µg/mL) and *C. lucens* (25.0/25.0 µg/mL) [34]. In the present study, against the same strain, CHEBRP presented MIC/MBC of 50.0 µg/mL, the same concentration as previously obtained for *C. oblongifolia*, *C. paupera* and *C. reticulta*, and lower than that obtained for *C. langsdorffii* and *C. pubiflora* [34]. These results underline the fact that natural products have strong anti-*H. pylori* activities and can contribute to fighting bacterial infection.

As the fight against bacterial infection is carried out through therapies that use a combination of antibiotics [7], it is interesting that any proposed therapeutic novelty evaluates the synergistic action of substances with standard antibiotics. In this sense, Nostro et al. [35] evaluated the synergistic activity of commercial propolis resin (Italy) in combination with clarithromycin against 25 strains of *H. pylori* (24 clinical isolates and one ATCC 43504), of which nine showed synergism, nine antagonism, and seven indifferent. In the present study, the combination of CHEBRP with tetracycline presented an indifferent result against the strains evaluated (*H. pylori* ATCC 43526 and clinical isolate).

The CHEBRP used in this study has among its compounds liquiritigenin, isoliquiritigenin, formononetin, vestitol, neovestitol, medicarpin, 7-O-methylvestitol, guttiferone E/xanthochymol, and oblongifolin B. Studies suggest that compounds such as medicarpin, vestitol and neovestitol commonly found in Brazilian red propolis samples and found in the CHEBRP used in the present study may be responsible for the antibacterial action of Brazilian red propolis [36,37]. However, studies also indicate that a synergistic effect between its compounds is related to biological properties presented by CHEBRP [38,39].

Fukai et al. [40] evaluated the anti-*H. pylori* activity of the compounds formononetin and vestitol, and it was determined that formononetin showed weak anti-*H. pylori* activity (MIC > 100 μg/mL) and vestitol good antibacterial activity (MIC 12.5 μg/mL) against *H. pylori* (ATCC 43526). The other six main compounds present in CHEBRP (liquiritigenin, isoliquiritigenin, neovestitol, medicarpin, 7-O-methylvestitol, guttiferone E/xanthochymol, and oblongifolin B) have not yet been reported in the literature to have anti-*H. pylori* activity. In the gastroprotective evaluation of compounds isolated from Brazilian red propolis in mice with ethanol-induced ulcers, Boing et al. [38] determined that the compound medicarpin is essential for the extract to present this biological property.

Due to promising anti-*H. pylori* activity in vitro from Brazilian red propolis in the present study, and the fact that the extract showed gastroprotective properties in previous studies [38,41], supports the realization of the anti-*H. pylori* activity in vivo performed.

Mendonça et al. [26] evaluated the gastroprotective activity of Brazilian red propolis, collected in Sergipe and its major compound being formononetin, in the reduction of gastric ulceration caused by indomethacin (100.0 mg/kg) in Wistar rats. The Brazilian red propolis used by the authors was able to reduce gastric ulceration by 87.34% and 100.0% with doses of 250.0 and 500.0 mg/kg. The CHEBRP used in the present study was collected in Bahia, and its components differ from the Brazilian red propolis used by Mendonça et al. [26] due to the geographical difference in propolis collection [22]. However, both the Brazilian red propolis, the one used in the present study and the one used by Mendonça et al. [26] showed gastroprotective action in in vivo assays using Wistar rats. Therefore, the data obtained in the present study are in agreement with the scientific literature.

In order to develop a vaccine against *H. pylori*, Mahboubi et al. [42] evaluated in C57BL/6 mice the action of Iranian propolis alone and in combination with outer inflammatory protein A (OipA), and OipA alone against *H. pylori* (clinical isolate). The concentration used by the authors of propolis was 10.0 mg/dose and of OipA 100.0 μg/dose. All vaccines used by the authors decreased the concentration of *H. pylori* (CFU/g) in the stomach of infected mice significantly compared to the unvaccinated control. However, when propolis was combined with OipA, the results were significantly greater than for propolis and OipA alone, and yet the effect of OipA was significantly smaller than for propolis, suggesting that some compound of Iranian propolis was antagonistic to OipA [42]. In the present study, in Wistar rats, concentrations of CHEBRP (18.25, 37.5, 75.0, 150.0 and 300.0 mg/kg) were used, and all concentrations of CHEBRP were able to significantly decrease the quantity of *H. pylori* (ATCC 43526 and clinical isolate) in the stomach (antrum) of rats when compared to the untreated control, corroborating earlier findings.

A clinical study evaluated the action of Brazilian green propolis in 18 patients who had confirmed *H. pylori* infection [43]. Participants received 20 drops of green propolis three times a day for seven days. However, it did not present promising results, which the authors attributed to the treatment with low dosage of Brazilian green propolis during

a short time of administration [43]. The same hypothesis can be raised in relation to the CHEBRP used in the present study, even though, all the concentrations evaluated, having significantly reduced the quantity of *H. pylori* in the antrum of the rats, did not completely eliminate the bacterial load in the tissue. More studies are needed to assess whether there is any dose–time relationship that influences anti-*H. pylori* in vivo activity of CHEBRP.

Many studies are still needed to better evaluate the biological properties of CHEBRP before it can be considered a therapeutic alternative against infection caused by *H. pylori*. However, the data obtained in the present study are a great advance on the way to better knowledge about the extract, since they are unprecedented.

## 4. Materials and Methods

### *4.1. Crude Hydroalcoholic Extract of Brazilian Red Propolis*

CHEBRP (submitted to the National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SISGEN, registered as AF234D8) was obtained from the Association of Beekeepers of Canavieiras (Cooperativa de Apicultores de Canavieiras, COAPER, Bahia, Brazil), a Brazilian municipality located in the southern region of Bahia, northeastern Brazil in March 2019. For extraction, Brazilian red propolis samples were frozen and minced. Two hundred grams of Brazilian red propolis were submitted to dynamic maceration at 30 °C and 120 rpm using a shaker incubator (INNOVA 4300, New Brunswick Scientific, Saint Albans, UK) with 70% hydroalcoholic ethanol solution. The extracts were concentrated under vacuum using a rotary evaporator and lyophilized to complete dryness.

The phytochemical characterization of CHEBRP was performed by high-performance liquid chromatography with a photodiode array detector (HPLC-PAD) on a Waters 1500 series setup (Milford, CT, USA). A Synergy Fusion RP-80 A column (150 × 4.6 mm, 4 µ; Phenomenex, Torrance, CA, USA) was used as a stationary phase, and water with 0.1% formic acid (A) and acetonitrile (B) as the mobile phase. The injections were performed on a gradient elution method of 10–100% of B (60 min), 100% of B (70 min), with a flow rate of 1 mL/min, at 40 °C.

Previously authentic standards from CHEBRP isolated by Aldana-Mejia et al. [44] were used to identify the major compounds on the sample. The standard compounds included liquiritigenin, formononetin, vestitol, neovestitol, medicarpin, 7-O-methylvestitol, guttiferone E/xanthochymol, and oblongifolin B. The purity of the standards was estimated to be greater than 96% by HPLC and NMR [44].

### *4.2. Bacterial Strains Used in the Assays*

All strains used are part of the culture collection of the Laboratory of Antimicrobial Testing (LEA) of the Federal University of Uberlândia (UFU), and are kept under cryopreservation (−80 °C) in Brucella broth (Difco Labs, Detroit, MI, USA) supplemented with 10.0% fetal bovine serum. One strain comes from the American Type Culture Collection (ATCC 43526), and a clinical isolate was isolated from clinical trials, in a previous study, from a patient with peptic ulcer caused by bacterial infection (clinical isolate).

### *4.3. Determination of Minimal Inhibitory and Bactericidal Concentrations*

The MIC is defined by the lowest concentration of the antimicrobial agent capable of inhibiting bacterial growth. For its determination, the microdilution method was carried out in a 96 well microplate, according to the methodology recommended by the Clinical and Laboratory Standards Institute (CLSI) [45], with adaptations, using resazurin (Sigma, St. Louis, MO, USA) as bacterial growth developer [46] and Brucella broth (Difco) supplemented with 10% fetal bovine serum.

Briefly, CHEBRP was dissolved in 5% DMSO to reach final concentrations ranging from 0.195 to 400.0 µg/mL. The bacterial inoculum was adjusted to produce a cell concentration equal to $5 \times 10^5$ CFU/mL. Tetracycline (Sigma) in concentrations ranging from 0.0115 to 5.9 µg/mL was used as a standard antibiotic and solubilized in water. Control of

5% DMSO was performed, and the solvent did not interfere with bacterial growth at this concentration. The plates were incubated in a $CO_2$ incubator (Panasonic Biomedical, Netherlands, Amsterdam) at 37 °C for 72 h in the atmosphere containing 10% $CO_2$. After the incubation period, 30.0 µL of a 0.01% aqueous resazurin solution was added to each well of the microplate to assess bacterial growth [46].

The MBC, defined as the lowest concentration capable of completely preventing bacterial growth, was performed in the MIC microplate, prior to the addition of resazurin. An aliquot of 10.0 µL was taken from all microplate wells, and inoculated onto Brucella agar (Difco) supplemented with 5.0% defibrinated horse blood and 10.0% fetal bovine serum. Then, the agar plates were incubated under the same conditions and time as the microplates described above. After the incubation period, it was found at the lowest concentration that no bacterial growth occurred. All assays were performed in triplicate.

### *4.4. Determination of Synergistic Activity*

Determination of synergistic activity was performed according to the protocol described by Chaturvedi et al. [47]. Concentrations of CHEBRP and tetracycline (Sigma) were combined in the standard MIC format against $5 \times 10^5$ CFU/mL of bacterial inoculum. To assess synergism, fractional inhibitory concentration index (FICI) values were calculated as previously established in the literature [47]. The index values were analyzed as follows: $\leq$0.5, synergism; >0.5 to <1.0, additive; $\geq$1.0 to <4.0, indifference; and $\geq$4.0, antagonism [48]. The assay was performed in triplicate.

### *4.5. Determination of Anti-H. pylori Activity in Wistar Rats*

For in vivo assessment of anti-*H. pylori* from CHEBRP against the two bacterial strains used, the method developed by Werawatganon [49], with adaptations, was performed. The evaluation was approved by the Ethics Committee on the Use of Animals of the Federal University of Uberlândia (Protocol n. 095/2019).

Briefly, 126 male Wistar rats weighing an average of 300 to 350 g were used in the trial. Initially, the animals were randomly divided into groups according to the treatment, *H. pylori* infection was induced in the animals through gavage with a bacterial inoculum adjusted to a coresponder from $10^8$ to $10^9$ CFU/mL for three days twice a day. After confirming the infection, performed by the OnSite *H. pylori* Ag rapid test (CTK Biotech, Inc. San Diego, CA, USA) in the animals' feces, the treatment was performed, also via gavage, with concentrations of CHEBRP (18,25, 37.5, 75.0, 150.0 and 300.0 mg/kg) solubilized in 1% DMSO and triple therapy (TT—amoxicillin 50.0 mg/kg, clarithromycin 25.0 mg/kg, and omeprazole 20.0 mg/kg) solubilized in water for seven days once a day. Each treatment group had seven rats and the necessary controls were also performed (inoculated, non-inoculated and solvent). The maximum volume of gavage received was 0.8 milliliters per rats.

After the treatment period, the animals were euthanized in a $CO_2$ chamber and the biological material of study (stomach) was collected, the stomach antrum was processed and divided into two parts, one for microbiological analysis (quantitative) and another for histopathological analysis (qualitative).

The microbiological analysis was performed through counting that determined the number of CFU/mL in the stomach tissue. The material was placed in Brucella broth (Difico) supplemented with 10.0% fetal bovine serum and vortexed for one minute. Afterwards, dilutions ranging from $10^0$ to $10^7$ were performed, and seeded on Brucella agar (Difico) supplemented with 5.0% horse blood and 10.0% fetal bovine serum, containing vancomycin (Sigma—5.0 mg), polymyxin B (Sigma—2.5.0 mg), and trimethoprim (Sigma—2.5 mg) to suppress the growth of other bacteria and fungi [50]. The plates were incubated at 37 °C for 72 h in a 10.0% $CO_2$ incubator. After this period, the count was performed which determined CFU/mL in the tissue.

For histopathological analysis, gastric tissue samples were fixed in 10.0% buffered formalin solution, dehydrated in ethanol solution, cleared in xylene and embedded in

paraffin. Blocks containing hearts were sectioned into 5.0 µm-thick sections and then placed on glass slides and routinely stained with hematoxylin and eosin (HE) and modified Giemsa stain [51].

To assess inflammatory infiltrate, activity, epithelial atrophy, and presence of metaplasia, slides were stained with hematoxylin and eosin (HE). The density of *H. pylori* was evaluated in sections stained with modified Giemsa. The type and severity of the inflammatory infiltrate and tissue damage were determined by two independent observers who were calibrated for the presence or absence of (i) inflammatory response, (ii) neutrophils, (iii) macrophages, (iv) lymphocytes, (v) plasma cells, (vi) foreign body giant cells, (vii) tissue damage, (viii) necrotic tissue, (ix) apoptotic bodies, (x) edema, (xi) epithelial atrophy, (xii) intestinal metaplasia, (xiii) density of *H. pylori*. These characteristics were scored for intensity: (−) absent, (+) mild, (++) moderate, (+++) severe, as previously described by the updated Sydney classification [27,28].

### *4.6. Statistical Analysis*

The data obtained in the microbiological evaluation (quantitative) were statistically analyzed by analysis of variance (ANOVA) using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA), with calculation of the F statistic and its respective *p*-value. In cases where $p < 0.05$, treatment means ($Log_{10}$) were compared using Dunnett's multiple comparisons test, with the calculation of the minimum significant difference for $\alpha = 0.05$.

## 5. Conclusions

Based on the data obtained in the present study, it can be concluded that the crude hydroalcoholic extract of red propolis has promising anti-*H. pylori* activity in vitro, due to its bactericidal capacity, at low concentrations, against standard and clinically isolated strains and not interfering with the effect of an antibiotic used in standard therapy. CHEBRP also shows promising anti-*H. pylori* activity in vivo, since the microbiological and histopathological studies performed showed the extract's ability to reduce bacterial load and modulate the immune response in the biological tissue of mice infected by the strains evaluated. However, more studies are needed to better analyze this biological property (e.g., antibiofilm activity, mechanisms of action), and our research group intends to continue this investigation.

**Author Contributions:** Conceptualization, M.B.S., L.F.L. and C.H.G.M.; methodology, M.B.S., L.F.L., J.A.A.-M., S.R.A., R.C.S.V., J.K.B. and C.H.G.M.; validation, S.R.A., R.C.S.V., J.K.B. and C.H.G.M.; formal analysis, M.V.S., J.K.B., C.H.G.M.; investigation, M.B.S., L.F.L., R.B.R., S.C.T., J.P.S.S. and J.A.A.-M.; resources, S.R.A., J.K.B. and C.H.G.M.; writing—original draft preparation, M.B.S. and L.F.L.; writing—review and editing, J.P.S.S., J.K.B., C.H.G.M.; supervision, C.H.G.M.; funding acquisition, S.R.A., J.K.B., C.H.G.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant 2017/04138-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES finance code 001, scholarship granted), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq grant 307974/2019-7; CNPq Scholarship 151962/2019-7).

**Institutional Review Board Statement:** The animal study protocol was approved by the Ethics Committee on the Use of Animals of the Federal University of Uberlândia (protocol 095/2019).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data generated and analyzed during this study are available from the corresponding author on reasonable request.

**Acknowledgments:** The authors are thankful to Sandra Gabriela Klein for the technical support.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from the authors.

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**CAPITULO III**

# Unveiling The Antibacterial Potential of Brazilian Red Propolis Against The Formation And Eradication of Biofilm of *Helicobacter Pylori*

Manuscrito a ser submetido para publicação no periódico Phytomedicine em 2024.

# UNVEILING THE ANTIBACTERIAL POTENTIAL OF BRAZILIAN RED PROPOLIS AGAINST THE FORMATION AND ERADICATION OF BIOFILM OF *Helicobacter pylori*

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ABSTRACT

*Background Helicobacter pylori* is a bacterium associated with gastrointestinal diseases. Treatment is challenging, and the emergence of antibiotic-resistant strains makes the development of alternative therapies necessary. Natural products, such as Brazilian red propolis (BRP), may be of interest due to their diverse bioactive compounds with pharmaceutical properties.

*Purpose:* To determine whether BRP has anti-*H. pylori* activity, particularly in inhibiting biofilm formation and eradicating biofilm.

*Methods:* BRP was used against the *H. pylori* (ATCC 43526) bacteria in the assays: time-kill, nucleotide leakage, biofilm formation inhibition (determination of the minimum inhibitory concentration of biofilm of 50% - $MICB_{50}$, and determination of cell viability), and biofilm eradication (determination of the minimum eradication concentration of biofilm of 99.9% - MBEC). The assay for standardization of biofilm formation by *H. pylori* (ATCC 43526) was also conducted.

