UNIVERSIDADE FEDERAL DO PAMPA

EDUARDA MONTEIRO FIDELIS

EXTRATO DA PITANGA ROXA (*Eugenia uniflora*): FRUTO BRASILEIRO COM POTENCIAL NEUROPROTETOR

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“Vai lá Duda…, o não tu já tens”.

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RESUMO

O estresse oxidativo (EO) é relatado como um fator importante em eventos bioquímicos precoces na patogênese de doenças neurodegenerativas, antes da morte celular e do avanço neurodegenerativo. Estudos sugerem que o uso terapêutico de extratos frutíferos pode ser apresentado como uma terapia alternativa com efeitos deletérios minimizados ou como uma terapia neuroprotetora. O extrato hidroalcoólico da pitanga roxa (*Eugenia uniflora*) (EPR) é uma fruta rica em compostos bioativos, com propriedades antioxidantes, anti-inflamatório e neuroprotetor. Neste contexto, a doença de Parkinson (DP) é um distúrbio neurodegenerativo progressivo sendo a segunda doença mais comum, caracterizada pela perda progressiva dos neurônios dopaminérgicos da substância negra pars compacta (SNpc) e de outras áreas do cérebro que podem ser correlacionadas com déficits nas funções olfativas, emocionais e de memória que precedem os sintomas motores clássicos na DP. Assim, o presente estudo revelou em uma revisão sistemática que a pitangueira é utilizada desde suas folhas aos frutos que a variante roxa do seu fruto, é a mais rara, apresentando o dobro do conteúdo fenólico em comparação com a variedade vermelha, ainda o EPR possui como composto majoritário cianidina 3-O-glicosídeo mas diversas propriedades farmacológicas ainda pouco exploradas. Nosso estudo sobre toxicidade aguda do EPR (2.000mg/kg, i.g) e toxicidade subcrônica do EPR (1.000mg/kg, i.g por 28 dias) não mostraram sinais toxicológicos nas doses testadas, a farmacocinética revelou que seus compostos bioativos possuem biodisponibilidade baixa, sugerindo que o efeito sinérgico desses compostos faz do EPR potencial farmacológico, de acordo com o experimento o EPR pode ser considerado seguro para o consumo. Nesse contexto, uma única administração prévia de EPR (1000mg/kg, i.g) mostrou-se segura e atenuou o EO induzido via administração intranasal (i.n.) pela neurotoxina 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP) (1mg/narina) em ratos. Nossos resultados demonstram também que o bulbo olfatório e a substância negra são as áreas mais afetadas após 6 horas de infusão de MPTP, observou-se um aumento precoce nos níveis de espécies reativas de oxigênio e de 4-hidroxi-2-nonenal, alterações que foram prevenidas pelo pré-tratamento com EPR. Além disso, o EPR previne a inibição da Na^+/K^+-ATPase pelo MPTP, mostramos assim, a capacidade antioxidantes e neuroprotetores do EPR contra a neurotoxicidade precoce do MPTP.

Palavras-Chave: Antioxidante; Pitanga roxa; MPTP intranasal; Doença de Parkinson.

ABSTRACT

Oxidative stress (OS) is reported to be an important factor in early biochemical events in the pathogenesis of neurodegenerative diseases, before cell death and neurodegenerative advancement. Studies suggest that the therapeutic use of fruit extracts can be presented as an alternative therapy with minimized deleterious effects or as a neuroprotective therapy. The hydroalcoholic of purple pitanga extract (*Eugenia uniflora*) (PPE) is a fruit rich in bioactive compounds, with antioxidant, anti-inflammatory and neuroprotective properties. In this context, Parkinson's disease (PD) is a progressive neurodegenerative disorder and is the second most common disease, characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars pacta (SNpc) and other areas of the brain that can be correlated with deficits in olfactory, emotional and memory functions that precede classic motor symptoms in PD. Thus, the present study revealed in a systematic review that pitangueira is used from its leaves to its fruits and that the purple variant of its fruit is the rarest, presenting twice the phenolic content compared to the red variety, even though the PPE has as the main compound cyanidin 3-O-glucoside but several pharmacological properties still little explored. Our study on acute toxicity of PPE (2.000mg/kg, p.o.) and subchronic toxicity of PPE (1.000mg/kg, p.o. for 28 days) did not show toxicological signs at the doses tested, pharmacokinetics revealed that its bioactive compounds have low bioavailability, suggesting that the synergistic effect of these compounds makes PPE pharmacological potential, according to the experiment, PPE can be considered safe for consumption. In this context, a single prior administration of PPE (1.000mg/kg, p.o.) proved to be safe and attenuated the OS induced via intranasal (i.n.) administration by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) (1mg/nostril) in rats. Our results also demonstrate that the olfactory bulb and substantia nigra are the most affected areas after 6 hours of MPTP infusion, an early increase in the levels of reactive oxygen species and 4-hydroxy-2-nonenal was observed, changes that were prevented by pretreatment with PPE. Furthermore, the PPE prevents the inhibition of $Na^{+/}K^{+}$-ATPase by MPTP, thus demonstrating the antioxidant and neuroprotective capacity of the PPE against the early neurotoxicity of MPTP.

Keywords: Antioxidant; Purple pitanga; intranasal MPTP; Parkinson's disease.

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LISTA DE ABREVIATURAS

i.g. - via gavagem / intragástrico

i.n. - via intranasal

LISTA DE SIGLAS

ATP - Adenosina trifosfato

Bax - Proteína X associada a bcl-2

Bcl-2 - Proteína intra-citoplasmática que inibe a apoptose

BHE - Barreira hematoencefálica

BO - Bulbo olfatório

CAT - Catalase

CEUA - Comitê de ética no uso de Animais

DA - Doença de Alzheimer

DAT - Transportador de dopamina

DP - Doença de Parkinson

EO - Estresse oxidativo

EROs - Espécies reativas de oxigênio

EPR -Extrato da pitanga roxa

GSH - Glutationa

GPX- Glutationa Peroxidase

H_2O_2 - Peróxido de hidrogênio

IL-1β - Interleucina-1β

IL-8 - Interleucina 8

L-DOPA - Levodopa

MAO - Monoamina oxidase

MAO-B - Monoamina oxidase B

MPP^+ - 1-metil-4-fenilpiridínio

MPPP - 1-metil-4-fenil-4-propionpiperidina

MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

MDA - Malondialdeído

mRNA - RNA mensageiro

Na^+K^+ATPase - Bomba sódio-potássio

NADPH - Nicotinamida-Adenina Dinucleotídeo Fosfato Trifosfopiridina

NF-κB - Fator Nuclear Kappa B

NPSH - Níveis de grupamentos tióis não-protéicos

O2• - Radical superóxido

OH• - Hidroxil

$p75^{NTR}$ - Receptor de neurotrofina p75

pró-BDNF - Precursor do fator neurotrófico derivado do cérebro

ROS - Reactive oxygen species

SOD - Superóxido dismutase

SN - Substância negra

SNC - Sistema nervoso central

SNpc - Substância negra pars compacta

TH – Tirosina hidroxilase

TNF- α - Fator de necrose tumoral-α

TrKB - Receptor de tropomiosina quinase B

TrKB-FL - Full TrKB

TrKB-T1 - Truncated TrKB isoform 1

VMAT - 2 - Transportador vesicular de monoamina do tipo 2

4-HNE - 4-hidroxi-2-nonenal

6-OHDA - 6-hidroxidopamina

SUMÁRIO

1 INTRODUÇÃO

A relação entre o homem e a natureza tem sido avaliada por muitos estudiosos, as plantas medicinais representam a forma mais antiga de medicina, utilizada há milhares de anos na medicina tradicional em muitos países ao redor do mundo (Marrelli, 2021). O consumo mundial de plantas como fitonutrientes e nutracêuticos teve crescimento, uma vez que a população tem fácil acesso a esses recursos para o tratamento de diversas doenças, além do seu baixo custo (Yadav *et al*., 2017; Villas-Boas *et al*., 2021).

No que diz respeito às plantas medicinais, o Brasil é uma fonte importante de diferentes espécies, devido à sua grande biodiversidade. O Brasil, país de dimensões continentais, hospeda uma das maiores diversidades de flora do mundo, e é por isso classificado entre os países megadiversos (BRASIL, 2024). Com seis grandes biomas continentais (Amazônia, Caatinga, Cerrado, Mata Atlântica, Pantanal e Pampa) (BRASIL, 2024), o país detém 46,9 mil espécies de plantas nativas (Walter *et al*., 2024). Devido a esta grande biodiversidade, uma variedade de espécies de frutas exóticas e nativas são sub exploradas, o que abre a oportunidade de estudos a fim de descobrir metabólitos secundários que tenham a capacidade para tratar diversas doenças (Silva *et al*., 2014; Ribeiro *et al*., 2018). No Rio Grande do Sul, o Bioma Pampa ocupa praticamente 178.243 km^2, o que corresponde a 63% do território estadual e a 2,07% do território nacional (BRASIL, 2024). O Bioma Pampa estende-se pelo Brasil, Uruguai e Argentina (Fig.1).

Fig. 1 - Mapa ilustrado do bioma pampa.

Fonte: Alexandre De Nadal.

Extratos naturais são uma fonte interessante para o tratamento de diversas condições de saúde (Chaachouay e Zidane, 2024). Extratos de frutas e outras partes vegetais, principalmente decoctos e infusões obtidas de folhas de plantas, são ricas fontes de compostos bioativos (Chaachouay e Zidane, 2024). Os compostos bioativos são compostos com benefícios nutricionais e geralmente são encontrados em plantas, bactérias e fungos. Eles são principalmente metabólitos secundários e podem ser amplamente categorizados em compostos fenólicos, alcalóides, pigmentos e fatores de crescimento (Kudanga *et al*., 2017), além de particular interesse devido às suas potentes propriedades anti-inflamatórias, antioxidantes, anticâncer e neuroprotetor sendo promotoras da saúde (Kumari *et al*., 2023). Devido a vasta oferta natural de compostos significativos podem ser de primordial importância para a expansão de novos medicamentos nutracêuticos (Abima *et al*., 2018).

Uma espécie Brasileira que merece destaque é a pitangueira (*Eugenia uniflora*), uma árvore frutífera com potencial para o desenvolvimento de medicamentos nutracêuticos por apresentar em seus frutos amarelo, vermelho e roxo grande diversidade de nutrientes e compostos bioativos (Denardin *et al*., 2015). Estes frutos apresentam atividade antioxidante (de Souza Cardoso *et al*., 2018), antiviral (Dewi *et al*., 2019), anti inflamatória (Bello *et al*., 2020), efeito neuroprotetor (Flores *et al*., 2020) e anticâncer (Roncato *et al*., 2018; Pereira *et al*., 2024), o que corrobora seu emprego na medicina popular para tratar enfermidades.

A variedade pitanga roxa é mais rara, se destacando por possuir o dobro do conteúdo fenólico em comparação com o tipo vermelho. Tambara e colaboradores (2018), identificaram no extrato da pitanga roxa (EPR) presença de miricetina, quercetina, ácido gálico. Sendo rico em antocianinas e derivados da cianidina, esse extrato apresenta um grande potencial antioxidante. Compostos fenólicos têm atraído um grande interesse, pois são potentes antioxidantes presentes nos alimentos como verduras e frutas (Gonzales *et al*., 2015).

O estresse oxidativo (EO) é relatado como um fator importante em eventos bioquímicos precoces na patogênese de doenças neurodegenerativas, antes da morte celular e do avanço neurodegenerativo (Aruoma, 1998), como a doença de Parkinson (DP). A DP é uma doença neurodegenerativa progressiva caracterizada patologicamente pela perda de neurônios dopaminérgicos na parte compacta da substância negra (SNpc) (Tansey *et al*., 2022) e em outras áreas do cérebro, que podem ser correlacionadas com déficits que precedem os sintomas motores clássicos na DP (Prediger *et al*., 2011). Atualmente, neurotoxinas como o 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) vem sendo utilizadas como modelo experimental da DP, no intuito de mimetizar características da doença para elucidação das suas causas e desenvolvimento de novas terapias (Dionísio *et al*., 2021).

O estudo de Borges e colaboradores (2025) demonstrou o efeito neuroprotetor do EPR e do extrato da pitanga vermelha, em retardar a paralisia induzida pelo peptídeo aβ1-42 em um modelo em *C. elegans*, assim como atenuar os sinais dopaminérgicos associados aos danos induzidos pelo metabólito do MPTP, o 1-metil-4-fenilpirimidina (MPP^+), nestes mesmos nematóides. De fato, o EPR mostra efeitos promissores em estudos com *C.elegans*, melhorando marcadores lipídicos e reduzindo o EO (Tambara *et al*., 2018; Roncato *et al*., 2019). No entanto, embora estes resultados tragam à luz a perspectiva do uso do EPR como um extrato nutracêutico promissor, os efeitos antioxidantes e neuroprotetores do EPR *in vivo* ainda são incipientes.

Diante do exposto, este estudo se justifica pela necessidade de desenvolvimento de protocolos e ensaios toxicológicos padrões para o uso seguro do EPR em mamíferos, bem como a avaliação do seu efeito antioxidante e neuroprotetor. Nesse sentido, o objetivo desta tese foi realizar uma revisão sistemática sobre a *Eugenia uniflora* e avaliar os possíveis efeitos toxicológicos do EPR e o seu potencial antioxidante e neuroprotetor frente ao estresse oxidativo agudo induzido pela neurotoxina MPTP em roedores.

APRESENTAÇÃO

Este documento apresentar-se-á dividido em três partes principais:

A **Parte I** abrange a revisão teórica da literatura, os objetivos, a justificativa e a hipótese que compõem esta tese.

A **Parte II** compreende a metodologia e os resultados obtidos na tese e está dividida em três subitens: 1) Artigo de revisão intitulado "Pitanga (*Eugenia uniflora L.*) as a source of bioactive compounds for health benefits: A review" e publicado em 2022, na *Arabian Journal of Chemistry*; 2) Manuscrito intitulado "Assessing the safety of purple pitanga extract (PPE) through toxicity studies *in vivo* and *in vitro* and computational analysis" a ser submetido na *ACS Chemical Health & Safety*; 3) Artigo original intitulado "Purple pitanga extract (*Eugenia uniflora*) attenuates oxidative stress induced by MPTP" e publicado em 2023, na *Metabolic Brain Disease*.

Por fim, a **Parte III** apresenta a discussão final, a conclusão, as referências bibliográficas e os anexos.

PARTE I

2. REVISÃO DE LITERATURA

2.1 A pitangueira (*Eugenia uniflora)*

O gênero *Eugenia uniflora* é considerado o quarto mais importante da família *Myrtaceae*, na qual estima-se que 350 espécies sejam nativas do Brasil (de Souza Cardoso *et al*., 2018). Além da importância ecológica, as espécies representativas da família *Myrtaceae* são classificadas morfologicamente como árvores ou arbustos, e seus frutos geralmente são esféricos, comestíveis (Auricchio e Bacchi, 2003) e apresentam grande potencial agroindustrial (Sardi *et al*., 2017).

A *Eugenia uniflor*a é popularmente chamada de pitangueira e é uma árvore nativa brasileira cultivada em vários países subtropicais (Sardi *et al*., 2017; Flores *et al*., 2020; de Paulo Farias *et al*., 2020). Seu fruto, a pitanga, que na sua maioria possui uma coloração vermelha quando madura, foi base para a origem do seu nome popular, em Tupy *ybápytanga* (*ybá* = fruto, *pytanga* = avermelhado) (Donadio *et al*., 2002).

A pitangueira apresenta diferentes variações de fruto, durante o amadurecimento o epicarpo do fruto pode evoluir de verde para amarelo, vermelho e finalmente para o roxo escuro (de Lira Júnior *et al*., 2007). A coloração do fruto no seu estágio final de maturação depende da variedade da árvore que pode ser do tipo amarela, vermelha ou roxa (Santos *et al*., 2021). A variedade predominante das pitangueiras é vermelha, por isso é mais comum encontrar os frutos vermelhos, mas a variedade amarela e roxa também é encontrada com frequência nas matas nativas (Celli *et al*., 2011). Os frutos são em forma de baga e possuem de 8 a 10 sulcos longitudinais na casca (Romagnolo e Souza, 2006), sendo compostos por 77% de polpa e 23% de semente (Köhler 2014). As folhas são identificadas como simples opostas, com pecíolos medindo aproximadamente 2 mm. As folhas novas apresentam coloração verde-acastanhada e consistência membranosa, enquanto as folhas adultas apresentam coloração verde-escura (Fouqué, 1981; Lorenzi, 1998). Suas flores crescem na base dos ramos, são compostas por 4 a 8 flores hermafroditas, de fragrância suave e que produzem pouco ou nenhum néctar, sendo compostas por 4 pétalas livres, de cor branco creme. As anteras são de cor amarelada e abundantes em pólen com estiletes brancos (Sanchotene 1989).

Os frutos são as partes mais utilizadas da pitangueira, podendo ser consumido *in natura* ou na preparação de sucos, sorvetes, doces e licores (Coradin *et al*., 2011). Ainda, suas folhas são utilizadas na medicina popular por meio de chá, principalmente para tratar distúrbios gastrointestinais (Auricchio e Bacchi, 2003). Vários estudos avaliaram a composição química

e eficácia terapêutica de extratos da pitanga e de outras partes da pitangueira. Os frutos da pitangueira demonstram propriedades biológicas interessantes (Moura *et al*., 2018), como anti-inflamatória (Soare *et al*., 2014); antiviral (Dewi *et al*., 2019); antibacteriana (Jovito *et al*., 2016); antioxidante (Helt *et al*., 2018) e neuroprotetora (Flores et a., 2020). Dessa forma, foi demonstrado que frutos e folhas de *E. uniflora*, principalmente os frutos vermelhos e roxos, possuem compostos antioxidantes em sua composição, como fenólicos, flavonoides e carotenoides que apresentam potencial efeito benéfico à saúde, o que indica seu alto valor como alimento funcional (Sobeh *et al*., 2020; Denardin *et al*., 2015; Tambara *et al*., 2018).

2.2 A pitanga roxa

A variedade roxa do fruto da pitangueira (Fig. 2) apresenta quantidades significativas de antocianinas, delfinidina 3-O-glicosídeo, pelargonidina 3-O-glicosídeo e derivado de cianidina, tendo como majoritário cianidina-3-O-glicosídeo (Tambara *et al*., 2018). Este composto majoritário demonstrou potencial para ligar e inibir pontos de controle imunológico que podem ativar a resposta imune no microambiente tumoral e induzir a morte de células cancerígenas (Mazewski *et al*., 2018).

Outros compostos presentes na pitanga roxa foram considerados biologicamente ativos, como miricetina que apresenta potencial inibidor da enzima ciclo-oxigenase-2 (COX-2) (Hiermann *et al*., 1998, Chen *et al*., 2001, Rattmann *et al*., 2012, Li *et al*., 2013), assim como reduz o acúmulo de EROs (Buchter *et al*., 2013). A quercetina contribui para a redução da supressão de TNF-ὰ e IL-1β (Sobeh *et al*, 2020), além de outros compostos ácido oleico e vitamina C (Bagetti *et al*., 2011) que fazem da pitanga roxa um promissor antioxidante.

Fig. 2 - Pitanga roxa, fruto da pitangueira (*Eugenia uniflora*).

Fonte: Site Capital mudas.

Os compostos bioativos desempenham ação antioxidante atuando como bloqueadores de radicais livres (Jayasena *et al*., 2013), reconhecidos pelo seu comportamento como agentes redutores e, às vezes, agindo como quelantes de metais (Joshi e Joshi, 2016). Devido a essas propriedades, estas moléculas são relacionadas à prevenção de doenças relacionadas ao estresse oxidativo, como doenças neurodegenerativas (Eghbaliferiz e Iranshahi, 2016).

Estudos utilizando o extrato da pitanga roxa (EPR) têm mostrado efeitos significativos. Denardin e colaboradores (2015) observaram, em células estreladas hepáticas ativadas (HSC), que concentrações crescentes do EPR que variaram de 5 a 100 μg/mL foram capazes de desencadear efeitos antiproliferativos e citotóxicos, demonstrado que o EPR é um bom candidato no tratamento da fibrose hepática, visto que essas células são pericitos específicos do fígado envolvidos no desenvolvimento de fibrose patológica (Denardin *et al*., 2017).

Além disso, o EPR aumentou notavelmente a sobrevivência do nematóide *Caenorhabditis elegans* (*C. elegans*), após diferentes situações de estresse oxidativo e também foi capaz de prolongar a vida útil em vermes mutantes N2 (tipo selvagem) e *mev-1*, modulando a expressão de SOD-3 e HSP-16.2, bem como aumentando a localização nuclear do DAF-16, o que demonstra o efeito antioxidante do extrato (Tambara *et al*., 2018). Neste mesmo modelo, Roncato e colaboradores (2019) observaram que os lipossomas convencionais são de grande vantagem para a administração oral de um EPR, que protegeu contra o aumento do teor de lipídios e triglicerídeos induzido pela dieta rica em colesterol em *C. elegan*s, sem causar quaisquer efeitos tóxicos.

O tratamento de até 100 μg CAE/mL do EPR por 48h prolongou a longevidade e reduziu o fenótipo *Muv* (multivulva) em verme mutante com ganho de função no gene *let-60*, que é uma cepa tumoral relacionada à via Ras. Ainda, foi observada redução na área *Muv*, promovendo aumento na postura de ovos. Além disso, o EPR aumentou a apoptose, processo necessário para eliminar células danificadas e tumorais e manter a viabilidade da prole (Pereira *et al*., 2024), demonstrando que EPR é promissor na redução da formação de tumores.

O efeito neuroprotetor do EPR e do extrato da pitanga vermelha foi demonstrado em um estudo onde observou-se a diminuição da paralisia induzida pelo peptídeo beta amilóide (fragmento 1-42) em um modelo de doença de Alzheimer em *C. elegans* (Borges *et al*., 2015), assim como a diminuição de comportamentos dopaminérgicos associados aos danos induzidos pelo metabólito do MPTP, o 1-metil-4-fenilpirimidina (MPP^+), em modelo de DP em *C. elegans*.

O potencial neuroprotetor do EPR também foi investigado contra danos na memória induzidos por MPTP em roedores. Durante 14 dias, diferentes doses do EPR foram

administradas após a administração intranasal (i.n) de MPTP, o extrato preveniu os efeitos prejudiciais do MPTP na memória social, memória de reconhecimento de objetos a curto e longo prazo, e na memória de trabalho. O EPR também atenuou o déficit motor induzido pelo MPTP, bem como os níveis de tirosina hidroxilase no estriado desses animais. O EPR bloqueou o aumento induzido pelo MPTP nos níveis do precursor do fator neurotrófico derivado do cérebro (pró-BDNF), TrkB.t1 e p75 ^{NTR} no hipocampo. Assim, este estudo sugere que o efeito neuroprotetor do extrato seja mediado, pelo menos em parte, pela regulação desta via de sinalização do BDNF no hipocampo (Savall *et al*., 2023). Sugerindo um potencial terapêutico do EPR em modelos de doença de Parkinson (DP) em roedores.

Esses resultados estão relacionados com a presença dos compostos bioativos do EPR (Borges *et al*., 2015; Tambara *et al*., 2018; Roncato *et al*., 2019; Savall *et al*., 2023), os quais proporcionam benefícios à saúde, como efeitos fisiológicos e imunológicos (Kumari *et al*., 2023). Neste contexto, o consumo de alimentos naturais tem se destacado por contribuir no manejo de doenças relacionadas à idade, como a DP (Evatt, 2007).

2.3 A doença de Parkinson

A DP foi descrita pela primeira vez pelo médico James Parkinson, quando publicou o estudo “An essay on the Shaking Palsy” em 1817. Neste estudo, foram descritos 6 casos de pacientes com sintomas motores semelhantes, consistindo de tremores de repouso, lentidão de movimentos (bradicinesia), perda da capacidade de mover os músculos voluntariamente, postura curvada e marcha festinante, porém com os sentidos preservados (Parkinson, 1817; Przedborski, 2017). Posteriormente, agregados proteicos foram identificados por Fritz Henrich Lewy em 1912, e então chamados corpos de *Lewy*. Os corpos de Lewy, são referência histopatológica na SNpc de enfermos da DP (Kumar *et al*., 2012). A formação desses corpos se dá principalmente pelas proteínas α-sinucleína e ubiquitina nos neurônios remanescentes. O acúmulo dessas proteínas pode surgir devido à falha do sistema ubiquitina-proteassoma na remoção de agregados proteicos patológicos (Dorszewska *et al*., 2021).

A maioria dos casos de DP tem uma etiologia multifatorial, resultante dos efeitos combinados de fatores ambientais e genéticos (Goldman *et al*., 2019). A exposição a produtos químicos tóxicos (Goldman, 2014; Racette *et al*., 2017) e lesões na cabeça podem aumentar o risco de DP (Kenborg *et al*., 2015), enquanto estilo de vida saudável pode diminuir o risco do seu desenvolvimento (Simon *et al*., 2019). Prevê-se que a prevalência da DP dobre entre 2005 e 2030, principalmente devido ao aumento da expectativa de vida em todo o mundo, apoiada

por cuidados de saúde de maior qualidade (Dorsey *et al*., 2007; Tysnes e Storstein, 2017).

Casos hereditários da DP estão relacionados a mutações em genes que codificam proteínas que regulam a função mitocondrial como LRRK2, PINK1 e DJ-1, proteínas responsáveis pela regulação da fissão e fusão mitocondrial, o que sugere que alterações na dinâmica mitocondrial contribuem para o processo neurodegenerativo observado na DP (Wang, 2011). Os estudos genéticos têm permitido esclarecer vários mecanismos envolvidos na fisiopatologia da doença, já que muitos dos genes associados com a doença são responsáveis pela codificação de proteínas envolvidas em vias moleculares alteradas nas formas esporádicas da doença (Kluss *et al*., 2019).

Carlsson *et al*. (1959) identificaram o papel da DA na DP utilizando a reserpina, um inibidor do transportador vesicular de monoaminas. Foi demonstrado que a administração de (L-DOPA), precursor da DA, revertia a redução dos níveis de dopamina e da atividade motora induzida pela reserpina. A L-DOPA foi testada em pacientes com DP, tornando-se o principal tratamento sintomático para DP (Fahn, 2015).

Um sistema de estagiamento para a DP de causa esporádica foi desenvolvido, demonstrando que a presença de agregados de α-sinucleína primeiramente acontece no núcleo dorsal motor e então ocorre a progressão para outras regiões como a SNpc (Braak *et al*., 2004). Ainda, o acúmulo de α-sinucleína nos terminais sinápticos causa prejuízo na liberação de neurotransmissores em neurônios dopaminérgicos, devido às alterações causadas na vesícula sináptica que impedem o reagrupamento das vesículas de endocitose (Nemani *et al*, 2010).

Assim, a DP é caracterizada como uma doença neurodegenerativa progressiva resultante da perda de neurônios dopaminérgicos na SNpc, resultando em sintomas motores como tremores, rigidez, bradicinesia e instabilidade postural, além de sintomas não motores como distúrbios do sono, depressão e alterações intestinais e cognitivas (Przedborski, 2017; Bang *et al*., 2021). A DP ainda permanece sem cura, mas evidências indicam o envolvimento do estresse oxidativo na sua etiologia (Trist *et al*., 2019).

2.4 Estresse Oxidativo

O estresse oxidativo (EO) é uma condição que se manifesta da desregulação da atividade redox celular, onde a produção de espécies reativas de oxigênio (EROs) supera a depuração realizada pelas defesas antioxidantes endógenas e exógenas (Trist *et al*., 2019). As principais EROs incluem espécies radicalares e não radicalares, sendo os radicais livres espécies químicas altamente instáveis e extremamente reativas produzidas durante o metabolismo celular. Eles

apresentam em seu orbital mais externo um ou mais elétrons desemparelhados que interagem em busca de um elétron para atingir a estabilidade (Barbosa *et al*., 2010). Esse processo de transferência de elétrons em excesso pode causar danos a níveis moleculares e celulares nos organismos vivos, causando o EO.

As EROs incluem o ânion superóxido (O2•-), peróxido de hidrogênio (H_2O_2), radicais hidroxila (OH•) e radicais peroxila e alcoxila, que são derivados de radicais livres de espécies de oxigênio fortemente reativos que ocorrem como um subproduto natural do metabolismo celular, principalmente a respiração mitocondrial (Milton e Sweeney, 2012). Por serem produtos universais do metabolismo aeróbico (Burgoyne *et al*., 2012) e componentes de sinalização celular, as EROs desempenham papéis na diferenciação celular e na manutenção da homeostase (Reczek e Chandel, 2015). No entanto, seu aumento tem sido associado a danos oxidativos no DNA e proteínas (Barnes, 2020) e à peroxidação de lipídios da membrana celular (Yoritaka *et al*., 1996).

Uma das principais fontes de EROs no cérebro são as mitocôndrias (Stefanatos e Sanz, 2018). Alterações na função mitocondrial são características do envelhecimento, no cérebro envelhecido o número de mitocôndrias danificadas produz níveis mais altos de EROs o que contribui para o colapso dos mecanismos redox (Chakrabarti *et al*., 2011). Cerca de 1 a 4% do consumo mitocondrial de O_2 é transformado em EROs (Ahmed *et al*., 2021). As mitocôndrias são as organelas responsáveis pela produção de ATP na célula (fosforilação oxidativa) e são extremamente importantes para o controle de processos celulares dependentes de ATP, essenciais para a viabilidade celular (Puntel *et al*., 2015).

Outras fontes de EROs incluem a monoamina oxidase B (MAO-B), que em níveis altos se torna a enzima predominante para metabolizar a dopamina (Zucca *et al*., 2014). A MAO-B é expressa nos astrócitos e não diretamente nas células dopaminérgicas, pela alta permeabilidade do H_2O_2, quando produzido nos astrócitos ele pode induzir efeitos tóxicos também nas células vizinhas como na SN (Dias *et al*., 2013). A produção de ERO catalisada pela MAO-B contribui para um aumento no dano mitocondrial relacionado à idade (Billings *et al*., 2019).

Esse processo gerado pela produção das ERO é inibido ou atenuado por substâncias antioxidantes que podem ser tanto exógenas como endógenas e enzimáticas ou não enzimáticas (Birben *et al*., 2012). Antioxidantes são “quaisquer substâncias presentes em baixas concentrações quando comparadas à do substrato oxidável que, atrasam ou inibem a oxidação deste substrato de maneira eficaz” (Sies, 1997).

O sistema de defesa enzimático inclui as enzimas Superóxido Dismutase (SOD),

Catalase (CAT) e Glutationa Peroxidase (GPx). A SOD catalisa a dismutação do ânion superóxido em H_2O_2 e oxigênio, o H_2O_2, por sua vez, pode ser convertido à H_2O pela GPx ou $O_2 + H_2O$ pela CAT (Georgieva *et al*., 2017). O sistema de defesa endógeno não enzimático inclui algumas moléculas, entre elas: a glutationa (GSH) e as vitaminas C e E (Marrocco *et al*., 2017; Silva *et al*., 2013). Quando estes antioxidantes são insuficientes para manter a homeostase celular, alimentos que contêm antioxidantes naturais, como algumas frutas, se tornam uma alternativa de suma importância (Zou *et al*., 2016; Rahal *et al*., 2014).

Nas membranas mitocondriais, os ácidos graxos poliinsaturados são alvos primários para o ataque de EROs, o que pode levar à peroxidação lipídica e geração de lipídios reativos (Xiao *et al*., 2017). O malondialdeído (MDA) é o principal produto natural da peroxidação lipídica, sendo um marcador bem estabelecido para triagem e monitoramento do EO (Buczyńska *et al*., 2021). Outro produto da peroxidação lipídica é o 4-hidroxi-2-nonenal (4-HNE), o qual possui vários efeitos citotóxicos como depleção dos níveis de GSH, disfunção de proteínas estruturais, redução das atividades enzimáticas e indução da morte celular (Liu *et al*., 2000).

A perda das funções fisiológicas como a redução das defesas antioxidantes contribui para processo de envelhecimento e para a progressão de doenças associadas à idade, como a DP (Li *et al*., 2021). A formação de EO é a característica da fase inicial da DP e ocorre antes mesmo da perda dopaminérgica. O acúmulo de espécies reativas de EROs está associado a modulações em processos celulares, como diferenciação, sinalização celular, motilidade, crescimento e apoptose na DP (Hemmati-DInarvand *et al*., 2019).

Em doenças neurodegenerativas como na DP, os altos níveis de 4-HNE e MDA estão relacionados ao EO (Hardas *et al*., 2013). A perda neuronal dopaminérgica no cérebro com DP resulta na diminuição dos níveis de GSH, importante antioxidante regulador de neurodegeneração associada à DP (Silva *et al*., 2013), e de altos níveis de ferro e cálcio na SNpc, que contribuem ainda mais para a formação do EO (Dorszewska *et al*., 2021). Da mesma forma, danos oxidativos locais e sistêmicos desencadeados por EROS podem promover a degeneração dos neurônios dopaminérgicos (Dorszewska *et al*., 2021) no corpo estriado, hipocampo e córtex (De Lau e Breteler, 2006; Braak *et al*., 2004).

