UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Estudo químico e avaliação anti-inflamatória, neuroprotetiva e toxicológica em camundongos das folhas de *Allophylus edulis* (A.St.-Hil., Cambess. & A. Juss.) Radlk.

SIDNEY MARIANO DOS SANTOS

Dourados – MS

2024

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ATA DA DEFESA DE TESE DE DOUTORADO APRESENTADA POR SIDNEY MARIANO DOS SANTOS, ALUNO DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE, ÁREA DE CONCENTRAÇÃO "FARMACOLOGIA".

Aos seis dias do mês de março do ano de dois mil e vinte e quatro, às treze horas e trinta minutos, em sessão pública, realizou-se na Universidade Federal da Grande Dourados, a Defesa de Tese de Doutorado intitulada **"Estudo químico e avaliação anti-inflamatória, neuroprotetiva e toxicológica em camundongos das folhas de Allophylus edulis (A.St.-Hil., Cambess. & A. Juss.) Radlk."**, apresentada pelo doutorando Sidney Mariano dos Santos, do Programa de Pós-graduação em Ciências da Saúde, à Banca Examinadora constituída pelos membros: Prof.ª Dr.ª Anelise Samara Nazari Formagio/UNESP (presidente/orientadora), Prof. Dr. Marcio Eduardo de Barros/UFGD (membro titular interno), Prof.ª Dr.ª Cláudia Andréa Lima Cardoso/UEMS (membro titular externo), Prof.ª Dr.ª Denise Brentan da Silva/UFMS (membro titular externo), Prof.ª Dr.ª Ana Carla Zarpelon Schutz/UFPR (membro titular externo). Iniciados os trabalhos, a presidência deu a conhecer ao candidato e aos integrantes da banca as normas a serem observadas na apresentação da Tese. Após o candidato ter apresentado a sua Tese, os componentes da Banca Examinadora fizeram suas arguições. Terminada a Defesa, a Banca Examinadora, em sessão secreta, passou aos trabalhos de julgamento, tendo sido o candidato considerado **APROVADO**. A Presidente da Banca atesta a participação dos membros que estiveram presentes de forma remota, conforme declarações anexas. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelos membros da Comissão Examinadora.

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DEDICATÓRIA

Dedico este trabalho a todos que reconhecem na ciência a força motriz capaz de resolver os mais complexos desafios que nosso mundo enfrenta.

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Muito obrigado.

Não é da natureza das coisas que qualquer homem faça uma descoberta repentina e violenta; a ciência avança passo a passo e cada homem depende do trabalho dos seus antecessores. Quando você ouve falar de uma descoberta súbita e inesperada - um raio em céu aberto - você pode sempre ter certeza de que ela cresceu pela influência de um homem ou de outro, e é a influência mútua que cria a enorme possibilidade de avanço científico. Os cientistas não dependem das ideias de um único homem, mas da sabedoria combinada de milhares de homens, todos pensando no mesmo problema e cada um fazendo a sua parte para contribuir para a grande estrutura de conhecimento que está sendo gradualmente erguida.

Sir Ernest Rutherford

The world breaks everyone, and
afterward, many are strong at the
broken places.

Ernest Hemingway

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO E REVISÃO DE LITERATURA

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ARTIGO II

ARTIGO III

LISTA DE ABREVIATURAS

^{1}H NMR	*Hydrogen-1 Nuclear Magnetic Resonance* (Ressonância magnética nuclear em relação ao hidrogênio-1)
5NAP	*AChE of Torpedo californica complexed with donepezil 17* (AChE de *Torpedo californica* complexada com donepezil 17)
ABTS	*2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt*) 2,2'-(Azinobis (3-etilbenzotiazolina-6-ácido sulfônico))
AChE	*Acetylcholinesterase* (Acetilcolinesterase)
AcSCh	*Acetylthiocholine* (Acetiltiocolina)
AE-1	*Vitexin 2''-O-rhamnoside* (Vitexina 2"-O-ramnosídeo)
AINEs	Anti-inflamatórios não-esteroides
ANOVA	*Analysis of variance* (Análise de variância)
BCRP	*Breast cancer resistance protein* (Proteína de resistência ao câncer de mama)
BHT	*Butylated hydroxytoluene* (Hidroxitolueno butilado)
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CE	*Catechin equivalent* (Equivalente de catequina)
CEUA	*Committee of Ethics on the Use of Animals* (Comitê de Ética no Uso de Animais)
CFA	*Complete Freund's adjuvant* (Adjuvante Completo de Freund)
CG/EM	Cromatografia Gasosa acoplada à Espectrometria de Massas
CIM	Concentração Inibitória Mínima
CL_{50}	Concentração Letal
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CONCEA	Conselho Nacional de Controle de Experimentação Animal (*National Council for Control of Animal Experimentation*)
COVID-19	Doença causada pelo vírus SARS-CoV-2
COX-1	Ciclooxigenase-1
COX-2	Ciclooxigenase-2
COXIBs	Inibidores da COX-2
DEXA	*Dexamethasone* (Dexametasona)
DI	*Discrimination index* (Índice de discriminação)
DL_{50}	Dose letal
DOP	*Delta Opioid Receptor* (Receptor peptídico opioide delta)

DPPH	*2,2-Diphenyl-1-picrylhydrazyl* (2,2-difenil-1-picril-hidrazila)
DTNB	*5,5-dithio-bis-(2-nitrobenzoic acid)* (Ácido 5,5′-ditiobis(2-nitrobenzoico))
DZ7	*Analogous to donepezil* 7 (Análogo do donepezil)
EAf	*Ethyl acetate fraction* (Fração acetato de etila)
EROs	Espécies reativas do oxigênio
ET	Equivalentes de Trolox
FINEP	Financiadora de Estudos e Projetos
FRAP	Poder antioxidante redutor férrico
FUNDECT	Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul
GABA-A	Receptores do ácido gama aminobutírico A
GAE	*Gallic acid equivalent* (Equivalente de ácido gálico)
GPCR	*G protein-coupled receptors* (Receptores acoplados à proteína G)
Hf	*n-Hexane fraction* (Fração n-hexano)
HMf	*Hidrometanol fraction* (Fração hidrometanólica)
IC_{50}	Metade da concentração inibitória máxima
ILAE	*Infusion of the leaves of A. edulis* (Infusão das folhas de *A. edulis*)
IL-1	Interleucina-1
IL-6	Interleucina-6
KOP	*Kappa-opioid receptor* (Receptor peptídico opioide kappa)
MDA	*Malondialdehyde* (Malondialdeído)
MeOD-D4	Metanol deuterado (*Deuterated methanol*)
MeOH	Metanol (*Methanol*)
MHC	*Major Histocompatibility Complex* (Complexo Principal de Histocompatibilidade)
MOP	*Mu-opioid receptor* (Receptor peptídico opioide mu)
MOR	*Morphine* (Morfina)
MW	*Molecular weight* (Peso molecular)
NHn	*Number of H-bond donors* (Número de doadores de ligação H)
NMDA	*N-methyl-D-aspartate* (Receptor N-metil-D-aspartato)
NMR	*Nuclear Magnetic Resonance* (Ressonância Magnética Nuclear)
nOH	*Number of H-bond acceptors* (Número de aceitadores de ligação H)
PBS	*Phosphate-buffered saline* (Tampão fosfato salino)
PCR	Proteína C-reativa

PDB	*Protein Data Bank* (Banco de dados de proteínas)
PGHS-1	*Prostaglandin-H-synthase 1* (Prostaglandina endoperoxidase sintase 1)
PGHS-2	*Prostaglandin-H-synthase 2* (Prostaglandina endoperoxidase sintase 2)
PRED	*Prednisolone* (Prednisolona)
QE	*Quercetin equivalent* (Equivalente de quercetina)
RMSD	*Root Mean Square Deviation* (Desvio Quadrático Médio da Raiz)
SARS-CoV-2	Coronavírus 2 causador da síndrome respiratória aguda grave
SisGen	Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (*National System for the Genetic Heritage and Associated Traditional Knowledge Management*)
TLC	*Thin-layer chromatography* (Cromatografia em camada delgada analítica)
TNF-α	*Tumor necrosis fator* (Fator de Necrose Tumoral Alpha)
TPSA	*Topological polar surface area* (Área de superfície polar topológica)
TRPA1	*Transient receiver potential cation channel A1* (Canal catiônico de potencial receptor transitório A1)

Estudo químico e avaliação anti-inflamatória, neuroprotetiva e toxicológica em camundongos das folhas de *Allophylus edulis* (A.St.-Hil., Cambess. & A. Juss.) Radlk.

RESUMO

A árvore nativa brasileira *Allophylus edulis* (A.St.-Hil., Cambess. & A. Juss.) Radlk., conhecida como "vacum", tem suas folhas popularmente utilizadas em quadros inflamatórios para o tratamento de dor de garganta, colecistite e febre, sendo descritas como ricas em flavonoides. Esta pesquisa teve como objetivos: **(1)** analisar a variação da composição química do óleo essencial de folhas de *A. edulis*; **(2)** realizar estudos químicos, histoquímicos, antioxidantes in vitro, farmacológicos in vivo (anti-inflamatório, anti-hiperalgésico e antinociceptivo) das folhas, com predições in silico da vitexina 2"-O-ramnosídeo; e **(3)** investigar os efeitos ansiolíticos, antiamnésicos e toxicidade subaguda das folhas, e a remodelagem molecular da vitexina 2"-O-ramnosídeo na acetilcolinesterase (AChE). No primeiro manuscrito **(1)**, as folhas frescas de *A. edulis* coletadas em Bonito e Dourados/MS (2018 a 2019) tiveram seus óleos essenciais extraídos e analisados. O rendimento variou de 0,07% a 0,6%, aumentando com temperaturas mais elevadas e durante a fase de inflorescência. Em Dourados, o principal componente foi o óxido de cariofileno (20 a 29%), e em Bonito, o α-zingibereno (25 a 45%). No segundo manuscrito **(2)**, a análise histoquímica revelou estruturas secretoras nas folhas frescas de *A. edulis*. Das folhas frescas foi obtida a infusão das folhas (ILAE), fracionada em frações que incluíram a hidrometanólica (HMf). Desta última a vitexina 2"-O-ramnosídeo foi isolada da passou por predição in sílico, apresentando baixa toxicidade prevista. Melhores efeitos antioxidantes foram observados em modelos de sequestro de radicais livres. A administração oral de ILAE (3, 30 e 100 mg/kg) e HMf (3 mg/kg) foi testada em modelos de inflamação aguda (induzido por carragenina), hiperalgesia mecânica, alodinia térmica e nocicepção (induzida por formalina), mostrando redução significativa dos efeitos induzidos. A ILAE (30 mg/kg), HMf (3 e 30 mg/kg) e vitexina 2"-O-ramnosídeo (AE-1, 3 mg/kg) foram testados em modelo de inflamação prolongada (induzido por Adjuvante Completo de Freund - CFA), resultando em redução significativa do edema, hiperalgesia mecânica e alodinia térmica, inclusive para o flavonoide isolado (AE-1). No terceiro manuscrito **(3)**, a administração oral de ILAE (3, 30 e 100 mg/kg) e HMf (3 mg/kg) foi avaliada em modelos de ansiedade e amnésia (induzida por escopolamina). Os tratamentos reduziram a ansiedade e melhoraram parâmetros relacionados à memória de curto prazo. A diminuição da atividade da AChE e peroxidação lipídica, juntamente com as interações intermoleculares da vitexina 2"-O-ramnosídeo com AChE (in silico), pode explicar, ao menos em parte, os resultados observados. A avaliação da

toxicidade oral em camundongos durante o período de tratamento de 28 dias indica baixa toxicidade em todos os parâmetros avaliados. Conclui-se que *A. edulis* possui diversidade de metabólitos, potencial anti-inflamatório, antinociceptivo e neuroprotetor.

Palavras-chave: Vacum. Flavonoides. Inflamação. Ansiedade. Escopolamina.

Chemical study and anti-inflammatory, neuroprotective and toxicological evaluation in mice of the leaves of ***Allophylus edulis*** **(A.St.-Hil., Cambess. & A. Juss.) Radlk.**

ABSTRACT

The Brazilian native tree *Allophylus edulis* (A.St.-Hil., Cambess. & A. Juss.) Radlk., known as "vacum", has its leaves commonly used for the treatment of inflammatory conditions such as sore throat, cholecystitis, and fever, described as rich in flavonoids. This research aimed to: **(1)** analyze the variation in the chemical composition of the essential oil from *A. edulis* leaves; **(2)** conduct chemical, histochemical, in vitro antioxidant, in vivo pharmacological studies (anti-inflammatory, anti-hyperalgesic, and antinociceptive) of the leaves, with in silico predictions of vitexin 2"-O-rhamnoside; and **(3)** investigate the anxiolytic, anti-amnesic effects, and subacute toxicity of the leaves, along with the molecular remodeling of vitexin 2"-O-rhamnoside on acetylcholinesterase (AChE). In the first manuscript **(1)**, fresh *A. edulis* leaves collected in Bonito and Dourados/MS (2018 to 2019) had their essential oils extracted and analyzed. The yield varied from 0.07% to 0.6%, increasing with higher temperatures and during the flowering phase. In Dourados, the main component was caryophyllene oxide (20 to 29%), and in Bonito, α-zingiberene (25 to 45%). In the second manuscript **(2)**, histochemical analysis revealed secretory structures in fresh *A. edulis* leaves. An infusion of the leaves (ILAE) was obtained from fresh leaves, fractionated into fractions, including hydromethanolic (HMf). From the latter, vitexin 2"-O-rhamnoside was isolated, undergoing in silico prediction, showing low predicted toxicity. Better antioxidant effects were observed in free radical scavenging models. Oral administration of ILAE (3, 30, and 100 mg/kg) and HMf (3 mg/kg) was tested in acute inflammation models (induced by carrageenan), mechanical hyperalgesia, thermal allodynia, and nociception (induced by formalin), showing a significant reduction in induced effects. The ILAE (30 mg/kg), HMf (3 and 30 mg/kg), and vitexin 2"-O-rhamnoside (AE-1, 3 mg/kg) were tested in a prolonged inflammation model (induced by complete Freund's adjuvant - CFA), resulting in a significant reduction in edema, mechanical hyperalgesia, and thermal allodynia, including for the isolated flavonoid (AE-1). In the third manuscript **(3)**, oral administration of ILAE (3, 30, and 100 mg/kg) and HMf (3 mg/kg) was evaluated in anxiety and amnesia models (induced by scopolamine). The treatments reduced anxiety and improved parameters related to short-term memory. The decrease in AChE activity and lipid peroxidation, along with the intermolecular interactions of vitexin 2"-O-rhamnoside with AChE (in silico), may explain, at least in part, the observed results. It is concluded that *A. edulis* has a diversity of metabolites and potential anti-inflammatory, antinociceptive, and neuroprotective properties.

***Keywords*:** Vacum. Flavonoids. Inflammation. Anxiety. Scopolamine.

SUMÁRIO

1 INTRODUÇÃO

A abordagem etnodirigida na busca de moléculas ou espécies vegetais com potencial farmacológico é descrita como aquela que considera a escolha de plantas de acordo com indicações de grupos populacionais específicos em contextos de uso relativos aos seus sistemas de saúde e doença. Inserimos neste contexto as pesquisas etnobotânicas e etnofarmacólogicas (RODRIGUES e OLIVEIRA, 2020).

A relação de povos e utilização de espécies vegetais, facilita a proposição e implementação de estratégias de melhoria da qualidade de vida e de conservação ambiental, de forma que a biodiversidade local está intimamente relacionada ao acesso à plantas medicinais. Por abrigar a maior biodiversidade do mundo, compreendendo mais de 45.000 espécies de plantas (20-22% do total existente no planeta), a flora brasileira apresenta uma grande variedade de plantas com propriedades medicinais proveniente especialmente do conhecimento tradicional de povos indígenas da América do Sul, com amplo potencial para descoberta de moléculas com propriedades farmacológicas (ALBUQUERQUE et al., 2014; DUTRA et al., 2016).

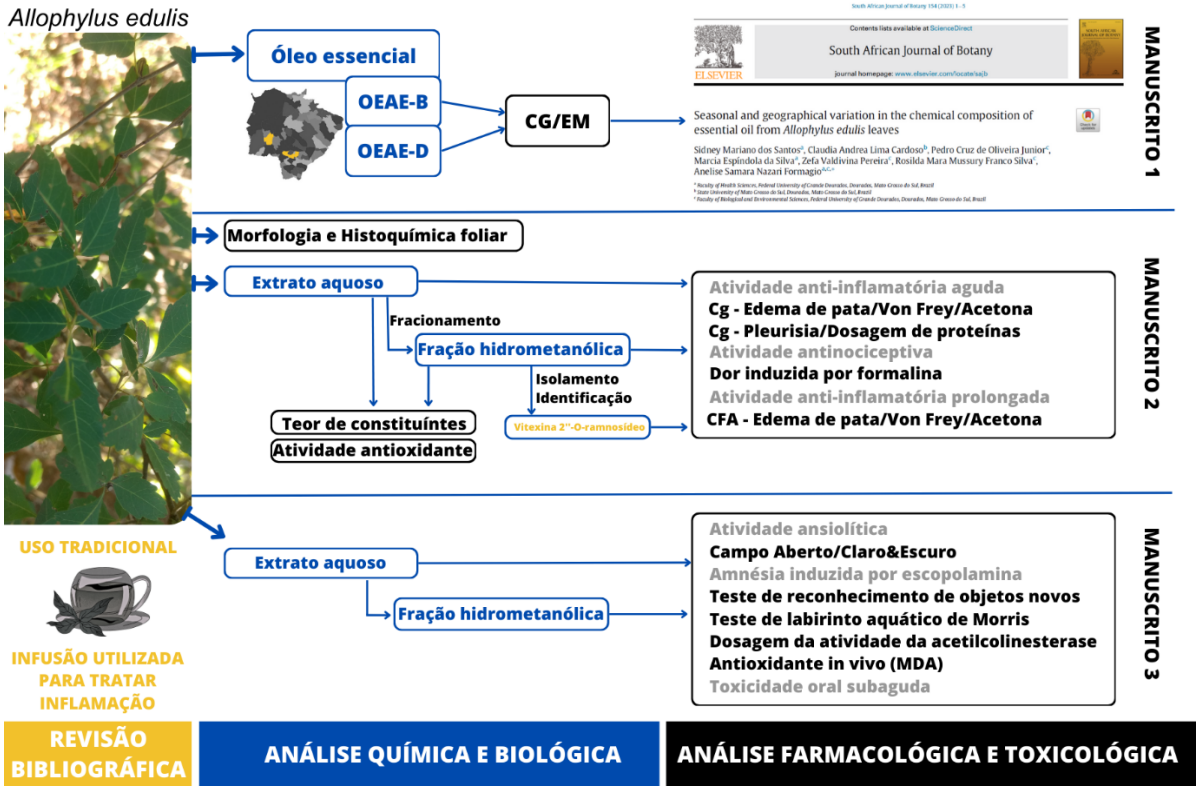
Considerando que o mercado global anual de medicamentos vale cerca de 1,1 trilhão de dólares, e que cerca de 50% dos medicamentos se originaram direta ou indiretamente de produtos naturais (25% de plantas) a enorme variedade da flora brasileira torna a descoberta de novas substâncias derivadas dessa fonte um processo contínuo e necessário (ATANASOV et al., 2015; CALIXTO, 2019; CHOPRA e DHINGRA, 2021).

Neste trabalho, o foco será destinado à *A. edulis* (Sapindaceae), uma planta nativa do Cerrado brasileiro que tem sido utilizada como planta medicinal por povos indígenas peruanos, argentinos e brasileiros na forma de infuso e maceração à frio das folhas, em casos de inflamações de garganta, febre e colecistite (ARISAWA et al., 1989; KÖRBES, 1995; KUJAWSKA e PIERONI, 2015; KUJAWSKA e SCHMEDA-HIRSCHMANN, 2022). Estudos utilizando extratos alcoólicos das folhas apresentam atividade antimicrobiana (TIRLONI et al., 2015), antioxidante (SCHMEDA-HIRSCHMANN et al., 2005), hepatoprotetiva (HOFFMANN-BOHM et al., 1992), potencial ionotrópico negativo (MATSUNAGA et al., 1997) e inibição da enzima conversora de angiotensina (ARISAWA et al., 1989). Para o extrato aquoso das folhas é reportado apenas atividade antioxidante pelo método de captura de radicais livres (DPPH) e bacteriostática frente à *Staphylococcus aureus* (TIRLONI et al., 2015).

Nosso grupo de pesquisa tem investigado o potencial anti-inflamatório do óleo essencial das folhas de *A. edulis*, com resultados que reforçam o uso medicinal, como em quadros inflamatórios. Com os primeiros resultados mostrando o potencial antiedematogênico e de diminuição da migração leucocitária induzida por carragenina, tanto do óleo essencial quanto do composto majoritário, viridiflorol (TREVIZAN et al., 2016). E mais tarde, resultados similares foram alcançados quando foi observada diminuição do edema de pata induzido por Carragenina e CFA, bem como diminuição da hiperalgesia mecânica e térmica utilizando óleos essenciais de diferentes localidades e com diferentes perfis químicos, majoritariamente compostos por óxido de cariofileno e α-zingibereno, também testados isoladamente (SANTOS et al. 2021). E mais recentemente, em uma avaliação mais aprofundada, Balsalobre et al. (2023) descreveu resultados similares ao evidenciar a diminuição do edema, hiperalgesia, infiltração leucocitária e nocicepção induzida por diferentes agentes, utilizando o óleo essencial das folhas de *A. edulis*.

Assim, no sentido de reforçar a pesquisa etnofarmacológica, visto a utilização das folhas na forma de infusão ou maceração aquosa a frio dessa espécie para tratar quadros inflamatórios (ARISAWA et al., 1989; KÖRBES, 1995; KUJAWSKA e PIERONI, 2015; KUJAWSKA e SCHMEDA-HIRSCHMANN, 2022), como ilustrado na **Figura 1**, o objetivo deste estudo foi investigar a composição química, e os efeitos anti-inflamatório (agudo e persistente), antinociceptivo e neurocomportamental (ansiedade e amnésia) da infusão das folhas de *A. edulis*. No processo de extração (infusão) das folhas para liberar seus metabolitos secundários, parte do óleo essencial presente nas folhas, também pode ser arrastado, assim, investigamos o efeito da sazonalidade sobre o perfil químico do óleo essencial de amostras de dois locais no Mato Grosso do Sul. E de forma complementar, estudos histoquímicos das folhas foram realizados para evidenciar as estruturas secretoras.

Figura 1. Design experimental do estudo.



Fonte: Elaborada pelo autor, 2023.

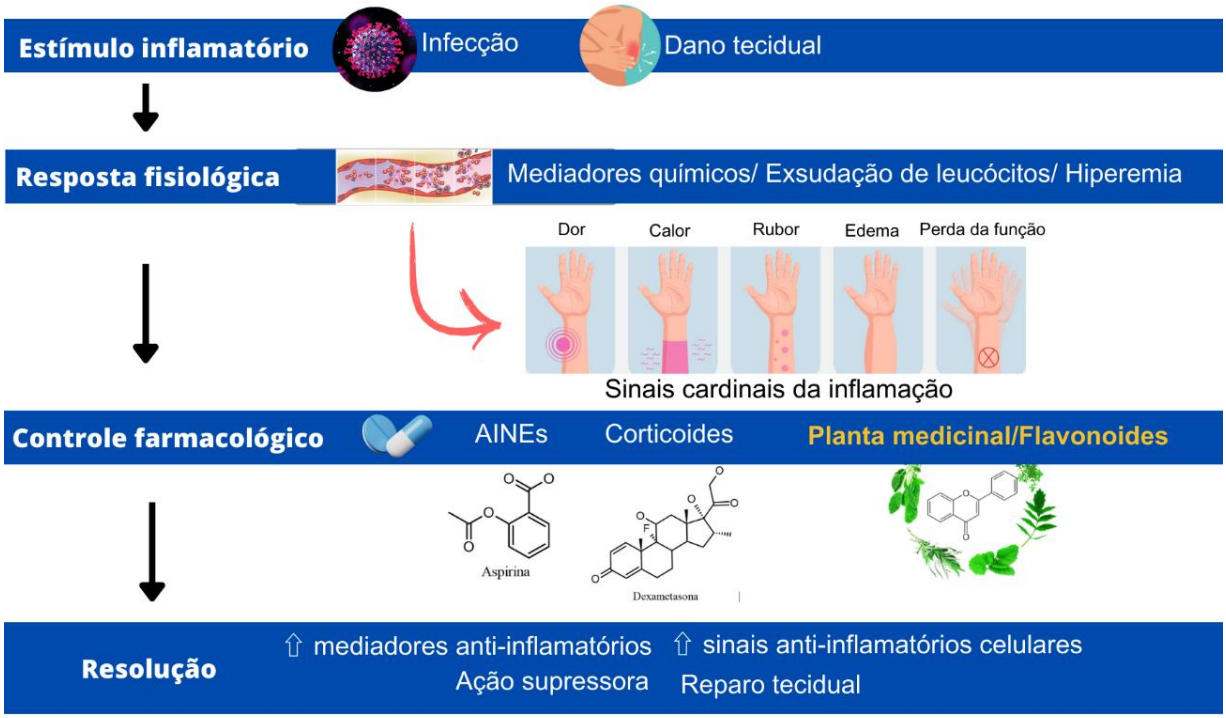
2 REVISÃO DE LITERATURA

2.1 Processo inflamatório, dor e controle farmacológico

O processo inflamatório representa uma intrincada reação do sistema imunológico diante de lesões celulares ou da invasão por agentes externos, tais como vírus ou bactérias. Seu propósito primordial é resguardar o organismo, desencadeando uma série coordenada de eventos biológicos destinados não apenas a eliminar a ameaça, mas também a promover a reparação dos tecidos afetados. Essa resposta imune, embora muitas vezes associada a sintomas como vermelhidão, calor e inchaço, reflete um intricado mecanismo de defesa essencial para manter a integridade e o equilíbrio fisiológico do corpo. Envolve diversos mediadores químicos (como prostaglandinas, leucotrienos, tromboxanos, histamina, ácido araquidônico, citocinas, quimiocinas e espécies reativas do oxigênio (EROs)) e células do sistema imunológico, como neutrófilos na fase aguda, e monócitos/macrófagos e linfócitos na fase tardia. A atração de células imunes e a produção de mediadores inflamatórios, por sua vez, levam ao desenvolvimento de respostas fisiológicas macroscópicas (sinais cardinais) como o rubor (vermelhidão) e calor, causados pela dilatação dos vasos sanguíneos; a dor, causada pelo aumento da sensibilidade local por ação de mediadores químicos; o edema, decorrente do

aumento da permeabilidade vascular; e a perda de função, causada pelos danos no tecido local, como mostra a **Figura 2** (KUMAR et al., 2010).

Figura 2. Processo fisiológico da inflamação e controle farmacológico.



Fonte: Adaptado de MEDZHITOV et al., 2008.

O processo inflamatório pode ser dividido em duas fases principais, que podem se sobrepor e não necessariamente são distintas, dependendo da causa e da intensidade da inflamação: aguda e crônica (GERMOLEC et al., 2018).

A inflamação aguda é a fase inicial, caracterizada pela rápida ativação das células imunes e pela liberação de mediadores químicos. Os principais mediadores incluem a histamina, liberada por basófilos e mastócitos, responsável por aumentar a permeabilidade dos vasos sanguíneos e permitir que as células de defesa alcancem a área inflamada. As prostaglandinas e leucotrienos produzidas por macrófagos, neutrófilos e células endoteliais, que aumentam a sensibilidade à dor, auxiliam na inibição da agregação plaquetária e promovem vasodilatação. Outros mediadores incluem a Proteína C-reativa (PCR) na facilitação da fagocitose pelos neutrófilos e macrófagos; Interleucina-1 (IL-1) de ação pró-inflamatória, por auxiliar na liberação de histamina, estimulação de linfócitos virgens e estimulação da síntese de PCR; Interleucina-6 (IL-6), envolvida na imunidade natural e hematopoiese; e Fator de Necrose Tumoral (TNF-α), responsável por ativar células inflamatórias, mediadores

inflamatórios e regulação da morte celular (YEUNG et al., 2018; VARELA et al., 2018; ROE, 2020).

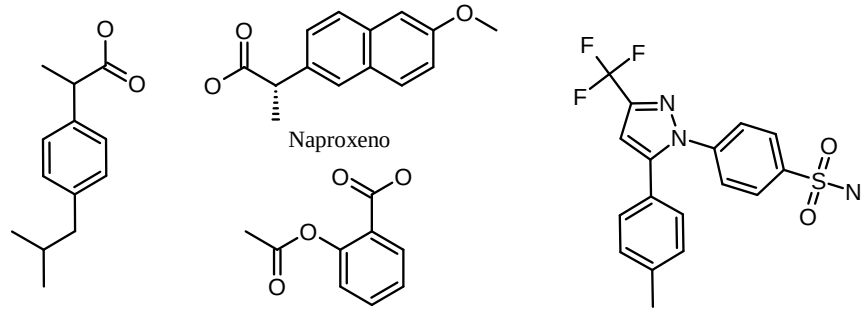
A inflamação crônica, por sua vez, é menos intensa, mas dura por um período prolongado. Pode ser causada por uma enormidade de condições, incluindo artrite, asma, aterosclerose, doenças autoimunes, diabetes e câncer, condições associadas ao envelhecimento ou por exposição prolongada a substâncias inflamatórias. Os mediadores químicos da inflamação crônica são semelhantes às da inflamação aguda, mas geralmente são liberados em quantidades maiores e por períodos mais longos resultando em danos teciduais e aumento do risco de desenvolvimento de outras patologias, como doenças cardíacas e câncer (GERMOLEC et al., 2018).

A resolução da inflamação é tipicamente definida como um processo estritamente regulado que restaura a homeostase tecidual e previne o desenvolvimento de doenças crônicas. Hoje sabe-se que as mudanças relativas à resolução da inflamação não se resumem à simples diminuição de sinais pró-inflamatórios, no entanto as intervenções farmacológicas continuam sendo a principal forma de restaurar a homeostase tecidual (FEEHAN e GILROY, 2019; PANEZAI e VAN DYKE, 2022).

Os anti-inflamatórios não esteroidais (AINEs) são uma classe de medicamentos que incluem ibuprofeno, naproxeno e aspirina, ilustrados na **Figura 3**. Os AINEs, em geral, bloqueiam enzimas reguladoras, como as prostaglandinas endoperoxidases sintase (PGHS-1 e PGHS-2, conhecidas popularmente como ciclooxigenase-1 e 2 (COX-1 e COX-2)), responsáveis pela produção de prostaglandinas, derivados do ácido araquidônico, que se origina dos fosfolipídios da membrana celular por meio da ação da fosfolipase A2. Por haver outros medicamentos desta classe, os mecanismos de ação podem incluir desde a inibição seletiva de COX-2 (chamados de COXIBs, como o celecoxibe), até a inibição de ambas as enzimas (maioria dos AINEs), resultando na inibição da produção de outros mediadores inflamatórios, como leucotrienos e citocinas. Esta classe de medicamento é bem tolerada quando utilizada por curtos períodos, com extensos benefícios terapêuticos. Mas, considerando que a ciclooxigenase-1 parece funcionar como uma enzima homeostática na maioria dos tecidos, permitindo a manutenção da função normal dos órgãos, incluindo mucosa gástrica, função renal e agregação plaquetária, o uso prolongado desta classe de medicamentos se relaciona com o aumento da incidência de toxicidade, que incluem eventos cardiovasculares como acidente vascular cerebral/infarto do miocárdio, úlcera péptica, retenção de líquidos; e até mesmo insuficiência renal aguda após um mês de uso por pacientes idosos. A presença desses efeitos adversos é, na verdade, um combustível na busca de novas alternativas farmacológicas, de

origem natural, e com menos efeitos adversos (SCHNEIDER et al., 2006; MARCUM e HANLON, 2010; BACCHI et al., 2012; PANCHAL e SABINA, 2023).

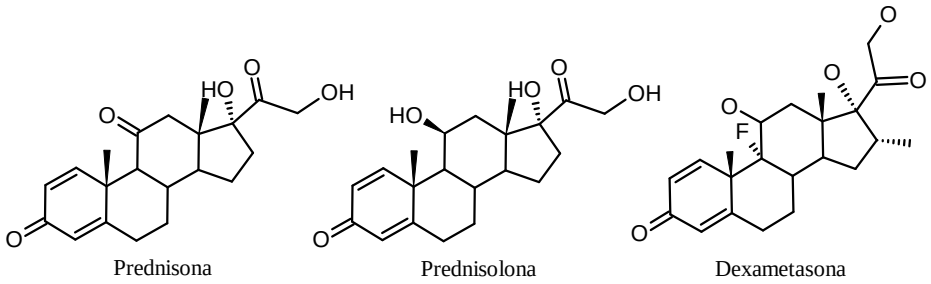
Figura 3. Medicamentos anti-inflamatórios da classe dos AINEs.



Fonte: Elaborada pelo autor, 2023.

Os anti-inflamatórios corticosteroides, também conhecidos como esteroides, são outra classe de medicamentos, como os glicocorticoides e mineralocorticoides que incluem fármacos como prednisona, prednisolona e dexametasona (**Figura 4**). Os corticosteroides iniciam a regulação positiva da lipocortina e da anexina A1, uma proteína que reduz a síntese de prostaglandinas e leucotrienos pela inibição da fosfolipase A2, além de inibirem a atividade da COX-2, reduzindo a síntese de prostaglandinas. De forma geral, esses agentes inibem os fatores de transcrição que controlam a síntese de diversos mediadores pró-inflamatórios, incluindo COX-2, óxido nítrico sintase induzível e citocinas pró-inflamatórias, incluindo TNF-α e interleucinas; além da diminuição de células inflamatórias como macrófagos, eosinófilos, linfócitos, mastócitos e células dendríticas, e a entrada desses leucócitos nos locais da inflamação. Devido à diversidade no mecanismo de ação dos corticoides, eles podem provocar uma ampla gama de efeitos adversos, que variam de leves a graves. Altas doses e o uso prolongado são os fatores de risco mais significativos para tais efeitos. Entre os impactos estão a supressão do eixo hipotálamo-hipófise, infecções graves, osteoporose, osteonecrose, miopatias, retenção de líquidos, edema, supressão adrenal, ganho de peso, hipertensão, arritmias, gastrite, diabetes, distúrbios psiquiátricos e do sono. Isso deixa claro as limitações das abordagens farmacológicas existentes, de forma que, buscar soluções com mecanismos de ação mais específicos pode representar uma alternativa na procura por agentes anti-inflamatórios com menor incidência de efeitos adversos (INGAWALE e MANDLIK, 2019; TIMMERMANS et al., 2019; HODGENS e SHARMAN, 2022).

