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Tese

Produtos naturais como potenciais agentes terapêuticos para o glioblastoma e neuroinflamação: estudos *in vitro* e *in vivo*

Natália Pontes Bona

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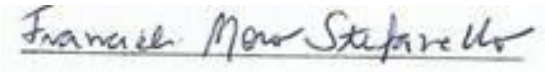

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“O que vale na vida não é o ponto de partida e sim a caminhada. Caminhando e semeando, no fim, terás o que colher.”

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Resumo

BONA, Natália Pontes. **Produtos naturais como potenciais agentes terapêuticos para o glioblastoma e neuroinflamação: estudos *in vitro* e *in vivo*.** 2022. 136f. Tese (Doutorado) - Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2022.

Os produtos naturais desempenham um importante papel na prevenção e tratamento de doenças, e são originados de várias fontes, incluindo as plantas. Dentre eles destacamos o composto isolado ácido tânico (AT) e a planta de *Cecropia pachystachya* (CEC). Ainda, o extrato aquoso de CEC apresenta entre seus compostos majoritários flavonoides C-glicosilados e O-glicosilados, a partir disso, foi desenvolvida uma fração enriquecida em flavonoides (FEF) que também foi alvo do presente estudo. Os produtos naturais citados apresentam atividades biológicas bem descritas na literatura, principalmente frente ao Sistema Nervoso Central (SNC) que abrange diversas doenças, dentre elas as que envolvem processos de neuroinflamação, como o câncer. Entre os tumores cerebrais, destaca-se o glioblastoma (GB), um tumor altamente agressivo e com um tratamento que permanece paliativo. Considerando a limitação terapêutica, é de extrema relevância a busca por novos alvos que visem um melhor prognóstico e melhor qualidade de vida dos pacientes. Frente a isso, o objetivo deste trabalho foi avaliar o efeito antiglioma *in vivo* do AT e do extrato de CEC, a atividade antitumoral do extrato de CEC e da FEF *in vitro* e o efeito neuroprotetor do extrato de CEC e da FEF em um modelo de neuroinflamação induzido por lipopolissacarídeo (LPS). Os resultados *in vitro* demonstraram uma redução da viabilidade e proliferação celular em linhagem de glioma de rato C6 e glioma humano U87, tanto do tratamento com extrato de CEC quanto da FEF. Além disso, pode-se observar alterações na migração celular de C6 e U87. Quanto ao modelo pré-clínico, houve redução do tamanho do tumor tanto nos animais tratados durante 15 dias com AT (50 mg/kg/dia) quanto com o extrato de CEC (200 mg/kg/dia), além de modulação de parâmetros de estresse oxidativo e a da sinalização purinérgica pelos tratamentos supracitados. Ainda, no modelo *in vivo* de neuroinflamação observou-se um efeito neuroprotetor nos animais tratados por 14 dias com o extrato de CEC (200 mg/kg/dia) e da FEF (50 e 100 mg/kg/dia) frente a parâmetros comportamentais, na modulação do estado redox e alterações nos níveis de TNF-α. Em vista disso, nossos resultados demonstram um potencial efeito antiglioma do AT e do extrato de CEC na modulação de vias que estão relacionadas a progressão tumoral, além de um potencial efeito neuroprotetor do extrato de CEC e da FEF em parâmetros associados ao processo de neuroinflamação, sendo assim importantes alvos no tratamento dessas doenças.

Palavras-chave: glioblastoma; neuroinflamação; ácido tânico; *Cecropia pachystachya;* flavonoides

Abstract

BONA, Natalia Pontes. **Natural products as potential therapeutic agents for glioblastoma and neuroinflammation: *in vitro* and *in vivo* studies.** 2022. 136f. Thesis (Doctorate) - Programa de Pós Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2022.

Natural products play an important role in the prevention and cure of diseases, originating from various sources, such as plants. Among them, we highlight the isolated compound Tannic Acid and the *Cecropia pachystachya* (CEC) plant. Also, the hydroalcoholic extract of CEC has among its major compounds C-glycosylated and O-glycosylated flavonoids, from which a flavonoid Enriched Fraction (FEF) was developed and used in the present study. The cited compounds present biological activities well described in the literature, mainly against the Central Nervous System (CNS). The central nervous system (CNS) encompasses several diseases, including diseases that involve neuroinflammation processes, such as cancer. Among the tumors, glioblastoma (GB) stands out, a highly aggressive tumor with a treatment that remains palliative. Considering the therapeutic limitation, it is extremely important to search for new targets that aim at a better prognosis and better quality of life for patients. In view of this, in this work we evaluated the *in vivo* antiglioma effect, the antitumor activity of CEC extract and FEF *in vitro* and the neuroprotective effect of CEC extract and FEF in a model of LPS-induced neuroinflammation. Our *in vitro* results demonstrated a reduction in cell viability and proliferation both from treatment with CEC extract and its FEF. In addition, we can observe changes in cell migration of C6 and U87. As for the preclinical model, there was a reduction in tumor size both in animals treated for 15 days with TA (50 mg/kg/day) and with CEC extract (200 mg/kg/day), in addition to modulating oxidative stress parameters and altering purinergic signaling. In addition, in the *in vivo* model of neuroinflammation, we observed a neuroprotective effect in animals treated for 14 days with the CEC extract (200 mg/kg/day) and its FEF (50 and 100 mg/kg/day) against behavioral parameters, in the modulation of redox status and changes in TNF-α levels. In view of this, our results demonstrate a potential antiglioma effect of TA and CEC extract in modulating pathways that are related to tumor progression, in addition to a potential neuroprotective effect of CEC extract and its FEF on parameters associated with the neuroinflammation process, thus being important targets in the treatment of these diseases.

Keywords: glioblastoma; neuroinflammation; tannic acid; *Cecropia pachystachya*; flavonoids

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Lista de Abreviaturas e Siglas

ADA – Adenosina Deaminase

ADO – Adenosina

ADP – Difosfato de Adenosina

AMP – Monofosfato de Adenosina

ANVISA – Agência Nacional de Vigilância Sanitária

AT – Ácido Tânico

ATP – Trifosfato de Adenosina

CAT – Catalase

CEC – *Cecropia pachystachya*

EROS – Espécies Reativas de Oxigênio

FEF – Fração Enriquecida em Flavonoides

GB – Glioblastoma

GST – Glutationa S-transferase

H_2O_2 – Peróxido de Hidrogênio

IDH – Isocitrato Desidrogenase

IL-6 – Interleucina 6

IL-10 – Interleucina 10

INCA – Instituto Nacional do Câncer

LPS – Lipopolissacarídeo

NTPDases – Ecto-Nucleosideo Trifosfato Difosfohidrolases

$O_2^{\bullet -}$ – Ânion Superóxido

OMS – Organização Mundial da Saúde

SNC – Sistema Nervoso Central

SOD – Superóxido Dismutase

SUS – Sistema Único de Saúde

TMZ – Temozolomida

UV – Ultravioleta

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1 INTRODUÇÃO

Atualmente já foram identificados mais de 277 tipos de doenças cancerígenas (HASSANPOUR & DEHGHANI, 2017). Em vista disso, o câncer é considerado um problema preocupante de saúde pública mundial, retratando-se como a segunda causa de morte do mundo. É definido por ser uma doença que atinge um amplo espectro tecidual, tornando a doença um grande desafio tanto para diagnóstico quanto para um tratamento eficaz (MEACHAM & MORRISON, 2013; FISHER, PUSZTAI; SWANTON, 2013).

Dentre os cânceres pertencentes ao Sistema Nervoso Central (SNC), destacam-se os gliomas, tumores mais prevalentes do cérebro e da medula. Histologicamente apresentam em comum diversas características de células gliais normais e sua nomenclatura normalmente se dá devido a essas compatibilidades. No entanto a origem dos gliomas ainda é uma incógnita, não é claramente elucidado se o surgimento se dá através de células gliais normais, percursores gliais ou neurais, células-tronco ou outros tipos celulares (MODREK, BAYIN, PLACANTONAKIS, 2014). A Organização Mundial da Saúde (OMS) classificou os principais grupos de tumores gliais subdividindo-os em diferentes graus de malignidade, os gliomas de grau IV, onde estão inseridos os glioblastomas (GB), os quais são caracterizados por apresentarem mecanismos de malignidade complexos, sendo os tumores mais agressivos e de alta letalidade pertencentes ao SNC (LOUIS et al., 2021).

Sabe-se que o GB é o astrocitoma primário mais comum e agressivo, responsável por mais de 60% de todos os tumores cerebrais em adultos (LEECE et al., 2017). Esses tumores são caracterizados por apresentarem particularidades histopatológicas como necrose e proliferação endotelial, o que confere ao GB o grau VI, sendo o grau mais elevado e grave quanto a classificação de tumores cerebrais da OMS (WIRSCHIN, GALANIS; WELLER, 2016). Atualmente os tratamentos são baseados em uma abordagem multimodal onde é realizada a ressecção cirúrgica máxima da massa tumoral, seguido de radioterapia e quimioterapia adjuvante com o fármaco padrão temozolomida (TMZ) (HAN et al., 2015). Apesar do progresso nos estudos, o GB ainda permanece sendo uma doença mortal, conferindo um baixo prognóstico e qualidade de vida para os pacientes, com uma sobrevida de aproximadamente 14 a 15 meses após o diagnóstico (TAKKAR et al., 2014).

No microambiente tumoral a inflamação é um componente persistente, contribuindo para a carcinogênese e progressão tumoral. No GB, uma característica comum é a necrose tecidual seguida de inflamação do microambiente. Essa inflamação juntamente com a necrose são típicas do GB, levando a uma maior resistência às terapias e um pior prognóstico para os pacientes (DECORDOVA et al., 2020; ALGHAMRI et al., 2021). Além disso, as células de GB expressam e secretam quimiocinas e citocinas imunossupressoras importantes, entre elas a interleucina 6 (IL-6) e interleucina 10 (IL-10), que agem através da infiltração de células imunes, induzindo um fenótipo celular pró-tumoral (YEO et al., 2021).

O sistema purinérgico é uma rota de comunicação comum entre células e está envolvido em diversos mecanismos neuronais e não neuronais, dentre eles a progressão tumoral, podendo levar à proliferação ou à morte celular (WHITE; BURNSTOCK, 2006; BURNSTOCK, 2016). Frente a isso, a sinalização purinérgica vem sendo um componente de estudo interessante frente ao GB.

O estado celular redox é o equilíbrio fisiológico entre os equivalentes oxidantes e reduzidos dentro dos compartimentos subcelulares, estes permanecem em equilíbrio dinâmico. Em condições fisiológicas normais as espécies reativas de oxigênio (EROS) são produzidas constantemente durante a respiração celular e atuam mediando a estimulação de diversas vias de sinalização considerando as condições ambientais (JABS, 1999). Devido aos diversos estágios apresentados pelo câncer, onde ocorre o desenvolvimento de alterações mutacionais e uma proliferação descontrolada, há um constante interesse no estudo da relação do estresse oxidativo na iniciação e progressão do câncer (KLAUNIG, 2018).

Considerando o baixo prognóstico dos pacientes acometidos pelo GB, e a falta de eficiência nos tratamentos disponibilizados, a busca por novos alvos terapêuticos tem ganhado visibilidade frente a comunidade científica. Produtos naturais são considerados importantes fontes de estudos, devido as suas ações multialvos e sua baixa toxicidade. Nesse contexto, destaca-se o ácido tânico (AT) e o extrato de *Cecropia pachystachya* Trécul (CEC).

O AT é um tanino hidrolisável encontrado em diversos alimentos como a uva, vinho tinto, café, chá verde, chá preto entre outros (CHUNG et al., 1998). Esse composto apresenta atividades biológicas bem descritas na literatura, como antioxidante e antidepressivo (LUDUVICO et al., 2020), antitumoral (BONA et al., 2019), antimicrobiana e antiviral (BUZZINI et al., 2008) além de apresentar efeitos

anti-inflamatórios (PERELSHTEIN et al., 2014). Ademais, nos últimos anos, um número crescente de trabalhos descreve novos mecanismos relacionados a atividade do AT e possível aplicação na prevenção do câncer e sensibilização a drogas convencionais utilizadas na terapia dessa patologia (BAER-DUBOWSKA et al., 2020).

O gênero *Cecropia* é amplamente distribuído na América Latina, apresentando em torno de 60 diferentes espécies, algumas dessas sendo amplamente utilizadas na medicina tradicional (BRANGO-VANEGAS et al., 2014). A CEC, popularmente conhecida como embaúba, é considerada uma planta medicinal, principalmente no que tange as folhas dessa planta (MACHADO et al., 2021). Já há diversos relatos na literatura quanto as atividades biológicas dos extratos de CEC, principalmente em doenças que atingem o SNC (GAZAL et al., 2014; GAZAL et al., 2015). Ainda, Ortmann e colaboradores (2016) demonstraram que a fração enriquecida em flavonoides (FEF) obtida do extrato de CEC exerce efeito tipo-antidepressivo e que a regulação da homeostase redox parece desempenhar um importante papel nesse efeito.

Diante da problemática exposta e dos benefícios já elucidados do AT, do extrato de CEC e de sua FEF, torna-se de extrema relevância a investigação desses produtos naturais em modelos de GB e de neuroinflamação.

2 OBJETIVO

2.1 Objetivo Geral

Avaliar o efeito antiglioma do ácido tânico (AT), do extrato de *Cecropia pachystachya* (CEC) e de sua fração enriquecida em flavonoides (FEF) e o efeito neuroprotetor do extrato de CEC e FEF.

2.2 Objetivos Específicos

Capítulo 1 – Artigo 1

a) Avaliar o efeito antiglioma do AT, em animais submetidos a um modelo pré-clínico de GB, frente aos seguintes parâmetros:

- Parâmetros de estresse oxidativo em cérebro, soro e plaquetas, como os níveis de nitritos, níveis de espécies reativas de oxigênio, conteúdo tiólico total, peroxidação lipídica, atividade de enzimas antioxidantes catalase, superóxido dismutase e glutationa S-transferase;
- Sinalização purinérgica, como atividade das enzimas NTPDase e 5'-nucleotidase em linfócitos, plaquetas e soro.

Capítulo 2 – Manuscrito 1

a) Avaliar o efeito antiglioma do extrato de CEC, em cultivo de células de glioma de rato (C6) e glioma humano (U87), frente aos seguintes parâmetros:

- Viabilidade celular através do teste do MTT ([3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina]);
- Proliferação celular através do teste da Sulforodamina B (SRB);
- Ensaio de migração celular pelo teste do risco.

b) Avaliar o efeito antiglioma do extrato de CEC, em animais submetidos a um modelo pré-clínico de GB, frente aos seguintes parâmetros:

- Parâmetros comportamentais, como o teste do campo aberto, plus-maze e reconhecimento de objetos;

- Análise histopatológica e quantificação do tumor;
- Toxicidade, através de análise dos parâmetros bioquímicos séricos;
- Parâmetros de estresse oxidativo em cérebro, soro e plaquetas, como os níveis de nitritos, níveis de espécies reativas de oxigênio, conteúdo tiólico total, peroxidação lipídica, atividade de enzimas antioxidantes catalase, superóxido dismutase e glutationa S-transferase;
- Sinalização purinérgica, como atividade das enzimas NTPDase e 5'-nucleotidase em linfócitos, plaquetas e soro;
- Avaliação dos níveis de citocinas inflamatórias IL-6 e IL-10.

Capítulo 3 – Manuscrito 2

a) Avaliar o efeito neuroprotetor do extrato de CEC e da FEF, em animais submetidos a um modelo de neuroinflamação induzido por lipopolissacarídeo (LPS), frente aos seguintes parâmetros:

- Parâmetros comportamentais, como o teste do campo aberto, labirinto em cruz elevada e reconhecimento de objetos;
- Parâmetros de estresse oxidativo em estruturas cerebrais, como córtex, hipocampo e estriado;
- Avaliação dos níveis de TNF-α.

Apêndice – Resultados Adicionais

a) Avaliar o efeito antiglioma da FEF, em cultivo de células de glioma de rato (C6) e glioma humano (U87), frente às seguintes análises:

- Viabilidade celular através do teste do MTT ([3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina]);
- Proliferação celular através do teste da Sulforodamina B (SRB);
- Ensaio de migração celular pelo teste do risco.

3 REVISÃO BIBLIOGRÁFICA

3.1 Câncer

O câncer engloba mais de 100 diferentes tipos de doenças malignas que apresentam em comum um crescimento desordenado de células, podendo invadir órgãos e tecidos adjacentes. Além disso, essas células, dividem-se de forma rápida e desordenada, além de serem agressivas e incontroláveis, formando tumores que podem se espalhar por outras regiões do corpo (INCA, 2022). O câncer é um preocupante problema de saúde pública, e são estimados mais de 20 milhões de novos casos até o ano de 2025, além de ser a segunda causa de morte no mundo (STEWART; WILD, 2014; WHO, 2020).

Segundo o Instituto Nacional do Câncer (INCA) é estimado para cada ano do triênio 2020-2022 um total de 625 mil novos casos de câncer. O câncer de pele não melanoma será o mais incidente (177 mil), sucessivamente apresenta-se o câncer de mama e próstata (66 mil cada), cólon e reto (41 mil), pulmão (30 mil) e estômago (21 mil) (INCA, 2021).

Mundialmente, em termos de incidência, cânceres que acometem o SNC são mais incidentes em homens do que em mulheres. No ano de 2018, foram descritos 296 mil novos casos entre homens e mulheres (BRAY et al., 2018; FERLAY et al., 2018). A estimativa trienal (2020-2022) para o Brasil é de 5.870 novos casos em homens e 5.220 novos casos em mulheres. Correspondendo a um risco de 5,61 novos casos a cada 100 mil homens e de 4,85 novos casos a cada 100 mil mulheres (INCA, 2021). Além disso é importante destacar que entre os dez tipos de câncer mais incidentes em mulheres estimados para 2020, os cânceres do SNC se fazem presente (INCA, 2021).

Os tratamentos convencionais mais utilizados são a intervenção cirúrgica, o uso de quimioterápicos e a radioterapia (WHO, 2022). A quimioterapia convencional ainda é a marca registrada no tratamento do câncer, os medicamentos são projetados com o objetivo de atingir não somente as células cancerosas, mas também as células normais. Diversos estudos vêm focando em terapias direcionadas as células cancerosas, com objetivo de inibir o crescimento celular, aumentando a morte celular e bloqueando a disseminação do câncer (BAUDINO, 2015). As terapias paliativas, ainda são consideradas primordiais, visando uma melhora na qualidade de vida do paciente e de suas famílias (WHO, 2022).

3.2 Gliomas

Os gliomas são os tumores primários malignos pertencentes ao SNC, englobando dois principais subtipos: os gliomas difusos e os não difusos (LOUIS et al., 2016; WESSELING & CAPPER, 2017). Ainda permanecem com sua origem desconhecida, uma das hipóteses é que o tumor se inicie em células estaminais ou progenitoras neurais neoplasicamente transformadas (RIEMENSCHNEIDER et al., 2010). Esses tumores são responsáveis por 24% de todos os tumores cerebrais primários do SNC (McNEILL, 2016). Como características, os gliomas apresentam um crescimento infiltrativo extenso, alta capacidade de neovascularização e a principal problemática, a resistência a terapias combinadas, além disso são tumores que apesar de apresentarem baixas taxas de incidência, demonstram altas taxas de mortalidade (LENTING et al., 2017; MA et al., 2018). Os gliomas estão no grupo dos tumores neuroepiteliais, correspondendo a 33% dos tumores primários e 79% dos tumores malignos do SNC (OSTROM et al., 2018).

Atualmente a Organização Mundial da Saúde (OMS) adotou uma nova abordagem para a classificação dos gliomas (Figura 1), dividindo-os de acordo com seu crescimento, faixa etária e o grau de malignidade conforme alterações genéticas apresentadas pelo paciente (LOUIS et al., 2021). Dentre os gliomas difusos do tipo adulto, destacamos o GB.

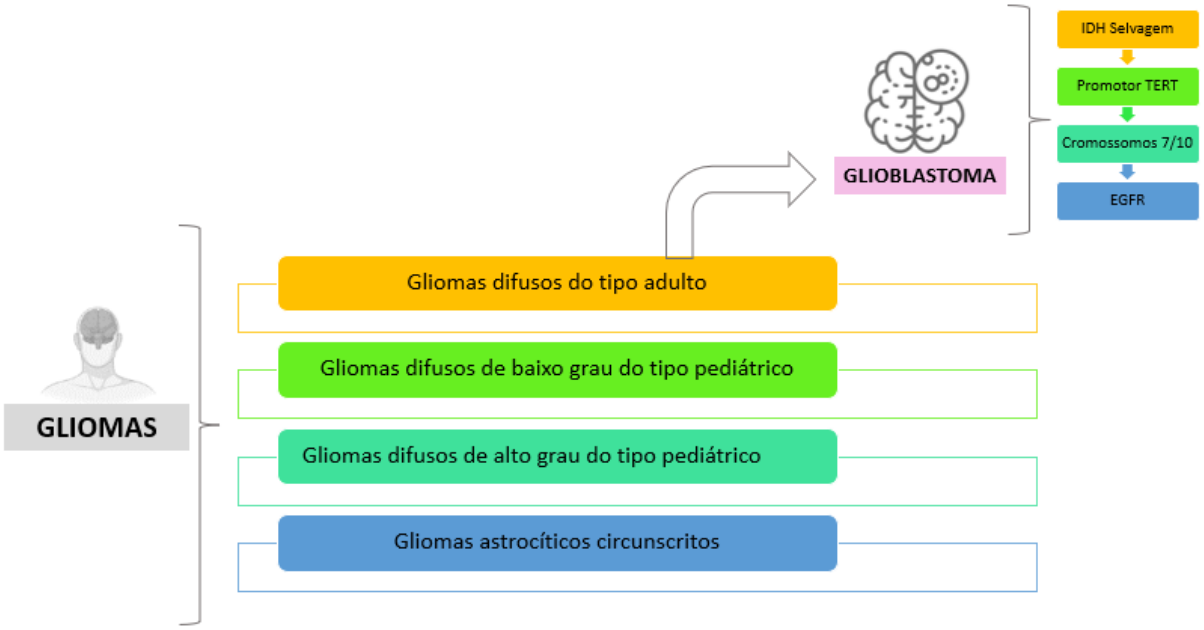

Figura 1. Nova classificação dos gliomas segundo a OMS (Figura desenvolvida pelo autor; Referenciado de Louis et al., 2021)

3.2.1 Glioblastoma

O GB (Figura 2) é o tumor cerebral primário maligno mais comum e agressivo, compreende aproximadamente 57% de todos os gliomas e 48% de todos os tumores malignos primários do SNC (OSTROM et al., 2018). É um glioma de grau IV caracterizados por possuírem uma população de células heterogêneas, geneticamente instáveis, com alta capacidade de infiltração e angiogênese e alta resistência à quimioterapia (WEN et al., 2008; VAN MEIR et al., 2010). Em contrapartida apresenta uma baixa capacidade de metástase, porém manifesta uma grande habilidade de infiltração pelo tecido cerebral (VAN MEIR et al., 2010). Uma das grandes preocupações é a gradual deterioração neurológica, devido ao seu rápido crescimento e alta capacidade infiltrativa, levando os pacientes à morte em até 15 meses (WU et al., 2013).

Nos Estados Unidos, no período de 2011 a 2015, a incidência média de GB por ano, estabelecida por idade é de 3,21 por 100.000 habitantes (OSTROM et al., 2018). A média de idade de diagnóstico é de 65 anos, porém idosos de 75 a 84 anos apresentam taxas mais elevadas. O GB é 1,58 vezes mais comum em homens do que em mulheres, levando em consideração uma incidência anual de 4,0 comparado com 2,53 por 100.000 habitantes, respectivamente (OSTROM et al., 2018).

Diversos estudos vêm sendo realizados para combater o GB, porém ele ainda permanece sendo uma doença de prognóstico insatisfatório e alto índice de mortalidade, conferindo aos pacientes uma sobrevida média de aproximadamente 14 a 15 meses após a realização do diagnóstico (THAKKAR et al., 2014). Devido a essa problemática, o GB se torna um problema fundamental de saúde pública (THAKKAR et al., 2014).

O GB é considerado o mais comum e agressivo tipo de câncer do SNC (FURNARI et al., 2007), sendo dividido em dois subtipos, caracterizando o tumor como primário ou secundário (MAHER et al., 2006). O GB primário já se manifesta clinicamente como um tumor de grau IV, já o GB secundário evolui a partir dos tumores de menores graus, pode levar de 5 a 10 anos para atingir o maior grau (MAHER et al., 2006). É possível observar em pacientes com tumores cerebrais, convulsões súbitas. Os pacientes com déficits neurológicos focais progressivos, provavelmente apresentam o crescimento de um tumor em uma região específica do cérebro. Já os sintomas não focais equivalem a vômitos, cefaleias, fadigas,

alterações cognitivas, desequilíbrio, transtorno de humor e distúrbio da marcha. Os pacientes também podem apresentar déficit neurológico agudo que se assemelha a um acidente vascular encefálico, porém esses sintomas aparecem com uma frequência bem menor (CHABNER; LONGO, 2015). Outro sintoma observado é o aumento da pressão intracraniana, que ocorre devido a um aumento muito pequeno do volume do tumor, que pode resultar em coma e morte (BEHIN et al., 2003).

A abordagem padrão para a grande parte dos tumores primários do SNC é a ressecção cirúrgica máxima, que irá permitir um diagnóstico histológico preciso do tumor. Para o GB, seguido da ressecção cirúrgica são realizadas seções de radioterapia (6 semanas) concomitantes com administrações diárias do fármaco padrão TMZ, posteriormente ainda são realizados mais 6 ciclos de TMZ como fase de manutenção (STUPP et al., 2014).

A intervenção cirúrgica ainda é fundamental para o diagnóstico e prevenção dos sintomas do tumor. Posteriormente, o paciente é submetido à radioterapia, considerada tratamento padrão após a ressecção ou biópsia. A radioterapia tem sido usada há muito tempo no tratamento do GB para melhorar o controle local e a sobrevida, e continua sendo uma modalidade essencial (STUPP et al., 2014). Na recorrência, uma das opções realizadas é a reirradiação, porém é apropriada apenas em circunstâncias selecionadas. Normalmente, isso seria aplicado em pacientes mais jovens com bom desempenho. Semelhante à cirurgia, não há ensaios clínicos randomizados que demonstrem benefício na sobrevida (CABRERA et al., 2016).

O quimioterápico TMZ tem sido administrado concomitantemente com a radioterapia, sendo considerado o padrão atual de tratamento para pacientes de até 70 anos de idade acometidos por GB (STUPP et al., 2014). O TMZ é um agente alquilante de DNA conhecido por induzir parada no ciclo celular em G2/M e eventualmente levar a apoptose (ALONSO et al., 2007). É possível observar uma melhora significante na sobrevida dos pacientes tratados com TMZ, com uma média de 2 a 5 anos de sobrevivência. Inicialmente o TMZ é administrado por 7 dias simultaneamente com a radioterapia, após a radiação são realizados 6 ciclos, que compreende 5 dias de administração do TMZ em um intervalo de 4 em 4 semanas (STUPP et al., 2014). Pacientes com GB tendem a receber uma dose de 150 mg/m^2 por dia (LEE, 2016). Ainda que o tratamento com TMZ aumente a sobrevida média dos pacientes, existe um rápido desenvolvimento de resistência a esse quimioterápico (TIEK et al., 2022).

Figura 2. Características gerais do glioblastoma (Figura desenvolvida pelo autor; Referenciado de: Ostrom et al., 2018). SNC - Sistema nervoso central

3.3. Neuroinflamação

A inflamação sistêmica é uma resposta fisiológica protetora a lesões e infecções. Esse processo é visto como um mecanismo de defesa que é desencadeado afim de proteger o organismo de patógenos (HODGE et al., 2005). Apesar dos efeitos de proteção da inflamação, alguns mecanismos inflamatórios comuns podem vir a contribuir para processos neurodegenerativos (ALLAN & ROTHWELL, 2003).

A inflamação crônica, no SNC, pode levar à liberação de citocinas pró-inflamatórias e moléculas neurotróficas pela ativação da micróglia astrocitária, em consequência disso ocorre uma acelerada ativação dos mecanismos de neurodegeneração (SOCHOCKA et al., 2017). A neuroinflamação é caracterizada por ser uma resposta inflamatória no SNC e apresenta algumas particularidades quanto a inflamação em outros órgãos. Na neuroinflamação as células gliais primárias são ativadas rapidamente em resposta a infecção, inflamação e lesão, a partir desse evento adquirem morfologia de macrófagos ativados que por consequência limitam a área da lesão através da fagocitose de células mortas e

liberação de citocinas pró-inflamatórias, como interleucina 1 (IL-1), interleucina 6 (IL-6), interleucina 18 (IL-18) e fator de necrose tumoral alfa (TNF-α) (STREIT, 2002).

Em tumores cerebrais, a inflamação pode gerar tumorigênese em consequência do aumento de alterações genéticas, que são resultado de EROS e nitrogênio secretadas por macrófagos. Durante o processo de resposta inflamatória, citocinas promovem o crescimento do tumor por meio da indução de angiogênese ou desencadeamento de cascatas de sinalização que ativam NFKB e STAT3. Além disso em um microambiente tumoral, a inflamação pode inibir ou promover o crescimento tumoral, isso irá depender da combinação de células imunes que estarão presentes e dos fatores de sinalização secretados (GALVÃO & ZONG, 2013). Os tumores e metástases cerebrais induzem alterações na remodelação do tecido cerebral que causam imunossupressão e desencadeiam uma resposta inflamatória no microambiente tumoral. Essas alterações colaboram com a invasão e metástase tumoral (ROESLER et al., 2021).

Uma das características comuns do GB é a necrose do tecido seguida de inflamação do microambiente. As células de GB expressam e secretam quimiocinas e citocinas imunossupressoras, incluindo IL-6, IL-10, fator de crescimento transformador (TGF-β), galectina-1 e prostaglandina-E, que atuam na infiltração de células imunes induzindo um fenótipo celular pró-tumoral. Esse quadro leva ao estímulo da proliferação de células de GB, migração, angiogênese e resistência ao tratamento (WATERS et al., 2019).

Em vista disso, torna-se de grande relevância o estudo de novos alvos terapêuticos que associem o GB e seu perfil neuroinflamatório.

3.4. Estresse Oxidativo

O estresse oxidativo é caracterizado por um desequilíbrio entre a produção de radicais livres e metabólitos reativos (oxidantes ou EROS) e sua eliminação pelos sistemas antioxidantes, que irão atuar de maneira protetiva. Esse desequilíbrio resultará em danos às biomoléculas e células (ĎURAČKOVÁ, 2010). As EROS são produzidas durante processos metabólicos intracelulares e compreendem o ânion superóxido ($O_2^{\bullet -}$), radical hidroxil ($OH^{\bullet}$) e peróxido de hidrogênio (H_2O_2) (SOSA et al., 2013) dentre outras. Estudos sugerem que as células cancerígenas quando comparadas com as células normais encontram-se sob um maior estresse oxidativo,

pois apresentam um aumento da atividade metabólica, um mau funcionamento mitocondrial e, consequentemente, um aumento na produção de EROS (WU; KASSIE; MERSCH-SUNDERMANN, 2005). Além disso, essas espécies podem desempenhar papel de mensageiras nas vias de transdução de sinal celular, promovendo assim o crescimento e a proliferação celular, contribuindo para a tumorigênese (BEHREND; HENDERSON; ZWACKA, 2003). O SNC apresenta uma alta atividade metabólica e um alto teor de ácidos graxos, em consequência disso, torna-se sensível aos danos oxidantes causados pelas EROS. Já é bem descrito na literatura que no microambiente do GB há um desequilíbrio no sistema redox celular (SALAZAR-RAMIRO et al., 2016).

