UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Caracterização química e estudos toxicológicos da própolis vermelha e da sua fonte botânica *Dalbergia ecastaphyllum* (L.) Taub.

Jennyfer Andrea Aldana Mejía

Ribeirão Preto
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Área de Concentração: Produtos Naturais e Sintéticos

Orientador: Prof. Dr. Jairo Kenupp Bastos

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A los cómplices de mis sueños, mis padres Pedro y Elena, y mis hermanos Sonia y Jorge.
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"La idea de que la ciencia sólo concierne a los científicos es tan anticientífica como es antipoético pretender que la poesía sólo concierne a los poetas".

Gabriel García Márquez.

RESUMO

ALDANA-MEJÍA, J. A. **Caracterização química e estudos toxicológicos da própolis vermelha e da sua fonte botânica *Dalbergia ecastaphyllum* (L.) Taub.** 2022. 122f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2022.

A própolis vermelha brasileira é o resultado da interação entre as abelhas *Apis mellifera* e duas espécies vegetais, sendo *Dalbergia ecastaphyllum* (L.) Taub. (Fabaceae), a fonte primária de isoflavonoides e de outros compostos fenólicos característicos. Esta variedade de própolis tem amplo uso tradicional e várias atividades biológicas relatadas, apresentando alto potencial no desenvolvimento de produtos farmacêuticos. Todavia, há poucos estudos que garantam segurança do seu uso. Neste sentido, este trabalho teve por objetivo realizar a caracterização química, a validação de método analítico e a avaliação do potencial toxicológico *in vitro* e *in vivo* da própolis vermelha proveniente de Canavieiras (Bahia). Para tanto, algumas técnicas cromatográficas foram utilizadas para a purificação dos compostos oriundos do extrato hidroalcóolico da própolis vermelha (EPV) e especroscópicas para a identificação dos compostos. Entre estas técnicas, a ressonância magnética nuclear que foi utilizada para aidentificação dos compostos liquiritigenina, calicosina, isoliquiritigenina, vestitol, neovestitol, medicarpina, biochanina A, 7-*O*-metilvestitol, oblongifolina B e uma mistura dos isômeros gutiferona E / xantoquimol. Posteriormente, foi desenvolvido e validado um método analítico por CLAE-DAD para a análise do extrato incluindo nove dos onze marcadores isolados, priorizando os isoflavonoides. O método apresentou linearidade, seletividade, precisão, exatidão e robustez, de acordo com os delineamentos dos guias de validação utilizados. Uma análise sazonal de amostras desta variedade de própolis também foi realizada, evidenciando maior concentração dos marcadores usados em períodos da estação chuvosa com maior umidade e menor radiação solar. No que se refere aos ensaios biológicos, foram avaliados a atividade citotóxica do EPV, do extrato das folhas (EFD) e do caule (ECD) de *D. ecastaphyllum* em linhagem de fibroblastos humanos (GM07492A), por meio do ensaio colorimétrico do XTT, sendo observada redução significativa da viabilidade celular em concentrações maiores ou igual a 78 µg/mL. A avaliação do potencial genotóxico dos EPV, ECD e EFD foi conduzida por meio do teste de micronúcleo em células V79 (fibroblastos de pulmão de hamster chinês) e do EPV em sangue periférico de camundongos. Os resultados revelaram a ausência de genotoxicidade dos extratos, tanto *in vitro* quanto *in vivo*. Um ensaio de toxicidade aguda por dose única também foi realizado, no qual se evidenciou que a dose letal média (DL_{50}) do EPV por administração oral em ratos *Wistar Hannover* (fêmeas) é superior a 2000 mg/kg. A partir dos resultados obtidos, foi realizado um ensaio de toxicidade subcrônica do EPV em ratos *Wistar Hannover* (machos e fêmeas), em doses repetidas de 1000 mg/kg durante 90 dias. Não foram observados sintomas de toxicidade imediata nos animais tratados com EPV, embora tenham sido observadas algumas alterações no peso relativo de alguns tecidos como fígado, tireoide e próstata, bem como em alguns dos parâmetros relacionados à função renal e hepática nos machos. Esses resultados reforçam a importância de que novos estudos sejam realizados de modo a investigar uma possível toxicidade mediada por um consumo prolongado da própolis vermelha.

Palavras-chave: Própolis vermelha; *Dalbergia ecastaphyllum*; citotoxidade; genotoxidade; toxicidade aguda/subcrônica; roedores.

ABSTRACT

ALDANA-MEJÍA, J. A. **Chemical characterization and toxicological studies of Brazilian red propolis and its botanical source *Dalbergia ecastaphyllum* (L.) Taub.** 2022. 122p. Thesis (Doctoral). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2022.

Brazilian red propolis results from the interaction between *Apis mellifera* bees and two plant species, being *Dalbergia ecastaphyllum* (L.) Taub. (Fabaceae), the primary source of isoflavonoids and other characteristic phenolic compounds. This variety of propolis has wide traditional use, and several biological activities reported, presenting a high potential in the development of pharmaceutical products. However, few studies guarantee safety in its use. In this sense, this work aimed to carry out the chemical characterization, the validation of the analytical method, and the evaluation of the *in vitro* and *in vivo* toxicological potential of red propolis from Canavieiras (Bahia). For that, some chromatographic techniques were used to purify compounds from the hydroalcoholic extract of red propolis (BRP), and spectroscopy to identify the compounds. Among these techniques is the nuclear magnetic resonance, identifying compounds such as liquiritigenin, calycosin, isoliquiritigenin, vestitol, neovestitol, medicarpin, biochanin A, 7-*O*-methylvestitol, oblongifolin B and a mixture of gutiferone E / xanthochymol isomers. Subsequently, an analytical method by HPLC-DAD was developed and validated to analyze the extract, including nine isolated chemical markers, prioritizing isoflavonoids. The method showed linearity, selectivity, precision, accuracy, and robustness according to the validation guides' designs. A seasonal analysis of samples of this variety of propolis was also performed, showing a higher concentration of markers used in periods of the rainy season with higher humidity and lower solar radiation. With regard to biological assays, the cytotoxic activity of BRP, leaf extract (DLE), and stem extract (DSE) of *D. ecastaphyllum* in a human fibroblast lineage (GM07492A) was evaluated employing the XTT colorimetric assay. Significant reduction in cell viability was observed at concentrations greater than or equal to 78 µg/mL. The genotoxic potential of BRP, DLE, and DSE was evaluated through the micronucleus test in V79 cells (Chinese hamster lung fibroblasts) and BRP in peripheral blood of mice. The results revealed the absence of the extracts' genotoxicity *in vitro* and *in vivo*. A single dose acute toxicity test was also performed. This trial showed that the mean lethal dose (LD_{50}) of BRP by oral administration in Wistar Hannover rats (female) is more significant than 2000 mg/kg. Based on the results obtained, a subchronic toxicity test of BRP was carried out in Wistar Hannover rats (male and female) at repeated doses of 1000 mg/kg for 90 days. No symptoms of immediate toxicity were observed in animals treated with BRP, although some changes were observed in the relative weight of some tissues, such as the liver, thyroid, and prostate, and some of the parameters related to renal and hepatic function in males. These results reinforce the importance of further studies to investigate possible toxicity mediated by prolonged consumption of red propolis.

Keywords: Red propolis; *Dalbergia ecastaphyllum*; cytotoxicity; genotoxicity; acute/sub-chronic toxicity; rodents.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ALT	Alanina aminotransferase (*alanine aminotransferase*)
ANOVA	Análise de Variância
ANVISA	Agência Nacional de Vigilância Sanitária
AST	Aspartato aminotransferase (*aspartate aminotransferase*)
ax	Axial
C_2D_6OS	Dimetilsulfóxido deuterado
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CD_3COCD_3	Acetona deuterada
CD_3OD	Metanol deuterado
CEUA	Comissão de Ética no Uso de Animais
CI	Índice de citotoxidade (*Cytotoxicity index*)
CL_{50} (LC_{50})	Concentração letal média
CLAE-DAD (HPLC-DAD)	Cromatografia líquida de alta eficiência acoplada a detector de Arranjo de Diodos (*High-performance* liquid chromatography *couppled to a diode-array detector*)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
d	Dupleto
dd	Duplo dupleto
ddd	Duplo duplo dupleto
DL_{50} (LD_{50})	Dose letal média
DMSO	Dimetilsulfóxido
DXR	Doxorrubicina
E	Erro (*error*)
ECD (DSE)	Extrato hidroalcóolico do caule de *Dalbergia ecastaphyllum* (*D. ecastaphyllum* stem extract)
EFD (DLE)	Extrato hidroalcóolico das folhas de *Dalbergia ecastaphyllum* (*D. ecastaphyllum* leaf extract)
EPV (BRP/BRPE)	Extrato hidroalcóolico da própolis vermelha (*Brazilian red propolis extract*)
eq	Equatorial
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FCFRP-USP	Faculdade de Ciências Farmacêuticas de Ribeirão Preto - USP
FFCLRP-USP	Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - USP
FOB	Bateria de observação funcional (*Functional Observational Battery*)
HCA	Análise de agrupamento hierárquico (*Hierarchical Cluster Analysis*)
HCT	Hematócritos (*hematocrit*)
HGB	Hemoglobina (*hemoglobin*)
HRESIMS	Espectrometria de massa de ionização por eletrospray de alta resolução (*High Resolution Electrospray Ionisation Mass Spectrometry*)
IC_{50}	Concentração de inibição média

J	Constante de acoplamento
LD	Limite de detecção (*limit of detection*)
LQ	Limite de quantificação (*limit of quantification*)
m	Multipleto
MCH	Hemoglobina corpuscular média (*Mean Corpuscular Hemoglobin*)
MCHC	Concentração de Hemoglobina Corpuscular Média (*Mean Corpuscular Hemoglobin Concentration*)
MCV	Volume corpuscular médio (*Mean Corpuscular Volume*)
MMS	Metanosulfonato de metila
MNBCs	Células Binucleadas Micronucleadas (*Micronucleated Binucleated Cells*)
MNPCEs	Micronúcleos em eritrócitos policromáticos (*Micronuclei in Polychromatic Erythrocytes*)
NC	Controle negativo (*negative control*)
NCEs	Eritrócitos normocromáticos (*normochromatic erythrocytes*)
NDI	Índice de divisão nuclear (*Nuclear division index*)
NMR (RMN)	*Nuclear magnetic resonance* (Ressonância magnética nuclear)
OECD	*Organisation for Economic Co-operation and Development*
PC	Controle positivo (*positive control*)
PCA	Análise de Componentes Principais (*Principal Component Analysis*)
PCEs	Eritrócitos policromáticos (*Polychromatic Erythrocytes*)
PEG	Polietilenoglicol (*Polyethylene glycol*)
PTFE	Politetrafluoretileno (Teflon)
R	Coeficiente de correlação (*correlation coefficient*)
R^2	Coeficiente de determinação (*determination coefficient*)
RBC	Hemácias (*Red Blood Cell*)
rpm	Rotações por minuto
RSD	Desvio padrão relativo (*relative standard deviation*)
s	Singleto
SC	Controle do solvente (*solvent control*)
sl	Singleto largo
SPFR	Herbário do Departamento de Biologia da FFCLRP-USP
T3	Triidotironina
T4	Tiroxina
TFA	Ácido trifluroacético
tR	Tempo de retenção (*retention time*)
TSH	Hormônio tireoestimulante
UV-Vis	Ultravioleta visível
XTT	Ensaio de citotoxidade pelo reagente 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazólio-5-carboxanilida
δ	Deslocamento químico

SUMÁRIO

1. INTRODUÇÃO

1.1. Própolis: aspectos gerais

A própolis é um produto natural resultante da coleta por parte das abelhas, de diversas resinas obtidas em diferentes órgãos de plantas, que são levadas para a colmeia para serem trituradas, umedecidas com saliva e secreções enzimáticas e misturados com a cera produzida pelas glândulas salivares destes insetos (Bankova et al., 2018; Cauich-Kumul & Segura Campos, 2019). Este é um dos três produtos apícolas derivados de espécies vegetais, junto com o mel e o pólen (Bankova et al., 2018). No geral, o estudo da própolis tem se centrado nas colmeias de abelhas da espécie *Apis mellifera* (Apidae), a qual é também produzida por outros grupos de abelhas, entre elas as abelhas tropicais sem ferrão (Apidae: Meliponini) (Drescher et al., 2019; Reyes-González & Zamudio, 2020).

O termo própolis foi proposto por Aristóteles, a partir das palavras "pro" ou antes e "polis" ou cidade, dando o significado de "defensor da cidade" por ser este um dos seus papeis biológicos dentro da colmeia (Martinotti & Ranzato, 2015). Após a sua produção, a própolis é usada para revestir as paredes internas da colmeia, selar furos e fendas no ninho, proteger a entrada contra ventos, chuvas e invasores, os quais são mortos e embalsamados com este material (Bankova et al., 2019; Cauich-Kumul & Segura Campos, 2019). Além disso, sabe-se que esta resina pode reduzir o risco do crescimento de fungos e bactérias, a transmissão de doenças e parasitas através da colônia, cumprindo, desta forma, um papel de imunidade social (Martinotti & Ranzato, 2015; Salatino et al., 2011). A própolis vem sendo considerada ainda como um importante composto no que se refere à saúde do sistema imunológico das próprias abelhas, reduzindo assim o risco de transmissão de doenças e parasitas através da colônia (Bankova et al., 2019; Salatino et al., 2011).

A própolis foi utilizada por culturas ancestrais; os egípcios deram uso como embalsamador, os gregos como ingrediente de perfumes e os romanos como conservante de vinhos (Martinotti & Ranzato, 2015). Ao longo do tempo, as propriedades medicinais tanto preventivas quanto curativas da própolis foram descobertas, e este produto foi tomando importância como antisséptico no tratamento de feridas, lesões cutâneas e úlceras, bem como na redução de edema e alivio da dor (Berretta et al., 2017; Martinotti & Ranzato, 2015).

O valor da própolis na medicina popular através dos anos fez com que no século XVII, a *London Pharmacopoeia* a considerasse oficialmente dentro da categoria de medicamento,

estabelecendo-se como um produto de alto impacto e aceitação, promovendo o interesse científico tanto por sua composição química, como por suas propriedades biológicas (Berretta et al., 2017).

1.2. Características e importância da própolis Brasileira

Para a produção da própolis, diferente do que se observa para outros produtos, as abelhas utilizam diferentes espécies de plantas, assim como diversas partes da planta, refletindo assim na complexidade química observada para este produto (Kasote et al., 2022). A própolis é composta por aproximadamente 50-70% de resinas, até 87% de ceras, 1-3% de compostos voláteis, até 5% de pólen, além de outras substâncias como metabólitos secundários, aminoácidos, vitaminas (B1, B2, B6, C e E), minerais (alumínio, antimônio, cálcio, césio, cobre, ferro, lantânio, manganês, mercúrio, níquel, prata, vanádio e zinco) e, por vezes, até alguns fragmentos de madeira (Cauich-Kumul & Segura Campos, 2019; Kasote et al., 2022; Martinotti & Ranzato, 2015).

Sólida à temperatura ambiente, sua composição química, aroma e cor variam de acordo com a região geográfica, fonte botânica, estação do ano e vegetação ao redor da colmeia. Mais de 300 metabólitos secundários diferentes já foram caracterizados na própolis, incluindo fenilpropanoides, chalconas, ésteres alifáticos, diidrochalconas, flavonoides, terpenoides entre outras substâncias (Jain et al., 2014; Martinotti & Ranzato, 2015).

As própolis provenientes de áreas tropicais, em especial as provenientes da América do Sul, são ricas em derivados prenilados do ácido *p*-cumárico, flavonoides, terpenos, isoflavonoides, benzofenonas e lignanas polipreniladas (Kasote et al., 2022). Num primeiro momento, baseado nas características físico-químicas e propriedades biológicas, foram identificados a partir da análise de 500 amostras, 12 tipos de própolis (Park et al., 2000). Recentemente, outras variedades deste produto foram caracterizados como o vermelho do mangue, o preto da Amazônia e o verde da Caatinga, indicando que ainda são necessários estudos para conhecer a magnitude da diversidade dos diferentes tipos de própolis brasileiros (Salatino et al., 2021).

Um dos tipos mais conhecidos de própolis tem a *Baccharis dracunculifolia* como sua origem botânica. Esta variedade de própolis, encontrada principalmente nas regiões sudeste e sul do Brasil, refere-se a própolis verde. Este tipo de própolis tem o artepelin C, a drupanina, e o ácido *p*-cumárico como seus principais marcadores (Bankova et al., 2019). Outras espécies vegetais que consistem em fonte botânica para outros tipos de própolis pertencem aos gêneros

Araucaria, *Populus*, *Pinus*, *Clusia*, *Vernonia*, *Mimosa*, *Eucalyptus* e *Dalbergia* spp, entre outras plantas (Kasote et al., 2022; Salatino et al., 2021).

Inúmeras propriedades, incluindo antimicrobiana, anticâncer, anti-inflamatória, antiviral, imunomodulatória, antisséptica e antioxidante, entre outras, têm sido associadas a utilização dos diferentes tipos de própolis. Tais propriedades tem proporcionado sua utilização como insumo para a indústria de produtos naturais, sendo encontrados nas formas de cápsulas, xaropes e cremes (Berretta et al., 2017; Salatino et al., 2021). Estes produtos, por sua vez, são altamente valorizados em países asiáticos, onde sua comercialização é lucrativa atingindo valores de até U$ 200 por quilograma do produto. O Brasil consiste no terceiro produtor mundial de própolis, abastecendo, por exemplo, aproximadamente 80% da demanda japonesa (Berretta et al., 2017).

1.3. Própolis vermelha brasileira: origem e composição química.

A própolis vermelha é produzida em países tropicais como México, Cuba, Venezuela, e Brasil (Mendonça-Melo et al., 2017). No território brasileiro, os manguezais do Nordeste com destaque para Alagoas e o sul da Bahia, são o principal foco de produção deste tipo de própolis (Vidal, 2021). Em 2008, foram publicados os primeiros trabalhos com amostras da própolis vermelha brasileira, as quais foram coletadas nos estados da Bahia, Sergipe, Alagoas, Pernambuco e Paraíba. Junto com as amostras de própolis foram coletados os exsudados da *Dalbergia ecastaphyllum* (L) (Fabaceae), espécie, até então, suspeita de ser sua fonte botânica (Daugsch et al., 2008; B. Silva et al., 2008).

A análise cromatográfica dos extratos, feitos a partir das amostras, revelaram que a composição química da própolis e dos exsudados da planta eram similares, concluindo que esta espécie era usada pelas abelhas para sua elaboração. A própolis vermelha e os exsudados de *D. ecastaphyllum*, fitoquimicamente, se caracterizam pela presença de flavononas, flavonóis, xantonas, triterpenos pentacíclicos, catequinas, chalconas, e benzofenonas polipreniladas (I. C. G. de Mendonça et al., 2015).

Entre os compostos identificados na própolis vermelha se encontram os flavonóis quercetina, retusapurpurina B e A (estes últimos responsáveis pela cor vermelha); a flavanona liquiritigenina; as isoflavonas daidzeína, formononetina, biochanina A e 7,4'-diidroxi-isoflavona; os isoflavonoides neovestitol, vestitol, e 4'-7-dimetoxi-2'-isoflavonol; o neoflavonoide dalbergina; a chalcona isoliquiritigenina; os pterocarpanos medicarpina e homopterocarpina; e as benzofenonas polipreniladas guttiferona E, xantochimol e

oblongifolinas (Alencar et al., 2007; Bueno-Silva et al., 2013; Daugsch et al., 2008; Mendonça-Melo et al., 2017; Oldoni et al., 2011; Piccinelli et al., 2011; B. Silva et al., 2008).

A mistura de flavonas, isoflavonoides e pterocarpanos é comum nas própolis vermelha brasileira e cubana. Estudos tem sugerido que estas própolis compartilham a *D. ecastaphyllum* como fonte botânica (Piccinelli et al., 2011). *D. ecastaphyllum* se distribui em ilhas do Caribe, e no Brasil está presente em restingas e manguezais, desde o estado de Santa Catarina (sul do Brasil) até as regiões nordeste e norte do Brasil (Salatino, 2018). Porém, a própolis vermelha brasileira tem revelado diferenças significativas tanto em sua composição química como em sua produção quando comparada a outros tipos de própolis vermelhas das regiões tropicais.

Ademais, novas interações planta-inseto que influenciam a produção de própolis vermelha no Brasil têm sido descobertas em apiários da região Nordeste. A resina avermelhada exsudada do caule de *D. ecastaphyllum* tem uma produção induzida pela eclosão de larvas do besouro *Agrilus propolis* (Buprestidae) (Migliore et al., 2022). Até o momento, não se tem conhecimento sobre este fenômeno e sua ocorrência em outras regiões tropicais produtoras de própolis vermelha no mundo.

Em relação a suas características químicas, a presença de benzofenonas polipreniladas detectadas nas amostras de própolis vermelha cubana e brasileira, mas ausentes na *D. ecastaphyllum*, tornaram-se o diferencial suscitando dúvidas sobre a possível participação de uma segunda fonte botânica na produção desta variedade de própolis. No que se refere a própolis cubana, por exemplo, *Clusia rosea* (Clusiaseae) foi indicada como a fonte botânica destes compostos (Cuesta-Rubio et al., 2002; Piccinelli et al., 2005). Já os estudos realizados no Brasil a partir das amostras provenientes da Bahia, revelaram que, a resina da espécie *Symphonia globulifera* L.f., consiste não só na fonte das benzofenonas polipreniladas, como de alguns de seus triterpenoides (Ccana-Ccapatinta et al., 2020).

1.4. Potencial biológico da própolis vermelha

São diversos os estudos relacionados à avaliação das atividades biológicas desta variedade de própolis. Entre o potencial biológico mais citado da própolis vermelha se encontra o antimicrobiano. Foi demonstrado que o extrato alcoólico é ativo contra *Staphylococcus aureus* (ATCC 25923) (Alencar et al., 2007; Daugsch et al., 2008; Oldoni et al., 2011), *Streptococcus sobrinus*, *S. mutans* e *S. aureus* (Bueno-Silva et al., 2013), *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Proteus mirabilis* (Righi et al., 2011) e *Escherichia coli* (Devequi-Nunes et al., 2018; Regueira et al., 2017; Righi et al., 2011).

Extratos da própolis também têm sido ativos contra diversas espécies de fungos do gênero *Candida* sp., tais como *C. albicans* (Botteon et al., 2021; F. Silva et al., 2019), *C. krusei* (F. Silva et al., 2019), *C. tropicalis* (Bezerra et al., 2015), *C. parapsilosis* e *C. glabrata* (L. S. de Mendonça et al., 2015). O extrato hidroalcoólico da própolis vermelha inibiu a atividade metabólica em biofilmes de *C. albicans*, com efeito semelhante à clorexidina (Martorano-Fernandes et al., 2020).

Atividade citotóxica significativa frente a células tumorais como HeLa (adenocarcinoma cervical) (Alencar et al., 2007; Silva Frozza et al., 2013), 5637 (bexiga) (Begnini et al., 2014), SF-295 (glioblastoma humano), OVCAR-8 (ovário) e HCT-116 (cólon) (I. C. G. de Mendonça et al., 2015; F. Silva et al., 2019), Hep-2 (carcinoma de laringe) (Silva Frozza et al., 2013), T24 (bexiga) e PC-3 (próstata) (Botteon et al., 2021) tem sido mencionada. A própolis também demonstrou ser citotóxica contra diferentes linhagens celulares de leucemia. Ressalta-se, no entanto, a atividade citostática encontrada contra as linhagens de leucemia K562 e Nalm16, promovendo a apoptose nas células K562 em resposta similar ao controle Gleevec (Franchi et al., 2012). Em ensaios *in vivo*, a administração oral do extrato da própolis a ratos na dose de 100 mg/Kg promoveu uma diminuição da multiplicidade tumoral da carcinogênese dérmica, demonstrando uma pontuação de malignidade significativamente menor do que aquela no grupo de animais não tratados (Pinheiro et al., 2014).

O extrato da própolis vermelha também exibiu significativa capacidade em neutralizar o radical DPPH (I. C. G. de Mendonça et al., 2015; Righi et al., 2011; Silva Frozza et al., 2014), assim como as suas frações em hexano, clorofórmio e acetato de etila (I. C. G. de Mendonça et al., 2015). Estudos de novos sistemas de liberação controlada e entrega a alvos específicos, também têm sido testados com a própolis vermelha brasileira. O extrato de própolis foi encapsulado em nanopartículas de sílica mesoporosa e a sua capacidade antioxidante também foi avaliada em relação a sua capacidade em neutralizar o radical DPPH. A atividade antioxidante da própolis vermelha foi mantida ao ser encapsulada, mostrando que estes novos sistemas de encapsulamento preservam as propriedades físico-químicas e farmacológicas deste produto (Laís et al., 2020).

Além disso, efeitos de proteção renal foram demonstrados por alguns estudos após episódios de insuficiência renal isquêmica aguda induzida (Da Costa et al., 2015) e de ablação (Teles et al., 2015). O extrato hidroalcoólico da própolis vermelha melhorou os parâmetros associados ao estresse oxidativo, lesões histológicas e inflamação do tecido.

Outros estudos evidenciaram ainda importante atividade anti-inflamatória do extrato da própolis vermelha. Ratos tratados oralmente com o extrato de própolis vermelha, apresentaram

inibição do edema de pata induzido por carragenina (Cavendish et al., 2015). As análises realizadas tanto em camundongos tratados por via intraperitoneal (Bueno-Silva et al., 2013), como em ratos tratados por via oral (Cavendish et al., 2015) demonstraram que a atividade anti-inflamatória do extrato pode estar relacionada a inibição da migração dos neutrófilos após a indução. Parte deste efeito pode ser atribuído à presença de neovestitol, vestitol e formononetina, que também mostraram atividade nos ensaios realizados. A própolis vermelha em formulação oral também tem se mostrado com potencial anti-inflamatório em pele de ratos expostos a radiação UVB (Batista et al., 2018).

Extratos hidroalcoólicos da própolis vermelha de diferentes localidades foram testados contra as formas promastigotas de *Leishmania braziliensis* e *L. infantum*, evidenciando atividade contra os dois protozoários de forma dose-dependente (Araujo et al., 2018; Regueira-Neto et al., 2018). Nanopartículas carregadas com extrato de própolis vermelha também mantiveram a atividade citotóxica sobre a forma promastigota de *L. braziliensis* (do Nascimento et al., 2016). A própolis vermelha também apresentou atividade citotóxica contra a forma epimastigota de *Trypanosoma cruzi*, sendo seletiva e segura para células de fibroblastos (Regueira-Neto et al., 2018).

Importante atividade gastroprotetora também tem sido atribuída à administração oral do extrato hidroalcóolico da própolis vermelha. Ensaios realizados tanto em ratos como em camundongos como modelo agudo de úlcera mostraram uma redução significativa nos danos à mucosa gástrica aliados à diminuição em alguns dos parâmetros relacionados ao estresse oxidativo (De Mendonça *et al*., 2020; Boeing et al., 2021). Sabe-se, ainda, que a incorporação de extratos hidroalcoólicos da própolis vermelha em filmes curativos à base de colágeno melhorou a cicatrização de queimaduras induzidas em ratos, sem efeitos tóxicos (De Almeida et al., 2013). Ademais, a administração oral destes extratos, em modelo murino, também favoreceu o processo de cicatrização, além de suprimir a resposta inflamatória durante o reparo tecidual (Corrêa et al., 2017).

1.5. Caracterização química da própolis vermelha: importância da validação de métodos analíticos

Nos últimos anos, muitas pesquisas vêm sendo desenvolvidas com o foco na avaliação das atividades biológicas da própolis vermelha brasileira. Sabe-se, contudo, que tais atividades estão diretamente associadas à sua composição química e a concentração dos seus constituintes. Todavia, a complexidade química, na maioria das vezes observada para os produtos de origem natural transformam sua utilização em um desafio no que diz respeito à sua utilização e ao

aproveitamento de todo seu potencial biológico (Indrayanto, 2018). Especificamente no caso da própolis, sabe-se que a área geográfica da coleta e a correlação entre as fontes botânicas e as condições meteorológicas são de grande importância na constituição final da resina (Bankova et al., 2018; Bueno-Silva et al., 2017; do Nascimento et al., 2019).

Nesta perspectiva, considerando o potencial da própolis vermelha brasileira na elaboração de produtos alimentícios e em formulações com potencial farmacológico, torna-se necessário o estabelecimento de métodos que permitam o seu controle de qualidade. Nesse sentido, a utilização de alguns critérios referentes à padronização dos perfis químicos das amostras é de extrema importância. Sem esta padronização não é possível certificar que os produtos possuem características aceitáveis, consistentes e estáveis entre e dentro de cada lote (Indrayanto, 2018). A quantificação de marcadores específicos, especialmente os biologicamente ativos, garante tanto a atividade farmacológica como a segurança dos produtos (Bankova et al., 2019; do Nascimento et al., 2019; Escriche & Juan-Borrás, 2018).

A necessidade do desenvolvimento e validação de métodos analíticos que possam ser aplicados para o controle de qualidade se torna de grande importância em produtos de origem natural (Indrayanto, 2018). Através da validação analítica, os métodos usados para quantificar substancias ativas podem ser considerados confiáveis e adequados (ANVISA, 2017; ICH, 2005). Dentro do desenvolvimento e da validação dos métodos analíticos, alguns critérios de aceitação devem ser considerados, tais como seletividade, faixa de trabalho (dos marcadores selecionados), linearidade, exatidão, precisão e limites de detecção e quantificação (Indrayanto, 2018). Guias sobre as metodologias a seguir para a validação de métodos analíticos têm sido emitidas por agencias de controle como a *International Conference on Harmonization* (ICH, 2005) e a Agência Nacional de Vigilância Sanitária (ICH, 2005).

Diversas técnicas analíticas têm sido utilizadas na busca da caracterização química da própolis vermelha, porém são poucos os trabalhos dedicados ao desenvolvimento de métodos para a quantificação dos compostos ativos. Estimativas sobre a quantidade dos compostos presentes na própolis vermelha brasileira têm sido realizadas através de CLAE-DAD (Bueno-Silva et al., 2017) e Cromatografia Líquida Acoplada à Espectrometria de Massas Triplo Quadrupolo (do Nascimento et al., 2019). Porém, nenhuma destas metodologias foi desenvolvida com o objetivo de ser referência no controle de qualidade de amostras de própolis vermelha, ficando apenas como uma aproximação ao estado dos marcadores em amostras selecionadas.

1.6. Segurança no uso da própolis vermelha brasileira

Apesar dos efeitos cientificamente comprovados da própolis vermelha, há poucos estudos tanto *in vitro* quanto *in vivo* avaliando a segurança do seu consumo. O estudo de Begnini *et al*. (2014) relata que o extrato alcoólico da própolis vermelha não apresenta seletividade citotóxica entre células tumorais e normais, mostrando IC_{50} de 95 µg/mL em células tumorais, inibindo em até 70% o crescimento de células de ovário de hamster chinês (CHO-K1). Silva Frozza *et al*. (2013) reportou que a IC_{50} do extrato da própolis vermelha é superior a 150 µg/mL em células embrionárias renais humana (Hek-283).

Em 2015, Silva e colaboradores, ao avaliarem a administração oral do extrato hidroalcóolico da própolis vermelha por meio de ensaios de toxicidade aguda de dose única (14 dias) e de toxicidade subaguda de doses repetidas (28 dias), demonstraram uma possível toxicidade induzida pela administração do extrato na dose de 300 mg/kg por peso corpóreo dos animais. Os autores consideraram que estas alterações podem ser indicativas de toxicidade à longo prazo, já que os efeitos foram evidenciados no ensaio de 28 dias (R. da Silva et al., 2015).

Mundialmente, a própolis tem sido categorizada como alimento junto a outros produtos apícolas, sendo matéria prima para elaboração de uma variedade de suplementos alimentares. No Brasil, muitos produtos à base de própolis têm sido registrados nos últimos anos. Entretanto, algumas informações são limitadas, prejudicando sua utilização segura (Berretta et al., 2017). Neste sentido, a Agência Nacional de Vigilância Sanitária (ANVISA), por meio da Resolução da Diretoria Colegiada - RDC nº 24 de 2011 (Resolução Da Diretoria Colegiada - Nº 24 de 14/06/2011, 2011), declarou que os produtos à base de própolis com alguma indicação terapêutica são considerados aptos para serem registrados como medicamentos específicos. Neste caso, para o registro dos produtos é necessária a apresentação do relatório de segurança e eficácia tanto pré-clínica como clínica ou mesmo de dados presentes na literatura científica que contemplem estas informações (com exceção dos produtos de uso tópico e na cavidade bucal). Além disso, estudos de genotoxidade, toxicidade de dose única (aguda) e de toxicidade de doses repetidas são contemplados nos guias vigentes da ANVISA para o desenvolvimento de medicamentos (ANVISA, 2019).

As propriedades científicas comprovadas da própolis vermelha confirmam o seu potencial para a elaboração de produtos medicinais. Contudo, poucos estudos atendem aos requisitos de segurança, tanto da própolis quanto de sua fonte botânica, esta última ainda menos estudada. Neste contexto, nossas análises têm por base o fato de que tanto as propriedades como as peculiaridades da própolis, aliadas a ausência de dados que possam fortalecer sua utilização

segura, a tornam alvo importante de pesquisa no que se refere a seleção das doses eficazes e seguras dos produtos elaborados a partir de sua matriz ou da sua fonte vegetal.

2. OBJETIVOS

2.1 Objetivo geral

Caracterizar, quimicamente, a própolis vermelha e investigar os seus possíveis efeitos tóxicos, bem como os de sua fonte botânica *Dalbergia ecastaphyllum*.

2.2 Objetivos específicos

- Obter o perfil químico dos extratos brutos da própolis vermelha e das partes aéreas de *D. ecastaphyllum* por CLAE-DAD;
- Submeter o extrato de própolis vermelha a diferentes técnicas cromatográficas visando o isolamento dos seus compostos majoritários;
- Desenvolver e validar um método cromatográfico em CLAE-DAD para a quantificação dos principais marcadores presentes em amostras de própolis vermelha;
- Avaliar as atividades citotóxicas *in vitro* dos extratos brutos da própolis vermelha e da sua fonte botânica por meio da determinação da viabilidade celular através do ensaio de XTT;
- Avaliar a atividade genotóxica *in vitro* dos extratos brutos da própolis vermelha e da sua fonte botânica através do ensaio de micronúcleo;
- Avaliar a atividade genotóxica *in vivo* do extrato bruto de própolis vermelha por meio do ensaio de micronúcleo em eritrócitos de camundongos;
- Avaliar as atividades toxicológicas *in vivo*, por meio de ensaios de toxicidade aguda por dose única (14 dias) e de toxicidade subcrônica em doses repetidas (90 dias) da própolis vermelha por via oral em modelo pré-clínico.

3. TRABALHOS RESULTANTES

CAPÍTULO 1. Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum*.

CAPÍTULO 2. A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and *Dalbergia ecastaphyllum*.

CAPÍTULO 3. Genotoxicity and Toxicological Evaluations of Brazilian Red Propolis oral ingestion in a Preclinical Rodent Model.

3.1 CAPÍTULO 1. Caracterização química e estudos toxicológicos *in vitro* da própolis vermelha e da sua fonte botânica *D. ecastaphyllum*

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Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum*

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ABSTRACT
Propolis is one of the most widely used products in traditional medicine. One of the most prominent types of Brazilian propolis is the red one, whose primary botanical source is *Dalbergia ecastaphyllum* (L.) Taub. Despite the potential of Brazilian red propolis for developing new products with pharmacological activity, few studies guarantee safety in its use. The objective of this study was the evaluation of the possible toxic effects of Brazilian red propolis and *D. ecastaphyllum,* as well as the cytotoxicity assessment of the main compounds of red propolis on tumoral cell lines. Hydroalcoholic extracts of the Brazilian red propolis (BRPE) and *D. ecastaphyllum* stems (DSE) and leaves (DLE) were prepared and chromatographed for isolation of the major compounds. RP-HPLC-DAD was used to quantify the major compounds in the obtained extracts. The XTT assay was used to evaluate the cytotoxic activity of the extracts in the human fibroblast cell line (GM07492A). The results revealed IC_{50} values of 102.7, 143.4, and 253.1 μg/mL for BRPE, DSE, and DLE, respectively. The extracts were also evaluated for their genotoxic potential in the micronucleus assay in Chinese hamster lung fibroblasts cells (V79), showing the absence of genotoxicity. The BRPE was investigated for its potential in vivo toxicity in the zebrafish model. Concentrations of 0.8−6.3 mg/L were safe for the animals, with a LC_{50} of 9.37 mg/L. Of the 11 compounds isolated from BRPE, medicarpin showed a selective cytotoxic effect against the HeLa cell line. These are the initial steps to determine the toxicological potential of Brazilian red propolis.

KEYWORDS. Red propolis, medicarpin, oblongifolin B, micronucleus, XTT, zebrafish.

INTRODUCTION

For centuries, propolis has played a vital role in the traditional medicine of ancient cultures, and even today, it plays an important role in the folk medicine of communities around the world.[1] Propolis is a natural product resulting from the interesting plant−insect interaction, given between bees and some plant species. During their manufacture, bees collect various substances from the buds or exudates of the plants and mix it with wax from their glands to produce a resinous material combined with some inorganic materials, resulting in propolis production.[2,3] This material performs essential biological functions within bee populations not only as physical protection of the hives but also against the growth and transmission of fungi, bacteria, and parasites through the colony.[3]

Different species of bees and plants can be interacting to produced propolis, and for this reason, propolis chemical composition can be complex and hugely dependent on the botanical sources available in the region.[4,5] There are different types of propolis in Brazil, based on their chemical composition and geographic location, but the green, brown, and red ones have been the most studied and commercialized.1 Brazilian red propolis is characteristically produced by *Apis mellifera* L. bees in the northeastern region and has *Dalbergia ecastaphyllum* (L.) Taub. (Fabaceae) as the primary recognized botanical source.[6,7] Our research group recently identified a second botanical source, a member of Clusiaceae family, namely, *Symphonia globulifera* L.f.[8] In conjunction, these studies revealed that *D. ecastaphyllum* is the botanical source of red propolis compounds, such as pterocarpans, flavanones, and isoflavones. At the same time, *S. globulifera* contributes mainly to triterpenoids and polyprenylated benzophenones that differentiate Brazilian red propolis from other types of red propolis in the world.

Brazilian red propolis and some of its isolated compounds have already been reported to possess different biological activities. The antimicrobial, antifungal, anti-inflammatory, antioxidant, and cytotoxic effects against tumor cell lines activities of red propolis and its compounds have been described.[9−12] Despite the positive biological effects of red propolis, there are few studies evaluating the safety of its consumption. *In vitro*, red propolis extract showed an IC_{50} superior to 150 μg/mL in human kidney embryonic cells (Hek-283).[13] However, in vivo, single-dose acute toxicity tests (14 days) and subacute toxicity (28 days) on rats, exposed to oral doses of red propolis hydroalcoholic extract showed signs of toxicity in both assays, with no lethal effects.[14] The authors considered these changes as possibly being indicative of long- term toxicity. Even with the potential of red propolis for the development of new drugs, propolis has been categorized as raw material for elaborating a variety of food supplements and other bee- derived products. However, some propolis products have a therapeutic indication, and according to some agencies responsible for public health, products based on propolis with medicinal use are suitable for being registered as drugs.[15,16] It is necessary to know both preclinical and clinical safety and efficacy data to register medicinal products. In this sense, studies with Brazilian red propolis are scarce, and those related to their botanical sources are even more meager.

In the present study, *in vitro* and *in vivo* assays were conducted for toxicological evaluation of Brazilian red propolis, as well as for its botanical source *D. ecastaphyllum*. In addition, the chemical characterization of red propolis was carried out.

MATERIAL AND METHODS

Propolis and Plant Material Collection and Extraction

The Brazilian red propolis, leaves, stems, and resin of *D. ecastaphyllum* were collected in Canavieras, Bahia, at the apiaries from the beekeepers association of Canavieiras (Cooperativa de Apicultores de Canavieiras - COAPER) in April of 2018. A plant specimen was deposited and identified by Milton Groppo as *Dalbergia ecastaphyllum* (L.) Taub. (SPFR 17771) at the Herbarium (Department of Biology, Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, FFCLRP, University of São Paulo, USP). The plant materials (stems and leaves) were dried and powdered using a knife mill. The plant resin was collected in small amount, and it was not enough to obtain extracts and run biological tests. It was used only for comparative HPLC chromatographic analysis. In order to remove wax and other materials and facilitate the extraction of medium to high polar compounds, red propolis (200 g) was frozen, ground, and macerated with ethanol−water 7:3,17,18 10 (w/v) percentage, using a shaker incubator at 30 °C and 120 rpm, for 24 h, repeating the process five times. Leaves and stems of *D. ecastaphyllum* were also macerated with ethanol−water 7:3 on the same w/v proportion, repeating the extraction three times. The extracts obtained were concentrated under vacuum using a Buchi rotary evaporator and were lyophilized.