Figura 4. Medicamentos anti-inflamatórios da classe dos corticoides.



Prednisona Prednisolona Dexametasona

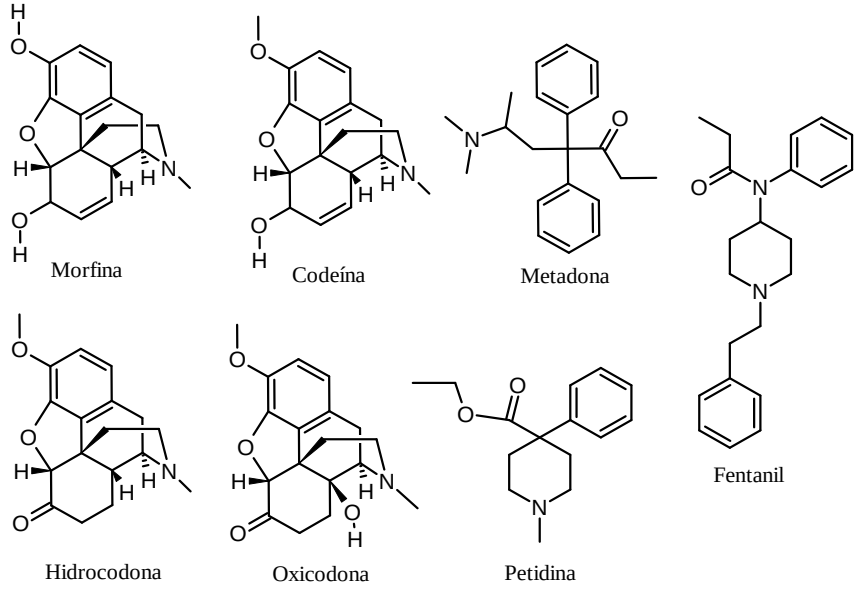
Fonte: Elaborada pelo autor, 2023.

O diálogo entre o sistema imunológico e os neurônios nociceptores é crucial na inflamação, seja ela aguda ou crônica. O adequado funcionamento do sistema nociceptivo reforça respostas comportamentais protetoras, como o equilíbrio ou retirada da pata (no teste de alodinia induzida por acetona) para evitar estímulos extremamente dolorosos. Em casos de lesão, o sistema nociceptivo adapta-se à vulnerabilidade causada, diminuindo limiares nociceptivos e facilitando respostas nocifensivas para proteger os tecidos. Neste estudo, a lesão tecidual será representada pela aplicação de agentes flogísticos como carragenina, CFA e formalina, que, ao ativar diretamente as fibras nociceptivas, induzem a secreção de citocinas pró-inflamatórias, reduzindo o limiar de dor. As manifestações comportamentais protetivas e adaptativas exploradas incluem a alodinia e a hiperalgesia. A alodinia é caracterizada pela dor devido a estímulo que geralmente não a provoca, como a alodinia ao frio induzida pela aspersão de acetona. Por outro lado, a hiperalgesia é definida pelo aumento da dor devido a um estímulo que normalmente seria doloroso, como a hiperalgesia causada por estímulo mecânico (VERMA et al., 2015; GREGORY et al., 2013).

Embora a dor em animais não possa ser diretamente medida, é crucial empregar modelos quantificáveis, sensíveis e específicos para avaliar a sensação de dor. Ao escolher parâmetros como hiperalgesia e alodinia, é importante ter em mente que qualquer reação a um estímulo doloroso não necessariamente indica uma sensação simultânea de dor. Isso acontece porque, mesmo ao classificar o tipo de estímulo como naturalmente causador ou não de dor, o que realmente medimos é o aumento ou a ausência de nocicepção. A nocicepção refere-se aos mecanismos moleculares, celulares e sistêmicos envolvidos no processamento de informações relacionadas à dor, sua amplificação ou depressão. Enquanto a dor é uma experiência sensorial e emocional desagradável associada a dano tecidual real ou potencial, ou descrita em termos de tal dano, ela permanece, em muitos aspectos, subjetiva (SANDKÜHLER, 2009; DEUIS et al., 2017; KALIYAPERUMAL et al., 2019).

A terapia utilizada para analgesia é feita com medicamentos que trazem insensibilidade à dor sem perda de consciência. Sendo os analgésicos mais comuns os anti-inflamatórios e os opioides. A analgesia causada pelos medicamentos anti-inflamatórios geralmente está relacionada com a inibição da COX, atenuando assim a síntese de prostaglandinas. Como já mencionado, as classes mais comuns de medicamentos anti-inflamatórios são os corticoides e AINEs (**Figura 3 e 4**). A farmacologia da dor utilizando opioides inclui medicamentos naturais como a morfina e codeína, e sintéticos e semissintéticos, como o fentanil, hidrocodona, petidina, metadona e oxicodona (**Figura 5)**. Os opioides são agentes analgésicos que atuam em receptores específicos no sistema nervoso central e periférico. Os principais mecanismos de ação incluem o agonismo dos receptores MOP (mu), DOP (delta) e KOP (kappa). A ligação dos opioides ao receptor MOP ativa vias inibitórias que reduzem a transmissão nociceptiva da periferia para o tálamo. Além disso, eles inibem diretamente neurônios nociceptivos na substância gelatinosa da medula espinhal e no tecido periférico. Os que se ligam ao receptor DOP proporcionam analgesia espinhal e supraspinal, com a redução da motilidade gástrica. Já os que se ligam ao receptor KOP causam analgesia espinhal, diurese e disforia. Os opioides, como tramadol e metadona, apresentam efeitos não opioides, incluindo atividade em outros receptores e sistemas de neurotransmissores, como NMDA, recaptação de serotonina e noradrenalina, e o sistema nociceptina/orfanina FQ. Esses efeitos podem contribuir para a analgesia, mas por atuarem em receptores no sistema nervoso central e periférico também estão associados a diversos efeitos adversos, como constipação, depressão respiratória, sedação, eventos cardiovasculares, imunossupressão, fraturas, desregulação hipotálamo-hipófise-adrenal, dependência, tolerância e overdose. O estudo de plantas medicinais visa buscar novas terapias analgésicas focadas na melhoria da eficácia e potência analgésica, bem como na redução de efeitos colaterais (PATHAN e WILLIAMS, 2012; BALDINI et al., 2012; LISTOS et al., 2019; PAUL et al., 2021).

Figura 5. Principais analgésicos opioides.



Fonte: Elaborada pelo autor, 2023.

2.1.1 Modelos animais na avaliação da inflamação e dor

Atualmente, a pesquisa com produtos naturais busca moléculas que inibam a síntese ou ação de mediadores inflamatórios, visando desenvolver adjuvantes ou substitutos com menos efeitos adversos em comparação aos fármacos anti-inflamatórios disponíveis (ARULSELVAN et al., 2016). O papel da farmacologia de plantas medicinais no estudo da inflamação é explorar novos fármacos por meio de modelos apropriados, elucidando os mecanismos de direcionamento das terapias anti-inflamatórias. O objetivo principal é aprimorar a qualidade de vida com soluções farmacológicas mais acessíveis, específicas e com menores efeitos adversos. Os modelos experimentais, embasados em princípios farmacológicos, devem oferecer uma representação fisiológica e clinicamente relevante, relacionando os efeitos observados no modelo pré-clínico aos resultados no cenário clínico, incluindo alterações imunológicas, bioquímicas e fisiológicas do processo inflamatório (WEBB, 2014; PATIL et al., 2019).

Os modelos animais de quadros inflamatórios, embora forneçam informações importantes sobre os possíveis aspectos clínicos subjacentes das doenças humanas, não refletem uma doença específica, mas certos aspectos das doenças em si, como a presença/ausência e/ou intensidade de alterações macroscópicas ou microscópicas resultantes do processo inflamatório. Essas alterações incluem desde o aumento da permeabilidade vascular e migração leucocitária observada no teste de pleurisia induzida por carragenina, até o surgimento de edema nos testes de edema de pata induzido por carragenina ou CFA, e sensibilização periférica e central observada no teste de von Frey/hiperalgesia mecânica, alodinia ao estímulo térmico e teste da formalina (nocicepção induzida por estímulo químico) (PATIL et al., 2019).

Neste estudo, a carragenina e o CFA foram empregados como agentes flogísticos. A carragenina, um mucopolissacarídeo das paredes celulares de algas vermelhas, atua como agente inflamatório ao ativar células como neutrófilos e macrófagos. Essas células liberam diversos mediadores inflamatórios, incluindo citocinas, proteínas de choque térmico e espécies reativas do oxigênio (EROs). Esses eventos culminam na sensibilização de receptores mecânicos/térmicos, desencadeando hiperalgesia e aumento da permeabilidade vascular, resultando na formação de edema na região da injeção. Para analisar as fases iniciais do processo inflamatório (até 24 horas), utilizamos como controle positivo anti-inflamatórios esteroidais, como prednisolona e dexametasona, devido à sua eficácia superior em reduzir o edema de pata induzido por carragenina em comparação aos AINEs (KOCHER et al., 1987; HENRIQUES et al., 1987; AL-SWAYEH et al., 2000; NECAS e BARTOSIKOVA, 2013).

O CFA consiste em uma suspensão de *Mycobacterium tuberculosis* inativada por calor, cujo mecanismo de ação está associado a um processo inflamatório prolongado, simulando a cronificação da inflamação. A liberação prolongada de anticorpos estimula coestimuladores de ativação de células T e B, bem como citocinas por células apresentadoras de antígenos, prolongando a expressão de complexos peptídeo-MHC (Complexo Principal de Histocompatibilidade) em suas superfícies. A injeção de CFA na pata do animal provoca edema nos tecidos periarticulares, com aumento progressivo na fase inicial da inflamação, atingindo o pico em 24 horas. Esse edema torna-se constante e pode persistir por pelo menos 7 dias. Além do edema, a injeção de CFA resulta em alterações como alodinia térmica e hiperalgesia mecânica, decorrentes da infiltração maciça de leucócitos, aumento dos níveis de quimiocinas e citocinas, incluindo IL-1β e TNF-α, assim como a liberação de EROs (STILS Jr., 2005; PATIL et al., 2019; MCCARSON e FEHRENBACHER, 2021).

Uma maneira de avaliar a hiperalgesia mecânica é por meio do teste de von Frey, que utiliza uma estrutura pontiaguda calibrada, comumente conhecida como analgesímetro ou von Frey digital, aplicada na pata do animal. Esse aparelho determina o limiar de força (em gramas) necessário para desencadear a retirada da pata em contato com o medidor. Quanto ao modelo de alodinia ao frio, ele envolve a indução da sensação de frio pela aspersão de acetona na pata do animal, com a subsequente medição da duração e intensidade dos comportamentos nociceptivos (DEUIS et al., 2017).

O teste da formalina, ou nocicepção induzida por formalina, envolve a injeção de formalina (formol diluído) no tecido subcutâneo da pata do animal. Após a aplicação, a formalina desencadeia uma resposta dolorosa aguda, seguida por uma fase crônica de dor, evidenciada por comportamentos nociceptivos como balanço e lambida da pata injetada. Na

primeira fase (1-10 minutos), também conhecida como fase neurogênica, a formalina ativa as fibras aferentes primárias, cuja excitação dos receptores é mediada pelo canal catiônico de potencial receptor transitório A1 permeável ao cálcio, denominado TRPA1. Além disso, a liberação de neurotransmissores como serotonina e substância P amplifica a transmissão da dor pelo sistema nervoso. Ao final da primeira fase, ocorre um período de quiescência chamado interfase, caracterizado por hiperpolarização e inativação transitória de neurônios, resultando na redução da corrente nos canais de cálcio dependentes de voltagem. Na segunda fase (15–60 minutos), muitas vezes descrita como fase inflamatória, a formalina continua ativando os neurônios nociceptores de maneira excitotóxica, além de desencadear a cascata inflamatória e aumentar a liberação de citocinas inflamatórias, como TNF-α e interleucinas, contribuindo para a sensação dolorosa (VANEGAS e SCHAIBLE, 2004; SAWYNOK e LIU, 2003; DUBIN e PATAPOUTIAN, 2010; HOFFMANN et al., 2022).

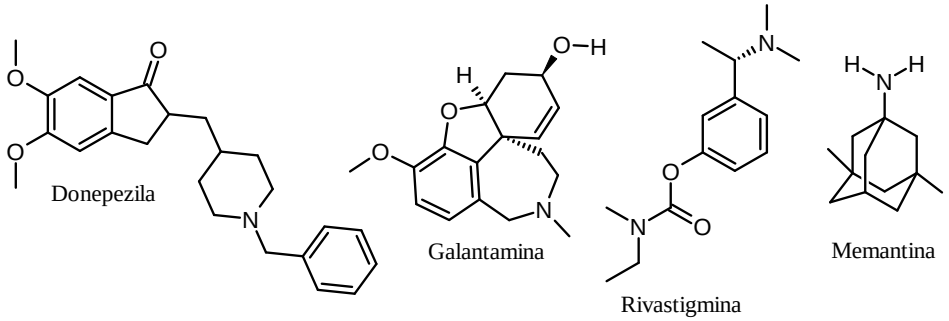
2.2 Demência e controle farmacológico

Doenças neurodegenerativas resultam da deterioração das células nervosas, prejudicando o funcionamento do sistema nervoso. A progressão dessas condições pode influenciar diversos aspectos, como locomoção, linguagem, percepção, cognição e memória, variando conforme as regiões cerebrais afetadas. A demência, uma manifestação proeminente nas doenças neurodegenerativas, caracteriza-se pela deterioração do funcionamento cognitivo, abrangendo pensamento, memória e raciocínio, interferindo substancialmente nas atividades diárias dos indivíduos afetados. Os sintomas clínicos da demência incluem desde a perda de habilidades mentais, até alterações emocionais e de personalidade, evidenciando o impacto abrangente dessa condição. A demência é um desafio de saúde pública em escala global, com sua prevalência crescendo especialmente entre os idosos, com estimativas que chegam em mais de 150 milhões de casos em 2050. Ainda que a doença de Alzheimer seja a mais conhecida, outras formas de demência incluem a demência vascular, demência com corpos de Lewy, a frontotemporal ou a coexistência destas (DENING e SANDILYAN, 2015; NICHOLS et al., 2022; ARANDA et al., 2021).

A doença de Alzheimer, correspondendo a 60–70% dos casos de demência, é caracterizada por níveis elevados de beta-amiloide e proteína tau fosforilada. Essas alterações estão associadas a perturbações no sistema colinérgico central, resultando em um declínio cognitivo irreversível e deterioração da memória. Atualmente, não há uma cura conhecida para a doença de Alzheimer, e o desafio persiste em retardar sua progressão. As abordagens terapêuticas concentram-se no alívio sintomático para desacelerar o avanço da doença. Os

tratamentos principais incluem inibidores da AChE (donepezila, rivastigmina e galantamina) e antagonistas do receptor *N*-metil-D-aspartato (NMDA), uma subfamília de receptores de glutamato, conforme apresentado na **Figura 6**. Infelizmente, esses medicamentos estão associados a numerosos efeitos adversos graves. Por esse motivo, alguns inibidores da AChE de primeira geração, como tacrina, velnacrina e fisostigmina, já foram retirados do mercado. Os inibidores de segunda geração, ainda presentes, cursam com efeitos adversos, incluindo náuseas, vômitos, anorexia e insônia, resultantes de suas ações não seletivas em diversos tecidos, tanto central quanto perifericamente (TAN et al., 2014; BRIGGS et al., 2016; LEI et al., 2021; FERRARI e SORBI, 2021; RUANGRITCHANKUL et al., 2021).

Figura 6. Medicamentos utilizados no tratamento do declínio cognitivo.



Fonte: Elaborada pelo autor, 2023.

As abordagens de tratamento existentes atualmente têm como objetivo principal diminuir a progressão clínica da doença e aliviar os sintomas. Os inibidores da AChE, utilizados na terapia sintomática para a doença de Alzheimer baseia-se na hipótese colinérgica, e visam compensar a redução de acetilcolina no cérebro, que, segundo a teoria inicial, se inicia no núcleo de Meynert. A degeneração dos neurônios colinérgicos está ligada a alterações na síntese da acetilcolina ou em sua recaptura pré-sináptica, resultando em um declínio gradual na capacidade de memória. Enquanto a terapia com inibidor de glutamato está fundamentada na hipótese excitotóxica. No entanto, os benefícios gerais dos inibidores da AChE e antagonistas do receptor NMDA são modestos para retardar o declínio do comportamento e a mudança clínica global dos pacientes. E considerando as sucessivas falhas no desenvolvimento de medicamentos para tratar doenças neurodegenerativas e sintomas demenciais, é pertinente a necessidade de buscar terapias alternativas principalmente para melhoria da qualidade de vida dos indivíduos acometidos pela demência, com medicamentos com ação específica, menor custo e menos efeitos adversos (TAN et al., 2014; FERRARI e SORBI, 2021).

O interesse em novas terapias para combater o declínio cognitivo aumentou durante e após a pandemia de COVID-19, devido ao impacto significativamente negativo nas pessoas afetadas pela demência, e ao surgimento de sintomas neurológicos em indivíduos sem demência. As alterações relacionadas à infecção pelo vírus levaram à hipótese de efeito neurodegenerativo como consequência da infecção pelo SARS-CoV-2. Descobertas diversas, que vão desde o aumento da resposta imune e o agravamento da neuroinflamação até a redução na espessura da substância cinzenta, correlacionam-se com um declínio cognitivo mais pronunciado durante e após o período de infecção. Apesar de os conhecimentos sobre a ligação entre neurodegeneração e infecção pelo SARS-CoV-2 não estarem completamente claros, é fundamental buscar alternativas que possam aprimorar o funcionamento cognitivo na farmacologia das síndromes demenciais (LIU et al., 2021; ŁUC et al., 2022; DING e ZHAO, 2023; OLIVERA et al., 2023).

2.2.1 Modelos comportamentais para avaliação do declínio cognitivo

Modelos animais de comprometimento cognitivo são essenciais para entender as bases neurais de aprendizagem e memória, além de testar alternativas aos tratamentos convencionais. Os modelos farmacológicos, especialmente focados nos receptores colinérgicos muscarínicos e nicotínicos, desempenham papel crucial na investigação das disfunções cognitivas, permitindo simular eficazmente síndromes específicas de comprometimento cognitivo (LEVIN e BUCCAFUSCO, 2006).

O modelo farmacológico amplamente utilizado é o da amnésia induzida por escopolamina, um antagonista do receptor muscarínico que bloqueia a neurotransmissão colinérgica, resultando em comprometimento da memória em roedores. Este comprometimento pode ser resultado de alguns dos efeitos da escopolamina, incluindo neuroinflamação (CHEON et al., 2021), interrupção da conectividade de circuitos colinérgicos, interrupções na amplitude e no alinhamento de ondas cerebrais teta (2–10 Hz) durante a codificação da memória no hipocampo (GEDANKIEN et al., 2023), estresse oxidativo (RAHIMZADEGAN e SOODI, 2018), disfunção mitocondrial e aumento da atividade da AChE (WONG-GUERRA et al., 2017). Assim, ao considerarmos um tratamento complementar eficaz, efeitos como a restauração da atividade do sistema colinérgico, inibindo a enzima AChE, efeito antioxidante e anti-inflamatório são fundamentais. Efeitos estes observados em muitas plantas medicinais (ULLAH et al., 2020), incluindo a *A. edulis* (UMEO et al., 2011; TIRLONI et al., 2015; SANTOS et al. 2021) e explorados nesta tese.

O teste de reconhecimento de objetos novos (*Novel object recognition test)* é uma ferramenta comportamental usada para avaliar a memória de reconhecimento em animais, especialmente a de objetos. Esse teste se baseia na tendência natural dos roedores de dedicar mais tempo à exploração de um objeto recém-introduzido em comparação com um objeto familiar. Além de medir a memória, o teste também é utilizado para avaliar comportamentos relacionados à ansiedade, como a preferência por novidades. Os parâmetros comportamentais analisados no teste incluem o tempo de exploração de objetos novos e familiares, a taxa de exploração e a preferência pelo objeto recém-introduzido (ENNACEUR e DELACOUR, 1988).

O teste do labirinto aquático de Morris (*Morris water maze test*), é uma ferramenta comportamental usada para estudar a aprendizagem, a memória espacial e a memória de longo prazo em roedores. Consiste em uma piscina (aparato) circular de água com pistas de localização nas laterais, onde o animal é colocado e deve encontrar uma plataforma submersa para escapar da água. O tempo de escape, também chamado de latência de fuga, é uma medida de aprendizado, pois avalia a capacidade dos animais de lembrar a localização da plataforma. O teste compreende um período de treinamento de quatro dias, durante o qual os animais aprendem a encontrar a plataforma e são condicionados a associar esse encontro com a saída do aparato. Outros parâmetros comportamentais avaliados incluem o tempo de escape, a distância percorrida, a velocidade e o tempo gasto no quadrante da plataforma após o período de aprendizagem (MORRIS, 1984).

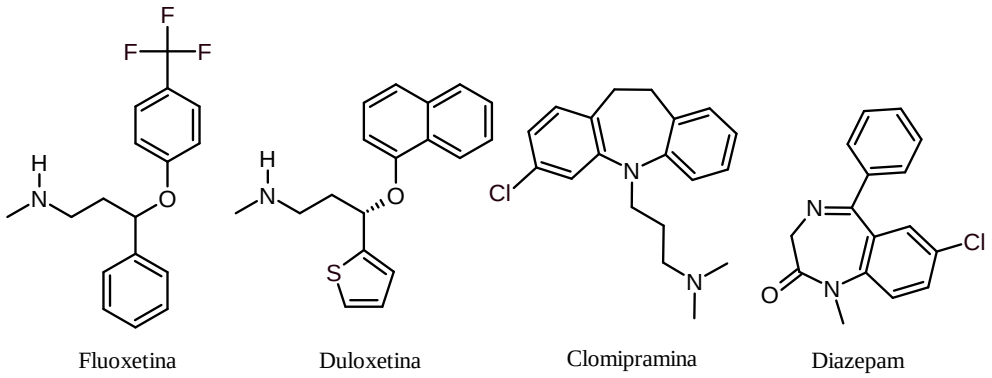
2.3 Ansiedade e controle farmacológico

A ansiedade é o transtorno psiquiátrico mais comum globalmente, afetando aproximadamente 4% da população. Este transtorno abrange condições como ansiedade generalizada, pânico, estresse pós-traumático, transtornos obsessivo-compulsivos e fóbicos. Em muitos casos, a ansiedade se manifesta de maneira debilitante, sendo inclusive um sintoma frequente em casos de demência (presente em 38% a 72% dos casos). No entanto, ela recebe relativamente pouca atenção e pesquisa em comparação com outros sintomas neuropsiquiátricos. Além da escassez de novas abordagens terapêuticas, apenas 60-85% dos pacientes com transtornos de ansiedade respondem efetivamente aos tratamentos existentes, que incluem abordagens farmacológicas e psicoterapêuticas. Além disso, esses pacientes enfrentam baixa recuperação e altas taxas de recorrência nos sintomas. A ansiedade pode causar uma diminuição na qualidade de vida do paciente e contribuir para o aumento da sobrecarga do cuidador, especialmente em casos de pacientes idosos. Além de impactar negativamente a cognição, o comportamento e a funcionalidade do paciente com demência, a ansiedade também

pode ser considerada um fator de risco para a progressão da demência (KWAK et al., 2017; GARAKANI et al., 2020; WHO, 2023).

A terapia ansiolítica atual inclui inibidores seletivos da recaptação da serotonina (como fluoxetina e sertralina), inibidores da recaptação da serotonina e norepinefrina (duloxetina e venlafaxina), antidepressivos tricíclicos (clomipramina e nortriptilina), agonistas dos receptores do ácido gama aminobutírico A (GABA-A) (benzodiazepínicos, como diazepam e lorazepam) e anti-histamínicos (hidroxizina), alguns destacados na **Figura 7**. Devido à ampla gama de mecanismos de ação e efeitos em diferentes sistemas, muitos efeitos adversos são relatados, incluindo náusea, dor de cabeça, boca seca, diarreia ou prisão de ventre, disfunção sexual, mudanças de peso, sedação, retenção urinária, arritmias, risco de mortalidade em caso de sobredosagem, tolerância e dependência. Diante desses desafios, torna-se essencial o contínuo desenvolvimento de medicamentos de origem natural baseados em neurorreceptores específicos, visando reduzir os efeitos adversos associados à terapia ansiolítica convencional (FARACH et al., 2012; LIU et al., 2015; GARAKANI et al., 2020).

Figura 7. Medicamentos ansiolíticos.



Fluoxetina Duloxetina Clomipramina Diazepam

Fonte: Elaborada pelo autor, 2023.

2.3.1 Modelos comportamentais para avaliação da ansiedade

Na avaliação do efeito ansiolítico em modelos animais, alguns testes são empregados para definir a eficácia de novas abordagens terapêuticas. Um desses testes é o claro/escuro (*Light/dark test*), utilizado para avaliar respostas de ansiedade não condicionadas em roedores. Fundamentado na aversão inata a áreas iluminadas e no comportamento exploratório espontâneo, o teste aplica estressores leves, como um ambiente novo e luz. O aparato consiste em uma zona escura (considerada segura) e uma zona iluminada (considerada aversiva), sendo que o tempo gasto em cada zona funciona como um parâmetro indicativo da presença ou

ausência de comportamento ansioso (CRAWLEY e GOODWIN, 1980; COSTALL et al., 1989).

Outro método de avaliação da ansiedade em roedores é o teste de campo aberto (*Open field test*). Este teste é empregado para avaliar os níveis gerais de atividade locomotora, ansiedade e motivação para explorar em roedores, utilizando também a aversão do animal a áreas expostas e mais iluminadas, como o centro do aparato. A arena do campo aberto possui paredes para evitar fugas e é dividida em zonas mais ou menos afastadas das laterais do aparato, incluindo a zona central e a zona periférica. Esse delineamento possibilita a medição de diversos padrões comportamentais, como a razão tigmotática (preferência por estar próximo às paredes), tempo gasto na zona central, cruzamentos de linhas, entradas na área central, exploração vertical por levantamento (rearing), além de comportamentos como autolimpeza, defecação e micção (HALL, 1934; KRAEUTER et al., 2019).

2.4 Abordagem etnodirigida

Novas abordagens terapêuticas complementares têm sido buscadas para o tratamento de condições inflamatórias, dor, síndromes demenciais e ansiedade, que incluem combater fatores como dor e inflamação, além de contribuir para o declínio cognitivo, como a modulação da neurogênese, sinalização cerebral, neuroinflamação (WANG et al., 2019), estresse oxidativo (ABORODE et al., 2022) e comportamentos ansiosos (KHAN et al., 2022). Essas alternativas abrangem dietas saudáveis, suplementação alimentar, exercícios físicos e meditação. Diversas plantas medicinais são consideradas para desempenhar um papel complementar na terapia da inflamação e dor, tais como *Ricinus communis* (Euphorbiaceae), *Curcuma longa* (Zingiberaceae), *Zingiber officinale* (Zingiberaceae), *Rosmarinus officinalis* (Lamiaceae). Para melhoria cognitiva, destacam-se *Bacopa monnieri* (Plantaginaceae), *Centella asiatica* (Apiaceae), *Ginkgo biloba* (Ginkgoaceae), *Cinnamomum zeylanicum* (Lauraceae) e *Panax ginseng* (Araliaceae). No âmbito da ansiedade, plantas como *Lavandula angustifolia* (Lamiaceae), *Melissa officinalis* (Lamiaceae), *Passiflora incarnata* (Passifloraceae), *Erythrina verna* (Fabaceae) e *Valeriana officinalis* (Caprifoliaceae) têm sido estudadas (GHASEMIAN et al., 2016; SHARMA e KUMAR, 2019; AREMU e PENDOTA, 2021; KENDA et al., 2022).

Há evidências crescentes de que os flavonoides presentes em alimentos vegetais e plantas medicinais beneficiam a saúde cognitiva, associados a taxas mais lentas de declínio cognitivo. Estudos destacam a relação positiva entre a ingestão desses compostos, como os encontrados em frutas vermelhas e outros alimentos (LETENNEUR et al., 2007; DEVORE et al., 2012), e melhorias na função executiva em casos de Alzheimer leve a moderada (KENT et

al., 2016). Embora a ingestão a curto prazo possa promover benefícios cognitivos agudos (WHYTE et al., 2021), sabe-se que a ingestão a longo prazo está associada a riscos mais baixos de doença de Alzheimer e demências relacionadas (SHISHTAR et al., 2020), além da manutenção da saúde cognitiva em geral (GODOS et al., 2020; YEH et al., 2021). As características protetivas da ingestão de flavonoides estão associadas a um declínio mais lento em várias áreas cognitivas (memória episódica, memória semântica, velocidade perceptual, memória de trabalho e de forma menos intensa, na habilidade visuoespacial), além de melhora na função executiva, com destaque para quercetina e canferol (JENNINGS et al., 2021; HOLLAND et al., 2023).

Os mecanismos neuroprotetores dos flavonoides em quadros demenciais envolvem a modulação de vias de sinalização neuronal, incluindo a inibição da AChE, a redução da agregação da proteína Tau, β-secretase, estresse oxidativo, inflamação e apoptose. Essas ações ocorrem por meio de vias que englobam quinases reguladas por sinal extracelular, fosfatidilinositol-3-quinase, fator nuclear kappa B, proteína quinase ativada por mitógeno, Fator Neurotrófico Derivado do Cérebro e sistemas enzimáticos antioxidantes endógenos. Essas alterações culminam no aprimoramento de mecanismos relacionados à plasticidade sináptica, redução da neuroinflamação e otimização do fluxo sanguíneo cerebrovascular, inclusive pela modulação da microbiota intestinal (WILLIAMS et al., 2008; CALDERARO et al., 2022; WANG et al., 2023).

Este trabalho pretende investigar os potenciais efeitos farmacológicos de *Allophylus edulis* no tratamento de inflamação, dor, declínio cognitivo e ansiedade. A planta é conhecida por sua riqueza em flavonoides e tem sido utilizada na medicina popular para tratar diversas condições patológicas. Sua aplicação pode representar uma alternativa promissora no desenvolvimento de soluções terapêuticas de origem natural, com potencial para minimizar efeitos adversos.

2.5 Família Sapindaceae e gênero *Allophylus*

Sapindaceae é composta por 141 gêneros e 2000 espécies, distribuídas principalmente em regiões tropicais e subtropicais, composta principalmente pelos gêneros *Allophylus* L. (250 espécies), *Cupania* L. (50 espécies), *Matayba* Aubl. (50 espécies), *Paullinia* L. (190 espécies), *Serjania* Mill. (230 espécies) e *Talisia* Aubl. (ACEVEDO-RODRÍGUEZ et al., 2010; ZINI et al., 2012). No território brasileiro estão catalogados 27 gêneros e 419 espécies, das quais 193 são endêmicas, com presença em todos os biomas do território nacional (COELHO, 2014).

Considerando a abundância de espécies de *Allophylus*, ela é, consequentemente, a que possui a maior relevância etnofarmacológica (JOLY, 2005; JUDD, 2008).

Diversas espécies de *Allophylus* são utilizadas na medicina popular (CHAVAN e GAIKWAD, 2013) e algumas com estudos químicos e efeitos farmacológicos evidenciados cientificamente (**Tabela 1**).

Tabela 1. Espécies de *Allophylus* e suas características etnofarmacológicas.

Espécie	Utilização popular (Parte/forma de uso)	Estudos farmacológicos	Estudos químico	Referência
A. africanus P. Beauv.	Dor de cabeça (F/V); malária (-/-); febre (F/D); conjuntivite (F/M); diarreia e vermicida (R e C/-); artrite (R, C e F/-); epilepsia e insanidade (F e R/D)	Antioxidante, antitumoral, anti-inflamatório e antimalárico	Terpenos, flavonoides, taninos, fitosteróis, alcaloides e glicosídeos cardioativos	IWU e ANYANWU, 1982; OLADOSU et al., 2013; BALOGUN et al., 2014; OLADOSU et al., 2015; CHAVAN E GAIKWAD, 2016; FERRERES et al., 2018; IBRAHIM et al., 2018; KINSOU et al., 2019; ZEUTSOP et al., 2022; RIBEIRO et al, 2023.
A. cobbe (L.) Raeusch.	Bandagem de fraturas ósseas (R e F/-); erupções cutâneas (F/S); diarreia (R/D); dor de estômago (F/M); e enxaguante bucal (F/-).	Antioxidante, antitumoral, antimicrobiano e citotóxico	Terpenos, ácidos fenólicos, cumarinas, fitosteróis, saponinas, ácidos graxos e alcanos	EDIRIWEERA e GRERUB, 2009; PUNEKAR e LAKSHMINARASIMHAN, 2011; BHAT et al. 2012; ISLAM et al., 2012; CHAVAN e GAIKWAD, 2013; CHAVAN e GAIKWAD, 2016; CHAVAN e GAIKWAD, 2017; GHAGANE et al., 2017; SANGSOPHA et al., 2019; THUSYANTHAN et al., 2022.
A. cominia (L.) Sw.	Diabetes (F/D); Resfriado comum, disenteria, dispepsia, hemoptise, dor de estômago, tétano, dor de dente, tuberculose, depurativo, perda de ferro e cólicas menstruais (-/-).	Antidiabético, inibidor da glicoproteína P (P-gp) e anti-inflamatório	Flavonoides, ácidos fenólicos, taninos, fitosteróis, antocianidinas e saponinas	RODRÍGUEZ et al., 2004; CHAVAN e GAIKWAD, 2016; OLIVA-HERNÁNDEZ et al., 2013; SANCHEZ et al., 2014; PÉREZ et al., 2017; SEMAAN et al., 2017; SEMAAN et a., 2018.
A. ferrugineus Taub.	Gases estomacais, tosse, lombrigas e febre (R/I).	-	-	CHAVAN e GAIKWAD, 2016.