O tecido nervoso é particularmente vulnerável ao estresse oxidativo devido à alta demanda por oxigênio, o que leva a ineficiência de seus mecanismos de defesa contra os radicais livres, em conjunto com uma alta concentração de íons metálicos que estão envolvidos nas reações redox (HALLIWELL, 2014). Essa circunstância dentro do SNC tem sido correlacionada ao desenvolvimento de doenças neurodegenerativas como Alzheimer e Parkinson e também a vários tumores cerebrais (GUTOWSKI et al., 2013; RUBATTU et al., 2013). O SNC apresenta uma alta atividade metabólica e um alto teor de ácidos graxos, em consequência disso, torna-se sensível aos danos oxidantes causados pelas EROS.

No GB ocorre um aumento nos níveis basais de EROS, que irão atuar como mediadores químicos regulando a transdução do sinal e protegendo as células malignas da apoptose. Com isso, alternativas terapêuticas com potencial antioxidante poderiam reduzir o estresse oxidativo, atuando na prevenção de danos celulares e na alta taxa de mutação acompanhada de uma instabilidade cromossômica, o que reduziria o ambiente imunossupressor (SALAZAR-RAMIRO et al., 2016).

Com isso, torna-se evidente que a regulação dos mecanismos de defesa de radicais livres celulares (Figura 3) é de extrema importância, pois protege o organismo contra as lesões oxidativas. Eles incluem os sistemas enzimáticos antioxidantes como a catalase (CAT), a superóxido dismutase (SOD) e a glutationa peroxidase (GPx), que atuarão eliminando as EROS conferindo proteção ao organismo dos efeitos nocivos causados pelas mesmas (OZBEN, 2007). Como o ambiente redox desempenha um papel importante na iniciação, progressão e regressão do tumor, terapias que atuem na modulação dos mecanismos redox, vem

sendo de extremo interesse na busca de novos alvos para o tratamento do câncer. Além do envolvimento do sistema redox no GB, a sinalização purinérgica também vem sendo investigada, visto que, já foi relatado a relação desse sistema com o crescimento e invasão do GB (BRAGANHOL et al., 2020).

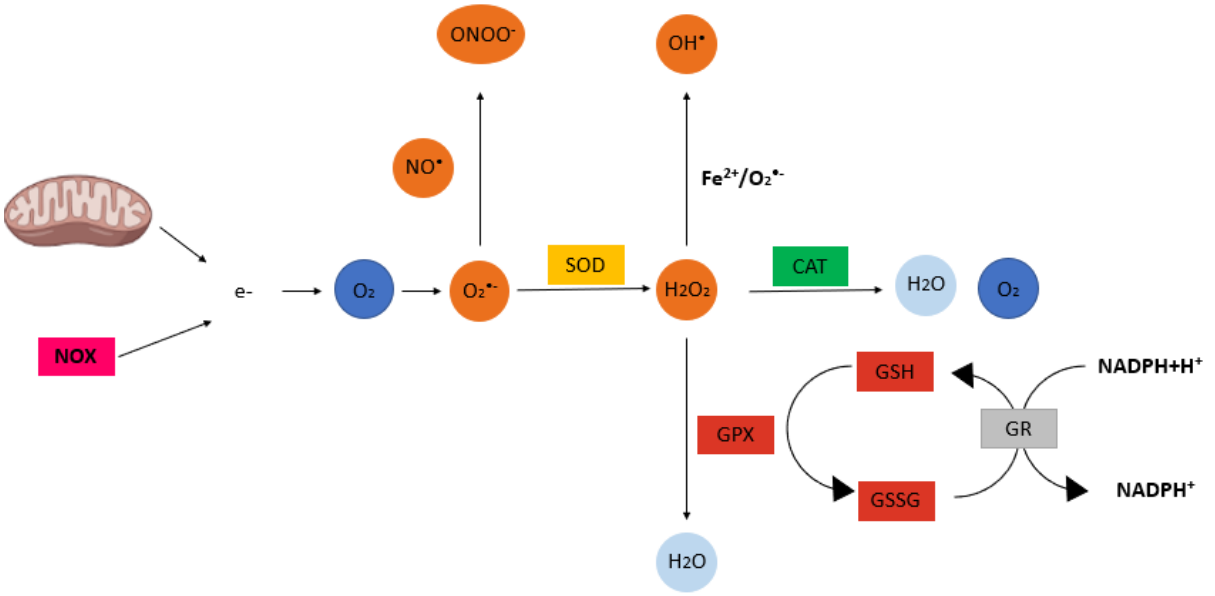

Figura 3. Ilustração da Homeostase redox. A cadeia transportadora de elétrons da mitocôndria e o complexo NADPH oxidase (NOX) que está ligado à membrana são fontes intracelulares importantes de espécies reativas de oxigênio (EROS). O ânion superóxido ($O_2^{\bullet-}$) é a principal fonte de ROS e pode ser rapidamente convertido em peróxido de hidrogênio (H_2O_2) pela enzima antioxidante superóxido dismutase (SOD) ou ainda formar peroxinitrito ($ONOO^-$) através da reação comóxido nítrico (NO•). O H_2O_2 pode ser catalisado ao radical hidroxila (OH•) ou pode ser convertido a H_2O e O_2 catalisado pelas enzimas antioxidantes catalase (CAT), glutationa peroxidase ou peroxiredoxinas. De modo a manter a homeostase redox, as células envolvem poderosos sistemas enzimáticos que eliminam EROS, entre elas a CAT, SOD, glutationa redutase (GR), glutationa reduzida (GSH) e glutationa oxidada (GSSG) (Figura desenvolvida pelo autor; Referenciado de SOSA et al., 2013)

3.5. Sistema Purinérgico

A neurotransmissão purinérgica (Figura 4) foi descrita por Burnstock em 1972, que atribuiu aos nucleotídeos e nucleosídeos de adenina a ação de mensageiros extracelulares, sendo responsáveis pela modulação da resposta celular (BURNSTOCK, 1972). O sistema purinérgico engloba três componentes principais, os nucleotídeos e nucleosídeos de adenina extracelulares, os receptores purinérgicos e as enzimas encarregadas pelos níveis extracelulares das moléculas referenciadas (YEGUTKIN, 2008).

Os nucleotídeos de adenina (trifosfato de adenosina – ATP, difosfato de adenosina – ADP e monofosfato de adenosina – AMP) e o seu correspondente

nucleosídeo, a adenosina, são uma importante classe de moléculas extracelulares pois desempenham diversos efeitos biológicos por meio da ativação de receptores purinérgicos (GIULIANI et al., 2019). Em condições fisiológicas normais, os nucleotídeos são encontrados no meio extracelular em concentrações baixas, porém quando o ATP se apresenta em altas concentrações pode-se observar a formação de poros nas membranas celulares, que irá promover mudanças osmóticas na célula e, por conseguinte levar a morte celular (DI VIRGILIO et al., 2001).

É sabido que o ATP intracelular é direcionado primariamente para processos energéticos, no entanto, quando exposto a episódios de injúria, dano celular, apoptose, entre outros, esse nucleotídeo se direciona para o meio extracelular, passando a ser considerada uma importante molécula sinalizadora de dano celular (YEGUTKIN, 2014). Os nucleotídeos e nucleosídeos de adenina, além da ação como neurotransmissores, podem atuar de diversas maneiras como agentes tróficos no SNC (RATHBONE et al., 1999). Além disso desempenham um importante papel no controle da proliferação, migração e diferenciação de astrócitos e neurônios (RYU et al., 2003). Quando os nucleotídeos são liberados para o espaço extracelular, ocorre uma interação com os receptores transmembrana ou sofrem hidrólise através da ação de ecto-enzimas (DI VIRGILIO et al., 2018).

Sabe-se que os nucleotídeos e nucleosídeos de adenina executam suas funções biológicas através da interação com duas famílias de receptores purinérgicos, denominados P1 e P2 e que são encontrados na superfície de diversas células, (RALEVIC; BURNSTOCK, 1988; JACOB et al., 2013). Os receptores P1 (A1, A2A, A2B e A3), são acoplados a proteína G e são sensíveis a adenosina (ROBSON et al., 2006). Já os receptores do tipo P2, apresentam preferência pelos nucleotídeos di e trifosfatados (ATP e ADP), e são subdivididos em duas subclasses, o P2X (ionotrópicos) e o P2Y (metabotrópicos). Os receptores P2X ainda são subdivididos em sete subtipos (1, 2, 3, 4, 5, 6 e 7), que são caracterizados por apresentarem o ATP como seu principal ligante, já os receptores P2Y apresentam 8 subtipos (1, 2, 4, 6, 11, 12, 13 e 14) e são sensíveis ao ADP, ATP (WHITE; BURNSTOCK, 2006).

Os eventos de sinalização induzidos pelos nucleotídeos de adenina extracelulares são controlados pela ação das ectonucleotidases, que são enzimas ancoradas na membrana celular ou localizadas no meio intersticial de maneira solúvel. Essas enzimas são divididas em quatro famílias, as ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPPs), as ecto-nucleosídeo difosfoidrolases (E-

NTPDases), as fosfatases alcalinas e ecto-5’-nucleotidases. Essas ecto-enzimas são caracterizadas por constituírem uma cascata enzimática altamente organizada, responsável pela regulação das vias de sinalização mediadas pelos nucleotídeos e nucleosídeos de adenina, sendo assim, agem controlando a quantidade e o tempo de degradação dos nucleotídeos (ZIMMERMANN et al., 2012). As E-NTPDases e a E-NPP iniciam a cadeia/cascata de sinalização, onde catalisam a hidrólise do ATP e do ADP gerando AMP. Por conseguinte, a enzima ecto-5’-nucleotidase hidrolisará a molécula de AMP formando adenosina, que será degradada pela ADA, gerando inosina a qual é convertida em hipoxantina, que em seguida é oxidada a xantina e por fim convertida em ácido úrico (YEGUTKIN, 2008).

O sistema purinérgico tem sido alvo de diversos estudos e está interligado à uma série de patologias diferentes apresentando relevantes ações terapêuticas, como na osteoporose, infarto do miocárdio, síndrome do intestino irritável, aterosclerose, diabetes e no câncer (BURNSTOCK, 2017). Dentre os constituintes do sistema purinérgico, um deles apresenta destaque e tem sido amplamente estudado frente a doenças do SNC, o receptor P2X7 (P2X7R), devido a sua ação na sinalização inflamatória e de ativação microglial (BURNSTOCK, 2017; BURNSTOCK, 2020).

O ATP está envolvido na estimulação e proliferação de linfócitos, além de fazer parte daprodução de citocinas pró-inflamatória, como as interleucinas (SEVIGNY et al., 2015). Já o ADP é o principal agonista envolvido no recrutamento e agregação plaquetária (KUMAR & SHARMA, 2009).

Estudos sugerem que alterações na ativação dos receptores purinérgicos e na atividade das enzimas envolvidas no metabolismo extracelular de nucleotídeos estejam envolvidas na patologia dos gliomas (BRAGANHOL et al., 2009). Além disso, ao contrário do que ocorre nos astrócitos, os gliomas apresentam uma baixa expressão e atividade de NTPDases, o que leva ao favorecimento de acúmulo de ATP no meio extracelular (WINK et al., 2003). Ainda, esse nucleotídeo demonstra uma indução de estímulo proliferativo nos gliomas, sendo citotóxico para células normais (MORRONE et al., 2005).

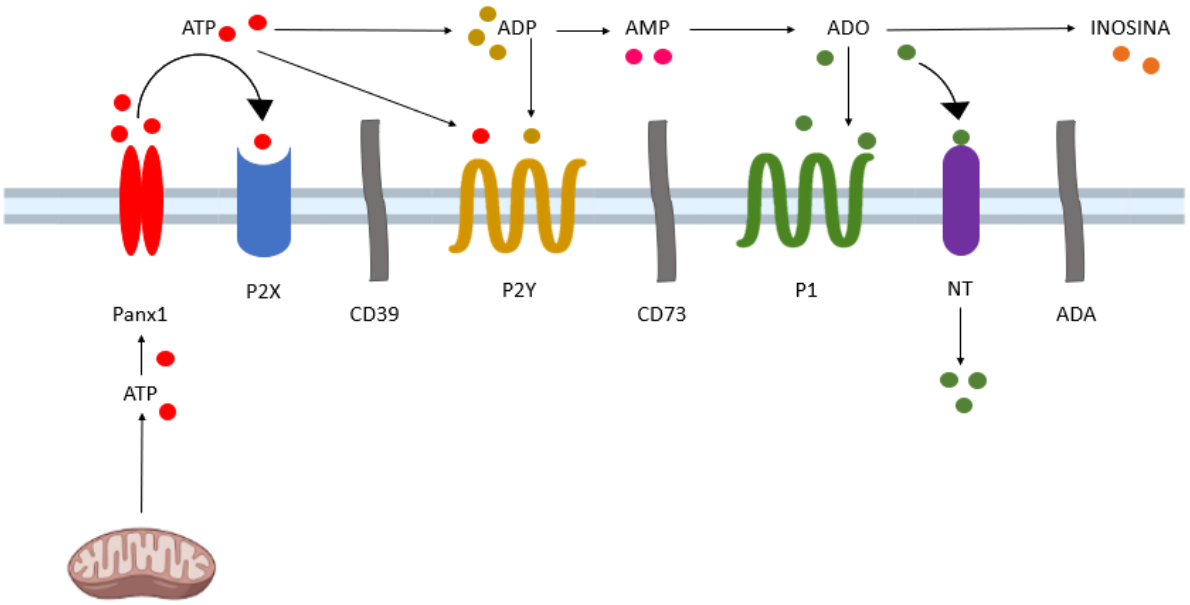

Figura 4. Sinalização Purinérgica. A ativação de receptores leva à abertura de canais e ocorre a liberação do trifosfato de adenosina (ATP). O ATP liberado promove a ativação dos receptores purinérgicos do tipo 2 (P2). As ectonucleotidases, como a ectoenzima trifosfato de nucleosídeo fosfohidrolase (CD39) e a ectoenzima 5'-nucleotidase (CD73) promovem a hidólise de ATP e a formação de adenosina (ADO), que irá ativar os receptores purinérgicos do tipo 1 (P1). A adenosina é neutralizada pela adenosina desaminase (ADA), que irá converter adenosina em inosina (Figura desenvolvida pelo autor; Referenciado de YEGUTKIN, 2008)

3.6. Fármacos Originados de Produtos Naturais

Historicamente, os produtos naturais desempenham um papel importante na prevenção e cura de diversas doenças (RAO et al. 2019). São originados de diferentes fontes e abrangem um número significativo de plantas e microorganismos (NEWMAN et al., 2000). Entre os anos de 1983 e 1994, foram aprovados 520 novos medicamentos, dentre eles 39% eram de produtos naturais ou derivados desses produtos e 60% a 80% dos antibacterianos e anticancerígenos eram derivados de produtos naturais (HARVEY, 2000). Os produtos naturais e/ou estruturas oriundas de produtos naturais continuam desempenhando um papel fundamental no processo de descoberta e desenvolvimento de novos fármacos (NEWMAN; CRAGG, 2012). Ao longo do período de 1940 a 2014, na área do câncer foram aprovadas 175 pequenas moléculas, 85 eram produtos naturais ou derivados deles (NEWMAN; CRAGG, 2016).

A toxicidade de fármacos bem estabelecidos consiste em um dos principais problemas na terapêutica do câncer, sendo necessária a busca de métodos

alternativos para o tratamento de neoplasias malignas. Neste contexto, as plantas apresentam-se como fontes importantes de compostos quimiopreventivos (KEUM et al., 2016). Cabe destacar que, na área de anticancerígenos, agentes vegetais como a vimblastina, vincristina, etoposídeo, paclitaxel, docetaxel, topotecano e irinotecano, estão entre os mais eficientes quimioterápicos atualmente disponíveis (NEWMAN; CRAGG et al., 2014).

3.7. *Cecropia pachystachya* Trécul

A Cecropia é uma das plantas amplamente utilizadas na medicina popular brasileira, pertencente à família Urticaceae, é conhecida popularmente como embaúba, imbaúba, umbaúba e embaúva. Apresenta uma maior recorrência nas regiões sul e sudeste do Brasil (BERG; ROSSELI, 2005; COSTA et al., 2011).

No extrato aquoso das folhas de CEC foi possível identificar como compostos majoritários o ácido clorogênico, isoorientina, orientina, isovitexina e isoquercitina (COSTA et al., 2011). O extrato de CEC apresenta diversas atividades bem elucidadas na literatura, como hipoglicemiante, antimalárica, antinociceptiva, citotóxica e leishmanicida (UCHÔA et al., 2009; ARAGÃO et al., 2010; ARAGÃO et al., 2013; CRUZ et al., 2013).

O extrato metanólico de CEC apresentou uma atividade anti-inflamatória *in vivo* em modelo de edema de orelha induzido pelo óleo de cróton. Nesse estudo, a dose de 300 mg/kg foi capaz de inibir o edema em 85% (ARAGÃO et al., 2013). Ainda, extrato metanólico de CEC *in vitro* demonstrou uma eficiente ação antioxidante nos ensaios de DPPH (1,1-difenil-1-picrilhidrazil) e TBARS (substâncias reativas ao ácido tiobarbitúrico) (VELÁZQUEZ et al., 2003; ARAGÃO et al., 2010). Pacheco et al. (2014) realizaram o tratamento com extrato metanólico em modelo *in vivo* de edema de orelha induzido pelo óleo de cróton e o tratamento demonstrou atividade anti-inflamatória tópica. O extrato etanólico das folhas de CEC também foi eficaz, *in vivo,* no tratamento da hipertensão, inflamação e doença renal progressiva. Os animais tratados apresentaram uma diminuição da pressão arterial sistólica e uma redução na taxa de filtração glomerular, além disso o tratamento com o extrato levou a redução na expressão e na atividade da arginase renal e reduziu o número de macrófagos no córtex renal (MAQUIAVELI et al., 2014). Ademais, um gel contendo extrato de acetato de etila das folhas de CEC demonstrou ação

antioxidante *in vitro* e cicatrizante de feridas em estudo com modelo animal (DUQUE et al., 2016).

Além disso, o extrato aquoso de CEC possui ações farmacológicas bem descritas na literatura, como atividade tipo-antidepressiva e tipo-ansiolítica, avaliada através do modelo animal de estresse crônico imprevisível. Esses efeitos foram atribuídos aos compostos majoritários identificados no extrato, como os flavonoides C-glicosídeos orientina e isovitexina, O-glicosídeo isoquercitrina e ácido clorogênico (GAZAL et al., 2014). Adicionalmente, Gazal e colaboradores (2015) demonstraram o papel neuroprotetor e antioxidante do extrato aquoso de CEC em modelo animal de mania induzida em ratos. É importante destacar que a toxicidade desse extrato foi avaliada em camundongos tratados pela via oral durante 16 dias e nenhuma alteração foi relatada no comportamento dos animais, bem como nenhuma mudança histológica ocorreu no rim e fígado (BIGLIANI et al., 2010).

A CEC é composta por diversas classes de compostos fenólicos, aos quais normalmente são atribuídas as atividades farmacológicas descritas para essa espécie. Os flavonoides são considerados os compostos majoritários presentes no extrato aquoso das folhas de CEC (COSTA et al., 2011).

Os flavonoides pertencem ao grupo de compostos fenólicos mais importante e diversificado entre os produtos de origem natural (SIMÕES et al., 2007). A maioria dos flavonoides possui uma estrutura química formada por 15 átomos de carbono constituído por dois anéis de benzeno ligados por uma cadeia com três carbonos entre elas e um oxigênio (Figura 6).

Dentre as diversas atividades biológicas relatadas pelos flavonoides, eles têm sido estudados devido sua atividade inibidora de células cancerosas, por desempenharem propriedades farmacológicas antioxidantes, controlando dessa forma a proliferação celular e bloqueando a oncogênese (AMADO et al., 2011). Além disso, os flavonoides vêm sendo relatados como importantes neuroprotetores (AIROLDI et al., 2018).

Considerando as ações biológicas já relatas do extrato de CEC e dos compostos fenólicos, foi elaborada uma fração enriquecida em flavonoides, como uma importante alternativa terapêutica na prevenção e reversão de doenças (ORTMANN et al., 2016). Estudos evidenciaram que a fração enriquecida em flavonoides obtida do extrato aquoso de folhas de CEC exerce efeito tipo-

antidepressivo *in vivo* e que a regulação da homeostase redox parece desempenhar um importante papel neuroprotetor (ORTMANN et al., 2016).

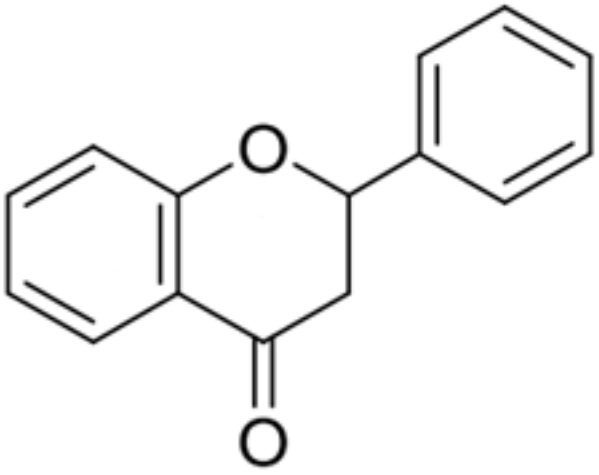

Figura 5. Estrutura química dos flavonoides (Fonte: O autor)

3.8. Ácido Tânico

Os taninos são pertencentes a um grupo dos polifenois (CHUNG & REED, 2012) originados do metabolismo secundário das plantas (BUTLER et al., 1984) e são caracterizados por serem polímeros fenólicos com uma alta solubilidade em água e por possuírem capacidade de precipitar proteínas (HASLAM, 1989). Os polifenois são caracterizados por constituírem um grande grupo de compostos orgânicos. São biossintetizados naturalmente por plantas e organismos marinhos. Entre os polifenois estão os flavonoides, ácidos fenólicos, e estilbenos. São de grande interesse da comunidade científica frente as suas propriedades biológicas (SILVA et al., 2020). Os ácidos fenólicos vêm sendo bastante estudados devido as suas diversas atividades descritas na literatura na prevenção de doenças como doenças cardiovasculares, osteoporose, diabetes mellitus, doenças neurodegenerativas e até no tratamento do câncer (SCALBERT et al., 2005; SILVA et al., 2020).

Pesquisas demonstram que vários taninos atuam como captadores de radicais, interceptando oxigênio ativo e formando radicais estáveis, e assim, por sua vez reduzindo o estresse oxidativo (SIMÕES, 2007). Os potenciais anticancerígenos

e antimutagênicos dos taninos, podem estar relacionados à sua atividade antioxidante, que consequentemente torna-se importante na proteção do dano oxidativo (CHUNG et al., 2010).

O AT (Figura 5) é um tanino natural pertencente ao grupo dos ácidos fenólicos que compreende uma molécula de glicose central, e possui a fórmula molecular $C_{76}H_{52}O_{46}$. Apresenta cinco cadeias formadas por duas moléculas de ácido gálico ligadas por esterificação, a molécula de glicose (AELENEI et al., 2009). É produzido a partir do metabolismo secundário das plantas e é encontrado em diversos alimentos, como lentilhas, chocolates, café, chá preto e chá verde, uvas, vinho tinto entre outros (KUO et al., 1992).

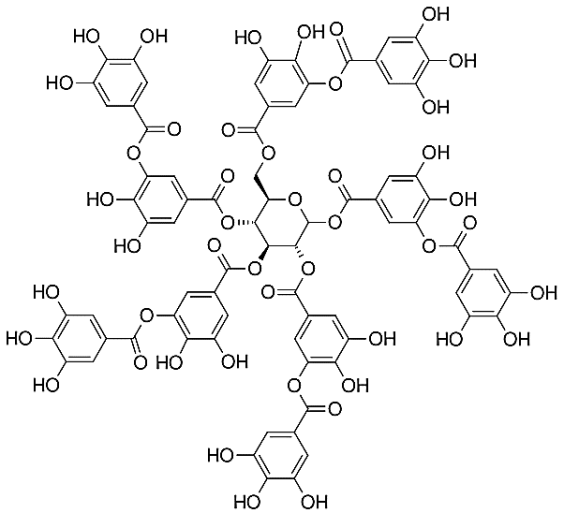

Figura 6. Estrutura química ácido tânico (Fonte: O autor)

Possui uma importante ação antioxidante devido a capacidade de quelar e sequestrar radicais livres (SAH et al., 1995). Estudos apontam os mecanismos envolvidos no AT, conferindo a ele diversas propriedades medicinais, como efeitos anticancerígenos, atuando no controle de células tumorais (HL-60 de leucemia humana) e induzindo apoptose (CHEN et al., 2009), além de demonstrar efeitos no SNC, como exercendo um papel antioxidante contra efeitos negativos causados pela isquemia cerebral (SEN et al., 2015). Além de ser caracterizado como um importante agente neuroprotetor, pois demonstra atividade antioxidante e anti-inflamatória em modelo animal de acidente vascular cerebral (ASHAFAQ et al., 2017). O AT

apresentou efeitos neuroprotetores em modelos animais de depressão e doença de Alzheimer (GERSZON et al., 2020; LUDUVICO et al., 2020).

Além disso, Zhang e colaboradores (2018) demonstraram que a AT induz a morte celular por apoptose em células de glioma humano (Hs 683), sugerindo que o mecanismo envolvido compreende a ativação de pró-caspase 3 e caspase 9 além da clivagem em poli (ADP-ribose) polimerase. Este resultado vem de encontro ao aumento do número de células sub-G1, indicativo de fragmentação de DNA e, com isso, morte celular apoptótica (WILLIAMS et al., 2012). O AT também foi descrito como indutor de parada no crescimento de células na fase G1, levando à morte celular por apoptose em células Jurkat T, sugerindo um mecanismo relacionado com a inibição da atividade do proteassoma (NAM et al., 2001).

4 RESULTADOS

Os resultados pertencentes a esta tese serão apresentados na forma de um artigo (Capítulo 1), dois manuscritos (Capítulo 2 e 3) e resultados adicionais (Apêndice). Os materiais e métodos, resultado e discussão encontram-se nos próprios artigos e manuscritos.

Ao fim desta tese, encontra-se uma discussão integrada com comentários gerais do artigo e manuscritos e por fim a conclusão e referências utilizadas para elaboração da mesma.

4.1. Capítulo 1 – Artigo 1

Tannic Acid Attenuates Peripheral and Brain Changes in a Preclinical Rat Model of Glioblastoma by Modulating Oxidative Stress and Purinergic Signaling

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ORIGINAL PAPER

Tannic Acid Attenuates Peripheral and Brain Changes in a Preclinical Rat Model of Glioblastoma by Modulating Oxidative Stress and Purinergic Signaling

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Abstract
Glioblastoma (GB) is a highly aggressive and invasive brain tumor; its treatment remains palliative. Tannic acid (TA) is a polyphenol widely found in foods and possesses antitumor and neuroprotective activities. This study aimed to investigate the effect of TA on oxidative stress parameters and the activity of ectonucleotidases in the serum, platelets, and lymphocytes and/or in the brain of rats with preclinical GB. Rats with GB were treated intragastrically with TA (50 mg/kg/day) for 15 days or with a vehicle. In the platelets of the animals with glioma, the adenosine triphosphate (ATP) and adenosine monophosphate (AMP) hydrolysis and the catalase (CAT) activity decreased. Besides, the adenosine diphosphate (ADP) hydrolysis, adenosine (Ado) deamination, and the reactive oxygen species (ROS) and nitrite levels were increased in glioma animals; however, TA reversed ROS and nitrite levels and AMP hydrolysis alterations. In lymphocytes from animals with glioma, the ATP and ADP hydrolysis, as well as Ado deamination were increased; TA treatment countered this increase. In the brain of the animals with glioma, the ROS, nitrite, and thiobarbituric acid reactive substance (TBARS) levels increased and the thiol (SH) levels and CAT and superoxide dismutase (SOD) activities were decreased; TA treatment decreased the ROS and TBARS levels and restored the SOD activity. In the serum of the animals with glioma, the ATP hydrolysis decreased; TA treatment restored this parameter. Additionally, the ROS levels increased and the SH and SOD activity decreased by glioma implant; TA treatment enhanced nitrite levels and reversed SOD activity. Altogether, our results suggest that TA is an important target in the treatment of GB, as it modulates purinergic and redox systems.

Keywords Cancer · Glioma · Polyphenols · Tannin · Ectoenzymes · Redox status

Introduction

Approximately 57% of all gliomas and 48% of primary brain cancers are glioblastoma (GB), and these can be subdivided into GB (IDH–wild-type) and GB (IDH mutant). Both conditions exist as astrocytic tumors with areas of microvascular proliferation and/or focal necrosis [1]. The known incidence is low as patients succumb to the disease even before the manifestation of clinical symptoms that include seizures, headache, nausea and vomiting, and neck stiffness associated with low back pain [2].

Even with the standard multimodal therapy adopted in cases of GB, including maximum safe resection, radiotherapy, and chemotherapy, the prognosis remains fatal with an average overall survival of 14 to 20 months [3]. The pathophysiology is complex and multifactorial and involves,

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among other mechanisms, redox status and inflammation with various signaling systems such as purinergic signaling [4–6].

It is well established that reactive oxygen species (ROS) control cell stability and thus influence different signaling pathways. Cellular antioxidant enzymatic and non-enzymatic mechanisms are highly impaired [4]. ROS generation in tumor cells affects the cell cycle and is involved in tumor progression and resistance. GB exhibits a high metabolic rate and produces high levels of ROS, and therefore, it is of interest to reduce the production of these species [4, 6]. However, it is established that high levels of ROS also act in the metabolic adaptation of these cells, ultimately playing an essential role in the resistance to cell death induced by oxidative stress [4, 7]. Thus, the search for therapeutic alternatives that act on different targets such as purinergic signaling is important.

Purinergic signaling is mediated by adenine nucleotides and nucleosides that are released by a variety of cell types in response to signs of stress and inflammatory conditions in the tumor microenvironment [8]. These molecules are metabolized by ectonucleotidases, including NTPDases, that hydrolyze ATP and ADP to AMP. Specifically, ecto-5'-nucleotidase converts AMP to adenosine (Ado), and adenosine deaminase (ADA) deaminates Ado in inosine [9, 10]. Therefore, these ectoenzymes play a crucial role in maintaining or halting purinergic signaling by controlling the extracellular levels of adenine nucleotides and nucleosides. In agreement with this knowledge, studies have suggested that ecto-5′-nucleotidase (known as CD73) silencing or enzyme inhibition decreases GB growth in vitro and in vivo [6].

Data from the literature have shown several changes in blood associated with GB, including vascular proliferation, cell growth, inflammation, the immune system, and coagulation [11]. Blood biomarkers of these changes are intrinsically related to the exacerbated production of ROS and also to changes in purinergic signaling [11].

A multitude of preclinical and clinical studies examining GB have explored promising therapies with targeted agents [12]. The limited efficiency and serious side effects associated with the use of conventional anticancer therapies have encouraged scientists to focus on the discovery and development of new anticancer agents that are derived from natural products [13, 14]. Tannic acid (TA), a naturally occurring plant polyphenol with anti-inflammatory, antioxidant, and neuroprotective properties, is also known to induce the death of cancer cells in various types of cancer [15–17]. Interestingly, it has recently been reported in an in vitro and preclinical study that TA can induce cell death due to apoptosis of the GB lineage in rats and humans [16, 18]. Bona et al. [16] demonstrated that TA treatment in vitro reduced the cell viability and proliferation of GBs and modulated the redox status, and the administration of TA also significantly decreased the size of the tumor in rats. Thus, the aim of this study was to investigate the protective effect of TA on oxidative stress parameters and on the activity of ectonucleotidases in serum, platelets, and lymphocytes and/or in the brain of rats subjected to a preclinical model of GB.

Materials and Methods

Chemical

Dulbecco's modified Eagle's medium (DMEM), Fungizone (amphotericin B), penicillin/streptomycin, 0.5% trypsin/EDTA solution, and fetal bovine serum (FBS) were obtained from Gibco BRL (Carlsbad, CA, USA). TA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals and solvents used for this study were of analytical or pharmaceutical grade.

