UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Estudo fitoquímico, desenvolvimento de método analítico e avaliação biológica de própolis marrom do sudeste brasileiro produzida por *Apis mellifera*

Victor Pena Ribeiro

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências Farmacêuticas para obtenção do Título de Doutor em Ciências.

Área de Concentração: Produtos Naturais e Sintéticos.

Orientado: Victor Pena Ribeiro

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"Não serei interrompida"

Marielle Franco

"Se não puder voar, corra. Se não puder correr, ande. Se não puder andar, rasteje, mas continue em frente de qualquer jeito".

Martin Luther King

RESUMO

RIBEIRO, V. P. Estudo fitoquímico, desenvolvimento de método analítico e avaliação biológica de própolis marrom do sudeste brasileiro produzida por *Apis mellifera*. 2022. 176f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2022.

A própolis tem sido objeto de diversos estudos farmacológicos devido às propriedades biológicas importantes como antioxidante e antiflamatória, entre outras, sendo uma importante alternativa terapêutica, do ponto de vista econômico, por ser farmacologicamente eficiente e de fácil obtenção. Os principais tipos de própolis Brasileiras são: verde, vermelha, amarela e marrom. Apesar da importância comercial, histórica e econômica da própolis brasileira, a composição química da própolis marrom tem sido escassamente investigada. Todas as propriedades biológicas atribuídas a própolis, são fortemente dependentes da concentração de constituintes ativos. Assim, a investigação química e a padronização dos produtos de própolis são fundamentais devido às variabilidades nos perfis químicos dos extratos. Diante disso, o objetivo do trabalho foi investigar a composição química, desenvolver e validar métodos cromatográficos para quantificação dos marcadores e avaliar a atividade biológica de amostras de própolis marrom produzida por *Apis mellifera* coletadas na região sudeste do Brasil. Amostras de própolis marrom foram coletadas no apiário Sol, localizado no município de Cabo Verde - MG, utilizando-se coletores de madeira vazados nas tampas das colmeias de *Apis Mellifera*. A partir da própolis, foi obtido o extrato bruto através de maceração com soluções hidroalcoolicas. O Extrato bruto foi submetido a partições com solventes de polaridade crescentes e posteriormente foi submetido a diferentes técnicas cromatográficas para isolamento dos compostos majoritários. As substâncias isoladas tiveram suas estruturas determinadas por meio de técnicas como RMN. Da fração fixa foram isoladas e identificadas 15 substâncias que foram utilizadas no desenvolvimento e validação de um método por HPLC/DAD. A validação levou em consideração parâmetros estabelecidos nos guias de agências como ANVISA e ICH. O extrato bruto foi avaliado quanto a citotoxidade celular frente a linhagens normais e tumorais, atividade frente a *Leishimania amazonenses* e *Plasmodium falciparum*. A avaliação citotóxica do extrato hidroalcoólico, não indicou efeito sobre a viabilidade das linhagens celulares normais (VERO e LLC-PK1), contudo, a própolis marrom exibiu efeito de toxicidade nas células tumorais, com os seguintes valores de IC_{50}: 80 µg/mL para SK-MEL; 82 µg/mL para KB; 71 µg/mL para SK-OV-3; e 64 µg/mL para BT-549. A própolis marrom apresentou atividade leishimanicida promissora, com IC_{50} de 1,8 µg/mL contra a forma promastigota de *L. amazonensis* e IC_{50} de 2,4 µg/mL contra a forma amastigota. Sobre a atividade antiplasmódica, a própolis marrom apresentou atividade contra cepas de *P. falciparum*, com IC_{50} de 18,1 µg/mL e 15,6 µg/mL, contra as cepas D6 e W2 respectivamente. A fração volátil da Propolis marrom, também foi considerada neste trabalho, para tanto a própolis foi submetida a hidrodestilação para obtenção da fração volátil. Essa fração foi fracionada por *spinning band distillation* e duas frações submetidas a cromatografia em coluna aberta para isolamento dos compostos majoritários. As substâncias isoladas tiveram suas estruturas determinas por meio de RMN e espectrometria de massas. Foram isoladas e identificadas 8 substâncias voláteis que foram utilizadas no desenvolvimento e validação do método por CG-DIC os quais foram considerados os parâmetros estabelecidos nos guias de validação da ANVISA e ICH. A fração volátil apresentou atividade leishmanicida significativa, com IC_{50}= 21,3 µg/mL contra formas amastigotas e IC_{50}= 25,1 µg/mL contra formas promastigotas de *Leishmania amazonensis*. Essa fração também apresentou efeito antibacteriano ao inibir o crescimento de *Streptococcus mutans e Staphylococcus aureus* a 25 µg/mL e 50 µg/mL, respectivamente, mas não foi citotóxico contra as linhagens AGP-01, He-

La e CHO-K1, com IC_{50}> 100 μg/mL. O extrato bruto e a fração volátil da própolis marrom, tiveram suas propriedades analgésica e anti-inflamatória avaliadas por meio do teste de formalina e hipernocicepção mecânica induzida por carragenina. O extrato hidroalcoólico e a fração volátil foram avaliados nas doses de 50, 100 e 200 mg/kg. No teste de formalina, o extrato bruto reduziu a resposta em 73 ± 7%, 83 ± 8%, e 89 ± 3% para a primeira fase e 48 ± 13%, 65 ± 9%, e 75 ± 6% para a segunda fase, respectivamente. A fração volátil reduziu a resposta em 75 ± 7%, 94 ± 5%, 99 ± 1% e 30 ± 7%, 53 ± 5%, 91 ± 5% para a primeira e segunda fase respectivamente. Para o teste de carragenina, o extrato hidroalcoólico e a fração volátil apresentaram reduções na sensibilização mecânica de 57 ± 7%, 67 ± 6%, 79 ± 5% e 62 ± 7%, 79 ± 9%, 88 ± 4% respectivamente, após 48 h. A indometacina e a morfina apresentaram inibições de 42 ± 4% and 88 ± 2%, respectivamente. No ensaio de movimento de cauda, os resultados mostraram evidências de atividades analgésicas; os animais tratados com extrato bruto aumentaram o limiar nociceptivo em 58±8% e 70±6% e 77±10%, respectivamente, para as doses avaliadas, e 55±6% e 73±7% e 82±14% para a fração volátil, em comparação com o controle. A coadministração de naloxona alterou o efeito antinociceptivo da morfina e da própolis, sugerindo que a própolis marrom tem ação no sistema nervoso central. Uma própolis marrom coletada na cidade de Angatuba – SP teve sua composição química elucidada, para tanto, foi obtido o extrato bruto através de maceração com soluções hidroalcoolicas que foi submetido a partições e posteriormente a diferentes técnicas cromatográficas para isolamento dos seus constituintes. As substâncias isoladas tiveram suas estruturas determinas por meio de RMN. A composição química dessa própolis se apresntou complexa e diferente das reportadas na literatura. Foram isoladas e identificadas 16 substâncias dessa própolis marrom, incluindo sete compostos fenólicos, um flavanonol, duas lignanas e seis ácidos/álcool diterpênicos. O perfil químico dessa própolis indica que *Pinus* spp., *Eucalyptus* spp. e *Araucaria angustifolia* podem ser sua principal fonte vegetal. A própolis marrom apresentou atividade significativa contra as cepas de *Plasmodium falciparum* D6 e W2 com IC_{50} de 5,3 e 9,7 μg/mL, respectivamente. A fração volátil dessa própolis também foi ativa com IC_{50} de 22,5 e 41,8 μg/mL, respectivamente. Entre os compostos, 1-O,2-O-digalloil-6-O-trans-p-cumaroil-β-D-glicopiranosídeo apresentou IC_{50} de 3,1 e 1,0 μg/mL contra as cepas D6 e W2, respectivamente, enquanto o ácido comúnico apresentou um IC_{50} de 4,0 μg/mL contra a cepa W2. A citotoxicidade foi determinada em quatro linhas de células tumorais (SK-MEL, KB, BT-549 e SK-OV-3) e duas linhas de células renais normais (LLC-PK1 e VERO). Matairesinol, 7-O-metil aromadendrina e ácido isopimárico apresentaram uma faixa de IC_{50} de 1,8 – 0,78 μg/mL, 7,3 – 100 μg/mL e 17-18 μg/mL, respectivamente, contra as linhagens de células tumorais, mas não foram citotóxicos contra linhagens celulares normais. O extrato bruto de própolis marrom apresentou atividade antimicrobiana contra *C. neoformans, Staphylococcus aureus* resistente à meticilina e *P. aeruginosa* em 29,9 μg/mL, 178,9 μg/mL e 160,7 μg/mL, respectivamente. A fração volátil inibiu o crescimento de *C. neoformans* em 53,0 μg/mL. A própolis marrom possui uma variedade química maior do que outros tipos de própolis brasileiras, uma vez que várias fontes botânicas possíveis são listadas. Portanto, a própolis marrom brasileira apresenta grande potencial e deve ser mais investigada para viabilizar sua produção e comercialização como produto eficaz e seguro para a saúde.

Palavras-chave: 1. Propolis Marrom; 2. Métodos analíticos; 3. Cromatografia líquida e gasosa; 4. Validação.

ABSTRACT

RIBEIRO, V. P. Phytochemical study, development of analytical methods and biological evaluation of brown propolis from southeastern Brazil produced by *Apis mellifera*. 2022. 176f. Thesis (Doutorado). School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2022.

Propolis has been the subject of several pharmacological studies due to important biological properties such as antioxidant and anti-inflammatory, among others, being an important therapeutic alternative, from the economic point of view, for being pharmacologically efficient and easy to obtain. The main types of Brazilian propolis are: green, red, yellow and brown. Despite the commercial, historical and economic importance of Brazilian propolis, the chemical composition of brown propolis has been scarcely investigated. All biological properties attributed to propolis are strongly dependent on the concentration of active constituents. Thus, chemical investigation and standardization of propolis products are fundamental due to the variability in the chemical profiles of the extracts. Therefore, the objective of this work was to investigate the chemical composition, develop and validate chromatographic methods to quantify the markers and evaluate the biological activity of samples of brown propolis produced by *Apis mellifera* collected in the southeastern region of Brazil. Samples of brown propolis were collected in the apiary Sol, located in the municipality of Cabo Verde - MG, using wooden collectors hollowed in the lids of Apis Mellifera hives. From the propolis, the crude extract was obtained through maceration with hydroalcoholic solutions. The crude extract was partitioned with solvents of different polarity and was subsequently subjected to different chromatographic techniques to isolate the major compounds. The isolated substances had their structures determined by means of techniques such as NMR. From the crude extract, 15 substances were isolated and identified, which were used in the development and validation of a method by HPLC/DAD. The validation took into account parameters established in the guides of agencies such as ANVISA and ICH. The crude extract was evaluated for cell cytotoxicity against normal and tumor lines, activity against Leishimania amazonenses and Plasmodium falciparum. The cytotoxic evaluation of the hydroalcoholic extract did not indicate an effect on the viability of normal cell lines (VERO and LLC-PK1), however, brown propolis exhibited a toxic effect on tumor cells, with the following IC_{50} values: 80 µg/mL for SK-MEL; 82 µg/ml for KB; 71 µg/ml for SK-OV-3; and 64 µg/ml for BT-549. Brown propolis showed promising leishmanicidal activity, with an IC_{50} of 1.8 µg/mL against the promastigote form of L. amazonensis and an IC_{50} of 2.4 µg/mL against the amastigote form. Regarding antiplasmodic activity, brown propolis showed activity against P. falciparum strains, with IC_{50} of 18.1 µg/mL and 15.6 µg/mL, against D6 and W2 strains, respectively. The volatile fraction of brown Propolis was also considered in this work, for which the propolis was submitted to hydrodistillation to obtain the volatile fraction. This fraction was fractionated by spinning band distillation and two fractions were subjected to open column chromatography to isolate the major compounds. The isolated substances had their structures determined by means of NMR and mass spectrometry. Eight volatile substances that were used in the development and validation of the method by GC-DIC were isolated and identified, which were considered the parameters established in the validation guides of ANVISA and ICH. The volatile fraction showed significant leishmanicidal activity, with IC_{50}= 21.3 µg/mL against amastigote forms and IC_{50}= 25.1 µg/mL against promastigote forms of Leishmania amazonensis. This fraction also showed an antibacterial effect by inhibiting the growth of Streptococcus mutans and Staphylococcus aureus at 25 µg/mL and 50 µg/mL, respectively, but was not cytotoxic against AGP-01, He-La and CHO-K1 strains, with IC_{50} > 100 µg/mL. The crude extract and the volatile fraction of brown propolis had their analgesic

and anti-inflammatory properties evaluated by means of the formalin test and mechanical hypernociception induced by carrageenan. The hydroalcoholic extract and the volatile fraction were evaluated at doses of 50, 100 and 200 mg/kg. In the formalin test, the crude extract reduced the response at 73 ± 7%, 83 ± 8%, and 89 ± 3% for the first phase and 48 ± 13%, 65 ± 9%, and 75 ± 6% for the second phase, respectively. The volatile fraction reduced the response by 75 ± 7%, 94 ± 5% and 99 ± 1% and 30 ± 7%, 53 ± 5%, and 91 ± 5% for the first and second phases. For the carrageenan test, the hydroalcoholic extract and the volatile fraction showed reductions in mechanical sensitization of 57 ± 7%, 67 ± 6% and 79 ± 5% and 62 ± 7%, 79 ± 9% and 88 ± 4% respectively, after 48 h. Indomethacin and morphine showed inhibitions of 42 ± 4% and 88 ± 2%, respectively. In the tail flick test, the results showed evidence of analgesic activities; animals treated with crude extract increased the nociceptive threshold by 58±8% and 70±6% and 77±10%, respectively, for the doses evaluated, and 55±6% and 73±7% and 82±14% for the volatile fraction, compared to the control. Co-administration of naloxone altered the antinociceptive effect of morphine and propolis, suggesting that brown propolis has an action on the central nervous system. A brown propolis collected in the city of Angatuba - SP had its chemical composition elucidated, for that, the crude extract was obtained through maceration with hydroalcoholic solutions that was subjected to partitions and later to different chromatographic techniques for isolation of its constituents. The isolated substances had their structures determined by means of NMR. The chemical composition of this propolis was complex and different from those reported in the literature. Sixteen substances from this brown propolis were isolated and identified, including seven phenolic compounds, one flavanonol, two lignans and six diterpenic acids/alcohol. The chemical profile of this propolis indicates that Pinus spp., Eucalyptus spp. and Araucaria angustifolia may be its main plant source. Brown propolis showed significant activity against Plasmodium falciparum D6 and W2 strains with IC50 of 5.3 and 9.7 μg/mL, respectively. The volatile fraction of this propolis was also active with IC50 of 22.5 and 41.8 μg/mL, respectively. Among the compounds, 1-O,2-O-digalloyl-6-O-trans-p-coumaroyl-β-D-glucopyranoside presented IC50 of 3.1 and 1.0 μg/mL against strains D6 and W2, respectively. , while comunic acid showed an IC50 of 4.0 μg/mL against the W2 strain. Cytotoxicity was determined in four tumor cell lines (SK-MEL, KB, BT-549 and SK-OV-3) and two normal renal cell lines (LLC-PK1 and VERO). Matairesinol, 7-O-methyl aromadendrin and isopimaric acid showed an IC50 range of 1.8 – 0.78 μg/mL, 7.3 – 100 μg/mL and 17-18 μg/mL, respectively, against the strains of tumor cells, but were not cytotoxic against normal cell lines. The brown propolis crude extract showed antimicrobial activity against C. neoformans, methicillin-resistant Staphylococcus aureus and P. aeruginosa at 29.9 μg/mL, 178.9 μg/mL and 160.7 μg/mL, respectively. The volatile fraction inhibited the growth of C. neoformans by 53.0 μg/mL. Brown propolis has a greater chemical variety than other types of Brazilian propolis, as several possible botanical sources are listed. Therefore, Brazilian brown propolis has great potential and should be further investigated to enable its production and commercialization as an effective and safe product for health.

Keywords: 1. Brown Propolis; 2. Analytical methods; 3. Liquid and gas chromatography; 4. Validation.

LISTA DE FIGURAS

1 INTRODUÇÃO

LISTA DE TABELAS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ANVISA	Agência Nacional de Vigilância Sanitária
A.R.	Área Relativa
CCD	Cromatografia em Camada Delgada
$CDCL_3$	Clorofórmio Deuterado
CG	Cromatografia Gasosa
CNPQ	Conselho Nacional de Desenvolvimento Científico e Tecnológico
D	Dupleto
d.i.	Diâmetro interno
DIC	Detector de ionização por chamas
Dd	Duplo dupleto
EM	Espectrometria de Massas
Hex	Hexano
ICH	*International Conference on Harmonization*
INMETRO	Instituto Nacional de Metrologia, Normalização e Qualidade Industrial
J	Constante de acoplamento
m/z	Razão massa carga
Pi	Padrão interno
Ppm	Partes por milhão
RMN	Ressonânica Magnética Nuclear
δ	Deslocamento químico

SUMÁRIO

1. INTRODUÇÃO

1. INTRODUÇÃO

1.1 Própolis

A própolis é um produto apícola resultante da interação inseto-planta. As abelhas coletam resina e outros materiais como goma, látex e outros de uma ampla variedade de fontes botânicas (Arruda et al. 2020). Esse material resinoso é digerido pelas abelhas com as enzimas da saliva e misturado à cera produzindo a própolis. O material resinoso vegetal é uma importante fonte de compostos bioativos, que serão incorporados à própolis, resultando em benefícios para a colmeia, como controle de infecções microbianas, imobilização e mumificação de invasores (Bankova et al. 2019).

O mecanismo de seleção de fontes botânicas pelas abelhas para produção de própolis não está totalmente elucidado, porém alguns estudos apontam para uma via de sinalização química realizada pelos compostos voláteis presentes nessas resinas (Rodrigues et al. 2020). Assim, a composição química da própolis será determinada pela flora disponível para as abelhas. Além disso, a própolis também está sujeita a fatores que causam variações químicas nas plantas, como tempo de coleta, temperatura, radiação, degradação, entre outros (Bankova et al. 2019). A própolis possui importância econômica devido às suas propriedades farmacológicas de grande utilidade para a humanidade. As atividades antioxidante (Andrade et al. 2017), citotóxica (Arruda et al. 2020), antimicrobiana (Silva et al. 2019), antinociceptiva e anti-inflamatória (Ribeiro et al. 2021) são atribuídas à própolis.

Diversas classes de compostos já foram relatadas nos mais diferentes tipos de própolis em todo o mundo. Geralmente, a própolis é classificada de acordo com sua coloração que está diretamente ligada a sua fonte botânica e consequentemente a sua composição química. Os principais tipos de própolis brasileira são verde, característica da região Sudeste, compostas principalmente por compostos fenólicos prenilados e flavonoides (Kocot et al. 2018); marrom, composta por ácidos fenólicos e diterpenos (Huang et al, 2014); e a vermelha, que é característica da região nordeste do Brasil, que é composta principalmente por isoflavonas e benzofenonas preniladas (Mendonça et al, 2015).

1.2 Propolis Brasileiras e a propolis Marrom

Os principais tipos de própolis Brasileira são: verde, vermelha, amarela e marrom. São assim classificadas devido ao aspecto dos seus extratos. A vermelha, caracteristicamente produzida na região nordeste, tem como fonte botânica a *Dalbergia ecastaphyllum* (L), membro da família Fabaceae (Mendonça-melo et al., 2017). Os estudos de Daugsch et al. (2008) e Silva et al. (2008) descreveram a *D. ecastaphyllum* como a principal fonte botânica

da própolis vermelha. Esses estudos confirmaram a presença de uma rica variedade de compostos fenólicos, tanto na própolis quanto na resina vegetal, tais como chalconas (como a isoliquiritigenina), flavonoides (luteolina, liquiritigenina), isoflavonas (formononetina, biochanina A), isoflavonas (vestitol, neovestitol, 7-O-metilvestitol), pterocarpanos (como a medicarpina, homopterocarpina, vesticarpana) e isoflavonas C30 (retusapurpurinas A e B).

Estudos fitoquímicos apresentaram quantidades substanciais de benzofenonas poliprenilladas, tais como gutifferona E e oblongifolina A na própolis vermelha brasileira (Fasolo et al., 2016) e importantes atividades biológicas atribuídas a própolis vermelha, como propriedades antimicrobianas e citotóxicas, têm sido correlacionados com a ocorrência de benzofenonas poliprenilladas (Rufatto et al., 2017; Trusheva et al., 2016). Estes resultados direcionaram Ccana-Ccapatinta et al. 2020 a novos estudos que possibilitaram identificar *Symphonia globulifera* L.f. (Clusiaceae) como a fonte botânica das benzofenonas poliprenilladas da própolis vermelha, principalmente gutiferona E e oblongifolina B, bem como a de triterpenoides.

Uma das própolis mais estudadas e comercializadas do Brasil é a própolis verde, proveniente do sudeste brasileiro, cuja principal fonte vegetal para sua produção é a *Baccharis dracunculifolia*, conhecida popularmente por alecrim-do-campo. A própolis verde brasileira possui composição química característica, pois contêm majoritariamente compostos fenólicos prenilados, como o artepelin C, bacarina e drupanina, assim como ácidos fenólicos, como o precursor biossintético destes compostos, o ácido p-cumárico (Costa et al. 2019).

O tipo marrom de própolis é produzido principalmente por apicultores das regiões sul e sudeste do Brasil (Figura 1).

Figura 1. (A) Disposição das colmeias em um apiário (Apiário Sol – Cabo verde/MG); **(B)** Coloração característica da Própolis marrom. (Fonte: Acervo Pessoal)

Apesar de alguns compostos químicos presentes na própolis marrom já terem sido identificados, bem como alguns dos seus efeitos biológicos já terem sido descritos, este tipo de própolis não tem sido bem estudada como as própolis vermelha e verde. Há ainda divergência na literatura quanto a sua origem botânica.

1.3 Importância econômica da própolis

A atividade apícola brasileira é um empreendimento promissor e tem ganhado força no mercado internacional uma vez que a enorme biodiversidade brasileira favorece a diversidade de produtos apícolas. Produções científicas comprovando as atividades farmacológicas da própolis brasileira têm contribuído para aumentar a competitividade e a demanda da própolis nacional e internacionalmente (Bereta et al. 2017).

A gama de propriedades biológicas da própolis brasileira tem despertado o interesse das indústrias farmacêutica, alimentícia e cosmética, principalmente devido ao seu potencial antimicrobiano e antioxidante com efeitos benéficos à saúde dos consumidores. As propriedades organolépticas características, como cheiro, cor e textura da maioria das própolis, são essenciais para a aceitação e comercialização do consumidor. Seus usos medicinais e como suplemento alimentar estão bem estabelecidos em várias partes do mundo, especialmente na Europa Oriental, China e Japão. O Brasil é um grande destaque no mercado internacional, movimentando milhões de dólares anualmente (Hata et al. 2012).

A CONAP-Brasil (Cooperativa Nacional de Apicultura) informou em 2020 que os mercados nacional e internacional de própolis aumentaram aproximadamente entre 40% e 50%, respectivamente, devido ao surto de covid-19. Estima-se que aproximadamente 90% da receita do CONAP seja proveniente das exportações de produtos apícolas, principalmente para países asiáticos, incluindo Japão, Coreia do Sul e Taiwan, registrando um aumento de 94% nas exportações em relação a 2019 (Belvedere, 2021).

Minas Gerais é o maior produtor de própolis do Estado brasileiro, responsável por 70% de toda a produção de própolis no Brasil com uma produção total estimada de 120 toneladas por ano, sendo 85% de própolis verde e 15% de própolis marrom (Riardo, 2021). Com o crescimento da demanda mundial por própolis, com exportação para Europa e Estados Unidos, outras regiões do Brasil também estão aumentando sua produção, como as regiões Nordeste e Sul. Para consolidar a própolis marrom no mercado internacional, mais estudos científicos devem ser realizados sobre a própolis de cada região, agregando valor a esse produto apícola brasileiro (Guimarães, 2021).

1.5 Desenvolvimento e validação de método analítico

Uma vez que produtos naturais são rotineiramente utilizados na medicina popular, a sua eficácia e segurança devem ser obrigatórias, havendo necessidade de realização de testes farmacológicos, toxicológicos e clínicos. A eficácia e segurança de produtos naturais acabados são diretamente dependentes da qualidade e da composição química da matéria-prima natural. Portanto, se faz necessário, além do perfil químico qualitativo a determinação quantitativa nos perfis fitoquímicos de plantas medicinais. Estas análises são mandatórias para assegurarem-se a segurança e eficácia de medicamentos naturais, as quais estão intrinsecamente ligadas à constância de perfis fitoquímicos (Govindaraghavan & Sucher, 2015).

O desenvolvimento tecnológico de um produto fitoterápico requer estudos prévios tais como: estudos botânicos, agronômicos, químicos e pesquisas sobre sua atividade biológica, o que o diferencia das plantas medicinais e das preparações utilizadas na medicina popular. No ponto de vista da qualidade, verifica-se que, para garantir um produto uniforme e eficaz é necessário que todos os insumos intermediários (planta *in natura*, tinturas, extratos secos, etc), bem como o produto final, sejam caracterizados através de seus constituintes químicos, e/ou atividades farmacológicas (Patil et al. 2014). Sendo assim, em muitos casos, o produto final pode ser padronizado por meio da quantificação de marcadores, que podem ser compostos químicos característicos de certa espécie, ou compostos presentes em grandes quantidades (Naz et al. 2014).

Um método analítico é caracterizado pelo seu desempenho, cujos parâmetros devem ser avaliados para que possam fornecer os valores de desempenho correto. Estes valores de desempenho devem estar em conformidade com os requisitos previamente definidos que o método analítico deve satisfazer. Todos os procedimentos analíticos exigem algum tipo de validação, independentemente do método. Durante as últimas duas décadas, a validação tem-se tornado tradicional para representar o desempenho de métodos analíticos (Ruiz-Angel et al. 2014).

O termo validação é definido como a avaliação sistemática de um procedimento analítico para demonstrar que este está sob as condições nas quais ele deve ser aplicado. A Agência Nacional de Vigilância Sanitária preconiza que a validação deve garantir, através de estudos experimentais, que o método atenda às exigências das aplicações analíticas, assegurando a confiabilidade dos resultados (ANVISA, 2017).

Com esta finalidade, métodos desenvolvidos utilizando-se técnicas cromatográficas, como a cromatografia gasosa (CG) e a cromatografia líquida de alta eficiência (CLAE) têm

sido largamente utilizados, tanto para o estudo fitoquímico, quanto na química analítica para o controle de qualidade de plantas medicinais, uma vez que proporcionam vantagens como a alta eficiência e rapidez (Kruve et al. 2015).

O desenvolvimento de um método analítico, a adaptação ou a implementação de um método conhecido envolvem um processo de avaliação que estime sua eficiência na rotina do laboratório, o qual é denominado de validação. A validação é imprescindível para garantir a confiabilidade dos resultados de um determinado procedimento analítico (Chauhan, Mittu e Chauhan, 2015).

No Brasil, há duas agências credenciadoras para verificar a competência de laboratórios de ensaios, a ANVISA (Agência Nacional de Vigilância Sanitária) e o INMETRO (Instituto Nacional de Metrologia, Normalização e Qualidade Industrial). Estes dois órgãos disponibilizam guias para o procedimento de validação de métodos analíticos. Na Europa, Estados Unidos e Japão, a ICH (International Conference on Harmonization) e a USP (The United States Pharmacopeia) definem parâmetros, requerimentos e, em alguns casos, também metodologias para processo de validação. Além destes, a IUPAC (International Union of Pure and Applied Chemistry) e a FDA (Food and Drug Administration) também têm propostos guias sobre validação de métodos. Estes guias e/ou requerimentos, de forma geral, exibem os parâmetros de desempenho a serem seguidos para validação de um método (Garcia et al. 2011).

O guia de validação da ANVISA foi escolhido para ser utilizado, pois o guia é aceito comercialmente, sendo assim, o método desenvolvido e validado neste trabalho poderá ser utilizado no controle de qualidade de propolis. Os parâmetros analíticos para a validação de métodos, conhecidos também como parâmetros de desempenho analítico são normalmente encontrados como: seletividade, linearidade, precisão, limite de quantificação, limite de detecção, exatidão e robustez (Kruve et al. 2015).

A Seletividade de um método refere-se à capacidade deste em distinguir um determinado analito presente em uma matriz complexa, sem interferência de outros componentes da mistura. Para tanto, a seletividade deve ser o primeiro passo a ser desenvolvido para validação de um método analítico. A seletividade é baseada nos parâmetros de separação e detecção, sendo que as técnicas cromatográficas hifenadas a detectores seletivos, como o espectômetro de massas, têm sido utilizadas em determinações de alta qualidade. Além disso, em técnicas cromatográficas, outros parâmetros de separação devem ser determinados e otimizados, tais como: resolução, fator de separação, fator de retenção, fator assimetria e número de pratos teóricos (ANVISA, 2017).

A Linearidade corresponde à capacidade de um método analítico fornecer resultados diretamente proporcionais à concentração da substância na amostra, dentro de uma faixa de aplicação, sendo obtida pela confecção de curva analítica utilizando padrões interno ou externo (ANVISA, 2017).

A Precisão é a habilidade do método em reproduzir resultados entre ensaios independentes, repetidos de uma mesma amostra, amostras semelhantes ou padrões, em condições definidas. Já a exatidão de um método representa a concordância entre o resultado de um determinado ensaio em relação ao valor de referência aceito como verdadeiro. A robustez de um método mede a habilidade que este apresenta frente a pequenas variações. Diz-se que um método é robusto quando ele não é afetado por uma modificação pequena e deliberada em seus parâmetros. As mudanças introduzidas refletem as alterações que podem ocorrer quando o método é transferido para outros laboratórios sendo executado por diferentes analistas utilizando equipamentos e/ou materiais de consumo fornecidos por diferentes fabricantes (ANVISA, 2017).

Todas as propriedades biológicas atribuídas a própolis, são fortemente dependentes da concentração de constituintes ativos. No entanto, a padronização dos produtos de própolis é fundamental devido às variabilidades nos perfis químicos dos extratos, como para outras plantas medicinais e produtos derivados (Bankova et al. 2019). Como mencionado, a localização e as correlações entre a fonte botânica e as condições meteorológicas são fatores essenciais que induzem mudanças no perfil químico. É fundamental estabelecer alguns critérios sobre a concentração dos compostos ativos necessários para atividades farmacológicas específicas (Nascimento et al. 2019). O desenvolvimento de métodos analíticos validados é necessário para determinar a qualidade dos produtos através da quantificação de substâncias bioativas. Não é possível sem a padronização de procedimentos analíticos confiáveis determinar a concentração de compostos bioativos para o controle de qualidade da matéria-prima, seus extratos e produtos derivados.

2. OBJETIVO

2.1 OBJETIVO GERAL

Investigar a composição química, desenvolver e validar métodos cromatográficos para quantificação dos marcadores e avaliar algumas atividades biológicas da própolis marrom produzida por *Apis mellifera* na região sudeste do Brasil.

2.2 OBJETIVOS ESPECÍFICOS

•Obter o perfil químico do extrato hidroalcoólico e das frações de própolis marrom por Cromatografia líquida de alta eficiência acoplada a Detector por Arranjo de Diodos (CLAE-DAD) e dos voláteis por Cromatografia Gasosa acoplada a Detector por Ionização por Chamas (CG-DIC);

•Submeter os extratos a diferentes técnicas cromatográficas, visando o isolamento dos marcadores para utilização como padrões cromatográficos e nas avaliações biológicas;

•Elucidar as estruturas químicas dos compostos isolados com o auxílio de métodos físicos de análise, como Ressonância Magnética Nuclear e Espectrometria de Massas;

•Desenvolver e validar método cromatográfico em CLAE-DAD e CG-DIC visando quantificar, os principais marcadores presentes nas amostras de própolis marrom;

•Realizar ensaios *in vitro* e *in vivo* com os extratos da própolis marrom para avaliar as atividades leishimanicida, citotóxica, antimicrobiana e anti-inflamatória.

3. TRABALHOS DESENVOLVIDOS

3.1. Capítulo 1 – Desenvolvimento e validação de um método por CLAE-DAD para análise de uma própolis marrom do sudeste Brasileiro e avaliação de suas propriedades biológicas

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A RP-HPLC-PDA method for analysis of a Brazilian southeast brown propolis and its biological properties.

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Abstract

Propolis is a natural product of great economic and pharmacological importance. The flora surrounding the bee hives is a determining factor in the composition of propolis and therefore in its biological and medicinal properties. Brown propolis is one of the most important types of propolis in Brazil, produced in the southeastern region. The ethanolic extract of a brown propolis sample from Minas Gerais state was chemically characterized for the subsequent development of a novel RP-HPLC method, validated according to the standards of regulatory agencies. The leishmanicidal, antimalarial and cytotoxic activities of this extract were assessed. The brown propolis was characterized by the presence of chemical markers that were also reported on green propolis such as ferulic acid, coumaric acid, caffeic acid, cinnamic acid, baccharin, artepillin and drupanin, indicating a probable origin on *Baccharis dracunculifolia*. The developed method proved to be reliable for the analysis of this sample. The brown propolis displayed significant activity against *Leishmania amazoniensis*, with IC_{50} of 1.8 µg/mL against the promastigote form and IC_{50} of 2.4 µg/mL against the amastigote form. The antimalarial assay against *Plasmodium falciparum* showed activity with IC_{50} of 18.1 µg/mL and 15.6 µg/mL against D6 and W2 strains, respectively. No cytotoxic effect was observed on non-tumoral kidney cell lines (VERO and LLC-PK1), however, in the tumoral lines SK-MEL (malignant melanoma), KB (human oral epidermal carcinoma), SK-OV-3 (human ovarian cancer cell line), and BT-549 (Breast cancer), the brown propolis extract showed toxic effect with IC_{50} values between 64-82 µg/mL.

Keywords: antimalarial activity, cytotoxicity, leishmanicidal activity, method validation, Brazilian propolis.

1. Introduction

Propolis is a bioactive bee product resulted from plant-insect interaction. The bees collect resin and other materials such as gum, latex and others from a wide range of botanical sources available to the bees (Arruda et al., 2020a). These resinous materials are digested by bees with saliva enzymes and mixed with beeswax producing propolis. The plant resinous material is an important source of bioactive compounds, which will be incorporated to propolis, resulting in benefits to the hive, such as control of microbial infections, immobilization and mummification of invaders (Bankova et al., 2018). The mechanism of botanical sources selection by bees for production of propolis has not been fully elucidated, however some studies point to a chemical signaling pathway performed by the volatile compounds present in these resins (Rodrigues et al., 2020).

Brazil is responsible for the production of different types of propolis, characteristic of each region. Green propolis, produced mainly in the southeastern region, has *Bacharis dracunculifolia* as its main botanical source (Beserra et al., 2021). The red propolis, from the north and northeast regions, has a striking color due to the resin of *Dalbergia ecastaphyllum* (Aldana-Mejía et al., 2021). Brazilian brown propolis is mainly found in the south and southeast regions and does not yet have a well-established botanical source. Some studies of brown propolis report the presence of *Pinus*, *Eucalyptus*, *Araucaria* resins, among others; generally associated with two or more botanical sources, even with *B. dracunculifolia* (de Freitas et al., 2011; Ribeiro et al., 2020). Thus, brown propolis has a high chemical complexity due to the diversity of resins that make up its composition.

In addition, propolis is also subject to factors that cause chemical variations in plants, such as time of collection, temperature, radiation, degradation, among others (Bankova et al., 2019). This can generate different types of propolis, with different biological properties. At this point, the use of analytical tools that allow a qualitative and quantitative analysis can be very useful.

Propolis has an economic importance due to its pharmacological properties of great use to humanity: antioxidant (Andrade et al., 2017), cytotoxic (Waller et al., 2017), antimicrobial (Silva et al., 2017), antinociceptive and anti-inflammatory (Ribeiro et al., 2021c) activities are attributed to brown propolis. These activities are dependent on the concentration of bioactive constituents. The standardization of propolis products is critical due to the variabilities in its chemical profile. Therefore, validated analytical method development is necessary to determine the quality of the products (Ribeiro et al., 2019). So, we developed and validated an

HPLC-PDA method for the detection and quantification of fifteen compounds presented in a Brazilian brown propolis, according to the standards of regulatory agencies like the International Conference on Harmonization (ICH) and Agência Nacional de Vigilância Sanitária (ANVISA) and evaluated the cytotoxicity, leishmanicidal and antiplasmodium properties of this propolis.

2. Material and methods

2.1. Propolis material

Brown raw propolis from *Apis mellifera* bees were collected from artificial hives in Cabo Verde – Minas Gerais, Brazil in May 2018. The impurities were removed and the propolis was stored in amber bottle and kept at -20 ºC freezer until its utilization.

2.2. Propolis hydroalcoholic extract

The brown propolis were crushed using a mill and subjected to dynamic maceration at 35 ºC and 140 rpm with ethanol-water 7:3 (v:v) and a ratio of 1:10 (w:v) of raw material/solvent. The solvent was filtered and renewed every 24 h, totaling three macerations. The obtained hydroalcoholic solutions were concentrated in a Buchi® rotary evaporator apparatus, and the resulting extract was lyophilized, furnishing the crude hydroalcoholic extract.

2.3. Isolation and identification of the standard compounds

The crude extract was suspended in a solution of MeOH:H_2O (6:4) and then partitioned with EtOAc and *n*-BuOH., resulting in three fractions. Each fraction was subjected to chromatographic procedures to isolation the markers present in the brown propolis. The isolation procedure is schematized in figure 1.

To obtain a satisfactory purity degree of the analytes to be used in the validation process (≥ 97%) the compounds obtained from the chromatographic columns were subjected to a Shimadzu® preparative HPLC, model Proeminence, with a UV detector model SPD-20A. The HPLC procedure was performed on a C-18 semi-prepared column (250 x 10 mm, 4 μm) with a pre-column (10 x 10 mm, 4 μm), both from the Phenomenex®. The mobile phase was composed of (A) water + 2% formic acid and (B) acetonitrile. The flow was adjusted to 4 mL/min, and the UV detector was set at 280 nm. The gradient used was as follows: 0.01−14.00 min, 20−60% B; 14.00−17.00 min, 60−20% B; and 17.00−20.00 min, 20−20% B.

The chemical structures of the isolated compounds were determined using 1D and 2D NMR analyses, compared with literature data. The obtained NMR spectra data were recorded in a Brucker – Advance DRX500 spectrometer, operating at 500 MHz, using $CDCl_3$ and CD_3OD from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The purity of the isolated compounds was estimated to be greater than 97% by high performance liquid chromatography (HPLC) and NMR.

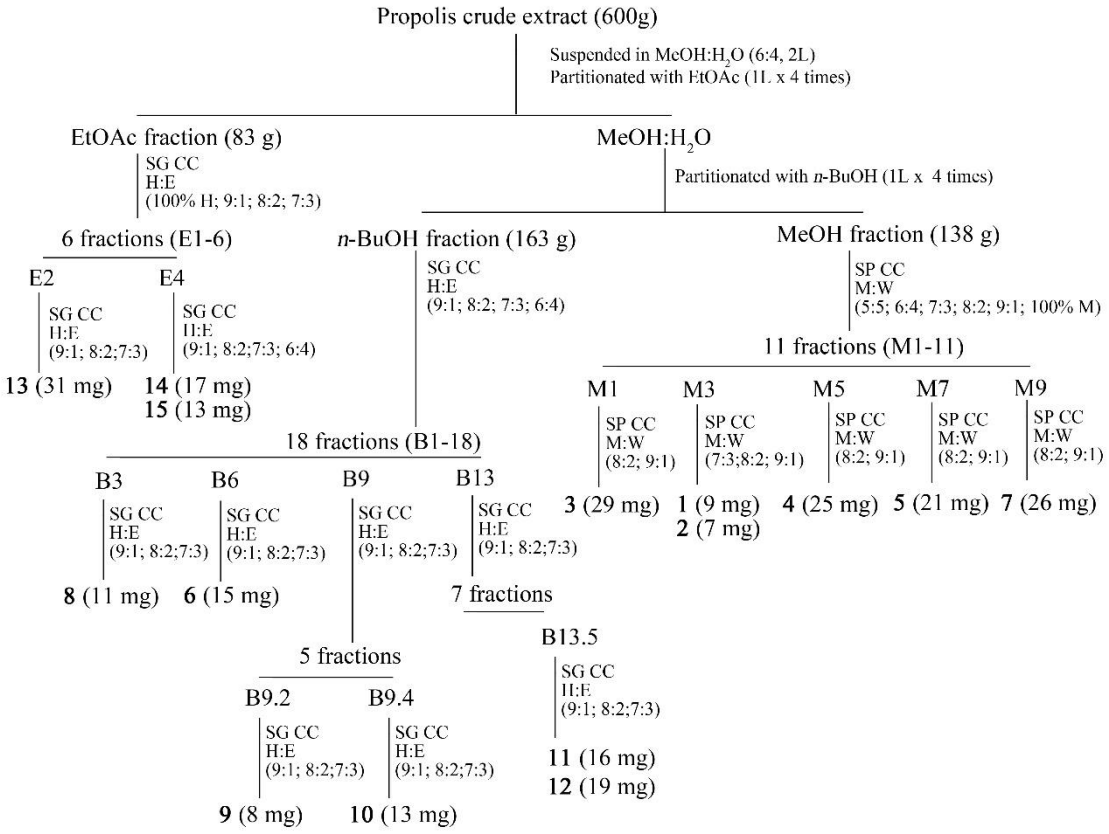

Figure 1. Schematic procedure of the isolation of Brown Propolis compounds.

2.4. RP-HPLC-PDA analysis

The analytical method was developed in a Waters 2695 HPLC instrument, with a 1525 binary solvent delivery system, a 2707 autosampler, and coupled to a 2998 photodiode array detector (PDA). The software Empower 3 was used as a controller of the analytical system and data processor. All experiments were performed in triplicate with temperature controller.

The column used was a C-18 (150 × 4.6 mm, 2.7 µm) column. The mobile phase consisted of water and 0.2% formic acid (solvent A) and acetonitrile (solvent B). The gradient condition was as follows: 15-20% of B in 12 min; 20-25% of B in 17 min; 25-50% of B in 30

min; 50-68% of B in 40 min; 68-100% of B in 45 min; going from 100→15% in 46 min., to reestablish the initial conditions until 50 min. Veratraldehyde was chosen as the internal standard. The set injection volume and oven temperature were 20 μL and 35 °C, respectively. The flow rate was 1 mL/min. The chromatograms were monitored at 280 and 315 nm and acquired at 280 nm.

2.5. Validation of the method

To assure the reliability of the method, validation parameters such as selectivity, linearity, limits of detection and quantification, accuracy, precision, recovery and robustness were evaluated. The method validation was performed according to International Conference on Harmonization (ICH, 2005) and the Brazilian National Health Surveillance Agency (ANVISA, 2017) guidelines. In the validation experiments, all the parameters were performed in triplicate. The selectivity of the method was established according to the separation efficiency between the chromatographic peaks defined by evaluation of the chromatographic resolution. The peaks were assigned according to their retention times with the authentic standards as well as based on UV spectra.

The parameters linearity, limit of detection, and limit of quantitation were assessed by the construction of an analytical curve of each target compound from 10 to 500 μg/mL. In each solution, veratraldehyde was added as the internal standard at 100 μg/mL. The solutions were injected in triplicate, for four consecutive days. The ratio between the area of each analyte and the IS, was the response used to plot the analytical curve. The concentration that generated a peak with an intensity equivalent to three times the baseline noise was considered the limit of detection, while for the limit of quantification, it was considered the concentration that showed a peak equivalent to ten times the baseline noise, calculated according to the equations: $LOD = (3.3\,SD)/IC$, $LOQ = (10\,SD)/IC$, where SD is the standard deviation of the intercept obtained from the calibration curves, and IC is the slope of the analytical curve.

For evaluation of precision, three levels of concentrations of the calibration curve (high 300, medium 100, and low 50 μg/mL) were selected to measure the repeatability of the results intra (same day) and interday (four consecutive days) by the relative standard deviation (RSD%) of the responses. The accuracy results were estimated by the comparison between the theoretical and real values of the same three concentration solutions.

To evaluate the recovery, 10 g of grounded brown propolis matrix were exhaustively extracted in a Soxhlet apparatus for 12 h, using 500 mL of 96% of ethanol. The propolis sample

was dried in an air-circulating oven at 50 °C for 4 h. The extract was concentrated, and both extract and matrix were analyzed by developed HPLC method. A standard solution containing coumaric acid, 5,7-dimethoxykaempferol and artepillin C was prepared at a final concentration of 100 μg/mL of each compound. After that, in 200 mg of this propolis biomass was spiked with the standard solution in three concentrations levels: 3 mL (300 μg/mL, high level), 1 mL (100 μg/mL, medium level), and 0.5 mL (50 μg/mL, low level). The spiked matrix was dried at room temperature. For the extraction a solution of EtOH:H_2O (7:3) was used. Veratraldehyde was added to the extraction solvent as the internal standard, and benzophenone was used as the secondary internal standard at 50 μg/mL. The extraction process was performed using a shaker incubator at 35 °C and 140 rpm for 120 min. The samples were filtered and analyzed by developed HPLC method to quantitate the standard compounds. The experiments were performed in quadruplicate, and the recovery percentage was calculated considering the theoretical and real concentration values.

The matrix effect was also evaluated, for that in eight different flasks each containing 200 mg the brown propolis biomass, a solution of coumaric acid, 5,7-dimethoxykaempferol and artepillin C at 10, 25, 50, 100, 200, 300, 400 and 500 μg/mL was added. One additional flask containing the propolis biomass without spiking the standards was used to determine the concentration of these compounds in the propolis. The extraction was performed and analyzed in the HPLC method. Using the areas of the standards peaks and the internal standard, the analytical curve in the matrix was plotted, which was compared to the analytical curve of the standards in solution. The angular coefficients of the two analytical curves were compared to verify the parallelism between them.

For robustness a Box, Hunter & Hunter factorial design was elaborated through the software Statistica 8 to measure the capacity of the method to quantitate the standards after small changes in the run conditions within three different levels. The parameters measured were the run temperature, wavelength and flow rate. The low, medium, and high levels were as follows: temperature, 33, 35 and 37 ºC; wavelength, 277, 280, and 283 nm and flow rate, 0.9, 1.0, and 1.1 mL/min.

2.6. Cytotoxic and antiparasitic evaluation

For the evaluation of cytotoxicity, four tumor cell lines (BT-549, SK-MEL, SK-OV-3, KB) and two normal cell lines (VERO and LLC-PK1), obtained from the American Type Culture Collection (ATCC, Rockville, MD), were used. Cell culture and treatment procedures

were those described previously by Ribeiro et al. (2021a). Viability was tested using a tetrazolium dye WST- 8. Control substances were included on the assay. On the tumoral cell lines DMSO (1%) was used as negative control and doxorubicin (DOX) as positive control. On the nontumoral cell lines DMSO was used as positive control (10%). The dose-response curves of cell viability were used to calculate de IC_{50} values of brown propolis.

The antileishmanial activity was tested against promastigote and amastigote forms of *Leishmania amazonensis* (MHOM/BR/PH8) grown in RPMI 1640 medium, as described by Arruda et al. (2020b). Promastigote forms of *L. amazonensis* (2 x 10^6 parasites/mL) were incubated in 96-well microtiter plates with VOBP previously dissolved in 1% dimethylsulfoxide (DMSO), for final concentrations of 3.12, 6.25, 12.5, 25 and 50 µg/mL. The bioassays were performed in triplicate, using a medium with 0.1% DMSO as a negative control and amphotericin B as a positive control.

Brown propolis extract was tested in vitro against two *Plasmodium falciparum* strains: D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) as described by Ribeiro et al. (2021a). Red blood cells (200 µL) infected with *P. falciparum* were added to a 96-well plate containing serially diluted samples (10 µL) of the extract, and then were incubated for 72 h (37°C, 90% N_2, 5% O_2, and 5% CO_2). The parasitic lactate dehydrogenase (pLDH) activity was measured by a colorimetric assay at 650 nm. As negative control was used DMSO, and as positive controls were included chloroquine and artemisinin. The software XLfit 4.2 was used to calculate the IC_{50} value of the extract.

3. Results and Discussion

In recent years, natural products have gained prominence and notoriety in the market, impacting the need for more research involving these products (Símaro et al., 2021). As there is an increase in demand in propolis production, combined with the emergence of new products from this natural product, it is necessary to use analytical tools capable of ensuring the quality of these products. One suitable analytical technique is chromatography, which is capable of performing both qualitative and quantitative analyses (Mangabeira da Silva et al. 2020). Propolis is a complex natural product rich in flavonoids and phenolic acids. To phenolics compounds with strong chromophore groups, UV-based detectors ensure a good selectivity and sensitivity in the identification and quantification of bioactive compounds, so it represent an ideal choice to develop quality control methods.

The phytochemical study of the sample from Minas Gerais revealed a high complexity on the chemical profile. As seen in figure 2, fifteen compounds were isolated from the hydroalcoholic extract. The sample was characterized by the presence of phenylpropanoids including: ferulic acid **(5)**; coumaric acid **(4)**; caffeic acid **(3)** and cinnamic acid **(7)**. Also, flavonoids and phenolics as: narigenin **(2)**; garbanzol **(6)**; dihydrokaempferide **(8)**; resokaempferol **(1)**; kaempferol 3,7,4'-trimethyl ether **(11)**; 5,7-dimethoxykaempferol **(12)**; 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran **(15)**. As well as cinnamic acid derivatives as baccharin **(14)**, artepillin C **(13)**, drupanin **(10)** e capilartemisin A **(9)**.

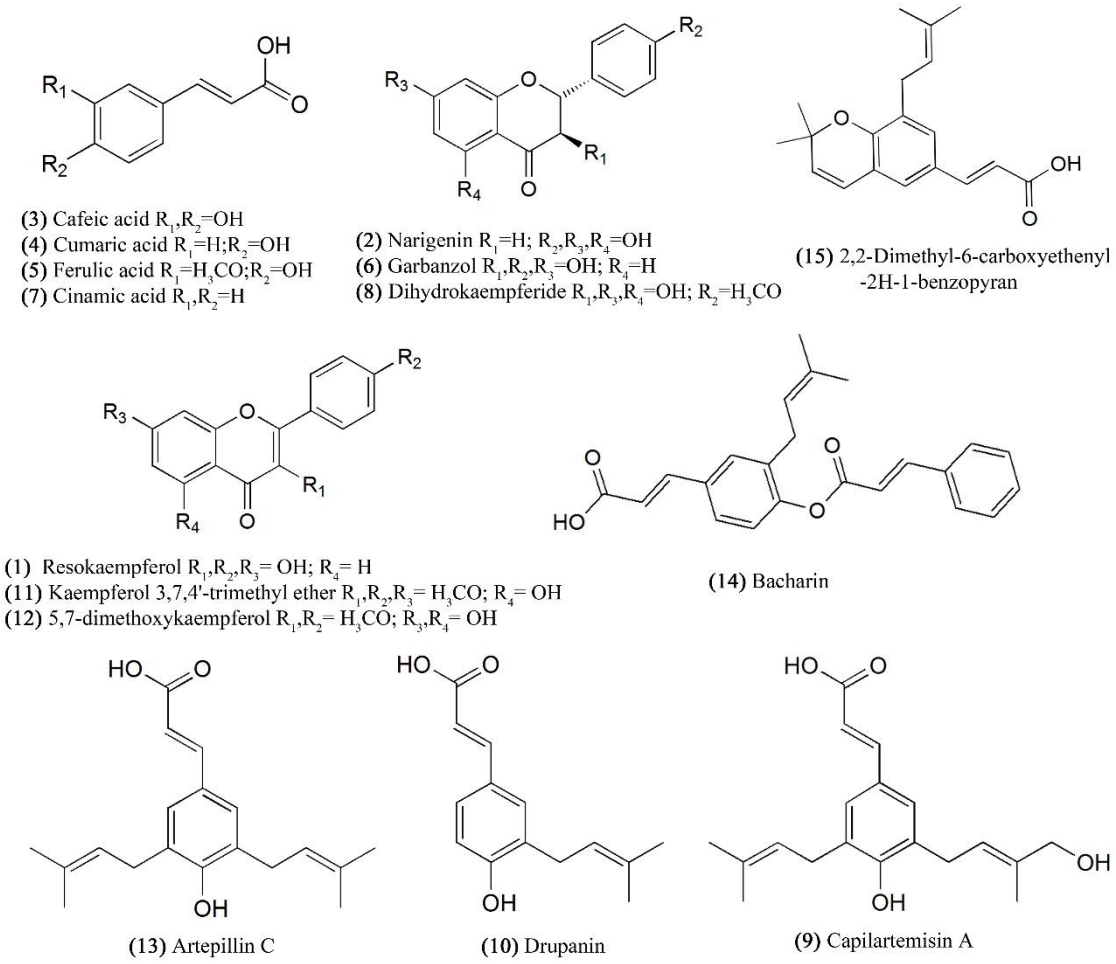

Figure 2. Isolated compounds from Brazilian brown propolis crude extract.