A. occidentalis (Sw.) Radlk.	Resfriado comum, tétano, dor de dente, tuberculose e depurativo (-/-).	-	-	CHAVAN e GAIKWAD, 2016.
A. racemosus Sw.	Icterícia (R/-)	-	-	CHAVAN e GAIKWAD, 2016.
A. rubifolius (Hochst. ex A. Rich.) Engl.	Infecções de feridas (F/P); cicatrização (F/-); Furúnculos (-/-).	-	-	MARWAH et al., 2007; CHAVAN e GAIKWAD, 2016.
A. serratus (Roxb.) Kurz	Fraturas ósseas (F/-); Tônico doce, refrescante e nutritivo (FR/-).	Gastroprotetivo	Terpenos, saponinas, alcanos e ácidos graxos	DHARMANI, et al., 2005; CHAVAN e GAIKWAD, 2013; CHAVAN e GAIKWAD, 2016; CHAVAN e GAIKWAD, 2017.
A. timoriensis (DC.) Blume	Mal-estar (-/-).	Antitumoral	-	BRADACS et al., 2010; CHAVAN e GAIKWAD, 2016.
A. zeylanicus L.	Fraturas e deslocamentos (-/-).	-	-	CHAVAN e GAIKWAD, 2016.
A. laevigatus (Turcz.) Radlk.	-	-	Terpenos e flavonoides	DAVID et al., 2004.
A. longipes Radlk.	-	-	Cumarinas e fitosteróis	ZHANG et al., 2012.

(-): Não descrito.

Partes: - (Não descrito), R (Raízes), C (Cascas), F (Folhas), e FR (Frutos).

Formas de uso: - (Não descrito), M (Moído), S (Suco), D (Decocção), I (Infusão), P (Pasta feita com folhas), e V (Vapor inalado/fumigado).

2.6 *Allophylus edulis* **(A.St.-Hil., Cambess. & A. Juss.) Radlk.**

2.6.1. Aspectos botânicos e étnicos

A origem etimológica da palavra *Allophylus* remete ao termo grego *állos* (de outro) e *phylus* (nação), referindo-se a uma situação geográfica, pois o material da descrição original do gênero era procedente do Ceilão (atual Sri Lanka) e *edulis* remete a sua característica edível, já que os frutos desta espécie são apreciados como alimento tanto pelo homem quanto por animais silvestres (REITZ, 1980; MARCHIORI, 1995).

Allophylus edulis A.St.-Hil., Cambess. & A. Juss. Radlk. possui as sinonímias *Allophylus cambessedei* Blume, *Allophylus edulis* var. *gracilis* Radlk., *Schmidelis edulis* A. St.-Hil., Cambess. & A. Juss, *Allophylus guaraniticus* (A. St.-Hil.) Radlk., *Schmidelia edulis* A.St.-Hil., Cambess. & A.Juss., *Schmidelia guaranitica* Griseb, *Urvillea seriana* Griseb. e *Nassavia terminalis* Vell. (SANO et al., 2008; POWO, 2023; COELHO, 2023).

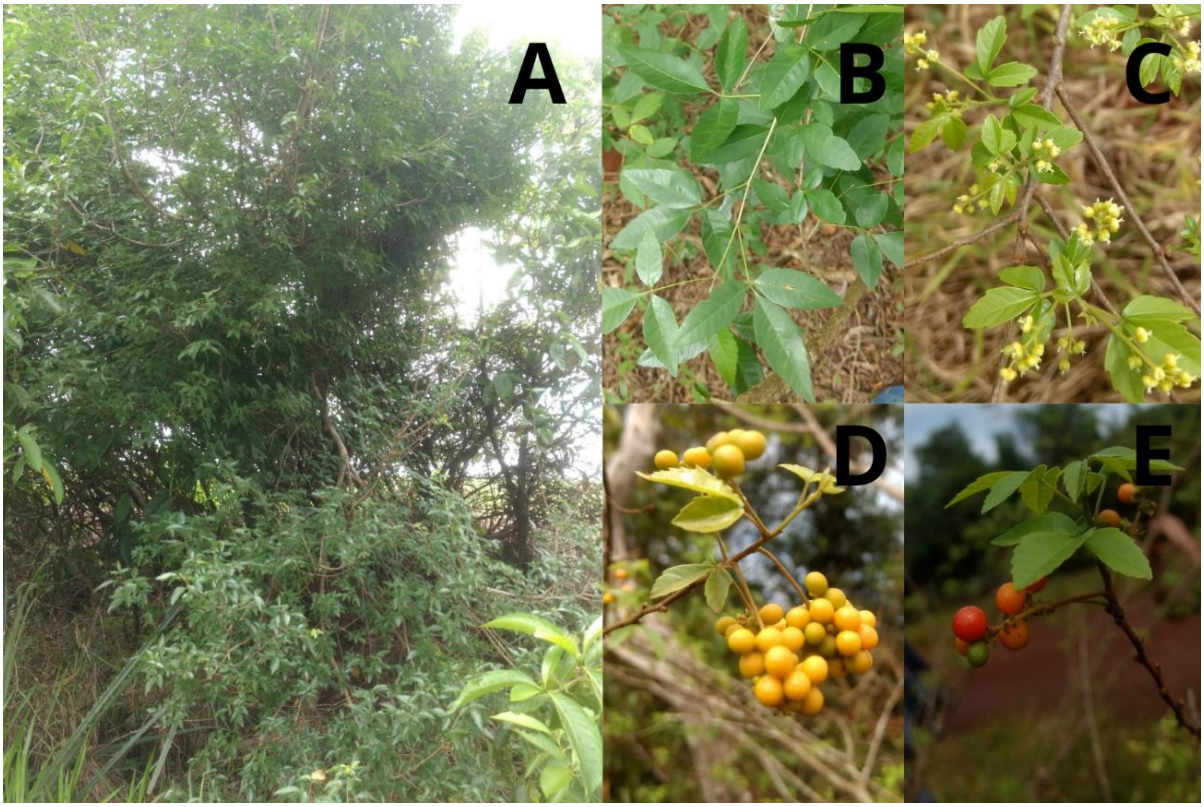
É uma espécie conhecida popularmente no Brasil pelos nomes de vacum, chal-chal, fruta-de-pombo, fruta-de-passarinho, olho-do-pombo, fruta-de-paraó, fruto-do-faraó, fruto-do-rei, grão-de-galo, três-folhas-do-mato, baga-de-morcego, pau-de-pedreira, cocu, murta-branca, vacunzeiro, murta-vermelha. Na argentina, por *frutilla*, *chalchal*, *albarillo*, *chanchal*, *cacú*, *caguy*, no Paraguai por *cochinillá*, *cochinillo*, *cocu* e por comunidades indígenas Guarani como *wakú*, *kokû* e *pykasu rembi'u* (ARISAWA et al., 1989; ABREU et al., 2005; KÖHLER et al., 2013; COELHO, 2023).

A distribuição geográfica dessa espécie compreende parte significativa da América do Sul, em países como as Guianas, Bolívia, Paraguai, Uruguai e Argentina (DÍAZ et al., 2014). No Brasil, a ocorrência se estende por áreas de Floresta Ombrófila Mista, Floresta Estacional Decidual e Semidecidual, e Cerradão; especialmente nos estados do Amazonas, Ceará, Bahia, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul (REITZ, 1980; DURIGAN et al., 2004; BACKES e IRGANG, 2004; LORENZI, 2016).

A espécie é descrita como árvore, de até 17m de altura, ramos cilíndricos, estriados, lenticelados, (**Figura 8A**). As folhas são trifolioladas e pecíolo subcilíndrico e os folíolos laterais menores que o central e cartáceos (**Figura 8B**). Inflorescências são axilares, não ramificadas, maiores ou menores que os pecíolos, menores que as folhas, raramente maiores. As flores possuem sépalas glabras em ambas as faces, e membranáceas. Pétalas são espatuladas, com ápice agudo a irregular (**Figura 8C**) (COELHO, 2014). A coloração do fruto varia de

acordo com o estágio de maturação, variando do verde-escuro e amarelo-laranja (**Figura 8D**) até o vermelho vivo, quando totalmente maduro (**Figura 8E**) (ABREU, 2005).

Figura 8. Imagem de *Allophylus edulis* (A), suas folhas (B), flores (C) e frutos (D e E).



Fonte: Elaborada pelo autor, 2023.

Em relação ao uso medicinal de *A. edulis*, Kujawska e Schmeda-hirschmann (2022) e Kujawska e Pieroni (2015) descrevem o uso das cascas do caule para icterícia; da fruta crua para aftas; e das folhas para limpeza do sangue, catapora, remédio frio contra superaquecimento do estômago e fígado, problema de digestão, azia, hepatite, hipertensão, ressaca, diurético e profilaxia, em preparados que englobam desde a maceração à frio, infusão até a decocção. Enquanto Arisawa et al., (1989) descreve o uso da planta sem processamento (*crude drug*) para o tratamento de problemas hepáticos, digestivos e colecistite, além de fazer referência ao uso do suco das folhas (maceração à frio) em casos de icterícia. O chá das folhas é utilizado em inflamações da garganta, febre, diabetes, problemas intestinais, diarreia e problemas digestivos, enquanto o decocto é utilizada para lavar feridas, contra a pressão alta (KÖRBES, 1995;

FRANCO e FONTANA, 2001; ALVES et al., 2008) e estimulante da secreção da bile para o duodeno (colagogo) (GRANDTNER e CHEVRETTE, 2013).

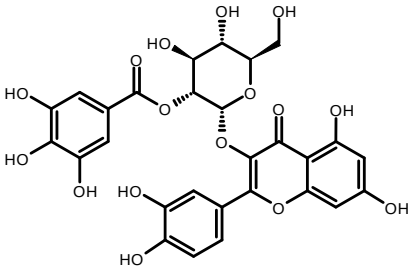
Kinupp et al. (2007) cita que existe aceitabilidade na utilização dos frutos como fruta de mesa, no entanto, pela perecibilidade, seu uso para a fabricação de licores, sucos e polpa congelada são mais indicados. Os frutos quando submetidos ao processo de fermentação produzem uma bebida vinosa conhecida como *aloja de chachal*, *chicha* ou *aloja*, sendo preparado com milho e consumida por índios peruanos, argentinos e brasileiros (REITZ et al., 1988; ABREU et al., 2005; CHEBEZ e MASARICHE, 2010).

Também há relato do uso das folhas desta espécie como refrescante, na forma de infusão fria conhecido como tereré, composta pelo ingrediente principal, de folhas de erva-mate (*Ilex paraguaiensis* St. Hil., da família Aquifoliaceae), acompanhado de capim-limão (*Cymbopogon citratus* (DC.) Stapf., da família Poaceae) e erva-luisa (*Aloysia citriodora* Palau, da família Verbenaceae) utilizadas popularmente em casos de hipertensão, ressaca, profilaxia e refrescante. O consumo na forma de maceração aquosa também é descrito em comunidades que conservam costumes da tradição Guarani tanto na Argentina quanto no Paraguai, com a preparação composta por *A. edulis* em conjunto com folhas de pitanga (*Eugenia uniflora* L., da família Myrtaceae) e gervãozinho-do-campo (*Verbena litoralis* Kunth, da família Verbenaceae) como refrescante. Ou ainda de *A. edulis* em conjunto com lantana-de-montevidéu (*Verbena montevidensis* Spreng., da família Verbenaceae), gervãozinho-do-campo (*V. litoralis* Kunth., da família Verbenaceae), quebra-pedra (*Phyllanthus niruri* L., da família Phyllanthaceae) e perpétua (*Gomphrena celosioides* Mart., da família Amaranthacea) em casos de problemas de digestão e ressaca (KUJAWSKA e PARDO-DE-SANTAYANA, 2015; KUJAWSKA, 2018; KUJAWSKA e SCHMEDA-HIRSCHMANN, 2022).

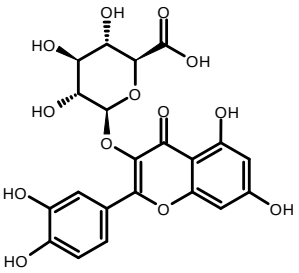
2.6.2 Composição química

O estudo químico das folhas (extratos alcoólicos), como ilustrado na **Figura 9**, reportam a presença de flavonoides (1-18), um ácido fenólico (19), isocumarinas (20-21), sesquiterpenos (22, 24), um diterpeno (28), um triterpeno (29), fitosterois (30-31), um açúcar (32) e uma glioxildiureida (33) (ARISAWA et al. 1989; HOFFMANN-BOHM et al. 1992; DÍAZ et al. 2008; DÍAZ et al., 2014). Arruda et al. (2018) também descreveu qualitativamente a presença de saponinas e flavonoides.

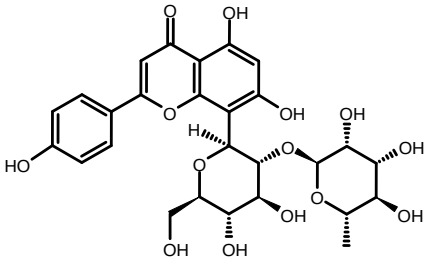
Figura 9. Estruturas químicas de substâncias isoladas de *A. edulis*.



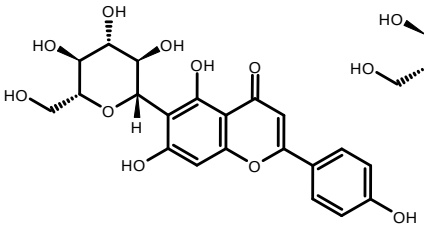
(1) Quercetina 3-*O*-(2”-*O*-galoil)-glicosídeo



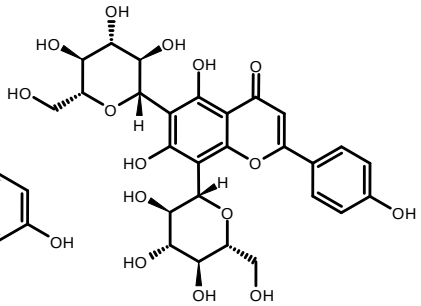
(2) Quercetina 3-*O*-β-D-glicuronídeo



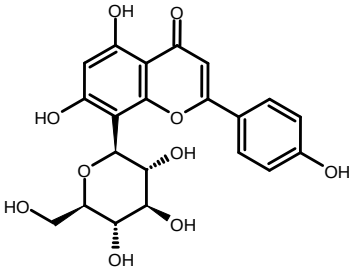
(3) Vitexina 2”-*O*-α-L-ramnosídeo



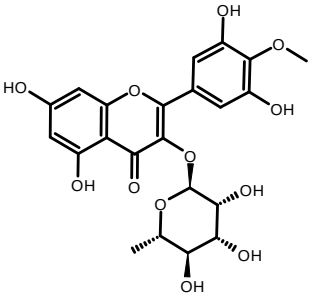
(4) Isovitexina



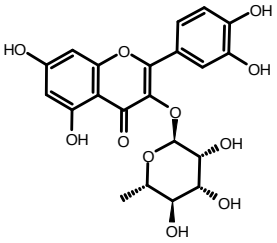
(5) Vicenina 2



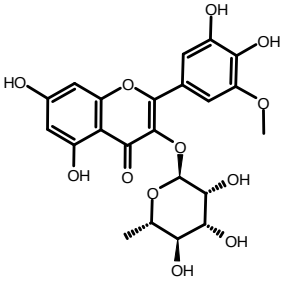
(6) Vitexina



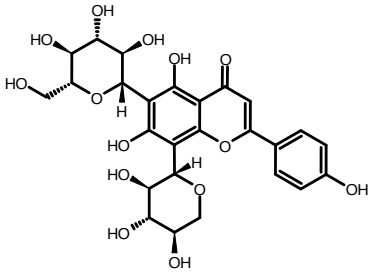
(7) Mearnsitrina



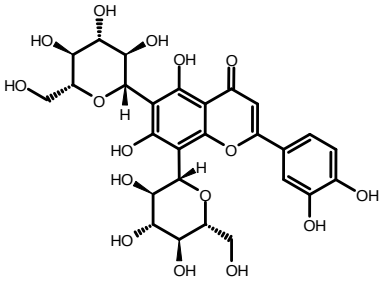
(8) Quercitrina



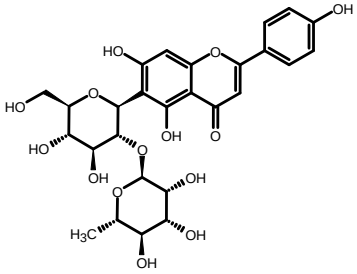
(9) Laricitrina 3-ramnosídeo



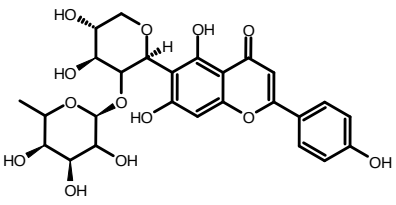
(10) Schaftosídeo



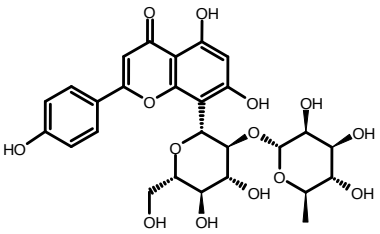
(11) Lucenina-2



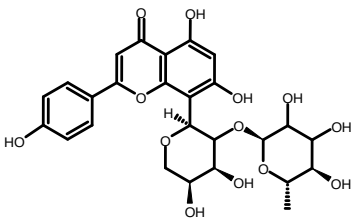
(12) Isovitexina 2-*O*-ramnosídeo



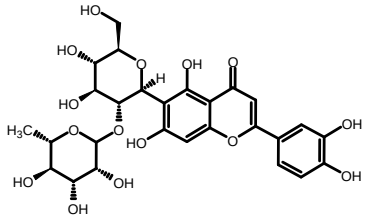
(13) Cerarvensina 2-*O*-ramnosídeo



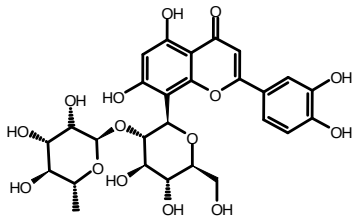
(14) Vitexina 2”-*O*-ramnosídeo



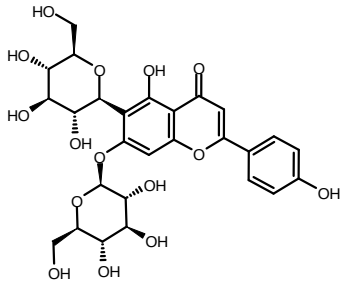
(15) Mollupentina 2''-*O*-ramnosídeo



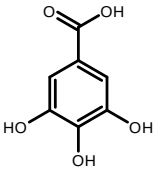
(16) Isoorientina 2''-*O*-ramnosídeo



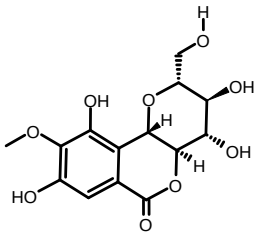
(17) Orientina 2''-*O*-ramnosídeo



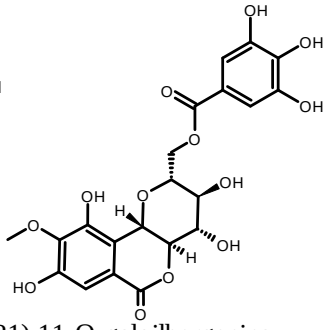
(18) Saponarina



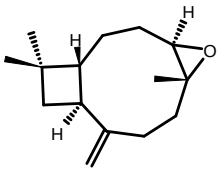
(19) Ácido gálico



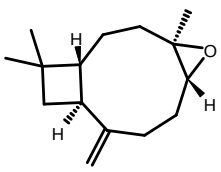
(20) Bergenina



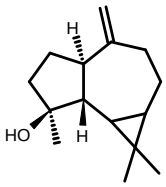
(21) 11-O-galoilbergenina



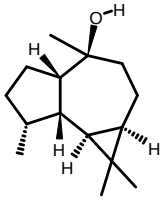
(22) 6,7-Epoxicariofileno



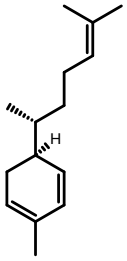
(23) Óxido de cariofileno



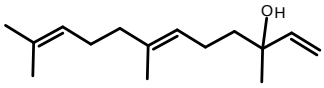
(24) Espatulenol



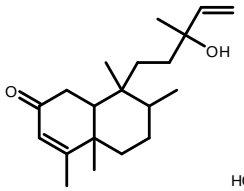
(25) Viridiflorol



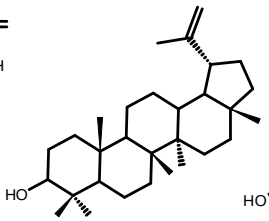
(26) α-Zingibereno



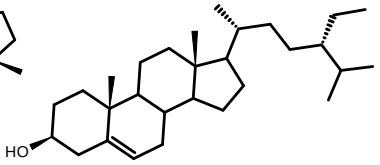
(27) E-Nerolidol



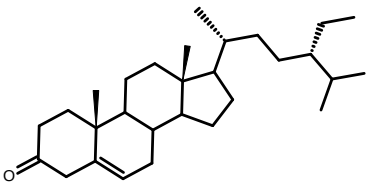
(28) Roseostaquenona



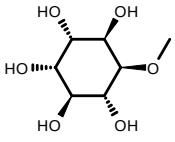
(29) Lupeol



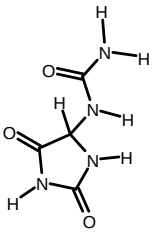
(30) Sitosterol



(31) Sitosterona



(32) L-quebrachitol



(33) D,L-Alantoína

Fonte: Elaborada pelo autor, 2023.

Em relação ao óleo essencial obtido das folhas de *A. edulis*, nosso grupo de pesquisa descreveu a presença de 41 substâncias, com predominância do sesquiterpeno viridiflorol (30,88%, **Figura 9**, 25) de amostras coletadas em Dourados/MS em março de 2015 (TREVIZAN et al., 2016). De forma complementar, Santos et al. (2021) também evidenciou a presença majoritária de sesquiterpenos nas amostras coletadas na cidade de Dourados e Bonito/MS, em julho de 2018, com predominância do óxido de cariofileno (29,5%, **Figura 9**, 23), em Dourados e α-zingibereno (45%, **Figura 9**, 26), em Bonito. O espécime coletado por Piekarski-Barchik et al. (2021) em Almirante Tamandaré/PR, apresentou 18 substâncias, sendo a mais abundante o sesquiterpeno E-nerolidol (59,44%, **Figura 9**, 27).

A utilização de *A. edulis*, bem como os componentes químicos de seus extratos têm sido direcionados para uma grande variedade de funções, que vão desde a busca por soluções sustentáveis na agricultura, até a busca por alternativas farmacológicas no tratamento de diversas condições patológicas.

2.6.3 Estudos biológicos

Produtos naturais são alternativas seguras para práticas agrícolas sem produtos químicos sintéticos, conhecida como agricultura sustentável. O interesse em compostos bioativos de plantas (biopesticidas) surge de sua eficácia no controle de pragas agrícolas, custo reduzido, biodegradabilidade, fácil disponibilidade e baixa toxicidade para organismos não-alvos (GODLEWSKA et al., 2021). No contexto da busca por pesticidas naturais e ecológicos, *A. edulis* foi estudada. O extrato etanólico dos galhos demonstrou ação repelente contra o pulgão-verde-do-pessegueiro (*Myzus persicae*) e joaninha (*Epilachna paenulata*) (CASTILLO et al., 2009). Além disso, ao utilizar o extrato etanólico e frações, observou-se atividade contra o pulgão-da-aveia (*Rhopalosiphum padi*) e a lagarta-do-algodão (*Spodoptera littoralis*) (DÍAZ et al., 2014). Neste último estudo, verificou-se que o extrato foi mais efetivo contra pulgões, enquanto as frações foram mais efetivas contra insetos mastigadores, indicando um possível sinergismo dos compostos do extrato na ação contra pulgões.

2.6.4 Estudos farmacológicos

A aplicação farmacológica dos extratos e frações de *A. edulis* estão descritas no desenvolvimento de estudos na pesquisa pré-clínica, desde ensaios primários e fundamentais envolvendo atividade antioxidante e antimicrobiana até a aplicação em condições patológicas mais complexas, como nos sistemas cardíaco, renal e hepático. Os estudos mais difundidos para

esta espécie são aqueles utilizando extrações alcoólicas (etanólicas e metanólicas), aquosas e hidrodestilação (óleos essenciais) de diversas partes da planta.

Com relação ao extrato etanólico, estudos utilizando as folhas relatam ausência de atividade antimicrobiana (300 mg/ml), frente à *Escherichia coli* e *Candida albicans*, e efeito bactericida frente à *S. aureus* (TIRLONI et al., 2015). Assim como atividade antioxidante in vitro pela eliminação de radicais livres (DPPH, IC_{50} 17,4 µg/ml), diminuição da peroxidação lipídica em eritrócitos (até 77%), e de proteção contra hemólise oxidativa de eritrócitos (TIRLONI et al., 2015). As folhas também foram responsáveis pela atividade inibidora da enzima conversora de angiotensina, de 34% (100 µg/ml) para o extrato e 53% (100 µg/ml) para a fração n-butanólica (ARISAWA et al., 1989). Mais recentemente foi relatado efeito nefroprotetivo em modelo de nefrotoxicidade induzido por gentamicina (GALEANO et al., 2023).

Ao utilizarem o extrato etanólico dos frutos como substrato de análise, foi observado aumento da atividade antioxidante pelo ensaio de DPPH (IC_{50} 46,4 de µg/ml), e atividade anticolinesterásica moderada (a partir de 100 µg) por meio de ensaio bioautográfico em microplaca (UMEO et al., 2011).

Os extratos metanólicos também são uma alternativa muito explorada nos estudos envolvendo *A. edulis*. Com relatos que remontam à Hoffmann-Bohm et al. (1992), que demonstrou até 79% de proteção contra toxicidade de tetracloreto de carbono e galactosamina em cultura primária de hepatócitos de rato, tanto do extrato metanólico quanto dos flavonoides isoladas, de *A. edulis* var. *edulis* e *A. edulis* var. *gracilis*, evidenciando potencial hepatoprotetivo. E Matsunaga et al. (1997), que sem descrever a parte da planta, reportou potencial ionotrópico negativo in vitro da fração acetato de etila, n-butanol e aquosa (0,3 mg/ml) no átrio esquerdo isolado de Cobaia (*Cavia porcellus*), ao observar inibição da contratilidade do átrio em 100% (fração acetato de etila), 79% (fração n-butanólica) e 83% (fração aquosa), baseado na resposta máxima.

Estudos envolvendo o extrato metanólico dos frutos, reportam apenas atividade antioxidante, utilizando o mesmo método de DPPH, com proteção máxima de 33% (100 µg/ml), e inibição menor que 50% (50 µg/ml) na inibição do ânion superóxido e xantina oxidase (SCHMEDA-HIRSCHMANN et al., 2005).

Ainda que as descrições etnofarmacológicas retratem o uso de preparações aquosas utilizando partes diversas de *A. edulis*, esta segue sendo uma das formas menos exploradas desta espécie, com relatos limitando-se à avaliação antimicrobiana e antioxidante. Com relação à

infusão das folhas, Arruda et al. (2018) relatou ausência de atividade inibitória pelo teste de disco difusão em ágar frente à *E. coli*, *S. aureus* e *C. albicans* em nenhuma das concentrações testadas (12,5-200 mg/ml). Ao utilizar o extrato aquoso obtido por maceração das folhas à frio (4°C), efeitos similares foram observados, com ausência de inibição do crescimento de *E. coli* e *C. albicans* (300 mg/ml), ainda que houvesse efeito bacteriostático contra *S. aureus* (TIRLONI et al., 2015). Nesse mesmo estudo, Tirloni et al. (2015) também reportou atividade antioxidante in vitro pela eliminação de radicais livres (DPPH, IC_{50} 45,8 µg/ml). No trabalho desenvolvido nesta tese, pretendemos utilizar a infusão das folhas não apenas pelo seu uso tradicional, mas também como uma maneira de reduzir o emprego de solventes químicos. A química verde tem crescido em resposta às questões ambientais e de segurança (SAHOO e BANIK, 2020). Com essa abordagem, almejamos minimizar o impacto ambiental decorrente da utilização de solventes, diminuindo a produção de subprodutos e resíduos. Ao mesmo tempo, buscamos aumentar a segurança e a saúde dos envolvidos na pesquisa, contribuindo para o desenvolvimento de estudos mais sustentáveis na área de farmacognosia.

Em relação ao óleo essencial obtido das folhas de *A. edulis*, nosso grupo de pesquisa tem explorado de forma extensiva, especialmente por seus efeitos em modelos de inflamação e hiperalgesia. De forma que os primeiros trabalhos remontam às amostras coletas no Cerrado sul-mato-grossense. Trevizan et al. (2016) realizou a avaliação em modelos animais utilizando carragenina como agente flogístico nos modelos de edema de pata e pleurisia tanto do óleo essencial quanto do composto majoritário, viridiflorol. Foi observado que, tanto o óleo essencial quanto o viridiflorol inibiram significativamente a pleurisia induzida por carragenina, reduzindo a migração de leucócitos totais em camundongos em 62% (30 mg/kg de óleo), 35% (100 mg/kg de óleo), 71% (3mg/kg de viridiflorol) e 57% (30 mg/kg de viridiflorol). Em adicional, este estudo descreve o efeito antioxidante pelos métodos de DPPH (IC_{50} 82,9 µg/ml para o óleo e 74,7 µg/ml para o viridiflorol) e ABTS (IC_{50} 44,3 µg/ml para o óleo, 57,5 µg/ml para o viridiflorol), bem como atividade antimicobacteriana contra *Mycobacterium tuberculosis* (Concentração Inibitória Mínima (CIM) de 157,5 mg/ml para o óleo essencial e 190,0 mg/ml para o viridiflorol). De forma complementar, Piekarski-Barchik et al. (2021) descreveu a atividade antioxidante nos testes de DPPH (105,6 mmol equivalentes de Trolox (ET) por 100 g de óleo essencial), ABTS (29,1 mmol de ET/100g) e FRAP (76.8 mmol de ET/100g) para o óleo essencial.

Da mesma maneira, Santos et al. (2021) e Balsalobre et al. (2023) encontraram efeitos anti-inflamatórios similares de perfis químicos distintos do óleo essencial de *A. edulis*.

Enquanto Santos et al. (2021) observou a diminuição do edema agudo de pata induzido por carragenina (inibição máxima de 89% nas doses de 30 e 100 mg/kg das amostras de óleo, e nas doses de 30 mg/kg de óxido de cariofileno e 40 mg/kg de α-zingibereno) quanto edema prolongado (até 12 dias), induzido por CFA (inibição máxima de ~50%). Neste caso os tratamentos também foram capazes de diminuir a hiperalgesia mecânica e térmica (alodinia ao frio). Balsalobre et al. (2023) descreveu a exposição oral às doses de 30, 100 e 300 mg/kg do óleo essencial e 30, 100 e 200 mg/kg de viridiflorol no modelo de nocicepção e edema induzido por formalina, com resultados de inibição máxima de 70% das manifestações de dor e 69% do edema de pata, ambos para a dose de 300 mg/kg do óleo essencial. Enquanto o viridiflorol inibiu as manifestações de dor em no máximo 70% e o edema em, no máximo 66%, ambos também na dose máxima testada, 200 mg/kg. A dose de 200 mg/kg continuou a mais efetiva ao utilizar zimosam para induzir inflamação articular, de forma que foi possível observar inibição, após 4 horas, de 63% da hiperalgesia mecânica, e 79% da formação de edema para o óleo essencial e 59% da hiperalgesia mecânica e 78% do edema para o viridiflorol. Ao final de 6 horas, ambos os tratamentos foram capazes de inibir até 69% de leucócitos totais e até 64% dos polimorfonucleares. Nesta mesma linha, foi observado diminuição da produção de óxido nítrico (máximo 86%) e extravasamento de proteínas (até 74%). A administração intraplantar de viridiflorol (300 μg/pata) também foi capaz de inibir a hiperalgesia mecânica (até 71%), térmica (até 88%) e edema de pata (até 81%) após 4h da injeção. Quando houve injeção intraplantar de viridiflorol (300 μg/pata) concomitante com TNF-α (100 pg/pata) ou dopamina (30 μg/pata), o viridiflorol foi capaz de inibir 72% e 53% da hiperalgesia mecânica, respectivamente, após 4 horas. Três horas após as aplicações, foi observada inibição de 31% (pata com TNF-α) e 83% (pata com dopamina) da formação do edema.