Ethical Approve Committee and Animals

Thirty male Wistar rats aged 60 days (250–300 g) were used for the preclinical protocol of GB. The experimental design was approved by the Animal Experimentation Ethics Committee on Animal Experimentation of the institution (CEEA 31292-2018). The animals were used in accordance with the Brazilian Guidelines for the Care and Use of Research Animals in Sciences (DBCA) and the National Council for Animal Control and Experimentation (CONCEA). Wistar rats were obtained from the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. The animals were maintained at a constant temperature (22 ± 1 °C) with a 12/12 h light/dark cycle with ad libitum access to food and water.

Experimental Protocol

C6 rat glioma cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured in DMEM with 5% FBS that was maintained at 37 °C and 5% CO_2 according to the method described by Bona et al. [16]. C6 cells were cultured, and after reaching 90% confluence at a ratio of 1×10^6, the cells were resuspended in 3 µL of DMEM and infused at a rate of 0.5 µL/min into the right striatum of the experimental animals (coordinates relative to bregma, 3.0 mm lateral, 0.5 later and 6 mm depth).

Five days after intracerebroventricular (icv) C6 cell implantation, the animals were divided into three groups that included (1) Sham (DMEM icv; vehicle), (2) GB (C6 cells icv) + Vehicle and (3) GB (C6 cells icv) + TA (50 mg/kg/day). The vehicle and TA groups were intragastrically administered the treatments for 15 days (Fig. 1). The TA was diluted with distilled water and the dose administered

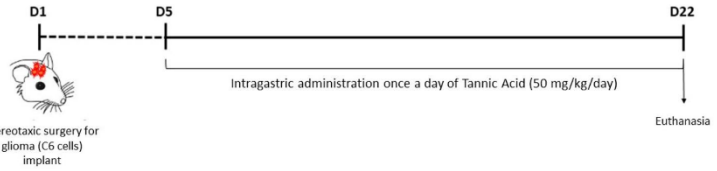

Fig. 1 Experimental design. Glioma implantation with C6 cells and treatment with tannic acid (TA) at 50 mg/kg/day for 15 days

was chosen based on previous data [19, 20]. At 21 days after tumor implantation, the animals were euthanized, and the brain, serum, platelets, and lymphocytes were collected for biochemical analysis [16].

Sample Preparation and Protein Determination

Serum

For serum, blood samples were collected in tubes without anticoagulant and subsequently centrifuged at 2500×*g* for 15 min at room temperature. The clot was removed, and the serum was stored at − 80 °C and then used for biochemical determinations. Protein levels were measured as previously described by Bradford [21].

Lymphocytes

Blood was collected with EDTA anticoagulant and diluted in an equal volume of saline for lymphocyte separation using Ficoll-Histopaque density gradients. After this, the lymphocyte preparation was washed with saline and centrifuged three times. The methodology described by Böyum [22] was employed to separate mononuclear cells; however, in the study performed by Jaques et al. [23], it was demonstrated that there is a high incidence of lymphocytes (about 95%) in these samples, and the amount of monocytes was practically insignificant. Therefore, we treated the samples as lymphocyte preparations. Protein concentrations were measured as described by Bradford [21].

Platelets

For platelet-rich plasma (PRP) preparation, total blood was collected using 0.120 M sodium citrate as an anticoagulant [24]. Then, it was centrifuged at 160×*g* for 10 min at room temperature. Next, PRP was centrifuged at 1400×*g* for 30 min and washed twice with 3.5 mM 4-(2-hydroxyethyl)-1 piperazine ethanesulfonic acid (HEPES) buffer (pH 7.0) and then resuspended in 200 µL of HEPES buffer for further analysis. Protein concentration was measured as described by Bradford [21].

Brain

After euthanasia by cardiac puncture, the brain was removed and stored at − 80 °C and used for determination of oxidative stress parameters. The brains were homogenized (1/10 w/v) using 20 mM sodium phosphate buffer (pH 7.4) containing 140 mM KCl. The homogenates were centrifuged at 2500×*g* for 10 min at 4 °C. The supernatants were collected and used for further analysis. The protein concentration was measured as described by Lowry et al. [25].

Ectonucleotidases Activity Assay

NTPDase and 5′-Nucleotidases Activities in Serum

NTPDase and 5-nucleotidase activity in the serum were determined according to Fürstenau et al. [26]. Serum samples were preincubated for 10 min at 37 °C in a reaction medium containing 112.5 mM Tris-HCl, pH 8.0. To initiate the reactions, ATP, ADP, and AMP were added to the medium at a final concentration of 3.0 mM and then incubated for 40 min. Finally, 10% TCA was used to halt the enzymatic reactions. The amount of inorganic phosphate (Pi) that was released was measured using the method described by Chan et al. [27]. Enzyme activity was expressed as nmol Pi released/min/mg of protein.

NTPDases Activities in Lymphocytes

This assay was performed as described by Leal et al. [28]. The intact lymphocyte cells suspended in saline solution were added to the reaction medium (0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose, and 50 mM Tris–HCl buffer, pH 8.0) and incubated for 70 min. The reaction was initiated by the addition of a substrate (ATP or ADP) and halted with TCA 10%. The released Pi was assayed using the method described by Chan et al. [27]. The specific activity was reported as nmol Pi released/min/mg of protein.

NTPDases and 5′-Nucleotidase Activities in Platelets

For the NTPDase enzymatic assay, platelets were preincubated for 10 min at 37 °C in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose,

and 50 mM Tris–HCl buffer at pH 7.4. For 5'-nucleotidase activity, 5 mM $CaCl_2$ was replaced with 10 mM $MgCl_2$ in the above-mentioned reaction medium. The reaction was initiated by the addition of ATP or ADP (final concentration of 1 mM) and AMP (final concentration of 2 mM). After 60 min of incubation, the assays were halted with 10% TCA [24, 29]. The released Pi was assayed using the method described by Chan et al. [27]. Enzymatic specific activities were reported as nmol Pi released/min/mg of protein.

Adenosine Deaminase (ADA) Activity

Adenosine Deaminase activity was measured by Giusti and Galanti [30] in serum, platelets, and lymphocytes. The samples were subjected to reaction with 21 mmol/L Ado (pH 6.5) and incubated at 37 °C for 60 min. Specific ADA activity is expressed as U/L. One unit (1 U) of ADA was defined as the amount of enzyme required to release 1 mmol ammonia/min from Ado under standard assay conditions.

Oxidative Stress Parameters Analysis

Reactive Oxygen Species (ROS)

The oxidation of DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) was measured. ROS formation was expressed as µmol DCF/ mg of protein according to the method of Ali et al. [31].

Nitrite

Nitrite was measured by the Griess reaction [32] and was assessed at 540 nm using sodium nitrite as the standard. The results are expressed as µM nitrite/mg of protein.

Total Thiol Content

Samples were added to PBS buffer (pH 7.4) containing EDTA and DTNB. One hour after incubation in the dark, DTNB reduced by thiol groups was oxidized (disulfide), and a yellow derivative (TNB) was generated and measured [33]. The results were reported as nmol TNB/mg of protein.

Thiobarbituric Acid Reactive Substances (TBARS)

For this assay, the samples were mixed with 10% TCA and centrifuged. The supernatant was collected, mixed with TBA (0.67%), and incubated in a dry block at 100 °C for 30 min [34]. TBARS levels were reported as nmol of TBARS/mg of protein.

Superoxide Dismutase (SOD)

This assay is based on the inhibition of superoxide-dependent adrenaline auto-oxidation to adenochrome as described by Misra and Fridovich [35]. The intermediate in this reaction is superoxide that is scavenged by SOD, and the absorbance was measured at 480 nm. SOD activity was reported as units/mg of protein.

Catalase (CAT)

The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored at 240 nm for 180 s at 37 °C as previously reported by Aebi [36]. CAT activity was reported as units/mg of protein.

Glutathione S-Transferase (GST)

Glutathione S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate as described by Habig et al. [37]. Activity was expressed as µmol GS-DNB/min/mg of protein.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc tests using GraphPad Prism 5.0 (Intuitive Software for Science, São Diego, CA, USA). Statistical significance was set at $P < 0.05$. All data are expressed as the mean ± standard error of the mean (SEM).

Results

TA50 Attenuates Oxidative and Purinergic Changes Observed in Serum

The results obtained in serum are presented in Table 1. Regarding ectonucleotidase activity, it was observed that the animals in the control group exhibited a decrease in ATP hydrolysis compared to sham group ($F_{2-17} = 11.05$, $P < 0.05$). However, treatment with TA50 was able to protect against this change ($F_{2-17} = 11.05$, $P < 0.001$). No significant alterations were observed in ADP ($F_{2-15} = 0.55$, $P > 0.05$) and AMP ($F_{2-17} = 0.60$, $P > 0.05$) hydrolysis, or Ado deamination ($F_{2-13} = 1.29$, $P > 0.05$).

In relation to oxidative stress parameters, it was observed that ROS production was enhanced in the control group compared to levels in sham animals, and treatment with TA50 was unable to significantly mitigate this increase ($F_{2-10} = 5.40$, $P > 0.05$). Nitrite levels were increased in the TA50 group compared to levels in

Table 1 Analysis of parameters of oxidative stress and purinergic system in serum rats submitted to implant (icv) of glioma C6 (control) and treated with tannic acid (TA) (50 mg/kg/day) for 15 days

Serum	Sham	Control	TA50
ATP hydrolysis	2.16 ± 0.3	1.35 ± 0.1$^{\#}$	2.70 ± 0.2***
ADP hydrolysis	3.18 ± 0.4	2.78 ± 0.2	3.22 ± 0.2
AMP hydrolysis	2.78 ± 0.3	2.52 ± 0.2	2.90 ± 0.1
Ado deamination	9.59 ± 1.8	10.34 ± 1.8	14.48 ± 0.4
ROS levels	43.78 ± 8.79	123.9 ± 27.03$^{\#}$	91.32 ± 16.4
Nitrite levels	6.81 ± 0.6	20.4 ± 5.01	71.15 ± 13.4***, ###
SH content	115.7 ± 27.9	60.65 ± 8.1$^{\#}$	50.41 ± 3.0$^{\#}$
SOD activity	11.35 ± 1.7	2.52 ± 0.72$^{\#\#\#}$	8.46 ± 0.64*
GST activity	2408 ± 773.4	2637 ± 802.8	2024 ± 701.1

Results of NTPDase and 5-nucleotidase activities are expressed in nmol Pi/min/mg of protein, and ADA activity is expressed as U/L. ROS levels are expressed as µmol DCF/mg of protein, nitrite levels are expressed as µM nitrite/mg of protein, SH content is expressed as nmol TNB/mg of protein, SOD activity is expressed as U/mg of protein, and GST is expressed as µmol GS-DNB/min/mg of protein. Values represent the mean ± SEM (n = 4–7)

$^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared to sham group and $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the control group

the sham and control groups ($F_{2-11} = 39.38$, $P < 0.001$). Additionally, the SH content was significantly reduced in the control and TA50 groups compared to that in the sham group ($F_{2-12} = 5.6$, $P < 0.05$). For SOD activity, it was demonstrated that TA50 ($F_{2-9} = 16.12$, $P < 0.01$) protected against the reduction in SOD activity caused by GB implants. No changes were observed in GST activity ($F_{2-12} = 0.13$, $P > 0.05$, Table 1).

TA 50 Protects Against Changes in Ectonucleotidases and Oxidative Parameters in Platelets

Figure 2 presents the hydrolysis of ATP, ADP, AMP and Ado deamination evaluated in the platelets. As presented in the Fig. 2, we observed a reduction in the ATP ($F_{2-13} = 15.68$, $P < 0.05$) and AMP ($F_{2-7} = 25.62$, $P < 0.01$) hydrolysis in the control group compared to those in the sham group. However, treatment with TA50 attenuated the decrease in the hydrolysis of these nucleotides. In contrast, there was an increase in ADP ($F_{2-11} = 7.32$, $P < 0.05$) hydrolysis and Ado ($F_{2-14} = 6.13$, $P < 0.01$) deamination in the control group compared to that in the sham, and the administration of TA50 protected ADP hydrolysis.

Figure 3 presents the oxidative stress parameters evaluated in the platelets. First, it was observed that the C6 implant enhanced ROS ($F_{2-9} = 10.17$, $P < 0.01$) and nitrite ($F_{2-10} = 7.16$, $P < 0.05$) levels in the control group compared to those in the sham group; however, treatment with TA50 reduced the increase in ROS ($P < 0.05$) and nitrite ($P < 0.05$) levels. Also, the treatment with TA50 protected against the reduction in CAT activity observed in the control group ($F_{2-12} = 6.10$, $P < 0.05$). No changes were observed in the SH content ($F_{2-13} = 0.43$, $P > 0.05$), SOD ($F_{2-11} = 0.22$, $P > 0.05$) and GST ($F_{2-10} = 0.06$, $P > 0.05$) activities.

TA50 Treatment Prevents Increased Hydrolysis of ATP and ADP, and the Degradation of Ado in Lymphocytes of Rats with Glioma

Figure 4 presents the hydrolysis of ATP and ADP, and Ado degradation evaluated in the lymphocytes. As demonstrated, an increase in ATP ($F_{2-18} = 18.67$, $P < 0.001$) and ADP ($F_{2-14} = 07.42$, $P < 0.01$) hydrolysis and in Ado deamination ($F_{2-23} = 15.33$, $P < 0.001$) was observed in the control group compared to levels in the sham animals; however, TA50 was able to protect against the hydrolysis of ATP ($P < 0.001$) and ADP ($P < 0.05$), as well as against Ado deamination ($P < 0.01$) (Fig. 4).

TA50 Protects Against Oxidative Damage in the Brain that is Induced by C6 Glioma Cell Implant

Figure 5 presents the oxidative stress parameters evaluated in the brain. ROS was enhanced in the control group when compared to sham ($F_{2-19} = 78.31$, $P < 0.001$). However, treatment with TA50 partially reversed this alteration ($P < 0.001$). There was an increase in the nitrite ($F_{2-18} = 42.15$, $P < 0.001$) levels in the control group compared to sham animals, and TA50 treatment did not protect against this enhance. An increase in the TBARS ($F_{2-21} = 9.2$, $P < 0.01$) levels in the control group was observed compared to levels in the sham group. However, treatment with TA50 reduced TBARS ($P < 0.05$) levels. There was a reduction in the SH content ($F_{2-21} = 10.99$, $P < 0.001$) and the SOD ($F_{2-18} = 14.27$, $P < 0.001$) and CAT ($F_{2-19} = 11.38$, $P < 0.01$) activities in the control group. The administration of TA50 ($P < 0.05$) reversed only the decrease in SOD activity. No changes were observed in GST activity ($F_{2-24} = 0.20$, $P > 0.05$) (Fig. 5).

Discussion

In this study, we demonstrated that the administration of TA in rats with GB modulates biochemical changes in both the brain and blood. The antitumor effects of TA have previously been reported in relation to several types of tumors [15, 17]; however, the protective role of TA against GB has

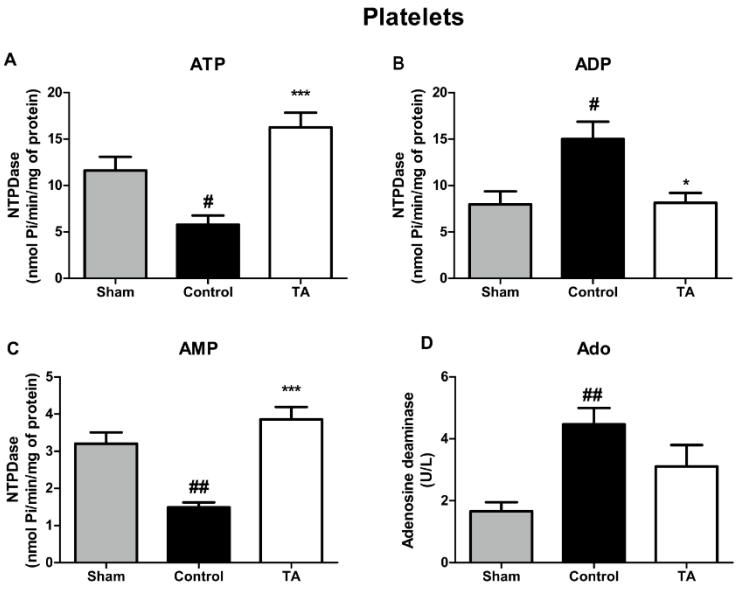

Fig. 2 Hydrolysis of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and adenosine (Ado) deamination in platelets of rats submitted to implant (icv) of glioma C6 (control) and treated with tannic acid (TA) (50 mg/kg/day) for 15 days. Results for NTPDase and 5′-nucleotidase activities are expressed in nmol Pi/min/mg of protein, and adenosine deaminase activity is expressed as U/L. Bars represent mean ± SEM (n = 5–7). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to sham group and $^{*}P < 0.05$, $^{***}P < 0.001$ compared to control group

only recently been demonstrated. Zhang et al. [18] demonstrated that TA treatment could inhibit the viability of human glioma cells (Hs 683 cells). Furthermore, the authors reported that the reduction in cell death was confirmed by the induction of apoptosis in cells treated with TA, and they revealed that the decrease in cell growth was concentration-dependent. Additionally, TA could activate pro-caspase 3 and caspase 9, thus implying the induction of apoptosis, as corroborated by the loss of mitochondrial membrane potential and increased intracellular ROS production by TA in a dose-dependent manner [18].

In agreement with the results of a previous study, Bona et al. [16] demonstrated that TA could reduce the cell viability and proliferation of rat C6 glioma by influencing apoptosis and cell cycle arrest, reducing colony formation and size, modulating cell migration/adhesion, and exhibiting antioxidant potential in vitro. Interestingly, the authors also demonstrated that the cytotoxic action of TA is selective for the glioma lineage, as TA does not alter the viability of healthy astrocytes [16]. Finally, the antitumor effect of TA in a preclinical model of GB has been reported, where the oral administration of TA for 15 days was capable of reducing the size of the tumor [16]. Thus, considering the promising effects of TA against GB, we investigated its role against oxidative and purinergic changes in a preclinical model of GB in rats.

Patients with GB suffer from an increased incidence of vascular thrombotic events, and therefore, the investigation of platelet function in the context of GB is relevant. It has also been demonstrated that tumor-educated platelets (TEPs) are potential biomarkers for cancer diagnosis [38–40]. Sol et al. [40] revealed that TEPs exhibit potential as a minimally invasive bio source for blood-based diagnostics and the monitoring of GB patients [40]. First, we demonstrated that there was a reduction in the hydrolysis of ATP and AMP, and this was followed by an increase in the hydrolysis of ADP and deamination of Ado in the GB group. This scenario converges to an accumulation of ATP and AMP, and this is followed by a reduction in extracellular levels of ADP and Ado. However, TA treatment protected against this change.

Platelets are known to be one of the main platelet-stimulating agonists (ADP). Additionally, ATP, ADP, and Ado can also modulate the vascular system through interactions with specific purinergic receptors. In this sense, it is known that the platelet membrane possesses the entire cascade of ectonucleotidases that are responsible for the hydrolysis of these molecules [41–43]. Thus, these enzymes play an important role in thromboregulation, and altered activities of these enzymes have been observed in various diseases.

The reduction of ATP hydrolysis by platelets may contribute to the accumulation of this molecule in the extracellular medium. It should be noted that high concentrations of ATP contribute significantly to an exacerbated pro-inflammatory environment through its connection with the P2 type receptor, particularly the P2X7 receptor [41, 43]. Regarding the

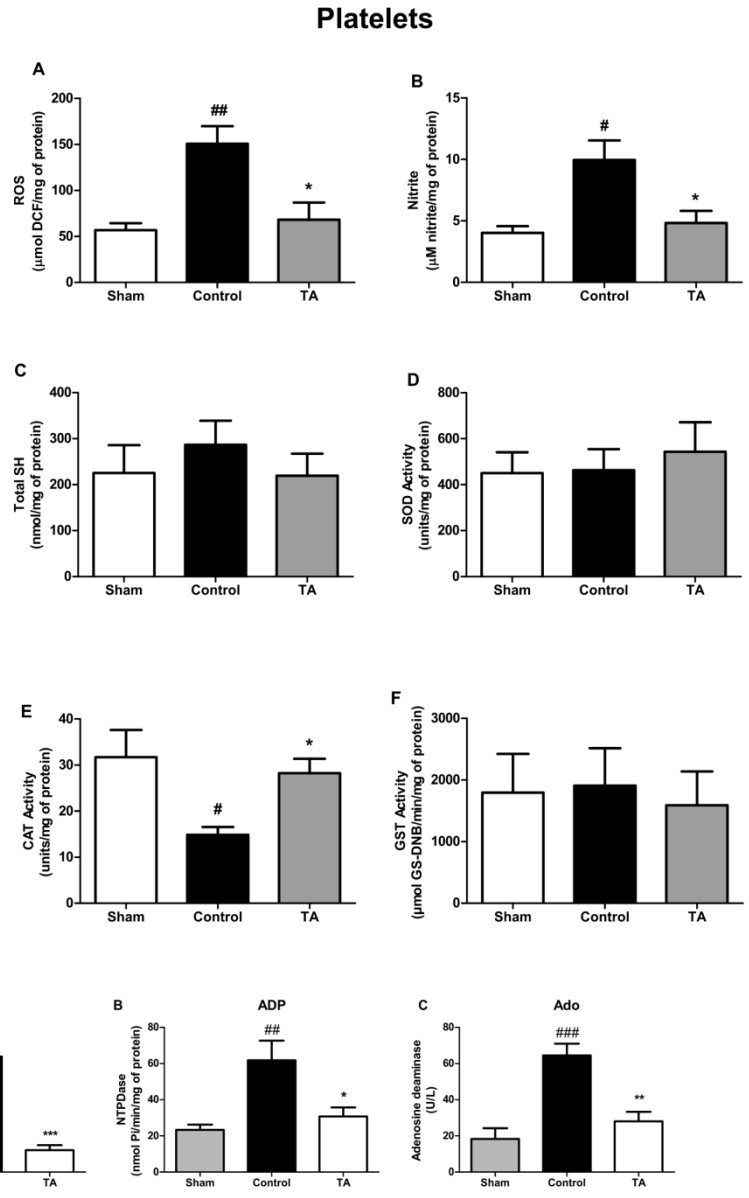

Fig. 3 ROS (**A**), nitrite (**B**), and total sulfhydryl content (**C**) levels and SOD (**D**), CAT (**E**), and GST (**F**) activities in platelets of rats submitted to implant (icv) of glioma C6 (control) and treated with tannic acid (TA) (50 mg/kg/day) for 15 days. ROS levels are expressed as µmol DCF/mg of protein, nitrite levels are expressed as µM nitrite/mg of protein, thiol content is expressed as nmol TNB/mg of protein, and SOD and CAT activities are expressed as U/mg of protein and GST as µmol GS-DNB/min/mg of protein. Bars represent mean ± SEM (n = 5–7). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to sham group and $^{*}P < 0.05$ compared to control group

Fig. 4 Hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP), and adenosine (Ado) deamination in lymphocytes of rats submitted to implant (icv) of glioma C6 (control) and treated with tannic acid (TA) (50 mg/kg/day) for 15 days. Results of NTPDase activities are expressed in nmol Pi/min/mg of protein, and adenosine deaminase activity is expressed as U/L. Bars represent mean ± SEM (n = 5–7). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to sham group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group

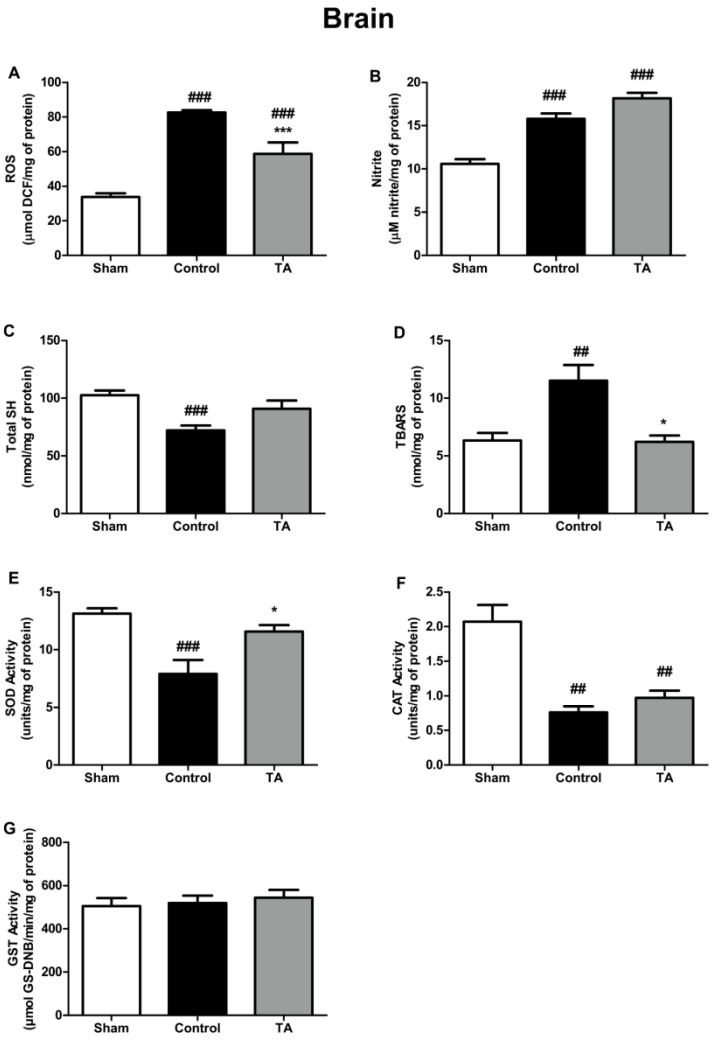

Fig. 5 ROS (**A**), nitrite (**B**), total sulfhydryl content (**C**), and TBARS (**D**) levels and SOD (**E**), CAT (**F**), and GST (**G**) activities in brain of rats submitted to implant (icv) of glioma C6 (control) and treated with tannic acid (TA) (50 mg/kg/day) for 15 days. ROS levels are expressed as µmol DCF/mg of protein, nitrite levels are expressed as µM nitrite/mg of protein, thiol content is expressed as nmol TNB/mg of protein, TBARS levels are expressed as nmol TBARS/mg of protein, SOD and CAT activities are expressed as U/mg of protein and GST as µmol GS-DNB/min/mg of protein. Bars represent mean ± SEM (n = 5–7). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to sham group and $^{*}P < 0.05$, $^{***}P < 0.001$ compared to control group

higher hydrolysis of ADP, these data could be explained in part due to high extracellular concentrations of ADP that would contribute to the activation of aggregation platelets. Additionally, the anti-inflammatory effects induced by Ado would be reduced, as in addition to the lower production of this nucleoside, there is also a greater degradation [6]. There is a negative correlation between thrombocytosis in the laboratory prior to surgery and overall survival in these patients [38, 44]. Thus, the protective effects of TA against these parameters are extremely relevant.

Another important observation that must be mentioned is that circulating platelets can affect the GB microenvironment by supplying oncopromoter and pro-angiogenic factors. Moreover, at the site of injury, platelets degranulate and release pro-angiogenic factors as S1P (sphingosine-1-phophate), a potent bioactive lipid that enhances cell proliferation and survival. Platelets can act through

two processes that include (1) the accumulation of huge reservoirs of biomolecules that results in the sequestering tumor-derived factors/signals (e.g., growth factors, cytokines, and nucleic acids) and thus the development of "tumor-educated" platelets and (2) the release of microparticles that are shuttled to tumors to regulate both anti- and pro-tumorigenic gene expression programs [45].

In the serum of animals that were not treated with TA, a reduction in ATP hydrolysis was observed, and this could contribute to the elevated levels of this nucleotide and also to deleterious actions in the blood. However, an increase in the hydrolysis of ATP, ADP, and Ado degradation was found in lymphocytes. Lymphocytes are important cells of the immune system, and tumor-infiltrating lymphocyte expression is detectable in the majority of GB samples [46]. Therefore, it is important to investigate the effect of TA on these cells. To date, knowledge regarding the functionality of peripheral lymphocytes in patients with GB is limited. However, in view of the potent effects of the purinergic system on both platelets and lymphocytes, it is important to analyze ectonucleotidases in these cells.

In GB, there is an impaired immune response that culminates in tumor progression. Additionally, the involvement of purinergic signaling is well known to influence the development of cancer, as it exerts a direct influence on cancer cells and mediates the modulation of immune cells, particularly by the Ado P1 receptors. Although several studies have been conducted to understand the role of supra-physiological concentrations of Ado in the tumor microenvironment, there is still no consensus regarding the pro- or anti-tumor role of the adenosinergic system. Most studies have shown the immunosuppressive effects of Ado (pro-tumor), while other studies have revealed that Ado can lead to the death of tumor cells [47]. In fact, targeting of the enzyme ecto-5'-nucleotidase (the main extracellular factor that produces Ado) has been discussed as an alternative treatment for solid tumors and for GB.

Additionally, ATP induces several cellular responses, including proliferation and differentiation in the GB lineage, mainly by the P2X7 receptor (P2X7R) that is upregulated in GB and is associated with increased tumor cell proliferation [47, 48]. Many of the effects of the purinergic system components found in the platelets, lymphocytes, and serum of rats with GB could represent changes in the tumor site. Thus, the results of this study that demonstrated that TA is capable of modulating the activity of ectonucleotidases are extremely important in maintaining the levels of extracellular purine compounds within homeostasis.

To date, the role of TA on ectonucleotidases in the GB model has not been investigated. However, the exact mechanism of TA still requires extensive study. The antioxidant effect of TA is one of the most elucidated mechanisms of this compound. In other types of cancer, the anticarcinogenic role of TA has already been demonstrated, and this role included the inhibition of lipid metabolism and the induction of ROS generation [17, 49], the induction of endoplasmic reticulum stress-mediated apoptosis [50], the modulation of cellular ROS and RNS levels, the downregulation of antioxidant expression [51], and the regulation of mitochondrial ROS levels [52].

In GB, oxidative damage is represented as changes in the redox status of tumor cells (generally activating ROS production) that would engender tumorigenesis by impeding DNA repair mechanisms, ultimately resulting in an accumulation of DNA damage and an increase in cell proliferation due to the increase in H_2O_2 and $O_2^{-\cdot}$. However, ROS induces damage to proteins, lipids, and DNA, thus resulting in genomic instability. The major sources of ROS production in tumor cells include NADPH oxidases (NOXs) and the electron transport chain in the mitochondria and endoplasmic reticulum from oxidoreductases and NOXs [4].

In agreement with previous literature data, this study demonstrated the presence of oxidative stress in the brain, platelets, and serum of animals with GB. The increase in the levels of ROS and nitrites that was followed by a reduction in the levels of thiol groups and the presence of lipid peroxidation in the GB group confirmed the damage caused by the reactive species. However, the reduction in the antioxidant enzyme activity (SOD and CAT) can contribute to the increase in ROS levels. Additionally, previous studies have demonstrated that high concentrations of H_2O_2 potentially lead to platelet aggregation. Furthermore, high concentrations of H_2O_2 can also induce damage to the platelet membrane. Importantly, TA protects against most oxidative changes [53]. In addition to the possible mechanisms by which TA restores redox status and reduces the viability and proliferation of tumors, this compound can also act at the molecular level in breast cancer cells by inhibiting NF-κB-mediated phenotype transition [54] and EGFR/STAT1/3, and by enhancing the p38/STAT1 signaling axis [55].