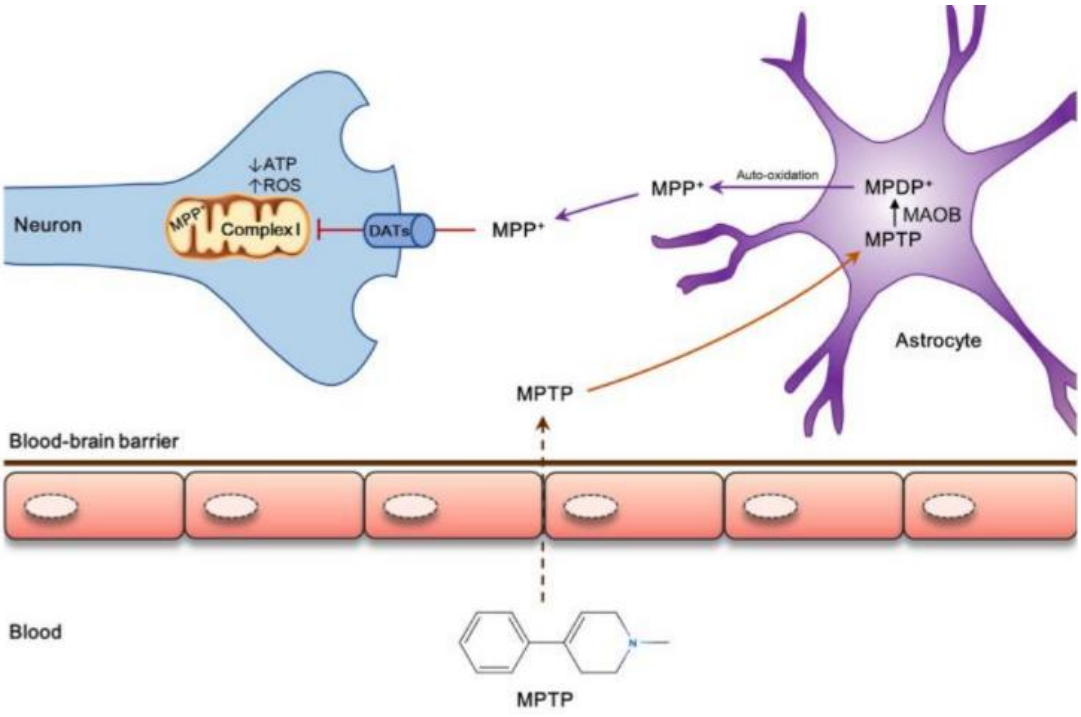
2.5 A neurotoxina MPTP: Um modelo em roedores para o estudo da DP

Possuindo um efeito comparável ao da heroína, o análogo sintético do opióide miperidina com o nome químico 1-metil-4-fenil-4-propionpiperidina (MPPP foi introduzido

como droga recreativa no final do século 20. No início dos anos 80, um grupo adicto de heroína foi internado com imobilidade corporal severa após o uso intravenoso de MPPP contaminada com um produto secundário, o MPTP (Langston *et al*., 1983). O diagnóstico mostrou que estes pacientes apresentavam parkinsonismo, que foi posteriormente confirmado pela resposta do tratamento com a L-DOPA e análise post-mortem (Langston, 2017).

O MPTP é uma protoxina com cinética complexa capaz de atravessar a barreira hematoencefálica (BHE) com facilidade devido à sua lipofilicidade (Zeng *et al*., 2018). Posteriormente, a enzima MAO-B secretada por células gliais (astrócitos) converte MPTP em um metabólito intermediário, 1-metil-4-fenil-2,3-diidropiridina (MPDP), uma molécula instável capaz de auto-oxidar-se e subsequentemente formar o metabolito tóxico final, 1-metil-4-fenilpiridino (MPP^+) (Fig.3) (Kato *et al*., 2003).

Fig. 3 - Metabolismo do MPTP.



Fonte: Adaptado de Dionísio *et al*., 2021.

Liberado no espaço extracelular, o MPP^+ adentra seletivamente os neurônios dopaminérgicos por meio do transportador de DA da membrana plasmática, a qual possui

afinidade (Meredith e Rademacher, 2011; Javitch *et al*., 1985). Dentro dos neurônios, o MPP^+ se acumula em vesículas sinápticas e por transporte passivo chega a matriz mitocondrial (Mustapha e Mat, 2021), quando o MPP^+ se acumula nas vesículas sinápticas, ligando-se ao transportador vesicular de monoamina tipo 2 (VMAT2), sequestrador de dopamina, e nas mitocôndrias de maneira dependente de energia (Schildknecht *et al*., 2017).

A perda do transporte de dopamina dependente de VMAT2 nas vesículas sinápticas aumenta a dopamina citosólica nos terminais pré-sinápticos, o que resulta em múltiplas reações oxidativas prejudiciais (Langston *et al*., 2017; Lohr *et al*., 2014). Nas mitocôndrias, o MPP^+ inibe o complexo I da cadeia transportadora de elétrons, o que reduz a síntese de ATP juntamente com a produção excessiva de ERO, como ânions superóxido (O2•-), peróxido de hidrogênio (H_2O_2) (Sriram *et al*., 1997). Essas ERO sobrecarregam o mecanismo de defesa antioxidante celular e causam danos às células nigroestriatais DA no SNpc e no corpo estriado por meio de peroxidação lipídica, danos ao DNA e ligação cruzada de proteínas (Zamanian *et al*., 2023).

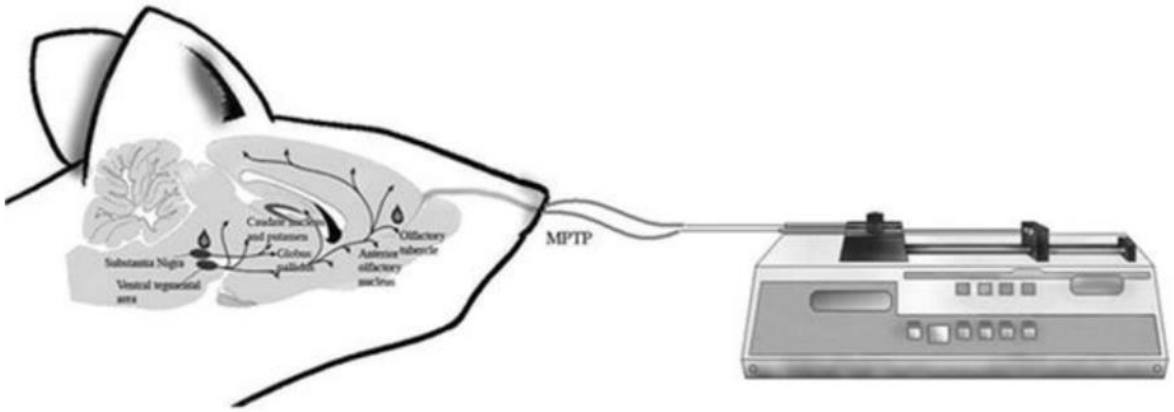
O papel das mitocôndrias na degeneração nigroestriatal dependente de MPTP implica fortemente a disfunção mitocondrial na DP esporádico, pacientes com DP apresentam diminuição da atividade do complexo I da cadeia respiratória no SN (Dionísio *et al*., 2021). Já em primatas e camundongos os terminais nervosos dopaminérgicos do corpo estriado são eventos precoces neurais associados ao dano induzido por MPTP (Marques *et al*., 2018; Mustapha *et al*., 2021), provavelmente devido ao maior conteúdo de DAT e VMAT2, elevada densidade mitocondrial e maior sensibilidade das mitocôndrias sinápticas à inibição do complexo I quando comparadas às mitocôndrias somáticas. (Dauer e Przedborski, 2003).

A administração via intranasal (i.n) bilateral de MPTP vem sendo proposta para o estudo da DP (Prediger *et al*., 2006; Franco *et al*., 2007; Prediger *et al*., 2012; Marques *et al*., 2018; Schamne *et al*., 2018). Este modelo tem como base a ‘Hipótese da Vetorização Olfatória” a qual estabelece que xenobióticos atingem o SNC através da sua entrada pela via olfatória (Fig. 4), por meio de nervos, vasos sanguíneos, fluido cerebrospinal e o sistema linfático, contornam os obstáculos impostos pela BHE e causam efeitos tóxicos, contribuindo para o surgimento de sinais semelhantes à doenças, como a DP (Doty, 2008; 2012).

Braak e colaboradores (2004) realizaram mapeamento da DP onde identificaram seis estágios neuropatológicos para a doença, essa visão temporal recebeu o nome de “Hipótese de Braak”. Esta hipótese traz o aparecimento de sintomas olfatórios iniciais, na qual a degeneração de neurônios no bulbo olfatório seria a responsável pelos distúrbios olfatórios observados nas fases prodrômicas da DP, passando por sintomas cognitivos (como prejuízo nas memórias

operacionais) e emocionais (depressão e ansiedade), até o aparecimento das alterações motoras clássicas (Sveinbjornsdottir, 2016). Nesse sentido, há uma expectativa no aumento de sequelas neuropsiquiátricas e na incidência de doenças neurodegenerativas nos próximos anos (de Erausquin *et al*., 2022).

Fig. 4 - Modelo de administração i.n. do MPTP.



Fonte: (Adaptado de Hami *et al*., 2016).

Estudos apontam que o uso deste modelo com uma única administração de MPTP em cada narina de roedores levou a um aumento significativo na geração de EROs foi observado no estriado após 6 horas (Marques *et al*., 2018); disfunção olfatória nos dias 1, 3, 7 e 14 após (Prediger *et al*., 2007); prejuízos cognitivos (Castro *et al*., 2013); comportamento tipo anedônico depressivo mais pronunciado nas fêmeas, também observados em ovariectomia (OVX) e fêmeas idosas (Schamne *et al*., 2018), prejuízo motor (Datta *et al*., 2019). Além disso, este modelo de DP induziu uma diminuição na expressão da enzima Tirosina Hidroxilase (TH), no bulbo olfatório, SNpc e estriado (Prediger *et al*., 2006; Franco *et al*., 2007, Castro *et al*., 2013; Savall *et al*, 2023) e um aumento do número de células (glial fibrillary acidic protein) GFAP positivas no estriado e SN, características do adoecimento. Além de alterar a sinalização de neurotrofinas no SNC, contribuindo para a disfunção sináptica (Savall *et al*, 2023).

Seguindo a linha temporal de alterações causadas pela infusão de MPTP, vale salientar que o EO mostrou-se tempo-dependente em neurônios dopaminérgicos, 6 horas após a infusão intranasal do MPTP é possível notar um aumento mais pronunciado nos níveis da peroxidação lipídica e na atividade GSH em estruturas como bulbo olfatório, estriado e córtex, retornando à condições normais dentro de 24 horas (Franco *et al*., 2007; Marques *et al*., 2018). A síntese neuronal de GSH depende de sua clivagem extracelular pela atividade da ÿ-glutamil

transpeptidase (GGT) (Pérez-Sala e Pajares, 2023). Assim, paralelamente à indução da síntese de GSH, um aumento na rota extracelular de GSH pode ser uma possível explicação para o grande aumento dos níveis de GSH total no OB no 6 h após o tratamento com MPTP. Sugerindo uma resposta adaptativa direcionada à síntese de GSH para evitar sua depleção (Olanow e Tatton, 1999). Curiosamente, essas respostas não foram observadas 2 ou 24 horas após a administração de MPTP, indicando que o aumento dos níveis de GSH, que provavelmente representa uma resposta compensatória aos efeitos pró-oxidativos do MPTP/MPP^+, é extremamente transitório (Franco *et al*., 2007; Prediger *et al*., 2011).

Assim, esse modelo representa uma interessante estratégia para testar novas estratégias e alvos moleculares no contexto da DP, como a busca por novos fitoterápicos com potencial antioxidante e neuroprotetor.

2.6 Compostos bioativos e suas propriedades

A natureza oferece uma fonte praticamente ilimitada de compostos com efeitos positivos para a saúde humana, conhecidos como bioativos naturais (Kussmann *et al*., 2023). Eles são principalmente metabólitos secundários e podem ser amplamente categorizados em compostos fenólicos, alcalóides, pigmentos e fatores de crescimento (Kudanga *et al*., 2017).

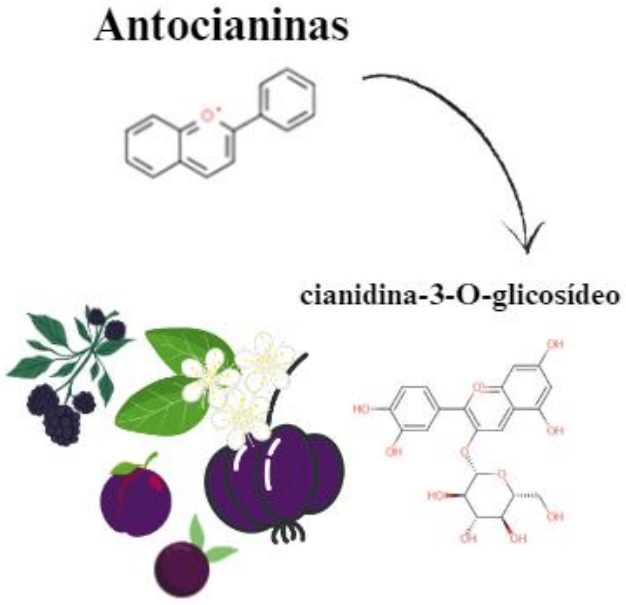
Compostos fenólicos podem ser encontrados em vegetais, sementes, frutas, nozes, vinho tinto, chá e muitas outras fontes alimentares (Eseberri *et al*., 2022), estruturalmente, são caracterizados por pelo menos um anel aromático com um ou mais grupos hidroxila ligados (Zdunczyk *et al*., 2002). Entre os diferentes tipos de compostos fenólicos, os ácidos fenólicos simples e os flavonóides são os mais abundantes na natureza (Pagnussatt et a., 2016). Como o ácido gálico, que representa a classe simples de ácido fenólico (Dai e Mumper, 2010) e está abundantemente disponível em várias partes das plantas, como frutas, sementes, folhas e madeira. Também é abundante em formas comestíveis, como amora, mirtilo, morango, castanha de caju, nozes, uvas, ameixas, jabuticaba entre outras (Reynertson *et al*., 2006; Daglia *et al*., 2014; Figueredo *et al*., 2018; Villas-Boas *et al*., 2021).

Os flavonóides são os polifenóis presentes nos vegetais, eles fornecem para plantas proteção contra agentes externos (radiação, UV, parasitas e vírus), regulam enzimas envolvidas no metabolismo celular e possuem propriedades antioxidantes (Manach *et al*., 2004). São encontrados principalmente nos vacúolos das células vegetais na forma de *C*-glicosídeos ou *O*-glicosídeos (Hussain *et al*., 2020). Eles são sintetizados através da via metabólica dos

fenilpropanóides e possuem 15 átomos de carbono dispostos em três anéis (C6-C3-C6) (do Nascimento *et al*., 2022). Os flavonóides são classificados em vários tipos, dependendo de sua estrutura química, grau de insaturação e oxidação do anel de carbono, incluindo flavonas, flavanonas, isoflavonas, flavonóis, chalconas, flavanóis e antocianinas (Chen *et al*., 2023).

Antocianinas são uma classe flavonóides polifenólicas naturais solúveis em água, responsável pelos pigmentos vegetais naturais, fornecem às plantas várias cores azul, roxo, vermelho e rosa (Khoo *et al*., 2017). A cor das antocianinas está intimamente relacionada com o valor do pH, pesquisas revelaram que tons vermelhos aparecem em condições ácidas e ficam azuis ou incolores à medida que o pH aumenta (Castañeda -Ovando *et al*., 2009; Liu *et al*., 2021). As antocianinas foram extraídas e isoladas de plantas, entre elas malvidina, pelargonidina, delfinidina, petunidina, peonidina e cianidina são amplamente distribuídas nas plantas (Wu *et al*., 2006). No conteúdo destas antocianinas, nas partes comestíveis das plantas, a cianidina se destaca por ocupar cerca de 50%. A cianidina confere a cor magenta (roxo-avermelhada) ao vegetal e é encontrada usualmente em bagas (De Brito *et al*., 2007), além de ser uma das antocianinas mais difundidas na natureza, sendo a cianidina-3-O-glicosídeo (Fig.5) a mais comum (Riaz *et al*., 2016).

Fig. 5 - Estrutura química da antocianina e cianidina-3-O-glicosídeo, presente nos frutos roxos.



Fonte: Autoria própria.

Vários estudos dirigiram-se a entender as atividades biológicas das antocianinas,

estudos farmacocinéticos *in vivo* com antocianinas, descobrindo que, em geral, a biodisponibilidade é baixa (Hahm *et al*., 2022), mas as propriedades físico-químicas de cada antocianina influenciam a sua biodisponibilidade. Estudos demonstraram que elas são absorvidas como glicosídeos em humanos e roedores (Milbury *et al*., 2002; Scalbert *et al*., 2002) e ao serem absorvidas podem atravessar a BHE e localizar-se em diferentes regiões cerebrais como córtex, hipocampo, corpo estriado e cerebelo (Andrés-Lacueva *et al*., 2005; Milbury e Kalt, 2010).

Em células, extratos provenientes de mirtilos, semente de uva, hibisco, groselha-negra e amora chinesa, ricos em antocianinas e proantocianidinas, foram mais eficazes em proteger neurônios dopaminérgicos da morte induzida por rotetona in *vitro* do que extratos contendo outros polifenóis Strathearn e colaboradores (2014). Kim e colaboradores (2010), observaram efeito neuroprotetor do extrato etanólico da amoreira, fruto rico em cianidina-3-O-glicosídeo que protegeu a neurotoxicidade em células SH-SY5Y estressadas por 6-OHDA. Ainda em células primárias mesencefálicas estressadas com 6-OHDA ou MPP^+, o pré-tratamento também protegeu os neurônios dopaminérgicos, mostrando uma ampla gama de concentrações efetivas na toxicidade induzida por MPP^+. Já em modelo subagudo de DP as antocianinas protegem os neurônios dopaminérgicos contra a exposição ao MPTP, regulando a geração de ROS e NO, reduzindo a expressão de Bcl-2 e Bax, a despolarização da membrana mitocondrial e a ativação da caspase-3.

As antocianinas mostraram, em modelo animal de doença de Alzheimer, reverter o comprometimento da aprendizagem e da memória induzidos por escopolamento através da diminuição da atividade acetilcolinesterase no hipocampo e córtex, aumentando a atividade de Na, K^+-ATPase e Ca^{2+}-ATPase, enzimas envolvidas na geração de potencial de membrana, excitabilidade neuronal, plasticidade sináptica e produção de espécies reativas de oxigênio (Gutierres *et al*., 2014).

O extrato de morango exerce um efeito protetor contra lesões gástricas induzidas pelo etanol, possivelmente ativando SOD e catalase e reduzindo as reações de peroxidação lipídica (Alvarez-Soarez *et al*., 2011). O extrato de antocianina de mirtilo atua contra hiperplasia prostática benigna em camundongos, quando usado em combinação com o pólen de *Brassica napus*, diminuindo os níveis de peroxidação lipídica, aumentando a capacidade de absorção de radicais de oxigênio e o conteúdo de GSH, e elevando a atividade da SOD e da GPx (Li *et al*., 2013).

Antocianinas extraídas de amoras reduzem pela metade a incidência de câncer de fígado induzido por N-nitrosodietilamina em ratos. O efeito protetor do extrato de antocianina contra

o câncer de fígado está relacionado à diminuição da expressão da enzima COX-2, que, pela via NF-κB (Fator Nuclear Kappa B), aumenta a expressão de enzimas antioxidantes, reduzindo a peroxidação lipídica (Liao *et al*., 2018). As antocianinas exercem efeitos protetores dificultando a apoptose induzida por estresse oxidativo mitocondrial, preservando os níveis de GSH, inibindo a oxidação da cardiolipina e prevenindo a fragmentação mitocondrial (Kelsey *et al*., 2011).

Os efeitos neuroprotetores das antocianinas estão intimamente ligados às suas capacidades antioxidantes e de eliminação de radicais (Zaa *et al*., 2023). A ação das antocianinas sobre a diminuição da produção de EROs pode estar associada tanto com a inibição direta quanto com a modulação dos mecanismos de defesa do organismo, como a GSH (Ereminas *et al*., 2017). Assim, frutos contendo antocianinas como o EPR se tornam uma potente alternativa para avaliar os efeitos do EO, como uma fonte natural de antioxidantes.

3. JUSTIFICATIVA E HIPÓTESE

O Brasil possui uma grande diversidade biológica que pode ser explorada para produzir extratos para aplicação terapêutica no controle e/ou prevenção de doenças crônicas e agudas (Denardin *et al*., 2015; Dutra *et al*., 2016). As frutas tropicais frescas são importantes fontes de carboidratos, vitaminas, minerais, fibras e antioxidantes (Feng *et al*., 2015), que ajudam a melhorar o sistema imunológico (West *et al*., 2018). Embora o maior número de espécies nativas brasileiras se encontre no bioma da Amazônia e do Cerrado, o bioma Pampa possui grande riqueza (Raseira, 2004).

O extrato da pitanga roxa fruto da região do pampa brasileira tem demonstrado, tanto *in* vivo como *in* vitro, efeitos antioxidante e neuroprotetor em modelos de doenças neurodegenerativas. Entretanto, parte das plantas medicinais nativas não passaram por nenhum tipo de estudo que avalie a sua farmacologia e toxicologia, havendo poucos dados científicos no que diz respeito à segurança de consumo dessas plantas pela população (Bednarczuk *et al*., 2010). Assim, apesar do potencial antioxidante e antiparkinsoniano em modelos experimentais já demonstrados na literatura, nenhum estudo avaliou os efeitos toxicológicos do EPR e seus efeitos antioxidantes e protetores frente à neurotoxinas em roedores.

Com base no exposto, este estudo se justifica pela necessidade de realizar ensaios toxicológicos para o uso seguro do extrato de *Eugenia uniflora* em mamíferos, além de avaliar os efeitos antioxidantes e neuroprotetores deste extrato. Assim, o objetivo desta tese foi realizar uma revisão sistemática sobre *Eugenia uniflora* e investigar a biossegurança do EPR, assim como seu potencial antioxidante e capacidade neuroprotetora. A hipótese avaliada neste estudo é que o EPR, por apresentar componentes bioativos, apresenta baixa toxicidade e atenue o estresse oxidativo induzido pela administração i.n. de MPTP em ratos.

4. OBJETIVOS

O objetivo geral desta tese foi compreender o estado da arte sobre a *Eugenia uniflora* e investigar a segurança e os efeitos do EPR sobre e às alterações oxidativas induzidas pela administração i.n. de MPTP em diferentes estruturas cerebrais em roedores.

4.1 Objetivos específicos

- Pesquisar, revisar e sintetizar as informações disponíveis até o momento sobre a *E. uniflora*, abrangendo aspectos como suas propriedades, usos, potenciais benefícios para a saúde e toxicidade;

- Investigar os efeitos toxicológicos e farmacocinéticos do EPR utilizando modelagem molecular para prever parâmetros ADMET (Absorção, Distribuição, Metabolismo, Excreção e Toxicidade) e em ensaios *in vitro* e *ex vivo*;

- Avaliar os efeitos da EPR sobre o estresse oxidativo agudo induzido pela administração i.n. de MPTP em bulbo olfatório, estriado e substância negra de ratos.

PARTE II

5. DESENVOLVIMENTO

O desenvolvimento desta tese de doutorado está apresentado sob a forma de artigo científico publicado na revista *Arabian Journal of Chemistry*; um manuscrito científico a ser submetido conforme às normas da revista *ACS Chemical Health & Safety*, ainda segundo artigo publicado na revista *Metabolic Brain Disease*. Desta forma, a metodologia adotada no desenvolvimento das pesquisas, assim como os resultados se encontram nestas seções, seguidos das referências bibliográficas.

5.1 Artigo Científico I

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REVIEW ARTICLE

Pitanga (*Eugenia uniflora L.*) as a source of bioactive compounds for health benefits: A review

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Abstract The pitangueira (*Eugenia uniflora*) is a tree native to Brazil but is cultivated in several subtropical countries. A great diversity of nutrients and bioactive compounds have been found in the leaves and fruits of *E. uniflora*, which supports its use in folk medicine to treat diseases such as stomach and intestinal disorders, fever and general inflammation. Antimicrobial, antiviral, antifungal and antioxidant effects on metabolism have been reported for this plant. This review discusses the phytochemical profile, toxicity and pharmacological action of *E. uniflora* leaves and fruits and points out that gaps in the literature that need to be investigated further. This review also discusses studies developed with *E. uniflora* demonstrating its promising therapeutic potential for several diseases with an apparent low toxicity in mammals. The compilation of the main pharmacological and toxicological results, as well as the phytochemical characterization of the varieties and constituents of *E. uniflora* are general aspects that this review attempts to demonstrate in order to contribute to the new approaches and developments to plant-derived natural product drug

Abbreviations: *A. salina*, *Artemia salina*; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid; ACE, aqueous crude extract; AOPP, protein oxidation products; AqF, aqueous fraction; aβ_{1-42}, amyloid β peptide fragment 1-42; *C. elegans*, *Caenorhabditis elegans*; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; *D. melanogaster*, *Drosophila melanogaster*; d.w, dry weight; DEX, dexamethasone; DMSO, dimethyl sulfoxide; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; *E. uniflora*, *Eugenia uniflora*; EAF, ethyl acetate fraction; EBV, Epstein-Barr virus; EC50, half maximal effective concentration; FRAP, Fe-reducing antioxidant potential; GAE, gallic acid equivalents; GSH, glutathione; HDPAF, high-polymerization Agave Frutans; HIV, human immunodeficiency virus; HPAF, high-performance Agave Frutans; i.c.v, intracerebroventricular; IC_{50}, half maximal inhibitory concentration; IL-1β, Interleukin-1 Beta; IL-8, Interleukin-8; iNOS, induced nitric oxide synthase; LD_{50}, lethal dose 50%; LOX, lipoxygenase; LPS, lipopolysaccharide; MD, maltodextrin; N2, wild type; NF-kB, nuclear factor-kB; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; PCA, air-dried extract; PCS, ethanolic sun-dried extract; ROS, reactive oxygen species; SOD-3, superoxidodismutase-3; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances; TNF-α, Tumor Necrosis Factor-Alpha; VLDL, very low-density lipoprotein

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discovery. However, further studies are required to establish the nutraceutical effects and uses of *E. uniflora* as an important and safe supplement for human health.

Contents

1. Introduction

The *Myrtaceae* family is one of the main families of commercial fruit trees in the world, comprising approximately 121 genera (de Paulo Farias et al., 2020). The genus *Eugenia* is considered the fourth most important genus of the Myrtaceae family, it is estimated that 350 species are native to Brazil (de Souza Cardoso et al., 2018), in addition to ecological importance, the representative species of the *Myrtaceae* family are aromatic plants with large agro-industrial potential (Sardi et al., 2017).

The genus *Eugenia* is used in folk medicine to treat wounds, flu, fever, cough, gout, hypertension, digestive and liver diseases, rheumatism, tonsillitis, sore throat, hemorrhoids and diarrhea (Arai et al., 1999, de Araujo et al., 2019, Araujo et al., 2021). The most studied species of *Eugenia* is *E. uniflora L.*, producer of pitanga (*E. uniflora L.*) (Malaman et al., 2011).

The pitangueira (*E. uniflora L.*) is a tree with a dense crown, measuring between 2 and 9 m in height, branched, with a rounded shape, persistent foliage and deep root system (Sanchotene 1989, Lorenzi 2008) (Fig. 1). It is found primarily in the south and southeast regions of Brazil. However, it is also cultivated in subtropical areas of Latin America (Bicas et al., 2011). Brazil has the largest germplasm bank of *E. uniflora* conserved *ex situ*, although not all information is available as there has been no complete evaluation and characterization.

The leaves of *E. uniflora* are identified as simple opposites, with a petiole measuring approximately 2 mm. When the leaves are new, they have a brownish-green color and a membranous consistency, whereas the adult leaves are dark green in color (Fouqué 1981, Lorenzi 1998). Due to complexities including variability, biotype, environmental factors, and the region in which it is located, it is challenging to complete a phenolic profile and characterize the components of the *E. uniflora* leaf (Costa et al., 2010).

The flowers grow at the base of branches after an age of approximately one year. They comprise 4 to 8 hermaphrodite flowers that have a mild fragrance and produce little or no nectar. The flower comprises 4 free petals, cream white in color. The anthers are yellowish in color and abundant in pollen with white stylets (Sanchotene 1989).

The pitanga is the popular name of the fruit from the pitangueira. The name has an indigenous origin being derived from the term Tupy '' pi'tãg which means red, referring to the most common color of the fruit (Donadio et al., 2002). However, this plant is also known as the Brazilian cherry or Suriname cherry (Fiuza et al., 2008). Generally, pitanga fruits are berry-shaped and have 8–10 longitudinal furrows in the skin (Romagnolo and Souza 2006). They are composed of 77% pulp and 23% seed and have a unique sweet, acid flavor with an intense aroma (Köhler 2014). The pitanga fruit can be consumed raw or used in juices, ice creams, sweets, liquors, and jellies (Coradin et al., 2011).

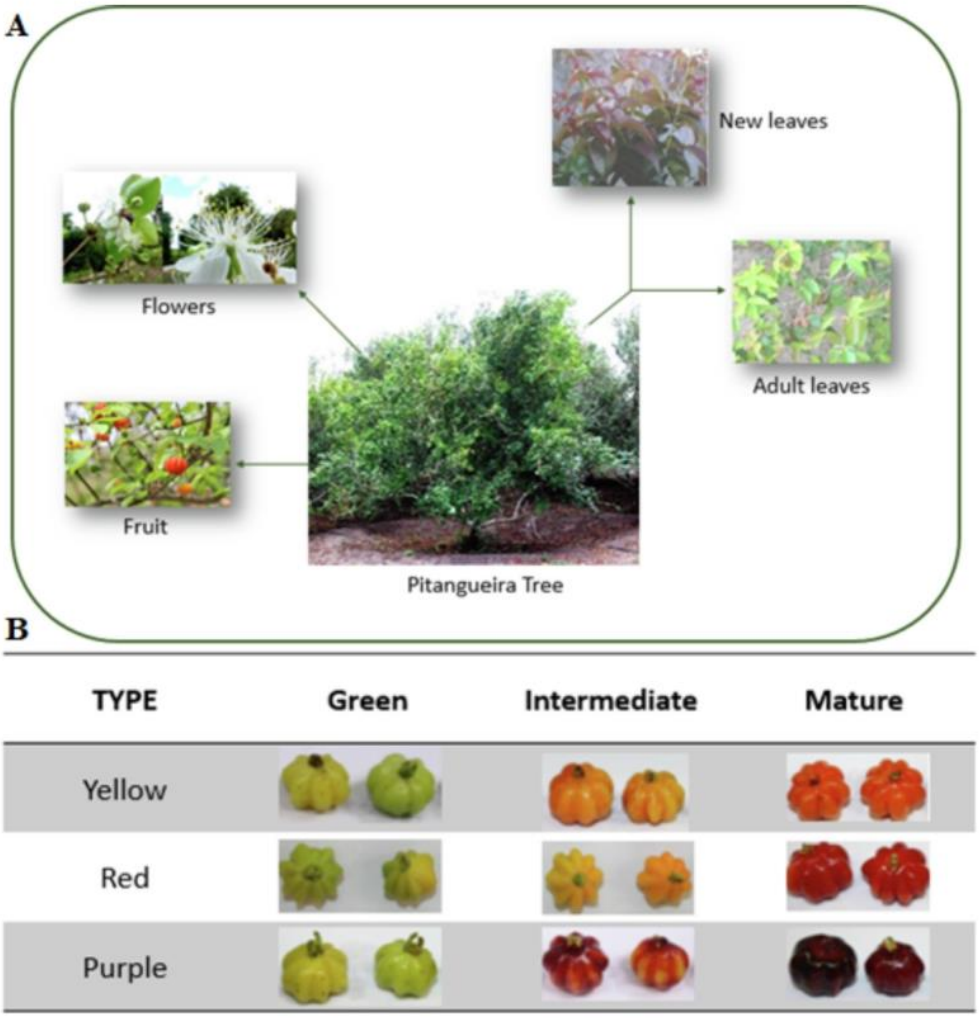

Fig. 1 The Pitangueira tree, its parts, varieties and different berries according to the stage of maturation and the variety. A) Images adapted from the Pitangueira Book (Júnior et al., 2007). B) Ripening stages of the different types of pitanga (*E. uniflora* fruit) (Souto, 2017).

Pitanga has different berries according to the stage of maturation and the variety. The extensive genetic diversity is primarily manifested in the color of the ripe fruit (Silva 2006, Bezerra et al., 2018). During ripening, the fruit's epicarp evolves from green to orange and red to dark purple (de Lira Júnior et al., 2007) (Fig. 1). Therefore, the fruits undergo changes not only in its color but also in its phytochemical constituents.

Several studies have evaluated the therapeutic efficacy of extracts derived from this plant through different extraction methods in several experimental models, however, this data from different varieties have never been analyzed altogether (Soares 2014, Falcao et al., 2018, Tambara et al., 2018). This narrative review was carried out as a literature review in 2021 and includes articles published from 1976 to 2021. Specialized databases (Web of Science, Scielo, Pubmed, Science Direct, Scopus and selected articles from Google Scholar), were used and included *E. uniflora, E. uniflora* purple, *E. uniflora* red, *E. uniflora* yellow as keywords for literature search. In this review, the phytochemical characteristics of *E. uniflora* will be addressed along with its toxic potential. In addition, the pharmacological properties of leaves and fruits of different varieties of *E. uniflora* will be described.

2. Physical-chemical characterization of *E. uniflora* varieties

It has been demonstrated that *E. uniflora* leaves contain constituents such as anthraquinones, steroids, triterpenes, flavonoids, saponin heterosides, and tannins (Brasileiro et al., 2006, Fiuza et al., 2008). Additionally, the fruits present a significant number of bioactive compounds, such as catechins, flavonols, proanthocyanidins, and carotenoids (Santos et al., 2003, Hoffmann-Ribani et al., 2009, Celli et al., 2011, Denardin et al., 2015, Souto 2017). The pulp presents low calories and contains calcium, phosphorus, iron, and vitamins B1, B2, and C (Helt et al., 2018).

Among the different types of pitanga, the red variety is richer in carotenoid compounds, such as beta-carotene and lycopene as well as in the flavonols myricetin, kaempferol and quercetin (Hoffmann-Ribani et al., 2009). In smaller quantities, these bioactive compounds are also found in yellow pitanga (Souto 2017). Generally, the higher amounts of carotenoids and phenolic compounds gives the purple variety the best antioxidant potential (Celli et al., 2011, Denardin et al., 2015).

It is noteworthy that many factors influence the phytochemical composition of these plants, such as environmental conditions or soil characteristics. For instance, the climate influences the carotenoid composition of pitanga fruit and its phenolic compounds (Robards and Antolovich 1997). A study of mature fruits from 12 randomly selected sister seedlings of purple pitanga demonstrated that there existed differences between the content of myricetin, quercetin, and lutein in the different samples, which exemplifies the variability in the constituents of the fruits belonging to the same plant (Griffis et al., 2013).

The ripening of the fruits is related to the presence and quantity of pigments, such as carotenoids, which may have increased synthesis according to the maturation stage and mainly due to the warm climate (Santos et al., 2003, Bagetti et al., 2011). In a study developed by Santos et al. (2003), the maturation stages of the fruits were classified into six, these maturation stages were compared using the red and purple pitanga varieties. The purple pitanga variety has six maturation stages, which is more than the red type, which has five (Dos Santos et al., 2018).