Artepillin C, baccharin, drupanin, and *p*-coumaric acid derivatives are identified as chemical markers of Brazilian green propolis, that has as main botanical source the species *Baccharis dracunculifolia* (Arruda et al., 2020a). Several studies previously reported the presence of *p*-coumaric acid, drupanin, artepillin C and baccharin on brown propolis extracts (Borges et al., 2016; do Nascimento Araújo et al., 2020).

The chemical similarity between green and some brown propolis samples was studied by Salomão et al. (2008), the authors conclude that in some locations, brown propolis could have a botanical origin on *B. dracunculifolia* and *Auraucaria* spp. The samples with two botanical sources presented as major compounds cinnamic acid derivatives, *p*-coumaric acid, caffeolquinic acid derivatives, and kaempferide. Instead, samples with a probably botanical origin on *Auraucaria* spp. resin, were enriched with coniferaldehyde derivatives (Sartori et al. 2021). Compounds as cinnamic acid and 2,2-dimethyl-6-prenyl-2H-1-benzopyran-6-propenoic acid, detected by HPLC-PDA analysis on green propolis samples by Rodrigues et al. (2016), were present on the studied sample. The similarity on the chemical profile with green propolis from *B. dracunculifolia*, indicate that the sample from Cabo Verde has a probable origin on this botanical source.

Samples from the southern are characterized by the presence of flavonols, caffeoyl-quinic acids, *p*-coumaric acid derivatives, benzoic acid derivatives and dihydroflavonols (Machado et al., 2021). Other reports, suggest the presence of additional secondary metabolites as acetylisocupressic acid (Fernandes et al., 2019), rutin, chlorogenic acid (Waller et al., 2017), chrysin, pinocembrin, galangin (Fabio et al., 2019) and diterpenes as isocupressic acid, (*E*)-communic acid, (*Z*)-communic acid and abietic acid (Santos et al., 2021; Tazawa et al., 2016).

A RP-HPLC-PDA method was established to the analysis of brown propolis samples. During the development of the analytical method, parameters as temperature, flow rate, gradient, chromatographic conditions such as column and mobile phase were optimized, aiming a good peak separation (Figure 3). Thus, the best resolution of peaks, were achieved with 60 min analysis. All the obtained parameters in the method validation process are in accordance with the validation guidelines of ICH (2005) and ANVISA (2017). The developed method resulted in a good peak separation of compounds from Brazilian brown propolis extract, with a good chromatographic resolution for all evaluated analytes (> 1.5). The UV spectra of each peak and its respective standard matched perfectly, indicating that each peak corresponds to the respective chromatographic standard.

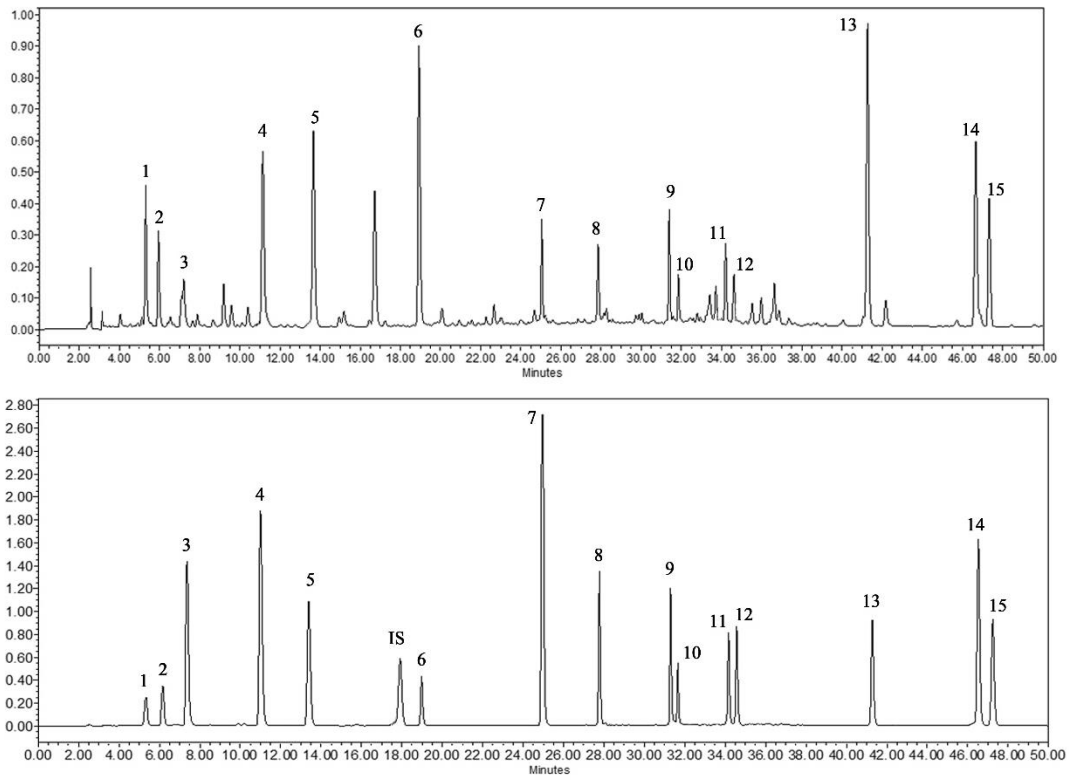

Figure 3. RP-HPLC-DAD chromatogram of the Brazilian brown propolis crude extract (A) and 1- Resokaempferol; 2- Naringenin; 3- Cafeic acid; 4- Coumaric acid; 5- Ferulic acid; IS- Veratraldehyde; 6- Garbanzol; 7- Cinamic acid; 8- Dihydrokaempferide; 9- Capilartemisin A; 10- Drupanin; 11- Kaempferol 3,7,4'-trimethyl ether; 12- 5,7-dimethoxykaempferol; 13- Artepillin C; 14- Baccharin; 15- 2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran (B).

For linearity assessment, solutions of the standards at eight different concentrations were analyzed by the RP-HPLC-PDA method furnishing the correlation (R) and determination (R^2) coefficients values obtained by an analytical curve of each standard statistically analyzed by minimum square method, and the equation for their concentration calculation. The R values of all analytical curves were above 0.99 giving a linearity according to validation guidelines. Besides, residual analyzes was performed, confirming homoscedasticity of the data. Also, the analytical curves do not have lack of fit ($p > 0.05$). In addition, the range obtained by limits of detection and quantification were satisfactory, indicating that the chromatographic method can detect and quantify the selected compounds at low concentrations (Table 1).

Table 1. Linearity, limits of detection and quantification of analytes.

Compound	λ (nm)	Equation	R^2	R	LD	LQ	Minimum Observed residual value	Maximum Observed residual value	Lack of fit *p* value
Resokaempferol	325,8	y = 0,0014x - 0,0025	0,9993	0.9996	1,4	4,3	-0.22058	3.49354	0,473468
Naringenin	334,2	y = 0,0029x - 0,0069	0,9975	0.9998	1,1	3,5	-1.64064	2.00453	0,160235
Cafeic acid	321,0	y = 0,008x + 0,005	0,9960	0.9979	1,9	5,9	-1.04380	1.17427	0,477220
Coumaric acid	311,5	y = 0,011x + 0,0423	0,9939	0.9969	1,5	4,7	-1.30497	0.76496	0,850241
Ferulic acid	321,0	y = 0,0068x - 0,0227	0,9974	0.9986	3,6	8,1	-2.19150	0.61820	0,228001
Garbanzol	327,0	y = 0,0018x - 0,0094	0,9974	0.9986	3,0	9,3	-1.00715	2.89495	0,910247
Cinamic acid	276,9	y = 0,016x + 0,265	0,9914	0.9956	3,8	9,6	-1.86322	1.63462	0,464964
Dihydrokaempferide	291,2	y = 0,0043x - 0,0091	0,9923	0.9961	3,0	9,3	-1.64832	3.19076	0,891103
Capilartemisin A	313,9	y = 0,0034x - 0,0081	0,9956	0.9977	2,9	8,9	-2.18570	2.31728	0,524094
Drupanin	313,9	y = 0,0015x - 0,0036	0,9973	0.9986	0,4	1,4	-0.61001	2.46272	0,068993
Kaempferol 3,7,4'-trimethyl ether	361,9	y = 0,0046x + 0,0101	0,9943	0.9971	2,1	6,6	-1.15541	1.95651	0,193089
5,7-dimethoxykaempferol	361,9	y = 0,0057x + 0,001	0,9944	0.9971	3,8	7,5	-1.32506	0.76748	0,558717
Artepillin C	313,9	y = 0,0043x - 0,0206	0,9964	0.9981	4,3	9,3	-3.11499	1.86287	0,734727
Baccharin	289,5	y = 0,0098x + 0,0548	0,9924	0.9961	4,0	9,4	-0.77263	1.22396	0,162892
2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran	319,8	y = 0,0078x - 0,0035	0,9962	0.9980	0,7	2,2	-0.47415	2.29946	0,681924

R^2: determination coefficient; R: correlation coefficient; LD: limit of detection (µg/mL); LQ: limit of quantification (µg/mL).

The method precision was evaluated regarding the repeatability (intraday precision) and intermediate precision (interday precision). The results showed low variation among the analyses, since both, intraday and interday precisions showed relative standard deviations (RSDs) lower than 5.27%. The accuracy results were approximately 100%, with variations among 91.30% and 109.02%, indicating that the analytical method furnished accurate results (Table 2). Therefore, the developed method can be considered accurate and precise once these parameters are in accordance with guidelines.

Table 2. Precision and accuracy of the method.

Compound	Level	Precision (RSD)		Accuracy (%)	E(%)	Recovery (%)
		Intraday	Interday			
Resokaempferol	Low	1.66	3.34	91.30 ± 0.9	1.86	-
	Medium	3.99	3.27	94.80 ± 2.40	1.53	-
	High	2.74	2.20	95.61 ± 0.33	1.08	-
Naringenin	Low	2.50	1.79	94.82 ± 2.20	1.35	-
	Medium	0.90	1.33	94.08 ± 0.86	0.93	-
	High	3.99	2.89	103.98 ± 2.62	-1.13	-
Cafeic acid	Low	0.04	0.61	97.84 ± 1.88	0.48	88.61 ± 1.94
	Medium	0.56	0.64	97.10 ± 0.85	0.13	92.44 ± 3.10
	High	0.05	1.78	103.77 ± 1.47	-0.70	90.83 ± 2.72
Coumaric acid	Low	1.59	0.94	95.06 ± 1.59	1.90	-
	Medium	0.04	0.54	95.73 ± 1.11	0.43	-
	High	3.19	1.99	105.60 ± 0.96	-0.34	-
Ferulic acid	Low	0.83	1.10	95.41 ± 1.12	0.02	-
	Medium	0.11	0.92	94.97 ± 1.91	1.97	-
	High	1.18	1.53	103.68 ± 1.93	-1.54	-
Garbanzol	Low	1.65	2.37	94.56 ± 1.42	0.19	-
	Medium	1.13	0.90	109.02 ± 0.57	-1.94	-
	High	3.36	2.40	99.13 ± 1.83	-0.11	--
Cinamic acid	Low	0.55	0.56	107.38 ± 2.77	-0.13	-
	Medium	3.58	1.61	106.23 ± 1.18	-1.74	-
	High	0.32	0.76	104.62 ± 1.24	-1.66	-
Dihydrokaempferide	Low	0.69	0.60	97.52 ± 1.25	0.85	-
	Medium	0.19	0.81	95.70 ± 2.30	1.70	-
	High	0.46	1.34	106.21 ± 1.91	1.17	-

	Low	0.61	0.46	96.52 ± 1.67	0.87	-
Capilartemisin A	Medium	0.79	1.59	94.47 ± 1.89	0.58	-
	High	2.86	1.91	104.13 ± 1.39	-0.01	-
	Low	3.04	5.27	95.74 ± 1.53	1.43	-
Drupanin	Medium	0.69	0.83	92.18 ± 2.09	1.66	-
	High	0.95	3.03	98.89 ± 4.07	0.28	-
	Low	2.06	1.80	98.35 ± 0.62	0.25	-
Kaempferol 3,7,4'-trimethyl ether	Medium	1.41	1.30	103.78 ± 0.32	-0.65	-
	High	1.07	1.74	106.04 ± 2.01	-1.47	-
	Low	2.76	1.96	97.40 ± 0.91	0.39	96.61 ± 2.95
5,7-dimethoxykaempferol	Medium	0.78	1.13	94.59 ± 0.52	0.75	89.79 ± 4.04
	High	0.65	1.31	105.41 ± 1.30	1.29	92.96 ± 3.38
	Low	3.41	4.66	100.61 ± 2.43	-1.40	93.12 ± 3.97
Artepillin C	Medium	3.63	3.07	92.71 ± 2.29	0.04	103.26 ± 3.17
	High	2.95	1.97	102.15 ± 1.43	1.63	100.90 ± 4.63
	Low	0.66	0.70	92.84 ± 1.91	1.43	-
Baccharin	Medium	0.29	0.50	100.64 ± 1.71	-1.80	-
	High	0.13	1.44	107.69 ± 2.17	1.61	-
	Low	2.03	2.07	93.76 ± 2.60	1.70	-
2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran	Medium	0.31	0.76	99.08 ± 0.61	0.29	-
	High	0.41	1.71	104.81 ± 1.47	-0.17	-
	Low	-	-	-	-	102.77 ± 2.98
Veratraldehyde (IS)	Medium	-	-	-	-	98.12 ± 1.85
	High	-	-	-	-	91.87 ± 2.69

RSD: relative standard deviation; E: error.

To the recovery method evaluation, a Soxhlet apparatus was used to perform the exhaustive extraction of brown propolis. The extract yield was 78.3%. It was found that recovery was good, between 93 and 107% at low, medium, and high concentrations. Thus, the optimized method can be considered reliable for performing the extraction of coumaric acid, 5,7-dimethoxykaempferol, artepillin C and other chemically similar phenolic compounds. As these phenolics are the major compounds in the extract of brown propolis and belongs to the same class as the other compounds, it was used as a representative for recovery studies. Furthermore, the recovery of the internal standard was, between 91 and 102%.

To evaluate the matrix effect in the quantitation of the phenolic compounds, the parallelism between the lines obtained from analytical curves of coumaric acid, 5,7-

dimethoxykaempferol and artepillin C in solution and in the crude extract after spiking the analytes in the same concentrations as those of the analytical curve was verified. To be parallel, two lines should present similar angular coefficients, which was observed statistically after applying the *t* test between the angular coefficients of the obtained analytical curves. The *p* value was >0.05. Therefore, no significant difference was observed between them, which confirms the parallelism between the lines and that there was no significant matrix effect in the quantitation of the samples, which is in accordance with ANVISA guidelines.

Finally, the robustness was evaluated by making small variations in four parameters of the method in two levels. The changes in run temperature, wavelength and flow rate did not lead to a significant variation in the concentration of the analyzed compounds RP-HPLC-PDA method ($p<0,05$). Consequently, the developed method proved to be robust.

The cytotoxic evaluation of the hydroalcoholic extract, indicated no effect on the viability of the normal cell lines (VERO and LLC-PK1), by contrast, brown propolis exhibited toxicity effect on tumoral cells, with the following IC_{50} values: 80 µg/mL for SK-MEL; 82 µg/mL for KB; 71 µg/mL for SK-OV-3; and 64 µg/mL for BT-549. Some studies suggest that higher levels of flavonoids and phenolic compounds on the samples are related with a strong cytotoxic potential (Hochheim et al., 2019). Isolated compounds present on Cabo Verde sample, have been tested against tumoral cell lines. Artepillin C and *p*-coumaric acid, exhibited IC_{50} values of 15.39 and 42.20 µM on AGP-01 cell line (Arruda et al., 2020b).

According to Ribeiro et al. (2021a), brown propolis from São Paulo State, and with a probably origin on *Pinus* spp., *Eucalyptus* spp. and *A. angustifolia* resins, also demonstrated a cytotoxic effect on tumoral cell lines. The hydroalcoholic extract presented IC_{50} values of 85 µg/mL for SK-MEL; 71 µg/mL for KB; 42 µg/mL for SK-OV-3; and 57 µg/mL for BT-549. No cytotoxic effect was observed on the non-tumoral cell lines assessed. The cytotoxic potential of brown propolis have been tested against other cell lines. A sample from Paraná State, has a promising cytotoxic potential on tumor cell lines SF-295 (glioblastoma cells, IC_{50} 27.91 µg/mL), HCT-116 (colon cancer cells, IC_{50} 19.43 µg/mL); OVCAR-8 (ovarian cancer cells, IC_{50} 26.97 µg/mL), with especial potential against HL-60 line (leukemia cells) with an IC_{50} of 9.44 µg/mL, and a selective index of 22.99 (Machado et al., 2016). However, other studies against the same cell lines, with samples from Santa Catarina, Rio Grande do Sul and Parana States, did not exhibited cytotoxic properties.

A range of biological effects are attributed to propolis, including the leishmanicidal property. The brown propolis used in this study showed promising leishimanicidal activity,

with IC_{50} of 1.8 µg/mL against the promastigote form of *L. amazonensis* and IC_{50} of 2.4 µg/mL against the amastigote form of *L. amazonensis* (Figure 4).

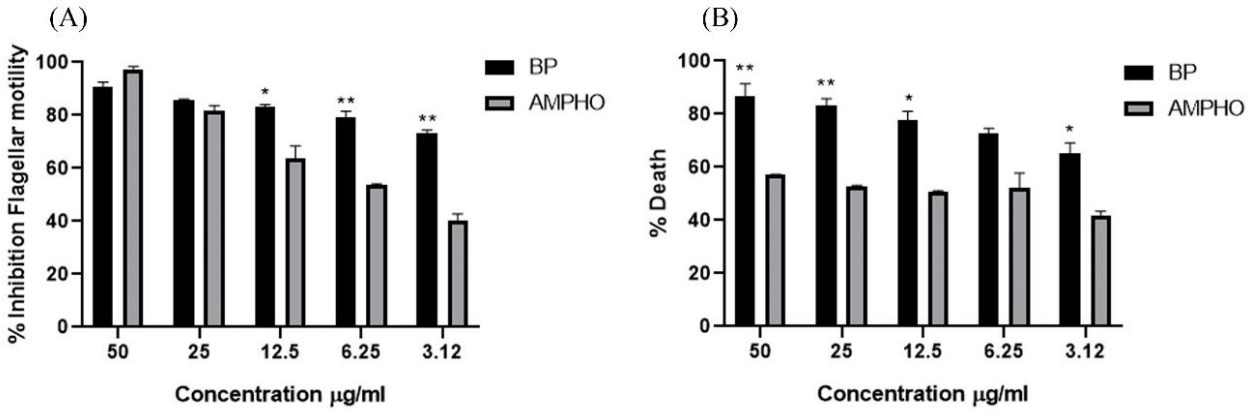

Figure 4. Leishimanicidal activity against promastigote forms (A) and amastigote forms (B) of *Leishmania amazonensis*. BP: Brazilian Brown Propolis; AMPHO: Amphotericin B. ** Significant difference from the control group, *t* test ($p < 0,05$).

According to Santana et al. (2014), Brazilian brown propolis from Piauí state, exhibit a leishmanicidal effect against *L. amazonensis* with a marked time-dependent inhibitory activity. The hydroalcoholic extract showed an IC_{50} value of 11.87 µg/mL after 24 h, 12.08 µg/mL after 24 h, and 4.64 µg/mL at 72 h. The *n*-hexane, dichloromethane and ethyl acetate fractions were also evaluated, finding, a leishmanicidal potential of the ethyl acetate fraction characterized by the presence of *p*-coumaric acid and 3,5-dihydroxybenzoic acid, with an IC_{50} of 8.83 µg/mL, after 72 h (Santana et al. 2014).

Dichloromethane fraction of brown propolis characterized by the presence of the triterpene lupeol by GC-MS analysis, also displayed a promising antileishmanial potential. An increase of 31% on the biological activity of the dichloromethane fraction, was observed with an IC_{50} of 4.96 µg/mL (Santana et al., 2014). Studies with the volatile oil of brown propolis from Minas Gerais, displayed a significant leishmanicidal activity, with IC_{50} of 21.3 µg/mL against amastigote forms and IC_{50} of 25.1 µg/mL against promastigote forms of *L. amazonensis*. This fraction was characterized by the presence of nerolidol (10.47%), spathulenol (7.41%), 1,8-cineole (7.03%), *β*-caryophyllene (6.14%), terpinen-4-ol (6.06%), *γ*-palmitolactone (5.73%), *α*-copaene (5.16%), and *γ*-muurolene (5.07%) (Ribeiro et al., 2021b).

Brown propolis from Cabo Verde was characterized by the presence of artepillin C, garbanzol, ferulic acid, coumaric acid, baccharin, resokaempferol, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran. In leishmanicidal assays, artepillin C and *p*-coumaric acid were active against *L. amazonensis* promastigotes, with IC_{50} values of 17.46 and 48.46 µM

(Arruda et al., 2020b). Also, *p*-coumaric acid were active against amastigotes, with IC_{50} lower than 3.12 µM. It is described that caffeic acid induces changes on plasma membrane and mitochondria of infected cells with *L. amazonensis*, promoting apoptosis (Zulhendri et al., 2021). Probably the presence of those secondary metabolites on the brown propolis from Minas Gerais, is generating the promising leishmanicidal effect observed expressed in those low IC_{50} values.

Other studies with Brown propolis from Cuba, indicated leishmanicidal activity against *Leishmania infantum* (Monzote et al., 2012). The methanolic extract from different locations, was evaluated on amastigotes, presenting IC_{50} between 7.5 – 22.2 µg/mL, close to the control mitelfosine with an IC_{50} of 7.7 µg/mL. Different from Brazilian propolis, the Cuban extracts were characterized by the major presence of nemorosone, a polycyclic polyprenylated acylphloroglucinol. Probably, the presence of chemical markers as artepillin C and *p*-coumaric on Cabo Verde sample can have a strong influence on the leishmanicidal activity observed on this study.

About the antiplasmodium activity, Cabo Verde brown propolis presented activity against *P. falciparum* strains, with IC_{50} of 18.1 µg/mL (1.8 of selectivity index - SI) and 15.6 µg/mL (2.1 SI), against D6 and W2 strains respectively. The IC_{50} for the chloroquine controls were: 0.02 µg/mL for D6, and 0.15 µg/mL for W2. And the IC_{50} values for the artemisinin were: 0.014 µg/mL for D6, 0.007 µg/mL for W2.

Brazilian brown propolis extract from a different location (Angatuba, São Paulo), revealed activity against D6 and W2 *P. falciparum* strains, with lower IC_{50} values (5.3 and 9.7 µg/mL, respectively) (Ribeiro et al., 2021a). The major compounds on the sample were the diterpenes 7-oxodehydroabietic acid, dehydroabietic acid, and isopimaric acid, however the extract also presented phenolic compounds, lignanes and flavanonols as caffeic acid, matairesinol, pinoresinol and 7-*O*-methylaromadendrin. This chemical profile was an indicative of the contribution of three botanical sources: *Pinus* spp., *Eucalyptus* spp. and *Araucaria angustifolia*.

Brown Cuban propolis from different locations were also effective against *P. falciparum* (chloroquine-susceptible), with IC_{50} values between 0.2-12.5 µg/mL, the lowest value was more effective than the control chloroquine with an IC_{50} of 0.3 µg/mL (Monzote et al., 2012). Wink (2012), comments that in *P. falciparum* IC_{50} values lower than 11 µM indicates potential of the sample for drug development, as already seen for some natural products as phenolics and terpenoids especially sesquiterpenes, diterpenes and triterpenes.

4. Conclusion

Brown propolis from Cabo Verde (Minas Gerais), was characterized by the presence coumaric acid, ferulic acid, cinamic acid, drupanin, artepillin C and baccharin, compounds also identified on green propolis from *B. dracunculifolia*, indicating a probable origin on this plant species. A RP-HPLC-PDA method was developed and validated for the analysis of brown propolis samples with the previously chemical fingerprint. The method agreed with the parameters established by the validation guidelines. The studied samples exhibited a promising leishmanicidal activity against *L. amazonensis*, and a moderate antimalarial effect against *P. falciparum*. Also, cytotoxic potential was observed against some tumoral cell lines, with no effect on non-tumoral strains.

5. Acknowledgements

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6. Disclosure statement

The authors reported no potential conflict of interest.

7. References

Aldana-Mejía, J.A., Ccana-Ccapatinta, G. V., Squarisi, I.S., Nascimento, S., Tanimoto, M.H., Ribeiro, V.P., Arruda, C., Nicolella, H., Esperandim, T., Ribeiro, A.B., de Freitas, K.S., da Silva, L.H.D., Ozelin, S.D., Oliveira, L.T.S., Melo, A.L.A., Tavares, D.C., Bastos, J.K., 2021. Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum*. Chem. Res. Toxicol. 34, 1024–1033. https://doi.org/10.1021/acs.chemrestox.0c00356

Andrade, J.K.S., Denadai, M., de Oliveira, C.S., Nunes, M.L., Narain, N., 2017. Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region. Food Res. Int. 101, 129–138. https://doi.org/10.1016/j.foodres.2017.08.066

ANVISA, 2017. Resolução da diretoria colegiada - RCD No. 166, de 24 de julho de 2017.

Arruda, C., Pena Ribeiro, V., Oliveira Almeida, M., Aldana Mejía, J.A., Casoti, R., Kenupp Bastos, J., 2020a. Effect of light, oxygen and temperature on the stability of artepillin C and p-coumaric acid from Brazilian green propolis. J. Pharm. Biomed. Anal. 178. https://doi.org/10.1016/j.jpba.2019.112922

Arruda, C., Ribeiro, V.P., Mejía, J.A.A., Almeida, M.O., Goulart, M.O., Candido, A.C.B.B., dos Santos, R.A., Magalhães, L.G., Martins, C.H.G., Bastos, J.K., 2020b. Green Propolis: Cytotoxic and Leishmanicidal Activities of Artepillin C, p-Coumaric Acid, and Their Degradation Products. Rev. Bras. Farmacogn. 30, 169–176. https://doi.org/10.1007/s43450-020-00043-3

Bankova, V., Bertelli, D., Borba, R., Conti, B.J., Barbosa Da, I., Cunha, S., Danert, C., Eberlin, M.N., Falcão, S.I., Inés, M., Moreno, N., Papotti, G., Popova, M., Santiago, K.B., Salas, A., Helena, A.C., Sawaya, F., Schwab, N.V., Sforcin, M., Simone-Finstrom, M., Spivak, M., Trusheva, B., Vilas-Boas, M., Wilson, M., Zampini, C., 2019. Standard methods for *Apis mellifera* propolis research. J. Apic. Res. 58, 1–49. https://doi.org/10.1080/00218839.2016.1222661

Bankova, V., Popova, M., Trusheva, B., 2018. The phytochemistry of the honeybee. Phytochemistry 155, 1–11. https://doi.org/10.1016/j.phytochem.2018.07.007

Beserra, F.P., Gushiken, L.F.S., Hussni, M.F., Ribeiro, V.P., Bonamin, F., Jackson, C.J., Pellizzon, C.H., Bastos, J.K., 2021. Artepillin C as an outstanding phenolic compound of Brazilian green propolis for disease treatment: A review on pharmacological aspects. Phyther. Res. 35, 2274–2286. https://doi.org/10.1002/ptr.6875

Borges, C.H.G.G., Cruz, M.G., Carneiro, L.J., da Silva, J.J.M.M., Bastos, J.K., Tavares, D.C., de Oliveira, P.F., Rodrigues, V., Veneziani, R.C.S.S., Parreira, R.L.T.T., Caramori, G.F., Nagurniak, G.R., Magalhães, L.G., Ambrósio, S.R., 2016. *Copaifera duckei* oleoresin and Its main nonvolatile terpenes: *in vitro* schistosomicidal properties. Chem. Biodivers. 13, 1348–1356. https://doi.org/10.1002/cbdv.201600065

de Freitas, A. da S., Barth, O.M., Sales, É. de O., Matsuda, A.H., de Almeida-Muradian, L.B., 2011. A palynological analysis of Brazilian propolis samples. J. ApiProduct ApiMedical Sci. 3, 67–74. https://doi.org/10.3896/ibra.4.03.2.01

do Nascimento Araújo, C., Mayworm, M.A.S., Yatsuda, R., Negri, G., Salatino, M.L.F., Salatino, A., Timenetsky, J., Campos, G.B., 2020. Chemical composition and antimycoplasma activity of a brown propolis from southern Brazil. J. Food Sci. Technol. 57, 4228–4235. https://doi.org/10.1007/s13197-020-04461-y

Fabio, G., Federica, C., Alfredo, F., Nicola, V., 2019. Recent advances in analytical approaches for the standardization and quality of polyphenols of propolis. J. Med. Plants Res. 13, 487–500. https://doi.org/10.5897/jmpr2019.6849

Fernandes, F.H., Guterres, Z.D.R., Corsino, J., Garcez, W.S., Garcez, F.R., 2019. Assessment of the mutagenicity of propolis compounds from the Brazilian cerrado biome in somatic cells of *Drosophila melanogaster*. Orbital 11, 307–313. https://doi.org/10.17807/orbital.v11i5.1418

Hochheim, S., Guedes, A., Faccin-Galhardi, L., Rechenchoski, D.Z., Nozawa, C., Linhares, R.E., Filho, H.H. da S., Rau, M., Siebert, D.A., Micke, G., Cordova, C.M.M. de, 2019. Determination of phenolic profile by HPLC–ESI-MS/MS, antioxidant activity, in vitro cytotoxicity and anti-herpetic activity of propolis from the Brazilian native bee Melipona quadrifasciata. Rev. Bras. Farmacogn. 29, 339–350. https://doi.org/10.1016/j.bjp.2018.12.010

ICH, I.C. on H., 2005. Validation of analytical procedures: text and methodology Q2(R1), in: Validation of Analytical Procedures. ICH, Geneva, p. 13.

Machado, C.S., Finger, D., Dias De Lima, T.C., Modesto, T., Monteiro, M.C., de Oliveira, F. de C.E., Pessoa, C., Torres, Y.R., 2021. Seeking the Regional Identity of Brown Propolis Produced in Southern Brazil and Linked to Total Levels of Bioactive Compounds and Biological Activity. ACS Food Sci. Technol. 1, 176–185. https://doi.org/10.1021/acsfoodscitech.0c00052

Machado, C.S., Mokochinski, J.B., Lira, T.O. de, de Oliveira, F. de C.E., Cardoso, M.V., Ferreira, R.G., Frankland Sawaya, A.C.H., Ferreira, A.G., Pessoa, C., Cuesta-Rubio, O., Monteiro, M.C., de Campos, M.S., Torres, Y.R., 2016. Comparative Study of Chemical Composition and Biological Activity of Yellow, Green, Brown, and Red Brazilian Propolis. Evidence-Based Complement. Altern. Med. 2016, 1–11. https://doi.org/10.1155/2016/6057650

Mangabeira da Silva, J.J., Pena Ribeiro, V., Lemos, M., Miller Crotti, A.E., Rogez, H., Kenupp Bastos, J., 2020. Reliable Methods for Analyses of Volatile Compounds of Copaifera Oleoresins Combining Headspace and Gas Chromatography. Chem. Biodivers. 17. https://doi.org/10.1002/cbdv.201900440

Monzote, L., Cuesta-Rubio, O., Fernandez, M.C., Hernandez, I.M., Fraga, J., Pérez, K., Kerstens, M., Maes, L., Cos, P., 2012. In vitro antimicrobial assessment of Cuban propolis extracts. Mem. Inst. Oswaldo Cruz 107, 978–984. https://doi.org/10.1590/S0074-02762012000800003

Ribeiro, V.P., Arruda, C., Aldana-Mejia, J.A., Bastos, J.K., Tripathi, S.K., Khan, S.I., Khan, I.A., Ali, Z., 2021a. Phytochemical, Antiplasmodial, Cytotoxic and Antimicrobial Evaluation of a Southeast Brazilian Brown Propolis Produced by Apis mellifera Bees. Chem. Biodivers. 18. https://doi.org/10.1002/cbdv.202100288

Ribeiro, V.P., Arruda, C., Alves, R., Magalh, L.G., 2020. Brazilian southeast brown propolis : gas chromatography method development for its volatile oil analysis , its antimicrobial and leishmanicidal activities evaluation. Phytochem. Anal. 1–8. https://doi.org/10.1002/pca.2988

Ribeiro, V.P., Arruda, C., da Silva, J.J.M., Aldana Mejia, J.A., Furtado, N.A.J.C., Bastos, J.K., 2019. Use of spinning band distillation equipment for fractionation of volatile compounds of Copaifera oleoresins for developing a validated gas chromatographic method and evaluating antimicrobial activity. Biomed. Chromatogr. 33, 1–12. https://doi.org/10.1002/bmc.4412

Ribeiro, V.P., Arruda, C., Mejía, J.A.A., Candido, A.C.B.B., dos Santos, R.A., Magalhães, L.G., Bastos, J.K., 2021b. Brazilian southeast brown propolis: gas chromatography method development for its volatile oil analysis, its antimicrobial and leishmanicidal activities evaluation. Phytochem. Anal. 32, 404–411. https://doi.org/10.1002/pca.2988

Ribeiro, V.P., Símaro, G.V., Mejia, J.A.A., Arruda, C., Bastos, J.K., 2021c. Anti-inflammatory and Antinociceptive Activities of the Hydroalcoholic Extract and the Volatile Fraction of Southeastern Brazilian Brown Propolis. Rev. Bras. Farmacogn. 31, 59–66. https://doi.org/10.1007/s43450-020-00122-5

Rodrigues, C.R.F., Plentz, L.C., Marcucci, M.C., Dihl, R.R., Lehmann, M., 2016. In vivo evaluation of mutagenic and recombinagenic activities of Brazilian propolis. Food Chem. Toxicol. 96, 117–121. https://doi.org/10.1016/j.fct.2016.07.037

Rodrigues, D.M., De Souza, M.C., Arruda, C., Pereira, R.A.S., Bastos, J.K., 2020. The Role of

Baccharis dracunculifolia and its Chemical Profile on Green Propolis Production by Apis mellifera. J. Chem. Ecol. 46, 150–162. https://doi.org/10.1007/s10886-019-01141-w

Salomão, K., Pereira, P.R.S., Campos, L.C., Borba, C.M., Cabello, P.H., Marcucci, M.C., De Castro, S.L., 2008. Brazilian propolis: Correlation between chemical composition and antimicrobial activity. Evidence-based Complement. Altern. Med. 5, 317–324. https://doi.org/10.1093/ecam/nem058

Santana, L.C.L.R., Carneiro, S.M.P., Caland-Neto, L.B., Arcanjo, D.D.R., Moita-Neto, J.M., Citó, A.M.G.L., Carvalho, F.A.A., 2014. Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of Leishmania amazonensis. Nat. Prod. Res. 28, 340–343. https://doi.org/10.1080/14786419.2013.856904

Santos, M.F.C., Oliveira, L.C., Ribeiro, V.P., Soares, M.G., Morae, G. de O.I., Sartori, A.G. de O., Rosalen, P.L., Bastos, J.K., Alencar, S.M., Veneziani, R.C.S., Ambrósio, S.R., 2021. Isolation of diterpenes from Araucaria sp Brazilian brown propolis and development of a validated HPLC method for its analysis. J. Sep. Sci. 1–21. https://doi.org/10.1002/jssc.202100374

Sartori, A.G.O., Spada, F.P., Ribeiro, V.P., Rosalen P.L., Ikegaki, M., Bastos, J.K., Alencar, S.M., 2021. An insight into the botanical origins of propolis from permanent preservation and reforestation areas of southern Brazil. Sci. Rep. 11, 22043. https://doi.org/10.1038/s41598-021-01709-1

Silva, R.P.D., Machado, B.A.S., De Abreu Barreto, G., Costa, S.S., Andrade, L.N., Amaral, R.G., Carvalho, A.A., Padilha, F.F., Barbosa, J.D.V., Umsza-Guez, M.A., 2017. Antioxidant, antimicrobial, antiparasitic, and cytotoxic properties of various Brazilian propolis extracts. PLoS One 12, 1–18. https://doi.org/10.1371/journal.pone.0173397

Símaro, G.V., Lemos, M., Mangabeira da Silva, J.J., Ribeiro, V.P., Arruda, C., Schneider, A.H., Wagner de Souza Wanderley, C., Carneiro, L.J., Mariano, R.L., Ambrósio, S.R., Faloni de Andrade, S., Banderó-Filho, V.C., Sasse, A., Sheridan, H., Andrade e Silva, M.L., Bastos, J.K., 2021. Antinociceptive and anti-inflammatory activities of Copaifera pubiflora Benth oleoresin and its major metabolite ent-hardwickiic acid. J. Ethnopharmacol. 271, 113883. https://doi.org/10.1016/j.jep.2021.113883

Tazawa, S., Arai, Y., Hotta, S., Mitsui, T., Nozaki, H., Ichihara, K., 2016. Discovery of a novel diterpene in brown propolis from the State of Parana, Brazil. Nat. Prod. Commun. 11, 201–205. https://doi.org/10.1177/1934578x1601100218

Waller, S.B., Peter, C.M., Hoffmann, J.F., Picoli, T., Osório, L. da G., Chaves, F., Zani, J.L., de Faria, R.O., de Mello, J.R.B., Meireles, M.C.A., 2017. Chemical and cytotoxic analyses of brown Brazilian propolis (Apis mellifera) and its in vitro activity against itraconazole-resistant Sporothrix brasiliensis. Microb. Pathog. 105, 117–121. https://doi.org/10.1016/j.micpath.2017.02.022

Wink, M., 2012. Medicinal Plants: A Source of Anti-Parasitic Secondary Metabolites. Molecules 17, 12771–12791. https://doi.org/10.3390/molecules171112771

Zulhendri, F., Chandrasekaran, K., Kowacz, M., Ravalia, M., Kripal, K., Fearnley, J., Perera, C.O., 2021. Antiviral, Antibacterial, Antifungal, and Antiparasitic Properties of Propolis: A Review. Foods 10, 1360. https://doi.org/10.3390/foods10061360

3.2. Capítulo 2 – Desenvolvimento e validação de método analítico por Cromatografia gasosa para análises se substâncias voláteis presentes na própolis marrom do sudeste brasileiro e

avaliação das propriedades antimicrobianas e leishimanicida.

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Article

Brazilian southeast brown propolis: gas chromatography method development for its volatile oil analysis, its antimicrobial and leishmanicidal activities evaluation.

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Abstract

Introduction: Propolis is widely used in folk medicine, and many factors can affect its chemical composition, including abiotic factors that can influence plants and bees. Therefore, analytical methods are powerful techniques in the quality control of such products.

Objective: Develop and validate an analytical method for quantifying volatile compounds in Brazilian Brown propolis, and evaluate its biological activities.

Methods: A CG-FID analytical method was validated, attending the parameters of international validation guidelines as ANVISA 2017 and ICH 2005, for quantification of compounds present in volatile oils from propolis. Evaluation of cytotoxic, antimicrobial, and leishmanicidal activities of the oil.

Results: The compounds 1,8-cineole, terpinen-4-ol, α-copaene, β-caryophyllene, γ-muurolene, nerolidol, spathulenol, and γ-palmitolactone were isolated from the volatile fraction of a Brazilian Brown Propolis and used in the method validation. All the validation parameters of the method were satisfactory. The volatile fraction displayed a significant leishmanicidal activity, with IC_{50}= 21.3 µg/mL against amastigote forms and IC_{50}= 25.1 µg/mL against promastigote forms of *Leishmania amazonensis*. The oil also displayed an antibacterial effect by inhibiting the growth of *Streptococcus mutans* and *Staphylococcus aureus* at 25 µg/mL and 50 µg/mL, respectively, but it was not cytotoxic against AGP-01, He-La and CHO-K1cell lines, with IC50> 100 µg/mL.

Conclusion: The GC-FID method can be a useful tool in the quality control of propolis material. The southeast brown propolis showed a high chemical complexity in its volatile fraction, which displayed leishmanicidal activity and bactericidal activity.

Keywords: Volatile oil; Southeast Brazilian Brown Propolis; Sesquiterpenes; Leishimanicidal; analytical method.

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1. Introduction

Propolis is a resin-type natural product produced by bees from parts of plants. In this way, the chemical composition of these products is closely related to the botanical source available to the bees. Bees produce propolis for protection and temperature maintenance of the hives, as well as to prevent contamination by bacteria or parasites (Kuropatnicki et al. 2013). For humankind, propolis has great importance since it is widely used in traditional medicine, mainly due to its anti-inflammatory properties, and it is also used mainly in the food and cosmetic industries due to its high antioxidant activity, making it of great economic importance (Machado et al. 2012).

Volatile oils can represent up to 10% of the composition of propolis, thus constituting a vital fraction that needs to be considered in the chemical and biological evaluation of propolis (Wagh, 2013). Volatile oil composition may vary due to several factors, including geographical localization, temperature, UV radiation, genetic variation of plants, storage and transportation conditions of propolis raw material, which can cause loss or degradation of the volatile compounds (Yang et al. 2010). These factors may compromise the chemical composition of volatile propolis compounds, making the analytical tools of great importance in the quality control of these products.

In addition to these factors, bees use the available natural plant resources, which generate a high variability in the propolis composition, and there is also a lack of international guidelines for quality standardizing of these products. Therefore, we proposed to develop and validate a method for qualitative and quantitative analysis of chemical markers present in the volatile oil of brown propolis produced in Minas Gerais, Brazil. Additionally, the leishmanicidal, cytotoxic and antimicrobial properties of the oil were evaluated.

2. Material and methods

2.1. Propolis material

Four samples of Brown raw propolis from *Apis mellifera* bees were collected from artificial hives in Cabo verde – Minas Gerais, Brazil. Two samples were collected in May 2018 (VOBP-I and VOBP-II), and two samples were collected in December 2018 (VOBP-III and VOBP-IV). After removing impurities such as parts of plants and insects, propolis was stored in an amber bottle and kept at -20 ºC freezer to prevent loss or degradation of its volatile components.

2.2. Volatile oil extraction

The Brown Propolis raw material was powdered and submitted to hydrodistillation for four hours using a Clevenger-type apparatus. The volatile oil was collected by decantation and dried with sodium sulfate, followed by filtration, and stored at -20 °C until analyzed.

2.3. Isolation and identification of the standard compounds

The volatile oil of brown propolis (VOBP-III) was subjected to fractionation by spinning band distillation in a method described by Ribeiro et al. (2019), which resulted in four fractions. Each fraction was subjected to chromatographic columns packed with silica gel 60H (Sigma-Aldrich) in the proportion of 1:150. The mobile phase used consisted of a gradient of hexane and ethyl acetate in increasing polarity of 98: 2, 95: 5, 90:10, and 50:50 (v/v). The elution of the compounds was monitored by thin-layer chromatography (CCD), revealed with anisaldehyde under heating.

The chemical structures of the isolated compounds were determined using GC-MS and 1H and 13C NMR analyses. The obtained NMR spectra data were recorded in a Brucker – Advance DRX500 spectrometer, operating at 500 MHz, using CDCl3 from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

2.4. Gas chromatography analysis

The Gas chromatographic analysis was performed using Agilent Technologies GC equipment, model 6890 N, equipped with a flame ionization detector (FID). A capillary column of fused silica HP-5 (5% phenyl-methyl-siloxane, length: 30 m, internal diameter: 0.32 mm, liquid film: 0.25 μm, Agilent®) was used. The carrier gas was hydrogen at a flow rate of 2.0 mL/min. The temperature setting was determined as follows: the initial oven temperature was set at 80 °C, held for 6 min and then increased to 120 °C with a rate of 2 °C/min held for 5 min; then increased to 130 ºC at 2 °C/min and kept for 6 min; then increased to 170 ºC at 2 °C/min held for 1 min; and finally increased to 280 ºC at 20 ºC/min, ending the analysis within 51 min. The injector and detector temperatures were set at 240 ºC and 280 ºC, respectively. The injection volume was 1 μl, with a split ratio of 50:1.

The chemical characterization of the oil was performed by gas chromatography-electron impact mass spectrometry. For that, a Shimadzu GCMS-QP2010 equipped with an automatic sampler AOC-20Si was used with RTX-5 MS chromatographic column (30 m x 0.25 mm x 0.25 μm, Restek). All mass spectra were recorded in the scan mode at 70 eV. Analytical conditions were set as 60 °C to 220 °C with a rate of 3 °C/min, which was held for 2 min, and

then from 220 °C to 290 °C with a rate of 10 °C/min. The injector and detector temperatures was set at 250 °C. Helium was used as carrier gas at 1 mL/min.

The identification of compounds was performed by calculating the retention index using homologous series of hydrocarbons (Sigma-Aldrich) (Mühlen and Von Marriott, 2011)., in comparison with the mass spectra of the samples with Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC), Wiley Library, National Institute of Standards and Technology (NIST) spectral libraries, and literature data.

2.5. Validation of the GC-FID method

Method validation was performed according to International Conference on Harmonization (ICH, 2005) and the Brazilian National Health Surveillance Agency (ANVISA, 2017) guidelines. In the validation experiments, all the parameters were performed in triplicate. All the standards solutions were prepared in hexane HPLC grade from Tedia® (Fairfield, OH, USA). The selectivity of the method was established according to the separation efficiency between the chromatographic peaks defined by evaluation of the following chromatographic parameters: retention time (Rt), separation factor (α), chromatographic resolution (Rs) and the number of theoretical plates (N).

Calibration curves were used to estimate the linearity. Solution with 5 mg/mL of the standard compounds was diluted and combined to achieve eight different concentrations (500, 400, 300, 200, 100, 50, 25 e 10 μg/mL), and 100 μg/mL of benzophenone were added as the internal standard. Limits of detection and quantification were estimated according to the equations established through baseline noise, and the equations are available in the supplementary material (ANVISA, 2017). The concentration that generated a peak with an intensity equivalent to three times the baseline noise was considered the limit of detection, while for the limit of quantification, it was considered the concentration that showed a peak equivalent to ten times the baseline noise.

Precision of the method was assessed by relative standard deviation trought measuring the repeatability of the results at three concentrations at high (300 μg/mL), medium (100 μg/mL) and low (50 μg/mL) levels of the analytical curve performed in the same day (intraday precision) and in three different consecutive days (interday precision). The accuracy results were estimated by the comparison between the theoretical and real values of the same three concentration solutions. The recovery was determined by standard addition method, a solution of 1 mg/mL of the oil was fortified with the standards at the three concentration levels

determined (300, 100 and 50 μg/mL). The recovery was expressed as the percentage of the difference between experimental response and nominal response.

The robustness of the method by CG-FID was determined from the experimental design for four nominal factors. They were analyzed at low levels (−1) and high levels (+1), and were tested with eight experiments, which were also evaluated at the three concentration levels, high (300 μg/mL), medium (100 μg/mL) and low (50 μg/mL). The following factors were evaluated: injector temperature (°C), initial oven temperature (°C), detector temperature (°C), and flow of the mobile phase (mL/min). The experimental designs were obtained and processed in the software STATSOFT Statistica™.

2.6. Antimicrobial, citotoxic and leishmanicidal activities evaluation

For the evaluation of cytotoxicity, three cell lines between the third and eighth passage (when the cells were resuspended) obtained from the Cell Bank of Rio de Janeiro were used, comprising two tumor cell lines AGP-01 (gastric cancer cells) and He-La (cervical cancer cells), as well as one noncancer cell line, CHO-K1. Doxorubicin (DOX) was used as the positive control for tumor cell lines and DMSO (10%) for the noncancer cell line. The VOBP activity was evaluated at concentrations of 100, 10, 1, and 0.6 μg/mL. The cell viability was determined using an XTT-based colorimetric assay.

The antileishmanial activity was tested against promastigote and amastigote forms of *Leishmania amazonensis* (MHOM/BR/PH8) grown in RPMI 1640 medium, as described by Arruda et al. 2020a. Promastigote forms of *L. amazonensis* (2 x 106 parasites/mL) were incubated in 96-well microtiter plates with VOBP previously dissolved in 1% dimethylsulfoxide (DMSO), for final concentrations of 3.12, 6.25, 12.5, 25 and 50 μg/mL. The bioassays were performed in triplicate, using a medium with 0.1% DMSO as a negative control and amphotericin B as a positive control.

For the evaluation of antimicrobial activity, the microplate microdilution method was used according to the Clinical and Laboratory Standards Institute (CLSI, 2012). The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the following microorganisms were evaluated: *Staphylococcus aureus* ATCC 6538, *Staphylococcus saprophyticus* ATCC 35552, *Streptococcus mutans* ATCC 25175, *Candida krusei* ATCC 6258, *Candida glabrata* ATCC 15126 and *Candida parapsilosis* ATCC 22019. The range concentrations were 400 to 0.195 μg/mL obtained by serial dilution. Tetracycline was used as a positive control for bacteria and amphotericin B for yeasts.

3. Results and Discussion

3.1. Chemical characterization

The brown propolis (VOBP-III), which was subjected to the hydrodistillation process, resulted in a high yield of volatile oil (1.4%). Several factors can influence the volatile oil yielding, including the period of collection, storage conditions, and type of propolis. The brown propolis volatile oil (VOBP-III) used in this study proved to be a complex mixture of volatile compounds, as depicted in the chromatogram obtained by CG-FID analysis (Figure 1). The VOBP was subjected to different chromatographic techniques to furnish the isolated compounds used in the validation process of the analytical method.

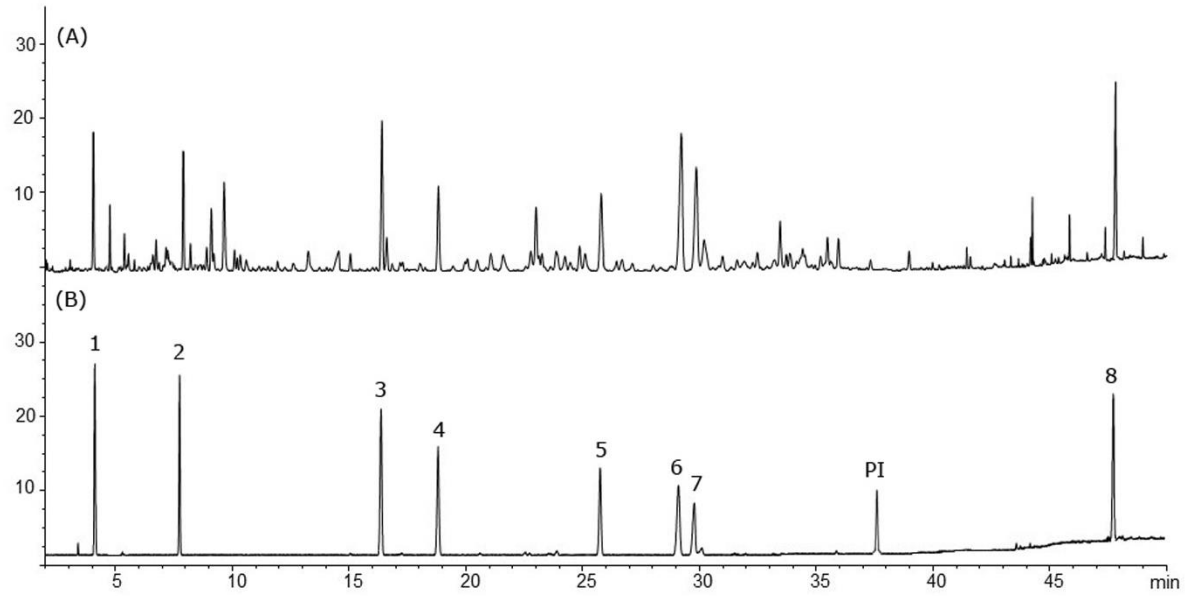

Figure 1. GC-FID chromatogram of the Brazilian southeast brown propolis volatile oil (A) and (1) 1,8-cineole, (2) terpinen-4-ol, (3) *α*-copaene, (4) *β*-caryophyllene, (5) *γ*-muurolene, (6) nerolidol, (7) spathulenol, (PI) benzophenone and (8) *γ*-palmitolactone compounds (B).