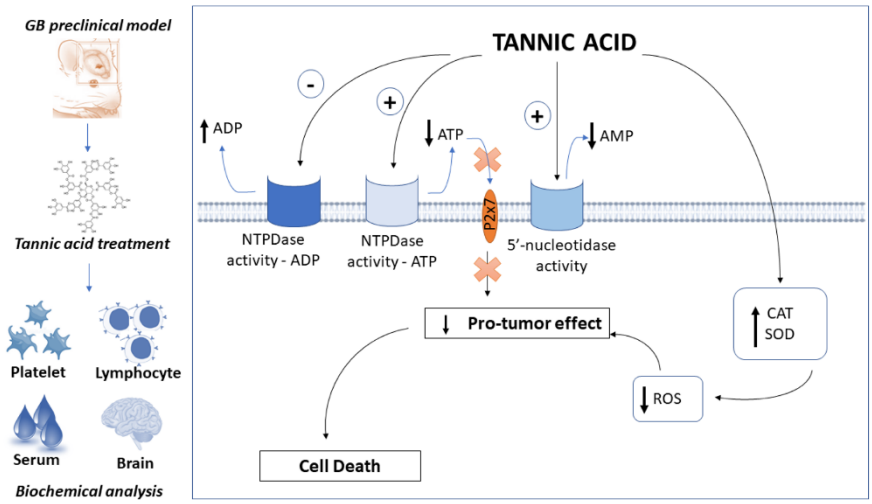

Fig. 6 Overview of possible mechanisms of action of tannic acid in serum, lymphocytes, platelets and brain of rats submitted to a preclinical model of glioblastoma.

Conclusion

It can be concluded that peripheral cells such as lymphocytes and platelets are an alternative that can be used to investigate changes caused by GB. In view of the important need to search for new therapeutic alternatives for GB, TA has been demonstrated to be a promising agent for the treatment of this pathology. In addition to being able to reduce the size of the tumor, it can modulate different pathways involved in the pathophysiology of GB, including purinergic signaling, through the modulation of ectonucleotidases and redox status, thus making it an interesting target for future studies in view of its multidirectional action (Fig. 6).

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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4.2. Capítulo 2 - Manuscrito 1

Antitumoral activity of *Cecropia pachystachya* extract in *in vitro* and *in vivo* model of rat glioma: brain and blood effects

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Antitumoral activity of *Cecropia pachystachya* extract in *in vitro* and *in vivo* model of rat glioma: brain and blood effects

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Abstract

The aim of this work was to investigate the antiglioma effect of *Cecropia pachystachya* (CEC) Trécul extract against C6 rat GB and U87 human GB *in vitro* and in a rat preclinical GB model. For *in vitro* tests, concentrations of 25-500 μg/mL were used at times of 24, 48 and/or 72 h. In the preclinical protocol, the animals received CEC extract at a dose of 200 mg/kg for 15 days. Our results demonstrated a promising effect *in vitro* of the CEC extract, both on cell viability and proliferation, reducing around 66% and 84%, respectively. Treatment with CEC extract decreased approximately 62% of tumor volume, not causing systemic toxicity to the animals. Furthermore, GB model induced a deficit in locomotion and memory and an anxiolytic-like behavior, but the treatment with extract was able to minimize these alterations. In platelets, serum and brain of animals with GB, there was an increase in the levels of reactive oxygen species (ROS), nitrites and thiobarbituric acid reactive substances (TBARS) and a decrease in the activity of antioxidant enzymes, the treatment with CEC extract was able to reverse these damages. In lymphocytes, platelets and serum, we observed changes in the activity of NTPDases, 5'-nucleotidase and adenosine deaminase, but the treatment with CEC extract was able to modulate the activity of these enzymes. Finally, we observed an increase in the levels of IL-6 and a decrease of IL-10 in the serum of animals submitted to the C6 glioma model; however, the treatment with CEC extract was able to reverse only the decrease in IL-10. Thus, in view of the results obtained, the aqueous extract of CEC becomes an important target for the treatment of GB, since in addition to decreasing the viability, proliferation and size of the tumor; it acts in the modulation of the redox and purinergic system.

Keywords: Glioblastoma; natural products; *Cecropia pachystachya*; toxicity; redox status; purinergic system

1. Introduction

Glioblastoma (GB) is a grade IV glioma characterized by being the most aggressive and common brain tumor in adults representing about 80% of malignant gliomas (Louis et al., 2016; Caragher et al., 2019; Hanif et al., 2017). Tumors characterized as grade IV, such as GB, are extremely aggressive and malignant, expressing an increase in mitral activity, a high capacity for angiogenesis, in addition to inducing necrosis and being resistant to chemotherapy (Fang et al., 2012).

The treatment used in patients affected by GB is first surgical resection followed by radiotherapy concomitant with chemotherapy with the standard drug temozolomide (TMZ) (Stupp et al., 2014). Even though there are therapeutic alternatives, GB remains a disease with a high mortality rate and an unsatisfactory prognosis, giving patients a survival of approximately 14 to 15 months after diagnosis (Thakkar et al., 2014). Taking this scenario into account, the development of new therapeutic strategies to help improve the prognosis of patients affected by GB becomes extremely relevant.

Although the main purpose of cancer treatments is to eliminate cancer cells without causing any effect on normal cells, most available treatments have limited efficacy and still exert actions on malignant and normal cells, which leads to adverse effects on patients. Given this scenario, it is extremely important to develop new therapeutic strategies that have fewer adverse effects and help improve the prognosis of patients affected by GB (Cragg et al., 2014; Gutiérrez-Rodríguez et al., 2018). In view of this, natural products become an important alternative for the treatment of this pathology.

GB has a high metabolic rate and basal levels of reactive oxygen species (ROS) playing an important role as chemical mediators in the regulation of signal transduction, protecting tumor cells from apoptosis and generating an immunosuppressive environment (Salazar-Ramiro et al., 2016). Therefore, the alteration in redox homeostasis is involved in the onset, progression and regression of tumors. GB progression is a multifactorial process due to numerous genetic and pathophysiological changes.

The dysregulation of purinergic signaling has been described as an important target of study for understanding the pathophysiological mechanisms of GB (Bergamin et al., 2019). Nucleotides and nucleosides that act on the purinergic system play important roles as neurotransmitters, but they can also act in different ways as CNS trophic agents (Rathbone et al., 1999). Unlike what occurs in astrocytes, gliomas have a low expression and activity of NTPDases, which favors the accumulation of adenosine triphosphate (ATP) in the extracellular environment (Wink et al., 2003). Still, this nucleotide demonstrates an induction of proliferative stimulus in gliomas, being cytotoxic to normal cells (Morrone et al., 2005).

GB is an extremely aggressive cancer, with a low estimate of survival and quality of life for the patient and there are still few studies related to the treatment of this type of neoplasm, which is still a major challenge for public health (American Brain Tumor Association, 2021). Natural products become important targets as adjuvants in the treatment of this pathology.

Cecropia pachystachya Trécul, popularly known as embaúba, is a fast-growing arboreal species widely found in all regions of Brazil (Pacheco et al., 2014). Studies demonstrate that the methanol extract of CEC performs biological functions well described as hypoglycemic, anti-inflammatory, antinociceptive and cytotoxic (Aragão et al., 2010; Aragão et al., 2013). Furthermore, authors highlight the hypotensive, cardiotonic and sedative effects of the aqueous extract of CEC (Consolini et al., 2005; Consolini et al., 2006). In addition, a pharmaceutical formulation containing *C. pachystachya* was recently developed which has proven anti-obesity, hepatoprotective and anti-hyperglycemic effects (de Campos et al., 2021). *C. pachystachya* becomes an important target in the treatment of various pathologies due to the antioxidant power conferred on the plant's leaves where phenolic compounds such as isoorientin, orientin and isovitexin are found (Ortmann et al., 2016). In view of the above, the present study aimed to investigate the antiglioma effect of *C. pachystachya* extract (CEC) on C6 rat glioma strain *in vitro* and its activity in a preclinical GB model, against behavioral, biochemical and oxidative stress parameters, as well as activity of ectonucleotidases.

2. Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), Fungizone (amphotericin B), penicillin/streptomycin, 0.5% trypsin/EDTA solution, and fetal bovine serum (FBS) were obtained from Gibco BRL (Carlsbad, CA, USA). TA, dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals and solvents used were of analytical or pharmaceutical grade.

2.2. Phytochemical

C. pachystachya leaves were collected. The extract of CEC was prepared by the aqueous infusion method according to the pre-protocol established by Gazal et al. (2014). The extract was dried by the spray dryer method under drying conditions of 160 ºC (inlet temperature) and 111 ºC (outlet temperature); feed flow at 5%; 100% air flow and 0.7 mm atomizer diameter. The crude extract was dried and stored at -20 ºC until use.

2.3. Animals

For use of the animals is in accordance with the Brazilian Guidelines for the Care and Use of Research Animals in Sciences (DBCA) and according to the National Council for Animal Control and Experimentation (CONCEA). The study was approved by the Animal Experimentation Ethics Committee on Animal Experimentation of Universidade Federal de Pelotas, under protocol number CEEA 31292-2018.

2.4. Cell culture protocol

C6 rat glioma cells and U87 human were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in DMEM supplemented with 10% FBS maintained at 37 °C containing 5% CO_2. A stock solution was made by dissolving extract of CEC in distilled water. The stock solution was then diluted in 10% FBS supplemented DMEM to obtain solutions of 25, 50, 100, 200, 300, 400, and 500 μg/mL. For cytotoxicity evaluation, C6 and U87 glioma cells were seeded in 96-well plates at 5 x 10^3 and 1.7 x 10^4 cells/well respectively and exposed to extract of CEC at the aforementioned concentrations for 24, 48 or 72 h. Cells exposed to DMEM were considered as control.

2.5. Cytotoxicity assays

The MTT assay was performed to determine cell viability (Mosmann et al., 1983). The medium was removed after treatment and a solution of MTT mixed with DMEM (0.5 mg/mL) was added. The C6 and U87 were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 90 min. Absorbance at 492 nm was then measured with a microplate reader (SpectraMax 190, Molecular Devices, San Jose, CA, USA). SRB assay was used for cell density determination based on the ability of the dye to bind to basic amino acids present in cellular proteins (Pauwels et al., 2003). First, the medium containing the treatment was removed and 50% trichloroacetic acid was used to fix cells, after, the cells were incubated at 4 °C for 45 min. After incubation, the dye was added, and cells were incubated in the dark at room temperature for 30 min. Absorbance at 530 nm was determined using a microplate reader (SpectraMax190). The results are expressed as percentage of the control cells.

2.8. Cell migration

The *in vitro* scratch assay was used to evaluate cell migration capability (Fernandes et al., 2015; Guerreiro et al., 2017). Cells were seeded in 24 well plates at a density of 1.5 x 10^5 cells per well. After confluence a pipette was used to create a scratch at the bottom of the plate on the cell monolayer. Debris was removed and treated medium was added. The C6 strain was treated at concentrations of 25, 50 and 100 μg/mL and U87 at concentrations of 0.5, 1 and 2 μg/mL. The photos were taken at 0, 2, 4, 6 and 24 h to observe the risk closing. Quantitative analysis of the cell-free area was performed using ImageJ software, after which the percentage of inhibition of cell migration was determined.

2.6. Glioma Implantation

C6 cells were cultured after reaching 90% confluency at a ratio of $1x10^6$ cells, resuspended in 3 µL of DMEM and infused at a rate of 0.5 µL/min in the right striatum of the animals (coordinates relative to bregma, 3.0 mm lateral,0.5 later and 6 mm depth) (Da Silveira et al., 2017). The animals were pre-anesthetized with intraperitoneal (i.p.) administration of ketamine and xylazine.

2.7. Experimental design

Five days after glioma implantation, the animals were divided into three groups: (1) Sham, (2) Control (Glioblastoma + Vehicle), (3) Treated (Glioblastoma + extract of CEC (200 mg/kg/day)). The treatment was given intragastrically for 15 days. After 21 days of tumor implantation, the animals were euthanized and the brain was removed, sectioned and fixed in formalin for further analysis. Blood samples were collected by cardiac puncture and the serum was separated for biochemical analysis. The body weight of the animals was monitored every 3 days.

2.8. Pathological analysis and tumor quantification

Three brain sections of each hematoxylin and eosin (HE) animal (4 µm thick in paraffin blocks) were analyzed by a pathologist. For quantification of tumor size, images were captured using a microscope attached to a camera (× 2 magnification; Nikon Eclipse TE300) and the tumor area (mm^2) was quantified using ImageJ software. Total tumor volume (mm^3) was calculated by multiplying the slice section and adding the segmented area (Da Silveira et al., 2017).

2.9. Behavioral parameters

2.9.1. Open field

The locomotor activity of the animals was quantified through the open field test. The apparatus consists of a box divided into 16 equal quadrants. The number of quadrants that the animal crosses when crossing with the four legs in a total of 5 min is counted (Walsh and Cummins, 1976).

2.9.2. Object recognition test

This test was carried out to assess the animals recent learning and memory. The apparatus used was the same as the open field test. This test is divided into two stages, the training stage consists of the disposition of two identical objects in the apparatus, in the test stage, which is carried out after 3 h of the training, one of the objects is replaced. At this stage, animals can explore a familiar object (A) and a new object (B). Exploration time is defined as the time animals spend sniffing or touching objects with their nose and / or forelegs for a total period of 5 min. The objects were of similar textures, colors and sizes, but had different shapes. An exploratory preference (%) was expressed by the ratio (T1 x 100)/T2, T1 = time spent in exploring the novel object (B); T2 = time total spent in exploring the two objects (A+B) (Dere et al., 2005).

2.9.2. Elevated plus maze test

The elevated plus-maze test assesses the anxiolytic-like behavior of animals and was based on Ziegler et al. (2005). The labyrinth consists of an elevated apparatus, 60 cm from the floor, composed of two open arms (50 cm long and 10 cm wide) and two closed arms (50 cm long, 10 cm wide and 40 cm high). walls), in the shape of a cross with a central square (10 cm). Individually, the animals are placed in the center of the apparatus, facing one of the open arms and from that point on, the time spent in the open arms of the apparatus is timed for a total time of 5 min.

2.10. Euthanasia and sample preparation for biochemical analysis, oxidative stress and purinergic parameters

After 21 days of tumor implantation, the animals will be euthanized to obtain the following samples:

Serum: Serum was obtained by collecting blood in tubes without the presence of anticoagulant. Blood was centrifuged at room temperature and the clot removed for serum collection and stored at -80°C for further analysis.

Lymphocytes: For lymphocyte separation, blood was collected in tubes with anticoagulant (EDTA) and diluted in saline with Ficoll-Histopaque density gradients, then washed with saline and centrifuged three more times. The separation was based on a protocol aimed at separating mononuclear cells through a method previously described by Boyum (1968) and Jaques et al. (2011).

Platelets: To obtain platelet-rich plasma, blood was collected in tubes containing sodium citrate (0.120 M) in the presence of anticoagulant, according to the methodology described by Lunkes et al. (2004). Subsequently, the plasma was centrifuged at room temperature and washed twice with 4-(2-hydroxyethyl)-1 ethanesulfonic acid piperazine (HEPES) and resuspended for further analysis.

Brain: For histology, the brain was removed, sectioned and fixed in formalin for further analysis. For oxidative stress analyses, the left hemisphere of the brain was separated and stored at − 80°C. The brain was homogenized (1/10 w/v) using 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenates were centrifuged at 2500 x g for 10 min at 4 °C. Supernatants were collected and used for further analysis.

2.11. Oxidative stress parameters

2.11.1. Protein determination

Protein concentration was determined using the method of Lowry et al. (1951).

2.11.2. Reactive Oxygen Species (ROS)

The production of intracellular ROS was determined through the oxidation of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA). ROS formation was determined according to Ali et al. (1992).

2.11.3. Nitrite levels

Nitrite levels are measured according to Stuehr and Nathan (1989) using the Griess colorimetric reaction. The absorbance is measured at 540 nm and the results are expressed as µM nitrite/mg of protein.

2.11.4. Total sulfhydryl content

For this assay, the samples are prepared with the addition of PBS buffer (pH 7.4), containing EDTA and DTNB. The total thiol content is measured through a reaction where the reduction of DTNB by oxidized thiol group (disulfide) occurs, leading to the generation of TNB (derived from yellow color). The results were expressed as nmol TNB/mg of protein (Aksenov & Markesbery, 2001).

2.11.5. Thiobarbituric acid reactive substances (TBARS) quantification

In this assay, lipid peroxidation was quantified according to Esterbauer and Cheeseman (1990). 10% trichloroacetic acid (TCA) was added to the samples. After centrifugation, the supernatant was collected, mixed with 0.67% thiobarbituric acid (TBA) and heated to 100 °C for 30 min. The results were expressed in nmol TBARS/mg of protein

2.11.6. Superoxide dismutase (SOD) activity

This assay is described by Misra and Fridovick (1972), based on the inhibition of adrenaline auto-oxidation. The absorbance is measured in a spectrophotometer at 480 nm and the SOD activity is expressed in units/mg of protein.

2.11.7. Catalase (CAT) activity

This test is described by Aebi (1984), based on the decomposition of H_2O_2, being monitored in a spectrophotometer at 240 nm by 180 seconds at 37 ºC. CAT activity is expressed as units/mg of protein.

2.11.8. Glutathione S-transferase (GST) activity

This assay is based on Habig et al. (1974), where GST activity is measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Enzyme activity is expressed as µmol GS-DNB min/mg of protein.

2.12. Ectonucleotidases activity assay

2.12.1. Protein determination

Protein concentration was determined using the method of Bradford (1976).

2.12.2. NTPDases and 5'-nucleotidase in serum

It was performed according to the pre-established protocol (Fürstenau et al., 2004). Serum samples were pre-incubated for 10 min at 37 °C in a reaction medium (112.5 mM Tris-HCl, pH 8.0). To start the reactions, ATP, adenosine diphosphate - ADP and adenosine monophosphate - AMP were added to the medium (3.0 mM) and incubated for 40 min. Finally, TCA (10%) was used to stop enzymatic reactions. The amount of inorganic phosphate (Pi) released was measured by the method of Chan et al. (1986). Enzyme activities were expressed as nmol Pi released/min/mg of protein.

2.12.3. NTPDases in lymphocytes

It was performed according to the protocol previously described by Leal et al. (2005). Intact lymphocyte cells suspended in saline solution were added to the reaction medium (0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris HCl buffer pH 8.0) and incubated for 70 min. The reaction was started after addition of ATP or ADP and stopped with (10% TCA). Released Pi was tested by the method previously described by Chan et al. (1986). Specific activity was reported as nmol Pi released/min/mg of protein.

2.12.4. NTPDases and 5'-nucleotidase in platelets

In the NTPDases assay, platelets were pre-incubated for 10 min at 37 °C in a reaction medium (5 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris-HCl buffer, pH 7.4). In determining the 5'-nucleotidase activity, $CaCl_2$ was replaced by $MgCl_2$ (10 mM) in the reaction medium mentioned above. The reaction was initiated by the addition of ATP or ADP (1 mM final concentration) and AMP (2 mM final concentration). They were incubated for 60 min and the assays were stopped by TCA (10%) (Lunkes et al., 1996; Pilla et al., 2004). Released Pi was tested by the method of Chan et al. (1986). Specific enzyme activities were reported as nmol Pi released/min/mg of protein.

2.12.5. Adenosine deaminase activity

This test was performed using the methodology previously described (Giusti and Galanti, 1988) in serum, platelets and lymphocytes from animals submitted to the GB model. The samples were submitted to the adenosine (Ado) reaction (21 mmol/L of Ado and pH 6.5) and incubated at 37 °C for 60 min. The specific activity of ADA was expressed in U/L. One unit (1U) of ADA was defined as the amount of enzyme required to release 1 mmol ammonia/min Ado under standard assay conditions.

2.13 Cytokines quantification

Cytokines Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were quantified by ELISA using commercial kits (R&D Systems) following the manufacturer's instructions. The presence and

concentration were evaluated through the color intensity measured by spectrometry in an ELISA microreader.

2.14 Statistical analysis

Results will be expressed as mean ± mean standard error. Statistical analyzes will be performed using Student's t test, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc or two-way ANOVA followed by Bonferroni's post-hoc. Differences will be considered significant when $P<0.05$.

3. Results

3.1. CEC decreases the proliferation and viability of C6 and U87 glioma cells

Treatment with the extract of CEC was added to C6 rat glioma cells and U87 human glioma cells at concentrations of 25 – 500 µg/mL at 24, 48 and 72 h. In C6 cells, it was possible to observe that treatment with extract of CEC was able to reduce both cell proliferation (Fig 1a-c) and cell viability (Fig 1d-f). We can observe that the cells exposed to the extract of CEC in the time of 48 h in the concentration of 500 µg/mL, reduced by 84% ($P<0.001$) the cell proliferation, presenting the greatest effect in relation to the tested concentrations and times. Regarding cell viability, we can observe the greatest reduction in 72 h in the concentration of 500 µg/mL, being 66.3% ($P<0.001$). In U87 cells it was possible to observe a similar behavior, where the extract of CEC reduced proliferation (Fig 1g-i) and cell viability (Fig 1j-l). At 72 h, at a concentration of 500 µg/mL there was an 88.5% ($P<0.001$) reduction in cell proliferation, demonstrating the greatest effect. In cell viability, the greatest reduction was observed, 94.7% ($P<0.001$) in the 48 h time at the concentration of 500 µg/mL.

3.2. CEC inhibits the migration of C6 glioma cells

To evaluate the influence of CEC on the migration of C6 and U87 glioma cells, a monolayer risk test was performed, with images captured at 0, 2, 4, 6 and 24 h after the risk and the risk measurement was performed, thus obtaining the percentage of cell migration. In the C6 cell line, within 24 h, it was possible to observe a risk closure in 78.65% in the control group, and the treatment with extract of CEC at a concentration of 25, 50 and 100 µg/mL, demonstrated an inhibition of cell migration in 49.25% ($P<0.01$), 51.25% ($P<0.001$), 51.54% ($P<0.001$), respectively (Fig 2a-c). Treatments with extract of CEC at concentrations of 0.5, 1 and 2 µg/mL in the U87 cell line showed no significant difference in cell migration ($P>0.05$) (Fig 2d-f).

3.3. CEC reduces behavioral changes caused by GB

In the open field test, it was possible to observe a significant difference between the groups regarding the total number of crossings ($F_{(2,34)} = 5.98$, $P<0.05$), demonstrating that the control group

showed an alteration in the animals' locomotion when compared to the Sham group, and that the group treated with the extract of CEC was able to attenuate this behavior (Fig 3a). In the object recognition test phase (Fig 3b) was observed that the control animals had a reduction in the total time spent exploring the new object when compared as a sham group and that the treatment with the extract of CEC was able to reverse this behavior ($F_{(2,32)} = 6.27$, $P<0.05$). In the elevated plus maze test (Fig 3c), was observed that the control group showed a significant reduction in the number of entries in the open arms of the apparatus when compared to the sham group, considering an anxiolytic behavior in this group. The extract of CEC was able to reverse this damage, increasing the number of entries in the open arms when compared to the control group ($F_{(2,37)} = 10.52$, $P<0.05$).

3.4. CEC reduces GB growth *in vivo* and does not cause toxicity in rats

After the *in vitro* antiglioma results obtained, we verified the antiglioma potential in a preclinical model of the disease. As shown in Fig 3c, it was possible to observe that the animals treated with extract of CEC showed a significant reduction in tumor size (295.1 ± 122.7) ($P<0.05$) when compared to the control group (772.3 ± 149.1). In addition, the control animals showed characteristics similar to patients with glioblastoma. Sham animals have normal parenchyma, neurons with characteristic morphology, absence of cell infiltrate, necrosis, vascular proliferation and cell atypia. Glioma animals showed a well-delimited tumor growth structure (Fig 4), with an area of central necrosis and vascular proliferation. Peritumoral inflammatory infiltrate, necrotic area and apoptosis are also observed. In addition, it is important to note that the treatment with extract of CEC did not demonstrate renal and hepatic toxicity in the treated animals (Table 1). Finally, regarding the weight gain of the animals (Fig 3d), we can observe that the control animals showed a significant weight loss when compared to the healthy animals ($P<0.01$) and extract was able to reverse this weight loss ($P<0.01$).

3.5. CEC modulates oxidative stress parameters in the brain, platelets and serum of animals with glioma

In fig 4 was demonstrated the parameters of oxidative stress assessed in the animals' brain. When comparing the control group with the sham, it was possible to identify that the C6 implant led to an increase in the levels of ROS ($F_{(2,20)} = 77.84$, $P<0.001$) (Fig 4a) and nitrites ($F_{(2,18)} = 29.40$, $P<0.001$) (Fig 4b), treatment with extract of CEC was able to reduce the increase in the levels of ROS ($P<0.001$) and nitrites ($P<0.001$). In addition, treatment with extract of CEC increased the activity of the CAT enzyme (Fig 4f) when compared to the control group ($F_{(2,18)} = 12.46$, $P<0.01$). No significant differences were observed in the treated animals compared to the control group in the SH ($F_{(2,20)} = 20.25$, $P>0.05$) and TBARS ($F_{(2,22)} = 20.69$ $P>0.05$) levels and in the SOD ($F_{(2,16)} = 17.54$, $P>0.05$) and GST ($F_{(2,24)} = 0.079$, $P>0.05$) activities.

As for platelets (Fig 5), an increase in the levels of ROS ($F_{(2,9)} = 12.33$, $P<0.01$) and nitrites ($F_{(2,10)} = 6.94$, $P<0.05$) was observed, treatment with extract of CEC was able to protect when the increased

levels of ROS (P<0.01) and nitrites (P<0.05). We did not observe a significant difference in the SH levels ($F_{(2,12)}$ = 0.42, P>0.05) and in the activity of the SOD enzymes ($F_{(2,11)}$ = 0.35, P>0.05), CAT ($F_{(2,9)}$ = 7.02, P>0.05) and GST ($F_{(2,9)}$ = 0.31, P>0.05).

Finally, the serum of the animals was analyzed (Fig 6), we observed an increase in the levels of ROS ($F_{(2,12)}$ = 7.96, P<0.01) and nitrites ($F_{(2,12)}$ = 16.86, P>0.001), whereas the treatment with extract of CEC was able to reduce the levels of ROS (P<0.05) and nitrites (P<0.01). In addition, we observed an increase in the activity of the enzymes CAT ($F_{(2,12)}$ = 27.73, P<0.001) and SOD ($F_{(2,10)}$ = 6.25, P<0.05) when compared to the control group. We did not observe a significant difference in the SH levels when compared as a control (P> 0.05) and in the GST activity ($F_{(2,13)}$ = 0.16, P>0.05).

3.6. CEC alters the activity of NTPDases, 5'-nucleotidase and adenosine deaminase in lymphocytes, platelets and serum

It was possible to observe, in the lymphocytes, an increase in the deamination of Ado ($F_{(2,20)}$ = 17.11, P<0.001), ATP ($F_{(2,14)}$ = 25.80, P<0.001) and ADP ($F_{(2,14)}$ = 35.71, P<0.001) hysrolysis in the control group when compared with the sham group. However, treatment with CEC was able to reverse the increase in hydrolysis of ATP (P<0.001) and ADP (P<0.001).

In platelets, it was shown an increase in Ado deamination ($F_{(2,13)}$ = 13.04, P<0.001) and ADP hydrolysis ($F_{(2,12)}$ = 9.23, P<0.01) and a decrease in ATP hydrolysis ($F_{(2,12)}$ = 24.54, P< 0.01) and AMP ($F_{(2,11)}$ = 6.31, P<0.05) in the control group compared with the sham group. Treatment with CEC only reversed the increase in hydrolysis of ADP (P<0.01), and no changes were found in Ado (P>0.05), ATP (P>0.05) and AMP (P>0.05).

Finally, in the serum, we observed a decrease only in the hydrolysis of ATP ($F_{(2,15)}$ = 6.53, P<0.05), with no significant changes in the deamination of Ado ($F_{(2,15)}$ = 0.22, P>0.05), hydrolysis of ADP ($F_{(2,15)}$ = 0.79, P>0.05) and AMP ($F_{(2,17)}$ = 0.34, P>0.05). Treatment with CEC was able to reduce the increase in ATP hydrolysis (P>0.05), but did not alter Ado (P>0.05) deamination, ADP (P >0.05) and AMP (P >0.05) hydrolysis.

3.7. CEC restores IL-10 levels in serum of animals with glioma

Figure 9 demonstrates the results obtained from IL-6 and IL-10 in animals submitted to the preclinical model of GB and treatment with the CEC extract. In the serum levels of IL-6, we observed a significant increase ($F_{(2,15)}$ = 37.43, P<0.001) in sham animals when compared to control animals, however the treatment with CEC extract was not able to reverse this alteration. As for IL-10 levels, we observed a reduction in the control group when compared to the sham group ($F_{(2,11)}$ = 6.629, P<0.05) and the animals treated with CEC extract were able to attenuate this change (P<0.05).

4. Discussion

GB is a highly aggressive tumor with an extremely low prognosis. Its appearance is suggested in the occurrence of malignant transformation of glial precursor cells or mature glia. Multimodal treatment (maximum surgical resection + radiotherapy + chemotherapy) is still considered palliative and the average patient survival does not exceed 15 months after diagnosis (Ladomersky et al., 2016). In this way, the search for new targets for the treatment of GB becomes extremely important, given the public health problem that this disease represents. In the present study, we aimed to evaluate the antiglioma effect of CEC extract in C6 cells and in a preclinical GB model. It is important to mention that CEC extract exerts neuroprotective properties in animal models of neuropsychiatric diseases (Gazal et al., 2014; 2015). In addition, our research group has focused on antiglioma treatments based on natural products, obtaining positive results both in *in vitro* tests and in preclinical GB model (Pedra et al., 2019; Bona et al., 2020).

The extract of CEC presents secondary metabolites such as terpenes, steroids and several classes of phenolic compounds, such as phenolic acids, flavonoids, catechins and procynidines. The phenolic compounds present in this plant are often associated with the pharmacological activities described for this species. Importantly, flavonoids are the major compounds found in the aqueous extract of CEC (Costa et al., 2011).

Due to the pharmacological properties of extract of CEC already described in the literature on CNS diseases, in the present study we investigated the antiglioma effects of this extract. *In vitro* data showed reduction of cell viability and proliferation caused by extract of CEC. In agreement with our findings, researches have demonstrated the anticancer effect of flavonoids, such as luteolin, a flavonoid found in different plants that have an anticancer action in several tumor types, such as lung, breast, GB, prostate, colon and pancreas cancer (Imran et al., 2019). This flavonoid acts by blocking the *in vitro* and *in vivo* action of these tumors, inhibiting the proliferation of tumor cells in addition to activating cell cycle arrest by inducing apoptosis through different signaling pathways (Imran et al., 2019).

Studying models that mimic physiological and behavioral conditions becomes extremely relevant to the scientific community. It is important that the animal model mimics as much as possible the characteristics of the human tumor. From the satisfactory *in vitro* results, we obtained the necessary support to carry out the pre-clinical GB model, through the inoculation of C6 rat glioma cells, seeking to verify the similarities with human GB. Our results demonstrated a significant reduction in tumor size in animals treated with extract of CEC at a dose of 200 mg/kg, administered once a day for 15 days. Corroborating these results, our research group has already shown promising results from tannic acid, a phenolic compound found in many foods. This compound also demonstrated a significant reduction in tumor size compared to the same C6 implant protocol (Bona et al., 2020). Furthermore, deficits in locomotion and memory were observed in animals with GB, in addition to an anxiolytic-like behavior. However, it is important to highlight that the treatment with

extract of CEC was able to alleviate these damages. It is well described in the literature that patients affected by GB present a very rapid neurological deterioration, leading to cognitive difficulties and behavioral changes (Omuro & Deangelis, 2013). Thus, we suggest that our model is able to mimic behaviors observed in the clinic and that our treatment with extract of CEC was able to minimize the behavioral changes caused by this disease.

The treatment performed in most neoplasms is chemotherapy. This treatment aims to destroy cancer cells through its systemic action. However, it is necessary to consider that standard chemotherapy drugs do not have specificity; they simply act on highly proliferating cells. As a result, there is toxicity and side effects from this treatment modality, as non-neoplastic cells also end up being affected (Bonassa, 2005). In view of this, it is essential to seek treatments that minimize these adverse effects and toxicity generated by standard chemotherapy. Our results showed that the treatment with extract of CEC did not show any change in the levels of ALT, AST, creatinine and urea, thus providing a safe use of the compound, as it did not show any toxicity at the systemic level.