Factors including the storage time that can influence pulp characteristics, causing reduction in the carotenoid amount (Lopes et al., 2005), plant part selection (for example leaf, whole fruit, fruit pulp or peel) (de Lima et al., 2002), pre-extraction steps (drying, grinding, pressing, etc.), and the extraction method (Figueirôa et al., 2013) must be well considered as they influence the quantity and stability of the phytochemicals (Kumar and Sharma 2018).

2.1. Characterization of the leaves

Although some studies have already demonstrated the effects of *E. uniflora*, little data in the literature presents the identification of the phytochemicals present in the plant's leaves. In addition, the knowledge about a possible correlation between phytochemicals and the biological activities of this plant is still insufficiently evidenced because of the lack of studies (Falcao et al., 2018).

A phytochemical analysis by Brasileiro et al. (2006) demonstrated that *E. uniflora* leaves contain alkaloids, triterpenes, tannins, flavonoids, and anthraquinones. Furthermore, high concentrations of volatile organic compounds germacrene B (9.60%), γ-elemene (7.97%), β-elemene (9.26%), germacrene D (6.46%), γ-muurolene (5.2%) and β-caryophillene (5.17%) were found in the leaves from purple pitangueira (Mesquita et al., 2017). Furthermore, Chang et al. (2011) demonstrated curzerene presence at 85.1%, the same amount reported by Costa et al. (2009).

Notably, substance from the pitangueira leaves of Rio de Janeiro (50.2%, Melo et al., 2007), Goiás (42.6%, brilliant red fruit pitangueira) (Costa et al., 2010), Goiás (34.8%), (Peixoto et al., 2019) and Nigeria (19.7%) (Ogunwande et al., 2005) were also found to be the primary component in essential oils.

Other classes of compounds are also present but to a lesser extent (less than 20% in the leaves), such as monoterpene hydrocarbons, oxygenated monoterpenes and oxygenated sesquiterpenes (Apel et al., 2004, Costa et al., 2010). The presence of flavonoids and phenolic compounds has been reported in the *E. uniflora* leaves ethanolic extract as described by Fiuza et al. (2008).

Table 1 Chemical components of different extracts of *E. uniflora* leaves.

Extract	Total phenolic (mg GAE.g^{-1})	Flavonoid content (mg QE.g^{-1})
Ethyl acetate fraction of leaves	2756 ± 15.5	84.3 ± 3.4
Butanol fraction of leaves	2492 ± 58.1	8.6 ± 0.9
Aqueous fraction of leaves	2445 ± 15.5	2.1 ± 0.07
Ethyl acetate fraction of leaves	2831 ± 23.0	31.2 ± 1.1

See Table adapted from Figuerôa et al. 2013. GAE = gallic acid equivalents and QE = quercetin equivalents.

Besides, Figuerôa et al. (2013) demonstrated in an experimental study using different extractors from *E. uniflora* leaves that the total phenolic content was similar between the different types of extracts. However, the flavonoid content showed substantial differences that can significantly influence the pharmacological properties of these extracts. Thus, it is noteworthy that the extraction method also markedly influences the phytochemical constitution of the extract (Table 1).

A recent study by Sobeh et al. (2020) showed a more comprehensive phytochemical profile of the ethyl acetate fraction (EAF) of red *E. uniflora* leaves, which presented an extensive variety of polyphenols, such as quercetin, myricetin, apigenin and kaempferol glucosides. This extract seems to present promising properties due to this composition. Other studies using methanol extract of the leaves of *E. uniflora* and LC-MS/MS chromatography have identified about 17 compounds among which are secondary metabolites: gallic acid 3-O-[6′-O-acetyl-β- D-glucoside], myricetin-3-O-β-D-glucoside, myricetin-3-O-β-D-galactoside, myricetin-3-O-[6′-O-β-N-acetyl galacto-side], myricetin-3-O-rhamnoside, myricetin-3-O-[3′-O-acetylrhamnoside], myricetin-3-O-[2′-O-acetyl-α-L-rhamno side], quercetin-3,5-dimethyl ether, ellagic acid, 2,3-hexahy droxydiphenoyl-D-glucose, gallic acid, methylgallate, 3-O-D-galloylglucose, 2-O-D-galloylglucose, gentistic acid 5-O-β-D-glucoside, gentistic acid 5-O-[6′ -O-β-D-galloyl glucoside], valoneic acid dilactone (Sobeh et al., 2019).

2.2. Purple E. uniflora fruits

Purple pitanga is the rarest variety but has a higher amount of total phenolics and therefore higher antioxidant properties than other pitanga types (Weyerstahl et al., 1988). The purple *E. uniflora* fruit has approximately twice the phenolic content compared to the red type (463 ± 16 to purple and 210 ± 3 % mg galic acid (GA). 100 g^{-1} to red fruit) This has promoted an increasing scholarly interest in this variety (Bagetti et al., 2011). The purple pitanga variety presents higher pH and higher total soluble solids and carbohydrates (Table 2). Furthermore, regarding fatty acid content, it has been described that oleic acid is present in a higher proportion than others (monounsaturated and polyunsaturated fatty acids). It has been found to have a high phenolic content in the methanolic extract, and a considerable amount of anthocyanins in the ethanolic extract has been detected (Bagetti

Table 2 Chemical characteristics and bioactive compounds found in the maturation of purple and red *E. uniflora* fruits.

Characteristics according to stage of maturation *E. uniflora* fruits						
Parameters	Maturation stages (purple / red type)					
	1	2	3	4	5	6
Diameter [A]	17.9 / 19.9	17.8 / 21.4	17.8 / 21.7	17.8 / 22.4	17.0 / 21.2	17.6 / nd
Length [A]	14.3 / 13.2	13.9 / 14.3	15.1 / 15.0	15.0 / 15.1	14.6 / 14.6	15.1 / nd
Fresh weight [B]	2.56 / 3.54	2.56 / 4.25	2.96 / 4.65	3.37 / 5.25	2.67 / 4.40	3.32 / nd
Dry matter [C]	26.26 / 19.02	24.7 / 19.44	22.36 / 18.26	23.51 / 17.83	22.86 / 18.81	24.41 / nd
Seeds [C]	35.26 / 27.61	28.20 / 29.69	27.64 / 23.86	30.93 / 20.41	34.26 / 25.04	38.23 / nd
Soluble solids [C]	8.53 / 8.13	9.73 / 8.96	10.76 / 10.33	12.53 / 11.00	10.56 / 12.56	13.04 / nd
Acidity [D]	1.66 / 0.86	1.74 / 1.23	1.98 / 1.61	1.55 / 1.58	1.73 / 1.57	1.64 / nd
pH	3.38 / 3.36	3.29 / 3.11	3.24 / 3.13	3.43 / 3.16	3.28 / 3.28	3.55 / nd
Vitamin C [E]	21.85 / 22.50	29.15 / 29.93	32.85 / 40.65	42.65 / 51.00	55.00 / 33.00	38.5 / nd
Total Anthocyanins [E]	0.00 / 0.00	2.40 / 0.09	9.40 / 0.29	16.30 / 0.51	21.60 / 0.98	29.60 / nd

Characteristics according to maturation stage of the purple and red pitanga. 1 – Green; 2 – Breaker (starting to change shell's color); 3 – Beginning of purple pigmentation; 4 – Partially purple; 5 – Completely purple; 6 – Dark purple. nd - values not described. Source: dos Santos et al., 2002. Data are expressed as [A] mm, [B] g, [C] %, [D] % citric acid and, [E] mg.100 g^{-1}.

et al., 2011). Accordingly, this can be associated with greater antioxidant capacity as demonstrated by the DPPH method (Celli et al., 2011).

The anthocyanins present in fruits such as pitanga are very promising for beneficial effects on human health. Some studies report that the intake of these compounds in humans is estimated to be 180–215 mg.day^{-1} in the USA (Kuhnau 1976). A profile characterization of anthocyanins in the ethanolic extract of purple pitanga revealed the presence of five anthocyanins: delphinidin 3-O-glucoside (99.65 ± 1.77 mg.100 g^{-1} lyophilized fruit), cyanidin 3-O-glucoside (512.01 ± 11.18 mg.100 g^{-1} lyophilized fruit), pelargonidin 3-O-glucoside (2.16 ± 0.13 mg.100 g^{-1} lyophilized fruit), cyanindin 3-O-pentoside (0.83 ± 0.07) and cyanidin derivative (5.16 ± 1.23 mg.100 g^{-1} lyophilized fruit) (Tambara et al., 2018). These anthocyanins seem to be very stable after freezing unlike other plant constituents (Lima et al., 2005). Furthermore, the peel has been found to contain more anthocyanins, flavonoids, and carotenoids compared to the pulp: Total Anthocyanins (mg.100 g^{-1}) – 26 ± 0, Total Flavonoids (mg.100 g^{-1}) – 18 ± 0, Total Carotenoids - (μ.g^{-1}) 111 ± 2 (de Lima et al., 2002).

2.3. Red E. uniflora fruit

The general properties of *E. uniflora* red fruit variety are characterized by lower pH, acidity, carbohydrates and phenolic content when compared to the purple type (Table 2). According to data from Denardin et al. (2015), a red pitanga has about 5.86 ± 0.03 ug of β-carotene.g^{-1}. Carotenoids are natural pigments produced during the photosynthetic process that provides coloring for flowers and fruits. These have some ingredients that can potentially prevent cardiovascular disease and cancer. Therefore, the investigation of these compounds has expanded in recent years (Yuan et al., 2015).

One study found the concentration at 0.086 ± 0.00 mg GAE.100 g^{-1} of ascorbic acid and the total phenolic content of the ethanolic extract at 433.84 ± 60.5 mg GAE.100 g^{-1} fw (fresh weight) as determined by the Folin-Ciocalteu method adapted from Swain and Hillis (Denardin et al., 2015). This difference in values in relation to Table 2 can be attributed to a series of processes related to the stage of maturation at harvest, genetic variants, post-harvest management, storage, and processing conditions (Szeto et al., 2002).

It is known that there is a difference in the quantification of these compounds according to the pitanga variety. However a difference is also found when using other methods for the detection of phenolic compounds. It is noteworthy that phenolic compounds are secondary metabolites widely found in vegetables, fruits, leaves and fruits and may vary depending on soil composition, temperature, season, etc. Therefore, phenolics composition varies across different studies (Sobeh et al., 2019).

A chromatographic analysis of the ethanolic extract of the red fruit performed on HPLC-DAD (wavelength at 280 nm and 360 nm) demonstrated the presence of gallic acid and derivatives, quercetin and derivatives, quercetin-3-b-D-glucoside, quercetin-3-rhammoside, kaempeferol derivative, cyanidin-3-glucoside, cyanidin derivative and malvidin derivative (Denardin et al., 2015).

2.4. Yellow E. uniflora fruit

Few data in the literature provide information about the components present in the yellow *E. uniflora* variety (fruits and leaves). Souto et al. (2017) assessed the phytochemical composition of the methanolic extract of the yellow *E. uniflora* fruit, which included the presence of quercetin, myricetin and its derivatives: 1) In Green stage (mg.100 g^{-1} d.w): quercetin (43.3 ± 3.4), quercetin-3-glucoside (26.4 ± 5.3), myricetin (62.4 ± 8.2), kaempferol (1.5 ± 0.4); 2) In mature stage (mg.100 g^{-1} d.w): quercetin (20.1 ± 2.1), quercetin-3-glucoside (6.7 ± 1.8), myricetin (25.0 ± 3.6), kaempferol (ND). Additionally, the content of flavonoids with respect to the stages of development do not show significant changes with myricetin being the majority compound corresponding to 48% of the total flavonoids followed by quercetin with up to 39% of the total compared to the other flavonols.

On the other hand, the phenolic content changed depending on the development stage of the fruit. In the green maturation stage, the values obtained were approximately 35 mg GAE.g^{-1} d.w (dry weight), in the intermediate stage approximately 25 mg GAE.g^{-1} d.w and in the mature fruit approximately

15 mg GAE.g^{-1} d.w. The presence of ellagic acid was also detected at the mature stage (around 485.1 mg.100 g^{-1} d.w). In the earlier maturation stages, carotenoids such as ß-carotene and lycopene are not present. They are produced at the ripest stage of the fruit before changing to the next color (7.4 ± 3.1 μg.g^{-1} dw; 20.5 ± 5.2 μg.g^{-1} dw, respectively), other compounds as citric, succinic and malic acids were also found in the yellow cherry as demonstrated by Souto et al. (2017).

The lack of studies on the yellow pitanga hinders the discussion of this topic, it is evident that the preferential research on the red and purple pitanga is due to the strong presence of phenolic compounds, anthocyanins and carotenoids, since they present some photoprotective ingredients that can potentially prevent diseases related to oxidative damage. The low amount of carotenoids in the yellow pitanga can be a limiting factor for studies with this variety.

2.5. Non-volatile and volatile compounds

The constituents of plants directly influence the characteristics of their fruits. The presence of volatile organic compounds (VOCs) is responsible for the aroma and consequently the flavor of fruits. Some studies also demonstrate the influence of non-volatile compounds in the flavor of these fruits (Bai et al., 2016). Many factors can influence the presence of these compounds, such as climatic and growing conditions, in addition to the ripening stage of the fruit (Kawahata et al., 1996). Some authors used different methodologies to identify volatile and non-volatile compounds present in the constituents of pitangueira, as shown in Table 3. In addition, Fig. 2 shows the structures of the main bioactive compounds present in the constituents of pitangueira.

The main limitation of these studies is that many do not report the variety of the fruit or plant from which the compound was isolated, as well as its constituents. Only one study that demonstrated the presence of non-volatile compound cyanidin-3-glucoside used liquid chromatography-mass spectrometry (LC/MS) (Brasileiro et al., 2006). Many authors used the gas chromatography–mass spectrometry (GC/MS) to isolate the volatile compounds (Oliveira et al., 2008, Soares 2014). Mesquita et al., (2017) determined the volatile profiling through solid-phase microextraction (HS-SPME), combined with gas chromatography-mass spectrometry (GC–MS).

In the different maturation stages, terpenoids (monoterpenes and sesquiterpenes) are found. However, the presence of esters only was determined in the last stages of maturation (da Silva et al., 2019). In addition, it was observed that the different varieties of *E. uniflora* are responsible for the variability in the volatile compounds present in the leaves of the plant. The orange[1] variety of pitanga presents a high amount of hydrocarbon monoterpenes in relation to the purple and red that present similar profile (Mesquita et al., 2017). Some compounds, such as selin-1,3,7(11)-trien-8-one, are also found in the fruit extract (Oliveira et al., 2008). The presence of selina-1,3,7(11)-trien-8-one, curzerene and atractylone, spathulenol and germacrone were identified in specifically in red-orange[1], red and purple, respectively. It has been identified that the increase in the presence of anthocyanins and tannins decrease during the maturation process of pitanga (Ramalho et al., 2019).

The differences of climate conditions can exert influence on the chemical constituents of *E. uniflora*. The prevalence of monoterpenes is related in the pitanga fruits, but differences in relation to majority compound are observed in fruits cultivated in Brazil and Cuba, with the presence of *trans*-b-ocimene (36.2%) and curzenene (38,9%), respectively (Pino and Correa 2003, Oliveira and Padilha 2007). In addition, different seasons in the same country have been shown to affect the constitution of essential oils in the leaves of the plant. In the dry season, the essential oil of the leaves of *E. uniflora* of the red–orange variety presented as major compounds spathulenol (10%) and caryophyllene oxide (4.1%). However, in the wet season, the main major constituent was seline-1,3,7(11)-trien-8-one epoxide (29%) (Costa et al., 2009).

3. *E. uniflora* toxicity

Little is known about the potential toxicity of *E. uniflora* extracts and the essential oils from this plant. It is known that the seeds are extremely resinous and should not be ingested. Additionally, it has been known to cause diarrhea in dogs fed with the whole fruits and the strong, and the spicy scent emanating from bushes being pruned has been known to irritate the respiratory tract of allergic people (Morton 1987).

In mice, a single oral administration of the essential oil obtained from the leaves of *E. uniflora* at different doses (10, 50, 100 and 200 mg.kg^{-1}) did not cause death in any subject. Therefore, the lethal dose 50% (LD_{50}) was estimated to be greater than 200 mg.kg^{-1} (Victoria et al., 2012). Besides, the single administration of the leaves' essential oil did not induce any signs of toxicity in mice, such as weight change or loss of appetite (Victoria et al., 2012). Similarly, Schmeda-Hirschmann et al. (1987) demonstrated that the hydro-alcoholic extract of the leaves did not produce any signs of acute or subacute toxicity in mice orally receiving doses of up to 4200 mg.kg^{-1}.

A toxicity test was performed 14 days after a single oral administration of *E. uniflora* hydro-alcoholic leaf extract at different concentrations (3.0, 3.6, 4.3, 5.2 and 6.2 g.kg^{-1}) in mice (Auricchio et al., 2007). The first deaths occurred in the first 24 h in the animals treated with the higher dose. By the fourth day, a small percentage of the animals treated with the two highest doses died. On the sixth day of observation, the surviving animals recovered and no difference in the behavior of treated animals was observed. At the end of the study, no differences were observed in the general appearance (color, size) of removed organs such as heart, lung, liver and kidneys. Furthermore, no alterations in the relative masses of these organs removed from treated animals were observed compared to controls. The LD_{50} estimated was 5.93 g.kg^{-1}. In another study assessing chronic exposure to crude extract of *E. uniflora* fruit at the dose of 200 mg.kg^{-1}.day^{-1} for 21 days, no signs of toxicity were observed in rats (de Souza Cardoso et al., 2018). Accordingly, it can be suggested that overall, *E. uniflora* fruit extract has low toxicity for mammals.

However, toxicity studies with the ethanolic leaf extract in *Artemia salina* (*A. salina*) have recently shown moderate toxi-

[1] Corresponds to yellow variety of pitanga.

Table 3 Volatile and non-volatile compounds present in the constituents of pitangueira

Pitanga varieties	Plant Constituent	Volatile compound	Authors
Purple-	Pulpleaf	oxidoselina-1,3,7(11)-trien-8-one	Soares et al., 2015; Weyerstahl 1988
Purple	Pulp	selina-1,3,7(11)-trien-8-one	Soares et al., 2015
Purple	Pulp	oxidoselina-1,3,7(11)-trien-8-one	Soares et al., 2015
-	Fruit	Propyl acetate	Oliveira et al., 2006[F3]
-	Fruit	Ethyl propionate	Oliveira et al., 2006
-	Fruit	Isobutyl acetate	Oliveira et al., 2006
-	Fruit	n-Butyl acetate	Oliveira et al., 2006
-	Fruit	3-Methyl butyl acetate	Oliveira et al., 2006
-	Fruit	1,5,8-p-Menthatriene	Oliveira et al., 2006
-	Fruit	*trans*-β-Ocimen	Oliveira et al., 2006
-	Fruit	p-Mentha-1,5,8-triene	Oliveira et al., 2006
-	Fruit	Rosefuran	Oliveira et al., 2006
-	Fruit	β-Ocimene	Oliveira et al., 2006
-	Fruit	Acetophenone	Oliveira et al., 2006
-	Fruit	Germacrene-D	Oliveira et al., 2006
-	Fruit	Caryophyllene oxide	Oliveira et al., 2006
-	Fruit	α-Thujene	Pino et al., 2003
-	Fruit	α -Pinene	Pino et al., 2003
-	Fruit	β-Pinene	Pino et al., 2003
-	Fruit	β -Myrcene	Pino et al., 2003
-	Fruit	λ-Terpinene	Pino et al., 2003
-	Fruit	Terpinolene	Pino et al., 2003; Silva et al., 2019
-	Fruit	Allo-ocimene	Pino et al., 2003
-	Fruit	β-Elemene	Pino et al., 2003; Silva et al., 2019
-	Fruit	β-Caryophillene	Pino et al., 2003
-	Fruit	λ-Elemene	Pino et al., 2003; Silva et al., 2019.
-	Fruit	β-Elemenone	Pino et al., 2003
-	Leaf	α-Terpinene	Weyerstahl 1988
-	Leaf	*p*-Cymene	Weyerstahl 1988
-	Leaf	*trans*-Ocimene	Weyerstahl 1988
-	Leaf	*cis*-Ocimene	Weyerstahl 1988
-	Leaf	Linalool	Weyerstahl 1988
-	Leaf	Curzerene	Weyerstahl 1988
-	Fruit	Dracunculifoliol	Silva et al., 2019
-	Fruit	Heptanol	Silva et al., 2019
-	Fruit	Hexenol	Silva et al., 2019
-	Fruit	Cedrenal	Silva et al., 2019
-	Fruit	Hexanal	Silva et al., 2019
-	Fruit	Isovaleraldehyde	Silva et al., 2019
-	Fruit	Ethyl acetate	Silva et al., 2019
-	Fruit	Ethyl butanoate	Silva et al., 2019
-	Fruit	Ethyl hexanoate	Silva et al., 2019
-	Fruit	Turmerone	Silva et al., 2019
-	Fruit	Aromadrendene	Silva et al., 2019
-	Fruit	Cadinene	Silva et al., 2019
-	Fruit	Carene	Silva et al., 2019
-	Fruit	Cymene	Silva et al., 2019
-	Fruit	Epizonarene	Silva et al., 2019
-	Fruit	Germacren	Silva et al., 2019
-	Fruit	Guaiene	Silva et al., 2019
-	Fruit	Limonene	Silva et al., 2019
-	Fruit	Maaliol	Silva et al., 2019
-	Fruit	Ocimene	Silva et al., 2019
-	Fruit	Sylvestrene	Silva et al., 2019
-	Fruit	Tricyclene	Silva et al., 2019
-	Fruit	Valerianol	Silva et al., 2019
Orange*, Red and Purple	Leaf	3-hexen-1-ol acetate	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	α -phellandrene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	methyl salicylate	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	(Z)-3-hexenyl butyratea	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	α-copaene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	α-gurjunene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	α-humulene	Mesquita et al., 2016

(*continued on next page*)

Table 3 (*continued*)

Pitanga varieties	Plant Constituent	Volatile compound	Authors
Orange*, Red and Purple	Leaf	allo-aromadendrene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	β-selinene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	γ-muurolene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	α-amorphene	Mesquita et al., 2016
Orange*, Red and Purple	Leaf	ledol	Mesquita et al., 2016
Non-Volatile compound			
Purple	Pulp	cyanidin-3-glucoside	Soares et al., 2015

* Orange pitanga corresponds to yellow variety.

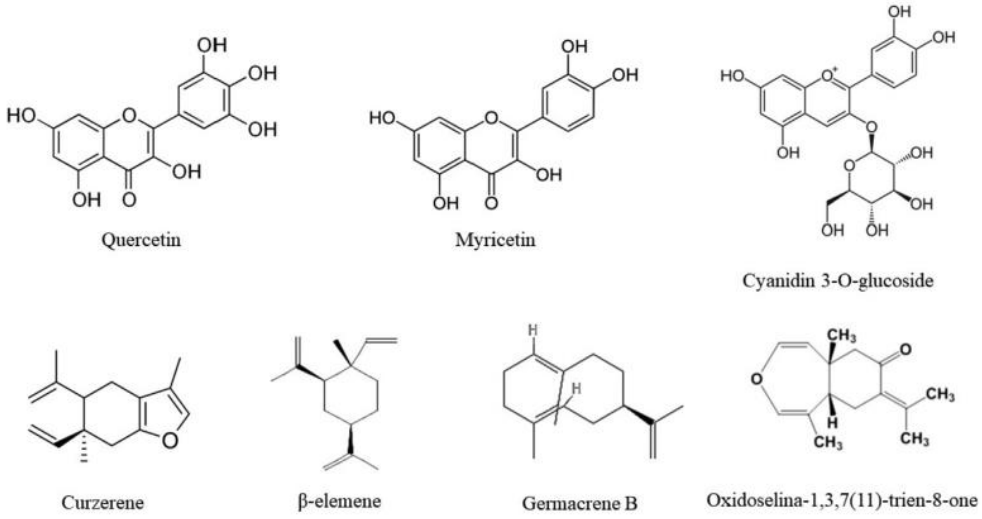

Fig. 2 The chemical structures of the main bioactive compounds present in the constituents of pitangueira.

city in this crustacean according to the criteria established by Déciga-Campos et al. (2007) with a lethal concentration 50% (LC_{50}) of 0.61 $mg.mL^{-1}$ (Bobadilla et al., 2018). Montanher et al. (2002) obtained an LC_{50} higher than 1 $mg.mL^{-1}$ for *E. uniflora* ethanolic extract in the same species. Additionally, Arcanjo et al. (2012) demonstrated that the ethanolic extract of the aerial parts contained a LC_{50} of 0.288 $mg.mL^{-1}$ in *A. salina*.

In *Drosophila melanogaster* (*D. melanogaster*), Da Cunha et al. (2015) demonstrated that a short period of exposure to low concentrations of essential oil from *E. uniflora* leaves induced mortality and locomotor deficits by inducing oxidative stress. Moreover, De Carvalho et al. (2017) recently demonstrated for the first time that *E. uniflora* essential oil can induce mitochondrial dysfunction as well as to compromise mitochondrial respiration in *D. melanogaster*.

In vivo studies using the nematode *Caenorhabditis elegans* (*C. elegans*) have demonstrated that animals treated with red and purple pitanga fruit ethanolic extract 1%(v/v) showed no signs of toxicity either in short- or long-term assessments (Borges 2015). Similarly, Tambara et al. (2018) also demonstrated that treatment with purple pitanga fruit extract did not alter the worms' survival and reproduction at different concentrations (5, 50, 100, 250 and 500 $\mu g.mL^{-1}$).

Overall, it seems that very little is known about the toxic effects of *E. uniflora* extracts in mammalian models. However in invertebrate animals, it seems that the extracts are more toxic and may even compromise cellular respiration, depending on the part used (fruit or leaf) and the extraction method used. This toxic outcome has been associated with the insecticidal activity of *E. uniflora* extract (Jung et al., 2013) due to the presence of flavonoids and tannins identified in the plant's hydroalcoholic extracts (Auricchio et al., 2007). Tannins significantly reduce the growth and survival of insects by inactivating digestive enzymes and creating a complex of difficult-to-digest tannin-proteins (Melo et al., 2007). Therefore, further studies are required to clarify the possible toxicological mechanisms of the components of this plant.

4. Biological effects of *E. uniflora*

4.1. Antibacterial activity

Infections caused by bacteria are a significant public health concern. Multi-resistant microorganisms are present in hospitals and in other places. The investigation of new molecules in natural products that can kill resistant microorganism

species presents a plethora of possibilities. This property has been found for *E. uniflora* and has been explored in different scenarios.

A study evaluating the *in vitro* antibacterial activity of the hydroalcoholic extract of ripe *E. uniflora* demonstrated that it reduced the biofilm formation of *Streptococcus mutants*, *Streptococcus oralis* and *Lactobacillus casei*, with a reduction in bleeding caused by gingivitis, which was attributed to its anti-inflammatory action due to the presence of flavonoids (Jovito Vde et al., 2016). *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus mitis*, and *Streptococcus oralis* are involved in the biofilm formation and consequently cause predisposition to cavities. The ethanolic extract of the mature and unripe fruits as well as leaves extracts showed antibacterial activity, with the exception of the essential oil derived from the leaves (Oliveira et al., 2008).

In addition, another study with the essential oil from *E. uniflora* leaves demonstrated antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes*. These bacteria are part of the gram-positive group, whereas no effect was observed in the gram-negative pathogens (Victoria et al., 2012). Similarly, the essential oils were found to have bactericidal effect against other gram-positive pathogens, such as *Bacillus subtilis*, *Streptococcus faecalis* and *Staphylococcus albus*. Whereas no effect on the gram-negative group was found (Thambi et al., 2013). In gram-negative bacterias (*E. coli)* this oil present inhibitory effect and increase the activity of antibiotic aminoglycosides (Pereira et al., 2017).

On the other hand, positive results of *E. uniflora* against gram-negative bacteria were reported with ethanolic extract of the leaves, and inhibition was effective against *Enterobacter cloacae*, *Escherichia coli, Pseudomonas aeruginosa* and *Serratia marcescens* (Table 4) (Fiuza et al., 2009). Furthermore, pitanga seed have been found to have biological properties against both gram-negative and gram-positive pathogens. The essential oil and seed extracts present similar antibacterial properties due to the presence of oxygenated groups in the structure of the isolated compounds such as terpenoids, which may be responsible for this potential (Santos et al., 2015).

E. uniflora seeds contain lecithin that binds to microorganisms such as bacteria and can inhibit the growth of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella spp* at

Table 4 Antibacterial activity in different samples of *E. uniflora.*

Sample	Concentration	Bacteria	Inhibition zone (mm)	Reference
HEEU	0.3 g.mL^{-1}	*S. mutans*	14.0	Jovito et al., 2016
HEEU	0.3 g.mL^{-1}	*S. oralis*	23.0	Jovito et al., 2016
HEEU	0.3 g.mL^{-1}	*L. casei*	26.0	Jovito et al., 2016
EOL	5 μg.mL^{-1}	*S. aureus*	11.0	Thambi et al., 2013
EOL	5 μg.mL^{-1}	*B. subtilis*	12.0	Thambi et al., 2013
EOL	5 μg.mL^{-1}	*S. faecalis*	12.0	Thambi et al., 2013
EOL	5 μg.mL^{-1}	*S. albus*	12.0	Thambi et al., 2013
EOL	0.875 g.mL^{-1}	*S. aureus*	25.0	Fiuza et al., 2008
EOL	0.175 g.mL^{-1}	*E. coli*	16.0	Fiuza et al., 2008
EOL	0.0043 g.mL^{-1}	*P. aeruginosa*	25.0	Fiuza et al., 2008
EUS	1.5 μg.mL^{-1}	*S. aureus*	20.0	(Oliveira et al., 2008)
EUS	1.5 μg.mL^{-1}	*P. aeruginosa*	18.6	(Oliveira et al., 2008)
EUS	1.5 μg.mL^{-1}	*Klebsiella sp*	19.6	(Oliveira et al., 2008)
EUS	1.5 μg.mL^{-1}	*E. coli*	12.0	(Oliveira et al., 2008)

HEEU = Hydroalcoholic extract of *E. uniflora* ripe fruit, EOL = Essential oil of *E. uniflora L.* leaves, EUS = *E. uniflora* seeds.

Table 5 Anti-proliferative effects of *E. uniflora* oils in human cancer cell lines and in human fibroblast cell line.

	Cell lines IC_{50}			
	HCT-116 Colon	AGP-01 Gastric	SKMEL-19 Melanoma	MRC-5 Human Fibroblast
E1	ND	ND	ND	ND
E2 [A]	16.26 (14.45–18.29)	12.60 (10.35–15.35)	12.20 (10.10–14.72)	10.27 (8.15–12.94)
E3 [A]	>25	>25	>25	>25
E4 [A]	9.28 (7.86–10.97)	8.73 (5.45–13.98)	15.42 (9.38–25.33)	14.95 (9.44–13.90)
E5 [A]	>25	>25	>25	>25
Curzerene [B]	9.18 (8.18–10.30)	8.04 (5.66–11.41)	5.17 (4.04–6.62)	11.45 (9.44–13.90)

The oils of five specimens (E1 to E5) that occur in the Brazilian Amazon were tested. Data are presented as IC_{50} values and 95% confidence intervals obtained by nonlinear regression for all cell lines, from three independent experiments. *ND = not determined, adapted from Figueiredo et al. 2019. Data are expressed as [A] μg/mL and [B] μM.

1.5 μg.mL^{-1} and *Bacillus subtilis, Streptococcus* spp. and *Escherichia coli* at 16.5 μg.mL^{-1}. This effect can be explained by a channel formation in the membrane cells, which affects cell permeability and leads to cell death (Table 4) (Oliveira et al., 2008) (see Table 5).

4.2. Antifungal activity

Candida spp. is the primary fungal pathogenic agent in humans. Despite belonging to the human microbiota, alteration in tissues in association with virulence factors allow this fungus to invade patients with lower immunologic resistance that are mostly affected. It was demonstrated that *E. uniflora* leaf extract could reduce biofilm formation in cells isolated from the oral cavity in transplanted patients. Various *Candida* fungal strains were evaluated such as *C. tropicalis, C. parapsilosis, C. glabrata, C. orthopsilosis, C. metapsilosis,* and *C. dubliniensis*. A study showed that pitanga could reduce adherence of human oral epithelial cells to the biofilm formation and alter the hydrophobicity of the cell surface of *Candida albicans*, suggesting that the antifungal effect of *E. uniflora* is due to gallic acid and myricitrin, phytochemicals that can act against *Candida spp per se* (Souza et al., 2018).

Another study on *E. uniflora* essential oil also demonstrated antifungal activity against *Candida spp*. When this oil was administered associated with the antifungal fluconazole, the authors did not observe a synergic effect. In fact, this combination compromised the effect of the drugs, whereas the isolated use of the plant or the antifungal were effective. Despite the incompatibility with fluconazole, antifungal effect of *E. uniflora* essential oil were attributed to interference with virulence factors of candidiasis and morphological changes caused through the formation of filamentary structures (Dos Santos et al., 2018). However, when the ethanolic extract was associated with the antifungal drug metronidazole, a combined effect against *C. tropicalis* was found; however, this synergic activity was not evidenced in other strains (Santos et al., 2013). Another virulence factor of *Candida* spp. is the secretion of hydrolytic enzymes which can be reduced by leaf extract from *E. uniflora* (Silva-Rocha et al., 2015).

Besides fungus that colonize the oral cavity, there are those that affect the skin and are responsible for dermatitis. Among these organisms is *Paracoccidioides brasiliensis*, the main organism responsible for systemic mycosis in Latin America. Essential oil from *E. uniflora* was able to inhibit the fungus growth at a concentration of 62.5 μg.mL^{-1}. This effect can be attributed to the presence of selinatrienone derivatives and curzerene compounds in this oil (Costa et al., 2010).

The studies describe the inhibitory effect of *E. uniflora* against biofilm formation and fungal growth. The presence of some compounds in the pitanga extract, such as oleic acid, can be responsible for this response. Muthamil and collaborators (2020) found that this compound is able to reduce the filament formation, induce alterations in ergosterol present in the cell membrane of *Candida spp*. In addition, the acid oleic affects the expression of genes (*asl-1, sap2, hwp1* and *cst20)* involved in the virulence response of *Candida spp*. The flavonols myricetin and quercetin, also present in this fruit, present effect in the biofilm formation of *P. aeruginosa* that acts on the phenazines, a molecules involved in the intracellular redox state and colony biofilm formation, promoting alterations in the electron transporter of this molecules (Pruteanu et al., 2020).