From the VOBP, eight compounds were isolated, which were identified according to NMR and MS analyses in comparison with the literature data. Two isolated compounds were monoterpenes identified as 1,8-cineol (5.9 mg) and terpinen-4-ol (6.1 mg); five were sesquiterpenes identified as *α*-copaene (7.3 mg), *β*-caryophyllene (6.7 mg), *γ*-muurolene (5.8 mg), nerolidol (11.7 mg) and spathulenol (8.4 mg); and one was identified as lactone *γ*-palmitolactone (5.6 mg) (Figures 1B and 2). The chemical shifts of each compound obtained by the NMR experiments are described in the Supplementary material.

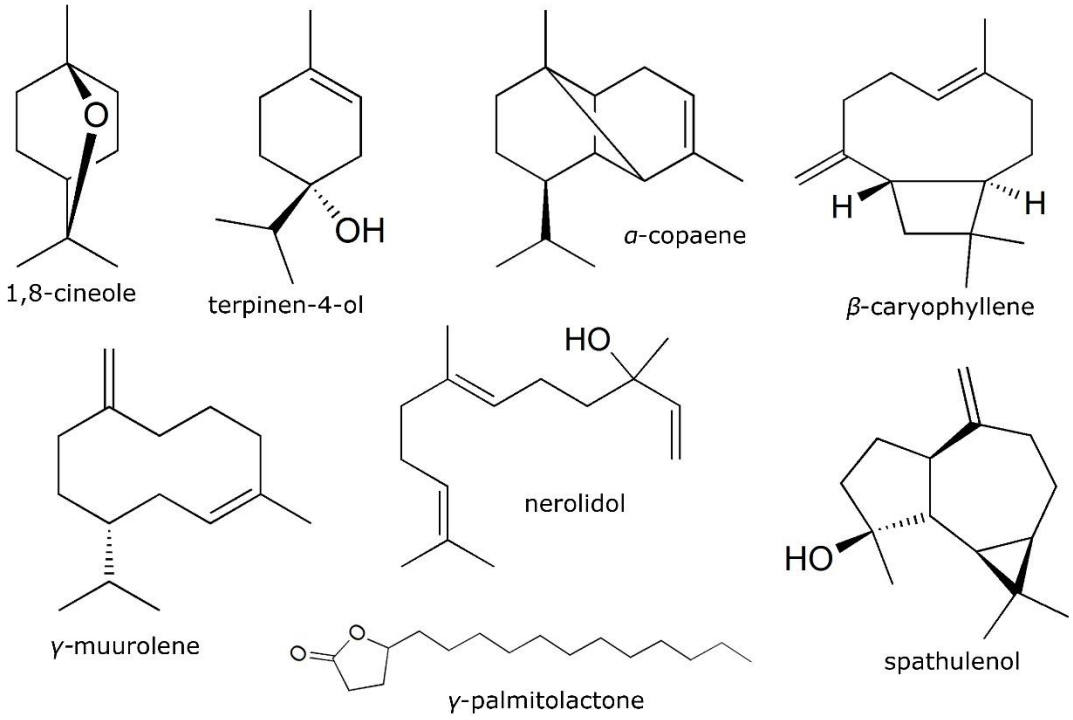

Figure 2. Isolated compounds from Brazilian southeast brown propolis volatile oil.

The chemical characterization of VOBP-III was performed by GC-MS, allowing to identify 56 compounds, for the identification of the compounds, the retention index was calculated, and the similarity range of the fragmentation pattern was observed (Table S1). The VOBP-III is composed mainly by sesquiterpenes (33.62%) and oxygenated sesquiterpenes (26.98%), along with oxygenated monoterpenes (18.99%), aromatic hydrocarbons (9.22%), lactones (6.15%), oxygenated hydrocarbons (1.72%), monoterpenes (0.89%), hydrocarbons (0.78%) and diterpenes (0.14%). Among the major compounds, it is possible to highlight 1,8-cineole (7.03%), acetophenone (3.62%), terpinen-4-ol (6.06%), Hydrocinnamyl alcohol (4.60%), *α*-copaene (5.16%), *β*-caryophyllene (6.14%), *γ*-gurjunene (3.01%), γ-muurolene (5.07%), nerolidol (10.47%), spathulenol (7.41%) and *γ*-palmitolactone (5.73%). Another characteristic of this propolis is the presence of a large number of minor constituents (<1%).

Considering that the chemical composition of propolis is directly linked to the vegetation surrounding the *Apis mellifera* beehives, it is possible to infer that this propolis was produced from different plant species. Other types of Brazilian propolis, such as green and red, have their respective botanical sources well defined, with *Baccharis dracunculifolia* being the botanical source of green propolis and *Dalbergia ecastophyllum* the botanical source of red propolis (Daugsch et al 2008).

Some studies have shown different types of brown-colored propolis with varied botanical sources, such as eucalyptus and araucaria, mainly in Brazil's southern region (Freitas et al. 2011). The propolis used in this study was collected in the southeast region, where the Atlantic forest predominates, a region with high biodiversity and significant predominance of *B. dracunculifolia* (Sousa et al. 2009). For this reason, it is the region with the most significant production of green propolis. Published works report that nerolidol and spathulenol are chemical markers of the volatile oil of *B. dracunculifolia*, and γ-muurolene, also found in this oil, is an excellent indication that the bees used this plant in the production of this propolis (Klopell et al. 2007). Likewise, cineol is a primary compound found in many *Eucalyptus* species that are also common in the southeastern region of Brazil (Silva et al. 2006).

Traditionally, propolis has been used in folk medicine to treat inflammatory disorders, especially the green type propolis (Ribeiro et al. 2018). Thus, this chemical diversity, comprising different types of propolis, can expand the range of its use in folk medicine, due to the pharmacological importance of its volatile compounds. α-Copaene and β-caryophyllene isolated from copaiba oils have shown cytoprotective activities (Aldana et al. 2019; Silva et al. 2020). Compounds such as cineole and terpineol displayed anti-inflammatory and antinociceptive effects (Santos and Rao 2000). In this way, it is possible to link new biological properties from the volatile fractions of propolis by new studies, which may corroborate its pharmacological uses.

Combined with pharmacological evaluation, studies involving the quality control of propolis are also important. Analytical methods are powerful tools in the quality control of natural products. Especially with propolis, several factors can affect its chemical composition, including abiotic factors that can influence plants and bees.

3.2. Method Validation

There are different techniques by gas chromatography for the analysis of volatile compounds. Gas chromatography coupled to flame ionization detector (GC-FID) is a low-cost and robust technique. Therefore, a GC-FID method was developed and validated, capable of quantifying the major volatile compounds present in the studied brown propolis. The ideal analytical method can perform the separation of analytes quickly and efficiently in compliance with specifications established by international guidelines. The composition of the obtained oil was very complex. Therefore, it was necessary to optimize the chromatographic parameters, including injector and detector temperatures and gas flow and oven temperature ramps, resulting in a 51 min of chromatographic analysis.

All the obtained parameters in the method validation process are following the validation guides of ICH and ANVISA. The results showed good selectivity of the method (Table 1). The number of theoretical plates (N) indicates excellent column efficiency. The separation factor and resolution were satisfactory for all analyzed compounds.

Table 1. Response for chromatographic parameters of GC-FID method in selectivity

	α		**Rs**		**N**	
Compound	**Mean ± SD**	**RSD (%)**	**Mean ± SD**	**RSD (%)**	**Mean ± SD**	**RSD (%)**
1,8-Cineole	1.84 ± 0.00	0.01	84.96 ± 0.96	1.13	261059 ± 6606	5.36
Terpinen-4-ol	2.08 ± 0.00	0.01	135.21 ± 1.34	0.99	374823 ± 3255	0.87
α-Copaene	1.14 ± 0.00	0.01	29.09 ± 0.23	0.79	767218 ± 11347	2.78
β-Caryophyllene	1.32 ± 0.00	0.02	66.53 ± 1.38	2.08	685843 ± 6369	0.93
γ-Muurolene	1.15 ± 0.00	0.01	40.11 ± 0.95	2.36	1101460 ± 7774	5.06
Nerolidol	1.02 ± 0.00	0.01	6.59 ± 0.13	1.98	1262859 ± 4864	2.76
Spathulenol	1.10 ± 0.00	0.02	35.49 ± 0.88	2.48	1463939 ± 0487	4.5
γ-Palmitolactone	1.44 ± 0.00	0.01	258.42 ± 3.89	1.5	2673091 ± 9315	4.19

α: separation factor; Rs: chromatographic resolution; N: number of theoretical plates; SD: standard deviation; RSD: relative standard deviation.

Linearity, limits of detection, and quantification data of the method were also determined. The correlation (R) and determination (R2) coefficients values calculated by regression analysis of all calibration curves were above 0.99 as recommended by ICH and ANVISA guidelines. The data's homoscedasticity was confirmed by the residual analysis and did not have a lack of fit ($p > 0.05$). Besides, the range obtained by limits of detection and quantification were satisfactory (Table 2).

Table 2. Linearity, limits of detection and quantification of the method

Compound	**Equation**	**R^2**	**R**	**LD**	**LQ**	**Residual**	**Lack of fit *P* value**
1,8-Cineole	y = 0.0126x - 0.0775	0.997	0.998	1.6	4.9	0.048	0.18
Terpinen-4-ol	y = 0.014x - 0.1181	0.997	0.998	3.7	5.4	0.029	0.27
α-Copaene	y = 0.0166x - 0.0916	0.997	0.998	2.7	6.3	0.054	0.17
β-Caryophyllene	y = 0.0147x - 0.0546	0.997	0.998	4.1	7.1	0.038	0.07
γ-Muurolene	y = 0.0134x - 0.0982	0.997	0.998	2.9	8.2	0.036	0.12
Nerolidol	y = 0.0128x - 0.1188	0.997	0.998	6.5	8.7	0.032	0.08
Spathulenol	y = 0.0095x - 0.0335	0.995	0.997	1.7	7.3	0.023	0.09
γ-Palmitolactone	y = 0.0260x - 0.0234	0.995	0.997	3.5	9.1	0.041	0.16

R^2: determination coefficient; R: correlation coefficient; LD: limit of detection; LQ: limit of quantification.

The method precision was evaluated regarding the repeatability (intraday precision) and intermediate precision (interday precision). The results showed low variation among the analyses since intraday and inter-day precisions showed relative standard deviations (RSDs) lower than 5%. The accuracy results were approximately 100%, with variations among 92.54% and 106.62%, indicating that the analytical method furnished accurate results (Table 3). The recovery parameter was considered good, between 89 and 107% at low, medium, and high concentrations. Therefore, the developed method can be considered accurate and precise once these parameters are following ANVISA and ICH guidelines.

Table 3. Precision and accuracy of GC-FID method

		Precision (RSD)			
Compound	**Level**	**Intraday**	**Interday**	**Accuracy (%)**	**Recovery (%)**
	Low	1.08	1.51	94.9 ± 3.0	90.4 ± 1.7
1,8-Cineole	Medium	1.10	1.14	97.3 ± 3.8	93.0 ± 3.2
	high	0.54	0.36	93.5 ± 2.7	91.9 ± 2.4
	Low	1.20	1.55	97.7 ± 2.2	103.7 ± 2.8
Terpinen-4-ol	Medium	1.08	1.52	93.8 ± 2.5	95.8 ± 2.1
	high	1.72	2.96	95.70 ± 1.8	92.3 ± 4.0
	Low	0.94	1.36	93.7 ± 0.8	101.7 ± 1.9
α-Copaene	Medium	1.27	1.90	102.3± 0.8	107.2 ± 3.9
	high	3.05	4.22	96.0 ± 1.2	98.7 ± 1.5
	Low	2.26	2.97	99.8 ± 2.7	92.6 ± 3.1
β-Caryophyllene	Medium	1.37	2.04	106.6 ± 2.5	96.9 ± 4.5
	high	2.16	1.75	95.7 ± 1.0	92.7 ± 3.3
	Low	0.35	0.44	97.1 ± 1.6	99.0 ± 4.2
γ-Muurolene	Medium	1.08	1.11	95.0 ± 1.1	98.7 ± 6.1
	high	2.55	2.08	94.8 ± 0.8	102.9 ± 3.9
	Low	0.09	0.14	100.0 ± 1.7	104.6 ± 2.1
Nerolidol	Medium	2.78	3.62	96.3 ± 0.1	97.8 ± 3.6
	high	1.17	1.62	95.6 ± 0.9	91.5 ± 2.4
	Low	0.57	2.13	92.5 ± 1.4	89.2 ± 2.8
Spathulenol	Medium	0.90	1.17	103.3 ± 1.9	94.9 ± 3.6
	high	4.78	5.92	94.2 ± 0.6	95.4 ± 4.2
	Low	0.14	0.14	95.2 ± 0.2	97.2 ± 4.2
γ-Palmitolactone	Medium	0.42	0.62	96.2 ± 2.2	100.9 ± 3.7
	high	0.59	0.90	94.2 ± 3.8	103.6 ± 4.0

RSD: relative standard deviation.

The robustness was evaluated by making small variations in four parameters of the method in two levels. The changes in the injector temperature, initial oven temperature, detector temperature and flow rate of the mobile phase did not lead to a significant variation in the concentration of the analyzed compounds by GC-FID method ($p<0,05$). The variation of the concentration of the analytes was lower than 20%, ranging between -9.76% and 11.32%. Therefore, the developed method proved to be robust.

The validated GC-FID method was used to quantify the analytes present in the four brown propolis samples collected (Table S2). In the sample VOBP-I, the major compound was terpinen-4-ol, while in the other samples, the major compounds were nerolidol and spathulenol. However, these compounds were not found in VOBP-I. The α-copaene was not identified in VOBP-II. It confirms the variations that propolis can undergo in chemical composition, especially in volatile compounds. The validated method can be extended for different propolis types since these analytes are also found in the botanical sources of other types of própolis.

3.3. Bioactivity evaluation

Regarding the biological activities, the VOBP showed significant leishmanicidal activity. It was possible to observe the inhibition of flagellar motility in the promastigote form of parasites when treated with VOBP in a dose-dependent manner with IC_{50}= 21.3 µg/mL. The amastigote form is the clinically relevant form of the parasite, and the oil was able to increase the parasite death with IC_{50}= 25.1 µg/mL (Figure 3). At the highest concentrations evaluated, the oil did not show significant differences compared to the positive control. At 50 µg/mL, the oil was more active than amphotericin B against the amastigote forms. The multiplication of the parasite's amastigote forms can lead to the lysis of the infected cells, releasing these parasites that will continue the infection cycle. During the evaluation of the amastigote forms, the macrophages' integrity was observed, demonstrating in addition to the leishmanicidal effect, low toxicity.

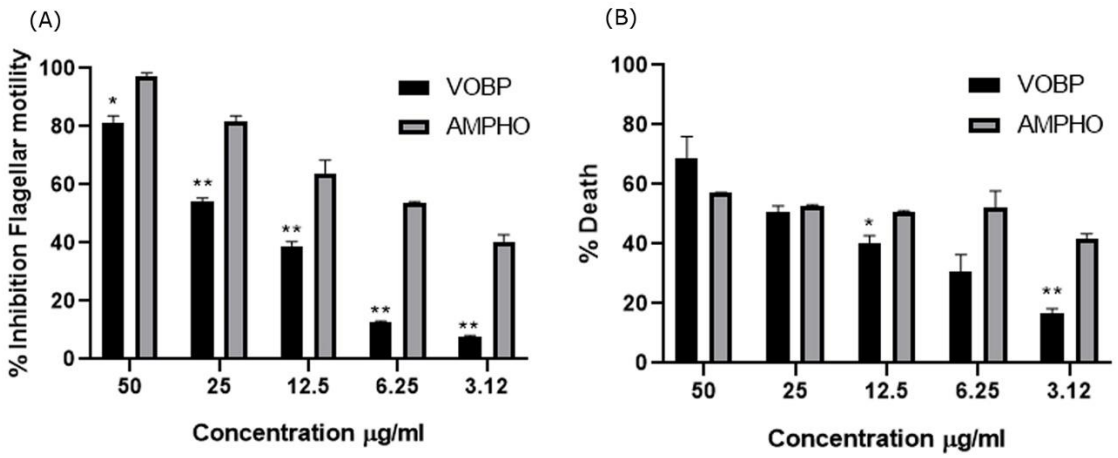

Figure 3. Leishimanicidal activity against promastigote forms (A) and amastigote forms (B) of *Leishmania amazonensis*. VOBP: volatile oil of Brazilian Brown Propolis; AMPHO: Amphotericin B. ** Significant difference from the control group, T test ($p > 0,05$).

Nerolidol, the major component of VOBP, was reported to display leishmanicidal activity, Ceole et al. (2017) observed structural changes, such as the reduction in the cell size and DNA degradation in the cells of *L. braziliensis* treated with the sesquiterpene nerolidol, which induced the death of the parasite. Santana et al. (2014) found an IC_{50}= 11.87 μg/mL against promastigote forms of *L. amazonenses* for the hydroalcoholic extract of brown propolis collected in northeastern Brazil, in which the main compound was the pentacyclic triterpenoid lupeol (25.77%), while the ethyl acetate fraction displayed an IC_{50}= 36.95 μg/mL.

Leishimanicidal activity has already been reported for propolis from various regions, such as Bolivian propolis, which presented an IC_{50} = 12.1 μg/mL against *L. amazonenses*, chemically described as rich in phenolic compounds (Nina et al. 2016). Bulgarian propolis presented a IC_{50}= 29.3 μg/mL against *L. amazonenses* (Machado et al. 2007). Arruda et al. (2020b) evaluated metabolites isolated from green propolis against *L. amazonenses*, where *p*-coumaric acid displayed IC_{50} <3.12 μM against amastigote forms of the parasite, thus evidencing the critical role of propolis against leishmaniosis.

The VOBP was not cytotoxic against the cell lines used in this study. The IC_{50} value was more significant than the highest concentration evaluated for the AGP-01, He-La, and CHO-K1 lines (IC_{50}> 100 μg/mL). The hydroalcoholic crude extract of propolis is generally rich in flavonoids and phenolic compounds, which provides a high antioxidant action for this propolis. Previous studies have shown that this antioxidant effect is related to the cytotoxic activity found in propolis, mediated via free radical-scavenging activity (Kocot et al. 2018). The volatile fraction by hydrodistillation excludes the phenolic compounds, such as flavonoids, leading to the decrease or absence of antioxidant activity.

The obtained volatile oil displayed antimicrobial activity against *S. mutans* and *S. aureus* at 25 μg/mL and 50 μg/mL, respectively, and showed a bactericidal effect against *S. mutans* at 50 μg/mL. The microbial growth of both *S. saprophyticus* and *C. parapsilosis* was inhibited at 100 μg/mL of the oil. VOBP displayed activity in the range of 200 to 400 μg/mL for the other evaluated microorganisms (Table S3).

Many terpenes present in VOBP have been reported to be active against a variety of microorganisms. Nerolidol was activity against several strains of *S. aureus* (Lee et al. 2014). *β*-Caryophyllene showed antibacterial activity against *S. mutans* by reducing the expression of gtf genes of this strain (Yoo and Jwa 2018). Five Greek propolis oils presented activity in the range of 4.1 to 6.5 μg/mL against *S. aureus,* and *α*-pinene was characterized as the principal constituent in four of the five samples (Melliou et al. 2007).

The volatile fractions of different propolis have been poorly explored chemically and as a source of bioactive compounds regarding many biological activities already reported for propolis crude extracts. The chemical diversity of propolis components is directly correlated with the plant biodiversity of its production area, making it a vast horizon yet to be explored. Volatile compounds are essential as an attractant of plant pollinators and many other essential roles for plant survival, as well as in propolis production as reported for green propolis production by Rodrigues et al. (2020). Therefore, the volatile compounds undoubtedly play a vital role in the production of propolis and its biological activities, which have not yet been fully elucidated.

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5. Disclosure statement

The authors reported no potential conflict of interest.

6. References

Aldana JA, Grandis RA, Nicolella H, Guissoni APP, Squarisi I, Arruda C, Ribeiro VP, Tavares DC, Barcelos GRM, Antunes LMG, Bastos JK. Evaluation of cytoprotective effects of compounds isolated from *Copaifera langsdorffii* Desf. against induced cytotoxicity by

exposure to methylmercury and lead. Nat Prod Res 2019;9:1-5. DOI: 10.1080/14786419.2018.1543673.

ANVISA, 2017. National Health Surveillance Agency. RE- 166.

Arruda C, Ribeiro VP, Almeida MO, Mejía JAA, Casoti R, Bastos JK. Effect of light, oxygen and temperature on the stability of artepillin C and *p*-coumaric acid from Brazilian green propolis. J Pharmaceut Biomed 2020a;178:112922. DOI: 10.1016/j.jpba.2019.112922.

Arruda C, Ribeiro VP, Mejía JAA, Almeida MO, Goulart MO, Candido ACBB, Santos RA, Magalhães LG, Martins CHG, Bastos JK. Green Propolis: Cytotoxic and leishmanicidal activities of artepillin C, *p*-coumaric acid, and their degradation products. Rev Bras Farmacogn 2020b. DOI: 10.1007/s43450-020-00043-3.

Ceole L, Cardoso M, Soares M. Nerolidol, the main constituent of *Piper aduncum* essential oil, has anti-*Leishmania braziliensis* activity. Parasitology 2017;144:1179-1190. DOI:10.1017/S0031182017000452.

Clinical and laboratory standards institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; 9th ed. Approved standard M07–A9. 2012;32(2).

Daugsch A, Moraes CS, Fort P, Park YK. Brazilian Red Propolis—Chemical Composition and Botanical Origin. Evid Based Complement Alternat Med 2008;5:435–441. DOI: 10.1093/ecam/nem057

Melliou E, Eleftherios S, Ioanna C. Volatile constituents of propolis from various regions of Greece – Antimicrobial activity. Food Chem 2007;103: 375-380.

Freitas AS, Barth OM, Sales EO, Matsuda AH, Muradian LBA. A palynological analysis of Brazilian propolis samples. Journal of ApiProduct and ApiMedical Science 2011;3:67-74. DOI: 10.3896/IBRA.4.03.2.01

ICH. Harmonized tripartite guideline. Validation of analytical procedures: Text and methodology Q2(R1). Geneva: International Conference on Harmonization 2005.

Klopell FC, Lemos M, Sousa JPB, Comunello E, Maistro EL, Bastos JK, Andrade SF. Nerolidol, an antiulcer constituent from the essential oil of *Baccharis dracunculifolia* DC (Asteraceae). Z Naturforsch C 2007;62:537-542. DOI: 10.1515/znc-2007-7-812.

Kocot J, Kiełczykowska M, Luchowska-kocot D, Kurzepa J, Musik I. Antioxidant potential of propolis, bee pollen, and royal jelly: Possible medical application. Oxid Med Cell Longev 2018;7074209. DOI: 10.1155/2018/7074209.

Kuropatnicki AK, Szliszka E, Krol W. Historical aspects of propolis research in modern times. Evid Based Complement Alternat Med 2013;964149, DOI: 10.1155/2013/964149.

Lee K, Lee JH, Kim SI, Cho M, Lee J. Anti-biofilm, anti-hemolysis, and anti-virulence activities of black pepper, cananga, myrrh oils, and nerolidol against *Staphylococcus aureus*. Appl Microbiol Biotechnol 2014;98:9447–9457. DOI: 10.1007/s00253-014-5903-4.

Machado GMC, Leon LL, Castro SL. Activity of Brazilian and Bulgarian propolis against different species of *Leishmania*. Mem Inst Oswaldo Cruz 2007;102:73-77. DOI: 10.1590/S0074-02762007000100012.

Machado JL, Assunção AKM, Silva MCP, Reis AS, Costa GC, Arruda DS, Rocha BA, Vaz MMOLL, Paes AMA, Guerra RNM, Berretta AA, Nascimento FRF. Brazilian green propolis: Anti-Inflammatory property by an Immunomodulatory activity. Evid Based Complement Alternat Med 2012;157652. DOI:10.1155/2012/157652.

Mühlen C, and Von Marriott PJ. Retention indices in comprehensive two-dimensional gas chromatography. Anal Bioanal Chem 2011;401:2351–2360. DOI:10.1007/s00216-011-5247-1

Nina N, Lima B, Feresin G, Giménez A, Salamanca CE, Schmeda-Hirschmann G. Antibacterial and leishmanicidal activity of Bolivian propolis. Lett Appl Microbiol 201;62:290-296. DOI:10.1111/lam.12543.

Ribeiro VP, Arruda C, El-Salam MA, Bastos JK. Brazilian medicinal plants with corroborated anti-inflammatory activities: a review. Pharm Biol 2018;56:253–268. DOI: 10.1080/13880209.2018.1454480.

Ribeiro VP, Arruda C, Silva JJM, Meíja JAA, Furtado NAJC, Bastos JK. Use of spinning band distillation equipment for fractionation of volatile compounds of *Copaifera* oleoresins for developing a validated gas chromatographic method and evaluating antimicrobial activity. Biomed Chromatogr, 2019;33:e4412. DOI: 10.1002/bmc.4412.

Rodrigues DM, Souza MC, Arruda C, Pereira RAS, Bastos JK. The Role of *Baccharis dracunculifolia* and its chemical profile on green propolis production by *Apis mellifera*. J Chem Ecol 2020;46:150–162. DOI: 10.1007/s10886-019-01141-w.

Santana LC, Carneiro SM, Caland-Neto LB, Arcanjo DD, Moita-Neto JM, Citó AM, Carvalho FA. Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of *Leishmania amazonensis.* Nat Prod Res 2014;28:340-343. DOI: 10.1080/14786419.2013.856904.

Santos FA, Rao VSN. Anti-inflammatory and antinociceptive effects of 1,8-cineole, a terpenoid oxide present in many plant essential oils. Phytother Res 2000;14:240-244. DOI:10.1002/1099-1573(200006)14:4<240::aid-ptr573>3.0.co;2-x.

Silva JJM, Ribeiro VP, Lemos M, Crotti AEM, Rogez H, Bastos JK. Reliable methods for analyses of volatile compounds of *Copaifera* oleoresins combining headspace and gas chromatography. Chem Biodivers, 2020;17:e1900440. DOI: 10.1002/cbdv.201900440.

Silva PHM, Otávio BJ, Silva Junior FG. Potential of eleven *Eucalyptus* species for the production of essential oils. Sci Agr 2006;63:85-89. DOI: 10.1590/S0103-90162006000100014.

Sousa JPB, Silva Filho AA, Bueno PCP, Gregório LE, Furtado NAJC, Jorge RF, Bastos JK. A validated reverse-phase HPLC analytical method for the quantification of phenolic compounds in *Baccharis dracunculifolia*. Phytochem Anal 2009;20:24-32. DOI:10.1002/pca.1087.

Yang C, Luo L, Zhang H, Yang X, Lv Y, Song H. Common aroma-active components of propolis from 23 regions of China. J Sci Food Agric 2010;90:1268–1282. DOI: 10.1002/jsfa.3969.

Yoo HJ, Jwa SK. Inhibitory effects of *β*-caryophyllene on *Streptococcus mutans* biofilm. Arch Oral Biol 2018;88:42-46. DOI: 10.1016/j.archoralbio.2018.01.009.

3.3. Capítulo 3 – Avaliação *In vivo* das atividades anti-inflamatória e antinociceptiva da própolis marrom do sudeste brasileiro.

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Article

Anti-Inflammatory and Antinociceptive Activities of the Hydroalcoholic Extract and the Volatile Fraction of Southeastern Brazilian Brown Propolis

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Author´s Contributions

VPR and JKB were responsible for the study's design. VPR, GVS and JAAM performed the anti-inflammatory and antinociceptive experiments. VPR and CA performed phytochemical analysis. JAAM helped with the treatment and discussion of the results. VPR wrote the manuscript, and JKB supervised the study and reviewed the manuscript. All the authors have approved the manuscript.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Animal Study

The experiment protocols were approved by the by the Ethics and Research Council on the Use of Animals of the School of Pharmaceutical Sciences of Ribeirão Preto of the University of São Paulo (Process nº 18.1.834.60.1, March 1, 2019). All experiments were carried out under the Ethical Principles of Animal Experimentation recommended by the Brazilian College of Animal Experimentation (COBEA).

Abstract

The analgesic and anti-inflammatory activities of a Southeastern Brazilian brown propolis were evaluated using the formalin test and carrageenan-induced mechanical hypernociception. The hydroalcoholic extract and the volatile fraction were evaluated at doses of 50, 100, and 200 mg/kg. In the formalin test, the crude extract reduced the response by 73 ± 7%, 83 ± 8%, and 89 ± 3% for the first phase and 48 ± 13%, 65 ± 9%, and 75 ± 6% for the second phase, respectively. The volatile fraction reduced the response by 75 ± 7%, 94 ± 5%, 99 ± 1%, and 30 ± 7%, 53 ± 5%, 91 ± 5% for the first and second phases. For the carrageenan test, the hydroalcoholic extract and volatile fraction showed mechanical sensitization reductions of 57 ± 7%, 67 ± 6%, 79 ± 5% and 62 ± 7%, 79 ± 9%, 88 ± 4% respectively, after 48 h. Indomethacin and morphine showed inhibitions of 42 ± 4% and 88 ± 2%, respectively. In the tail-flick assay, the results showed evidence of analgesic activities; the animals treated with crude extract increased the nociceptive threshold by 58 ± 8% and 70 ± 6% and 77 ± 10%, respectively, for evaluated doses, and 55 ± 6% and 73 ± 7% and 82 ± 14% for the volatile fraction, compared with control. The coadministration of naloxone altered the antinociceptive effect of morphine and propolis, suggesting that brown propolis has an action on the central nervous system.

Keywords: *Apis mellifera*, Essential oils, Sesquiterpenes, Opioid antagonist, Phenolic compounds, Tail-flick test

Introduction

Propolis is produced from resins collected from many parts of plants by bees for protection against invaders, microorganisms, and hives' temperature regulation (Simone-Finstrom and Spivak 2010). It is a millennial natural product used by humankind for medicinal purposes. Currently, propolis is used in traditional medicine due to its anti-inflammatory (Ribeiro et al. 2018), immunomodulatory (Machado et al. 2012), antimicrobial (Arruda et al. 2020a), among other properties. Its use is mainly supported in the food and cosmetic field due to its potent antioxidant activity (Kocot et al. 2018).

Apis mellifera bees use plant resins for the production of propolis. Considering that Brazil possesses several biomes with incredible biodiversity, different propolis types are produced from different plant sources; besides, it has been described considerable variations depending on the geographical location (Yang et al. 2010). Propolis is composed of resins (50%), waxes (30%), essential oils (10%), pollen (5%), and other substances (5%) (Kumazawa et al. 2002). Thus, the volatile compounds present in propolis represent a considerable fraction, which must be taken into account when evaluating its chemical composition and its biological activities.

The organism has a delicate regulatory system for the inflammatory response, and if this regulation is affected in any way, a pathological disorder can be installed (Medzhitov 2008). Drugs that control inflammatory responses are essential, and propolis has played an essential role due to its anti-inflammatory properties (Ribeiro et al. 2018). Therefore, the prest investigation explored the anti-inflammatory potential of propolis and its *in vivo* antinociceptive capacity by testing the hydroalcoholic extract and the volatile fraction of brown propolis collected in the Southeastern region of Brazil.

Material and Methods

Propolis material

Brown propolis type from *Apis mellifera* bees was collected in Cabo Verde (21°25'45.5"S 46°23'48.9"W), Minas Gerais, Brazil, in February 2018. The propolis raw material was stored in an amber bottle and kept at -20 ºC in a freezer to prevent its components' loss or degradation. The brown propolis (100 g) were crushed using a mill and subjected to maceration with ethanol-water 7:3 (v:v). The solvent was filtered and renewed every 24 h three times. The obtained hydroalcoholic solutions were concentrated in a rotary evaporator apparatus, and the resulting extract was lyophilized, furnishing 47 g of the crude extract.

Dereplication of the volatile compounds

The raw material was powdered (300 g) and submitted to hydrodistillation for 4 h using a Clevenger-type apparatus. The volatile fraction was obtained by the liquid-liquid partition of the entire distillate (hydrolate and oil) with ethyl acetate (three times 100 ml). Sodium sulfate was added to the organic solvent, filtered, and stored at -20 °C until analysis. The volatile compounds' characterization was carried out in a Shimadzu GC/MS – QP2010 equipped with automatic sampler AOC – 20Si with a RTX-5 MS chromatographic column (30 m × 0.25 mm × 0.25 μm, Restek). All mass spectra were recorded in the scan mode at 70 eV. Analytical conditions were set as 60 °C to 220 °C at a rate of 3 °C/min, setting the temperature for 2 min, and then from 220 to 290 °C at a rate of 10 °C/min. The injector and detector temperatures were set at 250 °C. Helium was used as carrier gas at 1 ml/min. The compounds were identified by comparison with the retention index of a homologous mixture of hydrocarbon series and the fragmentation standard with the Wiley 7.0 and NIST data system library.

HPLC analysis

The hydroalcoholic extract of brown propolis was analyzed in a Waters® high-performance liquid chromatography, with a binary pump system model 1525, automatic injector model 2707, diode array detector model 2998, and temperature controller. The data were acquired and

processed using the Empower 3 software. The method used in the analyses was developed and validated by Sousa et al. (2007). The mobile phase consisted of a gradient starting with the mixture of 75% of solvent A (water + methanol 5% + acetic acid 0,4%) and 25% of solvent B (acetonitrile), and finishing with 100% of B, over 60 min, with a flow rate of 1 ml/min, injection volume of 20 µl and temperature of 35 °C. The spectral data were collected over the 265–320 nm range of the absorption spectrum from the photodiode array detector, and the chromatograms were plotted at 280 nm. The identification of the compounds present in the samples was carried out by using authentic chromatographic standards either acquired in the market or isolated and following the retention time in the validated method, and by their UV absorption spectrum.

Animals

Male Balb/C mice 3-5 weeks, 20 ± 5 g were used. The animals were kept in rooms with controlled temperature (20-24 ºC), natural humidity (40-60%), and controlled cycles of 12 h light/dark, with food and water offered *ad libitum*. Before the experiments, the animals were acclimatized to the laboratory for at least 1 h. Euthanasia was performed with an anesthetic overdose of sodium phenobarbital by intraperitoneal administration.

Formalin-Induced Licking Response

Animals were injected subcutaneously (*s.c.*) with 20 µl of a 2.5% formalin solution into the right dorsal hind paw. The pain response was taken by the time (s) spent licking and biting the injected paw, which was measured for 30 min. Two distinct periods of intensive licking activity were identified and scored separately. The first period, phase one, was recorded for the first 5 min, after the injection of formalin, and the second period, phase two, was recorded between 15 and 30 min after the injection of the stimulus. Groups of mice (n = 6) were treated with

hydroalcoholic and volatile extracts (50, 100 and 200 mg/kg), indomethacin (10 mg/kg), morphine (5 mg/kg, *s.c.*) or vehicle (cremophor 10% solution), orally, 30 min before formalin injection. The controls were solubilized in saline (0.9%), each extract concentration was prepared separately, solubilized in cremophor, resulting in the concentrations described.

Carrageenan-Induced Hyperalgesia

For the mechanical hypernociception evaluation, the mice received an injection of 20 µl of carrageenan (300 µg/paw) under the surface of the right hind paw (Santos et al. 2010) for the induction of inflammatory pain. The mice (n = 6) were treated with hydroalcoholic and volatile extracts (50, 100 and 200 mg/kg), indomethacin (10 mg/kg), morphine (5 mg/kg, *s.c.*) or vehicle (cremophor 10% solution), 30 min before carrageenan injection. The mice were placed in clear plexiglas boxes (9 cm × 7 cm × 11 cm) individually on elevated wire mesh platforms to allow access to the ventral surface of the right hind paw. All groups were assessed employing von Frey filament (Stoelting, Chicago, IL, USA), up to 1, 2, 4, 6, 12, 24, and 48 h after carrageenan administration. The paw withdrawal response frequency was measured following 10 von Frey filament applications, applied from below, to the plantar surface of the right hind paw. The inhibition percentage was calculated using the formula: % inhibition = 100(1-At/Ac), where Ac represents the area under the curve in the control group and At the area under the curve in the group treated with the extract.

Nociception Evaluation

For measuring the nociceptive activity, a tail-flick apparatus (Insight, Brazil) was used. The distal half of the mice's tail was placed in an analgesia meter set at 50 °C. An automatic cut-off time (15 s) was adjusted to minimize possible tissue damage in the absence of withdrawal response. The animals were divided into different groups (n = 6) and pretreated with morphine

(5 mg/kg, *s.c.*), cremophor solution 10%, and with hydroalcoholic and volatile extracts (50, 100, and 200 mg/kg) of propolis. Studies were carried out with naloxone, the opioid receptor antagonist, to investigate the possible antagonistic effects of propolis. The animals (n = 6) were divided into different groups and treated with morphine (5 mg/kg, *s.c.*) + naloxone (1 mg/kg, *i.p.*), naloxone (1 mg/kg, *i.p.*) + hydroalcoholic extract (200 mg/kg) and naloxone (1 mg/kg, *i.p.*) + volatile extract (200 mg/kg). The flicking response (tail withdrawal) from the heat was taken as the endpoint. The tail-flick latencies were recorded before administering the drug and then at post-treatment time intervals of 30, 60, 120, and 180 min.

Statistical Analysis

Data were reported as mean ± s.e.m., and it was analyzed statistically by analysis of variance, followed by Bonferroni's test. Results with $p < 0.05$ were considered significant.

Results and Discussion

The hydroalcoholic extract of brown propolis yielded 47.3% of total raw propolis. The crude extract was analyzed by high-performance liquid chromatography, which displayed a very complex mixture of compounds (Fig. 1).

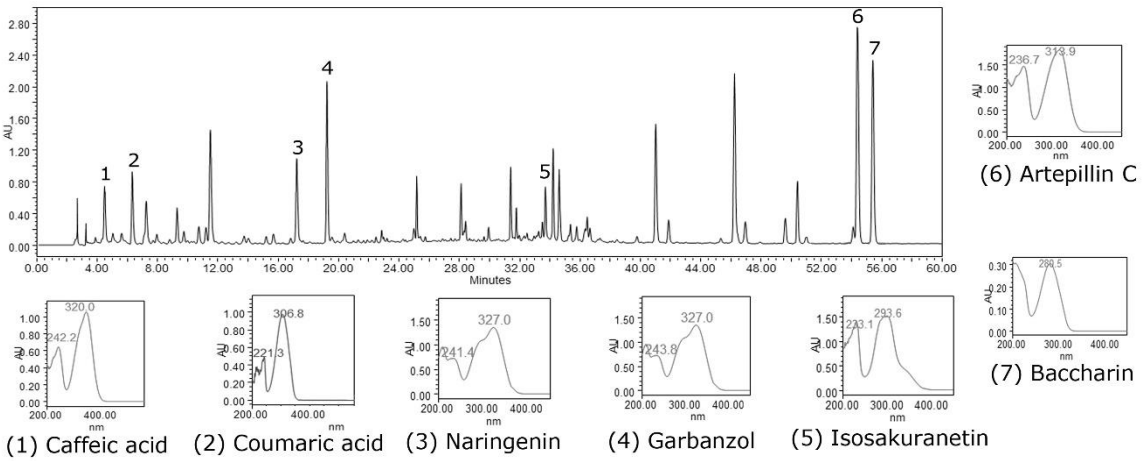
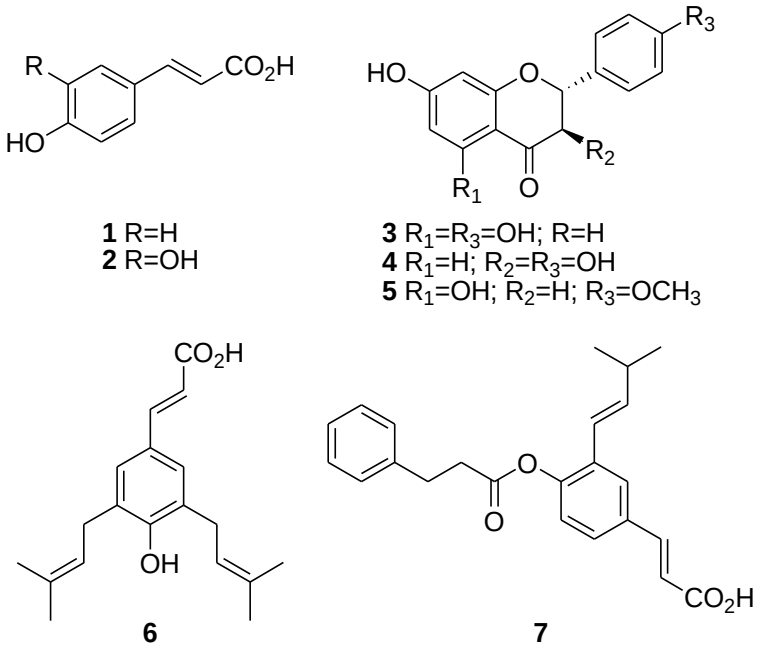


Figure 1. HPLC-DAD chromatogram of the hydroalcoholic extract of brown propolis and the UV spectra of the identified compounds.

The analytical method used to analyze brown propolis was previously validated to analyze green propolis (Sousa et al. 2007). However, it was possible to dereplicate common chemical constituents to both type of propolis, including caffeic acid (**1**; Rt = 4.19, λmax = 320 nm), coumaric acid (**2**; Rt = 5.91, λmax = 307 nm), isosakuranetin (**5**; Rt = 33.93, λmax = 293 nm), artepillin C (**6**; Rt = 54.40, λmax = 313 nm) and baccharin (**7**; Rt = 55.51, λmax = 280 nm). Authentic chromatographic standards and previously isolated and identified compounds were also used to identify the brown propolis sample compounds. It was possible to suggest, by comparing the UV absorbance spectra and the retention times after variations in the method, the presence of two flavonoids, naringenin (**3**; Rt = 17.42, λmax = 327) and garbanzol (**4**; Rt = 19.14, λmax = 327).



The volatile fraction was obtained through hydrodistillation (yield 0.9%) was characterized by CG-MS analysis (Table S1). The volatile fraction was also complex with 44 substances identified and mainlycomposed of monoterpenes and sesquiterpenes, such as nerolidol (12.51%), spathulenol (8.64%), cineole (7.42%), β-caryophyllene (5.52%), β-

bourbunene (5.14%), γ-bisabolene (5.11%), α-copaene (4.81%), terpineol (4.78%), acetophenone (4.57%) and α-chamigrene (4.04%).

During the first phase of the formalin test, both the hydroalcoholic extract and the volatile fraction significantly inhibited the animals' response time at all evaluated concentrations (Fig. 2a). At concentrations of 50, 100, and 200 mg/kg, the hydroalcoholic extract inhibited the painful process induced by the phlogistic agent in the neurogenic phase with inhibitions of 73 ± 7%, 83 ± 8%, and 89 ± 3%, respectively, while the volatile fraction inhibited 75 ± 7%, 94 ± 5% and 99 ± 1%, respectively, at the same concentrations. The positive controls indomethacin and morphine inhibited 66 ± 4% and 96 ± 1%, respectively. Throughout the second phase of the formalin test, all the hydroalcoholic extract doses inhibited the response time. The volatile fraction was active only at the concentrations of 100 and 200 mg/kg (Fig. 2b). The calculated inhibitions were 48 ± 13%, 65 ± 9%, and 75 ± 6% at 50, 100, and 200 mg/kg for the hydroalcoholic extract in the inflammatory phase. For the volatile fraction, the inhibitions were 30 ± 7%, 53 ± 5%, and 91 ± 5%, respectively, while for the controls indomethacin and morphine it showed 70 ± 3% and 99 ± 1% of inhibitions, respectively.

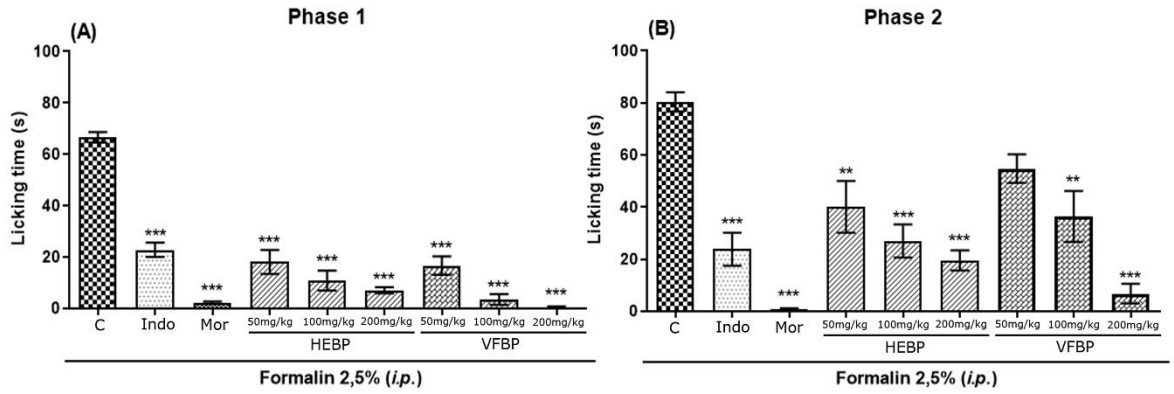


Figure 2. Effects of administration of indomethacin (Indo; 10 mg/kg), morphine (Mor; 5 mg/kg), brown propolis hydroalcoholic extract (HEBP) and brown propolis volatile fraction (VFBP) (50, 100, 200 mg/kg) on formalin injection (2.5%; 20 µl/paw) in mice. (A) 0-5'; 1st phase; neurogenic pain, (B) 15-30'; 2nd phase; inflammatory pain. Data represent the mean ± E.P.M. (n= 6). Significant difference from the control group, ANOVA, Bonferroni a posteriori test (***p <0.001; **p <0.01).

It was observed that during the evaluated period of 48 h, there was a mechanical hypernociception response induced by carrageenan, which was inhibited significantly by both the hydroalcoholic extract and the volatile fraction of the brown propolis in comparison with the control group, and in a dose-dependent manner (Fig. 3). The hydroalcoholic extract showed mechanical sensitization reductions of 57 ± 7%, 67 ± 6% and 79 ± 5% and the volatile fraction 62 ± 7%, 79 ± 9% and 88 ± 4% at 50, 100 and 200 mg/kg, respectively. The control groups treated with indomethacin and morphine showed inhibitions of 42 ± 4% and 88 ± 2%, respectively. Although indomethacin has anti-inflammatory activity, it could not significantly inhibit the hypernociceptive response at certain evaluated periods.

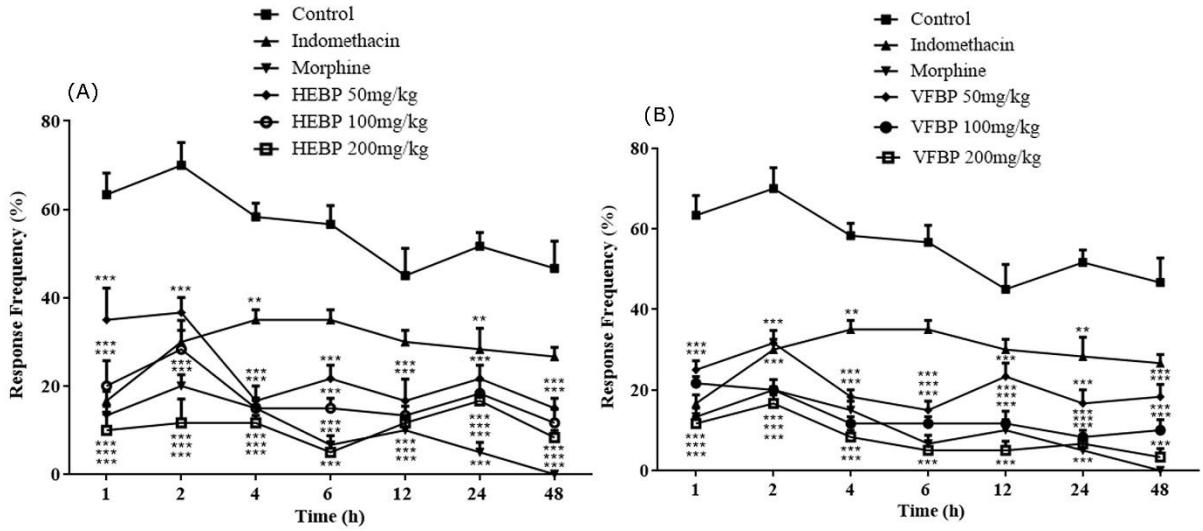


Figure 3. Response frequency of right hind paw withdrawal, evaluated in the control group of indomethacin (10 mg/kg), morphine (5 mg/kg), and in animals treated with brown propolis hydroalcoholic extract (HEBP) (A) and brown propolis volatile fraction (VFBP) (B) at concentrations of 50, 100, 200 µg/ml in different time intervals after i.pl injection carrageenan. Data represent the mean ± E.P.M. (n= 6). Significant difference from the control group, ANOVA, Bonferroni a posteriori test (***p <0.001; **p <0.01).

In the tail-flick test, the animals' treatment with the crude extract and the volatile fraction induced an increase of the nociceptive threshold in mice exposed to the heat source compared to the control (Fig. 4). Inhibitions of 58 ± 8% and 70 ± 6% and 77 ± 10% were observed, respectively, for doses of 50, 100 and 200 mg/kg to animals treated with crude extract, whereas for the volatile fraction, inhibitions of 55 ± 6% and 73 ± 7% and 82 ± 14%

were observed, respectively. Morphine also increased the nociceptive threshold of animals with a maximum inhibition of 76 ± 5%. The coadministration of naloxone, a non-selective opioid antagonist, with morphine and crude extract and the volatile fraction of propolis altered animals' latency time in response to thermal stimulus.