*Results:* In the time-kill assay at a concentration of 50 µg/mL, BRP was able to eliminate all *H. pylori* cells after 24 h of exposure. The nucleotide leakage assay showed no statistical differences between the control groups (only *H. pylori*) and the groups containing BRP at concentrations of 25 µg/mL and 50 µg/mL. *H. pylori* demonstrated the ability to form biofilm *in vitro* at a concentration of $10^9$ CFU/mL after 72 h of incubation. The $MICB_{50}$ of BRP against *H. pylori* was determined at a concentration of 15.6 µg/mL, and at concentrations of 500, 1000, and 2000 µg/mL, it killed all bacterial cells. The MEBC was determined at a concentration of 2000 µg/mL.

*Conclusion:* Due to the results obtained, it can be concluded that BRP has promising anti-*H. pylori* activity, being effective in inhibiting and eradicating the bacteria biofilm. Further studies are needed to better understand the mechanisms of action of BRP against *H. pylori*.

*Keywords*: *Helicobacter pylori*, Brazilian red propolis, natural products, antibiofilm, time-kill, nucleotide leakage

*Abbreviations:* BRP, Brazilian red propolis; $MICB_{50,}$ minimum inhibitory concentration of biofilm; MBEC, minimum eradication concentration of biofilm; SD, standard deviation; OD, optical density; CFU, colony forming units.

## Introduction

The Gram-negative bacillus *Helicobacter pylori* is a microaerophilic, flagellated bacterium capable of colonizing the mucosa of the gastric epithelium (Al-Fakhrany & Elekhnawy, 2023; Chmiela & Kupcinskas, 2019; Malfertheiner et al., 2023). Owing to its colonization site, the bacteria is associated with the development of various chronic gastric diseases, such as gastritis, gastric and duodenal ulcers, and is also a risk factor for the development of gastric cancer (Chmiela & Kupcinskas, 2019; Malfertheiner et al., 2023; Thrift, Wenker, & El-Serag, 2023).

In addition to its pathogenic potential, *H. pylori* exhibits high prevalence rates, with estimates suggesting that more than half of the global population is colonized by the bacteria (Hooi et al., 2017). It is not yet possible to determine the exact mechanism of its transmission; however, indications suggest that individuals are contaminated during childhood via oral-oral and fecal-oral routes. Once colonized, the individual becomes a carrier and may never exhibit any symptoms or develop pathologies (Cover & Blaser, 2009; Sharndama & Mba, 2022).

However, in cases where individuals become ill, the eradication of the bacteria is challenging. The standard gold treatment consists of triple therapy, involving a proton pump inhibitor (e.g., omeprazole) and two antibiotics (e.g., clarithromycin, tetracycline, and amoxicillin) (Malfertheiner et al., 2023). Nevertheless, this therapy presents high failure rates, which can be associated with its virulence factors that contribute to the development of bacterial resistance to the antibiotics used (Al-Fakhrany & Elekhnawy, 2023; Buzas & Birinyi, 2023; Malfertheiner et al., 2023).

Such virulence factors present in *H. pylori* (e.g., motility, toxin production, urease activity, and outer membrane proteins) are also responsible for the bacteria ability to colonize an environment considered hostile, such as the stomach (Al-Fakhrany & Elekhnawy, 2023; Sharndama & Mba, 2022). The capability of biofilm formation is a virulence factor present in *H. pylori*, which is one of the primary factors associated with the development of antibiotic resistance (Al-Fakhrany & Elekhnawy, 2023; Elshenawi, Hu, & Hathroubi, 2023; Hathroubi, Servetas, Windham, Merrell, & Ottemann, 2018; Krzyzek, Grande, Migdal, Paluch, & Gosciniak, 2020).

The development of antibiotic resistance by *H. pylori* is a serious health issue, recognized by the World Health Organization (WHO), which in 2017 published a list containing 12 antibiotic-resistant bacteria that pose a threat to human health. In this list, the development of new therapeutic agents capable of eradicating clarithromycin-resistant *H. pylori* is considered a high priority (Tacconelli et al., 2018).

However, even though there is a demand for new antibiotics capable of eliminating bacteria resistant to current antibiotics, and acknowledging that the biofilm-forming ability of the bacterium plays a significant role in the development of this resistance, the biofilm-forming ability of *H. pylori* is still under-explored in scientific research. On the other hand, there are studies on therapeutic options with antibiofilm properties. The vast majority of the studied agents that have shown promising results are natural products or derivatives. (Al-Fakhrany & Elekhnawy, 2023).

As the term implies, natural products are derived from natural sources, such as plants, animals, minerals, and microorganisms. Their scientific importance lies in being sources of unique active substances that possess biological properties (Newman & Cragg, 2020). Their use in healthcare is one of the oldest practices of human care, representing a body of folk knowledge passed down from generation to generation (Bernardini, Tiezzi, Laghezza Masci, & Ovidi, 2018).

Propolis is a natural product produced by bees, commonly known as "bee glue". It is formed through a combination of buds, flowers, and various plant exudates with bee saliva secretions, wax, and pollen (Sforcin & Bankova, 2011). Its composition is predominantly resin (50%), wax (30%), essential oils (10%), pollen (5%), and organic compounds (5%). Due to the seasonality of natural products, the compounds present in propolis can vary according to geographic location or time of collection (Lachance, Wetzel, Kumar, & Waldmann, 2012; Wieczorek et al., 2022). As a consequence, approximately 500 different chemical components have been identified in propolis from various sources and locations. Among these, significant compounds have been identified in propolis, such as flavonoids, phenolic compounds, alcohols, and terpenes (Hossain et al., 2022).

In Brazil, red propolis is the result of bees, *Apis mellifera*, collecting red exudates from the surface of the trunks of *Dalbergia ecastophyllum*, commonly known in Brazil as "*rabo de bugio*" (Batista et al., 2012; de Freitas et al., 2017; Moise & Bobis, 2020). Owing to its botanical source and the vast biodiversity of Brazil, Brazilian red propolis (BRP) contains compounds not found in propolis from other sources (Moise & Bobis, 2020). Literature reveals various biological properties attributed to BRP, such as antibacterial, antiparasitic, antioxidant, among others (de Freitas et al., 2017). Two studies have already reported promising anti-*H. pylori* activity of BRP against the sessile form of the bacteria (Mendonça et al., 2020; Santiago et al., 2022).

Therefore, considering that bacterial resistance is highly associated with the ability of bacterial biofilm formation (Al-Fakhrany & Elekhnawy, 2023), that natural products have already shown promising antibiofilm activity against *H. pylori* (Al-Fakhrany & Elekhnawy,

2023), that there is an urgent need for the development of new therapeutic options against *H. pylori* (Tacconelli et al., 2018), and that studies have already demonstrated promising anti-*H. pylori* activity of BRP against free-living forms of the bacteria (Mendonça et al., 2020; Santiago et al., 2022), it is encouraged to deepen this evaluation to determine if BRP also exhibits antibiofilm activity against *H. pylori*. For this purpose, the current study evaluated *in vitro* the potential of the crude hydroalcoholic extract of BRP against the formation and eradication of the biofilm of *H. pylori* (ATCC 43526).

## Methods

*Crude Hydroalcoholic Extract of Brazilian Red Propolis*

The crude hydroalcoholic extract of Brazilian Red Propolis (BRP) used in the present study was collected in the Brazilian municipality of Canavieiras, located in the southern region of Bahia, northeastern Brazil, in March 2019, by the Association of Beekeepers of Canavieiras (Cooperativa de Apicultores de Canavieiras, COAPER, Bahia, Brazil). The sample was submitted to the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), and registered as AF234D8. The processes of extraction, phytochemical characterization, and purity standards of the BRP used were previously described by Santiago et al. (2022).

*Bacteria used in the assays*

The *H. pylori* strain used is sourced from the American Type Culture Collection (ATCC 43526) and is part of the culture collection of the Laboratory of Antimicrobial Testing (LEA) at the Federal University of Uberlândia (UFU). The strain is preserved under cryopreservation at -80°C in Brucella broth (Difco Labs, Detroit, MI, USA) supplemented with 10.0% (v/v) bovine fetal serum and containing 20.0% (v/v) glycerol.

For bacterial cultivation, *H. pylori* was seeded on Brucella agar (Difco) containing 5% of defibrinated horse blood or in Brucella broth (Difco) supplemented with 10.0% bovine fetal serum. It was then incubated in a $CO_2$ incubator (Panasonic Biomedical, Netherlands, Amsterdam) at 37°C for 72 hours in an atmosphere containing 10.0% $CO_2$.

*Time-Kill*

The Time-Kill assay of BRP against *H. pylori* (ATCC 43526) was conducted in triplicate, as described by Sposito et al. (2019), with minor modifications. For the assay, the bacterial inoculum was adjusted to contain $5x10^5$ Colony Forming Units per milliliter (CFU/mL). BRP was added at concentrations of 25 and 50 µg/mL, and tetracycline, evaluated

at a concentration of 0.74 µg/mL, was used as a positive control. The bacterial inoculum without any treatment was used as a negative control. The assay was incubated in a $CO_2$ incubator at 37°C.

To perform the viable bacterial colony count, aliquots were removed at 0 and 30 min, and at 2, 4, 8, 12, 24, 32, 36, 48, 56, 60, and 72 h. The aliquots were diluted in a 1:10000 ratio and seeded on Brucella agar containing 5% defibrinated horse blood. The agar was incubated under the necessary conditions and after 72 hours, colony counting was conducted. The results were expressed as mean ± standard deviation (SD). The time-kill curve was constructed by plotting $\log_{10}$ CFU/mL against time using GraphPad Prism (version 8.0) software.

*Nucleotide Leakage*

To evaluate whether BRP causes damage to the cell membrane of *H. pylori*, the nucleotide leakage assay was performed. The assay was conducted as described by Chen and Cooper (2002), with modifications. Briefly, the bacterial inoculum was adjusted to achieve a final concentration of $5x10^5$ CFU/mL in three test tubes, one containing 25 µg/mL of BRP, another 50 µg/mL of BRP, and the third containing only the inoculum. Additionally, three tubes containing only broth and BRP at the evaluated concentrations were used as blanks. The tubes were incubated in a $CO_2$ atmosphere for 24 hours. After incubation, the contents of the tubes were filtered through 0.2 µm syringe filters, and the absorbance was measured at 260 and 280 nm using a microtiter plate reader (GloMax®, Promega, Madison, WI, USA). The absorbance values obtained were subtracted from their respective blanks by subtraction. The assay was performed in triplicate, and the results of optical density (OD) were graphically represented using GraphPad Prism (version 8.0) software.

*Standardization of Biofilm Formation*

The optimal inoculum concentration and incubation time for biofilm formation were determined using the biofilm formation standardization assay, adapting Sandberg, Maattanen, Peltonen, Vuorela, and Fallarero (2008) methodology for *H. pylori* in order to respect the nutritional and physiological characteristics of the bacteria. The bacteria were incubated in a $CO_2$ incubator for 3, 5, and 7 days at concentrations ranging from $10^6$ to $10^9$ CFU/mL in 96-well microplates.

After incubation, the contents were removed from all wells, and they were washed with Milli-Q water to ensure removal of planktonic cells. The adhered biofilm in the microplate was fixed with methanol for 20 minutes, after which the methanol was removed and crystal violet (0.2% v/v) was added to the wells for 10 minutes. The crystal violet was then removed and

excess washed off with tap water. After drying, 200 µL of 33% (v/v) acetic acid was added to the wells to solubilize the dye adhered to the cells. After 30 minutes, the reading was taken at 595 nm using a microtiter plate reader (GloMax®). The assay was carried out in triplicate and the OD values obtained were expressed graphically.

*Minimum biofilm inhibition concentration*

To evaluate the potential of BRP to inhibit biofilm formation of *H. pylori*, the study assessed the Minimum Inhibitory Concentration of Biofilm ($MICB_{50}$), which is the lowest concentration of the sample evaluated capable of inhibiting the formation of 50% or more of the biofilm (Wei, Campagna, & Bobek, 2006). Additionally, the assessment of cell viability was conducted through CFU count.

Two 96-well microplates were prepared identically for the assessment. Briefly, dilutions of BRP were made, with final concentrations ranging from 0.98 to 2000 µg/mL. Tetracycline was used as a positive control with concentrations ranging from 0.0115 to 5.9 µg/mL. An inoculum of *H. pylori* at a concentration of $10^9$ CFU/mL was added to all wells, and a control with only the inoculum and Brucella broth was also used. The microplates were incubated for 72 hours at 37ºC in a $CO_2$ incubator.

After incubation, the microplate designated for determining the $MICB_{50}$ underwent the process described above, standardized by Sandberg et al. (2008), for quantification of OD. The percentage of inhibition was calculated using the equation: $1-(At_{595nm}/Ac_{595nm})x100$; where $At_{595nm}$ and $Ac_{595nm}$ are the absorbances of the wells treated with BRP and the control, respectively (Wei et al., 2006).

The microplate designated for CFU counting had the contents of its wells aspirated, and was carefully washed with Milli-Q water to eliminate residual planktonic cells. After this, new broth was added to all wells and the microplate underwent a sonication process so that the adhered cells could be dislodged by vibration. Dilutions ranging from $10^{-1}$ to $10^{-7}$ were performed per well, and 50 µL of each dilution was plated on new Brucella agar containing 5.0% defibrinated horse blood, which was then incubated according to the needs of the bacteria. After incubation, the colonies were counted and the result expressed in $Log_{10}$ CFU/mL. All assays were performed in triplicate and the results were graphically represented.

*Minimum biofilm eradication concentration*

To determine if BRP has the ability to eradicate *H. pylori* biofilm, the minimum biofilm eradication concentration (MBEC) was assessed. This is defined as the lowest concentration of the sample capable of reducing the number of viable cells in the biofilm by at least 99.9% (Polonio, Mermel, Paquette, & Sperry, 2001). Additionally, the biomass was evaluated by obtaining the OD (Sandberg et al., 2008).

The assay to evaluate the eradication of *H. pylori* biofilm by BRP is similar to the one used for assessing biofilm formation inhibition, with the main difference being in the initial steps. Initially, only the bacterial inoculum ($10^9$ CFU/mL) is added to all wells of the microplates. This is then left to form and adhere as a biofilm to the microplate at 37ºC for 72 h in a $CO_2$ incubator. After this period, the contents of the wells are removed, and fresh broth containing varying concentrations of BRP (0.98 to 2000 µg/mL), tetracycline (0.0115 to 5.9 µg/mL) for testing the eradication potential of these substances on the established biofilm, or it contains only the broth (serving as a negative control, with the inoculum only) is carefully added to the wells. The microplates are then reincubated under the same conditions for an additional 72 hours.

After the incubation period, one microplate underwent the process previously described using crystal violet as a dye to obtain the OD (Sandberg et al., 2008). The other microplate underwent the same process, also previously described, to perform the count of CFU/mL. MEBC value was calculated by dividing the count of microorganisms obtained in the treatments by the count obtained in the negative control (inoculum only) and multiplying the result by 100 to obtain the percentage of biofilm eradication (Polonio et al., 2001). All assays were performed in triplicate and the results were graphically expressed.

*Statistical analysis*

The results of the nucleotide leakage assay were presented as means ± standard deviations. The normality of the data was assessed using the Shapiro-Wilk test. Since the data showed a normal distribution, it was then analyzed using one-way analysis of variance (ANOVA). Statistical differences were considered significant at $p < 0.05$.

**Results**

The Time-Kill curve of *H. pylori* (ATCC 43526) is represented in Figure 1. It was determined that after 24 h of exposure of the bacteria to 50 µg/mL of BRP, the CFU count was reduced to zero. When exposed to 25 µg/mL of BRP, even after 72 h, it did not eliminate bacterial growth. Tetracycline at 0.74 µg/mL, used as a positive control, eliminated the growth within 56 h of exposure.

The results obtained in the nucleotide leakage assay were represented in Figure 2 and demonstrate that BRP does not cause damage to the cell membrane of *H. pylori* (ATCC 43526), as no statistical differences were observed between the OD obtained with the treatments of 25 and 50 µg/mL of BRP compared to the OD obtained in the control (bacteria only).

In the assay for the standardization of biofilm formation, it was observed that after 3 days of incubation at an inoculum concentration of $10^9$ CFU/mL (Figure 3), *H. pylori* (ATCC 43526) formed a biofilm in satisfactory quantities for the continuation of the experiments. Therefore, this inoculum concentration and incubation time were selected for the continuation of the experiments on the inhibition of biofilm formation and biofilm eradication.

The BRP was able to inhibit 50% of the biofilm formation of the bacteria at a concentration of 15.6 µg/mL ($MICB_{50}$ value). Additionally, it was observed that at concentrations of 500, 1000, and 2000 µg/mL, there was no cell growth (Figure 4A). Tetracycline achieved the same action at a concentration of 0.0461 µg/mL, but was not able to eliminate viable cells at the evaluated concentrations (Figure 4B).

The data from the eradication assay are represented in Figure 5. It was determined that the MEBC of BRP is 2000 µg/mL. Furthermore, it is noticeable that in addition to eliminating 99.9% of the cells, BRP also achieved lower OD values, which represent the biofilm aggregate, compared to the control (Figure 1A). On the other hand, tetracycline also obtained lower OD values than the control; however, at the evaluated concentrations, it was not possible to determine an MEBC value, as cells were still viable at the highest concentration evaluated.