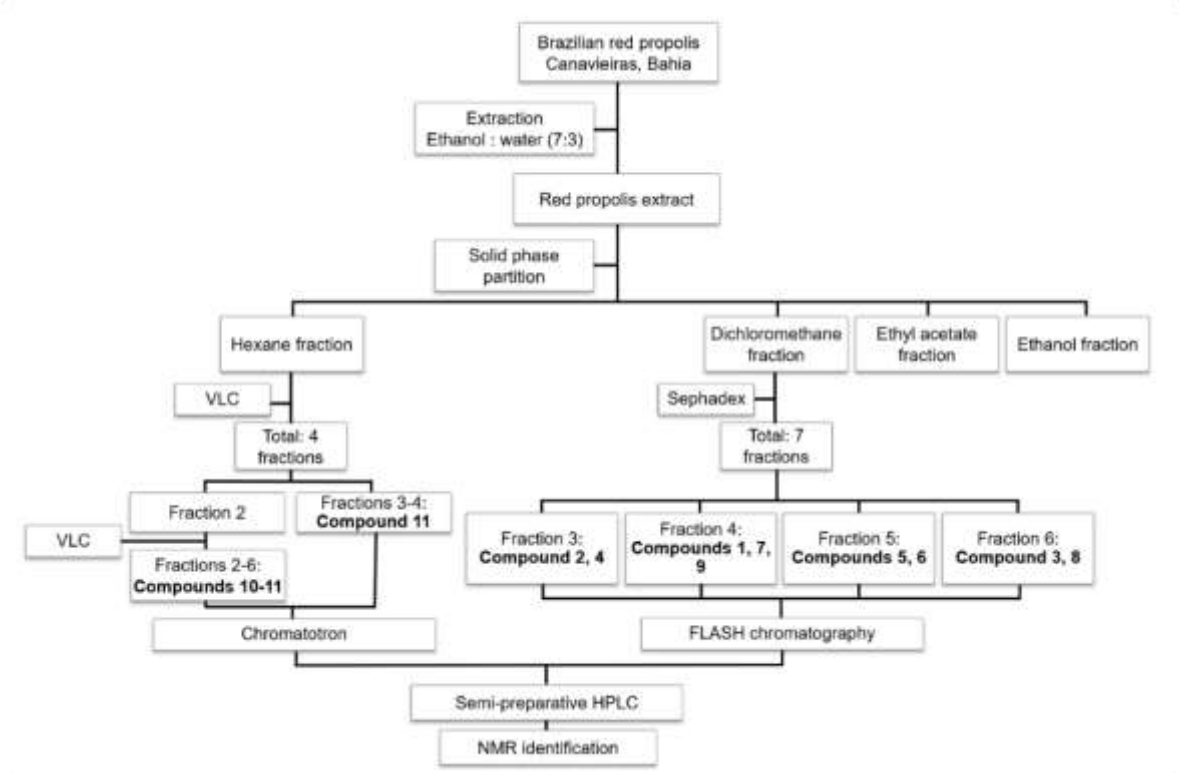
Brazilian Red Propolis Chemical Characterization.

The Brazilian red propolis extract (BRPE) was partitioned in a solid phase process, mixing 87 g of the extract with 200 g of microcrystalline cellulose. The mixture was placed on a glass column (13 × 11 cm^2 i.d.), for the extraction solvents of increasing polarity: hexanes (2 L), dichloromethane (2 L), ethyl acetate (2 L), and ethanol 98% (2 L). The fractions were concentrated under vacuum and lyophilized.

The hexane fraction (20 g) was submitted to a vacuum liquid chromatography (VLC) as described by Ccana-Ccapatinta.[8] The isolation of the major compounds was undertaken by using different chromatographic techniques (Scheme 3-1). The hexanes' fraction 2 was submitted to a VLC column under the same conditions. The enriched fractions with the mixture of the major compounds, and the hexane fractions 3 and 4 were submitted to a centrifugal thin-layer chromatography on a Chromatotron device (T-Squared Technology), under previously stablished conditions.[8] This procedure enabled the separation of compounds 10 and 11, which were submitted to a final purification through semipreparative HPLC.

The dichloromethane fraction (12.5 g) was fractionated on Sephadex LH-20 (25−1000 μm; Sigma-Aldrich) as a stationary phase (Scheme 3.1-2) on a glass column (30 × 4cm2 i.d.), using ethanol 96% as mobile phase. The process was repeated twice. The fractions were combined according to their thin layer chromatographic profile (TLC) affording seven fractions. Fractions 3−6 were submitted to flash chromatography using the Isolera Prime (Biotage) equipment. The stationary phase consisted of cartridges of silica gel Biotage SNAP Ultra C18 (12 or 30 g, HP-Sphere, 25 μm). The mobile phase consisted of ethanol (pump A) and acidified water (1:99, pump B), in gradient mode (80:20 to 20:80). After TLC, HPLC and semi- preparative HPLC purification were afforded compounds 1−9.

Scheme ¡Error! No hay texto con el estilo especificado en el documento.**-1.** Chromatographic Procedures to Fractionate the Brazilian Red Propolis Hydroalcoholic Extract (BRPE) and Isolation of Its Major Compounds.



Chromatographic Analyses, Compounds Purification, and Identification.

HPLC analyses of the resin, extracts, fractions, and isolated compounds were carried out using an Ascentis Express C18 column (2.7 μm, 150 × 4.60 mm2; Supelco) on an HPLC Waters 2695 chromatographer with a 2998 PDA as previously reported.[8]

Red propolis compounds were purified by semipreparative HPLC, using a Shimadzu preparative chromatographer, Proeminence model, with CBM-20A controller and SPD-20 A UV/vis detector. A Synergi Polar-RP (4 μm, 250 × 21.5 mm2, Phenomenex) column was used as a stationary phase. Acid−water (0.1:99.9, pump A) and acetonitrile (pump B), in gradient mode, with a flow rate of 10 mL/min, were employed as the mobile phase. Samples were prepared in concentrations between 300 and 400 mg/mL.

The gradient conditions for the purification of compounds from hexane fractions were: 70−100% of B in 30 min, maintaining 100% until 33 min, re-equilibrating from 100 to 70% of B in 35 min, and finishing in 70% of B in 37 min. Compounds of the dichloromethane fractions were purified using the same column at the same flow rate but with a different gradient condition: 40−80% of B in 25 min, reaching 100% of B in 27 min, isocratic 100% of B until 28 min, re- equilibrating from 100 to 40% of B in 33 min, ending with 40% of B in 35 min.

NMR spectra of the isolated compounds were acquired to identify the chemical structures on a Bruker-Avance DRX-500, DRX 400, and DRX 300 spectrometer (Bruker), operating at

500, 400, and 300 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, respectively. The compounds were dissolved in deuterated methanol (CD_3OD) with the addition of 1% trifluoroacetic acid (TFA), acetone (CD_3COCD_3), or DMSO (C_2D_6OS) according to their solubility. The NMR data were compared with the literature to confirm the structure of the isolated compounds, as reported below. Mass spectrometry data were obtained on an orbitrap mass spectrometer (Thermo Scientific), by direct injection, using negative ionization mode.

The quantitation of the major compounds in the extracts was performed for compounds 4−7 and 9, isolated in higher yielding after purification by HPLC-DAD. For that, a standard stock solution was prepared and diluted to achieve concentrations of 100, 80, 60, 40, 20, and 10 μg/mL, using benzophenone at 20 μg/mL as Internal Standard (IS). The chromatographic analyses were performed as previously reported.[8] DAD reading range was set at 220−600 nm; chromatograms were monitored at 275, and the area of the peaks was acquired at different wavelengths (249, 254, 280, and 285 nm), according to the maxima absorption of each compound. The ratio between the area of each standard and the IS was used to plot the analytical curves and obtained the regression equations. All the experiments were performed in triplicate.

NMR and LC-MS Data.

*Liquiritigenin (**1**)*. This compound is a yellow amorphous powder; UV λ_{max} = 276, 311 nm; ^{1}H and ^{13}C NMR data as reported by Zhao et al.[19] HRESIMS negative mode m/z 255.0663 [M − H] − (calcd for $C_{15}H_{11}O_4$, 255.0663).

*Calycosin (**2**).* This compound is a white powder; UV λ_{max} = 250, 290 nm; ^{1}H and ^{13}C NMR data as reported by Du et al.[20] HRESIMS negative mode m/z 283.0612 [M − H] − (calcd for $C_{16}H_{11}O_5$, 283.0612).

*Isoliquiritigenin (**3**).* This compound is a yellow powder; UV λ_{max} = 239, 376 nm; ^{1}H and ^{13}C NMR data as reported by Zhao et al.[19] HRESIMS negative mode m/z 255.0664 [M − H] − (calcd for $C_{15}H_{11}O_4$, 255.0663).

*Formononetin (**4**)*. This compound is a white powder; UV λ_{max} = 249, 302 nm; ^{1}H and ^{13}C NMR data as reported by Granados-Covarrubias and Maldonado[21] HRESIMS negative mode m/z 267.0664 [M − H] − (calcd for $C_{16}H_{11}O_4$, 267.0663).

*Vestitol (**5**)*. This compound is a white powder; UV λ_{max} = 280 nm; ^{1}H and ^{13}C NMR data as reported by Piccinelli et al.[22] HRESIMS negative mode m/z 271.0979 [M − H] − (calcd for $C_{16}H_{15}O_4$, 271.0976).

Neovestitol (**6**). This compound is a white powder; UV λ_{max} = 280 nm; ^{1}H and ^{13}C NMR data as reported by Franchin et al.[23] HRESIMS negative mode m/z 271.0978 [M − H] − (calcd for $C_{16}H_{15}O_4$, 271.0976).

*Medicarpin (**7**).* This compound is a white powder; UV λ_{max} = 285 nm; ^{1}H and ^{13}C NMR data as reported by Martínez-Sotres et al.[24] HRESIMS negative mode m/z 271.0970 [M − H] − (calcd for $C_{16}H_{15}O_4$, 271.0976).

*Biochanin A (**8**).* This compound is a white powder; UV λ_{max} = 260 nm; ^{1}H and ^{13}C NMR data as reported by Almeida et al.[25] HRESIMS negative mode m/z 283.0612 [M − H] − (calcd for $C_{16}H_{11}O_5$, 283.0612).

*7-O-methylvestitol (**9**)*. This compound is a white powder; UV λ_{max} = 280 nm; ^{1}H and ^{13}C NMR data as reported by Piccinelli et al.[22] HRESIMS negative mode m/z 285.1137 [M − H] − (calcd for $C_{17}H_{17}O_4$, 285.1132).

*Mixture of Guttiferone E and Xanthochymol (**10**).* This compound is a yellow amorphous powder; UV λ_{max} = 245, 354 nm; ^{1}H and ^{13}C NMR data as reported by Li et al.[26] HRESIMS negative mode m/z 601.3546 [M − H] − (calcd for $C_{38}H_{50}O_6$, 601.3529)

*Oblongifolin B (**11**).* This compound is a yellow amorphous powder; UV λ_{max} = 245, 354 nm; ^{1}H and ^{13}C NMR data as reported by Hamed et al.[27] HRESIMS negative mode m/z 601.3546 [M − H] − (calcd for $C_{38}H_{50}O_6$, 601.3529). HRESIMS negative mode m/z 601.3546 [M − H] − (calcd for $C_{38}H_{50}O_6$, 625.3529).

In Vitro Toxicological Evaluation of BRPE and *D. ecastaphyllum* Extracts.

Cell Lines and Cell Culture. In this study, different cell lines were used: normal cell lines = human lung fibroblast (GM07492A), human gingival fibroblast (FGH), and Chinese hamster lung fibroblast (V79) and tumor cell lines = human breast adenocarcinoma (MCF-7), human glioblastoma (U343), and human cervical adenocarcinoma (HeLa). The FGH and MCF-7 cells were kindly provided by Prof. Dr. Eliana Aparecida Varanda, Universidade Estadual Paulista, Araraquara Campus. The U- 343 cells were kindly provided by Prof. Dr. Mari Cleide Sogayar, Nucleus of Cellular and Molecular Therapy at the University of São Paulo, São Paulo Campus. The HeLa cell line were purchased from the Rio de Janeiro Cell Bank (BCRJ).

Cells were cultured in HAM F10 + DMEM medium (Sigma-Aldrich, 1:1), with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma- Aldrich), and 2.38 mg/mL Hepes (Sigma-Aldrich), at 37 °C with 5% CO_2. All *in vitro* assays were performed with three replicas per treatment and on three different days to ensure reproducibility.

Cytotoxic Assay.

The evaluations of the cytotoxicity of the BRPE, *D. ecastaphyllum* stem extract (DSE), and *D. ecastaphyllum* leaves extract (DLE) were carried out in GM07492A normal cells, with 24 h of exposition to the treatments, using a Cell Proliferation Kit II (Roche Diagnostics) according to the manufacturer's instruction guidelines. The extracts were dissolved in dimethyl sulfoxide (DMSO, 1%), to obtain maximum solubility of the samples and no interaction with the cytotoxicity test.[28] With the intention of increasing the concentration of the substances present on the extracts, concentrations ranging from 39.1 to 5000 μg/mL as recommended by the OECD were tested.[28] Moreover, DMSO 25% was used as positive control considering that doses greater than 1% can induce toxicity in some normal cell lines.[29] Negative control (no treatment) was also included.

The absorbance of the samples was determined by a multiplate reader (ELISA-Biochrom Asys) at 450 nm with a reference length of 620 nm. Cell viability was expressed as the concentration required to inhibit 50% of cell viability (IC_{50}). The IC_{50} was determined using GraphPad Prism 6.

Genotoxic Assay.

The *in vitro* micronucleus assay was performed following OECD 487 recommendations, using V79 cells for being a validated model for this test.30 Cell culture and treatment procedures were those described by Reis et al.[31] The concentrations of the extracts evaluated for genotoxic potential were determined from the XTT assay results, using cytotoxicity as the criterion of choice. Cells were exposed during 3 h to the following concentrations: 20, 40, and 60 μg/mL for BRPE; 30, 60, and 120 μg/mL for DSE; and 25, 50, and 100 μg/mL for DLE. Negative control (no treatment), solvent control (DMSO, 1%), and positive control (methylmethanesulfonate, MMS, Sigma-Aldrich, 44 μg/mL) groups were included as suggested by the protocol.[30]

For each treatment group, 3.000 binucleate cells were counted as established by Fenech.[32] The nuclear division index (NDI) and the cytotoxicity index (CI) were assessed according to Eastmond and Tucker[33] and Kirsch-Volders et al.,[34] respectively.

Zebrafish Acute Toxicity Assay of BRPE.

Male and female adult zebrafish (*Danio rerio*) (six months old, bodyweight 0.35 ± 0.18 g, body size 3.12 ± 0.70 cm) were purchased from a local commercial source. The assays involving adult zebrafish followed the OECD 203 recommendations,[35] and the experimental protocols used were submitted and approved by the Ethics Committee on the Use of Animals at the University of Franca (CEUA n° 1606040918).

Fish were maintained in stock aquaria with mineral water and aeration for 14 days before the assays, under standardized temperature (26.0 ± 2 °C), pH (7.31 ± 0.49), dissolved oxygen (60% air saturation), and photoperiod (12:12 h light/dark) conditions. The animals were fed twice a day with a commercially available nutritionally balanced artificial fish meal (Tetra Color Flakes, Tetra). Housing parameters were measured during BRPE exposure. The experiment was performed in glass containers filled with 2 L of exposure solution with constant aeration and without feeding.

Fish were exposed to different concentrations of the extract, at 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 mg/L, for 96 h in a static system. At the end of the exposure, the average lethal concentration was determined, correspondent to the concentration that causes lethality in 50% of the exposed organisms (LC_{50}). Two replications were performed, including males and females, chosen at random. The first repetition contained three animals per treatment group, and in the second repetition, four animals per treatment group. Thus, seven animals were used for each concentration evaluated. During the treatment period with the extract, the animals were observed at 24, 48, 72, and 96 h, after the beginning of the exposure, regarding the occurrence of mortality; morbidity; macroscopic, behavioral, swimming, fluctuation, and orientation changes; and other clinical changes (for example, respiratory changes).

Cytotoxic Assay of BRPE Isolated Compounds.

The cytotoxic activity of eight isolated compounds from BRPE was evaluated in four different cell lines by XTT assay in triplicate. In order to assess the selectivity of the substances, one nontumor cell line (FGH) and three tumor cell lines of a different type of tissues (MCF7, U-343, and HeLa) were selected. Cell culture and treatment procedures were those described

by Abrão et al.[36] The compounds 1, 3, 5, 6, 7, 9, 10, and 11 with a purity >96% were tested at concentrations ranging from 1.25 to 50 μg/mL. Negative control (no treatment), solvent control (DMSO 1%), and a chemotherapy drug as positive control (doxorubicin, DXR) treatments were included. Cell viability was expressed as IC_{50}. The selectivity index (SI) for the tumor cell lines was calculated (normal cells IC_{50}/tumor cells IC_{50}).

Statistical Analysis. The results were analyzed using GraphPad Prism version 6, using ANOVA and the Tukey test with 95% confidence to detect significant differences between treatments. For the determination of the LC_{50}, the graphical interpolation method was used.

RESULTS

Chemical Characterization of BRPE.

The HPLC-DAD analysis of the Brazilian red propolis extract displayed a complex chromatogram comprising many secondary metabolites. BRPE and DSE showed similar chromatographic profiles, but the relative intensity of the internal standard with the samples' peaks in the chromatograms showed higher intensity for the compounds in the BRPE sample (Figure 3.1-1). On the other hand, DLE displayed a different chromatographic profile compared to those of both BRPE and DSE (Figure 3.1-1 B, C). *D. ecastaphyllum* resin presented a highly similar chromatographic profile, compared to BRPE (Figure S. 8-1, Supporting Information), with higher intensity of the peaks of compounds 1−9, and absence of the chromatographic peaks eluting around 80 min. The extraction process used to obtain the hydroalcoholic extract of red propolis furnished 140 g, 43% of yield. The solid- phase extraction of the hydroalcoholic extract of red propolis furnished 23.8, 54.2, 4.3, and 3.6 g of the hexanes, dichloromethane, ethyl acetate, and aqueous ethanol fractions, respectively. The hexanes and dichloromethane fractions, obtained by the solid-phase partition process with BRPE, exhibited a predominance of different types of secondary metabolites. The hexanes fraction's chromatogram exhibits the prevalence of low polar compounds, and the dichloromethane fraction's chromatogram showed greater complexity, which is highly similar to the BRPE chromatographic profile. Hexanes fraction's chromatographic fractionation led to the isolation of polyprenylated benzophenones, comprising two major fractions, the mixture of guttiferone E (10) with xanthochymol and oblongifolin B (11) (Figures 3.1-1A and 3.1-2).

The chromatographic fractionation of the dichloromethane fraction led to the isolation of several phenolic compounds, including flavanones (liquiritigenin 1), isoflavones (calycosin 2, formononetin 4, biochanin A 8), chalcones (isoliquiritigenin 3), isoflavanes (vestitol 5, neovestitol 6, and 7-*O*-methylvestitol 9), and pterocarpans (medicarpin 7) (Figure 3.1-2).

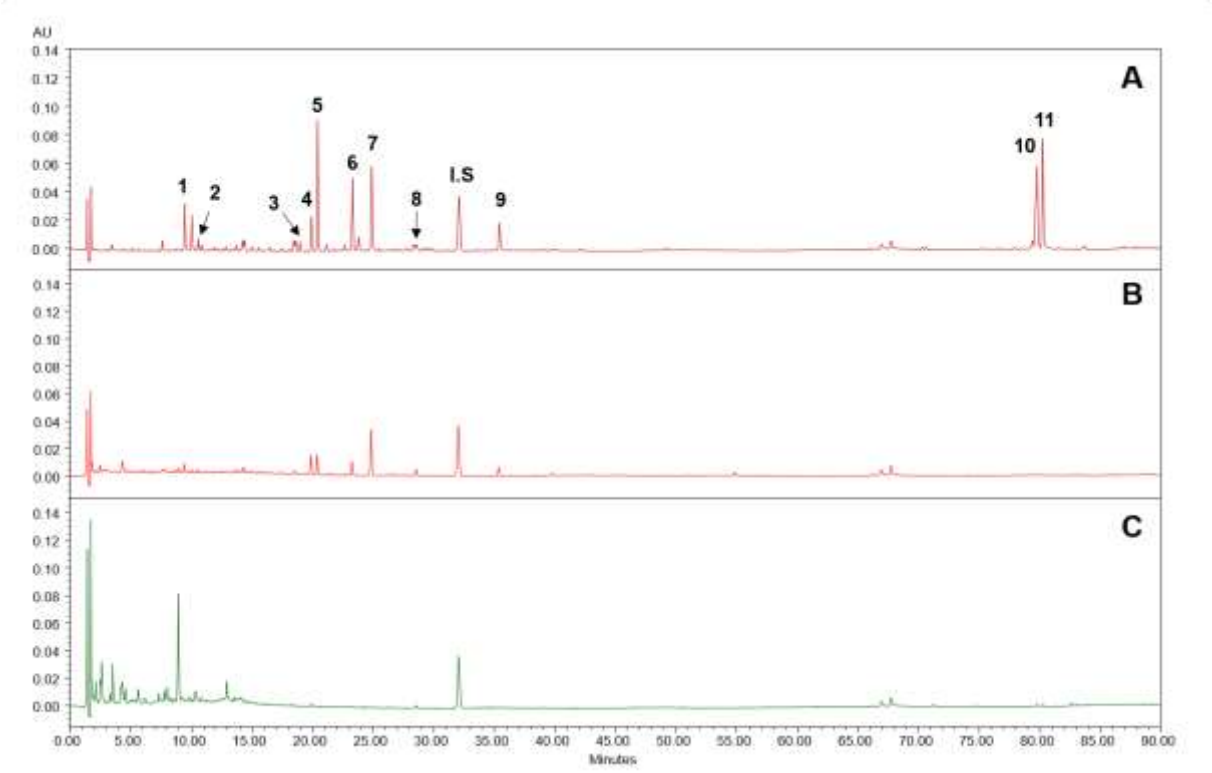


Figure 3.1-1. HPLC chromatographic profiles of the Brazilian red propolis and *D. ecastaphyllum* extracts: (A) Brazilian red propolis extract; (B) *D. ecastaphyllum* stems extract; (C) *D. ecastaphyllum* leaves extract. Identity of the isolated constituents: liquiritigenin (1), calycosin (2), isoliquiritigenin (3), formononetin (4), vestitol (5), neovestitol (6), medicarpin (7), biochanin A (8), 7-*O*-methylvestitol (9), mixture of guttiferone E and xanthochymol (10), oblongifolin B (11).

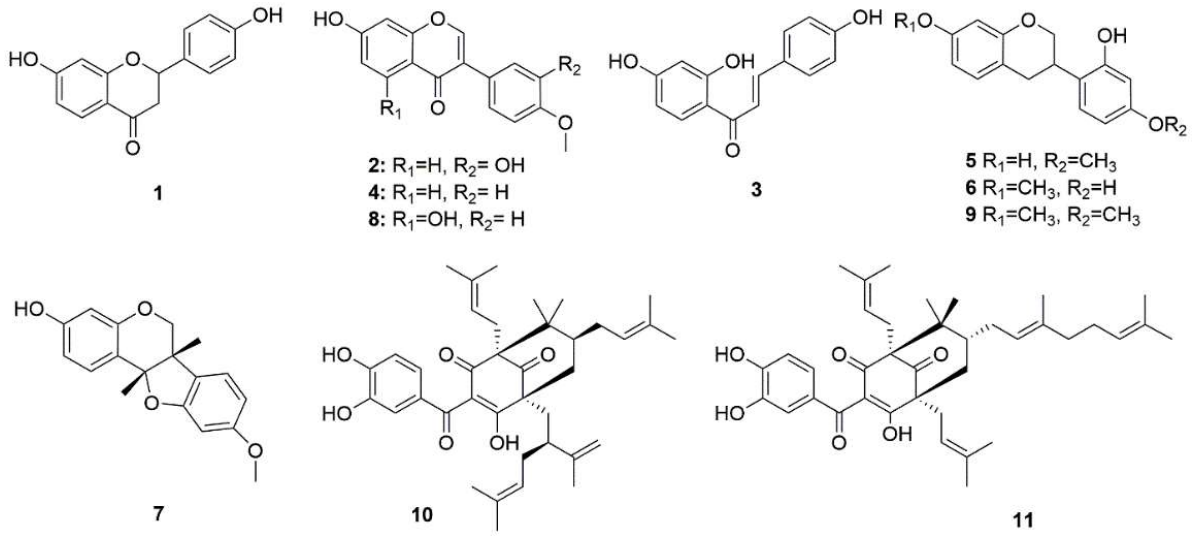


Figure 3.1-2. Chemical structures of the main constituents of the Brazilian red propolis extract: liquiritigenin (1), calycosin (2), isoliquiritigenin (3), formononetin (4), vestitol (5), neovestitol (6), medicarpin (7), biochanin A (8), 7-*O*-methylvestitol (9), mixture of guttiferone E and xanthochymol (10), oblongifolin B (11).

The quantification revealed major presence of vestitol in BRPE extract (65.06 ± 0.17 μg/mL), followed by medicarpin (48.72 ± 0.18 μg/mL), neovestitol (33.03 ± 0.70 μg/mL), 7-*O*-methylvestitol (16.12 ± 0.29 μg/mL), and formononetin (6.64 ± 0.01 μg/mL). In DSE, the quantitation showed major presence of medicarpin (28.98 ± 0.03 μg/mL), followed by vestitol (10.10 ± 0.02 μg/mL), neovestitol (6.40 ± 0.27 μg/ mL), 7-*O*-methylvestitol (5.79 ± 0.17 μg/mL), and formononetin (3.94 ± 0.01 μg/mL). In the DLE, the analyzed compounds were not detected (Figure 3.1-1).

In Vitro Toxicological Evaluation of BRPE and *D. ecastaphyllum* Extracts.

Cytotoxic Assay.

The three evaluated extracts reduced the cell viability significantly at concentrations of 78.1 μg/mL and greater (Figure 3.1-3). However, BRPE showed a lower IC_{50} (102.7 ± 2.6 μg/mL) in comparison with those of *D. ecastaphyllum* extracts (143.4 ± 14.9 and 253.1 ± 8.3 μg/mL for DSE and DLE, respectively).

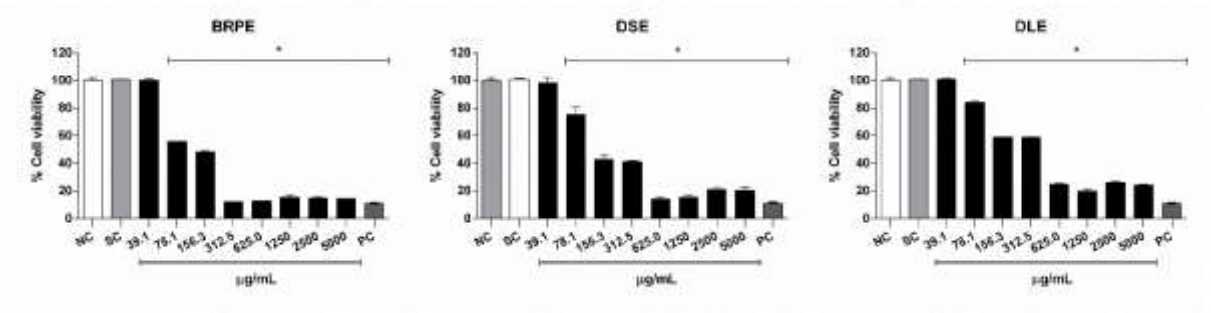


Figure 3.1-3. Effect of Brazilian red propolis (BRPE) and *D. ecastaphyllum* extracts (stems-DSE and leaves-DLE) on cell viability of GM07492A cells after 24 h of treatment. NC = negative control (culture medium); SC = solvent control (dimethyl sulfoxide, DMSO 1%); PC = positive control (DMSO 25%). Values are expressed as mean ± SD (n = 3). *Significantly different from the NC group, $p < 0.05$ ANOVA, and post Tukey test.

Genotoxic Assay.

No significant difference in the induction of micronuclei were observed among the groups treated with different concentrations of BRPE, DSE, and DLE in comparison with the negative control (Table 3.1-1). However significant reductions in NDI were obtained in treatments with BRPE 60 μg/mL (CI = 72.30%), DSE 120 μg/mL (CI = 90.77%), and DLE 100 μg/mL (CI = 52.30%), revealing cytotoxicity.

Zebrafish Acute Toxicity Assay of BRPE.

Abnormal behavior was observed on the treated animals at BRPE concentrations of 12.5−100 mg/L. Lack of balance, an accelerated operculum, and altered fluctuation were observed in some individuals, leading to mortality. However, in the lowest concentrations (0.8−6.3mg/L), there were no behavioral and physiological changes. The LC_{50} calculated was 9.37 mg/L.

Table 3.1-1. Frequencies of Micronucleated Binucleated Cells (MNBCs), Nuclear Division Index (NDI), and Cytotoxicity Index (CI)[a].

Treatment (µg/mL)	MNBCs[a]	NDI[b]	CI (%)
NC	3.33 ± 1.15	1.65 ± 0.03	-
DMSO (1%)	4.00 ± 1.00	1.57 ± 0.01	12.30
BRPE 20	5.33 ± 1.15	1.52 ± 0.10	20.00
BRPE 40	7.33 ± 1.53	1.41 ± 0.07[c]	36.92
BRPE 60	-	1.18 ± 0.02[c]	72.30
DSE 30	2.00 ± 1.00	1.64 ± 0.08	1.53
DSE 60	3.67 ± 1.15	1.57 ± 0.02	12.30
DSE 120	-	1.06 ± 0.07[c]	90.77
DLE 25	4.00 ± 1.00	1.41 ± 0.06	36.92
DLE 50	4.67 ± 0.58	1.44 ± 0.17	32.30
DLE 100	5.67 ± 1.53	1.31 ± 0.03[c]	52.30
PC (MMS, 44 µg/mL)	23.33 ± 4.16[c]	1.53 ± 0.12	18.46

[a] Obtained in V79 cells treated with BRPE, DSE, and DLE and their respective controls. MMS, methylmethanesulfonate. Values are mean ± SD. [b] 3000 binucleated cells were analyzed per treatment. [c]1500 cells were analyzed per treatment. [d] Significantly different from the negative control ($p < 0.05$).

Cytotoxic Assay of BRPE Isolated Compounds.

The IC_{50} values of BRPE isolated compounds obtained on nontumor and tumor cell lines and SI are presented in Table 3.1-2. Among the chemical constituents evaluated, oblongifolin B and vestitol were the most cytotoxic ones, with IC_{50} ranging from 37.28 to 42.34 µM and 90.12 to 110.21, but with no selectivity. Medicarpin showed a selective cytotoxic effect for the HeLa cell line (IC_{50} = 65.75 µM), with a selectivity index equivalent to 2.0.

Table 3.1-2. IC_{50} Values of BRPE Isolated Compounds Obtained in Non-Tumor and Tumor Cell Lines (µM)[a]

Compound	FGH	MCF7	U-343	HeLa
1	165,4 ± 4,3	> 175,0	> 175,0	> 175,0
3	167,8 ± 7,5	> 175,0	> 175,0	> 175,0
5	103,1 ± 7,9	96,6 ± 3,4[b]	110,2 ± 1,3	90,1 ± 4,9[b]
6	121,5 ± 2,8	132,3 ± 4,3	164,6 ± 1,5	162,6 ± 2,7
7	134,2 ± 4,8	163,6 ± 2,9	116,3 ± 0,4[b]	65,7 ± 4,5[ab]
9	155,7 ± 17,7	> 175,0	89,6 ± 2,0[b]	107,0 ± 2,8[b]
10	72,5 ± 4,6	61,1 ± 0,4[b]	79,9 ± 0,3	> 175,0
11	39,8 ± 1,7	37,3 ± 0,6[b]	40,2 ± 0,9	42,4 ± 2,4

[a] Liquiritigenin (**1**), isoliquiritigenin (**3**), vestitol (**5**), neovestitol (**6**), medicarpin (**7**), 7-*O*-methylvestitol (**9**), mixture of guttiferone E and xanthochymol (**10**), oblongifolin B (**11**). Normal cell line: Human gingival fibroblasts (FGH). Tumor cell lines: human breast adenocarcinoma (MCF7), human glioblastoma (U-343), human cervical carcinoma (HeLa). Values are presented µg/mL, as mean ± SD. [b] Selective index > 2.00. [c] IC_{50} value significantly different from the IC_{50} FGH cell line ($p < 0.05$).

DISCUSSION

Chemically, Brazilian and Cuban red propolis are characterized by the presence of flavones, flavanones, isoflavonoids, and pterocarpanes, compounds that were also identified in *D. ecastaphyllum* exudates, recognizing this species as their primary botanical source.[6,7,37] Brazilian red propolis chemical studies described the presence of isoliquiritigenin, medicarpin, biochanin A, liquiritigenin, medicarpin, vestitol, and neovestitol,[13,38] secondary metabolites isolated on this study.

However, the presence of polyprenylated benzophenones in the studied sample (Canavieiras, Bahia), also present in samples from Alagoas (Brazil) and absent in Cuban propolis,[22,37] suggested the presence of other botanical sources in the Brazilian red propolis. Recently, *Symphonia globulifera* L.f., belonging to Clusiaceae family, was reported as the botanical source of the polyprenylated benzophenones detected in the Brazilian red propolis samples.[8]

The chromatographic profiles of the analyzed extracts demonstrated similarity between the red propolis extract and stem extract from its primary botanical source *D. ecastaphyllum*. The similarity was expected when we consider the stems as the source of the red resin collected by the bees for the propolis production.[6] Formononetin (**4**), vestitol (**5**), neovestitol (**6**), and medicarpin (**7**) are present in both extracts, while the leaves extract from *D. ecastaphyllum* exhibit a different profile with the presence of high polar compounds appearing on the chromatogram.

Considering the chemical composition, and the association of its compounds to a wide variety of biological activities, Brazilian red propolis has the potential to be used beyond the traditional folk medicine and be incorporated in the natural products industry. Nowadays propolis has been categorized as a functional food or dietary supplement; however, some regulatory agencies declared that propolis products with therapeutic indication must be registered as specific drugs.[1,16,39] The registration of new drugs includes the presentation of preclinical and clinical reports of safety and efficacy, but clinical trials with propolis are scarce; some of them did not prioritize the evaluation of safety in the consumption or use of propolis.[1] Studies about Brazilian red propolis are even more scarce.

One of the first steps in the evaluation of a product safety is the *in vitro* evaluation of the toxicology potential. Several papers report the antiproliferative activity of red propolis in tumor cell lines, but a few have been developed with nontumor cells, which does not yet allow for the establishment of the safety and selectivity of this natural product.[40] Silva Frozza et al.[13] reported an IC_{50} for the red propolis extract >150 μg/mL in human kidney embryonic cells (Hek-283), but other studies reported safe use of Brazilian red propolis just in concentrations lower than 50 μg/mL.[40] According to Lopez et al.,[40] IC_{50} values in mouse embryo fibroblasts cells (BALB/c3T3) and human keratinocytes (HaCaT) exposed to hydroalcoholic extracts of Brazilian red propolis from different locations (Sergipe, Alagoas and Paraíba) presented some differences when evaluated by MTT and neutral red (NR) assays, which detect lysosome and mitochondrial cellular damage, respectively. The MTT and NR assays showed that samples from Sergipe and Alagoas reduced viability of 50% of BALB/c3T3 cells between 65 and 85 μg/mL, whereas the Paraiba sample presents this effect between 75 and 85 μg/mL. These results were also reported for the NR test of HaCaT cells. However, the MTT assay showed lower cytotoxic effect of Sergipe and Paraiba samples, with $IC_{50} > 170$ μg/mL, indicating the possible lysosome damage of the red propolis samples. The extracts used in these studies revealed the presence of liquiritigenin, formononetin, pinocembrin, medicarpin, biochanin A, and retusapurpurin B as major chemical constituents.

In this study, the red propolis extract showed cytotoxicity similar to that of the stem extract from its primary botanical source *D. ecastaphyllum*, which may be due to the similarity observed between the respective chromatographic profiles. The presence of the formononetin, vestitol, and neovestitol can be related to their lower IC_{50} value against GM07492A cells, in comparison with the *D. ecastaphyllum* leaves extract, which displays different chemical composition. In addition, the higher cytotoxicity of the Brazilian red propolis in comparison with the extracts from *D. ecastaphyllum* may be due to the presence of the benzophenones, which are from another botanical source, *S. globulifera*.

For the first time, we are reporting studies of the *in vitro* genotoxic potential of Brazilian red propolis. The results obtained showed the absence of genotoxicity of BRPE and its primary botanical source, *D. ecastaphyllum*. However, the cytotoxicity of the extracts was observed in the highest concentrations evaluated, with BRPE being more cytotoxic.

Regarding the acute toxicity assessment in fish, the results obtained revealed LC_{50} equivalent to 9.37 mg/L. There was a mortality of fish in the concentrations of 12.5 to 100 mg/L for BRPE. In addition to mortality, there was also a lack of balance, an accelerated operculum and altered fluctuation, with altered behavior in individuals. Thus, these concentrations may have affected the central nervous system, causing the swimming capacity to be compromised and the death of individuals. According to Hofer et al.,[41] some substances can cause epithelial hyperplasia, increasing the interlamellar distance and hindering the diffusion of oxygen, which results in respiratory stress and osmoregulatory failure. Thus, this change may be involved in the observed BRPE toxicity.

During the evaluations performed at 24, 48, 72, and 96 h after the beginning of the exposure, the animals showed agitation, loss of panting capacity, increased opercular beat, erratic swimming, and lethargic behavior. It is important to note that animal mortality was observed throughout the experimental period. Clinical signs of intoxication include: respiratory disorders (increased respiratory rate and search for surface oxygen) and later inactivity. Changing the opercular frequency is one of the signs of intoxication. Fish, as a defense of the organism, produce mucus on the surface of the branchial epithelium. Thus, this accumulation of mucus that the fish produce as a form of defense ends up impairing the diffusion of gases.[42,43] Consequently, in the present study, the increase in the opercular beat observed in zebrafish before death can be understood as a defense mechanism against the aggressive chemical agents present in the water.

Other studies observed that the red propolis of Alagoas, Brazil, was considered nontoxic by the acute toxicity test with the crustacean Artemia salina in the concentrations tested (200−1000 μg/mL).[44] Some papers show a good correlation between A. salina assays and acute oral toxicity tests with rodents.[45,46] Silva et al.[14] tested the toxicity of BRPE in rats for 14 days, and it was observed that oral administration at a dose of 300 mg/kg did not produce lethal effects; however, some symptoms of toxicity were observed on the first hours after administration.

Behavioral changes observed in fish may be due to the presence of some chemical markers of BRPE. According to Bugel et al.,[47] the flavonoid class's phytochemicals can induce neurobehavioral and endocrine-disrupting effects throughout development, becoming a relevant toxicological issue. These authors evaluated the toxicity of 14 flavonoids in the development of the fish larval embryo. Among these, formononetin and biochanin A, flavonoids present in BRPE, were evaluated. The results showed that formononetin led to changes in the behavioral test of larval photo motor response at concentrations of 25 and 50 μM and proved to be toxic for the development of zebrafish at concentrations of 5 and 10 μM. Biochanin A, on the other hand, at concentrations of 25 and 50 μM, resulted in the mortality of 100% of individuals in 72 h after fertilization. In addition, this flavonoid demonstrated developmental toxicity at concentrations of 5 and 10 μM. These studies prove that the toxicity

of BRPE observed in the present study may be due, in part, to the presence of formononetin and biochanin A in its chemical composition.

For the antiproliferative activity of the eight major isolated compounds from BRPE, oblongifolin B showed higher cytotoxicity against all cell lines, followed by vestitol, both with no selective effect. Xu et al.[48] also observed that oblongifolin B reduced the cell viability of human colorectal cancer cell lines (HT-29 and HCT-116), without significant negative effects in normal colon epithelium cells (CCD-18Co), showing selective activity.