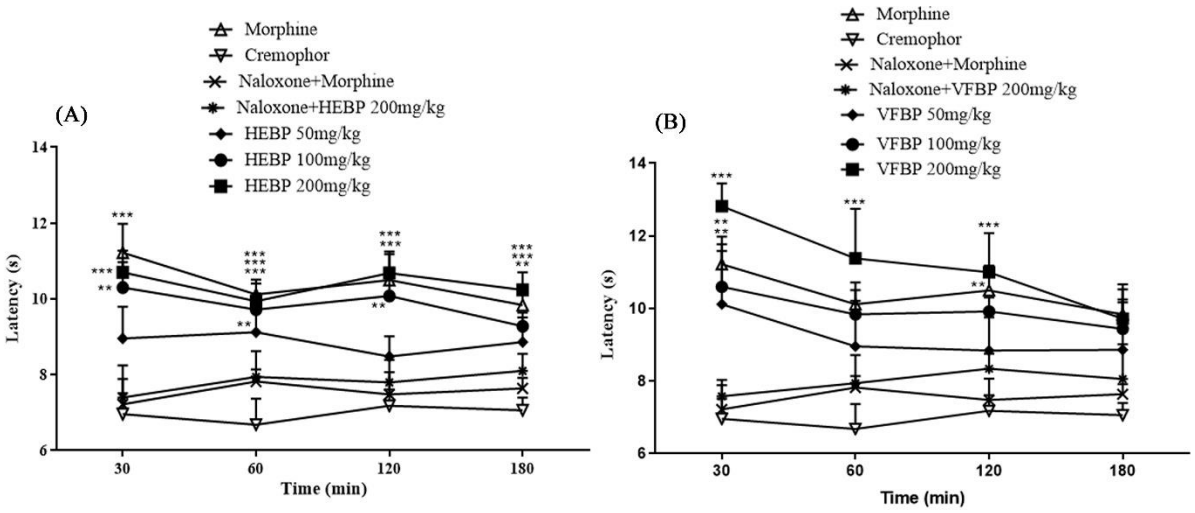


Figure 4. Latency time in response to thermal stimulus in the tail flick test in mice, evaluated in the group of morphine (5 mg/kg), cremophore (10%), naloxone (1mg/kg), and in animals treated with brown propolis hydroalcoholic extract (HEBP) (A) and brown propolis volatile fraction (VFBP) (B) at concentrations of 50, 100, 200 µg/ml in different time intervals. Data represent the mean ± E.P.M. (n= 6). Significant difference from the control group, ANOVA, Bonferroni a posteriori test (***p <0.001; **p <0.01).

Compounds such as artepillin C (**6**) and baccharin (**7**) were identified in the hydroalcoholic extract. These compounds are chemical markers of *Baccharis dracunculifolia* (Beserra et al. 2020). The identification of these compounds supports that *B. dracunculifolia*, a predominant species of the Southeastern Brazilian region, is one of the botanical sources used by bees in the production of this brown propolis (Arruda et al. 2020b). Compounds such as coumaric acid (**2**) and artepillin C (**6**) have already been identified in Brazilian brown propolis. Machado et al. (2016a) quantified them in brown propolis collected in the state of Santa Catarina and identified artepillin C (**6**) in different samples of brown propolis collected in the region of Rio Grande do Sul, both states in the Southern region of Brazil. Caffeic acid (**1**),

coumaric acid (**2**) and artepillin C (**6**) were also identified in brown propolis from Paraná, another Southern Brazilian state (Machado et al. 2016b).

The flavonoid naringenin (**3**) has been identified in several types of propolis from different locations of the world, such as Algeria and Croatia, which was also reported in several species of *Baccharis* (Ribeiro et al. 2013). Garbanzol (**4**) was identified in red propolis samples from the Northeastern regions of Brazil, and there are no reports of its occurrence in *Baccharis* species (Li et al. 2008), which reinforces the hypothesis of the presence of other botanical sources in the production of this type of brown propolis.

The volatile fraction corroborates the fact that the Southeastern brown propolis is produced from different plants. Lima et al. (2019) reported the volatiles' chemical composition present in a Southeastern Brazilian brown propolis, which was similar to the results for the propolis sample used in this study. Research involving the volatile oil of *B. dracunculifolia* showed nerolidol and spathulenol as its chemical markers, confirming that this plant is a source for propolis production. However, other major compounds present in the studied propolis sample were not found in *B. dracunculifolia* (Sousa et al. 2009). Cineol is mainly found in *Eucalyptus* spp., which is an abundant plant in the region where the propolis sample was collected (Silva et al. 2006), as well β-caryophyllene and α-copaene are reported in *Copaifera* sp., a tree from Brazilian flora (Ribeiro et al. 2019; Silva et al. 2020). Due to the biodiversity available to bees and this propolis's chemical complexity, it is possible to infer that bees also visited other plants.

In nociception model using formalin, the first phase (neurogenic) is characterized by the direct stimulation of nociceptors present on C and Aδ fibers releasing glutamate and substance P. The second phase (inflammatory) of nociception is related to the release of pro-inflammatory mediators, such as bradykinin, prostaglandin, histamine, and serotonin (Bars et al. 2001). The formalin test can be used to explain the mechanism of antinociceptive effect.

Drugs with central action, such as opioids (morphine), inhibit both phases of the formalin test, but peripherally acting drugs, such as indomethacin, inhibit only the late phase (Shibata et al. 1989). In this way, it is possible to suggest that the hydroalcoholic extract and volatile oils are acting by reducing inflammatory pain signaling processes, interfering with inflammatory mediators' action. The inhibition observed in the first phase also suggests a relation of the samples with the production of some neuropeptides as glutamate, generating an antinociceptive response.

Carrageenan is a phlogistic agent that induces inflammation, lesions in blood vessels, and kinins' release, generating inflammatory exudation and paw edema. In this regard, intraplantar injection of carrageenan in mice produces acute paw edema (Omote et al. 2001; Lopes at al. 2019). The immune response triggered by carrageenan involves the release of pro-inflammatory mediators, such as the cytokines TNF-α, IL-1β, IL-6, and nitric oxide through the activation of macrophages and endothelial cells, which are related to the development of mechanical hypernociception (Zhang et al. 2018). Thus, there is an exacerbated development of nociceptive sensory primary neurons' sensitivity to a thermal or mechanical stimulus (Fecho et al. 2007). The results showed that the hydroalcoholic extract and volatile oils reduced the hypernociceptive response, and this effect was noticed during the 48 h of the experiment.

The tail-flick test evaluates thermal nociception and central spinal involvement. The tail withdrawal occurs through a rapid, vigorous, and reflexive spinal origin movement in response to a thermal stimulus. Substances with antinociceptive activities that act by opioid-like mechanisms increase latency and response to this thermal stimulus (Eghianruwa et al. 2020). Propolis was able to increase the nociceptive threshold of mice exposed to the heat source. Central analgesic drugs are selective in the face of these painful thermal stimuli, whereas peripheral action analgesics have low influence against these stimuli. The activation of opioid receptors mediates the antinociceptive activity of morphine. Therefore, the

coadministration of an opioid antagonist alters the effect of this drug's nociceptive activity (Shibata et al. 1989). Thus, we can also suggest that propolis has an antinociceptive effect on the central nervous system since its effect was altered by administering an opioid receptor antagonist, naloxone.

Brazilian brown propolis hydroalcoholic extract reduced the lesions caused by the herpes virus (HSV-2) in mice by inflammatory and oxidative processes. Sartori et al. (2012) reported that propolis decreased reactive oxygen species, tyrosine nitration, and myeloperoxidase activity, promoting an anti-inflammatory action. Previous reports show that artepillin C (**6**) display anti-inflammatory activity by inhibiting neutrophil migration and activation of the pro-inflammatory mediator NF-κB (Paulino et al. 2008; Ikeda et al. 2011). Pinheiro et al. (2011) reported that the oil of *Pinus serpens*, in which the major compound is nerolidol, inhibits adhesion molecules responsible for binding and cellular migration. Therefore, it can be suggested that the volatile oils from the brown propolis may act by reducing cytokines that directly interfere in cell migration, consequently reducing the inflammatory process due to its chemical composition.

Conclusion

The Southeastern Brazilian brown propolis hydroalcoholic extract and volatile oils showed promising results in the nociceptive and anti-inflammatory assessment protocols. Besides, it should be highlighted that the volatile fraction at the highest tested concentration (200 mg/kg) inhibited both phases in the formalin test and the mechanical hypernociception, which was similar to those observed for morphine, an opioid with high analgesic power. Brown propolis showed significant antinociceptive activity, acting through the modulation of nociceptive transmission and as an anti-inflammatory. The results obtained in the pharmacological models corroborate the traditional use of propolis as an anti-inflammatory agent.

References

Araújo MJAM, Mattar NS, Reis AS, Serra ICPB, Fialho EMS, Assunção AKM, Dutra RP, Nogueira AMC, Libério SA, Guerra RNM, Lopes AS, Ribeiro MNS, Nascimento FRF (2011) Pharmacognostic and acute toxicological evaluation of *Scaptotrigona* aff. *postica* propolis extract in pre-clinical assays. Nat Prod Res 11:1037-1046. DOI: 10.1080/14786419.2010.482059.

Arruda C, Ribeiro VP, Almeida MO, Mejía JAA, Casoti R, Bastos JK (2020a) Effect of light, oxygen and temperature on the stability of artepillin C and *p*-coumaric acid from Brazilian green propolis. J Pharm Biomed 178:112922. DOI: 10.1016/j.jpba.2019.112922.

Arruda C, Ribeiro VP, Mejía JAA, Almeida MO, Goulart MO, Candido ACBB, Santos RA, Magalhães LG, Martins CHG, Bastos JK (2020b) Green propolis: cytotoxic and leishmanicidal activities of artepillin C, *p*-coumaric acid, and their degradation products. Rev Bras Farmacogn 30:169–176. DOI: 10.1007/s43450-020-00043-3.

Beserra FP, Gushiken LFS, Hussni MF, Ribeiro VP, Bonamin F, Jackson CJ, Pellizzon CH, Bastos JK (2020) Artepillin C as an outstanding phenolic compound of Brazilian green propolis for disease treatment: A review on pharmacological aspects. Phytother Res 1-13. DOI: 10.1002/ptr.6875.

Eghianruwa Q, Osoniyi O, Maina N, Wachira S, Imbuga M (2020) Evaluation of analgesic activities of extracts of two marine molluscs: *Tympanotonus fuscatus* var *radula* (Linnaeus) and *Pachymelania aurita* (Müller). J Pain Res 13:2739-2747. DOI:10.2147/JPR.S271458.

Fonseca DV, Salgado PRR, Carvalho FL, Salvadori MGSS, Penha ARS, Leite FC, Borges CJS, Piuvezam MR, Pordeus LCM, Sousa DP, Almeida RN (2016) Nerolidol exhibits antinociceptive and anti-inflammatory activity: involvement of the GABAergic system and proinflammatory cytokines. Fund Clin Pharmacol 30:14-22. DOI:10.1111/fcp.12166.

Freitas AS, Barth OM, Sales EO, Matsuda AH, Muradian LBA (2011) A palynological analysis of Brazilian propolis samples. JAAS 48:67-74. DOI: 10.3896/IBRA.4.03.2.01.

Ikeda R, Yanagisawa M, Takahashi N, Kawada T, Kumazawa S, Yamaotsu N, Nakagome I, Hirono S, Tsuda T (2011) Brazilian propolis-derived components inhibit TNF-α-mediated downregulation of adiponectin expression via different mechanisms in 3T3-L1 adipocytes. Biochim Biophys Acta 1810:695–703. DOI: 10.1016/j.bbagen.2011.04.007.

Kocot J, Kiełczykowska M, Luchowska-kocot D, Kurzepa J, Musik I (2018) Antioxidant potential of propolis, bee pollen, and royal jelly: possible medical application. Oxid Med Cell Longev. DOI:10.1155/2018/7074209.

Kumazawa S, Hayashi K, Kajiya K, Ishii T, Hamasaka T, Nakayama T (2002) Studies of the constituents of Uruguayan propolis. J Agric Food Chem 50:4777–4782. DOI: 10.1021/jf020279y.

Li F, Awale S, Tezuka Y, Kadota S (2008) Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. Bioorg Med Chem 16:5434-5440. DOI:10.1016/j.bmc.2008.04.016.

Lima VHM, Almeida KCR, Alves CCF, Rodrigues ML, Crotti AEM, Souza JM, Ribeiro AB, Squarisi IS, Tavares DC, Martins CHG, Miranda MLD (2019) Biological properties of volatile oil from Brazilian brown propolis. Rev Bras Farmacogn 29:807-810. DOI:10.1016/j.bjp.2019.07.004.

Lopes AH, Silva RL, Fonseca MD, Gomes FI, Maganin AG, Ribeiro LS, Cunha FQ, Alves-Filho JC, Zamboni DS, Franklin BS, Gombault A, Quesniaux VFJ, Couillin I, Ryffel B, Cunha T (2019) Carrageenan triggers NLRP3 inflammasome activation and IL-1β production by macrophages. SSRN Electronic Journal. DOI: 10.2139/ssrn.3405570.

Machado BAS, Silva RPD, Barreto GDA, Costa SS, Silva DFD, Brandão HN, Rocha JLC, Dellagostin OA, Henriques JAP, Umsza-Guez MA, Padilha FF (2016a) Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PLoS One 11:e0145954. DOI:10.1371/journal.pone.0145954.

Machado CS, Mokochinski JB, Lira TO, Oliveira FCE, Cardoso MV, Ferreira RG, Sawaya ACHF, Ferreira AG, Pessoa C, Cuesta-Rubio O, Monteiro MC, Campos MS, Torres YR (2016b) Comparative study of chemical composition and biological activity of yellow, green, brown, and red brazilian propolis. Evid-Based Compl Alt. DOI:10.1155/2016/6057650.

Machado JL, Assunção AKM, Silva MCP, Reis AS, Costa GC, Arruda DS, Rocha BA, Vaz MMOLL, Paes AMA, Guerra RNM, Berretta AA, Nascimento FRF (2012) Brazilian green propolis: anti-inflammatory property by an immunomodulatory activity. Evid-Based Compl Alt. DOI:10.1155/2012/157652.

Paulino N, Abreu SRL, Uto Y, Koyama D, Nagasawa H, Hori H, Dirsch VM, Vollmar AM, Scremin A, Bretz WA (2008) Anti-inflammatory effects of a bioavailable compound, artepillin C in Brazilian propolis. Eur J Pharmacol 587:296-301. DOI: 10.1016/j.ejphar.2008.02.067.

Pinheiro BG, Silva AS, Souza GE, Figueiredo JG, Cunha FQ, Lahlou S, Silva JK, Maia JG, Sousa PJ (2011) Chemical composition, antinociceptive and anti-inflammatory effects in rodents of the essential oil of *Peperomia serpens* (Sw.) Loud. J Ethnopharmacol 138:479-86. DOI: 10.1016/j.jep.2011.09.037.

Ribeiro PRV, Girão DC, Pimenta ATA, Braz-Filho R, Guedes MLS, Silveira ER, Lima MAS (2013) Clerodane and patchoulene terpenes as new constituents from *Baccharis salzmannii* DC. Biochem Syst Ecol 50:101-105. DOI: 10.1016/j.bse.2013.03.042.

Ribeiro VP, Arruda C, El-Salam MA, Bastos JK (2018) Brazilian medicinal plants with corroborated anti-inflammatory activities: a review. Pharm Biol 56:253–268. DOI: 10.1080/13880209.2018.1454480.

Ribeiro VP, Arruda C, Mejía JAA, Candido ACBB, Santos RA, Magalhães LG, Bastos, JK (2020) Brazilian southeast brown propolis: gas chromatography method development for its volatile oil analysis, its antimicrobial and leishmanicidal activities evaluation. Phytochem Analysis. DOI:10.1002/pca.2988.

Ribeiro VP, Arruda C, Silva JJM, Meíja JAA, Furtado NAJC, Bastos JK (2019) Use of spinning band distillation equipment for fractionation of volatile compounds of Copaifera oleoresins for developing a validated gas chromatographic method and evaluating antimicrobial activity. Biomed Chromatogr 33:e4412. DOI: 10.1002/bmc.4412.

Santos DA, Fukui MJ, Dhammika NNP, Khan SI, Sousa JP, Bastos JK, Andrade SF, Silva Filho AA, Quintão NL (2010) Anti-inflammatory and antinociceptive effects of *Baccharis dracunculifolia* DC (Asteraceae) in different experimental models. J Ethnopharmacol 127:543-50. DOI: 10.1016/j.jep.2009.09.061.

Santos FA, Rao VSN (2000) Anti-inflammatory and Antinociceptive Effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytother Res 14:240-244. Doi: 10.1002/1099-1573(200006)14:4<240::aid-ptr573>3.0.co;2-x.

Sartori G, Pesarico AP, Pinton S, Dobrachinski F, Roman SS, Pauletto F, Rodrigues LC, Prigol M (2012) Protective effect of brown Brazilian propolis against acute vaginal lesions caused by herpes simplex virus type 2 in mice: involvement of antioxidant and anti-inflammatory mechanisms. Cell Biochem Funct 30:1-10. DOI:10.1002/cbf.1810.

Silva JJM, Ribeiro VP, Lemos M, Crotti AEM, Rogez H, Bastos JK (2020) Reliable methods for analyses of volatile compounds of *Copaifera* oleoresins combining headspace and gas chromatography. Chem Biodivers 17:e1900440. DOI: 10.1002/cbdv.201900440

Simone-Finstrom M, Spivak M (2010) Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie 41:295–311. https://doi.org/10.1051/apido/2010016.

Sousa JPB, Bueno PCP, Gregório LE, Silva Filho AA, Furtado NAJC, Sousa ML, Bastos JK (2007) A reliable quantitative method for the analysis of phenolic compounds in Brazilian propolis by reverse phase high performance liquid chromatography. J Sep Sci 30:2656-2665. DOI:10.1002/jssc.200700228.

Yang C, Luo L, Zhang H, Yang X, Lv Y, Song H (2010) Common aroma-active components of propolis from 23 regions of China. J Sci Food Agric 90:1268–1282. DOI: 10.1002/jsfa.3969.

Zhang P, Bi RY, Gan YH (2018) Glial interleukin-1β upregulates neuronal sodium channel 1.7 in trigeminal ganglion contributing to temporomandibular joint inflammatory hypernociception in rats. J Neuroinflammation 15:117. DOI: 10.1186/s12974-018-1154-0.

3.4. Capítulo 4 – Estudo fitoquímico de uma amostra de própolis marrom do sudeste brasileiro e avaliação das atividades antiplasmódica, citotóxica e antimicrobiana.

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Article

Phytochemical, antiplasmodial, cytotoxic and antimicrobial evaluation of a Southeast Brazilian Brown Propolis produced by *Apis mellifera bees*.

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Abstract

Seven phenolic compounds (ferulic acid, caffeic acid, 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 3-hydroxy-4-methoxybenzaldehyde, 3-methoxy-4-hydroxypropiophenone and 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside), a flavanonol (7-*O*-methyl aromadendrin), two lignans (pinoresinol and matairesinol) and six diterpenic acids/alcohol (19-acetoxy-13-hydroxylabda-8(17),14-diene, totarol, 7-oxodehydroabietic acid, dehydroabietic acid, communic acid and isopimaric acid) were isolated from the hydroalcoholic extract of a Brazilian Brown Propolis and characterized by NMR spectral data analysis. The volatile fraction of brown propolis wascharacterized by CG-MS, composed mainly of monoterpenes and sesquiterpenes, being the major α-pinene (18.4%) and β-pinene (10.3%). This propolis chemical profile indicates that *Pinus* spp., *Eucalyptus* spp. and *Araucaria angustifolia* might be its primary plants source. The brown propolis displayed significant activity against *Plasmodium falciparum* D6 and W2 strains with IC_{50} of 5.3 and 9.7 μg/mL, respectively. The volatile fraction was also active with IC_{50} of 22.5 and 41.8 μg/mL, respectively. Among the compounds, 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside showed IC_{50} of 3.1 and 1.0 μg/mL against D6 and W2 strains, respectively, while communic acid showed an IC_{50} of 4.0 μg/mL against W2 strain. Cytotoxicity was determined on four tumor cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and two normal renal cell lines (LLC-PK1 and VERO). Matairesinol, 7-*O*-methyl aromadendrin, and isopimaric acid showed an IC_{50} range of 1.8 – 0.78 μg/mL, 7.3 – 100 μg/mL, and 17-18 μg/mL, respectively, against the tumor cell lines but they were not cytotoxic against normal cell lines. The crude extract of brown propolis displayed antimicrobial activity against *C. neoformans*, methicillin-resistant *Staphylococcus aureus*, and *P. aeruginosa* at 29.9 μg/mL, 178.9 μg/mL, and 160.7 μg/mL, respectively. The volatile fraction inhibited the growth of *C. neoformans* at 53.0 μg/mL. The compounds 3-hydroxy-4-methoxybenzaldehyde, 3-methoxy-4-hydroxypropiophenone and 7-oxodehydroabietic acid were active against *C. neoformans*, and caffeic and communic acids were active against methicillin-resistant *Staphylococcus aureus*.

Keywords: Brazilian Brown Propolis, Lignans, Diterpenes, Antimalarial, Cytotoxicity.

Introduction

Propolis is a natural resin produced by bees from plants materials, bees wax, and salivary secretion. Propolis has benefits for the hive, additionally, the plant metabolites present in the resin collaborate for a low incidence of microorganisms in the hive.[1] Propolis is widely used in folk medicine due to several biological properties, especially anti-inflammatory and antioxidant.[2] Besides, propolis is an essential material in the cosmetic and food industries. Thus, propolis is a natural product of outstanding chemical, biological and economic importance.[3]

Several classes of compounds have been reported for the different types of propolis worldwide. Propolis is generally classified according to its color in Brazil, and green propolis, rich in phenolic and prenylated phenolic compounds such as aromadendrin, kaempferide,

drupanin, baccharin, and artepillin C, stands out.[4] The brown type contains mainly phenolic acids and diterpenes, such as coniferylaldehyde, imbricatoloic acid, isocupressic acid, and communic acid.[5] The red type, characteristic of the northeast region of Brazil, contains mainly isoflavones and prenylated benzophenones, as vestitol, neovestitol, medicarpin, fomonometin, guttiferone E, xanthochymol and oblogifolin B.[6, 7]

Several biological properties were attributed to Brazilian brown propolis, such as leishmanicidal,[8] anti-inflammatory and nociceptive,[9] mutagenic and antimicrobial,[10] and antimycoplasma.[11] Some studies attribute the antimicrobial activity of brown propolis to terpenic compounds present in its extract,[12] while its antioxidant effect is related to the cytotoxic activity, mediated via free radical-scavenging activity by the phenolic compounds.[13] The pharmacological application of propolis, must be correlated with its chemical composition. Many factors can influence propolis chemical composition once bees use plant resin as the source. Therefore, propolis chemical composition is closely related to each region's ecology and flora.[14] Hence, identifying the botanical source of propolis is a crucial step for the use of this natural product safely and effectively in medicine.[15]

Prenylated phenylpropanoids, as 2,2-dimethyl chromene-6-propenoic acid and the 2,2-dimethyl-8-prenyl chromane-6-propenoic acid, and caffeoyl-quinic acids were characterized in a southern Brazilian brown propolis.[11] The labdane type diterpenic acids coniferylaldehyde, isocupressic acid and communic acid were isolated from a Brazilian brown propolis from Parana state.[12] The compounds lupeol, 2,3-dihydroxybenzofurane and coumaric acid were identified in a brown propolis sample from the northeast region of Brazil.[16] There are still many gaps in determining the chemical compositions and botanical sources of Brazilian brown propolis compared to both green and red ones. Filling these gaps is very important for developing medicinal products with therapeutical indications. Considering the importance of the chemical standardization of this valuable natural product and the great chemical diversity of brown propolis, we investigated the chemical composition of a type of Brazilian brown propolis and some of its biological properties. The studied propolis presents a huge chemical diversity, with different classes of metabolites, usually not reported in propolis samples from different plant sources. The obtained results might interest companies and beekeepers, bringing a significant contribution to the field.

Results and discussion

Chemical characterization

For the phytochemical investigation of brown propolis obtained in the region of Angatuba-SP, Brazil, the crude hydroalcoholic extract was partitioned and subjected to different chromatographic techniques. Sixteen compounds were isolated and identified from Brazilian Brown propolis, including seven phenolic compounds (ferulic acid, caffeic acid, 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 3-hydroxy-4-methoxybenzaldehyde, 3-methoxy-4-hydroxypropiophenone and 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside), a flavanonol (7-*O*-methyl aromadendrin), two lignans (pinoresinol and matairesinol) and six diterpenic acids/alcohol (19-acetoxy-13-hydroxylabda-8(17),14-diene, totarol, 7-oxodehydroabietic acid, dehydroabietic acid, communic acid, and isopimaric acid), figure 1. The compounds were identified by NMR and mass spectroscopic data analyses in comparison with the literature data.[17-27]

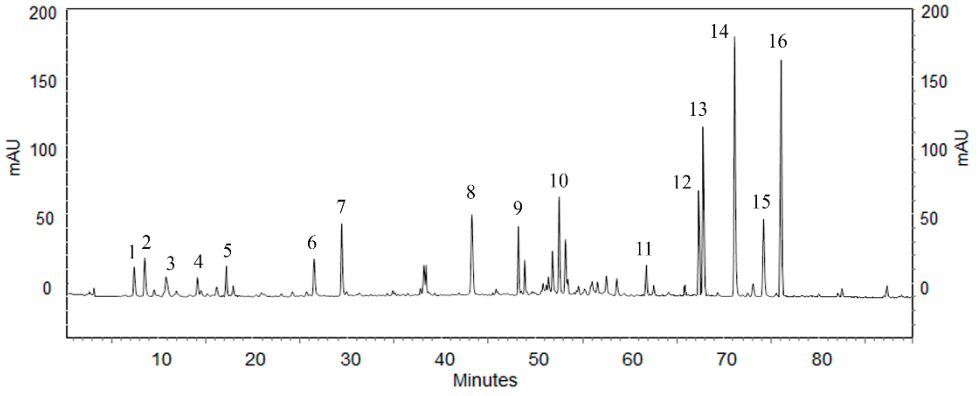
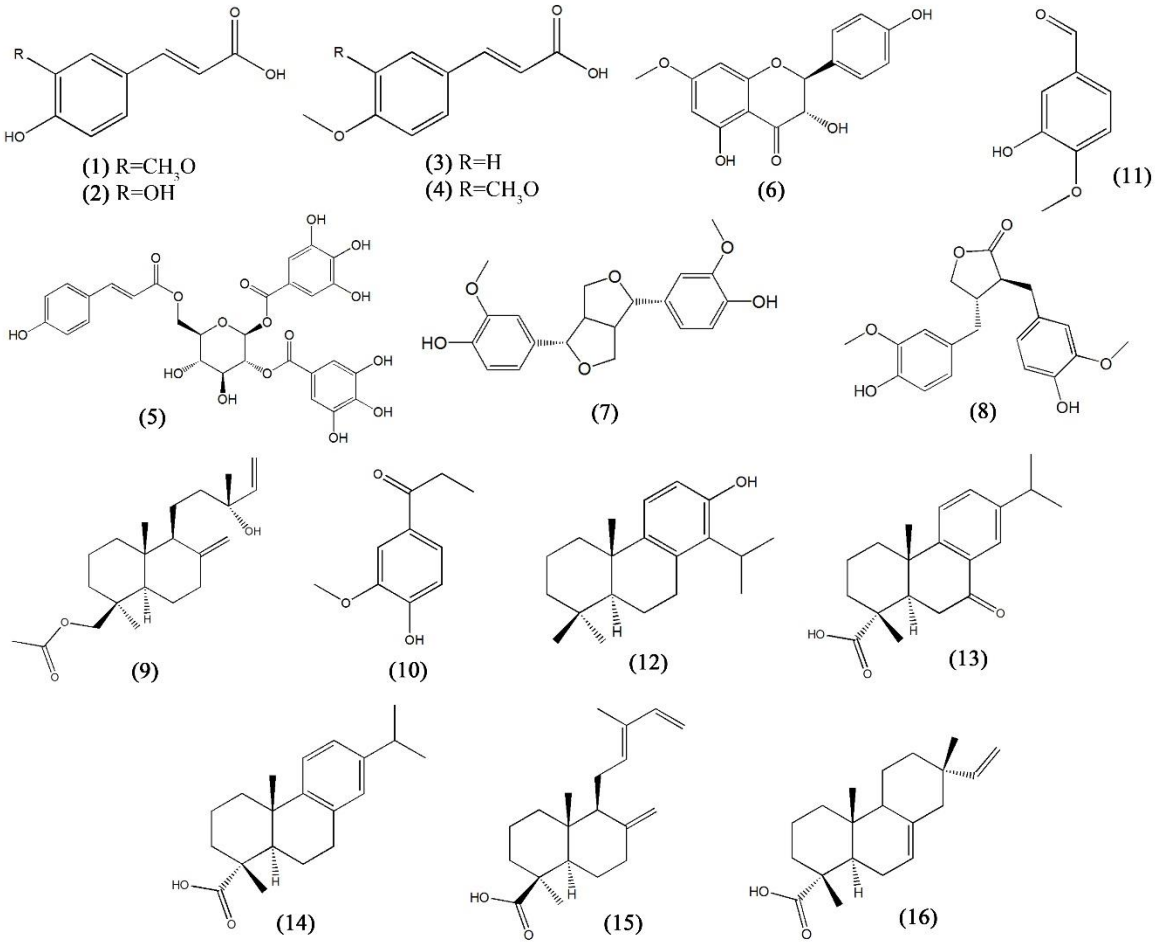


Figure 1. Chemical profile of Brazilian Brown propolis extract. Peak 1: 3,4-dimethoxycinnamic acid; Peak 2: ferulic acid; Peak 3: 4-methoxycinnamic acid; Peak 4: 3-hydroxy-4-methoxybenzaldehyde; Peak 5: 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside; Peak 6: 3-methoxy-4-hydroxypropiophenone; Peak 7: caffeic acid; Peak 8: matairesinol; Peak 9: pinoresinol; Peak 10: 7-*O*-methyl aromadendrin; Peak 11: 19-acetoxy-13-hydroxylabda-8(17),14-diene; Peak 12: totarol; Peak 13: 7-oxodehydroabietic acid; Peak 14: dehydroabietic acid; Peak 15: communic acid; Peak 16: isopimaric acid.

Some of the isolated phenolic compounds are common to other types of propolis. Ferulic acid and caffeic acid are found in brown, green, and red types of Brazilian propolis.[6,28] The other phenolic compounds were identified in many different types of propolis over the world. The compounds 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid and 3-hydroxy-4-methoxybenzaldehyde were isolated from a poplar-type of propolis.[29] 3-Methoxy-4-hydroxypropiophenone has not been reported in propolis samples. The flavanonol, 7-*O*-methyl aromadendrin and galloyl glucosides compounds have already been reported in several species of *Eucalyptus* and geopropolis samples from northeastern Brazil.[30] 7-*O*-Methyl aromadendrin and 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside were isolated from the

kino of *Eucalyptus citriodora*, a thick exudate that is formed in the cavities of the eucalyptus stems.[22]



The isolated labdane skeleton diterpenic acids are known as components of the oleoresin of some conifers, such as *Pinus* and *Araucaria*, both of these plants are growing in the southeast region in Brazil.[31] All these diterpene acids were identified in several oleoresins of *Pinus* species. The non-volatile fraction of oleoresin consists of a mixture of diterpenic acids and other compounds.[32, 33] Diterpenic resin acids are the most common compounds of *Pinus* oleoresin. The diterpene 19-acetoxy-13-hydroxylabda-8(17),14-diene has not been reported in propolis sample.

The diterpene communic acid and other labdane-type diterpenic acids were isolated from brown propolis from Paraná state of Brazil. These diterpenes occur in *Araucaria* species, a possible plant source of this propolis.[12, 34] Other compounds isolated from brown propolis used in this study were previously identified in Araucaria species, such as the lignans pinoresinol and matairesinol, described as major resin components from *Araucaria angustifolia* knots.[35] However, these lignans were also reported as the main compounds in *Pinus taeda* resin.[36] These findings corroborate with the hypothesis

that *Araucaria* and *Pinus* are the primary plant sources of this propolis. Pinoresinol was already related in Chinese propolis and Brazilian red propolis.[7] The lignan maitaresinol has not been reported in propolis samples.

The brown propolis used in this study was collected in Brazil's southeastern region, where the Atlantic forest biome predominates.[37] The southeastern Brazilian region is the leading producer of green propolis type, its botanical source *B. dracunculifolia* is characterized by flavonoids and prenylated phenolic compounds such as artepillin C, drupanin, and baccharin.[28] These compounds were not found in this brown propolis, revealing that *B. dracunculifolia* does not participate in its composition. In contrast to these findings, Ribeiro et al (2021) found markers of *B. dracunculifolia* in brown propolis from the southeastern region in Brazil.[8]

Some studies have correlated the presence of certain volatile compounds found in plants with the attraction of bees for propolis production.[38] Therefore, the volatile fraction of propolis is an essential indicator of bees' botanical source and should be taken into account in phytochemical studies of propolis. The volatile fraction of brown propolis, obtained by hydrodistillation, gave a high yield (2.3%).

The volatile fractions chemical characterization was carried out by GC-MS, allowing to identify 46 compounds (Table 1). The brown propolis of Angatuba-SP consisted mainly of monoterpenes (34.8%), followed by sesquiterpenes (30.8%), diterpenes (14.5%), acids (7.1%), and esters (5.6%) among others (4.1%). Among the major compounds, it is possible to highlight the monoterpenes α-pinene (18.4%) and β-pinene (10.3%), the diterpenes 13-epi-manoyl oxide (9.2%) and manool (5.2%), the sesquiterpenes β-caryophyllene (6.2%) and δ-cadinene (4.7%).

Table 1. Chemical composition of volatile fraction from Brazilian Brown Propolis

	Compound	% RA	RI $_{exp}$	RI $_{lit}$		Compound	% RA	RI $_{exp}$	RI $_{lit}$
1	2-Heptanone	0.1	886	888	**24**	β-Cubebene	0.6	1389	1390
2	α-Pinene	18.4	931	930	**25**	Methyleugenol	0.3	1400	1401
3	Camphene	0.6	952	953	**26**	α-Gurjunene	2.3	1412	1411
4	verbenene	0.2	970	972	**27**	β-Caryophyllene	6.2	1418	1418
5	β-Pinene	10.3	977	978	**28**	Dihydro-α-ionone	0.2	1422	1425
6	Caproic acid	6.3	981	981	**29**	Longifolene	0.8	1431	1430
7	α-Phellandrene	0.2	1000	1002	**30**	Aromandendrene	1.3	1436	1439
8	Eucaliptol	2.6	1025	1026	**31**	α-Humulene	0.7	1445	1446
9	Linalool oxide	0.1	1063	1065	**32**	γ-Muurolene	2.2	1478	1478
10	α-Terpinolene	0.2	1083	1084	**33**	δ-Selinene	0.2	1483	1485
11	Linalool	0.3	1105	1105	**34**	α-Muurolene	0.4	1490	1490
12	Isopinocarveol	0.3	1116	1117	**35**	Cadina-1(6),4-diene	0.2	1500	1500

13	Borneol	0.4	1149	1147	**36**	α-Amorphene	0.3	1506	1506
14	Phellandrene-8-α-ol	0.6	1151	1153	**37**	β-Selinene	0.4	1512	1510
15	Isobutyl methacrylate	2.1	1163	1162	**38**	γ-Cadinene	1.2	1516	1517
16	Octanoic acid	0.8	1177	1175	**39**	δ-Cadinene	4.7	1525	1524
17	Methyl chavicol	1.1	1196	1195	**40**	Spathulenol	3.6	1553	1553
18	α-Terpinyl acetate	2.7	1317	1317	**41**	Globulol	0.5	1577	1576
19	4-Vinyl-guaiacol	1.3	1329	1329	**42**	Bornyl acetate	0.6	1598	1595
20	α-Cubebene	0.2	1351	1349	**43**	epi-α-Cadinol	0.6	1642	1640
21	α-Copaene	1.6	1354	1353	**44**	Octadecane	0.7	1807	1810
22	Ylangene	0.2	1373	1373	**45**	Manool	5.2	1891	1893
23	β-Bourbonene	1.7	1381	1380	**46**	13-epi-Manoyl oxide	9.2	2006	2005
						Total	97.1		

RA: Relative area in the chromatograms, RI_{Exp}: calculated retention index, RI_{Lit}: literature retention index.

Nerolidol, spathulenol, and caryophyllene were the major compounds found in a brown propolis from cerrado biome in Midwest Brazil. This propolis was mutagenic, and its antimicrobial activities are not associated with DNA damage induction. The compounds nerolidol and spathulenol showed strong antimicrobial activity.[10, 39] Nerolidol, spathulenol, and acetophenone were reported as major compounds in a brown propolis sample from the southeast region of Brazil, which also exhibited strong antibacterial activity.[40] The presence of nerolidol and spathulenol in the volatile fraction of propolis generally indicates *B. dracuculifolia* as a botanical source, as these compounds are chemical markers of this plant and play an essential role in attracting bees.[38]

The volatile profile of Brazilian brown propolis of non-*B. dracuculifolia* type was also reported. Olegário et al. (2019) reported the Chemical characterization of Brazilian brown propolis from different regions.[41] In the brown propolis sample from Bahia and Minas Gerais state, the most abundant constituents were β-caryophyllene, humulene and δ-cadinene. In the propolis from the Paraná state, the most abundant compounds were α-pinene and β-pinene.[41] α-Pinene and β-pinene were also the main constituents of the volatile fraction of a southeast Brazilian brown propolis.[8] The volatile main compound manool was found in Greek propolis, and the compound 13-epi-manoyl oxide has not been reported in propolis samples.[42]

The volatile constituents of propolis play an essential role in its biological activities and can elucidate their botanical source. α-Pinene, β-pinene, β-caryophyllene, and manool are the major constituents of several *Pinus* species.[43] α-Pinene, 13-epi-manoyl oxide and β-caryophyllene are reported in *Eucalyptus* species.[44] The monoterpenes α-pinene and β-pinene were identified in *Araucaria angustifolia*, the representative specie of the Araucariaceae in Brazil.[45]

The volatile fraction's chemical characterization corroborates the findings in the phytochemical evaluation of the crude extract of Brown propolis. The identification of α-pinene and β-pinene reinforces the hypothesis that *Pinus, Eucalyptus* and *Araucaria angustifolia* are the primary botanical sources of this propolis. The propolis used in this study proved to be different in its volatile composition from other brown propolis evaluated in the same region,[8] thus demonstrating the tremendous chemical diversity of this bee product.

Biological evaluation

Brown propolis showed significant activity against *P. falciparum* strains, D6, and W2 with IC_{50} of 5.3 and 9.7 μg/mL, respectively (Table 2). The volatile fraction was also active with IC_{50} of 22.5 and 41.8 μg/mL for D6 and W2 strains, respectively. Among the pure compounds, 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside showed IC_{50} values of 3.1 and 1.0 μg/mL against D6 and W2 strains of *P. falciparum,* respectively, while communic acid displayed a IC_{50} of 4.0 μg/mL against W2 strain. The other isolated compounds did not show any antiplasmodial activity up to the highest concentration evaluated.

Chloroquine and artemisinin are used in malaria treatment, but there has been drug resistance and high toxicity, reinforcing the need to seek new antiplasmodial drugs. AlGabbani et al (2017) demonstrated that Saudi propolis samples showed significant antiplasmodial activity with the most effective dose of 100 mg/kg, and the propolis extract also reduced the oxidative damage and increased the level of some pro-inflammatory cytokines.[46] On the other hand, Indonesian propolis showed a weak antiplasmodial activity,[47] which indicates the high chemical diversity of different types of propolis.

Several polyphenolic compounds have been reported to exert a moderate antiplasmodial activity in some different *P. falciparum* strains.[48] The isolated compounds 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside presented a potent activity against *P. falciparum*. Glucopyranosides polyphenolic compounds isolated from *Albizia zygia* (Mimosaceae) exhibited significant activity against *P. falciparum.*[48] The results suggest the application of this class of compounds as antiplasmodial agents.

The crude extract and the volatile fraction displayed cytotoxicity against all the cancer cells evaluated, with IC_{50} range of 42 – 85 μg/mL, and 64 – 80 μg/mL, respectively, and were not cytotoxic against normal cells VERO and LLC-PK1 (Table 2). The isolated compound matairesinol was active against all the cancer cell lines evaluated with IC_{50} range of 0.78 – 1.8 μg/mL. 7-*O*-Methyl aromadendrin showed IC_{50} of 7.3 μg/mL against KB cell and IC_{50} of 25

µg/mL against BT-549 and SK-OV-3 cells. Isopimaric acid presented an IC_{50} of 18 µg/mL against all the cancer cell lines. All the evaluated compounds did not show cytotoxicity against normal cell lines.

Table 2. Antiplasmodial and cytotoxic activity of Brown propolis and isolated compounds

Extract/Compound	*P. falciparum* (µg/mL)				Cytotoxicity IC_{50} (µg/mL)					
	D6		W2							
	IC_{50}	**SI**	**IC_{50}**	**SI**	**Vero**	**SK-MEL**	**KB**	**BT-549**	**SK-OV-3**	**LLC-PK1**
BPCE	9.7	>4.9	5.3	>8.9	NC	85	71	57	42	NC
BPVF	41.8	>1.1	22.5	>2.1	NC	80	82	64	71	NC
Peak 5	3.1	>1.5	1.0	NA	NC	NC	>100	55	90	NC
Peak 10	NA		NA		NC	NC	7.3	25	25	NC
Peak 8	NA		NA		NC	1.6	1.3	1.8	0.78	NC
Peak 11	NA		NA		NC	NC	90	25	90	NC
Peak 12	NA		NA		NC	NC	>100	25	70	NC
Peak 13	NA		NA		NC	NC	>100	55	65	NC
Peak 14	NA		NA		NC	22	90	>100	>100	NC
Peak 15	NA		4.0	>1.2	NC	NC	>100	19	18	NC
Peak 16	NA		NA		NC	17	17	19	18	NC
Chloroquine	0.02	-	0.15	-	-	-	-	-	-	-
Artemisinin	0.014	-	0.007	-	-	-	-	-	-	-
Doxorubicin	-	-	-	-	NC	0.5	0.4	0.3	0.4	0.1

NA = not active up to 47.6 (for extract and fraction) or 4.76 µg/mL for pure compounds, NC= not cytotoxic up to 100 µg/mL, SI= Selectivity index

There are a significant number of reports linking propolis with the antitumor property. The mechanism of action is correlated with apoptosis and interference on cells metabolic pathways by propolis and its compounds, and also their antioxidant effect is associated with their cytotoxic activity.[49] Another mechanism attributed to propolis is the effect on the apoptotic process in cancer cells. Studies indicate that propolis induces apoptosis by releasing cytochrome c from mitochondria to the cytosol through the caspase cascade and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signal.[50]

Greek propolis presented cytotoxicity against human colon adenocarcinoma cells, HT-29, with the diterpene manool as the most active compound.[42] Brazilian green propolis also presented activity against cancer cells, such as AGP-01 and He-La.[4] The antitumor effect of water-soluble derivatives of propolis from Croatia and Brazil on carcinoma cells MCA and HeLa, and fibroblast cells V79 have been studied by Orsolic and Basic (2003).[51] Their results showed that the percentage of apoptotic MCA and HeLa cells increased after exposure to Brazilian and Croatian propolis, and the percentage of apoptotic V79 cells treated with both

Brazilian and Croatian propolis was smaller than in nontreated cells. These indicate the sensitivity to propolis among cancer and normal cells.[51]

The most pronounced cytotoxic effect was observed for matairesinol against a human ovarian cancer cell (SK-OV-3, IC_{50} = 0.78 μg/mL), although this compound also presented strong cytotoxicity against the other cell lines evaluated, with no higher selectivity among cancer cells. These results corroborate with previous reports, once matairesinol showed cytotoxicity against several cancer cell lines, such as HepG2, HL-60, K562, and AGC.[52] It was proved that matairesinol functions as an activator of the tumor necrosis factor-related apoptosis-inducing ligand at prostate cancer. This factor is selectively pro-apoptotic in cancer cells, with minimal toxicity to normal tissues.[53] Nectandrin B, a lignan epoxide as matairesinol presented anticancer activity by inhibiting DNA topoisomerases I and II, which are important molecular targets for anticancer drugs.[54]

The propolis hydroalcoholic extract and volatile fraction displayed antimicrobial activity against *C. neoformans* at 29.9 and 53 μg/mL, respectively, and the isolated compounds 19-acetoxy-13-hydroxylabda-8(17),14-diene, and totarol were active at 7.7 and 16.8 μg/mL, respectively. Dehydroabietic acid significantly inhibited *C. neoformans* growth with IC_{50} value of 2.2 ug/mL (Table 3). The microbial growth of MRS was inhibited at 15.2 and 10.9 μg/mL by matairesinol and isopimaric acid, respectively (Table 3).

Table 3. Antimicrobial activity of Brown propolis and isolated compounds

	IC_{50} (μg/mL)					
Extract/Compound	***C. albicans***	***C. neoformans***	***A. fumigatus***	***MRS***	***E. coli***	***P. aeruginosa***
BPCE	NA	29.9	NA	178.9	NA	160.7
BPVF	NA	53.0	NA	NA	NA	NA
Peak 5	NA	NA	NA	NA	NA	NA
Peak 10	NA	NA	NA	NA	NA	NA
Peak 8	NA	NA	NA	15.2	NA	NA
Peak 11	NA	7.7	NA	NA	NA	NA
Peak 12	NA	16.8	NA	NA	NA	NA
Peak 13	NA	NA	NA	NA	NA	NA
Peak 14	NA	2.2	NA	NA	NA	NA
Peak 15	NA	NA	NA	NA	NA	NA
Peak 16	NA	NA	NA	10.9	NA	NA
Amphotericin B	0.28	0.46	1.31	-	-	-
Meropenem	-	-	-	2.63	0.7.57	0.5.07

MRS: Methicillin-resistant *Staphylococcus aureus*. NA = not active up to 200 μg/mL for extracts/ fraction and 20 μg/mL for pure compounds.

Diterpenes are active compounds in several medicinal plants, with important biological activities. Diterpenes from different sources showed cytotoxicity against various cancer cell lines, anti-inflammatory, and antimicrobial activities.[55] The Brazilian brown propolis used in this study is mainly composed of diterpenes. The compounds of this chemical class showed the most promising antimicrobial activities. Bankova et al., (1996) showed that Brazilian brown propolis, composed of diterpenes, possess antibacterial activity, and no single component was more active than the whole extract.[12] This data contrast with our findings, once the isolated compounds showed high antibacterial activity than the crude extract.

Despite many studies reported in the literature showing the antimicrobial activity of propolis, the brown propolis from Angatuba-SP showed a weak antimicrobial activity against some evaluated microorganisms. This difference could be attributed to the tremendous chemical variations between propolis samples, enhancing the importance of phytochemical studies of propolis.

Conclusions

The primary botanical sources of the studied propolis might be *Pinus* spp., *Eucalyptus* spp, and *Araucaria angustifolia*. The compounds 3-methoxy-4-hydroxypropiophenone, 19-acetoxy-13-hydroxylabda-8(17),14-diene, maitaresinol and 13-epi-manoyl oxide are reported for the first time in a propolis sample. This study confirms the tremendous chemical variability of different Brazilian propolis. Besides, our results provide evidence for its potential medicinal use.

Experimental Section

General

1D and 2D NMR spectra were recorded on a Bruker Avance III-400 MHz spectrometer using CD_3OD or $CDCl_3$ as solvents, with residue solvent as an internal standard. Column chromatography was performed using a silica gel (40-63 µm, SiliaFlash®) or Sephadex LH-20. Analytical TLC was performed on Silica gel 60 on aluminum sheet (20 cm × 20 cm, 200 µm, Sorbtech). The detection was made under UV-254 nm and by spraying with 1% Vanillin in H_2SO_4-EtOH (10:90), followed by heating. Preparative TLC was carried out on Silica gel GF (20 cm × 20 cm, Analtech). The purification of the compounds was performed in a preparative HPLC (LaboAce, Japan Analytical Industry).

Propolis sample

Crude brown propolis, produced by *Apis mellifera* bees, was collected in Angatuba - São Paulo, Brazil. The sample was collected in April 2019. The propolis sample was scraped from beehives, packed in an amber glass bottle to avoid light degradation, and stored at -20 ^{o}C.

Extraction, isolation, and identification of compounds

The raw brown propolis (200 g) was grounded and successively extracted by maceration with 500 mL of hydroalcoholic solution (EtOH:H_2O, 7:3, v:v), for 48 h, with three consecutive extractions. The combined extracts were evaporated under reduced pressure at 40 °C, furnishing 96.3 g of crude extract. The crude hydroalcoholic extract was suspended in a solution of MeOH:H_2O (1:1, v:v, 300 mL) and then partitioned with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The solvents were removed under reduced pressure furnishing EtOAc (28.5g), *n*-BuOH (44.5g), and H_2O (9.7g) fractions.

The aqueous fraction was subjected to silica gel column chromatography (90 x 4 cm) using $CHCl_3$/MeOH (9:1, v:v) resulting in eight fractions (W1-8). Fraction W2 formed a precipitate, identified as ferulic acid (52.8 mg). Fraction W4 (1.8 g) was submitted to a Sephadex LH-20 column chromatography (90 cm × 3 cm) with MeOH to give caffeic acid (28.3 mg). Fraction W5 (1.3 g) was submitted to Sephadex LH-20 column chromatography (120 cm × 2 cm) using MeOH to give 4-methoxycinnamic acid (7.9 mg). Fraction W7 (0.9 g) was submitted to Sephadex LH-20 column chromatography (90 cm × 3 cm) using MeOH to give 3,4-dimethoxycinnamic acid (5.6 mg).

The butanolic fraction was submitted to Sephadex LH-20 column chromatography (120 cm × 5 cm) with MeOH/H_2O (5:5, 7:3, 8:2, 1:0) resulting in twelve fractions (B1-12). Fraction B3 (2.1 g) was chromatographed on Sephadex LH-20 column (120 cm × 2 cm) with MeOH resulting in four fractions ($B3_{1-4}$). Fraction $B3_3$ (0.3 g) was subjected to Sephadex LH-20 column chromatography (40 cm × 3 cm) with MeOH/H_2O (8:2, v:v) to give 1-*O*,2-*O*-digalloyl-6-*O*-trans-*p*-coumaroyl-β-D-glucopyranoside (4.9 mg). Fraction B5 (3.7 g) was subjected to Sephadex LH-20 column chromatography (90 cm × 4 cm) with MeOH resulting six fractions (F_{B5} 1-6). Fraction B5-2 (1.0 g) was subjected to Sephadex LH-20column chromatography (60 cm × 3 cm) with MeOH to give 7-*O*-methyl aromadendrin (21.5 mg). Fraction B7 (8.1 g) was subjected to Sephadex LH-20 column chromatography (90 cm × 4 cm) with MeOH resulting

nine fractions ($B7_{1-9}$). Fraction $B7_3$ (1.1 g) was subjected to Sephadex LH-20 column chromatography (60 cm × 3 cm) with MeO/H_2O (8:2, v:v) resulting four fractions ($B7_{3a-d}$). Fraction $B7_{3b}$ (0.1 g) was submitted to semipreparative HPLC with a GS310 (Jaigel, Japan) column and MeOH (8 mL/min) as mobile phase to give pinoresinol (9.4 mg). Fraction $B7_8$ (2.6 g) was subjected to Sephadex LH-20 column chromatography (90 cm × 4 cm) with MeOH/H_2O (8:2, v:v) resulting in six fractions ($B7_{8a-f}$). Fraction $B7_{8a}$ (0.9 g) was submitted to semipreparative HPLC with a GS310 (Jaigel, Japan) column and MeOH/H_2O (9:1, v:v) (5 mL/min) as mobile phase to give matairesinol (287.1 mg). Fraction B8 (0.8 g) was subjected to Sephadex LH-20column chromatography (40 cm × 3 cm) with MeOH resulting in three fractions ($B8_{1-3}$). Fraction $B8_2$ (0.09 g) was submitted to preparative TLC (20 cm × 20 cm) with $CHCl_3$/MeOH (9:1, v:v) as mobile phase to give 19-acetoxy-13-hydroxylabda-8(17),14-diene (7.0 mg). Fraction B11 (1.1 g) was submitted to Sephadex LH-20 column chromatography (40 cm × 3 cm) with MeOH/H_2O (8:2, v:v) to give 3-methoxy-4-hydroxypropriophenone (4.3 mg) and 3-hydroxy-4-methoxybenzaldehyde (3.9 mg).