**Discussion**

To the best of our knowledge, the results obtained in the present study regarding the properties of BRP against the bacteria *H. pylori* are unprecedented in the scientific literature. Although there are some studies that have evaluated the antibacterial properties of propolis samples from different locations (Baltas, Karaoglu, Tarakci, & Kolayli, 2016; Boyanova et al., 2003; Villanueva et al., 2015; Widelski et al., 2022) their antibiofilm property has not been explored.

In previous studies, our research group determined that the minimum bactericidal concentration (MBC) of BRP against *H. pylori* (ATCC 43526) is 50 µg/mL (Santiago et al., 2022). Due to this, this concentration was chosen for evaluating the *H. pylori* time-kill curve, with the BRP concentrations assessed being 50 µg/mL (1x MBC) and 25 µg/mL (1/2 MBC). Tetracycline was also used at the MBC concentration defined by the authors (0.74 µg/mL) (Santiago et al., 2022).

Therefore, our study determined that the time required for BRP at a concentration of 50 µg/mL to completely eliminate *H. pylori* (ATCC 43523) is 24 h, and its sub-bactericidal concentration (25 µg/mL) was not able to eliminate the bacteria even after 72 h of exposure. The antibiotic tetracycline at a concentration of 0.74 µg/mL took 56 h to eliminate the bacteria, which is 32 h more than what was necessary for BRP at 50 µg/mL.

Due to the lack of studies that have charted this time-kill curve profile for propolis samples, it is not possible to discuss the results of the present study with other authors. However, comparing with other natural products, Ye et al. (2015) used *Chenopodium ambrosioides* to determine the time-kill of *H. pylori* (NCTC11637), and complete elimination of the bacteria occurred in 24 h at concentrations of 1x and 2x the determined minimum inhibitory concentration (16000 µg/mL). Taking together the data from the present study and that of the authors (Ye et al., 2015), it can be suggested that natural products may exhibit bactericidal activity against *H. pylori* within the first 24 h of contact.

Some antibacterial agents have the property of causing irreversible damage to the bacterial cytoplasmic membrane. When the leakage of cytoplasmic content is detected, this release of intracellular content is used as an indicator of membrane integrity (Chen & Cooper, 2002). In the present study, no statistical differences were observed in the nucleotide leakage between the control and those treated with BRP at 25 and 50 µg/mL after 24 h of exposure, indicating that the bacterial death caused by BRP does not cause any damage to the membrane of *H. pylori* (ATCC 43526).

Another novel finding in the scientific literature presented by this study is about the antibiofilm property of BRP against *H. pylori* (ATCC 43526). No other studies were found that evaluated the antibiofilm action, even though there have been studies demonstrating the antibacterial potential of propolis in both *in vitro* and *in vivo* assays (Mendonça et al., 2020; Santiago et al., 2022). The biofilm-forming capability of *H. pylori* is one of the mechanisms associated with the development of antibiotic resistance, with the particularity being that the bacteria can change its classic bacillus morphology to coccoid-shaped, thus obtaining a more resistant biofilm (Elshenawi et al., 2023).

In the present study, the *H. pylori* strain (ATCC 43526) proved capable of forming biofilm at a concentration of $10^9$ after 72 h of incubation at 37°C in a $CO_2$ incubator. The biofilm formation inhibition assay determined that BRP can inhibit biofilm growth by 50% at a concentration of 15.6 µg/mL and completely eliminate viable cells at concentrations starting from 500 µg/mL. Due to the complexity of the bacterial community in biofilm, it is estimated that the dose of an antibacterial agent required to effectively eliminate the biofilm may be 10- to 1000-fold higher than the dose needed to eliminate the bacteria in its planktonic form (Fux,

Stoodley, Hall-Stoodley, & Costerton, 2003; Mooney et al., 2018; Richards & Melander, 2009). The current study corroborates this estimate, as the necessary concentration of BRP to completely eliminate the biofilm of *H. pylori* (ATCC 43526) was 500.0 µg/mL, and in other studies a BRP concentration of 50.0 µg/mL was sufficient to eliminate the planktonic cells of the same bacteria.

de Sa Assis et al. (2022) conducted a study evaluating the potential of the aqueous extract of Brazilian green propolis in inhibiting the formation of biofilm by bacteria associated with endodontic infections. The bacteria tested included *Fusobacterium nucleatum* (ATCC 25586), *Parvimonas micra* (ATCC 23195), *Prevotella intermedia* (ATCC 35406), *Porphyromonas gingivalis* (ATCC 33277), and *P. endodontalis* (ATCC 33563). The authors determined that the extract was effective in reducing both the biomass and the cellular viability of the evaluated bacteria at a concentration of 110000.0 µg/mL, with the exception of *P. micra*, for which the most effective concentration for reducing viability was 55000.0 µg/mL (de Sa Assis et al., 2022).

Other researchers, Meccatti et al. (2023), also investigated the inhibitory action on biofilm formation of Brazilian green propolis. They assessed the effectiveness of a combination of hydroethanolic extract of Brazilian green propolis (concentration of 5000.0 µg/mL) with *Cinnamomum verum* (concentration of 2200.0 µg/mL) against various strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The authors found that, in some cases, the combination of these two natural products was statistically more effective in inhibiting biofilm formation than the use of chlorhexidine, which was used as a positive control in the study (Meccatti et al., 2023).

The studies mentioned above by de Sa Assis et al. (2022) and Meccatti et al. (2023), regarding the antibiofilm action of Brazilian green propolis extracts against different bacterial species, combined with the results obtained in the present study on the antibiofilm property of BRP against *H. pylori* (ATCC 43526), highlight the biological potential of these extracts and how Brazilian biodiversity is capable of producing resources with active potential against various pathogenic bacteria.

When it comes to antibiofilm action, there are various opinions about which strategy is more relevant. Wei et al. (2006) argue that preventing biofilm formation is more effective, as it prevents the biofilm from becoming mature. On the other hand, Olson, Ceri, Morck, Buret, and Read (2002) contend that the efficacy of an agent in eliminating mature biofilm increases the success rate in the treatment of clinical diseases. In the present study, BRP showed promise against *H. pylori* (ATCC 43526) in both strategies, as it was capable of completely inhibiting the cellular viability of biofilm formation at a concentration of 500.0 µg/mL, and also of

completely eliminating the viability of mature biofilm in the eradication assay at a concentration of 2000.0 µg/mL.

The study by Meccatti et al. (2023), previously mentioned, which assessed the activity of Brazilian green propolis in combination with *C. verum* extract against the bacteria *A. baumannii* and *P. aeruginosa*, also evaluated the effect of this combination on mature biofilms of the same bacteria. In all the assays conducted, the authors determined that there was a significant reduction in mature biofilm density, when the concentration of green propolis extract in the mixture was 5000.0 µg/mL (Meccatti et al., 2023). In the current study, BRP achieved total eradication of mature *H. pylori* (ATCC 43526) biofilm at a concentration of 2000 µg/mL. Once again, the data obtained here, as well as by other authors (Meccatti et al., 2023), highlight that natural products from Brazilian sources are a source of bioactive compounds of great interest in combating pathogenic bacteria and their mechanisms of action.

Further investigation into the effects of BRP against *H. pylori* is necessary before it can truly become an alternative therapy in combating the bacteria. However, the results presented in this study contribute further to the advancement of this topic and bring unprecedented novelties.

## Conclusion

Based on the data obtained in this study, it can be concluded that BRP has promising antibiofilm activity against *H. pylori* (ATCC 43526) under the evaluated *in vitro* conditions, as it was capable of completely eliminating bacterial cells in both biofilm formation and eradication, and was able to inhibit 50% of biofilm formation. It is also determined that the time required for BRP to eliminate planktonic cells of *H. pylori* is 24 h. Furthermore, it can be suggested that the bacterial death of the planktonic cells does not cause damage to the cytoplasmic membrane, as indicated by the nucleotide leakage assay.

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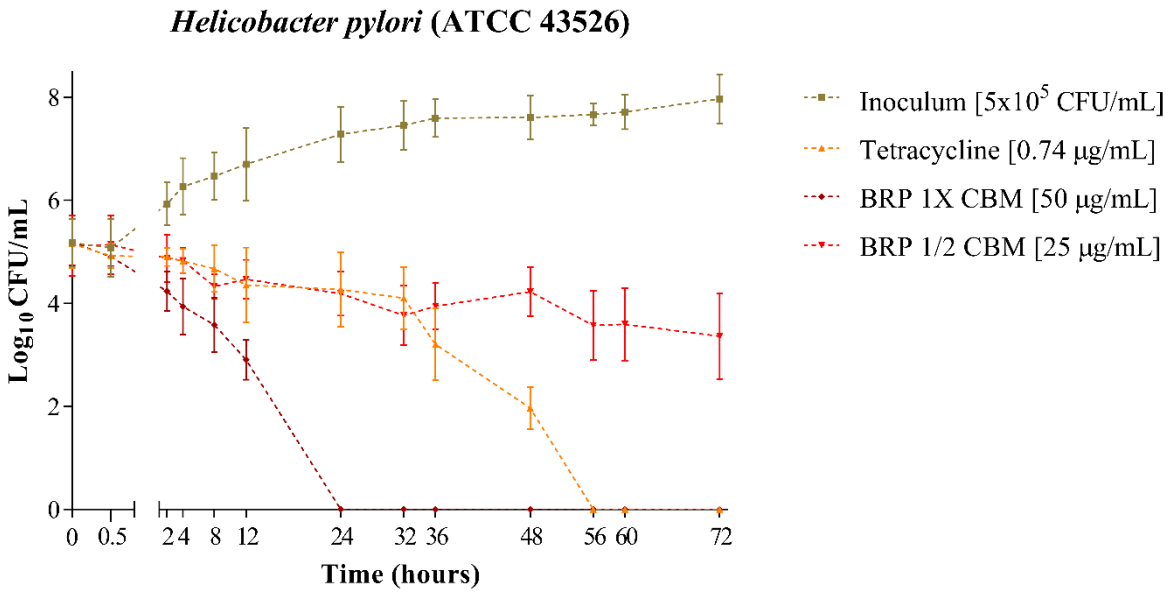



**Figure 1 –** Time-Kill curve of *Helicobacter pylori* (ATCC 43526) obtained after 72h of exposure to the treatments.

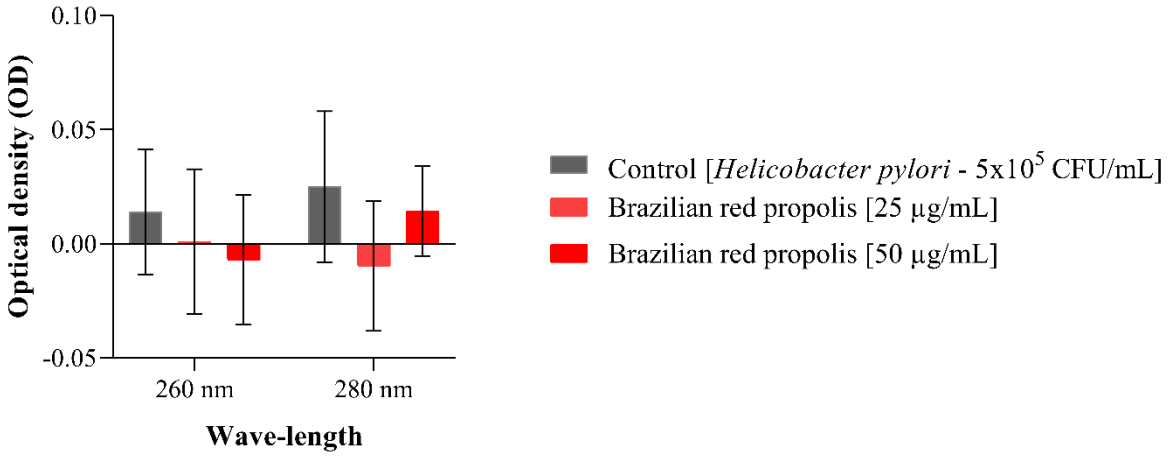



**Figure 2 –** Nucleotide leakage from *Helicobacter pylori* (ATCC 43526) observed after 24 h of exposure to treatments. No statistical differences were found between the treatments and the control.

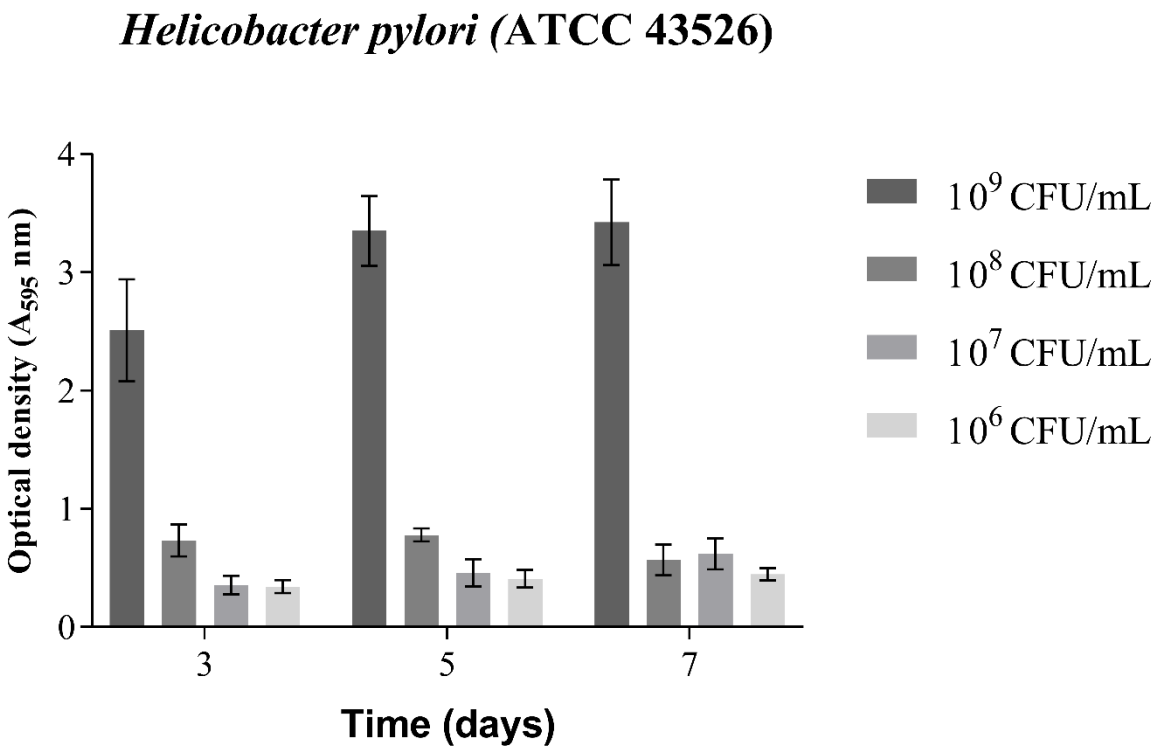



**Figure 3 -** Biofilm formation of *Helicobacter pylori* (ATCC 43526) obtained by optical density (595 nm) after 3, 5 and 7 days of incubation at inoculum concentrations of $10^6$, $10^7$, $10^8$ and $10^9$ CFU/ml.