Medicarpin showed a selective cytotoxic effect against the HeLa cell line. The benzophenone oblogifolin B (**10**) presented the lowest IC_{50} values against the tumor cell lines. Other polyprenylated benzophenones, such as guttiferone E, have been reported as potential lead compounds against tumor cell lines. IC_{50} values between 7.87 and 13.92 μM have been obtained against leukemia (CCRF−CEM, HL-60), breast cancer cells with resistance protein BCRP (MDA-MB-231- BCRP clone 23), nontransduced human glioblastoma multi- form (U87MG), human hepatocellular carcinoma cells (HepG2), and human wild-type colon cancer (p53+/+) cells.[49] The compound was also tested against normal hepatocytes (AML 12), showing an IC_{50} of 66.45 μM. Overall, the Brazilian red propolis benzophenones should be further studied as a promising class of antitumor compounds.

The results obtained in the present study revealed the similarity of red propolis's chemical profile with the stem of its primary botanical source *D. ecastaphyllum*. Also, characteristic markers of red propolis have been identified in the sample collected in the region of Canavieiras, Bahia, highlighting the presence of polyprenylated benzophenones and isoflavans. Our results are an initial step on the considerations of safe doses of Brazilian red propolis extract and of some of their constituents. The potential clinical benefits proven for red propolis and their secondary metabolites must not just be proved but also considered as secure, through the evaluation of their toxicological potential with *in vitro* research base. Additional *in vivo* studies should be undertaken to determine the efficacy and safety conditions for using Brazilian red propolis in human health, considering the nonclinical and preclinical toxicological evaluation results. Furthermore, some constituents of red propolis showed antiproliferative activity against tumor cell lines. Future investigations on its mechanisms of action will help obtain potential antitumor compounds, based on the present selective cytotoxicity against some of the tumor cell lines tested.

ASSOCIATED CONTENT

*ѕı Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00356. NMR data of isolated compounds (PDF)

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3.2 CAPÍTULO 2. Desenvolvimento e validação de um método por CLAE-DAD para análise de própolis vermelha e *D. ecastaphyllum*

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Short communication

A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and *Dalbergia ecastaphyllum*

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ABSTRACT

Propolis is a natural product produced from the interaction between bees and plants. Brazilian red propolis results from *Apis mellifera* collection of resins from two plant species, being *Dalbergia ecastaphyllum* (L.) Taub, Fabaceae, the primary botanical source, containing isoflavonoids and other characteristic phenolic compounds. Several biological activities of Brazilian red propolis and their isolated compounds have been described in the literature. However, to our knowledge, there are no validated analytical methods for the analysis and standardization of products derived from this type of propolis reported in the literature. We developed a reverse-phase high-performance liquid chromatography analytical method for the detection and quantification of nine red propolis chemical markers: liquiritigenin, calycosin, isoliquiritigenin, formononetin, vestitol, neovestitol, medicarpin, biochanin A, and 7-*O*-methylvestitol, present in Brazilian red propolis extracts and *D. ecastaphyllum*. The developed method was also applied to the analyses of *D. ecastaphyllum* samples and seasonal analysis of Brazilian red propolis. Good detection response, linearity, precision, and robustness were obtained by the method, being reliable for the quality control of Brazilian red propolis extracts, raw propolis, plant material, and their derived products. The red propolis chemical markers were present in *D. ecastaphyllum* stems at lower concentrations. The seasonal analysis of Brazilian red propolis extract showed higher phenolic compound concentration on periods of the rainy season with higher humidity and lower solar radiation.

Keywords: Isoflavonoids. Flavanones. Pterocarpans. Liquid chromatography. Validation.

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INTRODUCTION

Propolis is the result of one of the most interesting plant-insect interactions. Several species of bees and plants worldwide are involved in the production of this natural product. However, special attention has been focused on *Apis mellifera* honeybee [1].

The red type of propolis is characterized by chalcones, isoflavones, and pterocarpans [2–4]. However, red propolis is a complex group with several chemical composition differences [3–6], strongly influenced by the location and the correlations between the botanical source and the meteorological conditions [1,7,8].

Different chromatographic techniques have been used for the chemical characterization of red propolis. High-performance liquid chromatography with photodiode array detection (HPLC-PDA), HPLC-electrospray ionization tandem mass spectrometry (ESI/MS) [5] and Gas Chromatography-MS methods [3] studies revealed that red Cuban propolis could be classified into three groups, the red type, composed by isoflavonoids (medicarpin, formononetin, vestitol, among others), the brown Cuban type, with the prevalence of polyprenylated derivatives (nemorosone, scrobiculatones A and B) and the yellow type with aliphatic compounds (triterpenoids and steroids).

As a result of the comparison between Cuban and Brazilian red propolis by HPLC-diode array detection (DAD)-MS method [6], *D. ecastaphyllum* was confirmed as the source of the isoflavonoids of the Brazilian sample as previously observed by Reverse Phase-HPLC analysis [9,10]. Also, benzophenones and isoflavonoids were identified in Nigerian propolis samples by HPLC–UV–Evaporative Light Scattering Detection (ELSD), HPLC–High-Resolution Mass Spectrometry (HRMS), and GC–MS analysis, finding high similarity with Brazilian red propolis [4].

Red propolis was characterized but reports on the quantitation studies of their compounds are scarce. Quantification of red propolis total flavonoids and phenolic compounds using the Folin-Ciocalteu (FC) method has been used [7,11]. However, FC quantification is limited to the sample's reducing capacity but not the compounds' content [1]. In other cases, LC-Orbitrap-Fourier transform (FTMS) methods have been used to quantify some samples [7], but even with high sensitivity, these instruments can be expensive, limiting their use [4]. RP-HPLC-PAD methods were also employed to estimate the relative peak area of compounds on red propolis samples [8], which seems to agree with HPLC-ESI//MS detection data [5]. For phenolics with strong chromophore groups, UV-based detectors are an ideal choice to develop quality control methods that guarantee selectivity and sensitivity in the identification and quantification of active compounds [12], being an easy and suitable method to be employed.

Red propolis is one of the most prominent Brazilian propolis types, being the second most produced and commercialized type. Recent research indicated that the polyprenylated

benzophenones absent on *D. ecastaphyllum* but detected on Brazilian red propolis samples result from another interaction between bees and a second botanical source identified as *Symphonia globulifera*, belonging to the Clusiaceae family [13].

In the last decade, many researchers have corroborated the biological activities of Brazilian red propolis. These activities are strongly dependent on the concentration of active constituents. However, standardization of propolis products is critical due to the variabilities in the extracts' chemical profiles [2,14]. It is crucial to establish some criteria about the concentration of the active compounds required for specific pharmacological activities and the standardization of reliable analytical procedures for their quality control [2,7,15].

Considering the pharmacological potential of Brazilian red propolis extract, we developed and validated an RP-HPLC-DAD method to detect and quantify nine phenolic compounds according to regulatory agencies' standards [16,17]. The method was employed to quantify these compounds in the primary botanical source of Brazilian red propolis and to analyze de seasonal variations in the red propolis extracts of Canavieiras, Bahia.

MATERIAL AND METHODS

Plant material

Brazilian red propolis samples were produced in Canavieiras (Bahia) by the beekeepers association of Canavieiras (COAPER), from March 2019 to February 2020. Additionally, samples of stems and leaves of *D. ecastaphyllum* (L.) Taub., were collected in the same location. Resin samples were collected in March of 2019, and samples of roots, flowers, and seeds were collected in January of 2020. The plant specimen is deposited in the herbarium of the Biology Department at the University of Sao Paulo of Ribeirao Preto (SPFR 17771).

Reagents and chemicals

Propolis and plant extraction was performed with HPLC ethanol grade solvent purchased from Merck Millipore (Darmstadt, Germany), and water purified by a Milli-Q-plus system from Merck Millipore. The chromatographic method development used acetonitrile HPLC grade supplied by SK Chemicals and formic acid obtained from Synth. Benzophenone and veratraldehyde standards were purchased from Sigma-Aldrich.

The flavonoids and derivatives from Brazilian red propolis: liquiritigenin (**1**), calycosin (**2**), isoliquiritigenin (**3**), formononetin (**4**), vestitol (**5**), neovestitol (**6**), medicarpin (**7**), biochanin A (**8**), and 7-*O*-methylvestitol (**9**), were previously isolated and identified by our research group. The purity of the isolated compounds was estimated to be greater than 97 % by high-performance liquid chromatography (HPLC) and NMR.

Sample preparation

Red propolis and D. ecastaphyllum samples preparation

The propolis and plant samples were frozen and powdered. Twenty mg of each sample were transferred to a 5 mL Eppendorf flask, and 200 µL of 70 % aqueous ethanol were added.

Samples were submitted to ultrasound extraction for 30 min. Extracts were centrifuged at 1,500 × g, and the supernatants were filtered on a 0.45 um PTFE filter. The extraction process was repeated twice. The obtained extracts were dried on a speed vacuum. Solutions of 1 mg/mL of each extract were prepared by adding the internal standard (IS) at a final concentration of 20 µg/mL before HPLC analysis.

Chromatographic conditions

The chromatographic method was developed on a Waters 2695 HPLC instrument, coupled to a 2998 photodiode array detector (PDA), a 1525 binary solvent delivery system, and a 2707 autosampler. The software Empower 3 was used as a controller of the analytical system and data processor. All experiments were performed in triplicate. Chromatographic analysis was carried out on a Supelco Ascenti Express C-18 (150 × 4.6 mm, 2.7 µm) column. The mobile phase consisted of water (A) (0.1 % formic acid) and acetonitrile (B) in gradient condition as follows: 20→50 % of B in 40 min; 50→100 % of B in 41 min; 100 % of B until 45 min; going from 100→20 % in 46 min., to reestablish the initial conditions until 50 min. The chromatography was performed at a flow rate was of 1 mL/min at 40 ˚C with an injection volume of 10 µL. PDA reading range was set at 220–500 nm, chromatograms were observed at 275 and 315 nm, and acquired at different wavelengths (249, 254, 260, 276, 280, 285, 376), according to the maxima absorption of each compound.

Method validation

The chromatographic peaks were evaluated in comparison with authentic standards to determine the red propolis flavonoids' selectivity by analyzing the following parameters: retention time, peak area, resolution, and UV spectra. The linearity was undertaken by preparing a stock solution with nine isolated compounds, using benzophenone as IS at a final concentration of 20 µg/mL. Serial dilutions were made, achieving seven different concentrations (80, 60, 40, 20, 10, 5, and 1 µg/mL) to determine each compound's linear dynamic range. The solutions were injected in triplicate for four consecutive days. The ratio between each flavonoid standard and the IS areas was the response used to plot the analytical curve. The responses' linearity was evaluated by estimating the residual value of replicates (R), the coefficient of determination (R2), and the lack of fit test. The LOD and LOQ were calculated according to the guides [16]. Three levels of concentrations of the calibration curve (80, 40, and 10 µg/mL) were selected to measure the repeatability of the results on the same day and interday (four consecutive days). The method's robustness was determined by making changes in the wavelength, flow rate, and elution gradient, using a Box, Hunter & Hunter factorial design elaborated through the Statistica 8.

For the evaluation of the recovery, 11 g of grounded red propolis matrix were exhaustively extracted in a Soxhlet apparatus for 8 h, using 300 mL of 96 % of ethanol. A standard solution with three major compounds isolated from red propolis in higher yielding (vestitol, neovestitol, and medicarpin), was prepared at a final concentration of 40 µg/mL of each compound. Then, the dried matrix, free of compounds, was spiked with the standards solution in three concentration levels by adding to 100 mg of the matrix: 2 mL (80 µg/mL, high level), 1 mL (40 µg/mL, medium level), and 0.25 mL (10 µg/mL, low level) of the standard solution. Four

replicates were prepared for each level. The spiked matrix was dried at room temperature and then extracted using an aqueous ethanol solution (70 %), with benzophenone as IS at 20 µg/mL, and coumarin as secondary standard (20 µg/mL). The extraction process was performed using a shaker incubator at 30 °C and 120 rpm for 90 min. After extraction, samples were filtered and analyzed by HPLC. The recovery percentage was calculated based on the real and theoretical analytical responses.

Method application

Samples of *D. ecastaphyllum* were prepared as previously described and analyzed by the developed method to quantify the selected phenolic markers in the different organs of *D. ecastaphyllum*. The monthly samples of Brazilian red propolis, collected from March of 2019 to February of 2020, were prepared and analyzed for the seasonal analysis.

Multivariate analyses

A dataset including different concentrations of the nine quantified metabolites of Brazilian red propolis samples was transferred to a spreadsheet for multivariate analyses. The PCA analysis was carried out by using the "prcomp" function and the aid of "ggplot2" package for scatter plot assembling on R software (version 3.6). The hierarchical clustering heatmap was performed using the Euclidian distance and the Ward clustering method employing the package "gplots" for R software.

RESULTS AND DISCUSSION

Chromatographic conditions

Brazilian red propolis is composed of several secondary metabolites, highlight flavonoids (liquiritigenin), chalcones (isoliquiritigenin), pterocarpanes (medicarpin), isoflavones (formononetin), isoflavans (vestitol, neovestitol, and 7-*O*-methylvestitol), and prenylated benzophenones (mixture of guttiferone E/xanthochymol and oblongifolin B) [13].

The presence of the isomers guttiferone E and xanthochymol as mixture, discovered through the NMR spectrum analysis, led us to test several gradient methods. A long term, isocratic 100 % organic mobile phase elution showed efficacy on the separation of guttiferone E and xanthochymol peaks, with low resolution (Fig. S. 8-2, Supplementary material). *D. ecastaphyllum* phenolic compounds were selected to develop a simple method comprising several secondary metabolites classes, with a reduced time of analysis (Figs. 3.2-1 and 3.2-2).

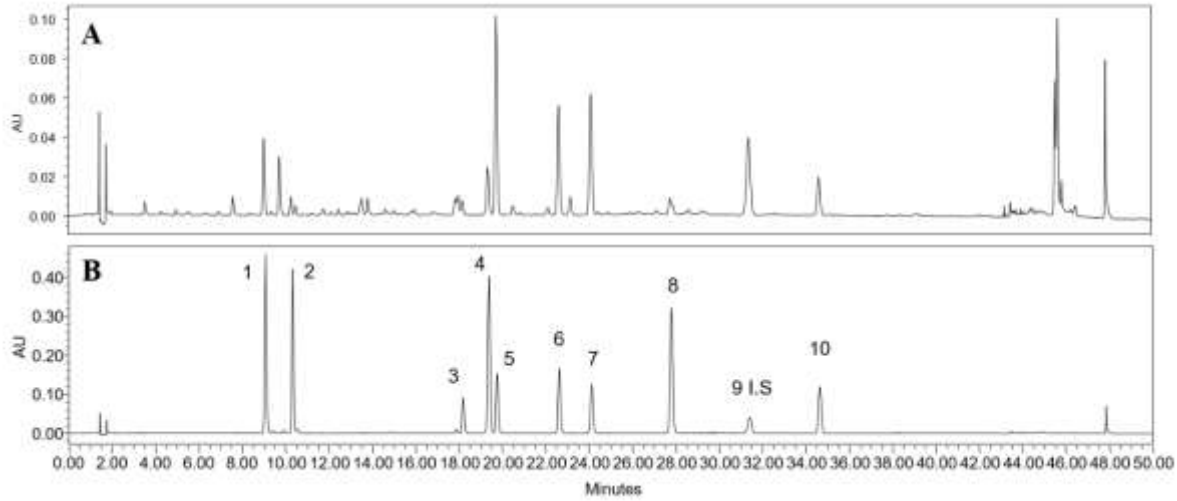

Figure 3.2-1. A) Chemical profile of Brazilian red propolis extract using the developed method. B) Brazilian red propolis standards used for the validation process: 1, liquiritigenin; 2, calycosin; 3, isoliquiritigenin; 4, formononetin; 5, vestitol; 6, neovestitol; 7, medicarpin; 8, biochanin A; 9, benzophenone (IS); 10, 7-*O*-methylvestitol.

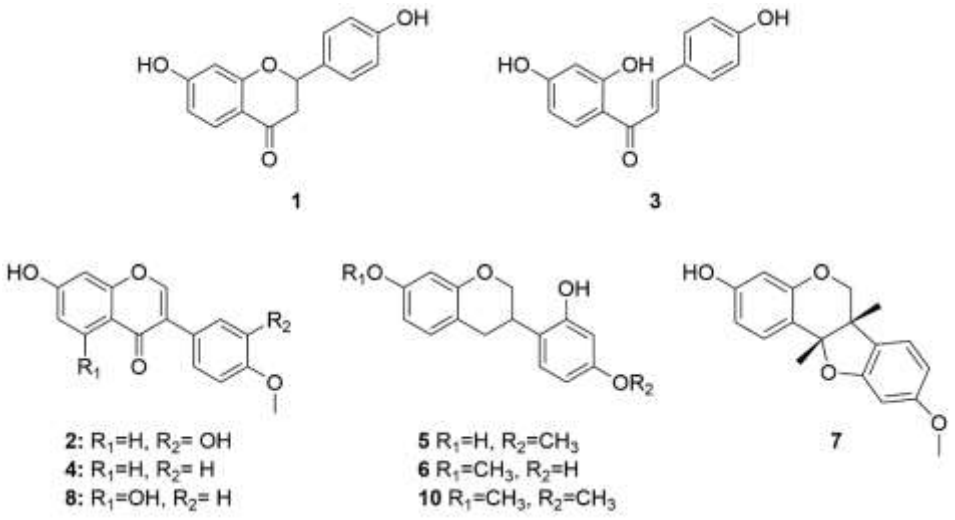

Figure 3.2-2. Chemical structures of the phenolic compounds present in Brazilian red propolis extract: 1, liquiritigenin; 2, calycosin; 3, isoliquiritigenin; 4, formononetin; 5, vestitol; 6, neovestitol; 7, medicarpin; 8, biochanin A; 10, 7-*O*-methylvestitol.

Validation of the HPLC method

The identification of the compounds of interest in the HPLC chromatographic profile was made by comparing the retention times and UV spectrum with authentic standards, with estimated purities higher than 97 %. Benzophenone was used as IS (Fig. 3.2-1A).

The correlation with retention times (tR) of the standards solution allowed to identify the following compounds in the Brazilian red propolis extract: peak 1, tR of 8.94 min, liquiritigenin, 1; peak 2, tR of 10.18 min, calycosin, 2; peak 3, tR of 18.12 min, isoliquiritigenin, 3; peak 4, tR of 19.31 min, formononetin, 4; peak 5, tR of 19.69 min, vestitol, 5; peak 6, tR of 22.58,

neovestitol, 6; peak 7, tR of 24.07, medicarpin, 7; peak 8, tR of 27.75, biochanin A, 8; peak 9, tR 31.35, benzophenone (IS) 9; and peak 10, tR 34.61, 7-*O*-methylvestitol, 10 (Figs. 3.2-1 and 3.2-2).

A linear correlation for all the compounds was observed, following the validation guidelines' recommended parameters [16,17] (Table 3.2-1). LDs between 0.09 to 0.35 µg/mL and QLs 0.29 o 1.07 µg/mL were calculated, indicating that the chromatographic method can detect and quantify the selected compounds at low concentrations. The precision parameter, presented as the relative standard deviation (RSD%) of the samples' analysis, were lower than 5.36 %, which indicated low variations of the replicates in the low (10 µg/mL), medium (40 µg/mL), and high (80 µg/mL) concentration levels (Table 3.2-2). In all cases, accuracy was greater than 99 %.

Table 3.2-1. 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
1	276	y= 0.0233x+0.001	0.9999	0.9999	0.17	0.52	-1.58411	1.24890	0.35
2	249	y= 0.036x+0.0004	0.9999	0.9999	0.35	1.07	-1.46373	1.04090	0.33
3	376	y= 0.0577x-0.0026	0.9999	0.9999	0.13	0.42	-1.31722	1.42100	0.87
4	249	y= 0.0518x-0.0007	0.9999	0.9999	0.20	0.63	-1.34801	1.18354	0.38
5	280	y= 0.0116x+0.0026	0.9999	0.9999	0.32	0.99	-3.26353	0.90352	0.34
6	280	y= 0.0134x+0.0004	0.9999	0.9999	0.22	0.66	-2.27299	1.44993	0.24
7	285	y= 0.0137x-0.002	0.9999	0.9999	0.20	0.60	-1.37082	1.15817	0.34
8	260	y= 0.0524x-0.001	0.9999	0.9999	0.16	0.49	-1.45705	1.06147	0.44
10	280	y= 0.0119x-0.0036	0.9999	0.9999	0.09	0.29	-1.58847	1.04423	0.06

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

Variations of wavelength, elution gradient, and mobile phase flow rate were tested to verify the method's sensitivity. The results suggested that variations in the wavelength cause significant changes in the isoflavonoid compounds' response on medium and high levels (Figs. S. 8-2–4, Supplementary material). The other evaluated variations or their interactions did not significantly affect the responses ($p > 0.05$). The extracted matrix was spiked with three major compounds (vestitol, neovestitol, and medicarpin) in three different concentration levels. For the isoflavonoids vestitol and neovestitol, the recovery percentage was above 97.7 % at the three levels, and for medicarpin, the recovery percentage was between 84.2–91.5% (Table 3.2-2). The hydroalcoholic extraction method was reliable for the isoflavonoids extraction used in the recovery studies.

Table 3.2-2. Precision and accuracy of the method.

Compound	Level	Precision (RSD)		Accuracy (%)	E(%)	Recovery (%)	% Compound content on Soxhlet extract (g/100mg)
		Intraday	Interday				
	Low	0.91	0.24	100.05 ± 0.82	0.06	-	
1	Medium	0.18	0.03	100.14 ± 1.39	0.14	-	0.02
	High	2.38	0.25	101.77 ± 1.73	1.77	-	
	Low	0.95	0.25	100.07 ± 0.98	0.07	-	
2	Medium	0.48	0.09	100.16 ± 1.34	0.16	-	0.01
	High	1.94	0.19	101.82 ± 1.75	1.82	-	
	Low	1.04	0.25	100.04 ± 0.95	0.04	-	
3	Medium	1.18	0.35	100.36 ± 1.49	0.36	-	0.07
	High	2.13	0.12	101.11 ± 1.94	1.11	-	
	Low	1.00	0.25	99.84 ± 1.04	-0.16	-	
4	Medium	0.78	0.19	100.09 ± 1.49	0.09	-	0.05
	High	2.13	0.19	101.86 ± 2.01	1.86	-	
	Low	1.10	0.31	100.05 ± 0.93	0.05	101.35 ± 1.36	
5	Medium	0.69	0.16	99.71 ± 1.30	-0.29	97.95 ± 6.21	0.08
	High	2.12	0.15	99.03 ± 6.79	-0.97	107.53 ± 1.57	
	Low	1.03	0.31	99.82 ± 0.96	-0.18	100.02 ± 1.45	
6	Medium	0.49	0.11	100.18 ± 1.22	0.18	97.75 ± 7.34	0.05
	High	2.05	0.25	100.07 ± 5.41	0.07	109.14 ± 1.61	
	Low	1.04	0.29	100.24 ± 0.93	0.24	84.29 ± 1.14	
7	Medium	0.63	0.10	100.73 ± 1.20	0.73	82.53 ± 5.44	0.10
	High	4.11	0.30	99.33 ± 3.11	-0.67	91.53 ± 1.95	
	Low	1.06	0.34	99.90 ± 0.92	-0.10	-	
8	Medium	0.60	0.09	100.14 ± 1.33	0.14	-	0.004
	High	2.32	0.19	101.22 ± 2.04	1.22	-	
	Low	1.13	0.37	99.96 ± 1.05	-0.04	-	
10	Medium	0.38	0.09	99.51 ± 1.44	-0.49	-	0.06
	High	5.36	0.55	100.24 ± 6.16	0.24	-	
	Low					92.34 ± 2.03	
I.S (9)	Medium	-	-	-	-	90,47 ± 0,95	-
	High					89,66 ± 0,66	

RSD: relative standard deviation; E: error.

Analysis of *D. ecastaphyllum* samples

Stems, leaves, roots, flowers, seeds, and resin of *D. ecastaphyllum* were processed and analyzed by using the developed method. The selected compounds were not detected in seeds, roots, and flower samples. On the other hand, there are some similarities among the chromatogram profile of leaves, stems, and resin (Fig. S. 8-6, Supplementary material). The

phenolic compounds are expressed as % g/100 g, as mean value ± standard deviation (Table 4-3).

The chromatographic profile of stems and resin of *D. ecastaphyllum* was similar, with differences only in the chromatographic peaks' intensity, with all the selected flavonoids detected in the resin sample. In previous studies, isoliquiritigenin [10], formononetin [10,18,19], medicarpin [9], biochanin A [18,19], daidzein, and pinocembrin [19] were detected as major compounds in *D. ecastaphyllum* resin, but it varied depending on its geographical location.

Our analysis revealed that the isoflavonoids vestitol, neovestitol, and the pterocarpan medicarpin were the major compounds in stems and resin samples (Table 4-3). Calycosin, biochanin A, and isoliquiritigenin were the compounds with lower concentrations in the samples. In contrast, the leaves extract was significantly different from the resin and the stem. Liquiritigenin was the only compound of the selected ones that could be detected in leaves. However, it was not possible to quantify it in the stem extract.

Table 3.2-3. Compounds content in samples of *D. ecastaphyllum* samples (% g/100g of dried raw material).

Compound	Compound content (% g/100g)		
	Stems	Leaves	Resin
1	ND	2.012 ± 0.025	1.896 ± 0.044
2	0.055 ± 0.001	ND	0.784 ± 0.004
3	0.119 ± 0.001	ND	1.743 ± 0.006
4	0.380 ± 0.0002	ND	2.506 ± 0.017
5	1.003 ± 0.002	ND	12.035 ± 0.144
6	0.642 ± 0.027	ND	8.981 ± 0.028
7	2.894 ± 0.003	ND	12.036 ± 0.022
8	0.076 ± 0.001	ND	0.382 ± 0.006
10	0.565 ± 0.017	ND	5.615 ± 0.011

ND: not detected.

Seasonal analysis of Brazilian red propolis

The validated method was used to analyze samples of red propolis from Canavieiras, Bahia, in Brazil's northeast region. Vestitol, medicarpin, and neovestitol were found during all the year at higher concentrations, and calycosin and biochanin A were found at lower concentrations in the samples (Fig. 3.2-3, Table S. 8-1, Supplementary material). March and April were the months with the highest concentration of the major compounds medicarpin (7) and vestitol (5), while August, September, and February had lower concentrations of these compounds in the Brazilian red propolis extract. September was a critical month with the lowest concentration of all compounds.

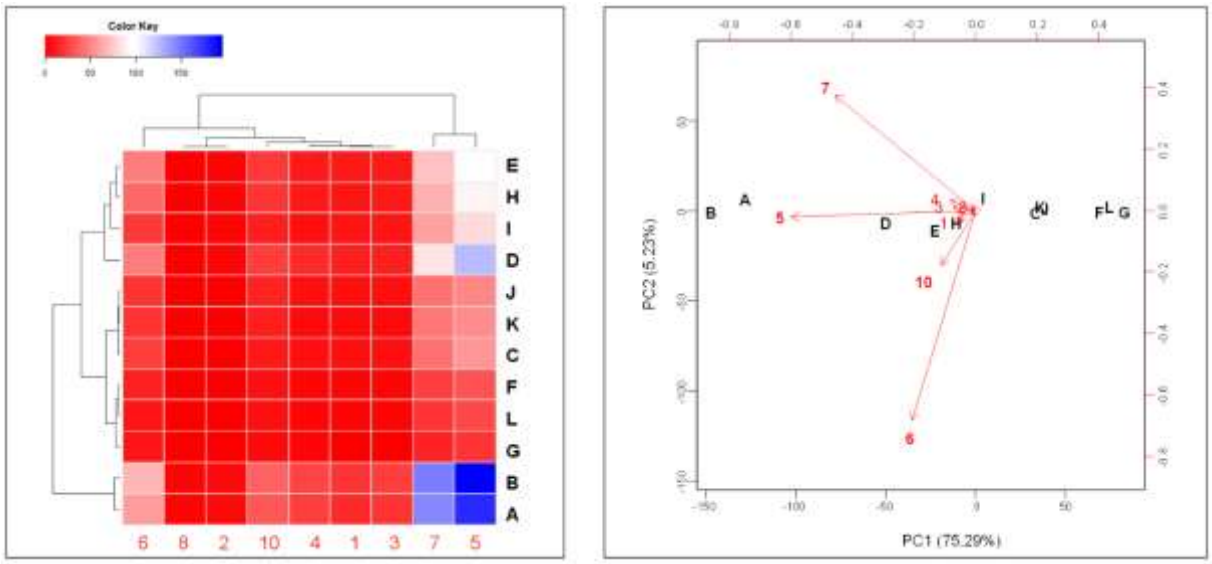

Figure 3.2-3. Multivariate analyses based on the concentrations of the main constituents in Brazilian red propolis: HCA dendrogram with heatmap (left) and PCA biplot (right). Numbers correspond to liquiritigenin (1), calycosin (2), isoliquiritigenin (3), formononetin (4), vestitol (5), neovestitol (6), medicarpin (7), biochanin A (8), and 7-*O*-methylvestitol (10). The analysis includes samples collected in Mar 2019 (A), Apr 2019 (B), May 2019 (C), Jun 2019 (D), Jul 2019 (E), Aug 2019 (F), Sep 2019 (G), Oct 2019 (H), Nov 2019 (I), Dec 2019 (J), Jan 2020 (K) and Feb 2020 (L).

The highest humidity was already correlated with the accumulation of some phenolic compounds in Brazilian red propolis samples from different northeast regions [7,8]. Bueno-Silva et al. [8] performed a seasonal estimation of the relative area of red propolis compounds from Maceio, Alagoas, and detected formononetin as the compound with the highest concentration in the samples followed by isoliquiritigenin. Vestitol and neovestitol were also detected all over the year, with prevalence in March and May, the region's rainy period. Likewise, samples collected in September presented the lowest concentrations of vestitol, neovestitol, and isoliquiritigenin. Meteorological conditions indicated higher precipitation and humidity in Canavieiras between May and October of 2019 (Table S. 8-2, Supplementary material).

Nascimento et al. [7] correlated two methods to quantify the chemical markers of red propolis of three apiaries of the state of Alagoas. The quantification by LC-UV-DAD and LC-ESI-Orbitrap- FTMS confirmed the accumulation of isoflavonoids and flavanones from May to September, a season associated with increased rain, humidity, and decreased in solar radiation. Conditions of high temperature and solar radiation between October and March decreased the isoflavonoids' concentrations and increased guttiferone. A possible preference of bees for *D. ecastaphyllum* resin in the rainy season [7] is hypothesized for the Brazilian red propolis production. Studies about the biological interaction between bees and the botanical species are important to clarify the bees' resin preferences and other biotic and abiotic conditions on red propolis production.

CONCLUSIONS

The developed HPLC-DAD method for detecting and quantifying Brazilian red propolis flavonoids displayed good linearity, precision, accuracy, and robustness according to the parameters established by international guidelines. The method is reliable for analyzing this class of compounds in red propolis extracts, *D. ecastaphyllum* extracts, and their derived products. The seasonal studies showed qualitative variations in phenolic compound amounts throughout the year.

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CRediT authorship contribution statement

Jennyfer A. Aldana-Mejía: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Gari V. Ccana-Ccapatinta:** Conceptualization, Methodology, Validation, Investigation, Visualization, Supervision, Project administration, Writing - review & editing. **Victor Pena Ribeiro:** Conceptualization, Validation, For- mal analysis, Visualization, Writing - review & editing. **Caroline Arruda:** Conceptualization, Validation, Formal analysis, Visualization, Writing - review & editing. **Rodrigo C.S. Veneziani:** Conceptualization, Supervision, Visualization, Writing - review & editing. **Sérgio Ricardo Ambrósio:** Conceptualization, Supervision, Visualization, Writing - review & editing. **Jairo Kenupp Bastos:** Conceptualization, Supervision, Visualization, Resources, Project administration, Funding acquisition, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114029.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.3 CAPÍTULO 3. Genotoxidade e avaliações toxicológicas da própolis vermelha brasileira em modelo pré-clínico

Manuscrito submetido ao periódico *Food and Chemical Toxicology*

Genotoxicity and Toxicological Evaluations of Brazilian Red Propolis oral ingestion in a Preclinical Rodent Model

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Abbreviations: BRP: Brazilian Red Propolis Extract; **DMSO**: Dimethyl sulfoxide. **FOB**: Functional observational battery; **HCT**: hematocrit; **HGB**: hemoglobin; **MMS**: Methyl methanesulfonate. **MNPCES**: Micronuclei in Polychromatic Erythrocytes; **NCEs**: Normochromatic erythrocytes; **NDI**: Nuclear Division Index; **OECD**: Organization for Economic Cooperation and Development; **PCEs**: Polychromatic Erythrocytes; **PEG**: Polyethylene glycol. **RBC**: red blood cell; **MCH**: mean corpuscular hemoglobin; **MCHC**: mean corpuscular hemoglobin concentration; **MCV**: mean corpuscular volume.

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Abstract

Brazilian red propolis is a natural product known due to its medicinal properties. The efficacy of this natural resin has been proved. However, few studies are reporting the safety of its use. We undertook the preclinical toxicity studies of Brazilian red propolis extract (BRP) in rodents, including genotoxicity, acute (single dose), and sub-chronic toxicities (90 days). No genotoxic effect of the BRP was detected in a range of 500-2000 mg/kg oral treatment of male Swiss mice. An acute toxicity test performed on Wistar Hannover rats (female) indicated no signs of toxicity of a single oral dose of 2000 mg/kg of BRP. The long-term oral toxicity performed with 1000 mg/kg of BRP on Wistar Hannover rats (males/females) altered water and food intake and the biometrics, hematological and biochemical parameters. Biochemical alterations in hepatic and renal parameters were detected only in the males. Despite the detection of biochemical alterations, no histopathological changes were detected in the organs of any group. In conclusion, BRP, at a higher dose, showed no signs of immediate toxicity. However, the obtained results suggest that the chemical composition and the intake of higher doses deserve special attention regarding possible toxicity.

Keywords: Natural Products; Brazilian Red Propolis; Genotoxicity; Acute/subchronic toxicity; Oral Toxicity; Rats.

INTRODUCTION

Several compounds of animal, micro-organisms, or plant origin have played an essential role in health care. Centuries of observations and experimental trials led to the identification of several natural medicines' preventive or curative effects (Bernardini et al., 2018). Herbal preparations are continuously being promoted in the alternative medicine of different countries, especially those with a strong base of traditional knowledge, and in developing countries where access to health services is limited (Aydin et al., 2016; Wang et al., 2021). Nowadays, the developed countries' natural products industry includes medicinal plants, cosmetics, and food supplements, among others. It is a growing market that has been seen and promoted as an alternative lifestyle.

This long-term history of the traditional use of natural products has been associated with being harmless. However, subsequent risks of continuous exposure to some herbal medicines have been discovered and confirmed at preclinical and clinical levels (Wang et al., 2021). One example is licorice extracts, with prolonged traditional use on foods and folk medicines. The glycyrrhizinate contained in this natural product, at a long-term exposition, has negativelly influences the cortisol inactivation (Aydin et al., 2016). Likewise, many other cases of herbal and dietary supplements intoxications with gastrointestinal symptoms, neurological effects, and hepatic and renal toxicity, among others, have been discovered from acute or chronic consumption (Aydin et al., 2016; Wang et al., 2021). Factors such as dose, duration of treatments, and chemical composition complexity are a challenge for studying the possible toxic effects of the products from natural sources (Aydin et al., 2016).

Propolis is one of the most outstanding products from natural sources in Brazil. Bees produce it as a result of a mixture of various natural substances, including plant resins, beeswax,

pollen, and inorganic compounds (de Carvalho et al., 2020; Salatino et al., 2021). Several Brazilian types of propolis are classified according to their geographical and botanical origins, bearing different chemical and organoleptic characteristics. Brazil has a large territory and is one of the major producers worldwide (Berretta et al., 2017). Brazilian red propolis is one of the most exciting types, riched in isoflavones, polyprenylated benzophenones, pterocarpans, and flavanones (Aldana-Mejía et al., 2021b). The red propolis extract has been related to antioxidant (do Nascimento et al., 2019), antimicrobial (Souza Silva et al., 2021), cytotoxic against tumor cell lines (de Carvalho et al., 2020; Silva et al., 2019), and gastroprotective (Boeing et al., 2021) activities, among others.

Studies on the possible toxic effects of red propolis consumption are scarce, despite the scientific support for its biological properties. In 2021, in tests carried out by our research group, the red propolis extract from Canavieiras (Bahia State) showed IC_{50} values of 102.7 µg/mL in the human fibroblast cell line (GM07492A) and no genotoxicity on lung fibroblasts cells (V79). In the *in vivo* assays, this sample was tested on the zebrafish model, exhibiting an LC_{50} of 9.37 mg/L (Aldana-Mejía et al., 2021b). However, studies performed with red propolis samples from different locations (Alagoas State) indicated toxic signs of the sub-acute oral treatment in Wistar male rats at 200 mg/kg (Silva et al., 2015). In this study, the chemical prevalence of isoflavones in the extract was related to changes in estrogenic activity, suggesting caution in ingesting red propolis.

Since some toxic effects, including reproductive/developmental toxicity and genotoxicity, may not be expressed in traditional use, preclinical studies are necessary to guarantee its safety, especially when clinical evidence and epidemiological data are unavailable (Aydin et al., 2016). We are reporting the *in vivo* toxicological assays with Brazilian red propolis extract to determine the possible effects on its oral intake.

MATERIAL AND METHODS

Red propolis collection and extraction

Brazilian red propolis was purchased from *Cooperativa de Apicultores de Canavieiras* (COAPER, Canavieiras, Bahia), in January of 2020. Red propolis (1.5 kg) was frozen, grounded, and macerated with ethanol-water (7:3) in a 1/10 crude propolis/solvent ratio. It was kept in a shaker incubator at 30 °C and 120 rpm for 24 h, and the process was repeated five times. The obtained extract was concentrated under vacuum and lyophilized. Its chemical characterization and quantification were previously reported by Aldana-Mejía et al. (2021b, 2021a) (Supplementary material).

Biological assays

Animals

The genotoxic assay was performed on male Swiss mice (*Mus musculus*) with approximately 30 g of body weight (b.w) (8-10 weeks; n = 36). The acute oral toxicity test was carried out on female Wistar Hannover rats (*Ratus norvegicus*), with approximately 210 g b.w. (6-8 weeks; n = 7). The sub-chronic toxicity test was conducted on male and female Wistar

Hannover rats at 35 - 40 days of age an average weight of 140 g (n = 50), from the Central Animal Facility of the Ribeirão Preto Campus of the University of São Paulo. Animals were kept in cages and placed in a ventilated environment with controlled temperature (24 °C), humidity (55 %), and light (12-h light/dark cycle). During the experiment, food and water were available *ad libitum*. The project was approved by the Ethics Committee on Animal Use of the University of Franca (n°. 9701030418).

Genotoxic assay

The *in vivo* genotoxic evaluation followed the OECD No 474 *Mammalian Erythrocyte Micronucleus Test* protocol (OECD 474, 2016). Male Swiss mice were selected and acclimated for seven days before the experiment. Animals were divided into five groups of six and treated with Brazilian red propolis extract (BRP) by gavage at 500, 1000, and 2000 mg/kg. Negative (no treatment), vehicle (DMSO), and positive control groups (MMS 25 mg/kg) were included. After 48 of administration, peripheral blood was collected by tail vein puncture.

A total of 4000 polychromatic erythrocytes (PCEs) were analyzed per animal to determine the frequency of micronuclei in polychromatic erythrocytes (MNPCEs). A total of 2000 normochromic erythrocytes (NCEs) per animal were analyzed to calculate the nuclear division index (NDI) through the equation NDI = [PCE/PCE+NCE].

Acute toxicity assay

Acute oral toxicity - up-and-down procedure (UDP) (OECD, 2008). This study was performed following the procedure described by OECD Nº 425 in *the Guideline for testing chemicals*. Female, young, healthy, nulliparous, non-pregnant Wistar Hannover rats were selected and acclimated for seven days before the experiment. After fasting for 12 h, a single animal was treated with a dose of the BRP by gavage, expected to be lower than the IC_{50} based on the *in vivo* micronucleus experiments. The animal was treated with a 1000 mg/kg dose, and after administration, feeding was suspended for another 3-4 h. The animal remained under observation during the first 30 min, and 2, 4, and 6 h after treatment, and periodically during 48 h.

In case of death or symptoms of animal toxicity, the protocol recommends reversing the dose; otherwise, the dose should be increased. With BRP, doses of 1000, 1500, and 2000 mg/kg were tested without mortality or symptoms of toxicity. Then, the limit dose of 2000 mg/kg was administered to four additional animals, which were treated individually at each 48 h, and monitored periodically until 14 days. Then, animals were euthanized, and macroscopic necropsy was performed.