The ethyl acetate fraction was submitted to silica gel column chromatography (120 cm × 4 cm) using hexanes/EtOAc (1:0, 9:1, 8:2, 7:3) as mobile phase furnishing five fractions (E1-5). Fraction E2 (2.4 g) was subjected to silica gel column chromatography (60 cm × 3 cm) with hexanes/EtOAc (7:3, v:v) to give totarol (6.2 mg) and 7-oxodehydroabietic acid (11.6 mg). Fraction E4 (5.7 g) was subjected to silica gel column chromatography (90 cm × 4 cm) with hexanes/EtOAc (1:0, 9:1, 8:2, 7:3) resulting in six fractions ($E4_{1-6}$). Fraction $E4_4$ (2.7 g) was submitted to silica gel column chromatography (40 cm × 3 cm) with hexanes/EtOAc (7:3, v:v) to give dehydroabietic acid (493.2 mg). Fraction $E4_5$ (0.7 g) was submitted to silica gel column chromatography (40 cm × 3 cm) with hexanes/EtOAc (7:3, v:v) to give communic acid (21.0 mg). Fraction E5 (3.5 g) (7:3, v:v) was submitted to silica gel column chromatography (60 cm × 3 cm) with hexanes/EtOAc to give isopimaric acid (472.8 mg).

CG/MS analysis of volatile fraction

Fifty grams of crude propolis were submitted to the hydrodistillation process using a Clevenger apparatus to analyze its volatile composition. Chemical characterization was carried out by gas chromatography coupled to mass spectrometry Shimadzu®, model QP-2010. The column used was RTX-5MS (RESTEK) (30 m x 0.25 mm x 0.25 um) with the flow rate at 1.3 mL-1. The initial oven temperature was 60 °C held 2 min, then increased to 220 C with a rate of 3 °C/min, held for 2 min, and then increased to 290 °C ramped 10 °C/min. The electronic

impact (EI) mass detector was operating at 70 eV under 250 °C, and the scan range was 35-500 *m/z*.

The identification of compounds was performed by calculating the retention index using homologous series of hydrocarbons (Sigma-Aldrich),[56] in comparison with the mass spectra of the samples reported in Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC), Wiley Library, National Institute of Standards and Technology (NIST) spectral libraries, and literature data.

Cytotoxicity evaluation

The cytotoxicity of crude extract, volatile fraction, and pure compounds was determined towards four tumor cell lines (BT-549, SK-MEL, SK-OV-3, KB) and two normal cell lines (VERO and LLC-PK1). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The assay was performed in 96-well tissue culture-treated microplates.[57] In brief, cells were seeded at a density of 25000 cells/well and incubated for 24 hrs. Test samples (at various concentrations) were added and cells were further incubated for 48 hrs. The cell viability was determined using a tetrazolium dye WST-8. Doxorubicin was used as a positive control, and DMSO was used as a negative (vehicle) control. IC_{50} values were determined from dose-response curves of the percentage decrease in cell viability against tested concentrations.

Antiplasmodial activity

The *in vitro* antiplasmodial activity was determined against two *Plasmodium falciparum* strains namely, D6 (chloroquine-sensitive) and W2 (chloroquine-resistant). The activity was measured by a colorimetric assay that determines the parasitic lactate dehydrogenase (pLDH) activity, as described earlier.[58] Briefly, red blood cells (200 µL) infected with *P. falciparum* in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL amikacin with 2% parasitemia and 2% hematocrit, were added to the wells of a 96-well plate containing serially diluted samples (10 µL) and incubated for 72 hours in an incubator that maintains a temperature of 37 °C and an environment of 90% N_2, 5% O_2, and 5% CO_2. The human packed red blood cells and human serum were obtained from Interstate Blood Bank Inc., Memphis, TN, USA. At the end of incubation, 20 µL of the incubation mixture was mixed with 100 µL of Malstat reagent and incubated at room temperature for 30 min followed by the addition of 20 µL of a 1:1 mixture of NBT:PES and incubation in the dark for one hour. The reaction was stopped by adding 5% acetic acid (100 µL) and the absorbance

was read at 650 nm. The IC_{50} values were calculated using XLfit 4.2. The antimalarial drugs, chloroquine, and artemisinin, were used as positive controls, with DMSO as the negative (vehicle) control.

Antimicrobial activity

The antimicrobial activity of crude extracts/fractions/compounds was evaluated against *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, *Aspergillus fumigatus* ATCC 204305, methicillin-resistant Staphylococcus aureus ATCC 1708 (MRS), *Escherichia coli* ATCC 2452, *Pseudomonas aeruginosa* ATCC BAA-2018, *Klebsiella pneumonia* ATCC 2146 and *Enterococcus faecium* (VRE) ATCC 700221. All microbial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Susceptibility testing was performed using a modified version of the CLSI methods.[59] Crude extracts were tested at 200, 40, 8µg/mL while pure compounds were tested at 20, 4, 0.8µg/mL. Inocula were prepared by correcting the OD_{630} of microbe suspensions in incubation broth [RPMI 1640 (2% dextrose/0.03% glutamine/MOPS @ pH 6.0) for *C. albicans*, Sabouraud Dextrose for *C. neoformans*, cation-adjusted Mueller-Hinton pH 7.0 for MRS, VRE, *E. coli*, *K. pneumonia* and *P. aeruginosa*, and RPMI 1640 broth (2% dextrose, 0.03% glutamine, buffered with 0.165M MOPS at pH 7.0) for *A. fumigatus* to afford recommended inocula as per CLSI protocol. 5% Alamar Blue™ was added in *A. fumigatus*, VRE, and MRS. Drug controls for bacteria and fungi were included in each assay. All organisms were read, at either 530nm or 544ex/590em for *A. fumigatus*, VRE, and MRS, using the Bio-Tek plate reader before and after incubation: MRS, VRE, *E. coli*, *K. pneumonia*, and *P. aeruginosa* at 35°C for 18-24h, *C. albicans*, and *A. fumigatus* at 35°C for 48h, *C. neoformans* at 35°C for 68-72h. The concentration of compound/fraction responsible for 50% growth inhibition (IC_{50}) was calculated using XLfit 4.2 software (IDBS, Alameda, CA) using fit model 201.

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Author Contribution Statement

Victor Pena Ribeiro: Conceptualization, performed experiments (extraction, fractionation, isolation, structural elucidation, and biological activities), data analysis, and wrote the draft of the manuscript. Caroline Arruda: Conceptualization, data analysis. Jennyfer Andrea Aldana Mejia: Conceptualization, data analysis. Jairo Kenupp Bastos: Conceptualization, supervision, funding acquisition, review & editing. Siddharth K.Tripathi: Performed the biological activities experiments, data analysis. Shabana I. Khan: Supervision, performed the biological activities experiments, data analysis, review & editing. Ikhlas A. Khan: Conceptualization, supervision, resources, project administration, funding acquisition, writing - review & editing. Zulfiqar Ali: Conceptualization, supervision, project administration, data analysis, writing - review & editing.

Disclosure statement

No potential conflict of interest was reported by the authors.

Short Twitter text

Lignans and Diterpenes from a Southeast Brazilian Brown Propolis with Potential Cytotoxic Antiplasmodial Activities by V. P. Ribeiro et al., University of São Paulo, Brazil.

References

[1] V. Bankova, M. Popova, B. Trusheva, 'The phytochemistry of the honeybee', *Phytochem.* **2018**, *155*, 1 – 11.

[2] V. P. Ribeiro, C. Arruda, M. A. El-Salam, J. K. Bastos, 'Brazilian medicinal plants with corroborated anti-inflammatory activities: a review', *Pharm. Biol.* **2018**, *56*, 253 – 268.

[3] A. A. Berretta, C. Arruda, F. G. Miguel, N. Baptista, A. P. Nascimento, F. Marquele-Oliveira, J. I. Hori, H. S. Barud, B. Damaso, C. Ramos, R. Ferreira, J. K. Bastos, 'Functional properties of Brazilian propolis: From chemical composition until the market', *in Superfood Funct. Food - An Overv. Their Process. Util., InTech.* **2017**, 55 - 98.

[4] C. Arruda, V. P. Ribeiro, J. A. A. Mejía, M. O. Almeida, M. O. Goulart, A. C. B. B. Candido, R. A. Santos, L. G. Magalhães, C. H. G. Martins, J. K. Bastos, 'Green propolis: Cytotoxic and leishmanicidal activities of artepillin C, *p*-coumaric acid, and their degradation products', *Rev. Bras. Farmacogn.* **2020**, *30*, 169 – 176.

[5] S. Huang, C. P. Zhang, K. Wang, G. Q. Li, F. L. Hu, 'Recent advances in the chemical composition of propolis', *Molecules.* **2014**, *19*, 19610 - 19632.

[6] J. A. Aldana-Mejía, G. V. Ccana-Ccapatinta, V. P. Ribeiro, C. Arruda, R. C. S. Veneziani, S. R. Ambrósio, J. K. Bastos, 'A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and *Dalbergia ecastaphyllum*', *J. Pharm. Biomed. Anal.* **2021**, *198*, 114029.

[7] J. A. Aldana-Mejía, G. V. C. Ccapatinta, I. S. Squarisi, S. Nascimento, M. H. Tanimoto, V. P. Ribeiro, C. Arruda, H. Nicolella, T. Esperandim, A. B. Ribeiro, K. S. Freitas, L. H. D. Silva, S. D. Ozelin, L. T. S. Oliveira, A. L. A. Melo, D. C. Tavares, J. K. Bastos, 'Nonclinical toxicological studies of Brazilian red propolis and Its primary botanical source *Dalbergia ecastaphyllum'*, *Chem. Res. Toxicol*. **2021**, *34*, 1024 – 1033.

[8] V. P. Ribeiro, C. Arruda, J. A. A. Mejía, A. C. B. B. Candido, R. A. Santos, L. G. Magalhães, J. K. Bastos, 'Brazilian southeast brown propolis: gas chromatography method development for its volatile oil analysis, its antimicrobial and leishmanicidal activities evaluation', *Phytochem. Anal*. **2020**, *32*, 404 – 411.

[9] V. P. Ribeiro, G. V. Símaro, J. A. A. Mejia, C. Arruda, J. K. Bastos, 'Anti-inflammatory and antinociceptive activities of the hydroalcoholic extract and the volatile fraction of southeastern Brazilian brown propolis', *Rev. Bras. Farmacogn*. **2021**, *31*, 59 – 66.

[10] F. H. Fernandes, Z. R. Guterres, I. M. P. Violante, T. F. S. Lopes, W. S. Garcez, F. R. Garcez, 'Evaluation of mutagenic and antimicrobial properties of brown propolis essential oil from the Brazilian Cerrado biome', *Toxicol. Rep*. **2015**, *2*, 1482 - 1488.

[11] C. N. Araújo, M. A. S. Mayworm, R. Yatsuda, G. Negri, M. L. F. Salatino, A. Salatino, J. Timenetsky, G. B. Campos, 'Chemical composition and antimycoplasma activity of a brown propolis from southern Brazil', *J. Food. Sci. Technol.* **2020**, *57*, 4228 – 4235.

[12] V. Bankova, M. C. Marcucci, S. Simova, N. Nikolova, A. Kujumgiev, S. Popov, 'Antibacterial diterpenic acids from Brazilian propolis', *Z. Naturforsch*. **1996**, *51*, 277 - 280.

[13] J. Kocot, M. Kiełczykowska, D. Luchowska-kocot, J. Kurzepa, I. Musik, 'Antioxidant potential of propolis, bee pollen, and royal jelly: Possible medical application', *Oxid. Med. Cell Longev*. **2018**, *7074209*, 1 - 29.

[14] R. Pavlovic, G. Borgonovo, V. Leoni, L. Giupponi, G. Ceciliani, S. Sala, A. Bassoli, A. Giorgi, 'Effectiveness of different analytical methods for the characterization of propolis: A case of study in northern Italy', *Molecules*. **2020**, *25*, 504 - 530.

[15] L. Stan, A. Liviu, D. Dezmirean, 'Quality criteria for propolis standardization', *Sci. Pap. Anim. Sci. Biotechnol.* **2011**, *44*, 137 – 140.

[16] L. C. L. R. Santana, S. M. P. Carneiro, L. B. Caland-Neto, D. D.R. Arcanjo, J. M. Moita-Neto, A. M.G.L. Citó, F. A. A. Carvalho, 'Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of *Leishmania amazonensis*', *Nat. Prod. Res*. **2014**, *28*, 340 - 343.

[17] L. M. López-Martínez, H. Santacruz-Ortega, R. E. Navarro, R. R. Sotelo-Mundo, G. A. González-Aguilar, 'A ^{1}H NMR Investigation of the Interaction between Phenolic Acids Found in Mango (*Manguifera indica* cv Ataulfo) and Papaya (*Carica papaya* cv Maradol) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) Free Radicals', *PLoS One*. **2015**, *10*, e0140242.

[18] W. Hidalgo, M. Kai, B. Schneider, '4-Methoxycinnamic acid – an unusual phenylpropanoid involved in phenylphenalenone biosynthesis in *Anigozanthos preissii*', *Phytochem*. **2015**, *117*, 476 - 481.

[19] Ł. Szeleszczuk, D. M. Pisklak, M. Zielińska-Pisklak, I. Wawer, 'Effects of structural differences on the NMR chemical shifts in cinnamic acid derivatives: comparison of GIAO and GIPAW calculations', *Chem. Phys. Lett*. **2016**, *653*, 35 - 41.

[20] A.W. Khan, S. Jan, S. Parveen, R. A. Khan, A. Saeed, A. J. Tanveer, A. A. Shad, 'Phytochemical analysis and enzyme inhibition assay of *Aerva javanica* for Ulcer', *Chem. Cent. J.* **2012**, *6*, 76.

[21] J. Ito, F. R. Chang, H. K. Wang, Y. K. Park, M. Ikegaki, N. Kilgore, K. H. Lee, 'Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis', *J. Nat. Prod*. **2001**, *64*, 1278 - 1281.

[22] M. O. Freitas, M. A. S. Lima, E. R. Silveira, 'Polyphenol compounds of the kino of *Eucalyptus citriodora', Quím. Nova*. **2007**, *30*, 1926 - 1929.

[23] J. Schmitt & M. Petersen, 'Pinoresinol and matairesinol accumulation in a *Forsythia* × *intermedia* cell suspension culture', *Plant Cell Tissue Organ. Cult*. **2002**, *68*, 91 – 98.

[24] W. Chiung, Su, J. Min, Fang, Y. Shia, Cheng, 'Labdanes from *Cryptomeria japonica*', *Phytochem*. **1994**, *37*, 1109 - 1114.

[25] A. Bernabeu, S. Shapiro, J. Villalaín, 'A MAS-NMR study of the location of (+)-totarol, a diterpenoid bioactive molecule, in phospholipid model membranes', *Chem. Phys. Lipids.* **2002**, *119*, 33 - 39.

[26] R. Tanaka, H. Ohtsu, S. Matsunaga, 'Abietane diterpene acids and other constituents from the leaves of *Larix kaempferi*', *Phytochem.* **1997**, *46*, 1051 - 1057.

[27] Y. H. Kuo, W. C. Chen, 'Chemical constituents of the pericarp *of Platycladus orientalis', J. Chin. Chem. Soc.* **1999**, *46*, 819 - 824.

[28] F. P. Beserra, L. F. S. Gushiken, M. F. Hussni, V. P. Ribeiro, F. Bonamin, C. J. Jackson, C. H. Pellizzon, J. K. Bastos, 'Artepillin C as an outstanding phenolic compound of Brazilian green propolis for disease treatment: a review on pharmacological aspects', *Phytother. Res*. **2020**, *35*, 2274 - 2286.

[29] P. Ristivojevi, J. Trifkovic, F. Andrić, D. Milojković-Opsenica, 'Poplar-type propolis: chemical composition, botanical origin and biological activity', *Nat.Prod. Comm*. **2015**, *10*, 1869 - 1876.

[30] S. A. Sousa, T. M. G. Silva, E. M. S. Silva, C. A. Camara, T. M. S Silva, 'Characterization of phenolic compounds by UPLC-QTOF-MS/MS of geopropolis from the stingless bee *Melipona subnitida* (jandaíra)', *Phytochem Anal.* **2018**, *29*, 549 – 558.

[31] S. Rezzi, A. Bighelli, V. Castola, J. Casanova, 'Composition and chemical variability of the oleoresin of *Pinus nigra* ssp. *laricio* from Corsica', *Ind. Crops Prod*. **2005**, *21*, 71 – 79.

[32] K. Ioannidis, E. Melliou, P. Magiatis, 'High-Throughput ^{1}H-nuclear magnetic resonance-based screening for the identification and quantification of heartwood diterpenic acids in four black pine (*Pinus nigra* Arn.) marginal provenances in Greece', *Molecules*. **2019**, *24*, 3603.

[33] G. V. Símaro, M. Lemos, J. J. M. Silva, V. P. Ribeiro, C. Arruda, A. H. Schneider, C. W. S. Wanderley, L. J. Carneiro, R. L. Mariano, S. R. Ambrósio, S. F. Andrade, V. C. B. Filho, A. Sasse, H. Sheridan, M. L. A. Silva, J. K. Bastos, 'Antinociceptive and anti-inflammatory activities of *Copaifera pubiflora* Benth oleoresin and its major metabolite ent-hardwickiic acid', *J. Ethnopharmacol*. **2021**, *271*, 113883.

[34] M. F. C. Santos, L. C. Oliveira, V. P. Ribeiro, M. G. Soares, G. O. I. Morae, A. G. O. Sartori, P. L. Rosalen, J. K. Bastos, S. M. Alencar, R. C. S. Veneziani, S. R. Ambrósio, 'Isolation of diterpenes from *Araucaria* sp Brazilian brown propolis and development of a validated HPLC method for its analysis', *J. Sep. Sci.* **2021**, 34169651.

[35] R. J. Anderegg, J. W. Rowe, 'Lignans, the major component of resin from *Araucaria angustifolia* Knots', *Wood Sci. Technol*. **1974**, *28*, 171 - 175.

[36] T. L. Eberhardt, M. A. Bernards, L. H. L. B. Davin, J. B. Wooten, N. G. Lewis, 'Lignification in cell suspension cultures of *Pinus taeda*. *In situ* characterization of a gymnosperm lignin', *J. Biol. Chem*. **1993**, *268*, 21088 - 21096.

[37] J. J. M. Silva, V. P. Ribeiro, M. L., A. E. M. Crotti, H. Rogez, J. K. Bastos, 'Reliable methods for analyses of volatile compounds of Copaifera oleoresins combining headspace and gas chromatography', *Chem. Biodivers*. **2020**, *17*, e1900440.

[38] D. M Rodrigues, M. C. Souza, C. Arruda, R. A. S. Pereira, J. K. Bastos, 'The role of *Baccharis dracunculifolia* and its chemical profile on green propolis production by *Apis mellifera', J. Chem. Ecol*. **2020**, *46*, 150 – 162.

[39] N. Arghiani, M.M. Matin, A.R. Bahrami, M. Iranshahi, A. Sazgarnia, F.B. Rassouli, 'Investigating anticancer properties of the sesquiterpene ferutinin on colon carcinoma cells, *in vitro* and *in vivo', Life Sci*. **2014**, *109*, 87 - 94.

[40] V. H. . Lima, K. C. Rodrigues, A. C. C. F. Alves, M. L. Rodrigues, A. E. M. Crotti, J. M. Souza, A. B. Ribeiro, I. S. Squarisi, D. C. Tavares, C. H. G. Martins, M. L. D. Miranda, 'Biological properties of volatile oil from Brazilian brown propolis', *Rev. Bras. Farmacogn.* **2019**, *29*, 807 – 810.

[41] L. S. Olegário, J. K. S. Andrade, G. R. S. Andrade, M. Denadai, R. L. Cavalcanti, M. A. A. P. Silva, N. Narain, 'Chemical characterization of four Brazilian brown propolis: an insight in tracking of its geographical location of production and quality control', *Food Res. Int.* **2019**, *123*, 481 - 502.

[42] H. Pratsinis, D. Kletsas, E. Melliou, I. Chinou, 'Antiproliferative Activity of Greek Propolis', *J. Med. Food*. **2010**, *13*, 286-290.

[43] E. Ioannou, A. Koutsaviti, O. Tzakou, V. Roussis, 'The genus *Pinus*: a comparative study on the needle essential oil composition of 46 pine species', *Phytochem. Rev*. **2014**, *13*, 741 – 768.

[44] P. K. Bett, A. L. Deng, J. O. Ogendo, S. T. Kariuki, M. K. Mugisha, J. M. Mihale, B. Torto, 'Residual contact toxicity and repellence of *Cupressus lusitanica* Miller and *Eucalyptus saligna* Smith essential oils against major stored product insect pests', *Ind. Crop. Prod.* **2017**, *110*, 65 - 74.

[45] J. J. Brophy, R. J. Goldsack, M. Z. Wu, C. J. R. Fookes, P. I. Forster, 'The steam volatile oil of *Wollemia nobilis* and its comparison with other members of the *Araucariaceae* (Agathis and Araucaria)', *Biochem. Syst. Ecol*. **2000**, *28*, 563 - 578.

[46] Q. AlGabbani, L. Mansour, Y. A. Elnakady, S. Al-Quraish, S. Alomar, E. M. Al-Shaebi, A. S. Abdel-Baki, '*In vivo* assessment of the antimalarial and spleen-protective activities of the Saudi propolis methanolic extract', *Parasitol. Res*. **2017**, *116*, 539 – 547.

[47] A. Syamsudin, R. M. Dewi, K. Kusmardi, 'Immunomodulatory and *In vivo* antiplasmodial activities of propolis extracts', *Am. J. Pharmacol. Toxicol*. **2009**, *4*, 75 - 79.

[48] R. R. Koagne, F. Annang, B. Cautain, J. Martín, G. P. Moreno, G. T. M. Bitchagno, D. G. Pacanowska, F. Vicente, I. K. Simo, F. Reyes, P. Tane, 'Cytotoxycity and antiplasmodial activity of phenolic derivatives from *Albizia zygia* (DC.) J.F. Macbr. (Mimosaceae)', *BMC Complement. Med. Ther*. **2020**, *20*, 1 - *8*.

[49] M. A. E Watanabe, M. K. Amarante, B. J. Conti, J. M. Sforcin, 'Cytotoxic constituents of propolis inducing anticâncer effects: a review', *JPP*. **2011**, *63*, 1378 – 1386.
[50] D. Sawicka, H. Car, M. H. Borawska, J. Nikliński, 'The anticancer activity of propolis', *Folia Histochem. Cytobiol.* **2012**, *50*, 25 – 37.
[51] N. Orsolic, I. Basic, 'Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity', *J. Ethnopharmacol*. **2003**, *84*, 265 – 273.
[52] E. Al-Sayed, T. Y. Ke, T. L. Hwang, S. R. Chen, M. Korinek, S. L. Chen, Y. B. Cheng, 'Cytotoxic and anti-inflammatory effects of lignans and diterpenes from *Cupressus macrocarpa*', *Bioorg. Med. Chem. Lett*. **2020**, *30*, 127127.
[53] E. Peuhu, A. Rivero-Müller, H. Stykki, E. Torvaldson, T. Holmbom, P. Eklund, M. Unkila, R. Sjöholm, J. E. Eriksson, 'Inhibition of Akt signaling by the lignan matairesinol sensitizes prostate cancer cells to TRAIL-induced apoptosis'. *Oncogene*. **2010**, *29*, 898 – 908.
[54] Y. K. Lee, C. S. Seo, C. S. Lee, K. S. Lee, S. J. Kang, Y. Jahng, H. W. Chang, .J. K. Son, 'Inhibition of DNA topoisomerases I and II and cytotoxicity by lignans from *Saururus chinensis*', *Arch. Pharm. Res*. **2009**, *32*, 1409 - 1415.
[55] I. P. Sousa, M. V. S. Teixeira, N. A. J. C. F. NA, 'An Overview of Biotransformation and Toxicity of Diterpenes', *Molecules*. **2018**, 23, 1387.
[56] C. Mühlen, P. J. V. Marriott, 'Retention indices in comprehensive two-dimensional gas chromatography', *Anal. Bioanal. Chem*. **2011**, *401*, 2351 – 2360.
[57] F. Zulfiqar, S. I. Khan, S. A. Ross, Z. Ali, I. A. Khan, 'Prenylated flavonol glycosides from *Epimedium grandiflorum*: cytotoxicity and evaluation against inflammation and metabolic disorder', *Phytochem. Lett*. **2017**, *20*, 160 - 167.
[58] Mallika Kumarihamy, Luiz H. Rosa, Natascha Techen, Daneel Ferreira, Edward M. Croom, Stephen O. Duke, Babu L. Tekwani, Shabana Khan, N. P. Dhammika Nanayakkara, 'Antimalarials and phytotoxins from *Botryosphaeria dothidea* identified from a seed of diseased *Torreya taxifolia*', *Molecules*. **2021**, *26*, 59 - 70.
[59] Clinical and Laboratory Standards Institute, methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard 9th ed., **2012**. Document M7-A9.

4. Discussão

Nos capítulos anteriores, foram apresentados estudos fitoquímicos, desenvolvimento e validação de métodos anaíliticos para análises qualitativas e quantitativas de amostras de própolis marrom coletadas da região sudeste do Brasil, foram apresentados também, diversas atividades biológicas que podem ser atribuídas a essas própolis.

Em relação a composição química, a própolis marrom coletada em Cabo Verde-MG, apresentou certa semelhança com a própolis verde em relação aos compostos fenólicos, compartilhando a mesma fonte botânica, contudo essa própolis marrom, não é constituída apenas de uma única fonte botânica, apresentando uma maior complexidade química. Essa semelhança pode estar relacionada ao fato da localização geográfica da produção dessas duas própolis, onde é abundante a *B. dracunculifolia*, fonte botânica da própolis verde

Compostos fenólicos já relatados na própolis verde brasileira foram encontrados em extratos padronizados de própolis marrom, como ácido p-cumárico, drupanina, artepillina C e bacarina, revelando semelhança no perfil fenólico entre esses dois tipos de própolis (Fonseca et al., 2011). Segundo Rodrigues et al.. 2016, além dos tradicionais marcadores químicos de própolis verde, a própolis marrom também pode apresentar menores concentrações de ácido propenoico e cinâmico (Rodrigues et al., 2016). Outros compostos fenólicos como crisina, pinocembrina, galangina, éster feniletílico do ácido cafeico e pinobanksin-3-O-acetato também foram identificados por análise de HPLC-UV-ESI-MS em amostras brasileiras de própolis marrom (Fabio et al., 2019).

Capilarartemisina A e derivados de ácido cafeoilquínico como ácido 3,4-di-OE-cafeoilquínico, ácido 3,5-di-OE-cafeoilquínico, dihidrocafeato de O-hexosil-cafeoil, ácido 4,5-di-OE-cafeoilquínico e Ácido OE-cumaroil-cafeoilquínico foram detectados no PB de Minas Gerais (região Sudeste) por análise LC-DAD-MS (Dembogurski et al. 2018). A própolis marrom de Mato Grosso também forneceu ácido acetilisocupressico, ácido diidro-p-cumárico, ácido cafeico e aromadendrina (Fernandes et al. 2019). Análise por UPLC-MS do extrato hidroalcoólico de BP do Rio Grande do Sul (região Sul) revelou a presença de rutina, ácido clorogênico, ácido ferúlico e ácido cafeico (Waller et al., 2017).

Uma amostra de própolis marrom do Paraná foi caracterizada quimicamente utilizando HPLC–DAD–ESI–MS/MS. A propolis foi fracionada com diferentes solventes em um sistema Soxhlet. Em todas as frações foram identificados artepillin C, bacarina e ácido 3-hidroxi-2,2-dimetil-8-prenil cromano-6-propenoico, embora a fração hexânica tenha sido enriquecida com derivados do ácido propenoico e cinâmico como drupanina; a fração hexanica apresentou derivados de ácido propenóico e cinâmico, e flavonóides como kaempferol e quercetina; a fração acetato de etila foi composta em flavonóides, ácidos clorogênicos e ácido quínico

esterificado por uma ou mais unidades de ácidos cinâmico, p-cumárico, cafeico ou ferúlico, enquanto que as frações metanol e aquosa continham derivados do ácido quínico e cafeoílo (Nascimento Araújo et al., 2020).

O extrato bruto hidroalcoólico de própolis marrom produzido por abelhas do tipo *Melipona quadrifasciata* foi analisado por HPLC–ESI-MS/MS. A amostra do Estado de Santa Catarina apresentou em sua composição as substâncias aromadendrina, ácido p-cumárico, naringenina, catequina, epicatequina e pinocembrina, que são encontradas em própolis do tipo verde e própolis amarela (Hochheim et al., 2019).

A composição química da própolis é altamente dependente das condições do local e dos constituintes químicos da fonte botânica. Esse fato pode corroborar com os achados reltados no capítulo 4. Os estudos fitoquímicos revelaram uma composição química bem diferente da própolis marrom coletada em Angatuba-SP. Essa própolis não apresentou os compostos fenólicos clássicos e derivados prenilados do ácido cinâmico encontrados na própolis de Cabo Verde-MG.

Outro centro de produção da própolis marrom é a região sul do Brasil, onde foram analisadas 44 amostras própolis marrom do Paraná e de Santa Catarina. Seus compostos foram identificados e quantificados comparando seu perfil químico com padrões autênticos (Machado et al., 2021). De acordo com este estudo, a própolis marrom do sul do Brasil é caracterizada quimicamente por ácidos cafeoilquínicos, derivados do ácido p-cumárico, flavonóis, seguidos por derivados de ácido benzóico e dihidroflavonóis (Machado et al., 2021).

Diterpenos como ácido isocupressico, ácido (E)-comúnico, ácido (Z)-comúnico e ácido abiético foram isolados de amostras de própolis marrom do Estado do Paraná (Alencar et al. 2021). Além disso, o ácido 13-epi-cupressico e o epi-13-torulosol foram isolados e identificados em resina de própolis marrom e Araucária (Santos et al., 2021). Tazawa et al. 2016 identificaram a presença do ácido 15-acetoxiisocupressico e isolaram um diterpeno não relatado, o rel-(5S,6S,8R,9R,10S,18R,19S)-18,19-epoxy-2-oxocleroda-3,12(E),14-trieno-6,18,19-triol 18,19-diacetato 6-benzoato em extrato etanólico de própolis marrom (Tazawa et al., 2016).

Recentemente a fração volátil de própolis tem sido alvo de diferentes grupos de pesquisa. O óleo essencial de própolis marrom tem sido caracterizado por diferentes estudos, mostrando diferenças no perfil químico da própolis de regiões distintas. No capítulo 2 apresentamos o isolamento e identificação de 8 substâncias majoritárias da própolis marromd e Cabo verde-MG, o que permitiu a validação do método analítico por CG-DIC. Já no capítulo

4 realizamos a caracterização química da própolis marrom de Angatuba-SP por espectrometria de massas acoplada a cromatografia gasosa.

A análise de microextração em fase sólida Head Space (HS-SPME) e GC-MS permitiu a identificação de mais de 315 compostos voláteis em própolis marrom da Bahia, Minas Gerais, Paraná e Sergipe (Olegário et al., 2019). Os terpenos foram a classe de compostos predominante em todas as amostras, seguido pelos aldeídos.

Os sesquiterpenos β-cariofileno e α-humuleno foram os compostos mais abundantes na amostra de própolis marrom da Bahia; acetofenona, (R)-α-pineno e (+)-δ-cadineno, foram predominantes na amostra do Paraná; D-limoneno e nonanal foram os compostos majoritários na amostra do estado de Sergipe (Olegário et al., 2019). A fração volátil da própolis marrom do Mato Grosso do Sul apresentou (E)-cariofileno, δ-cadineno, espatulenol, α-copaeno, (E)-nerolidol e aromadendreno, sendo viridifloreno e α-trans-bergamoteno as substâncias majoritárias (Fernandes et al., 2015).

Um dos principais gargalos nos estudos da própolis é a elucidação de suas fontes botânicas. A fonte botânica visitada pelas abelhas está diretamente relacionada à composição química da própolis, impactando suas propriedades farmacológicas. Muitas publicações sobre a própolis não descrevem seu tipo, fontes botânicas e até mesmo sua composição química, dificultando a padronização deste produto medicinal.

Diversas abordagens visam encontrar a provável fonte botânica da própolis, que ainda é pouco relatada na literatura. Uma das abordagens é a observação das abelhas em campo, um exemplo pode ser observado com a própolis vermelha, onde abelhas *Apis mellifera* coletam exsudatos vermelhos de *Dalbergia ecastaphyllum*, a fonte foi confirmada através da semelhança química entre o exsudato da planta e a própolis (Daugsch et al. 2008). Outra abordagem é a identificação de marcadores quimiotaxonômicos e a correlação desses marcadores com espécies botânicas próximas às colmeias, o que levou Ccana-Ccapatinta et al. 2020 a descrever *Symphonia globulifera* como a fonte das benzofenonas na própolis vermelha brasileira (Ccana-Ccapatinta et al. 2020). Alguns pesquisadores utilizam a palinologia para identificação botânica da própolis, contudo as abelhas visitam algumas plantas para coleta de néctar e outras plantas para coleta de resina, dificultando a identificação da fonte botânica para a produção da própolis (Freitas et al. 2011; Sartori et al. 2021; Ribeiro et al. 2022).

Na abordagem para identificação das possíveis fontes botânicas das própolis marrons utilizadas em nosso estudo, foi empregada a fitoquímica clássica para isolamento e disentificação das substâncias químicas presentes nessas amostras de própolis, então foram identificadas as espécies endêmicas das regiões de coletas e que possuem relatos da presença

dessas substâncias em sua composição química. Um exemplo foi a identificação dos derivados prenilados do ácido cumárico na própolis apresentada no capítulo 1, levando a identificação da *Baccharis dracunculifolia* como uma das fontes botânicas e a identificação dos diterpenos ácidos na própolis apresentada no capítulo 4, comumente encontrados nas resinas de Pinus e Araucária.

As origens botânicas de algumas própolis brasileiras já foram bem estabelecidas. A origem botânica da própolis verde brasileira é a *Baccharis dracunculifolia* e da própolis vermelha uma mistura de resinas de *Dalbergia ecastaphyllum* e *Symphonia globulifera*. Várias plantas têm sido descritas como responsáveis pela contribuição na composição da própolis marrom, como *Pinus* spp., *B. dracunculifolia*, *Eucalyptus* spp. e *Araucaria angustifolia* (Freitas et al. 2011; Ribeiro et al. 2021; Santos et al. 2021).

A *Baccharis dracunculifolia* DC (Asteraceae), popularmente conhecida como 'alecrim-do-campo', é amplamente distribuída na América do Sul desde o sudeste do Brasil até a Argentina e Uruguai (Beserra et al. 2021). É a principal fonte vegetal da própolis verde produzida no sudeste brasileiro. A própolis verde contém altos níveis de ácidos p-cumáricos prenilados, principalmente artepillin C e bacarina, e os compostos voláteis nerolidol e espatulenol, todos encontrados em *B. dracunculifolia* DC (Beserra et al. 2021).

Devido à sua localização geográfica, substâncias presentes em *B. dracunculifolia* são descritas em várias amostras de própolis marrom coletadas na região sudeste do Brasil, contudo, a coloração marrom característica dessas própolis, podem estar associadas com a associação de outras resinas de diferentes fontes botânicas. Os ácidos fenólicos de *B. dracunculifolia* e seus componentes voláteis como nerolidol e espatulenol são descritos em diversas amostras de própolis marrom (Dembogurski et al., 2018; Araújo et al., 2020; Ribeiro et al., 2021). Compostos não encontrados em espécies de Baccharis já foram identificados em estudos fitoquímicos dessas propolis, evidenciando a participação de outras plantas na produção da própolis marrom.

Ácidos diterpênicos encontrados em amostras de própolis do sudeste brasileiro também são relatados em espécies de coníferas como *Araucaria angustifolia*. Alguns estudos fitoquímicos da própolis marrom brasileira confirmaram *Araucaria angustifolia* como fonte vegetal primária (Santos et al. 2021; Ribeiro et al. 2021; Sartori et al. 2021). Em trabalho publicado por nosso grupo de pesquisa, foi relatado o isolamento desses diterpenos da própolis marrom brasileira de *Araucaria* sp. Durante a coleta de amostras de própolis em campo, foi possível observar as abelhas coletando o exsudato do tronco de *Araucaria angustifolia* para produção da própolis. As abelhas coletam o exsudato de *A. angustifolia,* armazenam na

corbícula da perna esquerda e levam para a colmeia (Figura 2). A participação da Araucária foi confirmada posteriormente através do estudo fitoquímico desta própolis (Santos et al. 2021).

Figura 2. Abelha *Apis mellífera* coletando resina de *Araucaria angustifólia* para produção de própolis marrom. (Fonte: Acervo Pessoal)

A participação de *A. angustifolia* na composição química da própolis se deve principalmente à presença de diterpenos ácidos, como ácido 13-epi-cupressico, ácido abiético e ácido comunico (Tazawa et al.2016; Santos et al. 2021). A partir de compostos voláteis, *A. angustifolia* apresenta o sesquiterpeno germacreno-D e os diterpenos hibaeno e filocladeno como componentes majoritários em sua fração volátil (Brophy et al., 2000). Algumas propriedades biológicas, como anti-inflamatórias e antimicrobianas dessa resina, são atribuídas aos diterpenos. Muitos diterpenos isolados da própolis marrom e *A. angustifolia* possuem atividade antimicrobiana (Bankova et al. 1996; Ribeiro et al. 2021).

Diterpenos de *Pinus* spp. e *Eucalyptus* spp também foram encontrados na própolis marrom. Quase todos os diterpenos isolados da própolis marrom descrita no capítulo 4, já foram encontrados em resinas de Pinus, como 19-acetoxi-13-hidroxiabda-8(17),14-dieno, totarol, ácido 7-oxodehidroabiético, ácido desidroabiético, ácido comúnico e ácido isopimárico. As lignanas pinoresinol e matairesinol também foram isoladas dessa mesma amostra de própolis, essas lignanas são descritas como compostos majoritários em "nó de pinho" de *A. angustifolia* e também são descritas como majoritários em resina de *Pinus taeda* (Anderegg & Rowe 1974; Eberhardt et al., 1993).

Muitas amostras de própolis marrom, contêm α-pineno e β-pineno como principais constituintes de sua fração volátil. Esses compostos são marcadores químicos do óleo volátil de Pinus, corroborando esta planta como fonte botânica de algumas amostras de própolis marrom (Ioannou et al. 2014). O totarol, um álcool diterpênico encontrado na própolis marrom, também foi identificado no óleo essencial de *Eucalyptus* spp. (Bett et al. 2017). Espécies de Eucalyptus também contribuem com alguns flavonóides e glicopiranosídeos na composição deste tipo de própolis (Freitas et al. 2007).

É fundamental identificar os compostos responsáveis pelas atividades biológicas para garantir a qualidade da própolis e seus produtos. O desenvolvimento de métodos analíticos validados é essencial para garantir seletividade, exatidão e precisão na quantificação dos compostos (Sawaya et al. 2011). Métodos analíticos foram desenvolvidos para analisar a própolis bruta e seus produtos comerciais. Alguns deles visam identificar os componentes químicos com atividades biológicas, principalmente compostos fenólicos utilizados como biomarcadores/padrões (Galeotti et al. 2019). É um desafio desenvolver métodos analíticos para análise de própolis, pois possui uma matriz complexa exigindo abordagens de métodos diferentes para analisar todas as classes de compostos presente na própolis (Pavlovic et al. 2020).

É necessário o uso de métodos analíticos validados para garantir o uso da própolis para humanos com qualidade, segurança e eficácia. A validação de método analítico é uma avaliação sistemática de um método desenvolvido em que vários experimentos são feitos para provar que o método é capaz de quantificar um composto em uma amostra. Alguns parâmetros são avaliados no processo de validação, como robustez, exatidão, precisão, seletividade, limite de detecção, limite de quantificação, linearidade e intervalo de análise. Todos esses parâmetros permitem a confiabilidade do método.

Nos capítulos 1 e 2 foram apresentados os resultados da validação de métodos analíticos para análises de própolis marrom. No primeiro, um método para análise de compompostos fenólicos por HPLC-DAD. No segundo, um método por CG-DIC para análise de substâncias voláteis presentes na própolis. Os métodos apresentados poderão auxiliar na verificação da qualidade deste produto em sua totalidade; permitindo que a comunidade científica, empresas privadas e agências reguladorass utilizem os dados apresentados no auxílio da investigação e regularização desse importante produto natural oriundo da biodiversidade brasileira.

Diversas doenças não possuem medicamentos disponíveis no mercado para seu tratamento, e algumas vezes os medicamentos disponíveis apresentam efeitos colaterais relevantes. Portanto, novos medicamentos devem ser desenvolvidos para o tratamento dessas

doenças. A própolis é usada desde os tempos antigos como agente de cura. E têm um enorme potencial para contribuir como fonte alternativa de terapia, seja como fitomedicamentos, ou como fonte de entidades químicas que tenham propriedades farmacológicas de interesse.

A própolis é frequentemente mencionada na literatura como agente antitumoral e imunomodulador. Nos últimos anos, ensaios *in vitro* e *in vivo* forneceram novas informações sobre seus mecanismos de ação, e foram compilados dados de vários laboratórios com foco em sua composição química, fontes botânicas e propriedades biológicas (Watanabe et al. 2011).

A avaliação citotóxica do extrato hidroalcoólico da própolis marrom coletada em Cabo Verde- MG, não indicou efeito sobre a viabilidade das linhagens celulares normais (VERO e LLC-PK1), contudo, a própolis marrom exibiu efeito de toxicidade nas células tumorais, com os seguintes valores de IC_{50}: 80 µg/mL para SK-MEL; 82 µg/mL para KB; 71 µg/mL para SK-OV-3; e 64 µg/mL para BT-549. A fração volátil dessa própolis não foi citotóxica contra as linhagens AGP-01, He-La e CHO-K1, com IC_{50}> 100 µg/mL.

Para a própolis marrom de Angatuba-Sp, a citotoxicidade foi determinada em quatro linhas de células tumorais (SK-MEL, KB, BT-549 e SK-OV-3) e duas linhas de células renais normais (LLC-PK1 e VERO). O extrato bruto e a fração volátil apresentaram citotoxicidade frente todas as células cancerígenas avaliadas, com intervalo de IC_{50} de 42 – 85 µg/mL e 64 – 80 µg/mL, respectivamente, e não foram citotóxicos contra células normais VERO e LLC-PK1. Dentre as substâncias isoladas, Matairesinol, 7-O-metil aromadendrina e o ácido isopimárico apresentaram uma faixa de inibição IC_{50} de 1,8 – 0,78 µg/mL, 7,3 – 100 µg/mL e 17-18 µg/mL, respectivamente, contra as linhagens de células tumorais, mas não foram citotóxicos contra linhagens celulares normais.

O extrato etanólico de uma própolis marrom do Ceará inibiu mais de 75% da proliferação de células cancerígenas humanas, como colorretal (HCT116), leucemia (HL60), próstata (PC3) e células cancerosas de melanoma murino (B16F10). O extrato hexânico da própolis marrom frente as mesmas células tumorais apresentaram valores de inibição entre 50 e 100% do crescimento celular e baixa citotoxicidade contra células normais, mostrando excelente atividade e seletividade frente a células cancerígenas PC3 e HL60 (Frota et al., 2021).

A própolis marrom do Paraná também apresentou atividade moderada contra células de câncer de ovário humano (OVCAR-8) e carcinoma colorretal (HCT-116), com 75% e 50% de inibição do crescimento celular, respectivamente. Machado et al. 2021 avaliou 44 tipos de própolis marrom da região sul com diferentes níveis de atividades antitumorais, variando de extratos inativos a extratos altamente ativos contra três células cancerígenas humanas (OVCAR-8, HCT-116 e SF-295) (Machado et al., 2021).

Existem 20 doenças tropicais negligenciadas (DTNs) listadas pela Organização Mundial da Saúde incluindo Leishmaniose e doença de Chagas (WHO, 2019). Essas DTN afetam pessoas que vivem em grande pobreza, correspondendo a 1,4 bilhão de pessoas em todo o mundo. Apesar disso, grandes indústrias farmacêuticas não apresentam grandes esforços e interesse em investir no desenvolvimento de novos medicamentos para tratar essas doenças, principalmente porque as pessoas acometidas por essas doenças, em sua grande maioria, se encontram em vulnerabilidade econômica e scoail, assim, não possuem recursos financeiros para pagar os medicamentos (Fitzpatrick et al, 2017).

Leishmania é um gênero de protozoários que compreende mais de 20 espécies. Em 2018, a leishmaniose foi considerada endêmica em aproximadamente 90 países. Atualmente, mais de um bilhão de pessoas vivem em áreas endêmicas, e 30.000 novos casos de leishmaniose visceral e mais de um milhão de novos casos de leishmaniose cutânea são relatados anualmente (WHO, 2021). O tratamento usual inclui antimoniais pentavalentes e anfotericina B, embora este tratamento tenha várias limitações e desvantagens, como toxicidade, necessidade de administração prolongada, resistência e efeitos colaterais (Tiwari et al, 2018).

O extrato bruto da própolis marrom de Cabo Verde – MG apresentou atividade leishimanicida promissora, com IC_{50} de 1,8 µg/mL contra a forma promastigota de *L. amazonensis* e IC_{50} de 2,4 µg/mL contra a forma amastigota. Ao passo que, a fração volátil da própolis marrom inibiu a motilidade flagelar nas formas promastigota de forma dose-dependente com IC_{50} = 21,3 µg/mL e também aumentou a morte das formas amastigotas com IC_{50} = 25,1 µg/mL.

Santana et al. (2014) relataram a atividade da própolis marrom contra as formas promastigota e amastigota de *L. amazonensis*, comparando diferentes frações obtidas pela partição do extrato bruto. O ensaio *in vitro* de inibição do crescimento da forma promastigota com a fração diclorometano contendo 2,3-dihidroxibenzofurano como composto majoritário apresentou o melhor resultado (IC_{50}= 3,22 µg/mL). A fração hidroalcoólica (IC_{50}= 4,64 µg/mL), a fração hexânica (IC_{50}= 4,79 µg/ml) e a fração acetato de etila (IC_{50}= 8,83 µg/mL) também foram ativas.

A atividade antimicrobiana da própolis marrom vem sendo estudada contra bactérias gram-positivas e gram-negativas, chamando a atenção de empresas farmacêuticas e alimentícias (Nedji, 2014). Além disso, a necessidade de desenvolver novos medicamentos com potencial antimicrobiano tem aumentado anualmente devido ao elevado número de novas resistências ao tratamento usual (Morrill et al. 2015).

A atividade antimicrobiana do extrato de própolis marrom foi observada contra as bactérias gram-positivas *Staphylococcus aureus* e *Enterococcus* spp. (CIM = 31,3 – 500 µg/mL), No entanto, não foi encontrada atividade contra bactérias gram-negativas, como *Klebsiella* spp. e *Escherichia coli* (Silva et al. 2017). Salomão e colaboradores relataram resultado semelhante, descrevendo a atividade do extrato de própolis marrom contra *Streptococcus pneumoniae* (CIM = 0,2 – 0,8 µg/mL) e *Staphylococcus aureus* (CIM = 1,6 – 52,4 µg/mL). Por outro lado, o extrato mostrou-se inativo contra *Klebsiella pneumoniae* uma bactéria gram-negativa (Salomão et al. 2007).

A fração volátil da própolis marrom também foi avaliada quanto a atividade antimicrobiana por Lima e colaboradores, e apresentou atividade contra *Helicobacter pylori*, *Mycobacterium tuberculosis* e *M. avium* com CIM = 3,25, 50,0 e 62,5 µg/mL, respectivamente. A tetraciclina apresentou CIM de 1,0 µg/mL contra *H. pilory* e a isoniazida apresentou CIM de 1,47 µg/mL contra ambos *M. tuberculosis* e *M. avium* (Lima et al. 2019).

Uma das principais atividades biológicas atribuída a própolis é a atividade anti-inflamatória. No capítulo 3 foi discutido sobre os resultados obtidos sobra a avaliação das atividades anti-inflamatória e antinociceptiva do extrato bruto e da fração volátil da própolis marrom. Foram observados diferentes níveis de inibição quando comparados as duas frações da própolis, é preciso levar em consideração a diferença entre a constituição química do extrato bruto e da fração volátil, também é preciso considerar que a própolis é uma mistura complexa, e as interações sinérgicas entre seus compostos também devem ser consideradas como um fator importante em suas atividades anti-inflamatórias.

5. Conclusão

- A própolis marrom de Cabo Verde-MG é quimicamente constituída por compostos fenólicos prenilados, ácidos fenólicos e flavonoides. *Bacharis dracunculifolia* é uma de suas fontes botânicas;
- A combinação das técnicas de cromatografila em fase líquida empregando coluna de vidro clássica e cromatografia líquida de alta eficiência acoplada com detector UV/Vis, permitiu o fracionamento e isolamento de 15 constituintes principais da própolis marrom de Cabo Verde-MG. Os dados experimentais de ressonância magnética nuclear unidimensional (^{1}H e ^{13}C) e bidimensionais (HSQC e HMBC), em comparação com dados da literatura permitiram a identificação das substâncias: Resokaempferol; Naringenina; Ácido cafeico; Ácido cumárico; Ácido Ferúlico; Garbanzol; Ácido Cinâmico; Dihidrocaempferide; Capilartemisina A; Drupanina; Kaempferol éter-3,7,4'-trimetílico; 5,7-dimetoxikaempferol; Artepillin C; Bacarina; 2,2-Dimetil-6-carboxietenil-2H-1-benzopirano;
- O método HPLC/DAD desenvolvido e otimizado para análise de quinze compostos fenólicos da própolis marrom apresentou parâmetros adequados de validação como especificidade, linearidade, precisão, precisão e limites de detecção e quantificação. Demostrando ser uma ferramenta analítica adequada para o controle de qualidade e auxiliar na determinação da composição química das amostras de própolis marrom;
- A fração volátil da própolis marrom obtida por hidrodestilação, demonstrou alta complexidade química, composta por majoritariamente por monoterpenos e sesquiterpenos. Com a utilização de diferentes técnicas, foi possível realizar o isolamento e identificação de 8 substâncias voláteis.
- Uma outra ferramenta analítica, o método CG-DIC foi desenvolvido e otimizado para quantificação de 1,8-cineol, terpinen-4-ol, α-copaeno, β-cariofileno, γ-muurolene, nerolidol, espatulenol e γ-palmitolactona em amostras de frações voláteis de própolis e apresentou parâmetros adequados de validação;
- A própolis marrom de Cabo verde-MG apresentou alto potencial leishimanicida frente a *L. amazonenesis* e potencial citotóxico frente as linhagens cancerígenas avaliadas SK-MEL; KB; SK-OV-3; BT-549. A fração volátil demonstrou potencial leishimanicida frente a *L. amazonenesis* e antimicrobiano frente aos microorganismos *Streptococcus mutans* e *Staphylococcus aureus*;
- O extrato hidroalcoólico de própolis marrom do sudeste brasileiro e os óleos voláteis apresentaram resultados promissores nos protocolos de avaliação nociceptiva e anti-

inflamatória. Além disso, deve-se destacar que a fração volátil na maior concentração testada (200 mg/kg) inibiu ambas as fases no teste da formalina e a hipernocicepção mecânica, que foi semelhante às observadas para a morfina, um opioide com alto poder analgésico. A própolis marrom apresentou significativa atividade antinociceptiva, atuando através da modulação da transmissão nociceptiva e como anti-inflamatório. Os resultados obtidos nos modelos farmacológicos corroboram o uso tradicional da própolis como agente anti-inflamatório.