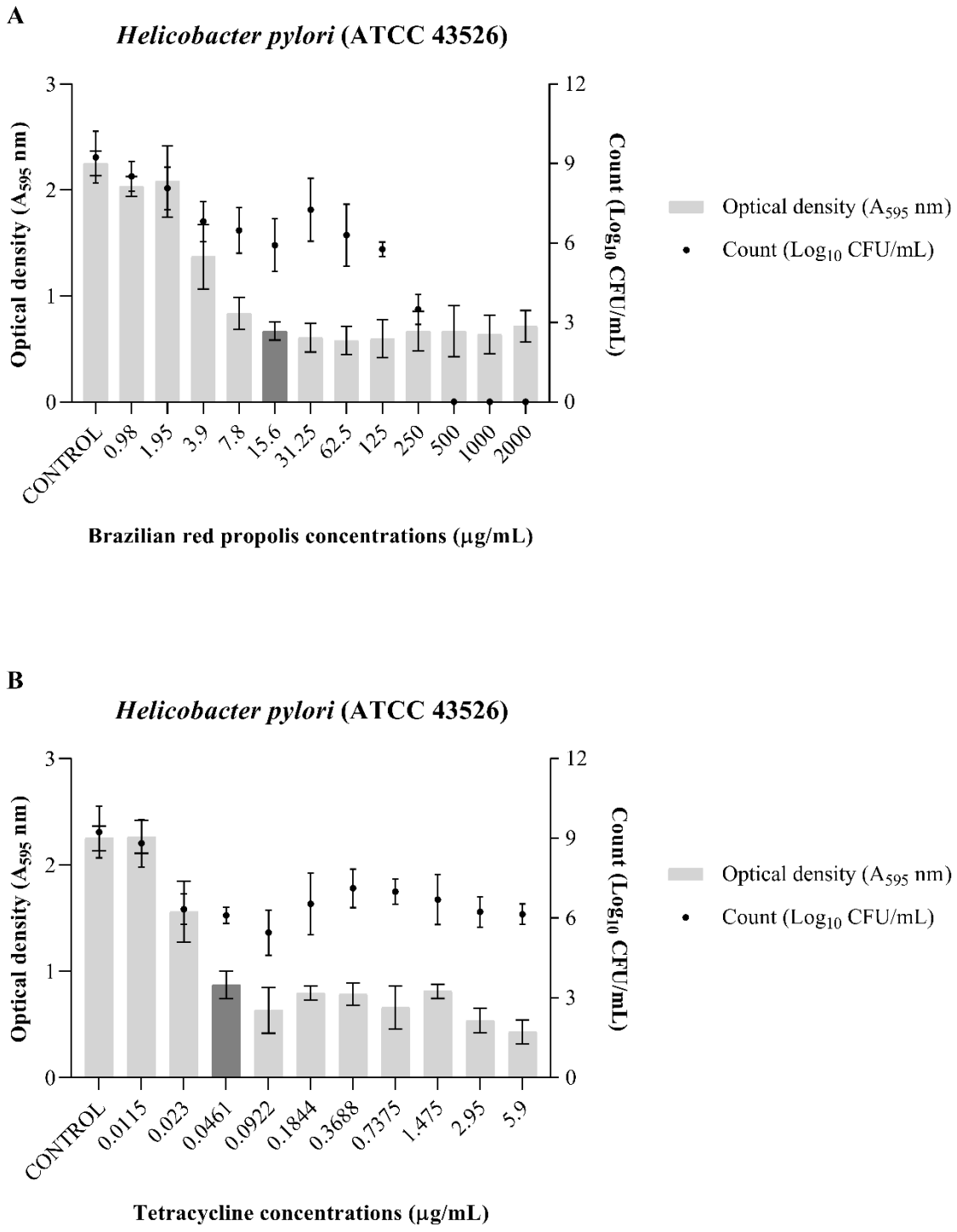



**Figure 4 -** Graphical representation of biofilm formation inhibition of *Helicobacter pylori* (ATCC 43526) as demonstrated by optical density (OD) and numbers of microorganisms ($Log_{10}$ CFU/mL). (A) Brazilian red propolis. (B) Tetracycline. The $MICB_{50}$ value is represented in dark gray.

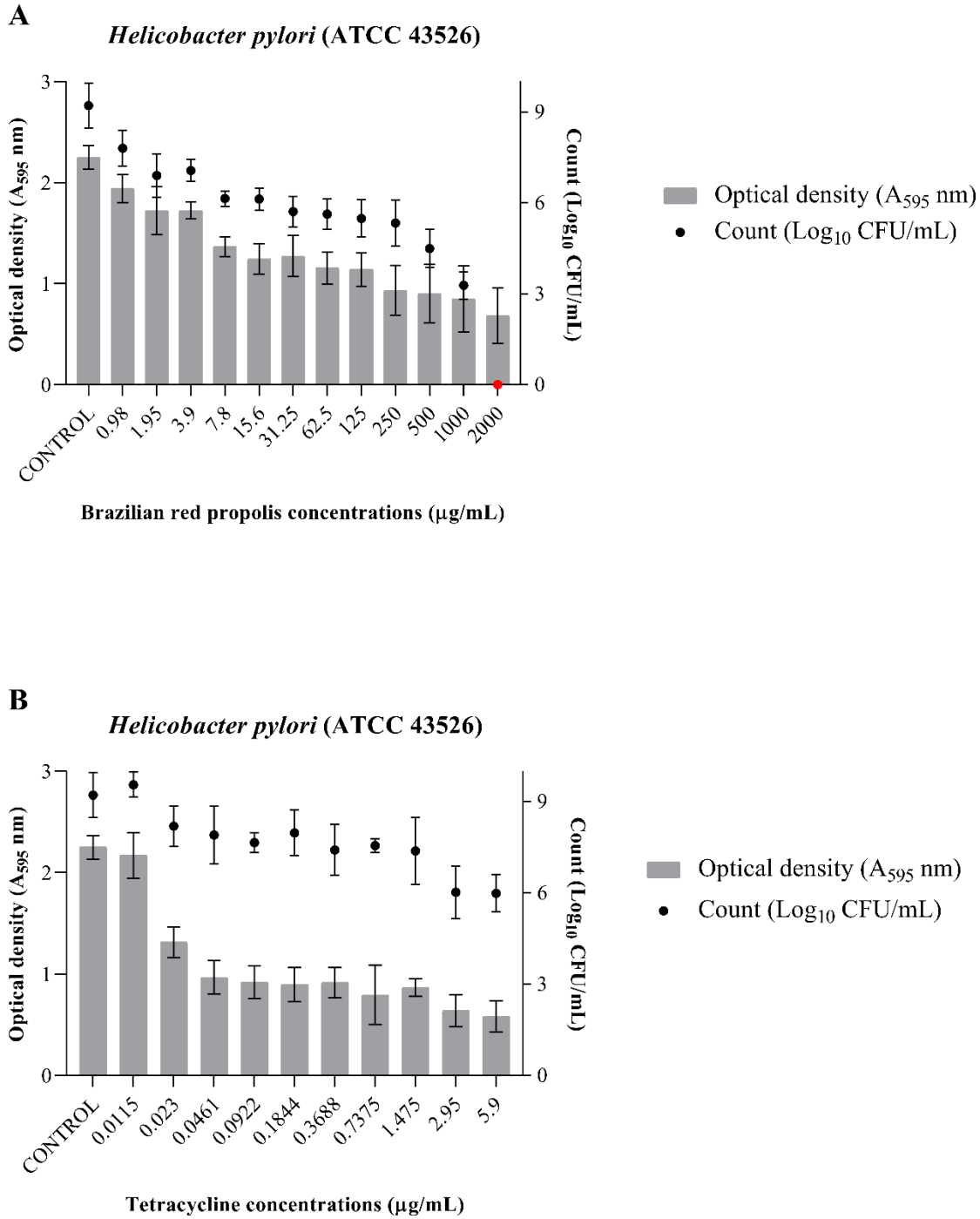



**Figure 5 -** Graphical representation of the eradication of *Helicobacter pylori* (ATCC 43526) biofilm as demonstrated by optical density (OD) and numbers of microorganisms ($Log_{10}$ CFU/mL). (A) Brazilian red propolis. (B) Tetracycline. The MEBC value is represented in red.

# CAPITULO IV

## Considerações Finais

Nas considerações finais deste estudo sobre a atividade anti-*H. pylori* da própolis vermelha, as seguintes conclusões são destacadas:

- Promissora Atividade Anti-*H. pylori in vitro*: Com base nos dados obtidos, pode-se concluir que o extrato hidroalcoólico bruto de própolis vermelha exibe uma atividade anti-*H. pylori* promissora *in vitro*. Sua capacidade bactericida, em concentrações baixas, tanto contra cepas padrão quanto isoladas clinicamente, sem interferir nos efeitos de um antibiótico utilizado na terapia padrão, destaca seu potencial como agente terapêutico contra a infecção por *H. pylori*.

- Promissora Atividade de inibição e erradicação de biofilme: Os ensaios voltados para avaliação da propriedade antibiofilme levam a conclusão de que a própolis vermelha brasileira é eficaz tanto na inibição da formação do biofilme, como na eliminação do biofilme maduro da *H. pylori*.

- Eficácia Anti-*H. pylori in vivo*: Os estudos microbiológicos e histopatológicos realizados demonstram a capacidade do extrato em reduzir a carga bacteriana e modular a resposta imune nos tecidos biológicos de ratos infectados. Esses resultados respaldam a promissora atividade anti-*H. pylori* do extrato em ambientes biológicos mais complexos, sugerindo um potencial valor terapêutico.

Novas perspectivas de agentes antibacterianos surgem a partir das conclusões deste estudo sobre a atividade anti-*H. pylori* da própolis vermelha, apontando para direções futuras promissoras no campo da terapia antibacteriana e da exploração de produtos naturais como fontes de compostos bioativos.

A constatação de que o extrato hidroalcoólico bruto de própolis vermelha exibe uma atividade anti-*H. pylori in vitro* promissora sugere uma potencial alternativa terapêutica contra infecções por *H. pylori*. Esta descoberta pode direcionar pesquisas para o isolamento e identificação de compostos específicos na própolis vermelha responsáveis por essa atividade, abrindo caminho para a formulação de novos agentes antibacterianos baseados em produtos naturais. Além disso, a eficácia anti-*H. pylori* demonstrada *in vivo* através da redução da carga bacteriana e da modulação da resposta imune em tecidos biológicos de ratos infectados aponta para uma potencial aplicação clínica da própolis vermelha como agente terapêutico. Esse resultado encoraja pesquisas clínicas mais aprofundadas para avaliar a segurança e eficácia da própolis vermelha em seres humanos, bem como para entender os mecanismos moleculares

subjacentes a esses efeitos. Esses resultados também podem estimular a busca por outros produtos naturais com atividades similares, explorando a riqueza da biodiversidade para a descoberta de novos agentes terapêuticos.

Portanto, este estudo enfatiza a importância de investigar e explorar produtos naturais como fontes de soluções terapêuticas inovadoras. As perspectivas delineadas pelas descobertas ressaltam a necessidade contínua de investigações interdisciplinares que incorporem a biologia, química e medicina para desvendar os segredos dos compostos bioativos e suas aplicações. À medida que a pesquisa avança, a esperança é que esses estudos não apenas nos levem a abordagens terapêuticas mais eficazes, mas também inspirem um compromisso renovado com a conservação da biodiversidade e a promoção da saúde humana.

# ANEXO

*pharmaceuticals*

MDPI

*Article*

# Polyalthic Acid from *Copaifera lucens* Demonstrates Anticariogenic and Antiparasitic Properties for Safe Use

**Mariana B. Santiago [1], Vinicius Cristian O. dos Santos [1], Samuel C. Teixeira [2], Nagela B. S. Silva [1], Pollyanna F. de Oliveira [3], Saulo D. Ozelin [3], Ricardo A. Furtado [3], Denise C. Tavares [3], Sergio Ricardo Ambrósio [3], Rodrigo Cassio S. Veneziani [3], Eloisa Amália V. Ferro [2], Jairo K. Bastos [4] and Carlos Henrique G. Martins [1,*]**

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**Abstract:** This study aimed at evaluating the potential of *Copaifera lucens*, specifically its oleoresin (CLO), extract (CECL), and the compound *ent*-polyalthic acid (PA), in combating caries and toxoplasmosis, while also assessing its toxicity. The study involved multiple assessments, including determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against cariogenic bacteria. CLO and PA exhibited MIC and MBC values ranging from 25 to 50 µg/mL, whereas CECL showed values equal to or exceeding 400 µg/mL. PA also displayed antibiofilm activity with minimum inhibitory concentration of biofilm ($MICB_{50}$) values spanning from 62.5 to 1000 µg/mL. Moreover, PA effectively hindered the intracellular proliferation of *Toxoplasma gondii* at 64 µg/mL, even after 24 h without treatment. Toxicological evaluations included in vitro tests on V79 cells, where concentrations ranged from 78.1 to 1250 µg/mL of PA reduced colony formation. Additionally, using the *Caenorhabditis elegans* model, the lethal concentration ($LC_{50}$) of PA was determined as 1000 µg/mL after 48 h of incubation. Notably, no significant differences in micronucleus induction and the NDI were observed in cultures treated with 10, 20, or 40 µg/mL of CLO. These findings underscore the safety profile of CLO and PA, highlighting their potential as alternative treatments for caries and toxoplasmosis.

**Keywords:** *Copaifera lucens*; *ent*-polyalthic acid; antibacterial; antibiofilm; antiparasitic; toxicity

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**Citation:** Santiago, M.B.; dos Santos, V.C.O.; Teixeira, S.C.; Silva, N.B.S.; de Oliveira, P.F.; Ozelin, S.D.; Furtado, R.A.; Tavares, D.C.; Ambrósio, S.R.; Veneziani, R.C.S.; et al. Polyalthic Acid from *Copaifera lucens* Demonstrates Anticariogenic and Antiparasitic Properties for Safe Use. *Pharmaceuticals* **2023**, *16*, 1357. https://doi.org/10.3390/ph16101357

Academic Editors: Chung-Yi Chen, Shengbao Cai and Jiangbo He

Received: 16 August 2023
Revised: 6 September 2023
Accepted: 22 September 2023
Published: 26 September 2023

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## 1. Introduction

The oral cavity comprises various microenvironments such as tooth surfaces and mucosal epithelium. In each of these oral cavity sites, one can find about fifty species of microorganism, each of these locations in the oral cavity will be inhabited by different bacterial species, due to the cellular tropism of each microbe, there are around a thousand species that are capable of colonizing and integrating the microbiota of the oral cavity. Changes in the resident microbial community lead to dysbiosis, affecting the composition of the community [1,2].

In the case of dental caries, a diet with excess carbohydrates and sugars promotes the production of extracellular polymeric substances (EPS), the matrix that integrates the

Metabolomics (2023) 19:68
https://doi.org/10.1007/s11306-023-02032-8

ORIGINAL ARTICLE

# Dereplication of *Lantana trifolia* L. leaves and fruits by UFLC-DAD-(+)-ESI-MS/MS and its antifungal and cytotoxic activities

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Received: 9 August 2022 / Accepted: 10 July 2023
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## Abstract

**Introduction** *Lantana trifolia* L. (Verbenaceae) is a shrubby plant. In folk medicine, its leaves are used in the form of infusions and syrups to treat angina, coughs, and colds; they are also applied as tranquilizer. Previous studies have reported the antimicrobial potential of the compounds present in *L. trifolia* leaves.

**Objectives** To report the anti-*Candida* activities of the fractions obtained from the fruits and leaves of two *L. trifolia* specimens.

**Methods** The *L. trifolia* fractions were submitted to UFLC-DAD-(+)-ESI-MS/MS, and the data were analyzed by using multivariate statistical tools (PCA, PLS-DA) and spectral similarity analyses based on molecular networking, which aided dereplication of the bioactive compounds. Additionally, NMR analyses were performed to confirm the chemical structure of some of the major compounds in the fractions.

**Results** The ethyl acetate fractions presented MIC values lower than 100 μg $mL^{-1}$ against the three *Candida* strains evaluated herein (*C. albicans*, *C. tropicalis*, and *C. glabrata*). Fractions FrPo AcOEt, FrPe AcOEt, and FrPe *n*But had MIC values of 1.46, 2.93, and 2.93 μg $mL^{-1}$ against *C. glabrata*, respectively. These values resembled the MIC value of amphotericin B, the positive control (0.5–1.0 μg $mL^{-1}$), against this same strain. Cytotoxicity was measured and used to calculate the selectivity index.

**Conclusion** On the basis of our data, the most active fractions in the antifungal assay were more selective against *C. glabrata* than against non-infected cells. The analytical approach adopted here allowed us to annotate 29 compounds, nine of which were bioactive (PLS-DA results) and belong to the class of phenolic compounds.

**Keywords** *Lantana trifolia* L. · Anti-*Candida* · Natural products · Molecular networking

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Published online: 24 July 2023

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# Potential in vitro anti-periodontopathogenic, anti-*Chikungunya* activities and in vivo toxicity of Brazilian red propolis

Nagela Bernadelli Sousa Silva[1], Jonathan Henrique de Souza[1], Mariana Brentini Santiago[1], Jhennyfer Rodrigues da Silva Aguiar[1], Daniel Oliveira Silva Martins[1,2], Rafael Alves da Silva[3], Igor de Andrade Santos[1], Jennyfer A. Aldana-Mejía[4], Ana Carolina Gomes Jardim[1,2], Reginaldo dos Santos Pedroso[5], Sergio Ricardo Ambrósio[6], Rodrigo Cássio Sola Veneziani[6], Jairo Kenupp Bastos[4], Regina Helena Pires[7] & Carlos Henrique Gomes Martins[1]✉

**Bacterial and viral infections are serious public health issue. Therefore, this study aimed to evaluate the antibacterial, antibiofilm and antiviral potential of the Brazilian Red Propolis (BRP) crude hydroalcoholic extract, fractions, and isolated compounds, as well as their in vivo toxicity. The antibacterial activity was evaluated by determining the Minimum Inhibitory Concentration and the antibiofilm activity by determining the Minimum Inhibitory Concentration of Biofilm ($MICB_{50}$). The viable bacteria count ($Log_{10}$ UFC/mL) was also obtained. The antiviral assays were performed by infecting BHK-21 cells with *Chikungunya* (CHIKV) *nanoluc*. The toxicity of the BRP was evaluated in the *Caenorhabditis elegans* animal model. The MIC values for the crude hydroalcoholic extract sample ranged from 3.12 to 100 µg/mL, while fractions and isolated compounds the MIC values ranged from 1.56 to 400 µg/mL.The BRP crude hydroalcoholic extract, oblongifolin B, and gutiferone E presented $MICB_{50}$ values ranging from 1.56 to 100 µg/mL against monospecies and multispecies biofilms. Neovestitol and vestitol inhibited CHIKV infection by 93.5 and 96.7%, respectively. The tests to evaluate toxicity in *C. elegans* demonstrated that the BRP was not toxic below the concentrations 750 µg/mL. The results constitute an alternative approach for treating various infectious diseases.**

Despite important advances in the health area, infectious diseases have constituted a serious public health issue over time[1]. One example is periodontitis, an inflammatory disease that affects tooth-supporting apparatus and which is caused by microorganisms present in dysbiosis plaque biofilms[2]. According to Mehrotra and Singh[3], about 2.6% of African Americans, 5% of Africans, 0.2% of Asians, 1% of North Americans, and 0.3% of South Americans have been diagnosed with periodontitis in its most severe form. Periodontal treatment is essential not only for dental parameters, but also to avoid other pathological conditions such as adverse reactions in pregnancy, cardiovascular and respiratory diseases, cancer, lupus, rheumatoid arthritis, diabetes mellitus, and chronic kidney disease[4]. Even if the illness can be treated with antibiotics, the infection can be aggravated in patients lacking treatment or in the presence of resistant periodontopathogenic bacteria[5].