2.6.5 Estudos de toxicidade

Plantas medicinais, embora geralmente consideradas seguras, não estão isentas de efeitos secundários ou toxicidade. Portanto, a avaliação dos parâmetros de toxicidade é essencial para garantir a segurança de uma planta cujos efeitos biológicos e farmacológicos são conhecidos (JITĂREANU et al., 2023). Neste sentido, o teste de toxicidade aguda utilizando larvas de *Artemia salina* apresentou baixa toxicidade na concentração de 200 mg/ml, quando utilizado o extrato aquoso das folhas (ARRUDA et al., 2018) e CL_{50} maior que 1000 μg/ml com extrato etanólico da polpa dos frutos (UMEO et al., 2011). A atividade antiproliferativa e genotóxica de extratos aquosos de folhas através do sistema teste de *Allium cepa* também foi

descrita, sendo observado atividade genotóxica apenas na concentração de 4 g/l (YAJÍA et al., 1999; PASQUALLI et al., 2015). Quanto ao estudo de toxicidade aguda em roedores (*Rattus norvegicus* - Wistar), a DL_{50} do extrato etanólico das folhas foi descrita como maior que 5 g/kg, pois demonstrou baixa toxicidade, considerando apenas o aumento no peso do fígado na maior dose testada (5 g/kg), sem quaisquer outras alterações relatadas (TIRLONI et al., 2015).

3 OBJETIVOS

GERAL

Investigar as propriedades químicas e farmacológicas das folhas de *A. edulis*, visando compreender sua composição química e seu potencial terapêutico em modelos de inflamação, ansiedade e declínio cognitivo, bem como a toxicidade oral em camundongos.

ESPECÍFICOS

ARTIGO 1

Avaliar a composição química do óleo essencial das folhas de *A. edulis* coletadas em Dourados/MS e Bonito/MS ao longo de quatro estações.

ARTIGO 2

Submeter as folhas a cortes histológicos, identificação das estruturas secretoras e avaliação histoquímica das secreções.

Preparar a infusão das folhas de *A. edulis* (ILAE) e fracionar as frações hexânica (Hf), acetato de etila (EAf) e hidrometanólica (HMf).

Quantificar os teores de compostos fenólicos totais, flavonoides, flavonóis e taninos condensados de ILAE, EAf e HMf.

Submeter a vitexina 2"-O-ramnosídeo à predição in silico de similaridade farmacológica (parâmetros de Lipinski), alvos farmacodinâmicos e toxicidade oral em roedores.

Avaliar a atividade antioxidante de ILAE, EAf e HMf por métodos de sequestro de radicais livres (DPPH e ABTS) e inibição da lipoperoxidação lipídica do β-caroteno.

Avaliar o efeito da administração oral de ILAE (3, 30 e 100 mg/kg) e HMf (3 mg/kg) em modelos de inflamação aguda (edema de pata e pleurisia induzidos por carragenina), hiperalgesia mecânica, alodinia térmica e nocicepção (induzido por formalina).

Avaliar o efeito da administração oral de ILAE (30 mg/kg), HMf (3 e 30 mg/kg) e AE-1 (3 mg/kg) em modelo de inflamação prolongada (edema de pata induzido por CFA), hiperalgesia mecânica e alodinia térmica.

ARTIGO 3

Avaliar o efeito da administração oral de ILAE (3, 30 e 100 mg/kg) e HMf (3 mg/kg) em modelos de ansiedade, incluindo o teste de campo aberto e claro/escuro.

Avaliar o efeito da administração oral de ILAE (3, 30 e 100 mg/kg) e HMf (3 mg/kg) em modelo de declínio cognitivo agudo e prolongado, utilizando o teste de reconhecimento de objetos e o teste do labirinto aquático de Morris, ambos induzidos por escopolamina.

Utilizar a estrutura cerebral dos modelos animais para avaliar a inibição da peroxidação lipídica e da atividade da AChE.

Realizar a redocagem molecular da vitexina 2"-O-ramnosídeo na AChE.

Avaliar a toxicidade oral em camundongos do tratamento de 28 dias com ILAE (30, 100 e 300 mg/kg).

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5 APÊNDICES

5.1 Artigo I: Seasonal and geographical variation in the chemical composition of essential oil from *Allophylus edulis* leaves

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Seasonal and geographical variation in the chemical composition of essential oil from *Allophylus edulis* leaves

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ABSTRACT

Allophylus edulis (Sapindaceae), also known as "chal-chal", "vacuum" or "cocu", is a tree widely found in Brazil whose leaves are still used in folk medicine and are rich in essential oil. The focus of this research was to investigate the chemical composition profiles of the essential oil from *A. edulis* leaves collected seasonally (the four seasons) in two cities. The *A. edulis* leaves were collected in winter (July) and spring (November) 2018 and summer (January) and autumn (May) 2019, in the cities of Bonito and Dourados, in the state of Mato Grosso do Sul, Brazil. The essential oils were extracted by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC–MS). The essential oil yield in this seasonal variation study consistently ranged between 0.07% and 0.6% (wet wt.) for all samples, increasing considerably with higher temperature and during the inflorescence stage. In the oil samples obtained from Dourados, the major components present were α-pinene, caryophyllene oxide, and viridiflorol with yields of 3.04–29.81% across all four seasons, being caryophyllene oxide the most abundant (20.1–29.81%). The major compound identified in the oil samples obtained from Bonito was α-zingiberene for all four seasons, though its concentrations were highest in summer (46.90%) and spring (45.05%). The chemical composition profiles of both cities' oil samples were similar, in that they shared four sesquiterpene compounds, caryophyllene oxide, germacrene D, E-caryophyllene and viridiflorol. The study highlighted that both seasonal and geographical variation can influence the chemical composition of essential oil from *A. edulis*.

1. Introduction

Allophylus edulis (A.St.-Hil., A. Juss. & Cambess.) Radlk., in the family Sapindaceae, popularly known as "chal-chal", "vacuum" or "cocu", is a tree (6–10 m in height) with flowering from September to November and fruiting between November and December (Arisawa et al., 1989; Körbes, 1995; Lorenzi, 1992). It is widely distributed in the Brazilian native flora as well as in Bolivia, Argentina, Guayanas and Uruguayan, with climates ranging from equatorial in the north and subtropical in the center-south (Díaz et al., 2014; Reitz, 1980). In Brazil, there is a description of its presence in all biomes, with the highest incidence in the Atlantic Forest, Pampa, and Cerrado by areas of Mixed Ombrophylous Forest, Deciduous and Semideciduous Seasonal Forest, and predominance in Latosols, Ultisols and Neosols (Durigan et al., 2004; Backes and Irgang, 2004; Lorenzi, 2016). Considering the diversity of biomes and environmental characteristics present in the different places where the plant is described, the phenology can change, flowering in February or July to November and fruiting from February to March, according to Fortunato and Quirino (2016).

In folk medicine, the leaves (cold maceration with water, infusion, and decoction) are used in the treatment of gastrointestinal disorders or as an anti-inflammatory (Körbes, 1995; Kujawska and Schmeda-Hirschmann, 2022). Biological effects for *A. edulis* with extracts obtained from leaves have reported, such as antioxidant (Tirloni et al., 2015; Piekarski-Barchik et al., 2021), antimicrobial (Tirloni et al., 2015), hepatoprotective (Hoffmann-Bohm et al., 1992), negative ionotropic potential (Matsunaga et al., 1997), anticholinesterase activity (Umeo et al., 2011) and angiotensin-converting enzyme inhibitory activity (Arisawa et al., 1989). Chemical studies of this species with extracts reported the presence of cyanolipids and triacylglycerols

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(Aichholz et al., 1997), flavonoids and coumarin (Arisawa et al., 1989), sesquiterpenes and phytosterols (Díaz et al., 2014), and L-quebrachitol (Díaz et al., 2008).

Investigations by our research group show that the essential oil of *A. edulis* leaves collected in March 2015, in Dourados, Mato Grosso do Sul State, Brazil, was 6.5% for which high concentrations of sesquiterpenes, with viridiflorol the main constituent found (30.88%) exhibited biological activities, such as anti-mycobacterial, anti-inflammatory and antioxidant activity (Trevizan et al., 2016). Later, a re-investigation of the plant's essential oil composition was done with leaves collected in July 2018, in Dourados and Bonito, which also reported sesquiterpenes as the major constituent; however, difference in chemical composition was noticed, highlighting caryophyllene oxide (29.5%) in Dourados, and α-zingiberene (45.0%) in Bonito, with viridiflorol present at a low concentration of 2.9% (Santos et al., 2021). This divergent composition could be due to several factors, such as environmental differences and the plant's growth stage. Nevertheless, both studies also reported biological activities, including anti-inflammatory activity.

Essentials oils are complex mixtures of several volatile secondary metabolites from plants, and it is known that intrinsic and extrinsic factors can change this secondary metabolism and, consequently, significantly alter the variation in chemical composition throughout the seasons of the year (Yang et al., 2018). Consequently, this temporal variation may, in part, influence the biological activity of the plant. Therefore, identifying how the combination of these factors might influence the chemical composition of an essential oil may be relevant not only for phytochemical prospecting of *A. edulis* but also for providing support to explore the active agents responsible for this plant's pharmacological action in future studies.

Here we report on the seasonal and geographical variation in chemical composition of the essential oil of *A. edulis* (leaves). This species was selected because it is widespread in the Brazil and still used in the practice of local traditional medicine.

2. Material and methods

2.1. Plant material and extraction of essential oil

Allophylus edulis leaves were collected in winter (July) and spring (November) 2018 and summer (January) and autumn (May) 2019, in two locations in the state of Mato Grosso do Sul, Brazil: Bonito (21° 15′56″S, 56°42′10″W) and Dourados (22°08′23″S, 55°08′16″W). The taxonomic identity of the plant was certified at the Herbarium of the Federal University of Grande Dourados (City of de Bonito; 6342 and City of Dourados, 6343). Access to the plant's samples was carried out in accordance with the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen-A51F665). The samples (400 g of fresh leaves) were subjected to hydrodistillation in a Clevenger-type apparatus for approximately 4 h, trapping the essential oil in n-hexane. The oil was dried over anhydrous sodium sulfate and stored at 4 °C until analyzed. The essential oil yields were expressed as weight of oil/weight of plant material.

2.2. Gas chromatography-mass spectrometry (GC/MS)

The analysis was performed using a gas chromatograph equipped with a mass spectrometer (GC/MS-QP2010 Ultra, Shimadzu, Kyoto, Japan). A DB-5 column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness) was used, with helium (99.999% purity) as the carrier gas at a flow rate of 1.0 mL min^{-1} and an injection volume of 1 μL (in the split mode, 1:10). The starting oven temperature was 50 °C, then heated to 280 °C at a rate of 3 °C min^{-1}. The injector temperature was set to 220 °C, and the temperature of the transfer line and the quadrupole detector was 280 °C. The MS scan parameters included an electron impact ionization voltage at 70 V, a mass range from 50 to 600 Da, and a scan interval of 0.3 s. The retention index was calculated using a mixture of linear alkanes (C8−C40) as an external reference. Compound identification was achieved by comparing the mass spectra of the samples with the spectra available in the NIST21 and WILEY229 libraries, as well referring to data found in published studies (Adams, 2007).

3. Results and discussion

To study how the collection period (time) and geography (space) influenced the chemical composition of the essential oil from *A. edulis* leaves, these were collected during the four yearly seasons (at 8 AM) at two locations - Bonito and Dourados, in the state of Mato Grosso do Sul, Brazil.

We reported that the essential oil yield consistently ranged between 0.07% and 0.6% (w/w) for all seasonal samples in both cities (Fig. 1). The yield was affected by seasonal changes. The highest amount of the essential oil in the *A. edulis* was found during springer (0.6%) which decreased in autumn to 0.07%. In Dourados and Bonito, springer and summer is quite hot with an average temperature 32−38 °C, that in part, also resulted in the highest essential oil content consisted of those months that received the highest light intensity (Fig. 1). As is known, increasing average monthly temperatures create an effect of temperature stress on aromatic plants and cause an increase in essential oil content. In springer (November) the plants were at the inflorescence stage, which may have favored the synthesis of essential oil, which differed from the autumn (May) sample (Fig. 1). This stage remarkably increased oil yield, to attract pollinators such as bees and other insects.

The variability of the chemical composition revealed over 20 organic volatile compounds, at relative concentrations of 1.00% to 29.81%, with a predominance of sesquiterpenes found as well as smaller amounts of non-oxygenated monoterpenes in both oil samples. These results agree with the literature on the chemical composition of essential oils from *A. edulis*, which is known to feature a predominance of sesquiterpenes (Trevizan et al., 2016; Santos et al., 2021). Other remaining constituents were present at concentrations of less than 0.1%. The main substances present in the essential oil of *A. edulis* leaves in the different seasons at the two different localities are shown in Table 1. This could be explained the synthesis stages and plant needs. Sesquiterpenes are larger, denser and less volatile molecules than the monoterpenes, which often have protective functions.

The chemical composition of the essential oil samples obtained from the *A. edulis* leaves varied according to the place of collection and across seasons. Those samples obtained from Dourados were distinguished by α-pinene, caryophyllene oxide and viridiflorol as major

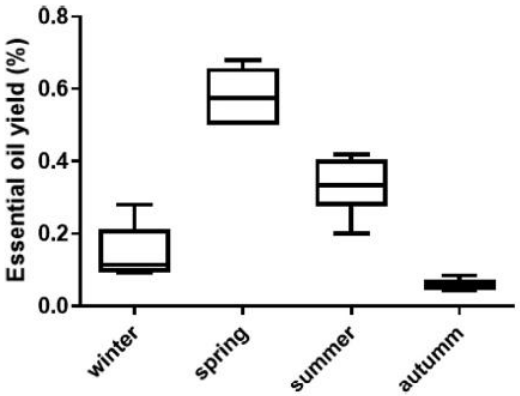

Fig. 1. Seasonal variation in the yield of essential oil from *A. edulis* leaves.

Table 1
Majority substances present in the essential oil of A. edulis leaves collected in the cities of Dourados and Bonito, MS, Brazil.

	Retention indexes			Essential oil (Dourados) (%)				Essential oil (Bonito) (%)			
Compound	Retention time	Determined	Literature	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Monoterpenes											
α-Pinene	7.001	938	932	3.04	22.29	11.12	10.01	0.2	0.9	–	–
Camphene	7.441	950	946	0.13	0.33	0.58	0.39	–	0.03	–	–
Thuja-2-4(10)-diene	7.623	955	953	0.07	0.21	0.29	0.47	–	–	–	–
Myrcene	8.885	992	988	–	0.34	0.19	–	–	–	–	–
σ-Cymene	10.115	1022	1022	–	0.26	0.2	–	–	–	–	–
Limonene	10.130	1026	1024	0.12	–	0.37	0.29	–	0.02	–	–
Sylvestrene	10.285	1026	1025	–	0.66	–	–	–	–	–	–
α-Campholenal	14.092	1121	1122	0.28	0.22	0.21	–	–	–	–	–
Trans-Pinocarveol	14.636	1135	1135	0.4	0.34	0.32	–	–	–	–	–
Cis-Verbenol	14.763	1138	1137	0.54	0.4	0.44	–	–	–	–	–
Trans-Verbenol	15.194	1143	1140	2.49	1.68	1.69	2.77	–	–	–	–
p-Mentha-1,5-dien-8-ol	15.304	1146	1166	1.18	1.12	1.1	1.53	–	–	–	–
Myrtenal	17.142	1194	1195	0.28	0.32	0.44	–	–	–	–	–
Myrtenol	17.194	1196	1194	0.24	0.16	–	0.32	–	–	–	–
Verbenone	17.633	1208	1202	0.66	0.46	0.64	0.76	–	–	–	–
Sesquiterpenes											
δ-Elemene	23.436	1337	1335	–	–	–	–	0.35	0.35	0.14	–
α-Copaene	25.185	1375	1374	1.65	1.85	–	3.12	0.36	–	–	0.17
β-Elemene	25.571	1389	1389	0.25	0.11	0.19	–	8.26	9.89	9.26	8.58
(E)-Caryophyllene	27.056	1420	1417	3.58	5.44	5.39	2.84	2.9	6.86	5.98	4.20
α-trans-Bergamotene	27.713	1436	1432	–	–	–	–	1.92	1.02	1.31	1.58
α-Humulene	28.397	1453	1452	1.03	1.82	1.4	1.0	–	0.73	0.4	0.93
9-epi-(E)-Caryophyllene	28.688	1461	1464	–	–	–	–	–	–	0.39	–
γ-Muurolene	29.336	1477	1478	0.29	–	0.21	0.25	–	–	–	12.83
ar-Curcumene	29.403	1480	1479	–	–	–	–	–	2.78	1.57	3.08
Germacrene D	29.528	1484	1484	1.84	11.76	11.5	1.55	1.24	11.23	6.91	1.45
γ-Himachalene	29.597	1487	1481	–	4.05	1.93	1.35	–	0.66	0.49	0.58
cis-Eudesma-6,11-diene	29.747	1488	1489	1.49	–	–	–	0.15	–	–	–
α-Zingiberene	30.157	1497	1493	1.89	–	–	–	27.55	45.0	46.9	25.86
Bicyclogermacrene	30.218	1498	1500	2.43	2.64	4.1	5.09	–	–	–	–
α-Muurolene	30.224	1501	1500	0.64	0.67	0.67	0.53	–	0.61	0.44	0.55
(E,E)-α-Farnesene	30.545	1506	1505	–	–	–	–	5.94	6.76	7.18	6.02
Cubebol	30.676	1510	1514	–	–	–	1	–	–	–	1.07
δ-Amorphene	31.113	1521	1511	–	3.47	–	–	–	0.46	0.55	–
δ-Cadinene	31.291	1522	1522	3.22	–	4	2.64	–	–	–	–
β-Sesquiphellandrene	31.296	1525	1521	–	–	–	–	12.75	6.73	8.9	5.84
trans-Cadina-1,4-diene	31.430	1533	1533	0.18	0.04	0.06	0.12	0.05	0.07	–	–
α-Agarofuran	32.064	1547	1548	1.56	1.64	1.3	–	–	–	–	–
Germacrene B	32.369	1558	1559	2.38	–	–	1.85	0.19	0.47	0.22	0.1
(E)-Nerolidol	32.778	1564	1561	–	–	–	–	–	0.73	0.61	0.74
Spathulenol	33.149	1578	1577	–	–	3.13	2.5	0.54	0.51	0.17	1.53
Germacrene D-4-ol	33.200	1579	1574	–	2.32	–	–	–	–	0.02	–
Caryophyllene oxide	33.888	1583	1582	28.48	20.1	29.81	22.88	1.55	1.06	1.17	1.54
Viridiflorol	34.044	1592	1592	2.8	2.5	8.66	14.47	1.04	1.62	1.38	0.08
Globulol	34.145	1580	1590	–	–	2.92	1.88	–	1	1.12	1.42
Humulene epoxide II	34.655	1613	1608	3.69	3.07	3.3	2.12	–	–	–	–
10-epi-γ-Eudesmol	34.978	1622	1622	1.71	5.62	1.2	–	0.06	–	–	–
epi-α-Muurolol	35.535	1645	1640	–	3.6	2.77	1.57	–	–	–	–
epi-α-Cadinol	35.556	1626	1638	–	–	–	–	–	1.62	1.14	–
α-Muurolol	35.762	1647	1644	–	–	0.83	0.5	3.92	1.3	1.57	–
Agarospirol	35.845	1646	1646	3.41	–	–	–	–	–	–	–
4-α-hydroxy-Dihydri agarofuran	35.979	1650	1651	1.25	–	–	–	–	–	–	–
α-Cadinol	36.356	1656	1652	4.52	3.66	3.56	1.55	3.43	3.52	1.28	–
14-hydroxy-9-epi-(E)-Caryophyllene	36.806	1668	1668	–	0.84	1.48	–	–	–	–	–
Occidenol	36.983	1676	1676	3.66	–	–	–	–	–	–	–
11-αH—Himachal-4-en-1-β-ol	37.490	1689	1699	–	–	–	–	6.55	–	1.99	–

(-) not determined.

components, ranging from 3.04% to 29.81% for all four seasons, in which caryophyllene oxide was most prevalent (20.1–29.81%) (Table 1). Those three major compounds accounted for 34.32–54.99% of the total oil composition over the different seasons. There was no fluctuation in caryophyllene oxide levels during the analysis period. The presence of α-pinene was highest during the spring (22.29%), with a much lower level in winter (3.04%), where the viridiflorol content peaked in autumn (14.47%), with lower levels obtained in both winter and spring (2.8% and 2.5%) (Table 1).

More intriguingly, however, was the difference in oil composition. Considering the major compounds identified in the oil samples obtained from Bonito, α-zingiberene was dominant all year, reaching its highest concentrations in summer (46.90%) and spring (45.05%). This level of concentration varied temporally, with lower levels obtained for the winter (27.55%) and autumn (25.86%) collection periods (Table 1). The other compounds present, namely β-elemene and β-sesquiphellandrene, also had relatively moderate concentrations that ranged from 8.26% to 9.86% and 5.84% to 12.75%, respectively (Table 1).

The level of concentration of the compounds present in essential oils was distributed unevenly among seasons. It is known, that all terpenoids are synthesized by the condensation of repetitive 5-carbon

isoprene units, isopentenyl pyrophosphate (IPP), and its isomer, dimethylallyl-pyrophosphate (DMAPP) (Singh and Sharma, 2015). The formation of IPP can occur by two biosynthetic routes: the classical or mevalonate pathway (MVA), responsible for the formation of sesquiterpenes, which occurs preferentially in the cytosol and whose precursors are pyruvate and acetyl coenzyme A. The formation of sesquiterpenes begins with the condensation of two pyrophosphate isoprenoids (IPP and DMAP), forms the geranyl pyrophosphate, precursor of monoterpenes, and units are added of the IPP, generating farnesyl pyrophosphate. These structures are modified by enzymes (hydroxylases, dehydrogenases, reductases and glycosyl, methyl and acyl transferases), which together generate a number of different compounds, that can be generated from rearrangement and cyclization, as showed this study, in different collection period, showing a significant variability, when the concentration of the α-pinene, viridiflorol and α-zingiberene varied in relation the temperature. Under unfavorable conditions, whose combinations of substrate preference and folding, transient stabilization of carbocations and controlled suppression of carbocations will lead to the production of others terpenes (Verma and Shukla, 2015; Rudolf and Chang, 2020).

These variations may be partially correlated with some characteristics of the access sites. Bonito city (Serra da Bodoquena National Park), biome consists mainly of deciduous forest and semideciduous seasonal forest so that the territory comprises features such as the Caatinga, Cerrado, Chaco and Atlantic Forests (Cáceres et al., 2007; Uetanabaro et al., 2007). The climate is characterized as Aw (tropical climate with a dry season in winter), according to the Köppen classification (Chagas et al., 2009). In Dourados, the climate is considered a transition between tropical and subtropical, and Köppen's classification is Cwa (humid temperate climate with dry winter and hot summer) (Souza et al., 2017). Similarities can be observed in the soil characteristics of the two sites. Soil pH varies between slightly acidic and neutral and has low fertility. In the city of Dourados, the soil is classified as Red Latosol with higher levels of aluminum, magnesium, and lower levels of potassium and sodium, as well as the calcareous soil of Bonito, classified as Red Argisol. Although the concentration of these elements does not clarify their bioavailability, since the nutrients may be present in non-assimilable forms, the similarity between soil characteristics may be responsible for the specific similarities in the oil composition (Lourente et al., 2011; Silva et al., 2013; Souza et al., 2017).

The chemical composition profiles of both cities' oil samples were similar, in that they shared four sesquiterpene compounds, caryophyllene oxide (1.06–29.80%), germacrene D (1.45–11.76%), E-caryophyllene (2.9–6.86%) and viridiflorol (0.08–14.47%) (Table 1), which can be suggested in part as chemical markers of the species.

Although there is no information about the dynamic accumulation of sesquiterpenes in *A. edulis*, it is possible to define this compound class as being the most expressive in the species, at least among the studied samples here. This interpretation is supported by comparing our results to those of Trevizan et al. (2016), Santos et al. (2021), and Piekarski-Barchik et al. (2021), who respectively reported viridiflorol, caryophyllene oxide and α-zingiberene, and (E)-nerolidol, as the major compounds in the analyzed samples, in addition to having larger amounts sesquiterpenes compared to other terpenes. The same pattern was observed for the essential oil samples obtained from *A. africanus*, which showed caryophyllene oxide as one of the major substances (Balogun et al., 2014). To our best knowledge, the present study is the first to report seasonal and geographical effects upon the chemical composition of essential oil from *A. edulis* leaves.

The variation observed in the concentration of chemical constituents of the essential oils from *A. edulis* can be attributed, in part, to environmental conditions, mainly water availability and temperature, which can change the relative proportions of compound in the oil composition profile. In this study, the concentration of terpenoids in the oil samples appeared to generally increase at higher temperatures (i.e., in spring and summer), with some exceptions to this pattern. Many factors could influence the seasonal variation of the essential oil composition, *i.e.* thermoregulatory, as the essential oil hydrophobic compounds could increase during the hot periods to protect the plant from desiccation and luminosity fluctuation rates (Kamatou et al., 2008; de Amaral et al., 2015). In this study, results showed a progressive increase in essential oil yield with increasing temperature, showed a significant positive correlation between essential oil content and in the concentration of chemical constituents with temperature, respectively.

This is in agreement, when a correlation was performed with the data obtained from the Weather and Climate Monitoring Center of the state of Mato Grosso do Sul (CEMTEC/SEMAGRO) (http://www.cemtec.ms.gov.br/) by Dourados and Bonito city, during spring (September to December) and summer (January to March) higher insolation (incidence of UV radiation), temperatures and precipitations were observed compared to autumn (March to June) and winter (June to September). Such conditions may provide an increase in the photosynthetic activity and growth of species, contributing with a greater amount of carbon skeletons to produce secondary metabolites (Santos et al., 2012).

These results agree with other studies in the literature that documented higher levels of terpenoids in spring and summer than in autumn and winter (Chen et al., 2014; Robles and Garzino, 2000; Rivoal et al., 2010; Silva et al., 2019).

4. Conclusion

The chemical composition and yield of the essential oil from *A. edulis* varied significantly during the collections, highlighting the presence of caryophyllene oxide (Dourados city) for all four seasons and α-zingiberene (Bonito) in summer and spring. In turn, there was a increase in essential oil yield and difference in chemical profiles with increasing temperature and during the inflorescence stage. Four sesquiterpene, caryophyllene oxide, germacrene, E-caryophyllene and viridiflorol were reported in all collections which in part may indicate the possibility of being a chemical marker of the species.

Declaration of Competing Interest

The authors declare that there were no conflicts of interest.

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5.2 Artigo II: Analysis of secretory structures, chemical composition, and anti-inflammatory properties of *Allophylus edulis* (A. St.-Hil., A. Juss. & Cambess.) Radlk leaves

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Analysis of secretory structures, chemical composition, and anti-inflammatory properties of *Allophylus edulis* (A. St.-Hil., A. Juss. & Cambess.) Radlk leaves

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Abbreviations: ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BHT, Butylated hydroxytoluene; CEUA, Committee of Ethics on the Use of Animals; CFA, Complete Freund's Adjuvant; CONCEA, National Council for Control of Animal Experimentation; DDMS, Herbarium of the Federal University of Grande Dourados; DEXA, Dexamethasone; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; EAf, ethyl acetate fraction; EC, Catechin equivalent; GAE, Gallic acid equivalent; Hf, n-hexane fraction; HMf, hydromethanol fraction; ILAE, Infusion of the leaves of *A. edulis*; MOR, Morphine; NMR, Nuclear Magnetic

Resonance; PBS, Phosphate buffered saline; PRED, Prednisolone; QE, Quercetin equivalent; SisGen, National System for the Genetic Heritage and Associated Traditional Knowledge Management; TLC, Thin-layer chromatography; UFGD, Federal University of Grande Dourados.

ABSTRACT

Ethnopharmacological relevance: Allophylus edulis, known as “vacum” in Brazil, is used in popular medicine for the treatment of inflammatory disease. However, there is no scientific evidence demonstrating this activity by infusion obtained from *A. edulis* leaves.

Aim of the study: This research aims to evaluate the chemical composition, potential antioxidant activity, anti-inflammatory, and antinociceptive properties of the infusion obtained from *A. edulis* leaves. Additionally, a detailed histochemical description of *A. edulis* leaf sections is provided.

Materials and Methods: Fresh *A. edulis* leaves underwent histochemical analysis. Another set of leaves was used to produce lyophilized infusion (ILAE) and the hydromethanolic fraction (HMf), along with the compound vitexin 2"-O-rhamnoside (AE-1). Chemical investigation (quantification of total phenols, flavonoids, flavonols and condensed tannins) of the ILAE and isolation of vitexin 2"-O-rhamnoside (AE-1) was performed. In silico methods predicted drug similarity and identified pharmacodynamic targets for vitexin 2"-O-rhamnoside, along with projecting its oral toxicity in rodents. The antioxidant activity of ILAE, HMf and ethyl acetate fraction (EAf) was evaluated by radical scavenging (2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS)) and lipid peroxidation (β-carotene/linoleic acid). The anti-inflammatory and anti-nociceptive properties of oral administration of ILAE (3, 30 and 100 mg/kg), hydromethanolic fraction HMf (3 and 30 mg/kg) and EA-1 (3 mg/ kg) were evaluated in the following models: carrageenan-induced acute paw edema/hyperalgesia and pleurisy, formalin-induced nociception and by Complete Freund’s Adjuvant (CFA) induced paw inflammation in mice.

Results: The ILAE and fractions has total phenols measuring ≤ 177 mg GAE/g, also identified in secretory structures. Additionally, it exhibited antioxidant activity by inhibiting free radicals (IC_{50} ≤ 28 μg/mL in DPPH and 61 μg/mL in ABTS) and lipid peroxidation (IC_{50} ≤ 195 μg/mL

in β-carotene/linoleic acid). The vitexin 2"-O-rhamnoside, a flavonoid with three Lipinski's rule violations and low predicted toxicity through in silico methods was obtained from hydromethanolic fraction. All oral doses of ILAE and HMf significantly reduced carrageenan-induced paw edema/hyperalgesia and leukocyte migration, particularly at a dose of 3 mg/kg for both ILAE and HMf. Among the samples, HMf (3 mg/kg) most effectively reduced formalin-induced manifestations, particularly during phase II. Similarly, both edema and hyperalgesia induced by CFA responded to treatments with ILAE (30 mg/kg), HMf (30 mg/kg) and AE-1 (3 mg/kg).

Conclusions: This study revealed the chemical compositions, mainly polyphenolic substances, of lyophilized infusion obtained from of *A. edulis* leaves. The antioxidant capacity, particularly in free radical inhibition, and the acute and prolonged anti-inflammatory, antihyperalgesic, and antinociceptive properties explains the popular use and the potential for novel therapies in inflammation-related pathologies.

Keywords: Vacum, Leaf Infusion, Vitexin flavonoid, Carrageenan, Complete Freund's Adjuvant.

1. Introduction

The use of medicinal plants serves as the primary therapeutic approach for a wide range of pathologies, especially within specific communities. Peruvian, Argentine and Brazilian traditional communities report the traditional use of leaves (aqueous maceration and/or infusion) from *Allophylus edulis* (A. St.-Hil., A. Juss. & Cambess.) Radlk. This plant (Sapindaceae family) is known as "vacum", "cocu" or "chal-chal", and is traditionally used to treat several inflammatory conditions, including sore throat, cholecystitis and fever (Arisawa et al., 1989; Körbes, 1995; Franco and Fontana, 2001).

In an attempt to demonstrate these effects, some studies have provided evidence that extracts from the leaves (aqueous, ethanolic, or methanolic), exhibit antimicrobial properties (Arruda et al., 2018), angiotensin-converting enzyme inhibition (Arisawa et al., 1989), antioxidative effects (Tirloni et al., 2015), hepatoprotective potential (Hoffmann-Bohm et al., 1992), and negative ionotropic properties (Matsunaga et al., 1997). Additionally, chemical studies on the leaves of *A. edulis* (methanolic extracts), have reported the isolation and characterization of phenolic acids, coumarins, and flavonoids (Arisawa et al., 1989; Hoffmann-Bohm et al., 1992).

In the same context, as part of efforts to validate the ethnobotanical use of *A. edulis* leaves for their anti-inflammatory effects, it was demonstrated anti-inflammatory activity for both the essential oil and its major components. The anti-inflammatory potential was assessed through various methods, revealing significant inhibition of leukocyte migration, edema, cold sensitivity, and mechanical hyperalgesia induced by agents including carrageenan, zymosan, tumor necrosis factor-α (TNF-α), dopamine (DOPA), Complete Freund's Adjuvant (CFA), and formalin-induced nociception (Trevizan et al., 2016; Santos et al., 2021; Balsalobre et al., 2023). However, to date, the anti-inflammatory actions of the leaf extract have not been evaluated, despite its traditional folk use.

This reinforces the importance of continuing studies with *A. edulis*, since maceration or infusion of the leaves are used to treat inflammation (Arisawa et al., 1989; Körbes, 1995). As such, controlling the duration and magnitude of inflammation plays a central role in mitigating the damage caused by these pathologies (Kaur and Singh, 2022). And knowing that there are few effective anti-inflammatory drugs with minimal side effects, there is an evident need for more effective drugs with low toxicity. One of the alternatives is the use of glycosylated flavonoids, abundant metabolites in *A. edulis* that have important pharmacological properties,

especially due to their solubility, stability, and bioactivity in the human body (Plaza et al., 2014; Xu et al., 2016).

Considering that the reports made by our research group showed anti-inflammatory, anti-arthritic and anti-hyperalgesic properties from its essential oil (apolar metabolites), this work aims to evaluate the anti-inflammatory, antioxidant and antinociceptive activity from the infusion leaves (polar) (ILAE) and major compound (AE-1) of *A. edulis* (**Fig. 1**). Additionally, we reported the histochemical analysis of leaf secretory structures, which aids species identification, and contributes for the quality control of herbal products.

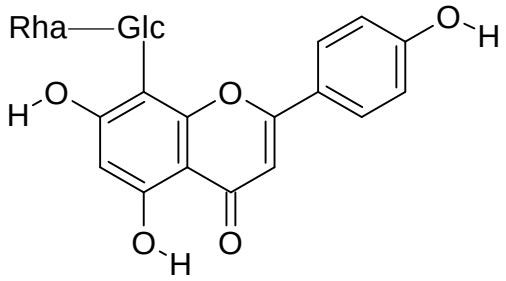


Fig. 1. Chemical structure of Vitexin 2"-O-rhamnoside isolated from *A. edulis*.