High amounts of ROS in cells react with various macromolecules, such as chromosomal and mitochondrial DNA, leading to genetic instability and damage that impair the functioning of DNA repair enzymes. These alterations lead to genetic instability and abnormal metabolic processes, which favor the oxidative environment and increase the rate of cell proliferation. Thus, targets for tumor treatment, aim at redox therapies with the aim of reducing oxidative stress, acting in the reduction of cell damage and increasing the mutation rate accompanied by a chromosomal instability that will result in the reduction of the immunosuppressive environment. Furthermore, it is known that therapies that modulate the redox system reduce resistance and moderate the high rate of cell proliferation, favoring apoptosis of tumor cells (Salazar-Ramiro et al., 2016). Our results showed an increase in ROS and nitrite levels in animals implanted with GB and our treatment with extract of CEC was able to reduce this increase in all analyzed structures. Furthermore, we observed an increase in the antioxidant enzyme CAT in the brain of animals treated with extract of CEC, a parameter that had been decreased in GB animals. In platelets, we did not observe changes in the face of antioxidant enzymes. In serum there was a decrease in the activity of the antioxidant enzymes SOD and CAT in the control animals and the treatment with the CEC extract was able to reduce this alteration. In the platelets, we can observe an increase in the levels of ROS and nitrites in the control animals when compared to the healthy animals and extract treatment was able to restore these levels. These results are in agreement with previous studies performed by our research group in which the animals were treated with tannic acid (Bona et al., 2022) and gallic acid (Pedra et al., 2022). We also observed a decrease in CAT enzyme activity in control animals when compared to healthy animals, but treatment with CEC extract was not able to prevent this damage. Polyphenols, mainly flavonoids, have an ideal archetype for scavenging radicals, being considered to be very efficient antioxidants. This antioxidant activity given to flavonoids depends on their structure and factors such as reactivity against other

antioxidants, ability to chelate metals from transition, solubility and interaction with membranes (Shen et al., 2022).

Researchers elucidate the involvement of the purinergic system in GB malignancy (Braganhol et al., 2009). Our results showed an increase in the deamination of Ado, as well as in the hydrolysis of ATP and ADP in lymphocytes in control animals and treatment with CEC extract was able to decrease these changes. In platelets, the control animals showed an increase in the deamination of Ado and ADP and a decrease in the hydrolysis of ATP and AMP, but the treatment with extract was able to reduce only the alteration caused in the deamination of Ado. In the serum of control animals, we can only observe a decrease in ATP hydrolysis, and the treatment with CEC extract modified this alteration. Data from the literature demonstrate that glioma presents a reduced activity and expression of NTPDases, as a consequence of this, the increase of ATP in the extracellular medium occurs, in astrocytes it occurs the opposite (Wink et al., 2003). ATP also induces a stimulus leading to proliferation in gliomas; on the other hand, it has a cytotoxic action for normal neuronal cells in various CNS pathologies, such as neurodegenerative and inflammatory pain (Morrone et al., 2005; Burnstock, 2008).

Furthermore, the CNS presents a strategic distribution of NTPDases, which leads to the assumption that they may be correlated with the neuroprotection and neurotransmission that microglial cells, astrocytes and neurons perform (Burnstock, 2008). Braganhol and colleagues (2009) suggest that the overexpression of NTPDase2 and the generation of ADP in the extracellular environment can induce changes in the interaction of glioma and the brain microenvironment, causing an increase in cell adhesion, recruitment of immune cells and platelets and in the production of cytokines. The role of the 5'-ectonucleotidase/CD73 enzyme is already well defined in the literature, presenting an important role in the progression of GB, since this enzyme is responsible for the production of Ado. Azambuja et al. (2019) demonstrated that Ado increased the viability in glioma cells. The down-regulation of this enzyme led to reduced migration and invasion of glioma cells, reducing the expression of metalloproteinase-2 and vimentin, in addition to reducing cell proliferation, relating these results to necrosis and blockade of the sub-G1 phase of the cell cycle. Furthermore, in an *in vivo* glioma model, inhibition of CD73 was able to slow the progression of GB, decreasing tumor size (Azambuja et al., 2019).

Some researchers have correlated the secretion of inflammatory mediators from peripheral blood and their expression in neoplastic cells (Samaras et al., 2007). Cytokines are regulatory proteins of high importance, as they control the growth and differentiation of normal and malignant cells (Rolhion et al., 2001). Our results also showed an increase in serum levels of IL-6 in animals implanted with C6 cells, but treatment with the CEC extract was not able to reverse this parameter. As for the serum levels of IL-10, we observed a reduction in the control animals and the treatment with CEC extract increased these levels.

5. Conclusion

The present study demonstrated that extract of CEC had a promising effect on rat glioma cells, with a concentration/time dependent effect. In addition, in a preclinical and GB model, it was able to reduce tumor volume without presenting systemic toxicity to animals, it was also able to modulate different pathways, such as the redox system and purinergic signaling, being an interesting target for further studies.

Conflict of interest statement: The authors declare that there is no conflict of interest.

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Figure captions

Fig 1 Effect of *Cecropia pachystachya* (CEC) on the proliferation (A–C and G–I) and viability (D–F and J–L) of C6 rat glioma cells after 24 (A and D), 48 (B and E), and 72 h (C and F) and U87 human glioma cells after 24 (G and J), 48 (H and K) and 72 h (I and L). Values are the means ± standard errors of the means from at least three independent experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's test. **P*<0.05, ***P*<0.01, and ****P*<0.001 versus the control group.

Fig 2 Effect of *Cecropia pachystachya* (CEC) on the cell migration of C6 rat glioma cells (A-C) and U87 human glioma cells (D-F) after different treatments and times 0, 2, 4, 6 and 24 h. Percentage cell migration was analyzed by two-way ANOVA followed by the Bonferroni post-hoc test (B). ***P*<0.01 and ****P*<0.001 versus the control group. Three independent experiments were performed in triplicate.

Fig 3 Effects of treatment with *Cecropia pachystachya* extract (200 mg/kg) on the behavioral tests in animals submitted to the C6 glioma model. The locomotor activity behavior was analyzed using the open field task on the number of total crossings (A). The non-spatial memory was analyzed using the object recognition task (B). The anxiolytic-like behavior of the animals was evaluated using the elevated plus maze test (C). Data are expressed as mean ± S.E.M. (n= 10-12). **P*<0.05, ***P*<0.01, and ****P*<0.001 versus the sham group. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 versus the control group.

Fig 4 C6 glioma cells were implanted intracerebroventricularly in adult rats and treatment with CEC extract (200 mg/kg/day) was started 5 days later and administered for 15 days. On the 22nd day the animals were euthanized and the brain was collected (A). The brain was sectioned three times, stacked and analyzed by HE. The HE images were captured using a camera coupled to a microscope (B) and the total volume (mm^3) was determined using the ImageJ software (C). Body weight of the animals submitted to the glioma implant were analyzed every 3 days (n = 10) (D)**.** Data were analyzed by Student's *t*-test. **P* < 0.05 versus the control group.

Fig 5 Histopathological analysis performed on sections stained by the hematoxylin and eosin technique obtained from implanted sham rat glioma. Images were captured under an optical microscope using 5X-40X magnification on 5 μm thick sections. The white arrows represent a well-delimited tumor area, the yellow arrows areas of necrosis and the red arrows areas of vascular proliferation.

Fig 6 Effects of treatment with *Cecropia pachystachya* extract (200 mg/kg) on oxidative stress parameters in brain animals submitted to the C6 glioma model. ROS (A), nitrite (B), total sulfhydryl content (C) and TBARS (D) levels and SOD (E), CAT (F) and GST (G). ROS levels were expressed as μmol DCF/mg of protein, nitrite levels as μM nitrite/mg of protein, thiol content as nmol TNB/mg of protein, TBARS levels as nmol TBARS/mg of protein, SOD and CAT activities as U/mg of protein GST as μmol GS-DNB min/mg of protein. Data are expressed as mean ± S.E.M (n = 5). ***P*<0.01 and ****P*<0.001 versus the sham group. ##*P*<0.01 versus the control group.

Fig 7 Effects of treatment with *Cecropia pachystachya* extract (200 mg/kg) on oxidative stress parameters in platelets animals submitted to the C6 glioma model. ROS (A), nitrite (B), total sulfhydryl content (C) and TBARS (D) levels and SOD (E), CAT (F) and GST (G). ROS levels were expressed as μmol DCF/mg of protein, nitrite levels as μM nitrite/mg of protein, thiol content as nmol TNB/mg of protein, TBARS levels as nmol TBARS/mg of protein, SOD and CAT activities as U/mg of protein GST as μmol GS-DNB min/mg of protein. Data are expressed as mean ± S.E.M. (n= 5). **P*<0.05 and ***P*<0.01 versus the sham group. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 versus the control group.

Fig 8 Effects of treatment with *Cecropia pachystachya* extract (200 mg/kg) on oxidative stress parameters in serum animals submitted to the C6 glioma model. ROS (A), nitrite (B), total sulfhydryl content (C) and TBARS (D) levels and SOD (E), CAT (F) and GST (G). ROS levels were expressed as μmol DCF/mg of protein, nitrite levels as μM nitrite/mg of protein, thiol content as nmol TNB/mg of protein, TBARS levels as nmol TBARS/mg of protein, SOD and CAT activities as U/mg of protein GST as μmol GS-DNB min/mg of protein. Data are expressed as mean ± S.E.M. (n= 5). **P*<0.05, ***P*<0.01, and ****P*<0.001 versus the sham group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 versus the control group.

Fig 9 IL- Effects of treatment with *Cecropia pachystachya* extract (200 mg/kg) on serum levels of IL-6 and IL-10 in serum in animals submitted to the C6 glioma model. IL-6 and IL-10 levels were expressed as pg/mL. Data are expressed as mean ± S.E.M. (n= 5-6). ****P*<0.001 versus the sham group. $^{\#}P$<0.05 versus the control group.

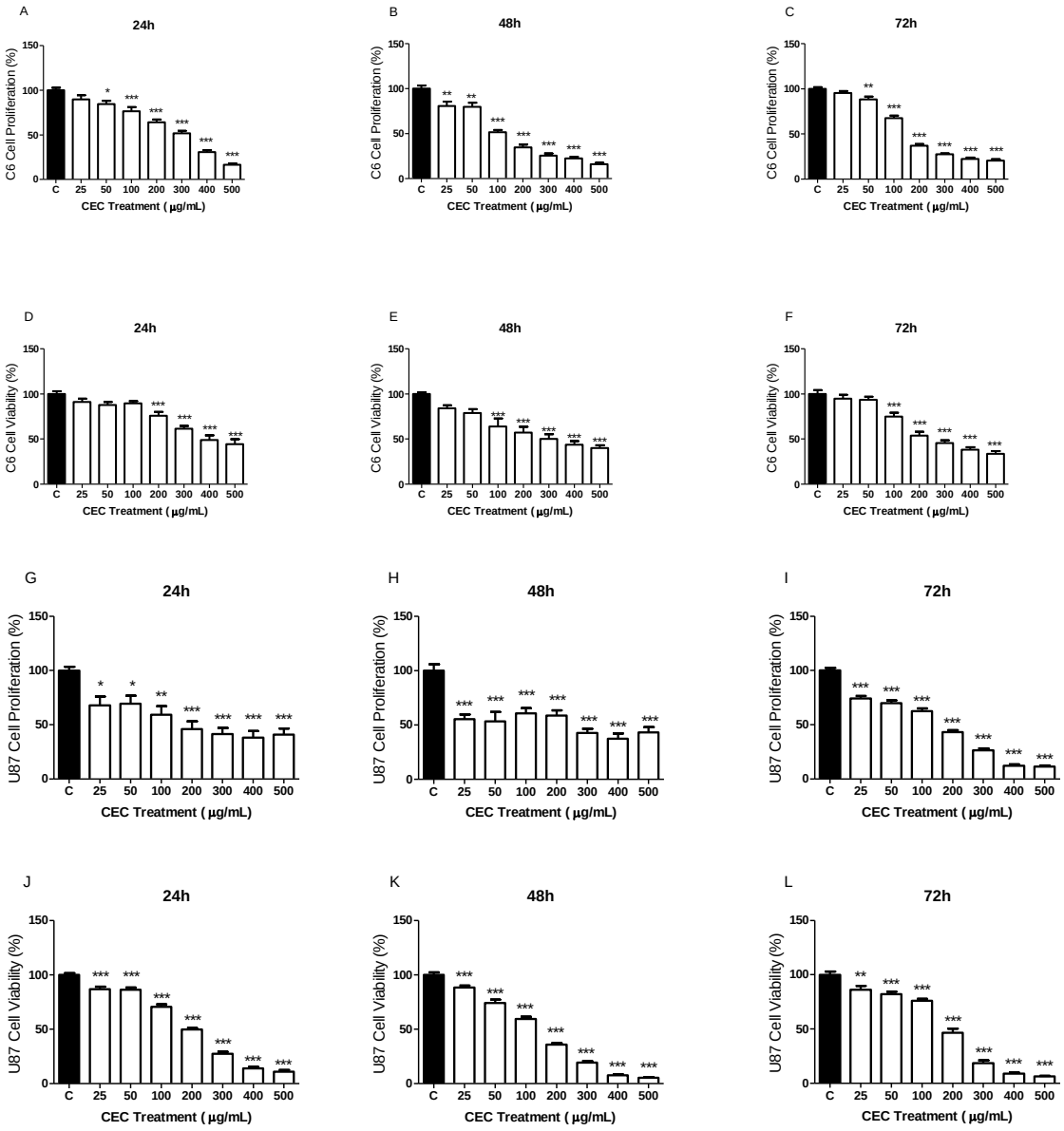
Table 1. Biochemical parameters in animals with glioma implant control and treated (CEC)

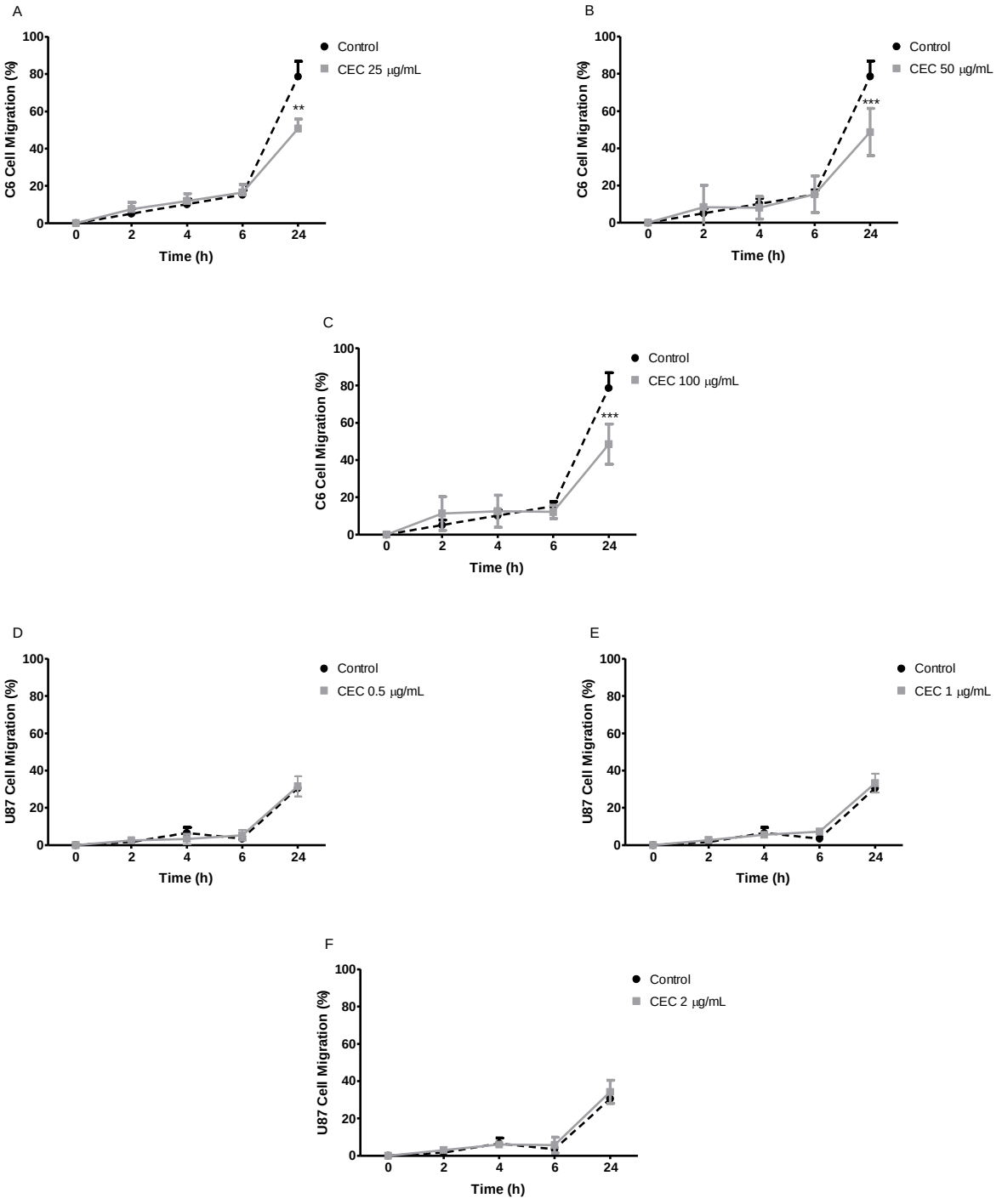
	Sham	**Control**	**CEC**
ALT (U/L)	53.2±4.46	50.17±2.75	53.63±5.83
AST (U/L)	199.2±20.47	237.8±7.86	188.8±18.48
Urea (mg/mL)	47.20±3.05	51.17±4.34	51.71±1.20
Creatinine (mg/mL)	0.46±0.04	0.47±0.03	0.38±0.01
Total protein (mg/mL)	6.16±0.18	5.98±0.17	6.06±0.06
Albumin (mg/mL)	3.4±0.10	3.26±0.09	3.15±0.05

Table 2 ATP, ADP and AMP hydrolysis and adenosine deaminase (ADA) activity in lymphocytes, platelets and/or serum of animals with glioma implant control and treated (CEC)

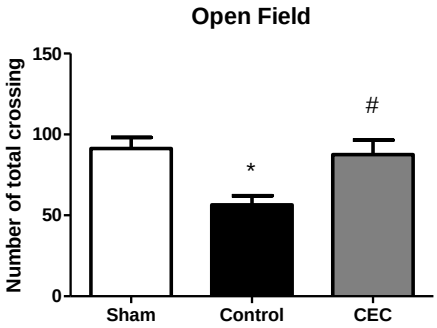
	Sham	Control	CEC
LYMPHOCYTES			
ADA	18.33±5.90	64.43±6.53***	45.77±3.48*
ATP	25.54±7.29	159.6±25.08***	6.87±1.66$^{\#\#\#}$
ADP	20.51±0.58	37.08±2.22***	9.51±3.53$^{\#\#\#}$
PLATELETS			
ADA	1.65±0.29	4.46±0.53**	4.43±0.39**
ATP	11.61±1.47	5.87±0.29**	2.47±0.58***
ADP	7.97±1.39	15.00±1.44*	5.88±1.83$^{\#\#}$
AMP	2.85±0.31	1.48±0.10*	2.29±0.39
SERUM			
ADA	9.59±1.80	10.34±1.80	8.37±2.63
ATP	2.28±0.28	1.29±0.11*	2.12±0.25$^{\#}$
ADP	3.18±0.40	2.78±0.22	2.63±0.22
AMP	2.78±0.33	2.52±0.18	2.54±0.21

Data are expressed as mean ± S.E.M. (n= 5). *P<0.05, **P<0.01 and ***P<0.001 versus the sham group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 versus the control group.

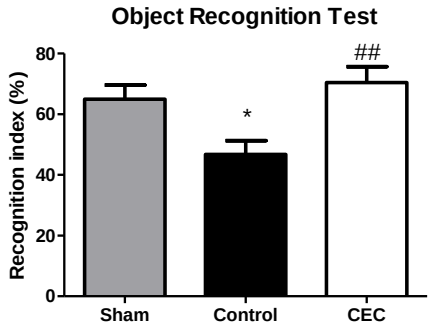

A
24h
C6 Cell Proliferation (%)
B
48h
C6 Cell Proliferation (%)
C
72h
C6 Cell Proliferation (%)
D
24h
C6 Cell Viability (%)
E
48h
C6 Cell Viability (%)
F
72h
C6 Cell Viability (%)
G
24h
U87 Cell Proliferation (%)
H
48h
U87 Cell Proliferation (%)
I
72h
U87 Cell Proliferation (%)
J
24h
U87 Cell Viability (%)
K
48h
U87 Cell Viability (%)
L
72h
U87 Cell Viability (%)
C 25 50 100 200 300 400 500
CEC Treatment (μg/mL)


A
C6 Cell Migration (%)
Time (h)
Control
CEC 25 μg/mL
**
B
C6 Cell Migration (%)
Time (h)
Control
CEC 50 μg/mL
***
C
C6 Cell Migration (%)
Time (h)
Control
CEC 100 μg/mL
***
D
U87 Cell Migration (%)
Time (h)
Control
CEC 0.5 μg/mL
E
U87 Cell Migration (%)
Time (h)
Control
CEC 1 μg/mL
F
U87 Cell Migration (%)
Time (h)
Control
CEC 2 μg/mL

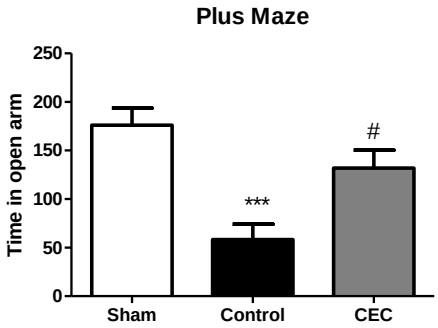
A

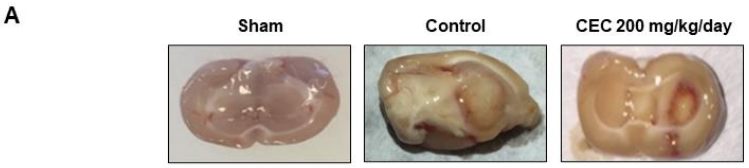

Open Field
Number of total crossing
150
100
50
0
*
#
Sham
Control
CEC

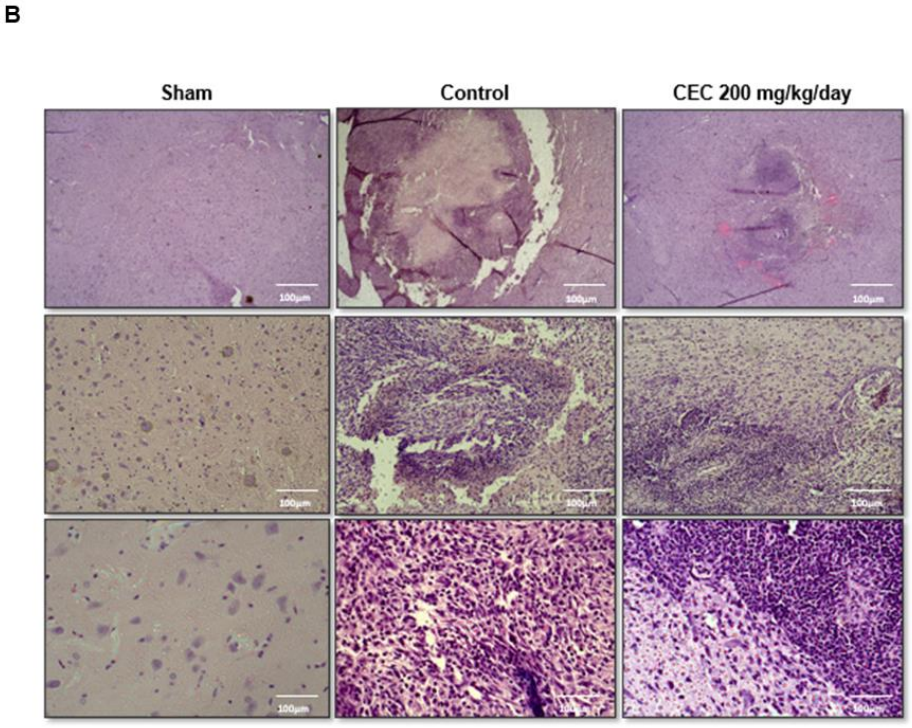
B

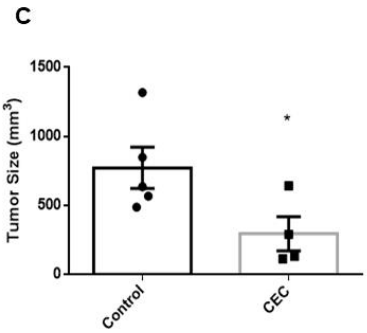

Object Recognition Test
Recognition index (%)
80
60
40
20
0
*
##
Sham
Control
CEC