Viral diseases also burden the global health system due to lack of vaccines and approved antivirals to combat important human viruses, including the *Chikungunya Fever*, caused by the *Chikungunya* virus (CHIKV)[6]. CHIKV was identified in 2014 and has become hyperendemic in Brazil[7]. This virus causes dengue-like symptoms such

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Inorganica Chimica Acta 549 (2023) 121421

Contents lists available at ScienceDirect

Inorganica Chimica Acta

journal homepage: www.elsevier.com/locate/ica

Research paper

# Crystal design, spectroscopic analyses and antibacterial study of new carbazate ligands and their Cu(II) complexes

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ARTICLE INFO

*Keywords:*
Carbazates
Copper complexes
Crystal structures
Hirshfeld surface
Antibacterial activity

ABSTRACT

The current work reports the synthesis and crystal structures of two carbazate ligands, 2-hydroxyacetophenone-methylcarbazate ($HL^1$) and 2-hydroxyacetophenone-benzylcarbazate ($HL^2$), and their Cu(II) complexes $[Cu(L^1)(CH_3OH)]_2$ (1) and $[Cu(L^2)]_n$ (2). All compounds were characterized by X-ray single-crystal analysis, spectroscopic and physicochemical methods. Intermolecular interactions were analyzed quantitatively with the 3D Hirshfeld surface and the 2D fingerprint plots. The dimeric complex (1) exhibits a $\mu_2$-oxo group located between the two metal ions, an *ONO*-donor system, and a coordinated methanol molecule with a square-pyramidal geometry to the metal ions. The polymeric complex (2) shows the Cu(II) atom tetracoordinate, bonded to two anionic carbazate, through the *ONO*-donor system of one molecule, and to a deprotonated nitrogen atom from the second molecule of the ligand. This one-dimensional network shows the metal center with a square planar geometry. The antibacterial properties of the free ligands and metal complexes have been evaluated against periodontopathogenic bacterial strains and were observed to increase the activity after the complexation of the carbazates.

## 1. Introduction

Studies of Schiff bases transition metal complexes are in constant evolution due to the several possibilities of applications. The preparation of the metal complexes is via the addition of the precursor metal salt and the Schiff base in appropriate and suitable experimental conditions. The Schiff bases are an important class of organic compounds widely studied due to their biological and pharmaceutical properties, such as antimicrobial, antifungal, and antimalarial activity.[1–5].

The carbazates are Schiff bases that have the potential to coordinate and form stable transition metal complexes with different geometries. They are obtained through a condensation reaction between an aldehyde or ketone and a primary amine. Generally, the donor atoms can coordinate with several different metal ions. There are many reported studies in the literature with similar Schiff bases, such as hydrazones, semicarbazones, dithiocabazates and their Ni(II), Cu(II), Co(II), Zn(II) complexes.[6–8] However, carbazate ligands and their metal complexes are not yet expansively explored. There are very few recent papers in the literature on this type of ligand.[9,10]

There is great interest in carbazate and its complexes due to its pharmacological and biological applications. These compounds also show cytotoxic potential against different cancer cells and are successful when tested against Gram-positive and Gram-negative bacteria.[1–3].

Anaerobic bacteria such as *Porphyromonas gingivalis*, *Actinomyces naeslundii*, and *Fusobacterium nuclatum* are part of the oral microbiota, which can be permanent or resident in the oral environment. When dysbiosis occurs in this system, bacteria proliferate and can cause infections, the most commonly associated with these bacteria being gingivitis, periodontitis, and endodontic infection. However, oral microorganisms are also associated with other chronic diseases e.g. inflammatory bowel disease, cancers, cardiovascular diseases, Alzheimer's disease, diabetes, rheumatoid arthritis, and preterm birth.[11].

In this sense, this work reports the study of new carbazate ligands and their dimeric and polymeric Cu(II) complexes. The compounds were analyzed by single crystal X-ray diffraction, FT-IR, UV–vis, NMR, Mass

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https://doi.org/10.1016/j.ica.2023.121421
Received 26 October 2022; Received in revised form 10 January 2023; Accepted 31 January 2023
Available online 2 February 2023
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*antibiotics*

MDPI

*Article*

# Physicochemical Profile, Antioxidant and Antimicrobial Activities of Honeys Produced in Minas Gerais (Brazil)

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**Abstract:** Honeys can be classified as polyfloral or monofloral and have been extensively studied due to an increased interest in their consumption. There is concern with the correct identification of their flowering, the use of analyses that guarantee their physicochemical quality and the quantification of some compounds such as phenolics, to determine their antioxidant and antimicrobial action. This study aims at botanical identification, physicochemical analyses, and the determination of total polyphenols, chromatographic profile and antiradical and antimicrobial activity of honey from different regions of Minas Gerais. Seven different samples were analyzed for the presence of pollen, and color determination. The physicochemical analyses performed were total acidity, moisture, HMF, reducing sugar, and apparent sucrose. The compound profile was determined by UHPLC/MS, the determination of total phenolics and antiradical activity (DPPH method) were performed by spectrophotometry, and minimum inhibitory and bacterial concentrations were determined for cariogenic bacteria. All honey samples met the quality standards required by international legislation, twenty compounds were detected as the main ones, the polyfloral honey was the only honey that inhibited all of the bacteria tested. Sample M6 (Coffee) was the one with the highest amount of total polyphenols, while the lowest was M4 (Cipó-uva). Regarding the antioxidant activity, M5 (Velame) had the best result and M4 (Cipó-uva) was the one that least inhibited oxidation. Of the polyfloral honeys, there was not as high a concentration of phenolic compounds as in the others. Coffee, Aroeira, Velame and Polyfloral have the best anti-radical actions. Betônica, Aroeira, Cipó-uva and Pequi inhibited only some bacteria. The best bacterial inhibition results are from Polyfloral.

**Keywords:** bee's honey; phenolic compounds; pollen

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**Citation:** Royo, V.d.A.; Oliveira, D.A.d.; Veloso, P.H.F.; Sacramento, V.d.M.; Olimpio, E.L.A.; Souza, L.F.d.; Pires, N.d.C.; Martins, C.H.G.; Santiago, M.B.; Alves, T.M.d.A.; et al. Physicochemical Profile, Antioxidant and Antimicrobial Activities of Honeys Produced in Minas Gerais (Brazil). *Antibiotics* **2022**, *11*, 1429. https://doi.org/10.3390/antibiotics11101429

Academic Editors: Malgorzata Dzugan and Miroslava Kačániová

Received: 1 October 2022
Accepted: 14 October 2022
Published: 18 October 2022

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## 1. Introduction

Honey is a viscous and aromatic product made by bees from the nectar of flowers or honeydew. A natural sweetener, it has a complex composition of carbohydrates and other substances such as organic acids, amino acids, proteins, minerals, vitamins, lipids, aromatic compounds, flavonoids, pigments, waxes, pollen grains, various enzymes and other phytochemicals [1,2]. The presence of phenolic compounds in a composition is related to its antioxidant capacity, in addition to the antibacterial activity and stimulating effect on the multiplication of probiotic bacteria [3–7].

*Orbital: Electron. J. Chem.* **2022**, 14(3), 176-181

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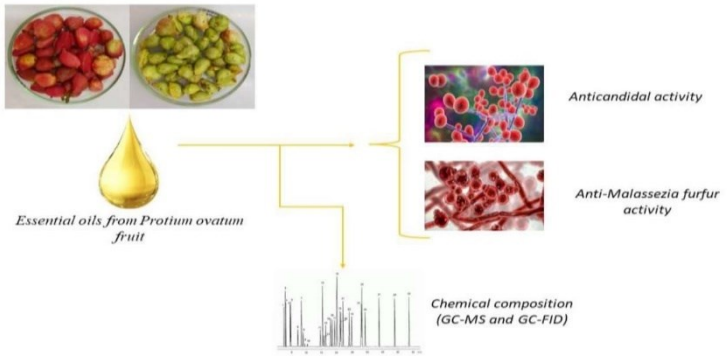
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# Antifungal Activities of Essential Oils from *Protium ovatum* Engl. Against *Malassezia furfur* and *Candida* Species

Gardênia Barbosa Carrijo Rodrigues [a], Cassia Cristina Fernandes [a], Suzana Maria Loures de Oliveira Marcionilio [a], Vitor de Paula Castro [b], Carmen Magaly Alvarez [b], Regina Helena Pires [b], Mariana Brentini Santiago [c], Carlos Henrique Gomes Martins [c], Reginaldo dos Santos Pedroso [c], and Mayker Lazaro Dantas Miranda* [d]

Fungal opportunistic infections have increased in recent decades due to the increase in the immunosuppressed patients and the indiscriminate use of antifungals. In Brazil, a country with the greatest biodiversity in the world, studies that seek new antifungals from natural sources have been stimulated. *Protium ovatum* Engl., belongs to the Burseraceae family and is a shrub tree found mainly in Brazil, in the Cerrado biome, and has medicinal, food and aromatic uses. This study aims to investigate the chemical composition and the anti-*Malassezia furfur* and anticandidal activities of essential oils (EOs) from *Protium ovatum* ripe fruit (RF-EO) and unripe fruit (UF-EO). The EOs antifungal activities were determined by microdilution broth methodology. GC-FID and GC-MS analyses showed that limonene, α-pinene and myrcene were the major components of both EOs. MIC values of RF-EO and UF-EO against *M. furfur* were 375 and 1500 µg/mL, respectively. RF-EO exhibited MIC values between 62.5 and 250 µg/mL while UF-EO was slightly active (> 1000 µg/mL) against *Candida* species. In addition, RF-EO showed antibiofilm activity against *Candida* species and was not toxic to *C. elegans* larvae. This study suggests that EOs from *P. ovatum* could be an alternative therapeutic option for infectious diseases caused by *M. furfur* and *Candida* species.

## Graphical abstract




## Keywords

Opportunistic infections
Plant products
Essential oils
Antifungal activity
Cerrado biome

## Article history

**Received** 25 Aug 2022
**Revised** 06 Oct 2022
**Accepted** 06 Oct 2022
**Available online** 06 Oct 2022

**Handling Editor:** Adilson Beatriz

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**An Acad Bras Cienc (2022) 94(Suppl. 4): e20220150 DOI 10.1590/0001-3765202220220150**
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

# Detection of Waterborne and Airborne Microorganisms in a Rodent Facility

LUIZ MARCIO DA SILVA, MARIANA B. SANTIAGO, PAULA AUGUSTA D.F. DE AGUIAR, SALVADOR B. RAMOS, MURILO V. DA SILVA & CARLOS HENRIQUE G. MARTINS

**Abstract:** This study aimed to evaluate the air and water contamination level and to identify the microbes isolated from a rodent facility located at the Federal University of Uberlândia, Minas Gerais, Brazil. Colony forming units (CFU) per milliliter was used for monitoring water quantitatively; CFU per cubic meter was used for air monitoring. The isolated colonies were identified for qualitative monitoring. Due to absence of specific parameters for these facilities, the results were analyzed according to Brazilian and international standards, depending on which best suited each sample. The mean total number of microorganisms in water ranged from 0.015 ± 0.02 to 0.999 ± 0.91 CFU/mL. The number of microorganisms in air ranged from 9.1 ± 4.6 to 351.56 ± 158.2 CFU/m³. Forty-one microorganisms identified in the samples obtained from the rodent facility were potentially pathogenic or opportunistic for animals and humans (e.g., *Corynebacterium* spp.). We concluded that the water and air samples were contaminated with potentially pathogenic or opportunistic microorganisms that can harm rodents and humans. On the basis of our observations, specific sanitary standards suitable for these facilities should be developed for controlling microbial contamination, which will prevent zoonosis and ensure the reliability of scientific results obtained from animal experiments.

**Key words:** waterborne, airborne, rodent facility, fungi, bacteria, microbial.

## INTRODUCTION

Biological contamination in facilities used for housing animals intended for scientific research prevents good laboratory quality from being achieved and maintained (Majerowicz 2019). Constantly surveilling the presence of microbes in these environments is required given that microorganisms can cause serious diseases in humans and animals and may negatively affect the quality of research conducted with these animals (Felasa 2014, Majerowicz 2019). Therefore, Brazilian animal facilities are designed to comply with regulations, legislation, and standards created to meet the needs of animal and scientific research (Politi et al. 2008, Brasil 2008).

Quality control programs aimed at maintaining animal health have been designed (Felasa 2014). However, the animal facility environment must also comply with quality control standards because water and air are sources of microbial contamination (Cincinelli & Martellini 2017, Dawson & Sartory 2000, Kauffmann-Lacroix et al. 2016, Rao et al. 1996, Strickland & Shi 2021, Zhou et al. 2020). Although animal facilities are known to be sources of contamination, the Brazilian legislation does not define specific standards for their environmental quality control.

Indoor air contamination occurs via bioaerosols, which, in most cases, are generated in the external environment and carried inside

Archives of Oral Biology 143 (2022) 105520

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

# Antibacterial activity of Brazilian red propolis and in vitro evaluation of free radical production

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ARTICLE INFO

*Keywords:*
Antibacterial agent
Cytotoxicity
Free radical
Propolis

ABSTRACT

*Objective:* This study aimed to evaluate the antibacterial activity of crude Brazilian red propolis (BRP) extract against anaerobic bacteria involved in primary endodontic infection. Additionally, we evaluate the cell viability and free radical production of human dental pulp fibroblasts (HDPF) in direct contact with mineral trioxide aggregate (MTA) and BRP.

*Design:* The Minimum Inhibitory Concentration, Minimum Bactericidal Concentration (MIC, MBC) and Minimum Inhibitory Concentration of Biofilm ($MICB_{50}$) of BRP against anaerobic endodontic pathogens were determined. HDPF were exposed to BRP10 (10 μg/mL), BRP50 (50 μg/mL), MTA extract (1:1, 1:2, 1:4 e 1:8), dimethyl sulfoxide 0.5% (DMSO), and cell culture medium (DMEM). The groups were tested for cell viability (MTT assay), and free radical production (reactive oxygen species - ROS, DCFH-DA probe and nitric oxide – NO, Griess reagent). The one-way ANOVA and Tukey's tests were employed at a significance level of 5%.

*Results:* MIC/MBC values of BRP performed antibacterial activity for *Parvimonas micra* (6.25/6.25 μg/mL), *Fusobacterium nucleatum* (25/25 μg/mL), *Prevotella melaninogenica* (50/100 μg/mL), *Prevotella nigrescens* (50/100 μg/mL), *Prevotella intermedia* (50/100 μg/mL), and *Porphyromonas gingivalis* (50/200 μg/mL). The $MICB_{50}$ values ranged from 1.56 to 50 μg/mL. BRP and MTA stimulated cell viability, emphasizing BRP10 ($p = 0.007$). Furthermore, it was observed that MTA 1:1, MTA 1:2, and BRP50 slightly increased ROS ($p < 0.001$) and NO production ($p = 0.008$, $p = 0.007$, and $p < 0.001$ respectively) compared to DMEM group.

*Conclusions:* BRP exhibits good antibacterial activity against endodontic pathogens, and both BRP and MTA promote the viability of HDPF without increasing NO and ROS production.

*Abbreviations:* BRP, Brazilian red propolis; MTA, mineral trioxide aggregate; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; HDPFs, Human dental pulp fibroblasts; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; NO, nitric oxide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate fluorescent probe; DMEM, Dulbecco's modified Eagle medium; VPT, Vital pulp therapy; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6 (IL-6); FBS, fetal bovine serum; MTT, methyl-thiazol-tetrazolium.

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https://doi.org/10.1016/j.archoralbio.2022.105520
Received 10 March 2022; Received in revised form 7 August 2022; Accepted 8 August 2022
Available online 13 August 2022
0003-9969/© 2022 Elsevier Ltd. All rights reserved.