2. Material and methods

2.1. Drugs and solvents

λ-Carrageenan, Complete Freund's Adjuvant (CFA), Prednisolone, Bradford Reagent, Quercetin hydrate, Catechin hydrate, Gallic Acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Butylated hydroxytoluene (BHT), silica gel GF (254) and Lipophilic sephadex resin (Sephadex LH-20) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TCL Silica gel 60 were purchased from Merck KGaA (Darmstadt, DE). Dexamethasone was purchased from EMS (Hortolândia, SP, BR). L-Ascorbic acid from Dinâmica (São Paulo, SP, BR). Morphine from Cristália (Itapira, SP, BR). Formalin from Cromato (Diadema, SP, BR). Turk's solution from Newprov (Pinhais, PR, BR). Methanol and n-Hexane from Neon (Suzano, SP, BR). Ethyl acetate from

Proquimios (Rio de Janeiro, RJ, BR). Methanol-D4 was used on Nuclear Magnetic Resonance (NMR) was LC grade and purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Additional other drugs and reagents used were of analytical grade.

2.2. Plant material

The *A. edulis* leaves were collected at the Medicinal Plant Garden of the Federal University of Grande Dourados (UFGD), in the city of Dourados (Mato Grosso do Sul, Brazil, 22°11'43.7"S 54°56'08.5"W), identified by Dr. Zefa Valdivina Pereira, and deposit in the UFGD herbarium under the code DDMS 342. Authorization to access the Brazilian genetic heritage was obtained by the National System for the Genetic Heritage and Associated Traditional Knowledge Management (SisGen-A51F665).

2.3. Histochemical analyses

Free-hand sections of fresh leaves of *A. edulis* were transversely sectioned and, subsequently submitted to different treatments to investigate the chemical composition of the secretions present in the glandular trichomes, ducts, laticifers and idioblasts. Various reagents were used for detection of classes of compounds and the reagents description/compounds are as follows: : (a) Sudan III to verify the presence of lipophilic compounds (Pearse, 1972); (b) Nadi reagent to detect terpenoids (David and Carde, 1964); (c) ferric chloride (Johansen, 1940) and potassium dichromate (Gabe, 1968) to reveal the presence of phenolic compounds; (d) vanillin – hydrochloric acid to evidence tannins (Mace and Howell, 1974); (e) Dragendorff's reagent (Svendsen and Verpoorte, 1983) to detect the presence of alkaloids and (f) oil red O reagent to detect latex (Pearse, 1968). Untreated sections were used as control. Slides were observed under bright field and the photomicrographs were obtained using an Olympus CX31 attached to a C7070 control unit.

2.4. Infusion preparation, fractioning, isolation, and NMR analysis

The infusion of *A. edulis* leaves (ILAE) was obtained by boiling distilled water (10 L), which was then placed over fresh chopped *A. edulis* leaves (1.0 kg). After infusing for 15-25 minutes, it was filtered and lyophilized (Lyophilizer Christ, Osterode am Harz, DE). The resulting *A. edulis* leaf infusion (ILAE, 40 g) was stored in a freezer at -5°C until needed for the experiments.

Part of the ILAE (38 g) was dissolved in MeOH: H_2O (1:1) and partitioned with n-hexane and ethyl acetate, to obtain the n-hexane (Hf, 7.6 g), ethyl acetate (EAf, 9.5 g) and hydromethanol (HMf, 13.7 g) fractions, which subsequently analyze by Thin-layer chromatography (TLC) plates (silica gel 60 or GF254), accomplished by UV irradiation at 254 and 366 nm, and/or by spraying with a H_2SO_4/MeOH (1:1), H_2SO_4/anisaldehyde/acetic acid (1:0.5:50 mL) solutions followed by heating at 100°C or dragendorff's solution. The HMf, resulting from partitioning, was subjected to column chromatography on Sephadex LH-20 eluted with H_2O, H_2O-MeOH 8:2, 6:4, 4:6, and 2:8, and MeOH. After preparative TLC ($CHCl_3$/MeOH 2:8), afforded **AE-1** (16 mg). The isolated compound was identified by comparing spectroscopic data (^{1}H NMR) with data from the literature (Chopin et al., 1977, Li et al., 2015). ^{1}H NMR (300 MHz), spectra were recorded on a Bruker Ascend 300 spectrometer (Bruker, Germany), in ppm, using Methanol (MeOD-D4) as solvent.

Vitexin 2"-O-rhamnoside (**AE-1**): ^{1}H NMR δ_H (300 MHz, MeOD): 8.04 (d, J= 7.8 Hz), 7.44 (d, J=7.8 Hz), 6.92 (s), 6.58 (s), 5.51 (d, J=4.5 Hz), 5.47 (d, J=4.8 Hz), 4.39-2.03 (m) sugar protons, 1.04 (m).

2.5. Quantification of constituents: total phenol content and polyphenolics

The ILAE, EAf and HMf were submitted to the quantification of constituents: **(A)** total phenols, measured at 760 nm using a UV spectrophotometer (Bel Photonics, Monza, IT), using 1 mg/ml (in methanol HPLC) of each sample, with results expressed as mg of gallic acid equivalent per gram of sample (mg GAE/g) with reference to the gallic acid (0.005-0.05 mg/mL) calibration curve ($y = 1.3516x + 0.1098$, $R^2=0.9802$) (Siddhuraju and Becker, 2003); **(B)** flavonoids were measured at 415 nm, using 2 mg/ml (in methanol HPLC) of each sample; **(C)** flavonols at 440 nm, using 2 mg/ml (in ethanol P. A.) of each sample, both expressed as mg of quercetin equivalent per gram of sample (mg QE/g) by reference to the quercetin (0.001 – 0.01 mg/mL) calibration curve ($y = 12.94x - 0.0148$, $R^2 = 0.9991$) and ($y = 26.143x + 0.3571$, $R^2 = 0.9885$), respectively (Aryal et al., 2019); and **(D)** condensed tannins were measured at 500 nm, using 10 mg/ml (in Methanol HPLC) of each sample, expressed as mg of catechin equivalent per gram of sample (mg CE/g) with reference to the catechin (0.02 – 0.2 mg/mL) calibration curve ($y = 1.5666x - 0.0412$, $R^2=0.9905$) (Hayat et al., 2020). The tests were carried out in triplicate.

2.6. Drug-likeness, pharmacodynamic targets and toxicity prediction of vitexin 2''-O-rhamnoside

The prediction of drug likeness and the identification of pharmacodynamic targets for vitexin 2''-O-rhamnoside (**AE-1**) were conducted utilizing the Molinspiration online server (https://www.molinspiration.com) and Swiss ADME (http://www.swissadme.ch/) (Daina et al, 2017), employing Lipinski's rule of five (Lipinski et al., 1997; Lipinski, 2000). The projection of rodent oral toxicity was performed via the Protox II servers (https://tox-new.charite.de/protox_II), estimating LD_{50} values in accordance with the methodology described by Banerjee et al. (2018).

2.7. Antioxidant activity

2.7.1. Radical scavenging activity

The ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging method, as described by Re et al. (1999) and Formagio et al. (2014), involved creating the ABTS radical by mixing ABTS (7.0 mM) and potassium persulfate (140 mM) and allowing in darkness at room temperature for 16 hours. The resulting ABTS+ solution was then diluted with ethanol (P.A) to achieve an absorbance of 0.700±0.05 at 734 nm. Different concentrations of ILAE, EAf, and HMf (0.6–0.05 mg/mL in methanol) were added to this solution, and the absorbances were measured after 6 minutes. BHT was used as positive control. The ABTS+ scavenging activity was calculated as: ABTS+ scavenging activity (%) = (Abs Sample - Abs Control/Abs Control) x 100. The results were expressed as IC_{50}.

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenging method, as described by Blois et al. (1958) and Formagio et al. (2014), used different concentrations (0.6–0.05 mg/mL in methanol) of ILAE, EAf and HMf mixed with DPPH (0.1 mM). After incubating in the dark at room temperature for 30 minutes, the absorbance was measured after 30 minutes at 515 nm using a spectrophotometer (Bel Photonics, Monza, IT). The experiments were conducted in triplicate and BHT was used as positive control. The percentage of DPPH inhibition was calculated as follows: I% = (Abs Sample - Abs Control / Abs Control) x 100. The results were reported as IC_{50}.

2.7.2. Lipid peroxidation assay

The antioxidant activity of ILAE, EAf and HMf was assessed using the β-carotene/ linoleic acid method, following Marco (1968) and Formagio et al. (2014). A solution of β-carotene was prepared (2 mg/mL of β-carotene in chloroform mixed with 20 μL of 99 % linoleic

acid and 200 μL of Tween 40). After eliminating chloroform, an emulsion was formed by vigorously stirring the solution with oxygen-rich distilled water. Aliquots of this emulsion were mixed with samples at various concentrations (0.01-1 mg/mL). Absorbance at 470 nm was immediately measured post-preparation. The solutions were then placed in a 50 °C water bath, and absorbance readings were taken every 20 minutes to track oxidation until the β-carotene coloration disappeared within 100 minutes. Antioxidant activity, measured as the percentage of bleaching inhibition, was determined using the formula %AA = 100-[(Ai − At)/(A'i − A't) × 100]. Ai = initial absorbance of the sample, At = after 100 minutes of incubation at 50 °C, A'i = initial absorbance of the control, and A't = control's after 100 minutes of incubation at 50 °C. The results were reported as IC_{50}. The assay was performed in triplicate.

2.8. Animals and ethical clearance

Experiments were performed on male and female Swiss mice (25-30 g), from the Central animal house facility of the Federal Faculty of Grande Dourados. Animals were housed in 30x20x13cm polypropylene cages at 22±2°C with a 12:12 h light-dark cycle, with free access to commercial pelleted food and water. The preparations of infusion and hydrometanolic fraction were solubilized in 0.9% saline solution and were given to the animals according to their weight. All animals were euthanized by cervical dislocation or lethal injection of ketamine (300 mg/kg) and xylazine (30 mg/kg), according to the Euthanasia Practice Guideline, Normative Resolution No. 37/2018 of the National Council for the Control of Animal Experimentation (CONCEA). The CONCEA also defined the parameters for handling animals, and the project received approval from the Committee of Ethics on the Use of Animals (CEUA) of the Federal University of Grande Dourados (n. 05.2021).

2.9. Anti-inflammatory activity

2.9.1. Paw edema, cold allodynia and mechanical hyperalgesia induced by carrageenan in mice paw

Male Swiss mice (n=5) were distributed in groups according to oral (gavage) treatments. Groups with different doses ILAE (3, 30 or 100 mg/kg), HMf (3 mg/kg), Dexamethasone (DEXA 1 mg/kg) or control (0.9 % saline solution) received the oral treatment. After 1 h, the inflammation was induced by injection of carrageenan (300 μg/paw, 50 μL in sterile 0.9 % saline) in the right paw and 50 μL of 0.9 % saline solution in the contralateral paw (Winter et al. 1962; Santos et al. 2021). The basal group (physiological control) received no treatment or injections. Edema was measured after 0.5, 1, 2 and 4 h with a paw plethysmometer (PANLAB Harvard), and the degree of edema was compared to the one in the left paw.

In this same experiment, the animals were subjected to the cold allodynia using acetone (Decosterd and Woolf, 2000), and mechanical hyperalgesia (Von Frey test) assessed using an electronic von Frey apparatus (Deuis et al., 2014). Both parameters were measured 3 and 4 h after carrageenan injection.

2.9.2. Pleural cell migration and protein exudation induced by carrageenan

Different groups of female Swiss mice (n=5) were treated orally at different doses of ILAE (3, 30 or 100 mg/kg), HMf (3 mg/kg), DEXA (1 mg/kg) or control (0.9 % saline solution). The naive group was treated orally and received intrapleural injection of sterile saline solution (0.9 %). Inflammation of the pleura (pleurisy) was induced by applying 100 μL of 1 % carrageenan into the mice's pleural cavity (Vinegar et al. 1973; Santos et al. 2021). After 4 h, euthanasia was performed by injection of ketamine/xylazine, and the thoracic cavity was washed with 1 mL of phosphate buffered saline (PBS), and the pleural exudate was collected. The exudate volume was measured and 20 μL are diluted in Turk's Liquid (1:20) and used to determine the total number of leukocytes present in a Neubauer chamber. Protein extravasation,

a portion of the exudates were centrifuged, and the protein concentrations were determined by the Bradford method (Bradford, 1976).

2.9.3. Spontaneous nociceptive response in the formalin model

Male Swiss mice (n=5) were treated orally at different doses ILAE (3, 30 or 100 mg/kg), HMf (3 mg/kg), or control (sterile saline solution 0.9 %). The positive control group received morphine (MOR 4 mg/kg) by intraperitoneal route. The animals received 20 μL of 2.5 % formalin solution by subplantar injection in the right paw. Immediately, were observed pain manifestations and timed from 0-5 min (first phase) and 15-40 min (second phase). Pain manifestations were considered as the time (s) that the animal spent licking, shaking, and holding the injected paw (Hunskaar and Hole, 1987; Sufka et al., 1998).

2.9.4. Paw edema, mechanical and cold hyperalgesia induced by Complete Freund's Adjuvant (CFA) in mice paw

Male Swiss mice (n=5) were treated orally at different doses ILAE (30 mg/kg), HMf (3 and 30 mg/kg), AE-1 (3 mg/kg), Prednisolone (PRED 3 mg/kg) or control (sterile saline solution 0.9 %). The basal group (physiological control) received no treatment or injections. Inflammation was induced by injection of a suspension of CFA (20 μL/right paw) and 0.9 % saline solution (20 μL) in the contralateral paw (Larson et al., 1986). Edema, cold sensitivity, and mechanical sensitivity were measured 3, 4, and 24 h after CFA injection, and the methodology used is described above.

2.10. Statistical analysis

Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Tukey’s test, and the differences were considered statistically significant when

$P < 0.05$. All statistical calculations and graphs were prepared using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA). The IC_{50} value is determined as the concentration of the sample at which the percentage inhibition reaches 50%.

3. Results

3.1. Histochemical analysis of leaf secretory structures

It has compound leaves, trifoliate, with serrated margin and acuminate apex. *A. edulis* leaves (**Fig. 2a, b**) presented four distinct types of secretory structures represented by ducts (**Fig. 2c, 2d, 2e, 2f, 3d, 3e, 3i, 3l**), glandular trichomes (**Fig. 2g, 2h**), laticifers (**Fig. 2e, 2i, 2j, 3g-i, 3k, 3m**) and idioblasts (**Fig. 2c-e, 2j, 3c, 3e, 3g-i, 3k-m**).

In the present study, secretory ducts were present in the midrib (**Fig. 2c-e, 3a, 3b**), lamina (**Fig. 2f**), petiole (**Fig. 3f, 3i**) and petiolule (**Fig. 3j, 3l**). They were commonly found in the collenchyma or near it (**Fig. 2c, 2d, 3d, 3e**). The secretion of the secretory ducts reacted positively with Sudan III (**Fig. 2e**) and with NADI reagent that confirm the presence of essential Iin blue) oil as the secretion storage in ducts (**Fig. 2d**). Glandular trichomes (**Fig. 2g, 2h**) were rare and found on the epidermis of leaves (midrib, lamina, petiole and petiolule). The secretion reacted with Sudan III and become red-orange (**Fig. 2h**) and turned blue in the NADI reaction, indicating the presence of essential oils.

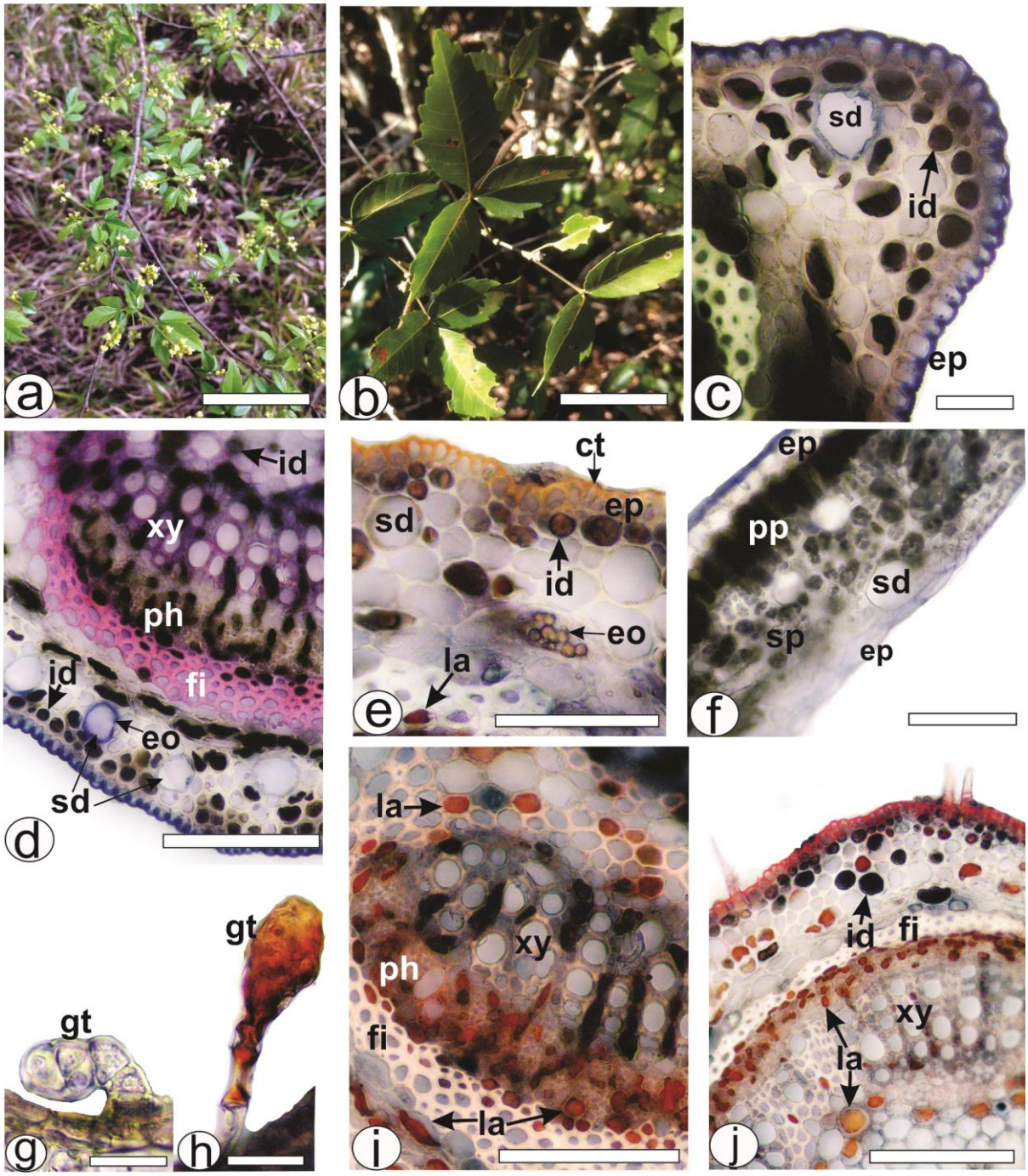


Fig. 2. Histochemical analysis of leaf secretory structures of *A. edulis*. Fresh material. (a) Plant in inhabit. (b) A twig. (c, d) Detection of secretory ducts and essential oil by NADI reagent in the midrib. (e) Exposure of secretory ducts and essential oils by Sudan III in the midrib. (f) Positive result for secretory ducts in the lamina by NADI reagent. (g) Glandular trichome without reaction. (h) Observation of lipophilic material in the glandular trichome using Sudan III. (i) Detection of latex in the laticifers of petiole by red oil O reagent. (j) Positive result of

the presence of latex in the laticifers of petiolule by red oil O reagent. [ct: cuticle, eo: essential oil, ep: epidermis, fi: fiber, gt: glandular trichome, id: idioblast, la: laticifer, ph: phloem, pp: palisade parenchyma, sd: secretory duct, sp: spongy parenchyma, xy: xylem]. Scale bars: a = 5 cm; b = 10 cm; d, e, f, i, j = 50 µm; c, g, h = 20 µm.

Laticifers were observed in great amount in the ground parenchyma of midrib (**Fig. 2e, 3d, 3e**), petiole (**Fig. 2i, 3h, 3i**) and petiolule (**Fig. 2j, 3m**). Specifically, laticifers were present in the collenchyma in the ground parenchyma and near the fibers (**Fig. 2e, 2j, 3g-i, 3k, 3m**), in the phloem (**Fig. 2i, 2j, 3d, 3g-i**), close to the xylem and in the pith (**Fig. 2i, 2j, 3h**). Latex reacted positively with red oil O (**Fig. 2i, 2j, 3i, 3m**) in the histochemical tests. Various chemical classes of metabolites were detected in the latex. Lipids were evidenced using Sudan III (**Fig. 2e**), phenolic compounds were evidenced with ferric chloride (**Fig. 3c, 3k**) and potassium dichromate (**Fig. 3g**). Tannins were detected with vanillin solution only in the laticifers of phloem (**Fig. 3d, 3h**). Alkaloids were distinguished using Dragendorff (**Fig. 3e**).

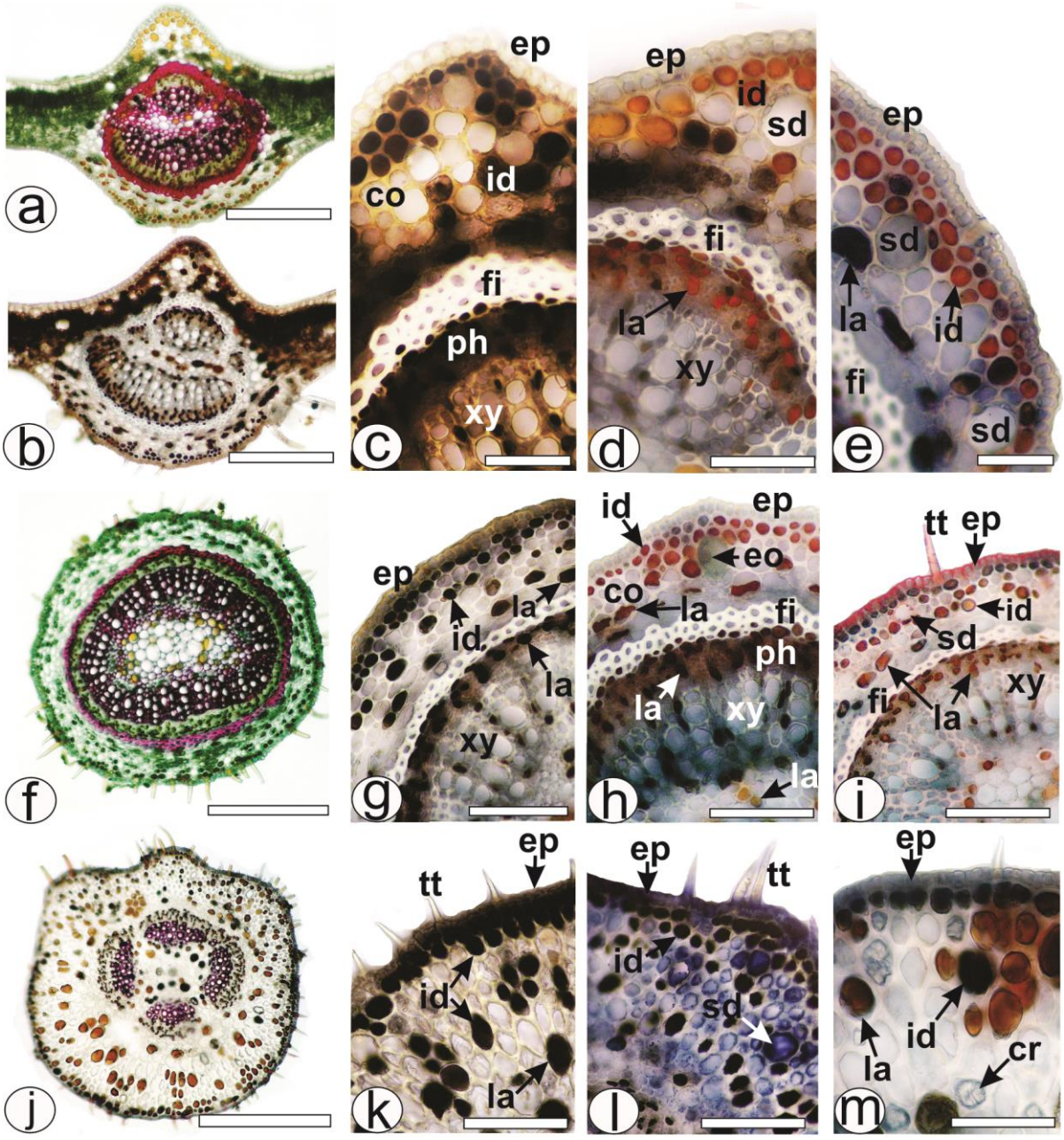


Fig. 3. Histochemical analysis of leaf secretory structures of *A. edulis*. Fresh material. (a-e) Midrib. (f-i) Petiole. (j-m) Petiolule. (a,f,j) Positive reaction of fibers and xylem with phloroglucinol/HCl. (b,c,k) Detection of phenolic compounds by ferric chloride solution. (d,h) Detection of tannins by vanillin. (e) Detection of alkaloids in the laticifers by Dragendorff. (g) Phenolic compounds in reaction with potassium dichromate solution. (i,m) Detection of latex in the laticifers by red oil O reagent. (l) Detection of essential oils in the secretory ducts by NADI reagent. [co: collenchyma, cr: crystal, eo: essential oil, ep: epidermis, fi: fiber, id:

idioblast, la: laticifer, ph: phloem, sd: secretory duct, tt: non-glandular trichome, xy: xylem]. Scale bars: f, j = 500 µm; a, b = 200 µm; c, g-i, k, l, m = 50 µm; d, e = 20 µm.

Secretory idioblasts were frequently observed in leaves of *A. edulis* (midrib, petiole and petiolule). They were found beneath the epidermis, forming rows (**Fig. 2c-e, 3c-e**), scattered in the collenchyma (**Fig. 2c-e, 2j, 3c-e**) and in the parenchyma cells of the xylem (**Fig. 2i**) and in small amount in the pith (**Fig. 2d**). The histochemical tests evidence the presence of lipids (**Fig. 2e**) and phenolic compounds (**Fig. 3c, 3g, 3k**) in the idioblasts. Vanillin was detected only in the idioblasts of the collenchyma (**Fig. 3d**), yet not found in the idioblasts present in the xylem (**Fig. 3h**). Laticifers contrast with idioblasts in terms of cell diameter, shape, color, localization of storage, and the chemical composition of the secretion (**Fig. 2e, 2i, 2j, 3d, 3e, 3h, 3i**). Idioblasts were frequently found in the leaves of *A. edulis* and formed extensive rows, whereas laticifers formed less extensive rows and were less abundant.

3.2. Chemical study

The infusion (ILAE) and fractions resulting from partitioning (Hf, EAf and HMf) showed moderate concentration of the total phenols ≤ 177 mg GAE/g, flavonoids ≤ 63 mg EC/g and condensed tannins ≤ 75 mg QE/g, (**Table 1**). The flavonol showed lower concentration in all samples (≤ 23 mg QE/g) (**Table 1**).

Table 1. Total phenol, flavonoids, flavonols and condensed tannins of *A. edulis*.

Metabolites	ILAE	Hf	EAf	HMf
Total phenols (mg GAE/g)	177.56±6.16	88.15±5.24	155.92±4.59	159.56±4.74
Flavonoids (mg QE/g)	37.29±0.18	24.36±0.39	46.37±0.18	63.10±0.20
Flavonols (mg QE/g)	5.22±0.11	6.39±0.07	23.71±0.09	21.63±0.42
Condensed tannins (mg CE/g)	37.78±1.12	46.48±1.01	75.53±1.31	63.88±1.50

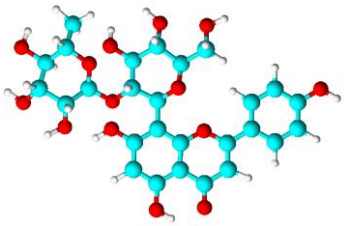
The values represent the means of three measurements ±standard deviation.

The HMf partitioning resulted in the isolation and identification of vitexin 2"-O-rhamnoside (**EA-1**). This compound is a flavone-C-glycosides, by the presence of two signals from hydrogen atoms located in ring A and C, corresponding to the H-6 (δ_H 6.58) and H-3 (δ_H 6.92) for the C-8-susbtituted apigenine skeleton. The ^{1}HNMR spectra of **EA-1** showed signals characteristics for a ring B the mono-oxygenated ring at C-4′, due the presence of ortho coupled aromatic hydrogens at δ_H 8.04 (d, J= 7.8 Hz, H-2'/H-6') and δ_H 7.44 (d, J=7.8 Hz, H-3'/H-5'). In addition, the sugar units, C-4 glucose-2'-O-ramnose were evidenced by the signals in δ_H 5.51 (d, J=4.5 Hz), 5.47 (d, J=4.8 Hz) and 4.39-2.03 (m) sugar protons.

3.3. Drug-likeness, pharmacodynamic targets and toxicity prediction of vitexin 2"-O-rhamnoside

The results are shown in **Table 2** and indicate that the vitexin 2"-O-rhamnoside exhibited three violations surpassing acceptable thresholds of MW (578.52 g/mol), H-bond acceptors (14), and hydrogen bond donors (9). The predicted oral bioavailability was 17%.

Table 2. Drug-likeness, pharmacodynamic targets and toxicity prediction of vitexin 2"-O-rhamnoside from *A. edulis.*



Lipinsk's parameter		Drug Likeness		Bioactive scores		Toxicity		Water solubility	
MW	578.52	**TPSA**	239.97	**GPCR ligant**	0.12	**Hepatot.**	Inactive	**Log S**	-2.82
Bioav.	0.17	**%ABS**	26.21	**Ion channel modulator**	-0.37	**Carcinog.**	Inactive		
Log P	-0.18			**Kinase inhibitor**	0.05	**Immunog.**	Active		

nOH	14		**Nuclear receptor ligant**	-0.01	**Cytot.**	Inactive	
NHn	9		**Protease inhibitor**	0.04	**LD_{50} (mg/kg)**	5000 (class 5)	
Violations	3		**Enzyme inhibitor**	0.35			

TPSA: topological polar surface area (< 140 Å2); %ABS: theoretical oral absorption percentage (% = 109 – [0.345 × TPSA]); MW: molecular weight (< 500 g/mol); Bioavailability score (> 0.1); LogP: octanol-water partition coefficient Log P (lipophilicity) (not > 5); nOH: number of H-bond acceptors (< 10); NHn: number of H-bond donors (not > 5); and Log S (solubility) (–1 to –5).

Considering positive values (>0.0) for plant molecules as a bioactivity predictor (Lata et al., 2023), the analysis of vitexin-2″-O-rhamnoside's pharmacodynamic behavior reveals its potential for greater bioactivity as a GPCR ligant, kinase, protease, and enzyme inhibitor (**Table 2**). When submitted to the server of oral toxicity analysis in rodents, exhibited class 5 toxicity, with a predicted LD_{50} of 5000 mg/kg and an accuracy of 67.38%. It showed no hepatotoxicity or toxicological parameters related to carcinogenicity, mutagenicity, and cytotoxicity. However, it demonstrated active immunotoxicity (**Table 2**).

3.4. Antioxidant activity

The results showed that ILAE, EAf and HMf have potent antioxidant activity by reducing the DPPH radical ($IC_{50} \leq 28.07$ µg/mL), highlighting the HMf with IC_{50} (15.1 µg/mL) (**Table 3**). Higher IC_{50} values were found in the assessment of lipid peroxidation assessed by the β-carotene/linoleic acid test, which showed values of $IC_{50} \leq 195.6$ µg/mL, highlighting also HMf (with IC_{50} of 55.44 µg/mL), when compared to BHT (IC_{50} = 13.03) (**Table 3**).

Table 3. Antioxidant activity of infusion (ILAE) and fractions (EAf and HMf) of *A. edulis* leaves.

Antioxidant activity	ILAE	EAf	HMf	BHT

	IC_{50} (µg/mL)			
DPPH	27.88±0.002	28.07±0.003	15.17±0.004	9.89±0.002
ABTS	40.55±0.029	25.15±0.039	61.80±0.013	9.75±0.004
β-carotene/linoleic acid	117.9±0.50	195.6±0.09	55.44±0.72	13.03±0.04

The values represent the means of three measurements ±standard deviation.