4.3. Antiparasitic activity

Parasitic diseases are significant and neglected health problems in emerging countries and can affect both humans and animals. Social and cultural factors are related to the parasite infections in humans caused by inadequate and unsanitary conditions. Antiparasitic drugs can be quite toxic and thus the search for natural products has been increasing (Wink 2012). Therefore, antiparasitic properties of native plants are particularly interesting for the population of less developed countries.

The essential oil from pitangueira leaves demonstrated antiparasitic activity (IC_{50} 6.10 ± 1.80 μg·mL^{-1}) against *Leishmania amazonensis* (*L. amazonensis)*, which indicates a higher efficacy than the drug pentamidine isethionate (IC_{50} 23.22 ± 9.04 μg·mL^{-1}). This oil is rich in sesquiterpenes, which causes alterations in the mitochondrial and plasmatic membrane of the parasite (Kauffmann et al., 2017). The *Leishmania* genus affects various regions worldwide. In the Americas, the phlebotomus of the genus *Lutzomyia* is the vector. The manifestations of Leishmaniasis include cutaneous sores (localized and disseminated), muco-cutaneous, and visceral ulcers or kala-azar (Torres-Guerrero et al., 2017).

Another study with *L. amazonensis* showed that *E. uniflora* essential oil caused 100% inhibition of promastigotes at the concentrations of 400, 200, and 100 μg·mL^{-1} after 72 h of exposure with an IC_{50} of 1.75 μg·mL^{-1}. As the parasite infects macrophages, a study evaluated the oil's effect in the cells infected with *L. amazonensis*. Pitanga essential essential oil was able to reduce parasite infection due to greater activation of macrophages, with increased phagocytosis capacity and lysosomal activity. It is believed that the terpene compounds act in the isoprenoid pathway, inhibiting one of the stages of ubiquinone biosynthesis, which is required for the advance of the parasite stages. This might explain the anti-*Leishmania* activity of this plant. The lipophilic constituents of essential oils derived these from plants can alter the constitution of cell barriers and lipids of the plasma membrane of the parasitic promastigote stage, leading to the release of cell contents and consequently the inhibition of cytoplasmic processes (Rodrigues et al., 2013).

Similar to *Leishmania*, *Trypanosoma cruzi* is transmitted by an insect, by blood transfusion, organ transplant or ingestion of contaminated food. The recovery rate following medicine treatment is high only in the acute phase of this disease and is less than 20% effective in the chronic phase. However, the drugs available for the treatment are highly toxic. Therefore, it is necessary to search for new alternatives with antiparasitic activity and safety. A study with the epimastigote form, found in the vector of this disease, showed that the pitanga leaf extract could inhibit 80% of the parasite at the concentration of 100 μg·mL^{-1}. This extract also showed low cytotoxic activity in J774 macrophage cells. Only 8% of the cells exposed to a concentration of 100 μg·mL^{-1} of the extract were unviable, and this toxicity was reduced to 0% with a concentration of 10 100 μg·mL^{-1}. These results indicate that *E. uniflora* has

anti-epimastigote potential and locytotoxicity (Costa et al., 2010).

The efficacy of the plant was evaluated also against other parasites of the genus *Trypanosoma* (*T. brucei*), responsible for anemia and damages to organs, such as liver and kidney. The leaves of *E. uniflora* demonstrated effectiveness against trypanosomal infection and the symptoms of this condition, such as anemia. The enzyme adenosine kinase (TbAK) was a target of the plant action (Abdelfattah et al., 2021). In addition, de Souza et al., (2017), demonstrated that components such as tannins and flavonoids of *E. uniflora* fractions are able to protect against the promastigote and amastigote forms of *Leishmania* spp.

Another study evaluated the effectiveness of the extract obtained from the *E. uniflora* leaves against the parasite *Trichomonas gallinae*, which is responsible for trichomoniasis that affects birds. However, the methanolic extract did not yield promising results when compared to the control drug metronidazole. When the $CHCl_3$ and EtOAc fractions of this extract were obtained and tested, an anti-parasitic potential similar to the drug was found (Ibikunle et al., 2011).

The anthelmintic activity depicted by *E. uniflora* extracts has been attributed to the presence of compounds with proven anthelmintic action, such as flavonoids, saponins, tannins and triterpenes. Different extracts of *E. uniflora* inhibited the hatchability of eggs of gastrointestinal sheep nematodes, with a percentage of inhibition ranging from 14.56 to 99.75%. The hydroalcoholic extracts were the most promising compared to the aqueous ones. The chemical composition was analyzed using qualitative phytochemical protocols to observe compounds with proven anthelmintic action, such as flavonoids, saponins, tannins and triterpenes. Thus, the results of study revealed that *E. uniflora* extracts were promising anthelmintic candidates (Castro et al., 2019).

4.4. Antiviral activity

Viruses are unicellular microorganisms that infect cells for survival, triggering an immunological response. For instance, the human immunodeficiency virus (HIV) causes immune system impairment by causing the death of cells involved in the defense mechanism. In this example, the continuous use of retroviral therapy is necessary to reduce the viral load. Notably, some plants can inhibit or reduce viral replication through different mechanisms (Mukhtar et al., 2008) which is the case of *E. uniflora*.

For instance, when tested against the Dengue Virus Serotype 2 in culture human cells (Huh7-it), *E. uniflora* leaf extract showed antiviral activity with an IC_{50} of 19.83 $\mu g.mL^{-1}$ (Dewi et al., 2019). It has been described that some flavonoids can inhibit the protease enzymes involved in viral replication (Qamar et al., 2017). For HIV treatment, the use of antiretroviral therapy presents efficacy but causes some side effects associated with the need for continuous administration. Notably, the methanolic and water extracts from *E. uniflora* leaves showed a replication inhibition at 100 $ug.mL^{-1}$ (94%) against HIV-1, promoting the inhibition of the protease required in replication and viral action (Kawahata et al., 1996).

Some viruses have long latency periods, therefore they activate during some immunocompromising condition. The Epstein-Barr virus (EBV) is one such virus, causing a condition known as herpes and mononucleosis. Its viral mechanism is associated with the presence of DNA polymerase enzyme that favors replication. The tannins isolated from *E. uniflora* leaf extract, Eugeniflorin D1 and D2, were found to have inhibitory effect against this virus at concentrations of 3.0 and 3.5 μM, respectively; the mechanism by which they conferred this effect was by blocking the DNA polymerase enzyme (Lee et al., 2000).

4.5. Anti-inflammatory effect

The anti-inflammatory capacity of *E. uniflora* extract has shown promising results as demonstrated by Bello et al. (2020) who used the ethanolic extract of the leaves in a model of acute formalin-induced inflammation in rats. In this study, the effects of *E. uniflora* at doses of 50, 100 and 200 $mg.kg^{-1}$ were similar to those of ibuprofen (100 $mg.kg^{-1}$, p.o), a standard non-steroidal anti-inflammatory drug (NSAID). This demonstrates the high effectiveness of ethanolic extract against inflammatory processes caused by acute injury. Additionally, the ethanolic extract was able to limit the area of secondary damage in the tissue and to accelerate the recovery of the soft tissue after the injury, leading to the reduction of inflammatory cells in the injured hind paw.

In a different experiment, Falcão et al. (2018) treated carrageenan-induced peritonitis in rats with crude extract, aqueous fraction (AqF) or EAF from *E. uniflora* leaves. They observed anti-inflammatory properties with reduced levels of IL-1β (Interleukin-1 Beta) following treatment with the crude extract in both fractions and at all doses used (50, 100 and 200 $mg.kg^{-1}$). Remarkably, the aqueous fraction also stood out due to the decrease in TNF-α (Tumor Necrosis Factor-Alpha) levels. In addition, the anti-inflammatory effect of the extracts and their fractions also showed the reduction of lipid peroxidation that occurred during treatment. This implies that the antioxidant effect would be mediated by an intracellular reduction in glutathione (GSH) consumption, which would cause, in part, increase in the anti-inflammatory potential.

Similarly, *E. uniflora* leaf extracts (aqueous, ethanolic and essential oil) were assessed by Schapoval et al. (1994). Using the carrageenan-induced rat paw edema test, the authors found that the ethanolic extract (300 $mg.kg^{-1}$, v.o) was more effective than the aqueous one, which was attributed to the presence of volatile or unstable substances that underwent changes during the time necessary for drying and/or extraction even at room temperature. Using a similar protocol of carrageenan-induced hind-paw edema, Sobeh et al. (2019) found that the methanolic extract from *E. uniflora* leaves decreased the thickness of the edema and reduced leukocyte migration to the peritoneal cavity. Furthermore, it was demonstrated for the first time that the extract inhibits cyclooxygenases (COX-1 and COX-2) and lipoxygenase (LOX) therefore reducing the production of pro-inflammatory mediators. Although the extract showed greater selectivity for COX-2, its LOX inhibitory activity was similar to that of the diclofenac.

Furthermore, Syama et al. (2019) demonstrated that the leaf ethanolic extract showed an important action in cell line RAW264.7 stimulated by lipopolysaccharide (LPS). The study revealed the extract's ability to inhibit enzymes that regulate inflammation, which was also confirmed by decrease in the

expression of COX-2 and nuclear factor-kB (NF-kB). *In vivo* carrageenan-induced chronic inflammatory studies in male Wistar rats demonstrated that doses of 250 and 500 mg.kg^{-1} ethanolic extract of leaves decreased the inflammatory response possibly through the inhibition of pro-inflammatory mediators. This could be attributed to the presence of gallic acid and dihydromyricetin that have proven anti-inflammatory property.

There are reports of *E. uniflora*'s influence in reducing infection induced by *Helicobacter pylori,* a bacteria that causes gastric ulcerative disorders that in more severe cases can develop into gastric cancer. Monteiro et al. (2019) demonstrated that leaf *E. uniflora* methanolic extract decreased inflammatory activity through the use of murine macrophage cell culture RAW 264.7 (ATCC TIB-71) stimulated by LPS. The results demonstrated a reduction in IL-6, TNF-α and nitric oxide (NO) levels at all concentrations tested. However, the concentration 100 μg.mL^{-1} reached the highest inhibition rate of about 83.19, 39.10 and 97.20%, respectively.

The anti-inflammatory potential of *E. uniflora* purple pulp juice has also been reported in gingival epithelial cells. It acts by attenuating the release of IL-8 (Interleukin-8) in non-stimulated and prostaglandin-*LPS*-stimulated cells, respectively (Soares 2014). In addition, important constituents present in the juice such as cyanidin-3-glucoside and oxidoselina-1,3,7(11)-trien-8-one were able to reduce CXCL8 mRNA expression by HGF-1 cells and IL-8 release, thereby revealing an anti-inflammatory potential of the volatile compound oxidoseline-1,3,7(11)-trien-8-one, reported for the first time in the literature.

Another study conducted by Schumacher et al. (2015) reported that the anti-inflammatory effects of *E. uniflora* leaf extract also contributed to improvement in type 1 diabetes mellitus induced in non-obese rats. Parameters such as decreased inflammatory cell infiltration associated with attenuating oxidative stress, increased levels of hepatic GSH and seric insulin indicate that these associated effects could help preserve insulin-producing pancreatic ß cells and inflammatory insult. However, the authors stated that a better explanation regarding the anti-inflammatory signaling process of the immune response is required.

In addition, the anti-inflammatory effects of these leaves were also confirmed using the Cecal Ligation and Puncture model in mice. The results indicated that 150 and 300 mg.kg^{-1} doses given orally 1 h before surgery and 6 h after the procedure managed to significantly decrease serum levels of TNF-α by 36.6–38.8%, and levels of IL −1β were also inhibited by 32.3–38.5% after 6 h of induction in the false surgical group. Another important product of cell inflammation was the decrease in induced nitric oxide synthase (iNOS) levels by septic mice ileum cells as *E. uniflora's* aqueous extract at all doses (75, 150 and 300 mg.kg^{-1}) decreased iNOS levels by 35.2, 33.6 and 75.2%, respectively. Furthermore, COX-2 expression was reduced by 12.7 and 62% after treatment with higher doses (Rattmann et al., 2012).

4.6. Anti-cancer activity

Anti-cancer activity is based on the cytotoxic potential of a molecule. As previously mentioned, *E. uniflora* extracts do not cause significant toxicity, especially when the organism or cells are healthy. However, tumorous cells present different membrane and metabolic characteristics, proliferating under oxidative stress and impaired antioxidant defense (Fry and Jacob 2006). Then, it is possible that altering redox status may trigger apoptotic cell death in carcinogenic cells. Therefore, several promising results have been described in this field.

Nuñez et al. (2018) demonstrated that the aqueous leaf extract of *E. uniflora* showed positive results in a human tumorous cell line derived from invasive cervical carcinoma, SiHa (HPV 16-positive). The data demonstrated that all concentrations of *E. uniflora* extract (0.5–20 mg.mL^{-1}) significantly inhibited the viability of the SiHa cell line in 24 h and 48 h, and this effect was prolonged at the highest doses of 10 mg.mL^{-1} 72 h after treatment. In addition, the migration of tumor cells significantly reduced after 24 h of treatment to 63.4% and in 48 h to 24.5%, and the capacity of tumor cell adhesion decreased in a dose-dependent manner (5, 10 and 20 mg.mL^{-1}). In this case, cell death due to apoptosis was also observed in tumor cells treated with *E. uniflor*a and mitigation of the migration, adhesion, colony formation and recovery capacities was observed even after treatment withdrawal without altering the normal cells viability.

Promising results of using *E. uniflora* against carcinogenic activity were found by Ismiyati et al. (2012) in the breast cancer T47D cell line treated with the extract obtained from the leaves. The results demonstrated an antiproliferative effect at 50 μg.mL^{-1} 48 h and 72 h hours after incubation. At 75 μg.mL^{-1}, the extract decreased cell proliferation during the entire incubation period (24 h, 48 h and 72 h). Additionally, a cytotoxic effect was also observed, which may have contributed to inhibiting the proliferation of T47D cells and inducing apoptosis.

The cancer cell lines HCT-116 (colon), AGP-01 (malignant gastric ascites) and SKMEL-19 (melanoma) were treated with different *E. uniflora* leaf oils in a study by Figueiredo et al. (2019). Despite the variations between oils, their constituents had common predominance and belonged to the classes of oxygenated sesquiterpenes (20.8–69.0%) and sesquiterpene hydrocarbons (18.0–53.9%). Figueiredo et al. (2019) demonstrated that curzerene, selin-1,3,7(11)-trien-8-one and selin-1,3,7(11)-trien-8-one epoxide were found in oil extract and all stood out as potential anti-cancer agents for lung, colon, stomach and melanoma tumors. Cytotoxic activity was demonstrated by the two types of oil against all tested HCT-116 cell lines. In addition, curzerene showed the most significant activity against melanoma cells (SKMEL-19), induced apoptosis at 5.0 and 10.0 μM compared to vehicle DMSO and exhibited a decrease in cell migration at 5.0 and 10.0 μM after 30 h of treatment.

Another study using MCF-7 cells (breast cancer cell line) demonstrated that *E. uniflora* essential oil presented an IC_{50} of 11.20 μg.mL^{-1} and a selectivity index of 6.82, making it an interesting candidate for further studies in terms of anti-carcinogenic properties. In this study, *E. uniflora* oil presented more than 80 compounds, the most predominant of which were from the oxygenated sesquiterpenes class known as spathulenol (15.8%), α-copaene (10.96%), muurola-4,10-dien-1-β-ol (9.3%), caryophyllene oxide (8.93%), alloaromadendrene (5.5%) and nootkatone (5.17%) (Sobeh et al., 2016).

Finally, Ogunwande at al. (2005) used essential oil extracted from fully grown leaves and fruit and found effects

on tumor cells of the human prostate (PC-3), liver (Hep G2) and breast (Hs 578 T). The results identified mostly sesquiterpenoid compounds in both oils. In the leaves, the compounds identified were curzerene (19.7%), selina-1,3,7 (11)-trien-8-one (17.8%), atractylone (16.9%) and furanodiene (9.6%), and those from the fruits were germacrone (27.5%), selina-1,3,7(11)-trien-8-one (19.2%), curzerene (11.3%) and oxidoselina-1,3,7(11)-trien- 8-one (11.0%). In this case, the volatile oils exhibited an excellent cytotoxic action in relation to the human cell lines of PC-3 (99.36% and 99.55%) and Hep G2 (99.71% and 99.96%), while completely inhibiting the growth of Hs 578 (100%) by percentage of fruit and leaf oil, respectively.

4.7. Neuroprotective effect

Studies evaluating *E. uniflora*'s neuroprotective potential are still relatively scarce. Da Da Silva et al. (2019) used the ethanol extract of *E. uniflora* leaves to observe its neuroprotective effect on memory impairment induced by intracerebroventricular injection of streptozotocin (STZ, i.c.v) in rats. The administration of STZ in rats is a model of sporadic Alzheimer's disease widely accepted for inducing impairment in memory and metabolic changes similar to those of patients. Therefore, rats that received leaf *E. uniflora* extract at doses of 300 and 1000 $mg.kg^{-1}$ for 30 days alternately, after i.c.v administration of STZ, demonstrated improvement in episodic memory and learning compared to the untreated animals. Additionally, the performance of the animals that received the extracts were better than untreated animals in both doses tested. The neuroprotective effects have been attributed to the antioxidant and anti-inflammatory properties that have also been described in previous studies using other species of the genus *Eugenia*. In this case, this study was the first to report potential neuroprotective effects of pitangueira leaves in this neurodegenerative disease model.

The antioxidant and anti-acetylcholinesterase actions of red pitanga extract chronically administered at a dose of 200 mg/kg was analyzed in male Swiss mice in a model of depression. Flores et al (2020) observed promising results regarding the prevention of the depressive effect induced by unpredictable chronic stress, provided by the regulation of acetylcholinesterase activity, reducing the production of reactive oxygen species in the prefrontal cortex and hippocampus and avoiding glutathione peroxidase in the hippocampus of treated animals. It is important to emphasize that the administration of the extract of the red pitanga produced neuroprotection similar to the classic antidepressant fluoxetine, which was used as a positive control. Therefore, these findings may suggest a potential role for the *E. uniflora* in the treatment of depressive disorders.

Neuroprotective effects of red and purple pulp extracts of *E. uniflora* were observed in models of Alzheimer's and Parkinson's diseases induced by amyloid β peptide fragment 1–42 ($a\beta_{1\text{-}42}$) and MPP+, respectively, in *C. elegans*. Borges et al. (2015) demonstrated that the effects induced by $a\beta_{1\text{-}42}$ were reduced in animals treated with both extracts. These could reduce the paralysis phenotype in *C. elegans*. The authors highlighted that purple pitanga extract presented a higher efficacy than red pitanga extract. Additionally, they also favored important genes of oxidative and thermal stress activation such as the transcription factors daf-16 and Nrf2/Skn-1/ inhibition pathways. In the neurodegeneration induced by MPP+, both red and purple pitanga extracts could reduce paralysis in *C. elegans*, but the red pitanga extract presented the highest percentages of reduction compared to the control.

Based on the data presented, *E. uniflora* is a promising natural product for the development of new drugs aiming at the central nervous system. The presence of anthocyanins, of which cyanidin-3-O-glucoside is the main compound present in pitanga, demonstrates the neuroprotective mechanism against the formation of free radicals and oxidative processes in the body, since these endogenous processes are related to the genesis of several neurodegenerative diseases.

However, a better understanding of the mechanism of action of the majority compounds such as flavonoids, terpenes and phenolic compounds is still needed, therefore, in addition to components for the development of new drugs, they can become a form of complementary treatment through the diet.

4.8. Antioxidant effect of Eugenia uniflora

Exogenous food antioxidants such as polyphenols and carotenoids can help to protect an organism against diseases associated with oxidative stress (Rahimi-Madiseh et al., 2017). Antioxidant activity of the chemical compound (s) or extract (s) vary according to the model used and also according to the ability of the phytochemical (da Cunha et al., 2016). Currently, several studies have investigated the antioxidant capacities of *E. uniflora* using *in vitro* and *in vivo* assays particularly because this potential might be responsible for several biological advantages attributed to *E. uniflora*.

4.8.1. In vitro antioxidant activity

An *in vitro* study performed on human keratinocyte cells (HaCaT cell) evaluated the protective activity of *E. uniflora* against oxidative stress induced by UVA irradiation (100 $J.cm^{-2}$). The cells were pre-treated with 50 $\mu g.mL^{-1}$ of methanolic extract obtained from leaves. High levels of reactive oxygen species (ROS) and p38 activation (mitogen-activated protein kinases) were found to have been reduced with concomitant increase in GSH levels compared to stressed cells. This protective response can be associated with the presence of total phenols in the *E. uniflora* extract, which have strong antioxidant capabilities (Sobeh et al., 2019).

Similarly, several studies have demonstrated the free radical scavenging activity of different *E. uniflora* extracts. The leaf extract has a high content of flavonoids (42.46 $mg.g^{-1}$) and showed a sequestering DPPH radical activity with an EC_{50} 185.47 $\mu g.mL^{-1}$. The extract also reduced the levels of lipid peroxidation (levels of reactive substances) in the TBARS assay to the baseline when induced with Fe^{2+} (Sobral-Souza et al., 2014). In another study, the leaf hydroalcoholic extract presented an EC_{50} of 14.19 $\mu g.mL^{-1}$ in DPPH assay and 19.75 $\mu g.mL^{-1}$ in the ABTS radical scavenger test. The results obtained with the TBARS *in vivo* tests and levels of advanced oxidation protein products (AOPP) demonstrate that treatment for four weeks with this extract reduced oxidative stress in the plasma of treated rats compared to the control group, highlighting the antioxidant activity of the extract (Peixoto et al., 2019).

In a study conducted with four native Brazilian fruits, the purple pitanga extract was found to have the highest DPPH

radical scavenger activity and also presented the highest FRAP (Fe-reducing antioxidant potential) followed by orange and red pulp seed extracts. These results were attributed to the antioxidant capacity of compounds such as quercetin, quercitrin, isoquercitrin and cyanidin derivatives (Denardin et al., 2015).

The leaf ethanolic extract (1–480 mg.mL^{-1}) inhibited Fe^{2+}-induced lipid peroxidation in rat brain and liver homogenates and eliminated the DPPH radical. Notably, this extract further presented a high content of some polyphenolic compounds such as quercetin, quercitrin, isoquercitrin, luteolin and ellagic acid, which might be at least partly responsible for its antioxidant effect (da Cunha et al., 2016).

The antioxidant compounds isolated from *E. uniflora* such as cyanidin-3-glycoside and delfinidin-3 glycoside are highly unstable and require technology to protect them from degradation. Microencapsulation is a technology used to protect active ingredients. Therefore, spray drying was used to evaluate High-performance Agave Frutans (HPAF) and High Degree of Polymerisation Agave Fructans (HDPAF) and maltodextrin (MD) as coating materials, respectively. The results showed that the highest yield and concentration of anthocyanins after drying and during storage were found at a ratio of 1:6 core: wall material. This study showed that the fraction of both fructans had encapsulation properties similar to that of MD. Moreover, HDPAF was more effective than MD in protecting antioxidants during drying and storage, and the total color change could be used as an indicator of anthocyanin degradation during storage (Ortiz-Basurto et al., 2017).

4.8.2. In vivo antioxidant activity

In an experimental model of insulin resistance induced by DEX (dexamethasone), 200 mg.kg^{-1}.day^{-1} of *E. uniflora* fruit extract was administered for 21 days. The extract was able to prevent lipid peroxidation and the formation of ROS in rat liver, suggesting its important antioxidant action in the experimental model (de Souza Cardoso et al., 2018). Additionally, Schumacher et al (2015), found that repeated consumption of aqueous leaf extract in a type 1 diabetes mellitus model in mice showed reduction in the rate of inflammatory infiltrate in pancreatic islets, with serum levels of insulin and hepatic GSH being maintained and seric lipid peroxidation and the risk of diabetes being reduced.

To better understand the effects of the fruit on aging and conditions related to oxidative stress, the ethanolic extract of the purple *E. uniflora* fruit pulp was tested in the alternative model *C. elegans*. Exposure to the extract showed improvement in survival after different situations of oxidative stress and was also seen to prolong the lifespan of N2 (wild type) and *mev-1* mutants, increasing the expression of SOD-3 and HSP-16.2 and the nuclear localization of DAF-16, which are alterations that promoted longevity by modulating antioxidant signaling (Tambara et al., 2018).

A study investigated the antioxidant effect of ethanolic sun-dried (PCS) and air-dried (PCA) extracts from *E. uniflora* leaves in rat brain and liver. The results indicated that while air-dried leaves significantly inhibited the formation of TBARS in liver and brain tissue homogenates, PCS did not. Subsequent investigations revealed that the phenolic content of PCS was significantly lower compared to PCA, thereby suggesting that air-drying should be used in the preparation of the extract as phenolics significantly contribute to the plant's antioxidant potential (Kade et al., 2008).

Meira et al., (2020) evaluated the antioxidant effects of the hydroalcoholic extract of *E. uniflora* leaves at the dose (200 mg / kg, p.o) in Wistar rats, 28 days before the induction of acute kidney injury (AKI) by bilateral renal ischemia for 45 min. Renal production of reactive oxygen species and apoptosis, SOD and catalase expression and activity were determined. Treatment with pitanga prevented the AKI-induced decrease in glomerular filtration rate and renal blood flow, as well as the increase in renal vascular resistance. The *E. uniflora* extract also prevented the increase in oxidative stress and apoptosis, probably due to the increased activity and expression of antioxidant enzymes. These results demonstrate a protective effect of pitanga extract on the development of AKI. This protective effect of *E. uniflora* extract on oxidative stress can be attributed to identified compounds, such as flavonoids, polyphenols and terpenes. These substances are able to reduce oxidative stress through similar mechanisms, such as increased SOD activity, catalase and glutathione peroxidase.

The active edible coatings and films produced by the addition of plant extracts and antimicrobial compounds are of interest to food packaging. In this sense, Chakravartula et al., (2020) sought to develop and characterize a film and film forming solution based on mixtures of cassava starch/chitosan (CS/CH) incorporated with cherry tree leaf extract of *E. uniflora L* (PE) and/or natamycin (NA) and studies their effects on selected physical properties, antioxidant and antifungal activity. The addition of PE did not affect the mechanical properties of the film, while NA significantly decreased the flexibility of the films due to changes in the behavior of the parafragile ductile biopolymer. Structural analysis by FTIR and XRD indicated interaction between the components, particularly the presence of new vibration peaks C]O and change in wavenumbers of the characteristic CS/CH mixture. The antioxidant activity of the films significantly increased with PE, although the combination of additives resulted in reduction of activity. Positive antifungal effect of films containing NA was observed against *Aspergillus flavus* and *Aspergillus parasiticus*, indicating potential for applications in active food packaging.

These promising data on antioxidant effects both *in vitro* and *in vivo* can be related to the accumulation of phenolic compounds such as anthocyanins present in *E. uniflora* extract as well as in leaves, which corroborates to the phytochemical characterization already described in this review.

4.9. E. uniflora effects on metabolism and TGI

E. uniflora leaves are rich in tannins and flavonoids (Auricchio and Bacchi 2003) and several studies have shown that tannin-rich species have been traditionally used for their gastroprotective properties (de Jesus et al., 2012). In fact, Souza and Costa (2017) demonstrated the gastroprotective effect of an aqueous fraction of hydroacetonic leaf extract of *E. uniflora* against several gastric ulcer models in mice; increase in the gastric mucus and reduced GSH levels were also found.

Gastric mucus is the first line of defense of the gastric mucosa. It is a transparent, viscous, elastic adherent gel made up of water and glycoproteins (Martins et al., 2015). The mucus layer is a physical barrier that adheres with bicarbonate

and protects the underlying mucosa from proteolytic digestion (Allen and Flemstrom 2005). Furthermore, it is known that NO is crucial in the defense of the gastric mucosa and is a biological mediator which regulates the secretion of mucus and blood flow (Falcao Hde et al., 2013). Souza and Costa (2018) demonstrated that an inhibition of NO synthase by L-NAME did not reverse the gastroprotection effect of the aqueous fraction of hydroacetonic leaf extract of *E. uniflora,* suggesting that NO synthesis is not critical to its gastroprotective activity. Thus, it seems that the primary mechanism of the action of leaf extract of *E. uniflora* is through increased gastric mucus.

In 2008, a hepatoprotective effect of leaves of *E. uniflora* against lipid peroxidation was demonstrated *in vitro*. This can be attributed to the antioxidant properties of *E. uniflora* extract (Kade et al., 2008). Another study conducted by Fiuza et al. (2009) showed a hepatopancreas action of crude ethanol extract and fractions in *Oreochromis niloticus L.* Recently, Sobeh et al. (2020) showed that the polyphenol-rich fraction from *E. uniflora* leaves has substantial hepatoprotective activities against acute liver injury in rats due to its antioxidant property.

Furthermore, a hypotensive activity in normotensive rats has been demonstrated following administration of 3 mg. kg^{-1} of dried leaves of a crude aqueous extract. This effect was associated with a direct vasodilating action in perfused resistance vessels and a slight diuresis at higher doses (120 mg dried leaves.kg^{-1}) (Consolini et al., 1999, Consolini and Sarubbio 2002). Additionally, a cardiovascular activity caused by aqueous crude extract (ACE) of *E. uniflora* was demonstrated in rats through β-adrenergic mechanisms. In this case, the release of catecholamines and Ca-blocking action might have produced this therapeutic effect (hypotension) and contributed to chronotropic and inotropic effects on the heart (Consolini and Sarubbio 2002).

In relation to metabolism, folk medicine reports the use of hydro-alcoholic extract of *E. uniflora* leaves to control the levels of triglycerides, very low-density lipoprotein (VLDL) cholesterol and uric acid in *Cebus paella,* monkeys (Ferro et al., 1988). Furthermore, Arai et al. (1999) showed that extracts from the leaves of *E. uniflora* had improved effects on postprandial hyperglycemia and hypertriglyceridemia, and these effects can be attributed to the inhibition of sugar and fat decomposition and reduction of glucose absorption. The red variety of *E. uniflora* fruit demonstrated effects against high levels of glucose, triacylglycerol, cholesterol and LDL cholesterol, as well as visceral fat and weight accumulation (Oliveira et al., 2017).

Additionally, Schumacher et al. (2015) studied the effects of continuous treatment with aqueous extract of dried leaves of *E. uniflora* in an experimental model of spontaneous type 1 diabetes mice. This treatment was found to reduce the incidence of type 1 diabetes, decreasing inflammatory cell infiltration and the oxidative stress and increasing hepatic GSH levels and serum insulin. This may indicate preservation of insulin-producing pancreatic β cells.

Another interesting effect was observed with respect to α-glucosidase activity. Even at low concentrations, the ethanolic extract of purple *E. uniflora* leaves inhibited almost 100% the activity of the aforementioned enzyme (IC_{50} 0.26 μg.mL^{-1}) (Vinholes and Vizzotto 2017). The *E. uniflora* fruit juice was also found to inhibit α-glucosidase activity in 69.47 ± 2.89 % at 1 mg.mL^{-1}. The total phenolic content of the juice was 367.00 ± 11.42 mg GAE.L^{-1} and was considered important to the inhibitory activity observed in the study (Siebert et al., 2020).

This inhibitory effect can be associated with the presence of fatty acids and derivatives. Unsaturated fatty acids such as oleic, linoleic, and linolenic acids and their methyl ester forms inhibit α-glucosidase enzyme through competitive mode inhibition (Su et al., 2013). Furthermore, phenolic compounds such as salicylic acid derivatives showed interaction with α-glucosidase enzyme (Chen et al., 2019). Therefore, the inhibitory properties of the ethanolic extract show promising results as the inhibition of this digestive enzyme is used to control type 2 diabetes mellitus.

Furthermore, *E. uniflora* fruit (red variety) standardized extract has a beneficial effect in rats submitted to metabolic syndrome induced by diet. This extract presented an antihyperglycemic, antihyperlipidemic and a neuroprotective role as it presented antioxidant and antidepressant-like effects (Oliveira et al., 2018). Moreover, liposomes loaded with an ethanolic extract of purple pitanga were found to reduce lipid accumulation induced by high cholesterol levels in *C. elegans* (Roncato et al., 2019).

It is evident that *in vitro* and *in vivo* studies prove that bioactive compounds present in pitanga can positively affect metabolism biomarkers. The mechanism of action against diabetes have been attributed to the phytochemicals and include modulation of carbohydrate metabolism, glucose homeostasis and insulin secretion, reducing oxidative stress, suppressing the formation of advanced glycation end products and protecting / regenerating pancreatic β-cells. Therefore, leaves and the fruits of *E. uniflora* have been showing therapeutic potential to be used in the treatment of diabetes and its comorbidities.

These results are interesting as they again point out that natural antimicrobial and antioxidant compounds have shown potential application for the production of active packaging and meet the growing demand from consumers, who are increasingly looking for food products with the lowest amount of artificial additives.

5. Majority compounds *E. uniflora* - possible mechanisms

As described in this review, several biological properties have been attributed to leaf, fuit and seeds extracts obtained from *E. uniflora* (Fig. 3). Some studies explored even further by investigating which components were responsible by these pharmacological effects and how they act in molecular targets. For instance, it has been demonstrated that pure phenolics delphinidin 3-O-glucoside and cyanidin-3-O-glucoside inhibit the viability of human colon cancer cells, HCT 116 and HT-29 by inducing apoptosis (Mazewski et al., 2019). These metabolites have shown potential for binding and inhibiting immunological checkpoints, PD-1 and PD-L1, which can activate the immune response in the tumor microenvironment and induce the death of cancer cells. Additionally, the flavonoids also induced apoptosis in the same cancer cell lineage by inhibiting tyrosine kinases (Mazewski et al., 2018).