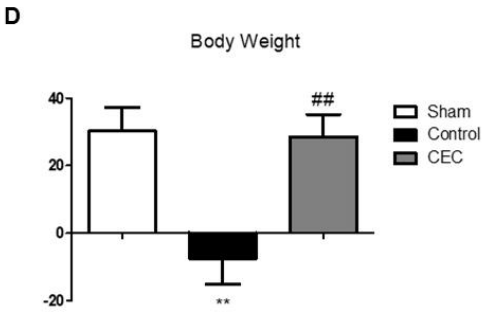
C

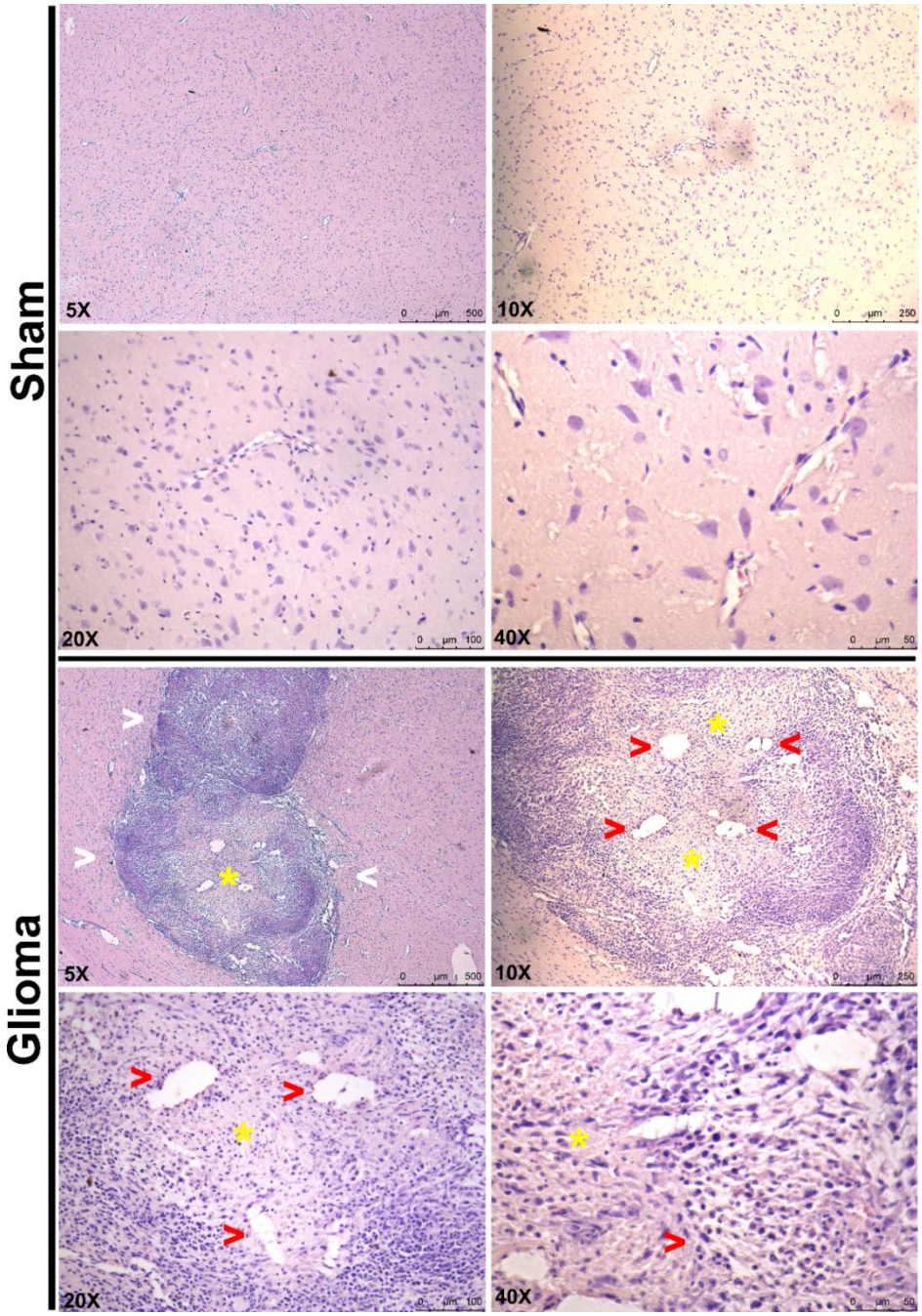

Plus Maze
Time in open arm
250
200
150
100
50
0
***
#
Sham
Control
CEC


A
Sham
Control
CEC 200 mg/kg/day

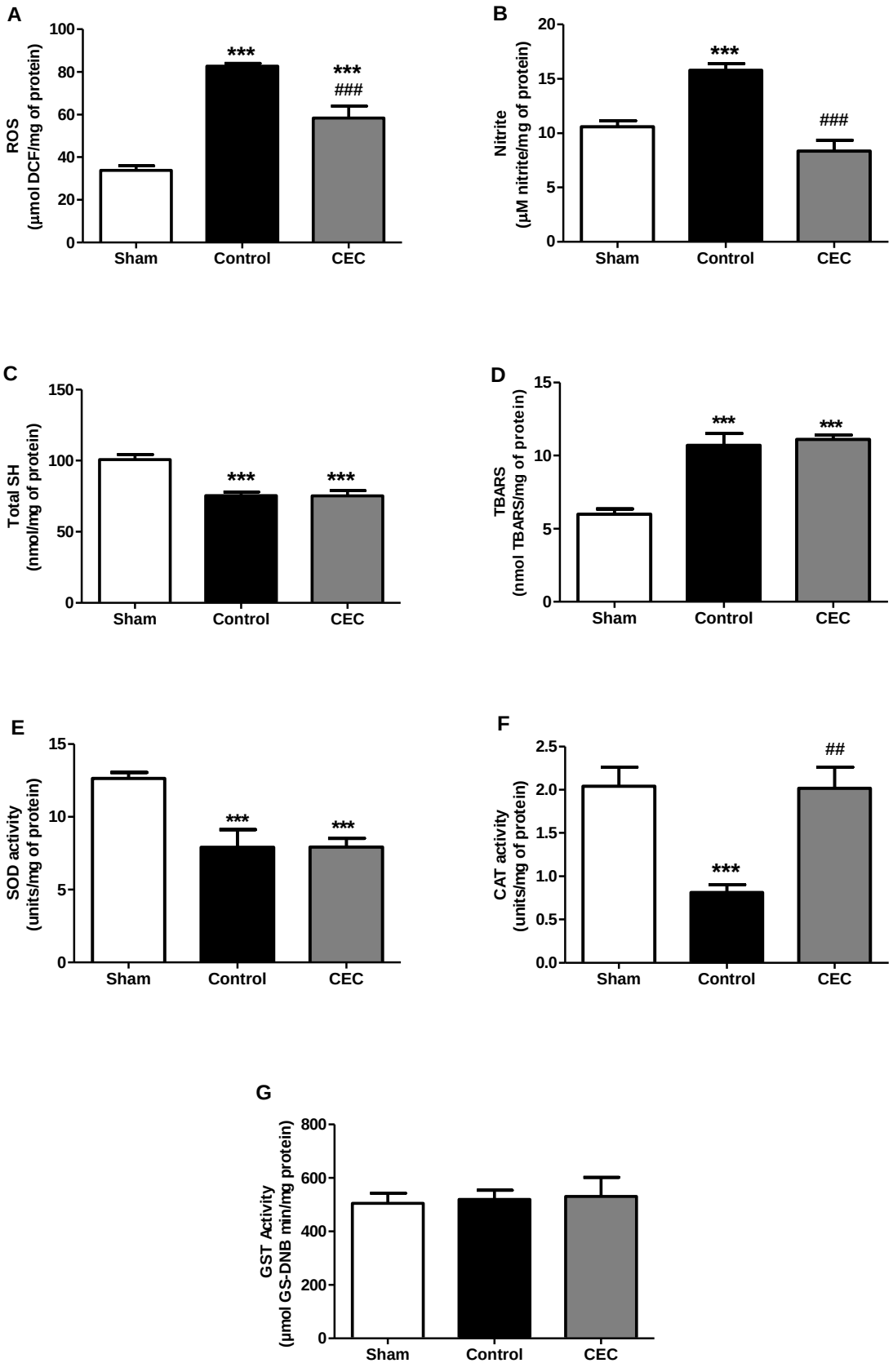

B
Sham
Control
CEC 200 mg/kg/day


C
Tumor Size (mm3)
1500
1000
500
0
*
Control
CEC

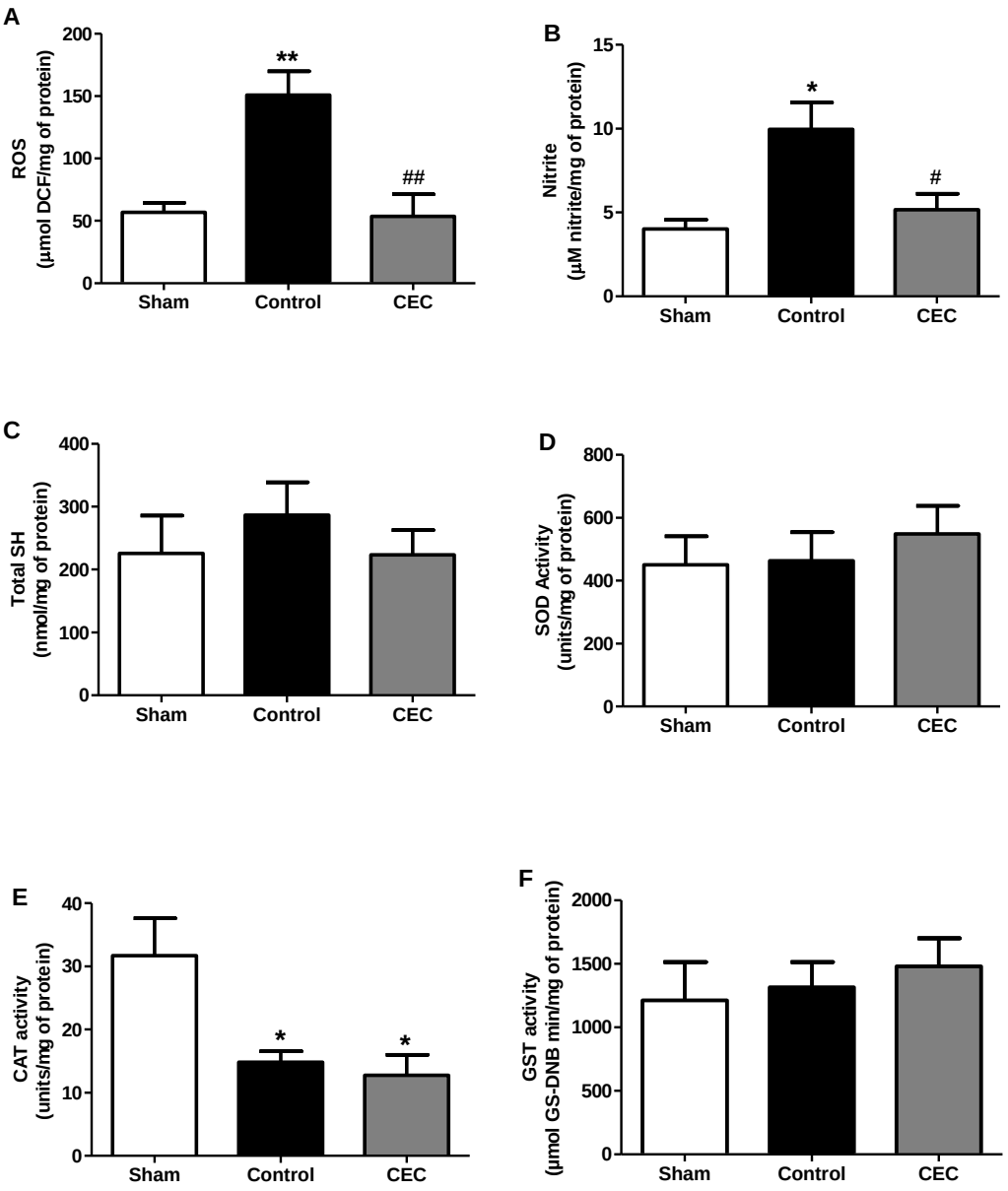

D
Body Weight
40
20
0
-20
##
**
Sham
Control
CEC


Sham
5X
10X
20X
40X
Glioma
5X
10X
20X
40X

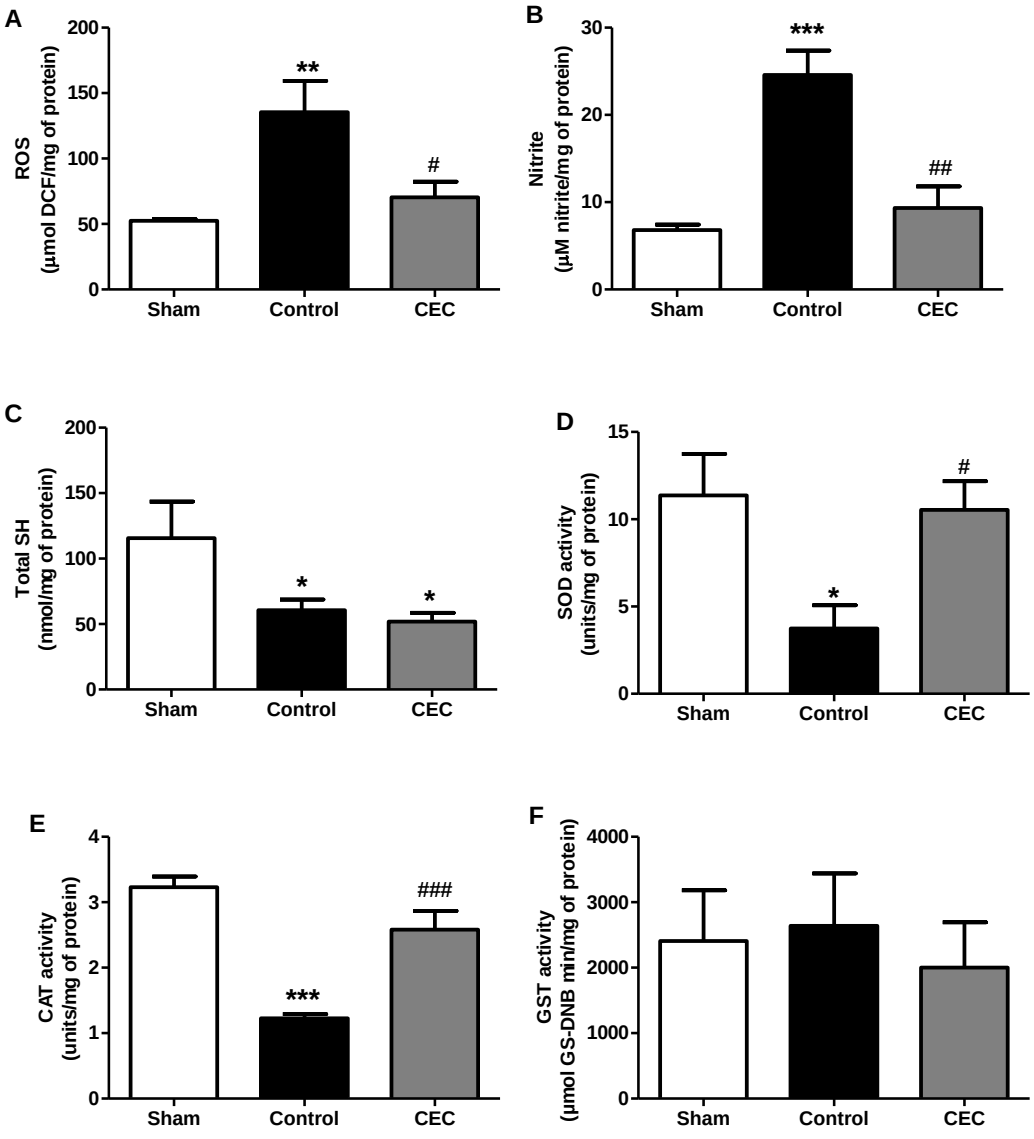
Brain

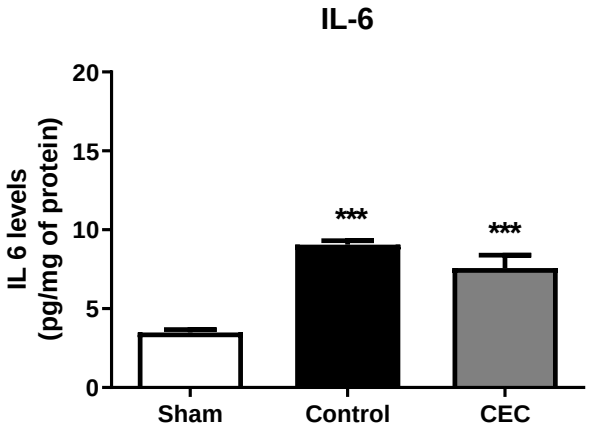

A
ROS
(µmol DCF/mg of protein)
100
80
60
40
20
0
***
***
###
Sham
Control
CEC
B
Nitrite
(µM nitrite/mg of protein)
20
15
10
5
0
***
###
Sham
Control
CEC
C
Total SH
(nmol/mg of protein)
150
100
50
0
***
***
Sham
Control
CEC
D
TBARS
(nmol TBARS/mg of protein)
15
10
5
0
***
***
Sham
Control
CEC
E
SOD activity
(units/mg of protein)
15
10
5
0
***
***
Sham
Control
CEC
F
CAT activity
(units/mg of protein)
2.5
2.0
1.5
1.0
0.5
0.0
##
***
Sham
Control
CEC
G
GST Activity
(µmol GS-DNB min/mg protein)
800
600
400
200
0
Sham
Control
CEC

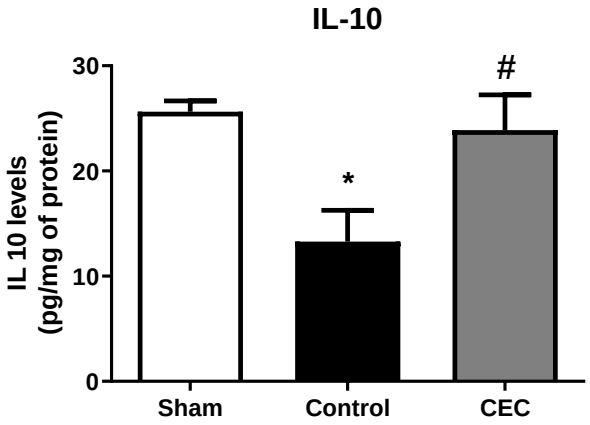
Platelets


A
ROS
(µmol DCF/mg of protein)
200
150
100
50
0
**
##
Sham
Control
CEC
B
Nitrite
(µM nitrite/mg of protein)
15
10
5
0
*
#
Sham
Control
CEC
C
Total SH
(nmol/mg of protein)
400
300
200
100
0
Sham
Control
CEC
D
SOD Activity
(units/mg of protein)
800
600
400
200
0
Sham
Control
CEC
E
CAT activity
(units/mg of protein)
40
30
20
10
0
*
*
Sham
Control
CEC
F
GST activity
(µmol GS-DNB min/mg of protein)
2000
1500
1000
500
0
Sham
Control
CEC

Serum


A
ROS
(µmol DCF/mg of protein)
200
150
100
50
0
**
#
Sham
Control
CEC
B
Nitrite
(µM nitrite/mg of protein)
30
20
10
0
***
##
Sham
Control
CEC
C
Total SH
(nmol/mg of protein)
200
150
100
50
0
*
*
Sham
Control
CEC
D
SOD activity
(units/mg of protein)
15
10
5
0
*
#
Sham
Control
CEC
E
CAT activity
(units/mg of protein)
4
3
2
1
0
***
###
Sham
Control
CEC
F
GST activity
(µmol GS-DNB min/mg of protein)
4000
3000
2000
1000
0
Sham
Control
CEC


IL-6
IL 6 levels
(pg/mg of protein)
20
15
10
5
0
***
***
Sham
Control
CEC


IL-10
IL 10 levels
(pg/mg of protein)
30
20
10
0
*
#
Sham
Control
CEC

4.3. Capítulo 3 - Manuscrito 2

Cecropia pachystachya extract and enriched flavonoid fraction protect against memory deficits, inflammation and oxidative damage in LPS challenged mice

Natália Pontes Bona, Mayara Sandrielly Soares de Aguiar, Luiza Spohr, Nathalia Stark Pedra, Francieli da Silva dos Santos, Juliane Torchelsen Saraiva, Fernando Lopez Alvez, Bernardo de Moraes Meine, Vânia Recart, Ingrid Vicente Farias, Caroline Flach Ortmann, Roselia Maria Spanevello, Flavio Henrique Reginatto, Francieli Moro Stefanello

Status: Submetido no periódico ***Neurotherapeutics***

Cecropia pachystachya extract and enriched flavonoid fraction protect against memory deficits, inflammation and oxidative damage in LPS challenged mice

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Abstract

Cecropia pachystachya (CEC) Trécul is a medicinal plant native to South and Central Americas used to treat asthma and diabetes, and a rich source of polyphenols, particularly flavonoids. Some studies also demonstrate several pharmacological properties, such as anti-inflammatory, antidiabetic and neuroprotective. In this study, we aimed to investigate the potential neuroprotective of CEC extract (200 mg/kg) and its enriched flavonoid fraction flavonoid-enriched fraction (EFF-CP) (50 and 100 mg/kg) in a model of lipopolysaccharide (LPS)-induced neuroinflammation. CEC and EFF-CP were administered intragastrically for 14 days and LPS (250 µg/kg) was administered intraperitoneally from the 8th to the 14th day of treatment. The HPLC analysis showed the presence of isoorientin, orientin, and isovitexin as major compounds. The pharmacological results demonstrated that CEC extract and its EFF-CP protected against LPS-induced short-term and long-term memory deficits. Also, the treatment with CEC and/or EFF-CP showed to be effective to protect against LPS-induced increases in reactive oxygen species, nitrites, total thiol and thiobarbituric acid reactive species in cerebral cortex, hippocampus and striatum. Moreover, CEC and EFF-CP restored superoxide dismutase and catalase activity that were reduced by LPS in the cortex, hippocampus and striatum. Finally, we observed an increase in TNF-α levels in cortex, striatum and hippocampus in LPS group, while CEC treatment reversed all these changes in cortex. In contrast, EFF-CP decreased this cytokine levels in all structures analyzed at both doses. The results, suggest that CEC extract and its EFF-CP are important therapeutic targets for the treatment of neurodegenerative diseases caused by neuroinflammation.

Keywords: *Cecropia pachystachya*; flavonoids; neuroinflammation; lipopolysaccharide; memory; redox status

1. Introduction

Cecropia pachystachya Trécul (Urticaceae) is a typical tree that occur in humid or semi-humid parts of the Neotropics. *C pachystachya* is native to Brazil, and can be known as ‘embaúba’or “umbaúba” [1, 2]. It is popularly known as a traditional medicinal plant, and is widely used to treat respiratory diseases [2,3] and renal diseases [4]. In addition, other pharmacological properties have already been associated to CEC, as anti-inflammatory, diuretic, anti-hypertensive [2], and anti-diabetic properties [2, 5].

Regarding the phytochemical composition of *C. pachystachya*, has already been reported the presence of flavonoids such as C-glycosylated (orientin, isoorientin and isovitexin) and O-glycosylated (isoquercitrin), in addition to chlorogenic acids, glycosylated iridoids, proanthocyanidins, organic acids, flavan-3-ols [6, 7]. It should be noted that flavonoids have already been reported in the literature as potent neuroprotectors [8], thus, the optimization of the *C. pachystachya* extract in a flavonoid-rich fraction (EFF-CP) becomes an interesting target of study against neuroinflammation.

In view of the wide range of biological effects of *C. pachystachya*, the study of its actions on neurological disorders becomes of great relevance. In this sense, it has been demonstrated that *C. pachystachya* prevented the depressive-like behavior as well as the oxidative damage in the brain of mice exposed to chronic unpredictable stress [6] and to chronic mild stress [9,10]. In addition, Gazal et al [11] demonstrated that *C. pachystachya* pretreatment prevents both behavioral and pro-oxidant effects of ketamine in a model of bipolar disorders [11]. These findings supporting its neuroprotective potential of *C. pachystachya* against behavioral and neurochemical dysfunctions. However, it is still poorly understood how *C. pachystachya* exerts the neuroprotective effects.

In this sense, considering the role of neuroinflammation in the pathophysiology of different neurological disorders, such as depression, bipolar disorders, Alzheimer's disease, Parkinson's disease and in brain tumors such as glioblastoma [12, 13, 14, 15], it is extremely important to investigate the effects of *C. pachystachya* in a neuroinflammation model

The induction of neuroinflammation through the peripheral administration of lipopolysaccharide (LPS) in mice is one of the most used biological models to investigate both the biochemical and molecular mechanisms of neuroinflammation,

as well as for pre-clinical assessment of nutraceuticals [13]. LPS is an endotoxin from membrane of bacteria able to induce a potent trigger of inflammation. When administered peripherally in mice, LPS leads to cellular and molecular changes that characterize neuroinflammation, such as, for example, induced astrocyte and microglia activation, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokine expression in the brain [13, 16].

Thus, the aim of the present study was to investigate the effects of *Cecropia pachystachya* extract (CEC) and EFF-CP against memory deficits, inflammation and oxidative damage in cerebral cortex, striatum and hippocampus from LPS challenged mice.

2. Methodology

a. Chemicals

LPS (E. coli strain O55:B5) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

b. Plant Material, Extraction and EFF-CP Preparation

Leaves of *Cecropia pachystachya* were collected in Torres, RS, Brazil. The extract (CEC) was prepared by the aqueous infusion method according to the protocol established by Gazal et al [17]. The extract was dried spray drying under conditions under the follow conditions: 160ºC (inlet temperature) and 111ºC (outlet temperature); 5% feed flow; 100% airflow and 0.7 mm atomizer diameter. The powdered extract was then stored at -20 ºC until use. The flavonoid-enriched fraction will be prepared according to the methodology described by Ortmann et al [9].

c. Animals

All experimental procedures involving animal were analyzed and approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, RS, Brazil (protocol number: CEEA 32979-2019). Adult male Swiss mice (60 days, 30–45 g) were obtained from the Central Animal House of Federal University of Pelotas, Pelotas, RS, Brazil. The animals were kept at constant temperature (22 ± 1°C) under the light/dark cycle and free access to water and food. All animal experiments were carried out in accordance with the National Institutes of Health

guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

d. Protocol procedure

Adult male Swiss mice (60 days old) will be divided into the following groups (n= 8-10 per group): I- control; II- LPS (250 µg/kg); III- LPS + CEC 200 mg/kg; IV- LPS+ EFF-CP 50 mg/kg and V- LPS + EFF-CP 100 mg/kg. The experimental protocol of neuroinflammation and treatment with CEC and EFF-CP lasted two weeks, with the animals receiving water or CEC 200 mg/kg or EFF-CP for 14 days at doses of 50 or 100 mg/kg orally (gavage). Between the 8th and 14th day, the animals in the groups also received vehicle (0.9% saline solution) or LPS (250 µg/kg) intraperitoneally. On the 14th and 15th, the animals were submitted to behavioral tests to assess locomotor activity and short- and long-term memory. After that, the animals were anesthetized and euthanized and samples were collected for biochemical tests (Fig 1).

e. Behavior tests

i. Locomotor activity

Locomotor behavior was evaluated using open-field test which was carried out to identify motor disabilities, which might influence the other behavioral tests performed. The floor of the arena it has 9 equal squares and placed in a sound free room. The animals were placed in the rear left square and allowed to explore freely for 5 min. The total number of squares crossed with all paws (crossing) was manually counted [18]. The apparatus was cleaned and dried with a 40% alcohol solution after testing with each mouse.

ii. Long-term memory test

This evaluation was performed by object recognition test. Mice were habituated to the experimental arena 24 h before the test. Objects, made of waterproof plastic, were placed on the sand floor. This test was performed in two stages. During training, mice were placed in the arena with two identical objects (A1 and A2) and allowed to explore for 5 min. The session was valid if mice explored each object for at least 30 s. Following this, mice were tested 24 h after training to evaluate their long-term memory. For the testing, one of the objects was changed to a different, novel object (B). The mice were introduced into the arena for 5 min and

allowed to explore freely. The positions of the objects (familiar or novel) were randomly exchanged. Exploration was defined as smelling or touching the object with the nose and/or forelegs. Sitting on or around the object was not considered exploratory behavior. The apparatus and the objects were cleaned and dried with a 40% alcohol solution after each mouse. In this task was evaluated the total exploration time (s) in the test. Exploratory preference time for the novel object expressed as an index evaluated in the object recognition test [19].

iii. Short-term memory test

This evaluation was performed by Y-maze test. The apparatus has three arms: start arm, in which rats were placed to start to explore (always open); novel arm, which was blocked during the first trial, but open during the second trial; and other arm (always open). Firstly, animals underwent training: they were placed in the start arm and could explore the start and other arm. The third arm (novel arm) remained closed during training. After 2 h, testing was performed. Mice were placed in the start arm with free access to all three arms for 5 min. The number of entries in each arm and time spent exploring each arm (expressed as a percentage of the time spent in each arm) was recorded [20]. The apparatus was cleaned with 40% alcohol solution and dried after each session with each animal.

f. Tumor Necrosis Factor-α (TNF-α) quantification

TNF-α level was determined with enzyme-linked immunosorbent assay (ELISA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The concentration of TNF-α was determined according to the manufacturer instructions and was expressed as pg/mg of protein.

g. Oxidative stress parameters

At the end of the experimental protocol, the animals were euthanized by deepening anesthetic using inhaled isoflurane, and the brain was quickly removed for separation of the cerebral cortex, hippocampus and striatum, which were then stored at -80 °C for subsequent biochemical analyzes. The structure regions were prepared by homogenizing with 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM

KCl (1/10 w/v). The homogenates were centrifuged at 2500 x g for 10 min at 4 °C. The supernatants were collected and used in oxidative stress analyses.

Reactive oxygen species (ROS): ROS production was detected by Ali et al [21] methodology which use the detection of the oxidation of DCFH-DA to fluorescent 2′,7′-dichlorofluorescein (DCF). Results were expressed as µmol DCF/mg of protein.

Nitrite: This parameter was evaluated by Griess reaction [22]. The sample was incubated with 51% sulfanilamide and 0.3% N-1-naphthylethylenediamine dihydrochloride at room temperature. Nitrite was measured using sodium nitrite as standard. Results were expressed as µM nitrite/mg of protein.

Thiobarbituric acid reactive substances (TBARS): This assay was based in the Esterbauer and Cheeseman [23] method, which firstly precipitates the proteins from the samples supernatant using 10% TCA. Posteriorly, TBA (0.67%) was added to supernatant and incubated in a dry block at 100 °C for 30 min. TBARS levels were reported as nmol of TBARS/mg of protein.

Total sulfhydryl content: For this assay, was used the supernatants, PBS buffer (pH 7.4) containing EDTA and for the reaction started, was added DTNB which was incubated for one hour in the dark. DTNB reduced by thiol groups was oxidized (disulfide) and a yellow derivative (TNB) was generated. The results were reported as nmol TNB/mg of protein [24].

Superoxide dismutase (SOD): This assay is based on the inhibition of superoxide-dependent adrenaline auto-oxidation to adenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD. SOD activity was measured, as previously described by Misra and Fridovich [25], and reported as units/mg of protein.

Catalase (CAT): The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored at 240 nm at 37 °C, as previously reported by Aebi [26]. CAT activity was reported as units/mg of protein.

Protein determination: Protein concentration was measured as previously described by Lowry et al [27].

h. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc tests using GraphPad Prism 5.0 (Intuitive Software for Science, São Diego, CA, USA). *P*<0.05 was considered statistically significant. All data were expressed as mean ± standard error of the mean (SEM).

3. Results

a. CEC and EFF-CP protect against LPS-induced short-term and long-term memory deficits

In the fig 2 are demonstrated the results about locomotor activity and short-term and long-term memory. Firstly, was showed that no changes was found regarding locomotor activity evaluated in the open-field test (Fig 2A, $F_{(4-41)}$= 5.47, *P*>0.05). In relation to long-term memory evaluated through the exploration time on the new object in the object recognition test, it was possible to observe that both CEC200 and EFF-CP50 and EFF-CP100 treatment were able to protect against LPS-induced long-term memory deficit (Fig 2B, $F_{(4-41)}$= 23.75, *P*<0.001). Also, CEC200, EFF-CP50 and EFF-*CP*10 treatments were able to protect against LPS-induced short-term memory deficit (Fig 2C, $F_{(4-30)}$= 4.16, *P*<0.05) evaluated through the exploration time in the new arm in the Y-maze deficit (Fig 2C, $F_{(4-30)}$= 4.16, *P*<0.05). No significant changes were observed regarding exploration time in the initial and other arms (Fig 2C, $F_{(4-30)}$= 4.16, *P*<0.05).

b. CEC and EFF-CP reduce TNF-α levels in the brain of mice exposed to LPS

In the fig 3 is possible to observed that LPS induced the enhanced in the TNF-α levels in the cerebral cortex (Fig 3, $F_{(4-15)}$= 23.82, *P*<0.001). However, both CEC200 (*P*<0.01) and EFF-CP50 (*P*<0.001) and EFF-CP100 (*P*<0.001) are able to reduce the levels of this cytokine (Fig 3, $F_{(4-15)}$= 23.82). In the striatum EFF-CP50 and EFF-CP100 were able to protect against the increase in the TNF-α levels caused by LPS administration (Fig 3, $F_{(4-16)}$= 33.97, *P*<0.001). Also, in the hippocampus EFF-CP50 (*P*<0.01) and EFF-CP100 (*P*<0.05) were able to protect against the increase in the TNF-α levels caused by LPS (*P*<0.001) administration (Fig 3, $F_{(4-16)}$= 16.22).

c. CEC200, EFF-CP50 and EFF-CP100 protect against oxidative damage in cerebral cortex

In fig 4 are the results of oxidative damage in the cerebral cortex. First, it is possible to observe a significant increase in the production of ROS caused by the administration of LPS (Fig 4A, $F_{(4-20)}$= 64.63, *P*<0.001). However, CEC200, EFF-CP50 and EFF-CP100 treatments were able to reduce ROS levels (Fig 4A, $F_{(4-20)}$= 64.63, *P*<0.001). Furthermore, it is possible to observe that both EFF-CP concentrations (Fig 4B, $F_{(4-20)}$= 8.46, *P*<0.01) were able to protect against the increase in nitrite levels caused by LPS (Fig 4B, $F_{(4-20)}$= 8.46, *P*<0.01).

On the other hand, LPS administration was able to reduce the total thiol content in the cerebral cortex (Fig 4C, $F_{(4-20)}$= 16.55, *P*<0.001). However, all treatment with CEC200 and with EFF-CP50 and EFF-CP100 were able to increase the levels of total thiol (Fig 4C, $F_{(4-20)}$= 16.55, *P*<0.001). Furthermore, it was shown that LPS is able to increase the levels of TBARS (Fig 4D, $F_{(4-20)}$= 10.18, *P*<0.01), while treatment with CEC200, EFF-CP50 and EFF-CP100 was able to reduce the lipid peroxidation (Fig 4D, $F_{(4-20)}$= 10.18, *P*<0.01).

Regarding the activity of antioxidant enzymes, it is possible to observe that there was a reduction in the activity of SOD (Fig 4E, $F_{(4-20)}$= 4.55, *P*<0.05) and CAT (Fig 4F, $F_{(4-20)}$= 13.22, *P*<0.05) caused by LPS compared to the control group. However, EFF-CP100 treatment was able to restore SOD (Fig 4E, $F_{(4-20)}$= 13.22, *P*<0.05) and CAT (Fig 4F, $F_{(4-20)}$= 13.22, *P*<0.001) activity.

d. CEC and EFF-CP protect against oxidative damage caused by LPD in the striatum

In striatum, it is possible to observe the results of the redox status in fig 5. There was an increase in the levels of ROS (Fig 5A, $F_{(4-20)}$= 17.77, *P*<0.001) and nitrites (Fig 5B, $F_{(4-20)}$= 8.91, *P*<0.001) caused by the administration of LPS in relation to the control group. However, treatment with CEC200, EFF-CP50 and EFF-CP100 is able to reduce both ROS (Fig 5A, $F_{(4-20)}$= 17.77, *P*<0.001, *P*<0.01, *P*<0.001 respectively) and nitrite (Fig 5B, $F_{(4-20)}$= 8.91, *P*<0.001, *P*<0.01, *P*<0.001 respectively) levels.

No changes were found regarding total thiol content in striatum in any experimental group (Fig 5c, $F_{(4-20)}$= 1.69, *P*>0.05). About lipid peroxidation, was possible to observed that LPS causes an increase in the TBARS levels (Fig 5D, $F_{(4-17)}$= 5.18, *P*<0.05) in the striatum, however, the treatment with EFF-CP100 was able to protect against this enhanced (Fig 5D, $F_{(4-17)}$= 5.18, *P*<0.01).

Furthermore, it was demonstrated that treatments with CEC200 (*P*<0.05), EFF-CP50 (*P*<0.01) and EFF-CP100 (*P*<0.05) were able to protect against the reduction of SOD activity caused by the LPS administration (Fig 5E, $F_{(4-20)}$= 5.59, *P*<0.05). Similar results were found regarding CAT activity, which was reduced in the LPS group (Fig 5F, $F_{(4-20)}$= 13.01, *P*<0.001) compared to the control group, while treatment with CEC200 (*P*<0.001) and EFF-CP100 (*P*<0.05) were able to protect against this alteration.

e. Protective effect of CEC and EFF-CP against LPS-induced changes in redox status

Fig 6 shows the results of the oxidative stress parameters evaluated in the hippocampus. It is possible to observe an increase in the production of ROS in the LPS group (Fig 6A, $F_{(4-20)}$= 16.96, *P*<0.05) compared to the control group, while the treatment with CEC200 (Fig 6A, $F_{(4-20)}$= 16.96, *P*<0.001) was able to protect against this increase. Furthermore, there was an increase in nitrite levels caused by the administration of LPS (Fig 6B, $F_{(4-20)}$= 18.23, *P*<0.001), however treatment with CEC200, EFF-CP50 and EFF-CP100 (Fig 6B, $F_{(4-20)}$= 18.23, *P*<0.001) were able to protect against this increase.

Furthermore, it was possible to demonstrate that the treatment with CEC200, EFF-CP50 and EFF-CP10 (Fig 6C, $F_{(4-17)}$= 69.94, *P*<0.001) were able to prevent the reduction of the total thiol content found in the LPS group in relation to the control group (Fig 6C, $F_{(4-17)}$= 69.94, *P*<0.001). No significant changes were found regarding the TBARS results in the hippocampus (Fig 6D, $F_{(4-20)}$= 3.84, *P*>0.05).