Sub-chronic toxicity assay

Experimental design

The sub-chronic assay was performed, considering the acute test results, with the limited dose allowed by the OECD No 408 protocol, *Guideline for testing of chemicals, Repeated Dose 90-day Oral Toxicity Study in Rodents* (OECD, 2018). A 1000 mg/kg b.w dose of the BRP was evaluated as a single dose level used in the trial. Animals of both sexes were separated into three experimental groups: vehicle (n = 10), BRP (n = 10), and satellite (n = 5). The BRP (1000 mg/kg) was administered daily by gavage for 90 days to the groups BRP and satellite. Animals

in the vehicle group received the same volume of polyethylene glycol (PEG 15%) for the same period. After 90 days, the satellite group underwent a 30-day recovery period without administration of the extract.

Clinical observations, body weight, water and food intake

Before the administration of the extract, all animals were physically evaluated to verify the presence of any abnormality. During the experimental period, the animals were monitored twice daily for symptoms that could suggest any toxicity-related physical (fur, skin, eyes, nose) and behavioral changes. The animals' weights were also recorded weekly, as well as their food and water consumption. The vehicle group continued to be monitored after the gavage period.

Functional observational battery (FOB)

Functional observational battery tests (FOB) were performed at the experimental 12th week for three days, with three animals/sex from each group, pre, and post-gavage. The same researcher performed the tests without knowledge of the animal group assignment, following the protocol established by Mathiasen and Moser (2018). Animals were studied for the following parameters: undisturbed observations (body position, locomotor activity, piloerection, ptosis, exophthalmos, unusual behavior, writhing, respiration, tremors, and twitches), handling behavior (reactivity to touch, abdominal tone, limb tone, aggressiveness to handler, pupil size, visual placement, grip strength, tail suspension, pinnae reflex, lacrimation, salivation, diarrhea, increased urination/defecation, vocalization), open field observations (ataxia, foot splay walking, Straub tail, increased locomotion, no exploration, tail pinch, and body temperature).

Euthanasia and clinical pathology

At the end of the experimental period for the vehicle and BRP groups (90 days) and the recovery period for the satellite group (120 days), animals were fasting overnight (8-12 h) before sample collection. The animals were weighed, anesthetized, and blood was collected via cardiac puncture and placed into non-heparinized tubes for biochemical analyses and EDTA tubes for hematological analyses.

Hematological parameters (red blood cells, hemoglobin, hematocrit, mean corpuscular volume, medium corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and counting of neutrophils, eosinophils, basophils, lymphocytes, and monocytes) were measured using a hematology autoanalyzer. Total leukocytes were counted by microscopic observation. Biochemical analyses were performed using commercial kits from Latest: albumin (No.19), total protein (No.99), glucose (No.133), total cholesterol (No.76), HDL (No.13), triacylglycerols (No.87), urea (No.27), creatinine (No.35), alanine aminotransferase (No.108), and aspartate aminotransferase (No.109). An automatic potentiometric electrolyte analyzer (STAT/ION) was used to determine serum sodium, potassium, and calcium. Levels of stimulating thyroid hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were determined using the electro-chemiluminescence immunoassay (ECLIA) with Cobas Elecsys 411(Roche Diagnostics GmbH, Mannheim, Germany).

Samples of vaginal smears were collected after the euthanasia to determine the estrous cycles. Samples were taken by flushing approximately 50 μL of saline solution in and out of

the vagina, and a drop was put on a slide. The samples were evaluated under light microscopy, and the cycle stage was determined based on the predominant cell type. In males, sperm material was collected from the epididymis to perform a program exam. The male epididymis was washed with 1 mL of saline solution. A drop of the sperm suspension was placed on a microscope slide to evaluate its motility and morphology under light microscopy. After diluting epididymal sperm to 1:5, approximately 20 μL of the solution was transferred to a Neubauer counting chamber. The sperm count was expressed as x 10^6 number of cells per milliliter.

A macroscopic evaluation of the animal organs was performed during the necropsy. Then, the brain and pituitary gland; thyroid; heart; thymus; liver; pancreas; kidneys and adrenals; spleen; uterus; ovaries; epididymis, and prostate with seminal vesicles were removed, weighed, and fixed in 10% neutral formalin. In addition, the spinal cord (cervical and middle thoracic), eyes, aorta, gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum cecum, colon), trachea, larynx, pharynx, lung, lymph nodes (mandibular and mesenteric), mammary glands, cervix, sciatic nerve, skeletal muscle (rectus femoris), bone with bone marrow (femur), skin, urinary bladder, and vagina were collected and fixed in the same solution.

Histopathologic procedures and microscopic examination

Microscopic examinations were performed on all tissues, but only liver and kidney tissue results are reported here. Morphometric analysis of tissues was obtained from the image using the microscopic system "Leica," ICC50W, Las EZ software version 3.4.0 (Leica Microsystems®, Germany). The organs were analyzed using a total magnification of 100×. Formalin-fixed and paraffin-embedded tissue sections were cut to a 5 μm thickness and stained with hematoxylin and eosin for histological examination.

Statistical analysis

All data were analyzed using the Kolmogorov–Smirnov normality test. For data that presented a normal distribution, a univariate analysis of variance (One-way ANOVA) was used, followed by Tukey's post-test. The non-parametric Kruskal-Wallis test and Dunn's post-test were used for data that did not follow a normal distribution. Data were expressed as mean ± standard deviation for the parametric data or minimum/median/maximum value for the non-parametric data. Differences were considered significant for $p < 0.05$. All analyses were performed using GraphPad Prism® software version 9 for Windows (San Diego, California, USA).

RESULTS

Genotoxic assay

The *in vivo* genotoxic evaluation showed that the concentrations of BRP administered to the animals did not affect the frequency of micronuclei or NDI. No significant difference between the treatments and the negative control was observed (Table 1).

Table 3.3-1. Frequencies of micronucleated polychromatic erythrocytes (MNPCE) in peripheral blood of mice treated with different doses of BRP and respective negative, positive (MMS 25 mg/kg b.w., i.p.), and solvent (DMSO 100%) controls.

Treatments (mg/kg b.w.)	MN[a]	NDI[b]
Negative control	8.40 ± 2.61	0.09 ± 0.03
DMSO 100%	7.00 ± 1.22	0.07 ± 0.02
BRP 500	9.20 ± 2.77	0.07 ± 0.03
BRP 1000	8.40 ± 0.89	0.07 ± 0.02
BRP 2000	10.40 ± 2.61	0.07 ± 0.03
MMS 25mg/kg	17.20 ± 2.95 *	0.07 ± 0.04

[a] 4000 PCE were analyzed per animal, for a total of 20.000 cells per group treatment.
[b] NDI = PCE/PCE+NCE. 2000 NCE were analyzed per animal, for a total of 10.000 cells per group treatment.
* $p < 0.05$ compared to negative control. One way-ANOVA and post-hoc Tukey test.
b.w.: body weight. **i.p.:** Intraperitoneal administration. **NDI:** Nuclear Division Index. **DMSO:** Dimethyl sulfoxide. **BRP:** Brazilian Red Propolis extract. **MMS:** Methyl methanesulfonate. **MN:** micronucleus.

Acute toxicity assay

After BRP extract (2000 mg/kg b.w.) oral administration, the five animals survived, without apparent symptoms of toxicity, even during the first hours after treatment. Necropsy revealed no pathological signs in the organs (liver, lungs, kidneys, heart, stomach, and spleen). According to OECD No 425 (2008), considering these results, the LD_{50} of red propolis in acute oral administration is more significant than 2000 mg/kg.

Sub-chronic toxicity assay

Clinical observations, body weight, water and food intake

No lethal effects on the animals were observed during the 90-days administration of BRP. Changes in feces were observed after one week of the experiment on the BRP and satellite groups; both were treated with 1000 mg/kg of propolis extract. A reddish coloration, pungent odor, and more liquid stools were observed on days 9-11 of oral administration. Nevertheless, during the experiment, no other symptoms that could be related to possible toxicity were observed.

Male body weight changes among the groups were detected from the third to 13th weeks of oral gavage (Figure 3.3-1, A). The satellite males had the lowest corporal weights, with no statistical difference from the BRP group. On the other hand, the differences in the body weight of the females were only in the initial weeks, with no statistical difference between the weight of these animals at the end of the experimental period (Figure 3.3-1, A). Also, it was observed a lower food intake in the male satellite group in comparison to the vehicle (Figure 3.3-1, B). The satellite group's food and water intake comparison at the end of the treatment (90 days) and the recovery period (120 days) are depicted in Figures 3.3-1B and 3.3-1C. The male BRP group presented a statistical difference in the increased water intake. The satellite group of females reduced water intake during the recovery period.

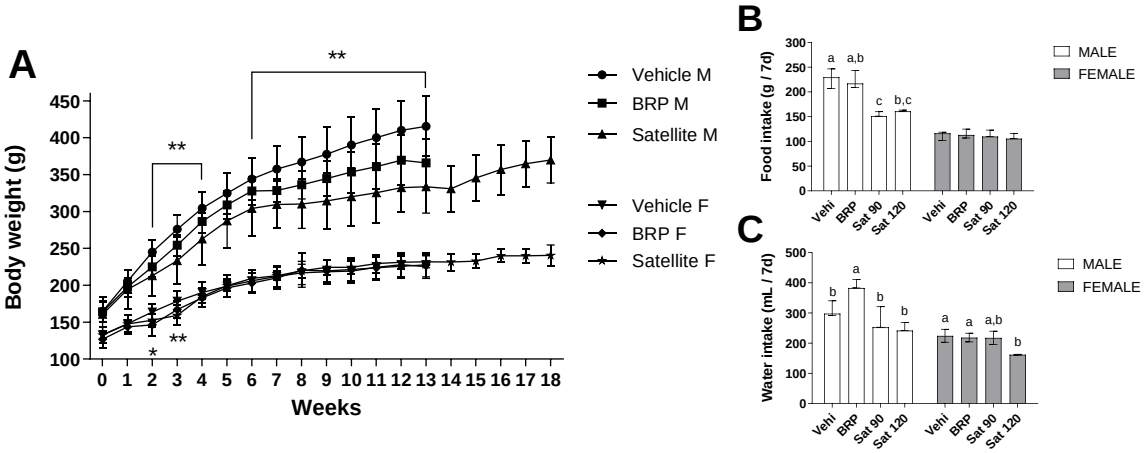

Figure 3.3-1. Weekly body weight (A), food intake (B) and water intake (C) of Wistar rats during the oral sub-chronic (90 days) toxicity assays of Brazilian red propolis. Data is presented as mean ± standard deviation (A), and minimum value / median / maximum values (B and C). Weeks 14-18: satellite group recovery period (30 day); n = 10 animals per group for vehicle and BRP; n = 5 for satellite group. Symbols indicate statistical difference between groups ($p < 0.05$) during this period: * Vehicle x BRP; ** Vehicle x Satellite. **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). **Vehicle**: Polyethylene Glycol 400 (15%). **M**: male. **F**: female.

Functional observational battery (FOB)

Observations from FOB were classified into excitation, coordination, sedation, and autonomic behaviors (Mathiasen and Moser, 2018), and the results are presented on a heatmap (Figure 3.3-2). The groups and the time of the observations are in columns. The intensity of the color indicates the increase (red) or decrease (blue) of the alterations, and most parameters were unaffected by the administration of BRP. No signs of immediate toxicity as tremors, stereotypy, convulsions, or diarrhea, were registered. Mild arousal after gavage, such as increased reaction to tail pinching, was observed in the propolis-treated groups (BRP and satellite) for both sexes. An increase in startle in propolis-treated males was another change observed during the analysis. In all groups, males showed an increase in vocalization and decreased locomotion activity after gavage. However, none of these behaviors were significantly altered among groups.

Increased vocalization, urination, and defecation are routine in handling rodents, and some of the observed changes might be related to the stress in these tests. However, even with manipulation stress, it was seen that the animals showed less exploratory activity in the field and fewer defensive body positions in front of the evaluator after oral treatment.

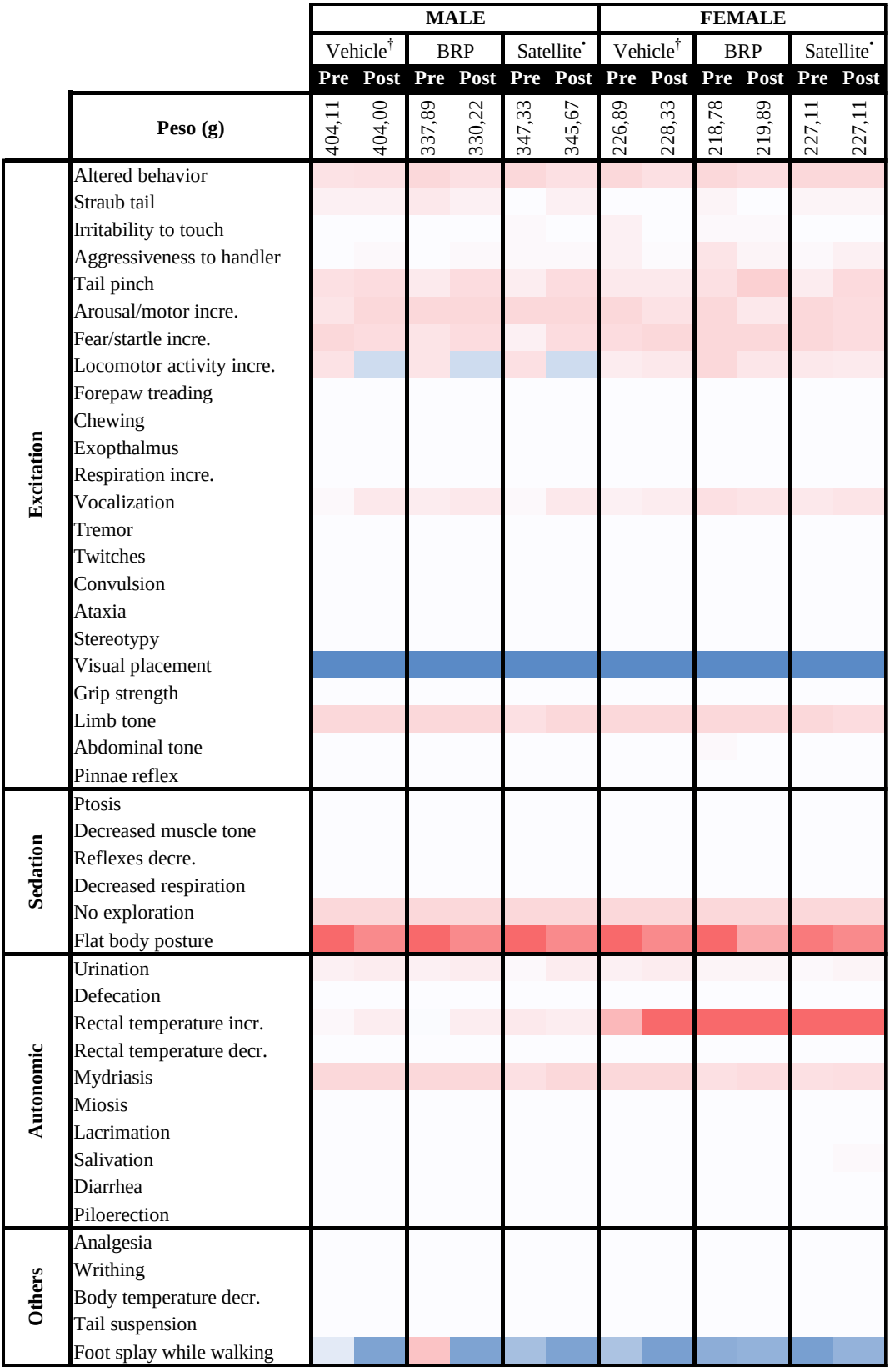

Figure 3.3-2. HeatMap representing behavioral alterations in rats (males and females) treated and not treated with 1000 mg/kg b.w. of Brazilian Red Propolis extract (BRP) by gavage for 90-days daily. Increase and decrease are represented by the red blue colors, respectively. † Vehicle: Polyethylene Glycol 400 (15%). • Satellite group treated with 1000 mg/Kg of BRP for 90 days, followed by 30 days of recovery before euthanasia.

Haematological analysis

Differences in male hematological parameters were detected (Table 3.3-2). An increase in MCV level was observed in the satellite group. The percentage of segmented neutrophils increased in the males treated with propolis, with and without a recovery period. On the BRP males, an increased-on reticulocyte counting was detected.

On the females, propolis treatment generated alterations in other parameters. Hemoglobin, MCV, segmented neutrophils, and total plasma protein levels were lower in the BRP group, with significant differences compared to the vehicle and satellite groups. Eosinophil's counting was augmented in the satellite group; on the other hand, the reticulocyte decreased in this group. The leucocyte counting increased in the BRP group.

Biochemical analysis

Variations on the biochemical parameters were mainly presented in the males. Significant changes were observed in some parameters related to liver and kidney function only in the groups that received the propolis extract (Table 3.3-3). The biochemical parameters related to renal function analysis revealed that urea levels increased in the male propolis and satellite groups, whereas creatinine decreased in the male BRP group. Regarding the parameters related to liver function, the alanine aminotransferase (ALT) activity increased in the BRP group, and the aspartate aminotransferase (AST) values increased in the male satellite group. The ALT level decreased in the female BRP group. Regarding the lipid profile, triglyceride levels decreased in the propolis group and satellite males. Also, total protein content decreased in animals of propolis and satellite groups of both sexes.

A decrease of the T4 in the males treated with propolis was observed (Table 3.3-4) in the thyroid hormonal levels. No changes were observed in the female's TSH, T3, and T4 serum concentrations.

Gross pathology, organ weights and histopathology

The relative organ weights of the male and female rats after 90 days of administration of BRP extract are depicted in Table 3.3-5. The administration of red propolis promoted a significant increase in the weight of liver tissue in the females, with no significant difference between the control and the satellite groups. The liver, pancreas, brain, thyroid, and prostate showed differences between the groups in the males. The administration of BRP increased liver and thyroid relative weights in the group without a recovery period. The relative pancreas weight also presented higher values in the groups that received propolis compared with the vehicle control. The relative weight of the brain and prostate was higher in the satellite group.

Table 3.3-2. Summary of haematologic parameters observed from sub-chronic oral administration of red propolis extract in Wistar Hannover rats.

Parameters	Sex	Experimental groups		
		Vehicle†	**BRP**	**Satellite•**
RBC (x10⁶/µL)	M	5.98 / 8.82 / 9.25	7.83 / 9.03 / 10.35	6.13 / 8.93 / 9.94
	F	8.46 ± 0.46	8.03 ± 0.63	8.19 ± 0.70
HGB (g/dL)	M	16.70 / 17.95 / 18.20	16.30 / 17.25 / 18.70	17.20 / 17.50 / 20.80
	F	**17.39 ± 0.75[a]**	**16.13 ± 0.55[b]**	**17.34 ± 0.83[a]**
HCT (%)	M	30.70 / 46.65 / 48.10	40.90 / 46.90 / 53.00	33.30 / 47.25 / 56.00
	F	46.50 ± 2.54	42.11 ± 4.06	44.84 ± 4.81
MCV (fL)	**M**	**52.00 ± 1.26[a,b]**	**51.90 ± 0.88[b]**	**53.60 ± 1.52[a]**
	F	**55.00 ± 1.63[a]**	**52.22 ± 1.79[b]**	**54.60 ± 2.07[a,b]**
MCH (pg)	M	18.9 / 20.10 / 28.10	18.10 / 18.75 / 21.70	19.20 / 20.30 / 28.20
	F	20.60 ± 1.12	20.18 ± 1.36	21.26 ± 0.87
MCHC (g/dL)	M	36.00 / 37.70 / 55.80	34.90 / 36.30 / 41.60	36.50 / 37.00 / 51.90
	F	35.40 / 37.50 / 40.00	36.10 / 36.80 / 45.10	36.60 / 37.50 / 42.30
Total leukocytes	M	4.90 ± 0.67	5.81 ± 1.17	4.85 ± 0.49
(10^3 cell/µL)	**F**	**3.76 ± 0.61[a,b]**	**4.28 ± 0.75[a]**	**2.87 ± 0.75[b]**
Segmented	**M**	**10.20 ± 5.36[b]**	**19.14 ± 4.22[a]**	**20.00 ± 4.32[a]**
neutrophils %	**F**	**23.80 ± 11.48[a]**	**9.50 ± 2.51[b]**	**19.50 ± 5.80[a,b]**
Eosinophil	M	0.00 / 0.00 / 0.00	0.00 / 0.00 / 4.00	0.00 / 0.00 / 3.00
(cell/µL)	**F**	**0.00 / 0.00 / 1.00[b]**	**0.00 / 1.00 / 2.00[a,b]**	**0.00 / 2.00 / 4.00[a]**
Lymphocyte %	M	84.17 ± 10.09	78.50 ± 7.84	72.40 ± 8.02
	F	76.83 ± 12.07	84.00 ± 6.38	78.40 ± 10.09
Monocyte %	M	3.00 ± 0.82	2.40 ± 1.51	3.00 ± 2.00
	F	1.00 / 2.00 / 3.00	1.00 / 2.00 / 2.00	1.00 / 1.00 / 5.00
Platelets (10^3 cell/µl)	M	315.00 / 387.00 / 492.00	51.70 / 428.00 / 557.00	350.00 / 425.00 / 559.00
	F	450.00 / 529.00 / 589.00	105.00 / 476.00 / 568.00	341.00 / 399.00 / 643.00
Total plasma	M	5.70 ± 0.41	5.78 ± 0.40	5.92 ± 0.36
protein (g/dL)	**F**	**5.40 / 6.00 / 6.20[a,b]**	**5.20 / 5.50 / 6.40[b]**	**6.00 / 6.50 / 6.50[a]**
Reticulocytes %	**M**	**0.58 ± 0.10[b]**	**1.48 ± 0.30[a]**	**1.16 ± 0.67[a,b]**
	F	**1.10 ± 0.34[b]**	**1.76 ± 0.33[a]**	**0.83 ± 0.21[b]**

Data submitted to the univariate analysis of variance (one-way ANOVA), followed by Tukey's post-test. Values are expressed as mean ± standard deviation. Nonparametric data analyzed by Kruskal–Wallis and Dunn's post-tests. In this case, data are presented as minimum value / median / maximum. Different superscript letters indicate statistically significant difference on the same line ($p < 0.05$). **M**: male. **F**: female. **PEG**: polyethylene glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). **RBC:** red blood cell; **HCT**: hematocrit; **HGB**: hemoglobin; **MCV**: mean corpuscular volume; **MCH**: mean corpuscular hemoglobin; **MCHC**: mean corpuscular hemoglobin concentration. † Vehicle: PEG 15%. • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia.

Table 3.3-3. Summary of biochemical parameters observed from sub-chronic oral administration of red propolis extract in Wistar Hannover rats.

Parameters	Sex	Experimental groups		
		Vehicle†	BRP	Satellite•
Albumin (µmol/L)	M	408.46 ± 28.14	397.31 ± 53.57	361.18 ± 56.26
	F	447.38 ± 35.99	428.62 ± 22.99	426.89 ± 48.27
Total protein (g/L)	**M**	**52.81 ± 2.15^{a}**	**48.63 ± 2.74^{b}**	**48.80 ± 2.38^{b}**
	F	**50.37 ± 4.81^{a}**	**48.37 ± 2.86^{a,b}**	**43.87 ± 4.10^{b}**
Glucose (mmol/L)	M	6.77 ± 1.18	6.87 ± 1.34	6.71 ± 1.06
	F	7.76 ± 1.47	7.08 ± 1.89	5.91 ± 1.09
Cholesterol total (mmol/L)	M	2.06 ± 0.34	1.80 ± 0.31	1.80 ± 0.24
	F	1.40 ± 0.30	1.61 ± 0.35	1.54 ± 0.31
Cholesterol HDL (mmol/L)	M	1.22 ± 0.21	1.05 ± 0.21	1.13 ± 0.15
	F	1.11 ± 0.21	1.09 ± 0.29	0.96 ± 0.19
Triacylglycerols (mmol/L)	**M**	**0.92 ± 0.17^{a}**	**0.71 ± 0.17^{a,b}**	**0.48 ± 0.11^{b}**
	F	0.45 ± 0.12	0.40 ± 0.06	0.47 ± 0.07
Urea (mmol/L)	**M**	**5.89 ± 0.86^{b}**	**8.01 ± 0.86^{a}**	**8.36 ± 1.05^{a}**
	F	5.50 ± 0.52	6.26 ± 1.02	6.21 ± 1.08
Creatinine (µmol/L)	**M**	**22.18 / 41.89 / 64.07^{a}**	**12.32 / 27.11 / 49.28^{b}**	**22.18 / 46.82 / 51.75^{a,b}**
	F	37.31 ± 7.98	45.41 ± 10.24	38.93 ± 8.95
ALT (U/L)	**M**	**22.41 ± 7.50^{b}**	**32.41 ± 6.06^{a}**	**28.62 ± 3.92^{a,b}**
	F	**26.80 ± 3.43^{a}**	**19.41 ± 4.07^{b}**	**27.99 ± 4.21^{a}**
AST (U/L)	**M**	**25.06 ± 4.78^{b}**	**31.17 ± 6.36^{b}**	**40.61 ± 5.26^{a}**
	F	37.29 ± 5.83	34.66 ± 6.59	32.13 ± 4.71
Sodium (mmol/L)	M	127.63 ± 10.27	122.63 ± 3.11	118.40 ± 4.16
	F	118.33 ± 6.71	121.71 ± 4.75	119.00 ± 10.91
Potassium (mmol/L)	M	82.00 / 97.00 / 110.0	89.00 / 94.00 / 95.00	89.00 / 94.50 / 97.00
	F	94.17 ± 5.78	96.14 ± 3.93	94.20 ± 8.20

Data submitted to the univariate analysis of variance (one-way ANOVA), followed by Tukey's post-test. Values are expressed as mean ± standard deviation. Nonparametric data analyzed by Kruskal–Wallis and Dunn's post-tests. In this case, data are presented as minimum value / median / maximum. Different superscript letters indicate statistically significant difference on the same line ($p < 0.05$). **M**: male. **F**: female. **PEG**: polyethylene glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). † Vehicle: PEG 15%. • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia.

Table 3.3-4. Summary of hormonal parameters observed from sub-chronic oral administration of red propolis extract in Wistar Hannover rats.

Parameters	Sex	Experimental groups		
		Vehicle†	**BRP**	**Satellite•**
TSH (µIU/mL)	M	0.04 ± 0.02	0.02 ± 0.01	0.04 ± 0.01
	F	0.03 ± 0.02	0.06 ± 0.01	0.07 ± 0.04
T3 (ng/dL)	M	144.67 ± 37.41	134.74 ± 7.65	126.02 ± 7.76
	F	156.38 ± 29.62	135.67 ± 7.02	128.33 ± 12.06
T4 (µg/dL)	**M**	**7.95 ± 0.68[a]**	**6.26 ± 1.06[b]**	**6.58 ± 0.47[b]**
	F	5.84 ± 1.36	7.05 ± 1.15	6.40 ± 0.10

Data submitted to the univariate analysis of variance (one-way ANOVA), followed by Tukey's post-test. Values are expressed as mean ± standard deviation. Nonparametric data analyzed by Kruskal–Wallis and Dunn's post-tests. In this case, data are presented as minimum value / median / maximum. Different superscript letters indicate statistically significant difference on the same line ($p < 0.05$). **M**: male. **F**: female. **PEG**: polyethylene glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). † Vehicle: PEG 15%. • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia.

Table 3.3-5. Summary of relative organ weights observed from sub-chronic oral administration of red propolis extract to Wistar Hannover rats.

Parameters	Sex	Experimental groups		
		Vehicle†	**BRP**	**Satellite•**
Hearth	M	0.25 ± 0.01	0.25 ± 0.02	0.26 ± 0.01
	F	0.30 ± 0.03	0.30 ± 0.02	0.30 ± 0.04
Thymus	M	0.17 ± 0.03	0.14 ± 0.03	0.15 ± 0.04
	F	0.21 ± 0.04	0.21 ± 0.04	0.21 ± 0.04
Liver	**M**	**2.82 ± 0.19[b]**	**3.41 ± 0.31[a]**	**2.92 ± 0.08[b]**
	F	**2.89 ± 0.33[b]**	**3.53 ± 0.50[a]**	**2.76 ± 0.51[b]**
Pancreas	**M**	**0.23 ± 0.07[b]**	**0.31 ± 0.04[a]**	**0.32 ± 0.05[a]**
	F	0.33 ± 0.04	0.34 ± 0.08	0.35 ± 0.14
Kidneys	M	0.66 ± 0.07	0.71 ± 0.06	0.69 ± 0.07
	F	0.70 ± 0.05	0.72 ± 0.07	0.71 ± 0.11
Adrenals	M	0.01 / 0.02 / 0.02	0.01 / 0.02 / 0.02	0.01 / 0.02 / 0.02
	F	0.02 / 0.03 / 0.04	0.03 / 0.03 / 0.04	0.03 / 0.03 / 0.04
Spleen	M	0.17 ± 0.03	0.18 ± 0.02	0.18 ± 0.01
	F	0.16 / 0.20 / 0.23	0.17 / 0.22 / 0.24	0.16 / 0.19 / 0.27
Brain	**M**	**0.53 ± 0.05[b]**	**0.58 ± 0.03[a,b]**	**0.59 ± 0.03[a]**
	F	0.82 / 0.91 / 1.07	0.42 / 0.86 / 0.98	0.75 / 0.88 / 0.94
Thyroid	**M**	**0.06 / 0.06 / 0.08[b]**	**0.07 / 0.08 / 0.09[a]**	**0.07 / 0.07 / 0.09[a,b]**
	F	0.06 / 0.10 / 0.16	0.08 / 0.09 / 0.11	0.09 / 0.10 / 0.12
Testis	M	0.81 / 0.96 / 1.14	0.83 / 1.03 / 1.27	0.72 / 0.98 / 1.03
Epididymis	M	0.39 ± 0.04	0.40 ± 0.05	0.35 ± 0.05
Prostate + vesicles	**M**	**0.56 / 0.63 / 0.80[b]**	**0.51 / 0.64 / 0.76[b]**	**0.67 / 0.77 / 0.94[a]**

Prostate	**M**	**0.23 ± 0.07[b]**	**0.24 ± 0.06[b]**	**0.41 ± 0.06[a]**
Uterus + ovaries	F	0.57 ± 0.16	0.44 ± 0.13	0.49 ± 0.17

Data submitted to the univariate analysis of variance (one-way ANOVA), followed by Tukey's post-test. Values are expressed as mean ± standard deviation. Nonparametric data were analyzed by Kruskal–Wallis and Dunn's post-tests. In this case, data are presented as minimum value / median / maximum. Different superscript letters indicate statistically significant difference on the same line ($p < 0.05$). **M**: male. **F**: female. **PEG**: polyethylene glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). † Vehicle: PEG (15%). • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia.

The biochemical analysis revealed changes in kidney and liver function in the male propolis treatment groups. However, the histological sections of both tissues stained by H&E showed that the propolis-treated animals presented histological aspects compatible with normality (Figure 3.3-3). Increased liver weight was observed in males and females of the propolis group. However, the histopathological analysis revealed the absence of changes. Among groups, no histological changes were observed in the brain, pancreas, thyroid, and prostate (data not shown).

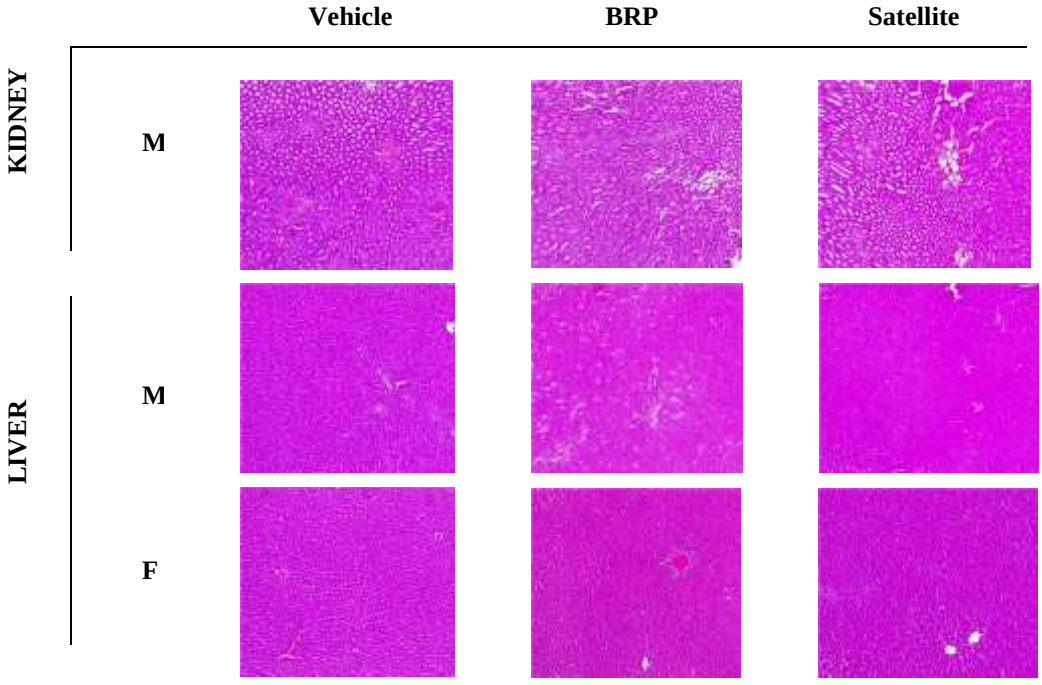

Figure 3.3-3. Representative photomicrographs of histological sections of liver and kidney tissue stained with Hematoxylin and Eosin (H&E) in rats (males and females) treated or not with 1000 mg/kg b.w. of Brazilian Red Propolis extract (BRP) by gavage for 90-days, daily. Vehicle: Polyethylene Glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). **M**: male. **F**: female. Final magnification of 100x.

Reproductive parameters

Regarding the analyses of male sperm function, a significant decrease in total sperm motility was observed in animals from the satellite group (Table 3.3-6). However, both morphology and sperm count were unchanged among the groups. The histological sections of

the epididymal tissue stained by H&E (data not shown) revealed that the animals that received the red propolis extract also presented a histological aspect compatible with the normality. The estrus phase estimation by vaginal cytology was determined at the euthanasia in the females (Table 7). Analysis of the vaginal smear of females indicated a prevalence of the estrus phase on the BRP and the metestrus phase on the satellite group. No histological alterations of sexual organ tissues were observed (data not shown).

Table 3.3-6. Effect of subchronic oral administration of red propolis hydroalcoholic extract (EPV 1000 mg/kg body weight) and vehicle (Control) on sperm function in Wistar Hannover rats.

Experimental groups	Motility (%)		Sperm count (x 10^6 cell/mL)	Morphology	
	Total	Progressive		% Normal	% Anormal
Vehicle	**90.83 ± 1.75[a]**	43.33 ± 4.71	7.69 / 9.73 / 10.28	80.43 / 85.71 / 91.00	9.00 / 14.29 / 19.57
BRP	**82.73 ± 11.88[a]**	44.13 ± 8.59	7.77 / 9.92 / 10.56	78.75 / 87.21 / 92.20	7.80 / 12.79 / 21.25
Satellite	**49.38 ± 15.99[b]**	35.00 ± 7.07	8.35 / 12.59 / 16.64	79.90 / 88.83 / 90.00	10.00 / 11.17/ 20.10

Data submitted to the univariate analysis of variance (one-way ANOVA), followed by Tukey's post-test. Values are expressed as mean ± standard deviation. Nonparametric data analyzed by Kruskal–Wallis and Dunn's post-tests. In this case, data are presented as minimum value / median / maximum. Different superscript letters indicate statistically significant difference on the same columns ($p < 0.05$). **M**: male. **F**: female. **PEG**: polyethylene glycol 400 (15%). **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day). † Vehicle: PEG 15%. • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia.

Table 3.3-7. Estrus phase estimation by vaginal cytology on female Wistar Hannover rats subchronic oral administration of red propolis hydroalcoholic extract (BRP 1000 mg/kg body weight) and vehicle (PEG 15%).

Phase (%)	Experimental groups		
	Vehicle†	BRP	Satellite•
Estrus	30	50	20
Metestrus	30	20	80
Diestrus	40	30	-
Proestrus	-	-	-

† Vehicle: PEG 15%. • Satellite group treated with 1000 mg/kg body weight/day of Brazilian red propolis for 90 days, followed by 30 days of recovery before euthanasia. **PEG**: polyethylene glycol 400; **BRP**: Brazilian red propolis extract (1000 mg/kg body weight/day).

DISCUSSION

Propolis is one of the Brazilian types of products with more significant distinction in the market, being the constituent or additive for toothpaste, mouth spray, immunity, energy enhancers, and wound healing formulations (Freires et al., 2016). Propolis-based products are mainly considered a supplement or functional foods, such as honey, royal jelly, and bee pollen, with more flexible regulatory affairs in most cases. However, some misleading information on efficacy and safety (Berretta et al., 2017).

Many researchers reported the potential of propolis and its isolated compounds for therapeutic use, making it necessary to consider and evaluate its potential adverse side effects (Braakhuis, 2019). Using *in vivo* models in rodents can be an effective way to observe the influence of different factors on the potential toxic effect of new drug candidates, such as administration route, exposure time, and pharmacokinetics (Aydin et al., 2016). The Brazilian health regulatory agency currently requires a series of non-clinical studies to develop drugs and herbal medicines, including genotoxic assays and acute and repeated dose toxicity tests (ANVISA, 2019).

It is the first report of *in vivo* genotoxicity study of Brazilian red propolis extract. The results indicated no *in vivo* genotoxic effect of BRP. An *in vitro* study with red propolis from the exact location was performed on Chinese hamster lung fibroblast (V79). Concentrations between 20-60 µg/mL were evaluated in the micronucleus test without any significant effect on the induction of micronuclei in the cell model. These studies indicated that Brazilian red propolis was innocuous in *vitro* and *in vivo* at the genetic level (Aldana-Mejía et al., 2021b).

The next stage of the toxicological study was the acute or single-dose toxicity assay. It is one of the first steps to determining the toxicological potential of new drugs, and it is vital in determining the doses in the subsequent repeated dose toxicity tests and the assignment of the LD_{50} dose (Aydin et al., 2016). The up-and-down procedure was followed to reduce the number of animals and as an efficient methodology to test a good range of concentrations of the red propolis extract.

A 1000 mg/kg b.w. dose was selected to start the essay, considering that previous *in vitro* and *in vivo* genotoxic studies indicated no BRP extract toxicity. There were no signs of toxicity, and then the dose was increased up to 2000 mg/kg. Even with this high dosage, no toxicity sign was observed. However, other acute toxicity studies indicated that the oral administration of Brazilian red propolis could have a toxic effect on Wistar rats. According to a previous report, 20 to 40% of the animals treated with a 300 mg/kg dose of the extract presented some depressor effects on the central nervous system, including decreased ambulation, somnolence, hyperventilation, tremor, and diarrhea in the first days after oral administration. All the symptoms disappeared after the third day after administration, and any animals were observed throughout the assay. The LD_{50} value of the extract on a single dose was greater than 300 mg/kg (Silva et al., 2015). However, the obtained results in our study indicated that this value could be higher (>2000 mg/kg).

The sub-chronic toxicity test was performed with the limit dose of 1000 mg/kg b.w., considering the results of the sub-acute assay. Silva et al. (2015) performed an orally sub-acute toxicity test for 28 days with red propolis extract from the state of Alagoas. This treatment revealed changes in the biometrics of the male animals that received the propolis extract. Lower

body mass was detected for the groups treated with propolis and those that passed by the recovery period. Unlike the Canavieiras red propolis sample used in this study, red propolis from Alagoas increased the body weight of males and females at the highest dose (200 mg/kg). This difference was significant in the females during the four weeks of treatment, compared to the control group, while for the males, it was significant during the last weeks of treatment. Also, in the first week of the sub-acute assay undertaken by Silva et al. (2015), the males that received 200 mg/kg dose increased their water intake and presented diarrhea. At 10 and 100 mg/kg extract, a small percentage of the animals were wheezing and with decreased ambulation.

The males with lower body weight also presented a lower food intake during the sub-chronic (90-days) experiment. Together with the biometrics, alterations of the hematological parameters were observed in the sub-chronic group treated with propolis. Other studies suggested changes in the MCHC and monocytes of Wistar rats treated with Brazilian red propolis compared to the control. On the other hand, RBC and hemoglobin increased in the groups treated with 10 and 100 mg/kg of the extract, being outside the usual parameters. MCV decreased, and MCHC increased in females compared to the control at the highest concentrations tested (200 mg/kg) (Silva et al., 2015).