Antioxidant ad anti-inflammatory actions depend on similar pathways, using cell line RAW 264.7 and inducing inflammatory response with lipopolysaccharide, it was possible to

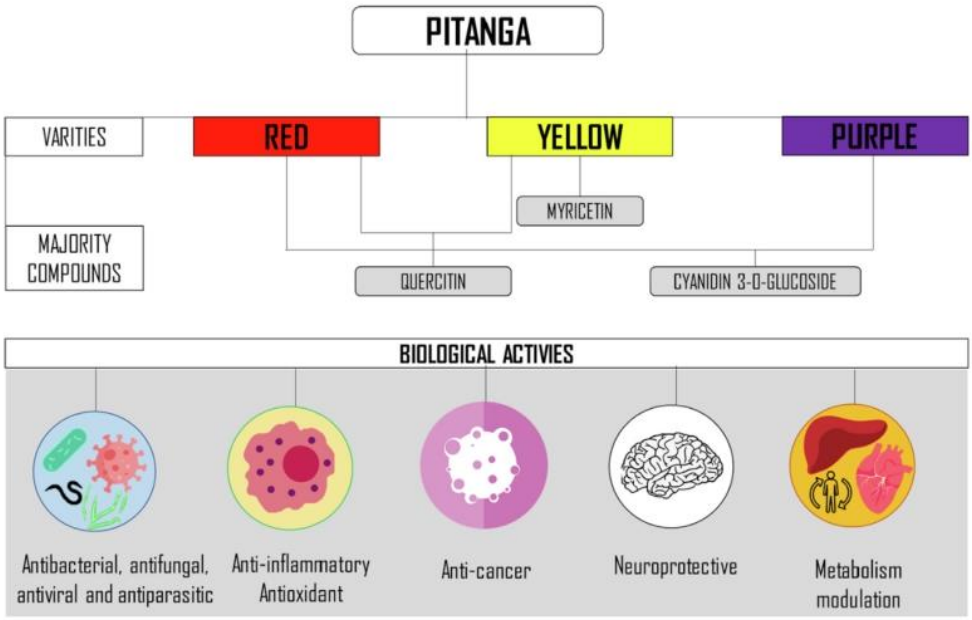

Fig. 3 The major bioactive compounds found in different pitanga varieties and their beneficial effects.

observe that Pelargonidin 3-O-glucoside arrests IkB-α activation and reduces JNK-AMPK phosphorylation, therefore reducing NF-κB signaling (Duarte et al., 2018). Reports demonstrate that myricetin has anti-inflammatory property and can inhibit IL-1β-induced inflammatory mediators in cells. Myricetin was also found to be a potential inhibitor of the COX-2 enzyme (Hiermann et al., 1998, Chen et al., 2001, Rattmann et al., 2012, Li et al., 2013). In *C. elegans* it was possible to observe that myricetin reduced the accumulation of ROS and also the aging pigment lipofuscin by modulating the transcription factor DAF-16, therefore prolonging worms lifespan (Buchter et al., 2013).

According to Lingaraju et al. (2016), kaempferol isolated from the leaf of Eugenia jambolana has anti-inflammatory effects that may be related to the decrease in the level of paw edema in rodents by reducing the activities of NO and MPO. It probably exerts anti-inflammatory effects through the suppression of TNF-α and IL-1β. This reduction in inflammation is also important for hepatoprotective effects that have been attributed to the presence of flavonoid, such as quercetin, myricetin, apigenin and kaempferol glycosides, which have antioxidant activity and had a broad spectrum of bioactivity, according by Sobeh et al. (2020).

A comparative data between majority bioactive compounds, pharmacological action and target tissue is shown in Table 6.

6. Technological potential of *E. uniflora*

In terms of innovation in the food and pharmaceutical areas, *E. uniflora* stands out for its wide application. According to De Araújo et al (2019) it was possible to verify patent applications related to this fruit including extraction processess, beverage preparations and herbal products, just to name a few.

For biological control, botanical insecticides that involve the use of essential oils can be a safe and eco-friendly option for insect control. In a study by Stenger et al., (2021) aiming to minimize productive losses in *Eucalyptus*, an important hardwood tree that is affected by the bronze insect *Thaumastocoris peregrinus*, it was possible to determine the efficacy of *E. uniflora*. In addition, authors described the selectivity of this oil on the parasitoid *C. noackae* and its parasitism in *T. peregrinus*. The essential oil showed insecticidal potential in adults, nymphs and eggs of *T. peregrinus*, and was safer for *C. noacka* when applied one day after parasitism than for pre-parasitism and 7 days after parasitism. The majoritary compounds found in the essential oil of *E. uniflora* were calame-10-one (20.20%), silfiperpherol-6-in-5-one (10.06%) and germacrone (6.61%).

Edible films are thin and flexible materials based on natural biopolymers and have additives generally recognized as safe (GRAS). Biopolymer-based films are biodegradable and, in this sense, they generate interest in replacing or reducing the use of synthetic plastic, which leads to serious problems of ecological accumulation. In this sense, the study by Tessaro et al, (2021), sought to evaluate the effect of incorporating a double emulsion (DE) water-in-oil-in-water (W/O/W) loaded with hydroethanolic extract of pitanga leaf in physicochemical, antimicrobial and antioxidant properties of films based on gelatine, chitosan and gelatine/chitosan mixture to guide the future application of these films as active food packaging. As a result, the incorporation of double emulsion W/O/W to encapsulate pitanga leaf hydroethanolic extract generated films with high antioxidant activity. However, only gelatin-based and DE films inhibited Staphylococcus aureus. The other films containing DE inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella ssp* only in the region of contact with the films. The incorporation of pitanga leaf hydroethanolic extract loaded DE affected the physical properties of gelatine, chitosan and gelatine/chitosan films. Overall, DE-added films were more intense in color and had higher roughness but less hydrophobicity and gloss than the controls on the airside surface. In addition, they presented an important barrier to UV/visible light, in addition to greater mechanical strength and stiffness, when

Table 6 Comparative table between majority bioactive compounds, pharmacological action and target tissue.

Majority Bioactive Compounds	Pharmacological activity/tissue	Experimental model	References
Sesquiterpenes compounds	1.1. antibacterial and anti-inflammatory/ Blood cell 1.2. Antibacterial/oral	*1.In vitro* and phase II clinical trial (children)	1. Jovito et al., 2016
	2. Antiparasitic/Cultivation of Leishmania promastigotes	2. *In vitro*	2. Kauffmann et al., 2017
	3. Anti-cancer/Cell culture	*3. In vitro*	3. Figueiredo et al., 2019
delphinidin 3-O-glucoside, cyanidin 3-O-glucoside	1. Antioxidant/brain, liver and kidneys	*1.In vitro* and *in vivo* (mice)	1. Oliveira et al., 2018;
pelargonidin 3-O-glucoside, myricetin, kaempferol, quercetin.	2. Antibacterial and antifungal/oral	*2.In vitro*	2. Thambi et al., 2013; Fiuza et al., 2019; Santo et al., 2013, 2015;Dos Santos et al., 2018;
	3. Antiparasitic/Cultivation of *Trichomonas gallinae*	*3. In vitro*	3. Ibikunle et al., 2011; Santos et al., 2012;
	4.Anti-inflammatory/ Paw	*4. In vitro* and *in vivo* (rats)	4. Bello et al., 2020; Falcão et al., 2018; Apel et al., 2004;
	5. Anti-cancer/Cell culture	*5. In vitro*	5. Núnes et al., 2018;Ismiyati et al. 2012;
	6. Neuroprotective/Brain	*6. In vivo* (rats)	6. Da Silva et al.,2019;Borges et al., 2015;
	7. Cardiovascular activity/ Heart and vessels	7.*In vivo* (rats)	7. Consolini et al., 1999;Consolini et al., 2002;
Gallic acid 3-O-[6′-O-acetyl-β-D-glucoside]	1. Anti-inflammatory, anti-nociceptive, antioxidant and anti-diabetic/central and peripheral	*1. In vivo (mice)*	1. Sobeh et al., 2019
Gallic acid and myricitrin,	1.Antifungical/mycological culture and Human Erythrocytes	*1.In vitro*	1.Souza et al., 2018;
	2. Anti-inflammatory/ Paw and Blood cell	*2. In vivo*	2. Syama et al., 2020
Selinatrienone derivatives and curzerene compounds	1.Antifungical/ mycological culture	*1.In vitro*	1.Costa et al., 2010;
Flavonoids: saponins, tannins and triterpenes	1. Anthelmintic/ Culture	*1. In vitro*	1. Castro et al., 2019;
	2. Antiviral/Culture	*2. In vitro*	2.Qamar et al., 2017; El Mekkawy et al., 2009; Lee et al., 2000;
	3. Antioxidant/*In vitro*	*3. In vitro*	3. Denardin et al., 2015; Peixoto et al., 2018;
	4.Antioxidant/*C.elegans*	*4.In vivo (C.elegans)*	4. Tambara et al., 2018;
	5. Gastroprotective properties/ Gastric mucosa	*5.In vivo (mice)*	5. de Jesus et al., 2012;Eric de Souza & Suzana da Costa, 2017; Falcão et al., 2013;
	6. Antihyperglycemic, antihyperlipidemic action/Blood	*6. Ex vivo (mice)*	6. Oliveira et al., 2017;
	7. Antihyperlipidemic action/*C.elegans*	*7. In vivo (C.elegans)*	7. Roncato et al., 2019;
Gallic acid; rutin and ellagic acid.	1. Anti-inflammatory and antidiabetic	1. *In vitro* and *in vivo (rats)*	1. Schumacher et al. 2015
	2. α-glucosidase activity	*2.Ex vivo (rats)*	2. Vinholes & Vizzotto, 2017;Siebert et al., 2020;
Quercetin, quercitrin, isoquercitrin, luteolin and ellagic acid	1. Antioxidant/ Human blood cells	1. *In vitro*	1. Da Cunha et al. 2016;
Phenolic, flavonoid, and anthocyanin	1. Antioxidant/Liver	1.*In vivo* (rats)	1. De Souza Cardoso et al., 2016;
	2. Hepatoprotective activities/Liver	2.In vivo (rats)	2.Sobeh et al., 2020;
	3. Beneficial action on hepatopancreas/ Liver and pancreas	3. In vivo (fish)	3. Fiuza et al., 2009;
Monoterpenes and sesquiterpenes	1.Metabolic actions/Blood	4. *Ex vivo* (rats);	1.Arai et al., 1999;Ferro et al.,1988;

compared to films without DE. These results demonstrated that the incorporation of the W/O/W emulsion encapsulating the pitanga hydroethanolic extract did not cause any deleterious effect on the properties of the films. The high barrier to UV/Vis light of these films is notable, suggesting an application for protecting lipid-rich foods.

A market that has been growing in recent years is that of functional drinks made from probiotics and kombucha (Kapp and Sumner 2019). Kombucha is a drink made by fermenting tea (usually black tea) and sugar, with a symbiotic culture of bacteria and yeast (SCOBY) which is a biofilm of microorganisms. Kombucha's popularity as a functional food is driven by its alleged health benefits, which include multiple functional properties such as anti-inflammatory potential and antioxidant activity (Martínez Leal et al., 2018). In this sense, Júnior et al. (da Silva Júnior et al., 2021) sought to evaluate traditional Kombuchás flavored with pitanga pulps. They reported lower sugar losses in flavored kombuchas, showing that pre-existing levels of glucose and fructose in fruits contributed to sweeter drinks. Acetic, butyric, citric, succinic and malic acids were identified, as well as terpenes such as curzerene and β-caryophyllene. High antioxidant activity was observed for fruit flavored kombuchas, and among the phenolics identified, epigallocatechin gallate was the most predominant component (over 63%). The most bioaccessible phenolics in flavored kombucha were caffeine (22.38–29.98%), catechin (17.61–23.48%) and hesperidin (22.43–28.47%). After a simulated gastrointestinal digestion, the phenolic contents decreased, influencing the significant drop in antioxidant capacity. The findings showed that pitanga contributes to diversifying and improving the chemical and bioactive characteristics of kombucha.

The quality of meat products varies throughout their shelf life (temperature, presence of oxygen and light, microbial activity). Meat deterioration is caused by lipid oxidation, which can cause undesirable effects, such as loss of essential fatty acids, flavor and discoloration, leading to changes in organoleptic properties (Zamuz et al., 2018). On the other hand, lipid reformulation by replacing a portion of animal fat with fat substitutes containing oils rich in n-3 PUFA can provide healthier characteristics to the food product, thus meeting the demands of health-conscious consumers. In order to improve that, red pitanga leaves extract was added to mutton burgers with fat replacement during storage (at 2 °C). The addition of pitanga extract did not change the proximate composition and acceptance of mutton hamburgers on day 0. The extract also delayed the discoloration of the hamburgers, conferring a more reddish intensity and delayed the oxidation of lipids and proteins over the storage time, decreasing TBARs and carbonyl values and demonstrating a high antioxidant activity on day 18. In addition, the n-6/n-3 ratio was higher in the pitanga haburgers, but within recommended levels (de Carvalho et al., 2019). Thus, results indicate that pitanga extract was effective against color deterioration and lipid and protein oxidation of the meat, without harming sensory characteristics, representing a promising alternative to replace synthetic antioxidants with natural products in lamb hamburgers.

A growing area of research with focus on environmental conservation and industrial development is that which seeks to reverse the toxic effects of environmental stressors (Dartora et al., 2011). In this sense, secondary metabolites from plants with antioxidant activity represent an interesting alternative. Cunha et al., (2019) evaluated the cytoprotective effect of the ethanol extract of *E. uniflora* leaves against mercury chloride. The ethanol extract of *E. uniflora* demonstrated a chelating effect against iron, and these results can be related with total phenols (1079 mg / g) and flavonoids (946.9 mg / g), detected and quantified by HPLC. The same extract showed cytoprotective against mercury and was non-toxic to *D. melanogaster*, with low mortality and low geotaxy inhibition, demonstrating that the extract can reduce the toxicity of this heavy metal against prokaryotic and eukaryotic organisms. From the results, we can conclude that phytocompounds from the ethanol extract of *E. uniflora*, possibly phenols and flavonoids, can be interesting agents to protect different organisms against heavy metal damage through a chelation or antioxidant mechanism.

During this session we could observe that the wide possible applications of *E. uniflora* in new products. Pitanga has great economic potential, due to the sensory characteristics that favor its commercial exploitation, plus the presence of phytochemicals that play an important role in the management of several chronic and degenerative diseases, in addition to representing a hotspot of technological innovation in food, cosmetics and pharmaceuticals.

7. Conclusion

The present review seeks to contribute to the literature, bringing more clarifications about *E. uniflora*. The results of the literature review have revealed that different parts of the pitangueira tree can be used for different purposes because they have a great number of volatile and non-volatile bioactive compounds in its composition.

The phytochemical profile is related to the cultivation of *E. uniflora* which depends on the climate, ripping, storage and preparation of extracts. All these factors can cause variation in the compounds present in the plant's fruits and leaves. The literature has shown that *E. uniflora* fruits and leaves, especially red and purple fruits, have antioxidant compounds in their composition, such as phenolics, flavonoids and carotenoids that have a potential beneficial effect on health which indicates its high value as a functional food.

Data on the toxicological potential of the plant's essential oil and extract are limited but studies have mentioned low toxicity in rodent rates. The application of *E. uniflora* shows a range of biological properties, such as antibacterial, antifungal, antiviral, anti-inflammatory, anti-oxidant, neuroprotective and hepatoprotective effects among others. However, there are no clinical studies on the effects of *E. uniflora* neither biological nor toxicological. Moreover, *in vivo* studies about *E. uniflora* are incipient. Although few studies in humans have reported the profile and biological activities of pitanga, scientific investigation on its phytochemical and biological properties must be conducted, including nutritional and phytochemical profiles in its different botanical parts.

The results obtained in *in vitro* and *in vivo* studies in different models of animals and described in the present review can thus encourage the use of *E. uniflora* in clinical trial, since the studies demonstrate its safety to mammals and the diverse promising effects. Essentially, the results highlight the beneficial potential of *E. uniflora* against several human comorbidities and its use as a nutraceutical, supplement or phytoterapy must be inserted into the pharmacopoiea.

Declaration of Competing Interest

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

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5.2 Manuscrito

ASSESSING THE SAFETY OF PURPLE PITANGA EXTRACT (PPE) THROUGH TOXICITY STUDIES IN VIVO AND IN VITRO AND COMPUTATIONAL ANALYSIS

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Abstract

Purple pitanga is a variety of fruit from the pitangueira (*Eugenia uniflora*), a native Brazilian tree, but cultivated in several subtropical countries. Purple pitanga extract (PPE) stands out for its richness in anthocyanins, flavonoids and carotenoids, which makes it possible to apply it in various models to evaluate its antioxidant, anti-inflammatory and neuroprotective effects. However, information on safe doses and use in mammals is still scarce. Therefore, this study investigated the effects of PPE *in vivo* and *in vitro*, as well as in silico of the compounds present in the extract. To determine the effects of PPE on C6 glioma cells were exposed to an PPE dose curve, 24 hours after the SRB assay showed that cellular prediction was significantly inhibited, with the LC50 estimated at 380µM. In the acute study, female Wistar rats were administered at a dose of (2.000 mg/kg, 24 hours) orally and observed for another 14 days. In the subacute study, in male and female rats, PPE was administered at the dose (1.000 mg/kg for 28 days), and distributed over an additional 14 days. They were not transmitted in both mortality tests and signs of toxicity, such as physiological, locomotor and there was no significant change in the weight of the organs. Biochemical markers do not signal signs of kidney or liver damage. Suggesting that the LD50 of PPE is greater than 2.000 mg/kg. In computational analysis of ADMET, cyanidin-3-O-glucoside (C3G), myrcetin (MYR), quercetin (QUER), and gallic acid (GA) investigated in parallel exhibit different profiles. MYR, QUER, and GA show moderate distribution between plasma and tissues, while C3G exhibits the highest permeability to the blood-brain barrier (BBB). But all they are not toxic in rats, and do not cause liver damage. Thus, our data suggest that the nutraceutical effects of PPE inhibit the growth of cancer cells and the doses of the extract administered acutely, as in the subacute toxicity model, did not show signs of toxicity and can be considered safe for consumption.

Keywords: Purple pitanga; Glioblastoma; Toxicity; Rats; Nutraceutical.

1. Introduction

Medicinal plants represent the oldest form of medicine, used for thousands of years in traditional medical practices in many countries around the world [1]. Due to their abundant natural reservoir of active compounds, medicinal plants are an interesting source of natural products for the treatment of various health conditions [2], thereby holding prime importance for the development of new medicines [3].

Herbal medicines contain phyto-constituent complexes and organic chemicals, including alkaloids, fatty acids, sterols, flavonoids, glycosides, saponins, terpenes, among others [4]. Within this context, the *Myrtaceae* family is one of the main commercial fruit families in the world, comprising approximately 121 genera [5]. Among these, the genus *Eugenia* is considered the fourth most important genus in the *Myrtaceae* family [6] with *E. uniflora L.* being the most studied species [7], This species produces the pitangueira (*Eugenia uniflora*), a brazilian native tree, but cultivated in several subtropical countries [8]. Its fruit, pitanga, exists in varieties such as orange, red and purple, with bothits pulp and peel containing valuable phytochemical properties [9]. Notably, the purple variety stands out by its richness in anthocyanins, flavonoids and carotenoids [10] which confer the ability to reduce pró-oxidative substances in the body [11].

Purple pitanga is widely used in folk medicine to treat fevers, digestive and liver diseases, rheumatism, tonsillitis, sore throat and diarrhea [12,13,14]. The beneficial effects of pitanga are related to important compounds such as Myricetin (MYR), which demonstrated potential as an inhibitor of the COX-2 enzyme [15], Cyanidin 3-O-Glucoside (C3G), which demonstrated potential for binding and inhibiting control points. immune responses, thus activating the immune response in the tumor microenvironment and inducing cancer cell death [16], Quercetin (QUER) demonstrated to inhibit caspase 3, preventing intrinsic apoptosis by regulating Bcl-2 and Bax

proteins [17], as well as Gallic acid (GA) which plays an anti-inflammatory role by inhibiting the NF-Kb pathway considered a prototype of the pro-inflammatory signaling pathway in the body [18].

Several studies have investigated the biological effects of purple pitanga extract (PPE) such as cytotoxicity of in different models, antiproliferative effects in cancer cell lines [19] and antioxidant in the nematode *Caenorhabditis Elegans* (*C.elegans*) [20]. Moreover, liposomal formulations have also been used as a delivery method for PPE and antilipidemic effects have been reported in this model [21], yet PPE prolonged longevity and reduced the Muv phenotype in mutant worm with gain of function in the let-60 gene, which is a tumor strain related to the HRas pathway [22]. In rodents, PPE showed a neuroprotective effect against memory impairment in a model induced by MPTP [23] and an antioxidant effect in a single dose of PPE in the same model, [24].

However, the consumption of natural extracts has led to growing concerns about the safety of their use. High intake of these compounds can potentiate harmful effects due to their diverse pharmacological properties, which can alter the drugs metabolism o, modulate the activity of environmental genotoxicants, and alter the activity of other important metabolizing enzymes [25]. To better utilize natural extracts, an assessment of toxicity patterns is necessary to ensure safety during its use [26]. Furthermore, the pharmacokinetic prediction of natural products is an important process to discovery and optimization of new pharmaceutical compounds [27]. Thus, acute and subacute toxicity tests are used to evaluate the toxicity or side effects of many natural extracts.

Therefore, the objective of this study was to evaluate the safety of PPE in cell culture and in rats submitted to experimental models of acute or subchronic toxicity. Additionally, we will

perform computational analysis to predict pharmacokinetics and toxicity of the main components of PPE.

2. Materials and methods

2.1 Preparation of Pitanga Purple Extract (PPE)

Pulp samples of purple pitanga (*E. uniflora*) were obtained from Embrapa Clima Temperado (Pelotas, RS, Brazil) and immediately frozen. A pool of fruits collected in the 2018/2019 harvest was frozen at -18 °C and transported to the Universidade Federal do Pampa (Uruguaiana, RS, Brazil). We thaw the fruits, remove the seeds and homogenize the pulp and rind. They were homogenized with an ultra-turrax homogenizer for 5 min in 95% ethanol (1:3,w/v). The homogenates were mixed for 30 min in a flask protected from light, at room temperature and centrifuged at 3000 rpm for 5 min, the supernatant was collected and the extraction procedure repeated once more with the residue. The pooled supernatants were dried on a rotary evaporator at a maximum temperature of 40°C. Samples were reconstituted in water and stored at -80°C [28]. Total phenolic compounds were quantified according to the Folin-Ciocalteu method adapted from Swain & Hillis [29]. Absorbance was measured at 725 nm and results were expressed as chlorogenic acid equivalents (CAE; µg/mL), serving as standard (purity 95%; Sigma Aldrich, St. Louis, MO, USA). PPE exhibited a total phenolic content of 11,252.56 mg chlorogenic acid equivalent per milliliter (CAE/mL).

2.2. *In vitro* cell assay

2.2.1 Cell culture

The C6 rat glioblastoma cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured until passage 210 and maintained in DMEM (Sigma, USA) containing antibiotics (0.5 U/mL penicillin/streptomycin) and supplemented with 5% fetal bovine serum (FBS). The cells were maintained at a temperature of 37°C, minimum relative humidity of 95% and an atmosphere of 5% CO_2.

Confluent cells were separated from culture flasks using 0.05% trypsin/EDTA, collected by centrifugation for 5 min (400× g , 23°C), suspended in complete medium supplemented with 10% FBS, and plated (9× 10 3 cells/cm 2) in 24-well plates or 25 cm 2 flasks, depending on each parameter evaluated. After the cells reached 80% confluence, the culture medium was removed and the cells were incubated in the presence of 1000µM, 500µM, 250µM, 125µM, 62.5µM, 31.25µM, prepared in DMEM with 5% FBS (the pH was adjusted to 7.4 with NaOH) for 24 to 37°C in a 95% air/5% CO_2 incubator. Control cells were incubated for the same periods of time in DMEM supplemented with 5% FBS.

2.2.2 Cellular cytotoxicity assay using Sulforhodamine B

Sulforhodamine B (SRB) assays were used to determine cell proliferation. The assay was performed as previously described for [30] with some modifications [31]. After treatment with different concentrations of PPE, the culture medium was removed, the cells were washed three times with PBS and 500 µl of PBS/4% paraformaldehyde were raised. After 15 min, the inserted cells were stained with SRB. Subsequently, cells were washed with Milli-Q® water to remove unbound stain. The culture plates were air-dried and the protein-bound sulforhodamine was solubilized in 1% SDS. Absorption was measured using a

spectrophotometric microtiter plate reader (Spectra Max M5, Molecular Devices) at 560 nm. This absorption was linearly proportional to the number of cells.

2.2..4 Measurement of apoptosis of C6 Hoechst 33342/PI cells

C6 cells were seeded in a 24-well bottom culture plate at a density of 1x10(4) cells/well. After treatment with different concentrations of PPE, cells from each group were washed once with PBS and then 2µl of *Hoechst 33342* was added and gently mixed. The cells were then placed in the dark at room temperature for 10 to 15 min. PBS was then used to wash the cells once. PI solution (50 µl) was added after washing the cells with PBS for 2 more times, followed by gentle mixing. Cells at respective concentrations were incubated in the dark at room temperature for another 10–15 min. Randomly selected images were captured using an EVOS Floid cell imaging station (Thermo Fisher Scientific Inc.) and density was evaluated using the NIH ImageJ software.

2.3. *In vivo* assay

2.3.1 Animals

The analyzes were performed on rodents (Wistar lineage), males and females (250-300g), with three months of age. The animals were kept in a room with a 12-hour light/dark cycle, at a temperature of 22 ± 2 °C. The animals were obtained from the Federal University of Santa Maria, Brazil. The experiments were carried out between 8:00 am and 10:00 am in a noise-free room. The animals were used in accordance with the National Institutes of Health guide to the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and every effort was made to minimize animal suffering and reduce the number of used

animals. The protocol was approved by the Committee for the Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil (Protocol 008/2021).

2.3.2 Acute oral toxicity study

The acute oral toxicity of PPE was evaluated in female rats according to OECD Guidelines 423 [32] (Fig.1). The animals were divided into two groups: *Control (C) and PPE 2000mg/kg (PEE2000)* (N=6/group). Control group received a vehicle (saline solution) (3 ml/kg, orally) and PPE2000 group received a single oral dose of 2000 mg/kg of PPE. The open field test was performed 24 hours after PPE administration. During this period signs of mortality and general toxicity, such as tremors, salivation, diarrhea were recorded.

Following the same guideline, to observe possible late toxicity effects, it is necessary to use a satellite group, which consists of repeating these two groups and observing them for 14 days after the administration of the PPE. Thus, two new groups were created: *Satellite (S)* (saline, 3 ml/kg, orally) and *SPPE2000* (PPE, 2000 mg/kg, orally) (N=3/group). After 14 days of treatment, the animals underwent the same procedures mentioned above for the evaluation of general toxicity, behavioral test and blood and organ collection.

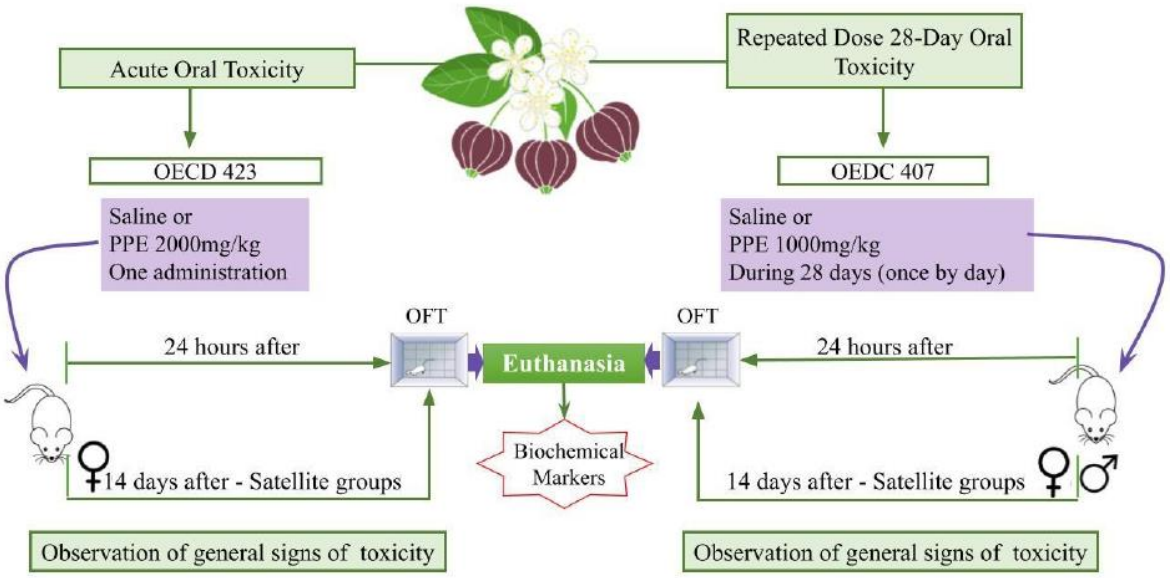


Fig 1. Experimental design of *in vivo* toxicity studies. Timelines illustrating the two protocols (acute and subchronic). Source: Figure created by the author.

2.3.3 Oral subchronic toxicity study

The subchronic toxicity of PPE at a dose of 1000 mg/kg was evaluated according to OECD Guidelines 407 [34] (Fig. 1). The animals were divided into 4 groups: (*Control/Female; PPE1000/Female; Control/Male* and *PPE1000/Male*, N=6/group). Control groups received vehicle (saline solution (3 ml/kg), orally) and groups treated received an oral dose of 1000 mg/kg of PPE for 28 days.

Mortality and general toxicity were observed at two-day intervals. General signs of toxicity, including loss of body weight, water and food consumption, tremors, salivation, diarrhea and loss of body hair were recorded over 28 days. The open field test was performed at the end of treatment, after which the animals were anesthetized with isoflurane and blood from the cardiac ventricle was collected using EDTA as an anticoagulant. Organs such as the liver, spleen and kidney were removed and weighed.

As mentioned before, to observe possible effects of late toxicity, a satellite group was used with observation for another 14 days. Thus, new groups were created: Satellite (S) (*S/Female; SPPE1000/Female*; *S/Male* and *SPPPE1000/Male*) (saline solution, 3 ml/kg, orally) and SPPE1000 (PPE, 1000 mg/kg, oral route) (N=6/group). Similarly, the mortality, signs of general toxicity, consumption of food and water and open field test e samples collect were performed in the satellites groups.

2.3.4 Open-field test

Spontaneous locomotor activity was measured in the open-field test according to the method of [35]. The floor of the open field was divided into nine squares. Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a 5-minutes session. The apparatus was cleaned after each individual section of the rat.

2.3.5. Tissues dissections and plasma general marker of toxicity

At the end of the protocols, the animals were fasted overnight, euthanized with isoflurane, and killed by exsanguination. Blood was collected from the cardiac ventricle using EDTA. The organs (liver, spleen, kidneys) were quickly removed and weighed. The relative organ weight of each animal was then calculated according [36]. Plasma enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels were measured using commercial kits (BIOCLIN).

2.3.6 Pharmacokinetic prediction of absorption, distribution, metabolism, excretion and toxicity (ADMET)

Pharmacokinetic prediction of absorption, distribution, metabolism, excretion and toxicity (ADMET) was predicted for the major compound C3G Myrcetin, Quercetin and Gallic Acid of PPE; the composite was designed using ChemDraw Ultra 12.0 and later converted to a SMILE sequence using Open Babel GUI. ADMET data were evaluated: intestinal absorption, distribution, with assessment of permeability parameters in the central nervous system (CNS) and blood-brain barrier (BBB) and total clearance to analyze the excretion of the compound. Regarding toxicity parameters, acute oral toxicity in rats was evaluated at a lethal dose of 50% (LD50), oral toxicity in rats at a dose with the lowest observed adverse effect (LOAEL), AMES and hepatotoxicity.

2.3.6 Statistical analysis

The generated data were analyzed by GraphPad Prism (version 8) and are expressed as mean ± standard error of the mean (SEM). Analysis of statistical significance between the experimental groups was performed using one-way analysis of variance (ANOVA). Student's *t* test was used to compare results between groups, followed by Tukey's test, when necessary. p values < 0.05 were considered statistically significant.

3. Results

3.1 Cell viability evaluation

To determine the lethal concentration that reduces cell viability by 50% (LC50), we performed a toxicity assay on C6 cells using the SRB method (Fig. 2). Our results indicated

that a concentration of 250 µM of PPE (*p=0.0272*) and 500 µM of PPE (*p=0.0003*) reduced C6 cell viability by 61.34% and 37.64% compared to the control, respectively. The assay estimated the LC50 to be approximately 380 µM.

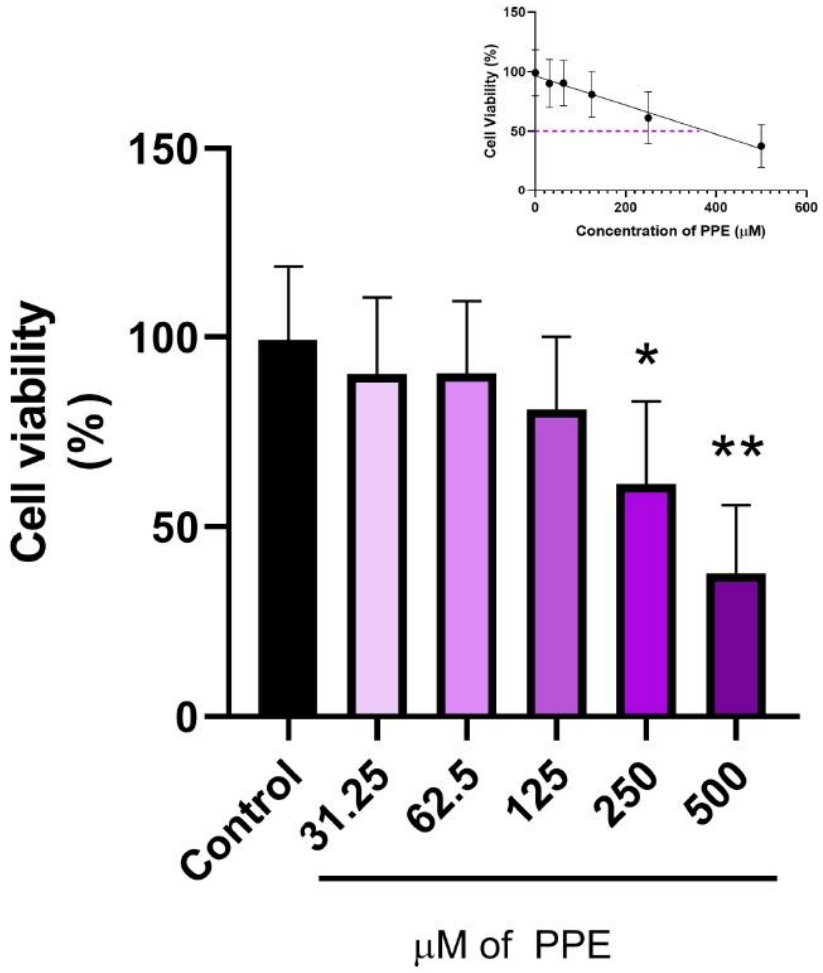


Fig 2. Cell viability curve of the C6 glioblastoma line arranged in a 24-well plate, incubated with different concentrations of PPE evaluated by the SRB assay. Data are expressed as mean ± standard error (one-way ANOVA). *$p<0.05$ and **$p<0.01$ compared to untreated control cells.