- A partir do estudo fitoquímico da própolis marrom de Angatuba-Sp foi possível verificar a variabilidade química desse produto natural. A partir dessa própolis marrom, foram isolados sete compostos fenólicos (ácido ferúlico, ácido cafeico, ácido 4-metoxicinâmico, ácido 3,4-dimetoxicinâmico, 3-hidroxi-4-metoxibenzaldeído, 3-metoxi-4-hidroxipropiofenona e 1-O,2-O-digalloil-6- O-trans-p-cumaroil-β-D-glicopiranosídeo), um flavanonol (7-O-metil aromadendrina), duas lignanas (pinoresinol e matairesinol) e seis ácidos/álcool diterpênicos (19-acetoxi-13-hidroxilabda-8(17),14-dieno, totarol, ácido 7-oxodehidroabiético, ácido desidroabiético, ácido comúnico e ácido isopimárico).
- A própolis marrom apresentou atividade significativa contra as cepas de *Plasmodium falciparum* D6 e W2. A substância matairesinol, isolado da própolis marrom de Angatuba-SP demontrou ter potencial citotóxico promissor frente a diferentes linhagens celulares tumorais.

6. Referências

Anderegg, R. J.; Rowe, J. W. Lignans, the Major Component of Resin from Araucaria Angustifolia Knots. *Holzforschung* 1974, *28* (5), 171–175.

Andrade JKS, Denadai M, Oliveira CS, Nunes ML, Narain N. Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region. Food Research International, 101, 129-138, 2017.

ANVISA: Ministério da saúde Agência Nacional de Vigilância Sanitária. RDC No 166 2017.

Arruda C, Ribeiro VP, Almeida MO, Mejía JAA, Casoti R, Bastos JK. Effect of light, oxygen and temperature on the stability of artepillin C and *p*-coumaric acid from Brazilian green propolis. J Pharmaceut Biomed, 178:112922, 2020.

Bankova V, Bertelli D, Borba R, Conti BJ, Barbosa I, Cunha S, Danert C, Eberlin MN, Falcão SI, Inés M, Moreno N, Papotti G, Popova M, Santiago KB, Salas A, Helena AC, Sawaya F, Schwab NV, Sforcin M, Simone-Finstrom M, Spivak M, Trusheva B, Vilas-Boas M, Wilson M, Zampini C. Standard Methods for *Apis Mellifera* Propolis Research. J. Apic. Res.58, 1–49, 2019.

Bankova, V.; Marcucci, M. C.; Simova, S.; Nikolova, N.; Kujumgiev, A.; Popov, S. Antibacterial Diterpenic Acids from Brazilian Propolis. *Zeitschrift fur Naturforsch. Sect. C - J. Biosci.* 1996, *51* (5–6), 7–10.

Belvedere. Conap: exports increase in the pandemic https://www.jornalbelvedere.com.br/index.php/estilo/saude/item/1653-conap-exportacoes-aumentam-na-pandemia (accessed Nov 24, 2021).

Berretta AA, Arruda C, Miguel FG, Baptista N, Nascimento AP, Oliveira FM, Hori JI, Barud HS, Damaso B, Ramos C, Ferreira R, Bastos JK. Functional properties of brazilian propolis: from chemical composition until the market chemical composition until the market. Superfood and Functional Food - An Overview of Their Processing and Utilization, n. March, p. 45, 2017.

Beserra, F. P.; Gushiken, L. F. S.; Hussni, M. F.; Ribeiro, V. P.; Bonamin, F.; Jackson, C. J.; Pellizzon, C. H.; Bastos, J. K. Artepillin C as an Outstanding Phenolic Compound of Brazilian Green Propolis for Disease Treatment: A Review on Pharmacological Aspects. Phyther. Res. 2021, 35 (5), 2274–2286.

Brophy, J. J.; Goldsack, R. J.; Wu, M. Z.; Fookes, C. J. R.; Forster, P. I. The Steam Volatile Oil of Wollemia Nobilis and Its Comparison with Other Members of the Araucariaceae (Agathis and Araucaria). *Biochem. Syst. Ecol.* 2000, *28* (6), 563–578.

Ccana-Ccapatinta, G. V.; Mejía, J. A. A.; Tanimoto, M. H.; Groppo, M.; Carvalho, J. C. A. S. de; Bastos, J. K. Dalbergia Ecastaphyllum (L.) Taub. and Symphonia Globulifera L.f.: The Botanical Sources of Isoflavonoids and Benzophenones in Brazilian Red Propolis. Molecules 2020, 25 (9), 2060.

Chauhan, A.; Mittu, B.; Chauhan, P. Analytical method development and validation: a concise review. Analytical & Bioanalytical. v. 6, n. 1, p. 1-5, 2015.

Costa P, Boeing T, Somensi LB, Cury BJ, Espíndola VL, França TCS, et al. Hydroalcoholic extract from Baccharis dracunculifolia recovers the gastric ulcerated tissue, and p -coumaric acid is a pivotal bioactive compound to this action. BioFactors 2019:biof.1503.

Daugsch A, Moraes CS, Fort P, Park YK. Brazilian red propolis—Chemical composition and botanical origin. Evidence-Based Complement. Altern. Med, 5, 435–441, 2008.

Daugsch, A.; Moraes, C. S.; Fort, P.; Park, Y. K. Brazilian Red Propolis - Chemical Composition and Botanical Origin. Evidence-Based Complement. Altern. Med. 2008, 5 (4), 435–441.

Dembogurski, D. S.; Silva Trentin, D.; Boaretto, A. G.; Rigo, G. V.; da Silva, R. C.; Tasca, T.; Macedo, A. J.; Carollo, C. A.; Silva, D. B. Brown Propolis-Metabolomic Innovative Approach to Determine Compounds Capable of Killing Staphylococcus Aureus Biofilm and Trichomonas Vaginalis. Food Res. Int. 2018, 111 (February), 661–673.

Dembogurski, D. S.; Silva Trentin, D.; Boaretto, A. G.; Rigo, G. V.; da Silva, R. C.; Tasca, T.; Macedo, A. J.; Carollo, C. A.; Silva, D. B. Brown Propolis-Metabolomic Innovative Approach to Determine Compounds Capable of Killing Staphylococcus Aureus Biofilm and *Trichomonas Vaginalis*. *Food Res. Int.* 2018, *111* (February), 661–673.

Eberhardt, T. L.; Bernards, M. A.; He, L.; Davin, L. B.; Wooten, J. B.; Lewis, N. G. Lignification in Cell Suspension Cultures of Pinus Taeda. In Situ Characterization of a Gymnosperm Lignin. *J. Biol. Chem.* 1993, *268* (28), 21088–21096.

Elian Guimarães. With therapeutic properties, the search for green propolis increases in the pandemic
https://www.em.com.br/app/noticia/economia/2021/06/26/internas_economia,1280710/com-propriedades-terapeuticas-busca-por-propolis-verde-aumenta-na-pandemia.shtml (accessed Nov 24, 2021).

Fabio, G.; Federica, C.; Alfredo, F.; Nicola, V. Recent Advances in Analytical Approaches for the Standardization and Quality of Polyphenols of Propolis. J. Med. Plants Res. 2019, 13 (19), 487–500.

Fasolo D, Bergold AM, Poser G, Teixeira HF. Determination of benzophenones in lipophilic extract of Brazilian red propolis, nanotechnology-based product and porcine skin and mucosa: Analytical and bioanalytical assays. J. Pharm. Biomed. Anal. 124, 57–66, 2016.

Fernandes, F. H.; Guterres, Z. D. R.; Corsino, J.; Garcez, W. S.; Garcez, F. R. Assessment of the Mutagenicity of Propolis Compounds from the Brazilian Cerrado Biome in Somatic Cells of Drosophila Melanogaster. Orbital 2019, 11 (5), 307–313

Fernandes, F. H.; Guterres, Z. D. R.; Violante, I. M. P.; Lopes, T. F. S.; Garcez, W. S.; Garcez, F. R. Evaluation of Mutagenic and Antimicrobial Properties of Brown Propolis Essential Oil from the Brazilian Cerrado Biome. Toxicol. Reports 2015, 2, 1482–1488.

Fitzpatrick, C.; Nwankwo, U.; Lenk, E.; Vlas, S. J. de; Bundy, D. A. P. An Investment Case for Ending Neglected Tropical Diseases. In Major Infectious Diseases; Holmes, K. K., Bertozzi, S., Bloom, B. R., Jha, P., Eds.; World Bank Group, 2017; pp 411–432.

Fonseca, M. J. V.; Fonseca, Y. M.; Marquele-Oliveira, F.; Vicentini, F. T. M. C.; Furtado, N. A. J. C.; Sousa, J. P. B.; Lucisano-Valim, Y. M. Evaluation of the Potential of Brazilian Propolis against UV-Induced Oxidative Stress. Evidence-based Complement. Altern. Med. 2011, 2011.

Freitas, A. da S.; Barth, O. M.; Sales, É. de O.; Matsuda, A. H.; de Almeida-Muradian, L. B. A Palynological Analysis of Brazilian Propolis Samples. J. ApiProduct ApiMedical Sci. 2011, 3 (2), 67–74.

Freitas, M. O.; Ponte, F. A. F.; Lima, M. A. S.; Silveira, E. R. Flavonoids and Triterpenes from the Nest of the Stingless Bee Trigona Spinipes. J. Braz. Chem. Soc. 2008, 19 (3), 532–535.

Frota, V. M.; Dias, F. M. F.; do Nascimento, M. F.; da Silva Mendonça, L.; dos Santos Moita, E. C.; dos Santos Gomes, L. C.; Leal, A. L. A. B.; Barreto, H. M.; Silva, M. F. S.; dos Santos Fontenelle, R. O.; Gomes, G. A.; do Vale, J. P. C.; da Silva Julião, M. S.; Bandeira, P. N.; Pessoa, C. do Ó.; dos Santos, H. S.; Zocolo, G. J.; Rodrigues, T. H. S. Inhibitory Activity of Brown Propolis Extracts on a Norfloxacin-Resistant Strain of Staphylococcus Aureus. Rev. Bras. Farmacogn. 2021, 31 (2), 249–255.

Garcia, P. L.; Buffoni, E.; Gomes, F. P.; Quero, J. L. V. Analytical Method Validation. In: AKYAR, I. (Ed) Wide Spectra of Quality Control. Rijeka-Croácia: In Tech, p. 3-20, 2011.

Govindaraghavan, S. & Sucher, N.J. Quality assessment of medicinal herbs and their extracts: Criteria and prerequisites for consistent safety and efficacy of herbal medicines. Epilepsy & Behavior, v. 45, p. 1-9, 2015.

Hata, T.; Tazawa, S.; Ohta, S.; Rhyu, M.-R.; Misaka, T.; Ichihara, K. Artepillin C, a Major Ingredient of Brazilian Propolis, Induces a Pungent Taste by Activating TRPA1 Channels. *PLoS One* 2012, *7* (11), e48072.

Hochheim, S.; Guedes, A.; Faccin-Galhardi, L.; Rechenchoski, D. Z.; Nozawa, C.; Linhares, R. E.; Filho, H. H. da S.; Rau, M.; Siebert, D. A.; Micke, G.; Cordova, C. M. M. de. Determination of Phenolic Profile by HPLC–ESI-MS/MS, Antioxidant Activity, in Vitro Cytotoxicity and Anti-Herpetic Activity of Propolis from the Brazilian Native Bee Melipona Quadrifasciata. Rev. Bras. Farmacogn. 2019, 29 (3), 339–350.

Huang S, Zhang CP, Wang K, Li GQ, Hu FL. Recent Advances in the Chemical Composition of Propolis. Molecules, 19:19610-19632, (2014).

Ioannou, E.; Koutsaviti, A.; Tzakou, O.; Roussis, V. The Genus Pinus: A Comparative Study on the Needle Essential Oil Composition of 46 Pine Species. Phytochem. Rev. 2014, 13 (4), 741–768.

Kocot J, Kiełczykowska M, Luchowska-kocot D, Kurzepa J, Musik I. Antioxidant Potential of Propolis, Bee Pollen, and Royal Jelly: Possible Medical Application. Oxidative Medicine and Cellular Longevity, 2018:7074209, 2018.

Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M. L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I. Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II. Analytica Chimica Acta. v. 870, n. 1, p 1-28, 2015.

Lima, V. H. M. de; Almeida, K. de C. R.; Alves, C. C. F.; Rodrigues, M. L.; Crotti, A. E. M.; Souza, J. M. de; Ribeiro, A. B.; Squarisi, I. S.; Tavares, D. C.; Martins, C. H. G.; Miranda, M. L. D. Biological Properties of Volatile Oil from Brazilian Brown Propolis. Rev. Bras. Farmacogn. 2019, 29 (6), 807–810.

Machado, C. S.; Finger, D.; Dias De Lima, T. C.; Modesto, T.; Monteiro, M. C.; de Oliveira, F. de C. E.; Pessoa, C.; Torres, Y. R. Seeking the Regional Identity of Brown Propolis Produced in Southern Brazil and Linked to Total Levels of Bioactive Compounds and Biological Activity. ACS Food Sci. Technol. 2021, 1 (2), 176–185.

Machado, C. S.; Finger, D.; Dias De Lima, T. C.; Modesto, T.; Monteiro, M. C.; de Oliveira, F. de C. E.; Pessoa, C.; Torres, Y. R. Seeking the Regional Identity of Brown Propolis Produced in Southern Brazil and Linked to Total Levels of Bioactive Compounds and Biological Activity. ACS Food Sci. Technol. 2021, 1 (2), 176–185.

Maria Angélica Ehara Watanabe, Marla Karine Amarante, Bruno José Conti, José Maurício Sforcin, Cytotoxic constituents of propolis inducing anticancer effects: a review, Journal of Pharmacy and Pharmacology, Volume 63, Issue 11, November 2011, Pages 1378–1386

Mendonça ICG, Porto ICCM, Nascimento TG, Souza NS, Oliveira JMS, Arruda RES, Mousinho KC, Santos AF, Basílio-Júnior ID, Parolia A, Barreto FS. Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. BMC Complementary and Alternative Medicine, 15:357, 2015.

Mendonça-Melo L, Mota E, Lopez B, Sawaya A, Freitas L, Jain S, Batista M, Araújo E. Chemical and genetic similarity between *Dalbergia ecastaphyllum* and red propolis from the Northeastern Brazil. Journal of Apicultural Research, 56, 32–39, 2017.

Morrill, H. J.; Pogue, J. M.; Kaye, K. S.; LaPlante, K. L. Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. Open Forum Infect. Dis. 2015, 2 (2), 2633851.

Nascimento Araújo, C.; Mayworm, M. A. S.; Yatsuda, R.; Negri, G.; Salatino, M. L. F.; Salatino, A.; Timenetsky, J.; Campos, G. B. Chemical Composition and Antimycoplasma Activity of a Brown Propolis from Southern Brazil. *J. Food Sci. Technol.* 2020, *57* (11), 4228–4235.

Nascimento, T. G.; dos Santos Arruda, R. E.; da Cruz Almeida, E. T.; dos Santos Oliveira, J. M.; Basílio-Júnior, I. D.; Celerino de Moraes Porto, I. C.; Rodrigues Sabino, A.; Tonholo, J.; Gray, A.; Ebel, R. A. E.; Clements, C.; Zhang, T.; Watson, D. G. Comprehensive Multivariate

Correlations between Climatic Effect, Metabolite-Profile, Antioxidant Capacity and Antibacterial Activity of Brazilian Red Propolis Metabolites during Seasonal Study. Sci. Rep. 2019, 9 (1), 1–16.

Naz, S.; Vallejo, M.; García, A.; Barbas, C. Method validation strategies involved in non-targeted metabolomics. Journal of chromatography A. v. 1353, n. 1, p. 99 – 105, 2014.

Nedji, N.; Loucif-Ayad, W. Antimicrobial Activity of Algerian Propolis in Foodborne Pathogens and Its Quantitative Chemical Composition. Asian Pacific J. Trop. Dis. 2014, 4 (6), 433–437.

Olegário, L. S.; Andrade, J. K. S.; Andrade, G. R. S.; Denadai, M.; Cavalcanti, R. L.; da Silva, M. A. A. P.; Narain, N. Chemical Characterization of Four Brazilian Brown Propolis: An Insight in Tracking of Its Geographical Location of Production and Quality Control. Food Res. Int. 2019, 123 (April), 481–502.

Patil, R.; Deshmukh, T.; Patil, V.; Khandelwal, K. Review on analytical method development and validation. Research and Reviews: Journal of Pharmaceutical Analysis. v. 3, n. 3, p. 1-10, 2014.

Paulo Riardo. The heated propolis market in Brazil. https://www.diariox.com.br/economia/o-aquecido-mercado-de-propolis-no-brasil/18875/ (accessed Nov 24, 2021).

Pavlovic, R.; Borgonovo, G.; Leoni, V.; Giupponi, L.; Ceciliani, G.; Sala, S.; Bassoli, A.; Giorgi, A. Effectiveness of Different Analytical Methods for the Characterization of Propolis: A Case of Study in Northern Italy. Molecules 2020, 25 (3).

Ribeiro, V. P; Arruda, C.; El-Salam, M. A.; Bastos, J. K. Brazilian medicinal plants with corroborated anti-inflammatory activities: a review, Pharmaceutical Biology, 2018, 56:1, 253-268.

Ribeiro VP, Símaro GV, Mejia JAA, Arruda C, Bastos JK. Anti-inflammatory and Antinociceptive Activities of the Hydroalcoholic Extract and the Volatile Fraction of Southeastern Brazilian Brown Propolis. Rev. Bras. Farmacogn. 31, 59–66, 2021.

Ribeiro, V. P.; Arruda, C.; Aldana-Mejia, J. A.; Bastos, J. K.; Tripathi, S. K.; Khan, S. I.; Khan, I. A.; Ali, Z. Phytochemical, Antiplasmodial, Cytotoxic and Antimicrobial Evaluation of a Southeast Brazilian Brown Propolis Produced by Apis Mellifera Bees. Chem. Biodivers. 2021, 18 (9).

Ribeiro, V. P.; Arruda, C.; Mejía, J. A. A.; Candido, A. C. B. B.; dos Santos, R. A.; Magalhães, L. G.; Bastos, J. K. Brazilian Southeast Brown Propolis: Gas Chromatography Method Development for Its Volatile Oil Analysis, Its Antimicrobial and Leishmanicidal Activities Evaluation. *Phytochem. Anal.* 2021.

Ribeiro, V.P., Ccapatinta, G.V.C., Aldana-Mejia, J.A., Berretta, A.A., Moraes, L.A.B. and Bastos, J.K. (2022), Chemical characterization of Brazilian propolis using automated Direct Thermal Desorption–Gas Chromatography-Mass Spectrometry. J Sci Food Agric. 2022.

Ribeiro, V. P.; C. Arruda, J. A. Aldana-Mejia, J. K. Bastos, S. K. Tripathi, S. I. Khan, I. A. Khan, Z. Ali. Phytochemical, Antiplasmodial, Cytotoxic and Antimicrobial Evaluation of a Southeast Brazilian Brown Propolis Produced by *Apis mellifera* Bees. Chem. Biodiversity 2021, 18, e2100288.

Rodrigues DM, Souza MC, Arruda C, Pereira RAS, Bastos JK. The Role of *Baccharis dracunculifolia* and its chemical profile on green propolis production by *Apis mellifera*. J Chem Ecol, 146:150–162, 2020.

Rodrigues, C. R. F.; Plentz, L. C.; Marcucci, M. C.; Dihl, R. R.; Lehmann, M. In Vivo Evaluation of Mutagenic and Recombinagenic Activities of Brazilian Propolis. Food Chem. Toxicol. 2016, 96, 117–121.

Rufatto LC, Santos DA, Marinho F, Henriques JAP, Ely MR, Moura S. Red propolis: Chemical composition and pharmacological activity. Asian Pac. J. Trop. Biomed, 7, 591–598, 2017.

Ruiz-Angel, M. J.; García-Alvarez-Coquea, M. C.; Berthod, A.; Carda-Broch, S. Are analysts doing method validation in liquid chromatography? Journal of Chromatography A. v. 1353, n. 1, p. 2-9, 2014.

Salomão, K.; Pereira, P. R. S.; Campos, L. C.; Borba, C. M.; Cabello, P. H.; Marcucci, M. C.; de Castro, S. L. Brazilian Propolis: Correlation between Chemical Composition and Antimicrobial Activity. Evidence-Based Complement. Altern. Med. 2008, 5 (3), 317–324.

Santana, L. C. L. R.; Carneiro, S. M. P.; Caland-Neto, L. B.; Arcanjo, D. D. R.; Moita-Neto, J. M.; Citó, A. M. G. L.; Carvalho, F. A. A. Brazilian Brown Propolis Elicits Antileishmanial Effect against Promastigote and Amastigote Forms of Leishmania Amazonensis. Nat. Prod. Res. 2014, 28 (5), 340–343.

Santos, M. F. C.; Oliveira, L. C.; Ribeiro, V. P.; Soares, M. G.; Morae, G. de O. I.; Sartori, A. G. de O.; Rosalen, P. L.; Bastos, J. K.; Alencar, S. M.; Veneziani, R. C. S.; Ambrósio, S. R. Isolation of Diterpenes from Araucaria Sp Brazilian Brown Propolis and Development of a Validated HPLC Method for Its Analysis. J. Sep. Sci. 2021, 1–21.

Sartori, A. G. O.; Papa Spada, F.; Pena Ribeiro, V.; Rosalen, P. L.; Ikegaki, M.; Kenupp Bastos, J.; de Alencar, S. M. An Insight into the Botanical Origins of Propolis from Permanent Preservation and Reforestation Areas of Southern Brazil. *Sci. Rep.* 2021, *11* (1), 1–7.

Sawaya, A. C. H. F.; Barbosa da Silva Cunha, I.; Marcucci, M. C. Analytical Methods Applied to Diverse Types of Brazilian Propolis. Chem. Cent. J. 2011, 5 (1), 1–10.

Silva BB, Rosalen PL, Cury JA, Ikegaki M, Souza VC, Esteves A, Alencar SM. Chemical composition and botanical origin of red propolis, a new type of brazilian propolis. Evidence-Based Complement. Altern. Med, 5, 313–316, 2008.

Silva FRG, Matias TMS, Souza LIO, Matos-Rocha TJ, Fonseca SA, Mousinho KC, Santos AF. Phytochemical screening and *in vitro* antibacterial, antifungal, antioxidant and antitumor activities of the red propolis Alagoas. Brazilian Journal of Biology, 79, 452–459, 2019.

Silva, R. P. D.; Machado, B. A. S.; De Abreu Barreto, G.; Costa, S. S.; Andrade, L. N.; Amaral, R. G.; Carvalho, A. A.; Padilha, F. F.; Barbosa, J. D. V.; Umsza-Guez, M. A. Antioxidant, Antimicrobial, Antiparasitic, and Cytotoxic Properties of Various Brazilian Propolis Extracts. PLoS One 2017, 12 (3), 1–18.

Tazawa, S.; Arai, Y.; Hotta, S.; Mitsui, T.; Nozaki, H.; Ichihara, K. Discovery of a Novel Diterpene in Brown Propolis from the State of Parana, Brazil. *Nat. Prod. Commun.* 2016, *11* (2), 201–205.

Tiwari, N.; Gedda, M. R.; Tiwari, V. K.; Singh, S. P.; Singh, R. K. Limitations of Current Therapeutic Options, Possible Drug Targets and Scope of Natural Products in Control of Leishmaniasis. Mini-Reviews Med. Chem. 2017, 18 (1), 26–41.

Trusheva B, Popova M, Bankova V, Simova S, Marcucci MC, Miorin P, Da F, Pasin R, Tsvetkova I. Bioactive constituents of brazilian red propolis. Evidence-Based Complement. Altern. Med, 3, 249–254, 2006.

Waller, S. B.; Peter, C. M.; Hoffmann, J. F.; Picoli, T.; Osório, L. da G.; Chaves, F.; Zani, J. L.; de Faria, R. O.; de Mello, J. R. B.; Meireles, M. C. A. Chemical and Cytotoxic Analyses of Brown Brazilian Propolis (Apis Mellifera) and Its in Vitro Activity against Itraconazole-Resistant Sporothrix Brasiliensis. Microb. Pathog. 2017, 105, 117–121.

World Health Organization, W. Leishmaniasis https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (accessed Nov 24, 2021).

World Health Organization. Control of Neglected Tropical Diseases https://www.who.int/data/gho/data/themes/neglected-tropical-diseases (accessed Nov 24, 2021).

7. Apêndices

7.1. CAPÍTULO 1 – Desenvolvimento e validação de um método por CLAE-DAD para análise de uma própolis marrom do sudeste Brasileiro e avaliação de suas propriedades biológicas.

Artigo 1. RIBEIRO VP, MEJIA JAA, ARRUDA C, PAGOTTI MC, MAGALHÃES LG, KHAN SI, KHAN IA, ALI Z, BASTOS JK. A RP-HPLC-PDA method for analysis of a Brazilian southeast brown propolis and its biological properties, manuscript to be submitted in the Journal *Food Analytical Methods.*

Tabela 7.1. Dados experimentais dos espetros de ^{1}H e ^{13}C para os compostos isolados do Extrato bruto da própolis marrom

	Ac. cafeico		**Ac. cumárico**		**Ac. ferúlico**		**Ac. cinâmico**	
Posição (C/H)	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ 1H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	-	128,3	-	124,4	-	126,6	-	133
2	7,07 (*d*)	112,7	7,42 (*dd*)	128,2	7,09 (*d*)	123,5	7,54 (*m*)	126,3
3	-	147,2	6,78 (*dd*)	113,9	-	114,3	7,35 (*m*)	128,5
4	-	149,8	-	158,2	-	148,3	7,35 (*m*)	127,1
5	6,81 (*d*)	117	6,78 (*dd*)	113,9	6,97 (*d*)	147	7,35 (*m*)	128,5
6	6,96 (*dd*)	123,4	7,42 (*dd*)	128,2	7,14 (*dd*)	109,4	7,54 (*m*)	126,3
7	7,55 (*d*)	147,6	7,57 (*d*)	143,8	7,75 (*d*)	146,8	7,62 (*d*)	143,5
8	6,24 (*d*)	116	6,25 (*d*)	112,7	6,34 (*d*)	114,7	6,43 (*d*)	115,6
9	-	171,6	-	168,1	-	171,3	-	167,5
10					3,98 (s)	55,9		

	Capilartemisina A		**Drupanina**		**3,4',7-Trimetilkaempferol**		**7,4'-Dimetoxikaempferol**	
Posição (C/H)	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	-	127,2	-	127,2				
2	7,19 (*d*)	126,2	-	130,6	-	156,2	-	158
3	-	128,9	7,27 (*s*)	129,5	-	136	-	137,8
4	-	154,8	-	158,2	-	176	-	180
5	-	128,5	6,76 (*d*)	116,2	-	160,7	-	160,6
6	7,15 (*d*)	126,2	7,26 (*d*)	128,3	6,42 (*d*)	98,6	6,38 (*d*)	98,7
7	1,54 (*d*)	145,7	6,22 (*d*)	145,9	-	164	-	164,7
8	6,22 (*d*)	114,2	7,56 (*d*)	115,6	6,16 (*d*)	93,5	6,70 (*d*)	93,4
9	-	169,8	-	168,1	-	160,4	-	161,5
10	3,39 (*d*)	67,4	3,37 (*d*)	28,9	-	103,1	-	104,5
11	5,61 (*t*)	122,8	5,32 (*t*)	123,2	-	146,2	-	145,6
12	-	136	-	132,9	8,60 (*d*)	128,8	8,57 (*d*)	127,9
13	1,76 (*s*)		1,79 (*s*)	25,8	7,13 (*d*)	114,5	7,09 (*d*)	113,8
14	3,99 (*s*)		1,79 (*s*)	17,8	-	160,5	-	160,7
15	3,33 (*d*)	121,8			7,13 (*d*)	114,5	7,09 (*d*)	113,8
16	5,32 (*t*)				8,60 (*d*)	128,8	8,57 (*d*)	127,9
17	-	132,7			3,80 (*s*)	48,9	3,86 (*s*)	56,8

18	1,76 (*s*)		3,13 (*s*)	55,3	3,91 (*s*)	56,5
19	1,72 (*s*)		3,12 (*s*)	56		

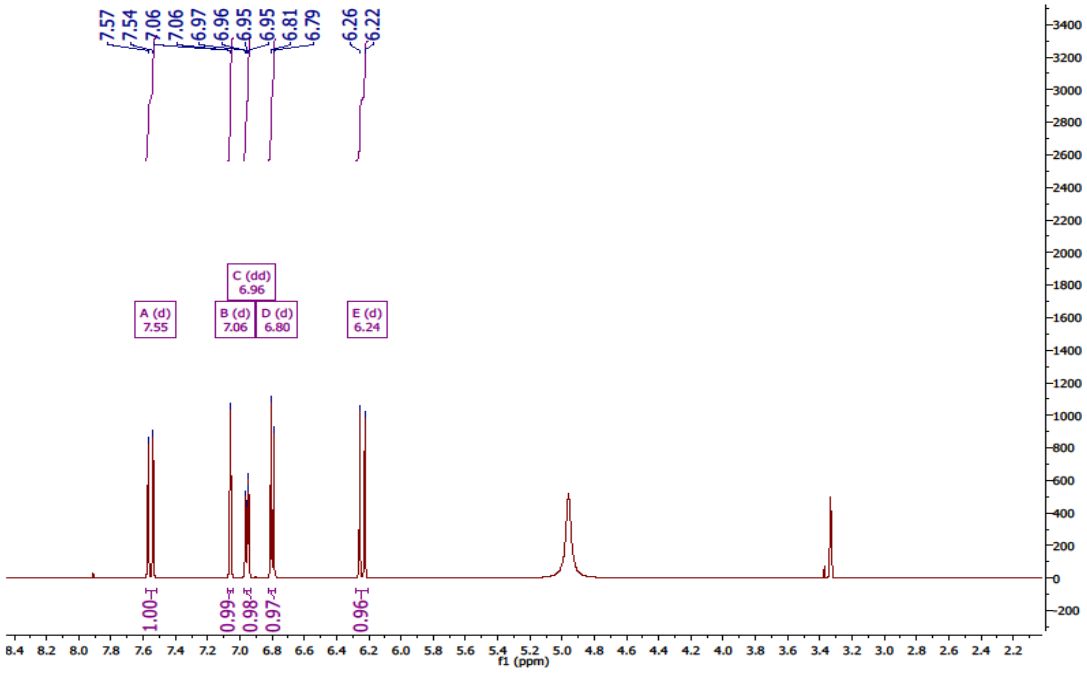
	Resokaempferol		Narigenina		Garbanzol		Dihidrokaempferide	
Posição (C/H)	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	-						-	198,3
2	-	158,3	5,02 (*dd*)	83,1	5,10 (*d*)	67,6	-	101,8
3	-	145.5	3,39-2,92 (*m*)	42,7	5,12 (*d*)	74,6	-	165,3
4	-	173,8	-	194,8	-	199	5,91 (*d*)	96,3
5	7,55 (*d*)	126,2	-	163	7,51 (*d*)	121,9	-	168,7
6	6,94 (*dd*)	113,6	6,60 (*s*)	95,4	6,91 (*dd*)	113,4	5,95 (*d*)	97,3
7	-	163	-	164,8	-	167,5	-	164,4
8	(6,27 (*d*)	105,2	6,60 (*s*)	94,2	6,75 (*d*)	106,3	5,02 (*d*)	84,7
9	-	152,8	-	162,3	-	148,3	4,53 (*d*)	73,6
10	-	115,4	-	101	-	113,5	-	130,2
11	-	119,1	-	128,9	-	126,4	7,46 (*d*)	130,5
12	7,05 (*d*)	121,7	7,97 (*d*)	127,3	7,02 (*d*)	115,1	6,90 (*d*)	114,8
13	6,78 (*d*)	114,3	6,93 (*d*)	114,5	6,19 (*d*)	114	-	161,6
14	-	152	-	157	-	146,2	6,90 (*d*)	114,8
15	6,78 (*d*)	114,3	6,93 (*d*)	114,5	6,19 (*d*)	114	7,46 (*d*)	130,5
16	7,05 (*d*)	121,7	7,97 (*d*)	127,3	7,02 (*d*)	115,1	-	55,7

	ArtepelinC		Bacarina		DCBEN	
Posição (C/H)	**δ ^{1}H (ppm)**	**δ 13C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	-	127,7	-	134	-	126,3
2	7,2 (*s*)	126,4	7,38 s	126,5	7,2 (*s*)	122
3	-	128,4	-	134,4	-	114
4	-	155,4	-	146,3	-	153,3
5	-	128,4	6,98 (*d*)	120,8	-	129,8
6	7,2 (*s*)	126,4	7,32 (*d*)	122,9	7,1 (*s*)	129,9
7	7,7 (*d*)	147,4	7,74 (*d*)	140	7,68 (*d*)	147,2
8	6,3 (*d*)	114,2	6,39 (*d*)	117,1	6,27 (*d*)	121
9	-	172,8	-	171,7	-	172,1
10	3,36 (*d*)	29,5	3,15 (*d*)	30,9	6,3 (*d*)	122
11	5,31 (*t*)	121,3	5,19 (*m*)	132	5,6 (*d*)	131,1
12	-	135,2	-	130,2	-	77
13	1,79 (*s*)	17,9	1,66 (*s*)	25,7	1,44 (*s*)	28,2
14	1,78 (*s*)	25,8	1,75 (*s*)	17,9	1,44 (*s*)	28,1

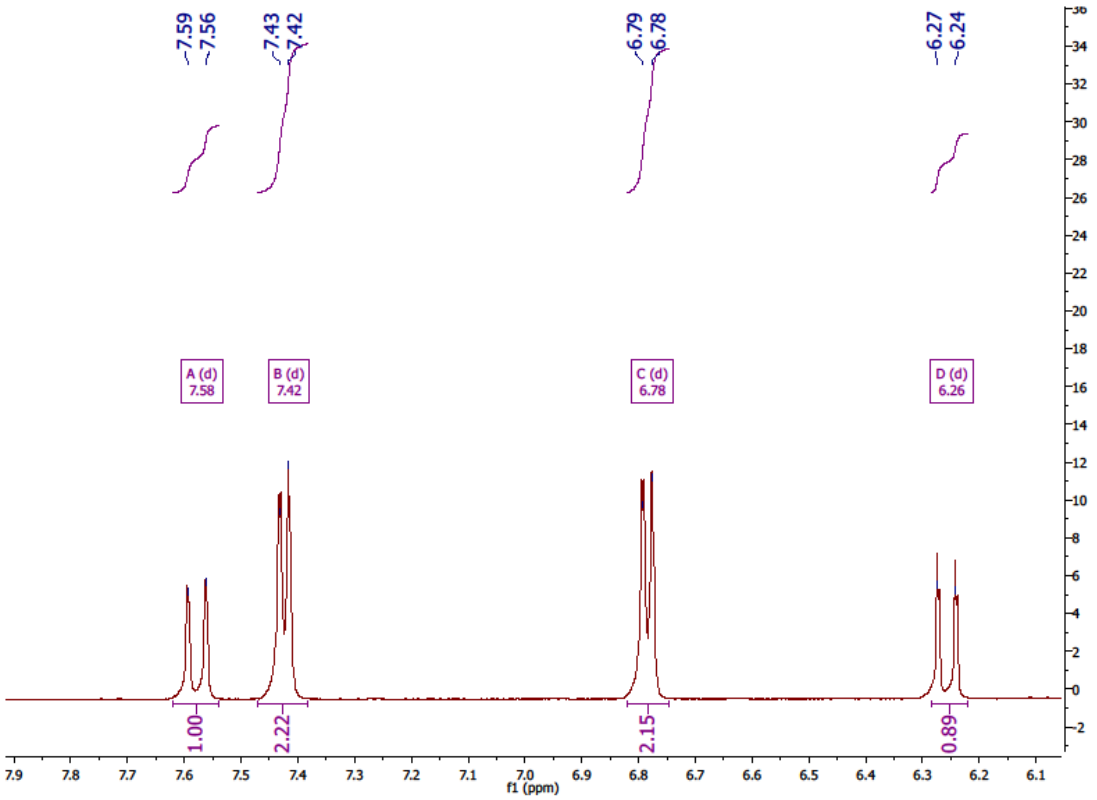
15	3,36 (*d*)	29,5	-	134	3,27 (*d*)	28,3
16	5,31 (*t*)	121,3	7,3-7,2 (*m*)	128,4	5,2 (*t*)	124,4
17	-	135,2	7,3-7,2 (*m*)	128,6	-	132,7
18	1,78 (*s*)	25,8	7,3-7,2 (*m*)	126,9	1,73 (*s*)	25,8
19	1,79 (*s*)	17,9	7,3-7,2 (*m*)	128,6	1,75 (*s*)	17,9
20			7,3-7,2 (*m*)	128,4		
21			3,10 (*t*)	140		
22			2,94 (*t*)	117,1		
23				171		

(*s*) simpleto, (*d*) dupleto, (*dd*) duplo dupleto, (*m*) multipleto e (*t*) tripleto

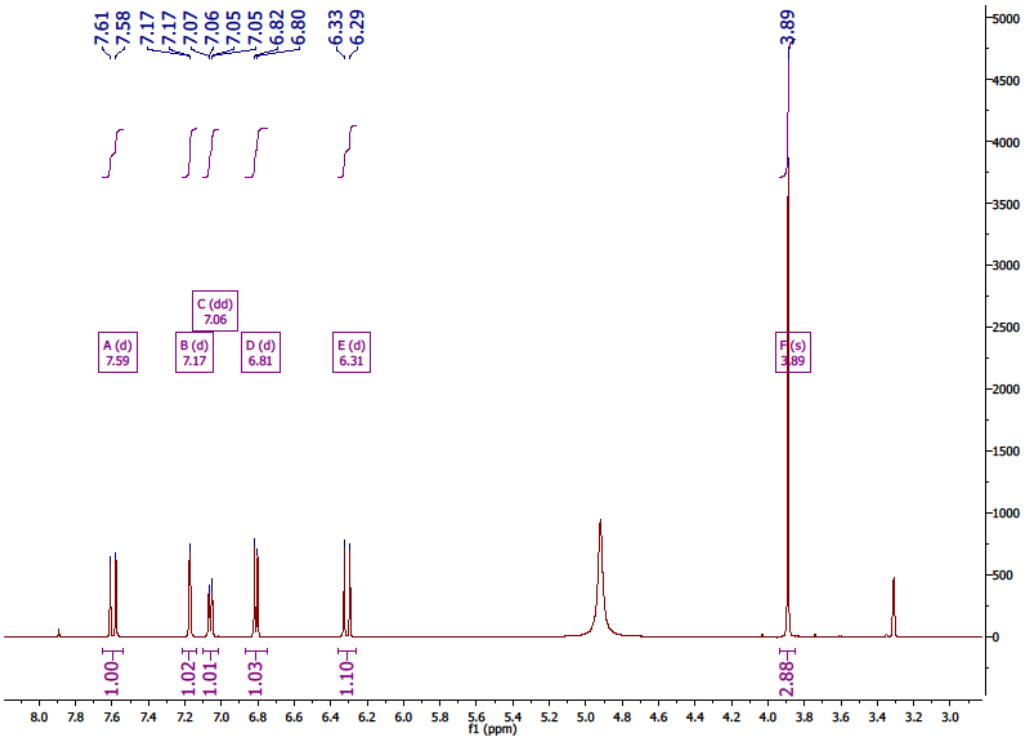
Apêndice 7.1.1 Espectro de RMN ^{1}H do composto Acido cafeico em CD_3OD (500 MHz).



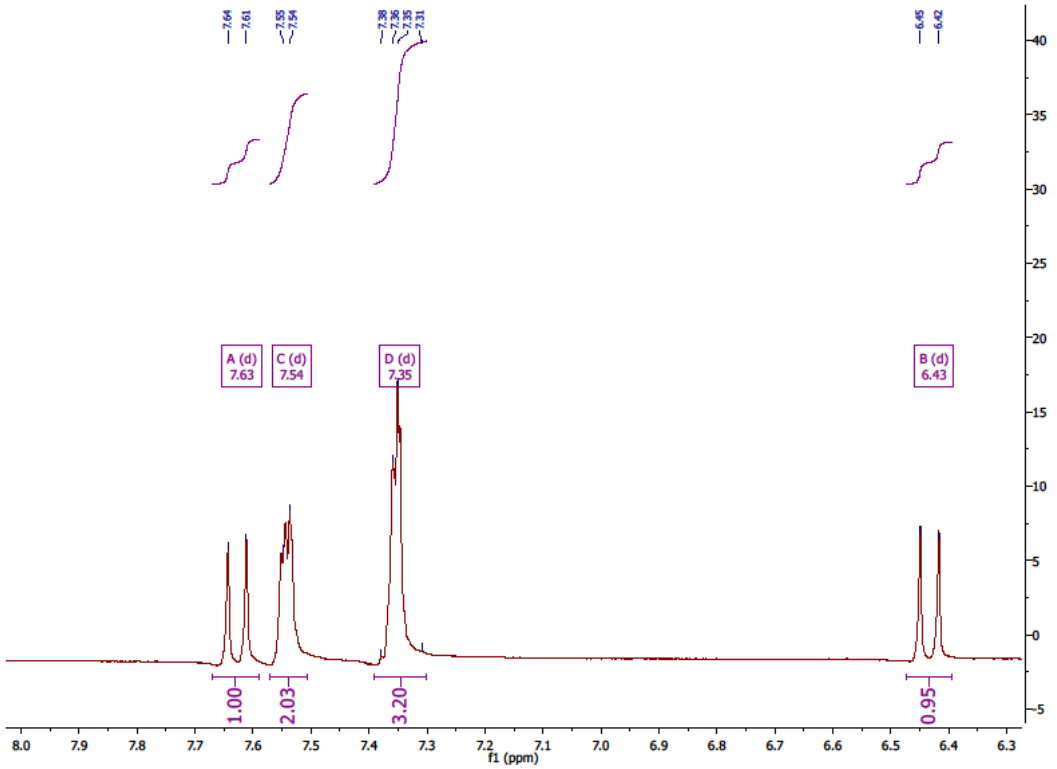
Apêndice 6.1.2. Espectro de RMN ^{1}H do composto Ácido cumárico em CD_3OD (500 MHz).



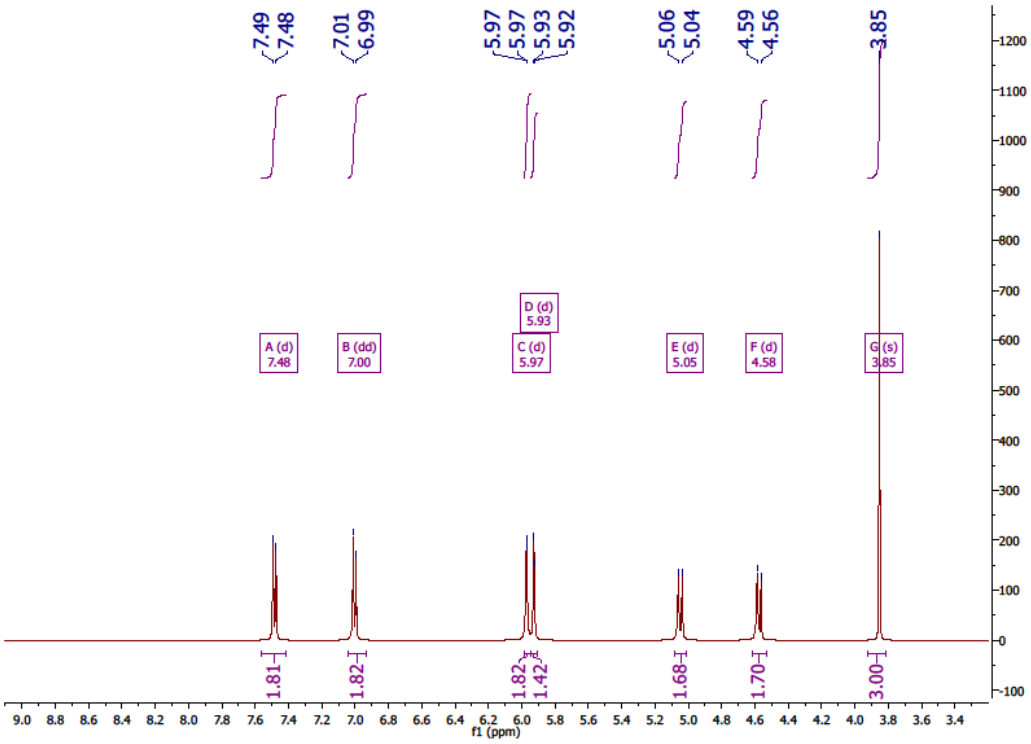
Apêndice 6.1.3. Espectro de RMN ^{1}H do composto Ácido ferúlico em CD_3OD (500 MHz).



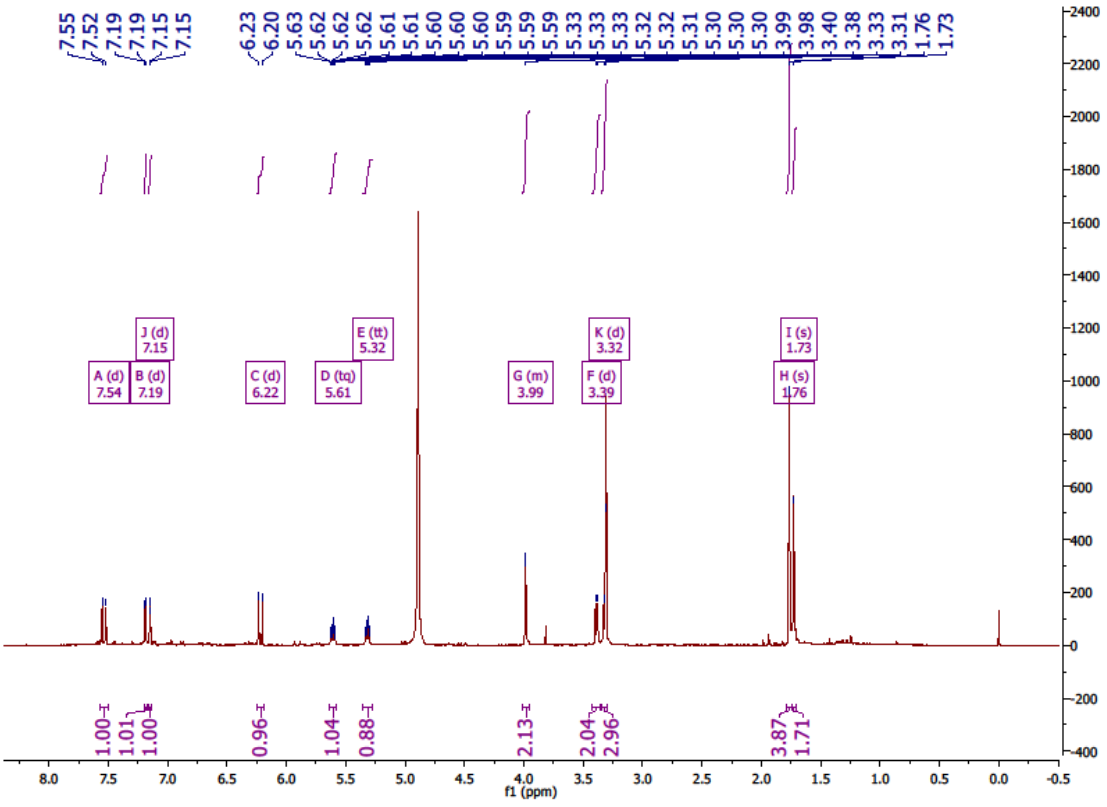
Apêndice 6.1.4. Espectro de RMN ^{1}H do composto Ácido cinâmico em CD_3OD (500 MHz).



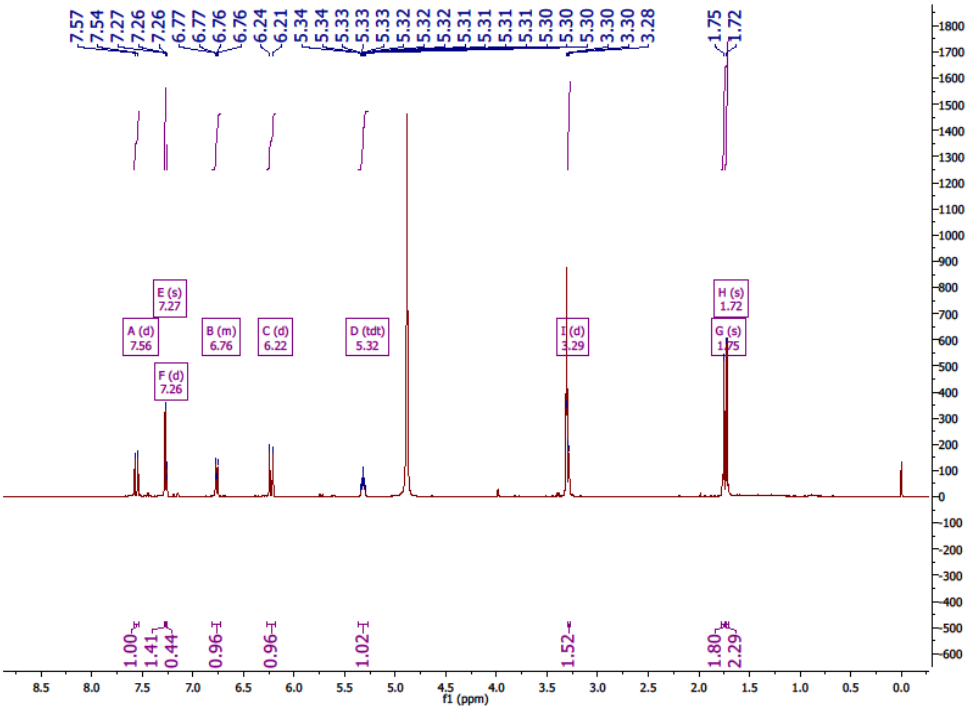
Apêndice 6.1.5. Espectro de RMN ^{1}H do composto Dihidrokaempferide em CD_3OD (500 MHz).



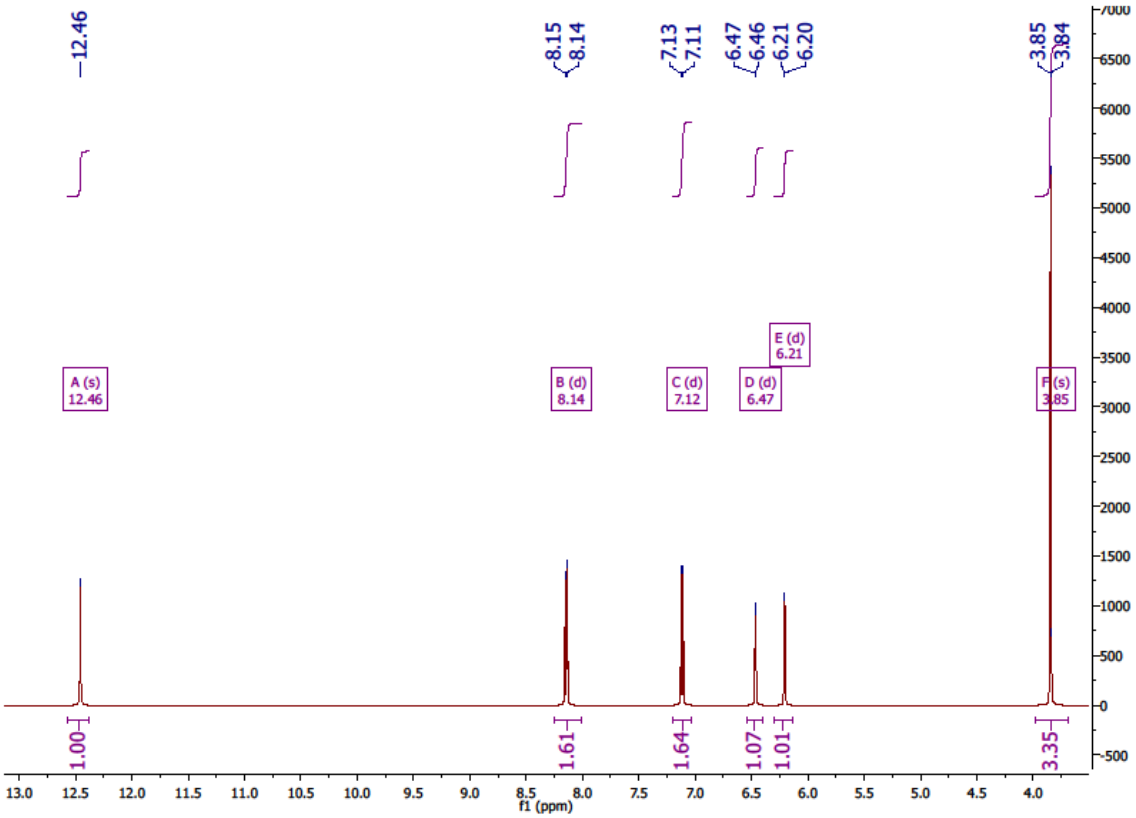
Apêndice 6.1.6. Espectro de RMN ^{1}H do composto capilartemisina A em CD_3OD (500 MHz).