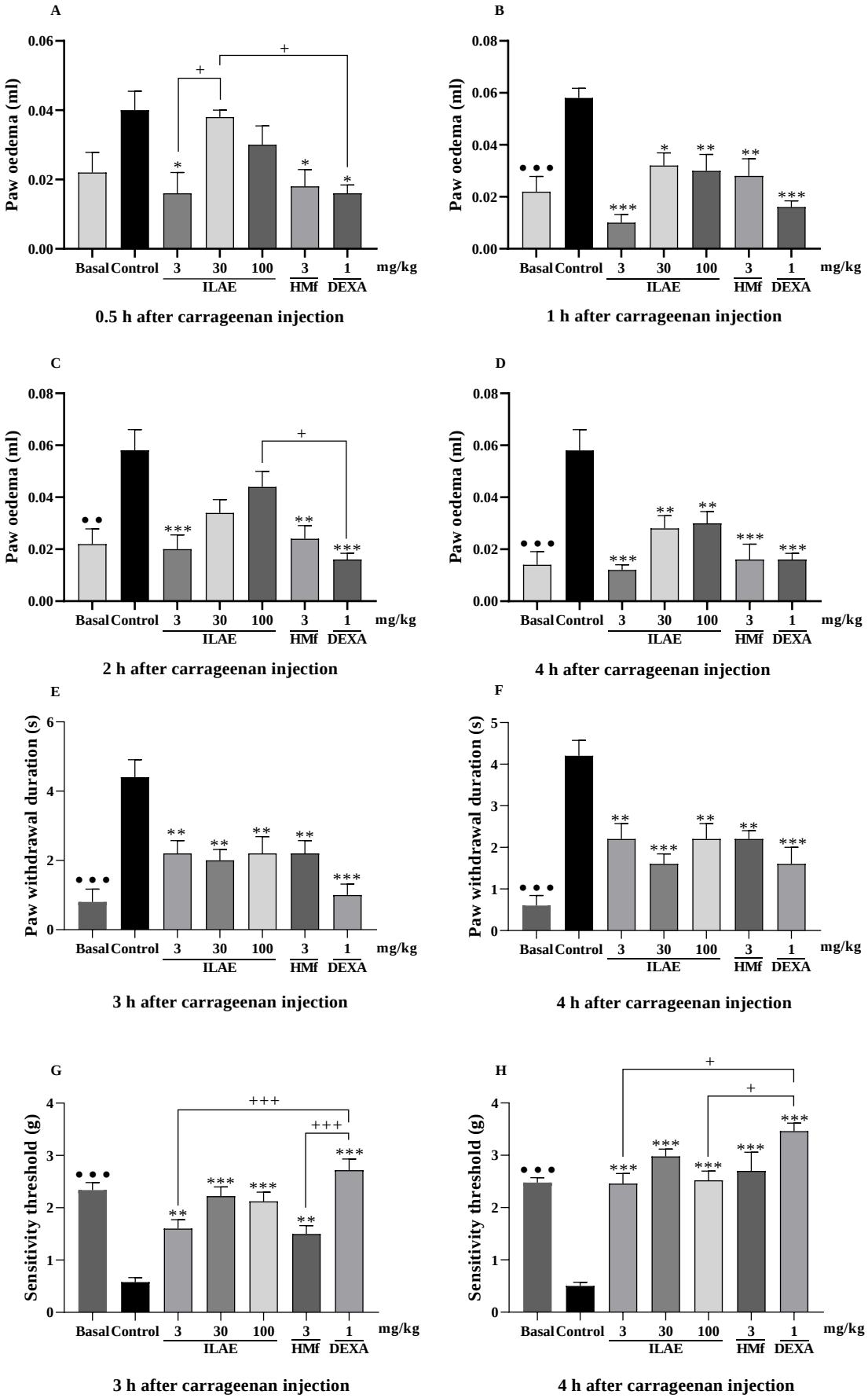
3.5. Anti-inflammatory, anti-hyperalgesic and antinociceptive activity of ILAE, HMf and/or AE-1

In the carrageenan-induced paw edema inflammatory model, the oral exposure to ILAE (3 mg/kg) and HMf (3 mg/kg) at 0.5 h significantly reduced edema formation by 60.0 and 55.0%, respectively, compared to the control (both $P<0.05$). The positive control (DEXA 1 mg/kg) also significantly reduced edema formation by 60.0% compared to the control ($P<0.05$). When comparing ILAE and HMf doses, there was no statistical difference, except for the 3 and 30 mg/kg ILAE doses ($P<0.05$). This lack of statistical difference persisted when comparing with DEXA, except for the 30 mg/kg ILAE dose ($P<0.05$). At 1 h after carrageenan injection (**Fig. 4B**), ILAE (3 mg/kg) demonstrated inhibition of edema formation at 82.7% ($P<0.001$), while HMf (3 mg/kg) exhibited a 51.7% inhibition ($P<0.01$). In contrast, DEXA showed a substantial inhibition of 72.4% ($P<0.001$), all relative to the control. No statistical differences were observed between ILAE and HMf treatments, as well as in comparison with DEXA (**Fig. 4B**). After 2 h (**Fig. 4C**), ILAE (3 mg/kg) and HMf (3 mg/kg) significantly reduced edema formation by 65.5% ($P<0.001$) and 58.6% ($P<0.01$), respectively, compared to the control. In parallel, DEXA (1 mg/kg) exhibited an inhibition of 72.4% ($P<0.001$) relative to the control. No statistical differences were observed between ILAE and HMf treatments. The only differences found were between ILAE (100 mg/kg) and DEXA (1 mg/kg) treatments, with $P<0.05$ (**Fig. 4C**). In the final evaluation at 4 h (**Fig. 4D**), ILAE (3 mg/kg) and HMf (3 mg/kg) demonstrated a statistically significant effect with 79.3 and 72.4% inhibition of paw edema

compared to the control ($P<0.001$), respectively. In a similar vein, DEXA (1 mg/kg) also exhibited a significant effect with 72.4% inhibition of paw edema compared to the control ($P<0.001$). No statistically significant differences were observed between treatments, as well as in comparison with DEXA (**Fig. 4D**).

In the carrageenan paw model, the acetone-induced allodynia and oral administration of ILAE and HMf significantly reduced responses to cold stimuli, by 50.0% (ILAE 3 and 100 mg/kg, and HMf 3 mg/kg) and 54.0% (ILAE 30 mg/kg) at 3 h (**Fig. 4E**), compared to the control group (all with $P<0.01$). After 4 h (**Fig. 4F**), the observed reduction was 47.6% (ILAE 3 and 100 mg/kg, and HMf 3 mg/kg, $P<0.01$) and 61.9% (ILAE 30 mg/kg, $P<0.001$), compared to control. Treatment with DEXA (1 mg/kg) exhibited a decrease of 77.2% at 3 h and 61.9% at 4 h (**Fig. 4E, F**) compared to the control group (both with $P<0.001$). No statistically significant differences were observed between ILAE and HMf, as well as between both and DEXA at 3 and 4 h (**Fig. 4E, F**).

In the carrageenan paw model, the mechanical hyperalgesia was evidenced after 3 and 4 h from carrageenan injection. (**Fig. 4G**). The ILAE inhibited mechanical sensitivity by 63.7% (3 mg/kg, $P<0.01$), 73.8% (30 mg/kg, $P<0.001$), and 72.6% (100 mg/kg, $P<0.001$), while HMf (3 mg/kg, $P<0.01$) inhibited by 61.3% compared to the control group. At 4 h (**Fig. 4H**), ILAE treatments (3, 30, and 100 mg/kg) inhibited mechanical sensitivity by 79.6, 83.2, and 80.1%, respectively, and HMf (3 mg/kg) by 81.4%, all compared to the control group ($P<0.001$). The positive control, DEXA, showed a significant reduction at all time points (78.6% after 3 h and 85.4% after 4 h), with $P<0.001$, compared to the control group (**Fig. 4G, H**). When comparing treatments, no statistical difference was found between ILAE and HMf doses at 3 and 4 hours. In contrast, compared to DEXA, the positive control exhibited statistical differences from ILAE (3 mg/kg) and HMf (3 mg/kg) at 3 hours ($P<0.001$) and from ILAE (3 and 100 mg/kg) at 4 hours ($P<0.05$) (**Fig. 4G, H**).


A
Paw oedema (ml)
0.06
0.04
0.02
0.00
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
0.5 h after carrageenan injection
B
Paw oedema (ml)
0.08
0.06
0.04
0.02
0.00
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
1 h after carrageenan injection
C
Paw oedema (ml)
0.08
0.06
0.04
0.02
0.00
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
2 h after carrageenan injection
D
Paw oedema (ml)
0.08
0.06
0.04
0.02
0.00
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
4 h after carrageenan injection
E
Paw withdrawal duration (s)
6
4
2
0
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
3 h after carrageenan injection
F
Paw withdrawal duration (s)
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Basal Control 3 30 100 3 1 mg/kg
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4 h after carrageenan injection
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Sensitivity threshold (g)
4
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1
0
Basal Control 3 30 100 3 1 mg/kg
ILAE HMf DEXA
4 h after carrageenan injection

Fig. 4. Effect of oral ILAE (3, 30 and 100 mg/kg), HMf (3 mg/kg), DEXA (1 mg/kg), or the vehicle (control) administration on (A, B, C and D) carrageenan-induced paw edema; (E and F) acetone-induced cold allodynia, and (G and H) mechanical hyperalgesia in mice. Each point represents the mean±SEM of 5 animals. The (•) symbol indicates the significant differences between basal and control group (••P<0.01 and •••P<0.001), while the (*) symbol indicates the significant differences between treated groups compared with control group (*P<0.05, **P<0.01 and ***P<0.001), and (+) symbol indicates the significant differences between treated groups (+P<0.05 and +++P<0.001). Differences between groups were analyzed by one-way ANOVA followed by the Tukey's test.

Oral treatment with ILAE (3 and 30 mg/kg) and HMf (3 mg/kg) significantly reduced leukocyte counts by 45.1, 52.0, and 53.4%, respectively, compared to the control, with P<0.01 **(Fig. 5A**). The positive control, DEXA (1 mg/kg), decreased leukocyte migration by 79.2%, with P<0.001, while Naïve group decreased by 66.3%, with P<0.001 (**Fig. 5A**). Differences between treatments were found only between ILAE (100 mg/kg) and DEXA (1 mg/kg), with P<0.05 (**Fig. 5A**). Protein exudation decreased more significantly in treatment with ILAE (30 mg/kg) by 63.3% (P<0.001), while ILAE (3 mg/kg) and HMf (3 mg/kg) decreased by 52.1 and 58.2%, respectively, compared to control (P<0.01) (**Fig. 5B**). The DEXA (1 mg/kg) decreased by 66.6% compared to the control, with P<0.001, while Naïve group decreased by 64.1%, with P<0.001 (**Fig. 5B**). No differences between treatments were found.

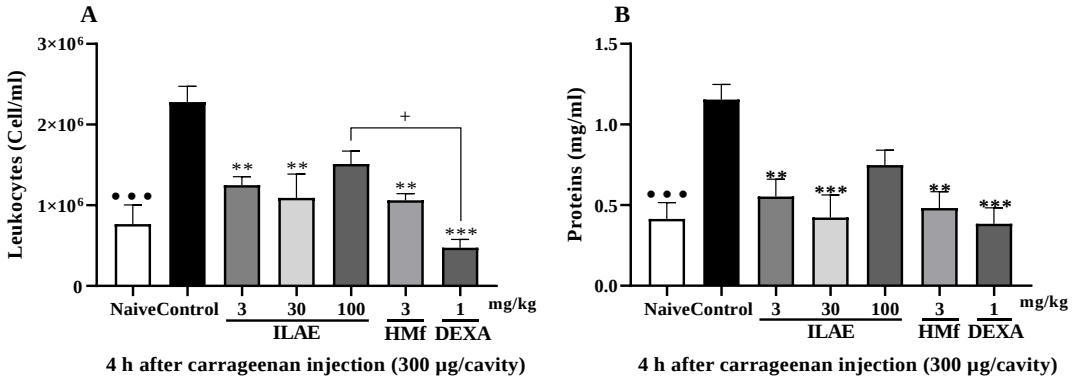

Fig. 5. Effect of oral treatment with ILAE (3, 30 and 100 mg/kg), HMf (3 mg/kg), in carrageenan-induced pleurisy model, measured by (A) total leukocyte count and (B) protein dosage. The other induced groups received DEXA (1 mg/kg, p.o.), or the vehicle (control). The data are represented as the means±SEM of 5 animals. The (•) symbol indicates the significant differences between naive and control group (•••P<0.001), while the (*) symbol compares the treated groups with the control group (**P<0.01 and ***P<0.001), and the (+) symbol indicates the significant differences between treated groups (+P<0.05). Differences between groups were analyzed by one-way ANOVA followed by the Tukey's test.

In the phase I of formalin induced nociception, no significant effects were verified when animals were treated with HMf or ILAE (**Fig. 6A**). In the phase II of formalin induced nociception the HMf (3 mg/kg) significantly reduced pain by 52.6% (P<0.001), while ILAE (3, 30, and 100 mg/kg) reduced pain by a maximum of 21.8% (P<0.05), and the positive control (MOR) caused a reduction of 76.1% (P<0.001) when compared to control group. The comparison between treatments showed a statistical difference among all groups with P<0.001, except between ILAE doses, which were not significant. The comparison did not include the MOR group (4 mg/kg) due to the difference in administration routes.

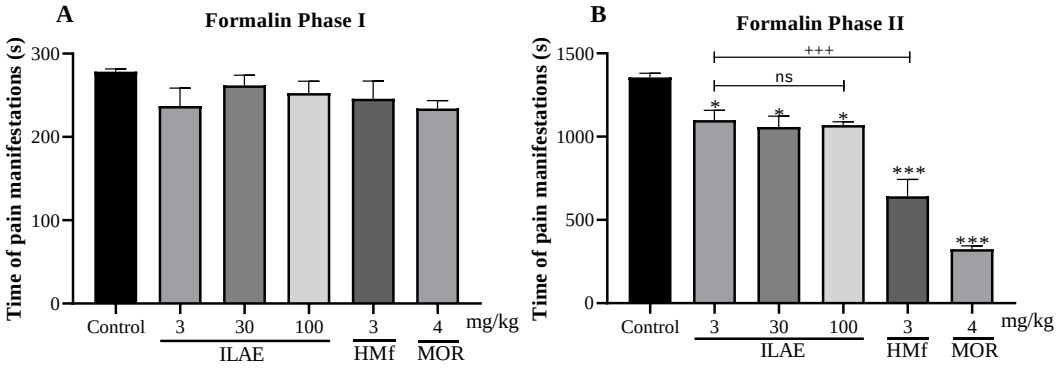

Fig. 6. Effect of treatment with ILAE (3, 30, and 100 mg/kg, p.o.), HMf (3 mg/kg, p.o.), and MOR (4 mg/kg, i.p.) on formalin-induced nociception. The nociception was measured by pain manifestations in the (A) first phase and (B) second phase after the injection of a 2.5% formalin solution in the right paw. Each column represents the mean±SEM of 5 animals. The (*) symbol indicates the significant differences between treated groups compared with control group (*P<0.05 and ***P<0.001), and (+) symbol indicates the significant differences between treated groups (except the MOR group) (+++P<0.001). “ns” indicates not significant (P>0.05). One-way ANOVA followed by the Tukey’s test was used to analyze the differences between groups.

During the evaluation of CFA-induced paw edema at various intervals, ILAE (30 mg/kg), HMf (30 mg/kg), and AE-1 (3 mg/kg) demonstrated reductions in paw edema by 74.5%, 35.5%, and 50.8% at 3 h; 71.8%, 39.0%, and 48.4% at 4 h; and 61.9%, 36.9%, and 40.4% at 24 h, respectively, compared with the control (all with P<0.001). In contrast, PRED (3 mg/kg) exhibited a more substantial reduction, with 89.1% at 3 h, 85.4% at 4 h, and 79.5% at 24 h, all relative to the control (all with P<0.001) (**Fig. 7A-C**). When comparing *A. edulis* treatments, all showed a statistical difference with P<0.001, except between HMf (30 mg/kg) and AE-1 (3 mg/kg), which exhibited P<0.05 at 3 h and no statistical difference at 4 and 24 h (**Fig. 7A-C**). In comparison with the positive control (PRED 3 mg/kg), only ILAE (30 mg/kg)

showed no significant difference at 3 and 4 h after CFA injection. And the remaining ones that showed statistical differences had a significance level of P<0.001 (**Fig. 7A-C**).

When assessing acetone-induced allodynia (**Fig. 7D-F**) and mechanical hyperalgesia models (**Fig. 7G-I**) influenced by CFA, all treatments demonstrated statistically significant inhibition compared to the control, except for HMF (3 mg/kg). The ILAE (30 mg/kg), HMf (30 mg/kg), and AE-1 (3 mg/kg) showed inhibition of acetone-induced allodynia by 88.2%, 70.5%, and 88.1% at 3 hours; 88.8%, 83.3%, and 88.7% at 4 hours; and 90.4%, 85.7%, and 90.3% at 24 hours, respectively, all with P<0.001. In contrast, PRED (3 mg/kg) exhibited a higher inhibition, reaching 92.1% at 3 hours, 92.6% at 4 hours, and 93.6% at 24 hours, all relative to the control, with P<0.001 (**Fig. 7D-F**). Comparison of treatments with *A. edulis* revealed that all treatments differed from the HMf group (3 mg/kg), with a P<0.001 in most comparisons. Only HMf (3 mg/kg) differed from the positive control (P<0.001) at 3, 4, and 24 h after CFA injection (**Fig. 7D-F**).

In the mechanical hyperalgesia model following CFA injection, ILAE (30 mg/kg), HMf (30 mg/kg), and AE-1 (3 mg/kg) exhibited inhibitions of hyperalgesia by 82.1%, 77.7%, and 80.3% at 3 hours; 68.9%, 59.0%, and 65.3% at 4 hours; and 75.9%, 70.4%, and 76.3% at 24 hours, respectively, all with P<0.001. In contrast, PRED (3 mg/kg) demonstrated a higher inhibition of 87.0% at 3 hours, 79.2% at 4 hours, and 84.9% at 24 hours, relative to the control, with P<0.001 (**Fig. 7G-I**). Group comparisons indicated that all treatments differed from the HMf group (3 mg/kg) with P<0.001. When compared with the positive control, all treatments showed a significant difference with P<0.001, except ILAE (30 mg/kg), which exhibited a smaller difference with P<0.01 at 3 h after CFA injection (**Fig. 7G-I**).

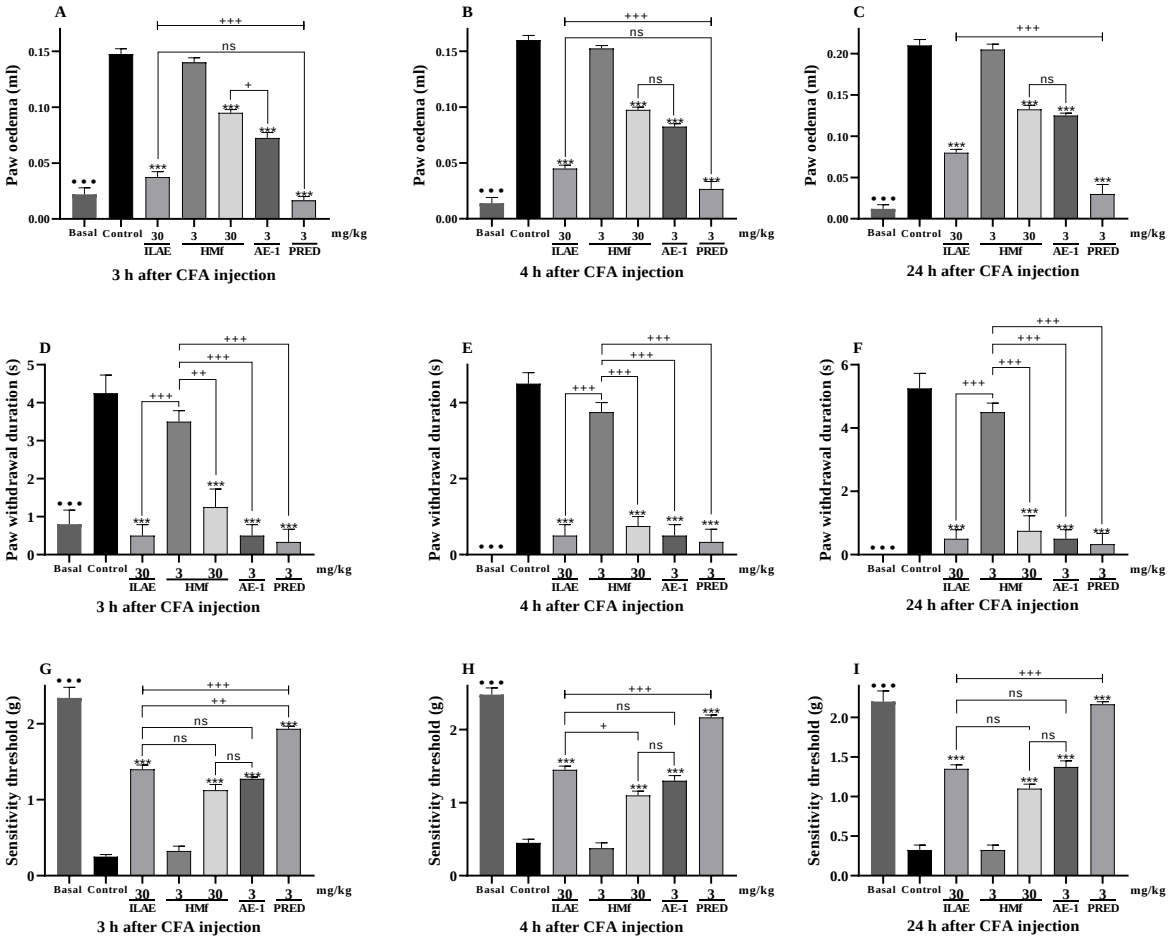

Fig. 7. Effect of oral treatment with ILAE (30 mg/kg), HMf (3 and 30 mg/kg), AE-1 (3 mg/kg), PRED (3 mg/kg), or vehicle (control) on (A, B and C) CFA-induced paw edema; (D, E and F) acetone-induced cold allodynia, and (G, H and I) mechanical hyperalgesia in mice, by 3, 4 and 24 h after of CFA injection. Each point represents the mean±SEM of 5 animals. The (•) symbol indicates the significant differences between basal and control group (•••P<0.001), while the (*) symbol indicates the significant differences between treated groups compared with control group (***P<0.001), and (+) symbol indicates the significant differences between treated groups (+P<0.05, ++P<0.01 and +++P<0.001). “ns” indicates not significant (P>0.05). Differences between groups were analyzed using one-way ANOVA followed by the Tukey’s test.

4. Discussion

This study provides the first description of secretory structures and their chemical components in *A. edulis* leaves. In addition, it reveals the acute anti-inflammatory and antinociceptive effects, along with prolonged anti-inflammatory benefits, resulting from the infusion of *A. edulis* leaves. Our results contribute significantly by aiding in the identification to the plant material and to the elucidation of the ethnobotanical use of this plant in traditional South American medicine for the treatment of inflammatory diseases (Körbes, 1995).

Allophylus edulis is naturally distributed throughout South America in biomes such as the Amazon, Caatinga, Cerrado, Atlantic Forest, and Pantanal (Forzza et al., 2010). It is described as a tree with an erect trunk reaching up to 17 meters in height (Backes and Irgang, 2004), it features trifoliate leaves (**Fig. 2b**) that are cartaceous consistency, with lateral leaflets smaller than the terminal leaflets (Lorenzi, 2016). Histological sections of the leaves revealed secretory structures, including ducts, glandular trichomes, laticifers, and idioblasts (**Fig. 2, 3**). Some of these have been previously identified in *Allophylus* species and other genera within the Sapindaceae family (Arambarri et al., 2006; Cunha Neto et al., 2017; Medina et al., 2021). Laticifers and idioblasts, identified in *Allophylus sericeus* (Cambess.) Radlk., were stored in the pith and phloem (Medina et al., 2021). In *A. edulis*, these structures, when treated with dyes, reveal the presence of essential oils, latex, phenolic compounds, tannins, and alkaloids (**Fig. 2, 3**). Medina et al. (2021) reported that the main components of latex in the Sapindaceae family are the lipid fraction, with the predominant compounds being terpenes (essential oils and resins), carbohydrates (mucilage), proteins, and phenolic compounds. In addition, the detection of alkaloids (**Fig. 3e**), even in a qualitative context, seems to be a novel observation for this species, as they have only been found in the latex of the genus *Paullinia* (Medina et al., 2021) and in the hydromethanolic extract of the leaves of *Allophylus africanus* P. Beauv. (Ibrahim et al., 2018).

Our research group has extensively investigated the presence of essential oils in *A. edulis* leaves. This exploration is driven by the chemical diversity of terpenes, as well as their potential applications in pain and inflammatory models. The results, presented in studies by Trevizan et al. (2016), Santos et al. (2021), and Santos et al. (2023), showed promising results regardless of terpene quantity and quality.

The presence of phenolic and polyphenolic compounds in the secretory structures was revealed by partitioning of the leaf infusion and spectrophotometric quantification of these secondary metabolites. Both the leaf infusion and the hexane, ethyl acetate, and hydromethanolic fractions displayed high levels of total phenolics, flavonoids, flavonols, and condensed tannins (**Table 1**).

Our results showed amounts higher than those observed by Tirloni et al. (2015), who described lower concentrations of total phenolics and flavonoids in the aqueous extract of leaves, possibly due to the low extraction temperature (4°C) they used. In our study, the extraction of phenol compounds and flavonoids may have been enhanced in the leaf infusion because traditional extractions yield higher amounts of total polyphenols at temperatures above 60°C (Antony and Farid, 2022). Based on the histological results, it is possible to infer those phenolic compounds in the idioblasts of the midrib (**Fig. 3c**), petiole (**Fig. 3g**), and petiolule (**Fig. 3k**) can be extracted at higher temperatures. Previous studies using alcoholic extracts have also reported the isolation of phenolic compounds, specially flavonoids (Arisawa et al., 1989; Hoffmann-Bohm et al. 1992; Díaz et al., 2008; Díaz et al., 2014; Arruda et al., 2018).

Given the elevated levels of all quantified constituents, the hydromethanolic fraction was selected for chromatographic column fractionation, which revealed the presence of vitexin 2"-O-rhamnoside (**EA-1**). This derivative of vitexin has an alpha residue -L-rhamnosyl attached at the 2" position of the glycosidic unit. This compound has been previously identified in alcoholic extracts of *A. edulis*, showing both angiotensin-converting enzyme inhibitory

(Arisawa et al., 1989) and hepatoprotective effects (Hoffmann-Bohm et al., 1992). Aqueous extracts of *Allophylus africanus*, with a significant amount of vitexin 2"-O-rhamnoside, also show anti-inflammatory potential (Ferreres et al., 2018). Other species with an abundance of this substance and its analogues have shown potential antinociceptive (Strada et al., 2017), anti-inflammatory (Hong et al., 1996; Rosa et al., 2016; Nascimento et al., 2021; Li et al., 2021), antioxidant (Ying et al., 2008; Wei et al., 2014; Wang et al., 2019), and immunomodulatory effects (Wang et al., 2022).

In an attempt to understand some of the mechanisms involved in the activity of this compound, we subjected it to in silico tests for drug similarity, identification of pharmacodynamic targets and oral toxicity in rodents. In silico predictive methods offer an alternative approach to streamline preclinical drug development, resulting in reduced time, cost, and dependence on animal testing. Lipinski's Rule, also known as the "*Rule-of-Five*", serves as a molecular descriptor that is reliably informative and predictive regarding whether a chemical compound will exhibit pharmacological or biological activity as an orally active drug in humans. Here, we observed three violations of Lipinski's Rule, which states that two or more violations by a compound indicate inadequate solubility and/or permeability (Lipinski et al., 1997; Lipinski, 2000). Although bile salts significantly enhancing the intestinal absorption of vitexin 2"-O-rhamnoside (Xu et al., 2008), this detail does not alter its poor bioavailability (Gao et al., 2016), which is attributed to the first-pass effect through the intestine, with hepatic and gastric first-pass effects described as nearly negligible. This Information is consistent with the high levels of TPSA (239.97 $Å^2$) (**Table 2**), indicating poor intestinal absorption, when lower than 140 $Å^2$ is desirable (Ibrahim et al., 2021). The potential to act as a GPCR ligand, kinase, protease, and enzyme inhibitor and the absence of predicted oral toxicity, are consistent with reports indicating the absence of cytotoxicity on human adipose-derived stem cells (Wei et al., 2014), inhibition of breast cancer resistance protein (BCRP) (Pick et al., 2011), and

immunomodulatory activity (Wang et al., 2022). Low toxicity of natural compounds is critical to ensure that therapeutic treatments are effective without causing harmful side effects, thereby improving the overall safety and tolerability of the potential medications.

The presence of phenolic and polyphenolic compounds, along with the potential of the isolated compound, encourages bioguided investigation, given that these substances possess antioxidant and anti-inflammatory properties (Arulselvan et al., 2016). This study marks the first exploration of the anti-inflammatory effects of *A. edulis* leaves infusion, shedding light on its traditional medicinal use in Brazil for the treatment of inflammatory diseases (Körbes, 1995). And considering the connection between oxidative stress and several pathologies, including inflammation, we evaluated the antioxidant capacity of the leaf infusion and its fractions. The results showed lower IC_{50} values through free radical scavenging methods (≤ 28.07 μg/mL, DPPH) and higher IC_{50} values in lipid peroxidation (IC_{50} ≤195.6 μg/mL, β-carotene/linoleic acid test). These results are similar to those found in the aqueous extract analyzed by Tirloni et al. (2015), which showed an IC_{50} of 45.8 μg/mL (DPPH). The antioxidant potential was also observed in the evaluation of the ethanolic (Umeo et al., 2011) and methanolic (Schmeda-Hirschmann et al., 2005) extracts of *A. edulis* fruits. This property is largely attributed to the presence of flavonoids, which have the ability to scavenge free radicals by forming less reactive phenoxyl flavonoid radicals due to their hydrogen atom donating ability. These properties are closely related to the distribution of hydroxyl and methoxy radicals as the presence of electron-donating or withdrawing groups in the aromatic system directly influences the redox potential of flavonoids (Arora et al., 1998). The presence of flavonoids, such as vitexin derivatives may also support the endogenous antioxidant defenses during a chronic inflammatory process (Lorizola et al., 2018).

To evaluate the anti-inflammatory effects of *A. edulis* leaves, we used experimental models of carrageenan and CFA-induced paw edema, carrageenan-induced leukocyte

migration, acetone-induced cold allodynia, and mechanical hyperalgesia in mice. These models were selected based on the methodology outlined by Santos et al. (2021), who used the essential oil from the leaves of the same species.

The acute anti-inflammatory effects of leaf infusion (ILAE) and HMf (phenolic compounds and flavonoids-rich fraction) were evaluated using carrageenan-induced inflammation models. Carrageenan, a mucopolysaccharide, triggers inflammation by activating genes for cytokines and promoting the migration of immune system cells (Myers et al., 2019). The carrageenan-induced inflammatory response is a local process characterized by the cardinal signs typical of inflammation, including redness, heat, pain and edema. These manifestations result from increased blood flow to the inflamed site, driven by changes in the local microvasculature, resulting in the extravasation of fluids, plasma proteins, and leukocytes, as well as proinflammatory cytokines (Pober and Sessa, 2015). Both ILAE (3 mg/kg) and HMf (3 mg/kg) reduced paw edema development time (**Fig. 4A-D**) during the early (up to 2 h) and late (3–4 h) phases of carrageenan-induced inflammation. And recognizing the efficacy of the treatments in reducing edema formation, a process resulting from microvascular changes, evaluated and observed reduced leukocyte migration (**Fig. 5A**) and protein extravasation (**Fig. 5B**), especially at the lowest doses, both of ILAE (3 and 30 mg/kg) and HMf (3 mg/kg). Although leukocyte migration is a critical mechanism of inflammatory response, chronic inflammation can exacerbate the intensity and duration of the process. The observed regulatory effect on leukocyte infiltration in the pleura may be attributed to the changes instigated by the flavonoids contained in the treatments, such as changes in leukocyte rolling ability, adhesion, and transmigration (Suyenaga et al., 2014; Werner et al., 2014). The presence of a 2, 3 double bond and the 4-keto group of the C ring, have been identified as key requirements for the inhibition of adhesion molecule expression (Lotito and Frei, 2006). This effect may be in line with the in vivo antioxidant capacity (Wang et al., 2022) and the protective effect on endothelial

cells and injured cardiac myocytes (Zhu et al., 2006), described for the vitexin 2"-O-rhamnoside (AE-1) from *A. edulis*. Although ILAE doses did not exhibit dose-dependent anti-inflammatory activity in acute assessments (paw edema and pleurisy), they generally did not significantly differ from the positive control (DEXA 1 mg/kg). Both ILAE and HMf were administered oral, suggesting potential similarities with dexamethasone, such as inhibition pro-inflammatory cytokine production (including IL-1 and IL-6), prostaglandin E2, and histamine, along with cellular and vascular effects (Madamsetty et al., 2022).

Carrageenan-induced inflammation action produces chemical mediators responsible for activating and sensitizing peripheral nociceptors, resulting in subtle changes that cause pain hypersensitivity (cold allodynia and mechanical hyperalgesia) (Li et al., 2012). While this is an adaptive response aiming to sensitize the injured area (and adjacent uninjured tissue causing secondary hyperalgesia), promoting vigilance, and preventing new injury (Jensen and Finnerup, 2014). In our study of acute inflammation induced by carrageenan and treated with *A. edulis*, we observed the ability of the treatments to consistently reduce cold allodynia by acetone spray (**Fig. 4E, F**) and mechanical hyperalgesia in the von Frey test (**Fig. 4G, H**) across doses tested, with no statistical differences between them. The effects of all treatments on cold allodynia were comparable to the positive control (DEXA 1 mg/kg) at both 3 and 4 hours after carrageenan injection (**Fig. 4E, F**). However, when evaluating the efficacy of the treatments in reducing sensitivity to mechanical stimuli (von Frey test), although all showed statistically significant effects compared to the control, there were differences compared to the positive control at 3 hours (ILAE 3 mg/kg and HMf 3 mg/kg) and 4 hours (ILAE 3 and 100 mg/kg) after carrageenan injection (**Fig. 4G, H**). Nevertheless, the consistent impact of treatments across various doses indicates that even smaller doses can effectively reduce sensitivity to both the cold stimulus of acetone and the mechanical stimulus of the von Frey test.

Previous studies by our research group have documented analogous results with essential oil, particularly at doses of 30 and 100 mg/kg, indicating a consistent reduction in edema formation and responsiveness to cold-induced and mechanical stimuli (Santos et al., 2021). Regardless of the chosen extraction method (polar or non-polar), the plant retains its anti-inflammatory properties. In extraction by infusion, we are also able to partially extract an amount of essential oil by adding the plant and leaving the mixture remains in a smothered reserve for a few minutes. Thus, leaf infusion over essential oil can result in higher yields of products derived from *A. edulis* without compromising the anti-inflammatory effect of the plant. The yield of essential oil ranges from 0.07 to 0.6% (Santos et al., 2023), while we observed a yield of 4%, contributing to the standardization of *A. edulis*-derived products.