Previous studies compared the hematological data on Wistar rats (de Kort et al., 2020; Giknis and Clifford, 2008), and the decrease of female reticulocytes in the satellite group led to a lower counting compared to the average level. Also, the platelet counting was lower compared to the reference level in all the groups for both sexes. Even with the observed differences, the other parameters were within the normal range for Wistar rats. However, alterations in the hematological parameters were observed among animals of different breeders. Moreover, it is recommended to compare these effects with untreated control animals rather than reference intervals in the preclinical safety studies (Kampfmann et al., 2012).

There were differences between the effects between our study and the study performed by Silva et al. (2015). The tested red propolis samples were from different locations, and the chemical composition may vary. Variations in the metabolite profile are associated with the biological effects of propolis (do Nascimento et al., 2019; Regueira et al., 2017). The difference in the obtained results also suggests that the duration of the experiments, mode of administration, and the dose used may have been crucial in achieving those effects.

Studies of the red propolis protective effect on acetic acid-induced ulcerative colitis in Wistar rats indicated that increased doses of the treatment did not exert a beneficial response, and this was attributed to the promotion of a pro-oxidant activity due to the enhancement of the concentration of chemical constituents (Barbosa Bezerra et al., 2017). Daidzein, formononetin, and biochanin A were identified in the Alagoas red propolis sample. Isoliquiritigenin and liquiritigenin were suggested to be present as the main compounds of the extract. Also, polyprenylated benzophenones were proposed to be present in the sample. In the Canavieiras' sample, the isoflavones vestitol and neovestitol, and the pterocarpane medicarpin were identified as main compounds (Aldana-Mejía et al., 2021b, 2021a). Also, the polyprenylated benzophenones guttiferone E/xanthochymol and oblongifolin B were the primary metabolites in the sample (Ccana-Ccapatinta et al., 2020). Do Nascimento et al., 2019 reported a strong correlation between guttiferone E and total compounds concentration associated with higher antioxidant activity of Brazilian red propolis extract.

According to the literature, the general safe dosage recommendation for propolis is 1.4 mg/kg/day for humans. However, practical dosing taking 400 to 1500 mg/day was reported (Alvarenga et al., 2021). Although our study aimed to administer a dose considered extreme, in a search using the expression "red propolis consumption by the population" in google search, it was observed that Brazilian red propolis products are suggested to be consumed in different doses according to the pharmaceutical presentation. For liquid extract, it is suggested to consume 110 mg/mL dose (1 mL of a 11% w/v extract), while for capsule presentation, it is indicated to consume a 500 mg/day dose, equivalent to two capsules/day containing 250 mg each of propolis extract. For example, a person weighing 70 kg would range between 1.57 - 7.14 mg/kg/day, which is more than 100 times lower compared to the dose used on the 90-day assay.

Differences in the biochemical results of the previous toxicity studies with red propolis were noticed, especially in the parameters related to kidney and liver function. In the acute toxicity study carried out with red propolis from Alagoas (Silva et al., 2015), significant increases in serum levels of creatinine, total protein, and albumin were detected in male rats treated orally with 200 mg/kg of the extract. Increased creatinine and urea plasma concentrations can be associated with renal toxicity. However, this association can only be confirmed with biochemical, gross pathology, and histopathology analysis (Gad, 2014).

Regarding the BRP sub-chronic treatment, augmentation of water intake and urea levels were observed in the BRP male group without recovery, indicating a possible sign of toxicity. However, serum creatinine levels decreased in the group, and kidney weight and histopathological analysis did not present differences from the control. Some studies recommend caution about propolis ingesting in the renal-risk population. A case of a patient with cancer in the bile duct that developed acute renal failure was correlated to propolis consumption (5 mL three times/day). Possible interference of propolis on kidney perfusion was suggested. However, propolis composition and the mechanism of renal failure induction were not identified (Li et al., 2005).

According to Gad (2014), changes in liver color or size at necropsy, increased ALT and AST activity decreased plasma total protein concentration, and histopathological changes are classical indicatives of liver toxicity. As previously reported, the 90-day oral ingestion of propolis also caused alteration in some of these parameters. Increases in ALT activity in male rats treated orally with 200 mg/kg of Brazilian red propolis extract were previously reported. However, the possible cause of this effect was not reported (Silva et al., 2015). According to the literature, the administration of some isoflavones can affect the biochemical parameters of the rodent’s model. The highest doses of the treatment (500 and 1000 mg/kg) increased lipid peroxidation (LPO) and decreased the antioxidant peptide glutathione (GSH) levels (Singh et al., 2014). In mice, oral administration of genistein, a metabolic product from biochanin A, increased levels of ALT and AST, inducing liver tissue degeneration by oxidative stress. Genistein, biochanin A, daidzein, and formononetin are isoflavones identified in Brazilian red propolis samples (Corrêa et al., 2017).

No correlations between ALT and AST activity were found with histopathologic liver injury by the BRP treatment. According to Ozer et al. (2008), although serum ALT measurements are of clinical importance, serum increased levels of this enzyme in the absence of liver histomorphological changes can be considered a false positive or a potential indicator

of a prodromal effect. Similarly, AST increased levels can be directly related to hepatic injury. ALT and AST enzymatic levels can also arise from extra-hepatic injury, mainly associated with skeletal and cardiac muscle disorders (Ozer et al., 2008; Smith et al., 2013). However, as suggested by Luo et al. (2014), additional studies are necessary for more specific analyses with other enzymatic markers, including alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) serum activity, serum concentration of total bilirubin (TBIL) and bile acids (BAs).

Regarding the hormonal levels in males, T4 levels were affected by the treatment with red propolis. The red propolis sample was mainly composed of medicarpin, isoliquiritigenin, vestitol, and isoflavones as formononetin (Morsy et al., 2021). In studies performed with Wistar rats, treatments with isoflavones (genistein and daidzein) decreased serum T4 levels (Šošić-Jurjević et al., 2014). In other animal models, the red propolis also induced changes in the thyroid hormonal levels. Diet supplementation with BRP (3 g/day) on pregnant sheep decreased T3 and T4 serum levels.

The role of the isoflavones on the adverse effects concerning thyroid function is a debate in the scientific community. Some studies revealed that isoflavones negatively affect thyroid peroxidase (TPO) activity, the enzyme involved in the synthesis of T3 and T4 in rodents (Otun et al., 2019). Compounds such as daidzein, genistein, and flavonoids such as rutin and quercetin negatively influence T3 and T4 serum levels in rodents (Di Dalmazi and Giuliani, 2021; Pistollato et al., 2018). The effect of BRP treatment was sex-dependent because only the males that received the propolis treatment presented a reduction in the T4 levels without a recovery of hormone levels after 30 days without administration of BRP.

Thyroid-related disruption can involve multiple targets that can cause alterations in the hormonal serum levels, and the probable disruption mechanisms are different between animal species (Pistollato et al., 2018). In the case of rats, the circulating thyroid hormones are determined by the binding affinity of T4 to albumin or transthyretin in plasma, which is much lower than the binding affinity between T4 and thyroxine-binding globulin in human plasma. Subsequently, the T4 excretion and production rate increase in rats. According to Esch and Lehmann (2020), these differences do not allow extrapolations between the effects displayed by rodent models to possible alterations in humans.

Isoflavones are called phytoestrogens because they can act as endocrine disruptors (Křížová et al., 2019). These compounds can actively bind to estrogen receptors, interfering with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural hormones in the body responsible for reproduction (Cederroth et al., 2012). As seen in the results section, effects on the reproductive parameters of males were detected, specifically on the sperm motility of animals treated with propolis. According to Esch and Lechmann (2020), the exposition of rodents to isoflavones presented some unclear results respecting the reproductive parameters; even when some studies pointed out no effects of the long-term adult exposition to isoflavones, other researchers reported that the exposition from conception to adulthood, make the males susceptible to decrease in sperm production.

Wistar rats treated with extracts of *Leucaena leucocephala* (3.5 g/kg/day), a plant rich in phytoestrogens, showed a decrease in motility, viability, and total sperm count (Retana-Márquez et al., 2016). According to the authors, a decrease in androgen receptors (AR) in the epididymis caused by phytoestrogens may reduce antioxidant protection, leading to an increase in lipid peroxidation. Similar results were reported in assays with isolated isoflavones. The

reduction of sperm motility in Wistar rats chronically exposed to genistein at a 1 mg/kg oral dose has been reported. This effect was attributed to the capacity of the isoflavone to reduce the expression of α-glucosidase, an enzyme that sustains sperm motility (Eustache et al., 2009).

The intake of genistein influences the morphology of testes and epididymis, as well as the number of epididymal spermatozoa, besides a negative influence on the body mass of the animals (Piotrowska et al., 2011). Other researchers also described the influence of oral treatment with daidzein and genistein at a dosage of 200 mg/kg on young male Wistar rats. Likewise, prostate weight increased significantly in the satellite male group. However, no structural damage could be correlated during the histopathological analysis of male sexual tissues. Conversely, several studies report the therapeutic effects of phytoestrogens against prostate cancer (Semenov et al., 2021).

Analysis of the vaginal smear of females indicated a prevalence of some phases in the groups treated with the BRP. According to Goldman et al. (2007) a single smear can be of limited value in determining the status of the reproductive system of rodents in toxicological studies, but it can be helpful. In female control, animals under the same environmental conditions and without stimuli are expected to show cytological asynchronicity of cycles within the group (Goldman et al., 2007; Smith et al., 2015). In toxicant-exposed animals, a greater homogeneity of cells across the group can be a consequence of a possible effect of the treatment on the endocrine function (Goldman et al., 2007). In the present study, no histological alterations in sexual organ weights, ovarian and uterine histology, and thyroid hormone levels were observed in the females.

Some of the detected alterations caused by the sub-chronic exposure to BRP were more pronounced in the satellite group. There might be a delayed toxic effect of the BRP treatment, especially concerning possible liver injury and disruptive endocrine and reproductive functions. However, some recovery studies revealed that after the dosing phase, toxic indicators do not necessarily represent delay toxicity but are a consequence of prolonged exposure to agents that have long half-lives (Pandher et al., 2012).

Pharmacokinetics studies determining the half-life of BRP samples in blood, tissues, and excreta are essential to evaluate the actual state of the toxicity concerning delayed or result of a lengthy exposition, as well as their mechanisms. The measure of oxidative stress biomarkers can also be a way to determine if the observed alterations would result from a possible metabolism activation of the BRP metabolites into products that can exert or promote toxicity by a mechanism that the execution of the used protocol cannot determine.

CONCLUSIONS

The administration of Brazilian red propolis hydroalcoholic extract does not seem to have triggered signs of immediate toxicity. However, there were relative weight variations in the liver, thyroid, and prostate, along with alterations in the parameters related to renal and hepatic functions in male animals. These results suggest that the chemical composition of red propolis -isoflavones, isoflavans, pterocarpanes, and polyprenylated benzophenones-, associated with long-term high dosage, deserve special attention regarding possible toxicity mediated by their ingestion. These alterations are also related to sex since the most significant alterations were

observed in males. Usually, people take between 1.6 - 7.1 mg/kg/day for a short period, which is more than 100 times lower than the dose used on the 90-day assay. Even though, further studies are needed to identify the possible mechanism associated with the observed changes in red propolis 90-day toxicological assay with 1000 mg/kg/day.

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CRediT authorship contribution statement

Jennyfer Andrea Aldana-Mejía: conceptualization; data curation; formal analysis; funding acquisition; investigation; writing - original draft methodology; writing - review & editing. **Aline Mayrink Miranda:** conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing - review & editing. **Gari Vidal Ccana-Ccapatinta:** data curation; methodology. **Luciana Silva de Araújo:** data curation; methodology. **Victor Pena Ribeiro:** data curation; methodology. **Caroline Arruda:** data curation; methodology. **Samuel Nascimento:** data curation; methodology. **Iara Squarisi:** data curation; methodology. **Tábata Esperandim:** data curation; methodology. **Karoline S. de Freitas:** data curation; methodology. **Saulo D. Ozelin:** conceptualization; data curation; methodology. **Denise Crispim Tavares:** conceptualization; data curation; project administration; resources; supervision. **Fernando Silva Ramalho:** conceptualization; data curation; methodology; project administration; resources; supervision. **Jairo Kenupp Bastos:** funding acquisition; supervision; validation; visualization; writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4. DISCUSSÃO

A própolis vermelha consiste em uma mistura complexa de compostos químicos entre os quais se destacam metabólitos secundários provenientes das diversas fontes botânicas que as abelhas visitam. No caso da própolis vermelha de Canavieiras (Bahia) foi possível o isolamento de 11 compostos presentes na amostra em estudo, como mencionado no capítulo 1. Entre estes se encontram as substâncias fenólicas, tais como flavanonas (liquiritigenina), isoflavonas (calicosina, formononetina, biochanina A), chalconas (isoliquiritigenina), isoflavanas (vestitol, neovestitol e 7-*O*-metilvestitol) e pterocarpanas (medicarpina). Análises cromatográficas comparativas entre a própolis vermelha, a resina, extratos das folhas, do caule, das raízes e das flores da espécie *D. ecastaphyllum*, evidenciaram que as substâncias fenólicas mencionadas estão presentes na resina da planta (capítulo 2). Com exceção da liquiritigenina, os outros compostos também estão presentes no caule. Todavia, as outras partes analisadas da planta como as flores, as raízes e as sementes não apresentaram a presença destes compostos.

O terpenoide metil-*o*-orselinato, flavonóis como a quercetina e as retusapurpurina A e B (responsáveis pela cor vermelha da própolis), a isoflavona daidzeína, o neoflavonoide dalbergina e o pterocarpano homopterocarpina (Alencar et al., 2007; Bueno-Silva et al., 2013; Daugsch et al., 2008; Oldoni et al., 2011; B. Silva et al., 2008), já foram identificados em amostras de própolis vermelha de outras regiões. Righi e colaboradores (2011) também identificaram alcanos, tais como *n*-tricosano, *n*-pentacosano, *n*-heptacosano, *n*-nonacosano, *n*-hentriacontano e *n*-tritiacontane no extrato hexânico da própolis vermelha, bem como α-amirina, lupeol, metilguaiacol, *trans*-anetol, resorcinol, anisilacetona, *cis*-asarone e farnesol, reconhecendo a riqueza de compostos de baixa polaridade nas amostras de própolis vermelha brasileiras. Triterpenoides como β-amirina e glutinol também têm sido isolados da própolis vermelha de Canavieiras (Ccana-Ccapatinta et al., 2020). Acilcloroglucinóis policiclícos poliprenilados conhecidos como benzofenonas polipreniladas, oblongifolina B e a mistura dos isómero gutiferona E e xantoquimol também foram identificados nas amostras em estudo. Estas substâncias foram identificadas na resina da espécie *S. globulifera* por Ccana-Ccapatinta (2020), confirmando esta espécie como a segunda fonte botânica da própolis vermelha brasileira.

A seleção destas fontes botânicas é de grande importância para a comunidade de abelhas, considerando que a própolis, além de representar um meio de proteção física da colmeia, tem

como propósito a defesa da imunidade da comunidade. De acordo com Salatino e Salatino (2017), as plantas utilizadas como fonte botânica na produção da própolis precisam fornecer material com composição química compatível com o papel de barreira contra agentes patogênicos. Sendo assim, a seleção da resina, provavelmente, está baseada em seu potencial antimicrobiano (Borba & Spivak, 2017; Pusceddu et al., 2019). Embora haja muitas lacunas a serem respondidas sobre os fatores que desencadeiam os processos de seleção das fontes botânicas pelas abelhas, para Pusceddu e colaboradores (2019), esta escolha só é possível pela capacidade que estes insetos possuem de se guiar por odores específicos que as conduzem a uma fonte de resina vegetal rica em metabolitos ativos.

Até o momento, os mecanismos relacionados a forma como as abelhas são guiadas e como selecionam as fontes botânicas não têm sido completamente esclarecidos. Porém, algumas hipóteses têm sido propostas, sendo que uma sugere que as abelhas são capazes de aprender estímulos tácteis, o que facilitaria na escolha de resinas adequadas. O látex e outros exsudados das plantas, por exemplo, podem solidificar em contacto com o ar, tornando-se resistentes para a manipulação das abelhas. Sendo assim, a seleção da resina pode estar condicionada a matérias com barreiras suficientemente macias e suaves para serem cortados (botões apicais e primórdios foliares) ou raspados da superfície da planta (exsudados) pelas abelhas, que possuem mandíbulas delicadas em comparação a insetos mastigadores (Salatino & Salatino, 2017).

Esta hipótese fundamenta, pelo menos em parte, a seleção das fontes da própolis brasileira, onde as abelhas selecionaram exsudados secretados como resposta à lesão do besouro *Agrilus propolis*, como foi descrito recentemente por Migliore *et al*. (2022) para o caso da *D. ecastaphyllum*. Neste caso, os besouros adultos depositam seus ovos em fendas na casca de *D. ecastaphyllum* e, após a eclosão dos ovos, as larvas cavam um túnel no interior para se alimentar da casca, criando galerias em forma de “D”. As larvas maduras eventualmente formam pupas nas placas externas da casca e os adultos emergem induzindo a produção da resina pela planta. Esta resina é coletada pelas abelhas. No caso de *S. globulifera* a resina é relatada como parte das flores, dos frutos e do caule da espécie (Lima et al., 2018).

Tem sido observado que esta busca de resinas botânicas específicas é de grande importância na colmeia, já que tanto o número de abelhas forjadoras de resina como a procura das resinas é influenciada pelo ataque de patógenos na colônia (Pusceddu et al., 2019; Simone-Finstrom & Spivak, 2012). A coleta e o depósito de resinas vegetais nas paredes interiores do ninho, além de funcionar como camada protetora contra infecção (Borba & Spivak, 2017), estabiliza a composição microbiana e reduz a diversidade taxonômica do microbioma na colmeia, favorecendo a composição de simbiontes intestinais favoráveis para as comunidades

(Saelao et al., 2020). Além da proteção antimicrobiana, a seleção das resinas que compõem a própolis é fundamental para reduzir a necessidade de manter o sistema imunológico das abelhas adultas altamente ativado quando o inseto não se encontra exposto a algum patógeno (defesa imune constitutiva) (Borba & Spivak, 2017).

Esta seleção das fontes botânicas para a elaboração da própolis tem uma implicação direta sobre os efeitos terapêuticos para o ser humano. Muitas das propriedades biológicas atribuídas à própolis vermelha brasileira estão relacionadas com a sua composição fenólica. Alguns estudos atribuem as atividades antimicrobianas, antifúngicas e antioxidantes às isoflavonas, flavanonas, chalconas e pterocarpanos presentes na própolis (Regueira et al., 2017). Para a utilização de produtos à base de própolis, especialmente aqueles relacionados aos cuidados de saúde, além de ser necessário realizar uma caracterização química para garantir a presença/estabilidade dos compostos bioativos, é importante padronizar os mesmos para garantir qualidade, segurança e eficácia aos consumidores (Fabio et al., 2019).

Vários estudos têm focado na caracterização química da própolis vermelha de diferentes localidades do Brasil, com destaque para as provenientes do estado de Alagoas. Nesse sentido, alguns pesquisadores realizaram quantificações de alguns marcadores específicos da própolis vermelha brasileira, através de diferentes técnicas. No entanto, até o momento, estudos relacionados ao desenvolvimento e validação de métodos analíticos para este tipo de própolis não tinham sido publicados. Neste sentido, no presente trabalho, foi desenvolvido um método analítico por meio de CLAE-DAD, para a análise da própolis vermelha.

O método desenvolvido evidenciou boa resolução na separação dos compostos selecionados. As curvas de calibração apresentaram correlação linear para todos os compostos, de acordo com os parâmetros recomendados nas diretrizes de validação (ANVISA, 2017; ICH, 2005). Os resultados indicaram que o método cromatográfico detectou e quantificou os compostos fenólicos da própolis vermelha brasileira em baixas concentrações com precisão, exatidão e robustez. Tendo em vista a qualidade e a precisão das análises por este método, o mesmo foi utilizado como uma ferramenta na análise sazonal da própolis vermelha da Cooperativa de Apicultores de Canavieiras (COAPER, Bahia). Os resultados revelaram que vestitol, medicarpina e neovestitol foram os compostos majoritários ao longo do ano, enquanto calicosina e biochanina A foram os que estiveram presentes em menores quantidades. De modo geral, os compostos quantificados apresentaram concentrações maiores nos meses de abril, junho e outubro de 2019 e menores quantidades nos meses de maio e setembro de 2019, bem como fevereiro de 2020. As amostras produzidas em março e abril apresentaram as maiores

concentrações dos compostos, enquanto o mês de setembro foi o mais crítico, apresentando os valores mais baixos.

Em diferentes localidades da região nordeste, alta umidade tem sido associada com o aumento nas concentrações de compostos fenólicos na própolis vermelha brasileira (Bueno-Silva et al., 2017; do Nascimento et al., 2019). Bueno-Silva *et al* (2017) realizaram análise sazonal de própolis vermelha coletada em Maceió (Alagoas), detectando formononetina como a substância majoritária nas amostras analisadas, seguido pela isoliquiritigenina. Vestitol e neovestitol também foram detectados ao longo do ano com prevalência nos meses de março e maio, que correspondem à estação chuvosa. Ainda assim, amostras coletadas em setembro, apresentaram as menores concentrações de vestitol, neovestitol e isoliquiritigenina durante o ano. As condições meteorológicas indicaram um período de altas precipitações e umidade na região de Canavieiras entre os meses de maio e outubro de 2019.

Nascimento *et al* (2019), correlacionaram dois métodos para quantificar os marcadores químicos da própolis vermelha em apiários do estado de Alagoas. A quantificação por CL-UV-DAD e CL-IES-Orbitrap-EMTF confirmou o acúmulo de isoflavonoides e flavanonas entre maio e setembro, temporada associada com o aumento das chuvas, umidade e diminuição da radiação solar. Por outro lado, condições em que prevaleceram as altas temperaturas e a radiação solar, período que corresponde aos meses de outubro e março, diminuíram as concentrações dos isoflavonoides, aumentando as quantidades de gutiferona.

Duas teorias sobre a variação sazonal na própolis vermelha brasileira têm sido sugeridas. A primeira sugere a indução do metabolismo dos fenilpropanoides na temporada de chuva, devido ao estresse causado nas plantas ao interagir com os insetos associados à produção de resina, levando a maior produção de isoflavonoides. A segunda teoria sugere a possível preferência das abelhas por *D. ecastaphyllum* na época das chuvas (Dixon & Paiva, 1995; do Nascimento et al., 2019). Considerando a ausência de flavanonas e isoflavonoides na resina de *S. globulifera* (Ccana-Ccapatinta et al., 2020), a última hipótese se mostra plausível. Estudos sobre a interação biológica entre abelhas e fontes botânicas são de grande importância no esclarecimento da preferência das abelhas na escolha de resinas, assim como a influência de outras condições bióticas e abióticas na produção da própolis.

Ainda nesta perspectiva, o conhecimento da influência sazonal na composição química da própolis também se mostra importante, principalmente no que se refere ao estudo das suas atividades biológicas, que são dependentes da concentração de constituintes ativos. Contudo, a padronização dos produtos à base de própolis é crítica devido as oscilações nos perfis químicos dos extratos (Bankova et al., 2019; Berretta et al., 2017). A própolis é um constituinte ou aditivo

para uma grande variedade de produtos. O registro destes tipos de produtos, na maioria das vezes, segue protocolos regulamentares mais flexíveis e, como consequência, informações sobre eficácia e segurança não são completamente esclarecidos (Berretta et al., 2017). No Brasil, a regulamentação atual, recomenda que os produtos à base de própolis com alguma indicação terapêutica sejam registrados como medicamentos específicos e, portanto, para seu registro é necessária a inclusão de dados referentes à segurança, incluindo ensaios citotóxicos, genotóxicos, testes de toxicidade aguda e de dose repetida (ANVISA, 2019; Resolução Da Diretoria Colegiada - Nº 24 de 14/06/2011, 2011).

Para tanto, neste estudo foram realizados ensaios *in vitro* e *in vivo* visando preencher estas lacunas referentes à segurança no consumo da própolis vermelha brasileira. O primeiro ensaio realizado foi o da citotoxidade, revelando que, tanto os extratos da própolis vermelha propriamente dita, quanto os de sua fonte botânica, *D. ecastaphyllum*, levaram à redução significativa da viabilidade celular em concentrações maiores a 100 µg/mL. Ressalta-se que o EPV foi significativamente mais citotóxico do que os extratos de sua fonte botânica. De acordo com a literatura, a própolis vermelha apresenta significativa atividade antiproliferativa em linhagens celulares tumorais (Alencar et al., 2007; Li et al., 2008; Nunes et al., 2009; Silva Frozza et al., 2013). Kamiya *et al*. (2012) relataram seletividade na atividade citotóxica da própolis vermelha, sendo que o extrato hidroalcoólico induziu apoptose em células de câncer de mama (MCF-7), sem efeito negativo na viabilidade celular de fibroblastos humanos em concentrações entre 0,1-20 µg/mL. Da mesma forma, Frozza e colaboradores (2013) verificaram citotoxidade do extrato hidroalcoólico da própolis vermelha em células embrionárias renais epiteliais (Hek-293), com IC_{50} superior a 150 µg/mL. Tais resultados são indicativos de que haja uma toxicidade seletiva em comparação a algumas linhagens tumorais testadas.

Por outro lado, ensaios de toxicidade realizados por Lopez e colaboradores (2015), revelaram variações nos valores de IC_{50} (65-200 µg) em células BALB/c3T3 (fibroblastos de embrião de camundongo) e HaCaT (queratinócitos humanos) expostas a extratos hidroalcóolicos de própolis vermelha de diferentes localidades (Sergipe, Alagoas e Paraíba). O estudo destaca que a própolis vermelha de Sergipe e Paraíba exerceu um efeito citotóxico sobre as membranas celulares na linhagem HaCaT. Os autores sugerem que concentrações menores do que 50 µg/mL sejam utilizadas. As própolis analisadas no estudo apresentaram como marcadores majoritários os compostos biochanina A, pinocembrina e formononetina (Lopez et al., 2015). Na própolis vermelha proveniente de Canavieiras usadas neste estudo, os compostos biochanina A e formononetina estavam presentes na amostra, porém em baixas concentrações.

Contudo, para Lopez e colaboradores (2015), poucos desses estudos têm sido desenvolvidos com células não tumorais, o que não permite estabelecer ainda algumas questões sobre a segurança e a seletividade da própolis vermelha.

No presente trabalho, tanto a própolis quanto sua fonte botânica apresentaram valores de IC_{50} superiores a 100 µg/mL, sendo usado como método de avaliação da toxicidade o ensaio de XTT, que avalia o dano mitocondrial. As diferenças nos valores de IC_{50} eram esperadas, considerando a diferença no perfil químico entre os extratos. Já o extrato do caule de *D. ecastaphyllum* manteve algumas similaridades nas bandas cromatográficas com o extrato da própolis, apresentando resultados similares entre estas duas amostras. Contudo, o caule apresentou menor citotoxidade. Já o perfil cromatográfico do extrato das folhas de *D. ecastaphyllum* foi diferente dos outros extratos, aumentando o valor de IC_{50} até 253 µg/mL.

Os compostos isolados a partir do extrato de própolis vermelha também foram testados contra células de linhagens tumorais e normais, visando conhecer o seu potencial seletivo. O vestitol e a oblongifolina B apresentaram efeito citotóxico nas linhagens, sem seletividade para as células tumorais. A medicarpina por outro lado apresentou ser seletiva para células de Hela, com um valor IC_{50} de 65,75 µM. Na literatura, destacam-se os estudos relacionados ao potencial antitumoral de compostos isolados ou presentes na própolis vermelha brasileira, porém são poucas as informações disponíveis acerca de sua segurança.

A isoliquiritigenina também foi avaliada, com IC_{50} superior a 1000 µg/mL, não apesentou toxicidade em células hepáticas humanas (LO_2), ressaltando sua segurança de uso (H.-H. Yang et al., 2017). A liquiritigenina, por outro lado, não apresentou toxicidade contra células derivadas do hipocampo de camundongos (HT22). Pelo contrário, tratamentos com 50 µM da substância induziram a proliferação celular em células tratadas com glutamato, neurotransmissor que induz dano celular causando morte progressiva das células neuronais, base de algumas doenças neurodegenerativas (E.-J. Yang et al., 2013).

Frações de própolis vermelha, compostas majoritariamente por medicarpina e 7-*O*-metilvestitol, foram seletivas, reduzindo a viabilidade em células tumorais de câncer de cólon (HT-29 e HCT-116), com menores efeitos citotóxicos na linhagem não tumoral Vero (células epiteliais renais de macaco), com IC_{50} superiores a 100 µg/mL (Santos, 2016). A oblongifolina B reduziu a viabilidade celular das linhagens HT-29 e HCT-116 a 5 µM, sem efeitos negativos significativos em células de epitélio normal de cólon (CCD-18Co), evidenciando seletividade na atividade biológica (Xu et al., 2010).

A partir dos resultados obtidos foi possível observar, *in vitro*, efeito inócuo em células normais resultantes do tratamento com a própolis vermelha e com a sua fonte botânica, em

termos de viabilidade celular. Todavia, as técnicas usadas não eram adequadas para identificar se os tratamentos tinham potenciais efeitos tóxicos no material genético do modelo utilizado. Desta forma, para tentar sanar esta limitação, posteriormente, foram realizados os ensaios de genotoxidade *in vitro*. Foram conduzidos ensaios de micronúcleo em linhagem celular normal, revelando que nas condições experimentais utilizadas, as amostras não apresentaram efeito genotóxico. Contudo, os extratos revelaram citotoxidade nas maiores concentrações avaliadas no ensaio, sendo o EPV mais citotóxico em relação aos extratos da fonte botânica.

Considerando que no modelo celular *in vitro* os extratos da própolis de Canavieiras não apresentaram efeitos negativos sobre a viabilidade celular ou mesmo sobre o material genético de linhagens celulares normais, foi proposta a realização de estudos *in vivo* em roedores. Tais ensaios tinham por objetivo corroborar o potencial toxicológico sistêmico da própolis vermelha, por meio da administração oral. A escolha da via de administração se deve ao uso tradicional desta própolis pela população.

O primeiro ensaio foi o de genotoxidade do EPV em camundongos *Swiss*. O ensaio do micronúcleo em sangue periférico demostrou que as concentrações administradas aos animais, não tiveram efeito na frequência de micronúcleos nem no IDN, sem diferença significativa dos tratamentos contra o controle negativo. Diferentes concentrações foram testadas, sendo a concentração de 2000 mg/kg de peso corporal o limite estabelecido pelo protocolo. A administração do extrato, mesmo na concentração mais alta, não causou efeitos negativos sobre modelo utilizado. Nossos resultados corroboram os encontrados em outros estudos que avaliaram os efeitos mutagênicos de amostras de extratos aquosos de própolis vermelha originárias do estado de Sergipe em larvas de *Drosophila melanogaster*, sem encontrar efeitos negativos significativos (Cardoso et al., 2015).

Considerando a segurança encontrada a nível citotóxico e genotóxico dos tratamentos com o EPV, foi conduzido um estudo de toxicidade aguda. Para este ensaio, após a utilização de doses menores que não apresentaram efeitos tóxicos aparentes nos animais, também foi selecionada a dose limite permitida pelo protocolo (2000 mg/kg). Após administração oral da dose limite, os animais tratados sobreviveram sem sintomas aparentes de toxicidade. A necropsia não revelou sinais patológicos nos tecidos (fígado, pulmões, rins, coração, estômago e baço). Nesta perspectiva, de acordo com a OECD 425 (OECD, 2008), considera-se que a DL_{50} da própolis vermelha em administração oral seja superior a 2000 mg/kg.

Silva *et al*. (2015) também realizaram ensaios de toxicidade aguda do extrato hidroalcoólico da própolis vermelha da cidade de Marechal (Alagoas) em ratos. Aos animais foi administrado uma dose única de 300 mg/kg. Os animais foram observados durante 14 dias.

Neste trabalho, apesar de não terem sido observados efeitos letais nos animais, sinais de toxicidade foram observados, sendo que 80% dos animais apresentaram sonolência, diminuição da ambulação, arquejamentos e tremedeira nos primeiros 30 min. Após 12 e 24 h os animais apresentaram diarreia. A partir do terceiro dia não foram observados mais sintomas de toxicidade. Contudo, nenhum efeito significativo no peso corpóreo nem nas necropsias foi observado após os 14 dias.

Posteriormente, foi realizado o ensaio de toxicidade subcrônica de doses repetidas durante 90 dias, na dose limite de 1000 mg/kg de peso corpóreo, conforme orientações do protocolo. Durante o período experimental de três meses não foram observadas mortes dos animais. Nos grupos tratados com a dose de 1000 mg/kg do extrato de própolis (EPV e satélite), as fezes apresentaram-se com coloração vermelha e consistência amolecida uma semana após o início do experimento, alteração essa observada em apenas um único dia. Nenhum outro sintoma de toxicidade aparente foi observado nos animais.

O tratamento com a própolis promoveu alterações em alguns dos parâmetros biométricos dos animais (machos) que receberam o extrato da própolis. Estes animais apresentaram menor massa corporal quando comparados ao grupo veículo. Uma redução da ingestão alimentar por parte dos animais machos do grupo satélite ao longo do experimento também foi observada. Desta forma, a redução observada em relação a massa corporal dos animais, pode estar relacionada diretamente a este fator. Por outro lado, não foram observadas diferenças no que diz respeito ao peso das fêmeas.

Silva *et al.* (2015), realizaram um teste de toxicidade subaguda de 28 dias de tratamento oral com extrato de própolis vermelha do estado de Alagoas. Ao contrário da amostra de Canavieiras utilizada pelo nosso grupo, a amostra de Alagoas aumentou o peso corporal de machos e fêmeas na maior concentração do tratamento (200 mg/kg). Também, no ensaio subagudo realizado por Silva *et al.* (2015), na primeira semana do experimento, os machos que receberam a dose de 200 mg/kg, apresentaram aumento na ingestão de água, período no qual também apresentaram alguns sinais tóxicos como diarreia. Com as doses de 10 e 100 mg/kg do extrato, uma pequena percentagem dos animais apresentou diminuição da ambulação e sibilância.

Alterações dos parâmetros hematológicos a partir do tratamento subcrônico com a própolis vermelha, também foram observadas conforme relatado no capítulo 3. No estudo realizado com a amostra de Alagoas também foi possível observar que os animais tratados com a própolis mostraram alterações dos parâmetros hematológicos como a contagem de monócitos, hemácias,

e a concentração média de hemoglobina corpuscular, em grupos tratados com 10 e 100 mg/kg do extrato (Silva *et al*., 2015).

Alterações bioquímicas relativas ao funcionamento hepático e renal também foram observadas a partir do ensaio subcrônico. Aumento nos níveis de creatinina e ureia plasmáticos podem estar associados com toxicidade, enquanto mudanças na atividade ALT e AST, são indicativos de toxicidade hepática (Gad, 2014). Neste estudo, o aumento no consumo de água e do nível de ureia no grupo tratado com própolis sem recuperação, apontavam para uma possível toxicidade decorrente do tratamento. No entanto, o nível de creatinina diminuiu no grupo e a avaliação biométrica e histopatológica do tecido renal, não apresentou diferenças significativas quando comparado ao grupo controle. Alterações também foram observadas na atividade da alanina aminotransferase (ALT) e da aspartato aminotransferase (ALT) nos animais tratados com o extrato. Contudo, tais alterações não foram observadas quando da análise estrutural do tecido hepático para ambos os sexos.

As mudanças observadas na atividade destas enzimas devem ainda ser consideradas no momento de se determinar a segurança do consumo da própolis vermelha. Os níveis séricos de ALT são de importância clínica; os níveis séricos aumentados dessa enzima na ausência de alterações histomorfológicas hepáticas podem ser considerados falsos positivos ou um potencial indicador de um efeito prodrômico (Ozer et al., 2008). Ensaios adicionais de medição de outros marcadores enzimáticos como fosfatase alcalina, concentração de bilirrubina e ácidos biliares seriam necessários para determinar um possível efeito negativo no fígado (Luo et al., 2014).

De acordo com Silva *et al*. (2015), o tratamento agudo com a própolis vermelha de Alagoas aumentou os níveis séricos de creatinina, proteína total e albumina em fêmeas tratadas com 200 mg/kg do extrato por via oral. Neste ensaio, um aumento significativo foi observado na atividade da ALT. Contudo, os autores não encontraram alterações morfológicas teciduais que pudessem confirmar os efeitos do tratamento.

Considerando que as amostras são de locais e períodos de coleta diferentes, o perfil metabólico também pode variar, sendo responsável pelas diferenças muitas vezes observadas entre os estudos. Na amostra de própolis vermelha de Alagoas, por exemplo, daidzeína, formononetina e biochanina A foram identificadas, enquanto isoliquiritigenina e liquiritigenina, foram sugeridas como presentes, sendo os compostos majoritários do extrato. Além disso, benzofenonas polipreniladas foram propostas para estarem presentes na amostra. Na amostra de Canavieiras, utilizada neste estudo, as isoflavonas vestitol e neovestitol e a pterocarpana medicarpina foram identificadas como substâncias majoritárias. Foram identificadas também

as benzofenonas polipreniladas gutiferona E/xantoquimol e oblongifolina B, como metabólitos principais presentes na amostra (Ccana-Ccapatinta et al., 2020).

Correlações entre a concentração de gutiferona E e atividade antioxidante foram identificadas em extratos de própolis vermelha brasileira (do Nascimento et al., 2019). Porém, o aumento da concentração de substâncias químicas em alguns sistemas pode gerar também efeitos negativos. Em estudos sobre o efeito protetor da própolis vermelha na colite ulcerativa induzida em ratos *Wistar*, o aumento da concentração da dose induziu efeito pro-oxidante, atribuído ao aumento da concentração dos metabólitos secundários presentes na amostra (Barbosa Bezerra et al., 2017).

Em nosso estudo, a amostra utilizada no ensaio de toxicidade subcrônica foi coletada no verão. Nesta época do ano, como mencionado anteriormente, a própolis apresenta aumento na concentração de benzofenonas. Também é importante considerar que alguns compostos pertencentes ao grupo das isoflavonas têm induzido alterações bioquímicas em roedores. Camundongos tratados oralmente com genisteína (500 e 1000 mg/kg) apresentaram níveis elevados da atividade enzimática de ALT e AST, aumento da peroxidação lipídica (LPO) e diminuição do peptídeo antioxidante glutationa (GSH), induzindo a degeneração do tecido hepático por indução do estresse oxidativo (Singh et al., 2014). A genisteína, biochanina A, daidzeína e formononetina são isoflavonas que têm sido identificadas nas amostras de própolis vermelha brasileira (Corrêa et al., 2017). Na amostra utilizada no presente estudo, não foi identificada a presença de genisteína ou daidzeína. Contudo, estes compostos são provenientes da mesma rota biossintética da biochanina A e da formononetina identificadas na própolis de Canavieiras. Tais compostos referem-se a um grupo de metabólitos secundários com semelhança estrutural. Sendo assim, é provável que os processos de ativação metabólica, os efeitos sistêmicos e os mecanismos de excreção apresentem algumas semelhanças.

Acerca desta hipótese, cita-se o fato de como algumas isoflavonas têm sido relacionadas como “interruptores” da atividade endócrina. Isoflavonas como daidzeína e genisteína e mesmo alguns flavonoides como rutina e quercetina têm sido relacionados a efeitos negativos nos níveis séricos de T3 e T4 em roedores (Di Dalmazi & Giuliani, 2021; Pistollato et al., 2018; Šošić-Jurjević et al., 2014). De acordo com estes estudos, as isoflavonas podem ter efeito negativo na enzima peroxidase tireoidiana (TPO), envolvida na síntese das enzimas envolvidas nas sínteses de T3 e T4 (Otun et al., 2019).

No presente estudo, foram observadas alterações no nível de tiroxina (T4) nos machos tratados com a própolis vermelha, sem recuperação no grupo satélite. Em outros estudos, realizados a partir de modelos experimentais diferentes, foi observado que a suplementação

através da dieta com a própolis vermelha diminuiu os níveis dos hormônios T3 (triiodotironina) e T4 (tiroxina) em ovelhas. A amostra era composta majoritariamente por medicarpina, isoliquiritigenina, vestitol e isoflavonas como a formononetina (Morsy et al., 2021).

Alguns dos parâmetros reprodutivos também foram alterados com o tratamento da própolis vermelha, tanto em machos quanto em fêmeas. Nos machos houve diminuição da motilidade espermática nos animais que receberam o tratamento com a própolis e nas fêmeas foi observada a prevalência de algumas fases do ciclo estral. Estas variações também podem estar associadas à presença de isoflavonas presentes na amostra. As isoflavonas são capazes de se ligar ativamente aos receptores de estrogênio, interferindo na síntese, secreção, transporte, metabolismo, ação de ligação ou eliminação de hormônios naturais no organismo que são responsáveis pela reprodução (Cederroth et al., 2012).