After that, the cells were double stained with Hoechst 33342 and PI. PPE at concentrations of 125µM (*p=0.0001*), 250µM (*p=0.0001*) and 500µM *(p=0.0001*) promoted death in C6 glioblastoma cells when compared to the control (Fig. 3).

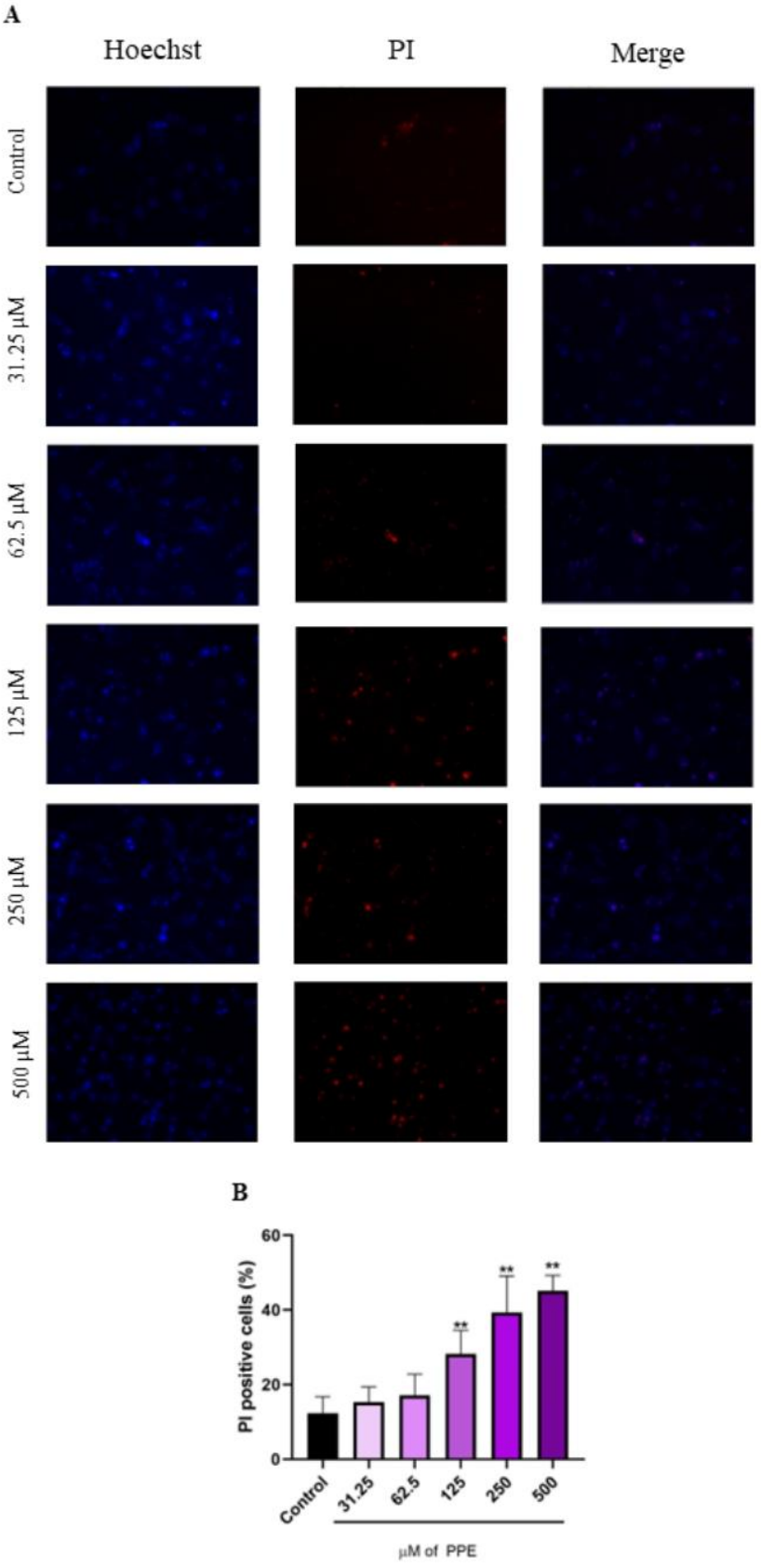

A
Hoechst
PI
Merge
Control
31.25 µM
62.5 µM
125 µM
250 µM
500 µM
B
PI positive cells (%)
60
40
20
0
**
**
**
Control
31.25
62.5
125
250
500
µM of PPE

Fig 3. Effects of different PPE concentrations induce cell death in the C6 cells. **A**) Cells were double stained with Hoechst 33342 and PI, and prediction was evaluated under fluorescence specificity. Typical photographs of control cells (Control) and after PPE exposed (31.25 to 500μM). Cells positive for Hoechst 33342, exhibiting blue fluorescence, considered viable cells. PI-stained cells exhibiting red fluorescence were considered necrotic. **B**) Quantitative analysis of cells undergoing necrotic. Data are expressed as mean ± standard error (one-way ANOVA). $*p<0.05$ and $**p<0.01$ compared to untreated control cells.

3.2 Acute oral toxicity assessment in female rats

The oral administration of PPE at a dose of 2,000 mg/kg did not produce any signs of toxicity, such as mortality, tremors, salivation, or diarrhea, in animals within 24 hours. Similarly, these signs were not observed after 14 days in the SPPE group when compared to the satellite group. No statistically significant differences in body weight gain were found between the animals during the 14 days after the last administration of PPE. Thus, no weight gain difference was observed among the SPPE2000 groups in comparison to the control group (SControl) (table 1).

After 24 hours, exposure in PPE2000 did not affect the number of crossings or animal rearing in the open field test. Furthermore, no significant changes were observed in crossing and rearing between the SControl and SPPE2000 groups (table 1). No statistically significant differences in vital organ weights were observed in rats twenty-four hours after PPE administration or later in the protocol (table 1). Likewise, biochemical toxicity parameters in the blood, such as AST, ALT, and creatinine, were not altered by exposure to PPE2000 when compared to the control group, nor in SPPE2000 compared to the SControl group (table 1).

Table 1. Effects of acute PPE administration on physiological and biochemical parameters.

Acute oral toxicity		24 hours after treatment			14 days after treatment		
		Control	**PPE 2000**	***P* value**	**SControl**	**SPPE 2000**	***P* value**
General toxicity sings	**Body gain (g/period)**	2.33±0.21	1.80±0.20	*0.1039*	33.50±4.59	32.50±1.38	*0.8391*
	Food consumption (g/day/rat)	25.58±0.00	24.25±0.00	N/A	25.57±1.70	25.40±1.42	*0.9416*
	Water intake (ml/day/rat)	28.33±0.00	28.00±0.00	N/A	26.06±1.34	28.24±1.73	*0.3447*
Open field test	**Crossing**	87.33±8.51	93.17±7.93	*0.9584*	90.92±4.99	83.67±10.64	*0.9245*
	Rearing	42.83±6.11	38.80±3.32	*0.9016*	37.17±0.60	37.20±4.17	*0.9999*
Relative organs weight	**Liver (g%)**	3.53±0.48	3.95±0.09	*0.6976*	4.26±0.14	5.19±0.14	*0.1339*
	Kidney (g%)	0.76±0.03	0.81±0.03	*0.7014*	0.94±0.02	0.97±0.04	*0.8814*
	Spleen (g%)	0.29±0.02	0.31±0.01	*0.5759*	0.33±0.01	0.35±0.0	*0.0757*
Plasmatic marker of toxicity	**AST (U/L)**	105.98±33.3	150.71±27.3	*0.6624*	105.7±11.1	97.0 ±7.7	*0.3116*
	ALT(U/L)	27.42±8.6	17.16±4.1	*0.4837*	23.96±8.7	21.38±5.7	*0.9907*
	Creatinine (mg/dL)	0.898±0.22	0.665±0.26	*0.8962*	0.907±0.33	0.710±0.13	*0.9187*

Values expressed as mean ± SEM, n = 6 animals/group. g/period (gram per period); mg/dL (milligram per deciliter); g/dL (gram per deciliter); g% (grams per percentage of organs); UI/L (International Units per Liter). N/A (not available) since data from the group 24 hours later only presents a measure of food consumption and water intake.

3.3 Subchronic oral toxicity assessment in male and female rats

No mortality was observed of animals (male and female rats) during the 28-day period of oral administration of PPE. The animals did not change their general appearance during the observation period. Morphological features (skin, eyes and nose) appeared normal at the end of treatment. No tremors, salivation, diarrhea, lethargy, or unusual behavior such as walking backwards were observed; gait and posture, reactivity to stimuli or sensory stimuli.

Table 2 shows the effect of administering PPE for 28 days and the subsequent 14 days, considering gender differences. One-way ANOVA analysis of body weight data in rats showed no significant difference at 28 days in the PPE1000 group when compared to the Control group. Likewise, no significant difference was observed in late gain between the SPPE2000 groups compared to SControl. Similar results were observed in male rats treated with PPE, which did not show any difference in weight gain neither during the 28 days of treatment nor on the 14th day after the last administration of the extract when compared to their respective control group.

Food and water consumption showed no significant differences in female rats treated with PPE1000 compared to the control group (table 2). Over the 28-day treatment period and the subsequent 14 days, neither food intake nor water intake differed significantly of control groups. These results were consistent in males, with no significant differences in food intake or water intake between the treated and control groups.

As shown in table 2, spontaneous locomotor activity females approached in open field showed no difference in the number of crossing or the number of rearing in PPE1000 rats in relation to the control. Likewise, locomotor activity was not changed after 14 days in

SPPE1000 compared to the satellite groups. Similar results were observed in male rats, subchronic treatment with PPE1000 did not change in locomotion in early and late protocol (table 2).

There were no significant differences in organ weights between the control and treated groups for both male and female rats compared to the control group. Results of biochemical studies (table 2) indicated that there were no significant changes in serum levels for AST and ALT activity and creatinine levels in rats treated with PPE1000 for 28 days compared to the control group. Furthermore, biochemical parameters also remained unchanged in the SPPE1000 groups even 14 days later. Male rats treated for 28 days or observed for 14 days showed no significant difference between the groups.

Table 2. Effects of subchronic PPE administration on physiological and biochemical parameters.

Subchronic oral toxicity		SEX	28 days treatment			14 days after treatment		
			Control	PPE 1000	*P* value	SControl	SPPE 2000	*P* value
General toxicity sings	Body gain (g)	F	55.00±5.45	54.00±5.84	*0.9986*	89.20±3.02	86.20±3.1	*0.9655*
		M	52.00±4.61	50.00±2.50	*0.9954*	113.6±10.15	102.6±4.22	*0.5895*
	Food consumption (g/day)	F	25.26±0.59	26.11±0.29	*0.4822*	27.51±0.33	27.48±0.35	*0.9999*
		M	24.93±0.36	25.50±0.16	*0.6413*	26.07±0.42	27.16±0.33	*0.1414*
	Water intake (ml/day)	F	22.64±0.47	23.68±0.36	*0.2085*	25.47±0.26	26.14±0.30	*0.5611*
		M	31.06-±0.21	32.08±0.18	*0.0706*	33.61±0.38	33..48±0.24	*0.9872*
Open field test	Crossing	F	46.80±3.17	36.80±4.79	*0.8167*	83.50±7.70	76.20±1.46	*0.9691*
		M	52.00±4.20	67.40±4.69	*0.3439*	68.20±6.70	70.20±4.87	*0.9999*
	Rearing	F	47.00±6.90	30.60±4.83	*0.4153*	46.80±3.17	36.80±4.80	*0.6266*
		M	40.20±4.40	39.60±5.66	*0.9999*	27.00±6.72	33.00±5.93	*0.9930*
Relative organs weight	Liver (g%)	F	3.81±0.16	3.69±0.16	*0.9556*	3.39±0.19	3.40±0.16	*0.9999*
		M	3.87±0.20	3.66±0.17	*0.7799*	3.59±0.16	3.58±0.03	*0.9999*
	Kidney (g%)	F	0.84±0.01	0.82±0.04	*0.9772*	0.81±0.04	0.83±0.06	*0.9930*
		M	0.77±0.02	0.81±0.04	*0.7398*	0.74±0.02	0.75±0.02	*0.9711*
	Spleen (g%)	F	0.27±0.02	0.30±0.01	*0.7005*	0.33±0.03	0.26±0.01	*0.2295*
		M	0.24±0.01	0.25±0.01	*0.9990*	0.74±0.02	0.76±0.02	*0.9222*
Plasmatic marker of toxicity	AST(U/L)	F	160.4±43.18	174.4±25.6	*0.9999*	143.1±45.7	71.5±8.0	*0.9951*
		M	150.1±25.4	109.1±46.4	*0.3422*	179.4±81.4	131.8±54.8	*0.9999*
	ALT(U/L)	F	25.12±5.00	31.40±4.92	*0.9999*	27.33±9.27	43.07±24.43	*0.6855*
		M	30.26+5.37	32.88+9.07	*0.9349*	30.03+5.62	27.93+7.40	*0.9979*
	Creatinine (mg/dL)	F	0.700±0.341	1.280±0.773	*0.9094*	0.800±0.432	2.160±1.52	*0.9137*
		M	0.396±0.062	0.423±0.049	*0.9999*	0.370±0.037	0.396±0.062	*0.9989*

Values expressed as mean ± SEM, n = 5 animals/group. M denotes male and F denotes female. g/period (gram per period); mg/dL (milligram per deciliter); g/dL (gram per deciliter); g% (grams per percentage of organs); U/L (International Units per Liter).

3.4 Pharmacokinetic prediction of absorption, distribution, metabolism, excretion and toxicity of the compound Cyanidin-3-glucoside, Myrcetin, Quercetin and Gallic Acid

According to the data presented in table 3, it was possible to predict that the partition coefficient (log P) of QUER is the highest (1.988), followed by GA (0.5016), C3G (0.382), and MYR (0.1943), indicating that all compounds possibly have a good permeability-solubility balance. Additionally, the data showed that the water solubility of the compounds varies: QUER has the lowest solubility (-3.416 log mol/L), while C3G and MYR have higher solubility (-2.892 log mol/L), and GA has the highest solubility (-1.914 log mol/L) [49].

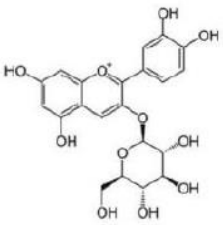
The anthocyanin C3G showed good intestinal absorption (80.614%), as did QUER (75.236%), MYR (43.334%), whereas GA had less absorption (40.154%). The VDss (Volume of Distribution at steady state) values indicate the different distributions of the compounds in the human body. MYR has a high VDss of 1.552 logL/kg, indicating extensive tissue distribution. QUER, with a VDss of 0.106 logL/kg and C3G, with a VDss of 0.011 logL/kg, shows moderate distribution between plasma and tissues. Conversely, GA has a low VDss of -0.27 logL/kg, indicating it remains more concentrated in plasma with limited tissue distribution (table 3). The compounds showed limited permeability to the BBB: C3G (-0.013 log BB), MYR (-1.811 log BB), QUER (-1.357 log BB) and GA (-1,424 log BB), these values indicate that, although all the compounds have low permeability to the BBB, C3G is the most penetrative among them. In this sense, they have low permeability to the CNS, where we have C3G (-1.915 log PS), MYR (-4.376 log PS), QUER (-3.467 log PS) and GA (-4.131 log PS) [64]

(table 3). Furthermore, MYR and QUER are substrates of P-glycoprotein, which could contribute to the limited distribution of these compounds in the CNS [64] .

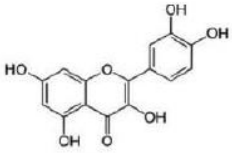
Regarding metabolism, the results of the prediction of C3G and QUER show that they are potential inhibitors of CYP1A2 enzymes. C3G has a very low excretion, eliminating a total of -36.914 log mL/min/kg, MYR (0.303 log mL/min/kg), QUER (0.637 lg mL/min/kg) has moderate total elimination as well as GA (0.625 log mL/min/kg) (table 3).

The AMES test for compounds indicates tolerable doses of these compounds in humans are C3G (0.438 log/mg/kg/day), MYR (0.454 log/mg/kg/day), QUER (1.119 log/mg/kg/day) and GA (0.519 log/mg/kg/day). In rodents, acute oral toxicity showed LD50 values for C3G (2.482 mol/kg), MYR (2.537 mol/kg), QUER (2.054 mol/kg), and GA (3.241 mol/kg). In chronic toxicity studies in rats, the values were: C3G (13.821 log mg/kg_bw/day), MYR (3.386 log mg/kg_bw/day), QUER (3.138 log mg/kg_bw/day), and GA (3.241 log mg/kg_bw/day). Finally, none of the evaluated compounds showed hepatotoxicity (table 3).

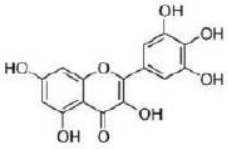
Table 3. ADMET parameter values of Cyanidin-3-glucoside, Myrcitin, Quercetin and Gallic acid generated by the pkCSM Pharmacokinetics server.



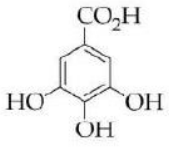
Cyanidin 3-O-glucoside



Myricetin



Quercetin



Gallic Acid

Parameters / models	*Cyanidin-3 -glucoside*	*Myrcetin*	*Quercitin*	*Gallic Acid*
Molecular weight	449.388	464.379	302.238	170.12
Log P	0.382	0.1943	1.988	0.5016
Rotatable bonds	4	3	1	1
Acceptors	10	12	7	4
Donors	8	8	5	4
Surface area	179.740	183.901	122.108	67.135
Water solubility (log mol/L)	-2.892	-2.892	-3.416	-1.914
Intestinal absorption Human (% absorbed)	80.614	43.334	75.236	40.154
P-glycoprotein substrate	No	Yes	Yes	No
VDss human (log L/kg)	0.011	1.552	0.106	-0.27
BBB permeability (log BB)	-0.013	-1.811	-1.357	-1.424
CNS permeability (log PS)	-1.915	-4.376	-3.467	-4.131
CYP2D6 substrate	No	No	No	No
CYP3A4 substrate	No	No	No	No
CYP1A2 inhibitor	Yes	No	Yes	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No
Total clearance (log mL/min/kg)	-36.914	0.303	0.637	0.625
AMES toxicity	Yes	No	Yes	No
Max. tolerated dose human (log/mg/kg/day)	0.438	0.454	1.119	0.519
Oral rat acute toxicity LD50 (mol/kg)	2.482	2.537	2.054	2.03
Oral Rat chronic Toxicity LOAEL (log mg/kg_bw/day)	13.821	3.386	3.138	3.241
Hepatotoxicity	No	No	No	No

4. Discussion

Medicinal plants continue to be frequently used without scientific basis, based only on their popular knowledge perpetuated for generations. However, many medicinal plants also

have adverse effects [37,38], raising concerns about the potential toxic effect resulting from the use of such medicinal plants.

The C6 glioma cell line is often used as a glioblastoma model due to its close simulation of the characteristics of human glioma, including rapid expansion and invasiveness [39]. Glioblastoma, a very aggressive brain tumor, originates in the glial cells of the brain, which provide support and insulation between neurons, and is a primary area of interest in the field of neuro-oncology due to its physiological rapidity [40]. In our study, PPE reduced the viability of the rat C6 glioma cell line, with the LC50 estimated at 380μM of PPE. The Hoechst 33342/PI assay confirmed cells death of C6 cells from 125 μM.

In line with our results, Denardin and colleagues [19] observed that increasing concentrations of PPE ranging from 5 to 100 μg/mL were capable of triggering antiproliferative and cytotoxic effects in activated hepatic stellate cells, demonstrating that PPE is a good candidate in the treatment of liver fibrosis, as that these cells are liver-specific pericytes involved in the development of pathological fibrosis [28]. The safety and pharmacological effects of PPE were evaluated using an *in vivo* model of cancer in *C. elegans*, which presents a hyperplasia phenotype due to the gain of function of let-60, a homolog to human Ras. The PPE treatment effectively mitigates the hyperplastic phenotype associated with let-60 activation, suggesting potential therapeutic benefits in conditions involving Ras pathway dysregulation [22]. This underscores the relevance of our findings in exploring the therapeutic potential of PPE in Ras-related cancers.

According to OECD 423[32], acute toxicity is generally carried out at a high dose that is responsible for the development of mortality and morbidity in experimental animals. In our study, as there was no previous report on PPE toxicity, we used a dose of 2.000 mg/kg, which did

not kill any treated animals, nor did it show any clinical signs of toxicity. Furthermore, weight and food consumption were not affected by the fruit extract, suggesting that the extract has no effect on normal behavior or appetite suppression. The weight of the vital organs did not show any changes, justifying the safety of the extract, since the weight of the organs is an important index of the pathophysiological state of humans and animals (Pedraza and Oliveira, 2021). These results are in line with those observed in the purple fruit of the same genus, *Eugenia jambolana Lam,* at a dose of 2.000 mg/kg, no difference was observed between the groups [41].

Animals treated for 24 hours and observed 14 days later did not have the number of crossings and reproductions reduced in the open field test, showing that the PPE had no stressful effect on the rats. Stressors have a complex effect on outdoor behavior, with physical stressors decreasing activity and emotional stressors increasing activity [42,43]. Savall and collaborators (2023) [23], did not observe locomotor differences in animals that received PPE at a dose of 2.000 mg/kg for 14 days, showing that the extract does not affect spontaneous locomotion within 24 hours and after 14 days of a single dose, as well as daily administration. for 14 days.

Biochemical data are essential for a complete toxicological assessment of a specific substance, allowing to determine the occurrence of toxic effects in specific tissues (OECD, 2008). Regarding biochemical parameters, animals treated with PPE 2.000 mg/kg did not show signs of kidney or liver damage, evidenced by values compatible with normality of kidney function indicators such as creatinine and liver function indicators such as ALT, AST. This implies that PPE did not alter any mechanism in the body, as the liver and kidneys are important organs to be affected in any toxic reactions, thus ensuring the safety of the extract [44]. Thus, according to OCED 423, PPE can be classified as category 5 with LD50 above 2.000

mg/kg. This illustrates that the no-observable-adverse-effect dose level of PPE is above 2.000 mg/kg.

Based on initial results on acute toxicity, determination of short-term toxic potential can be determined by repeated dose oral toxicity protocol [37]. In the study carried out over 28 days, the 1.000mg/kg dose of PPE did not produce any marked changes in female and male rats, as evidenced by the absence of toxic symptoms and no change in water/food intake or weight gain. The animal models for behavioral and pharmaceutical testing are used in several fields of research [45]. Locomotor behavior in females and males was not compromised by the daily dose of 1.000mg/kg of PPE in 28 days and 14 days after the last dose. This data is interesting, since the behavior was maintained in both groups, showing that PPE did not cause a late toxicological effect. Organ weights revealed that PPE at the dose used did not produce organ swelling, atrophy or hypertrophy in males and females. Biochemical parameters ALT, AST and creatinine were also not affected by ingested PPE. The lack of significant changes in the levels of these markers are functional indicators of the liver and kidneys [46]. Yele and Veeranjaneyulu [41], obtained similar results at a dose of 1.000mg/kg of *Eugenia jambolana Lam*, in both groups.

In vitro and *in vivo* results serve as the basis for drug design, many early-stage drug discovery programs focus on identifying molecules that bind to a target of interest. Although potency is a determining factor in these early stages, ultimately pharmacokinetic and toxicity properties determine whether it will ever increase its efficacy and therapeutic success [47]

In this sense, we selected four bioactive compounds present in PPE, C3G, MYR, QUER and GA [20], in order to predict ADMET using the pkCSM web server. The four molecules were found to have a molecular weight of less than 500 Da and a log P value did not exceed 3.

However, while the hydrogen bond acceptors were checked and met the recommended value, both MYR and C3G exceeded it by 5. In this case, both QUER and GA meet the Lipinski rule criteria, whereas C3G meets only 3 criteria. The Lipinski rule suggests that molecules meeting these criteria are likely to exhibit good bioavailability and are therefore promising drug candidates [48].

Furthermore, the polar surface area that is linked to oral absorption or membrane permeability of QUER is <140, proving that it has weak polarity and will be absorbed more easily by the body [49]. The water solubility of the molecules proved to be high, as it is greater than -6 log mol/L. The prediction of ADMET and similarity of compounds to drugs according to Lipinski's Rule, indicates that a molecule that violates two or more Lipinski's Rule is not orally active, the rule consists of calculating molecular properties such as log P, polar surface area, number of hydrogen bond donors, number of hydrogen bonds, acceptors and molecular weight, which can help predict the oral action of the respective pharmacological compound. acceptors and 10 hydrogen bond donors [50]. However, Lipinski's Rule is not the only standard for determining the viability of phytochemicals [49].

Other ADMET parameters indicate that C3G has a higher percentage of intestinal absorption, in fact, anthocyanin glycosides undergo rapid absorption in the small intestine, which is followed by rapid metabolism and excretion in bile and urine, both in intact form and in derivatives metabolized [51], just as quercetin showed a high percentage. The absorption process is influenced by the physicochemical properties of the substance, such as solubility, particle size of the drugs as well as the physiological and pathological state [52]

MYR and QUER are substrates for P-glycoprotein, an ATP-dependent efflux protein located in several tissues, including those lining the intestine and the BBB [53]. It is also

involved in the processes of modulating absorption and distribution, and may contribute to the occurrence of pharmacokinetic drug interactions (Broccatelli et al., 2011). The volume of distribution of MYR and GA is greater, which means that it has greater distribution in body tissues, while the permeability of the BBB and the CNS is low in the molecules studied here.

The CYP1A2 enzyme can be inhibited by C3G and QUER, this inhibition can result in a decrease in the biotransformation of the drugs it metabolizes. In vitro studies have shown that some phytochemicals, namely coumarins, saponins, flavonoids and anthocyanins, have inhibitory activities on CYP isoforms [55,56,57], these molecules can be classified as a weak inhibitor of cytochrome P450, since only one isoform was inhibited [58]. Still, in the AMES test, they showed toxicity that could lead to genotoxicity [59]. It is important to highlight that the analysis of these molecules did not demonstrate hepatotoxicity, as well as other toxicity parameters, such as LD50 and the level of no observed adverse effect, are also at acceptable levels. It is important to note that when multiple compounds within the extract interact to enhance each other's therapeutic properties, leading to more potent biological effects [60]. Furthermore, some polyphenols can increase the bioavailability or stability of others, increasing their overall effectiveness [61]. These data support the safe development of PPE studies.

7. Conclusions

The present investigation showed *in vitro* that PPE demonstrates antiproliferative activity in the glioblastoma model, with the synergistic effects of its combined compounds. *In vitro* it does not present significant toxic effects in considerably large volumes, and does not show signs of toxicity in the acute oral toxicity model or in the 28-day repeated dose model. Just as no

physical or biochemical changes were observed, which attests to the safety of PPE and its applicability in the treatment of diseases, its compounds corroborate its use as a new source of bioactive components, both as food and as a possible nutraceutical.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. CRediT: **Simone Pinton** Conceptualization; Methodology; Formal analysis; Resources; Writing - Review & Editing; Supervision; Project administration; Data Curation; Funding acquisition. **Eduarda M. Fidelis** Formal analysis; Validation; Investigation; Data Curation; Writing - Original Draft; Visualization; Project administration. **Anne S. P. Savall** Formal analysis; Investigation; Data curation. **Jhuly D. Mello** Formal analysis; Investigation; Data curation. **Cristiane C. Denardin** Extract synthesis; Writing - Review & Editing. **Fábio Klamt** Support in the development of cell studies; Writing - Review and Editing. **Victor Cortez** Formal analysis; Investigation; Data curation. **Jessica E. Batista** Formal analysis; Investigation; Data curation. **Suzan G. Rosa** Formal analysis; Investigation; Data curation; Writing - Review & Editing.

Notes

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5.3 Artigo Científico II

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ORIGINAL ARTICLE

Purple pitanga extract (*Eugenia uniflora*) attenuates oxidative stress induced by MPTP

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Abstract
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been widely used due to its specific and reproducible neurotoxic effect on the nigrostriatal system, being considered a convenient model of dopaminergic neurodegeneration to study interventions therapeutics. The purple pitanga (*Eugenia uniflora*) is a polyphenol-rich fruit with antioxidant and antidepressant properties, among others. Therefore, this study investigated the effect of purple pitanga extract (PPE) on acute early oxidative stress induced by intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration in rats. Male Wistar rats were pre-treated orally with PPE (1000 mg/kg) or vehicle. After 24 h, MPTP (0.1 mg/10μL/nostril) or vehicle was administered bilaterally into the animal's nostrils, and 6 h later, the olfactory bulb (OB), striatum (ST), and substantia nigra (SN) were collected to evaluate the oxidative stress parameters. Our findings revealed that OB and SN were the most affected areas after 6 h of MPTP infusion; an early increase in reactive oxygen species (ROS) levels was observed, while pretreatment with a single dose of PPE prevented this increment. No differences in thiobarbituric acid reactive species (TBARS) and 3-nitrotyrosine (3-NT) formation were observed, although 4-hydroxy-2-nonenal (4-HNE) levels increased, which is the most toxic form of lipid peroxidation, in the MPTP group. The PPE pretreatment could prevent this increase by increasing the NPSH levels previously decreased by MPTP. Furthermore, PPE prevents the Na+/K+ATPase strongly inhibited by MPTP, showing the neuroprotective capacity of the PPE by inhibiting the MPTP-generated oxidation. Thus, we demonstrated for the first time the antioxidant and neuroprotective effects of PPE against the early MPTP neurotoxicity.

Keywords Brazilian cherry · Antioxidant · Neuroprotection · Parkinson's Disease

Introduction

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a pro-neurotoxin that damages dopaminergic neurons and is largely used to model Parkinson's disease (PD) (Langston et al., 1983). MPTP is converted into the astrocytes into 1-methyl-4-phenylpyridinium ion (MPP^+), from where it can diffuse extracellularly and be selectively transported to dopaminergic neurons via the dopamine transporter, inducing inhibitory effects on mitochondrial complex I activity (Gainetdinov et al. 1997). Increased oxidative stress due to reduced mitochondrial activity is a common feature in neurodegenerative disorders and MPTP-induced models (Dauer and Przedborski 2003; Prediger et al. 2011; Zhu et al. 2019).

The single intranasal MPTP administration is a well-established Parkinson's disease (PD) model (Prediger et

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al. 2011). Its transport from the nose to the central nervous system (CNS) occurs through nerves, blood vessels, cerebrospinal fluid, and the lymphatic system, bypassing the blood-brain barrier (Dhuria et al. 2010). Importantly, intranasal MPTP infusion affects the degeneration in dopaminergic neurons and mitochondrial dysfunction, oxidative stress, and glutamatergic excitotoxicity in a time-dependet manner (Franco et al. 2007; Prediger et al. 2011; Marques et al. 2018). In this way, changes in the oxidative stress markers are more pronounced in the olfactory bulb (OB) and dopaminergic structures at 6 h (h) after intranasal MPTP administration compared to other evaluated time points (2 h to 7 days) (Franco et al. 2007). In this way, oxidative stress appears to act as the catalyst for the progression of PD (Abou-Sleiman et al. 2006). Many of the neurological changes exhibited by rats treated intranasally with MPTP are similar to those observed in the early stages of PD when there is not yet significant motor impairment (Prediger et al. 2011).

Indeed, elevated levels of oxidative stress have been observed in patients diagnosed with early-stages PD, preceding significant dopaminergic neuronal loss (Ferrer et al., 2011). Catecholamine-rich brain regions, such as dopaminergic areas, are favorable for free radical production (Mosley et al., 2006). Under normal conditions, monoamine oxidase regulates dopamine metabolism, although it can suffer auto-oxidation to produce H_2O_2 and dopamine quinones in pathological situations and aging (Saura et al. 1997). The dopaminergic metabolism can exacerbate oxidative stress, leading to inflammation and tissue damage (Dias et al. 2013). Therefore, oxidative stress is one of the main drivers of the degenerative cascade underlying all forms of PD (Dauer and Przedborski 2003; Blesa et al. 2015).

Although dopaminergic drugs are well-established for PD treatment, their therapy is compromised by side effects and reduced efficacy after long-term treatment (Zhang et al. 2019). Moreover, they only treat the symptoms, not the disease or its progression. Trials for potential new agents are often conducted after patients exhibit unmistakable motor symptoms, indicating that significant neurodegeneration has already taken place (Stoker & Barker, 2020). In recent years, many researchers have investigated the role of various natural products in treating PD, some of which have proved to be more reliable than typical synthetic drugs (Amro et al., 2018).

The pitanga or Brazilian cherry (*Eugenia uniflora*) is the fruit of Brazilian native tree cultivated in various subtropical countries and known for its antioxidant, anti-inflammatory, and neuroprotective properties, among others (Sardi et al., 2017; Fidelis et al. 2022). Notably, the purple variety of pitanga is rich in anthocyanins, flavonoids, and carotenoids (Lima et al. 2005). Studies suggest that metabolites such as phenolic compounds, flavonoids, anthocyanins and others present in fruits and plants play a preventive nutritional role by providing antioxidant protection to the body, mitigating oxidative stress caused by free radicals in cells (Hertog et al. 1992; Paganga et al. 1999; Rechner et al. 2002). Recently, our research group demonstrated that purple pitanga ethanolic extract (PPE) was able to block the effects of MPTP on memory impairment by modulating the BDNF pathway (Savall et al. 2023). Therefore, we investigated the protective effects of PPE on acute and initial cell damage induced by MPTP in different brain structures of rats.

Material and method

Animals

The analyses were carried out using three-month-old male Wistar rats (250–300 g) from the Federal University of Santa Maria (UFSM, Brazil) animal facility. The animals were kept in a room with a 12-h light/dark cycle (lights on at 7:00 am) at a room temperature of 22 ± 2 °C. All experimental procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications, 8th edition, 2011). Every effort was made to minimize animal suffering and reduce the number of animals used. The protocol was approved by the Committee for the Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil (Protocol nº. 010/2021).