Research, Society and Development, v. 11, n. 7, e40111730059, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i7.30059

# Antifungal and antioxidant activities and chemical constituents from *Pluchea sagittalis*

**Atividades antifúngica e antioxidante e constituintes químicos de *Pluchea sagittalis***

**Actividades antifúngicas y antioxidantes y constituyentes químicos de *Pluchea sagittalis***

Received: 05/06/2022 | Reviewed: 05/17/2022 | Accept: 05/24/2022 | Published: 05/29/2022

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**Abstract**

*Pluchea sagittalis* (Asteraceae) is widely distributed in South America and used as a medicinal plant to treat various diseases. The objectives of the work were to evaluate the antioxidant and antifungal activities of the ethanol extract and fractions from the aerial parts of *P. sagittalis*, as well as to identify the chemical constituents of the most active sample by liquid chromatography coupled to mass spectrometry with electrospray ionization (LC-MS-ESI). The ethanolic extract obtained by maceration of aerial parts showed relevant activity in DPPH free radical scavenging ($IC_{50}$ = 33.9 µg $mL^{-1}$) and presented an anti-*Candida* effect with MIC values of 93.75 µg $mL^{-1}$ for *C. albicans* and 187

Polyhedron 225 (2022) 116073

Contents lists available at ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

# Antibacterial and antifungal activities *in vitro* of a novel silver(I) complex with sulfadoxine-salicylaldehyde Schiff base

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ARTICLE INFO

*Keywords:*
Silver(I)
Sulfadoxine
Schiff base
DFT studies
Antimicrobial activity

ABSTRACT

Skin wound infections can be considered one of the most common causes of death in burned patients. The raising reports of multidrug resistant microorganisms reveals a complicated condition for the treatment of burn wound injuries, establishing urgency in the development of novel antimicrobial agents. Considering this scenario, the present work describes the synthesis, antibacterial and antifungal activities of a sulfadoxine-salicylaldehyde Schiff base (SFX-SL), and of its novel dimeric silver(I) complex (AgSFX-SL). Elemental analysis indicated a 1:1 metal:ligand molar composition for the AgSFX-SL complex, with the minimum formula $AgC_{19}H_{17}N_4O_5S$. Mass spectrometric (HRMS) data reveal the existence of dimeric species of the complex in solution. Infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopic studies suggest coordination of the Schiff base to silver(I) by the nitrogen and oxygen atoms of the sulfonamide group. The structure of the SFX-SL Schiff base was determined by single crystal X-ray diffraction. Molecular modeling confirms the proposition of a dimeric structure for the AgSFX-SL complex as early indicated by mass spectrometric data. The AgSFX-SL complex showed an effective antimicrobial activity over pathogens including Gram-positive *Staphylococcus aureus* (multidrug resistant), *S. epidermidis*, *Cutibacterium acnes*, and Gram-negative *Pseudomonas aeruginosa* and *Burkholderia cepacia*, with minimum inhibitory concentration (MIC) values in the range 12.0–48.0 $\mu mol \cdot L^{-1}$. Additionally, the AgSFX-SL complex was active against *Candida albicans* with a MIC value of 2.8 $\mu mol \cdot L^{-1}$. Electrophoresis assays showed that the AgSFX-SL complex does not interact with protein models lysozyme and bovine serum albumin (BSA).

## 1. Introduction

Burn wounds in their diverse forms are characterized by the accumulation of injured tissues and cells, becoming a potential target for infections by pathogenic microorganisms such as Gram-negative *Pseudomonas aeruginosa* and Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strains, and fungi [1]. These infections can complicate the treatment process and, consequently, the recovery of affected individuals, increasing time, cost of treatment and mortality [2]. The World Health Organization (WHO) estimates >180,000 deaths annually due to burns, establishing itself as a global public health issue.

With the increasing reports of resistant microorganisms, this scenario could be aggravated, since the development of new antimicrobial agents is much slower than the emergence of new resistant microorganisms [3]. In this case, metallodrugs have demonstrated potential as novel antimicrobial agents exhibiting a multitarget mode of action on different cellular structures. The metal-based agents act by promoting membrane disruption, formation of reactive oxygen species (ROS), denaturation of essential proteins and enzymes, and interruption on cell division process. This set of possible cellular targets can overcome the resistance mechanisms in emerging microorganisms and thus promote patient recovery [4,5].

One of the strategies for metallodrug design is based on the combination of bioactive molecules with well-established activities, such as

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https://doi.org/10.1016/j.poly.2022.116073
Received 8 May 2022; Accepted 27 July 2022
Available online 2 August 2022
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*pharmaceuticals*

MDPI

*Article*

# Anticariogenic Activity of Three Essential Oils from Brazilian Piperaceae

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**Abstract:** The current trend toward using natural food additives, cosmetics, and medicines has motivated industries to substitute synthetic compounds for natural products. Essential oils (EOs) from medicinal plants are a well-known source of chemical compounds that display several interesting biological activities, including antimicrobial action. In this study, we investigated the antibacterial activity of EOs extracted from three Piperaceae species collected in the Brazilian Amazon region against a representative panel of cariogenic bacteria. The minimum inhibitory concentration (MIC) of the essential oils extracted from *Peperomia pellucida* (PP-EO), *Piper marginatum* (PM-EO), and *Piper callosum* (PC-EO) was determined against *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. salivarius*, *S. sobrinus*, *Enterococcus faecalis*, and *Lactobacillus casei* by using the microplate microdilution method. PM-EO, PC-EO, and PP-EO displayed antibacterial activity against all the tested cariogenic bacteria. PM-EO displayed the best inhibitory activity, with MIC values ranging from 50 to 500 μg/mL. The lowest MIC values were obtained for PM-EO against *S. mitis* (MIC = 75 μg/mL), *Lactobacillus casei* (MIC = 50 μg/mL), and *S. mutans* (MIC = 50 μg/mL). Gas chromatography mass spectrometry (GC-MS) analysis allowed the chemical composition of all the EOs to be identified. The main constituents of PM-EO, PC-EO, and PP-EO were 3,4-(methylenedioxy)propiophenone, α-pinene, and dillapiole, respectively. Finally, the compounds that were exclusively detected in PM-EO are highlighted. Our results suggest that PM-EO may be used in products for treating dental caries and periodontal diseases.

**Keywords:** antibacterial activity; oral pathogens; *Piper callosum*; *Piper marginatum*; *Peperomia pellucida*

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**Citation:** Carvalho, Ê.S.; Ayres, V.F.S.; Oliveira, M.R.; Corrêa, G.M.; Takeara, R.; Guimarães, A.C.; Santiago, M.B.; Oliveira, T.A.S.; Martins, C.H.G.; Crotti, A.E.M.; et al. Anticariogenic Activity of Three Essential Oils from Brazilian Piperaceae. *Pharmaceuticals* **2022**, *15*, 972. https://doi.org/10.3390/ph15080972

Academic Editor: Michael Danilenko

Received: 30 June 2022
Accepted: 3 August 2022
Published: 6 August 2022

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## 1. Introduction

The current trend toward consuming minimally processed products has encouraged the development of alternative natural additives that can perform the same functions as synthetic additives. In this scenario, essential oils (EOs) extracted from medicinal plants have been screened for their safe and eco-friendly applications in the pharmaceutical, cosmetic, and food industries [1]. EOs are mixtures of volatile compounds produced by the specialized metabolism of plants to carry out survival functions [2]. Interesting biological properties, such as antimicrobial, antioxidant, anti-inflammatory, and anticancer activities, have been attributed to the complex chemical constitution of EOs [3]. Terpenoids, especially monoterpenes and sesquiterpenes, are the main chemical constituents of EOs and have been proven to play a key role in inhibiting pathogens [4]. Specifically, monoterpenoids affect

Anaerobe 76 (2022) 102588

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

# Chalcones with potential antibacterial and antibiofilm activities against periodontopathogenic bacteria

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ARTICLE INFO

*Article history:*
Received 13 September 2021
Received in revised form
2 May 2022
Accepted 19 May 2022
Available online 24 May 2022

*Keywords:*
Chalcone
Anaerobic bacteria
Periodontitis
Antibacterial activity
Antibiofilm activity

ABSTRACT

*Objectives:* Periodontitis is a pathology resulting from complex interaction of microorganisms in the dental biofilm with the host's immune system. Increased use of antibiotics associated with their inappropriate use has increased resistance levels in anaerobic bacteria. Therefore, identifying new antimicrobial compounds, such as chalcones, is urgent. This study evaluates the antibacterial activity and the antibiofilm activity of 15 chalcones against the periodontopathogenic bacteria *Prevotella nigrescens* (ATCC 33563), *P. oralis* (ATCC 33269), *Peptostreptococcus anaerobius* (ATCC 27337), *Actinomyces viscosus* (ATCC 43146), *Porphyromonas asaccharolytica* (ATCC 25260), and *Fusobacterium nucleatum* (ATCC 25586).
*Methods:* The compounds were evaluated by minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) tests.
*Results:* Compounds **1**–**6** showed good antibacterial and antibiofilm activities against most of the evaluated bacteria: MIC was lower than or equal to 6.25 μg/mL, biofilm biomass was reduced by 95%, and the compounds at concentrations between 0.78 and 100 μg/mL totally inhibited cell viability. Among the tested chalcones, **3** stood out: it was effective against all the bacteria, as revealed by the MIC and MBIC results.
*Conclusions:* Our results have consolidated a base for the development of new studies on the effects of the tested chalcones as agents to combat and to prevent periodontitis.
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## 1. Introduction

The oral microbiota represents an important portion of the human microbiota: it contains over >750 bacterial species. Microbial films are usually formed on the tooth surface, and some bacteria in these structures may cause dental complications, including endodontic cavities and periodontal diseases [1].

Periodontitis is an oral disease that affects the periodontal ligament and the alveolar bone. It results from a complex interaction between microorganisms in the dental biofilm (e.g., *Prevotella melaninogenica*, *Fusobacterium nucleatum*, and *P. nigrescens*) and the host's immune system [2,3]. Several bacteria may colonize the periodontal area; i.e., the periodontal pockets, and once the bacterial biofilm is formed, it produces virulence factors, such as enzymes and toxins, which assist in evasion of the immune system and allow periodontitis to develop [4]. The biofilm is composed of an extracellular matrix that confers resistance to antimicrobials and helps to evade the immune system. The extracellular matrix promotes biofilm persistence in chronic diseases such as periodontitis [4].

If left untreated, periodontitis may lead to tooth loss, greatly impacting the patient's quality of life [5]. In most cases, mechanical biofilm debridement, antiseptic rinses, and surgery are used to treat periodontal diseases. Some cases also require the use of systemic

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https://doi.org/10.1016/j.anaerobe.2022.102588
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**Brazilian Journal of Biology**
ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)
https://doi.org/10.1590/1519-6984.230533
Original Article

# Chemical composition and biological activities of essential oil from flowers of *Psidium guajava* (Myrtaceae)

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Received: October 29, 2019 – Accepted: January 30, 2020 – Distributed: August 31, 2021
(With 1 figure)

## Abstract

*Xylella fastidiosa* is a plant-pathogenic bacterium that lives inside host xylem vessels, where it forms biofilm which is believed to be responsible for disrupting the passage of water and nutrients. *Pectobacterium carotovorum* is a Gram-negative plant-specific bacterium that causes not only soft rot in various plant hosts, but also blackleg in potato by plant cell wall degradation. Chagas disease, which is caused by *Trypanosoma cruzi*, has been commonly treated with nifurtimox and benzonidazole, two drugs that cause several side effects. As a result, the use of natural products for treating bacterial and neglected diseases has increased in recent years and plants have become a promising alternative to developing new medicines. Therefore, this study aimed to determine, for the first time, the chemical composition of essential oil from *Psidium guajava* flowers (PG-EO) and to evaluate its *in vitro* anti-*Xylella fastidiosa*, anti-*Pectobacterium carotovorum*, anti-*Trypanosoma cruzi* and cytotoxic activities. PG-EO was obtained by hydrodistillation in a Clevenger apparatus while its chemical composition was determined by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Major compounds identified in PG-EO were α-cadinol (37.8%), β-caryophyllene (12.2%), nerolidol (9.1%), α-selinene (8.8%), β-selinene (7.4%) and caryophyllene oxide (7.2%). Results showed that the PG-EO had strong trypanocidal activity against the trypomastigote forms of *Trypanosoma cruzi* ($IC_{50}$ = 14.6 µg/mL), promising antibacterial activity against *X. fastidiosa* (MIC = 12.5 µg/mL) and *P. carotovorum* (MIC = 62.5 µg/mL), and moderate cytotoxicity against $LLCMK_2$ adherent epithelial cells in the concentration range ($CC_{50}$ = 250.5 µg/mL). In short, the PG-EO can be considered a new source of bioactive compounds for the development of pesticides and trypanocide drugs.

*Keywords:* Neglected tropical diseases; bactericide efficacy; guava; α-cadinol; $LLCMK_2$.

# Composição química e atividades biológicas do óleo essencial das flores de *Psidium guajava* (Myrtaceae)

## Resumo

*Xylella fastidiosa* é uma bactéria patogênica que vive dentro dos vasos do xilema hospedeiro, onde forma um biofilme responsável por interromper a passagem de água e nutrientes. *Pectobacterium carotovorum* é uma bactéria Gram-negativa que causa não só podridão macia em várias plantas hospedeiras, mas também canela-preta na batata por degradação da parede celular da planta. A doença de Chagas, causada pelo *Trypanosoma cruzi*, é comumente tratada com nifurtimox e benzonidazol, duas drogas que causam vários efeitos colaterais. Como resultado, o uso de produtos naturais para o tratamento de doenças bacterianas e negligenciadas aumentou nos últimos anos e as plantas continuam sendo uma alternativa promissora para o desenvolvimento de novos medicamentos. Portanto, este estudo teve como objetivo determinar, pela primeira vez, a composição química do óleo essencial de flores de *Psidium guajava* (PG-EO) e avaliar suas propriedades anti-*Xylella fastidiosa*, anti-*Pectobacterium carotovorum*, anti-*Trypanosoma cruzi* e citotóxica *in vitro*. PG-EO foi obtido por hidrodestilação em um aparelho Clevenger, enquanto sua composição química foi determinada por cromatografia em fase gasosa com detecção por ionização por chama (CG-DIC) e por

Journal of *Fungi*

MDPI

*Article*

# Identification of Substances Produced by *Cercospora brachiata* in Absence of Light and Evaluation of Antibacterial Activity

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**Abstract:** Cercospora *brachiata* is a phytopathogenic fungus. To know more about the metabolites produced by this fungus, the objective of this work was to identify, isolate and characterize substances present in extracts of the growth broth and mycelium, using gas chromatography with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). It was also objective to evaluate the antibacterial activity of the extracts. Among the compounds identified, fatty acids, esters, and steroids can be highlighted. The main compounds identified are 9-hexadecenoic, hexadecenoic, oleic, octadecanoic, lauric, myristic, palmitic, doceno-13-enoic, stearic, linoleic, and nonadecanoic acids present in almost all extracts. For the antibacterial activity, the broth microdilution method was used. The ethyl acetate extract of the mycelium presented inhibitory concentrations (MICs) against the bacterium *Actinomyces naeslundii* (100 μg $mL^{-1}$) and *Streptococcus sanguinis* (200 μg $mL^{-1}$). Finally, two steroids were isolated and identified in the hexane extract of mycelium: ergosta-6,22-dien-3β,5α,8α-triol and brassicasterol.

**Keywords:** *Cercospora brachiata*; secondary metabolites; antibacterial activity; fungus; oral bacteria

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**Citation:** Felisbino, J.K.R.P.; Vieira, B.S.; de Oliveira, A.; da Silva, N.A.; Martins, C.H.G.; Santiago, M.B.; Munoz, R.A.A.; Cunha, L.C.S.; Sousa, R.M.F. Identification of Substances Produced by *Cercospora brachiata* in Absence of Light and Evaluation of Antibacterial Activity. *J. Fungi* **2021**, *7*, 680. https://doi.org/10.3390/jof7090680

Academic Editors: Jasmina Glamočlija and Dejan Stojković

Received: 31 July 2021
Accepted: 19 August 2021
Published: 24 August 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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## 1. Introduction

Fungi can be classified according to the type of host. Fungi that use host plants are called endophytic plants which do not cause plant diseases, while phytopathogens cause many types of diseases in theirs hosts [1], which is the case of some *Cercospora* species [2].

Phytopathogenic fungi can produce toxic substances. The toxins, therefore, are secondary metabolites produced by fungi, usually of low molecular mass (<1000 Da), that may be related to the infection process in their hosts [3].

Some fungi are also harmful to plants for being necrotrophic causing plant cells death during plant tissue colonization. One of the genera of necrothophic fungi is *Cercospora*, which produces toxins such as cercosporin involved in the pathogenicity of these fungi, that can be activated by light [4,5], causing lesions in leaves, stems, and fruits. One of the plants parasitized by one species of *Cercospora*, known as *Cercospora coffeicola*, is coffee, whose productivity can be reduced by up to 30% for this pathogen [6,7].