No entanto, os estudos relacionados à análise de parâmetros reprodutivos em modelos animais expostos à isoflavonas ainda não são conclusivos. Para alguns autores as isoflavonas não são capazes de induzir efeitos negativos a longo prazo em machos. Em outros estudos, a exposição desde a concepção até a idade adulta, torna os machos suscetíveis à diminuição da produção de espermatozoides (Esch & Lehmann, 2020).

Ratos *Wistar* tratados com extratos vegetais ricos em fitoestrogênios de *Leucaena leucocephala* (3,5 g/kg/dia) apresentaram redução da contagem espermática, além de apresentar menor motilidade e viabilidade. O efeito foi atribuído à diminuição dos receptores androgênicos no epidídimo, reduzindo a proteção antioxidante e aumentando a possibilidade de peroxidação lipídica (Retana-Márquez et al., 2016). Resultados semelhantes foram relatados em ensaios com isoflavonas isoladas. Uma redução da motilidade espermática em ratos *Wistar* que foram expostos cronicamente à genisteína na dose oral de 1 mg/kg foi observada. Esse efeito foi atribuído à capacidade da isoflavona em reduzir a expressão de α-glicosidase, uma enzima relacionada a motilidade espermática (Eustache et al., 2009).

Por outro lado, nas fêmeas tratadas com a própolis vermelha proveniente de Canavieiras, houve prevalência de algumas fases do ciclo estral. Esta situação é atípica considerando que em animais controle fêmeas, sob as mesmas condições ambientais e sem estímulos, espera-se uma assincronia citológica de ciclos dentro do grupo (Goldman et al., 2007; Smith et al., 2015). Pelo contrário, uma maior homogeneidade de células em todo o grupo pode ser consequência de possível efeito do tratamento sobre a função endócrina, em animais expostos a substâncias tóxicas (Goldman *et al*., 2007). No entanto, como mencionado anteriormente, neste estudo, não foram observadas alterações histológicas dos tecidos sexuais. Histologia ovariana e uterina,

assim como os níveis dos hormonais tireoidianos das fêmeas apresentaram-se dentro da normalidade.

Conforme mencionado, algumas das alterações promovidas pela administração subcrônica do extrato da própolis vermelha foram mais pronunciadas nos animais dos grupos satélites. Isto levaria a pensar que alguns dos possíveis efeitos adversos do tratamento poderiam ser tardios. Estudos de exposição a agentes tóxicos revelam que sintomas de toxicidade no período de recuperação após a fase de administração, não necessariamente são indicadores de toxicidade tardia, mas sim consequência da exposição prolongada a agentes com meia-vida de longa duração (Pandher et al., 2012).

Para tanto, estudos de farmacocinética da própolis vermelha, assim como a avaliação de biomarcadores do estresse oxidativo, são de grande importância na avaliação dos possíveis mecanismos relacionados aos efeitos observados. Nesta perspectiva, seria possível sugerir doses eficazes e seguras dos produtos elaborados a partir da própolis vermelha, aproveitando assim todo o seu potencial biológico.

5. CONCLUSÕES

- Marcadores característicos da própolis vermelha foram identificados na amostra coletada na região de Canavieiras, Bahia, destacando-se a presença majoritária de isoflavanas (metilvestitol, vestitol e neovestitol), pterocarpanos (medicarpina), flavanonas (liquiritigenina), isoflavonas (calicosina, formononetina e biochanina A), chalconas (isoliquiritigenina) e benzofenonas polipreniladas, oblongifolina B e a mistura dos isómeros gutiferona E/xantoquimol.
- A análise qualitativa do perfil cromatográfico da própolis vermelha brasileira por CLAE-DAD foi desenvolvida e otimizada para análise das isoflavanas, pterocarpanos, flavanonas, isoflavonas e chalconas da amostra. O método foi validado cumprindo com os parâmetros de especificidade, linearidade, precisão, exatidão e robustez, mostrando ser uma ferramenta analítica adequada para o controle de qualidade de amostras de própolis vermelha e da sua principal fonte botânica *D. ecastaphyllum*.
- Estudos de sazonalidade indicaram que as substâncias vestitol, medicarpina e neovestitol são majoritárias em amostras de própolis vermelha da região de Canavieiras (Bahia) ao longo do ano. Por outro lado, biochanina A e calicosina se apresentaram como os metabólitos em menor concentração nas amostras. No entanto, a concentração destas substâncias pode ser influenciada por fatores climáticos, sendo que nos períodos de alta precipitação pluviométrica e umidade (maio e outubro), os compostos fenólicos tendem a ter sua concentração aumentada na própolis vermelha.
- A própolis vermelha e a sua principal fonte botânica *D. ecastaphyllum,* em altas concentrações, apresentam efeito citotóxico significativo sobre a linhagem celular normal GM07492A, apresentando valores de IC_{50} superiores a 100 µg/mL. Os compostos isolados da própolis vermelha apresentaram atividade citotóxica contra linhagens tumorais, com baixos valores de IC_{50}, porém com baixa seletividade para células normais. A medicarpina foi a única substância que apresentou efeito citotóxico seletivo para a linhagem tumoral HeLa.
- O extrato de própolis vermelha proveniente de Canavieiras e o da sua fonte botânica *D. ecastaphyllum* não apresentaram efeito genotóxico sobre a linhagem de células normais V79. Da mesma forma, o extrato de própolis não apresentou efeito genotóxico *in vivo* em

camundongos *Swiss* machos, por ingestão oral em doses que chegaram até a dose limite de 2000 mg/kg.

- Nos ensaios *in vivo* de toxicidade aguda com dose única de 2000 mg/kg, não foram observados sintomas de toxicidade em fêmeas *Wistar*, mostrando segurança de seu uso nestas condições. Já no ensaio de toxicidade subcrônica em doses repetidas de 1000 mg/kg durante 90 dias em ratos *Wistar*, ambos os sexos, a administração não parece ter desencadeado sinais de toxicidade imediata, embora tenham sido observadas algumas alterações no peso relativo de alguns tecidos como fígado, tireoide e próstata, bem como em alguns dos parâmetros relacionados à função renal e hepática nos machos. Esses resultados sugerem que a composição química da própolis vermelha associada a altas dosagens, merece atenção especial quanto a possível toxicidade mediada por sua ingestão. Essas alterações também parecem estar relacionadas ao sexo, uma vez que as maiores alterações foram observadas nos machos. No entanto, para que o potencial da própolis vermelha seja devidamente explorado e para que seu consumo seja seguro, são necessários novos estudos para identificar o possível mecanismo associado às alterações observadas para determinação das doses seguras para uso humano e animal.

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APÊNDICES

Apêndice 1. CAPÍTULO 1. Material Suplementar

Artigo: Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum*

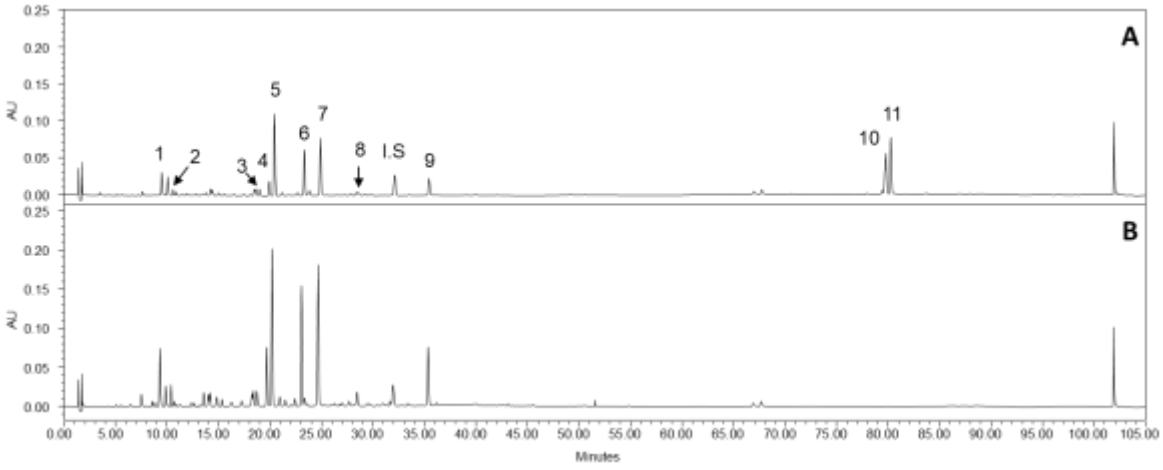


Figure S. 0-1. HPLC chromatographic profiles of the Brazilian red propolis (A) and *D. ecastaphyllum* resin (B). Identity of the isolated constituents: liquiritigenin (1), calycosin (2), isoliquiritigenin (3), formononetin (4), vestitol (5), neovestitol (6), medicarpin (7), biochanin A (8), oblongifolin B 7-*O*-methylvestitol (9), mixture of guttiferone E and xanthochymol (10), oblongifolin B (11).

NMR data.

Liquiritigenin (1): ^{1}H NMR (300 MHz, CD_3OD) δ 2.65 (dd, *J* = 16.9, 2.8 Hz, 1H, H-3_{eq}), 3.01 (dd, *J* = 16.9, 13.2 Hz, 1H, H-3_{ax}), 5.31 (dd, *J* = 12.9, 2.2 Hz, 1H, H-2), 6.34 (d, *J* = 2.1 Hz, 1H, H-8), 6.48 (dd, *J* = 8.7, 2.0 Hz, 1H, H-6), 6.81 (d, *J* = 8.5 Hz, 2H, H-3', H-5'), 7.29 (d, *J* = 8.4 Hz, 2H, H-2', H-6'), 7.70 (d, *J* = 8.7 Hz, 1H, H-5). ^{13}C NMR (101 MHz, CD_3OD) *δ* 44.8 (C-3), 80.9 (C-2), 103.8 (C-8), 111.7 (C-6), 114.9 (C-10), 116.2 (C-3', C-5'), 128.98 (C-2', C-6'), 129.8 (C-5), 131.2 (C-1), 158.8 (C-4'), 165.4 (C-9), 166.6 (C-7), 193.5 (C-4).

Calycosin (2): ^{1}H NMR (300 MHz, C_2D_6OS) δ 3.79 (s, 3H, 4'-OCH_3), 6.86 (d, *J* = 1.9 Hz, 1H, H-8), 6.93 (dd, *J* = 8.8, 1.9 Hz, 1H, H-6), 6.94 (s, 2H, H-5', H-6'), 7.05 (s, 1H, H-2'), 7.97 (d, *J* = 8.8, 1H, H-5), 8.28 (s, 1H, H-2). ^{13}C NMR (101 MHz, C_2D_6OS) δ 55.7 (4'-OCH_3), 102.1

(C-8), 111.9 (C-5'), 115,2 (C-6), 116,4 (C-10), 116,63 (C-2'), 119.70 (C-6'), 123,3 (C-3), 124,71 (C-1'), 127,3 (C-5), 146.0 (C-3'), 147.5 (C-4'), 153.0 (C-2), 157.4 (C-9), 162.6 (C-7), 174.6 (C-4).

Isoliquiritigenin (3): ^{1}H NMR (300 MHz, CD_3COCD_3) δ 6.37 (d, J = 2.4 Hz, 1H, H-3'), 6.47 (dd, J = 8.8, 2.4 Hz, 1H, H-5'), 6.93 (d, J = 8,6 Hz, 2H, H-3, H-5), 7.73 (d, J = 8.4 Hz, 2H, H-2, H-6), 7.80 (d, J = 11.1 Hz, 2H, H-α, H-β), 8.12 (d, J = 8.9 Hz, 1H, H6'). ^{13}C NMR (101 MHz, CD_3COCD_3) δ 103.65 (C-3'), 108.6 (C-5'), 114.4 (C-1'), 116.7 (C-3, C-5), 118.2 (C-α), 127.4 (C-1), 131.6 (C-2, C-6), 133.1 (C-6'), 145.0 (C- β), 160.9 (C-4), 165.4 (C-2'), 167.4 (C-4'), 192.7 (C=O).

Formononetin (4): ^{1}H NMR (400 MHz, C_2D_6OS) δ 3.78 (s, 3H, 4'-OCH_3), 6.88 (d, J = 2.2 Hz, 1H, H-8), 6.95 (dd, J = 8.8, 2.3 Hz, 1H, H-6), 6.97 -7.02 (m, 2H, H-3', H-5'), 7.49-7.54 (m, 2H, H-2', H-6'), 7.98 (d, J = 8.8 Hz, 1H, H-6), 8.35 (s, 1H, H-2). ^{13}C NMR (101 MHz, C_2D_6OS) δ 55.1 (4'-OCH_3), 102.1 (C-8), 113.6 (C-3', C-5'), 115.2 (C-6), 116.6 (C-10), 123.1 (C-3), 124.2 (C-1'), 127.3 (C-5), 130.1 (C-2', C-6'), 153.1 (C-2), 157.4 (C-9), 158.9 (C-4'), 162.6 (C-7), 174.6 (C-4).

Vestitol (5): ^{1}H NMR (500 MHz, CD_3COCD_3) δ 2.80 (ddd, J = 15.6, 5.2, 2.0 Hz, 1H, H-4_{eq}), 2.96 (ddd, J = 15.6, 11.0 Hz, 1H, H-4_{ax}), 3.40-3.55 (m, 1H, H-3), 3.71 (s, 3H, 7-OCH_3), 3.97 (t, J = 10.1 Hz, 1H, H-2_{ax}), 4.23 (ddd, J = 10.3, 3.5, 2.1 Hz, 1H, H-2_{eq}), 6.29 (d, J = 2.4 Hz, 1H, H-8), 6.37 (dd, J = 8.2, 2.5 Hz, 1H, H-6), 6.41 (dd, J = 8.5, 2.5 Hz, 1H, H-5'), 6.50 (d, J = 2.5 Hz, 1H, H-3), 6.89 (d, J = 8.2 Hz, 1H, H-5), 7.04 (d, J = 8.4 Hz, 1H, H-6'). ^{13}C NMR (125 MHz, CD_3COCD_3) δ 31.0 (C-4), 32.6 (C-3), 55.4 (7-OCH_3), 70.5 (C-2), 102.5 (C-3'), 103.7 (C-8), 105.7 (C-5'), 108.8 (C-6), 114.3 (C-10), 120.9 (C-1'), 128.7 (C-6'), 131.0 (C-5), 156.0 (C-9), 156.0 (C-9), 156.7 (C-2'), 157.5 (C-2'), 160.4 (C-4').

Neovestitol (6): ^{1}H NMR (500 MHz, CD_3COCD_3) δ 2.81 (ddd, J = 15.7, 5.2, 2.0 Hz, 1H, H-$4_{eq,}$), 2.97 (ddd, J = 15.7, 11.1, 1.1 Hz, 1H, H-4_{ax}), 3.42-3.52 (m, 1H, H-3), 3.73 (d, 3H, 7-OCH_3), 3.98 (t, J = 10.2 Hz, 1H, H-2_{ax}), 4.26 (ddd, J = 10.3, 3.5, 2.1 Hz, 1H, H-2_{eq}), 6.35 (td, J = 4.1, 2.4 Hz, 2H, H-6, H-8), 6.43 (dd, J = 8.4, 2.6 Hz, 1H, H-5'), 6.47 (d, J = 2.5 Hz, 1H, C-3'), 6.94 (d, J = 8.3 Hz, 1H, H-6'), 6.97 (d, J = 8.3 Hz, 1H, H-5). ^{13}C NMR (125 MHz, CD_3COCD_3) δ 31.1 (C-4), 32.6 (C-3), 55.4 (7-OCH_3), 70.7 (C-2), 102.1 (C-8), 103.6 (C-3'),

107.6 (C-6), 107.8 (C-5'), 115.6 (C-10), 119.5 (C-1'), 128.7 (C-6'), 131.0 (C-5), 156.8 (C-2'), 156.7 (C-2'), 157.9 (C-4'), 160.1 (C-7).

Medicarpin (7): ^{1}H NMR (300 MHz, CD_3COCD_3) δ 3.51 – 3.63 (m, 2H, H-6a, H-6b), 3.73 (s, 3H, 9-OCH_3), 4.18-4.35 (m, 1H, H-6), 5.50 (d, *J* = 5.9 Hz, 1H, H-11a), 6.36 (d, *J* = 2.4 Hz, 1H, H-4), 6.38 (d, *J* = 2.3 Hz, 1H, H-10b), 6.44 (dd, *J* = 8.2, 2.3 Hz, 1H, H-8), 6.56 (dd, *J* = 8.4, 2.4 Hz, 1H, H-2), 7.22 (d, *J* = 8.2 Hz, 1H, H-7), 7.32 (d, *J* = 9.0 Hz, 1H, H-1). ^{13}C NMR (101 MHz, CD_3COCD_3) δ 40.4 (C-6a), 55.7 (OCH_3), 67.1 (C-6), 79.42 (C-11a), 97.2 (C-10b), 103.9 (C-4), 106.8 (C-8), 110.5 (C-2), 112.8 (C-11b), 120.4 (C-6b), 125.8 (C-7), 133.1 (C-1), 157.7 (C-4a), 159.6 (C-3), 161.8 (C-10a), 162.09 (C-9).

Biochanin A (8): ^{1}H NMR (500 MHz, CD_3COCD_3) δ 3.83 (s, 4'-OCH_3, 3H), 6.29 (d, *J* = 2.2 Hz, 1H, H-8), 6.42 (d, *J* = 2.1 Hz, 1H, H-6), 6.99 (d, *J* = 8.8, 2H, H-3', H-5'), 7.54 (d, *J* = 8.7 Hz, 2H, H-2', H-6'), 8.19 (s, 1H, H-2). ^{13}C NMR (126 MHz, CD_3COCD_3) δ 55.6 (4'-OCH_3), 94.5 (C-8), 99.9 (C-6), 106.2 (C-10), 114.5 (C-3', C-5'), 123.8 (C-1'), 124.2 (C-3), 131.1 (C-2', C-6'), 154.5 (C-2), 159.1 (C-9), 160.7 (C-4'), 163.9 (C-5), 165.1 (C-7), 181.6 (C-4).

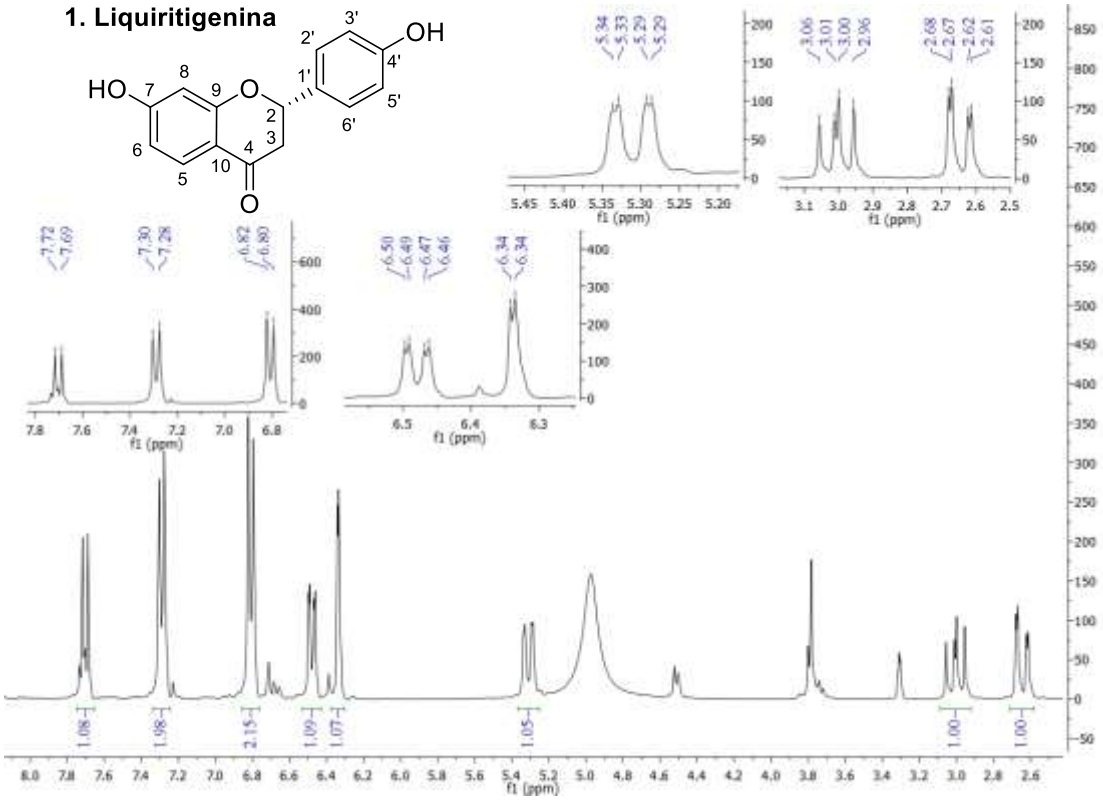
7-*O*-methylvestitol (9): ^{1}H NMR (300 MHz, CD_3COCD_3) δ 2.83 (ddd, *J* = 15.8, 5.3, 2.0 Hz, 1H H-4_{eq}), 2.99 (m, 1H, H-4_{ax}), 3.49 (tdd, *J* = 10.3, 5.3, 3.5 Hz, 1H, H-3), 3.71 (s, 3H, 7-OCH_3), 3.72 (s, 3H, 4'-OCH3), 4.00 (t, *J* = 10.1 Hz, 1H, H-2ax), 4.26 (ddd, *J* = 10.3, 3.5, 2.0 Hz, 1H, H-2eq), 6.35 (d, *J* = 2.6 Hz, 1H, H-8), 6.42 (dd, *J* = 5.0, 2.6 Hz, 1H, H-5'), 6.45 (dd, *J* = 4.8, 2.5 Hz, 1H, H-6), 6.50 (d, *J* = 2.5 Hz, 1H, H-3'), 6.98 (d, *J* = 8.4 Hz, 1H, H-5), 7.05 (d, *J* = 8.5 Hz, 1H, H-6'). ^{13}C NMR (101 MHz, CD_3COCD_3) δ 31.00 (C-4), 32.6 (C-3), 55.4 (4'-OCH_3), 55.5 (7-OCH_3), 70.6 (C-2), 102.1 (C-8), 102.5 (C-3'), 105.7 (C-5'), 107.6 (C-6), 115.4 (C-10), 120.8 (C-1'), 128.8 (C-6'), 131.0 (C-5), 156.1 (C-2'), 156.7 (C-9), 160.1 (C-4'), 160.4 (C-7).

Mixture of guttiferone E and xanthochymol (10): ^{1}H NMR (CD_3OD and 1% of TFA, 500 MHz) δ 0.98 (s, 3H, H-23), 1.16 (s, 3H, H-37), 1.49 (bs, 4H, H-6, H-28), 1.57 (d, *J* = 1.0 Hz, 3H H-38), 1.60 (s, 3H, H-33), 1.64 (s, 3H, H-20), 1.68 (s, 3H, H-22), 1.69 (s, 3H, H-21), 1.73 (s, 3H, H-27), 1.79-1.95 (m, 2H, H-29), 1.99-2.05 (m, 4H, H-7_{eq}, H-24, H-34), 2.07-2.14 (m, 1H, H-24), 2.26 (d, *J* = 14.0 Hz, 1H, H-7ax), 2.57 (m, 2H, H-17, H-30), 2.71 (dd, *J* = 13.0, 9.5 Hz, 1H, H-17), 4.46 (d, *J* = 9.6 Hz, 1H, H-32), 4.50 (s, 1H, H-32), 4.84 – 4.89 (m, 1H, H-35), 5.00-5.09 (m, 2H, H-18, H-25), 6.72 (d, *J* = 8.5 Hz, 1H, H-15), 6.99 (dd, *J* = 8.2, 2.1 Hz,

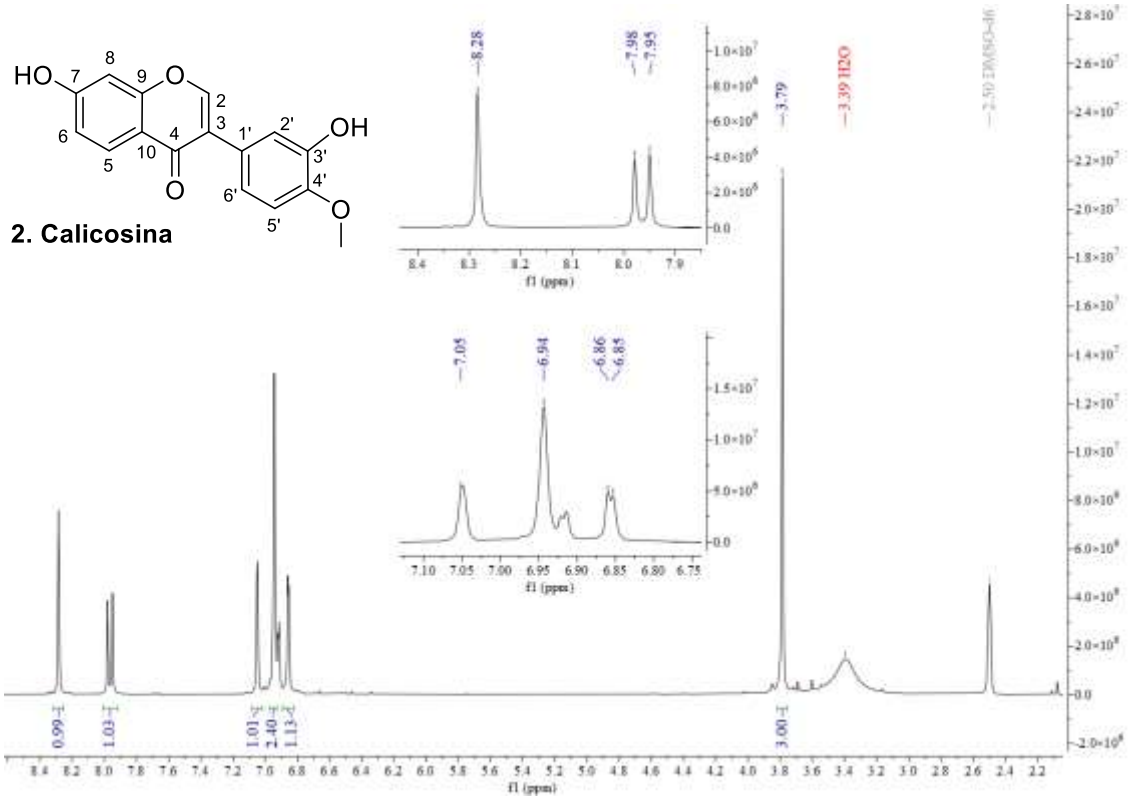
1H, H-16), 7.20 (d, *J* = 2.1 Hz, 1H, H-12). ^{13}C NMR (125 MHz, CD_3OD) δ 17.8 (C-38), 18.1 (C-33), 18.2 (C-28), 18.3 (C-21), 23.2 (C-22), 25.5 (C-22), 26.0 (C-37), 26.4 (C-20), 27.0 (C-17), 27.4 (C-23), 30.2 (C-24), 33.4 (C-34), 36.7 (C-29), 43.8 (C-7), 45.1 (C-30), 47.9 (C-6), 50.2 (C-5), 59.8 (C-8), 69.7 (C-4), 112.5 (C-32), 115.1 (C-15), 117.3 (C-12), 119.3 (C-2), 121.2 (C-18), 124.0 (C-35), 125.1 (C-16), 125.4 (C-25), 129.5 (C-11), 132.6 (C-36), 133.5 (C-26), 135.8 (C-19), 146.1 (C-13), 149.3 (C-31), 152.3 (C-14), 194.3 (C-3), 195.6 (C-10), 196.1 (C-1), 210.6 (C-9).

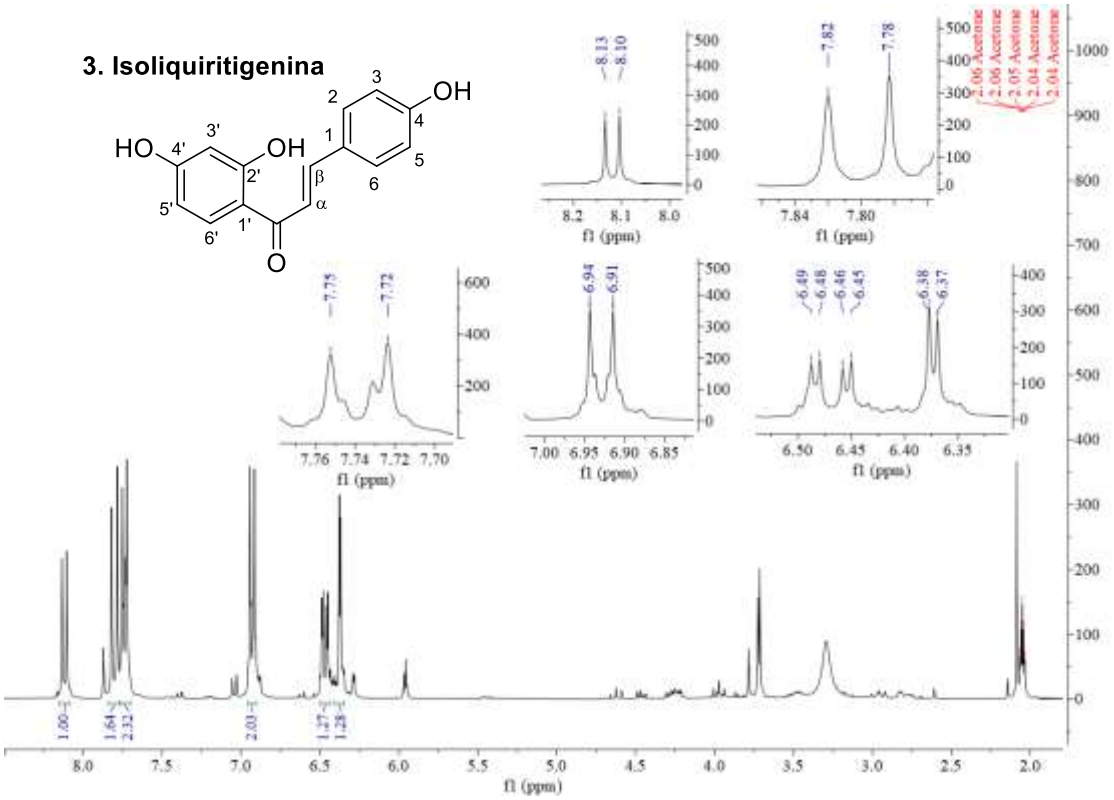
Oblongifolin B (11): ^{1}H NMR (500 MHz, CD_3OD) *δ* 0.79 (s, 3H, H-22), 1.17 (s, 3H, H-23), 1.47 (t, *J* = 13.34 Hz, 1H, H-7_{ax}), 1.54 (s, 3H, H-28), 1.57 (s, 3H, H-38), 1.60 (s, 1H, C-6), 1.64 (s, 3H, H-32), 1.66 (s, 3H, H-37), 1.67 (s, 3H, H-33), 1.69 (s, 3H, H-21), 1.71 (s, 3H, H-20), 1.75 (d, *J* = 9.9 Hz, 3H, H-24), 1.99 (dd, *J* = 17.8, 5.4 Hz, 3H, H-27), 2.05 (d, *J* = 6.8 Hz, 3H, H-7, H-34), 2.13 (d, *J* = 11.6 Hz, 1H, H-24), 2.48 (dd, *J* = 13.9, 5.5 Hz, 1H, H-29), 2.54 (dd, *J* = 13.9, 8.5 Hz, 1H, H-29), 2.61 (d, *J* = 13.1 Hz, 1H, H-17), 2.71 (dd, *J* = 13.5, 8.6 Hz, 1H, H-17), 4.95-5.02 (m, 1H, H-25), 4.92 (s, 1H, H-18), 5.03 – 5.09 (m, 1H, H-35), 5.12 – 5.20 (m, 1H, H-30), 6.70 (d, *J* = 8.3 Hz, 1H, H-15), 6.96 (dd, *J* = 8.3, 2.1 Hz, 1H, H-16), 7.19 (d, *J* = 2.1 Hz, 1H, H-12). ^{13}C NMR (126 MHz, CD_3OD) *δ* 16.4 (C-22), 16.5 (C-28), 17.8 (C-38), 18.2 (C-33), 18.3 (C-21), 23.8 (C-23), 25.9 (C-37), 26.0 (C-32), 26.2 (C-20), 26.3 (C-34), 27.4 (C-17), 29.4 (C-24), 31.4 (C-29), 40.7 (C-27), 43.2 (C-7), 44.1 (C-6), 48.5 (C-5), 63.6 (C-8), 69.6 (C-4), 115.8 (C-15), 117.4 (C-12), 118.8 (C-2), 120.9 (C-30), 121.1 (C-18), 123.8 (C-25), 124.9 (C-35), 125.1 (C-16), 129.8 (C-11), 132.2 (C-36), 135.2 (C-31), 135.3 (C-19), 137.8 (C-26), 146.0 (C-13), 152.3 (C-14), 192.5 (C-3), 194.5 (C-10), 196.4 (C-1), 208.8 (C-9).

Apêndice 2. Estrutura química e espectro de RMN de ^{1}H (CD_3OD; 300 MHz) do composto liquiritigenina (1).

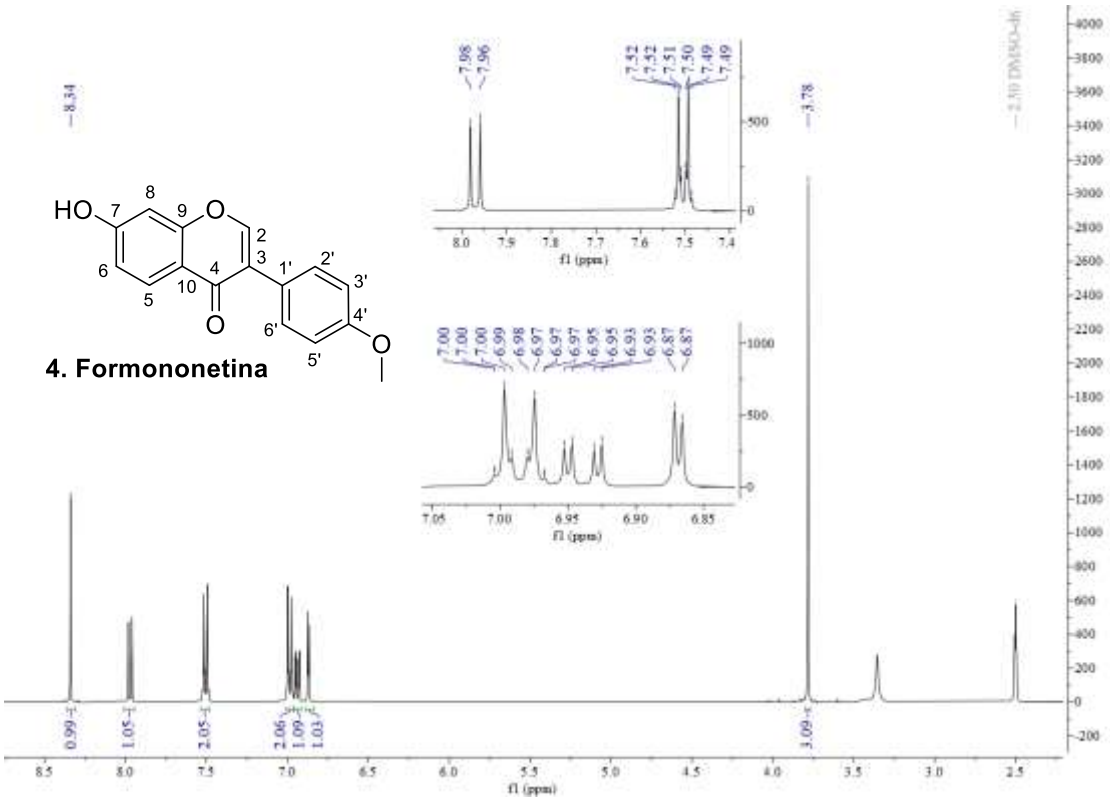


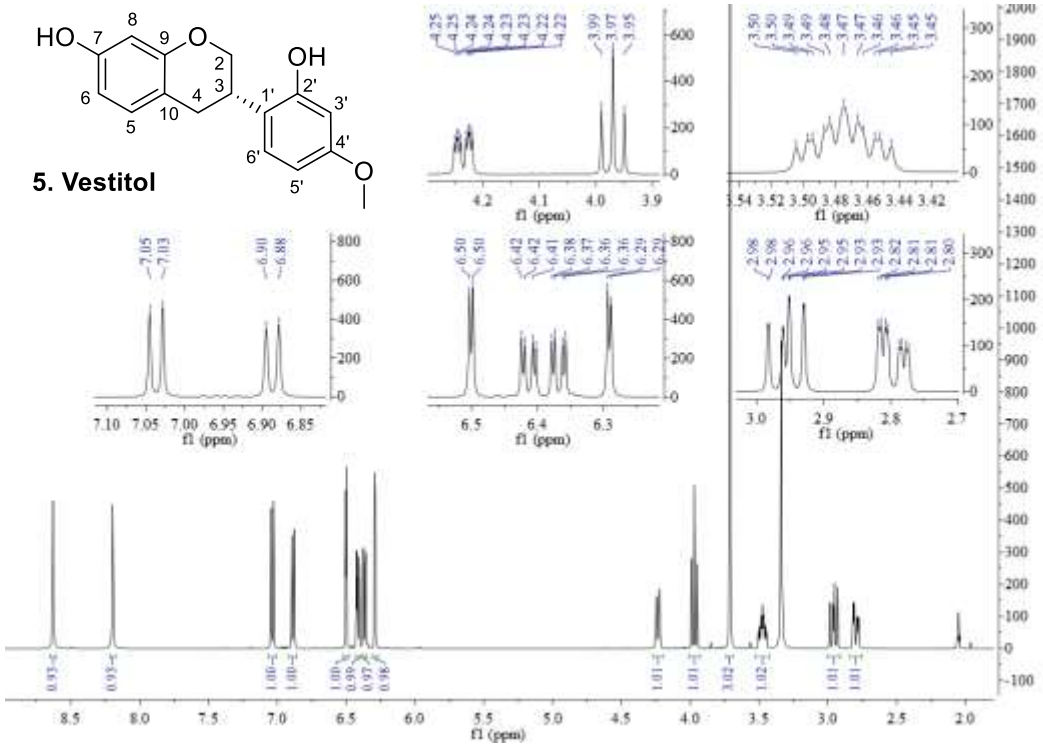
Apêndice 3. Estrutura química e espectro de RMN de ^{1}H dos compostos calicosina (2; C_2D_6OS; 300 MHz) e isoliquiritigenina (3; CD_3OD; 300 MHz).