Preparation of purple pitanga ethanolic extract

The species *Eugenia uniflora* was identified using the herbarium voucher ECT450, and samples of purple pitanga pulp were obtained from the Brazilian Agricultural Research Corporation - Embrapa (Pelotas, RS, Brazil) and immediately frozen. A pool of fruits collected in the 2018/2019 harvest was frozen at -18 °C and transported to the Universidade Federal do Pampa (Uruguaiana, RS, Brazil). We thawed the fruits, removed the seeds, and homogenized the pulp and rind. They were then homogenized with an ultra-turrax homogenizer for 5 min in 95% ethanol (1:3, w/v). The homogenates were mixed for 30 min in a flask protected from light at room temperature and centrifuged at 3000 rpm for 5 min; the supernatant was then collected, and the extraction procedure was repeated once more with the residue. The pooled supernatants were dried in a rotary evaporator at a maximum temperature of 40 °C. The samples were reconstituted in water and stored at -80 °C (Denardin et al. 2015; Tambara et al. 2018).

The PPE used has already been characterized and reported elsewhere (Tambara et al. 2018). The main active compounds identified are delphinidin 3-O-glucoside (99.65 ± 1.77 mg/100 g lyophilized fruit), pelargonidin 3-O-glucoside (2.16 ± 0.13 mg/100 g of lyophilized fruit), cyanidin 3-O-pentoside (0.83 ± 0.07/100 g of lyophilized fruit), cyanidin derivative (5.16 ± 1.23 mg/100 g of lyophilized fruit), and cyanidin 3-O-glucoside (512.01 ± 11.18 mg/100 g lof yophilized fruit) as the major compounds. The PPE dose of 1000 mg/kg was established based on a previous study by our research group (Savall et al. 2023) and on toxicological studies that demonstrated that this dose is safe for the animals in this protocol (data not shown).

Experimental design

The animals were randomly divided into four experimental groups (n = 7–9/group): (1) control, (2) MPTP, (3) PPE, and (4) PPE + MPTP. The control and MPTP groups received vehicle (0.9% NaCl, 3 mL/kg by intragastric gavage [i.g.]), while the PPE-treated groups received a 1000 mg/kg dose (i.g.). Twenty-four h after vehicle or PPE treatment, the rats were bilaterally infused (intranasally [i.n.]) with MPTP or saline, as described elsewhere (Prediger et al. 2006).

The animals were then briefly and lightly anesthetized with 0.96% isoflurane (0.75 CAM), and a polyethylene tube (PE 50) was introduced approximately 10 mm into the animal's nostril. Then, 10 µL of MPTP HCl (Sigma Chemical Co., USA) (1 mg/nostril) or vehicle (0.9% NaCl; 10 µL/nostril) was administered into each nostril of the animal at an infusion rate of 10 µL/min with a 10-µL microsyringe (Hamilton, USA) attached to an infusion pump (Insight, Brazil) (Marques et al., 2018; Sampaio et al., 2017). Six hours after MPTP administration, the animals were killed by decapitation, and their OB, striatum (ST), and substantia nigra (SN) were quickly dissected for biochemical experiments (Marques et al. 2018).

Oxidative stress parameters

Samples were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v). The homogenates were centrifuged at 2400 rpm for 10 min at 4 °C, and the low-speed supernatant fraction (S1) was used for the following analyses (except for the immunocontent of 4-hydroxy-2-nonenal [4-HNE] and 3-nitrotyrosine [3NT]).

Reactive oxygen species quantification

Reactive oxygen species (ROS) levels were quantified using a protocol outlined elsewhere (Ali et al. 1992). Oxidation of dichlorodihydrofluorescein diacetate to fluorescent dichlorofluorescein (DCF) was measured to determine intracellular ROS production. The results were expressed as arbitrary units (AU) of DCF's fluorescence.

Thiobarbituric acid reactive species formation

Thiobarbituric acid reactive species (TBARS) formation was measured using a previously developed method to determine lipid peroxidation levels (Ohkawa et al. 1979). The tissue supernatant was briefly mixed with 15% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS was reported in nmol TBARS/mg of protein. The protein content in sample homogenates was quantified as demonstrated elsewhere using bovine serum albumin (1 mg/mL) as a standard (Bradford 1976).

Immunocontent of 4-HNE and 3NT

The tissues were homogenized on ice in tissue protein extraction reagent T-PER (Thermo Fisher Scientific, Massachusetts, USA) supplemented with EDTA and phosphatase v Cocktail (Thermo Fisher Scientific, Massachusetts, USA). The supernatant was obtained after centrifuging at 12,000 rpm at 4 °C for 10 min. To avoid binding in non-specific sites, nitrocellulose membranes were blocked by soaking in 5% (w/v) non-fat dry milk for 60 min before antibodies incubation. The immunocontent of 4-HNE and 3NT was determined by the dot-blot technique according to Da Costa Sobral et al. (2021) using the primary antibodies for 4-HNE (1:1000, Sigma-Aldrich, #SAB5202472), 3-NT (1:200, Thermo Fisher Scientific, #A-21,285), and peroxidase-linked secondary antibody (anti-mouse 1:5000, Santa Cruz Biotechnology, #sc-2005; anti-rabbit 1:10000, Sigma-Aldrich, #A0545). Immunoreactivity was detected by Clarity ECL (Bio-Rad) in a ChemiDoc™ MP Image System (Bio-Rad) and measured using the ImageJ software. The 4-HNE levels were expressed as % of control.

Non-protein thiols levels

The non-protein thiol (NPSH) levels were determined as described elsewhere (Ellman, 1959). S1 was mixed (1:1) with 10% trichloroacetic acid to determine NPSH. After centrifugation, the protein pellet was discarded, and the free -SH groups were determined in the clear supernatant. An aliquot of the supernatant was added in 1 M potassium phosphate buffer, pH 7.4, and 10 mM DTNB. The color reaction was measured at 412 nm, and the NPSH levels were expressed as mol NPSH/g tissue.

Sodium-potassium pump activity

The Sodium-potassium pump (Na^+/K^+ATPase) activity according to a previous study (Fiske and Subba, 1925). Briefly, 3 mM MgCl, 125 mM NaCl, 20 mM KCl, and 50 mM Tris-HCl pH 7.4 were used in a final volume of 500 mL. The reaction was initiated by adding ATP to a final concentration of 3.0 mM. To obtain the ouabain-sensitive activity, the samples were made under the same conditions with the addition of 0.1 mM of ouabain. The samples were incubated at 37 °C for 30 min, and the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. The Na^+/K^+ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain). Released inorganic phosphate (Pi) was measured spectrofluorimetrically at 650 nm, and Na^+/K^+ATPase activity was expressed as nmol Pi/mg protein/min. The protein content was quantified according to Bradford (1976).

Statistical analysis

Results are expressed as mean ± SD standard deviation (SD). Comparisons between groups were performed by two-way analysis of variance (ANOVA), followed by Tukey's test when appropriate. Pearson's correlation was applied to associate the different variables developed with exposure to MPTP. Probability values below $p < 0.05$ were considered statistically significant. Statistical analyzes were performed using GraphPad Prism 8 software (San Diego, CA, USA).

Results

To access the putative antioxidant effect of PPE on the MPTP-induced oxidative stress, ROS, TBARS, 3-NT, 4-HNE, and NPSH levels were measured. Regarding ROS levels, two-way ANOVA revealed a significant effect of MPTP in increasing ROS generation in SN homogenates (~85%) ($F_{(1, 24)} = 9.334$; $p = 0.005$) (Fig. 1C); however, this was not completely prevented by PPE treatment. In addition, ROS levels were not altered by MPTP or PPE treatments in the OB ($F_{(1, 24)} = 0.400$; $p = 0.533$) (Fig. 1A) and ST ($F_{(1, 24)} = 0.0509$; $p = 0.823$) (Fig. 1B).

The TBARS levels and 4-HNE immunocontent were also used as lipid peroxidation markers in different rat brain structures. No changes were found in TBARS levels in the OB, ST, or SN (Table 1). Nevertheless, 4-HNE immunocontent analyses revealed alterations in the three structures (Fig. 2). Two-way ANOVA analysis showed a significant MPTP × PPE interaction ($F_{(1, 24)} = 7.183$; $p = 0.0131$) in OB (Fig. 2A). *Post-hoc* comparisons showed that MPTP

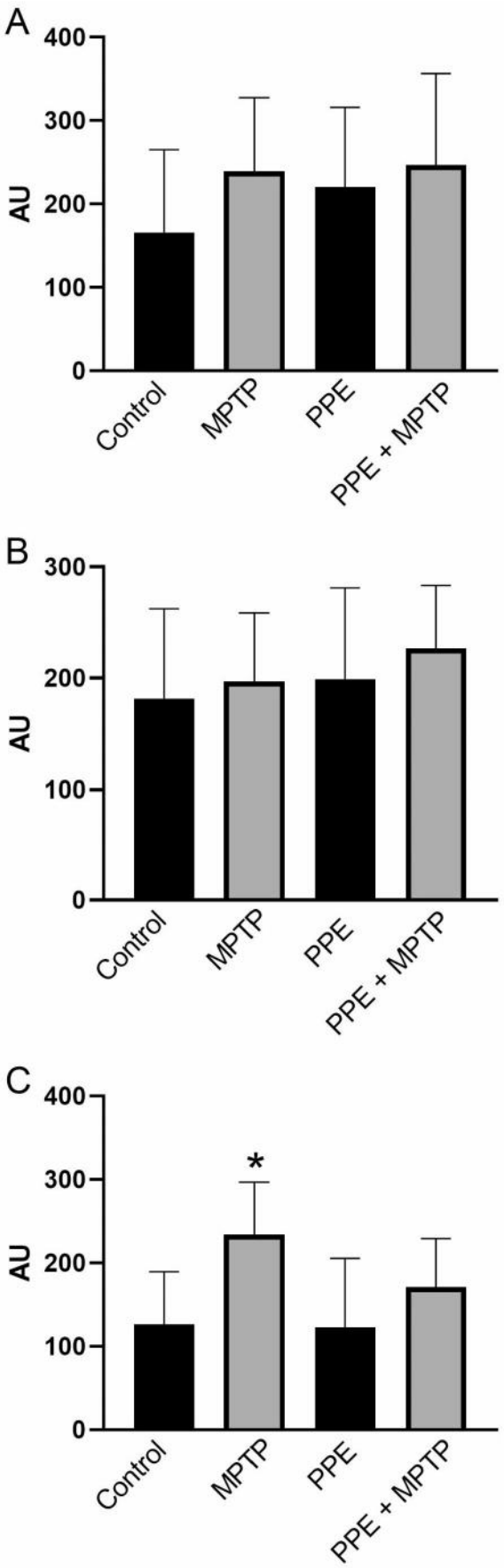

Fig. 1 Effect of prior PPE administration on reactive oxygen species (ROS) levels in rats subjected to intranasal MPTP infusion. **(A)** Olfactory bulb, **(B)** striatum (ST), and **(C)** substantia nigra. Data are presented as mean ± SD (n = 7). *$p < 0.05$ compared to the control group (two-way ANOVA followed by Tukey's test)

increased 4-HNE levels (~56%) ($p = 0.0139$), while PPE pretreatment prevented the MPTP-induced increase in OB ($p = 0.0087$). Two-way ANOVA analysis revealed a significant main effect of MPTP ($F_{(1, 24)} = 8.900$; $p = 0.0065$) on 4-HNE in the ST (Fig. 2B), a decrease in 4-HNE levels was observed in the ST of the MPTP group when compared to the control group ($p = 0.0168$) (Fig. 2B). In addition, a significant main effect of PPE ($F_{(1, 24)} = 5.978$; $p = 0.022$) on 4-HNE levels was found in the SN (Fig. 2C), the levels of 4-HNE were increased in rats of the PPE + MPTP group when compared to those in the control group in the ($p = 0.0350$) (Fig. 2C).

In this way, 3-NT immunocontent — a product of tyrosine nitration — was measured in OB, ST, and SN as an indirect marker of nitrogen reactive species presence, such as peroxynitrite anion and nitrogen dioxide (Da Costa Sobral et al. 2021). However, data analysis of 3-NT levels demonstrated that MPTP infusion and/or PPE treatment did not alter 3-NT levels in all brain structures evaluated (Table 1).

Two-way ANOVA of NPSH levels, an indicator of the antioxidant status, yielded a significant MPTP × PPE interaction ($F_{(1, 24)} = 12.35$; $p = 0.0018$) (Fig. 3A). The prior administration of PPE prevented NPSH depletion in the OB in MPTP-infused rats (~40%) ($p = 0.0053$ when comparing MPTP to PPE + MPTP groups) (Fig. 3A). There was no difference for striatal NPSH level among groups (Fig. 3B). Nevertheless, a significant MPTP × PPE interaction ($F_{(1, 24)} = 4.438$; $p = 0.0458$) was observed in SN. MPTP administration increased NPSH levels in the SN of rats ($p = 0.0051$) and the pretreatment with PPE in blocking this effect ($p = 0.043$) (Fig. 3C).

As for the Na^+/K^+ATPase activity (Fig. 4), two-way ANOVA showed a significant MPTP × PPE interaction in the OB ($F_{(1, 30)} = 5.491$; $p = 0.0259$) and in the SN ($F_{(1, 30)} = 6.398$; $p = 0.0171$). The intranasal MPTP infusion significantly reduced the Na^+/K^+-ATPase activity in the OB (~90%) ($p = 0.0169$), and the PPE pretreatment attenuated the Na^+/K^+-ATPase inhibition induced by intranasal MPTP administration in the OB ($p = 0.0034$) (Fig. 4A). Similarly, MPTP inhibited the Na^+/K^+ATPase activity in the SN (~65%) ($p = 0.0035$), and PPE attenuated this nigral change ($p = 0.0214$) (Fig. 4C). No significant differences were observed in the striatal Na^+/K^+ATPase activity ($F_{(1, 30)} = 1.879$; $p = 0.1806$) (Fig. 4B).

Lastly, in order to shed more light on how intranasal MPTP administration inferred with multiple variables that are similar or different from each other, a correlation analysis was performed to infer potential relationships. The 4-HNE levels correlated negatively with Na^+/K^+ATPase activity in the OB homogenate ($r = -0.7718$, $p = 0.0033$) (Fig. 5A), and a significant negative correlation was observed in the SN ($r = 0.5620$, $p = 0.0456$) (Fig. 5B) in the ratio between ROS levels and Na^+/K^+ATPase activity.

Discussion

Little is known about the neurodegenerative mechanisms in PD, albeit evidence has pointed to oxidative stress being a key factor in the complex degenerative cascade underlying dopaminergic loss (Blesa et al. 2015; Jin et al. 2018; Kumar and Kurup 2002). Oxidative stress arises from cellular redox activity dysregulation, in which ROS production outweighs the clearance by endogenous antioxidant defenses (Trist et al. 2019). Moreover, the brain is susceptible to oxidative damage due to its high oxygen consumption and lipid abundance (Stefanatos and Sanz 2017).

The single i.n. administration of MPTP is a well-established model of PD (Prediger et al. 2011), which results in neurochemical damage patterns similar to those seen in the PD patients (Schildknecht et al., 2017; Bloem, Okun, Klein, 2021). Franco et al. (2007) observed time-dependent alterations in oxidative stress markers in the brain of rats 6, 12, 24, and 168 h after a single intranasal MPTP infusion (1 mg/nostril). Such changes were mainly observed in the OB

Table 1 Effect of prior PPE administration on thiobarbituric acid reactive substance (TBARS) and 3-nitrotyrosine (3-NT) levels in the olfactory bulb (OB), striatum (ST), and substantia nigra (SN) of rats subjected to intranasal MPTP infusion

	TBARS[a]			3-NT[b]		
	OB	ST	SN	OB	ST	SN
Control	256.8 ± 81.4	240.2 ± 63.7	200.6 ± 56.8	100.0 ± 5.5	100.0 ± 9.7	100.0 ± 16.2
MPTP	208.8 ± 66.3	161.5 ± 49.8	250.9 ± 99.8	103.2 ± 6.1	102.5 ± 9.1	96.0 ± 5.3
PPE	261.6 ± 50.0	174.4 ± 42.1	260.6 ± 76.7	103.6 ± 17.7	96.2 ± 8.2	90.9 ± 11.5
PPE + MPTP	250.9 ± 107.3	215.0 ± 143.8	238.8 ± 87.2	108.4 ± 22.5	93.1 ± 10.0	101.8 ± 11.4
Two-way ANOVA Interaction results	$F_{(1, 24)} = 0.389$; $p = 0.538$	$F_{(1, 24)} = 3.436$; $p = 0.076$	$F_{(1, 24)} = 2.365$; $p = 0.254$	$F_{(1,24)} = 0.0173$; $p = 0.896$	$F_{(1,24)} = 0.635$; $p = 0.433$	$F_{(1,24)} = 0.0175$; $p = 0.895$

[a]Values expressed in nmol TBARS/mg of protein. [b]Values of 3-NT immunocontent were expressed as % of control

Data are presented as mean ± SD (n = 7)

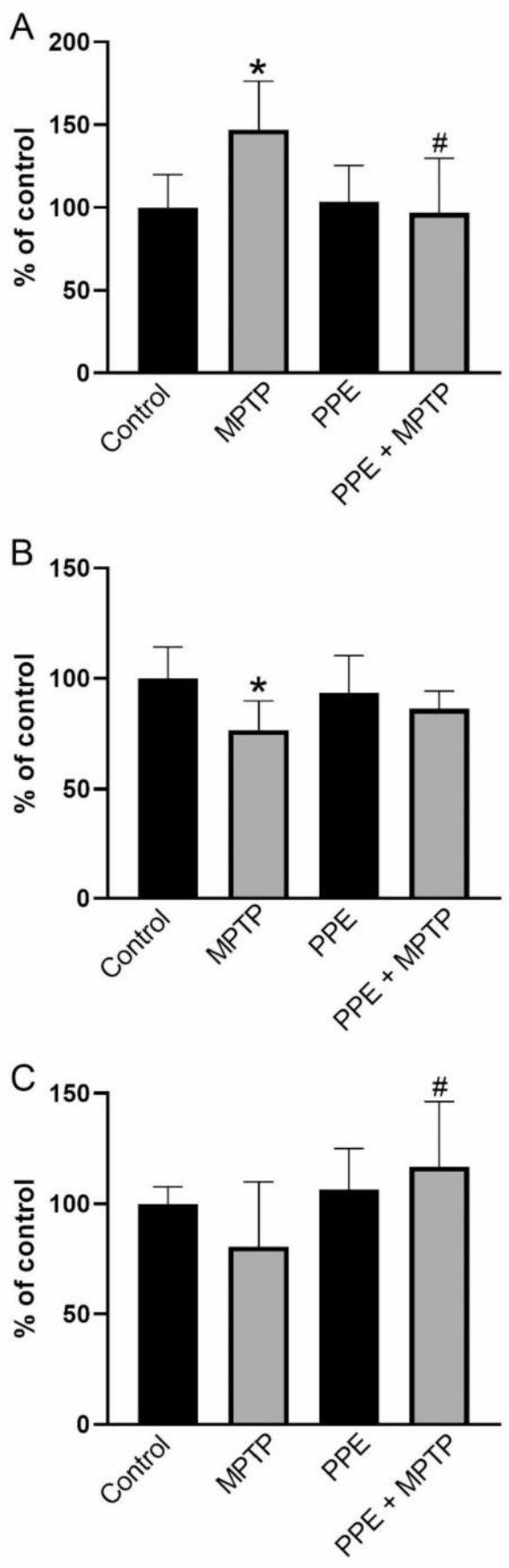

Fig. 2 Effect of prior PPE administration on 4-hydroxyl-2-nonenal (4-HNE) levels in rats subjected to intranasal MPTP infusion. **(A)** Olfactory bulb, **(B)** striatum (ST) and **(C)** substantia nigra. Data are presented as mean ± SD (n = 7). *$p < 0.05$ compared to the control group. #$p < 0.05$ compared to the MPTP group (two-way ANOVA followed by Tukey's test)

6 h after MPTP administration (Franco et al. 2007). Additionally, Marques et al. (2018) found early striatal MPTP toxicity due to increased ROS generation, imbalanced antioxidant defenses, and reduced mitochondrial respiratory chain complex I activity 6 h after intranasal administration. In fact, MPTP-treated mice showed significant ATP depletion within 2 h of administration in both the ST and SN (Jiao et al. 2015), indicating that MPTP can quickly inhibit the electron transport chain. Thus, oxidative stress appears to be one of the primary events in the MPTP neurotoxicity mechanism, although it may not be directly responsible for the death of dopaminergic neurons (Prediger et al. 2011).

Given that MPTP-treated rodents show oxidative stress in different brain structures at 6 h after administration and in order to find new therapeutic approaches for early intranasal MPTP-induced toxicity, this study demonstrated, for the first time, that a single pre-dose of PPE protect rats from MPTP-induced oxidative stress in the SN, ST, and OB. Our findings revealed that oxidative changes are more pronounced in the OB and SN in the early stage of MPTP neurotoxicity when administered intranasally. In fact, the initial toxic effects of MPTP occur in areas with a major accumulation of MPP^+ (Kadar et al. 2014). Therefore, it is plausible that the OB, which is the target structure of MPTP administration, is more affected, as well as the SN through the nigro-olfactory projection (Höglinger et al. 2015).

Nonetheless, MPTP decreased striatal 4-HNE levels and increased 4-HNE immunocontent in the OB. The PPE treatment prevented the MPTP-caused increase in the olfactory 4-HNE levels. Notwithstanding, it is important to emphasize that PPE pretreatment also prevented NPSH alterations in the OB and SN, highlighting its antioxidant potential against MPTP-induced oxidative stress. The antioxidant effect of PPE may be related to the presence of quercetin, given that research has shown that it can decrease striatal 4-HNE immunoreactivity and increase the activities of antioxidant enzymes in an MPTP model (Lv et al. 2012).

4-HNE, one of the end-products of lipid peroxidation, is highly reactive and can induce cytotoxicity (Zheng et al. 2014). Indeed, the increased 4-HNE production alters dopamine transport, contributing to dopamine loss and, consequently, PD pathogenesis (Di Domenico et al. 2017). The physiological effects of 4-HNE depend on its intracellular concentration and enzyme metabolization, which are carried out by alcohol dehydrogenase, aldehyde dehydrogenase, aldo-keto reductases, cytochrome P450s, and glutathione

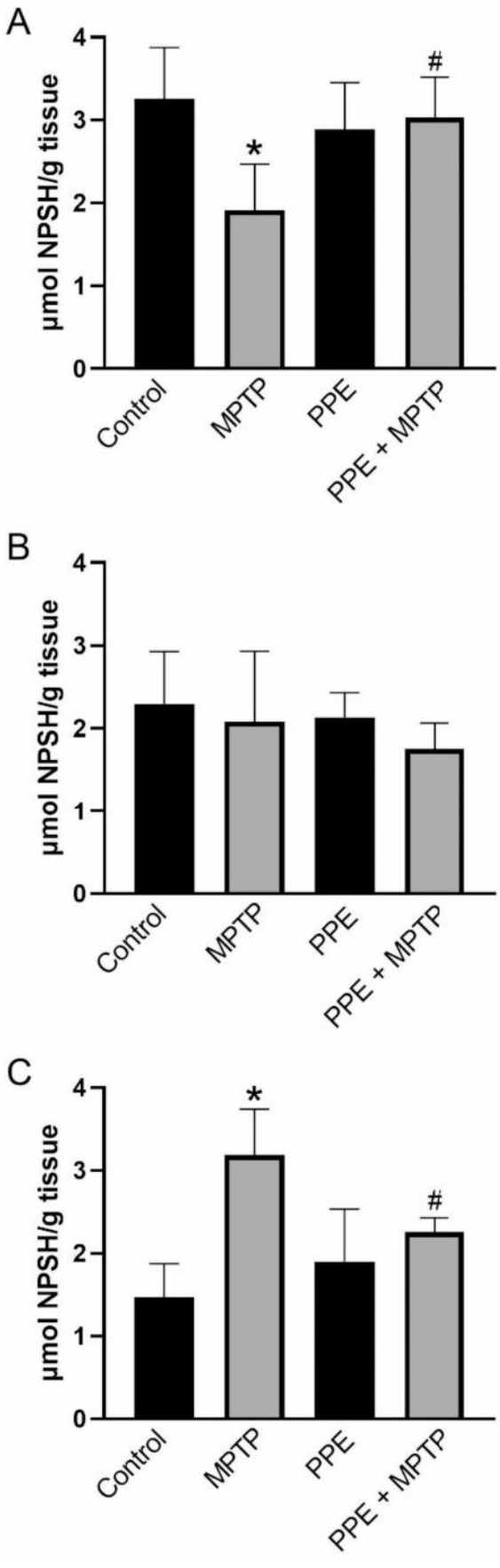

Fig. 3 Effect of prior PPE administration on non-protein thiol (NPSH) levels in rats subjected to intranasal MPTP infusion. **(A)** Olfactory bulb, **(B)** striatum (ST), and **(C)** substantia nigra. Data are presented as mean ± SD (n = 7). $^{*}p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the MPTP-treated group (two-way ANOVA followed by Tukey's test)

S-transferases (Zheng et al. 2014). Thus, since 4-HNE can be metabolized and detoxified by cells, it is plausible that glutathione S-transferase may act on 4-HNE and GSH conjugation in the OB, which may also explain the lower NPSH levels induced by MPTP in the olfactory region. Notably, NPSH (GSH mostly) may be the first barrier against ROS, thereby being essential for cell survival (Halliwell 2001).

Regarding the increase in 4-HNE levels in the SN in the MPTP + PPE group, this result suggests a synergistic effect of the administrations. It is possible that the dose used in this study provided a high concentration of compounds that could contribute to the oxidation of macromolecules. This is because the pro-oxidant or antioxidant activity of natural compounds, such as flavonoids, depends on the redox state of their biological environment (Skibola and Smith 2000).

In addition to 4-HNE, malondialdehyde (MDA) is another secondary product of lipid peroxidation (Morales et al., 2019; Lv et al. 2012) and, although we observed variations in 4-HNE levels in different brain structures induced by MPTP, we did not detect significant differences in TBARS levels among the groups. We believe that some factors may have contributed to the lack of treatment effects in this assay, including the chosen time window for marker analysis and the sensitivity of the employed methodology. The TBARS method is a colorimetric technique used to detect thiobarbituric acid-reactive substances (TBA), including MDA and other aldehydes. While the TBARS method is widely accepted as a marker for lipid damage, it does not exclusively detect MDA, and other compounds can potentially limit this quantification (Morales et al., 2019).

The role of ROS in mediating MPTP neurotoxicity remains unclear. It is known that the hemostasis of the GSH antioxidant system is important for the detoxification of exogenous and endogenous substances. Levels of GSH in the CNS are altered after administration of MPTP in rats (Dringen et al. 2000). Franco et al. (2007) demonstrated a significant increase in total GSH levels in the OB, ST and hippocampus after 6 h of MPTP infusion, but this was not observed at 2 and 24 h. Corroborating these results, Prediger and colleagues demonstrated that within 6 h, it was possible to observe that the MPTP disrupts GSH homeostasis and induces peroxidative damage in the rat brain. Furthermore, structures such as the OB, ST, hippocampus, and prefrontal cortex, exhibited significant changes in ROS levels (Prediger et al. 2011). In the present study, we observed a significant decrease in NPSH levels in the olfactory bulb

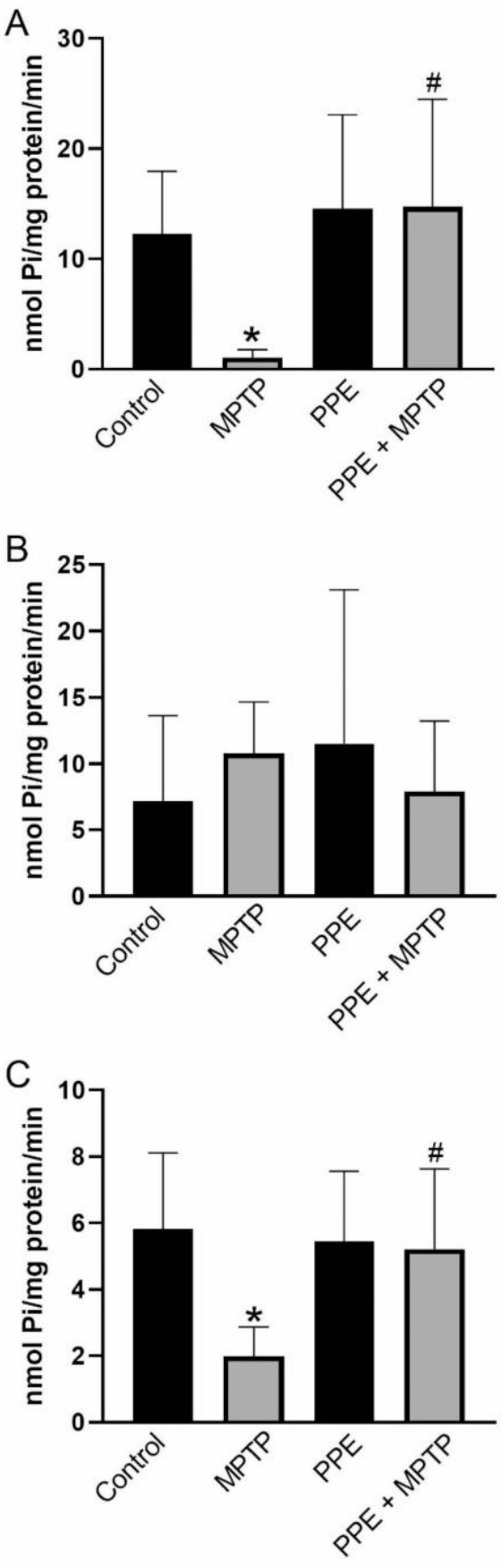

Fig. 4 Effect of previous PPE administration on Na+K+ATPase activity in rats subjected to intranasal MPTP infusion. **(A)** Olfactory bulb, **(B)** striatum (ST), and **(C)** substantia nigra. Data are presented as mean ± SD (n = 8–9). *$p < 0.05$ compared to the control group. #$p < 0.05$ compared to the MPTP-treated group (two-way ANOVA followed by Tukey's test)

(OB) and an increase in NPSH levels in the substantia nigra (SN) in the MPTP group. These changes were prevented by PPE administration. Just as increased NPSH levels can suggest an adaptive response to the rise in ROS (Li et al., 2017), decreased NPSH levels may indicate a reduction in the cell's antioxidant capacity to combat the oxidative stress (Brandão et al. 2009). It's evident that different brain structures exhibited distinct responses to the neurotoxin MPTP. The variation in oxidative stress responses in different brain structures is due to the complex interplay between functional characteristics, lipid composition, neuronal density, metabolic demands, and the specific antioxidant capacity of each brain region (Olufunmilayo et al. 2023). For instance, the hippocampus, amygdala, prefrontal cortex, and cerebellar granular cells are considered to be the brain structures most susceptible to oxidative stress (Wang and Michaelis 2010).

Moreover, our study demonstrates, for the first time, the effect of intranasal MPTP administration in reducing Na^+/K^+ATPase activity in the OB and SN. Na^+/K^+ATPase plays a crucial role in maintaining cellular homeostasis and is an ion pump necessary for neural excitability and neurotransmission (Xie and Askari 2002), although its reduced activity has been associated with aging (Cao et al. 2021). Na^+/K^+ATPase is a sulfhydryl enzyme sensitive to oxidative stress, meaning ROS may modify the enzyme structure, inhibiting its activity and promoting its degradation (Liu et al., 2018). In fact, a negative correlation between 4-HNE levels and Na^+/K^+ATPase activity in the OB and ROS levels and Na^+/K^+ATPase activity in the SN reinforces the vital role of the oxidative pathway in Na^+/K^+ATPase function. Lastly, it is noteworthy that the PPE attenuated/prevented the exacerbated production of ROS/4-HNE in the SN and OB, which could prevent Na^+/K^+ATPase activity inhibition. These results corroborate the study by Lv and collaborators (2012), who demonstrated that MPTP intraperitoneal administration inhibited Na^+/K^+ATPase activity and that pretreatment with quercetin increased this enzyme's activity in the mouse brain tissue.

PPE is rich in flavonoids, which are substances with a high antioxidant capacity (Celli et al. 2011). Cyanidin 3-O-glycoside, an anthocyanin, is a major compound of PPE and possibly related to its antioxidant effect (Tambara et al. 2018). This compound is promising for developing new drugs targeting a neuroprotective mechanism since the formation of free radicals and oxidative processes are

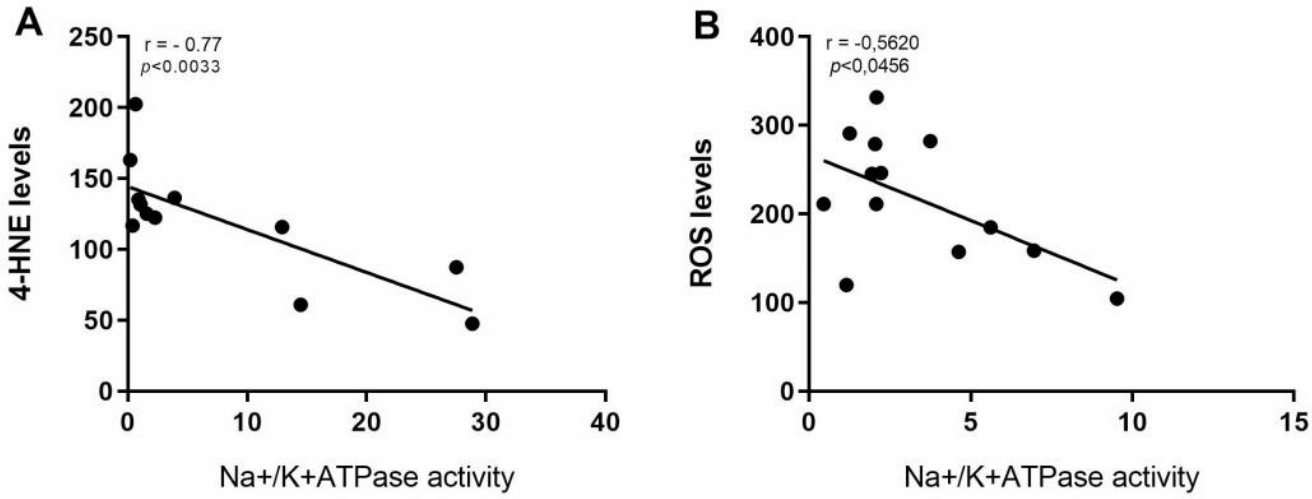

Fig. 5 Correlation between 4-HNE/ROS levels and Na^+/K^+ATPase activity after PPE and MPTP treatments. Each dot represents one pair comprising the **(A)** 4-HNE immunocontent and Na^+/K^+ATPase activity obtained in the olfactory bulb, and **(B)** ROS levels and Na^+/K^+ATPase activity in the substantia nigra from the same animal (Pearson's correlation)

included in the genesis of various degenerative diseases (Fidelis et al. 2022). In addition, anthocyanin- and proanthocyanidin-rich botanical extracts are known to alleviate neurodegeneration in cellular models of PD by enhancing mitochondrial function (Strathearn et al., 2014). Regarding this, it is possible that these compounds could soon serve as complementary and/or preventive therapies to delay the progression of PD. However, it is important to highlight, for proper data interpretation, that the reported data refer to a prior treatment with PPE. PPE prevents the onset of oxidative stress following MPTP administration, potentially leading to a decrease or delay in neurotoxicity induced by MPTP. Another hypothesis, which requires more detailed studies for confirmation, is that PPE might directly interfere with MPTP metabolism, thus inhibiting the production of MPP + and, consequently, its neurotoxic cascade.