When examining treatments for their impact on formalin-induced nociception (**Fig. 6A, B**), no antinociceptive effects were observed in the first phase, characterized by the activation of primary afferent fibers stimulated by TRPA1-mediated nociceptor activation (transient receiver potential cation channel A1). The observed effect in the second phase may be linked to vascular and cellular changes noted in earlier tests, potentially involving the reduction of inflammatory mediators in the formalin-induced lesion, as well as facilitating formaldehyde dilution and elimination in the animal's tissue. This process diminishes the substance's quantity and its excitotoxic effect (Hoffmann et al., 2022). Treatments rich in flavonoids operate through a physiological mechanism that reduces neuroinflammatory, cellular, bioenergetic, and oxidative stress markers (Basu and Basu, 2020), thereby restoring homeostasis to injured tissues. An alternative explanation is the direct interaction of treatments with opioid receptors, inducing an anesthetic effect similar to that observed with HMf (3 mg/kg) (**Fig. 6B**). This effect is documented in flavonoid studies (Higgs et al., 2013), limiting the transient excitability of demyelinated C and Aδ fibers, restored only after the interphase caused by formalin (Heinke et al., 2011; Alizadeh et al., 2014).

To determine the prolonged CFA induction after verifying the effectiveness of treatments within 4 hours following carrageenan injection (**Fig. 4A-D**). The CFA-induced paw edema is recognized as a model of chronic or persistent inflammation resulting from the continuous release of antibodies stimulating phagocytosis, cytokine secretion by mononuclear phagocytes, and the expression of costimulators for T cell activation and proliferation (Billiau and Matthys, 2001). To explore the potential for sustained effects, the intermediate dose of ILAE (30 mg/kg) was retained, given the absence of differences among the analyzed doses in carrageenan models. In investigating the persistence of HMf effects during the prolonged inflammatory process, the dose of HMf (30 mg/kg) was introduced. Furthermore, vitexin 2"-O-rhamnoside (AE-1), derived from *A. edulis*, was tested to explore any potential correlation between its presence and the observed pharmacological effects.

The effect of *A. edulis* was evident at doses of ILAE (30 mg/kg), HMf (30 mg/kg), and AE-1 (3 mg/kg) observed at 3, 4, and 24 hours post-CFA injection **(Fig. 7A-C**), revealing distinct differences in edema inhibition among treatment doses. The lack of statistical significance, compared to the positive control (PRED 3 mg/kg), was observed only in the comparison with ILAE (30 mg/kg) at 3 and 4 h after CFA injection (**Fig. 7A, B**). This suggests a prolonged and more effective result, similar to the glucocorticoid, associated with to the infusion of the leaves in their entirety (ILAE), and not only in the substances selected from the hydromethanolic fraction (HMf), or even the isolated flavonoid vitexin 2"-O-rhamnoside (AE-1), although they present antiedematogenic action when compared to the control (**Fig. 7A-C**). These results indicate that the anti-edematogenic effect of *A. edulis* lasts longer than 4 hours in animal models.

Similar results were observed when evaluating of treatment effects on cold-induced allodynia (**Fig. 7D-F**) and mechanical hyperalgesia (**Fig. 7G-I**). At 3, 4, and 24 h post-CFA injection, all treatments differed from the control group, except for HMf (3 mg/kg), was

statistically different from all other treatments in both assessments. This makes it the only dose without a significant effect in both tests (**Fig. 7D-I**). When evaluating cold allodynia, ILAE (30 mg/kg), HMf (30 mg/kg), and AE-1 (3 mg/kg) showed a statistically similar effect to the positive control (PRED 3 mg/kg) at all time points analyzed (**Fig. 7D-F**). In contrast, with respect to mechanical hyperalgesia, all *A. edulis* treatments were statistically different from the positive control, although there were minimal statistical differences between them, specifically in the doses of ILAE, HMf, and AE-1, except for HMf (3 mg/kg) (**Fig. 7G-I**).

These results demonstrate the prolonged effect of *A. edulis* treatment, except for HMf (3 mg/kg), all other treatments maintained stable effects throughout the period, reducing edema, cold allodynia and mechanical hyperalgesia. These findings are consistent with previous test results shown here, suggesting that doses ≤30 mg/kg of ILAE are more effective in treating edema, allodynia, and hyperalgesia induced by both acute (**Fig. 4A-H**) and prolonged (**Fig. 7A-I**) inflammatory agents. Notably, while HMf at a 3 mg/kg dose effectively attenuates the effects of carrageenan, it is noteworthy that only the 30 mg/kg dosage shows statistically significant effects in the CFA models. These effects are consistent with observations from our research group while assessing the impact of *A. edulis* essential oil (30 mg/kg) in the CFA model. The antiedematogenic effect persisted consistently from 1h to 9 days post-CFA injection. Meanwhile, the reduction in mechanical hyperalgesia remained virtually unchanged until the twelfth day, and the decrease in cold-induced allodynia exhibited a transient effect (Santos et al., 2021).

The well-documented antiedematogenic and analgesic effects of flavonoid-rich samples are noteworthy (Ferraz et al., 2020). While flavonoids alone can reduce inflammation and inflammatory pain (Iannitti et al., 2020), their positive impact on the inflammatory agents carrageenan and CFA may be related not only to the direct decrease of inflammatory factors or oxidizing enzymes (Afridi et al., 2019) but also to changes in amino acid metabolism, crucial

in pain transmission (Zhang et al., 2020). This includes the inhibition of regulatory enzymes, antioxidant properties, influence on arachidonic acid metabolism, and genetic and cellular modulation (Yahfoufi et al., 2018; Fraga et al., 2019; Maleki et al., 2019).

This investigation presents initial evidence of efficacy in reducing paw edema, cold-induced allodynia, mechanical hyperalgesia and nociception using the infusion of *A. edulis* leaves and HMf.

5. Conclusions

In conclusion, this study unveils the chemical diversity of secretory structures of *A. edulis* leaves, highlighting the prevalence of polyphenolic substances in the leaf infusion. In vitro studies underline its antioxidant capacity, especially in the inhibition of free radical. Treatments involving leaf infusion and hydromethanolic fraction demonstrate acute and prolonged anti-inflammatory and antihyperalgesic and antinociceptive activities. Notably, the isolated flavonoid vitexin 2"-O-rhamnoside shows prolonged anti-inflammatory effects. These effects explain the popular use of this plant as an anti-inflammatory and could facilitate the development of novel therapies for inflammation-related pathologies.

Conflict of interest

The authors declare no conflict of interest.

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5.3 Artigo III: Anxiolytic, antiamnesic and toxicological assessment of *Allophylus edulis* (A. St.-Hil., A. Juss. & Cambess.) Radlk. leaves

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Therapeutic potential, subacute toxicological analyses and in silico studies of compound and extracts from the leaves of *Allophylus edulis* **(A. St.-Hil., A. Juss. & Cambess.) Radlk.**

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Therapeutic potential, subacute toxicological analyses and in silico studies of compound and extracts from the leaves of *Allophylus edulis* (A. St.-Hil., A. Juss. & Cambess.) Radlk.

Background: *Allophylus edulis*, a plant native to the Brazilian Cerrado, renowned for its antioxidant and anti-inflammatory properties, and abundant in flavonoids, remains unexplored in terms of its anxiolytic potential, its ability to counteract cognitive impairment induced by scopolamine as well as the subacute toxicity of the leaf infusion. **Objectives:** This study explores the pharmacological effects of *A. edulis* leaf infusion (ILAE 3, 30, and 100 mg/kg) and the hydromethanolic fraction (HMf 3 mg/kg), focusing on anxiolytic effects (light/dark and open field tests) and short-term memory protection (new object recognition test), as well as spatial learning memory (Morris water maze test). Additionally, we assess the antioxidant and acetylcholinesterase inhibitory impact on the mouse brain. In silico analysis examines the interaction capacity of the compound abundant in ILAE. Subacute toxicity evaluation of ILAE (30, 100, and 300 mg/kg) aims to establish the safety of leaf infusion. **Results:** Although treatments minimally inhibit anxious behavior, they exhibit the ability to partially restore short-term memory impairment induced by scopolamine in the object recognition test. Neuroprotective effects observed may be attributed to antioxidant impact and the reduction in acetylcholinesterase (AChE) activity in the brain homogenate of treated mice. Molecular re-docking of vitexin 2"-O-rhamnoside (AE-1) reveals hydrogen bonding interactions, both conventional and unconventional, along with π-stacking interactions with the enzyme. Oral toxicity evaluation in mice during the 28-day treatment period indicates low toxicity across the assessed parameters. **Conclusion:** These findings contribute significantly to characterizing *A. edulis* as a medicinal plant with neuroprotective properties.

Keywords: vacum; anxiety; amnesia; acetylcholinesterase; flavonoid; scopolamine.

1. Introduction

Vacum (scientifically known as *Allophylus edulis*, Sapindaceae) is a South American tree that thrives in several biomes, including the Amazon, Caatinga, Cerrado, Atlantic Forest, and Pantanal (Forzza et al., 2010). It is native to countries such as the Guianas, Bolivia, Paraguay, Uruguay, and Argentina (Díaz et al., 2014). This species is used in traditional Guarani practices and adopted by migrants in the region, wich is commonly prepared as an infusion, decoction, or cold maceration drink known as tereré (often combined with *Ilex paraguariensis* (Aquifoliaceae) and other species). *A. edulis* is used medicinally for gastrointestinal, hepatic, diuretic, hypertensive, and anti-inflammatory disorders (Arisawa et al., 1989; Körbes, 1995; Franco and Fontana, 2001; Alves et al., 2008; Kujawska and Pardo-De-Santayana, 2015; Kujawska and Pieroni, 2015; Kujawska and Schmeda-Hirschmann, 2022).

The aqueous, alcoholic extracts, and/or essential oils have demonstrated antioxidant (Tirloni et al., 2015; Piekarski-Barchik et al., 2021), nephroprotective (Galeano et al., 2023), anticholinesterase effects in vitro (Umeo et al., 2011), hepatoprotective (Hoffmann-Bohm et al., 1992), negative ionotropic in vitro (Matsunaga et al., 1997), and anti-inflammatory properties (Trevizan et al., 2016; Santos et al., 2021; Balsalobre et al., 2023). These effects are attributed to polyphenolic substances, especially flavonoids, which are abundant in A. edulis leaves (Arisawa et al., 1989; Hoffmann-Bohm et al., 1992; Díaz et al., 2008; Díaz et al., 2014).

Neurodegenerative diseases, such as Alzheimer's, becoming increasingly prevalent worldwide, particularly among the elderly, causing memory loss, anxiety, and dementia. The changes associated with disturbances in the central cholinergic system led to an irreversible cognitive decline. Current dementia treatments, such as acetylcholinesterase (AChE) inhibitors, namely donepezil, rivastigmine, and galantamine, are symptomatic but associated with side effects, including nausea, vomiting, anorexia, and insomnia (Tan et al., 2014; Briggs et al., 2016; Lei et al., 2021; Ferrari and Sorbi, 2021; Ruangritchankul et al., 2021).

Numerous medicinal plants, including *A. edulis* with its flavonoids, such as quercetin, vitexin, and isovitexin, have been cited for reducing symptoms of cognitive decline, attenuating neuroinflammation, improving cholinergic and glutamatergic communication, and decreasing tau protein phospholylaton (Li et al., 2022).

The aim of this study was to investigate the effects of *A. edulis* leaf infusion (ILAE) and hydromethanolic fraction (HMf) on the cognitive performance in mice using light/dark, open field, novel object recognition and Morris water maze tests. In addition, AChE inhibition and lipid peroxidation in brain homogenate, were measured. Study of the molecular re-docking in

AChE were carried out with the main constituent (vitexin 2"-O-rhamnoside) obtained of the HMf. Also, this study aims to determine the subacute toxicity of the infusion of *A. edulis* leaves.

2. Material and methods

2.1. Drugs and solvents

Scopolamine hydrobromide, Bradford reagent, (5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (AcSCh) and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid and thiobarbituric acid were purchased from Dinâmica (São Paulo, SP, BR). Ketamine and xylazine were purchased from Syntec (Santana de Parnaíba, SP, Brazil). Other drugs and reagents used were of analytical grade.

2.2. Plant material, infusion, fractioning, and isolation of vitexin 2''-O-rhamnoside (AE-1)

The leaves of *A. edulis* were collected from the Medicinal Plant Garden at the Federal University of Grande Dourados (UFGD) the city of Dourados (Mato Grosso do Sul, Brazil, 22°11'43.7"S 54°56'08.5"W). Dr. Zefa Valdivina Pereira identified and deposited in the herbarium of the UFGD 342. The authorization to access the Brazilian genetic heritage was obtained through the National System for the Genetic Heritage and Associated Traditional Knowledge Management (SisGen-A51F665). The infusion of *A. edulis* leaves (ILAE), hydromethanol fraction (HMf) and vitexin 2"-O-rhamnoside (AE-1) were obtained as shown in a previous study (unpublished data).

2.3. Animals and ethical approval

Adult male and female Swiss mice (25-30 g) were provided by the Central Animal Care Facility of the Federal University of Grande Dourados. The animals were housed in polypropylene cages measuring 30x20x13 cm. Housing conditions maintained a temperature of 22±2°C with a 12:12 h light-dark cycle, and the mice had free access to water and standard pelleted food. Euthanasia was performed by administering a lethal injection of ketamine (300 mg/kg) and xylazine (30 mg/kg) or cervical dislocation, *according to the* euthanasia practice guideline outlined in Normative Resolution No. 37/2018 of the National Council for the Control of Animal Experimentation (CONCEA). The parameters for animal handling were also established by CONCEA, and the project was approved from the Committee of Ethics on the Use of Animals (CEUA) at the Federal University of Grande Dourados (approval number 05.2021).

2.4. Experimental Design - Animal grouping and drug administration for scopolamine-induced amnesia and brain collection for antioxidant and AChE activity analysis

Two days before the start of the experiment, the animals were acclimatized in the laboratory. After a 2-day acclimation period, mice were distributed into 6 groups (n=6) and treated once daily for 16 days. Groups received oral a 0.9% saline solution (control) and three doses of ILAE (3, 30, and 100 mg/kg) and HMf (3 mg/kg). A scopolamine group was also used, and treatment consisted of an intraperitoneal injection (once daily) of a solution containing scopolamine (1 mg/kg, i.p.) from day 11 to day 16. All groups underwent light/dark, open field, novel object recognition (NOR) and Morris water maze (MWM). For the NOR and MWM tests, an additional group was included, receiving only scopolamine (1 mg/kg, i.p.). For the ILAE and HMf memory test analyses, mice also received a daily injection of scopolamine (1 mg/kg, i.p.) from day 11 to day 16, administered 0.5 hours prior to the experiment to induce amnesia. On day 16, after completion of the tests, all animals were sacrificed, and their brains were isolated for subsequent determination of AChE activity and antioxidant levels using the MDA method. The complete scheme is shown in **Figure 1**.

2.5. Behavioral tests for anxiety

2.5.1. Light/dark box test

The light/dark transition test (Crawley and Goodwin, 1980; Costall et al., 1989) consisted of two rectangular compartments, one light and one dark, made of white and dark materials. The dimensions were 40x40 cm for the light compartment and 40x33 cm for the dark compartment, interconnected by a slit (6 x 6 cm). On day 9, after 1 hour of treatment, the animals' behavior was recorded for 5 minutes (using ANY-maze version 7.2, Stoelting Co. USA). Parameters included total time spent in the light and dark compartment. After each trial, the floor of the apparatus was cleaned using a 15% ethanol solution.

2.5.2. Open field test

One hour after treatment (day 10) the mice were individually placed in the center of the open field (62 cm diameter circular apparatus) (Hall, 1934), and allowed to explore freely for 5 minutes under standard room lighting conditions. The assessment of anxiety was based on the mouse's preference for the open areas of the field. This revealed that open areas are anxiogenic, although their innate curiosity drives exploration. Parameters analyzed included the

thigmotactic ratio, time spent in the central zone, and the number of line crossings, all automatically recorded using ANY-maze version 7.2 (Stoelting Co. USA). In addition, the number of rearings and fecal boli were manually evaluated. After each trial, the floor of the apparatus was cleaned with a 15% ethanol solution.

2.6. Behavioral tests for learning and memory

2.6.1. Novel object recognition (NOR) test

The day before the test, animals were allowed to freely acclimatize to the test apparatus for 10 min without any objects present. The apparatus comprised of a 40x40 cm polypropylene box (Ennaceur and Delacour, 1988). On the test day (day 11), two identical objects were placed inside the box, allowing mice a 10-minute exploration period. Subsequently, the animals received oral treatment, followed by scopolamine administration (1 mg/kg, i.p.) after 30 minutes. One hour after treatment, the animals returned to the apparatus for another 10 minutes, during which the objects were replaced – one new and one identical to the familiar object. Time spent with the novel object was measured using ANY-maze version 7.2 (Stoelting Co. USA) and included instances in which mice touched the object with their nose or directed their nose toward the object at a minimum distance of 2 cm. The discrimination index (DI) was then calculated based on these observed parameters, reflecting improved working memory if increased. DI = [(time spent on new object - time spent on old object)/ (time spent on new object + time spent on old object)].

2.6.2 Morris water maze test

The circular tank (65 cm in diameter and 30 cm in height) , was divided into four quadrants, with the water temperature maintained at 25 ± 1 °C. A submerged platform (1 cm below the water surface) was placed in one quadrant, referred to as the target quadrant/ platform quadrant (Morris, 1984). The apparatus was equipped with a video camera and a tracking system (ANY-maze version 7.2, Stoelting Co., USA) to record the performance of the mice. The acquisition test, which lasted from day 12 to day 15, consisted of three daily trials starting from different quadrants. The mice were given 60 seconds to locate the hidden platform; if unsuccessful, they were gently led to the platform and allowed to remain for 20 seconds. After finding the platform, they spent an additional 10 seconds on it for familiarization. The platform remained stationary, while starting points varied randomly. Escape latency served as the measure for acquisition or learning. In day 16, the platform was removed for a 60-second probe trial. Mice were placed in the apparatus opposite to the target quadrant, and number of platform

zone entries, time spent in platform zone, and distance travelled until first entry into the platform zone were assessed.

2.7. AChE activity and lipid peroxidation

2.7.1. Determination of acetylcholinesterase activity

On the last day (day 16) of the study, all mice were anesthetized with ketamine (300 mg/kg) and xylazine (30 mg/kg), before decapitation for brain extraction. To prepare the brain homogenate, the brain was homogenized and crushed in a 5% weight/volume phosphate buffer solution (0.01 M, pH 7.4) using a TURRAX-type homogenizer (Marconi, Brazil). The homogenate was then centrifuged (6000 rpm) for 20 minutes. An aliquot (10 µl) of the supernatant was collected, and the protein concentration was estimated using Bradford reagent (Bradford, 1976). A mixture of 25 µl of brain homogenate, 150 µl of potassium phosphate buffer (0.1 M, pH 7.4), 100 µl of DTNB (0.01 M, pH 7), and 20 µl of acetylthiocholine iodide (0.075 M), was added to a microplate (iMark, Bio-Rad Laboratories, USA), and absorbances were read at 415 nm for 7 minutes, with readings taken every 30 seconds (Ellman et al., 1961) and the specific activity was calculated using the formula: AChE activity = (ΔA x total volume x 60)/13.6 x AcSCh volume x protein of tissue (mg/ml)), were: ΔA is the change in absorbance per minute; and 13.6 represents the TNB molar absorption coefficient. All the reactions were performed in triplicate and expressed as hydrolysis of acetylthiocholine in µmol per hour per milligram protein (µmol AcSCh/h/mg of protein).

2.7.2. Determination of lipid peroxidation inhibition

On the last day (day 16) of the assessments, based on the methodology developed by Stocks et al. (1974), total mice brain homogenate was obtained by homogenization crushing with phosphate-buffered saline using a TURRAX-type (Marconi, Brazil). The homogenate was centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was collected and further diluted (1:3, v/v) in phosphate-buffered saline. Subsequently, 3 ml of the homogenate was mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged for at 3000 rpm for 20 min. After discarding the precipitated proteins, the sample was incubated in a water bath at 37°C for 1 hour. After this incubation, 1 ml of 0.67% thiobarbituric acid was added, and the mixture was kept in a water bath at 100°C for 15 minutes. The reaction mixture was then cooled in an ice bath and read at 532 nm using a UV spectrophotometer (Bel Photonics, Monza, IT). The percentage of lipid peroxidation inhibition was calculated using the following formula: %I =

(Control group Abs - Sample Abs) / Control group Abs x 100. All the reactions were performed in triplicate.

2.8. Molecular Re-docking

Molecular anchoring tests were performed using the three-dimensional structure of vitexin 2"-O-rhamnoside (AE-1) obtained from PubChem website (https://pubchem.ncbi.nlm.nih.gov/compound/Vitexin-2_-O-rhamnoside) and the structure of the AChE (AChE, PDB: 5NAP, resolution 2.17 Å) complexed with a non-chiral inhibitor analogous to donepezil (DZ7). The simulations were carried out using the Gold (2020.3.0) program (Jones et al., 1997), following the steps: (i) removal of all water molecules from the enzyme structure; (ii) the ligand binding site DZ7 was used in the automatic function of the program to recognize the ligand cavity of interest; (iii) radius of 15Å, together with the genetic search algorithm and the ChemPLP ranking function. Finally, 10 scans were simulated to establish the re-docking protocol.

For molecular docking simulations, this was increased to 100 runs per ligand and 5 scans in total. Score values were determined as the simple arithmetic mean of the five scans plus the population standard deviation value of the measurements taken. Interactions of vitexin 2"-O-rhamnoside (AE-1) with the receptor were analyzed using the software Discovery Studio Visualizer version 21.1.0.20298 (BIOVIA, San Diego, CA, USA).

2.9 Subacute Oral Toxicity Study

The evaluation of oral toxicity in mice was performed according to OECD Guideline 407 (58 OECD, 2008). Thirty-two female Swiss mice (n=8) were divided into 4 groups. These groups received different oral treatments for 28 days: a negative control group (0.9% saline solution) and treatment groups administered with ILAE (30, 100, or 300 mg/kg) solubilized in 0.9% saline solution. Behavioral and physiological changes were closely monitored throughout the 28-day period. At the end of the experiment, euthanasia was performed by cervical dislocation, and macroscopic and histopathological evaluations of organs were performed. Blood samples were collected, and hematological parameters (erythrocyte and platelet counts, total and differential count of leukocytes, hematocrit, hemoglobin, and hematimetric indexes), and biochemical parameters (glucose, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, albumin, total cholesterol) were evaluated.

2.9. Statistical analysis

Statistical comparisons were made using a one or two-way analysis of variance (ANOVA) followed by Tukey's test, and the differences were considered statistically significant when $P<0.05$. All statistical calculations and graphs were generated using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Behavioral tests for anxiety

Anxiety-like behavior was first assessed using the light/dark test (**Figure 2**). Animals treated with ILAE (30 and 100 mg/kg) and HMf (3 mg/kg) showed a significant reduction in the time spent in the dark zone, with values of 19.28, 20.57 and 18.99%, respectively compared to control (**Figure 2A**). The anxiolytic effect was also presented based on the time spent in the light zone, which increased for treatments with ILAE. An increase of 31.9% (ILAE, 30 mg/kg), 33.7% (ILAE, 100 mg/kg) and 31.94% (HMf, 3 mg/kg) was observed (**Figure 2B**). There were no significant differences among treatments.

Anxiety related to locomotor performance was assessed using the open field test (**Figure 3**). A significant decrease in the thigmotactic ratio was observed in ILAE (30 mg/kg) and HMf (3 mg/kg), with values of 60.59 and 56.44%, respectively, compared with control (**Figure 3A**). This trend also extended to the time spent in the centre zone, which was higher at doses of 30 mg/kg of ILAE (47.97%) and 3 mg/kg of HMf (49.36%) compared to control (**Figure 3B**). When comparing treatments, significant differences were found only when ILAE (100 mg/kg) was compared with ILAE (30 mg/kg) and HMf (3 mg/kg), with $P<0.05$ for both (**Figure 3B**). There was an increase line crossing, more significant at ILAE (3 mg/kg) and HMf (3 mg/kg) by 48.66 and 55.18%, respectively, when compared to control (**Figure 3C**). When comparing treatments, significant differences were found in the comparison between ILAE (100 mg/kg) and HMf (3 mg/kg), with $P<0.001$ (**Figure 3C**). And a decrease in spontaneous rearing, significantly with ILAE (30 mg/kg), by 40.07%, compared to control (**Figure 3D**). In addition, a decrease in defecation was observed in ILAE (100 mg/kg) and HMf (3 mg/kg) of 47.91 and 65.27%, respectively, compared to control (**Figure 3E**). When comparing the treatments, they were all differed from each other ($P<0.001$), with no significance only in the comparison between ILAE (100 mg/kg) and HMf (3 mg/kg) (**Figure 3E**).

3.2 Behavioral tests for cognitive function

Short-term memory was assessed using the novel object recognition test (**Figure 4**). Scopolamine was able to induce a decrease in memory compared to the control group, which

was observed both in the shorter time spent exploring the novel object (**Figure 4A**) and in the lower discrimination index (**Figure 4B**). The effect of scopolamine was reversed by treatments with ILAE (3 and 30 mg/kg) and HMf (3 mg/kg), with increase in 71.31, 67.32 and 67.44%, respectively, when compared with scopolamine (**Figure 4A**), allowing to recall the familiar object and to devote more time to explore the novel object, as observed by the increased discrimination index (**Figure 4B**). No significant differences were found among treatments.

Spatial learning and long-term memory were assessed using the Morris water maze test (**Figure 5**). The post-hoc test revealed that scopolamine alone significantly increased escape latency values from the day 13 onwards ($P < 0.01$), indicating a decrease in platform recall, when compared to control group (**Figure 5A**). At day 15 treatments with ILAE (3 and 30 mg/kg) and HMf (3 mg/kg) show a statistically significant decrease in escape latency compared to the scopolamine group (**Figure 5A**). On probe day, long-term memory retention was assessed. Scopolamine injection alone reduced the number of platform quadrant entries (43.39%) (**Figure 5B**) and the time spent in the platform quadrant (63.12%) (**Figure 5C**) compared to the control group, indicating memory loss in the mice. A significant improvement in memory conservation was observed only in incread (41.17%) of platform quadrant entries by HMf (3 mg/kg) treatment compared to scopolamine (**Figure 5B**). A significant increase (62.25%) in the distance traveled to reach the platform quadrant for the first time was also observed in the scopolamine group compared to the control group (**Figure 5D**). All treatments of ILAE were able to reduce this distance, highlighting the doses of 3 and 100 mg/kg (79.17 and 83%, respectively), when compared to the scopolamine group (**Figure 5D**). There were no statistically significant differences between treatments.

The treatments with ILAE (3 mg/kg) and HMf (3 mg/kg) showed inhibition of lipid peroxidation of 62.44 and 62.12%, respectively, in comparison to the scopolamine group (19.12%) (**Figure 6A**). The scopolamine group showed displayed a significant increase of 34.77% of the AChE activity, when compared to the control group (**Figure 6B**). All treatment with ILAE (3, 30 and 100 mg/kg) and HMf (3 mg/kg) significantly reduced AChE activity in the homogenate obtained from brains of mice and the observed inhibitions were of 47.52, 30.59, 25.52 and 44.49%, respectively (**Figure 6B**). The treatment with ILAE (3 mg/kg) ($P<0.001$) showed significant differences when compared to the ILAE (30 and 100 mg/kg) (**Figure 6B**). The treatment with HMf (3 mg/kg) showed significant differences in relation to ILAE (100 mg/kg) ($P<0.001$) and ILAE (100 mg/kg) ($P<0.01$) (**Figure 6B**).

3.3 Molecular Re-docking

An illustration of the three-dimensional (3D) modeled molecular surface structure of vitexin 2"-O-rhamnoside within the binding site of 5NAP (AChE enzyme) is shown (**Figure 7A**). A focused examination of the docked compound superimposed on DZ7 confirms that the flavonoid occupies binding pockets analogous to those of DZ7 (**Figure 7B**). In the DZ7 binding site of the AChE enzyme, vitexin 2"-O-rhamnoside participates in seven hydrogen bonding interactions, six of which are conventional and one unconventional, and three π-stacking interactions.

The crucial residues in the AChE enzyme binding site (**Figure 7B**) indicate hydrogen bonding interactions with the glucitol moiety (TYR121 and TYR70), the rhamnosyl moiety (TYR334), A ring atoms (SER286), and C ring atoms (PHE288 and ARG289). Additionally, carbon-hydrogen bonds form between C ring atoms and ILE287. Furthermore, π-stacking interactions are observed between the B ring and PHE331, as well as between the A and C rings with TRP279.

These intermolecular interactions mentioned are important for anchoring the ligand in the binding site and are in accordance with some characteristics present in that cavity: binding site cavity has few residues containing hydrophobic regions (**Figure 8A**), and almost no regions containing residues with electrical charges (**Figure 8B**). The region of the enzyme in which vitexin 2"-O-rhamnoside is anchored has more hydrogen bond donor and acceptor regions and regions containing more aromatic residues (**Figure 8C** and **8D**, respectively).

3.4 *Subacute Oral Toxicity Study*

In the 28-days subacute toxicity animal model, daily oral administration of ILAE (30, 100 and 300 mg/kg) did not result in mortality or any observable signs of toxicity. A summary of the physiological parameters observed in mice exposed to ILAE is represented in **Table 1**. Body weight values showed no significant difference at baseline, and this consistency maintained after 28 days of ILAE administration. The results of weight gain of treated groups were also not statistically different from to the control group. The results of food consumption were statistically similar in all groups, water consumption was significantly different from the control at doses of 100 mg/kg ($P<0.01$) and 300 mg/kg ($P<0.001$), resulting in decreased consumption of 19.3% and 22.1%, respectively. After 28 days of ILAE administration, vital organs (brain, liver, kidneys, spleen, heart, and lungs) were weighed. Statistically significant changes in organ weight were observed only in the liver, with a decrease of 4.8% (ILAE 30 and 100 mg/kg) and a 9.5% decrease (ILAE 300 mg/kg), with P values of <0.01, <0.05, and <0.001, respectively (**Table 1**).

The oral administration of ILAE's induced changes in only one hematological parameter, as summarized in **Table 1**, indicating that platelet counts were significantly different from the control group only at doses of 30 mg/kg and 300 mg/kg, with $P<0.05$ and $P<0.001$, respectively. The 30 mg/kg dose showed a 7.0% decrease, while the 300 mg/kg dose showed a 15.1% increase. Among the biochemical parameters, only AST levels demonstrated a statistically significant change. The ILAE (100 mg/kg) showed an increase of 33.7% ($P<0.001$), while the ILAE (300 mg/kg) showed a decrease of the 26.4% ($P<0.001$).

4. Discussion

This study presents, for the first time, an exploration of *A. edulis* properties on cognitive abilities in a mouse model. The models used in this study aim to mimic natural anxiety and manifestations akin to neurodegenerative conditions, such as amnesia, seen in dementia, particularly Alzheimer's disease. Dementia is characterized by a progressive decline in cognitive function (Andersson and Stone, 2023). Anxiety, whether associated to dementia or not, is known to increase the risk of cognitive decline (Sun et al., 2023). Consequently, may be beneficial not only for the well-being of individuals with dementia but also for the prevention of dementia itself.

Rodents naturally exhibit anxiolytic behaviors, with protective mechanisms that result in a preference for less exposed areas near walls in an open and lit arena (Horev et al., 2007). The evaluation of the anxiolytic effect performed out on the ninth day of treatment, using the light/dark test (**Figure 2**), revealed a preference for the brighter and generally more aversive area, compared to the control group. Complementarily, the treatments were also able to provoke anxiolytic behaviors in the open field test on the tenth day of treatment, with no differences among treatments (**Figure 3**). The anxiolytic effect to all doses is evident from the fact that the treated animals successfully navigated the anxiety-inducing open fields and generally showed a greater ability to explore, with few statistical differences among treatments. There was an observable increase in horizontal locomotor activity, measured by line crossings (**Figure 3C**). These data reflect an increase in general motor activity, although no difference in distance traveled was observed among groups (data not shown). Conversely, a decrease in vertical locomotor activity, indicated by spontaneous rearing (sum of supported and unsupported rearing), was significantly at higher doses ILAE (30 and 100 mg/kg) (**Figure 3D**), reflecting lower directed exploration. In addition, a decrease in defecation, considered a parameter of emotional stress was more evident in ILAE (100 mg/kg) and HMf (3 mg/kg) (**Figure 3E**).

Compounds with an affinity for the GABA receptor are believed to play a significant role in anxiety (Felice et al., 2022). Given that *A. edulis* is a polyphenol-rich plant (Arisawa et al., 1989; Hoffmann-Bohm et al., 1992), particularly flavonoids, many of which are known to interact with benzodiazepine sites on GABA receptors (Karim et al., 2018; Ríos et al., 2022), it is plausible that this contributes to anxiety relief. And although it is an undesirable effect of several anxiolytics, the negative ionotropic effect on the guinea pig atrium reported by Matsunaga et al. (1997) in fractions of the methanolic extract of *A. edulis* may be associated with the observed anxiolytic effect here. This is because, although they act on GABA receptors, different subtypes of receptors generate distinct responses (Rudolph and Knoflach, 2011).

From the eleventh day of treatment with ILAE and HMf, memory and spatial learning tests were performed by inducing amnesia with scopolamine. Scopolamine model used is a method to simulate cognitive decline by disrupting learning and impairing of short (NOR test) and long (MWM test) term memory in rodents. This is done by inhibiting of muscarinic receptors in the brain, which prevents memory encoding (Gedankien et al., 2023), inducing neuroinflammation (Cheon et al., 2021), causes oxidative stress (Rahimzadegan and Soodi, 2018), and increases acetylcholinesterase activity (Wong-Guerra et al., 2017).