Fungi may also produce compounds capable of preventing the growth of bacteria. This activity is associated with the fact that fungi produce secondary metabolites capable of inhibiting the action of bacteria and even of other fungi, as in the case of extracts from cultures of *Cercospora* spp. isolates [8]. Bacteria used to test the antibacterial activity herein

Journal of Molecular Structure 1246 (2021) 131261

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

# Synthesis, spectroscopic characterization and *in vitro* antibacterial and antiviral activities of novel silver(I) complexes with mafenide and ethyl-mafenide

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ARTICLE INFO

*Article history:*
Received 19 June 2021
Revised 1 August 2021
Accepted 5 August 2021
Available online 8 August 2021

*Keywords:*
Silver(I)
Mafenide
Ethyl-mafenide
DFT studies
Antibacterial activity
Antiviral agents

ABSTRACT

Two novel silver(I) complexes with the antibacterial sulfa drugs mafenide (Maf) and ethyl-mafenide (eMaf) are presented in this article. Elemental analysis indicated the 1:1 metal:ligand compositions $AgC_7H_9N_2O_2S$ for Ag-Maf and $AgC_8H_{11}N_2O_2S$ for Ag-eMaf. Infrared (IR), and $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectroscopic analyses indicated coordination of the sulfa drugs to silver(I) by the nitrogen and oxygen atoms of the deprotonated sulfonamide ($SO_2NH$) group in a bidentate chelate mode. The proposition of the coordination mode of the sulfa drugs to silver(I) was also supported by a combination of experimental and theoretical (DFT) data. The Ag-Maf and Ag-eMaf complexes exhibited antibacterial activity over *Staphylococcus aureus*, *Burkholderia cepacia*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* aerobic bacteria, with MIC (minimum inhibitory concentration) in the range 21.3–170 μmol $L^{-1}$, being comparable to the commercial drug silver-sulfadiazine (SSD). Antiviral studies against Chikungunya virus (CHIKV) showed that the complexes exhibit anti-CHIKV activity at non-cytotoxic concentrations to the host cells. Biophysical studies based on gel electrophoresis indicated that the complexes do not interact with albumin and lysozyme, which suggests that proteins are not the main targets of the compounds.

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Dedicatory: This work is dedicated to the memory of Professor Oswaldo Luiz Alves (1947-2021) from the Institute of Chemistry of the University of Campinas-UNICAMP, Brazil.

- New silver(I) complexes with mafenide and ethyl mafenide are presented.
- Spectroscopic studies suggest bidentate coordination of the ligands to silver.
- Theoretical data confirms ligand coordination to silver by nitrogen and oxygen atoms.
- The complexes exhibit antiviral activity over Chikungunya virus.
- The complexes were active over aerobic bacteria at concentrations 21.3–170 μmol $L^{-1}$.

## 1. Introduction

Infectious diseases (ID) (re)emergence in the last years has become a case of concern to public health systems around the globe [1]. From diseases caused by bacteria to those from viral, fungal and parasite origin, ID account annually for about 4.3 million deaths worldwide [2].

Bacterial infections, for instance, are prone to be the main cause of deaths in the next years, with current antibiotics estimated to lose their effectiveness due to the emergence of resistant strains, indicating the imperativeness of the development of

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https://doi.org/10.1016/j.molstruc.2021.131261
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Natural Product Research
Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

# Qualitative analysis of the acetogenins from *Annona coriacea* (Annonaceae) leaves by HPLC-Q-Orbitrap and their antibacterial potential against oral pathogens

Ana Paula Terezan , João Gabriel M. Junqueira , Vinícius G. Wakui , Lucilia Kato , Cecília Maria A. Oliveira , Carlos Henrique G. Martins , Mariana B. Santiago & Vanessa Gisele P. Severino

**To cite this article:** Ana Paula Terezan , João Gabriel M. Junqueira , Vinícius G. Wakui , Lucilia Kato , Cecília Maria A. Oliveira , Carlos Henrique G. Martins , Mariana B. Santiago & Vanessa Gisele P. Severino (2020): Qualitative analysis of the acetogenins from *Annona coriacea* (Annonaceae) leaves by HPLC-Q-Orbitrap and their antibacterial potential against oral pathogens, Natural Product Research, DOI: 10.1080/14786419.2020.1803312

**To link to this article:** https://doi.org/10.1080/14786419.2020.1803312

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Published online: 10 Aug 2020.

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Revista Brasileira Multidisciplinar
ISSN 1415-3580
e-ISSN 2527-2675
http://revistarebram.com/index.php/revistauniara

# ROTAVIRUS AND THE EMERGENCE OF NEW GENOTYPES: A NARRATIVE REVIEW

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**Palavras-chave**

Rotavírus
Genótipos
Vacinação
Diarréia

**Keywords**
Rotavirus
Genotypes
Vaccination
Diarrhea

**Resumo**

A infecção por rotavírus, diretamente relacionada à qualidade de vida da população, pode culminar na morte, principalmente de crianças. Esta revisão discute a prevalência e distribuição de genótipos de rotavírus, com foco na variação genotípica do vírus após a implementação de vacinas e a infecção cruzada entre espécies animais e humanas. Foi pesquisada a literatura publicada de janeiro de 2006 a julho de 2017, utilizando o banco de dados Web of Knowledge e os termos de pesquisa "rotavirus", "genotype", "prevalence post vaccine", e "emerging genotypes". Observou-se que os genótipos predominantes mudaram em todos os continentes e que alguns genótipos ainda estão emergindo. Duas hipóteses para essa mudança global são a variabilidade genética do vírus e o surgimento de genótipos resistentes a vacinas. Além disso, o vírus pode facilmente infectar várias espécies de animais que não o ser humano, como evidenciado por relatos de infecção cruzada de cepas, que serviram de alerta para a geração de novos genótipos de vírus. As ações intersetoriais que abrangem não apenas o setor da saúde, mas também todo o setor socioeconômico, incluindo o governo, pesquisadores, professores, agentes de saúde e comunidades, contribuem para diminuir os gastos relacionados à saúde e reduzir a mortalidade causada pelo rotavírus, melhorando assim indicadores de saúde e promoção da saúde em todo o mundo.

**Rotavirus and the emergence of new genotypes: a narrative review**

Rotavirus infection, which is directly related to the population's quality of life, can culminate in death, mainly of children. This review discusses the prevalence and distribution of rotavirus genotypes, focusing on the genotypic variation of the virus after vaccines were implemented and cross-infection between animal and human species took place. We conducted a search of the literature from January 2006 to July 2017 by using the Web of Knowledge database and the search terms "rotavirus", "genotype", "prevalence post vaccine", and "emerging genotypes". The predominant genotypes changed in all the continents, and some genotypes are still emerging. There are two hypotheses for this global change: the genetics of the virus is variable, and vaccine-resistant genotypes have emerged. In addition, the virus can easily infect several animal species other than humans, as evidenced by reports of cross-infection of strains, which have served as a warning that new virus genotypes have been generated. Inter-sectoral actions that encompass not only the health sector, but also all the socio-economic sector including the government, researchers, teachers, health agents, and communities have contributed to reduce the health-related costs and mortality due to rotavirus infection, thereby improving health indicators and promoting health worldwide.

**Recebido em:** 09/07/2020
**Aprovação final em:** 25/08/2020
**DOI:** https://doi.org/10.25061/2527-2675/ReBraM/2020.v23i3.840

RBCTI
Revista Brasileira de Ciência, Tecnologia e Inovação

DOI: https://doi.org/10.18554/rbcti.v5i2.4359

Artigo

# Chemical profile of the twigs of *Ozoroa obovata* by HPLC-MS-ESI and antimicrobial activity

*Perfil químico dos galhos de Ozoroa obovata por CLAE-EM-IES e atividade antimicrobiana*

Domingos A. João[1,7]; Tiara C. Silva[1]; Diego G. Prado[1]; Carlos H. G. Martins[2]; Mariana B. Santiago[3]; Luiz R. Goulart[4]; Mário M. Martins[4]; Francisco J. T. Aquino[5]; Alberto de Oliveira[5]; Raquel M. F. Sousa[5]; Luís C. S. Cunha[6]; Sérgio A. L. Morais[5]

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**ABSTRACT:** In Mozambique, a large part of the population depends on plants for the treatment of various diseases. However, some of them have been little studied in relation to chemical and biological aspects. Among these species, *Ozoroa obovata* is widely used in traditional medicine in Mozambique. The factors that influence the use of medicinal plants in developing countries are mainly cultural habits, the inefficiency of the health system and the high cost of medicines. This work aimed to study the chemical composition and evaluate the antimicrobial activity of the twigs of *O. obovata*. Through the analysis by liquid chromatography coupled to mass spectrometry with electrospray ionization (HPLC-MS-ESI), it was possible to propose that in the ethanolic extract of the twigs, most of the constituents belong to the classes of phenolic acids and flavonoid. The phenolic compounds were represented by quinic, gallic and protocatechuic acids and five anacardic acids, whereas flavonoids were represented by mangiferin, taxifolin and quercetin mono and dihexoside. The extract was evaluated against bacteria in the oral cavity and showed moderate activity against *Streptococcus mutans*, *S. mitis* and *Porphyromonas gingivalis* (minimum inhibitory concentration - MIC 400 µg $mL^{-1}$). In relation antifungal test, the extract showed activity against *Candida albicans* with MIC of 3000 µg $mL^{-1}$. The biological results indicated that the twigs of *O. obovata* have bioactive metabolites with antimicrobial potential.
**Keywords:** antimicrobial activity, phenolics, flavonoids, *Ozoroa obovata*.

***RESUMO:*** *Em Moçambique, grande parte da população depende das plantas para o tratamento de várias doenças. No entanto, algumas delas foram pouco estudadas com relação aos aspectos químicos e biológicos. Dentre essas espécies, Ozoroa obovata é amplamente utilizada na medicina tradicional de Moçambique. Os fatores que influenciam o uso de plantas medicinais nos países em desenvolvimento são principalmente os hábitos culturais, a ineficácia do sistema de saúde e o elevado custo dos medicamentos. Este trabalho teve como objetivo estudar a composição química e avaliar a atividade antimicrobiana dos galhos de O. obovata. A partir da análise por cromatografia líquida acoplada a espectrometria de massas com ionização por electrospray (CLAE-EM-IES) foi possível propor que no extrato etanólico dos galhos, a maioria dos constituintes pertence as classes dos ácidos fenólicos e flavonoides. Os compostos fenólicos foram representados pelos ácidos quínico, gálico, protocatecuico e cinco ácidos anacárdicos, enquanto que os flavonoides foram representados por mangiferina, taxifolina e quercetina mono e dihexosídeo. O extrato foi avaliado frente as bactérias da cavidade bucal e mostrou moderada atividade contra Streptococcus mutans, S. mitis e Porphyromonas gingivalis (concentração inibitória mínima - CIM 400 µg $mL^{-1}$). Com relação ao ensaio antifúngico, o extrato etanólico apresentou atividade contra Candida albicans com CIM de 3000 µg $mL^{-1}$. Os resultados biológicos indicaram que os galhos de O. obovata possuem metabólitos bioativos com potencial antimicrobiano.*
***Palavras chave:*** *atividade antimicrobiana, fenólicos, flavonoides, Ozoroa obovata.*

International Journal of
*Molecular Sciences*

MDPI

*Article*

# Fragmentation Study, Dual Anti-Bactericidal and Anti-Viral Effects and Molecular Docking of Cobalt(III) Complexes

**Laísa de P. Fernandes [1], Júlia M. B. Silva [2], Daniel O. S. Martins [2], Mariana B. Santiago [2], Carlos H. G. Martins [2], Ana C. G. Jardim [2], Guedmiller S. Oliveira [1], Marcos Pivatto [1], Rafael A. C. Souza [1], Eduardo de F. Franca [3], Victor M. Deflon [4], Antonio E. H. Machado [5,6] and Carolina G. Oliveira [1,*]**

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Received: 21 October 2020; Accepted: 3 November 2020; Published: 7 November 2020




**Abstract:** Considering our previous findings on the remarkable activity exhibited by cobalt(III) with 2-acetylpyridine-*N(4)*-R-thiosemicarbazone (Hatc-R) compounds against *Mycobacterium tuberculosis*, the present study aimed to explored new structure features of the complexes of the type $[Co(atc–R)_2]Cl$, where R = methyl (Me, **1**) or phenyl (Ph, **2**) ($^{13}C$ NMR, high-resolution mass spectrometry, LC–MS/MS, fragmentation study) together with its antibacterial and antiviral biological activities. The minimal inhibitory and minimal bactericidal concentrations (MIC and MBC) were determined, as well as the antiviral potential of the complexes on chikungunya virus (CHIKV) infection in vitro and cell viability. $[Co(atc\text{-}Ph)_2]Cl$ revealed promising MIC and MBC values which ranged from 0.39 to 0.78 μg/mL in two strains tested and presented high potential against CHIKV by reducing viral replication by up to 80%. The results showed that the biological activity is strongly influenced by the peripheral substituent groups at the *N*(4) position of the atc-$R^{1-}$ ligands. In addition, molecular docking analysis was performed. The relative binding energy of the docked compound with five bacteria strains was found in the range of −3.45 and −9.55 kcal/mol. Thus, this work highlights the good potential of cobalt(III) complexes and provide support for future studies on this molecule aiming at its antibacterial and antiviral therapeutic application.

**Keywords:** cobalt(III) complexes; biological application; mass spectrometry; molecular docking

## 1. Introduction

The clinical achievement of inorganic drugs, such as cisplatin and its derivates chemotherapeutics agents, has significantly improved the study of metal ions on the biological system for the treatment

Article

BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902019000418371

# Chemical composition and *in vitro* antibacterial activity of essential oils from *Murraya paniculata* (L.) Jack (Rutaceae) ripe and unripe fruits against bacterial genera *Mycobacterium* and *Streptococcus*

**Flávia Fernanda Alves da Silva[1], Cassia Cristina Fernandes[1], Mariana Brentini Santiago[2], Carlos Henrique Gomes Martins[2], Tatiana Manzini Vieira[3], Antônio Eduardo Miller Crotti[3], Mayker Lazaro Dantas Miranda[4*]**

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This study aims to investigate chemical composition of essential oils from *Murraya paniculata* (L.) Jack (Rutaceae) ripe and unripe fruits and determine their *in vitro* antibacterial activity. Essential oils were extracted by hydrodistillation from *Murraya paniculata* (L.) Jack ripe and unripe fruits collected in the *Cerrado*, in Rio Verde, southwestern Goiás, Brazil. They were analyzed by gas chromatography with flame ionization detector (GC-FID) and by gas chromatography-mass spectrometry (GC-MS). Sesquiterpenes, which represent the most abundant class of compounds in oils, predominated in both ripe and unripe fruits. Major constituents of essential oils extracted from ripe fruits (RF-EO) were β-caryophyllene (21.3%), α-ylangene (13.3%), germacrene-D (10.9%) and α-zingiberene (9.7%) whereas the ones of unripe fruits (UF-EO) were sesquithujene (25.0%), α-zingiberene (18.2%), germacrene-D (13.1%) and α-copaene (12.7%). *In vitro* antibacterial activity of essential oils was evaluated in terms of its minimum inhibitory concentration (MIC) values by the broth microdilution method in 96-well microplates. Both essential oils under investigation showed moderate anti-streptococcal activity against the following bacteria: *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. sobrinus* and *S. salivarius*. MIC values ranged between 100 and 400 µg/mL. Regarding the antimycobacterial activity, essential oils from *M. paniculata* (L.) Jack unripe and ripe fruits were active against *Mycobacterium kansasii* (MIC = 250 µg/mL), moderately active against *M. tuberculosis* (MIC = 500 µg/mL) and inactive against *M. avium* (MIC = 2000 µg/mL). This study was pioneer in revealing similar chemical profiles of both essential oils extracted from *Murraya paniculata* (L.) Jack unripe and ripe fruits, besides describing their *in vitro* anti-streptococcal and antimycobacterial activities.

**Keywords:** *Murraya paniculata* (L.) Jack. Rutaceae. Anti-streptococcal activity. *Mycobacterium kansasii*. Antibacterial agent. Antimycobacterial activity.

## INTRODUCTION

Chemical studies of essential oils have been widely carried out due to their several biological applications (Properzi *et al.*, 2013). Therefore, this study aims to evaluate antibacterial activity of essential oils from

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Phytochemistry Letters 40 (2020) 109–120

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

# Antifungal and cytotoxicity activities and new proanthocyanidins isolated from the barks of *Inga laurina* (Sw.) Willd

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ARTICLE INFO

*Keywords:*
*Inga laurina*
Proanthocyanidins
Antifungal activity
Cytotoxicity
Total phenolics

ABSTRACT

The *Inga* genus comprises approximately 300 species that can be found from Mexico to northern Argentina. In folk medicine, *Inga* species are used to treat various diseases. The species *Inga laurina* is widely found in the Brazilian flora; however, there are few studies about its biological activity and chemical composition. The main purpose of this study was to identify and isolate the chemical constituents of *Inga laurina* barks and to evaluate the antifungal and cytotoxic activities. The total content of phenolics, proanthocyanidins, and flavonoids from the barks of *Inga laurina* were performed by spectrophotometric methods and the ethyl acetate (EAF) and *n*-butanol (BF) fractions showed the best results. Eleven compounds were identified in EAF by HPLC-ESI(−)-MS/MS, which showed good antifungal activity with MIC values of 23.4 and 46.8 μg $mL^{-1}$, evaluated by broth microdilution method. Five new compounds of the genus *Inga* were isolated for the first time. Three of these compounds were isolated and reported on the literature for the first time: a proanthocyanidin B-type, gallocatechin-(4α→8)-4'-*O*-methylgallocatechin (**XI**) and two proanthocyanidins A-type, gallocatechin-(2→*O*→7,4→8)-4'-*O*-methylgallocatechin (**XII**) and gallocatechin-3-*O*-galloyl-(2→*O*→7,4→8)-4'-*O*-methylgallocatechin (**XIII**). The chemical study of the plant bark showed that this species is rich in phenolic compounds and it has great potential for the discovery of new bioactive compounds.