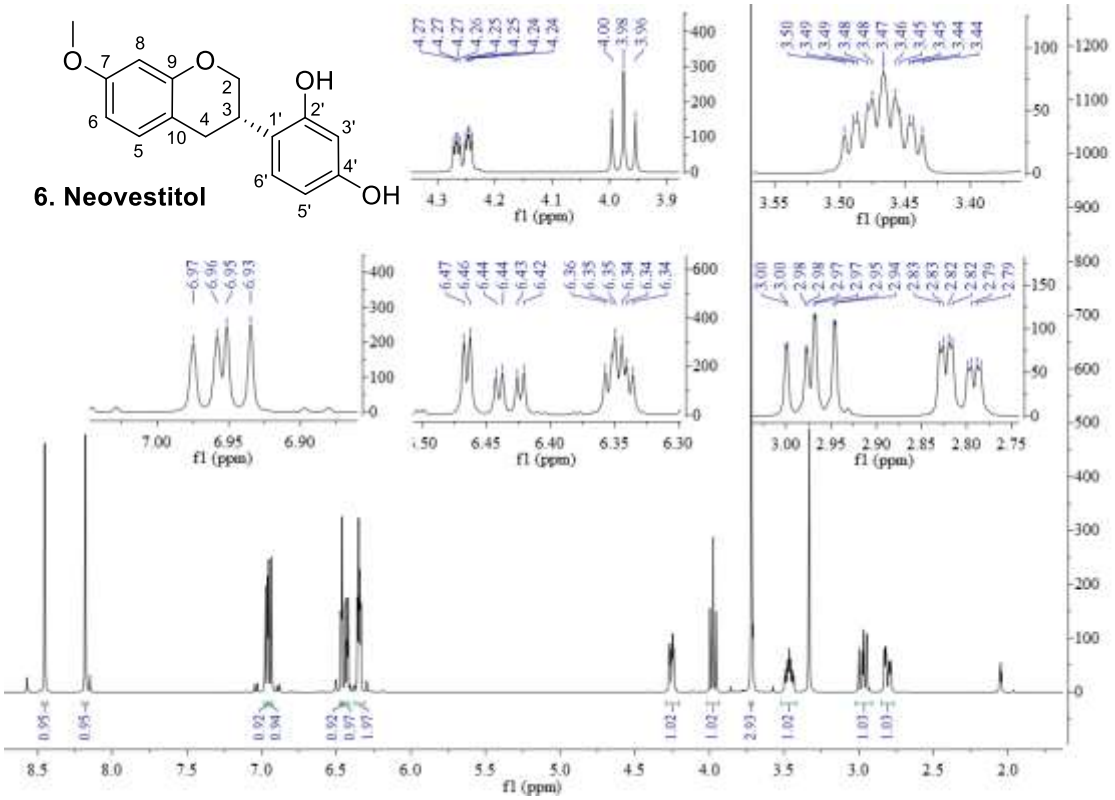


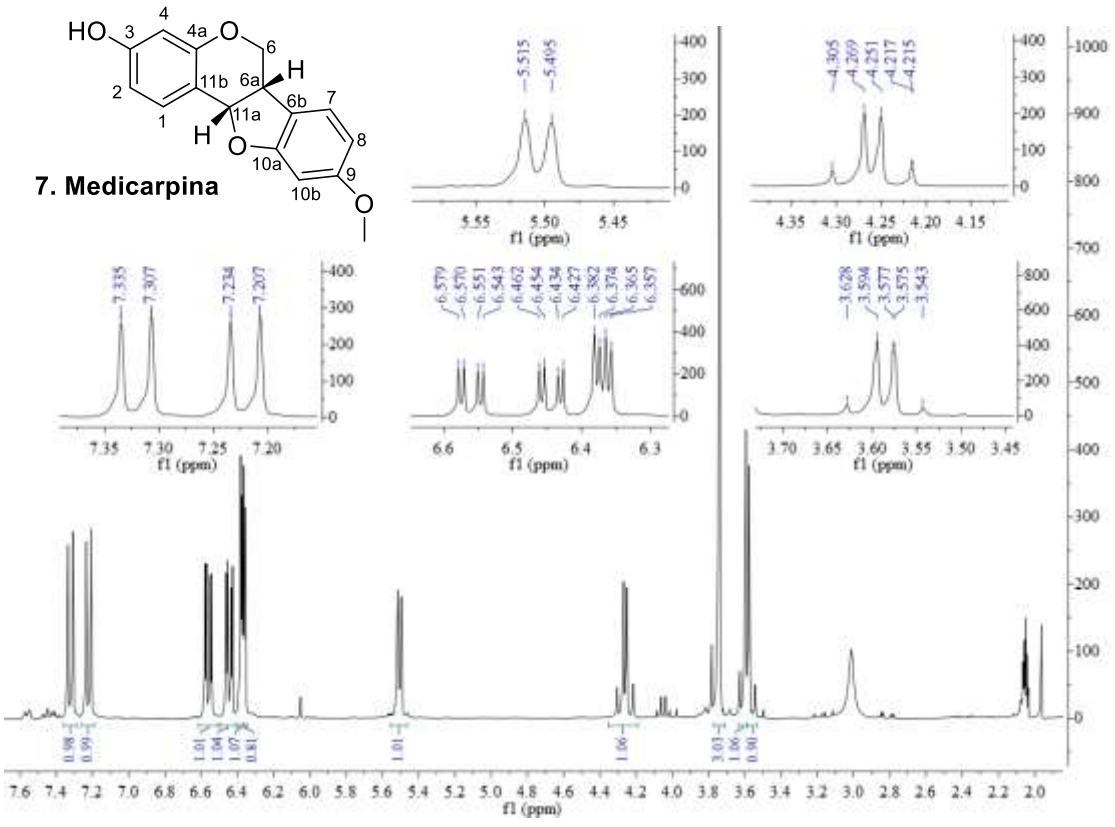
Apêndice 4. Estrutura química e espectro de RMN de ^{1}H dos compostos formononetina (4; C_2D_6OS; 400 MHz) e vestitol (5; CD_3COCD_3; 500 MHz).



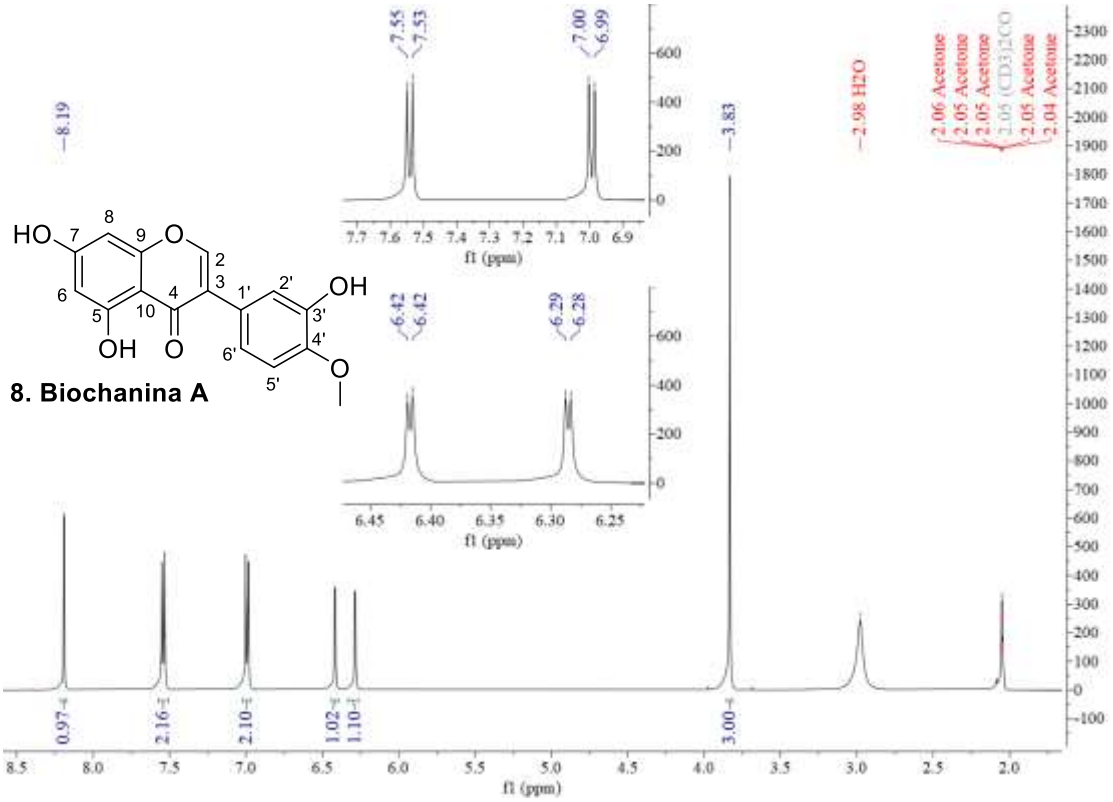


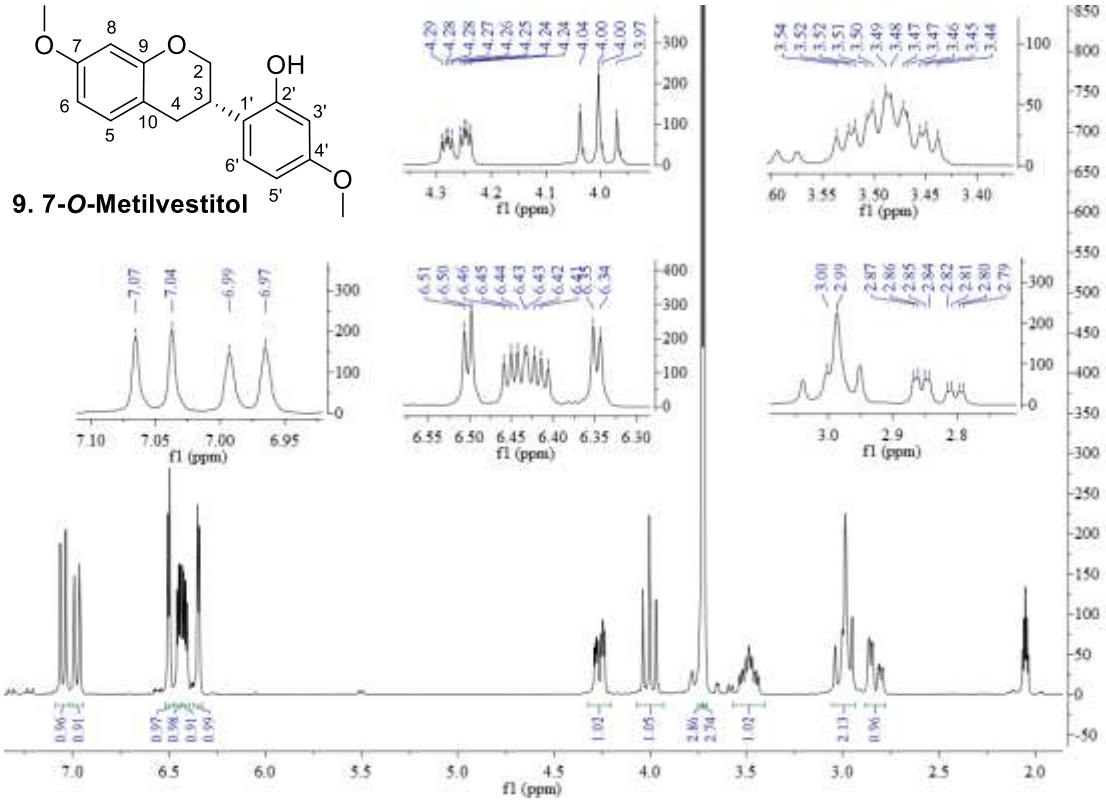
Apêndice 5. Estrutura química e espectro de RMN de ^{1}H dos compostos neovestitol (6; CD_3COCD_3; 500 MHz) e medicarpina (7; CD_3OD; 300 MHz).



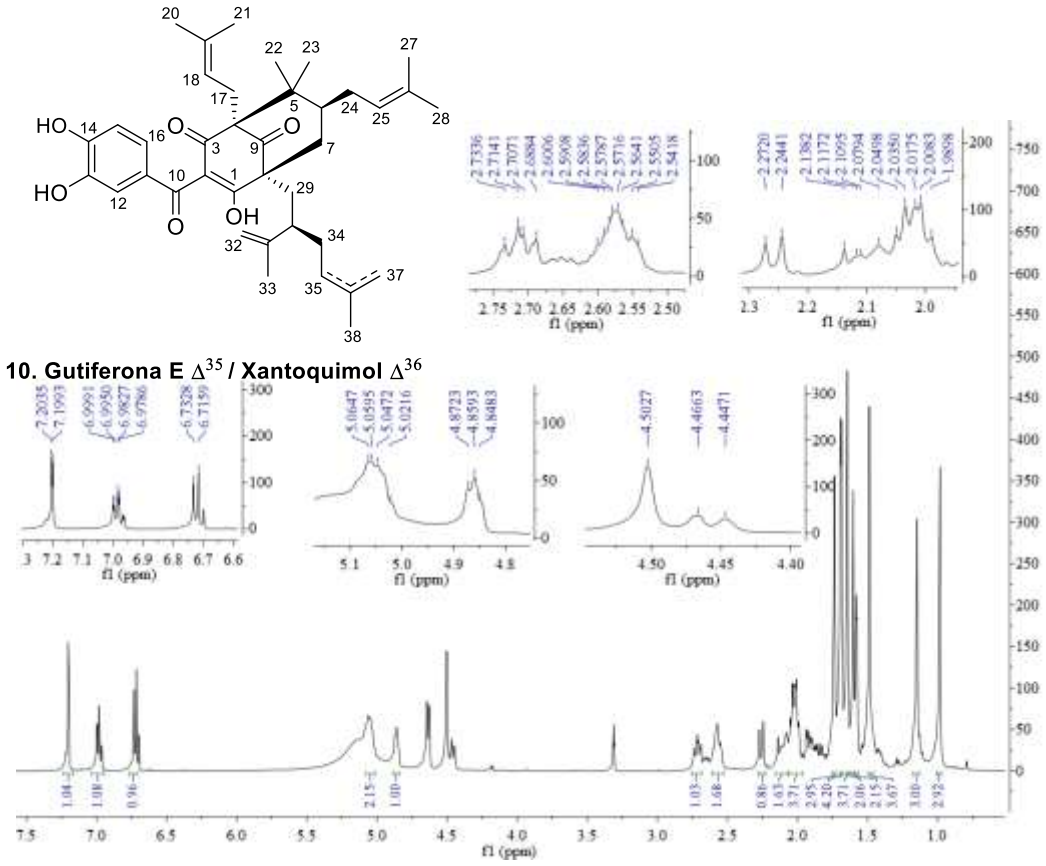


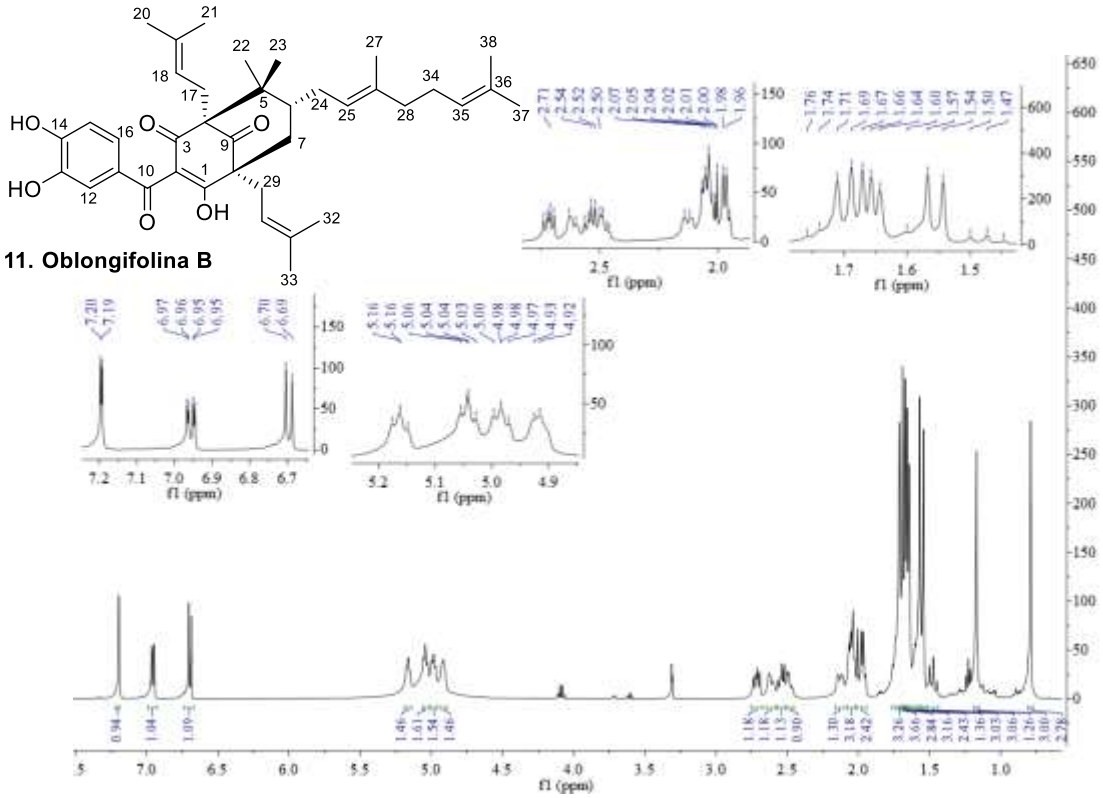
Apêndice 6. Estrutura química e espectro de RMN de ^{1}H (CD_3COCD_3; 500 MHz) dos compostos e biochanina A (8) e 7-*O*-metilvestitol (9)





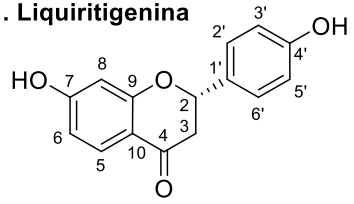
Apêndice 7. Estrutura química e espectro de RMN de ^{1}H (CD_3OD com adição de 1% de ATF; 500 MHz) dos compostos gutiferona E / xantoquimol (10) e oblongifolina B (11).



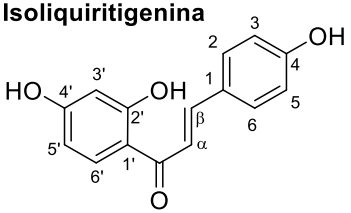


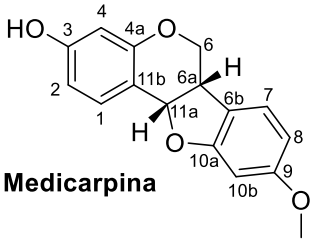
Apêndice 8. Dados de RMN de ^{1}H e ^{13}C dos compostos liquiritigenina (1), isoliquiritigenina (3), e medicarpina (7).

1. Liquiritigenina



3. Isoliquiritigenina

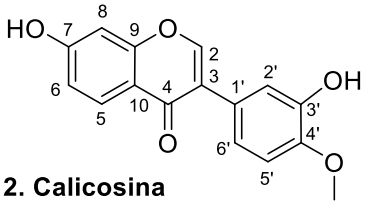




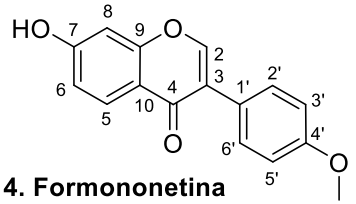
7. Medicarpina

	Liquiritigenina		**Isoliquiritigenina**			**Medicarpina**	
Carbono	**$^{1}H\delta$(ppm)***	**$^{13}C\delta$(ppm)***	**$^{1}H\delta$(ppm)$^{+}$**	**$^{13}C\delta$(ppm)$^{+}$**	**Carbono**	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**
1			-	127,4	**1**	7,32 (d, *J* = 9,0 Hz; 1H)	133,0
2	5,31 (dd, *J* = 12,9; 2,2 Hz; 1H)	80,9	7,73 (d, *J* = 8,4 Hz; 2H)	131,6	**2**	6,56 (dd, *J* = 8,4; 2,4 Hz; 1H)	110,5
3	eq 2,65 (dd, *J* = 16,9; 2,8 Hz; 1H) ax 3,01 (dd, *J* = 16,9; 13,2 Hz; 1H)	44,8	6,93 (d, *J* = 8,6 Hz; 2H)	116,6	**3**	-	159,6
4	-	193,5	-	160,8	**4**	6,36 (d, *J* = 2,4 Hz; 1H)	103,9
5	7,70 (d, *J* = 8,7 Hz; 1H)	129,8	6,93 (d, *J* = 8,6 Hz; 2H)	116,6	**4a**	-	157,7
6	6,48 (dd, *J* = 8,7; 2,0 Hz; 1H)	111,7	7,73 (d, *J* = 8,4 Hz; 2H)	131,6	**6**	α 4,35-4,19 (m; 1H) β 3,63-3,51 (m; 2H)	67,0
7	-	166,6			**6a**	3,63-3,51 (m; 2H)	40,3
8	6.34 (d, *J* = 2,1 Hz; 1H)	103,8			**6b**	-	120,4
9	-	165,4			**7**	7,22 (d, *J* = 8,2 Hz; 1H)	125,8
10	-	114,9			**8**	6,44 (dd, *J* = 8,2; 2,3 Hz; 1H)	106,8
1'	-	131,2	-	114,4	**9**	-	162,0
2'	7,29 (d, *J* = 8,4 Hz; 2H)	128,9	-	165,4	**10a**	-	161,7
3'	6,81 (d, *J* = 8,5 Hz; 2H)	116,2	6,37 (d, *J* = 2,4 Hz; 1H)	103,6	**10b**	6,38 (dd, *J* = 2,3 Hz; 1H)	97,2
4'	-	158,8	-	167,4	**11a**	5,50 (d, *J* = 5,9 Hz; 1H)	79,4
5'	6,81 (d, *J* = 8,5 Hz; 2H)	116,2	6,47 (dd, *J* = 8,8; 2,4 Hz; 1H)	108,5	**11b**	-	112,8
6'	7,29 (d, *J* = 8,4 Hz; 2H)	128,9	8,12 (d, *J* = 8,9 Hz; 1H)	133,1	**OCH_3**	3,76 (s, 3H)	55,7
α			7,80 (d, *J* = 11,8 Hz; 2H)	118,2			
β			7,80 (d, *J* = 11,8 Hz; 2H)	145,0			
C=O				192,7			
	***300 e 101 MHz, MeOH-d_4**		**$^{+}$300 e 101 MHz, Acetona-d_6**			**$^{\pm}$300 e 101 MHz, Acetona- d_6**	

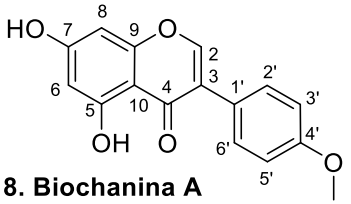
Apêndice 9. Dados de RMN de ^{1}H e ^{13}C dos compostos calicosina (2), formononetina (4) e biochanina A (8).



2. Calicosina



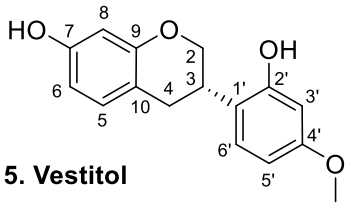
4. Formononetina



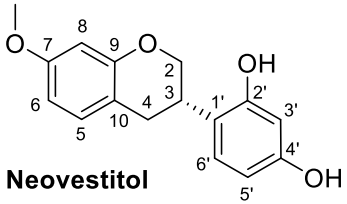
8. Biochanina A

	Calicosina		**Formononetina**		**Biochanina A**	
Carbono	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**
2	8,28 (s; 1H)	153,0	8,35 (s; 1H)	153,1	8,19 (s; 1H)	154,5
3	-	123,3	-	123,1	-	124,2
4	-	174,6	-	174,6	-	181,6
5	7,97 (d; *J* = 8,8; 1H)	127,3	7,98 (d; *J* = 8,8 Hz; 1H)	127,3	-	163,9
6	6,93 (dd; *J* = 8,8; 1,9 Hz, 1H)	115,2	6,95 (dd; *J* = 8,8; 2,3 Hz; 1H)	115,2	6,42 (d; *J* = 2,1 Hz; 1H)	99,9
7	-	162,6	-	162,6	-	165,1
8	6,86 (d; *J* = 1,9 Hz; 1H)	102,1	6,88 (d; *J* = 2,2 Hz; 1H)	102,1	6,29 (d; *J* = 2,2 Hz; 1H)	94,5
9	-	157,4	-	157,4	-	159,1
10	-	116,4	-	116,6	-	106,2
1'	-	124,7	-	124,2	-	123,8
2'	δ 7,05 (s; 1H; H-2')	116,6	7,49-7,54 (m; 2H)	130,1	7,54 (d; *J* = 8,7 Hz; 2H)	131,1
3'	-	146,0	6,97 -7,02 (m; 2H)	113,6	6,99 (d; *J* = 8,8; 2H)	114,5
4'	-	147,5	-	158,9	-	160,7
5'	6,94 (sl; 2H)	111,9	6,97 -7,02 (m; 2H)	113,6	6,99 (d; *J* = 8,8; 2H)	114,5
6'	6,94 (sl; 2H)	119,7	7,49-7,54 (m; 2H)	130,1	7,54 (d; *J* = 8,7 Hz; 2H)	131,1
7-OCH_3	-	-	-	-	-	-
4'-OCH_3	3,79 (s; 3H)	55,7	3,78 (s; 3H)	55,1	3,83 (s; 3H)	55,6
	$^{\pm}$300 e 101 MHz, DMSO- d_6		**$^{\pm}$400 e 101 MHz, DMSO- d_6**		**$^{\pm}$500 e 101 MHz, Acetona- d_6**	

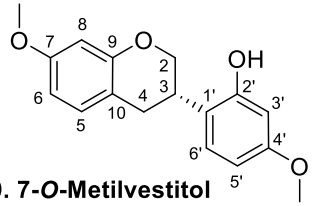
Apêndice 10. Dados de RMN de ^{1}H e ^{13}C dos compostos vestitol (5), neovestitol (6) e 7-*O*-metilvestitol (9).



5. Vestitol



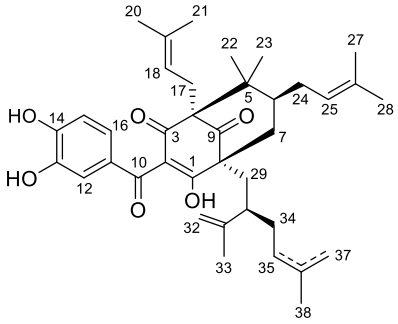
6. Neovestitol



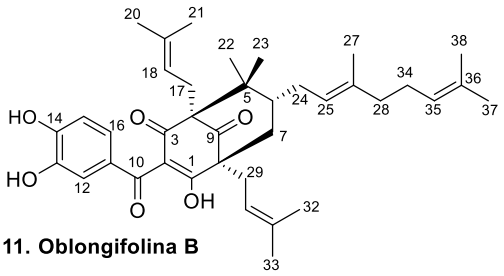
9. 7-*O*-Metilvestitol

	Vestitol		**Neovestitol**		**Metilvestitol**	
Carbono	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**	**$^{1}H\delta$(ppm)$^{\pm}$**	**$^{13}C\delta$(ppm)$^{\pm}$**
2	ax 3,97 (t, *J* = 10,1 Hz; 1H) eq 4,23 (ddd, *J* = 10,3; 3,4; 2,0 Hz; 1H)	70,5	ax 3,98 (t, *J* = 10,2 Hz; 1H) eq 4,26 (ddd, *J* = 10,3; 3,5; 2,1 Hz; 1H)	70,7	ax 4,00 (t, *J* = 10,1 Hz; 1H) eq 4,26 (ddd, *J* = 10,03; 3,5; 2,0 Hz; 1H)	70,5
3	3,55-3,40 (m; 1H)	32,6	3,52-3,42 (m; 1H)	32,5	3,49 (tdd, *J* = 10,3; 5,3; 3,5 Hz; 1H)	32,5
4	ax 2,96 (dd; *J* = 15,5; 10,9 Hz; 1H) eq 2,79 (ddd, *J* = 15,6; 5,1; 1,7 Hz; 1H)	31,0	ax 2,97 (ddd; *J* = 15,7; 11,1; 1,1 Hz; 2H) eq 2,81 (ddd, *J* = 15,7; 5,2; 2,0 Hz; 1H)	31,0	ax 2,99 (m; 2H) eq 2,83 (ddd, *J* = 15,8; 5,3; 2,0 Hz; 1H)	31,0
5	6,89 (d, *J* = 8,2; 1H)	130,9	6,97 (d, *J* = 8,3; 1H)	131,0	6,98 (d, *J* = 8,4 Hz; 1H)	131,0
6	6,37 (dd; *J* = 8,2; 2,5 Hz; 1H)	108,7	6,35 (td; *J* = 4,1; 2,4 Hz; 2H)	107,5	6,45 (dd; *J* = 4,8; 2,5 Hz; 1H)	107,6
7	-	157,4	-	160,0	-	160,8
8	6,29 (d, *J* = 2,4 Hz; 1H)	103,6	6,35 (td; *J* = 4,1; 2,4 Hz; 2H)	102,1	6,35 (d, *J* = 2,6 Hz; 1H)	102,0
9	-	156,0	-	156,1	-	156,7
10	-	114,3	-	115,5	-	115,4
1'	-	120,9	-	119,5	-	120,8
2'	-	156,7	-	156,7	-	156,1
3'	6,50 (d; *J* = 2,4 Hz; 1H)	102,5	6,47 (d; *J* = 2,5 Hz; 2H)	103,6	6,50 (d; *J* = 2,5 Hz; 2H)	102,5
4'	-	160,3	-	157,9	-	160,1
5'	6,41 (dd, *J* = 8,5; 2,5 Hz; 1H)	105,6	6,43 (dd, *J* = 5,0; 2,6 Hz; 1H)	107,7	6,42 (dd, *J* = 5,0; 2,6 Hz; 1H)	105,7
6'	7,04 (d, *J* = 8,5 Hz; 1H)	128,7	6,94 (d, *J* = 8,5 Hz; 1H)	128,7	7,05 (d, *J* = 8,5 Hz; 1H)	128,7
7-OCH$_3$	-	-	3,73 (s, 3H)	55,4	3,71 (s, 3H)	55,4
4'-OCH$_3$	3,71 (s, 3H)	55,3			3,72 (s, 3H)	55,3
	$^{\pm}$300 e 101 MHz, Acetona-d_6		**$^{\pm}$500 e 101 MHz, Acetona-d_6**		**$^{\pm}$500 e 101 MHz, Acetona-d_6**	

Apêndice 11. Dados de RMN de ^{1}H e ^{13}C dos compostos gutiferona E / xantoquimol e oblongifolina B.



10. Gutiferona E Δ^{35} / Xantoquimol Δ^{36}



11. Oblongifolina B

	Gutiferona E		Oblongifolina B	
Carbono	**$^{1}H\delta$(ppm)***	**$^{13}C\delta$(ppm)***	**$^{1}H\delta$(ppm)$^{+}$**	**$^{13}C\delta$(ppm)$^{+}$**
1	-	196,1	-	196,4
2	-	119,3	-	118,8
3	-	194,3	-	192,5
4	-	69,7	-	69,6
5	-	50,2	-	48,5
6	1,49 (s, 4H)	47,9	1,60 (m, 1H)	44,1
7	eq 2,05-1,99 (m, 4H) ax 2,26 (d, *J* = 14,0 Hz, 1H)	43,8	eq 2,05 (m, 3H) ax 1,47 (t, *J* = 13,34 Hz, 1H)	43,2
8	-	59,8	-	63,6
9	-	210,6	-	208,8
10	-	195,6	-	194,5
11	-	129,5	-	129,8
12	7,20 (d, *J* = 2,1 Hz, 1H)	117,3	7,19 (d, *J* = 2,1 Hz, 1H)	117,4
13	-	146,1	-	146,0
14	-	152,3	-	152,3
15	6,72 (d, *J* = 8,8 Hz, 1H)	115,1	6,70 (d, *J* = 8,3 Hz, 1H)	115,8
16	6,99 (dd, *J* = 8,2; 2,1 Hz, 1H))	125,1	6,96 (dd, *J* = 8,3; 2,1 Hz, 1H)	125,1
17	2,57 (m, 2H) 2,71 (dd, *J* = 13,0; 9,5 Hz, 1H)	27,0	2,71 (dd, *J* = 13,5; 8,6; 1H) 2,61 (d, *J* = 13,1 Hz, 1H)	27,4
18	5,09-5,00 (m, 2H)	121,2	4,92 (m, 1H)	121,1
19	-	135,8	-	135,3
20	1,64 (s, 6H)	26,4	1,71 (s, 3H)	26,2
21	1,69 (s, 3H)	18,3	1,69 (s, 3H)	18,3
22	1,68 (s, 3H)	23,2	0,79 (s, 3H)	16,4
23	0,98 (s, 6H)	27,4	1,17 (s, 3H)	23,8
24	2,05-1,99 (m, 4H) 2,14-2,07 (m, 1H)	30,2	2,13 (d, *J* = 11,6 Hz, 1H) 1,75 (d, *J* = 9,9 Hz; 1H)	29,4
25	5,09-5,00 (m, 2H)	125,4	4,98 (m, 1H)	123,8
26	-	133,5	-	137,8
27	1,73 (s, 3H)	25,5	1,99 (dd, *J* = 17,8; 5,4 Hz, 2H)	40,7
28	1,49 (s, 4H)	18,2	1,54 (s, 3H)	16,5
29	1,95-1,79 (m, 2H)	36,7	2,54 (dd, *J* = 13,9; 8,5 Hz, 1H) 2,48 (dd, *J* = 13,9; 5,5 Hz, 1Hz)	31,4
30	2,57 (m, 2H)	45,1	5,16 (m, 1H)	120,9
31	-	149,3	-	135,2
32	4,50 (s, 1H) 4,46 (d, *J* = 9,6 Hz, 1H)	112,5	1,64 (s, 3H)	26,0
33	1,60 (s, 3H)	18,1	1,67 (s, 3H)	18,2
34	2,05-1,99 (m, 4H)	33,4	2,05 (m, 3H)	26,3

35	4,89-4,84 (m, 1H)	124,0	5,05 (m, 1H)	124,9
36	-	132,6	-	132,2
37	1,16 (s, 3H)	26,0	1,66 (s, 3H)	25,9
38	1,57 (d, *J* =1,0 Hz, 2H)	17,8	1,57 (s, 3H)	17,8
***500 MHz e 125 MHz, MeOH-d_4 com adição de 1% de ácido trifluroacético (TFA)**				

Apêndice 12. Capítulo 2. Material Suplementar.

Artigo: A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and *Dalbergia ecastaphyllum.*

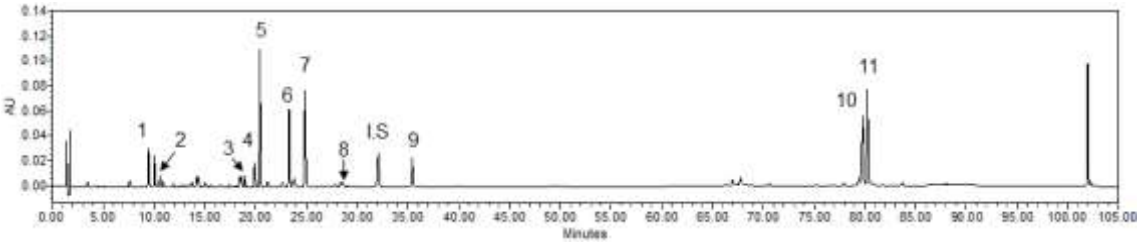
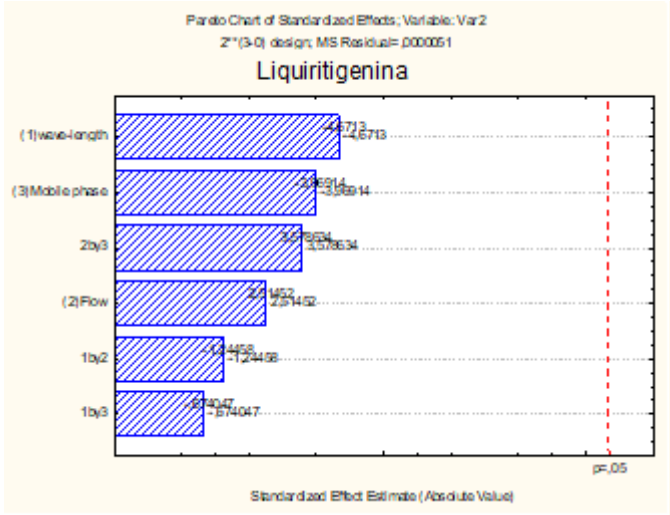
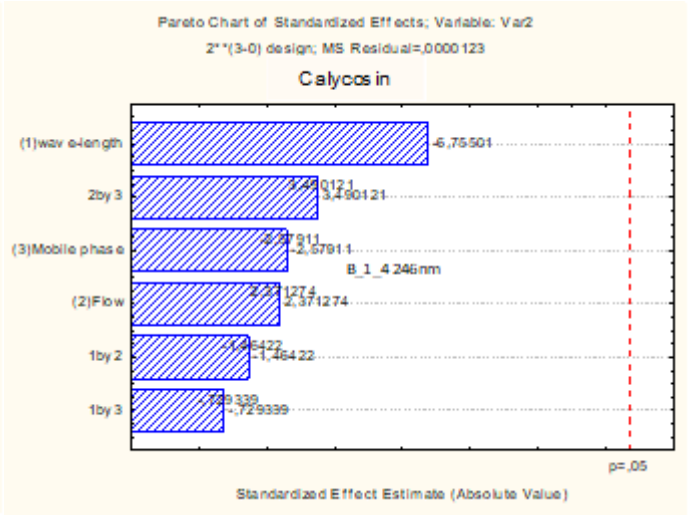
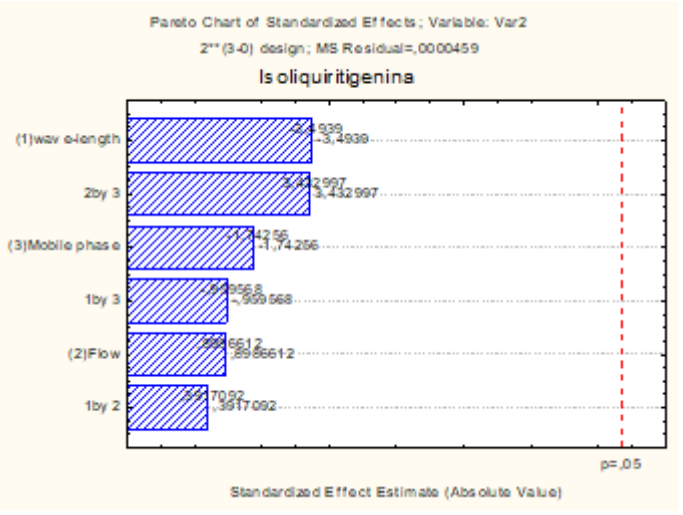
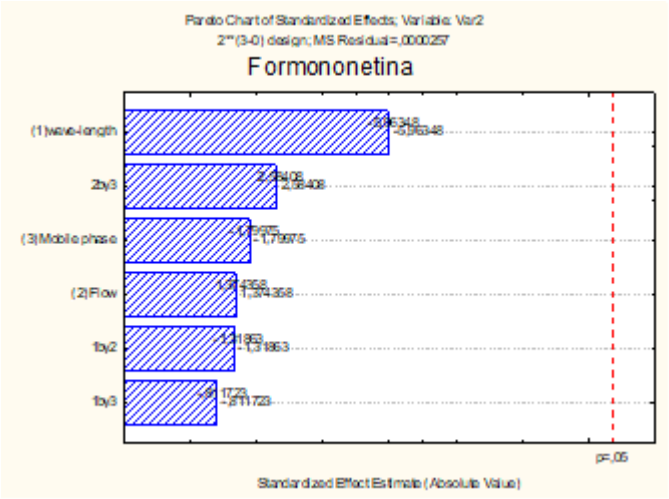


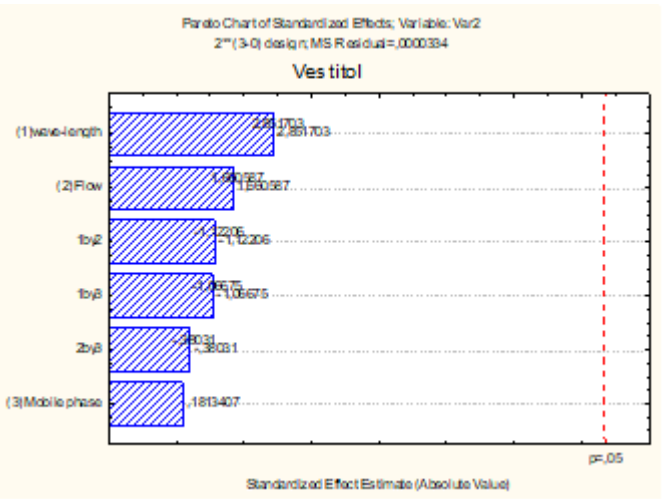
Figure S. 0-2. HPLC chromatographic profile of Brazilian red propolis extract. Identity of the isolated constituents: liquiritigenin (**1**), calycosin (**2**), isoliquiritigenin (**3**), formononetin (**4**), vestitol (**5**), neovestitol (**6**), medicarpin (**7**), biochanin A (**8**), 7-*O*-methylvestitol (**9**), mixture of guttiferone E and xanthochymol (**10**), oblongifolin B (**11**). Column: Ascentis Express C18 column (2.7 µm, 150 × 4.60 mm). Mobile phase: water with 0.1% formic acid (A) and acetonitrile (B). Flow rate: 1.0 mL/min in a gradient elution mode: 20→50% B in 40 min, 50→100% B in 90 min, 100% B (isocratic) until 95 min, 100→20% B until 100 min, 20% B (isocratic) up to 105 min. Benzophenone (20 µg/mL) as the internal standard (IS).