In addition, there was a reduction in the activity of SOD (Fig 6E, $F_{(4-20)}$= 17.49, *P*<0.01) and CAT (Fig 6F, $F_{(4-20)}$= 17.06, *P*<0.05) enzymes in the LPS group compared to the control*, however treatment with CEC200 (P<0.05),* EFF-CP50 *(P<0.001) and* EFF-CP100 (*P*<0.001) were able to protect against this reduction in SOD and CAT activity.

4. Discussion

The brain or spinal cord are response for the inflammatory process when the brain's innate immune system is activated by insults such as injury, inflammation, toxin exposure such as LPS, and during aging. So, neuroinflammation can result in cognitive impairment and neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and glioblastoma [28, 29, 30]. In this sense, considering the neurodegenerative consequences of neuroinflammation, it is important to study the pathophysiological mechanisms of this process and possible therapeutic alternatives.

LPS promoting neuroinflammation upon binding with the stimulation of Toll-like receptor-4 (TLR4) receptors present in astrocytes and microglia. The stimulation of TLR4 by LPS, active two signaling pathways: (1) the myeloid differentiation factor-88 (MyD88)-dependent and (2) MyD88-independent pathways (Sangaran et al., 2021). LPS is able to binds to CD14/TLR4/myeloid differentiation protein 2 (MD2) complex of pattern-recognition receptors and degrade inhibitory kappa B (IκB), resulting in the translocation of the transcription factor, nuclear factor-kappa B (NF-κB), that expresses a wide variety of genes involved in the immune response [31].

The activation of TLR4/NF-κB signaling pathway induces the upregulation of pro-inflammatory cytokines and mediators, promoting the neuroinflammation mechanisms which induces neuronal apoptosis by intrinsic or extrinsic pathway and results in the secretion of inflammatory cytokines and chemokines, as well ROS and nitrogen species (RNS) [28, 29,31]. Also, one of the main mediators of the deleterious effects caused by LPS is the TNF-α, which are increased in brain from the animals that received peripheral injection of LPS [13, 32].

Corroborating previous data in the literature, it was shown that LPS administration caused an increase in TNF-α concentration in all brain structures evaluated. TNF-α can mediate the neuroinflammation through binding to the TNF receptor, with consequent formation of the complex I followed by activation of the NFκB pathway. This process generates exacerbated production of ROS and RNS since in the neuroinflammation there are an enhanced in the expression and activity of inducible oxide nitric synthase (iNOS) and NADPH oxidase 2 (NOX2) of glia cells [13; Fisher et al., 2015).

Thus, as mentioned above, the increase in the production of ROS and nitrites, which is an intermediary for the formation of RNS, can be explained through the following possible mechanisms after TNF-α signaling: increased activity of iNOS and NOX2, by the process of apoptosis, as well as reduction of the activity of the antioxidant enzyme SOD and CAT, as demonstrated in the present study [13, Fisher et al., 2015). Also, it was observed a reduction in SH levels, which may be associated with possible protein damage, in addition to an increase in lipid peroxidation, as observed through TBARS levels enhanced. These events together characterize the oxidative damage in the studied brain structures, with a possible reduction in brain cellular integrity and function in the LPS group.

Although LPS causes an increase in TNF-α levels and induces oxidative stress, both CEC200 and EFF-CP at doses of 50 and 100 mg/kg were able to protect against neuroinflammation, since they reduced the TNF levels and prevented most of the changes observed in the oxidative stress parameters evaluated in the three brain structures. Mendonça et al [5] demonstrated that the CEC extract present chlorogenic acid, isoorientin, orientin, and isovitexin as major compounds. Also, the author related that the total phenolic and tannin contents were, respectively, 305.6±0.80 and 144.6±19.04 mg of gallic acid equivalent/g of extract in the CEC extract [5].

The compounds found in CEC extract and EFF-CP may be able to act on two fronts, in the case of the inflammatory process. That is, (1) by inhibiting the release of TNF-α and (2) as an antioxidant. Studies in the literature have already shown that, gallic acid [33,34], isoorientin [35], orientin [36], isovitexin [37], and some tannins, such as tannic acid [38], are able to suppressed the TNF-α induced inflammatory responses by decreasing phosphorylation of two critical inflammatory signaling proteins, NF-kappa-B inhibitor alpha (IκBα) and/or nuclear factor-κB (NF-κB). Also, the antioxidant effects of phenolic compounds, as found in the CEC extract, are well established, for example these compounds are able to modulate iNOS and NOX2 activity, and thus, reduced ROS production; it has ROS scavenging activity; capacity of chelate metal ions; positively modulates of the activity of the antioxidant enzymes SOD, CAT and glutathione peroxidase (GPx) [13, 39, 40, 41].

Therefore, considering the previous evidence of the beneficial effects of phenolic compounds, especially C-glycosyl flavonoids and the extract of *C. pachystachya*, the standardization of an enriched-flavonoid fraction EFF-CP

becomes an interesting alternative [9, 10]. In this way, Ortmman et al [10] demonstrate fifteen flavonoids, including isoorientin (43.46 mg/g), orientin (23.42 mg/g) and isovitexin (17.45 mg/g) as major C-glycosyl flavonoids. Also, EFF demonstrated a rich matrix of C-glycosyl flavonoids, mainly luteolin and apigenin derivatives. Thus, the purification of EFF-CP is extremely important because it can minimize possible undesirable effects of other compounds present in the CEC, in addition to contributing to the synergistic effect of phenolic compounds, and thus potentiating their neuroprotective effects 9, 10].

It has been demonstrated that LPS-induced neuroinflammation and oxidative stress-mediated neuronal apoptosis by promoting ROS generation and caspase activation as well as behavioral and memory impairments [42]. Corroborating these findings, LPS administration was shown to cause short-term and long-term memory impairment possibly associated with TNF-α -mediated signaling and oxidative damage. Nonetheless, CEC protected against long-term deficit, while EFF-CP at both concentrations protected against short-term and long-term memory.

It is demonstrated that neural progenitors in the hippocampus respond rapidly by reducing their proliferative profile in a neuroinflammatory context. It has even been demonstrated that LPS, promoting a net reduction in the hippocampal neurogenic process and reduced proliferation in the dentate gyrus [43]. Thus, the deleterious effects caused by LPS administration, such as oxidative damage and neuroinflammation, contribute to reduced neurogenesis in the hippocampus, which can lead to cognitive and memory impairment, as demonstrated by object recognition and maze Y tests. Another important point is that in striatum proinflammatory cytokines, as TNF-α, are involved in the decrease in dopamine neurotransmission could lead to neurodegeneration [44].

Together, the neuroinflammation process associated with oxidative damage in the cerebral cortex, hippocampus and striatum causes cognitive and memory changes in animals. Thus, compounds with the ability to protect against the cascade of neurochemical changes caused by LPS, such as CEC and EFF-CP, are of great relevance as they can prevent the process of neurodegeneration and cognitive and memory impairment.

5. Conclusion

The present study demonstrated that *C. pachystachya* extract and its flavonoid-enriched fraction are important therapeutic targets in the treatment of diseases involving the neuroinflammation process. Since they demonstrated a promising effect against alterations in behavioral parameters, modulating oxidative stress parameters and reestablishing TNF-alpha levels in LPS challenged mice

Conflict of interest statement: The authors declare that there is no conflict of interest.

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Figure captions

Fig 1. Experimental scheme of treatment with *Cecropia pachystachya* extract and Enriched Flavonoid Fraction in an animal model of neuroinflammation.

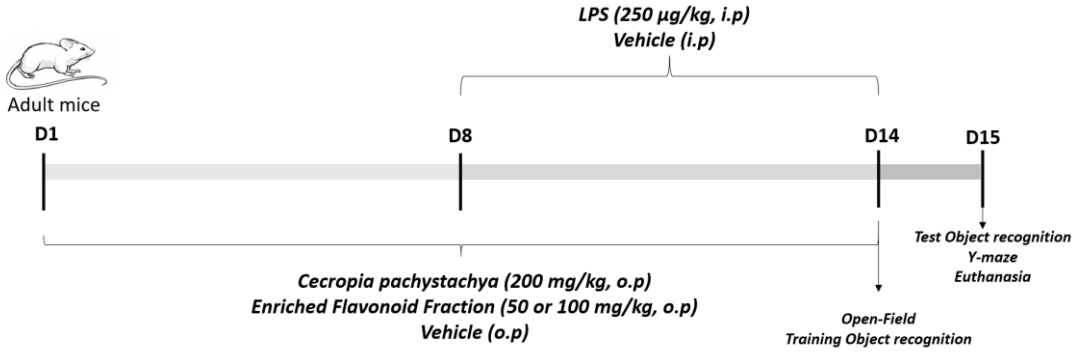
Fig 2. Effect of *Cecropia pachystachya* (CEC) extract (200 mg/kg) and Enriched Flavonoid Fraction (EFF) (50 and 100 mg/kg) on locomotion (A) and long (B) and short (C)-term memory of mice submitted to experimental model of lipopolysaccharide (LPS, 250 μg/kg)-induced neuroinflammation. One-way ANOVA, and post-hoc Tukey multiple comparisons tests were performed. Bars represent mean and ± SEM. $^{\#}$ P<0.05; $^{\#\#\#}$ P<0.001 compared to vehicle/saline group. * P<0.05; *** P<0.001, compared to vehicle/LPS group (n = 8–10 per group).

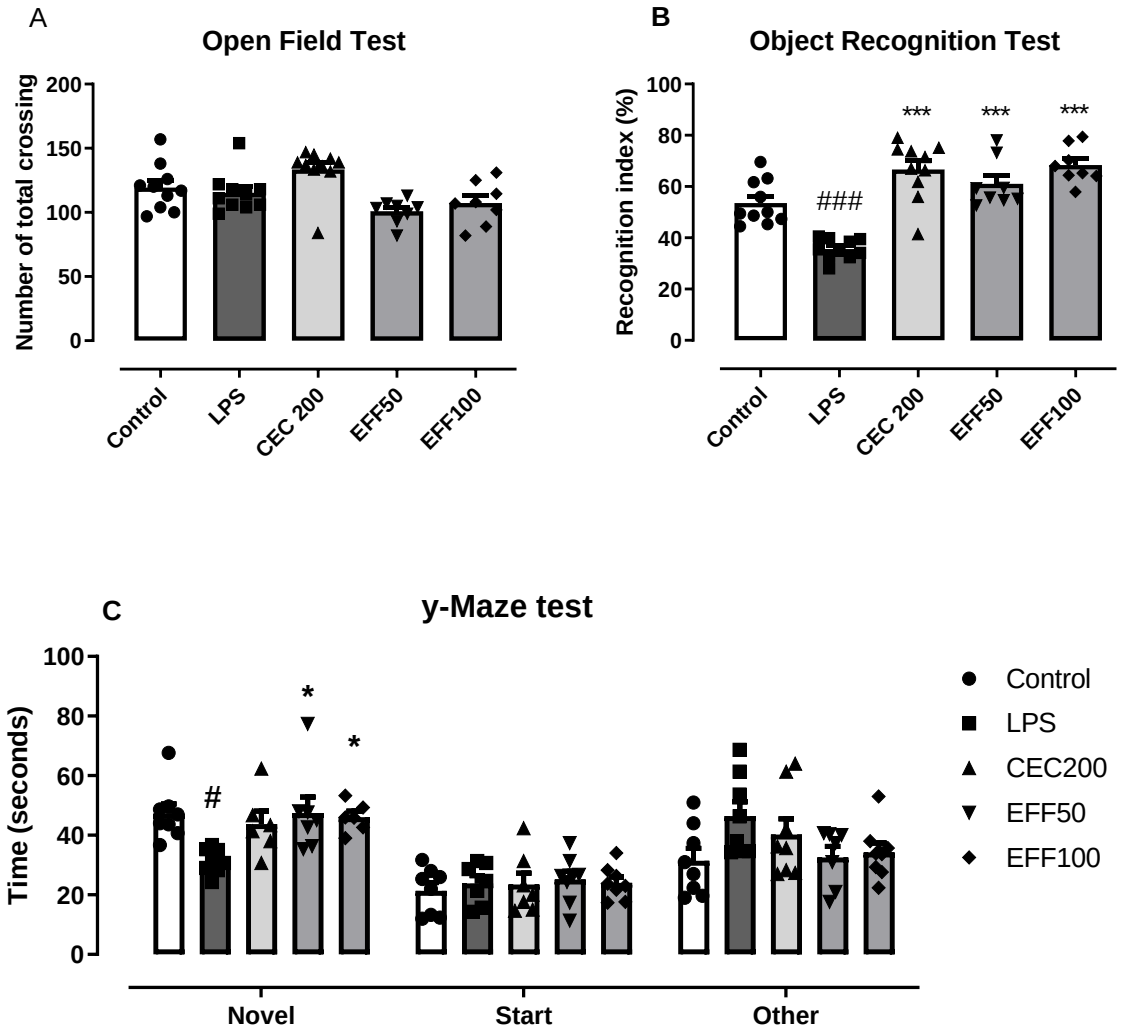
Fig 3. Effect of *Cecropia pachystachya* (CEC) extract (200 mg/kg) and Enriched Flavonoid Fraction (EFF) (50 and 100 mg/kg) on tumor necrosis factor alpha (TNF-α) levels in cerebral cortex, striatum and hippocampus of mice submitted to experimental model of lipopolysaccharide (LPS, 250 μg/kg)-induced neuroinflammation. One-way ANOVA, and post-hoc Tukey multiple comparisons tests were performed. Bars represent mean and ± SEM. $^{\#\#\#}$ P<0.001 compared to vehicle/saline (control) group. * P<0.05; **P<0.01; *** P<0.001, compared to vehicle/LPS group (n = 4-5 per group).

Fig 4. Effect of *Cecropia pachystachya* (CEC) extract (200 mg/kg) and Enriched Flavonoid Fraction (EFF) (50 and 100 mg /kg) on reactive oxygen species - ROS (A), nitrite (B), total thiol content - SH (C) and thiobarbituric acid reactive species - TBARS (D) levels and on the activity of superoxide dismutase - SOD (E) and catalase - CAT (F) in cerebral cortex of mice submitted to experimental model of lipopolysaccharide (LPS, 250 μg/kg)-induced neuroinflammation. One-way ANOVA and post-hoc Tukey multiple comparisons tests were performed. Bars represent mean and ± SEM. $^{\#}$ P<0.05; $^{\#\#}$ P<0.01; $^{\#\#\#}$ P<0.001 compared to vehicle/saline (control) group. * P<0.05; **P<0.01; *** P<0.001, compared to vehicle/LPS group (n = 5 per group).

Fig 5. Effect of *Cecropia pachystachya* (CEC) extract (200 mg/kg) and Enriched Flavonoid Fraction (EFF) (50 and 100 mg/kg) on reactive oxygen species-ROS (A), nitrite (B), total thiol content - SH (C) and thiobarbituric acid reactive species - TBARS (D) levels and on the activity of superoxide dismutase - SOD (E) and catalase - CAT (F) in striatum of mice submitted to experimental model of lipopolysaccharide (LPS, 250 μg/kg)-induced neuroinflammation. One-way ANOVA and post-hoc Tukey multiple comparisons tests were performed. Bars represent mean and ± SEM. $^{\#}$ P<0.05; $^{\#\#}$ P<0.01; $^{\#\#\#}$ P<0.001 compared to vehicle/saline (control) group. * P<0.05; **P<0.01; *** P<0.001, compared to vehicle/LPS group (n = 5 per group).

Fig 6. Effect of *Cecropia pachystachya* (CEC) extract (200 mg/kg) and Enriched Flavonoid Fraction (EFF) (50 and 100 mg /kg) on reactive oxygen species-ROS (A), nitrite (B), total thiol content - SH (C) and thiobarbituric acid reactive species - TBARS (D) levels and on the activity of superoxide dismutase - SOD (E) and catalase - CAT (F) in hippocampus of mice submitted to experimental model of lipopolysaccharide (LPS, 250 μg/kg)-induced neuroinflammation. One-way ANOVA and post-hoc Tukey multiple comparisons tests were performed. Bars represent mean and ± SEM. $^{\#}$ $P<0.05$; $^{\#\#}$ $P<0.01$; $^{\#\#\#}$ $P<0.001$ compared to vehicle/saline (control) group. * $P<0.05$; ** $P<0.01$; *** $P<0.001$, compared to vehicle/LPS group (n = 5 per group).


LPS (250 µg/kg, i.p)
Vehicle (i.p)
Adult mice
D1
D8
D14
D15
Test Object recognition
Y-maze
Euthanasia
Cecropia pachystachya (200 mg/kg, o.p)
Enriched Flavonoid Fraction (50 or 100 mg/kg, o.p)
Vehicle (o.p)
Open-Field
Training Object recognition


A
Open Field Test
Number of total crossing
200
150
100
50
0
Control
LPS
CEC 200
EFF50
EFF100
B
Object Recognition Test
Recognition index (%)
100
80
60
40
20
0
###
***
***
***
Control
LPS
CEC 200
EFF50
EFF100
C
y-Maze test
Time (seconds)
100
80
60
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20
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*
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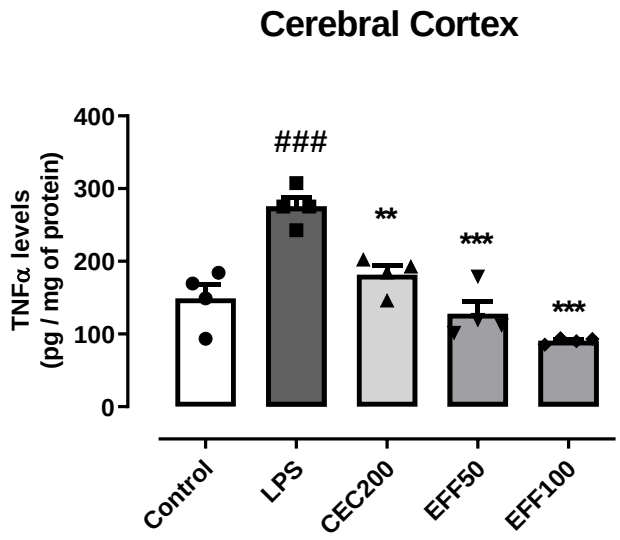
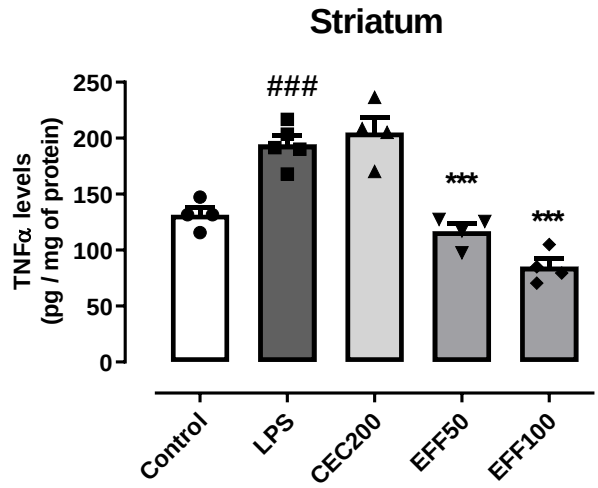
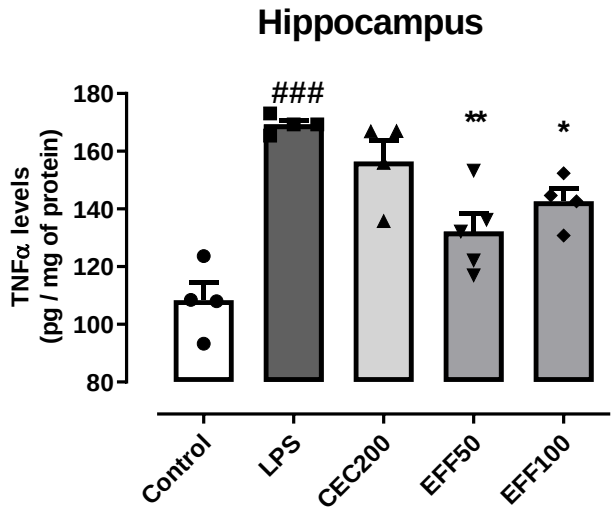
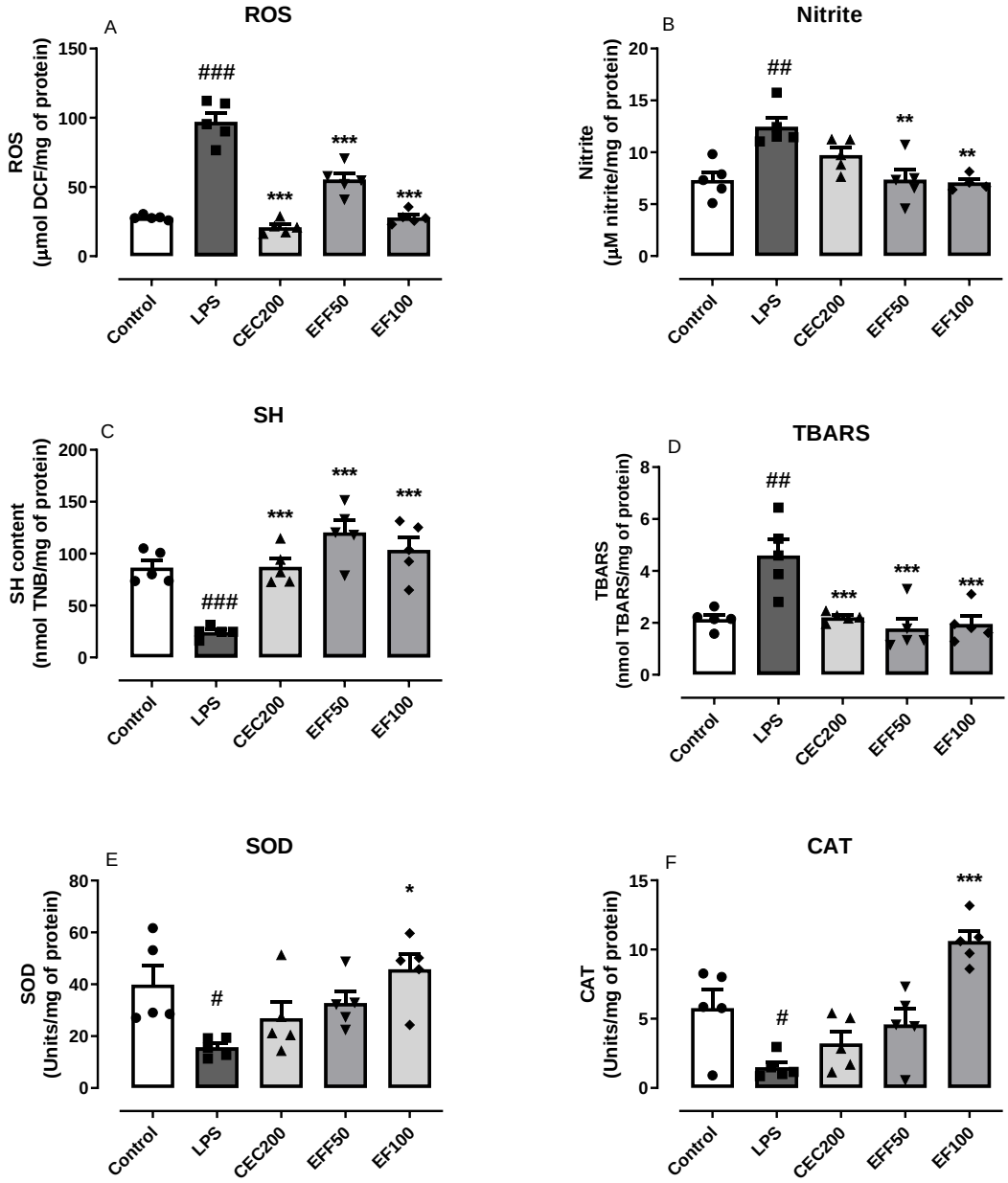
Novel
Start
Other
Control
LPS
CEC200
EFF50
EFF100


Cerebral Cortex
TNFα levels
(pg / mg of protein)
400
300
200
100
0
###
**
***
***
Control
LPS
CEC200
EFF50
EFF100

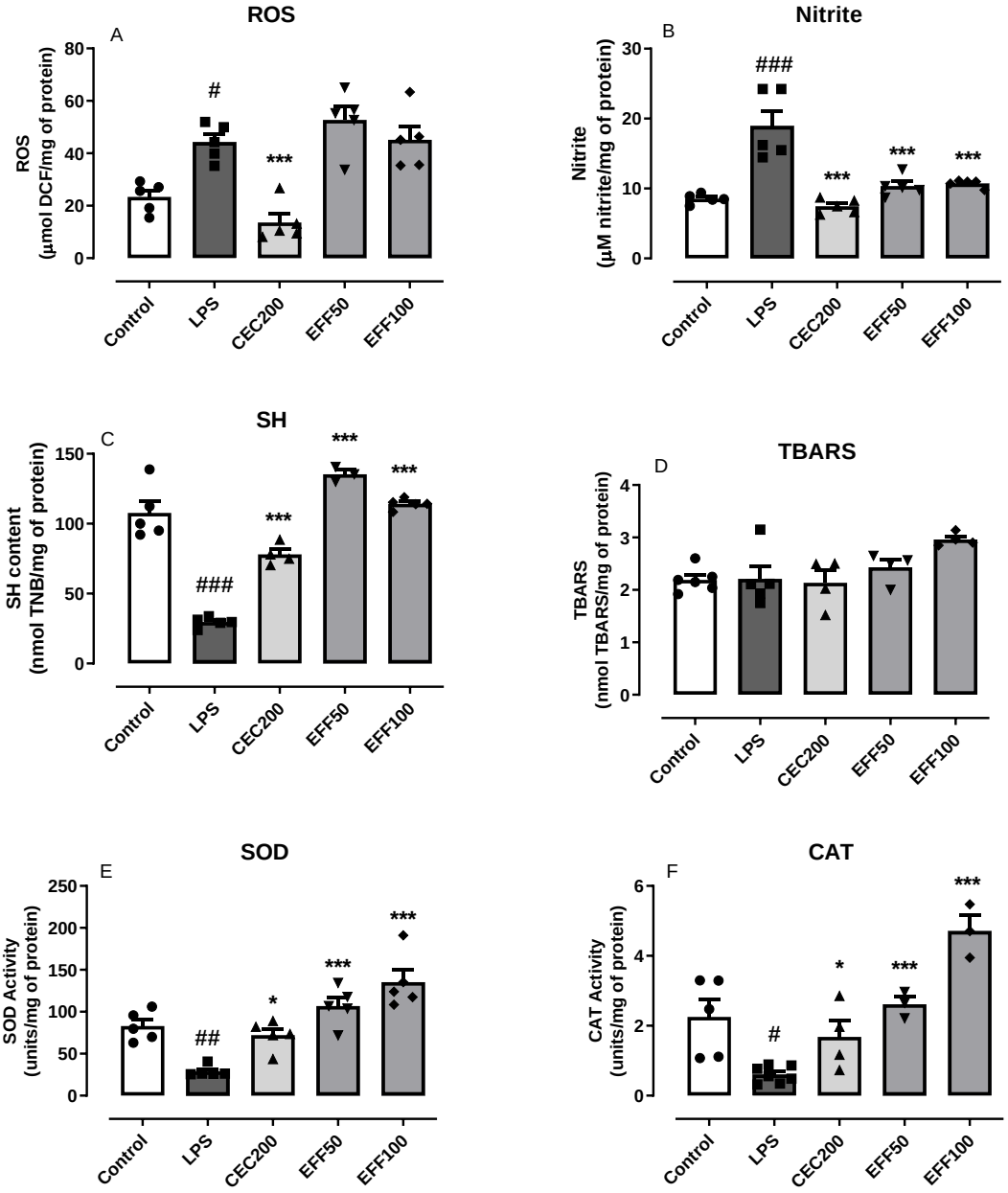

Striatum
TNFα levels
(pg / mg of protein)
250
200
150
100
50
0
###
***
***
Control
LPS
CEC200
EFF50
EFF100


Hippocampus
TNFα levels
(pg / mg of protein)
180
160
140
120
100
80
###
**
*
Control
LPS
CEC200
EFF50
EFF100

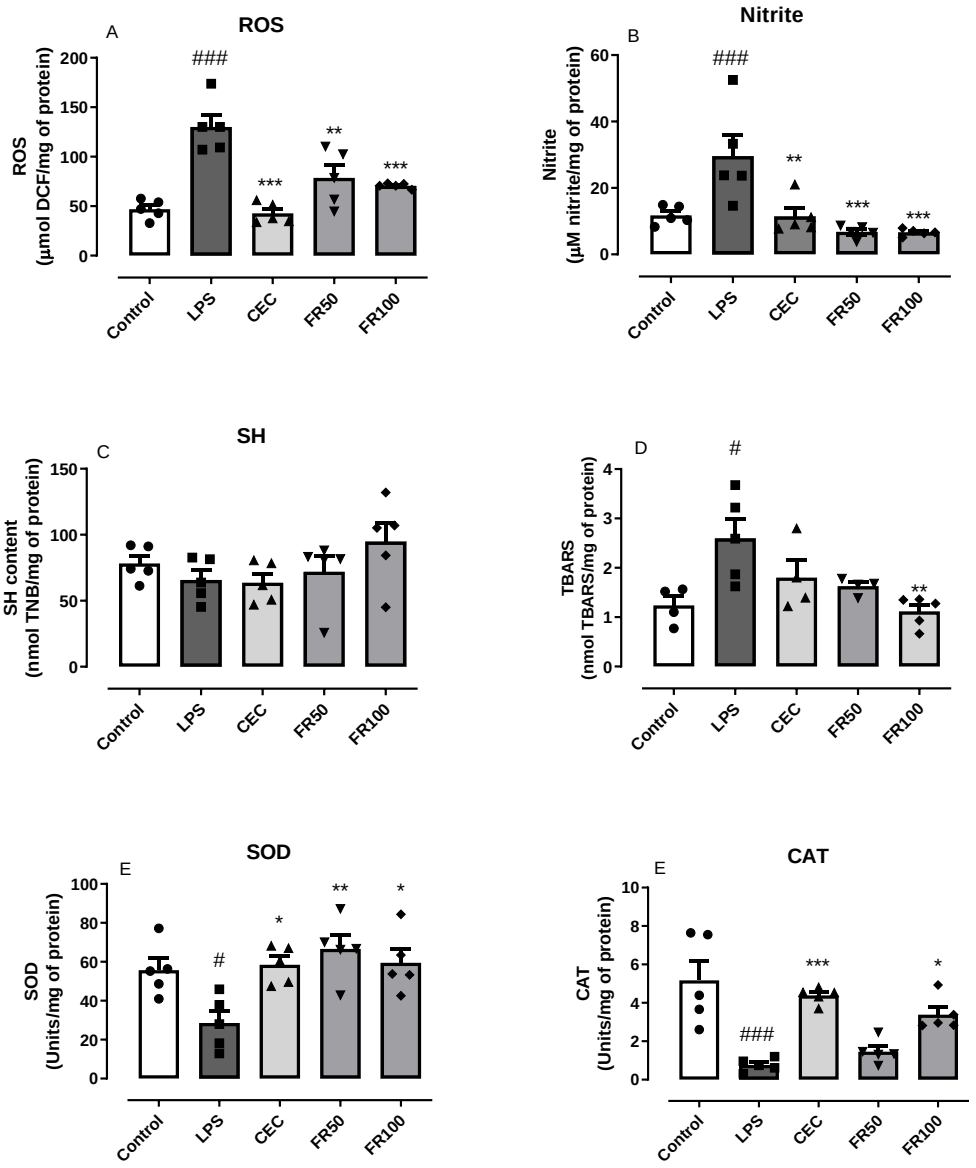
Cerebral Cortex



Hippocampus



Striatum


A
ROS
ROS
(µmol DCF/mg of protein)
###
**
***
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Control
LPS
CEC
FR50
FR100
B
Nitrite
Nitrite
(µM nitrite/mg of protein)
###
**
***
***
C
SH
SH content
(nmol TNB/mg of protein)
D
TBARS
(nmol TBARS/mg of protein)
#
**
SOD
SOD
(Units/mg of protein)
#
*
**
*
CAT
CAT
(Units/mg of protein)
###
***
*

5 DISCUSSÃO INTEGRADA

O câncer é designado por ser um conjunto de várias doenças de alta complexidade onde estão envolvidos processos entre diferentes tipos de tecidos que interagem entre si de forma heterogênea (INCA, 2022). Sendo considerado um problema mundial de saúde pública, que nas últimas décadas vêm gerando uma movimentação significativa da comunidade científica na busca de novas terapias para essa patologia. O câncer é um grande desafio, conforme dados do *National Cancer Institute* (NCI), para o ano de 2021 foram estimados 1.898.160 novos casos de câncer diagnosticados, sendo que 608.570 casos levariam os pacientes a morte nos Estados Unidos (NCI, 2021).