We assessed spontaneous recognition capacity by observing animals' innate tendency to respond more to a new stimulus than to a familiar one (NOR test). Notably, the administration of scopolamine alone reduced mice's ability to differentiate the novel object from the familiar one, significantly decreasing both the time spent on the novel object (**Figure 4A**) and the discrimination index (**Figure 4B**). It is known that flavonoids demonstrate neuroprotective activity, even at low concentrations in the brain (Figueira et al., 2017). We observed a neuroprotective effect on short-term memory, showed at the treatment ILAE (3 and 30 mg/kg) and HMf (3 mg/kg) (**Figure 4)**, indicating that a dose-dependent effect does not occur, and that low doses can improve short-term memory impairment induced by scopolamine.

When we subjected the animals to spatial learning in the MWM test scopolamine-induced, we observed no improvement in cognitive performance during acquisition in the first three days of training, with a slight improvement on the fourth day (**Figure 5A**). The *A. edulis* treatments were ineffective in mitigating cognitive decline in long-term memory, as demonstrated by the animals' inability to recall the platform over the 16-day period. *A. edulis* was able to improve short-term memory more effectively in the new object recognition test **(Figure 4)**, whereas in long-term memory it was more effective only in the distance travelled until first entry into the platform quadrant **(Figure 5D)**.

After completing behavioral assessments, brain homogenate was used to evaluate lipid peroxidation and AChE activity. And all treatments demonstrated discrete potential in inhibiting lipid peroxidation (**Figure 6A**). Inhibiting the formation of MDA, the final product generated by the decomposition of arachidonic acid and other larger polyunsaturated lipids, serves as a predictor of the antioxidant capacity of *A. edulis*. This capability, whether in inhibiting free radicals or lipid peroxidation, has been previously reported for alcoholic extracts (Tirloni et al., 2015), attributed to high concentrations of polyphenols known for their reducing action (Kumar and Goel, 2019).

While biological factors such as oxidative stress, neuroinflammation, and protein accumulation are linked to the pathogenesis of neurodegenerative diseases, it is essential to emphasize that the foundation of memory processing heavily relies on the cholinergic system. This system plays a crucial role in maintaining acetylcholine levels (Newman et al., 2012). This dependence becomes particularly evident when considering that one of the mechanisms scopolamine-induced amnesia in rodents involves a specific increase in AChE activity, thereby manipulating acetylcholine. The scopolamine group displayed elevated levels of AcSCh hydrolysis (**Figure 6B**), reinforcing the notion that this is one possible mechanism inducing amnesia in rodents, manipulating acetylcholine. Memory impairment due to reduced acetylcholine levels can be mitigated with AChE inhibitors as demonstrated when mice were treated with ILAE and HMf (**Figure 6B**). The AChE inhibition using a bioautographic assay had previously been described for the ethanolic extract of *A. edulis* fruits (Umeo et al., 2011). Still, this marks the first description of AChE activity inhibition in animal models. The reduction in AChE activity may, at least partially, account for the effects observed in short-term memory during object recognition (**Figure 4A, B**), yet it is insufficient to enhance the parameters of long-term memory and spatial learning **(Figure 5**). The benefits of treatments aiming to inhibit AChE include improving the cholinergic anti-inflammatory signal (Shaked et al., 2009) and inhibiting motor neuron loss (Gotkine et al., 2015), enabling application in various pathologies.

The neuroprotective efficacy of flavonoid-rich samples can be attributed, in part, to the ability of these metabolites to influence various systems, as antioxidant defenses, and promotion of neuronal connectivity (Bakoyiannis et al., 2019; Cheatham et al., 2022). New molecules have been constantly sought, aiming to inhibit AChE in silico (Das et al., 2017). AChE is considered one of the enzymes with the highest catalysis rates, including the catalysis of acetylcholine. The catalytic site is formed by three amino acids (SER203, GLU334, and HIS447), but there are also peripheral binding sites. So that interaction can happen with both

sites (Peitzika and Pontiki, 2023). In this context, the fractionation of HMf resulted in the isolation of the glycosylated flavonoid, vitexin 2"-O-rhamnoside, which may partly corroborate the effect of *A. edulis*. Thus, to explain the possible effect, study of the re-docking was established using the GOLD program, in which the genetic search algorithm was able to reinsert the ligand DZ7 in AChE with a low deviation from the crystallographic pose, determined in terms of RMSD = 0.43±0.03. The structure of vitexin 2"-O-rhamnoside was inserted into the molecular docking simulations, whose average score was 78.87±2.08.

With molecular redocking we observed seven hydrogen bond interactions, six of which are conventional and one unconventional, and three π-stacking interactions. As well as few residues containing hydrophobic regions, or with electrical charges, despite having more hydrogen bond donor and acceptor regions and regions containing aromatic residues. It is possible to note that, predominantly, interactions involving π systems occur between amino acid residues containing aromatic groups and the aromatic rings of the flavonoid portion of the ligand. Hydrogen bonds occur both with polar regions of the flavonoid portion and with the polar regions of the sugar portion, resulting mainly from hydroxyl groups (OH).

This study marks the inaugural exploration of the toxicological potential of *A. edulis* leaf infusion. Previously, only acute toxicity evaluations in rats have been reported, indicating low toxicity with a single dose of 2 and 5 g/kg of the ethanolic leaf extract, exhibiting an LD_{50} greater than 5g/kg (Tirloni et al., 2015). In this study, we present the first evidence of low toxicity associated with leaf infusion at doses of 30, 100, and 300 mg/kg.

The only parameters that statistically differed from the control group were the reduction in liver weight and altered AST values, indicating a potential impact on hepatic metabolism – a finding also observed by Tirloni et al. (2015). The increase in AST values at a dose of 100 mg/kg is a sign of hepatotoxicity. A dose of 300 mg/kg, in turn, revealed much lower values compared to the control, which combined with the more intense decrease in liver weight (9.5%), is possible that this reflects the loss of functional mass associated with atrophy, also characteristic of acute injury (Cattley and Cullen, 2013). Additionally, while platelet count relative values differed statistically from the control, they remained within the reference values for mice (O'Connell et al., 2015).

5. Conclusions

Our study investigated the effects of *A. edulis* leaf infusion (ILAE) and hydromethanolic fraction (HMf) on mice cognitive performance using various behavioral tests. The ILAE demonstrated significant anxiolytic effects and protected short-term memory. The results linked

cognitive benefits to *A. edulis*'s antioxidant properties and AChE inhibition. Molecular re-docking studies with vitexin 2"-O-rhamnoside from HMf supported these findings. Additionally, we evaluated the subacute toxicity of ILAE over 28 days, confirming its low toxicity and safety for potential therapeutic use.

Conflict of interest

The authors declare no conflict of interest.

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List of tables

Table 1. Parameters evaluated in the subacute oral toxicity study of *A. edulis* infusion.

Parameters	Control	ILAE		
		30 mg/kg	100 mg/kg	300 mg/kg
Body weight gain and food and water consumption				
Initial BW (g)	26.88±0.54^{a}	26.25±0.92^{a}	26.25±0.88^{a}	26.00±0.84^{a}
Final BW (g)	28.13±0.95^{a}	28.50±0.86^{a}	27.00±0.73^{a}	27.13±1.07^{a}
BW gain (g)	1.25±0.64^{a}	2.25±0.64^{a}	0.75±0.64^{a}	1.12±0.69^{a}
Food intake (g/day)	3.72±0.09^{ab}	3.79±0.03^{b}	3.64±0.06^{abc}	3.48±0.05^{ac}
Water intake (mL/day)	6.86±0.35^{a}	6.45±0.33^{ab}	5.53±0.18^{bc}	5.34±0.16^{c}
Organ weight				
Brain (g/10g BW)	0.15±0.00^{a}	0.14±0.00^{a}	0.16±0.00^{a}	0.16±0.00^{a}
Liver (g/10g BW)	0.42±0.01^{a}	0.40±0.00^{b}	0.40±0.01^{b}	0.38±0.00^{b}
Kidney (g/10g BW)	0.13±0.00^{a}	0.13±0.00^{a}	0.13±0.00^{a}	0.12±0.00^{a}
Spleen (g/10g BW)	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
Heart (g/10g BW)	0.05±0.00^{a}	0.05±0.00^{a}	0.06±0.00^{a}	0.05±0.00^{a}
Lung (g/10g BW)	0.07±0.00^{a}	0.07±0.00^{a}	0.07±0.00^{a}	0.08±0.00^{a}
Hematological parameters				
WBC (10^3/µL)	2.82±0.30^{a}	4.92±0.89^{a}	5.88±0.93^{a}	4.67±0.36^{a}
RBC (10^6/µL)	8.40±0.18^{a}	8.68±0.45^{a}	9.79±0.34^{a}	9.14±0.22^{a}
HBG (g/dL)	12.76±0.28^{a}	13.30±0.52^{a}	14.47±0.39^{a}	14.14±0.50^{a}
HCT (%)	40.83±0.95^{a}	42.12±2.32^{a}	47.50±1.95^{a}	44.17±1.33^{a}
MCV (fL)	48.51±0.32^{a}	48.48±0.34^{a}	48.42±0.35^{a}	48.24±0.47^{a}
MCH (pg)	15.18±0.05^{a}	15.40±0.31^{a}	14.82±0.19^{a}	15.44±0.26^{a}
MCHC (g/dL)	31.26±0.07^{a}	31.75±0.65^{a}	30.61±0.46^{a}	31.98±0.30^{a}
PLT (10^3/µL)	1,069.12±52.23^{a}	994.14±89.74^{b}	1,084.00±72.03^{a}	1,231.00±39.11^{c}
MPV (fL)	6.48±0.10^{a}	6.58±0.06^{a}	6.55±0.06^{a}	6.40±0.07^{a}
LYM (%)	38.12±1.27^{a}	16.00±1.25^{a}	16.00±2.08^{a}	17.71±1.40^{a}
EOS (%)	1.12±0.12^{a}	1.14±0.14^{a}	1.12±0.12^{a}	1.14±0.14^{a}
MON (%)	2.50±0.46^{a}	1.71±0.36^{a}	2.12±0.29^{a}	1.14±0.50^{a}
NEUT (%)	58.25±1.16^{a}	81.14±1.05^{a}	80.75±2.28^{a}	80.00±1.48^{a}
Biochemical parameters				

CR (mg/dL)	0.16±0.02[a]	0.12±0.02[a]	0.10±0.00[a]	0.20±0.00[a]
TC (mg/dL)	61.60±5.63[a]	53.61±4.14[a]	54.70±1.44[a]	50.20±4.56[a]
ALB (g/L)	23.02±1.88[a]	19.75±2.61[a]	21.36±1.27[a]	16.90±1.52[a]
URE (mg/dL)	45.10±2.45[a]	38.40±3.11[a]	26.85±1.41[a]	36.90±2.32[a]
ALT (U/L)	36.35±6.69[a]	32.64±2.46[a]	20.15±1.87[a]	15.43±2.27[a]
AST (U/L)	184.20±29.06[a]	181.44±15.25[a]	246.45±12.90[b]	135.45±17.71[c]
GI	116.25±7.02[a]	106.75±5.57[a]	119.87±3.52[a]	108.37±6.19[a]

ALB: albumine; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BW: body weight; CR: creatinine; EOS: eosinophils; GI: glycaemic index; HBG: hemoglobin; HCT: hematocrit; LYM: lymphocytes; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MON: monocytes; MPV: mean platelet volume; NEUT: neutrophils; PLT: platelet count; RBC: red blood cell count; TC: total cholesterol; URE: urea; WBC: white blood cell count. Mean ± standard error of 8 animals. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA followed by Tukey).

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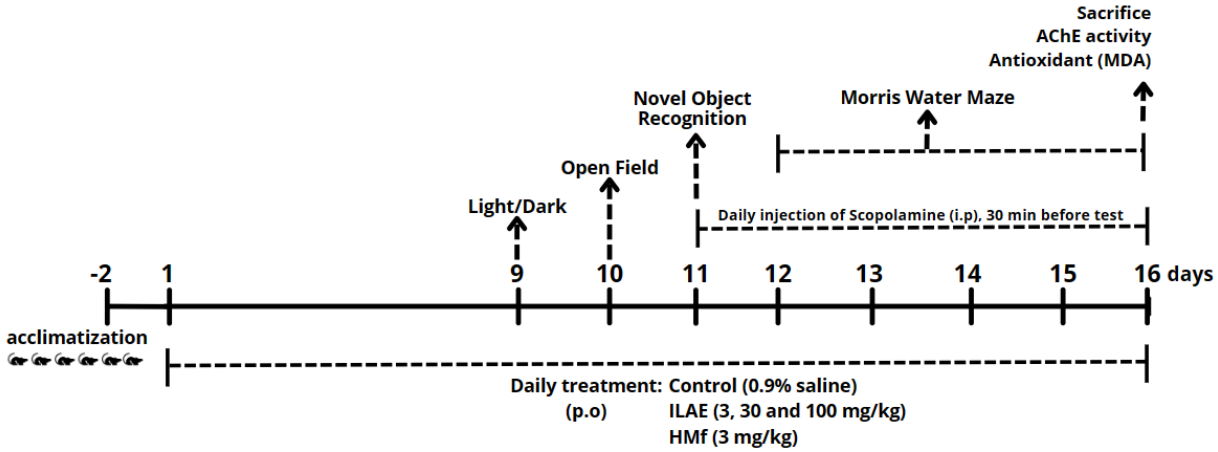

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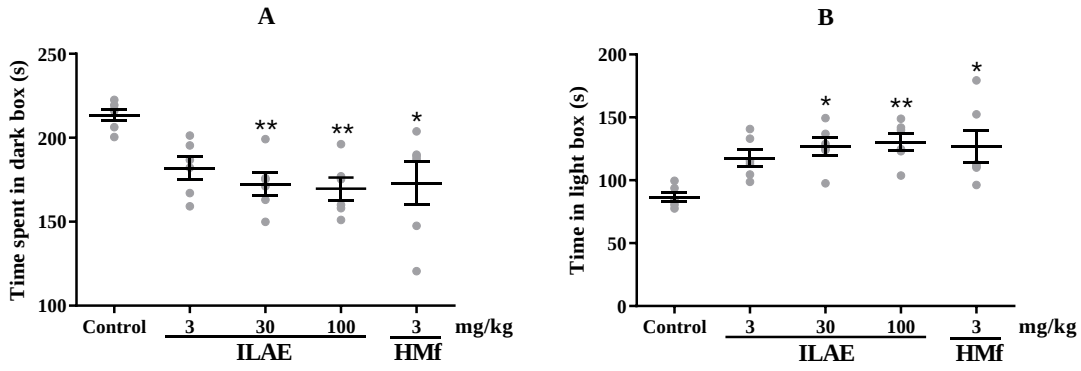

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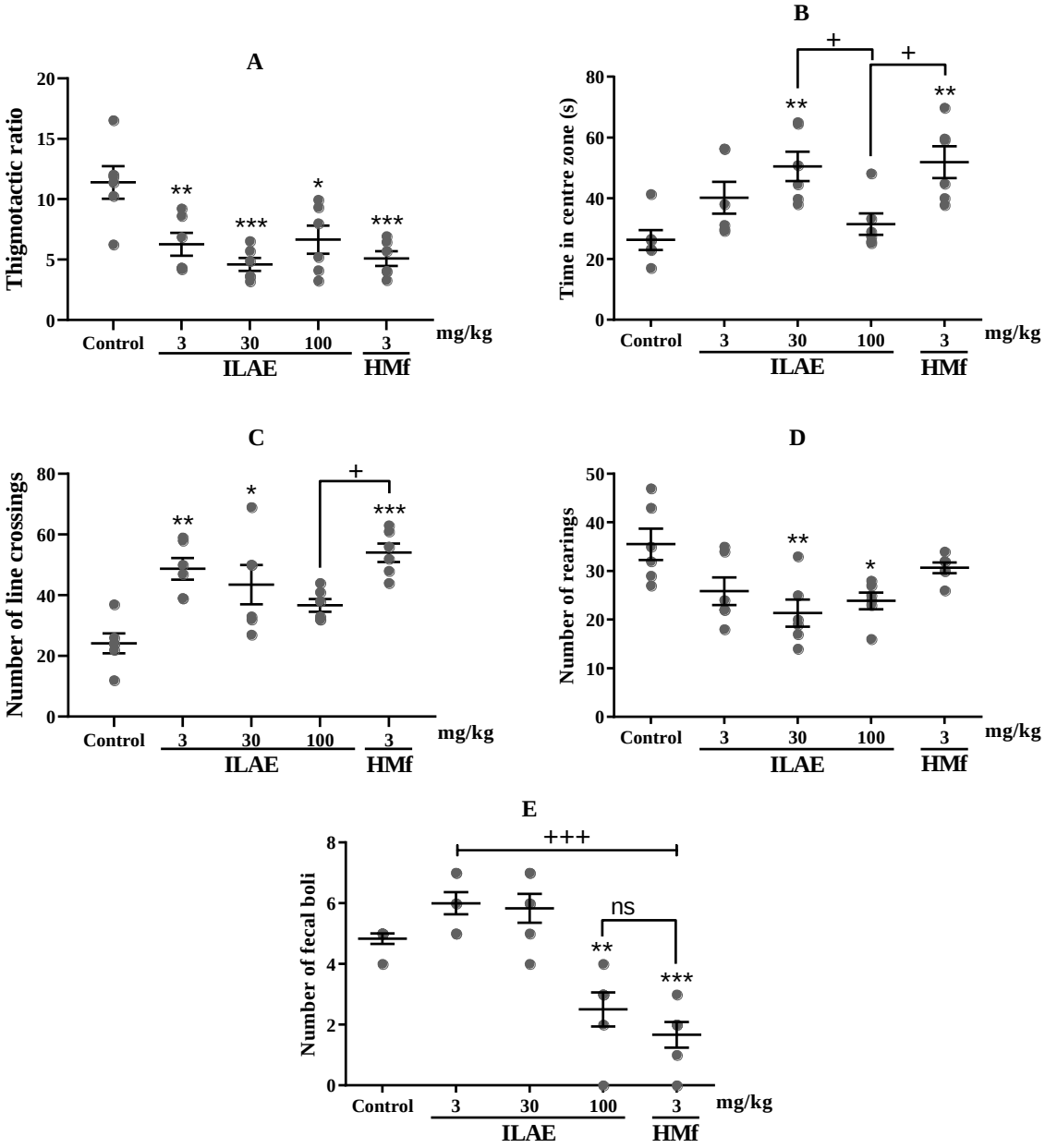

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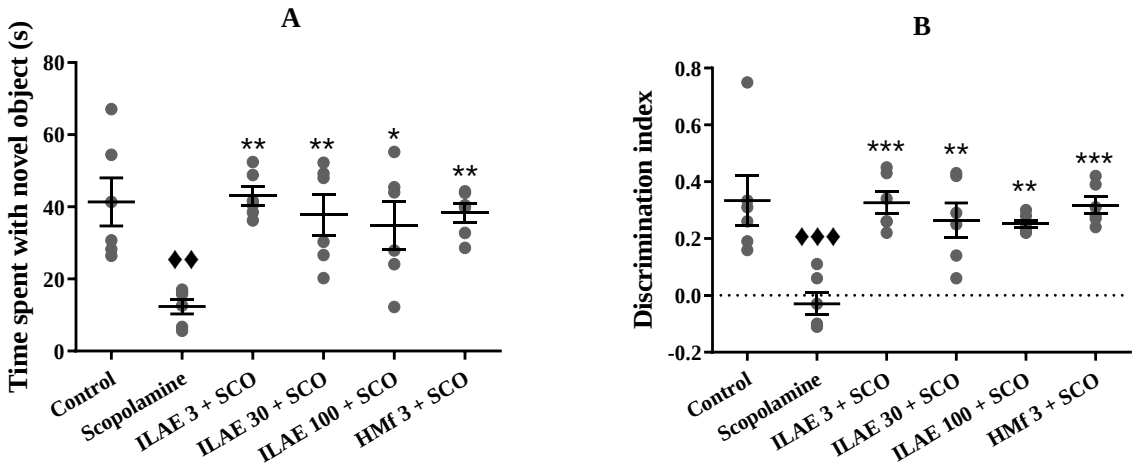

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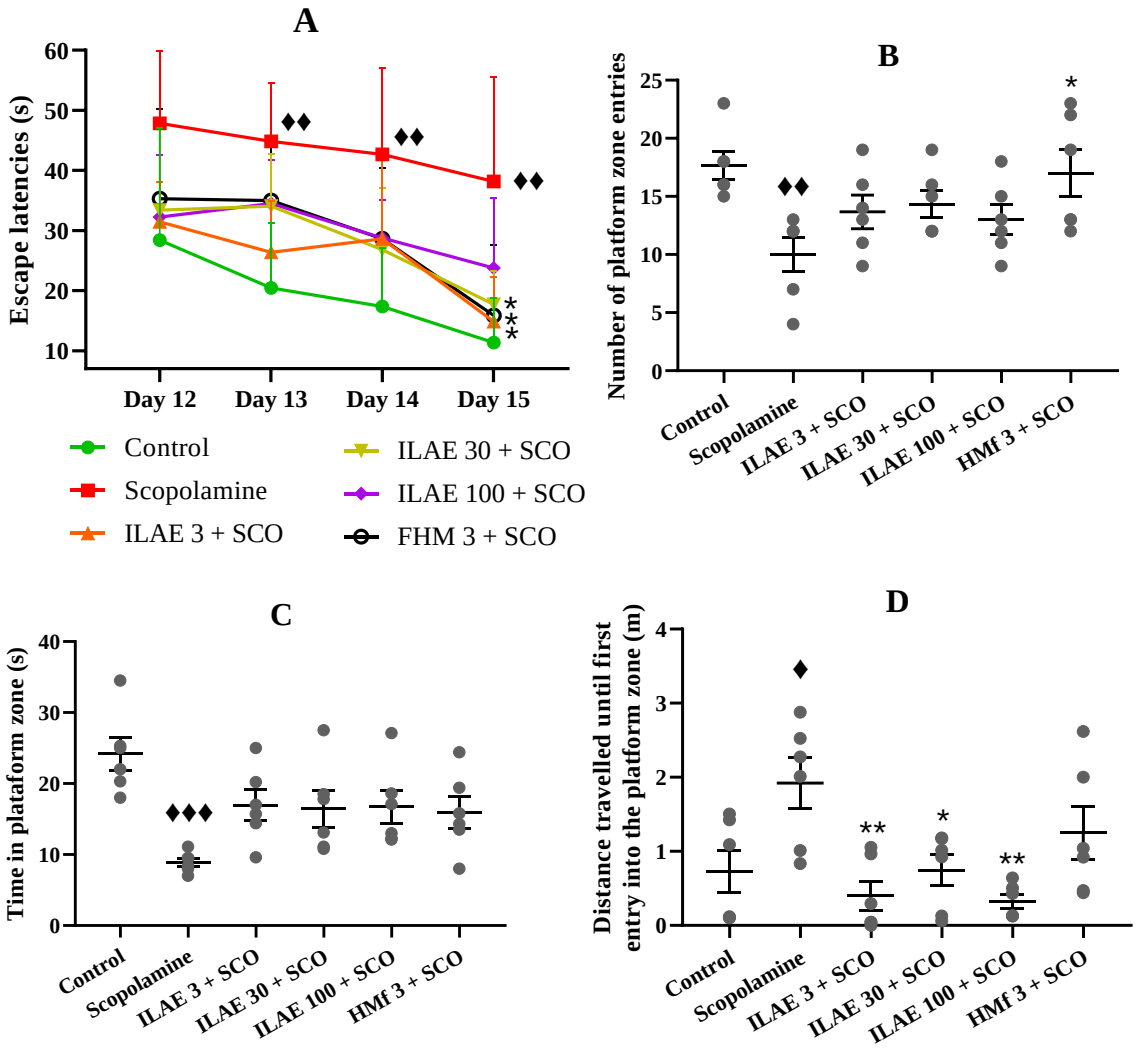

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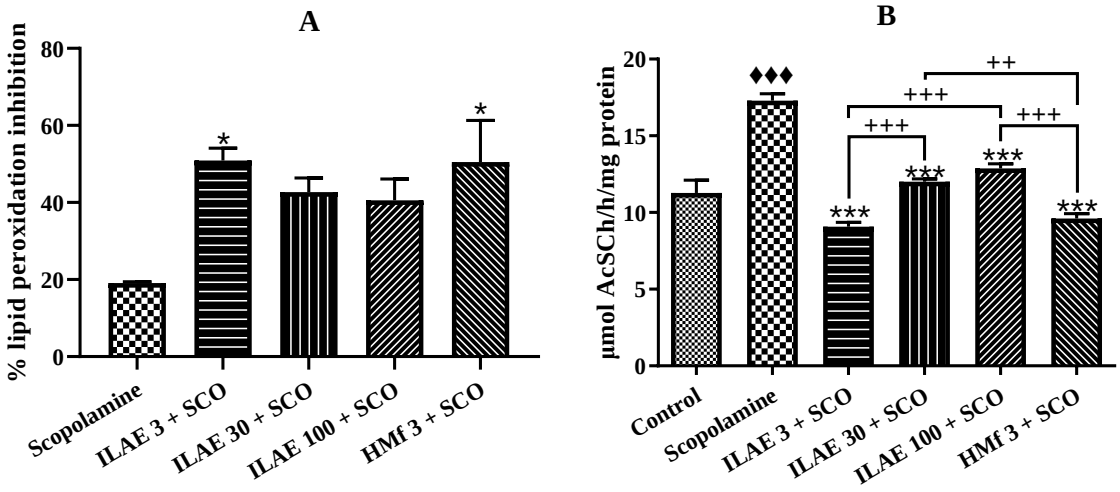

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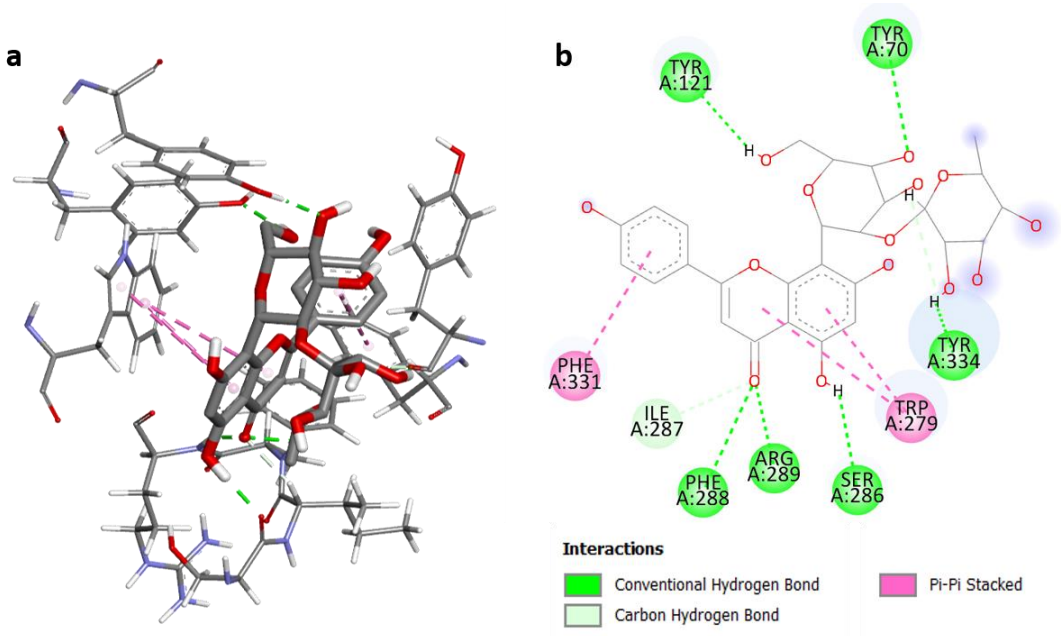

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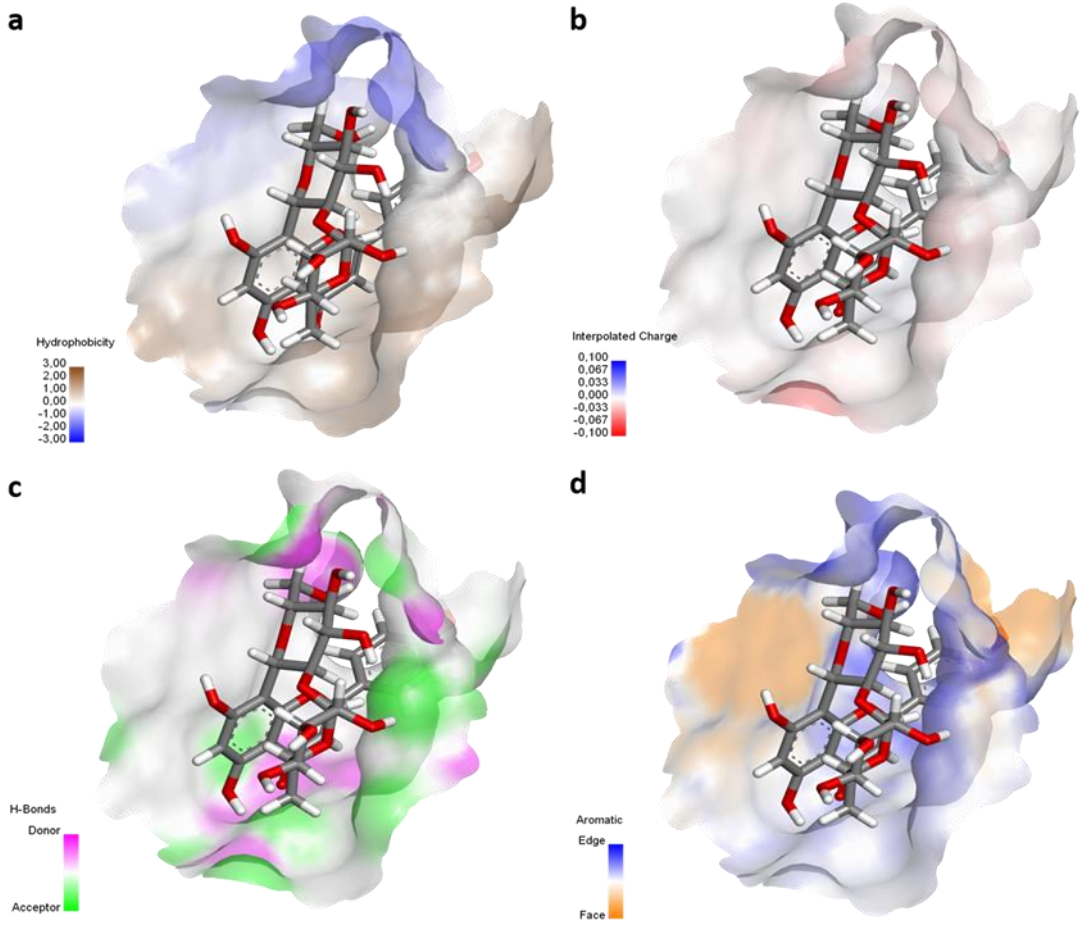

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6 CONCLUSÃO FINAL

Este estudo proporciona insights valiosos sobre os efeitos farmacológicos das folhas de *Allophylus edulis*, destacando sua riqueza em constituintes bioativos. A análise da variação sazonal e geográfica revelou influências significativas na composição química dos óleos essenciais, enquanto a infusão das folhas demonstrou marcados efeitos farmacológicos, incluindo propriedades antioxidantes, anti-inflamatórias, antinociceptivas, ansiolíticas e neuroprotetoras na memória de curto prazo. O isolamento do vitexin 2"-O-ramnosídeo da fração hidrometanólica (HMf) evidenciou propriedades promissoras, destacando-se na redução do edema/hiperalgesia e na inibição da migração de leucócitos. Os tratamentos orais, notavelmente com ILAE e HMf, demonstraram eficácia na atenuação de diversas respostas fisiológicas, inclusive ansiedade e melhorias na memória de curto prazo, assim como baixa toxicidade. Estas descobertas contribuem significativamente para a compreensão das potencialidades terapêuticas das folhas de *A. edulis*, abrindo caminho para futuras investigações e aplicações clínicas.

7. ANEXO

7.1 Parecer de aprovação do comitê de ética (original)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA

Dourados-MS, 05 de Abril de 2022.

CERTIFICADO

Certificamos que a proposta intitulada ***"Potencial farmacológico e toxicológico de Allophylus edulis (A.St.-Hil., A.Juss. & Cambess.) Radlk."***, registrada sob o protocolo de nº 05.2021, sob a responsabilidade de *Anelise Samara Nazari Formagio e Sidney Mariano dos Santos* – 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), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal Da Grande Dourados, em reunião de 18/06/2021.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*06/04/2022 a 30/12/2023*
Espécie/linhagem/raça	*Mus musculus*
Nº de animais	*216*
Peso/idade	*25-30 g/ 45 dias*
Sexo	*168 machos e 48 fêmeas*
Origem	*Biotério Central – UFGD*

Melissa Negrão Sepulvida
Coordenadora CEUA

Comissão de Ética no Uso de Animais – CEUA/UFGD – Rua João Rosa Góes, 1761 – Vila Progresso. Dourados/MS. E-mail: ceua@ufgd.edu.br

7.2 Parecer de aprovação do comitê de ética (atualizado após adendo)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA

Dourados-MS, 16 de agosto de 2023

CERTIFICADO

Certificamos que o projeto intitulado "***Potencial farmacológico e toxicológico de Allophylus edulis (A.St.-Hil., A.Juss. & Cambess.) Radlk.***", protocolo 05.2021, sob a responsabilidade de ***Anelise Samara Nazari Formagio***, - 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) - encontra-se de acordo com os preceitos da lei no. 11.794, de 8 de outubro de 2008, do Decreto no 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal da Grande Dourados (UFGD).

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DANIELA TORRES CANTADORI
Data: 16/08/2023 14:19:51-0300
Verifique em https://validar.iti.gov.br

Daniela Torres Cantadori
Coordenadora em exercício CEUA/UFGD