Conclusion

Our findings demonstrate that intranasal MPTP induces a redox imbalance and that these disturbances affect the evaluated structures differently, likely caused by time variations, as reported elsewhere. Furthermore, this study showed that the pretreatment with PPE prevented the early effects of intranasal MPTP administration. The OB, the target structure of MPTP delivery, and the SN, the main dopaminergic structure affected in early PD, showed the most prominent responses toward the harmful effects of MPTP. Interestingly, a single administration of PPE prevented MPTP-induced redox shifts, including the effect on Na^+/K^+ATPase activity inhibition. These neuroprotective effects may be related to a plethora of compounds contained in the PPE, especially the presence of anthocyanin cyanidin-3-O-glycoside, the major compound in the extract.

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PARTE III

6. DISCUSSÃO GERAL

A OMS define plantas medicinais como espécies vegetais das quais podem ser obtidos produtos terapêuticos para uso humano (OMS, 2024) e, mesmo que o uso de plantas e frutos no tratamento de enfermidades seja uma prática milenar, o interesse por fitoterápicos aumentou significativa e globalmente nas últimas décadas (Dutra *et al*., 2016). Estudos diversos têm abordado o uso de produtos naturais na prevenção e tratamento de doenças (Pohl e Lin, 2018) e neste contexto, esta tese explorou o potencial farmacológico e toxicológico do EPR e os efeitos sobre as alterações oxidativas induzidas em diferentes estruturas do cérebro quando exposto a administração intranasal de MPTP, uma neurotoxina que induz neurodegeneração dopaminérgica (Ferrucci *et al*., 2021).

Primeiramente realizamos uma revisão narrativa sobre as propriedades e os efeitos da *Eugenia uniflor*a em diferentes contextos, coletamos dados em artigos publicados de 1976 a 2021, os quais estão compilados no **Artigo I**. Com os dados expostos neste artigo, foi possível observar que diferentes partes da *Eugenia uniflor*a, que incluem folhas e frutos, apresentaram propriedades farmacológicas interessantes, como antioxidante, anti-inflamatória, anti-hipertensiva, anti-microbiana, antiparasitária e antidiarreica (Almeida *et al*., 1995; Consolini *et al*., 1999; Victoria *et al*., 2012; Denardin *et al*., 2014; Soares, 2014). Nestes estudos, pode-se concluir que as propriedades farmacológicas das diferentes partes da *Eugenia uniflor*a devem-se, principalmente, pela sua propriedade antioxidante.

A pitanga roxa é a variedade mais rara dos frutos da pitangueira, mas possui maior quantidade de compostos fenólicos totais e, portanto, maiores propriedades antioxidantes do que outros tipos de pitanga (Weyerstahl *et al*., 1988; Celli *et al*., 2011; Denardin *et al*., 2015). A pitanga roxa mostrou capacidade antioxidante significativa em estudos in *vitro* e in *vivo*, esses resultados foram atribuídos a compostos como quercetina, mircetina, isoquercitrina e derivados de cianidina (Denardin *et al*., 2015; Tambara *et al*., 2018). Os resultados encontratos se relacionam com a presença de antocianinas, das quais a cianidina-3-O-glicosídeo é o principal composto presente na pitanga roxa edemonstra o mecanismo protetor contra processos oxidativos no organismo.

O potencial antiinflamatório do suco da polpa de pitanga roxa também foi relatado em células epiteliais gengivais, atenuando a liberação de IL-8 (Interleucina-8) em células não estimuladas e estimuladas por prostaglandina (Soares 2014). Além disso, importantes constituintes presentes no suco, como cianidina-3-glicosídeo e oxidoselina-1,3,7(11)-trien-8-ona, foram capazes de reduzir a expressão do mRNA de CXCL8 pelas células HGF-1 e a

liberação de IL-8, revelando assim o potencial antiinflamatório do composto volátil oxidoselina-1,3,7(11)-trien-8-ona.

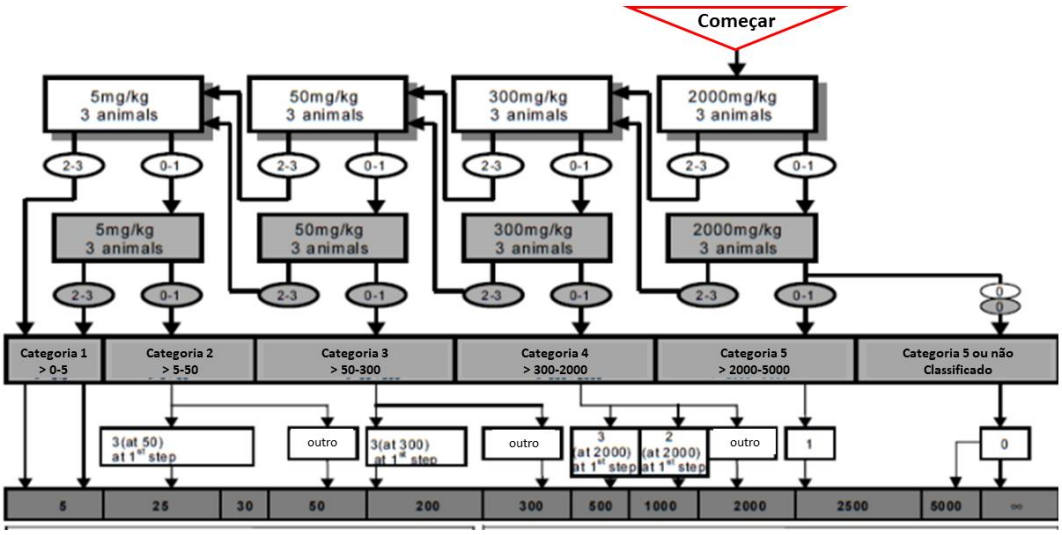
O EPR, o qual foi obtido pelo mesmo método e das mesmas árvores do extrato usado neste estudo, revelou a presença de cinco antocianinas: delfinidina 3-O-glicosídeo (99,65±1,77mg.100g $^{-1}$ fruta liofilizada), cianidina 3-O-glicosídeo (512,01 ± 11,18 mg 0,100g $^{-1}$ de fruta liofilizada), pelargonidina 3-O-glicosídeo (2,16 ± 0,13 mg,100g $^{-1}$ de fruta liofilizada), cianindina-3-O-pentosídeo (0,83 ± 0,07) e derivado de cianidina (5,16±1,23mg.100 g $^{-1}$ fruta liofilizada) (Tambara *et al*., 2018). Estas antocianinas parecem ser muito estáveis após o congelamento, ao contrário de outros constituintes das plantas (Lima *et al*., 2005).

Em relação ao perfil toxicológico do EPR, na presente tese, demonstramos no **Manuscrito I** que a exposição ao EPR em células C6 (glioma) induziu morte celular a partir de 125 µg CAE/mL em 24 horas. Estudos anteriores *in vitro* mostraram que o EPR diminuiu a viabilidade de células estreladas hepáticas ativas, tratadas com 50 e 100 µg/ml por 48 e 72 horas (Denardin *et al*., 2014), apresentando efeitos antiproliferativos e citotóxicos. Ainda, o EPR, em concentrações entre 5 a 100 µg/mL, apresentaram um aumento na expressão de mRNA da proteína 7 relacionada à autofagia nestas mesmas células (Denardin *et al*., 2017). Esses dados sugerem que os compostos presentes no EPR, podem fazer o extrato ser potencialmente útil na elaboração de drogas antineoplásicas (quimiopreventivo). Outros estudos demonstraram os efeitos antioxidantes, antiproliferativos e citotóxicos de compostos naturais incluindo quercetina, miricetina, ácido gálico e cianidina-3-glicosídeo (Kawada *et al*. 1998; Li *et al*., 2009; Sharma *et al*., 2022; Chen et a., 2005; Abdullah *et al*., 2023).

No **Manuscrito I** também demonstramos que, baseados no guia 423 da OECD para toxicidade oral aguda, uma única dose de 2.000mg/kg do EPR foi administrada em ratas fêmeas. Em 24 horas após a administração do EPR, bem após 14 dias, foi possível determinar que uma única dose do EPR não gerou sinais toxicológicos nem mortalidade. No teste de campo aberto não foram observados efeitos do EPR na função motora e atividade exploratória das ratas. O teste de campo aberto é um modelo clássico utilizado para investigar a atividade exploratória e comportamento emocional de roedores (Pellow *et al*., 1985; Prut e Belzung, 2003). Na mesma dose, Savall e colaboradores (2023) não observaram toxicidade do extrato ao longo de 14 dias de administração, ainda a atividade locomotora não foi afetada. O EPR não interferiu no consumo de água e ração, não mostrou diferença no peso dos animais, não havendo diferença no peso dos órgãos e análises bioquímicas. Assim, a dose letal estimada (LD50) do EPR foi considerada superior a 2.000 mg/kg, sendo classificada como uma droga segura, de categoria 5

ou sem classificação (Fig.6).

Fig 6. - Procedimento de teste com dose inicial de 2.000 mg/kg de peso corporal.



Fonte: Adaptado de OECD 423.

Para o estudo de toxicidade oral subcrônica, utilizou-se o guia OECD 407, onde a administração do EPR foi realizada por 28 dias, na dose de 1.000 mg/kg/dia, os sinais de toxicidade foram avaliados logo após o término do tratamento (24hs) e também tardiamente (14 dias). Os resultados coletados nestes protocolos sugerem que o uso repetido (28 dias) do EPR a 1.000 mg/kg não induz efeitos tóxicos significativos. De acordo com os critérios do G*lobally Harmonized Classification System*, o extrato pertence à classe 5, apresentando baixíssima toxicidade aguda ou atividade atóxica. Nossos resultados pela primeira vez sugerem que a exposição prolongada ao EPR não teve efeito tóxico *in vivo*, portanto, esta abordagem amplia o perfil de segurança do extrato e indica o potencial de componentes seguros que podem ser úteis para o desenvolvimento de novos medicamentos.

A interação entre farmacocinética, toxicidade e potência é crucial para medicamentos eficazes (Pires *et al*., 2015). Encontramos na modelagem computacional informações sobre quatro compostos presentes no EPR, cianidina 3-O-glicosídeo, miricetrina, quercetina e ácido gallico (Tambara *et al*., 2018). A modelagem sugere que os compostos apresentam alta solubilidade em água, dentre eles, o cianidina-3-O-glicosídeo apresenta maior percentual de absorção intestinal, de fato, os glicosídeos das antocianinas sofrem absorção rápida no intestino delgado, que é seguida por rápido metabolismo e excreção na bile e na urina, tanto na forma intacta quanto em derivados metabolizados (Kumkum *et al*., 2024). A quercetina também

apresentou um percentual elevado de solubilidade.

No entanto, a mircetina e a queratina são substratos para a glicoproteína-P, uma proteína da família de transportadores ABC, que atua como barreira fisiológica uma vez que expulsar toxinas e xenobióticos para o exterior das células (Lakshmie e Srivall., 2012, Sharom, 2011), localizada em vários tecidos, incluindo aqueles que revestem o intestino e a BHE (Reis *et al*., 2015), o que pode contribuir para uma menor disponibilidade destes compostos.

O processo de absorção é influenciado por propriedades físico-químicas da substância como por exemplo, a solubilidade, tamanho de partículas dos fármacos assim como o estado fisiológico e patológico (Leblanc *et al*., 2004). A distribuição da mircetina é a maior entre as moléculas analisadas, o que significa que ela tem maior distribuição nos tecidos corporais, enquanto a permeabilidade da BHE e do SNC é baixa nas moléculas aqui estudadas. A distribuição de fármacos é limitada pela permeabilidade capilar em cada tecido, a qual é determinada pela estrutura dos capilares e pela natureza química do fármaco (Paul, 2019).

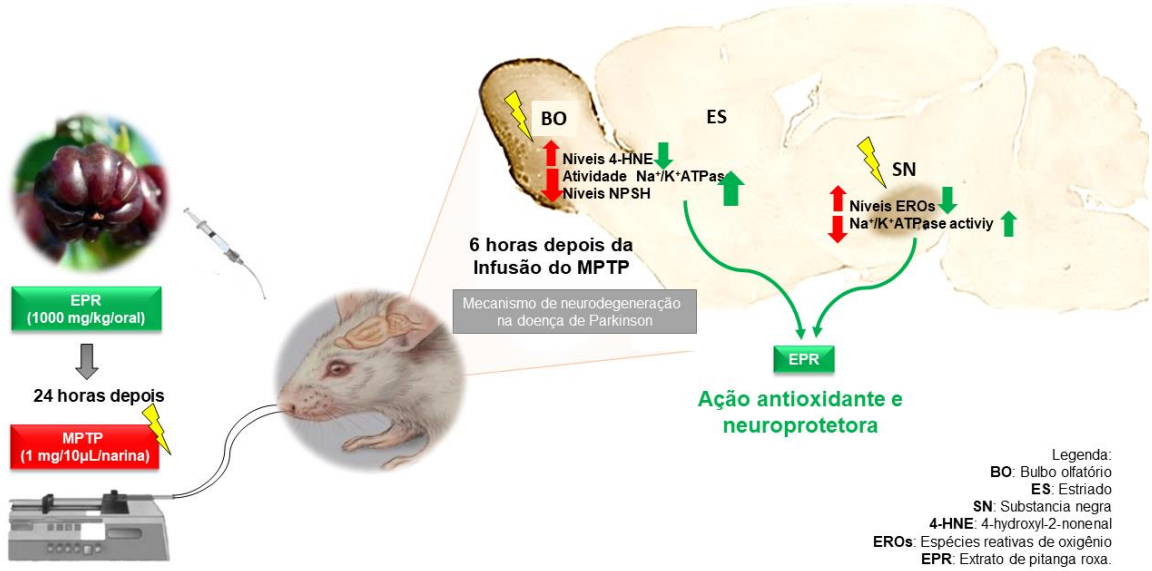
Segundo os dados computacionais, a enzima CYP1A2 poderia ser inibida pela cianidina-3-O-glicosídeo e quercetina, e sua inibição poderia resultar na diminuição da biotransformação dos medicamentos por ela metabolizados. Estudos *in vitro* demonstraram que alguns fitoquímicos, nomeadamente cumarinas, saponinas, flavonóides e antocianinas, têm atividades inibitórias nas isoformas do CYP (Kim *et al*., 1997; Kimura *et al*., 2010; Sand *et al*., 2010), essas moléculas podem ser classificadas como um inibidor fraco do citocromo P450, uma vez que apenas uma isoforma foi inibida (Johnson *et al*., 2013). Ainda no teste AMES, cianidina-3-O-glicosídeo e quercetina apresentaram toxicidade, o que pode levar à genotoxicidade (Kramer *et al*., 2007). É importante ressaltar que a análise dessas moléculas não indicaram hepatotoxicidade ou outros parâmetros de toxicidade relevantes. E, embora a análise computacional dos efeitos das moléculas tenha sido feita separadamente, esses dados dão suporte para o desenvolvimento seguro de estudos com o EPR.

Por fim, demonstramos no **Artigo II** o efeito antioxidante do EPR frente a neurotoxicidade induzida pela administração intranasal de MPTP. Com base no estudo de toxicidade apresentado no **Manuscrito I**, selecionamos a dose de 1.000mg/kg do EPR para esse estudo. Nossas descobertas demonstram que o pré-tratamento com PPE possui efeitos neuroprotetores significativos contra o MPTP intranasal. Este estudo revelou que o PPE preveniu os efeitos iniciais da administração de MPTP, incluindo o desequilíbrio redox e a inibição da atividade da Na+/K+ATPase.

O EPR atenuou/preveniu a produção exacerbada de ROS/4-HNE no SN e BO, o que poderia impedir a inibição da atividade da Na^+/K^+ATPase. Com base nos nossos resultados,

demonstramos que o EPR previne contra o aparecimento de EO após a infusão i.n. de MPTP (Fig.7). O BO, alvo da administração de MPTP, e a SN, principal estrutura dopaminérgica afetada no início da doença de Parkinson, apresentaram as respostas mais proeminentes aos efeitos nocivos do MPTP. E neste contexto, uma única administração de PPE foi eficaz em prevenir as mudanças redox induzidas pelo MPTP.

Fig. 7 - Efeito do pré-tratamento do EPR previne contra o aparecimento de EO após a infusão i.n. de MPTP.



Fonte: Autoria própria.

Os efeitos neuroprotetores contra o desequilíbrio redox no tecido cerebral dos ratos expostos ao MPTP, podem ser atribuídos às propriedades antioxidantes dos compostos presentes no PPE. Assim, a hipótese que confirmamos no **Artigo I** é que o PPE previne o início do estresse oxidativo após a administração de MPTP, potencialmente resultando em uma diminuição ou atraso na neurotoxicidade induzida pelo MPTP. Mas também é preciso considerar a hipótese, que requer estudos mais detalhados para confirmação, de que o PPE poderia interferir diretamente no metabolismo do MPTP, inibindo assim a produção de MPP^+ e, consequentemente, sua cascata neurotóxica.

7. CONCLUSÕES

Nesta tese, compilamos e discutimos os inúmeros potenciais farmacológicos do uso de diferentes partes de produtos derivados da *Eugenia uniflora*, apresentando-se como um produto natural versátil e multi-alvo, o qual apresenta entre as suas propriedades, efeitos antioxidantes e antiinflamatórios. Em relação ao EPR, um extrato produzido da variedade mais rica em compostos como antocianinas, flavonóides e carotenóides, dentre as variedades dos frutos da pitangueira, destacamos a sua baixa toxicidade em mamíferos, sendo classificado na categoria de menor toxicidade *in vivo*. Por fim, destacamos o potencial antioxidante e neuroprotetor do EPR, uma vez que ele foi capaz de proteger diferentes estruturas cerebrais do estresse oxidativo induzido por uma neurotoxina, o MPTP, que induz neurodegeneração dopaminérgica através de um mecanismo de geração de estresse oxidativo.

8. PERSPECTIVA

- Avaliar os efeitos do EPR sobre as alterações motoras e não motoras em fêmeas e fêmeas ovariectomizadas expostas i.n. ao MPTP;
- Investigar os mecanismos envolvidos no efeito farmacológico do EPR em ratas fêmeas controle e fêmeas ovariectomizadas expostas i.n. MPTP;
- Investigar os efeitos do EPR sobre alterações intestinais induzidas pela exposição ao MPTP i.n., utilizado como modelo animal para o estudo dos sintomas não motores da DP.

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ANEXOS I

Os anexos deste documento de qualificação incluem as cartas de aprovação do CEUA/UNIPAMPA e as regras da revista *The Journal of Nutritional Biochemistry*.

CERTIDÃO

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 010/2021

Título: Estudo das alterações neurocomportamentais e neuroquímicas induzidas pela administração intranasal de MPTP em ratas ovariectomizadas.

Data da aprovação: 30/04/2021

Período de vigência do projeto: 01/05/2023

Pesquisadores(a): Simone Pinton

Campus: Uruguaiana

Telefone: (55) 991336520

E-mail: simonepinton@unipampa.edu.br

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie / Linhagem / Raça	Ratos Wistar
N° de animais	217 fêmeas e 58 machos
Peso / Idade	Fêmeas 350-400 g, machos 250-300g / 160 fêmeas de 60 dias, 57 fêmeas e 58 machos de 90 dias
Sexo	Fêmeas e Machos
Origem	Biotério Central da UFSM ou UFPEL

Assinado eletronicamente por **CATIA ALINE VEIVERBERG, PROFESSOR DO MAGISTERIO SUPERIOR**, em 30/04/2021, às 19:12, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

A autenticidade deste documento pode ser conferida no site https://sei.unipampa.edu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **0515467** e o código CRC **05153DFF**.

ANEXO II

Comprovante de depósito de patente

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

22/06/2023 870230053670 13:53

29409162305311709

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2023 012562 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 09341233000122
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
Endereço: Av. General Osório, 1139
Cidade: Bage
Estado: RS
CEP: 96400100
País: Brasil
Telefone: 5332405406
Fax:
Email: nit@unipampa.edu.br

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): Efeito neuroprotetor do extrato da pitanga roxa (Eugenia uniflora)

Resumo: A presente invenção insere-se no campo da Saúde na área de Farmacologia. A tecnologia em questão refere-se ao extrato da pitanga roxa para uso na prevenção e tratamento de desordens neurodegenerativas, de humor e contra o prejuízo na memória. Os compostos bioativos presentes nestes extratos dos frutos da pitangueira roxa conferem a presente invenção potente efeito neuroprotetor, com grande potencial para aplicação em fármacos fitoterápicos e/ou nutracêuticos.

Figura a publicar: Fig 1

Dados do Inventor (72)

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Estado: RS
CEP:
País: BRASIL
Telefone:
Fax:
Email: cristianedenardin@unipampa.edu.br

Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	Pgto GRU 200.pdf
Declaração de período de graça	1_s2.0_S0024320523003454_main.pdf
Desenho	Desenhos.pdf
Resumo	Resumo.pdf
Relatório Descritivo	Relatório Descritivo.pdf
Reivindicação	Reivindicações.pdf

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de Divulgação Anterior Não Prejudicial

☑ Artigo 12 da LPI - Período de Graça.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

__ SIAFI2023-DOCUMENTO-CONSULTA-CONDOC (CONSULTA DOCUMENTO)_____
07/06/23 20:51 NS USUARIO : ASSUNÇÃO
DATA EMISSAO : 07Jun23 VALORIZACAO : 07Jun23 NUMERO : 2023NS004994
UG/GESTAO EMITENTE: 154359 / 26266 - FUNDACAO UNIVERSIDADE FEDERAL DO PAMPA
FAVORECIDO : 183038 / 18801 - INSTITUTO NACIONAL DA PROPRIEDADE INDUS
TITULO DE CREDITO : 2023RP000587 DATA VENCIMENTO : 06Jul23

OBSERVACAO
Registro INPI (Serviço de Patentes) - Nº documento: 29409162305311709. Serviço : 200-Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT. Processo SEI nº 23100.011230/2023-76.

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LANCADO POR : 97681458034 - ASSUNÇÃO UG : 154359 07Jun23 20:50
PF1-AJUDA PF3=SAI PF4=ESPELHO PF12=RETORNA

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DATA EMISSAO : 07Jun23 VALORIZACAO : 07Jun23 NUMERO : 2023NS004994
UG/GESTAO EMITENTE: 154359 / 26266 - FUNDACAO UNIVERSIDADE FEDERAL DO PAMPA
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TITULO DE CREDITO : 2023RP000587 DATA VENCIMENTO : 06Jul23

L	EVENTO	INSCRICAO	CLAS.CONT	CLAS.ORC	V A L O R
01	511005	2022NE000137	332320100	33914710	70,00
02	521214	2022NE000137	213120400	33914710	70,00
03	401005	2022NE000137		33914710	70,00

LANCADO POR : 97681458034 - ASSUNÇÃO UG : 154359 07Jun23 20:50
PF1-AJUDA PF3=SAI PF4=ESPELHO PF5=EVENTO/CONTA PF12=RETORNA

ANEXO III

Normas da revista ao qual o manuscrito 1 será submetido.

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- **Reviewers**. Recommended reviewers should be experts in the subject matter of the manuscript and not be anyone who is or has been a former adviser/advisee, colleague in the same institution, research collaborator, and/or co-author of articles and patents or in any other way has a conflict of interest.
- **Authors**. All authors and their respective email addresses should be entered into ACS Paragon Plus. Corresponding author(s) are designated with an asterisk. The ACS Ethical Guidelines in Appendix 1 must be read and understood by all authors.
- **Title**. The title should use Title Case and avoid words like “New,” “Novel,” “First,” “Outstanding,” “Excellent,” “Exceptional,” “Green,” or other similar descriptive words unless appropriately supported.
- **Table of Contents (TOC) graphic**. Provide a TOC graphic at the end of the manuscript, included in the manuscript file, or the file may be uploaded separately. Ensure compliance with the TOC requirements outlined in Appendix 2.
- **Manuscript**. Manuscript text should be submitted in one-column format and double-spaced, using headings and subheadings (without numbers, references, or acronyms in the headings). Ensure compliance with the length guidelines, scope, and text component requirements listed in this document.
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- **References**. References should be cited within the text by consecutive numbers. Arabic numbers in the order of their first citation in the text are required. The corresponding numbers must be inserted at the appropriate locations in the text as superscript close to the last character and outside any punctuation marks. The references should adhere to the format described in the Manuscript Preparation section.
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Figure S1.

Scope of the Journal

While safety permeates all ACS journals, it benefits from its preeminence in ACS Chemical Health & Safety where its authors influence chemical safety and guide tomorrow's science. Chemical safety research can be transformational and innovative, and it can also focus on practice and be narrowly scoped. This includes foundational datasets or variations on well-studied themes because they hold value to those working with hazardous materials. *ACS Chemical Health & Safety* provides the opportunity to publish across all these areas. As a discipline, chemical health and safety does not limit itself to laboratories and chemical facilities, and our authors are more than just chemists and chemical engineers; they are scientists with broad research backgrounds and members of the public from many disciplines, including environmental safety and health professionals, industrial hygienists, public health professionals, safety policy makers, human factors specialists, and more.

Sample topics that span the journal's scope include:

- Fundamental chemical safety research and datasets Emerging technologies (e.g., AI/machine learning, virtual reality, green chemisty, decarbonization, advancements in personal protective equipment (PPE), etc.)Analysis of previously published safety datasetsData from field testing and real-world simulationsInvestigations of new inherently safer materials and proceduresEffectiveness of chemical health and safety training, tools, or resourcesScientific studies aimed at reducing chemical exposure and improving laboratory and/or industrial working conditions
- Chemical health safety and security warnings Near-miss or incident case studies and accident investigationsCommentaries on current safety and security issuesCommunications on techniques and engineering solutions that enhance safe and secure working environmentsReviews, commentaries, and methodologies that address the life cycle of hazardous materials and related techniques.
- Chemical risk assessment and management Risk assessment methodologies and applied case studiesCase studies demonstrating best practices in risk management approaches in a variety of settings (e.g., academic laboratories, industrial research and development laboratories, industrial manufacturing, storage, and transportation)Chemical safety and security decision making, including economic considerationsLaboratory designUse of chemicals in other academic settings (e.g., art or theatre departments, research hospitals, etc.)
- Other topics that influence chemical safety and guide tomorrow's science New emerging threats (e.g., COVID impacts on researchers and utilizing chemicals to mitigate associated risks)Occupational safety and health related to chemical exposuresProcess safety and security associated with the management of bulk hazardous materialRegulatory updates, requirements, and implementationEmergency response and planningDevelopment and management of safety culturesSafety commentaries or case studies from other disciplines that provide lessons and insights that could be adapted for chemical safetyThe scientific study of chemical safety training, teaching, and learning

Manuscripts relating to commercial products are welcome, provided that they are not advertisements.

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Methods/Protocols - Methods/Protocols feature safety methods and protocols related to the entire chemistry community. The journal aims to provide one-stop access to both novel and improvised safety methods as well as standard techniques, such that this content is peer reviewed and can be made more visible and easily discoverable. Methods/Protocols should have sufficient data (where applicable) and a comprehensive review of relevant sources. Authors are asked to include videos and photographs of experiments or apparatus as well as sufficient details to ensure reproducibility. There is no set length and manuscripts may take many forms depending on the content (e.g. short practical safety applications, validation of safety methods, or comprehensive protocol summaries).

The following order of presentation is preferred:

- Abstract, TOC graphic, keywords
- Introduction
- Materials, methods, or theory used
- Procedure
- Safety-specific sections, including: Hazard and risk assessmentHierarchy of controlsEmergency preparednessPolicy, regulation, and code considerations
- Results and discussion
- Conclusions
- Acknowledgements, supporting information description
- References

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While this document will provide basic information on how to prepare and submit the manuscript as well as other critical information about publishing, we also encourage authors to visit the ACS Researcher Resources for additional information on everything that is needed to prepare (and review) manuscripts for ACS journals and partner journals, such as

- Mastering the Art of Scientific Publication, which shares editor tips about a variety of topics including making your paper scientifically effective, preparing excellent graphics, and writing cover letters.
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The title should clearly and concisely reflect the emphasis and content of the manuscript and be accessible to a broad audience. The title should not contain esoteric terms, symbols, trademark names, institution names, abbreviations, or uncommon acronyms, and part or series numbers. The title should use Title Case and avoid words like “New,” “Novel,” “First,” “Outstanding,” “Excellent,” “Exceptional,” “Green,” or other similar descriptive words unless appropriately supported. Indicate the audience and the setting if that is significant. A well-crafted title aids in successful information retrieval and supports search engine optimization.

Author List

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The abstract should be approximately 250 words or fewer and summarize the important points made in the manuscript. Describe briefly and clearly the purpose of the work, the principle results and/or major conclusions. The abstract should not include words like “New,” “Novel,” “First,” “Outstanding,” “Excellent,” “Exceptional,” “Green,” or other similar descriptive words unless rigorously supported. Include the abstract text in the manuscript file. No cited literature or display elements should appear in the abstract. A well-written abstract aids in successful information retrieval and is one of the initial aspects considered closely by Editors.

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Text should be presented in one column with numbered pages and organized using headings and subheadings (without numbers, references, or acronyms in the headings). Abbreviations and acronyms should be used sparingly and should be defined at their first occurrence. Other than headings, present the text in black. Whenever possible, use systematic nomenclature as recommended by IUPAC for chemical compounds and SI units, including in table column headings. Present analyzed data in an accurate, complete, yet concise manner. Express results with indications of their reliability. This includes appropriate use of significant figures, as well as statistical parameters (e.g., standard deviation, p-values indicating statistical significance, and measures of effect size). Terms, variables, and symbols should be defined within the text (rather than in a list of abbreviations). The Journal does not publish appendices; such material should be removed from the main text of the manuscript and uploaded as separate Supporting Information.

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Supporting Information

All nonessential figures, tables, and procedures should be included as SI and uploaded as a separate file (PDF recommended). Supporting Information must be submitted at the same time as the manuscript. The availability of SI should be mentioned with a description in the manuscript. The SI should be formatted with a cover sheet listing authors, manuscript title, and a table of

contents where appropriate. SI pages must be numbered consecutively, starting with page S1. Tables and Figures should be numbered starting with Table S1 and Figure S1.

References

A thorough literature review should be conducted, and the submission should be placed within the context of previously published work, including that which has appeared in the Journal. Citations and references should follow the publication style found in The ACS Style Guide. Titles are required for all works cited; please provide complete publication information, including an issue number where applicable, and a DOI. Unpublished work that has been cited should be uploaded for editorial review. Reference call-out numbers in the text should be superscripted sequential Arabic numerals. Journal names are abbreviated according to the Chemical Abstracts Service Source Index (CASSI). Page ranges for articles as well as book citations should also be provided. Rather than providing URLs in the main text of the manuscript, add a citation for each discrete URL and include it sequentially in the References section with an "accessed" statement: "(accessed [Month] 20XX)." References to resources only in a language other than English will be largely inaccessible to readers; including sufficient references to English-language resources will benefit readers and increase the value of the manuscript. Textual material that might otherwise constitute a footnote or endnote must be incorporated into the References section and presented using complete sentences.

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Resolution

Digital graphics pasted into manuscripts should have the following minimum resolutions:

- Black and white line art, 1200 dpi
- Grayscale art, 600 dpi
- Color art, 300 dpi

Size

Graphics must fit a one- or two-column format. Single-column graphics can be sized up to 240 points wide (3.33 in.) and double-column graphics must be sized between 300 and 504 points (4.167 in. and 7 in.). The maximum depth for all graphics is 660 points (9.167 in.) including the caption (allow 12 pts. For each line of caption text). Lettering should be no smaller than 4.5 points in the final published format. The text should be legible when the graphic is viewed full-size. Helvetica or Arial fonts work well for lettering. Lines should be no thinner than 0.5 point.

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Color may be used to enhance the clarity of complex structures, figures, spectra, and schemes, etc., and color reproduction of graphics is provided at no additional cost to the author. Graphics intended to appear in black and white or grayscale should not be submitted in color.

Type of Graphics

Table of Contents (TOC)/Abstract Graphic

Consult the Guidelines for Table of Contents/Abstract Graphics for specifications.
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Figures

A caption giving the figure number and a brief description must be included below each figure. The caption should be understandable without reference to the text. It is preferable to place any key to symbols used in the artwork itself, not in the caption. Ensure that any symbols and abbreviations used in the text agree with those in the artwork.

Charts

Charts (groups of structures that do not show reactions) may have a brief caption describing their contents.

Tables

Each table must have a brief (one phrase or sentence) title that describes the contents. The title should be understandable without reference to the text. Details should be put in footnotes, not in the title. Tables should be used when the data cannot be presented clearly in the narrative, when many numbers must be presented, or when more meaningful inter-relationships can be conveyed by the tabular format. Tables should supplement, not duplicate, information presented in the text and figures. Tables should be simple and concise.

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Each scheme (sequences of reactions) may have a brief caption describing its contents.

Chemical Structures

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ANEXO IV

Participações em projetos e publicações

Neuroscience Letters 749 (2021) 135697

Contents lists available at ScienceDirect

Neuroscience Letters

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

Potential role of a newly AChE reactivator in the depressive-like behavior induced by malathion

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ARTICLE

Evaluation of curcumin-loaded polymeric nanocapsules with different coatings in chick embryo model: influence on angiogenesis, teratogenesis and oxidative stress

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brain sciences

MDPI

Article

Nanoencapsulated Curcumin: Enhanced Efficacy in Reversing Memory Loss in An Alzheimer Disease Model

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Neuroprotective effect of *Eugenia uniflora* against intranasal MPTP-induced memory impairments in rats: The involvement of pro-BDNF/ $p75^{NTR}$ pathway

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