Os gliomas, são os tumores mais comuns do SNC, com características e diferentes graus de malignidade. Além disso representam 80% de todos os tumores cerebrais malignos pertencentes ao SNC (OMURO & DEANFELIS, 2013). Segundo o NCI, eram estimados para 2022, 25.050 novos casos de tumores do SNC, desses, 18.280 mortes eram estimadas (NCI, 2022). Dentre os gliomas, destacamos o GB, um glioma incurável, de grau IV, altamente prevalentes, representado 45% dos tumores malignos do SNC (BUSH et al., 2017).

O tratamento para esse tipo de tumor é considerado multimodal, onde é realizada a ressecção cirúrgica, associada da radioterapia e quimioterapia com o fármaco padrão TMZ (STUPP et al., 2010). Apesar de todas as estratégias de tratamento atualmente adotadas, os pacientes apresentam uma sobrevida extremamente baixa, sendo de aproximadamente 15 meses (BATASH et al., 2017). Em vista disso, torna-se desafiador estabelecer uma terapia que melhore a qualidade de vida e prognóstico dos pacientes acometidos por esse tumor. A comunidade científica tem dado uma grande atenção aos produtos naturais, visto que eles são capazes de agir em múltiplas vias e apresentam baixa toxicidade.

Dentre esses produtos naturais, destacamos o AT, um polifenol abundantemente encontrado na natureza, é considerado um importante alvo de estudo pois é um dos principais exemplos de tanino que podem ser extraídos eficientemente de fontes naturais com alta eficiência. Além disso, apresenta diversas atividades bem descritas na literatura frente a doenças neurodegenerativas e cânceres (SCALBERT et al., 2015).

Estudos demonstram o efeito antioxidante do AT em modelos de doenças que acometem o SNC, como na doença de Alzheimer (GERSZON et al., 2020) e na depressão (LUDUVICO et al., 2020). Além disso, nosso grupo de pesquisa demonstrou que o AT é um importante agente na terapia do glioma, atuando tanto *in* vitro, reduzindo a viabilidade e proliferação celular de linhagem de glioma de rato C6, atuando na parada do ciclo celular, induzindo a apoptose e apresentando um potencial antioxidante, sendo importante ressaltar que o AT teve uma ação seletiva, visto que alterou a viabilidade apenas das células tumorais, não alterando a mesma em cultivo primário de astrócitos (BONA et al., 2020). O AT ainda foi capaz de reduzir significativamente o tamanho do tumor em animais com GB sem causar toxicidade renal e hepática (BONA et al., 2020). A partir desses resultados, investigamos o potencial do AT em duas diferentes vias de sinalização, o estresse oxidativo e a sinalização purinérgica em um modelo pré-clínico de GB, resultados que estão demonstrados no capítulo 1 dessa tese.

Os animais receberam AT em uma dose de 50 mg/kg/dia pela via intragástrica, durante 15 dias. Houve uma modulação na via purinérgica, visto que, nas plaquetas dos animais com GB observamos uma redução na hidrólise de ATP e AMP seguida de um aumento na hidrólise de ADP e desaminação de ADO, demonstrando então um acúmulo de ATP e AMP e uma redução nos níveis extracelulares de ADP e ADO em conttrapartida os animais tratados com AT foram capazes de proteger contra essas alterações. No soro dos animais com GB, houve uma redução na hidrólise de ATP. Já nos linfócitos, observamos um aumento na hidrólise de ATP, ADP e degradação de ADO. Os resultados demonstrados frente a sinalização purinérgica, sugeriram que o AT é capaz de modular a atividade das ectonucleotidases, sendo de grande relevância a sua investigação.

O efeito antioxidante do AT é bastante elucidado na literatura. Nossos resultados demonstraram uma alteração no estado redox no cérebro, plaquetas e soro dos animais GB. Houve um aumento nos níveis de EROS e nitritos, e uma diminuição nos níveis de grupos tióis além da presença de peroxidação lipídica. Além disso, observamos uma redução na atividade das enzimas antioxidantes SOD e CAT, o que contribui diretamente para o aumento dos níveis de EROS. É importante ressaltar que os animais tratados com AT foram capazes de reverter a maioria das alterações, restaurando assim o estado redox. O AT é um polifenol antioxidante natural bem elucidado na literatura que ganhou extrema atenção na

última década no campo científico biomédico, visto que apresenta propriedades bioquímicas relevantes (BALDWIN & BOOTH, 2022). Esse composto consiste em uma unidade central de glicose ligada a dez moléculas de ácido gálico, sendo considerada uma estrutura simples (AELENEI et al., 2009). Corroborando com nossos resultados, recentemente um estudo envolvendo o ácido gálico foi publicado em um modelo pré-clínico de GB, demonstrando um efeito antiglioma importante, tanto *in vitro*, diminuindo a viabilidade e proliferação celular quanto *in vivo* reduzindo o tamanho do tumor e modulando os parâmetros de estresse oxidativo e sinalização purinérgica (PEDRA et al., 2022).

Outro produto natural que vem sendo alvo de diversos estudos é a *C. pachystachya*. Essa planta é conhecida popularmente como “embaúba” e tem sido utilizada na medicina tradicional como um importante diurético, além disso também é utilizada como tratamento de asma, tosse, hipertensão, diabetes e inflamação (PACHECO et al., 2014). Em vista disso, o manuscrito 1 teve como objetivo investigar o efeito antiglioma *in vitro* e em modelo pré-clínico de GB do extrato aquoso de CEC. Primeiramente observamos que o extrato de CEC foi capaz de diminuir significativamente a viabilidade e proliferação tanto das células de glioma de rato C6 quanto de glioma humano U87. Além disso uma inibição da migração celular foi notada nas células de C6 tratadas com extrato de CEC. O protocolo pré-clínico trouxe resultados relevantes, como a alterações comportamentais dos animais com GB. Os animais com GB, demonstraram uma diminuição na capacidade locomotora, ainda foi observado um déficit de memória a curto prazo e um comportamento do tipo ansioso. Porém é importante ressaltar que os animais tratados com o extrato de CEC (200 mg/kg/dia) foram capazes de atenuar esses danos causados pelo GB, restaurando assim a capacidade locomotora e de memória dos animais, além de ser sugerido como um importante ansiolítico, visto que diminuiu o comportamento do tipo ansioso dos animais. Uma das grandes problemáticas do GB, é que apesar de ter uma baixa incidência ele confere um mau prognóstico e como consequência disso os pacientes apresentam uma baixa qualidade de vida e uma alteração cognitiva elevada (GUNTUKU et al., 2016).

Além disso, os animais implantados com as células de GB e não submetidos ao tratamento apresentaram características típicas do tumor, como área de necrose, infiltrado inflamatório peritumoral e apoptose, além de apresentarem um crescimento da massa tumoral expressivo, em contrapartida, nos animais tratados com extrato de

CEC houve uma redução significativa da massa tumoral. Outra alteração típica observada foi a redução de peso nos animais controle, e uma recuperação desse peso foi observada nos animais tratados com o extrato. Em vista desses resultados o extrato de CEC torna-se um importante alvo no tratamento do glioma, ressaltando que além dos resultados positivos frente aos parâmetros comportamentais e a redução da massa tumoral, o extrato ainda não demonstrou citotoxicidade renal e nem hepática, sugerindo assim uma segurança do seu uso.

Além disso, investigamos o perfil antioxidante do extrato de CEC. No hemisfério esquerdo cerebral, onde estava localizado o tumor e também nas plaquetas e soro observamos um estresse oxidativo exacerbado, com o aumento dos níveis de EROS e nitritos seguido de uma diminuição no conteúdo tiólico total e evidência de peroxidação lipídica, e também de uma diminuição nas enzimas antioxidantes SOD e CAT. No cérebro, o tratamento com o extrato de CEC foi capaz de prevenir o aumento de EROS e nitritos e restabelecer a atividade da enzima CAT. No soro dos animais também observamos um reestabelecimento na atividade das enzimas SOD e CAT e nos níveis de EROS e nitritos. Nas plaquetas houve um reestabelecimento dos níveis de EROS e nitritos, porém o extrato de CEC não foi capaz de reverter a atividade das enzimas antioxidantes.

Outa via avaliada no nosso trabalho foi a sinalização purinérgica, via que está diretamente envolvida com a malignidade do GB (DEBOM et al., 2021). Nossos resultados demonstraram uma modulação na sinalização purinérgica, visto que nos animais implantados com GB e que não receberam tratamento houve um aumento na hidrólise de ADO, ATP e ADP em linfócitos e o tratamento com extrato de CEC foi capaz de prevenir esse aumento. Nas plaquetas observamos um aumento na hidrólise de ADO e ADP e diminuição na hidrólise de ATP e AMP, o tratamento com extrato de CEC foi capaz de reduzir apenas o aumento na hidrólise de ADO. Por fim, em soro, observamos apenas uma diminuição na hidrólise de ATP e o tratamento com o extrato foi capaz de modular essa alteração.

As citocinas inflamatórias estão diretamente associadas ao microambiente tumoral. Nossos resultados relataram um aumento nos níveis séricos de IL-6 em animais controle, porém o tratamento com extrato de CEC não foi capaz de reverter esse aumento. Quando aos níveis séricos de IL-10, observamos uma redução nos níveis séricos nos animais glioma e o tratamento com extrato de CEC foi capaz de aumentar esses níveis. A IL-6 vem sendo de grande interesse científico frente a

biologia do tumor cerebral, visto que contribui para a progressão maligna, radiorresistência e resistência a apoptose no câncer (CHANG et al, 2005). Já a expressão de IL-10 tem sido amplamente associada ao potencial de invasão do tumor (NITTA et al., 1994).

Em vista dos resultados apresentados no manuscrito 1, demonstrando o efeito antiglioma do extrato de CEC *in vitro* e em modelo pré-clínico de GB, podemos sugerir que esse composto é de grande relevância sendo um importante alvo terapêutico para o tratamento do GB.

Um dos componentes presentes na patologia do câncer é a inflamação, ela age contribuindo para a carcinogênese e progressão tumoral. Uma característica típica do GB é a necrose tecidual acompanhada de inflamação no microambiente tumoral (ALGHAMARI et al., 2021). O processo de neuroinflamação está associado com a etiologia e agravamento de diversas doenças do SNC, em vista disso a busca por novos alvos terapêuticos que atuem nesse processo torna-se de extrema relevância. Os flavonoides são um grupo de substâncias polifenólicas naturais que estão abundantemente encontradas em vegetais, frutas grãos e chás. Caracterizados por serem metabólitos secundários de plantas, os flavonoides desempenham um papel essencial em diversos processos biológicos, sendo importantes alvos para o tratamento de doenças (SHEN et al., 2022). Visto os benefícios já elucidados de CEC, Ortmann et al (2017) elaborou uma fração enriquecida em flavonoides C-glicosil. A FEF se apresenta como uma importante alternativa, visto que, o efeito sinérgico dos flavonoides presentes na mesma pode agir eficientemente em doenças do SNC. No manuscrito 2 desta tese, demonstramos o efeito neuroprotetor do extrato de CEC (200 mg/kg/dia) e da FEF (50 e 100 mg/kg/dia) em um modelo de neuroinflamação induzido por LPS (250 μg/kg).

Os animais LPS demonstraram um déficit de memória de curto e longo prazo, bem como níveis elevados de TNF-α. O tratamento com extrato de CEC e FEF, em ambas as doses, foi capaz de reverter o déficit de memória a longo prazo, porém a memória de curto prazo foi restabelecida apenas nos animais tratados com FEF. Quanto aos níveis de TNF-α, avaliados em córtex cerebral, estriado e hipocampo, o extrato de CEC foi capaz de reverter os danos causados apenas em córtex cerebral, já a FEF em ambas as dosses reverteu o dano nas três estruturas testadas.

Os parâmetros de estresse oxidativo foram avaliados nesse trabalho, onde observamos uma alteração no *status* redox, visto que houve um aumento significativo nos níveis de EROS e nitritos, uma diminuição no conteúdo tiólico total e a presença de peroxidação lipídica nos animais LPS. Em consequência disso, observou-se uma diminuição na atividade das enzimas antioxidantes CAT e SOD. O tratamento com extrato de CEC e a FEF em ambas as doses foram capazes de reestabelecer os níveis de EROS, nitritos e tiois, além de recuperar a atividade das enzimas antioxidantes SOD e CAT.

Em vista dos resultados obtidos, podemos sugerir que tanto o extrato de CEC quanto a sua fração rica em flavonoides são importantes alvos terapêuticos frente a doenças que envolvem processos de neuroinflamação.

Levando em consideração os resultados positivos da FEF obtidos no manuscrito 2 desta tese, testes *in vitro* com esse composto foram realizados em linhagem de glioma de rato C6 e glioma humano U87. É importante ressaltar que essa tese é a primeira a trazer resultados em modelo pré-clínico de tumor utilizando o tratamento com o extrato de CEC e que ainda existem poucos relatos na literatura sobre modelos *in vitro* e *in vivo* utilizando a FEF. Os resultados *in vitro* com a fração em linhagem de glioma foram descritos no anexo 2 desta tese.

A FEF demonstrou ser um potencial alvo antiglioma, visto que diminuiu significativamente a proliferação e viabilidade celular. Ademais, foi capaz de inibir a migração de células de glioma C6. Mais testes ainda serão realizados para melhor elucidar os mecanismos envolvidos no efeito da FEF *in vitro*.

O GB possui uma incidência relativamente baixa, porém apresenta um mau prognóstico o que afeta a qualidade de vida dos pacientes, tornando a terapia desse tumor algo desafiador. Mesmo sendo submetidos ao tratamento, os pacientes ainda apresentam uma sobrevida extremamente baixa. Em vista disso, há uma constante busca por novos agentes terapêuticos que possam auxiliar na terapia do GB. Neste contexto, nossos resultados demonstraram uma ação neuroprotetora relevante do AT, extrato de CEC e da FEF, sendo assim, podemos sugerir esses produtos naturais como potenciais alvos para o tratamento dessa doença.

6 CONCLUSÃO

Em nosso estudo foi possível observar que o AT atua modulando tanto o estado redox quanto as alterações observadas no sistema purinérgico. É importante ressaltar que esses resultados são complementares a estudos já realizados pelo grupo, onde já havia sido comprovada a atividade *in vitro* do composto frente a viabilidade e proliferação celular, além de também ter modulado o estado redox em células de glioma C6. Além disso, o AT demonstrou ser um importante agente antiglioma, visto que reduziu o tamanho do tumor em um modelo *in vivo*, além de não ter apresentado toxicidade para os animais.

Os produtos naturais vêm sendo amplamente estudados e em vista disso, avaliamos os efeitos de CEC e de fração rica em flavonoides oriunda desse extrato. O efeito antiglioma de CEC *in vitro* foi satisfatório, visto que reduziu significativamente tanto a proliferação quanto a viabilidade celular, além de ter inibido as células de glioma C6, é importante ressaltar que a FEF demonstrou o mesmo comportamento nos testes *in vitro*. No modelo pré-clínico de GB, apenas o extrato bruto de Cecropia foi utilizado, devido ao pouco rendimento da FEF. Observamos um efeito antiglioma significativo no extrato de CEC, visto que reduziu o tamanho do tumor significativamente, não apresentou toxicidade para os animais e modulou parâmetros de estresse oxidativo e sinalização purinérgica e restaurou os níveis de IL-10. É importante destacar que esse é o primeiro trabalho *in vivo* onde o efeito antitumoral do extrato de CEC é investigado.

Por fim, avaliamos o efeito protetor do extrato de CEC e da FEF em um modelo *in vivo* de neuroinflamação induzida por LPS. Observamos que ambos foram capazes de reestabelecer alterações em parâmetros comportamentais, na homeostase redox, bem como nos níveis de TNF-α causadas pela exposição à toxina.

Em vista dos nossos resultados podemos sugerir que os produtos naturais estudados são importantes alvos no tratamento de doenças que envolvem processos de neuroinflamação como o GB.

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APÊNDICE

Resultados realizados em parceria com a Universidade Lusófona de Portugal.

Análise de citotoxicidade e migração celular de células de glioma de rato (C6) e glioma humano (U87) expostas à Fração Enriquecida em Flavonoides obtida do extrato de *Cecropia pachystachya*.

Após as análises *in vitro* realizadas com o extrato de CEC, foram investigados os efeitos antiglioma da Fração Enriquecida em Flavonoides obtidas desse extrato. Foram realizadas análises de viabilidade, proliferação e migração celular, utilizando metodologias previamente descritas no manuscrito 1. Essas análises foram realizadas em parceria coma Universidade Lusófona de Portugal.

Para análise da citotoxicidade foram realizados testes de viabilidade e proliferação celular. O tratamento com FEF foi adicionado às linhagens de glioma de rato C6 e glioma humano U87 em concentrações de 25 – 500 μg/mL nos tempos de 24, 48 e 72h. Na linhagem de C6, observamos que o tratamento com FEF foi capaz de reduzir tanto a proliferação (**Figura 1a-c**) quanto a viabilidade celular (**Figura 1d-f**). Foi possível observar que as células de C6 expostas a FEF no tempo de 24h na concentração de 300 μg/mL, foi capaz de reduzir a proliferação celular em 86% (P<0,001), apresentando seu maior efeito nessa concentração e tempo testados. Quanto a viabilidade celular, observamos a maior redução no tempo de 72h na concentração de 300 μg/mL, sendo de 72,1% (P<0,001). Nas células de U87 expostas a FEF, observamos a redução mais significativa no tempo de 24 h na concentração de 200 μg/mL, sendo de 85,7% (P<0,001). Na viabilidade celular observamos a maior diferença no tempo de 48h na concentração de 200 μg/mL, sendo uma redução de 91,1% (P<0,001).

Afim de avaliar a influência da FEF na migração celular de células de glioma C6 e U87, foi realizado o teste do risco e foram capturadas imagens nos tempos de 0, 2, 4, 6 e 24h para obter a porcentagem de migração das células. Na linhagem de C6, observamos um fechamento do risco, no tempo de 24h, em 78,65% no grupo controle e o tratamento com FEF na concentração de 25 e 50 μg/mL, demonstrou uma inibição da migração celular no tempo de 24h em 58,15% (P<0,001) e 86,24%

(*P*<0,001), respectivamente (**Figura 2a e b**). Já na concentração de 100 μg/mL, houve uma inibição da migração celular nos tempos de 4, 6 e 24h em 96,58% (*P*<0,001), 95,99% (*P*<0,001) e 84,83% (*P*<0,001), respectivamente (**Figura 2c**). Os tratamentos com a FEF nas concentrações de 0,5, 1 e 2 μg/mL na linhagem de U87 não apresentaram diferença significativa frente a migração celular (*P*>0,05) (**Figura 2d-f**).

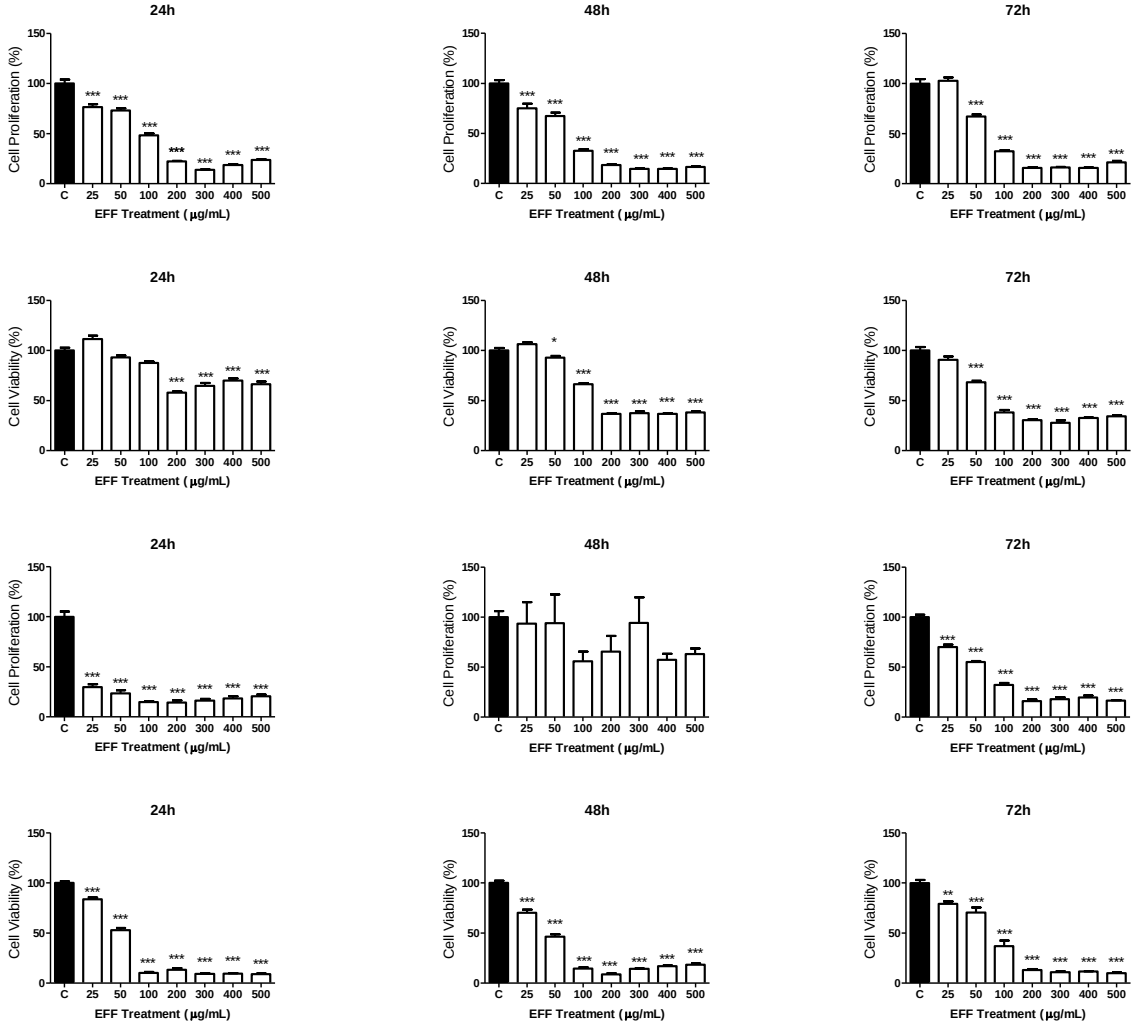

Figura 1. Efeito da Fração Enriquecida em Flavonoides (EFF) na proliferação (A–C e G–I) e viabilidade (D–F e J–L) de células de glioma de rato C6 nos tempos de 24 (A e D), 48 (B e E), e 72 h (C e F) e células de glioma humano U87 nos tempos de 24 (G e J), 48 (H e K) e 72h (I e L). Os valores são expressos como média ± erro padrão das médias de pelo menos três experiências independentes realizadas em triplicata. Os dados foram analisados por ANOVA de uma via seguido pelo teste de Tukey. *P<0,05, **P<0,01 e ***P<0,001 comparados ao grupo controle.

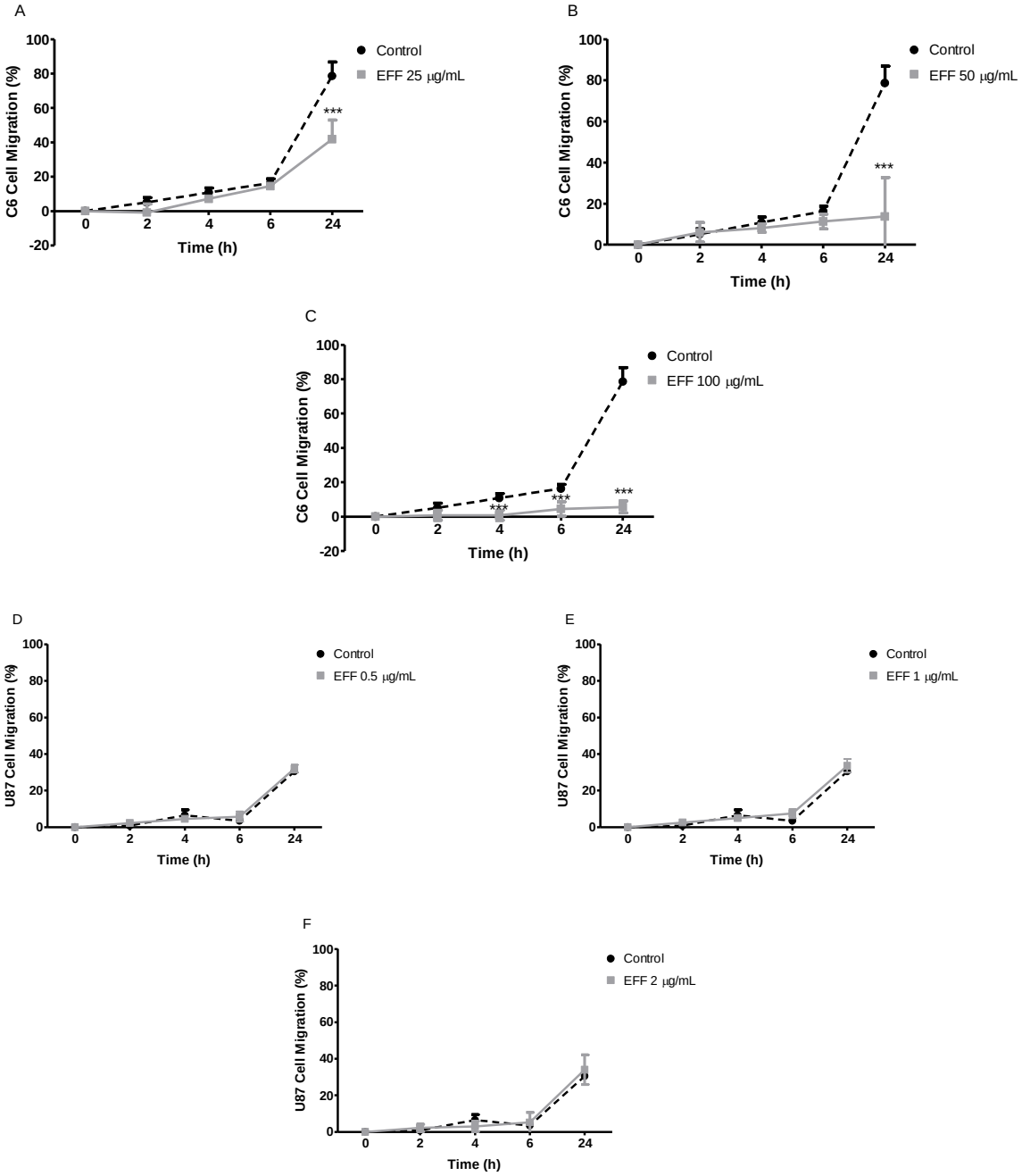

Figura 2. Efeito da Fração Enriquecida em Flavonoides (EFF) na migração celular de células de glioma de rato C6 (A-C) e células de glioma humano U87 (D-F) após diferentes tratamentos e tempos de 0, 2, 4, 6 e 24h. A porcentagem de migração celular foi analisada por ANOVA de duas vias seguida do teste de Bonferroni. ****P*<0,001 comparado com o grupo controle. Três experimentos independentes foram realizados em triplicata.

ANEXO A

Carta de aprovação na Comissão de Ética em Experimentação Animal (CEEA)

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **104/2018/CEEA/REITORIA**
PROCESSO Nº 23110.031292/2018-18

Certificado

Certificamos que a proposta intitulada **"Avaliação das atividades antitumoral e antioxidante de produtos naturais em modelo pré - clínico de glioblastoma multiforme"** processo número 23110.031292/2018-18, de responsabilidade de Francieli Moro Stefanello- que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua complementação pela Comissão de Ética em Experimentação Animal, em reunião de 10/09/2018.

Finalidade	(X) Pesquisa ( ) Ensino	
Vigência da autorização	15/09/2018 a 01/07/2020	
Espécie/linhagem/raça	*Rattus norvegicus*/Wistar	
N° de animais	296	
Idade	80 RN	216 com 60 dias
Sexo	Machos e Fêmeas	Machos
Origem	Biotério Central - UFPel	

Código para cadastro **CEEA 31292-2018**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX**, **Médico Veterinário**, em 14/09/2018, às 11:36, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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ANEXO B

Carta de parecer do Comitê de Ética em Experimentação Animal 2

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **108/2019/CEEA/REITORIA**
PROCESSO Nº 23110.032979/2019-51

Certificado

Certificamos que a proposta intitulada "**Produtos naturais na prevenção de alterações neuroquímicas e comportamentais em modelo animal de depressão induzido por lipopolissacarídeo**", registrada com o nº 23110.032979/2019-51, sob a responsabilidade de **Francieli Moro Stefanello** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de **11 de setembro de 2019**.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	18/09/2019 a 01/09/2022
Espécie/linhagem/raça	*Mus musculus*/Swiss
Nº de animais	384
Idade	60 dias
Sexo	Machos
Origem	Biotério Central - UFPel

Código para cadastro nº **CEEA 32979-2019**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX**, **Médico Veterinário**, em 18/09/2019, às 10:03, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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ANEXO C

Artigo Publicado

Neurochemical Research (2022) 47:1541–1552
https://doi.org/10.1007/s11064-022-03547-7

ORIGINAL PAPER

Tannic Acid Attenuates Peripheral and Brain Changes in a Preclinical Rat Model of Glioblastoma by Modulating Oxidative Stress and Purinergic Signaling

Natália Pontes Bona[1] · Mayara Sandrielly Pereira Soares[2] · Nathalia Stark Pedra[2] · Luiza Spohr[2] · Francieli da Silva dos Santos[1] · Alana Seixas de Farias[1] · Fernando Lopez Alvez[2] · Bernardo de Moraes Meine[1] · Karina Pereira Luduvico[1] · Roselia Maria Spanevello[2] · Francieli Moro Stefanello[1]

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ANEXO D

Carta de submissão do manuscrito 2 (capítulo 3) no periódico

NERX-D-22-00488 - Submission Notification to co-author - [EMID:69637cf84f71d3f1] Caixa de entrada ×

Neurotherapeutics (NERX) <em@editorialmanager.com> 18:58 (há 4 horas)
para mim

Re: "Cecropia pachystachya extract and enriched flavonoid fraction protect against memory deficits, inflammation and oxidative damage in LPS challenged mice"

Full author list: Natália Pontes Bona; Mayara Soares de Aguiar; Luiza Spohr; Nathalia Stark Pedra; Francieli da Silva dos Santos; Juliane Torchelsen Saraiva; Fernando Lopez Alvez; Bernardo Moraes Meine; Vânia Recart; Ingrid Vicente Farias; Caroline Flach Ortmann; Flavio Henrique Reginatto; Roselia Maria Spanevello; Francieli Moro Stefanello

Dear MSc Bona,

You are receiving this email because you have been listed as an author on a manuscript recently submitted to Neurotherapeutics entitled: "Cecropia pachystachya extract and enriched flavonoid fraction protect against memory deficits, inflammation and oxidative damage in LPS challenged mice".

The corresponding author for the submission process is: Dr. Dr Mayara Soares de Aguiar.

If you are not aware of this submission, or if you should not be listed as a co-author, please contact the journal office by clicking on the contact us link available in the EM banner. If we do not hear back from you within a week, we will assume you agree with your co-authorship.

With kind regards,
Springer Journals Editorial Office
Neurotherapeutics