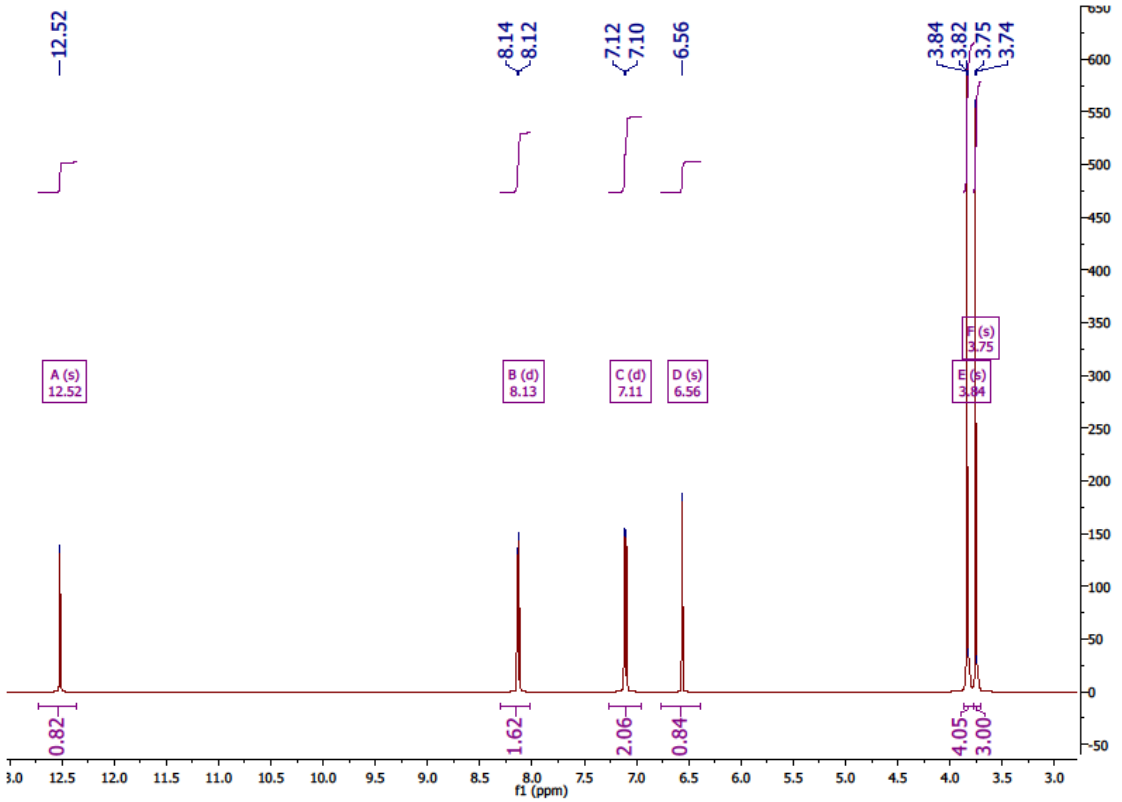
Apêndice 6.1.7. Espectro de RMN ^{1}H do composto drupanina em $CDCl_3$ (500 MHz).



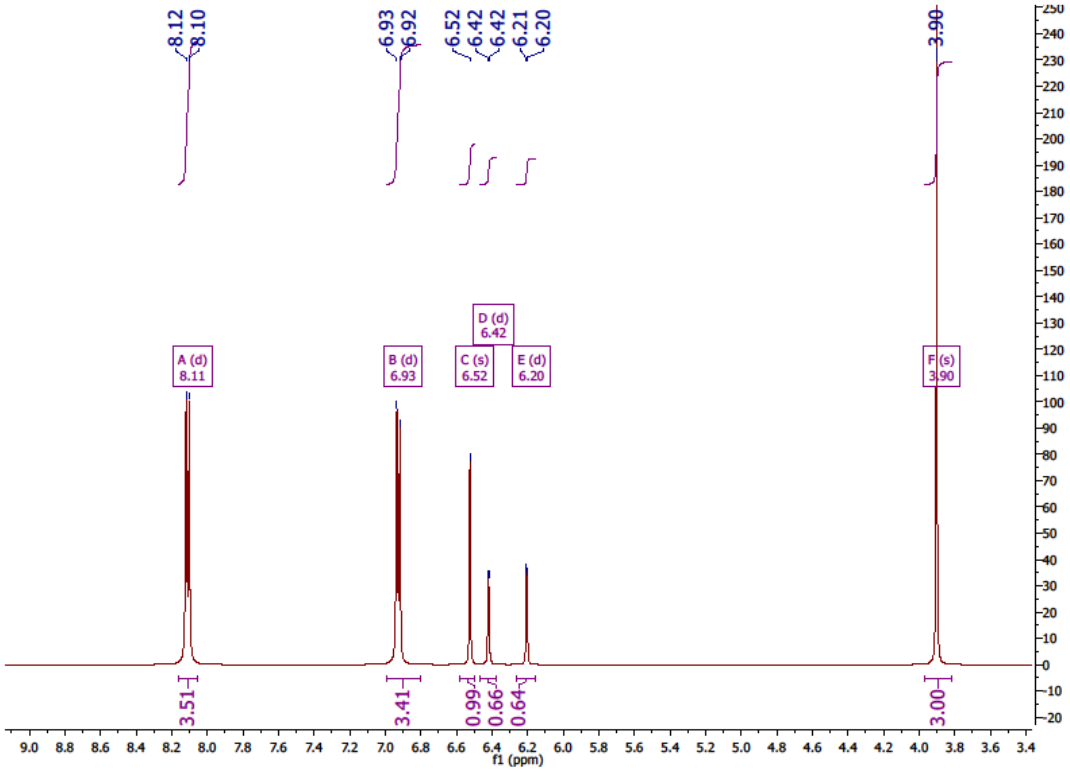
Apêndice 6.1.8. Espectro de RMN ^{1}H do composto 3,4',7-Trimetilkaempferol em $CDCl_3$ (500 MHz).



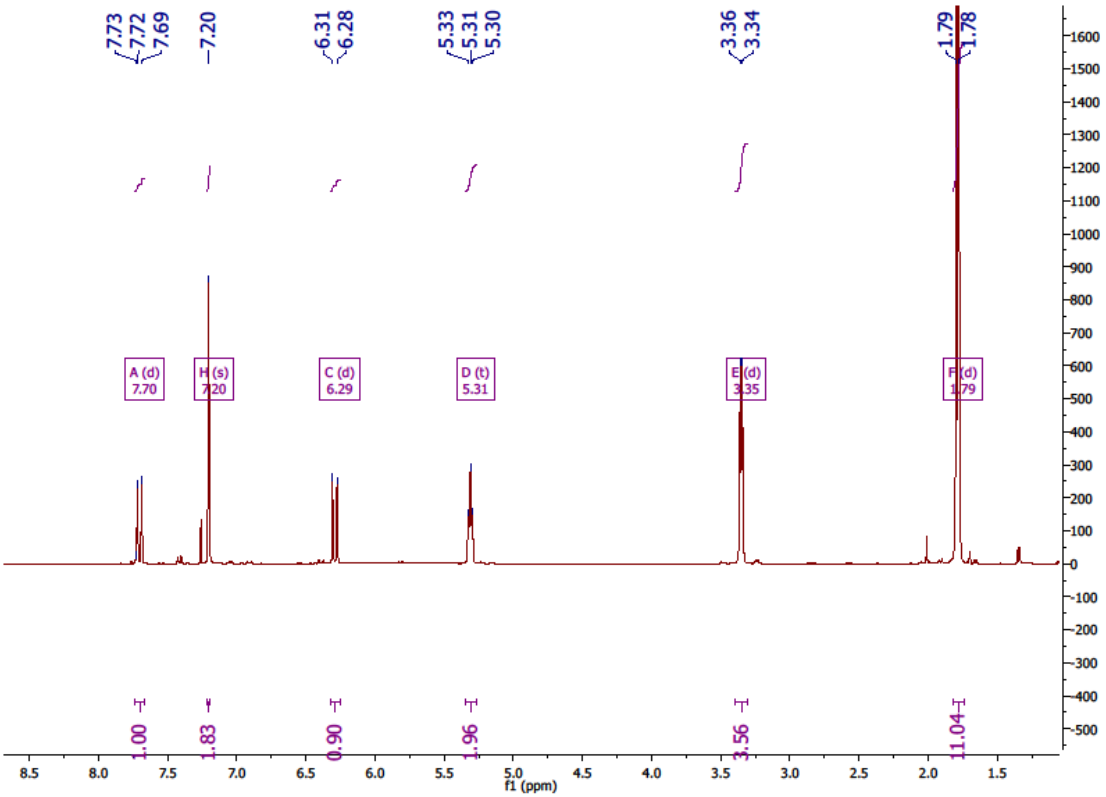
Apêndice 6.1.9. Espectro de RMN ^{1}H do composto 7,4'-Dimetoxikaempferol em CD_3OD (500 MHz).



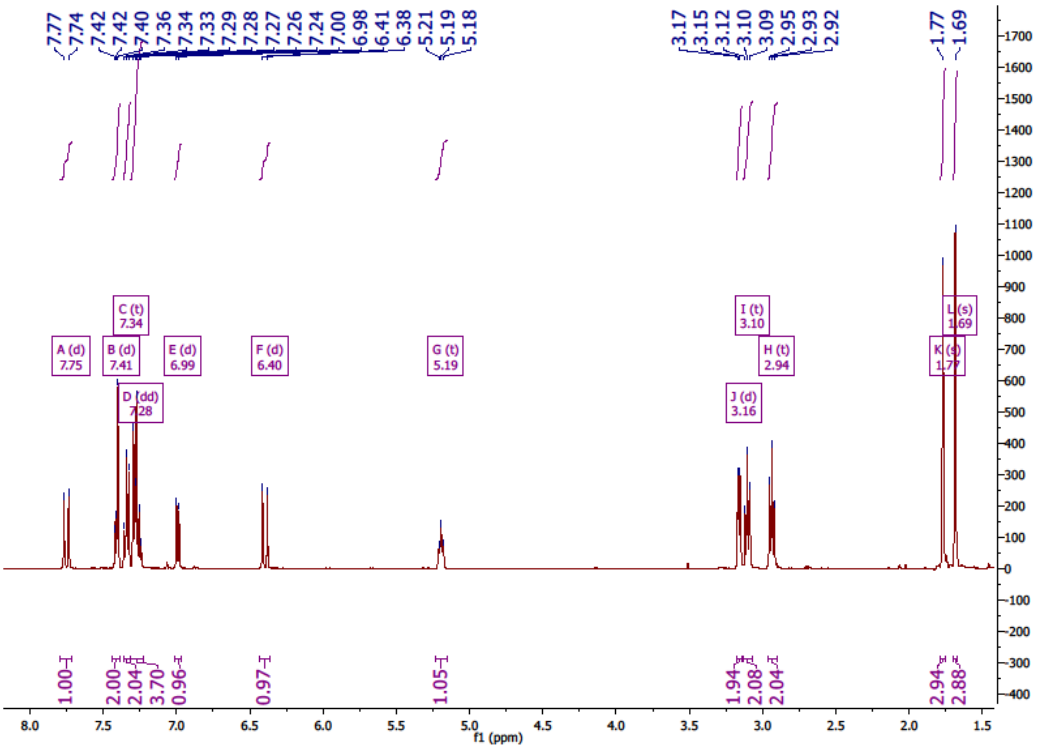
Apêndice 6.1.10. Espectro de RMN ^{1}H do composto garbanzol em CD_3OD (500 MHz).



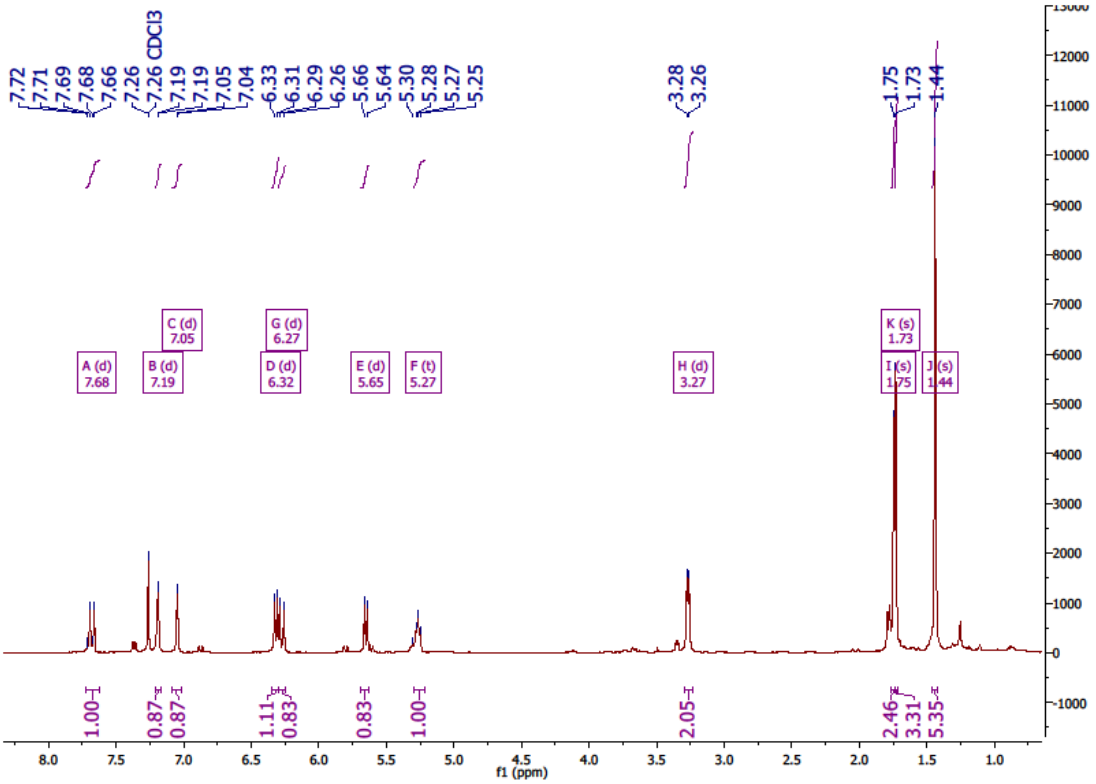
Apêndice 6.1.11. Espectro de RMN ^{1}H do composto artepelinC em $CDCl_3$ (500 MHz).



Apêndice 6.1.12. Espectro de RMN ^{1}H do composto bacarina em $CDCl_3$ (500 MHz).



Apêndice 6.1.13. Espectro de RMN ^{1}H do composto 2,2-Dimetil-6-carboxietenil-2H-1-benzopirano em $CDCl_3$ (500 MHz).


7.72
7.71
7.69
7.68
7.66
7.26 CDCl3
7.26
7.19
7.19
7.05
7.04
6.33
6.31
6.29
6.26
5.66
5.64
5.30
5.28
5.27
5.25
3.28
3.26
1.75
1.73
1.44
A (d) 7.68
B (d) 7.19
C (d) 7.05
D (d) 6.32
E (d) 5.65
F (t) 5.27
G (d) 6.27
H (d) 3.27
I (s) 1.75
J (s) 1.44
K (s) 1.73
1.00
0.87
0.87
1.11
0.83
0.83
1.00
2.05
2.46
3.31
5.35
f1 (ppm)

6.2. CAPÍTULO 2 – Desenvolvimento e validação de método analítico por Cromatografia gasosa para análises se substâncias voláteis presentes na própolis marrom do sudeste brasileiro e avaliação das propriedades antimicrobianas e leishimanicida.

Artigo 2. RIBEIRO VP, ARRUDA C, MEJÍA JAA, CANDIDO ACBB, SANTOS RA, MAGALHÃES LG, BASTOS JK. Brazilian southeast brown propolis: gas chromatography method development for its volatile oil analysis, its antimicrobial and leishmanicidal activities evaluation. Phytochemical Analysis. 2021; 32: 404– 411

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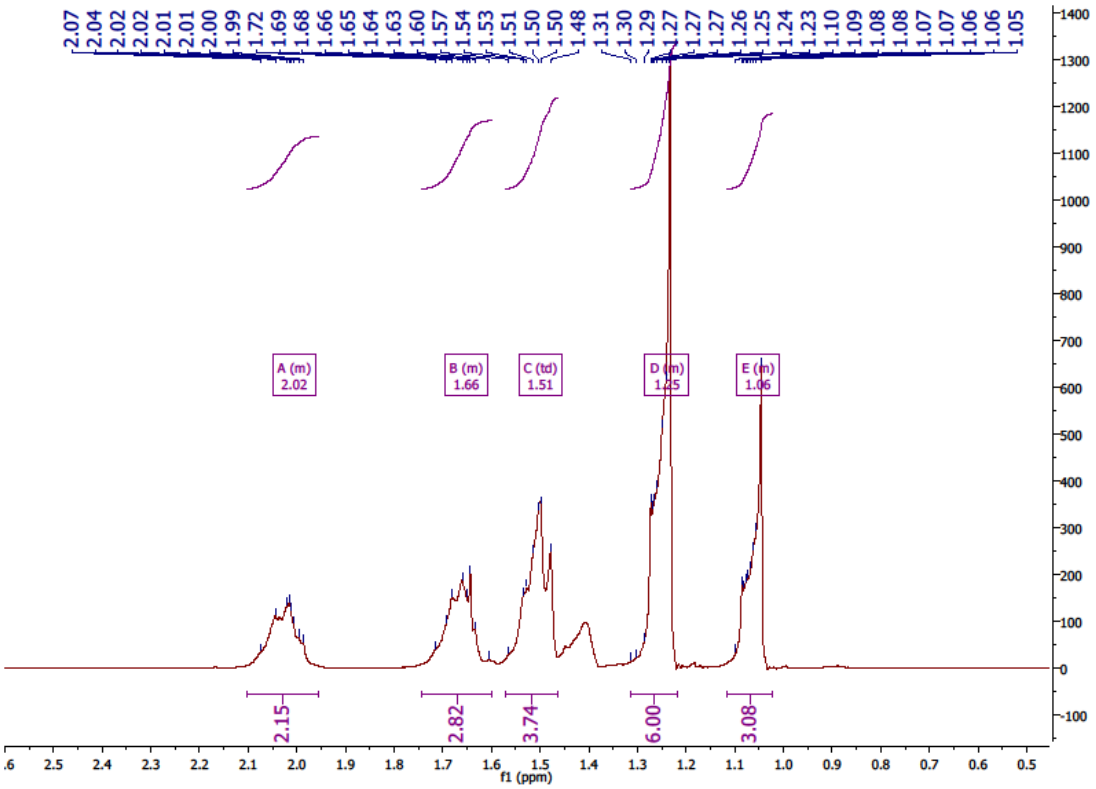
Tabela 6.2. Dados experimentais dos espetros de ^{1}H e ^{13}C para os compostos isolados da fração volátil da Própolis marrom

	1,8-cineol		Terpinen-4-ol		α-copaeno		β-cariofileno	
Posição	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	-	69,8	-	71,7	1,43 (*m*)	44,7	1,67 (*m*)	53,6
2	1,52-2,04 (*m*)	31,5	2,12 (*m*)	36,7	1,72, 2,10 (*m*)	30	1,49, 1,55 (*m*)	28,4
3	1,52-2,04 (*m*)	22,8	5,33 (*m*)	118,4	5,21 (*m*)	116,1	1,91, 2,0 (*m*)	40
4	1,43 (*m*)	32,9	-	133,8	-	143	-	135,5
5	1,52-2,04 (*m*)	22,8	1,94 (*m*)	27	2,18 (*m*)	54,2	5,3 (*m*)	124,3
6	1,52-2,04 (*m*)	31,5	1,60 (*m*)	30,8	1,49 (*m*)	36,2	2,09, 2,30 (*m*)	29,4
7	1,07 (*sl*)	27,5	1,50 (*m*)	34,6	1,27 (*m*)	44,3	2,06, 2,19 (*m*)	34,8
8	-	76,6	0,96 (*s*)	16,8	0,97, 1,58 (*m*)	23	-	154,7
9	1,26 (*sl*)	28,9	0,96 (*s*)	16,8	0,96, 1,51 (*m*)	36,9	2,34 (*m*)	48,5
10	1,26 (*sl*)	28,9	1,71 (*s*)	23,8	-	39,1	1,57, 1,65 (*m*)	40,4
11					0,84 (*s*)	21,8	-	33
12					1,73 (*m*)	32,2	0,99 (*s*)	22,6
13					0,79 (*s*)	19,6	0,96 (*s*)	30,1
14					0,79 (*s*)	19,6	1,64 (*s*)	16,32
15					1,59 (*s*)	19,9	4,81, 4,93 (*s*)	111,6

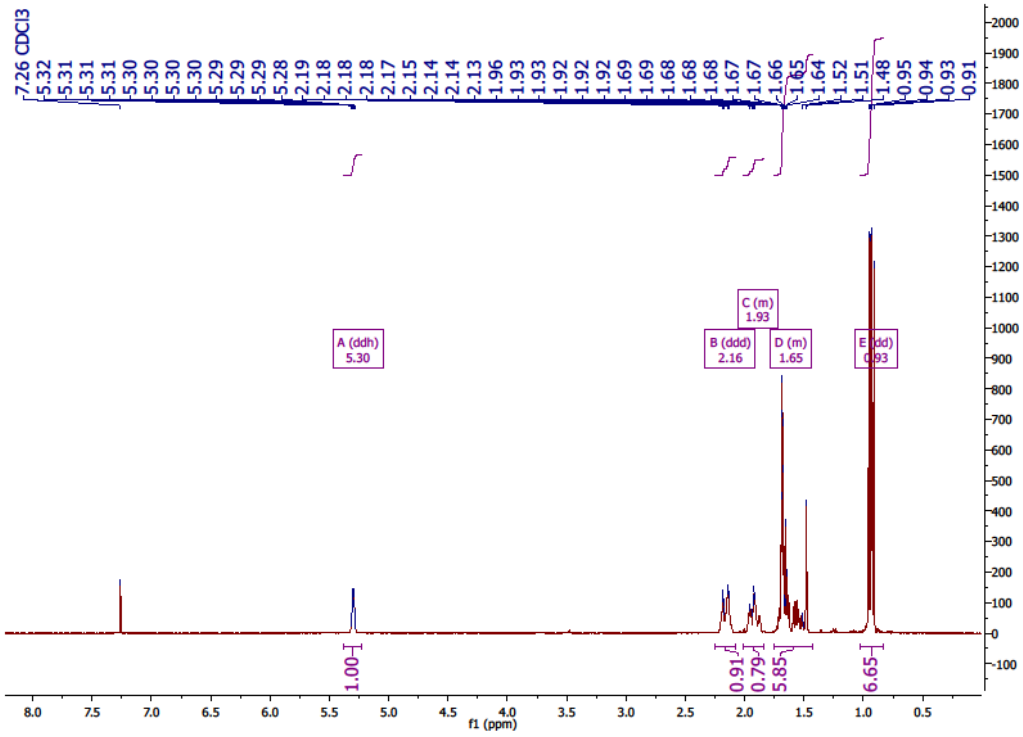
	Germacreno D		Nerolidol		Spatulenol		γ-Palmitolactona	
Posição	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
1	5,24 (*dd*)	121,4	5,08-5,01 (*m*)	111,6	2,38 (*dd*)	53,4	-	172,1
2	1,95-2,09 (*m*)	25,6	5,8 (*dd*)	144,7	1,99-1,68 (*m*)	26,6	3,41-3,58 (*m*)	30,9
3	1,95-2,09 (*m*)	39,7	-	70,6	1,53-1,77 (*m*)	41,7	1,94-2,26 (*m*)	28
4	-	145	1,38-1,60 (*m*)	41,4	-	80,9	5,27 (*m*)	76,18
5	5,96 (*dd*)	124,2	2,05 (*m*)	22,6	1,53 (*m*)	54,3	1,55 (*m*)	32,6
6	5,16 (*m*)	131,4	5,21 (*m*)	124,1	0,83 (*m*)	29,9	1,18 (*sl*)	23,7
7	2,15 (*m*)	73,5	-	133,5	0,91 (*m*)	27,4	1,18 (*sl*)	28,6
8	1,55-1,59(*m*)	26,6	1,89 (*m*)	26,4	1,53-1,52 (*m*)	24,7	1,18 (*sl*)	28,6
9	1,95-2,09 (*m*)	42,08	2,19 (*m*)	123,9	2,38 (*dd*)	38,8	1,18 (*sl*)	28,6
10	-	135,5	5,27 (*m*)	130,5	-	153,4	1,18 (*sl*)	28,6
11	1,95-2,09 (*m*)	27,8	-	25,5	-	20,1	1,18 (*sl*)	28,6
12	1,28 (*s*)	22	1,54 (*s*)	16,8	1,14 (*s*)	28,6	1,18 (*sl*)	28,6
13	1,28 (*s*)	19,6	1,54 (*s*)	15,8	1,14 (*s*)	16,3	1,18 (*sl*)	28,3
14	1,60 (*s*)	16	1,6 (*s*)	27,3	1,32 (*s*)	28,7	1,18 (*sl*)	28,7
15	5,10(d)/5,07(*d*)	111,6	1,38 (*s*)		4,80-5,0 (*d*)	106,2	1,18 (*sl*)	21,6
16							0,80 (*sl*)	13

(*s*) simpleto, (*d*) dupleto, (*dd*) duplo dupleto, (*m*) multipleto e (*sl*) sinpleto largo.

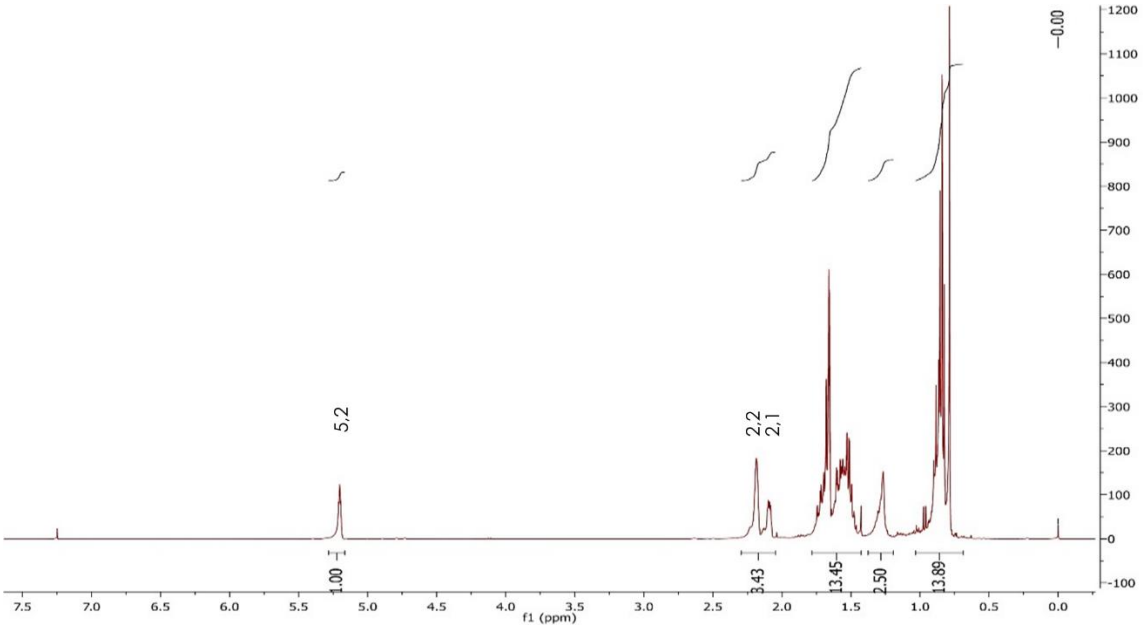
Apêndice 6.2.1. Espectro de RMN ^{1}H do composto 1,8-cineol em $CDCl_3$ (500 MHz).



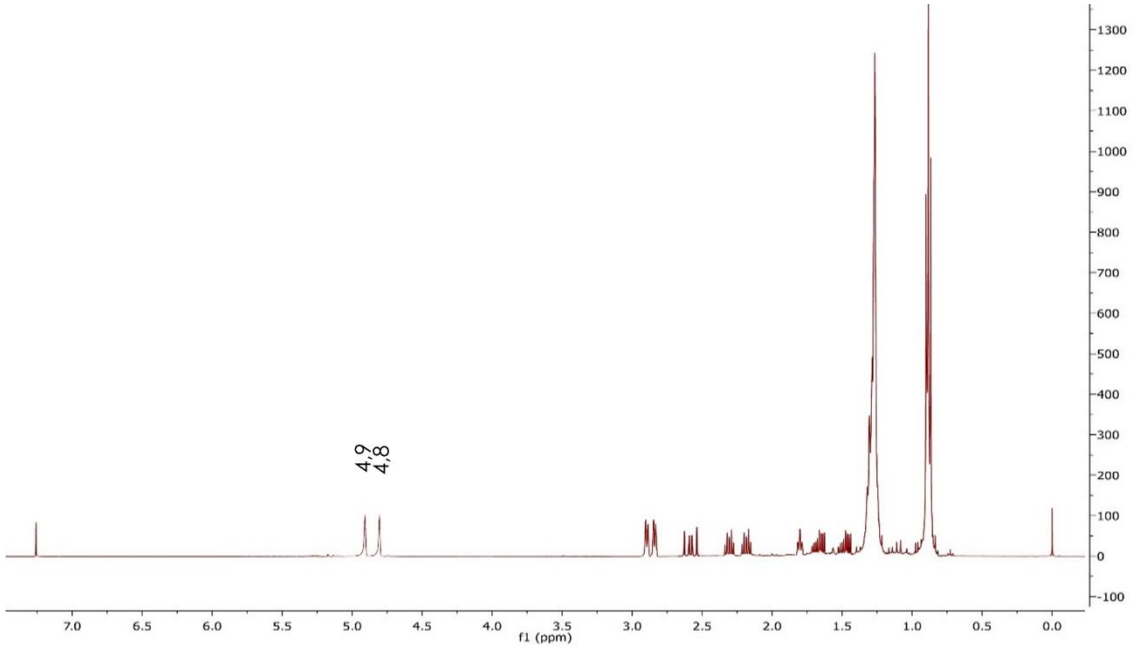
Apêndice 6.2.2. Espectro de RMN ^{1}H do composto Terpinen-4-ol em $CDCl_3$ (500 MHz).



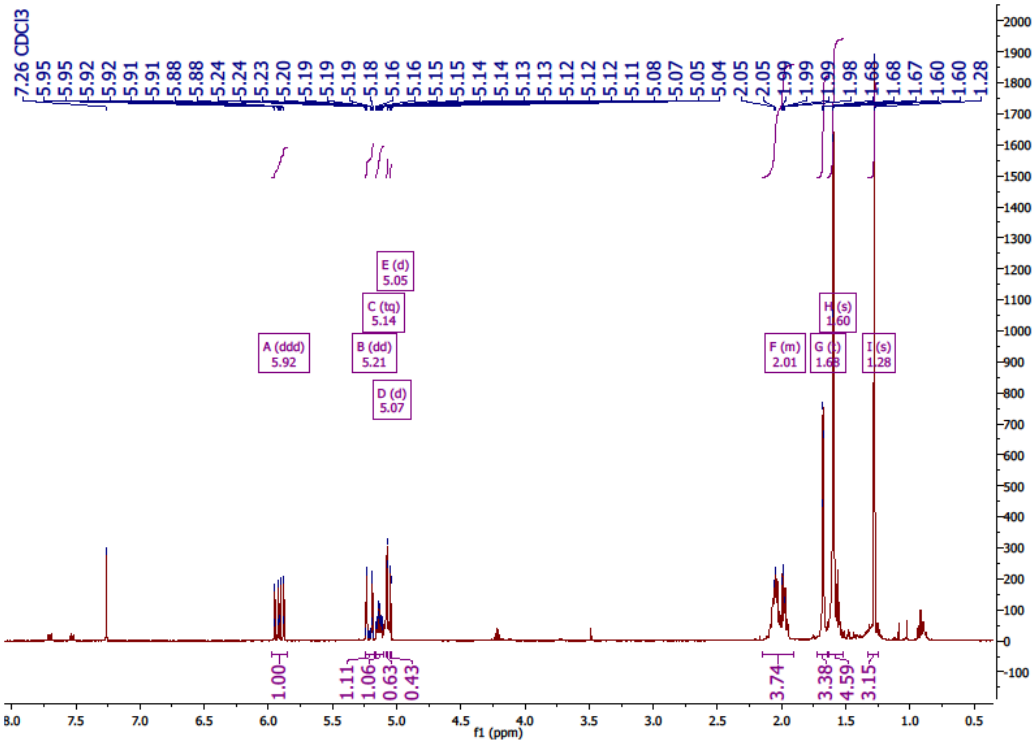
Apêndice 6.2.3. Espectro de RMN ^{1}H do composto α-copaeno em $CDCl_3$ (500 MHz).



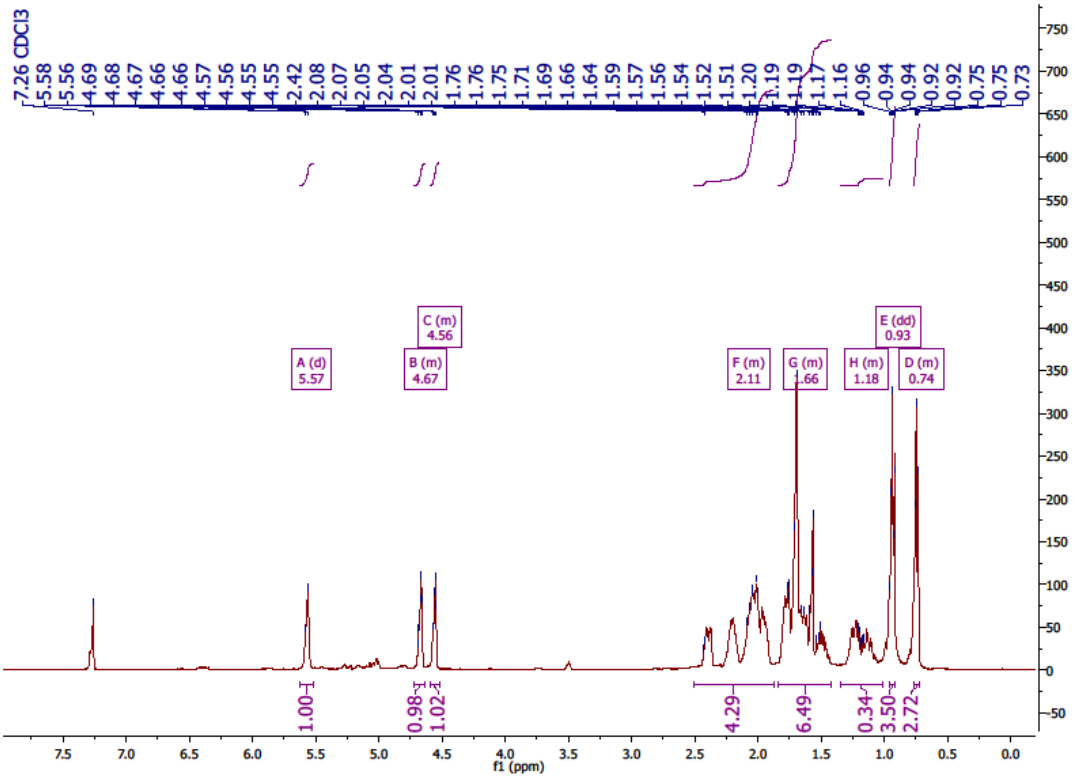
Apêndice 6.2.4. Espectro de RMN ^{1}H do composto β-cariofileno em $CDCl_3$ (500 MHz).



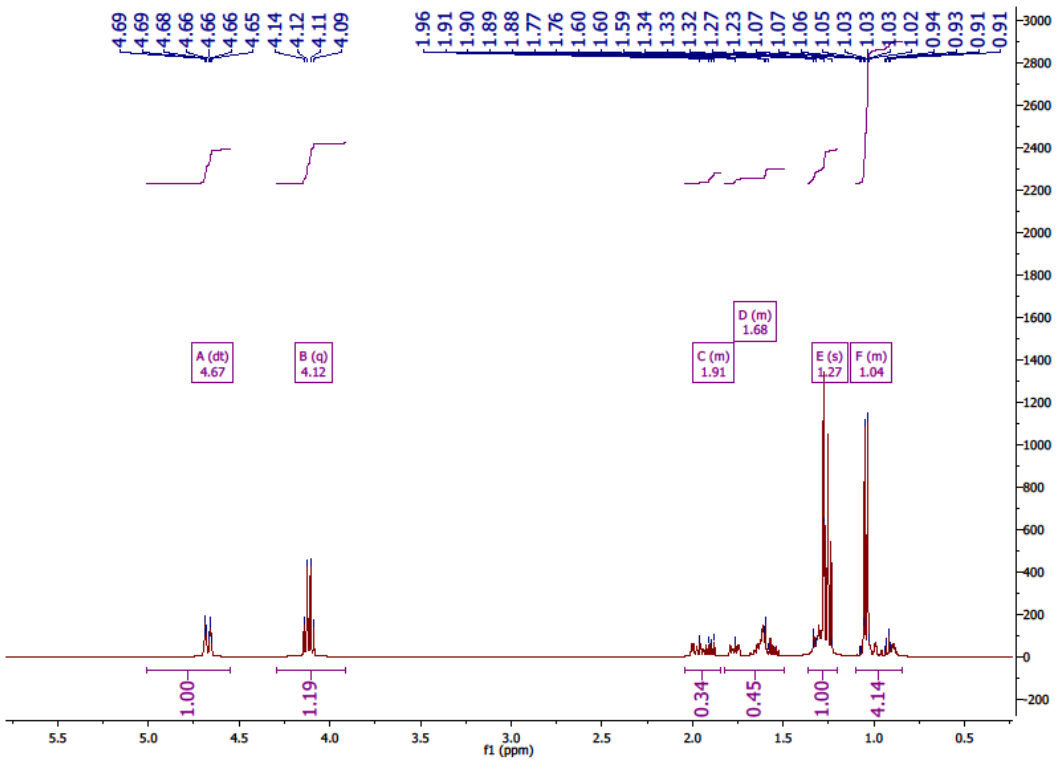
Apêndice 6.2.5. Espectro de RMN ^{1}H do composto Germacreno D em $CDCl_3$ (500 MHz).



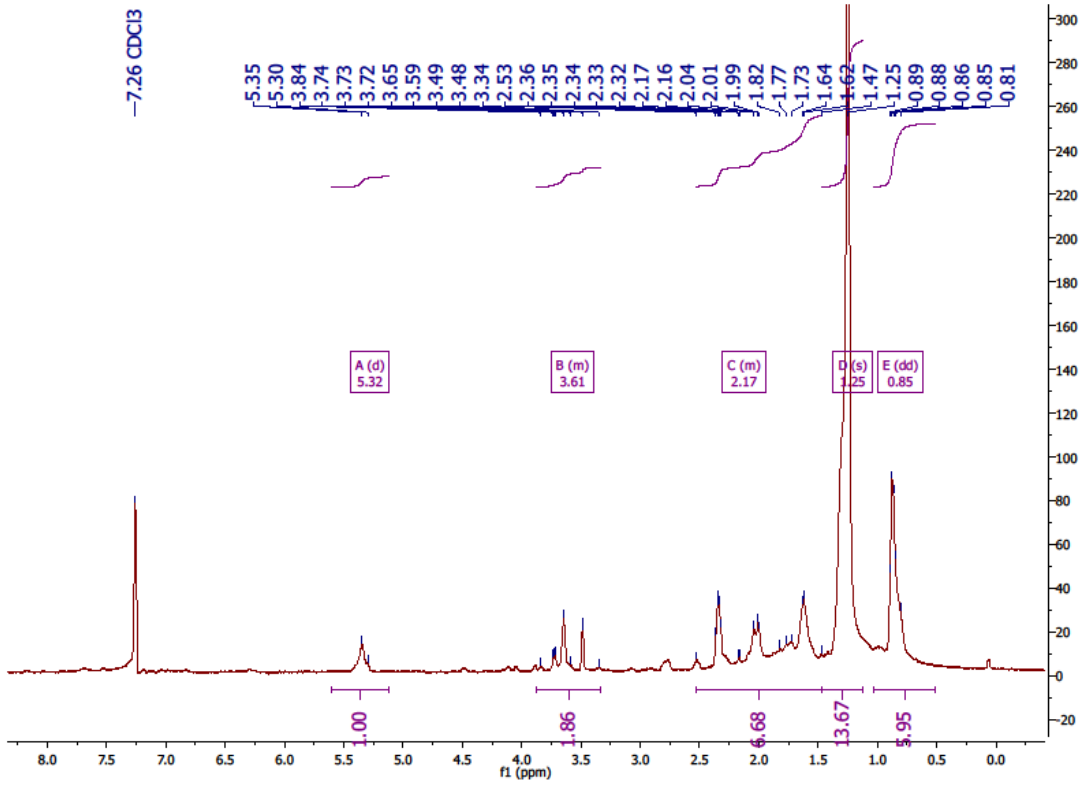
Apêndice 6.2.6. Espectro de RMN ^{1}H do composto nerolidol em $CDCl_3$ (500 MHz).



Apêndice 6.2.7. Espectro de RMN ^{1}H do composto espatulenol em $CDCl_3$ (500 MHz).



Apêndice 6.2.8. Espectro de RMN ^{1}H do composto γ-Palmitolactona em $CDCl_3$ (500 MHz).



6.3. CAPÍTULO 3 – Avaliação *In vivo* das atividades anti-inflamatória e antinociceptiva da própolis marrom do sudeste brasileiro.

Artigo 3. RIBEIRO VP, SÍMARO GV, MEJIA JAA, ARRUDA C, BASTOS JK. Anti-inflammatory and Antinociceptive Activities of the Hydroalcoholic Extract and the Volatile Fraction of Southeastern Brazilian Brown Propolis. Brazilian Journal of Pharmacognosy. 2021; 31: 59–66

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Faculdade de Ciências Farmacêuticas de Ribeirão Preto
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Colaborador: Guilherme Venâncio Símaro.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da Autorização	01/03/2019 a 01/05/2020
Espécie/Linhagem/Raça	Camundongo isogênico Balb/C
Nº de animais	80
Peso/Idade	15-21 g / 4 semanas
Sexo	Macho
Origem	Biotério Central do Campus USP de Ribeirão Preto

Ribeirão Preto, 28 de outubro de 2021.

Danielle Palma de Oliveira

Danielle Palma de Oliveira
Coordenadora da CEUA-FCFRP

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6.4. CAPÍTULO 4 – Estudo fitoquímico de uma amostra de própolis marrom do sudeste brasileiro e avaliação das atividades antiplasmódica, citotóxica e antimicrobiana.moagem das folhas de C. langsdorffii.

Artigo 4. RIBEIRO VP, ARRUDA C, ALDANA-MEJIA JA, BASTOS JK, TRIPATHI SK, KHAN SI, KHAN IA, ALI Z. Phytochemical, Antiplasmodial, Cytotoxic and Antimicrobial Evaluation of a Southeast Brazilian Brown Propolis Produced by *Apis mellifera* Bees. Chemistry & Biodiversity. 2021; 18, e2100288

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Tabela 6.4. Dados experimentais dos espectros ^{1}H e ^{13}C para os compostos isolados do extrato bruto de própolis marrom.