## 1. Introduction

The Fabaceae family, to which the *Inga* genus belongs, is the third largest family of Angiosperms, behind Asteraceae and Orchidaceae. The plants of this family are widely distributed in Brazil and can be found in several states, such as Amazonas, Ceará, Goiás, Minas Gerais and Paraná. This family has 222 genus and 2807 species present in the country and 15 genus and 1508 species are endemic (Pereira et al., 2019). Among the several species found in the Cerrado, the genus *Inga* belongs to this family and has several species that present biological activities. Some species of *Inga* have already been studied and the chemical and biological review show that this genus is promising in the search for new active substances for drug manufacturing (Álvarez et al., 1998; Dias et al., 2010; Lokvam et al., 2006; Pinto et al., 2010; Pistelli et al., 2009; Silva et al., 2007; Souza et al., 2007; Vivot et al., 2001).

In previous studies with *Inga* genus, the phenolic compounds were the main substances identified. In the leaves of *I. umbellifera*, flavonoids and proanthocyanidins derived from catechin were isolated (Lokvam et al., 2004). From *I. goldmani*, a trimeric proanthocyanidin and *trans*-4-methoxypipecolic acid was isolated from *I. paterno* (Lokvam and Kursar, 2005; Morton et al., 1991). From the leaves and barks of *I. edulis* metabolites derived from catechin, myricetin, quercetin, and terpenes derived from stigmasterol were isolated (Tchuenmogne et al., 2013).

The phenolic compounds are a large class of metabolites found in plants and they have a broad structural diversity. More than 8000 chemical structures are known, from simple compounds like phenolic acids to more complex compounds like tannins. They are involved in plant defense mechanism, protecting them from UV radiation and

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https://doi.org/10.1016/j.phytol.2020.10.001
Received 29 May 2020; Received in revised form 18 August 2020; Accepted 2 October 2020
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Biomedicine & Pharmacotherapy 129 (2020) 110467

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

# Assessment of the antibacterial, antivirulence, and action mechanism of *Copaifera pubiflora* oleoresin and isolated compounds against oral bacteria

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ARTICLE INFO

*Keywords:*
*Copaifera pubiflora*
Dental caries
Endodontic infection
Antibacterial activity
Acid resistance
Cell membrane integrity

ABSTRACT

The microorganisms that constitute the oral microbiome can cause oral diseases, including dental caries and endodontic infections. The use of natural products could help to overcome bacterial resistance to the antimicrobials that are currently employed in clinical therapy. This study assessed the antimicrobial activity of the *Copaifera pubiflora* oleoresin and of the compounds isolated from this resin against oral bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays provided values ranging from 6.25 to > 400 μg/mL for the *C. pubiflora* oleoresin and its isolated compounds. The fractional inhibitory concentration index (FICI) assay showed that the oleoresin and chlorhexidine did not act synergistically. All the tested bacterial strains formed biofilms. $MICB_{50}$ determination revealed inhibitory action: values varied from 3.12–25 μg/mL for the oleoresin, and from 0.78 to 25 μg/mL for the *ent*-hardwickiic acid. Concerning biofilm eradication, the *C. pubiflora* oleoresin and hardwickiic acid eradicated 99.9 % of some bacterial biofilms. Acid resistance determination showed that *S. mutans* was resistant to acid in the presence of the oleoresin and *ent*-hardwickiic acid at pH 4.0, 4.5, and 5.0 at all the tested concentrations. Analysis of DNA/RNA and protein release by the cell membrane demonstrated that the oleoresin and hardwiickic acid damaged the bacterial membrane irreversibly, which affected membrane integrity. Therefore, the *C. pubiflora* oleoresin and *ent*-hardwickiic acid have potential antibacterial effect and can be used as new therapeutic alternatives to treat oral diseases such as dental caries and endodontic infections.

## 1. Introduction

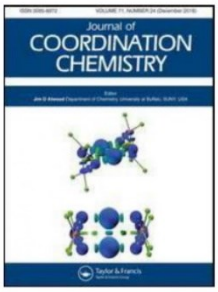
Dental caries, endodontic infections, and periodontitis are the most common oral infectious diseases [1,2].

*S. mutans* is one of the main etiological agents of human dental caries, and it has important virulence attributes: it produces large quantities of organic acids (acidogenicity) from metabolized carbohydrates, it survives at low pH (aciduricity), and it synthesizes extracellular glucan-homopolymers from sucrose. These homopolymers play a critical role in initial biofilm attachment, colonization, and accumulation on tooth surfaces [3,4]. *S. mutans* is a Gram-positive bacterium that colonizes the supragingival plaque in humans. It is the main bacterial species associated with the early stage of bacterial biofilm formation [5].

From a microbiological viewpoint, carious lesions result from an ecological imbalance imposed by frequent carbohydrate consumption [6] and have recently been defined as a polymicrobial dysbiosis [2]. Dental caries is related to increased frequency of dietary sugar intake. Sugars are rapidly metabolized, consequently acidifying the local environment. The low pH generated within the biofilm promotes acidogenic and aciduric bacterium growth and predominance [7]. These bacteria include *Streptococcus mutans* with tolerance to low pH [8]. A decrease in the pH of dental plaque to a critical level (to 5.5) culminates in an imbalance between enamel remineralization and

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https://doi.org/10.1016/j.biopha.2020.110467
Received 10 April 2020; Received in revised form 19 June 2020; Accepted 24 June 2020
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Journal of Coordination Chemistry

ISSN: 0095-8972 (Print) 1029-0389 (Online) Journal homepage: https://www.tandfonline.com/loi/gcoo20

# Transition metal complexes with 2-acetylpyridine-ethylcarbazate: noncovalent interactions in their structures and antimicrobial studies

Claudia C. Gatto, Eduardo de A. Duarte, Gabriela S. Liarte, Thayná S. Silva, Mariana B. Santiago & Carlos H. G. Martins

**To cite this article:** Claudia C. Gatto, Eduardo de A. Duarte, Gabriela S. Liarte, Thayná S. Silva, Mariana B. Santiago & Carlos H. G. Martins (2020): Transition metal complexes with 2-acetylpyridine-ethylcarbazate: noncovalent interactions in their structures and antimicrobial studies, Journal of Coordination Chemistry, DOI: 10.1080/00958972.2020.1777408

**To link to this article:** https://doi.org/10.1080/00958972.2020.1777408

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Inorganica Chimica Acta 508 (2020) 119632

Contents lists available at ScienceDirect

Inorganica Chimica Acta

journal homepage: www.elsevier.com/locate/ica

Research paper

# Copper(II) and zinc(II) complexes with Hydrazone: Synthesis, crystal structure, Hirshfeld surface and antibacterial activity

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ARTICLE INFO

*Keywords:*
Cu(II) and Zn(II) complexes
Hydrazone
Crystal structure
Antibacterial activity

ABSTRACT

The present study reports the synthesis and characterization of six Cu(II) and Zn(II) complexes with 2-cetylpyridinenicotinichydrazone (HL). The characterization of the complexes were applied by conductivity measurements and spectroscopic techniques (FT-IR, UV–Vis, ESI(+)-MS and NMR $^1$H). Four complexes have been studied by single crystal X-ray diffraction, $[Cu(L)_2]$ (1), $[Zn(L)_2]$ (2), $[CuCl_2(HL)]$ (3) and $[CuBr_2(HL)]$ (4). Important interactions upon the molecular packing were also performed by the analysis of their Hirshfeld surfaces and compared to the 2D-fingerprint plots. The characterizations indicates the formation of mononuclear Cu (II) and Zn(II) complexes with the hydrazone ligand coordinated to the metal ions in tridentate mode through the *NNO* chelating system. The antibacterial activity of HL and its metal complexes was tested against cariogenic bacteria strains.

## 1. Introduction

When it comes to clinical relevance for microbiology, the oral cavity is one of the most important habitats. The oral microbiota is colonized by various microorganisms including bacteria, fungi and viroses. Among these microorganisms that make up the microbiota, bacteria stand out due to their ability to cause oral diseases such as caries and endodontic infections [1]. Dental caries is one of the most prevalent infectious diseases and still a major health problem worldwide [2]. Therefore, one of the challenges of science is to develop new drugs that are effective and safe for clinical therapy [3].

Hydrazones are organic compounds characterized by the presence of the $R_2C = NNHR$ group, which possess applications in organic synthesis, analytical chemistry and medicine [4–6]. These compounds can have different heteroatoms in its structure, which enables the use of hydrazones in coordination chemistry as multidentated ligands to the synthesis of metal complexes with pharmacological and catalytic properties [7–13].

Copper and zinc are known to be present in live organisms as trace metals, responsible for important biological processes, such as oxidoreduction and electron transfer reactions, oxygen transport, acid-base processes, etc. [14–16]. Actually, metal complexes based on hydrazones ligands attracted scientific interest due their broad pharmacological profile, with antitumor, antimicrobial and antitubercular potential [17–21]. In previous work Cindric et al. demonstrated that the complexation of copper(II) ion to benzhydrazone's ligands improved the cytotoxic activity of the free ligands against THP-1 and HepG2 cells lines [22]. Rmanovic et al. reported previously two zinc(II) complexes with hydrazone ligand based on Girard's T reagent, which showed antimicrobial potential against different microorganisms strains [23].

In this work, we describe the synthesis and characterization of six copper(II) and zinc(II) complexes based on 2-acetylpyridinenicotinichydrazone ligand (HL), which were characterized by FT-IR, electronic spectroscopy, mass spectrometry, $^1$H NMR and CHN elemental analysis. The crystal structure of four complexes was elucidated by single crystal X-ray diffraction and analyzed by Hirshfeld Surface. Furthermore, the antibacterial potential of the synthesized compounds was evaluated against bacteria present in the oral cavity.

## 2. Experimental section

### 2.1. Materials, methods and instruments

The solvents and reagents were purchased from commercial sources and used without purification. The molar conductivities were measured in an ION DDS 12DW microprocessor conductimetric cell with platinum electrode, using $1x10^{-3}$ M solutions of the complexes in DMSO at room temperature. The FT-IR spectra of the compounds were obtained from KBr pellets, using a FT-IR Varian 640 spectrometer in the range of 4000–400 $cm^{-1}$. Electrospray ionization mass spectrometry analysis

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https://doi.org/10.1016/j.ica.2020.119632
Received 27 February 2020; Received in revised form 25 March 2020; Accepted 26 March 2020
Available online 29 March 2020
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Australian Journal of Crop Science AJCS

AJCS 14(02):236-243 (2020) ISSN:1835-2707
doi: 10.21475/ajcs.20.14.02.p1876

# *In vitro* evaluation of anticaries, antimycobacterial, antileishmanial and cytotoxic activities of essential oils from *Eremanthus erythropappus* and of α-bisabolol, their major sesquiterpene

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**Abstract**

Interest in researches into medicinal plants and therapeutic effects of essential oils (EOs) on humans has increased over the last few years. *Eremanthus erythropappus*, known as *candeia*, is a Brazilian aromatic herbaceous plant whose α-bisabolol-rich oil has been used in several cosmetic preparations. This paper reports *in vitro* anticaries, antimycobacterial, antileishmanial and cytotoxic activities of EOs from *E. erythropappus* leaves (EL-EO) and stalks (ES-EO), besides α-bisabolol, their main sesquiterpene. EL-EO and ES-EO were extracted by hydrodistillation and analyzed by GC-FID and GC-MS. α-Bisabolol, *cis*-α-bisabolene and β-bisabolene were identified as their major constituents. Antibacterial activity of EOs was evaluated against eight standard strains of pathogens from the American Type Culture Collection (ATCC) by determining minimum inhibitory concentrations (MICs) with the use of the microdilution method. Antibacterial activity was evaluated against *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. sobrinus*, *S. salivarius*, *Mycobacterium tuberculosis*, *M. avium* and *M. kansasii*. EL-EO, ES-EO and α-bisabolol exhibited high leishmanicidal activity against promastigote forms of *Leishmania amazonensis*; $IC_{50}$ values were 9.22 µg/mL, 6.00 µg/mL and 3.12 µg/mL, respectively. The 50% cytotoxic concentrations ($CC_{50}$) of EL-EO, ES-EO and α-bisabolol against mouse peritoneal macrophages were 24.65 µg/mL, 8.87 µg/mL and 1021.00 µg/mL, respectively. These results suggest that EOs from *E. erythropappus* seem to be very promising for the development of new bactericidal and leishmanicidal agents.

**Keywords:** *Eremanthus erythropappus,* essential oils, genus *Mycobacterium*, α-bisabolol, *Leishmania amazonensis*, *Streptococcus mutans.*
**Abbreviations:** GC-FID - gas chromatography-flame ionization detector; GC-MS - gas chromatography-mass spectrometry; EOs - essential oils; MIC - minimum inhibitory concentration; DMSO - dimethylsulfoxide; NCCLS - National Committee for Clinical Laboratory Standards.

## Introduction

The family Asteraceae, which has about 1,300 genera and 23,000 species, comprises mostly herbs, shrubs and small trees with leaves and stalks whose secretory structures, such as trichomes, secrete essential oils (EOs) (Rios, 2015). These EOs, especially the ones from *Eremanthus erythropappus* (Asteraceae), are rich in α-bisabolol, a fact that gives them great economic importance, because this sesquiterpene is used for the formulation of several cosmetic products (Scolforo et al., 2016). Extracts and EOs from *E. erythropappus* have been known for their important biological activities, such as antimicrobial ones against several pathogens – *Alternaria carthami*, *Rhizoctonia solani*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Candida spp.* and *Cryptococcus spp.* –, besides antinociceptive, anti-inflammatory, schistosomicidal, antiedematogenic and antiulcerogenic ones (Hillen et al., 2012; Nascimento et al., 2007; Silvério et al., 2013; Santos et al., 2015; Sousa et al., 2008a; Almeida et al., 2012; Keles et al., 2010). However, anticaries, antimycobacterial and

Chemistry & Biodiversity 10.1002/cbdv.202200097

Chem. Biodiversity

# Antibacterial Activity of Essential oils Against Oral Pathogens

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This updated review article covers the literature between 2011 and 2021 on the antibacterial activity of EOs against the main bacteria that cause caries and periodontal diseases. The criteria to classify the *in vitro* antibacterial activity of EOs is updated and the most promising results are addressed.

**Keywords**: *caries, oral bacteria, periodontal diseases, terpenes, volatile organic compounds*

## Introduction

Essential oils (EOs) are complex mixtures of volatile hydrophobic compounds obtained from different parts of plants, mainly leaves and flowers. EOs are generally rich in monoterpenes, sesquiterpenes, phenylpropanoids, esters, aldehydes, phenols, ethers, and peroxides.[1] However, several the chemical composition of EOs may vary with geographical location, climatic conditions, time of collection or variations between individuals of the same species (*i.e.*, chemotypes). These variations may also affect the toxicity and biological effects of EOs.[2]

Over the last decade, the antimicrobial activity of EOs has been extensively reviewed.[3-6] Because the term "antimicrobial activity" is a generic term that is used to refer to antibacterial or even antifungal activity,[7] the antibacterial activity of EOs against bacteria present in the oral cavity has not been highlighted previously. These bacteria cause caries and periodontal diseases, such as gingivitis and periodontitis.

Dental caries is defined as localized destruction of the tooth hard tissues due to acidic by-products of bacterial carbohydrate fermentation. These by-products reduce the pH values to decrease to a critical value, which culminates in tooth demineralization and so in the teeth cavitation.[8] *Streptococcus mutans* is one of the main etiological agents of human dental caries: it can colonize and persist on the tooth surface and metabolize different types of carbohydrates, using them to form biofilms on the tooth surface under low pH conditions.[9, 10] However, oral diseases generally have polymicrobial origin, and together with *S. mutans*, other acid-resistant bacteria can cause cavities, including *S. sobrinus*, *S. oralis*, *S. sanguinis*, *Lactobacillus* spp., and *Actinomyces* spp.[11] Studies have suggested that *S. mutans* represents only 0.02-0.73% of the bacterial community, which reinforces the polymicrobial nature of caries and the hypothesis that the entire flora of acidogenic/aciduric bacteria contributes to the carious process.[12, 13]

Periodontal disease encompasses gingivitis and periodontitis. Gingivitis is an inflammatory process that occurs in the soft tissues surrounding the teeth (gum) as an immune response to plaque that forms on the tooth. Periodontitis manifests similarly to gingivitis and destroys the structures that support the teeth, including the periodontal ligament, bones, and soft tissues. Because periodontitis is more aggressive and results in tooth loss, it is the most significant of the two diseases.[14] More than 300 species of bacteria are found in periodontal infections. Among the bacteria that are considered periodontopathogens we can highlight *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Fusobacterium nucleatum*.[15] Periodontitis is a highly prevalent disease; in the United States of America between 2009 until 2012, its prevalence is estimated at 45.9%, 8.9% of which correspond to severe periodontitis.[16] In 2019, an event coordinated by the



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