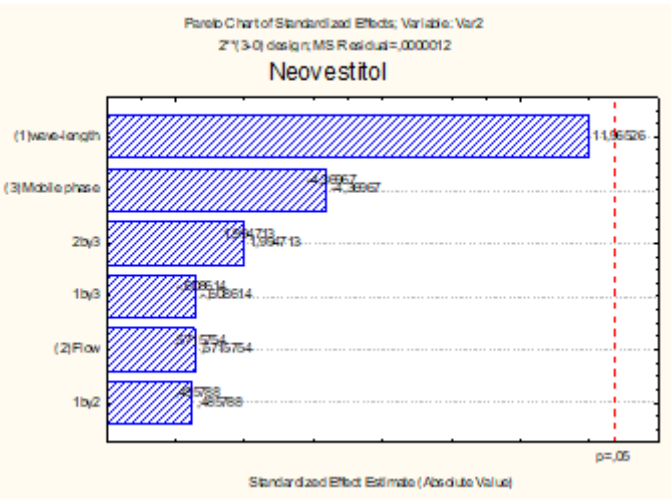

Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000051
Liquiritigenina
(1)wave-length
(3)Mobile phase
2by3
(2)Flow
1by2
1by3
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000123
Calycosin
(1)wavelength
2by3
(3)Mobile phase
(2)Flow
1by2
1by3
B_1_4246nm
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000459
Isoliquiritigenina
(1)wavelength
2by3
(3)Mobile phase
1by3
(2)Flow
1by2
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000257
Formononetina
(1)wavelength
2by3
(3)Mobile phase
(2)Flow
1by2
1by3
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000334
Vestitol
(1)wavelength
(2)Flow
1by2
1by3
2by3
(3)Mobile phase
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000012
Neovestitol
(1)wavelength
(3)Mobile phase
2by3
1by3
(2)Flow
1by2
p=,05
Standardized Effect Estimate (Absolute Value)

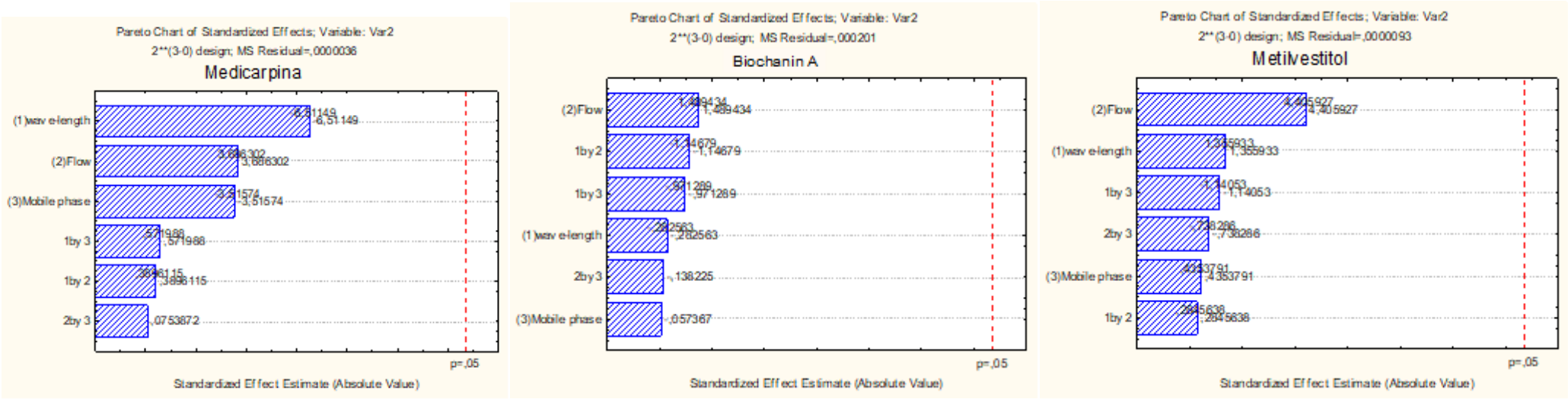
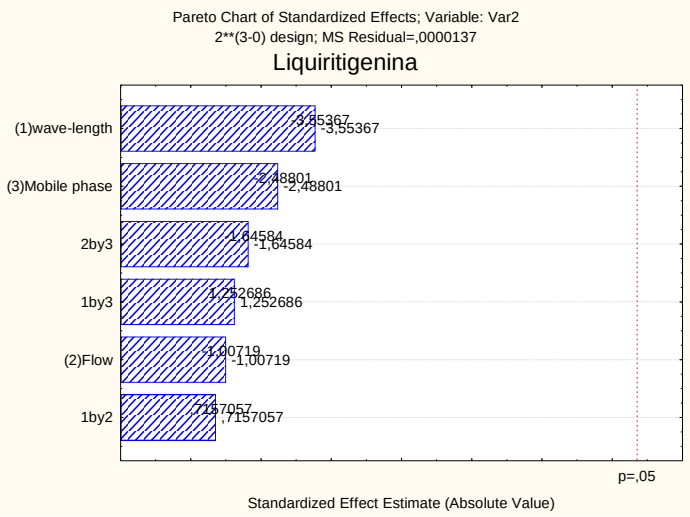
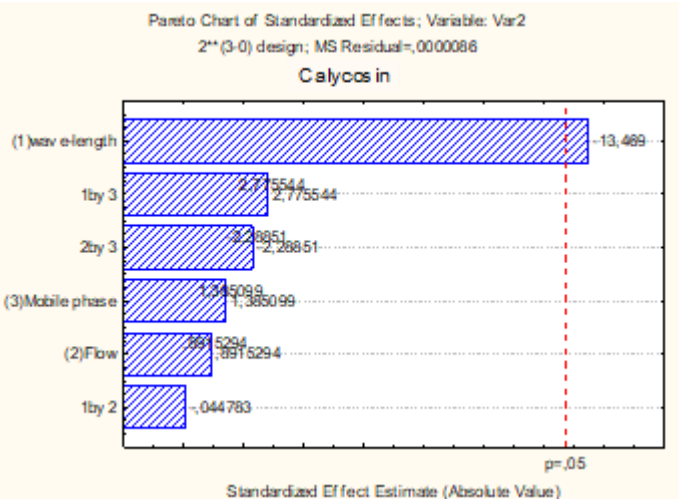
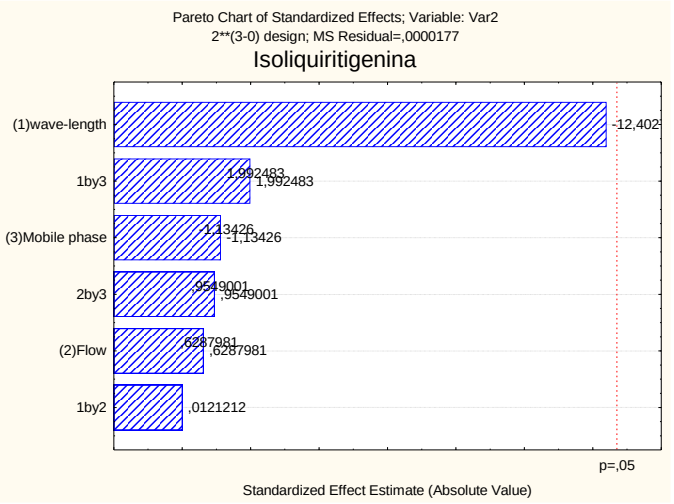
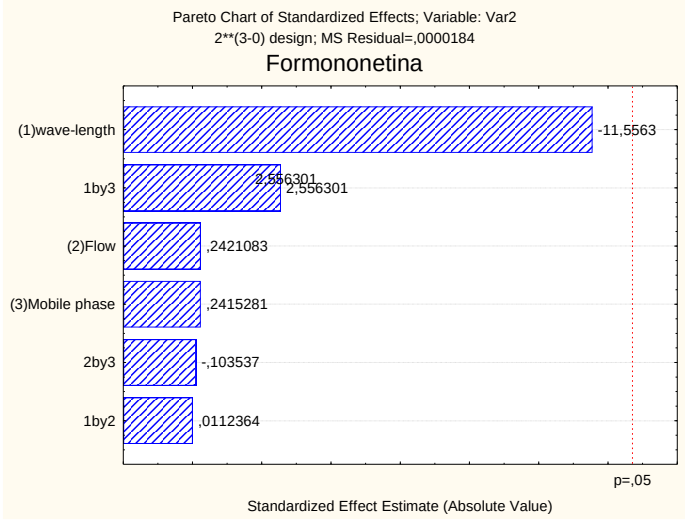


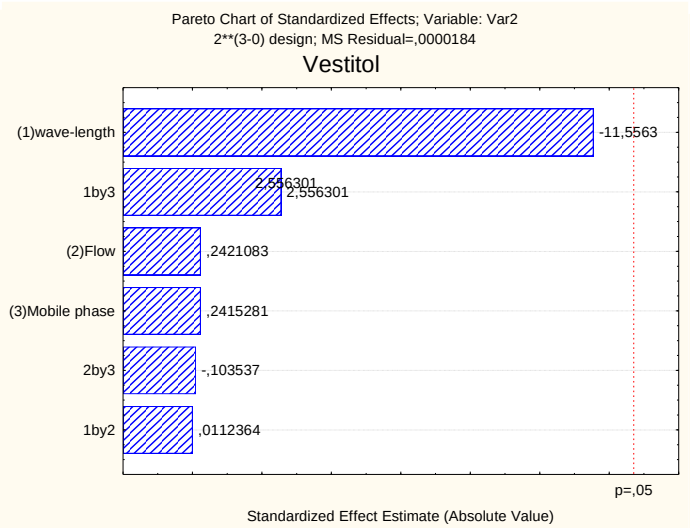
Figure S. 0-3. Pareto charts of wavelength, flow rate, and elution gradient changes, and their interactions, for the evaluation of the robustness of the method at the low level (10 µg/mL).

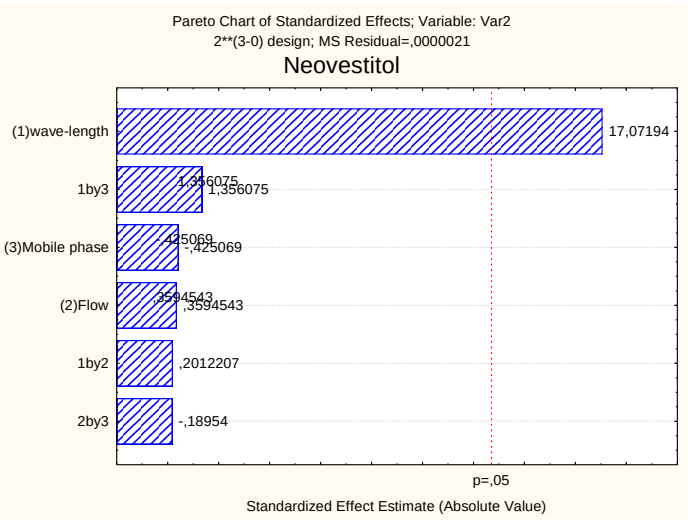

Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000137
Liquiritigenina
(1)wave-length
(3)Mobile phase
2by3
1by3
(2)Flow
1by2
-3,55367
-2,48801
-1,64584
1,252686
-1,00719
,7157057
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000086
Calycosin
(1)wave-length
1by3
2by3
(3)Mobile phase
(2)Flow
1by2
-13,489
2,775544
-2,28851
1,385099
,8915294
-,044783
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000177
Isoliquiritigenina
(1)wave-length
1by3
(3)Mobile phase
2by3
(2)Flow
1by2
-12,402
1,992483
-1,13426
,9549001
,6287981
,0121212
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000184
Formononetina
(1)wave-length
1by3
(2)Flow
(3)Mobile phase
2by3
1by2
-11,5563
2,556301
,2421083
,2415281
-,103537
,0112364
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000184
Vestitol
(1)wave-length
1by3
(2)Flow
(3)Mobile phase
2by3
1by2
-11,5563
2,556301
,2421083
,2415281
-,103537
,0112364
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000021
Neovestitol
(1)wave-length
1by3
(3)Mobile phase
(2)Flow
1by2
2by3
17,07194
1,356075
,425069
,3594543
,2012207
-,18954
p=,05
Standardized Effect Estimate (Absolute Value)

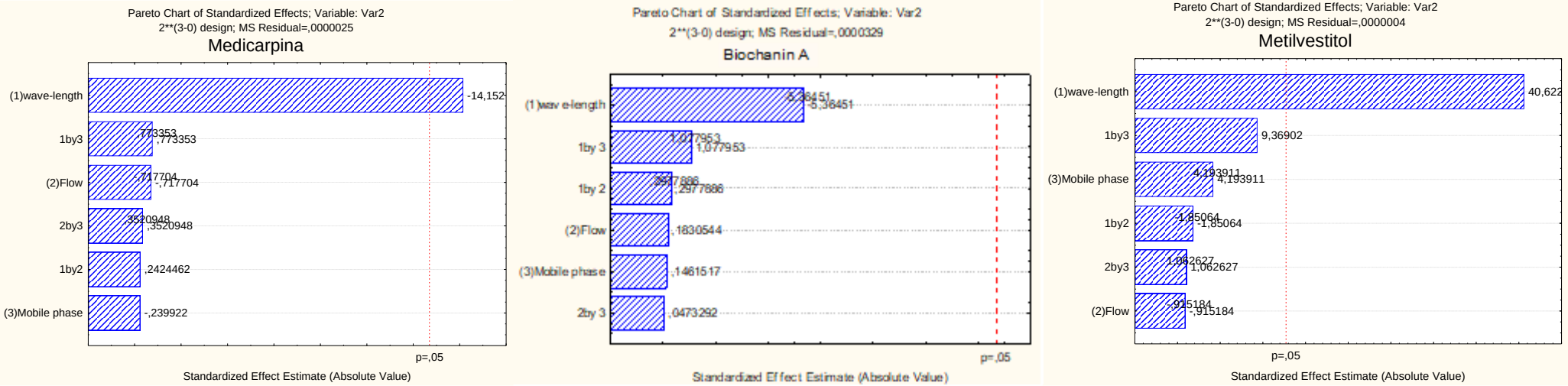
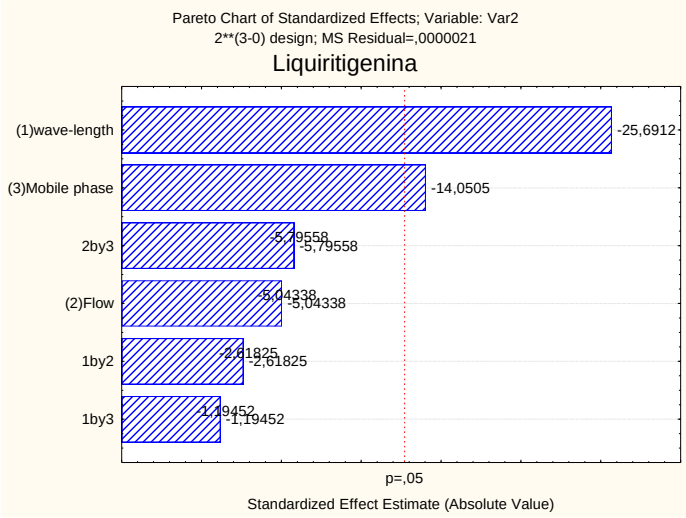
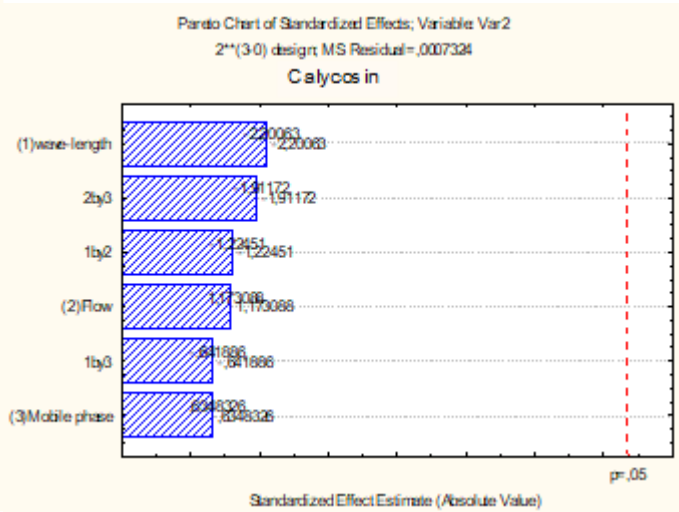
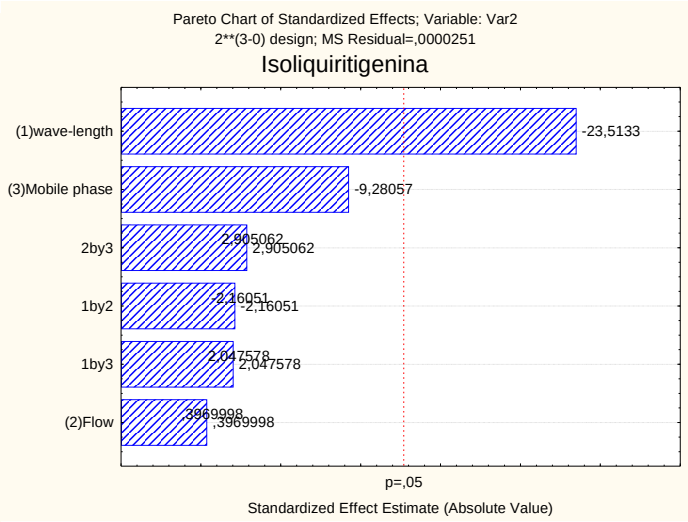
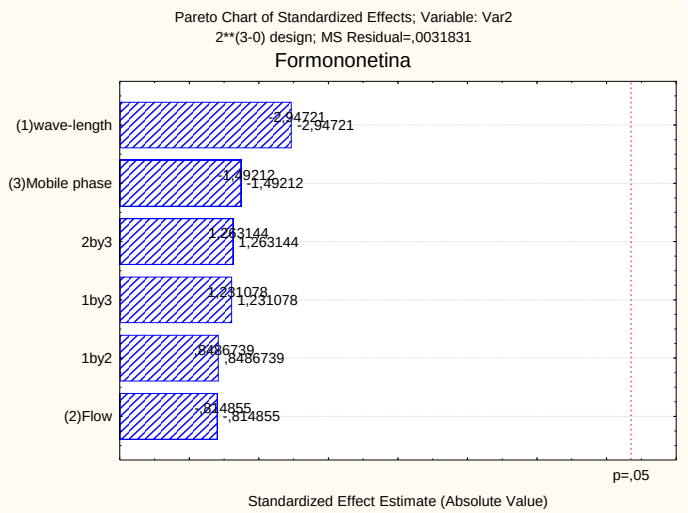


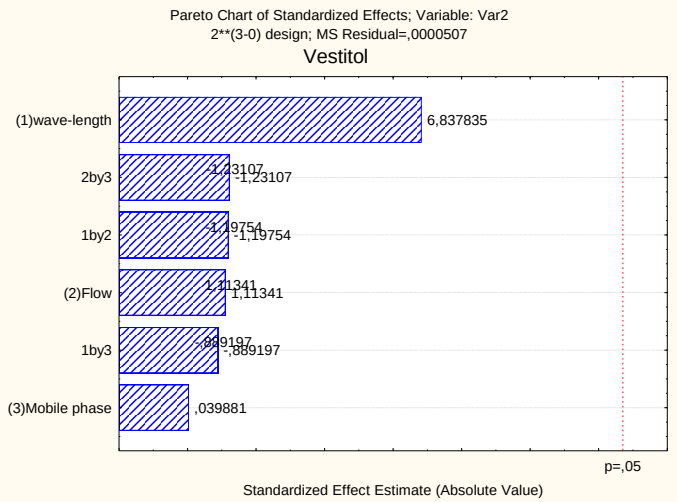
Figure S. 0-4. Pareto charts of wavelength, flow rate, and elution gradient changes, and their interactions, for the evaluation of the robustness of the method at the medium level (20 µg/mL).

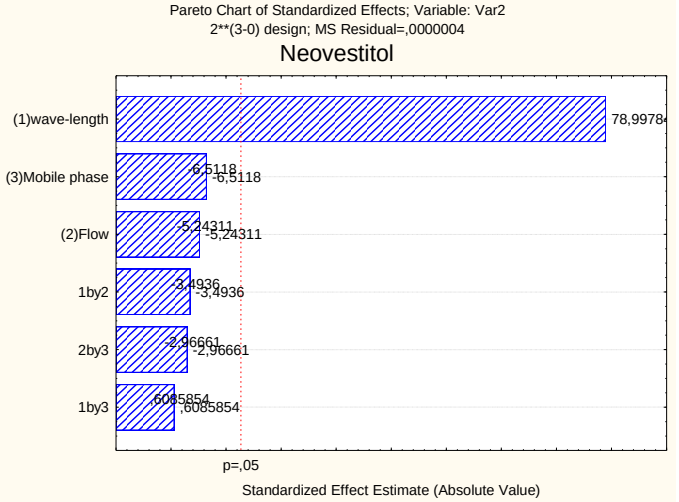

Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000021
Liquiritigenina
(1)wave-length
(3)Mobile phase
2by3
(2)Flow
1by2
1by3
-25,6912
-14,0505
-5,79558
-5,04338
-2,61825
-1,19452
p=,05
Standardized Effect Estimate (Absolute Value)

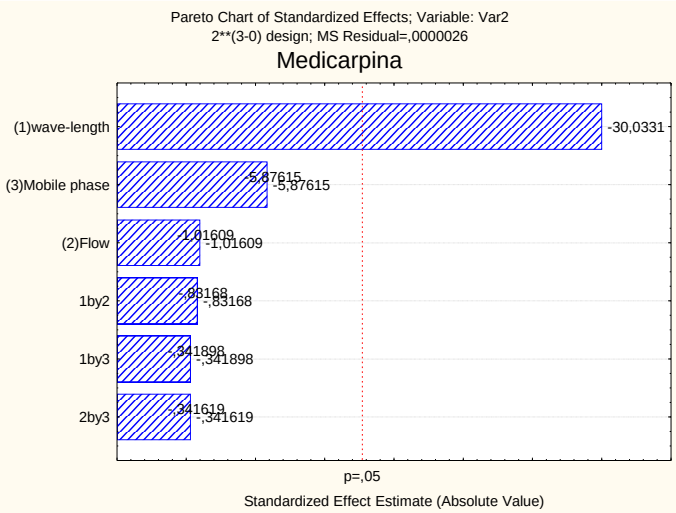

Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0007324
Calycosin
(1)wave-length
2by3
1by2
(2)Flow
1by3
(3)Mobile phase
p=,05
Standardized Effect Estimate (Absolute Value)

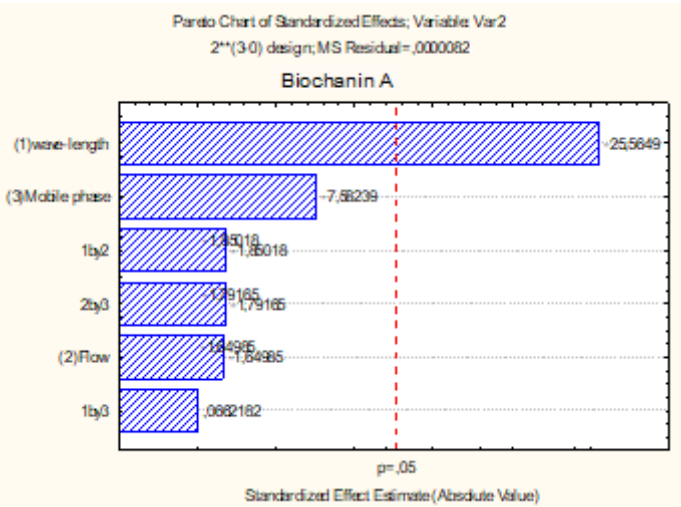

Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000251
Isoliquiritigenina
(1)wave-length
(3)Mobile phase
2by3
1by2
1by3
(2)Flow
-23,5133
-9,28057
2,905062
2,16051
2,047578
,3969998
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0031831
Formononetina
(1)wave-length
(3)Mobile phase
2by3
1by3
1by2
(2)Flow
2,94721
1,49212
1,263144
1,231078
,8486739
,814855
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000507
Vestitol
(1)wave-length
2by3
1by2
(2)Flow
1by3
(3)Mobile phase
6,837835
1,23107
1,19754
1,11341
,889197
,039881
p=,05
Standardized Effect Estimate (Absolute Value)


Pareto Chart of Standardized Effects; Variable: Var2
2**(3-0) design; MS Residual=,0000004
Neovestitol
(1)wave-length
(3)Mobile phase
(2)Flow
1by2
2by3
1by3
78,9978
6,5118
5,24311
3,4936
2,96661
,6085854
p=,05
Standardized Effect Estimate (Absolute Value)





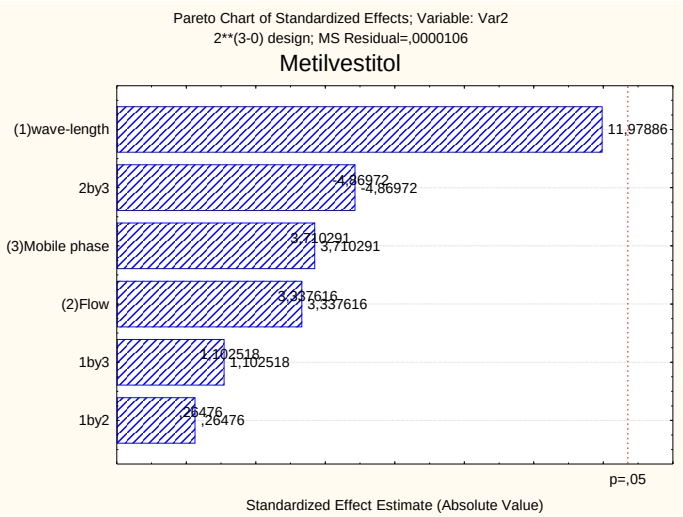


Figure S. 0-5. Pareto charts of wavelength, flow rate, and elution gradient changes, and their interactions, for the evaluation of the robustness of the method at the high level (40 µg/mL).

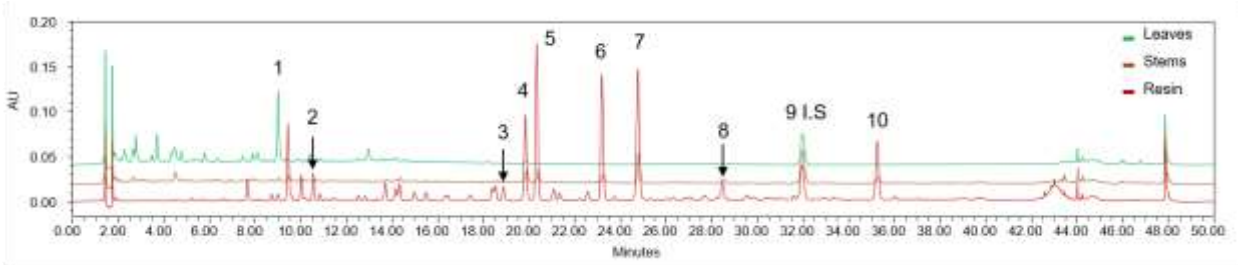


Figure S. 0-6. Chemical profiles of *D. ecastaphyllum*'s leaves (green), stems (brown) and resin (red).

Table S. 0-1. Compounds content in samples of red propolis samples (% g/100 g of dried raw material).

Compound	Compound content (% g/100g)											
	Mar 2019	**Apr 2019**	**May 2019**	**Jun 2019**	**Jul 2019**	**Aug 2019**	**Sep 2019**	**Oct 2019**	**Nov 2019**	**Dec 2019**	**Jan 2020**	**Feb 2020**
1	1.748 ± 0.027	2.069 ± 0.024	0.638 ± 0.006	1.220 ± 0.006	1.060 ± 0.161	0.348 ± 0.023	0.179 ± 0.005	0.995 ± 0.005	0.824 ± 0.001	0.659 ± 0.002	0.589 ± 0.002	0.295 ± 0.001
2	0.573 ± 0.004	0.512 ± 0.274	0.210 ± 0.003	0.405 ± 0.004	0.305 ± 0.048	0.085 ± 0.003	0.056 ± 0.003	0.350 ± 0.002	0.235 ± 0.001	0.212 ± 0.004	0.178 ± 0.004	0.125 ± 0.0054
3	2.087 ± 0.004	2.409 ± 0.014	0.705 ± 0.013	1.323 ± 0.007	1.091 ± 0.167	0.353 ± 0.0002	0.210 ± 0.0003	1.106 ± 0.005	0.903 ± 0.004	0.620 ± 0.002	0.604 ± 0.004	0.304 ± 0.011
4	2.489 ± 0.029	2.640 ± 0.035	0.753 ± 0.006	1.607 ± 0.015	1.124 ± 0.170	0.374 ± 0.003	0.238 ± 0.004	1.021 ± 0.004	0.769 ± 0.001	0.769 ± 0.003	0.612 ± 0.014	0.347 ± 0.001
5	17.713 ± 0.053	19.455 ± 0.115	5.771 ± 0.062	12.319 ± 0.042	9.875 ± 1.535	3.157 ± 0.003	2.140 ± 0.008	9.162 ± 0.031	8.263 ± 0.014	5.253 ± 0.011	5.442 ± 0.032	2.824 ± 0.007
6	5.989 ± 0.010	6.894 ± 0.022	2.406 ± 0.029	4.812 ± 0.016	4.697 ± 0.731	1.385 ± 0.009	0.997 ± 0.001	3.918 ± 0.013	2.273 ± 0.007	2.025 ± 0.018	2.107 ± 0.009	0.964 ± 0.005
7	14.233 ± 0.044	14.611 ± 0.057	4.389 ± 0.037	8.603 ± 0.011	7.317 ± 1.146	2.394 ± 0.012	1.493 ± 0.016	6.655 ± 0.026	6.205 ± 0.016	4.318 ± 0.004	4.590 ± 0.018	2.114 ± 0.004
8	0.376 ± 0.005	0.341 ± 0.011	0.101 ± 0.017	0.222 ± 0.022	0.176 ± 0.025	0.060 ± 0.003	0.034 ± 0.004	0.167 ± 0.006	0.146 ± 0.009	0.122 ± 0.0002	0.113 ± 0.013	0.062 ± 0.0003
10	3.445 ± 0.005	3.833 ± 0.023	1.173 ± 0.054	2.430 ± 0.012	2.356 ± 0.360	0.733 ± 0.031	0.533 ± 0.016	2.074 ± 0.016	1.351 ± 0.003	1.551 ± 0.003	1.225 ± 0.009	0.734 ± 0.001

Table S. 0-2. Meteorological conditions during the time series between March 2019 and February 2020.

Data	Total precipitation (mm)	Global radiation (KJ/m²)	Maximum temperature (°C)	Minimum temperature (°C)	Relative humidity (%)
March 19	65,80	1463,00	26,81	25,85	82,15
May 19	86,20	1223,61	26,00	25,04	83,57
June 19	176,20	1116,98	23,59	22,60	84,80
July 19	79,40	1153,02	22,27	21,29	84,79
August 19	199,40	1183,37	22,26	21,25	86,37
September 19	175,60	1323,29	23,24	22,22	84,87
October 19	82,00	1571,91	24,30	23,24	84,03
November 19	30,40	1749,93	25,64	24,48	81,56
December 19	110,40	1693,13	26,03	24,83	82,53
January 20	36,40	1626,30	26,61	25,54	82,70
February 20	0,00	1650,31	26,80	25,70	83,71

Automatic Surface Observation Meteorological Station of Una Meteorological Station located 47 km from Canavieiras. The data were extracted from the Weather Data Storage Section (SADMET). National Institute of Meteorology (INMET) from the Brazilian Ministry of Agriculture. Brasilia: Accesed on 1 November 2020.

ANEXOS

Anexo 1. Aprovação comitê de ética no uso de animais.

UNIFRAN
Universidade
de Franca

Comissão de Ética no
Uso de Animais

DECLARAÇÃO

Declaramos que a PROPOSTA intitulada "Estudos não clínicos de toxicologia da própolis vermelha ", protocolada sob o CEUA nº 9701030418, sob a responsabilidade de **Denise Crispim Tavares** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade de Franca (CEUA/UNIFRAN) na reunião de 11/06/2018.

We certify that the proposal "No clinical toxicology studies of red propolis ", utilizing 42 Heterogenics mice (42 males), 92 Rattus norvegicus (males and females), protocol number CEUA 9701030418, under the responsibility of **Denise Crispim Tavares** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Franca's University (CEUA/UNIFRAN) in the meeting of 06/11/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 07/2018 a 12/2019 Área: Ciências Biológicas/mutagênese

Origem:	Biotério Central USP Ribeirão Preto						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	4 a 6 semanas	N:	42
Linhagem:	Balb/c			Peso:	25 a 30 g		
Origem:	Biotério Central USP Ribeirão Preto						
Espécie:	Rattus norvegicus	sexo:	Machos e Fêmeas	idade:	4 a 6 semanas	N:	92
Linhagem:	Wistar			Peso:	120 a 200 g		

Local do experimento: Biotério da Universidade de Franca

Franca, 07 de agosto de 2018

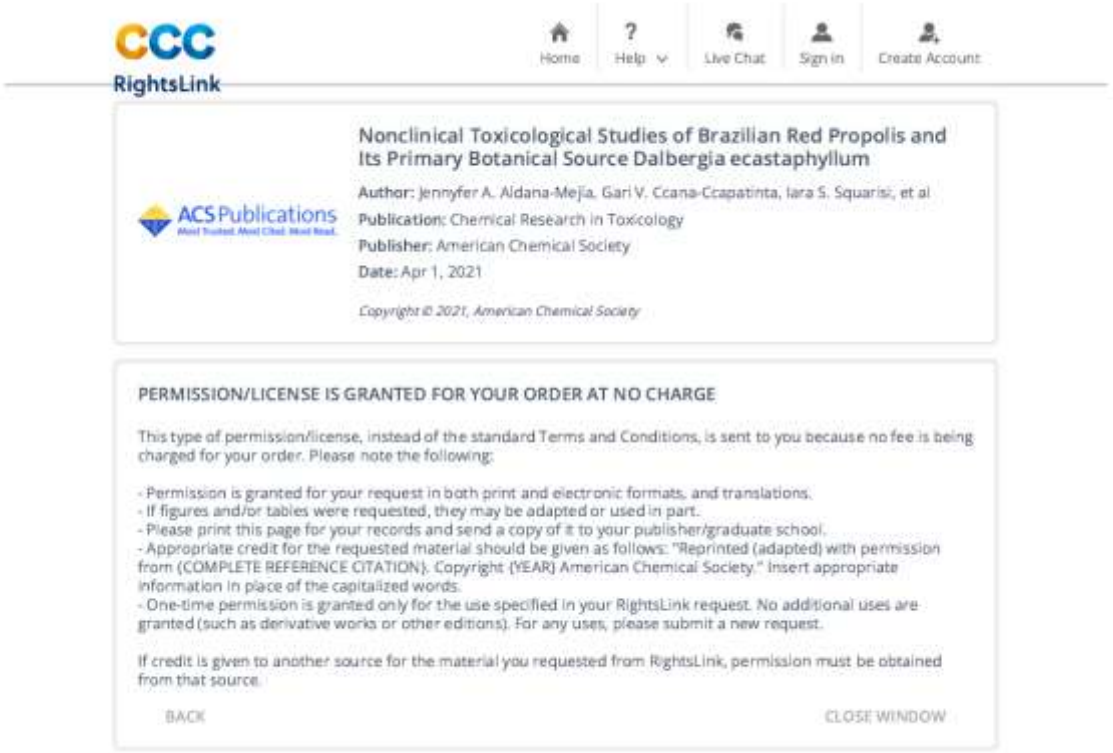
Prof. Dr. Ewaldo de Mattos Junior
Coordenador da Comissão de Ética no Uso de Animais
Universidade de Franca

Prof. Dr. Daniel Paulino Junior
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade de Franca

OBS: Este documento contempla apenas a aprovação da PROPOSTA, desta forma, o CERTIFICADO está vinculado a entrega do relatório final podendo inclusive haver alteração no PARECER FINAL.

Av. Dr. Armando Salles Oliveira, 201 - Pq. Universitário - CEP: 14404-600 Franca/SP - tel: 55 (16) 3711-8904 / fax: 55 (16) —
Horário de atendimento: 2ª, 4ª e 6ª (7 às 12h) / 3ª e 5ª (13h às 17h) : e-mail: ceua@unifran.edu.br
CEUA N 9701030418

Anexo 2. Autorização de uso artigo: Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source *Dalbergia ecastaphyllum* (Capítulo 1).



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Nonclinical Toxicological Studies of Brazilian Red Propolis and Its Primary Botanical Source Dalbergia ecastaphyllum

Author: Jennyfer A. Aldana-Mejía, Gari V. Ccana-Ccapatinta, Iara S. Squarisi, et al

Publication: Chemical Research in Toxicology

Publisher: American Chemical Society

Date: Apr 1, 2021

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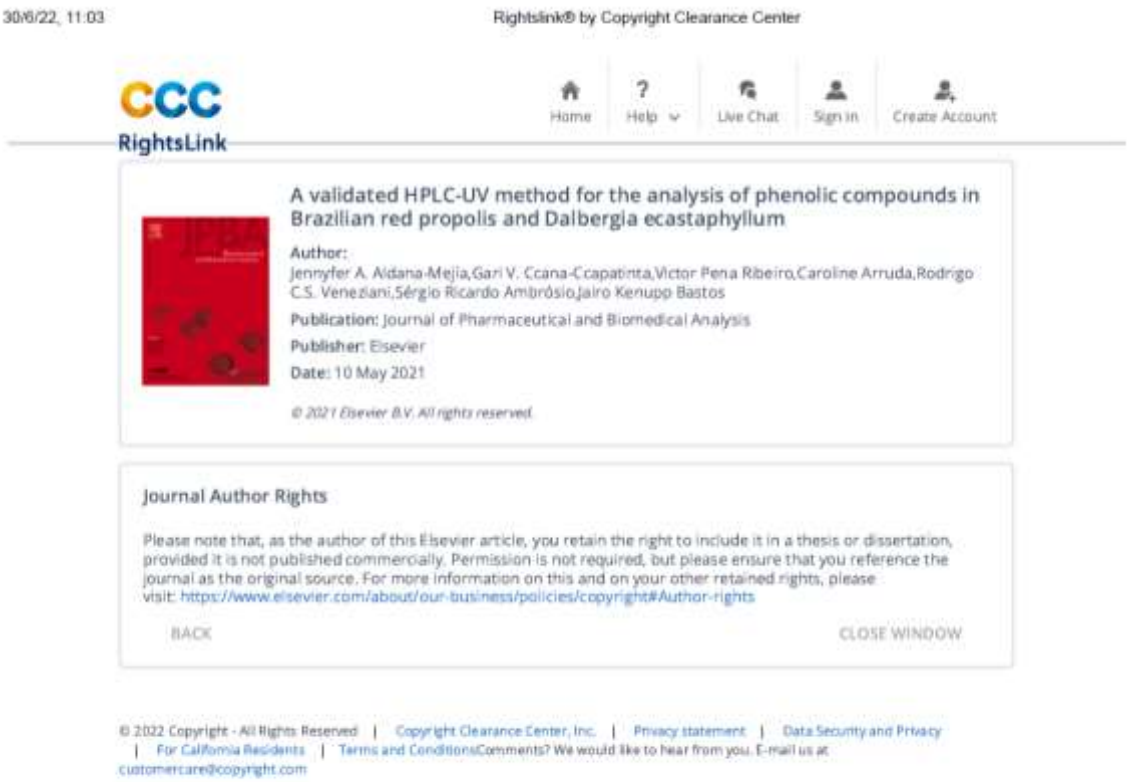
Anexo 3. Autorização de uso artigo: A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and *Dalbergia ecastaphyllum* (Capítulo 2).



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A validated HPLC-UV method for the analysis of phenolic compounds in Brazilian red propolis and Dalbergia ecastaphyllum

Author:
Jennyfer A. Aldana-Mejía,Gari V. Ccana-Ccapatinta,Victor Pena Ribeiro,Caroline Arruda,Rodrigo C.S. Veneziani,Sérgio Ricardo Ambrósio,Jairo Kenupp Bastos

Publication: Journal of Pharmaceutical and Biomedical Analysis

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Anexo 4. Comprovante submissão artigo: Genotoxicity and Toxicological Evaluations of Brazilian Red Propolis oral ingestion in a Preclinical Rodent Model (Capítulo 3).

Jennyfer Andrea Aldana Mejía <j.aldana@usp.br>

Confirm co-authorship of submission to Food and Chemical Toxicology

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Corresponding Author: Dr Jairo Kenupp Bastos
Co-Authors: Jennyfer Andrea Aldana-Mejía, MSc.; Aline Mayrink Miranda, PhD; Gari Vidal Ccana-Ccapatinta, PhD; Luciana Silva de Araújo, MSc.; Victor Pena Ribeiro, PhD; Caroline Arruda, PhD; Samuel Nascimento; Iara Squarisi; Tábata Esperandim; Karoline Soares de Freitas, MSc.; Saulo D. Ozelin, MSc.; Denise Crispim Tavares, PhD; Fernando Silva Ramalho, PhD
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