	Ferulic acid		Cafeic acid		Methoxycinnamic acid		Dimethoxycinnamic acid	
Posição	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**
1	-	126,6	-	128,3	-	133	-	156.0
2	7,06 (*d*, *J* = 8.2 Hz, 1H)	123,5	7,04 (*d*, *J* = 2.0 Hz, 1H)	112,7	7,43 (*m*, 1H)	126,3	6,92 (*dd*, *J* = 8.2, 1.9 Hz, 1H)	128.5
3	-	114,3	-	147,2	6,28 (*m*, 1H)	118,5	-	133.0
4	-	148,3	-	149,8	-	159,1	-	157.5
5	6,81 (*dd*, *J* = 8.2, 2.3 Hz, 1H)	147	6,78 (*d*, *J* = 8.2 Hz, 1H)	117	6,28 (*m*, 1H)	118,5	6,77 (*d*, *J* = 8.1 Hz, 1H)	128.5
6	7,18 (*s*, 1H)	109,4	6,93 (*dd*, *J* = 8.2, 2.0 Hz, 1H)	123,4	7,43 (*m*, 1H)	126,3	7,03 (*d*, *J* = 2.0 Hz, 1H)	116.3
7	7,60 (*d*, *J* = 15.9 Hz, 1H)	146,8	7,53 (*d*, *J* = 15.9 Hz, 1H)	147,6	7,60 (*d*, J = 15.9 Hz, 1H)	143,5	7,49 (*d*, *J* = 15.9 Hz, 1H)	143,9
8	6,34 (*d*, *J* = 15.8 Hz, 1H)	114,7	6,22 (*d*, *J* = 15.8 Hz, 1H)	116	6,28 (*d*, J = 15.9 Hz, 1H)	115,6	6,25 (*d*, *J* = 16.0 Hz, 1H)	116.5
9	-	171,3	-	171,6	-	167,5	-	167.5
10	3,98 (*s*, 3H)	55,9	-	-	3,88 (*s*, 3H)	55,9	3,98 (*s*, 3H)	58.6
11							3,98 (*s*, 3H)	58.0
	19-Acetoxy-13-hydroxylabda-8(17),14-diene		**Totarol**		**7-Oxodehydroabietic acid**		**Dehydroabietic acid**	
Posição	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**
1	5.23-5.24 (*dd*, *J* = 17.4, 1.6 Hz, 1H)	115.7	0.99 (*s*, 3H)	22.1	1.29 (*s*, 3H)	23.4	-	37.9
2	5.89 (*dd*, *J* = 17.4, 10.8 Hz, 1H)	144.3	0.99 (*s*, 3H)	22.1	3.12 (*p*, *J* = 6.9 Hz, 1H)	36.3	-	18.5
3	-	73.5	-	32.6	1.29 (*s*, 3H)	23.4	-	36.9
4	1.41 (*s*, 3H)	28.6	1.56-1.31 (*m*, 1H)	42.0	-	145.7	-	47.4
5	1.44 (*m*, 2H)	42.4	1.53-1.43 (*m*, 1H)	18.2	7.23 (*d*, *J* = 8.2 Hz, 1H)	130.3	-	44.6
6	1.29 (*m*, 2H)	19.7	2.00-1.75 (*m*, 1H)	38.7	7.29 (*dd*, *J* = 8.2, 2.2 Hz, 1H)	126.2	-	21.8
7	2.14 (*m*, 1H)	56.4	-	38.2	-	149.0	-	30.0
8	-	148.1	-	143.6	-	37.4	-	134.7
9	4.88-4.63 (*m*, 2H)	109.1	6.57 (*d*, *J* = 7.9 Hz, 1H)	124.9	1.44 (*s*, 3H)	24.5	-	146.6
10	2.01-1.91 (*m*, 2H)	36.4	6.32 (*d*, *J* = 8.2 Hz, 1H)	112.5	1.82-1.57 (*m*, 2H)	38.1	-	36.9
11	1.41-1.16 (*m*, 2H)	21.9	-	151.8	1.49-1.39 (*m*, 2H)	17.9	7.17 (*d*, *J* = 8.2 Hz, 1H)	124.1
12	1.99 (*m*, 1H)	58.1	-	130.9	1.75-1.50 (*m*, 2H)	37.1	7.00 (*dd*, *J* = 8.1, 2.0 Hz, 1H)	123.9
13	-	85.1	-	133.8	-	45.8	-	145.7
14	1.5 (*s*, 3H)	22.5	2.9-2.8 (*m*, 1H)	28.4	1.29 (*s*, 3H)	20.5	6.89 (*d*, *J* = 1.4 Hz, 1H)	126.9
15	-	170.3	1.69-1.44 (*m*, 1H)	19.2	-	181.0	2.82 (*m*, 1H)	33.5
16	2.61 (*s*, 3H)	21.3	1.55 (*m*, 1H)	50.0	2.65 (*m*, 1H)	50.2	1.21 (*d*, *J* = 1.7 Hz, 1H)	24.0
17	1.76-1.51 (*m*, 2H)	39.0	2.87 (*m*, 1H)	27.2	2.62-2.38 (*m*, 1H)	35.7	1.21 (*d*, *J* = 1.7 Hz, 1H)	24.0
18	1.49-1.39 (*m*, 2H)	15.5	1.20 (*s*, 3H)	21.2		196.9	-	184.7
19	1.49-1.24 (*m*, 2H)	39.1	1.20 (*s*, 3H)	21.2	-	132.1	1.23 (*s*, 3H)	25.1

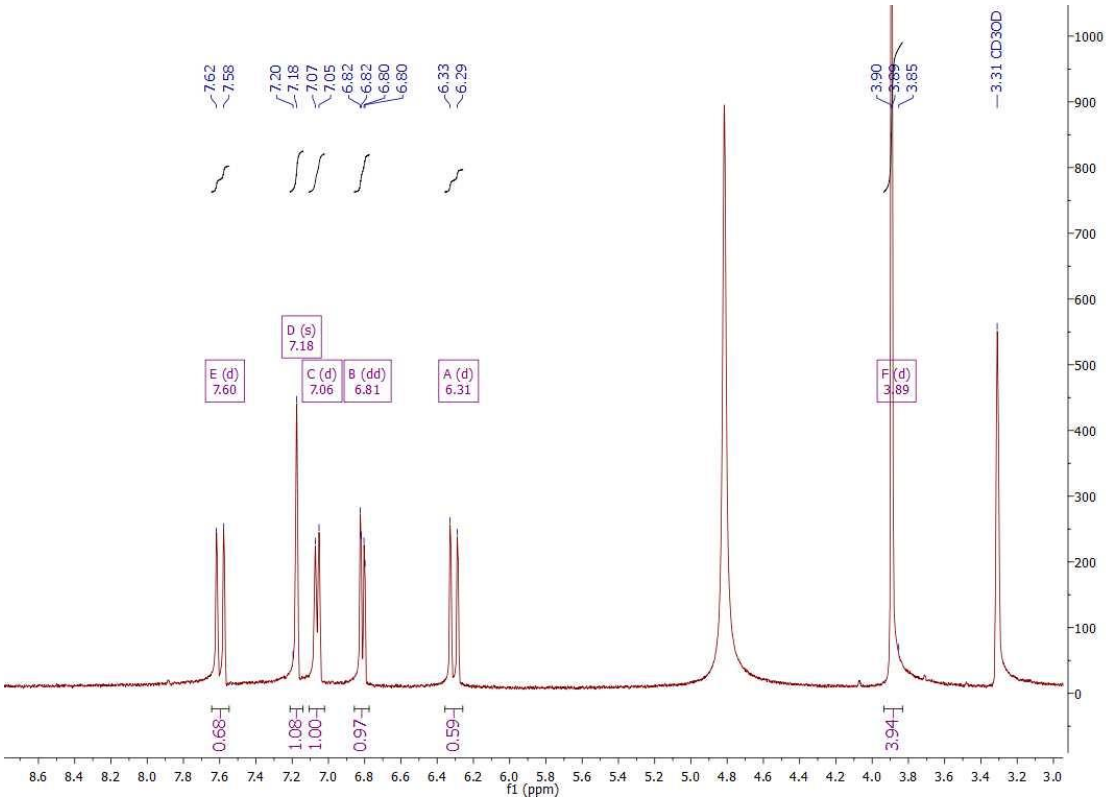
20	-	36.0	1.45 (*s*, 3H)	25.0	7.68 (*d*, *J* = 2.2 Hz, 1H)	125.9	1.23 (*s*, 3H)	16.2

	3-Hydroxy-4-methoxybenzaldehyde		**3-Methoxy-4-hydroxypropiophenone**		**Pinoresinol**		**7-O-Methyl Aromadendrin**	
Posição	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**
1	9.73 (*s*, 1H)	191.9	-	10.7	-	135.2	5.03 (*d*, *J* = 11.6 Hz, 1H)	83.8
2	-	128.2	-	191.9	6.95 (*d*, *J* = 1.6 Hz, 1H)	112.4	4.58 (*d*, *J* = 11.6 Hz, 1H)	72.0
3	7.43 (*d*, *J* = 1.4 Hz, 1H)	124.4	6.95 – 6.90 (*m*, 1H)	128.2	-	150.5	-	196.9
4	6.96 – 6.90 (*m*, 1H)	113.9	7.59 (*d*, *J* = 2.0 Hz, 1H)	124.4	-	148.9	-	102.6
5	-	158.2	-	113.9	6.77 (*d*, *J* = 8.1 Hz, 1H)	117.5	-	159.3
6	-	143.8	-	158.2	6.81 (*dd*, *J* = 8.1, 1.6 Hz, 1H)	121.1	6.08 (*dd*, *J* = 16.6, 2.3 Hz, 2H)	93.5
7	7.41 (*d*, *J* = 1.9 Hz, 1H)	112.7	7.57 (*d*, *J* = 2.0 Hz, 1H)	143.8	4.71 (*d*, *J* = 4.6 Hz, 1H)	8.9	-	162.3
8	3.91 (*s*, 3H)	59.4	3.92 (*s*, 3H)	112.7	3.11-3.17	56.6	6.89 – 6.82 (*m*, 2H)	93.0
9			3.00 (*q*, *J* = 7.3 Hz, 2H)	59.4	3.84 (*dd*, *J* = 9.5, 3.7 Hz, 1H)	73.9	-	167.1
10			1.18 (*t*, *J* = 7.3 Hz, 3H)	33.7	4.23 (*dd*, *J* = 6.7, 9.4 Hz, 1H)	73.9	-	133.3
11					3.85 (*s*, 3H)	57.8	7.38 (*d*, *J* = 8.4 Hz, 1H)	128.6
12							7.13 (*d*, *J* = 21.5 Hz, 1H)	116.1
13							-	157.4
14							7.13 (*d*, *J* = 21.5 Hz, 1H)	116.1
15							7.38 (*d*, *J* = 8.4 Hz, 1H)	128.6
16							3.83 (*s*, 3H)	55.9

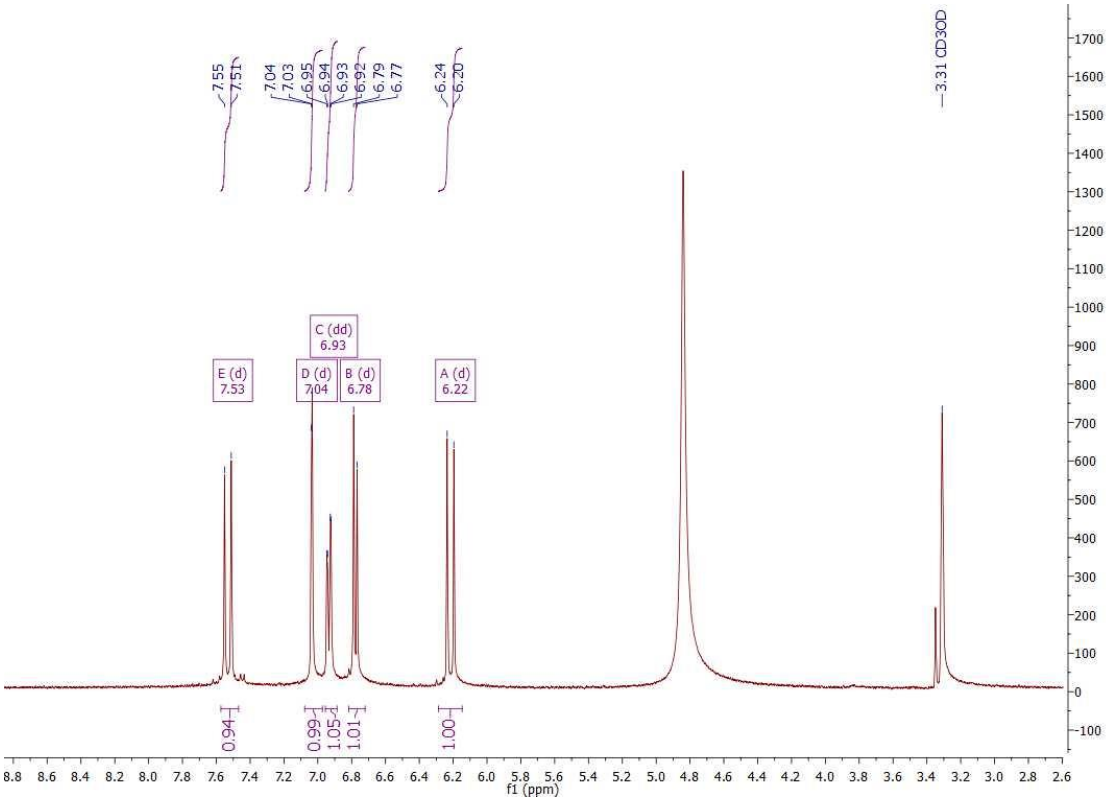
	Communic Acid		**Isopimaric acid**		**1-O,2-O-digalloyl-6-O-trans-p-coumaroyl-β-dglicopiranoside**		**Matairesinol**	
Posição	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**	**δ 1H (ppm)**	**δ 13C (ppm)**
1	5.00-4.81 (*d*, *J* = 17.4 Hz, 1H)	113.1	1.53-1.43 (*m*, 2H)	18.7	5,88 (*d*, *J* = 8,6 Hz)	94.2	-	132.2
2	6.25 (*dd*, *J* = 17.3, 10.7 Hz, 1H)	141.1	1.56-1.31 (*m*, 2H)	39.1	5,17 (*t*, *J* = 8,6 Hz)	74.1	6.66 (*d*, *J* = 1.9 Hz, 1H)	115.1
3	-	133.8	-	35.2	3,82 (*t*, *J* = 9,2 Hz)	76.7	-	150.7
4	5.33 (*t*, *J* = 6.6 Hz, 1H)	133.9	1.04 (*s*, 3H)	14.5	3,68 (*d*, *J* = 9,2 Hz)	71.4	-	147.7
5	1.71 (*s*, 3H)	12.6	1.93 (*m*, 1H)	52.1	3,82 (*m*, 1H)	75.9	6.69 (*d*, *J* = 8.1 Hz, 1H)	117.4
6	2.09-1.84 (*m*, 2H)	23.9	1.42-1.17 (*m*, 2H)	20.5	4,57 (*d*, *J* = 11,3 Hz)	64.3	6.57 (*dd*, *J* = 8.1, 1.9 Hz, 1H)	124.4
7	2.18 (*m*, 1H)	56.6	1.38-1.13 (*m*, 2H)	36.4	-	120.1	2.81 (*dd*, *J* = 14.1, 6.9 Hz, 1H)	36.6
8	-	148.0	-	37.0	7,05 (*s*, 1H)	110.7	2.87 (*dd*, *J* = 14.0, 5.5 Hz, 1H)	36.6
9	5.11-4.92 (*d*, *J* = 1.7 Hz, 1H)	108.1	1.30 (*s*, 3H)	23.4	-	146.6	2.65 (*ddd*, *J* = 8.1, 6.9, 5.5 Hz, 1H)	49.0
10	2.01-1.91 (*m*, 2H)	38.1	5.70 (*dd*, *J* = 17.5, 10.8 Hz,	150.1	-	140.7	-	183.2
11	1.42-1.17 (*m*, 2H)	25.1	5.07-5.02 (*dd*, *J* = 10.7, 1.4	112.6	-	166.6	3.78 (*s*, 3H)	57.7
12	1.72 (*m*, 1H)	56.0	2.04-1.79 (*m*, 2H)	46.3	-	121.4	-	133.0
13	-	44.0	-	135.6	7,11 (*s*, 1H)	110.3	-	114.5
14	1.33 (*s*, 3H)	28.0	5.37 (*dd*, *J* = 5.8, 2.3 Hz, 2H)	121.8	-	146.6	-	150.6
15	-	183.0	2.04-1.79 (*m*, 2H)	23.4	-	140.0	-	147.7
16	2.01-1.76 (*m*, 2H)	37.4	1.76 (*m*, 1H)	44.7	-	168.5	6.67 (*d*, *J* = 7.9 Hz, 1H)	117.6
17	1.53-1.43 (*m*, 2H)	18.7	-	46.8	-	127.2	6.50 (*dd*, *J* = 7.5, 1.8 Hz, 1H)	123.6
18	1.56-1.31 (*m*, 2H)	39.2	1.33 (*s*, 3H)	17.2	7,40 (*d*, J = 8,6 Hz)	131.4	2.48-2.56 (*m*, 2H)	40.2
19	-	39.3	-	183.0	6,77 (*d*, J = 8,6 Hz	116.9	2.45-2.52 (m, 1H)	43.8

20	0.64 (*s*, 3H)	14.6	2.01-1.76 (*m*, 2H)	37.5	-	161.4	3.91 (*dd*, *J* = 9.0, 8.1 Hz, 1H)	74.3
21					6,30 (*d*, J = 16,0 Hz)	114.7	4.15 (*dd*, *J* = 9.1, 7.4 Hz, 1H)	74.3
22					7,63 (*d*, J = 16,0 Hz)	147.5	3.76 (*s*, 3H)	57.7
23					-	168.4		

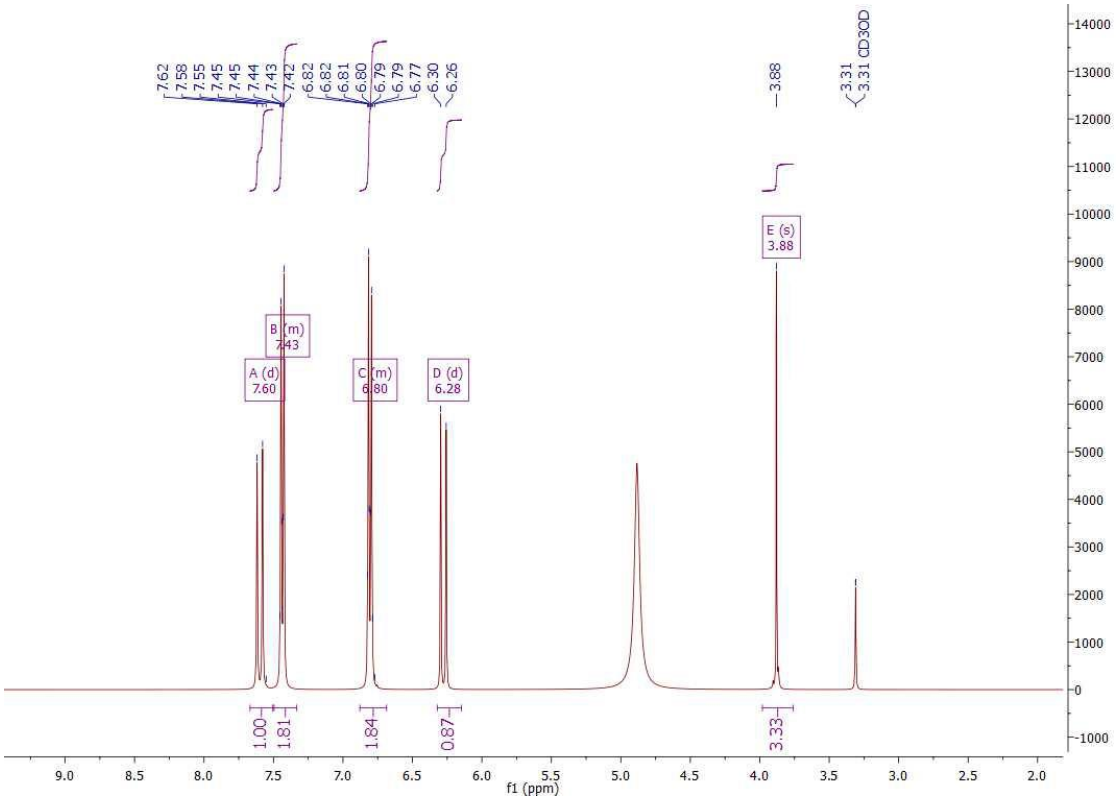
Apêndice 6.4.1. Espectro de ^{1}H NMR de ácido ferúlico em CD_3OD (400 MHz).



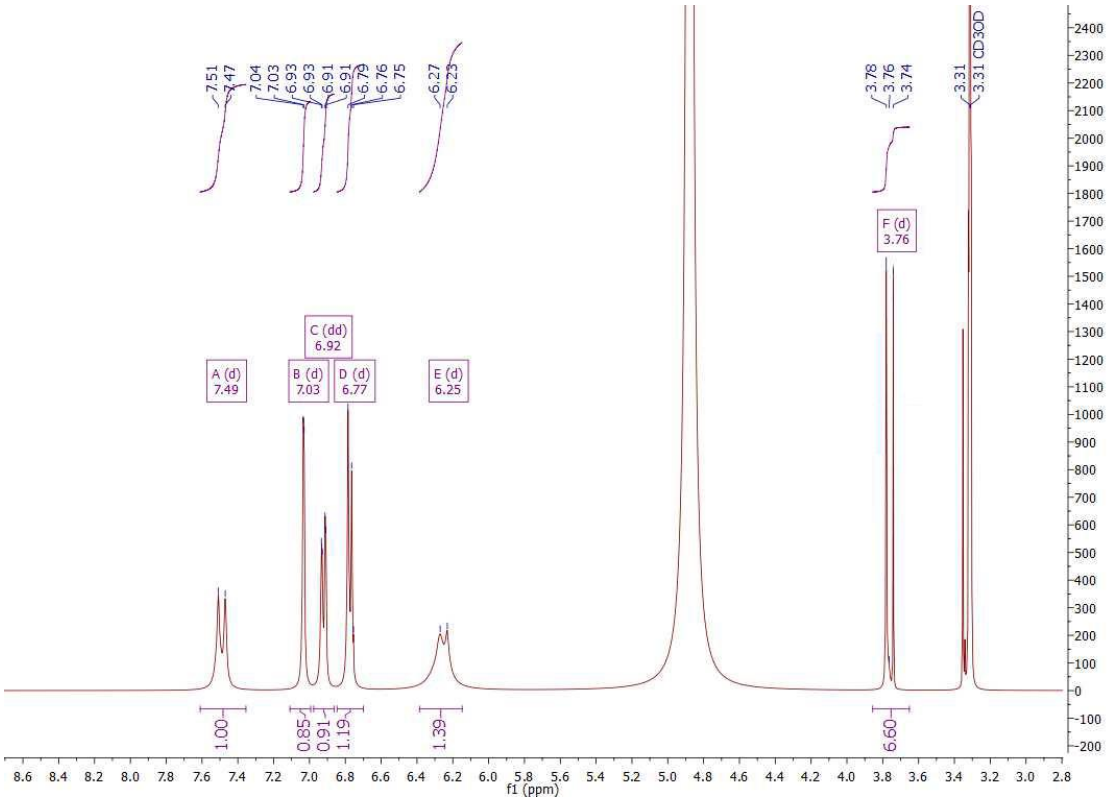
Apêndice 6.4.2. Espectro de ^{1}H NMR de ácido cafeico em CD_3OD (400 MHz).



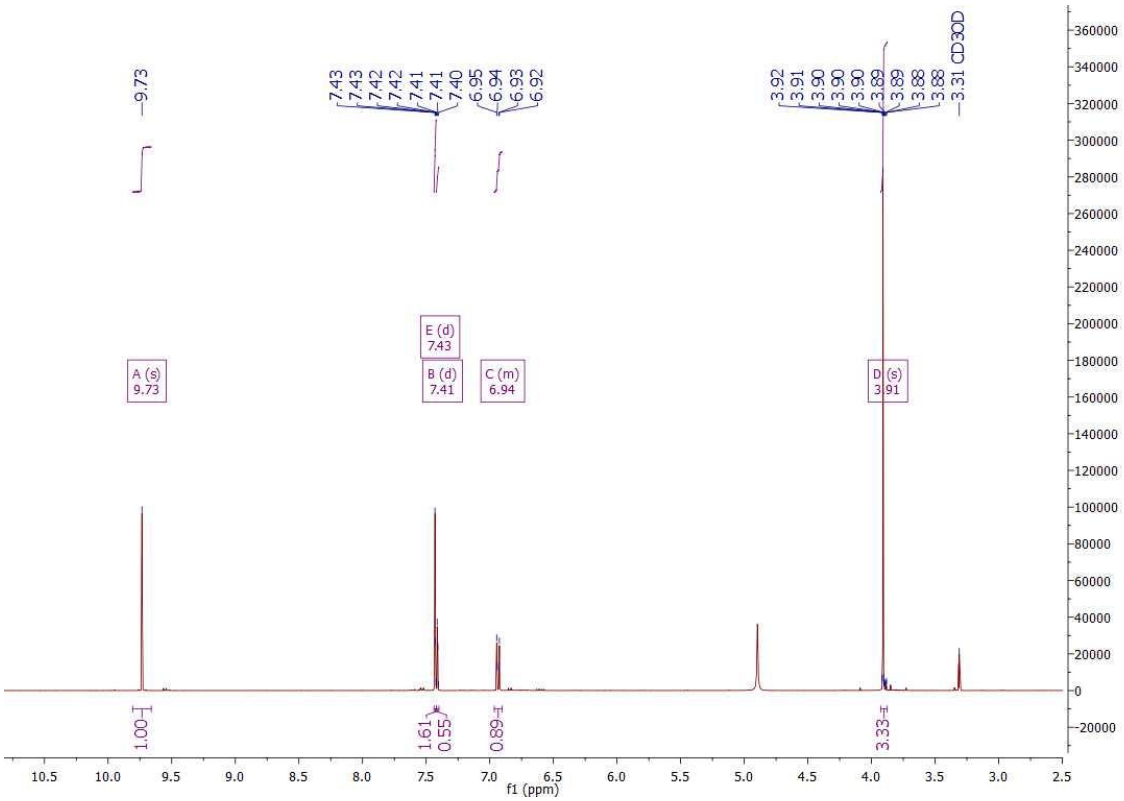
Apêndice 6.4.3. Espectro de ^{1}H NMR de ácido metoxicinâmico em CD_3OD (400 MHz).



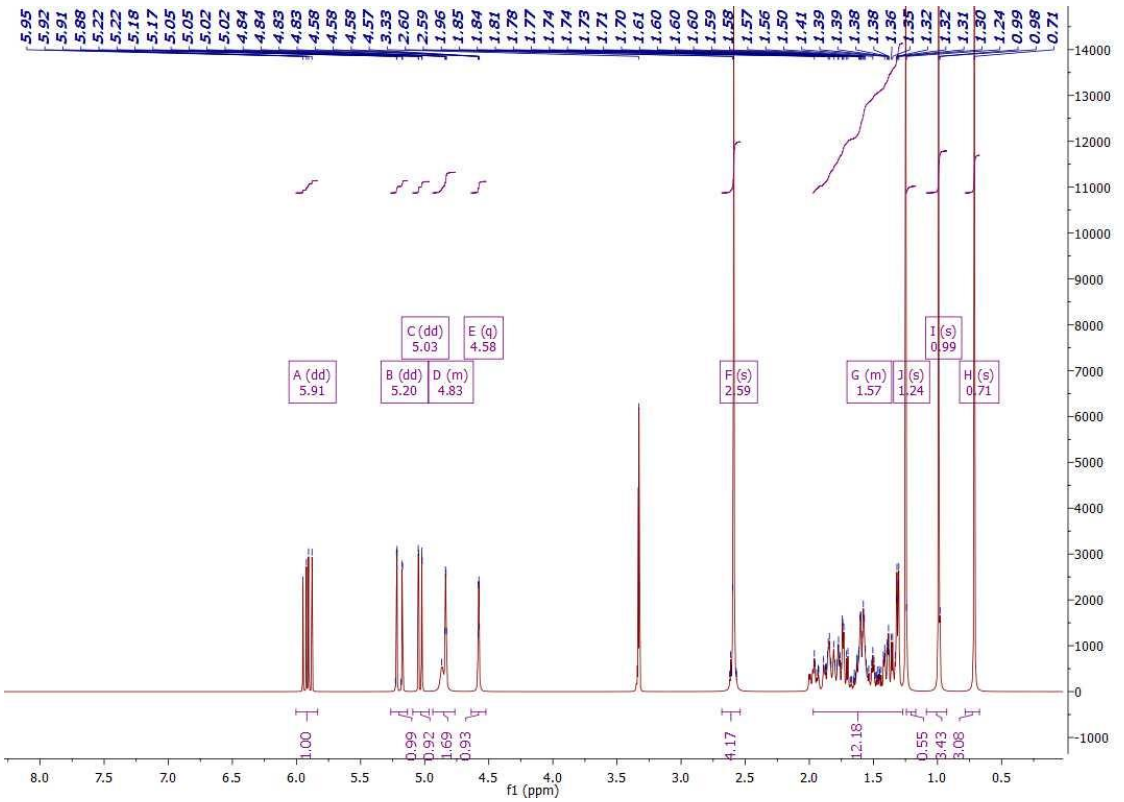
Apêndice 6.4.4. Espectro de ^{1}H NMR de ácido dimetoxicinâmico em CD_3OD (400 MHz).



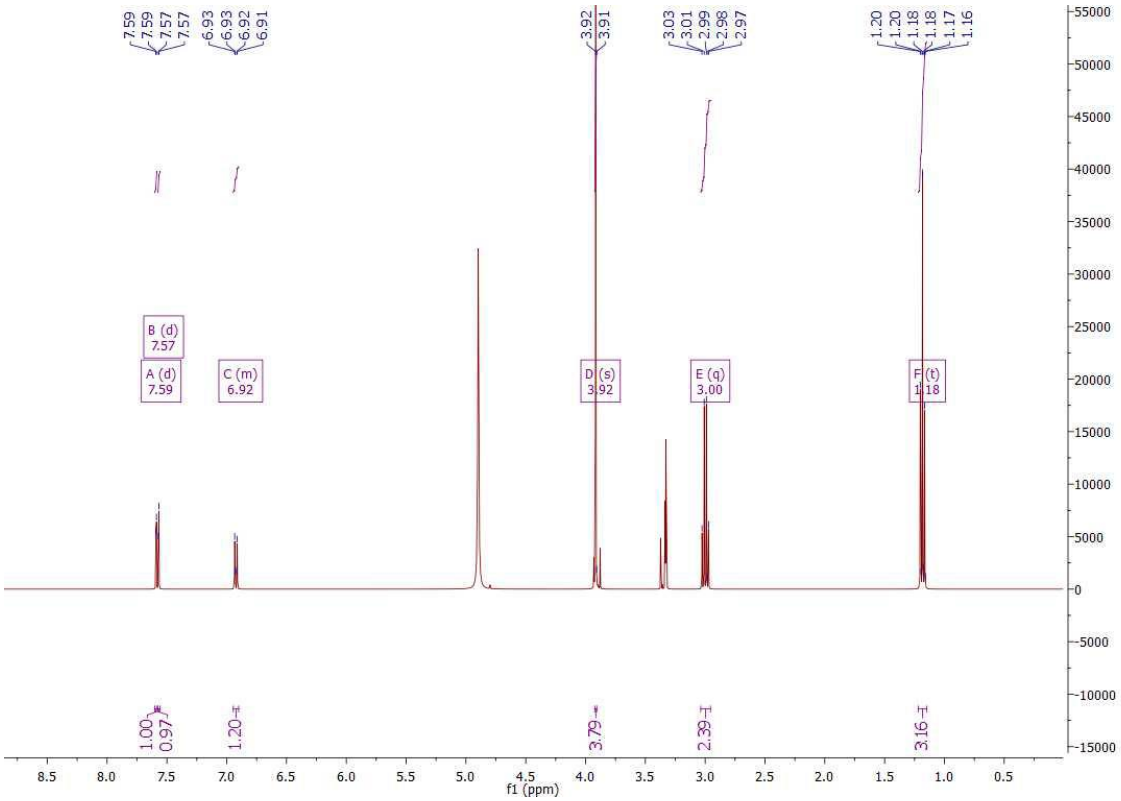
Apêndice 6.4.5. Espectro de ^{1}H NMR de 3-Hidroxi-4-metoxibenzaldeído em CD_3OD (400 MHz).



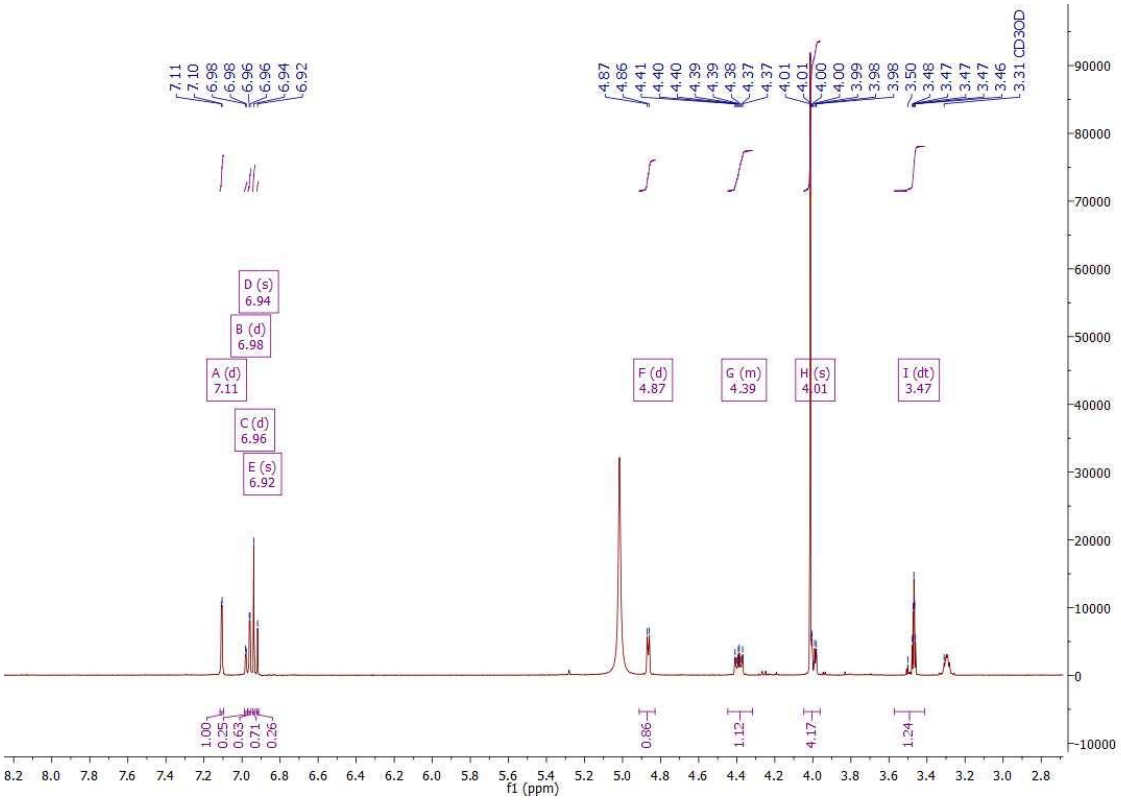
Apêndice 6.4.6. Espectro de ^{1}H NMR de 19-Acetoxi-13-hidroxilabda-8(17),14-dieno em CD_3OD (400 MHz).



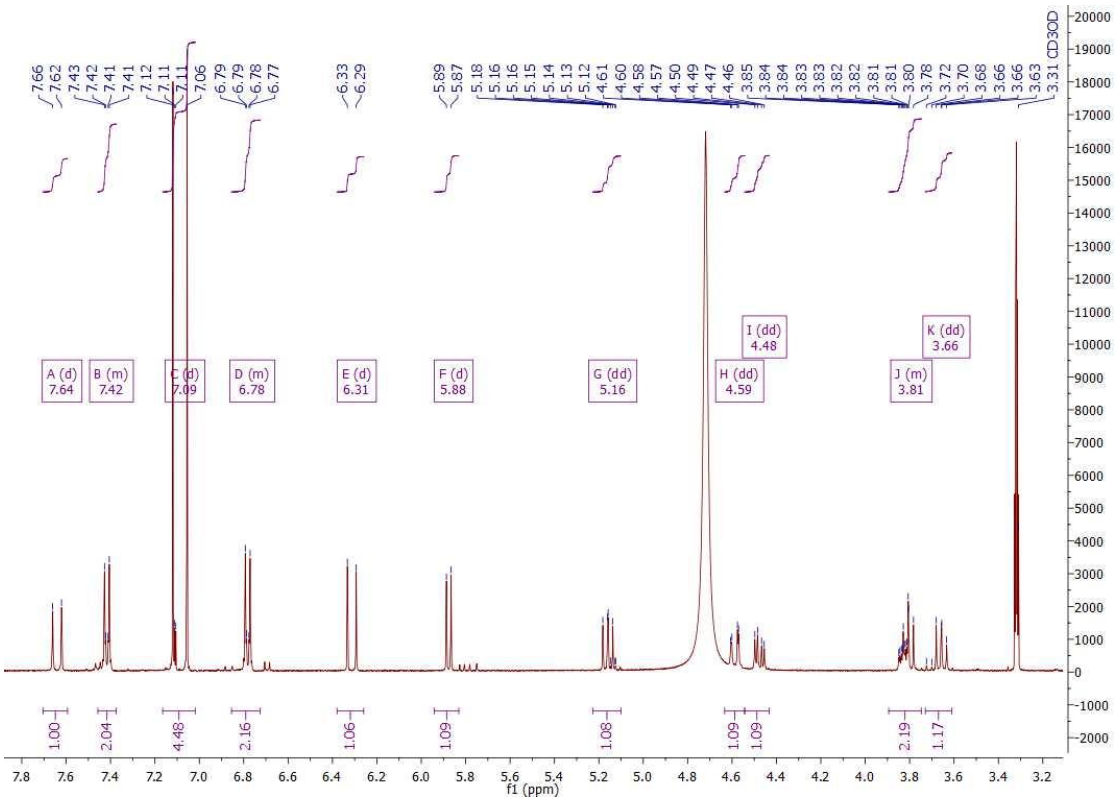
Apêndice 6.4.7. Espectro de ^{1}H NMR de 3-Metoxi-4-hidroxipropiofenona em CD_3OD (400 MHz).



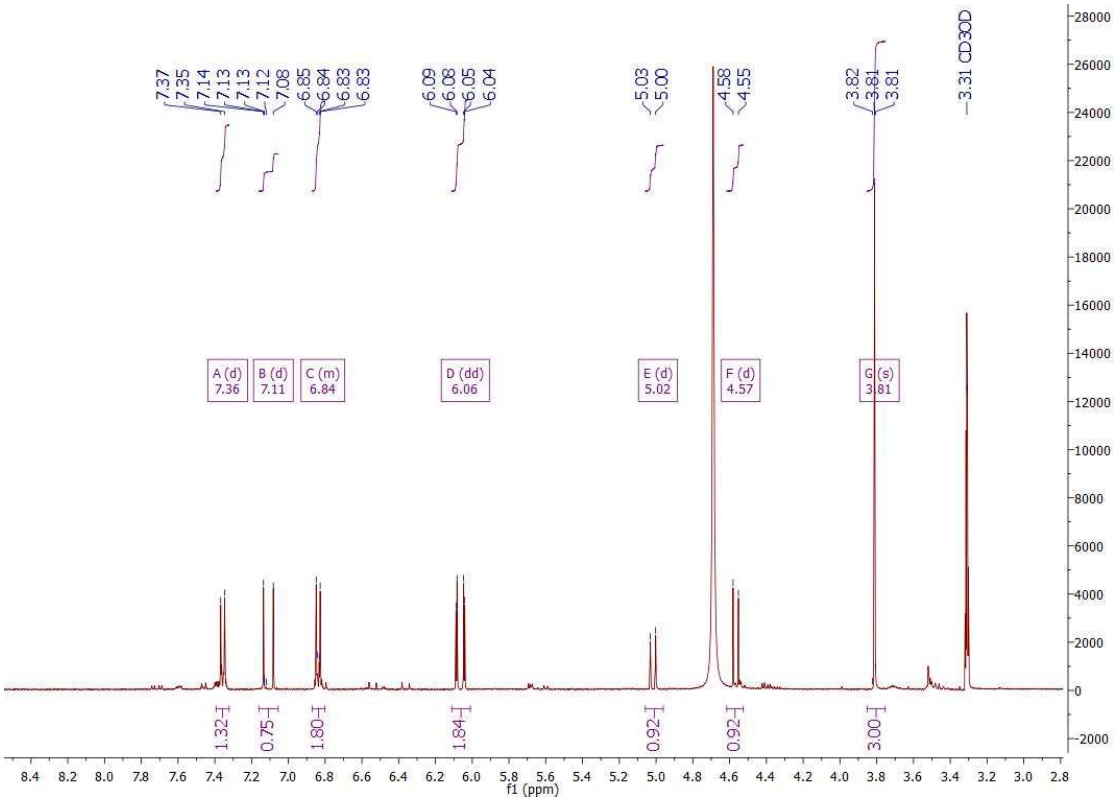
Apêndice 6.4.8. Espectro de ^{1}H NMR de Pinoresinol em CD_3OD (400 MHz).



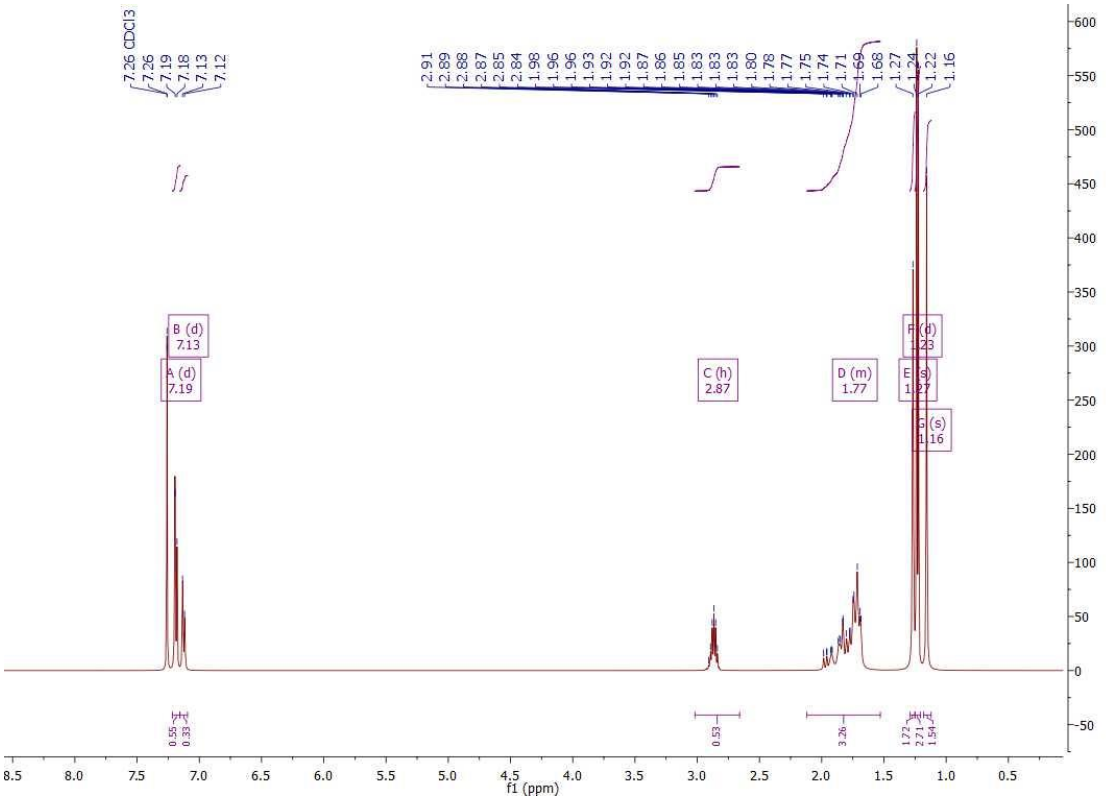
Apêndice 6.4.9. Espectro de ^{1}H NMR de 1-O,2-O-digalloil-6-O-trans-p-cumaroil-β-dglicopiranosídeo em CD_3OD (400 MHz).



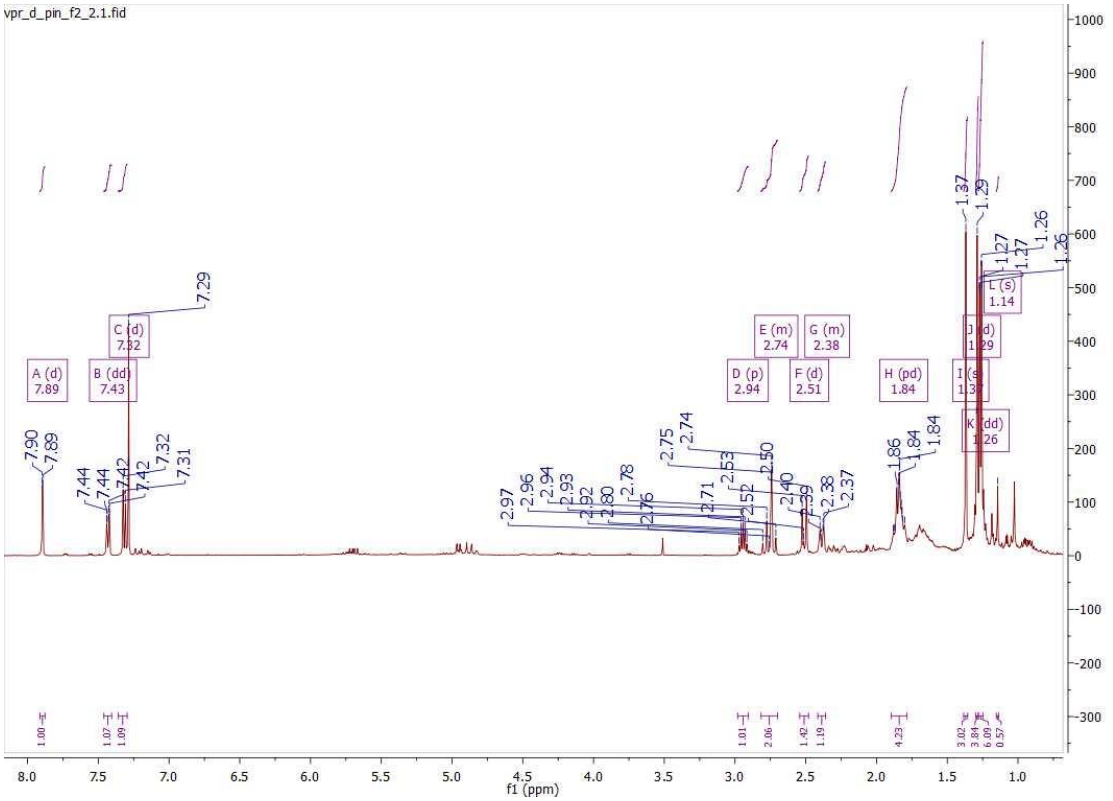
Apêndice 6.4.10. Espectro de ^{1}H NMR de 7-O-Metil Aromadendrina em CD_3OD (400 MHz)**.**



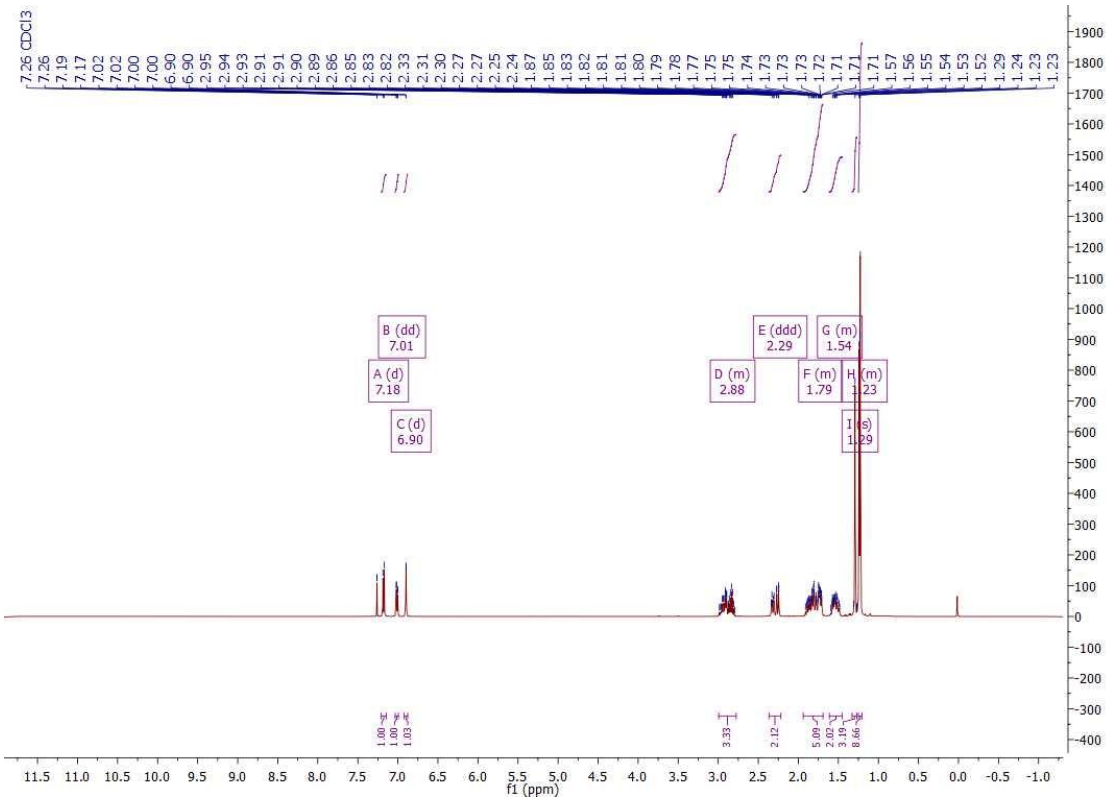
Apêndice 6.4.11. Espectro de ^{1}H NMR de Totarol em $CDCl_3$ (400 MHz).



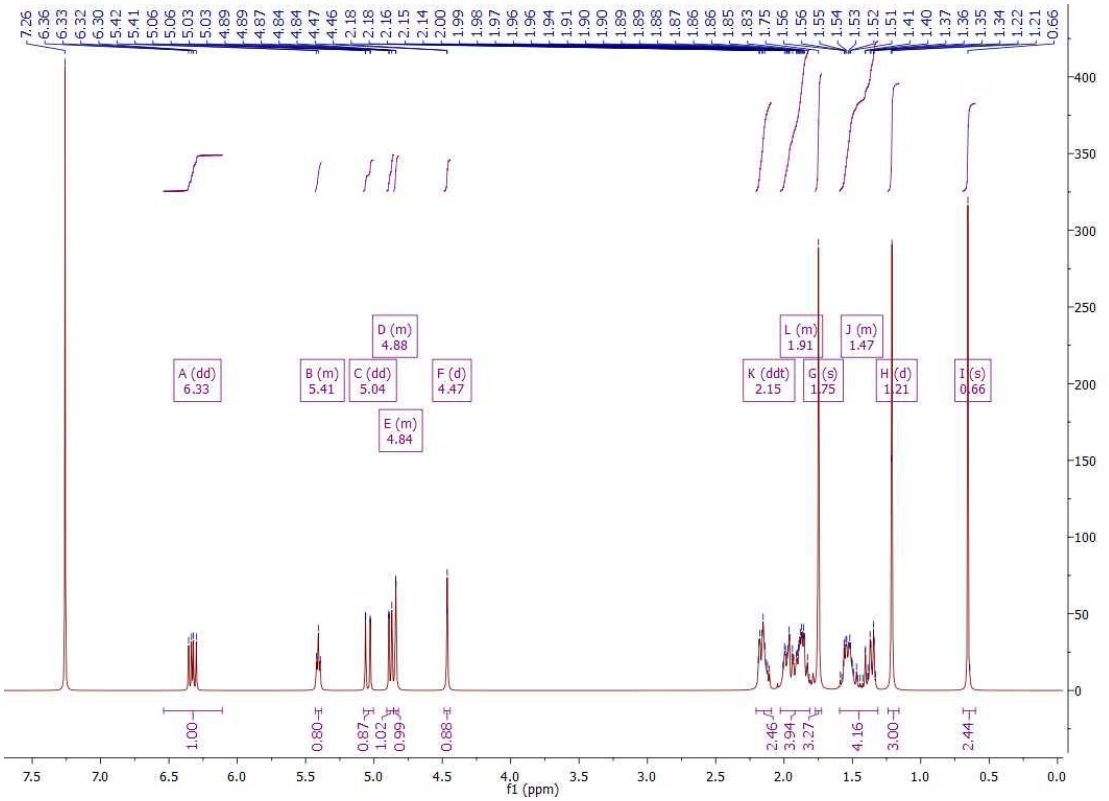
Apêndice 6.4.12. Espectro de ^{1}H NMR de ácido 7-oxodesidroabiético em $CDCl_3$ (400 MHz).



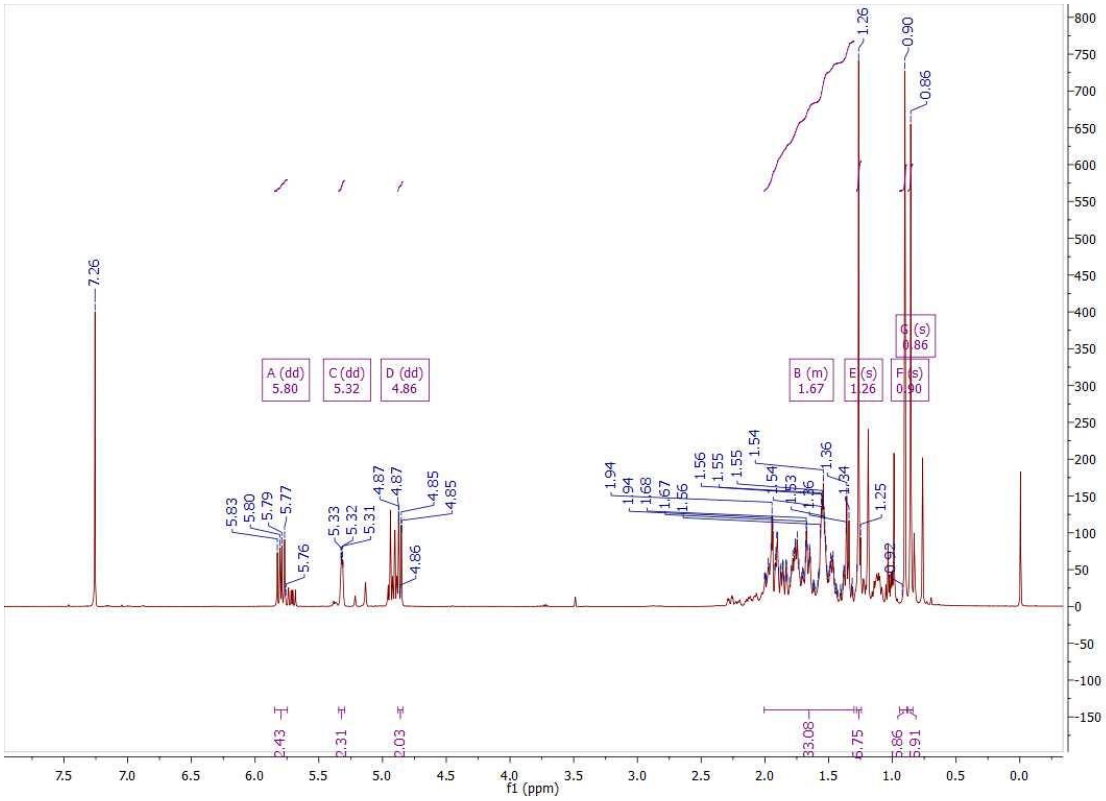
Apêndice 6.4.13. Espectro de ^{1}H NMR de ácido desidroabiético em $CDC1_3$ (400 MHz).



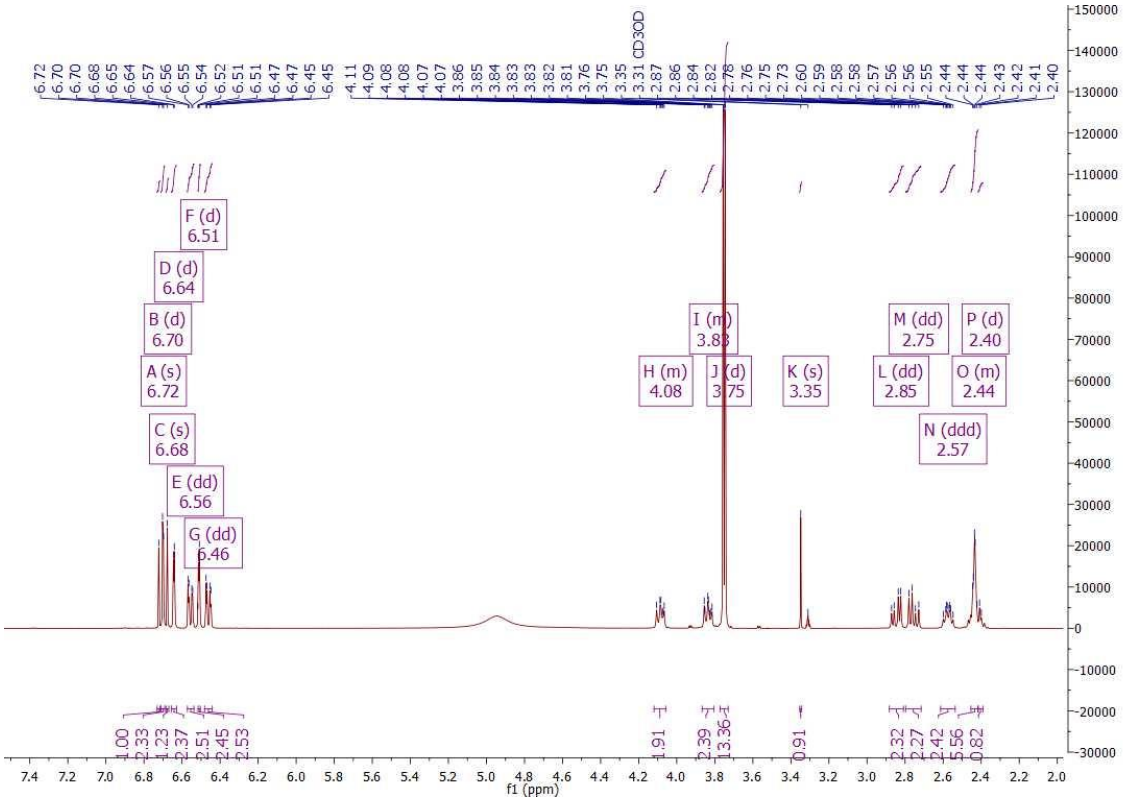
Apêndice 6.4.14. Espectro de ^{1}H NMR de Ácido Comum em $CDCl_3$ (400 MHz).



Apêndice 6.4.15. Espectro de ^{1}H NMR de ácido isopimárico em $CDCl_3$ (400 MHz)**.**



Apêndice 6.4.16. Espectro de ^{1}H NMR de Matairesinol em CD_3OD (400 MHz)**.**

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